

1 **Emerging single cell endothelial heterogeneity supports**
2 **sprouting tumour angiogenesis and growth**

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37 **SUMMARY**

38 Blood vessels supplying tumors are often dysfunctional and generally heterogeneous. The
39 mechanisms underlying this heterogeneity remain poorly understood. Here, using
40 multicolor lineage tracing, *in vivo* time-lapse imaging and single cell RNA sequencing in
41 a mouse glioma model, we identify tumour-specific blood endothelial cells that originate
42 from cells expressing the receptor for colony stimulating factor 1, *Csf1r*, a cytokine which
43 controls macrophage biology. These *Csf1r* lineage endothelial cells (CLECs) form up to
44 10% of the tumour vasculature and express, besides classical blood endothelial cell
45 markers, a gene signature that is distinct from brain endothelium but shares similarities
46 with lymphatic endothelial cell populations. *in silico* analysis of pan-cancer single cell
47 RNAseq datasets highlights the presence of a comparable subpopulation in the
48 endothelium of a wide spectrum of human tumours. We show that CLECs actively
49 contribute to sprouting and remodeling of tumour blood vessels and that selective
50 depletion of CLECs reduces vascular branching and tumour growth. Our findings indicate

51 that a non-tumour resident Csf1r-positive population is recruited to tumours,
52 differentiates into blood endothelial cells to contribute to vascularization and, thereby,
53 tumour growth.

54

55

56 **INTRODUCTION**

57 Endothelial cells form a single cell layer lining the inner walls of blood vessels and play
58 critical roles in organ homeostasis and disease progression. Once formed, following
59 embryonic and early post-natal development, blood vessels retain a high level of
60 adaptability to meet changing metabolic or hemodynamic requirements or enable further
61 tissue growth including in tumours. How local vascular networks respond to these
62 adaptive challenges, rapidly expand, remodel and reestablish homeostasis remains a
63 timely research topic, with many open questions still unresolved. In particular in
64 pathologies, knowledge of the precise nature and origin of new vessel formation,
65 endothelial activation and differentiation steps, and of the mechanisms driving vessel
66 dysmorphia, or preventing effective revascularization in ischemic diabetic complication
67 is critical for new therapeutic approaches. New blood vessel formation in the adult was
68 long believed to arise exclusively by sprouting and proliferation of endothelial cells from
69 local blood vessels without any de novo differentiation from progenitor cells. However,
70 Asahara and coworkers isolated mononuclear cells from human peripheral blood and
71 identified circulating endothelial progenitor cells (EPCs) ¹. These cells were shown to
72 derive from the bone marrow and contribute to endothelial cells in blood vessels in
73 hindlimb ischemia and tumour xenograft mouse model ^{2,3}. By fluorescent in situ
74 hybridization with sex chromosome-specific probes in patients with cancer after bone
75 marrow transplantation, Peters and coworkers demonstrated that bone marrow-derived

76 stem cells contribute to endothelial cells in human tumour endothelium ⁴. The resulting
77 concept of vasculogenesis in the adult raised the prospect of novel therapeutic approaches
78 for ischemic vascular disease and for targeting tumour angiogenesis ^{5,6}. Interestingly,
79 EPCs also contributed to newly forming lymphatic vessels ^{7,8}. A vast number of
80 publications since have supported or challenged these initial discoveries, and the
81 occurrence, origin and significance of EPCs continues to be a matter of debate ⁹⁻¹².
82 Whereas EPCs and other circulating progenitor cells were long thought to origin from the
83 bone marrow, recent studies challenged this idea and suggested the existence of a
84 different yet unknown vascular niche for endothelial progenitor cells that can contribute
85 to blood vessel formation¹³.

86

87 **RESULTS**

88 *A **Csf1r** cell lineage contributes to blood vascular endothelial cells during glioma*
89 *growth in mice.*

90 In order to study dynamic macrophage recruitment and blood vessel patterning *in vivo*
91 during glioma growth, we adapted a surgical cranial window model for two-photon
92 microscopy allowing repeated visualization of the same animal over time. We generated
93 spheroids of syngeneic C57BL/6 mouse glioma cells (CT2A or GL261) ^{14,15} modified to
94 stably express blue fluorescent protein (BFP), and injected them into the mouse cortex,
95 followed by implantation of a glass coverslip ¹⁶. By crossing transgenic *Csf1r* (*Colony*
96 *stimulating factor 1 receptor*)– specific tamoxifen-inducible Cre driver mice ¹⁷ with the
97 *mTmG* Cre reporter mouse line ¹⁸, we induced membrane-targeted GFP expression in
98 *Csf1r*-expressing cells and used this stable lineage trace to follow the cell population over
99 time (Fig. 1a, b). As expected, the vast majority of tumour associated macrophages were
100 labelled by GFP in line with their *Csf1r*-dependent recruitment and expansion¹⁹.

101 Unexpectedly, however, through the combination of longitudinal live two-photon
102 imaging and lineage tracing, we discovered GFP-positive cells contributing to the tumour
103 vasculature during glioma growth (Fig. 1c). These *Csf1r* lineage endothelial cells
104 (CLECs) frequently emerged at tip cell positions of the growing vasculature, heading
105 blood vessel sprouts in the mouse glioma (Fig. 1d, Supplementary Movie1). Additionally,
106 CLECs were found bridging adjacent capillaries during anastomosis (Fig. 1e).
107 Fluorescent dextran injection confirmed that CLECs contributed to - and were
108 incorporated in the endothelial lining of - functionally perfused blood vessels including
109 lumenized sprouts headed by endothelial tip cells (Fig. 1f). Counterstaining on fixed
110 samples identified that all cells forming blood vessels including the lineage-traced CLECs
111 expressed typical endothelial markers (CDH5, CD31) and lacked macrophage and
112 myeloid cell markers (F4/80, CD45) (Fig. 1g, h, Extended Data Fig. 1). BFP was not
113 expressed in CLECs, demonstrating that they were not derived from glioma cells (Fig.
114 1g).

115 To gain more insight into the nature and significance of CLECs, we isolated and
116 quantified the cell population in the mouse glioma model. By 4 weeks of tumour
117 development (close to the specified humane endpoint of 5 weeks) up to 10% of the tumour
118 vasculature was comprised of cells carrying the *Csf1r* lineage trace. Similar frequencies
119 were found in tumours derived from either of the mouse glioma cell lines, CT2A (Fig. 2a,
120 b), or GL261 (Fig. 2c). Polyclonal antibody staining indicated that *Csf1r* may also be
121 expressed in some brain capillary endothelial cells^{20,21}. Recent single cell sequencing of
122 adult mouse brain endothelial cells however did not confirm this notion²², suggesting that
123 CLECs are unlikely to originate from brain endothelium. Indeed, tamoxifen mediated
124 activation of our transgenic *Csf1r* lineage trace did not label brain endothelial cells in
125 mice lacking tumours, sham operated as well as in the healthy brain parenchyma

126 surrounding implanted tumours (Fig. 2d, e, f). *Csf1r* is important for the embryonic
127 development of microglia and its expression is also detected in adult microglia by single
128 cell RNA sequencing. Surprisingly however, *Csf1r*-Mer-iCre-Mer mediated
129 recombination of the Cre-Reporter expression was also not observed in microglia of the
130 healthy adult mouse brain. Irrespective of this apparent discrepancy between reported
131 microglial *Csf1r* expression and the lack of *Csf1r*-Mer-iCre-Mer mediated recombination
132 in microglia, these data also exclude microglia as a potential source of CLECs.
133 Furthermore, we observed CLECs also in tumours originating from B16F1 melanoma
134 cells implanted under the skin; thus, this *Csf1r*-lineage endothelial cell contribution not
135 only occurs in brain tumours with microglia in their environment, but also in other tumour
136 types and locations (Fig. 2g, h). Notably, we also found *Csf1r*-lineage lymphatic
137 endothelial cells in B16F1 skin melanomas indicating that CLECs can contribute to both
138 blood and lymphatic vessels (Extended Data Fig. 2).

139 ***CLECs do not originate from hematopoietic niche in bone-marrow or spleen.***

140 Given the abundant labelling of macrophages and the important role of *Csf1r* in the
141 recruitment of bone-marrow derived monocytes and differentiated macrophages, we
142 asked whether CLECs are also derived from the bone-marrow. To test this possibility, we
143 generated bone marrow chimeras. Flow cytometric analysis confirmed 97.3 ± 1.6 %
144 reconstitution of wild-type recipient bone marrow by transplanted *Csf1r*-Mer2.Cre^{mTmG}
145 bone marrow after 8 weeks post transplantation (Extended Data Fig. 3). Analysis of
146 glioma blood vessels in these bone marrow chimeras (BM^{*Csf1r*-Mer2.Cre-mTmG}) failed to
147 identify bone marrow derived CLECs, although the numbers of tumour macrophage were
148 similar to controls (Fig. 3a). Conversely, chimeras of wild-type donor bone marrow
149 transplanted into *Csf1r*-Mer2.Cre^{mTmG} recipients (*Csf1r*-Mer2.Cre^{mTmG}::BM^{WT}),
150 displayed similar CLECs numbers in glioma vessels as observed earlier in non-chimeric

151 *Csf1r-Mer2.Cre^{mTmG}* mice (Fig. 3b). These results were confirmed by intravital imaging
152 in the glioma of *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* (Fig. 3c, Supplemental Movie 2). Thus, the
153 bone marrow chimera and lineage tracing experiments strongly suggest that CLECs do
154 not originate from bone marrow-derived cells, nor do they represent previously suggested
155 transdifferentiation events from bone marrow-derived macrophages. Surgical removal of
156 the spleen also did not affect CLECs numbers, further ruling out spleen-derived
157 macrophages as source of CLECs (Extended Data Fig. 4).

158 ***CLECs regulate vascular patterning and support glioma growth.***

159 In late-stage gliomas, CLECs preferentially localized to the peripheral tumor area,
160 correlating with the zone of most active angiogenesis (Extended Data Fig. 5). Real-time
161 intravital imaging of mouse gliomas also revealed that CLECs incorporation into vessels
162 is very dynamic. This implied that CLECs might have unique and potentially transient
163 functions during tumour vessel formation. To gain a better understanding of CLECs
164 behaviour during tumour growth, we developed dual-recombination combinatorial
165 genetics as a tool for CLECs tracing^{23,24}. To achieve selective recombination in CLECs,
166 we used the *Cdh5* endothelial driver for expression of the Dre recombinase, and the same
167 transgenic mouse line as above expressing the tamoxifen inducible form of the Cre
168 recombinase under control of the *Csf1r* transgene. The combined action of these distinct
169 recombinases, Dre and Cre, allowed for deletion of two stop cassettes flanked by RoxP
170 and LoxP sites, respectively, located upstream of a tdTomato expression cassette (Fig.
171 4a). Using this strategy, we could selectively trace tdTomato-positive CLECs in mouse
172 gliomas following tamoxifen exposure (Fig. 4b). Note that the appearance of labelled
173 CLECs and blood vessel labelling differs from the images of the mTmG line, as the dual
174 Dre/Cre tdTomato reporter used lacks a general label of unrecombined cells. Fluorescent
175 dextran injection instead was used to label vascular lumen, highlighting dtTomato

176 positive CLECs that line the vessel lumen. As expected, counterstaining on fixed samples
177 demonstrated that CLECs express the prototypical endothelial marker, ERG (ETS-related
178 gene) (Fig. 4c). In order to assess whether CLECs contribute functionally to tumour
179 growth and vascular patterning in glioma, we used the same dual recombination strategy
180 to selectively ablate CLECs (Fig. 4d). In this model, CLECs expressed both GFP as well
181 as the diphtheria toxin receptor after tamoxifen induction enabling depletion of GFP-
182 positive CLECs by administration of diphtheria toxin (Fig. 4e, h). Strikingly, CLECs
183 ablation significantly decreased glioma growth (Fig. 4g, i). CLECs ablation also
184 decreased the vascular network length and the number of bifurcations in tumour vessels
185 (Fig. 4j, k, l), indicating that CLECs may promote tumour growth by supporting vessel
186 branching.

187 ***CLECs express a unique set of markers***

188 To further understand the nature of tumour CLECs (T_CLECs), we performed single-cell
189 RNA sequencing (scRNA-seq) on isolated T_CLECs in the mouse glioma model. Single-
190 cell transcriptome analysis using Smart-seq2²⁵ identified that the gene expression pattern
191 of T_CLECs was similar to lineage-negative tumour endothelial cells (T_EC), but not
192 to tumour macrophages (T_MAC), as determined by UMAP (Uniform Manifold
193 Approximation and Projection) analysis (Fig. 5a). Single-cell differential expression
194 (SCDE) analysis²⁶ confirmed that T_CLECs expressed typical endothelial markers, but
195 lacked macrophage and myeloid cell markers (Fig. 5b). Unexpectedly, however,
196 T_CLECs showed no *Csf1r* expression, despite carrying the *Csf1r*-lineage marker (Fig.
197 5c). Beyond the overlapping gene expression pattern between T_CLECs and T_EC,
198 SCDE analysis identified a unique signature that can distinctly define the T_CLECs
199 population; RNAs coding for 24 genes including three types of cell surface proteins
200 (*Aqp1*: Aquaporin 1, *Fabp4*: Fatty acid binding protein 4, *Kcnj8*: Potassium voltage-

201 *gated channel subfamily j member 8*) were significantly enriched in T_CLECs (Fig. 5d;
202 Extended Data Fig. 6; Supplementary Table 1). *Aqp1* is highly expressed in vascular
203 endothelial cells and was reported to contribute to tumour growth and angiogenesis ²⁷.
204 Staining of lineage traced T_CLECs on fixed mouse glioma samples confirmed AQP1
205 expression (Extended Data Fig. 7).

