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(54) Title: MICROENGINEERED TRANSPLANTATION OF HUMAN SOLID TUMORS FOR IN VITRO STUDIES OF CAR T IMMUNOTHERAPY

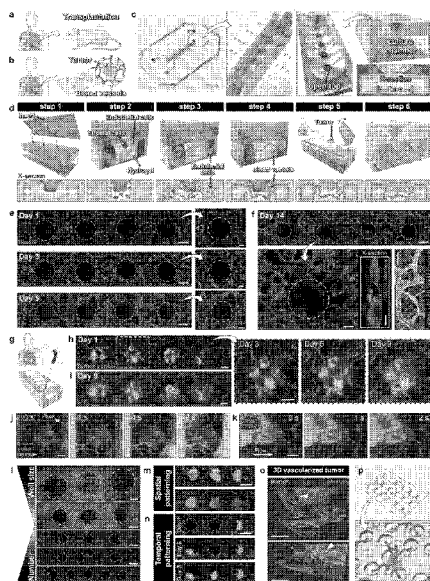


FIG. 1

(57) Abstract: A microfluidic chip for in vitro culture, comprising: a chamber, the chamber comprising a primary channel and at least one side channel adjacent thereto, the chamber having a bottom surface and a top surface, the chamber being configured as an open-top chamber having at least one aperture extending through the top surface and in fluid communication with the primary channel; and a separator disposed between the primary channel and the at least one side channel, the separator extending from the bottom surface of the chamber toward the top surface of the chamber; and optionally, an insert, the insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture. A method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip and a sample cell perfused to the primary channel.

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MICROENGINEERED TRANSPLANTATION OF HUMAN SOLID TUMORS
FOR IN VITRO STUDIES OF CAR T IMMUNOTHERAPY

RELATED APPLICATIONS

[0001] The present application claims priority to and the benefit of United States patent application no. 63/616,019, “Microengineered Transplantation Of Human Solid Tumors For In Vitro Studies Of CAR T Immunotherapy,” filed December 29, 2023. All foregoing applications are incorporated herein by reference in their entireties for any and all purposes.

TECHNICAL FIELD

[0002] The present disclosure relates to the field of in vitro tissue models and to the field of microfluidics.

BACKGROUND

[0003] Despite its proven efficacy for hematologic malignancies, chimeric antigen receptor (CAR) T cell therapy has met with limited success in treating solid tumors. Although this represents a significant clinical challenge in cancer immunotherapy, preclinical efforts to assess and improve the efficacy and safety of CAR T therapy are hindered by our limited ability to model, probe, and modulate the complexity of cancer-immune interactions in solid tumors.

SUMMARY

[0004] Provided here is, inter alia, a microengineered platform for in vitro reproduction, real-time imaging, and in-depth analysis of malignant human solid tumors during CAR T therapy. Using a compartmentalized device with an externally accessible interface, this system makes it possible to engineer three-dimensional organotypic constructs composed of human tumor explants that can be vascularized and perfused with blood-borne immune cells in a controlled manner. As an example, first presented is a microphysiological model of adeno-carcinoma tumors in the human lung infused with CAR-T cells to demonstrate its capabilities to reproduce the three essential steps of

targeting solid tumors, including CAR T cell recognition of tumor-associated antigens, infiltration, and survival and effector function in the tumor microenvironment. Using flow cytometry and single-cell RNA sequencing, one can probe how the phenotype of CAR T cells changes during their recruitment and persistence. Furthermore, one can use the sequencing data to discover a therapeutic target that can be pharmacologically inhibited in the model to significantly increase CAR T cell trafficking and their anti-tumor effects, for which are presented specific biomarkers identified by untargeted metabolomics analysis. This disclosure thus provides a powerful approach for mechanistic studies and preclinical screening of adoptive cell therapies for cancer and other complex diseases.

[0005] In meeting the described long-felt needs, the present disclosure provides, among other things, a microfluidic chip for in vitro culture, comprising: a chamber, the chamber comprising a center channel and at least one side channel adjacent thereto, the chamber having a bottom surface and a top surface, the chamber being configured as an open-top chamber having at least one aperture extending through the top surface; and a separator separating the center channel and the at least one side channel, the separator extending from the bottom surface of the chamber toward the top surface of the chamber; and optionally, an insert, the insert having at least one projection configured to extend into the chamber when the projection is placed into register with the one aperture.

[0006] The present disclosure also provides a method, comprising: contacting a tumor cell residing in the center channel of a microfluidic chip according to the present disclosure and a cell perfused to the center channel.

[0007] Further provided is a microfluidic chip for in vitro culture, comprising: a chamber, the chamber comprising a primary channel and at least one side channel adjacent thereto, the chamber having a bottom surface and a top surface, the chamber being configured as an open-top chamber having at least one aperture extending through the top surface and in fluid communication with the primary channel; and a separator disposed between the primary channel and the at least one side channel, the separator extending from the bottom surface of the chamber toward the top surface of the chamber; and optionally, an insert, the insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture.

[0008] Also disclosed is a method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip according to the present disclosure

(e.g., according to any one of Aspects 1-11) and a sample cell perfused to the primary channel. Example such solid tumor cells and sample cells are described elsewhere herein; a sample cell can be a CAR T cell.

[0009] Further provided is a microfluidic chip, comprising: a primary channel; at least one side channel, the at least one side channel being in fluid communication with the primary channel; a separator disposed between the primary channel and the at least one side channel; a vascular bed, the vascular bed crossing the separator so as to place the at least one side channel into fluid communication with the primary channel. As described elsewhere herein, such a microfluidic chip can receive explanted material from a subject, for example tumor material.

[0010] Additionally disclosed is a method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip as described herein (e.g., according to any one of Aspects 16-21) and a sample cell perfused to the primary channel.

[0011] Also provided is a method, comprising: placing solid tumor tissue in a primary channel, effecting vascularization of the solid tumor tissue such that a vascular bed places the tissue into fluid communication with at least one side channel; and perfusing a material into the at least one side channel such that the material is communicated via the vascular bed to the solid tumor tissue.

BRIEF DESCRIPTION OF THE DRAWINGS

[0012] In the drawings, which are not necessarily drawn to scale, like numerals may describe similar components in different views. Like numerals having different letter suffixes may represent different instances of similar components. The drawings illustrate generally, by way of example, but not by way of limitation, various aspects discussed in the present document. In the drawings:

[0013] FIGs. 1a-1p: A microengineered platform for in vitro transplantation and prolonged maintenance of human solid tumors. FIGs. 1a,b, Conceptual illustration of solid tumor transplantation in vivo (FIG. 1a) and in our microengineered device (FIG. 1b) designed to reconstitute the tumor-vascular interface. Created with Biorender.com. FIG. 1c, Photos and illustration of the open-top device with a compartmentalized chamber design. Scale bars, 5 mm (left) and 250 μ m (bottom-right). FIG. 1d, Illustration of experimental procedure to construct vascularized and perfusable tumor constructs in the

device. FIGs. 1e,f, Representative fluorescence micrographs showing blood vessel development in the device over time. The dashed white circles show the location of closed culture wells. Scale bars, 100 μm (single-well view in FIG. 1e and FIG. 1f, image of vessel and human lung fibroblast in FIG. 1f), 250 μm (multi-well view in FIGs. 1e and f), and 5 μm (inset in FIG. 1f). FIGs. 1g,h,i, Time-lapse fluorescence images of vascularization of primary lung cancer explant. Scale bars, 200 μm . FIGs. 1j,k, Time-lapse fluorescence micrographs showing directional vascular perfusion of tumor constructs cultured for 10 days using 70-kDa FITC Dextran FIG. 1 (j) and 1- μm fluorescent microbeads (FIG. 1k). Scale bars, 200 μm (FIG. 1j) and 100 μm (FIG. 1k). FIG. 1l, Examples of different array designs with different numbers or sizes of culture wells (shown with dashed white circles). Red in the images shows fluorescence of blood vessels embedded in the culture scaffold. Scale bars, 500 μm . FIGs. 1m,n, Demonstration of controlling the spatial distribution (FIG. 1m) or timing (FIG. 1n) of in vitro tumor transplantation into the culture-well array. Tumors shown in m were color-coded to represent different types/compositions. Scale bars, 500 μm . FIG. 1o, 3D imaging reconstruction of the vascularized tumor showing the engineered microvessels wrapping around the tumor construct and forming close contact with the surface of tumor (top, arrowhead), and can also penetrate into the tumor (bottom, arrowhead). Scale bars, 100 μm . FIG. 1p, Photos of a device containing an array of 18 individually accessible and controllable transplantation units to scale up the production of vascularized solid tumor constructs. Scale bar, 5 mm.

[0014] FIGs. 2a-2u: In vitro modeling of CAR T cell-tumor interactions. FIG. 2a, Illustration of three essential steps of tumor-directed CAR T cell trafficking modeled in the tumor-on-a-chip. FIG. 2b, Experimental workflow to model the interaction of mesothelin-targeted CAR T cells with mesothelin-overexpressing xenograft lung tumors (meso-tumors). Created with Biorender.com. FIGs. 2c,d, Representative confocal images demonstrating rapid vascularization and growth of control (FIG. 2c) and meso-tumors (FIG. 2d) over 11 days. The lower panels in each figure show a close-up of tumor marked with a dashed square in the upper image. Scale bars, 250 μm . FIG. 2e, Micrographs of meso-CAR T cells adherent to blood vessels associated with meso-tumors on day 0 of CAR T infusion. Scale bars, 50 μm (left, right) and 250 μm (middle). FIG. 2f, Time-lapse images of CAR T cell extravasation and migration towards tumors. The images were taken

24 hours after CAR T cell infusion. Scale bars, 20 μm . FIGs. 2g,h, Confocal images showing the accumulation of CAR T cells in meso-tumors over time. Scale bars, 200 μm (FIG. 2g) and 500 μm (FIG. 2h). FIGs. 2i,j,k, Quantification of tissue area occupied by CAR T cells (FIG. 2i), the percentage of CAR T cells found within tumors (FIG. 2j), and tumor area normalized to that prior to CAR T cell infusion (FIG. 2k). Data are presented as mean $\pm$ SEM. $**P < 0.01$ ($n \geq 4$). FIGs. 2l-o, Micrographs of meso-CAR T cells infused into control tumors at various time points. Scale bars, 50 μm (left, right) and 100 μm (middle) (FIG. 2l), 20 μm (FIG. 2m), 200 μm (FIG. 2n), and 500 μm (FIG. 2o). FIG. 2p, Immunostaining of ICAM-1 expression (left) and quantification of tissue area stained positive for ICAM-1 (right). Scale bars, 250 μm . FIG. 2q, Measurement of secreted human IL-2 and IFN- γ . Data are presented as mean $\pm$ SEM ($n \geq 4$). FIG. 2r, Visualization of apoptotic tumor cells at Day 5 post CAR T cell infusion (left) and quantification of caspase 3 expression and LDH release (right). Data are presented as mean $\pm$ SEM ($n \geq 3$). Scale bars, 100 μm . s, Illustration of experimental procedure to harvest whole tumor constructs from culture chambers. Scale bar, 5 mm. FIGs. 2t,u, Histological sections of CAR T cell-infused meso- (FIG. 2t) and control tumors (FIG. 2u). Scale bars, 100 μm and 20 μm (zoom-in view). The yellow dashed polygons in the rightmost images show the outline of tumors. CAR T cells derived from two healthy donors were tested in this study.

[0015] FIGs. 3a-3f: Flow cytometric analysis of CAR T cell phenotype. FIG. 3a, Illustration of workflow for separate analysis of CAR T cells harvested from tumors and those from the surrounding stroma. CAR T cells in the stroma are pseudo-colored blue and shown with white arrowheads in the phase contrast image. Scale bars, 250 μm (top, middle) and 25 μm (bottom). FIG. 3b, Quantification of CAR T cell proportions in the tumor and stromal compartments. Data are presented as mean $\pm$ SEM. $*P < 0.05$ and $**P < 0.01$ ($n \geq 3$). FIGs. 3c-f, Representative flow cytometry plots and quantification of surface marker expression by CAR T cells, including CD69 (FIG. 3c), PD-1 (FIG. 3d), CD45RO and CD62L (FIG. 3e), and CD103 (FIG. 3f). Data are presented as mean $\pm$ SEM. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, and $****P < 0.0001$ ($n \geq 3$). CAR T cells derived from one healthy donor were tested.

[0016] FIGs. 4a-4n: Single-cell RNA sequencing analysis of microengineered tumor model. FIG. 4a, UMAP projection of cell populations in the CAR T cell-treated meso-tumor model at Day 6 post-infusion. Endos represent endothelial cells. FIG. 4b,

UMAP plots of cell type-specific markers. FIG. 4c, UMAP clusters annotated with their location within the model. FIG. 4d, UMAP projection of 11 color-coded clusters representing distinct cell subpopulations and their phenotypes in the meso-tumor model (top). Each cluster is marked with a box label indicating the number of the cluster (e.g., M-1) and the type of cells (e.g., Tumor). Underneath the box label is the annotation of cellular phenotype. The plot below the UMAP projection shows cluster-specific, relative proportions of cells quantified by their location (tumor vs. stroma). e-g, UMAP plots depicting the expression levels and spatial distribution of genes used to identify the subpopulations of tumor cells (FIG. 4e), fibroblasts (FIG. 4f), and CAR T cells (FIG. 4g). FIG. 4h, Violin plots showing the expression of select gene signatures by individual CAR T cell clusters. $***P < 1e-7$ vs. other clusters. i, UMAP projection of 9 color-coded clusters representing distinct cell subpopulations and their phenotypes in the control tumor model (top). Plot of cluster-specific, relative proportions of cells quantified by their location (bottom). FIG. 4j, Phenotype-specific comparison of the fraction of CAR T cells between meso-tumor and control groups. FIGs. 4k,l, Violin plots of select markers and gene signatures by tumor-infiltrating activated effector CAR T cells from the meso-tumor (M-11) and control (C-9) models. $***P < 1e-7$. FIG. 4m, Heatmap showing SingleR scores for CAR T cells in the meso-tumor model computed against each CD8 T cell subtype described in the GSE99254 reference dataset of human endogenous tumor-infiltrating T cells from NSCLC patients. Each vertical line represents a single cell. FIG. 4n, UMAP projection of CAR T cell clusters in the meso-tumor model annotated using labels from the reference human dataset. CAR T cells derived from one healthy donor were tested.

[0017] FIGs. 5a-5j: Analysis of ligand-receptor interactions in CAR T cell-treated tumors. FIG. 5a, Conceptual diagram illustrating pairwise mapping of directional ligand-receptor interactions between different cell types in the engineered tumor constructs. FIG. 5b, Chord diagrams showing an overview of intercellular communication and the number of identified interactions in the meso-tumor (top) and control (bottom) groups. Each chord represents a bundle of paired and statistically significant ligand-receptor interactions between a particular pair of cell types. The width of the chord indicates the number of interacting ligand-receptor pairs. FIGs. 5c-j, Chord diagrams of cell pair-specific ligand-receptor interactions with adjusted p-values < 0.05 and total mean

expression > 0.35 (FIGs. 5c,e,g,i) and violin plots comparing the expression of interacting genes of interest (FIGs. 5d,f,h,j).

[0018] FIGs. 6a-6i: CAR T cell treatment of transplanted human mesothelioma explants. FIG. 6a, Workflow of in vitro transplantation of primary tumor explants surgically resected from mesothelioma patients #1-#4. The photos at bottom show tumor slices in a 24-well plate (arrowheads, left) and minced, suspended explants in a conical tube ready for transplantation (right). Scale bars, 5 mm (left) and 1 mm (right). FIG. 6b, Representative fluorescence images of tumor transplants during vascularization. Tumor explants were stained with calcein AM. Scale bars, 200 μ m. FIGs. 6c,d, Micrographs of tumor constructs after the infusion of meso-CAR T cells. Scale bars, 100 μ m. FIG. 6e, Quantification of compartment-specific proportion of CAR T cells. Data are presented as mean $\pm$ SEM (n = 4). FIGs. 6f,g, Representative flow plots and quantification of CD45RO/CD62L (f) and CD69 (g) expression by CAR T cells harvested from the tumor constructs at Day 6 post-infusion. Data are presented as mean $\pm$ SEM (n = 4). FIG. 6h, UMAP plot of CAR T cells grouped into 3 clusters of transcriptionally distinct states as labeled (top). UMAP plots showing the expression level and distribution of 9 select canonical gene markers (bottom). FIG. 6i, Violin plots comparing the expression of gene signatures associated with T cell phenotype across all subpopulations. FIG. 6j, Gene Ontology (GO) enrichment analysis of CAR T cell subpopulations. CAR T cells derived from one healthy donor were tested in this study.

[0019] FIGs. 7a-7g: Effects of DPP4 inhibition on CAR T cell trafficking into composite lung tumor spheroid. FIG. 7a, Visualization of CAR T cell-endothelial cell interactions mediated by CXCR3/DPP4-CXCL10/CXCL11 (left) and violin plots comparing the expression of these mediators across all cell types (right). FIG. 7b, Experimental timeline for CAR T cell infusion and drug treatment. FIG. 7c, Representative confocal micrographs of single tumor spheroids treated with CAR T cells and different concentrations of LAF237. Blood vessels are not shown in these images. Scale bars, 250 μ m. FIG. 7d, Representative images of CAR T cell-infused tumors at Day 26. Scale bars, 200 μ m. e, Quantification and comparison of tumor area (top) and CAR T cell-occupied hydrogel area (bottom). Shaded in grey is a period of CAR T cell infusion and daily LAF237 treatment. Data are presented as mean $\pm$ SEM (n $\geq$ 3). FIGs. 7f,g, Quantification of CXCL10 and CXCL11 levels (f) and the activity of DPP4 (FIG. 7g) in

device effluent. Shaded in grey is a period of CAR T cell infusion and daily LAF237 treatment. Data are presented as mean $\pm$ SEM ($n \geq 3$). CAR T cells derived from two healthy donors were tested in this study.

[0020] FIGs. 8a-8m: Metabolomic analysis of lung tumors treated with CAR T cells and LAF237. FIG. 8a, Workflow of untargeted, global profiling of metabolites in vascular perfusate. FIG. 8b, Plot of PLS-DA scores. $N = 4$ for each group. FIG. 8c, Heatmap of metabolites whose levels of expression were significantly altered by LAF treatment. $N = 4$ for each group. The source of metabolites is color-coded in their labels – blue, amino acid metabolism; red, carbohydrate metabolism; green, nucleotide metabolism. The color gradient of the scale bar indicates the relative abundance of metabolites, with red and blue indicating higher and lower concentrations, respectively. $p < 0.05$ was considered significant. FIG. 8d, Plot of significantly changed metabolic pathways identified by pathway impact analysis of significantly upregulated metabolites in the high-dose LAF237 group. FIGs. 8 e-g, Comparison of normalized concentrations of select metabolites across all conditions. FIG. 8h, ROC curves for biomarker prediction models to identify metabolites that can differentiate more efficacious CAR T cell treatment with high-dose LAF237 from control CAR T cell treatment without LAF237 at Day 16 post-infusion. Different numbers of constituent metabolite features per model are indicated by different colors. FIG. 8i, List of top metabolites yielded by the 25-feature prediction model shown in h and ranked according to their predictive accuracy. FIG. 8j, Comparison of normalized concentrations of metabolites selected from FIG. 8i. FIG. 8k, ROC curves for biomarker prediction models to identify metabolites that can differentiate more efficacious CAR T cell treatment with high-dose LAF237 from control treatment at all time points post-infusion. FIG. 8l, List of top metabolites yielded by the 15-feature prediction model shown in FIG. 8k and ranked according to their predictive accuracy. Common metabolites that appear in both FIG. 8i and FIG. 8l are labelled blue. FIG. 8m, Comparison of normalized concentrations of metabolites selected from FIG. 8l. Boxplots show minimum, 25th percentile, mean, 75th percentile, and maximum. $N = 4$ for each group. Note that post-hoc pairwise comparison was calculated between the high-dose (high) and control (ctrl) groups at all time points with $*p < 0.05$.

[0021] FIGs. 9a-9h: In vitro modeling and in vivo validation of enhanced CAR T trafficking through CCR2 modification. FIG. 9a, Conceptual diagram illustrating (top) a

mismatch between tumor-secreted chemokine CCL2 and its receptor on CAR T cells and (bottom) a strategy to enhance CAR T trafficking through modification of CCR2 expression on CAR T cells. FIG. 9b, Experimental workflow to model the interaction of CCR2 modified meso-CAR T cells with mesothelin-expressing xenograft mesothelioma tumors (EMMeso-tumors) in the engineered vascular niche using human lung microvascular endothelial cells (HMVEC-L). Scale bar, 250 μ m. FIG. 9c, Representative images of EMMeso-tumors prior to and post-infusion of non-transduced donor T cells, meso-CAR T cells, and CCR2 modified meso-CAR T cells. Scale bars, 250 μ m. These three types of T cells were all derived from one healthy donor. FIGs. 9d,e, Quantification and comparison of (FIG. 9d) T cell-occupied hydrogel area and (FIG. 9e) tumor area at different time points. Data are presented as mean $\pm$ SEM (n = 3-6). Shaded in grey is a period of T cell infusion. FIG. 9f, Experimental workflow to validate the interaction of CCR2 modified meso-CAR T cells with EMMeso-tumors in vivo. Created with Biorender.com. These three types of T cells were all derived from the same donor and T cells derived from additional two healthy donors were tested in this in vivo study. FIGs. 9g,h, Representative flow cytometry plots and quantification of tumor infiltrated human CD3⁺ T cells from different treatment groups at Day 5 post IV injection. Data are presented as mean $\pm$ SEM. N = 3. FIG. 9i, Tumor growth curve post IV injection of different types of T cells. *P < 0.05 (n $\geq$ 3).

[0022] FIGs. 10a-10k: Single-cell trajectory analysis of CAR T and tumor cells in the meso-tumor model. FIG. 10a, UMAP plot showing the subpopulations of CAR T cells in the meso-tumor model re-clustered for trajectory analysis. FIG. 10b, Transition of CAR T cell phenotype over pseudotime. FIGs. 10c-h, Pseudotime dynamics of key marker expression plotted by cell type (FIGs. 10c,e,g) and location (FIGs. 10d,f,h). i, UMAP plots of tumor cell clusters in the meso-tumor model (left) and pseudotime trajectories (right). FIGs. 10j,k, Dynamic expression of select markers by tumor cells over the course of pseudotime.

[0023] FIGs. 11a-11i: Transplantation and CAR T treatment of human mesothelioma explants with tissue-relevant microvasculature. FIG. 11a, Workflow of construction of vascular bedding using human pulmonary microvascular endothelial cells (HMVEC-L) and in vitro transplantation of primary tumor explants surgically resected from mesothelioma patient #6. The photos at bottom show tumor slices and minced,

suspended explants in a conical tube ready for transplantation. Scale bars, 5 mm (left) and 1 mm (right). FIG. 11b, Typical fluorescent images showing the vascularization of tumor explants using HMVEC-L over time. Scale bars, 250 μ m. FIG. 11c, Typical fluorescent images showing vascularized tumor constructs post-infusion with control NTD T cells or meso-CAR T cells. Scale bars, 250 μ m. FIG. 11d, Quantification and comparison of area of T cells infiltrated into the gel per individual tumor nest. Data are presented as mean $\pm$ SEM. (n = 10-11). FIG. 11e, Count and comparison of T cells infiltrating into stroma and tumor compartments by flow cytometry. FIGs. 11f,g, Representative flow cytometry plots of surface marker expression of CD45RO and CD62L by (FIG. 11f) meso-CAR T cells only, and (FIG. 11g) CD8⁺ T cells. FIG. 11h, Comparison of number of CD45RO⁺ CD62L⁻ CD8⁺ T cells. FIG. 11i, Comparison of intensity of cell tracker labelled T cells. T cells derived from an additional healthy donor to the one used in FIG. 6 were tested in this study.

[0024] FIGs. 12a-12b: FIG. 12a. The open-top microdevice consists of three layers including a three-lane culture chamber, an open-top ceiling, and an insert. FIG. 12b. To fabricate the device, degassed PDMS prepolymer is dispensed onto 3D printed molds and cured at 65°C for 2 hours (Step 1). The fully cured PDMS slabs are then peeled off the molds (Step 2) and assembled to generate an open-top device in which the protruding features of the insert are fit into the open wells of the device ceiling (Step 3).

[0025] FIG. 13: Time-lapse images demonstrating the injection of cell-hydrogel mixture into the middle chamber of the device. Injection occurs while the open wells in the ceiling of the device are covered by the insert (shown as a square at the center). For visualization purposes, the mixture solution was dyed black in these images.

[0026] FIGs. 14a-14b: FIGs. 14a-14b, Quantification of (FIG. 14a) the total branch length per microdevice and (FIG. 14b) the average diameters of microvessels during the vascularization process in our microengineered platform. Data are presented as mean $\pm$ SEM (n = 8-17).

[0027] FIG. 15: Histological sections of squamous cell carcinoma in human lung cancer (left) and head and neck cancer (right). Cancer lesions are seen with microscopic tumor nests in the vascularized stroma.

[0028] FIGs. 16a-16b: FIGs. 16a-16b, Fluorescence images of lung tumor spheroids composed of A549 human lung adenocarcinoma cells (FIG. 16a) and human

colorectal cancer organoids (FIG. 16b) embedded and vascularized in the in vitro transplantation microdevice.

[0029] FIGs. 17a-17d: FIGs. 17a-17c. Comparison of wild-type A549 cells (Ctrl-A549) and those transduced to overexpress mesothelin (Meso-A549). FIG. 17a, Fluorescence images show mesothelin expression (red) by GFP-expressing A549 cultured in 2D monolayers (top row) and in A549-CDX tumors (bottom row). FIG. 17b, Data in the quantification of mesothelin expression are presented as mean $\pm$ SEM ($n \geq 6$). FIG. 17c, Mesothelin and GFP expression on both A549 cell lines was further confirmed and quantified by flow cytometry. FIG. 17d, Schematic representation of the mesothelin-binding chimeric receptors.

[0030] FIG. 18: Fluorescence time-lapse images of meso- (left) and control (right) tumors in the device prior to (days 1 to 11) and after (days 14 to 19) CAR T cell infusion. Scale bars, 500 μ m. CAR T cells derived from one healthy donor were tested.

[0031] FIGs. 19a-19d: FIG. 19a, Representative confocal micrographs of single A549-CDX tumor nest of different initial sizes and the corresponding vascular network formation. Scale bar, 200 μ m. FIGs. 19b-d, Quantification and comparison of (FIG. 19b) average diameter of vessels, (FIG. 19c) number of vessel junctions, and (FIG. 19d) percentage of vessel covered area over time based on different sizes of tumor. Data are presented as mean $\pm$ SEM ($n = 3-14$).

[0032] FIGs. 20a-20d: Array screening of chemokine expression of (FIG. 20a) vessel only control, (FIG. 20b) control A549 CDX tumor, and (FIG. 20c) Meso-A549 CDX tumor in the engineered model by collecting device effluent prior to CAR T cell infusion. FIG. 20d, Relative comparison of expression levels of each chemokine examined.

[0033] FIGs. 21a-21c: FIG. 21a, Representative confocal micrographs of single tumors treated with NTD T cells or meso-CAR T cells. Scale bar, 250 μ m. FIGs. 21b,c, Quantification of (FIG. 21b) T cell area and (FIG. 21c) normalized tumor area over time. Data are presented as mean $\pm$ SEM ($n \geq 3$). CAR T cells derived from one healthy donor were tested.

[0034] FIGs 22a-22d: FIG. 22a, Histological sections and H&E staining of CAR T cell-infused meso- and control tumors. Scale bars, 100 μ m. The green dashed polygons in the images show the outline of tumors. Tissue sections were cut from similar locations

of respective tissue blocks. FIGs. 22b-d, Quantification of (FIG. 22b) tumor area, (FIG. 22c) effective diameter, and (FIG. 22d) circularity of tumor nests. Data are presented as mean $\pm$ SEM (n = 3-4).

[0035] FIG. 23: Flow cytometry is applied to the single cell suspensions derived from our model to identify the phenotypic states and changes of CAR T cells post-infusion. The live singlet lymphocytes are first gated for CD3⁺ CD8⁺ T cells, and then gated based on the CAR expression to identify meso-CAR T cells. Within meso-CAR T cells, we further gate using CD45RO and CD62L as markers for central memory cells (CD45RO⁺ CD62L⁺) and effector memory cells (CD45RO⁺ CD62L⁻); using CD103 as positive marker for tissue resident cells; using CD69 as positive marker for activated and tissue resident cells; using PD-1 as marker for recently activated cells.

[0036] FIG. 24: Heatmap showing top 20 differentially expressed genes for each cluster in the meso-tumor group.

[0037] FIG. 25: GO enrichment analysis for each cluster in the meso-tumor group. A Fisher's exact test was performed and corrected by the calculation of False Discovery Rate (FDR), with the enrichment cutoff at FDR $p < 0.05$.

[0038] FIG. 26: Violin plots showing select top differentially expressed genes by individual clusters identified in the meso-tumor model.

[0039] FIG. 27: UMAP plots showing the expression levels and spatial distribution of select genes for tumor and stromal cells in the meso-tumor group.

[0040] FIG. 28: Violin plots depicting the expression of select genes of interest in fibroblast subtypes from meso-tumor and control groups. Genes including TGFB1, TGFB2, SMAD2, FAP, ACTA2, SPARC, COL1A1, and FN1 are significantly upregulated in the subpopulations of fibroblast in which the majority of cells are located in the tumor compartment (i.e., M-6 and C-4) as shown in FIGs. 4d, 4i. Additional genes associated with fibroblasts in tumors in vivo such as HGF, FGF7, DCN, TWIST1, and LTBP1 are also upregulated in M-6 and C-4.

[0041] FIG. 29: UMAP plots showing the expression level and spatial distribution of select T cell genes.

[0042] FIG. 30: Violin plots showing the expression of gene signatures and individual marker genes by the subpopulations of CAR T cells in the meso-tumor group.

*** $P < 1e-7$ against M-11 for LEF1 and SELL, *** $P < 1e-7$ against M-9 for MIR155HG, *** $P < 1e-7$ against other clusters for the rest of genes.

[0043] FIG. 31: UMAP plots showing the expression level and spatial distribution of select genes in CAR T cells associated with 4-1BB signaling (left) and T cell activation (right).

[0044] FIG. 32: Heatmap showing top 20 differentially expressed genes for each cluster in the control group.

[0045] FIGs. 33a-33c: FIG. 33a, Violin plots comparing the expression of select genes by activated effector CAR T cells in the stromal and tumor compartments in the meso-tumor (M-11) and control (C-9) groups. FIGs. 33b,c, Comparison of gene expression by CAR T cell infiltrates in the control and meso-tumor groups. ** $P < 0.0005$, *** $P < 1e-6$ against meso-tumor and control tumor for MKI67, TCF7, SELL, and LEF1. *** $P < 1e-7$ against other groups for AGR2 and SERPINA1.

[0046] FIG. 34: Pseudotime dynamics of additional transcription factors expressed by cancer cells in the meso-tumor group.

[0047] FIGs. 35a-35e: FIGs. 35a,b, Separate UMAP plots showing the clustering of cells from the control (FIG. 35a) group and meso-tumor (FIG. 35b) group by cell type (top) and by location of origin (bottom). FIGs. 35c-e. Violin plots showing the cluster-specific expression of interacting gene pairs, including CCL3-IDE (FIG. 35c), GRN-EGFR (FIG. 35d), and CXCL10-CXCR3 (FIG. 35e).

[0048] FIG. 36: FIGs. 36a-b. Chord diagram comparison of significant ligand-receptor interactions between fibroblasts and endothelial cells (FIG. 36a) and CAR T cells and fibroblasts (FIG. 36b). FIGs. 36c-e. Violin plots showing the expression of interacting gene pairs, including (FIG. 36c) CD38-PECAM1, (FIG. 36d) TGFB1-EGFR, and (FIG. 36e) ITGAL-ICAM1 by major cell types.

[0049] FIGs. 37a-37d: FIGs. 37a-d. Violin plots comparing the expression of interacting gene pairs, including CD38-PECAM1 (FIG. 37a), TGFB1-EGFR (FIG. 37b), SPP1-CD44 (FIG. 37c), and ITGAL-ICAM1 (FIG. 37d) by individual clusters shown in FIG. 29.

[0050] FIGs. 38a-38c: FIG. 38a, Example image showing human mesothelioma explants embedded in the vascularized stroma. Scale bar, 200 μm . This image was taken 6 days after transplantation. FIG. 38b, Micrographs of transplanted and vascularized

mesothelioma tissues 2 days post CAR T cell infusion. The white dotted lines show the outline of tumor explants. Scale bar, 200 μm . FIG. 38c, Comparison of PD-1 expression by CAR T cells. Data are presented as mean $\pm$ SEM ($n \geq 4$). CAR T cells derived from one healthy donor were tested.

[0051] FIG. 39: UMAP plots showing the expression levels and spatial distribution of selected T cell genes.

[0052] FIGs. 40a-40d: FIGs. 40a-d, Quantification and comparison of (FIG. 40a) the percentage of vessel covered area, (FIG. 40b) the total branch length per microdevice, (FIG. 40c) the total number of junctions per microdevice, and (FIG. 40d) the average diameters of microvessels during the vascularization process between using CDX tumor from mouse and using patient-derived explant in our microengineered platform. Data are presented as mean $\pm$ SEM ($n = 4-18$).

[0053] FIGs. 41a-41e: FIG. 41a, Count and comparison of T cells infiltrating into stroma and tumor compartments by flow cytometry. FIGs. 41b,c, Representative flow cytometry plots of surface marker expression of CD45RO and CD62L by (FIG. 41b) meso-CAR T cells only, and (FIG. 41c) CD8⁺ T cells from both NTD T cell and meso-CAR T cell groups. FIG. 41d, Comparison of number of CD45RO⁺ CD62L⁻ CD8⁺ T cells. FIG. 41e, Comparison of intensity of cell tracker labelled T cells. CAR T cells derived from one healthy donor were tested.

[0054] FIGs. 42a-42b: FIG. 42a, Representative confocal micrographs of CAR T cell-infused composite lung tumor spheroids treated with different concentrations of LAF237 during long-term culture. Scale bars, 250 μm . FIG. 42b, Comparison of CAR T cell trafficking at Day 26. Data are presented as mean $\pm$ SEM ($n \geq 4$).

[0055] FIGs. 43a-43b: FIG. 43a. Representative confocal micrographs of composite lung tumor spheroids during daily treatment with LAF237 in the absence of CAR T cells. FIG. 43b. Quantification and comparison of normalized tumor area over time. Data are presented as mean $\pm$ SEM ($n = 3-6$).

[0056] FIGs 44a-44e: FIG. 44a. Development of gold nanoparticle (GNP) approach modified with valine-specific aptamer for the binding and detection of valine-proline dipeptide that is truncated off the intact CXCL10 by DPP4. b. UV-vis measurement of the aptamer modified GNPs after mixing with device effluent samples from the experiment in FIG. 8 at various time points post-infusion of CAR T cells. Less

peak shift in the group with high dose of DPP4 inhibitor, in comparison to the control group, indicates reduced truncation of intact CXCL10. FIG. 44c. Raman measurement confirming the presence of valine and proline on the surface of aptamer modified GNPs from the measurement in (FIG. 44b). FIG. 44d. Western blot measurement showing the intact CXCL10 protein control (first column), the artificially truncated CXCL10 protein control generated by incubation with DPP4 (second column), the increased truncation of CXCL10 in the non-treated control group from the experiment in FIG. 8 at day 8 post-infusion (third column), and preservation of intact CXCL10 in the high-dose DPP4 inhibition group from the experiment in FIG. 8 at day 8 post-infusion (fourth column). FIG. 44e. Unprocessed scan of western blots presented in (FIG. 44d).

[0057] FIGs 45a-45d: FIG. 45a, Experimental timeline for CAR T infusion and drug treatment. FIG. 45b, Representative images of single tumor spheroid treated with CAR T cells, LAF237 at 1000 nM, anti-CXCR3 antibody, or isotype-matched control antibody. Scale bar, 250 μ m. FIGs. 45c,d, Quantification of (FIG. 45c) T cell area and (FIG. 45d) normalized tumor area over time. Data are presented as mean $\pm$ SEM ($n \geq 3$). CAR T cells from one healthy donor were tested.

[0058] FIGs. 46a-46e: FIG. 46a, Visualization of CAR T-endothelial cell interactions mediated by CD38-PECAM1 and LTB-LTBR. FIG. 46b, Experimental timeline for CAR T infusion and drug treatment. FIG. 46c, Representative images of single tumor spheroid treated with CAR T cells and Daratumumab or Baminercept. Blood vessels are not shown in these images. Scale bar, 250 μ m. FIGs. 46d,e, Quantification of (FIG. 46d) T cell area and (FIG. 46e) normalized tumor area over time. Data are presented as mean $\pm$ SEM ($n \geq 3$). CAR T cells derived from one healthy donor were tested.

[0059] FIG. 47: The color in the heatmap indicates the relative abundance of metabolites with red and blue representing higher and lower abundance, respectively.

[0060] FIG. 49: Heatmap of top 50 metabolites identified by the partial least squares discriminant analysis (PLS-DA). The color in the heatmap indicates the relative abundance of metabolites with red and blue representing higher and lower abundance, respectively.

[0061] FIG. 49: Overview of top 25 significantly enriched metabolite sets from the analysis of 65 significantly upregulated metabolites identified in the high-dose LAF237 group.

[0062] FIGs. 50a-50f: FIGs. 50a,b, ROC curves for six biomarker prediction models, with increasing numbers of constituent metabolite features as indicated by the color legend, to identify biomarkers that can differentiate more efficacious CAR T cell treatment with high-dose LAF237 from control CAR T cell treatment without LAF237 at day 16 post-infusion (FIGs. 50a,c,e) and all time points (FIGs. 50b,d,f). FIGs. 50c,d, Predictive accuracy of biomarker models with increasing numbers of features. The most accurate biomarker model is highlighted with a red dot. FIGs. 50e,f, Predicted class probabilities for all samples using selected biomarker models of (FIG. 50e) 25 features and (FIG. 50f) 15 features. The classification boundary is at the center ($x=0.5$, dotted line).

[0063] FIG. 51: Boxplots show minimum, 25th percentile, mean, 75th percentile, and maximum. $N = 4$ for each group. Note that post-hoc pairwise comparison is only calculated between the high-dose and control groups at all time points with $*p < 0.05$.

[0064] FIGs. 52a-52e: FIG. 52a, Quantification of CCR2 expression among non-transduced donor T cells, meso-CAR T cells, and meso-CAR T cells with lentiviral transduction of CCR2. FIG. 52b, Quantification of tumor weight and numbers of tumor infiltrated human CD3⁺ T cells at day 33 post IV injection of three types of T cells. Data are presented as mean $\pm$ SEM ($n = 9$). FIG. 52c, Quantification of CCR2 expression between meso-CAR T cells with and without transient expression of CCR2 through electroporation delivery of CCR2 mRNA. FIG. 52d, Quantification of T cell-occupied hydrogel area at day 6 post-infusion of CAR T cells in the engineered EMMeso tumor chip model. Data are presented as mean $\pm$ SEM ($n = 3-5$). FIG. 52e, Quantification of tumor infiltrated human CD3⁺ T cells from different treatment groups at day 5 post IV injection. Data are presented as mean $\pm$ SEM ($n = 3-5$). T cells derived from three healthy donors were tested in this study.

DETAILED DESCRIPTION OF ILLUSTRATIVE EMBODIMENTS

[0065] The present disclosure may be understood more readily by reference to the following detailed description of desired embodiments and the examples included therein.

[0066] Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art. In case

of conflict, the present document, including definitions, will control. Preferred methods and materials are described below, although methods and materials similar or equivalent to those described herein can be used in practice or testing. All publications, patent applications, patents and other references mentioned herein are incorporated by reference in their entirety. The materials, methods, and examples disclosed herein are illustrative only and not intended to be limiting.

[0067] The singular forms “a,” “an,” and “the” include plural referents unless the context clearly dictates otherwise.

[0068] As used in the specification and in the claims, the term "comprising" can include the embodiments "consisting of" and "consisting essentially of." The terms “comprise(s),” “include(s),” “having,” “has,” “can,” “contain(s),” and variants thereof, as used herein, are intended to be open-ended transitional phrases, terms, or words that require the presence of the named ingredients/steps and permit the presence of other ingredients/steps. However, such description should be construed as also describing compositions or processes as "consisting of" and "consisting essentially of" the enumerated ingredients/steps, which allows the presence of only the named ingredients/steps, along with any impurities that might result therefrom, and excludes other ingredients/steps.

[0069] As used herein, the terms “about” and “at or about” mean that the amount or value in question can be the value designated some other value approximately or about the same. It is generally understood, as used herein, that it is the nominal value indicated $\pm 10\%$ variation unless otherwise indicated or inferred. The term is intended to convey that similar values promote equivalent results or effects recited in the claims. That is, it is understood that amounts, sizes, formulations, parameters, and other quantities and characteristics are not and need not be exact, but can be approximate and/or larger or smaller, as desired, reflecting tolerances, conversion factors, rounding off, measurement error and the like, and other factors known to those of skill in the art. In general, an amount, size, formulation, parameter or other quantity or characteristic is “about” or “approximate” whether or not expressly stated to be such. It is understood that where “about” is used before a quantitative value, the parameter also includes the specific quantitative value itself, unless specifically stated otherwise. Unless indicated to the contrary, the numerical values should be understood to include numerical values which are

the same when reduced to the same number of significant figures and numerical values which differ from the stated value by less than the experimental error of conventional measurement technique of the type described in the present application to determine the value.

[0070] Unless indicated to the contrary, the numerical values should be understood to include numerical values which are the same when reduced to the same number of significant figures and numerical values which differ from the stated value by less than the experimental error of conventional measurement technique of the type described in the present application to determine the value.

[0071] All ranges disclosed herein are inclusive of the recited endpoint and independently of the endpoints. The endpoints of the ranges and any values disclosed herein are not limited to the precise range or value; they are sufficiently imprecise to include values approximating these ranges and/or values.

[0072] As used herein, approximating language can be applied to modify any quantitative representation that can vary without resulting in a change in the basic function to which it is related. Accordingly, a value modified by a term or terms, such as “about” and “substantially,” may not be limited to the precise value specified, in some cases. In at least some instances, the approximating language can correspond to the precision of an instrument for measuring the value. The modifier “about” should also be considered as disclosing the range defined by the absolute values of the two endpoints. For example, the expression “from about 2 to about 4” also discloses the range “from 2 to 4.” The term “about” can refer to plus or minus 10% of the indicated number. For example, “about 10%” can indicate a range of 9% to 11%, and “about 1” can mean from 0.9-1.1. Other meanings of “about” can be apparent from the context, such as rounding off, so, for example “about 1” can also mean from 0.5 to 1.4.

[0073] Further, the term “comprising” should be understood as having its open-ended meaning of “including,” but the term also includes the closed meaning of the term “consisting.” For example, a composition that comprises components A and B can be a composition that includes A, B, and other components, but can also be a composition made of A and B only. Any documents cited herein are incorporated by reference in their entireties for any and all purposes.

[0074] Any embodiment or aspect provided herein is illustrative only and does not limit the scope of the present disclosure or the appended claims. Any part or parts of any one or more embodiments or aspects can be combined with any part or parts of any one or more other embodiments or aspects.

[0075] Despite its proven efficacy for hematologic malignancies, chimeric antigen receptor (CAR) T cell therapy has met with limited success in treating solid tumors. Although this represents a significant clinical challenge in cancer immunotherapy, preclinical efforts to assess and improve the efficacy and safety of CAR T therapy are hindered by our limited ability to model, probe, and modulate the complexity of cancer-immune interactions in solid tumors. Provided here is, inter alia, a microengineered platform for in vitro reproduction, real-time imaging, and in-depth analysis of malignant human solid tumors during CAR T therapy. Using a compartmentalized device with an externally accessible interface, this system enables one to engineer three-dimensional organotypic constructs composed of human tumor explants that can be vascularized and perfused with blood-borne immune cells in a controlled manner.

