SUPPLEMENTARY FIGURES

Knudson, et al. Mechanisms involved in IL-15 superagonist enhancement of anti-PD-L1 therapy

Antibody	**Clone**	**Company**
FoxP3	R16-715	BD Biosciences
CD62L	MEL-14	BD Biosciences
CD44	IM7	BD Biosciences
CD3ε	2C11	BD Biosciences
CD247	MIH5	BD Biosciences
CD11b	M1/70	BD Biosciences
IFNγ	XMG1.2	BD Biosciences
TNFα	MPG-XT22	BD Biosciences
CD8α	53-6.7	ThermoFisher Scientific
Ki67	SolA15	ThermoFisher Scientific
FoxP3	FJK-16s	ThermoFisher Scientific
NKp46	29A1.4	ThermoFisher Scientific
NKG2D	CX5	ThermoFisher Scientific
CD279	J43	ThermoFisher Scientific
CD8β	53-5.8	Biolegend
Ly6C	HK1.4	Biolegend
Ly6G	1A8	Biolegend
CD49b	DX5	Biolegend
CD45.2	104	Biolegend
CD4	RM4-4	Biolegend
CD4	RM4-5	Biolegend
Granzyme B	GB11	Invitrogen

Table S1. List of flow cytometry antibodies used for analysis of murine immune cell populations.

Cell Population	**Flow Cytometry Gating Strategy**
$CD8^+$ T Cells	Live/$CD45.2^+$/$CD3\varepsilon^+$/$CD8\alpha^+$
$CD4^+$ T Cells	Live/$CD45.2^+$/$CD3\varepsilon^+$/$CD4^+$/$FoxP3^-$
$CD4^+$ T_{reg}	Live/$CD45.2^+$/$CD3\varepsilon^+$/$CD4^+$/$FoxP3^+$
NK Cells	Live/$CD45.2^+$/$CD3\varepsilon^-$/$CD49b^+$
G-MDSC/Granulocytes	Live/$CD45.2^+$/$CD11b^+$/$Ly6C^{lo}$/$Ly6G^{hi}$
M-MDSC/Monocytes	Live/$CD45.2^+$/$CD11b^+$/$Ly6C^{hi}$/$Ly6G^{lo}$
$CD45^+$ Cells	Live/$CD45.2^+$
$CD45^-$ Cells	Live/$CD45.2^-$

Table S2. Flow cytometry gating strategy used for identification of murine immune cell populations.

Figure S1

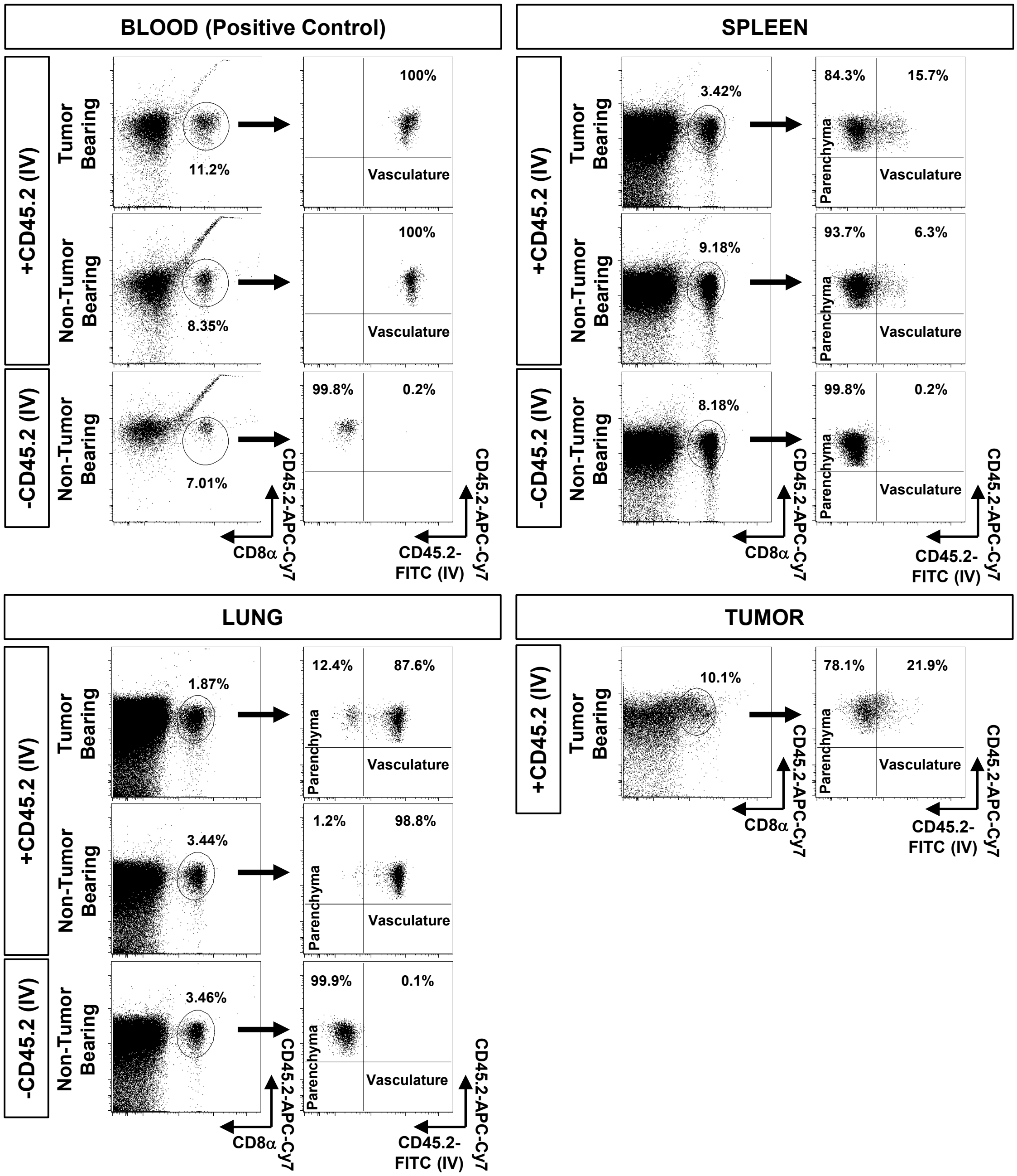
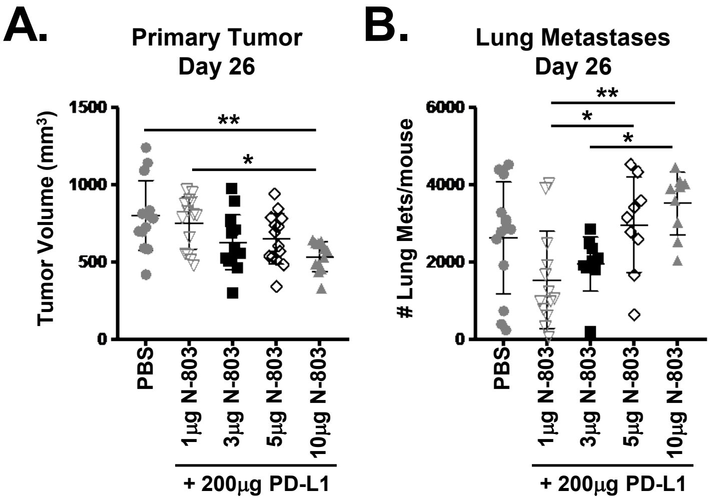


