Imaging CAR T-cell kinetics in solid tumors: Translational implications

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SUMMARY

Success in solid tumor chimeric antigen receptor (CAR) T-cell therapy requires overcoming several barriers, including lung sequestration, inefficient accumulation within the tumor, and target-antigen heterogeneity. Understanding CAR T-cell kinetics can assist in the interpretation of therapy response and limitations and thereby facilitate developing successful strategies to treat solid tumors. As T-cell therapy response varies across metastatic sites, the assessment of CAR T-cell kinetics by peripheral blood analysis or a single-site tumor biopsy is inadequate for interpretation of therapy response. The use of tumor imaging alone has also proven to be insufficient to interpret response to therapy. To address these limitations, we conducted dual tumor and T-cell imaging by use of a bioluminescent reporter and positron emission tomography in clinically relevant mouse models of pleural mesothelioma and non-small cell lung cancer. We observed that the mode of delivery of T cells (systemic versus regional), T-cell activation status (presence or absence of antigen-expressing tumor), and tumor-antigen expression heterogeneity influence T-cell kinetics. The observations from our study underscore the need to identify and develop a T-cell reporter—in addition to standard parameters of tumor imaging and antitumor efficacy—that can be used for repeat imaging without compromising the efficacy of CAR T cells in vivo.

INTRODUCTION

The efficacy of chimeric antigen receptor (CAR) T-cell therapy in solid tumors is limited by inefficient tumor T-cell infiltration and inadequate proliferation and accumulation of T cells, which are secondary to a lack of uniform and strong expression of antigen on cancer cells.¹,² The infiltration of adoptively transferred T cells into solid tumors is currently assessed by analyzing biopsy specimens. However, as the performance of serial and multiple biopsies of metastatic sites is not feasible, the ability to interpret antitumor responses to immunotherapy is limited. Although flow cytometric-, immunohistochemical-, and polymerase chain reaction-based assays can detect the presence of CAR T cells in peripheral blood and tissue samples,³ our understanding of CAR T-cell kinetics is limited by the lack of a dynamic imaging modality. To optimize the efficacy of CAR T-cell immunotherapy and gain insights into site-specific responses, it is critical to develop a noninvasive imaging modality that enables the monitoring of CAR T-cell trafficking in real time.

Immune checkpoint inhibitor agents are associated with improved survival in patients with solid tumors, which is achieved through the reactivation of T-cell responses.⁴ In addition, immune checkpoint inhibitor agents have been shown to rescue exhausting CAR T cells and promote their functional persistence and antitumor efficacy.⁵,⁶ A portion of patients receiving immunotherapy, both cell-based and checkpoint-blockade therapy, show responses on traditional imaging; however, in the majority of patients, noninvasive assessment of response to immunotherapy (by computed tomography [CT] and positron emission tomography [PET]) provides an inaccurate or incomplete picture, despite the use of several different assessment criteria, such as RECIST 1.1, mRECIST, and iRECIST.⁷,⁸ Some patients experience initial “progression” of disease, as evidenced by increasing tumor volume or the appearance of new lesions on CT, followed by a favorable response to treatment.⁹–¹¹ This phenomenon, called “pseudoprogression,” does not necessarily represent tumor cell growth but rather may reflect infiltrating immune cells and an appropriate inflammatory reaction. Similarly, some patients treated with induction immunotherapy experience tumor enlargement after therapy or the absence of visible regression on imaging; however, they demonstrate tumor cell death of up to 90% and extensive immune cell infiltration on pathological assessment.¹² A noninvasive imaging modality that can reliably detect and quantify immune cell infiltration in such cases would be beneficial.

An ideal noninvasive imaging method would facilitate the imaging of CAR T-cell trafficking, infiltration, and accumulation in intended and unintended organs following the administration of CAR T cells.

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Pulmonary sequestration and toxicity following systemic administration of nonspecifically activated T cells (activated as a result of the manufacturing process) or due to off-target recognition are areas of concern. Regional administration of CAR T cells has been shown to promote antitumor efficacy against solid tumors within the pleural and peritoneal cavities and against intrathoracic and intracranial metastases. Investigation of CAR T-cell kinetics following regional versus systemic administration can provide insights into the trafficking and accumulation of administered CAR T cells at tumor sites.

