Doxil Synergizes with Cancer Immunotherapies to Enhance Antitumor Responses in Syngeneic Mouse Models

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Abstract

Based on the previously described roles of doxorubicin in immunogenic cell death, both doxorubicin and liposomal doxorubicin (Doxil) were evaluated for their ability to boost the antitumor response of different cancer immunotherapies including checkpoint blockers (anti–PD-L1, PD-1, and CTLA-4 mAbs) and TNF receptor agonists (OX40 and GITR ligand fusion proteins) in syngeneic mouse models. In a preventative CT26 mouse tumor model, both doxorubicin and Doxil synergized with anti–PD-1 and CTLA-4 mAbs. Doxil was active when CT26 tumors were grown in immunocompetent mice but not immunocompromised mice, demonstrating that Doxil activity is increased in the presence of a functional immune system. Using established tumors and maximally efficacious doses of Doxil and cancer immunotherapies in either CT26 or MCA205 tumor models, combination groups produced strong synergistic antitumor effects, a larger percentage of complete responders, and increased survival. *In vivo* pharmacodynamic studies showed that Doxil treatment decreased the percentage of tumor-infiltrating regulatory T cells and, in combination with anti–PD-L1, increased the percentage of tumor-infiltrating CD8$^+$ T cells. In the tumor, Doxil administration increased CD80 expression on mature dendritic cells. CD80 expression was also increased on both monocytic and granulocytic myeloid cells, suggesting that Doxil may induce these tumor-infiltrating cells to elicit a costimulatory phenotype capable of activating an antitumor T-cell response. These results uncover a novel role for Doxil in immunomodulation and support the use of Doxil in combination with checkpoint blockade or TNFR agonists to increase response rates and antitumor activity.

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Introduction

Immunotherapy is a promising new area of cancer therapeutics. Several immunotherapies are being evaluated preclinically as well as in clinical trials and have demonstrated promising activity [1–4]. One challenge that remains is that not all patients respond despite the durable effect these therapies can have. This is likely due to an immunosuppressive tumor microenvironment and/or poor immunogenicity of patient's tumors. To increase the response rate of tumors to immunotherapy, rational combination approaches of different cancer immunotherapies have been investigated, including combining mediators of checkpoint blockade (i.e., anti–PD-1 and PD-L1) and TNFR-family agonists (i.e., OX40) with small-molecule drugs [5–11]. Although significant progress has been achieved in the evaluation of combination therapies preclinically, there remains a great need for rational testing of immunotherapies in combination settings, in particular with established

cancer treatments, and translation of novel combinations with improved activity into the clinic.

Doxorubicin is a widely used chemotherapeutic drug for patients with sarcoma, lung, breast, and other cancers. Previously, doxorubicin has been well characterized as a DNA intercalator and an inhibitor of topoisomerase II [12]. Other mechanisms of action of doxorubicin

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that are reported are DNA cross-linking, interference with DNA strand separation, free-radical formation, helicase activity, and direct membrane effects [13]. Doxorubicin thus has been viewed as a cytotoxic agent with direct cell-killing effects on tumor cells. More recently, doxorubicin has been established as an inducer of immunogenic cell death and has been shown to increase IFN gamma production and induce dendritic and T-cell tumor infiltration in mouse models [14–20].

Based on these immunomodulatory effects, we hypothesized that doxorubicin, or liposomal doxorubicin (Doxil), could potentiate the antitumor activity of immunotherapeutic agents in syngeneic mouse models. Doxil is an approved drug for paclitaxel- and platinum-resistant ovarian carcinoma and Kaposi's sarcoma. In preclinical models, Doxil has been shown to have more antitumor activity; therefore, comparison of this drug to free doxorubicin was explored in this study [21,22]. Here, we demonstrate that both doxorubicin and Doxil synergize with several T-cell–targeted immunotherapies in two syngeneic mouse models. Importantly, combination activity was durable, led to high rates of complete response (CR), and generated immunological memory in mouse models. Furthermore, the results reveal for the first time that Doxil has effects on dendritic and immature myeloid cells in tumors, as well as on $CD8^+$ T cells and regulatory T cells (Tregs).

