SUPPLEMENTAL MATERIALS AND METHODS

Flow Cytometry Cell Suspension Preparation and Staining

Single-cell suspensions were obtained through digestion with RPMI/Enzyme solution (Tumor Dissociation Kit; Miltenyi Biotec, Bergisch Gladbach, Germany), followed by mechanical dissociation using gentleMACS tissue dissociator (Miltenyi Biotec). The single-cell suspensions (diluted to 1.0×10^7 cells/mL) were incubated for 20 minutes at 4 °C with anti-mouse CD16/32 (BioLegend, San Diego, California) to block the Fc receptors and stained with the following anti-mouse antibodies (1:500 dilution; BioLegend) for 1 hour at 4 °C in the dark: CD45 Peg CP/Cy5.5 (30-F11), CD3 AF488 (17A2), CD4 Brilliant violet 605 (RM4-4), CD8 Brilliant violet 785 (53-6.7), CD137 PE (17B5), I-A/I-E PE/Cy7 (M5/114.15.2), NK1.1 APC/Cy7 (S17016D), CD134 APC (OX-86), CD279 APC-R700 (29F.1A12), Ly-6G PE-eFluor 610 (1A8-Ly6g), CD11b Super Bright 645 (M1/70), and CD11c Super Bright 780 (N418). Stained cells were fixed with BD stabilizing fixative (BD Biosciences, Franklin Lakes, New Jersey) and resuspended in 200 μL of fluorescence-activated cell sorting buffer (2% [v/v] fetal bovine serum in phosphate-buffered saline).

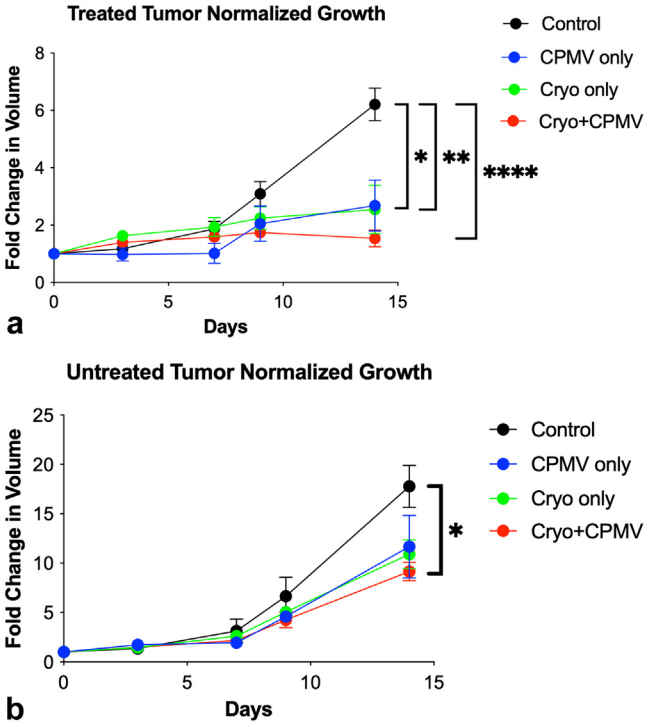

Figure E1. Normalized tumor growth of the treated and untreated tumors. **(a)** The fold change in the primary tumor volume over 2 weeks compared with the baseline volume demonstrated growth suppression of the treated tumor by cryoablation only (Cryo) (green) and cowpea mosaic virus (CPMV) (blue) alone or combined, cryoablation plus cowpea mosaic virus (Cryo + CPMV) (red), versus control (black). **(b)** The fold change in the untreated tumor volumes over time compared with the baseline demonstrated growth suppression of the untreated tumor in the Cryo + CPMV (red) group versus control (black); the single-arm groups showed a trend toward growth retardation that did not meet statistical significance. *$P < .05$, **$P < .01$, and ****$P < .0001$.

