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Identification of druggable regulators of cell secretion via a kinome-wide screen and high-throughput immunomagnetic cell sorting

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6 **Isolation of tumour-reactive lymphocytes from peripheral blood via microfluidic immunomagnetic**
7 **cell sorting**

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36
37 **The clinical use of tumour-infiltrating lymphocytes for the treatment of solid tumours is hindered by**
38 **the need to obtain large and fresh tumour fractions, which is often not feasible in patients with**
39 **unresectable tumours or recurrent metastases. Here, we show that circulating tumour-reactive**
40 **lymphocytes (cTRLs) can be isolated from peripheral blood at high yield and purity via microfluidic**
41 **immunomagnetic cell sorting, allowing for comprehensive downstream analyses of these rare cells.**
42 **We observed that CD103 is strongly expressed by the isolated cTRLs, and that in mice with**
43 **subcutaneous tumours, tumour-infiltrating lymphocytes isolated from the tumours and rapidly**
44 **expanded CD8⁺ CD103⁺ cTRLs isolated from blood are comparably potent and respond similarly to**
45 **immune checkpoint blockade. We also show that CD8⁺ CD103⁺ cTRLs isolated from the peripheral**
46 **blood of patients and co-cultured with tumour cells dissociated from their resected tumours resulted**
47 **in the enrichment of interferon- γ -secreting cell populations with T-cell-receptor clonotypes**
48 **substantially overlapping those of the patients' tumour-infiltrating lymphocytes. Therapeutically**
49 **potent cTRLs isolated from peripheral blood may advance the clinical development of adoptive cell**
50 **therapies.**

51
52 The autologous transplantation of tumour-infiltrating lymphocytes (TILs) expanded from resected tumours
53 has become a promising therapeutic modality in the clinic¹. TIL-based adoptive cell therapy (ACT) has
54 notable advantages over other allogenic and engineered cell therapies because of its inherent heterogeneity
55 that maximizes the tumour-recognizing T cell receptors (TCRs) while minimizing off-tissue effects². The
56 clinical outcomes obtained with TILs to date are extremely encouraging – long-term complete responses
57 have been observed in subsets of melanoma patients³. Despite the positive outcomes from pioneering
58 clinical trials, the applicability of TIL-mediated ACT has primarily been demonstrated for metastatic
59 melanoma, where resectable metastatic lesions are often large (> 3 cm in diameter), providing an optimal
60 source material for TIL isolation⁴. However, for other solid tumours, such large lesions are not readily
61 accessible. Moreover, in some cases excisional surgery may not be an option for patients due to a
62 substantial risk or rapid tumour progression⁵. Recent studies have explored the possibility to apply TIL ACT
63 to other solid tumours, such as renal carcinoma⁶, cervical cancer⁷, and breast cancer⁸. But limited
64 functionality of TILs^{6,8} and reduced response rates⁷ were observed. Hence, new approaches that deliver
65 active autologous cells are needed.

66
67 Advances in organoid development and deep sequencing have facilitated the generation of TIL-like tumour-
68 reactive lymphocytes (TRLs) via the co-culture of peripheral blood lymphocytes with tumour-derived

69 organoids⁹ or peptide pools from tumour-derived neoantigens¹⁰. However, these approaches still require a
70 resection of primary tumour cells harvested using invasive surgical procedures. In addition, establishing
71 organoids⁹ and the synthesis of neoantigen-derived peptides¹⁰ takes several weeks to complete. Overall, the
72 requirement of tumour biopsy and lengthy workflow limits the translational value of these approaches as an
73 alternative to TILs.
74

75 Prior work has confirmed the presence of TRLs in circulation at a very low frequency¹¹. Given the intriguing
76 possibility that tumour-reactive cells could be isolated non-invasively from blood, several studies have sought
77 to characterize and isolate rare circulating TRLs (cTRL). Studies from multiple groups further substantiated
78 the presence of cTRL in lung¹², and melanoma patients¹³, even prior to immunotherapy¹⁴. However,
79 personalized neoantigen-derived multimers were used to identify and isolate such a rare population from
80 circulation^{13–17}, which requires the characterization of tumour neoantigens through invasive biopsy. In
81 addition, a recent study on peripheral T cell dynamics after immunotherapy revealed that a subset of
82 peripheral lymphocytes shares clonotypes with TILs, and that their expansion is highly correlated with
83 response to treatment¹⁸.
84

85 As multimer-based cTRL isolation may be difficult to translate into the clinic, the identification of more
86 generalized markers for cTRLs is an important goal. Based on the surface markers that are known to
87 influence the phenotypic properties of TILs, prior studies suggested that cTRLs may exhibit high expression
88 levels for immune checkpoint markers such as PD-1¹⁹ or CD39²⁰. Yet, a more comprehensive analysis,
89 ideally using an unbiased screening method, is needed to identify a biomarker that offers clear discrimination
90 between cTRLs and their non-tumour reactive counterpart. The rarity of cTRLs remains a key challenge for
91 the comprehensive analysis and therapeutic application of cTRLs, as most molecular approaches require at
92 least several thousand cells as the input and millions of cells are required to test therapeutic efficacy. With
93 levels as low as 0.002% in peripheral T cell populations¹⁴, it is extremely difficult to enrich cTRLs with high
94 purity and recovery for downstream analysis.
95

96 Here we describe a microfluidic approach that efficiently isolates cTRLs from blood circulation for rapid
97 expansion and cellular therapy (Fig. 1a). Our approach is non-invasive and appears applicable to a variety of
98 solid tumours on the basis of studies in mouse tumour models, and may therefore provide a practical
99 alternative to existing TIL-mediate ACT. Our approach is built on immunomagnetic-cell-sorting technology²¹
100 reported previously but now optimized for identifying and isolating cTRLs in PBMCs. With the high recovery
101 and purity established, we identified CD103 as a molecular signature for cTRLs. Compared to immune
102 checkpoint markers like PD-1 and CD39, CD103 is widely expressed by the tumour-reactive population in
103 circulation. This population has a tissue-resident-like (T_{im} -like) phenotype and has the capability to re-enter
104 blood circulation from primary tumours and accumulate in secondary tumours. We subsequently show that
105 the cTRLs can be expanded through a feeder-based rapid expansion protocol (REP) and that they have
106 strong therapeutic potency in multiple adoptive-cell-transfer models in mice. We also confirmed that the
107 enrichment based on CD8⁺CD103⁺ yields higher tumour reactivity in a small cohort of patient specimens by
108 comparing the level of interferon gamma (IFN- γ) secretion and clonal similarity.
109
110

111 Results

112 **Presence of cTRLs in circulation during tumour progression.** We first sought to confirm the presence
113 and fate of cTRLs in animal models. To confirm the presence of cTRLs, we pursued the isolation of cTRLs
114 using this approach in animal models with two defined highly immunogenic epitopes - chicken ovalbumin
115 (OVA₂₅₇₋₂₆₄, SIINFEKL) in C57BL6 models and influenza A hemagglutinin (HA₅₃₃₋₅₄₁, IYSTVASSL) in Balb/c
116 models. Tumour cells with/without the expression of these defined epitopes were injected subcutaneously.
117 Blood and tumour were collected at the mid-late stage (defined by 300 – 800 mm³ tumour size) of tumour
118 development and CD8⁺ T cells were labelled by corresponding multimers and antibodies accordingly
119 (Extended Data Fig. 1). Flow cytometric analysis indicated that mice bearing tumours expressing
120 immunogenic epitopes exhibited a higher degree of immune response and slower growth of the tumours
121 (Supplementary Fig. 1). The upregulated immune response produced a significantly higher fraction of TRLs
122 specific to OVA/HA epitopes in tumour and blood (Extended Data Fig. 1 and Supplementary Fig. 2), which
123 matches a previous observation for melanoma patients¹⁴. As expected, the percentage of TRLs is extremely
124 low in blood (0.05% - 0.45% in CD8⁺ T cells) – this highlights the intrinsic rarity of cTRLs.
125

126 Various recent reports suggest that the cTRLs have memory phenotypes,^{18,22,23} which are believed to
127 contribute to the adaptive immune response against similar pathogens, through homing to the infected sites
128 for direct killing.²⁴ With this in mind, we established a tumour transplantation model to determine the
129 destination of cTRLs in blood. In brief, an OVA-expressing tumour from the donor CD45.2 mice was
130 transplanted as a whole to the host CD45.1 mice bearing a secondary (2nd) tumour with/without OVA
131 epitopes. Two tumours were allowed to grow simultaneously and the 2nd tumours were collected at the end

132 for flow cytometric analysis (Supplementary Fig. 3). For all of the specimens analysed, we observed a small
133 portion of CD45.2⁺ cells presented in the 2nd tumour. Since the only source of CD45.2⁺ T cells is the TILs
134 within the transplanted tumour, this observation provides direct evidence that the TILs can enter the
135 circulation and migrate to distant organs. In addition, we noticed that the 2nd tumours with OVA epitopes
136 attract at least a 5-fold higher percentage of CD45.2⁺ T cells compared to OVA-free WT tumours
137 (Supplementary Fig. 3) – this suggests the migration and the accumulation of cTRLs are antigen-driven.
138 Sites with similar patterns of infection (e.g. same immunogenic epitope) attract more cTRLs to re-infiltrate
139 and reside. Taken together, the data collected indicates that the cTRLs commonly present in blood during
140 tumour development, migrate towards and accumulate specifically in distal tumours (Fig. 1b).
141

142 **High-performance cell isolation to enable cTRL profiling.** The use of blood as source material and
143 considerable levels of tumour specificity make cTRLs an ideal route to non-invasively acquire tumour-
144 targeting lymphocytes for immunotherapy. However, there is a critical issue preventing the implementation of
145 this idea, which is the use of major histocompatibility complex (MHC) multimers for cTRL identification during
146 isolation. MHC multimers are derived from tumour neoantigens through invasive biopsy and off-the-shelf
147 reagents are not available for individual patient samples. To develop a multimer-independent isolation
148 workflow, it is important to comprehensively profile cTRLs and understand their unique clonal and molecular
149 signatures (e.g. surface protein expression) that can serve as a specific biomarker for isolation.
150

151 Given that cTRLs are extremely rare (as low as 0.002%)¹⁴ and difficult to analyse without purification, it is
152 challenging to isolate rare cells efficiently with high levels of purity and recovery using conventional cell
153 sorting techniques. For instance, fluorescence-activated cell sorting (FACS) cannot robustly discriminate rare
154 cells when the target population is fewer than 0.2%^{25,26}. Special protocols have been developed to increase
155 the detection limits of flow cytometry to 0.001% by collecting a very large number of events (e.g. 1×10^9)^{27,28}
156 but this is not practical for real-world implementation. In addition, FACS typically loses 50 – 70% of target
157 cells due to bad droplet formation or incorrect scanning^{29,30}. The low performance of cell sorting may
158 substantially impact the results of downstream molecular assays, such as TCR sequencing²¹. For example,
159 our previous study has shown that FACS could only recover 684 clonotypes from isolated TILs while other
160 purification approaches could recover up to 64,155 clonotypes simultaneously²¹.
161

162 With this in mind, we hypothesized the introduction of a high-performance cell sorting technology would help
163 to deconvolute the clonal and molecular profiles of cTRLs by effectively isolating these cells with high purity
164 and recovery levels. The high-quality molecular profile of cTRLs would further contribute to the identification
165 of multimer-independent biomarkers for cTRL isolation. We adapted a microfluidic system developed for
166 sorting TILs from solid tumours based on surface markers²¹ to the isolation of cTRLs from blood, firstly based
167 on multimer binding for molecular profiling and later on based on identified surface markers that emerged
168 from profiling studies. The system achieved up to 10-fold higher throughput and recovery compared to FACS
169 while maintaining similar purity.
170

171 The overall workflow of the tumour reactivity-mediated cell labelling and sorting strategy is illustrated in
172 Figure 2a. Lymphocytes are treated with MHC multimers mimicking a defined tumour epitope to selectively
173 isolate a subset of TRLs with putative tumour reactivity. The multimers are conjugated with a fluorophore,
174 which is used as a linker to attach magnetic nanoparticles (MNPs). To separate the multimer-binding
175 lymphocytes, or tumour-reactive lymphocytes, magnetically labelled cell mixtures are processed with a
176 microfluidic device sandwiched by arrays of magnets (Supplementary Fig. 4). The device contains multiple
177 capture zones that can spatially separate cells with different degrees of magnetization – a higher degree of
178 magnetization results in the capture in a compartment close to the inlet (Supplementary Fig. 5)^{31,32}. In the
179 case of multimer-mediated labelling, the TRLs are captured here while the non-TRL populations are
180 captured in a different compartment. Captured cells can be easily and efficiently recovered from the device
181 compartments when the external magnets are removed. Recovered cells are highly viable and suitable for
182 downstream culture and analysis^{25,26}. Details of the working principle of the device can be found in the
183 supporting discussion (SD). As previously reported²¹, the microfluidic cell sorting outperforms commercial
184 cell sorting techniques when recovering rare cell populations. For multimer-mediated labelling and sorting, it
185 is worth noting that the purity of FACS post-isolation is better than microfluidics (89% vs 75%, Extended Data
186 Fig. 2), which may be beneficial to purity-focused applications such as TCR derivation. However, our
187 microfluidic approach achieved up to 10-fold higher cell recovery compared to FACS and therefore is more
188 suitable for molecular assay and cellular therapies. We pursued the isolation of cTRLs using this approach in
189 the defined epitopes model described above. We utilized a workflow consisting of negative capture of CD8⁺
190 lymphocytes, followed by positive selection multimers through immunomagnetic cell sorting (Fig. 2b). The
191 purity of isolation is 76% for OVA (Extended Data Fig. 2) and 84% for HA multimers (Fig. 2c), respectively.
192

193 The high yield and purity from microfluidic sorting enabled us to perform direct TCR sequencing on the rare
194 cTRL populations (Fig. 2d). We identified over 1,000 clonotypes (defined by the unique CDR3 sequence) in

195 the cTRLs from 10 – 15 mL pooled mouse whole blood. Mapping of V-J usage reveals a high level of
196 similarity between cTRLs and intratumoural TILs, rather than the non-reactive portion of peripheral blood
197 mononuclear cells (PBMCs). In terms of the TCR repertoire, cTRLs cover 30% - 85% of the top 50 clones
198 presented in TILs, 3 – 8 times higher than the coverage observed in non-cTRL PBMC (~10%, Fig. 2e). We
199 also analysed the percentage of top-20 TIL clones by fraction in cTRLs and non-cTRL CD8⁺ PBMC and
200 observed a notable enrichment of TIL clones in cTRLs (Tab. 1). Taken together, these data suggest that the
201 cTRLs exist in the circulation during disease progression and share a higher degree of clonal similarity with
202 intratumoural TILs.
203

204 The high yield and purity from microfluidic sorting also allowed RNA sequencing (RNAseq) to be performed
205 on the rare cTRL population. For the OVA-reactive cTRLs isolated from the melanoma model, a tissue-
206 resident memory (T_{rm}) phenotype was detected with strongly upregulated expression of *ITGAE* compared to
207 non-cTRL bulk CD8⁺ cells (Fig. 3a, left) and upregulated expression of *ZFP683*, a transcriptional hallmark of
208 T_{rm} (Fig. 3a, right). In addition, cTRLs expressed a higher level of exhaustion, cytotoxicity, and activation
209 markers compared to other dedicated T cell subpopulations in PBMC, including naïve T cells (T_{naïve},
210 CD8⁺CD45RA⁺) and memory T cells (T_{mem}, CD8⁺CD45RA⁻). This further revealed that cTRLs have a partially
211 exhausted yet activated tissue-resident memory phenotype. Gene set enrichment analysis (GSEA) also
212 suggests that the cTRLs have statistically significant upregulation of T cell activation and TCR signaling
213 pathways (Supplementary Fig. 6). We observed a similar trend of marker expression in HA-reactive cTRLs
214 isolated from the colon cancer model (Supplementary Fig. 8). By overlaying the differentially expressed
215 genes, we identified 12 shared upregulated genes. Genes that are known to be transiently or permanently
216 upregulated during TCR-MHC interaction (e.g. *GGT133*, and *CD8A34*) were eliminated from this subset. One
217 of the strongest hits observed was *ITGAE*, a gene that encodes CD103. In fact, the expression of CD103 on
218 healthy PBMCs is relatively low at the protein level, according to a comprehensive mass cytometry study³⁵.
219 This further suggests the utility of CD103 as a specific marker for cTRL isolation.
220

