Efficacy and safety of glycosphingolipid SSEA-4 targeting CAR-T cells in solid tumors

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Abstract

Chimeric antigen receptor (CAR) T-cell immunotherapies for solid tumors face critical challenges such as heterogeneous antigen expression. We characterized SSEA-4 cell-surface glycolipid as a target for CAR-T cell therapy. SSEA-4 is mainly expressed during embryogenesis but is also found in several cancer types making it an attractive tumor-associated antigen. Anti-SSEA-4 CAR-T cells were generated and assessed pre-clinically *in vitro* and *in vivo* for anti-tumor response and safety. SSEA-4 CAR-T cells effectively eliminated SSEA-4 positive cells in all tested cancer cell lines whereas SSEA-4 negative cells lines were not targeted. In vivo studies using NSG mice and the high-grade serous ovarian cancer cell line OVCAR4 demonstrated a remarkable and specific anti-tumor response at all CAR-T cell doses used. At high T cell doses, CAR-T cell-treated mice showed signs of health deterioration after a follow-up period. However, severity of toxicity was reduced with delayed onset when lower CAR-T cell doses were used. Our data demonstrate the efficacy of anti-SSEA-4 CAR-T therapy; however, safety strategies, such as dose-limiting and antibody-dependent cellular cytotoxicity, should be implemented for its potential clinical translation.

Introduction

Cancer immunotherapies aimed at harnessing the immune system to fight refractory malignancies are rapidly evolving and have a promising potential. Based on the cancer immunosurveillance hypothesis (Ochsenbein, 2002), chimeric antigen receptor T (CAR-T) cell therapies targeting CD19 (Greenbaum *et al*, 2020) have shown remarkable success in acute lymphoblastic and B-cell lymphomas (Waldman *et al*, 2020). In CAR-T cell therapy, patients' T cells are genetically engineered *ex vivo* to express synthetic single-chain variable fragment (scFv) chimeric antigen receptors (CAR) against tumor antigens and subsequently infused back into patients, mounting a cytotoxic anti-tumor response upon antigen recognition (Walsh *et al*, 2019).

In contrast to hematological malignancies, CAR-T cell therapies for solid tumors have produced limited efficacy and currently face multifactorial challenges for successful clinical translation. Solid tumors grow in functional association with the tumor microenvironment (TME) (Anderson & Simon, 2020). Typically composed of immune and stromal cells, blood vessels, and extracellular matrix (ECM), the TME constitutes a physical barrier and hostile immunosuppressive environment that CAR-T cells must overcome to target cancer cells (Rafiq *et al*, 2020; Rodriguez-Garcia *et al*, 2020). Furthermore, solid tumors are heterogeneous and express a diversity of tumor-associated antigens (TAA), which impair single-antigen CAR-T cell efficacy and lead to antigen escape and tumor relapse. Addressing these challenges in CAR-T cell therapy for solid tumors is thus necessary, and innovative strategies, such as the identification of actionable tumor antigens and cancer stem-like cell (CSC) targeting, are currently under active investigation (Newick *et al*, 2017; Wagner *et al*, 2020; Yamamoto *et al*, 2019).

CAR-T cells can trigger life-threatening toxicities, including cytokine release syndrome (CRS), neurotoxicity, and on-target off-tumor activity due to TAA expression in normal tissues (Sterner & Sterner, 2021). Management strategies for CAR-T cell therapy toxicities involve systemic immunosuppression, CAR-T cell depletion using antibody-dependent cytotoxicity (ADCC), and CAR-T cell dose evaluation to prevent potential off-targeting (Brandt *et al*, 2020; Brudno & Kochenderfer, 2016; Dasyam *et al*, 2020). Analogous to normal stem cells (SCs), CSCs are a rare and persistent subpopulation of cancer cells with a strong ability to self-renew and produce heterogeneous progenitor cells that comprise the bulk of the tumor. CSCs have been identified in various malignancies, including ovarian, pancreatic, and gastric cancers (Addeo *et al*, 2021; Askan *et al*, 2021; Terraneo *et al*, 2020), and are considered drivers of tumorigenesis, metastasis, and cancer

progression owing to their therapeutic resistance. Accordingly, CSCs constitute a potential key target for CAR-T cell therapy (Alhabbab, 2020; Prager *et al*, 2019).

Stage-specific embryonic antigen-4 (SSEA-4) is a developmentally regulated glycosphingolipid (GSL). SSEA-4 expression has been reported to be restricted mainly to early embryogenic stages and pluripotent SCs and is mostly absent in differentiated cells and tissues (Russo *et al*, 2018). GSLs play essential roles in cell adhesion, proliferation, and signal transduction. Consistently, malignant transformation encompasses cellular GSL re-programming (Munkley & Elliott, 2016). In functional association with tumorigenesis and cancer progression (Sivasubramaniyan *et al*, 2015), SSEA-4 is expressed in ovarian, breast, lung, pancreatic, and brain cancer cells, as well as in breast and ovarian CSCs (Ho *et al*, 2017; Parte *et al*, 2018), thereby making SSEA-4 an attractive target antigen for CAR-T cell therapy (Firuzi *et al*, 2019; Lou *et al*, 2014a; Soliman *et al*, 2020). In this study, we analyzed the expression of SSEA-4 in different human tumor tissues, as well as in cultured ovarian, pancreatic, and gastric cancer cell lines, and tested SSEA-4-targeting CAR-T cells pre-clinically *in vitro* and *in vivo* for their therapeutic efficacy and safety.

Results

Characterization of SSEA-4 expression in human cancer specimens and cell lines.

SSEA-4 expression has been demonstrated in various types of cancer (Sigal *et al*, 2022). To corroborate and extend previous findings, we stained formalin-fixed paraffin-embedded (FFPE) clinical gastric cancer samples. We observed only a low signal or complete absence of SSEA-4 immunoreactivity in all studied samples (Supplementary Fig. S1A). Next, we investigated whether the lack of specific staining was due to the organic solvent treatment normally used during staining of FFPE samples. In fresh frozen (FF) tumor samples, we detected strong SSEA-4 immunoreactivity in several cancer types, including gastric cancer (Fig. 1A-C). In contrast, the treatment of FF tumor tissue sections with a standard xylene/ethanol series resulted in SSEA-4 signal loss (Supplementary Fig. S1B). This finding indicates that the organic solvent treatment is not suitable for SSEA-4 detection. To confirm the specificity of the anti-SSEA-4 antibody, we stained FF pancreatic cancer samples with soluble SSEA-4 hexaose (blocking glycan). This resulted in the loss of the SSEA-4 signal, which was specific for SSEA-4, as the closely related glycolipid, SSEA-3, did not block SSEA-4 immunoreactivity (Supplementary Fig. S1C). SSEA-4 expression was heterogeneous in FF tissues from ovarian (OC), pancreatic (PC), and gastric (GC) cancers (Fig. 1A-C). In OC, 19 of 22 samples (86%) expressed SSEA-4 with variable intensity and extent (Fig. 1A). In some samples,

SSEA-4 expression was observed in both tumor cells and the surrounding stroma, whereas in others, its expression appeared to be restricted to the stroma. Focusing on OC, and for a more precise analysis, we performed double immunofluorescence staining for SSEA-4 and tumor cell-specific PAX8, which revealed the localization of SSEA-4 in tumor cells or stroma (Fig. 1A). In PC, 19 of 23 samples (83%) expressed SSEA-4 (Fig. 1B). Tumor cell and acinar cell immunoreactivity were also detected. Similar to OC cases, some samples showed SSEA-4 signals only in the stroma. In GC, eight of the nine samples (89%) were positive for SSEA-4, with expression mostly localized to tumor cells (Fig. 1C).

To find suitable SSEA-4 expressing cell models, we analyzed SSEA-4 expression in a variety of cancer cell lines using FACS. Some cell lines representing the aforementioned cancer types were either highly positive (OVCAR4, HPAC, and MKN45; OC, PC, and GC, respectively) or very low/negative (Caov-3, MIA PaCa-2, and MKN28; OC, PC, and GC, respectively) for SSEA-4 (Fig. 1D). The FACS results were further confirmed by immunofluorescence staining of OC cell lines (Supplementary Fig. S1D).

Generation and *in vitro* characterization of SSEA-4 specific CAR-T cells.

To obtain optimal expression of a long RNA encoding multiple gene products and to fulfil regulatory requirements in future clinical trials, a previously described anti-SSEA-4 CAR vector (Lin *et al*, 2021) was modified by replacing the cytomegalovirus (CMV) promoter with EF-1α (human elongation factor-1 alpha) and ampicillin (AmpR) cassette with kanamycin (KanR). For cell selection, tracking, and CAR-T cell depletion by cetuximab-mediated ADCC, a truncated human epidermal growth factor receptor (EGFRt) (Wang *et al*, 2011) (Fig. 2A) was inserted downstream of the CD28 and CD3zeta sequences (costimulatory/activation domains). A poly A-like sequence in EGFRt was also removed. Anti-SSEA-4 CAR-T cells (hereinafter referred to as CAR-T) were generated from healthy donor-derived $CD4^+$ and $CD8^+$ T cell populations, transduced at efficiencies ranging from to 35-90% (Fig. 2B and C), and expanded for 10-12 days with $CD4^+/CD8^+$ ratios of ≈ 1 (Fig. 2D and E). Non-transduced T-cells from the same donor (NT-T) were used as control.

