Supplementary methods

Cell proliferation. EO771 breast tumor cells overexpressing DLL1 (EO771-L1) or mock control (EO771-R1) (2000 cells/well) were seeded onto a 96-well plate with five duplications. After 24 hrs, 48 hrs, or 72 hrs of culture, cell proliferation was determined by using a Cell Counting Kit-8 (CCK-8) (Beyotime, China) according to the manufacturer's instruction. Briefly, 10 µl of CCK-8 solution was added to each well and cells were further incubated for 3 hrs at 37 °C. The absorbance value (OD450 value) was measured at a wavelength of 450 nm with an automatic microplate reader. Cell proliferation of LAP0297 lung cancer cells overexpressing DLL1 (LAP-L1) or mock control (LAP-R1) was determined by the same way.

Cell apoptosis. EO771-L1, EO771-R1, LAP-L1, and LAP-R1 tumor cells were seeded onto a 6-well plate, $3x10^5$ cells/well. After 48 hrs of culture, cell apoptosis was analyzed by using an Annexin V-FITC Apoptosis Assay kit (Beyotime, China) according to the manufacturer's instruction. Briefly, after resuspending in the binding buffer, cells were incubated with 5 µl of annexin V-FITC and 5 µl of 7-aminoactinomycin D (7-AAD) for 20 minutes in the dark under room temperature. Data were collected by using a Gallios flow cytometer (Beckman) and analyzed with Kaluza software (version 1.3). $7AAD^-Annexin\ V^-$: viable cells; $7AAD^-Annexin\ V^+$: early apoptotic cells; $7AAD^+Annexin\ V^+$: later apoptotic cells.

The isolation of tumor-infiltrating $CD8^+$ T cells and TAMs. Twenty-one EO771-R1 and twenty-nine EO771-L1 tumor tissues were digested with DMEM containing collagenase type 1A (1.5 mg/ml, Sigma-Aldrich), hyaluronidase (1.5 mg/ml, Sigma-Aldrich), and DNase (20 U/ml, Sigma-Aldrich) at 37°C for 45 minutes. The single cell suspensions were pooled together and tumor-infiltrating immune cells were enriched by using CD45-microbeads following the manufacturer's instruction (Miltenyi, Germany). $CD8^+$ T cells and TAMs were isolated by flow sorting from enriched cells stained with FITC-CD8a, PE-F4/80, PE-Cy7-CD45, APC-Gr1 and APC-Cy7-CD11b (BioLegend, USA).

Permeability assay. Tumor vessel permeability assay was performed according to previous publications with minor modifications(1, 2). Briefly, when EO771-R1, EO771-L1, LAP-R1 and LAP-L1 tumors reached 6-8 mm in diameter, mice were *i.v.* injected with 2.5 mg/mouse Rhodamine B-Dextran (70 KDa, Catalog R9379-1G, Sigma-Aldrich) 30 minutes and 200 µg/mouse Hoechst 33342 (Ho33342) 3 minutes before tumor harvest. Then mice were systemically perfused with 4% PFA and the tumors were removed and fixed by 4% PFA at room temperature for 2 hours, followed by incubation overnight with 30% sucrose at 4°C. The tissues were then embedded in Tissue-Tek O.C.T. compound and stored at -80°C.

PX478 treatments. PX478 (Catalog S7612-100 mg, Selleck) is a HIF1α antagonist which is able to eliminate tumor tissue hypoxia (3, 4). EO771-R1 and EO771-L1 breast tumor cells ($2x10^5$ cells/mouse) were orthotopically inoculated in mammary fat pad of female C57BL/6 mice. When tumors were palpable, mice were randomly divided into four groups and treated with PX478 (10 mg/kg) or PBS, gavage daily, every 5 dosages with two-day break. The tumor size was measured every three days using a caliper, and the tumor volume was estimated by the formula [(long axis) × (short axis)^2 × π/6]. At the end the experiment, mice were *i.v.* injected with 1.2 mg/mouse pimonidazole 25 minutes and 200 μg/mouse Hoechst 33342 5 minutes before tumor harvest. Tumor fixation and tumor vessel analysis were conducted as described previously in the main text.

References

1. J. S. Park *et al.*, Normalization of Tumor Vessels by Tie2 Activation and Ang2 Inhibition Enhances Drug Delivery and Produces a Favorable Tumor Microenvironment. *Cancer Cell* **30**, 953-967 (2016).
2. P. L. Olive, D. J. Chaplin, R. E. Durand, Pharmacokinetics, binding and distribution of Hoechst 33342 in spheroids and murine tumours. *Br. J. Cancer* **52**, 739-746 (1985).
3. S. Welsh, R. Williams, L. Kirkpatrick, G. Paine-Murrieta, G. Powis, Antitumor activity and pharmacodynamic properties of PX-478, an inhibitor of hypoxia-inducible factor-1alpha. *Mol. Cancer. Ther.* **3**, 233-244 (2004).
4. J. J. Jacoby *et al.*, Treatment with HIF-1alpha antagonist PX-478 inhibits progression and spread of orthotopic human small cell lung cancer and lung adenocarcinoma in mice. *J. Thorac. Oncol.* **5**, 940-949 (2010).

Supplementary figures and figure legends:

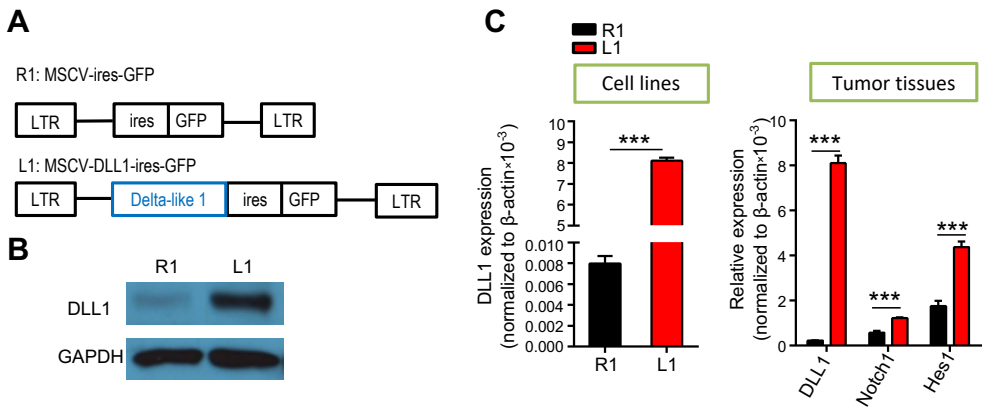

Fig. S1. Overexpression of DLL1 in EO771 breast tumor cells and tumor tissues. (**A**) DLL1 expression vector. Murine delta like 1 (*Dll1*) gene was cloned into a retrovirus plasmid MSCV-ires-GFP (R1). The successful insertion of *Dll1* gene in the MSCV-DLL1-ires-GFP (L1) was confirmed by restriction enzyme digestion and DNA sequencing. (**B**) Immunoblot analysis of DLL1 protein in EO771 tumor cells. Stably infected EO771 breast tumor cell lines EO771-R1 (R1) and EO771-L1 (L1) were obtained by flow sorting. The fractions of membrane proteins were extracted from EO771-L1 or EO771-R1 and then analyzed by western blotting. (**C**) The transcription of *Dll1*, *Notch1* and *Hes1* genes in EO771 tumor cells and tumor tissues was determined by qPCR (n=3 in the left panel and n=6 in the right panel). The experiments were repeated three times independently. Significance was determined by unpaired two-tailed Student's *t*-tests. All data are presented as means ± SEM. ****P*<0.001.

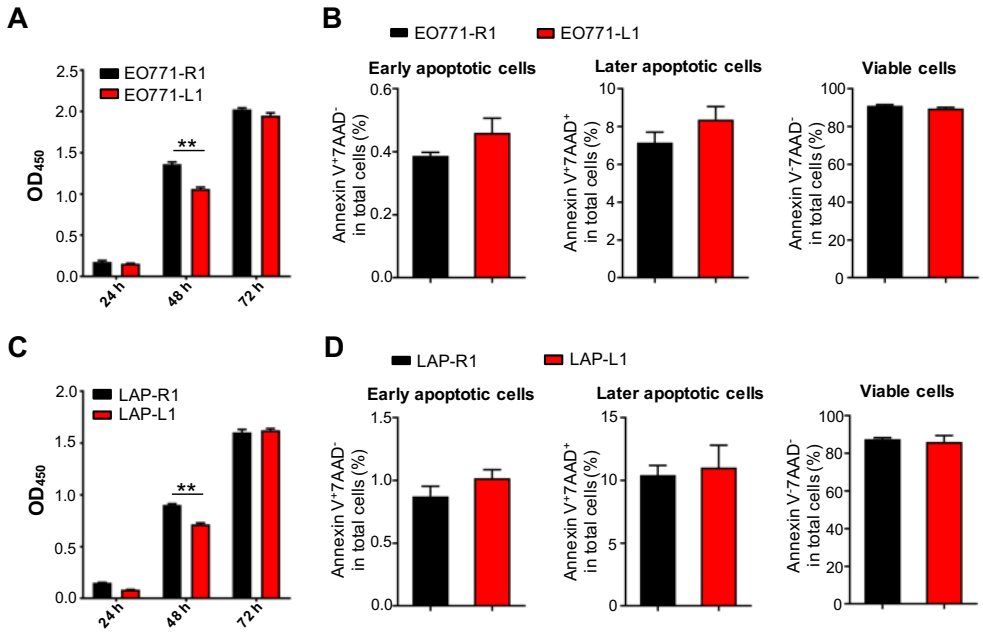

Fig. S2. Overexpression of DLL1 in EO771 or LAP0297 tumor cells slightly suppressed cell proliferation at 48 hrs of culture. (**A**) The proliferation of EO771-R1 and EO771-L1 tumor cells at 24, 48, and 72 hrs of culture. (**B**) The apoptosis of EO771-R1 and EO771-L1 tumor cells at 48 hrs of culture. (**C**) The proliferation of LAP-R1 and LAP-L1 tumor cells at 24, 48, and 72 hrs of culture. (**D**) The apoptosis of LAP-R1 and LAP-L1 tumor cells at 48 hrs of culture. $7AAD^-Annexin\ V^-$: viable cells; $7AAD^-Annexin\ V^+$: early apoptotic cells; $7AAD^+Annexin\ V^+$: later apoptotic cells. Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of two independent experiments with similar results. All data are presented as means ± SEM. $^{**}P<0.01$.

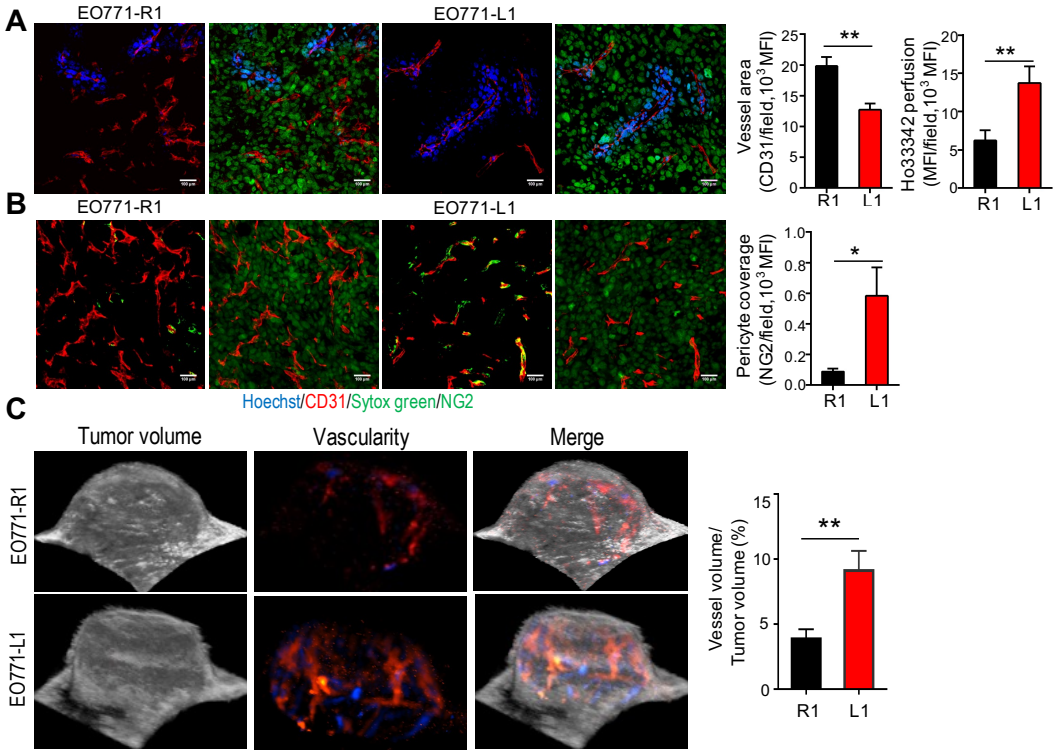

Fig. S3. Elevated levels of DLL1 in the tumor microenvironment (TME) induces EO771 tumor vascular normalization. EO771 murine breast tumor cells overexpressing DLL1 (EO771-L1 or L1) or mock control (EO771-R1 or R1) (2×10^5 cells) were orthotopically inoculated into the mammary fat pad of female C57BL/6 mice. Mice were intravenously (*i.v.*) injected with 200 μg/mouse Hoechst 33342 (Ho33342) 5 minutes before tumor harvest. Tumor blood vessel perfusion and vessel density (**A**) as well as pericyte coverage (**B**) in EO771-R1 and EO771-L1 breast tumors were analyzed. Scale bars: 100 μm. Ho33342 (blue), Hoechst 33342 perfused area; CD31 (red), endothelial cells; NG2 (green), pericytes; and Sytox green (green), counterstained for tumor tissue; MFI, mean fluorescence intensity. (**C**) The percentages of global tumor blood volume in 3D tumor volume were analyzed. Murine ultrasonographic imaging was conducted to measure global tumor vessel perfusion by using 3D imaging motor and color Doppler mode. The blue and red colors represent different blood flow directions. Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of three (in A and B) or two (in C) independent experiments with similar results, n=8-10 mice per group. All data are presented as means ± SEM. **P*<0.05, ***P*<0.01.

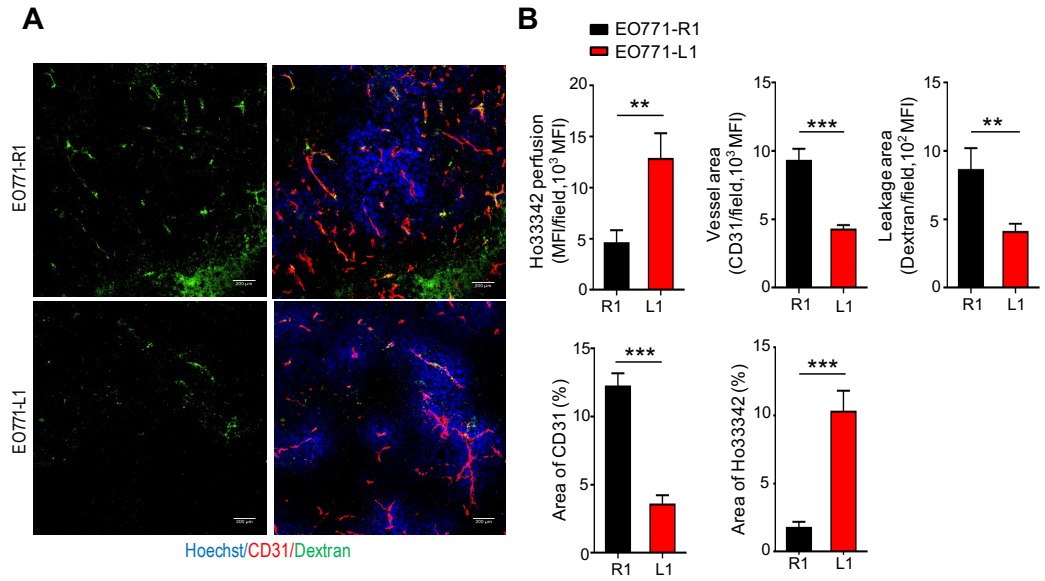

Fig. S4. Elevated levels of DLL1 in the TME reduces vessel leakage in EO771 tumors. EO771-R1 and EO771-L1 tumors were prepared as described in Fig. S3. Mice were *i.v.* injected with 2.5 mg/mouse Rhodamine B-Dextran (70 KDa) 30 minutes and 200 μg/mouse Hoechst 33342 (Ho33342) 3 minutes before tumor harvest. (**A**) Representative images of tumor blood vessel leakage and perfusion. (**B**) Tumor blood vessel perfusion, leakage and vessel density were analyzed in EO771-R1 and EO771-L1 breast tumors. Scale bars: 200 μm. Ho33342 (blue), Hoechst 33342 perfused area; CD31 (red), endothelial cells; Dextran (green), blood vessel leakage; MFI, mean fluorescence intensity. Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of two independent experiments with similar results, n=8-10 mice per group. All data are presented as means ± SEM. ***P*<0.01, ****P*<0.001.

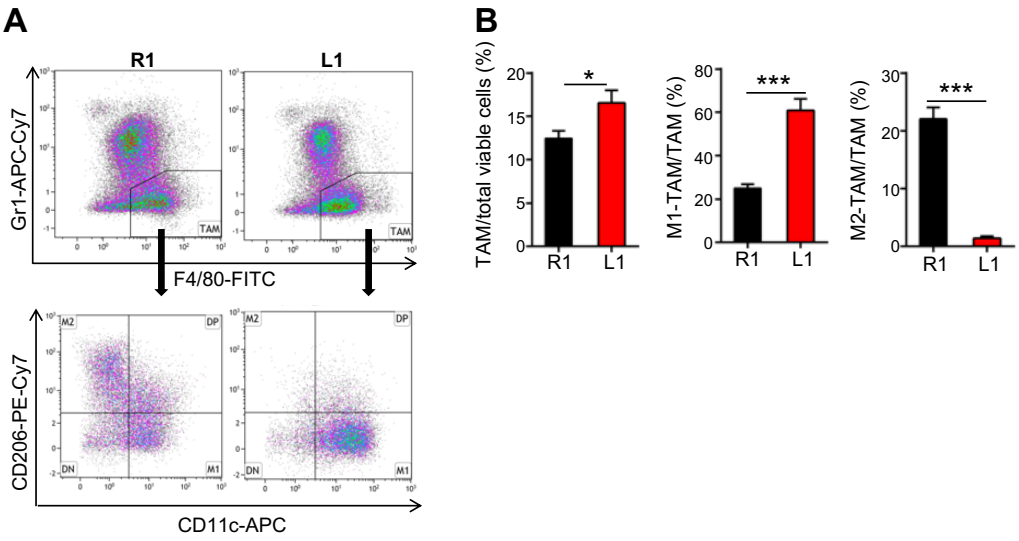

Fig. S5. Elevated levels of DLL1 in the TME polarizes tumor-associated macrophages (TAMs) from an M2- to M1-like phenotype. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. The proportions and phenotypes of TAMs were analyzed by flow cytometry. $CD45^+CD11b^+Gr1^-F4/80^+$ TAMs were further analyzed as an M1-like ($CD11c^+CD206^-$) or M2-like ($CD11c^-CD206^+$) phenotype. The representative flow cytometric figures were showed in (**A**) and the statistic analyzed data were shown in (**B**). Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of three independent experiments with similar results (n=7-9 mice per group). All data are presented as means ± SEM. **P*<0.05, ****P*<0.001.

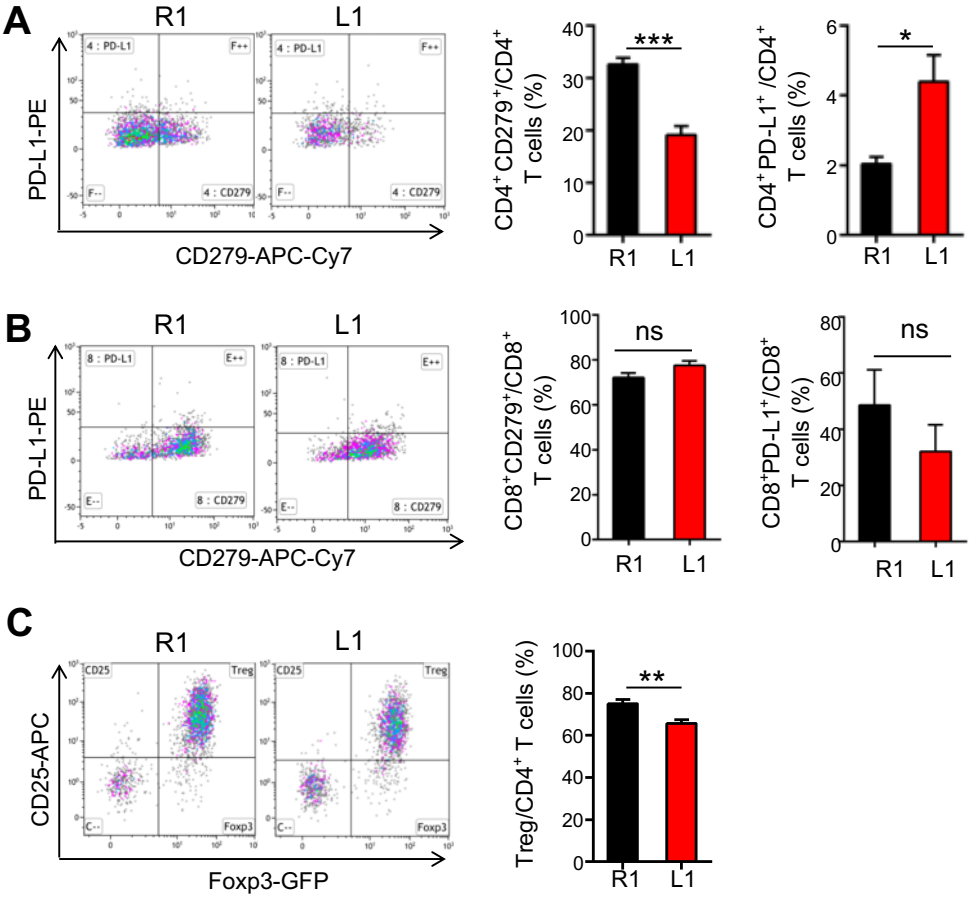

Fig. S6. Elevated levels of DLL1 in the TME decreases the proportions of immunosuppressive immune cell populations in EO771 breast tumors. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. Tumor-infiltrating immune cells were analyzed by flow cytometry as described in Fig. 3. (**A**) The proportions of tumor-infiltrating $CD4^+PD1(CD279)^+$ and $CD4^+PDL1^+$ T cells. (**B**) The proportions of tumor-infiltrating $CD8^+PD1^+$ and $CD8^+PDL1^+$ T cells. (**C**) The proportions of tumor-infiltrating Treg cells ($CD4^+CD25^+Foxp3\text{-}GFP^+$). Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of three independent experiments with similar results (n=7-9 mice per group). All data are presented as means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001. ns, no significant difference.

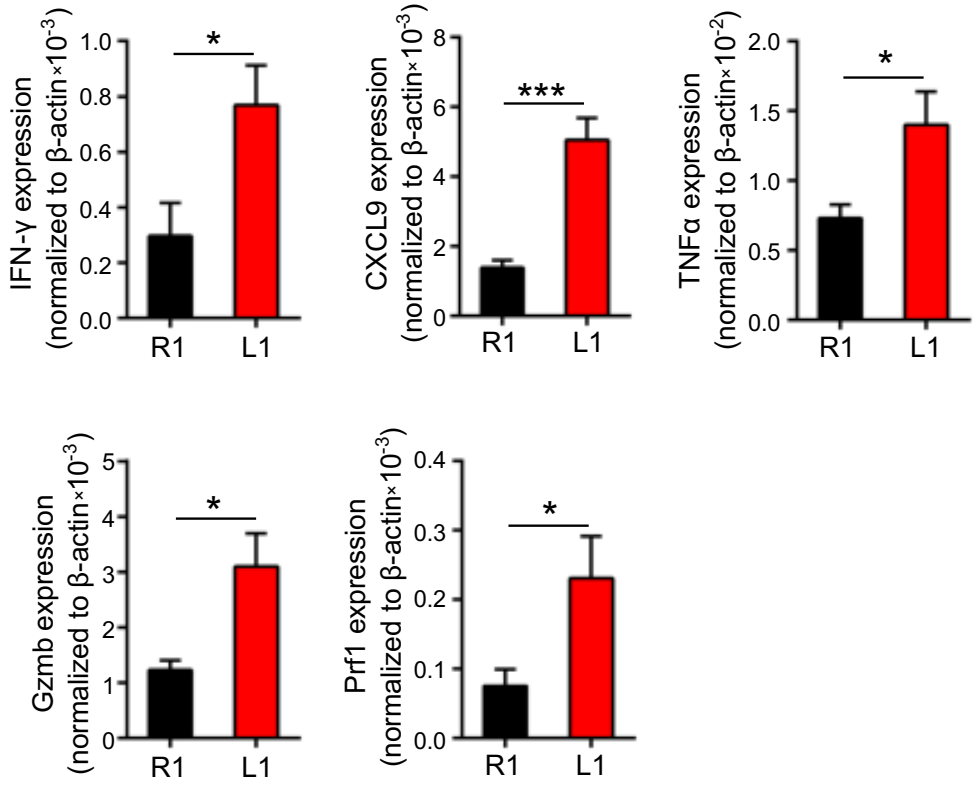

Fig. S7. Increased DLL1 levels in the TME upregulates expression of genes related to antitumor immune responses in EO771 breast tumor tissues. EO771-R1 (R1) and EO771-L1 (L1) breast tumors were prepared as described in Fig. S3. Tumor tissues were harvested on Day 14 post tumor cell inoculation. The transcription of *Ifng, Cxcl9*, *Tnfa*, *Gzmb*, and *Prf1* genes was determined by qPCR. Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of three independent experiments (n=7-9 mice per group). Data are presented as means ± SEM. **P*<0.05, ****P*<0.001.

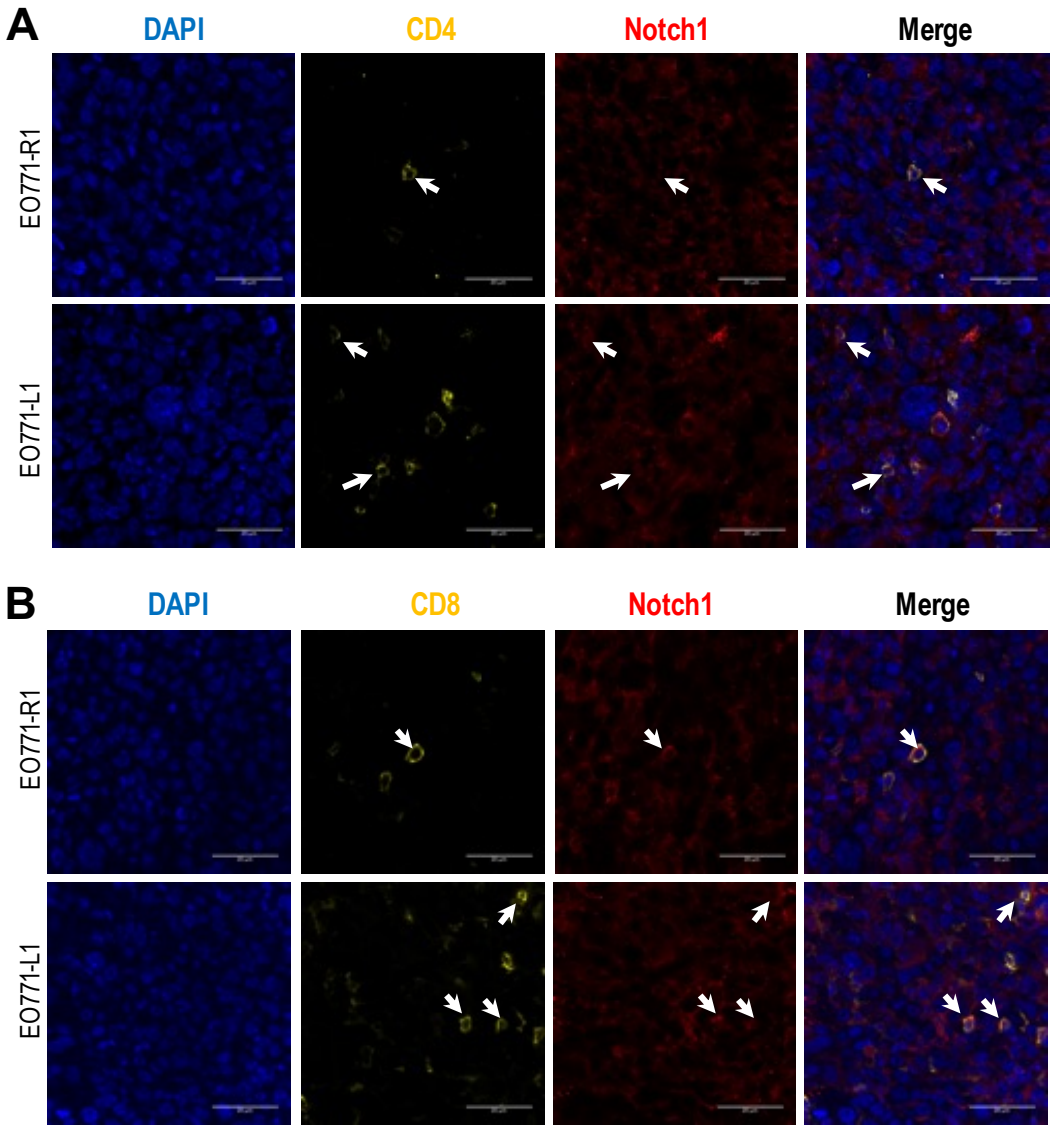

Fig. S8. Activated Notch1 staining is observed in CD4$^+$ and CD8$^+$ cells in EO771-L1, but rarely in EO771-R1, breast tumors. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. Anti-CD4, anti-CD8 and anti-activated Notch1 antibodies were used to stain the activation of Notch1 in CD4$^+$ (**A**) and CD8$^+$ (**B**) cells in frozen tumor tissues of EO771-R1 and EO771-L1. Representative images were showed. DAPI stained the cell nucleus. White arrows showed the indicated staining.

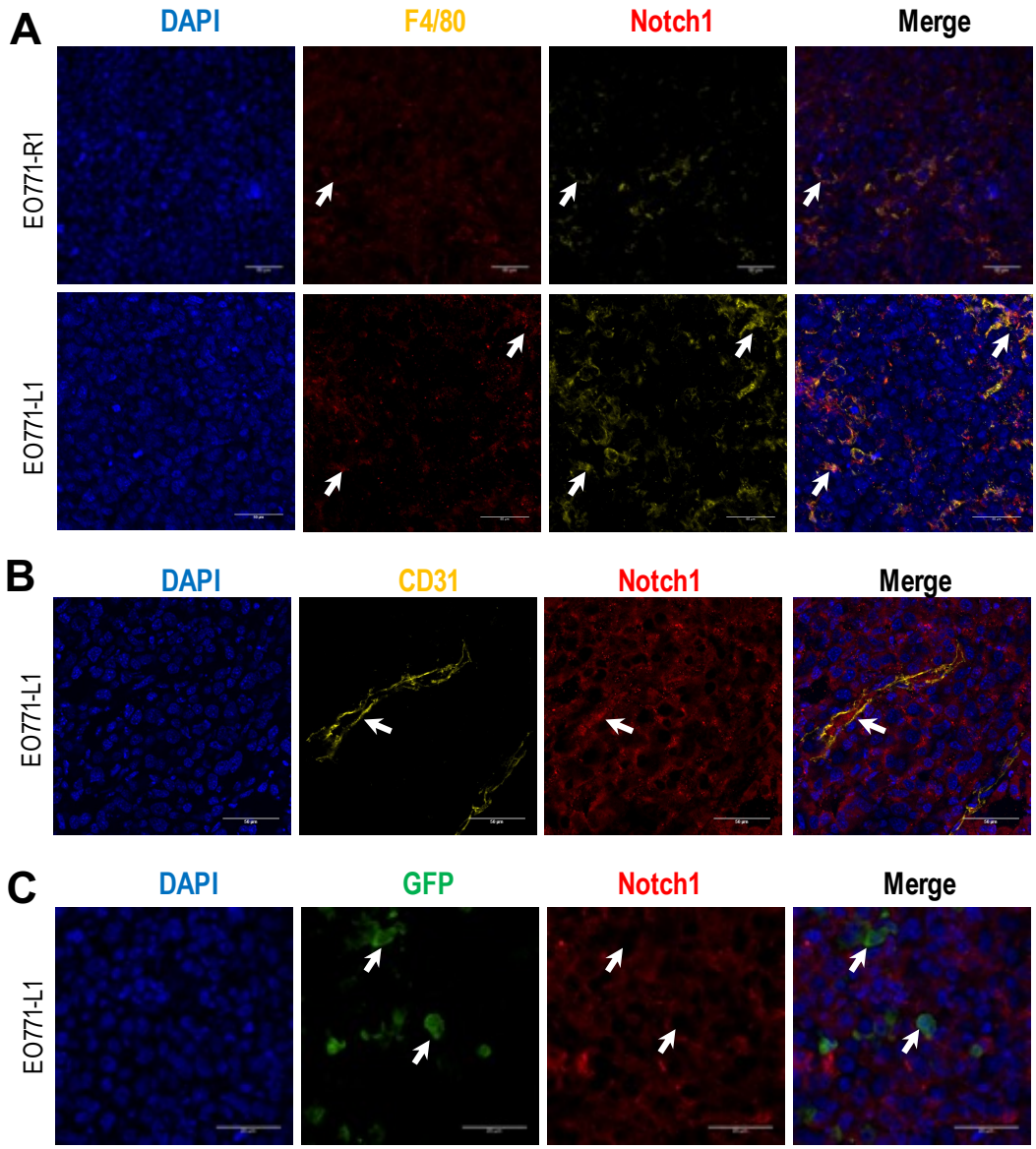

Fig. S9. Activated Notch1 staining is observed in F4/80$^+$ cells and CD31$^+$ endothelial cells in EO771-L1, but rarely in EO771-R1, breast tumors, neither in GFP$^+$ tumor cells. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. Anti-F4/80, anti-CD31 and anti-activated Notch1 antibodies were used to stain the activation of Notch1 in macrophages (**A**) and endothelial cells (**B**) in frozen EO771 tumor tissues. Representative images were showed. GFP represented EO771-L1 tumor cells (**C**); DAPI stained the cell nucleus; white arrows showed the indicated staining.

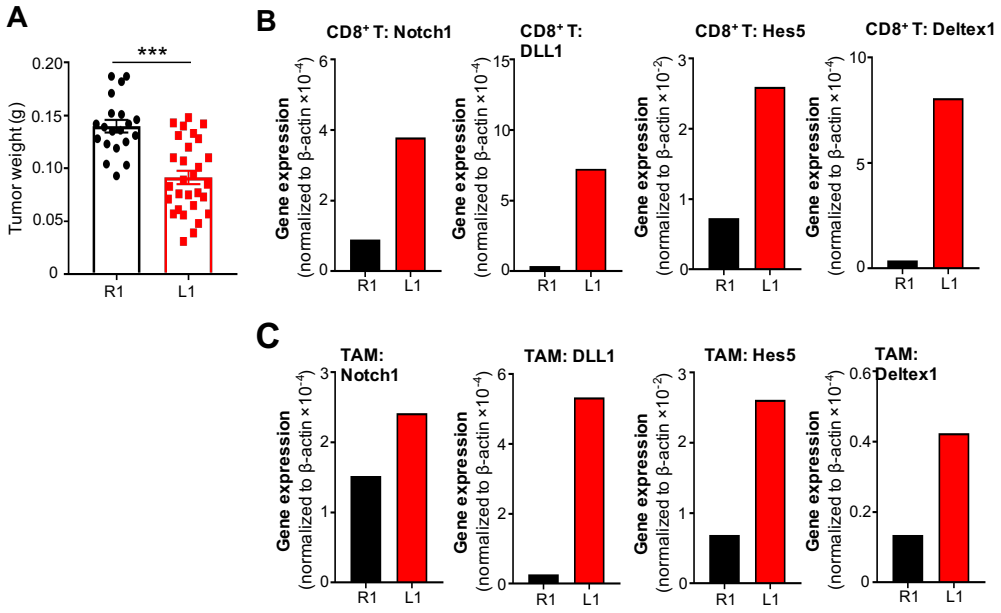

Fig. S10. DLL1 elevation in the TME upregulates the transcription of Notch target genes in tumor-infiltrating CD8⁺ T cells and TAMs in EO771 breast tumors. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. EO771-R1 tumor tissues (n=21) and EO771-L1 tumor tissues (n=29) were digested to obtain single cell suspension and then pooled together, respectively. Tumor-infiltrating immune cells were enriched by CD45 microbeads, then CD8⁺ T cells and TAMs were isolated by flow sorting. (**A**) The weight of EO771-R1 and EO771-L1 tumors. Significance was determined by unpaired two-tailed Student's *t* tests. Data are presented as means ± SEM. ***P<0.001. The transcription of *Notch1*, *Dll1*, *Hes5*, *Deltex1* in CD8⁺ T cells (**B**) and TAMs (**C**) were analyzed by qPCR.

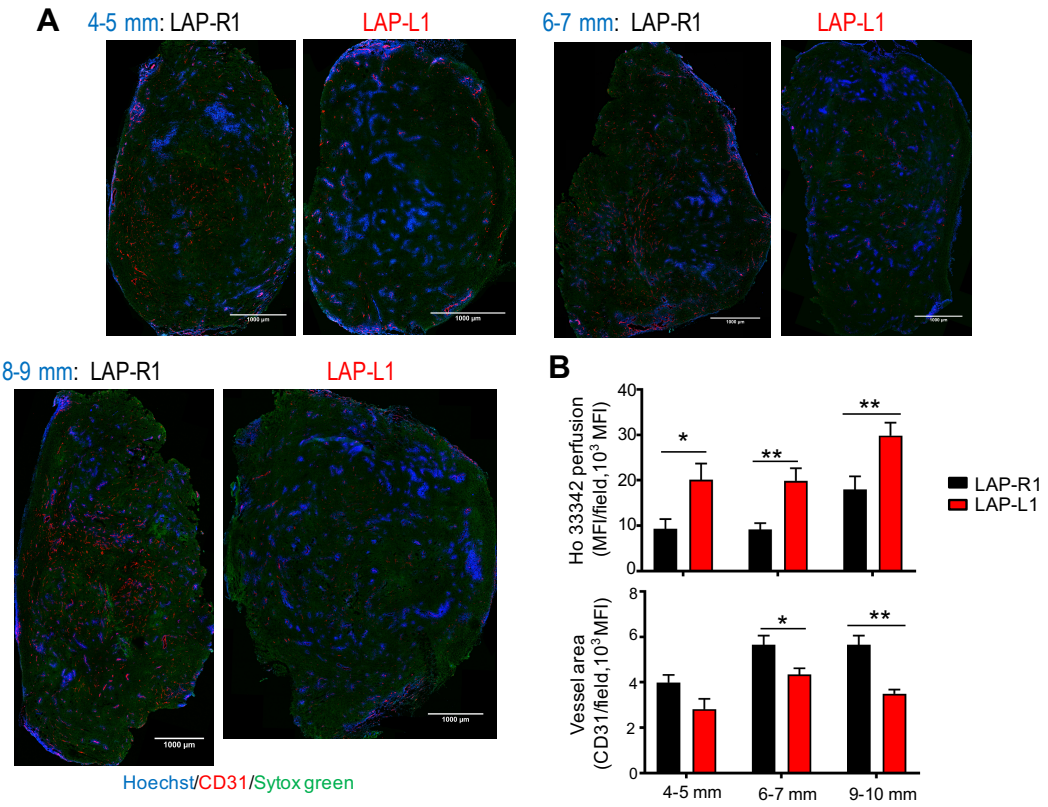

Fig. S11. DLL1 elevation in the TME induces long-term tumor vascular normalization in LAP0297 lung tumor model. LAP0297 murine lung cancer cells overexpressing DLL1 (LAP-L1) or mock control (LAP-R1) (2×10^5 cells) were inoculated subcutaneously in the right flank of female FVB mice. Tumor tissues were harvested when their sizes reached 4-5 mm, 6-7 mm, and 8-9 mm in diameter, respectively. Vessel perfusion over the entire cross-section of tumor tissues was assessed by confocal microscopy. (**A**) Representative whole tumor tissue perfusion images. Scale bars: 1000 μm. (**B**) Vessel perfusion and vessel density in indicated sizes of LAP-R1 and LAP-L1 tumors. Ho33342 (blue), Hoechst 33342 perfused area; CD31 (red), endothelial cells; and Sytox green (green), counterstained for tumor tissue. Significance was determined by unpaired two-tailed Student's *t*-tests. Each group had 8-10 mice. **P*<0.05, ***P*<0.01.

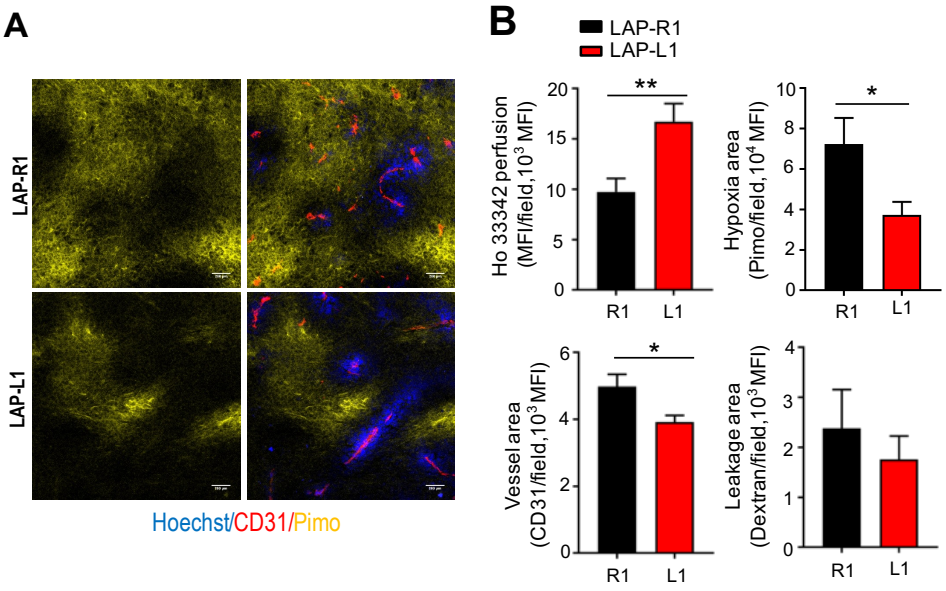

Fig. S12. Elevated levels of DLL1 in the TME decrease tissue hypoxia without changing vessel leakage in LAP0297 lung tumors. LAP-R1 and LAP-L1 tumors were prepared as described in Fig. S11. Mice were *i.v.* injected with 1.2 mg/mouse pimonidazole 25 minutes, 2.5 mg/mouse Rhodamine B-Dextran (70 KDa) 30 minutes, or 200 µg/mouse Hoechst 33342 (Ho33342) 3 minutes before tumor harvest. (**A**) Representative figures showing Pimo staining and Ho33342 perfusion. (**B**) The statistical analysis of tumor vessel perfusion (Ho33342), tumor hypoxia (Pimo) and vessel density (CD31) in LAP-R1 and LAP-L1 tumors. Scale bars: 200 µm. Ho33342 (blue), Hoechst 33342 perfused tumor area; CD31 (red), endothelial cells; Pimo (yellow), hypoxic tumor area; MFI, mean fluorescence intensity. Significance was determined by unpaired two-tailed Student's *t*-tests. Data are from one experiment representative of two independent experiments with similar results, n=8-10 mice per group. All data are presented as means ± SEM. **P*<0.05, ***P*<0.01.

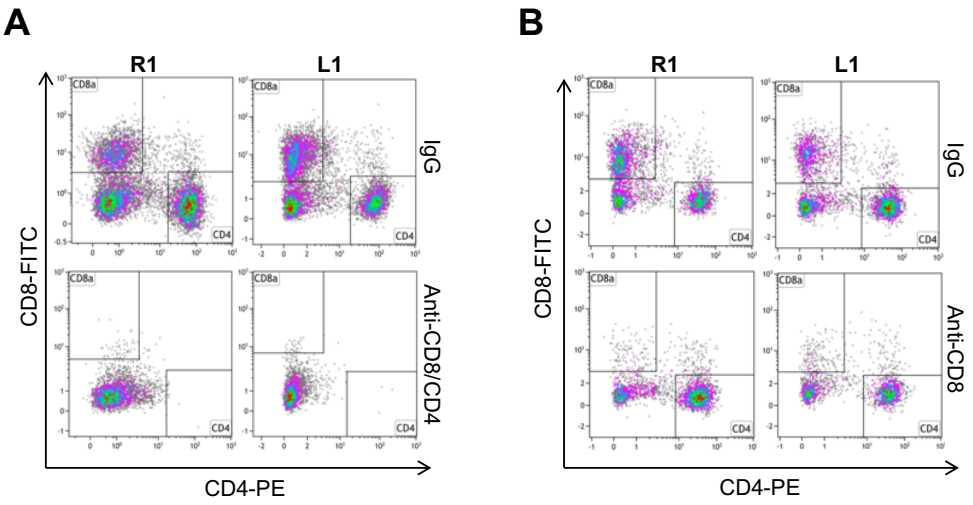

Fig. S13. The successful depletion of T cells was determined by flow cytometry at the end of the experiments. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. $CD4^+$ and $CD8^+$ T cell depletion or $CD8^+$ T cell depletion prior to tumor cell inoculation was conducted as described in Fig. 5 and Fig. S15, and the representative flow cytometric figures were shown in (**A**) and (**B**), respectively.

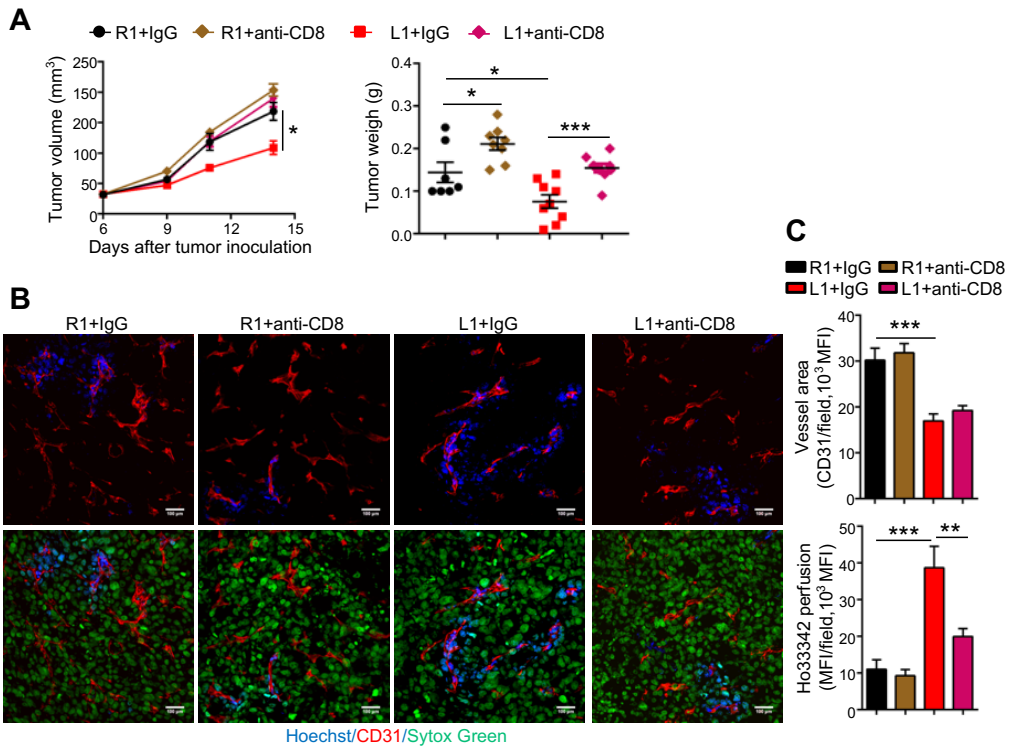

Fig. S14. *In vivo* depletion of CD8⁺ T cells reverses the improvement of vessel perfusion and the inhibition of tumor growth induced by DLL1 overexpression in EO771 breast tumor model. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. Mice were randomly assigned to 2 groups and treated with an anti-CD8 antibody, or control IgG (200 μg/mouse each) on days 6 (the corresponding tumor sizes were 3-4 mm in diameter), 8, and 14 post tumor cell inoculation, respectively. (**A**) The tumor growth curves and tumor weight. (**B, C**) Tumor blood vessel perfusion and vessel density in EO771-R1 and EO771-L1 breast tumors without or with CD8⁺ T cell depletion. Significance was determined by one-way ANOVA. Data are from one experiment representative of two independent experiments with similar results (n=7-9 mice per group). Data are presented as means ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.001.

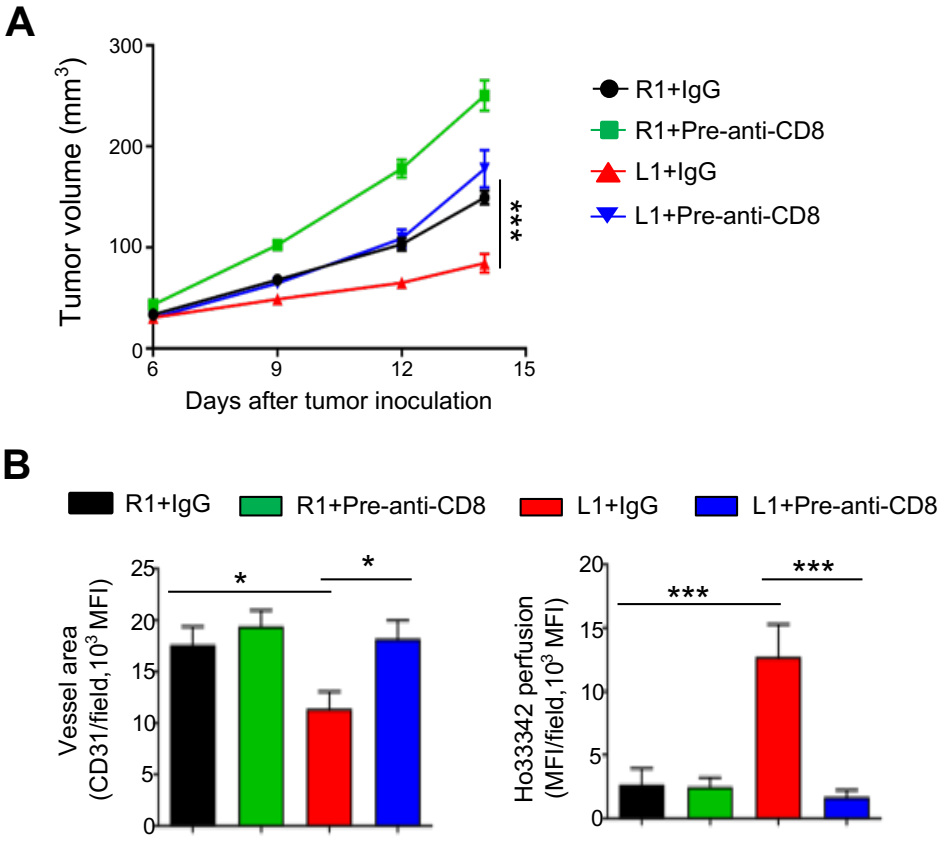

Fig. S15. *In vivo* depletion of CD8$^+$ T cells prior to tumor cell inoculation completely reverses the effects of DLL1 overexpression on tumor blood vessels and tumor growth in EO771 breast tumor model. Female C57BL/6 mice were randomly assigned to 2 groups and treated with an anti-CD8 antibody or control IgG (200 μg/mouse each) on days 0, 2, and 8. EO771-R1 and EO771-L1 breast tumor cells were inoculated on Day 1. Tumor size and tumor blood vessel perfusion were recorded and analyzed as described in Fig. S3. (**A**) The tumor growth curves. (**B**) Tumor blood vessel perfusion and vessel density in EO771-R1 and EO771-L1 breast tumors without or with prior CD8$^+$ T cell depletion. Significance was determined by one-way ANOVA. Data are from one experiment representative of two independent experiments with similar results (n=8-9 mice per group). Data are presented as means ± SEM. **P*<0.05, ****P*<0.001.

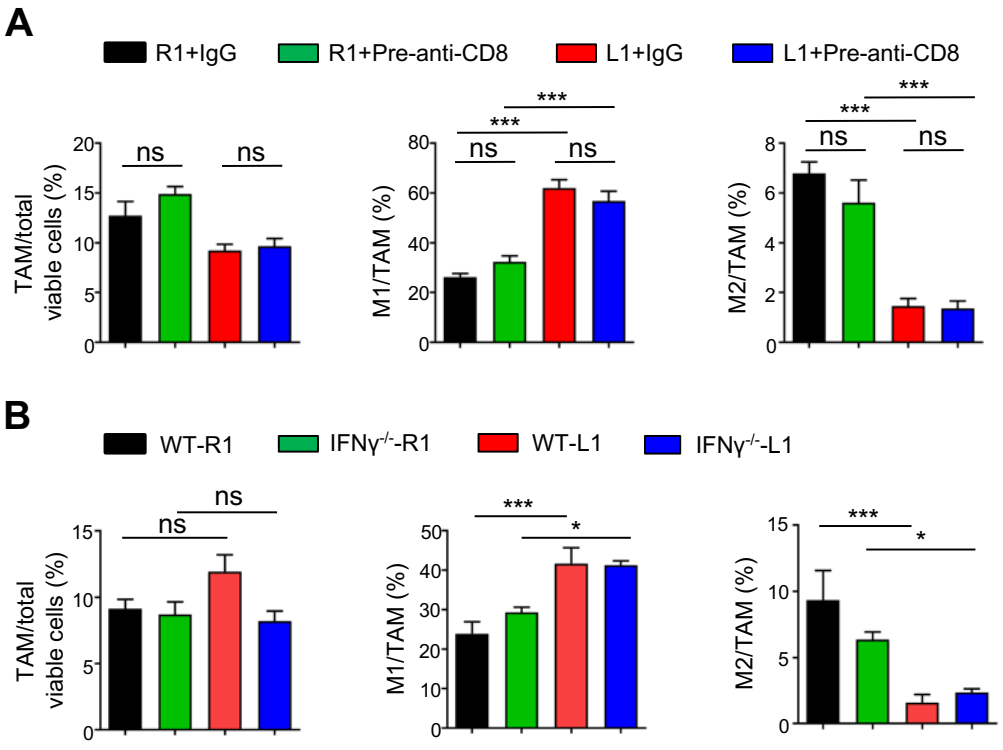

Fig. S16. CD8⁺ T cell depletion or mouse IFNγ deficiency does not alter the impacts of DLL1 overexpression on TAM polarization in EO771 breast tumors. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. The phenotypes of TAMs analysis were performed as described in Fig. S5. (**A**) The proportions of M1- and M2-TAMs without or with $CD8^+$ T cell depletion prior to EO771 tumor cell inoculation. (**B**) The proportions of M1- and M2-TAMs in EO771 breast tumors in WT or $IFN\gamma^{-/-}$ mice. Significance was determined by one-way ANOVA. Data are from one experiment representative of two independent experiments with similar results (n=7-9 mice per group). Data are presented as means ± SEM. **P*<0.05, ****P*<0.001.

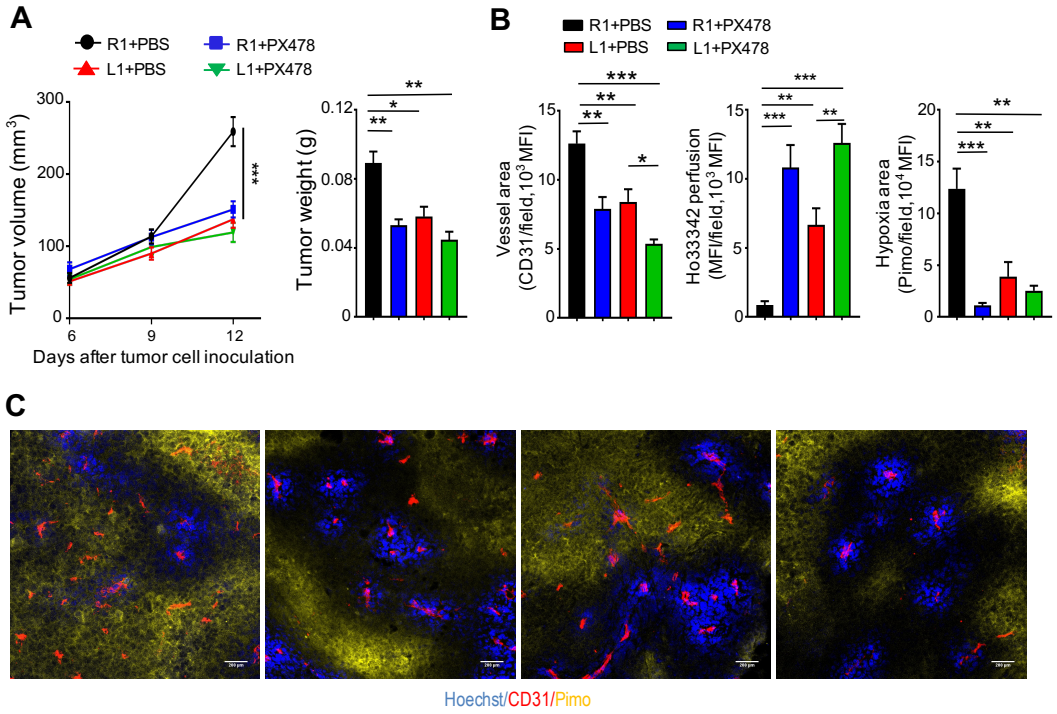

Fig. S17. Anti-hypoxia PX478 treatments alone indue tumor vascular normalization, but not enhancing the antitumor effects of DLL1 overexpression in EO771 tumors. EO771-R1 and EO771-L1 breast tumors were prepared as described in Fig. S3. When tumors were palpable, mice were randomly divided into four groups and treated with PX478 (10 mg/kg) or PBS, gavage daily, every 5 dosages with two-day break. Mice were *i.v.* injected with 1.2 mg/mouse pimonidazole (Pimo) 25 minutes and 200 μg/mouse Hoechst 33342 (Ho33342) 5 minutes before tumor harvest. (**A**) The tumor growth curves and tumor weight. (**B**) The statistical analysis of tumor vessel density (CD31), vessel perfusion (Ho33342), and tumor tissue hypoxia (Pimo). (**C**) Representative figures showing Pimo staining and Ho33342 perfusion. Scale bars: 200 μm. Ho33342 (blue), Hoechst 33342 perfused tumor area; CD31 (red), endothelial cells; Pimo (yellow), hypoxic tumor area; MFI, mean fluorescence intensity. Significance was determined by one-way ANOVA. Data are from one experiment representative of two independent experiments with similar results, n=8-10 mice per group. All data are presented as means ± SEM. *P<0.05, **P<0.01, ***P<0.001.

Table S1: Primers used for qPCR analysis.

Gene	Primer	Sequence (5'-3')
mβ-actin	Forward	ATCGTGCGTGACATCAAAGA
	Reverse	ACAGGATTCCATACCCAAGAAG
mNotch1	Forward	AAGGCTTCAGTGGCCCTAAT
	Reverse	TGTGGGACAGACACAGGAAA
mDll1	Forward	TTGCTTCAATGGAGGACGAT
	Reverse	ACACTTGGCACCGTTAGAACA
mHes1	Forward	GGCCTCTGAGCACAGAAAGT
	Reverse	GTGTTAACGCCCTCACACG
mHes5	Forward	TCGGGACCGCATCAACA
	Reverse	CAGGTAGCTGACGGCCATCT
mDeltex1	Forward	TGAGGATGTGGTTCGGAGGT
	Reverse	CCCTCATAGCCAGATGCTGTG
mCxcl9	Forward	AGTGTGGAGTTCGAGGAACC
	Reverse	GAGTCCGGATCTAGGCAGG
mTnfa	Forward	CCGATGGGTTGTACCTTGTC
	Reverse	CGGACTCCGCAAAGTCTAAG
mGzmb	Forward	TCATGCTGCTAAAGCTGAAGAGT
	Reverse	TTCCCCAACCAGCCACATAG
mIfng	Forward	CCAAGTTTGAGGTCAACAACCC
	Reverse	GGGACAATCTCTTCCCCACC
mPrf1	Forward	GTCGCATGTACAGTTTTCGCC
	Reverse	GGGCTGTAAGGACCGAGATG