Electronic Supplementary Material (ESI) for Nanoscale.

Hydrogel-mediated Delivery of Celastrol and Doxorubicin Induces Synergistic Effect on Tumor Regression *via* Upregulation of Ceramides

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Table S1. IC_{50} (mean ± SD) for celastrol and doxorubicin in CT-26, HCT-8, DLD-1 and HCT-116 colon cancer cell lines.

Colon Cancer Cell Lines	IC_{50}	
	Celastrol (μM)	**Doxorubicin (μM)**
CT26	0.46 ± 0.17	0.35 ± 0.15
DLD-1	0.45 ± 0.06	0.49 ± 0.24
HCT-8	0.71 ± 0.06	0.23 ± 0.21
HCT-116	0.74 ± 0.16	0.92 ± 0.04

Table S2. Different sphingolipid species with corresponding precursor ion Q1 and product Q3 ions.

Sphingolipid Class	Species Targeted	Precursor ion, Q1	Product ion, Q3
Ceramides	C14:0	510.4	264.2
	C16:0	538.5	264.2
	C18:0	566.5	264.2
	C20:0	594.5	264.2
	C22:0	622.4	264.2
	C24:0	650.6	264.2
	C24:1	648.9	264.2
Glucosylceramides	C16:0	700.7	264.2
	C18:0	728.7	264.2
	C20:0	756.6	264.2
	C22:0	784.6	264.2
	C24:0	812.6	264.2
	C24:1	810.9	264.2
Lactosylceramides	C16.0	862.7	264.2
	C24.0	974.8	264.2
Sphingomyelins	C16.0	703.5	184.1
	C18.0	731.6	184.1
	C20.0	759.6	184.1
	C22.0	787.6	184.1
	C24.0	815.9	184.1

Table S3. Fold change in different ceramides, glucosylceramides, lactosylceramides, and sphingomyelin species on different treatments.

		Fold Change over Untreated Tumor Tissues		
Sphingolipid Class	**Species Targeted**	**C-Gel**	**D-Gel**	**CD-Gel**
Ceramides	C14:0	0.99	1.27	1.65
	C16:0	0.78	0.94	1.16
	C18:0	0.87	0.98	1.66
	C20:0	0.89	0.87	1.53
	C22:0	0.88	0.63	1.27
	C24:0	0.84	0.55	1.13
	C24:1	0.82	0.67	0.91
Glucosylceramides	C16:0	0.80	0.77	0.71
	C18:0	0.80	0.77	0.68
	C20:0	0.76	0.79	0.61
	C22:0	0.72	0.61	0.52
	C24:0	0.65	0.55	0.44
	C24:1	0.72	0.75	0.48
Lactosylceramides	C16.0	1.53	1.03	1.14
	C24.0	1.15	0.90	0.58
Sphingomyelins	C16.0	0.89	0.91	0.87
	C18.0	0.96	0.86	1.01
	C20.0	0.98	0.98	0.92
	C22.0	0.91	0.90	0.93
	C24.0	0.83	0.83	0.88

Table S4. Fold change in gene expression along with p value for genes of the sphingolipid pathway upon different drug treatments as compared to untreated mouse tissues.

