Received: 11 May 2017 | Revised: 20 June 2017 | Accepted: 21 June 2017

DOI: 10.1002/ajh.24836

RESEARCH ARTICLE

WILEY AJH

Sickle cells produce functional immune modulators and cytotoxics

Chiao-Wang Sun[1] | Li-Chen Wu[1] | Peter L. Knopick[2] | David S. Bradley[2] | Tim Townes[1] | David S. Terman[1]

[1]Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, School of Medicine, Birmingham, Alabama; [2]Department of Biomedical Sciences, University of North Dakota School of Medicine and Health Sciences, Grand Forks, North Dakota

Correspondence
David S. Terman, Department of Biochemistry and Molecular Genetics, University of Alabama at Birmingham, 720 20th Street South, Kaul Building, Room 502, Birmingham, Alabama 35294.
Email: dst@sbcglobal.net

Funding information
Grant/Award Number: NIH R01HL057619; Azore Inc. (non-profit). Dr. Terman is recipient of the Doctors Cancer Research Foundation prize for 2016. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Abstract

Sickle erythrocytes' (SSRBCs) unique physical adaptation to hypoxic conditions renders them able to home to hypoxic tumor niches *in vivo*, shut down tumor blood flow and induce tumoricidal responses. SSRBCs are also useful vehicles for transport of encapsulated drugs and oncolytic virus into hypoxic tumors with enhanced anti-tumor effects. In search of additional modes for arming sickle cells with cytotoxics, we turned to a lentiviral β-globin vector with optimized Locus Control Region/β-globin coding region/promoter/enhancers. We partially replaced the β-globin coding region of this vector with genes encoding T cell cytolytics, perforin and granzyme or immune modulating superantigens SEG and SEI. These modified vectors efficiently transduced $Sca^{+}ckit^{-}Lin^{-}$ hematopoietic stem cells (HSCs) from humanized sickle cell knockin mice. Irradiated mice reconstituted with these HSCs displayed robust expression of transgenic RNAs and proteins in host sickle cells that was sustained for more than 10 months. SSRBCs from reconstituted mice harboring SEG/SEI transgenes induced robust proliferation and a prototypical superantigen-induced cytokine reaction when exposed to human CD4+/CD8+ cells. The β-globin lentiviral vector therefore produces a high level of functional, erythroid-specific immune modulators and cytotoxics that circulate without toxicity. Coupled with their unique ability to target and occlude hypoxic tumor vessels these armed SSRBCs constitute a potentially useful tool for treatment of solid tumors.

1 | INTRODUCTION

The formation and persistence of hypoxic niches within solid tumors constitutes a major cause of treatment resistance, defective drug transport, and aggressive malignant progression.[1–4] While many tumor targeting agents have been developed, ranging from small molecules to antibodies and nanoparticles, once administered into the circulation only minute fractions actually reach hypoxic tumor niches largely due to restrictions imposed by endothelial cells, aberrant rheology and other in vivo barriers.[5–7] This has prompted a search for therapeutics that can target hypoxic tumor niches and deliver a tumoricidal payload. For this important task we turned to the sickle erythrocyte. This cell is unique in mammals because under conditions extant in the hypoxic recesses of solid tumors sickle hemoglobin (HbS) polymerizes and multiple surface adhesion receptors are upregulated.[8] Intravital microscopy of tumors growing in the dorsal skin window indicates that transfused human sickle erythrocytes' (SSRBCs) rapidly target hypoxic tumor niches, shut down tumor blood flow and produce anti-tumor responses.[8] A clonogenic tumor cytotoxicity assay confirmed a potent synergy between HbS-derived heme and endogenous pro-oxidants in tumor cell eradication.[8]

Our previous studies sought to arm the SSRBCs with cytotoxics that could be directed to the tumor to enhance their tumoricidal effect. To this end, we showed that cytotoxic drugs could be physically encapsulated in SSRBCs and programed ex vivo to discharge 4 times more drug cargo into hypoxic tumors relative to normal RBCs and free drug.[9] Likewise, SSRBCs loaded with oncolytic reovirus directed the virus to melanoma in vivo and exhibited increased tumoricidal effectiveness relative to similarly treated normal RBCs and free virus.[10] While osmosis-based encapsulation allows the entrapment of large quantities of biologics it also injures cell membranes and alters the circulatory kinetics of the modified RBCs. Physical entrapment is confined to a limited group of agents and release at the targeted site can be unpredictable.[11,12] Likewise, virus adsorbed to SSRBCs runs the risk of exchange with higher affinity receptors and neutralization by seroreactive neutralizing antibodies.[10]

To obviate these concerns, we turned to a genetic method to load SSRBCs with immune modulators/cytotoxics by transduction of sickle hematopoietic stem cells with a lentiviral β-globin vector. Studies in transgenic mice demonstrated that coordinated interaction of several regulatory sequences including Locus Control Region (LCR) sequences HS2, HS3, and HS4 linked with beta-globin gene promoter, proximal enhancer and coding sequences directed beta-globin expression at levels equivalent to endogenous genes.[13–16] Incorporation of these LCR beta-globin transgenes into lentiviral vectors permitted efficient transduction of hematopoietic stem cells and subsequent expression of transgenic beta-globin genes in erythroid cells.[17,18] While a β-globin lentiviral vector demonstrated correction of the anemic phenotype in β-thalassemic and humanized sickle cell mice,[17,19–22] there have been no reports of functional incorporation of heterologous transgenes or autologous cytotoxics with tumoricidal activity for use in anti-tumor therapy.