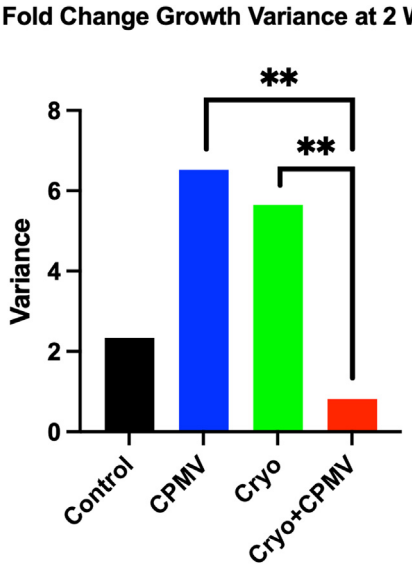

Figure E2. Cryoablation plus cowpea mosaic virus (Cryo + CPMV) was associated with decreased variance in tumor response compared with cryoablation only (Cryo) or cowpea mosaic virus (CPMV) alone. Variance of tumor volume was significantly lower in the Cryo + CPMV group than in either the CPMV or Cryo groups. Analyzed using the F-test of equality of variances and Tukey post hoc test. The asterisks indicate statistical significance: $^{**}P < .01$.

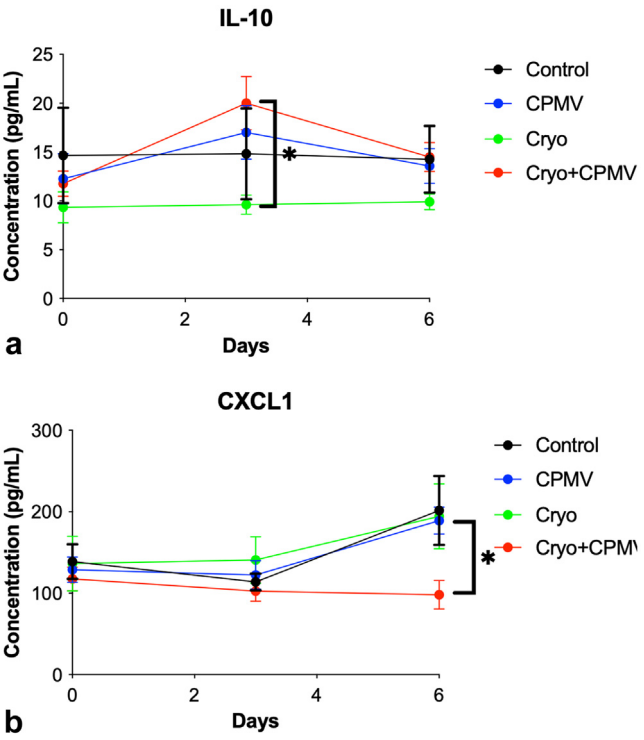

Figure E3. The serum levels of immunosuppressive cytokines interleukin (IL)-10 and CXCL1 differed among groups at select time points. Other serum cytokines and chemokines assayed showed no differences. **(a)** At Day 3, the IL-10 level significantly increased in the serum of cryoablation plus cowpea mosaic virus (Cryo + CPMV)–treated mice compared with that in cryoablation only (Cryo)–treated mice (20.0 pg/mL ± 2.7 vs 9.6 pg/mL ± 1.0, $P = .03$). This difference did not persist at Day 6. **(b)** At Day 6, the CXCL1 level significantly decreased in the serum of Cryo + CPMV–treated mice compared with that in the cowpea mosaic virus (CPMV) group (98.2 pg/mL ± 17.6 vs 189.0 pg/mL ± 16.6, $P = .01$). The asterisks indicate statistical significance: $^{*}P < .05$.

