Additional file 1

Additional Methods.

Conjugation, radiolabeling and quality control

ERY974, KLH/CD3, KLH/KLH and IgG4 were conjugated with tertrafluorphenol-N-succinyl desferal-Fe (N-suc-Df; ABX) as described before (1, 2). In short, antibodies were purified using Vivaspin-2 30,000 MWCO PES centrifugal concentrators (Sartorius) in 0.9% NaCl (Braun). After pH adjustment to 9.0 using 0.1 M Na_2CO_3, a 4-fold excess of N-suc-Df was added for 30 minutes. Subsequently Fe^{3+} was removed using EDTA and the solution was purified using PD-10 column (GE Healthcare) and 0.9% NaCl as eluent. Quality of conjugated antibody was assessed using size exclusion high-performance liquid chromatography as described before (1), using a TSKgel G3000SW_{XL} column (Tosoh). Radiolabeling of antibodies with [^{89}Zr]Zr-oxalate (PerkinElmer) was performed as described before (2). After 1 hour incubation, radiochemical purity was above 95% for all experiments and purification was not performed. Molar activity for all experiments was 72.8 MBq/nmol, unless stated otherwise.

Binding to GPC3 and CD3ε was tested using an ELISA based method. Recombinant human GPC3 (10088-H08H; Sino Biologicals Inc.) or CD3ε (10977-H08H; Sino Biologicals Inc.) were diluted in 0.05M Na_2CO_3 to a concentration of 0.1 µg/mL. Nunc-Immuno 96 well MicroWell MaxiSorp plates (Thermo Fisher Scientific) were coated with 100 µL recombinant protein at 4°C overnight. Wells were washed with 0.05% Tween20 in phosphate buffered saline (PBS; 140 mM/L NaCl, 9 mM/L Na2HPO4, 1.3 mM/L NaH2PO4, pH 7.4, UMCG). Next, wells were blocked with 0.5% bovine serum albumin (BSA), 0.05% Tween20 in PBS for 2 hours at

room temperature (RT). After blocking, wells were incubated with a concentration series (0.02 nM – 137.4 nM) of mAb diluted in 0.5% BSA/0.05% Tween20/PBS for 1 hour at RT. Subsequently, wells were washed three times with 0.05% Tween20/PBS followed by 1 hour incubation at RT of rabbit anti-human IgA, IgG, IgM, Kappa, Lambda HRP (1:8000; Agilent DAKO). Again, wells were washed three times with 0.05% Tween20/PBS followed by addition of 100 µL substrate SureBlue Reserve TMB microwell substrate (KPL Inc.). Reaction was stopped with 1 M hydrochloric acid (UMCG) and absorbance at 450 nm was determined with a microplate reader (Bio-Rad).

T cell activation potency was determined using a co-culture of HepG2 cells with Jurkat cells that express a luciferase reporter driven by a Nuclear Factor of Activated T cells response element (Jurkat-NFAT; Promega). In a 96-well plate, 12,500 HepG2 cells and 75,000 Jurkat-NFAT effector cells were incubated overnight at 37°C with a concentration of ERY974 or N-suc-Df-ERY974 ranging from 0.05 pM to 137.4 nM. After incubation, 75 µL Bio-Glo reagent (Promega) was added and bioluminescence was determined with a Synergy plate reader (Biotek).

Internalization of [^{89}Zr]Zr-N-suc-Df-ERY974

To determine internalization of [^{89}Zr]Zr-N-suc-Df-ERY974, 10^6 HepG2 cells were incubated with 50 ng [^{89}Zr]Zr-N-suc-Df-ERY974 in 1 mL medium on ice for 1 hour. After initial binding, unbound [^{89}Zr]Zr-N-suc-Df-ERY974 was washed three times using 1% human serum albumin in PBS. Next, cells were incubated at 4°C or 37°C for 1, 2, or 4 hours. After incubation, cell membranes were stripped with 1 mL stripping buffer (0.05 M glycine, 0.1 M NaCl, pH 2.8) at 4°C. Radioactivity of the cell pellet (internalization) was expressed as percentage of radioactivity initially bound to cells.