[0076] As an exemplary illustration of the disclosed technology, first presented is a microphysiological model of adeno-carcinoma tumors in the human lung infused with CAR-T cells to demonstrate its capabilities to reproduce the three essential steps of targeting solid tumors, including CAR T cell infiltration, recognition of tumor-associated antigens, and survival and effector function in the tumor microenvironment. Using flow cytometry and single-cell RNA sequencing, one can probe how the phenotype of CAR T cells changes during their recruitment and persistence. Furthermore, one can use the sequencing data to discover a therapeutic target that can be pharmacologically inhibited in the model to significantly increase CAR T cell trafficking and their anti-tumor effects, for which we also present specific biomarkers identified by untargeted metabolomics analysis. Finally, the present disclosure demonstrates the capability of the disclosed microengineered platform to model outcomes of solid tumor responses to an armored CAR T modification strategy in vivo. The present disclosure accordingly provides an approach for mechanistic studies and preclinical screening of adoptive cell therapies for cancer and other complex diseases.

[0077] By exploiting the destructive potential of the immune system in the fight against malignant cells, cancer immunotherapy has revolutionized the field of oncology

over the last decade. Among the key strategies of cancer immunotherapy is to use antigen-directed cytotoxicity of T lymphocytes to target and destroy cancer cells, which is known as adoptive T cell therapy. As one of the main modalities of this approach, chimeric antigen receptor (CAR) T cell therapy uses autologous or allogenic T cells genetically engineered to express synthetic CARs that can bind to target molecules on the surface of cancer cells to induce antigen-specific cytotoxicity. By using CD19-targeted CAR T cells, for example, this method demonstrated highly promising anti-cancer effects in patients with malignant B lymphocytes, leading to the recent approval of CAR T therapies for treating several types of leukemia and advanced B-cell lymphoma.

[0078] In contrast to the clinical success of CAR T therapy for hematologic malignancies, limited progress has been made in translating this technology into an effective treatment for solid tumors, which account for more than 90% of all cancer fatalities. Unlike blood cancer cells that express common tumor-specific antigens, malignant cells in most solid tumors lack unique surface markers and are often enriched with antigens that are highly heterogenous and also found on normal cells at lower levels. This makes it difficult to identify disease-specific target antigens required for the efficacy and safety of CAR T therapy. Another challenge arises from the local environment surrounding solid tumors, termed the tumor microenvironment (TME). Representing a complex, dynamic ecosystem composed of various cell types and extracellular components, the TME forms a physical barrier to trafficking of circulating CAR T cells from the bloodstream and their infiltration into tumor tissues. The TME also produces various molecular signals that can attenuate or inhibit immune responses of CAR T cells by suppressing their activation, proliferation, and long-term survival necessary to achieve effective and persistent anti-tumor function.

[0079] With increasing efforts to develop more effective CAR T therapies for solid tumors, understanding the biological underpinnings of these critical challenges has emerged as an area of intense investigation in preclinical research of cancer immunotherapy. What is generally required in these types of studies is to use live tumor-bearing animals, most commonly mice, to model the complexity of TME in human solid tumors and examine the interaction of circulating CAR T cells with malignant cells in vivo. As a common method, for example, xenograft models established by transplanting human tumor cell lines or patient-derived tumors into immunodeficient or humanized mice

allow sustained engraftment of human cancer cells, providing a platform to assess anti-tumor activity of infused human CAR T cells in vivo. To overcome the limitations of xenograft models due to their deficient or incomplete immunity, researchers have also developed syngeneic and transgenic models in which the behavior of murine CAR T cells in mouse tumors can be studied in vivo in the context of a fully competent immune system, which has proven useful for hypothesis-driven mechanistic investigation of cancer-immune interactions.

[0080] Despite considerable progress in animal studies, our ability to model CAR T therapy in vitro remains rudimentary. Given the complexity of solid tumors in the native environment, this is understandable but it also represents missed opportunities to develop potentially powerful preclinical technologies that can complement the capabilities of existing animal models. As illustrated by the failure of xenograft models to predict efficacy and cytokine-induced toxicities of CAR T cells reported in recent human clinical trials, the ability to generate data that are more translatable to human conditions is becoming increasingly important in preclinical studies of cancer immunotherapy. In principle, use of human cells readily possible in in vitro approaches would be advantageous for meeting this capability by providing a means to create human-relevant models of solid tumors and their responses to CAR T cells. Another important issue in preclinical research of CAR T therapy using animal models is the fact that CAR T cells are “living drugs”. Unlike traditional pharmacological agents, administered CAR T cells migrate, proliferate, undergo phenotypic changes, and persist, all of which are guided by their interactions with various biological signals from TME. Studies indicate that this complex, dynamic nature of therapy poses great challenges to the design of clinically relevant animal models and in-depth analysis of key biological processes underlying therapeutic effects and toxicities to produce pharmacology and toxicology data. In vitro techniques cannot fully resolve this problem but may complement some of the limitations of animal studies by permitting the development of preclinical models that are constructed and manipulated in a much more controllable manner and can be directly observed, accessed, and interrogated for in-depth analysis with greater flexibility.

[0081] Creating in vitro systems that can realize these advantageous capabilities, however, remains a challenge. Efforts to develop physiologically relevant, realistic in vitro models of CAR T therapy are greatly burdened by the difficulty of recapitulating the

complexity of human solid tumors in vivo. This problem is further complicated by another critical requirement of such models, which is to emulate a complex sequence of dynamic events that occur during the recruitment of CAR T cells and their interactions with cancer tissue.

[0082] Motivated by these challenges, provided here is a bioengineering approach that combines the advanced capabilities of microengineered cell culture with the inherent complexity of solid tumors in vivo to model cancer-immune interactions in vitro. The disclosed technology makes it possible to transplant living tumors from xenograft models or human patients into engineered vascular beds to generate vascularized human malignant solid tumor constructs that can be sustained for prolonged periods and perfused with human CAR T cells in a controlled manner. Using this platform, demonstrated here are i) in vitro modeling and direct visualization of the entire process of CAR T trafficking and anti-tumor activities and ii) phenotypic and transcriptomic interrogation of CAR T cells in tumor tissues using flow cytometry and single-cell RNA sequencing. Moreover, the present disclosure provides a strategy to enhance the efficacy of current CAR T therapies by presenting a druggable target discovered by analyzing ligand-receptor interactions of our model that can be pharmacologically modulated to significantly increase tumor-specific CAR T cell trafficking. Finally, the disclosure shows the use of the model to discover metabolic biomarkers of therapeutic efficacy that can be useful for continuous monitoring of this treatment strategy.

[0083] Results

[0084] Construction of microengineered platform for in vitro transplantation of solid tumors

[0085] Tumor transplantation is an established technique used for the study of tumorigenesis and metastasis in which malignant tumor tissues are surgically removed from donors and implanted into anatomically appropriate sites in recipient animals (FIG. 1a). Essential for the success of this method is engraftment of tumor transplants, which is achieved by their anastomosis with the host vasculature and subsequent vascular perfusion. Our approach is based on the same principle with the exception that tumor tissues are transplanted into microengineered recipient devices with externally accessible living stroma that contains a three-dimensional (3D) network of perfusable human blood vessels (FIG. 1b).

[0086] To enable this in vitro transplantation, provided is a 3D culture system in a compartmentalized microdevice that in some embodiments can comprise individually addressable three parallel cell culture chambers defined by two microfabricated rails protruding from the bottom surface (FIG. 1c). Another design feature of this system is a longitudinal array of funnel-shaped holes in the ceiling of the device, which provide direct access to the culture chambers from the external environment (FIG. 1c, FIG. 12). This open-top design allows for i) the production of microengineered 3D tissue reminiscent of the vascularized stroma surrounding solid tumors and ii) on-demand transplantation of tumor explants into the vascularized stromal constructs.

[0087] An exemplary first step of this sequential process is to block the open-top access in the device ceiling using a micropatterned insert designed to fit into the funnel-shaped wells (Step 1 in FIG. 1d). Next, an extracellular matrix (ECM) hydrogel precursor solution containing suspended human endothelial cells (e.g., human umbilical vein endothelial cells - HUVEC, and human lung microvascular endothelial cells - HMVEC-L) and stromal cells is injected into the middle chamber using an independent fluidic access port and solidified to embed and grow the cells in 3D with culture media in the side chambers (Step 2 in FIG. 1d, FIG. 13). After 24 hours of culture, the side chambers are seeded with human endothelial cells (HUVEC or HMVEC-L) (Step 3 in FIG. 1d). Designed to mimic de novo blood vessel formation during vasculogenesis, this culture configuration induces the formation of tubular structures by the endothelial cells in the hydrogel scaffold and their assembly into an interconnected 3D vascular network that anastomoses with the endothelial lining of the side chambers (Step 4 in FIG. 1d). The microengineered vascular bed constructed using this technique can be maintained in the device until tumor explants become available for in vitro transplantation. This procedure begins with the removal of the insert from the device ceiling, which is followed by injection of tumor tissues cut into an appropriate size into the funnel-shaped wells and then by injection of acellular ECM solution to cover and seal the wells (Step 5 in FIG. 1d). Over the next few days after gelation, the tumor implants embedded in the microengineered stroma become vascularized by the surrounding blood vessels, which also makes the tumors accessible and perfusable from the side chambers (Step 6 in FIG. 1d).

[0088] For an example, non-limiting proof-of-concept demonstration, we first generated a 3D tissue construct emulating the vascularized stroma in the human lung by culturing primary human lung fibroblasts and human endothelial cells (HUVECs) in a fibrin hydrogel in the middle chamber (FIG. 1e, 1f). In this environment, the endothelial cells became elongated to form thin sprouts initially but over a period of 5 days, these structures adjoined together to create a network of connected endothelial tubes throughout the scaffold (FIG. 1e). This vascular network continued to develop steadily over time as evidenced in the increasing branch length and average diameter of microvessels (FIG. 14). The self-assembled microvasculature remained stable for prolonged periods (over 14 days) without a loss of structural integrity and vascular connectivity (FIG. 1f). This microengineered construct was then used for in vitro transplantation of lung tumors surgically removed from lung cancer patients (FIG. 1g). Time-lapse analysis of the model in this experiment showed directional growth of existing blood vessels in the surrounding area towards the tumor deposited into the open well within 3-5 days of transplantation and as a result, the entire implant was enveloped by the vasculature by day 9 (FIG. 1h). In any given device, this in vitro vascularization of transplanted human lung tumors was observed across the entire array of open wells (FIG. 1i). Importantly, when a pressure difference was generated across the hydrogel, it was possible to flow media between the side chambers through the microengineered blood vessels, making it possible to perfuse the vascularized tumors and their local microenvironment in a controlled manner (FIGs. 1j, 1k).

[0089] The multi-well design of the disclosed platform allows for implantation and simultaneous vascularization of multiple tumors within the same construct. This is useful for modeling microscopic clusters of tumor cells surrounded by vascularized stroma, known as tumor nests, which have been recognized as an important microarchitectural feature of various types of malignant solid tumors in vivo (FIG. 15). Because the size and relative position of the wells are readily adjustable during device fabrication, this design also provides a means to pattern the spatial arrangement of transplanted tumors with a high degree of controllability (FIG. 11). Individual accessibility of the open wells adds to this capability by permitting spatiotemporal variation of cell/tissue types deposited into the wells for studies that require, for example, the inclusion of different types of solid tumors or a combination of malignant and normal tissues within

the same vascularized construct (e.g., in vitro modeling of cancer metastasis) (FIGs. 1m, 1n). 3D imaging reconstruction revealed details of the spatial arrangement of tumor-vascular interface and demonstrated that the engineered microvessels in the model of vascularized solid tumors indeed are wrapping around the tumor construct, forming close contact with the surface of tumor, and can also penetrate into the tumor (FIG. 1o). Finally, our platform can be further engineered to enable the formation and maintenance of the vascularized tumor constructs in an array format for increased experimental throughput (FIG. 1p) and can also be used for in vitro vascularization of tumor spheroids or organoids (FIG. 16). The molds for the fabrication of device layers can be 3D printed. The fabrication and assembly of the arrayed microdevices are simple and straightforward, and does not require any specialized equipment, materials, or expertise other than standard microfluidic techniques.

[0090] In vitro modeling, direct visualization, and analysis of CAR T cell-tumor interactions

[0091] Next, the disclosure provides the capabilities of the disclosed platform for the study of how CAR T cells interact with the microengineered tumor transplants. The focus of this work was to demonstrate the use of the system as an in vitro platform to model and probe three essential steps of CAR T cell trafficking, including i) their extravasation and infiltration into solid tumors, ii) recognition of tumor-associated antigens by infused CAR T cells, and iii) their survival and persistent anti-tumor function (FIG. 2a), which also represent three critical challenges of CAR T therapies for solid tumors.

[0092] To demonstrate the proof-of-principle of this approach, a complementary pair of malignant human lung tumors and CAR T cells (FIG. 2b) was used – lung tumors formed in a cell line-derived xenograft (CDX) model using A549 human lung adenocarcinoma cells engineered to overexpress mesothelin (FIG. 17), a tumor-associated antigen expressed at significantly higher levels in various types of cancer, and healthy donor-derived T cells transduced to express mesothelin-targeted CARs (referred to as meso-CAR T cells hereafter) (FIG. 17), which are being tested in ongoing clinical trials for immunotherapy of solid tumors. In this study, CAR T cells derived from three healthy donors were tested. When the mesothelin-overexpressing tumors (referred to as meso-tumors hereafter) were transplanted into a microengineered vascular bed, they were

vascularized within 7 days and exhibited rapid growth over time, as illustrated by their enlargement over the course of 11 days (FIG. 2c, FIG. 18). Similar patterns of tumor vascularization and growth were observed in the control group containing human lung tumor explants from a CDX model established using wild-type A549 cells that express very low levels of mesothelin (referred to as control tumors hereafter) (FIG. 2d, 17, 18). Of note, the initial sizes of transplanted tumors appeared to affect their vascularization (FIG. 19), with tumors of larger initial sizes (i.e., $>400\ \mu\text{m}$) achieving similar rate but higher extent of vascularization over time in comparison to tumors of smaller sizes (i.e., $<200\ \mu\text{m}$).

[0093] By day 13, a tumor mass was achieved in both meso-tumor and control groups, at which time a single dose of meso-CAR T cells was administered into the models. The CAR T cells infused into the side chamber of the meso-tumor-containing devices immediately flowed into the 3D vasculature in the hydrogel but during perfusion, many of these cells adhered to the blood vessels surrounding the tumors and began to spread and migrate along the vascular lumen, displaying the phenotype of activated T cells (FIG. 2e). Over the next 24 hours, these adherent cells were observed to undergo diapedesis across the endothelium and extravasate into the surrounding stroma, which was followed by directional migration towards the tumors (FIG. 2f). On the next day, the tumors were seen with a large number of CAR T cell infiltrates, and their number continued to increase over time during 6-day culture post infusion (FIGs. 2g, 2i, 2j). Notably, the projected area of these tumors remained relatively constant during this period, indicating arrested tumor growth (FIGs. 2h, 2k, 18).

[0094] These results were in contrast to what was observed in the control group. After a single dose of meso-CAR T cell infusion under identical conditions, the vast majority of the administered cells flowed through the tumor-associated vasculature without establishing firm attachment (FIG. 2l). A small number of T cells were detected within the vascular network 24 hours post infusion but most of them showed round morphology and remained at their original locations without any noticeable motility (FIG. 2m). In this group, the number of CAR T cells in the tumors did not change over time (Figs. 2i, 2j, 2n), and their effects on tumor growth were negligible as shown by a 6-fold increase in the tumor area by the end of 6-day culture following cell infusion, indicating a lack of cytotoxicity of meso-CAR T cells against control tumors (FIGs. 2k, 2o).

[0095] To gain more insight into this significant difference, we first measured endothelial expression of intercellular adhesion molecule-1 (ICAM-1). Compared to those associated with control tumors, the blood vessels present in the meso-tumors were seen with significantly more robust expression of ICAM-1 (FIG. 2p), supporting the observation of increased CAR T cell adhesion to the vasculature. By sampling device effluent, we also analyzed the concentration of interleukin-2 (IL-2) and interferon-gamma (IFN- γ), which are well-known markers of T cell activation and cytotoxic activity.

[0096] ELISA revealed significant production of both cytokines in the CAR T cell-treated meso-tumor model, whereas the control group did not yield any measurable amounts (FIG. 2q). This result was corroborated by significant cell death in the meso-tumor group evidenced by substantially increased caspase-3 expression by tumor cells and LDH release into the vascular perfusate (FIG. 2r). Chemokines secreted in both tumor groups were also profiled by collecting device effluent prior to the infusion of CAR T cells (FIG. 20). No relative difference was observed between control and meso-tumor groups in the expression of 34 out of 38 chemokines examined. There were, however, relative increases in the expression of RANTES (CCL5), MCP-1 (CCL2), Ck β 8-1 (CCL23), and CXCL16 in the meso-tumors, which may contribute to the increased trafficking of T cells.

[0097] The transplantation of CDX tumor explants may introduce mouse cells into the model. Although the meso-CAR construct (SS1 scFv) of the T cells only recognizes human mesothelin but not mouse mesothelin and in principle would not elicit xenogeneic reactions against mouse cells, it is possible that xenogeneic effect may still be present due to the intrinsic TCR signaling of T cells such as after recognizing the xenogeneic MHC molecules. Given that previous studies generally reported very low levels of T cell responses to xenogeneic cells, however, another study was performed to confirm any potential xenogeneic effects due to the use of CDX tumor explants and to also confirm the effect of CAR modification on the antigen-dependent T cell-tumor interactions. For this study, the experiment was modified where the meso- and control tumors were treated with not only meso-CAR T cells but also non-transduced T cells (NTD T) both of which were derived from the same healthy donor (FIG. 21). In contrast to the continuously increasing trafficking and infiltration of meso-CAR T cells post infusion to the meso-tumors which was accompanied by controlled tumor growth, there was minimal trafficking and infiltration of NTD T cells over time regardless of their infusion

to meso- or control tumors. Corresponding tumor growth post infusion of NTD T cells was continued in a similar fashion as the non-treated tumor group. Thus, the potential xenogeneic responses of T cells in our model appeared to be negligible. These results also confirmed that the significant meso-CAR T-meso-tumor interactions observed are attributable to the CAR modification of infused T cells.

[0098] For further analysis, a technique to peel apart the layers of the sealed culture device and harvest the vascularized tumor tissues intact after CAR T cell infusion and incubation (FIG. 2s) was developed. After the delamination process, more than 95% of the tumor nests remained attached to the tissue construct sheet as confirmed by gross and microscopic examination of the harvested tissues. The tumor shape and integrity were also fully preserved in both tumor groups as evidenced in the H&E staining and analysis (FIG. 22). Consistent with the observation on-chip, the tumor area and effective diameter of control tumors were significantly higher than those of the meso-tumors by the end of CAR T incubation, while tumor nests from both groups remained similarly and highly circular in shape. This method permitted immunohistological examination of the entire tumor constructs, which provided direct evidence of extensive infiltration and accumulation of CD8⁺ T cells in the meso-tumors (FIG. 2t). The tumor-killing effects of these CAR T cell infiltrates were evident from the fragmented morphology of tumor cells (inset, FIG. 2t). In contrast, accumulation of CD8⁺ cells in the control group was only observed along the tumor boundaries with very few cells visible in the tumor mass (FIG. 2u), approximating the spatial characteristics of T cell exclusion reported in solid tumors *in vivo*.

[0099] The models described here clearly show significant differences in immune responses of CAR T cells caused by their differential interactions with lung tumors expressing significantly different levels of mesothelin. These data verify the principle of the mesothelin-targeted approach in this study but they also demonstrate the application of the microengineered tumor transplants to emulate and visualize CAR T cell trafficking and persistence in solid tumors in a more physiologically relevant experimental setting.

[00100] Spatially-defined analysis of CAR T cell phenotypes

[00101] Given that the ability of T cells to localize to tumor sites and carry out their anti-tumor function is reflected in their phenotypes, next was investigated whether

and how meso-CAR T cells infused into our models change their phenotype in the microengineered tumor constructs. The analysis was conducted in a spatially defined manner to distinguish the T cell population within the tumors from the one in the surrounding vascularized stroma to interrogate their characteristics separately. This was achieved by isolating only the tumors from the construct through the openings of the wells 6 days post infusion and dissociating them to retrieve CAR T cell infiltrates (FIG. 3a). Subsequently, the remaining tissues without the tumors were harvested from the devices and processed to release CAR T cells in the stroma (FIG. 3a). In this study, CAR T cells derived from one healthy donor were tested.

[00102] Using flow cytometry, the numbers of CAR T cells in different regions of the construct were measured. Both meso-tumor and control groups showed substantially higher abundance of CAR T cells in the stroma in which over 90% of the infused cells were found (FIG. 3b), illustrating tumor resistance to infiltration by CAR T cells. Compared to control tumors, however, a significantly larger fraction of CAR T cells was found within the tumor mass in the meso-tumor group (FIG. 3b), which was consistent with the general trend of CAR T cell trafficking shown by imaging analysis (FIG. 2). Importantly, 35% of these CAR T cells in the meso-tumors expressed CD69 – a marker for T cell activation and tissue retention – and this was almost a 4-fold increase from the percentage of CD69+ cells in the original population infused into our model (9.9%) (FIG. 3c, 23). CD69-expressing CAR T cells were also found in the vascularized stroma of meso-tumors but the fraction of these cells was significantly lower (15.3%) and comparable to what was measured in the control group (FIG. 3c). Although the control tumor infiltrated CAR T cells also contained a significant fraction of CD69+ cells (25%), their absolute number was insignificant compared to that of meso-tumor infiltrated CAR T cells that were CD69+ (FIG. 3b,c). Flow cytometric analysis of programmed death-1 (PD-1), which is another established marker of T cell activation that allows for identification of tumor-reactive T cells, showed similar patterns of compartment-specific differential expression between the groups (FIG. 3d).

[00103] To further characterize the phenotypic states of CAR T cells in the meso-tumors, it was then examined whether they express markers of central memory (CD62L+ CD45RO+) and effector memory (CD62L- CD45RO+) T cells. This analysis revealed a substantial increase in the fraction of CD62L- CD45RO+ CAR T cells from

21.7% in the infused original population to 57.7% in the meso-tumors 6 days post infusion (FIG. 3e), indicating that the majority of the tumor-infiltrating CAR T cells in this model assumed the phenotype of effector memory T cells (T_{EM}). Although this relative increase in the T_{EM} population was also noted in control tumors, the extent of increase was not as pronounced and the number of T_{EM} cells was significantly limited in comparison to that of meso-tumor infiltrated CAR T_{EM} cells, and a substantial fraction of the cells (39.4%) expressed the markers of central memory T cells (T_{CM}) (FIG. 3e). In both meso-tumor and control groups, the preferential differentiation of CAR T cells into T_{EM} was not observed in the stromal compartment (FIG. 3e). Our characterization also showed that the population of CD103⁺ tumor-reactive tissue-resident memory T cells (T_{RM}) was negligible or accounted for minute fractions of CAR T cells in the stromal compartment surrounding the meso-tumors or in the control model, similar to the infused original population (FIG. 3f). However, despite subtle differences in the fractions of CD103⁺ CAR T cells between the meso-tumor compartment and stromal compartment or the control model, comparison to the infused original population clearly suggested the phenotypic alterations of a significant fraction of CAR T infiltrates in the meso-tumors towards T_{RM} cells (FIG. 3f) which have been shown to play roles in anti-tumor activities of T cells and their persistence.

[00104] The trends of T cell activation and differentiation described here demonstrate the ability of the tumor antigen-encountered CAR T cells to induce immediate effector function but also retain their memory for persistence and increase their potential for longevity. Notably, the cancer-induced CAR T cell differentiation into T_{EM} and T_{RM} observed in the meso-tumor model is similar to what occurs in tumor-infiltrating lymphocytes (TILs) in mesothelioma and early-stage non-small cell lung cancer (NSCLC) patients. Taken altogether, the results of the analysis demonstrate probing progressive phenotypic changes of CAR T cells during the course of their trafficking, anti-tumor function, and persistence in our microengineered tumor models.

[00105] Single-cell RNA sequencing analysis of CAR T cells in solid tumors

[00106] To develop a more in-depth understanding of tumor-CAR T interactions in our models, single cell RNA sequencing (scRNA-seq) of the entire CAR T cell-infused tumor constructs was performed. For this work, cells were harvested from the microengineered tissues at Day 6 post infusion in a compartment-specific manner

described above. CAR T cells derived from one healthy donor were tested. Overall, there was only a small, insignificant percentage of reads that aligned to the mouse genome and was excluded for analysis.

[00107] Unsupervised clustering of the sequencing data from the meso-tumor model using uniform manifold approximation and projection (UMAP) yielded four clusters corresponding to three distinct groups of cells, including i) tumor cells identified by their expression of mesothelin (MSLN) and other genes indicative of their epithelial origin (e.g., AGR2) as well as their association with lung cancer (e.g., EGFR), ii) CAR T cells that express known T cell markers such as CD3E and IL7R, and iii) a mixed population of endothelial cells and fibroblasts distinguished by their expression of endothelial markers (e.g., PECAM1, VWF) and genes involved in ECM synthesis (e.g., COL1A1, TIMP1) and contractility (e.g., ACTA2, TAGLN) (FIGs. 4a, 4b, FIG. 24). These different cell types were confirmed by gene ontology enrichment analysis (FIG. 25). Spatial mapping of the identified clusters showed that the entire tumor cell population was associated only with tumor masses, whereas T cells, fibroblasts, and endothelial cells were found in both stromal and tumor compartments (FIG. 4c).

[00108] Further analysis of cellular phenotypes in the meso-tumor model revealed a total of 11 different subpopulations (FIG. 4d). Specifically, the tumor cell clusters were composed of 5 subtypes. Among them are tumor cells that express hypoxia-inducible genes (e.g., NDRG1, EGLN3, HILPDA) (M-3 in FIG. 4d; FIG. 4e, FIGs. 24, 26, 27) or genes implicated in the survival of cancer cells, such as SERPINA1 (M-2 in FIG. 4d; FIG. 4e, FIGs. 24, 26, 27), which reflects the behavior of tumor cells in the engineered tumor microenvironment. Importantly, immune attack by CAR T cells in this model was demonstrated by a tumor cell cluster that was uniquely identified by increased expression of WARS and GBP1 known to play a critical role in cellular responses to interferon- γ and other cytokines and by GO analysis that indicated tumor necrosis factor-mediated signaling (M-1 in FIG. 4d; FIG. 4e, FIGs. 24-27). In case of fibroblasts in the stroma, UMAP analysis showed two subpopulations. One of these clusters was characterized by the markers of perivascular cells that regulate vascular remodeling during cancer angiogenesis (e.g., ANGPT1, COL4A2) (M-6 in FIG. 4d; FIG. 4f, FIGs. 35-39), corroborating tumor vascularization as an important feature of our model.

[00109] The CAR T cell group contained three clusters, all of which were enriched with CD8⁺ T cells (FIGs. 4d, 4g, FIGs. 24-26, 29). These subpopulations, however, exhibited distinct transcriptomic signatures reflecting different activation states and phenotypes during CAR T cell recruitment to meso-tumors. Specifically, the results showed resting meso-CAR T cells residing predominantly in the stroma that displayed markers of central memory CD8⁺ T cells (e.g., LEF1, SELL, TCF7) (M-9 in FIG. 4d; FIGs. 4g, 4h, 24-26, 29-31). These cells also expressed genes that are inducible by inflammatory cytokines (e.g., IL32, CD48, ZFP36L2) (FIG. 4g, 24-26, 29-31). The neighboring cluster showed an abundance of early activated cells that also expressed genes associated with cell motility and chemotaxis (e.g., CXCR4, RGS1)⁷⁰ (M-10 in FIG. 4d; FIGs. 4g, 4h, FIGs. 24-26, 29-31). Given that these cells were located in the stromal region, the cluster M-10 likely represents CAR T cells undergoing infiltration and directional migration towards tumors.

[00110] Activated CAR T cells were also found in Cluster M-11, and the majority of these cells were localized to tumor masses and expressed high levels of cell-cycle genes (e.g., TOP2A, CENPF, MKI67) (FIGs. 4d, 4g, 4h, FIGs. 24-26, 29-31), illustrating their identity as tumor-infiltrating CAR T cells undergoing proliferation presumably due to antigen stimulation and activation. The phenotype of this population as effector T cells was further verified by effector gene signatures and by GO analysis that indicated its capacity for interferon-gamma signaling and production (FIGs. 25, 30). Compared to those in the other CAR T cell clusters, these cells also expressed significantly higher levels of gene markers associated with ER stress response such as AGR2 that can be induced by the hostile microenvironment of solid tumors during CAR T cell infiltration (FIG. 4h).

[00111] Comparing these results to transcriptomic features of the control tumor model revealed notable differences. As demonstrated by the smaller number of clusters in the tumor cell population, the phenotypic variability of tumor cells was greatly reduced compared to the meso-tumor model, and a large fraction of the cells were characterized by patterns of gene expression known to promote cancer progression (e.g., MUC5AC, CEACAM6) (C-1 in FIG. 4i, FIG. 32). In the case of CAR T cells, similar clustering was observed but the relative abundance of the subpopulations was different from that in the meso-tumor group. For instance, the early activated cell type (C-8 in FIG.

4i) accounted for a substantially larger proportion of the CAR T cell population, whereas the activated effector CAR T cells (C-9 in FIG. 4i) were present in much lower relative abundance (FIG. 4j), consistent with our imaging and flow cytometry data demonstrating reduced CAR T cell trafficking in the control tumors (FIGs. 2, 3). In comparison to those in the meso-tumor model, the tumor-infiltrating effector CAR T cells in this group also showed higher levels of naïve T cell markers and lower expression of MKI67 and SERPINA1 (FIG. 4k, FIG. 33), indicating their reduced effector function, proliferative capacity, and persistence.

[00112] To further examine the phenotypic difference of tumor-infiltrating CAR T cells between the two groups, we used the sequencing data to compare groups of gene markers and transcription factors associated with T cell differentiation into effector memory (T_{em}) or terminal effector T cells (T_{eff}). This analysis showed that when compared to the control group, CAR T cells isolated from the meso-tumors expressed higher levels of genes upregulated in T_{em} (e.g., SOCS1, IL2RA, CCL3) and lower levels of genes known to be downregulated in T_{em} relative to T_{eff} (e.g., GNLY, GZMH, KLRB1) (FIG. 4l, FIG. 33), suggesting skewed differentiation of CAR T cell infiltrates towards T_{em} in the meso-tumors.

[00113] Finally, we attempted to investigate how our findings from the meso-tumor model compare the behavior of T cells infiltrating into malignant solid tumors of human lungs in vivo. To this end, we performed automated, unbiased cell type annotation of our CAR T cell sequencing data using SingleR in conjunction with published scRNA-seq of human TILs isolated from treatment-naïve NSCLC patients as a reference dataset (FIG. 4m). Importantly, the results of this analysis were consistent with the marker-based manual classification and characterization of CAR T cells described above. In the meso-tumor model, for example, most of the cells in Cluster M-9 – a subpopulation of CAR T cells that were less activated and expressed higher levels of naïve markers – were recognized by SingleR as CD8_C1-LEF1, which represents patient naïve T cells mostly from the peripheral blood (FIG. 4n). In comparison, a large portion of the cells in Clusters M-10 and M-11, especially tumor-infiltrating CAR T cells in M-11, were annotated as either CD8_C5-ZNF683 (tissue-resident memory T cells) or CD8_C6-LAYN (exhausted T cells), both of which originate from patient tumors (FIG. 4n). Very few T cells were identified as CD8_C3-CX3CR1 effector cells or CD8_C4-GZMK “pre-

exhausted” T cells, suggesting the lack of terminal effector differentiation and thus potential for persistence of CAR T cells (FIGs. 4m, 4n).

[00114] Single-cell trajectory analysis of CAR T and tumor cells

[00115] Having identified distinct cell populations in the CAR T cell-infused meso-tumor transplants, we next sought to probe the dynamics of how the transcriptional profiles of CAR T and tumor cells in this model are regulated during their interactions. In this study, single-cell trajectory analysis was performed using Monocle3 to examine how the expression levels of top-ranked genes indicative of different cellular phenotypes change over pseudotime.

[00116] First, CAR T cell data were isolated and re-clustered them for pseudotime analysis (FIG. 10a). The results showed the transition of CAR T cell phenotype from resting T cells to early activated T cells to proliferative effector T cells to cytotoxic effector T cells along the pseudotime trajectory (FIGs. 10a, 10b). Concurrent with this progressive phenotypic alteration was biphasic regulation of gene markers associated with T cell activation (e.g., GZMB, CCL3, IL2RA, IFNG). These markers began to increase with the emergence of early activated CAR T cells in the stromal compartment and peaked when the cells exhibited the phenotype of proliferative effector T cells within the tumors, which was followed by a plateau or gradual decrease with the progression of the cytotoxic activities of the CAR T cells while they were still in the tumors (FIGs. 10c, 10d). Over the same period of pseudotime, the activated effector CAR T cells in the tumors showed monotonically increasing expression of stress markers, such as AGR2, IGFBP1, S100P, and SPP1 (FIGs. 10e, 10f), demonstrating exposure and responses to increasing cellular stress in the CAR T cells as they gain effector function, enter the local environment of solid tumors, and engage cancer cells.

[00117] Among transcription factors with significant correlation with the phenotypic changes of CAR T cells were KLF2 and TXNIP (FIGs. 10g, 10h). In contrast to the increasing expression of activation-associated genes, both of these markers exhibited almost monotonically decreasing patterns of gene expression, suggesting that the downregulation of KLF2 and TXNIP may be required for antigenic activation of CAR T cells due to their established roles in regulating T cell effector functions and glucose consumption. Increases in the expression of CREM and DDIT3 along the pseudotime trajectory were also found to be closely associated with the transition of CAR T cells

towards effector lineages (FIGs. 10g, 10h), consistent with their reported role as negative regulators of effector functions.

[00118] Pseudotime trajectories generated by the analysis of cancer cell populations in the meso-tumors demonstrated the sequential change of their phenotype from responding to stimulation by IFN- γ to promotion of survival to hypoxically stressed to apoptotic or apoptosis-resistant states (FIG. 10i). This transition was associated with several gene markers and transcription factors whose expression was differentially regulated with pseudotime. For instance, one of the features of the cells at the beginning of the pseudotime trajectory was their highest expression of genes known to be activated by IFN- γ (e.g., STAT1, WARS) (FIG. 10j), verifying their phenotype as tumor cells stimulated by effector CAR T cell-produced IFN- γ . With progressive changes in the state of the tumor cells, these gene markers showed a cycle of downregulation and subsequent recovery in their expression with the emergence of the apoptotic phenotype towards the end of the trajectory (FIG. 10j). This trend was reversed in the expression of SERPINA1, FOS, JUN, and other transcription factors associated with the promotion of survival in responses to external stimulation such as IFN- γ (FIG. 10j, FIG. 34).

[00119] Another notable feature of the tumor cell population was the induction of genes implicated in tumor cell apoptosis or inhibition of cancer progression (e.g., MTRNR2L8, NDRG1, IGFBP3) and their continuous increase along the pseudotime trajectory prior to entering the apoptotic states (FIG. 10k), illustrating persistent deleterious effects of tumor-infiltrating CAR T cells. Interestingly, transcriptomic signatures of tumor cells also included markers that promote tumor development and progression (e.g., MUC5AC, NEAT1) (FIG. 10k, FIG. 34), which may be interpreted as a mechanism to protect/recover stressed and apoptotic tumor cells affected by CAR T cells. Increased induction of these genes was indeed visible at the late stages of phenotypic transition as the tumor cells moved towards the apoptotic state (FIG. 10k, FIG. 34).

[00120] Interrogation of ligand-receptor interactions in CAR T cell-infused lung tumors

[00121] For more in-depth analysis of the biological complexity of our models, the crosstalk of different cell populations present in the microengineered tumor transplants was investigated. This study was carried out using CellPhoneDB to predict

enriched intercellular communication mediated by ligand-receptor complexes from single-cell sequencing data (FIG. 5a).

[00122] The results showed that the meso-tumor and control groups each contained more than 150 ligand-receptor interactions with statistically significant ($p < 0.05$) cell-type specificity (FIG. 5b, FIG. 35). In both models, activated CAR T cells and tumor cells were found to be major contributors to these interactions as demonstrated by substantially large numbers of ligand-receptor interactions involving these cell populations (FIG. 5b). Pairwise analysis of the crosstalk between the two cell types showed several directional interactions shared by the meso-tumor and control groups. Among them were APP-CD74, PTPRC-MRC1, and CD58-CD2 (purple chords in FIG. 5c), all of which are known to be involved in mediating T cell adhesion to tumor cells. The data also predicted the interaction of SPP1 secreted by tumor cells with receptors, CD44 and PTGER4, on T cells (blue chords in FIG. 5c), which play a critical role in the suppression and activation of T cell signaling in the TME in vivo, respectively. In comparison to the control group, however, the meso-tumor model contained substantially greater numbers and extent of interactions involved in functional activation and cytotoxicity of “on-target” T cells that are mediated by binding of T cell-produced ligands to their receptors on cancer cells (e.g., CCL3-IDE, IL13-TMEM219, TNFSF10-TNFRSF10B) (red chords in FIG. 5c, FIG. 5d, FIG. 35).

[00123] Pairwise comparison of the tumor and endothelial cell populations revealed ligand-receptor signaling implicated in endothelial adhesion of tumor cells (e.g., CD38-PECAM1) in both the meso-tumor and control groups (purple chords in FIG. 5e), indicating close physical association between these two cell types at the tumor-vascular interface. Also identified in this cell pair were directional interactions of endothelial ligands, such as GRN, AREG, and TGFB1, with EGFR on the tumor cells (blue chords in FIG. 5e, FIG. 5f, FIG. 35), which has been shown to promote tumor growth and transformation. The interacting pair of MIF-EGFR known to inhibit EGFR signaling was also present in both groups (blue chords in FIG. 5e) but the number of these signaling events was relatively lower than that of interactions that lead to tumor-promoting effects.

[00124] Without being bound to any particular theory or embodiment, the data suggested that the interaction between fibroblasts and tumor cells may promote mutual survival and tumor progression in both the meso-tumor and control groups (FIG.

5g, 5h). This was exemplified by predicted binding of GRN and AREG from fibroblasts with EGFR on tumor cells, which is known to stimulate the growth of lung cancer cells (blue chords in FIG. 5g). SPP1 produced by tumor cells was shown to contribute to fibroblast expression of CD44 (green chord in FIG. 5g), which has been described as the phenotype of cancer-associated fibroblasts in vivo. The fibroblasts were also involved in signaling with CAR T cells mediated by interacting pairs of CD2-CD58 and ITGAL-ICAM1 (FIGs. 36, 37). Without being bound to any particular theory or embodiment, this suggests direct intercellular contact between the two cell types, which may happen during the migration of extravasated CAR T cells through the stromal compartment.

[00125] Analysis of the CAR T cell-endothelial cell pair in the meso-tumor and control groups identified ligand-receptor complexes known to mediate endothelial adhesion of T cells (e.g., LTB-LTBR, CD38-PECAM1, ITGAL-ICAM1) (purple chords in FIG. 5i, FIG. 37) but as expected, the number and variety of these interactions were substantially greater in the meso-tumor constructs, supporting the observation of increased CAR T cell trafficking into the meso-tumors. On a related note, the data also revealed directional signaling between CXCL10 and CXCL11 – endothelial chemokines induced by IFN- γ stimulation – and CXCR3 on CAR T cells (red chords in FIG. 5i). These interactions, which serve to mediate the recruitment of T cells and other immune cells, were absent in the control group (FIGs. 5i, 5j, FIG. 35).

[00126] Transplantation and CAR T cell infusion of tumor explants from mesothelioma patients

[00127] Having demonstrated the capabilities of the platform using CDX lung tumors genetically engineered to overexpress mesothelin, next was investigated whether this technology could be applied to the analysis of unmodified, native tumor explants isolated from cancer patients. To make this study directly relevant to our proof-of-concept demonstration, mesothelioma was selected as a model disease. Malignant mesothelioma is an aggressive form of cancer in which overexpression of mesothelin by tumor cells has been reported in over 80% of patients. This important feature of the disease has led to the development of targeted therapeutic approaches using meso-CAR T cells, many of which are being evaluated in several ongoing clinical trials. In this study, CAR T cells derived from two healthy donors were tested.

[00128] Malignant tumor tissues used in this study were obtained from surgical resections of multiple patients with locally advanced mesothelioma and were processed for in vitro transplantation into the vascularized bed of our device (FIG. 6a). Following transplantation, the blood vessels in the surrounding tissue grew into the open wells and covered the primary mesothelioma explants over time, generating fully vascularized and perfusable tumor constructs within a week (FIG. 6b). This vascular network formation surrounding the patient-derived tumor explants was similar to the vascularization of CDX tumors, as evidenced by the significant increases in the percentage of vessel covered area, total branch length, and vessel diameters over time in both groups (FIG. 40). The rate of vascularization of patient tumor explants appeared to be greater than that using CDX tumors especially at later time points post transplantation of tumor constructs, resulting in significantly more connected and larger vessels. When meso-CAR T cells were introduced into the constructs through the perfusable vasculature, many of the infused cells were distributed in the tumor masses, and this spatial localization indicative of CAR T infiltration appeared to increase with time (FIGs. 6c, 6d). In any given construct, T cell recruitment was observed across the tumor array but we also noted inter-tumor variability in the extent of this response (FIG. 38), which may be explained by heterogenous architecture and cellularity inherently present in mesothelioma tumors in vivo.

[00129] Flow cytometry of meso-CAR T cells isolated from this model showed spatially distinct cellular distribution. Compared to the tumor compartment, the surrounding vascularized stroma was seen with greater numbers of CAR T cells (FIG. 6e). The majority of the cells in the stroma were characterized as central memory T cells (CD45RO⁺ CD62L⁺), whereas the effector memory phenotype (CD45RO⁺ CD62L⁻) was more prevalent in the tumor-infiltrating CAR T cell population (FIG. 6f). CD69⁺ cells with the characteristics of activated and tissue-resident T cells were found in both the tumor and stromal compartments in similar abundance (FIG. 6g). The effector memory cells, tissue-resident cells, and PD-1⁺ CAR T cells in this model were present in significantly higher proportions than was measured in the original CAR T cell population prior to infusion (FIGs. 6f, 6g, FIG. 38). However, the differences in the proportions of these cellular phenotypes between tumor and stroma compartments were subtle in comparison to the spatially distinct cellular phenotypes in the meso-tumors (FIG. 3) –

without being bound to any particular theory, this may be attributable to and reflect the intrinsic variation of mesothelin expression on the primary explants in comparison to the uniformly and highly expressed mesothelin antigens on the meso-tumors.

[00130] ScRNA-seq analysis of the harvested CAR T cells revealed three clusters representing different subpopulations of T cells in the UMAP plot (FIG. 6h). Two of these clusters (C1 and C2 in FIG. 6h) displayed higher expression of naïve marker genes, such as LEF1 and TCF7, indicating the phenotype of central memory T cells (FIGs. 6h, 6i, FIG. 39). The other cluster (C3 in FIG. 6h) was enriched with T cells with substantially more robust expression of marker genes associated with T cell activation and effector function (e.g., GZMA, GZMB, IL2RA, IFNG, MIR155HG) (FIGs. 6h, 6i, FIG. 39). The distinguishing features of this group also included upregulation of cell-cycle genes (e.g., MKI67, TOP2A, CENPF) (FIGs. 6h, 6i), which was also shown by GO enrichment analysis (FIG. 6j). The activated and proliferative phenotype of this CAR T cell population was further demonstrated by increased expression of inhibitory receptor genes and related transcription factors (e.g., CTLA4, LAG3, HAVCR2) (FIG. 6h, FIG. 39).

[00131] To demonstrate the capability to engineer more tissue-specific vascular niche, vascular bedding was constructed with human pulmonary microvascular endothelial cells for the transplantation of additional primary mesothelioma explants and the treatment with meso-CAR T cells (FIG. 11a). Following transplantation, fully vascularized and perfusable tumor constructs were generated typically within ten days (FIG. 11b). In comparison to the infused NTD T cells, meso-CAR T cells were seen trafficking through the tumor vascular niche into the tumor masses and surrounding regions at a significantly higher rate and extent (FIG. 11c, 11d). Spatial analysis at day 6 post-infusion, with flow cytometry of NTD T or meso-CAR T cells isolated from the stroma and tumor compartments, confirmed higher abundance of meso-CAR T cells in both locations and especially more significant infiltration of meso-CAR T cells in the tumor masses than NTD T cells (FIG. 11e, FIG. 41a). The majority of the tumor-infiltrating CAR T cells were characterized as effector memory phenotype (CD45RO+ CD62L-) with higher abundance than those in the injection product and stroma-retained CAR T cells (FIG. 11f, FIG. 41b), a trend consistent with the mesothelioma tumor-infiltrating CAR T cells described above (FIG. 6f). Next, only the CD8+ T cells were

gated to assess and compare the phenotypic state between CAR T and NTD T cells. This analysis revealed that the tumor-infiltrating CD8 T cells in the CAR T cell group were significantly enriched with effector memory phenotype, at a higher fraction than those from the injection product and stroma-retained T cells (FIG. 11g, FIG. 41c). Although this relative increase in the effector memory population was also noted in the NTD T cell group, the number of effector memory T cells especially those post-infiltration into the tumor masses was not as pronounced (FIG. 11g, 11h, FIG. 41c, d). Further comparison of the intensity of cell tracker labelled T cells from both stroma and tumor compartments showed consistently decreased intensity of the majority of cells in the CAR T cell group (FIG. 11i, FIG. 41e), indicating more activation and proliferation of the meso-CAR T than NTD T cells.