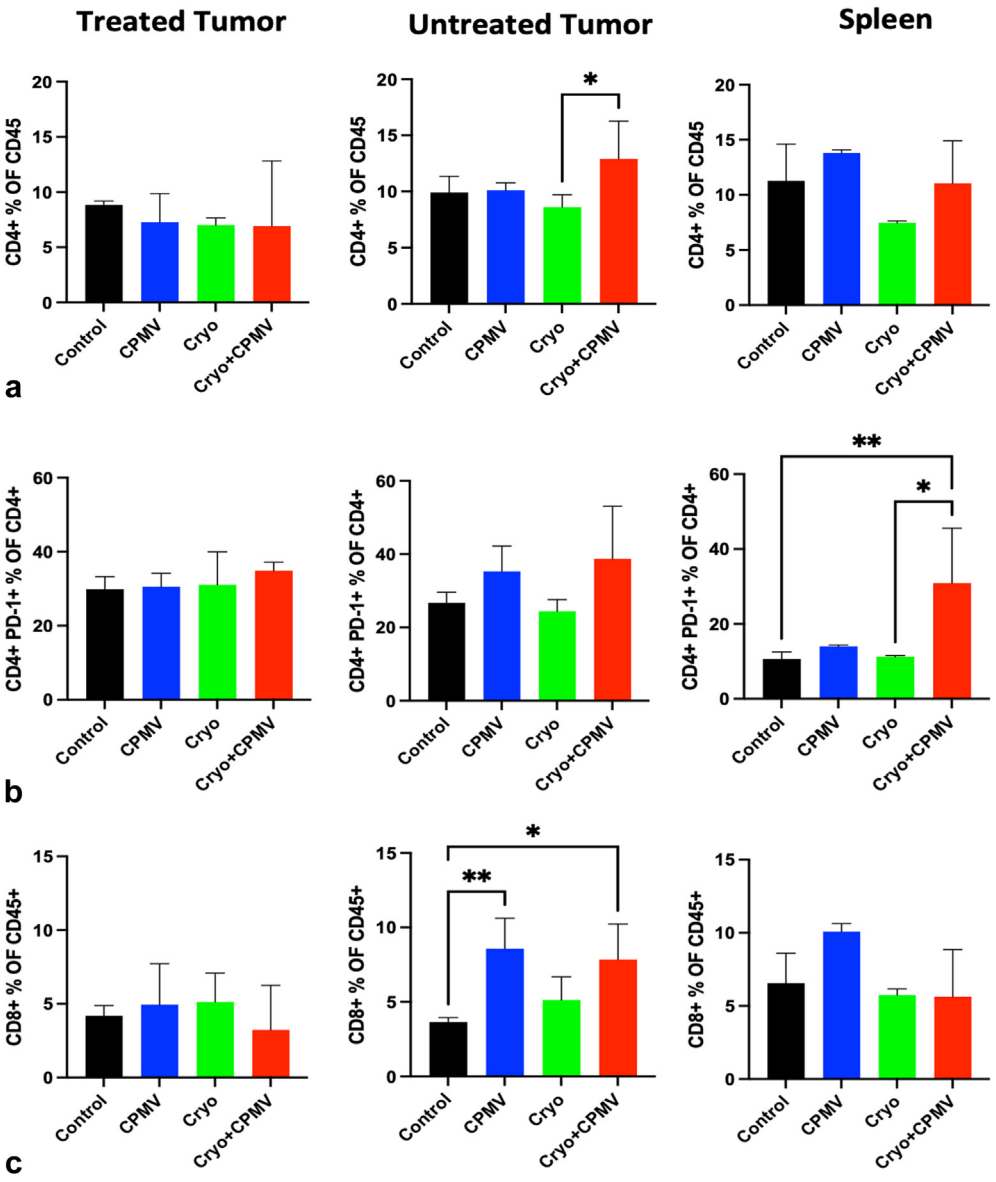

Figure E4. Cowpea mosaic virus (CPMV) and cryoablation (Cryo), alone or in combination, exerted varying effects on the populations and subpopulations of T cells and natural killer (NK) cells in the treated tumor, untreated tumor, and spleen. Flow cytometry was used to analyze the proportion of **(a)** $CD4^+$ T cells, **(b)** PD-1–expressing $CD4^+$ T cells ($CD4^+/PD\text{-}1^+$), **(c)** cytotoxic T cells ($CD8^+$), **(d)** activated cytotoxic T cells (CD8+/CD137+), **(e)** PD-1-expressing cytotoxic T cells ($CD8^+/PD\text{-}1^+$) T cells, and **(f)** NK cells among either all leukocytes ($CD45^+$), $CD4^+$ cells, or $CD8^+$ cells as indicated. Immune cell enrichment was analyzed using 1-way analysis of variance with Tukey post hoc tests. Treated tumors: There were no differences in the proportion of T or NK cells among any of the groups except for the **(d)** activated cytotoxic T cells, which were suppressed in the Cryo and CPMV groups compared with those in controls. Untreated tumors: Cryoablation plus cowpea mosaic virus (Cryo + CPMV) demonstrated enrichment of **(a)** $CD4^+$ T cells (vs the Cryo group), **(c)** cytotoxic $CD8^+$ T cells (vs the control group), **(e)** PD-1 expressing cytotoxic T cells (vs the Cryo group), and **(f)** NK cells (vs the control group). CPMV alone demonstrated enrichment of **(c)** cytotoxic T cells (vs the control group) but a suppression of the **(d)** activated subset of cytotoxic T cells (vs the control group). CPMV alone also demonstrated an enrichment of **(f)** NK cells (vs the control and Cryo groups). Spleens: CPMV again demonstrated an inhibition of **(d)** activated cytotoxic T cells, which was reversed with either Cryo or Cryo + CPMV. Additionally, **(f)** NK cells were increased in the Cryo + CPMV group compared with those in the control and CPMV groups, which differed with the untreated tumor data where CPMV generally increased NK cells. PD-1 expression of **(b)** $CD4^+$ and **(e)** $CD8^+$ cells increased with Cryo + CPMV, suggesting systemic immune cell exhaustion. The asterisks indicate statistical significance: $^*P < .05$, $^{**}P < .01$, and $^{****}P < .0001$.

