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A rational mouse model to detect on-target off-tumor CAR T cell toxicity

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ABSTRACT

Off-tumor targeting of human antigens is difficult to predict in preclinical animal studies and can lead to serious adverse effects in patients. To address this, we developed a mouse model with stable and tunable human HER2 (hHER2) expression on normal hepatic tissue and compared toxicity between affinity-tuned HER2 CAR T cells (CARTs). In mice with hHER2-high livers, both the high-affinity (HA) and low-affinity (LA) CARTs caused lethal liver damage due to immunotoxicity. Mice with hHER2-low livers, LA-CARTs exhibited less liver damage and lower systemic levels of IFN-γ than HA-CARTs. We then compared affinity-tuned CARTs for their ability to control a hHER2-positive tumor xenograft in our model. Surprisingly, the LA-CARTs outperformed the HA-CARTs with superior antitumor efficacy in vivo. We hypothesized that this was due in part to T cell trafficking differences between LA and HA-CARTs and found that the LA-CARTs migrated out of the liver and infiltrated the tumor sooner than the HA-CARTs. These findings highlight the importance of T cell targeting in reducing toxicity of normal tissue and also in preventing off-tumor sequestration of CARTs, which reduces their therapeutic potency. Our model may be useful to evaluate various CARTs that have conditional expression of more than one scFv.
INTRODUCTION

Chimeric antigen receptor (CAR)-modified T cell therapy has emerged as an effective treatment for blood malignancies and is currently being developed to treat solid tumor cancers. A key aspect of the anti-tumor effectiveness of CAR T cell therapy is its ability to target and kill malignant cells by recognizing cell surface antigens. CAR T cell therapy can result in severe adverse effects due to on-target, off-tumor toxicity, which arises in patients that have target antigen expressed on both tumor and healthy tissue. This expression pattern is typical for the vast majority of target antigens used in CAR T cell therapies. One such antigen is HER2, which is an attractive target for CAR T cell therapy since it can be overexpressed 40-100-fold in tumors and thus, has long been targeted therapeutically using monoclonal antibodies such as trastuzumab (Herceptin) (1). A HER2 CAR T cell therapy based on the trastuzumab sequence was used to treat a patient with colorectal cancer, but unfortunately off-tumor targeting of their cardiopulmonary system caused lethal toxicity (2). This adverse effect was not foreseen based on clinical studies of trastuzumab or in preclinical animal experiments.

Current FDA guidelines for preclinical animal testing of cellular therapies require the use of a relevant animal model capable of eliciting a biological response that would reasonably predict the expected human response (3). In many cases, animal models used for pharmacological testing of therapeutic human CAR T cells cannot adhere to this standard due to variability in cross-species reactivity to non-human target antigens. Therefore, preclinical animal studies have little chance of identifying potential adverse events in humans and often offer a false sense of safety (4). This limitation highlights the need for an animal model that expresses human targets in normal tissue to better predict off-tumor toxicity and improve the safety profile of immune therapies.

On-target, off-tumor toxicity can be reduced by using CAR targeting systems that can improve the recognition of tumor cells (5). One such strategy is to affinity-tune CARs so they detect tumor cells with a high density of surface antigens and do not react against normal cells that have low antigen
densities (6). Recently, a low affinity CD19 CAR T cell therapy was reported to exhibit promising therapeutic outcomes in patients with mostly low burden relapsed/refractory pediatric B cell acute lymphoblastic leukemia (7). The activity of CAR T cells is dependent on the strength and duration of activation signals transmitted through the antigen receptor (8, 9) and by the antigen density on target cells (10, 11). Genetically tuning the affinity of a CAR can be done by altering the scFv binding region via mutagenesis (12) or by recombining heavy and light chains (13). Recent in vivo studies have used tumors as surrogates for normal tissue to compare off-tumor toxicity between affinity-tuned CAR T cells (11, 14). Lower affinity CAR T cells demonstrated improved safety by bypassing tumors with low antigen expression while maintaining recognition and elimination of tumors with high antigen expression. However, neither of these mouse models had human antigen expression on normal mouse tissue and therefore on-target, off-tumor toxicity was not measured directly. Our first aim was to develop a mouse model that contains human antigen targets on both tumor and normal tissue. Our second aim was to assess the therapeutic index of affinity-tuned CAR T cells. Our study showed that the lower affinity CAR T cells resulted in less off-tumor toxicity and improved tumor control. We concluded that this model is an effective preclinical tool to assess the consequences of tumor recognition by CAR T cells permitting improved prediction of the safety and efficacy of CAR T cells than conventional tumor xenografts.
RESULTS

Human tumor antigens can be expressed in murine livers using AAV8 gene delivery

Human T cells respond differently than mouse T cells, thus most preclinical studies use human CAR T cells in immune-deficient mice (e.g. NSG mice) (15). The antigen recognition portion of CARs is commonly derived from monoclonal antibodies that are highly specific for their cognate antigen but are usually not species cross-reactive between mice and humans. The absence of human targets in mice poses a problem for testing on-target, off-tumor toxicity of CAR T cells in preclinical mouse models. To address this limitation, we genetically introduced human tumor antigen targets into mouse livers using two different methods of gene transfer.