		C-Gel		D-Gel		CD-Gel	
	Gene	FC[a]	*P* value[b]	FC[a]	*P* value[b]	FC[a]	*P* value[b]
1.	*Spt1*	2.79	< 0.01	1.85	< 0.05	0.28	<0.0001
2.	*Spt2*	2.00	< 0.0001	0.45	< 0.05	0.5	< 0.05
3.	*Kdsr*	0.66	< 0.01	0.26	< 0.0001	0.61	< 0.05
4.	*Cers1*	1.13	NS	0.48	< 0.01	4.9	< 0.005
5.	*Cers2*	0.65	< 0.05	0.57	< 0.05	0.33	< 0.0001
6.	*Cers4*	0.34	< 0.0001	0.47	0.05	2.61	< 0.0001
7.	*Cers5*	0.70	< 0.05	0.96	NS	0.52	< 0.0001
8.	*Cers6*	0.57	< 0.05	0.53	< 0.05	2.16	< 0.05
9.	*Degs1*	0.85	NS	1.48	NS	3.19	< 0.0001
10.	*Degs2*	0.20	< 0.0001	0.44	< 0.01	1.27	NS
11.	*Asah1*	1.16	NS	1.08	NS	0.65	< 0.01
12.	*Asah2*	0.70	< 0.0001	0.66	< 0.01	0.74	< 0.01
13.	*Asah3*	0.28	< 0.0001	0.53	< 0.05	2.7	< 0.05
14.	*Acer3*	0.21	< 0.0001	0.20	< 0.0001	0.28	< 0.0001
15.	*Sphk1*	0.48	< 0.05	0.13	< 0.0001	0.78	< 0.05
16.	*Sphk2*	1.59	NS	1.19	NS	0.46	< 0.0001
17.	*Sgpl1*	3.84	< 0.01	4.54	< 0.05	6.20	< 0.01
18.	*Sms1*	0.61	< 0.0001	0.33	< 0.005	0.26	< 0.0001
19.	*Sms2*	1.64	< 0.01	1.40	< 0.01	0.69	NS
20.	*Smpd1*	1.62	NS	1.10	NS	0.8	< 0.05
21.	*Smpd2*	0.38	< 0.01	0.17	< 0.0001	1.94	NS
22.	*Smpd3*	1.41	< 0.05	0.59	< 0.01	1.52	NS
23.	*Smpd5*	2.82	< 0.05	0.49	< 0.01	1.48	NS
24.	*Cerk*	0.44	< 0.01	0.45	< 0.005	0.95	NS
25.	*Spp1*	0.37	< 0.005	0.41	< 0.01	0.31	< 0.0001
26.	*Ugcg*	0.46	< 0.01	0.41	< 0.01	0.44	< 0.0001
27.	*Gba1*	1.99	< 0.05	0.72	< 0.05	0.94	NS
28.	*B4galt6*	0.64	< 0.01	0.91	NS	0.52	< 0.01
29.	*Glb1*	2.56	< 0.0001	1.43	NS	1.94	< 0.05

a: FC: means fold change in expression for a particular gene as compared to untreated tumor tissues. b: *p* value calculated by Student's *t* test for a given fold change.

Table S5. List of primers (mouse) used for validation of gene expression by Real-Time PCR.

Gene	Forward Primer (5' to 3')	Reverse primer (5' to 3')
Spt1	CCATCTGGATTTAGAAGAGCGC	GCCGCACTGTCCACAAAGATG
Spt2	GACTTTGTGTCCTTGTATCAGG	TTCCCTGTGTACTTGAATGACC
Kdsr	GCAGGGCAGTAATCACTACCA	GGCTTCACCTCCATCTGCAG
Asah1	AATAACACTTGGGTTGTCAC	TAG GAT ACC CAG ATA ACC AC
Asah2	CACAAAGATTCAGGAAATCACTGG	TGAGGAGACCCCGTGCATTC
Cers1	CGACCTGTGCCTCCTGTCA	CTGGAGGACAGACCGCTGT
Cers2	GATCCTACACTGCACGATGATA	CTTCTATCAGCTTTCCAGTTATG
Cers4	CTGTGAAGCCTGCTGGAGGT	TCAGCACCAAGGCAGGCTATG
Cers5	TGGCCAATTATGCCAGACGTGAG	GGTAGGGCCCAATAATCTCCCAGC
Cers6	GCA TTC AAC GCT GGT TTC GAC	TTCAAGAACCGGACTCCGTAG
Sphk1	GCTTTGTTGCTGACGTGGACC	CCACTATGCTGGGTACGAGC
Sphk2	CAGGGGACCAGGAAATCAC	GTCTGTATGAGGTTGAAGGAC
Acer1	CAGAGTACAAGAAGATTAGGGATG	CAGGTAGTAGAAGTGAATCCGCT
Acer3	CTGTGGACCGCAAAGGCTAT	GTATTCCAGAACTCAGCGACGA
Spp1	GGATGAGTCTGATGAGACCGT	GCATCAGGATACTGTTCATCAG
Sgpl1	GTTTTCCAGTTCTGACTTCAGG	GTGTCTACGCATCTCCAAGCA
Sgms1	CTGTTCTCCGAAGCTCTTTGG	GTTAAGCATGACGTGTGGCC
Sgms2	CTCCGCTCCCAGACAAGTTTT	CCACTATTGACTTGTAACGCAG
Smpd1	GCGCTGGTTCTGGCTCTGTT	CATTGGGCTCCTTCTTCAGCC
Smpd2	GTCTCTGAGTTCCACGTCTGC	TTCCAGTGGGCTAAAGTAGAGG
Smpd3	GGTCTACGGTTGTCATGGTTG	CTTCCCACCTGCACCTTGAG
Smpd5	CAGTCCAGGTACTAACCCAGG	GAATATGTCCATCCTCAACGGG
Cerk	CTTGTCCTCGGAGCCCTGG	GAAGTCGAACTGGTCCTCCTG
Glb1	GAGAATGAGTACGGGTCCTAC	GTGTGATATTGTTGCCTGTTCC
Gba	CATTTCCCGTGACCTAGGGCC	GGCTATCCCGTAATGATGCTGTG
Ugcg	CCAGAATGATCAGGTGGACC	CAGGCCACAGCATAATCAAG
Degs1	GAGATCCTGGCAAAGTATCCAG	GACCCATTTCCAGTCCAAGTC
Degs2	GGCCTATGCCCTTTGGTGGCT	GGGTACTTGGCGAGCATCTC
B4gatl6	GTCATCGAACAGACCGGCAC	CAAAGGTCATCGTCCTCGCC
β-actin	TCTACGAGGGCTATGCTCTCC	GGATGCCACAGGATTCCATAC