206 ***The T_CLEC state is enriched for “Tip-like” and a lymphatic gene signature***

207 Gene set enrichment analysis (GSEA) identified that the T_CLEC state shows significant
208 enrichment for a Tip-cell-like associated gene expression program (Fig. 5e), thus
209 supporting the morphological observations and their functional importance for branching
210 and tumour growth. Intriguingly, GSEA identified that T_CLECs were also enriched for
211 lymphatic gene signatures (Fig. 5e). AUCell analysis ²⁸ of scRNA-seq data from multiple
212 human primary tumours identified subpopulations of endothelial cells that express the
213 T_CLECs marker gene set (Fig. 5f-k, 442 out of 7314 ECs are candidates: 6.0%;
214 Supplementary Table 2). These *bona-fide* T_CLECs were highly enriched in tip cell,
215 neophalanx cell, lymphatic endothelial cell subpopulations and particularly abundant in
216 human pancreatic cancer (Fig. 5k). Neophalanx endothelial cells, which express two pro-
217 angiogenesis markers, *Glul* (*Glutamine synthetase*) and *Cxcl12* (*C-X-C motif chemokine*
218 *ligand 12*) ^{29,30} have recently been identified in lung tumour-associated endothelial cell
219 studies ^{31,32}, and were proposed to line neo-vessels after termination of vessel sprouting.
220 Together this data suggests that based on the transcriptional state, T_CLECs might have
221 a supportive role in tumor vessel sprouting and remodeling. Detailed comparison with
222 recent single cell RNA seq data on tumour endothelial cells in mouse and human tumours
223 ³¹ confirmed the Tip-like gene signature of T_CLECs, but also identified significant
224 similarities with lymphatics (Fig. 5k). The latter is particularly surprising given that our

225 perfusion experiments with dextran clearly demonstrated that T_CLECs in the glioma
226 model line blood vessels, not lymphatics.

227 *Acute recombination identifies a CLEC lineage in lymph nodes*

228 The transcriptional similarities between T_CLECs and lymphatic endothelial cells are
229 noteworthy as blood endothelial cells, in particular in the brain environment, and
230 lymphatic EC represent highly differentiated cell types. During vertebrate embryogenesis,
231 lymphovenous specification sees common progenitor cells diverge in their differentiation
232 to establish mature blood endothelium and lymphatic endothelium³³. The apparent
233 similarities could therefore suggest that T_CLECs represent an immature endothelial cell
234 state with partially overlapping profiles of both immature blood and lymphatic cells. In
235 this case, the expression of lymphatic genes would signify a return to an earlier cell state
236 or potentially some level of transdifferentiation between the two endothelial cell states.

237 The latter would imply a distant source of lymphatic endothelium as possible origin of
238 CLECs. Indeed, when searching for CLECs in all organs, we observed selective
239 endothelial labelling in lymph nodes, notably restricted to the periphery where specialized
240 lymphatic endothelial cells form the floor of the subcapsular sinus (SCS, Fig. 6a). Bone-
241 marrow transplantation to eliminate the abundant Csf1r-lineage macrophage population
242 highlighted the specific labelling of CLECs in lymph nodes (Fig. 6b).

243 To gain more insight into the emergence of T_CLECs, we asked whether acute
244 recombination could indicate their possible origin. Acute induction experiments (24h
245 after single tamoxifen injection) illustrated that the Csf1r-expressing progenitors of this
246 tumour endothelial cell population do not reside locally in pre-existing brain or the
247 associated brain tumour vasculature (Extended Fig. 8). Close inspection however
248 revealed GFP-positive endothelial cells with significant labelling 24h post-injection in
249 the floor endothelial cells of the SCS of lymph nodes (Fig. 6c). The mTmG Cre-reporter

250 switches from red fluorescent protein expression to mEGFP expression upon
251 recombination. Therefore, cells that acutely recombine, carry both GFP and Tomato
252 expression until the latter is fully degraded. Strikingly, floor SCS endothelial cells
253 exhibited both GFP and simultaneous Tomato expression, demonstrating recent
254 recombination. FACS analysis confirmed the presence of double positive EC in lymph
255 nodes, but not in brain or brain tumours. In fact, double positive cells for GFP and Tomato
256 were not observed in the brain tumours at any stage, excluding the possibility of any acute
257 or continuous local recombination of T_CLECs within the tumour microenvironment.
258 Prox-1 staining confirmed the lymphatic nature of CLECs in the lymphnode, and
259 demonstrated that only a subpopulation of lymphatic endothelial cells in the lymph node
260 carry the *Csfr1*-lineage trace, the cells in the floor of the SCS (Fig. 6d).

261 We decided to perform scRNA seq also on endothelial cells isolated from lymph nodes,
262 assess gene expression and perform pseudotime analysis to understand differentiation of
263 T_CLECs, T_EC, LN_CLECs (GFP positive) and LN_EC (GFP negative) (Fig. 7a-f).
264 We used the same Smart-seq2 platform for comparison to sequence another 384 cells of
265 lymph node origin. After quality control (Fig. 7a), 89 cells EC, not *Csfr1* lineage EC, and
266 104 cells expressing GFP (Fig. 7b) in the lymph nodes were selected for further analysis.
267 Comparative analysis with recent mouse lymph node scRNA data³⁴ confirmed that the
268 LN_CLEC co-register with identified SCS floor cells (Extended Data Fig. 9a).

269 Unlike T_CLECs, LN_CLECs also expressed *Csf1r*, in line with our finding of acute
270 recombination in the SCS floor cells.

271 UMAP based dimension reduction placed T_CLECs and T_EC closely together, as before,
272 and also LN_CLEC and LN_EC co-cluster, although two additional distinct LN_EC
273 clusters appear highly distinct and thus unrelated to any of the other cell types (Fig. 7c).
274 Interestingly, some T_CLEC cluster with the LN_CLEC, suggesting that there is at least

275 some overlap in gene expression between the Csf1r lineage cells in the brain tumour and
276 in the lymph nodes. Looking at relative expression levels of all genes in a heatmap
277 comparing the lineage positive and negative EC from lymph nodes and tumours
278 highlighted that differential expression is more prevalent than any overlap between
279 tumour EC and LN EC (Fig. 7d). However, there are small number of genes like Pde4d,
280 Robo1 and Itga9 that show higher expression in the two lineage positive populations in
281 LN and tumour, compared to the lineage negative cells (Extended Data Fig. 9b). Itga9 is
282 well known to be important in lymphatic development³⁵, confirming again that T_CLECs
283 carry some gene expression patterns normally associated with lymphatic EC.
284 Nevertheless, despite the fact that only LN_CLEC appear to acutely recombine,
285 differential gene expression analysis does not provide clear evidence for a lineage
286 relationship between LN_CLEC and T_CLEC.

287

288 ***T_EC and T_CLECs “co-evolve” during tumour growth, suggesting distinct origin***

289 To gain more insight into the differentiation state and possible route of differentiation,
290 we next performed pseudotemporal ordering using Monocle³⁶ (Fig. 7f). The Monocle
291 inferred trajectory suggested equally distributed pseudotime values for T_CLECs and
292 T_EC then LN_EC and finally LN_CLECs. When measuring single-cell gene expression
293 diversity as surrogate for differentiation potential with the CytoTRACE algorithm³⁷
294 (higher score means more undifferentiated, stem-like), we observed again equal scores
295 between T_CLECs and T_EC but lower cytoTRACE values for LN_EC and LN_CLECs
296 (Fig. 7e-f). The equal scores between T_CLECs and T_EC argue against a role of T_EC
297 as precursors for T_CLECs, independently confirming that T_CLECs are unlikely
298 emerging through differentiation from the pre-existing brain tumour vasculature.
299 However, the LN_CLECs display the lowest cytoTRACE scores (most differentiated).

300 Based on the cytoTRACE analysis, and its logic of developmental potential, it would
301 seem unlikely that LN_CLEC are the origin of T_CLEC (Fig. 7e-f). Moreover, it appears
302 that both lineage positive and negative EC in the lymph nodes are more differentiated
303 than endothelial cells in the tumour. It is noteworthy that Monocle was designed for
304 developmental questions, which are strictly unidirectional. Interestingly, when plotting
305 CytoTRACE scores onto the Monocle trajectory (Fig. 7f), the right side shows higher
306 scores (T_CLEC and T_EC) and the left side (darker blue) lower scores (LN_CLECs
307 lowest), being in line with pseudotime. This again predicts that LN_CLECs represent an
308 end state rather than the origin. In summary, these data predict that during tumour growth,
309 the T_EC and T_CLECs coevolve from a distinct origin to then share many aspects of
310 their gene expression and biology, yet with a few very distinct gene profiles.

311

312 **DISCUSSION**

313 In this study, using inducible *Csf1r* lineage tracing in adult mice intended to study
314 macrophage recruitment during glioma development, we identify a distinct endothelial
315 cell lineage that forms up to 10% of the tumour vasculature in adult mice. Selective
316 experimental depletion of this *Csf1r* endothelial cell lineage demonstrated a role in
317 promoting vascular branching and tumour growth. Through a combination of genetic
318 lineage tracing, longitudinal live two-photon imaging, and single cell analysis, we find
319 that these cells arise during tumour progression, adopt dynamic endothelial phenotypes
320 including tip cell characteristics, and express common endothelial as well as unique
321 markers that can also be found in patient-derived tumour endothelium.

322 *Csf1r* is a well-known regulator of monocyte/ macrophage differentiation ^{38,39}, but is
323 also important for yolk sac hematopoiesis and progenitor cell differentiation. Klotz and
324 coworkers reported that *Csf1r*-expressing cells in the mouse embryo contribute to *Prox1*-

325 positive lymphatic endothelial cells in the cardiac lymphatic vasculature⁴⁰. Moreover, a
326 recent report identified that *Csf1r*-expressing erythro-myeloid progenitors from the early
327 embryonic yolk-sac could give rise to intraembryonic endothelial cells in mouse
328 development⁴¹. *Csf1r* is also expressed in EPCs, such as colony forming unit-Hill cells
329 and circulating angiogenic cells⁴². In the adult however, *Csf1r* is not expressed in the
330 mature endothelium of blood and lymphatic vessels, but continues to drive recruitment
331 and expansion of myeloid cells in inflammation, ischemia and tumour growth⁴³.
332 Intriguingly, *Csf1r* itself is no longer expressed in the *Csf1r* lineage cells within the
333 tumour and acute recombination of endothelial cells 24h after tamoxifen injection fails to
334 label cells in the tumour vasculature. These results raise the possibility that a *Csf1r*-
335 *expressing cell population* of endothelial nature or potential is present elsewhere in the
336 body, recombines upon tamoxifen injection and is recruited to the tumour vasculature
337 where it differentiates into blood endothelium to contribute to tumour angiogenesis.
338 Alternatively, the fact that *Csf1r* expression is not detected in isolated tumour CLECs
339 could also indicate that the Cre-line is leaky and gives rise to labelled cells through
340 stochastic recombination. In that case, one would expect the labelled cells to populate any
341 part of the vasculature and be otherwise indistinguishable from the unlabelled tumour EC.
342 Single cell analysis however identifies a unique gene expression signature beyond the
343 large number of genes that are co-expressed in T_CLEC and T_EC, and demonstrates
344 that T_CLEC are distinctly enriched in tip-cell and neo-phalanx cell populations. Thus,
345 labelled cells show distinct phenotypes, location and expression patterns, ruling out
346 purely stochastic recombination events. Furthermore, such stochastic recombination
347 within the tumour vasculature would necessitate that we identify acutely labelled cells
348 marked by co-expression of Tomato and GFP. As we failed to identify such cells at any
349 timepoint during tumour growth, and also could detect the emergence of T_CLECs in

350 mice only injected with tamoxifen before tumour implantation, the cumulative evidence
351 would suggest that T_CLECs originate outside of the tumour and also that the progenitor
352 population resides outside of the brain. Despite all our efforts, the present work fails to
353 provide ultimate and definitive proof of the true origin of T_CLECs, but arrives at a clear
354 direction and working hypothesis for future studies. In search of their origin and a
355 potential progenitor population, we imaged and analysed all organs by FACS. We
356 performed bone-marrow transplantation, performed splenectomy and examined the entire
357 mouse by fluorescence stereomicroscopy after clearing to identify regions of
358 recombination. The results of bone-marrow transplantation effectively ruled out EPC or
359 any other known progenitors that may be found in circulation, including
360 transdifferentiation from macrophages. Four independent observations placed a spotlight
361 on lymphatics, and the lymph nodes in particular. First, the only *bonafide* Csf1r lineage
362 endothelial cell population that we could identify outside of the tumours, including after
363 complete reconstitution of the bone marrow by wildtype bone marrow, was found in the
364 subcapsular sinus of the lymph nodes. Here the floor cells, specialized lymphatic
365 endothelial cells that we identify as GFP and Csf1r positive line the capsule in lymph
366 nodes in close apposition to the Csf1 expressing ceiling cells. Second, after acute
367 tamoxifen injection the cells in the floor of the subcapsular sinus are uniquely double
368 positive for Tomato and GFP, demonstrating acute and likely local recombination. Third,
369 single cell RNAseq of T-CLECs identified lymphatic markers including the lymphatic
370 master regulator Prox1 co-expressed with blood endothelial markers, and SCDE placed
371 T_CLECs close to lymphatic endothelial cells from various mouse tumour models in
372 recently published datasets. And finally, CLECs can also be found in lymphatics in
373 subcutaneous tumour models and in skin. These independent observations clearly make

374 the SCS floor cells prime candidates for the origin of T_CLECs, but future work will be
375 necessary for a possible direct demonstration of this link.

376 In developmental studies, lineage relationships have recently been studied by scRNAseq
377 data sets through pseudotime analysis. In particular the CytoTRACE analysis can be
378 useful to estimate the degree of differentiation of cell populations and thereby also predict
379 their stemness³⁷. Our comparative analysis of endothelial cells isolated from tumour and
380 lymph nodes assigned the highest differentiation scores to cells in the lymph node, and
381 lower values to the EC in tumour, including T_CLECs. Accordingly, the cells in the floor
382 of the SCS that carry the lineage label would rather be assumed at the end of a
383 differentiation process rather than progenitors for other populations such as the T_CLECs.
384 Whether however such algorithms are suitable to determine the potential of endothelial
385 cells to become activated and possibly transdifferentiate is unclear. In particular given the
386 altered environmental context of EC within a growing tumour compared to a vascular bed
387 in homeostasis. The combined monocle³⁶ and cytoTRACE analysis would fit with a
388 general level of dedifferentiation of endothelial cells within the tumour microenvironment,
389 and therefore a co-evolution of the cell states of both T_EC and T_CLEC. It is likely that
390 pseudotemporal ordering of endothelial cells in a reactivation state will always show the
391 reactivated cells, like in the tumour environment as less differentiated. Indeed, recent
392 work studying the effect of chronic hypoxia on brain endothelium illustrated the wide
393 range of reactivation, in particular noting a strong increase in tip cell population amongst
394 the brain endothelial cells⁴⁴. The current assumption states that these new tip cells arise
395 under hypoxia from pre-existing brain endothelial cells, and possibly emerge from
396 particular endothelial subpopulations. In the absence of lineage tracing, an alternative
397 possibility, raised by our current observation in tumour angiogenesis, could be that new
398 tip cells arise at least in part elsewhere, and are recruited to the hypoxic vessel areas.

