In vivo Imaging of Nanoparticle Labeled CAR T-cells

Louise Kiru, Aimen Zlitni, Aidan Michael Tousley, Guillermo Nicolás Dalton, Wei Wu, Famyrah Lafortune, Anna Liu, Kristen May Cunanan, Hossein Nejadnik, Todd Sulchek, Michael Eugene Moseley, Robbie G. Majzner, and Heike Elisabeth Daldrup-Link.

1 Department of Radiology, Molecular Imaging Program at Stanford, Stanford University, CA, 94305, USA
2 Department of Pediatrics, Stanford University, CA, 94305, USA
3 Department of Biomedical Engineering, Georgia Institute of Technology, Atlanta, GA, 30332, USA
4 Hospital of the University of Pennsylvania, Philadelphia, PA 19104, USA
5 Stanford Cancer Institute, Stanford University, CA, 94305, USA

*Corresponding author: heiked@stanford.edu

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CONTENTS

1. The labeling efficiency of mechanoporation and co-incubation
2. Population doubling time of the anti B7-H3 CAR T-cells
3. Longitudinal in vivo study design
4. Caliper measurements of the osteosarcomas
5. Histology images for the longitudinal in vivo model
6. Quantitative analysis of the bioluminescence imaging
7. Dose-dependent accumulation of the iron labeled CAR T-cells in osteosarcomas
8. Tumor oxygenation measurements using photoacoustic imaging
9. In vitro photoacoustic characterization of ferumoxytol and the iron labeled T-cells
Methods

1. The labeling efficiency of mechanoporation and co-incubation.

The T-cells were labeled with ferumoxytol using a custom-designed microfluidics cell labeling device. The anti B7-H3 CAR T-cells (8 × 10^6/mL) were either co-incubated with ferumoxytol (10 mg iron/mL) for 10 minutes or passaged through the microfluidics device for 10 minutes. The cells were washed twice with 1× PBS and the level of iron was measured by inductively coupled plasma - optical emission spectrometry (ICP-OES).

2. Population doubling time of the anti-B7-H3 CAR T-cells.

The population doubling time was determined by seeding unlabeled or ferumoxytol labeled anti-B7-H3 CAR T-cells (1 × 10^5 cell/mL) in six well plates (Corning Incorporated, Corning, NY). The cells were counted using a Neubauer chamber (Fisher Scientific, Hampton, NH) at 24-hour intervals on day 0 to day 9. A plot of the number of cells as a function of the time in days was generated using the Poisson regression model to provide the growth rate. The population doubling time was calculated using the following equation;

\[ T_d = \ln2/\Lambda_p \]

where \( T_d \) is the population doubling time, which represents the time required for the population to double the number of cells and \( \Lambda_p \) is the exponential growth rate.

3. Longitudinal in vivo study design.

Initially, (i) human T-cells were genetically modified to express the anti B7-H3 CAR construct using a retroviral vector (ii) the genetically modified T-cells underwent mechanical labeling with ferumoxytol through mechanoporation using a microfluidics device and (iii) the ferumoxytol labeled B7-H3 CAR T-cells were administered through the tail vein in tumor bearing mice. Subsequently, MRI, PAT, MPI and BLI imaging were performed sequentially.
after the administration of the anti B7-H3 CAR T-cells labeled with ferumoxytol to monitor the localization of the T-cells noninvasively. For the longitudinal in vivo study, fifteen of the eighteen NOD scid gamma animals were injected with human MG63 osteosarcomas at Day 0. Pre-contrast MRI and PAT images were acquired on Day 28 post tumor injection. Animals were divided in three groups that received different treatments and a fourth group that was a control group. Group one was osteosarcoma bearing animals injected with the ferumoxytol labeled B7-H3 CAR T-cells (tumor + CAR + iron). Tumor bearing animals in group two received unlabeled B7-H3 CAR T-cells (tumor + CAR). Osteosarcoma bearing animals in group three were animals infused with ferumoxytol labeled non-transduced (NT) T-cells (tumor + NT + iron). Animals in the fourth group were not inoculated with tumors, however they were injected with ferumoxytol labeled B7-H3 CAR T-cells (CAR + iron). Subsequently, animals were imaged sequentially over three weeks using MRI, PAT and MPI.

4. Caliper measurements of the osteosarcomas.

Tumor volume was determined by measuring the greatest longitudinal diameter (length), the largest transverse diameter (width) and the distance between the exterior tumor edge and the mouse’s body (height) using an external caliper. Measurements were acquired prior to tumor inoculation (W0) and every week for six weeks following tumor injection. The tumor volume was calculated using the ellipsoidal formula; tumor volume = \( \pi/6 \times L \times W \times H \).

