Tumor Endothelium FasL Establishes a Selective Immune Barrier Promoting Tolerance in Tumors

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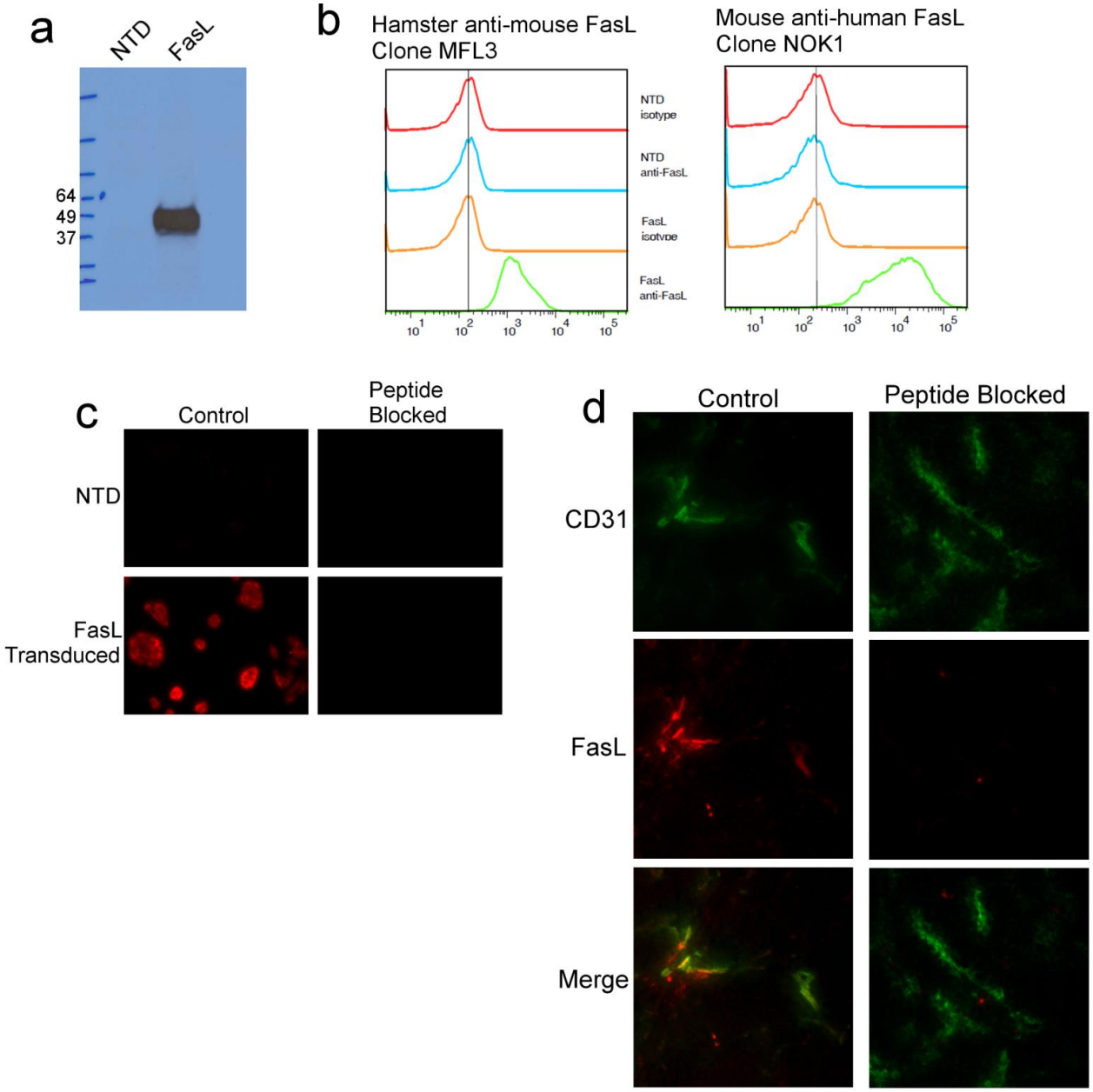
Supplementary Table 1

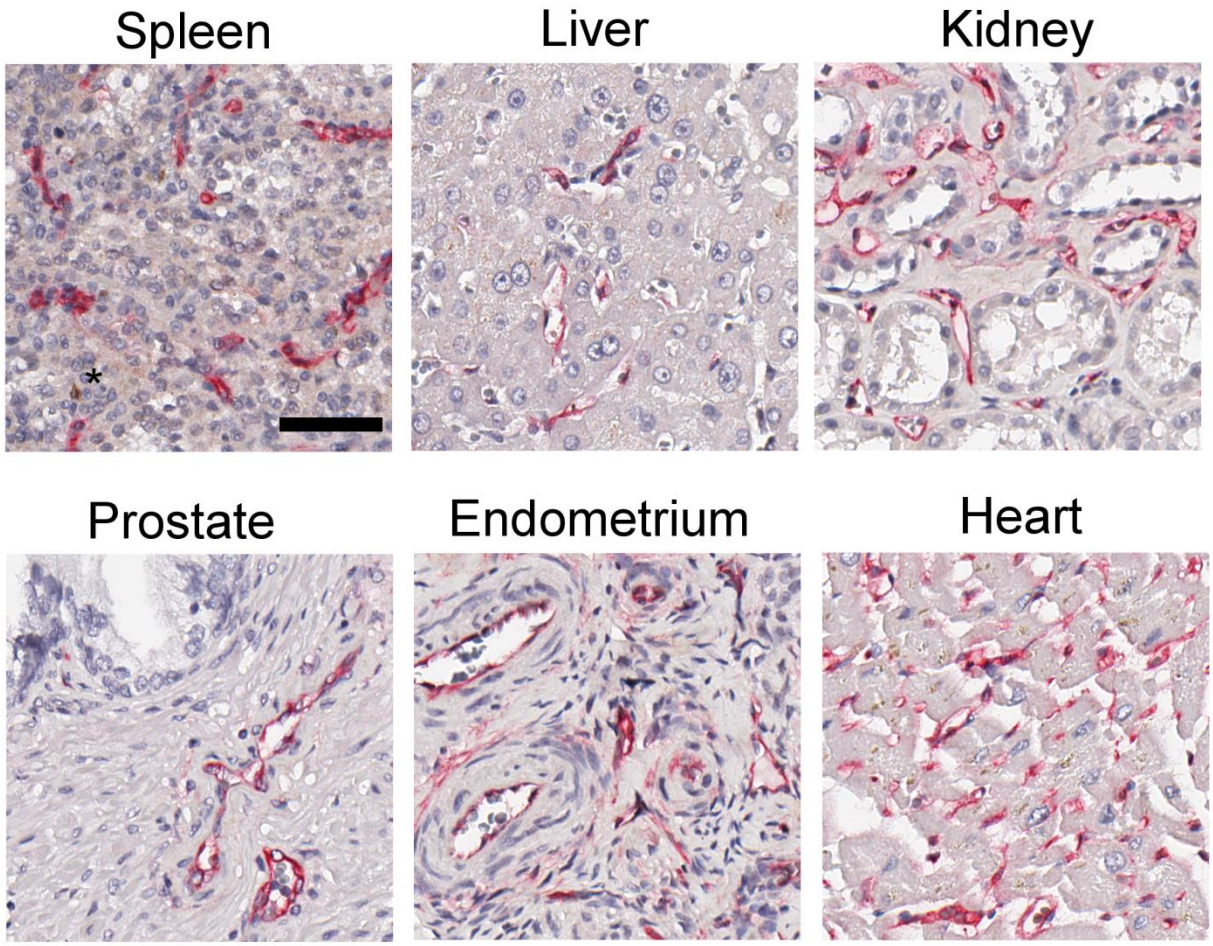
Characteristics of Ovarian Cancer Patients	
No. Patients	53
Age, years	59.6 ±11.7
Overall survival	39.3 ±19.5
Disease-Free Survival	24.6 ±18.0
Stage Group at Diagnosis	
I	0
II	3 (5.6%)
III	42 (79.2%)
IV	6 (11.3%)
no determination	3 (5.6%)
Debulking	
Optimal	29 (54.7%)
Suboptimal	21 (39.6%)