To assess CAR-T anti-tumor response and specificity, we performed *in vitro* cytotoxicity assays in SSEA-4^+ OVCAR4 (OC), HPAC (PC), and MKN45 (GC), as well as in SSEA-4^- Caov-3 (OC), MIA PaCa-2 (PC), and MKN28 (GC) human cell lines. The specificity and efficacy of CAR-T cells were evaluated at different T-cell doses in comparison with NT-T and GFP (mock) CAR-T cells. CAR-T cells killed SSEA-4^+ OC, PC, and GC cells efficiently (Fig. 2F) but did not show any significant cytotoxic activity against SSEA-4^- cells. NT-T and GFP CAR-T cells showed no effect on any cell

type tested, further corroborating the specific SSEA-4 targeted anti-tumor response of the CAR-T cells. The CAR-T cell response was dose-dependent and detectable at E:Ts (effector : target cell ratios) as low as 0.12 in all SSEA-4^{+} cell lines tested. In contrast to NT-T cells, the interaction of CAR-T cells with OVCAR4 cells, but not with Caov-3 cells, induced T-cell proliferation (Fig. 3A-C), indicating T-cell activation in response to SSEA-4. A significant increase in IFNγ and IL2 secretion was also observed in CAR-T cells, but not in NT-T cells, when co-cultured with OVCAR4 cells (Fig. 3D and E), further supporting the specific SSEA-4 -induced functional activation of CAR-T cells. To evaluate a potential safety strategy, we assayed ADCC *in vitro* using a cetuximab antibody targeting EGFRt (Brandt *et al.*, 2020). CAR-T cells were efficiently depleted by NK cells in response to two different concentrations of cetuximab, but not when NK + CAR-T cells were co-cultured without cetuximab (Fig. 3F).

SSEA-4 CAR-T cells produce robust anti-tumor response in the OVCAR4 xenograft model.

Next, we evaluated the *in vivo* efficacy of SSEA-4 CAR-T cells in ovarian cancer cell xenografts. NSG (NOD scid gamma) female mice were implanted intraperitoneally (i. p.) with 2x10^{6} luciferase- and GFP-expressing OVCAR4 cells. After 13 days, mice were infused intravenously with T cell doses of 1x10^{6}, 2x10^{6} and 3x10^{6} CAR-T cells (hereinafter referred as 1M, 2M and 3M), corresponding to 2.9x10^{6}, 5.7x10^{6} and 8.6x10^{6} of total T cells based on 35% lentivirus transduction efficiency in the cell batch used in this experiment. Control mouse groups were infused with PBS or an NT-T cell dose equal to the total T cell number in the CAR-T groups (Supplementary Fig. S2A). Thirty-two days after T-cell infusion, CAR-T cell-treated mice in all dose groups presented a remarkable and specific reduction in tumor burden, in contrast to PBS- and NT-T cell-treated mice showing continuous tumor growth (Fig. 4A-D). However, mice treated with the 3M dose of NT-T cells displayed a similar reduction in tumor burden as the CAR-T cell-treated group, revealing an upper threshold of ≈ 5-6x10^{6} total T cells for the specific anti-tumor response in the OVCAR4 i. p. xenograft model (Fig. 4D). All mice remained in apparently good body scoring conditions (BCS ≈ 3) during the 32-day experiment, except for 3M CAR-T mice, which showed a trend for weight loss at the later stages. (Supplementary Fig. S2B). T cell activation was confirmed by measuring IFNγ levels in post-mortem mouse blood samples. IFNγ secretion was significantly elevated in mice treated with 1M, 2M and 3M CAR-T cell doses compared to mice infused with PBS or NT-T cells. In line with the anti-tumor response of NT-T cells at high doses, elevated levels of IFNγ were also detected in 3M NT-T cell-treated mice (Supplementary Fig. S3A). Bioluminescence imaging (BLI) of the post-mortem organs corroborated the specific reduction in tumor burden in mice treated with 1M, 2M and 3M CAR-T doses, showing

a substantially reduced signal in all organs examined when compared to PBS- and NT-T-treated mice. The cancer cell signal in our OVCAR4 xenograft model was preferentially associated with the spleen, pancreas, ovaries, intestine, and stomach. Similar to the whole-body BLI results, mice treated with the 3M NT-T cell dose showed reduced organ BLI signal (Supplementary Fig. S3B and C). In contrast to OVCAR4-engrafted CAR-T mice, human gene expression, including *ACTB*, *TIM-3*, *LAG-3*, and *PD-1*, was detected at very low levels in post-mortem blood samples of OVCAR4-engrafted PBS-treated control mice (Supplementary Fig. S4A-C), thereby indicating that the detection of human gene expression in OVCAR4-engrafted CAR-T mice originated mostly from human T cells, and further allowed for an approximate analysis of the CAR-T exhaustion phenotype in mice. As shown in (Supplementary Fig. S4D-F), the expression of *TIM-3*, *LAG-3* and *PD-1* T cell exhaustion markers in CAR-T mice was dose-dependent and significantly elevated at 3M doses compared to 1M and 2M doses. Altogether, the data indicated that at lower doses (1M and 2M), anti-SSEA-4 CAR-T cells were highly effective, with a robust and specific anti-tumor response and reduced exhaustion phenotype in the OVCAR4 xenograft models.

SSEA-4 CAR-T cells cause long-term adverse effects.

Although there was no apparent toxicity in mice in the above-described 32-day efficacy *in vivo* study, modest signs of toxicity were observed in mice treated with the 3M CAR-T cell dose. We therefore carried out a long-term (70-day), safety, efficacy, and survival assay *in vivo* using 1M and 2M CAR-T cell doses, corresponding to 1.1×10^6 and 2.2×10^6 T cells based on 90% lentivirus transduction in the cell batch used in this experiment (Supplementary Fig. S5A). As previously described, tumor-bearing control mice were infused with PBS or an NT-T cell dose corresponding to the total number of T cells in the CAR-T groups. Tumor-free mice treated with a 1M dose were used to assess the potential toxicity of CAR/NT-T cells in the absence of OVCAR4 cells.

Corroborating the anti-tumor response of SSEA-4 CAR-T cells, both CAR-T doses led to a reduction in tumor burden compared to PBS treatment. While the 1M dose NT-T mice showed no significant reduction in their tumor burden, the NT-T 2M dose produced a significant anti-tumor response at the later stages of the 70-day treatment (Fig. 4E and F, Supplementary Fig. S5B and C). Consistent with the anti-tumor activity of CAR-T cells, mice treated with both 1M and 2M doses showed elevated levels of IFNγ and TNFα (Fig. 4G and H). All mice remained in good health during the first 35-40 days of treatment. However, thereafter, some tumor-bearing CAR-T cell-treated mice showed weight loss, inactivity, and an overall low BCS (≈ 1-2). Three out of 10 mice with 1M dose, and 5 out of 10 mice with 2M dose, had to be sacrificed before the experimental endpoint (Supplementary Fig. S5

and S6), translating in 70% and 50% survival rates, respectively (Fig. 4I). PBS, and the remaining CAR-T and NT-T mice were active and showed a good overall condition (BCS ≈ 3) and normal activity without signs of health deterioration. Similarly, tumor-free mice treated with CAR-T and NT-T cells remained healthy and active during the 70-day study period (Supplementary Fig. S6). Altogether, our data demonstrated the specific anti-tumor response of SSEA-4 CAR-T cells, even at relatively low doses. At high doses, CAR-T cells produced apparent toxicities in mice, thus precluding clinical translation at this stage.

SSEA-4 targeting of normal mouse cells as a potential cause for the *in vivo* adverse events during long-term SSEA-4 CAR-T cell treatment.

To examine the possible on-target off-tumor effects in mice treated long-term with SSEA-4 CAR-T cells, we analyzed the expression of SSEA-4 in normal mouse tissues. Prominent SSEA-4 expression was detected in the kidneys, stomach, and intestine and, to a lesser extent, in the brain and heart. The SSEA-4 signal was sparse in the lungs, whereas the spleen, pancreas, and liver were mostly devoid of SSEA-4 (Supplementary Fig. S7).

Histopathological analysis of post-mortem tissues was performed on tissues with high (kidney and intestine) or moderate (lung) SSEA-4 expression. The analysis included the estimation of lymphocyte infiltration and tissue damage, each graded on a scale from 0 to 3. Analysis of the H&E-stained sections revealed perivascular/peribronchiolar lymphocyte infiltrates in the lungs, and in the intestinal wall of the CAR-T-treated mice compared to the NT-T-treated mice (average grading 0 *vs* 1.43, respectively for mice treated with 2M doses; Supplementary Fig. S8A). Signs of aspiration pneumonia were observed in three CAR-T cell-treated and prematurely euthanized mice. However, there was no apparent, histologically appreciable, direct damage to the parenchymal cells (Supplementary Fig. S8A).