5. Histology images for the longitudinal in vivo model.

On Day 7 post treatment, osteosarcomas were harvested from the animals injected with the ferumoxytol-labeled anti B7-H3 CAR T-cells (tumor + CAR + iron), unlabeled anti B7-H3 CAR T-cells (tumor + CAR) or labeled NT T-cells (tumor + NT + iron), fixed in 4% paraformaldehyde in 1× PBS, embedded in paraffin and 5 \( \mu \)m sections were cut using a
microtome (Leica, Wetzlar, Germany). The paraffin-embedded sections were stained with hematoxylin and eosin (H&E) and CD3 immunostaining using an anti-CD3 antibody (1:150, AbCam, Cambridge, MA). Fixed sections were also stained for 30 min in 2% potassium ferrocyanide and 2% hydrochloric acid to detect iron using the Perl’s Prussian blue stain and counterstained with 0.5% eosin solution (Sigma-Aldrich, Oajville, ON). The slides were dehydrated and a cover slip was placed on them for imaging. The BZ-X700 series fluorescence microscope (Keyence, Itasca, IL) was used to acquire the images.

6. Quantitative analysis of the bioluminescence imaging.

Bioluminescence was acquired for animals administered with anti B7-H3 CAR-nLuc T-cells at 1h following the administration of either $2 \times 10^7$ or $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells or $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells. Animals were intraperitoneally administered with $\approx 0.25$ mg/kg of the furimazine ($\approx 40\times$ dilution of NanoGlo substrate, Promega, Madison, WI) in 100 μl of sterile PBS and the animals were imaged using the Lago-X Imaging System (Spectral Instruments Imaging, Tucson, AZ) with an exposure time of 30 seconds. The photon radiance was assessed by drawing region of interest (ROIs) on the whole body of the animals using XQuartz 2.8.1 software (Aura Imaging Software, Spectral Instruments Imaging. Tucson, AZ).

7. Dose-dependent accumulation of the iron labeled CAR T-cells in osteosarcomas.

Twelve osteosarcomas bearing NOD scid gamma animals were separated into three groups that were treated with B7-H3 CAR-nLuc T-cells. The tumor + CAR + iron $2 \times 10^7$ group ($n = 5$) was comprised of animals injected with $2 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells. The tumor + CAR + iron $1 \times 10^7$ group ($n = 4$) consisted of animals injected with $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells and the tumor + CAR $2 \times 10^7$ group ($n =$
3) contained animals injected with $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells. A spoiled gradient recalled echo sequence was used for imaging the animals pre-treatment, on Day 1 and 3 post treatment. The spoiled gradient recalled echo sequence had a TR of 85.8 ms, TE of 1.8 ms $\alpha$: 85°; a FOV of 4 cm $\times$ 4 cm and a slice thickness of 0.75 mm.

8. Tumor oxygenation measurements using photoacoustic imaging.

The photoacoustic system was connected to a LZ-250 transducer (center frequency= 21MHz) held by a 3D stepper motor. Cross-sectional two-dimensional (2D) and 3D images (thickness = 10 mm, increment = 0.125 mm) of the right tibia were acquired. During photoacoustic imaging, nanosecond laser pulses were delivered at specific wavelengths based on the imaging mode used. For ferumoxytol imaging, photoacoustic images were collected using the “nano-stepper” mode, where each slice of the 3D scan was excited at 680, 705, 855 and 924 nm wavelengths. For tumor oxygenation assessment, photoacoustic images were collected using the “oxy-hemo” mode and the laser irradiation was at 750 and 850 nm for each slice. Photoacoustic imaging was conducted before (pre), one week after injection of the anti B7-H3 CAR T-cells (Week 1) and two weeks post treatment (Week 2). Photoacoustic imaging has the additional advantage of providing quantitative measurements of tumor oxygenation as a biomarker of response. The percentage oxygen saturation (% SO$_2$) measurements were conducted using the “OxyZated” tool (VisualSonics, Inc., Canada) and the threshold was set to 20% for all experiments. Photoacoustic images were collected before (pre), one week after treatment (Week 1) and two weeks post treatment (Week 2) using the “oxy-hemo” mode and the laser irradiation was at 750 and 850 nm for each slice.
9. **In vitro photoacoustic characterization of ferumoxytol and iron labeled T-cells.**

A serial dilution of ferumoxytol (30, 15, 1.5, 0.15, 0.015 and 0 mg/mL) or ferumoxytol labeled CAR T-cells \((2 \times 10^6, 2 \times 10^5, 2 \times 10^4, 2 \times 10^3)\) and controls were prepared in 1× PBS. A suspension of the cells was infused in a tube phantom submerged in degassed water. All samples were measured in a Vevo PHANTOM imaging chamber and imaging was conducted using the Vevo2100 LAZR imaging system. Photoacoustic measurements were collected using the photoacoustic spectra mode (excitation from 680 – 970 nm) and a calibration curve was assessed at 680 nm excitation (photoacoustic max. excitation). Ten frames per sample were collected and photoacoustic signal was averaged. Experiments were done in triplicates and a calibration curve was created from the average of those triplicates.
**Supplementary Fig. 1. The labeling efficiency of mechanoporation and co-incubation.**

Iron level measurements by ICP-OES demonstrated that the labeling of anti B7-H3 CAR T-cells with ferumoxytol using the microfluidics device was significantly greater compared to co-incubation of the T-cells with ferumoxytol (0.80 ± 0.08 versus 0.09 ± 0.06, p < 0.0001).