[00132] Taken altogether, these results are consistent with the behavior of CAR T cells observed in the CDX tumor model and demonstrate the feasibility of engineering the tumor-vascular interface for in vitro investigation of CAR T cell trafficking into malignant solid tumor explants.

[00133] Discovery of therapeutic targets to promote CAR T cell trafficking

[00134] Extensive evidence shows that the interaction of circulating immune cells with the endothelium of tumor-associated blood vessels is the first critical step of their recruitment into TME. Based on this established understanding, one can reason that i) the ligand-receptor interaction data from scRNA-seq would allow one to delve into the crosstalk between CAR T cells and the vasculature of lung tumors in our model and that ii) insights from this study enable new strategies to pharmacologically modulate this interaction for the purposes of increasing CAR T cell trafficking into solid tumors.

[00135] For this work, endothelial-CAR T cell interactions were examined in the microengineered meso-tumor constructs used for our proof-of-concept demonstration, with the goal of identifying molecular mediators that might have a significant influence on CAR T cell trafficking. One of the top-ranked and notable interactions revealed by this analysis was the crosstalk between CXCL10/11 and CXCR3/DPP4, which were expressed at the highest levels by endothelial cells and CAR T cells, respectively (FIG. 7a). Binding of CXCL10/11 produced by IFN- γ -stimulated endothelium to CXCR3 expressed on T cells is a well-known interaction that induces endothelial trafficking of T cells and their chemotaxis but it was interesting that

CXCL10/11 was also interacting with DPP4, a ligand secreted by T cells and certain types of fibroblasts that can also bind to CXCL10/11 and cleave the N-terminal peptides of these chemokines, impairing their ability to mediate T cell recruitment and chemotaxis. Based on this finding, pharmacological inhibition of DPP4 was considered as a strategy to preserve the bioactivity of CXCL10/11 and promote the trafficking of meso-CAR T cells, which has not been investigated previously.

[00136] To test this idea, meso-tumor infusion model was modified as follows. Mesothelin expression by A549 cells was decreased from 88% to 44% by reducing the fraction of mesothelin-positive cells in the engineered tumor spheroid constructs, in order to more closely approximate the pathophysiological levels of mesothelin expression in malignant lung tumors in vivo. Second, the regimen of CAR T cell infusion was changed from a single dose of highly concentrated cells to three doses with lower cell density separated by 6-7 days to simulate the typical regimen of fractionated administration of engineered human TCR/CAR T cells for efficacy testing in preclinical and clinical settings (FIG. 7b). Lastly, the total culture period was extended to 26 days to allow for longer-term monitoring and assessment of CAR T cell activities. LAF237 (Vildagliptin) was used as a pharmacological inhibitor of DPP4 in the experiments – LAF237 is a potent DPP4 inhibitor previously developed for the treatment of type 2 diabetes. Given the short half-life of this drug, our model was administered with a single daily dose of LAF237 for the entire duration of experiments after CAR T cell infusion (from days 9 to 26) (FIG. 7b).

[00137] Prior to the introduction of CAR T cells, the composite lung tumor spheroids transplanted into the vascularized bed continued to grow in size over time (FIG. 7c, FIG. 42). Following the perfusion of the tumor constructs with meso-CAR T cells, this trend appeared to stop as illustrated by the slightly reduced tumor area between days 12 and 18 but they began to grow again after the second infusion and subsequently underwent rapid enlargement (top row in FIG. 7c, FIG. 7e, FIG. 42). Similar growth patterns were observed in CAR T cell-infused tumors treated with 50 nM of LAF237, a dose representing the lower end of clinically-relevant plasma concentrations of this drug (middle row in FIG. 7c, FIG. 7e, FIG. 42). When a higher dose (1000 nM) of LAF237 was used, however, the tumors began to shrink shortly after the first infusion of CAR T cells, after which their size remained relatively constant (bottom row in FIG. 7c, FIG. 7e,

FIG. 42). Of note, LAF237 alone without CAR T cell infusion did not have any significant effects on tumor growth, regardless of its concentration (FIG. 43).

[00138] Immunofluorescence imaging of tumor constructs at Day 26 revealed a marked difference in the distribution of infused T cells between these groups, which was highlighted by much more extensive recruitment and retention of T cells in the tumor compartment and its vicinity when the model was treated with 1000 nM of LAF237 (FIGs. 7d, 7e, FIG. 42). Measurement of intra-stromal and intra-tumoral T cell area over time showed that significant T cell trafficking and infiltration occurred in all three groups after the first infusion but in the absence of or at the low dose of LAF237, the abundance of CAR T cells in the constructs remained largely unchanged even after the second and third infusions (FIG. 7e). In contrast, the higher dose of the drug permitted a continuous increase in the T cell area (FIG. 7e), suggesting the ability of LAF237 to potentiate therapeutic effects of additional doses of CAR T cells after the initial infusion.

[00139] For further characterization, mediators of endothelial recruitment of CAR T cells were measured in device effluent collected from the vascular compartment of the model. According to this analysis, the concentration of CXCL10 and CXCL11 spiked due to the first infusion of CAR T cells and then gradually decreased over time in all three groups (FIG. 7f). However, the levels of these chemokines in the drug-treated constructs were higher, especially at the beginning and towards the end of post-infusion culture, compared to those measured in the untreated control model (FIG. 7f), supporting the observation of increased CAR T cell recruitment in these groups.

[00140] This ELISA analysis can, however, detect both the cleaved and intact forms of CXCL10 and CXCL11 and may therefore not provide accurate measurement of their contribution to CAR T cell trafficking. To address this, CXCL10 was used as an example and a customized gold nanoparticle platform modified with valine-specific aptamer was developed that can target the dipeptides cleaved from intact CXCL10. Results from UV-vis and Raman measurement suggested reduced truncation of CXCL10 in the group treated with high dose of LAF237, in comparison to the control group, at multiple time points post-infusion of CAR T cells, which was confirmed by western blotting (FIG. 44). DPP4 activity was then evaluated in the same effluent samples. Data from the untreated control group indicated that the level of DPP4 activity greatly increased by the first CAR T cell infusion and remained elevated throughout culture (FIG.

7g). When the model was administered with LAF237, however, the measured activity of DPP4 was lowered significantly in a dose-dependent manner, resulting in 35.4% (50 nM) and 89% (1000 nM) reduction in average activity during post-infusion culture when compared to the control group (FIG. 7g). Interestingly, this is consistent with the previous notion that LAF237 inhibits DPP4 at micromolar concentrations in spite of the IC₅₀ at 4-8 nM level.

[00141] Given the range of proteins that can be targeted by DPP4 and to confirm its regulatory roles in chemokine signaling in this study, it was sought to examine the effect of signal blocking of CXCL10 and CXCL11 on CAR T cell trafficking mediated by DPP4 inhibition. Because CXCR3 is the unique chemokine receptor for both CXCL10 and CXCL11, it was chosen to pharmacologically block CXCR3 as a way of targeting the signaling of CXCL10/CXCL11 (FIG. 45a). Significantly promoted trafficking of T cells (~3-fold increase from control) and shrinking of tumor spheroids were observed when the model was administered with LAF237 (1000 nM) alone or plus an isotype-matched control antibody (FIG. 45b, c, d). However, the treatment with anti-CXCR3 antibody, despite the treatment with LAF237 (1000 nM) as well, reduced the T cell trafficking down to the baseline level and only controlled the tumor growth minimally in a similar fashion as the no-treatment control group (FIG. 45b, c, d). This abrogation of promoted trafficking by DPP4 inhibition indicates that DPP4 and its inhibition mediate the trafficking of CAR T cells through the regulation of CXCL10/CXCL11-CXCR3 signaling axis.

[00142] Verifying the principle of our proposed therapeutic approach, these data suggest that LAF237 may have beneficial effects on meso-CAR T cell therapy of malignant solid tumors in the human lung by suppressing the activity of DPP4 to promote the recruitment of infused CAR T cells to the tumor-associated vasculature and their infiltration into tumor masses.

[00143] In addition to the CXCR3/DPP4-CXCL10/CXCL11 interactions, also identified were two other top-ranked interactions of interest between CAR T and endothelial cells that are suggested to be involved in the physical binding and trafficking of CAR T cells through endothelium, including CD38-PECAM1 (CD31) and LTB-LTBR (FIG. 46a). CD38 has been implicated in mediating the adhesion and trafficking of leukocytes through its ligand CD31, and LTB-LTBR signaling has been shown to control the homing of T cell progenitors. However, the effect of these interactions on CAR T

cells has not been directly examined. Additionally, the fact that these two interactions rank higher than the ITGAL (LFA-1)-ICAM1 interaction, one of the most classical and crucial interactions determining the formation of firm adhesions to endothelium during T cell trafficking, also provided motivation to verify their influence on CAR T cell trafficking. For this verification study, the same composite tumor spheroid infusion model as described above was used; pharmacological inhibition of CD38 and LT β with Daratumumab (Dara) and Baminercept (Bami), respectively, were used in the experiments as a way to examine their influence on CAR T cell trafficking (FIG. 46b). Daratumumab is an FDA-approved potent CD38 inhibitor previously developed for the treatment of multiple myeloma and Baminercept is a potent LT β inhibitor previously developed for the treatment of autoimmune diseases such as Rheumatoid Arthritis.

[00144] Fluorescence imaging of tumor constructs and T cells post-infusion revealed a marked difference in the overall trafficking of infused T cells between groups of various treatment (FIG. 46c). Similarly significant recruitment and retention of T cells in the tumor compartment and its vicinity were observed by day 4 post-infusion, when the model was treated with meso-CAR T cells only or meso-CAR T cells plus IgG isotype control antibody. In contrast, treatment with meso-CAR T cells plus Dara or Bami at either high or low dosage resulted in a general decrease of T cell area (FIG. 46d). Particularly, when treated with higher doses of Dara or Bami the T cell area decreased by ~50% compared to the groups with treatment of meso-CAR T cells only or meso-CAR T cells plus IgG isotype control antibody, confirming the significant regulating effects of blocking of CD38-CD31 and LT β -LT β R interactions on CAR T trafficking. Analysis of tumor showed relatively constant tumor sizes over time post-infusion for groups treated with CAR T cells only, CAR T cells plus IgG isotype control, and CAR T cells plus low dose Dara or Bami (FIG. 46e). When the higher doses of Dara and Bami were used, however, the tumors began to grow despite the infusion of meso-CAR T cells, with a similar growth pattern as that of the meso tumor only group.

[00145] Based on these findings, two potential therapeutic strategies aiming at promoting CAR T cell trafficking are provided. First, increasing expression of CD38 and LT β individually or simultaneously on CAR T cells may be achieved through genetic modification or mRNA delivery to stimulate the interactions of CD38-CD31 and LT β -LT β R between CAR T and endothelial cells as a trafficking promotion strategy. Second, it

is known that CAR T cells and indeed all adoptively transferred T cells after systemic injection are first sequestered in non-tumoral sites such as lung, liver, and bone marrow. Without being bound to any particular theory or embodiment, this could affect the safety and efficacy of CAR T therapies because extra dosage maybe required due to prolonged sequestering and suboptimal trafficking towards tumor. Given that CD31 and LT β R proteins are prevalently expressed in endothelial cells, the regulation of CAR T trafficking through CD38-CD31 and LT β -LT β R interactions would be applicable throughout the circulation system. Thus, sequestering of adoptively transferred CAR T cells may be reduced through local delivery of Daratumumab or Baminercept to non-tumoral sites as a potential therapeutic manipulation or “anti-adhesive” therapy to increase the presence of CAR T cells in the circulation for more trafficking to solid tumors.

[00146] Overall, CAR T cells derived from four healthy donors were tested in this study.

[00147] Discovery of therapy biomarkers through metabolomic analysis of tumor-on-a-chip

[00148] Having demonstrated the therapeutic potential of inhibiting DPP4 in meso-CAR T treatment, the possibility of developing biomarkers that indicate the efficacy of this method was then explored. In light of increasing evidence pointing to the importance of metabolism in cancer immunotherapy, a goal was to identify metabolic signatures that can be correlated with the progression and outcome of meso-CAR T therapy coupled with the use of LAF237 in our model. To this end, we conducted untargeted, global metabolomic profiling of our model using vascular perfusate collected from the side chambers (FIG. 8a). Specific conditions considered in this study included media only, pre-infusion, CAR T cell infusion only (ctrl), CAR T cell infusion with 50 nM LAF237 (low), and CAR T cell infusion with 1000 nM LAF237 (high) – the CAR T cell-infused groups were examined at defined time points (days 2, 7, 11, and 16 post-infusion) (FIG. 8b).

[00149] This analysis revealed 205 differentially regulated metabolites with statistical significance (False Discovery Rate (FDR) < 0.05) across all conditions (FIG. 47). Further analysis by partial least squares discriminant analysis (PLS-DA) identified a fraction of these differentially regulated metabolites that can be used to distinguish individual conditions with different time points and doses of LAF237 (FIG. 8b; FIG. 48).

Through subsequent multivariate analysis, we identified 96 metabolites whose concentrations in the vascular perfusate were significantly altered by LAF237 treatment (FIG. 8c). The hierarchical clustering-based heatmap showed that 65 of these metabolites were present in higher abundance in the drug-treated groups compared to control (CAR T cell infusion only), whereas the other 31 metabolites were downregulated due to LAF237 (FIG. 8c). According to metabolic pathway impact analysis, the upregulated metabolites had effects on biological processes involved in beta-alanine metabolism, alanine, aspartate and glutamate metabolism, citrate cycle, pyrimidine metabolism, and pantothenate and CoA biosynthesis (FIG. 8d, FIG. 49), suggesting significant changes in central carbon metabolism due to DPP4 inhibition.

[00150] To highlight a few key findings, certain products of carbohydrate metabolism, such as cis-aconitic acid in the TCA cycle, were detected at significantly elevated levels at day 16 post-infusion when the model was treated with a higher concentration (1000 nM) of LAF237 shown to be effective for promoting CAR T cell trafficking in our experiments (FIG. 8e). At the same time point, the higher dose treatment was also correlated with increased metabolism of amino acids and nucleic acids. This was demonstrated by significantly greater production of intermediate metabolites of these pathways at day 16 post-infusion, including L-proline, fumaric acid, beta-alanine, and xanthine (FIG. 8f). The data also revealed a negative correlation between the production of pyruvic acid and drug treatment. Compared to control, the concentration of pyruvic acid in the LAF237-treated models was consistently lower and in case of the higher-dose group, this drug-induced depletion of pyruvic acid became statistically significant at all time points after CAR T cell infusion (FIG. 8g).

[00151] These results prompt one to ask whether the differentially regulated metabolites found in the analysis would have any value as molecular indicators of the efficacy of inhibiting the activity of DPP4 during meso-CAR T therapy. For systematic investigation of this question, biomarker analysis was performed to identify metabolite-based signatures that can be used to quantitatively distinguish the CAR T cell-infused group receiving an efficacious dose (1000 nM) of LAF237 from that without LAF237 treatment. In this analysis, we generated the receiver operating characteristic (ROC) curves with increasing numbers of constituent model features for the last time point of measurement (day 16 post-infusion) to predict combinatorial biomarkers of efficacy (FIG.

8h, FIG. 50). The top five metabolites predicted by the model with the highest predictive accuracy included 1,2,3-propanetricarboxylic acid, 2-hydroxybutyric acid, 3-acetyl-2,7-naphthyridine, 3-nitrotyrosine, and L-cystine (FIG. 8i). In particular, higher efficacy of CAR T therapy due to 1000 nM LAF237 was predicted to correlate with significantly lower levels of 1,2,3-propanetricarboxylic acid, 2-hydroxybutyric acid, 3-acetyl-2,7-naphthyridine, and L-cystine or increased production of 3-nitrotyrosine and 2-hydroxyethanesulfonate at day 16 post-infusion (FIG. 8j, FIG. 51).

[00152] When the analysis was extended to all time points measured in our experiments (days 2, 7, 11, and 16 post-infusion), the model predicted pyruvic acid, ibudilast, vanilpyruvic acid, zymonic acid, and 2-hydroxybutyric acid as the five most significant biomarkers (FIGs. 8k, 8l, FIG. 50). In case of pyruvic acid and 2-hydroxybutyric acid, for example, their concentrations in the CAR T cell-infused group with 1000 nM LAF237 was substantially lower than that in control at days 11 and 16 post-infusion, whereas the same condition/group was seen with a significant increase in the level of ibudilast at days 7 and 16 post-infusion (FIGs. 8g, 8m). Notably, the two sets of predictions made for day 16 post-infusion only (FIG. 8i) and all time points (FIG. 8l) shared several metabolites that were upregulated (5-methoxytryptophan, ibudilast, vanilpyruvic acid) or downregulated (2-hydroxybutyric acid, inosine, glutaminyll glutamine, 2-aminoacrylic acid) due to administration of 1000 nM LAF237 during CAR T cell infusion (FIG. 51).

[00153] In vitro modeling of in vivo responses to armored CAR T cells

[00154] To demonstrate the capability of the disclosed platform to model solid tumor-CAR T interactions in vivo, a further study was performed for testing the effect of an armored CAR T strategy for enhancing their trafficking towards solid tumors.

[00155] Chemokines and chemokine receptors (CCR) are involved during most of the T cell trafficking processes through endothelium including activation on the endothelial surface, secondary adhesion, and extravasation. The mismatch between chemokines secreted from tumor cells and the CCRs expressed on T cells therefore results in suboptimal trafficking. It has been suggested that additional modifications of chemokine receptor expression on CAR T cells could be harnessed to address the chemokine-receptor mismatch issue, enhance T cell trafficking, and ultimately improve their anti-tumor efficacy. For example, CCR2 transduction of CAR T cells targeting

neuroblastoma cells that express chemokine CCL2 has been shown to achieve impressive enhancement of CAR T infiltration into solid tumors and increased anti-tumor activities in vivo.

[00156] It has also been shown that CCR2-modified CAR T cells showed enhanced trafficking towards M108 mesothelioma tumor in an animal model. To further validate the microengineered platform, in vitro and in vivo studies were conducted using a different mesothelioma cell line called EMMeso, which expresses mesothelin on its surface and secretes CCL2 (CCR2 ligand) and has been previously characterized to be treatment-resistant, for pairing with the meso-CAR + CCR2 T cell therapy (FIG. 9a,b). This EMMeso tumor transplantation was similar to that of the A549 lung-tumor model. After EMMeso CDX tumor explants were transplanted into the vascular bedding with lung microvascular endothelial cells, three types of T cells were infused for direct visualization and comparison including NTD T cells, meso-CAR T cells, and meso-CAR + CCR2 T cells (with constitutively expressed CCR2) (FIG. 9b, FIG. 52). These three types of T cells were always derived from the same donor and T cells derived from two healthy donors were tested.

[00157] Imaging analysis of labelled tumor constructs and T cells at day 1 and day 5 post-infusion showed a trend toward increased trafficking over time of both meso-CAR and meso-CAR + CCR2 T cells (FIG. 9c,d). However, analysis at day 5 post-infusion revealed a significant difference in the trafficking of infused T cells among these groups of T cell treatment, with much more extensive recruitment and retention of T cells in the tumor compartment and its vicinity when the model was infused with meso-CAR + CCR2 T cells (regardless of constitutive or transient expression of CCR2) in comparison with infusion of meso-CAR only T or NTD T cells (FIG. 9c,d, FIG. 52). Prior to the introduction of control NTD T cells and meso-CAR T cells, the EMMeso tumors transplanted into the vascular bedding started to grow in size over time (FIG. 9b,c,e). Following the infusion of the tumor constructs with NTD T cells, they continued to grow and underwent rapid enlargement (left column in FIG. 9c,e). In contrast, when the meso-CAR or meso-CAR + CCR2 T cells were infused, tumor growth began to slow down after the infusion (middle and right column in FIG. 9c,e). The analysis of tumor area over time also appeared to show a trend of delayed anti-tumor effect from meso-CAR + CCR2 T

cells, where the control of tumor growth occurred at day 5 post-infusion - a later time point than that with the infusion of meso-CAR only T cells (FIG. 9e).

[00158] To compare what was observed using the microengineered platform, next evaluated was the trafficking and anti-tumor activity of each type of T cells after injection into established tumors in mice (FIG. 9f). EMMeso cells were injected into the flanks of three groups of NSG mice and tumors were allowed to grow to 100 - 200 mm³ in size. This tumor establishment typically takes about four weeks, after which the tumors are highly vascularized. At this point, mice were injected with either (i) NTD T cells (as many to match the total numbers injected in the meso-CAR group), (ii) 5 million meso-CAR positive T cells, or (iii) 5 million meso-CAR + CCR2 positive T cells. These three types of T cells were derived from the same donor and T cells derived from two additional healthy donors were tested in this in vivo study.

[00159] At day 5 post tail IV injection, 3 tumors from each group were harvested, digested, and subjected to FACS analysis (FACS analysis of digested tumor was used instead of in vivo imaging because, based on experience, very few T cells traffic to the tumor at this early time point). The abundance of human CD3⁺ T cells present in the tumors injected with NTD T cells or meso-CAR T cells was very low (400-700 cells per million) (FIG. 9g,h). In contrast, >10-fold more meso-CAR + CCR2 T cells were found in those tumors (FIG. 9g,h). In a separate experiment using the same mouse model, we monitored the tumor growth over an extended period of time and observed significant differences in the tumor volume and weight (FIG. 9i, FIG. 52). The tumors from NTD T cell group grew steadily reaching a size of >1000 mm³ by day 33 post-injection of T cells. In contrast, tumor sizes and weight were significantly smaller in both meso-CAR and meso-CAR + CCR2 T cell groups compared with the NTD T cell group, correlating well with the significantly increased infiltration of T cells from both meso-CAR T groups (FIG. 9i, FIG. 52). Interestingly, despite similar tumor sizes and weight in both meso-CAR T cell groups by the end of experiment, a delayed anti-tumor effect was observed with the injection of meso-CAR + CCR2 T cells similar to the delayed anti-tumor effect observed in the microengineered model. This comparison of responses to a CAR T modification strategy thus demonstrates the capability of our microengineered platform to reproduce the key outcomes of solid tumor-CAR T interactions in vivo, including not only CAR T trafficking but also their anti-tumor dynamics.

[00160] Discussion

[00161] Clinical efficacy of CAR T therapy against solid tumors requires not only antigen-directed cytotoxicity of CAR T cells but also their ability to infiltrate into and recognize target tumors and to survive for prolonged periods to carry out their desired anti-tumor function. By providing an approach to recreate, directly observe, and interrogate key aspects of these required processes, the disclosed technology enables in vitro modeling and systematically controlled preclinical studies of tumor-immune interactions in physiologically relevant contexts of malignant human tissues, which currently remains a significant challenge. Emulating the principle of tumor transplantation, the disclosed microengineered system permits incorporation of in vivo tumor explants into a pre-formed, easily accessible vascular bed to generate 3D tumor constructs containing microvasculature that can be perfused with blood-borne immune cells to simulate CAR T cell infusion during immunotherapy. The demonstrations provided herein establish the use of the tumor model as an in vitro platform for in-depth investigation of tumor-immune interactions.

[00162] By leveraging the power of organ-on-a-chip technology, the present disclosure shows that microengineered devices can be used to culture malignant cells and other relevant cell types in physiologically relevant 3D environments to capture the characteristics of solid tumors. Existing 2D approaches present only an oversimplified approximation of the vascular-tumor interface in vivo. The present disclosure adds to the field by i) introducing a bioengineering workflow that enables new opportunities to harness and synergistically combine the inherent biological complexity of in vivo-derived malignant solid tumors with precision and high controllability of microengineered systems to create more realistic, human-relevant models of cancer immunotherapy and ii) demonstrating the proof-of-principle of how this technology can be used specifically to model and probe the critical steps of CAR T therapy. Moreover, the present disclosure illustrates the use of this approach to generate in vitro data useful in, for example, the development, preclinical evaluation, and mechanistic studies of CAR T therapies and related treatment strategies, as well as discovery of potential clinical biomarkers for these methods. Even in comparison to the mouse model in vivo with CDX, patient-derived xenograft (PDX), or tumor organoid transplantation, the disclosed microengineered model demonstrates unique and key differences and benefits for the study of human CAR T

therapy. CAR T cell functions are mediated by their interactions with not only the tumor cells but also stromal cells such as resident fibroblasts and endothelial cells. Although mouse tumor models using PDX or tumor organoid transplantation may represent more faithful signatures of personal malignancy for CAR T targeting, the interactions between human CAR T cell and mouse stromal cells may be limited by strict species specificity such as the insensitivity of mouse cells to human IFN γ , which makes the tumor associated mouse stroma unfit for the investigation of stromal-dependent regulation of anti-tumor functions of CAR T cells. In contrast, the disclosed model system enables not only closer monitoring and in-depth analysis with high resolution but also the formation of human tissue-specific vasculature permitting the investigation of human relevant signaling. The breadth and depth of the present disclosure highlights advantages of tumor-on-a-chip technology and represent an advance in the state-of-the-art.

[00163] The data demonstrate that this system has the capacity to recapitulate the behavior of T cells reported in animal or clinical studies of immunotherapy. For example, the enrichment of CAR T cells in the tumors compared to the injection product and their upregulation of the inhibitory receptor PD1 following tumor infiltration in our meso-tumor model (FIG. 3d) match the results of murine studies using meso-CAR T cells. Significant increases in PD1+ CAR T cells and the dominance of effector memory CAR T cells post infiltration into meso-tumors (FIGs. 3d, 3e) are also the findings of our study that have been described in TILs from lung cancer patients. Moreover, unbiased cell type annotation of our sequencing data using published reference dataset demonstrated the correlation of tissue compartment-specific phenotypes of CAR T cells in the meso-tumor model with those of T cells in lung cancer patients (FIGs. 4m, 4n). These results provide evidence supporting the physiological relevance of the disclosed model.

[00164] Demonstrating the use of the microengineered CAR T therapy model for applications in drug development is another key accomplishment of the disclosed technology. Through scRNA-seq-based interrogation of intercellular interactions in this model, we identified DPP4 as a druggable target to promote CAR T cell trafficking into human lung tumors. Our proof-of-principle experiments using a pharmacological inhibitor of DPP4 (LAF237) showed significantly increased trafficking and tumor infiltration of meso-CAR T cells and arrested tumor growth due to high-dose

drug treatment, verifying the therapeutic potential of our proposed approach. LAF237, also known as Vildagliptin, is an anti-hyperglycemic drug that increases the activity of incretin hormones by inhibiting DPP4. Given that vildagliptin is already in clinical use for the treatment of type 2 diabetes, these results provide a rationale for exploring the possibility of repurposing this drug as a new strategy to augment the efficacy of CAR T therapies for solid tumors. Because CXCR3-CXCL10/11-DPP4 signaling is conserved across many different types of cancer, this method has applicability to other clinical conditions of solid malignancies.

[00165] We note that studies have shown increased lymphocyte recruitment into mouse melanoma and hepatocellular carcinoma due to DPP4 inhibition^{156,157}. Based on the ligand-receptor interaction analysis of scRNA-seq data, our investigation of DPP4 was motivated and conducted independently of this previous work and provides new evidence verifying the efficacy of this principle in the context of treating malignant human solid tumors using human CAR T cells. Given the significant interspecies difference in the activity of T cells and DPP4, the human-relevant data generated in our study adds significant value to existing knowledge. From a broader perspective, these results contribute to ongoing efforts to address the rapidly increasing need for new strategies to augment and maximize the therapeutic efficacy of CAR T treatment for solid tumors. Unlike CD19-targeted CAR T cells used for blood cancers, CAR T cells designed for treating solid tumors undergo limited homeostatic expansion after infusion due to the physical and molecular barriers created by the immunosuppressive TME that prevent their recognition of target antigens. Clinical and animal studies have also shown the distribution of infused adoptively transferred T cells not only in tumor sites but also in lymphoid and other organs such as the bone marrow, lymph nodes, spleen, liver, and lungs¹⁶¹. These become problematic by limiting the number of tumor-infiltrating CAR T cells necessary for effective treatment. Because increasing the dose of CAR T cells to boost anti-tumor activity can lead to lethal toxicities, enhancing their ability to efficiently home to target tumors is emerging as one of the most promising approaches towards more efficacious and safer CAR T therapy of solid tumors. Our work shows how these types of studies could be designed and carried out in vitro to identify new therapeutic targets and evaluate the effects of their pharmacological modulators.

[00166] As an extension of this study, a set of metabolites was discovered whose levels were correlated with the efficacy of LAF237 treatment during meso-CAR T therapy in our model. The significance of this work can be discussed in the context of metabolic alterations in the immunosuppressive tumor microenvironment and clinical challenges associated with monitoring the progression of CAR T therapy. To address our limited understanding of tumor immune evasion by metabolic reprogramming, emerging studies have shed light on the molecular mechanisms of metabolic regulation of CAR T effector function and persistence by individual metabolites. For example, studies have shown significant correlations between CAR T cell function in patients and fumarate hydratase levels in the microenvironment¹⁶⁶, and that inosine can serve as an alternative carbon source for CAR T cell function when glucose supply is restricted. These results illustrate the potency of metabolic regulation of immune functions and the value of monitoring metabolite changes to infer CAR T cell performance in vivo. Although analysis of tumor biopsies provides accurate clinical measurement of the CAR T activities, the invasiveness and complexity of this procedure make it impractical for routine clinical monitoring. Therefore, the identification of correlative and specific metabolite biomarkers may complement current methods for routine clinical assessment of therapy progression, including qPCR and flow cytometry of blood samples, to provide additional value in metabolic measurement and monitoring of CAR T therapy and related treatment.

[00167] The differentially regulated metabolites identified in this study have not been described previously in the context of immunotherapy and may provide candidates for the development of clinical biomarkers for meso-CAR T therapy of lung tumors coupled with LAF237 treatment.

[00168] The treatment of EMMeso tumors with meso-CAR +CCR2 T cell therapy was tested in the engineered model and in the mouse model independently, yet both studies generated similar results with regard to CAR T trafficking and anti-tumor dynamics by the same modification strategy. The discrepancy in the time scale of delayed anti-tumor effect of meso-CAR + CCR2 T cells between in vitro and in vivo observations may be partially attributed to the scaling nature of respective models, with the T cell infusion into the microengineered niche approximating a local delivery of T cells to the tumor in vivo, thus quicker responses. The capability of our engineered platform to reproduce key outcomes of CAR T treatment in vivo highlights its application to other

studies, such as the mechanism of delayed anti-tumor effects or the general effects of CCR2 modification on CAR T cell functions. Another observation of value is the similar anti-tumor efficacy between meso-CAR and meso-CAR + CCR2 T cells in the EMMeso tumor models. Interestingly, our previous study testing meso-CAR T + CCR2 T cell therapy on a different M108 mesothelioma tumor model in vivo showed not only increased trafficking of but also increased end-point anti-tumor efficacy by the modified CAR T cells, likely attributable to the higher expression of CCL2 by the M108 cell line and ~10-fold higher number of tumor infiltrated T cells than those in the EMMeso tumor. Other factors that may contribute to the discrepancy in anti-tumor efficacy between these two models include the growth rate of tumor and production of immunosuppressive factors. Preliminary data indicate that CCR2 modified CAR T cells that infiltrated into EMMeso tumors acquire an exhaustion-like phenotype, that is hypothesized to result from prolonged exposure to CCL2 ligands and calcium flux contributing to exhaustion.

[00169] Finally, the utility and potential of our technology are not limited to investigating CAR T therapy and can be to in vitro modeling and preclinical assessment of cancer immunotherapies using other types of cells, such as TCR T cells, CAR NK cells, and CAR macrophages. Based on previous findings that tumor responses to immune checkpoint blockade (ICB) therapies require sufficient tumor-infiltrating lymphocytes, increasing efforts are being made to develop more advanced treatment strategies that combine different modalities of cancer immunotherapy (e.g., CAR T-ICB therapy). The disclosed platform provides a useful tool for these types of studies by enabling combinatorial screening of different cells/drug compounds to identify conditions that promote synergistic effects.

[00170] Methods

[00171] Device fabrication and assembly

[00172] The open-top microdevice used for in vitro transplantation of tumor explants (FIG. 1, FIG. 12) consists of three device layers of micropatterned poly(dimethylsiloxane) (PDMS), which were fabricated separately using conventional soft lithographic techniques. The mold for the culture chamber was fabricated in a negative photoresist (SU-8 2100, MicroChem, USA) using photolithographic techniques to create positive relief structures on a prime-grade 4-inch silicon wafer (Wafer World Inc., USA) per manufacturer recommended protocols. Additional molds for the open-top ceiling and

the removable insert layers were produced by stereolithographic 3D printing (Protolabs, USA). To fabricate the micropatterned PDMS device layers, Sylgard 184 silicone elastomer base (Dow Corning, USA) and the curing agent were mixed at a ratio of 10:1 (w/w), cast over the photolithographically prepared or 3D printed molds, degassed under vacuum for 30 minutes, and cured at 65°C for 2 hours. Cured PDMS layers were then cut and released from the molds and punched to generate fluidic access ports. Holes were made through an additional blank PDMS slab and used as media reservoirs. Following fabrication of the device layers, the three-lane culture chamber was aligned and bonded to the open-top ceiling layer as shown in FIG. 12 using uncured PDMS as an adhesive material. The assembled open-top microdevice array was baked at 65°C for 2 hours to fully cure the PDMS glue and was stored at room temperature until use.

[00173] Cell culture

[00174] Human non-small cell lung cancer cell line A549 cells (CCL-185, ATCC) were used as model tumor cells for studying meso-CAR T therapy. Since wild-type A549 cells only have minimal expression of mesothelin, lentiviral transduction of A549 cells was applied for stable expression of high levels of human mesothelin (FIG. 17). The transduced cell line was referred to as meso-A549 in this study. A human mesothelioma cell line derived from a patient's tumor, EMP (parental), was transduced with a lentivirus to stably express human mesothelin and thus named EMMeso as previously described. EMMeso tumor cells were also used for the study of meso-CAR T therapy and strategy of chemokine receptor modification. Wild-type A549 cells, meso-A549 cells, and EMMeso cells were also transduced to stably express green fluorescent protein (GFP). A549 and EMMeso cells, normal human lung fibroblasts (hLF; CC-2512, Lonza), human umbilical vein endothelial cells (HUVEC; cAP-0001RFP and cAP-0001GFP, Angio-Proteomie), and human lung microvascular endothelial cells (HMVEC-L; CC-2527, Lonza) were cultured and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum (FBS), hLF media supplemented with growth factors included in the FGM-2 BulletKit (CC-3132, Lonza), endothelial cell media supplemented with growth factors included in the EGM-2 BulletKit (CC-3162, Lonza), and endothelial cell media supplemented with growth factors included in the EGM-2 MV BulletKit (CC-3202, Lonza), respectively. These cells were used between passages 2 and 3 for all experiments.

[00175] Tissue engineering of blood vessels

[00176] The assembled open-top microdevice array and additional device layers containing the removable inserts and media reservoirs were first sterilized by exposing to high-power ultraviolet (UV) light (Electro-lite ELC-500) for 30 minutes. Subsequently, the insert layer was fit into the wells of the open-top ceiling, after which the culture chamber was filled and incubated with 2 mg/ml (0.2% w/v in 10 mM Tris-HCl buffer, pH 8.5) of sterile dopamine hydrochloride solution at room temperature for 2 hours to form a surface coating of poly(dopamine) (PDA) on PDMS for enhanced adhesion to hydrogel scaffolds. The PDA-coated microdevices were kept sterile until use.

[00177] To construct a vascular bed for in vitro transplantation of solid tumors, 15 μ l of fibrin gel pre-polymer solution was injected through the inlet access port into the middle lane of the culture chamber. The pre-polymer solution contained fibrinogen at a final concentration of 9 mg/ml (F8630, Sigma), thrombin at 1 U/ml (T7513, Sigma), aprotinin at 0.25 U/ml (A1153, Sigma), endothelial cells at $2.5\text{--}5 \times 10^6$ cells/ml for both HUVEC and HMVEC-L, and fibroblasts at 4×10^6 cells/ml. The microdevice was then transferred to a cell culture incubator to induce fibrin gelation at 37°C for 10 minutes. Upon gelation, the complete endothelial cell growth media (EGM-2 or EGM-2 MV depending on the experiment) were introduced to the side channels of the culture chamber through media reservoirs. Next day, the side channels were filled with fresh EGM-2 or EGM-2 MV media containing a suspension of endothelial cells at 1×10^7 cells/ml to form endothelial lining on the channel walls and the exposed surfaces of the hydrogel scaffold. The endothelial cell growth media in the reservoirs were changed every other day during the subsequent culture period.

[00178] In vivo xenograft tumor model and evaluation of CAR T therapy

[00179] All animal experiment protocols were approved and conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania. Six to ten weeks old female NOD/scid/IL2 $\gamma^{-/-}$ (NSG) mice were bred in and obtained from the University of Pennsylvania Stem Cell and Xenograft Core. The mice were housed under specific pathogen-free conditions in microisolator cages and given ad-libitum access to sterilized food and acidified water. An equal number of 1×10^6 wild-type A549 cells and meso-A549 cells in PBS solution were injected into the left and right flanks of NSG mice, respectively. For experiments using EMMeso tumor

model, a total of 2×10^6 EMMeso tumor cells were subcutaneously injected in the flanks of NSG mice in PBS. For all experiments, tumor volume was monitored and measured over time using calipers for the whole length of the experiments. Tumor volumes were calculated using the formula $0.5 \times (\text{length}) \times (\text{width})^2$. After tumors were established (100-200 mm³ usually after 3-4 weeks), the mice carrying the tumors were sacrificed, and the xenograft tumors were harvested and precision-cut sliced using a Compressstome vibrating microtome (Precisionary Instruments LLC) with aseptic techniques. The sliced xenograft tumors were maintained in RPMI 1640 supplemented with 10% FBS at 37°C with 5% CO₂ and were used for in vitro transplantation to the microengineered devices on the same day. The in vivo xenograft experiments were repeated more than eleven times in independent fashion.

[00180] For in vivo evaluation of CAR T therapy, mice were randomly assigned to one of three treatment groups after establishment of tumors: (i) as many millions of non-transduced donor (NTD) T cells as total meso-CAR T cells injected per mouse, (ii) 5×10^6 meso-CAR positive T cells, and (iii) 5×10^6 meso-CAR positive T cells either transduced with CCR2 lentivirus (constitutive expression of CCR2b), or electroporated with CCR2-mRNA (transient expression of CCR2b). Each group contained 3-10 mice. T cells were injected intravenously (tail-vein) in 100 µL of PBS. After T cell injection, experiments were ended at day 5 or day 33 post-injection. For experiments ending at day 5 post IV injection, tumors were then harvested, micro-dissected, and digested in a solution of BD Horizon™ Dri Tumor & Tissue Dissociation Reagent (BD Biosciences) at 37°C for 30 minutes with frequent agitation. Digested tumors were then filtered through 70 µm nylon mesh cell strainers and washed twice in 1% BSA/DPBS/2 mM EDTA (without Ca⁺⁺/Mg⁺⁺), and red blood cells were lysed if needed (BD Pharm Lyse; BD Biosciences). Cells were strained again through a 30 µm nylon mesh cell strainer to obtain single-cell suspensions.

[00181] Acquisition of human patient tumors

[00182] Operative tumor biopsy samples were obtained from non-small cell lung cancer (NSCLC) and malignant pleural mesothelioma (MPM) patients undergoing potentially curative surgery (n = 2) and pleurectomy (n = 7), respectively. The study was approved by the Institutional Review Board at the University of Pennsylvania and informed consent was obtained from all patients. The patient tumor explants were sliced

and maintained as described above within 2 hours of resection, without freezing or overnight “rest”.

[00183] Transplantation and retrieval of tumor constructs

[00184] To prepare for in vitro transplantation, the sliced tumor explants from both patients and xenografts were further processed by mincing into small pieces using razor blades (Hi-Stainless, Feather). The minced explant pieces were then filtered to select for diameters between 100 and 400 μm and suspended in complete endothelial cell growth media for the next step. To implant patient and xenograft tumor explant pieces, the inserts layer was removed first to expose the open-top wells (FIG. 1). A 5 μl of EGM-2 or EGM-2 MV media suspension containing around 10 tumor explant pieces was then added to the wells. The injected tumor tissues were allowed to settle into the open-top wells overnight, after which a mixture of fibrinogen and thrombin at the same concentrations was added to seal the open-top wells. To examine the viability of patient tumor explants, we used Live/Dead™ Viability/Cytotoxicity Kit (L3224, ThermoFisher Scientific). The labeled cells in the tumor explants were examined using a laser scanning confocal microscope (LSM 800, Zeiss). To retrieve tumor tissues for compartment-specific analysis, the acellular fibrin gel seal was first carefully removed to expose the embedded tumor in the open-top wells. The tumors were then separated from the surrounding stroma using fine-point tweezers, aspirated, and pooled together for subsequent analysis. The tumor compartments are defined as the original space of the open-top wells that later contain the tumor constructs and have the same diameter as the open-top wells (mostly 500 μm unless otherwise noted). The stroma compartments are defined as the whole of the gel construct minus the tumor compartments.

[00185] Evaluation of vascular perfusability

[00186] To test the perfusability of the microengineered vascular network in our model, we used 70-kDa FITC-dextran (46945-100MG-F, Sigma-Aldrich) and fluorescently labeled 1- μm microbeads (FluoSpheres; F-8821 and F-8823, ThermoFisher) as flow tracers for visualization. To generate flow through the vasculature, the cell culture media in the media reservoirs were aspirated, after which either a FITC-dextran solution (50 $\mu\text{g}/\text{ml}$ in PBS) or a suspension of microbeads (1:10,000 dilution in PBS) was injected into one of the side channels to generate pressure gradient across the vascularized hydrogel scaffold. Vascular perfusion was then visualized and monitored using a laser

scanning confocal microscope (LSM 800, Zeiss) with a 10×/0.45 objective (C-Apochromat, water immersion, Zeiss). Time-lapse and Z-stack confocal images were acquired for 2 minutes and processed using the ZEN software (Zeiss).

[00187] Generation and modification of meso-CAR T cells and on-chip evaluation of their activity

[00188] The plasmid design for mesoCAR and CCR2b, the lentivirus packaging, and T cell activation, expansion, and cryopreservation have been previously described. Briefly, the single-chain Fv domain of the anti-mesothelin antibody (scFv SS1), originally provided by Dr. Ira Pastan (National Cancer Institute/NIH, Bethesda, MD), was subcloned into the lentiviral vector pELNS that was driven by the EF1 α (eukaryotic translation elongation factor 1 alpha) promoter. All CAR constructs contained a CD8 hinge and transmembrane domain, 4-1BB costimulatory domain, CD3 ζ signaling domain, and fluorescent reporter GFP or mCherry to evaluate transduction efficiency (i.e., SS1BBz mesoCAR as detailed in FIG. 17). Primary human T cells were obtained from a total of eight healthy volunteer donors at the Human Immunology Core at the University of Pennsylvania following leukapheresis by negative selection using RosetteSep kits (Stem Cell Technologies). All specimens were collected under a University Institutional Review Board-approved protocol, and written informed consent was obtained from each donor. T cells were cultured in RPMI 1640 supplemented with 10% FCS (R10) and stimulated with magnetic microbeads coated with anti-CD3/anti-CD28 at a 1:3 cell to bead ratio without the addition of exogenous IL-2. Approximately 24 hours after activation, T cells were transduced with the lentiviral vectors encoding SS1BBz mesoCAR at a MOI of ~5. T cells were counted and fed with complete RPMI media (R10) every 2 days and once appeared to become quiescent, as determined by both decreased growth kinetics and cell size, they were cryopreserved for long-term storage.