To further corroborate the histopathological findings, we performed IF staining of T cells (CD3) and CAR-T cells (anti-EGFR) in the lungs, kidneys, and intestine (Supplementary Fig. S8B). Consistent with H&E staining, the most prominent T cell deposits were observed in the lungs of both CAR- and NT-T cell-treated mice. CAR-T cells were also detected in the intestinal wall of CAR-T cell-treated mice. NT-T cells were not observed in NT-T-treated mice in the tissues analyzed.

Discussion

CAR-T cell therapy approaches in solid tumors face multifactorial challenges, including identification of actionable tumor antigens (Sterner & Sterner, 2021). To tackle this obstacle, we conducted a pre-clinical CAR-T cell study using SSEA-4 as a TAA in an ovarian cancer model.

SSEA-4 has been shown to be expressed in pluripotent SCs, and developmentally regulated (Russo *et al.*, 2018). Consistent with its role in proliferation and migration, aberrant expression of SSEA-4 confers tumor cells with malignant stem-like traits such as proliferation, drug resistance, and metastasis (Magalhães *et al*, 2017; Munkley & Elliott, 2016). Thus, SSEA-4 reprogramming of tumor cells provides an attractive target for CAR-T therapies in solid tumors. In line with this, CAR-T cells targeting glycans such as MUC1 and GD2 have shown high therapeutic potential against triple-negative breast cancer and glioblastoma (Prapa *et al*, 2021; Zhou *et al*, 2019). Moreover, SSEA-4 CAR-T cells enable targeting of tumor cells with potential stem-like properties (CSCs), which are closely associated with tumor heterogeneity and relapse (Ho *et al.*, 2017; Lou *et al*, 2014b; Prager *et al.*, 2019). Clinical trials using CAR-T cells against CSC markers include CD133, CD171, and c-Met for ovarian, brain, and breast cancers and neuroblastoma (Masoumi *et al*, 2021).

Confirming previous results (Sigal *et al.*, 2022), SSEA-4 expression was detected in most of the cases of ovarian, pancreatic, and gastric cancer tissues. Based on the positive SSEA-4 expression in several cancer types, anti-SSEA-4 CAR-T cells were generated and functionally characterized for specific and effective anti-tumor response and safety.

SSEA-4 CAR-T cells showed specific and effective anti-tumor responses *in vitro* in SSEA-4^{+} ovarian, pancreatic, and gastric tumor cell lines. CAR-T cells produced elevated levels of IFNγ and IL-2 in killing assays with ovarian cancer cells and, corroborating the specific interaction with SSEA-4, CAR-T cell proliferation was induced in co-cultures with SSEA-4^{+} ovarian cancer cells. Current strategies to overcome CAR-T cell therapy challenges involve engineering CAR-T cells with powerful anti-tumor activity (Sterner & Sterner, 2021). In this respect, SSEA-4 CAR-T cells demonstrated effective SSEA4^{+} targeting, even at very low E:T ratios in all *in vitro* cancer models.

High-grade serous ovarian carcinomas (HGSOC), represented by OVCAR4 cells in this study, constitute the most common and lethal gynecologic malignancies, typically forming immunologically "cold" tumors that are unresponsive to immune checkpoint blockage (Wu *et al*, 2021). To date,

responses to CAR-T therapies in HGSOCs have been relatively poor, mainly due to the deficient anti-tumor response of T cells and poor infiltration capacity in the tumor tissue (Wagner *et al.*, 2020). In our *in vivo* OVCAR4 xenograft models, SSEA-4 CAR-T cells produced an effective anti-tumor response at dose levels ranging from 1M to 3M, thereby indicating that they could infiltrate into the tumor tissue and develop potent anti-tumor activity.

In a long-term safety assessment, SSEA-4 CAR-T cells produced severe adverse events in some mice, manifested by abnormal inactivity and progressive weight loss, indicating a dose-limiting toxicity for SSEA-4 CAR-T cells. Notably, in our OVCAR4 xenograft model, lower CAR-T cell doses produced an effective anti-tumor response, which was similar to that observed at higher CAR-T cell doses, but with a delayed onset and reduced severity of adverse effects. Moreover, 1M dose CAR-T cell-treated mice with moderate weight loss at the early stages of the treatment progressively regained weight to normal levels and remained active and in good health condition (BCS ≈ 3) until the end of the study. The severity of the CAR-T cell associated adverse events is related, among the others, to CAR-T cell potency and dose (Zhou *et al*, 2020). Increasing the number of CAR-T cells has been shown to enhance tumor clearance but may also increase the risk of toxicities (Mehta *et al*, 2021). In line with this, strategies to prevent and manage toxicities include treatment with low or split doses of CAR-T cells (Green *et al*, 2018; Jiang *et al*, 2018). Based on our results, it is conceivable that, at very low doses or with a lower affinity scFv, SSEA-4 CAR-T cells could exert a potent anti-tumor response with minimal toxicity.

Targeting CSCs with CAR-T cell therapy constitutes a promising strategy for tumor eradication (Cui *et al*, 2021; Masoumi *et al.*, 2021). However, the expression of CSC antigens may also occur in normal stem cells, thus increasing the risk of on-target off-tumor toxicities. In this regard, infiltrated SSEA-4 CAR-T cells in the SSEA-4 positive mouse intestinal wall may have impaired the normal absorption function of the gut, even though no direct damage to the epithelium was detected. Similarly, prominent lymphocyte infiltration was observed in the lungs of the CAR-T cell-treated mice. Accumulation of SSEA-4 CAR-T cells in the lungs and intestine might have produced severe inflammation, thereby affecting the normal respiratory and digestive functions in CAR-T-treated mice.

Tumor-free mice treated with CAR/NT-T cells at 1M dose showed no signs of health deterioration and remained active and healthy throughout the study. The absence of toxicities in these mice, and in contrast to tumor-bearing mice, strongly suggests that an OVCAR4-dependent over-stimulated

SSEA-4 CAR-T cell immune response caused serious adverse effects. NT-T cells at 3M dose, and to some extent at 2M dose, also reduced OVCAR4 tumor burden, thereby demonstrating a CAR-independent anti-OVCAR4 response. Further supporting this notion, our *in vitro* results showed a dose-dependent anti-OVCAR4 response in NT-T cells. Moreover, a recent study using SSEA-4 CAR-T cells in a pancreatic NSG mouse xenograft model showed no anti-tumor response for NT-T cells at doses up to 5M (Lin *et al.*, 2021). Taken together, these data indicate that OVCAR4 cells may be particularly prone to elicit CAR-independent, high tumor-specific T cell reactivity (Disis *et al*, 2009). Accordingly, at high CAR-T cell doses, the occurrence of both CAR-dependent and CAR-independent interactions with OVCAR4 tumor tissues could have resulted in over-stimulated T-cell activation, proliferation, inflammation, and damage to SSEA-4 positive normal tissues.

In conclusion, this study demonstrated the efficacy of SSEA-4 CAR-T cells in an HGSOC pre-clinical model. However, SSEA-4 CAR-T cell therapy produced severe adverse effects; therefore, further assessment and/or toxicity management strategies are required for its clinical translation. Alternative strategies could involve lowering the CAR-T cell dose or scFv affinity, ADCC, equipping CAR-T cells with combinatorial antigen recognition (Choe *et al*, 2021; Kloss *et al*, 2013) such as SSEA-4 and mesothelin (Schoutrop *et al*, 2021), and/or sigmoidal antigen-density threshold (Hernandez-Lopez Rogelio *et al*, 2021) using low-affinity synthetic Notch receptors to control the expression of the high-affinity anti-SSEA-4 CAR.

Materials and methods

Tissue processing and staining. Fresh frozen HGSC tumor samples were embedded in Tissue-Tek OCT compound (Sakura Finetek), frozen, and cut into 8-µm cryosections. Frozen sections were placed in 4% PFA in PBS for 10 min and washed with PBS for 10 min. For immunofluorescence staining sections were blocked with TNB blocking buffer (0.1 M Tris–HCl, pH 7.5; 0.15 M NaCl; 0.5% (w/v) blocking reagent (PerkinElmer, Cat # FP1020) for 30 min at RT. The sections were incubated overnight at 4 °C in a humidified chamber with SSEA4 antibody (1:100, GTX48037, Genetex; SSEA-4 hexaose [Elicity #GLY131-90%] blocking glycan was used as a negative control at 100 µM). After washes with TNT (0.1 M Tris-Cl, pH 7.5; 0.15 M NaCl; 0.1% (v/v) Tween 20), sections were incubated with PAX8 antibody (1:250, #10336-1-AP, Proteintech) and anti-mouse Alexa Fluor Plus 555 secondary antibody in TNB for 90 min and washed with TNT. The sections were then incubated with anti-rabbit Alexa Fluor Plus 488 secondary antibody in TNB for 90 minutes, washed with TNT, and rinsed with PBS. Slides were mounted with VECTASHIELD Antifade

Mounting Medium containing DAPI. Confocal micrographs of immunofluorescence staining were obtained using a confocal microscope (LSM 800) with a C-Apochromat 40×, 1.2 NA water objective lens, and a Plan-Apochromat 20×, 0.8 NA objective lens (Carl Zeiss). For immunohistochemistry, sections were blocked with 3% BSA in PBS for 30 min and incubated with SSEA-4 antibody (GTX48037, Genetex) overnight, followed by incubation with Brightvision poly HRP-anti-mouse antibody, washing, DAB solution, and hematoxylin staining using standard protocols.