One method of gene transfer used adeno-associated virus serotype 8 (AAV8) that contained a truncated human HER2 gene (hHER2) and a fluorescent reporter (Katushka), which was then transduced into murine hepatocytes by intravenous tail vein injection (Figure 1A). The expression of the fluorescent reporter in murine hepatocytes was detected by ex vivo imaging of livers, which showed greater fluorescent intensity in the livers of mice that received a higher number of genomic copies (GCs) of AAV8 (Figure 1B). Similarly, the expression of the human HER2 antigen was detected by IHC and showed widespread expression with a more pronounced perivascular staining pattern (Figure 1C). HER2 staining of hepatocytes was quantified, and we observed a positive correlation with the number of GCs of AAV8 and the percentage of positive hepatocytes, thus demonstrating that the level of expression can be regulated through AAV8 dosing (Figure 1D). Our results confirm and extend previous in vivo studies that showed transgene expression levels in hepatocytes directly correlate with AAV8 dosage (16).
**Transgene transposition by the piggyBac transposon system can create stable human antigen expression in murine livers**

We next wanted to explore the suitability of transgene transposition using a transposon system delivered to hepatocytes in vivo by hydrodynamic injection. The advantages to this system are that it requires no virus production, generates no viral antigens, has a large cargo delivery size that could accommodate multiple antigen transgenes and genetically integrates the transgene into the cell genome for stable expression. The piggyBac (PB) and Sleeping Beauty (SB) transposons are movable genetic elements that can efficiently transpose vector DNA into mammalian genomes through a “cut-and-paste” mechanism that is effective in genetically modifying cells in vivo (Figure 1E adapted from (17)). Although either the PB or SB system could be used in our model, we chose the piggyBac system because it allows for a larger transgene cargo size (18, 19). We replaced the Katushka reporter from our AAV8 construct with the IRFP720 protein since near-infrared fluorescent proteins are preferable for deep-tissue imaging (20). Injection of non-transposon plasmid DNA resulted in transient episomal expression that was ill-suited for prolonged mouse toxicity studies (Supplemental Figure 1). To test whether stable transgene expression could be achieved via the transposon system, mice were injected with 10ug of the fluorescent reporter plasmid DNA pPB7-IRFP720 either with or without 10ug of the piggyBac transposase plasmid DNA pCMV-HypPBase (Figure 1E). The murine livers were harvested 8 weeks post-injection and IRFP720 expression was measured by IVIS imaging, which showed fluorescence in the mice that was dependent on injection with the piggyBac transposase plasmids (Figure 1F). One liver from each group was enzymatically digested and dissociated hepatocytes were isolated and then analyzed by flow cytometry to detect fluorescence in individual hepatocytes (Figure 1G). We observed more IRFP720 expression in the mouse that received both the transposon and transposase plasmids versus the control mouse that only received the transposon plasmid, as evidenced by the proportion of positive
cells (20% versus 2%, respectively) and by the geometric mean fluorescent intensity (MFI of 242 vs 124, respectively).

Next, we performed a longitudinal study of the piggyBac gene editing system to evaluate the long-term kinetics of transgene expression (Figure 2A). The versatility of this system allowed us to customize the pattern of hepatic protein expression by using different combinations and concentrations of plasmids. We created three groups of mice that differed in their hydrodynamic tail vein injections of human HER2 transposon DNA, luciferase transposon DNA and piggyBac transposase DNA to ask if we could maintain stable expression of the transgenes at various levels. Group 1 received injections of luciferase transposon DNA and human HER2 transposon DNA but without transposase DNA. Group 2 received injections that combined luciferase transposon DNA, human HER2 transposon DNA and the piggyBac transposase DNA. Group 3 received the same plasmid combination as group 2 but at 1/10th the DNA concentration.

Human HER2 mRNA expression from the harvested murine livers was evaluated by real-time PCR using Taqman assays for human HER2, which was normalized to liver RNA using the mouse housekeeping gene, HPRT (Figure 2B). Relative expression is shown as $2^{-\Delta Ct}$, where $\Delta Ct$ is calculated as Ct HPRT minus Ct HER2, and was calculated for the mice that received all three of the plasmids (i.e., groups 2 and 3) and also for control mice that did not receive human HER2 transposon DNA, which were used to establish background Ct levels. This showed that a higher level of HER2 expression was achieved in the mice from Group 2 versus mice from Group 3 and thus confirmed that we could regulate antigen levels by the amount of transfected DNA, which is consistent with previous studies (18). We observed a positive correlation in the livers between luciferase radiance and HER2 mRNA expression suggesting that the HER2 and the luciferase transposon plasmids were transfected with equal efficiency and thus luciferase measurements were indicative of HER2 antigen levels (Figure 2C).
Weekly IVIS imaging of the murine liver was measured to determine longitudinal luciferase expression. This imaging revealed that Group 1 mice initially had the highest BLI levels (i.e. high concentration of the transposon DNA but no transposase DNA) but then bioluminescence was found to decrease dramatically during the first two weeks and then continued to decline during the following 6 months (Figure 2, D and E). The mice from groups 2 and 3, which received the additional transposase plasmid, showed a luciferase expression pattern that decreased markedly in the first week but then increased steadily before stabilizing approximately one month later (Figure 2, D and E). A comparison of groups 2 and 3 showed the group that received the higher concentration of DNA had a higher final average bioluminescent signal (i.e. transgene expression), which was consistent with the previous human HER2 mRNA expression results. Our findings demonstrate that either the piggyBac transposon system or AAV8 can be used to effectively obtain stable human antigen expression in the mouse liver and at predetermined levels.