In the current study, we replaced the first exon, first intron and part of the second exon of the beta-globin transgene in the lentiviral vector with genes encoding several biologics including staphylococcal superantigens SEG/SEI and granzyme/perforin. The former are powerful T cell stimulants that have been implicated in anti-tumor effects versus advanced non-small cell lung cancer while the latter are the major cytotoxics used by T cells to generate tumor cytolytic effects.[23–28] We hypothesized that the powerful β-globin LCR and downstream promoter/enhancers would drive transcription of these genes. Since expression of the human β-globin genes is confined to erythroid cells during defined stages of development, we reasoned that the tumoricidal transgenes would be translated in erythroid progenitors, precursors and reticulocytes and, therefore, emerge at high levels in mature erythroid cells. Here we show that insertion of several immunomodulators or cytolytic transgenes into the LCR β-globin lentiviral vector and subsequent transduction of sickle mouse hematopoietic stem cells results in erythroid-specific expression of functional transgenic cytotoxics in mature SSRBCs of transplanted hosts. Coupled with their ability to target hypoxic tumors and shut down tumor blood flow, SSRBCs armed with cytotoxics constitute a potentially useful tool for tumor killing.

2 | METHODS

2.1 | Mice

All animal procedures were approved by the UAB and UND Institutional Animal Care and Use Committees or the Animal Use Committees in compliance with the Guide for the Care and Use of Laboratory Animals. Transgenic sickle cell knock-in mice (B6;129-Hba$^{tm1(HBA)Tow}$ Hbb$^{tm2(HBG1,HBB/J)Tow}$/Hbb$^{tm3(HBG1,HBB)Tow}$/J) were obtained from a breeding colony at UAB. C57BL/6J mice were obtained from Jackson Laboratories (Bar Harbor, Me).

2.2 | Beta globin lentiviral vector preparation

Nucleic acid sequences encoding SEG and SEI were obtained from Aldevron, Fargo, ND. The full length lentiviral β-globin vector containing a transgene is modified from Levasseur et al.,[18] by substituting the transgene for the B^{AS3}-globin anti-sickling gene. In this vector, a transgene is substituted for the coding region of the β-globin gene under control of the β-globin promoter (266 bp), the PstI 3′ globin enhancer (260 bp) and a 375-bp *Rsal* fragment deletion of IVS2. The portion of the β-globin locus which includes the enhancer is found in the second intron of the β-globin gene as well as part of the third exon of *B*-globin and the enhancer located 3′ to the human *B*-globin gene, poly A sequence and a β-globin Kozak sequence are retained for coding stabilization. Also retained in the vector are the LCR of DNase I hypersensitivity regions (HS2, 3 and 4). The β-globin promoter/enhancer (2.3-kb) along with a tumoricidal transgene of choice were subcloned into the pWPT-GFP vector replacing the EF1a promoter and green fluorescent protein (GFP). This self-inactivating (SIN) vector contains a deletion in the U3 region of the 3′ long terminal repeat (LTR) from nucleotide 418 to nucleotide 18 that inhibits all transcription from the LTR. A biologic transgene replaces the coding region, exon 1 and 2, of the β-globin gene. The lentiviral-globin LTR contains in addition to the 3′globin enhancer, the β-globin promoter, β-globin gene, a 3′ SIN deletion, ψ packaging signal, splice donor and acceptor sites, Rev-responsive element, RRE, cPPT/CTS indicating central polypurine tract or DNA flap/central termination sequence and WPRE specifying woodchuck hepatitis virus post-transcriptional regulatory element. DNase 1 hypersensitive sited (HS) fragments 5′ HS4, 3, and 2 are amplified by polymerase chain reaction (PCR) from a 22-kb fragment of the LCR. Nucleotide coordinates from GenBank accession no. U01317 are: HS4 592–1545, HS3 3939¬5151, and HS2 8013–9215. The entire HS4 3.2 β-globin gene construct is verified by sequencing.

2.3 | Vector production

Vectors were produced by transient transfection into 293T cells as previously described[29] with the following modifications. A total of 2.5 × 10^6 293T cells were seeded in 10-cm–diameter dishes containing Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) 24 hours prior to transfection. Forty micrograms plasmid DNA was used for transfection of one 10-cm dish. The DNA cocktail contained 5 μg envelope-coding plasmid pMDG, 15 μg of the packaging plasmid pCMVdR8.91 (which expresses Gag, Pol, Tat, and Rev) and 20 μg SIN transfer vector with genes of interest. Transfection medium was removed after 14 to 16 hours and replaced with DMEM/F12 without phenol red (Invitrogen, Carlsbad, CA) containing 2% FBS. Virus- containing supernatant was collected after an additional 24 hours, cellular debris are cleared by low-speed centrifugation, and filtered through a low protein-binding 0.22-μm polyether sulfone filter (Millipore, Bedford, MA). The virus was concentrated 1000-fold by one round of centrifugation at 26,000 rpm for 90 minutes at 8°C using an SW-28 rotor (Beckman, Palo Alto, CA), resuspended into serum-free stem cell growth medium (SCGM) (Cellgenix, Freiburg, Germany) and allowed to incubate on ice for 4 hours before aliquot storages at −80°C. Virus titer was determined by infecting murine erythroleukemia (MEL) cells, with an EF1a-GFP control virus, and assaying GFP

positive populations in the cultures by FACS analysis to determine the physical state of viral numbers.