Cancer cell culture. OVCAR4 and Caov3 (human ovarian adenocarcinoma; RRID:CVCL_1627 and LGC Nordic ATCC HTB-75, respectively), HPAC and MIA PaCa-2 (human pancreatic adenocarcinoma; LGC Nordic, ATCC CRL-2119, and CRL-1420, respectively), and MKN45 and MKN28 (human gastric adenocarcinoma; RRID:CVCL_0434 and RRID:CVCL_1416, respectively) were cultured in RPMI medium (Roswell Park Memorial Institute 1640. #BE12-167F, Gibco) supplemented with 10% fetal bovine serum (FBS, qualified Brazil. #10270106 Gibco), 2 mM L-glutamine (#BE17-605E, Gibco), 100 U/mL penicillin, and 100 µg/mL streptomycin (#DE17-602E, Gibco) and subcultured in phosphate-buffered saline (1X; PBS. #MT21040CV, Gibco) and trypsin-EDTA solution (#BE17-161E; Lonza, Basel, Switzerland). HEK293T cells (human embryonic kidney; LGC Nordic ATCC CRL-3216) were cultured in DMEM high glucose (#BE12-614Q, Lonza), supplemented with 2 mM L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin, and sub-cultured with PBS and trypsin. All the cell lines were authenticated using the Genome Analysis Infrastructure (HiLIFE; Helsinki Institute of Life Science) and assayed at low passage numbers. All cells were incubated at 37°C in 5% CO2 and routinely tested for mycoplasma-free status (Eurofins Genomic Europe, Germany).

Flow cytometry analysis. 0.5-1x10^6 Cells were labeled per tube in staining buffer (1% BSA/2 mM EDTA/PBS). Cells were fixed with 4% PFA for 10 min (where appropriate) at room temperature, 3x washed in buffer (2 mM EDTA/PBS), blocked for 30 min in staining buffer, and subsequently stained with primary and secondary antibodies in 100 µL volume, with 3x washes in between where appropriate, and after staining. The cells were resuspended in 200 µL wash buffer and analyzed using BD Accuri C6 Flow Cytometer Analyzer and Accuri Cflow Plus software. The gating strategy followed FSC-A *vs* SSC-A size and granularity, FSC-H *vs* FSC-A doublets, and corresponding FL *vs* FSC-A marker detection. Negative controls included isotypes, not stained samples, and where appropriate NT-T cells were used. The following antibodies were used in this study: Alexa Fluor 488 anti-human SSEA-4 (#330412, clone MC-813-70, Mouse IgG3 κ, dilution 1:100, Biolegend); Alexa Fluor 647 anti-human SSEA-4 Antibody (#330408, clone MC-813-70, Mouse IgG3 κ, dilution 1:100,

Biolegend); Cetuximab (#A2000, Human IgG1, dilution 1:100, Selleckchem); Alexa fluor 488 Goat anti-Human IgG Secondary (#A-11013, dilution 1:100, Invitrogen); APC anti-human CD4 Antibody (#357408, Rat IgG2b κ, dilution 1:20, Biolegend); FITC anti-human CD8 Antibody (#344704, Mouse IgG1 κ, dilution 1:20, Biolegend); Alexa Fluor 647 anti-human CD3 Antibody (#300322, Mouse IgG2a κ, dilution 1:100, Biolegend); APC Rat IgG2a κ Isotype Ctrl (#400512, dilution 1:20, Biolegend); FITC Mouse IgG κ Isotype Ctrl (#00108, dilution 1:20, Biolegend); APC Mouse IgG2a κ Isotype Ctrl (#981906, dilution 1:100, Biolegend).

Immunofluorescence. OVCAR4 and Caov-3 cells were grown on glass coverslips at low confluence for 24 h. Next, the cells were fixed with 4% PFA (#HL96753.1000, without methanol, Histolab), blocked with staining buffer for 30 min, stained with Alexa Fluor 647 anti-human SSEA-4 antibody (dilution 1:100) overnight at 4ºC, 3x washed with PBS, and counterstained with Hoechst 33342 (#H21492, Invitrogen). The slides were mounted with mowiol (#81381; Sigma-Aldrich). Slides were visualized with Axio Imager (Zeiss upright epifluorescence microscope) with an EC Plan Neofluar 40x 1.3 NA water objective lens and Plan-Apochromat 63x 1.4 NA oil objective lens (Zeiss). Images were acquired using Hamamatsu Orca Flash 4.0 LT B&W camera for fluorescence and Zeiss Zen 2 software.

Luciferase/GFP expression. Mammalian gene expression lentiviral vector pLV-EF1A>Luc2:T2A:Puro:F2A:TurboGFP (#VB210127-1246ann, VectorBuilder) was used to produce luc2/tGFP carrying lentivirus. The expression vector and packaging plasmids, pLP1, pLP2, and pLP/VSVG, were transfected into HEK293T cells. The viral supernatant was collected after 72 h, 0.45µm filtered, and precipitated with PEG-it (#LV825A-1, System Biosciences). The virus precipitates were resuspended in PBS and stored at -80°C. Tumor cells were transduced with a MOI of 10 for 24 h using Vectofusin-1 (10 µg/mL; #130-111-163, Miltenyi Biotec), with the following selection in Puromycin 1-2 µg/ml (#P8833, Sigma-Aldrich).

Generation of anti-SSEA-4 and anti-GFP CAR vector. The anti-SSEA-4 scFv was designed by CHO Pharma USA Inc. and inserted into a 2nd generation CAR lentivector containing the CD28z co-stimulatory/activation domains; EGFRt sequence was cloned downstream of the CD28z sequence (Creative Biolabs; US). For anti-GFP CAR-T cells, anti-SSEA-4 scFv was replaced with anti-GFP scFv (Creative Biolabs). The CMV promoter was replaced with EF-1α for optimal expression of long RNA encoding multiple gene products, and the AmpR cassette was replaced with KanR to fulfil regulatory requirements in pre-clinical assessment and clinical trials (FIMEA; Finnish Medicines

Agency) (Oxford Genetics; UK). VIVE Biotech (ES) identified and removed a poly A-like sequence in EGFRt.

Generation of human anti-SSEA-4 CAR-T cells. Human blood samples (~ 40 ml) were obtained from healthy donors at the Finnish Red Cross Blood Services (FRCBS) and subsequently diluted 1:4 in PBS. Peripheral blood mononuclear cells (PBMCs) were separated by density gradient centrifugation using Ficoll-Paque Premium (#17-5442-02; GE Healthcare). PBMCs were washed 3x with PBS, filtered with a 30 µm cell strainer (#43-50030-03, Pluriselect), and cryopreserved in cryotubes (#177280, Nunc) in LN2 at $20x10^6$ cells/ml in 10% DMSO (cell culture reagent, #02196055-CF, MPBio)-Albunorm (200 g/l, #054343, Octopharma). CAR-T cells were generated following the Miltenyi protocol using TexMACS medium (#130-097-196), human CD4+ and CD8+ T-cell isolation kits (#130-096-533 and #130-096-495, respectively), T Cell TransAct human (#130-111-160), human IL-7/IL-15 cytokines (#130-095-362 and #130-095-764, respectively), and Vectofusin-1 (#130-111-163, Miltenyi Biotec). Briefly, PBMCs were thawed in a 37°C water bath for a few minutes, washed 2x with TexMACS medium, and incubated in TexMACS for 3 h. Subsequently, $CD4^+$ and $CD8^+$ T cells were isolated, combined, and activated/expanded in TexMACS medium supplemented with TransAct, IL7 (500 IU/mL), and IL-15 (84 IU/mL). After 24 h, CD4+ and CD8+ T cells were transduced with anti-SSEA-4 CAR lentivirus with a MOI of 5 for 24 h, using Vectofusin-1 (10 µg/mL) and spinoculation (800 × g for 30 min at 32°C). Subsequently, the culture medium was replaced with TexMACS supplemented with cytokines, and the cells were cultured for an additional 8-10 days, with half of the media changed every 2-3 days. For anti-GFP (mock) CAR-T cells, CD4+ and CD8+ T cells were transduced with anti-GFP CAR lentivirus.

Cytotoxicity assays. Cytotoxic killing of target cells was assessed using Thermo Scientific Black 96-Well Immuno Plates. Tumor cells were plated at $0.5x10^4$ (OVCAR4) and $1x10^4$ (Caov-3, HPAC, MIA Paca-2, MKN45, and MKN28) cells/well. All target cells expressed the Firefly luciferase (FLuc) and turboGFP (tGFP) reporter genes to facilitate detection. After overnight culture, anti-SSEA4, anti-GFP CAR-T, and NT-T cells were added at the indicated E:T (effector : target cell ratio), and incubated for 72 h. Cell viability was measured using the Steady-Glo Luciferase Assay System (Promega) as indicated by the manufacturer in FLUOstar Omega Filter-based multi-mode microplate reader (BMG LabTech). Tumor cell-killing efficacy is represented as % cell viability relative to untreated cultures (E:T=0).

