RESEARCH ARTICLE

Improved Efficacy of Neoadjuvant Compared to Adjuvant Immunotherapy to Eradicate Metastatic Disease

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ABSTRACT

Immunotherapy has recently entered a renaissance phase with the approval of multiple agents for the treatment of cancer. Immunotherapy stands ready to join traditional modalities, including surgery, chemotherapy, radiation, and hormone therapy, as a pillar of cancer treatment. Although immunotherapy has begun to have success in advanced cancer treatment, its scheduling and efficacy with surgery to treat earlier stages of cancer and prevent distant metastases have not been systematically examined. Here, we have used two models of spontaneously metastatic breast cancers in mice to illustrate the significantly greater therapeutic power of neoadjuvant, compared with adjuvant, immunotherapies in the context of primary tumor resection. Elevated and sustained peripheral tumor-specific immune responses underpinned the outcome, and blood sampling of tumor-specific CD8$^+$ T cells immediately prior to and post surgery may provide a predictor of outcome. These data now provide a strong rationale to extensively test and compare neoadjuvant immunotherapy in humans.

SIGNIFICANCE: We demonstrate the significantly greater therapeutic efficacy of neoadjuvant, compared with adjuvant, immunotherapies to eradicate distant metastases following primary tumor resection. Elevated and sustained peripheral tumor-specific immune responses underpinned the outcome, and blood sampling of tumor-specific CD8$^+$ T cells immediately prior to and post surgery may provide a predictor of outcome. *Cancer Discov; 6(12); 1382–99. ©2016 AACR.*

See related commentary by Melero et al., p. 1312.

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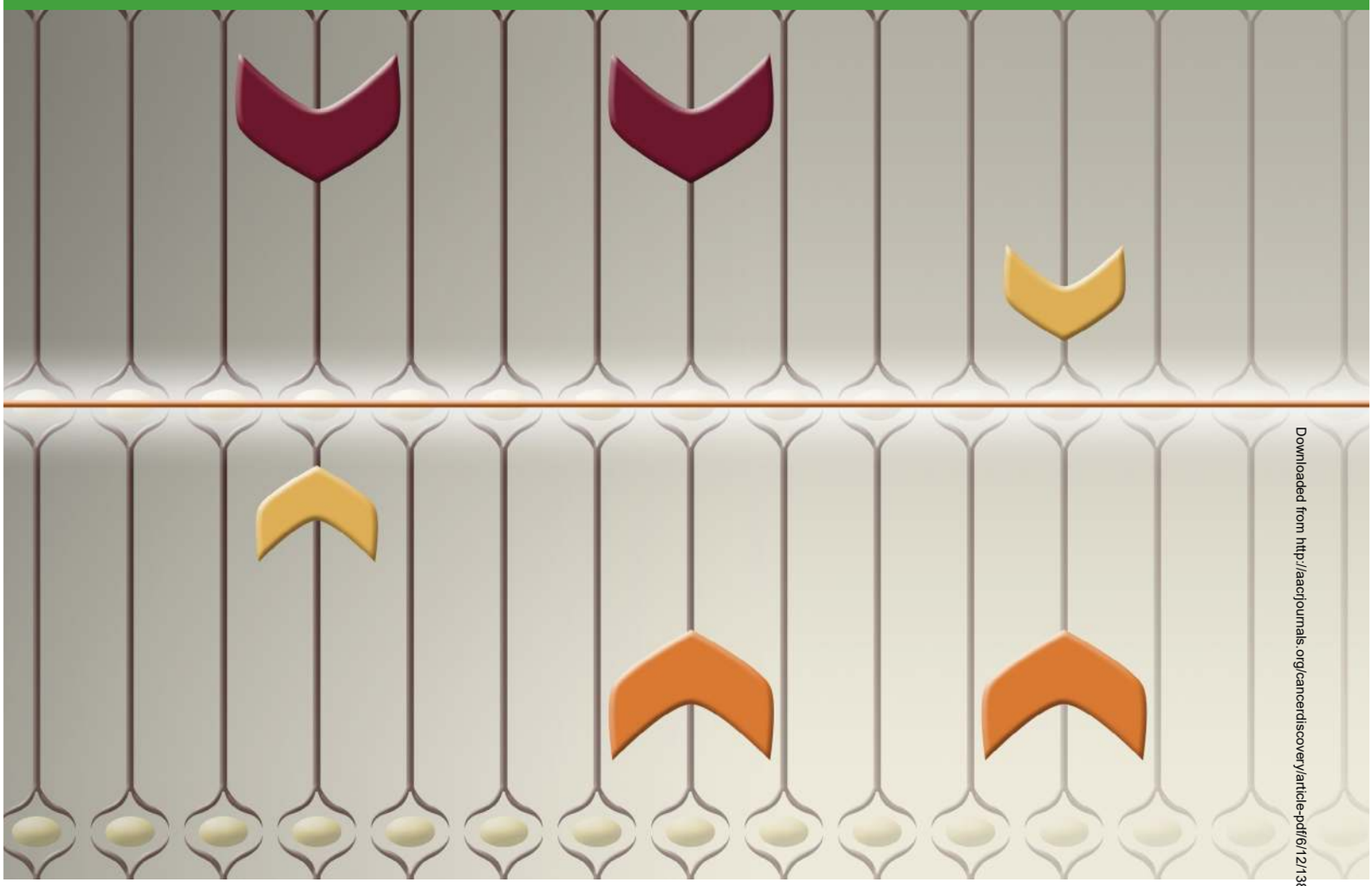

INTRODUCTION

Cancer surgery remains the most effective single modality for curing patients if staging reveals that the tumor can be removed by local resection (1). Unfortunately, only half of all patients with cancer undergo surgery with curative intent, with most relapsing from metastases. Thus, the standard schedule of cancer therapy for patients generally involves surgery followed by adjuvant therapies. Cancer immunotherapies that target the T-cell checkpoint receptors cytotoxic T-lymphocyte–associated protein 4 (CTLA4) and programmed cell death 1 (PD-1) and programmed cell death 1 ligand (PD-L1) are revolutionary new therapies able to cause long-term tumor regression and potential cures in advanced cancers (2). These therapies are predicted to be used to treat a large proportion of patients with advanced cancers over the next 10 years and most likely will be used in patients at earlier stages of disease, where surgery is potentially curative but sometimes fails due to occult distant metastases (3). Thus, how to best combine immunotherapies with surgery to reduce disease recurrence is a very meaningful question for the treatment of resectable tumors.

Neoadjuvant therapy refers to the systemic treatment of cancer prior to definitive surgical therapy (i.e., preoperative therapy). The primary objective of neoadjuvant therapy is to improve surgical outcomes in patients for whom a primary surgical approach is technically not feasible. Furthermore, systemic therapy administered to patients with nonmetastatic invasive cancer (e.g., of the breast) is also intended to reduce the risk of distant recurrence, because metastatic disease post surgery kills a significant proportion of patients with cancer. Although it was hypothesized that overall survival would be improved with earlier initiation of systemic therapy in patients with breast cancer at risk of distant recurrence, clinical studies have not yet demonstrated a survival benefit for preoperative versus postoperative delivery of systemic therapy such as chemotherapy (4–6). Although anti-CTLA4 and anti–PD-1/PD-L1 are FDA approved for the treatment of advanced metastatic malignancies, including melanoma, non–small cell lung carcinoma (NSCLC), and renal cell carcinoma (RCC; refs. 7–11), their efficacy in adjuvant settings is currently being evaluated. The efficacy of adjuvant anti-CTLA4 has been studied in a randomized phase III trial where it was compared with placebo (EORTC 18071; ref. 12), and a relapse-free survival advantage was seen compared with placebo in patients with resected stage III cutaneous melanomas, although overall survival data are not yet mature (13). Given its favorable therapeutic index, the efficacy of adjuvant anti–PD-1 is also

being assessed in a number of clinical trials (13). In one study, adjuvant anti–PD-1 plus vaccine in patients with resected stage IIIC and IV melanoma reported encouraging relapse-free survival data (14). Clinically, there have been studies reporting that neoadjuvant therapies including chemotherapies, targeted therapies, and immunotherapy (ipilimumab) given as a neoadjuvant could improve outcome in the management of patients with multiple different solid tumors (15–18). However, whether immunotherapies will be more efficacious when given in a neoadjuvant setting compared with an adjuvant setting is unknown; a head-to-head comparison study has only now just opened (NCT02519322).

Theoretically, neoadjuvant immunotherapy might prime an effective systemic immunity, which could be potentially effective in eradicating residual metastatic disease after the primary tumor is surgically removed. To study how the therapeutic efficacy of different scheduling regimens of surgery and immunotherapy affected metastases and survival, we utilized two models of triple-negative breast cancer (TNBC), the 4T1.2 and E0771 breast carcinoma cell lines. Following 4T1.2 tumor inoculation in the mammary fat pad, and prior to extensive primary tumor growth, mice develop extensive metastases in the lungs, liver, bones, and brain, among other organs (19). Similarly, mice inoculated with E0771 develop lethal metastases in the lungs prior to excision of the primary tumor (20). Previously, the primary tumor has been surgically resected, and these mice were then treated with the agent of interest to assess how adjuvant therapy affected metastases and survival. These two preclinical tumor models are generally utilized to mimic a clinical setting of surgery and adjuvant therapy of residual metastatic disease. In contrast, carcinogen and genetically modified mouse models of cancer do not offer this opportunity, because few truly metastasize, and metastasis is generally minimal relative to primary tumor size with late resection becoming impractical. Here, we demonstrate the significantly greater therapeutic power of neoadjuvant compared with adjuvant immunotherapies in the context of primary tumor resection in two models of TNBC.

RESULTS

Enhanced Efficacy of Neoadjuvant Treg Depletion to Eradicate Metastases

Depletion of regulatory T cells (Treg) represents the simplest manipulation and most effective immunotherapy to relieve tumor-induced immunosuppression, given it suppresses the antitumor activity of different immune cell types (21). We have previously reported conditional Treg depletion alone can eradicate a proportion of established experimental tumors and those arising from *de novo* carcinogenesis (22). This therapy was used initially as a "proof of principle" to answer the question of whether neoadjuvant immunotherapy was more effective compared with adjuvant immunotherapy. We injected 4T1.2 tumors into BALB/c FOXP3-GFP-DTR mice (FOXP3-DTR) where all Tregs (defined as $CD4^+$ $FOXP3^+$) express GFP, allowing for detection, and express human diphtheria toxin (DT) receptor, which allows for their conditional depletion following DT treatment (ref. 23; Fig. 1A). Our data demonstrated that a proportion of mice that received neoadjuvant or adjuvant Treg depletion had significantly improved long-term survival (>250 days) compared with the control group that received PBS, where all mice died by day 100 (Fig. 1A). More striking was the result from the neoadjuvant Treg-depleted group, where almost all mice (19/20) displayed long-term survival compared with adjuvant Treg-depleted mice (5/20; Fig. 1A).

