Mutual regulation of tumour vessel normalization and immunostimulatory reprogramming

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Blockade of angiogenesis can retard tumour growth, but may also paradoxically increase metastasis1,2. This paradox may be resolved by vessel normalization3, which involves increased pericyte coverage, improved tumour vessel perfusion, reduced vascular permeability, and consequently mitigated hypoxia4. Although these processes alter tumour progression, their regulation is poorly understood. Here we show that type 1 T helper (Th1) cells play a crucial role in vessel normalization. Bioinformatic analyses revealed that gene expression features related to vessel normalization correlate with immunostimulatory pathways, especially T lymphocyte infiltration or activity. To delineate the causal relationship, we used various mouse models with vessel normalization or T lymphocyte deficiencies. Although disruption of vessel normalization reduced T lymphocyte infiltration as expected4, reciprocal depletion or inactivation of CD4+ T lymphocytes decreased vessel normalization, indicating a mutually regulatory loop. In addition, activation of CD4+ T lymphocytes by immune checkpoint blockade increased vessel normalization. Th1 cells that secrete interferon-γ are a major population of cells associated with vessel normalization. Patient-derived xenograft tumours growing in immunodeficient mice exhibited enhanced hypoxia compared to the original tumours in immunocompetent humans, and hypoxia was reduced by adoptive Th1 transfer. Our findings elucidate an unexpected role of Th1 cells in vasculature and immune reprogramming. Th1 cells may be a marker and a determinant of both immune checkpoint blockade and anti-angiogenesis efficacy.

To better understand angiogenesis, we examined angiogenesis-related genes in breast cancer using the METABRIC database5. Among 377 genes, 30 positively and 27 negatively correlate with survival, and are defined as good- and poor-prognosis angiogenesis genes (GPAGs and PPAGs), respectively (Supplementary Table 1a, b), which together stratify patients with different prognoses (Fig. 1a, b). Single metrics defined by (∑GPAGs − ∑PPAGs) or principal component analysis are prognostic in multiple breast cancer datasets (Supplementary Table 1c–f), suggesting that different aspects of angiogenesis may play opposing roles in tumour progression.

GPAGs are mostly related to heterotypic cell–cell adhesion and smooth muscle cell proliferation (Fig. 1c, Supplementary Table 2a, b). Pericytes and smooth muscle cells share gene expression programs and may be ontologically related6. Pericyte recruitment is often regulated by common pathways such as pericyte proliferation, and is pivotal to vessel normalization7. Thus, GPAGs may reflect vessel normalization. In contrast, PPAGs are mostly related to extracellular matrix disassembly and hypoxia (Fig. 1c, Supplementary Table 2a, c), processes regulated by mechanisms opposing vessel normalization7.

We further tested the correlation between GPAGs and vessel normalization in liver cancer. CD31+ tumour-associated endothelial cells (TECs) or the matched CD31+ normal endothelial cells from the same patient were profiled (Extended Data Fig. 1a). Compared to normal endothelial cells, TECs express decreased GPAGs and increased PPAGs (Extended Data Fig. 1b). In the GSE20017 dataset, (∑GPAGs − ∑PPAGs) inversely correlates with invasive vasculature (Extended Data Fig. 1c). Thus, (∑GPAGs − ∑PPAGs) is a vessel normalization indicator.

In breast cancer, GPAGs correlate with immune response pathways (Fig. 1d, Supplementary Table 3), especially T-cell receptor (TCR) signalling (Fig. 1e, f). Similarly, in the GSE51401 dataset, (∑GPAGs − ∑PPAGs) in TECs correlated with TCR signatures in non-TECs from the same tumours (Extended Data Fig. 1d, e).

To investigate the relationship between T lymphocytes and vessel normalization, we examined mammary tumours in various host strains that lacked pericytes or T lymphocytes. We orthotopically transplanted E0771 mouse tumour cells into mice expressing both NG2-CreERT2 (tamoxifen-inducible Cre recombinase under the control of the NG2 also known as Cspg4) promoter/enhancer) and Cre-inducible diphtheria toxin receptor (mice denoted as Peri(Dd)). Upon tamoxifen and diphtheria toxin treatment, NG2+ pericytes were significantly reduced (Extended Data Fig. 2a, b), which caused a decrease in total infiltrating immune cells, consistent with previous findings8. T lymphocytes were particularly decreased, whereas CD11b+CD11c− cells remained unchanged (Extended Data Fig. 2c, d), suggesting that vessel normalization preferentially promotes infiltration of T lymphocytes.

To investigate any reciprocal effects of T lymphocytes on vessel normalization, we transplanted E0771 cells into animals with knockout of CD4 (CD4KO), CD8 (CD8KO) and T-cell receptor (TCRKO, lacking both CD4+ and CD8+ T lymphocytes). Tumours were removed at similar time points and were similar sizes. Flow cytometry analysis revealed significant effects of CD8KO on TEC frequency and of CD4KO on the ratio of TECs to pericytes (Fig. 2a). TCRKO exhibited both of these effects, suggesting that vascular proliferation and vessel normalization are distinct processes. Immunofluorescence staining of pericytes and TECs validated these conclusions (Fig. 2b). E0771 tumours growing in CD4KO or TCRKO mice, but not in CD8KO mice, exhibited increased vessel permeability (Fig. 2c), consistent with pericyte deficiency. Circulating tumour cells were increased in all three models including CD8KO (Fig. 2d), suggesting permeability-independent, CD8-specific mechanisms.
including (1) increased intravasation due to increased vascular frequency (Fig. 2a) and (2) increased circulating tumour cell survival due to loss of cytotoxic T lymphocytes. Pulmonary metastases correlated with frequency of circulating tumour cells, and were increased in all three models. The effects of CD4KO and CD8KO on metastases were additive, as TCRKO mice had the highest metastatic burden (Fig. 2e).

Figure 1 | The dichotomy of angiogenesis-related genes supports the ‘vessel normalization theory’ and links good prognosis angiogenesis genes to T-cell signalling. a, b, Hierarchical clustering of prognosis-related angiogenesis genes reveals two clusters of patients, and disease-free survival of the two clusters of patients. c, Pathways associated with GPAGs/PPAGs. Numbers of pathways shown in parentheses. d, GSEA reveals an association between Immune Response pathway and GPAGs. e, Top pathways associated with leading subset genes in d, f. Scatter plot showing the correlation between TCR signalling genes and GPAG/PPAG signatures in METABRIC discovery and validation datasets (n = 1,992 patients). P values are determined by log-rank tests (b), random permutation (d), hypergeometric test (e), and Student’s t-test (f). False discovery rate or q values are determined by Benjamini–Hochberg adjustment (d, e).