Supplementary Figure 1



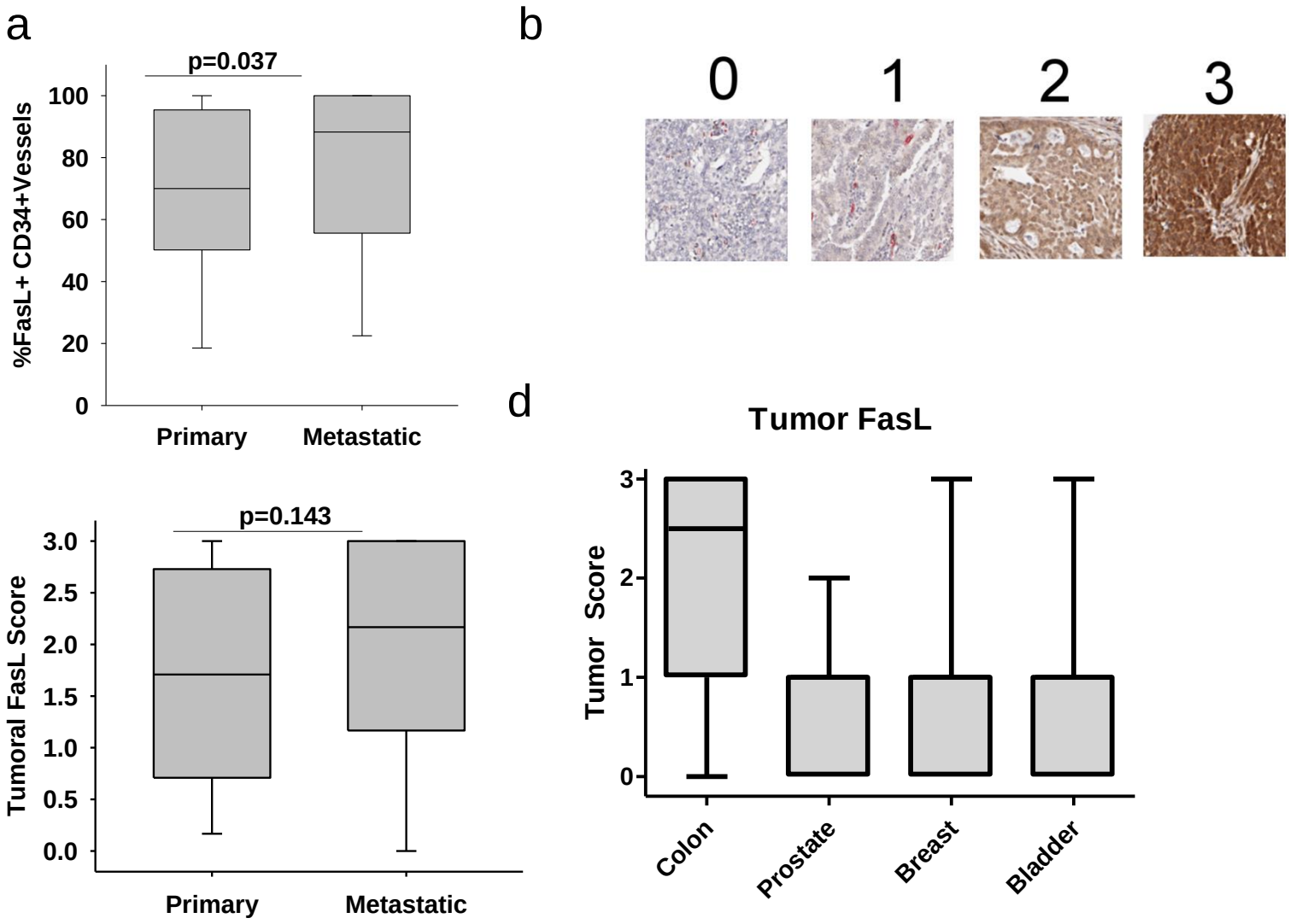
Supplementary Figure 1. Confirmation of antibody specificity. (a) Western blot against control (nontransduced, NTD) and human FasL transduced human ovarian cancer cell (OVCAR5) lysates using the mouse anti-human FasL monoclonal antibody clone G247-4 . (b) Flow cytometry analysis of mouse (left panel, ID8-VEGF) and human (right panel, OVCAR5) ovarian cancer cell lines that were transduced with either mouse or human FasL or control cells. Flow cytometry was carried out as indicated in the methods using a 3-step staining procedure with the antibody clones indicated in the figure. (c) Immunocytochemistry of control (NTD) and FasL transduced ID8-VEGF cells using the polyclonal rabbit anti-mouse FasL antibody indicated in the methods. Staining was carried out normally, or using antibody that had been preincubated with the immunizing peptide. (d) Dual immunohistochemical staining of CD31 and FasL of 5 week ID8-VEGF tumors. Staining was performed using the rabbit anti-mouse FasL polyclonal antibody either normally or using antibody that had been preincubated with the FasL immunizing antibody.