**Human antigens in the murine liver can be targeted by CAR T cells**

We next tested whether the human HER2 antigen that was expressed in the mouse livers could provoke on-target toxicity following infusion of anti-HER2-CAR T cells. HER2 CAR T cells with two different scFvs (4D5, high-affinity; 4D5-5, low-affinity) were IV infused into mice that had hepatic expression of human HER2 antigen, which was established using our piggyBac transfection method. To detect a HER2-independent T cell response in the livers, we included one negative control group that expressed HER2 but received untransduced T cells and a second negative control group that received HER2-CAR T cells but in HER2-negative mice. The presence of CAR T cells in the murine livers were measured one week after injection using a CAR DNA Taqman assay, which was normalized against a mouse PTGER2 Taqman assay. HER2-CAR T cell (HA or LA) DNA was elevated only in mice that had human HER2 expression (Figure 3A). The mice that had CAR T cells present in their livers also had elevated hepatic expression of human IFN-γ mRNA, as measured using a Taqman assay normalized
against mouse HPRT mRNA (Figure 3B). The serum cytokine levels in the four groups revealed that only mice that were injected with HER2-CAR T cells and expressed hepatic human HER2 antigen had elevated human cytokines associated with T cell activation (i.e. IFN-γ, GM-CSF, IL-2, IL-5 and MIP-1b), which suggests antigen-dependent activation and no response to hydrodynamic injection (Figure 3C). Serum levels of IFN-γ and GM-CSF were significantly higher in the mice that received the high versus low affinity CAR T cells, consistent with increased activation. To demonstrate that hepatic antigen expression delivered by AAV8 could also elicit an antigen-specific immune response, mice were injected with HER2-AAV8 and then infused with luciferase-expressing HER2 CAR T cells (Supplemental Figure 2). Mouse livers were analyzed by bioluminescent imaging and IHC, which showed an infiltration of HER2 CAR T cells. These results confirmed that the human antigen we delivered to the liver can act as a target for CAR T cells and that presence of this human antigen was sufficient for CAR T cell infiltration and activation. We concluded that either AAV8 or hydrodynamic tail vein injection of transposon DNA were suitable methods for promoting an antigen-dependent immune response in hepatocytes.

**Off-tumor toxicity can be reduced using a lower affinity CAR T cell**

The amount of toxicity in HER2-expressing livers was compared between mice that received a high-affinity versus a low-affinity CAR T cell (4D5 and 4D5-5 scFv, respectively). We postulated that the high-affinity CAR would cause more liver damage than its low-affinity counterpart when hepatic HER2 expression was low since the HER2 levels would be below the limit of detection for the low-affinity CAR T cells. We also hypothesized that when HER2 expression in the murine livers was high, hepatocytes would be recognized by both the high and low-affinity CAR T cells and thus the degree of liver damage would be equivalent. To test our assumption, we injected one group of mice with a high dose of HER2-AAV8 to create livers in mice with high antigen levels while another group of mice received a comparatively low dose of HER2-AAV8 to generate low antigen livers (Figure 4A). To determine if the AAV8 viral antigens alone would elicit T cell-mediated toxicity, a HER2-negative control group received a
high dose of AAV8 that expressed GFP instead of HER2 and then these mice were injected with high- 
affinity HER2-CAR T cells. As predicted, there was severe toxicity in mice with high antigen levels due to 
both the high and low-affinity CAR T cells as observed by markedly increased mortality (Figure 4B), and 
post-mortem analysis of liver pathology (Supplemental Table 1). In addition to liver damage, cytokine 
release as shown in Figure 3C may have also contributed to morbidity in these mice. In contrast, the 
high-affinity CAR T cells were nonlethal in mice that lacked hepatic HER2 expression, excluding 
xenoreactivity as a cause of morbidity (Figure 4B).

Toxicity was decreased in mice with low antigen levels as seen by reduced mortality (Figure 4C), 
and liver pathology (Supplemental Table 1). The low antigen mice had no significant difference in 
mortality between affinity-tuned CAR T cells (Figure 4C), but more liver damage was caused by the high 
versus low-affinity CAR T cells, according to elevated serum ALT levels (Figure 4D). The mean ALT from 
the mice that received high affinity CAR Ts is 84 U/L with a range of 64 to 118 U/L versus the negative 
control mice with a mean ALT of 46 U/L and a range of 34 to 66 U/L. The resulting fold change for the 
high affinity CART group is about double the normal values, which would be considered serious in 
humans. According to Hy’s law, as a rule of thumb, drug induced hepatocellular injury that is three 
times or greater above the upper limit of normal presents a high risk of fatal drug-induced liver injury. 
Toxicity effects were also apparent by mouse weight loss, which was profound in the high antigen 
groups that received either the low or high-affinity CAR T cells and also occurred in the low antigen mice 
that were infused with the high but not low-affinity CAR T cells (Figure 4E). Differences in abundance 
between the high and low-affinity CAR T cells were assessed by bioluminescence imaging (BLI) of their 
luciferase reporter gene (Figure 4F). In the HER2-negative control mice (i.e. AAV8-GFP group), the 
number of high-affinity HER2 CAR T cells remained constant during the first 2 weeks after T cell injection 
indicating an absence of antigen-dependent activation (Figure 4F). In the low antigen mice, we initially 
observed an increase in abundance via luminescence for both the high and low-affinity CAR T cells
followed by a decrease after 4 days by the low-affinity CAR T cells. In contrast, the high-affinity CAR T cells continued to increase until day 8 and remained higher than the low-affinity CAR T cells until dropping to equivalent values by day 22. This suggests that the high-affinity CAR T cells remained activated longer due to prolonged antigen recognition. All three groups had similar upward trends in T cell abundance starting at day 22, which was presumably due to xenogeneic GVHD. Overall, the low-affinity CARs were better able to distinguish between low and high-antigen density tissues in our safety model.