Immunohistochemistry

Formalin-fixed paraffin-embedded 4 µm tissue slides were stained with immunohistochemistry using 2 µg/mL rabbit monoclonal GPC3 antibody (SP86; Abcam) or isotype control (EPR25A; Abcam), followed by rabbit EnVision HRP (Agilent). Human placenta and HepG2 tumor of [^{89}Zr]Zr-N-suc-Df-ERY974 injected huNOG mice were used as positive control tissue (Additional file 1 Fig. S10A). For CD3, tissues were stained using 0.15 µg rabbit monoclonal CD3 antibody (SP162; Abcam) or isotype control (EPR25A; Abcam), followed by rabbit EnVision HRP (Agilent). Human liver and HepG2 tumors of [^{89}Zr]Zr-N-suc-Df-ERY974 injected mice were used as positive control tissue (Additional file 1 Fig. S10B). CD3+ cells were quantified using positive cell detection using QuPath (3).

Flow cytometry

HepG2, TOV-21G and SK-HEP-1 cells were harvested and suspended in 20 µg/mL of ERY974 or human IgG4 in 0.5% fetal bovine serum (FBS)/2 mM EDTA/PBS. Cells were incubated for 1 hour at 4°C, subsequently washed twice with 0.5% FBS/2 mM EDTA/PBS and incubated with PE-labeled goat anti-human IgG (1:50; Thermo Fisher Scientific) at for 1 hour 4°C. After two more washes with 0.5% FBS/2 mM EDTA/PBS, cells were measured using a BD Accuri C6 flow cytometer (BD Biosciences).

Additional Figure legends S1-S10

Fig. S1. Human CD3+ engraftment in huNOG mice. Percentage of human CD3+ of human CD45+ cells in the experimental groups involving huNOG mice.

Fig. S2. *In vitro* characteristics of N-suc-Df-conjugated tracers. (A) Representative binding curve of N-suc-Df-ERY974 and ERY974 binding to human GPC3 protein. **(B)** Representative binding curve of N-suc-Df-ERY974 and ERY974 binding to human CD3ε protein. **(C)** Potency of ERY974 and N-suc-Df-ERY974 to activate reporter T cells upon co-culture with HepG2 cells. **(D)** Internalization up to 4 h of [^{89}Zr]Zr-N-suc-Df-ERY974 in HepG2 cells at 4 and 37 °C (n = 3). **(E)** Representative binding curve of N-suc-Df-KLH/CD3 and N-suc-Df-KLH/KLH to human GPC3 protein. **(F)** Representative binding curve of N-suc-Df-KLH/CD3 and N-suc-Df-KLH/KLH to human CD3ε protein**.**

Fig. S3. Tumor characteristics of HepG2, TOV-21G and SK-HEP-1. (A) Hematoxylin and eosin (H&E), autoradiography and glypican-3 (GPC3) staining of HepG2, TOV-21G and SK-HEP-1 xenografts. Scale bar length represents 5 mm for HepG2, 1 mm for TOV-21G and 2.5 mm for SK-HEP-1, and 250 µm for the zoomed slides. Autoradiography and H&E were performed on the same slide. For each cell line, flow cytometry was performed using ERY974 as primary antibody (black), including IgG4 as control (red; right panel). **(B)** SDS-PAGE autoradiography of different individual HepG2 (left), TOV-21G (middle) and SK-HEP-1 (right) lysates and corresponding plasma samples. + represents activity matched [^{89}Zr]Zr-N-suc-Df-ERY974 tracer from injected solution. kDa = kilodalton.

Fig. S4. Influence of FcγR binding and radioactive dose on biodistribution of different tracers in mice. (A) Spleen uptake at 168 h after administration of 10 µg of [^{89}Zr]Zr-N-suc-Df-ERY974 (n = 6), [^{89}Zr]Zr-N-suc-Df-KLH/CD3 (n = 5), [^{89}Zr]Zr-N-suc-Df-KLH/KLH (n = 6) and [^{89}Zr]Zr-N-suc-Df-IgG4 (n = 5) expressed as median % injected dose per gram (%ID/g) with