Materials and Methods

Antibodies, Reagents, and Cell Lines

CT26 cells were obtained from ATCC (Manassas, VA) and were grown with RPMI 1640 medium supplemented with 10% fetal bovine serum. MCA205 cells were obtained from Agonox (Portland, OR) and grown in the same growth medium. Following receipt of cell lines, both cell lines were reauthenticated using STR-based DNA profiling and multiplex polymerase chain reaction (IDEXX Bioresearch, Columbia, MO). Antibodies obtained from BioXCell (West Lebanon, NH) included the following: anti–PD-1 (RMP1-14), anti–PD-L1 (10 F.9G2), anti–CTLA-4 (9D9), and mouse IgG2b control (MPC-11). Mouse OX40 ligand fusion protein (OX40L FP), mouse GITR ligand fusion protein (GITRL FP), and rat IgG2a isotype control antibodies were produced by MedImmune. Doxil, gemcitabine, and oxaliplatin were purchased from Bluedoor Pharma (Rockville, MD), and doxorubicin was obtained from Henry Schein Inc. (Melville, NY).

Animal Studies

Cells were grown in monolayer culture, harvested by trypsinization, and implanted subcutaneously into the right flank of 6- to 8-week-old female Balb/C (CT26) or C57BL/6 (MCA205), or 4- to 6-week-old athymic female nude mice (Harlan, Indianapolis, IN). For the CT26 tumor model, 5×10^5 cells were implanted in the right flank using a 26-gauge needle. For the MCA205 tumor model, 2.5×10^5 cells were implanted. All antibodies, OX40L FP, GITRL FP, gemcitabine, and oxaliplatin were dosed via intraperitoneal injection. Doxil and doxorubicin were dosed via intravenous injection. In some studies, isotype controls were administered to mice as a cocktail of rat IgG2a and mouse IgG2b. At the beginning of treatment, mice were randomized either by tumor volume (established-tumor studies) or by body weight (preventative studies). The number of animals per group ranged from 10 to 12 animals per group as determined based on sample size calculations using nQuery software. Both tumor and body weight measurements

were collected twice weekly, and tumor volume was calculated using the equation $(L \times W^2)/2$, where L and W refer to the length and width dimensions, respectively. Error bars were calculated as the standard error of the mean. The general health of mice was monitored daily, and all experiments were conducted in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care and MedImmune Institutional Animal Care and Use Committee guidelines for humane treatment and care of laboratory animals. Kaplan-Meier statistical analysis was performed using the log-rank test using GraphPad Prism. The log-rank (Mantel-Cox) test was used to compare survival curves (Prism 6.03). The Bonferroni method was used to adjust the 0.05 alpha level for multiple comparisons. Reported P values are two-sided P values.

Pharmacodynamic Study

MCA205 cells (2.5×10^5) were implanted in the right flank of 6- to 8-week-old C57BL/6 female mice. When tumors reached an average of ~250 mm^3, mice were randomized in groups of three and were dosed with Doxil (5 mg/kg), anti–PD-L1 (20 mg/kg), or a combination of Doxil with anti–PD-L1 on day 0. A second dose of anti–PD-L1 was given on day 3 and Doxil again on day 7. On day 8, all mice were euthanized and tissues were collected from mice. Red blood cells were lysed with ACK solution (Life Tech, Carlsbad, CA). Tumors were cut into 2-mm^3 pieces and digested for 20 minutes at 37°C with 200 U/ml of Collagenase type 3 (Worthington, Lakewood, NJ) and 0.25 mg/ml of DNase (Sigma-Aldrich, St. Louis, MO). One to two million cells were loaded per well in a 96-well plate and stained with Live/Dead Blue (Life Tech, Carlsbad, CA) and antibodies to CD11b (BD Clone M1/70), CD11c (Biolegend Clone n418), CD80 (Biolegend Clone 16-10A1), Ly6G (Biolegend Clone 1A8), Ly6C (Biolegend HK1.4), CD45 (Ebioscience Clone 30-F11), MHC-II (Biolegend Clone M5/114.15.2), CD4 (Biolegend Clone RM4-5), CD8 (BD Clone RPA-T8), T-bet (Biolegend Clone 4B10), and FOXP3 (Ebioscience Clone FJK-16S) in FACS buffer (PBS +0.5% FBS and 2 mm of EDTA). For FOXP3 detection, a FOXP3 transcription kit was used (Ebioscience, San Diego, CA). Cells were stained at 4°C for 20 minutes, washed, and fixed with 4% paraformaldehyde. To assess expression of PD-L1, CT26, and MCA205, cells were harvested from flasks using StemPro Accutase (Life Technologies, Carlsbad, CA). Cells were stained with anti–PD-L1 APC antibody (10F.9G2, Biolegend) or rat isotype IgG2b APC (RTK4530, Biolegend) for 20 minutes at 4°C. Sample data were acquired on a BD Fortessa (BD, San Jose, CA), and data were analyzed using Flowjo (Treestar, Ashland, OR).