Tracking CAR T cells in vivo may be completed by directly or indirectly labeling CAR T cells. Direct labeling (e.g., indium labeling, detectable by scintigraphy) comes with an inherent disadvantage, which is that detection over time is relatively short, as the signal produced is diluted as cells divide. Indirect labeling, which involves genetically engineering CAR T cells to express a reporter gene, may provide long-lasting detection. CAR T cells are tracked by administering the reporter gene’s substrate, resulting in a signal detectable by optical imaging, PET-CT, or scintigraphy, depending on the reporter gene chosen. Several groups have investigated the use of immunoPET imaging, and a first-in-human study demonstrated that clinical response on a per-lesion level correlated with the PET signal produced. In this study, we use an optical luciferase-based reporter gene as it is highly sensitive in translational studies and can be serially imaged at relatively low cost. We also investigate the use of a PET-avid reporter, which provides three-dimensional (3D) organ-specific resolution.

In the present study, we investigated the imaging of adoptively transferred CAR T cells in well-characterized murine models of human mesothelioma and non-small cell lung cancer. Using optimized protocols, we characterized CAR T-cell trafficking, infiltration, and accumulation in these solid tumor models with variable antigen expression after systemic versus regional administration of CAR T cells. By dual imaging of cancer and CAR T cells, we demonstrate the benefit in interpreting the antitumor response.

**RESULTS**

**Standard curves of imaging with enhanced firefly luciferase (effLuc)-expressing CAR T cells and firefly luciferase (ffLuc)-expressing cancer cells in vitro and in vivo**

We used a second-generation human mesothelin (MSLN)-specific CAR with a CD28/CD3ζ domain (M28z) with a retroviral vector (Figure 1A). To facilitate imaging, M28z CAR T cells were cotransduced with an additional retroviral construct encoding effLuc, which allows for the monitoring of CAR T cells by bioluminescence imaging (BLI). The transduction efficiency of T cells was confirmed by flow cytometric analysis of green fluorescent protein (GFP) expression for M28z and of Thy1.1 expression for effLuc (Figure 1B). M28z transduction efficiency across experiments varied between 45% and 60%, as indicated by GFP expression (Figure 1B, upper panel), and effLuc transduction varied between 10% and 30%, as indicated by Thy1.1 expression (Figure 1B, lower panel). EffLuc did not change MSLN-specific CAR T-cell cytolysis as measured by cytotoxicity assay (chromium release assay; Figure S1).

We next developed a standard curve for effLuc function in vitro and in vivo to determine the minimal and maximal T-cell dose for reliable signal and to accurately quantify the accumulation of T cells. For in vitro standardization, T-cell doses ranging from 2 × 10^5 to 6 × 10^6 were incubated with D-luciferin before imaging. The standard curve demonstrated a linear correlation between photon counts and effLuc-expressing CAR T cells (Figure 1C). For in vivo standardization, T-cell doses ranging from 1 × 10^5 to 1 × 10^6 were injected intrapleurally into nonobese diabetic (NOD)/severe combined immunodeficient (SCID)/γ_−null (NSG) mice. Mice were imaged after CAR T-cell injection, and the standard curve again showed a positive linear correlation between the number of injected T cells and the signal intensity (photons per second) (Figure 1D).

To optimize tumor cell imaging, we used mesothelioma MSTO-211H cells retrovirally transduced to express the GFP/ffLuc fusion protein (MSTO G). Transduction was validated by flow cytometric analysis for GFP+ cells. To generate antigen-positive cells expressing MSLN on the cell surface, MSTO G cells were transduced with the human MSLN variant 1 subcloned into an SFG retroviral vector (MSTO GM). GFP and MSLN expression were quantified by flow cytometric analysis (Figure 1E). In vitro, a linear positive correlation was observed between the number of MSTO GM tumor cells and the BLI signal (photons per second) (Figure 1F). We then performed in vivo standardization to confirm that ffLuc expression and the BLI signal accurately reflected the tumor burden in our pleural mesothelioma mouse model. MSTO GM cells (1 × 10^5–1 × 10^6) were injected intrapleurally into NSG mice (Figure 1G). A positive linear correlation between the number of injected cells and the signal intensity was observed.