**An increase in off-tumor targeting is associated with a delay in tumor CAR T infiltration and a decrease in anti-tumor efficacy**

We next wanted to test our affinity-tuned CAR T cell treatments for their tumor control in a mouse model that simulated a common clinical scenario, in which antigen is overexpressed in a patient’s tumor but is also found at lower levels in some of their healthy tissue. To accomplish this, mice were engrafted with a high HER2-expressing tumor xenograft and additionally injected with a low dose of HER2-AAV8 to produce a low HER2-expressing liver (Figure 5A). The mice were then infused with either high or low-affinity CAR T cells. Surprisingly, the mice that received the low-affinity CAR T cells showed significantly better anti-tumor efficacy than the ones that were treated with the high-affinity CAR T cells (Figure 5, B and C). Tumor size was visualized by the expression of the fluorescent reporter IRFP720, which was congruent with our caliper measurements (Figure 5C and Supplemental Figure 3).

To investigate whether differences in tumor control between the two groups were due to differences in T cell abundance and/or trafficking, we observed luciferase-expressing T cells using in vivo imaging (Figure 6A). Slight differences in overall abundance between the high and low-affinity CAR T cells were seen by whole body BLI measurements in the first week and then became comparable throughout the rest of the experiment (Figure 6B). Migratory behavior between the T cell treatment
groups was similar at day 1, in which the high and low-affinity CAR T cells were both seen in the liver while the T cells in the control group were observed in the spleen (Figure 6C). However, by day 8 there were striking differences in the trafficking of the CAR T cells as the high-affinity CAR T cells remained in the mouse livers whereas the low-affinity CAR T cells had emigrated from the liver and homed to the tumor. By day 12, the high-affinity CAR T cells had left the liver and infiltrated the tumor, while the low-affinity CAR T cells had already caused measurable tumor regression. By day 43, the low-affinity CAR T cell group had no measurable tumors by either fluorescent reporter expression or caliper readings (Figure 5, B and C). Conversely, the high-affinity CAR T cell group at day 43 had detectable tumor in 4 of the 6 mice and less tumor infiltration of CAR T cells in those tumors compared to earlier timepoints. In tumor-bearing mice without hepatic antigen expression, no difference between affinity-tuned CAR T cells was observed in trafficking (Supplemental Figure 4) or tumor control (11). Thus, in our mouse model the low-affinity CAR T cells were better able to discriminate between low-antigen healthy tissue and high-antigen tumor tissue, which resulted in a better therapeutic outcome.
DISCUSSION

On-target, off-tumor toxicities from CAR T cell therapies can have serious clinical consequences that are difficult to predict in current animal models because of the specificity of therapeutic CARs for human antigens. Our study showed that the improved targeting by the low-affinity CAR T cells resulted in less off-tumor toxicity and improved tumor control than the high-affinity CAR T cells. However, the lower-affinity CAR T cells would be less effective at controlling tumors with low antigen expression than the higher-affinity CAR T cells. This was seen clinically with a low-affinity Herceptin-based CAR, which showed a safer profile in sarcoma patients but had only modest clinical activity (21). This is an unavoidable consequence of enhancing the tumor specificity of CAR T cells via its antigen receptor. We concluded that our current off-tumor model is an effective preclinical tool to assess tumor recognition by CAR T cells that better predicts the therapeutic index of CAR T cells compared to tumor xenografts alone.

The liver is a suitable organ for testing CAR T cell toxicity because it tolerates damage well and it is well perfused. Hepatocytes have the added benefit of providing easy access to antigen since they lack basement membranes (22). In addition, hepatocytes are convenient cells to express human antigens as they have a high tropism for AAV8 (23) and they can be transfected in vivo by hydrodynamic gene delivery (24). Both methods for gene delivery were used in our study with equal effect although there are advantages and disadvantages to each method. Liver-targeted gene transfer in mice using AAV8 has the advantages of being highly efficient for both dividing and nondividing cells, it is technically easy to administer via intravenous or intraperitoneal injections, provides stable expression in adult mice, and has minimal toxicity and immunogenicity (25). However, limitations to using AAV8 include their small packaging capacity (<5kb), the dilution of transgene expression in replicating cells, and the cost and time of viral production. In comparison, the hydrodynamics-based method coupled with the piggyBac transposon system allows for efficient gene transfer, has a large packaging capacity (>200kb), forms
genomic integration resulting in stable expression, and the transgene is delivered by naked plasmid DNA so it does not introduce viral antigens or require virus production (26). The disadvantage to using the hydrodynamics-based method is the increased technical difficulty and physiological stress on the mouse than standard intravenous injections (27).