Statistical Analysis for Synergy

Statistical analysis for synergy was performed using a Bliss independence model [23]. The model is described as follows. If the rate of total tumor regression due to drug A alone is r_a and the rate due to drug B alone is r_b, then the expected rate of total tumor regression due to drug A and drug B in combination is $r_{Bliss} = r_a + r_b - r_a r_b$ assuming that the two drugs are Bliss independent. The difference between the observed total tumor regression rate r_{ab} and the expected rate is defined as the synergy index:

$$I = r_{ab} - r_{Bliss}$$

Then the variance of the synergy index can be written as

$$var(I) = var(r_{ab}) + var(r_{Bliss})$$

Further,

$$var(r_{Bliss}) = var(r_a) + var(r_b) + var(r_a r_b) - 2cov(r_a + r_b, \ r_a r_b)$$
$$var(r_a r_b) = var(r_a)var(r_b) + r_a^2 var(r_b) + r_b^2 var(r_a)$$
$$cov(r_a + r_b, \ r_a r_b) = r_a var(r_b) + r_b var(r_a)$$
$$var(r_{ab}) = \frac{r_{ab}(1 - r_{ab})}{n_{ab}}$$
$$var(r_a) = \frac{r_a(1 - r_a)}{n_a}$$
$$var(r_b) = \frac{r_b(1 - r_b)}{n_b}$$

where n_{ab}, n_a, and n_b are the respective sample sizes of the combination experiment and two monotherapy experiments. The two drugs are said to be synergistic if

$$\frac{I}{\sqrt{var(I)}} > Z_{0.95}$$

where $Z_{0.95}$ is the 95% percentile of standard normal distribution.