Figure 2 | Depletion of CD4+–TLs decreases tumour vessel pericyte coverage and increases metastasis. a, Flow cytometry quantification of E0771 tumour-infiltrating endothelial cells (CD31+) and pericytes (NG2+) (wild type (WT), n = 11; CD4KO, n = 9; CD8KO, n = 12; TCRKO, n = 8). b, Staining and quantification of endothelial cells (green) attached by pericytes (red) (n = 4 per group; scale bar, 50 μm). c, Normalized absorbance of Evans blue in tumours (BC (blank control), n = 5; WT, n = 11; CD4KO, n = 11; CD8KO, n = 12; TCRKO, n = 12). d, Blood circulating tumour cell frequencies from tumour-bearing mice (WT, n = 11; CD4KO, n = 11; CD8KO, n = 12; TCRKO, n = 12). e, Lung metastatic area from haematoxylin and eosin images (WT, n = 14; CD4KO, n = 14; CD8KO, n = 12; TCRKO, n = 8; scale bar, 1 mm). Mean ± s.e.m. shown. Smaller dots are values from individual fields (b) and whole lung sections (e). Outlined circles are mean values taken over multiple fields/sections from the same mouse. P values were calculated by comparing individual animals using two-tailed unpaired Student’s t-test (a–c, e) or two-tailed unpaired Mann–Whitney U-test (d). NS, not significant.
We further validated the effects mediated by CD4⁺ T lymphocytes (CD4⁺-TLs) on vessel normalization in other models with varying baseline CD4⁺-TL frequencies. CD4KO in 4T1 tumour-bearing mice led to decreased pericyte coverage and alterations related to vessel normalization disruption, including enhanced hypoxia, increased dextran leakage, and reduced lectin perfusion (Extended Data Fig. 3a–g). We also used anti-CD4 antibodies to deplete CD4⁺-TLs. This can be administered in an acute manner (Extended Data Fig. 3h) to delineate roles of CD4⁺-TLs in different metastasis stages. A regimen that restricts anti-CD4 effects to orthotopic tumours increased metastasis formation (Extended Data Fig. 3i–k), showing that anti-metastasis functions of CD4⁺-TLs begin in orthotopic tumours.

Another model, AT3, has a lower baseline T lymphocyte infiltration (Extended Data Fig. 3l). CD4KO resulted in reduced pericyte coverage and enhanced hypoxia (Extended Data Fig. 3m, n). However, dextran leakage and lectin perfusion efficiency were not altered (Extended Data Fig. 3o, p), which may be due to the already low level of CD4⁺-TLs. Two further models, T11 and T1, from a collection of p53-null tumour lines, had high and low baseline CD4⁺-TL infiltration, respectively (Extended Data Fig. 3q). CD4KO significantly increased hypoxia in T11, but did not affect the already high hypoxia level in T1 (Extended Data Fig. 3r).

To determine whether CD4⁺-TLs also regulate vessel normalization in normal tissues, we examined mammary glands and lungs of tumour-free mice. There was no difference in vascular structures between wild-type and CD4KO mice (Extended Data Fig. 4a, b). Wounded tissues trended towards lower pericyte coverage in CD4KO mice (Extended Data Fig. 4c). As tumour microenvironment may resemble wound healing, the vessel normalization effects of CD4⁺-TLs may have initially evolved to cope with wound healing.

We next investigated whether CD4⁺-TL activation is required for vessel normalization. We conditionally knocked out class II major histocompatibility complex (MHC-II) by combining mice expressing Tie2-Cre (in which Tie2 also known as Tek) promoter drives expression of Cre recombinase) and H2Abfox/foxp (M-IIKO). Because Tie2 is required for post-natal haematopoiesis, MHC-II was deleted in professional antigen-presentation cells, but not in the thymus, leaving positive selection intact (Extended Data Fig. 5a–e). Although 5–10% of pericytes and <1% of TECs also express MHC-II, they only account for 0.1% of all MHC-II⁺ cells and are unlikely to influence the immune microenvironment (Extended Data Fig. 5f, g). This conditional knockout reduced activated CD4⁺-TLs, decreased the ratio of effector memory to naive cells, but did not alter total numbers of CD4⁺-TLs (Extended Data Fig. 5h–j). Moreover, among CD4⁺-TL subsets, only interferon-γ (IFNγ)⁺ T helper 1 cells were substantially decreased (Extended Data Fig. 5k).

Tumours in M-IIKO mice phenocopied those in CD4KO or PeriDel mice with decreased pericyte coverage (Extended Data Fig. 6a), increased vascular leakiness (dextran and Evans blue assays, Extended Data Fig. 6b, c), decreased lectin perfusion (Extended Data Fig. 6d), increased hypoxia (nuclear HIF-1α and pimonidazole staining, Extended Data Fig. 6e, f), and decreased infiltration of immune cells except for neutrophils, which were increased (Extended Data Fig. 6i).

To study molecular mechanisms, we performed RNA sequencing (RNA-seq) on TECs (Extended Data Fig. 7a), the central cell type that...
integrates signals from T lymphocytes, pericytes and cancer cells in this vasculature remodelling process. Indeed, CD4\(^+\)-TLs (TREG cells) regulate multiple transcriptional alterations to TECs, including reduced immune response gene signature, lower GPAGs and higher PPAGs (Extended Data Fig. 9a, b), accompanied by other alterations, including increased dextran leakage (Extended Data Fig. 9c, d), possibly leading to increased pericyte coverage (Extended Data Fig. 8a). Thus, CD4\(^+\)-TLs regulate multiple pathways related to vessel normalization in TECs.

To determine whether activated T lymphocytes co-localize with TECs, we adoptively transferred both stimulated CD4\(^+\)-TLs expressing tdTomato red fluorescent protein (tdRFP\(^+\)) and tdRFP\(^-\) CFSE\(^-\) naive CD4\(^+\)-TLs into tumour-bearing TCRKO mice (Extended Data Fig. 8a). Compared to naive CD4\(^+\)-TLs (tdRFP\(^-\) CFSE\(^-\)), stimulated CD4\(^+\)-TLs (tdRFP\(^+\) CFSE\(^-\)) were more frequent in tumours (Extended Data Fig. 8b), and predominantly co-localized with lectin\(^+\) vessels (Extended Data Fig. 8c, d), suggesting direct cross-talk between stimulated CD4\(^+\)-TLs and TECs.

We reasoned that immune checkpoint blockade (ICB) should be immunostimulatory and thereby enhance vessel normalization. We used CD8\(^+\)\(^\#\) mice to separate CD4\(^+\)-TLs from CD8\(^+\)-TL-mediated cytotoxicity. Interestingly, even without CD8\(^+\)-TLs, combinatorial inhibition of PD1 and CTLA4, two clinically relevant ICB targets\(^+\), significantly delayed orthotopic E0771 tumour growth. In size-matched tumours, ICB led to increased pericyte coverage (Fig. 3a, b) and decreased pulmonary metastasis (Fig. 3c).

In addition to tumour size-matched experiments, we performed time-matched experiments (Extended Data Fig. 9a, b) to understand how a certain duration of ICB alters tumour microenvironment. Although the number of total immune cells appeared unaffected (Extended Data Fig. 9c), the number of CD4\(^+\)-TLs increased (Extended Data Fig. 9d), accompanied by other alterations, including increased dendritic cells and decreased neutrophils (Extended Data Fig. 9e, f). Among CD4\(^+\)-TLs, the percentage of regulatory CD4\(^+\)-TLs (T\(_{REG}\) cells) decreased, and the effector memory/naive CD4\(^+\)-TL ratio increased (Extended Data Fig. 9g, h). Intracellular cytokine staining showed that T\(_{H1}\) cells are the major population that is increased by ICB (Extended Data Fig. 9i, j), accompanied by other vessel normalization hallmarks (Fig. 3d–g), indicating T\(_{H1}\)-mediated vessel normalization.