CFSE-dilution assay T cell proliferation. CAR and NT-T cells were labeled with CFSE (#C34554, CellTrace CFSE Cell Proliferation Kit Molecular Probes) according to the manufacturer's instructions and at 1µM working concentration. $2.5x10^5$ CAR or NT-T cells were incubated with SSEA-4 and SSEA-3 (#GLY131 and #GLY122, respectively, Elicityl-Oligotech) at 200 µM for 48 h in 24 well plate (Nunc). $5x10^5$ CAR and NT-T cells CFSE-labeled were incubated with or without $1x10^6$ OVCAR4 and Caov-3 cells for 48 h in RPMI-10% FBS-Pen/Strep and L-Glut medium in 10 cm cell culture dishes (Nunc). CFSE dilution was analyzed using flow cytometry on $CD3^+$-gated cells; isotype/unstained cultures were used as $CD3^-$ control gating, and CFSE-unlabeled CAR and NT-T cells were used as gates for negative CFSE detection. Cell proliferation was assessed in relation to the corresponding CFSE-labeled CAR and NT-T cells cultured in the absence of cancer cells.

Cetuximab-based antibody-dependent cellular cytotoxicity. Human NK cells were isolated from PBMCs using the NK Cell Isolation Kit, human (#130-092-657, Miltenyi), according to the manufacturer's instructions, and subcultured for 72 h in NK MACS medium supplemented with 5% human serum male AB plasma (#H4522, Sigma-Aldrich) and human IL-2 IS (200 IU/ml, # 130-097-746). Next, $0.5x10^6$ NK cells were incubated with $1x10^6$ CAR-T cells in the presence of 5-10 µg/ml human cetuximab. After 72 h, the cultures were washed and stained sequentially with CD3 and 1 µg recombinant protein L PE-conjugate (#11044-H07E-P, SinoBiological) following the manufacturer's recommendations. CAR-T cell viability was assessed using flow cytometry on $CD3^+$-gated T cells, and isotype/unstained cultures were used for $CD3^-$ control gating.

Cytokine secretion in *in vitro* assays. Secretion of human IFNγ was assessed from the supernatants of cytotoxic assays where cancer and T cells were co-cultured for 72 h in a 24-well format ($0.5x10^5$ CAR and NT-T cells co-cultured with $1x10^5$ OVCAR4 cells), and mouse plasma samples, using ELISA MAX Deluxe Set human (IFNγ #430104, IL-2 # 431804, and IL-6 #430504, BioLegend) according to the manufacturer's instructions. The absorbance was measured at 450 nm using FLUOstar reader.

Animals and ethics statement. All mice were housed in the Laboratory Animal Center facility, Biomedicum 1, Helsinki University, under pathogen-free conditions and in ambient rooms with a 12 h light/dark cycle, filtered air at 22 ± 2°C temperature, and 55 ± 15% relative humidity. Mice were caged in groups of two or three per IVC cage (Green-Line mice. Emerald. Tecniplast) on autoclaved ASPEN wood chip bedding, with ad libitum access to laboratory chow (Tekland Global Rodent Diet;

T2916CMI.16) and sterile water. Animal experiments in this study were approved by the Finnish National Animal Experiment Board (Eläinkoelautakunta ELLA. Approved license: ESAVI/10548/2019) and conducted in accordance with the Finnish Act on the Protection of Animals Used for Scientific or Educational Purposes, and the University of Helsinki guidelines.

Animal studies and bioluminescence imaging. Female NSG NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ mice (4–5 weeks old) were purchased from The Jackson Laboratory (Scanbur). 2x10^{6} OVCAR4 Fluc-tGFP cells, in 100 µL PBS, were injected intraperitoneally into isoflurane-anesthetized mice (Attane vet, 100 mg/g) and grown for 13 days. Subsequently, CAR and NT-T cells were infused intravenously (d0) at the specified T-cell dose in 100 µl PBS. *In vivo* CAR-T cell dose is defined in this study as the specific number of CAR-T cells and was calculated based on the transduction percentage. Equal numbers of NT-T cells were used for each cell dose (Supplementary Fig. S2 and S5). For the no-treatment control group, mice were infused with 100 µL of PBS. Tumor progression was monitored by BLI with CoA D-luciferin sodium salt substrate (#bc218, Synchem) injected intraperitoneally at 150 mg/kg. Bioluminescence images were acquired 10 min after D-luciferin injection with the SPECTRAL Lago X Imaging System and analyzed with Aura Imaging Software v3.1.0 (Spectral Instruments Imaging). Mice were monitored at regular intervals for behavior, activity, feeding, and weight. At the end of the experiment, the mice were anesthetized with ketamine-xylazine (#511485, Intervet; #148999, Orion Pharma) and blood samples were collected via cardiac puncture in EDTA capillary tubes (#076012, Kabe Labortechnik). Plasma and blood cells were separated by centrifugation (500 × g for 5 min at 4°C) and stored at -80°C. Organs/tissues were collected post-mortem, imaged for bioluminescence, and fixed/stored in 4% PFA (without methanol) at 4°C.

Cytokine secretion in *in vivo* assays. Human cytokine secretion was assessed in mouse plasma samples using the Human XL Cytokine Luminex Performance Panel Premixed Kit (#FCSTM18, R&D Systems), according to the manufacturer's instructions.

RNA isolation and qPCR. Mouse blood cells were pelleted by centrifugation, washed with 1x PBS at 4°C, resuspended in Trisure (#BIO-38032, Bioline), and homogenized with high-density zirconium oxide beads in a PowerLyzer (Mo Bio). Chloroform was added, mixed, and the samples were centrifuged at 11000 × g for 10 min at 4ºC °C. The upper aqueous phase was subsequently used for RNA isolation using the NucleoSpin RNA, Mini it for RNA purification (#740955.50, Macherey-Nagel), and cDNA was synthesized using iScript Reverse Transcription Supermix (#1708841, Bio-Rad). Gene expression was assessed using TaqMan assays (Thermo Fisher), human ACTB

(Hs99999903_m1), human LAG-3 (#Hs99999160_m1), human TIM-3/HAVCR2 (#Hs00958618_m1), human PD-1/PDCD1 (#Hs1550088_m1), and mouse Gapdh (Mm03302249_g1), according to the manufacturer's instruction in 384 well plates (4ti-0382; 4titude) format, and the LightCycler 480 PCR platform (Roche Diagnostics). Ct values above 35 were considered not detected, and the relative fold change in expression was calculated using the delta-delta Ct method.

Histopathology. Tissue samples were shipped in 10% formalin solution at RT to HistoWiz Inc. (US) for processing and histological examination by a certified pathologist.

Ethics. Samples from patients with metastatic HGSC were obtained at The Karolinska University Hospital under protocols approved by The Swedish Ethical Review Agency (Etikprövningsmyndigheten; 2016/119731/1, 2016/2060-32). All methods for the use of human samples were carried out in accordance with relevant guidelines and regulations. Informed consent was obtained from all the patients. For gastric and pancreatic cancer samples, the study has the approval of the Helsinki University Hospital Ethics Committee (document number HUS 226/E6/06, additional petition TMK02 § 66/2013 and HUS/1223/2021). Permission to use archive material for this retrospective study without individual informed consent from the patients has been granted by the Finnish National Supervisory Authority for Welfare and Health (document number 1004/06.01.03.01/2012) and the Finnish Medicines Agency Fimea (document number FIMEA/2021/006901).

Statistics. *In vitro* experiments were independently performed at least three times, and at least three mice per donor were compiled for *in vivo* experiments unless otherwise specified in the figure legends. Data processing was performed using Microsoft Excel 2016, and graphing and statistical analyses were performed using GraphPad Prism 9.0 Windows 64-bit software (San Diego, CA, USA). Data are reported as mean ± standard deviation, unless otherwise specified. Statistical analysis was performed using one- or two-way analysis of variance with Tukey's correction for multiple comparisons and Student's *t* test, where appropriate, as indicated in the figure legends. Statistical significance was set at $p < 0.05$. *p*-values are indicated in the figure legends.

Data availability. The data generated in this study are available upon request from corresponding authors.

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Conflict of interest disclosure

This study may lead to the development of licensed products. The authors have fully disclosed these interests to eLife journal and have an approved plan in place to manage any potential conflicts arising from this arrangement. (S.L.: Beigene: Consultancy; Pfizer: Consultancy; Incyte: Consultancy; Roche: Consultancy, honoraria, research funding*; Orion Pharma: Consultancy; Novartis: Consultancy, honoraria, research funding*; Bayer: Research funding*; Celgene: Consultancy, research funding*; Genmab: Research funding*, Gilead: Consultancy*; CHO Pharma USA: Consultancy, research funding. P.M.O.: CHO Pharma USA: Consultancy, research funding; Orion Corporation: CRO work*; Gertrude Biomedical Pty Ltd: CRO work and research funding*. (*not related to this study).

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Authors' contributions

H.J.M. and **M.H**. Conceptualization, formal analysis, validation, investigation, methodology, writing-original draft, writing-review and editing. **E.E.** Formal analysis, validation, investigation, methodology, writing-review and editing. **K.K.**, **J.W.**, **L.M-G.** and **A.R.** Resources, investigation,

methodology, writing-review and editing. **T.R.J.** and **J.J.** Investigation, methodology. **P.L** and **K.L**. Resources, writing-review and editing. **H.S.**, **P.P.**, **J.W.C.**, **S.S.** and **C.H.** Resources, writing-review and editing. **P.M.O.** and **S.L.** Conceptualization, resources, formal analysis, funding acquisition, validation, writing-original draft, writing-review and editing.

