Research Article

Lung Tumor Microphysiological System with 3D Endothelium to Evaluate Modulators of T-Cell Migration

Katrina M. Wisdom1, Johnny Suijker2, Lenie van den Broek2, BanuPriya Sridharan1, Taraka Sai Pavan Grandhi1, Aaron Cheng3, Mahdi Lamb4, Steven A. Titus5, Derek Poore5, Niyant Shah5, Shih-Hsun Cheng1, Edward Kim6, Sue Griffin6, and Jason Ekert7

1In Vitro In Vivo Translation, Research, Pharmaceutical R&D, GSK, Collegeville, PA, USA; 2Mimetas B.V., Oegstgeest, The Netherlands; 3Genomic Sciences, Research, Pharmaceutical R&D, GSK, Collegeville, PA, USA; 4Genomic Sciences, Research, Pharmaceutical R&D, GSK, Stevenage, UK; 5Immuno-Oncology, Research, Pharmaceutical R&D, GSK, Collegeville, PA, USA; 6Immuno-Oncology, Research, Pharmaceutical R&D, GSK, Stevenage, UK

Abstract

Lung cancer is a leading cause of death worldwide, with only a fraction of patients responding to immunotherapy. The correlation between increased T-cell infiltration and positive patient outcomes has motivated the search for therapeutics promoting T-cell infiltration. While transwell and spheroid platforms have been employed, these models lack flow and endothelial barriers, and cannot faithfully model T-cell adhesion, extravasation and migration through 3D tissue. Presented here is a 3D chemotaxis assay, in a lung tumor-on-chip model with 3D endothelium (LTOC-Endo), to address this need. The described assay consists of a HUVEC-derived vascular tube cultured under rocking flow, through which T-cells are added; a collagenous stromal barrier, through which T-cells migrate; and a chemoattractant/tumor (HCC0827 or NCI-H520) compartment. Here, activated T-cells extravasate and migrate in response to gradients of rhCXCL11 and rhCXCL12. Adopting a T-cell activation protocol with a rest period enables proliferative burst prior to introducing T-cells into chips and enhances assay sensitivity. In addition, incorporating this rest recovers endothelial activation in response to rhCXCL12. As a final control, we show that blocking ICAM-1 interferes with T-cell adhesion and chemotaxis. This microphysiological system, which mimics in vivo stromal and vascular barriers, can be used to evaluate potentiation of immune chemotaxis into tumors while probing for vascular responses to potential therapeutics. Finally, we propose translational strategies by which this assay could be linked to preclinical and clinical models to support human dose prediction, personalized medicine, and the reduction, refinement, and replacement of animal models.

1 Introduction

Although immunotherapy has shown great promise, immune cell infiltration in many indications and/or sub-indications remains challenging, leading to mixed clinical outcomes (Oelkrug and Ramage, 2014; Zhang et al., 2019; van der Woude et al., 2017). Patients with “inflamed” tumors, in which immune cells are inhibited but in close contact with tumor cells, typically respond better to cancer immunotherapy and experience poorer outcomes if their tumors are “immune excluded”, in which cytotoxic T-cells have accumulated in the tumor stroma but are not able to reach the tumor cells, or “immune desert”, in which cytotoxic T-cells are absent from both the tumor nest and stroma (Hegde and Chen, 2020; Tokito et al., 2016). By contrast, patients experience poorer outcomes if their tumors are “immune excluded”, in which cytotoxic T-cells have accumulated in the tumor stroma but are not able to reach the tumor cells, or “immune desert”, in which cytotoxic T-cells are absent from both the tumor nest and stroma (Hegde and Chen, 2020; Tokito et al., 2016). Numerous factors, such as lack of chemokine gradients, reduced integrin activation, increased density of tumor stroma, and abnormal vasculature, as well as immune-suppressive soluble factors and immune cells (e.g. regulatory T-cells) are hypothesized to contribute to T-cell exclusion from the tumor microenvironment (Oelkrug and Ramage, 2014; Zhang et al., 2019; van der Woude et al., 2017; Tokito et al., 2016; Chen and Mellman, 2013). Given that a...
high presence of cytotoxic T-cells in tumors is correlated with improved patient survival, there is a strong need to enhance T-cell migration into the 3D tumor microenvironment and infiltration into solid tumors, thereby enhancing the effectiveness of immunotherapies (Oelkrug and Ramage, 2014; Zhang et al. 2019; van der Woude et al., 2017; Tokito et al., 2016; Chen and Mellman, 2013). Despite the clear rationale to address this aspect of the cancer-immunity cycle, there are limited potential therapeutics available to address it (Chen and Mellman, 2013).

While preclinical *in vivo* models have ushered in pivotal treatments in cancer immunotherapy (e.g., anti-CTLA-4 and anti-PD-(L)1), the limited translatability of preclinical models is a key challenge for the development of many immunotherapies (Hegde and Chen, 2020). Genetically engineered mouse models are considered to be the closest representation of human cancers, but mechanistic studies are challenging in whole animal models, and differences in species-specific immunology and disease progression hamper their clinical translatability (Hegde and Chen, 2020; Metstas and Hughes, 2004). Furthermore, increasing global attention on ethical issues with animal research has bolstered support for initiatives to refine, reduce, and replace animal models (Levy, 2012).

*In vitro*, Transwell migration systems have been employed to investigate modulators of cell migration and chemotaxis. However, the effects of chemotactic triggers on migrating cells over long time windows remains challenging in these platforms due to gravity and gradient instability (Sip et al., 2014; Boyd, 1962; Zhang et al., 2016). Furthermore, these platforms are unable to recapitulate some aspects of the tumor microenvironment. Transwell membranes with rigid pores are unable to model dynamic cell extravasation through living, responsive vasculature or 3D cell migration through viscoelastic and mechanically plastic pores of extracellular matrix (Wisdom et al., 2018). Furthermore, as chemotaxis takes place along the z-axis in these assays, large confocal z-stacks, which may be time and data intensive to acquire and process, may be necessary to obtain single cell-resolution migration information. Alternatively, 3D spheroids are valuable for modeling T-cell infiltration into tumor nests (Herter et al., 2017; Rodrigues et al., 2020; Booj et al., 2019). However, optical clearing is necessary in order to image inside spheroids beyond 200 μm, which can only be done as an endpoint analysis. Furthermore, spheroid assays do not always model the extracellular matrix of solid tumors, even though dense stromal matrix is known to physically prevent infiltration into human lung tumors (Salmon et al., 2012). Additionally, growing evidence suggests that T-cells exhibit distinct kinds of motility dependent on both their activation state and features of their microenvironment (i.e. migration through 3D ECM vs. tumor nest infiltration) (Krummel et al., 2016). For these reasons, infiltration studies with spheroids alone may not be sufficient to model the stromal constituents contributing to antitumor immunity in “immune excluded” and “immune desert” tumors.

While Transwell and spheroid models can be informative and high throughput, they lack a living endothelial barrier and vascular flow. For this reason, these platforms cannot be used to model extravasation, an early stage of T-cell chemotaxis into tumors. There is a need for an integrated complex *in vitro* model to investigate multiple stages of T-cell chemotaxis, including T-cell adhesion, extravasation, and migration through a 3D stromal barrier, to evaluate therapeutics that can enable T-cells to overcome these barriers and directly contact tumor cells. For maximum utility in drug discovery and development, it should be phenotypic-screening amenable, offering single cell resolution readouts without being time and data intensive to image or analyze.