Our data suggest a positive feedback loop between T\(_{H1}\) and vessel normalization. Disruption of vessel normalization through pericyte depletion decreased T lymphocyte infiltration (Extended Data Fig. 2). Reciprocally, CD4\(^+\)-TL knockout/inactivation reduced vessel normalization (Fig. 2, Extended Data Fig. 6), whereas activation improved vessel normalization (Fig. 3). Molecularily, T\(_{H1}\)-characteristic cytokine IFN\(_{\gamma}\) and surface molecule CD40L increased expression of the genes...
encoding endothelial adhesion molecules, ICAM1 and SELE (Extended Data Fig. 10a), which mediate immune cell infiltration\(^1\). IFN-γ also increases endothelial VEGFA, and increased CXCL9, CXCL10 and CXCL11 (Extended Data Fig. 10a, b), which encode the chemokines that recruit T\(\text{H}\)_1 cells (ref. 13) and stimulate pericyte recruitment\(^1\). To test the role of pericytes in this loop, we transferred CD45\(^+\) T\(\text{H}\)_1 cells into CD45\(^+\) mice (Extended Data Fig. 10c). This procedure improved pericyte coverage (Extended Data Fig. 10d), and increased infiltration of host CD45\(^+\) immune cells including CD4\(^+\)-T\(\text{H}\)_1 cells, macrophages and dendritic cells (Extended Data Fig. 10e, f). Neutrophils exhibited an opposite trend (Extended Data Fig. 10g), similar to previous experiments (Extended Data Figs 5l, 9e, f). Pericyte depletion attenuated these effects (Extended Data Fig. 10e, f), confirming the role of pericytes in this positive feedback loop (Extended Data Fig. 10h).

Patient-derived xenograft (PDX) models provide an opportunity to establish the causal role of T\(\text{H}\)_1 cells in vessel normalization for human tumours, because tumours were transferred from an immunocompetent (original patients) to an immunodeficient (animals) environment. We analysed gene expression profiles of PDX-original tumour pairs of multiple cancer types. GPAGs and PPAGs were not comparable owing to inter-species differences. Mouse stroma cell genes could not be captured by human microarray platforms, or were removed when aligned to human genomes in RNA-seq analyses. However, hypoxia-related signatures in human cells can reflect vessel normalization, which were indeed enriched in PDX tumours as compared to the matched or un-matched human tumours (Fig. 4a, b). We first used human MDA-MB-231 tumours for adoptive transfer of T\(\text{H}\)_1 cells. Tumours with T\(\text{H}\)_1 transfer had higher expression of IFN-γ (Fig. 4c), confirming successful infiltration. T\(\text{H}\)_1 cells induced vessel normalization, as indicated by decreased hypoxia and vessel leakiness, pruning of large/dilated vessels, and improved perfusion efficiency (Fig. 4d–g).

Molecularly, Angpt2 and other VEGF-signature genes\(^1\) decreased (Fig. 4h). We also applied this strategy to nine PDX models, seven of which responded to T\(\text{H}\)_1 cell transfer with reduced hypoxia. The two non-responders expressed VEGF signatures at a lower baseline level, and correspondingly displayed a low starting level of pimonidazole (Fig. 4i), suggesting that the lack of response is due to initially low VEGF signalling and/or hypoxia.

Finally, we investigated whether the connection between CD4\(^+\)-TLs and vessel normalization can be generalized to other cancer types. In the TCGA database, we analysed nine solid tumour types. In seven of these, estimated CD4\(^+\)-TL infiltration (CIBERSORT\(^1\)) and hypoxia were inversely correlated. In eight, CD4\(^+\)-TL infiltration and (∑GPAGs − ∑PPAGs) were positively correlated (Fig. 4j).

Although T lymphocytes have previously been linked to angiogenesis\(^1\)–\(^3\), their effect on vessel normalization has not been appreciated. Therefore, our finding adds to the growing list of T lymphocyte functions. Multiple lines of evidence show that stimulated T lymphocytes and decreasing neutrophils. At a molecular level, this mutually regulatory loop is orchestrated in part by IFN-γ and CD40L, through their effects on multiple downstream pathways. Our results argue that ICB efficacies may be achieved partially through T\(\text{H}\)_1-cell-mediated vessel normalization. A recent finding demonstrated that IFN-γ signalling is essential for therapeutic responses to ICB therapies\(^2\). Anti-angiogenesis therapies have also been extensively tested in the clinic\(^2\). The clinical outcomes of these therapies may partially depend on their effects on vessel normalization and the consequent reprogramming of immune microenvironment. It is conceivable, for instance, that improving vessel normalization will increase immune cell infiltration and enhance the efficacies of immunotherapies such as anti-PD-1 or anti-CTLA4 antibody treatments. Thus, combinatorial interventions that simultaneously perturb both processes may present promising strategies for potential synergy.

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**Supplementary Information** is available in the online version of the paper.

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**Author Contributions** L.T. and X.H.-F.Z. developed the concepts, analysed data and designed the experiments. L.T. performed the experiments. I.S.K. and T.W. sorted TECs for RNA-seq; K.S., A.G. and H.W. performed MATQ-seq; N.P. performed LC-MS/MS; L.E.D. and X.Z. prepared PDX; F.S. assisted epifluorescence imaging. T.L.P., S.A.M., A.S.M., W.K.D., C.Z. and M.T.L. developed methodology and interpreted the data. X.H.-F.Z., L.T., H.C.L. and A.G wrote the paper. X.H.-F.Z. supervised the project.

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METHODS

**Cell lines and cell culture.** E0771 cells (CH3 Biosystems) were cultured in RPMI-1640 medium (HyClone, supplemented with 10 mmol 1−1 HEPES (Gibco)), 4T1 cells (Michigan Cancer Foundation), AT3 cells (gift of S. I. Abrams at Roswell Park Cancer Institute) and MDA-MB-231 cells (ATCC) were cultured in DMEM/High Glucose medium (HyClone). All media contained 10% FBS (Gibco), 100 IU ml−1 penicillin/streptomycin (Lonza), and 25 ng ml−1 amphotericin B (Lonza).

**Animals used.** C57BL/6J (wild type, B6), B6.129S2-Cd4tm1Mak/J (CD4KO), B6, B6.129S2-Cd8a1Mak/J (CD8KO), B6, B6.129S2-Tcrα1Mak/J (TCRKO), B6, B6.Cg-Tg(Rosa26Sor)4CngI-CiTomato/Red2Rosa-tdT, B6, C57BL/6-Git(Rosa2A)265om(Tg-CAG-GFP/CFP)C1Sta/8381 (GFP/CFP), B6, B6(Cg-Tgtg(Ros-ACTdRI)-Esra*) Baki/kj (NgG2CreERT2), B6, B6.Cg-Tg(Tek-Cre)1Ewy/J (TekCcre, B6), B6.129X1-H2-Ab1tm1Kow/J (ROSA-H2-Aβ, B6), BALB/c and NOD.Cg-Pkd1tm1j IfgRm1Wol Sffj (NSG) (all from Jackson Laboratory), and athymic nude mice (from EnviGo Systems). For TH1-skewed stimulation, 10^6 cells were incubated at 37 °C with 5% CO_2 for 96 h.

**In vivo treatment.** Mice were injected with 1 mg tamoxifen (Sigma) each day for 4 consecutive days. For diphtheria-toxin-mediated cell depletion, 100 µg kg−1 of diphtheria toxin (Sigma) were injected intraperitoneally as indicated in the appropriate figure legends.