[00189] When needed, T cells were thawed and let rest overnight before being used for any experiment. To induce transient expression of CCR2 in the T cells, an mRNA molecule was designed and optimized for electroporation. After the overnight resting of the cells, they were washed twice with PBS and resuspended at 10⁸ T cells/mL in Optimem (Gibco), then they were electroporated using 1 μ g of CCR2 mRNA per 10⁶ T cells in each cuvette. The ECM 830 Square Wave Electroporation System was used at 500 V, 700 μ sec, and 1 pulse. Cells were rested for 24h and the expression of CAR and CCR2

(CCR2-APC, clone K036C2, Biolegend) were assessed by flow cytometry before being injected either in the mice or in the microengineered devices.

[00190] To model meso-CAR T therapy in our vascularized model, the cryopreserved T cell injection product that contained NTD T or meso-CAR T cells (with or without CCR2 expression) were thawed and recovered overnight at a density of 1×10^6 cells/cm²/ml in complete RPMI media (R10) prior to their infusion. Next day, the recovered T cells were first labelled with a fluorescent dye at a final concentration of 1 μ M (CellTracker Deep Red, ThermoFisher) and resuspended in EGM-2 or EGM-2 MV media. The NTD T and meso-CAR T cell suspension were then injected into the vascularized tumor models through one of the side microchannels at $\sim 150 \mu$ l of an equal cell density for each individual microdevice. The injected T cells were perfused through the vascular network and the flow of T cells was maintained overnight by continuous tilting on a rocker inside a cell culture incubator, resulting in a volumetric flow rate of 675.52 μ L/min and wall shear stress of 6.59 dynes/cm² at maximum. Next day, the microdevices with infused T cells were washed with fresh EGM-2 or EGM-2 MV media to remove non-attached T cells. The remaining NTD T or meso-CAR T cells were cultured for about 6 days. The microdevices with infused T cells were imaged every two days using a laser scanning confocal microscope (LSM 800, Zeiss) to monitor the dynamics of T cell trafficking and tumor growth. To capture specific events such as extravasation and infiltration into tumor masses, time-lapse and Z-stack confocal images were acquired for 25 to 45 minutes and processed using ZEN software (Zeiss). The time-series confocal images were processed in ImageJ (National Institutes of Health) and analyzed various endpoints described in the paper.

[00191] Histology and immunofluorescence staining

[00192] The vascularized tumor tissues were individually washed with PBS and fixed in 10% normal buffered formalin in situ overnight at 4°C. The corresponding microdevices were individually delaminated by carefully peeling off the open-top ceiling layers. The fixed tumor constructs together with the gel construct were carefully released from the culture chambers, transferred to pre-labeled tissue cassettes, and submerged in ethanol for dehydration and subsequent paraffin embedding. Care was taken to ensure that the shape and integrity of tumor constructs were preserved during the delamination

process. Thin tissue sections with a thickness of 5 μm were cut from the paraffin-embedded tissue blocks.

[00193] For staining with Hematoxylin and Eosin (H&E), the slides containing paraffin sections were deparaffinized and rehydrated by immersing the slides sequentially into 3 $\times$ Xylene, 2 $\times$ 100% ethanol, 95-90-80-70% ethanol, and distilled water. The slides were then immersed in 10 mM citric acid buffer (pH 6.0) and incubated in a microwave oven for 15 minutes. After gentle rinse, the slides with tissue sections were blocked with a protein blocking agent and immersed in Hematoxylin followed by rinsing with deionized water. The slides were further immersed in Eosin for 30 seconds and dehydrated in 95% ethanol-100% ethanol-xylene solutions. Stained tissue sections were covered with coverslip slides using PermountTM and stored until imaging.

[00194] For immunofluorescence staining, paraffin on the tissue section slides was cleared with xylene, and the slides were rehydrated through descending concentrations of ethanol. The slides were then treated with 3% H_2O_2 /methanol for 30 minutes, followed by pretreatment with Antigen Unmasking solution (Vector Labs H3300) in a pressure cooker (Biocare Medical). After cooling, the slides were blocked in Sudan Black (199664-25G, Sigma-Aldrich) for 30 minutes at RT, rinsed in 0.1M Tris Buffer, and blocked with 2% fetal bovine serum for 15 minutes. After removing the block solution, the slides were incubated overnight at 4°C with CD8 antibody (RB-9009-PO, Thermo) at a 1:500 dilution, rinsed, and then incubated with anti-rabbit polymer secondary prediluted (K4003, DAKO) for 30 minutes at RT. Following rinsing, the slides were incubated with the TSA biotin complex (NEL7490B001, Perkin Elmer) at 1:50 for 10 minutes at RT and incubated with Alexa 488 Streptavidin secondary (A21370, Life Technologies) at 1:200 dilution for 30 minutes at RT. After rinsing, the slides were treated in preheated 5% SDS (CS-5585-28, Denville Scientific) for 7 minutes at 55°C, rinsed, and blocked again with 2% fetal bovine serum before incubating with CD31 antibody (M0828, Dako) at 1:200 dilution for 1 hour at RT. After rinsing, the slides were incubated with Alexa Fluor 594 goat anti-mouse secondary antibody (A11032, Invitrogen) for 30 minutes, rinsed, and incubated with Cytokeratin antibody (Z0662, Dako) at 1:1000 dilution for 1 hour at RT. Subsequently, the slides were rinsed, incubated with Alexa Fluor 647 goat anti-rabbit secondary antibody (A21245, Invitrogen) for 30 minutes, counterstained with DAPI, and rinsed again before coverslipping with Prolong Gold

(P36930, Life Technologies). After drying, the slides were scanned at 20X using an Aperio IF slide scanner (Leica Biosystems) and imaged at 60X using a laser scanning confocal microscope (LSM800, Zeiss).

[00195] Flow cytometry

[00196] To evaluate the expression of CAR T cell surface markers by flow cytometry, the tumor constructs were carefully separated from the stroma as described above and then removed and pooled together for subsequent processing. The sample suspension was digested using a tumor digestion buffer (TTDR, BD Horizon) according to the manufacturer's instructions and incubated on a rocker in a cell culture incubator for one hour with vigorous pipetting every 10 minutes. After removing the tumor constructs, the gel stroma containing T cells and stromal cells remained intact in the microdevices. To isolate T cells and other cells from the stroma region, the gel stroma remaining in the microdevices was separately infused with the same digestion buffer (TTDR, BD Horizon) and incubated in the incubator for 30 minutes to digest and disintegrate the stroma gels. When the gel stroma were digested into fragmented pieces, they were aspirated out of the microdevices together with all of the liquid content and pooled together. This stroma sample suspension was then added with more of the same digestion buffer up to a total of 10 mL, and incubated for another 30 minutes to obtain single cell suspensions. All cell suspensions were passed through 70 μ m nylon mesh cell strainers to remove undigested clusters and multiplets.

[00197] All samples were stained and analyzed using standard flow cytometry. Single-cell suspensions from all samples were stained with Live/Dead cell stain (Invitrogen) for 10 minutes at 4°C. For staining of human cell surface marker proteins, the cells were incubated at 4°C for 30 minutes in staining buffer (2% FBS in PBS) with the following fluorescently-labeled antibodies based on the manufacturer's recommendations. The antibodies used include CD3 (clone HIT3a, 300310), CD45RO (clone UCHL1, 304246), CD103 (clone Ber-ACT8, 350221), PD-1 (clone EH12.2H7, 329924), CD62L (clone DREG-56, 304830), CD69 (clone FN50, 310926) purchased from Biolegend; CD8 (clone RPA-T8, 563795) purchased from BD Horizon. For experiments evaluating CAR T therapy using in vivo xenograft tumor model, 1×10^6 cells from the digested single-cell suspensions were placed in standard fluorescence-activated cell sorting (FACS) tubes and were stained for human CD45-BV421 (clone HI30) and/or

CD3-FITC (clone HIT3a) or CD3-PE/Cy7 (clone UCHT1) antibodies (all from Biolegend). Labeled cells were washed and resuspended in FACS buffer for flow cytometric analysis using LSRFortessa Cell Analyzer (BD Biosciences). Subsequent computer analysis was done using FlowJo software (v10.2). Negative gating was based on a "fluorescence minus one" (FMO) strategy.

[00198] Single-cell reverse transcription, library preparation, and sequencing

[00199] To prepare for single-cell RNA sequencing analysis, 6 to 12 tissue samples from each array microdevice were pooled together. Similar to the preparation for flow cytometry, the tumor explants were carefully separated from the stroma and removed from the culture chambers. Single cell suspensions were obtained using the same tissue digestion buffer used above (TTDR, BD Horizon). The isolated cells were filtered, manually counted, and prepared at the desired cell concentration of 800-1000 cells/ μ l. Finally, 20,000 cells were collected and washed with PBS, and then loaded onto a Chromium Single Cell Chip (10x Genomics) according to the manufacturer's instructions for co-encapsulation with barcoded beads at a target capture rate of 10,000 individual cells per sample.

[00200] The capture of RNA transcripts in the barcoded cells and reverse transcription of cDNA were performed using the manufacturer's standard protocols. The Agilent TapeStation High Sensitivity D5000 ScreenTape was used for QC of generated cDNA. The barcoded cDNA was converted into single-cell RNA-seq libraries for sequencing using the Chromium Single Cell 3' Reagent Kit v3 (10x Genomics) according to the manufacturer's instructions. The Agilent TapeStation High Sensitivity D1000 ScreenTape was used for QC of prepared libraries for sizing (bp) and concentration. The final libraries from four samples (i.e., control tumor, control stroma, meso-tumor, and meso-stroma) were pooled together and sequenced by Illumina NovaSeq sequencer with a SP v1.5 FlowCell (100 cycles). The pooled libraries were sequenced to a target depth of at least 20,000 mean reads per cell.

[00201] Computational analysis of single-cell RNA sequencing data

[00202] *Data pre-processing and quality control*

[00203] The raw FASTQ files containing sequence reads were pre-processed in the CellRanger pipeline (10X Genomics, v3.1.0). Reads produced from the

gene expression profiling were aligned to the human GRCh38 reference genome, which was additionally customized to include the reference sequences of GFP that tagged the tumor cells and of RFP and SS1 scFv that were contained in the mesoCAR construct. The feature-barcode matrices were generated using default parameters for each sample, which record the number of unique molecular identifiers for each gene within each cell barcode. The quality of cells was assessed based on the proportion of mitochondrial gene counts and the number of genes detected per cell. Low-quality cells were filtered out if the proportion of mitochondrial gene counts was higher than 20%. In addition, only cells with number of features/genes larger than 200 but less than 5000 were kept for subsequent analysis to avoid capture of possible doublet or multiplet.

[00204] *Single-cell data integration and clustering analysis*

[00205] The R package Seurat (v3.2.0) was used to filter out low-quality cells, normalize the raw counts data to account for sequencing depth, scale and identify highly variable features, cluster upon dimensionality reduction, and integrate samples. Briefly, the gene-count matrix was first normalized by cell-specific size factor, log-transformed, and then scaled to unit variance and zero mean. Samples from the same experiment were integrated using the merge function for cell type identification and direct comparisons. Principal components were calculated using the data decomposition technique of Latent Semantic Indexing (LSI) and used to reduce the dataset into two dimensions. Unsupervised hierarchical clustering of cells was generated and visualized using Uniform Manifold Approximation and Projection (UMAP) in Seurat. Differential-expression tests for all cells were performed using FindAllMarkers function. Genes with $\log_2$ -fold changes >0.25 , expression in at least 25% of cells in tested groups, and adjusted $p < 0.05$ were regarded as significantly differentially expressed genes (DEGs). Clusters were labelled based on the expression of the top DEGs as well as the canonical markers for each cell type.

[00206] *Gene Ontology (GO) analysis*

[00207] For each cluster, GO analysis was conducted by comparing top 100 genes that were highly differentially expressed and all the other genes in the dataset. GO analysis was performed using PANTHER Overrepresentation test (Released 20200728) (PANTHER version 16.0 Released 2020-12-01) with default parameters (Fisher's exact

test; cut off at False Discovery Rate $p < 0.05$). The results were visualized with heatmap using Morpheus (<https://software.broadinstitute.org/morpheus>).

[00208] *Comparison of CAR T cell clusters with external human single-cell gene signatures*

[00209] To assess the physiological relevance of CAR T cell phenotypes observed in our microengineered tumor constructs, we compared our CAR T cell cluster annotations against a published human single-cell RNA-seq dataset containing T cells isolated from tumors, adjacent normal tissues, and peripheral blood of lung cancer patients. The single-cell profiles of 12,346 T cells from 14 patients were obtained under GEO accession number GSE99254. The R package SingleR (v1.4.1) was used for comparison following standard procedures. The raw count matrices were downloaded from the publication and then normalized and clustered in Seurat following standard workflow. The same cell annotations from the reference publication were maintained on a cell name/barcode basis. Note that both the reference and our subject dataset were subsetting for CD4 and CD8 T cells, which were separately compared. SingleR calculates correlations between subject and reference cells using variable genes in the reference dataset to assign cellular identity to each cell in our subject dataset. The SingleR comparison outputs an UMAP clustering of our CAR T cells with the predicted T cell annotation from the reference dataset, and a heatmap of prediction scores of each cell within the subject dataset towards the corresponding cell type from the reference dataset.

[00210] *Single-cell trajectory analysis*

[00211] Monocle 3 (v1.0.0) was used for the analysis of pseudotime transition of cell transcriptional states. In this analysis, cells were ordered in pseudotime as a measure of progress through biological processes based on their transcriptional similarities. The aggregated Seurat object after dimensional reduction as constructed above was converted to a cell_data_set object in Monocle. Single cells of each cell type were visualized in the UMAP space with similar clustering to that in Seurat. CAR T cell clusters and tumor cell clusters were subsetting for pseudotime trajectory analysis, separately. The principal graphs were generated along each trajectory to represent possible paths that cells can take in response to emerging cues in the tumor microenvironment. Cell-wise pseudotime was calculated based on its position along the principal graphs after manual selection of root-node for each trajectory. The root nodes for CAR T cell trajectory

were selected from principal points with highest expression of T cell naivness markers (i.e., SELL, TCF7, and LEF1). For tumor cell trajectories, root nodes were assigned to the earliest principal points for pseudotime computation. Moran's I test was performed to identify genes with expression that are trajectory-dependent using the `graph_test` function. The smoothed gene marker expression along pseudotime was generated by the `plot_genes_in_pseudotime` function with a natural spline used to fit the gene expression along pseudotime. Top ranked genes with monotone increase or decrease of expression and biphasic trend of expression along the pseudotime were selected for comparison.

[00212] *Analysis of inter-lineage interactions*

[00213] CellPhoneDB 2.0 was used to identify enriched ligand-receptor interactions from our scRNA-seq datasets involved in soluble factor- or direct binding-mediated signaling of inter-lineage interactions in our microengineered tumor tissues. CellPhoneDB is a manually curated public repository of ligands, receptors and their interactions, integrated with a statistical framework for inferring cell-cell communication networks from single-cell transcriptomic data. The normalized count matrix with metadata column consisting of annotations of cell identity and other default parameters were provided as input. The interaction analysis was limited to the control tumor and meso-tumor models, separately. We estimated the potential interaction between two cell types mediated by a specific ligand-receptor pair by determining the mean of the average expression of the receptor in one cell type and the interacting ligand in the other cell type. To examine the statistical significance of such estimated interaction, random permutations were applied on the cell type cluster labels of individual cells for 1000 times. The p value of the likelihood of cell type specificity was estimated by the number of permutations that had interaction mean larger than the real mean value. The cutoff was set with the mean expression greater than 0.1 and p value smaller than 0.05. To precisely identify the specific interactions only between cells, the output matrix of significantly interacting ligand-receptor pairs was filtered to remove all direct integrin-ECM interactions. From those remaining, we prepared chord diagrams of interactions between each pair of interacting cell types and generated an overarching web using the Circlize package in R. In the overarching web, each chord represents the bundle of all significant interactions between a particular pair of cell types. The chord width represents the total number of ligand-receptor pairs in each pair of interacting cell types. For closer assessment, all the

directional interactions between ligand-receptor pairs within each interacting cell pair were also visualized in chord diagrams. The ligand-receptor pairs with total mean < 0.35 were neglected for visualization. In the directional chord diagrams, each chord represents the maximum of all significant means from all paired clusters for each interacting gene pair within each pair of interacting cell types. In this case, the chord width indicates the level of total mean of interaction between each specific ligand-receptor pair.

[00214] Therapeutic manipulation of CAR T cell trafficking

[00215] To construct the composite tumor spheroids with reduced mesothelin expression, the wild type A549 and meso-A549 cells were mixed at 1:1 ratio, suspended in RPMI 1640 supplemented with 20% FBS at a density of 1×10^4 cells/mL, and dispensed to 96-well ultralow attachment spheroid microplates (Corning) with 100 μ L of cell suspension per well. The tumor spheroids were generated and maintained in the microplates for two to three days prior to collection and in vitro transplantation. The DPP4 inhibitor - LAF237 (Vildagliptin, PharmaForm LLC) - was used to inhibit the enzymatic activity of DPP4. LAF237 was used at final concentrations of 50 nM and 1,000 nM. The DPP4 inhibitor was diluted in EGM-2 media to the target final concentrations and administered to the tumor-chip after the infusion of meso-CAR T cells. Fresh EGM-2 media with different concentrations of DPP4 inhibitors were separately prepared and changed daily until the end of culture. For evaluation of DPP4 activity, the spent cell culture media from individual microdevices were collected daily prior to routine media change. DPP4 activity was measured with the DPPIV-Glo Protease Assay (Promega) per manufacturer's instructions.

[00216] The anti-human CXCR3 antibody (clone 49801, MAB160, R&D Systems) was used to neutralize the chemotaxis mediated by functional signaling of CXCR3. The anti-hCXCR3 and its IgG₁ isotype control antibody (clone 11711, MAB002, R&D Systems) were used at final concentrations of 10 μ g/mL. The CAR T cells suspended in EGM-2 MV media were incubated with the anti-hCXCR3 and isotype control antibodies, as well as LAF237, at final concentrations for one hour at room temperature prior to the initial infusion to the microdevices. Fresh EGM-2 MV media with final concentrations of anti-hCXCR3, isotype control, and LAF237 were separately prepared and changed daily until the end of culture.

[00217] The human CD38 inhibitor - Daratumumab (HY-P9915, MedChemExpress) and the lymphotoxin β receptor IgG fusion protein - Baminercept (HY-P99459, MedChemExpress) were used to inhibit the interactions between CD38 and its ligand CD31 and between lymphotoxin β and its receptor, respectively. Daratumumab and Baminercept were used at final concentrations of 0.5 $\mu\text{g/mL}$ and 10 $\mu\text{g/mL}$. Their human IgG₁ isotype control antibody (HY-P99001, MedChemExpress) was used at final concentration of 10 $\mu\text{g/mL}$. Daratumumab, Baminercept, and isotype control antibody were diluted in EGM-2 MV media to the target final concentrations and administered to the tumor-chip together with the infusion of meso-CAR T cells. Then, fresh EGM-2 MV media with different concentrations of Daratumumab, Baminercept, and isotype control antibody were separately prepared and changed daily until the end of culture.

[00218] ELISA, LDH and chemokine profiler assays

[00219] To analyze and quantify the secretion of IL-2 and IFN γ from the activated T cells, the cell culture media from individual microdevices were collected at specified time points. Human IL-2 DuoSet ELISA kit (DY202-05, R&D Systems) and human IFN-gamma DuoSet ELISA kit (DY285B-05, R&D Systems) were used to measure the concentrations of IL-2 and IFN γ , respectively. Each assay was performed and analyzed following the manufacturer's instructions. Briefly, 100 μL of cell culture samples or standards was added per well upon plate preparation and incubated for 2 hours at RT. Subsequently, the wells were washed four times with 200 μL of manufacturer-provided wash buffer and incubated with 100 μL of respective detection antibodies for another 2 hours at RT. After washing, 100 μL of Streptavidin-HRP was added per well and incubated for 20 minutes at RT in the dark. After washing, 100 μL of substrate solution was added per well and incubated for another 20 minutes at RT in the dark. Finally, 50 μL of stop solution was added per well, and the plate was measured for optical densities of each well using the absorbance mode of a microplate reader (Infinite M200, Tecan).

[00220] For measurement of CXCL10 and CXCL11 dynamics in the engineered tumor constructs, the cell culture media from individual microdevices were collected at specified time points and analyzed using human CXCL10/IP-10 Quantikine ELISA kit (DIP100, R&D Systems) and human CXCL11/I-TAC Quantikine ELISA kit (DCX110, R&D Systems) following the manufacturer's instructions. Briefly, 100 μL of cell culture samples or standards in respective assay diluent was added per well and

incubated for 2 hours at RT. The wells were washed four times with 200 µl of manufacturer-provided wash buffer and incubated with 200 µl of respective conjugates for another 2 hours at RT. After washing, 200 µl of substrate solution was added per well and incubated for 30 minutes at RT in the dark. Finally, 50 µl of stop solution was added per well, and the plate was measured in a microplate reader (Infinite M200, Tecan).

[00221] To analyze and quantify the cytotoxicity of CAR T cells, the cell culture media from at least three individual microdevices per group were collected on day 5 post infusion of CAR T cells and analyzed using a lactate dehydrogenase (LDH) assay kit (ab102526, abcam) following manufacturer's instructions. 50 µl of cell culture samples or standards diluted in assay buffer and another 50 µl of reaction mix were added per well in sequence and mixed thoroughly. The output optical densities were measured immediately using a microplate reader (Infinite M200, Tecan) in a kinetic mode, every 3 minutes, for a total of 1 hour.

[00222] For profiling the relative expression of commonly studied chemokines between the control and meso-tumors in our model, the cell culture media from individual microdevices were collected one day prior to the infusion of T cells and analyzed using the human chemokine array G1 kit (AAH-CHE-G1-8, Ray Biotech) following the manufacturer's instructions. Samples were analyzed without dilution and the slide was imaged with a laser scanning confocal microscope (LSM 800, Zeiss).

[00223] Measurement of intact and truncated forms of CXCL10

[00224] *Preparation of aptamer modified gold nanoparticle*

[00225] Colloidal gold nanoparticles (GNP) capped with citrate were synthesized by seed-mediated growth. In brief, gold nanoparticles were grown from citrate-capped seed particles. The seed particles were synthesized by reacting 100 ml of 0.01 wt % HAuCl₄ solution and 3 mL of 1 wt % citrate solution at 99 °C for 30 min. 100 ml of 0.01 wt % HAuCl₄ solution was treated with 4 mL of seed solution and 400 µl of 1 wt % citrate solution at 99 °C for 30 min. Aptamer that specifically binds to valine was obtained from BIONEER (South Korea) and modified with thiol group to bind onto the surface of gold nanoparticles. For the preparation of GNP-aptamer, 10 ml of aqueous solution including ca. 14×10^{13} aptamers was mixed with 990 ml of gold nanoparticles solution (ca. 9×10^{10} nanoparticles/ml) and the mixture was kept at 4 °C for 24 hr.

[00226] *Absorbance and Raman measurements of GNP-aptamer after mixing with samples*

[00227] The absorbance spectra of GNP-aptamer were taken by Cary 5000 UV-Vis-NIR spectrophotometer after the mixing with filtered samples collected from device effluent in FIG. 8. For Raman measurements, GNP-aptamer was collected by centrifugation (4000 rpm, 10 min) after the mixing. Next, the collated GNP-aptamer was dropped and dried onto silicon wafer. Raman spectra were measured by using a micro-Raman system with a spectrometer SR-303iA (Andor Technology), 785 nm laser module I0785SR0100B (Innovative Photonic Solution Inc.), and Olympus BX-53 M TRF microscope (Olympus). The integration time was set to 5 s.

[00228] *Immuno-precipitation*

[00229] Immunoprecipitation of CXCL10 from device effluent samples was performed following the manufacturer's protocol. Briefly, 50 µl of Dynabeads™ Protein G magnetic beads (10007D, Thermo Fisher) were placed on a magnet to separate the beads from the solution. After removing supernatant, the beads were incubated with 200 µl of rabbit anti-CXCL10 monoclonal antibody (MA5-32674, Invitrogen) solution at a concentration of 20 µg/ml for 1 hour and washed on the magnet to remove unbound antibodies. Then 550 µl of the collected samples was added to the bead-antibody complex and incubated for 2 hours at room temperature to ensure the antigen binding. To elute the target antigen from the beads, the complex was incubated in 20 µl of elution buffer for 2 min and separated from the magnet. The pH of collected eluates was adjusted by adding 1 M Tris-HCl pH 7.5 solution for Western blotting analysis. To generate the truncated CXCL10 solution as a control, recombinant human CXCL10 (266-IP, R&D systems) was incubated at a final concentration of 1 µg/ml with recombinant DPP4 (D3446, Sigma) at a final concentration of 2 U/ml in a 100 mM Tris-HCL pH 8 solution for 2 h at 37°C.

[00230] *Western blotting*

[00231] Western blotting was performed. Briefly, 25 µl of protein samples were resolved by 20% SDS-PAGE and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skim milk for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies against CXCL10 (MAB266-100, R&D systems). After three washes, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 hour at room

temperature. Protein bands were visualized using ECL Western Blotting Substrate (Pierce) and quantified using Fiji/ImageJ software.

[00232] Metabolomics analysis

[00233] *Supernate metabolite extraction*

[00234] The spent media samples were collected from individual microdevices at each time point and individually frozen for storage at -80°C . A 5 μL of media was added to 120 μL of 25:25:10 (v/v/v) acetonitrile:methanol:water solution at -20°C , vortexed for 10 seconds, and put on ice for at least 5 minutes. The resulting extract was centrifuged at $16,000 \times g$ for 20 minutes at 4°C , and the supernatant was transferred to tubes for liquid chromatography-mass spectrometry (LC-MS) analysis. A procedure blank was generated identically without spent media, which was used later to remove background ions.

[00235] *Metabolite measurement by LC-MS*

[00236] Metabolites were analyzed using a Vanquish Horizon UHPLC System (Thermo Scientific) coupled to an Orbitrap Exploris 480 Mass Spectrometer (Thermo Scientific). Waters XBridge BEH Amide XP Column (particle size, 2.5 μm ; 150 mm (length) $\times$ 2.1 mm (i.d.)) was used for hydrophilic interaction chromatography (HILIC) separation. Column temperature was kept at 25°C . Mobile phases A = 20 mM ammonium acetate and 22.5 mM ammonium hydroxide in 95:5 (v/v) water:acetonitrile (pH 9.45) and B = 100% acetonitrile were used for both ESI positive and negative modes. The linear gradient eluted from 90% B (0.0–2.0 min), 90% B to 75% B (2.0–3.0 min), 75% B (3.0–7.0 min), 75% B to 70% B (7.0–8.0 min), 70% B (8.0–9.0 min), 70% B to 50% B (9.0–10.0 min), 50% B (10.0–12.0 min), 50% B to 25% B (12.0–13.0 min), 25% B (13.0–14.0 min), 25% B to 0.5% B (14.0–16.0 min), 0.5% B (16.0–20.5 min), then stayed at 90% B for 4.5 min. The flow rate was used at 0.15 mL/min. The sample injection volume was 5 μL . ESI source parameters were set as follows: spray voltage, 3200 V or -2800 V , in positive or negative modes, respectively; sheath gas, 35 arb; aux gas, 10 arb; sweep gas, 0.5 arb; ion transfer tube temperature, 300°C ; vaporizer temperature, 35°C . LC-MS data acquisition was operated under full scan polarity switching mode for all samples. The full scan was set as: orbitrap resolution, 120,000 at m/z 200; AGC target, $1\text{e}7$; maximum injection time, 200 ms; scan range, 60–1000 m/z .

[00237] *Data analysis*

[00238] LC-MS raw data files (.raw) were converted to mzXML format using ProteoWizard (version 3.0.20315). EL-MAVEN (version 0.12.0) was used to generate a peak table containing m/z, retention time, and intensity for the peaks. Default parameters were used for peak picking except for the following: mass domain resolution, 5 ppm; time domain resolution, 10 scans; minimum intensity, 10,000; and minimum peak width, 5 scans. The resulting peak table was exported as a .csv file. Peak annotation of untargeted metabolomics data was performed using NetID with default parameters. Statistical analyses were performed using MetaboAnalyst 5.0. Cut-off for significant change was set to $FDR < 0.05$.

[00239] Immunofluorescence analysis

[00240] For in situ immunofluorescence staining, cell-containing gel constructs in our microdevices were washed with PBS, fixed with 4% paraformaldehyde (Electron Microscopy Sciences) for 15 minutes at RT. The fixed cells were then permeabilized with 0.25% Triton X-100 in PBS for 15 minutes and blocked with 3% bovine serum albumin in PBS (Sigma Aldrich) for 1 hour at RT. After washing, the cells were incubated overnight at 4°C with primary antibodies against CD3 for T lymphocytes (pre-diluted, rabbit monoclonal 2GV6, 790-4341/05278422001, Roche), mesothelin for target antigen (1:50, mouse monoclonal G-1, sc-271540, Santa Cruz Biotechnology), cleaved caspase-3 for apoptotic cells (1:200, rabbit polyclonal Asp175, 9661S, Cell Signaling Technology), pan-cytokeratin for tumor cells (1:250, mouse monoclonal AE1/AE3+5D3, ab86734, Abcam), CD31 for endothelial cells (1:200, mouse monoclonal JC/70A, ab9498 or ab215911, Abcam), and Phalloidin for cellular actin filaments (1:100, A22287, ThermoFisher Scientific). After washing, the cells were then incubated with secondary antibodies (Goat anti-Rabbit IgG H&L (Alexa Fluor 488), 1:500, A32731, ThermoFisher Scientific; Goat anti-Mouse IgG H&L (Alexa Fluor 488), 1:500, A32723, ThermoFisher Scientific; Goat anti-Mouse IgG H&L (Alexa Fluor Plus 555), 1:500, A32727, ThermoFisher Scientific; Goat anti-Rabbit IgG H&L (Alexa Fluor Plus 555), 1:500, A32732, ThermoFisher Scientific; Goat anti-Mouse IgG H&L (Alexa Fluor 647), 1:500, A21236, ThermoFisher Scientific) overnight at 4°C or for 2 hours at RT. Finally, cell nuclei were counterstained with DAPI (D1306, ThermoFisher Scientific). Fluorescence images of the cells were acquired using a laser scanning confocal microscope (LSM 800, Zeiss) and processed using ZEN software (Zeiss) and ImageJ

(National Institutes of Health). 3D imaging reconstruction was performed using Imaris (Bitplane). Characterization of vessels such as the analysis of branch length and average diameters were performed using AngioTool2 and Vessel Analysis plugins in ImageJ.

[00241] Statistical analysis

[00242] Sample size for each experiment was determined on the basis of a minimum of $n = 3$ independent microdevices for each experimental group. Data were analyzed with Student's t -test and with one-way and two-way ANOVA followed by appropriate post-hoc testing for multigroup pairwise comparisons in GraphPad Prism v8.2, unless noted otherwise. Results were presented as mean $\pm$ standard error of the mean (SEM). Statistical significance of the analyzed data was attributed to values of $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ as determined by respective analyses.

[00243] Data availability The scRNA-seq datasets generated and analyzed during the current study are available at the NCBI Gene Expression Omnibus, under accession number GSE240121.

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[00436] Aspects

[00437] The following Aspects are illustrative only and do not limit the scope of the present disclosure or the appended claims. Any part or parts of any one or more Aspects can be combined with any part or parts of any one or more other Aspects.

[00438] Aspect 1. A microfluidic chip for in vitro culture, comprising: a chamber, the chamber comprising a primary channel and at least one side channel adjacent thereto, the chamber having a bottom surface and a top surface, the chamber being configured as an open-top chamber having at least one aperture extending through the top surface and in fluid communication with the primary channel; and a separator disposed between the primary channel and the at least one side channel, the separator extending from the bottom surface of the chamber toward the top surface of the chamber; and optionally, an insert, the insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture.

[00439] The chamber can be formed, for example, of a plastic or other working material. The chamber can be transparent. As shown in exemplary FIGs. 1c-1d, the

chamber can comprise a primary channel and a separator – which can be a rail – that separates the primary channel from at least one side chamber.

[00440] Also as shown in exemplary FIGs. 1c-1d, the chamber can comprise an open top having wells formed therein. The presence of well is not a requirement, however, although wells can facilitate the introduction of material – such as tumor cells – to the primary channel of the chip.

[00441] Aspect 2. The microfluidic chip of Aspect 1, further comprising a cell-laden medium within the primary lane, the cell-laden medium optionally comprising a hydrogel or even a hydrogel precursor. As but one example, the foregoing can comprise an extracellular matrix (ECM) hydrogel precursor solution containing suspended human endothelial cells (e.g., human umbilical vein endothelial cells - HUVEC, and human lung microvascular endothelial cells - HMVEC-L).

[00442] Aspect 3. The microfluidic chip of Aspect 2, wherein the cell-laden medium defines a depression, the depression in fluid communication with the at least one aperture, the depression optionally in at least partial register with the at least one aperture. As shown in FIG. 1d, the depression can take on the form of a projection that contacts the hydrogel. Without being bound to any particular theory or embodiment, the hydrogel can be solidified about the projection.

[00443] Aspect 4. The microfluidic chip of any one of Aspects 1-3, wherein any one or more of stromal cells or endothelial cells are disposed within the primary channel.

[00444] Aspect 5. The microfluidic chip of any one of Aspects 1-4, wherein endothelial cells are disposed within the at least one side channel. As shown in exemplary FIG. 1d, the endothelial cells can participate in the formation of a vascular bed.

[00445] Aspect 6. The microfluidic chip of any one of Aspects 1-5, further comprising solid tumor tissue disposed within the primary channel. Such solid tumor tissue can be, for example, tissue explanted from a subject, though this is not a requirement.

[00446] Aspect 7. The microfluidic chip of any one of Aspects 1-6, further comprising a vascular bed disposed in the primary channel. The vascular bed can place the primary channel into fluid communication with the at least one side channel. In this way, one can perfuse the at least side channel with any one or more of cells, agents, or other materials, which are then communicated via the vascular bed to the primary channel.

As shown in FIG. 1d, the vascular bed can connect cells or another construct – such as a solid tumor – that resides in the primary channel to the at least one side channel. In this way, one can introduce material to the side channel which is then perfused to cells or other construct of interest in the primary channel.

[00447] Aspect 8. The microfluidic chip of Aspect 7, wherein solid tumor tissue is disposed within the primary channel and wherein the vascular bed places the solid tumor tissue into fluid communication with the at least one side channel.

[00448] Aspect 9. The microfluidic chip of any one of Aspects 1-8, wherein (i) the at least one side channel comprises endothelial cells disposed therein, (ii) a plurality of CAR-T cells is disposed within the at least one side channel, or both (i) and (ii).

[00449] Aspect 10. The microfluidic chip of any one of Aspects 1-9, further comprising an insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture. An example such insert is shown in FIG. 1d. As shown, an insert can include cones or other projections that extend therefrom, which projections in turn obstruct apertures formed in the culture chamber of FIG. 1c. In this way, a user can form a vascularized 3D tissue (which can be, for example, reminiscent of the vascularized stroma surrounding solid tumors, which vascularized tissue can then receive transplantation of tumor explants or other material).

[00450] Aspect 11. The microfluidic chip of any one of Aspects 1-10, wherein the at least one side channel is a first channel disposed along a first side of the primary channel, and wherein the microfluidic chip further comprises a second channel, the second channel disposed along a second side of the primary channel. As an example, at least a portion of at least one of the first channel and the second channel can be parallel to the primary channel, as shown in FIG. 1c. A side channel can comprise one or more branches, inlets, outlets, flow control features, and the like. In this way, one can introduce a material – such as CAR T cells in a medium – into a side channel, which material is then perfused to the primary channel, and excess materials is then collected by the second channel.

[00451] Aspect 12. A method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip according to any one of Aspects 1-11 and a

sample cell perfused to the primary channel. Example such solid tumor cells and sample cells are described elsewhere herein; a sample cell can be a CAR T cell.

[00452] Aspect 13. The method of Aspect 12, further comprising predicting a cell-cell interaction between the solid tumor cell and the sample cell.

[00453] Aspect 14. The method of Aspect 12, further comprising determining a cell-cell interaction between the solid tumor cell and the sample cell. Such determining can comprise, for example, identifying the presence, absence, or level of one or more biomarkers associated with the cell-cell interaction. Such determining can also, for example, comprise visualization, spectroscopy, and other imaging.

[00454] Aspect 15. The method of any one of Aspects 12-14, wherein the sample cell is a CAR-T cell.

[00455] Aspect 16. A microfluidic chip, comprising: a primary channel; at least one side channel, the at least one side channel being in fluid communication with the primary channel; a separator disposed between the primary channel and the at least one side channel; a vascular bed, the vascular bed crossing the separator so as to place the at least one side channel into fluid communication with the primary channel. As described elsewhere herein, such a microfluidic chip can receive explanted material from a subject, for example tumor material.

[00456] Aspect 17. The microfluidic chip of Aspect 16, further comprising a medium disposed within the primary channel, the vascular bed being disposed within the medium, the medium optionally comprising a hydrogel. Exemplary hydrogels are described herein.

[00457] Aspect 18. The microfluidic chip of any one of Aspects 16-17, further comprising tumor tissue disposed within the primary channel.

[00458] Aspect 19. The microfluidic chip of Aspect 18, wherein the tumor tissue comprises tumor tissue explanted from a subject.

[00459] Aspect 20. The microfluidic chip of any one of Aspects 18-19, further comprising a CAR T cell contacting the tumor tissue.

[00460] Aspect 21. The microfluidic chip of any one of Aspects 16-19, further comprising a CAR T cell disposed within any one or more of the at least one side channel and the primary channel.

[00461] Aspect 22. A method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip according to any one of Aspects 16-21 and a sample cell perfused to the primary channel.

[00462] Aspect 23. The method of Aspect 22, further comprising predicting a cell-cell interaction between the solid tumor cell and the sample cell.

[00463] Aspect 24. The method of Aspect 22, further comprising determining a cell-cell interaction between the solid tumor cell and the sample cell.

[00464] Aspect 25. The method of any one of Aspects 22-24, wherein the sample cell is a CAR-T cell.

[00465] Aspect 26. A method, comprising: placing solid tumor tissue in a primary channel, effecting vascularization of the solid tumor tissue such that a vascular bed places the tissue into fluid communication with at least one side channel; and perfusing a material into the at least one side channel such that the material is communicated via the vascular bed to the solid tumor tissue.

[00466] Aspect 27. The method of Aspect 26, wherein the vascular bed is present in the primary channel to receive the solid tumor tissue.

[00467] Aspect 28. The method of any one of Aspects 26-27, further comprising monitoring interaction between the material and the solid tumor tissue.

[00468] Aspect 29. The method of claim 28, wherein the monitoring comprises collecting at least one biomarker.

[00469] Aspect 30. The method of any one of Aspects 26-29, wherein the solid tumor tissue is explanted from a subject.

What is Claimed:

1. A microfluidic chip for in vitro culture, comprising:
 - a chamber,
 - the chamber comprising a primary channel and at least one side channel adjacent thereto,
 - the chamber having a bottom surface and a top surface,
 - the chamber being configured as an open-top chamber having at least one aperture extending through the top surface and in fluid communication with the primary channel; and
 - a separator disposed between the primary channel and the at least one side channel,
 - the separator extending from the bottom surface of the chamber toward the top surface of the chamber; and
 - optionally, an insert,
 - the insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture.
2. The microfluidic chip of claim 1, further comprising a cell-laden medium within the primary lane, the cell-laden medium optionally comprising a hydrogel.
3. The microfluidic chip of claim 2, wherein the cell-laden medium defines a depression, the depression in fluid communication with the at least one aperture, the depression optionally in at least partial register with the at least one aperture.
4. The microfluidic chip of any one of claims 1-3, wherein any one or more of stromal cells or endothelial cells are disposed within the primary channel.
5. The microfluidic chip of any one of claims 1-4, wherein endothelial cells are disposed within the at least one side channel.

6. The microfluidic chip of any one of claims 1-5, further comprising solid tumor tissue disposed within the primary channel.
7. The microfluidic chip of any one of claims 1-6, further comprising a vascular bed disposed in the primary channel.
8. The microfluidic chip of claim 7, wherein solid tumor tissue is disposed within the primary channel and wherein the vascular bed places the solid tumor tissue into fluid communication with the at least one side channel.
9. The microfluidic chip of any one of claims 1-8, wherein (i) the at least one side channel comprises endothelial cells disposed therein, (ii) a plurality of CAR-T cells is disposed within the at least one side channel, or both (i) and (ii).
10. The microfluidic chip of any one of claims 1-9, further comprising an insert having at least one projection configured obstruct access to the aperture when the projection is placed into register with the one aperture.
11. The microfluidic chip of any one of claims 1-10, wherein the at least one side channel is a first channel disposed along a first side of the primary channel, and wherein the microfluidic chip further comprises a second channel, the second channel disposed along a second side of the primary channel.
12. A method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip according to any one of claims 1-11 and a sample cell perfused to the primary channel.
13. The method of claim 12, further comprising predicting a cell-cell interaction between the solid tumor cell and the sample cell.
14. The method of claim 12, further comprising determining a cell-cell interaction between the solid tumor cell and the sample cell.
15. The method of any one of claims 12-14, wherein the sample cell is a CAR-T cell.

16. A microfluidic chip, comprising:
- a primary channel;
 - at least one side channel,
 - the at least one side channel being in fluid communication with the primary channel;
 - a separator disposed between the primary channel and the at least one side channel;
 - a vascular bed,
 - the vascular bed crossing the separator so as to place the at least one side channel into fluid communication with the primary channel.
17. The microfluidic chip of claim 16, further comprising a medium disposed within the primary channel, the vascular bed being disposed within the medium, the medium optionally comprising a hydrogel.
18. The microfluidic chip of any one of claims 16-17, further comprising tumor tissue disposed within the primary channel.
19. The microfluidic chip of claim 18, wherein the tumor tissue comprises tumor tissue explanted from a subject.
20. The microfluidic chip of any one of claims 18-19, further comprising a CAR T cell contacting the tumor tissue.
21. The microfluidic chip of any one of claims 16-19, further comprising a CAR T cell disposed within any one or more of the at least one side channel and the primary channel.
22. A method, comprising: contacting a solid tumor cell residing in the primary channel of a microfluidic chip according to any one of claims 16-21 and a sample cell perfused to the primary channel.

23. The method of claim 22, further comprising predicting a cell-cell interaction between the solid tumor cell and the sample cell.
24. The method of claim 22, further comprising determining a cell-cell interaction between the solid tumor cell and the sample cell.
25. The method of any one of claims 22-24, wherein the sample cell is a CAR-T cell.
26. A method, comprising:
 - placing solid tumor tissue in a primary channel,
 - effecting vascularization of the solid tumor tissue such that a vascular bed places the tissue into fluid communication with at least one side channel;
 - and
 - perfusing a material into the at least one side channel such that the material is communicated via the vascular bed to the solid tumor tissue.
27. The method of claim 26, wherein the vascular bed is present in the primary channel to receive the solid tumor tissue.
28. The method of any one of claims 26-27, further comprising monitoring interaction between the material and the solid tumor tissue.
29. The method of claim 28, wherein the monitoring comprises collecting at least one biomarker.
30. The method of any one of claims 26-29, wherein the solid tumor tissue is explanted from a subject.