**In vivo CAR T-cell kinetics in the presence or absence of tumor**

Experiments were conducted to define the in vivo kinetics of adoptively transferred, systemically administered CAR T cells in the absence or presence of antigen-expressing tumors. We intravenously injected 1 × 10^6 M28z-effLuc CAR T cells into NSG mice without tumor. On serial imaging, peak signal intensity was detected in the lungs 4 h after CAR T-cell injection. The BLI signal intensity in the lungs gradually decreased up to 24 h, but T cells were sequestered in the lungs for up to 70 h (Figure 2A). At 14 h after administration, CAR T cells were observed to accumulate in the bone marrow, liver, spleen, and cervical lymph nodes. We then performed a second experiment, this time using mice in which flank tumor had been established with 1 × 10^6 antigen-expressing MSTO GM cells injected subcutaneously. At 14 days after the establishment of flank tumor, mice were treated with intravenous administration of 1 × 10^6 M28z CAR T cells. These mice had an initial accumulation of CAR T cells in the lungs on BLI (Figure 2B); signal intensity then decreased over a 24-h period, accompanied by an increase in the flank signal intensity by day 3 after CAR T-cell administration. By day 6, there was a noted increase in the signal intensity, reflecting the accumulation of M28z CAR T cells into the antigen-positive flank tumor. These results demonstrate CAR T-cell trafficking to the antigen-expressing tumor site after initial pulmonary sequestration as well as subsequent proliferation and accumulation at the tumor site, which is consistent with our
previously published results from immunohistochemical analysis of harvested organs.\textsuperscript{6,16,28}

\textit{In vivo} CAR T-cell kinetics in relation to the intensity of tumor-antigen expression

Having demonstrated that, at 72 h after treatment, CAR T cells accumulate within tumors expressing high levels of tumor antigen, we next questioned whether the density of antigen expression influences CAR T-cell kinetics, particularly given that human lung adenocarcinoma expresses MSLN heterogeneously.\textsuperscript{25,26} To explore this, we used our model of lung adenocarcinoma,\textsuperscript{6,29} which expresses MSLN at either high, low, or heterogeneous levels (Figures 3A–3C). High-antigen-expressing tumors, composed of 1:1 A549GM and A549M cells, expressed MSLN in all cells, and 50% of cells were fFluc\textsuperscript{+} (A549GM), allowing for the regression of tumors with high-antigen-expressing cells to be assessed (Figure 3B). Imaging of tumors with low (1:1 A549G:A549E) or heterogeneous (1:1 A549G:A549M) antigen expression specifically assessed the regression of low- or high-antigen-expressing tumor cells in the presence of low- or high-antigen-expressing tumor cells (Figure 3B). Mice bearing tumors with low, heterogeneous, or high antigen expression subsequently received a single low dose of CAR T cells (5 × 10\textsuperscript{4}, 1 × 10\textsuperscript{5}, and 1 × 10\textsuperscript{6}) followed by intraperitoneal injection of D-luciferin (n = 2–3 mice per dose).

\textbf{Figure 1.} \textit{In vitro} and \textit{in vivo} standardization of CAR T-cell and cancer-cell imaging

(A) Construct map of second-generation MSLN-targeted construct with the CD28 co-stimulatory domain (M28z) and effLuc construct, with GFP and Thy1.1 as reporters. (B) Flow cytometric plots of untransduced (UT) and M28z (GFP\textsuperscript{+}), effLuc (Thy1.1\textsuperscript{+}), and dual-transduced M28z-effLuc (GFP\textsuperscript{+}, Thy1.1\textsuperscript{+}) T cells. (C) \textit{In vitro} imaging standard curve of M28Z-effLuc CAR T cells (5 × 10\textsuperscript{5}–5 × 10\textsuperscript{6}) after addition of D-luciferin. (D) \textit{In vitro} imaging standard curve of intrapleurally administered M28Z-effLuc CAR T cells (1 × 10\textsuperscript{5}, 5 × 10\textsuperscript{5}, and 1 × 10\textsuperscript{6}) after intraperitoneal injection of D-luciferin (n = 3 mice per dose). Images were taken using the Xenogen IVIS 100 imaging system. (E) Flow cytometric plots showing the transduction efficiency of luciferase (GFP\textsuperscript{+}) and MSLN (MSLN\textsuperscript{+}), compared with fluorescence-minus-one (black histogram). (F) \textit{In vitro} imaging standard curve of mesothelioma cells with GFP with MSLN (MSTO GM) (1.5 × 10\textsuperscript{5}–2 × 10\textsuperscript{6} cells). (G) \textit{In vivo} imaging standard curve of intrapleurally injected MSTO GM cells (1 × 10\textsuperscript{5}, 1 × 10\textsuperscript{6}, and 1 × 10\textsuperscript{7}) followed by intraperitoneal injection of D-luciferin (n = 2–3 mice per dose).
environment of heterogenous antigen expression, tumor cells with low antigen expression had early regression. Both low- and heterogeneous antigen-expressing tumors eventually relapsed.