Results

To test the hypothesis that doxorubicin or Doxil may potentiate antitumor effects of immunotherapeutic agents, CT26 tumor-bearing Balb/C mice were treated with varying doses of these drugs alone and in combination with either anti–PD-1 or anti–CTLA-4 antibodies. In this study, drugs were administered after tumor cell implant but before formation of palpable tumors. Given that this was a preventative study, the doses of anti–PD-1 and anti–CTLA-4 antibodies administered were suboptimal as higher doses of these antibodies in this setting produced strong antitumor responses (data not shown). Figure 1A shows the growth of CT26 tumors in untreated mice. Mice treated with a mixture of isotype controls (rat IgG2a + mouse IgG2b) had no effect on tumor growth (Figure 1B). Compared to doxorubicin, Doxil had more potent antitumor activity at a 4-mg/kg dose (Table 1). Indeed, all mice treated with Doxil at its maximum tolerated dose (5 mg/kg) had a CR. A reduced dosage of Doxil at 1 mg/kg had nearly equivalent antitumor activity as doxorubicin at 4 mg/kg (Figure 1, C and D). Although anti–PD-1
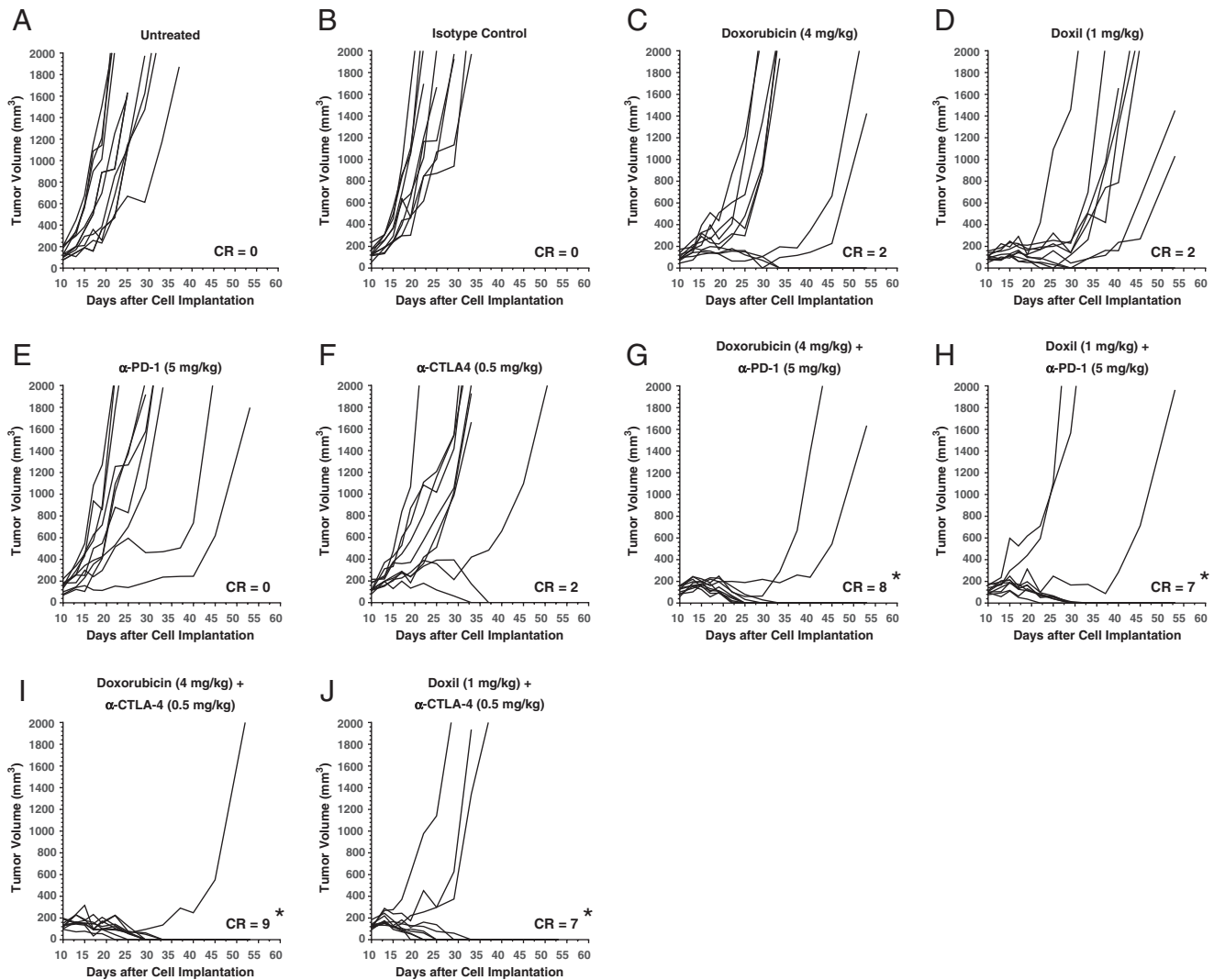
Figure 1. Synergy of doxorubicin or Doxil in combination with α-PD-1 and α-CTLA-4 antibodies in the CT26 tumor model. CT26 cells were implanted into Balb/C mice. Four days after cell implantation, mice were randomized by body weight and dosed with Doxil on days 4, 11, and 17; doxorubicin on days 4, 8, and 12; and anti–PD-1 or anti–CTLA-4 on days 10, 14, 17, and 21. The groups were as follows: (A) untreated, (B) isotype controls (rat IgG2a + mouse IgG2b; 5/0.5 mg/kg), (C) doxorubicin (4 mg/kg), (D) Doxil (1 mg/kg), (E) α-PD-1 (5 mg/kg), (F) α-CTLA-4 (0.5 mg/kg), (G) doxorubicin + α-PD-1 (4/5 mg/kg), (H) Doxil + α-PD-1 (1/5 mg/kg), (I) doxorubicin + α-CTLA-4 (4/0.5 mg/kg), and (J) Doxil + α-CTLA-4 (1/0.5 mg/kg). *P < .005 (Bliss independence test).

