Supplemental Material

CpG-1826 immunotherapy potentiates anti-tumor and anti-tumor immune responses to metronomic cyclophosphamide in a preclinical glioma model

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List of supplemental materials:

Fig. S1. **GL261 tumor immune response to CpG-1826 negatively correlates with tumor volume**. Shown are Pearson correlation coefficients and p-values for comparisons between: expression of the macrophage marker F4/80, determined by qPCR, and normalized tumor volume (3 days after a third or fourth CpG-1826 injection, as in Fig. 1 and Fig. 2) (A); and percent CD45+ (immune) cells in tumor tissue, determined by flow cytometry, and normalized tumor volume (3 days after a third CpG-1826 injection, with the first CpG-1826 injection given on day 3) (B). Data are for CpG-1826 treatment groups shown in Fig. 1A and Fig. 2C.

Fig. S2. CPA treatment increases markers for tumor-infiltrating immune cells. Data shown are based on qPCR analysis of RNA isolated from tumors excised 6 or 12 days after a single injection of CPA from tumors represented in Fig. 2A and Fig. 2C. The untreated tumor data shown here is the same data presented in Fig. 4. Untreated group includes pooled tumors from day 6 and day 12. Values shown are mean + SE values for n individual mice per group: untreated, n=11; 45 mg/kg CPA on day 6, n=3; 45 mg/kg CPA on day 12, n=7; 90 mg/kg CPA on day 6, n=3; 90 mg/kg CPA on day 12, n=8. Significance for comparisons to untreated tumors is indicated by: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p<0.0001$. Comparison of 45 vs. 90 mg/kg CPA on day 12 was determined by two-tailed t-test (dagger symbol), $p<0.05$.

Fig. S3. Tumor-infiltrating immune cells increase in GL261 tumors 6 and 12 days after CPA treatment. Flow cytometry data for tumors excised on treatment day 12 (Fig. 2C) were analyzed for increases in the indicated tumor-infiltrating immune cells using the indicated immune cell markers. Data for untreated and day 12 CPA treatment are the same as shown in Fig. 5. Mean values are indicated by horizontal lines, for: untreated, n=8; 45 mg/kg CPA day 6, n=3; 90 mg/kg CPA day 6, n=3; 45 mg/kg CPA day 12, n=7; and 90 mg/kg CPA day 12, n=8. Significance is indicated by: *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; and ****$p<0.0001$. 45 mg/kg CPA and 90 mg/kg CPA at either day 6 or day 12 were compared to each other by two-tailed t-test (dagger symbol), $p<0.05$.

Fig. S4. **Flow cytometry gating**. Representative flow cytometry analysis with gating for data presented in Fig. 5 and Fig. S3.

Fig. S5. Immunohistochemical staining for CD68. Representative images from immunohistochemical staining for CD68 in GL261 tumor cryosections. 2 representative 10x magnification images per group, from different tumors, were selected.

Fig. S6. Quantification of CD68 immunohistochemical staining. CD68 immunostaining was quantified using NIH ImageJ. (A) CD68 immunostaining signals (representative images shown in Fig. S5) were quantified and data are presented as mean $\pm$ SE values for n=3-4 tumors per group. Data are based on the data for individual tumors shown in panel B. *, $p<0.5$, **, $p < 0.01$ compared to the untreated tumor group by two-tailed t-test. (B) CD68 immunostaining signals for individual tumors, which are numbered 1 to 19, as shown underneath each bar. The 90 mg/kg CPA + CpG group includes 3 tumors (bars 17, 18, 19); an additional independent tumor region was analyzed from tumors 18 and 19, and is represented by bars 20 and 21, respectively. Values shown in panel B are mean $\pm$ SD values based on n=7-15 independent images of each tumor.