A separate experiment was designed to assess the imaging of CAR T cells in the above-mentioned mouse models with variable antigen expression (Figures 3D–3F). In mice that received non-MSLN-targeted control CAR T cells, T cells failed to accumulate at the tumor site (Figures 3F and 3G), resulting in tumor progression. In mice with tumors with high antigen expression—both tumors with homogeneous high antigen expression and tumors with heterogeneous antigen expression—CAR T cells accumulated at the tumor site rapidly (Figures 3F and 3G). T-cell imaging (Figure 3F) and corollary tumor imaging (from the separate experiment of Figures 3A–3D) demonstrate that robust and early antitumor efficacy may correlate with CAR T-cell accumulation. Furthermore, as tumor with high antigen expression was eradicated, the CAR T-cell signal within the chest began to regress. In tumors with low antigen expression, CAR T-cell accumulation began early but progressed slowly. This slow accumulation was correlated with the observed delay in antitumor efficacy.

Regional administration of CAR T cells enhances early, robust CAR T-cell accumulation, which correlates with increased antitumor potency

Having demonstrated that CAR T-cell accumulation and potency depend on tumor-antigen density, we next postulated that the enhanced efficacy of regionally administered CAR T cells may be directly related to increased early accumulation of CAR T cells. Regionally delivered CAR T cells without antigenic stimulation (Figures 4A–4C) persisted in the pleural space for 5 days. There was no evidence, by T-cell imaging, of proliferation or egress, regardless of dose, until day 6 (Figure 4C). In mice with established antigen-positive orthotopic mesothelioma, a single dose of regionally delivered CAR T cells
accumulated within the tumor and rapidly proliferated (Figures 4D and 4E). We observed a 6-fold increase in CAR T-cell imaging intensity 2 days after delivery (Figure 4F). In contrast, CAR T cells failed to accumulate in pleural tumors within 5 days after a single dose of systemically administered T cells. When T-cell imaging was compared with tumor imaging from a separate in vivo experiment (Figure 4F), CAR T-cell accumulation correlated with antitumor efficacy. Interestingly, as tumor was eradicated after regional delivery, CAR T cells continued to persist in the chest until at least 8 days after administration.

Dual imaging of CAR T cells and tumor by different imaging modalities

Given that concurrent imaging of tumor and T cells provided valuable insights into the clinical response to CAR T cells, we next investigated whether different reporter genes on T cells and tumor cells could be used to reliably assess antitumor efficacy. We cotransduced T cells with M28z CAR and HSVtk (herpes simplex virus thymidine kinase) (Figure 5A), a reporter gene that metabolizes a PET-CT-based reporter probe, $^{18}$F-FEAU (2′-fluoro-2′-deoxy-1-β-d-arabinofuranosyl-5-ethyl-uracil). Mice with established effLuc+ orthotopic mesothelioma subsequently received regionally delivered HSVtk-M28z CAR T cells (Figure 5B), HSVtk-M28z CAR T-cell transduction efficiency was quantified by flow cytometry (Figure 5C). Reporter function was demonstrated by radiotracer uptake assay, and there was no difference in antigen-specific cytotoxicity in vitro as measured by chromium release assay (Figure S2B). Mice were subsequently imaged using bioluminescence imaging and PET-CT. Again, we observed that tumor regression (Figure 5D, left, BLI images) was correlated with CAR T-cell accumulation (Figure 5D, right, PET images). Furthermore, PET-CT allows for analysis and 3D characterization of organ-specific CAR T-cell accumulation.

DISCUSSION

The kinetics of pharmacotherapeutic agents play an important role in determining dose, assessing toxicity, and developing regimens in combination with other agents. CAR T cells are “living drugs,” as the dose expands within the body, traffics differentially to multiple organ sites, accumulates in tumors depending on the intensity of antigen expression, and remains in the body for a long duration. Furthermore, unlike chemical agents, whose serum concentration can be used as a biomarker to predict efficacy and toxicity, solid tumor CAR T-cell therapy has no such marker available. Whereas chemical agents are manufactured and delivered uniformly to all patients, autologous CAR T cells are manufactured for each individual patient, with variable phenotype and transduction percentages. It is not feasible to predict secondary expansion and recirculation of CAR T cells after antigen-specific proliferation at different metastatic sites. Interpretation of CAR T-cell therapy response in solid tumors has been inconsistent, reflecting in part the lack of techniques to measure T-cell distribution and efficacy in vivo. Noninvasive imaging of CAR T-cell therapy using a reporter transgene is a useful method to address these limitations.