2.4 | Introduction of granzyme/perforin and SEG/SEI into the β-globin lentiviral vector

Construction of the polycistronic genes construct using PTV1 2A sequences and fusion PCR was performed essentially as described[30] (Supporting Information Figure S1). Briefly, mouse Adrb2 cDNA (Clone 1349283; Thermo Scientific) was PCR amplified and modified with primers Adrb2-F and Adrb2-R to contain a 50 bp homology to human HIFlα 5′ UTR and a Kozak consensus sequence. At 3′ end the Adrb2 stop codon was eliminated and replaced with nucleotides (nt) from PTV1 P2A to form a 22-nt overlap with the 50 bp 5′ of the Gzmb amplicon. Mouse Gzmb cDNA (Clone 3592898; Thermo Scientific) was PCR amplified and modified with primers Gzmb-F (the first 20 amino acids from ATG was removed) and Gzmb-R to overlap with the 3′ end of the Gzmb amplicon and to append 2A nt sequences. The Gzmb stop codon was eliminated and replaced with nt from PTV1 T2A that forms a 22-nt overlap with the 50-nt 5′ of the Prfl amplicon. Mouse Prfl cDNA (Clone 40130577; Thermo Scientific) was PCR amplified and modified with primers Prfl-F and Prfl-R to overlap with the 3′ end of the Gzmb amplicon and to append T2A nt sequences upstream of the Prfl ATG. At the 3′ end, the Prfl stop codon was retained and *Swa* I restriction sites were added. After PCR the individual amplicons were gel purified and used in a three-element fusion PCR at a 1:100:1 (Adrbl/Gzmb/Prf 1) molar ratio along with primers Adrb2-F and Prfl-R to produce a 3,764-bp amplicon containing the polycistronic genes. The polycistronic gene was gel purified and cloned into the general cloning vector pBS-SK+ (Stratagene) using the *Smal* restriction sites (enzymes from New England Biolabs) to produce pJS-AGP and sequenced for authenticity. The human HIF-la promoter and 5′ UTR was amplified by PCR with 50 bp overlap to 5′ of Adrb2-Gzmb-Prfl polycistron gene, and assembly the HIF-lα-Adrb2-Gzmb-Prfl is by fusion PCR. Subsequently, the polycistron was subcloned into a *Swal* site in the lentiviral vector to produce the HIF-1α-Adrb2-Gzmb-Prf 1 polycistronic lentiviral vector which was sequenced for authenticity

By the same strategy a second polycistronic lenitviral vector HIF-la-SEG-SEI was produced. PCR reactions were performed using PrimeStar polymerase (Takara, Otsu, Japan, http://www.takara.co.jp). SEG and SEI coding sequences were connected with a short picornavirus 2A peptide sequence. All of the oligonucleotides used in this study were synthesized by Integrated DNA Technologies (Coralville, IA) and all DNA gel extractions were performed using QIAquick Gel Extraction Kits (Cat. No. 28706).

2.5 | Stem cell transduction and transplantation

For stem cell transplantation studies in mice bone marrows from SS mice are isolated from the femurs and tibias and $ckit^{+}lin^{-}Sca^{+}$ erythroid progenitor cells, purified by Stem Cell Technology mouse *Sca1*+ positive selection kit, catalogue #18756, and transfected with tumoricidal transgenes SEG and SEI as described above and resuspended at 1×10^{7} cells/mL in Stem Span medium containing 1x Penicillin Streptomycin, mSCF, mTPO and mFLT3 at 50 ng/mL each. *Sca1*+ donor bone marrow cells 1×10^{6} in 150 μL of RPMI 1640 medium were injected via the retro-orbital into acidified water treated C57BL/6J recipient mice irradiated with two doses of 600 rad or 6 Gy, 4 hours apart. The chimeras were kept in autoclaved cages, with 1.1 g neomycin sulfate/liter (Sigma N6386) in the drinking water for 2 weeks, after which sterile drinking water was used. They were used after a 3 month rest period to allow for full reconstitution.

2.6 | FACS analysis, hemoglobin electrophoresis

Fifty microliters of whole blood were washed in 500 μL PBS. Cell pellets were resuspended in 100 μL lysis solution (5 mM sodium phosphate, 0.5 mM EDTA, pH 7.4) and incubated on ice for 15 minutes. One tenth volume of 10% NaCl was added and the sample was centrifuged at maximum speed in an Eppendorf microfuge (Hamburg, Germany). Approximately 1 μL of the supernatant was analyzed on an isoelectric focusing (IEF) gel. IEF was performed using the Isothermal Controlled Electrophoresis system (Fisher Scientific, Pittsburgh, PA) with precast agarose IEF gels (RESOLVE from PerkinElmer, Helsinki, Finland). Hemoglobin bands were quantitated by densitometry with a BioRad (Hercules, CA) GS-800 scanner using Quantity One software (BioRad).

2.7 | RT-PCR and western analysis

Total RNA was extracted from blood of mice transplanted with tumoricidal transgenes using Qiagen RNeasy Micro Kit (Cat No 74004), followed by DNaseI treatment with Invitrogen DNase I kit (Cat No 18080–051). cDNA was synthesized with Invitrogen SuperScript III Frist-Strand Synthesis System kit according manufacturer's protocol. PCR was performed on a DNA Engine Tetrad PTC-220 thermal cycler with TaKaRa LA Taq DNA polymerase 30 cycles reaction.