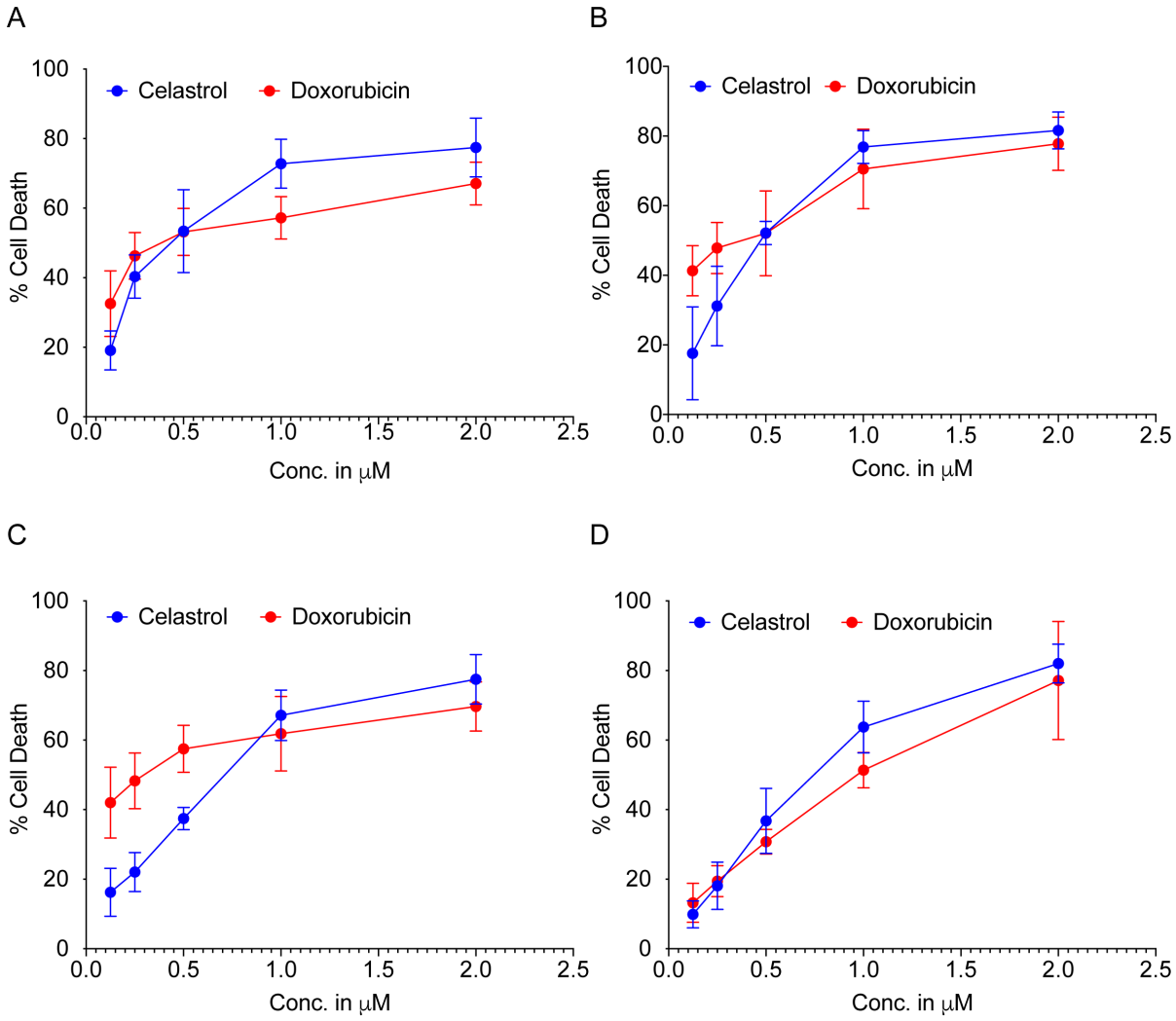


Figure S1. Cytotoxicity profiles of celastrol and doxorubicin against different colon cancer cells (A) CT26, (B) HCT-8, (C) DLD1 and (D) HCT116 cells.

