SUPPLEMENTARY INFORMATION AND FIGURE LEGENDS

Materials and Methods

Mice

FVB mice were obtained from Charles River Laboratory (Calco, Milan, Italy). Breeding pairs of FVB/MMTV-PyMT mice were a kind gift from Dr Zena Werb, UCSF, USA and established as a colony in the animal facility in the Medical School, University of Sheffield. The strain is maintained by breeding hemizygous males with FVB wild type females. The four main stages of tumor progression described previously for this model of mammary tumor development *(1)* were confirmed in our colony. In brief these classifications were: *alveolar hyperplasia* (4-6 wks), the pre-neoplastic stage, basement membrane still intact with numerous branching alveoli at the terminal ductal lobular unit; *adenoma* (7-8 wks), the advanced stage of pre-malignancy, the outline of the tumor is still well defined; *early adenocarcinoma* (8-12 wks) a state of early malignancy showing enlarged cells and a loss of basement membrane although still relatively well defined; *late adenocarcinoma* (10-12 wks) the advanced malignancy, displaying extensive neoplasia and solid sheets of epithelial cells throughout. Mammary tumors were excised from 10-12 weeks old mice and found to be late stage carcinoma with no detectable signs of pulmonary metastasis. For all experiments, one tumour per mouse from the same mammary gland (mammary gland three) was used for the analyses.

FVB/*Tie2*-HSV-tk transgenic mice were generated as described previously by De Palma et al. (2). All procedures involving mice were conducted in accordance with either: (i) UK Home Office regulations regarding the use and care of experimental animals and were approved by the University of Sheffield Ethical Committee, or (ii) the Animal Care and Use Committee of the Fondazione San Raffaele del Monte Tabor (IACUC 324, 335) and communicated to the Ministry of Health in Italy and local authorities in Milan.

Immunohistochemical labeling of tumor hypoxia

Bound pimonidazole (PIMO) was detected in tumor sections using Hypoxyprobe™-1MAb1, a monoclonal antibody IgG_1 (Millipore, Consett, UK). PIMO labelling was then quantified across whole tumor sections using a random point scoring system based on that described by Smith et al. (3). Briefly, a 25-point Chalkley grid eye-piece graticule was used to scan across whole tumor sections (50-120 fields per tumor, as determined by section size) using a x20 microscope objective and x10 eyepiece. For each region, the number of points out of 25 falling on hypoxic cells was determined and the summed number of points falling on these areas for each tumor was expressed as % of the total number of points (i.e. % of the whole tumor area).

Histological assessment of tumor necrosis

Five micron, paraffin wax sections were cut, de-waxed, re-hydrated and stained with haematoxylin and eosin to enable areas of tumor necrosis to be readily visualized using morphological criteria - reduced cellular density, pale cytoplasm and pyknotic nuclei or completely disrupted cells, with or without red blood cell infiltration. Necrosis was then quantified using Chalkley point counting as described above and expressed as a % of the tumor area.

Immunofluorescent analysis of F4/80, TIE2, CD31, MMP9 & CXCL12

Frozen tumor sections (7 μm) were blocked with 15% rabbit or goat serum in Tris-buffered saline containing 0.5% Tween-20 (TBST) for 30 mins. This was followed by incubation with the following primary antibodies: rat anti-mouse F4/80-FITC (1:25; Abcam), rat anti-mouse TIE2-PE (1:50; eBioscience), rabbit anti-mouse MMP9 (1:500; a gift from Dr Zena Werb at UCSF, USA), rat anti-mouse CD31-APC (1:50; eBioscience) and rabbit anti-mouse CXCL12 (1:50; Novus Biologicals Ltd., Cambridge, UK) or their IgG-matched isotype controls for 30 mins at room temperature. Following three washes in TBST, Alexa 647-conjugated anti-rabbit secondary antibody (1:500; Invitrogen) was applied to sections exposed previously to unconjugated primary antibodies (i.e. MMP9 or CXCL12) for 30 mins. All sections were then stained with 30 nM DAPI for 2 mins at room temperature, and overlayed with Invitrogen's Prolong Gold Antifade. They were then viewed using a Zeiss confocoal microscope.

The total number of all F4/80$^+$ TAMs, F4/80$^+$TIE2$^-$ TAMs and F4/80$^+$TIE2$^+$ TEMs in 5 randomly selected, viable areas of frozen tumor sections was counted using a 20x objective. For the analysis of MMP9 expression by TEMs/TAMs, the number of MMP9$^+$F4/80$^+$TIE2$^+$ and F4/80$^+$MMP9$^+$TIE2$^-$ cells per field of view were counted (at 20x magnification) and the average expressed as the number of cells per field of view. Luminal area of CD31$^+$ vessels was defined as the product of the longest vessel diameter and its orthogonal counterpart. To do this, cursors were positioned manually for diameter measurements, using Zeiss confocal software and a x20 objective. An average CD31$^+$ luminal area was then calculated per tumor and results expressed as a mean of the average values +/– S.E.M for the whole group. This measure provided a means of quantifying changes in luminal area with treatment rather than a precise measure of luminal area of individual vessels, which were highly irregular in shape. To quantify CXCL12 staining, Z-stacks of five consecutive optical sections per tumor were captured using a Zeiss LSM 510 laser scanning confocal microscope. A vertical stack was merged using ImageJ software and CXCL12 fluorescence intensity measured by selecting the entire field of view and calculating average grayscale values of all pixels; 4-6 randomly selected fields of view at 20x from at least 6 tumors were included in the analysis.