2.8 | RBC lysates and functional testing of superantigen transgenes

Lysates of peripheral blood erythrocytes from C57BL/6 mice reconstituted with hematopoietic stem cells (HSCs) from sickle cell knockin mice containing the β-globin vector incorporating SEG and SEI transgenes were prepared by three alternating freeze-thaw cycles in 2.0 mL of medium, followed by centrifugation to remove cell debris. Lysates were assayed for biologic activity by measuring the proliferative response of human PBMCs. Normal donor PBMCs were collected in EDTA tubes and isolated via Ficoll-Paque gradient. These cells were treated with Cell Proliferation Dye eFluorR 450 (eBioscience) according to manufacturer instructions. Cells were seeded in a 96 well round bottom plate in 200 uL DMEM supplemented with 10% HI FBS (ATLANTA Biologicals), HEPES, and Pen/Strep (Corning). Cells were either stimulated with recombinant SEG/SEI (0.5 ug each), 1 μL RBC lysate, or incubated in media alone for 3 days at 37°C in 5% CO_2. Cells were subsequently stained with Ghost Dye™ Red 710 (TONBO Biosciences), αCD3 FITC (OKT3; TONBO Biosciences), αCD4 PerCP-

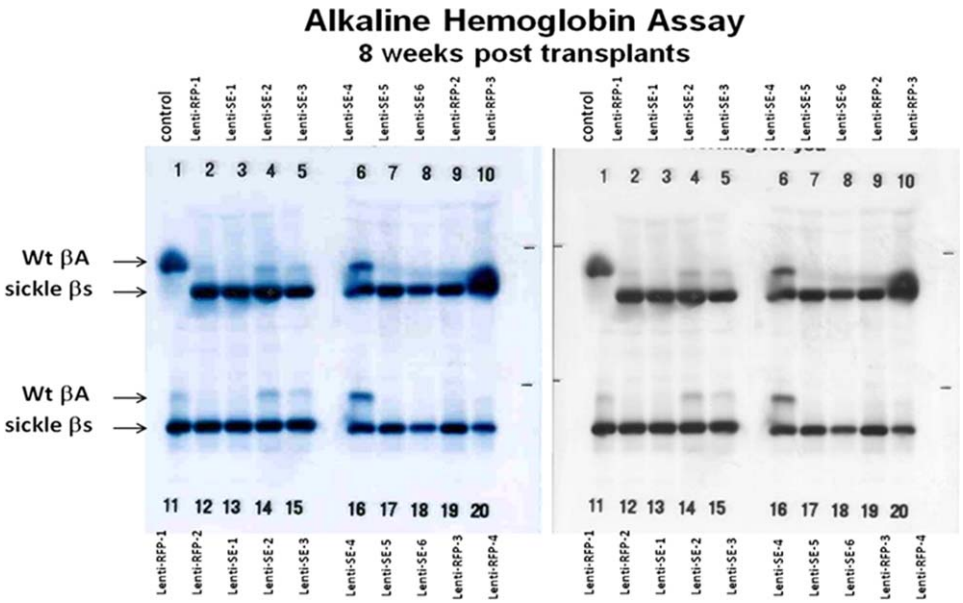


FIGURE 1 Alkaline hemoglobin of erythrocytes from mice reconstituted with Sca+ HSCs from SS knockin mice showing the presence of hemoglobin S [Color figure can be viewed at wileyonlinelibrary.com]

Cyanine5.5 (SK3; TONBO Biosciences), αCD8 PE (OKT8; TONBO Biosciences).

2.9 | Toxic shock model

We used a variant of the toxic shock model described by Miethke et al.[30] C57BL/6 mice were transplanted with hematopoietic stem cells from sickle cell knockin mice transduced with SEG/SEI or GFP integrated into the β-globin vector. The reconstituted mice were rested for 12 weeks after transplant and then injected i.p. with 10^6 splenocytes from healthy 8 week old C57BL/6 mice. Survival was assessed 24 hours later.

2.10 | Statistics

We deployed the one-wayANOVA with a Tukey's multiple comparison post test for statistical analysis of studies depicted in Figure 4A.

3 | RESULTS

3.1 | RFP, superantigen and hemoglobin S are expressed in irradiated C57BL/6 mice reconstituted with hematopoietic stem cells transduced with the lentiviral β-globin vector

We determined whether peripheral blood erythrocytes in mice reconstituted with hematopoietic stem cells from sickle cell mice transduced with the lentiviral vector encoding RFP or superantigen SEG could express these proteins along with hemoglobin S. Initially we cloned the RFP gene into the β-globin lentiviral vector and used it to transduce hematopoietic cells from sickle cell knockin mice. Irradiated mice reconstituted with these cells produced RFP in their peripheral blood (Supporting Information Figure S2A). Approximately 25% of the RFP+ erythrocytes demonstrated a sickle morphology indicative of sickle cells. Likewise, FACS analysis of peripheral blood erythrocytes from irradiated mice reconstituted with HSCs from sickle cell knockin mice transduced with SEG demonstrated expression of SEG in 31.7% of peripheral blood erythrocytes (Supporting Information Figure S2B). Alkaline hemoglobin electrophoresis in these mice demonstrated the presence of hemoglobin S but not hemoglobin a β-globin chains (Figure 1). Reconstituted mice bearing SEG and SEI in their SSRBCs 16 weeks after transplant still demonstrated 5% of SSRBCs with sickle morphology in their peripheral blood with no significant aberrations in other blood components (Supporting Information Figure S3). Mice reconstituted with HSCs from sickle cell knockin mice harboring the β-globin lentiviral vector loaded with granzyme and perforin genes showed robust expression of granzyme and perforin in Western blots 16 weeks after transplantation (Figure 2). These data indicate that RFP, SEG,