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Figure legends

Figure 1. Analysis of SSEA-4 expression in tissues and cancer cell lines. A-C. SSEA-4 immunohistochemistry in (A) Ovarian, (B) pancreatic, and (C) gastric cancer patient samples. Ovarian cancer samples were also immunofluorescently stained with antibodies against SSEA-4 (red) and PAX8 (green) (A. lower panel). Representative samples are shown. **D.** Flow cytometry plots show SSEA-4 expression in human cancer cell lines. OVCAR4 (ovarian), HPAC (pancreatic) and MKN45 (gastric) were positive for SSEA-4, and Caov-3 (ovarian), MIA PaCa-2 (pancreatic) and MKN28 (gastric) were negative for SSEA-4. Unstained/isotype antibody-stained cells were used for gating the SSEA-4 negative cells.

Figure 2. Anti-SSEA-4 CAR design, T cell transduction, and CAR-T cell anti-tumor response *in vitro*. A. Schematic representation of the SSEA-4 CAR lentivector. EF1-a promoter driving the expression of CSF2RA (colony stimulating factor receptor alpha 2) signal peptide followed by anti-SSEA-4 V_L-(GGGGS)3-V_H scFv linked by the CD28 hinge to CD28-CD3z co-stimulatory and signaling domains. EGFRt gene is located downstream and separated by the T2A self-cleaving peptide. **B**. Flow cytometry plots showing percentage of transduced T cells assessed by detection of EGFRt expression using cetuximab antibody. Unstained T cells were used for gating the EGFRt negative cells and non-transduced (NT) T cells as a control. **C**. Quantification of transduction

efficiency of the cells in B. ***p=0.002*. **D**. Flow cytometry plots showing the proportion of $CD4^+/CD8^+$ T cells in the cultures 8 days post transduction. Unstained/isotype T cells were used for gating the CD4/CD8 negative cells and NT-T as a control. **E**. Quantification of the $CD4^+/CD8^+$ quantification of the cells in D. **F.** *In vitro* cytotoxicity assays using co-cultures of SSEA-4 CAR-T, NT-T, and anti-GFP CAR-T cells at the indicated doses with SSEA-4^+ (left panel; OVCAR4, HPAC, MKN45) and SSEA-4^- (Caov-3, MIA PaCa-2 and MKN28; right panel) cancer cells for 72 h. Cancer cell killing efficacy was assessed by luminescence emission from the cancer cells, and is represented on the y-axis as the percentage of cell viability relative to untreated (E:T, effector : target cell ratio = 0) cultures. N=3. Mean ± SD. One-way ANOVA with Tukey's multiple comparison tests. (**F**) * Indicates significant differences with T cell dose = 0. ***p=0.0001; *p≤0.03*. Not significant, n.s., differences were attributed to *p≥0.05.*

Figure 3. Characterization of anti-SSEA-4 CAR-T cells *in vitro*. A. Flow cytometry histograms of CFSE (Carboxyfluorescein succinimidyl ester) cell proliferation assays showing CAR-T cell proliferation upon interaction with SSEA-4^+ OVCAR4 cells in contrast to SSEA-4^- Caov-3 after 48 h. NT-T cells showed no proliferation in OVCAR4 and Caov-3 co-cultures. **B** and **C**. CFSE quantification of cells in A. **D** and **E**. Levels of IFNγ (**D**) and IL-2 (**E**) detected by ELISA from the supernatants of OVCAR4 +/- NT-T or SSEA-4 CAR-T cells at 72 h. **F**. Antibody-dependent cellular cytotoxicity (ADCC) assay. SSEA-4 CAR-T cells were co-cultured with or without Natural Killer (NK) cells and Cetuximab antibody at the indicated concentrations. Quantification of SSEA-4 CAR-T cells was performed by flow cytometry using Protein L staining. (**B** and **C**) N=2. (**D-F**) N=3. Mean ± SD. One-way ANOVA with Tukey's multiple comparison tests. (**B**) **p=0.019;* (**D-F**) ****p=0.0001* and ***p=0.001*. Not significant differences, n.s., were attributed to *p≥ 0.05*.

Figure 4. Anti-tumor response and survival in *in vivo* study. A-D. Short-term anti-tumor response of SSEA-4 CAR-T cells in NSG mice implanted intraperitoneally with $2x10^6$ FLuc-expressing OVCAR4 cells. (**A**) Representative BLI images of mice treated with 1M, 2M and 3M T cell doses at the indicated time points (days; d). **B-D**. BLI kinetics in the mice treated with SSEA-4 CAR-T at the indicated doses and compared to NT-T cells and PBS. N=5 for PBS, 2M and 3M groups. N=2 for NT-T 1M group. N=3 for 1M CAR-T group. Mean ± SD. One-way ANOVA with Tukey's multiple comparison tests. (**B**) ***p=0.0018* and ***p=0.0004,* (**C**) ***p=0.0009,* (**D**) ****p=0.0001*. **E** and **F**. Long-term assay anti-tumor response of SSEA-4 CAR-T cells in NSG mice**.** (**E** and **F**) BLI kinetics in the mice treated with SSEA-4 CAR-T at the indicated doses and compared to NT-T cells and PBS. N=10 for all groups, except for NT-T 1M dose N=8. (**E**) ****p=0.0001 and **p=0.0035,* (**F**)

****$p=0.0001$ and ***$p=0.0004$. **G** and **H.** Quantification of IFNγ and TNFα using ELISA in the post-mortem mouse blood plasma samples of the long-term assay. N=10, except for control group (N=4), NT-T 1M (N=8), and CAR-T 1M group (N=9). **I**. Probability of survival for PBS and CAR/NT-T dose mice groups after 70-day treatment. (M=1x10^6).

Supplementary Figure S1. SSEA-4 staining specificity and sensitivity to xylene. A. SSEA-4 immunohistochemistry in paraffin-embedded gastric cancer tumor microarrays. **B**. Upper panel sections were treated with standard xylene/ethanol series used in paraffin embedded section treatment, whereas lower panel samples were untreated. **C**. Consecutive sections of fresh frozen pancreatic cancer patient samples were stained with anti-SSEA-4 antibody. The staining was blocked with SSEA-4 hexaose (middle panel) or SSEA-3 (right panel). **D.** Representative immunofluorescence micrographs of SSEA-4 (red) in OVCAR-4 cells (high expression) and Caov-3 cells (no expression). Nucleus (blue; Hoechst 33342). N=3.

Supplementary Figure S2. Short-term *in vivo* study, dose-response, and weight monitoring. A. Treatment groups and used T cell doses. **B**. Weight monitoring in the indicated mice groups. CAR-T 3M treated mice showed a trend of mild weight loss. (M=1x10^6).

Supplementary Figure S3. IFNγ measurement and organ BLI of the short-term *in vivo* study. A. IFNγ quantification by ELISA in post-mortem mouse blood plasma. **B**. Representative BLI images of isolated organs from mice treated with PBS, NT-T, and CAR-T cells at the indicated T cell doses after 32 days. **C**. Organ BLI quantification. N=5 for PBS, 2M and 3M groups. N=2 for NT-T 1M group. N=3 for 1M CAR-T group. Mean ± SD. One-way ANOVA with Tukey's multiple comparison tests. (**A**) ***$p=0.0005$, ****$p=0.0001$. (**C**) *$p=0.04$, **$p\leq0.001$ to PBS group. Not significant, n.s., differences were attributed to $p\geq0.05$. (M=1x10^6).

Supplementary Figure S4. CAR-/NT-T cell exhaustion marker quantification in the short-term *in vivo* study. A. Expression of human *ACTB* gene in mouse blood cell samples measured by qPCR. *huACTB* Ct values were above the detection limit (ND) in PBS samples, but below the detection limit in CAR-T treated mice samples at the indicated doses. **B**. Expression of human *TIM-3*, *LAG-3*, and *PD-1* genes in blood samples of PBS-treated mice measured by qPCR. Mouse *Gapdh* was used as positive control. Ct values for human *TIM-3*, *LAG-3*, and *PD-1*were above the detection limit. **C**. Expression of human *TIM-3*, *LAG-3*, and *PD-1* genes in blood samples of CAR-T treated mice at the indicated cell doses measured by qPCR. **D-F**. *TIM-3*, *LAG-3*, and *PD-1* fold gene expression in

samples of CAR-T treated mice at the indicated CAR-T cell doses. N=3. Mean ± SD. One-way ANOVA with Tukey's multiple comparison tests. (**A**) ****$p=0.0078$, ****$p=0.0001$. (**D**) *$p=0.0281$, ***$p=0.0001$, ****$p=0.0001$. (**E**) **$p=0.004$. (**F**) **$p=0.082$, ****$p=0.0001$. No significant differences were attributed to $p\geq0.05$. (M=1x10^6).