221 To further evaluate this finding, circulating CD8⁺ lymphocytes were isolated from mice bearing B16F10 and
222 AE17 cancer cell lines expressing the OVA epitope. Flow cytometric analysis indicated that CD103 is widely
223 expressed by the OVA-reactive cTRLs (Fig. 3b). Collectively, about 30% of the CD8⁺CD103⁺ cells in
224 circulation are OVA-reactive (Fig. 3c), which yields up to 50-fold enrichment of OVA-reactive T cells. It is
225 perhaps not surprising that CD103⁺ helps to define tumour-reactive populations in bulk T cells as previous
226 studies have suggested CD103, alone^{36,37} or together with other markers³⁸, defines T cells with elevated
227 potency for adoptive cell therapy and immune checkpoint blockade. However, it is interesting that such T_{rm}-
228 like phenotype, which is believed to reside in non-lymphoid tissue³⁹, is present in circulation. Therefore, we
229 further confirmed the signature of migrating and migrated cTRLs in the aforementioned tumour
230 transplantation model (Supplementary Fig. 3) by cytometry by time of flight (CyTOF). During migration, over
231 80% of the cTRLs (defined as the multimer-binding CD3⁺CD8⁺ T cells) were CD103⁺ (Fig. 3d and 3e). And
232 CD103⁺ cTRLs consist of a large portion of PD-1⁺, CD39⁺ or CD69⁺ cells (Supplementary Fig. 9). Post
233 migration, compared to the CD45.1⁺ endogenous T cells, CD45.2⁺ cTRLs still had higher expression of
234 CD103⁺, CD69⁺, and PD-1⁺ (Fig. 3f and 3g), suggesting they retained the T_{rm}-like, activated phenotypes after
235 migration. Indeed, this observation is consistent with the emerging evidence showing T_{rm} enter circulation to
236 increase the overall immune response^{40,41}. Mechanistically, CD103 is an integrin protein that binds to E-
237 cadherin and governs the formation of cell protrusions/filopodia⁴², an essential component for initiating cell
238 migration⁴³. This points to the critical role of CD103 in cell motility and moreover, CD103⁺ TRLs are reported
239 to have elevated energetic potential and greater migration capacity⁴⁴. Hence, the phenotypic properties of
240 cTRLs are consistent with prior observations of CD103⁺ T cells.
241

242 **Expansion and administration of cTRLs for ACT.** The use of high-performance microfluidics together with
243 the newly identified biomarker CD103 provides a simple approach to isolate cTRLs from PBMC for adoptive
244 cell therapy. We firstly optimized the microfluidic cell sorting based on CD103 and achieved up to 16-fold
245 higher cell recovery (73.3% vs 4.5%) compared to FACS while retaining similar purity (Extended Data Fig.
246 3). Subsequently, we adapted the rapid expansion protocol (REP) of rare tumour-reactive TILs⁴⁵ and
247 achieved up to 2000-fold expansion of cTRLs in 10 days (Supplementary Fig. 10 and 11), yielding the final
248 number of cTRLs around 0.1 – 4 million per mouse. We did not compare the yield of FACS-sorted samples
249 since its low recovery did not grant robust cell growth during REP. The cTRLs maintained their T_{rm}-like
250 phenotypes upon expansion (Supplementary Fig. 12), including a high expression of critical surface (CD103
251 and CD69) and intracellular cytotoxic markers (IFNG and GZMB). It is also worth noting that about 70% of
252 the cTRLs are PD-1^{med}TIM3⁻, indicating a partially exhausted yet tumour-reactive phenotype^{46,47}. An *in vitro*
253 killing assay revealed that expanded cTRLs lysed 65% of the cells in 24 hrs (Supplementary Fig. 13). Such
254 killing potency is comparable to TILs. We also elaborated a continuous antigen exposure model⁴⁸ to assess
255 the degree of exhaustion (Supplementary Fig. 13). Compared to TILs subjected to REP, expanded cTRLs
256 showed greater killing potency over 3 rounds of antigen exposure, suggesting a strong anti-exhaustion

257 phenotype. This result is in line with a recent pan-cancer analysis suggesting that the CD8⁺ZNF683⁺ T_{rm}
258 subpopulations have a lower frequency of terminally exhausted T cells⁴⁹.

259

260 We next characterized the therapeutic efficacy of cTRLs *in vivo* using multiple animal models. We first
261 benchmarked the therapeutic potency of expanded TILs, cTRLs, and CD8⁺CD103⁻ PBMC populations with
262 subcutaneous B16F10 melanoma (Fig. 4a – 4b, Supplementary Fig. 14 and 15) and LLC1 NSCLC model
263 (Extended Data Fig. 4). We confirmed that cTRLs have improved therapeutic potency compared to
264 CD8⁺CD103⁻ PBMCs. It is worth noting that we expanded the CD8⁺CD103⁻ PBMCs using a feeder-free
265 protocol due to their abundance. The feeder-free protocol may cause more severe activation-induced cell
266 death (AICD) and/or fratricide compared to feeder-based protocol⁵⁰. However, it is unlikely that the selective
267 expansion of CD8⁺CD103⁻ PBMCs will impact their anti-tumour potency given their low tumour reactivity
268 post-isolation (Fig. 3b and 3c).

269

270 The potency of cTRLs is comparable to the TILs isolated from solid tumours – both extending median
271 survival by 40% - 50%. This suggests that the TCR repertoire of the cTRLs, although not as diverse as the
272 TILs, exhibits sufficient tumour-reactive TCRs for tumour killing. In addition, we verified the feasibility of
273 treating metastases with cTRLs using an induced metastasis model by tail-vein injection of 4T1 cells (Fig. 4c
274 – 4d) – the group treated by cTRLs had a 30% increase in the median survival and a 60% reduction in the
275 lung metastases (Extended Data Fig. 5) compared to the CD8⁺CD103⁻ PBMC group. It is worth noting that
276 no primary tumour was involved during the cTRL isolation and reintroduction – this suggests that the use of
277 cTRLs is applicable to a broader group of patients, including individuals with unresectable tumours or who
278 underwent surgery (without TIL isolation/expansion) and went to developed recurrent metastasis. Taken
279 together, our findings indicate that the cTRLs have notable therapeutic potency and could be used in
280 conjunction with a more practical harvesting process. However, we observed that monotherapy using cTRLs
281 was insufficient for long-term tumour management, with less than 20% of the mice in this study experienced
282 a complete response (CR) at study endpoint.

283

284 To further improve the therapeutic efficacy of cTRLs, we next tested the combination of cTRLs with immune
285 checkpoint blockade (ICB). We hypothesized that the cTRLs would respond to the ICB considering their PD-
286 1^{med}TIM3⁻ partially exhausted phenotype. In addition, existing studies showed that the percentage of
287 CD8⁺CD103⁺ T_{rm} predicts response to ICB therapy – further supporting this rationale^{36,37}. To test this
288 hypothesis, we elaborated a MC38 colon cancer model in immunocompromised mice (RAG^{-/-}) and treated
289 them with the combination of cTRLs and anti-PD-1 blockade (Extended Data Fig. 6), to solely test the effects
290 of ICB on infused cTRLs. As anti-PD-1 blockade requires the T cells to function, we observed little difference
291 between the untreated and anti-PD-1 groups in RAG^{-/-} backgrounds that resulted in no mature T cells.
292 Monotherapy of cTRLs produced transient tumour control for about 2 weeks but underwent rapid tumour
293 progression after this time point. A cocktail of anti-PD-1 and cTRLs was observed to exhibit durable tumour
294 control over 4 weeks and extended the median survival by 100% compared to the untreated group. IHC
295 analysis comparing the resected tumours from the cTRLs and the cocktail revealed a large difference in the
296 number of infiltrated CD8⁺ T cells (1.2% vs 3.2%). Taken together, this immunocompromised model provides
297 direct evidence that the cTRLs are responsive to ICB and the cocktail of cTRLs and ICB can achieve long-
298 lasting tumour control.

299

300 In addition to direct tumour killing, T_{rm} is also known to utilize other mechanisms for tumour control, such as
301 the new recruitment of other immune cells⁵¹. To gain insights related to these indirect processes, we used a
302 MC38 model in immunocompetent mice (CD45.1⁺) and treated them with the CD45.2⁺ cTRLs and ICB (Fig.
303 5a). With intact endogenous immunity, the cocktail of cTRLs and ICB achieved remarkable tumour control,
304 with 5 out of 5 mice surviving at study endpoint and 4 mice exhibiting complete response (CR). To better
305 understand how cTRLs and ICB altered the tumour microenvironment (TME), pathway enrichment was
306 performed on the upregulated genes from gene expression analysis (Fig. 5b). Although both treatments
307 upregulated the overall adaptive immune response, we noticed that the type of enriched immune pathways
308 was different between the treatments, with PD-1 ICB significantly altering the PD-1/L1 pathways and
309 enhancing the immune effector functions, while cTRLs promoted migration and activation of other immune
310 cells, including granulocytes, B cells, and NK cells. The cocktail of PD-1 ICB and cTRLs further boosted the
311 differentiation of helper T cells and phagocytosis. IHC analysis on CD4 and CD208 (DC-LAMP) further
312 confirmed this observation at the protein level (Fig. 5c). Taken together, these data indicate that cTRLs
313 prompt additional immune cells to infiltrate while ICB can improve the functionality of infiltrated immune cells
314 – hence co-administration of cTRLs and ICB shows synergistic effects. In addition, it is worth noting that the
315 antitumour immunity against the MC38 tumour is durable as observed through tumour rechallenge
316 experiments conducted 3 months post initial therapy (Fig. 5d). Flow cytometric analysis using a MC38
317 derived multimer (SIIVFNLL) confirmed the presence of tumour-reactive memory T cells of both CD45.1⁺ and
318 CD45.2⁺ origin, providing direct evidence that the cTRLs participate in the establishment of long-term anti-
319 tumour immunity.

320

321 In addition to ICB, we also confirmed that the cocktail of cTRLs and co-stimulatory molecules (e.g. GITR) can
322 yield synergistic effects when treating mouse mesothelioma (Fig. 5e). The combination of anti-GITR
323 antibodies and cTRLs yields 47% extended median survival compared to untreated animals. CyTOF analysis
324 revealed that the administration of cTRLs promoted the infiltration of endogenous CD4 and CD8 cells (Fig. 5f
325 and Supplementary Fig. 16), similar to what we have observed in the MC38 model. The cocktail of cTRLs
326 and anti-GITR antibodies upregulated the frequency of CD8⁺ and CD8⁺PD-1⁺ cytotoxic T cells and reduced
327 the number of CD4⁺CD25⁺ regulatory T cells (Supplementary Fig. 16), generating a more proinflammatory
328 TME for better tumour control. This observation matches with the intrinsic function of T_{rm} cells – that is to
329 secrete proinflammatory cytokines at the diseased site to trigger downstream adaptive immune response⁵².
330 We also investigated the phenotype of intratumoural cTRLs at endpoint (Fig. 5g). Compared to endogenous
331 CD8⁺CD45.1⁺ cells, CD45.2⁺ cTRLs still maintained high expression of CD103. Taken together, we conclude
332 that the T_{rm}-like phenotype permits the expanded cTRLs a strong ability to not only perform direct killing on
333 tumour cells, but also to bolster the endogenous intratumoural adaptive immune response.

334

335 **Isolation and validation of human cTRLs.** To investigate the presence of cTRLs in human specimens, we
336 acquired paired malignant pleural effusion (MPE) samples and PBMCs from 6 immunotherapy-naïve
337 patients' samples. We co-cultured the bulk PBMCs with CD45⁺ depleted MPE-derived tumour cells and
338 measured the fraction of IFN-γ secreting cells in CD8⁺CD103⁻ and CD8⁺CD103⁺ subpopulations by
339 intracellular flow cytometry post 12 - 24 hours (Fig. 6a). The panel for flow cytometry included CD103 FMO
340 (fluorescence minus one) and unstimulated (PBMC alone) controls for the precise identification of CD103⁺
341 populations (Fig. 6b). We observed a small portion of interferon gamma (IFNγ) secreting cells in the
342 unstimulated control (5.3% for CD103⁺, 7.4% for CD103⁻). When stimulated by MPE-derived tumour cells, a
343 notable portion of CD103⁺ T cells exhibited IFNγ secretion (51%) while the percentage of IFNγ secreting
344 CD103⁻ T cells remained roughly unchanged (8.4%). This observation suggested that the IFNγ secretion in
345 CD103⁺ T cells is tumour-dependent. Hence, the expression of CD103 may also define cTRL population in
346 human specimens (Fig. 6c).

347

348 To gain more insight on the tumour reactivity of human CD8⁺CD103⁺ cTRLs, we acquired a small cohort of
349 patients with a variety of solid tumour (N = 20). The major tumour types in this study included colon,
350 mesothelioma, lung and breast (Fig. 7a). We isolated CD8⁺CD103⁺ cTRLs from PBMCs and co-cultured
351 them with dissociated tumour cells (DTCs) or MPE-derived cancer cells and assessed their tumour reactivity
352 by IFNγ secretion (Fig. 7b – 7c). Compared to the bulk CD8⁺ T cells, we consistently observed a global
353 increase of IFN-γ secretion after CD103-mediated isolation, suggesting that sorting based on CD8⁺CD103⁺
354 helped to define and enrich for a tumour-reactive T cell subpopulation. We found the presence of cTRLs is
355 common in immunotherapy-naïve PBMC – this is in line with the previous study reporting neoantigen-
356 reactive T cells can be detected prior to immunotherapy¹⁴. In addition, we assessed the tumour reactivity of
357 the isolated CD8⁺CD103⁺ cTRLs across the DTCs from different patients (Supplementary Fig. 17). We found
358 that IFNγ secretion of cTRLs was patient-specific – the isolated cTRLs experienced the highest IFNγ
359 secretion on autologous tumour cells. This highlights the patient-specific tumour reactivity of cTRLs at the
360 phenotypic level.

361

362 In a subset of patients, we also isolated the TILs, cTRLs, and CD8⁺CD103⁻ PBMC for TCRseq using a
363 workflow similar to Fig. 1B. Among three sequenced samples, we consistently observed similarity between
364 the major clones presented in TILs and cTRLs (Fig. 7d – 7e). For example, in the case of CA03, cTRLs
365 share 4 major clones with TILs – TRAV2/TRAJ37, TRAV25/TRAJ37, TRAV20/TRAJ53, and TRAV5/TRAJ18
366 (Fig. 7D). Over 25% of the cTRLs belong to one of these four clonotypes. In contrast, only 0.6% of the
367 CD8⁺CD103⁻ PBMC fall into these clonotypes. Overall, similar to mouse samples, CD8⁺CD103⁺ cTRLs cover
368 about 35 - 60% of the top 50 clones (ranked by fractions) presented in CD8⁺ TILs (Fig. 7e). The significant
369 overlap of TCR proves the tumour reactivity of CD8⁺CD103⁺ cTRLs from the perspective of clonal analysis.
370 This is in line with a set of prior studies reporting that the positive expression of CD103 defines the tumour-
371 reactive TILs across human breast⁵³, lung⁵⁴, cervical⁵⁵, and oral cancer⁵⁶.

372

373 Bioinformatic analyses also predicted their strong therapeutic potential of human CD8⁺CD103⁺ cTRLs. For
374 example, the TIMER (Tumour IMMune Estimation Resource) algorithm⁵⁷ reveals that the expression of
375 CD103 has strong correlation with a high level of immune infiltration in many cancer types (Supplementary
376 Fig. 18) using predicted immune cell fraction from bulk RNAseq⁵⁸. In addition, the TIDE (Tumour Immune
377 Dysfunction and Exclusion) algorithm⁵⁹ on the mRNA-seq data across 194 cohorts of solid tumours shows
378 that the upregulated expression of intratumoural *ITGAE* correlates with statistically lower death risk and good
379 prognosis over multiple cancer types (Supplementary Fig. 19). Furthermore, a meta-analysis involving 2,824
380 patients reports that patients with intratumoural CD103⁺ immune cells were associated with favorable
381 survivals⁶⁰. Taken together, our clonal and phenotypic analyses showcased that the expression of CD103
382 defines the cTRL population in human PBMC.

383

384 **Outlook**

385 Thus far, the production of therapeutic TILs has required an accessible tumour lesion for excisional biopsy
386 as source material for TIL isolation and expansion. However, such lesions are not always available from
387 patients and, moreover, surgery on patients bearing unresectable cancers can pose a substantial risk⁵. The
388 discovery of cTRLs in blood circulation highlights a new strategy for isolating therapeutic cells for ACT. The
389 minimal invasiveness of blood collection makes the cTRL acquisition a more feasible and amenable process
390 for the patients compared to TIL therapy. In addition, our analyses reveal that the cTRLs have sufficient
391 coverage of dominant clones in TILs and are primed to target similar tumour microenvironment. These
392 unique characteristics grant their therapeutic potency against metastatic tumours. Hence, the implementation
393 of cTRLs would greatly extend the applicability of adoptive cell therapy and may provide a new treatment
394 option for late-stage patients with unresectable and/or metastasized tumours.

395

396 Another outstanding issue for TIL therapy is the low CR rates (< 20%) in non-melanoma cancer in the clinic.
397 At present, it is unclear which phenotypes of TILs should be used and how often should TILs be
398 administered to deliver a persistent therapeutic outcome⁶¹. We noticed that the cTRLs were responsive to
399 immune checkpoint blockade (ICB) and co-stimulatory molecules – the combination of ACT and ICB
400 achieved 80% CR rate in mouse colon cancer models. Considering the ease of collection/administration of
401 cTRLs, the cocktail of ACT and ICB may hold promise as an immune-oncology combination. In addition,
402 although the enrichment based on CD103 results in relatively pure CD8⁺ cTRL populations, the best marker
403 combinations for cTRL isolation may require a more comprehensive comparison. Also, it remains to be
404 explored if the dose of 'pure' cTRLs is sufficient to achieve durable responses in patients. Indeed, recent
405 studies suggest that a more diverse and memory-like cell population may perform better on a long-term
406 basis⁶². Therefore, it may be necessary to pool cTRLs with other T cell subpopulations to deliver durable
407 therapeutic outcomes.