399 More work on vascular single cell analysis will be required to establish the fundamental
400 principles of endothelial activation and their interpretation for the different tissue
401 challenges that require vascular adaptations.

402 Angiogenesis is essential for tumour growth, and contributes to metastasis ^{45,46}. Early
403 hopes that targeting tumour angiogenesis may prove uniquely selective and effective as
404 anti-tumour treatment have, however, been disappointing. Intense research efforts
405 therefore currently focus on understanding reasons and mechanisms for resistance to anti-
406 angiogenic therapy, and identifying new cellular and molecular targets to effectively
407 modify the tumour vasculature ⁴⁷. Intravital imaging shows that T_CLECs arise during
408 tumour progression, adopt endothelial phenotypes with tip cell characteristics and line
409 perfused vascular tubes. Consistent with this observation, AUCell analysis of scRNA-seq
410 data in human samples identifies that T_CLECs signatures are highly enriched in tip cell
411 subpopulation. A recent study using single-cell transcriptome analysis revealed that anti-
412 VEGF (vascular endothelial growth factor) treatment reduces the proportion of
413 endothelial tip-like cells in a tumour xenografts ⁴⁸. How CLECs affect the outcome of
414 anti-angiogenic treatment will be important to investigate in the future. Considering that
415 T_CLECs prominently express *Vegfr3*, even more so than regular endothelial cells in the
416 tumour, we can speculate that T_CLECs are resistant to anti-VEGFR2 treatment, and
417 possibly escape treatment through the activity of VEGFR3. If so CLECs may adversely
418 affect anti-angiogenic therapy targeting VEGFR2 selectively. Tumour blood vessels are
419 generally highly tortuous and the branching pattern is greatly different from normal blood
420 vessels. Our results from the selective targeting of CLECs in the mouse glioma model
421 show that CLECs ablation decreases both vessel branching and tumour growth. Thus, in
422 the absence of CLEC, the dysmorphic vascular phenotype in tumours appears exacerbated,
423 suggesting that CLECs support the formation of a highly branched vascular network,

424 which in turn better promotes tumour growth. Future work will be required to identify the
425 true origin of T_CLECs and to extend this functional analysis to fully appreciate the
426 consequence and opportunities to target or utilize CLECs for therapeutic approaches.

427

428

429 **METHODS**

430 ***Mice***

431 Mice expressing both the *Csf1r-Mer-iCre-Mer* transgene (*Csf1r-Mer2.Cre*)¹⁷ and the
432 *Rosa^{mTmG}* Cre recombination reporter¹⁸ were generated by breeding. Mice carrying the
433 Endothelial specific *Cdh5-Dre* mice²⁴ were mated with *Csf1r-Mer2.Cre* mice and
434 *Rosa26-RSR-LSL-tdTomato* mice²³ to obtain *Cdh5-Dre::Csf1r-Mer2.Cre::Rosa26-RSR-*
435 *LSL-tdTomato* mice (*DreMer2.Cre^{tdTomato}*) that allows combinatorial genetics as a tool for
436 CLECs tracing. Endothelial specific *Cdh5-Dre* mice were mated with *Csf1r-Mer2.Cre*
437 mice and *Rosa26-RSR-mTmG-DTR* mice to obtain *Cdh5-Dre::Csf1r-Mer2.Cre::Rosa26-*
438 *RSR-mTmG-DTR* mice (*DreMer2.Cre^{mTmG-DTR}*) that allows for selective tracing and
439 ablation of CLECs. *Rosa26-RSR-mTmG-DTR* mice were designed by Dr. Fabio Stanchi
440 and generated by the team of Dr. Ralf Kühn at the Max Delbrück Center in Germany. All
441 mouse strains were maintained on C57BL/6J background. Animal housing and all
442 experimental procedures were approved by the Institutional Animal Care and Research
443 Advisory Committee of the KU Leuven (085/2016).

444 ***Cell lines***

445 CT2A, GL261 glioma cell lines and B16F1 melanoma cell line were cultured in DMEM
446 (Life Technologies) supplemented with 10% FBS (Life Technologies), 1% penicillin/
447 streptomycin (Life Technologies) and 1% glutamine (Life Technologies). Spheroids were
448 obtained by seeding the glioma cells for 48h on non-adherent culture dishes. Spheroids

449 of 200- 250µm were selected for implantation.

450 ***Intravital imaging on mouse gliomas***

451 Surgery for tumor implantation, installation of cranial windows and *in vivo* imaging were
452 performed as described previously¹⁶. Briefly, 8– 12-weeks-old mice were anesthetized
453 with ketamine/ xylazine and a craniotomy was performed on the parietal bone. Spheroids
454 of CT2A or GL261 cells modified to express the blue fluorescent protein TagBFP⁵⁰ were
455 injected in the exposed parietal brain cortex, which was then sealed by cementing a glass
456 coverslip to the bone surrounding the craniotomy. After surgery, mice were injected
457 intraperitoneally with tamoxifen (65 µg/g body weight) every 5 days. For CLECs
458 depletion, mice were injected intraperitoneally with diphtheria toxin (4 ng/g body weight)
459 the next day after tamoxifen induction. Longitudinal *in vivo* imaging of growing tumors
460 was performed in mice under isoflurane anesthesia using a SP8 upright microscope (Leica
461 Microsystems) equipped with a HCX IRAPO L25x/0.95 water objective and a Titanium:
462 Sapphire laser (Vision II, Coherent Inc.) tuned at 925 nm. Humane end-point of
463 experiments was applied if animals lost 15-20% of their original weight or shown evident
464 signs of distress.

465 ***Immunofluorescence imaging***

466 Mice anesthetized with ketamine/ xylazine were perfused through the heart with 15 ml of
467 ice-cold PBS, followed by 10 ml of 2% PFA in PBS. Tumour tissues or organs were
468 harvested and fixed overnight in 4% PFA. For tumour tissues, sections (200 µm-thick)
469 were prepared with a vibratome 650V (Thermo Scientific). For organ samples, frozen
470 sections (10 µm-thick) were prepared with a cryostat NX70 (Thermo Scientific). For
471 immunofluorescence imaging studies, the PFA-fixed sections were blocked and
472 permeabilized in TNBT (0.1 M Tris, pH 7.4, 150 mM NaCl, 0.5% blocking reagent from
473 Perkin Elmer, 0.5% Triton X-100) for 4h at room temperature. Sections were incubated

474 with antibodies against CDH5 (1:25, AF1002, R&D), CD31 (1:100, ab28364, Abcam),
475 F4/80 (1:100, MF48000, Invitrogen), CD45 (1:100, 550539, BD Pharmingen), CD169
476 (1:100, 142401, Biolegend), PROX1 (1:200, 11-002, Angiobio), AQP1 (1:4000, AB2219,
477 Millipore), LYVE1 (1:100, 50-0433-80, eBioscience) diluted in TNBT buffer overnight
478 at 4°C, washed in TNT buffer (0.1 M Tris pH 7.4; 150 mM NaCl, 0.5% Triton X-100)
479 and incubated with an Alexa Fluor conjugated antibodies (1:200, ThermoFisher
480 Scientific). Sections were washed and mounted in fluorescent mounting medium (Dako).
481 Images were obtained with a Leica TCS SP8 confocal microscope.

482 ***Flow cytometric analysis***

483 Mice were anesthetized with ketamine/ xylazine, then tumour tissues or organs were
484 harvested and incubated with PBS containing 1mg/ml collagenase I (Gibco), 2 mg/ ml
485 Dispase I (Gibco), 100ug/ml DNase I (Roche) and 2 mM CaCl₂ for 30 min at 37 °C. After
486 incubation, the digested tissue was passed through a cell strainer and then washed by PBS
487 including 2% FBS. For red cell exclusion, we incubated samples with red cell lysis buffer
488 (Sigma) for 5 min at 37 °C and then washed by PBS including 2% FBS and 2 mM EDTA.
489 Cells were stained with the following monoclonal antibodies: PE/ Cy7 anti-CD45
490 (552848, BD), APC anti-CD31 (561814, BD). Data acquisition was performed with BD
491 FACSVerse and analysis was performed with BD FACSuite software, and FlowJo
492 software.

493 ***Mouse melanoma model***

494 Mice (8–12-weeks-old) were anesthetized with ketamine/ xylazine and then 1x10⁶ B16F1
495 melanoma cells in PBS were implanted into the right flank region of the mice. B16F1
496 melanomas were removed from mice 12 days after implantation for immunofluorescent
497 imaging studies and flow cytometric analysis. Mice were injected intraperitoneally with
498 tamoxifen (65 µg/g body weight) 4 time/ 2 weeks before tumour implantation and then

499 intraperitoneally with tamoxifen (65 µg/g body weight) every 5 days after tumour
500 implantation.

501 ***Single cell isolation and RNA-sequencing***

502 Single cells were sorted (BD FACSAriaIII) in 96 well plates (VWR) containing 2 µL of
503 PBS including 0.2% Triton X-100 and 4U of RNase inhibitor (Takara) per well. Plates
504 were properly sealed and spun down at 2000 g for 1 min before storing at –80°C. Whole
505 transcriptome amplification was performed with a modified SMART-seq2 protocol as
506 described previously ²⁵, using 20 instead of 18 cycles of cDNA amplification. PCR
507 purification was realized with a 0.8:1 ratio (ampureXP beads:DNA). Amplified cDNA
508 quality was monitored with a high sensitivity DNA chip (Agilent) using the Bioanalyzer
509 (Agilent). Sequencing libraries were performed using the Nextera XT kit (Illumina) as
510 described previously ²⁵, using 1/4th of the recommended reagent volumes and 1/5th of
511 input DNA with a tagmentation time of 9 min. Library quality was monitored with a high
512 sensitivity DNA chip (Agilent) using the Bioanalyzer (Agilent). Indexing was performed
513 with the Nextera XT index Kit V2 (A-D). Up to 4 x 96 single cells were pooled per
514 sequencing lane. Samples were sequenced on the Illumina NextSeq 500 platform using
515 75bp single-end reads.

516 ***scRNA-sequencing data analysis***

517 BAM files were converted to merged, demultiplexed FASTQ files, cleaned using fastq-
518 mcf (ea-utils r819), and QC checked with FastQC (0.11.4). Reads were then mapped to
519 the mouse genome (mm18) using STAR (2.4.1b) and quantified with Subread (1.4.6-p2).
520 Cells with less than 100,000 reads and/or 500 genes expressed, more than 20%
521 mitochondrial reads, and less than an average expression level of 3.0 of about 80
522 housekeeping genes ⁵¹ were discarded. 469 cells passed these stringent quality criteria.
523 Winsorized Highly Variable genes (HVGs) were identified for tumor derived

524 macrophages (T_MACs), endothelial cell (T_EC) and *Csf1r* lineage endothelial cells
525 (T_CLECs)²⁶. These cells were then clustered based on HVG expression using non-
526 negative matrix factorization as dimension reduction approach (run=40, rank=10, in MeV
527 4.8.1). The “best fit” (numbers of clusters) was chosen based on the highest cophenetic
528 correlation coefficient. Next, Single-cell Differential Expression analysis (SCDE) was
529 performed between the different NMF-clusters using the global gene expression matrix
530²⁶. Expression values were library-size-factor-normalized (DESeq) and log2 transformed
531 (log2+1). T_MAC, T_EC, T_CLEC and LN_CLECS, LN_EC, T_CLEC, T_EC cells
532 were projected into a two-dimensional space using Uniform Manifold Approximation and
533 Projection (dims=15, resolution = 0.4) based on 5k most variable features (Seurat
534 pipeline)⁵². Monocle (2.14) based trajectory analysis was performed on T_CLEC, T_EC,
535 LN_CELC and LN_EC (total=454 cells) taking into account n=790 m3Drop genes. The
536 same data set was subjected to CytoTRACE analysis using the online tool
537 (<https://cytotrace.stanford.edu/>)³⁷.

538

539 *AUCell analysis of human scRNA-seq data in multiple tumours*

540 The droplet-based scRNA-seq data of 4 human tumour types were downloaded from
541 publicly available sources including lung cancer (ArrayExpress: E-MTAB-6149 and E-
542 MTAB-6653)⁵³, PDAC (GSA: CRA001160)⁵⁴, liver cancer (GEO: GSE125449)⁵⁵, and
543 BCC (GEO: GSE123814)⁵⁶. These datasets were processed and clustered per tumour type
544 using Seurat (v3.0.4) package, and the endothelial cell clusters were identified using
545 marker genes (*CLDN5*, *VWF*, *PECAMI*). The resulting endothelial cell data were pooled
546 together and aligned using anchor-based canonical correlation analysis (CCA) to mitigate
547 the difference related to distinct tumor types and scRNA-seq technologies. Initial graph-
548 based clustering identified 10 clusters including 3 potential doublet clusters as predicted

549 by DoubleFinder (v2), which were subsequently removed before second round of
550 clustering. The resulting 8 clusters consisting of 7314 endothelial cells were annotated
551 based on known marker genes, and their CLECs signature were calculated using AUCell
552 package (v1.6.1). The threshold of AUC score was chosen at the peak of the right modal,
553 and the percentage of putative CLECs were calculated for different subtypes and their
554 tumour type of origin.

555 *Generation of bone marrow chimeras*

556 Recipient 8-10-weeks-old mice were lethally irradiated (9.5 Gy) and then intravenously
557 injected with 1×10^7 bone marrow cells from donor mice 16 h later. Tumour implantation
558 experiments were initiated 8 weeks after bone marrow reconstitution. Reconstitution rate
559 was determined using flow cytometric analysis in the peripheral blood.

560 *Surgical removal of the spleen*

561 Mice (8–12-weeks-old) were anesthetized with ketamine/ xylazine and then the spleen
562 was gently taken out from the connective tissue, while cauterizing associated vessels. The
563 incision was thereafter sutured. These procedures were performed as described previously
564 ⁵⁷.