Immune tolerance is an important function of the liver to prevent an autoimmune response to food-derived and microbial antigens from the digestive system. As the high-affinity CAR T cells spent more time in the livers compared to the low-affinity CAR T cells, they had longer exposure to the liver microenvironment, which in addition to sequestration, could potentially explain their decreased tumor clearing capacity. Hepatic immune suppression has been extensively reported in various animal models, including humans (28-30). Mechanistically, hepatic tolerization has been attributed to inhibitory ligand expression and immunosuppressive cells (31), T-cell exhaustion (32, 33), elimination of CD8+ T by suicidal emperipolesis (34) and elimination of CD4+ T cells by enclysis (35). Our mouse model was devised to study off-tumor toxicity of human CAR T cells, but it may also serve as a useful tool to elucidate the mechanisms of liver tolerance.

Immune tolerance of autoreactive T cells is not unique to the liver and has been observed to a lesser extent in other organs such as the skin, the lung (36), immune privileged sites (37) and the gastrointestinal tract (38). It is unknown whether these or other tissues may contribute to on-target, off-tumor tolerance in immune cell therapies. At the very least, off-tumor targeting by immune cell therapies can cause immune cells to be sequestered and delayed from reaching their intended tumor targets, as was demonstrated in this study. This effect is in addition to the toxicity that on-target, off-tumor targeting can cause to healthy tissue. Taken together, these effects highlight the need to reduce off-tumor targeting by developing better targeting strategies for CAR T cells (5) or by modifying the route of CAR T cell delivery to avoid damage to healthy tissue (39).
In summary, we have developed a mouse model to test on-target, off-tumor toxicity of CAR T cells that recognize human antigens in normal murine livers. Another use of our model would be to test various forms of CAR T cells that have conditional expression of two CAR molecules (40, 41), as our model would permit the assessment of on- and off-switching kinetics. In future experiments, gene editing of the liver can be used in conjunction with antigen gene delivery to further manipulate target hepatocytes and study effector cell interactions (42, 43).
METHODS

Cell lines, primary human lymphocytes and primary mouse hepatocytes

The cell lines SKOV3 and HEK293T were obtained from ATCC and tumor cell lines were regularly validated to be Mycoplasma free. SKOV3 cells were authenticated by autosomal DNA profiling in 2018 by the University of Arizona Genetics Core (Tucson, Arizona, USA). The University of Pennsylvania Human Immunology Core (Philadelphia, PA) provided the human primary CD3+ T cells from healthy donors. Primary lymphocytes were stimulated with Dynabeads coated with CD3 and CD28 stimulatory antibodies (Life Technologies) at a 1:3 cell to bead ratio as previously described (44).

Expanded T cells were cryopreserved on day 10 following activation in a solution of 90% FCS and 10% DMSO. Cells were cultured in R10 (RPMI 1640 media supplemented with 10% FCS, 100-U/ml penicillin, 100 µg/ml streptomycin sulfate, 10 mM Heps; Invitrogen) in a 37°C and 5% CO2 incubator. Cells were transduced with lentiviral vectors containing CAR constructs approximately 24 hours following stimulation at a MOI of 3.

Primary mouse hepatocytes were dissociated following the non-perfusion method described previously (45) except that enzymatic digestion was done using the Liver Dissociation Kit (MACS Miltenyi Biotec) and liver homogenates were treated with the Debris Removal Solution (MACS Miltenyi Biotec) instead of Percoll. Hepatocytes were analyzed immediately by flow cytometry or cryopreserved in a solution of 90% FCS and 10% DMSO.

HER2 scFVs

We have previously described the HER2 CARs (11). The parental scFv has a high affinity with $K_D = 0.58\text{nM}$, $K_a = 2.95\times10^5$ and $K_d = 1.71\times10^{-4}$. The low affinity scFv has a $K_D$ of 1119nM and dissociation rate constant ($K_a$) that is too rapid to measure.
Virus production

AAV8

The AAV8 vector used to construct pENN.AAV.TBG.HER2/Neu-T2A-Katushka and a HER2-negative control AAV8 vector AAV8.TBG.PI.eGFP.WPRE.bGH (catalog # AV-8-PV0146) were obtained from the Penn Vector Core. Customized AVV8 vector was manufactured by the Penn Vector Core. Supplemental Figure 5, A and B shows schematic diagrams of the AAV8 vectors used in this study.

Lentivirus

DNA for the low or high-affinity scFv (4D5-5 and 4D5, respectively) were linked to the CD8 transmembrane domain and 4-1BB and CD3 zeta intracellular signaling domains. These were subcloned into pTRPE lentiviral vectors that co-expressed the click-beetle-red (CBR) luciferase gene. A CAR-negative lentiviral vector was used as a negative control and contained CBR and GFP. Supplemental Figure 5, C, D and E shows schematic diagrams of the lentiviral vectors used in this study. The plasmids were transformed into Stbl3 chemically competent cells (Life Technologies) and plasmid DNA was isolated using a PureLink HiPure Plasmid Maxiprep kit (Life Technologies). Lentivirus was produced as described previously (46).