Supplementary Figure 2



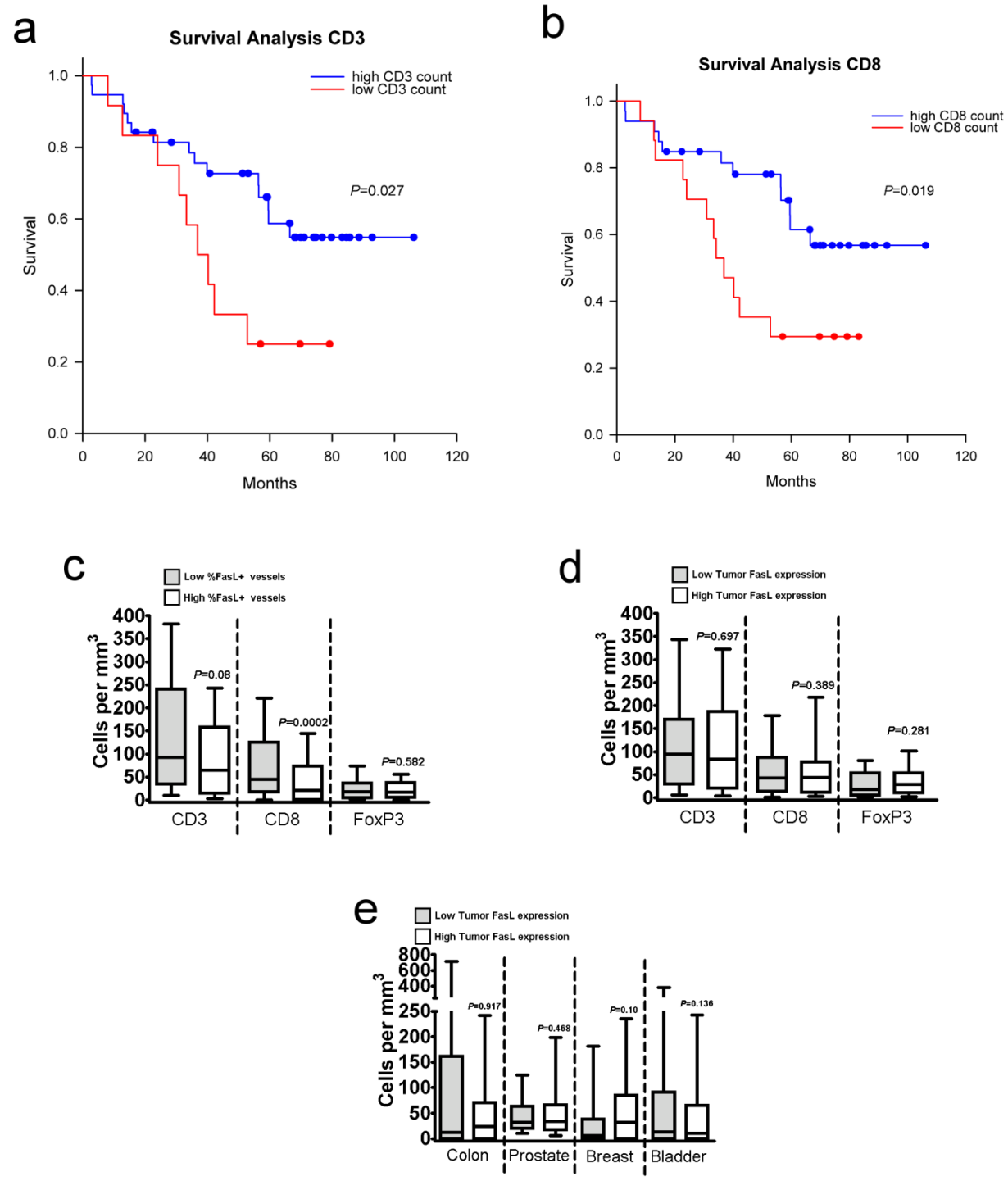
Supplementary Figure 2. Samples were stained with CD34 (red) and FasL (brown) as described for Figure 1. Original magnification is 400x and scale bar is 50μM. Asterisk denotes FasL+ lymphoid cell.

Supplementary Figure 3



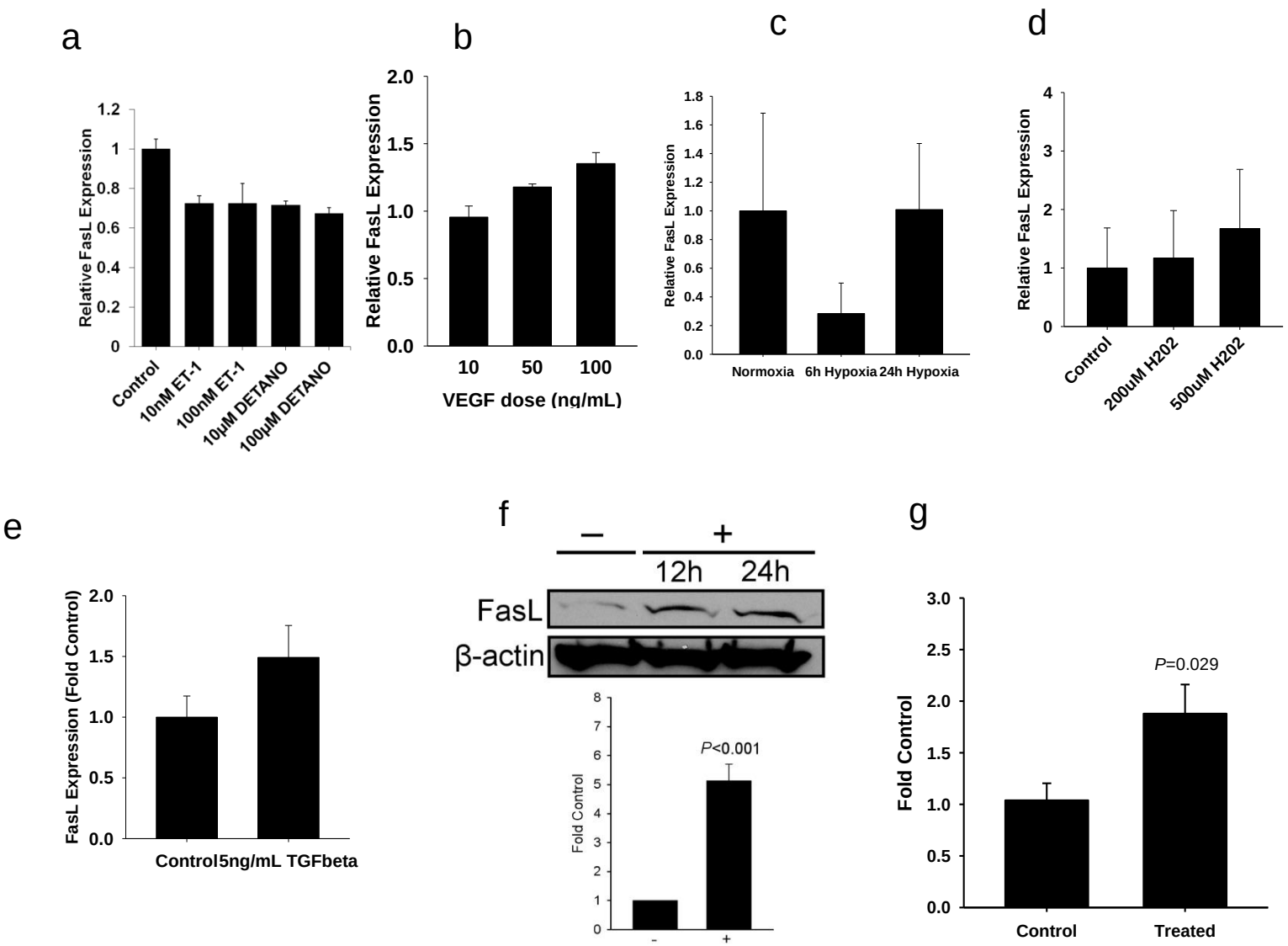
Supplementary Figure 3. Analysis of data presented in Figure 1. (a) Percentage of CD34+ vessels that are FasL+ in primary and metastatic lesions. (b) Expression of tumoral FasL. Numbers indicate the tumor scoring used. (c) Expression of tumoral FasL from primary and metastatic ovarian cancer samples, scored on a scale from 0-3. (d) Expression of tumoral FasL in the indicated tumor types. Data were analyzed by Mann-Whitney U test.

