

1 **Title: Human immunocompetent Organ-on-Chip platforms allow safety**
2 **profiling of tumor-targeted T-cell bispecific antibodies**

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23 **Abstract:** Traditional drug safety assessment often fails to predict complications in humans,
24 especially when the drug targets the immune system. Here, we show the unprecedented
25 capability of two Organs-on-Chips to evaluate the safety profile of T-cell bispecific antibodies
26 (TCBs) targeting tumor antigens. Although promising for cancer immunotherapy, TCBs are
27 associated with an on-target, off-tumor risk due to low levels of expression of tumor antigens in
28 healthy tissues. We leveraged in vivo target expression and toxicity data of TCBs targeting folate
29 receptor 1 (FOLR1) or carcinoembryonic antigen (CEA) to design and validate human
30 immunocompetent Organs-on-Chips safety platforms. We discovered that the Lung-Chip and
31 Intestine-Chip could reproduce and predict target-dependent TCB safety liabilities, based on
32 sensitivity to key determinants thereof, such as target expression and antibody affinity. These
33 novel tools broaden the research options available for mechanistic understandings of engineered
34 therapeutic antibodies and assessing safety in tissues susceptible to adverse events.

35

36 **Introduction**

37 Cancer immunotherapy has received intense attention over the past two decades owing to
38 the promise of delivering durable cures by harnessing the cytotoxic potential of the immune
39 system against tumor cells(1-3). However, although impressive improvement in long-term
40 survival has been reported(4-6), only a fraction of patients responds. Furthermore, the systemic
41 immunomodulation mediated by these drugs often elicits immune-related adverse events (irAEs),
42 including skin and liver toxicity, colitis and pneumonitis, limiting their broad clinical application
43 in battling cancer(7-9).

44 T-cell engaging bispecific antibodies (TCBs) are a novel class of cancer
45 immunotherapeutic agents that have the potential to improve on the clinical efficacy and safety
46 of traditional immunotherapy(10, 11). TCBs exert their anti-tumor activity by simultaneously
47 binding to a cancer surface antigen and the CD3 T-cell receptor, thereby both activating the latter
48 and physically crosslinking it to target cells(12). This synthetic immunity approach is
49 particularly favorable for targeting less immunogenic, neo-antigen-lacking tumors, as T cells can
50 be recruited and activated independently of their T cell receptor specificity. This strictly tumor-
51 targeted immunomodulation is also expected to reduce the systemic inflammatory toxicities
52 associated with traditional immunotherapies. The therapeutic potential of TCBs is exemplified
53 by the large number of molecules targeting solid and blood tumors, which are currently in
54 various stages of clinical evaluation(13, 14).

55 Although TCBs hold the promise for a safer therapeutic option, they are not risk-free.
56 The antigens targeted are rarely exclusive to the tumor, but are also often expressed, albeit at
57 lower levels, in normal tissues, rendering TCBs subject to “on-target, off-tumor” safety
58 liabilities. This is particularly true for epithelial tumor antigens as they are frequently targeted in

59 solid tumor indications. For example, a BiTE targeted to the epidermal growth factor receptor
60 (EGFR) produced severe liver and kidney toxicities in non-human primates, in line with EGFR
61 expression in these organs, and led to the termination of the animals(15, 16). Clinical adverse
62 events were reported in a recent Phase I study evaluating an Epithelial Cell Adhesion Molecule
63 (EpCAM)-targeted BiTE as a therapy for a variety of epithelial carcinomas. Consistent with the
64 expression of EpCAM in the gastrointestinal tract, the molecule triggered severe diarrhea and
65 ultimately prevented escalation to efficacious doses and the identification of a therapeutic
66 window(17, 18). Reliable human TCB safety evaluations at the preclinical stage are therefore of
67 vital importance to ensure that well-tolerated and efficacious therapeutics reach patients.

68 Traditional rodent-based preclinical models are often ill-suited for predicting cancer
69 immunotherapy-mediated adverse events in humans in part because of the fundamental
70 differences in the immunological responses between the species(19). In the EpCAM example
71 mentioned above, the severity of the diarrhea elicited by the treatment was not predicted by
72 preclinical studies in mice(20). Moreover, an increasing number of TCBs target human-specific
73 antigens that lack expression in animals, rendering preclinical animal studies uninformative for
74 safety and efficacy assessments(12). Indeed, the development of preclinical models that better
75 translate to human immunity is regarded as one of the top current challenges of cancer
76 immunotherapy(21).

77 While human-relevant cell-based models of tissues and organs promise to bridge this gap,
78 conventional in vitro two-dimensional (2D) models fail to provide the complexity required to
79 model the biological mechanisms of immunotherapeutic effects. These conventional 2D systems
80 typically lack the immune components needed to simulate TCB responses. Furthermore, their
81 reductive microenvironment, devoid of essential cellular, biochemical, and biophysical factors

82 found in the native organ, precludes the expression of TCB targets at physiologically relevant
83 levels and patterns, crucial for capturing TCB pharmacology and safety liabilities.

84 Organ-on-Chip models aim to overcome these limitations by combining micro-
85 engineering with cultured primary human cells to recreate the complex multifactorial
86 microenvironment and functions of native tissues and organs(22). The tissue microenvironment
87 in vivo provides the external signals that help drive cellular differentiation towards mature
88 phenotypes. Organs-on-Chips model key functional aspects of tissue-level physiology such as
89 epithelial and microvascular tissue-tissue interfaces, and physiologically relevant mechanical
90 forces, have been shown to more accurately capture in vivo-relevant phenotypes(23-25). The
91 enhanced tissue maturation promoted by Organs-on-Chips could help ensure organ-specific
92 expression of TCB targets, while the modularity of these devices and the possibility for
93 controlled circulation of molecules and immune cells could better capture the functional
94 interactions between TCBs, immune cells and target-expressing cells that occur in patients.
95 Motivated by these advantages, we set out to evaluate Organs-on-Chips as platforms for the
96 assessment of on-target, off-tumor TCB safety risks in human organs, using a panel of targeting
97 and non-targeting molecules, and leveraging in vivo target expression and toxicity data. We
98 found that these systems could reproduce and predict target-dependent TCB safety liabilities,
99 showing sensitivity to key determinants thereof, such as target expression and antibody affinity.

100 **Results**

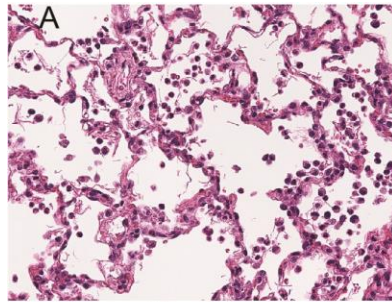
101 As a starting point for our method development and validation, we used molecules under
102 current preclinical development. We focused on a T-cell bispecific antibody generated to bring
103 Folate Receptor 1 (FOLR1) expressing tumor cells in close proximity to CD3 expressing
104 cytotoxic T-cells (Fig. S1A) (26). FOLR1 is overexpressed in many solid, epithelial-derived

105 tumors including ovarian, lung and breast cancer(27), but is also expressed to a lower degree on
106 normal epithelial cells as found in the lung and kidneys (28). While a high affinity FOLR1-TCB
107 (FOLR1(Hi) TCB) was efficacious in human breast cancer patient-derived xenograft models
108 (Fig. S1B), severe on-target toxicity in the lung of cynomolgus was observed (29). Clinical signs
109 of severe respiratory inflammation appeared as early as 24 hours post-dosing, and pro-
110 inflammatory cytokines IL-6, IL-2, and IL-8 were elevated in the blood of affected animals, and
111 importantly coincided with an increase of inflammation markers. Histopathology assessment
112 revealed leukocytic infiltrates in lung tissue indicative of immune mediated toxicity (Fig. 1A).
113 Further immunohistochemistry (IHC) studies (Fig. 1 B-D) indicated low relative expression
114 (compared to high FOLR1 expression in the ovarian carcinoma cell line, HeLa) of the FOLR1
115 target antigen in lung alveolar epithelial cells of cynomolgus lung tissue suggesting an adverse
116 event largely driven by on-target toxicity. Importantly, IHC analysis of FOLR1 expression in the
117 human lung revealed similar levels and patterns of the antigen as in the cynomolgus (Fig. 1 E-F),
118 extending the threat of safety liabilities to patients in the clinical setting. Bearing in mind the
119 toxicological profile of FOLR1(Hi) TCB in cynomolgus and the expression of FOLR1 in both
120 species lung tissue, we identified the lung as an at-risk organ in patients and accordingly set out
121 to evaluate a human Alveolus Lung-Chip model as a platform for FOLR1(Hi) TCB toxicology
122 assessment.

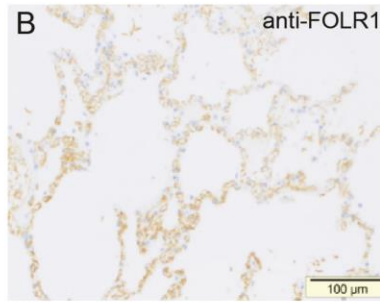
123 Alveolus Lung-Chips were seeded with human adult lung primary alveolar epithelial
124 cells on top of an extra-cellular matrix coated porous membrane that separates two parallel,
125 fluidic microchannels (Fig.1G and Fig. S2). On the opposite side of the membrane, human
126 primary lung microvascular cells were seeded to form a lower tubular vascular channel as
127 described previously(30). A mature Alveolus Lung-Chip model was obtained after five days of

128 liquid-liquid culture (LLI) followed by establishment of an air-liquid-culture (ALI) for a further
129 five days. To evaluate FOLR1 expression in chips, we combined RNA sequencing,
130 immunofluorescence and flow cytometry analyses. FOLR1 gene expression was even over time
131 as shown by quantification of RNA transcripts (Fig.1H). We also confirmed FOLR1 protein
132 expression in mature chips (Fig.1I). Flow cytometry-mediated quantification allowed us to
133 estimate the cell surface expression of FOLR1 within the Alveolus Lung-Chip at an average of
134 ~10,000 molecules expressed per cell (Fig.1J). For comparison, the high FOLR1 expressing
135 ovarian carcinoma HeLa cell line displayed an average of ~450 000 molecules per cell when
136 cultured on chip (Fig. S3B), confirming the difference observed in IHC between healthy and
137 tumor cells.

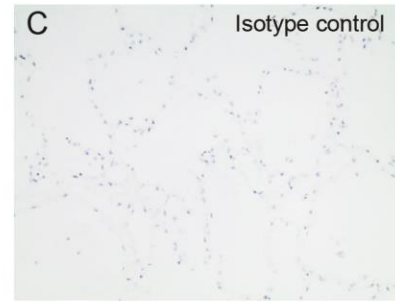
138 To render the device immunocompetent and capable of simulating on-target TCB-
139 mediated immunomodulation, we added peripheral mononuclear blood cells (PBMC) isolated
140 from human whole blood to the epithelial channel in direct contact with the mature alveolar
141 epithelium (Fig. S2). Introduction of T cells is required to engage the CD3 arm of FOLR1(Hi)
142 TCB thereby allowing its mode of action. The sequence of events we aimed to reproduce in
143 chips are the crosslinking of T cells to the FOLR1 expressing target cells mediated by the TCB,
144 subsequent T-cell activation and cytolytic synapse formation resulting in cytotoxic granules
145 release (granzymes and perforin) and consequent target cell apoptosis. Early T-cell cytokine
146 release ($\text{TNF}\alpha$, $\text{IFN}\gamma$) should be followed by later cytokine release from epithelial cells and
147 monocytes (IL-6 , $\text{IL1}\beta$, IL-8) combined with strong physical attachment of immune cells to the
148 FOLR1-expressing lung epithelium via the TCB. Thus, we selected and optimized experimental
149 readouts that would enable us to monitor these steps in the Alveolar Lung-Chip.



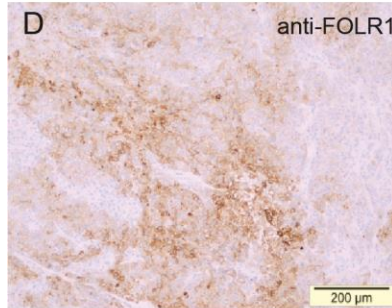
Cynomolgus, 10 µg/kg single IV injection



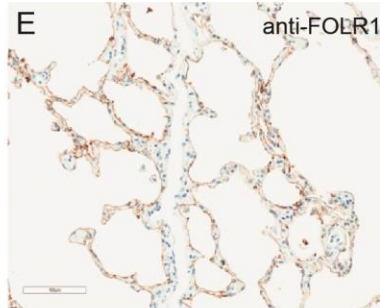
Cynomolgus, healthy lung



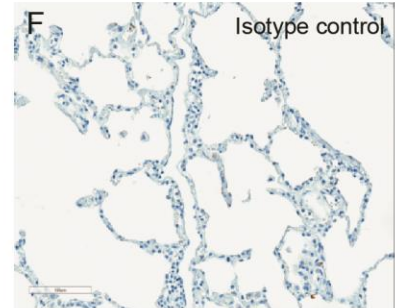
Cynomolgus, healthy lung



HeLa, positive control

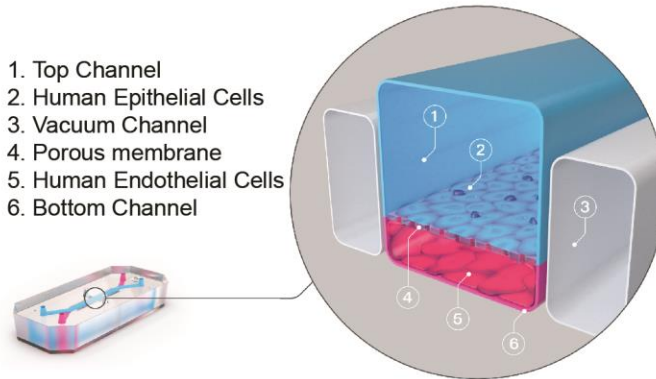


Human, healthy lung

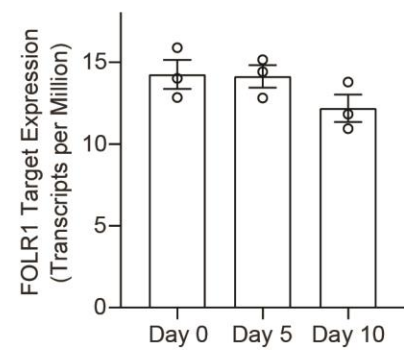


Human, healthy lung

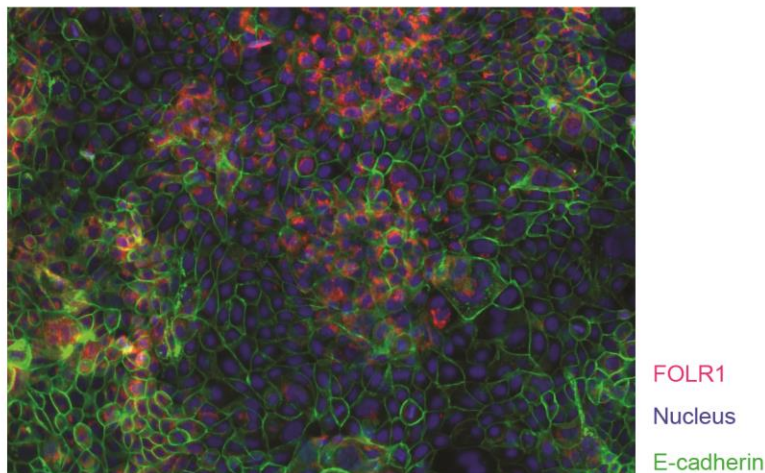
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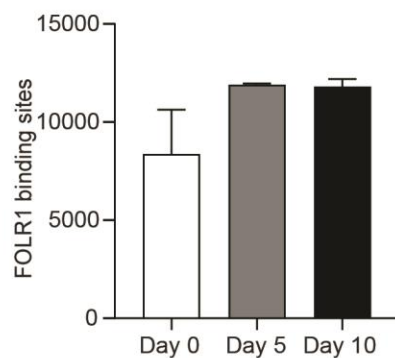
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151 **Fig. 1. FOLR1 expression in the alveolar epithelium of cynomolgus and humans underlies on-target**
152 **off-tumor toxicities of FOLR1-TCB and can be recreated in a human alveolus lung-chip.** (A) IHC of
153 pre-clinical, cynomolgus lung tissue 24 hours after intravenous single-dose administration of high-
154 affinity FOLR1-TCB (FOLR1(Hi), 10 µg/kg), demonstrating leukocytic infiltration (dark purple cells) and
155 inflammation. (B) Expression of FOLR1 protein in healthy cynomolgus lung tissue stained with antibody
156 targeting FOLR1. (C and F) Isotype controls of FOLR1 staining in healthy cynomolgus and human lung
157 tissues, respectively. (D) High FOLR1 expression displayed in human ovarian carcinoma HeLa cell line
158 for comparison to: (E) Histopathological staining of primary healthy human lung tissue for FOLR1. G)
159 Schematic of Alveolus Lung-Chip to model human FOLR1 on-target toxicities. Alveolus Lung-Chip
160 design is composed of a top microfluidic channel (1) seeded with primary adult human alveolar cells (2)
161 cultured to maturity with air-liquid interface (ALI). The top, epithelial channel is separated with a
162 flexible, porous membrane (4) from a bottom, vascular channel seeded with primary lung microvascular
163 cells (5,6). Mechanical stretching is applied via pneumatic actuation of parallel vacuum channels (3). (H)
164 RNAseq expression levels of FOLR1 gene in cultured alveolar epithelial cells on Day 0 (before seeding),
165 5 or 10 after seeding and differentiation on the Alveolus Lung-Chip (n=3, ± SEM). (I) Representative
166 immunofluorescent staining of chip epithelium (Nuclei, blue) at Day 10 of culture expressing the tight
167 junction marker E-cadherin (green) and FOLR1 target antigen (red). Images taken at 40x magnification.
168 (J) Estimation of surface FOLR1 binding site expression via flow cytometry of harvested chip epithelial
169 cells at Day 0 (before seeding), 5 and 10 (n=2-4, ± SEM).