Recent developments in organ-on-chip technologies have been encouraging, but many of these early models are low throughput, made of polydimethylsiloxane (PDMS) (a hydrophobic material known to nonspecifically adsorb proteins), and contain artificial membranes (de Haan et al., 2021). The MIMETAS 3-lane 40 Organoplate® is a platform containing 40 chips per plate, no PDMS, and phase guide technology, which enables membrane-free material separation. Recently, this platform was used to investigate monocyte-to-endothelium adhesion, neutrophilic migration, and 3D T-Cell chemotaxis in a melanoma model (de Haan et al., 2021). Building upon these models, we established a lung tumor-on-chip model with 3D endothelium (“LToC-Endo”) to investigate immune cell chemotaxis in response to chemokines and antibody treatments. Here we show that activated T-cells in the LToC-Endo model adhere, extravasate, and migrate in response to gradients of rhCXCL11 and rhCXCL12 (referred to throughout the manuscript as “CXCL11” and “CXCL12”). The model can simultaneously discriminate between angiogenic (CXCL12) and non-angiogenic (CXCL11) chemotaxins, based on the observation of endothelial sprouting. Using this assay, we show functional differences between T-cells activated using different approaches, and can inhibit migration by perturbing canonical endothelial receptor-T-cell receptor interactions.

2 Materials and Methods

2.1 Cell culture and media

All human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol. Human Umbilical Vein Endothelial Cells (HUVECs) (Lonza, product pooled from multiple donors) were cultured in complete human endothelial medium (Cell Biologies), expanded, and bio-banked in aliquots. HUVECs in all studies were used at or before passage 5. HCC0827 cells (licensed from the University of Texas Southwestern) were cultured in RPMI (Gibco) + 5% FBS (Gibco). NCI-H520 (ATCC) cells were cultured RPMI + 10% FBS. Primary human T-Cells (AllCells, StemExpress, or BioIVT) were thawed in one of the following media solutions, as indicated in the studies described: either AIM V medium (Gibco) containing 20 IU/mL of IL-2 (Miltenyi) or RPMI + 10% FBS. For activated T-Cells, 1:500 TransAct (Miltenyi), which utilizes polyclonal stimulation to activate T-cells via CD3 and CD28, was added to the medium. Activated-only T-cells were cultured for 48 hours (either with or without 1:500 TransAct) prior to use in assay. Activated-rested T-Cells were cultured for 72
t hours in 1:500 TransAct, followed by a 48-hour rest period, during which time the medium was washed out via centrifugation and replaced with RPMI + 10% FBS. Please refer to Tables S2-S4 for additional information on cells and reagents.

2.2 T-cell isolation
For Fig. 2-3 and Fig. S2-4, T-cells were obtained directly from AllCells and shipped to the MIMETAS research facility. For Fig. 4-6 and Fig. S5-8, T-cells were isolated from StemExpress or BioIVT leukopaks internally at GSK. Leukopaks were received and stored at 4°C overnight (approx. 16h). First, peripheral Blood Mononuclear cells (PBMCs) were isolated using a Custom PBMC Isolation Kit (Miltenyi), using magnetic beads to isolate out erythrocytes and granulocytes on magnetically charged cell selection columns while eluting PBMCs. T-cells were then isolated from the PBMCs using a standard Pan-T Isolation kit (Miltenyi) using manufacturer protocols. T-cells were cryopreserved in CS10 (BioLife Solutions) in a rate-controlled freezer over the course of one hour, and transferred to LN2 storage. Please refer to Tables S3-S5 for additional information on cells and reagents.

2.3 T-cell chemotaxis and migration assay
Mimetas 3-lane 40 Organoplates® (MIMETAS) were used for these studies. To seed the plates with collagen (Day -2, indexed to T-cell addition day), 50 μL of DPBS was added into the observation port to facilitate making chip filling visible. To form the extracellular matrix barrier, rat tail collagen-I (Cultrex) was mixed with HEPES and 37 g/L NaHCO3 in a 8:1:1 ratio to form a 4 mg/mL collagen-I solution. These components were mixed well > 20 times, being careful not to generate bubbles. Within 10 minutes, 1.8 μL gel solution was seeded into each chip using an automatic repeater pipette (Sartorius). The Organoplate® was then placed in a humidified incubator (37°C, 5% CO2) for 15 minutes to allow polymerization of the collagen-1 gel. 30 μL PBS was then added into the gel inlet to hydrate the ECM layer prior to returning the plate to the incubator. To form the 3D endothelium, HUVECs were trypsinized, resuspended in endothelial medium, counted using an automated cell counter (ViCell Blu, Beckman Coulter), and resuspended to a cell seeding density of 10^6 cells/mL. PBS was removed from the gel inlets, and 2 μL of cell suspension was deposited into the top inlet port using the automatic repeater pipette. Cell suspension was regularly mixed in order to ensure homogenous cell seeding density. After, 50 μL of endothelial medium was added to the same top medium inlet in which the cells were deposited. The Organoplate® was placed with the lid forming a 75 degree angle against the plate stand, and left in this orientation for around 3 hours in order to allow cells to attach. After cell attachment, 50 μL of endothelial medium was added into the top medium outlet. The plate was then placed on the OrganoFlow®, set to an inclination of 7° and an interval of 8 minutes, in a humidified incubator.

On Day -1, tumor cells or empty medium were seeded into the bottom channel using a different seeding strategy. Tumor cells (HCC0827 or NCI-H520) were trypsinized, resuspended in endothelial medium, counted, and resuspended to a cell seeding density of 10^6 cells/mL. 2 μL of cell suspension was then deposited into the bottom inlet port using an automatic repeater pipette. Cell suspension was regularly mixed in order to ensure homogenous cell seeding density. The Organoplate® was placed with the lid forming a 75 degree angle against the plate stand, but here with the plate rotated 180 degrees from the previous HUVEC seeding step (i.e. top of the plate on the bottom, touching the incubator shelf), and left in this orientation for around 3 hours in order to allow cells to attach. After, 50 μL of endothelial medium was added into the inlet of the bottom perfusion channel, and placed back on the OrganoFlow® rocker.