Figure S1. Validation of intravascular CD45-antibody labeling for the discrimination of vascular- versus parenchymal-resident immune cells in the spleen, lung, and primary tumor. 4T1 tumor implant was performed as in Figure 1. Intravascular labeling of all hematopoietic cells using an anti-CD45.2-FITC antibody was performed. Dot plots show examples of $CD8^{+}$ T cell populations in the blood (positive control), spleen, lung, and tumor from i.v.-labeled 4T1 tumor-bearing mice and i.v.-labeled and i.v.–non-labeled naïve (non-tumor bearing) control mice after subsequent staining with anti-CD45.2-APC-Cy7 and immune related markers. Lung parenchyma-resident immune cells were identified as CD45.2-APC-$Cy7^{+}$/CD45.2-$FITC^{-}$ and lung vascular-resident immune cells were identified as CD45.2-APC-$Cy7^{+}$/CD45.2-$FITC^{+}$. Data representative of 5 independent experiments, n=4-5 mice/group per experiment.

Figure S2



C.

Lung Metastases

# Lung Mets	PBS (n=13)	1μg N-803+ 200μg PD-L1 (n=13)	3μg N-803+ 200μg PD-L1 (n=10)	5μg N-803+ 200μg PD-L1 (n=9)	10μg N-803+ 200μg PD-L1 (n=9)
≥ 4000	3/13 (23%)	1/13 (8%)	0/10 (0%)	2/9 (22%)	3/9 (33%)
≤ 4000	10/13 (77%)	12/13 (92%)	0/10 (0%)	7/9 (78%)	6/9 (67%)
≤ 3000	7/13 (54%)	11/13 (85%)	10/10 (100%)	4/9 (44%)	2/9 (22%)
≤ 2000	**4/13 (31%)**	**10/13 (77%)**	**5/10 (50%)**	**2/9 (22%)**	**0/9 (0%)**
≤ 1000	**3/13 (23%)**	**6/13 (46%)**	**1/10 (10%)**	**1/9 (11%)**	**0/9 (0%)**
≤ 500	2/13 (15%)	2/13 (15%)	1/10 (10%)	0/9 (0%)	0/9 (0%)
≤ 100	1/13 (8%)	1/13 (8%)	0/10 (0%)	0/9 (0%)	0/9 (0%)
0	0/13 (0%)	0/13 (0%)	0/10 (0%)	0/9 (0%)	0/9 (0%)
Mean	2630	1545	1949	2972	3520
% Reduction		41%	26%	-13%	-33%

