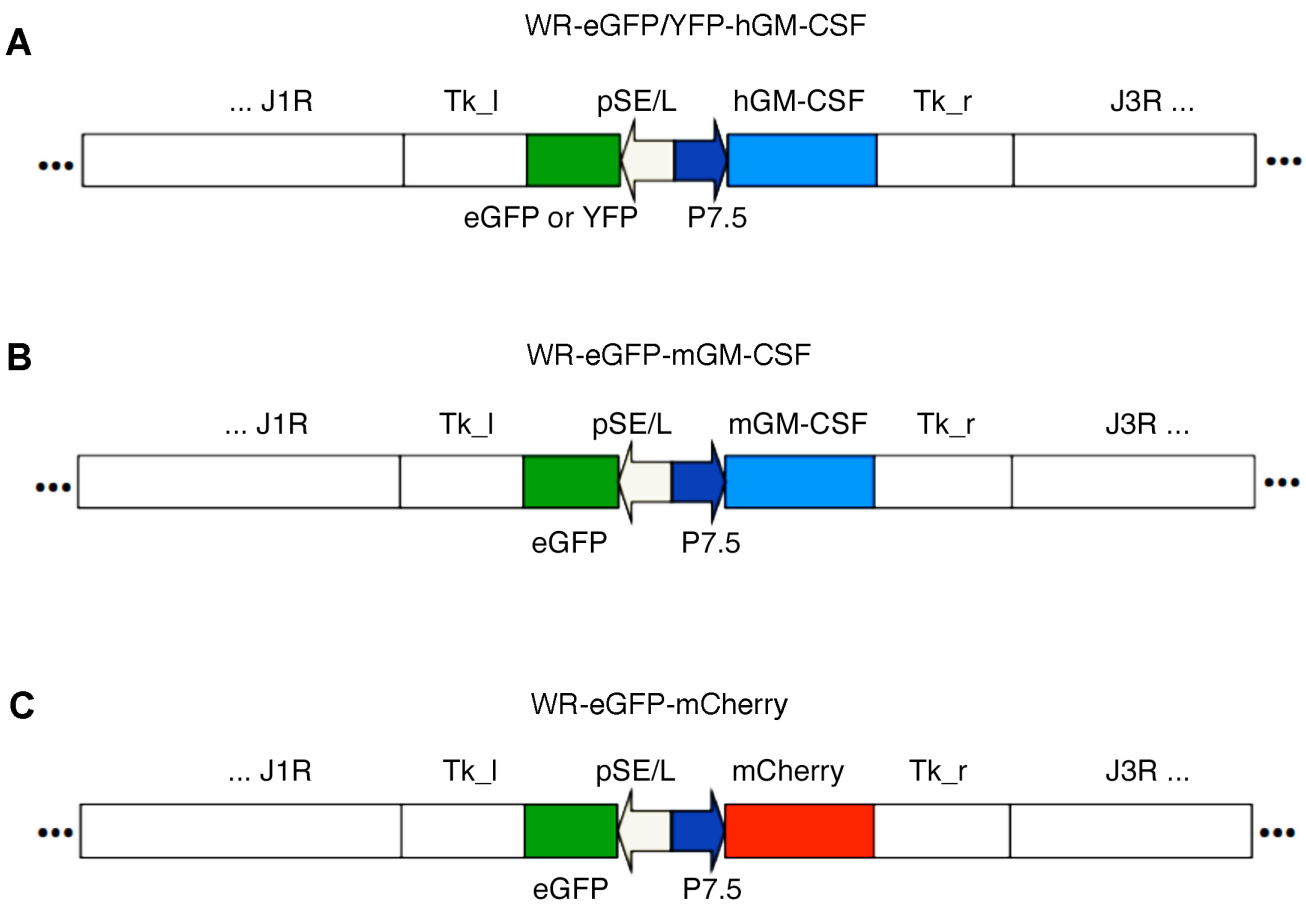
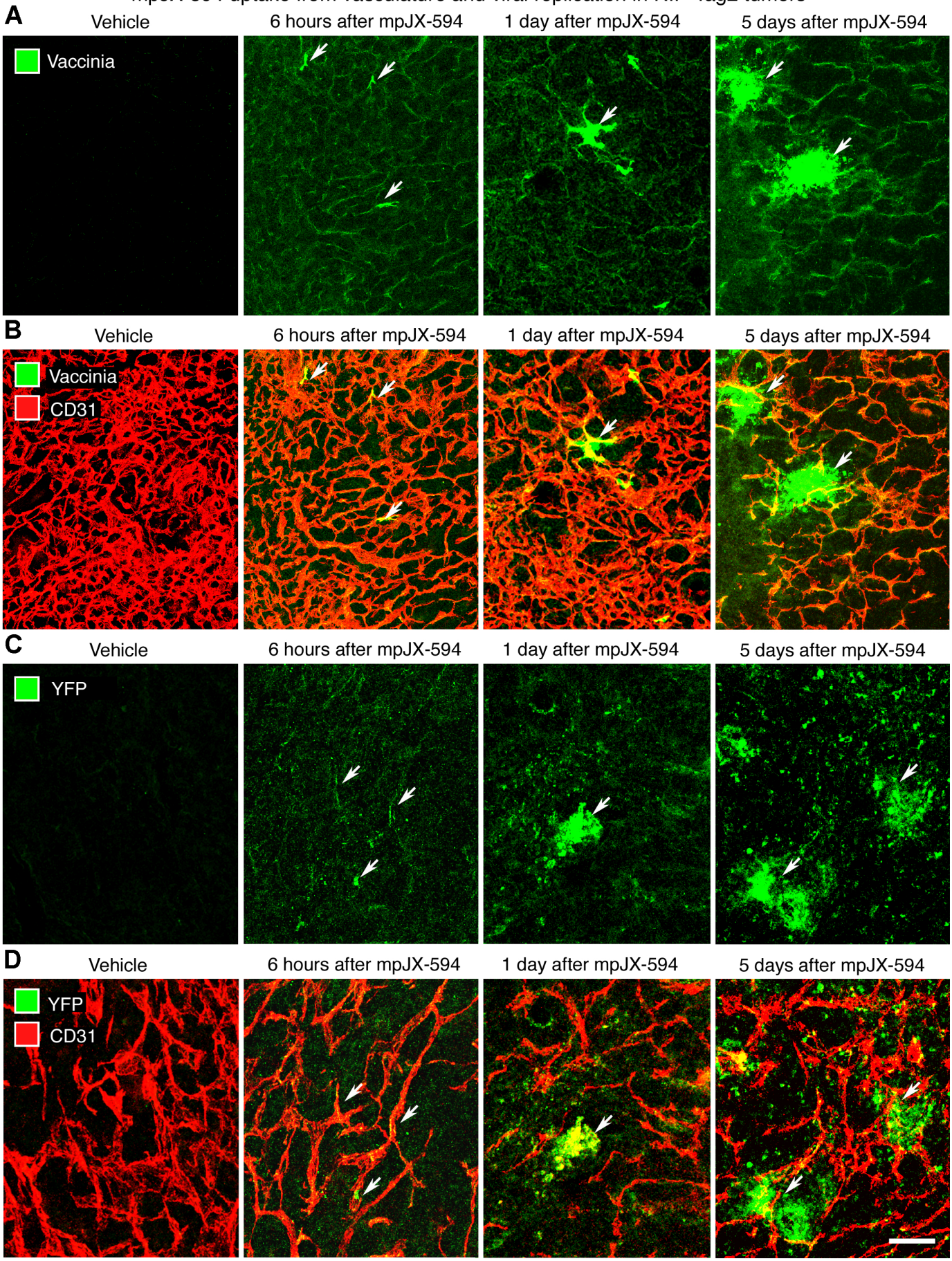
mpJX-594 vaccinia virus variants used to test contribution of GM-CSF expression