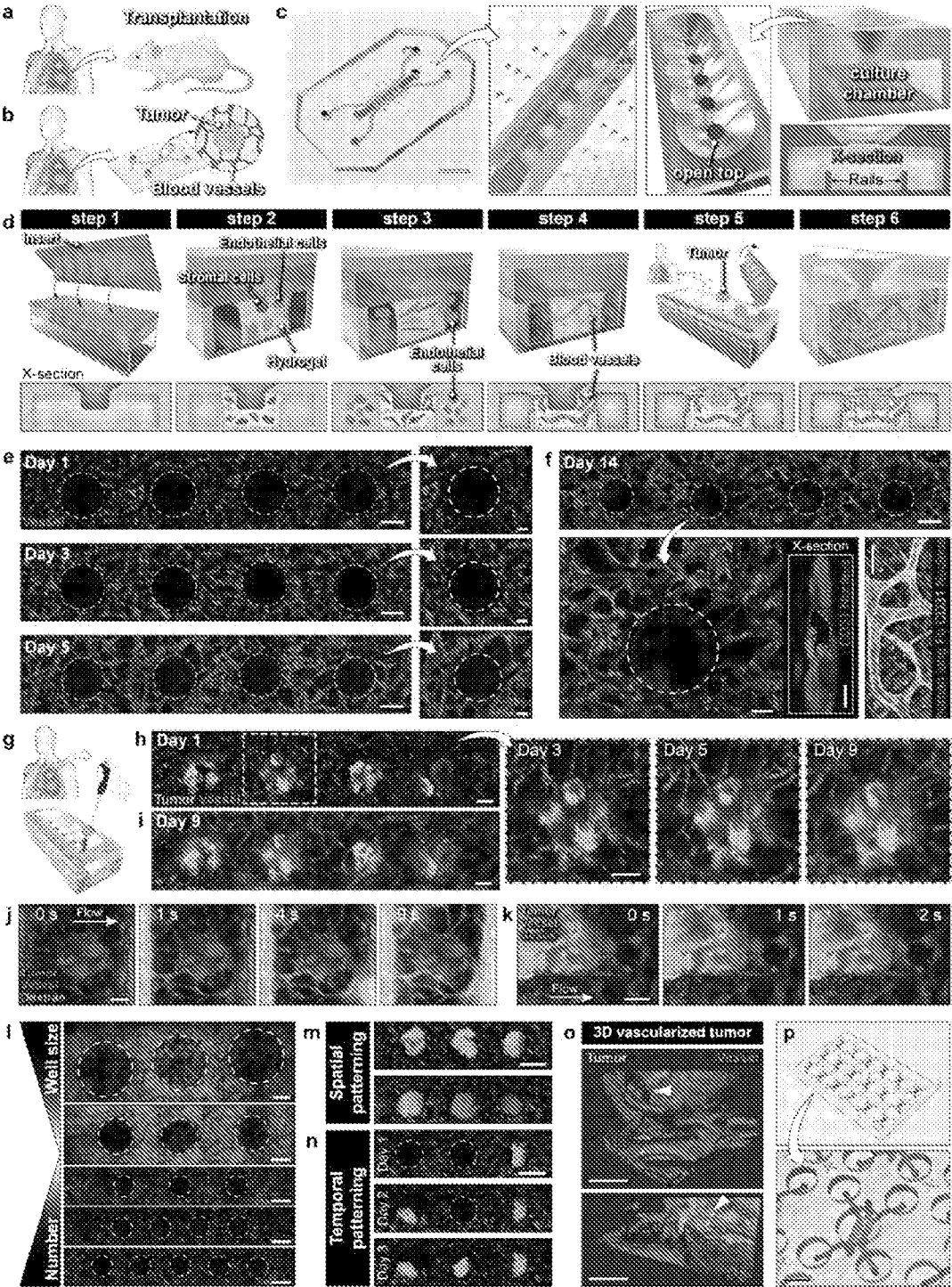


FIG. 1

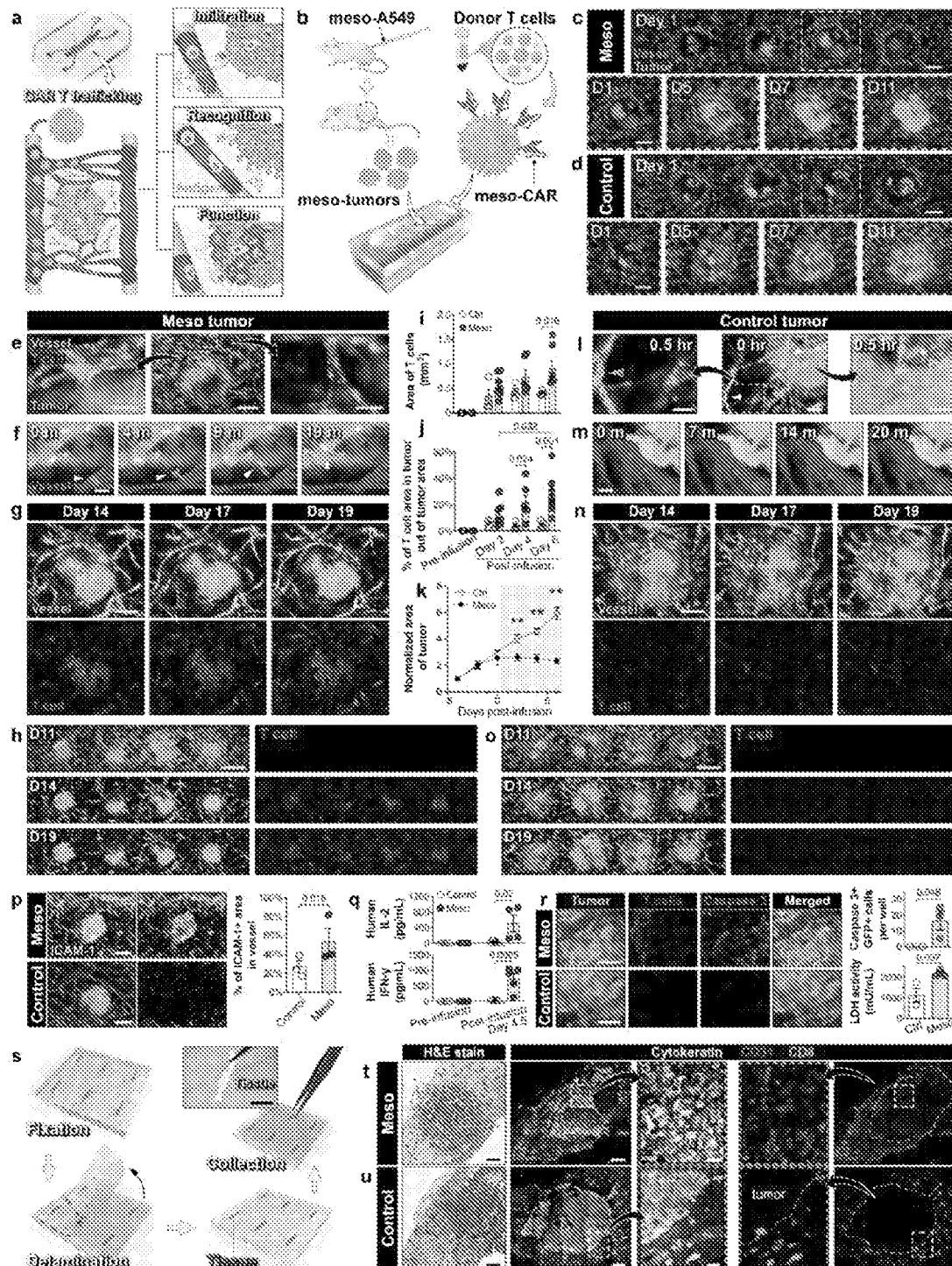


FIG. 2

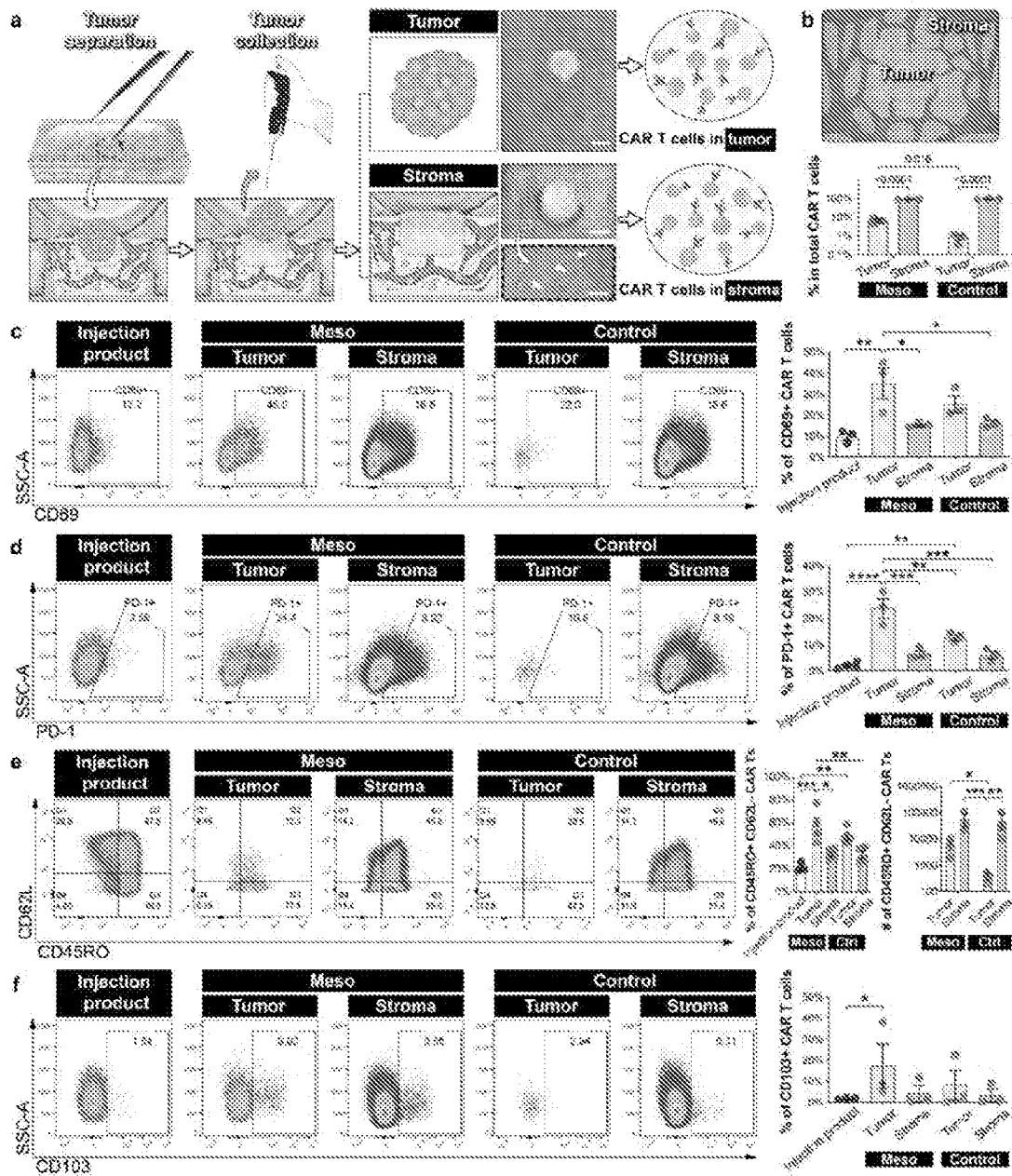


FIG. 3

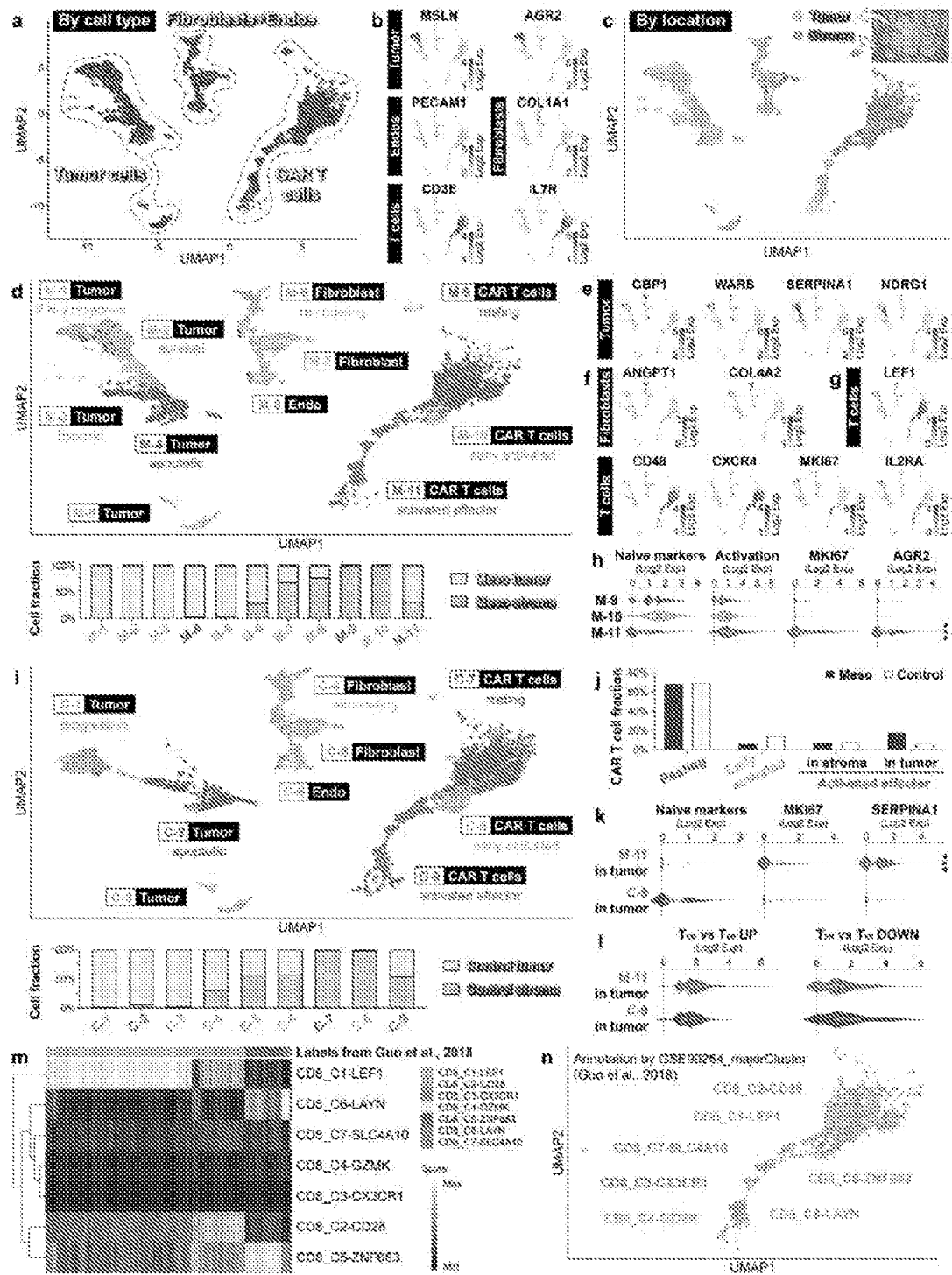


FIG. 4

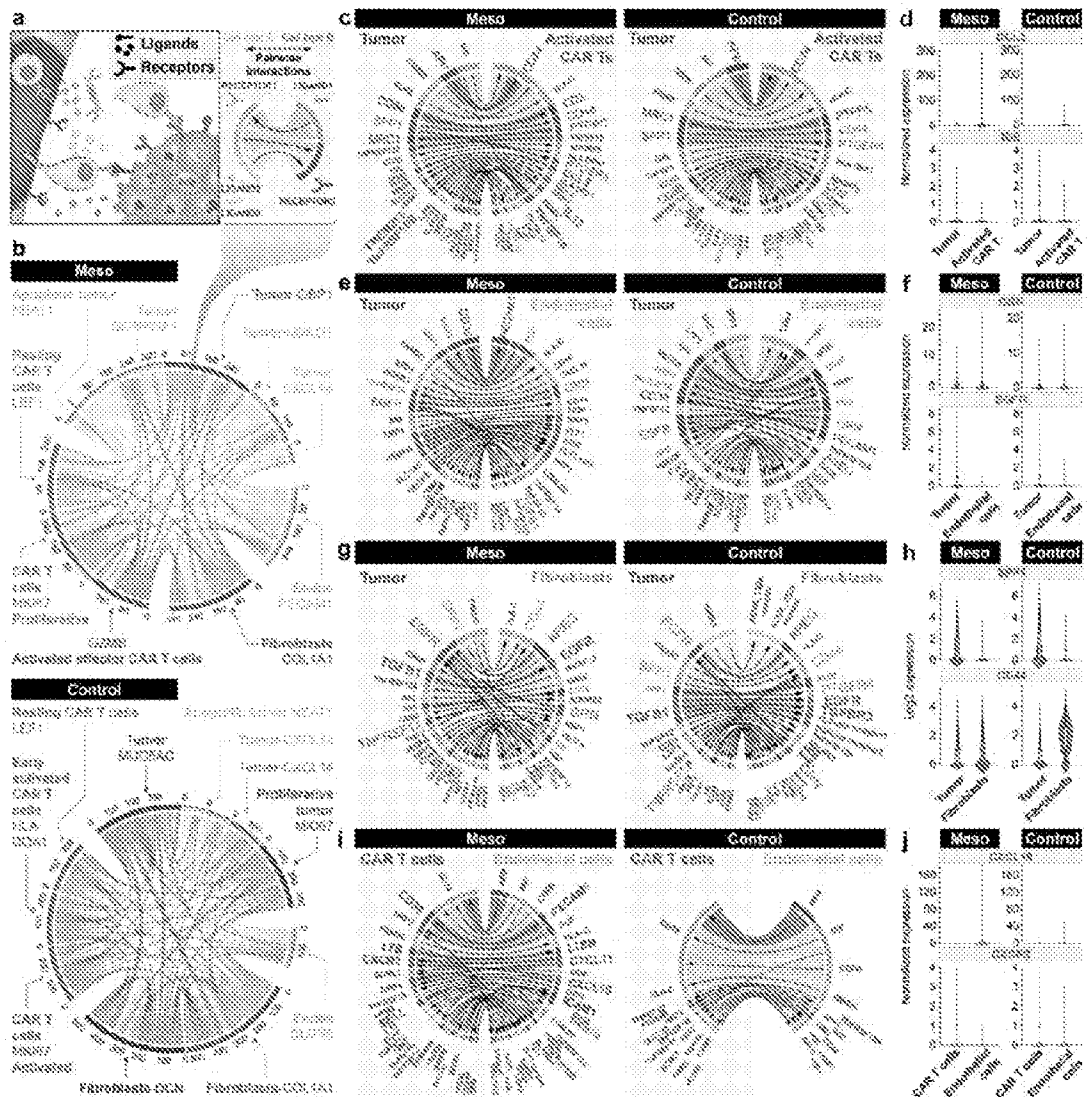


FIG. 5

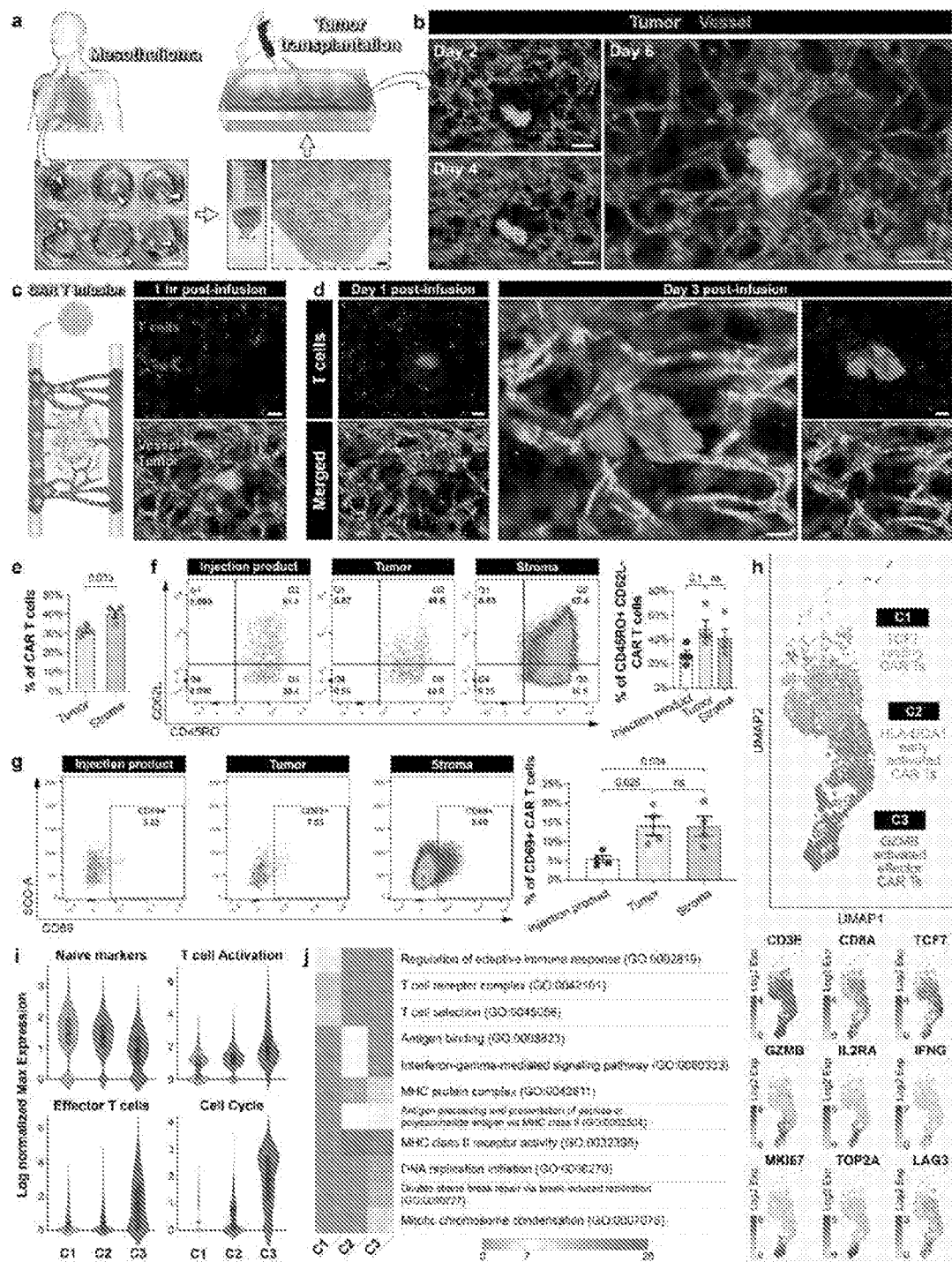


FIG. 6

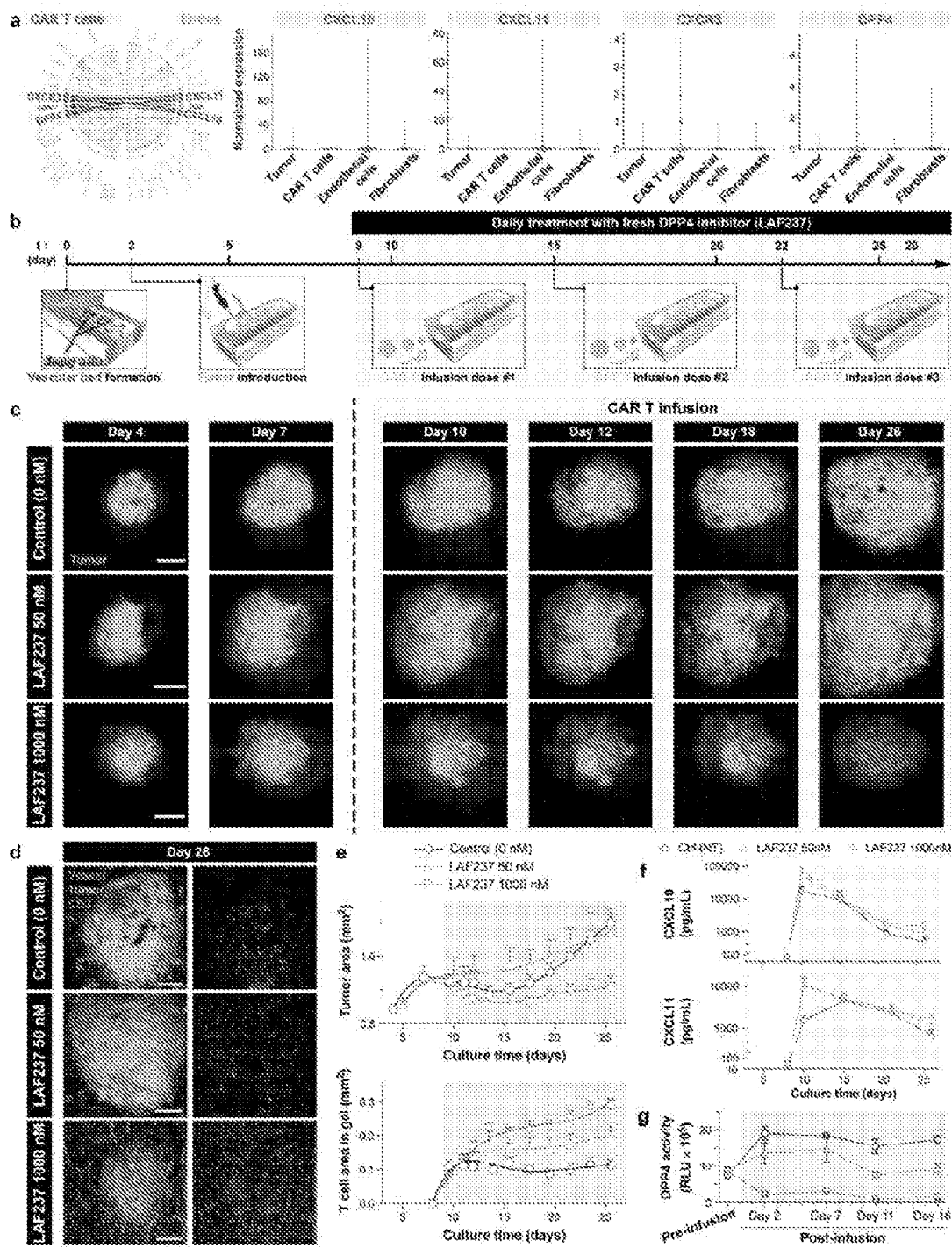


FIG. 7

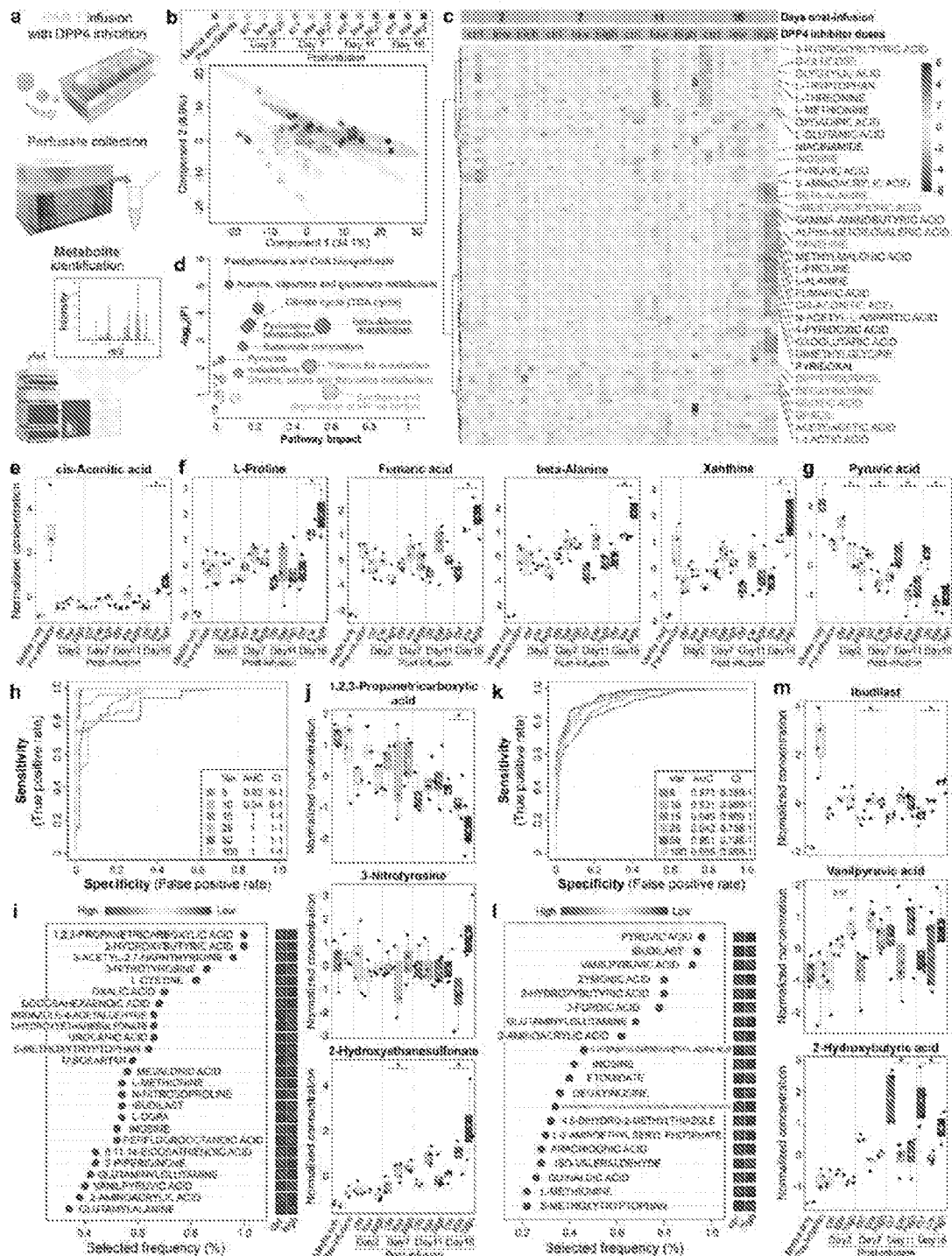


FIG. 8

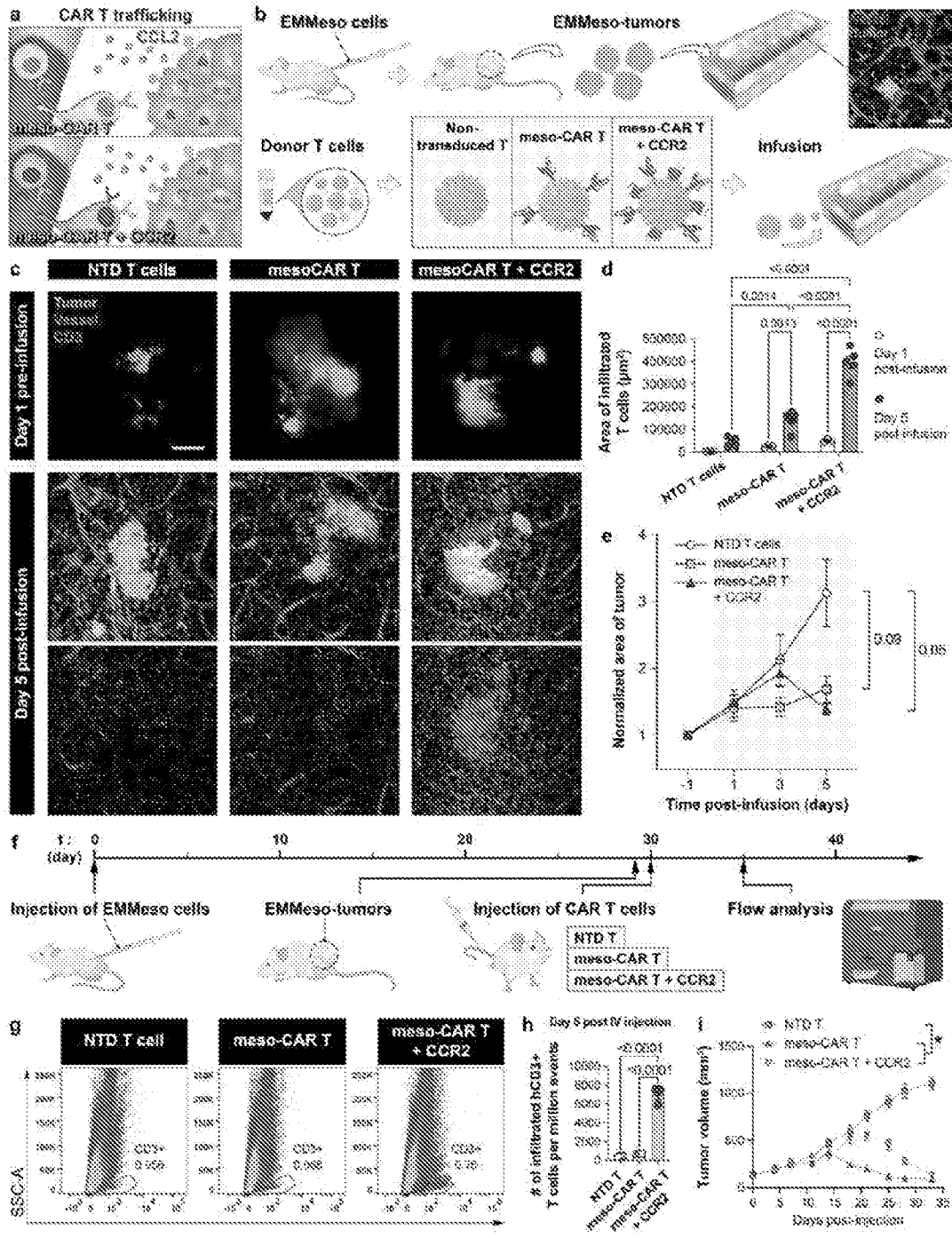


FIG. 9

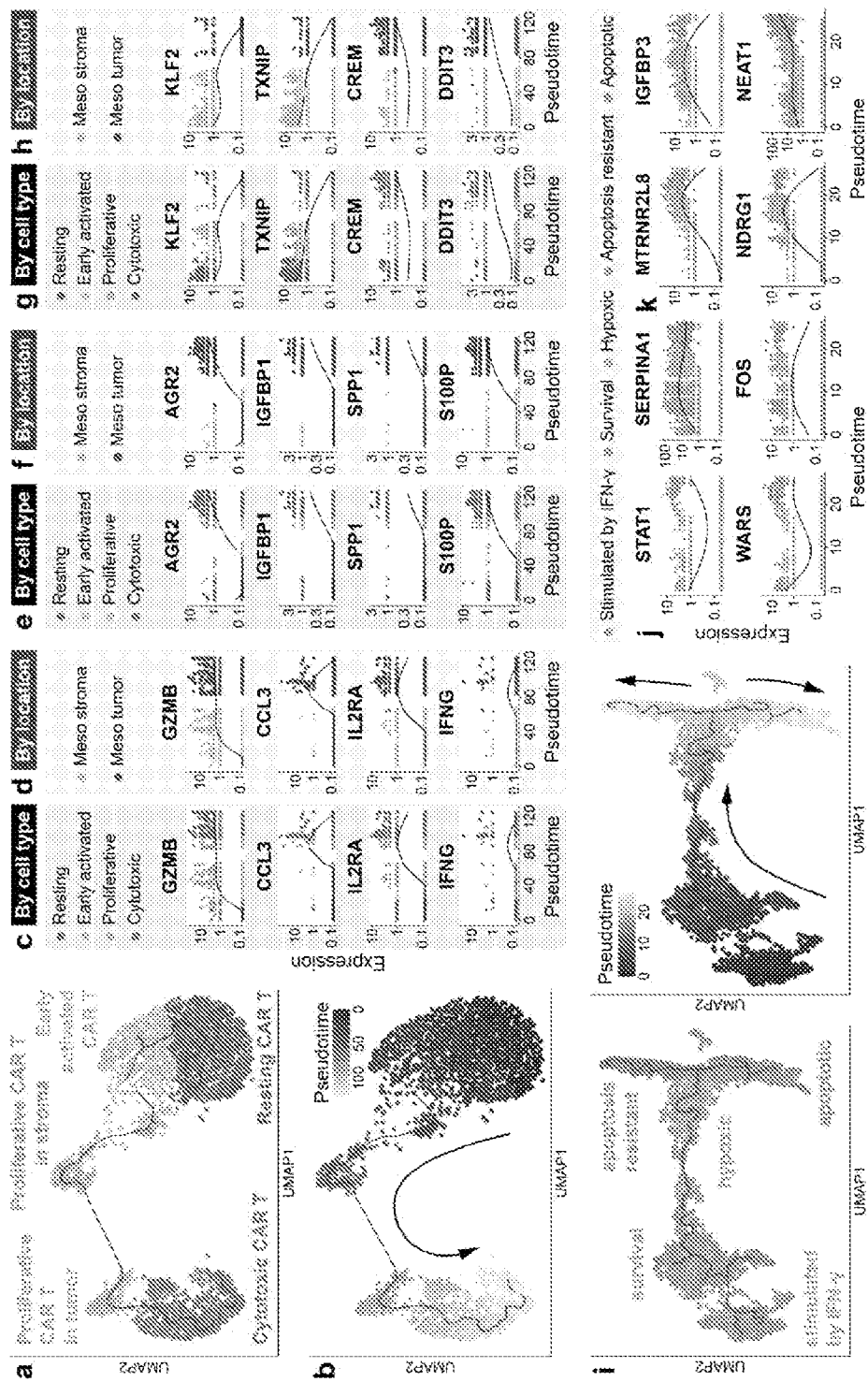


FIG. 10

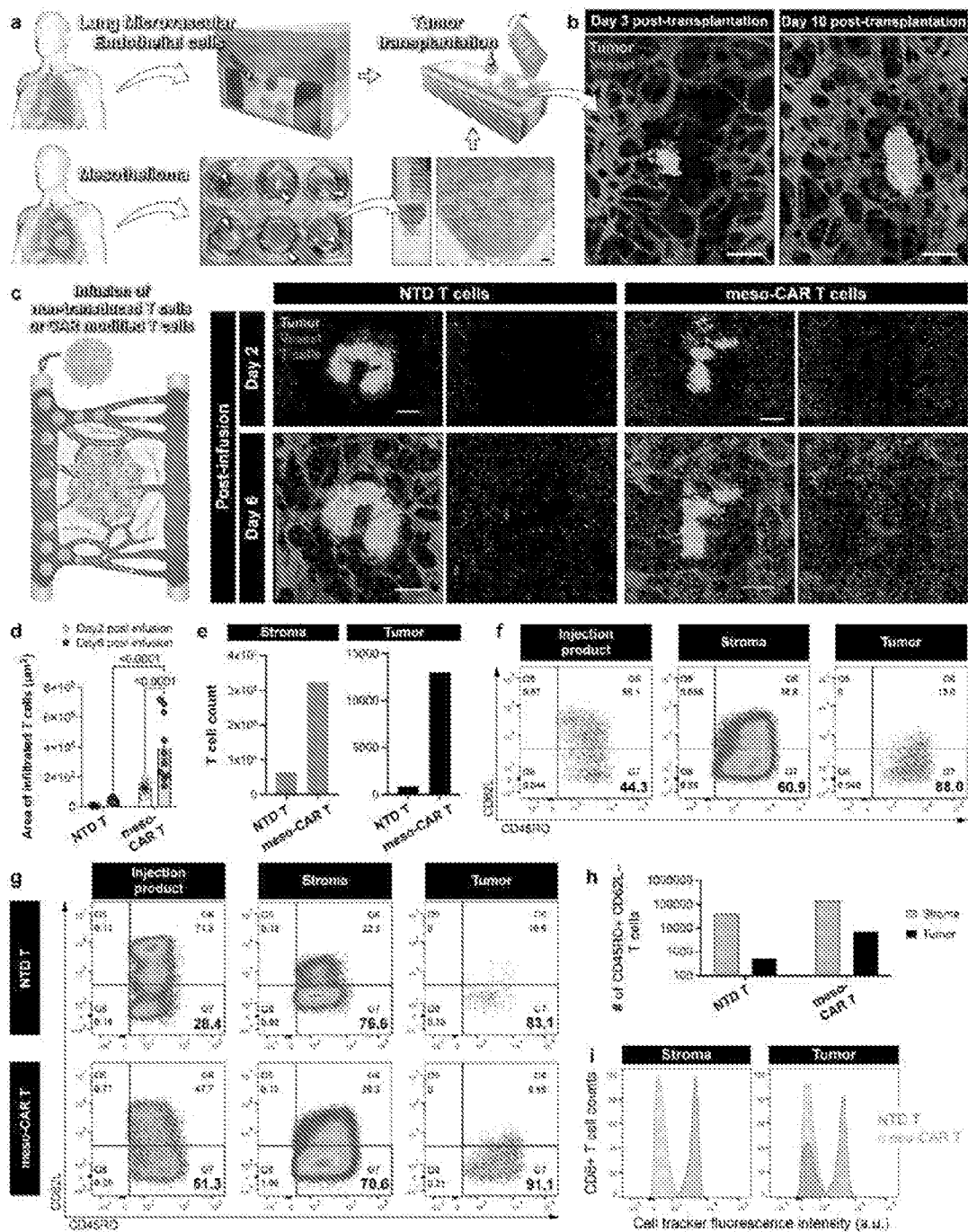


FIG. 11

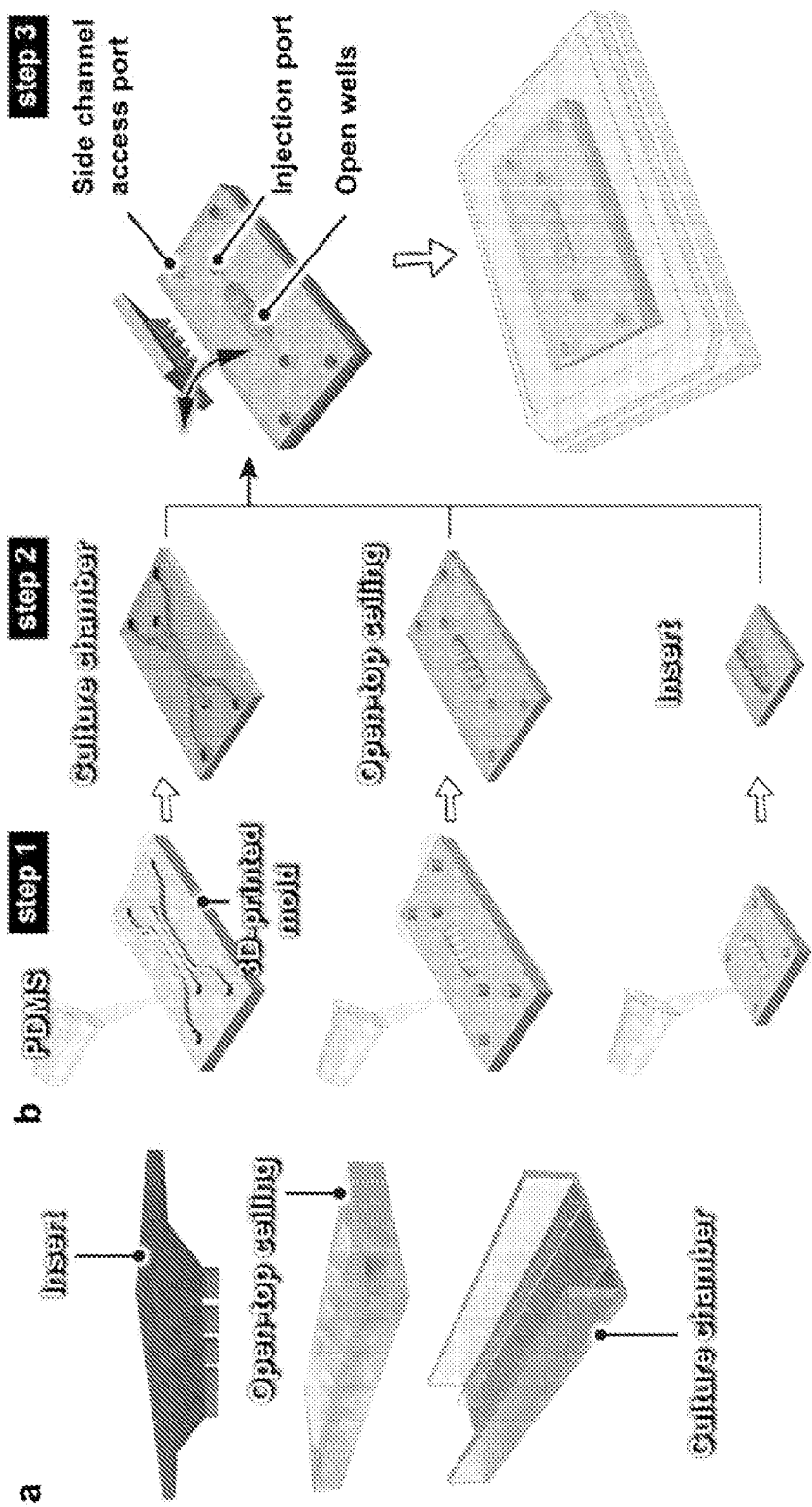


FIG. 12

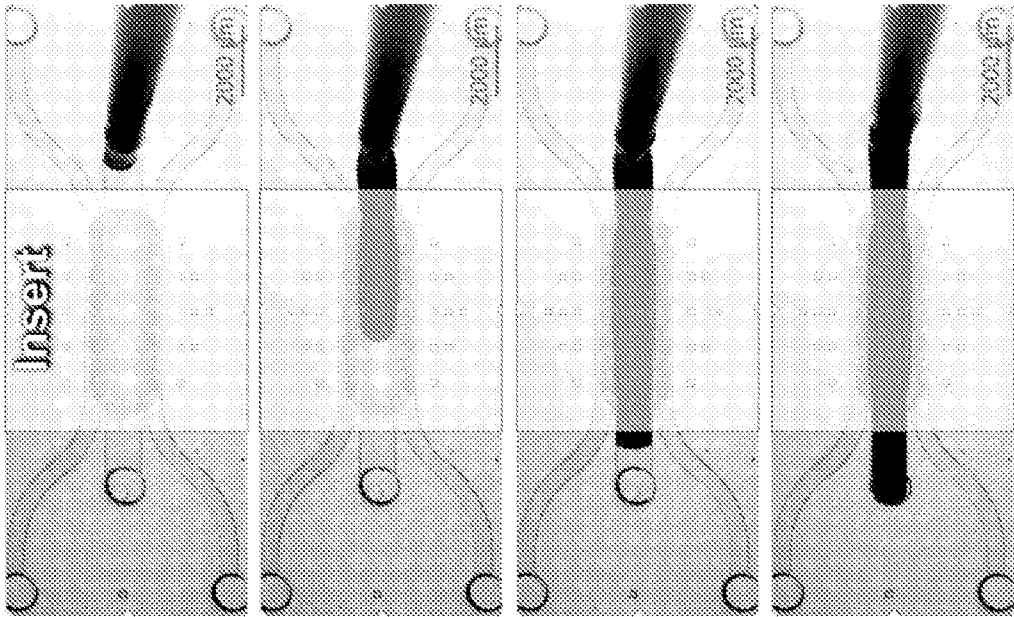


FIG. 13

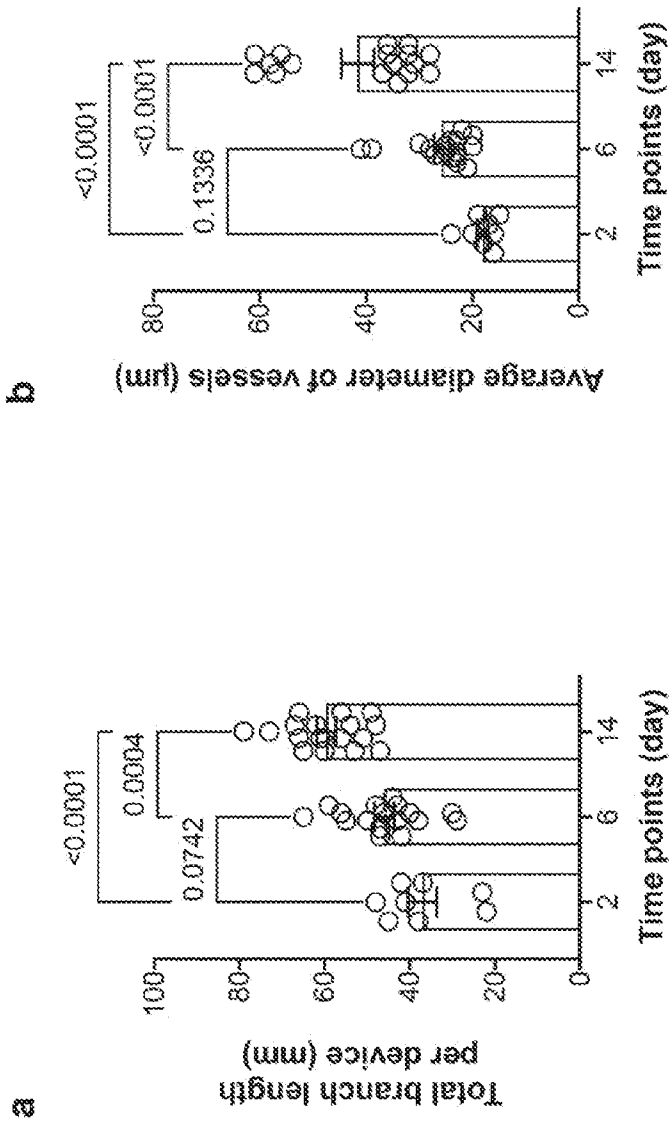


FIG. 14

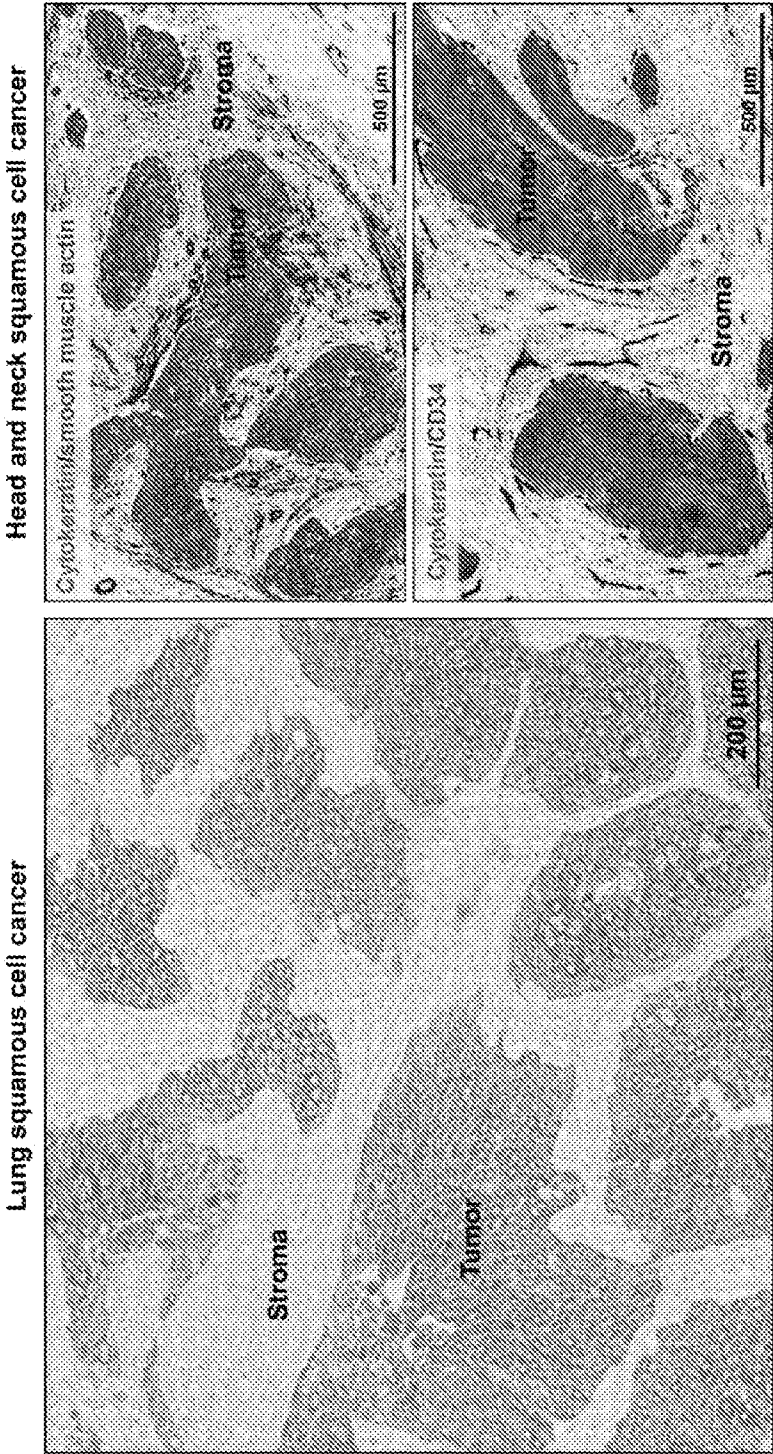


FIG. 15

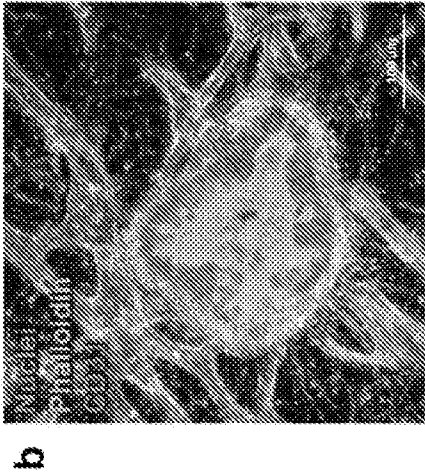
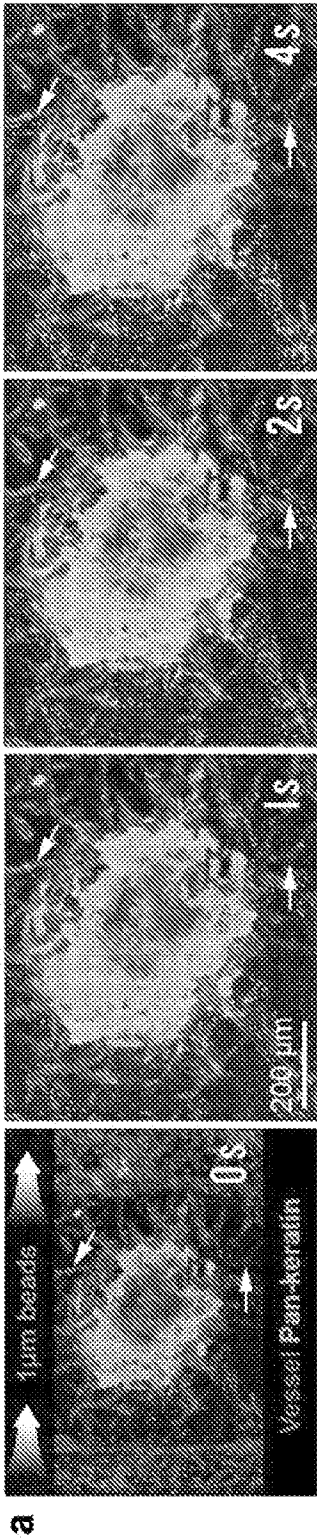