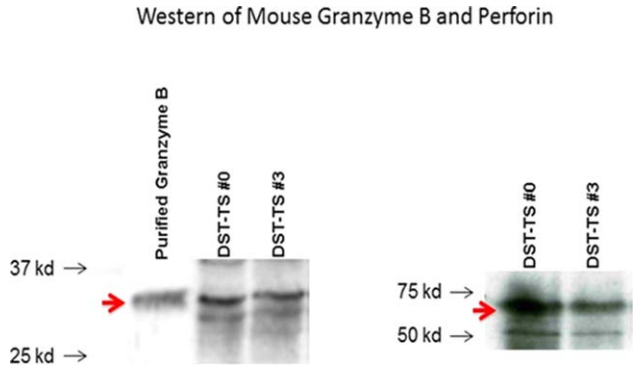


FIGURE 2 Western Blot showing granzyme B and perforin protein expression in peripheral blood SSRBCs three months after transplantation of Lin^-ckit^+ $Sca\text{-}1^+$ HSCs transduced with the β-globin lentiviral harboring granzyme B and perforin transgenes [Color figure can be viewed at wileyonlinelibrary.com]

FIGURE 3 RTPCR analysis of RBCs from mice 16 weeks after reconstitution with $lin^{-}ckit^{+}Sca\text{-}1^{+}$ HSCs from sickle cell knockin mice transduced with β-globin vector containing the granzyme and perforin or SEG and SEI transgenes

granzyme and perforin transgenes are efficiently and durably expressed in reconstituted hosts along with hemoglobin S.

3.2 | RNA synthesis of SEG, SEI, granzyme and perforin in SSRBCs of mice reconstituted with polycistronic β-globin lentiviral vectors

Erythrocytes from mice reconstituted with HSCs from sickle cell knockin mice containing the lentiviral β-globin vectors encoding SEG and SEI or granzyme and perforin demonstrated robust expression of RNA encoding these proteins 16 weeks after transplantation (Figure 3). This indicates durable transcription of the transgenes and translation of transgenic mRNA in SS erythroid cells for at least 16 weeks after transplant.

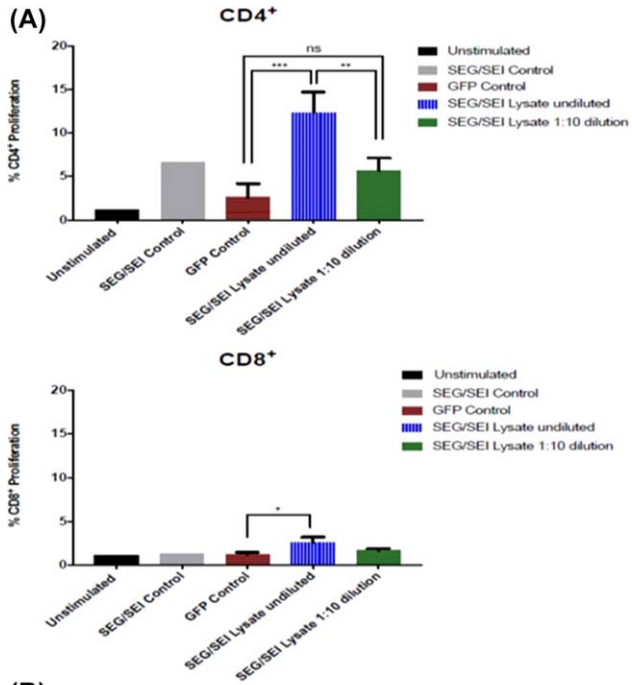
3.3 | SEG and SEI retain superantigenic function in reconstituted hosts

Lysates of erythrocytes from mice reconstituted with HSCs from sickle cell knockin mice transduced with the β-globin lentiviral vector encoding staphylococcal superantigens SEG and SEI or GFP transgenes were examined for their ability to activate T cell proliferation against human peripheral blood lymphocytes. The latter is a fundamental property of superantigens. Results show that lysates induced a 10 fold mitogenic increase in PMBCs relative to the GFP control (Figure 4A). Likewise, mice reconstituted with SEG and SEI or GFP transgenes were injected i.p. with 10^6 splenocytes from normal syngeneic mice 10 weeks after transplantation. All five mice reconstituted with SEG/SEI exhibited death at 24 hours typical of superantigen-induced toxic shock whereas 5 GFP control mice were alive in good condition at this time (Figure 4B). These results indicate that mice harboring SEG and SEI superantigenic proteins from HSC transgenes are immunologically unresponsive to these proteins and that their superantigenic function surfaces when exposed to human T cells. Notably, ten mice expressing SEG/SEI 10 months after transplantation did not display the syndrome of inanition, cachexia and weight loss associated with chronic exposure to superantigens.[31,32] This suggests that the exposure to SEG and SEI early in hematopoietic regeneration rendered these hosts tolerant to the superantigens. Six mice expressing transgenic granzyme A, B and perforin were alive 10 months after transplantation showing no constitutional signs or tissue injury. Ten months after transplantation autopsies of 3 mice from groups expressing SS hemoglobin together with transgenic SEG/SEI, granzyme A, B/perforin or GFP controls showed no evident injury to lungs, kidneys or liver. Splenomegaly observed in the SEG/SEI and granzyme/perforin groups was comparable to that noted in the GFP controls.