408

409 Taken together, our study provides new evidence supporting the presence of CD8⁺ tumour-reactive
410 lymphocytes in circulation and highlights the usefulness of such a population for cancer immunotherapy.
411 Future studies shall focus on verifying the therapeutic potency of cTRLs in humanized models. In addition, a
412 recent study revealed the presence of CD4⁺ tumour-reactive lymphocytes in circulation⁶³. Biomarkers for
413 CD4⁺ cTRLs is an additional area of importance considering the critical roles of CD4⁺ T cells in the long-term
414 success of ACT⁶⁴.

415

416

417 **Methods**

418 **Cell culture.** B16F10 mouse melanoma cells (RRID: CVCL_0159), CT26 mouse colon cancer cells (RRID:
419 CVCL_7254), and LLC1 mouse lung cancer cells (RRID: CVCL_4358) were purchased from ATCC
420 (Manassas, VA) and cultured in Roswell Park Memorial Institute 1640 (RPMI-1640) medium supplied with
421 10% fetal bovine serum (FBS, Wisent). AE17 mouse mesothelioma (RRID: CVCL_4408) were purchased
422 from ECACC (Porton Down, England) and maintained in RPMI-1640 with 10% FBS. MC38 mouse colon
423 cancer cells (RRID: CVCL_0A68) and KPCY 6419c5 mouse pancreatic cancer cells (RRID: CVCL_YM21)
424 were purchased from Kerafast (Boston, MA) and cultured in Dulbecco's Modified Eagle Medium (DMEM,
425 high-glucose) with 10% FBS. B16F10^{OVA}, and AE17^{OVA} cells were established by lipofection using
426 lipofectamine 3000 (Thermo) and SIINFEKL-GFP-puro plasmid (#102944, Addgene, Watertown, MA),
427 followed by two rounds of 5-day puromycin selection. CT26^{HA} cells were established as described
428 previously.⁶⁵ 4T1-Luc2 mouse breast cancer (RRID: CVCL_A4BM) were purchased from ATCC and
429 maintained in RPMI-1640 with 10% FBS and 8 µg/mL blasticidin (BLA477.100, BioShop, Canada).

430

431 **Mouse models for cTRL analysis and isolation.** All animal experiments were carried out in accordance
432 with the protocol approved by the Animal Care Committee of Northwestern University and/or University
433 Health Network. Female C57/BL6J strains of mice at 6 to 8 weeks of age were purchased from the Jackson
434 Laboratory (Bar Harbor, ME). Female Balb/c strains of mice at 6 to 8 weeks of age were purchased from
435 Jackson Laboratory. All mice are maintained at the animal facility under a 12/12 dark cycle.

436

437 For B16F10^{OVA} models, 5 x 10⁵ B16F10^{OVA} or B16F10 cells were engrafted subcutaneously (s.c.) on the right
438 flank of C57BL6J mice. On day 14, mice were euthanized for blood, spleen, and tumour isolation. For
439 CT26^{HA} models, 5 x 10⁶ CT26^{HA} or CT26 cells were engrafted s.c. on the right flank of Balb/c mice. On day
440 18, mice were euthanized for blood, spleen and tumour isolation. For AE17^{OVA} models, 3 x 10⁶ cells were
441 engrafted s.c. on the right flank of C57BL6J mice. On day 16, mice were euthanized for blood and tumour
442 isolation. For MC38 models, 5 x 10⁶ cells were engrafted s.c. on the right flank of C57BL6J mice. On day 21,
443 mice were euthanized for blood and tumour isolation. For KPCY 6419c5 models, 2 x 10⁶ cells were engrafted
444 s.c. on the right flank of C57BL6J mice. On day 21, mice were euthanized for blood and tumour isolation. For

445 4T1-Luc2 models, 2 x 10⁵ 4T1-Luc2 cells were injected intravenously (i.v.) to Balb/cJ mice. On day 21, mice
446 were euthanized for blood isolation.
447

448 The mouse blood was collected through cardiac puncture. The blood was lysed in 1X RBC lysis buffer (00-
449 4333-57, Thermo) for 5 min at RT and used for downstream flow cytometric analysis or magnetic isolation.
450 The spleens were mechanically dissociated by cell scrapers through 70 µm cell strainers. Dissociated
451 splenocytes were lysed by 1X RBC lysis buffer for 3 mins at room temperature (RT) and used for
452 downstream flow cytometric analysis or magnetic isolation. The tumours were sectioned into 2 – 4 mm²
453 pieces and dissociated with the mouse tumour dissociation kit (150-096-730, Miltenyi) using the gentleMACS
454 C tubes (130-093-237, Miltenyi Biotec, Germany) and gentleMACS Dissociator (130-093-235, Miltenyi),
455 according to manufacturer's protocol. Cell mixture was filtered twice by 70 µm strainers prior to downstream
456 flow cytometric analysis or magnetic isolation.
457

458 For flow cytometric analysis, cells were stained by fluorescently labelled multimers at 2 – 8 °C for 30 min,
459 followed by the staining of CD8α-APC at RT for 20 min in 1X phosphate-buffered saline (PBS) with 1%
460 bovine serum albumin (BSA, A9418, Sigma). Stained samples were analysed by an acoustic flow cytometer
461 (Attune NxT, Thermo) with a 2-laser 7-colour setup. Due to the rarity of tumour-specific T cells, at least
462 500,000 events were recorded for each sample. Acquired data were analysed by FlowJo software (FlowJo
463 LLC., Ashland, OR).
464

465 For magnetic isolation, cells were stained by untouched mouse CD8 cells kit (11417D, Thermo) and sorted
466 by the MATIC chips at the flow rate of 16 mL/hr. Negative portions were collected and stained by
467 fluorescently labelled multimers targeting OVA or HA at 2 – 8 °C for 30 min, followed by anti-fluorophore
468 magnetic particles with 50 nm diameter (Miltenyi) at RT for 20 min in 1X PBS with 1% BSA. Labelled cell
469 samples were sorted by the MATIC chips using the flow rate of 4 mL/hr to capture tumour-reactive T cell
470 population. For CD103-mediated sorting, negative portions collected from the first run were stained by anti-
471 CD103 APC at RT for 20 mins in 1X PBS with 1% BSA, followed by anti-APC magnetic particles (130-090-
472 855, Miltenyi) at RT for 20 mins in 1X PBS with 1% BSA. Labelled cell samples were sorted by the MATIC
473 chips using the flow rate of 10 mL/hr to capture CD103⁺ cells. Details of chip setup was describe in the
474 section of 'chip fabrication and operation'.
475

476 **Chip fabrication and operation.** The MATIC microfluidic cell sorter was fabricated using the protocol
477 described before.²¹ In brief, the master mold was 3D printed by a stereolithographic 3D printer (Microfluidics
478 Edition 3D Printer, Creative CADworks, Canada) using the "CCW master mold for PDMS" resin (Resinworks
479 3D, Canada) with the layer thickness of 25 µm. The chips were made by casting PDMS (Sylgard 184, Dow
480 Chemical, Midland, MI) on printed molds, followed by 30-min incubation at 100 °C. Cured PDMS replicas
481 were peeled off, punched and plasma bonded to thickness no. 1 glass coverslips (260462, Ted Pella,
482 Redding, CA) to finish the chip. Prior to use, the chips were conditioned by 1X PBS with 1% sterile Pluronic
483 F68 (24040032, Thermo) to reduce non-specific cell capture. During experiments, each chip was
484 sandwiched by arrayed N52 NdFeB magnets (D14-N52, K&J Magnetics, Pipersville, PA) and connected to a
485 digital syringe pump (Fusion 100, Chemyx, Stafford, TX) for fluidic processing.
486

487 To form the sorting setup for mouse samples, the modules with 200 µm thickness were chosen to form a
488 binary setup, which is able to separate cells into 2 populations (on-chip, effluent) based on its degree of
489 magnetic labelling. For the isolation of cTRLs, the flow rate was optimized to 16 mL/hr and 4 mL/hr for the
490 first (CD8 negative selection) and second run (multimer positive selection) by a preliminary run using RBC-
491 lysed blood samples. For the CD103-mediated second runs, the flow rate was set to 10 mL/hr. For the
492 isolation of TILs, the flow rate was set to 16 mL/hr, as optimized before.²¹ For the CD45RA-mediated
493 isolation of naïve and memory T cells, the flow rate was set to 32 mL/hr, as optimized before.²¹
494

495 To form the sorting setup for human samples, the modules with 200 µm thickness were chosen to form a
496 binary setup. For the isolation of cTRLs, the flow rate was set to 24 mL/hr and 12 mL/hr for the first (CD8
497 negative selection) and second runs (CD103 positive selection). For the isolation of TILs, the flow rate was
498 set to 24 mL/hr.
499

500 **Comparison of isolation efficiency.** The comparison of isolation efficiency among FACS, MACS, and
501 MATIC is carried out by spiked-in blood samples. OT-1 CD8⁺ mouse cytotoxic T cells were isolated from the
502 spleen of OT-1 mice (C57BL/6-Tg(TcrαTcrβ)1100Mjb/J, 003831, Jackson) using a magnetic separation kit
503 (130-096-543, Miltenyi Biotec). Isolated T cells were activated and expanded with CD3/CD28 beads (130-
504 093-627, Miltenyi or 11452D, Thermo) at the cell/bead ratio of 1:1 for the first 3 days in Iscove's Modified
505 Dulbecco's Medium (IMDM) and cultured up to 6 days supplied with 10% FBS and 100 ng/mL mouse
506 interleukin 2 (130-120-662, Miltenyi) prior to the spike-in experiment. The spike-in sample is generated by
507 spiking 0.5% of OT-1 cells into the RBC lysed blood from tumour-free C57BL6 mice. Spike-in samples were

508 stained by PE labelled multimers targeting OVA at 2 – 8 °C for 30 min, followed by anti-PE magnetic
509 particles with 50 nm diameter (Miltenyi) at RT for 20 min in 1X PBS with 1% BSA (for MACS and MATIC and
510 only). Labelled cell samples were sorted by the FACS based on the intensity of PE channel and MACS or
511 MATIC based on the degree of magnetic labelling. FACS was performed by a BD FACS Aria IIIu by gating
512 FSC/SSC and PE channels. MACS was performed by MACS LS columns (130-042-401, Miltenyi) using the
513 QuadroMACS separator (130-091-051, Miltenyi). MATIC was performed using the condition described in the
514 section of 'chip fabrication and operation'. Purity of unsorted and sorted samples was assessed by an
515 acoustic flow cytometer (Attune NxT). Recovery of sorted samples was calculated based on the number of
516 recorded PE⁺ cells, normalized to the unsorted sample.

517
518 **TCR sequencing.** Isolated mouse CD8⁺ T cell populations were centrifuged to form cell pellets and
519 submitted to MedGenome (Foster City, CA) for bulk TCR sequencing using a SMARTer mouse TCR a/b
520 profiling workflow (634402, TakaraBio, Japan). Isolated human CD8⁺ T cell populations were centrifuged to
521 form cell pellets and submitted to MedGenome for bulk TCR sequencing using a SMARTer human TCR a/b
522 profiling workflow (635016, TakaraBio). After library preparation, sequencing is performed using the 600
523 cycle kit on an Illumina MiSeq platform (San Diego, CA). Data generated is demultiplexed and trimmed.
524 MiXCR (version 2.1.11) software was used to align the reads to the TCR clonotypes and assemble the final
525 CD3 and full-length clonotypes. VJ gene usage was also extracted from the MiXCR data, including VJ gene
526 usage.

527
528 **RNA sequencing.** Isolated CD8⁺ T cell populations were centrifuged to form cell pellets and submitted to
529 MedGenome (Foster City, CA) for bulk mRNA sequencing using a SMART-seq v4 workflow in an ultra-low
530 input fashion (634888, TakaraBio, Japan). Prepared libraries were sequenced by an Illumina HiSeq platform.
531 Alignment was performed using STAR (v2.7.3a) aligner. Reads mapping to ribosomal and mitochondrial
532 genome were removed before performing alignment. The raw read counts were estimated using HTSeq
533 (v0.11.2). Read counts were normalized using DESeq2 to get the normalized counts. Additionally, the
534 aligned reads were used for estimating expression of the genes using cufflinks (v2.2.1). Differential gene
535 expression was calculated based on normalized counts.

536
537 **Cytometry by time of flight (CyTOF).** Single-cell suspension from TIL migration model were cryopreserved
538 immediately without culture and activation. Cells were thawed and submitted to the centre for advanced
539 single cell analysis (CASCA) at the SickKids Research Institute for cytometry by time of flight (CyTOF). Cells
540 were stained using the protocol recommended by Fluidigm (San Francisco, California) and examined by a
541 Helios CyTOF system. The antibodies used in CyTOF were listed in Supplementary Table 3. Acquired data
542 were processed by FlowJo v10.5 (FlowJo LLC, Ashland, OR) by gating center, width, residual, and 193Lr-
543 DNA2 channel. Built-in tSNE and plugins (FlowSOM and Cluster Explorer) were used for high-dimensional
544 analysis.

545
546 **Expansion of cTRLs.** For standard TILs isolated from the tumour, the TILs were cultured on 24-well or 12-
547 well plates for 3 days at the density of 0.5 - 1 x 10⁶ cells/mL in IMDM supplied with 10% FBS, 2X GlutaMAX
548 (35050061, Thermo), 500 ng/mL mouse interleukin 2. TILs were activated with CD3/CD28 beads at the
549 cell/bead ratio of 1:1 for the first 3 days. TILs were subcultured every 2 – 3 days to maintain the cell density
550 of 1 x 10⁶ cells/mL.

551
552 For cTRLs isolated from the blood, the TILs were co-cultured with the feeder CD45.1⁺ CD8⁺ T cells isolated
553 from the spleen, at the donor:feeder ratio of 1:50 – 1:100 in IMDM with 10% FBS, 2X GlutaMAX, 500 ng/mL
554 mouse interleukin 2, 25 - 50 ng/mL mouse interleukin 15 (566302, Biolegend, San Diego, CA) and 10 - 20
555 ng/mL recombinant mouse TGF-beta (763104, Biolegend). The feeder cells were isolated from spleen as
556 described above, expanded with CD3/CD28 beads in IMDM with 100 ng/mL mouse interleukin 2 for 3 days
557 and received 30 – 35 Gy irradiation through a Cs-137 source to inhibit proliferation prior to co-culture. During
558 co-culture, cTRLs were activated with CD3/CD28 beads (130-093-627, Miltenyi or 11452D, Thermo) for the
559 first 3 days and received medium renewal at day 3, 5 and 7. Post day 7, cTRLs were subcultured every 2 – 3
560 days to maintain the cell density of 1 x 10⁶ cells/mL.

561
562 Fold of expansion was calculated based on the ratio of CD45.2/CD45.1 staining through flow cytometry. All
563 cultured TILs were cryopreserved at day 10 – 14 with a freezing medium containing 90% FBS 10% DMSO.
564 TILs were thawed 1-2 days prior to downstream *in vitro* assays and *in vivo* transplantation.

565
566 **Adoptive transplantation.** For B16F10 models, female C57/BL6J strains of mice at 6 to 8 weeks of age
567 were s.c. engrafted with 5 x 10⁵ B16F10^{OVA} on the right flank of mice. On day 7, 1 x 10⁶ TILs, CD8⁺CD103⁻
568 PBMC, or cTRLs (CD8⁺CD103⁺) were injected intravenously (i.v.). All mice received the injection of 10 µg
569 IL-2 injection on day 8, 9 and 10. For LLC1 models, female C57/BL6J strains of mice at 6 to 8 weeks of
570 were s.c. engrafted with 2 x 10⁶ LLC1 on the right flank of mice. On day 7, 1 x 10⁶ TILs, CD8⁺CD103⁻ PBMC,

571 or cTRLs (CD8⁺CD103⁺) were injected intravenously (i.v.). All mice received the injection of 10 µg IL-2
572 injection on day 8, 9 and 10.

573

574 For MC-38 models, female Rag1KO C57/BL6J strains of mice (B6.129S7-Rag1^{tm1Mom}/J, 002216, Jackson) or
575 CD45.1 C57BL6J mice at 6 to 8 weeks of age were s.c. engrafted with 5 x 10⁶ MC-38 cells on the right flank
576 of mice. On day 7, some groups of mice received 1 x 10⁶ cTRLs (CD8⁺CD103⁺) i.v. All mice received the
577 injection of 10 µg IL-2 on day 8, 9, and 10. Some groups of mice received the intraperitoneal injection (i.p.)
578 injection of 10 mg·kg⁻¹ anti mPD-1 antibody (*In vivo* grade, BE0273, BioXCell, UK) every 2 days for 2 weeks.
579 For the MC-38 rechallenge models, naïve or cured CD45.1 C57BL6J mice at 18 to 20 weeks of age were
580 s.c. engrafted with 10 x 10⁶ MC38 cells on the left flank of mice.

581

582 For AE17 models, female CD45.1 C57BL6J mice at 6 to 8 weeks of age were s.c. engrafted with 3 x 10⁶
583 AE17 cells on the right flank of mice. On day 7, some groups of mice received 1 x 10⁶ cTRLs (CD8⁺CD103⁺)
584 i.v. All mice received the injection of 10 µg IL-2 on day 8, 9, and 10. Some groups of mice received the
585 intraperitoneal injection (i.p.) injection of 10 mg·kg⁻¹ anti mGTR antibody (*In vivo* grade, BE0063, BioXCell)
586 every 3 days for 2 weeks.