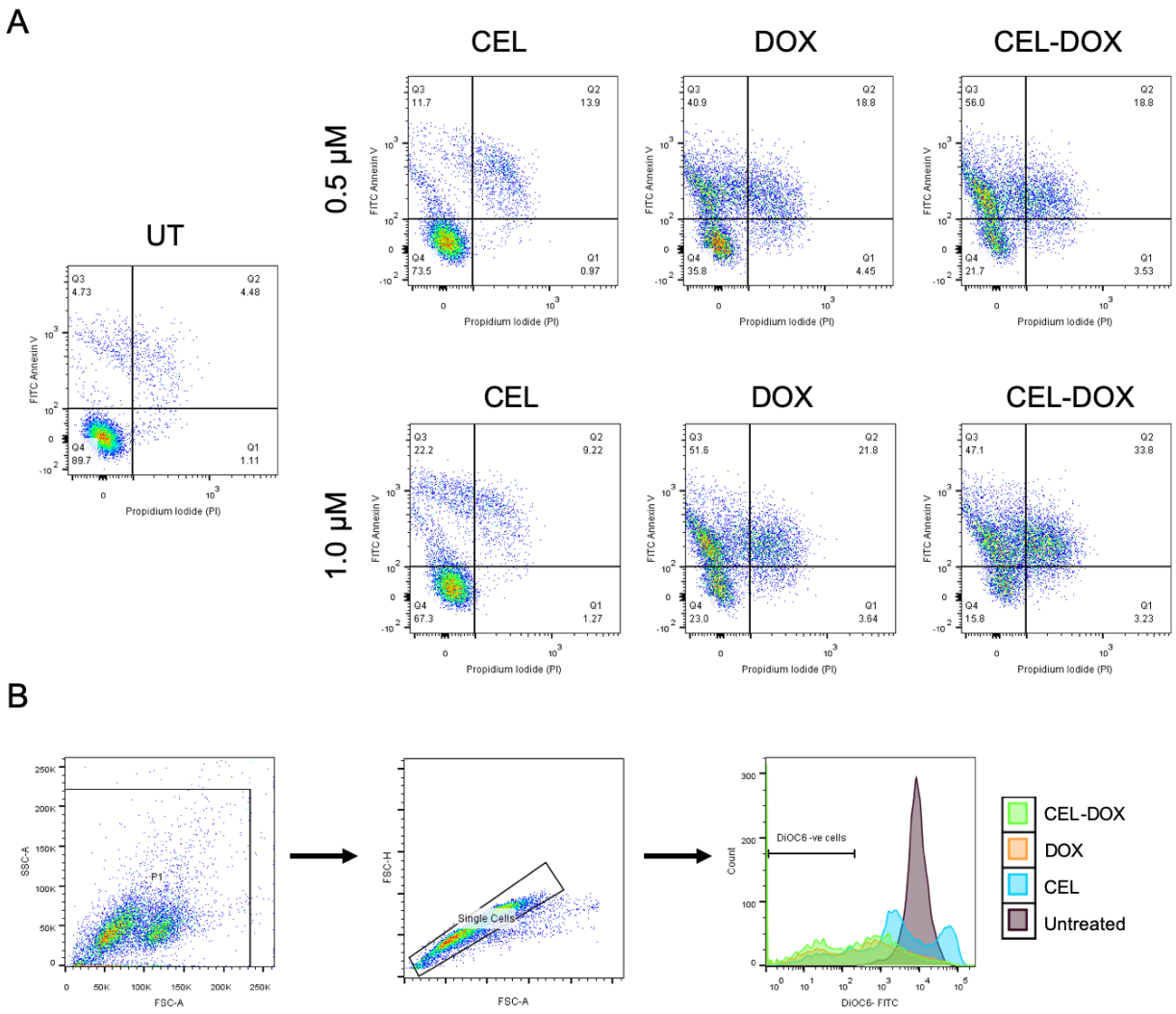


Figure S2. (A) Representative flow cytometry scans showing the staining of CT26 with Annexin-FITC and propidium iodide after treatment with CEL, DOX, and combination of CEL and DOX. (B) Representative