Flow cytometry analysis of tumors

Tumors were minced and then digested in DMEM medium containing 10 mg/ml collagenase type IV (Fischer Scientific Ltd., Poole, UK). The mixture was incubated for 60 min at 37°C under gentle

agitation at 250 RPM. Following incubation, cells were filtered through a 70 µm nylon mesh, washed then resuspended in Hank's balanced salt soluation (HBSS) containing 0.5% bovine serum albumin (BSA). Cells were blocked with Fc-receptor block (Miltenyi, Bergisch Gladbach, Germany) for 30 min, prior to the addition of antibodies: F4/80-FITC (1:25; eBioscience), TIE2-PE (1:50; eBioscience), and anti-CXCR4 (R&D Systems). To reveal anti-CXCR4 antibodies, in some experiments the cells were washed and further incubated with Alexa 647-conjugated anti-rabbit secondary antibody (1:500; Invitrogen). Analysis was performed using a BD Biosciences LSRII (BD Biosciences, Oxford, UK) and FlowJo software (Tree Star, Inc.). Compensation was calculated by CompBeads (BD Biosciences) according to the manufacturer's instructions. Gates were drawn around a viable cell population and marker$^+$ cells determined using the appropriate isotype controls. Experiments were repeated at least three times.

Non-Linear Mixed Effects (NLME) model used for the data in Figure 2E

Analysis of tumor growth was performed in R-statistical software (version 2.9.1; see http://www.R-project.org). Significance level was chosen at $\alpha = 0.05$. Briefly, tumor volume observed over time (N_{tij}) provides a measure of tumor evolution, while time and treatment group represent the independent variables. We fitted the data using a Non-Linear Mixed Effects (NLME) model to account for unobserved random effects that may influence the pattern of tumor responses to various treatments.

The NLME is given by the following formula:

$$\mathrm{Log}_2\,(N_{tij}) = \beta_0 + \beta_{1j} group_j + \beta_2 time + \beta_{3j} time * group_j + \upsilon_i + \varepsilon_{tij}$$

Where N_{tij} provides a measure of tumor evolution under logarithmic transformation of tumor volume measurements; υ_i is a subject-specific component, constant over time; ε_{tij} is an error component that varies throughout time. Tumor volume is used after logarithmic transformation. The model assumes random intercepts and time-group interactions. Coefficient estimates were obtained from loglinear fits.

References cited above:

1. Guy CT, Cardiff RD, Muller WJ Induction of mammary tumors by expression of polyomavirus middle T oncoprotein: a transgenic mouse model for metastatic disease. *Mol Cell Biol* 1992; 12:954-961.
2. De Palma M, Venneri MA, Galli R Tie2 identifies a hematopoietic lineage of proangiogenic monocytes required for tumor vessel formation and a mesenchymal population of pericyte progenitors. *Cancer Cell* 2005; 8:211-226.
3. Smith K A, Hill S A, Begg A C, Denekamp J. Validation of the fluorescent dye Hoechst 33342 as a vascular space marker in tumours. *Br J Cancer*, 57: 247-253, 1988.

Supplementary Figure Legends

Supplementary Figure 1. Flow cytometry analysis of TEMs. Enzymatically dispersed, saline- and CA4P-treated MMTV-PyMT tumors show increase in F4/80^{+}TIE2^{+} TEMs 24 hs after 50 mg/kg CA4P. N = 5-6 mice per group.

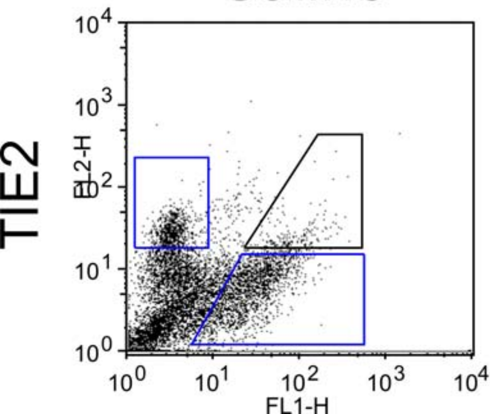
Supplementary Figure 2. Differential expression of MMP9 by TEMs and TIE2^{-} TAMs in MMTV-PyMT and N202 mammary tumors. (**A**) Frozen MMTV-PyMT tumor sections stained with anti-F4/80 (green), anti-TIE2 (red) and anti-MMP9 (white). Yellow arrows highlight MMP9^{+} TEMs. (**B**): Total number of MMP9^{+} TEMs/field (left panel) and percentage (%) of TAMs and TEMs expressing detectable MMP9 (right panel). All data are means +/– SEMs. **(C)** Frozen N202 tumor sections stained with anti-F4/80 (green), anti-TIE2 (red) and anti-MMP9 (white). Yellow arrows highlight MMP9^{+} TEMs. (**D**)**.** The total number of MMP9^{+} TEMs/field (left panel) and the % of TAMs and TEMs expressing detectable MMP9 (right panel). All data are means +/– SEMs. Scale bars = 100 µm. N=5 mice per group. *P <0.05, **P <0.01, ***P <0.001.