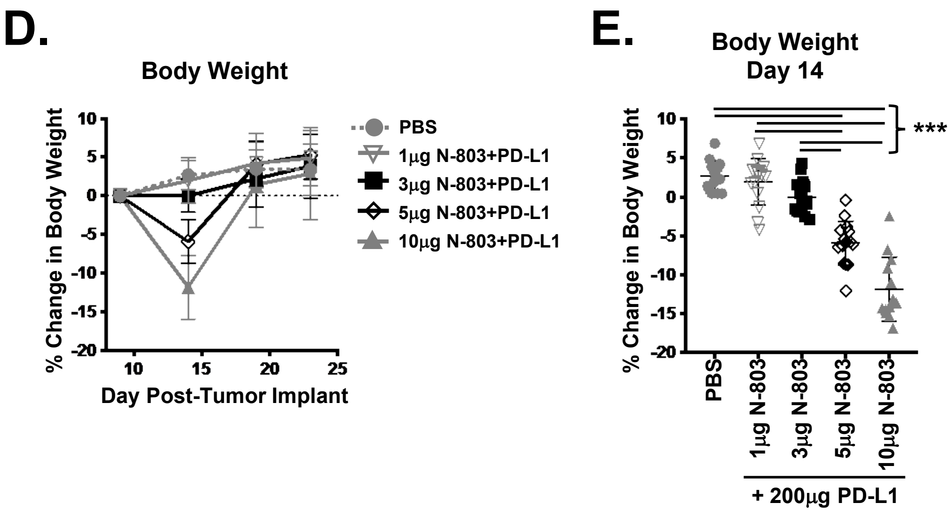
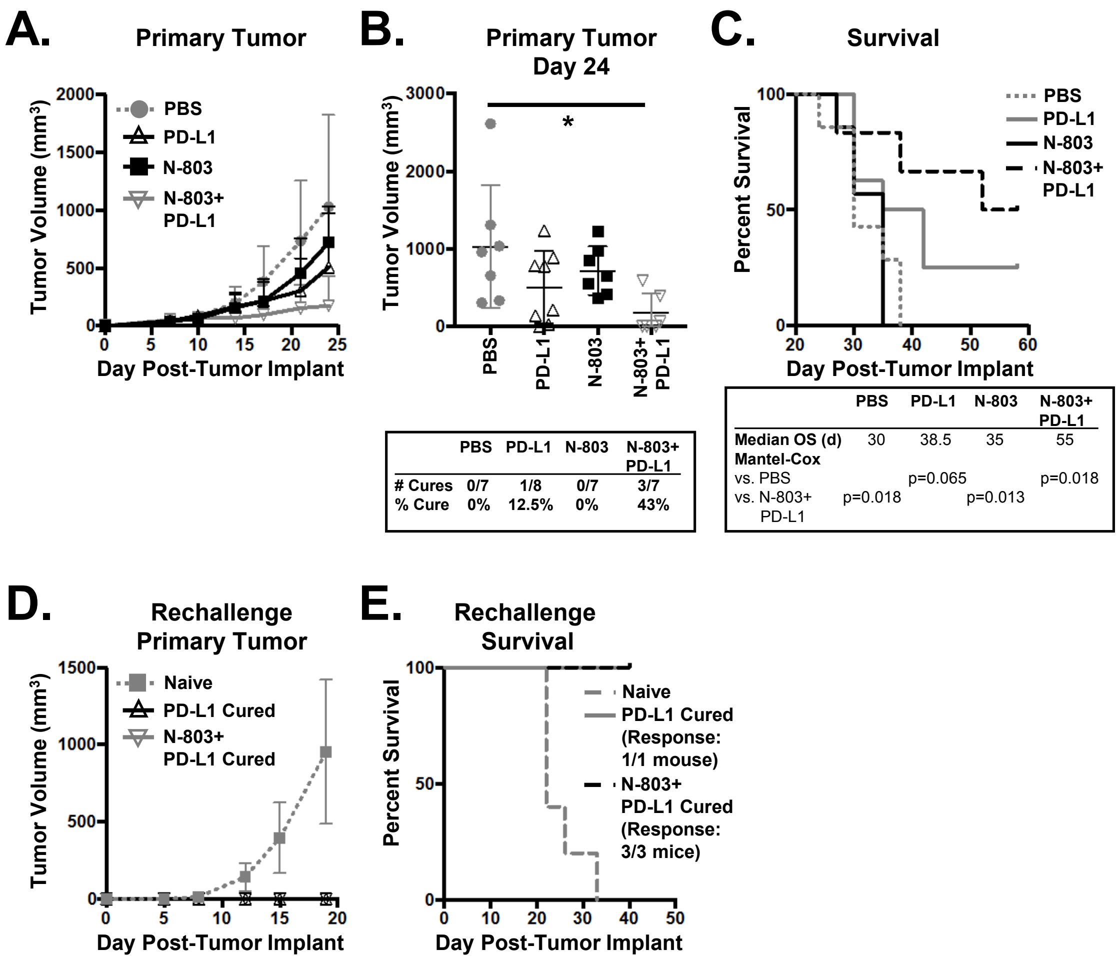


Figure S2. Dose escalation of N-803 in combination with a clinical relevant dose of αPD-L1. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 9 and 13 with 1μg, 3μg, 5μg, or 10μg N-803 (s.c) and 200μg αPD-L1 (i.p.) on days 9, 11, and 13. Graphs show tumor volumes of individual mice **(A)** or number of lung metastases in individual mice at day 26 post-tumor implant **(B)** as mean±SD. **(C)** Table shows distribution of lung metastases per mouse. **(D-E)** Graphs show % reduction in body weight (versus day 9 pre-treatment) over the course of the experiment **(D)** or of individual mice at day 14 (1 day post-final treatment) **(E)**. Data from 1 experiment, n=9-13 mice.