flow cytometry scans showing DiOC6(3)-stained CT26 cells after treatment with CEL (0.5 μM), DOX (0.5 μM), and combination of CEL and DOX (0.5 μM each).

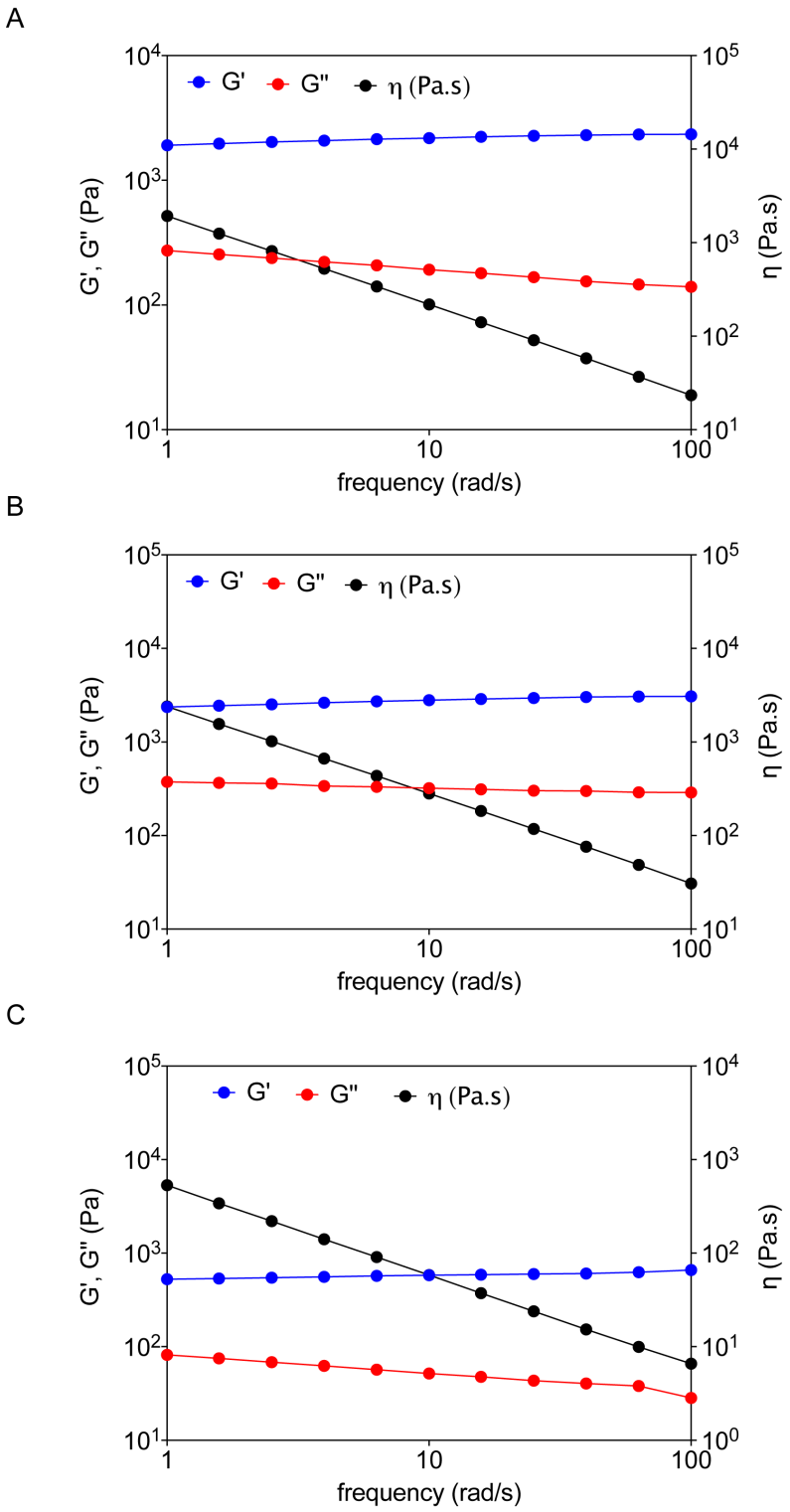
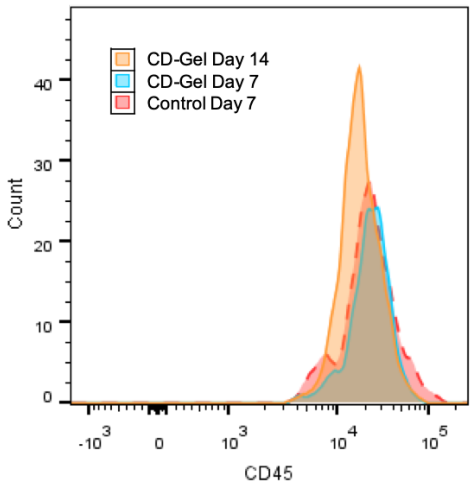


