Supplementary Data

Supplementary Methods

Quantitative PCR analysis

Total RNA was isolated from lung tissue or *in vitro* cultured 4T1.2 using RNAzol (Sigma-Aldrich) as per the manufacturer's instructions. For cDNA synthesis, 1 µg of total RNA was reverse transcribed in 20 µl reaction containing 500 µmol/l dNTP mix, 500 nmol/L OligodT, 200 U of Tetro Reverse Transcriptase and 20 U of Ribosafe RNase Inhibitor (Bioline) at 45°C for 50 min. The reactions were heated at 85°C for 5 minutes, cDNA product was diluted to 10 ng/ml and 2 µl of cDNA was subjected to 10 µl real-time PCR reaction. Real-time PCR was performed in duplicates with SensiFAST SYBR Lo-ROX Kit (Bioline) and primers (Sigma-Aldrich) on Applied Biosystems ViiA 7 Real-Time PCR system by using cycling conditions as follows: 50°C for 2 min, 95°C for 10 min, and then 40 cycles of 95°C for 15 s, 60°C for 1 min. Primers for mouse gp70-F: TGACCTTGTC CGAAGTGACC, gp70-R: TAGGACCCAT CGCTTGTCTT. HPRT-F: CCTCATGGACTGATTATGGACAG

HPRT-R: TCAGCAAAGAACTTATAGCCCC. gp70 expression was normalized to the housekeeping gene HPRT.

Supplemental Table 1 – List of antibodies used for flow cytometry

Antibody	Fluorochrome	Clone	Company
anti-CD45.2	A780	104	eBioscience
anti-CD45.2	FITC	104	eBioscience
anti-CD4	BV605	RM4-5	Biolegend
anti-CD8α	BV711	53-6.7	Biolegend
anti-TCRβ	PE-Cy5.5	H57-597	eBioscience
anti-CD44	PE	IM7	Biolegend
anti-CD62L	A780	MEL-14	eBioscience
anti-CD69	PE-Cy7	H1.2F3	Biolegend
H-2L^{d} tetramer to peptide SPSYVYHQF	APC	MuLV env gp70 423-431	NIH
anti-Ki67	eF450	Sol185	eBioscience
anti-Foxp3	AF488	FJK-16s	eBioscience
Zombie Aqua			Biolegend
anti-IFNγ	BV421	XMG1.2	Biolegend
anti-IFNγ	PE	XMG1.2	Biolegend
anti-TNF	PE	MP6-XT22	Biolegend
anti-TNF	BV605	MP6-XT22	Biolegend
Rat IgG1	PE	RTK2071	Biolegend

Supplementary Figure Legends

Supplementary Fig. S1. Neoadjuvant immunotherapy is more effective at reducing metastatic burden. A-B, Groups of BALB/c Foxp3-DTR mice (n = 5/grp) were injected with 5 x 10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice were treated with neoadjuvant or adjuvant DT (250 ng/mouse, i.p) on either day 10 or 16 respectively, while the control group received PBS on both day 10 and 16. All mice had their primary tumors resected on day 13. On day 30, all mice were sacrificed and their lungs collected. **A,** Representative H&E stained sections of lungs from the indicated groups of mice (scale bar 2 mm). Arrows indicate metastases. **B**, Quantification of gp70 gene expression in lungs from the indicated groups of mice. Levels of gp70 from *in vitro* passaged 4T1.2 tumor cells were also quantitated. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method using HPRT as housekeeping gene for normalization. Data presented as mean ± SEM. Experiment was performed once with significant differences between groups determined by one-way ANOVA with exact p values shown.