**In vitro CD4+/-TLs activation culture medium (RPMI-1640 supplemented with 0.2% heat-inactivated FBS, 100 µg ml−1 of anti-CD3: antibodies (5 µg ml−1 in PBS; eBioscience) at 4 °C. Next, 2 × 10^6 naive CD4+/-TLs cells were cultured in 100 µl per well of CD4+/- TL activation culture medium (RPMI-1640 supplemented with 5% heat-inactivated FBS, 100 IU ml−1 penicillin/streptomycin, and 25 ng ml−1 amphotericin B, 55 mmol 1−1 β-Mercaptoethanol (Sigma), 10 ng ml−1 IL-2 (R&D Systems). For Tg1−skewed stimulation, 10 µg ml−1 anti-IL-4 antibody (clone 11B11) and 20 ng ml−1 IL-12 (both from ThermoFisher) were added to the medium. The cells were incubated at 37 °C with 5% CO_2 for 96 h.

**Thymus dissociation.** Animals used. Mice were euthanized, and both thymuses overlying the heart were removed, minced into smaller pieces, and transferred to C-Tubes. Liberase TH/DNase I (RPMI-1640 medium containing 0.05% w/v of Liberase TH and 100 µl−1 of DNase I (both from Sigma)) were used for enzyme solution. The thymus samples were mechanically disrupted using the ‘m_spleen_01’ program. The reactions were halted using albumin-rich buffer (RPMI-1640 medium containing 5% bovine serum albumin (BSA) (Miltenyi)). A single-cell suspension was obtained by filtering through a 70-μm filter.

**Cell lines and cell culture.** E0771 cells were cultured from with 2 ml 1−1 EDTA (Versene, Lonza). 4T1 cells were cultured in 100 µg ml−1 penicillin/streptomycin (HyClone). The cells were washed twice with PBS (Lonza), counted, then resuspended in 1:1 solution of PBS and Matrigel (Phenol Red-Free and growth factor reduced; BD Bioscience). Mice were anaesthetized, and a small incision was made on the shaved abdomen to reveal the mammary fat pad. The incision was then closed using the ‘m_spleen_01’ program. The reactions were halted using albumin-rich buffer (RPMI-1640 medium containing 5% bovine serum albumin (BSA) (Miltenyi)). A single-cell suspension was obtained by filtering through a 70-μm filter.

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**Primary tumour resection.** Primary tumour growth was monitored every two days by measuring tumour length (L) and width (W). Tumour volume (V) was then calculated using the formula, V = π L W^2 / 6.
was used to eliminate the effect of residual haem pigment in the blood: corrected absorbance (A) = \(0.260\ \text{nm} - 0.055\ \text{nm} - (1.426 \times 0.75\ \text{nm} + 0.003)\).

For the clonogenic assay, blood was collected by whole-body perfusion into 15 ml EDTA-coated conical tubes and centrifuged at 350g for 5 min. The cell pellet was incubated with RBC lysis buffer on ice for 8 min, washed twice with PBS, then resuspended in RPMI complete medium (containing 10% FBS, 100 IU ml\(^{-1}\) penicillin/streptomycin, and 25 mg ml\(^{-1}\) amphotericin B). After incubating at 37°C with 5% CO\(_2\) overnight, the cell culture medium was discarded, the plate was washed three times with fresh cell culture medium was added. After culturing for an additional 8 days, the cell culture medium was removed and the plate was washed once with PBS. Then, the cells were fixed with 2% PFA for 20 min, stained with 1% Crystal Violet Solution (Sigma) for 1 min and washed with water. The plate was dried at 20°C, and then scanned at 1,200 dpi, using GelCount (Oxford Optronix). The number of colonies was counted manually and the colony areas were measured using ImageJ. The calibration of the circulating tumour cells based on clonogenic sizes can be found in Supplementary Table 5.

**Lung metastasis quantification.** Haematxolin and eosin (H&E) staining and India Ink assay were used to quantify lung metastasis.

For H&E staining, mice were euthanized and were injected with 5 ml 10% neutral buffered formalin into the trachea. The lungs were removed, fixed with 10% neutral buffered formalin and paraffin embedded. For each lung sample, six 3-μm paraffin-embedded sections at 300-μm intervals were used for H&E staining. Images of whole-lung sections were captured using PathScan Enabler IV (Meyer), and metastases were quantified using the texture segmentation algorithm of MATLAB v2014b.

For the India Ink assay, mice were euthanized and injected with 5 ml India Ink (15% v/v diluted in PBS; Hardy Diagnostics) into the trachea. The lungs were then removed and fixed with Fekete’s solution (for 1 h, mix 880 ml 70% ethanol, 80 ml 37% formaldehyde, and 40 ml glacial acetic acid) overnight at 4°C. The lung metastasis nodules were counted manually.

**Pimonidazole staining and blood vessel leakage analysis.** For hypoxia studies, 2 mg pimonidazole (Hypoxprobe) was injected intravenously and was left to circulate for 20 min before tumour resection. Staining of tumour sections was performed with a Hypoxprobe Plus Kit according to supplier’s protocol.

For the evaluation of vessel leakiness, mice were given 1 mg of 70-kDa fluorescent hydrazide labelled dextran (Thermo Fisher) and 50μg Dylight 649 labelled tomato Lectin (Lycopersicon esculentum; Vector Labs). After 10 min, the mouse was subjected to whole-animal perfusion as described in the ‘Evans blue assay’ section. The tumours were carefully excised and cut into two halves along the maximum diameter. Blood halves were fixed with 4% PFA overnight at 4°C, and were paraffin embedded or frozen.

**Flow cytometry.** Single-cell suspensions were prepared as described in the ‘Tumour dissociation’ section. The cells were incubated for 15 min at 4°C with anti-mouse Fc-block CD16/32 antibody (clone 93, eBioscience, 1:100), in PBS/FBS. ‘Tumour dissociation’ section. The cells were incubated for 15 min at 4°C with anti-goat (both from Thermo Fisher). Stained sections were visualized by a Carl Zeiss Axioskop2 plus microscope using 40 objectives, or scanned with a Zeiss Axioscan.Z1. The whole-section imaging was performed by an independent researcher who was blind to sample group allocation.

**Cell sorting and RNA isolation for RNA-seq.** To obtain tumour-associated CD31+ endothelial cells, E0771 breast tumours were dissociated into single-cell suspensions by a similar method as described in the ‘Tumour dissociation’ section, except that a Mouse Tumour Dissociation Kit (Miltenyi) was used instead of collagenase III, to obtain a higher yield and increased viability of CD31+ cells. Also, CD31 MicroBeads (Miltenyi) were used to enrich for tumour endothelial cells through magnetic cell separation as per manufacturer’s protocol. Dead cells and debris were excluded by FCS, SSC and 7AAD staining profiles. All single-sorting experiments were performed using an Aria Cell Sorter (BD Biosciences). The CD31+ cells were sorted directly into TRIzol LS (Thermo Fisher), and RNA was extracted using the standard protocol for TRIzol RNA Extraction. After RNA purification, MATQ-seq was performed to amplify the whole transcriptome. The input and yield of each sample was determined by qPCR after 2nd strand synthesis. To obtain similar cDNA yield and decrease PCR bias, we adjusted the PCR cycle number for each sample based on the qPCR results. Sequencing libraries were prepared from 1 ng of purified double-strand cDNA with the Illumina Nextera XT DNA Library Prep Kit per supplier’s protocol. Cluster generation was performed using the Illumina Nextseq 500/550 high output v2 kits and sequenced on the Illumina Nextseq 500 equipment.