In the present study, we used two different imaging techniques to efficiently quantify CAR T-cell kinetics and biodistribution: BLI and PET-CT. The reporters we investigated in this study are limited to use in preclinical models; our observations form a strong rationale to develop T-cell imaging markers that can be used in the clinic. Given the small size of T cells, their low metabolism compared with cancer cells, and the depth of human organs, the ideal translational reporter system will be able to generate a high signal intensity, will lack immunogenicity, and will have no effect on the efficacy of CAR T cells. Although HSVtk has been used in the imaging of biological agents, including oncolytic viruses, our results demonstrate that HSVtk-M28z CAR T cells have less efficacy than M28z CAR T cells alone (Figure S2). Increasing the expression of HSVtk negatively affects the viability of CAR T cells, whereas decreasing the expression of HSVtk limits the sensitivity of T-cell detection by imaging.

In addition to the need to develop an ideal reporter system to image T cells, the imaging method needs to be validated in a clinically
relevant model. The pleural mesothelioma mouse model we used in this study clinicopathologically and radiologically resembles malignant pleural mesothelioma in patients. Owing to the location of the pleural tumor (directly beneath the chest wall in both mice and patients), imaging sensitivity is high by both BLI and PET scan, even with a relatively small amount of T cells; in contrast, the depth of the nodule, especially when small, in solid organs such as the liver and lung may limit detection. However, in the metastatic lung cancer model used in our study, owing to the diffuse nature of metastases established by tail vein injection, the sensitivity of T-cell imaging was high, enabling characterization of CAR T-cell kinetics.

It has been proposed that the kinetics of antigen-activated T cells and nonantigen-activated T cells may differ. Our experiments, which were conducted in mice with and without tumor, demonstrate varied kinetics. Several mechanisms may underlie this variation, including upregulation of the T-cell chemokine receptor following antigen activation; a tumor-induced chemokine gradient that promotes T-cell infiltration; higher numbers of T cells following T-cell proliferation, with improved kinetics; and a migration gradient that is induced by activated CAR T-cell–secreted chemokines. Systemically administered CAR T cells are sequestered in the lungs, as evidenced by a peak signal at 4 h that steadily decreased up to 24 h. The persistence of the signal within the lungs for 3 days is consistent with results from studies that used prostate-specific membrane antigen CAR T cells. The presence of an antigen-expressing distant tumor did not promote higher exit of CAR T cells from the lungs. This observation prompts analysis of the kinetics of different phenotypes of cells from lungs to peripheral circulation. Nonspecific activation of CAR T cells resulting from manufacturing may contribute to pulmonary sequestration as well. As the protocols for manufacturing change, in vivo T-cell kinetics should be investigated to ensure that pulmonary sequestration is not prolonged, which, in addition to compromising efficacy, may also lead to untoward effects. Regionally administered CAR T cells, irrespective of tested doses, remain in the pleural cavity in the absence of tumor for at least 6 days without signal change followed by regression. In contrast, in the presence of antigen-expressing pleural tumors, signal intensity rapidly increases, possibly related to antigen-activated CAR T-cell proliferation. Signal intensity decreases following tumor regression, and there is a corresponding decrease in T-cell signal intensity with subsequent tumor eradication.

We have demonstrated that CAR T-cell accumulation is tumor antigen dependent. Tumors with high antigen expression exhibit robust early accumulation of CAR T cells, with rapid eradication of the tumor. In the setting of low antigen expression, however, CAR T cells accumulate relatively slowly (as observed on T-cell imaging), which accounts for the initial observation of tumor progression followed by slow tumor regression and eventual tumor relapse. However, in the presence of tumors with high antigen expression, CAR T cells do proliferate, with increasing signal intensity and subsequent tumor regression. These observations further highlight the need for timing of imaging and serial imaging—along with dual tumor and T-cell imaging—to accurately assess the kinetics of CAR T cells. One word of caution: we observed that the efficacy of CAR T cells is compromised with repeat imaging. It is advisable to investigate antitumor efficacy (tumor imaging for...
regression or progression and survival) and T-cell imaging for T-cell kinetics separately in 2 different experiments. In addition, to perform repeat T-cell imaging, the reporter system must have adequate sensitivity with minimal or no effect on T-cell efficacy, as T cells relative to tumor cells are small, few in number, and become exhausted.