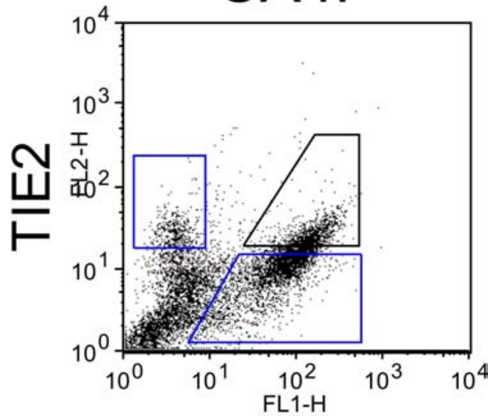
Supplementary Figure 3. CXCL12 expression analysis in the tumors. CXCL12 immunoflorescence (red) was increased in MMTV-PyMT tumors 24h after injection with 50 mg/kg CA4P. N = necrosis. Bar =100 µm.

Supplementary Figure 4. Effects of CA4P on tumor blood vessels and infiltrating neutrophils. (**A**) The appearance, number and luminal size of CD31^{+} blood vessels, and (**B**) Gr1^{+} cells/field in N202 tumors excised from mice bearing bone marrow transplants from *Tie2*-tk transgenic mice and given three daily injections of saline or 50mg/kg CA4P alone, or 50mg/kg CA4P and either GCV (to deplete TEMs) or saline. In panel A, vessels were examined 24h after final injection, whereas in panel B*, left panels:* 24h and *Right panels:* 72h after the final injection. Bars = 200 µm. All data are means +/- SEMs. N=6 mice per group. *P <0.05, **P <0.01, ***P <0.001.

Supplementary Figure 5. **Effects of conditional TEM depletion and/or CA4P on the growth of N202 tumors.** Wild-type FVB mice were transplanted with transgenic *Tie2*-tk BM cells 6 weeks before N202 tumor cell injection. Thirteen days after N202 tumor challenge, transplanted mice were treated with GCV (100 mg/kg) daily for 12 days or remained untreated. Nineteen days (day 19) after N202 tumor cell inoculation, GCV-injected and non-GCV injected mice were randomly allocated to receive CA4P (50mg/kg) or saline vehicle for 3 days. Means +/- SEMs. Statistical analysis performed at day 24 using Two-way ANOVA with Bonferroni post-test. $P < 0.001$ for Saline vs. GCV or GCV + CA4P; $P < 0.05$ for Saline vs. CA4P (n = 7 mice/group).