**In vitro treatment of IFNγ and soluble CD40 ligand (sCD40L).** The HUVEC and TIME cell lines were plated at 3,000 cells cm\(^{-2}\) in culture plate containing VEGF endothelial complete medium (LifeLine) for 10h. The cells were then treated with IFNγ (1,000 U ml\(^{-1}\); R&D Systems) for 20h or sCD40L (100 ng ml\(^{-1}\); Tonbo) for 4h before RNA isolation. The expression levels of VEGFA, ICAM1, SELK, CXCL9, CXCL10 and CXCL11 were measured using quantitative PCR with reverse transcription (qRT–PCR).

**RNA isolation and qRT–PCR.** For cells cultured in vitro, RNA isolation was performed with the Quick-RNA MiniPrep Plus Kit (Zymo). Reverse transcription was performed using the High-Capacity RNA-to-cDNA Master Mix (Applied Biosystems), and qRT–PCR was performed using PerfeCTa SYBR Green FastMix (Quanta) in a 7500 Real time PCR system (Applied Biosystems).

The xenograft tumors were snap-frozen with liquid nitrogen immediately after resection, and homogenized with Precellys Lysing kits (Bertin Instruments). Next, total RNA was extracted using standard TRIzol RNA extraction protocol. The cDNA was synthesized with SuperScript III First-Strand Synthesis System (Thermo Fisher), and qRT–PCR was performed using PerfeCta SYBR Green FastMix in a CFX Connect Real-Time PCR Detection System (BioRad).

Samples were amplified by 40 cycles of 10s at 95°C and 30s at 60°C. Results were calculated by the change-in–changeover threshold (ΔΔG) method as follows (relative to the reference gene control B2M and Gapdh, encoding 3-2-microglobulin and glyceraldehyde phosphate dehydrogenase, respectively) – ΔΔG = (ΔG treatment – ΔG control), where ΔG = Ctarget – ΔCref. The sequences of primers used can be found in Supplementary Table 6.

**Immunofluorescence staining.** To prepare frozen sections, the fixed tissues were incubated in 30% sucrose in PBS (w/v) overnight, and frozen in optimum cutting temperature gel (Tissue-Tek; Sakura). Sections of 6-μm thickness were cut on Leica CM1850 cryotome.

For paraformaldehyde blocks, 3-μm thick sections were deparaffinized and treated with a heat-induced antigen retrieval Tri-Epiderm EDTA (pH 9.0) solution. To reduce the background, the sections were immersed in the 100 mmol l\(^{-1}\) NH\(_4\)Cl for 10 min and washed with running water for 5 min.

Slides were blocked in PBS with 10% normal serum (Jackson Laboratory), 1% BSA, and 0.3% Triton X-100 (Millipore) for 1 h at 20°C. Primary antibodies were incubated overnight at 4°C in the blocking solution. Secondary antibodies were added into the blocking solution and incubated for 1 h at 20°C. Nuclei were stained with 1 μg ml\(^{-1}\) DAPI (4,6-diamidino-2-phenylindole; Thermo Fisher), for 2 min at 20°C. Excess antibodies were removed by washing for 5 minutes with PBS/FBS. Tissue sections were dehydrated with 85% ethanol for 5 min, then 100% ethanol for 5 min, then xylene. Also, CD31 MicroBeads (Miltenyi) were used to enrich for tumour endothelial cells through magnetic cell separation as per manufacturer’s instructions. The slides were then washed and coverslips were mounted, using ProLong Gold anti-fade reagent (Thermo Fisher). Primary antibodies included: anti-PDGFR3 (ab32570), anti-CD31 (ab28364), anti-Hif-1α (EPR16897; all from Abcam); anti-CD31 (clone MEC13.3), anti-mouse panendothelial cell antigen (clone MECA-32), anti-VCAM1 (clone 429; all from Biogeneg); anti-CD31 (clone 1C11, 4G1), anti-CD45 (MECA-14, 3A10), anti-CD44 (IM7), anti-CD45 (30-F11), anti-CD62L (MEL14), anti-F4/80 (BM8) (all from Tonbo); anti-SiglecF (ES5-2440, BD Biosciences).

For intracellular staining of the regulatory T cells (Treg), cells and T helper cells, CD45 MicroBeads (Miltenyi) were used to enrich tumour-infiltrating immune cells. Tumour cells were detected using FoxP3/Transcription Factor Staining Buffer Set (eBioscience) as per manufacturer’s instructions. For cytotoxic intracellular staining, 0.5 × 10\(^6\) enriched tumour-infiltrating immune cells were resuspended in 0.1 ml RPMI complete medium (RPMI-1640 medium containing 10% heat inactivated FBS, 100 IU ml\(^{-1}\) penicillin/streptomycin, 55 μmol l\(^{-1}\) 3-mercaptopetanol, 1 × Protein Transport Inhibitor Cocktail (eBioscience), and were incubated at 37°C with 5% CO\(_2\) for 5h. The T helper cells (Teff1, Tef2 and Tef17) were detected using Intracellular Fixation and Permeabilization Buffer Set (eBioscience) as per manufacturer’s instructions. Labelled cells were analysed immediately or fixed in 1.5% PFA. 7AAD (eBioScience) or Ghost Dye (Tonbo) was used as viability dyes for fresh or fixed cells, respectively.

Absolute cell numbers were quantified using liquid counting beads (BD Biosciences). Data were acquired using a FACs CantoII (BD Biosciences) and analysed with either Flowjo v10.0 or FACs Diva v8.0 software.

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For pericyte coverage quantification, vessels were counted manually in five independent ~0.4 mm² fields at ×20, and percentage of vessels covered by pericytes = pixels of CD31⁺ cells attached by NG2⁺ cells / total pixels of CD31⁺ cells.

For analysis of leakiness in addition to Evans blue assay, the diffused dextran area per section was quantified using CellProfiler.

Vessel area and density were quantified by analysis of more than 1,000 vessels from each mouse. The vessel density and colocalization were analysed automatically using CellProfiler.

The computational modelling of T H 1-skewed CD4⁺-TLs localization was performed with R programming language. To generate a simulated placement of CD4⁺-TLs for null hypothesis, two random anchor points were placed on the edge of the tumour section, and a random point located between the two anchor points was chosen. The number of simulated points was equal to the number of actual T H 1-skewed CD4⁺-TLs in the section in each round of simulation. The mean Euclidean distances between simulated points to the nearest lectin⁺ vascular structure was measured, and mean distances generated from 10,000 rounds of simulation defined a distribution of mean distances one would observe for non-preferentially localized CD4⁺-TLs in relation to lectin⁺ tumour-associated vessels. This was then compared to the mean distance distribution between actual CD4⁺-TLs and lectin⁺ vessels on each tumour section.

Bioinformatic analysis. The RNA-seq reads were mapped and quantified using the STAR RNA-seq aligner (v2.4.1d)30 and RSEM software package (v1.2.28)29, respectively. Mouse genome and gene annotation (GRCm38.83) was supplied at the genome index generation step. ‘DEseq2’ R package30 was used to normalize the STAR RNA-seq aligner (v2.4.1d) 28, and RSEM software package (v1.2.28) 29, vessels. This was then compared to the mean distance distribution between actual CD4⁺-TLs and lectin⁺ vessels on each tumour section.