interquartile range. **(B)** Spleen weight of [^{89}Zr]Zr-N-suc-Df-ERY974 (n = 6), [^{89}Zr]Zr-N-suc-Df-KLH/CD3 (n = 5), [^{89}Zr]Zr-N-suc-Df-KLH/KLH (n = 6) and [^{89}Zr]Zr-N-suc-Df-IgG4 (n = 5) expressed as median weight in mg with interquartile range. **(C)** Spleen weight of NOG mice injected with 10 µg of [^{89}Zr]Zr-N-suc-Df-ERY974 labeled with 5 MBq (A_m: 14.6 MBq/nmol) at 72 h (n = 2), 120 h (n = 2) and 168 h (n = 12) after administration expressed as median weight with interquartile range (IQR). A_m = molar activity. **(D)** Spleen uptake of NOG mice injected with 10 µg of [^{89}Zr]Zr-N-suc-Df-ERY974 labeled with 1 MBq (A_m: 14.6 MBq/nmol; n = 6) or 5 MBq (A_m: 72.8 MBq/nmol; n = 12) at 168 h expressed as median % injected dose per gram with IQR. **E)** Spleen weight of NOG mice injected with 10 µg of [^{89}Zr]Zr-N-suc-Df-ERY974 labeled with 1 MBq (A_m: 14.6 MBq/nmol; n = 6) or 5 MBq (A_m: 72.8 MBq/nmol; n = 12) at 168 h expressed as median weight with IQR. **(F)** Radioactivity dose of the spleen of NOG mice injected with 10 µg of [^{89}Zr]Zr-N-suc-Df-ERY974 labeled with 1 MBq (A_m: 14.6 MBq/nmol n = 6) or 5 MBq (A_m: 72.8 MBq/nmol; n = 12) at 168 h expressed as median dose with IQR. **(G)** Hematoxylin and eosin (H&E; 400x) staining of a NOG mice spleen injected with 1 MBq (A_m: 14.6 MBq/nmol) or 5 MBq (A_m: 72.8 MBq/nmol) of [^{89}Zr]Zr-N-suc-Df-ERY974 at 168 h after tracer administration. Scale bar length represents 250 µm. **(H)** Uptake of [^{89}Zr]Zr-N-suc-Df-ERY974 in spleen, bone, liver and blood in NOG (n = 6) and BALB/c^{nu} (n = 6) at 168 h after tracer administration expressed as median % injected dose per gram of tissue (%ID/g) with interquartile range (IQR). **(I)** Uptake of [^{89}Zr]Zr-N-suc-Df-ERY974 in spleen in NOG (n = 6) and BALB/c^{nu} (n = 6) at 168 h after tracer administration expressed as median % ID/g with IQR. **(J)** Spleen weight of NOG (n = 6) and BALB/c^{nu} (n = 6) mice at 168 h after tracer administration expressed as median weight with IQR. **(K)** Pooled data of [^{89}Zr]Zr-N-suc-Df-ERY974 uptake in

spleen, femur, cortical femur, femur bone marrow of NOG (n = 18) and BALB/c^{nu} (n = 6) mice at 168 h after administration expressed as median %ID/g with IQR.

Fig. S5. Dose escalation of [^{89}Zr]Zr-N-suc-Df-ERY974 in immunodeficient NOG mice bearing different tumor xenografts. (**A**) *Ex vivo* biodistribution of [^{89}Zr]Zr-N-suc-Df-ERY974 in HepG2 at 168 h post injection with 10 µg in (n = 12), 2000 µg (n = 6), or 1000 µg GPC3 bivalent (n = 3), and in TOV-21G with 10 µg (n = 6) or 2000 µg (n = 2). Doses higher than 10 µg were supplemented with non-labeled ERY974 or GPC3 bivalent antibody. Data is expressed as median %ID/g with interquartile range (IQR). ** $P \leq 0.01$ (Mann-Whitney U). **(B)** Uptake of [^{89}Zr]Zr-N-suc-Df-ERY974 dose groups in blood expressed as median %ID/g with IQR. *$P \leq$ 0.05 (Mann-Whitney U). **(C)** Tumor-to-blood ratio of [^{89}Zr]Zr-N-suc-Df-ERY974 dose groups expressed as median with IQR. *$P \leq 0.05$; ** $P \leq 0.01$ (Mann-Whitney U). **(D)** Uptake of [^{89}Zr]Zr-N-suc-Df-ERY974 dose groups in liver expressed as median %ID/g with IQR. *$P \leq$ 0.05 (Mann-Whitney U).