565 *Vessel morphology analysis*

566 Confocal images of the tumour vasculature were analyzed for two-dimensional vessel
567 area, network length and number of network bifurcation points. The tdTomato and GFP
568 channels were merged and a Gaussian blur filter as well as Otsu thresholding was applied
569 to obtain binary vessel mask for sectional area estimation. To obtain the vessel network,
570 a semi-automatic multicut workflow from ilastik ⁵⁸ was applied to all images. The
571 segmented vessels were skeletonized and converted into a network graph with nodes and
572 edges by using the Python package ImagePy ⁵⁹. The lengths of network edges were
573 summed up to obtain the total network length for each image, all nodes with a degree of

574 three or more were counted as bifurcations.

575 *Statistics*

576 Statistical analyses of data were performed with GraphPad Prism 7.0 software. All data
577 are shown as the means \pm standard deviations. Differences were assessed using a two-
578 tailed unpaired Mann-Whitney's U test or two-tailed unpaired Welch's correction.

579

580 **AUTHOR CONTRIBUTIONS**

581 KM led and conceived the project, designed and performed experiments, analysed and
582 interpreted data. FR, FS, TM, WG, LH, JQ performed experiments and analyzed data.
583 DL, BZ, CB, JM and HG analyzed data. HG conceived and supervised the project. KM
584 and HG wrote and edited the manuscript, with contribution by all authors.

585

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596 excellent technical assistance.

597

598 **CODE AVAILABILITY**

599 The computational code (R) used for scRNA-seq analysis is based on already published
600 pipelines (see Material and Methods) and available upon request.

601

602 **DATA AVAILABILITY**

603 All scRNA-seq data are available at Gene Expression Omnibus under accession number
604 GSE157507.

605

606 **STATISTICS**

607 Differences were assessed using either a two-tailed unpaired Mann-Whitney's or two-
608 tailed unpaired t-test with Welch correction. All planned comparisons were considered
609 stand-alone and were not corrected for multiple testing.

610

611 **REFERENCES**

- 612 1 Asahara, T. *et al.* Isolation of putative progenitor endothelial cells for
613 angiogenesis. *Science* **275**, 964-967, doi:10.1126/science.275.5302.964 (1997).
- 614 2 Shi, Q. *et al.* Evidence for circulating bone marrow-derived endothelial cells.
615 *Blood* **92**, 362-367 (1998).
- 616 3 Asahara, T. *et al.* Bone marrow origin of endothelial progenitor cells responsible
617 for postnatal vasculogenesis in physiological and pathological neovascularization.
618 *Circ Res* **85**, 221-228, doi:10.1161/01.res.85.3.221 (1999).
- 619 4 Peters, B. A. *et al.* Contribution of bone marrow-derived endothelial cells to
620 human tumor vasculature. *Nat Med* **11**, 261-262, doi:10.1038/nm1200 (2005).
- 621 5 Rafii, S., Lyden, D., Benezra, R., Hattori, K. & Heissig, B. Vascular and
622 haematopoietic stem cells: novel targets for anti-angiogenesis therapy? *Nat Rev*
623 *Cancer* **2**, 826-835, doi:10.1038/nrc925 (2002).

- 624 6 Rafii, S. & Lyden, D. Therapeutic stem and progenitor cell transplantation for
625 organ vascularization and regeneration. *Nat Med* **9**, 702-712,
626 doi:10.1038/nm0603-702 (2003).
- 627 7 Religa, P. *et al.* Presence of bone marrow-derived circulating progenitor
628 endothelial cells in the newly formed lymphatic vessels. *Blood* **106**, 4184-4190,
629 doi:10.1182/blood-2005-01-0226 (2005).
- 630 8 Kerjaschki, D. *et al.* Lymphatic endothelial progenitor cells contribute to de novo
631 lymphangiogenesis in human renal transplants. *Nat Med* **12**, 230-234,
632 doi:10.1038/nm1340 (2006).
- 633 9 Purhonen, S. *et al.* Bone marrow-derived circulating endothelial precursors do not
634 contribute to vascular endothelium and are not needed for tumor growth. *Proc*
635 *Natl Acad Sci U S A* **105**, 6620-6625, doi:10.1073/pnas.0710516105 (2008).
- 636 10 Kerbel, R. S. *et al.* Endothelial progenitor cells are cellular hubs essential for
637 neoangiogenesis of certain aggressive adenocarcinomas and metastatic transition
638 but not adenomas. *Proc Natl Acad Sci U S A* **105**, E54; author reply E55,
639 doi:10.1073/pnas.0804876105 (2008).
- 640 11 Basile, D. P. & Yoder, M. C. Circulating and tissue resident endothelial progenitor
641 cells. *J Cell Physiol* **229**, 10-16, doi:10.1002/jcp.24423 (2014).
- 642 12 Psaltis, P. J. & Simari, R. D. Vascular wall progenitor cells in health and disease.
643 *Circ Res* **116**, 1392-1412, doi:10.1161/CIRCRESAHA.116.305368 (2015).
- 644 13 Fujisawa, T. *et al.* Endothelial Progenitor Cells Do Not Originate From the Bone
645 Marrow. *Circulation* **140**, 1524-1526,
646 doi:10.1161/CIRCULATIONAHA.119.042351 (2019).
- 647 14 Martinez-Murillo, R. & Martinez, A. Standardization of an orthotopic mouse
648 brain tumor model following transplantation of CT-2A astrocytoma cells. *Histol*

- 649 *Histopathol* **22**, 1309-1326, doi:10.14670/HH-22.1309 (2007).
- 650 15 Zagzag, D. *et al.* Expression of hypoxia-inducible factor 1alpha in brain tumors:
651 association with angiogenesis, invasion, and progression. *Cancer* **88**, 2606-2618
652 (2000).
- 653 16 Stanchi, F., Matsumoto, K. & Gerhardt, H. Imaging Glioma Progression by
654 Intravital Microscopy. *Methods Mol Biol* **1862**, 227-243, doi:10.1007/978-1-
655 4939-8769-6_16 (2019).
- 656 17 Qian, B. Z. *et al.* CCL2 recruits inflammatory monocytes to facilitate breast-
657 tumour metastasis. *Nature* **475**, 222-225, doi:10.1038/nature10138 (2011).
- 658 18 Muzumdar, M. D., Tasic, B., Miyamichi, K., Li, L. & Luo, L. A global double-
659 fluorescent Cre reporter mouse. *Genesis* **45**, 593-605, doi:10.1002/dvg.20335
660 (2007).
- 661 19 Mathivet, T. *et al.* Dynamic stroma reorganization drives blood vessel dysmorphia
662 during glioma growth. *EMBO Mol Med* **9**, 1629-1645,
663 doi:10.15252/emmm.201607445 (2017).
- 664 20 Raivich, G. *et al.* Regulation of MCSF receptors on microglia in the normal and
665 injured mouse central nervous system: a quantitative immunofluorescence study
666 using confocal laser microscopy. *J Comp Neurol* **395**, 342-358,
667 doi:10.1002/(sici)1096-9861(19980808)395:3<342::aid-cne6>3.0.co;2-2 (1998).
- 668 21 Jin, S. *et al.* Interleukin-34 restores blood-brain barrier integrity by upregulating
669 tight junction proteins in endothelial cells. *PLoS One* **9**, e115981,
670 doi:10.1371/journal.pone.0115981 (2014).
- 671 22 Vanlandewijck, M. *et al.* A molecular atlas of cell types and zonation in the brain
672 vasculature. *Nature* **554**, 475-480, doi:10.1038/nature25739 (2018).
- 673 23 Liu, Q. *et al.* Lung regeneration by multipotent stem cells residing at the

- 674 bronchioalveolar-duct junction. *Nat Genet* **51**, 728-738, doi:10.1038/s41588-019-
675 0346-6 (2019).
- 676 24 Tang, M. *et al.* Simultaneous quantitative assessment of two distinct cell lineages
677 with a nuclear-localized dual genetic reporter. *J Mol Cell Cardiol* **146**, 60-68,
678 doi:10.1016/j.yjmcc.2020.07.002 (2020).
- 679 25 Picelli, S. *et al.* Full-length RNA-seq from single cells using Smart-seq2. *Nat*
680 *Protoc* **9**, 171-181, doi:10.1038/nprot.2014.006 (2014).
- 681 26 Kharchenko, P. V., Silberstein, L. & Scadden, D. T. Bayesian approach to single-
682 cell differential expression analysis. *Nat Methods* **11**, 740-742,
683 doi:10.1038/nmeth.2967 (2014).
- 684 27 Saadoun, S., Papadopoulos, M. C., Hara-Chikuma, M. & Verkman, A. S.
685 Impairment of angiogenesis and cell migration by targeted aquaporin-1 gene
686 disruption. *Nature* **434**, 786-792, doi:10.1038/nature03460 (2005).
- 687 28 Aibar, S. *et al.* SCENIC: single-cell regulatory network inference and clustering.
688 *Nat Methods* **14**, 1083-1086, doi:10.1038/nmeth.4463 (2017).
- 689 29 Eelen, G. *et al.* Role of glutamine synthetase in angiogenesis beyond glutamine
690 synthesis. *Nature* **561**, 63-69, doi:10.1038/s41586-018-0466-7 (2018).
- 691 30 Zhang, M. *et al.* CXCL12 enhances angiogenesis through CXCR7 activation in
692 human umbilical vein endothelial cells. *Sci Rep* **7**, 8289, doi:10.1038/s41598-017-
693 08840-y (2017).
- 694 31 Goveia, J. *et al.* An Integrated Gene Expression Landscape Profiling Approach to
695 Identify Lung Tumor Endothelial Cell Heterogeneity and Angiogenic Candidates.
696 *Cancer cell* **37**, 21-36 e13, doi:10.1016/j.ccell.2019.12.001 (2020).
- 697 32 Rohlenova, K. *et al.* Single-Cell RNA Sequencing Maps Endothelial Metabolic
698 Plasticity in Pathological Angiogenesis. *Cell Metab* **31**, 862-877 e814,

- 699 doi:10.1016/j.cmet.2020.03.009 (2020).
- 700 33 Yang, Y. & Oliver, G. Development of the mammalian lymphatic vasculature.
701 *The Journal of clinical investigation* **124**, 888-897, doi:10.1172/JCI71609 (2014).
- 702 34 Fujimoto, N. *et al.* Single-cell mapping reveals new markers and functions of
703 lymphatic endothelial cells in lymph nodes. *PLoS biology* **18**, e3000704,
704 doi:10.1371/journal.pbio.3000704 (2020).
- 705 35 Bazigou, E. *et al.* Integrin-alpha9 is required for fibronectin matrix assembly
706 during lymphatic valve morphogenesis. *Developmental cell* **17**, 175-186,
707 doi:10.1016/j.devcel.2009.06.017 (2009).
- 708 36 Trapnell, C. *et al.* The dynamics and regulators of cell fate decisions are revealed
709 by pseudotemporal ordering of single cells. *Nature biotechnology* **32**, 381-386,
710 doi:10.1038/nbt.2859 (2014).
- 711 37 Gulati, G. S. *et al.* Single-cell transcriptional diversity is a hallmark of
712 developmental potential. *Science* **367**, 405-411, doi:10.1126/science.aax0249
713 (2020).
- 714 38 Tushinski, R. J. *et al.* Survival of mononuclear phagocytes depends on a lineage-
715 specific growth factor that the differentiated cells selectively destroy. *Cell* **28**, 71-
716 81, doi:10.1016/0092-8674(82)90376-2 (1982).
- 717 39 Dai, X. M. *et al.* Targeted disruption of the mouse colony-stimulating factor 1
718 receptor gene results in osteopetrosis, mononuclear phagocyte deficiency,
719 increased primitive progenitor cell frequencies, and reproductive defects. *Blood*
720 **99**, 111-120, doi:10.1182/blood.v99.1.111 (2002).
- 721 40 Klotz, L. *et al.* Cardiac lymphatics are heterogeneous in origin and respond to
722 injury. *Nature* **522**, 62-67, doi:10.1038/nature14483 (2015).
- 723 41 Plein, A., Fantin, A., Denti, L., Pollard, J. W. & Ruhrberg, C. Erythro-myeloid

- 724 progenitors contribute endothelial cells to blood vessels. *Nature* **562**, 223-228,
725 doi:10.1038/s41586-018-0552-x (2018).
- 726 42 Hirschi, K. K., Ingram, D. A. & Yoder, M. C. Assessing identity, phenotype, and
727 fate of endothelial progenitor cells. *Arterioscler Thromb Vasc Biol* **28**, 1584-1595,
728 doi:10.1161/ATVBAHA.107.155960 (2008).
- 729 43 Mantovani, A., Marchesi, F., Malesci, A., Laghi, L. & Allavena, P. Tumour-
730 associated macrophages as treatment targets in oncology. *Nat Rev Clin Oncol* **14**,
731 399-416, doi:10.1038/nrclinonc.2016.217 (2017).
- 732 44 Heng, J. S. *et al.* Hypoxia tolerance in the Norrin-deficient retina and the
733 chronically hypoxic brain studied at single-cell resolution. *Proceedings of the*
734 *National Academy of Sciences of the United States of America* **116**, 9103-9114,
735 doi:10.1073/pnas.1821122116 (2019).
- 736 45 Folkman, J. Tumor angiogenesis: therapeutic implications. *N Engl J Med* **285**,
737 1182-1186 (1971).
- 738 46 Ferrara, N. & Kerbel, R. S. Angiogenesis as a therapeutic target. *Nature* **438**, 967-
739 974, doi:10.1038/nature04483 (2005).
- 740 47 Welte, J., Loges, S., Dimmeler, S. & Carmeliet, P. Recent molecular discoveries
741 in angiogenesis and antiangiogenic therapies in cancer. *J Clin Invest* **123**, 3190-
742 3200, doi:10.1172/JCI70212 (2013).
- 743 48 Zhao, Q. *et al.* Single-Cell Transcriptome Analyses Reveal Endothelial Cell
744 Heterogeneity in Tumors and Changes following Antiangiogenic Treatment.
745 *Cancer Res* **78**, 2370-2382, doi:10.1158/0008-5472.CAN-17-2728 (2018).
- 746 49 Bazigou, E. *et al.* Genes regulating lymphangiogenesis control venous valve
747 formation and maintenance in mice. *The Journal of clinical investigation* **121**,
748 2984-2992, doi:10.1172/JCI58050 (2011).