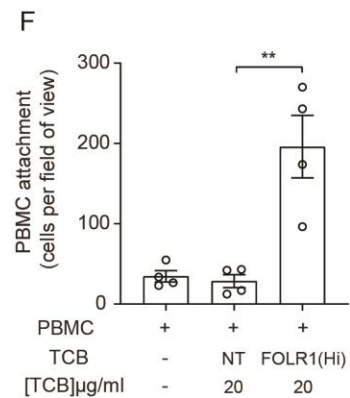
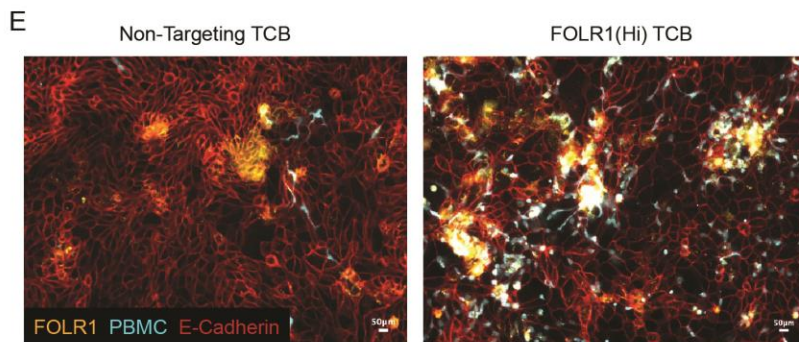
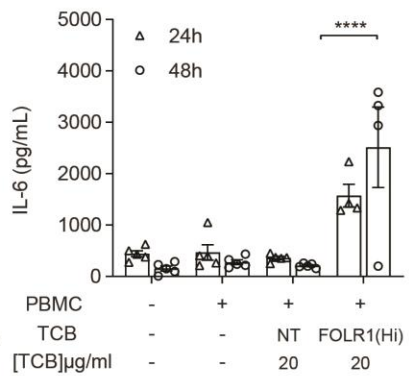
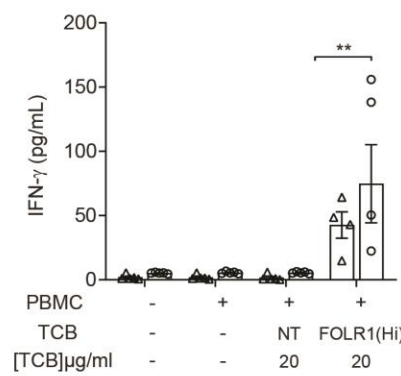
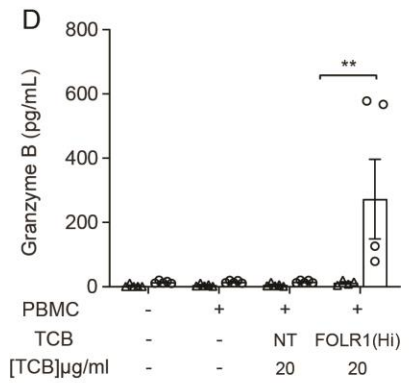
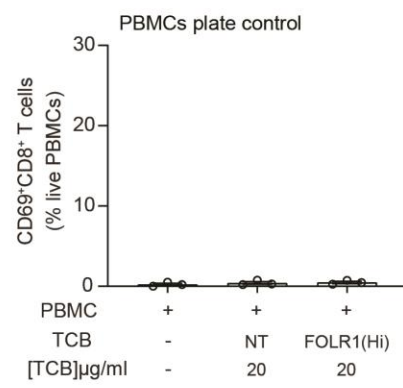
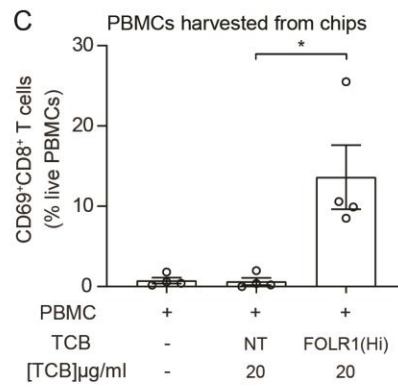
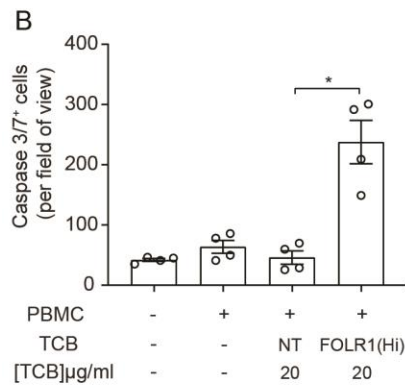
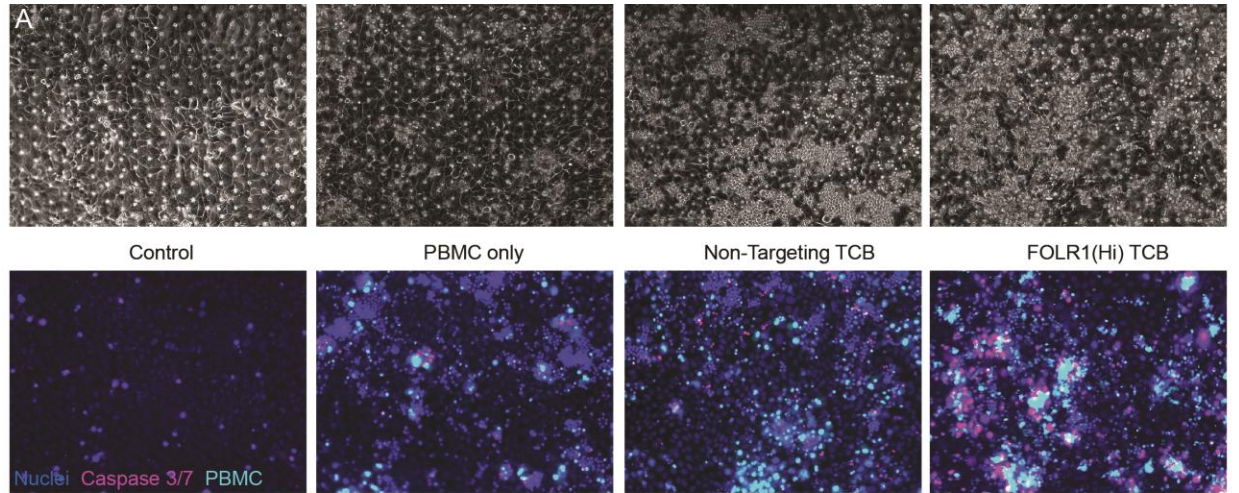
170 Figure 2A shows representative brightfield and fluorescent images of the Alveolus Lung-
171 Chip at 48 hours after the administration of immune cells. Compared to the chips treated with a
172 non-targeting (NT) TCB control antibody, FOLR1(Hi) TCB treated chips presented an increased
173 apoptosis of the alveolar epithelium (Fig. 2B). Consistently, FOLR1(Hi) TCB treatment led to
174 increased T cell activation (as evidenced by CD69 upregulation in CD8⁺ T cells) in the presence
175 of target-expressing cells (Fig. 2C), but not in PBMC only (Fig. 2C right panel). Supernatants

176 collected from the outlet reservoir of the epithelial channels at 24 and 48 hours post-treatment
177 were measured for multiplex cytokines (Fig. 2D), revealing a significantly increased secretion of
178 IFN γ at 24 hours and 48 hours that correlated with increased granzyme B and IL-6 at 48 hours in
179 response to FOLR1(Hi) TCB.

180 Interestingly, we noticed a higher number of immune cells in the FOLR1(Hi) TCB
181 condition compared to the NT control, possibly due to a combination of T cell proliferation and
182 increased attachment of T cells (Fig. 2A). Quantification of the immune cell presence in fixed
183 chips confirmed increased attachment to the target epithelium in the FOLR1(Hi) TCB condition
184 (Fig. 2 E, F), which is consistent with the TCB mode of action, whereby immune cells are
185 crosslinked to target cells. Importantly, these immune cells appeared to accumulate at sites of
186 high FOLR1 expression, suggesting a direct link between the amount of target antigen and
187 priming of T cells. Together, these data suggest that the Alveolus Lung-chip successfully
188 replicates aspects of the FOLR1 (Hi) TCB-mediated toxicity observed in cynomolgus, and
189 suggests that the human lung would be subject to similar safety liabilities.

190 In light of the toxicity risk predicted above, and hoping to define a potential therapeutic
191 window of FOLR1(Hi) TCB, we performed the same study with chips seeded with the high
192 FOLR1 expressing ovarian carcinoma cell line, HeLa, previously used to assess drug
193 efficacy(26). Although no effects were seen at the lowest concentration, all concentrations
194 starting from 2 ng/mL induced significant T-cell activation (measured at 48 hours), cancer cell
195 apoptosis (from 24 hours onwards) and strong cytokine release, as expected from the high level
196 of FOLR1 expression in HeLa cells (Fig. S3). Thus, we efficiently killed tumor cells at a much
197 lower concentration than was needed to induce damage to the healthy alveolar epithelial cells.

198



200 **Fig. 2. The immunocompetent Alveolus Lung-Chip recapitulates TCB-mediated on-target off-tumor**
201 **toxicity.** Isolated PBMCs were pre-incubated for 1 hour with high-affinity FOLR1 TCB (FOLR1(Hi)) or
202 non-targeting TCB control (NT) and introduced to the epithelial channel of differentiated Alveolus Lung-
203 Chips and rested for 3 hours prior to initiation of media perfusion. The established co-culture with
204 immune cells was then maintained for 48 hours under flow with fresh media. (A) Representative
205 brightfield (top) and immunofluorescent images (bottom) of Alveolar Lung-Chip epithelium (nuclei, blue)
206 48 hours after addition of PBMC (cyan). The control group did not have PBMC administered. The
207 FOLR1(Hi) group showed higher levels of PBMC attachment and caspase-3/7 positive, apoptotic cells
208 (magenta) (B) Quantification of apoptotic caspase-3/7 positive cells collected on live chips (n=4). (C)
209 Flow cytometry analysis of PBMC harvested from chips or plates for percentage of live, CD69⁺ activated
210 CD8⁺ T cells (n=4 approx. 10,000 cells per chip) after 48 hours (n=4). PBMC cultured on plates after 48
211 hours incubation showed overall low activation levels without attachment to epithelium. (D) Multiplex
212 cytokine analysis of epithelial channel supernatants at 24 and 48 hours after PBMC introduction (n=4).
213 (E) Immunofluorescent staining of FOLR1 target expression (yellow) in epithelium (E-cadherin, red) of
214 chips administered with NT control (left) and FOLR1(Hi)-treated (right) PBMC (cyan). Increased
215 accumulation of PBMC and co-localization with FOLR1 signal was observed in FOLR1(Hi) group. (F)
216 Quantification of immunofluorescent images confirmed increased PBMC in the FOLR1(Hi) group (n=4).
217 Statistical analysis was conducted by one-way ANOVA (B, C, D, F) and was defined as *P < 0.05,
218 **P<0.01, and ***P<0.001. Errors bars represent ± SEM.

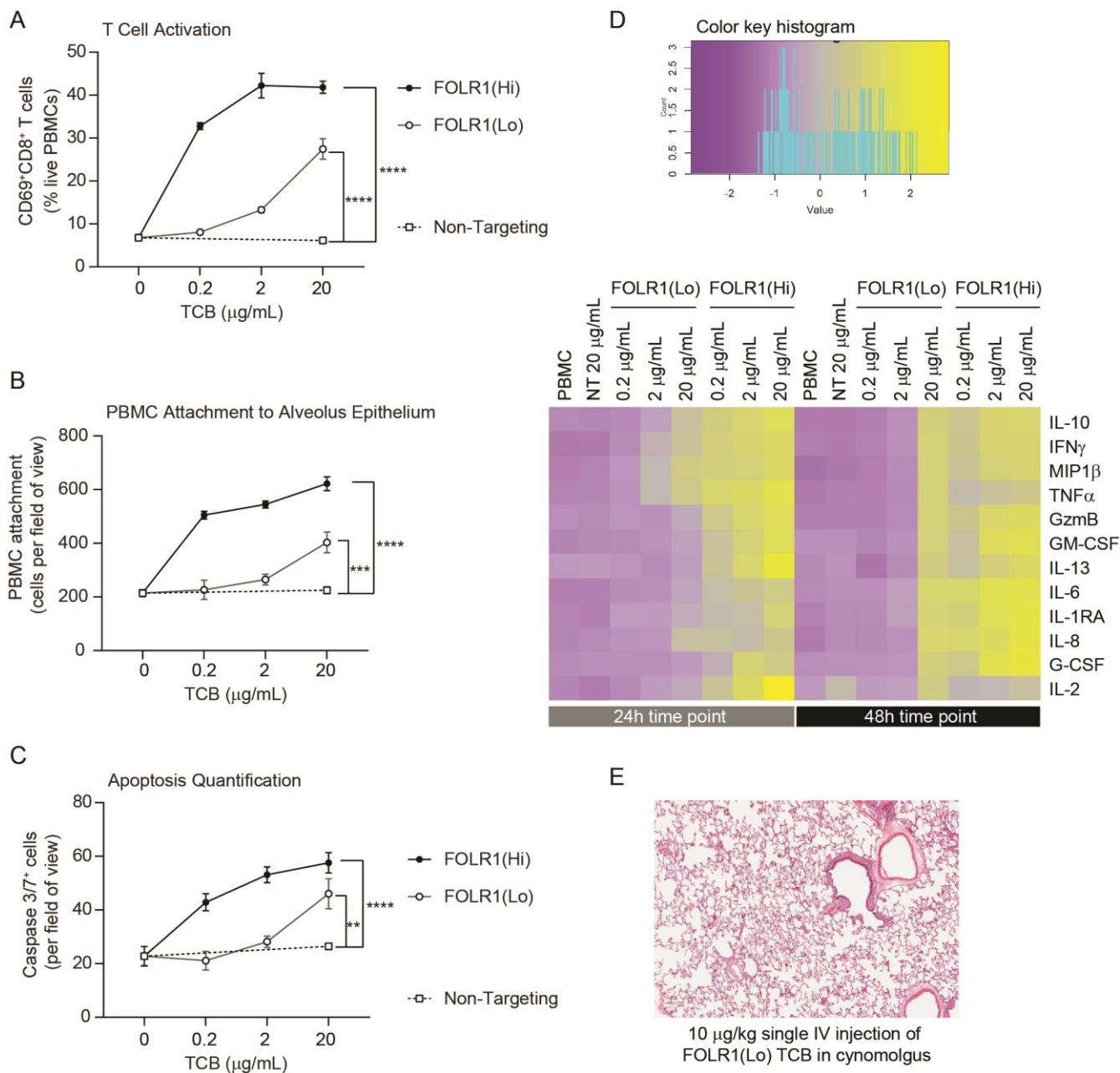
219 Although the data described above suggested that a therapeutic window for FOLR1(Hi)
220 TCB could be determined, we leveraged the chip to instead identify a safer molecule. In
221 particular, we utilized an antibody with lower monovalent affinity for the FOLR1 target, referred
222 to as FOLR1(Lo) TCB making use of avidity mediated selectivity gain (Fig.S1 C-D). As a result
223 of that design, FOLR1(Lo) TCB presented a lower binding to FOLR1-expressing HeLa cells
224 while retaining a potent killing activity in coculture assays (Fig. S1E-F). We profiled the two

225 TCBs, FOLR1(Hi) and FOLR1 (Lo), in the immunocompetent Alveolus Lung-Chip following
226 the workflow and readouts described above, and found that FOLR1(Hi) TCB induced a
227 significant increase in all the readouts starting at the 0.2 µg/mL concentration whereas
228 FOLR1(Lo) TCB showed a response only at the highest concentration of 20 µg/mL and to a
229 much lower magnitude than the high affinity molecule (Fig. 3A-D). These results demonstrated
230 that the cellular responses on the platform are sensitive to differences in antibody affinity and
231 recapitulates the biology associated with the mode of action of TCBs. Following these in vitro
232 observations, the lower affinity FOLR1 TCB was tested in a cynomolgus toxicology study and
233 none of the animals experienced the lung inflammation observed with FOLR1(Hi) TCB (Fig.
234 3E), confirming the safer profile predicted by the Alveolus Lung-Chip.