Gaining early insight into which tumor sites may progress or relapse by tumor imaging and the accumulation of CAR T cells by the use of T-cell imaging are of critical importance in the era of clinical immunoncology. Solid tumors express antigen at varying densities within or between metastatic nodules. This situation of variable expression is further complicated by the presence of unique tumor microenvironments, which may exhibit immunoinhibitory features. The sensitivity of CT scan to detect treatment failure may be limited, particularly in the early phases of the treatment course. We may therefore use T-cell imaging concurrently to quantify the immune kinetics at each tumor site and to characterize nodules earlier as either “immune-hot” or “immune-cold.” The T-cell reporters used in preclinical models an in the clinic are highlighted in Table 1.

There are several limitations to our study. We used immunodeficient mouse models. While these models commonly used in preclinical studies of CAR T-cell therapy allow the investigation of human constructs and human T cells, for direct translation, a comprehensive tumor immune microenvironment is lacking. Immunosuppressive mechanisms derived from tumor-associated macrophages or myeloid-derived stem cells, for example, found in immunocompetent models play a key role in compromising the efficacy of adoptively transferred T cells, which may limit their proliferation, activation status, and kinetics. These mechanisms are not assessed in our study. Another limitation is the use of one type of CAR T-cell that targets MSLN. Further studies should be conducted to ensure that the kinetics we observe are applicable to CAR T cells with different antigen targets. Furthermore, owing to its optical nature and expected immunogenicity to a foreign protein, the luciferase reporter used in our system is not translatable to humans. Experiments using ef-fluc are also limited by the use of a single reporter to assess either tumor or T cells, as they cannot be quantified or localized simultaneously. This could be overcome by using a different reporter, as in experiments using HSVtk, but the impact on in vivo function must be assessed before translation. Transducing cells with HSVtk may result in compromised antitumor efficacy due to the untoward effects of HSVtk overexpression (Figure S2). Others have identified candidate reporters with an acceptable immunotolerance profile that does not compromise antitumor efficacy (Table 1). However, optimization, translational studies, and clinical trials for many of these alternative reporter genes have not yet been performed.

In summary, our study underscores the importance of T-cell imaging, even in preclinical studies, to elucidate the differential kinetics related to tumor sites, T-cell activation status, mode of delivery, and heterogeneity of tumor-antigen expression. To advance adoptive T-cell therapy, it is critical to identify a T-cell reporter that can be detected on imaging even in deep, solid organs, that is not immunogenic, and that does not limit the antitumor efficacy of T cells.

MATERIALS AND METHODS

Tumor cells
MSTO-211H (human pleural mesothelioma) and A549 (human non-small cell lung cancer) cells were obtained from the American Type Culture Collection. To facilitate flow cytometric detection and noninvasive in vivo BLI, MSTO-211H and A549 cells were retrovirally transduced to express GFP-fluc. These cells were then transduced with the human MSLN variant 1 (isolated from the human ovarian cancer cell line OVCAR-3) subcloned into an SFG retroviral vector to generate MSTO MSLN+ GFP-fluc+ or A549 MSLN+ GFP-fluc+.
Gammaretroviral vector construction and viral production

Construction and generation of the MSLN-specific CAR and control prostate-specific membrane antigen CAR have been described.\textsuperscript{16} To facilitate the expression of a GFP reporter gene (\textit{hrGFP}), bicistronic CARs were constructed by use of an internal ribosomal entry site (Figure 1A). The effLuc reporter gene was provided and described by P. Hwu (M.D. Anderson Cancer Center, Houston, TX).\textsuperscript{27} Constructs were transfected into 293T H29 packaging cell lines, and viral supernatants were used to transduce and generate stable 293T RD114 cell lines.

Human T-cell transduction by CARs

T-cell transduction from healthy human volunteer donors (\(n = 5\)) under an institutional review board approval protocol with CAR was performed as previously described.\textsuperscript{16} Two separate spinoculations were performed to transduce effLuc and M28z. Cells were maintained with 20 U/mL interleukin-2 (IL-2) (Life Technologies, Carlsbad, CA) administered every 48 h. Transduction efficiencies were determined by flow cytometry (as described below). Transduced cells were either used immediately in experiments or cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 20 U/mL interleukin-2. T-cell transduction efficiencies were determined by flow cytometric analysis (described below). Transduced cells were either used immediately in experiments or cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 U/mL penicillin, 100 \(\mu\)g/mL streptomycin, and 20 U/mL IL-2.