4 | DISCUSSION

Here, we determined whether a β-globin lentiviral vector could be useful for induction of erythroid specific expression of biologically active tumoricidal transgenes. To this end, we showed that hematopoietic stem cells transduced with transgenes encoding cytotoxics perforin and granzyme and immunomodulating superantigens SEG and SEI are able to reconstitute irradiated hosts and exhibit high level expression and function of the transgene products in their peripheral blood erythrocytes along with hemoglobin S. Functional activity of these proteins together with hemoglobin S was retained for up 10 months post-transplant.

We and others have shown that the lentiviral β-globin vector used herein is under the control of the three powerful erythroid specific DNase I Hypersensitive Site (HS) sequences in the Locus Control Region[13–16] plus enhancers located downstream of the gene and in the second intron.[33] The transgene(s) replaces the β-globin at the first exon, the first intron and part of the second exon. Importantly, the transgenes contain their own stop codon therefore excluding β-globin production. When used to transduce murine $ckit^{+}$ lin^{-} Sca^{+} HSCs from sickle cell donors, the β-globin vector is incorporated into the genome of the transduced cell whereby the powerful erythroid specific regulatory sequences described above direct high-level expression of the transgenes specifically in erythroid cells. It is therefore unlikely that



(B)

Toxic shock in Transgenic Mice Expressing Erythroid-derived Superantigens		
	Syngeneic normal splenocytes injected i.p into:	
	SS (SEG/SEI) mice	SS (GFP) mice
Survival 24 hrs after syngeneic normal splenocytes injection	0/5*	5/5
*Autopsies showed hemorrhagic inflammation in the abdominal cavity		

FIGURE 4 (**A**) Lysates of erythrocytes from mice reconstituted with SEG/SEI exhibit significant T cell proliferation relative to mice reconstituted with GFP control protein. For percentage of live CD4+ cells $^{**}P < .001$, $^{**}P < .01$. For percent live-CD8+ cells $^{*}P = .01$. (**B**) depicts outcomes after nomal splenocyte injection into mice transplanted with SS stem cell transduced with the β-globin vector encoding SEG/SEI or similar stem cells transduced with the β-globin vector encoding GFP [Color figure can be viewed at wileyonlinelibrary.com]

transcription in *ckit*$^{+}$ *Lin*$^{-}$ *Sca*$^{+}$ stem cells driven by the powerful erythroid specific Locus Control Region in the β-globin vector would generate transgenes in the neutrophil or monocytes lineage. Indeed, the RFP uptake in peripheral blood smears of the RFP transplanted mice was limited to the RBCs sparing the morphologically larger neutrophils and mononuclear cells. Although the tumoricidal transgenes and betaS-globin genes are simultaneously expressed and produced in the transplanted hosts the endogenous betaS-globin genes are separate and distinct from the transgenes residing in the β-globin vector.

With respect to functional chimerism of the transgenes and hemoglobin S in the transplanted hosts, transgenic superantigen products retained immune activity in reconstituted hosts evidenced by a strong T cell mitogenic response in lysates of SSRBCs harboring SEG and SEI and the induction of superantigen-mediated toxic shock when these mice were exposed to normal syngeneic T cells. Alkaline hemoglobin electrophoresis showed that betaS-globin chain synthesis was intact in these mice indicating that transgenic protein synthesis did not silence endogenous betaS-globin chain production. Sixteen weeks after transplant these mice breathing room air exhibited sickle morphology in 5% of mature RBCs indicative of underlying functional hemoglobin S polymerization; of course, 100% of the RBCs from these mice sickle if deoxygenated. Moreover, Western blot and RNA gels from SSRBCs of the transplanted mice demonstrated the presence of the transgenic proteins and their genetic origin in the transplanted SSRBCs. In addition, we noted HbS and transgene product synthesis for up to 10 months after bone marrow reconstitution indicating the durability of the transgene and endogenous sickle gene expression. Collectively, these data show that the β-globin lentiviral vector encoding superantigen proteins is integrated into the genome of HSCs and functionally expressed simultaneously with HbS after long-term reconstitution.

The β-globin-transduced HSCs used for reconstitution do not appear to damage host cells or affect their survival. Indeed, several mice reconstituted with superantigens or granzyme and perforin survived for 10 months after transplantation and showed no signs of acute/chronic toxicity or tissue injury at autopsy. Granzymes and perforin are intracellular serine proteases and the major cytolytics used by T cells and NK cells in killing viruses and tumor cells. They are secreted by T cells and NK cells after cell to cell contact and work synergistically in cytolysis using partially overlapping mechanisms. Granzyme B activates caspase 3 and mitochondrial membranes while granzyme A interacts with nuclear elements.[27,28] Any leakage of these cytolytic products from circulating SSRBCs did not appear to induce acute or chronic toxicity likely because granzyme in plasma is degraded by blood borne protease inhibitors antithrombin III and α-2-macroglobulin.[34] Thus, despite their cytolytic properties, mice bearing granzyme and perforin transgenes in their SSRBCs showed durable transgene expression with no untoward acute or chronic effects of these proteins.

