Science Advances
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Supplementary Materials for

Breast tumors interfere with endothelial TRAIL at the premetastatic niche to promote cancer cell seeding

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This PDF file includes:

Figs. S1 to S11

Fig. S1

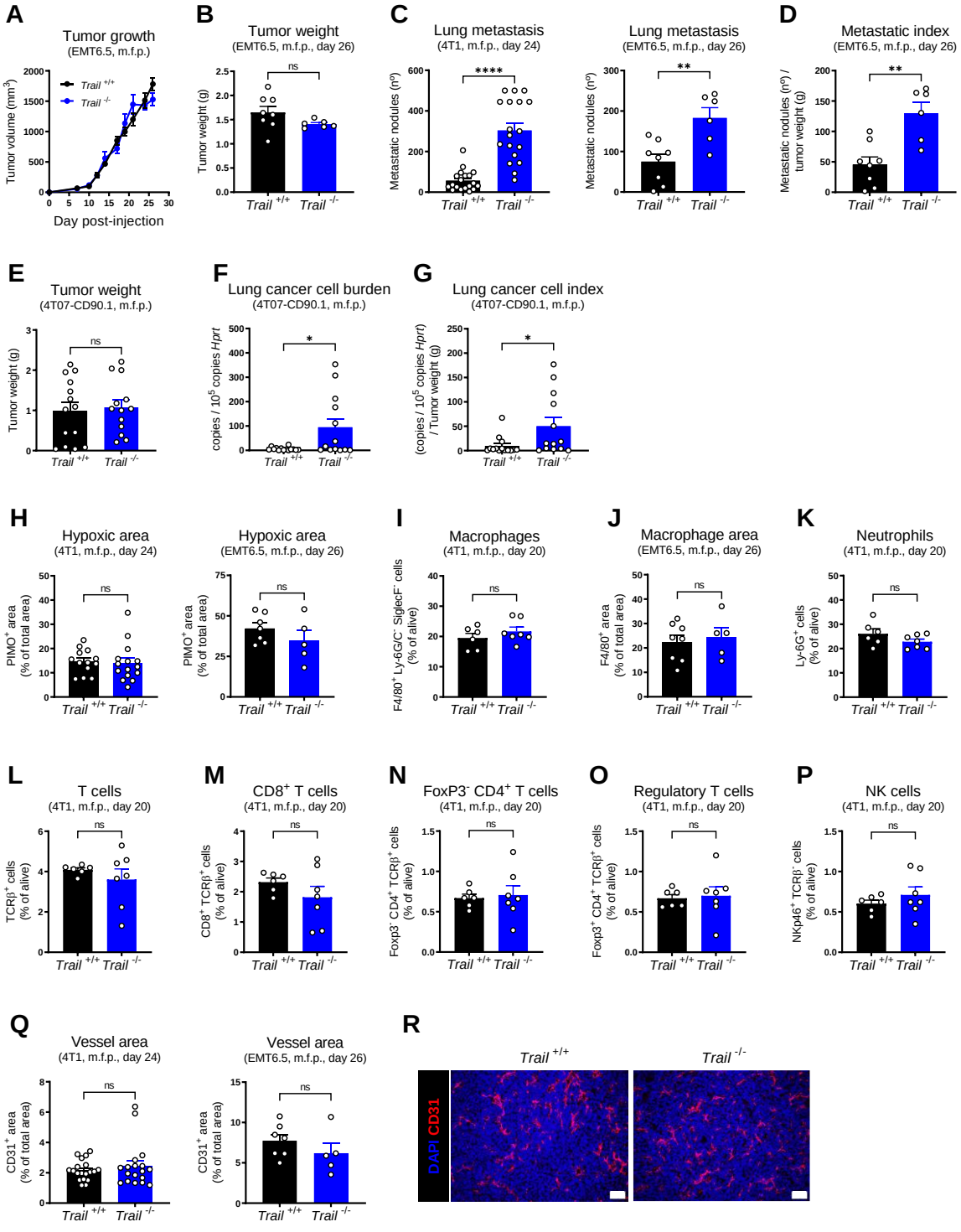
A
Tumor growth
(EMT6.5, m.f.p.)
Tumor volume (mm3)
Trail +/+
Trail -/-
Day post-injection
B
Tumor weight
(EMT6.5, m.f.p., day 26)
Tumor weight (g)
ns
C
Lung metastasis
(4T1, m.f.p., day 24)
Metastatic nodules (n°)
****
Lung metastasis
(EMT6.5, m.f.p., day 26)
**
D
Metastatic index
(EMT6.5, m.f.p., day 26)
Metastatic nodules (n°) / tumor weight (g)
**
E
Tumor weight
(4T07-CD90.1, m.f.p.)
F
Lung cancer cell burden
(4T07-CD90.1, m.f.p.)
copies / 10^5 copies Hprt
*
G
Lung cancer cell index
(4T07-CD90.1, m.f.p.)
(copies / 10^5 copies Hprt) / Tumor weight (g)
H
Hypoxic area
(4T1, m.f.p., day 24)
PIMO+ area (% of total area)
Hypoxic area
(EMT6.5, m.f.p., day 26)
I
Macrophages
(4T1, m.f.p., day 20)
F4/80+ Ly-6G/C- SiglecF- cells (% of alive)
J
Macrophage area
(EMT6.5, m.f.p., day 26)
F4/80+ area (% of total area)
K
Neutrophils
(4T1, m.f.p., day 20)
Ly-6G+ cells (% of alive)
L
T cells
(4T1, m.f.p., day 20)
TCRβ+ cells (% of alive)
M
CD8+ T cells
(4T1, m.f.p., day 20)
CD8+ TCRβ+ cells (% of alive)
N
FoxP3- CD4+ T cells
(4T1, m.f.p., day 20)
Foxp3- CD4+ TCRβ+ cells (% of alive)
O
Regulatory T cells
(4T1, m.f.p., day 20)
Foxp3+ CD4+ TCRβ+ cells (% of alive)
P
NK cells
(4T1, m.f.p., day 20)
NKp46+ TCRβ- cells (% of alive)
Q
Vessel area
(4T1, m.f.p., day 24)
CD31+ area (% of total area)
Vessel area
(EMT6.5, m.f.p., day 26)
R
Trail +/+
Trail -/-
DAPI CD31