On Day 0, T-cells or empty medium controls were seeded into the OrganoPlate®. T-cells were harvested gently, centrifuged at 300 g for 5 minutes, counted, and incubated in dye solution, either 2.5 μM CellTracker Orange CMRA (ThermoFisher) or 1:1000 NucLight Rapid Red (Sartorius), in AIM V medium. Cells were dyed at a concentration of 106 cells/mL, with no more than 3e6 cells per Falcon tube. Conicals of cells in dye solutions were wrapped in foil and placed in an incubator for 30 mins. Halfway through the incubation period, the tubes were inverted several times to gently mix. T-cells were then centrifuged and pelleted to wash out the stain, and resuspended in Complete Assay Medium containing AIM V Medium, 20 IU/mL IL-2, 5 ng/mL VEGF and 5 ng/mL bFGF. Cells were then counted and diluted to desired concentration in Complete Assay Medium in order to deliver the number of T-cells per chip indicated in these studies in 50 μL of medium. At this stage, the top medium inlets and outlets were aspirated. 50 μL of T-cell density solution was added into the top medium inlet, and 50 μL Complete Assay Medium was added into the top medium outlet. Then, the bottom medium inlet and outlets were aspirated, and replaced with 50 μL medium each containing specified chemokine trigger or control medium solutions. For studies corresponding to Fig. 3-5, a half-volume medium re-addition was implemented, in which 25 μL of additional Complete Assay medium were added into the top channel inlet and outlet, and 25 μL of chemokine trigger solution were added into the bottom channel inlet and outlet. For antibody blocking experiments, vehicle alone (PBS), IgG1 antibody control (30 μg/mL, R&D Systems), ICAM-1/CD54 (10μg/mL, R&D Systems) blocking antibody, or VCAM-1/CD106 (30μg/mL, R&D Systems, BBAS) blocking antibody was added into the top channel inlets and outlets at the same time as chemotactic trigger addition into the bottom compartment (Day 0) and also with the half medium refresh (Day 2). Please refer to Tables S2-S4 for additional information on equipment and reagents utilized.

2.4 Imaging and T-cell quantification
For data obtained in Figures 1-3 and Supplementary Figures 1-6, images were acquired using a spinning disc confocal and migrating T-cells were quantified using a custom FLII macro as previously described (de Haan et al., 2021).

1 doi:10.14573/altex.2208121s
For data obtained in Figures 4-6 and Supplementary Figures 6-8, imaging was performed either on EVOS microscope or a GE InCell 6500 high content confocal imaging system. Two confocal z-stacks were acquired per chip (the right and left sides), with the same z-stack size used across each plate. All stacks were converted into single maximum intensity projection images of equal size and used for analysis. Analyses were performed using ImageJ or using a custom python script. For analysing migration distance of T-cells and the number that successfully migrate, a python script was developed which utilised the open-source scikit-image library (Van Der Walt et al., 2014). This analysis pipeline was run in two stages: to accurately identify the PhaseGuidesTM from the brightfield image, and therefore the channel boundaries, and also to identify nuclei that had been stained with DAPI. In order to identify PhaseGuidesTM, a synthetic image that mapped out the position of the PhaseGuidesTM was used as a template to convolve along the image in order to find the position that looked most similar to the distribution of PhaseGuidesTM. To increase the accuracy of this approach, the synthetic image was a 1 pixel-width image with intensity bands that are similar to a vertical cross-section of the PhaseGuidesTM (as it is 1 pixel wide, this is less affected by rotation). Fast Normalized Cross Correlation was used for template matching and this led to a processed image with ideally a single horizontal line that had been rotated as per the rotation of the plate. Finding the maximum intensity (and therefore the highest correlation) along the x-axis enabled binarizing the image and then edge detection was used. The original positions of PhaseGuidesTM were then mapped back to this line. Separately, blob detection was used and the distances from the blobs was measured using a signed distance function (i.e., distances are negative if they are behind the line, and positive if they are in front). This meant that channels could be identified just by the sign of the distances. Once the channels had been assigned to each nuclei, it was also possible then to count the number of nuclei in chamber. To assist in detecting the PhaseGuidesTM illumination correction was performed retroactively by estimating the illumination profile using a low-pass filter (using a Gaussian kernel with a large sigma)(Dey, 2019).

2.5 Barrier integrity assays
The barrier integrity of HUVEC endothelial tubes embedded within the tumor-on-chip platform was evaluated before and after the addition of T-cell compatible assay media as previously described (Trietsch et al., 2017), and the procedure is detailed within the supplement of this publication (de Haan et al., 2021). Here, the top chip inlets and outlets were perfused with 0.5 mg/mL 20 kDa or 155 kDa FITC Dextran (Sigma). Please refer to Table S4 for additional information on equipment and reagents. The apparent intrinsic permeability, or $P_{app}$ was quantified using the following formula,

$$P_{app} = \frac{-(V_p V_D)}{(V_R + V_D) \Delta t} \cdot \ln \left( \frac{C_R(t)}{C(t)} \right)$$

where $V_p$ is the volume in the receiver channel, $V_D$ is the volume in the donor channel, $A$ is area of the ECM interface with the donor channel, $C_R(t)$ is the fluorescence intensity in the receiver channel at time point t, $C(t)$ is the average concentration of the dye in the system at time point t (Soragni et al., 2023). Estimates of the permeability of the vasculature component alone were obtained by subtracting the permeability of the completely filled chips (stroma with tumor and HUVEC tube) from the permeability of the partially filled chips (stroma with tumor, but no HUVEC tubes). Comparison to physiological measurement of tumor vasculature utilized published literature (Dewhirst and Secomb, 2017).

2.6 Flow cytometry
Cells were plated at 300,000 cells/well in 96-well U-bottom plates (Corning). Plates were spun down (300g, 5 mins), washed 1x with 200 μL DPBS (Life Technologies), and spun down again (300g, 5 mins) to remove supernatant. For live/dead staining, live/dead dye was resuspended as per manufacturer protocol and diluted in PBS at 1:100 dilution. 50 μL of diluted live/dead solution was added to plate wells, mixed thoroughly, and incubated at room temperature for 15 minutes in the dark. Samples were washed 1X with 150 μL PBS and spun down (300g, 5 mins) to remove supernatant. For Fc blocking and primary antibody staining, 10 μL of Fc block (Milenyi) were added to each well and incubated for 10 mins in the dark at room temp. Then, 90 μL of antibody cocktail (see details in Tables S6 and S7 on antibodies and reagents), prepared in FACS Buffer (Becton Dickenson) were added to each well and mixed. Samples were incubated for 30 mins at 4°C, wrapped in foil to protect from light. Wells were then washed 1X with 100 μL FACS Buffer and 1X with 200 μL FACS Buffer. Plate was then spun down (300g, 5 mins) and supernatant removed. For sample fixation, 100 μL CytoFlx fixation buffer (Becton Dickenson) was added to the wells and incubated for 25 mins at room temperature, wrapped in foil to protect from light. Samples were then washed 1X with 100 μL FACS Buffer and 1X with 200 μL FACS Buffer, spun down (300g, 5 mins) and supernatant removed. Samples were resuspended in 250 μL FACS Buffer and mixed well. Plates were stored at 4°C until being read on the cytometer. Staining for compensation controls was conducted on the day of flow analysis as follows. One drop of UltraComp eBeads (eBiosciences) were incubated with 2 μL of the appropriate antibody for 30 mins at room temperature protected from light. For Aqua LIFE/DEAD dye compensation control, 2 drops ArC beads (Life Technologies) were incubated with 2 μL of Live/Dead dye for 30 mins, at room temperature, protected from light. After incubation, beads were washed with flow buffer (500 μL), centrifuged (300g, 5 mins) and resuspended in 400μL of fresh flow buffer. One drop of ArC negative beads were added to the Aqua tube, and then compensation was run. Flow cytometry was