Supplementary Figure 4



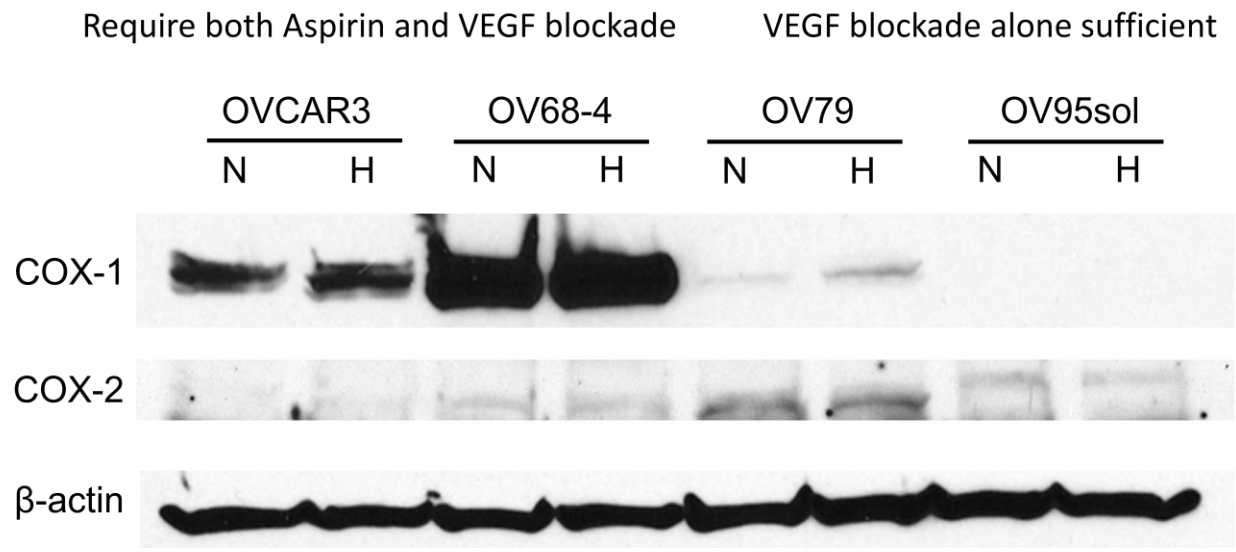
Supplementary Figure 4. Survival of ovarian cancer patients based on intratumoral (a) CD3 and (b) CD8 T cells. High/low determinations based on intratumoral cell counts divided at bottom tertile. *P* values determined by log-rank test. Intratumoral T cell counts based on (c) FasL vessel expression and (d) tumoral FasL expression. (e) CD8 T cell number based on tumoral FasL expression for the indicated tumor types. *P* values determined by Student's t test.

Supplementary Figure 5



Supplementary Figure 5. Effect of factors from the tumor microenvironment on FasL expression in human endothelial cells. HMVEC cells were place in low serum over night, then treated with the indicated compounds. (a) Endothelin-1 (ET-1) and nitric oxide donor (DETANO) slightly reduce FasL expression. (b) VEGF has a modest effect on endothelial FasL at high doses. (c) No effect of hypoxia (1.5% O_2) on endothelial expression of FasL. (d) Hydrogen peroxide has a modest effect on endothelial FasL. (e) Treatment with 5ng/mL TGFβ for 24hrs. All data are presented as means +/- SEM. (f) HMVEC were treated with PGE2, VEGF, and IL-10 as in Fig. 3d and FasL expression was determined by western blot. (n=4). (g) qRT-PCR data from HMVEC treated with PGE2, VEGF, and IL-10 for 6hrs.

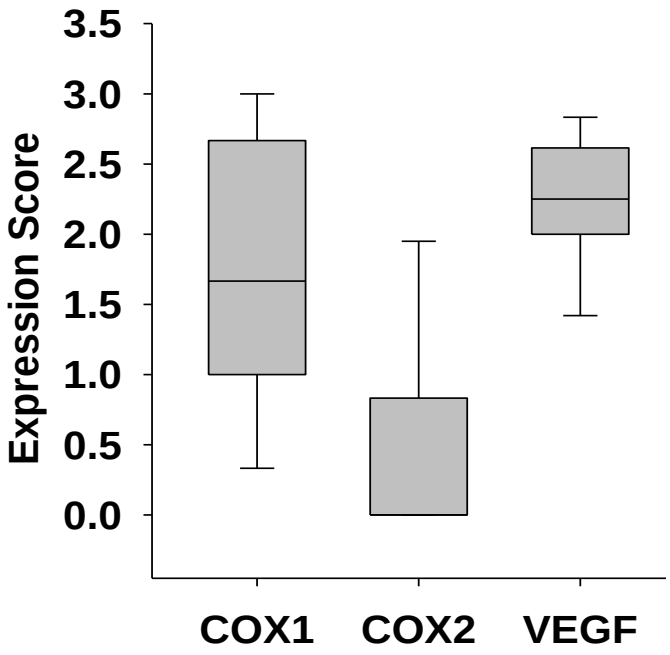
Supplementary Figure 6



Supplementary Figure 6. Human ovarian cancer cell lines were exposed to either normoxia or hypoxia (1.5% O_2) for 24 hrs and protein lysates were prepared and analyzed by western blotting.