Fig. S1

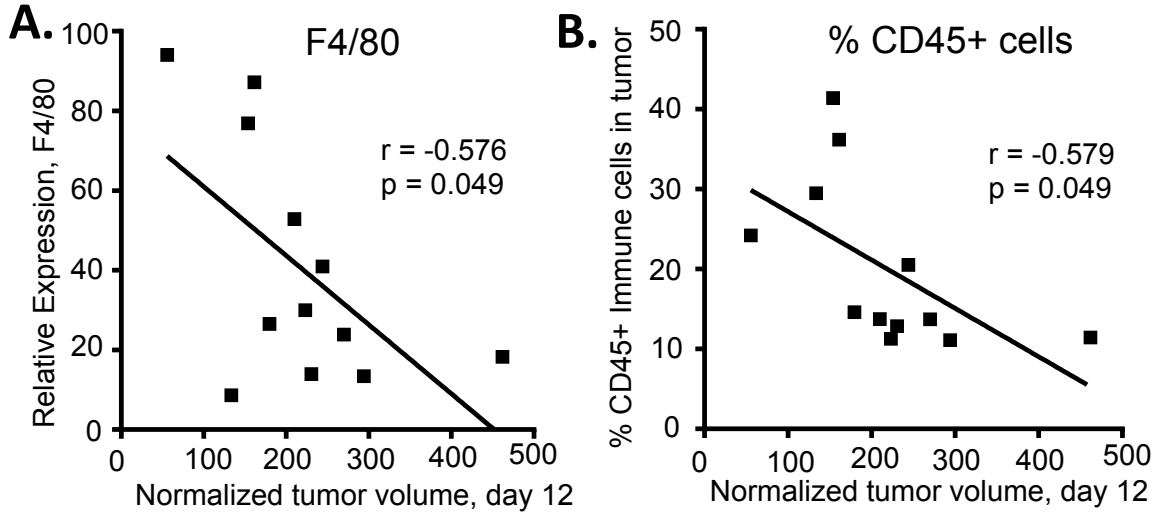

A.
F4/80
Relative Expression, F4/80
r = -0.576
p = 0.049
Normalized tumor volume, day 12
B.
% CD45+ cells
% CD45+ Immune cells in tumor
r = -0.579
p = 0.049
Normalized tumor volume, day 12