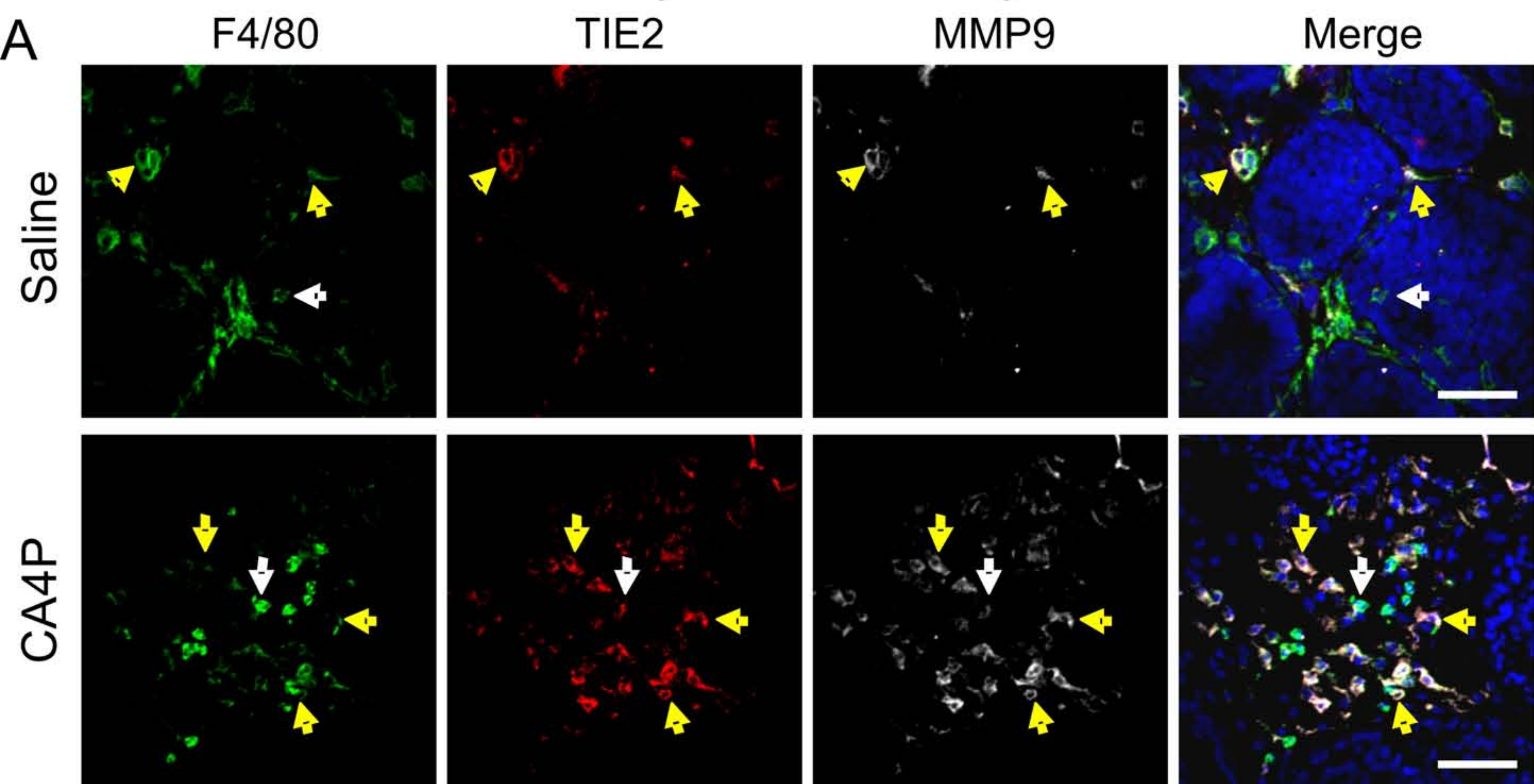
Suppl. Fig. 1

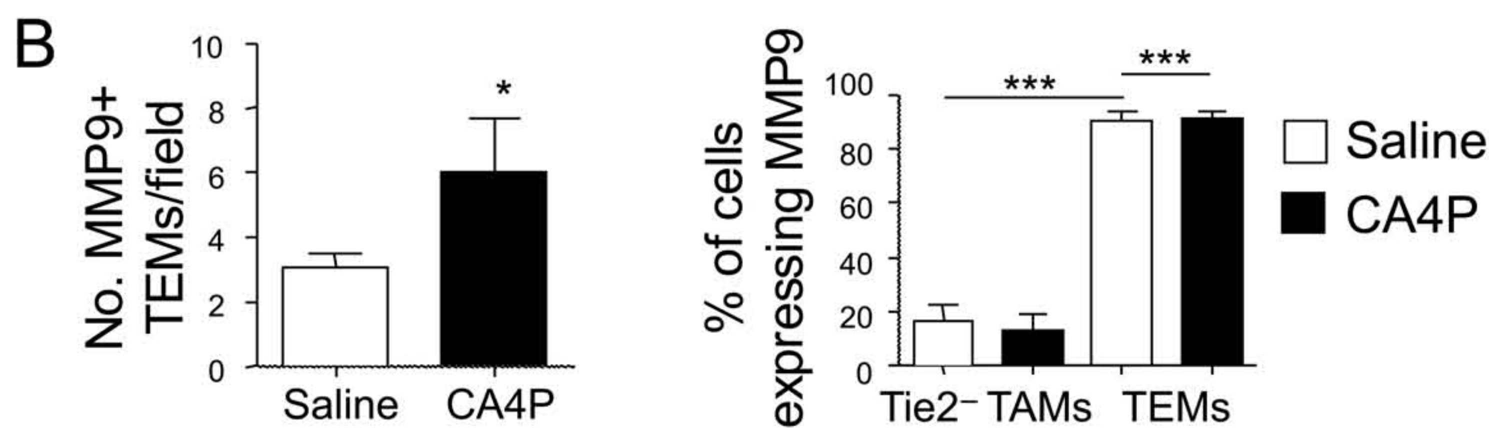




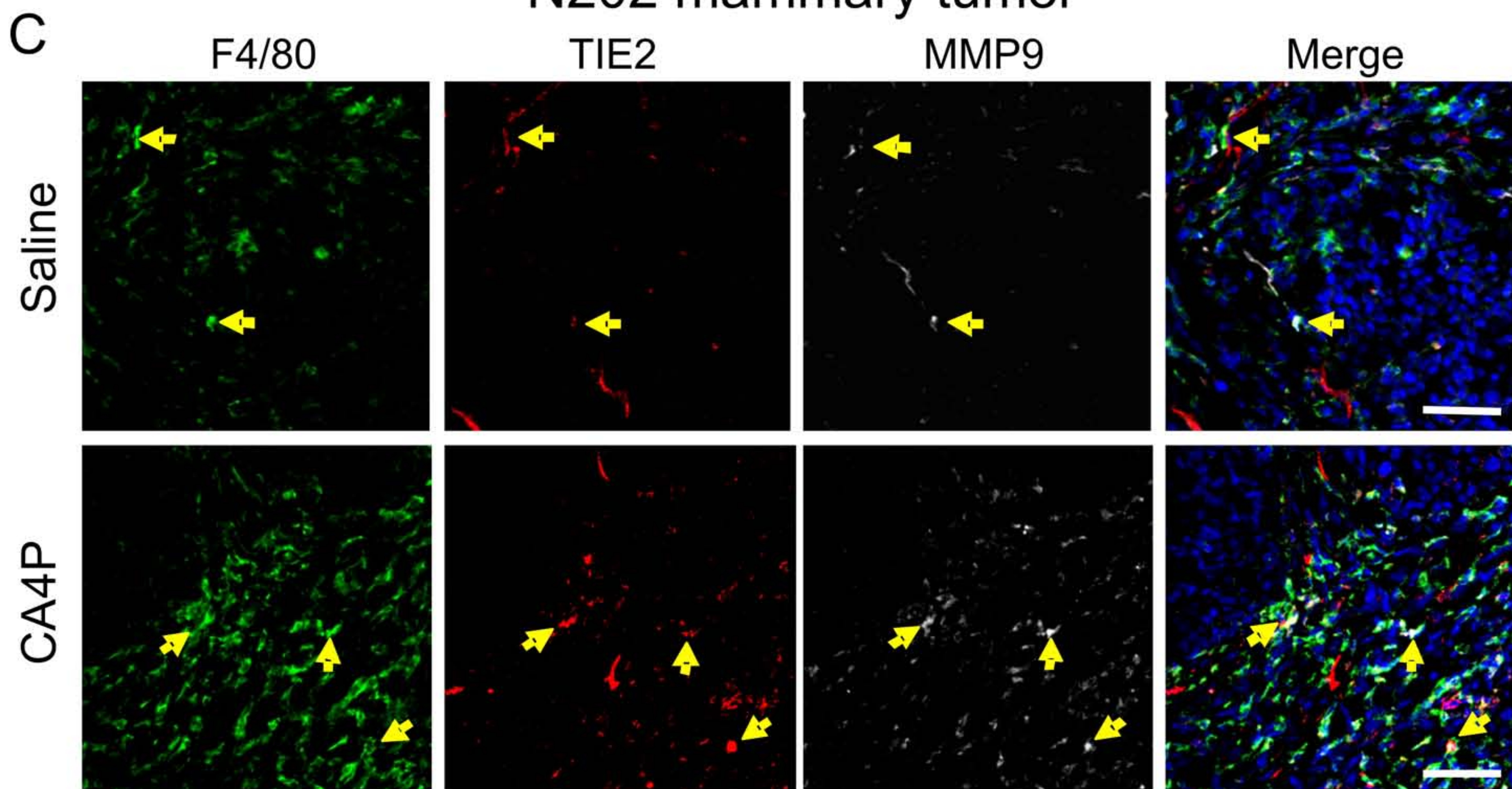
MMTV-PyMT mammary tumor