Flow cytometric analysis

Data were collected on either a LSRFortessa or LSR II (BD Biosciences) and analyzed using FlowJo software (FlowJo LLC). Cell suspensions were stained with a fixable live/dead violet stain (L34955, Invitrogen) in PBS prior to surface antibody staining in FACS buffer. T cells were stained using the antibodies for anti-human CD4-BV510 (317444, Biolegend), anti-human CD8-APC (17-0086-42, eBioscience). CAR positivity was assessed using either recombinant ErbB2/HER2-Fc Chimera (1129-ER-050, R&D Systems) with anti-Human IgG-Fc-DyLight® 650 (ab98622, Abcam) or with biotinylated
recombinant protein L (29997, Thermo Fisher) and anti-biotin-PE (12-9895-82, eBioscience). Primary mouse hepatocytes were gated using the gating schema described previously (47) and fluorescence from IRFP720 protein was detected in the AF100 spectrum (Supplemental Figure 6).

In vivo xenograft and gene transfer mouse studies

Immunodeficient NOD/scid/Ii2ry−/− (NSG) mice were purchased from The Jackson Laboratory and then bred, housed and handled at the University of Pennsylvania in pathogen-free conditions according to institutional guidelines. In vivo gene transfer of hepatocytes was performed using either tail vein injections of AAV8 or hydrodynamic tail vein injections of DNA. Mice received tail vein injections of AAV8 at the genomic copies shown in 200ul of PBS. For hydrodynamic tail vein transfections of hepatocytes, mice received 1.5ml of PBS in under 10 seconds with the amount of DNA shown in each figure as described previously (48). Supplemental Figure 7 shows schematic diagrams of the piggyBac vectors used in this study, which include the piggyBac transposase vector, pCMV-hyPBase that was obtained from the Wellcome Trust Sanger Institute. (49) and the piggyBAC transposon plasmid, PB007 SPB-007 that was obtained from Transposagen.

To establish xenografts of SKOV3 cells that expressed the fluorescent reporter IRFP720, adult male or female NSG mice were subcutaneously injected while under anesthesia with 5 x 10^6 SKOV3 cells resuspended in Matrigel (Corning) and diluted with equal parts PBS. Fluorescent imaging of the IRFP720 reporter gene in SKOV3 cells was done on an IVIS Spectrum imaging system (PerkinElmer). Bioluminescent imaging of T cells that expressed the click-beetle-red luciferase reporter gene was performed 10 minutes after IP injection of 123mg/kg D-Lucifer (PerkinElmer) on an IVIS Spectrum imaging system (PerkinElmer). Mice were euthanized if the tumor volume exceeded 4.2 cm^3, if tumor ulceration exceeded 25% of the surface area of the tumor, or when they became moribund. Tumor
The volumes were calculated from caliper measurements using the formula \( V = \frac{1}{2} \times L \times W \times W \), where \( L \) is length and \( W \) is width.

**Serum levels of cytokines and liver enzymes**

Mouse peripheral blood was collected either by retro-orbital bleeding or by intracardiac puncture at the time of euthanasia. Blood samples were left to clot, centrifuged and then the resulting serum was collected and stored at \(-80^\circ\text{C}\). Serum levels of mouse ALT were measured by ELISA at the University of Pennsylvania’s Veterinary Hospital, and human cytokines were measured on the Luminex panel (HSTCMAG28SPMX21, Millipore), which was performed by the University of Pennsylvania’s Human Immunology Core.

**Nucleic Acid isolation and quantitative PCR analysis**

For nucleic acid isolation, fresh frozen liver samples were thawed and immediately homogenized using Lysing Matrix D beads in a Fastprep homogenizer. DNA and RNA was isolated from the liver homogenate using an AllPrep DNA/RNA/Protein Mini kit (Qiagen) and quantified by NanoDrop.

For quantitative PCR (qPCR) analysis, RNA was treated with exonuclease and DNAse to remove genomic DNA, plasmid DNA or AAV8 DNA from the liver samples prior to cDNA synthesis. cDNA was generated from RNA using a High Capacity first-strand synthesis kit (Applied Biosystems). Either CAR T cell cDNA or genomic DNA were detected using a custom Taqman assay with the following primer and probe set: Forward primer, 5’- CCAGAAGAGAGAAGGAGATG - 3’; Reverse primer, 5’- GCTCGTTAGAGCTGGTCT -3’; probe, 5’- FAM-TGAGGTAAGGTCCAGGAGCG -3’. Reverse transcriptase negative controls were used for quantification of cDNA and only those samples that were negative were used in our analysis. The ABI gene expression assays for human IFN-\(\gamma\), Hs00989291, human Erb2/HER2, Hs01001580, human HPRT1, Hs99999909, and mouse HPRT, Mm00446968 were used. HPRT was used to normalize gene expression since it was found to be consistently expressed in
the liver in the setting of inflammation and tissue repair (50, 51). DNA content was normalized against PTGER gDNA as described previously (52). All qPCR assays were performed using a ViiA 7 realtime PCR system (Applied Biosystems). Each assay was done twice in duplicate and \( \Delta Ct \) was calculated by \( \Delta Ct_{\text{sample}} - \Delta Ct_{\text{reference}} \) (53).

**Immunohistochemistry**

Liver and tumor tissues were formalin-fixed and then sent to the University of Pennsylvania’s Cancer Imaging Core for paraffin embedding and sectioning. Slides were immunostained with anti-HER2 (AMAb90627, Sigma) or anti-CD8 (RB9009-PO, Thermo Fisher) antibodies and counterstained with Hematoxylin, which was performed by the Pathology Core Laboratory at The Children’s Hospital of Philadelphia. For stain quantification, slides were digitally scanned using an Aperio Scanscope and analyzed using Aperio ImageScope software (Release 6).