The improved survival of mice treated with neoadjuvant Treg depletion was also demonstrated by the lack of observable micrometastases in the lungs of these mice (0/5; as measured by IHC), when harvested 30 days after tumor inoculation compared with PBS-treated mice (4/5; $P < 0.05$, Fisher exact test; Supplementary Fig. S1A). Although micrometastases were also observed in a proportion of the adjuvant Treg-depleted group (3/5), this was not statistically significant when compared with the neoadjuvant Treg-depleted group (Supplementary Fig. S1A). In an attempt to determine differences in 4T1.2 tumor burden between the adjuvant and neoadjuvant Treg-depleted groups, we performed q-PCR in the lungs of these mice harvested 30 days after tumor inoculation to measure for gp70 expression as a more sensitive method to detect the presence of 4T1.2 tumors (Supplementary Fig. S1B). Envelope glycoprotein (gp70), encoded by the endogenous murine leukemia virus (MuLV), is universally expressed in a range of mouse cancer cell lines, including 4T1.2, but is generally silent in normal mouse tissues (24). Although detectable levels of gp70 were measured in a proportion of the control group (4/5) or those that received adjuvant Treg depletion (3/5), it was undetectable in the neoadjuvant Treg-depleted group (0/5); the relative gp70 gene expression levels were not significantly different (Supplementary Fig. S1B). Repeat experiments might allow early differences in tumor burden between the neoadjuvant-treated and adjuvant-treated groups to become clearer, but our data in Fig. 1A demonstrate strong proof of principle that neoadjuvant immunotherapy positively affected survival outcomes.

Given that complete Treg depletion is currently not feasible in the clinic, we next asked if similar effects could be obtained when Tregs were depleted using anti-CD25 mAbs (Fig. 1B). Prior to the generation of FOXP3-DTR mice, Tregs were depleted in mice using anti-CD25, as they highly expressed CD25 (IL2Rα). In the clinic, FDA-approved denileukin diftitox, an engineered protein combining IL2 and DT which binds to the IL2 receptor, has been used as a strategy to reduce Treg numbers in patients. Importantly, results from Fig. 1B validated our hypothesis that neoadjuvant immunotherapy was more efficacious at generating long-term survivors. Forty percent of mice (8/20) in the neoadjuvant anti-CD25–treated group survived long term compared with those that received adjuvant anti-CD25 (10%; 2/20). Although the proportion of long-term survivors in the neoadjuvant anti-CD25–treated group was lower compared with that of the neoadjuvant Treg-depleted mice, this result was still striking, considering only one dose of treatment was administered. In contrast, adjuvant anti-CD25–treated mice displayed no statistically enhanced survival over mice that received cIg (Fig. 1B). Importantly, this improved efficacy of neoadjuvant Treg depletion on metastases and long-term survival depended critically on resection of the primary tumor (Fig. 1C). Neoadjuvant Treg depletion of mice that received no surgery did not survive long term, due to eventual outgrowth of the primary tumor (Fig. 1C).

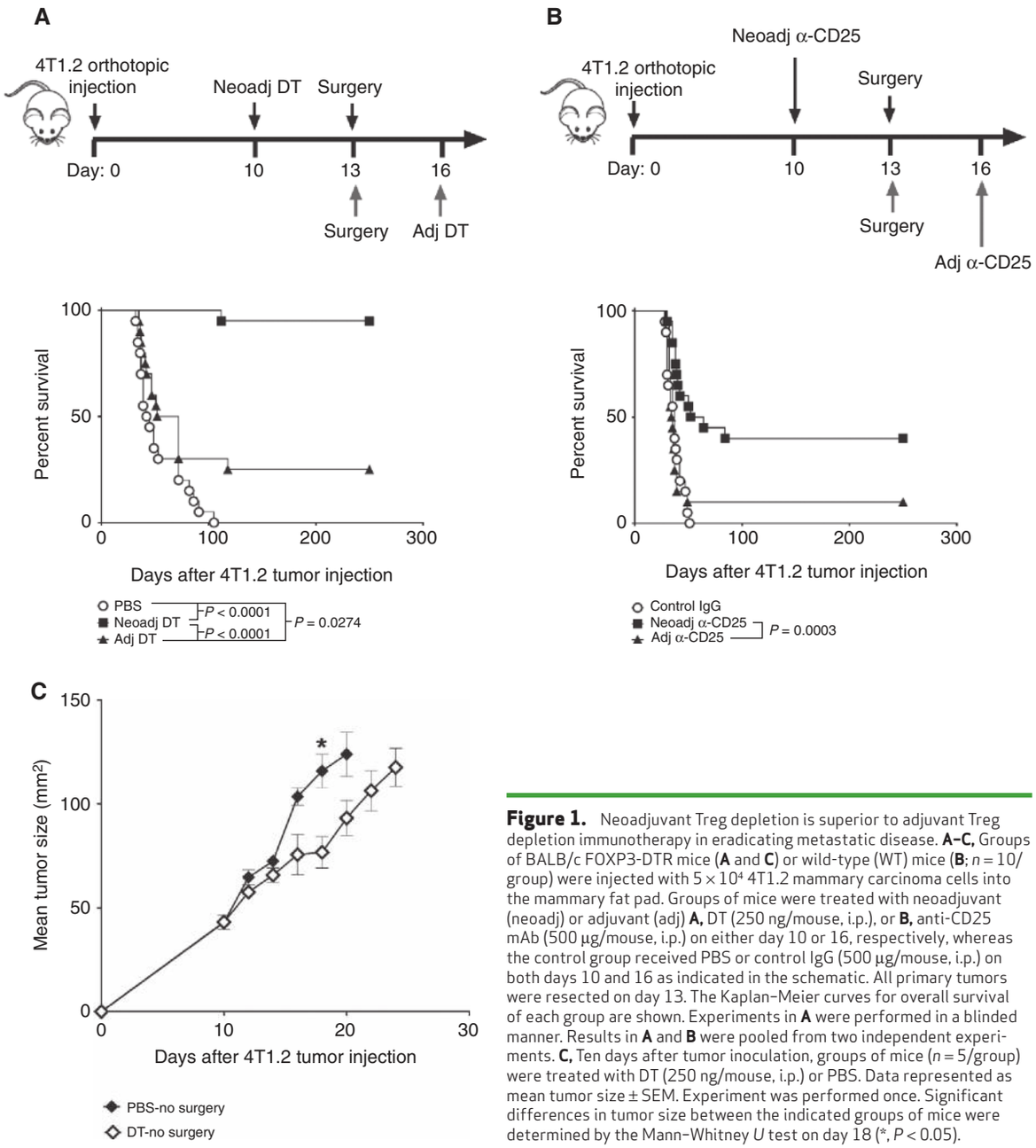

Figure 1. Neoadjuvant Treg depletion is superior to adjuvant Treg depletion immunotherapy in eradicating metastatic disease. **A–C,** Groups of BALB/c FOXP3-DTR mice (**A** and **C**) or wild-type (WT) mice (**B**; $n = 10$/group) were injected with 5×10^4 4T1.2 mammary carcinoma cells into the mammary fat pad. Groups of mice were treated with neoadjuvant (neoadj) or adjuvant (adj) **A,** DT (250 ng/mouse, i.p.), or **B,** anti-CD25 mAb (500 μg/mouse, i.p.) on either day 10 or 16, respectively, whereas the control group received PBS or control IgG (500 μg/mouse, i.p.) on both days 10 and 16 as indicated in the schematic. All primary tumors were resected on day 13. The Kaplan–Meier curves for overall survival of each group are shown. Experiments in **A** were performed in a blinded manner. Results in **A** and **B** were pooled from two independent experiments. **C,** Ten days after tumor inoculation, groups of mice ($n = 5$/group) were treated with DT (250 ng/mouse, i.p.) or PBS. 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 the Mann–Whitney *U* test on day 18 (*, $P < 0.05$).

Enhanced Efficacy of Neoadjuvant Anti–PD-1 and Anti-CD137 to Eradicate Metastases

To determine if the above findings were specifically due to Treg depletion or whether these also applied to immunotherapies currently being used clinically, we next compared the efficacy of neoadjuvant and adjuvant administration of anti–PD-1 in 4T1.2 tumor–bearing BALB/c mice (Fig. 2A). In the clinic, anti-human PD-1 antibodies (pembrolizumab and nivolumab) are FDA approved and have produced 20% to 50% objective response rates in a range of cancer types, including melanoma, renal cancer, and NSCLC (2). Clinical activity for anti–PD-1 and anti–PD-L1 was recently reported in trials of patients with TNBC, suggesting a broader group of cancers may benefit from checkpoint blockade (25). 4T1.2 is a mouse model of TNBC and, similarly, we have previously demonstrated that adjuvant anti–PD-1 given after 4T1.2 tumor resection can minimally extend survival, although no long-term survivors were obtained (26). In this experiment, we further delayed commencement of treatment to assess how anti–PD-1 affected heavier metastatic burden (Fig. 2A). Although neoadjuvant anti–PD-1–treated mice displayed significantly longer survival compared with those that received adjuvant anti–PD-1, no mice survived long term (Fig. 2A).

It is now obvious from both preclinical models and recent clinical trials that combination approaches may be required

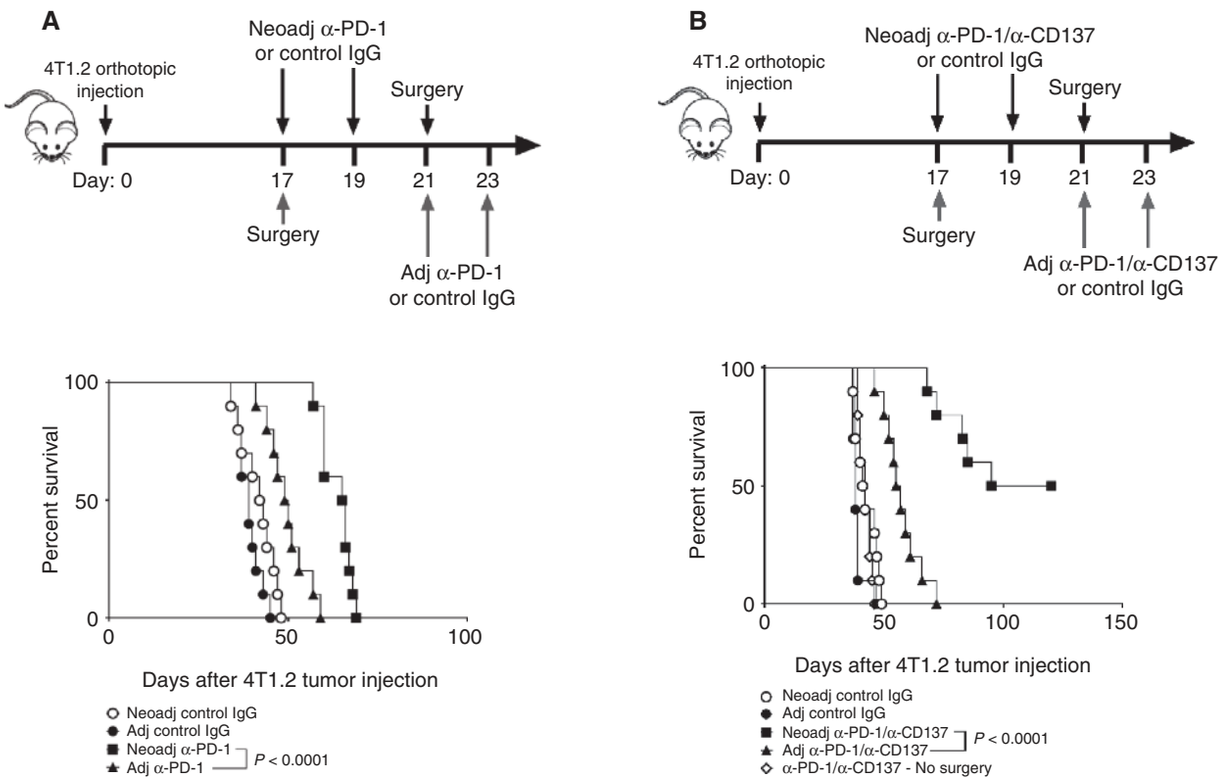

Figure 2. Neoadjuvant compared with adjuvant anti–PD-1 + anti-CD137 therapy is more efficacious in eradicating metastatic disease. **A** and **B,** Groups of BALB/c WT mice ($n = 10$/group) were injected with 2×10^4 4T1.2 mammary carcinoma cells into the mammary fat pad. As indicated in the schematic, some groups of mice were treated with neoadjuvant (neoadj) **A,** anti–PD-1 mAb or control IgG (all 100 μg/mouse, i.p.), or **B,** 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 with all primary tumors resected on day 21 except for the no-surgery group. In other groups, mice were resected of their tumors on day 17 and treated with adjuvant (adj) **A,** anti–PD-1 mAb or control IgG (all 100 μg/mouse, i.p.), or **B,** 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 21 and 23. (*continued on following page*)