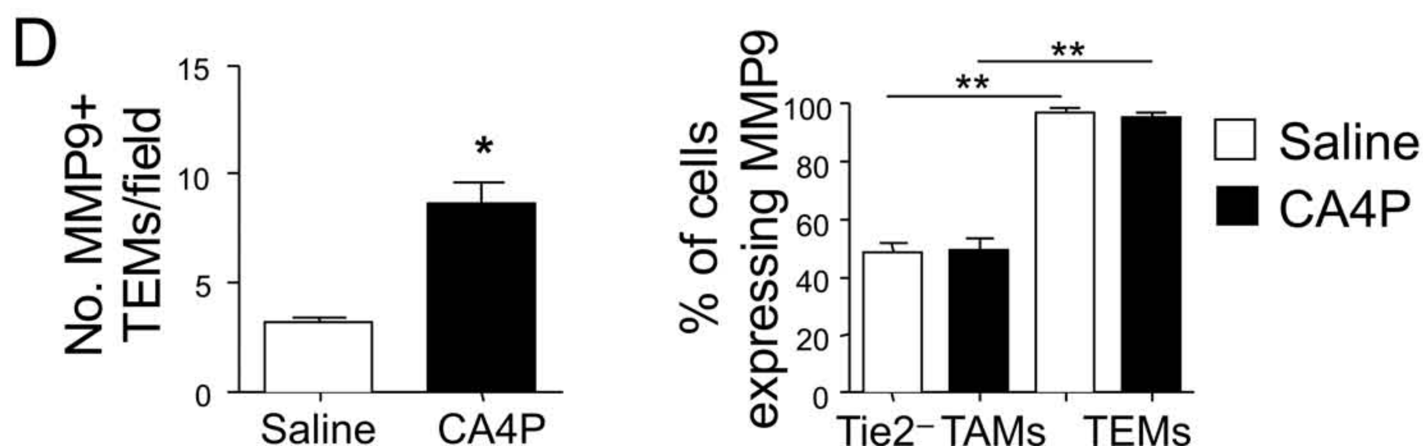
Suppl. Fig. 2





N202 mammary tumor

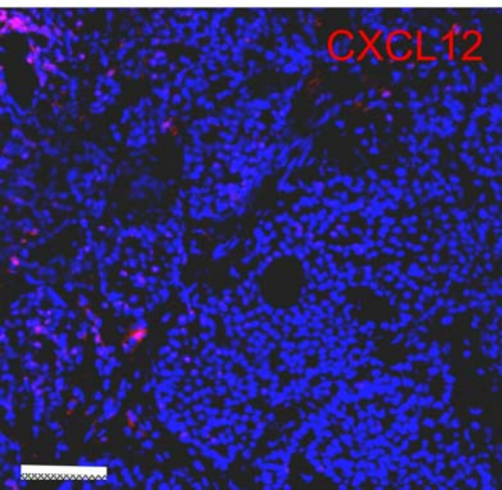


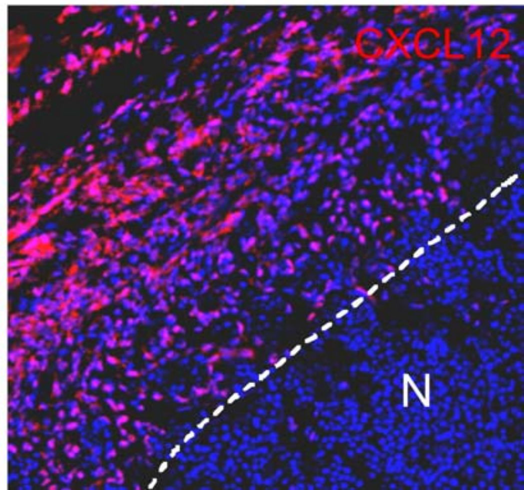


Suppl. Fig. 3