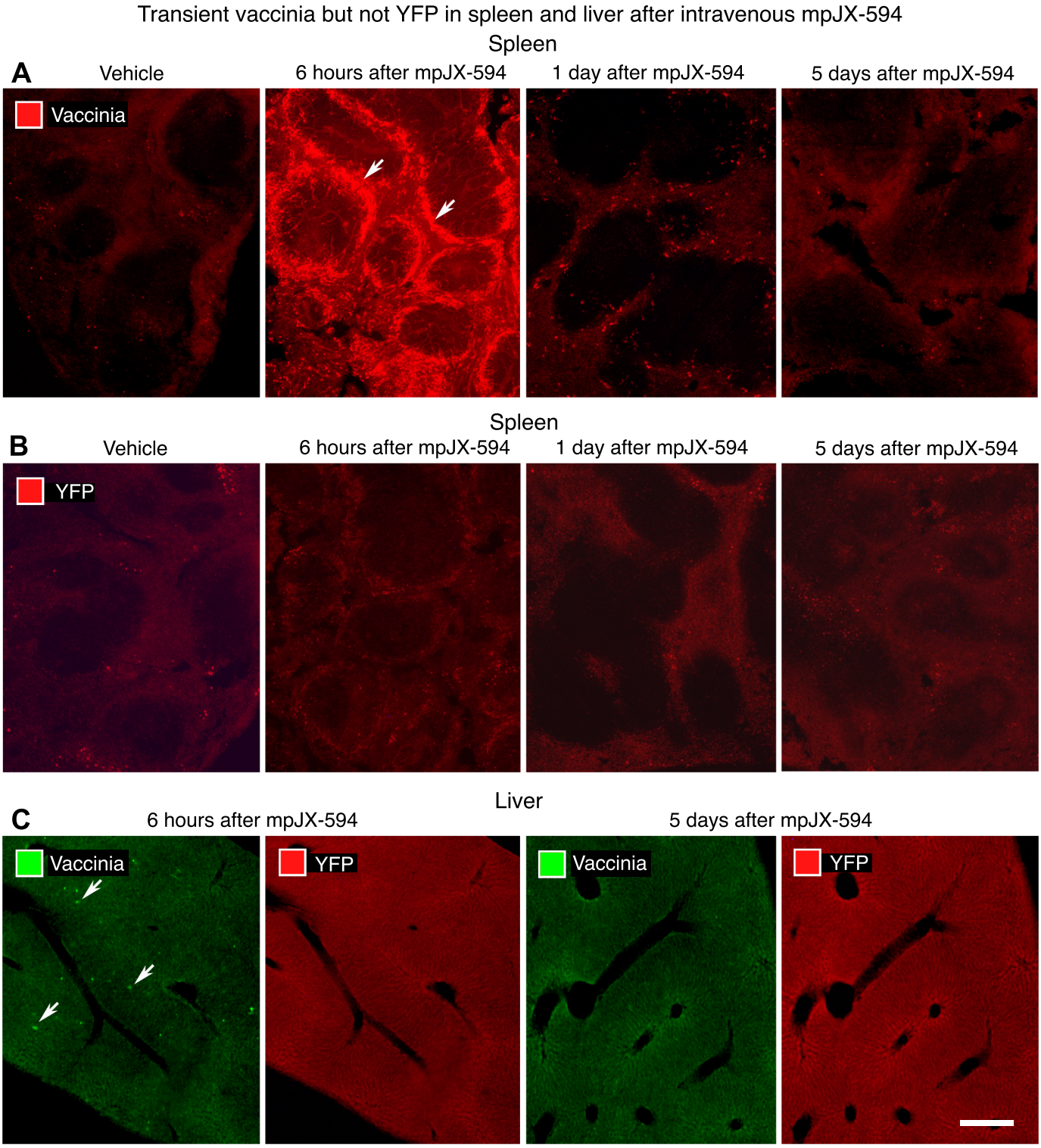
Supplemental Figure 1

Supplemental Figure 1. Three versions of mouse prototypes of JX-594 (mpJX-594) differing by the insertion cassette used to disrupt vaccinia thymidine kinase gene. All viruses were based on the Western Reserve (WR) strain, a mouse-adapted Wyeth strain vaccinia that was isolated through serial passage in mouse brain. WR-TK(-)-eGFP/YFP vaccinia viruses that express transgenes for (**A**) human GM-CSF, (**B**) mouse GM-CSF, or (**C**) mCherry instead of GM-CSF were generated by disrupting the vaccinia thymidine kinase gene (Tk) with an insertion cassette containing eGFP (enhanced green fluorescent protein) or YFP (yellow fluorescent protein) driven by a synthetic early/later promoter (pSel) and human or mouse GM-CSF or mCherry transgene driven by the p7.5 early/late promoter. J1R, gene for virion protein required for morphogenesis; J3R, gene for multifunctional poly-A polymerase subunit, cap methyltransferase, and transcription elongation factor.



Supplemental Figure 2

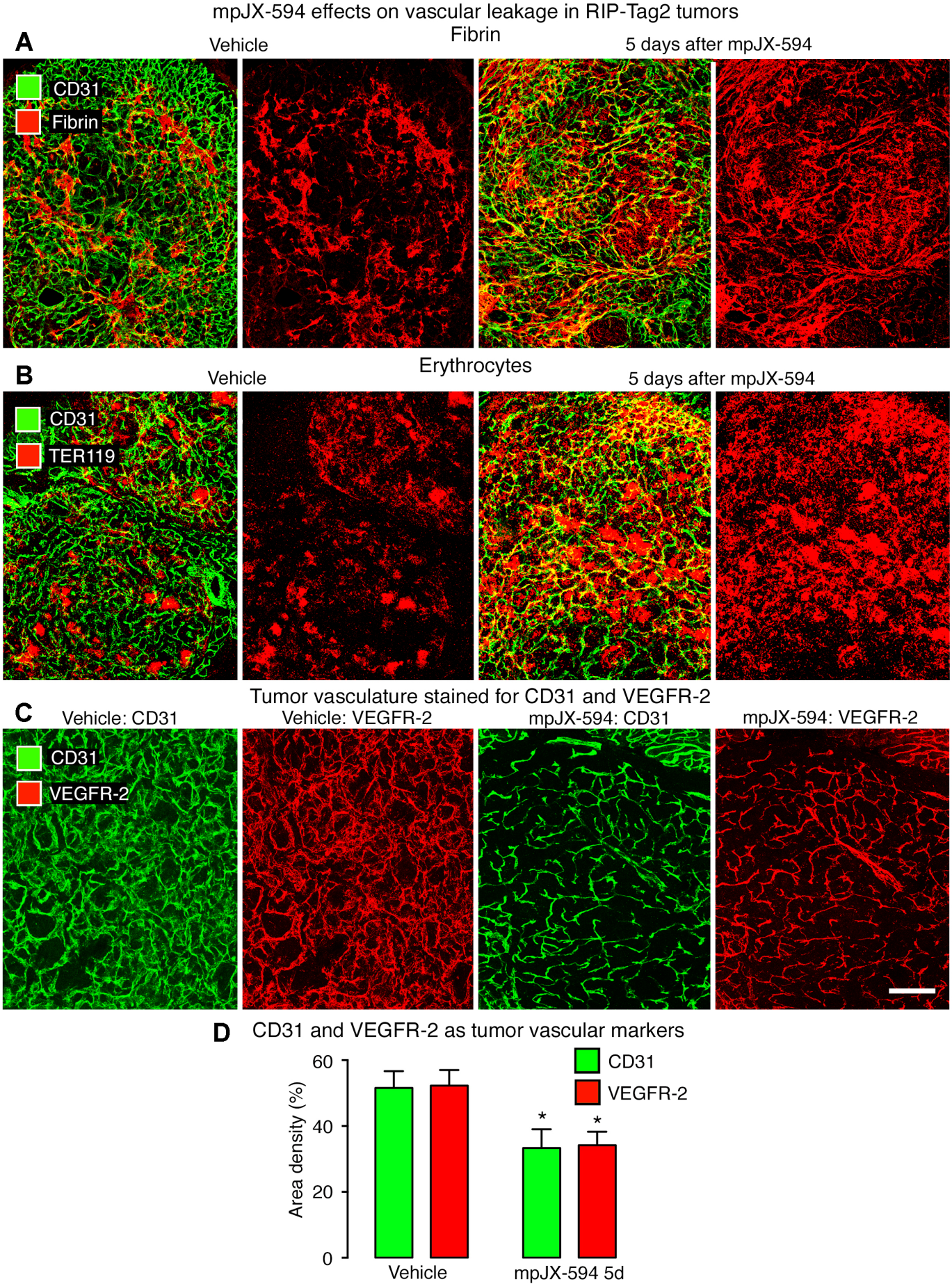
Supplemental Figure 2. mpJX-594 uptake from vasculature and replication in RIP-Tag2 tumors. A, B: Confocal micrographs of vaccinia immunoreactivity (green) in RIP-Tag2 tumors. No vaccinia is present after iv injection of vehicle, but 6 hours after mpJX-594, faint vaccinia is uniformly visible in blood vessels (**B**, CD31, red). Some vessels have strong staining (arrows). At 1 day, vaccinia is also located in small groups of cells near tumor vessels (arrow). At 5 days, larger patches of vaccinia are present (arrows). **C, D:** Yellow fluorescent protein (YFP, green) is absent after vehicle, but is visible in tumor vessels (**D**, CD31, red) at 6 hours after mpJX-594 (arrows), and in vessels and extravascular cells (arrows) at 1 and 5 days. Scale bar in D applies to all images: 100 μm in A, B and 50 μm in C, D.