Fig. S1. Related to **Endogenous TRAIL restrains cancer cell colonization and metastasis independently of DR5 expression in cancer cells**

(**A-D**), Tumor growth (**A**), tumor weight (**B**), number of ink-contrasted lungs (**C**) and metastatic index (**D**) 24 days (4T1) or 26 days (EMT6.5) after orthotopic m.f.p. injection of 4T1 or EMT6.5 cells in constitutive TRAIL WT (*Trail*$^{+/+}$) and KO (*Trail* $^{-/-}$) mice.

(**E**), Tumor weight after orthotopic m.f.p. injection of 4T07-CD90.1 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**F**), mRNA expression of *dLNGFR* in perfused whole lung tissue after orthotopic m.f.p. injection of 4T07-CD90.1 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**G**), mRNA expression of *dLNGFR* normalized by tumor weight in perfused whole lung tissue after orthotopic m.f.p. injection of 4T07-CD90.1 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**H**), Histological analysis of PIMO^{+} hypoxic area 24 days (4T1) or 26 days (EMT6.5) after orthotopic m.f.p. injection of 4T1 or EMT6.5 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**I**), FACS analysis of the percentage of tumor-infiltrating macrophages 20 days after orthotopic m.f.p. injection of 4T1 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**J**), Histological analysis of F4/80^{+} macrophage area 26 days after orthotopic m.f.p. injection of EMT6.5 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**K-P**), FACS analysis of the percentage of tumor-infiltrating neutrophils (**K**), total T cells (**L**), CD8^{+} T cells (**M**), Foxp3^{-} CD4^{+} T cells (**N**) Foxp3^{+} CD4^{+} regulatory T cells (**O**) and NK cells (**P**) 20 days after orthotopic m.f.p. injection of 4T1 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice.

(**Q,R**), Histological analysis of CD31^{+} tumor vessel area 24 days (4T1) or 26 days (EMT6.5) after orthotopic m.f.p. injection of 4T1 or EMT6.5 cells in *Trail*$^{+/+}$ and *Trail* $^{-/-}$ mice. Scale bar = 50μm. Abbreviations: mammary fat pad (m.f.p.). All graphs show mean±SEM. ns=not significant; **p<0,01; ****p<0,0001.