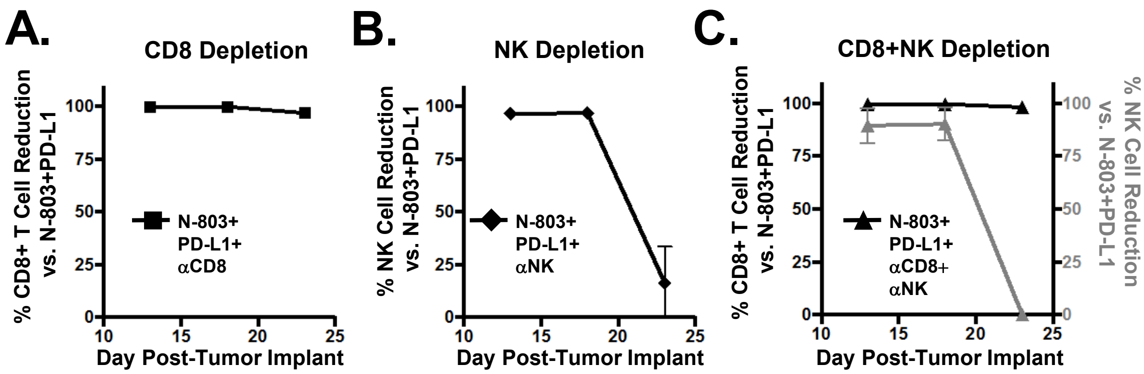
Figure S3

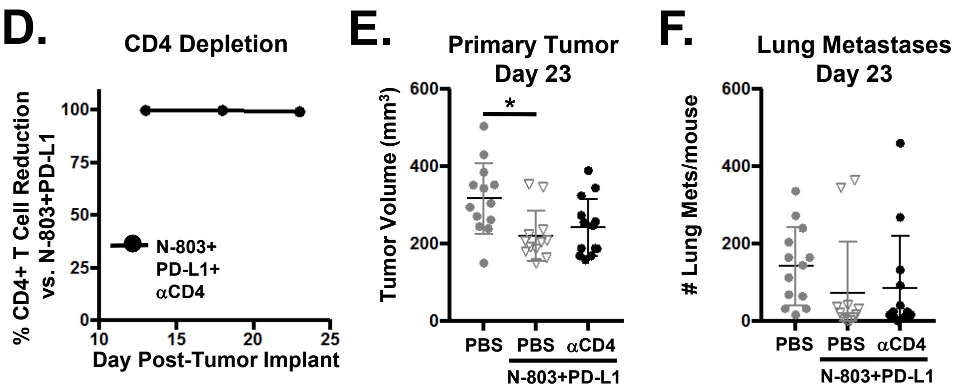


	PBS	PD-L1	N-803	N-803+ PD-L1
# Cures	0/7	1/8	0/7	3/7
% Cure	0%	12.5%	0%	43%

	PBS	PD-L1	N-803	N-803+ PD-L1
Median OS (d)	30	38.5	35	55
Mantel-Cox				
vs. PBS		p=0.065		p=0.018
vs. N-803+ PD-L1	p=0.018		p=0.013	

Figure S3. Combination of N-803+αPD-L1 reduces MC38-CEA primary tumor burden and increases survival. (A-C) 5x10[5] MC38-CEA tumor cells were implanted into the flank of female C57BL/6-CEA mice. When tumor volumes reached ~50mm^3, mice were treated at days 7 and 11 with 1μg N-803 (s.c.) and/or 200μg αPD-L1 (i.p.) on days 7, 9, and 11. Primary tumor growth curves **(A)** and tumor volumes of individual mice at day 24 (inset: % cured mice) **(B)** show mean±SD. **(C)** Survival curves (inset: mOS) show % survival. **(D-E)** At least 1 month after tumor cure (day 55), cured mice and paired naïve C57BL/6-CEA were implanted with 5x10[5] MC38-CEA tumor cells. **(D)** Primary tumor growth curves show mean±SD. **(E)** Survival curves (inset: # mice with memory response) show % survival. Data are from 1 independent experiment, n=7-8 mice.





G. Lung Metastases

# Lung Mets/ mouse	PBS (n=13)	N-803+ PD-L1 (n=12)	N-803+ PD-L1+αCD4 (n=13)
≤ 500	13/13 (100%)	12/12 (100%)	13/13 (100%)
≤ 200	9/13 (69.2%)	10/12 (83.3%)	11/13 (84.6%)
≤ 100	3/13 (23%)	10/12 (83.3%)	10/13 (76.9%)
≤ 50	**3/13 (23%)**	**10/12 (83.3%)**	**9/13 (69.2%)**
≤ 10	0/13 (0%)	5/12 (41.7%)	2/13 (15.4%)
0	0/13 (0%)	2/12 (16.7%)	1/13 (7.6%)
Mean	142.2	72.7	85.3
% Reduction	0%	49%	40%

Figure S4. (A-C) Depletion efficiency of CD8 and NK cell depletions. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 13 and 17 with N-803 and αPD-L1 on days 13, 15, and 17. CD8-expressing cells and NK cells were depleted on days 10, 11, 12, 16, and 19 using 100μg anti-CD8 and/or 25μl anti-asialo-GM1 (i.p.). Depletion efficiency of $CD8^+$ T cells **(A)**, NK cells **(B)**, or $CD8^+$ T and NK cells **(C)** was examined in the blood on days 13, 18, and 23 by flow cytometry. Data combined from 2 independent experiments, n=3 mice/group per experiment. **(D-G) $CD4^+$ T cells do not contribute to the anti-tumor efficacy of N-803+αPD-L1 combination.** Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 13 and 17 with N-803 and αPD-L1 on days 13, 15, and 17. CD4-expressing cells were depleted on days 10, 11, 12, 16, and 19 using 100μg anti-CD4. **(D)** CD4 depletion efficiency in the blood of 3 mice per group was determined on days 13, 18, and 23 by flow cytometry. Graphs show tumor volumes of individual mice **(E)** or number of lung metastases in individual mice at day 23 post-tumor implant **(F)** as mean±SD. **(G)** Table shows distribution of lung metastases per mouse. Data from 1 experiment, n=11-13 mice.