FIG. 16

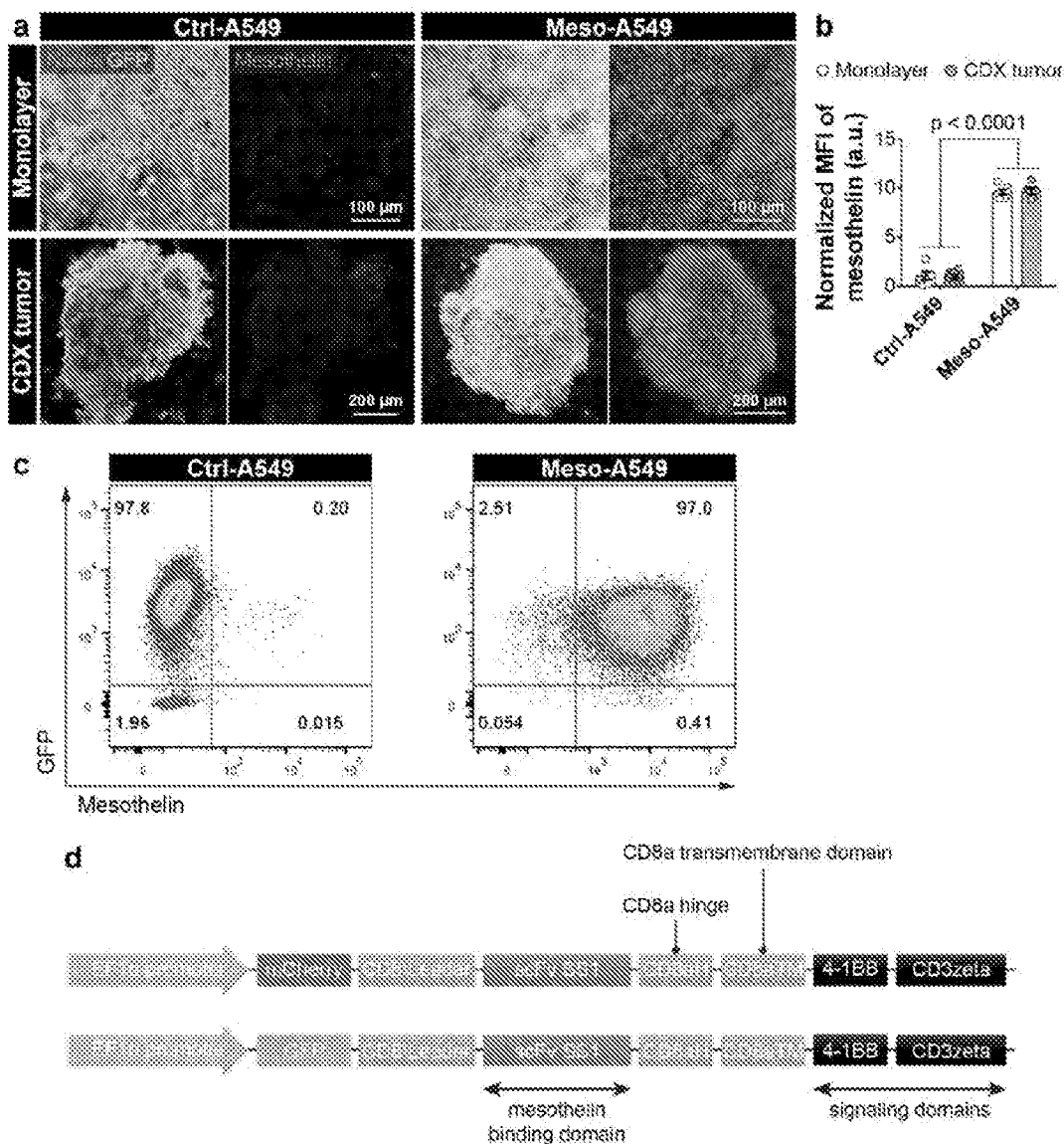


FIG. 17

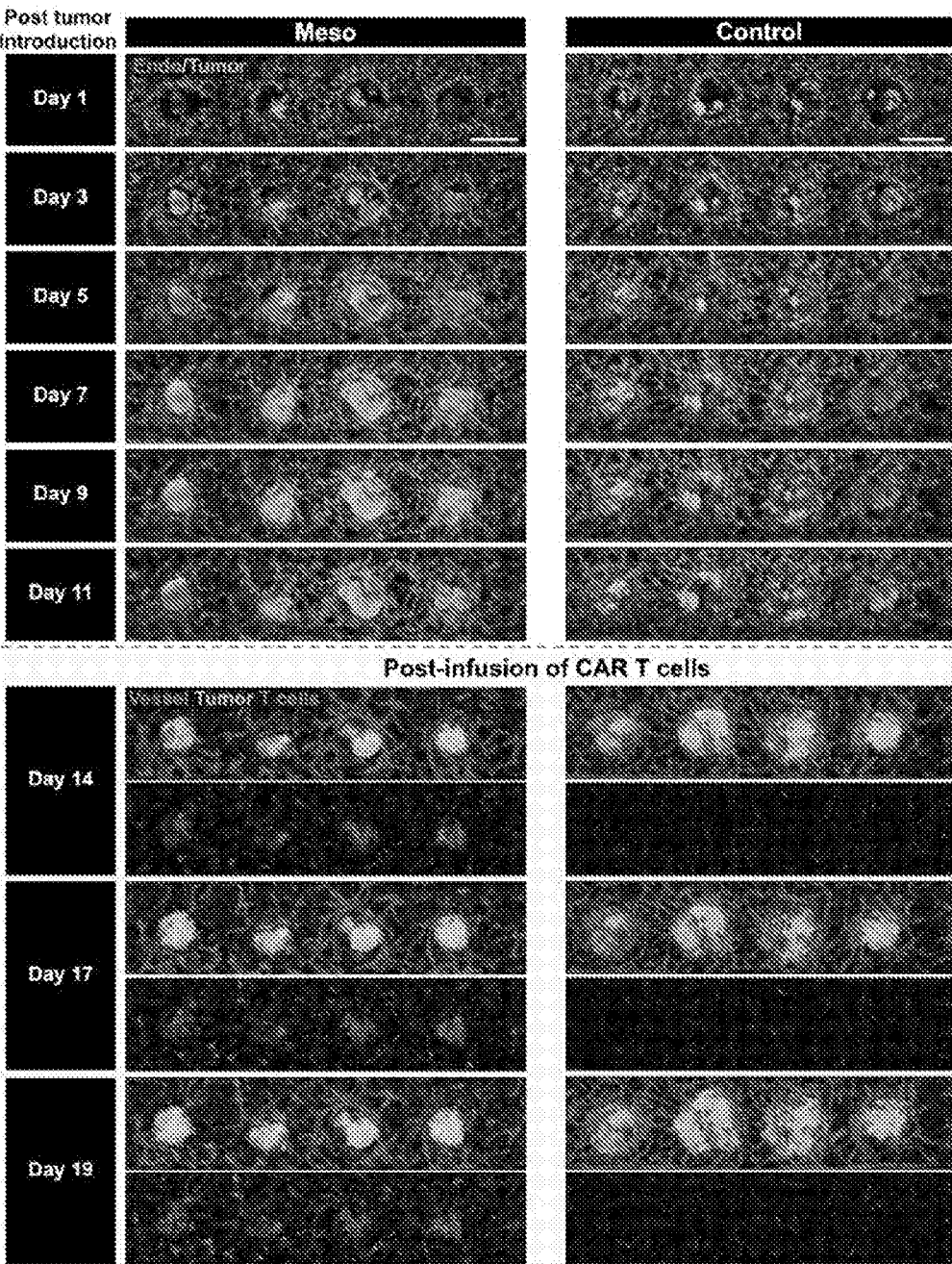


FIG. 18

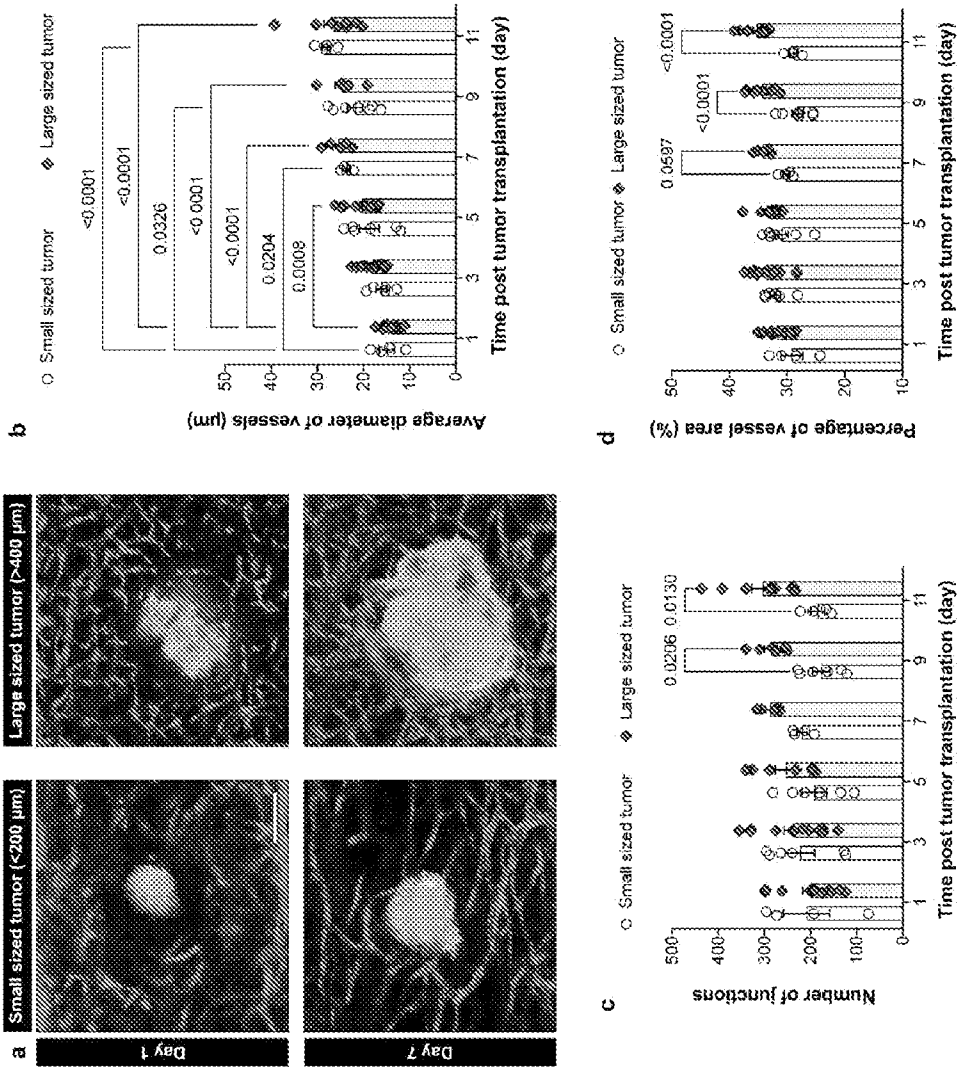


FIG. 19

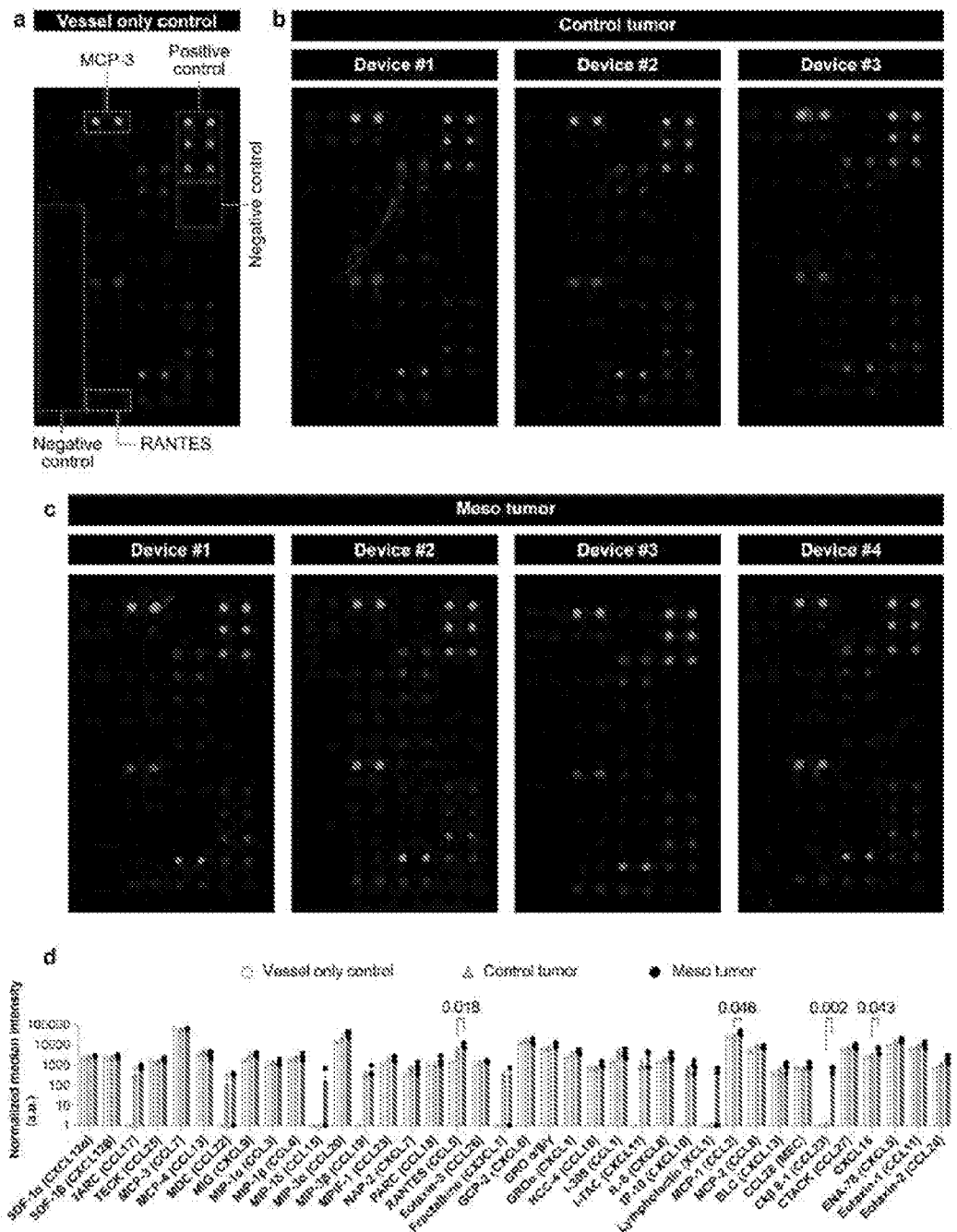


FIG. 20

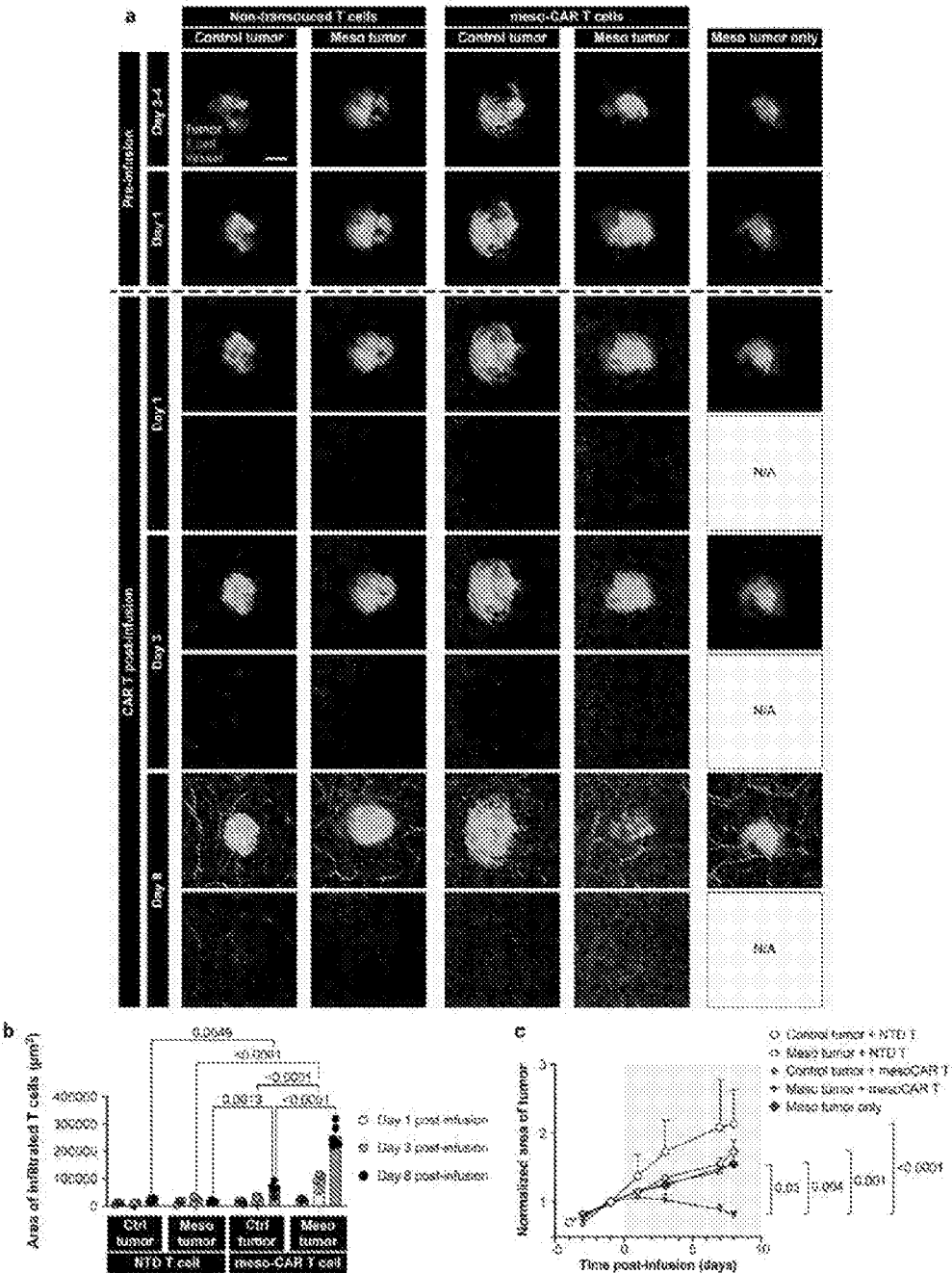


FIG. 21

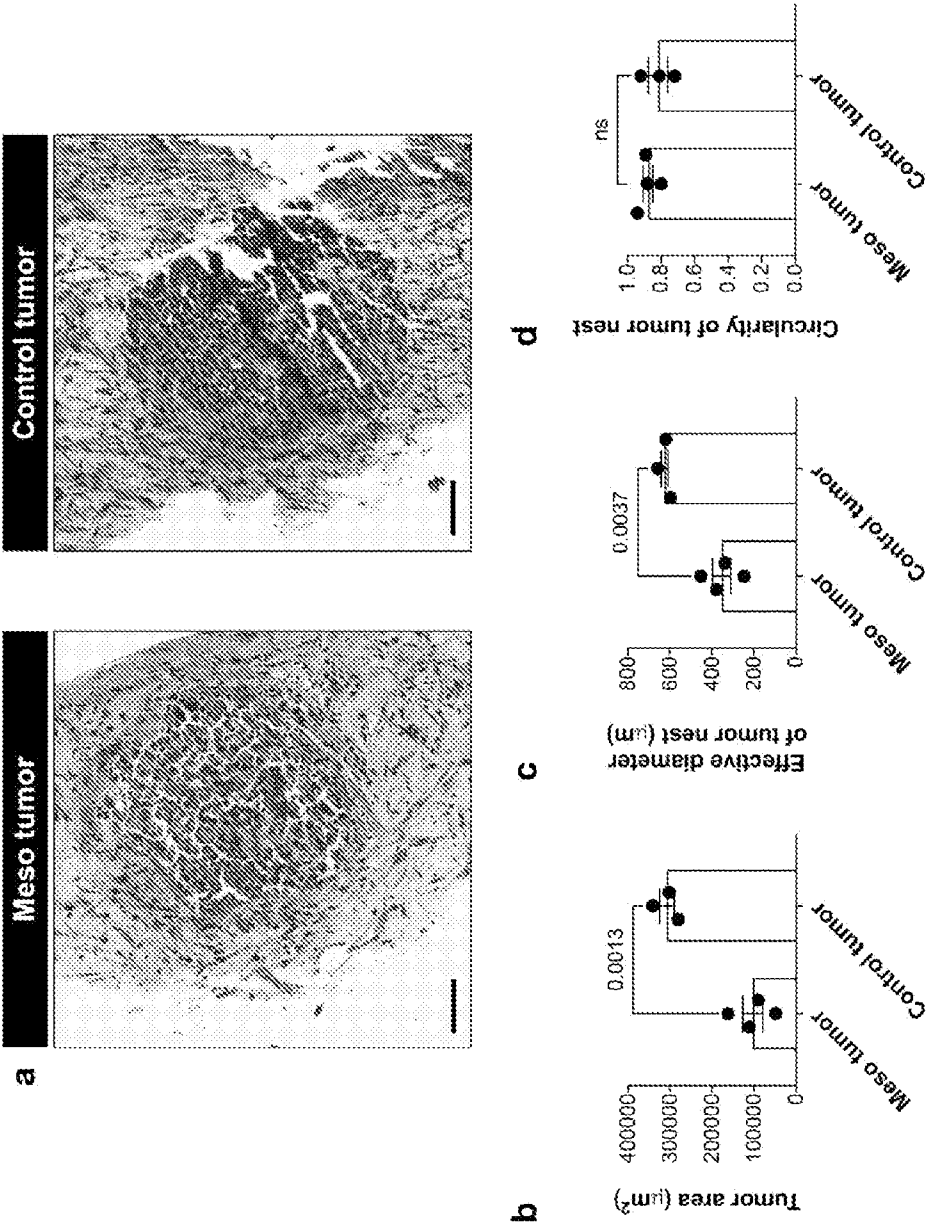


FIG. 22

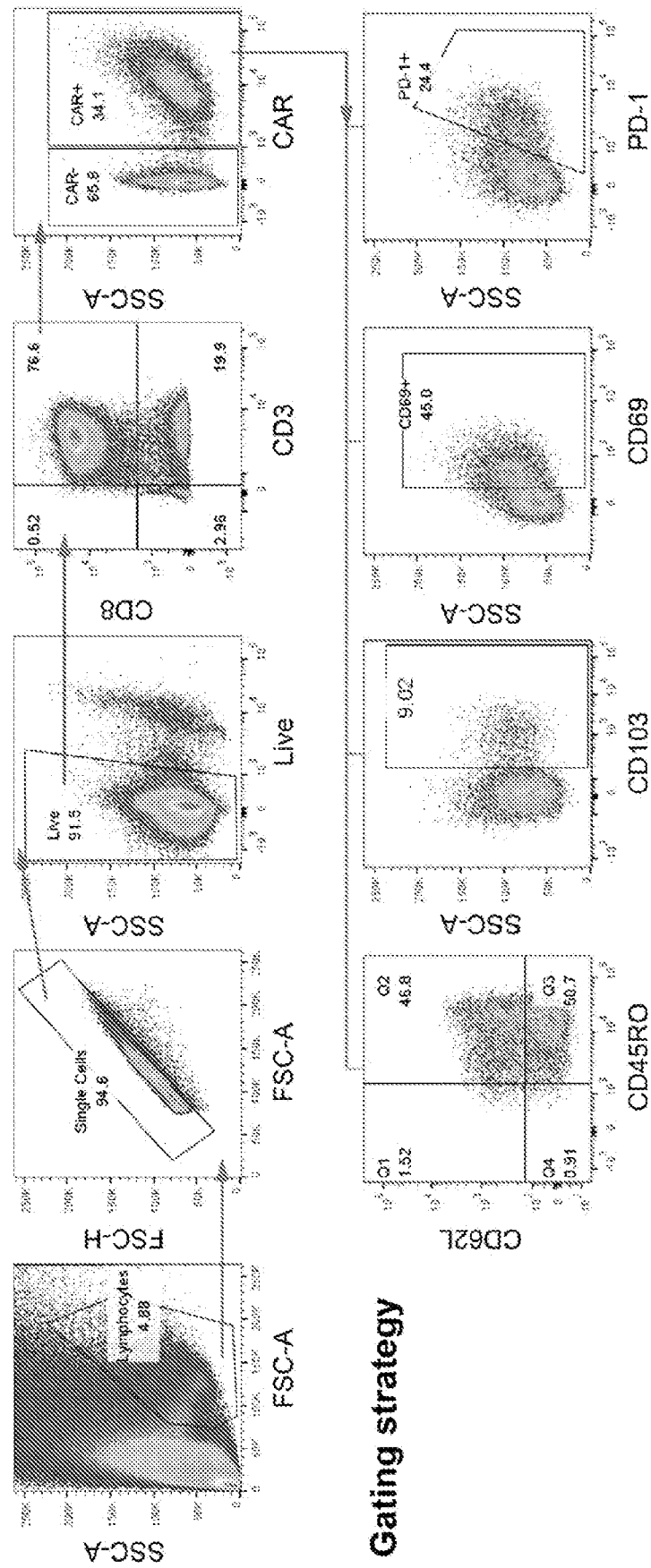


FIG. 23

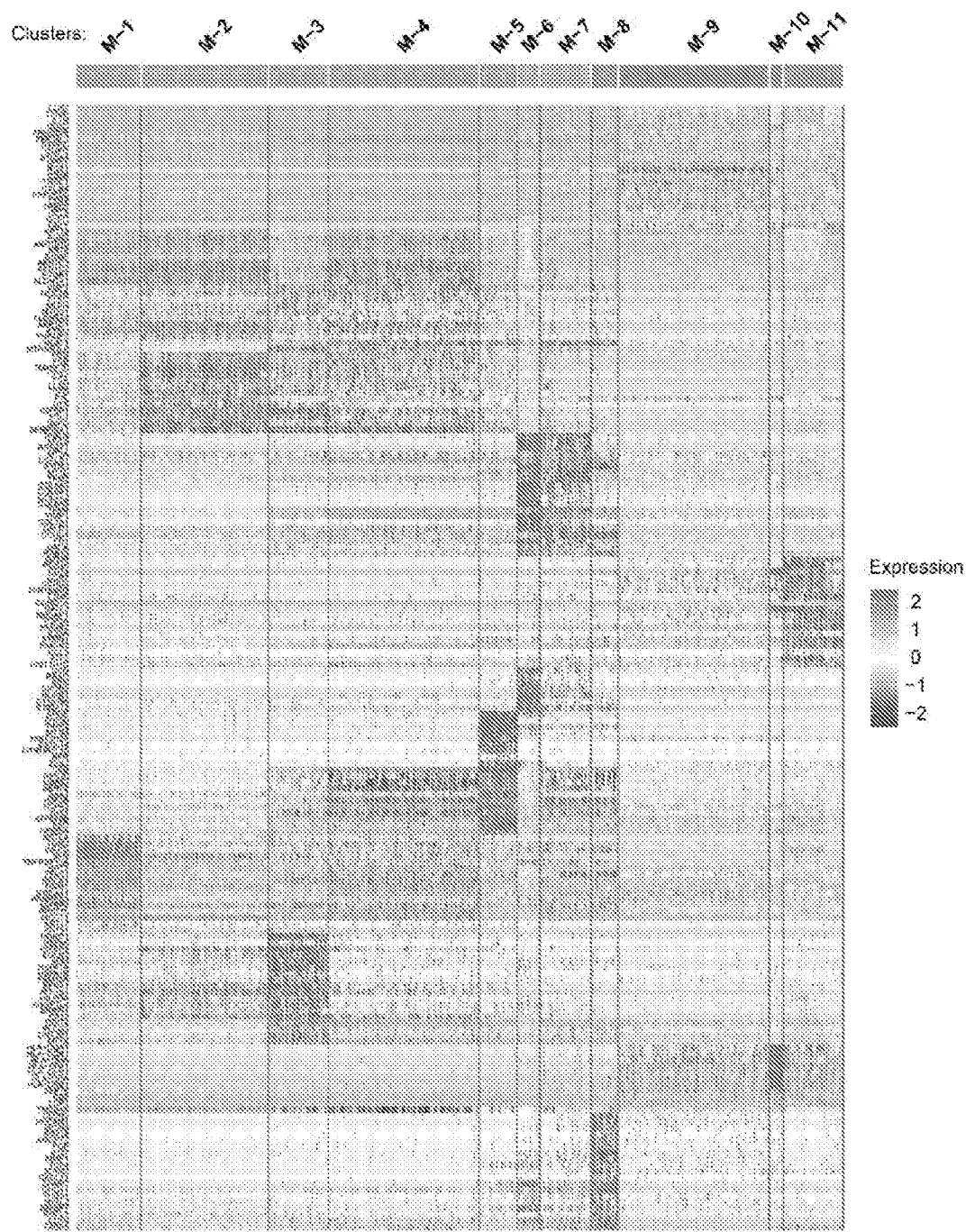


FIG. 24

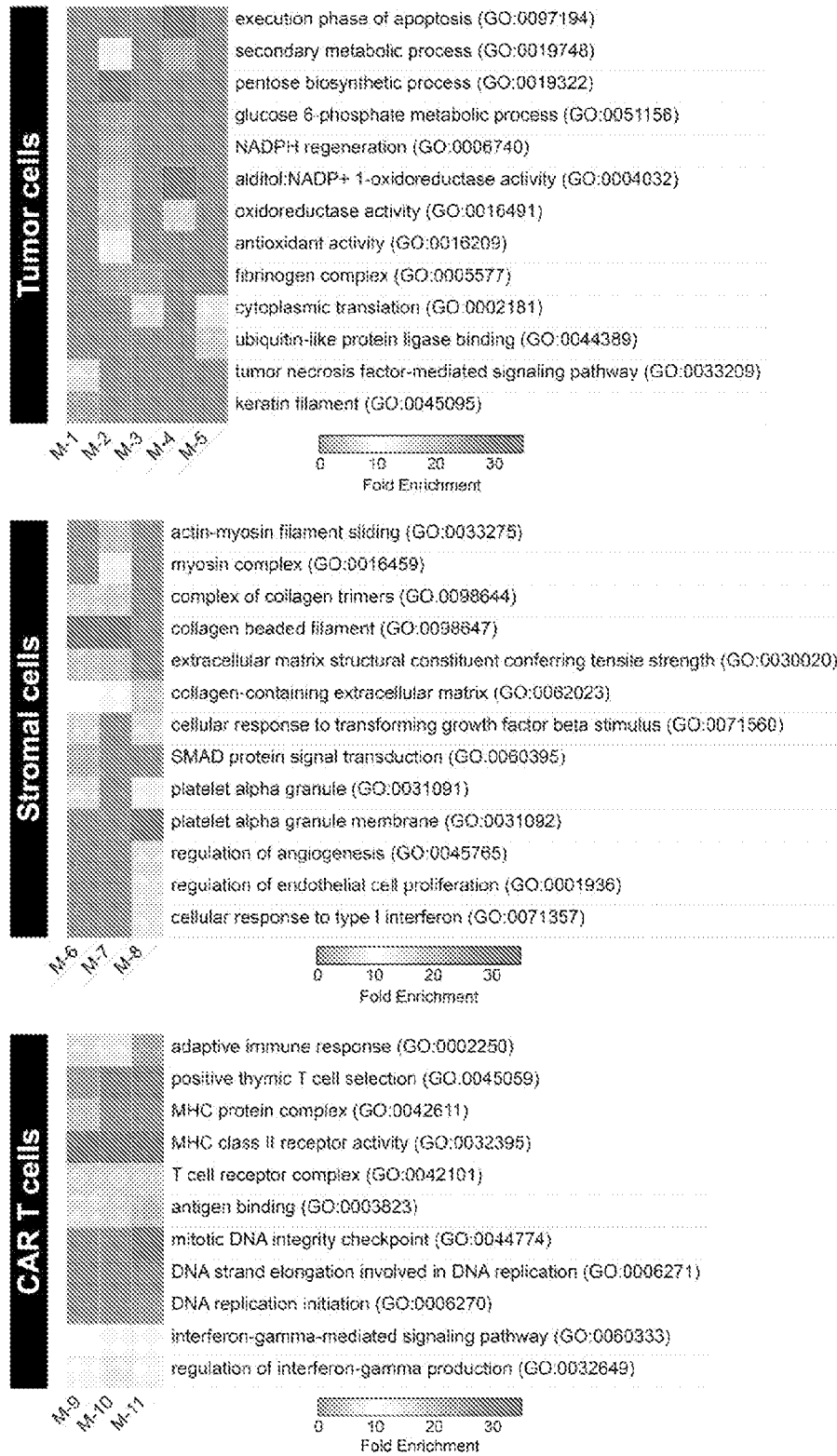


FIG. 25

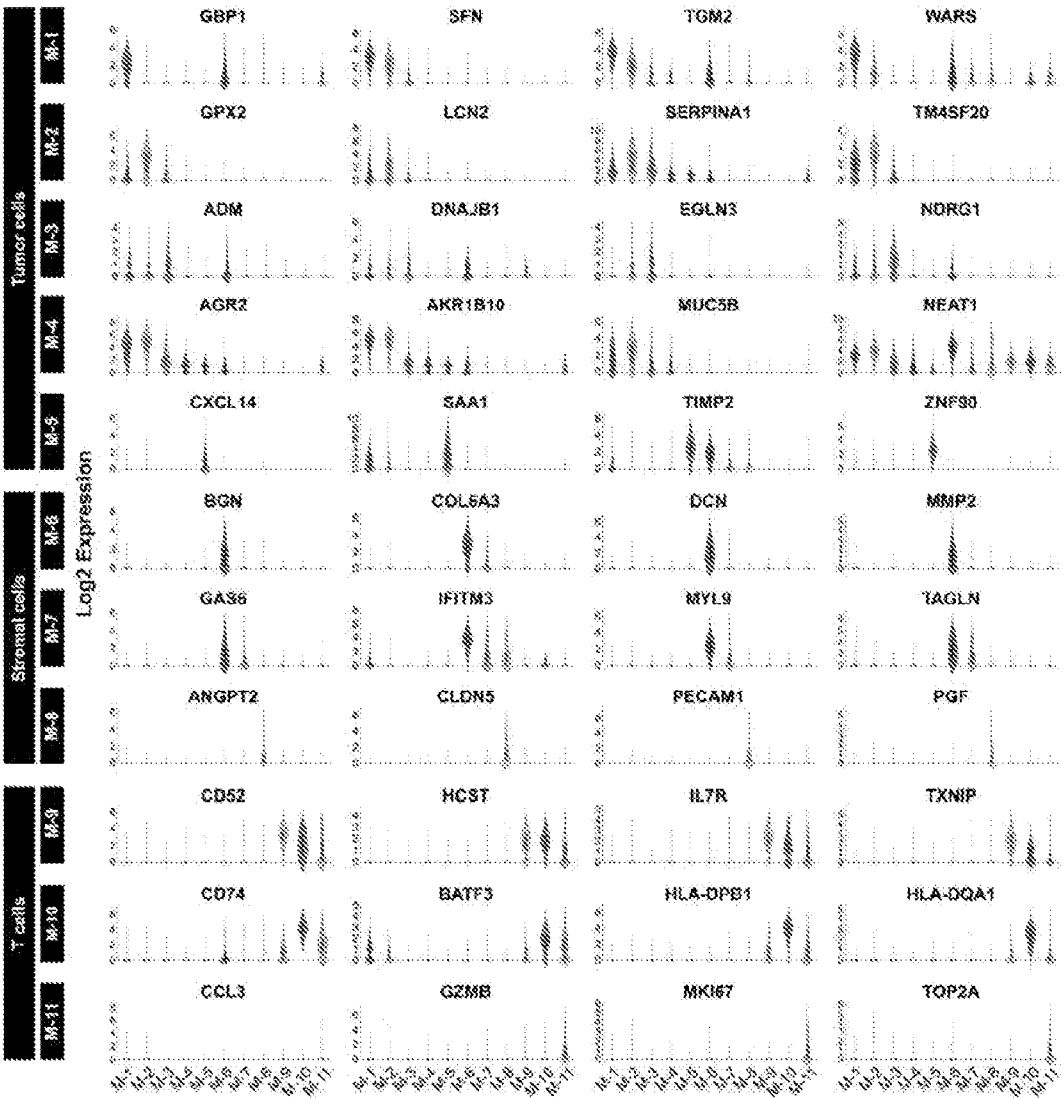


FIG. 26

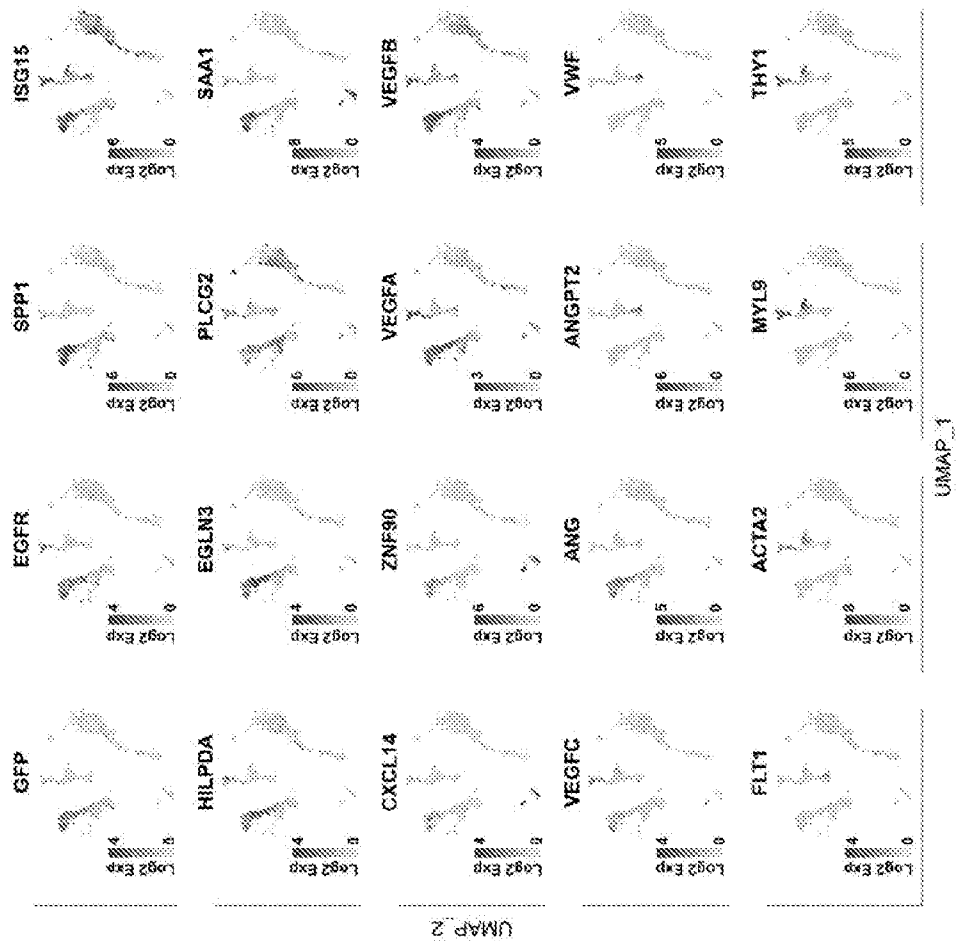


FIG. 27