587

588 Tumour growth was monitored twice a week starting from day 5 for 40 days. Tumour size was measured by
589 a caliper using the modified ellipsoid formula: 0.5*(Length*Width²). Mice were euthanized when the tumour
590 size exceeds the ethical limits (> 1000mm³). At the endpoint, tumours were isolated, fixed by 10% formalin
591 (HT501320, Sigma), paraffin-embedded, sectioned with 5 µm thickness, and stained by anti CD8α, CD45R,
592 CD4 and CD208 antibody (See Table. S5 for details) for immunohistochemical analysis of the degree of
593 infiltration. Stained slides were whole-slide scanned by an Aperio digital slide scanner (Leica, Wetzlar,
594 Germany) and quantified by Halo software (Version 3.0311, Indica Labs, Albuquerque, NM) for the number
595 of infiltrated cells (CD4⁺ or CD8⁺ for T cells, CD45R⁺ for B cells, CD208⁺ for dendritic cells) in solid tumours
596 using build-in random forest classifier and cytonuclear analyser (Version 2.0, Indica Labs).

597

598 For 4T1-Luc2 model, female NU/J (002019, Jackson) strains of mice at 6 to 8 weeks of age were i.v.
599 engrafted with 2 x 10⁵ 4T1-Luc2 cells. On day 10, mice received 1 x 10⁶ cTRLs (CD8⁺CD103⁺) or
600 CD8⁺CD103⁻ PBMC cells i.v. All mice received the injection of 10 µg IL-2 on day 11, 12, and 13. Growth of
601 metastasis was monitored twice a week starting from day 9 by bioluminescence (IVIS Spectrum, 124262,
602 Perkin Elmer, Waltham, MA). Mice were i.p. injected with 3 mg of D-Luciferin potassium salt (LUCK-100,
603 GoldBio, St. Louis, MO) 10 – 15 min prior to imaging as determined by the standard curve of Luciferin. 1 –
604 120s exposure with medium or low binning was used for the image acquiring. Acquired data were processed
605 by the IVIS Live Imaging software (Perkin Elmer) following the manufacturer's protocol. To quantify the exact
606 of metastases, lungs were isolated at the end point from different groups. Collected lungs were fixed by 10%
607 formalin, paraffin-embedded, sectioned and H&E stained. Stained slides were submitted to the Centre of
608 Phenogenomics at the Mount Sinai Hospital (Toronto, Canada) for histopathology. Slides were evaluated by
609 licensed veterinary pathologists and the exact number of micro metastases per layer were reported.

610

611 **Ethics statement and patient samples.** For colorectal cancer samples, the dissociated tumour samples
612 and paired PBMC were purchased from Discovery Life Science (DLS, Huntsville, AL). The cases we
613 purchased were at stage II – III with more than 40% CD45⁺ cells in dissociated tumour samples. Basic
614 information and percentage of CD45⁺ cells of each sample was examined by DLS at the time of banking
615 and provided as a PDF sheet.

616

617 For lung cancer samples, all experiments were approved by the Research Ethics Board (REB) at the Toronto
618 General Hospital Research Institute (TGHRI). All individuals have provided written consent and the protocol
619 was approved by the TGHRI. All blood samples were collected in standard K2-EDTA tubes (02-657-32, BD)
620 and stored in 2 – 8 °C for up to 6 hrs before gradient centrifuge in Ficoll Paque (GE17-1440-02, Sigma).
621 Isolated PBMCs were stored in CryoStor CS10 freezing medium (07930, Stem Cell Technologies, Canada)
622 under a liquid nitrogen condition before use. Resected tumour were dissociated using a human tissue
623 dissociation kit (130-095-929, Miltenyi), filtered twice through 100 µm stainers, and cryopreserved in liquid
624 nitrogen before use. Maglianant pleural effusions (MPE) were obtained via thoracentesis, lysed by RBC lysis
625 buffer, and filtered twice through 100 µm stainers and cryopreserved in liquid nitrogen before use.
626 For TCR sequencing, CD8⁺ T cells in cryopreserved dissociated tumour cells (DTCs) were enriched by CD8-
627 mediated MATIC following the protocol described in the section 'chip fabrication and operation'. CD8⁺CD103⁻
628 and CD8⁺CD103⁺ cells were purified from cryopreserved PBMCs using MATIC following the same protocol.
629 Isolated cells were centrifuged to form cell pellets for downstream sequencing.

630

631 For the co-culture experiments, leukocytes in DTCs were depleted by the MACS-mediated selection against
632 CD45 using LD columns (130-042-901, Miltenyi) using the QuadroMACS separator. CD8⁺CD103⁺ cells were
633 purified from cryopreserved PBMCs using MATIC following the protocol described in the section of 'chip

634 fabrication and operation'. Bulk or purified cells were co-cultured with leukocyte-depleted DTCs for 12 - 24
635 hrs in the medium consisting 50% of IMDM, 50% of ImmunoCult-XF T cell expansion medium (10981, Stem
636 Cell Technologies), 5% human AB serum (BP2525100, Fisher Scientific, Waltham, MA), 250 - 500 ng/mL
637 recombinant human interleukin 2 (78036.2, Stem Cell Technologies), 1% Penicillin/Streptomycin (15140122,
638 Thermo) and 5 µg/mL Brefeldin A (B7651, Sigma). The percentage of interferon gamma (IFN-γ) secreting
639 cells was assessed by the flow cytometry by gating the populations of CD8⁺/IFN-γ⁺.
640

641 **Statistical analysis.** Results were shown by Prism GraphPad (Version 9.1.0, GraphPad Software, San
642 Diego, CA) as an average ± standard deviation unless specified elsewhere. Each dot represents a biological
643 replicate. P value was calculated by the build-in analysis function of Prism GraphPad.
644

645 **Reporting Summary.** Further information on research design is available in the Nature Research Reporting
646 Summary linked to this article.
647
648
649

650 **Data availability**

651 The main data supporting the results in this study are available within the paper and its Supplementary
652 Information. The RNAseq data is available from the gene expression omnibus (GEO,
653 <https://www.ncbi.nlm.nih.gov/geo/>) under the access code **CODE**. The unprocessed TCR sequencing files
654 and CyTOF data are too large to be publicly shared, yet they are available from the corresponding author on
655 reasonable request. Source data are provided with this paper.
656

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814

815 **Author contributions**

816 Z.W and S.O.K conceived and designed the experiments. Z.W performed cell isolation, flow cytometry and
817 CyTOF. S.A performed the animal study. M.L performed RNA extraction and qPCR. H.W extracted the OVA
818 plasmid and assisted with the animal study. L.W maintained the AE17 cell lines. L.W, F.B-Z, N.S, S.B and
819 S.K managed patient-sample collection, distribution and administration. All authors discussed the results,
820 analysed the data and contributed to the preparation and editing of the manuscript.

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Competing interests

S.O.K and Z.W have a filled patent application using parts of the data reported in this article. S.O.K has a patent “Device for capture of particles in a flow” US10073079 licensed to Cellular Analytics. A.J.R.M is a paid consultant for Cellular Analytics. M.D.P. received personal fees from Actelion, AstraZeneca, Bayer, Bristol Myers Squibb, Merck, and Roche outside of the submitted work. S.O.K received research funds from Amgen through a sponsored research agreement. The other authors declare no competing interests.

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852 **Fig. 1 | Non-invasive collection of tumour-reactive cells in blood circulation for cancer**
853 **immunotherapy.** **a**, Schematic showing conventional invasive TIL therapy (left, it requires excisional surgery
854 to obtain large tumour lesion) compared to non-invasive circulating tumour-reactive lymphocytes (cTRL)
855 therapy (right, it requires blood collection only). **b**, Schematic showing the trafficking dynamics of cTRLs
856 between tumours.
857

858 **Fig. 2 | Isolation of tumour-reactive TRLs in blood circulation.** **a**, Working principle of tumour reactivity-
859 mediated microfluidic cell sorting for analysis. Cells were firstly magnetically labelled based on their TCR
860 reactivity with tumour antigen-derived MHC multimers. Magnetically labelled cells were separated from their
861 counterparts by microfluidic cell sorting for downstream analysis. **b**, Workflow of the identification via defined
862 epitope models. CD8⁺ T cells in PBMC were classified as tumour-reactive and non-reactive populations
863 based on their reactivity with multimers. Classified cells were compared to intratumoural CD8⁺ TILs for clonal
864 analysis. **c**, Quantitation of the sorting performance based on antibody and multimer through microfluidic
865 sorting. **d**, Comparison of clonal similarity among TIL, cTRL, and PBMC by V-J usage profile. **e**, Analyses of
866 the coverage of top 50 clones between each population. Unpaired t-test, mean ± s.d., each dot represents a
867 biological replicate.
868

869 **Fig. 3 | Molecular and phenotypic signature of cTRL during and post migration.** **a**, Volcano plots
870 showing the differential expression (DE) of genes when comparing the normalized counts from non-cTRL
871 CD8⁺ PBMC and cTRL identified by multimer-based sorting from the B16 model. Key genes for CD8⁺ T cells
872 were presented as a heatmap alongside. **b**, Identification of tumour-reactivity in circulating CD8⁺CD103⁺
873 populations. **c**, Quantitation of tumour-reactive fraction in circulating CD8⁺CD103⁺ populations. **d**, CyTOF
874 analysis of multimer-binding cTRLs. The expression levels of CD103, CD39, PD-1, and CD69 were
875 examined. **e**, Quantitation of the expression level of CD103, CD39, PD-1 and CD69 in cTRLs during
876 migration. **f**, CyTOF analysis of CD45.2⁺ cTRLs and CD45.1⁺ TILs for the expression of CD103 in AE17
877 models. **g**, Quantitation of CyTOF data for the expression of CD103, CD69 and PD-1 in CD45.2⁺ cTRLs and
878 CD45.1⁺ TILs. Unpaired t-test, mean ± s.d., each dot represents a biological replicate.
879

880 **Fig. 4 | cTRLs exhibit significant levels of activity against primary and metastasized tumours in**
881 **murine models.** **a**, Workflow of the animal study. cTRL, CD8⁺CD103⁻ PBMC and TIL were expanded 1 – 2
882 weeks *in vitro* before adoptive cell transfer. IL-2 was given daily for the first 3 days post cell transfer to boost
883 lymphocyte proliferation. **b**, Quantitation of tumour size, survival rate, and percentage of infiltrated CD8⁺ cells
884 in s.c. B16 models WT C57BL6 mice treated by different T cells (n = 5). **c**, Representative bioluminescence
885 images treated by different T cells (n = 6) in induced 4T1 metastasis models in nude mice. **d**, Quantitation of
886 the total flux and survival rate in induced 4T1 metastasis models. Unpaired t-test, mean ± s.d., each dot
887 represents a biological replicate.
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889 **Fig. 5 | Synergistic effects of cTRLs and ICB/costimulatory molecules.** **a**, Quantitation of tumour size,
890 survival rate, and percentage of infiltrated CD8⁺ cells in s.c. MC38 models treated by different therapeutic
891 modalities (n = 5). **b**, Enriched pathways from upregulated RNAs reveal that αPD-1 and cTRLs generate
892 different impacts to the immune responses within the tumour microenvironment in s.c. MC38 models. **c**,
893 Quantitation of CD4⁺ T cells and CD208⁺ dendritic cells post different therapeutic modalities. **d**, Rapid tumour
894 rejection and formation of long-lasting TRLs were observed in cTRL-cured mice. **e**, Quantitation of tumour
895 size, survival rate, and percentage of infiltrated CD8⁺ cells in s.c. AE17 models treated by different
896 therapeutic modalities (n = 5). **f**, Quantitation of lymphocyte subpopulations at the endpoint of treatment in
897 endogenous (CD45.1⁺) populations in s.c. AE17 models. **g**, Quantitation of CD103 expression in transferred
898 cTRLs (CD45.2⁺) and endogenous lymphocytes (CD45.1⁺) in s.c. AE17 models. Unpaired t-test, mean ± s.d.,
899 each dot represents a biological replicate.
900

901 **Fig. 6 | CD103⁺ defines cTRL population in human PBMC.** **a**, Workflow of the co-culture assay to study
902 the relationship between tumour-reactivity and CD103 on human PBMCs. **b**, Representative flow cytometric
903 profile of IFN-γ secreting populations according to CD103 expression. This specific set of images is from
904 PE95. **c**, Quantitation of IFN-γ secreting populations in CD8⁺CD103⁺ and CD8⁺CD103⁻ cells across the
905 patient cohort of malignant pleural effusion (MPE). Unpaired t-test, mean ± s.d., each dot represents a
906 technical replicate.
907

908 **Fig. 7 | CD8⁺CD103⁺ cTRLs are phenotypically and clonally tumour-specific.** **a**, Workflow of the co-
909 culture assay to examine the level of tumour specificity of isolated cTRLs. **b**, Representative flow cytometric
910 profile of IFN-γ secreting populations in co-cultured populations. This specific set of images is from PE86. **c**,
911 Quantitation of IFN-γ secreting populations across a set of 18 patient samples. Tumour cells used in a co-
912 culture model to induce IFN-γ secretion were harvested either from tumour tissue (red) or malignant pleural
913 effusions (blue). Fold enrichment was calculated by comparing the percentage of IFNγ⁺ cells in bulk CD8⁺
914 and CD8⁺CD103⁺ populations post co-culture. **d**, Analysis of the coverage of top 50 TIL clones in cTRLs