There was no significant difference in the level of iron in the anti B7-H3 CAR T-cells co-incubated with ferumoxytol compared to the unlabeled anti B7-H3 CAR T-cells (0.09 ± 0.06 versus 0.082 ± 0.004, p < 0.0001). N ≥ 3 per group. Data represents mean ± SEM. Statistical comparisons were performed using the one way ANOVA with the Tukey’s HSD test.
Supplementary Fig. 2. Population doubling time of the anti B7-H3 CAR T-cells. Growth curves indicating that the ferumoxytol labeled anti B7-H3 CAR T-cells doubled at a faster rate than the unlabeled anti B7-H3 CAR T-cells. The doubling time of the ferumoxytol labeled anti B7-H3 CAR T-cells (2.57 days) was 1.35-fold faster than the unlabeled anti B7-H3 CAR T-cells (3.46 days). Additionally, the population doubling time was significantly different between the unlabeled and labeled anti B7-H3 CAR T-cells at Day 6 (4.58 × 10^5 ± 1.92 × 10^4 versus 5.83 × 10^5 ± 3.33 × 10^4, p = 0.03), Day 7 (5.18 × 10^5 ± 1.30 × 10^4 versus 9.35 × 10^5 ± 3.77 × 10^4, p < 0.0001), Day 8 (6.23 × 10^5 ± 3.38 × 10^4 versus 1.01 × 10^6 ± 3.94 × 10^4, p < 0.0001) and Day 9 (6.85 × 10^5 ± 8.67 × 10^4 versus 1.07 × 10^6 ± 2.62 × 10^4, p < 0.0001) post labeling. Data represents mean ± SEM and N = 3 per group. Statistical comparisons were performed using an unpaired student t-test.
Supplementary Fig. 3. Longitudinal \textit{in vivo} study design. A timeline showing the sequential MRI, MPI and PAT imaging of the osteosarcoma bearing mice and controls prior to infusion of the T-cells, one day post T-cell injection and every week post treatment over three-weeks.
Supplementary Fig. 4. Caliper measurements of the osteosarcomas. Analysis of the tumor volume (mm\(^3\)) acquired prior to the tumor cell injection and six weeks post injection in the tumor + CAR + iron, tumor + CAR and tumor + NT + iron groups. Tumor cells were not injected in the CAR + iron group. Week 0 to 3 represents tumor volume prior to T-cell infusion, while week 4 to 6 shows the tumor volume post infusion of the CAR or NT T-cells. Tumor + CAR + iron group (n = 5); animals injected with ferumoxytol-labeled CAR T-cells, tumor + CAR group (n = 5); animals injected with unlabeled CAR T-cells and tumor + NT + iron group (n = 5); animals injected with non-transduced (NT) ferumoxytol-labeled T-cells. Data represents mean ± SEM. Statistical comparisons were performed using the one-way ANOVA with the Tukey’s HSD test.
Supplementary Fig. 5. Histology images for the longitudinal in vivo model.