Fig. S2

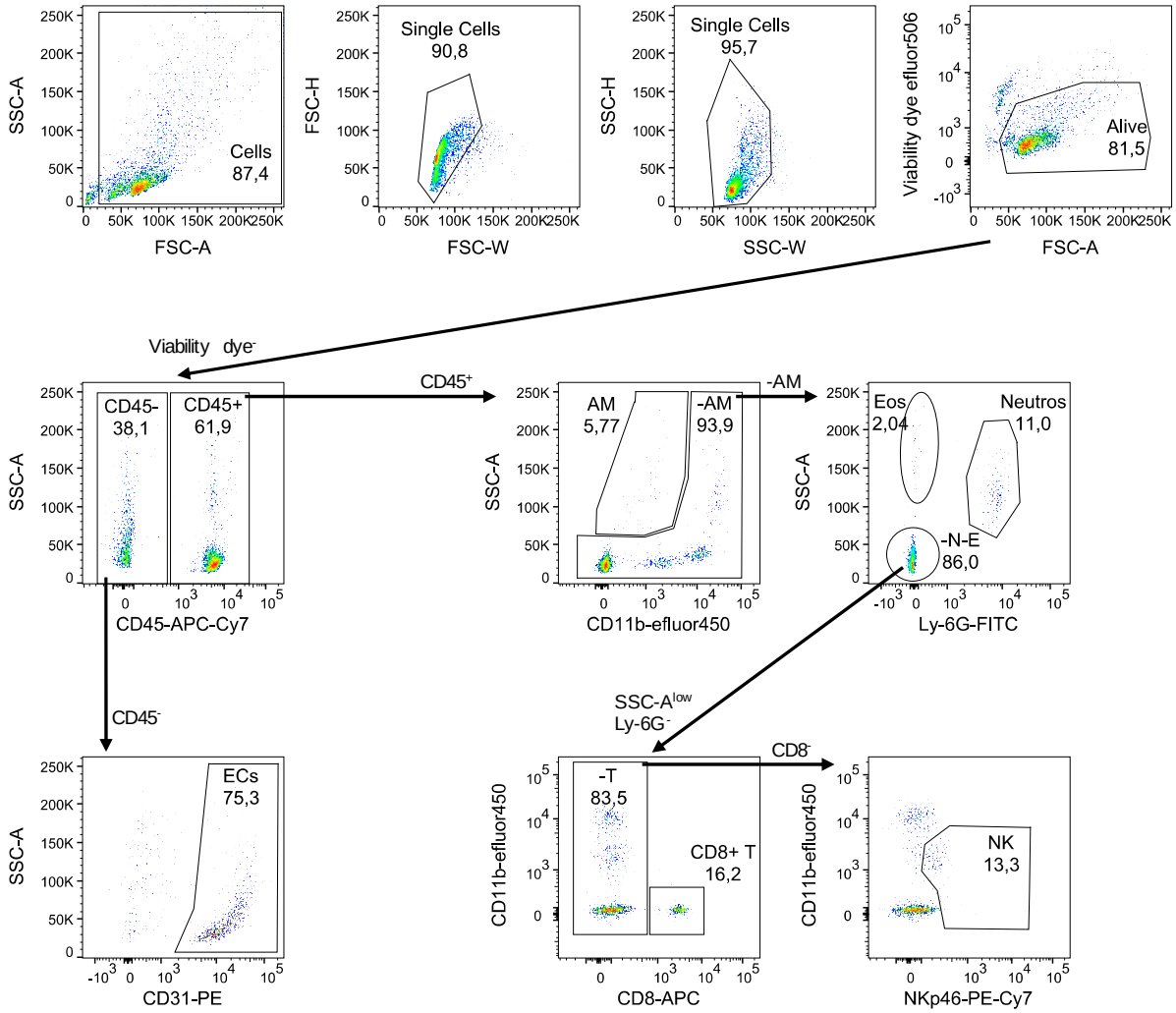

Fig. S2. Gating strategy for lung cell populations

Fig. S3

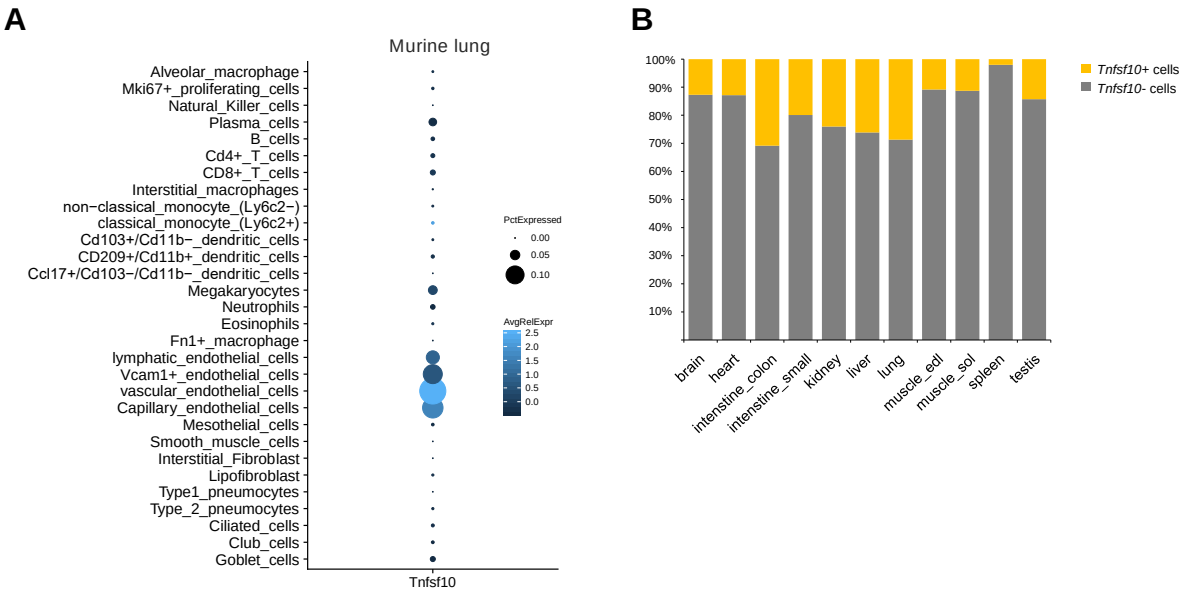

Fig. S3. Related to **ECs in the pre-metastatic lung (and liver) are the main source of TRAIL** (**A**), Dotplot showing the percentage of cells expressing *Tnfsf10* using dot size and the average expression level of *Tnfsf10* based on unique molecular identifier (UMI) counts. Rows represent hierarchically clustered cell types, demonstrating similarities of transcriptional profiles.
(**B**) Percentage of organ-specific cells expressing *Tnfsf10*.