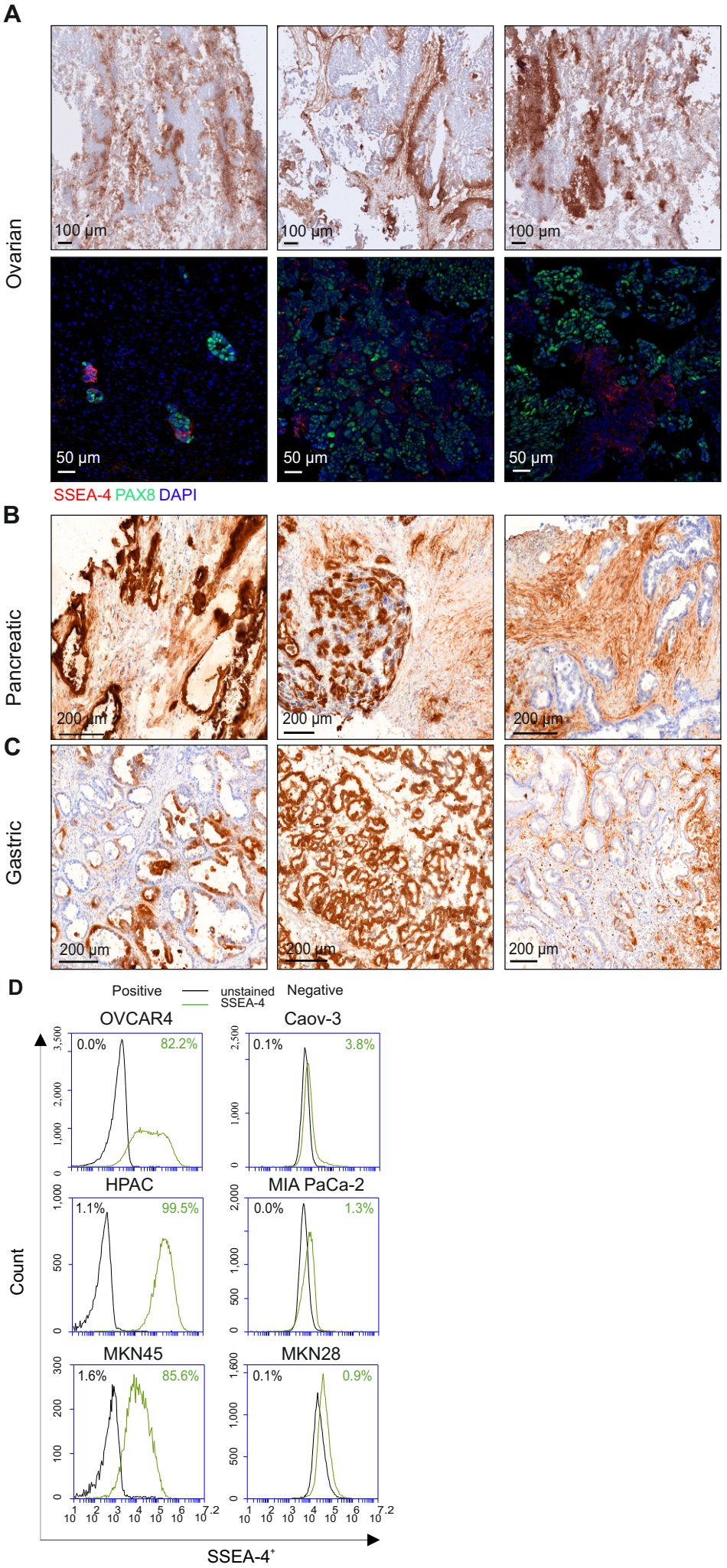
Supplementary Figure S5. Anti-tumor response and survival in the long-term *in vivo* study. A. Treatment groups and used T cell doses. **B**. Representative BLI images of mice treated with PBS, 1M, and 2M NT or CAR T cells doses at the indicated time points (days; d). (M=1x10^6).

Supplementary Figure S6. Weight monitoring during the long-term *in vivo* study. Mice treated with 2M CAR-T dose showed pronounced weight loss and health deterioration at earlier time points than mice treated with 1M CAR-T dose. PBS, tumor-free CAR/NT-T, and NT-T groups did not show weight loss and remained healthy during the duration of the experiment. Unless otherwise indicated, mice groups are tumor-bearing. (M=1x10^6).

Supplementary Figure S7. Expression of SSEA-4 in normal mouse tissues. Immunofluorescence detection of SSEA-4 (green) in the indicated tissues of healthy 10 weeks old (C57BL/6) mouse using anti-SSEA-4 antibody.

Supplementary Figure S8. Histopathological analysis of mice after long-term *in vivo* study treated with 2M dose. A. H&E staining of mouse tissues from CAR/NT-T treated mice shows prominent lymphocyte infiltrates in the CAR-T treated mice lungs. (M=1x10^6). **B.** CD3- and EGFR-positive CAR-T cells in tissues after long-term survival study. Indicated tissues were stained with HOECHST 33342 for nuclei (blue), anti-EGFR to detect CAR-T cells (middle panel; green) and anti-CD3 for T cells (right panel, white). Left panel shows merged image showing all three channels. Arrows highlight CAR-T and CD3 positive cells, whereas arrowheads highlight erythrocytes with intense background staining.

Figure 1.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C
Gastric
200 µm
200 µm
200 µm
D
Positive
unstained
SSEA-4
Negative
OVCAR4
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Caov-3
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3.8%
HPAC
1.1%
99.5%
MIA PaCa-2
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85.6%
MKN28
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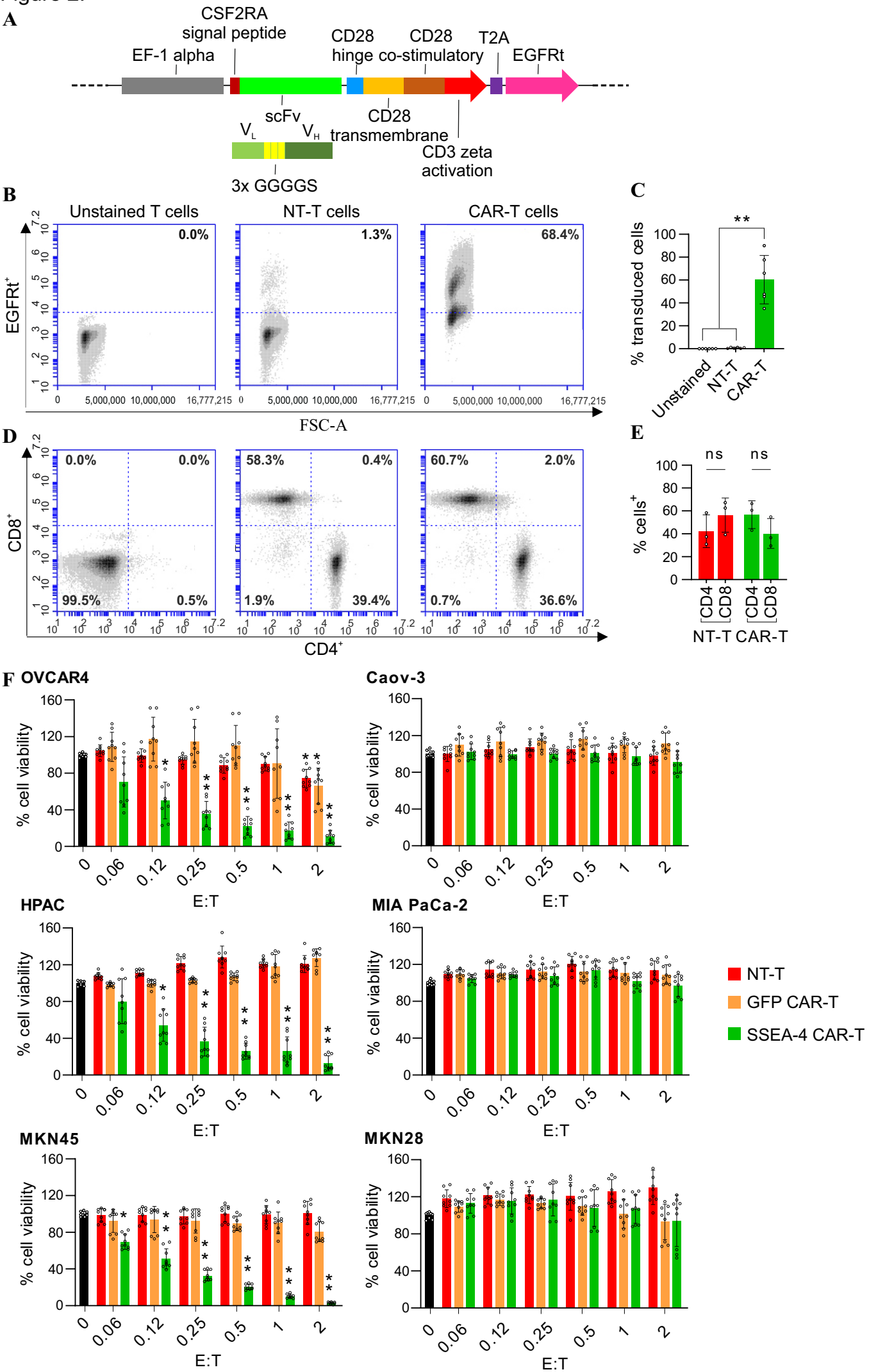
0.9%
Count
SSEA-4+