SEG and SEI are staphylococcal enterotoxins originating from the enterotoxin gene cluster and display the typical hallmarks of superantigens such as potent TCR vβ-specific T cell mitogenicity/activation.[23,25] It appears that these highly immunogenic and demonstrably mitogenic superantigens are broadly expressed in host SSRBCs for 10 months after transplantation without evoking acute or chronic superantigen-induced toxicity.[31,32] Indeed, these mice appeared to be immunologically unresponsive to or tolerant of the heterologous superantigen cargo. This tolerance was broken by exposure of the mature SSRBCs to normal murine T cells in vivo.[30] Such tolerance to SEG and SEI in reconstituted mice is likely to have occurred during early hematopoietic reconstitution. Indeed, similar HSC-mediated tolerance to other proteins has been observed in the course of stem cell transplantation.[35,36] Thus, despite their potent immunogenicity, foreign superantigens SEG and SEI introduced into SSRBCs do not induce tissue injury or untoward immune responses in the transplanted host.

This technology could be adapted for surface expression of transmembrane cargo such as enzymes, receptors, transporters, chemokines and anti-autoimmune peptides. Extracellular release of SSRBC transgenic proteins can occur during autohemolysis when sickle cells are trapped in

the hypoxic tumor vasculature. Such autohemolysis and cargo release can be programed to occur in the tumor microvessels using *ex vivo* time dependent photooxidation carried out before administration of the SSRBCs to the patient.[9] Alternatively, the cargo from the SSRBC or its precursors may be released by linking transgene synthesis to a host vesicular transport system that is activated upon synapse with a target cell. The β-globin lentiviral vector therefore provides a potentially broad and flexible platform for expression and delivery of a wide range of erythroid-based therapeutics.

In conclusion, we show that the β-globin lentiviral vector is capable of arming mature SSRBCs with native and foreign tumoricidal transgenes that are robustly expressed in transplanted hosts without toxicity. Coupled with the inherent ability to target tumors and shut down tumor blood flow, SSRBCs armed with cytotoxics constitute a potentially useful tool to augment their tumoricidal effectiveness.

ACKNOWLEDGMENTS

This work was supported by NIH R01HL057619, Azore (NP) Foundation. Dr. Terman is recipient of the Doctors Cancer Research Foundation prize for 2016. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Conceived and designed the study, analyzed the data and wrote the paper: David S. Terman.

analyzed the data and edited the manuscript: Tim Townes.

Performed the experiments in Figures 1-3. Supplementary Figures 1-3 and analyzed the data: Chiao-Wang Sun and Li-Chen Wu.

Performed the experiment in Figure 4: Peter L. Knopick and David S. Bradley.

REFERENCES

[1] Brown JM, William WR. Exploiting tumour hypoxia in cancer treatment. *Nat Rev Cancer*. 2004;4:437–447.

[2] Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer*. 2011;11:393–410.

[3] Bristow RG, Hill RP. Hypoxia, DNA repair and genetic instability. *Nat Rev Cancer*. 2008;8:180–192.

[4] Minchinton AI, Tannock IF. Drug penetration in solid tumours. *Nat Rev Cancer*. 2006;6:583–592.

[5] Greish K. Enhanced permeability and retention of macromolecular drugs in solid tumors: a royal gate for targeted anticancer nanomedicines. *J Drug Target*. 2007;15:457–464.

[6] Hait WN, Hambley TW. Targeted cancer therapeutics. *Cancer Res*. 2009;69:1263–1267.

[7] Park K. Programmed sickle cells for targeted delivery to hypoxic tumors. *J Control Release*. 2013;171:258–259.

[8] Terman DS, Viglianti BL, Zennadi R, et al. Sickle erythrocytes target cytotoxics to hypoxic tumor microvessels and potentiate a tumoricidal response. *PLoS One*. 2013;8(1):e52543. doi:10.1371/journal.pone.0052543

[9] Choe S, Terman DS, Rivers AE, Rivera J, Lottenberg R, Sorg BS. Drug-loaded sickle cells programmed ex vivo for delayed hemolysis target hypoxic tumor microvessels and augment tumor drug delivery. *J Control Release*. 2013;171:184–192.

[10] Sun CW, Willmon C, Wu L-C, et al. Sickle cells abolish melanoma tumorigenesis in hemoglobin S knockin mice and augment the tumoricidal effect of oncolytic virus in vivo. *Front Oncol*. 2016;6:166. doi:0.3389/fonc.2016.00166

[11] Biagiotti S, Paoletti MF, Fraternale A, Rossi L, Magnani M. Drug delivery by red blood cells. *IUBMB Life*. 2011;6:621–631.

[12] Godfrin Y, et al. International seminar on the red blood cells as vehicles for drugs. *Expert Opin Biol Ther*. 2012;12:127–133.

[13] Grosveld F, van AG, Greaves DR, Kollias G. Position-independent, high-level expression of the human beta-globin gene in transgenic mice. *Cell*. 1987;51:975–985.

[14] Behringer RR, Ryan TM, Reilly MP, et al. Synthesis of functional human hemoglobin in transgenic mice. *Science*. 1989;245:971–973.

[15] Forrester W, Novak U, Gelinas R, Groudine M. Molecular analysis of the human beta-globin locus activation region. *Proc Natl Acad Sci USA*. 1989;86:5439–5443.