235 To compare the chip format to its 2D equivalent, we benchmarked the Alveolus Lung-
236 Chip against transwell inserts coated with alveolar epithelial cells and endothelial cells on
237 opposing sides (Fig. S4). In the transwell environment, quantification of TCB-dependent
238 immune cell attachment and apoptosis did not show differences between the control and FOLR1
239 TCBs treatment conditions. T-cell activation and T-cell specific cytokines were elevated,
240 possibly due to prolonged interactions of the immune cells with the epithelium. Also, the
241 transwell version did not capture differences between FOLR1 antibodies: FOLR1(Lo) TCB led
242 to a higher amount of granzyme B and IFN γ release than the chips, which is inconsistent with the
243 absence of toxicity observed in vivo. Given the higher concordance of the results produced by
244 the Alveolar Lung-Chip compared with the transwell counterpart, we propose that the novel
245 immunocompetent Alveolus Lung-Chip platform can faithfully evaluate TCB on-target, off-
246 tumor risks and presents a superior value to the existing alternatives.

247

248



249 **Fig. 3. Dose Response and TCB Affinity-Dependent Effects Displayed in Immunocompetent Alveolus**
 250

251 **Lung-Chips.** (A) Flow cytometry analysis of PBMC harvested from epithelial channel and assessed for
 252 CD69⁺ activated CD8⁺ T cells (n=4 approx. 10,000 cells per chip). (B) Quantification of
 253 immunofluorescent images of prelabelled PBMC that remained attached after harvest from chip
 254 epithelium) (n=4). (C) Immunofluorescent image quantification of caspase-3/7⁺ apoptotic epithelial cells
 255 at 48 hours time point. The NT control group displayed no increase in T-cell activation, PBMC

256 *attachment, or apoptotic cells with increasing dose, while the FOLR1(Lo) group showed a significant*
257 *increase at 20 µg/mL and the FOLR1(Hi) group displayed an increase from 0.2 µg/mL in a dose-*
258 *dependent manner (n=4). (D) Heat map displaying multiplex cytokine analysis of chip epithelial channel*
259 *supernatant at 24 and 48 hours post-treatment. (E) Histological lung tissue section from pre-clinical*
260 *cynomolgus study of intravenous FOLR1(Lo) (10 µg/kg), 24 hours after administration. Statistical*
261 *analysis was conducted by one-way ANOVA (A, B, C) and was defined as ** $P < 0.01$, *** $P < 0.001$ and*
262 ***** $P < 0.0001$. Errors bars represent \pm SEM.*

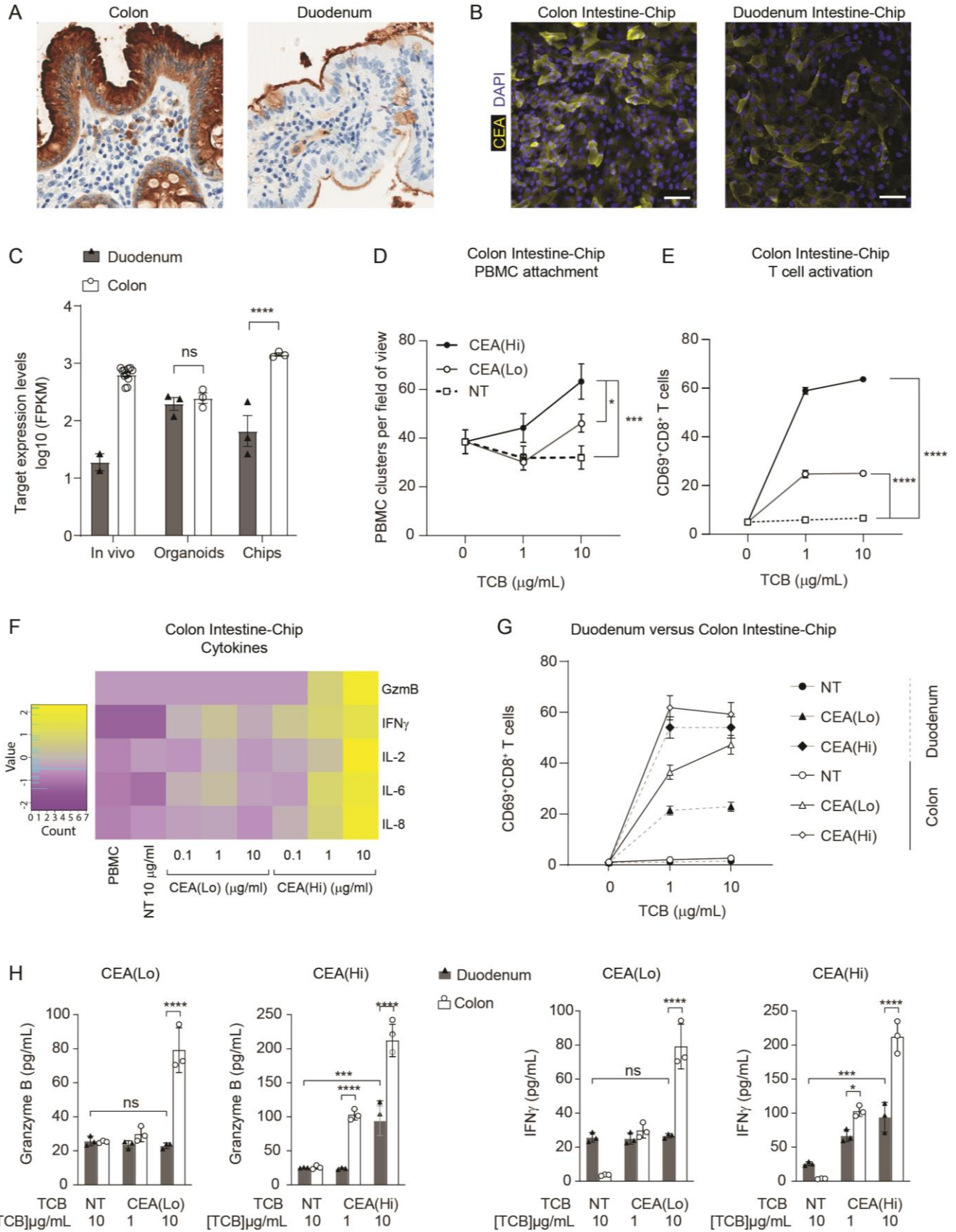
263 To demonstrate the broad applicability of the model for testing target-mediated TCB
264 safety risks, we extended the methodology to a second target and a second Organ-Chip model. In
265 this example, we focused on TCBs targeting carcinoembryonic antigen (CEA), which is
266 overexpressed in a range of solid tumors, including colorectal cancer(31). We have created TCBs
267 binding to CEA with high or low affinity – CEA(Hi) and CEA(Lo) TCB, respectively (Fig.
268 S5A), and are currently evaluating them as therapies for a range of solid tumors. Indeed, we have
269 found that CEA(Hi) and CEA(Lo) TCB are potent mediators of tumor cell lysis and T cell
270 activation in vitro (Fig. S5B, C), and exhibit robust anti-tumor activity in humanized mice
271 engrafted with CEA-expressing tumors (Fig. S5D).

272 Aside from solid tumors, however, CEA is expressed in the gastrointestinal tract(32-34).
273 Immunohistochemistry analysis of primary human intestinal samples confirmed high expression
274 of CEA in the colon. By comparison, small intestinal expression was lower and confined to the
275 apical side of the enterocytes (Fig. 4A). The substantial target presence in the gastrointestinal
276 tract implicates this system as an at-risk organ, motivating us to assess potential intestinal
277 toxicities triggered by CEA-engaging TCBs. CEA is a human-specific protein, making mice and
278 cynomolgus preclinical toxicology models unsuitable for the assessment of toxicities caused by
279 CEA-targeting TCBs. Indeed, our antibodies showed lack of cross-reactivity to cynomolgus

280 monkey CEA, which underscores the need for human-relevant models in addressing this
281 question (Fig. S5E). Therefore, we leveraged the recently developed and characterized Colon
282 and Duodenum Intestine-Chips, which combine the two most advanced approaches in the field of
283 intestinal modeling – primary human organoids and Organs-on-Chips(23, 24). Briefly, primary
284 human colon intestinal organoids are dissociated and seeded within an Organ-Chip (Fig. S6 and
285 S7A), where they form a tight, polarized barrier, containing the full range of mature intestinal
286 cell types. We have previously shown that the inclusion of luminal flow, peristaltic motion, and
287 an endothelial layer enhances the maturation and physiological fidelity of the barrier, compared
288 with organoids in conventional 3D culture(35).

289 To qualify the Intestine Chip as a platform for TCB safety assessment, we set out to
290 determine whether it 1) supports physiologically-relevant target expression and 2) can
291 successfully capture target-mediated TCB toxicity. Immunofluorescence analysis revealed robust
292 expression of CEA in the Colon Intestine-Chip epithelium, whereas expression in the Duodenum
293 Intestine-Chip appeared weaker and localized to the apical surface (Fig. 4B). Quantification of
294 transcript levels obtained from RNAseq data confirmed the higher expression of CEA in the
295 Colon Intestine-Chip, compared with the Duodenum Intestine-Chip (Fig. 4C). Thus, the Intestine
296 Chip successfully recapitulates CEA expression in the intestine, including the regional variations
297 between intestinal segments observed *in vivo*. Of note, differences in CEA expression are not
298 reproduced in colon and duodenum organoids cultured in the conventional 3D format (Fig. 4C),
299 suggesting that the Intestine-Chip is more suitable for modeling CEA-mediated toxicities,
300 compared with simpler models.

301



303 **Fig. 4. Application of Colon Intestine-Chip as Model of CEA-TCB-Mediated Adverse Effects.** (A) IHC
304 of human colon and duodenum tissue stained with anti-CEA (brown coloration) demonstrating difference
305 in regional expression. (B) Representative immunofluorescent micrograph depicting CEA expression in
306 the epithelial compartment of the Colon Intestine-Chip and Duodenum Intestine-Chip. (C) RNA-seq
307 expression levels of the CEA gene expression in healthy human tissues (in vivo), 3D organoids, and chips
308 at Day 8 of culture (n=3). (D) Colon-Chip epithelial channels were administered with PBMC treated
309 with/without low and high affinity (CEA(Lo) and CEA(Hi)) TCB (0.1-10 $\mu\text{g}/\text{mL}$), or Non-targeting (NT)
310 TCB (10 $\mu\text{g}/\text{mL}$). Co-culture was maintained under flow for 72 hours. Quantification of
311 immunofluorescent images collected live indicate multiple clusters of PBMC settled throughout epithelial
312 structures. Statistical analysis was conducted by one-way ANOVA and was defined as * $P < 0.05$ and
313 *** $P < 0.001$. Errors bars represent \pm SEM. (E) $\text{CD}69^+$ Activation of $\text{CD}8^+$ T cells of harvested PBMC
314 measured by flow-cytometry (n=3 \pm SEM). (F) Heat map of multiplex cytokine panel from epithelial
315 channel supernatants. Data (D-F) from terminal endpoint 72 hours after administration (n=3). (G)
316 Colon- and Duodenum-Chips were administered with PBMC with low and high-affinity (CEA(Lo) and
317 CEA(Hi)) TCB treatment from 0-10 $\mu\text{g}/\text{mL}$, along with Non-targeting (NT) control. Flow cytometry
318 analysis of harvested PBMC from chips 72 hours post-treatment to measure levels of activated
319 $\text{CD}69^+ \text{CD}8^+$ T cells (n=3 \pm SEM). (H) Multiplex cytokine analysis of supernatant collected from
320 epithelial channels of Colon and Duodenum-Chips after 72 hours of treatment (n=3 \pm SEM).

321 Next, we evaluated the Intestine-Chip for its ability to capture the toxic effects CEA(Hi)
322 and CEA(Lo) TCB, and expected differences therein, owing to differential binding affinity. We
323 focused first on the Colon Intestine-Chip, bearing in mind the higher levels of target in the native
324 organ and the chip model. Flow cytometry-mediated quantification verified the cell surface
325 expression of CEA within this model, revealing an average of 100,000 molecules expressed per
326 cell (Fig. S7B). Based on previous data suggesting that CEA-targeting TCBs trigger immune cell
327 activation and cancer cell killing above a threshold of 10,000 CEA molecules ((36), we expect to

328 detect on-target TCB toxicity in the Colon Intestine-Chip. Importantly, immunohistochemistry
329 analysis of organoid-derived intestinal barriers cultured in a conventional static transwell showed
330 patchy CEA expression, which appeared weaker than that observed in the native colon (Fig.
331 S7C). Consistently, we estimated an average of about 2000 CEA surface binding sites per cell in
332 this system (Fig. S7D), which is dramatically lower than the expression recorded in the Colon
333 Intestine-Chip.

334 To render the Intestine-Chips capable of simulating an immune response, we took an
335 approach analogous to that of the Lung-Chip: PBMC and TCBs were introduced using the top
336 fluidic channel of the Colon Intestine-Chip, affording direct contact with the epithelium and
337 enabling engagement with the target (Fig. S6). Epithelial cell death, immune cell attachment and
338 activation were monitored as readouts of on-target TCB safety liabilities. Unlike the Alveolus
339 Lung-Chip, TCB treatment did not lead to increased epithelial cell killing. Nonetheless, we did
340 observe significant changes in all of the other readouts of TCB-mediated toxicity. Both CEA(Hi)
341 and CEA(Lo) TCB induced dose-dependent increase in PBMC attachment (Fig. 4D) and
342 activation, as evidenced by CD69 upregulation (Fig. 4E) and the release of pro-inflammatory
343 cytokines, including granzyme B, IFN γ and IL-6 (Fig. 4F). As expected, CEA(Hi) TCB triggered
344 higher PBMC attachment, activation and cytokine release compared with CEA(Lo) TCB,
345 confirming that the model is sensitive to differences in antibody affinity, which would be
346 expected to translate into differential toxicity outcomes in the clinic. Importantly, we observed
347 no activation upon treatment with an antibody that engages the CD3 receptor on T cells but
348 cannot bind to CEA (NT TCB). Likewise, the CEA-targeting TCBs failed to induce activation of
349 PBMC only, in the absence of target tissue (Fig. S7E). Together, these data confirm that the

350 effects observed in the chips are target- and mode of action-dependent, ruling out non-specific
351 PBMC activation and cytokine release mediated by CD3 engagement only.