Gene set enrichment analysis (GSEA)13 and KEGG Orthology-Based Annotation System (KOBAS) v2.0 (ref. 32; http://kobas.cbi.pku.edu.cn/) were used for the genome index generation step. ‘DEseq2’ R package30 was used to normalize the gene expression matrix.

Gene set enrichment analysis (GSEA)13 and KEGG Orthology-Based Annotation System (KOBAS) v2.0 (ref. 32; http://kobas.cbi.pku.edu.cn/) were used to perform the functional enrichment analysis as indicated in the appropriate figure legend and Methods text. The enriched gene ontology terms were visualized using REVIGO31 (http://revigo.irb.hr). The single-sample GSEA projection (ssGSEA)32 was used to calculate separate enrichment scores for each pairing of a sample and a gene set in Molecular Signatures Database (MSigDB). ssGSEA (v7) and GSEA (v15.2) analyses were performed using GenePattern (http://genepattern.broadinstitute.org/). GSEA was performed using 10,000 iterations collapsing probes to the highest value if necessary.

For paired analysis between PDXs and the original patient tumours, a pimonidazole-based hypoxia signature was obtained from a 32-gene set that correlates with higher pimonidazole staining in human tumours33. Bladder cancer data were obtained from GSE67312. Breast cancer data were combined from GSE32531 and GSE14685, and only orthotopic xenograft samples were included. Liver cancer data were obtained from GSE55828. Ovarian cancer data were obtained from GSE56920. For unpaired analysis of breast cancer PDXs, data from GSE44412 were used. In addition to the pimonidazole signature, two other hypoxia-related signatures were used: a 26-gene hypoxia signature that predicts benefit from hypoxia-modifying therapy34, and a 13-gene VEGF signature associated with metastasis15. In analysing the correlation between CD4⁺-TL number with hypoxia signature and (Σ GPAGs – Σ PPAGs) in different solid tumour types, the percentages of CD4⁺-TLs were estimated by CIBERSORT of the TCGA RNA-seq dataset. TCGA RNA-seq data were downloaded from UCSC Cancer Genome Browser (https://genome-cancer.ucsc.edu/), and used as input for CIBERSORT to estimate the relative abundance of CD4⁺-TLs (naïve CD4⁺ T cells, memory resting CD4⁺ T cells and memory activated CD4⁺ T cells) among 22 leukocyte compositions. We used 1,000 permutation and disabled quantile normalization.

Statistical methods. Sample sizes are denoted in figures or figure legends, and refer to number of animals unless otherwise noted. Data are generally expressed as mean ± s.e.m. In box-whisker plots, the upper and lower hinges correspond to the first and third quartiles, and the upper and lower whiskers are highest and lowest values that are within 1.5 × IQR (interquartile range) of the hinge.

In most in vitro experiments, group sizes were determined based on the results of preliminary experiments and no statistical method was used to predetermine sample size. Experiments were repeated twice independently, and the data are combined and presented. Extended Data Fig. 3i is the only exception: one representative experiment from two is shown. For the dot plots of immunostaining results, smaller dots without an outline are values from individual fields (~0.4 mm² fields at ×20), and circles that are outlined represent mean values taken over multiple fields from the same mouse, unless otherwise noted. The statistical tests were performed by comparing the individual animals. All in vitro experiments were repeated independently for three times (batches). The P values in these in vivo experiments were determined on the basis of biological replicates (with technical replicates averaged within each biological replicate).

All samples that met proper experimental conditions were included in the analysis. Data were analysed with Prism 7 software (GraphPad) or R programming language (v3.2.0). Statistical significance was determined using a two-tailed Mann–Whitney U-test or Student’s t-test. F-test was conducted before Student’s t-test to compare the variance of two samples. Welch’s correction is applied to Student’s t-test if the null hypothesis of equality of variances is rejected. Comparison of tumour-growth curves were assessed by two-way analysis of variance (ANOVA). Survival analyses were evaluated by Kaplan–Meier curves and the log-rank (Mantel-Cox) test. P values lower than 0.05 were considered statistically significant.

Code availability. The source codes and data of bioinformatics analysis were deposited to https://github.com/lintian0616/vesselNormalization. All other Source Data are provided with the online version of the paper.

Data availability. The RNA-seq data have been submitted to the Gene Expression Omnibus under accession number GSE89758.
Extended Data Figure 1 | Evaluation of GPAGs and PPAGs in hepatocellular carcinoma links T-cell activity with tumour vessel normalization. a, Schematic diagram for the bioinformatic analysis. MACS, magnetic-activated cell sorting; NEC, normal endothelial cells; TEC, tumour-associated endothelial cells. The numbers of patients are denoted in parentheses.

b, GPAG and PPAG signatures of NECs versus TECs. 

c, Comparison of GPAG and PPAG signatures in tumour with vascular invasion (n = 40 patients) or without vascular invasion (n = 95 patients).