ALTEX, accepted manuscript
published June 16, 2023
doi:10.14573/altex.2208121

4
Fig. 1: T-cell chemotaxis assay development, medium evaluation, and barrier analysis in the LToC-Endo model
(a) Experimental setup and timeline of platform seeding with Collagen-1, endothelial cells, and tumor cells. (b) Representative images of the platform seeded in monoculture and coculture configurations, with endothelial medium and triculture assay medium, on day 0. The transition from endothelial medium to triculture assay medium occurs on day 0 to mimic T-cell seeding at that time point. Refer to Fig. S11 for information on the different assay media formulations considered. (c) and (e), Barrier integrity assay fluorescence images, where white shows 20 kDa FITC dextran presence. (d) and (f), permeability coefficient measurements for different configurations of the assay, for day 0 and day 2, respectively. Data show measurements per chip for n=2, or n>2 for those used for statistical testing, within-experiment, technical replicate chips per condition. Bars indicate means, and error bars indicate SD. Data was square root transformed prior to statistical testing to account for unequal SD. Outcomes are indicated for statistical tests comparing barrier diffusivity among the conditions tested (One-way ANOVA, ***p< 0.001, ****p<0.0001). (g) and (h), ELISA data of CXCL12 concentration in the bottom and top channels 48h after 150 nM CXCL12 is first introduced into the bottom channels of the chips. Data show measurements per chip for n = 2 chips per condition. The bars represent means and error bars indicate SD, and error bars indicate standard deviations. The scale bars in (b), (c), and (e) are 100 µm. Please refer to Fig. S1g1 for permeability measurements utilizing 155 kDa FITC dextran and other data obtained within the same study as those in panels (b) through (f).
conducted on the LSR Fortessa X-20 (Becton Dickinson), and data was analyzed using FlowJo 10.6.2. Please refer to Table S7 for more information on reagents.

2.7 **Immunocytochemistry**

Cell cultures in the MIMETAS OrganoPlate® were fixed in 3.7% formaldehyde (Sigma) after 48h, 72h, or 120h in culture and immunostained as previously described (de Haan et al., 2021). Hoechst 33342 (Thermo Fischer Scientific) was used to stain nuclei. Please refer to Table S4 for more information on primary and secondary antibodies utilized.

2.8 **Statistical analysis**

Statistical analyses were performed using GraphPad Prism version 8.1.2 (332) for Windows, GraphPad Software, San Diego, California USA. Data were tested for homogeneity in standard deviations, and were square root transformed if needed. Statistically significant differences between means of two or more groups were evaluated using one-way ANOVA (equal variance) or Brown-Forsythe and Welch ANOVA (Gaussian, unequal variance), with multiple comparisons corrected using Dunnett’s, Tukey’s, or Sidak’s. Differences were considered significant if p < 0.05.

3 **Results and discussion**

3.1 **Tumor barrier limits chemokine diffusion throughout tumor chips with 3D endothelium**

We developed a lung tumor-on-chip model with 3D endothelium ("LToC-Endo") in the MIMETAS 3-lane 40 Organoplate® using three human cell types: pooled donor human umbilical vein endothelial cells (HUVEC), non-small cell lung carcinoma cells (HCC0827), and primary T-cells. First, we established a collagen-1 extracellular matrix barrier. Then, we seeded endothelial cells in the top channel of the Organoplate® against this barrier on day -2 and cultured the chips under rocking flow (Fig. 1a). The following day (day -1), we seeded tumor cells and by day 0, we observed that both the endothelial cells and tumor cells formed tubules in the top and bottom lanes, respectively (Fig. 1b).

To evaluate the diffusivity of both the endothelial and tumor barriers in the LToC-Endo, we performed two different assays. First, using an imaging-based barrier integrity assay, we added 20 kDa fluorescent dextran (approximately the size of chemotactic chemokines) on day 0 into the top endothelial channel, and observed dextran flow through the chips over time with fluorescence microscopy (Fig. 1c, Fig. S1). By comparing permeability coefficients throughout different chip configurations, we noticed that the 3D endothelial tube readily allowed diffusion, and was comparable to no-cell chip controls (Fig. 1c-f, Fig. S1). We repeated these studies with larger fluorescent dextran (155 kDa) and found similar results (Fig. S1g). In both cases, the tumor tubule formed a more diffusion-limiting barrier within the device than did endothelial cells on their own, explaining why the combination of barriers is also significantly more diffusion-limiting than the endothelial barrier alone (Fig. 1c-f, Fig. S1g). This finding was corroborated by an ELISA-based permeability assay, in which CXCL12 chemokine was added into the bottom channel, such that it flowed into the tumor tubule or empty channel and then diffused upward through the chip (Fig. 1g,h). Media sampling over time revealed that in the no-tumor version of the assay, chemokine was detectable in the top channel as early as 4 hours, and increased markedly by the 48h time point, with a gradient remaining by this time. By contrast, with a tumor tubule, we could not detect any chemokine in the top channel after 48 hours, at which point the chemokine concentration in the top channel was comparable to, or less than, that of the no-tumor assay after only 4 hours. Altogether, these diffusion studies support that this assay models leaky tumor vasculature near a diffusion-limiting NSCLC tumor. When we compared the permeability of the vasculature barrier modeled in vitro within these tumor chips to the permeability of leaky tumor vasculature found in vivo, we estimate that the in vitro vascular barriers are roughly one order of magnitude leakier than physiologically relevant permeability estimates (Fig. S1h).

We note that the initial formation of the HUVEC endothelial tubule requires specialized media containing fetal bovine serum, an animal-derived product. To make the LToC-Endo amenable to T-cell addition, we adopted an assay medium that was xeno-free once the initial tubule was successfully formed, we explored the impact of an assay medium switch on day 0 and then evaluated the corresponding platform permeability in addition to endothelial cell number and phenotype (Fig. 1b,e, and f show images and data using final assay medium selected; Fig. S1 shows all media evaluated). We selected AIM V medium, supplemented with 5 ng/mL recombinant human VEGF (165 isoform) and bFGF, based on its ability to promote markers of endothelial tube stability without appreciably changing barrier diffusion properties and its being free from fetal bovine serum. This enables a fully xeno-free model, which can both avoid ethical issues around animal products as well as unwanted immunogenicity caused by species mismatch.

3.2 **Activated, but not naïve, T-cells migrate in response to chemokine gradients in the tumor chips with 3D endothelium**

Next, we used the LToC-Endo model to study the effect of activation status, T-Cell seeding density, tumor barrier presence, and chemoattractant type on T-cell chemotaxis. On day 0, we seeded either naïve or activated primary human T-cells into the endothelial channel of the tumor chip, along with recombinant CXCL11 or CXCL12 in the bottom tumor channels (Fig. 2a). While naïve T-Cells did not migrate into the ECM compartment (Fig. S2e), activated T-Cells migrated into the ECM compartment in a seeding
Fig. 2: T-cell seeding density, chemokine type and dose, and tumor barrier presence regulate activated T-cell migration in the LToC-Endo model

(a) Experimental setup and timeline with platform seeding of extracellular matrix, endothelial cells, tumor cells, and activated T-cells.

(b) Representative phase contrast and fluorescence images of T-cell migration through the collagen barrier of the tumor on chip, in response to chemokines CXCL11, CXCL12, and vehicle controls. Images depict data using a T-cell seeding density of 15,000 cells/chip.