Saline

CA4P

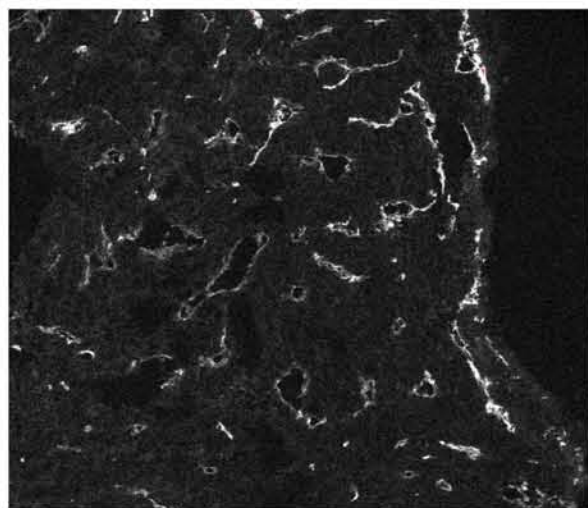


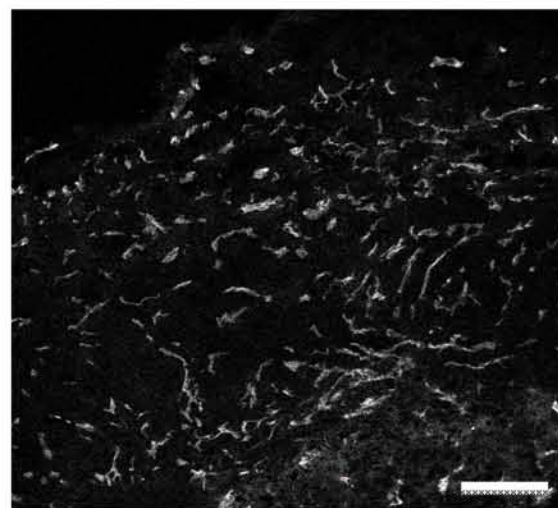


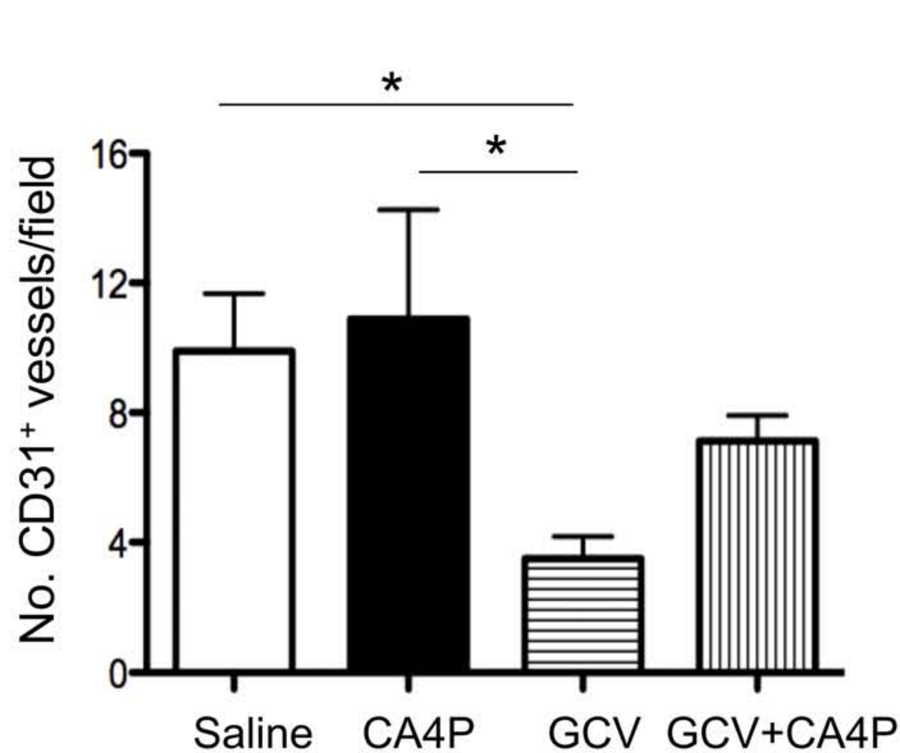
A

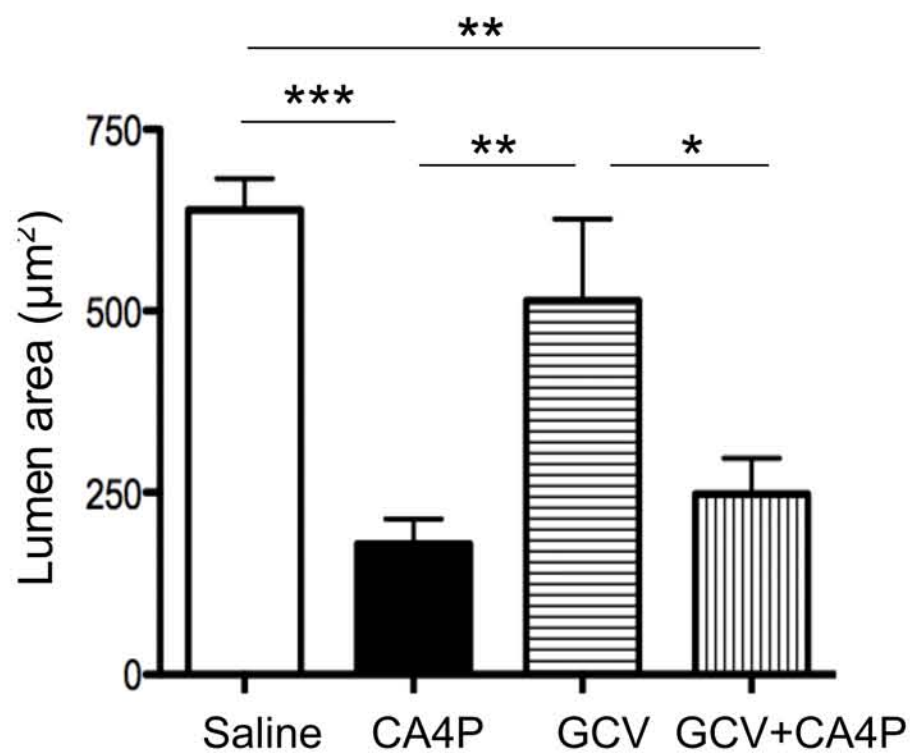