915 and PBMC populations among 3 colon cancer patients (CA01 – CA03). **e**, Comparison of clonal similarity
916 among TILs, cTRLs and PBMC by V-J usage profile. cTRLs contains four TIL-derived major clones.
917 Unpaired t-test, mean \pm s.d. For **c**, each dot represents a technical replicate. For **d**, each dot represents a
918 biological replicate.
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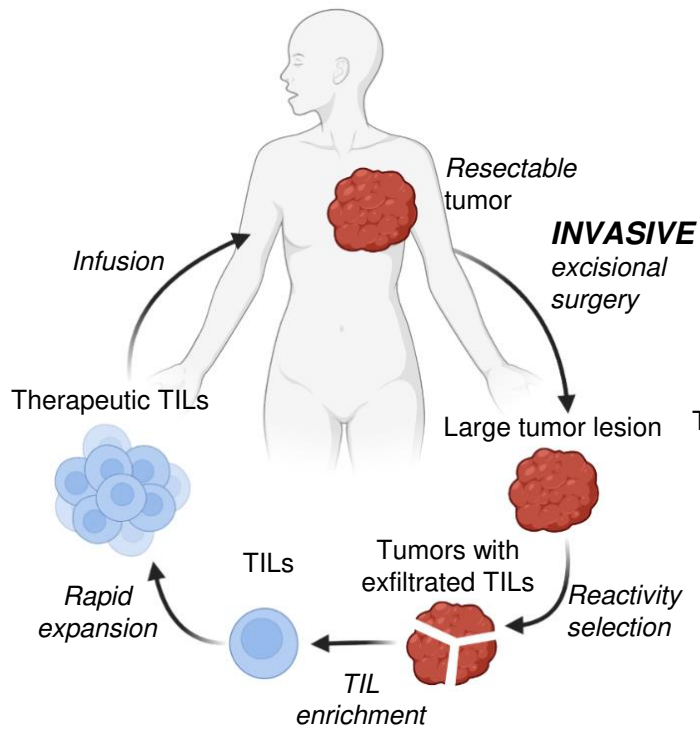
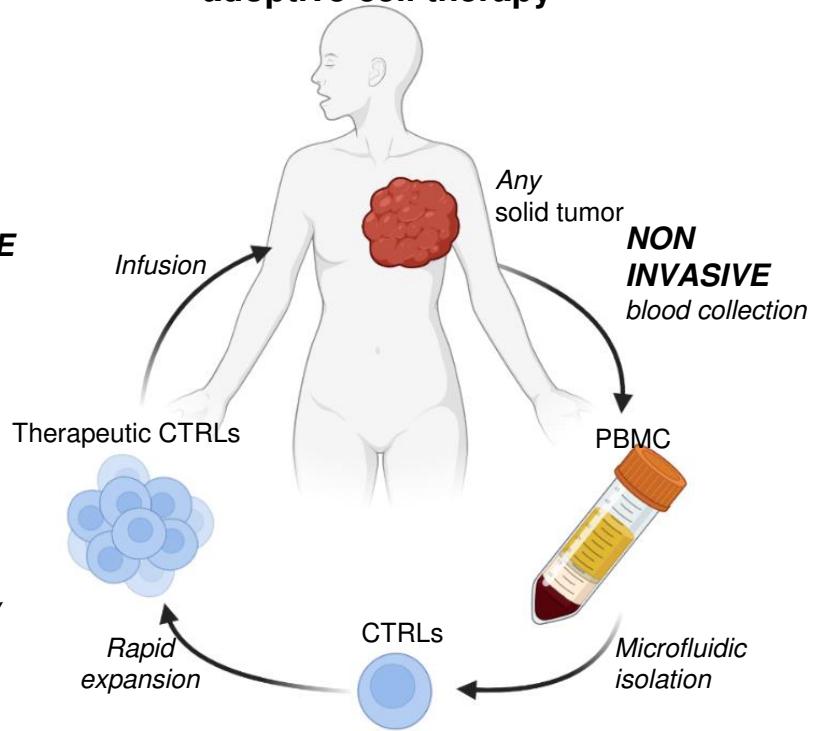
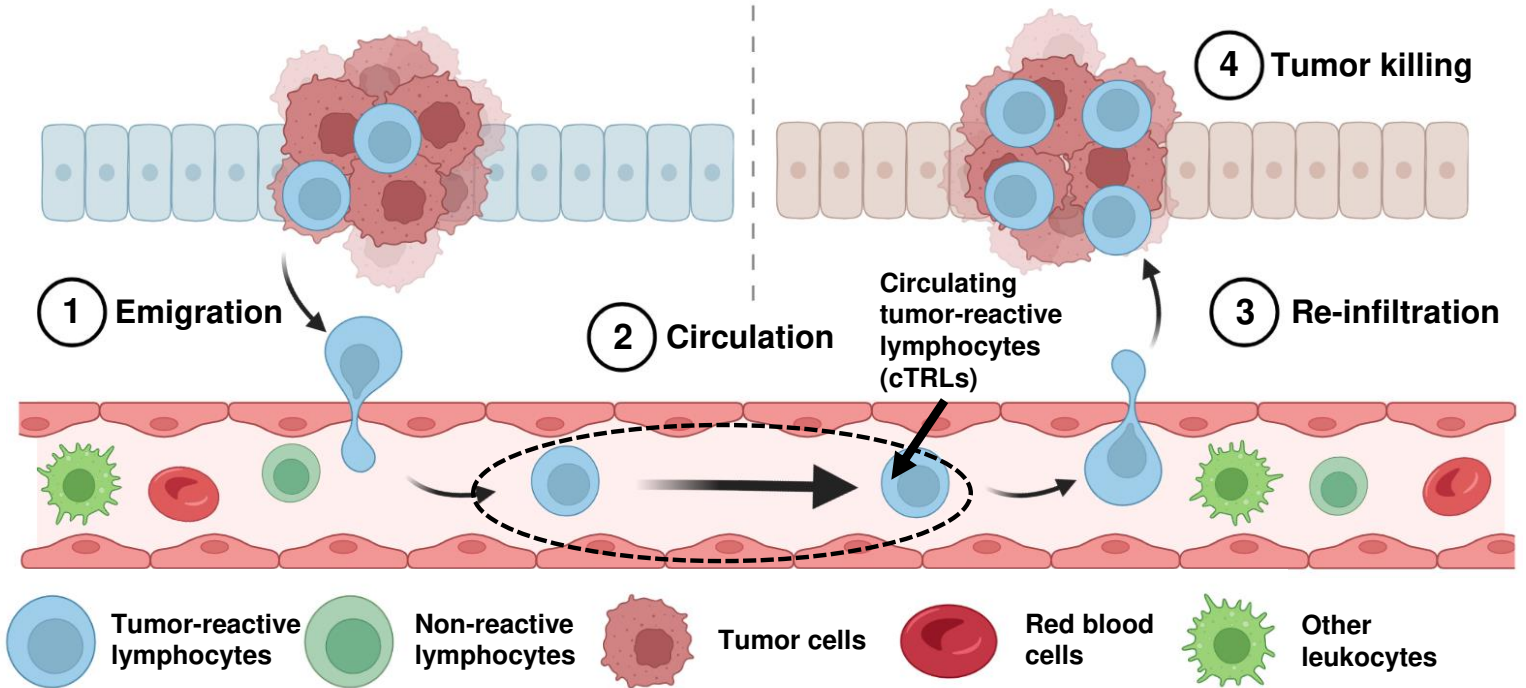
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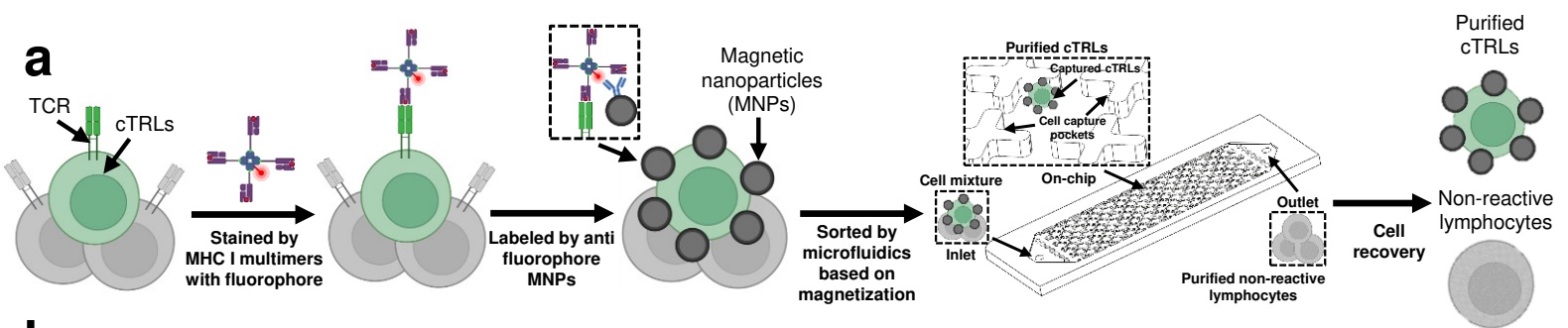
Table 1 | Fraction of top-20 TIL clones (by abundance) in cTRL and non-cTRL CD8+ PBMC.

CDR3 sequence	Fraction in TIL	Fraction in cTRL	Fraction in non-cTRL CD8+ PBMC
CAASVSGSFNKLTF	1.68%	0.06%	ND*
CAVSEAGSFNKLTF	1.29%	5.22%	ND
CAWSLSGTTSAETLYF	1.20%	ND	ND
CTCSADLGGFYAEQFF	1.19%	0.19%	ND
CTCSADRGGGYAEQFF	1.03%	3.98%	ND
CAMERPSSGQKLVF	1.01%	ND	ND
CAMREGGSNAKLTF	1.00%	1.20%	ND
CATDINQGGSAKLIF	0.99%	1.23%	ND
CAMREGMPNYNVLYF	0.88%	1.17%	ND
CAMREGGTGGYKVVV	0.86%	0.39%	ND
CAMSTGNYKYVF	0.80%	4.83%	0.07%
CASGVSGPDYTF	0.77%	1.14%	ND
CAVNTGNYKYVF	0.70%	0.15%	ND
CAVSMPSGSWQLIF	0.66%	ND	ND
CAMREANTGANTGKLTF	0.62%	4.92%	ND
CILRVDGPNYNVLYF	0.55%	1.07%	ND
CASNQGGSAKLIF	0.54%	1.40%	ND
CAAINNYAQGLTF	0.54%	1.14%	ND
CAMREGVGSALGRLHF	0.52%	0.17%	ND
CASSDVTGAYEQYF	0.50%	0.17%	ND

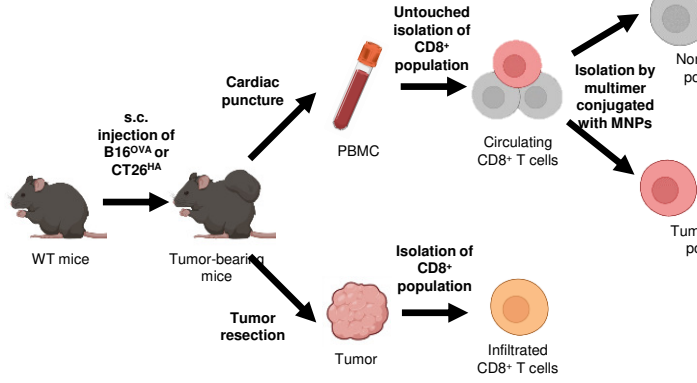
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*ND, Not detected.

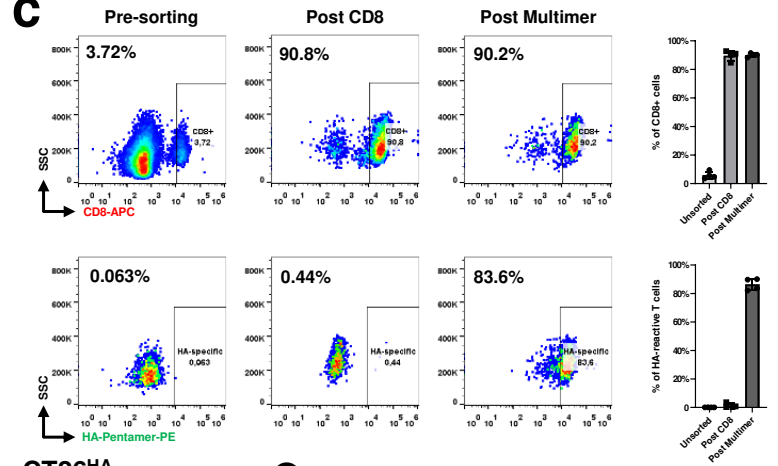
a**TIL-mediated adoptive cell therapy****cTRL-mediated adoptive cell therapy****b****Primary tumor site****Metastatic tumor site**



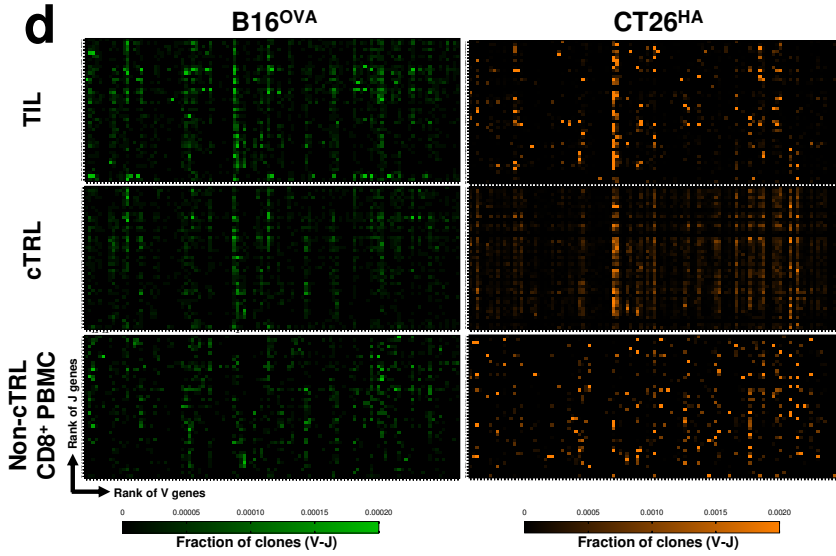
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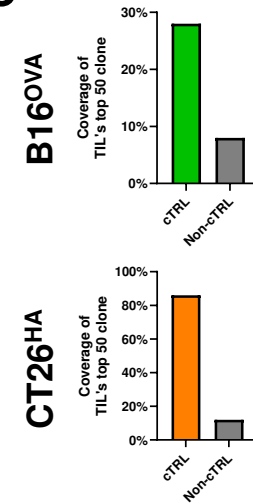
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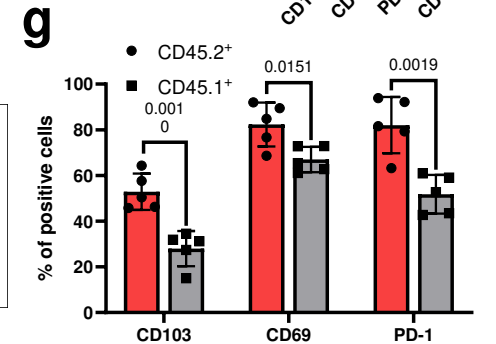
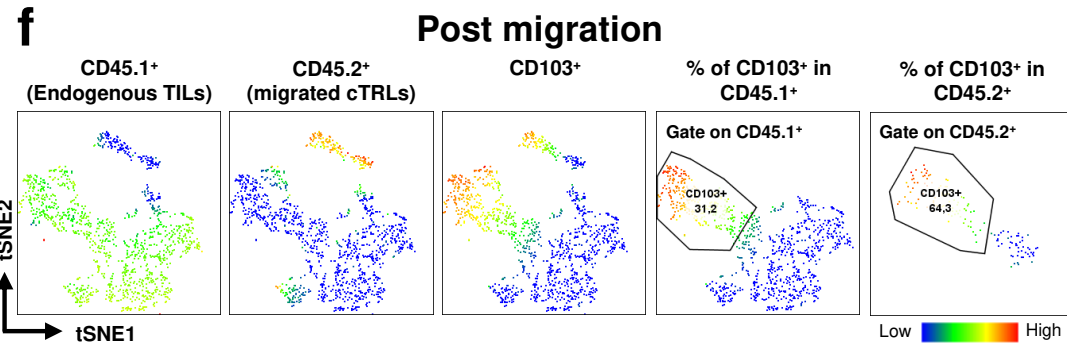
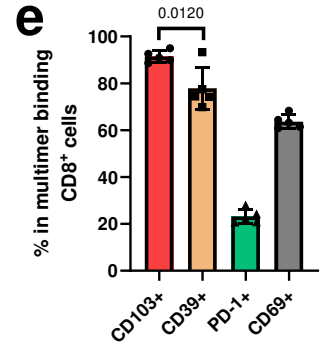
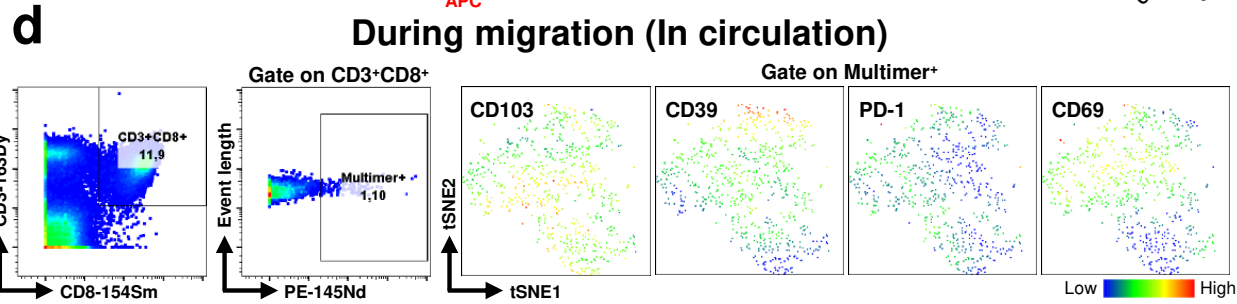
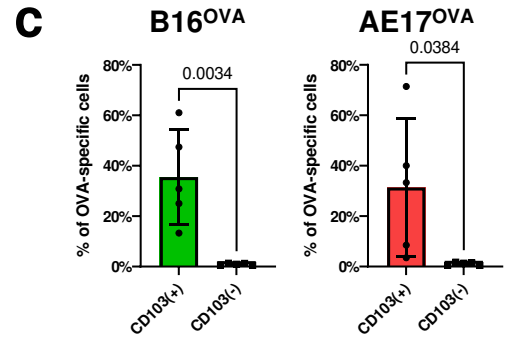
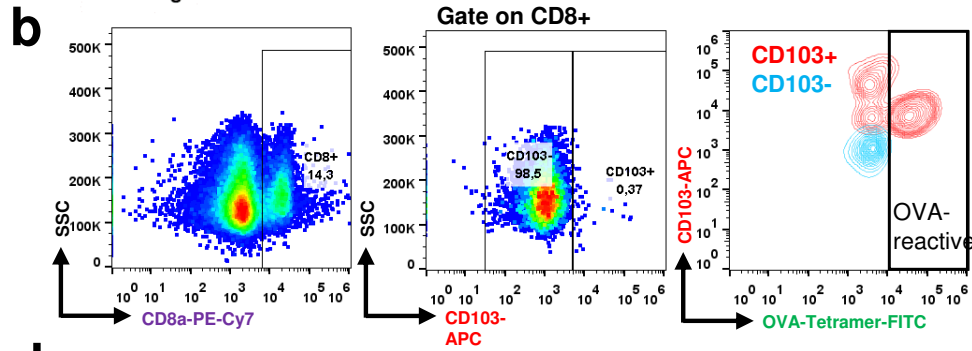
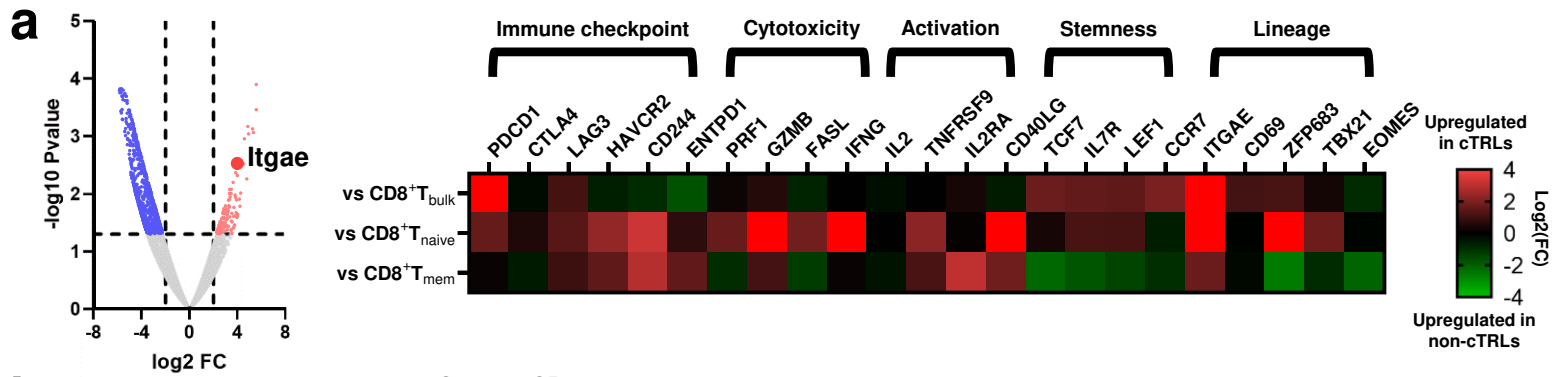