(c) Number of migrated T-cells, by seeding density and over time, in CXCL11 and CXCL12 and vehicle controls, with data points indicating means of n = 3 chips and error bars indicating SD. 48-hour time point data, from black boxes in (c), are highlighted in (d), with bars indicating means of n = 3 within-experiment technical replicate chips per condition and error bars indicating SD. Results shown for Welch’s t-tests to accommodate unequal variances (** p < 0.01, *** p < 0.001).

(e) Representative fluorescent images of T-cell migration, for chips by dose of CXCL12 chemokine, with and without tumor barriers, at the day 2 time point for two independent experiments evaluating dose-response to CXCL12 chemokine. (f) Number of migrated T-cells by CXCL12 dose, with and without tumor barriers, at Day 1 and Day 2 time points. Day 1 data for tumor and no tumor conditions are overlapping. Markers indicate means of n = 6-7 chips per condition and error bars indicate SEM. Significant differences between CXCL12 dosages and respective vehicle controls are shown (Brown-Forsythe and Welch ANOVA tests, corrected for multiple comparisons, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001). Scale bars in (b) and (e) are 100 µm. Panels (b) through (d) show data from a single experiment, whereas panels (e) and (f) show representative images and data pooled from two other independent, biological repeat experiments, performed in the same lab, on different days (please refer to Fig. S3a and S3b for data shown separately). Images and data in green boxes (b, d, e, and f) show the same condition amongst the first study and combined second and third studies. Please refer to Fig. S2 and Fig. S4 for data obtained within the same experiment as the data shown in panels (b) through (d); refer to Fig. S3 for additional data obtained within the same experiments as shown in (e) and (f). These two studies were conducted using Donor 1 T-cells.
density-dependent manner by the day 2 time point after T-cell seeding, in response to both chemokines (Fig. 2b-d). We observed significant differences between chemokine and vehicle control chips (Fig. 2d).

We then evaluated the role of chemoattractant dose and tumor presence on T-cell presence in endothelial tubes and migration. T-cell presence in the endothelial tube did not increase with CXCL12 concentration, although it did increase with time at all doses tested (Fig. S3c1). Meanwhile, all doses of CXCL12 tested, regardless of tumor presence, led to increases in T-cell chemotaxis compared to control chips, with the no-tumor version of the assay leading to greater overall T-cell migration (Fig. 2e,f, Fig. S3a,b1). We especially noticed in no-tumor conditions an elevated baseline level of migration even in the absence of chemokine, compared to the with-tumor assay (Fig. 2e,f, Fig. S3a,b1). One potential disease-relevant explanation for this could be soluble inhibitory factors secreted by the tumor cells. However, another explanation could be asymmetry of media consumption in the no-tumor version of the assay (i.e. endothelial cells and T-cells only, and present in the top channel, with a cell-free bottom channel), which may lead to a nutrient gradient that initiates nonspecific T-Cell migration even in the absence of recombinant chemokine. What is more, ELISA-based diffusion studies repeated with T-cells support that the no-tumor version of the assay is more permissive to chemokine diffusion at all doses of chemokine tested (Fig. S3d,e1). Thus, more effective chemokine diffusion may also explain why T-cell response saturates at lower doses in the no-tumor assay (37.5 nM CXCL12) compared to the with-tumor version of the assay (150 nM CXCL12) (Fig. 2f, Fig. S3a,b1). We note that throughout the assay we did not observe T-cells entering the tumor compartment; only a limited number of T-cells in the no-tumor condition migrated all the way into the bottom compartment of the chips (Fig. 2e).

3.3 Presence and activation status of T-cells influence endothelial activation in response to CXCL12

In the LToC-Endo, we observed notable differences in endothelial tube response to CXCL12 depending on the presence of activated T-cells. While CXCL12 drives migration or angiogenic sprouting of HUVECs with naïve T-cells (Fig. 3a, top row) or when T-cells were absent (Fig. 3b,c), we did not observe pervasive endothelial cell activation when introducing activated T-cells (Fig. 3a, bottom row). CXCL12 is a known driver of T-cell chemotaxis, but it is also a crucial regulator of angiogenesis. It acts by increasing VEGF-A production in endothelial cells, which then upregulates their CXCR4 expression, enhances responsiveness to CXCL12, and contributes to an amplifying angiogenic signaling loop (Salcedo et al., 1999; Staller et al., 2003; Romagnani et al., 2004). CXCL12 also promotes angiogenesis through Akt activation via atypical CXCR7 receptors, which are overexpressed only in stressed endothelial cells (Zhang et al., 2017). Under pro-angiogenic signaling, the endothelium responds by increasing endothelial wall permeability, destabilizing the vessel wall, and increasing expression of leukocyte adhesion receptors, in addition to increasing endothelial cell proliferation and migration (Romagnani et al., 2004; Distler et al., 2003; Hunt and Jurd, 1998; Strieter et al., 2005). It is possible that these CXCL12-mediated endothelial events indirectly contribute to the observed window in T-cell migration (Fig. 2b-f), in addition to the direct effect of CXCL12 driving T-cell chemotaxis. By contrast, CXCL11 is an angiostatic chemokine, known to counterbalance the vascular changes described above (Romagnani et al., 2004). Therefore, as expected we do not see angiogenic sprouting in response to this chemokine in the assay (Fig. 3a,b).

The reduction in migration and angiogenic sprouting responsiveness to CXCL12 suggests that the 3D endothelium in the LToC-Endo may be under stress with the addition of activated T-cells. Images of the 3D endothelium, 3 days after activated T-cell addition, show large holes that are suggestive of endothelial stress (Fig. 3a). Abundant T-cell proliferation is suspected to play a role, as both in-chip and off-chip T-cells exhibit an expected, post-activation proliferative burst (Fig. S41), leading to a higher effective number of T-cells than initially seeded. As a consequence, the rapidly proliferating T-cells may not only be contributing to endothelial stress at long time points, but also diluting live cell dye, all of which may contribute to the plateau or decline in migrated T-cells after 2 days, which was seen both here (Fig. 2c) and in a previous study (de Haan et al., 2021).