Saline

CA4P

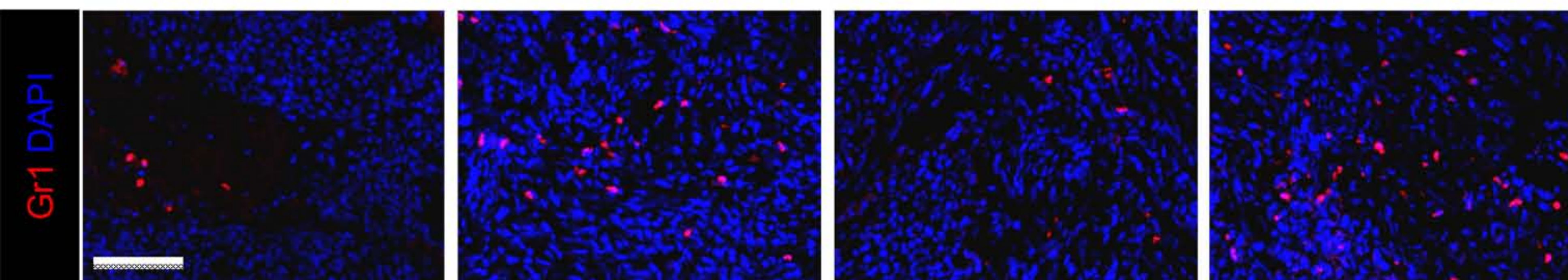


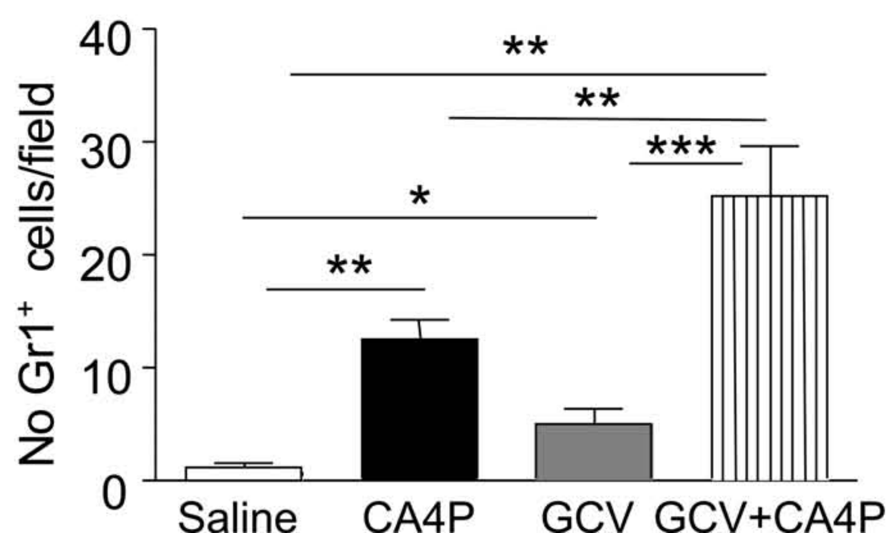


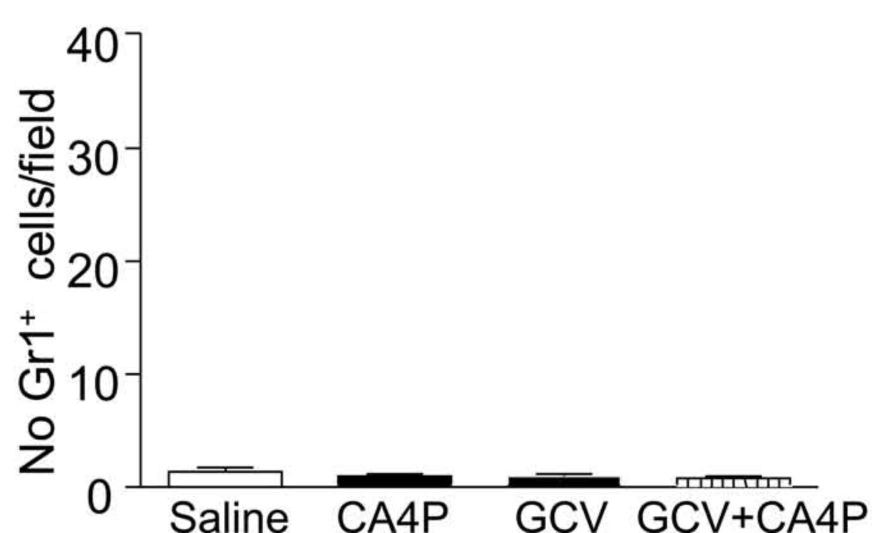