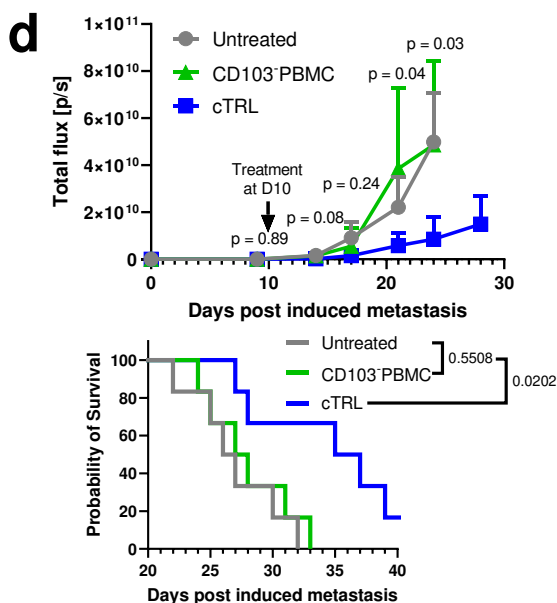
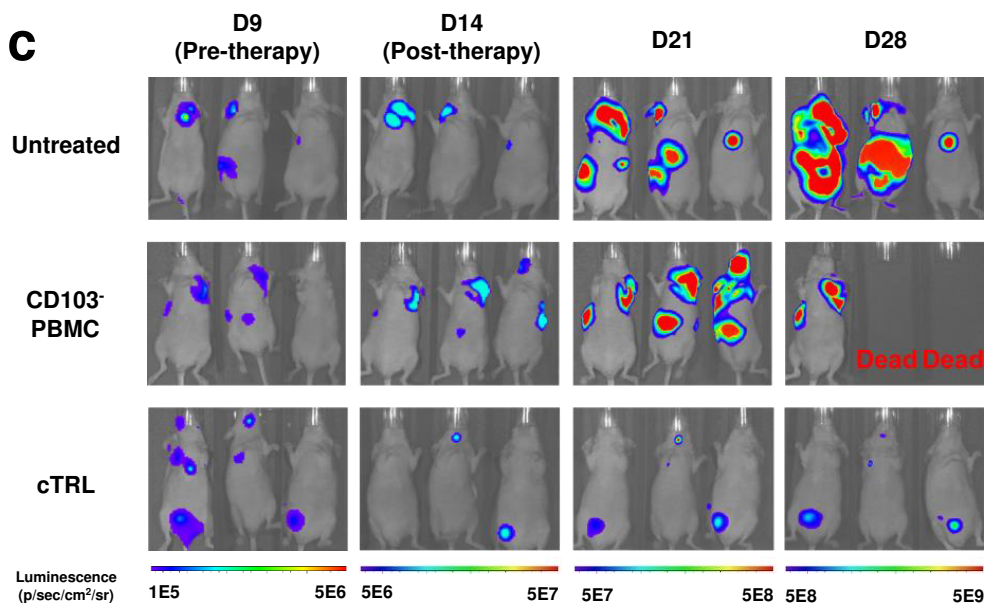
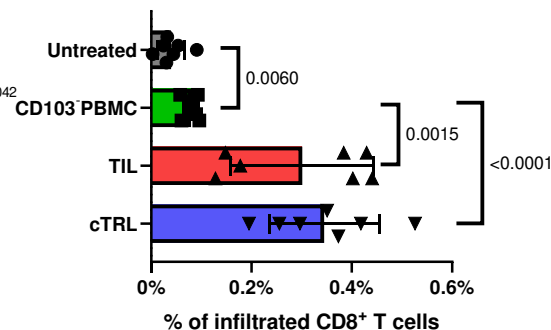
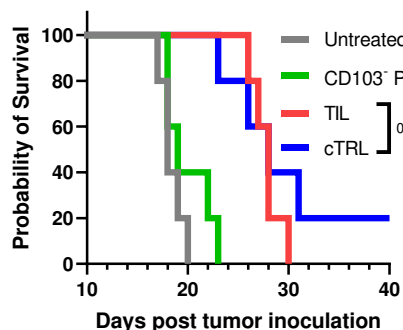
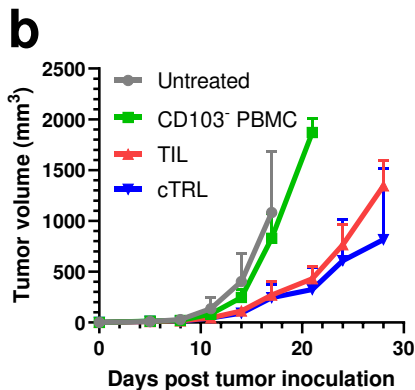
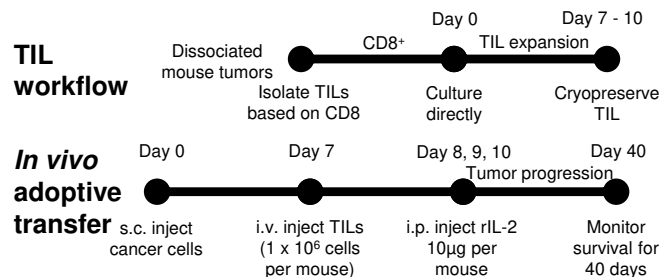
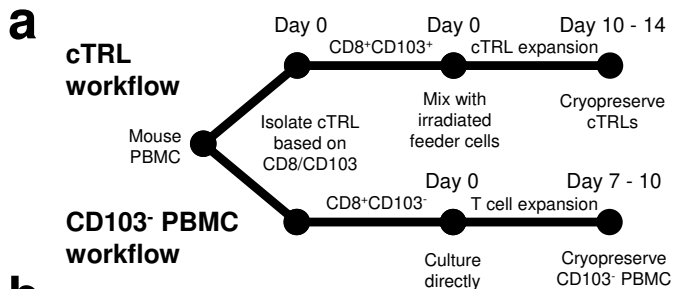
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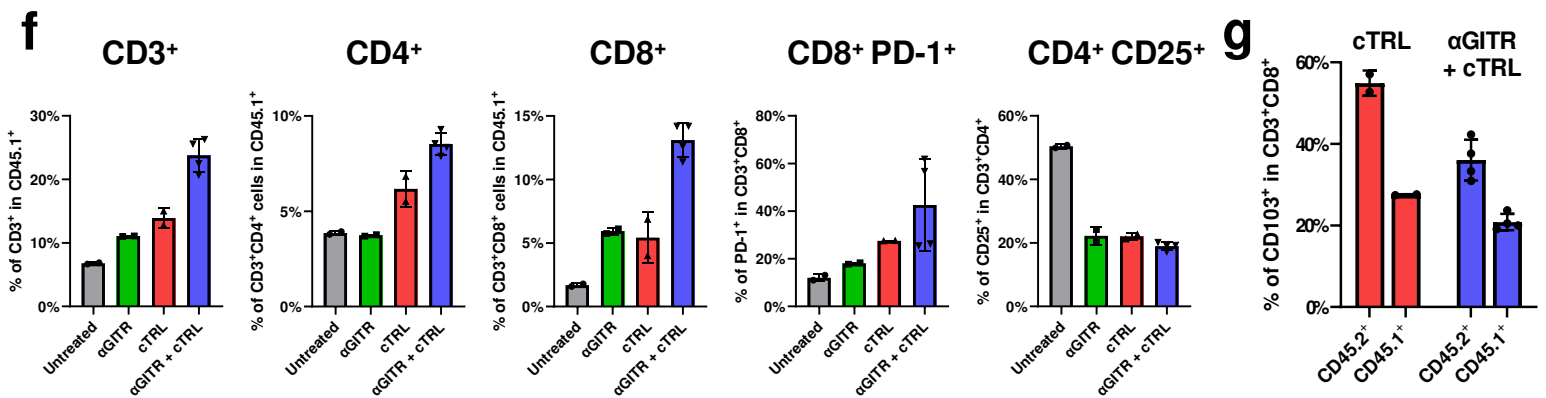
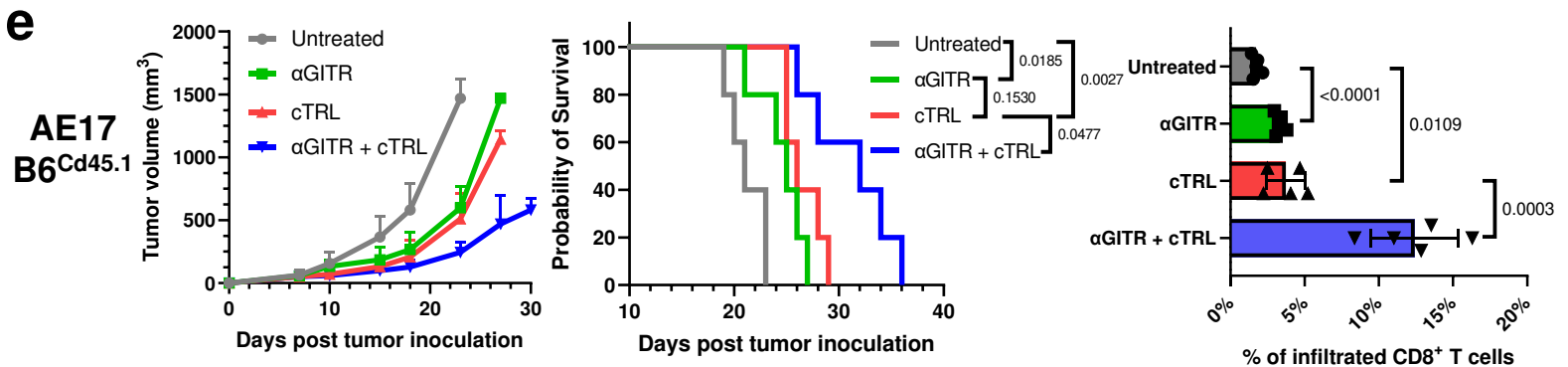
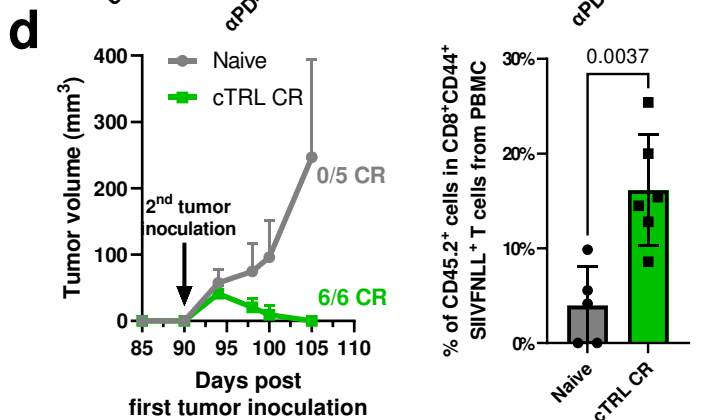
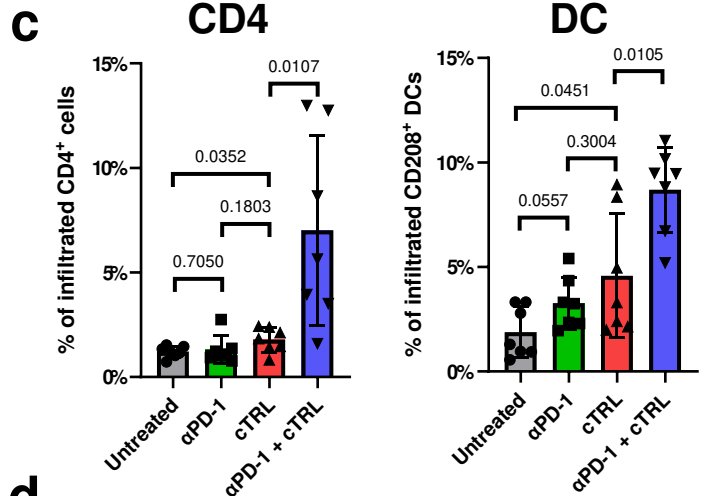
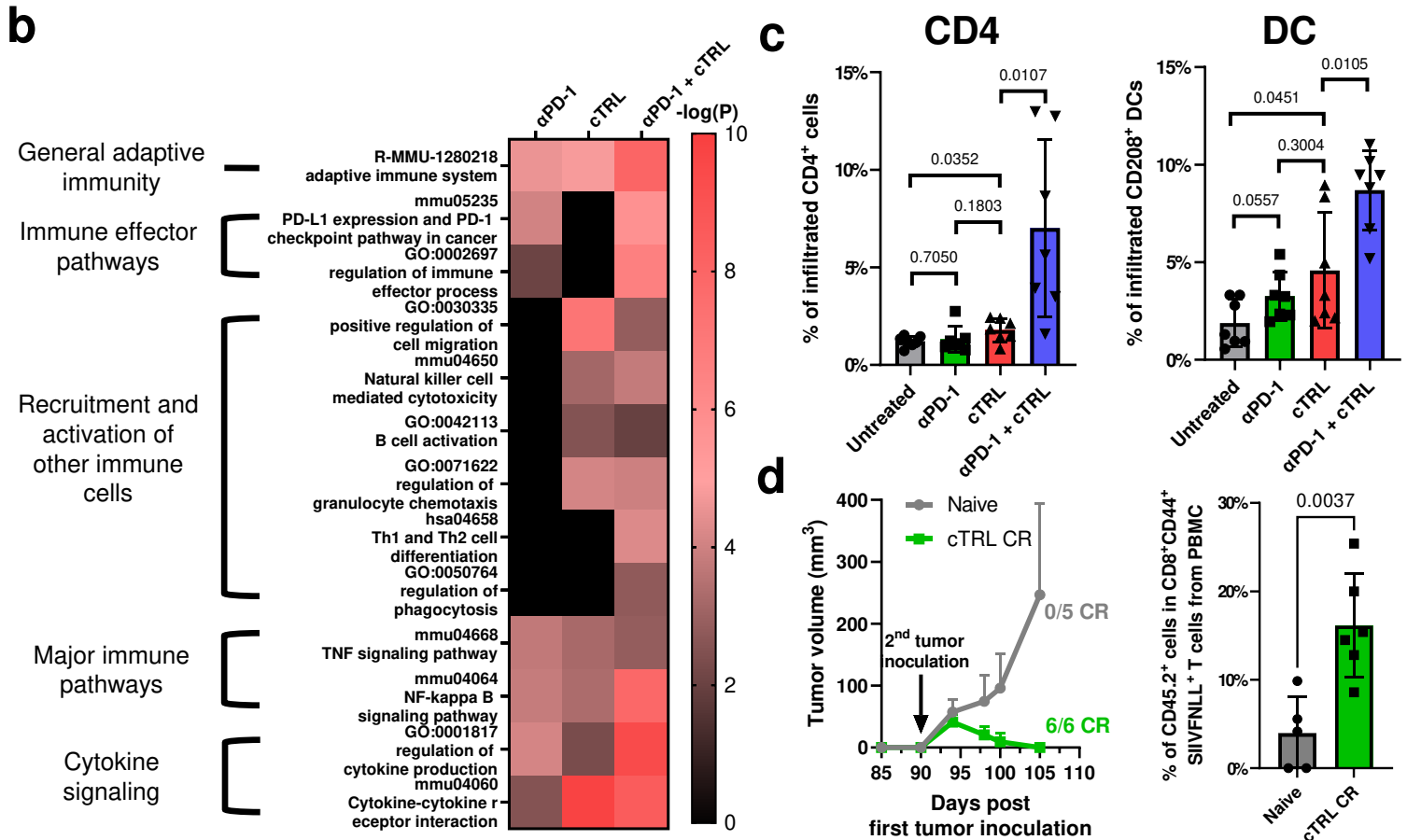
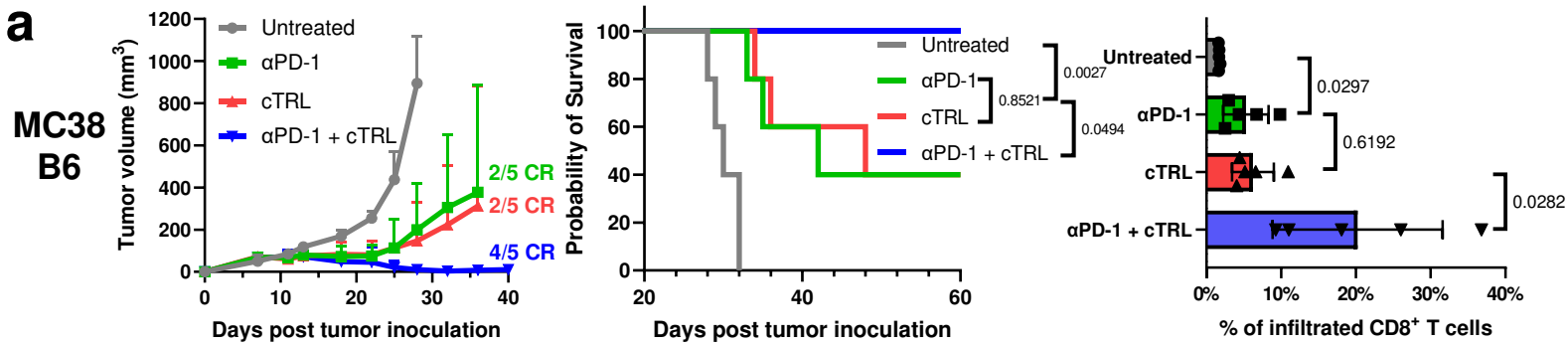