**Statistics**

All growth curves, mean fluorescence intensity, and engraftment plots were plotted using Prism 7 (GraphPad Software). For comparisons of 2 groups, 2-tailed unpaired t tests were used. One-way ANOVA with Tukey post hoc test was used for comparison of 3 or more groups in a single condition. Statistical analysis for tumor volume and weight change was performed using 2-way repeated-measures ANOVA. Kaplan-Meier survival data were analyzed using a log rank (Mantel-Cox) test. Correlation was estimated by calculation of 2-tailed Pearson coefficients and significance. The statistical test used for each figure is described in the corresponding figure legend. Data was transformed when needed to normalize variance. Symbols (* or +) indicate statistical significance as follows: *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \) and ****\( P < 0.0001 \).
Study approval

Animal studies were carried out by approval of the Institutional Animal Care and Use Committee (IACUC) of the University of Pennsylvania, Philadelphia.

Author Contributions

M.C., J.A.F., and C.H.J. designed experiments. M.C., C.S., T.D., and K.G. performed experiments. M.C., C.S. and E.B. analyzed data. J.S. and Y.Z. provided technical expertise and provided reagents. M.C. wrote the manuscript. C.S., J.S., J.A.F. and C.H.J. edited the manuscript.

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Figure 1. Hepatic gene transfer of human HER2 by AAV8 delivery and transposase gene editing. (A) Design of the AAV8 vector, which includes a truncated human HER2 gene (hHER2) and a fluorescent reporter gene (Katushka) that are expressed by the liver-specific TBG (thyroid hormone-binding globulin) promoter. Mice were IV injected with AAV8 and their livers were harvested 1 month later. (B) IVIS imaging of fluorescence in ex vivo livers harvested from mice that received either no genomic copies (GCs), 1.5e+10 GCs, or 1.5e+12 GCs of AAV8. (C) Immunohistological assessment of human HER2 expression in mice that received either 0, 1.5e+10 or 1.5e+12 GCs of AAV8. Darker HER2 stained cells have a perivascular pattern (black arrow) and are less frequent than the fainter HER2 stained cells (white arrows) (Scale bars, 400 μm and 200 μm). (D) Mean hepatocytes ± SEM were quantified for dark or faint HER2 staining by digitizing the IHC images using ImageScope and then analyzed using Aperio imaging software. Each group contained 4 mice except for 1.5e+12 GC, which had an n of 1. (E) Overview of gene editing using the piggyBac transposase system. The transposon vector contained a fluorescent reporter gene (IRFP720) that was expressed using the liver-specific TBG (thyroid hormone-binding globulin) promoter. (F) Imaging of 6 mouse livers harvested 2 months after injection with the IRFP720 fluorescent reporter plasmid and either with or without the PiggyBac transposase DNA vector. (G) Detection of fluorescent reporter expression by flow cytometry in isolated mouse hepatocytes representative of the livers shown in (F).
Figure 2. Murine expression of human antigen is stable and tunable using piggyBac transposase gene transfer. (A) Design of the luciferase and truncated human HER2 (hHER2) transposon vectors and the transposase vector used in the experiment. Group 1 mice were injected with 5ug of the hHER2 transposon plasmid and 5ug of the luciferase transposon plasmid, but no transposase plasmid. Group 2 mice were injected with 5ug of the hHER2 transposon plasmid, 5ug of the luciferase transposon plasmid, and 10ug of the transposase plasmid, hyPBase. Group 3 mice were injected with the same plasmids as group 2 but at 1/10th the DNA concentration. (B) Comparison of human HER2 mRNA expression between mice that received higher versus lower concentrations of piggyBac transposon and transposase plasmids. Human HER2 RNA was measured in murine livers using real-time PCR and normalized to mouse HPRT expression to calculate 2^ΔCt values. All data are shown as means ± SD (n = 4 – 7 mice per group). A two-tailed Mann-Whitney test of ΔCt values was used for statistical analysis. (C) Comparison of human HER2 DNA content and luciferase expression in the murine livers after hydrodynamic DNA injections (n = 15 mice). (D) In vivo imaging of luciferase expression in mice that either received piggyBac transposon but not transposase plasmid (group 1) or mice injected with either a higher or lower dose of PiggyBac transposon and transposase plasmid (groups 2 and 3 respectively). (E) Mean ± SEM radiance over time with n = 9 mice per group. A two-way repeated measures ANOVA with Tukey’s multiple correction test was used for statistical analysis. Statistical significance for group 2 versus either group 1 (*) or group 3 (+) is denoted as */+P < 0.5, **/++P < 0.01.