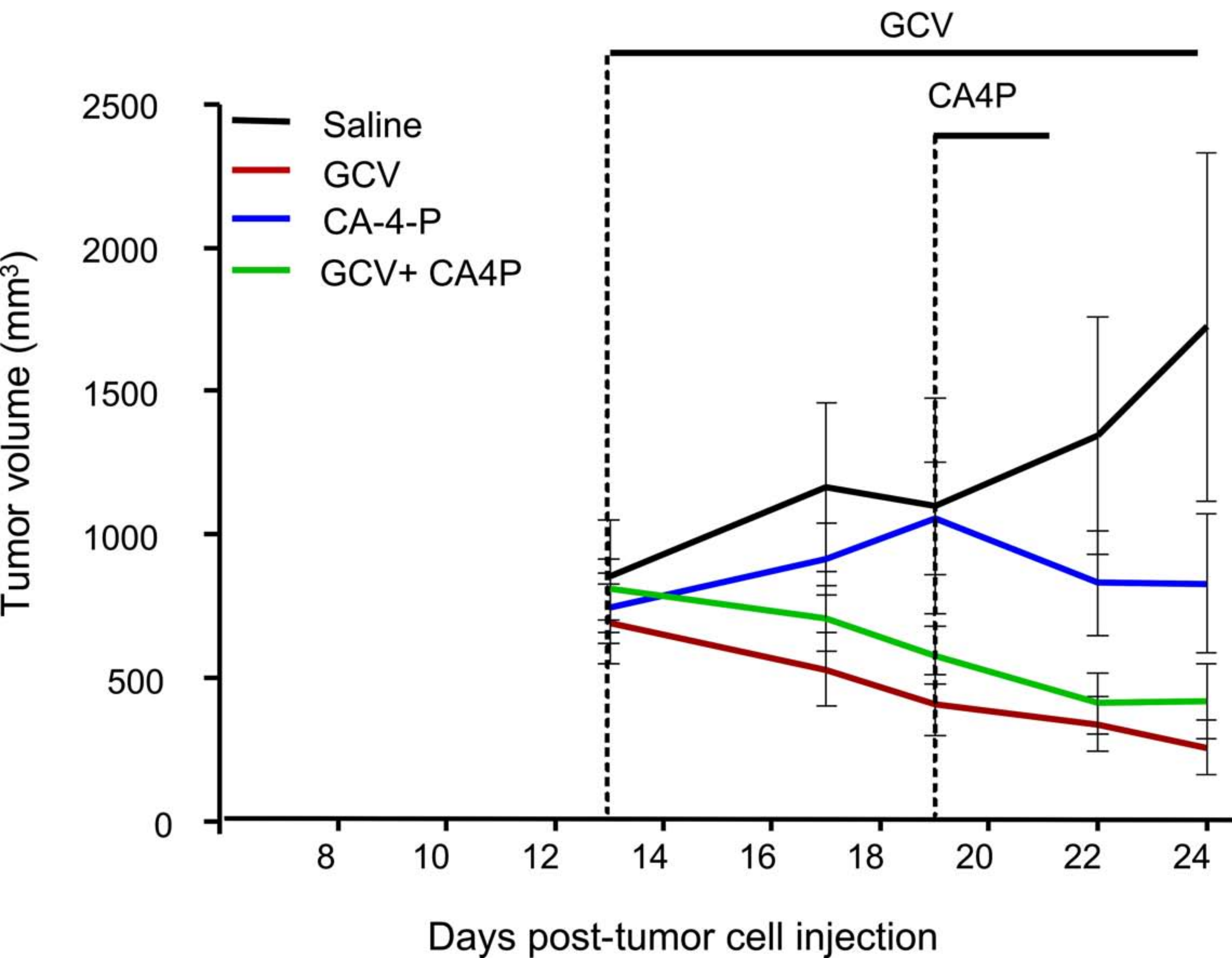


B








GCV
CA4P
Saline
GCV
CA-4-P
GCV+ CA4P
Tumor volume (mm3)
2500
2000
1500
1000
500
0
8
10
12
14
16
18
20
22
24
Days post-tumor cell injection