Fig. S4

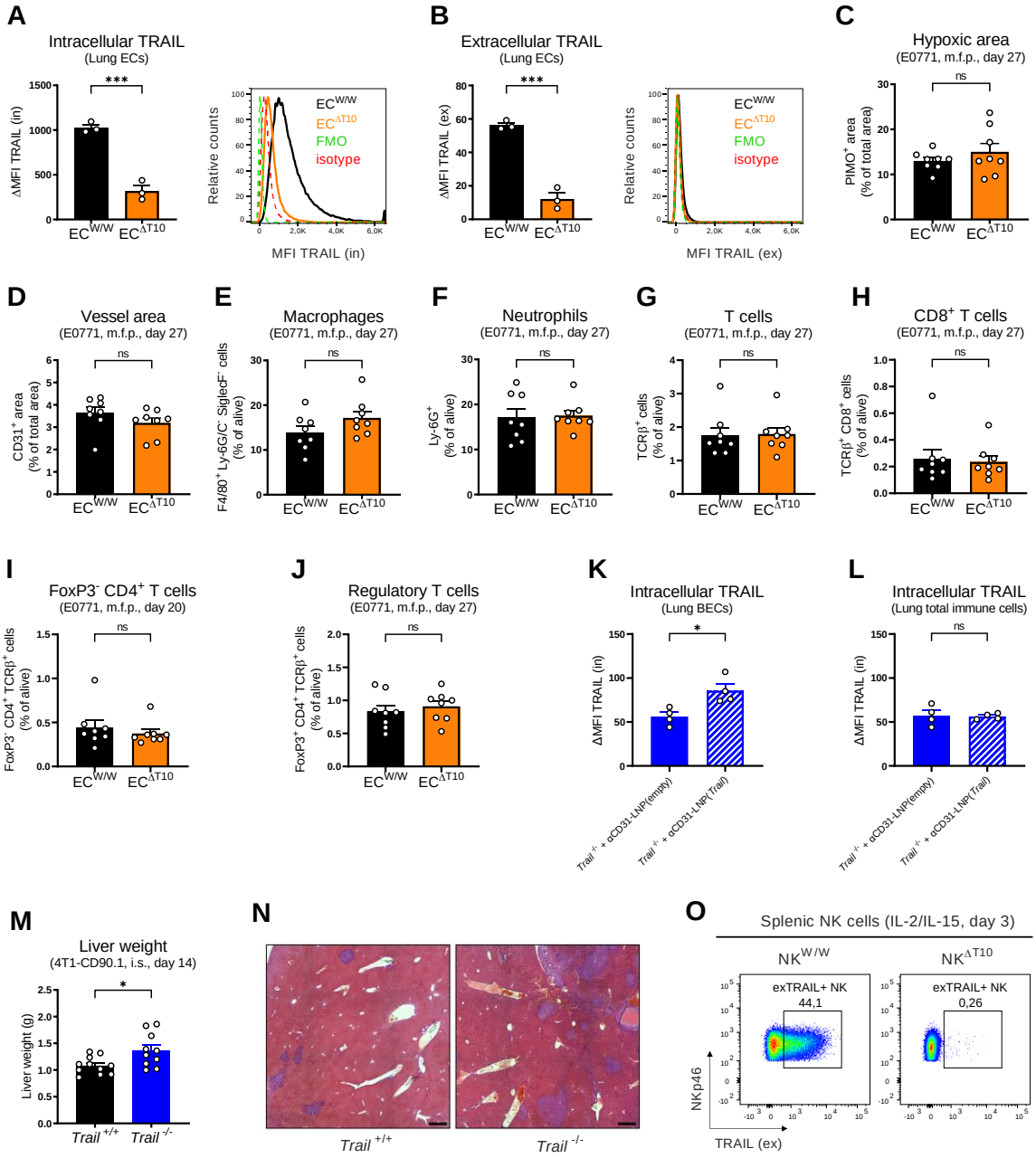
A
Intracellular TRAIL
(Lung ECs)
ΔMFI TRAIL (in)
***
EC^W/W
EC^ΔT10
Relative counts
EC^W/W
EC^ΔT10
FMO
isotype
MFI TRAIL (in)
B
Extracellular TRAIL
(Lung ECs)
ΔMFI TRAIL (ex)
***
EC^W/W
EC^ΔT10
Relative counts
EC^W/W
EC^ΔT10
FMO
isotype
MFI TRAIL (ex)
C
Hypoxic area
(E0771, m.f.p., day 27)
PIMO+ area
(% of total area)
ns
EC^W/W
EC^ΔT10
D
Vessel area
(E0771, m.f.p., day 27)
CD31+ area
(% of total area)
ns
E
Macrophages
(E0771, m.f.p., day 27)
F4/80+ Ly-6G/C- SiglecF- cells
(% of alive)
ns
F
Neutrophils
(E0771, m.f.p., day 27)
Ly-6G+
(% of alive)
ns
G
T cells
(E0771, m.f.p., day 27)
TCRβ+ cells
(% of alive)
ns
H
CD8+ T cells
(E0771, m.f.p., day 27)
TCRβ+ CD8+ cells
(% of alive)
ns
I
FoxP3- CD4+ T cells
(E0771, m.f.p., day 20)
FoxP3- CD4+ TCRβ+ cells
(% of alive)
ns
J
Regulatory T cells
(E0771, m.f.p., day 27)
FoxP3+ CD4+ TCRβ+ cells
(% of alive)
ns
K
Intracellular TRAIL
(Lung BECs)
ΔMFI TRAIL (in)
*
Trail-/- + αCD31-LNP(empty)
Trail-/- + αCD31-LNP(Trail)
L
Intracellular TRAIL
(Lung total immune cells)
ΔMFI TRAIL (in)
ns
Trail-/- + αCD31-LNP(empty)
Trail-/- + αCD31-LNP(Trail)
M
Liver weight
(4T1-CD90.1, i.s., day 14)
Liver weight (g)
*
Trail +/+
Trail -/-
N
Trail +/+
Trail -/-
O
Splenic NK cells (IL-2/IL-15, day 3)
NK^W/W
NK^ΔT10
exTRAIL+ NK
44,1
exTRAIL+ NK
0,26
NKp46
TRAIL (ex)

Fig. S4. Related to **EC-derived TRAIL at the pre-metastatic niche restrains early metastatic colonization**

(**A,B**), FACS analysis of intracellular (**A**) or extracellular (**B**) TRAIL protein on lung ECs from induced $EC^{W/W}$ and $EC^{\Delta T10}$ mice. $\Delta MFI = MFI_{stained} - MFI_{isotype}$ (**A**) or $\Delta MFI = MFI_{stained} - MFI_{FMO}$ (**B**).

(**C**), Histological analysis of $PIMO^+$ hypoxic area 27 days after orthotopic m.f.p. injection of E0771 cells in induced $EC^{W/W}$ and $EC^{\Delta T10}$ mice.

(**D**), Histological analysis of the $CD31^+$ tumor vessel area 27 days after orthotopic m.f.p. injection of E0771 cells in induced $EC^{W/W}$ and $EC^{\Delta T10}$ mice.

(**E-J**), FACS analysis of the percentage of tumor-infiltrating macrophages (**E**), neutrophils (**F**), total T cells (**G**), $CD8^+$ T cells (**H**), $Foxp3^-$ $CD4^+$ T cells (**I**), and $Foxp3^+$ $CD4^+$ regulatory T cells (**J**) 27 days after orthotopic m.f.p. injection of E0771 cells in $EC^{W/W}$ and $EC^{\Delta T10}$ mice.

(**K,L**), FACS analysis of intracellular TRAIL protein on lung BECs (**K**) or total $CD45^+$ immune cells (**L**) from *Trail*$^{-/-}$ mice. Anti-CD31-LNP/empty or anti-CD31-LNP/*Trail* (8µg/mouse) were injected i.v. 48h and 24h before sacrifice. $\Delta MFI = MFI_{stained} - MFI_{isotype}$.

(**M**), Liver weight 14 days after intrasplenic (i.s.) injection of 4T1-CD90.1 cells in *Trail*$^{+/+}$ and *Trail*$^{-/-}$ mice.