Fig. S2

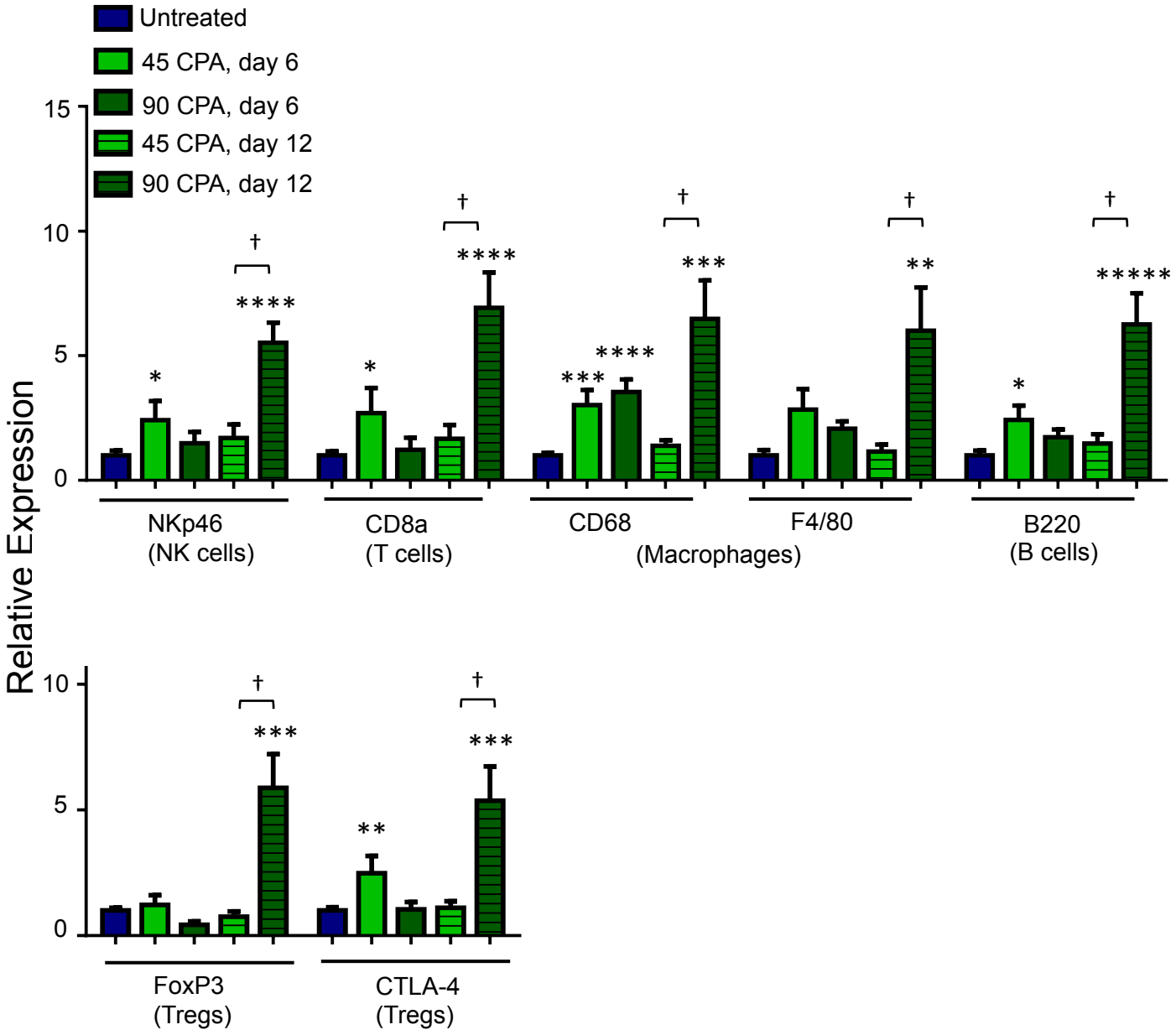

Untreated
45 CPA, day 6
90 CPA, day 6
45 CPA, day 12
90 CPA, day 12
Relative Expression
15
10
5
0
NKp46
(NK cells)
CD8a
(T cells)
CD68
(Macrophages)
F4/80
B220
(B cells)
FoxP3
(Tregs)
CTLA-4
(Tregs)

Fig. S3

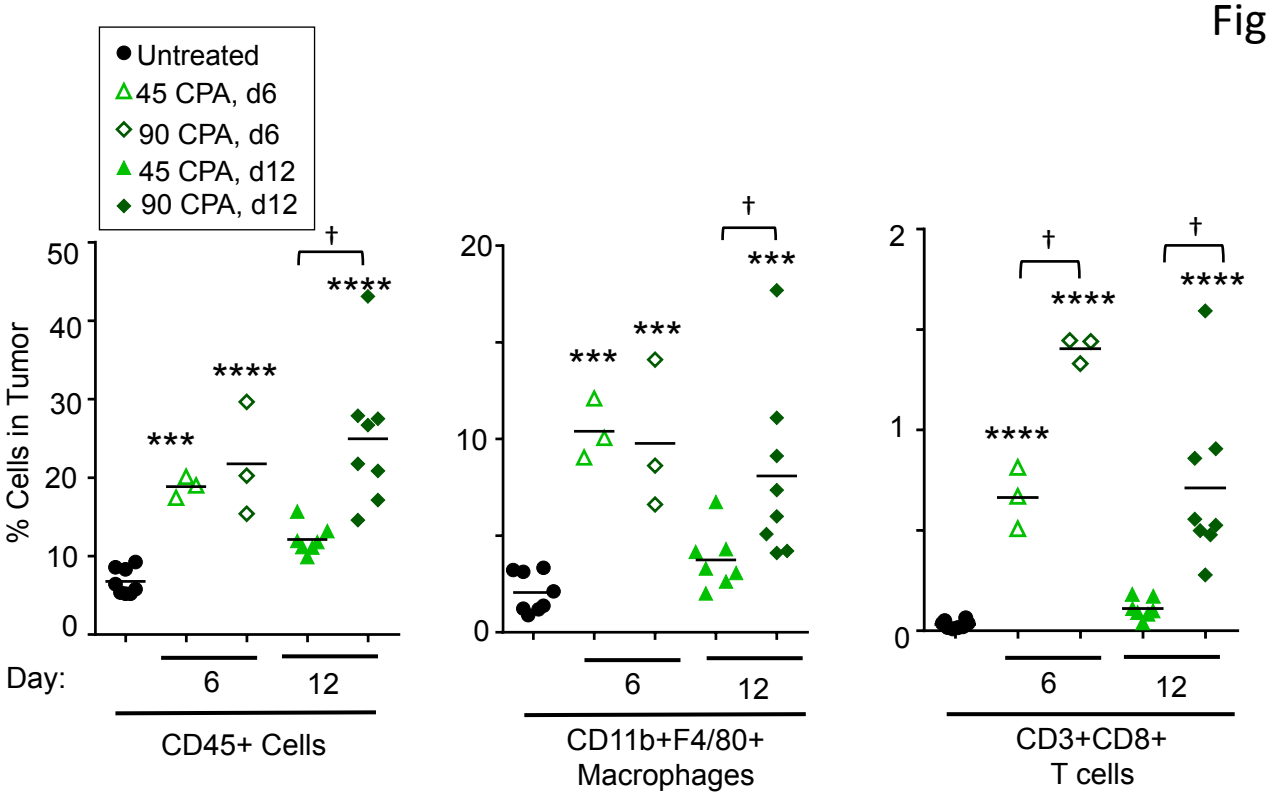

Untreated
45 CPA, d6
90 CPA, d6
45 CPA, d12
90 CPA, d12
% Cells in Tumor
Day:
6
12
CD45+ Cells
CD11b+F4/80+
Macrophages
CD3+CD8+
T cells
***
****
†