- 749 50 Subach, O. M. *et al.* Conversion of red fluorescent protein into a bright blue probe.
750 *Chem Biol* **15**, 1116-1124, doi:10.1016/j.chembiol.2008.08.006 (2008).
- 751 51 Tirosh, I. *et al.* Dissecting the multicellular ecosystem of metastatic melanoma by
752 single-cell RNA-seq. *Science* **352**, 189-196, doi:10.1126/science.aad0501 (2016).
- 753 52 Stuart, T. *et al.* Comprehensive Integration of Single-Cell Data. *Cell* **177**, 1888-
754 1902 e1821, doi:10.1016/j.cell.2019.05.031 (2019).
- 755 53 Lambrechts, D. *et al.* Phenotype molding of stromal cells in the lung tumor
756 microenvironment. *Nat Med* **24**, 1277-1289, doi:10.1038/s41591-018-0096-5
757 (2018).
- 758 54 Peng, J. *et al.* Single-cell RNA-seq highlights intra-tumoral heterogeneity and
759 malignant progression in pancreatic ductal adenocarcinoma. *Cell Res* **29**, 725-738,
760 doi:10.1038/s41422-019-0195-y (2019).
- 761 55 Ma, L. *et al.* Tumor Cell Biodiversity Drives Microenvironmental
762 Reprogramming in Liver Cancer. *Cancer Cell* **36**, 418-430 e416,
763 doi:10.1016/j.ccell.2019.08.007 (2019).
- 764 56 Yost, K. E. *et al.* Clonal replacement of tumor-specific T cells following PD-1
765 blockade. *Nat Med* **25**, 1251-1259, doi:10.1038/s41591-019-0522-3 (2019).
- 766 57 Cortez-Retamozo, V. *et al.* Origins of tumor-associated macrophages and
767 neutrophils. *Proc Natl Acad Sci U S A* **109**, 2491-2496,
768 doi:10.1073/pnas.1113744109 (2012).
- 769 58 Berg, S. *et al.* ilastik: interactive machine learning for (bio)image analysis. *Nat*
770 *Methods* **16**, 1226-1232, doi:10.1038/s41592-019-0582-9 (2019).
- 771 59 Wang, A., Yan, X. & Wei, Z. ImagePy: an open-source, Python-based and
772 platform-independent software package for bioimage analysis. *Bioinformatics* **34**,
773 3238-3240, doi:10.1093/bioinformatics/bty313 (2018).

774

775 **FIGURE LEGENDS**

776 **Fig. 1: Csf1r lineage endothelial cells (CLECs) contribute to perfused, dynamically**

777 **sprouting and remodeling blood vessels in mouse glioma.** a, Csf1r-Mer2.CremTmG:

778 a mouse model for Csf1r-lineage tracing combining the tamoxifen-inducible Cre-driver

779 under control of the Csf1r-promoter and the mTmG Cre-reporter. b, Timing schedule of

780 glioma implantation, tamoxifen induction and in vivo imaging. c-f, Longitudinal

781 intravital imaging of the tumour vasculature in CT2A mouse glioma of Csf1r-

782 Mer2.CremTmG by cranial window. Scale bar: 50 μ m. c, In vivo imaging in 3 weeks

783 glioma. Endothelial cells in red (tdTomato), Csf1r-labeled cells in green (GFP) and

784 glioma cells in blue (BFP). d, Detail image of outlined square in Fig. 1c illustrating tracing

785 of GFP-positive cells. White arrowheads, macrophages; blue arrowhead, CLECs at tip

786 position. e, In vivo imaging of CLECs in 2 weeks glioma. Blue arrowheads, tip of

787 endothelial cells. f, Visualization of blood vessels in 3 weeks glioma by Intravenous

788 injection of dextran with texas red dye. Yellow arrowhead, bridging CLECs; orange

789 arrowhead, tube formation by CLECs; blue arrowhead, CLECs at tip position. g-h,

790 Counterstaining on PFA-fixed 4 weeks CT2A glioma sections of Csf1r-Mer2.CremTmG

791 with indicated antibodies. Yellow arrows, CLECs. Scale bar: 25 μ m.

792 **Fig. 2: Up to 10% of intra-tumour endothelium is comprised of CLECs not only in**

793 **glioma, but also in other tumour types and locations.** a-i, Quantification of the

794 population of CLECs in glioma of Csf1r-Mer2.CremTmG mice. a, Gross appearance of

795 sham control and 4 weeks CT2A glioma (Upper panel). Purple arrowhead, the area of

796 capillary injection; blue arrowhead, glioma. Scale bar: 5 mm. Flow cytometric analysis

797 of CLECs in sham control and 4 weeks CT2A glioma (Lower panel). b, In 3 weeks CT2A

798 glioma (n= 9), CLECs constitute $2.1 \pm 1.0\%$ of total endothelial cells (CD45-CD31+); in
799 4 weeks CT2A glioma (n= 5), they make up $7.5 \pm 1.6\%$ of total endothelial cells. c, In 3
800 weeks GL261 glioma (n= 5), CLECs constitute $1.5 \pm 1.6\%$ of total endothelial cells. d,
801 Gross appearance of normal brain, sham control and 3 weeks CT2A glioma. Purple
802 arrowhead, area of capillary injection. Scale bar: 5 mm. e-f, Quantification of the
803 population of CLECs by flow cytometric analysis in brain and tumour samples. Bars
804 represent mean \pm s.d. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Two-tailed
805 unpaired Mann-Whitney' s U test. i-j, Analysis of CLECs in B16F1 melanoma in Csf1r-
806 Mer2.CremTmG mice. g, Counterstaining on PFA-fixed section of day12 melanoma with
807 CDH5 antibody. Yellow arrows, CLECs. Scale bar: 50 μ m. h, CLECs constitute $2.8 \pm$
808 0.75% of total endothelial cells in day 12 melanoma (n=4).

809 **Fig. 3: CLECs do not originate from the bone marrow.** a-b, Tamoxifen induction
810 timing. b, Quantification of the population of tumour macrophagens (T_MACs:
811 CD45+GFP+) and tumour CLECs (T_CLECs: CD45-CD31+GFP+) by flow cytometric
812 analysis in tumour samples (n=4). Bars represent mean \pm s.d. c, Fluorescent images of
813 PFA-fixed 3 weeks CT2A glioma de of Csf1r-Mer2.CremTmG mice within 24 hours after
814 tamoxifen induction. Scale bar: 100 μ m. d-e, Flow cytometric analysis of CT2A-glioma
815 in bone marrow chimeras. d, BMCsf1r-mer2.CremTmG: C57BL/6J mice are used as
816 recipients and Csf1r-Mer2.CremTmG mice used as donors. Within the sorted myeloid
817 cell population (BFP-CD45+), $32.3 \pm 12.1\%$ are GFP-positive in Csf1r-Mer2.CremTmG
818 mice (n= 10), and $33.8 \pm 6.9\%$ are GFP-positive in bone-marrow transplanted BMCsf1r-
819 Mer2.CremTmG mice (n= 12). Among endothelial cell population (BFP-CD45-CD31+),
820 $4.3 \pm 1.9\%$ are GFP-positive in Csf1r-Mer2.CremTmG mice (n= 10), whereas no GFP-
821 positive endothelial cells are found in BMCsf1r-Mer2.CremTmG mice (n= 12). e, Csf1r-

822 Mer2.CremTmG:: BMWT: Csf1r-Mer2.CremTmG are used as recipients and C57BL/6J
823 as donors. Of the sorted myeloid cell population, $43.4 \pm 11.3\%$ GFP-positive cells are
824 found in Csf1r-Mer2.CremTmG mice (n= 6), whereas $0.1 \pm 0.1\%$ GFP-positive cells are
825 found in Csf1r-Mer2.CremTmG:: BMWT mice (n= 4). Of the endothelial cell population,
826 $10.5 \pm 9.7\%$ GFP-positive cells are found in Csf1r-Mer2.CremTmG mice (n= 6), and 9.3
827 $\pm 3.7\%$ GFP-positive cells are found in Csf1r-Mer2.CremTmG:: BMWT mice (n= 4).
828 Bars represent mean \pm s.d. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Two-tailed
829 unpaired Mann-Whitney' s U test. e, Longitudinal intravial imaging of 2 weeks CT2A
830 glioma of Csf1r-Mer2.CremTmG and Csf1r-Mer2.CremTmG :: BMWT mice. White
831 arrows, CLECs. Scale bar: 50 μ m.

832 **Fig. 4: Dual recombination lineage tracing and inducible depletion model identifies**
833 **a role for CLECs in tumour blood vessel patterning supporting glioma growth.** a,
834 Cdh5-Dre: Csf1r-Mer2.Cre: RSR-LSL-tdTomato (DreMer2.CretdTomato): a genetic
835 mouse model for CLEC selective tracing by dual recombination. b-c, Analysis in
836 DreMer2.CretdTomato. b, Longitudinal intravital imaging of the tumour vasculature in 3
837 weeks CT2A mouse glioma by Intravenous injection of FITC dextran. Yellow
838 arrowheads, CLECs. Scale bar: 50 μ m. c, ERG antibody counterstaining on PFA-fixed
839 sections of 3 weeks CT2A glioma. Yellow arrows, CLECs. Scale bar: 50 μ m. d, Cdh5-
840 Dre: Csf1r-Mer2.Cre: RSR-mTmG-DTR (DreMer2.CremTmG-DTR): a genetic mouse
841 model for CLEC selective tracing and inducible ablation. DTR, diphtheria toxin receptor.
842 e-m, Analysis in DreMer2.CremTmG-DTR. e, Longitudinal intravital imaging of the
843 tumour vasculature in 3 weeks CT2A mouse glioma. Mer2.CremTmG, Csf1r-
844 Mer2.CremTmG. Yellow arrowheads, CLECs. f, Timing schedule of glioma implantation,
845 tamoxifen induction and diphtheria toxin (DT) treatment. g, Gross appearance of 3 weeks

846 CT2A gliomas. Scale bar: 5 mm. h, Confocal images of PFA-fixed 3 weeks CT2A glioma
847 sections. Yellow arrows, CLECs. Scale bar: 50 μ m. i, Tumour weight at 3 weeks CT2A
848 glioma. j-l, Analysis of PFA-fixed 3 weeks CT2A glioma sections. j, Tumour blood
849 vessels area. k, Network length of tumour blood vessels. l, Number of bifurcations of
850 tumour blood vessels. Bars represent mean \pm s.d. * p <0.05, ** p <0.01. Two-tailed unpaired
851 Mann-Whitney's U test.

852 **Fig. 5: Single-cell analysis identifies a unique signature that defines the tumour**
853 **CLECs.** a-d, Single-cell RNA sequencing (scRNA-seq) analysis of tumour macrophages
854 (T_MACs: CD45+GFP+, 79 cells), tumour endothelial cell (T_ECs: CD45-CD31+GFP-,
855 78 cells), tumour CLECs (T_CLECs: CD45-CD31+GFP+, 141 cells) in 4 weeks CT2A
856 glioma of Csf1r-Mer2.CremTmG mice. b, Gene expression of typical endothelial cell
857 markers, macrophages and myeloid cell markers. c, Csf1r gene expression. d, Three
858 characteristic genes expressed in T_CLECs. e, Enrichment of Tip-cell signature in
859 T_CLECs compared to T_ECs as shown by GSEA. f-k, Analysis of scRNA-seq data of
860 human endothelial cells from multiple primary tumours. f, UMAP plot of human
861 endothelial cell subtypes. g, UMAP plot of human endothelial cells colour-coded for the
862 tumour type of origins. h, UMAP plots with marker gene expression for each EC subtype.
863 i, Heatmap of marker gene expression for each EC phenotype (each cell as a column and
864 each gene as a row). j-k, AUCCell analysis of 24 T_CLECs markers. j, T_CLECs
865 subpopulation indicated (AUC>0.165) by red colored dots. k, Cell type-specific (left) and
866 tumour type-specific (right) populations of T_CLECs. BCC, basal cell carcinoma; PDAC,
867 pancreatic ductal adenocarcinoma.

868 **Fig. 6: CLECs are linked to Prox1-positive lymphatic endothelium of the**
869 **subcapsular sinus floor in lymph nodes.** a, Fluorescent images of the indicated organs

870 of Csf1r-Mer2.CremTmG mice after multiple tamoxifen induction. Scale bar: 50 μ m. b,
871 Quantification of the population of CLECs in Csf1r-Mer2.CremTmG mice by flow
872 cytometric analysis. Of the endothelial cell population (CD45-CD31+), $9.8 \pm 2.1\%$ GFP-
873 positive cells are found in the axillary lymph node of Csf1r-Mer2.CremTmG mice (n=
874 10). c, Counterstaining on PFA-fixed Inguinal lymph node of Csf1r-Mer2.CremTmG::
875 BMWT mice using PROX1 antibody within 24 hours after tamoxifen induction. Yellow
876 arrowheads, GFP+tdTomato+PROX1+ cells in the floor of the subcapsular sinus. Scale
877 bar 25 μ m. d, Fluorescent images of PROX1 antibody counterstaining on PFA-fixed
878 inguinal lymph node of Csf1r-Mer2.CremTmG::BMWT mice after multiple rounds of
879 tamoxifen induction. Scale bar: 100 μ m. Detailed image of square outlined in d. Yellow
880 arrowheads, GFP+Prox1+ cells; blue arrowheads, GFP+Prox1- cells. h-k, Analysis of
881 CT2A glioma in Prox1-CreERT2mTmG mice.

882 **Fig. 7: CLEC and EC heterogeneity in tumor and lymph node.** a, Quality measures
883 of 4 SMARTseq2 scRNA-seq. libraries: Csf1r-lineage endothelial cells (CLECs) and
884 endothelial cells (ECs) isolated from tumor (T_) and lymph node (LN_). b, Violin plots
885 show GFP expression per single cell. c, Uniform manifold approximation and projection
886 (UMAP) of T_CLECs, T_EC, LN_CLECs, LN_EC. d, Heatmap shows the 20 most
887 characteristic markers per cell population. e, UMAP colored by gene expression diversity
888 (CytoTRACE score) and predicted ordering by CytoTRACE. f, Monocle based trajectory
889 analysis of four cell populations, colored by pseudotime and CytoTRACE score.