Figure S3. Frequency sweep studies of C-Gel (A), D-Gel (B) and CD-Gel (C).

A



B

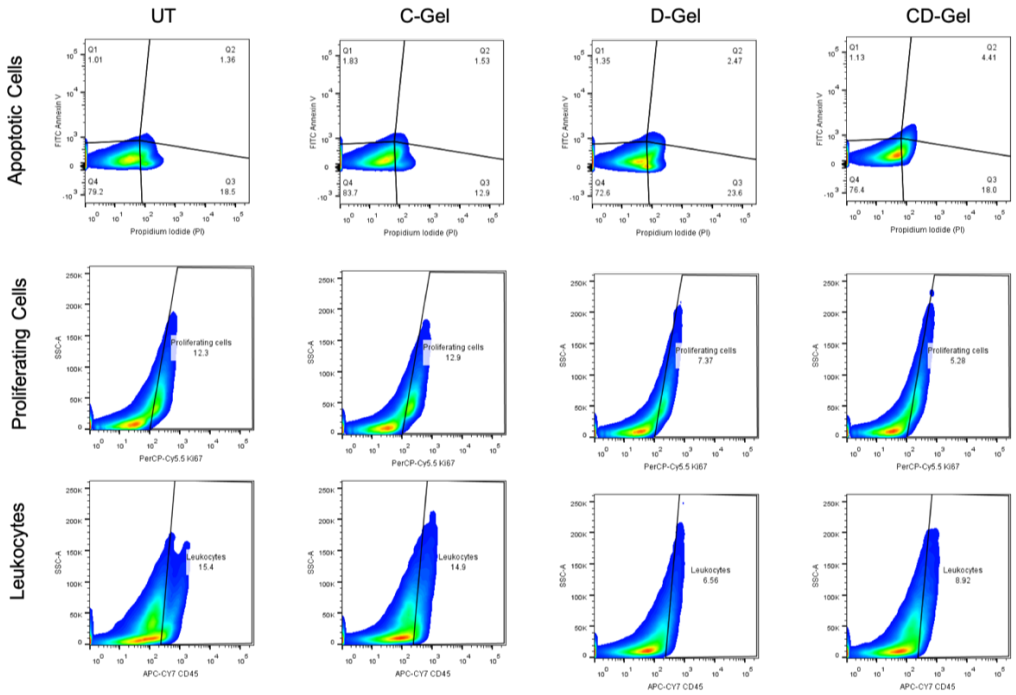


Figure S4. (A) Representative flow cytometry scans showing infiltration of $CD45^+$ cells at implant site after different durations. (B) Representative flow cytometry scans showing the staining of single cells isolated from CT26 tumor-bearing mice for quantification of apoptotic cells, proliferating cells, and leukocytes after treatment of tumor-bearing mice with different treatment regimens.