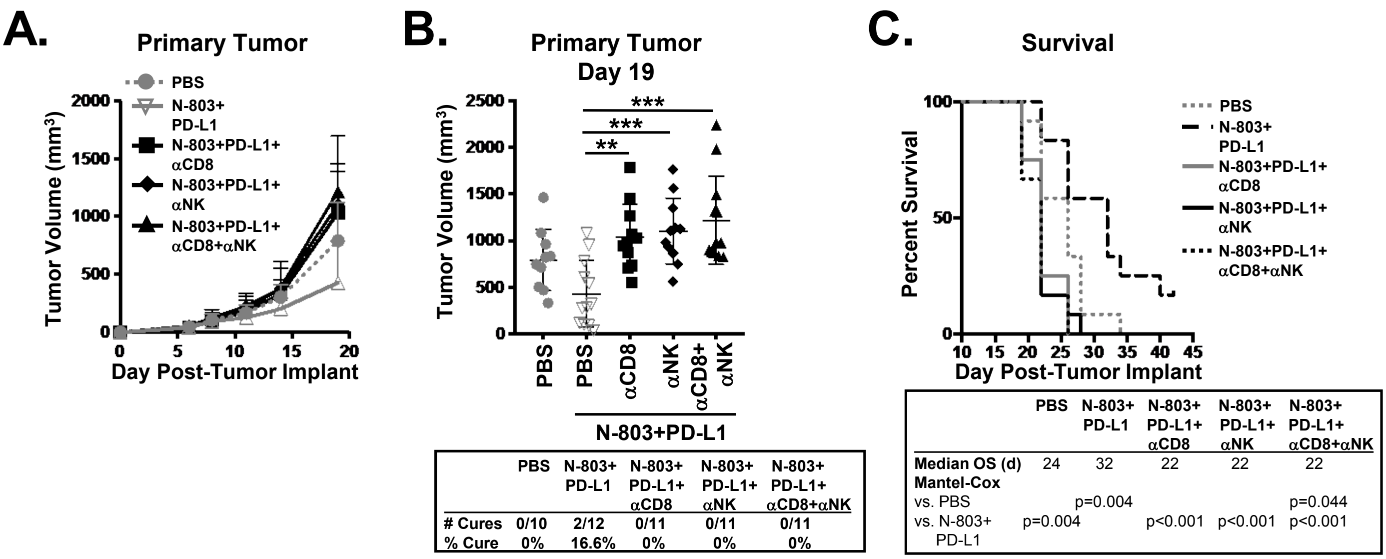


Figure S5. CD8⁺ T cells and NK cells are responsible for MC38-CEA anti-tumor efficacy. $3x10^5$ MC38-CEA tumor cells were implanted into the flank of female C57BL/6-CEA mice. When tumor volumes reached ~$50mm^3$, mice were treated at days 8 and 12 with 1μg N-803 (s.c.) and/or 200μg αPD-L1 (i.p.) on days 8, 10, and 12. CD8-expressing cells and NK cells were depleted on days 6, 7, 8, 11, and 14 using 100μg anti-CD8 and/or 25μl anti-asialo-GM1 (i.p.). Primary tumor growth curves **(A)** and primary tumor volumes of individual mice at day 19 (inset: % cured mice) **(B)** show mean±SD. **(C)** Survival curves (inset: mOS) show % survival. Data are from 1 independent experiment, n=10-12 mice.

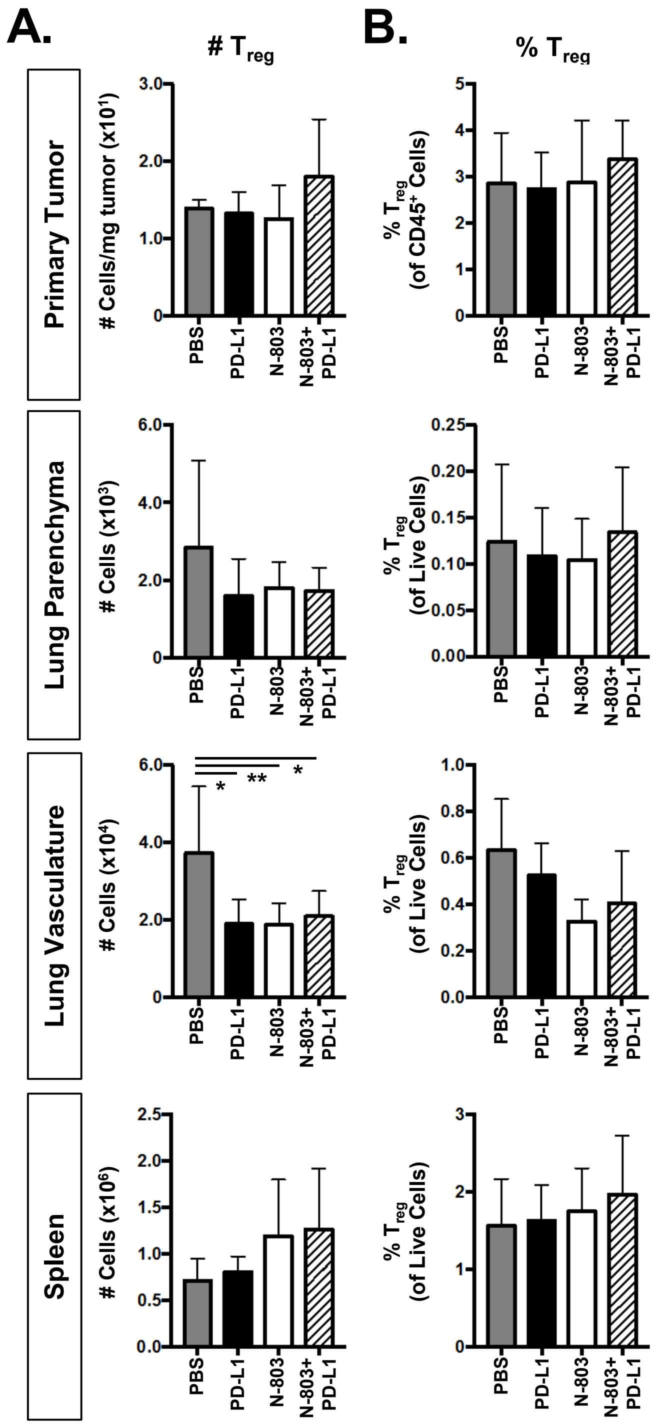


Figure S6. N-803+αPD-L1 combination decreases CD4⁺ T cell and T_{reg} numbers in the lung vasculature. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 9 and 13 with N-803 and/or αPD-L1 on days 9, 11, and 13. Graphs show $CD4^+$ T_{reg} number (**A**) and frequency **(B)** in the primary tumor, lung parenchyma and vasculature, and spleen 24 hours after the last treatment (mean±SD). Data combined from 2 independent experiments, n=5 mice/group per experiment.