Supplementary Fig. S2. Late neoadjuvant Treg depletion is more effective than adjuvant Treg depletion in eradicating metastatic disease. Groups of BALB/c Foxp3-DTR mice were injected with 5 x 10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice were treated with late neoadjuvant or adjuvant DT (250 ng/mouse, i.p) on day 16. Primary tumors were resected on day 13 for the Adj DT-treated group and day 19 for the Late NeoAdj DT-treated group as indicated. Data pooled from 3 experiments (which includes Fig.3A) for the Late NeoAdj DT-treated group (n = 28 in total) and 7 experiments (which includes Fig. 1A, 3A, Supplementary Fig. S6) for the Adj DT-treated group (n= 59 in total). The Kaplan-Meier curves

for overall survival of each group are shown. Significant difference between indicated groups was determined by log-rank sum test with exact p values shown.

Supplementary Fig. S3. Efficacy of neoadjuvant immunotherapy is dependent on surgical resection of the primary tumor. Groups of BALB/c Foxp3-DTR mice (n = 10/grp) were injected with 2 x 10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice were treated with neoadjuvant anti-PD-1 and anti-CD137 mAb (100 μg/mouse of each mAb, i.p.) or control IgG (200 μg/mouse, i.p.) on days 17 and 19. All groups of mice received sham surgery on day 21 as indicated. **A,** The Kaplan-Meier curves for overall survival of each group are shown. Significant differences between indicated groups were determined by log-rank sum test with exact p values shown. **B,** 18 days after 4T1.2 injection, primary tumor growth was measured every 2 days. Data represented as mean tumor size ± SEM. Experiment was performed once. Significant differences in tumor size between the indicated groups of mice were determined by Mann-Whitney U test on day 30 (****p <0.0001).

Supplementary Fig. S4. Neoadjuvant compared to adjuvant chemotherapy does not lead to improved survival benefit. Groups of BALB/c WT mice (n = 10/grp) were injected with 2 x 10^4 4T1.2 mammary carcinoma cells into the mammary fat pad. As indicated, some groups of mice received neoadjuvant paclitaxel (PAC)(10 mg/kg, i.p.) or PBS on days 17 and 21 and all primary tumors were resected on day 21. Other groups of mice had their primary tumors resected on day 17 and treated with adjuvant PAC (10 mg/kg, i.p.) or PBS on days 21 and 25. The Kaplan-Meier curves for overall survival of each group are shown. Experiment was performed once.

Significant differences between indicated groups were determined by log-rank sum test with exact p values shown.

Supplementary Fig. S5. Long-term survivors following neoadjuvant immunotherapy treatment are tumor free and display immunological memory. A, Quantification of gp70 gene expression in lungs from tumor-free mice from neoadjuvant Treg depleted group 250 days after 4T1.2 tumor inoculation (n = 4). Levels of gp70 from *in vitro* 4T1.2 tumor cells were also quantitated. Gene expression was quantified by the $2^{-\Delta\Delta Ct}$ method using HPRT as housekeeping gene for normalization. Data presented as mean ± SEM. **B,** 250 days after 4T1.2 tumor cell injection, tumor-free mice from the neoadjuvant DT group (n = 5) were treated with a combination of anti-CD4, anti-CD8β and anti-asGM1 (100 μg/mouse of each mAb, i.p.) every week for 4 weeks. The Kaplan-Meier curve for overall survival of the group is shown. **C,** 250 days after 4T1.2 tumor cell injection, age-matched naive BALB/c Foxp3-DTR mice (n = 6) or tumor-free mice from the neoadjuvant Treg-depleted group (n = 5) were injected s.c. with 5 x 10^4 4T1.2 mammary carcinoma cells in the right flank. Data represented as mean tumor size ± SEM. Significant differences in tumor size between the groups were determined by Mann-Whitney U test on day 26 with exact p value shown. **D,** 250 days after 4T1.2 tumor cell injection, tumor-free mice from the neoadjuvant DT group or age-matched naive BALB/c Foxp3-DTR mice (n = 4/grp) were injected with 5 x 10^4 4T1.2 cells in the fourth right mammary fat pad. 13 days later, mammary fat pads were harvested and photographed as shown. **E,** 250 days after 4T1.2 tumor cell injection, tumor-free mice from the neoadjuvant DT group (n = 5) or age-matched naive BALB/c Foxp3-DTR mice (n = 4) were injected i.v. with 2 x 10^5 4T1.2 tumor cells. 14 days

later, lungs were harvested and metastatic burden quantitated. Mean ± SEM are shown. Significant difference between indicated groups was determined by unpaired Welch's t-test with exact p value shown. Representative H&E stained sections of lungs from the indicated groups of mice are also shown (scale bar 2 mm). All experiments were performed once.