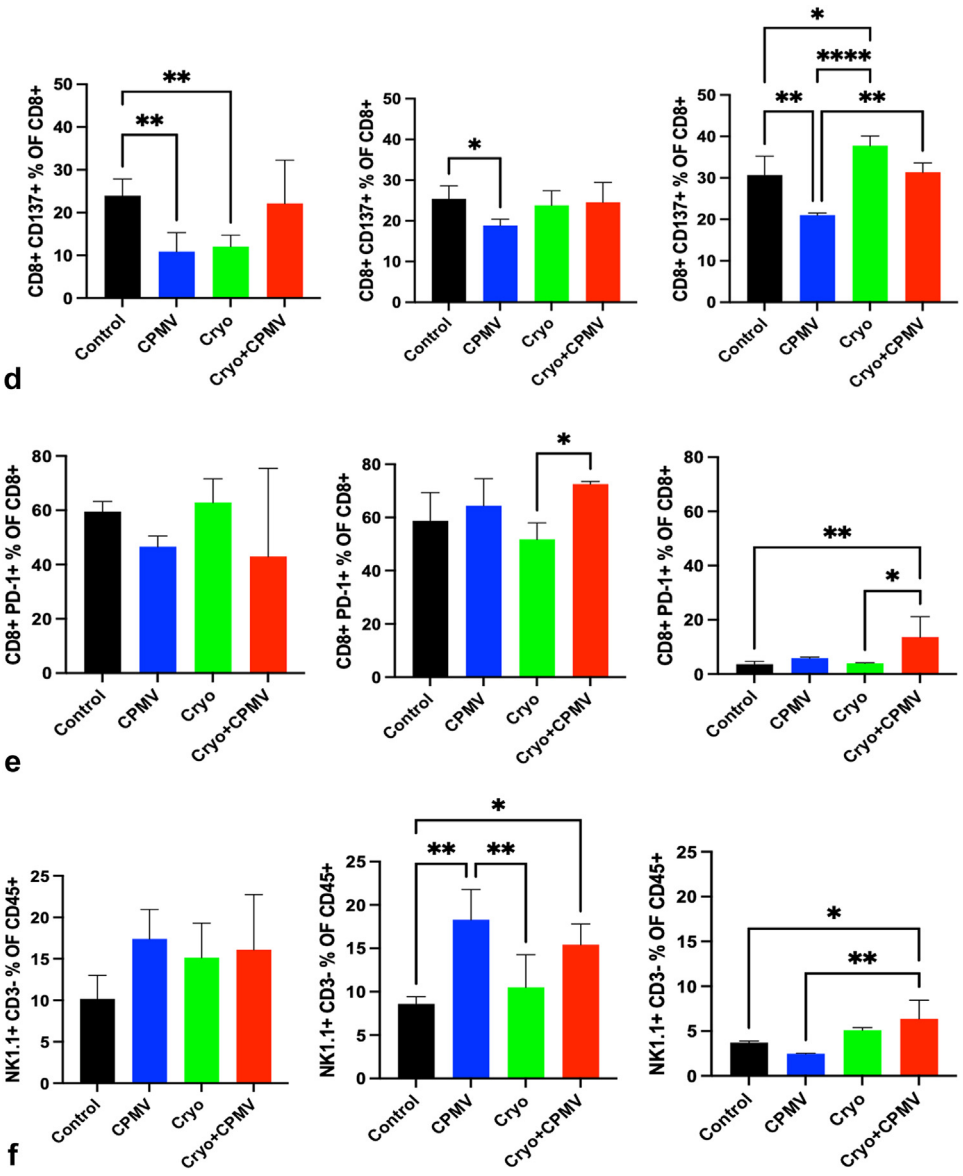

Figure E4. (Continued)

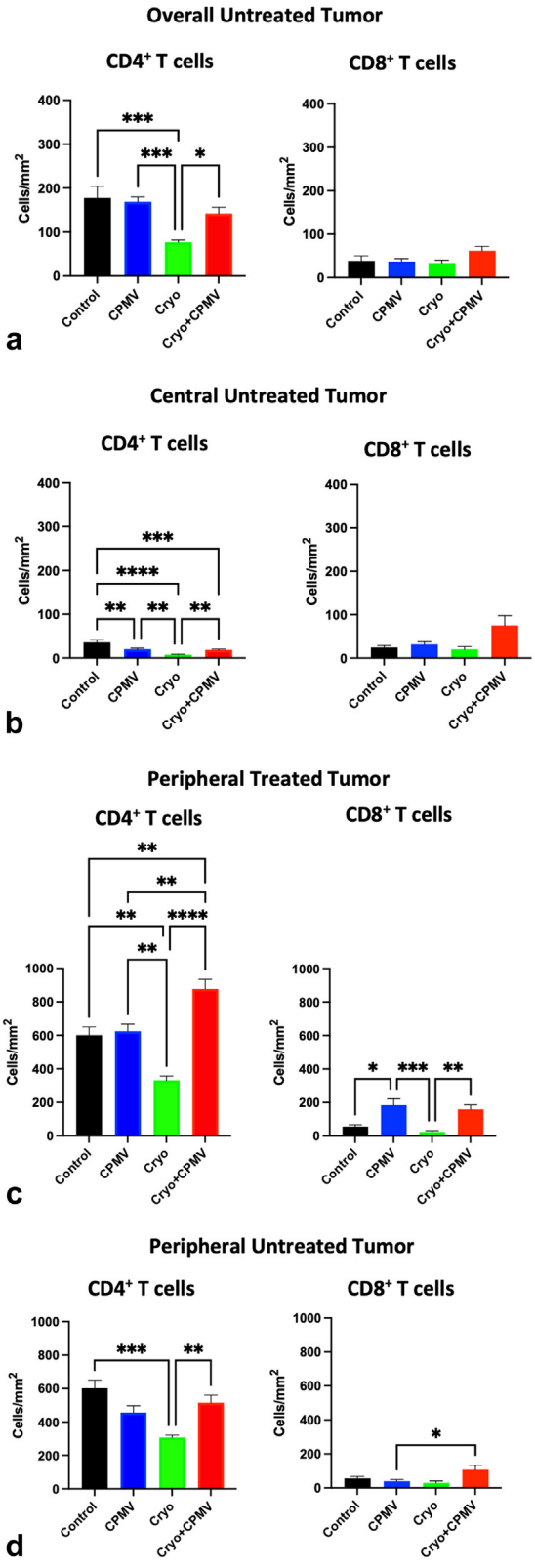

Figure E5. Supplementary immunohistochemistry data. The levels of $CD4^+$ and $CD8^+$ T cells in the untreated tumor **(a)** overall and **(b)** centrally as well as peripheral T cell levels in the **(c)** treated tumor and **(d)** untreated tumor are given. There is a relative paucity of $CD4^+$ T cells in the untreated tumor after cryoablation (Cryo) compared with those in all other groups. Centrally, all treatment groups had reduced $CD4^+$ cells with those in the control group. Although there was a trend for increased $CD8^+$ T cells overall and centrally, this did not reach statistical significance. The peripheral cell counts were generally in line with overall results. Cells were counted from 5–10 low-power fields from 3 sections from each mouse. Immune cell levels were analyzed using 1-way analysis of variance with Tukey post hoc tests. The asterisks indicate statistical significance: $^*P < .05$, $^{**}P < .01$, $^{***}P < .001$, and $^{****}P < .0001$. CPMV = cowpea mosaic virus; Cryo + CPMV = cryoablation plus cowpea mosaic virus.