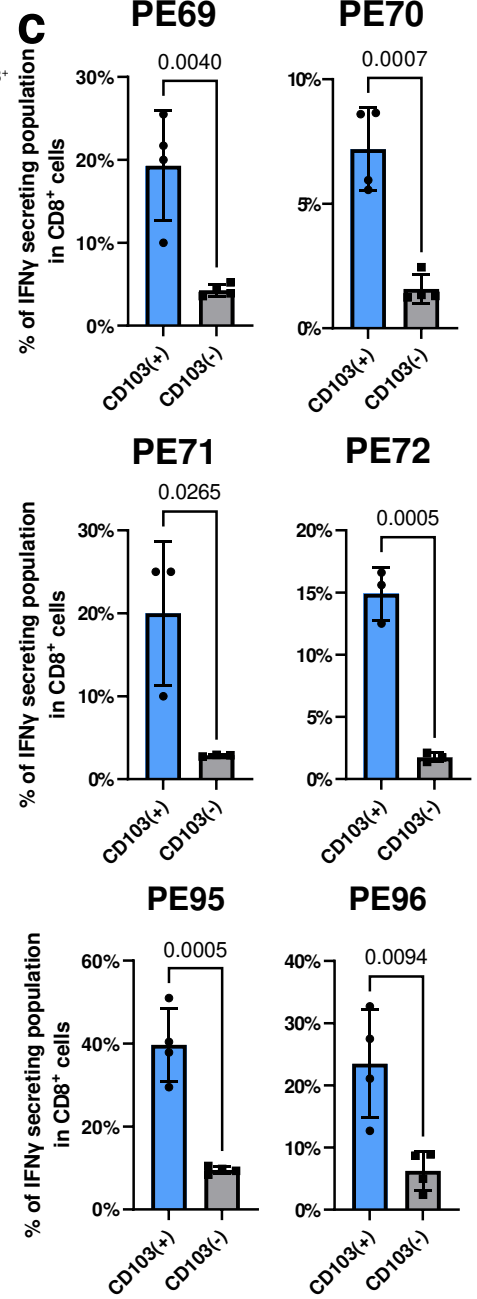
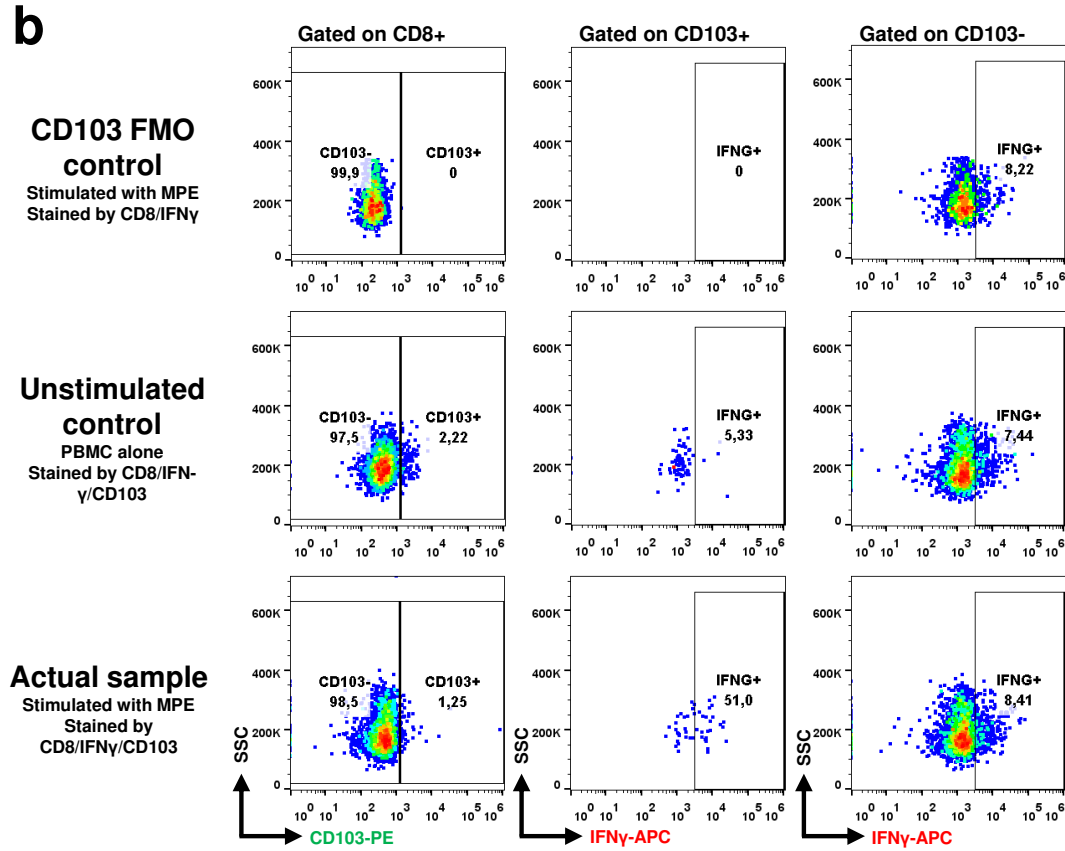
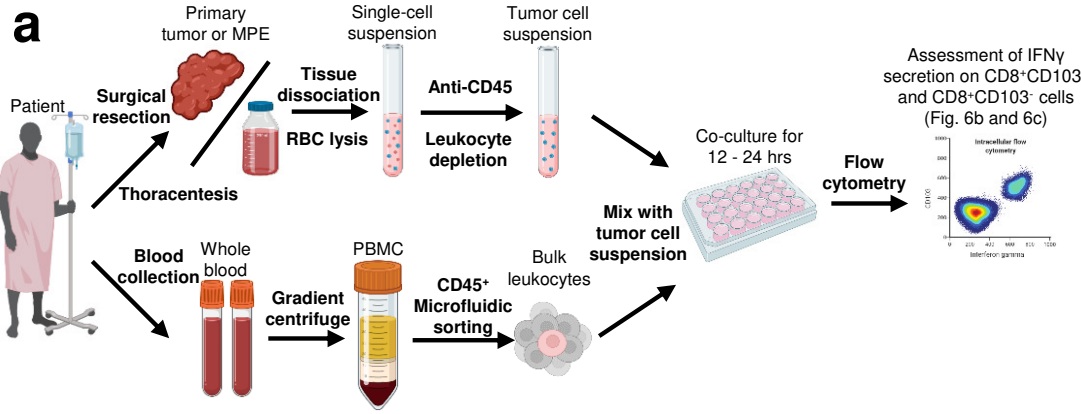
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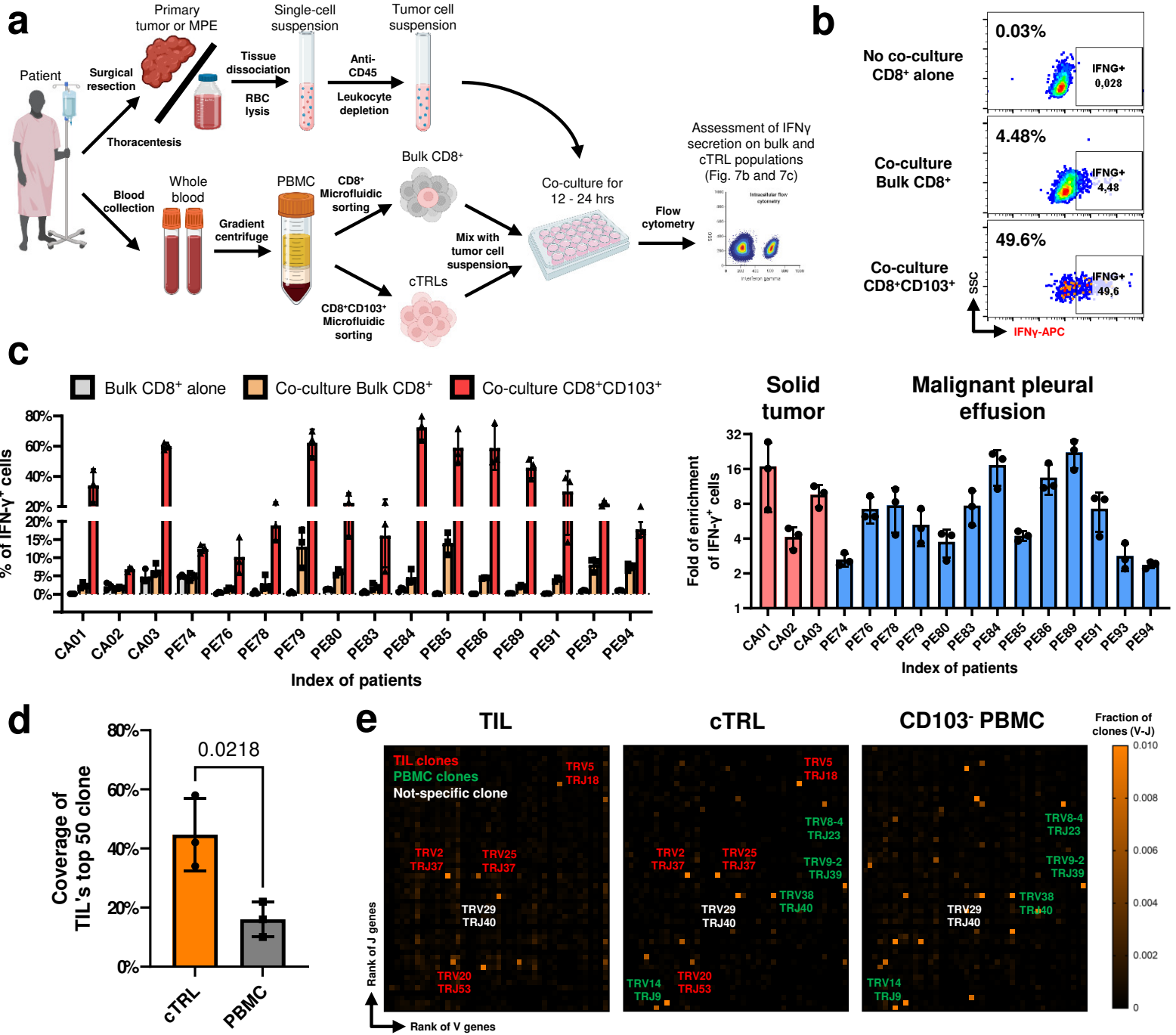


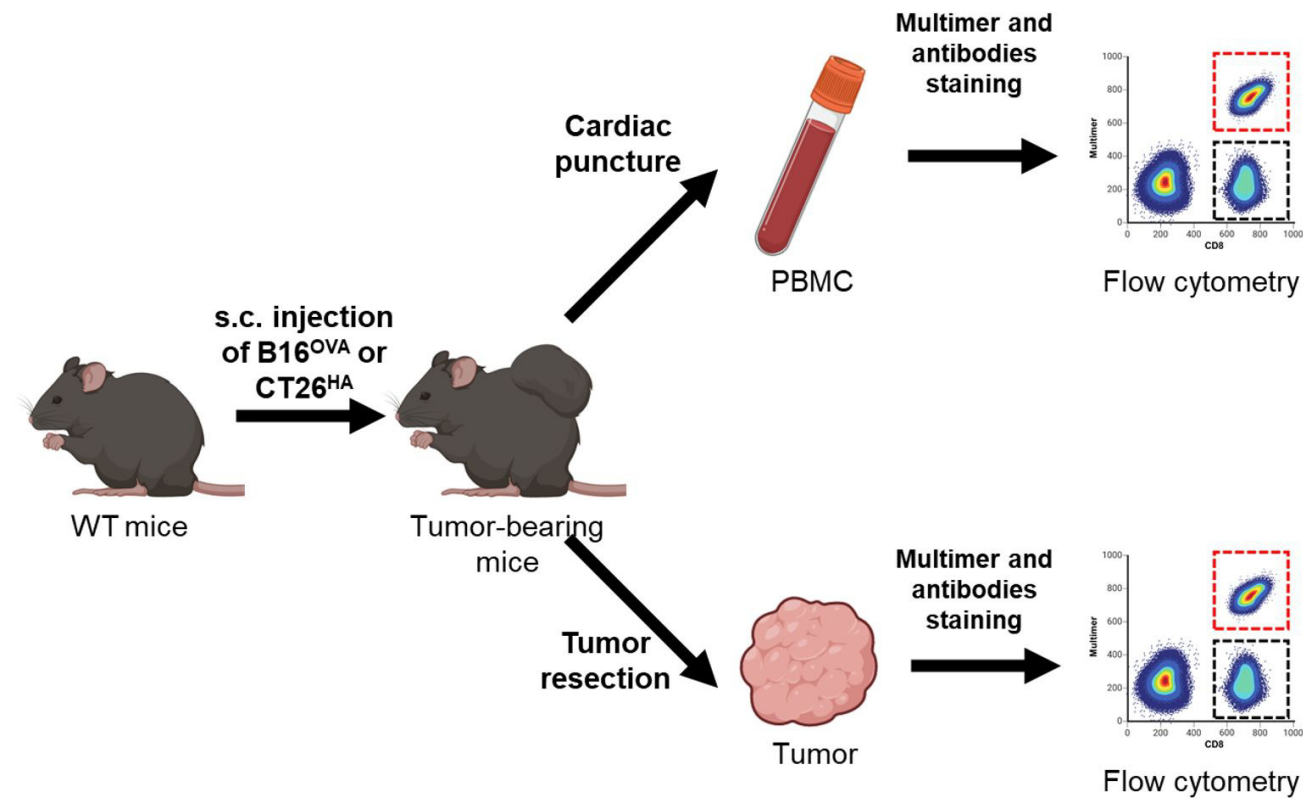
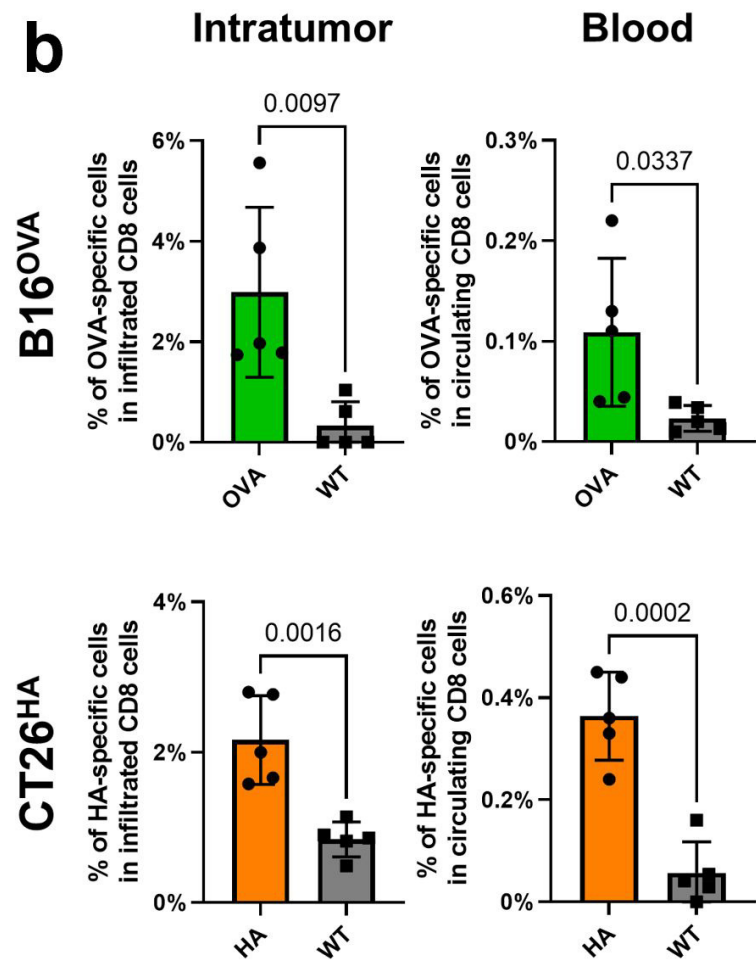