Supplemental Figure 3

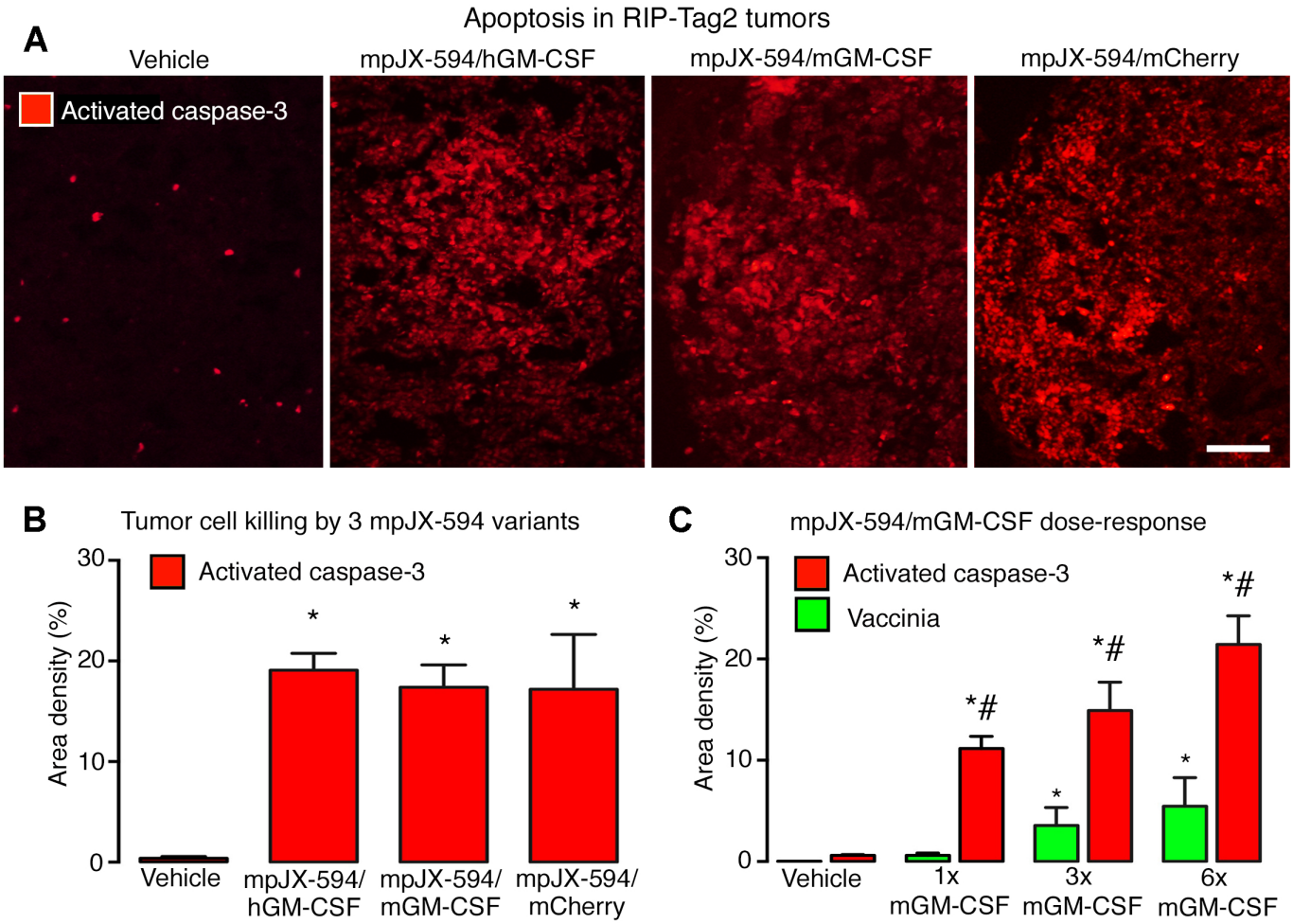
Supplemental Figure 3. mpJX-594: transient vaccinia but not YFP in spleen and liver.

Confocal micrographs of spleen or liver of RIP-Tag2 mice after iv injection of vehicle or mpJX-594; tissue prepared 6 hours, 1 day, or 5 days later. **A:** Spleen stained for vaccinia immunoreactivity (red). No vaccinia is evident after injection of vehicle as expected, but vaccinia is conspicuous (arrows) in splenic red pulp at 6 hours after mpJX-594, but little or none is present at 1 or 5 days. **B:** Staining of spleen for yellow fluorescent protein (YFP, red) as an indication of mpJX-594 viral replication. No YFP staining is evident at any time point. **C:** Liver stained for vaccinia (green) and YFP (red). Faint vaccinia staining is evident in liver at 6 hours (**C** left, arrows) but not at 5 days (**C** right). No YFP is visible at either time point. Scale bar in C applies to all images: 160 μm.