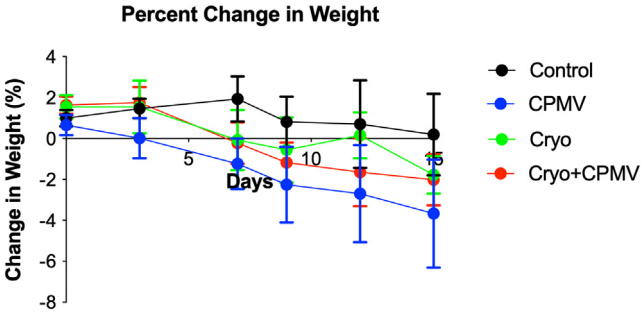

Figure E6. Percent change in weight from baseline. Cowpea mosaic virus (CPMV)-treated and cryoablation plus cowpea mosaic virus (Cryo + CPMV)–treated mice tended to weigh less than control mice; however, this difference did not reach statistical significance. Time course data were analyzed using repeated-measures 2-way analysis of variance.

Table E1. Fold Change in the Mean Serum Chemokine/Cytokine Levels

Cytokine/chemokine	Fold change in the mean level							
	Control		CPMV		Cryo		Cryo + CPMV	
	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6	Day 3	Day 6
IFN-γ	0.96	0.74	0.90	1.04	1.05	0.87	1.22	1.17
IL-1 β	7.69	9.00	4.93	5.05	4.85	7.84	5.76	4.82
IL-2	1.01	0.94	1.00	1.07	1.00	1.03	0.87	1.10
IL-4	0.99	0.95	1.08	1.02	1.52	1.08	1.54	0.45
IL-5	1.00	0.97	0.90	0.83	0.86	1.08	1.05	1.05
IL-10	**1.01**	**0.96**	**1.34**	**0.80**	**1.03**	**1.06**	**1.7**	**1.23**
IL-12p70	0.32	0.38	0.99	0.53	0.61	0.53	1.01	0.62
IL-13	1.02	0.90	0.88	0.17	1.42	1.37	0.90	0.77
CXCL1	**0.82**	**1.45**	**0.95**	**1.46**	**1.03**	**1.43**	**0.87**	**0.83**
TNF-α	0.99	1.34	1.22	1.49	1.32	1.16	1.13	1.23

Note–Significant differences were found for the IL-10 and CXCL1 levels, which are highlighted in bold.
Cryo = cryoablation; CPMV = cowpea mosaic virus; IFN-γ = interferon-γ; IL = interleukin; TNF-α = tumor necrosis factor α.

Table E2. Fold Change in Primary Tumor Volume Descriptive Statistics

	Control	CPMV	Cryo	Cryo + CPMV
No. of subjects	8	8	8	10
Minimum	4.601	0.0635	0.229	0.662
Maximum	9.223	6.645	7.403	3.171
Range	4.662	6.412	7.175	2.509
Mean	6.331	3.159	2.543	1.575
SD	1.528	2.554	2.377	0.903
SEM	0.54	0.903	0.840	2.221

Note–Descriptive statistics for the data on the volumes of treated tumor 2 weeks after treatment initiation that are presented in Figure 2 of the manuscript.
Cryo = cryoablation; CPMV = cowpea mosaic virus.

Table E3. Fold Change in Secondary Tumor Volume Descriptive Statistics

	Control	CPMV	Cryo	Cryo + CPMV
No. of subjects	5	5	6	8
Minimum	12.18	6.675	6.290	5.238
Maximum	23.78	23.95	17.07	14.03
Range	11.60	12.27	10.78	8.792
Mean	17.79	11.67	10.92	9.155
SD	4.763	7.083	3.593	2.589
SEM	2.130	3.168	1.467	0.9153

Note–Descriptive statistics for the data on the untreated tumor 2 weeks after treatment initiation that are presented in Figure 3 of the manuscript.
Cryo = cryoablation; CPMV = cowpea mosaic virus.