Supplementary Figure 7

a



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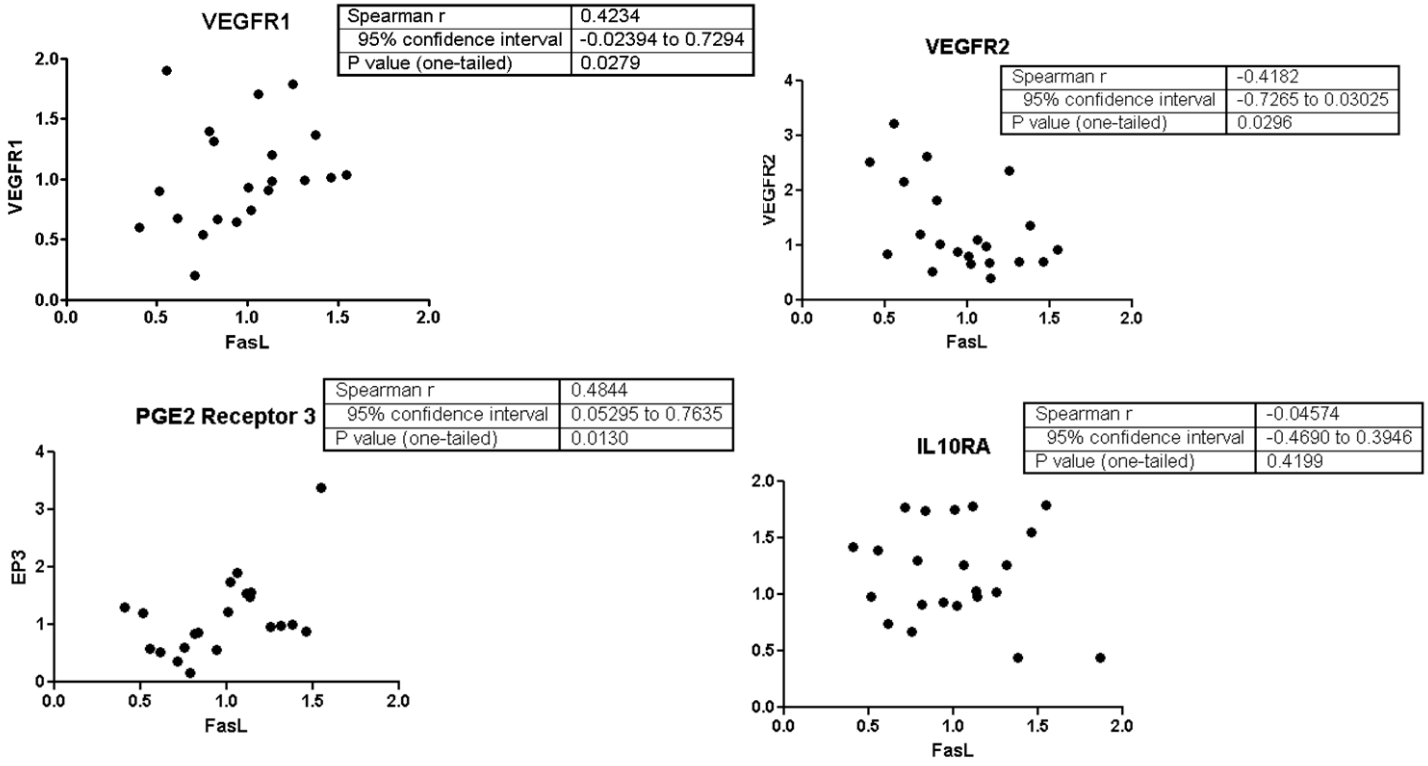
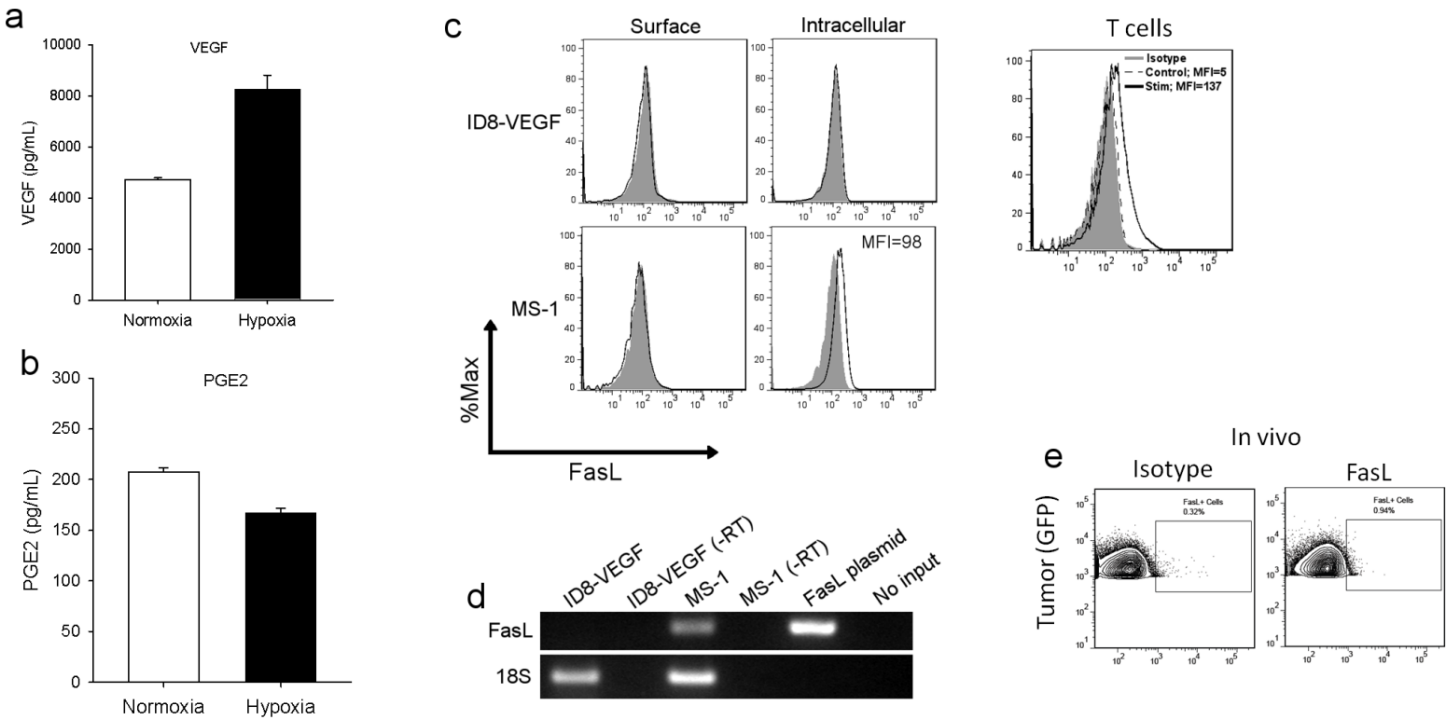


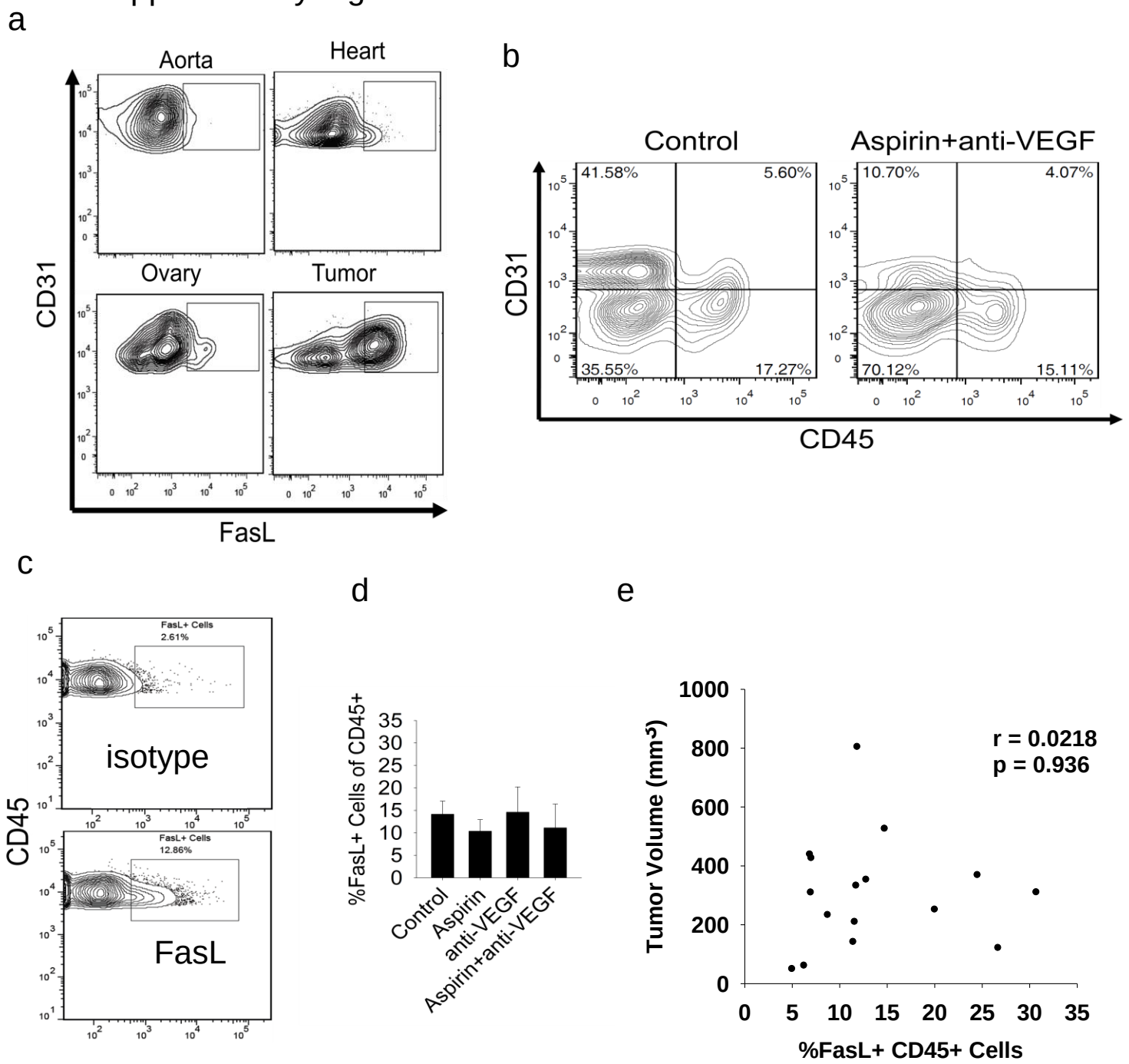
Supplementary Figure 7. (a) Quantification of TMAs depicted in Figure 4. (b) Correlation of mRNA expression from endothelial cells originally described by Bukanovich, et al. Nat Med 2008. There was no correlation between any of the additional PGE2 receptor subtypes.