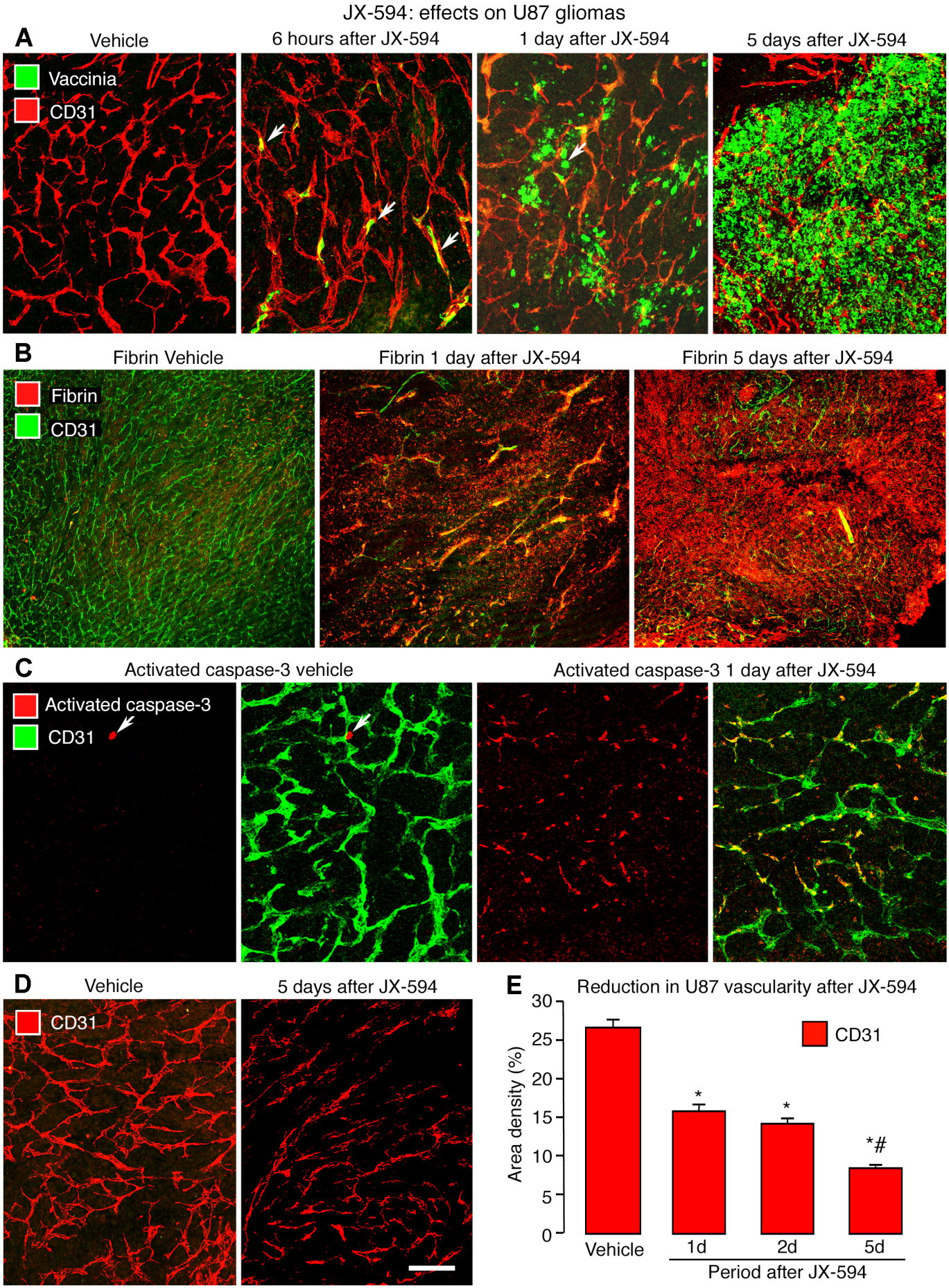
Supplemental Figure 4

Supplemental Figure 4. mpJX-594: effects on extravasation of fibrin and erythrocytes in RIP-Tag2 tumors. **A, B:** Regions of extravasated fibrin/fibrinogen (red, **A**) and erythrocytes (TER119 staining, red, **B**) are scattered in RIP-Tag2 tumors of control (vehicle) mice but are much greater and more widespread at 5 days after mpJX-594. Most erythrocytes in the vehicle-treated tumor are concentrated in blood lakes, which are separate from the vasculature (CD31, green) and are not washed free of blood by vascular perfusion of fixative (4). **C:** Comparison of tumor vasculature co-stained for CD31 and VEGFR-2 at 5 days after iv injection of vehicle or mpJX-594. The tumor vasculature is similar when assessed by CD31 or VEGFR-2. Tumors are highly vascular after vehicle and less vascular after mpJX-594. **D:** Measurements of tumors co-stained for CD31 and VEGFR-2 show similar tumor densities assessed under baseline conditions (vehicle) and similar reductions at 5 days after mpJX-594. ANOVA. *$P < 0.05$ for difference compared to vehicle (n = 5 mice/group). Scale bar in D applies to all images: 200 μm.