Fig. S6. *Ex vivo* biodistribution of different tracers in different mice models at 168 h after tracer administration. **(A)** Biodistribution of 10 µg [^{89}Zr]Zr-N-suc-Df-ERY974 in NOG (n = 12) and huNOG (n = 5) mice expressed as median % injected dose per gram of tissue (% ID/g) with interquartile range (IQR). **(B)** Biodistribution of 10 µg [^{89}Zr]Zr-N-suc-Df-KLH/CD3 in NOG (n = 5), huNOG (n = 4), or huNOG mice co-injected with 10 µg ERY974 (n = 3) expressed as median % ID/g with IQR. **(C)** Biodistribution of 10 µg [^{89}Zr]Zr-N-suc-Df-KLH/KLH in NOG (n = 6), huNOG (n = 6), or huNOG mice co-injected with 10 µg ERY974 (n = 3) expressed as median % ID/g with IQR.

Fig. S7. Binding to peripheral blood mononuclear sites of huNOG mice injected with [^{89}Zr]Zr-N-suc-Df-ERY974, [^{89}Zr]Zr-N-suc-Df-KLH/CD3 or [^{89}Zr]Zr-N-suc-Df-KLH/KLH. Percentage of bound tracer to peripheral blood mononuclear cells (PBMCs) isolated from blood from huNOG mice injected with [^{89}Zr]Zr-N-suc-Df-ERY974 (n = 3), [^{89}Zr]Zr-N-suc-Df-KLH/CD3 (n = 4) or [^{89}Zr]Zr-N-suc-Df-KLH/KLH (n = 4).

Fig. S8. CD3 immunohistochemistry in HepG2 tumors of huNOG mice injected with [^{89}Zr]Zr-N-suc-Df-ERY974, [^{89}Zr]Zr-N-suc-Df-KLH/CD3 or [^{89}Zr]Zr-N-suc-Df-KLH/KLH. (A) Intratumoral (top panel; scale bar length represents 100 µm) and stromal (bottom panel; scale bar length represents 100 µm) CD3+ T cells in HepG2 tumors (middle panel; scale bar length represents 5 mm) of huNOG mice injected with [^{89}Zr]Zr-N-suc-Df-ERY974, [^{89}Zr]Zr-N-suc-Df-KLH/CD3 or [^{89}Zr]Zr-N-suc-Df-KLH/KLH. **(B)** Quantification of T cell infiltrations expressed as CD3+ cells/mm^2. Lines represent median with interquartile range. *P < 0.05.

Fig. S9. CD3 immunohistochemistry in HepG2 tumors of huNOG mice co-injected with ERY974. (A) Intratumoral CD3+ T cells in HepG2 tumors of huNOG mice injected with [^{89}Zr]Zr-N-suc-Df-KLH/CD3 or [^{89}Zr]Zr-N-suc-Df-KLH/KLH co-injected with ERY974. Scale bar length represents 100 µm. **(B)** Quantification of CD3+ T cells expressed as CD3+ cells/mm^2.

Fig. S10. Immunohistochemical staining validation. **(A)** Glypican 3 (GPC3) or isotype control staining on human placenta tissue or huNOG HepG2 tumors. Scale bar length represents

100 µm for placenta and 2.5 mm for HepG2 tumor. **(B)** CD3 or isotype control staining on human liver or huNOG HepG2 tumors. Scale bar length represents 50 µm for liver and 500 µm for HepG2 tumor.

References

1. Warnders FJ, Waaijer SJ, Pool M, Lub-de Hooge MN, Friedrich M, Terwisscha van Scheltinga AG, et al. Biodistribution and PET imaging of labeled bispecific T cell-engaging antibody targeting EpCAM. J Nucl Med. 2016;57(5):812-7.

2. Verel I, Visser GW, Boellaard R, Stigter-van Walsum M, Snow GB, van Dongen GA. ^{89}Zr immuno-PET: comprehensive procedures for the production of ^{89}Zr-labeled monoclonal antibodies. J Nucl Med. 2003;44(8):1271-81.

3. Bankhead P, Loughrey MB, Fernandez JA, Dombrowski Y, McArt DG, Dunne PD, et al. QuPath: open source software for digital pathology image analysis. Sci Rep. 2017;7(1):16878.