Supplementary Figure 8



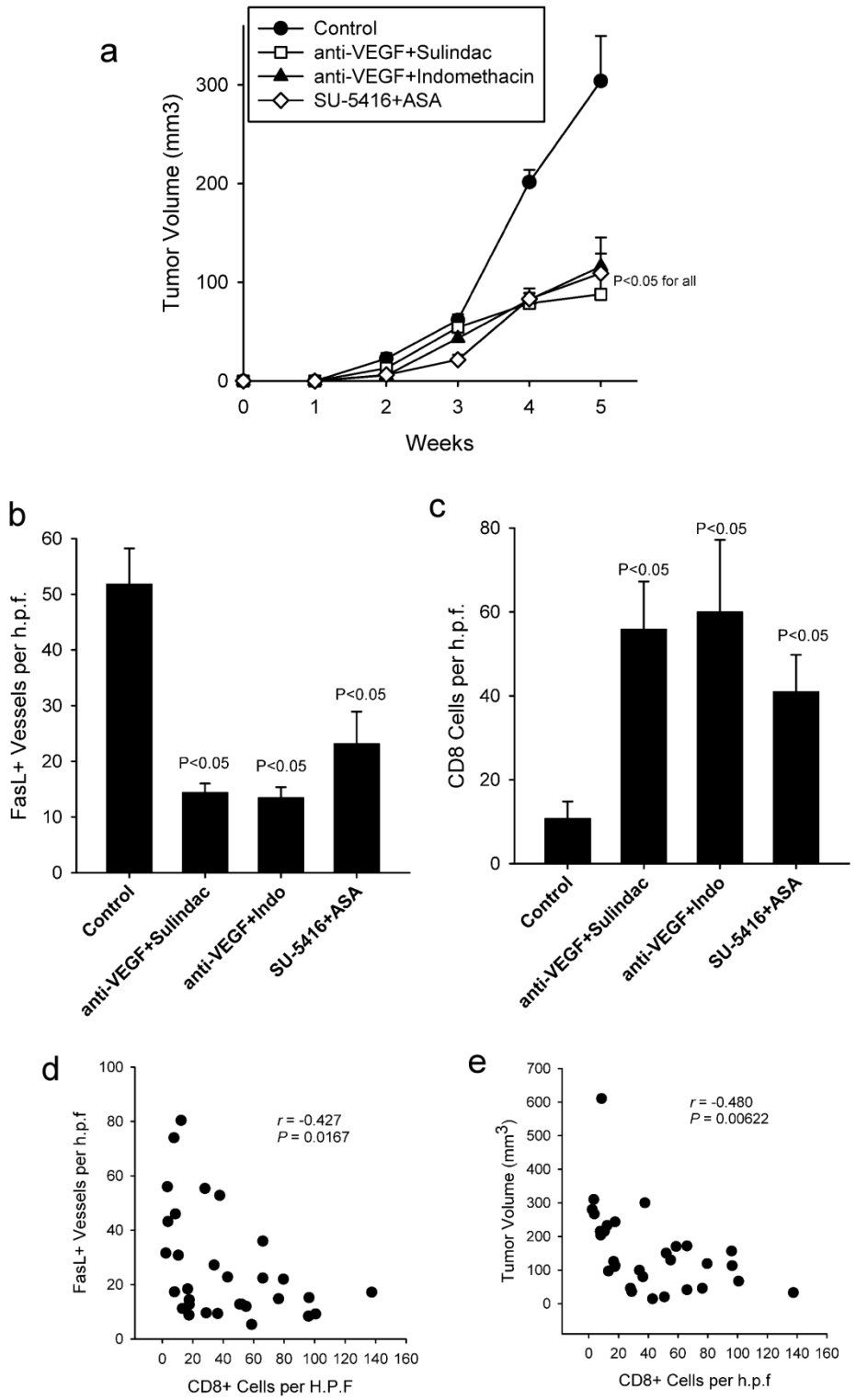
Supplementary Figure 8. ID8-VEGF cells were exposed to either normoxia or hypoxia (1.5% O_2) for 24 hrs and supernatants were analyzed by ELISA for (a) VEGF and (b) PGE2. (c) ID8-VEGF cells and a mouse endothelial cell line MS-1 were analyzed for FasL by flow cytometry. 24 hr PMA-Ionomycin activated T cells were used as a positive control. (d) ID8-VEGF cells and MS-1 cells were analyzed for FasL expression by RT-PCR. (e) Tumors from ID8-VEGF cells expressing GFP do not express FasL in vivo. Flow plots generated after gating on GFP+ cells