(**N**), Representative images of H&E staining of livers 14 days after intrasplenic (i.s.) injection of 4T1-CD90.1 cells in *Trail*$^{+/+}$ and *Trail*$^{-/-}$ mice. Scale bar = 500µm.

(**O**), FACS analysis of extracellular TRAIL protein in splenic NK cells from tumor-free $NK^{W/W}$ and $NK^{\Delta T10}$ mice cultured *ex vivo* with of IL-2 and IL-15 (100ng/ml) for 3 days.

Abbreviations: median fluorescence intensity (MFI), fluorescence minus one control (FMO), mammary fat pad (m.f.p.), blood-vessel endothelial cells (BECs), intravenous (i.v.). All graphs show mean±SEM. ns=not significant; *p<0,05; ***p<0,001.

Fig. S5

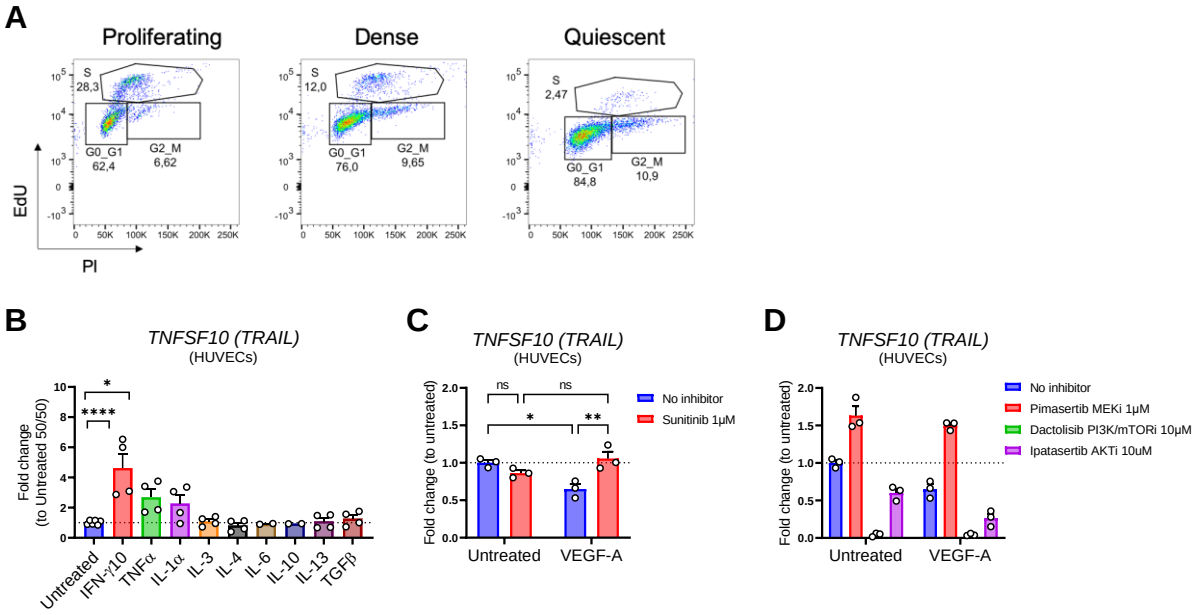

Fig. S5. Related to **TRAIL expression is a trait of endothelial cell quiescence**

(**A**), FACS analysis of the cell cycle showing the percentage of cells in G0/G1 phase (PI^{2n} EdU^{-}), S phase (EdU^{+}) and G2/M phase (PI^{4n} EdU^{-}) in proliferating, dense and quiescent HUVECs. EdU was added for the final 5h of the culture.

(**B**), mRNA expression of *TNFSF10* in quiescent HUVECs treated with IFN-γ (10ng/ml), TNFα (20ng/ml), IL-1α (5ng/ml), IL-3 (20ng/ml), IL-4 (20ng/ml), IL-6 (20ng/ml), IL-10 (150ng/ml), IL-13 (25ng/ml) or TGFβ (20ng/ml) for 17h.

(**C**), mRNA expression of *TNFSF10* in quiescent HUVECs treated with VEGF-A (50ng/ml) and/or sunitinib (1μM) for 17h.

(**D**), mRNA expression of *TNFSF10* in quiescent HUVECs treated with VEGF-A (50ng/ml) and/or the MEK inhibitor Pimasertib (1μM), the PI3K/mTOR inhibitor Dactolisib (10μM) or the Akt inhibitor Ipataserib (10μM) for 17h.

All graphs show mean±SEM. ns=not significant; *p<0,05; **p<0,01; ****p<0,0001.

Fig. S6

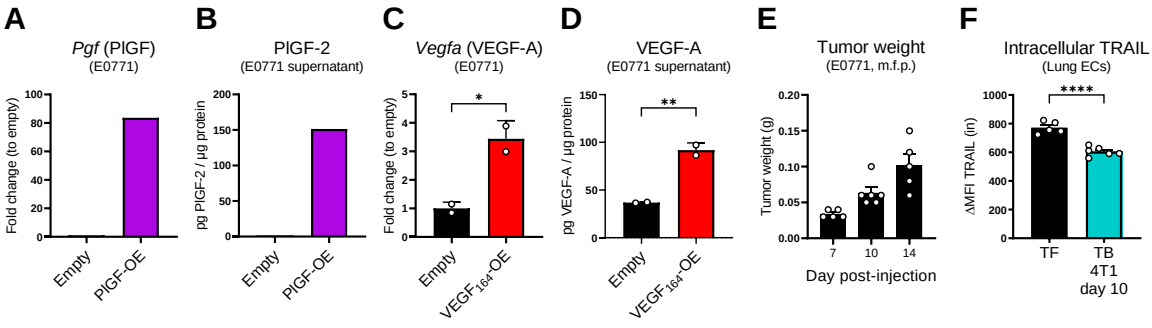

Fig. S6. Related to **TRAIL expression is downregulated by tumor-derived factors during pre-metastatic niche formation**

(**A**), mRNA expression of *Pgf* (PlGF) in PlGF-OE E0771 cells.
(**B**), Protein levels of PlGF-2 in the supernatant of PlGF-OE E0771 cells, normalized to total protein content.
(**C**), mRNA expression of *Vegfa* (VEGF-A) in VEGF-A_{164}-OE E0771 cells.
(**D**), Protein levels of VEGF-A in the supernatant of VEGF-A_{164}-OE E0771 cells, normalized to total protein content.
(**E**), Tumor weight after orthotopic m.f.p. injection of E0771-dLNGFR cells in WT mice.
(**F**), FACS analysis of intracellular TRAIL protein in lung ECs from tumor-free mice or 10 days after orthotopic m.f.p. injection of 4T1 cells in WT mice.
Abbreviations: overexpressing (OE). All graphs show mean±SEM. ns=not significant; *p<0,05; **p<0,01; ****p<0,0001.