890 **Extended Data Fig. 1: CLECs in mouse glioma express endothelial cell markers, but**
891 **lack macrophage and myeloid cell markers.** Counterstaining on PFA-fixed 4 weeks
892 CT2A glioma sections of Csf1r-Mer2.CremTmG with indicated antibodies. Lower panels

893 show the detail images of outlined squares in upper panels. Yellow arrowheads, CLECs;
894 blue arrowheads, macrophages. Scale bar: 25 μ m.

895 **Extended Data Fig. 2: CLECs contribute to lymphatic endothelium in the tumour**
896 **environment.** Fluorescent images of counterstaining on PFA-fixed section of day 12
897 B16F1 melanoma under ear skin of Csf1r-Mer2.CremTmG mice using LYVE1 antibody.
898 Lower panel indicates detail image of yellow outlined square in upper panel. Yellow
899 arrow heads, CLECs. Scale bar: 100 μ m.

900 **Extended Data Fig. 3: Reconstitution rate in bone marrow chimeras.** Reconstitution
901 rate was determined in peripheral blood 8 weeks after bone marrow transplantation.
902 TdTomato-positive cells in blood of: C57BL/6J ($0.3 \pm 0.1\%$, n= 6), Csf1r-
903 Mer2.CremTmG ($99.2 \pm 0.5\%$, n= 7), BMCsf1r-Mer2.CremTmG ($97.3 \pm 1.6\%$, n= 13).
904 Bars represent mean \pm s.d. ****p<0.0001. Two-tailed unpaired Mann-Whitney' s U test.

905 **Extended Data Fig. 4: CLECs do not originate from spleen.** Surgical removal of
906 spleen (splenectomy) was performed 3 weeks before glioma injection. CLECs isolated
907 from CT2A glioma of sham control Csf1r-Mer2.CremTmG mice (n= 5) constitute $1.5 \pm$
908 0.8% of total tumour endothelial cell population, compared to $1.5 \pm 0.5\%$ found in Csf1r-
909 Mer2.CremTmG with splenectomy (n= 4). Bars represent mean \pm s.d. Two-tailed
910 unpaired Mann-Whitney' s U test.

911 **Extended Data Fig. 5: CLECs locate at peripheral area of mouse glioma.** a,
912 Vibratome section (200 μ m-thick) of 4 weeks CT2A glioma of Csf1r-Mer2.CremTmG::
913 BMWT mice. Scale bar: 1 mm. b, Fluorescent images of area outlined in a. Yellow
914 arrowheads, CLECs. Scale bar: 100 μ m.

915 **Extended Data Fig. 6: 24 tumour CLECs markers in mouse glioma.** SCDE analysis
916 of scRNA-seq data of tumour macrophages (T_MACs: CD45+GFP+, n= 79), tumour
917 endothelial cells (T_EC: CD45-CD31+GFP-, n= 78), tumour CLECs (T_CLECs: CD45-
918 CD31+GFP+, n= 141) in 4 weeks CT2A glioma in Csf1r-Mer2.CremTmG mice. Bars
919 represent mean \pm s.d. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001. Two-tailed
920 unpaired t-test with Welch correction.

921 **Extended Data Fig. 7: CLECs in glioma express AQP1.** Counterstaining on PFA-fixed
922 CT2A glioma of Csf1r-Mer2.CremTmG::BMWT mice using AQP1 antibody. Yellow
923 arrowheads, T_CLECs; blue arrowheads, T_EC. Scale bar: 25 μ m.

924 **Extended Data Fig. 8: CLECs in glioma cannot be labelled by GFP within 24 hours**
925 **after tamoxifen induction.** a-b, Tamoxifen induction timing. b, Quantification of the
926 population of tumour macrophages (T_MACs: CD45+GFP+) and tumour CLECs
927 (T_CLECs: CD45-CD31+GFP+) by flow cytometric analysis in tumour samples (n=4).
928 Bars represent mean \pm s.d. c, Fluorescent images of PFA-fixed 3 weeks CT2A glioma de
929 of Csf1r-Mer2.CremTmG mice within 24 hours after tamoxifen induction. Scale bar: 100
930 μ m.

931 **Extended Data Fig. 9: CLEC and EC heterogeneity in tumor and lymph node .**
932 a, Heatmap shows the expression of n=91 fLECs markers in LN_CLEC, LN_EC,
933 T_CLEC and T_EC single cells. b, Violin plots show the expression of 3 CLEC markers:
934 Pde4d, Robo1 and Itga9.

935 **Supplemental Movie 1: Time-lapse *in vivo* imaging of Csf1r lineage endothelial tip**
936 **cell in CT2A glioma of Csf1r-Mer2.Cre^{mTmG} mice.** Imaging was performed over 4 hours
937 (1 frame/ 10 min).

938

939 **Supplemental Movie 2: Time-lapse *in vivo* imaging of *Csflr* lineage endothelial tip**
940 **cell in CT2A glioma of *Csflr-Mer2.Cre^{mTmG}::BM^{WT}*.** Imaging was performed over 50
941 minutes (1 frame/ 2.5 min).

942

943 **Supplemental Table 1: Details of gene expression analysis.** Seurat based differential
944 analysis (non-parameteric Wilcoxon rank sum test) was performed by comparing cell
945 types amongst each other (green columns) and the different Seurat clusters (yellow
946 columns). p_val: p_val (unadjusted). avg_logFC: log fold-change of the average
947 expression between the two groups. Positive values indicate that the feature is more
948 highly expressed in the first group. pct.1: The percentage of cells where the feature is
949 detected in the first group. pct.2: The percentage of cells where the feature is detected in
950 the second group. p_val_adj: Adjusted p-value, based on Bonferroni correction using all
951 features in the dataset.

952

953 **Supplemental Table 2: Marker genes for EC subtypes of multiple tumour types.**
954 p_val: p_val (unadjusted). avg_logFC: Average log (fold change). pct.1: Percentage of
955 expressed cells in current cluster. pct.2: Percentage of expressed cells in other cluster.
956 p_val_adj: Adjusted p-value. cluster: Cluster name. gene: Gene name. log2fc: log₂ (fold
957 change). Log2pct: log₂ ((pct+0.005)/(pct2+0.005)).

958

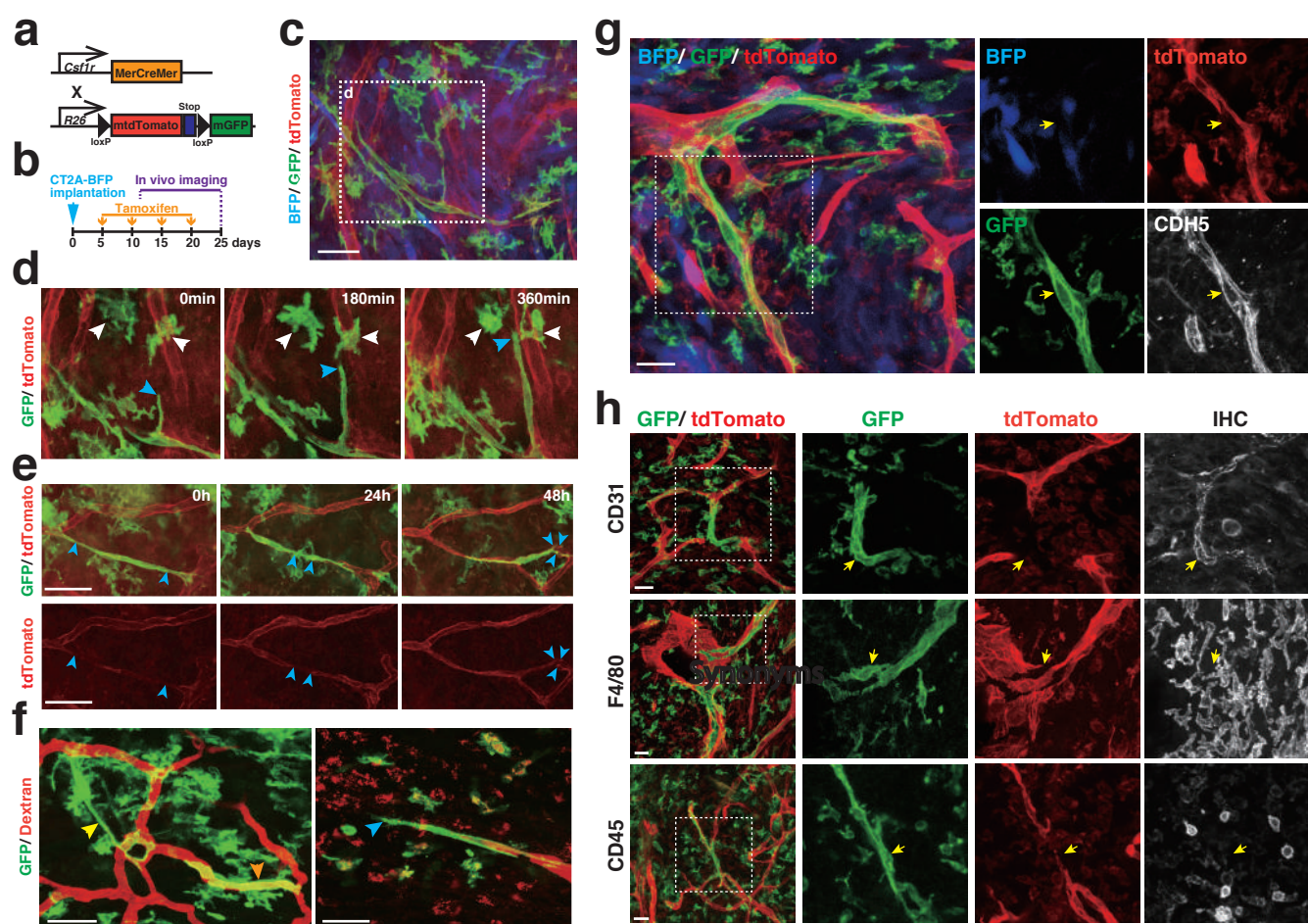


Fig. 1: *Csf1r* lineage endothelial cells (CLECs) contribute to perfused, dynamically sprouting and remodeling blood vessels in mouse glioma. a, *Csf1r-Mer2.Cre^{mTmG}*: a mouse model for *Csf1r*-lineage tracing combining the tamoxifen-inducible Cre-driver under control of the *Csf1r*-promoter and the *mTmG* Cre-reporter. b, Timing schedule of glioma implantation, tamoxifen induction and in vivo imaging. c-f, Longitudinal intravital imaging of the tumour vasculature in CT2A mouse glioma of *Csf1r-Mer2.Cre^{mTmG}* by cranial window. Scale bar: 50 μ m. c, In vivo imaging in 3 weeks glioma. Endothelial cells in red (tdTomato), *Csf1r*-labeled cells in green (GFP) and glioma cells in blue (BFP). d, Detail image of outlined square in Fig. 1c illustrating tracing of GFP-positive cells. White arrowheads, macrophages; blue arrowhead, CLECs at tip position. e, In vivo imaging of CLECs in 2 weeks glioma. Blue arrowheads, tip of endothelial cells. f, Visualization of blood vessels in 3 weeks glioma by Intravenous injection of dextran with texas red dye. Yellow arrowhead, bridging CLECs; orange arrowhead, tube formation by CLECs; blue arrowhead, CLECs at tip position. g-h, Counterstaining on PFA-fixed 4 weeks CT2A glioma sections of *Csf1r-Mer2.Cre^{mTmG}* with indicated antibodies. Yellow arrows, CLECs. Scale bar: 25 μ m.