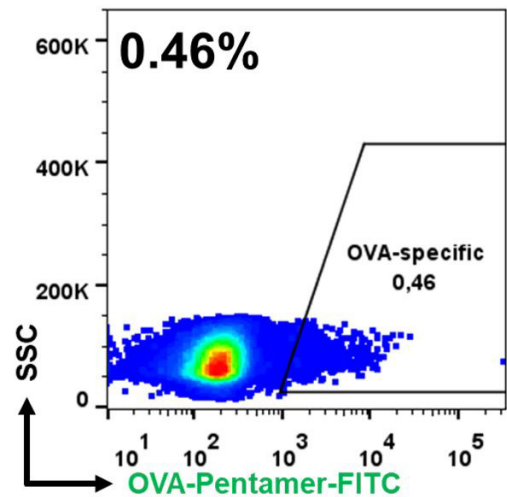




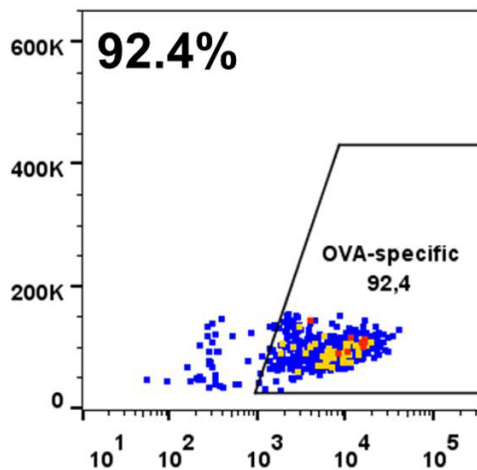


a**b**

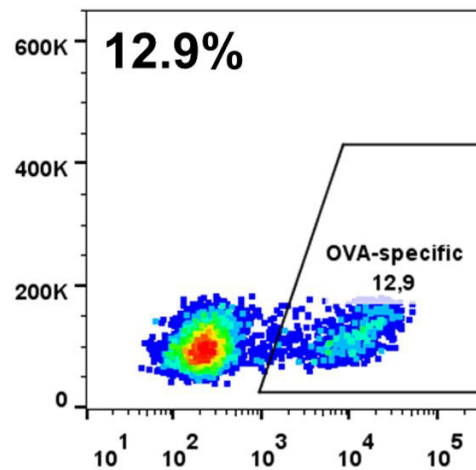
Pre-sorting



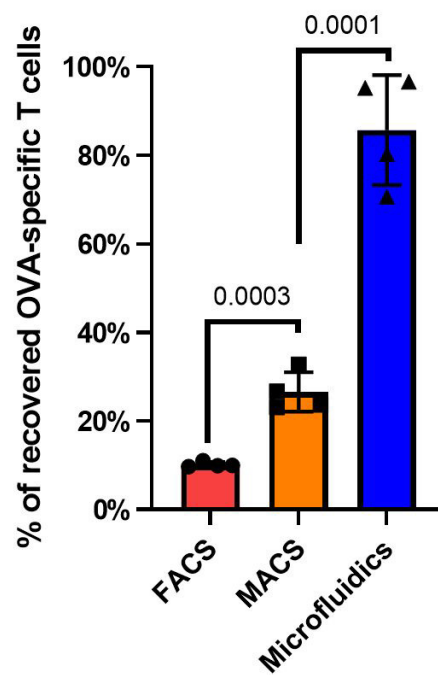
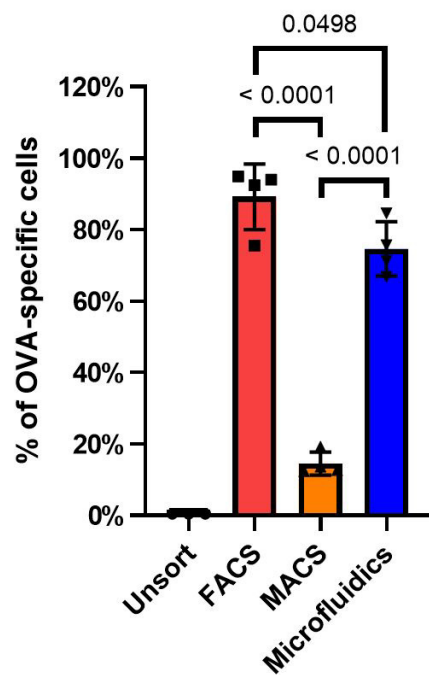
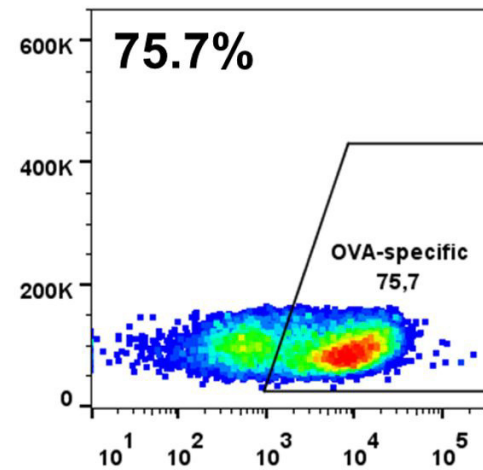
FACS



MACS



Microfluidics

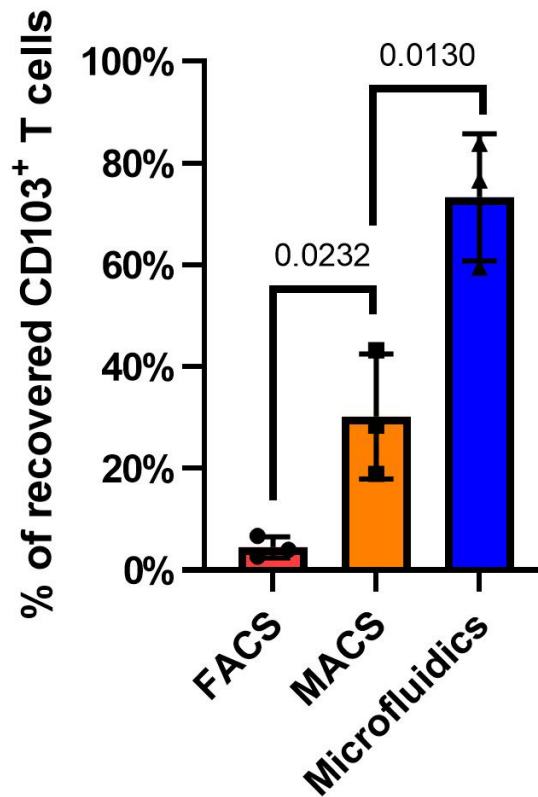
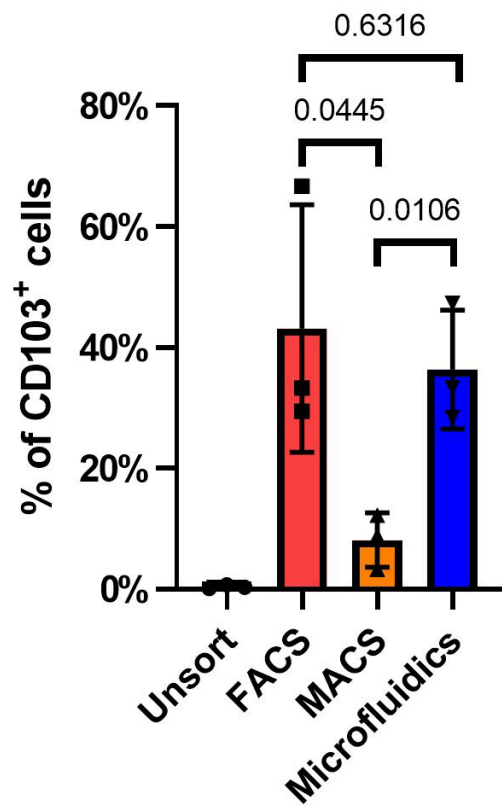
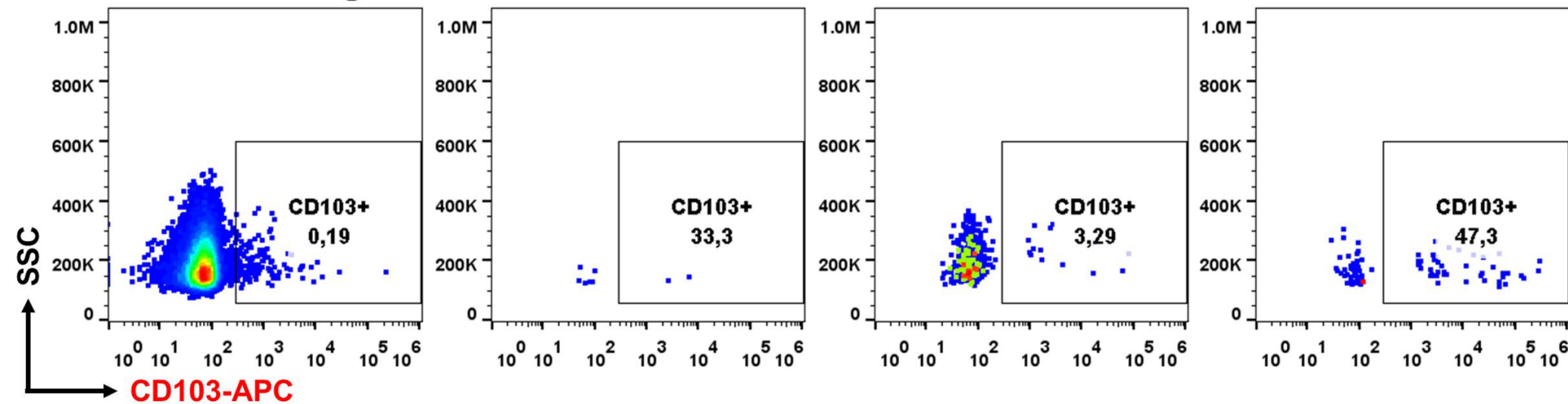


Pre-sorting

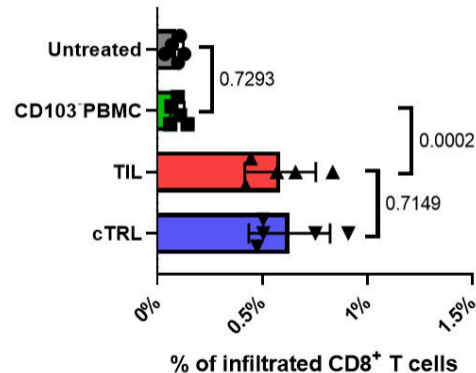
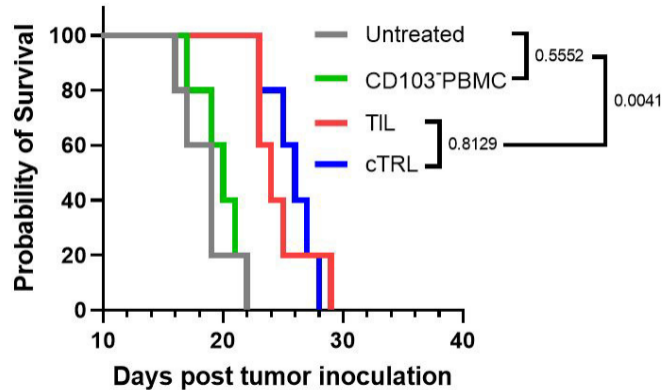
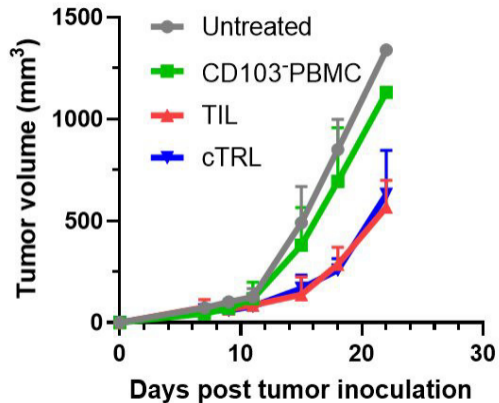
FACS

MACS

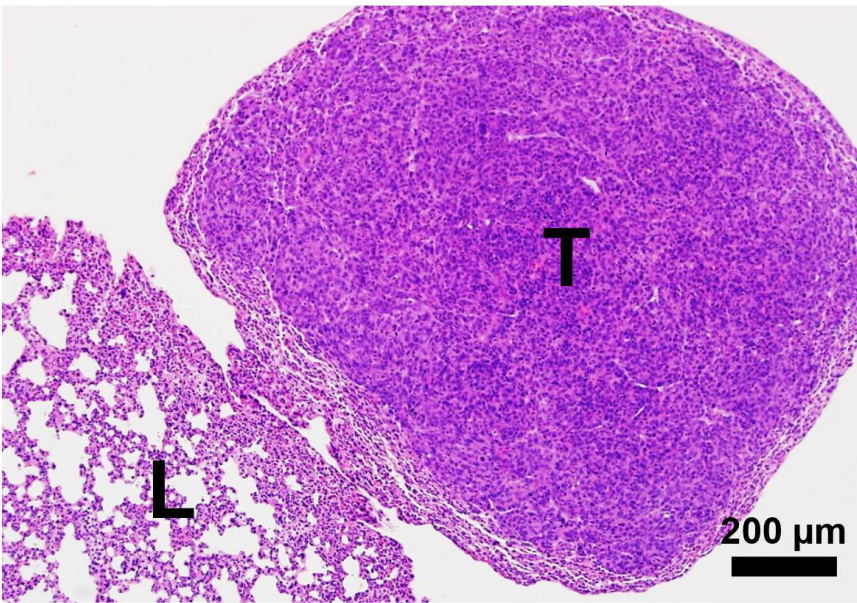
Microfluidics



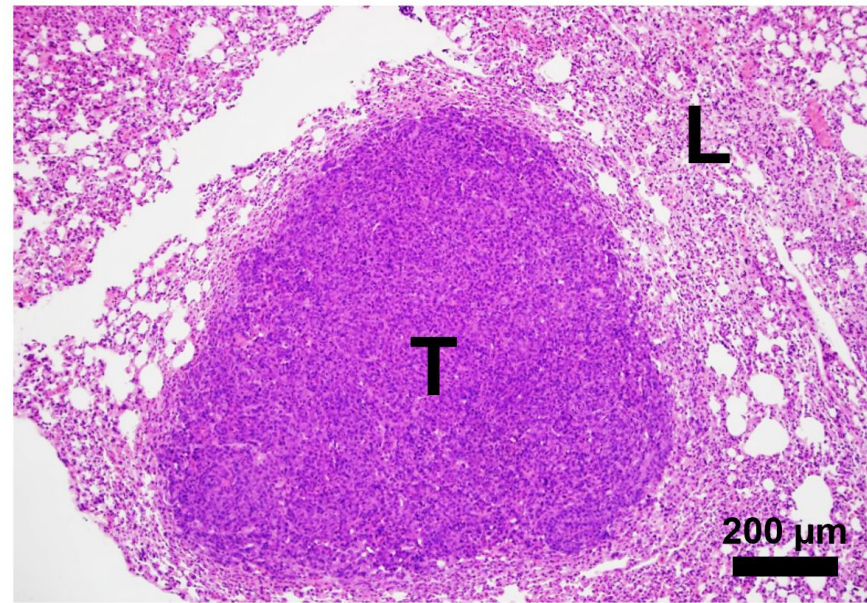
LLC-1 B6



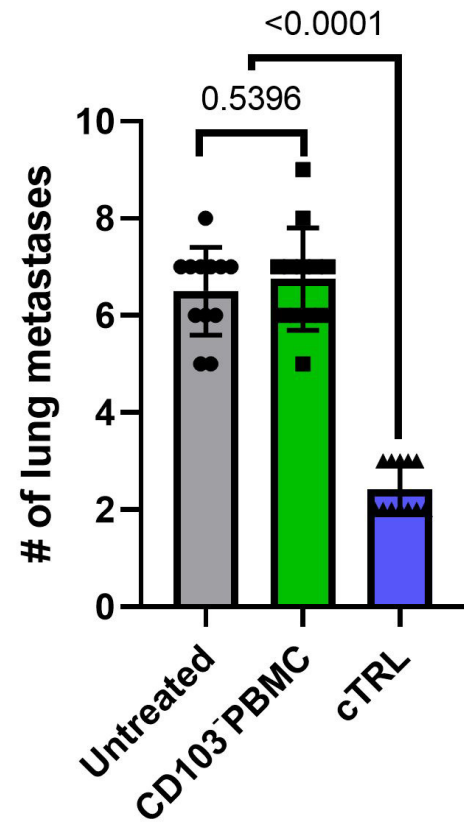
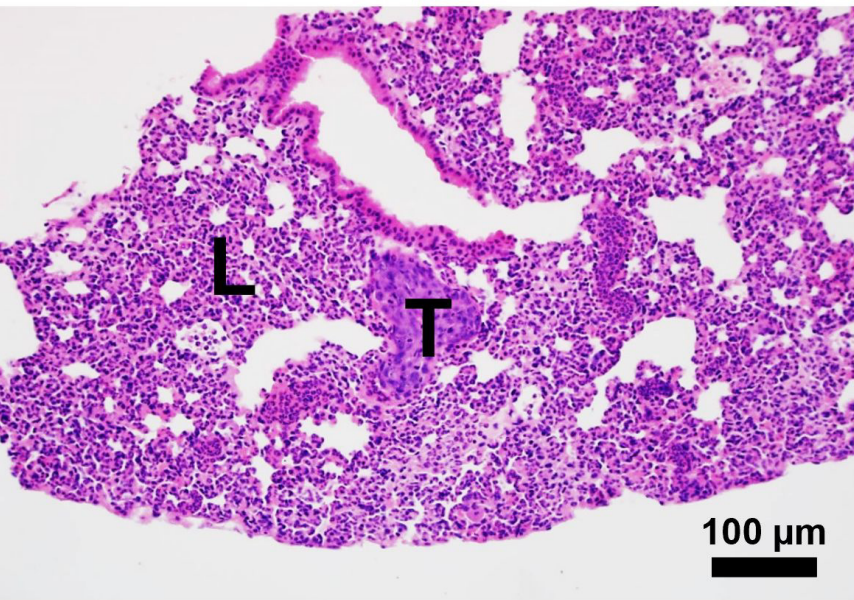
Untreated



CD103⁻ PBMC



cTRL



MC38 B6^{Rag1-/-}