d, Pathways in the non-TEC cells that positively correlate with higher ($\sum$GPAGs − $\sum$PPAGs) in the paired TECs. FDR, false discovery rate; NES, normalized enrichment score. e, GSEA mountain plot showing a strong association between ($\sum$GPAGs − $\sum$PPAGs) in the TEC and T-cell activation signalling in the paired non-TEC cells. Data are presented as means ± s.e.m. for dot plots. Data are obtained from GSE51401 (b, d, e), GSE20017 (c). P values were calculated using two-tailed paired Student's t-test (b), two tailed unpaired Mann–Whitney U-test (c), or a permutation-based approach with Benjamini–Hochberg multiple testing correction (d, e).
Extended Data Figure 2 | NG2<sup>+</sup> cell-depleted mice display decreased immune infiltration in E0771 tumours. a, Schematic of the experimental design. b, Quantification of tumour-infiltrating NG2<sup>+</sup> cells (NG2-CreERT<sup>M</sup>;tdRed, n = 3; NG2-CreERT<sup>M</sup>; tdRed;iDTR, n = 4). c, Flow cytometry gating strategy for tumour-infiltrating leukocytes. d, Flow cytometric quantification showing the decreased infiltration of TLs (CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup>), B cells (B220<sup>+</sup>), and dendritic cells (CD11b<sup>+</sup>CD11c<sup>+</sup>), but the percentage of CD11b<sup>+</sup>CD11c<sup>-</sup> cells remains unchanged (WT, n = 10; PeriDel, n = 5). Data are presented as means ± s.e.m. P values are determined by two-tailed paired Student’s t-test (b, d).
Extended Data Figure 3 | The effect of CD4⁺-TLs on promoting vessel normalization is dependent on the number of cells. a, Flow cytometric plots of CD3⁺ cells of 4T1 tumour from wild-type and CD4KO mice. Five animals were examined in each group. Representative plots are shown. b–g, Quantification of tumour vascular normalization markers including pericyte coverage (b), vessel density and vessel length (c), VE-cadherin expression (white arrow heads show vessels without VE-cadherin expression) (d), hypoxia measured by pimonidazole staining (e), lectin perfusion efficiency (f), and dextran leakage (g) (n = 5 per group; scale bars, 50µm (b, d, f, g), 1 mm (e)). h, Top, schematic of the experimental design. Bottom, the two doses of antibody deplete CD4⁺-TLs for 2 weeks. One point represents one mouse. Whole blood was collected for the flow cytometric analysis. i, Dot plots showing that tumours were resected at similar size/weight. j, Representative whole-animal bioluminescence images showing spontaneous 4T1 metastasis in anti-IgG or anti-CD4 treated mice. Dots representing mice with detected metastases are labelled with a red boundary. k, Kaplan–Meier curves showing the metastasis-free frequency of 4T1 tumour-bearing mice treated with anti-IgG or anti-CD4. n = 10 and 9 for anti-IgG and anti-CD4 groups, respectively (i–k). l, Top, flow cytometry quantification of tumour-infiltrating CD4⁺-TLs across three murine tumour models (4T1, n = 4; E0771, n = 5; AT3, n = 4). The tumours were resected at similar size/weight (around 1 g) from the same batch of experiments. Bottom, a table summarizing the results from vessel normalization assays. The number indicates the fold change increased (red) or decreased (green) in wild-type mice compared to CD4KO mice. In E0771 model, RNA-seq reveals an increase in expression of genes encoding extracellular matrix and adhesion molecules in wild-type mice over CD4KO mice. m–p, Quantification of tumour vascular normalization markers (n = 5 per group; scale bars, 50 µm (m, o, p); n = 10 per group; scale bars, 1 mm (n)). q, Scatter plot showing the Cd4 and Ilng gene expression levels of different p53⁻/⁻ mouse breast tumour models. The Cd8 gene expression and ER status are denoted by the colours of the dots and dot outlines, respectively. Two models chosen for hypoxia measurement are highlighted. r, Hypoxia quantification for T1 and T11 tumours in wild-type and CD4KO background as measured by pimonidazole staining (T1: n = 5 per group; T11: WT, n = 5; CD4KO, n = 8; scale bars, 1 mm). Data are presented as means ± s.e.m. Animal numbers used in i–k are denoted in (k). P values are determined by two-tailed unpaired Student’s t-test (b–g, i, m–p, r), Fisher’s exact test (j) and log-rank test (k). NS, not significant.
Extended Data Figure 4 | Comparison of pericyte coverage of normal tissues and wound tissues in different T-cell-deficient backgrounds.

a, Quantification of pericyte coverage of blood vessels in the mammary gland. As NG2 is also expressed by adipocytes, PDGFRβ was used as the marker for pericyte (n = 4 per group; scale bars, 50 μm; inset, 20 μm).

b, Representative fluorescent images and flow cytometric quantification of pericyte coverage of lung (n = 5 per group; scale bars, 50 μm).

c, Quantification of pericyte coverage of normal skin tissues and skin wound tissues (WT, n = 10; CD4KO, n = 10; CD8KO, n = 9; scale bars, 50 μm). Different immunodeficient backgrounds are denoted by the colours of the dots, and the strain information is denoted by the colours of point outlines. The points with arrows are the represented images of wound tissues in the left. Data are presented as means ± s.e.m. P values were calculated using non-parametric one-way ANOVA (Kruskal–Wallis) test (b, c).
Extended Data Figure 5 | Immune profiling on M-IIKO mice shows that CD4+ T cell activation is inhibited. a, b, Flow cytometry quantifications validate that M-IIKO mice have decreased MHC-II expression in tumour-infiltrating immune cells (CD45+), including macrophages (MΦ, CD45+CD11b−Ly6G−F4/80+), dendritic cells (DC, CD45+CD11b−Ly6G−F4/80+CD11c+), and B cells (CD45+B220+) (control, n = 10; M-IIKO, n = 11). c, Flow cytometry gating of suspension cells dissociated from thymus, characterized as CD45+EpCAM− immune cells and CD45−EpCAM+ epithelial cells. d, e, Quantification of MHC-II expression of thymus showing that MHC-II expression is inhibited in immune cells but preserved in epithelial cells in M-IIKO mice (control, n = 7; M-IIKO, n = 6). f, Quantification of different types of tumour-infiltrating stroma cells (n = 10 per group). g, Quantification of MHC-II expression in different cell types (control, n = 10; Tie2Cre;H2AbfloxP/floxP, n = 5; M-IIKO, n = 11). h, Quantification of T cells in spleens from 5–6-week-old female mice showing that the number of T cells is independent of MHC-II expression on Tie2Cre+ cells (control, n = 7; M-IIKO, n = 6). i, Quantification of activated CD4+ T cells and effector CD4+ T cells from tumours of similar sizes. Activated CD4+ T cells (CD4+CD3+CD44+FoxP3−; TREG: CD4+CD3+CD4+CD25+FoxP3+; effector memory cell: CD44+CD62L−; naive CD4+ T cells: CD44−CD62L+) (n = 11 per group). j, The percentages of CD4+ T cell activation markers in spleen showing a similar pattern as in tumour (i) (control: n = 7; M-IIKO, n = 6). k, Quantification of different E0771 tumour-infiltrating T helper cells (IFNγ+ TH1, IL4+ TH2 and IL17A+ TH17) (n = 11 per group). l, Quantification of E0771 tumour-infiltrating CD4+ T cells, macrophages, dendritic cells, B cells and neutrophils (CD45+CD11b+Ly6Ghi) (n = 11 per group). Data are presented as means ± s.e.m. The genetic backgrounds of mice are denoted with different colours shown on the right of l. Wild type, Tie2Cre and H2AbfloxP/floxP were combined as a control group. P values were calculated using two-tailed unpaired Student’s t-test (a, b, d–f, h–k) or two-tailed unpaired Mann–Whitney U-test (i). NS, not significant.
Extended Data Figure 6 | Inhibition of MHC-II-mediated CD4+ TL activation phenocopies the depletion of CD4+ TL or NG2+ pericytes with regard to tumour vessel normalization and hypoxia.

a, Immunofluorescence quantification of percentage endothelial cells (CD31+/MECA-32+) attached by pericytes (NG2+) (n = 4 per group), and flow cytometry quantification of endothelial cell to pericytes ratio (control, n = 10; M-IIKO, n = 11). b, c, Quantification of tumour-vasculature leakiness as measured by dextran (WT, n = 5; M-IIKO, n = 4; CD4KO, n = 5; scale bars, 50 μm) and Evans blue (WT, n = 11; M-IIKO, n = 8), respectively. d, Quantification of perfusion efficiency with lectin (WT, n = 5; M-IIKO, n = 4; CD4KO, n = 5; scale bars, 50 μm). e, f, Quantification of tumour hypoxia with HIF-1α (WT, n = 5; M-IIKO, n = 4; CD4KO, n = 4; PeriDel, n = 3; scale bars, 50 μm) and pimonidazole (WT, n = 5; M-IIKO, n = 4; CD4KO, n = 5; PeriDel, n = 8; scale bars, 1 mm).