3.4 Alternate T-cell activation protocol impacts T-cell phenotype and enhances functional response

We hypothesized that the introduction of a rest period, mimicking the time lag between T-cell activation and homing to a tumor site in vivo (Chen and Mellman, 2013), would allow us to overcome the proliferative burst prior to seeding activated T-cells. Additionally, we switched to a live nuclear dye which we expected would be stable over longer culture periods. We performed the assay side-by-side with activated or activated-rested T-cells. As expected, the activated-rested T-cells, which undergo proliferative burst during the 2-day rest, increase in number by 3-4x prior to seeding, compared to the activated-only T-cells (Fig. S4a1). Surprisingly, in spite of the lack of in-chip proliferative burst, more activated-rested T-cells were present in the endothelium and migrated in greater numbers in response to CXCL11 (~4x more on average) and CXCL12 (~2x more on average), compared to activated-only T-cells (Fig. 4a-d). To better understand these changes, we profiled T-cells prepared using both approaches for expression of CXCR3 and CXCR4, the cognate receptors for CXCL11 and CXCL12. We saw that the introduction of a rest period following a T-cell activation enhanced CXCR3 and CXCR4 expression in all T-cell subsets compared to activation-only T-cells, increasing the overall proportion of double positive (CXCR3+CXCR4+) T-cells from ~30-50% to ~85% (Fig. S5b, Tab. S11). Furthermore, we observed that the rest period led to more central memory (CD45RO-CCR7+) and effector memory (CD45RO-CCR7-) T-cell phenotypes, indicating a more durably activated state (Mahnke et al., 2013) (Fig. S5c1, Tab. S11). Our incorporation of an additional control in these studies allowed us to attribute changes in T-cell phenotype to differences in activation regimen, rather than culture medium, as this was also changed (Fig. S5b,c1, Tab. S1). We note that due to logistical constraints of performing this study, the activated-only T-cells were activated for 72 hours while the activated-rested T-cells were activated for 48 hours, prior to restocking both populations for 48 hours. While we have made the assumption that this difference in activation time period is not the source of variation in T-cell response based on prior experience (data not shown here), an additional study would be needed.
Fig. 3: Migration and sprouting of 3D endothelium in response to CXCL12 in the LToC-Endo model
(a), Hoechst and CD31 staining of the indicated conditions, for naïve and activated T-cells, low and high T-cell seeding density, and CXCL11 and CXCL12 chemokines, on day 3. With no T-cells in the chips, (b) CD31 staining depicting 3D endothelium response to control, CXCL12, or CXCL11 conditions after 3 days in culture and (c) brightfield images showing endothelial response to CXCL12 or media control, with and without tumor cells, after 3 days in culture. In (a) through (c), middle channel width is 350 μm as indicated by the vertical bars. Panel (a) was obtained in the same experiment as shown in Fig. 2b-d and Fig. S2; panels (b) and (c) were obtained in the same experiment as shown in Fig. 2e-f and Fig. S3. These two studies were conducted with Donor 1 T-cells.
Fig. 4: Activated-rested T-cell protocol enhances T-cell adhesion and chemotaxis, and restores CXCL12-driven endothelial activation, in the LToC-Endo
Representative brightfield and fluorescent images of T-cells (15k per chip) within the endothelial tubule and migration into the ECM compartment in response to the chemokine and dose indicated, at Day 2 time point, for (a) Activated-Only T-cells (AIMV) and (c) Activated-Rested T-cells (RPMI). White arrow indicates evidence of endothelial sprouting. Refer to Fig. S5 for flow cytometry data from this experiment that includes an additional Activated-Only (RPMI) condition. Scale bars in (a) and (c) are 100 µm. In (b) and (d), quantifications of migrated T-cells and T-cells within the endothelial tube for both T-cell preparation protocols, respectively. Markers indicate mean T-cell numbers per chip (n = 4 same-donor technical replicates per condition), bars indicate mean T-cell numbers per condition, and error bars indicate SD. Statistical testing was performed on square root transformed data to satisfy criteria of equal standard deviations. Significant differences between chemokines and respective vehicle alone controls are shown (One-way ANOVA corrected for multiple comparisons, *p<0.05, *** p<0.0001). This study was conducted with Donor 2 T-cells.
to show this definitively. Under this assumption, these data suggest that implementing a T-cell culture protocol with activation followed by a rest period enables the introduction of T-cells that are more sensitive and responsive into the tumor-on-chip assay.

With respect to endothelial tube responsiveness to CXCL12 chemokine, another key difference emerged when switching from an activated-only to activated-rested T-cell culture protocol. With the activated-only T-cells, a lack of response previously shown (Fig. 3a) was reproduced (Fig. 4a) in an independent experiment, using a separate donor’s T-cells. By contrast, in activated-rested T-cell chips, we observed endothelial migration in response to CXCL12 by 48h (Fig. 4c, white arrow). This endothelial response to CXCL12 with activated-rested T-cells appears to match more closely the endothelial response to CXCL12 with the naïve and no-T-cell conditions (Fig. 3). These data lead us to infer that adding rested T-cells minimizes the stress on 3D endothelial tubules caused by the addition of highly proliferative T-cells, and may preserve more physiologically relevant responsiveness of the 3D endothelium to angiogenic cues.

3.5 Assay timeline extension is facilitated by alternate T-cell activation protocol

Given that an activated-rested T-cell protocol allowed us to circumvent proliferative burst in-chip, preserve 3D endothelium responsiveness to activation, and mitigate live cell dye dilution, we hypothesized that we could extend the assay timeline. Repeating the assay with activated-rested T-cells, we compared day 2 and day 5 numbers of T-cells migrating and number of T-cells within the endothelial tubes. While the activated-only T-cell version of the assay results in a decline in T-cell chemotaxis after day 2 (Fig. 2c) and complete endothelial dissolution by day 5 (Fig. S6d), the activated-rested T-cell version of the assay shows higher levels of T-cell migration and residence within the endothelial tubules, as well as more intact endothelium, by day 5 (Fig. 5 for images and chemotaxis data, Fig. S6 for endothelial tube brightfield images). While by day 2 we observe comparable levels of T-cells within endothelial tubules between control and chemokine conditions, by day 5 we observe significantly fewer in the CXCL12 condition. This may reflect that although higher numbers of T-cells are present within the endothelial channel over time in all conditions, a significant number have migrated due to extravasation in the CXCL12 condition (Fig. 5d).

For further assay characterization, we focused on CXCL11, which was determined to be the clearer positive control given the confounding endothelial sprouting introduced by CXCL12. Using CXCL11, we confirmed that with this new T-cell activation strategy and extended timeline, T-cell presence within the endothelial compartment and migration still scales with T-cell seeding density (Fig. S7b), as observed in prior studies (Fig. 2c). We also observed that the absence of a HUVEC tubule diminishes migrating T-cells down to nearly zero, significantly below even basal infiltration levels in the presence of HUVEC tubules, but no chemokine (Fig. S7b). These data suggest that although these in vitro HUVEC tubules are leakier than their physiological counterparts, they still play a critical role in T-cell migration, likely by providing endothelial receptors as anchors for T-cell arrest in the rocking platform, in order to initiate transmigration.

Finally, to demonstrate versatility of the LToC-Endo assay, we repeated the T-cell activated-rested protocol using an additional non-small cell lung carcinoma cell line (NCI-H520), using two different T-cell donors (Fig. S8a). We did observe variability between T-cell donors throughout these studies (see Fig. 2f vs. Fig. 4b for Donor 1 to Donor 2 comparison, 150 nM CXCL12, activated only; see green boxes in Fig. S8 for Donor 2 to Donor 3 comparison, 300 nM CXCL11, activated-rested). However, our studies support that the adoption of an activated-rested T-cell culture protocol and a long-lasting, live nuclear dye can enable a sufficient assay timeline extension to observe significant differences between vehicle and chemokine conditions for a variety of experimental conditions: with and without tumor barriers; HCC087 and NCI-H520 tumor cells; high and low T-cell and tumor cell seed densities; multiple T-cell donors (Figs. 5, S7, S8a).

3.6 T-cell chemotaxis in tumor on chips requires ICAM-1

Finally, we evaluated the ability of this tumor on chip microphysiological system to recapitulate mechanisms of T-extravasation. Migration into the tumor microenvironment requires chemokine-induced polarization of T-cells and attachment to the endothelium through VCAM-1/ICAM integrin activity (Melder et al., 1995; Riegler et al., 2019). Therefore, we repeated this assay using CXCL11 as the chemotactic stimulus, and added blocking antibodies against endothelial receptors VCAM-1 and ICAM-1 or isotype controls at the same time as adding chemotactic triggers.