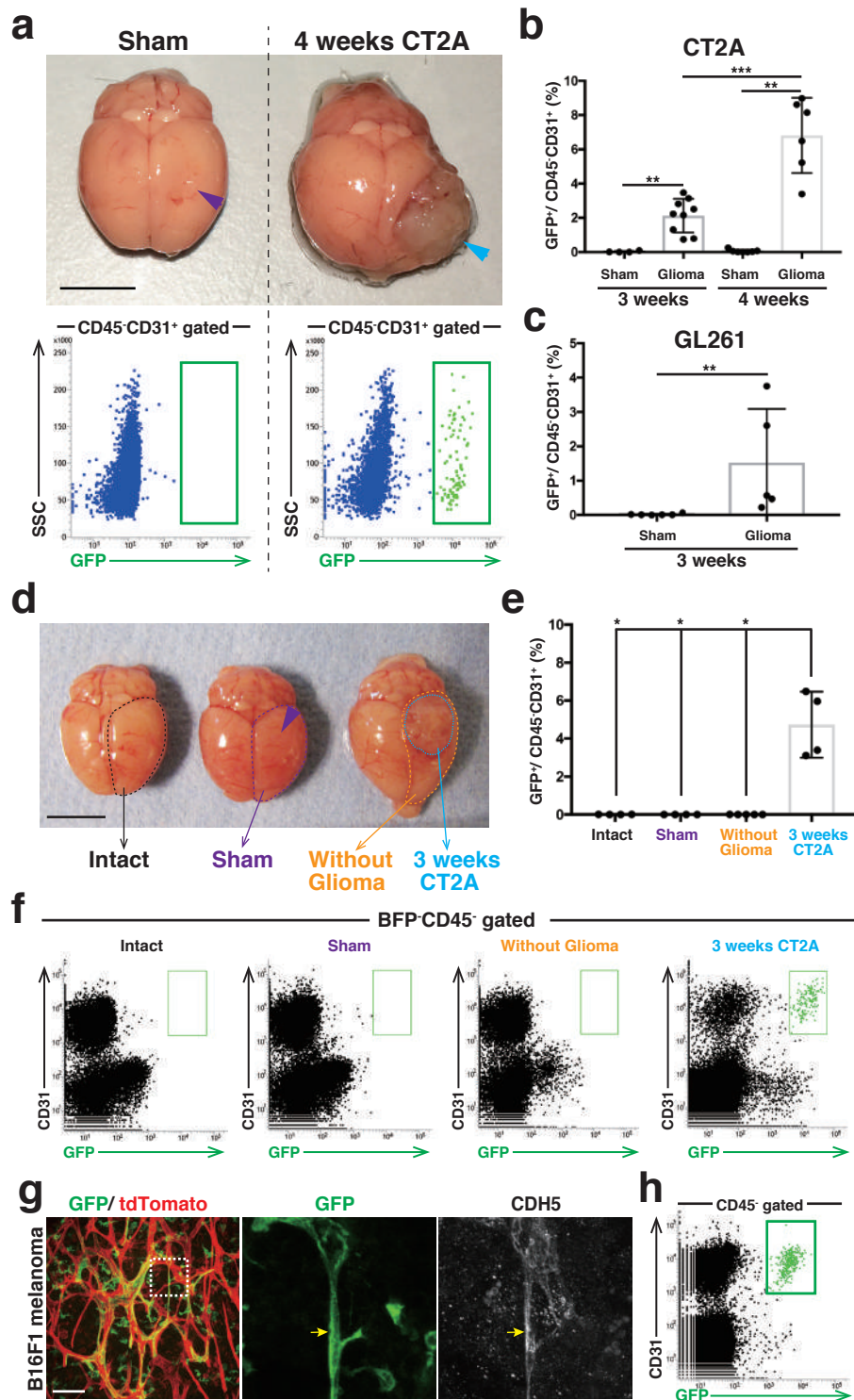


Fig. 2: Up to 10% of intra-tumour endothelium is comprised of CLECs not only in glioma, but also in other tumour types and locations. a-f, Quantification of the population of CLECs in glioma of *Csf1r-Mer2.Cre^{TM6G}* mice. a, Gross appearance of sham control and 4 weeks CT2A glioma (Upper panel). Purple arrowhead, the area of capillary injection; blue arrowhead, glioma. Scale bar: 5 mm. Flow cytometric analysis of CLECs in sham control and 4 weeks CT2A glioma (Lower panel). b, In 3 weeks CT2A glioma (n= 9), CLECs constitute $2.1 \pm 1.0\%$ of total endothelial cells (CD45-CD31+); in 4 weeks CT2A glioma (n= 5), they make up $7.5 \pm 1.6\%$ of total endothelial cells. c, In 3 weeks GL261 glioma (n= 5), CLECs constitute $1.5 \pm 1.6\%$ of total endothelial cells. d, Gross appearance of normal brain, sham control and 3 weeks CT2A glioma. Purple arrowhead, area of capillary injection. Scale bar: 5 mm. e-f, Quantification of the population of CLECs by flow cytometric analysis in brain and tumour samples. Bars represent mean \pm s.d. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Two-tailed unpaired Mann-Whitney's U test. g-h, Analysis of CLECs in B16F1 melanoma in *Csf1r-Mer2.Cre^{TM6G}* mice. g, Counterstaining on PFA-fixed section of day12 melanoma with CDH5 antibody. Yellow arrows, CLECs. Scale bar: 50 μ m. h, CLECs constitute $2.8 \pm 0.75\%$ of total endothelial cells in day 12 melanoma (n=4).

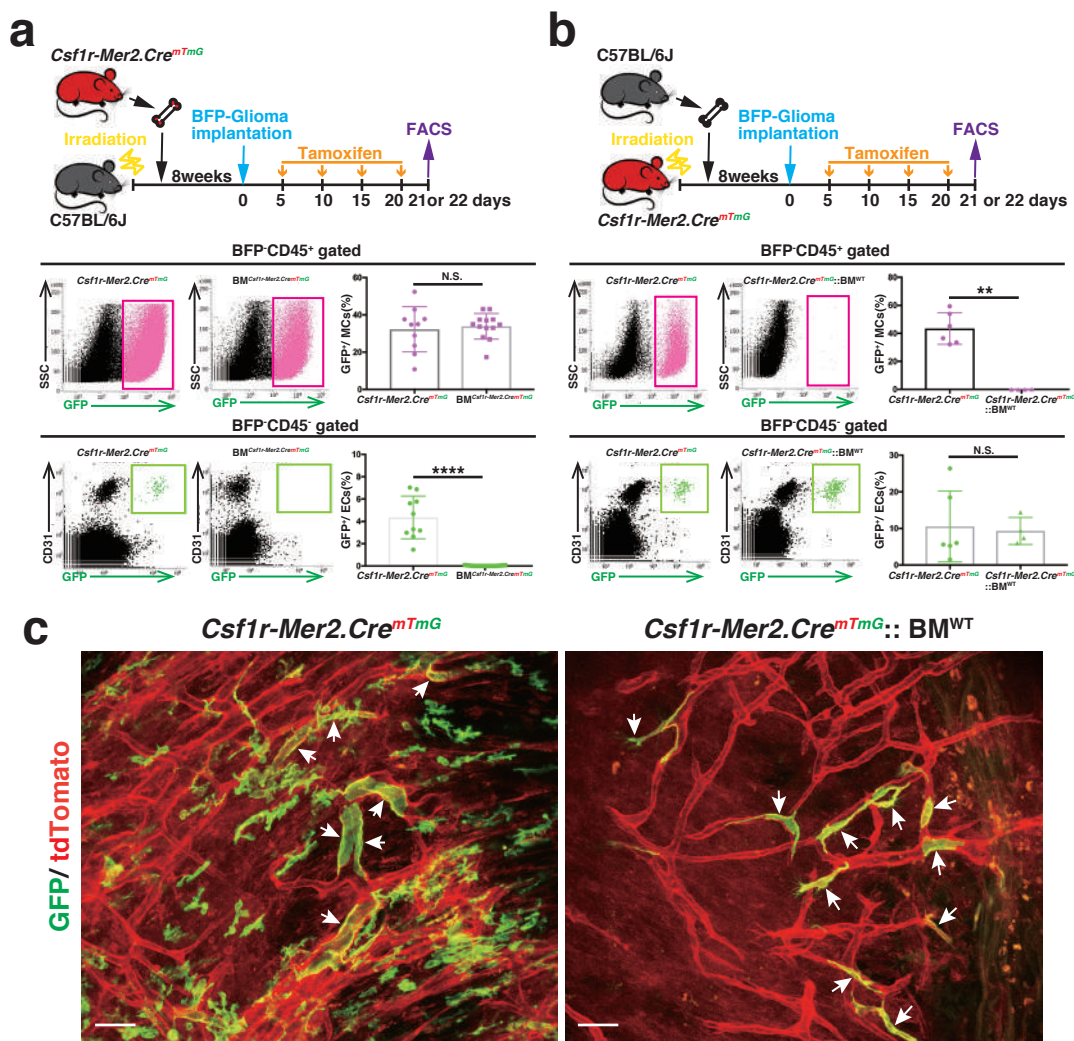


Fig. 3: CLECs do not originate from bone marrow. a-b, Flow cytometric analysis of CT2A-glioma in bone marrow chimeras. a, *BM^{Csf1r-mer2.CremTmG}*: C57BL/6J mice are used as recipients and *Csf1r-Mer2.Cre^{mTmG}* mice used as donors. Within the sorted myeloid cell population (BFP-CD45⁺), 32.3 ± 12.1% are GFP-positive in *Csf1r-Mer2.Cre^{mTmG}* mice (n= 10), and 33.8 ± 6.9% are GFP-positive in bone-marrow transplanted *BM^{Csf1r-Mer2.CremTmG}* mice (n= 12). Among endothelial cell population (BFP-CD45⁺CD31⁺), 4.3 ± 1.9% are GFP-positive in *Csf1r-Mer2.Cre^{mTmG}* mice (n= 10), whereas no GFP-positive endothelial cells are found in *BM^{Csf1r-Mer2.CremTmG}* mice (n= 12). b, *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}*: *Csf1r-Mer2.Cre^{mTmG}* are used as recipients and C57BL/6J as donors. Of the sorted myeloid cell population, 43.4 ± 11.3% GFP-positive cells are found in *Csf1r-Mer2.Cre^{mTmG}* mice (n= 6), whereas 0.1 ± 0.1% GFP-positive cells are found in *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* mice (n= 4). Of the endothelial cell population, 10.5 ± 9.7% GFP-positive cells are found in *Csf1r-Mer2.Cre^{mTmG}* mice (n= 6), and 9.3 ± 3.7% GFP-positive cells are found in *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* mice (n= 4). Bars represent mean ± s.d. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001. Two-tailed unpaired Mann-Whitney' s U test. c, Longitudinal intravital imaging of 2 weeks CT2A glioma of *Csf1r-Mer2.Cre^{mTmG}* and *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* mice. White arrows, CLECs. Scale bar: 50 μ m.

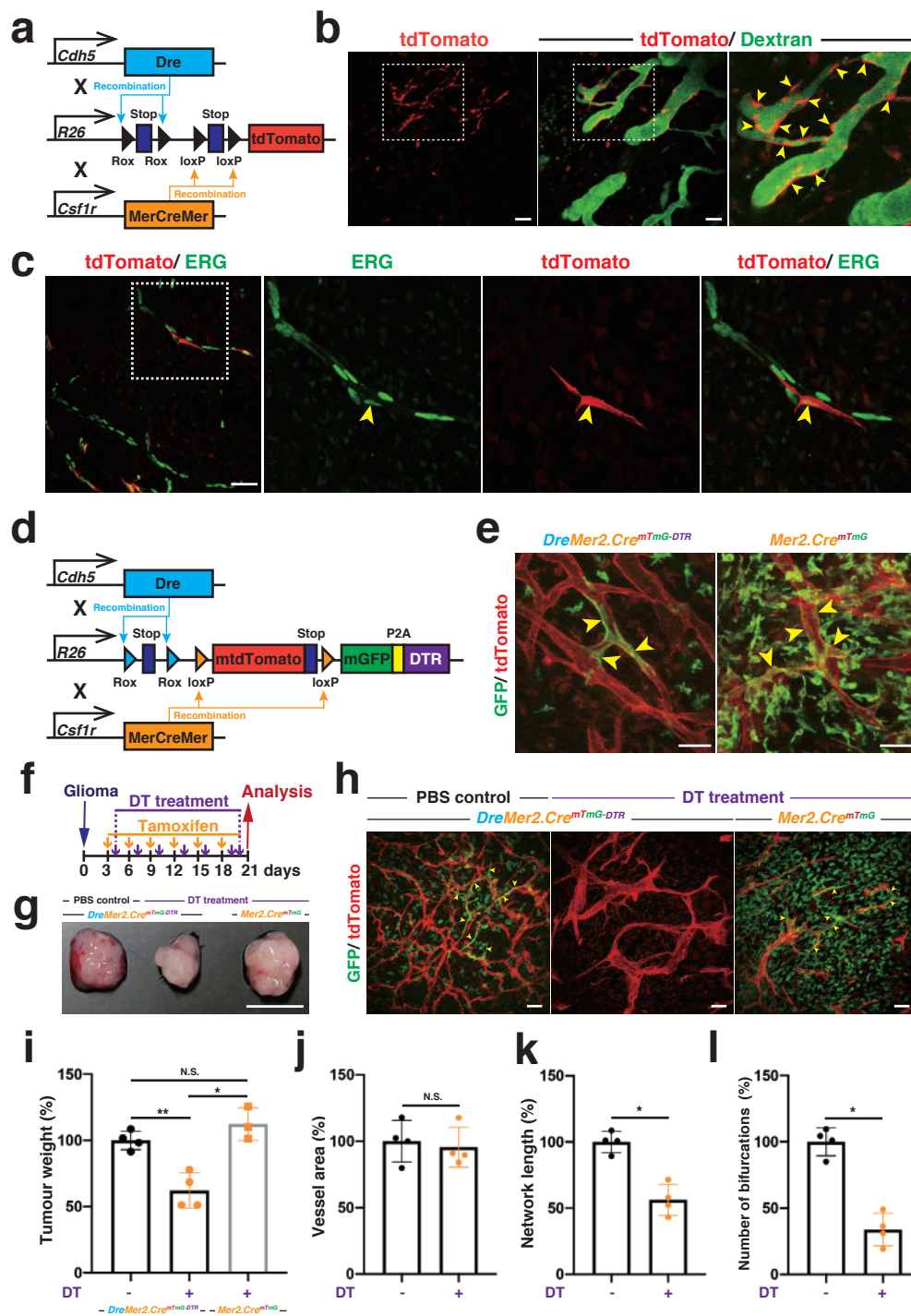


Fig. 4: Dual recombination lineage tracing and inducible depletion model identifies a role for CLECs in tumour blood vessel patterning supporting glioma growth. a, *Cdh5-Dre: Csf1r-Mer2.Cre: RSR-LSL-tdTomato (DreMer2.Cre^{tdTomato})*: a genetic mouse model for CLEC selective tracing by dual recombination. b-c, Analysis in *DreMer2.Cre^{tdTomato}*. b, Longitudinal intravital imaging of the tumour vasculature in 3 weeks CT2A mouse glioma by Intravenous injection of FITC dextran. Yellow arrowheads, CLECs. Scale bar: 50 μ m. c, ERG antibody counterstaining on PFA-fixed sections of 3 weeks CT2A glioma. Yellow arrows, CLECs. Scale bar: 50 μ m. d, *Cdh5-Dre: Csf1r-Mer2.Cre: RSR-mTmG-DTR (DreMer2.Cre^{mTmG-DTR})*: a genetic mouse model for CLEC selective tracing and inducible ablation. DTR, diphtheria toxin receptor. e-l, Analysis in *DreMer2.Cre^{mTmG-DTR}*. e, Longitudinal intravital imaging of the tumour vasculature in 3 weeks CT2A mouse glioma. *Mer2.Cre^{mTmG}, Csf1r-Mer2.Cre^{mTmG}*. Yellow arrowheads, CLECs. f, Timing schedule of glioma implantation, tamoxifen induction and diphtheria toxin (DT) treatment. g, Gross appearance of 3 weeks CT2A gliomas. Scale bar: 5 mm. h, Confocal images of PFA-fixed 3 weeks CT2A glioma sections. Yellow arrows, CLECs. Scale bar: 50 μ m. i, Tumour weight at 3 weeks CT2A glioma. j-l, Analysis of PFA-fixed 3 weeks CT2A glioma sections. j, Tumour blood vessels area. k, Network length of tumour blood vessels. l, Number of bifurcations of tumour blood vessels. Bars represent mean \pm s.d. * $P < 0.05$, ** $P < 0.01$. Two-tailed unpaired Mann-Whitney' s U test.