Supplementary Figure 9



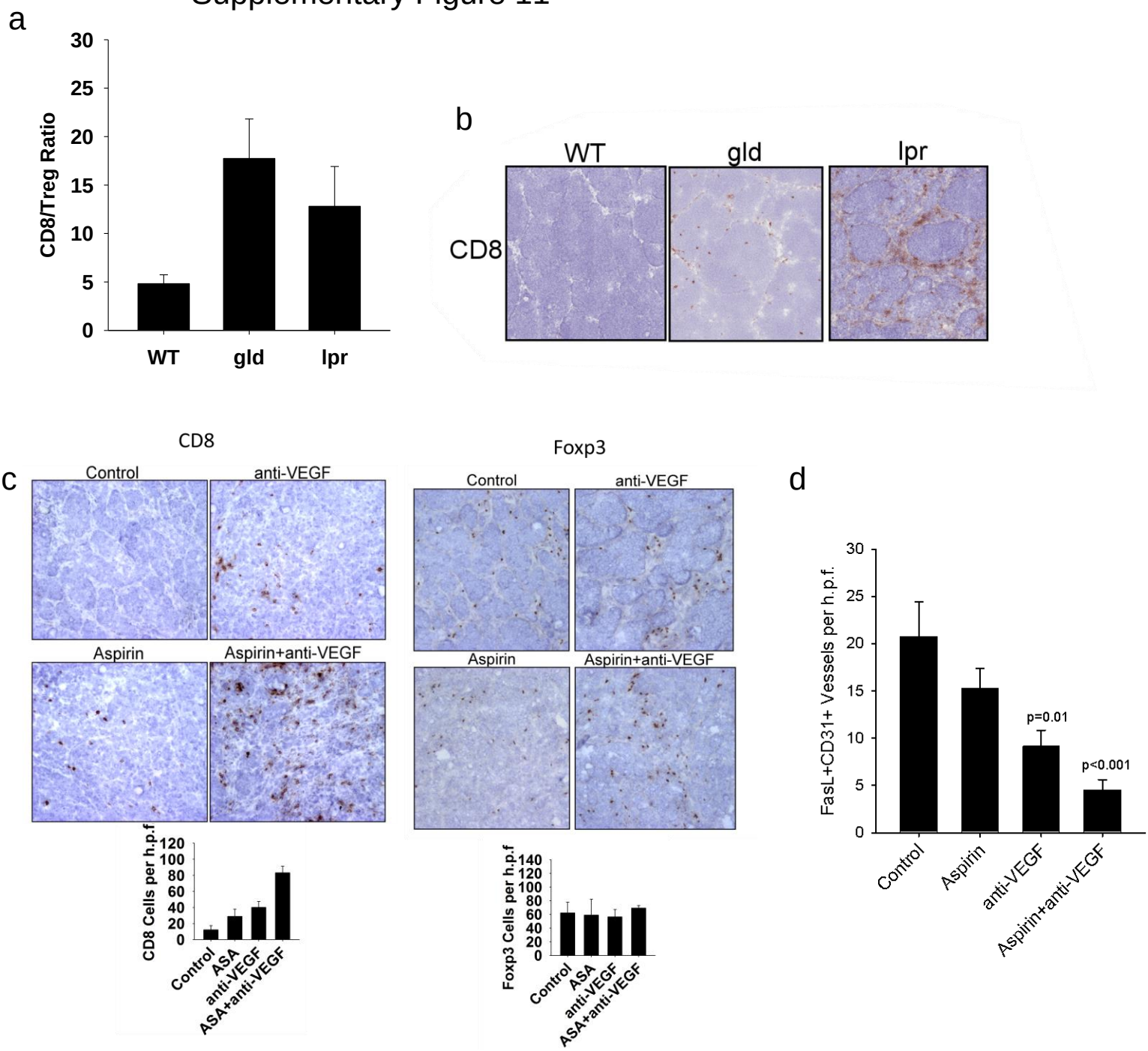
Supplementary Figure 9. (a) Tissues from the indicated mouse organs were analyzed for expression of FasL on endothelial cells by flow cytometry. Flow plots were generated from CD31+CD45- gate. Here, tumor was from i.p. tumor generated from ID8-VEGF cells. (b) Mice bearing s.c. ID8-VEGF tumors were treated as indicated in Figure 3. Flow plots were generated after gating on FasL+ cells. (c) Expression of FasL+ on CD45+ cells from control ID8-VEGF tumors. (d) Expression of FasL on CD45+ cells from animals bearing ID8-VEGF tumors treated as described in methods with the following compounds. Data are means +/- SEM. (e) Scatter plot of tumor volume and %FasL+ CD45+ cells from d.

Supplementary Figure 10



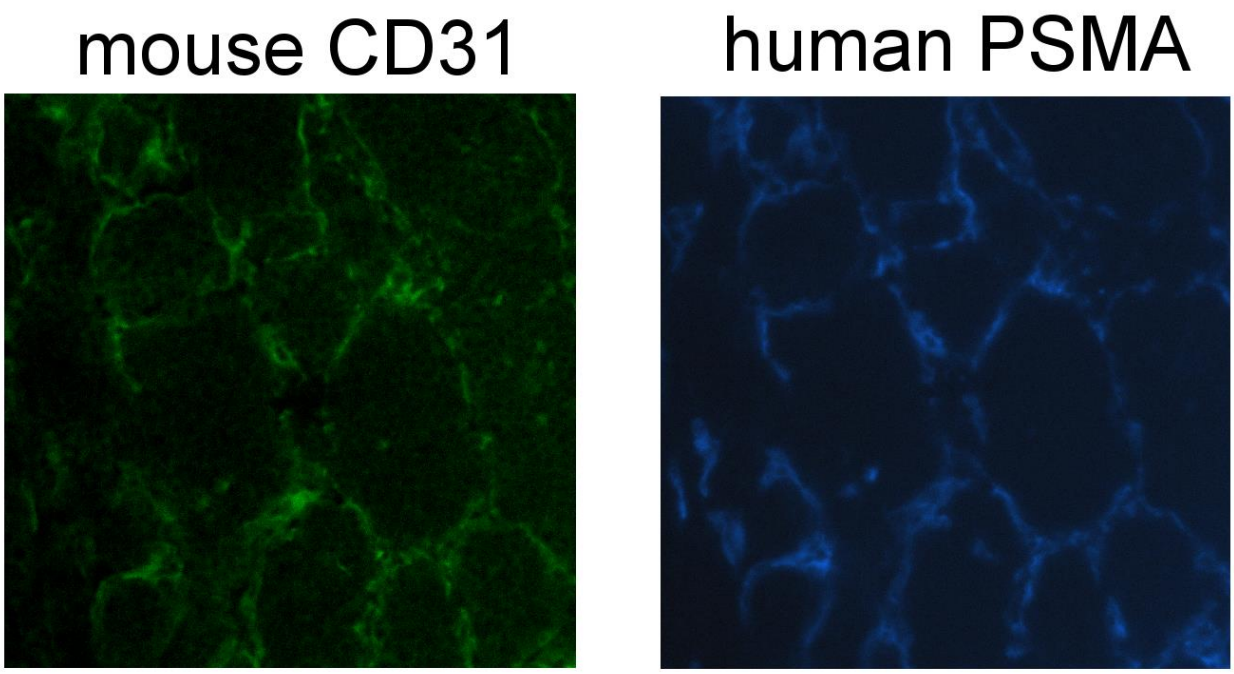
Supplementary Figure 10. Mice with ID8-VEGF tumors were treated for 5 weeks with either standard ASA+anti-VEGF (100mg/kg/day and 2mg/kg/wk) or 2mg/kg/day Indomethacin or 20mg/kg/day Sulindac or 1mg/kg/day SU-5416. (a) growth curves. (b) FasL expression (c) CD8 T cell infiltration of tumor treated as indicated. Correlation between CD8+ cells and (d) FasL+ vessels and (e) tumor volume.

Supplementary Figure 11



Supplementary Figure 11. (a) Ratio of CD45+CD3+CD8+ cells to CD45+CD3+CD4+CD25+FoxP3+ cells in s.c. ID8-VEGF tumors grown on WT, gld and lpr mice as determined by flow cytometry. (b) CD8 cells in mice from s.c. ID8-VEGF tumors grown on WT, gld and lpr mice. Original maginification is 100x. (c) CD8 and FoxP3 cells by IHC from mice treated as indicated for Figure 4. Original maginification is 200x. >10 random fields were counted per animal. (d) Number of FasL+vessels for the given treatments. Data are means from 2 independent biological experiments.