[16] Ryan TM, R.R. Behringer RR, Martin NC, Townes TM, Palmiter RD, Brinster RL. A single erythroid-specific DNase I super-hypersensitive site activates high levels of human beta-globin gene expression in transgenic mice. *Genes Dev*. 1989;3:314–323.

[17] Pawliuk R, Westerman KA, Fabry ME, et al. Correction of sickle cell disease in transgenic mouse models by gene therapy. *Science*. 2001;294:2368–2371.

[18] Levasseur DN, Ryan TM, Pawlik KM, Townes TM. Correction of a mouse model of sickle cell disease: lentiviral/antisickling beta-globin gene transduction of unmobilized, purified hematopoietic stem cells. *Blood*. 2003;102:4312–4319.

[19] Puthenveetil G, Scholes J, Carbonell D, et al. Successful correction of the human beta-thalassemia major phenotype using a lentiviral vector. *Blood*. 2004;104:3445–3453.

[20] Urbinati F, Hargrove PW, Geiger S, et al. Potentially therapeutic levels of anti-sickling globin gene expression following lentivirus-mediated gene transfer in sickle cell disease bone marrow CD34+ cells. *Exp Hematol*. 2015;43:346–351. doi:10.1016/j.exphem.2015.01.009

[21] Lisowski L, Sadelain M. Locus control region elements HS1 and HS4 enhance the therapeutic efficacy of globin gene transfer in -thalassemic mice. *Blood*. 2007;110:4175–4178.

[22] May C, Rivella S, Callegari J, et al. Therapeutic haemoglobin synthesis in [beta]-thalassaemic mice expressing lentivirus-encoded human [beta]-globin. *Nature*. 2000;406:82–86.

[23] Jarraud S, Peyrat MA, Annick Lim A, et al. *egc*, A highly prevalent operon of enterotoxin gene, forms a putative nursery of superantigens in *Staphylococcus aureus*. *J Immunol*. 2001;166:669–677.

[24] Terman DS, Bohach G, Vandenesch F, Etienne J, Lina G, Sahn SA. Staphylococcal superantigens of the enterotoxin gene cluster (EGC) for treatment of stage IIIb non-small cell lung cancer with pleural effusion. *Clin Chest Med*. 2006;27:321–334.

[25] Terman DS, Serier A, Dauwalder O, et al. Staphylococcal entertotoxins of the enterotoxin gene cluster (egcSEs) induce nitrous oxide- and cytokine dependent tumor cell apoptosis in a broad panel of human tumor cells. *Front Cell Infect Microbiol*. 2013;3:38. doi:10.3389/fcimb.2013.00038

[26] Ren S, Terman DS, Bohach G, et al. Intrapleural staphylococcal superantigen induces resolution of malignant pleural effusions and a

survival benefit in non-small cell lung cancer. *Chest*. 2004;126:1529–1539.

[27] Voskoboinik I, Whisstock JC, Trapani JA. Perforin and granzymes: function, dysfunction and human pathology. *Nat Rev Immunol*. 2015;15:389–400.

[28] Voskoboinik I, Dunstone MA, Baran K, Whisstock JC, Trapani JA. Perforin: structure, function, and role in human immunopathology. *Immunol Rev*. 2010;235(1):35–54. doi:10.1111/j.0105-2896.2010.00896.x.

[29] Holst J, Szymczak-Workman AL, Vignali KM, Burton AR, Workman CJ, Vignali DA. Generation of T-cell receptor retrogenic mice. *Nat Protoc*. 2006;1:406–417.

[30] Miethke T, Duschek K, Wahl C, Heeg K, Wagner H. Pathogenesis of the toxic shock syndrome: T cell mediated lethal shock caused by the superantigen TSST-I Eur. *Eur J Immunol*. 1993;23:1494–1500.

[31] Marrack P, Blackman M, Kushnir E, Kappler J. The toxicity of staphylococcal enterotoxin B in mice is mediated by T cells. *J Exp Med*. 1990;171:455–446.

[32] McCormack JE, Callahan JE, Kappler J, Marrack PC. Profound deletion of mature T cells in vivo by chronic exposure to exogenous superantigens. *J Immunol*. 1993;150:3785–3794.

[33] Behringer RR, Hammer RE, Brinster RL, Palmiter RD, Townes TM. Two 3′ sequences direct adult erythroid-specific expression of human beta-globin genes in transgenie mice. *Proc Natl Acad Sci*. 1987;84:7056–7060.

[34] Spaeny-Dekking EH, Kamp AM, Froelich CJ, Hack CE. Extracellular granzyme A, complexed to proteoglycans, is protected against inactivation by protease inhibitors. *Blood*. 2000;95:1465–1471.

[35] Chang AH, Stephan MT, Sadelain M. Stem cell–derived erythroid cells mediate long-term systemic protein delivery. *Nat Biotechnol*. 2006;24:1017–1021.

[36] Zheng J, Umikawa M, Zhang S, et al. Ex vivo expanded hematopoietic stem cells overcome the MHC barrier in allogeneic transplantation. *Cell Stem Cell*. 2011;9:119–130.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

How to cite this article: Sun C-W, Wu L-C, Knopick PL, Bradley DS, Townes T, Terman DS. Sickle cells produce functional immune modulators and cytotoxics. *Am J Hematol*. 2017;92:981–988. https://doi.org/10.1002/ajh.24836