Representative histological images of paraffin-embedded tumor sections obtained from mice on Day 7 post-treatment stained with H&E (left panel), anti-CD3 antibody (middle panel) and Prussian blue-DAP (right panel). The tumor cells are represented by the dark hematoxylin-stained nuclei, human CD3⁺ cells are stained brown and Prussian blue-stained iron labeled cells are visible in the labeled tumor + CAR + iron group (yellow arrows). Scale bars are 50 μm, magnification is X40. NT = non transduced. Tumor + CAR + iron group (n = 5); animals injected with ferumoxytol-labeled CAR T-cells, tumor + CAR group (n = 5); animals injected with unlabeled CAR T-cells, tumor + NT + iron group (n = 5); animals injected with non-transduced ferumoxytol-labeled T-cells and CAR + iron group (n = 3); animals without tumors injected with ferumoxytol-labeled CAR T-cells.
Supplementary Fig. 6. Quantitative analysis of the bioluminescence imaging. The photon radiance at 1h post injection was significantly greater in the animals intravenously administered with $2 \times 10^7$ ferumoxytol-labeled anti B7-H3 CAR-nLuc T-cells compared to animals injected with $1 \times 10^7$ ferumoxytol-labeled anti B7-H3 CAR-nLuc T-cells ($p = 0.02$). The photon radiance in the animals treated with $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells was significantly lower compared to animals treated with $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells ($p = 0.04$). Tumor + CAR + iron $2 \times 10^7$ group ($n = 5$); animals injected with $2 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells, Tumor + CAR + iron $1 \times 10^7$ group ($n = 4$); animals injected with $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells, Tumor + CAR $2 \times 10^7$ group ($n = 3$); animals injected with $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells. Data represents mean ± SEM. Statistical comparisons were performed using the one-way ANOVA with the Tukey’s HSD test.
Supplementary Fig. 7. Dose-dependent accumulation of the iron labeled CAR T-cells in osteosarcomas. Representative coronal T1-weighted MR images of the tibiae of tumor bearing animals injected with either $2 \times 10^7$ or $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells or $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells. The iron labeled B7-H3 CAR-nLuc T-cells were visualized in the tibiae of animals administered with $2 \times 10^7$ or $1 \times 10^7$ (orange arrows) ferumoxytol-labeled B7-H3 CAR-nLuc T-cells. Tumor + CAR + iron $2 \times 10^7$ group (n = 5); animals injected with $2 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells, Tumor + CAR + iron $1 \times 10^7$ group (n = 4); animals injected with $1 \times 10^7$ ferumoxytol-labeled B7-H3 CAR-nLuc T-cells, Tumor + CAR $2 \times 10^7$ group (n = 3); animals injected with $2 \times 10^7$ unlabeled B7-H3 CAR-nLuc T-cells.
Supplementary Fig. 8. Tumor oxygenation measurements using photoacoustic imaging.

a Tumor oxygenation images from representative animals. Scale bar is 10 mm. b Quantitative region of interest measurements showing the percentage oxygen saturation (%SO₂) pre-treatment as well as Week 1 and Week 2 post treatment. There was a significant decrease in the percentage oxygen saturation in Week 2 compared to the pre-treatment images in the animals injected with the ferumoxytol labeled B7-H3 CAR T-cells (p = 0.01). Tumor + CAR + iron group (n = 5); animals injected with ferumoxytol-labeled CAR T-cells, tumor + CAR group (n = 5); animals injected with unlabeled CAR T-cells and tumor + NT + iron group (n = 5); animals injected with non-transduced (NT) ferumoxytol-labeled T-cells. Data represents mean ± SEM. Statistical comparisons were performed using the one-way ANOVA with the Tukey’s HSD test.
Supplementary Fig. 9. *In vitro* photoacoustic characterization of ferumoxytol and the iron labeled T-cells. a Overlay (top), photoacoustic (middle) and ultrasound (bottom) images of phantoms containing different concentrations of ferumoxytol mixed in ficoll. Data was calculated by drawing regions of interest (light blue) shown in the ultrasound images. b Quantification of the photoacoustic signal in the phantoms. There was no significant difference in the photoacoustic signal of the background compared to 4.7 µg/mL and 9.4 µg/mL (0.18 ± 0.02 versus 0.22 ± 0.01, p > 0.99 and 0.33 ± 0.02, p = 0.37). However, the photoacoustic signal was significantly higher in phantoms containing 18.8 µg/mL (0.45 ± 0.01, p = 0.04), 37.5 µg/mL (0.71 ± 0.06, p = 0.0001), 75.0 µg/mL (0.86 ± 0.11, p < 0.0001), 150 µg/mL (1.77 ± 0.02, p < 0.0001) and 300 µg/mL (2.67 ± 0.12, p < 0.0001) compared to the background (0.18 ± 0.02). This indicates that the detection limit is 18.8 µg/mL of iron. c Calibration curve at an excitation of 680 nm showing the $R^2 = 0.97$. d Quantification of the photoacoustic signal in solutions containing $2 \times 10^6$, $2 \times 10^5$, $2 \times 10^4$, $2 \times 10^3$ ferumoxytol.
labeled anti B7-H3 CAR T-cells or $2 \times 10^6$ unlabeled anti B7-H3 CAR T-cells infused in a tube phantom. The photoacoustic signal was significantly greater in the solution containing $2 \times 10^6$ ($0.55 \pm 0.02$, $p < 0.001$) ferumoxytol labeled anti B7-H3 CAR T-cells compared to solutions containing $2 \times 10^5$ ($0.32 \pm 0.01$), $2 \times 10^4$ ($0.30 \pm 0.01$), $2 \times 10^3$ ($0.28 \pm 0.002$) or $2 \times 10^6$ ($0.28 \pm 0.01$) unlabeled anti B7-H3 CAR T-cells. Data represents mean $\pm$ SEM and $N \geq 3$. Statistical comparisons were performed using the one way ANOVA with the Tukey’s HSD test.