352 We then took advantage of the Duodenum Intestine-Chip, which faithfully mimics the
353 lower CEA expression observed in the native small intestine (Fig. 4B, C), to explore whether the
354 system is sensitive to variations in target expression. Indeed, we observed differences in on-
355 target toxicity governed by target abundance: CD69 expression and cytokine release induced by
356 both TCBs were significantly attenuated in the Duodenum Intestine-Chip, in line with the lower
357 target expression (Fig. 4E-F). The reduction was more extensive in the case of CEA(Lo) TCB,
358 which induced minimal increase in CD69 expression and no cytokine release, relative to the non-
359 targeting TCB. CEA(Hi) TCB induced PBMC activation and cytokine release which, while
360 lower than those observed in the Colon Intestine-Chip, were significantly elevated compared
361 with the non-targeting control, suggesting that the high affinity molecule may pose safety risks
362 even in tissues with low target expression as described for the high affinity FOLR1 molecule in
363 the lung.

364 **Discussion**

365 Here, we describe an Organs-on-Chips based approach for the assessment of TCB
366 toxicity in the lung and the intestine. We demonstrate that the Alveolus Lung-Chip successfully
367 recapitulated FOLR1 TCB-mediated lung toxicities observed in cynomolgus monkeys, and
368 instructed the design of a second-generation molecule, whose favorable lung safety profile
369 predicted by the chip model was verified in vivo. The Intestine-Chip model captured the
370 liabilities of a TCB targeting a human-specific antigen, thus filling the gap left by the lack of
371 cross-reactivity in animals and suitable animal models overall. The model likewise displayed
372 sensitivity to TCB affinity and predicted differential, target expression-dependent toxicity

373 outcomes between different intestinal regions. Both models were able to shed light into the
374 toxicity mechanisms of the molecules, clearly decoupling target-mediated effects from T cell
375 activation through CD3 engagement only, which has been shown to be an additional mode of
376 TCB-induced adverse events(37). It is worth mentioning that both the Lung-Chip and the
377 Intestine-Chip demonstrated an advantage over conventional models for this application: the
378 Alveolus Lung-Chip was found to report more specific TCB-responses and provide additional
379 important readouts compared with transwell-based approaches, whereas the Intestine-Chip
380 supported more physiologically relevant target expression, compared with both 3D organoids
381 and primary intestinal barrier grown in transwells.

382 Owing to the absence of predictive early-stage assays, “on-target, off-tumor” TCB safety
383 liabilities have in some cases been only detected either in late-stage preclinical models (non-
384 human primates) or as life-threatening adverse events in the clinic. Advanced human cellular
385 models that capture the immunopathology of TCB-induced adverse events would aid the iterative
386 antibody design at the early stage, thus ensuring favorable safety profiles before entering clinical
387 trials, reducing attrition rates and ultimately expediting the application of these potentially life-
388 saving therapies. Importantly, the mechanistic insights into on-target, off-tumor toxicities and
389 design opportunities afforded by these platforms are not restricted to T cell engagers like TCBs,
390 but also apply to chimeric antigen receptor T (CART) cell therapy, bearing in mind their similar
391 modes of action. For instance, a human epidermal growth factor receptor 2 (Her2) CART
392 therapy, intended to treat a patient with colorectal cancer, led to lethal toxicity through off-tumor
393 cardiopulmonary targeting(38). To improve its efficacy-safety profile, this therapy was affinity-
394 tuned to detect tumor cells with a high density of surface antigens, while sparing normal cells
395 with lower antigen expression(39). A mouse model expressing human Her2 was used to confirm

396 the safer profile of the low affinity CART(40). Using the Lung- and Intestine-Chips, we similarly
397 identified a reduced risk of healthy tissue targeting with a low affinity TCB for both the FOLR1
398 and CEA targets. However, in contrast to the humanized mouse models, these in vitro tools are
399 fully human, applicable to various targets and faster to generate, making them a promising
400 alternative for antibody/CART preclinical safety testing, format selection and optimization.

401 Furthermore, the immune-competent Organs-Chips described here can bridge preclinical
402 research to clinical application, by aiding the discovery of early predictive biomarkers of
403 TCB/CART toxicity in patients, which would certainly help to anticipate and manage life-
404 threatening adverse events. While no universally predictive markers of adverse events associated
405 with these therapies are currently accepted and validated, the phenotypic outcomes observed in
406 the chips closely match some of the few clinical indicators that have been proposed. For
407 example, early elevation of specific serum cytokines, including IFN- γ and IL-6 was found to
408 precede the development of severe cytokine release syndrome in response to CART therapy(41,
409 42). The release of IFN- γ and IL-6 was consistently observed in both the Alveolus Lung and
410 Colon Intestine-Chip upon treatment with high-affinity FOLR1 and CEA TCB, which led to on-
411 target toxicity. The detection of such clinically relevant biomarkers exemplifies the translational
412 value of our platform. Going forward, the system could be coupled with, for example, unbiased
413 proteomic analyses of chip supernatants and transcriptomic dissection of the effector and target
414 cell pools and used to uncover early novel predictors or TCB-mediated adverse events.

415 Another attractive future development of our platform is its use to evaluate the
416 therapeutic window of a therapy. We tested the same molecule in a healthy Lung-Chip versus a
417 cancer-Chip and found a 1000-fold difference in the concentration of TCB required to kill target
418 cells (Fig. S3). As a next step, an all-in-one platform could combine a healthy population with a

419 tumor one in the same chip as previously described(43), thus capturing safety liabilities triggered
420 by tumor lysis itself. Although efficacy-safety interactions are clinically relevant, these two
421 aspects are often evaluated independently prior to phase I clinical studies, owing largely to the
422 absence of fitting preclinical tools. Recent advances in organoid technology have proven the
423 concept feasible in the context of standard chemotherapies. Patient-derived organoids have been
424 shown to recapitulate patient responses in the clinic and could be implemented in personalized
425 medicine programs of metastatic gastrointestinal cancers(44). Follow-up studies with larger
426 cohorts confirmed the potential of patient-derived organoids to be adopted as in vitro companion
427 diagnostics(45, 46). The coupling of healthy and tumor biopsy-derived organoids with
428 immunocompetent Organ-Chip technology may provide the ideal setup for a combined efficacy
429 and safety assessment. In the near future, one can envision the application of these tools in a
430 more personalized fashion if standardization permits testing at the individual level.

431 In conclusion, we describe novel human immunocompetent models of the lung and
432 intestine and validate them as platforms for TCB safety profiling, outlining how these systems
433 could reduce the reliance on animal-based safety assessments, enable educated antibody format
434 selection, shed light into the mechanistic underpinnings of toxicities, and support the
435 identification of clinical biomarkers. Going forward, the concepts we introduced here can be
436 expanded to address the persisting gap in modeling immune-related toxicities, associated with,
437 for example, immune checkpoint blockade(47, 48). Considering their systemic and multifactorial
438 immunopathology, modeling these processes accurately would likely require the incorporation of
439 resident immune cells and lymphoid structures, as well as modalities that support the simulation
440 of T cell trafficking and tissue infiltration.

441

442 **Materials and Methods**

443 *Construction of FOLR1 and CEA targeted molecules*

444 FOLR1 and CEA-targeted TCB molecules were generated in the 2+1 format (two target binding
445 Fabs and one CD3 binding Fab). Heterodimerization of these bispecific antibodies is achieved by
446 using the ‘knob-into-hole’ technology(49) in which the FOLR1 Fab is N-terminally fused to the
447 CD3-Fc knob chain (head-to-tail configuration) and a second FOLR1 Fab is fused to the Fc hole
448 chain. These antibodies lack Fc effector functions due to the insertion of the PG LALA
449 mutations (P329G; L234A, L235A; (50)). In the FOLR1 TCB molecule, a common light chain
450 was used for both, the FOLR1 and CD3 Fab. Two versions of the FOLR1 TCB were generated
451 which differ in affinity. Clone 16D5 reveals high affinity (nM) whereas a variant of this clone,
452 carrying two amino acid changes (D52dE and W96Y according to Kabat numbering), exhibits an
453 affinity in the μM range.

454 The CEA TCB were generated analogously but use different light chains for CEA and CD3 Fabs.
455 To avoid light chain mispairing the CrossMabVH-VL technology(51) was applied generating a
456 VH/VL crossover in the CD3 Fab and a corresponding crossed light chain (VHCL). In addition,
457 charged residues were introduced in the constant kappa and CH1 domains of the CEA Fabs to
458 furthermore force correct light chain pairing. Two versions of the CEA targeted TCB were
459 generated which differ in affinity.

460 The genes for all chains of the TCB molecules were inserted into separate mammalian
461 expression cassettes by standard recombinant DNA technologies and expressed either transiently
462 in HEK293 cells or in stable CHO clones. Purification of bispecific TCB molecules was
463 performed according to standard Protein A affinity and size exclusion methods.

464 *Patient-derived xenograft (PDX) model*

465 All mice were maintained under specific pathogen-free condition with daily cycles of 12 hours
466 light/12 hours darkness. The animal facility has been accredited by the Association for
467 Assessment and Accreditation of Laboratory Animal Care (AAALAC). All animal studies were
468 performed in accordance with the Federation for Laboratory Animal Science Associations
469 (FELASA). The animal studies were approved by and done under license from the Government
470 of Upper Bavaria (Regierung von Oberbayern; Approval number: Az 55.2.1.54-2532.0-10-16).
471 We have complied with all relevant ethical guidelines and regulations. Animals were maintained
472 for 1 week after arrival to get accustomed to the new environment and for observation. Daily
473 continuous health monitoring and weekly body weight measurement was conducted.

474 Female NSG mice were injected intraperitoneal with 15 mg/kg of Busulfan followed one day
475 later by an intravenous injection of 1×10^5 human hematopoietic stem cells isolated from cord
476 blood. At week 14-16 after stem cell injection mice were bled sublingual and blood was analyzed
477 by flow cytometry for successful humanization. Efficiently engrafted mice were randomized
478 according to their human T cell frequencies into the different treatment groups. The human
479 breast cancer patient derived HER2+ ER- xenograft model BC_004 was purchased from
480 OncoTest (Freiburg, Germany). Tumor fragments were digested with Collagenase D and DNase
481 I (Roche), counted and 2×10^6 BC004 cells were injected in total volume of 20 μ l PBS into the
482 mammary fat pad of humanized NSG mice. Treatment with FOLR1 TCB started once weekly at
483 a dose of 0.5 mg/kg when tumor size reached approximately. 400 mm³ (day 28). Control group
484 received histidine buffer (Vehicle). All mice were injected intravenously with 200 μ l of the
485 appropriate solution.

486 Alternatively for CEA TCB assessment, mice were injected with 1×10^6 MKN45 cells and treated
487 once weekly (CEA(Hi) TCB) or twice weekly (CEA(Lo) TCB) at a dose of 2.5mg/kg when
488 tumor size reached approximately 150 mm^3 (day7). Control group received a histidine buffer
489 (Vehicle). All mice were injected intravenously with 200 μl of the appropriate solution.

490 **Alveolus Lung-Chip:**

491 *Immunohistochemistry cynomolgus monkey and human tissues*

492 Immunohistochemical staining for FOLR1 distribution in cynomolgus monkey or human
493 formalin-fixed, paraffin-embedded tissues was carried out on a Discovery XT automated slide
494 stainer using a mouse anti-human monoclonal antibody for FOLR1 (Novocastra Clone BN3.2;
495 Leica Biosystems, Wetzlar, Germany) at 15 $\mu\text{g}/\text{ml}$ after antigen retrieval with Cell Conditioning
496 1 (CC1, Ventana Medical Systems Inc.). As secondary Antibody was used a donkey anti-mouse
497 biotinylated polyclonal IgG (Jackson Immunoresearch Lab, cat: 715-065-151) at 6 $\mu\text{g}/\text{ml}$. DAB
498 Map Kit (Ventana 760-124) was used as a detection system. Xenograft tumors from FOLR1-
499 expressing HeLa cells were used as a positive control.

500 *Cell Culture*

501 Human alveolar epithelial cells (Cell Biologics, Accegen) were cultured using SABM medium
502 (Lonza) supplemented with growth factor kit and 5% v/v fetal bovine serum (FBS) in a T-25
503 flask coated with Gelatin (ATCC) until they reach 90% confluency.

504 Human microvascular lung endothelial cells (HMVEC-L) (Lonza) were cultured in EBM-2
505 Basal Medium supplemented with EGM-2 MV Microvascular Endothelial Cell Growth Medium
506 and 1% v/v Pen-Strep (ThermoFisher) according to manufacturer's instructions.

507 Peripheral blood mononuclear cells (PBMC) were isolated from fresh human buffy coat
508 (Research Blood Components) using immunomagnetic negative selection (Stem Cell
509 Technologies) and cultured in RPMI-1640 (Gibco) supplemented with 10% v/v FBS
510 (ThermoFisher) and 1% v/v Pen-Strep (ThermoFisher) or cryo-preserved in FBS containing 10%
511 dimethyl sulfoxide (DMSO) before use.

512 *Alveolus Lung-Chip*

513 The design and fabrication of Organ-Chips has been previously described(52). Briefly, the S-1
514 Chips are composed of transparent polydimethylsiloxane (PDMS) containing two parallel
515 microchannels: an epithelial channel (1 x 1 mm) and vascular channel (200 μm x 1 mm)
516 separated by a porous membrane. The chip protocol was performed according to the
517 manufacturer's instructions (Alveolus Lung-Chip Culture Protocol, Emulate Inc.). S-1 chip
518 microchannels were functionalized to covalently attach extracellular matrix proteins (ECM)
519 before seeding using ER solutions (Emulate Inc.). Chip channels were then coated with a mixture
520 of ECM in Dulbecco's phosphate-buffered saline (DPBS): 200 $\mu\text{g}/\text{mL}$ human placenta collagen
521 type IV (Sigma-Aldrich) and 30 $\mu\text{g}/\text{mL}$ fibronectin (Gibco) for the vascular channel; and 200
522 $\mu\text{g}/\text{mL}$ human placenta collagen type IV (Sigma-Aldrich) and 30 $\mu\text{g}/\text{mL}$ human plasma
523 fibronectin (Corning) and 5 $\mu\text{g}/\text{mL}$ human placenta laminin (Sigma-Aldrich) for the epithelial
524 channel. Chips were then incubated overnight at 37°C for coating and channels were washed
525 next day with their respective growth medium before seeding. Human Alveolar Epithelial Cells
526 (HPAECs) were seeded at a density of 0.5×10^6 cells/mL following protocols (Alveolus Lung-
527 Chip Culture Protocol, Emulate Inc.). Human Microvascular Lung Endothelial Cells (HMVEC-
528 L) were seeded at a density of 5×10^6 cells/mL following the Alveolus-Lung Chip protocol
529 (Emulate Inc.). Air-Liquid Interface is introduced on day 5 of culture following the protocol and

530 maintained for 4 days. On the day before dosing, hydrocortisone was removed from the bottom
531 channel growth medium.

532 *PBMC Administration*

533 After 4 days of culture under air-liquid interface, culture medium was re-introduced in the
534 epithelial channel before dosing with PBMC-TCB. 500 μ L of dosing media (M199 +2% v/v
535 FBS) was added to the epithelial inlet reservoir. Liquid -Liquid interface was re-introduced at
536 1000 μ L/h for 5 minutes on the epithelial channel, keeping the vascular channel at 0 after which
537 the flow was switched to 30 μ L/h in both channels.