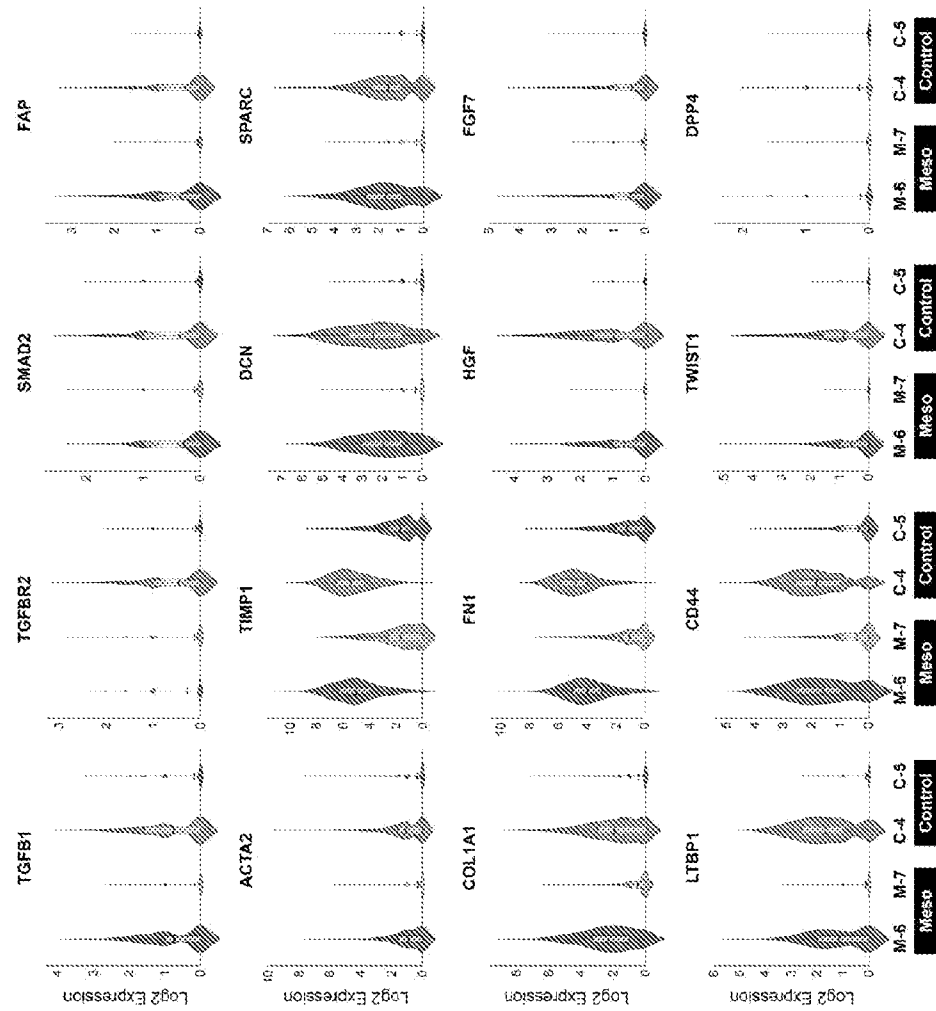


FIG. 28

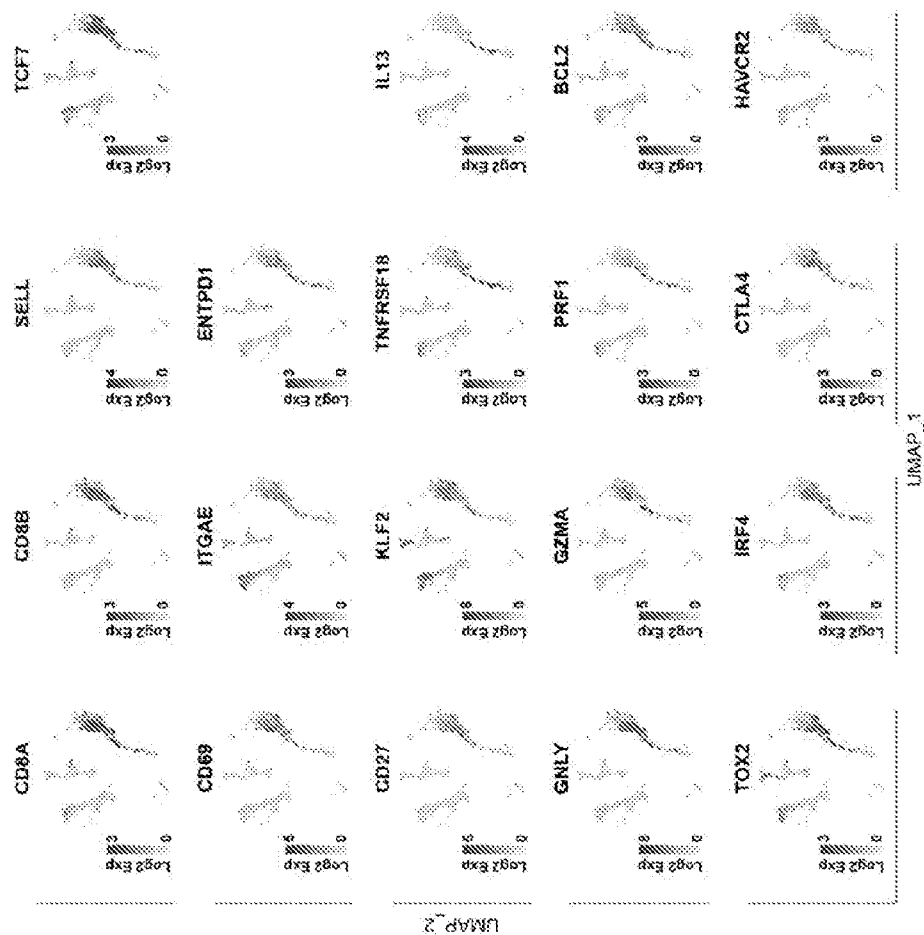


FIG. 29

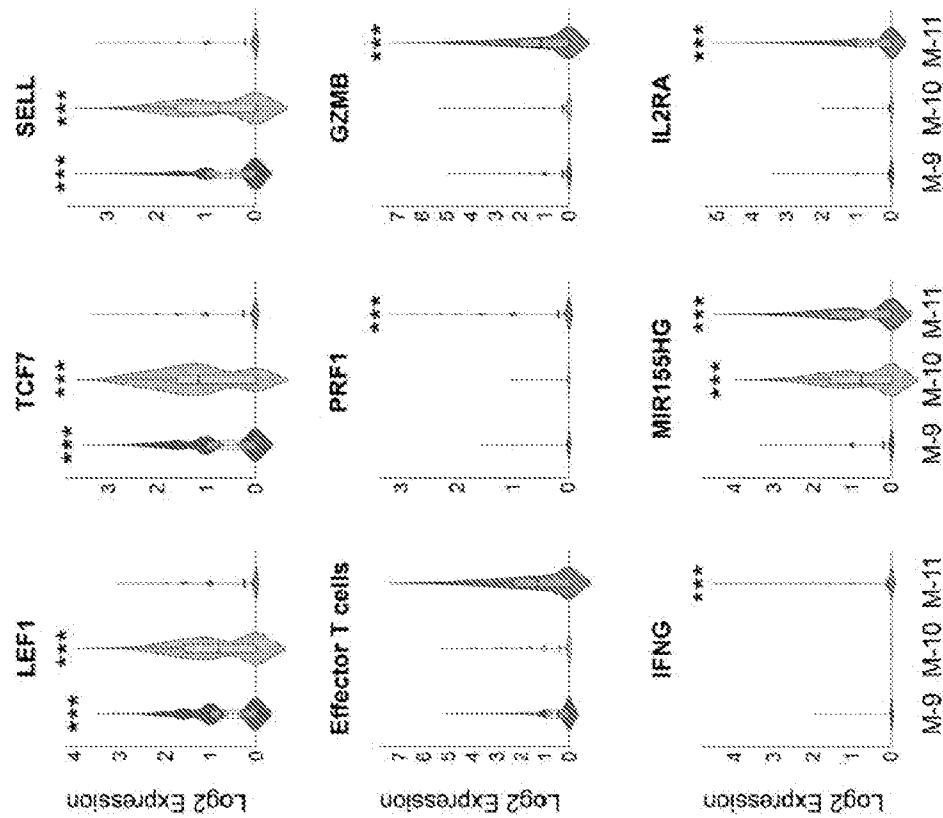


FIG. 30

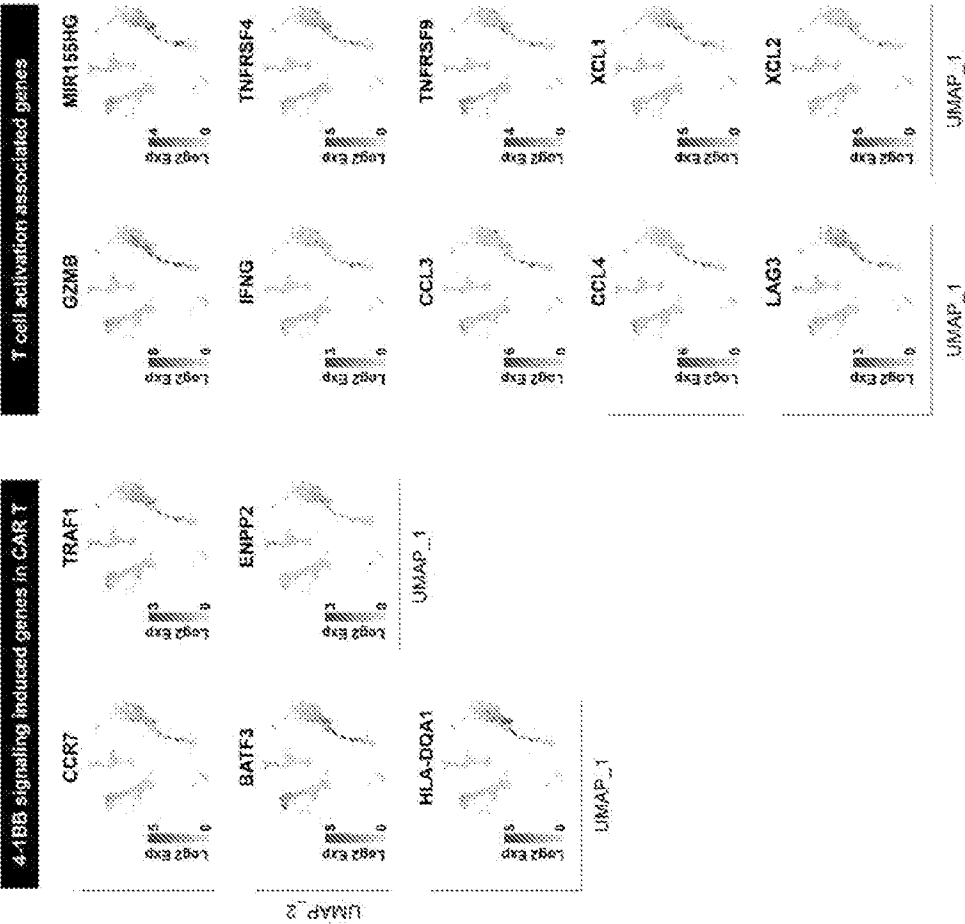


FIG. 31

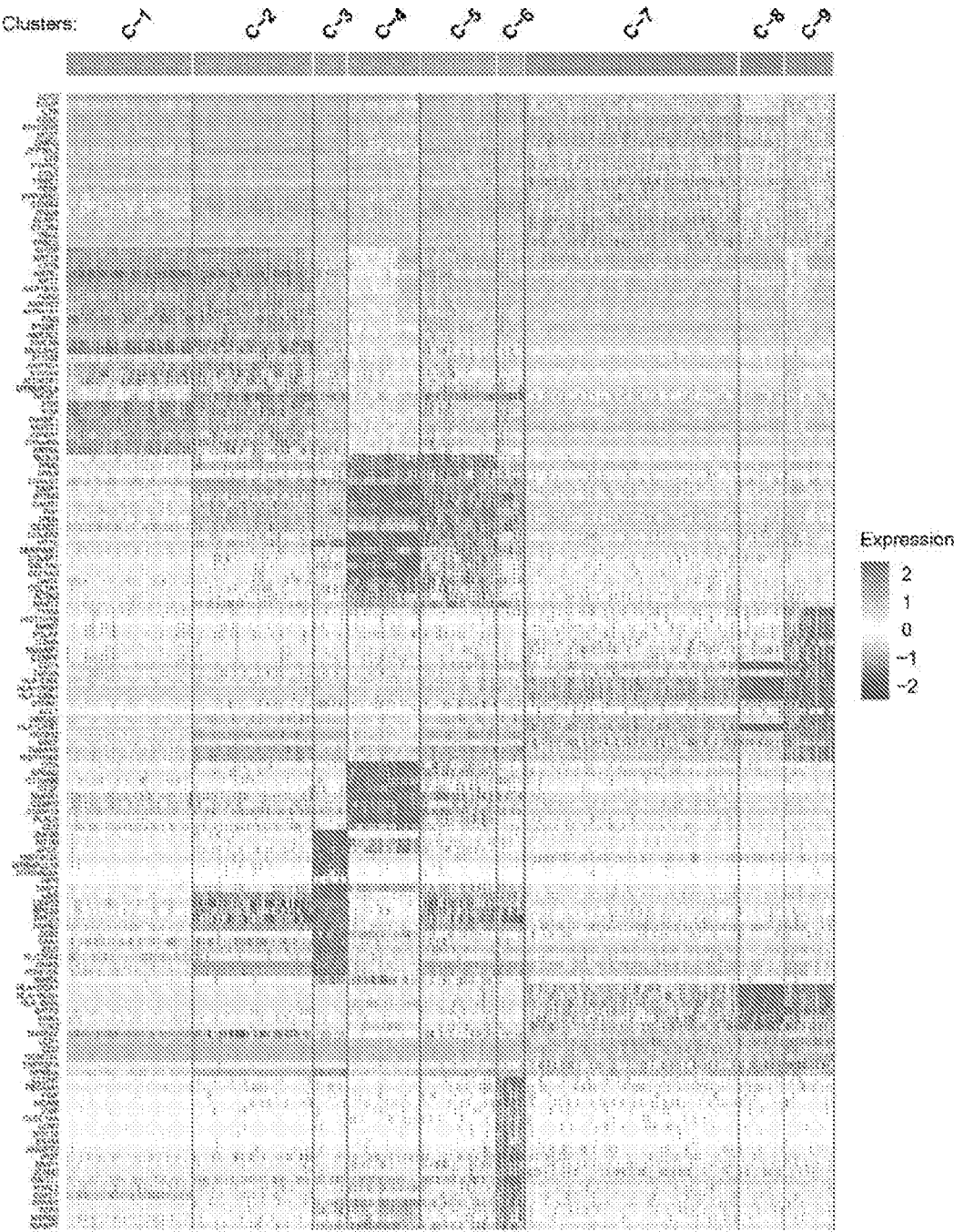


FIG. 32

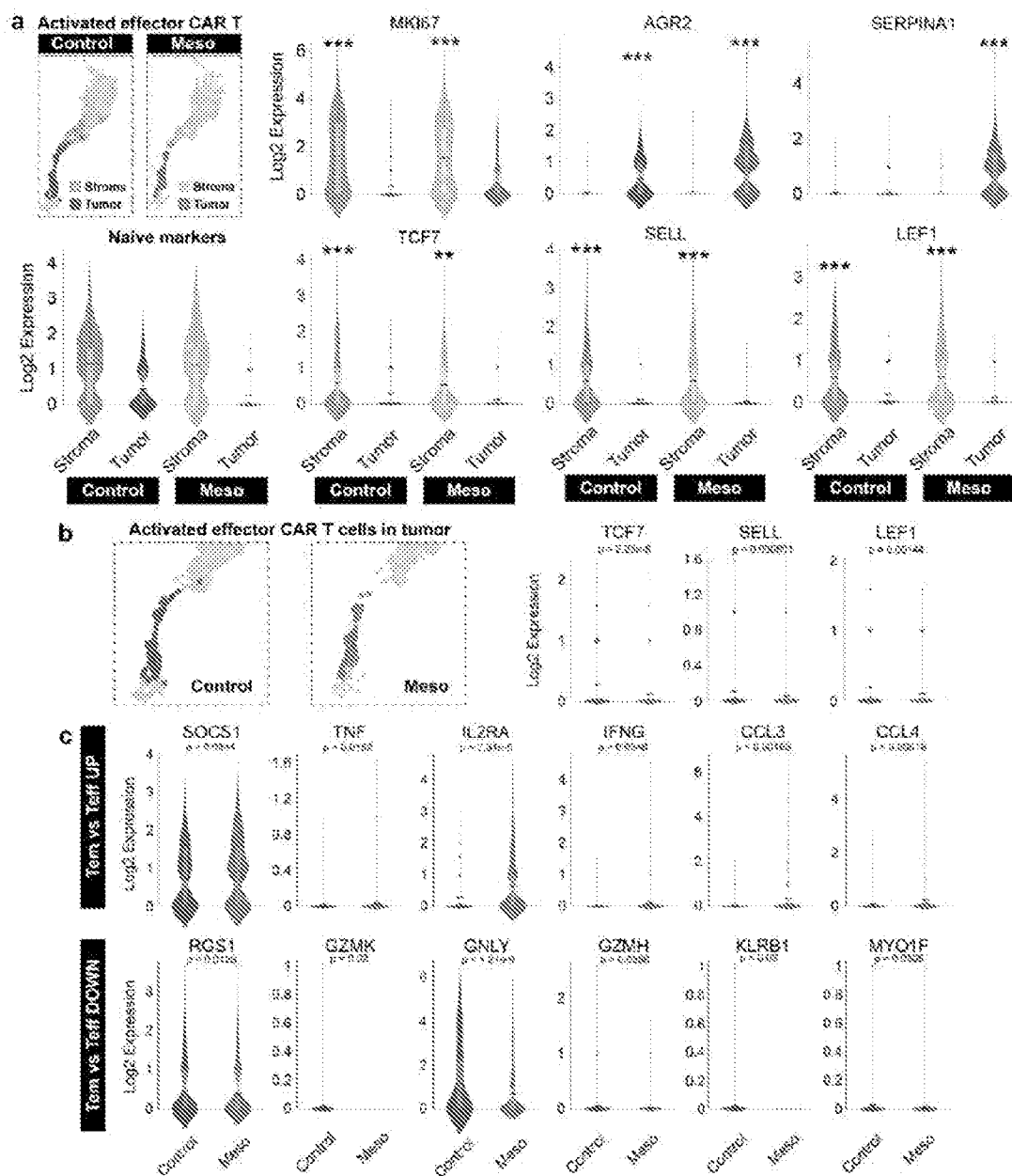


FIG. 33

◆ Stimulated by IFN-γ ◆ Survival ◆ Hypoxic ◆ Apoptosis resistant ◆ Apoptotic

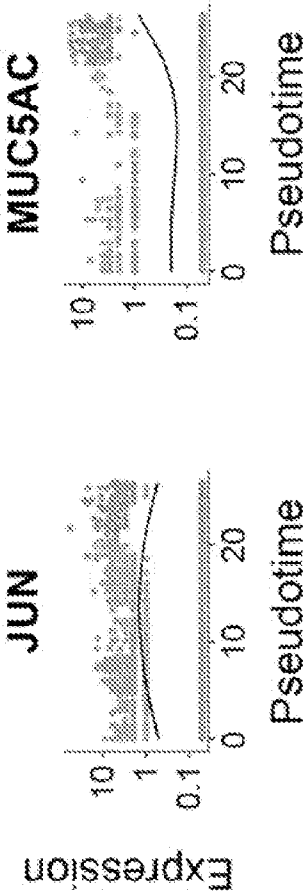


FIG. 34

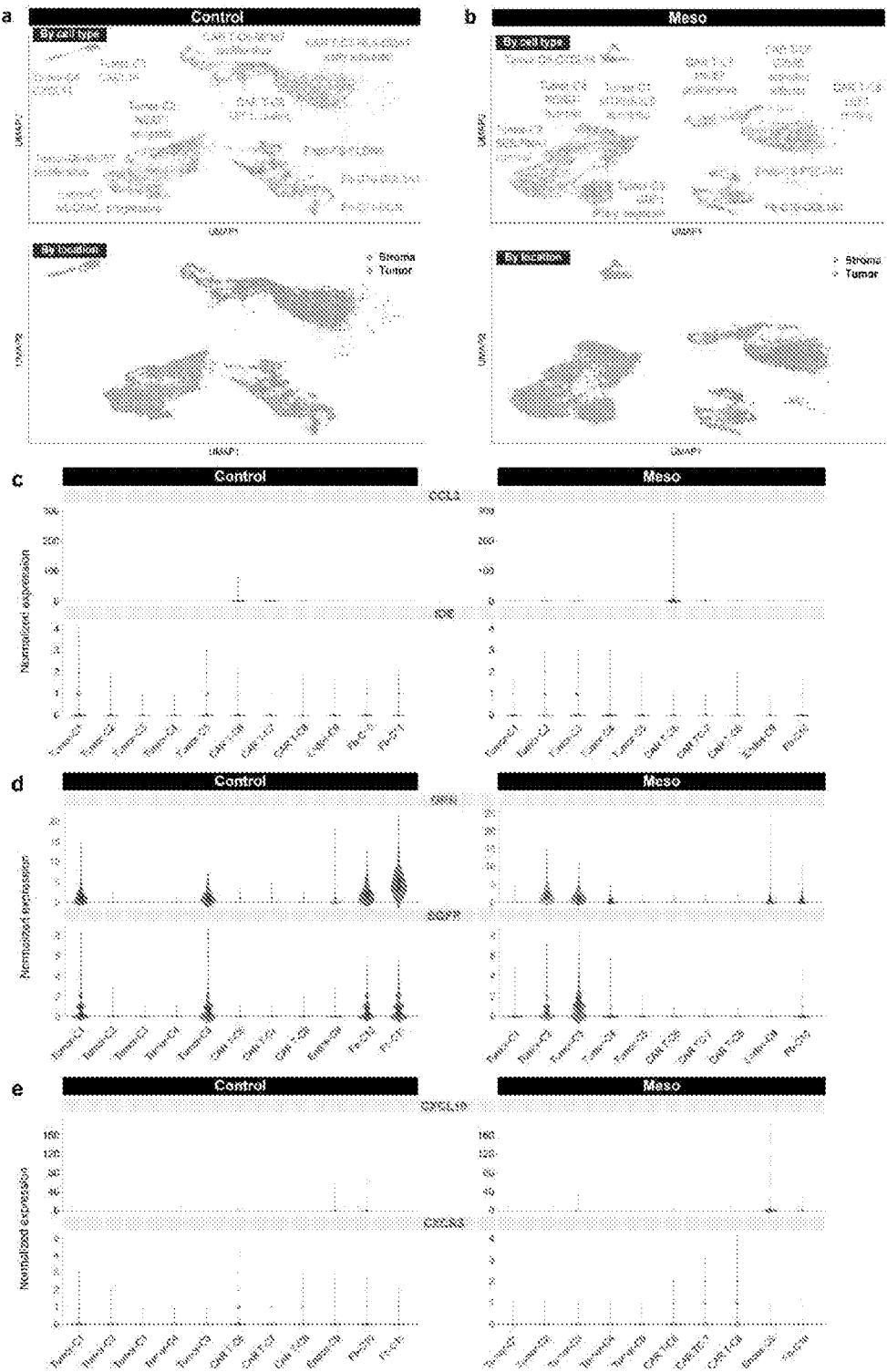
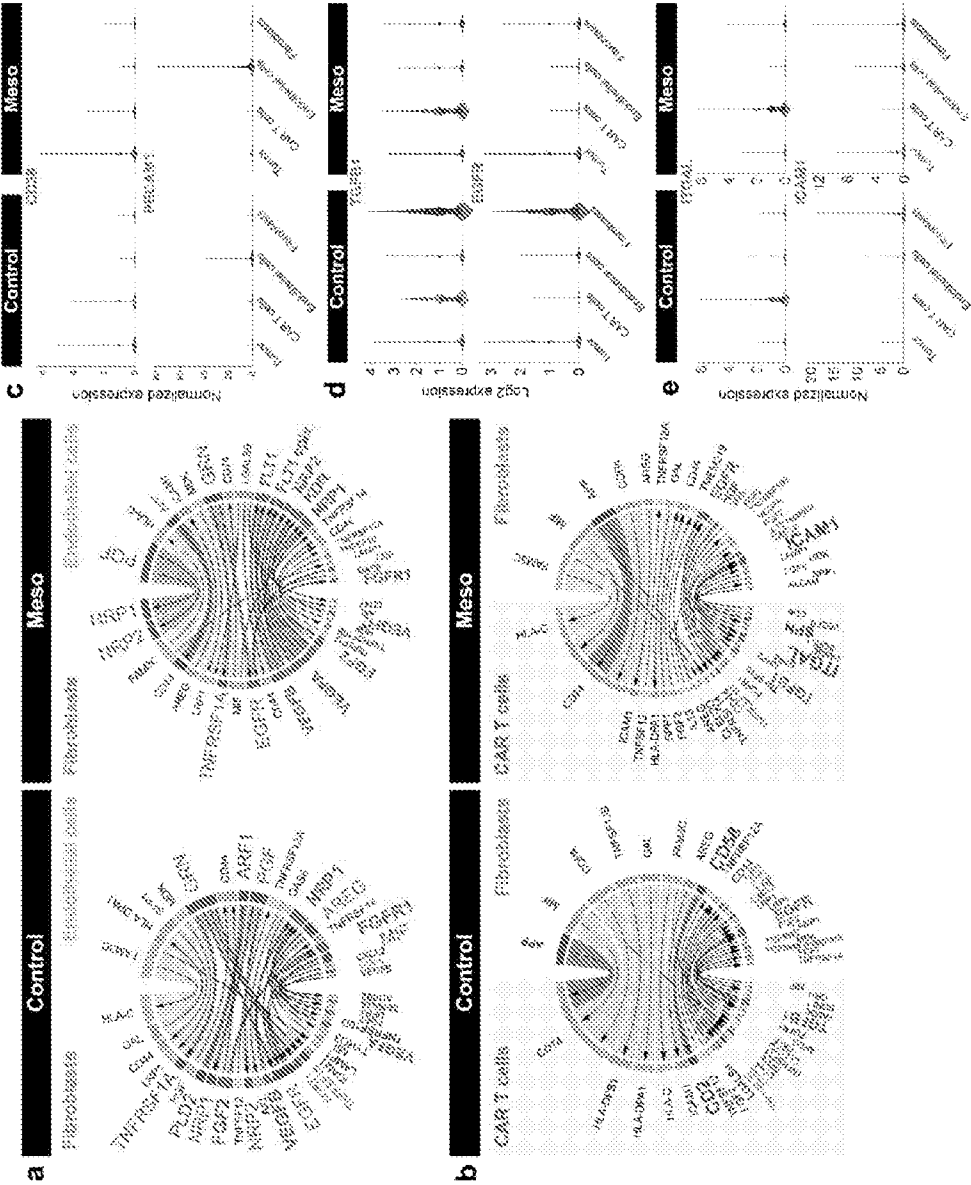


FIG. 35



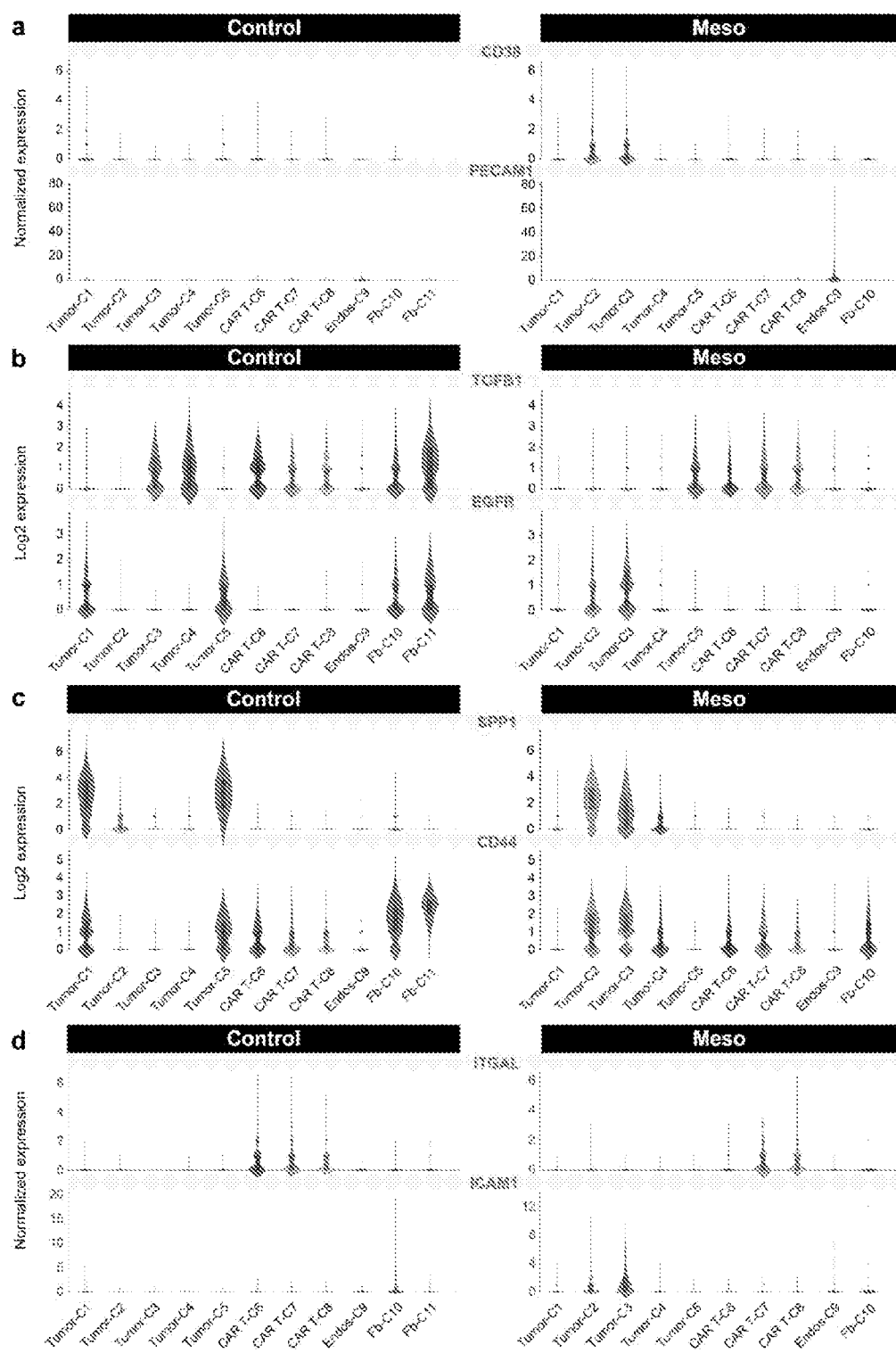
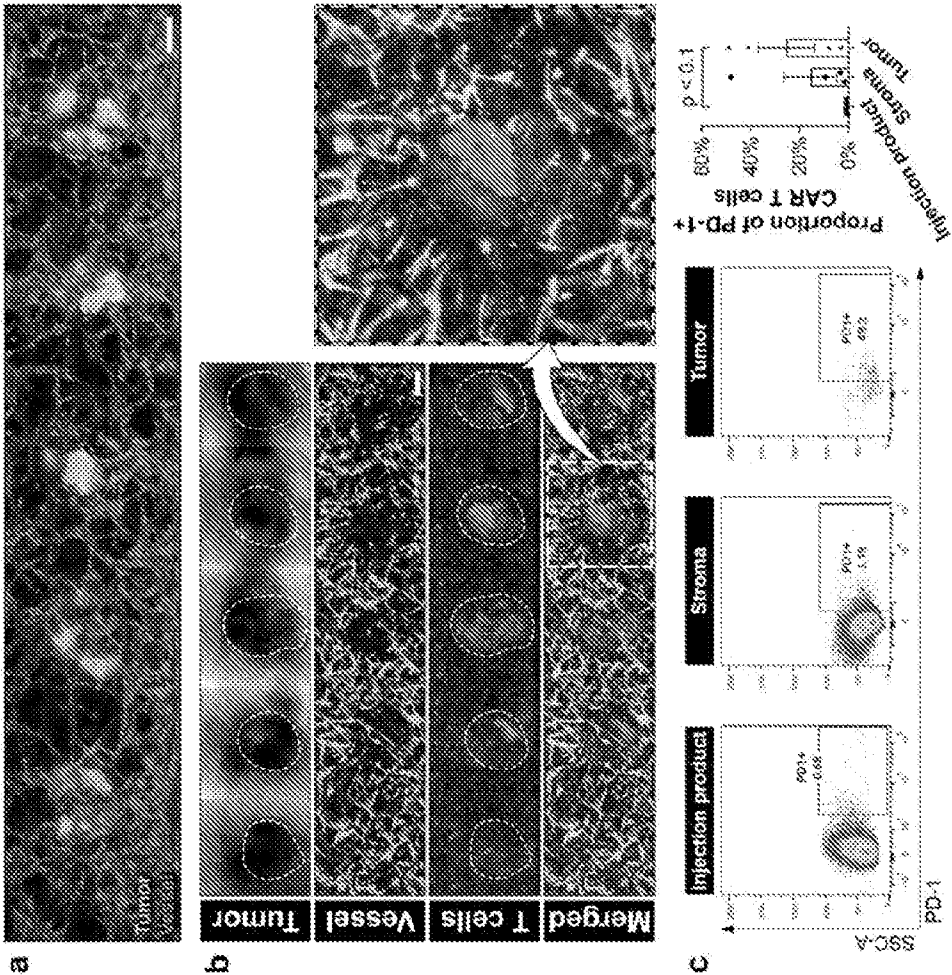