Figure S7

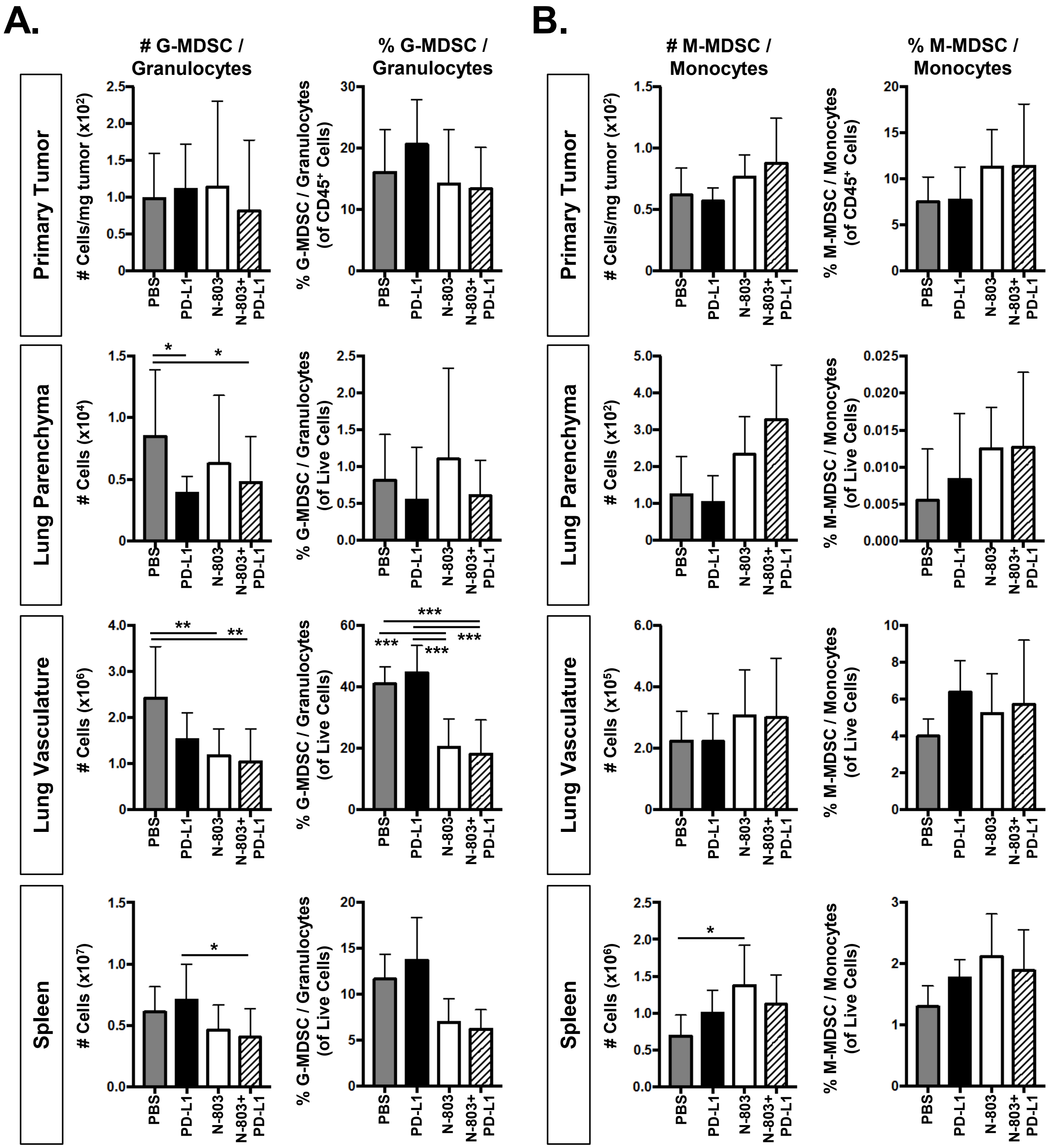


Figure S7. N-803+αPD-L1 combination reduces G-MDSC numbers in the lung vasculature. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 9 and 13 with N-803 and/or αPD-L1 on days 9, 11, and 13. Graphs show G-MDSC/Granulocyte **(A)** or M-MDSC/Monocyte **(B)** cell number (left panels) and frequency (right panels) in the primary tumor, lung parenchyma and vasculature, and spleen 24 hours after last treatment (mean±SD). Data combined from 2 independent experiments, n=5 mice/group per experiment.