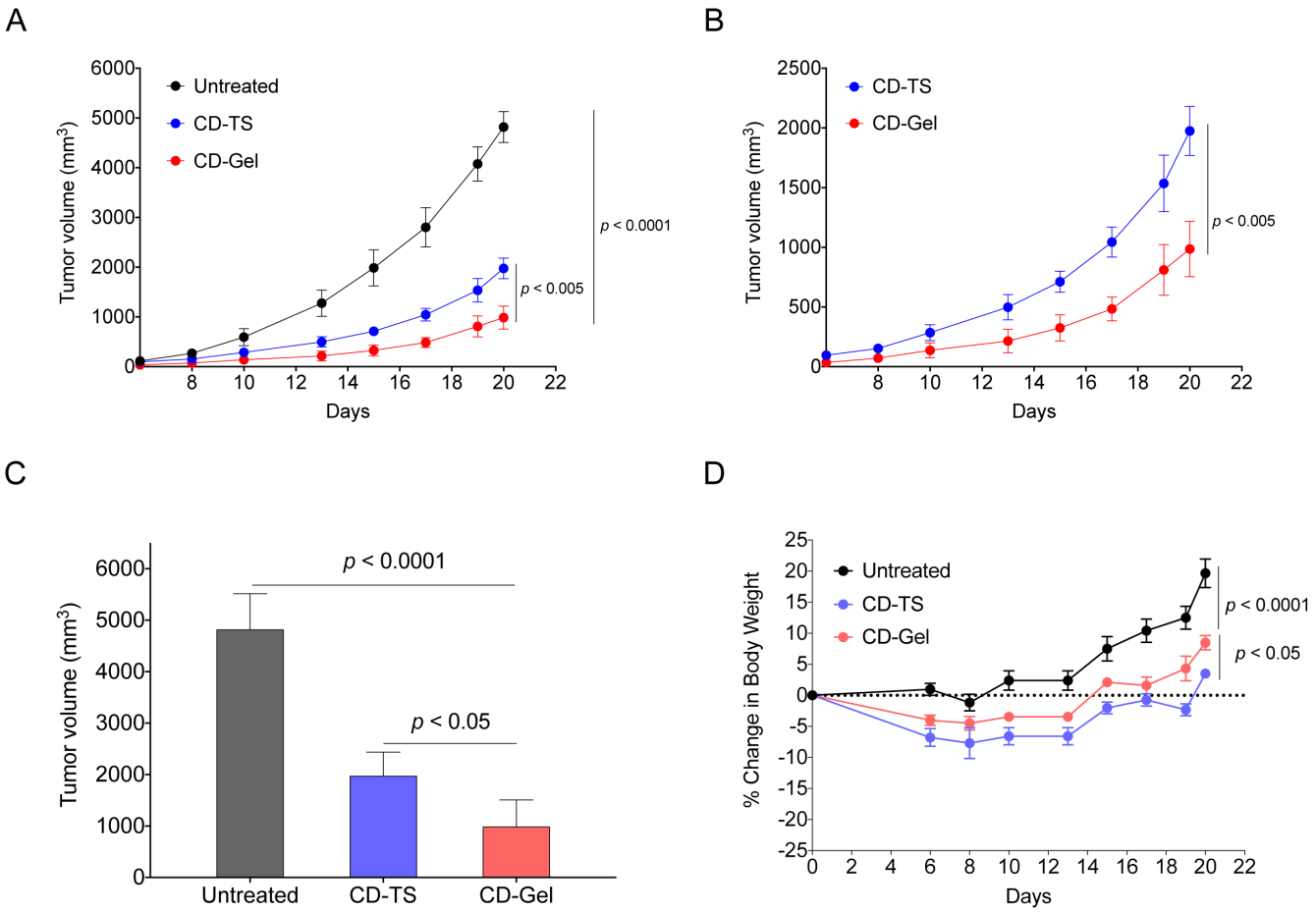


Figure S5. (A, B) Tumor growth kinetics (mean ± SEM, n = 6/group) of CT26 tumor-bearing mice after treatment with CD-TS and CD-Gel in comparison to untreated mice. (C) Final tumor volume (mean ± SEM, n = 6/group) on day 20. (D) Change in body weight of mice.