Figure 2.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3x GGGGS
B
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CAR-T cells
0.0%
1.3%
68.4%
EGFRt+
FSC-A
C
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D
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0.0%
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2.0%
99.5%
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CD8+
CD4+
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ns
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CD4
CD8
NT-T CAR-T
F OVCAR4
Caov-3
HPAC
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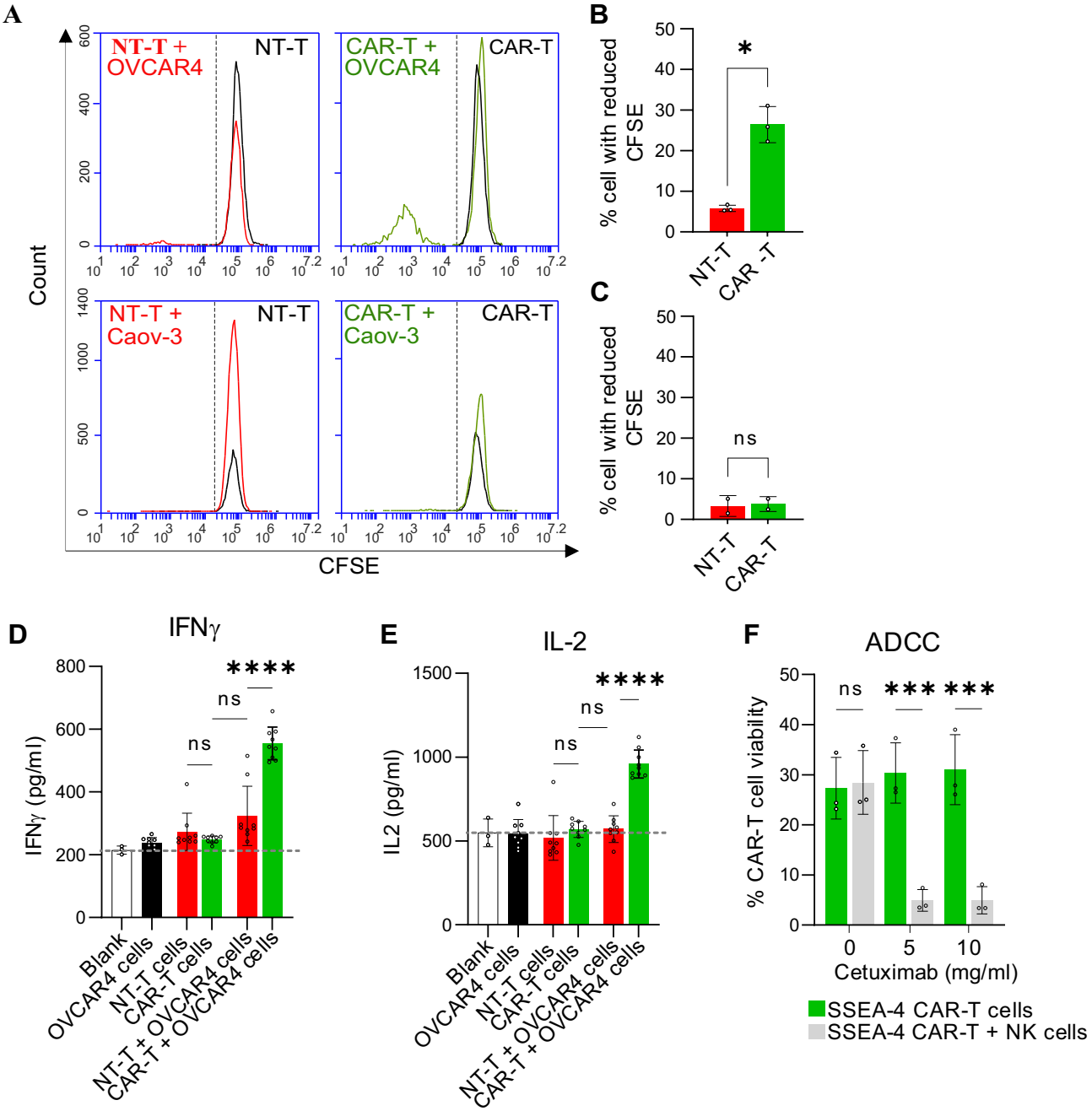
E:T
NT-T
GFP CAR-T
SSEA-4 CAR-T

Figure 3.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NT-T
CAR-T + OVCAR4
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NT-T + Caov-3
NT-T
CAR-T + Caov-3
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Count
CFSE
B
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NT-T
CAR -T
C
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NT-T
CAR-T
D
IFNγ
IFNγ (pg/ml)
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Blank
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NT-T + OVCAR4 cells
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E
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Blank
OVCAR4 cells
NT-T cells
CAR-T cells
NT-T + OVCAR4 cells
CAR-T + OVCAR4 cells
F
ADCC
% CAR-T cell viability
ns
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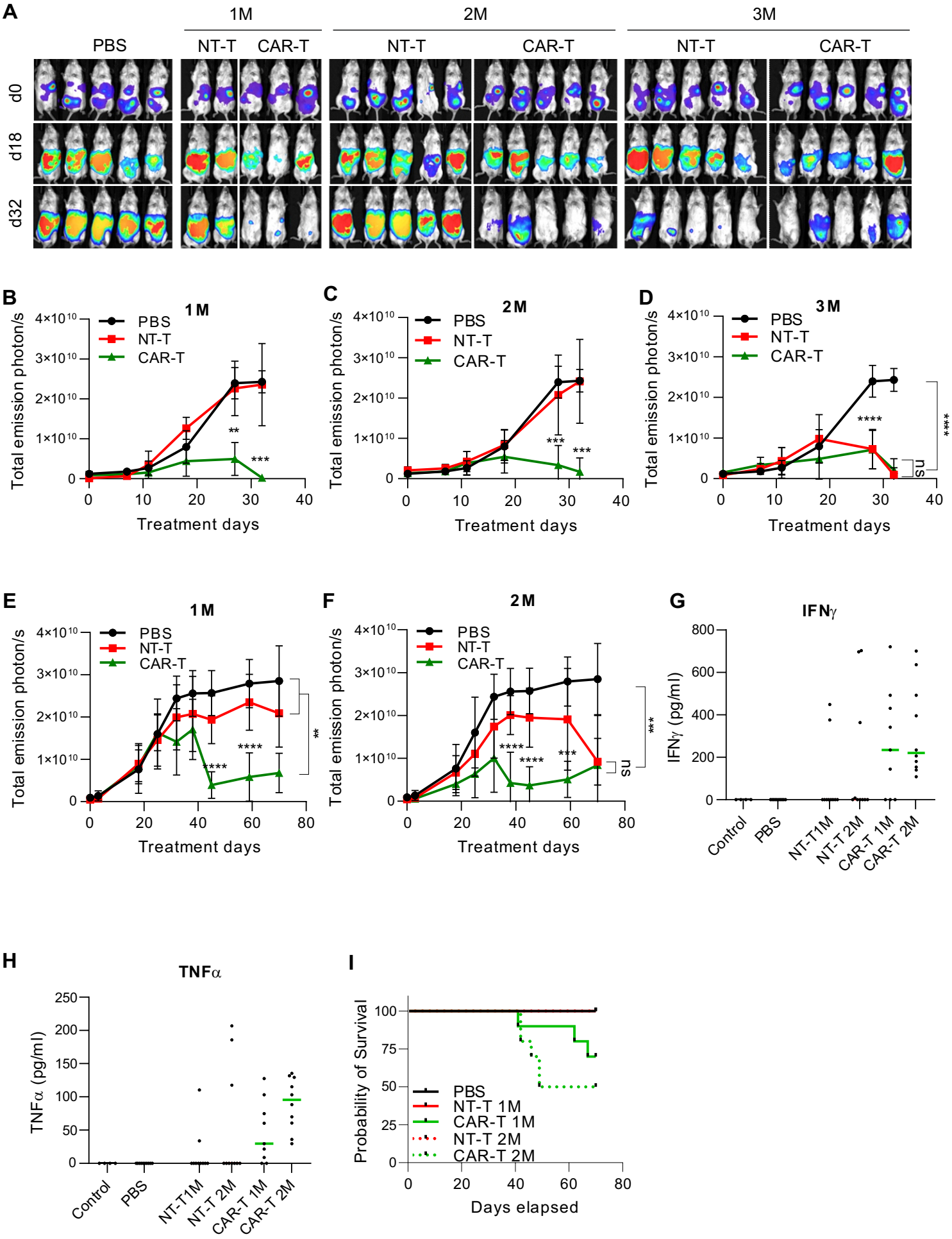
Cetuximab (mg/ml)
SSEA-4 CAR-T cells
SSEA-4 CAR-T + NK cells

Figure 4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3M
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CAR-T
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1 M
C
2 M
D
3 M
Total emission photon/s
PBS
NT-T
CAR-T
Treatment days
E
1 M
F
2 M
G
IFNγ
IFNγ (pg/ml)
Control
PBS
NT-T1M
NT-T 2M
CAR-T 1M
CAR-T 2M
H
TNFα
TNFα (pg/ml)
I
Probability of Survival
PBS
NT-T 1M
CAR-T 1M
NT-T 2M
CAR-T 2M
Days elapsed