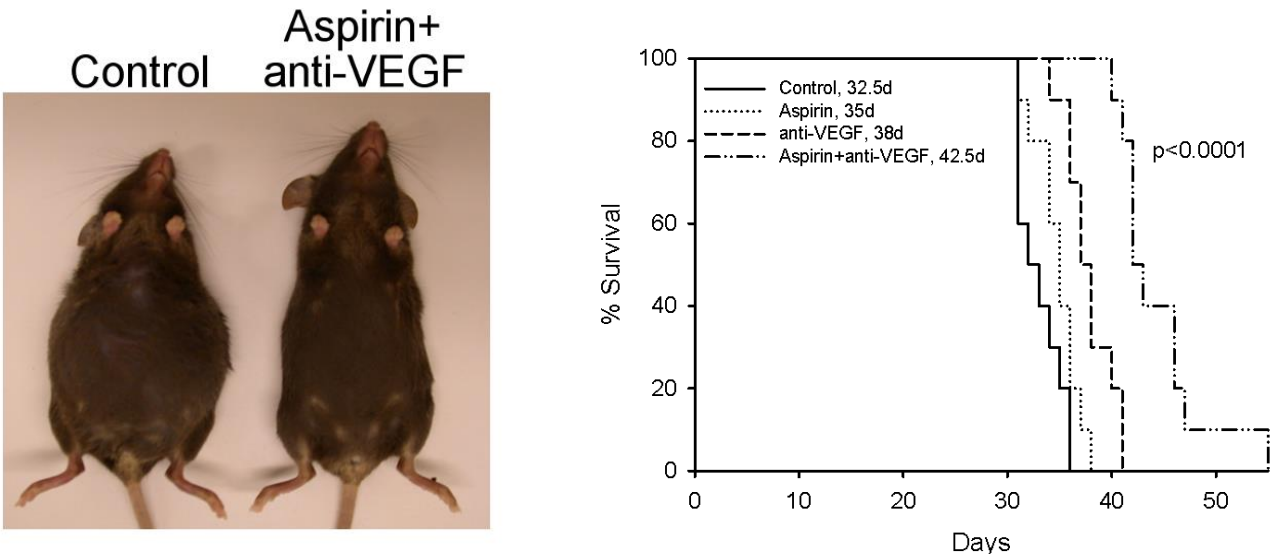
Supplementary Figure 12



Supplementary Figure 12. MS1 cells transduced with the human gene prostate-specific membrane antigen (PSMA) and co-injected with ID8-VEGF cells. Tumor were excised and immunofluorescence was performed using a FITC-conjugated anti-mouse CD31 antibody and an anti-human PSMA antibody followed by secondary staining with goat anti-human alexafluor 350 antibody.

Supplementary Figure 13

a



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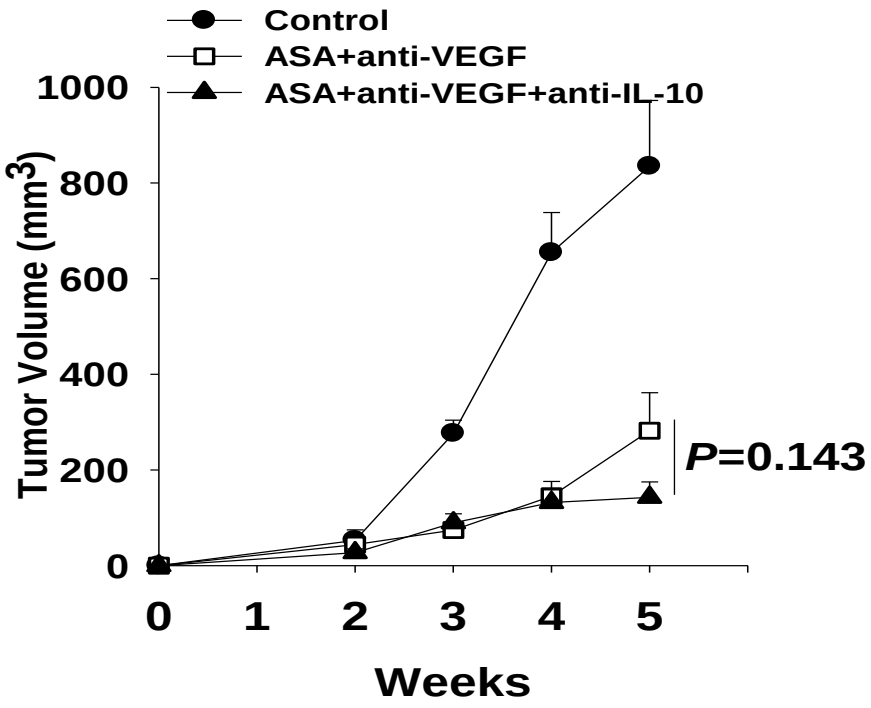
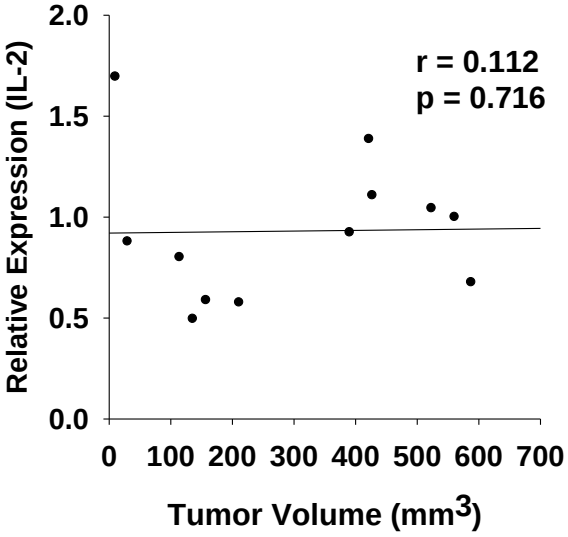


Supplementary Figure 13. (a) Survival of mice with i.p. ID8-VEGF tumors treated for 5 wks with Aspirin and/or anti-VEGF-A antibody. Data are pooled from 2 independent experiments, n=10 total animals per group. *P* value determined by log-rank test. Image captured at 5 weeks. (b) Mice bearing ID8-VEGF tumors were treated with Aspirin (ASA) and anti-VEGF antibody as indicated in the methods, or with the addition of 50ug twice weekly injections of anti-IL-10 antibody (JES5-2A5).

Supplementary Figure 14

a



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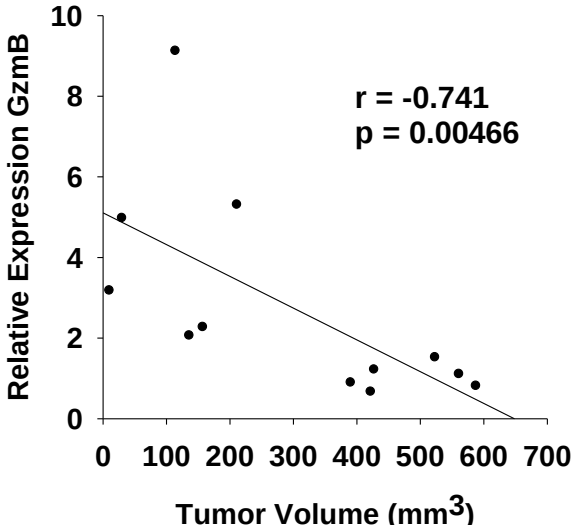

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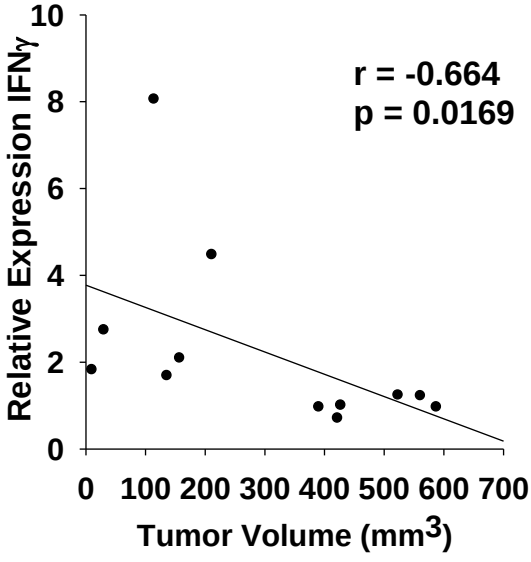

Supplementary Figure 14. Correlation of mRNA expression of tumor volume to (a) IL-2, (b) granzyme B, and (c) IFNg in mice from Figure 5f.