Supplemental Figure 5

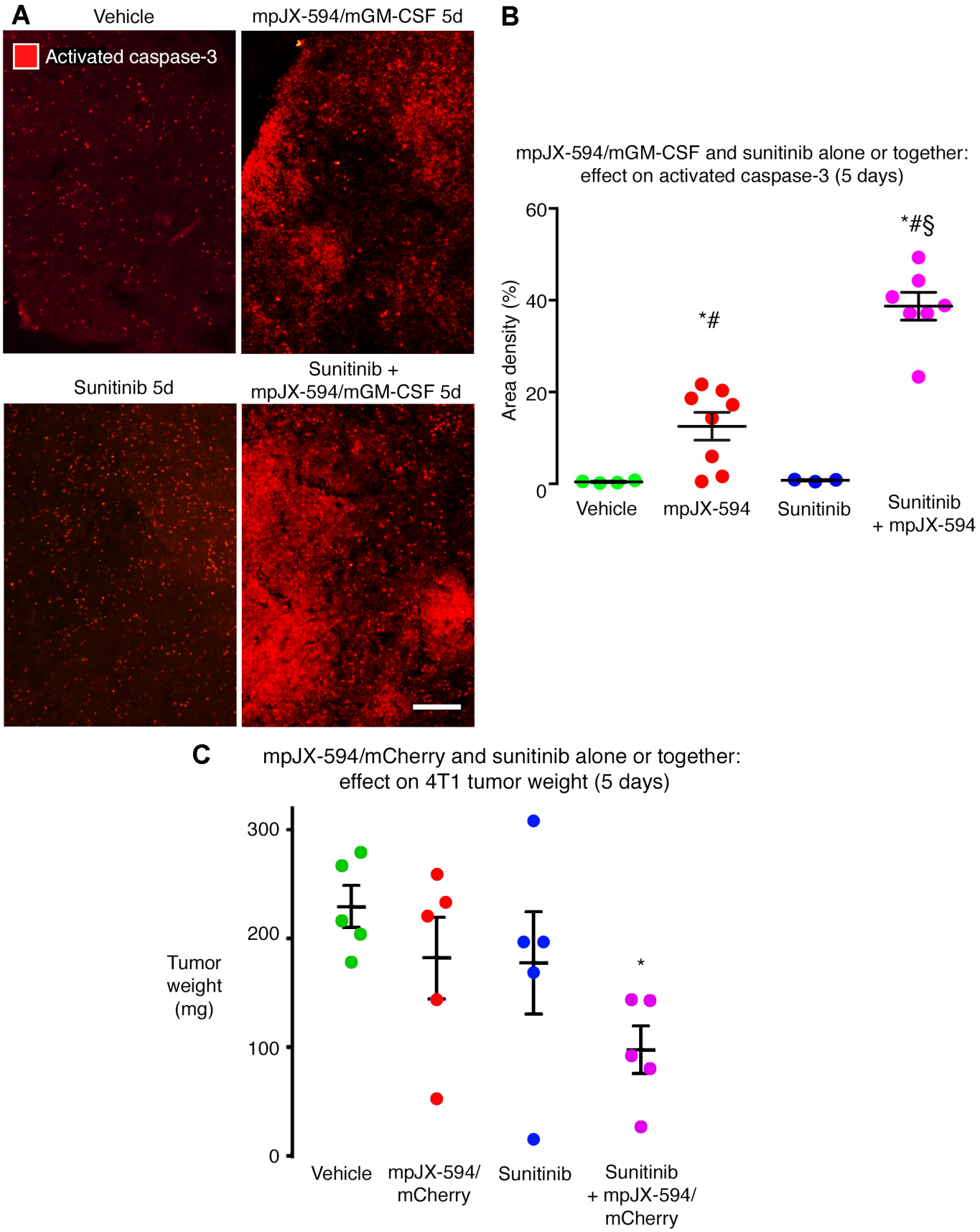
Supplemental Figure 5. Antitumor effects of mpJX-594 variants expressing human GM-CSF, mouse GM-CSF, or no GM-CSF and dose-response. A: Confocal micrographs of apoptotic cells (activated caspase-3, red) in tumors of RIP-Tag2 mice at 5 days after iv injection of mpJX-594 variants that express human GM-CSF, mouse GM-CSF, or no GM-CSF (mCherry). **B:** Measurements of activated-casepase-3 staining in tumors show similar increases in apoptotic cells at 5 days after all three mpJX-594 variants compared to vehicle-treated controls. ANOVA. * $P < 0.05$ compared to vehicle (n = 4-5 mice/group). **C:** Differences in area densities of activated caspase-3 (red) and vaccinia (green) in RIP-Tag2 tumors at 5 days after doses of 1, 3, or 6 times the standard dose (10^7 pfu) of mpJX-594/mGM-CSF. ANOVA. $P < 0.05$ compared to corresponding value for * vehicle or # vaccinia (vehicle, n = 3; 1x mpJX-594, n = 4; 3x mpJX-594, n = 5; 6x mpJX-594, n = 3). Scale bar in A applies to all images: 200 μm.