538 Frozen PBMC after thawing were suspended overnight at 4×10^6 cells/mL in complete RPMI-
539 1640 medium with 10% v/v FBS. The viability of PBMC was determined by using trypan blue
540 exclusion assay. PBMC were allowed to rest overnight at 37°C. The following day, PBMC were
541 stained using cell tracker green (ThermoFisher) according to the manufacturer's instructions.
542 PBMC dosing solutions (Dosing media: M199 (ThermoFisher) + 2% v/v FBS) were prepared by
543 incubating cell suspensions at 2×10^6 cells/mL in media containing TCBs at different
544 concentrations for an hour at 37°C prior to administration. After the incubation period, the
545 epithelial channel inlets were aspirated and 500 μ L of dosing solution was added to the inlet.
546 PBMC were administered to the chips at 1000 μ L/h for 5 minutes. After PBMC administration,
547 the system was left static for 3 hours before starting flow at 30 μ L/h with fresh dosing media
548 (M199 + 2% v/v FBS) without TCBs in the epithelial channel and custom ALI media without
549 hydrocortisone (Alveolus Lung-Chip Protocol, Emulate Inc.) in the vascular channel.

550 *Target Expression*

551 For quantification of target expression, HPAECs (Day 0, Day 5, Day 10 of chip culture) were
552 recovered using TrypLE Express Enzyme (Gibco) at 37°C for 10 minutes. Epithelium from
553 Alveolus Lung-Chips cultured to Day 5 and Day 10 was obtained by filling both channels with
554 TrypLE solution incubating at 37°C until complete dissociation was achieved using gentle
555 pipetting. The dissociated epithelium was collected from the epithelial channel and digestion was
556 quenched using SAGM culture medium with 2% v/v FBS. All single-cell samples were
557 distributed at 0.5×10^6 cells/mL for live staining with monoclonal mouse anti-human FOLR1
558 IgG1 (LS Bio) in DPBS with 2% v/v FBS (Sigma). Secondary staining for target was performed
559 using QIFIKIT® (BIOCYTEX) anti-mouse IgG, along with mouse IgG1 Isotype FOLR1 (L.S
560 Bio) for secondary control and provided calibration and standard beads. Samples were run with
561 BD FACSCelesta™ flow cytometer (BD Biosciences), and data analyzed using FlowJo V10
562 software (FlowJo).

563 *RNA Isolation*

564 Total RNA was isolated from the Alveolus Lung-Chip using TRIzol reagent (Sigma) following
565 manufacturer's instructions and flash frozen in liquid nitrogen. Samples were sent to GENEWIZ
566 for sequencing.

567 *RNA Sequencing Bioinformatics*

568 The RNA sequencing was performed using the Illumina TruSeq paired-end sequencing platform
569 with read length 2x150 bp and sequencing depth ~28M paired end reads/sample. To remove poor
570 quality adapter sequences and nucleotides, we trimmed the sequence reads using the
571 Trimmomatic v.0.36. The STAR (Spliced Transcripts Alignment to a Reference) aligner v.2.5.2b
572 was used to map the trimmed reads to the Homo sapiens reference genome GRCh38 (available
573 on ENSEMBL) and generate the BAM files. Using the featureCounts from the Subread package

574 v.1.5.2 we calculated the unique gene hit counts. Only unique reads that fell within exon regions
575 were counted. Note that since a strand-specific library preparation was performed, the reads were
576 strand-specifically counted. Using the gene hit counts and the corresponding gene lengths we
577 calculated the FPKM (Fragments Per Kilobase of exon per Million reads mapped) gene
578 expression levels.

579 *Live Staining and Imaging*

580 For timepoints T= 24 and 48 hours after PBMC-TCB administration, effluents were collected for
581 further analysis and pod inlets were aspirated. NucView405® Caspase-3 Enzyme, fluorescent
582 caspase 3/7 substrate for detecting apoptosis by live staining (Biotium) at 2 μ M was prepared
583 with dosing medium (M199 +2% v/v FBS). 500 μ L of the live stain was added to the epithelial
584 channel inlet reservoirs. Epithelial channel of the chips was flowed at 1000 μ L/h for 5 minutes
585 while setting the vascular channel to 0. Flow was then reset to 30 μ L/h for both the channels for
586 30 minutes, fresh media was then flushed through after incubation. Chips were then transferred
587 to fluorescent microscope (Olympus IX83 Inverted Microscope) one at a time for live imaging.
588 Additional brightfield images were captured using the Echo Revolve microscope.

589 *Flow Cytometry*

590 PBMC were harvested from the Alveolus Lung-Chip epithelial channel at T=48 hours (terminal
591 timepoint) after dosing with PBMC-TCB, by repeated washing using 200 μ L tips by blocking the
592 chip inlet. PBMC from each chip was transferred to a V bottom 96 well-plate and washed with
593 DPBS + 1% v/v FBS solution before staining with surface markers. Master mix of surface
594 markers was prepared in Brilliant Buffer solution (BD BioSciences) which consisted of anti-
595 human Alexa Fluor® 700 anti-human CD3 (BioLegend, cat. 300324), Brilliant Violet 785™
596 anti-human CD4 (BioLegend, cat. 317442) and Brilliant Violet 650™ anti-human CD69

597 (BioLegend, cat. 310934). Harvested PBMC was stained with the prepared master mix for 20
598 minutes at 4°C and fixed using 1% v/v paraformaldehyde in DPBS for 15 minutes at room
599 temperature. Samples collected were then washed with DPBS + 1% v/v FBS solution and stored
600 in 4°C and read within 3 days.

601 Sample data was acquired using BD FACSCelesta™ flow cytometer (BD BioSciences) and
602 data was analyzed using FlowJo V10 software (FlowJo).

603 *Immunofluorescence microscopy*

604 For alveolar epithelial cell staining, samples were fixed with 4% paraformaldehyde (Electron
605 Microscopy Sciences) for 20 minutes at room temperature. Samples were then washed twice
606 with DPBS and perfused with 100 mM glycine to quench autofluorescence for 30 minutes at
607 room temperature, then rinsed with DPBS and permeabilized with 0.1% v/v Triton-X for 10
608 minutes and blocked with 1% v/v BSA and 5% v/v Normal Donkey serum in DPBS for 30
609 minutes. Samples were then stained with primary antibodies overnight at 4°C, with the following
610 primary antibodies diluted 1:100 in 2% v/v BSA in DPBS and then rinsed twice with DPBS
611 before staining with secondary antibodies diluted 1:200 in 2% v/v BSA in DPBS for 2 hours in
612 the dark at room temperature, and counterstained with NucBlu (ThermoFisher) following the
613 manufacturer's instructions. The primary antibodies used were rabbit polyclonal anti-E-Cadherin
614 (abcam), mouse monoclonal anti-human FOLR1 IgG1 (LS Bio). Secondary antibodies used were
615 donkey anti mouse or rabbit Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647-conjugated
616 antibodies (Abcam), goat anti-mouse IgM (Heavy chain) Alexa Fluor 488 (ThermoFisher, A-
617 21042). Immunofluorescence microscopy was performed using an Inverted Olympus IX83
618 microscope and Echo Revolve (Echo). At least 5 fields of view were taken per chip along the co-
619 culture channel.

620 *Image Analysis*

621 Image analysis was performed using ICY software (BioImage Analysis Lab, Institut Pasteur) to
622 quantify PBMC attachment (CellTracker Green) to the alveolus epithelium and apoptotic
623 (NucView405 Caspase-3) alveolar epithelial cells.

624 *Cytokine Analysis*

625 At T= 24 and 48 hours after PBMC-TCB administration, effluents were collected from Alveolus
626 Lung-Chip Pod outlets. Effluents were then immediately frozen at -80°C until measurement.
627 Measurement of cytokines for Alveolus Lung-Chip (GranzymeB, IFN γ , IL-2, IL-6, IL-8, IL-10,
628 IL-13, IL1RA, TNF α , MIP-1 β , G-CSF, GM-CSF) was performed using customized Invitrogen
629 ProcartaPlex multiplex immunoassays (reference PPX-12-MXNKRV6). Each kit contained a
630 black 96-well plate (flat bottom plate), antibody-coated beads, detection antibody, streptavidin-
631 R-phycoerythrin (SAPE), reading buffer and universal assay buffer. In addition, standards with
632 known concentration were provided to prepare a standard curve. According to the Invitrogen
633 Publication Number MAN0017081 (Revision B.0 (33)), the assay workflow was the following.
634 After adding the beads into the flat bottom plate, the beads were washed using a flat magnet and
635 an automated plate washer (405TS microplate washer from Biotek). Then standards and
636 samples diluted with a universal buffer were added into the plate and a first incubation started for
637 2h. After a second wash, detection antibodies were added. After 30 min incubation and a wash,
638 SAPE was added. Finally, after 30min incubation and a last wash, the beads were resuspended in
639 the reading buffer and the plates were ready for analysis.

640 The data was acquired with a Luminex™ instrument, BioPlex-200 system from Bio-Rad. Using
641 the Certificate of Analysis provided with the kit, bead region and standard concentration value
642 S1 for each analyte of the current lot were entered in the software, BioPlex Manager. Plotting the

643 expected concentration of the standards against the Mean Fluorescent Intensity (MFI) generated
644 by each standard, the software generated the best curve fit and calculated the concentrations of
645 the unknown samples (in pg/mL). The data were then exported in Excel and plotted in Graphpad
646 Prism.

647 *Surface Plasmon Resonance*

648 The avidity of the interaction between the anti-FOLR1 T cell bispecifics and the recombinant
649 folate receptors was determined as described below. Recombinant biotinylated monomeric Fc
650 fusions of human and cynomolgus Folate Receptor 1 (FOLR1-Fc, produced in house) were
651 directly coupled on a SA chip using the standard coupling instruction (Biacore, Cytiva). The
652 immobilization level was about 200-300 RU. The anti-FOLR1 T cell bispecifics were passed at a
653 concentration range from 11.1 to 900 nM with a flow of 30 μ L/minutes through the flow cells
654 over 180 seconds. The dissociation was monitored for 240 or 600 seconds. The chip surface was
655 regenerated after every cycle using a double injection of 30 sec 10 mM Glycine-HCl pH 1.5.
656 Bulk refractive index differences were corrected for by subtracting the response obtained on
657 reference flow cell immobilized with recombinant biotinylated murine IL2R Fc fusion (unrelated
658 Fc fused receptor). The binding curves resulting from the bivalent binding of the T cell
659 bispecifics were approximated to a 1:1 Langmuir binding (even though it is a 1:2 binding) and
660 fitted with that model to get an apparent KD representing the avidity of the bivalent binding. The
661 apparent avidity constants for the interactions were derived from the rate constants of the fitting
662 using the Bia Evaluation software (Cytiva).

663 The affinity of the interaction between the anti-FOLR1 T cell bispecifics and the recombinant
664 folate receptors was determined as described below. For affinity measurement, direct coupling of
665 around 12000 resonance units (RU) of the anti-human Fab specific antibody (Fab capture kit,

666 Cytiva) was performed on a CM5 chip at pH 5.0 using the standard amine coupling kit (Cytiva).
667 Anti-FOLR1 T cell bispecifics were captured at 20 nM with a flow rate of 10 μ l/min for 40 sec,
668 the reference flow cell was left without capture. Dilution series (12.3 to 3000 nM) of human and
669 cyno Folate Receptor 1 Fc fusion were passed on all flow cells at 30 μ l/min for 240 sec to record
670 the association phase. The dissociation phase was monitored for 300 s and triggered by switching
671 from the sample solution to HBS-EP. The chip surface was regenerated after every cycle using a
672 double injection of 60 sec 10 mM Glycine-HCl pH 2.1. Bulk refractive index differences were
673 corrected for by subtracting the response obtained on the reference flow cell 1. The affinity
674 constants for the interactions were derived from the rate constants by fitting to a 1:1 Langmuir
675 binding using the Bia Evaluation software (Cytiva).

676 *Binding of FOLR1-targeted TCBS to human FOLR1-expressing tumor cells*

677 Experiments were performed with n=4 chips per condition and 8 to 10 fields of view per chip.
678 All graphs are plotted as group means (individual points displayed if n<5 samples per group) \pm
679 SEM. Statistical significance ($p < 0.05$) was determined by One-way or Two-way ANOVA using
680 Tukey's multiple comparison test.

681 *Assessment of FOLR1 TCBS binding to human FOLR1 expressed on HeLa cells*

682 The binding of FOLR1 TCBS to human FOLR1 was assessed on HeLa cells. Briefly, cells were
683 harvested, counted, checked for viability and resuspended at 2×10^6 cells/ml in FACS buffer (100
684 μ l PBS 0.1% BSA). 100 μ l of cell suspension (containing 0.2×10^6 cells) was incubated in round-
685 bottom 96-well plates for 30 minutes at 4°C with different concentrations of the bispecific
686 antibodies (30 pM - 500 nM). After two washing steps with cold PBS 0.1% BSA, samples were
687 re-incubated for further 30 minutes at 4°C with FITC-conjugated AffiniPure F(ab')₂ Fragment
688 goat anti-human IgG Fcγ Fragment Specific secondary antibody (Jackson Immuno Research Lab

689 PE # 109-096-098). After washing the samples twice with cold PBS, samples were resuspended
690 in PBS 0.1% BSA and analyzed on a FACS Canto II (Software FACS Diva). Binding curves
691 were obtained using GraphPadPrism6.

692 *TCB-mediated lysis of tumor cells in vitro*

693 T-cell killing mediated by FOLR1 TCBs was assessed on HeLa (high FOLR1) cells. Human
694 PBMC were used as effectors and the killing was detected at 24 hours of incubation with the
695 bispecific antibodies. Briefly, target cells were harvested with Trypsin/EDTA, washed, and
696 plated at a density of 25,000 cells/well using flat-bottom 96-well plates. Cells were left to adhere
697 overnight. Peripheral blood mononuclear cells (PBMC) were prepared by Histopaque density
698 centrifugation of enriched lymphocyte preparations (buffy coats) obtained from healthy human
699 donors. Fresh blood was diluted with sterile PBS and layered over Histopaque gradient (Sigma,
700 #H8889). After centrifugation (450 x g, 30 minutes, room temperature), the plasma above the
701 PBMC-containing interphase was discarded and PBMC transferred in a new falcon tube
702 subsequently filled with 50 ml of PBS. The mixture was centrifuged (400 x g, 10 minutes, room
703 temperature), the supernatant discarded and the PBMC pellet washed twice with sterile PBS
704 (centrifugation steps 350 x g, 10 minutes). The resulting PBMC population was counted (ViCell)
705 and stored in RPMI1640 medium containing 10% FCS and 1% L-alanyl-L-glutamine (Biochrom,
706 K0302) at 37°C, 5% CO₂ in cell incubator until further use. For the killing assay, the antibody
707 was added at the indicated concentrations (range of 0.01 pM – 10 nM in triplicates). PBMC were
708 added to target cells at final E:T ratio of 10:1. Target cell killing was assessed after 24 hours of
709 incubation at 37°C, 5% CO₂ by quantification of LDH released into cell supernatants by
710 apoptotic/necrotic cells (LDH detection kit, Roche Applied Science, #11644793001). Maximal
711 lysis of the target cells (= 100%) was achieved by incubation of target cells with 1% Triton X-

712 100. Minimal lysis (= 0%) refers to target cells co-incubated with effector cells without
713 bispecific construct.

714 *Statistics*

715 Experiments were performed with n=4 chips per condition and 8 to 10 fields of view per chip.
716 All graphs are plotted as group means (individual points displayed if n<5 samples per group) ±
717 SEM. Statistical significance ($p < 0.05$) was determined by One-way or Two-way ANOVA using
718 Tukey's multiple comparison test unless specified otherwise.

719 **Intestine-Chip**

720 *Binding of CEA-targeted TCBs to human CEA-expressing tumor cells*

721 MKN45 (DSMZ ACC 409) cells were harvested using Cell Dissociation Buffer, washed once
722 with PBS and re-suspended in FACS buffer (PBS + 0.1% BSA). 200,000 cells were seeded into
723 a 96 well round bottom plate, the assay plate was centrifuged at 400xg for 4 min and the
724 supernatant was removed. Antibody dilutions were prepared in FACS-buffer to cover a final
725 concentration range of 0.03 nM – 500 nM (1:4 dilution steps). Cells were incubated with
726 CEA(Hi) TCB and CEA(Lo) TCB for 30 min at 4°C. FACS plates were washed twice with 150
727 µl FACS buffer and incubated with 25 µl of the FITC-labeled AffiniPure F(ab')₂ Fragment Goat
728 Anti-Human IgG secondary antibody (Jackson Immuno Research, 109-096-008; pre-diluted 1:40
729 with FACS buffer) for another 30 min at 4°C. After two washing steps with FACS buffer, cells
730 were fixed in FACS buffer, containing 2 % paraformaldehyde for 30 min at 4°C. Finally
731 fluorescence was measured using BD FACS Canto II. EC50 values were calculated using
732 GraphPadPrism.