Data are presented as means ± s.e.m. P values were calculated using two-tailed unpaired Student’s t-test (a–f).
Extended Data Figure 7 | RNA-seq further supports that CD4+ -TLs promote tumour vessel normalization. a, RNA-seq experiment design. FACS, fluorescence-activated cell sorting; MATQ-seq, multiple annealing and tailing-based quantitative sequencing. b, t-distributed stochastic neighbour embedding (t-SNE) analysis of tumour-associated endothelial cells based on RNA-seq profiles of different transgenic mice. Different genetic backgrounds are denoted with different colours. c, ssGSEA projection of RNA-seq data validated the downregulation of ‘Immune Effector Process’ pathway (GO:0002697) in CD4+ -TL-deficient group. d, Analyses on RNA-seq data validated the downregulation of GPAGs and upregulation of PPAGs in CD4+ -TL-deficient group. e, f, Gene expression analysis of Vegfa and Angpt1/Angpt2 in tumour-associated CD31+ cells from different genetic backgrounds of mice. g, GSEA mountain plots showing increased biological activities in the tumour-associated vessel isolated from CD4+ -TL-competent backgrounds. h, Heat map summarizing the top 20 genes upregulated in tumour-associated CD31+ cells isolated from CD4+ -TL-competent genetic background, compared to that from CD4+ -TL-deficient background. i, Analysis of sphingolipid metabolic process signature (GO:0006665) for tumour-associated CD31+ cells from different genetic background. j, Sphingolipid metabolite profiling of sphingolipid associated metabolites on whole E0771 tumour lysates from mice of different T-cell-deficient backgrounds (WT, n = 5; CD4KO, n = 5; CD8KO, n = 6; TCRKO, n = 5). AC, acid ceramidase; ASMase, acid sphingomyelinase; FA, fatty acid; P-choline, phosphatidylcholine; SPHK, sphingosine kinase. All genotypes are divided into two groups based on CD4 status. CD4KO, TCRKO and conditional knockout of H2Ab are deficient of CD4+ -TL, and the others are not. The two groups have n = 9 and 10 animals, respectively. Data are presented as means ± s.e.m. Animal numbers used (b–i) are denoted in (b). P values were calculated using two-tailed unpaired Mann–Whitney U-test (c–f, i), two-tailed one-way analysis of variance (ANOVA) (j) or permutation (g). NS, not significant.
Extended Data Figure 8 | Spatial relationships between activated CD4⁺-TLs and lectin⁺ tumour-associated endothelial cells. a, Schematic of the experimental design. b, A table showing the counts of naive CD4⁺-TLs (tdRed⁺ CFSE⁻) and activated CD4⁺-TLs (tdRed⁺) in whole cross sectional area of five animals (n = 5). c, The violin plots showing the kernel probability density of the distances of naive and activated CD4⁺-TLs to the nearest lectin⁺ endothelial cells. Smaller dots without an outline are distances of individual CD4⁺-TL, and larger circles that are outlined represent mean distances taken over all CD4⁺-TLs in the section from the same mouse. CD4⁺-TLs from the same mouse are denoted with the same colour. The P value was calculated using one sample Student’s t-test by comparing the mean distances of activated CD4⁺-TLs from individual mouse with the mean distance of all naive CD4⁺-TLs (dashed horizontal line) (n = 5 mice). d, Top, mosaic scanning images of whole tumour sections. Representative areas are magnified and naive CD4⁺-TLs (yellow) are pinpointed with arrowhead. Bottom, solid lines show the distribution of distances between CD4⁺-TLs and lectin⁺ endothelial cells in whole tumour sections. The mean distances observed are shown as a vertical straight line. For comparison, dashed lines show the probability distribution of mean distances between endothelial cells and computer-simulated random dots. P values were calculated using a permutation-based approach. More detailed information about image simulation is described in the Methods.
Extended Data Figure 9 | ICB therapy promotes T<sub>H1</sub> differentiation of CD4<sup>+</sup>-T Ts and induces further immune reprogramming. a, Schematic of the experimental design. b, ICB leads to CD4<sup>+</sup>-T Ts dependent tumour growth inhibition, measured by tumour weight at Day 15 after E0771 injection. c, d, Total number of immune cells (c) and T cells (d) in tumours from different groups. Although the number of pan tumour-infiltrating immune cells (CD45<sup>+</sup>) is not changed, the number of CD4<sup>+</sup>-T Ts increased after immune checkpoint blockade therapy. The number of different immune cells (rows) is shown for each tumour (columns) after control or checkpoint blockade treatment. The weight of each tumour is shown (top panel). Row-side annotations show P values comparing between CD8<sup>KO</sup> (anti-IgG) and CD8<sup>KO</sup> (anti-PD1 and anti-CTLA4) groups (far left column), and between CD8<sup>KO</sup> (anti-PD1 and anti-CTLA4) and TCR<sup>KO</sup> (anti-PD1 and anti-CTLA4) (far right column) (EM T, effector memory T cells). e, A heat map summarizing changes to tumour-infiltrating immune components after ICB therapy. f, Quantification of different subsets among CD45<sup>+</sup>CD11b<sup>+</sup> cells showing the effect of ICB on innate immune microenvironment (eosinophil: CD45<sup>+</sup>CD11b<sup>+</sup>SiglecF<sup>+</sup>). g, h, Quantification of the percentage of T<sub>REG</sub> cells among total CD4<sup>+</sup>-T Ts, and the ratio of effector memory CD4<sup>+</sup>-T Ts to naive CD4<sup>+</sup>-T Ts after ICB in CD8 knockout mice. i, Quantification of the percentage of different CD4<sup>+</sup> T helper cells. j, Percentage of IFN<sub>γ</sub><sup>+</sup> cells in CD4<sup>+</sup> or CD4<sup>+</sup>- cells among all the CD45<sup>+</sup> tumour-associated immune cells, indicating CD4<sup>+</sup>-T Ts make up the majority of IFN<sub>γ</sub><sup>+</sup> cells. Data are presented as means ± s.e.m. Animal numbers used in (b–j) are denoted in a. P values were calculated using two-tailed unpaired (b–i) or paired (j) Student's t-test. NS, not significant.
Extended Data Figure 10 | Molecular and cellular mechanisms that contribute to tumour immunostimulatory reprogramming positive feedback loop. a, b, Quantitative RT–PCR analysis showing the effect of IFNγ and sCD40L on the mRNA levels of adhesion molecules, VEGFA (a), and T cell attractant chemokines (b). The experiments were repeated independently for three times (batches) with technical duplicates each time. c, Schematic of the experimental design and hypothetical model. d, Tumours resected on days 12–13 after injection of E0771 have similar size/weight, and the effect of T H1 adoptive transfer on vessel normalization as measured by the CD31+ endothelial cells to NG2+ pericytes ratio. e, Flow cytometry quantification CD45.1+ adoptive transferred T H1 cells, and CD45.2+ host immune cells. f, Characterization and quantification of CD45.2+ host immune cells showing that T H1-mediated immune infiltration is partially dependent on pericyte coverage. g, Effect of T H1 adoptive transfer and pericyte depletion on CD11b+Ly6G+ immune cells demonstrating different pattern with other tumour-infiltrating immune cells as from f. h, Schematic summary of CD4+ T L-mediated vessel normalization, and subsequent formation of positive feedback loop through cell–cell interaction, cytokine production and increased pericyte coverage. Checkpoint blockade therapy and antigen presentation enhance T H1-skewed CD4+ T L activation and promote the vessel normalization/ immunostimulatory reprogramming positive feedback loop. Data are presented as means ± s.e.m. Animal numbers used in (d–g) are denoted in (c). P values were calculated using two-tailed unpaired Student’s t-test based on biological replicates (a, b, d–g). Technical replicates are averaged within each biological replicate (a, b).