Fig. S7

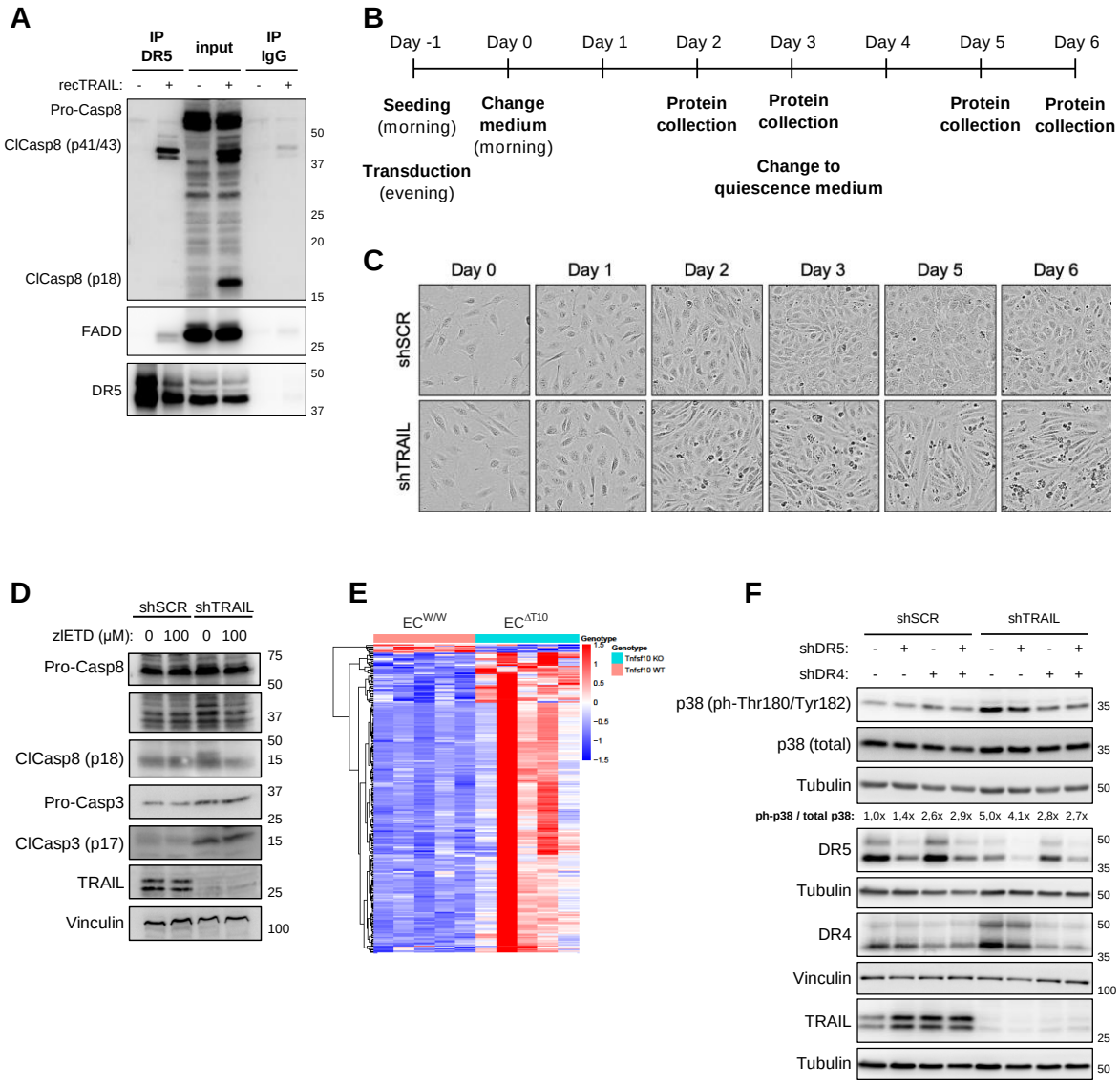
A
IP DR5
input
IP IgG
recTRAIL: - + - + - +
Pro-Casp8
ClCasp8 (p41/43)
ClCasp8 (p18)
FADD
DR5
50
37
25
20
15
25
50
37
B
Day -1
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Seeding (morning)
Change medium (morning)
Transduction (evening)
Protein collection
Protein collection
Change to quiescence medium
Protein collection
Protein collection
C
Day 0
Day 1
Day 2
Day 3
Day 5
Day 6
shSCR
shTRAIL
D
shSCR shTRAIL
zIETD (μM): 0 100 0 100
Pro-Casp8
ClCasp8 (p18)
Pro-Casp3
ClCasp3 (p17)
TRAIL
Vinculin
75
50
37
50
15
37
25
15
25
100
E
EC^W/W
EC^ΔT10
Genotype
Tnfsf10 KO
Tnfsf10 WT
F
shSCR
shTRAIL
shDR5: - + - + - + - +
shDR4: - - + + - - + +
p38 (ph-Thr180/Tyr182)
p38 (total)
Tubulin
ph-p38 / total p38: 1,0x 1,4x 2,6x 2,9x 5,0x 4,1x 2,8x 2,7x
DR5
Tubulin
DR4
Vinculin
TRAIL
Tubulin
35
35
50
50
35
50
35
100
25
50

Fig. S7. Related to **EC-specific depletion of TRAIL triggers apoptosis**

(**A**), Co-immunoprecipitation of DR5 or IgG control in quiescent HUVECs treated with Super*Killer*TRAIL (recTRAIL, 1µg/ml) for 30min, showing protein levels of pro-caspase-8, cleaved caspase-8 (clCasp8), FADD, and DR5. Input = whole cell extract.