733 *TCB-mediated lysis of tumor cells in vitro*

734 TCB-induced lysis of CEA-positive target cells was assessed using MKN45 (DSMZ ACC 409)
735 cells. Human PBMCs were used as effectors and the killing was detected at 24 hours and 48
736 hours of incubation with the bispecific antibodies. Briefly, target cells were harvested with
737 Trypsin/EDTA, washed, and plated at a density of 30 000 cells/well using flat-bottom 96-well
738 plates. Cells were left to adhere overnight. For the killing assay, the antibody was added at the
739 indicated concentrations (range of 6 pM – 100 nM for CEA(Lo) TCB and 1.3 pM - 20 nM for
740 CEA(Hi) TCB in triplicates). PBMCs were added to target cells at final ratio to tumor cells of
741 10:1. Target cell killing was assessed after 48h of incubation at 37°C, 5% CO₂ by quantification
742 of LDH released into cell supernatants by apoptotic/necrotic cells (LDH detection kit, Roche
743 Applied Science, #11 644 793 001). Maximal lysis of the target cells (= 100%) was achieved by
744 incubation of target cells with 1% Triton X-100. Minimal lysis (= 0%) refers to target cells co-
745 incubated with effector cells without bispecific construct.

746 *Quantification of T cell activation in response to TCB treatment*

747 Tumor cell lysis assay plates were centrifuged (400xg for 4 min), cells were resuspended,
748 washed with FACS buffer and incubated with 25 µl of the diluted CD4/CD8/CD69 antibody mix
749 for 30 min at 4°C (e.g. PE/Cy7 anti-human CD4 #557852, FITC anti-human CD8 #555634, APC
750 anti-human CD25 #555434, as indicated). Cells were washed twice to remove unbound antibody,
751 and finally resuspended in 200 µl FACS buffer containing PI (propidium iodide) to exclude dead
752 cells for the FACS measurement. Fluorescence was measured using BD FACS CantoII.

753 *Assessment of TCB binding to human- or cynomolgus monkey-derived CEA*

754 HEK293T cells were transiently transfected to overexpress either human or cynomolgus monkey
755 CEACAM5 were harvested using Cell Dissociation Buffer, washed once with PBS and re-
756 suspended in FACS buffer (PBS + 0.1% BSA). 100,000 cells were seeded into a 96 well round

757 bottom plate, the assay plate was centrifuged at 400xg for 4 min and the supernatant was
758 removed. Antibody dilutions were prepared in FACS-buffer to cover a final concentration range
759 of 7.6 pM– 500 nM (1:4 dilution steps), respective 125 nM and 500 nM of the positive control
760 antibody binding to cynomolgus monkey CEACAM5 (clone 28A9, internal production, ID
761 AB03195). Cells were incubated with CEA(Lo) TCB or positive reference molecule for 30 min
762 at 4°C. FACS plates were washed twice with 150 µl FACS buffer and incubated with 25 µl of
763 the FITC-labeled AffiniPure F(ab')₂ Fragment Goat Anti-Human IgG secondary antibody
764 (Jackson Immuno Research, 109-096-008; pre-diluted 1:40 with FACS buffer) for another 30
765 min at 4°C. After two washing steps with FACS buffer, cells were stained with a live/dead dye
766 (DAPI, diluted in PBS) for 30 min at 4°C. After a final washing step with FACS buffer,
767 fluorescence was measured using a BD FACS CantoII.

768 *Cell Culture*

769 Human colon organoid cultures (colonoids) were established from biopsies obtained during
770 surgical procedures utilizing methods developed by the laboratory of Dr. Hans Clevers(53). De-
771 identified biopsy tissue was obtained from healthy adult subjects who provided informed consent
772 at Johns Hopkins University and all methods were carried out in accordance with approved
773 guidelines and regulations. All experimental protocols were approved by the Johns Hopkins
774 University Institutional Review Board (IRB). Routine expansion of colonoids was performed by
775 embedding isolated intestinal crypts in droplets of growth factor–reduced Matrigel (Corning) and
776 cultured in Human IntestiCult™ Organoid Growth Medium (StemCell) supplemented with 10
777 mmol/L Y-27632 (Sigma), 5 mmol/L CHIR99021 (ReproCell) and 50 mg/mL primocin
778 (InvivoGen). After 3 days, colonoids Y-27632 and CHIR99021 supplements were removed.
779 Colonoids were passaged every 7 days.

780 Human Large Intestine Microvascular Endothelial Cells (cHIMEC) (Cell Systems) were thawed
781 at passage 5 and cultured in Endothelial Cell Growth Medium (EGM-2MV) (PromoCell)
782 supplemented with Endothelial Cell Growth Medium MV2 Supplement Pack (PromoCell) and
783 1% v/v primocin (InvivoGen).

784 Peripheral blood mononuclear cells (PBMC) were isolated from fresh human buffy coats using
785 immunomagnetic negative selection (Stem Cell Technologies) and cultured in RPMI-1640
786 (Gibco) supplemented with 10% v/v FBS and 1% v/v Pen-Strep or cryo-preserved in FBS
787 containing 10% dimethyl sulfoxide (DMSO) before use.

788 *Colon Intestine-Chip Culture*

789 The design and fabrication of Organ-Chips has been previously described (52). Briefly, the S-1
790 Chips are composed of transparent polydimethylsiloxane (PDMS) containing two parallel
791 microchannels: an epithelial channel (1 x 1 mm) and vascular channel (200 μm x 1 mm)
792 separated by a porous membrane. S-1 chip microchannels were functionalized to covalently
793 attach extracellular matrix proteins (ECM) before seeding using ER solutions (Emulate Inc.)
794 following provided protocols (Basic Organ-Chip Culture Protocol, Emulate Inc.). Chip channels
795 were then coated with a mixture of ECM in Dulbecco's phosphate-buffered saline (DPBS): 200
796 $\mu\text{g}/\text{mL}$ human placenta collagen type IV (Sigma-Aldrich) and 30 $\mu\text{g}/\text{mL}$ fibronectin (Gibco) for
797 the vascular channel; and 200 $\mu\text{g}/\text{mL}$ human placenta collagen type IV (Sigma-Aldrich) and
798 100 $\mu\text{g}/\text{mL}$ Matrigel (Corning) for the epithelial channel. Chips were then incubated overnight at
799 37°C for coating and channels were washed the next day with their respective growth medium.
800 Human colonic microvascular endothelial cells (cHIMECs) were then seeded into the vascular
801 channel at a density of 8×10^6 million cells/mL. After 1.5h, the chips were inverted and cHIMECs
802 at a density of 8×10^6 million cells/mL were seeded again creating a contiguous vascular tube.

803 Colonoids were recovered from Matrigel and fragmented as reported previously (24).
804 Fragmented colonoids were suspended in organoid expansion medium at a density of 2-3 culture
805 wells per chip and seeded onto the membrane of the epithelial channel.
806 The following day, vascular and epithelial channels were washed with EGM-2MV and organoid
807 expansion medium, respectively, and connected in Pod portable modules (Basic Research Kit;
808 Emulate, Inc). The Human Emulation System (Emulate Inc.) was continuously perfused at 30
809 $\mu\text{L}/\text{hour}$ for both channels with 2% cyclic stretching (0.15 Hz) from day 2-5, then with 10%
810 cyclic stretching (0.15 Hz) until day 8 of culture. Supplements were removed from the epithelial
811 channel media after day 2 of culture.

812 *PBMC Administration*

813 Twenty-four hours prior to PBMC-TCB administration, freshly isolated or thawed PBMC were
814 suspended at 4×10^6 cells/mL in complete RPMI-1640 medium. The viability of PBMC was
815 determined by using trypan blue exclusion assay. The acceptance criteria for PBMC viability
816 was $>85\%$ to proceed to the next experimental step. PBMC were allowed to rest overnight at
817 37°C . The following day, PBMC dosing solutions were prepared by incubating cell suspensions
818 in media containing TCBs at different concentrations for four hours at 37°C prior to
819 administration. Epithelial channel TCB dosing media was also prepared by adding TCBs to
820 organoid growth media. After the incubation period, the Pod epithelial channel inlets were
821 aspirated and $500 \mu\text{L}$ of dosing solution (approx. 2×10^6 PBMC cells) was added to the inlet.
822 PBMC were administered to the chips at $1000 \mu\text{L}/\text{h}$ for 10 minutes. After PBMC administration,
823 dosing media with and without TCBs was then added to the epithelial channel inlet and perfused
824 through the chip. The vascular channels were perfused with EGM2-MV complete growth media.

825 *Target Expression*

826 For quantification of target expression, colonic organoids (Day 0 of chip culture) were recovered
827 from Matrigel following standard procedure, then digesting in TrypLE Express Enzyme (Gibco)
828 in DPBS at 37°C for 10 minutes to single cells. Epithelium from Colon Intestine-Chips cultured
829 to Day 5 and Day 8 from the same organoid culture was obtained by filling both channels with
830 TrypLE solution in PBS and incubating at 37°C for 20 minutes or until complete dissociation
831 was achieved using gentle pipetting. The dissociated epithelium was collected from the epithelial
832 channel and digestion was quenched using Advanced DMEM/F-12 (Gibco). All single-cell
833 samples were distributed at 5×10^5 cells/mL for live staining with mouse anti-human CEA IgG
834 (Santa Cruz) in DPBS with 2% FBS (Sigma). Secondary staining for target was performed using
835 QIFIKIT® (BIOCYTEX) anti-mouse IgG, along with mouse IgG1 Isotype CEA (BioLegend) for
836 secondary control and provided calibration and standard beads. Samples were run with BD
837 FACSCelesta™ flow cytometer (BD Biosciences), and data analyzed using FlowJo V10
838 software (FlowJo).

839 *RNA isolation*

840 Total RNA was isolated from Intestine-Chips using TRIzol reagent (Sigma) following
841 manufacturer's instructions and flash frozen in liquid nitrogen. Samples were sent to GENEWIZ
842 for sequencing.

843 *RNA Sequencing Bioinformatics*

844 The RNA sequencing for colon and duodenum samples was performed using the Illumina
845 TruSeq paired-end sequencing platform with read length 2x150 bp and sequencing depths ~54M
846 and ~64M paired end reads/sample respectively. To remove poor quality adapter sequences and
847 nucleotides, we trimmed the sequence reads using the Trimmomatic v.0.36. The STAR (Spliced
848 Transcripts Alignment to a Reference) aligner v.2.5.2b was used to map the trimmed reads to the

849 Homo sapiens reference genome GRCh38 (available on ENSEMBL) and generate the BAM
850 files. Using the featureCounts from the Subread package v.1.5.2 we calculated the unique gene
851 hit counts. Note that only unique reads that fell within exon regions were counted. Using the
852 gene hit counts and the corresponding gene lengths we calculated the FPKM (Fragments Per
853 Kilobase of exon per Million reads mapped) gene expression levels. For comparison to tissue,
854 we used previously published datasets(54).

855 *Live Staining and Imaging*

856 For timepoints T=0, 12, 48 and 72 hours after PBMC-TCB administration, effluents were
857 collected for further analysis and Pod inlets were aspirated. CellEvent™ Caspase-3/7 Green live
858 staining detection reagent (Thermofisher Scientific) at 2 μM was prepared and added to the
859 epithelial and vascular channels in order to visualize an apoptotic T-cell killing response. Pod
860 inlets were aspirated and 300 μL of live staining solution was added to each respective inlet.
861 Chips were flowed at 1000 μL/hr for 10 minutes to flush, then flow was paused to incubate the
862 stain at 37°C for 30 minutes. Fresh media was flushed through after incubation and chips were
863 transferred to a confocal laser-scanning microscope (Inverted Zeiss LSM 880, Zeiss) in small
864 groups for live imaging.

865 *Flow Cytometry*

866 PBMC were harvested from Colon Intestine-Chip epithelial channels at the final timepoint after
867 administration. PBMC were washed with DPBS and stained with 2 μM live/dead™ Fixable
868 Yellow Dead Cell Stain (ThermoFisher) and washed in DPBS. PBMC were then fixed with BD
869 Cytotfix (BD Biosciences) fixation solution, washed in DPBS and either resuspended in 90%
870 FBS (Sigma) + 10% DMSO solution and frozen at -20°C until use or stained immediately.
871 Samples to be stained for surface markers were washed in DPBS and resuspended in Cell

872 Staining Buffer (Biolegend). Surface marker stains were prepared in BD /Cytoperm™ solution
873 (BD Biosciences) and consisted of anti-human CD3 APC-Cy7 (BioLegend), anti-human CD4
874 Brilliant Violet 786 (BioLegend), anti-human-CD8-PE/Dazzle-594 (BioLegend), and anti-human
875 CD69 APC (BioLegend).

876 Sample data was acquired using the BD FACSCelesta™ flow cytometer (BD Biosciences), and
877 data analyzed using FlowJo V10 software (FlowJo).

878 *Immunofluorescence microscopy*

879 Colon Intestine-Chip and Transwell samples were fixed with 4% paraformaldehyde (PFA)
880 (Electron Microscopy Sciences). Samples were then washed twice using DPBS and perfused
881 with a 0.3 M glycine in DPBS (Sigma) solution to remove residual PFA. Chips were cut in half
882 and stored in DPBS and 0.05% sodium azide. Samples were stained overnight at 4°C with the
883 following primary antibodies diluted in CytoPerm/Wash buffer (BD Biosciences): recombinant
884 rabbit anti-CEA (Abcam) for samples without TCB treatment and monoclonal rat anti-CD45
885 (Invitrogen). After overnight incubation, the chips were washed three times in DPBS and nuclei
886 were counter-stained with DRAQ5™ (Thermofisher Scientific) and secondary antibody
887 DyLight™ 405 AffiniPure Donkey Anti-Rat IgG (H+L) (Jackson ImmunoResearch) diluted in
888 Perm/wash buffer. For samples without TCB treatment, the rabbit anti-CEA was stained with the
889 secondary antibody, donkey anti-rabbit Alexa Fluor-555 (Invitrogen). For samples with TCB
890 treatment, a secondary goat anti-human Alexa Fluor-555 (Invitrogen) was used as the target sites
891 were bound with anti-human TCB after administration. Remaining live imaging signal from
892 CellEvent™ Detection Reagent was also imaged for all samples.

893 Confocal laser-scanning microscopy was performed using an Inverted Zeiss LSM 880 (Zeiss). At
894 least three fields of view were taken per chip, from separate random locations along the co-

895 culture channel. Widefield tile images were also acquired on Axio Observer.Z1 (Zeiss) (n=5, per
896 chip co-culture channel).

897 *Image Analysis*

898 Image analysis was performed using the image analysis suite Fiji (National Institute of Health) to
899 quantify PBMC attachment to the Colon Intestine-Chip epithelium. Cell Event Green positive
900 signal was visualized using confocal laser-scanning microscopy and quantified in Fiji.

901 *Analysis of Cytokines*

902 At T=0, 24, 48 and 72 hours after PBMC-TCB administration, effluents were collected from
903 Colon Intestine-Chip Pod inlets and outlets. Effluents were centrifuged to remove debris and
904 then frozen at -20°C until measurement. Measurement of cytokines for Colon Intestine-Chip
905 (IFN γ , TNF α , Granzyme-B, IL-2, IL-4, and IL-8) was performed using customized Invitrogen
906 ProcartaPlex multiplex immunoassays (reference PPX-12-MXNKRV6). Each kit contained a
907 black 96-well plate (flat bottom plate), antibody-coated beads, detection antibody, streptavidin-
908 R-phycoerythrin (SAPE), reading buffer and universal assay buffer. In addition, standards with
909 known concentration were provided to prepare a standard curve. According to the Invitrogen
910 Publication Number MAN0017081 (Revision B.0 (33)), the assay workflow was the following.
911 After adding the beads into the flat bottom plate, the beads were washed using a flat magnet and
912 an automated plate washer (405TS microplate washer from Biotek). Then standards and
913 samples diluted with a universal buffer were added into the plate and a first incubation started for
914 2h. After a second wash, detection antibodies were added. After 30 min incubation and a wash,
915 SAPE was added. Finally, after 30min incubation and a last wash, the beads were resuspended in
916 the reading buffer and the plates were ready for analysis.