Fig. S4

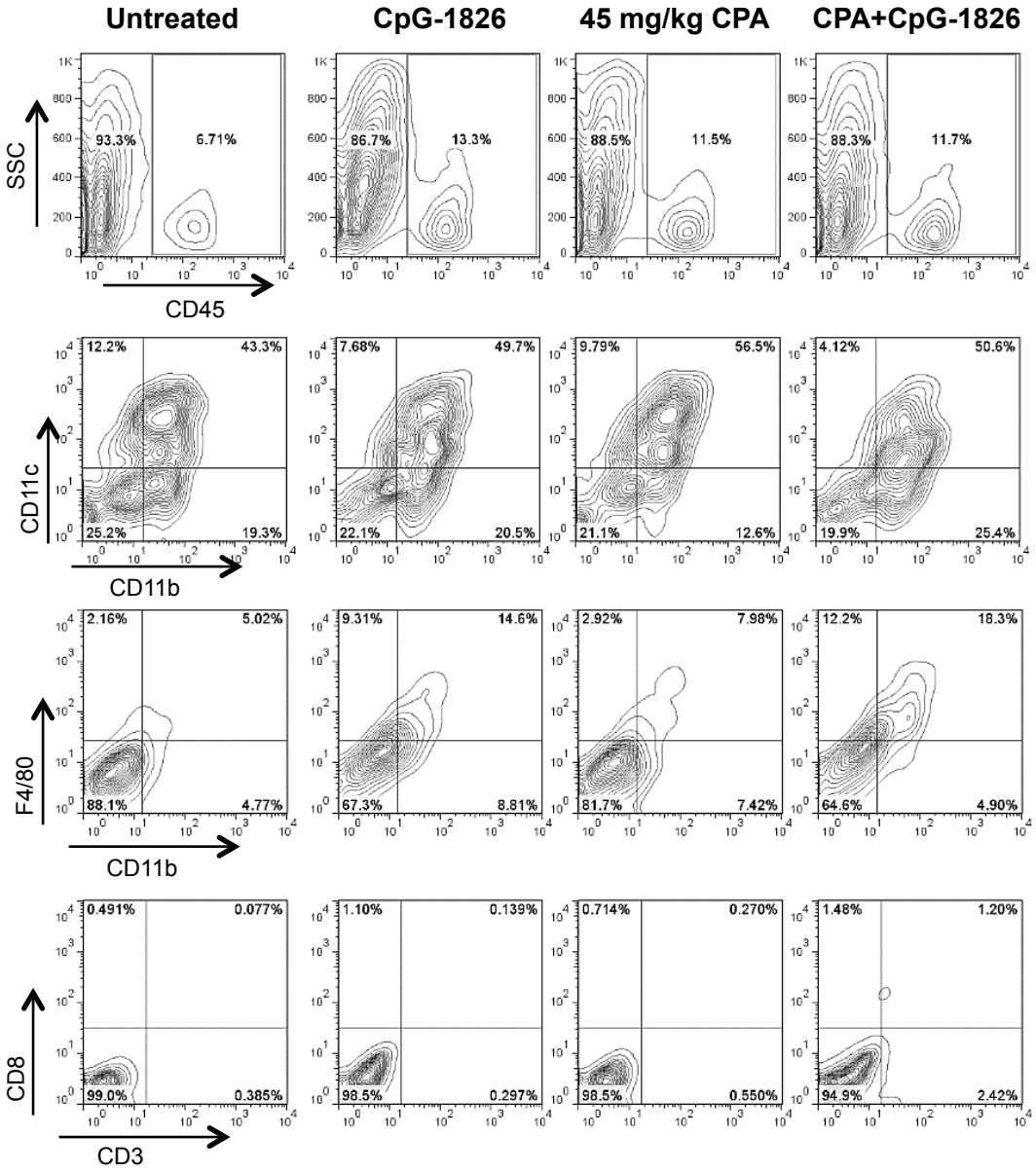

Untreated
CpG-1826
45 mg/kg CPA
CPA+CpG-1826
SSC
CD45
93.3%
6.71%
86.7%
13.3%
88.5%
11.5%
88.3%
11.7%
CD11c
CD11b
12.2%
43.3%
25.2%
19.3%
7.68%
49.7%
22.1%
20.5%
9.79%
56.5%
21.1%
12.6%
4.12%
50.6%
19.9%
25.4%
F4/80
CD11b
2.16%
5.02%
88.1%
4.77%
9.31%
14.6%
67.3%
8.81%
2.92%
7.98%
81.7%
7.42%
12.2%
18.3%
64.6%
4.90%
CD8
CD3
0.491%
0.077%
99.0%
0.385%
1.10%
0.139%
98.5%
0.297%
0.714%
0.270%
98.5%
0.550%
1.48%
1.20%
94.9%
2.42%