Supplemental Figure 6

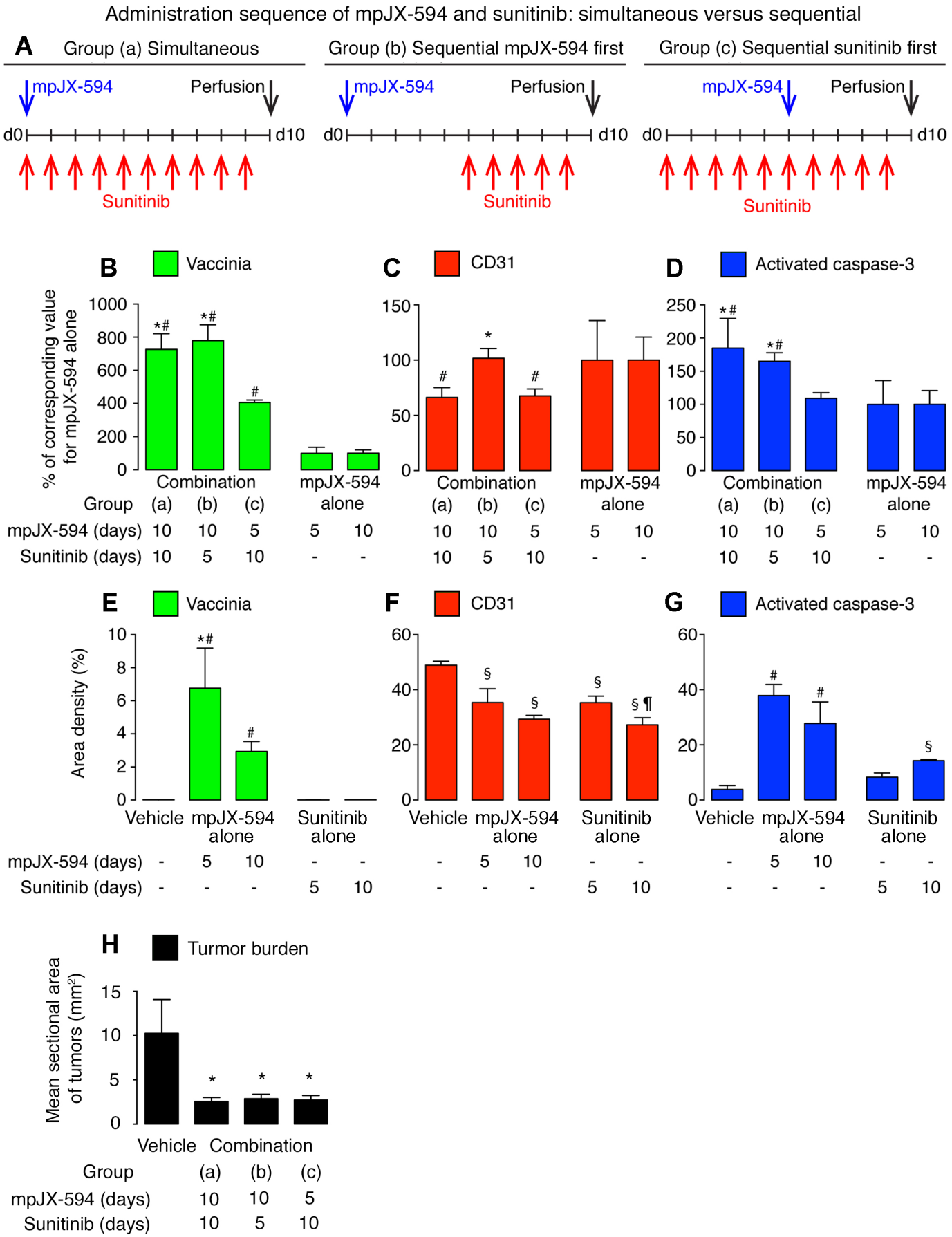
Supplemental Figure 6. JX-594: effects on U87 glioma xenografts. A: Confocal micrographs show the amount and distribution of vaccinia staining (green) in and around the vasculature (CD31, red) of human U87 tumor xenografts in nude mice at 6 hours, 1 day, or 5 days after iv injection of JX-594. Control tumor (vehicle) without vaccinia shown for comparison. Arrows point to vaccinia staining in blood vessels. **B:** Little extravasated fibrin (red) in a U87 tumor at baseline compared to conspicuous perivascular fibrin at 1 day and widespread fibrin at 5 days after JX-594. **C:** One apoptotic cell (activated caspase-3, red, arrow) near a blood vessel (CD31, green) in a vehicle-treated U87 tumor compared to numerous apoptotic cells in or near blood vessels in a U87 tumor 1 day after JX-594. **D:** Blood vessels (CD31, red) of U87 tumor at baseline compared to 5 days after JX-594. Tumor vessels are sparse and narrow or fragmented after JX-594. **E:** Reduction of vascularity of U87 tumors from 1 to 5 days after JX-594. ANOVA. $P < 0.05$ for differences compared to * vehicle or to # 2 days or less after JX-594 (n = 5 mice/group). Scale bar in D applies to all images: 100 μm for A, C, D; 400 μm for B.