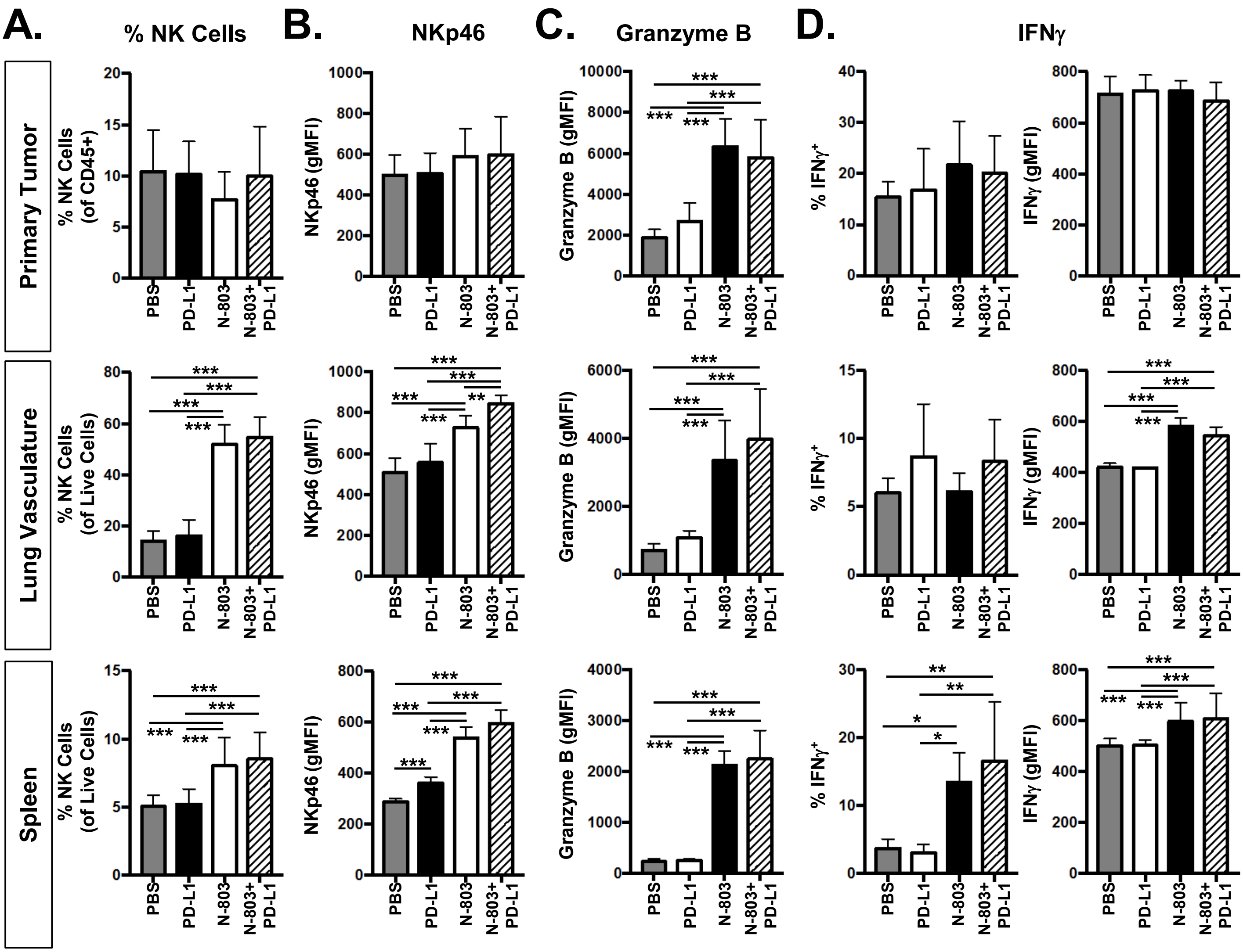


Figure S8. N-803 monotherapy and combination of N-803+αPD-L1 promotes an activated NK cell phenotype and increases NK function. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 9 and 13 with N-803 and/or αPD-L1 on days 9, 11, and 13. **(A-C)** NK cells were examined by flow cytometry in the primary tumor, lung vasculature, and spleen 24 hours after the last treatment. Graphs show NK cell frequency **(A),** expression of NKp46 (gMFI) in NKp46^{+} NK cells **(B)**, and expression of Granzyme B (gMFI) in NK cells **(C)**. **(D)** Immune cells were stimulated with 50ng/ml PMA+500ng/ml ionomycin for 4 hours. Graphs show frequency of IFNγ^{+} NK cells (left panel) and production of IFNγ (gMFI) (right panel) by NK cells. All graphs show mean±SD. Data combined from 2 independent experiments, n=5 mice/group per experiment.

Figure S9

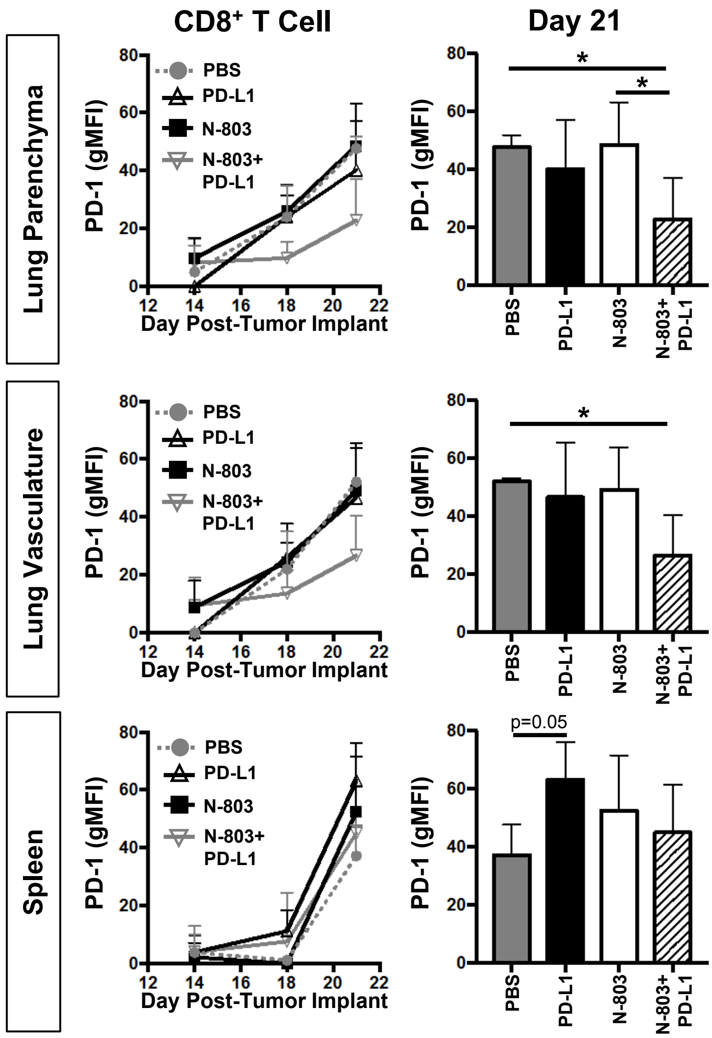


Figure S9. CD8$^+$ T cell expression of PD-1 is significantly reduced in lung parenchyma and vasculature after N-803+αPD-L1 treatment. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 9 and 13 with N-803 and/or αPD-L1 on days 9, 11, and 13. The primary tumor was resected at day 15. CD44^{hi} CD8$^+$ T cells were examined by flow cytometry for PD-1 expression in the lung parenchyma and vasculature and spleen at days 14, 18, and 21. Graphs of PD-1 expression (gMFI) of CD44^{hi} CD8$^+$ T cells at days 14, 18, and 21 (left panels) or day 21 only (right panels) show mean±SD. Data are from 1 experiment, n=5 mice.