Figure 3. CAR T cells recognize cognate human antigen in mice. Human HER2 antigen was expressed in mouse hepatocytes following piggyBac gene transfer. Mice were then injected with $2.5 \times 10^6$ anti-human HER2 CAR T cells (either HA-CAR or LA-CAR) and livers were harvested one week later for analysis. Control groups included mice that had hepatic HER2 expression but untransduced T cells and mice that received HER2 CAR T cells but lacked hepatic HER2 expression due to empty transposon vector transfections. (A) HER2 CAR T cells were detected in mouse livers ($n = 4 - 8$) by performing real-time PCR assays for CAR DNA. $2^{-\text{DCt}}$ values for CAR T cells were calculated using PCR assays that amplify the CAR intracellular signaling domain, 4-1BBz-CD3z and were normalized to mouse PTGER2 genomic DNA content. (B) Expression of human IFN-γ mRNA from T cells was measured in murine livers ($n = 4 - 8$) using real-time PCR and normalized to mouse HPRT expression. Kruskal-Wallis test with Dunn’s multiple comparisons was used for statistical analysis of real-time PCR data. (C) Systemic cytokine release by T cells was detected in mouse serum ($n = 4 - 8$) by Luminex assay. A two-way ANOVA with Tukey’s multiple comparison test was performed and comparisons are shown between all groups and the UTD group (*) or between the HA-CAR and LA-CAR groups (+). Statistical significance is denoted as *P < 0.5, **P < 0.01, ***P < 0.001 ****/****P < 0.0001, ns – not significant.
Figure 4. Legend on next page.
Figure 4. CAR T cells cause lethal on-target, off-tumor toxicity in mice. (A) Overview of the experimental design for comparing on-target, liver toxicity between affinity-tuned HER2 CAR T cells. Two groups of mice received either $1.5 \times 10^{10}$ or $7.5 \times 10^{11}$ GCs of HER2-AAV8 and then were infused with either $5 \times 10^{6}$ high affinity (HA) or low affinity (LA) HER2-CAR T cells. A control group of mice received $4 \times 10^{11}$ GCs of GFP-AAV8 (ie. no HER2) and $5 \times 10^{6}$ HA CAR T cells. An $n = 6$ mice per group is shown in each panel unless stated otherwise. (B) Survival curves of mice that received the $7.5 \times 10^{11}$ GCs of HER2-AAV8 and then CAR T cell injection. Statistical analysis was performed using a log-rank Mantel-Cox test. (C) Survival curves of mice that received the $1.5 \times 10^{10}$ GCs of HER2-AAV8 and then CAR T cell injections. (D) Liver function profile as determined by serum ALT levels collected 25 days post-T cell injection. Mean ALT ± SEM in mice ($n = 4$ - $6$) that received $1.5 \times 10^{10}$ GCs of HER2-AAV8 and either HA-CAR or LA-CAR. A one-tailed unpaired two-sample t-test of ALT was used for statistical analysis. (E) Weight change shown by percent change from initial weight ± SD in mice that received either $7.5 \times 10^{11}$ (dashed lines) or $1.5 \times 10^{10}$ (solid lines) GCs of HER2-AAV8 and then either HA-CAR or LA-CAR. (F) Mean total flux ± SD for whole body bioluminescence imaging (BLI) of T cell luciferase. A two-way repeated measures ANOVA with Bonferroni multiple comparison test was used for statistical analysis of weight change and BLI. Statistical significance for (D-F) is denoted as *$P < 0.5$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$. 
**Figure 5. The low affinity CAR has better tumor control than the high affinity CAR when antigen is also expressed in normal tissue.** (A) Overview of the experimental design for comparing HER2+ tumor control between affinity-tuned HER2 CAR T cells. All mice received $1.5 \times 10^{10}$ GCs of HER2-AAV8 and were implanted with $5 \times 10^{6}$ HER2+ SKOV3 tumor cells, then three groups were injected with either $5 \times 10^{5}$ high affinity (HA) or low affinity (LA) HER2-CAR T cells or no CAR control T cells. The HER2+ tumor cells, SKOV3, were genetically modified to express the fluorescent reporter, IRFP720, for in vivo imaging. Tumor xenograft fluorescence is shown in a yellow to red spectrum. (B) Lateral views of fluorescent tumor imaging. (C) Mean tumor volume ± SEM measured by calipers in $n = 6$ mice per group. A two-way repeated measures ANOVA with Bonferroni multiple comparisons test was used for statistical analysis. Statistical significance is denoted as *$P < 0.5$, ****$P < 0.0001$.
Figure 6. Low affinity CAR T cells spend less time off-tumor than high affinity CAR T cells. In vivo CAR T cell kinetics were captured using IVIS imaging for n = 6 mice per group. (A) T cells were engineered to express a luciferase gene for in vivo luminescent imaging. The dorsal views of the mice that were kept in the same order as in Figure 5b and luminescence intensity is shown in a blue to red spectrum. In addition to luciferase expression, the T cells contained either no CAR expression (negative control), or they were engineered to express a high affinity (HA) or low affinity (LA) HER2 CAR. (B) Whole body bioluminescent imaging (BLI) of T cell luciferase. Statistical significance for HA-CAR versus LA-CAR (*) or HA-CAR vs No CAR (+) was compared by two-way repeated measures ANOVA with a Tukey’s multiple comparison test. (C) Spatial luciferase expression was measured along a line that starts in the upper left thorax (point A) and ends in the lower right abdomen (point B). Luminescence from the spleen, liver and tumor appear at the beginning (~0 – 1.5 cm), middle (~1 – 3 cm) and end (~2.5 – 4 cm) of the line respectively. Mean luminescence along the line was compared between groups by two-way repeated measures ANOVA with Bonferroni’s multiple comparisons test. Statistical significance is denoted as *P < 0.5, **P < 0.01, ns – not significant.