Sunitinib amplification of mpJX-594 antitumor actions: independence of GM-CSF



Supplemental Figure 7

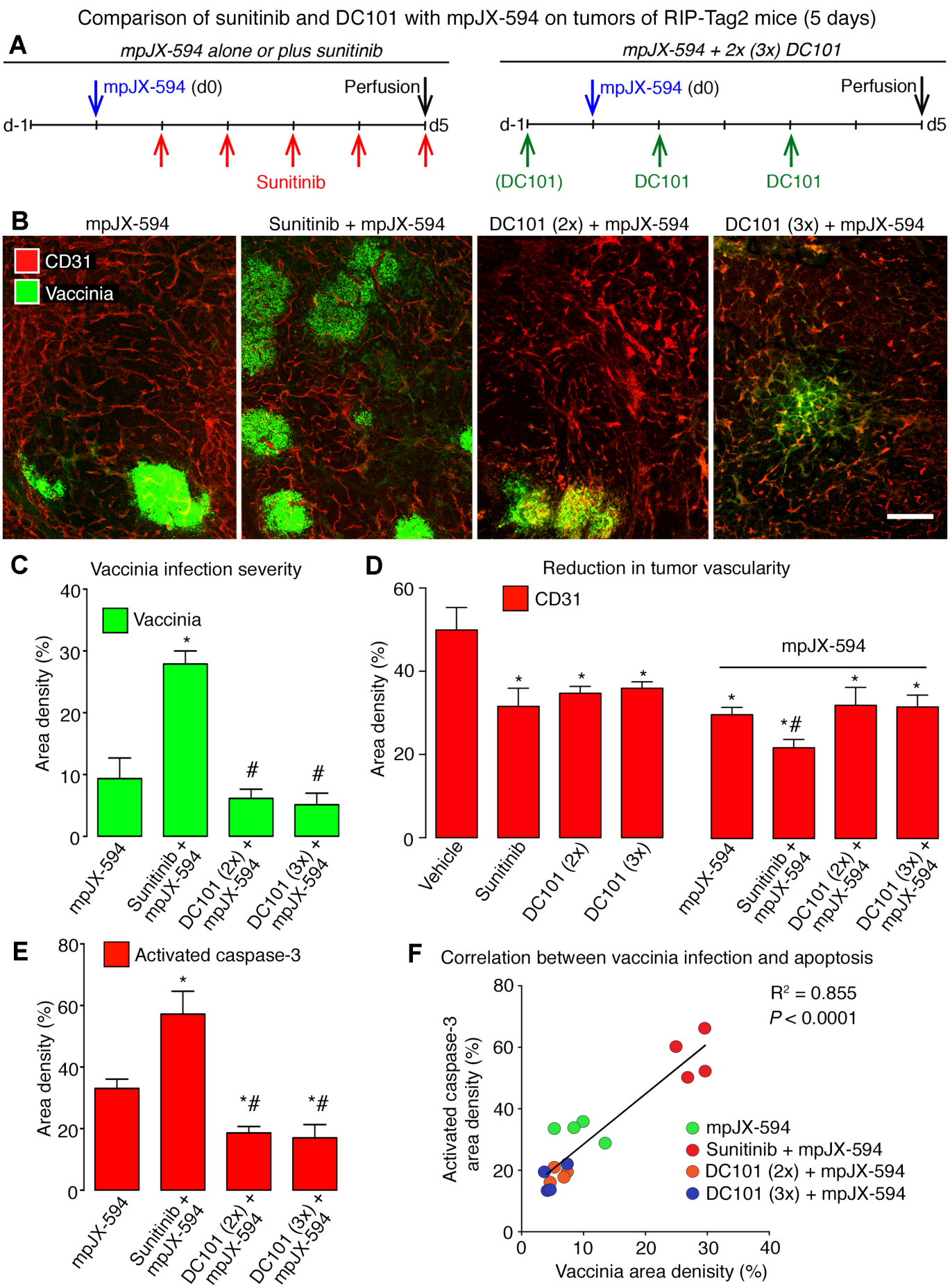
Supplemental Figure 7. Sunitinib amplification of mpJX-594 antitumor actions on tumors independent of GM-CSF. A: Confocal micrographs comparing sparse apoptotic cells (activated caspase-3, red) in RIP-Tag2 tumors at 5 days after vehicle or sunitinib with extensive apoptosis after mpJX-594/mGM-CSF (mouse GM-CSF) and even more widespread apoptosis after the virus plus sunitinib. **B:** Measurements of treatment-related differences in activated-caspase-3 staining in tumors from the four groups of mice shown in A. ANOVA. $P < 0.05$ compared to * vehicle, to # sunitinib alone, or to § virus alone (vehicle, n = 4; virus, n = 8; sunitinib, n = 3; virus plus sunitinib, n = 7). **C:** Weight of 4T1 mouse mammary carcinomas implanted subcutaneously in BALB/c mice after treatment with vehicle, mpJX-594/mCherry (no GM-CSF), sunitinib, or virus plus sunitinib over 5 days. One dose of virus was injected iv and daily sunitinib was started on day 0. 4T1 tumors treated with virus plus sunitinib were significantly smaller than the controls. ANOVA. * $P < 0.05$ compared to vehicle (n = 5 mice/group). Scale bar in A: 200 μm.