917 The data was acquired with a Luminex™ instrument, BioPlex-200 system from Bio-Rad. Using
918 the Certificate of Analysis provided with the kit, bead region and standard concentration value
919 S1 for each analyte of the current lot were entered in the software, BioPlex Manager. Plotting the
920 expected concentration of the standards against the Mean Fluorescent Intensity (MFI) generated
921 by each standard, the software generated the best curve fit and calculated the concentrations of
922 the unknown samples (in pg/mL). The data were then exported in Excel and plotted in Graphpad
923 Prism.

924 *Statistics*

925 Experiments were performed with at least triplicates for each chip sample per group. Brightfield
926 images of chips were collected including at least three fields of view per chip at various points
927 throughout the co-culture area of the Intestine-Chips. All graphs are plotted as group means
928 (individual points displayed if $n < 10$ samples per group) \pm SEM. Statistical significance ($p <$
929 0.05) was determined via One-way or Two-way ANOVA using Tukey's multiple comparisons
930 unless specified otherwise.

931 *Immunohistochemistry human tissues*

932 Immunohistochemical staining for CEA expression in formalin-fixed, paraffin-embedded human
933 intestinal tissues was carried out on a Discovery Ultra automated slide stainer using a rabbit anti-
934 human monoclonal antibody for CEA (Clone T84.66, Roche Glycart AG, Switzerland) at 2.23
935 $\mu\text{g/ml}$ after antigen retrieval with Cell Conditioning 1 (CC1, Ventana Medical Systems Inc.) on
936 tissues. As secondary Antibody was used a donkey anti-rabbit biotinylated polyclonal IgG
937 (Jackson ImmunoResearch Lab, cat: 711-065-152) at 5 $\mu\text{g/ml}$ and DAB Map Kit (Ventana 760-
938 124) was used as detection system.

939 **Supplementary Materials**

940 Fig. S1. Anti-tumor potency and efficacy of FOLR1-targeted TCBs.

941 Fig. S2. Experimental outline of Alveolus-Chip model.

942 Fig. S3. HeLa Lung-Chip Produces On-Target T-cell Killing Response.

943 Fig. S4. Comparison of T cell Killing Response of Transwell Culture to Alveolus Lung-Chip.

944 Fig. S5. Anti-tumor potency and animal cross-reactivity of CEA-targeted TCBs.

945 Fig. S6. Experimental outline of Intestine-Chip model

946 Fig. S7. Intestine-Chip CEA expression, comparison to conventional models and target-

947 independent PBMC activation of CEA-targeted TCBs.

948

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1100 application (US WO/2021/001435A1) submitted by Hoffmann-LaRoche and Emulate that covers
1101 “Method for Assessing a Compound Interacting with a Target on Epithelial Cells” **Data and**
1102 **materials availability:** All analyzed data and materials are available in the main text. Raw data

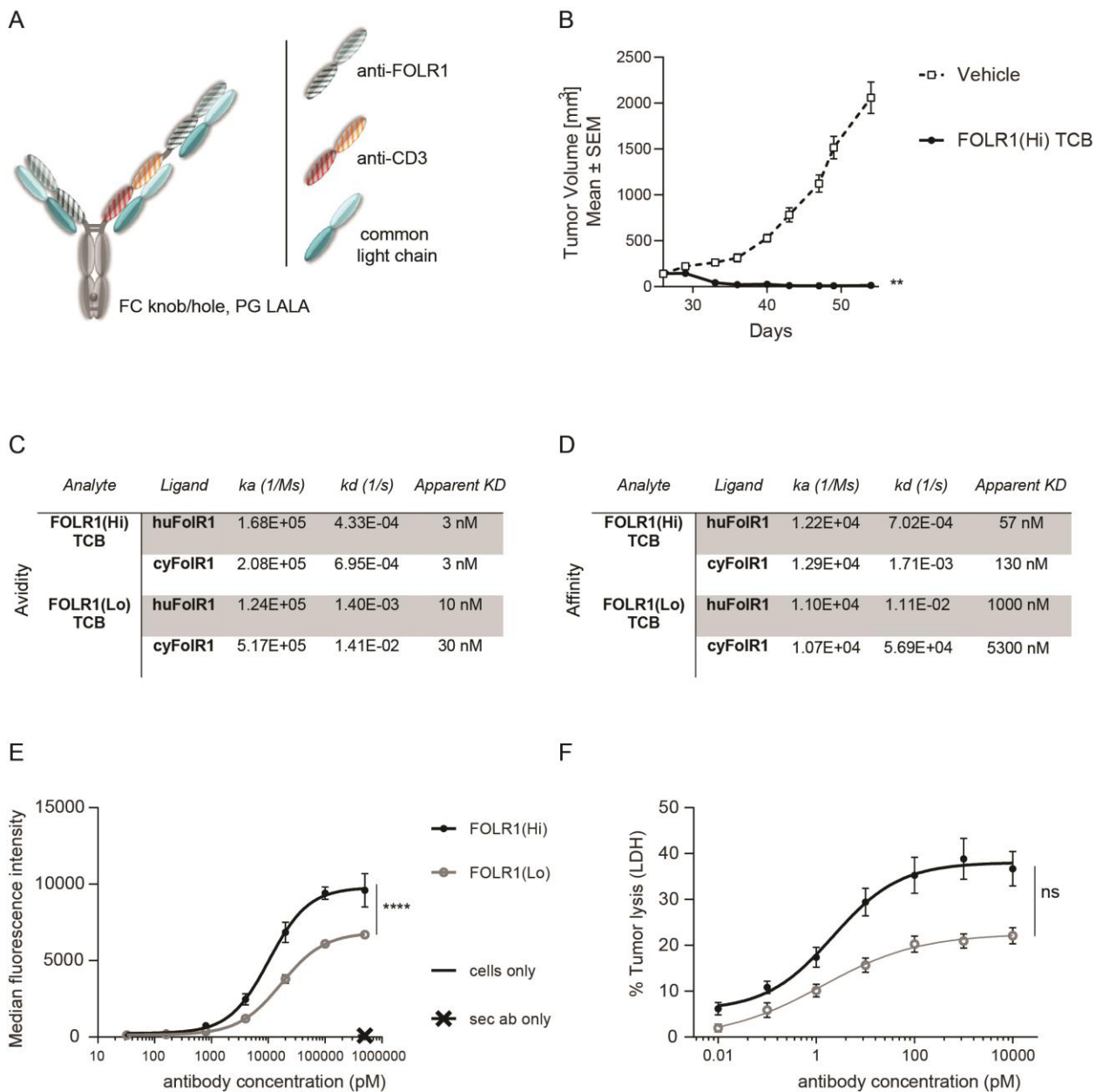
1103 supporting the findings of this study are available from the corresponding author upon

1104 reasonable request.

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1107 **Supplementary Materials:**



1108

1109 **Figure S1: Anti-tumor potency and efficacy of FOLR1-targeted TCBs.** A) “Knobs-into-holes”

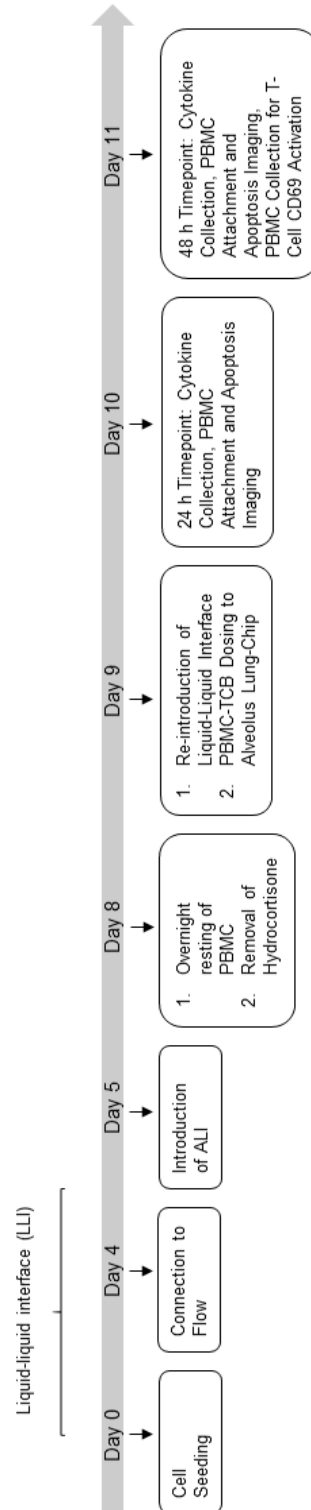
1110 technology was used for the generation of heterodimeric molecules and PG LALA mutations were

1111 inserted to prevent FcγR binding. The resulting FOLR1-specific asymmetric 2:1 T cell bispecific antibody

1112 with a molecular format that incorporates bivalent binding to FOLR1 and monovalent binding to CD3e is

1113 described thereafter as FOLR1-TCB. B) Tumor growth inhibition curves of breast PDX BC004 model in

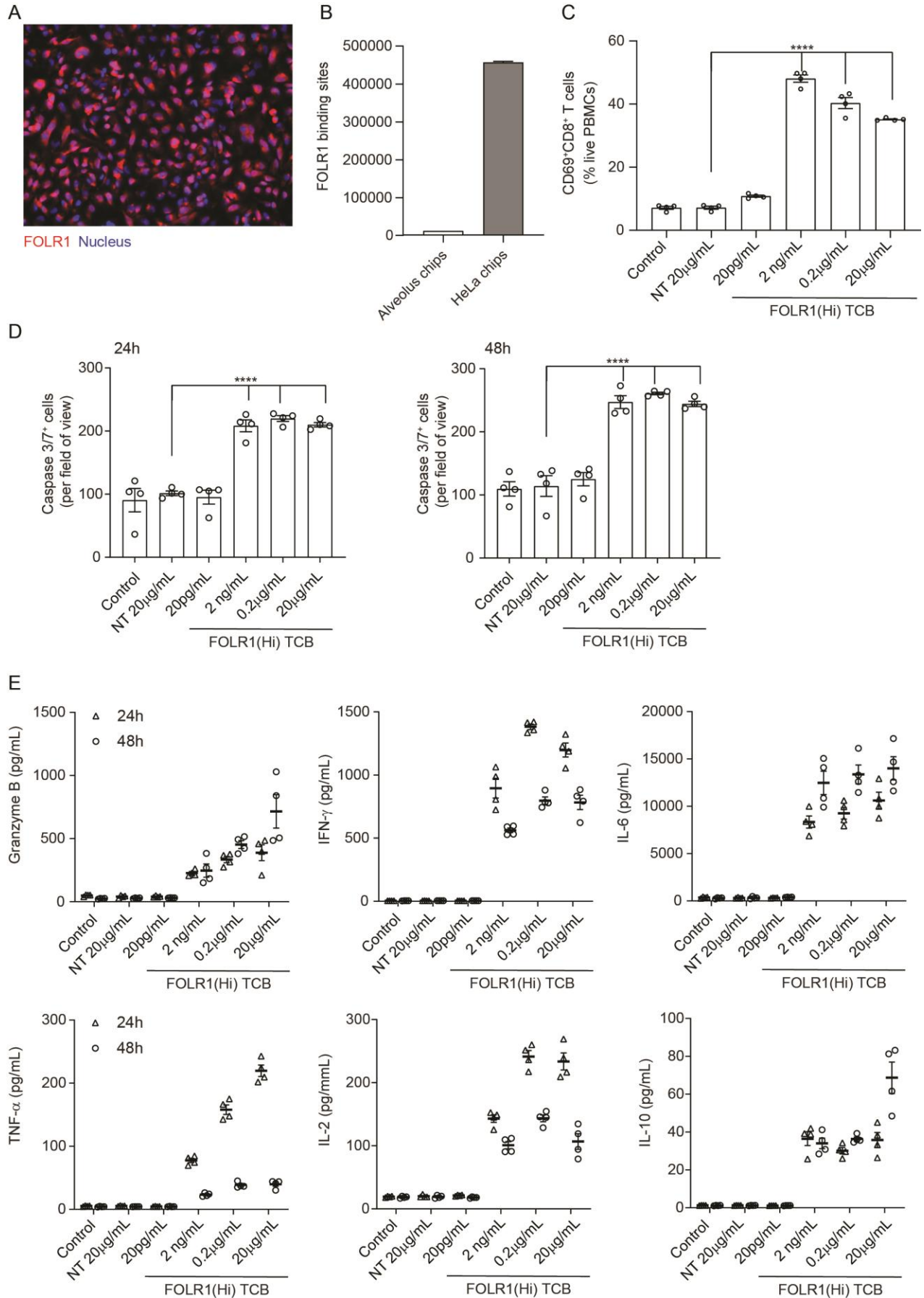
1114 CD34⁺ HSC humanized NSG mice (HSC-NSG). Humanized mice (n = 9 per group) were weekly
1115 intravenously injected with FOLR1-TCBs (0.5 mg/kg) or vehicle once the tumor size reached 150 mm³
1116 (day 26). Each dot represents the mean tumor volume ± SEM. Efficacy was evaluated by measuring the
1117 reduction of the mean tumor volume at day 62 relative to vehicle control. Statistical analysis was done
1118 using an unpaired t-test. C) Bivalent binding (avidity with apparent KD) of FOLR1-TCBs on human and
1119 cynomolgus FOLR1 as determined by SPR. D) Monovalent binding (affinity) of FOLR1-TCBs on human
1120 and cynomolgus FOLR1 as determined by SPR. E) Binding of FOLR1-TCBs to human FOLR1 expressed
1121 on HeLa cells revealed the higher binding of FOLR1(Hi) TCB compared to FOLR1(Lo) TCB, consistent
1122 with the observed higher affinity for FOLR1. F) Treatment of HeLa cells with both TCBs in presence of
1123 PBMC led to a concentration-dependent HeLa cell killing.
1124