By day 5 of T-cell incorporation into the platform, we observe that ICAM-1 blocking antibody treatment significantly reduces the number of T-cells migrating into the extracellular matrix in response to CXCL11 down to control (i.e. no-chemokine) levels, while VCAM-1 blocking antibody does not (Fig. 6a,b). The median migration distance of T-cells in chips with ICAM-1 blocking antibody is significantly reduced compared to those with isotype control (Fig. 6c). These trends hold in with-tumor and without-tumor versions of the assay (Fig. 6a-c). We observe that the addition of IgG control antibody significantly impacts the number of T-cells adhering to the endothelium, even in the absence of chemokine (Fig. 6d). In the presence of CXCL11, and in the with-tumor assay condition, we observe a significantly lower number of T-cells adhering to the endothelium using ICAM-1 blocking antibody. Altogether, these data suggest that blocking ICAM-1 is sufficient to block chemokine-induced T-cell adherence, extravasation, and chemotaxis.

It is unclear why VCAM-1 blocking did not result in decreased adhesion and chemotaxis. In preclinical animal models, VCAM-1 density and tumor perfusion are predictive of T-cell migration and treatment response to adoptively transferred and endogenous T-cells (Riegler et al., 2019). However, blocking VCAM-1 is only marginally effective at blocking T-cell adhesion to endothelial cells in vivo. By contrast, combined blocking of CD49d/integrin-α4 (a VCAM-1 binding partner), and CD18/integrin β2 (an ICAM binding partner) offers substantially improved blocking, with this cocktail shown to prevent T-cell mediated tumor rejection (Riegler et al., 2019).
Fig. 5: Activated-Rested T-cells enable an extended assay endpoint. (a) and (b). Representative brightfield (BF) and fluorescent images of T-cell migration in response to the indicated chemokines and doses, on days 2 and 5, for assay with 15k T-cells seeded and with tumor barrier. Scale bars are 100 µm. (c). Quantifications of migrated T-cells and (d) T-cells within the endothelial tubes on days 2 and 5. In (c), statistical testing was performed on square root transformed data to satisfy criteria of equal SD. In (c) and (d), significant differences between chemokines and respective vehicle controls are shown (One-way ANOVA corrected for multiple comparisons, *p< 0.05, ** p<0.01, *** p < 0.001, **** p<0.0001). Please refer to Fig. S6 for bright field images of endothelial tubes obtained from this experiment. This study was conducted with Donor 2 T-cells.
Fig. 6: T-cell extravasation and chemotaxis in response to CXCL11 are dependent on ICAM-1 endothelial receptor in the tumor on chip platform

(a) Representative fluorescent images of T-cell migration in response to chemokine or vehicle control, with additional treatment as indicated with blocking antibody or IgG control. Images show day 5 assay data both without and with tumor barrier. Scale bar is 100 µm. Day 5 quantifications of (b) mean migrated T-cell number, (c) median migrated distance, and (d) mean T-cell number within the endothelial tubes. In (b-d), markers indicate metrics per chip (n = 4 within-experiment, technical replicates per condition), whereas bars indicate means per condition, across all technical replicates, and error bars indicate SD. Significant differences between chemokines and respective vehicle alone controls, with or without antibody treatments, are shown (One-way ANOVA corrected for multiple comparisons, *p< 0.05, ** p<0.01, *** p<0.001, **** p<0.0001). In (b), statistical testing was performed on square root transformed data to satisfy criteria of equal standard deviations. This study was conducted with Donor 2 T-cells.
In conclusion, we developed a microfluidic lung tumor on chip assay with a 3D endothelium (LToC-Endo) perfused with rocking flow to evaluate modulators of T-cell extravasation and migration through 3D extracellular matrix in a non-small cell lung carcinoma (NSCLC) context. Due to the orientation of the platform, T-cell chemotaxis takes place across the x-y plane. This orientation readily facilitates snapshots of T-cell chemotaxis profiles across the stromal matrix, making the assay amenable to phenotypic screening and migration time point analysis. To more wholistically capture T-cell migration strategies, future studies could incorporate tumor spheroids into the bottom compartment of the platform, enabling distinct modes of migration, i.e. directed chemotaxis through 3D ECM in addition to Brownian “hunt and kill” tumor nest infiltration, to be modeled within the same platform (Krummel et al., 2016).

In alignment with a need for future work highlighted previously (de Haan et al., 2021), we extended the assay timeline and improved the assay window by introducing a rest period after T-cell activation and selecting a long lasting, live nuclear dye. Similar to in vivo, activated T-cells in the LToC-Endo extravasate and migrate in response to chemotactic gradients, and the living endothelial barrier responds to pro-angiogenic cues through sprouting. We have also shown the dependence of T-cell migration on the presence of non-small cell lung carcinoma cells and on ICAM-1 endothelial receptors. Although the portion of T-cells migrating through ECM appears small compared to the total number of T-cells deposited into each chip (15,000 for the majority of studies performed here), we note that the observation portion of the endothelial tubule only depicts a fraction (~7%) of the space, by volume, into which T-cell solution is deposited within the upper chamber of these microfluidic chips. We refer to the diagram shown in Fig. 1a to emphasize that the inlet and outlet ports, in addition to the diagonal lanes through which fluids wick in order to reach the observation channel, are not included in imaging or computational analysis. Therefore, if 15,000 T-cells are deposited into the inlet port of the microfluidic chip, and we assume ideal microfluidic wicking and uniform diffusion of the cell solution across the upper chamber of the chip, we would expect only about 1000 T-cells to be present in the observation channel. Indeed, we find that in the control conditions depicted in Fig. 6d, at the conclusion of a 5-day experiment, we observe around 1000 T-cells present in the observation portion of the endothelial tubule in both the with-tumor and without-tumor conditions. Furthermore, within this fraction of cells in the observation window of the 3D endothelial tubule, our data (using specific and nonspecific adhesion blocking molecules) suggest that up to half of the T-cells can make sufficient contact with the endothelial cells lining the tubule to achieve arrest on this 3D cell layer. Of the several hundred T-cells that may achieve arrest, it is reasonable to expect up to a few hundred of them to successfully extravasate through the endothelial barrier and migrate through the extracellular matrix barrier.

While animal models typically recapitulate immune cold tumors, the LToC-Endo and described chemotaxis assay can also recapitulate features of immune-excluded tumors (i.e. angiogenesis, immune 3D migration into stroma) (Hegde and Chen, 2020). Given differences in chemokines present and antigen-presenting functions of endothelial cells between human and animal models (Hegde and Chen, 2020), this assay has potential to serve as a valuable tool for probing humanized tumor-immune-endothelial multicellular interactions Additionally, this in vitro assay simultaneously offers the ability to observe compound efficacy (i.e. T-cell adhesion and migration) with safety (i.e. drug induced vascular injury, exacerbated angiogenesis in the tumor microenvironment (TME)), bringing safety information earlier into the discovery research pipeline.