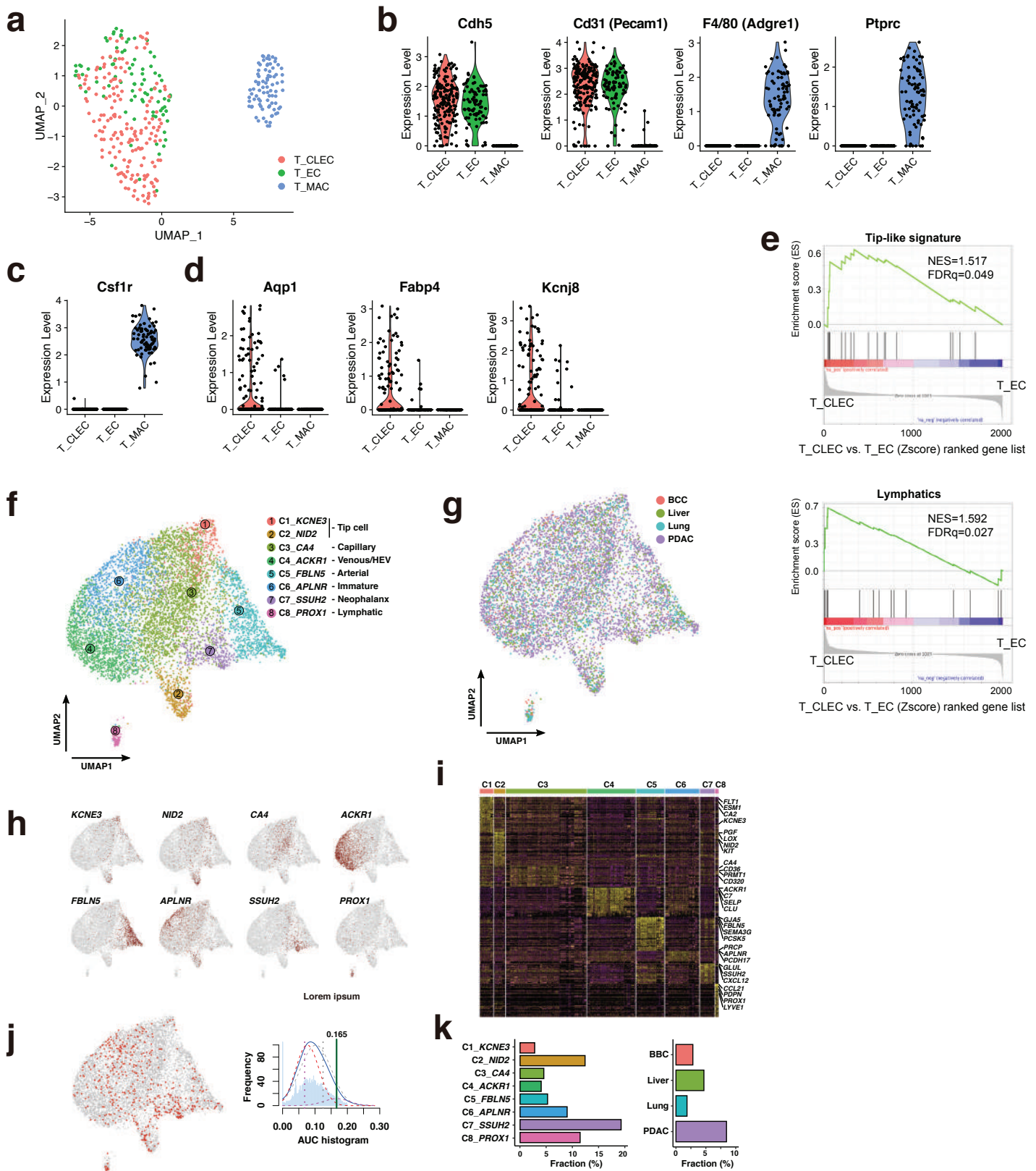


Fig. 5: Single-cell analysis identifies a unique signature that defines the tumour CLECs. a-d, Single-cell RNA sequencing (scRNA-seq) analysis of tumour macrophages (T_MACs: CD45⁺GFP⁺, 79 cells), tumour endothelial cell (T_EC: CD45⁺CD31⁺GFP⁺, 78 cells), tumour CLECs (T_CLECs: CD45⁺CD31⁺GFP⁺, 141 cells) in 4 weeks CT2A glioma of *Csf1r-Mer2.Cre^{mtmG}* mice. b, Gene expression of typical endothelial cell markers, macrophages and myeloid cell markers. c, *Csf1r* gene expression. d, Three characteristic genes expressed in T_CLECs. e, Enrichment of Tip-cell and lymphatic signatures in T_CLECs compared to T_EC as shown by GSEA. f-k, Analysis of scRNA-seq data of human endothelial cells from multiple primary tumours. f, UMAP plot of human endothelial cell subtypes. g, UMAP plot of human endothelial cells colour-coded for the tumour type of origins. h, UMAP plots with marker gene expression for each EC subtype. i, Heatmap of marker gene expression for each EC phenotype (each cell as a column and each gene as a row). j-k, AUC analysis of 24 T_CLECs markers. j, T_CLECs subpopulation indicated (AUC>0.165) by red colored dots. k, Cell type-specific (left) and tumour type-specific (right) populations of T_CLECs. BCC, basal cell carcinoma; PDAC, pancreatic ductal adenocarcinoma.

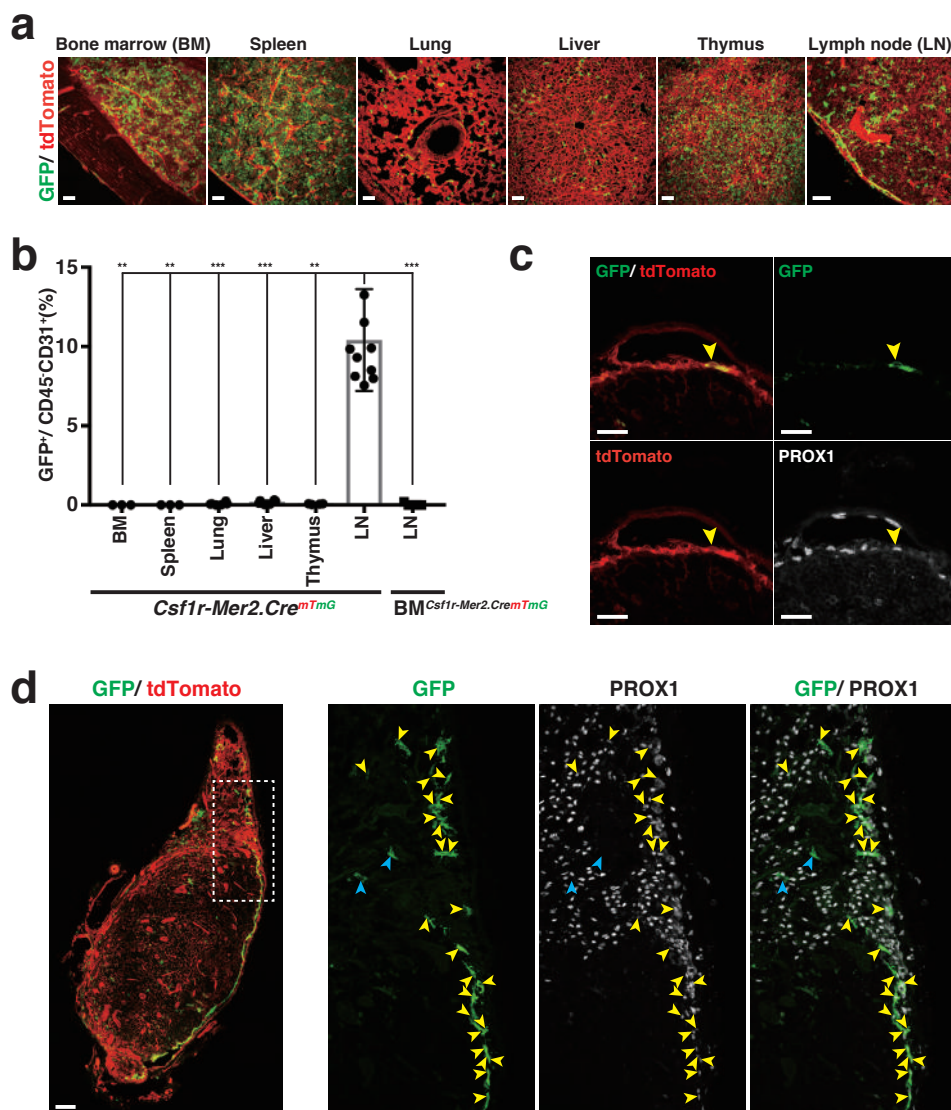


Fig. 6: CLECs are linked to *Prox1*-positive lymphatic endothelium of the subcapsular sinus floor in lymph nodes. a, Fluorescent images of the indicated organs of *Csf1r-Mer2.Cre^{mTmG}* mice after multiple tamoxifen induction. Scale bar: 50 μ m. b, Quantification of the population of CLECs in *Csf1r-Mer2.Cre^{mTmG}* mice by flow cytometric analysis. Of the endothelial cell population (CD45⁺CD31⁺), 9.8 \pm 2.1% GFP-positive cells are found in the axillary lymph node of *Csf1r-Mer2.Cre^{mTmG}* mice (n= 10). c, Counterstaining on PFA-fixed Inguinal lymph node of *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* mice using PROX1 antibody within 24 hours after tamoxifen induction. Yellow arrowheads, GFP⁺tdTomato⁺PROX1⁺ cells in the floor of the subcapsular sinus. Scale bar 25 μ m. d, Fluorescent images of PROX1 antibody counterstaining on PFA-fixed inguinal lymph node of *Csf1r-Mer2.Cre^{mTmG}::BM^{WT}* mice after multiple rounds of tamoxifen induction. Scale bar: 100 μ m. Detailed image of square outlined in d. Yellow arrowheads, GFP⁺Prox1⁺ cells; blue arrowheads, GFP⁺Prox1⁻ cells.

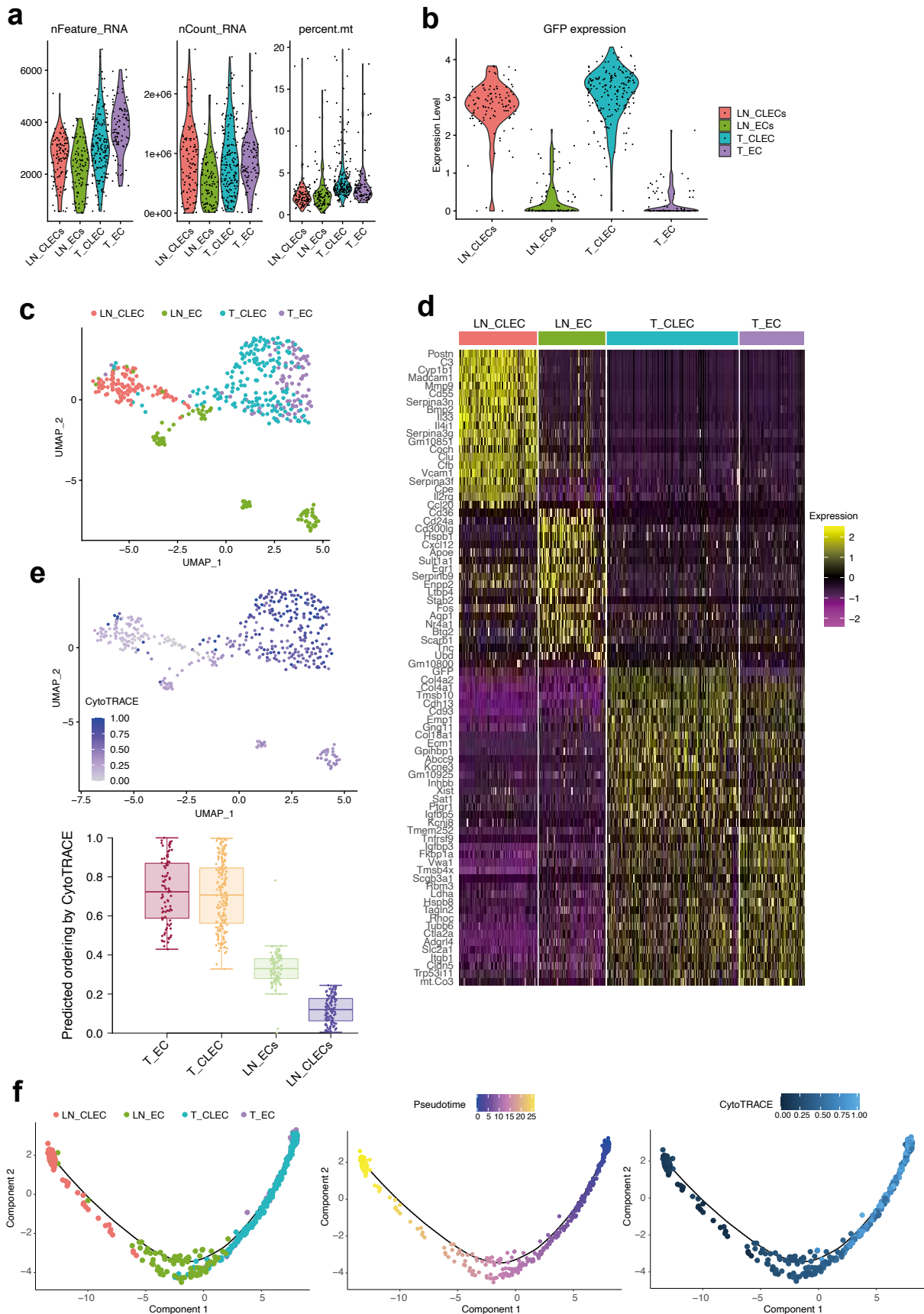


Fig. 7: CLEC and EC heterogeneity in tumor and lymph node .

a, Quality measures of 4 SMARTseq2 scRNA-seq libraries: Csf1r-lineage endothelial cells (CLECs) and endothelial cells (ECs) isolated from tumor (T_) and lymph node (LN_). b, Violin plots show GFP expression per single cell. c, Uniform manifold approximation and projection (UMAP) of T_CLECs, T_EC and LN_CLECs, LN_EC. d, Heatmap shows the 20 most characteristic markers per cell population. e, UMAP colored by gene expression diversity (CytoTRACE score) and predicted ordering by CytoTRACE. f, Monocle based trajectory analysis of four cell populations, colored by pseudotime and CytoTRACE score.