Figure S10

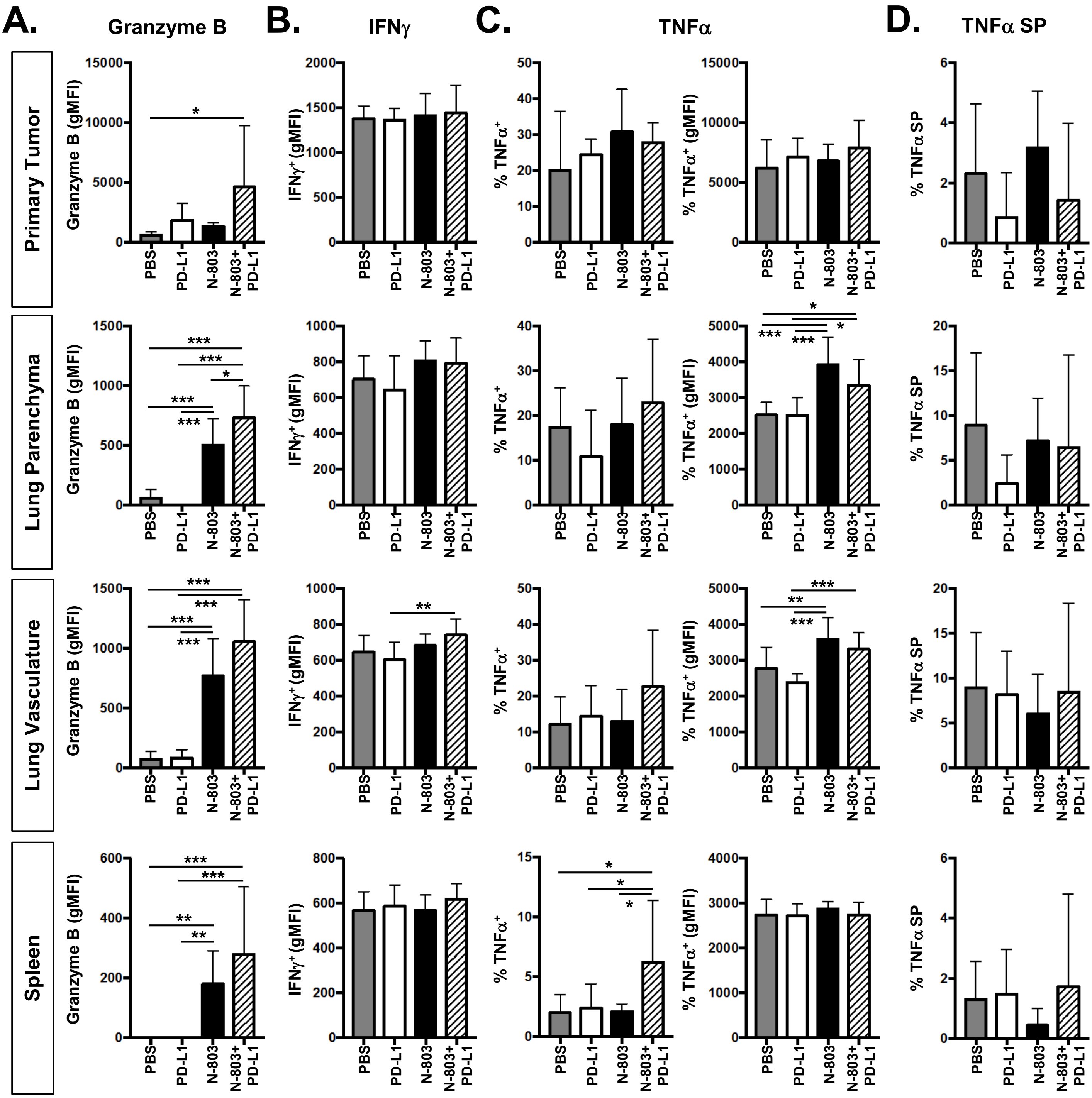


Figure S10. Combination of N-803+αPD-L1 increases effector function of CD8+ T cells. Mice were implanted with 4T1 tumors as in Figure 1 and treated at days 12 and 16 with N-803 and/or αPD-L1 on days 12, 14, and 16. CD8+ T cell effector cytokine and molecule production were examined by flow cytometry in the primary tumor, lung parenchyma and vasculature, and spleen 24 hours after last treatment. **(A)** Graphs show production of Granzyme B (gMFI) by CD44hi CD8+ T cells. **(B-D)** Immune cells were stimulated with 1μg/ml αCD3+1μg/ml αCD28 for 4 hours. Graphs show production of IFNγ (gMFI) by CD44hi CD8+ T cells **(B)**, frequency of TNFα+ CD44hi CD8+ T cells **(C, left panel)** or production of TNFα (gMFI) by CD44hi CD8+ T cells **(C, right panel)**, or frequency of TNFα-single producing (SP) CD44hi CD8+ T cells **(D)**. All graphs show mean±SD. Data combined from 2 independent experiments, n=5 mice/group per experiment.