FIG. 37



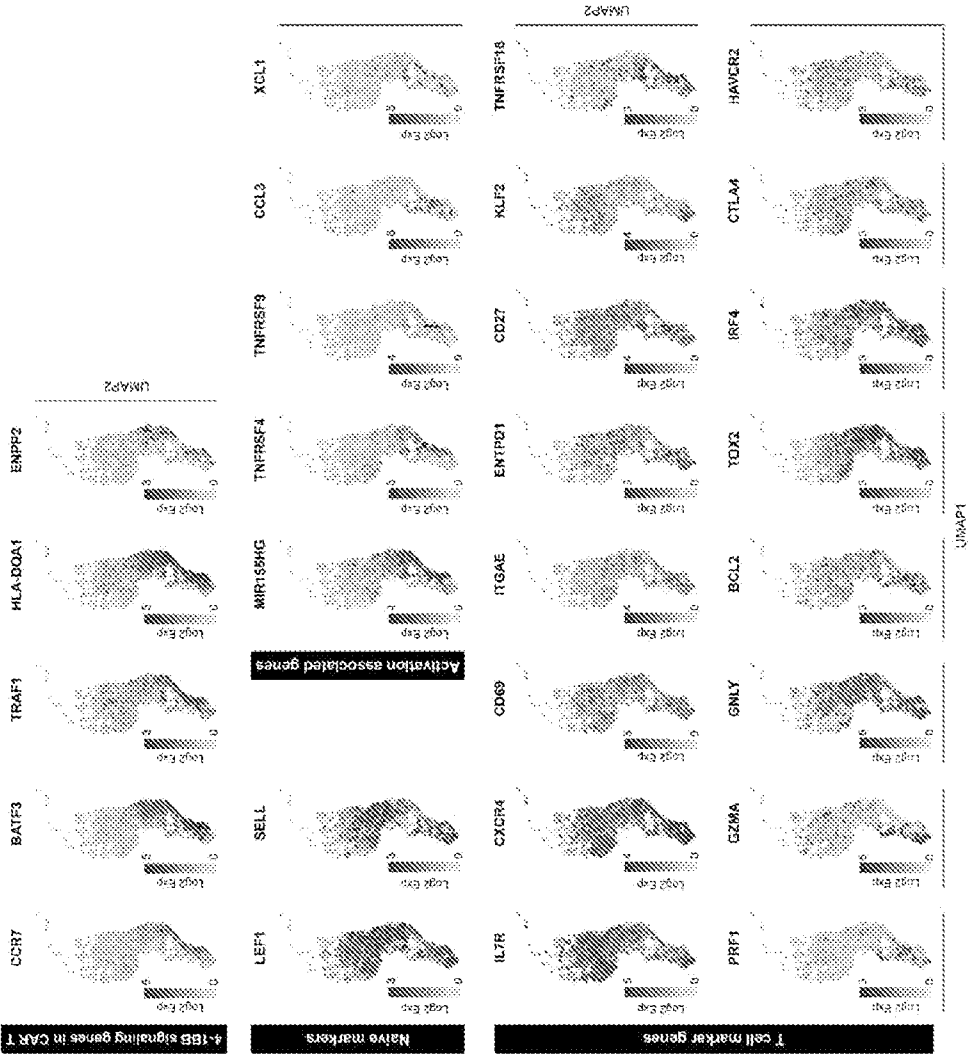


FIG. 39

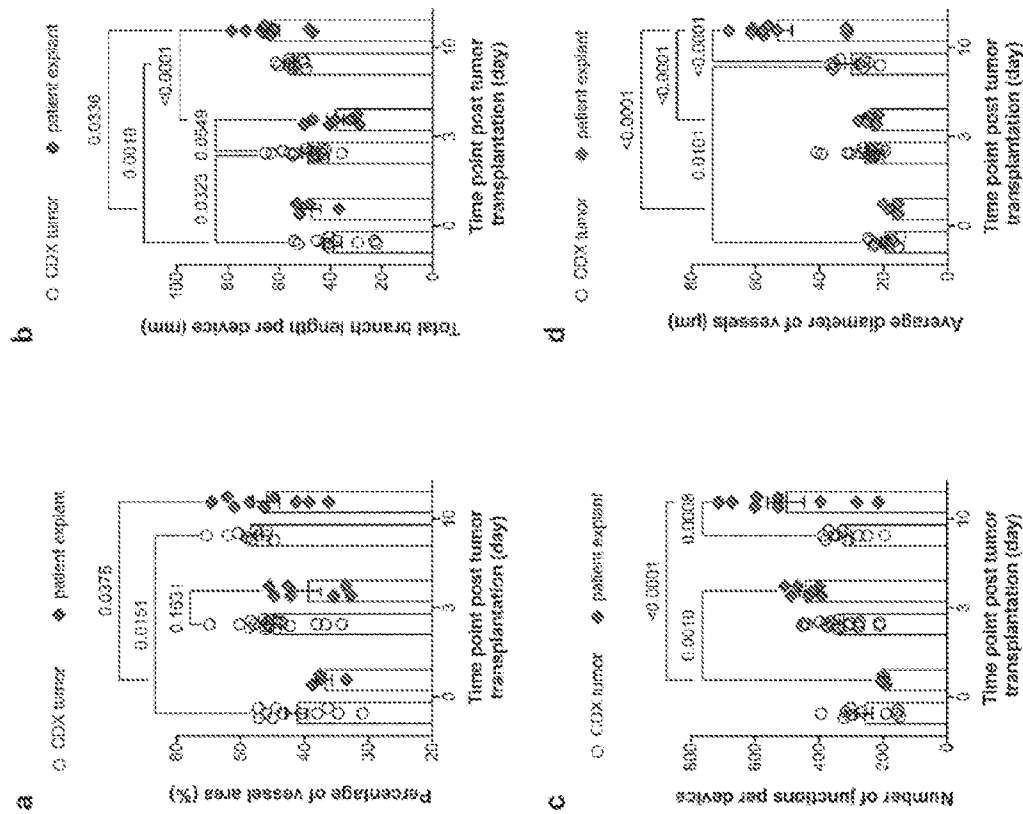


FIG. 40

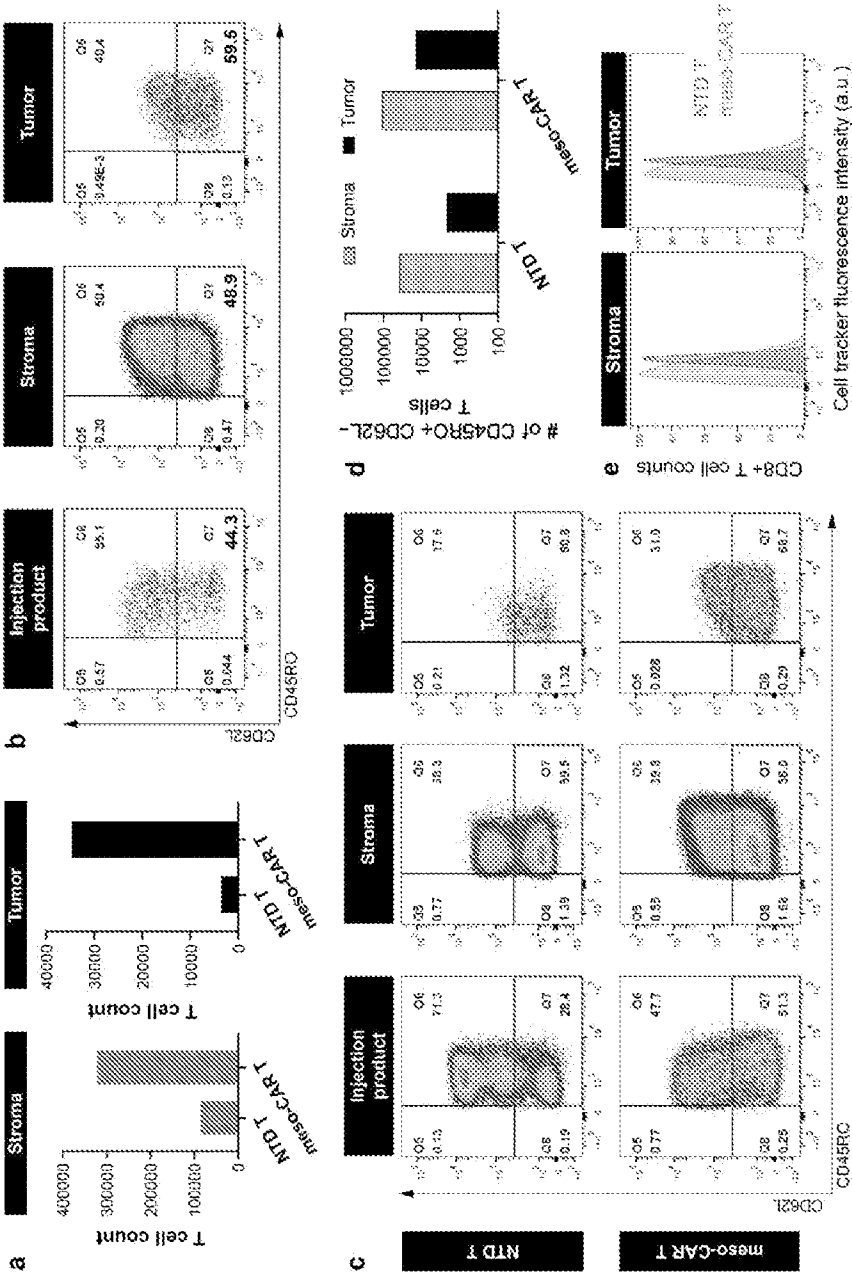


FIG.41

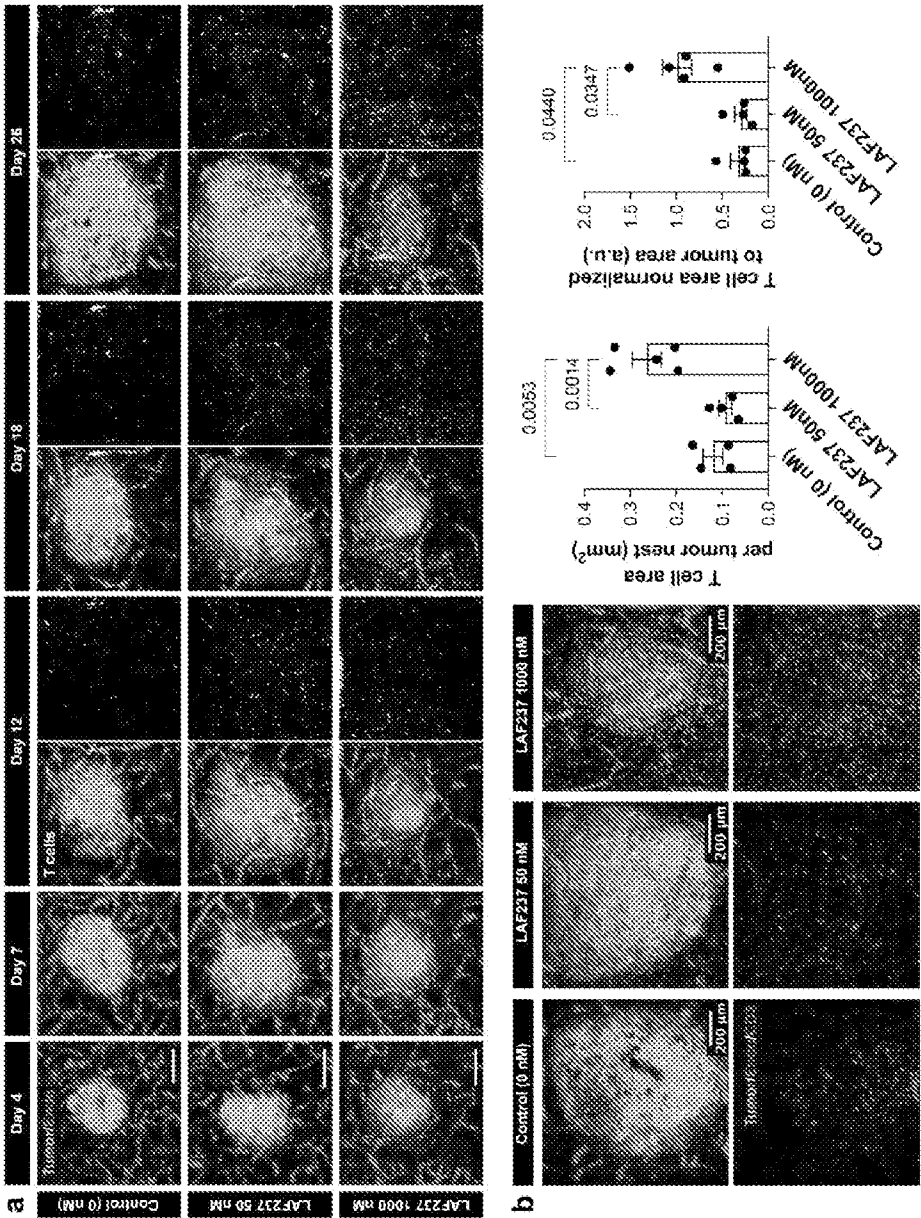


FIG. 42

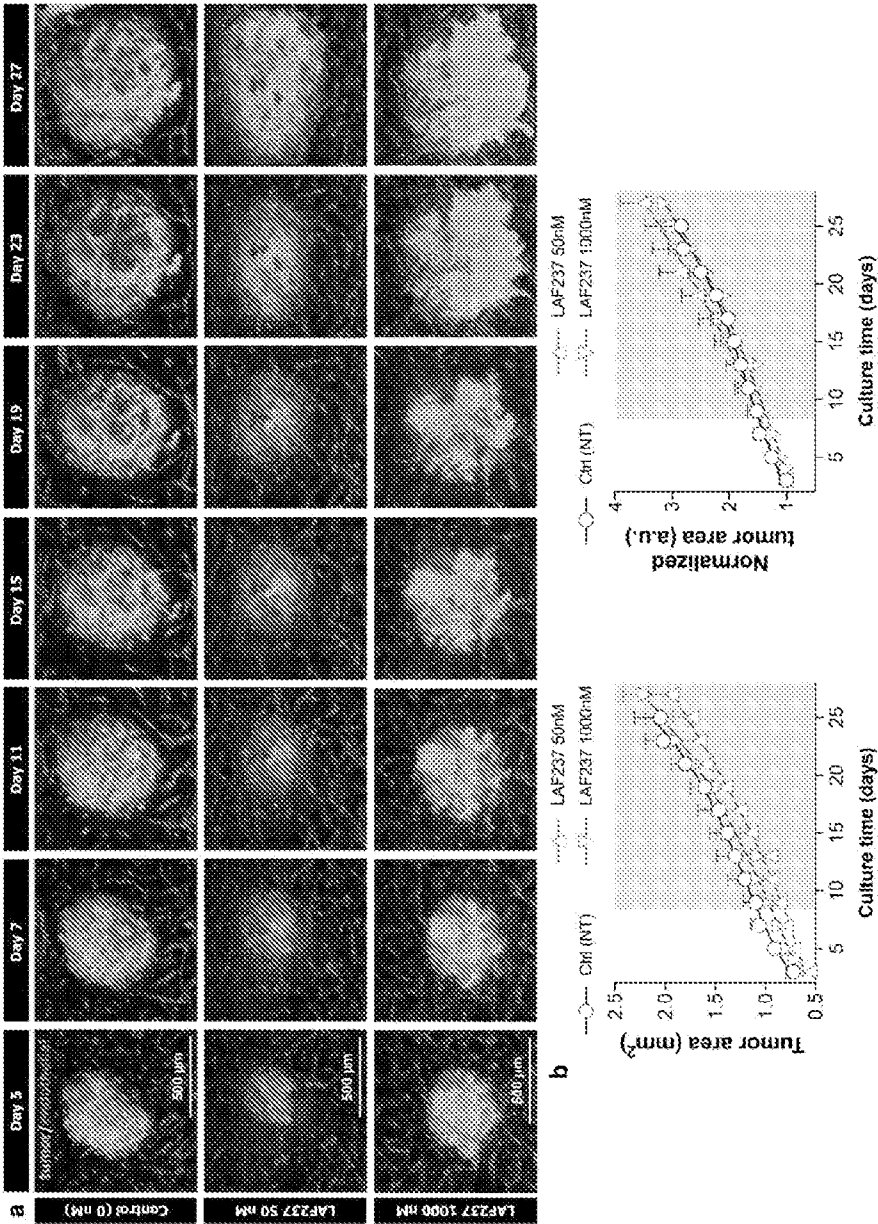


FIG. 43

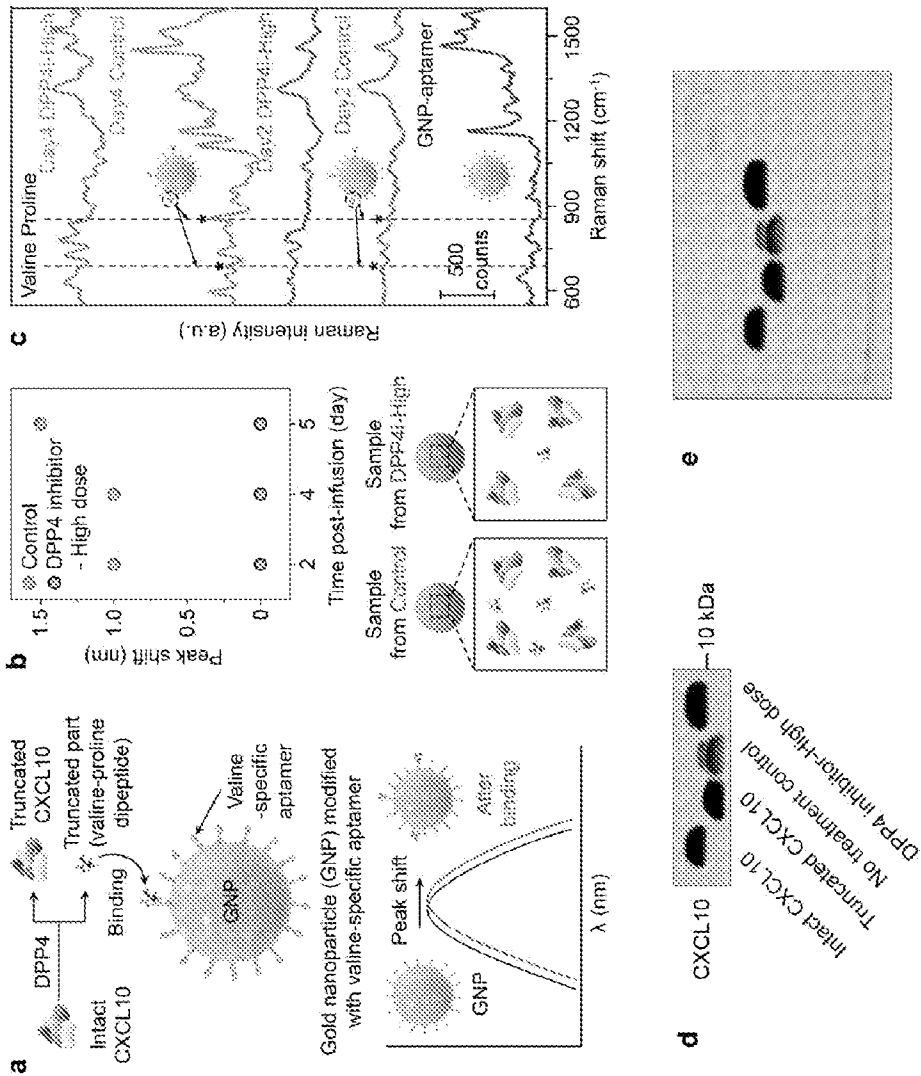


FIG. 44

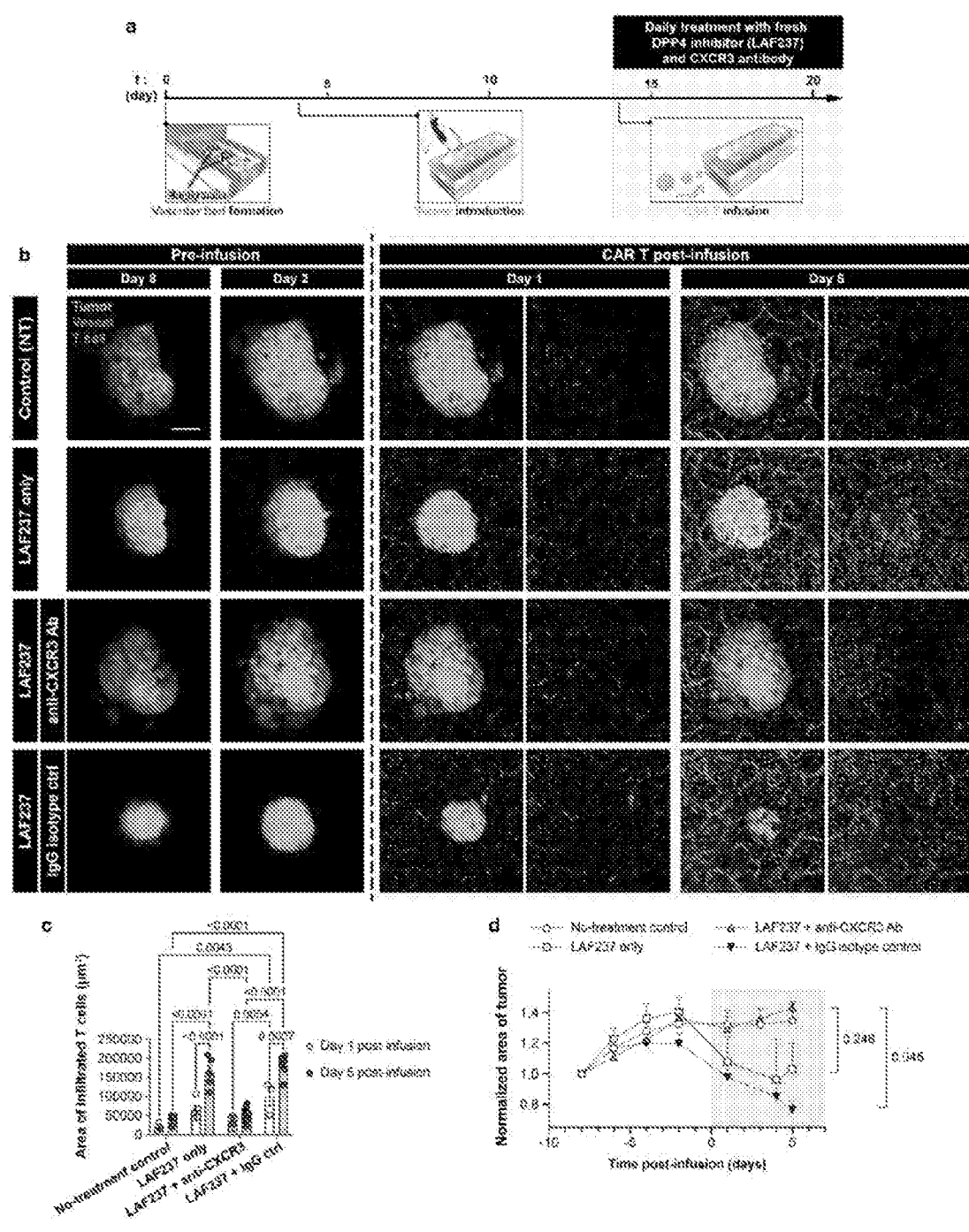
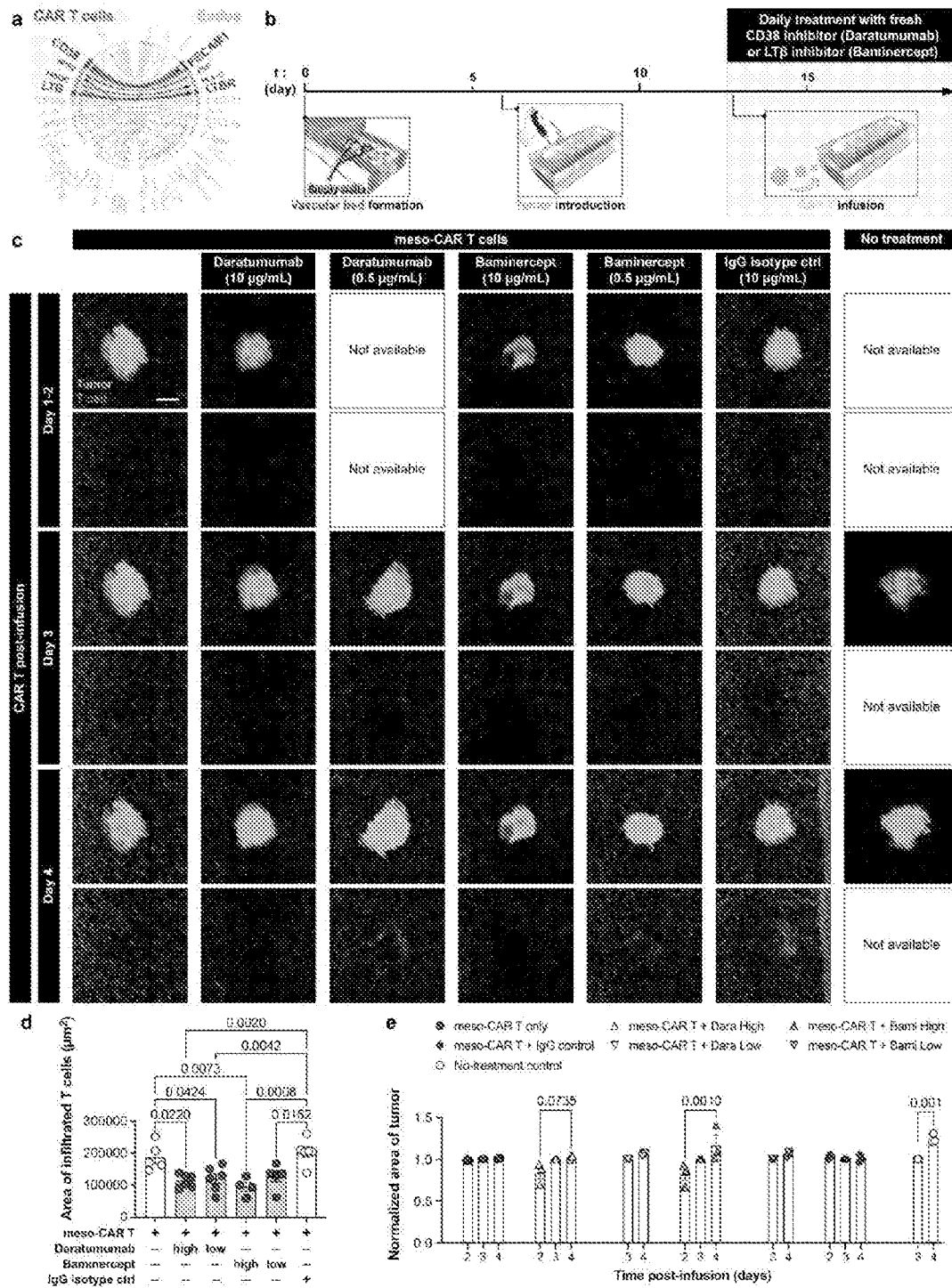


FIG. 45



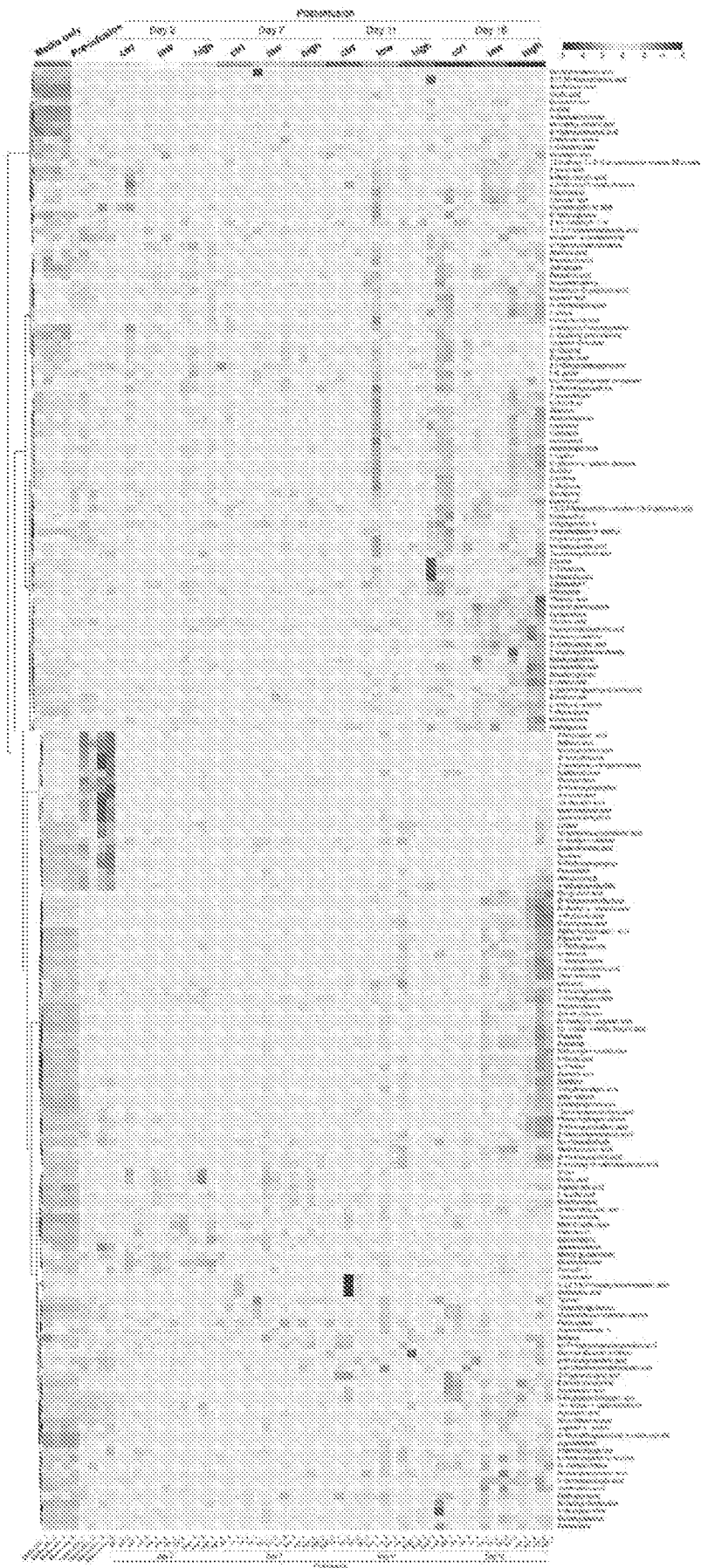


FIG. 47

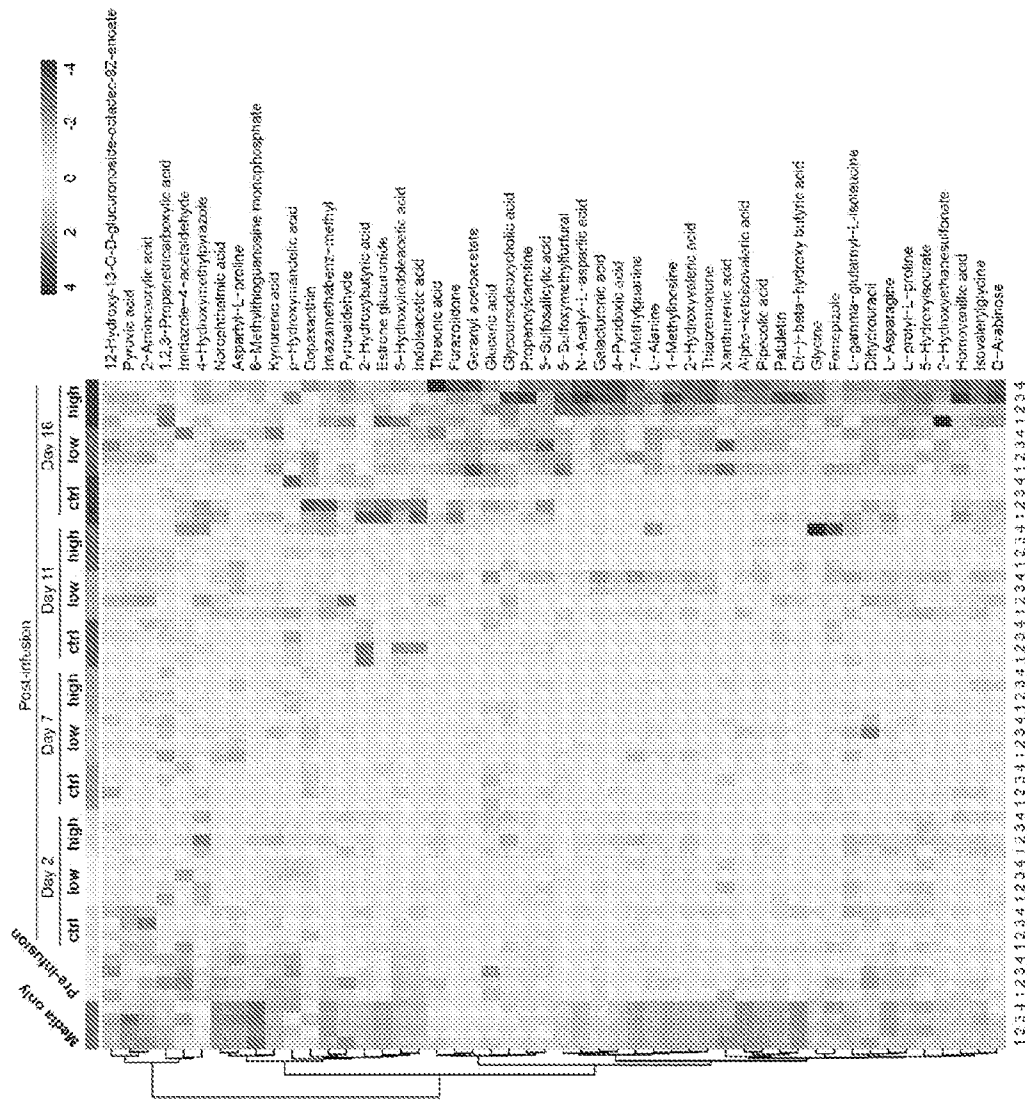


FIG. 48

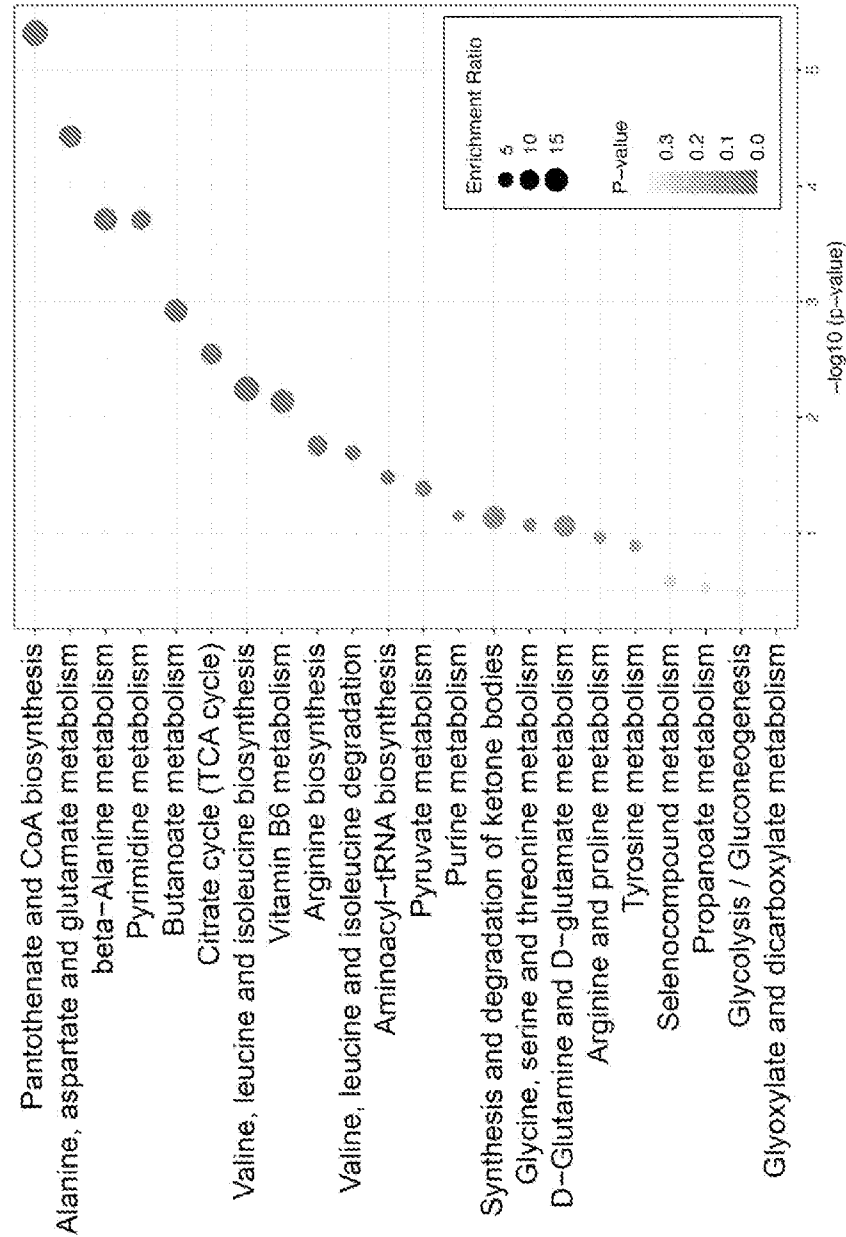


FIG. 49

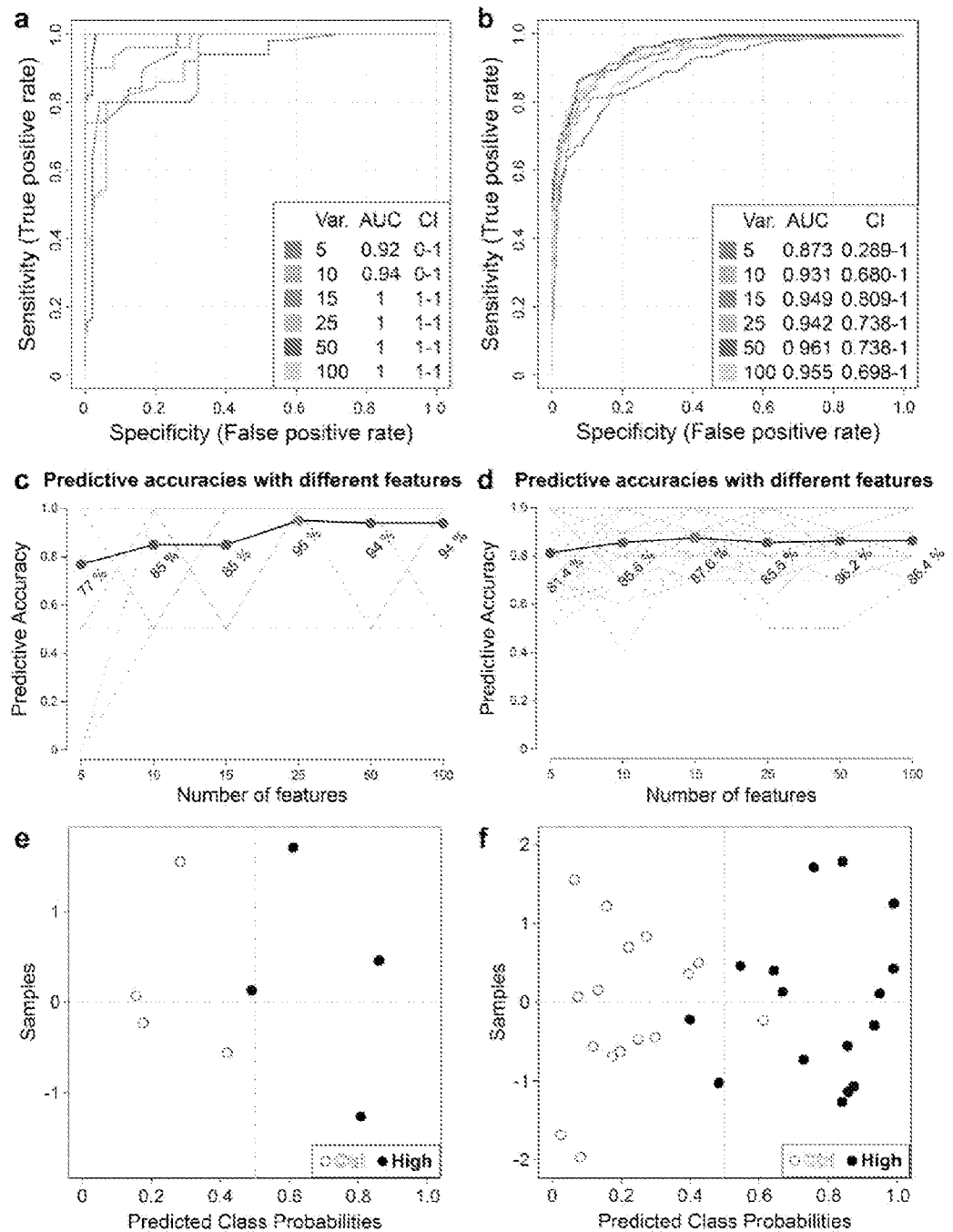


FIG. 50

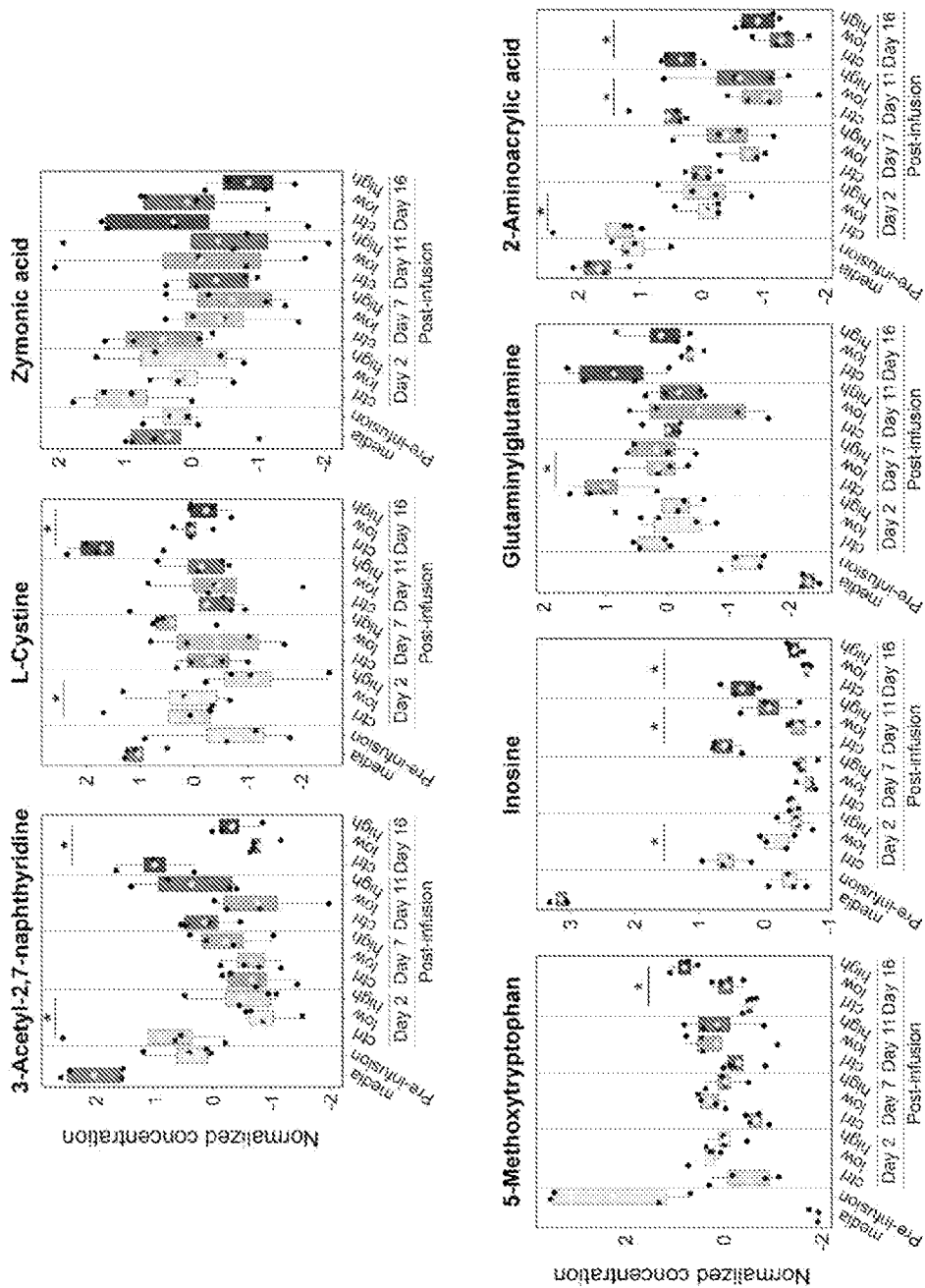


FIG. 51

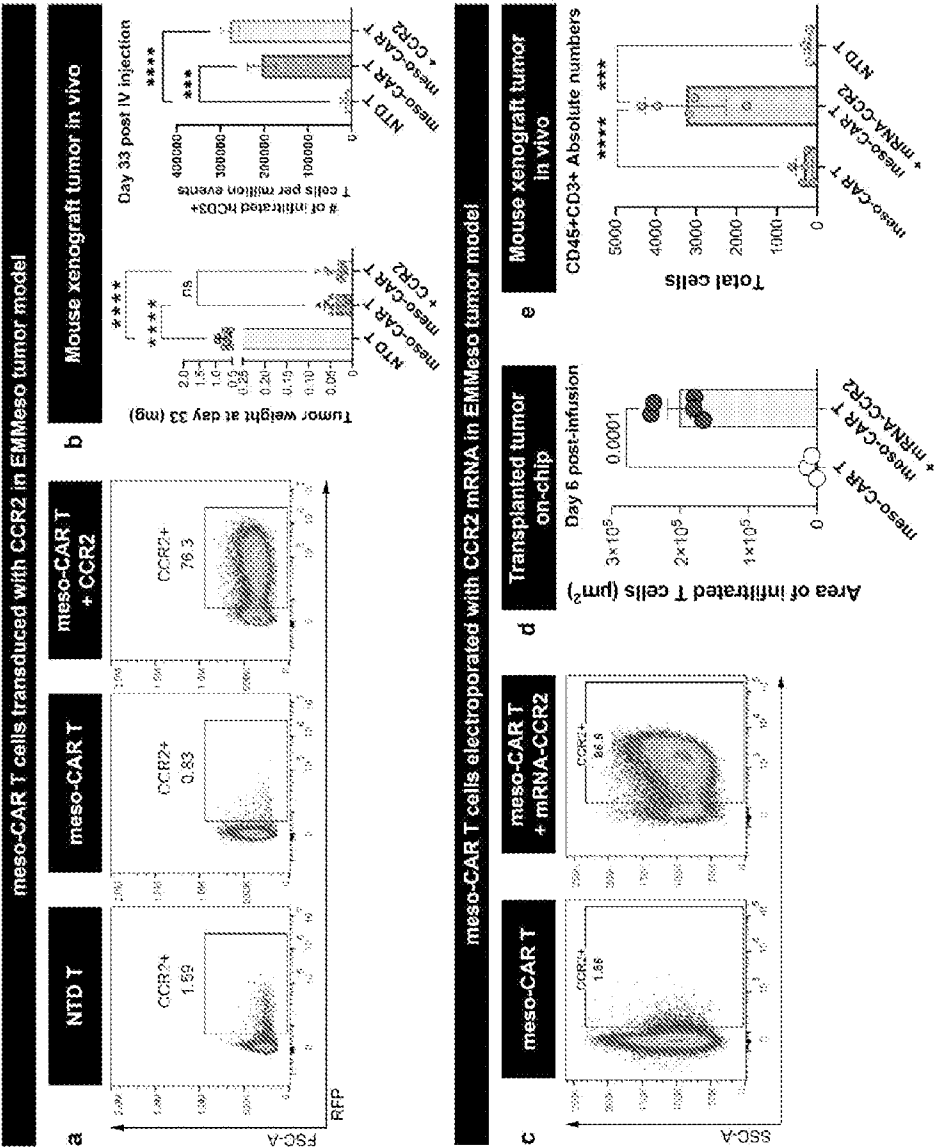


FIG. 52

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/062077

A. CLASSIFICATION OF SUBJECT MATTERIPC: *C12M 1/00* (2025.01); *C12M 1/12* (2025.01)CPC: *C12M 21/08*; *C12M 23/16*; *C12M 29/10*; *C12M 25/14*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

See Search History Document

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

See Search History Document

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

See Search History Document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2023/0159872 A1 (President and Fellows of Harvard College)25 May 2023 (25.05.2023) Entire Document	1-4
A	US 2017/0088807 A1 (Board of Regents, The University of Texas System)30 March 2017 (30.03.2017) Entire Document	1-4
A	US 2014/0186414 A1 (Ingber et al.)03 July 2014 (03.07.2014) Entire Document	1-4
X	Paek, et al. "Microphysiological engineering of self-assembled and perfusable microvascular beds for the production of vascularized three-dimensional human microtissues." ACS nano 13.7 (13 June 2019): 7627-7643. Abstract, pg 7629 col 1 para 2 – col 2 para 1; pg 7630 col 1 para 1; Figure 1; Figure 2.	1-4



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

“A” document defining the general state of the art which is not considered to be of particular relevance

“D” document cited by the applicant in the international application

“E” earlier application or patent but published on or after the international filing date

“L” document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

“O” document referring to an oral disclosure, use, exhibition or other means

“P” document published prior to the international filing date but later than the priority date claimed

“T” later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

“X” document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

“Y” document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

“&” document member of the same patent family

Date of the actual completion of the international search

26 February 2025 (26.02.2025)

Date of mailing of the international search report

30 April 2025 (30.04.2025)

Name and mailing address of the ISA/US

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Telephone No. PCT Help Desk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US2024/062077

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☒ Claims Nos.: **5-15, 20-25, 30**
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1.

Group I: Claims 1-4, directed to a microfluidic chip configured as an open-top chamber.

Group II: Claims 16-19, directed to a microfluidic chip comprising a vascular bed crossing a separator.

Group III: Claims 26-29, directed to a method comprising placing solid tumor tissue in a primary channel.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.: **1-4**

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.