for optimally effective and broadly applicable cancer immunotherapy (27–29). CD137 is a costimulatory receptor selectively expressed on activated T and natural killer (NK) cells (30), and anti-CD137 has been shown to be particularly effective in mouse tumor models. Furthermore, in clinical trials, anti-CD137 alone has demonstrated some efficacy with combinations currently being tested in a number of solid tumors and hematologic malignancies (31). Thus, we next set up an experiment similar to Fig. 2A to test the antitumor efficacy of neoadjuvant or adjuvant administration of anti–PD-1 in combination with anti-CD137 (Fig. 2B). Validating our hypothesis, 50% of 4T1.2 tumor–bearing mice that received neoadjuvant anti–PD-1 + anti-CD137 displayed long-term survival compared with those that received adjuvant anti–PD-1 + anti-CD137 (no survivors; Fig. 2B). As before, we demonstrated that the efficacy of neoadjuvant anti–PD-1 + anti-CD137 to affect metastases and generation of long-term survivors depended on removal of the primary tumor (Fig. 2B, no surgery group), as primary growth was initially suppressed but relapsed (data not shown), similar to Fig. 1C. To confirm the generality of the findings observed with the 4T1.2 model, we also set up similar experiments using the E0771 mammary carcinoma cell line injected into the mammary fat pad of C57BL/6 WT mice (ref. 20; Fig. 2C and D). Again, neoadjuvant anti–PD-1 + anti-CD137 treatment resulted in 40% (4/10) of E0771 tumor–bearing mice surviving long-term compared with no survivors among those that received adjuvant anti–PD-1 + anti-CD137 (Fig. 2C). Similarly, when surgeries were performed on the same day, neoadjuvant compared with adjuvant anti–PD-1 + anti-CD137–treated mice had significantly improved long-term survival (6/9; 67% vs. 0%; Fig. 2D). Interestingly, unlike the 4T1.2 tumor model (Fig. 2A), adjuvant anti–PD-1 alone was ineffective, and neoadjuvant anti–PD-1 did not extend overall survival (Fig. 2D). In contrast, neoadjuvant anti-CD137 alone significantly prolonged survival of treated mice compared with a group treated with adjuvant anti-CD137 (Fig. 2D). Overall, we demonstrated the superior efficacy of neoadjuvant, compared with adjuvant, administration of four different immunotherapies in two spontaneous mouse models of metastases. These data were very impressive because the 4T1.2 tumor is highly spontaneously metastatic and widely acknowledged as extremely difficult to eradicate once the primary tumor is established (19). This result contrasted with all our previous studies using this model, where various adjuvant immunotherapies improved survival following primary tumor resection, but long-term survival was rarely obtained (19, 26, 32, 33).

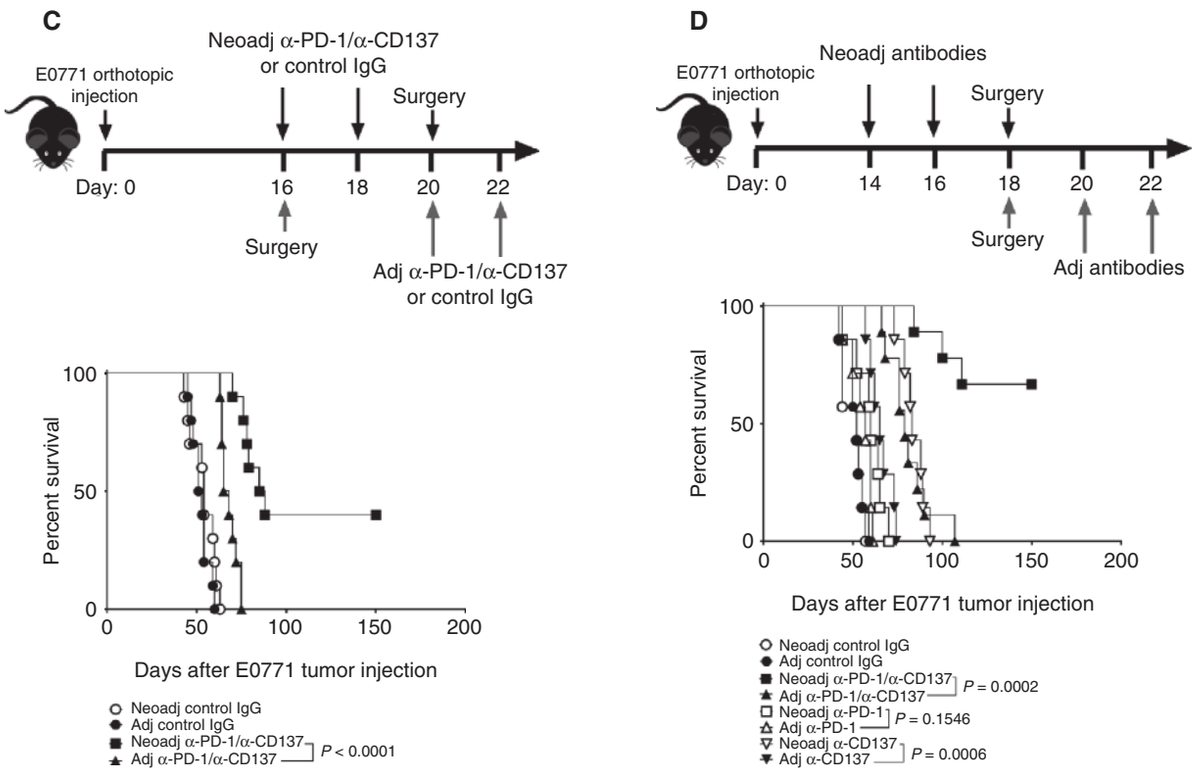

Figure 2. ***(Continued)*** **C** and **D,** Groups of C57BL/6 WT mice were injected with 5×10^4 E0771 mammary carcinoma cells into the mammary fat pad. **C,** As indicated in the schematic, groups of mice ($n = 10$/group) received neoadjuvant anti–PD-1 and anti-CD137 mAb (100 μg/mouse of each mAb, i.p.) or control IgG (200 μg/mouse, i.p.) on days 16 and 18, and their primary tumors were resected on day 20. Other groups of mice had their primary tumors resected on day 16 and were treated with adjuvant 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 20 and 22. **D,** As indicated in the schematic, groups of mice ($n = 7$–9/group) received neoadjuvant anti–PD-1 mAb or anti-CD137 mAb alone or in combination (100 μg/mouse of each mAb, i.p.) or control IgG (200 μg/mouse, i.p.) on days 14 and 16. Other groups of mice were treated with adjuvant anti–PD-1 mAb or anti-CD137 mAb alone or in combination (100 μg/mouse of each mAb, i.p.) or control IgG (200 μg/mouse, i.p.) on days 20 and 22. All groups of mice had their primary tumors resected on day 18. 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.

Improved Efficacy of Neoadjuvant Immunotherapy Is Not Due to Differences in Metastatic Burden

We next wanted to eliminate the possibility that the improved efficacy of neoadjuvant immunotherapy was simply due to differences in metastatic burden in the mice at the time of treatment. We thus set up a series of experiments where we varied the schedule of neoadjuvant and adjuvant immunotherapy administration (Fig. 3). First, delaying neoadjuvant DT to the same day as mice receiving adjuvant DT (day 16) still resulted in 35% (7/20) long-term survivors compared with 5% (1/20) of adjuvant Treg-depleted mice (Fig. 3A). When we pooled all experiments where mice were treated with either late neoadjuvant DT (3 experiments) or adjuvant DT (7 experiments), far more long-term survivors were observed in the late neoadjuvant DT-treated (14/28) compared with adjuvant DT-treated groups (9/59; Supplementary Fig. S2). Additionally, we also demonstrated that the metastatic burden (as measured by gp70 expression levels) was similar in the lungs of 4T1.2 tumor–bearing mice at the time when they would normally receive neoadjuvant or adjuvant DT (i.e., day 10 or 16, respectively; data not shown). We performed q-PCR to detect gp70 expression as a measure of 4T1.2 tumor burden, given that no observable micrometastases were detectable by hematoxylin and eosin (H&E) staining, and observed no significant differences (data not shown). Next, when we shifted the schedule of surgery and adjuvant Treg depletion to the same time points as when neoadjuvant Treg depletion followed by surgery was given (i.e., days 10 and 13), neoadjuvant Treg depletion was still superior (Fig. 3B). Similarly, whether neoadjuvant or adjuvant anti–PD-1 alone or anti–PD-1 + anti-CD137 therapies were administered on the same day (Fig. 3C) or surgeries were performed on the same day (Fig. 3D), we still observed a significant proportion of mice surviving long term following neoadjuvant, but not adjuvant, immunotherapy. Simply, surgery-related effects were insufficient for the efficacy of neoadjuvant anti–PD-1 + anti-CD137 therapy, because neoadjuvant therapy followed by sham surgery did not enable long-term survivors (Supplementary Fig. S3A and S3B).

Our finding that immunotherapy worked more effectively in a neoadjuvant setting raised the question as to whether chemotherapy would also be more effective when given in a

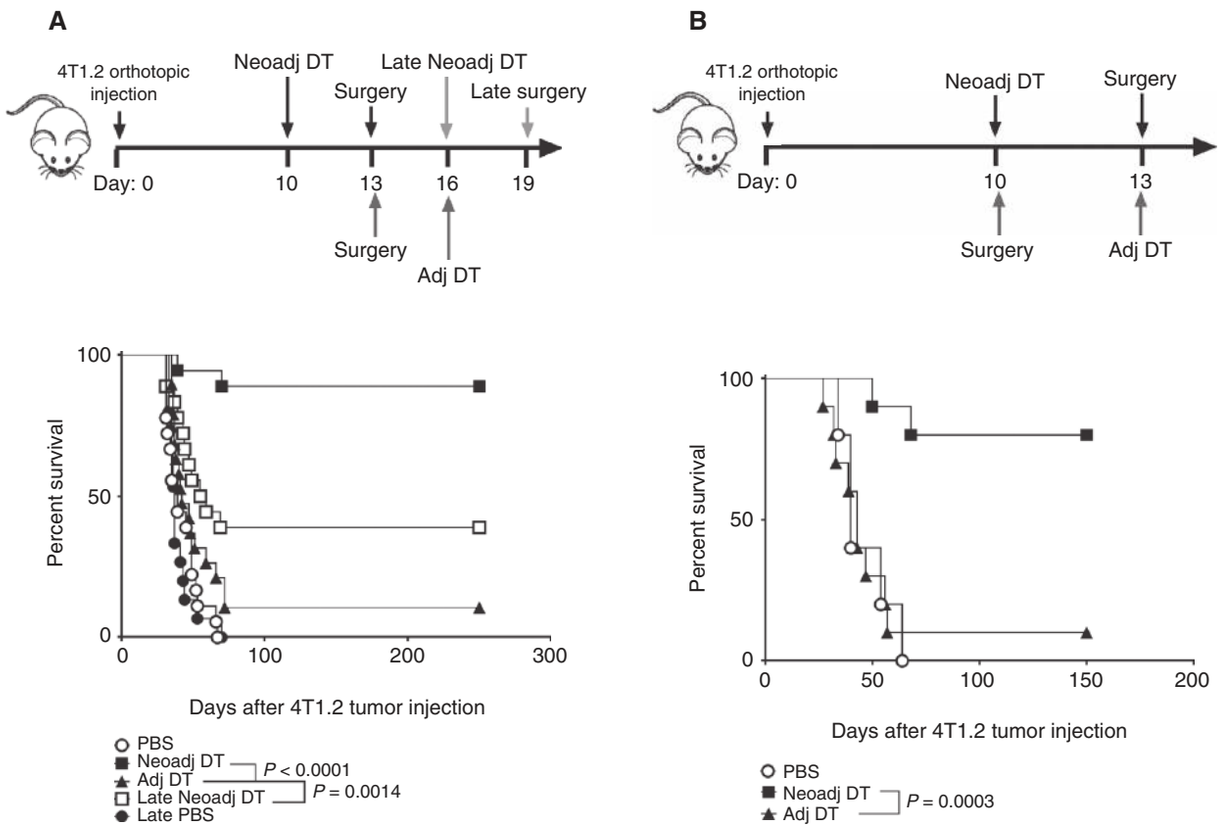

Figure 3. Improved efficacy of neoadjuvant immunotherapy is not due to differences in metastatic burden. **A** and **B,** Groups of BALB/c FOXP3-DTR mice were injected with 5×10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. **A,** Groups of mice ($n = 10$/group) were treated with neoadjuvant (neoadj) or adjuvant (adj) DT (250 ng/mouse, i.p.) on either day 10 or 16, respectively, whereas the control group received PBS on both days 10 and 16. Additionally, some groups of mice received neoadjuvant DT (250 ng/mouse, i.p.) or PBS on day 16 (late neoadj DT/PBS group). All primary tumors were resected on day 13 or 19 as indicated. **B,** Groups of mice ($n = 5$–10/group) were treated with neoadjuvant or adjuvant DT (250 ng/mouse, i.p.) on either day 10 or 13, respectively, whereas the control group received PBS on both days 10 and 13. Neoadjuvant and adjuvant DT-treated mice had their primary tumors resected on day 13 or 10, respectively, as indicated in the schematic. (*continued on following page*)

neoadjuvant setting in this model (Supplementary Fig. S4). We chose paclitaxel given its use in the treatment of women with metastatic breast cancer and its adjuvant activity in the 4T1.2 model post surgery (34). Interestingly, mice given neoadjuvant paclitaxel displayed no significant benefit over mice that received adjuvant paclitaxel, despite the chemotherapy being partially effective in both settings and prolonging survival (Supplementary Fig. S4). These data suggest that not all effective cancer therapies benefited from neoadjuvant scheduling.

Long-term Survivors Following Neoadjuvant Immunotherapy-Treated Groups Are Cured

We next performed a series of experiments to determine if long-term surviving mice in the neoadjuvant Treg-depleted mice were cured or harbored dormant tumors (Supplementary Fig. S5). No detectable levels of gp70 were found in the lungs of these mice (>250 days after tumor challenge; Supplementary Fig. S5A). We have previously demonstrated in a methylcholanthrene (MCA)-induced model of tumor dormancy that the equilibrium period can exist for much of the life of the mouse (hundreds of days) and is actively controlled by the immune system (35, 36). Depletion of CD8/CD4 T cells and/or neutralization of IFNγ in mice with dormant MCA-induced tumors resulted in their rapid outgrowth. To examine this, another cohort of long-term survivors from the neoadjuvant DT-treated group was depleted of NK and T cells over a period of 4 weeks. No reduction in survival was observed, suggesting no latent tumor cells were present in these mice (Supplementary Fig. S5B). As an alternative approach, to assess whether long-term surviving mice were cured because they had developed a strong protective memory response, another cohort of these surviving mice or naïve age-matched FOXP3-DTR mice were challenged with 4T1.2 tumors either subcutaneously or in the mammary fat pad (on the opposite flank; Supplementary Fig. S5C and S5D), or injected i.v. for experimental lung metastases (Supplementary Fig. S5E). In all experiments, these long-term survivors did not develop progressively growing tumors or metastases compared with controls. Collectively, our data clearly demonstrated that neoadjuvant DT-treated long-term survivors were free of

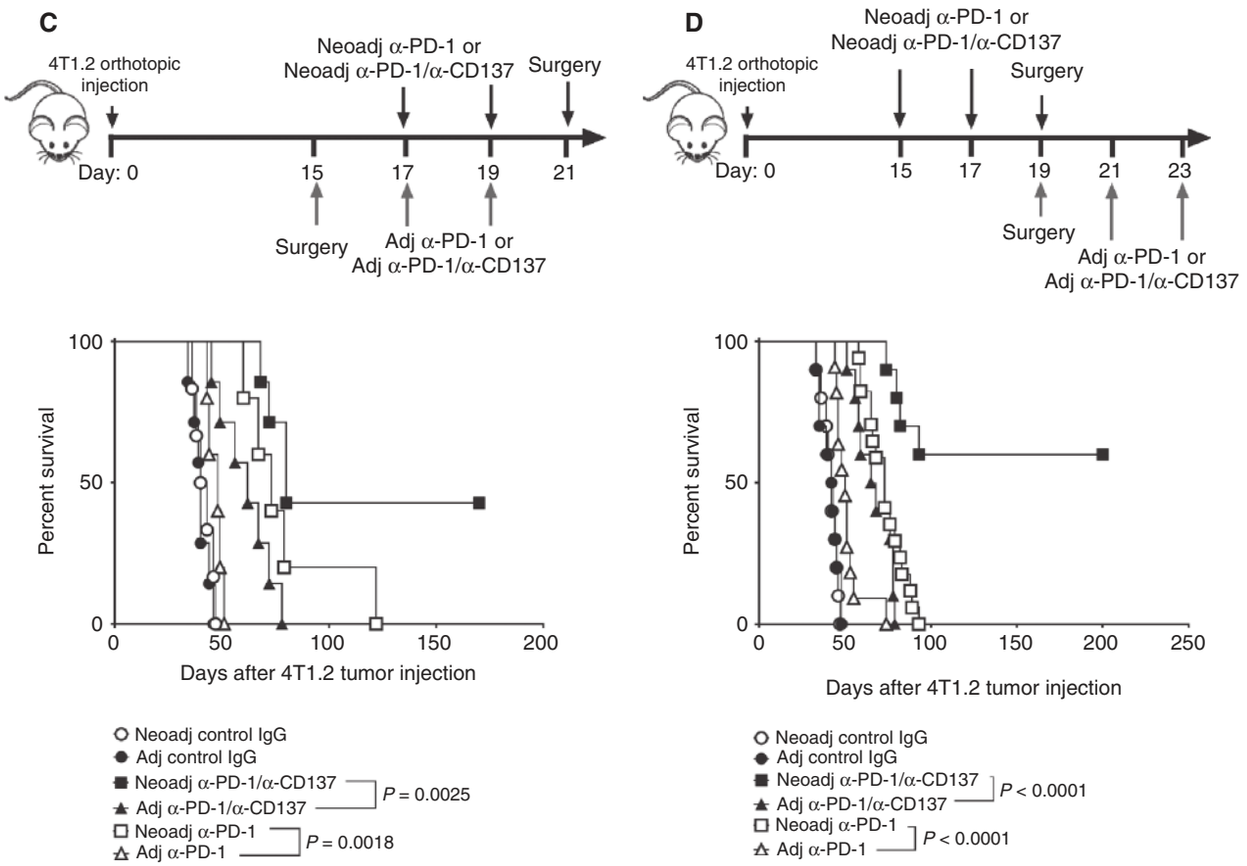

Figure 3. ***(Continued)*** **C** and **D,** Groups of BALB/c WT mice were injected with 2×10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. **C,** Groups of mice ($n = 5$–7/group) were treated with neoadjuvant (neoadj) or adjuvant (adj) anti-PD-1 mAb or control IgG (all 100 μg/mouse, i.p.) alone, or 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 with all primary tumors resected on day 21 (neoadjuvant groups) or day 15 (adjuvant groups). **D,** As indicated in the schematic, groups of mice ($n = 10$–17/group) were treated with neoadjuvant anti-PD-1 mAb or 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 15 and 17. Other groups of mice were treated with adjuvant anti-PD-1 mAb or 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 21 and 23. All groups of mice had their primary tumors resected on day 19. The Kaplan–Meier curves for overall survival of each group are shown. All experiments were performed once except for those shown in **A,** which is pooled from two experiments. Significant differences between indicated groups were determined by log-rank sum test with exact *P* values shown.

residual metastatic disease and had developed tumor-specific memory immune responses.

Neoadjuvant Immunotherapy Depends on CD8$^+$ T Cells and IFNγ

We next examined the effector pathways and immune cells that were involved in the protective effect of neoadjuvant anti–PD-1 + anti-CD137 treatment using gene-targeted mice or depleting/neutralizing antibodies (Fig. 4). In both 4T1.2 and E0771 tumor models, the efficacy of neoadjuvant therapy was dependent on IFNγ because there were no long-term survivors when it was neutralized (Fig. 4A and B). In contrast, loss of perforin did not greatly affect the efficacy of neoadjuvant immunotherapy, as a significant proportion of mice still survived long term (Fig. 4A and B). Our experiments also suggested all three immune cell types, CD8, CD4, and NK cells, were required, although the relative importance of each subset varied depending on the tumor type and therapy (Fig. 4C; Supplementary Fig. S6).

Neoadjuvant Immunotherapy Increases Tumor-Specific CD8$^+$ T Cells in Peripheral Blood and Organs

We next dissected the mechanism by which neoadjuvant immunotherapy induced better antitumor immunity compared with adjuvant treatment. Given the importance of CD8$^+$ T cells in both 4T1.2 and E0771 tumor models, we first asked if their proportion was changed and whether it could be detected in the blood early after treatment. As we did not observe any changes in the proportion of total CD8$^+$ T cells between the neoadjuvant and adjuvant Treg-depleted or anti–PD-1/CD137–treated groups (data not shown), we next determined whether changes could be detected if we measured for tumor-reactive gp70 tetramer-specific CD8$^+$ T cells, as previously reported (Fig. 4D; ref. 37). Prior to therapy on day 10, tumor-bearing mice had similar levels of peripheral blood gp70 tumor–specific CD8$^+$ T cells among the different groups. Strikingly, 4 days after neoadjuvant anti–PD-1 + anti-CD137 therapy, we observed a strong increase in tumor-specific CD8$^+$ T cells. Although this decreased following

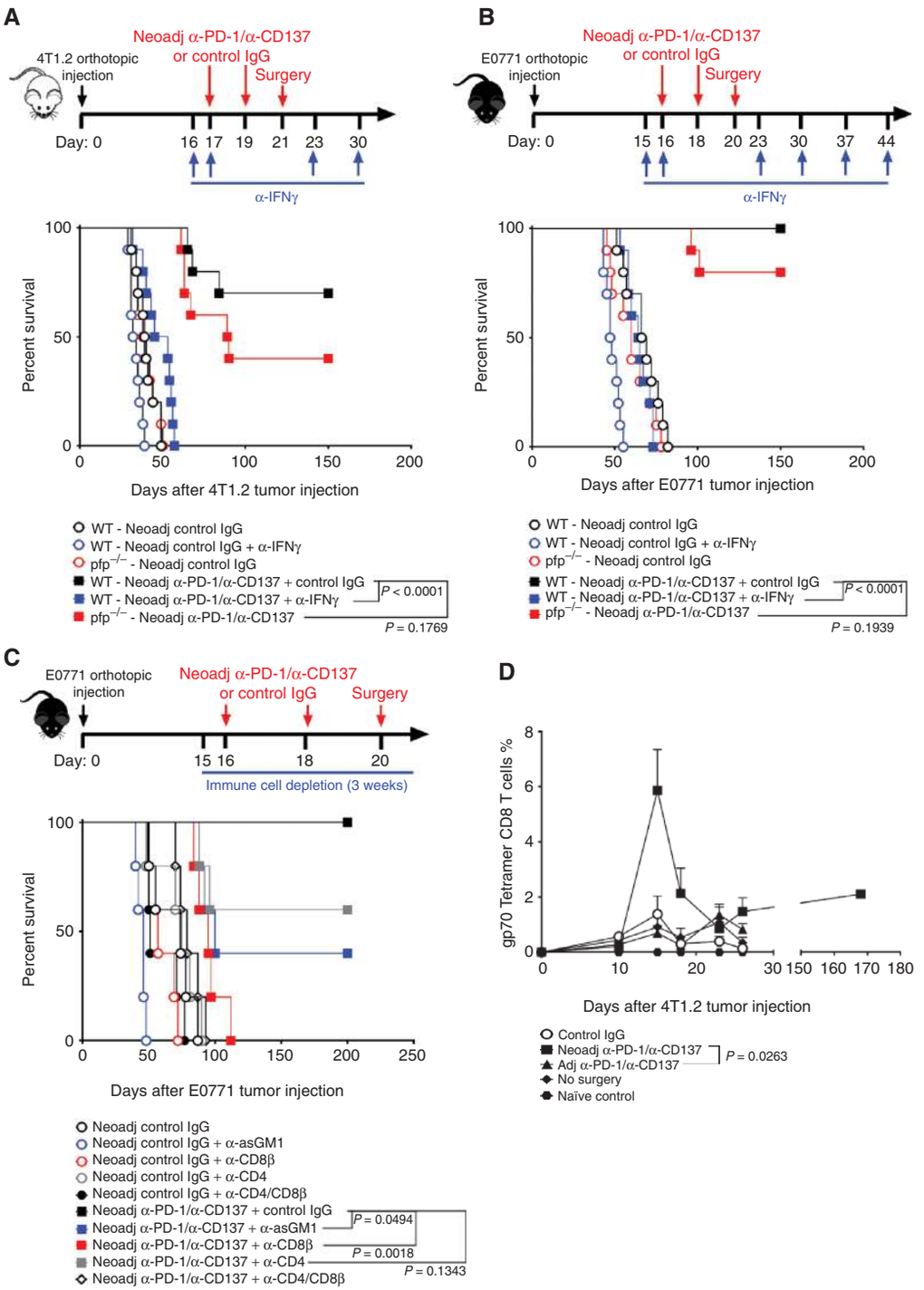

Figure 4. Efficacy of neoadjuvant immunotherapies depends on CD8+ T cells and IFNγ. **A–D,** Groups of BALB/c or C57BL/6 WT or gene-targeted mice were injected with 2×10^4 (**A**) or 5×10^4 (**D**) 4T1.2 or 5×10^4 E0771 (**B** and **C**) mammary carcinoma cells into the mammary fat pad. **A–C,** groups of mice received neoadjuvant (neoadj) anti–PD-1 and anti-CD137 mAb (100 μg/mouse of each mAb, i.p.) or control IgG (200 μg/mouse, i.p.) followed by surgery as indicated in the schematics. Additionally, some groups of mice were treated with control IgG or anti-IFNγ mAb (250 μg/mouse, i.p.; **A** and **B**) or anti-CD4, anti-CD8β, or anti-asGM1 mAbs (all 100 μg/mouse each, i.p.) alone or in combination (**C**). Experiments were all performed once. Significant differences between indicated groups were determined by a log-rank sum test with exact *P* values shown. **D,** Groups of mice ($n = 3$–4/group) were treated with neoadjuvant or adjuvant (adj) anti–PD-1 and anti-CD137 mAb (100 μg/mouse of each mAb, i.p.) on either days 11 and 13 or days 19 and 21, respectively, whereas the control group received control IgG (200 μg/mouse, i.p.) on days 11, 13, 19, and 21. All primary tumors were resected on day 16, except for the no-surgery group. A naïve 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 is shown. Data, mean + SEM. Data are representative of two independent experiments. Significant differences between neoadjuvant anti–PD-1/CD137 (day 15) and adjuvant anti–PD-1/CD137 (day 23; i.e., 4 days after their respective therapy) were determined by an unpaired Student *t* test with exact *P* value indicated.

resection of the primary tumor on day 16, the levels remained high and, surprisingly, tumor-specific CD8+ T cells were still detectable more than 170 days after tumor challenge. In contrast, we did not observe this same magnitude of increase in tumor-specific CD8+ T cells 4 days after receiving adjuvant therapy (Fig. 4D). Likewise, we observed similar kinetics in mice that received neoadjuvant Treg depletion compared with mice that received adjuvant Treg depletion and a striking persistence of tumor-specific CD8+ T cells over a long period of time (Supplementary Fig. S7).

To confirm if this expansion of tumor-specific CD8+ T cells in the blood was also observed in organs, we set up a similar experiment as Fig. 3C, where neoadjuvant or adjuvant anti–PD-1 + anti-CD137 was given on days 17 and 19, and these mice and their respective controls were culled on day 21. Blood, spleen, as well as organs such as the lung and liver to which 4T1.2 metastasized were harvested, and the presence of gp70 tumor–specific CD8+ T cells was measured (Fig. 5). In all organs, the numbers (Fig. 5A–D) and proportion (data not shown) of tumor-specific CD8+ T cells were significantly

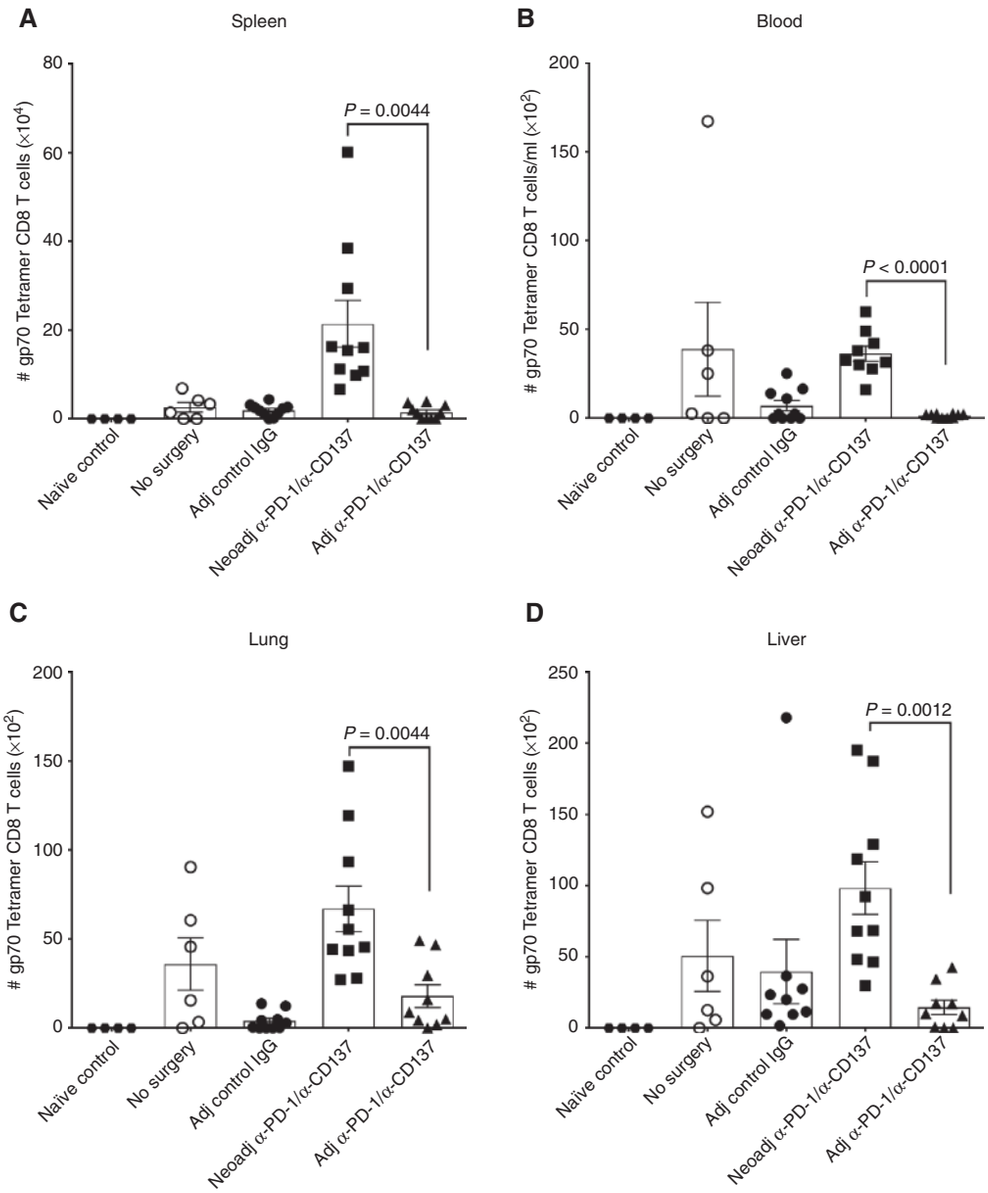

Figure 5. Neoadjuvant anti–PD-1 + anti-CD137 therapy leads to systemic expansion of gp70 tumor–specific CD8+ T cells in peripheral blood and organs. **A–D,** Groups of BALB/c WT mice were injected with 5×10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice ($n = 3$–5/group) were treated with neoadjuvant (neoadj) or adjuvant (adj) 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 with primary tumors resected on day 15 (for the adjuvant-treated groups). A naïve control group ($n = 2$) was also included in each experiment. Mice were sacrificed on day 21 and their spleens, blood, lungs, and livers were collected and single-cell suspensions generated for flow cytometry. Gating on live CD45.2+ cells of lymphocyte morphology, the absolute numbers of gp70 tetramer+ CD8+ TCRβ+ cells in the indicated organs were determined. Each symbol represents a single mouse. Data, mean ± SEM. Data are pooled from two experiments with significant differences between neoadjuvant- and adjuvant-treated groups determined by an unpaired Welch *t* test with exact *P* value shown.

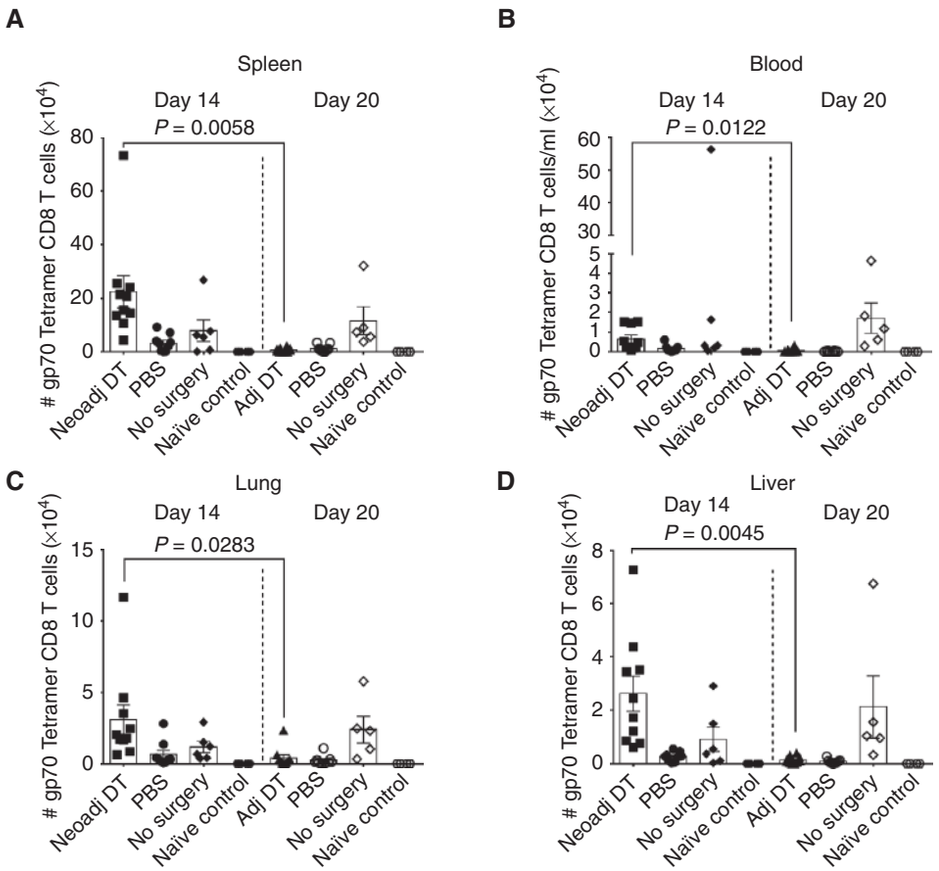

Figure 6. Neoadjuvant Treg depletion leads to systemic expansion of gp70 tumor–specific CD8⁺ T cells with effector function. **A–H,** Groups of BALB/c FOXP3-DTR mice ($n = 2$–5/group) were injected with 5×10^4 4T1.2 mammary carcinoma cells in the mammary fat pad. Groups of mice were treated with neoadjuvant (neoadj) or adjuvant (adj) DT (250 ng/mouse, i.p.) on either day 10 or 16, respectively, whereas the control group received PBS on both days 10 and 16. All primary tumors were resected on day 13, except for the no-surgery group. A naïve control group ($n = 2$) was also included in each experiment. Mice were sacrificed on days 14 and 20, respectively, and their spleens, blood, lungs, and livers were collected and single-cell suspensions generated for flow cytometry. Gating on live CD45.2⁺ cells of lymphocyte morphology, absolute numbers (**A–D**) of gp70 tetramer⁺ CD8⁺ TCRβ⁺ cells in the indicated organs were determined. Each symbol represents a single mouse. (*continued on following page*)

higher in the neoadjuvant versus the adjuvant anti–PD-1 + anti–CD137-treated group. Similar increases in numbers and proportion of gp70 tumor–specific CD8⁺ T cells across various organs were also observed in neoadjuvant but not in adjuvant Treg-depleted mice (Fig. 6A–D; Supplementary Fig. S8A and data not shown). In this experiment, neoadjuvant or adjuvant Treg-depleted mice and their respective controls were culled on day 16 or 20, respectively (i.e., 4 days after their respective DT treatment, to allow for immunologic effects induced by the therapy to be fairly compared), and organs harvested for flow cytometry analysis (Fig. 6A–D). Interestingly, in both experiments, mice that received no treatment or PBS control and no surgery generally had higher levels of gp70 tumor–specific CD8⁺ T cells in their organs compared with similar groups that had their primary tumor excised (Fig. 5B and C and Fig. 6A–D; day 20). This suggests that the primary tumor may contribute to the systemic increase of these tumor-specific T cells; however, importantly, the effector function of tumor-specific T cells in the no-surgery group was poor compared with neoadjuvant immunotherapy-treated and tumor-resected groups (Fig. 6E–G). We also observed increased numbers of gp70 tumor–specific CD8⁺ T cells in the resected primary tumors of neoadjuvant Treg-depleted (Supplementary Fig. S8B) or anti–PD-1 + anti–CD137-treated (Supplementary Fig. S8C) mice compared with resected tumors that did not receive therapy. In the same experiment, we observed that the tumor-specific CD8⁺ T cells from the neoadjuvant Treg-depleted group displayed an effector/memory phenotype (CD44⁺ CD62L⁻) in the lungs (Fig. 6H), blood, and liver (Supplementary Fig. S9A and S9B); were proliferative, as measured by Ki67 staining (Fig. 6E; Supplementary Fig. S9C and S9D); and produced IFNγ (Fig. 6F; Supplementary Fig. S9E) and TNF (Fig. 6G; Supplementary Fig. S9F) as measured by intracellular cytokine staining.

Tumor-Specific CD8⁺ T Cells Are a Biomarker of Outcome

Interestingly, we observed mice that had high levels of tumor-specific CD8⁺ T cells in blood when measured early after neoadjuvant immunotherapies were predicted to survive long term (Fig. 7). In the groups that received neoadjuvant Treg depletion (Fig. 7A) or anti–PD-1 + anti-CD137

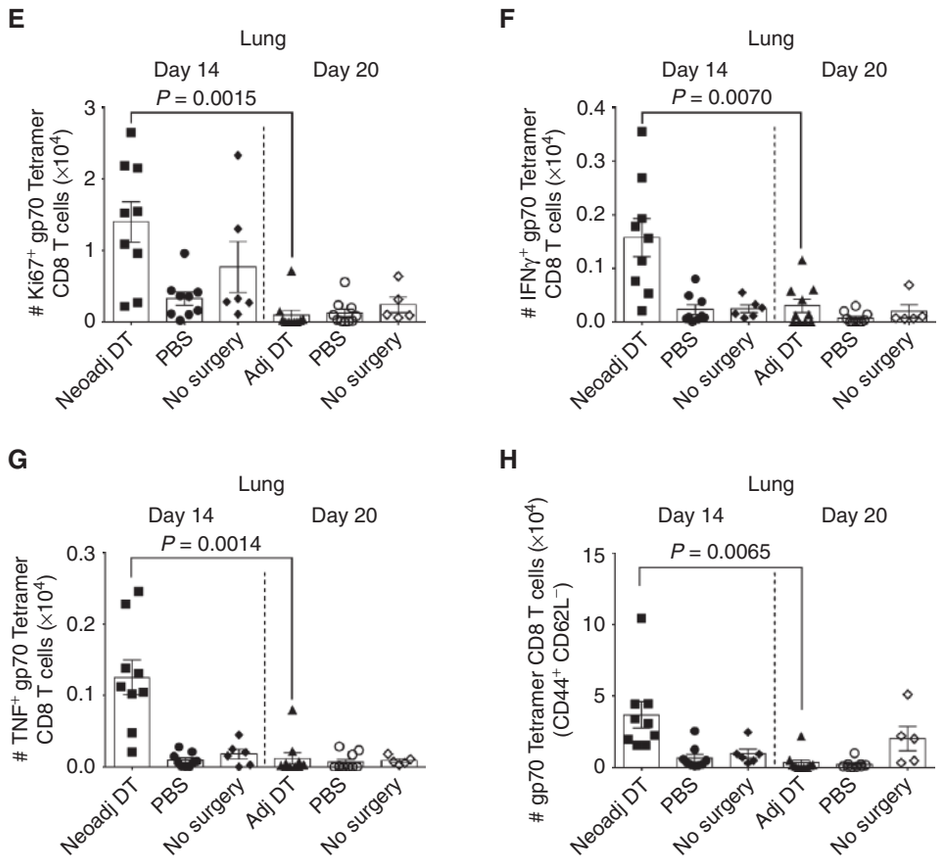

Figure 6. ***(Continued)*** **E–H,** Gating on live CD45.2$^+$ cells of lymphocyte morphology, absolute cell numbers of gp70 tetramer$^+$ CD8$^+$ TCRβ$^+$ T cells in the indicated organ that were Ki67$^+$ (**E**), IFNγ$^+$ (**F**), TNF$^+$ (**G**), or CD44$^+$ CD62L$^-$ (**H**) are shown. Each symbol represents a single mouse. Data, mean ± SEM. Data are pooled from two experiments. Significant differences between neoadjuvant (day 14) and adjuvant (day 20) DT groups (i.e., 4 days after their respective therapy) were determined by an unpaired Welch *t* test with exact *P* value shown.

(Fig. 7B), the majority of mice that displayed the highest levels of tumor-specific CD8$^+$ T cells at day 14 or 15 (i.e., 4 days after neoadjuvant therapy) survived greater than 100 days and generally went on to become long-term survivors. In contrast, neoadjuvant-treated mice that had lower levels of tumor-specific CD8$^+$ T cells did not survive more than 100 days. Similarly, adjuvant-treated mice generally had lower levels of tumor-specific CD8$^+$ T cells compared with neoadjuvant-treated groups when measured at day 20 (i.e., 4 days after adjuvant therapy). The magnitude of tumor-specific CD8$^+$ T cells as a predictor of long-term survival was more clearly observed in mice that received neoadjuvant anti–PD-1 + anti-CD137 therapy (Fig. 7B), perhaps reflecting the stronger dependency on CD8$^+$ T cells for this therapy (Fig. 4C). Indeed, for this therapy, we observed a correlation between survival outcomes and the level of peripheral tumor-specific CD8$^+$ T cells, although the relationship was not exactly linear (Fig. 7C). Interestingly, we also observed that gp70 tumor–specific CD8$^+$ T cells in the blood maintained their effector memory or central memory phenotype over the life of these mice (Fig. 7D and E). Intriguingly, it seemed to suggest if the level of tumor-specific CD8$^+$ T cells reached a certain threshold (~7%), these mice would most likely survive long term. Thus, improved antitumor efficacy induced by neoadjuvant immunotherapy may be due to its ability to significantly increase the number and maintenance of tumor-specific CD8$^+$ T cells, and the early increase is a biomarker of outcome, particularly in CD8$^+$ T cell–dependent therapies.

DISCUSSION

This report has demonstrated the improved efficacy of neoadjuvant immunotherapy to eradicate metastatic disease in two preclinical models of TNBC following surgical resection. The orthotopic 4T1.2 and E0771 tumors represent the two best models where surgery and lethal metastases can be assessed in the context of neoadjuvant versus adjuvant therapy in a robust and timely manner. In genetically engineered tumor models (GEM), primary tumors grow to an unethical and unresectable size before there are any significant metastases. Other *de novo* models of cancer, including breast cancer, do not significantly metastasize, and not in a way that the primary tumor can be resected and survival monitored. Importantly, this proof of principle of improved efficacy on neoadjuvant

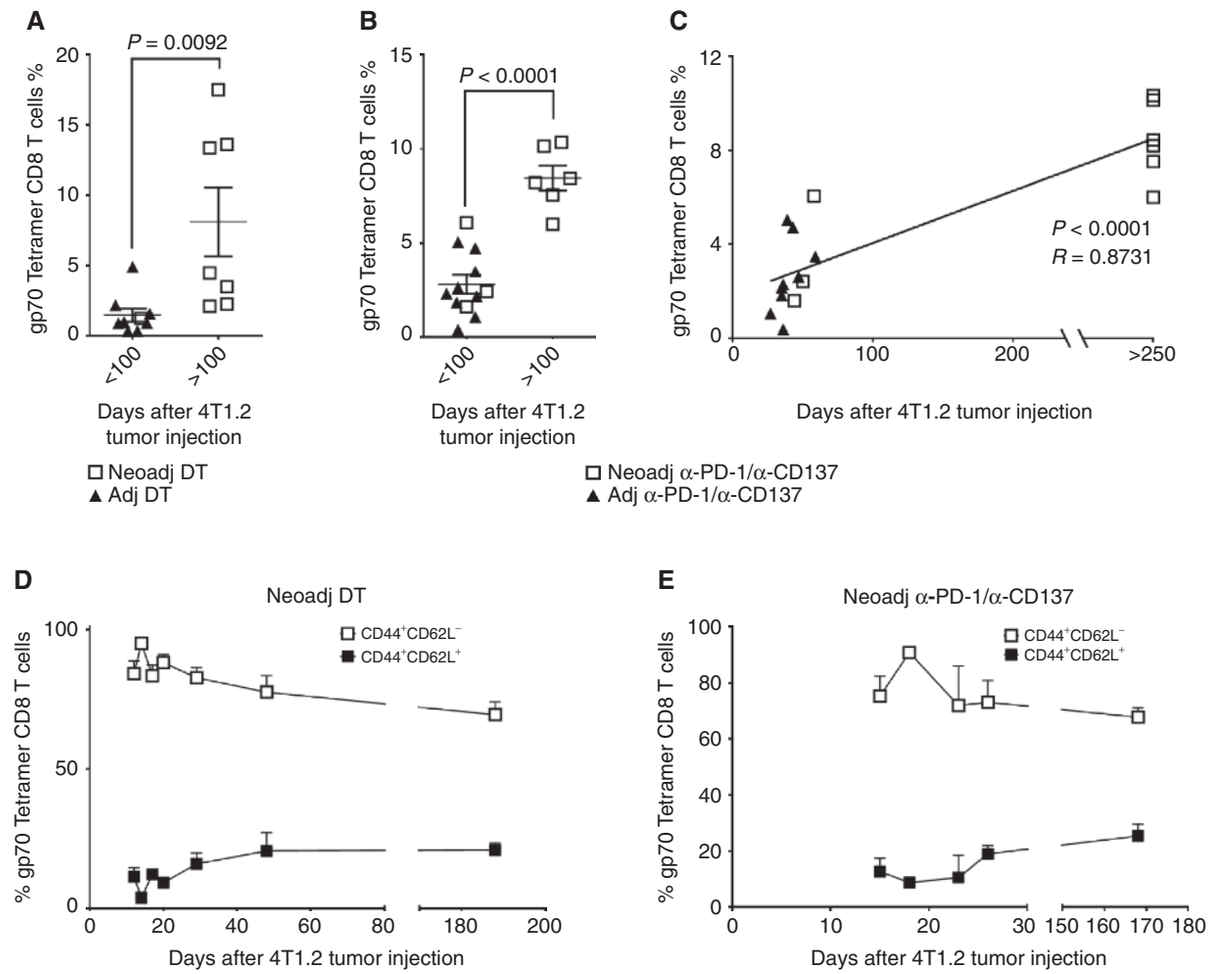

Figure 7. Tumor-specific CD8+ T cells are a biomarker of outcome. **A** and **B,** Level of gp70 tetramer+ CD8+ TCRβ+ cells from individual mice 4 days after receiving neoadjuvant (neoadj) or adjuvant (adj) immunotherapy (as indicated), grouped based upon whether they survived greater or less than 100 days after 4T1.2 tumor challenge. Data, mean ± SEM. **C,** linear regression and correlation between the level of gp70 tetramer+ CD8+ TCRβ+ cells from individual mice 4 days after receiving neoadjuvant or adjuvant anti–PD-1/CD137 and survival. **A–C,** Each symbol represents a single mouse; data derived from Fig. 4D and Supplementary Fig. S8. Data are pooled from two experiments with significant differences between groups that survived less than or greater than 100 days after tumor challenge determined by an unpaired Student *t* test with exact *P* value shown. **D** and **E,** In the same experiment as described and shown in Fig. 4D and Supplementary Fig. S8, gating on live CD45.2+ cells of lymphocyte morphology, the proportion of gp70 tetramer+ CD8+ TCRβ+ T cells in the peripheral blood that are CD44+ CD62L− or CD44+ CD62L+ are shown. Data, mean + SEM. Data are representative of two independent experiments.

immunotherapy was demonstrated using four different immunotherapies: complete Treg depletion, anti-CD25, or anti–PD-1 alone or in combination with anti-CD137. Indeed, by deliberately performing the neoadjuvant and adjuvant immunotherapies using a variety of different schedules, we provided validation that neoadjuvant immunotherapies were superior. Neoadjuvant immunotherapy that was given at the same time as the adjuvant immunotherapy or further delayed was still more efficacious in inducing long-term survivors.

In our study, neoadjuvant chemotherapy (paclitaxel) in our preclinical model did not improve overall long-term survival when compared with adjuvant chemotherapy, similar to reported clinical studies (6). Although paclitaxel does not cause immunogenic cell death (ICD) and priming of host dendritic cells (DC) and T cells (38), it still has immunomodulatory activity such as inducing proinflammatory cytokine secretion from macrophages, which can result in immune cell activation (39). Our data suggest that the improved survival benefit of neoadjuvant therapy may be restricted to therapies that activate T cell antitumor immunity. Chemotherapeutic agents such as doxorubicin that induce ICD alone or in combination with immunotherapy can now be tested in a neoadjuvant setting in these models to answer this question.

Recently, a number of clinical trials have commenced to assess the efficacy of neoadjuvant immunotherapies (clinicaltrials.gov); however, only one phase II trial ($n = 40$) is directly comparing neoadjuvant and adjuvant anti–PD-1, alone or in combination with anti-CTLA4, in patients with resectable melanoma (NCT02519322). In this trial, the primary and secondary aims are to assess the pathologic and immunologic response in the resected tumor. Given that overall survival data can take 3 to 5 years to obtain, our data now provide strong impetus to set up clinical trials that are powered to determine whether neoadjuvant immunotherapy

can further improve survival outcomes over adjuvant immunotherapy. Unlike our study, patients in this trial who receive neoadjuvant treatment will also receive adjuvant treatment following resection of their tumors. From our study, a short course of neoadjuvant immunotherapy induced long-term survivors in a proportion of treated mice. We can now compare in our model if further increase in the proportion of long-term survivors can be obtained by maintaining treatment post-surgery in mice given neoadjuvant immunotherapy. If we observe similar efficacy, it may suggest a short course of treatment is sufficient and thus may potentially limit the severity of immune-related adverse-event toxicities associated with long-term administration of combination immunotherapies. Alternatively, in humans, another consideration would be whether neoadjuvant immunotherapy might be likely to cause serious immune-related adverse events in the first few cycles and thus potentially prevent timely surgery. These are interesting complexities, and it is likely that adjuvant immunotherapy will keep its place as a treatment regime, particularly for cancer types where delaying of surgery is not advised. In the near future, should neoadjuvant immunotherapy prove to have utility, one would envisage that these patients would further receive continued immunotherapy following surgery. Nevertheless, the optimal patient scheduling and dosing comparisons will take considerable time to be validated, and the mouse model offers the opportunity to test these features of neoadjuvant immunotherapy further.

In our study, we have treated 4T1.2 tumor–bearing mice with widespread metastases to mimic clinically advanced-stage cancers. These mice have multiple organs involved and extensive disease can be quantitated by colony formation, histopathology, or other means within 2 weeks of primary tumor implantation, and these mice die within weeks of the primary surgery if untreated (19). Although immune checkpoint inhibitors to date have shown efficacy in bulky disease, neoadjuvant immunotherapy may induce even greater efficacy when used in locoregionally advanced cancers that are surgically operable but have a high risk of relapse. This could include TNBC (as we have shown here), bladder cancer, epithelial ovarian carcinoma, or renal cell carcinoma, of which a proportion has already responded to immune checkpoint inhibitors. Although anti-CTLA4 + anti–PD-1 is currently the only FDA-approved combination immunotherapy, our data with anti–PD-1 and anti-CD137 certainly provide a compelling rationale to test this combination in a neoadjuvant setting. Anti–PD-1 and anti-CD137 combinations are currently being tested in a number of clinical trials against advanced solid tumors and advanced B-cell non-Hodgkin lymphoma (NCT02253992 and NCT02179918). In addition, although we have clearly demonstrated the improved efficacy of neoadjuvant over adjuvant immunotherapy in two preclinical models of breast cancer, confirming these findings in other tumors of different tissue origin will broaden these findings. The challenge is finding additional preclinical tumor models that can mimic a clinical setting of surgical resection and spontaneous metastasis.

The increased efficacy of neoadjuvant immunotherapy to induce a greater proportion of long-term survivors compared with adjuvant immunotherapy may be explained by its ability to increase the numbers of gp70 tumor–specific T cells that were proliferating, displayed an effector/memory phenotype, and produced IFNγ and TNF in peripheral blood and various organs early after treatment. There are a number of mechanisms by which this could be mediated. One possibility is that the release of tumor-specific antigens from dying tumor cells may act as a vaccine to further prime and expand tumor-specific T cells in the primary tumor before they are released into the periphery. To test this, the proliferative capacity of gp70 tumor–specific $CD8^+$ T cells in the primary tumors compared with those in the periphery (e.g., blood and lung) can be measured during the first 3 days following neoadjuvant therapy. Alternatively, tumor antigens from dying primary tumor cells may "shower" into the periphery where they are processed, presented by antigen-presenting cells to prime and expand T cells systematically. Demonstrating this will be experimentally challenging, as tumor antigens will have to be marked to allow for their tracking in the periphery, and this will be complicated by their origin potentially being from both the primary tumor and the metastases. Finally, the role of the primary tumor itself in modulating immunologic changes systematically also has to be examined. The primary tumor potentially plays a dual role. The primary tumor has to be excised at some point, because it grows and continually seeds new metastases, and neoadjuvant immunotherapy alone cannot eradicate it. Yet its presence is required for the expansion of tumor-specific T cells following neoadjuvant immunotherapy, and we did not observe this same magnitude of tumor-specific T-cell expansion in adjuvant-treated mice. Notably, untreated mice that did not have their primary tumor resected also displayed an increase in tumor-specific T cells over time, but this was limited and proved insufficient, and these T cells displayed poor effector function compared with T cells from neoadjuvant-treated mice that had their primary tumors resected. To determine how resection of the primary tumor affects expansion of tumor-specific T cells, we are currently examining the kinetics of tumor-specific T cells in mice that received no therapy and no surgery compared with those that received neoadjuvant immunotherapy with or without surgery. More recently, a study reported that the efficacy of anti–PD-1 and anti-CD137 combination therapy required cross-priming of tumor antigens by BATF3-dependent DCs (40). Future experiments where neoadjuvant anti–PD-1 + anti-CD137 therapy is performed in BATF3-deficient mice will allow us to answer questions such as whether BATF3-dependent DCs are required for the efficacy of neoadjuvant immunotherapy and the requirement of the primary tumor for cross-priming. Nevertheless, elucidating the major reasons the tumor-specific $CD8^+$ T cells increase so clearly in the neoadjuvant scheduling is a complex immunologic issue and will take considerably more experimentation to determine.

In addition to affecting the quantity of tumor-specific T cells, neoadjuvant immunotherapy may also affect their quality. A striking observation of our study was the high proportion of gp70 tumor–specific $CD8^+$ T cells that persisted in the blood of long-term survivors (>170 days after tumor inoculation) following neoadjuvant Treg depletion or anti–PD-1 + anti-CD137 therapy. This is in contrast to what has been reported for acute viral infection where approximately 90% to 95% of effector $CD8^+$ T cells die following resolution

of infection, leaving 5% to 10% of these T cells to further differentiate into a memory T-cell population (41). Among these memory T cells, CD62L^{lo} CCR7^{lo} effector/memory and CD62L^{hi} CCR7^{hi} central/memory T cells become enriched in nonlymphoid or lymphoid organs, respectively, and over time, these effector/memory T cells are thought to convert to central/memory T cells which have self-renewing capacity that does not depend on antigen for their maintenance (41). In our study, we saw an opposite pattern among the memory T cells in the blood of our neoadjuvant immunotherapy–treated long-term survivors (>170 days after tumor inoculation) where ~70% were CD44$^+$ CD62L$^-$ and ~30% were CD44$^+$ CD62L$^+$, although staining with CCR7 is required to validate their effector or central memory phenotype. Future studies will determine if this ratio of effector to central memory T cells is specific to the blood or also present in other sites of metastasis such as the lung and liver, as well as in the lymph nodes, spleen, and bone marrow, where central memory T cells generally localize. Future studies will now aim to fully characterize these gp70 tumor–specific memory CD8$^+$ T cells on a transcriptional, phenotypic, and functional level to understand how they stably persist in such high proportion in long-time survivors.

In addition to measuring clinical objectives, neoadjuvant immunotherapy allows access to blood and tumor tissue prior to and after the initiation of treatment, and thus immunomonitoring and identification of potential biomarkers that may be predictive of therapeutic response. In our studies, elevated gp70 tumor–specific CD8$^+$ T cells in the blood early after neoadjuvant immunotherapy that was sustained were a predictor of survival outcomes. In humans, tumor-specific mutant neoantigens (TSMA, or neoantigens) have been demonstrated to be critical targets of antitumor immune responses (42). These antigens are derived from oncogenic viral proteins or abnormal proteins that arise as a consequence of somatic mutations or posttranslational modifications and are foreign to the host and not subjected to central tolerance (43). With recent advances in the field of genomics and bioinformatics, neoantigens can now be more easily and rapidly identified to generate MHC tetramers (42) to identify these tumor-specific T cells. In our preclinical study, gp70 likely serves as a tumor-specific neoantigen and probably represents the immunodominant epitope toward which the majority of CD8$^+$ T-cell responses are directed. Although tumor-bearing mice may not be initially centrally tolerized to gp70 (44), the tumor clearly grows unimpeded, resulting in eventual death if no major intervention is provided. Thus, gp70 represents a relevant neoantigen in the mouse spontaneous 4T1.2 tumor model, but clearly other tumor-associated and tumor-specific antigens have to be identified and tetramers generated in order for them to be assessed in the neoadjuvant context. The restriction currently faced is the lack of additional suitable metastatic models, but at least neoadjuvant priming of peripheral tumor-specific T cells can be assessed in other primary tumors pre- and post-surgery, regardless of their lack of metastasis.

Although it is currently not clear how many different neoantigen-specific CD8$^+$ T cells are required for an effective clinical response, tumor-specific T cells reactive to one to three neoantigens have generally been reported (45–48), although T cells from a single patient with reactivity to seven neoantigens have been measured (49). Similarly, we can utilize next-generation deep sequencing and epitope prediction algorithms (43) to help identify other non-gp70 tumor–specific T cells and assess how neoadjuvant immunotherapy shapes their kinetic of response and their antitumor contribution in future experiments. In humans, CD4$^+$ T cells specific for MHC II–restricted tumor neoantigens have been identified, and their infusion into one patient was able to induce sustained tumor regression (48, 50). Given that depletion of CD4$^+$ T cells negated the efficacy of neoadjuvant immunotherapy in our study, their tumor specificity can also be evaluated, albeit with more challenges (42). In future clinical trials comparing neoadjuvant versus adjuvant immunotherapy, it will be important to identify and measure tumor-specific T cells in the blood and resected tumors of these patients to determine whether they can act as biomarkers to distinguish patients who will derive long-term benefit. If validated, this biomarker may also identify patients who will require more intervention. Positive trial results will further revolutionize the field of cancer immunotherapy and improve outcomes for patients with cancer.

METHODS

Mouse Strains

C57BL/6J and BALB/c wild-type (WT) mice were purchased from the Walter and Eliza Hall Institute for Medical Research. BALB/c FOXP3-DTR-GFP mice were generated by backcrossing C57BL/6 FOXP3-DTR-GFP mice (kindly provided by Dr. Geoffrey Hill; ref. 23) ten generations to BALB/c WT mice. C57BL/6 or BALB/c perforin-deficient (pfp$^{-/-}$) and BALB/c FOXP3-DTR mice were all bred and maintained at the QIMR Berghofer Medical Research Institute. Female mice greater than 8 weeks old were used in all experiments, which were approved by the QIMR Berghofer Medical Research Institute Animal Ethics Committee.

Cell Culture

BALB/c-derived 4T1.2 mammary carcinoma cells were cultured in RPMI-1640 containing 10% FCS, penicillin/streptomycin, and L-glutamine as previously described (51). The C57BL/6 E0771 mammary carcinoma cell lines were cultured in DMEM containing 10% FCS, penicillin/streptomycin, and L-glutamine, as previously described (51). All cell lines were obtained between 2000 and 2008 and routinely tested negative for *Mycoplasma*. Cell line authentication was not routinely performed.

Antibodies and Reagents

Purified anti-mouse PD-1 mAb (RMP1-14), anti-mouse anti-CD137 (3H3), control IgG (2A3), anti-CD4 (GK1.5), anti-CD8β (53.5.8), anti-IFNγ (H22; all from BioXCell), and anti-asGM1 (Wako Chemicals) were used in the schedule and doses as indicated. To deplete Tregs, BALB/c FOXP3-DTR-GFP mice were injected i.p. with 250-ng DT (Sigma Aldrich; cat. No. D0564) and used in the schedule as indicated.

Experimental Tumor Models/Treatments

In the preclinical 4T1.2 breast cancer tumor model, following tumor inoculation in the mammary fat pad, mice develop metastases in the lungs, liver, bones, and brain, among other organs (19). The primary tumor is surgically resected, and these mice are then treated

with the agent of interest to assess how adjuvant therapy affects metastasis and survival. Similar to the 4T1.2 tumor model, injection of E0771 into the mammary fat pad of mice results in their metastasizing to the lungs, resulting in death even when the primary tumor is resected (20).

For spontaneous metastasis and post-surgery survival experiments, the indicated dose of 4T1.2 or E0771 tumor cells suspended in 50 μL PBS were always inoculated into the fourth left mammary fat pad of female FOXP3-DTR or WT mice. The mean tumor size at the time of resection was ~40 to 80 mm^2. Some experiments were performed in a blinded fashion as indicated, and three different investigators performed the experiments. During surgery, mice were anesthetized before their primary tumors including the draining lymph nodes were resected, and their wounds closed with surgical clips. Mice were monitored for symptoms of illness with changes to weight, posture, activity, and fur texture, and euthanized when clinical symptoms reached the cumulative limit outlined by animal ethics. In some experiments where paclitaxel was used, the dose and schedule was as previously described (34). For s.c. or mammary fat-pad rechallenge experiments, long-term survivors were always injected with the indicated dose of tumor cells on the opposite flank.

Flow Cytometry Analysis

Tumors, blood, spleen, lung, and liver were harvested from mice and processed for flow cytometry analysis as previously described (22). For surface staining, tumor-infiltrating lymphocyte or immune cell suspensions were stained with antibodies and respective isotype antibodies in the presence of anti-CD16/32 (2.4G2) to block FcR. The list of antibodies used is described in the Supplementary Methods (see Supplementary Table S1). To stain for Ki67, samples were fixed and permeabilized with a FOXP3 Fixation/Permeabilization Kit (eBioscience). To measure intracellular cytokine staining, single-cell suspensions were incubated for 4 hours in complete RPMI with monensin and brefeldin A (eBioscience). Samples were then surface stained before being fixed/permeabilized (BD CytoFix/CytoPerm Kit) and stained with anti-IFNγ and anti-TNF or fluorescence minus one control for IFNγ staining and isotype control for TNF staining. To determine absolute counts in samples, liquid counting beads (BD Biosciences) were added directly before samples were run on the flow cytometer. All data were collected on a Fortessa 4 (Becton Dickinson) flow cytometer and analyzed with FlowJo v10 software (Tree Star, Inc.).

Histology

Mouse tissues were perfusion fixed in 10% neutral buffered formalin overnight, processed routinely, and embedded in paraffin. Sections (8 μm) were cut and stained with H&E. H&E-stained tissue sections were imaged by using Aperio Scanscope AT (Leica) and analyzed by Aperio ImageScope.

Statistical Analysis

The animal numbers used for all experiments are outlined in the corresponding figure legends. Groups of 5 to 10 mice per experiment were generally used. No statistical method was used to predetermine sample size. No method of randomization was used to allocate animals to experimental groups except for Fig. 4D, Figs. 5–7, and Supplementary Figs. S7–S9. Across all experiments, we did not exclude samples or mice from the analysis with the exception of two samples due to technical flaws in execution. The investigators were not blinded to the group allocation during the experiment and/or when assessing the outcome except for Fig. 1A and Supplementary Fig. S6. Statistical analysis was performed using GraphPad Prism software. Comparison of different groups was carried out using either an unpaired Student *t* test, unpaired Welch *t* test, one-way ANOVA or Mann–Whitney *U* test. In some experiments, to determine what immunologic effects were induced 4 days after respective neoadjuvant or adjuvant therapy, significance had to be performed on different days. We tested for the assumption of unequal variances using the Bartlett test. In instances where we found unequal variances, we used the Welch *t* test. Kaplan–Meier analyses with a log-rank sum test were used for animal survival experiments. We did not adjust for all multiple comparisons between the survival curves because we were only concerned with a limited number of comparisons between groups. Data were considered to be statistically significant where the *P* value was equal to or less than 0.05.

Disclosure of Potential Conflicts of Interest

M.J. Smyth reports receiving a commercial research grant from Bristol-Myers Squibb and other commercial research support from MedImmune. M.W.L. Teng has received speakers bureau honoraria from Merck Sharp & Dohme. No potential conflicts of interest were disclosed by the other authors.

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Development of methodology: J. Liu, S.F. Ngiow, M.J. Smyth
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Liu, S.J. Blake, M.C.R. Yong, A. Young, J.S. O'Donnell, M.J. Smyth
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Liu, S.F. Ngiow, J.S. O'Donnell, M.J. Smyth, M.W.L. Teng
Writing, review, and/or revision of the manuscript: J. Liu, S.J. Blake, S.F. Ngiow, A. Young, J.S. O'Donnell, M.J. Smyth, M.W.L. Teng
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Liu, M.C.R. Yong, H. Harjunpää, K. Takeda, S. Allen, M.W.L. Teng
Study supervision: M.J. Smyth, M.W.L. Teng

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