Supplementary Fig. S6. Efficacy of neoadjuvant Treg depletion depends on T and NK cells. Groups of BALB/c Foxp3-DTR mice (n = 5-10/grp) were injected with 5 x 10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. As indicated, groups of mice were treated with neoadjuvant or adjuvant DT (250 ng/mouse, i.p.) on either day 10 or 16 respectively, while the control group received PBS on both day 10 and 16. All primary tumors were resected on day 13. In some groups, mice were additionally treated with anti-CD4, anti-CD8β or anti-asGM1 mAbs (all 100 μg/mouse each, i.p.) alone or in combination on day 9, 10, 16, 23 and 30. The Kaplan-Meier curves for overall survival of each group are shown. Results pooled from 2 independent experiments and performed blinded.

Supplementary Fig. S7. Kinetics of gp70 tumor-specific $CD8^+$ T cells in peripheral blood following neoadjuvant or adjuvant Treg depletion. Groups of BALB/c Foxp3-DTR mice (n =3-4/grp) were injected with 5 x 10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice were treated with neoadjuvant or adjuvant DT (250 ng/mouse, i.p.) on either day 10 or 16 respectively, while the control group received PBS on both day 10 and 16. All primary tumors were resected on day 13 except for the no surgery group. A naive mouse was also included for each experiment. Peripheral blood was collected from all groups of mice at the indicated time point for flow cytometry. Gating on live $CD45.2^+$ cells of lymphocyte

morphology, the proportion of gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ cells are shown. Data presented as mean + SEM. Data representative of two independent experiments.

Supplementary Fig. S8. Neoadjuvant immunotherapy increases tumor-specific CD8$^+$ T cells in the peripheral organs and primary tumor. A, Gating strategy and representative gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ T cells staining as gated on live CD45.2$^+$ of lymphocyte morphology in the same experiment as described and shown in Figure 6. **B,** In the same experimental set-up as Figure 6A-D, single cell suspensions from primary tumors resected on day 13 were generated for flow cytometry. The frequency, absolute numbers and representative FACs plot of gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ T cells gated on live CD45.2$^+$ lymphocytes are shown. Each symbol represents a single mouse. Data pooled from 2-3 experiments with significant differences between neoadjuvant DT and surgery groups determined by unpaired Welch's t-test with exact p value shown. **C,** In the same experiment as Figure 4D, single cell suspensions from primary tumors resected on day 16 were generated for flow cytometry. The frequency, absolute numbers and representative FACs plot of gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ T cells gated on live CD45.2$^+$ lymphocytes are shown. Each symbol represents a single mouse. Data pooled from 2 experiments with significant differences between neoadjuvant treatment and surgery groups determined by unpaired Welch's t-test with exact p value shown.

Supplementary Fig. S9. Neoadjuvant compared to adjuvant immunotherapy increases the number of tumor-specific CD8$^+$ T cells with effector phenotype. A-B, In the same experiment as described and shown in Figure 6E-F, the absolute numbers of gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ T cells that were CD44$^+$ CD62L$^-$ in the blood (**A**) and liver (**B**), Ki67$^+$ in the spleen (**C**) and liver

(**D**), IFNγ^+ (**E**) or TNF$^+$ (**F)** in the spleen are shown. Each symbol represents a single mouse. Data pooled from 2 experiments with significant differences between neoadjuvant DT (day 14) and adjuvant DT (day 20) groups determined by unpaired Welch's t-test with exact p value shown.