(**B**), Global scheme of gene silencing experiments in HUVECs, showing the time points of protein collection for the time course analysis.

(**C**), Representative contrast-phase images in HUVECs silenced for TRAIL (shTRAIL) or its scrambled control (shSCR).

(**D**), Protein levels of pro-caspase-8, cleaved caspase-8 (clCasp8), pro-caspase-3, cleaved caspase-3 (clCasp3) and TRAIL in shSCR and shTRAIL HUVECs at day 3 treated with the caspase-8-specific inhibitor zIETD (100µM) since day 1.

(**E**), Heatmap of differentially expressed genes in sorted lung ECs from tumor-free induced $EC^{W/W}$ and $EC^{\Delta T10}$ mice.

(**F**), Protein levels of p38 (total and ph-Thr180/Tyr182), DR5, DR4 and TRAIL in shSCR and shTRAIL HUVECs in which DR5, DR4, both or none, were silenced. For each condition, the densitometry fold change towards shSCR is indicated.

Fig. S8

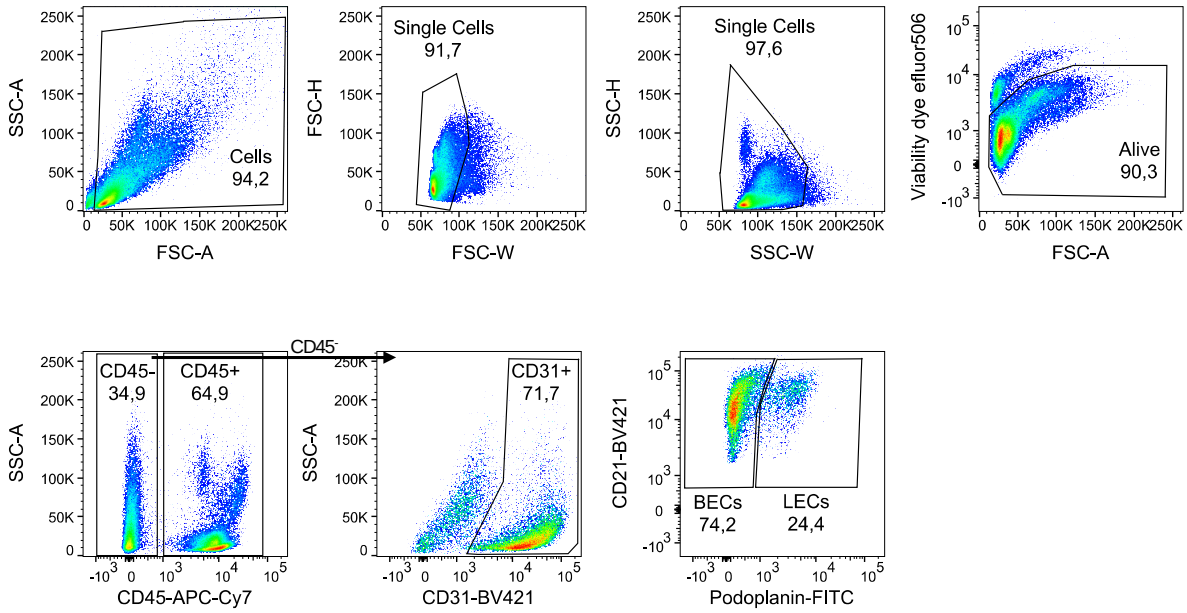

Fig. S8. Gating strategy for lung blood-vessel and lymphatic-vessel ECs (LECs)

Fig. S9

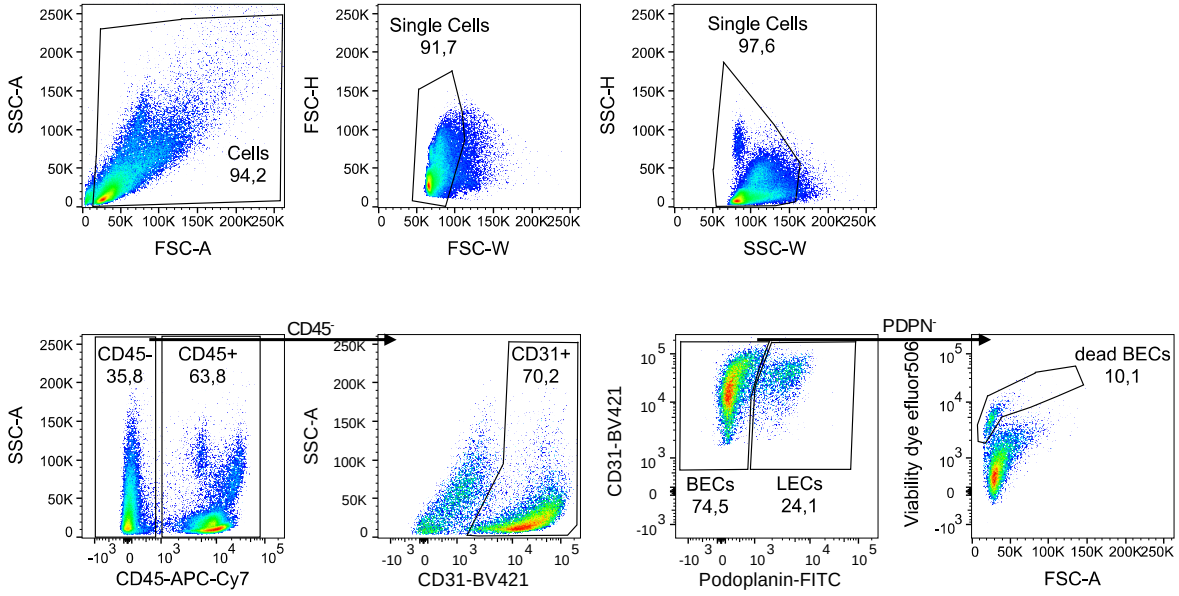

Fig. S9. Gating strategy for dead lung blood-vessel ECs (BECs)