Similar to what has been shown for an angiogenesis assay using this platform (van Duinen et al., 2020), the next step will be to evaluate the reproducibility and robustness in the LToC-Endo. Establishing a positive control with a clinically meaningful 2-5 fold window, yet without angiogenic side-effects, would be ideal based on prognostic differences between immune phenotypes in cancer tumors (Pagès et al., 2005) and in alignment with robust assay design (Iversen et al. 2006). Further work is needed to validate the translatability of the assay by using standard of care molecules and comparing outcomes to clinical responses (Baran, 2022). Moreover, there is a need to identify the T-cell subtypes that potential therapeutics successfully induce to migrate; in this case, enhancement of CD8+ cytotoxic T-cells is would be desirable.

There are many opportunities for adding increased physiological relevance and complexity into this platform, starting with the extracellular matrix. For this initial study, rat-tail collagen, an animal product, was utilized due to its availability, well-characterized physicochemical properties for reliable chip-filling, and batch-to-batch consistency. In the future, synthetic biomaterials could replace this animal-derived biomaterial. Alternatively, human material or in situ, tumor-generated stroma may more accurately recapitulate the donor-specific, heterogeneous niche encountered by T-cells. However, we note that the variability of these more complex materials will likely be higher, the sourcing more challenging, and the physicochemical/microfluidic properties of these materials will need to be evaluated. While lung tumors are not generally considered to be amongst the most fibrotic tumors, future adaptation of this system toward the most dense, fibrotic tumor types (i.e. breast and pancreatic) may require much higher density extracellular matrix than what was utilized in this study (Piersma et al., 2020).Regarding stromal thickness, it is expected that the distance between a tumor and parent blood vessel not only varies between patients, but also throughout the course of cancer progression. Mathematical modelling suggests that the distance between a tumor and its parent blood vessel (between 500-2500 μm, as seen in animal models) impacts the growth trends of vascular tumors by changing the spatiotemporal dynamics of angiogenesis (Akbarpour Ghazani et al., 2020). Future studies using this, or a similar microfluidic platform, could evaluate the role of these distance in vitro as a means to validate this or similar mathematical models and inform treatment strategies for patient-specific tumors.

Additional future directions for the LToC-Endo involve incorporating the stromal cells that perpetuate immune suppression in the TME, such as cancer-associated fibroblasts, myeloid derived suppressor cells, and tumor-associated macrophages (Mariathasan et al., 2018; Joyce and Fearon, 2015; Brennes et al., 2011). It will also be important to evaluate how
other immune cell types (i.e. T-regulatory cells, natural killer cells, and B-cells (van der Woude et al., 2017)) migrate into the TME in response to chemotactic cues and compounds, and to include a tumor cell killing component into the assay. After additional characterization and validation, our hope is that the LToC-Endo complex in vitro model can serve as a valuable tool to study multicellular and cell-extracellular matrix mechanisms of immune suppression, screen for drug candidates that target these processes to improve patient responses to immunotherapies.

We note that the initial culture of the HUVEC endothelial tube in this assay is not animal-free, as it requires media supplemented with fetal bovine serum. Recent studies suggest that human platelet lysate could serve as an animal-free replacement for fetal bovine serum in endothelial cell culture (Peters et al., 2022). This should be additionally investigated in a 3D microfluidic platform such as the one described here, where functional assessment (i.e. barrier integrity) could be evaluated in addition to features such as morphology, proliferation, and apoptosis. Furthermore, given the estimate that the tumor vasculature modeled here is about one order of magnitude leakier than tumor vasculature in vivo (Fig. S1h) and (Dewhirst and Secomb, 2017), and that these measurements are somewhat noisy, it would be beneficial to adopt new developments in media optimization and barrier permeability measurement techniques (Ehlers et al., 2023), and consider automated methods of filling these multi-component tumor chips, to both reduce the variability and improve the resolution of these measurements while reducing the permeability of the in vitro vasculature to more closely mimic vasculature in vivo.

For this complex in vitro model to support the refinement, reduction, and replacement of animal immuno-oncology models, whether classical syngeneic (i.e. MC38, 4T1) or ‘humanized’ mouse tumor models(Zitvogel et al., 2016), further validation studies are needed which incorporate additional human and animal tumor cell lines and primary, single-donor, tissue specific endothelial cells (compared to pooled donor HUVECs), which would enable consideration of HLA-matching in selection of T-cell. As these studies should be guided by a translational strategy, we propose that noninvasive imaging techniques serve as a translational link to align imaging-based pharmacodynamic (PD) timepoint readouts between complex in vitro and in vivo models of immune migration. Noninvasive imaging techniques can detect and monitor anatomical, functional, metabolic, or molecular-level changes within the body of animals with minimal pain, distress, or premature termination (Beckmannand and Ledermann, 2017), and can do so in a temporal and spatial manner. For example, migration and infiltration of specific T-cell populations (e.g. CD8+) can be tracked into specific organs, tumors, or tumor-draining lymph nodes over time within a single animal (Rashidian et al., 2017). In this way, noninvasive imaging can enable comprehensive, longitudinal immune response datasets to be derived from fewer animals, thereby increasing the statistical power of the data gathered by reducing experimental variation (Weissleder et al., 2016; Rashidian et al., 2019; Alsaid, H. et al., 2021; Tavaré et al., 2014). This is in contrast to traditional methods requiring animals to be sacrificed at given time points, i.e. using histology and flow cytometry (Zitvogel et al., 2016). Instead of relying exclusively on these informative yet endpoint-requirement techniques, which now include scRNAseq (Rashidian et al., 2019), they could instead be employed as-needed to verify or supplement noninvasive longitudinal imaging. Ideally, these noninvasive in vivo approaches would translate to evolving clinical imaging techniques, which are expected to gather similar longitudinal immune migration data, monitor therapeutic response in individual patients, and enable precision oncologic medicine (Weissleder et al., 2016; Rashidian et al., 2017, 2019).

With this translational strategy in mind, an imaging-based, humanized, immune 3D migration complex in vitro model such as the LToC-Endo could be well suited to establish an in vitro/in vivo correlation in the future. Longitudinal, imaging-based T-cell migration datasets, gathered per-chip, per-animal, and per patient, could then be used to calibrate silico models, enable better in vivo response prediction, refine the selection of candidates to progress into animal studies, and ultimately provide better medicines to patients.

References


ALTEX, accepted manuscript
published June 16, 2023
doi:10.14573/altext.2208121

doι:10.1016/j.immuni.2013.07.012


**Conflicts of interest**
J.S. and L.v.d.B are employees of Mimetas BV. K.M.W., B.S., T.S.P.G., A.C., M.L., S.T., A.G., S.-H.C., D.P., N.S., S.G., and J.E. are employees of GSK, or were at the time of producing this work. The OrganoPlate® is a registered trademark of Mimetas BV.

**Author contributions**

**Acknowledgements**
We gratefully acknowledge John Lowman for his facilitation of the collaborative research agreement. Thank you to Kimberly Nadwodny, Helena Rannikmae, and Ed Gimmi for their data integrity and patents reviews. We also acknowledge Maggie Connelly for isolating T-cells for these studies. Thank you to Jeremy Waigt for providing helpful feedback on the presentation of this work and the manuscript. This work was funded by GSK.

**Data availability**
The datasets generated during and analyzed during the current study are available from the corresponding author upon reasonable request.

**Ethics statement**
The human biological samples were sourced ethically, and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol.