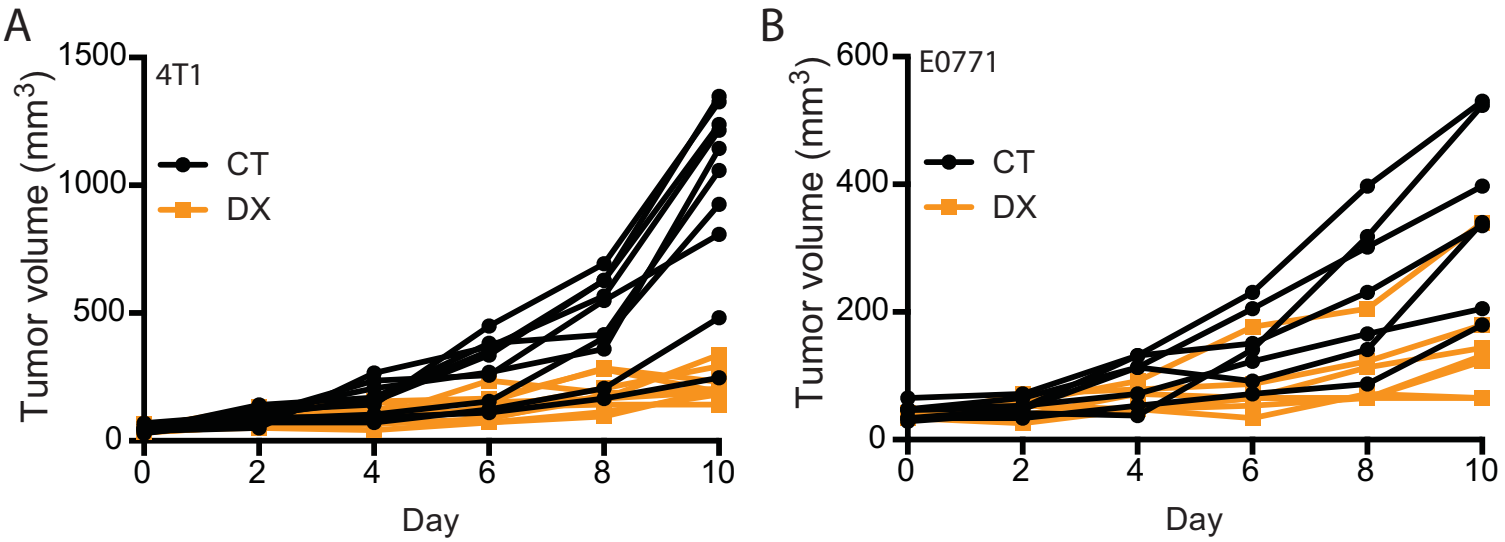
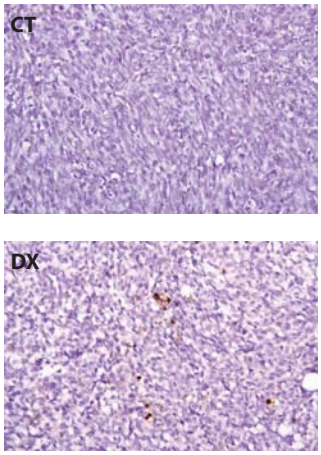
Supplementary Figure 1: A-B) Tumor growth curves for individual tumors. Once tumors reached ~40 mm^3 (Day 0) mice were treated with control IgG (CT) or DX-2400 (DX) for up to day 10, at which time mice were sacrificed. Individual tumor growth curves in each group (CT, black; DX, orange) are shown for the 4T1 **(A)** and E0771 **(B)** tumor models. n = 10 per group for the 4T1 model and 7 per group for the E0771 model.

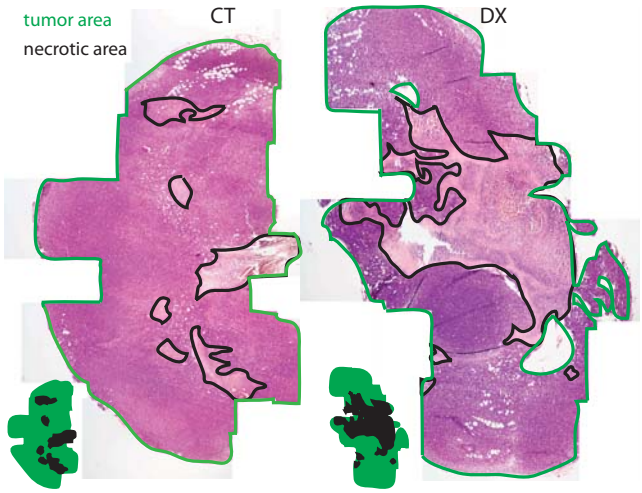

A
4T1
CT
DX
Tumor volume (mm3)
1500
1000
500
0
0
2
4
6
8
10
Day
B
E0771
CT
DX
Tumor volume (mm3)
600
400
200
0
0
2
4
6
8
10
Day

Supplementary Figure 2: Representative images used for analyses of apoptosis, necrosis, and proliferation (PCNA). All images are from 4T1 tumors. **A**) Day 4 Apoptag staining. **B**) Day 10 H&E staining (necrosis). **C-D**) An example of how the tumor area and necrotic area were determined is shown (for analysis areas were drawn in imageJ but here were drawn in illustrator for example only). Proliferation (PCNA) was assessed at day 4 (**C**) and 10 (**D**).

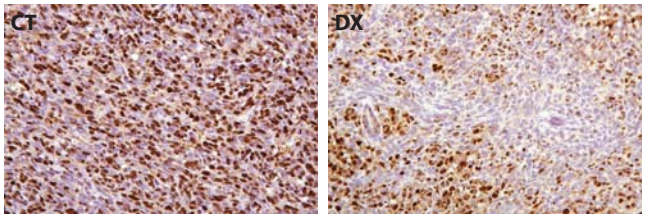
A) ApopTag (day 4)



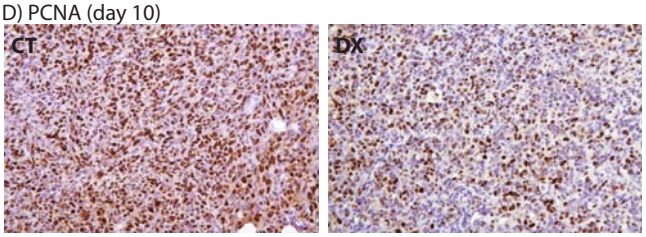
B) H&E (necrosis, day 10)



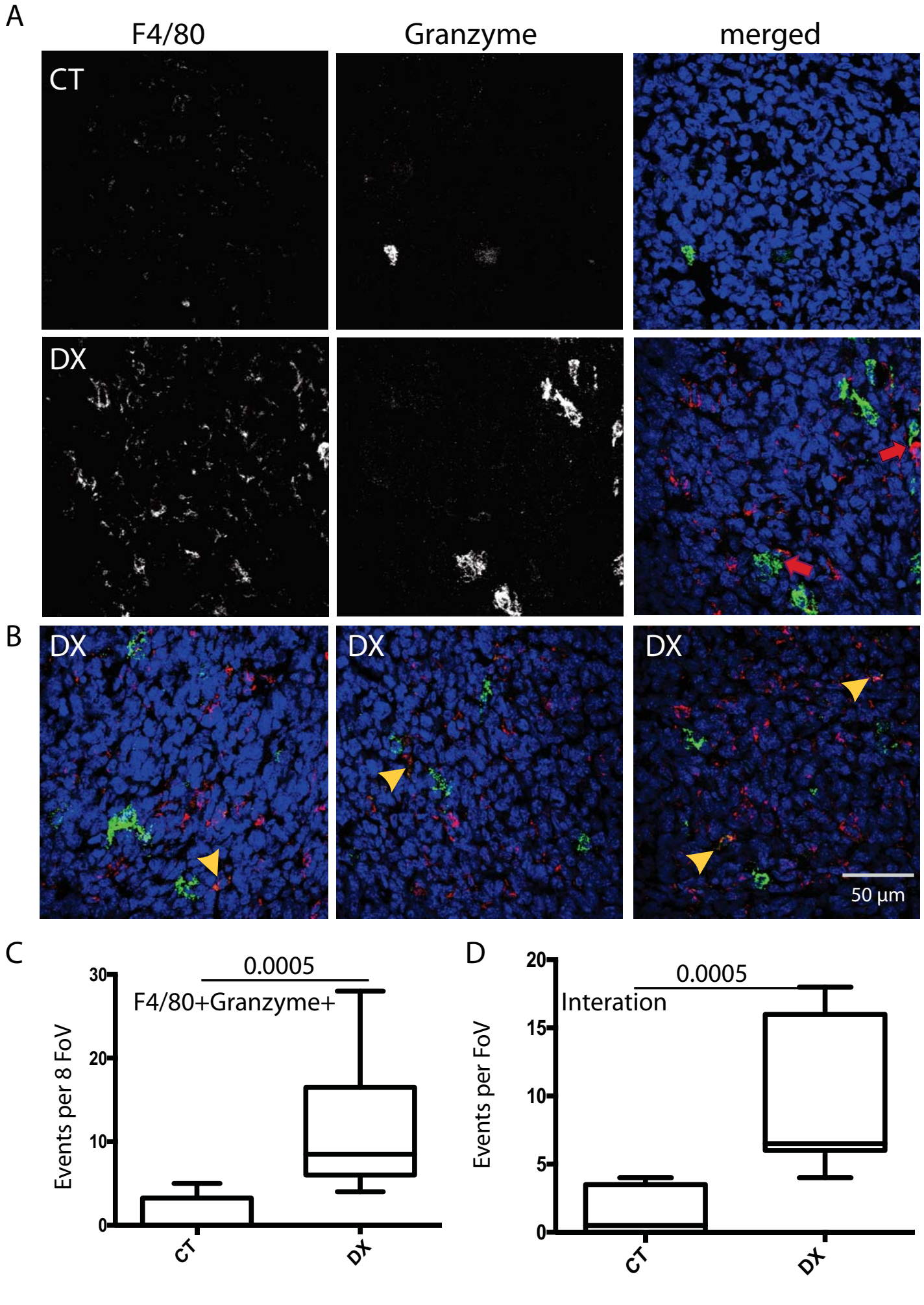
C) PCNA (day 4)



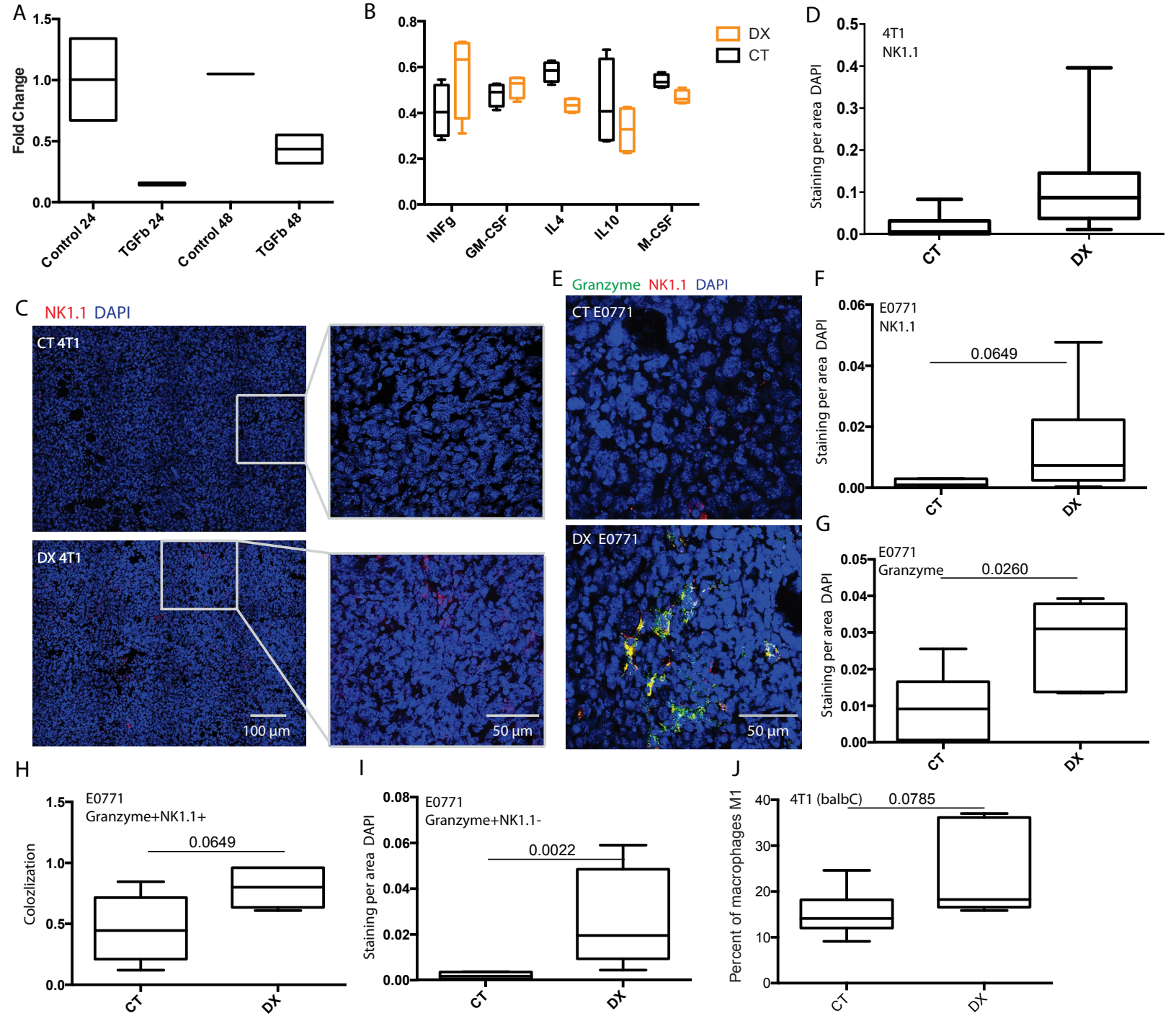
D) PCNA (day 10)



Supplementary Figure 3: Representative images of granzyme B and F4/80 double stain. All images are from day 4 4T1 tumors. **A)** Single color images and merged image for control IgG (CT) and DX-2400 (DX) treated tumors. Red arrows on the DX merged image in panel A indicate instances of "interactions", i.e. a close association between an $F4/80^{+}$ cell and a granzyme B positive cell. **B)** Additional examples of DX-2400 treated tumors are shown with yellow arrows indicating granzyme B and $F4/80^{+}$ double positive cells. **C**) Quantification of the interactions (seen in Panel A, red arrows). **D**) Quantification of granzyme B and F4/80 double positive population**.** Data are presented as median with the interquartile range (box) and maximum and minimum values (whiskers). n = 8 per group. P values determined by two-sided Mann-Whitney test.

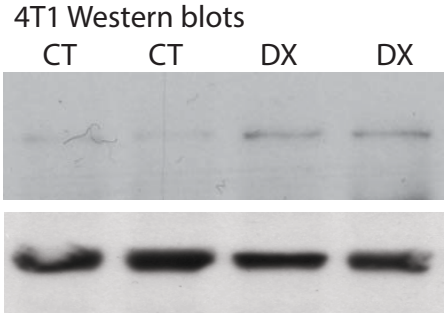

A
F4/80
Granzyme
merged
CT
DX
B
DX
DX
DX
50 µm
C
0.0005
F4/80+Granzyme+
Events per 8 FoV
30
20
10
0
CT
DX
D
0.0005
Interation
Events per FoV
20
15
10
5
0
CT
DX

Supplementary Figure 4: A) Decreases in iNOS production by macrophages following treatment with TGFβ. **B)** Data of normalized densitometry of cytokines changed by DX-2400 treatment after 6 days in 4T1 tumors. **C-D)** In 4T1 tumors at day 4, NK cell numbers were increased by DX-2400 treatment. **E-G)** Similar changes in NK cells (**F**) and granzyme B (**G**) were seen in E0771 at day 4. **H)** Moreover, there was also a significant increase in the number of granzyme B positive $NK1.1^-$ cells (likely indicative of increased cytotixic $CD8^+$ T cell activity). **I)** Flow cytometry (see Supplementary Methods for protocols) data for 4T1 tumors from BlabC mice confirming a trend towards a shift in M1 phenotype (here CD11c was used as an M1 marker). Data are presented as median with the interquartile range (box) and maximum and minimum values (whiskers). Data for two (A) or three (B) replicates are shown, n = 8 (CT) and 9 (DX) for panel C, n = 6 per group for panels F-I. P values determined by two-sided Mann-Whitney test.


A
Fold Change
Control 24
TGFb 24
Control 48
TGFb 48
B
DX
CT
INFg
GM-CSF
IL4
IL10
M-CSF
D
4T1
NK1.1
Staining per area DAPI
CT
DX
C
NK1.1 DAPI
CT 4T1
DX 4T1
100 µm
50 µm
E
Granzyme NK1.1 DAPI
CT E0771
DX E0771
50 µm
F
E0771
NK1.1
0.0649
Staining per area DAPI
G
E0771
Granzyme
0.0260
H
E0771
Granzyme+NK1.1+
0.0649
Colozlization
I
E0771
Granzyme+NK1.1-
0.0022
J
4T1 (balbC)
0.0785
Percent of macrophages M1

Supplementary Figure 5: iNOS at day 6 was also increased although this was not as strong as the effects seen at day 4. **A)** Western blot of iNOS at day 6 in 4T1 tumors and match densitometry measurements to the right. **B)** Immunofluorescent staining for iNOS at days 4 (as presented in the manuscript) in addition to days 6 and 10. **C)** We show that by day 10, iNOS levels are no-longer significantly altered by DX-2400 in the 4T1 model. At this time-point, the endogenous (CT) levels of iNOS are higher than at day 4. Thus, the failure of DX-2400 to further increase iNOS in these late-stage 4T1 tumors also supports the suggestion that already high levels of iNOS (as seen in E0771 tumors) are less susceptible to a further induction by DX-2400. Data are presented as median with the interquartile range (box) and maximum and minimum values (whiskers). Panel **C** shows both 4T1 and E0771 models on the same axis to illustrate the difference in the endogenous iNOS production of these two tumor models. In **B)** n = 10 per group day 4, n = 5 per group day 6, n = 8 per group day 10. In **C)** n = 10 per group for 4T1 and 6 per group for E0771. P values determined by two-sided Mann-Whitney test.

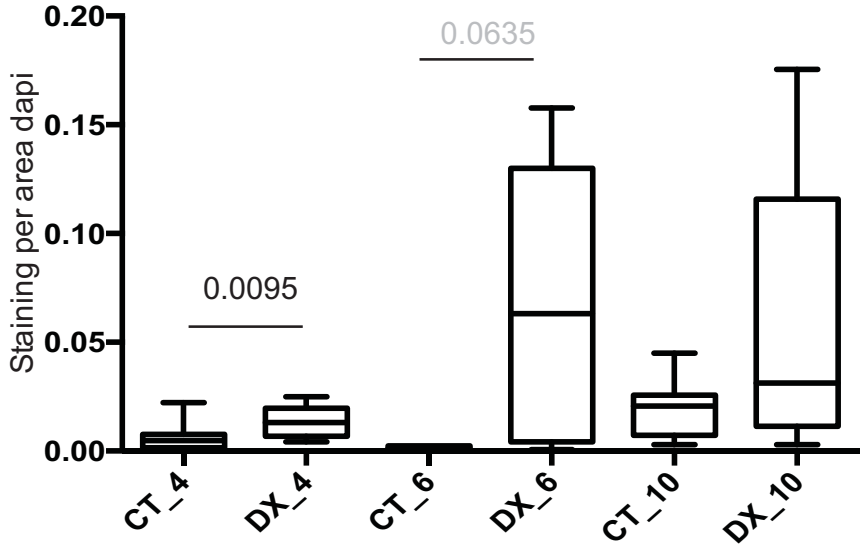
A



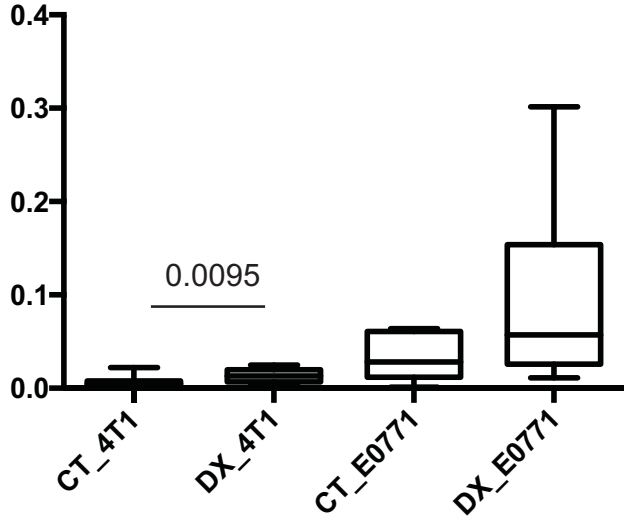
Normalised densitometry

	CT	CT	DX	DX
iNOS	1.00	0.88	6.92	6.51

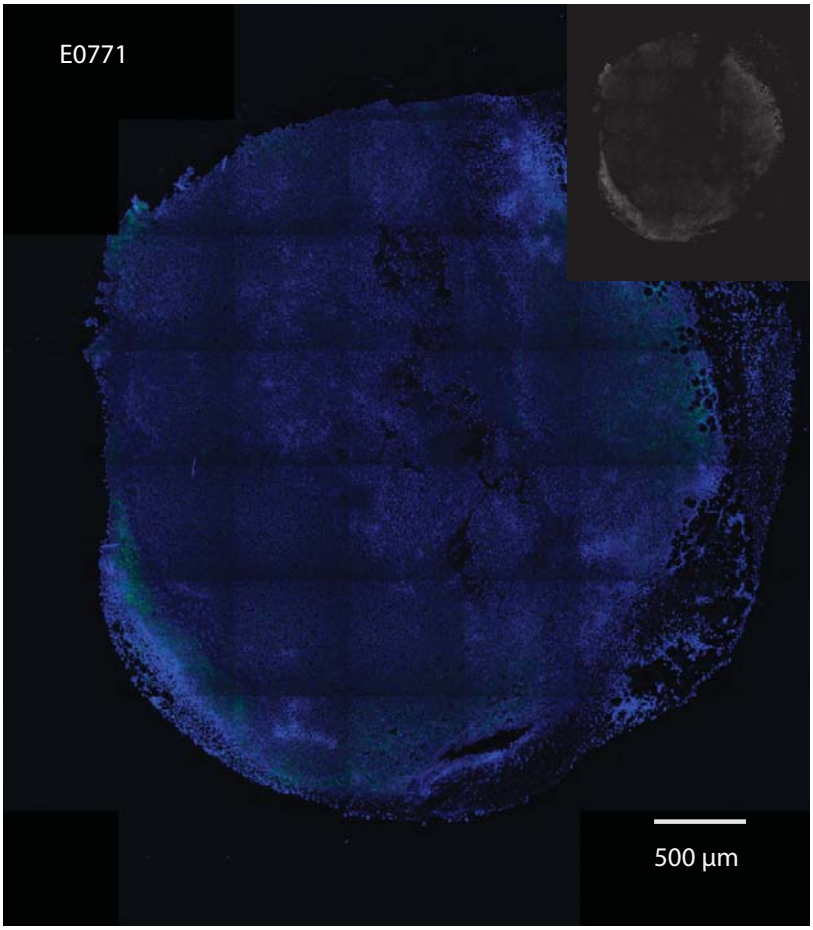
B

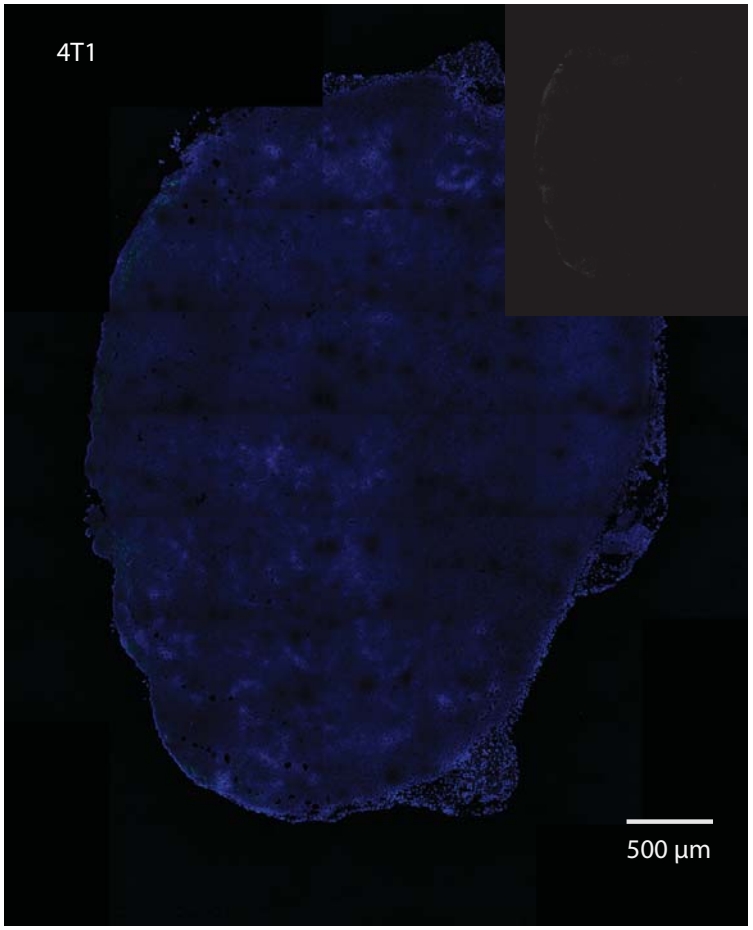


C

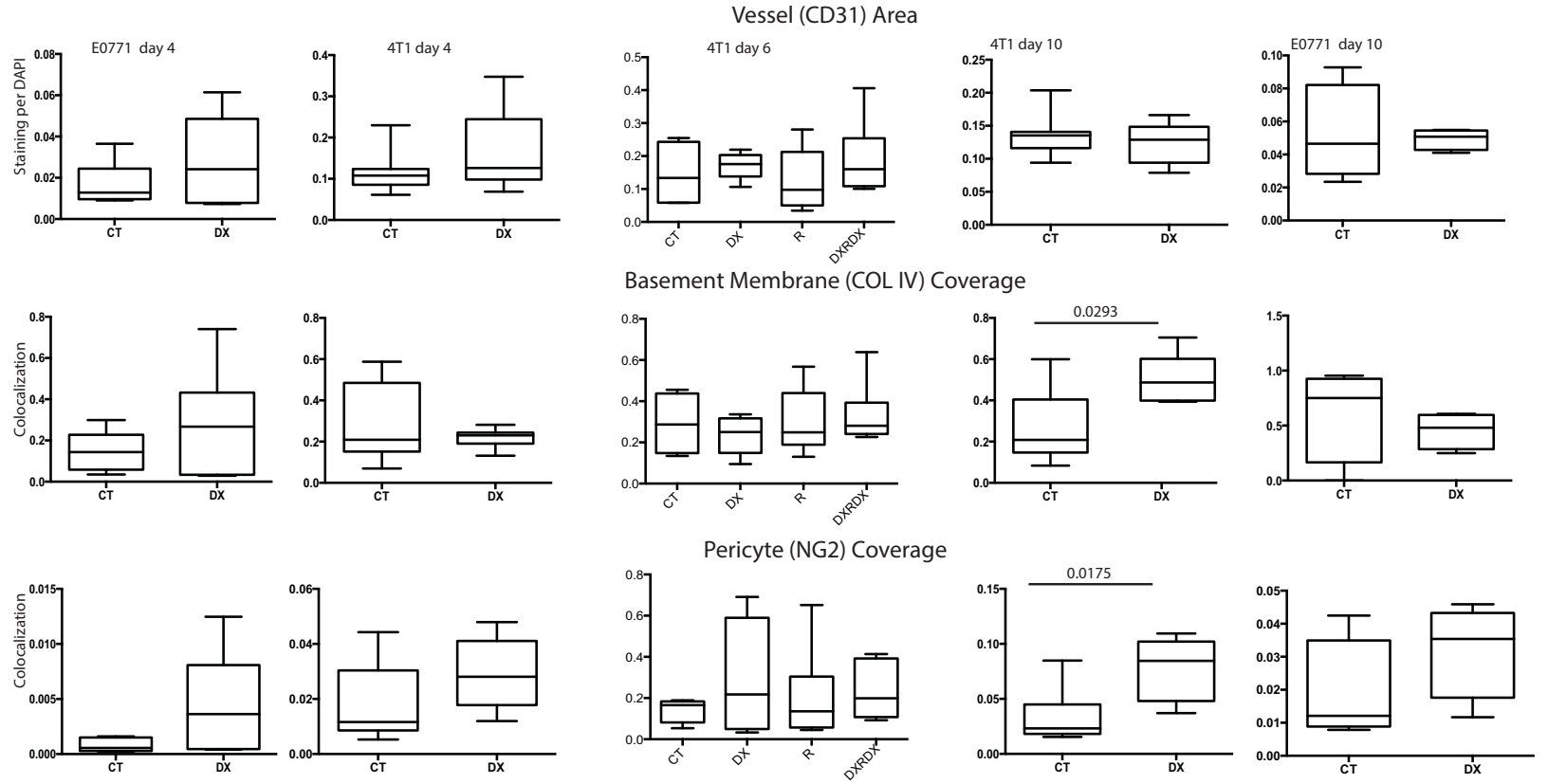


Supplementary Figure 6: Representative control E0771 and 4T1 tumors showing notable differences in endogenous iNOS expression in these two models.


E0771
500 µm

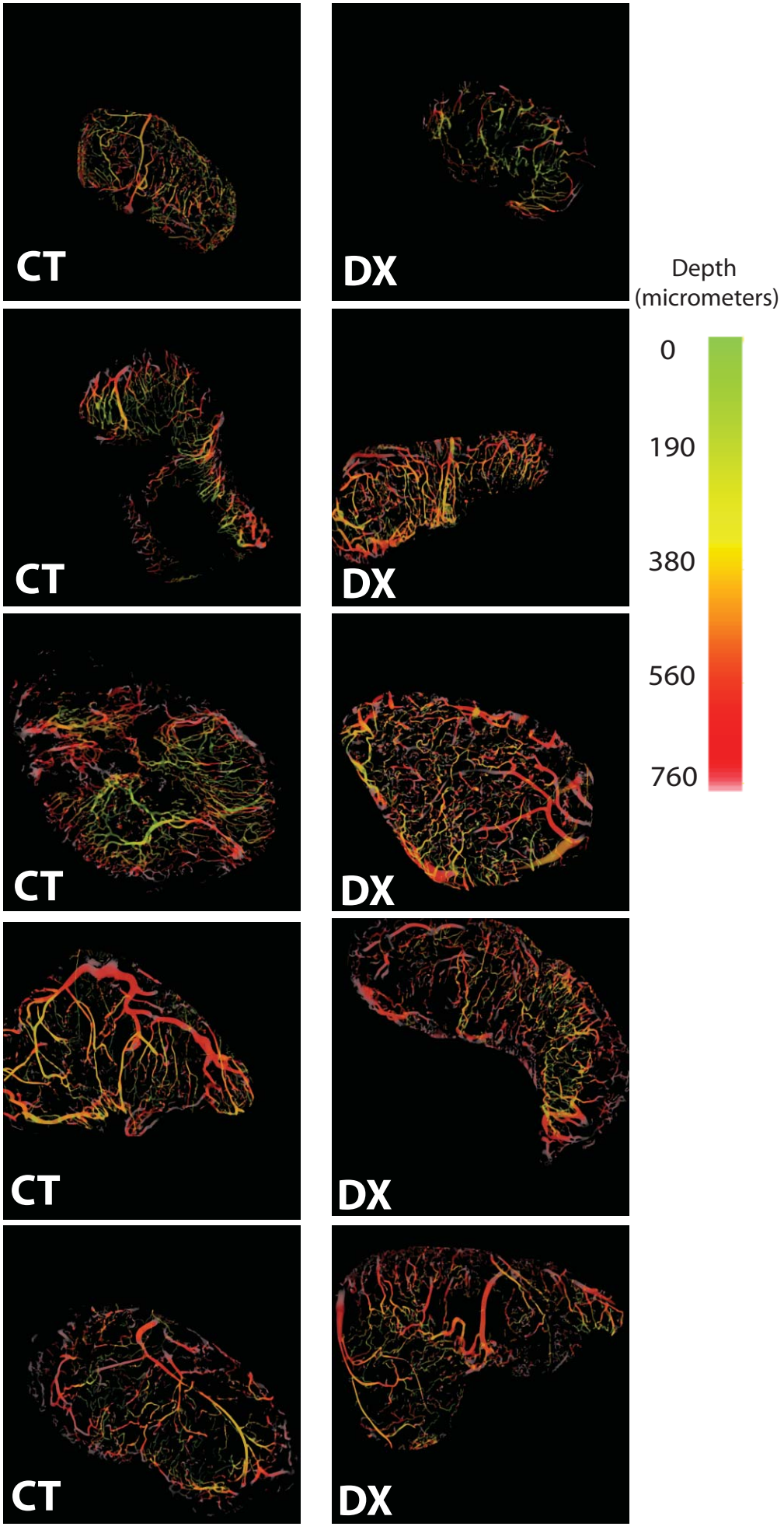

4T1
500 µm

Supplementary Figure 7: Representative OFDI images 4 days after the initiation of control (CT) or DX-2400 (DX) treatment of 4T1 tumors grown in BALB/c mice.

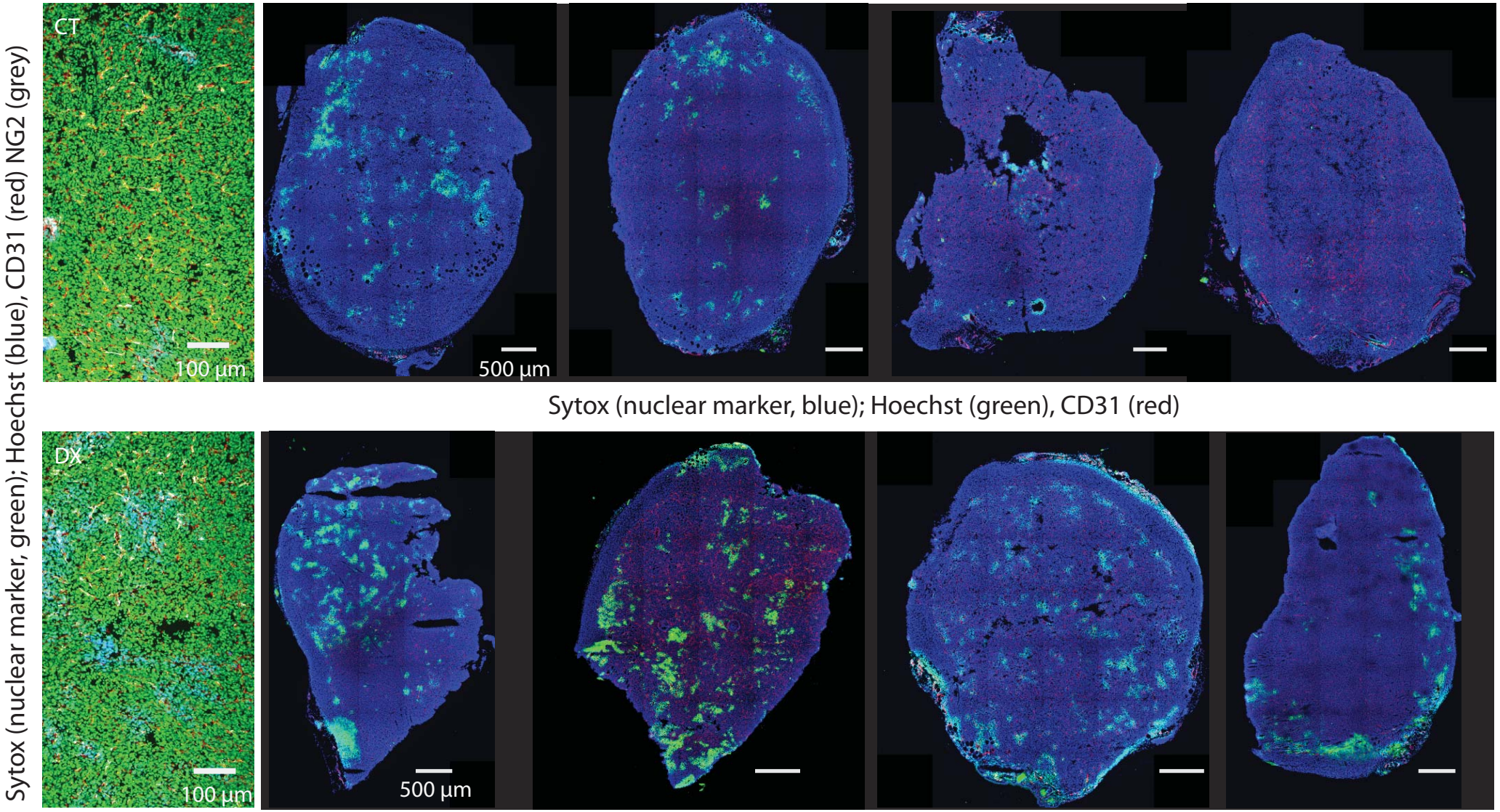

Vessel (CD31) Area
E0771 day 4
4T1 day 4
4T1 day 6
4T1 day 10
E0771 day 10
Staining per DAPI
CT
DX
R
DXRDX
Basement Membrane (COL IV) Coverage
Colocalization
0.0293
Pericyte (NG2) Coverage
0.0175

Supplementary Figure 8: Data of vessel (CD31), BM (Col IV) and pericyte (NG2) coverage from 4T1 and E0771 tumors. Control (CT), DX-2400 (DX), irradiation (R), or combination (DXR). Tumor type and day are indicated in the top graph of each row and are the same for each corresponding graph below. The first column shows all data for CD31 staining are, the second data for BM coverage and the third for pericyte coverage. Data are presented as median with the interquartile range (box) and maximum and minimum values (whiskers). Sample size (n) for E0771 day 4 n = 6 per group, E0771 day 10 n = 4 per group, 4T1 day 4 n = 6-7 per group, 4T1 day 6 n = 4-6 per group, and 4T1 day 10 n = 6-8 per group. P values determined by two-sided Mann-Whitney test.

OFDI images from control (CT) and DX-2400 (DX) treated tumors at day 4

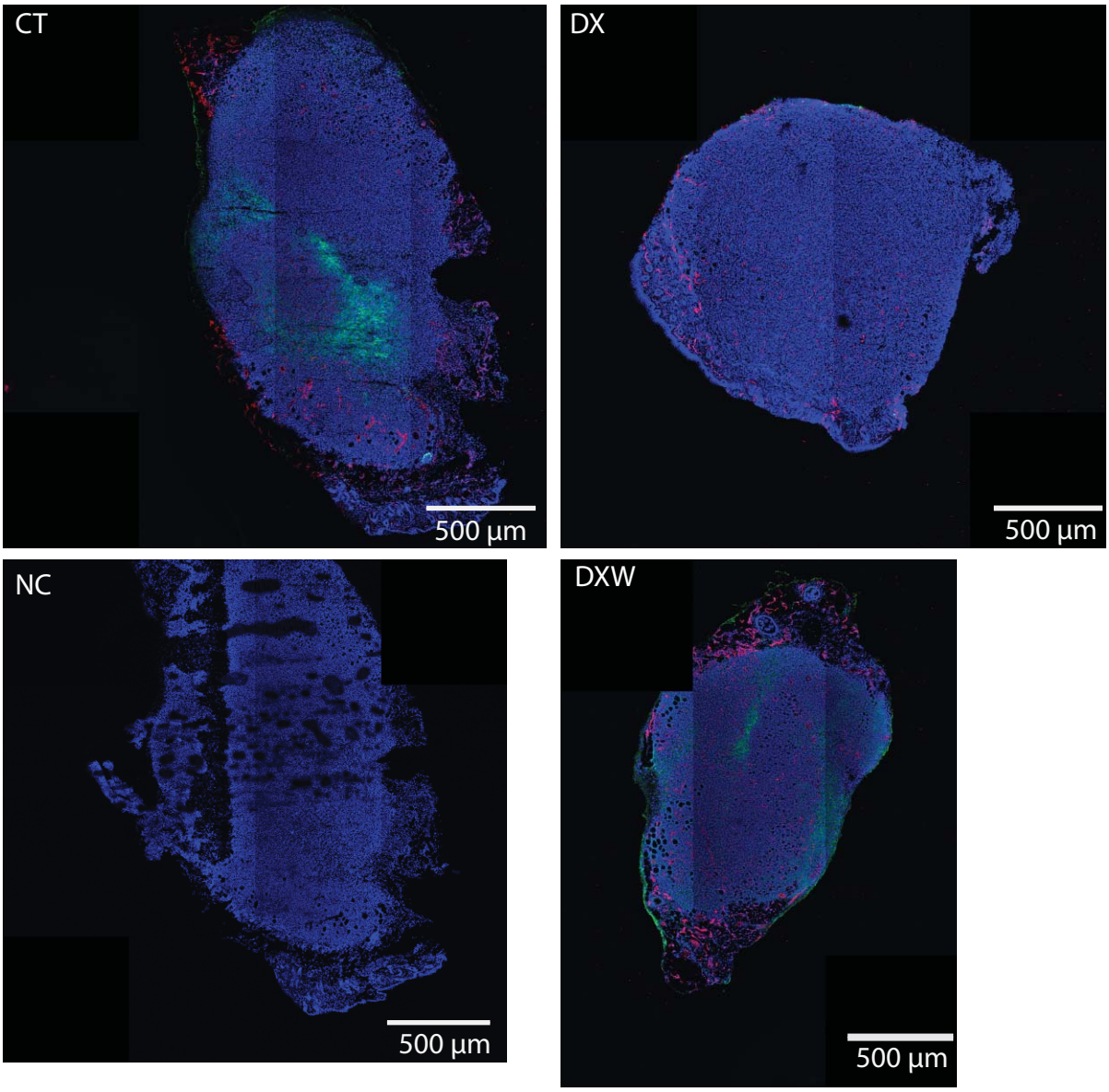


Supplementary Figure 9: Representative images of day 4 4T1 tumors in nude mice perfused by Hoechst. Two high magnification images show Hoechst (blue) in control and treated tumors also stained with SYTOX (nuclear marker, green), CD31 (vessels, red) and NG2 (pericytes, grey). The scale bar in the bottom right of each high magnification image indicates 100 μm. In addition, four low magnification tumor-wide mosaics also illustrate the general increase in Hoechst with DX-2400 treatment – in these images to allow better visualization of hoechst across the tumor hoechst had been pseudocolored green and SYTOX to blue (CD31 is shown in red and pericytes have been omitted).

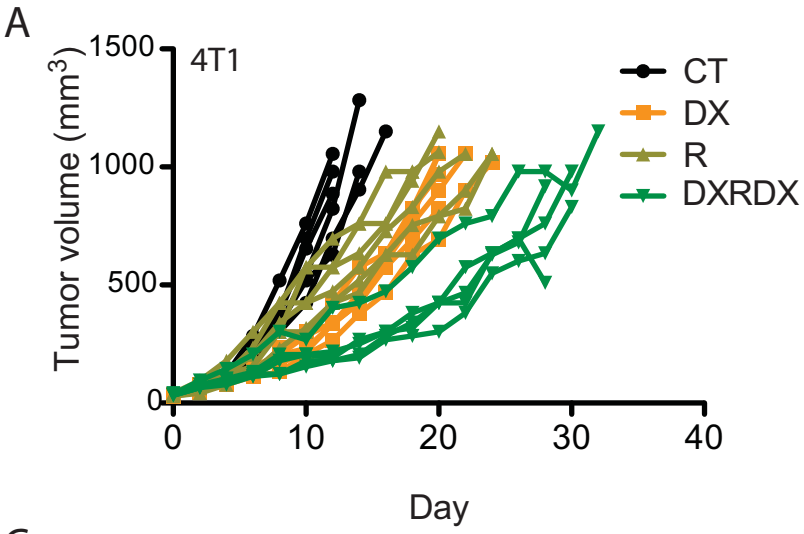

CT
100 µm
500 µm
Sytox (nuclear marker, blue); Hoechst (green), CD31 (red)
Sytox (nuclear marker, green); Hoechst (blue), CD31 (red) NG2 (grey)
DX
100 µm
500 µm

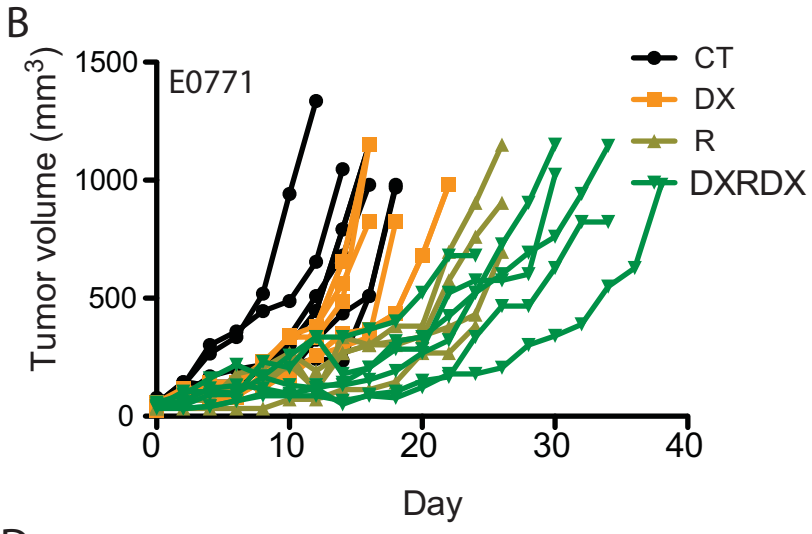
Supplementary Figure 10: Representative images of CA9 staining in 4T1 tumors in nude mice at day 4. Mosaic images for control (CT), DX-2400 (DX), DX-2400 in combination with 1400W (DXW), and a negative staining control (no secondary antibody; NC) are shown. Scale bar in the bottom right of each image indicates 500 μm.

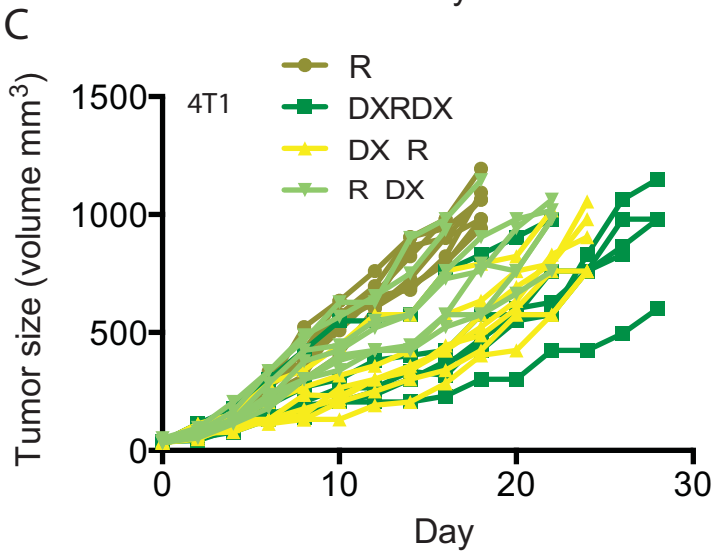
CA9 (green), CD31 (red), DAPI (blue)

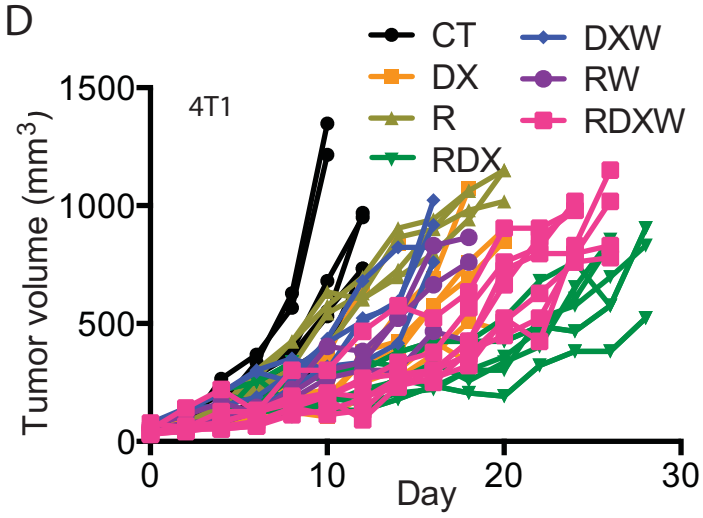


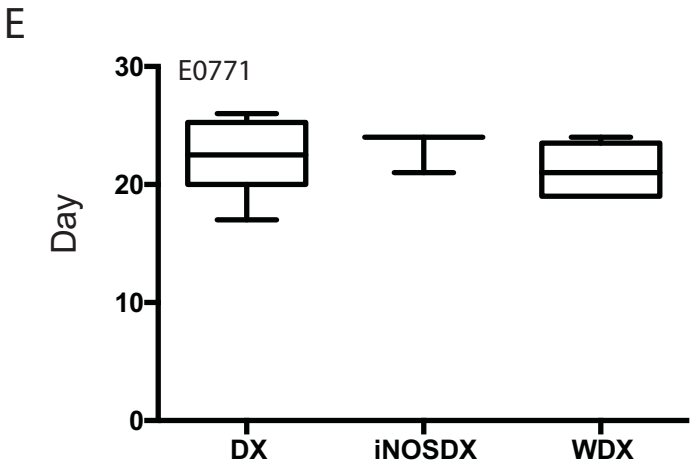
Supplementary Figure 11: A-F) Tumor growth curves for individual mice and days to grow to 800 mm^3. Mice were treated with IgG control (CT), DX-2400 (DX), radiation (R), and the combination (DXRDX) for 4T1 tumors **(A)** and E0771 tumors **(B)**. The effects of 10 DX-2400 injections (DXRDX), was also compared with 3 initial injections of DX-2400 (DX_R; DX on days 0, 2, and 4 with radiation on days 4, 5, and 6) and 7 post-irradiation injections (R_DX) as well as treatments with 1400W in combination with each monotherapy (DXW and RW) and with the triple combination (DXRDXW) and, for E0771 tumors, in iNOS knockout mice (iNOSDX). Tumor growth curves for 4T1 tumors in nude mice to assess importance of the timing of DX-2400 treatments in relation to radiation **(C)**. Mean tumor growth curves for 4T1 tumors in nude mice treated with established treatments and with combinations of 1400W **(D)**. The time (days) taken for E0771 tumors to grow to 800 mm^3 **(E)** and the matching tumor growth curves **(F)** following treatment with DX-2400 alone or in combination with 1400W or in iNOS-/- mice (i.e. lacking host iNOS). Data are shown for individual mice, or in the case of panel E as median with the interquartile range (box) and maximum and minimum values (whiskers). Panel A, n = 5-6 per group, Panel B, n = 4-6 per group, Panel C, n = 5-8 per group, Panel D = 4-7 per group, except RW (n = 3), panel E and F n = 3-5 per group.

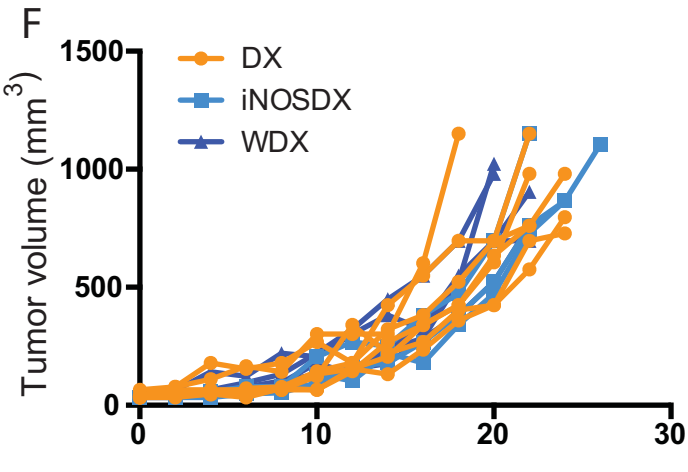

A
4T1
Tumor volume (mm3)
1500
1000
500
0
0
10
20
30
40
Day
CT
DX
R
DXRDX


B
E0771
Tumor volume (mm3)
1500
1000
500
0
0
10
20
30
40
Day
CT
DX
R
DXRDX

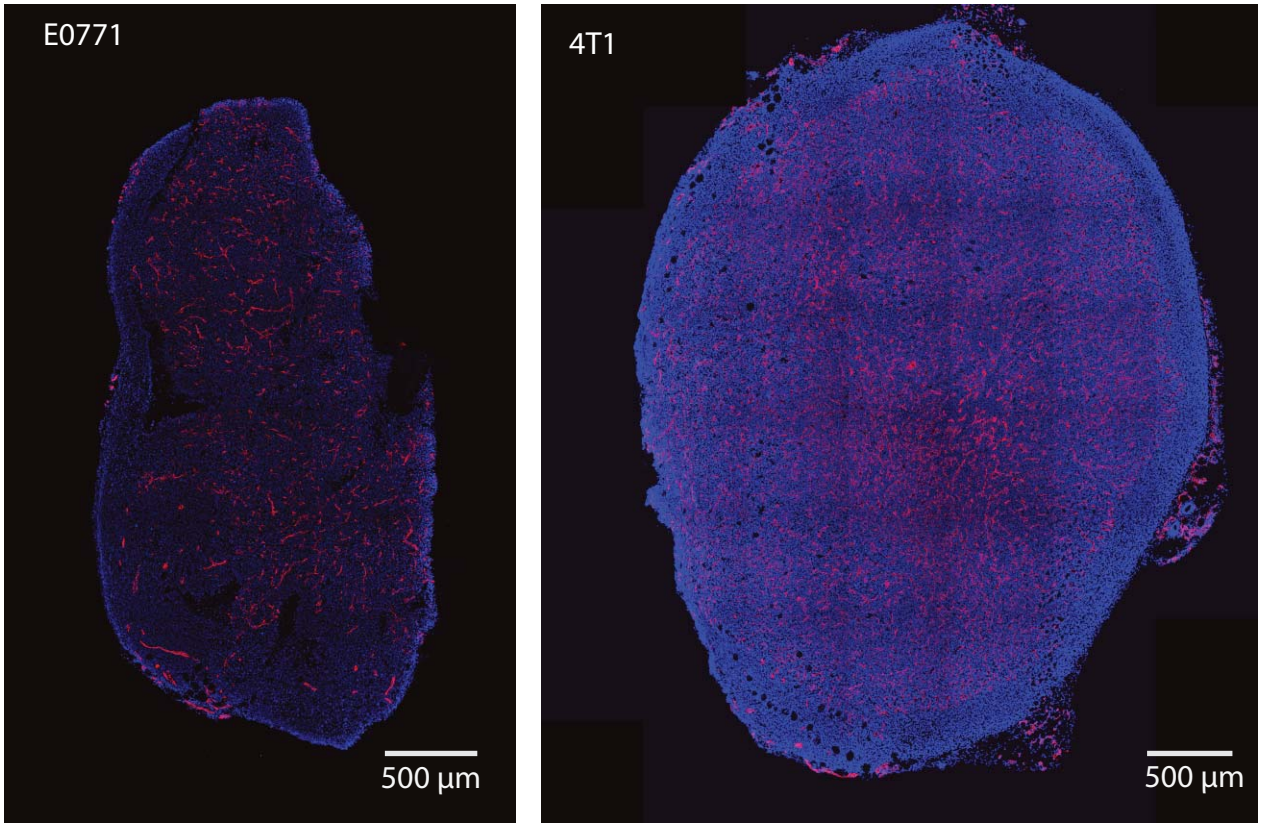

C
4T1
Tumor size (volume mm3)
1500
1000
500
0
0
10
20
30
Day
R
DXRDX
DX R
R DX


D
4T1
Tumor volume (mm3)
1500
1000
500
0
0
10
20
30
Day
CT
DX
R
RDX
DXW
RW
RDXW


E
E0771
Day
30
20
10
0
DX
iNOSDX
WDX


F
Tumor volume (mm3)
1500
1000
500
0
0
10
20
30
DX
iNOSDX
WDX

Supplementary Figure 12: Representative control E0771 and 4T1 tumors showing notable differences in endogenous (untreated) vascular density.


E0771
500 µm
4T1
500 µm