Fig. S10

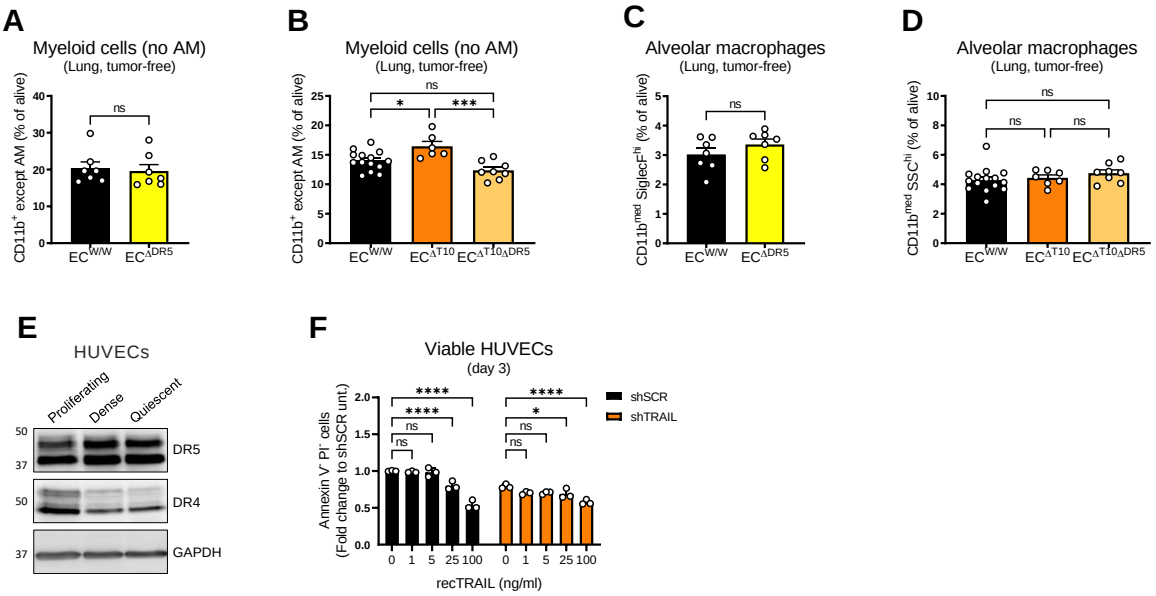

Fig. S10. Related to **Endothelial DR5 favors metastatic spread**

(**A-D**), FACS analysis of the percentage or number of myeloid cells excluding alveolar macrophages (AM) (**A,B**), and AM (**C,D**) in perfused lungs from tumor-free induced $EC^{W/W}$, $EC^{\Delta DR5}$ and $EC^{\Delta T10\Delta DR5}$ mice. **Fig. 7Q,U** previously showed dataset relative to $EC^{\Delta T10}$.

(**E**), Protein levels of DR5 and DR4 in proliferating, dense or quiescent HUVECs.

(**F**), FACS analysis of viable (Annexin V^- PI^-) shSCR or shTRAIL HUVECs treated with Super*Killer*TRAIL (recTRAIL, at the indicated doses) for 48h (from day 3 to day 5).

All graphs show mean±SEM. ns=not significant; *p<0,05; ***p<0,001; ****p<0,0001.

Fig. S11

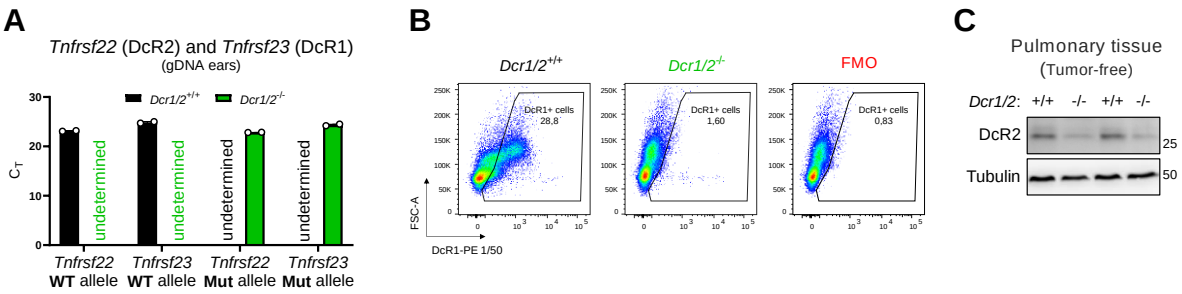

Fig. S11. Related to **Endothelial TRAIL decoy receptors (DcRs) favor metastatic spread**

(**A**), Allelic discrimination of wild type and mutant *Tnfrsf22* and *Tnfrsf23* allele determined by real-time PCR using specific LNA hybridization probes. The figure shows representative threshold cycle (C_T) values from constitutive DcR1/2 WT (*Dcr1/2*$^{+/+}$) and KO (*Dcr1/2*$^{-/-}$) mice.
(**B**), FACS analysis of extracellular DcR1 protein on viable cells from *Dcr1/2*$^{+/+}$ and *Dcr1/2*$^{-/-}$ mice.
(**C**), Protein levels of DcR2 in whole lung tissue lysates of *Dcr1/2*$^{+/+}$ and *Dcr1/2*$^{-/-}$ mice.