Orthotopic pleural mesothelioma mouse model and intrapleural administration of CAR T cells

To develop the orthotopic mouse model of pleural mesothelioma, female NSG mice (Jackson Laboratory, Taconic, NY) at 6–10 weeks of age were anesthetized and the chest was opened through a left thoracotomy incision. The pleural cavity was injected with 0.05 mL of 1.5 × 10^6 effLuc–engineered CAR T cells in 3% dextrose solution. Tumor burden was evaluated by bioluminescence imaging on days 7, 14, and 21.

Table 1. T-cell imaging reporters used in preclinical models and in the clinic

| Imaging modality | Reporter | Substrate | T cells identified in | Use in humans | Reference |
|------------------|----------|-----------|-----------------------|---------------|-----------|
| Bioluminescence imaging | firefly luciferase | D-luciferin | mesothelioma, leukemia, lymphoma | – | 16,27 |
| | enhanced firefly luciferase | D-luciferin | mesothelioma, lymphoma | – | 16,27 |
| | Gaussia luciferase | coelenterazine | mesothelioma, lymphoma | – | 16,26 |
| | Renilla luciferase | coelenterazine | urothelial carcinoma, lymphoma | – | 50,61 |
| | click beetle red luciferase | D-luciferin | urothelial carcinoma | – | 50,61 |
| | HSV-tk | 18F-FHBG, 124I-FIAU, 18F-FEAU | leukemia, lymphoma, mesothelioma, glioma | Yes | 62,63,65 |
| | human deoxyctydine kinase, hdCK3mut | 18F-FEAU | prostate cancer, melanoma (M202 tumor) | – | 66,67 |
| | 64Cu-conjugated murine antibody | direct | lymphoma | – | 68 |
| | human norepinephrine transporter | 18F-MFBG, 124I-MIBG | lymphoma | yes | 60,70 |
| | human somatostatin receptor type 2 | 68Ga-DOTATOC | anaplastic thyroid cancer | – | 71 |
| | 68Zr-anti-CD4 and -CD8 cytdiabodies | direct | stem cell transplantation | – | 72 |
| | 68Zr-oxime | direct | glioblastoma, prostate cancer | yes | NCT03142204 |
| | 18F-AraG | direct | patients receiving radiation therapy or immunotherapy | yes | NCT03065764, NCT02760225 |
| | 68Zr-labeled pembrolizumab | direct | NSCLC, melanoma | yes | NCT02453984 |
| | prostate-specific membrane antigen | 18F-DCFpyL | leukemia | – | 73 |
| | 68Zr-labeled atezolizumab | direct | bladder cancer, NSCLC, breast cancer | yes | NCT03853187 |
| | human norepinephrine transporter | 123I-MIBG | EBV lymphoma, glioma | yes | 60,70 |
| | indium 111-oxine | direct | melanoma, colon, breast, and lung cancer | yes | 76 |
| | DOTA antibody reporter 1 | 86Y-AABD or 77Lu-AABD | leukemia | – | 77 |

EBV, Epstein-Barr virus; NSCLC, non-small cell lung cancer.
age were used. All of the procedures were performed under approved Institutional Animal Care and Use Committee protocols. Mice were anesthetized using inhaled isoflurane and oxygen and were administered bupivacaine for analgesia. Intrapleural injection of $1 \times 10^7$ tumor cells in 200 μL serum-free media via a right thoracic incision was performed to establish orthotopic malignant pleural mesothelioma tumors, as previously described. In total, $5 \times 10^4$–$2 \times 10^6$ transduced T cells were adoptively transferred into tumor-bearing mice 11–21 days after tumor injection; specifically, T cells were suspended in 200 μL serum-free media and were injected into the thoracic cavity of mice by direct pleural injection or systemically by tail vein injection. Assessment of tumor burden and T-cell trafficking was performed using BLI, as described below.

All of the mice were monitored every 15 min for the first hour following infusion of CAR T cells or tumor cells, and at least once daily for signs of toxicity. Humane endpoints included >20% body weight loss, change in physical appearance (e.g., ruffled hair), lethargy or persistent recumbency, loss of a righting reflex, or any condition interfering with daily activity. Once humane endpoints were reached, mice were sacrificed immediately. Mice were assessed for toxicity by trained animal workers, and they were blinded to the treatment groups to avoid subjective bias.