Fig. S5

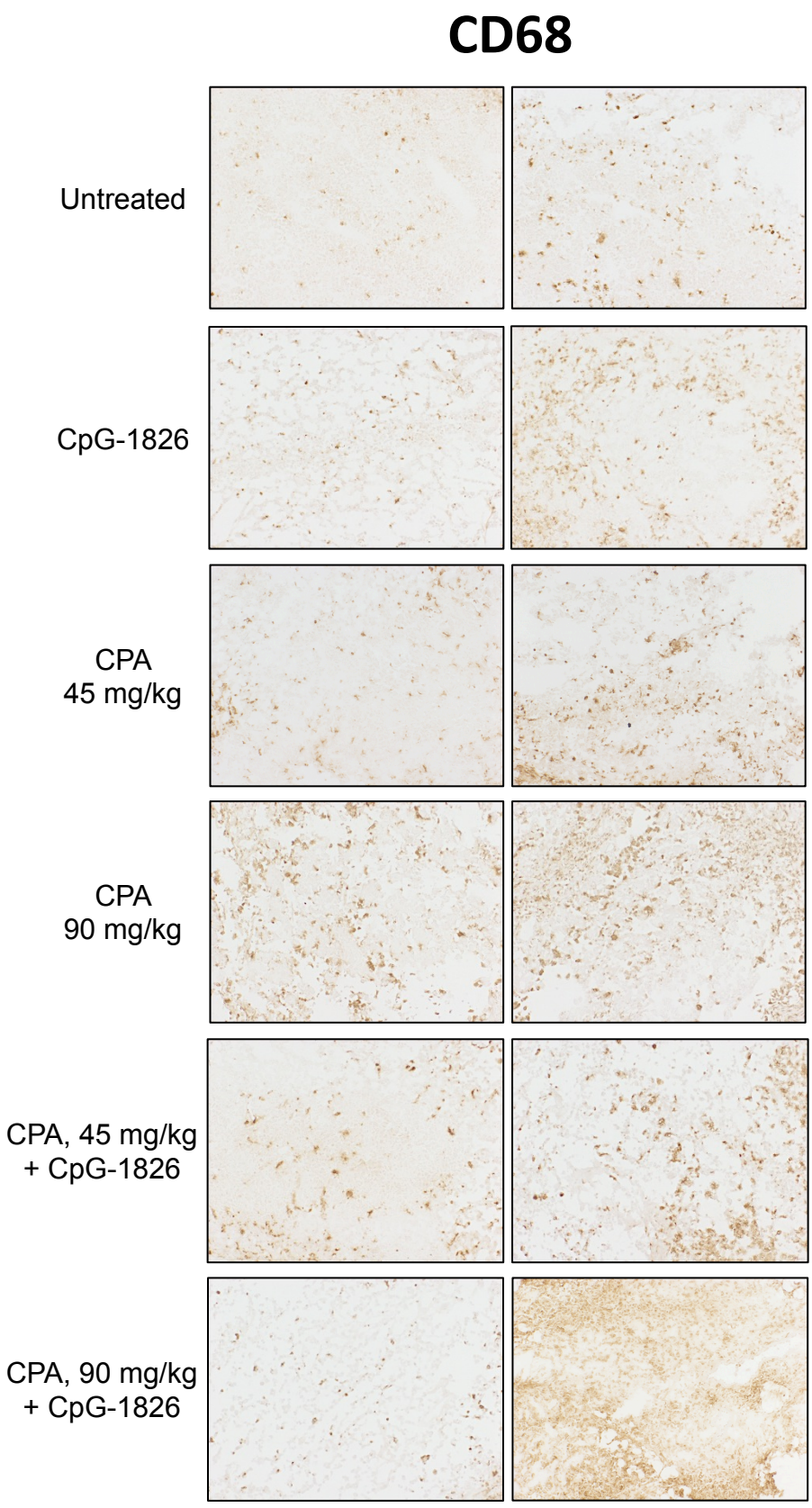
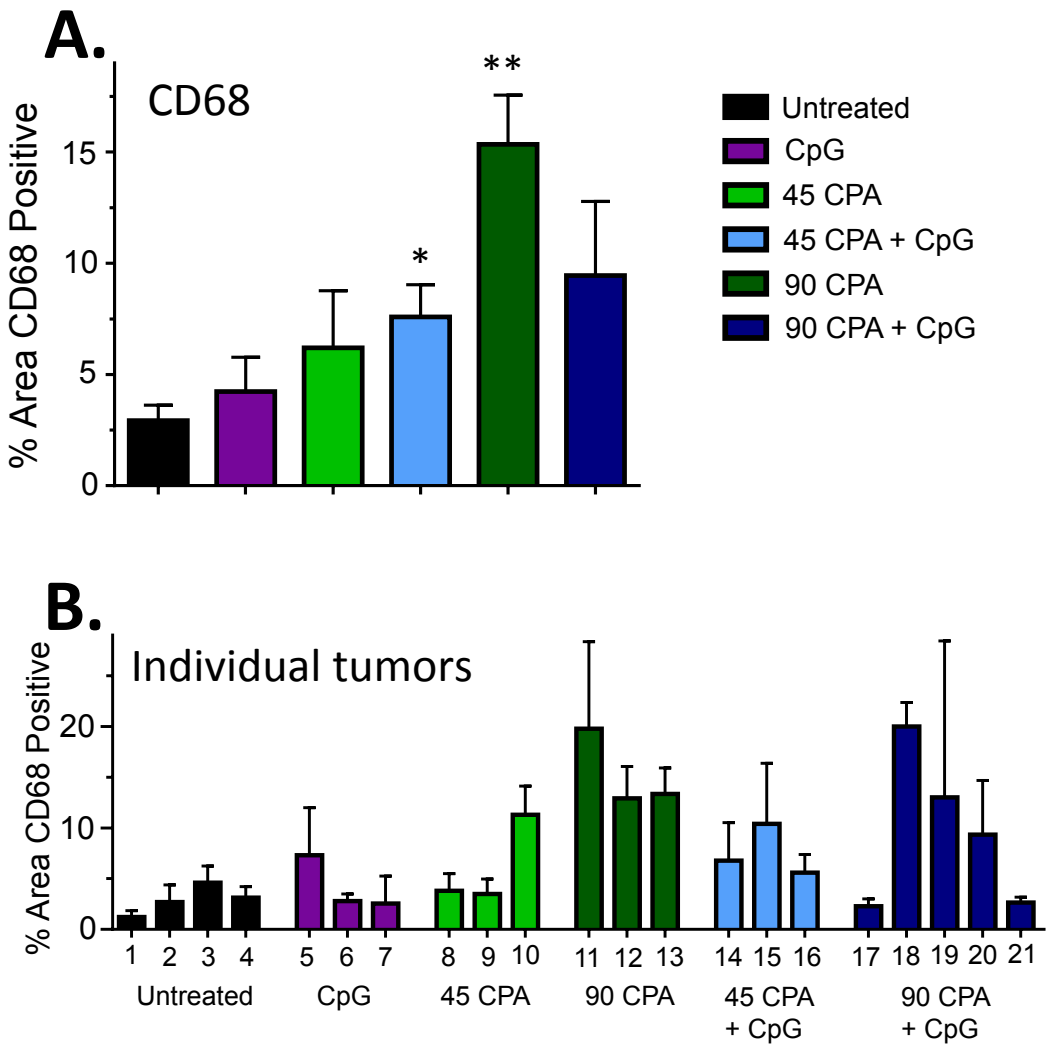

CD68
Untreated
CpG-1826
CPA
45 mg/kg
CPA
90 mg/kg
CPA, 45 mg/kg
+ CpG-1826
CPA, 90 mg/kg
+ CpG-1826

Fig. S6


A.
CD68
% Area CD68 Positive
0
5
10
15
*
**
Untreated
CpG
45 CPA
45 CPA + CpG
90 CPA
90 CPA + CpG
B.
Individual tumors
% Area CD68 Positive
0
10
20
1 2 3 4
5 6 7
8 9 10
11 12 13
14 15 16
17 18 19 20 21
Untreated
CpG
45 CPA
90 CPA
45 CPA + CpG
90 CPA + CpG