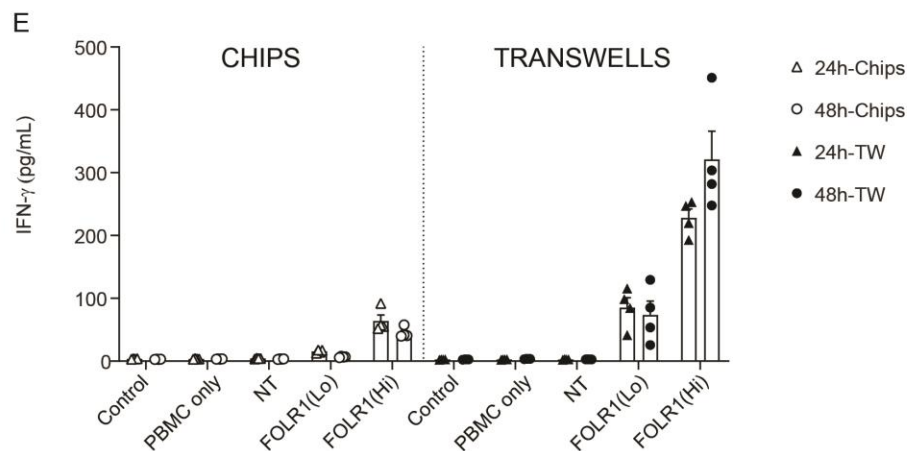
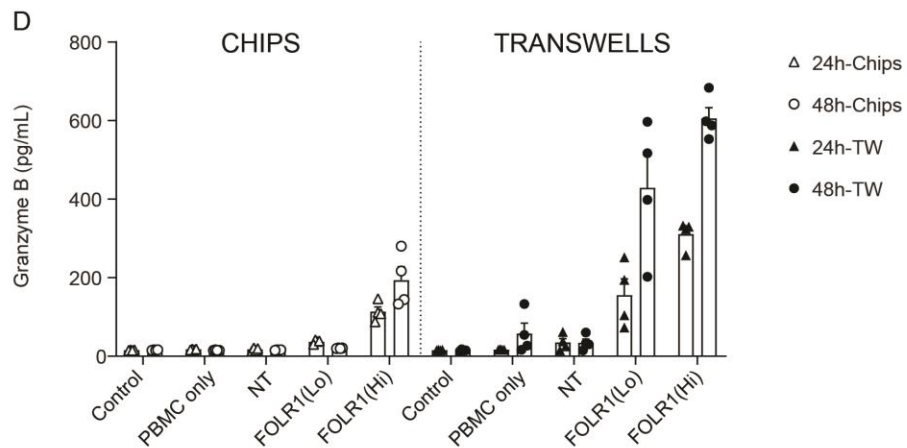
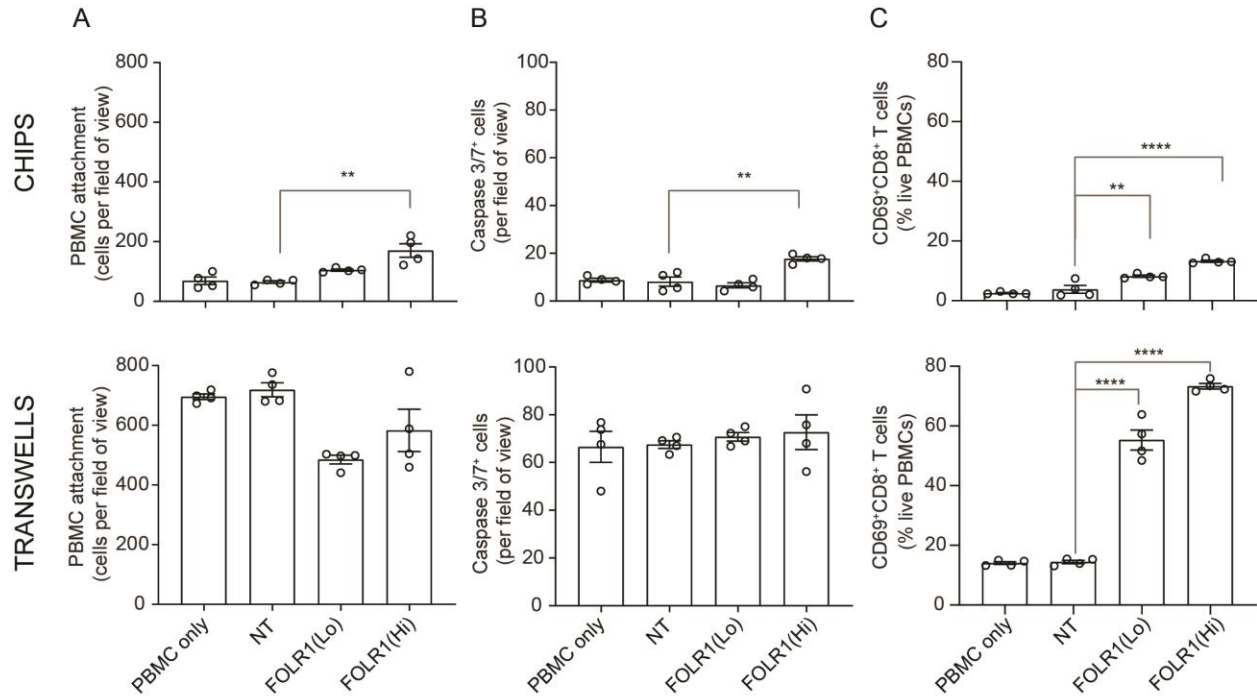
1125

1126 **Figure S2: Experimental outline of Alveolus-Chip model.**

1127



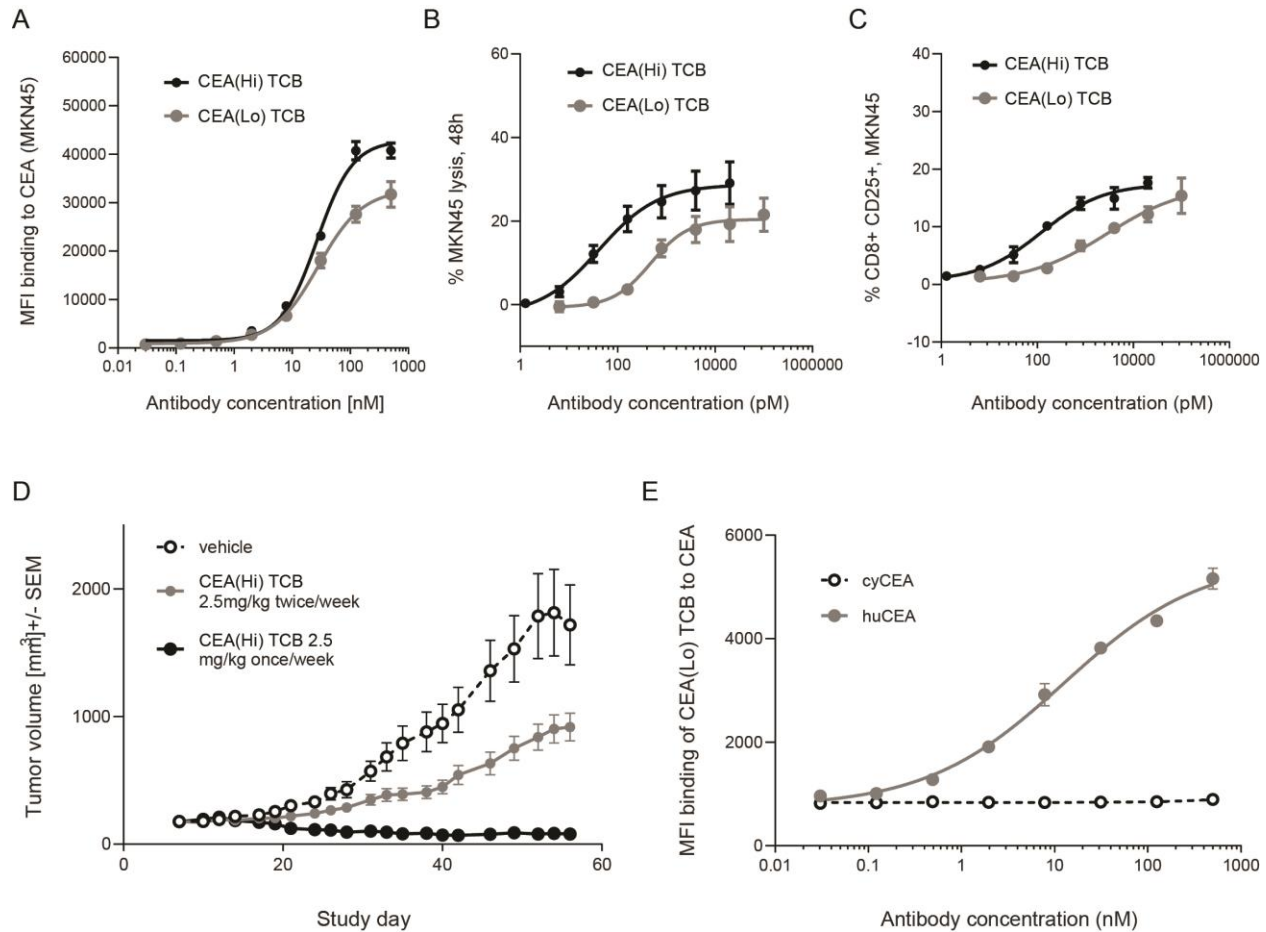
1129 **Figure S3. HeLa Lung-Chip Produces On-Target T-cell Killing Response.** Ovarian carcinoma HeLa
1130 cells were seeded into epithelial channels of chips, with lung microvascular cells in vascular channels,
1131 and cultured under liquid-liquid interface (LLI) to confluency. A) Representative microscopy picture of
1132 immunostaining of FOLR1 (red) in mature HeLa chips and Dapi nuclear counterstain in blue B) Flow
1133 cytometry quantification of FOLR1 target sites on alveolar epithelial cells or HeLa cells after culture on
1134 chips for 5 days (n=2, \pm SEM). C) Chips were administered with PBMC with high-affinity FOLR1(Hi)
1135 TCB at 20 pg/mL- 20 μ g/mL concentration or Non-targeting (NT) TCB (20 μ g/mL) (also included
1136 PBMC-deficient control). T-cell activation (CD69⁺/CD8⁺) was measured on harvested PBMC from
1137 epithelial channels through flow cytometry on live cells (n=4). D) Analysis of apoptotic, caspase-3/7⁺
1138 epithelial cell signal from live imaging at 24- (left) and 48-hours (right) post PBMC administration (n=4).
1139 E) Multiplex cytokine analysis from supernatants collected from epithelial channels at 24- and 48-hours
1140 (n=4). Statistical analysis was conducted by one-way ANOVA (B, C) and was defined as ****P<0.0001.
1141 Errors bars represent \pm SEM.
1142



1144 **Figure S4. Comparison of T cell Killing Response of Transwell Culture to Alveolus Lung-Chip.**

1145 Transwells were cultured in parallel to Alveolus Lung-Chips and both were introduced to PBMC
1146 with/without low-affinity (FOLR1(Lo)) and high-affinity (FOLR1(Hi)) TCB or Non-targeting (NT)
1147 control. A, B, C) Comparison of chips (top graphs) and transwells (bottom graphs): A) live imaging
1148 analysis of PBMC attachment to epithelium, B) live imaging analysis of apoptotic caspase-3/7⁺ epithelial
1149 cells, and C) flow cytometry analysis of live harvested PBMC for T cell activation CD69⁺ and killer CD8⁺
1150 surface markers. Data from 48 hours after PBMC administration (n=4). D, E) Multiplex cytokine analysis
1151 of D) Granzyme B and E) IFN- γ from supernatant collected from epithelial channels 24 and 48 hours
1152 after PBMC administration (TW, transwells). Statistical analysis was conducted by one-way ANOVA (A,
1153 B, C) and was defined as **P<0.01 and ****P<0.0001. Errors bars represent \pm SEM.

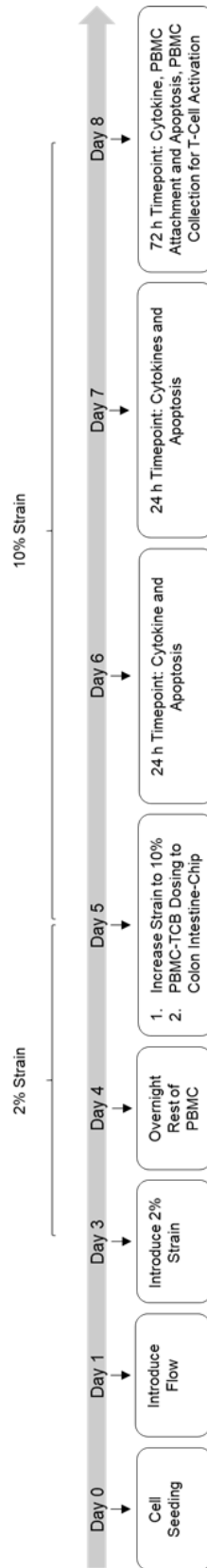
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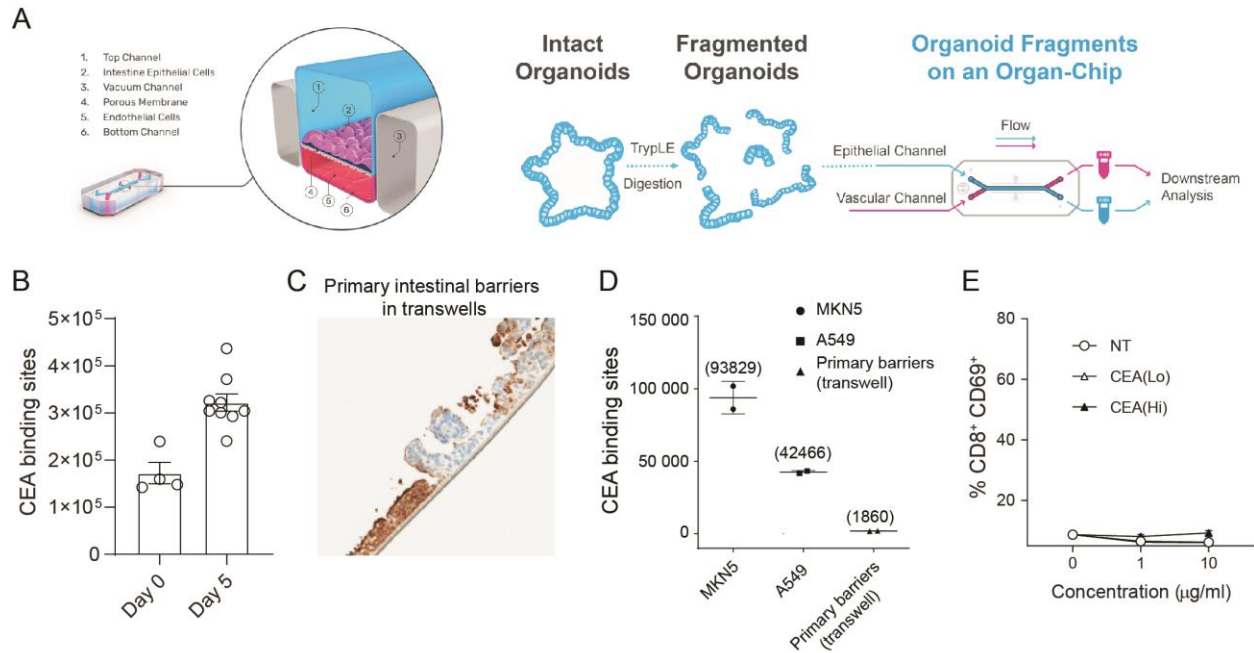
1156 **Figure S5. Anti-tumor potency and animal cross-reactivity of CEA-targeted TCBs.** A) Both TCB
1157 molecules displayed concentration-dependent binding to human CEA-expressing gastric cancer cell line
1158 MKN45. CEA(Hi) TCB showed stronger binding, consistently with its higher affinity for CEA.
1159 Treatment with both TCBs led to concentration-dependent B) MKN45 cancer cell killing and C) T cell
1160 activation, with the higher affinity molecule producing a stronger effect. D) Effect of CEA-targeted TCBs
1161 on tumor progression in CD34⁺ HSC humanized NSG mice (HSC-NSG mice), engrafted with tumor-
1162 forming MKN45 cells. E) Assessment of binding of CEA(Lo) TCB to CEA derived from humans or
1163 cynomolgus monkeys. Data are represented as mean values, with SEM.

1164



1165

1166 **Figure S6. Experimental outline of Intestine-Chip model.**



1167

1168 **Figure S7. Intestine-Chip CEA expression, comparison to conventional models and target-**

1169 **independent PBMC activation of CEA-targeted TCBs.** A) Diagram of Colon and Duodenum-Intestine

1170 chip seeding, beginning with fragmented primary human organoids seeded into the epithelial channel of

1171 the chip. Primary intestinal endothelial cells, either colon or small-intestinal depending on corresponding

1172 epithelial tissue, are seeded into the vascular channel and the chip is cultured to maturity under flow and

1173 mechanical deformations. B) Estimation of apical target sites in Colon-Chip epithelium through flow

1174 cytometry at Day 0 (organoids before seeding, n=4 wells) and Day 5 of culture (n=8). C)

1175 Immunohistochemistry analysis of CEA (brown coloration) in a conventional, static model of the

1176 intestinal barrier: organoid-derived intestinal cell seeded on ECM-coated transwell membranes. D) Flow

1177 cytometry-based quantification of CEA binding sites expressed by intestinal barriers cultured in

1178 transwells. High CEA-expressing cancer cell lines MKN5 and A549 serve as positive controls. E)

1179 Treatment of PBMC with CEA-targeted TCBs in the absence of target does not induce activation,

1180 confirming target-dependent mode of toxicity observed in the Intestine-Chips.

1181