**Non-small cell lung cancer mouse model and systemic administration of CAR T cells**

To develop the mouse model of non-small cell lung cancer, female NSG mice at 6–10 weeks of age were used. All of the procedures were performed under approved Institutional Animal Care and Use Committee protocols, and mice were monitored as described above. Tumors with low antigen expression were established by the use of a mixture of 1:1 A549E (low MSLN) and A549G (ffLuc+, low MSLN) cells. Tumors with heterogenous antigen expression were established by the use of a mixture of 1:1 ffLuc+ A549G (low MSLN, ffLuc+) and A549M (MSLN+, ffLuc negative) cells. Tumors with high antigen expression were established by the use of a mixture of 1:1 A549GM (ffLuc+, MSLN+) and A549M cells. In total, $1 \times 10^6$ tumor cells in 200 μL serum-free media were injected via the dorsal tail vein. Twenty-one days after tumor cell inoculation, CAR T cells were administrated as described above. Assessment of tumor burden and CAR T cells was performed using BLI, as described below.

**Quantitative tumor and T-cell BLI**

BLI was used in vivo to assess tumor burden in tumor-bearing mice. Animals were injected with a single intraperitoneal dose of 150 mg/kg D-luciferin. Mice were then imaged with the Xenogen IVIS 100 imaging system (Caliper Life Sciences, Boston, MA) 15 min after D-luciferin injection. Images were acquired for 5–30 s. BLI data were analyzed using Living Image software (version 4.31; Caliper Life Sciences); BLI signal is reported as total flux (photons per second). Individual mouse signals were determined by averaging the dorsal and ventral BLI signals for each animal using Microsoft Excel (Microsoft, Seattle, WA); the resulting data were analyzed using GraphPad Prism software (GraphPad Software, San Diego, CA).

BLI was also used to visualize in vivo trafficking of adoptively transferred CAR T cells. T cells transduced with an effLuc reporter gene (provided by P. Hwu) or HSVtk were transferred into mice by a single intraperitoneal or intravenous injection. These T cells could then be visualized by injecting the T-cell–bearing mice with a single intraperitoneal dose of 150 mg/kg D-luciferin and imaging the animals for 120 s at 20 min after injection, again using the Xenogen IVIS 100 imaging system.

**PET imaging**

For PET-CT imaging, mice were injected with 150 μCi $^{18}$F-FEAU via the lateral tail vein. Sixty minutes after injection, PET and CT images were collected using a micro-PET-CT hybrid Inveon scanner (Siemens, Munich, Germany). Three-dimensional reconstructions were made using Inveon Research Workplace image analysis software (version 4.0; Siemens).

**Radiotracer uptake assay**

Cells were seeded in 15-cm culture plates at a density of $2 \times 10^6$ cells/mL and grown until 50% confluence. The cells were harvested by scraping after periods of incubation (30, 60, and 120 min), and centrifuged at 1,250 × g. Cell pellets were weighed and reconstituted in solubilization buffer (Soluene-350, PerkinElmer) and scintillation buffer (Insta-Fluor Plus; PerkinElmer). Using a TriCarb 1600 $β$-spectrometer (Packard), cell pellets were assayed for radioactivity concentration with standard $^3$H-channel counting. Harvested cell-to-medium concentration ratios were determined and reported (dpm/g cells)/(dpm/mL medium). The radiochemical purity of each compound was checked by high-performance liquid chromatography to ensure >98% purity.

**Flow cytometry**

Expression of M28z and effLuc CAR was detected using GFP and Thy1.1 APC-conjugated F(ab)2 anti-human immunoglobulin G1 (IgG1), respectively. Anti-human antibodies against CD3 (BD Biosciences, San Jose, CA) were used. Stained cells were processed using a FACScan instrument (BD Biosciences), and data were analyzed using FlowJo software (version 6.0; TreeStar, Ashland, OR).

**Supplemental Information**

Supplemental information can be found online at https://doi.org/10.1016/j.omto.2021.06.006.

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AUTHOR CONTRIBUTIONS

Conceptualization, P.S.A.; methodology, J.V.-V., V.P., A.M., and P.S.A.; validation, J.V.-V., A.M., V.P., and P.S.A.; investigation, J.V.-V., M.S.S., J.K.S., R.Y.B., M.M., V.P., A.M., and P.S.A.; formal analysis, J.V.-V., A.M., J.K.S., R.Y.B., M.S.S., H.R.H., M.M., V.P., and P.S.A.; writing – original draft, M.S.S., H.R.H., J.V.-V., and P.S.A.; writing – review & editing, all authors; visualization, J.K.S., R.Y.B., M.M., M.S.S., H.R.H., S.B., V.P., and P.S.A.; supervision, P.S.A.; funding acquisition, P.S.A.

DECLARATION OF INTERESTS

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