Supplemental Figure 8

Supplemental Figure 8. **mpJX-594 and sunitinib: effects of administration sequence**.

A: Treatment regimens used to compare sequence of mpJX-594 and sunitinib administration on efficacy in RIP-Tag2 mice: *Simultaneous* (a), *mpJX-594-first* (b), and *Sunitinib-first* (c). **B-D:** Area densities of vaccinia (**B**), CD31 (**C**), and activated caspase-3 (**D**) are expressed as percentages of the corresponding value for mpJX-594 alone for 10 days *(Simultaneous* and *mpJX-594-first*) or 5 days (*Sunitinib-first*). **E-G:** Treatment-related differences in area density of vaccinia (**E**), CD31 (**F**), and activated caspase-3 (**G**) in tumors after vehicle (-/-), mpJX-594 alone, or sunitinib alone for 5 or 10 days. $P < 0.05$ in B-D compared to * *Sunitinib-first* (group c) or to # mpJX-594 alone for 5 or 10 days. $P < 0.05$ in E-G compared to * 10-day mpJX-594 group, # vehicle and sunitinib groups, § vehicle group, or ¶ 5-day sunitinib group. Student's *t*-test comparison of group (b) in panel C and of 10-day mpJX-594 group in panel E to other groups; otherwise ANOVA (n = 4-5 mice/group). **H:** Measurements of tumor burden, assessed as mean sectional areas of RIP-Tag2 tumors in four treatment groups, show smaller values after mpJX-594 plus sunitinib regardless of administration sequence. ANOVA. * $P < 0.05$ compared to vehicle (n = 4-5 mice/group).



Supplemental Figure 9

Supplemental Figure 9. Sunitinib compared to DC101 in combination with mpJX-594. A: Treatment protocols for administration of sunitinib or DC101 with mpJX-594 to RIP-Tag2 mice over 5 days. mpJX-594 injected iv on day 0. Sunitinib given by gavage daily on days 1 through 5. DC101 given on days 1 and 3 (2x) or as a priming dose on day -1 followed by doses on days 1 and 3 (3x). **B:** Confocal micrographs of tumors show patches of strong vaccinia staining (green) after mpJX-594 alone and more widespread vaccinia after mpJX-594 plus sunitinib. By comparison, vaccinia is weaker after mpJX-594 plus DC101 (2x or 3x). Tumor vessels marked by CD31 (red). **C:** Measurements of vaccinia staining in tumors after mpJX-594 show 3-fold increase after sunitinib but not after DC101. ANOVA. $P < 0.05$ compared to * mpJX-594 alone or to # mpJX-594 plus sunitinib (n = 4 mice/group). **D:** Measurements of tumor vascular density at 5 days after vehicle, sunitinib, or DC101, with or without mpJX-594. Only sunitinib plus mpJX-594 resulted in greater reduction in vascular density than other treatments. $P < 0.05$ compared to * vehicle or # all other groups (n = 3-6 mice/group). **E:** Treatment-related differences in activated caspase-3 in tumors. ANOVA. $P < 0.05$ compared to * mpJX-594 alone or # mpJX-594 plus sunitinib (n = 4 mice/group). **F:** Linear regression shows significant correlation between vaccinia and activated caspase-3 staining in tumors from the four treatment groups with consistently greater staining for activated caspase-3 than vaccinia. Scale bar in B applies to all images: 200 μm.