Table 1. Doxorubicin or Doxil in Combination with PD-1 or CTLA-4 Antibodies Produce a Large Percentage of CRs

Treatment	No. of CRs
Untreated	0/10
Isotype controls (5/0.5 mg/kg)	0/10
Anti–PD-1 (5 mg/kg)	0/10
Anti–CTLA-4 (0.5 mg/kg)	2/10
Doxorubicin (4 mg/kg)	2/10
Doxorubicin (1 mg/kg)	0/10
Doxil (5 mg/kg)	10/10
Doxil (4 mg/kg)	8/10
Doxil (1 mg/kg)	2/10
Anti–PD-1/doxorubicin (5/4 mg/kg)	8/10
Anti–PD-1/doxorubicin (5/1 mg/kg)	5/10
Anti–PD-1/Doxil (5/4 mg/kg)	9/10
Anti–PD-1/Doxil (5/1 mg/kg)	7/10
Anti–CTLA-4/doxorubicin (0.5/4 mg/kg)	9/10
Anti–CTLA-4/doxorubicin (0.5/1 mg/kg)	2/10
Anti–CTLA-4/Doxil (0.5/4 mg/kg)	10/10
Anti–CTLA-4/Doxil (0.5/1 mg/kg)	7/10

The number of CRs from all of the groups from the experiment in Figure 1 is shown. Doxil was more active than doxorubicin, and doxorubicin or Doxil combined with PD-1 or CTLA-4 antibodies produced a strong antitumor response.

and anti–CTLA-4 treatment had low to moderate antitumor activity as single agents (Figure 1, E and F), both antibodies exhibited synergistic antitumor effects when combined with doxorubicin or Doxil (Figure 1, G–J). When anti–PD-1 was combined with doxorubicin (4 mg/kg), the number of complete responders increased from two to eight animals (Figure 1G). Similarly, the combination of doxorubicin with anti–CTLA-4 increased the number of complete responders from two to nine animals (Figure 1I). Similar results were obtained when anti–PD-1 and anti–CTLA-4 were combined with Doxil at 1 mg/kg (Figure 1, H and J). The number of complete responders per group for the entire study is shown in Table 1. These data demonstrate that both doxorubicin and Doxil are synergistic with anti–PD-1 and CTLA antibodies and suggest that this feature is inherent in doxorubicin itself, as utilization of liposomal doxorubicin had similar synergistic activity as the free drug.

To determine if mice which obtained a CR with Doxil treatment alone or in combination with anti–PD-1 or anti–CTLA-4 displayed immunological memory, these animals were rechallenged with live CT26 cells 70 days after the initial treatment. Whereas CT26 cells grew in all 10 out of 10 naive, untreated mice (Figure 2A), mice that achieved CR with Doxil showed widespread tumor rejection with 9 out of 10 mice rejecting tumor (Figure 2B). Eight out of 10 mice treated with Doxil + anti–CTLA-4 and 9 out of 9 mice with Doxil + anti–PD-1 also rejected tumors (Figure 2, C and D). These results demonstrate that treatment with Doxil as a single agent as well as with Doxil in combination with checkpoint inhibitors resulted in tumor-specific immunological memory.

Although both Doxil and doxorubicin were active in a preventative CT26 tumor model, we also were interested in whether these drugs were effective in inhibiting established CT26 tumors and whether the activity of these drugs was different in immunocompetent compared with immunocompromised mice. T-cell–deficient athymic nude mice and immunocompetent Balb/C mice both bearing CT26 tumors were treated with these drugs at their maximum tolerated doses when tumors reached approximately 200 mm^3. In this experiment, doxorubicin did not elicit antitumor activity in either immunocompromised or immunocompetent mice (Figure 3, A and B). In contrast, Doxil treatment showed robust

antitumor activity in immunocompetent mice bearing established CT26 tumors (Figure 3B) but much less activity in immunodeficient mice (Figure 3A), demonstrating that Doxil activity is dependent on a functional immune system. To assess whether other chemotherapeutic agents could produce similar effects, immunodeficient and immunocompetent mice with CT26 tumors were administered either oxaliplatin, a DNA cross-linking agent, or gemcitabine, a nucleoside analog. Oxaliplatin demonstrated increased antitumor activity in immunocompetent mice (Supplemental Figure 1B) compared with immunodeficient mice (Supplemental Figure 1A), consistent with previous reports [24]. In contrast, gemcitabine had significant antitumor activity in both immunodeficient as well as immunocompetent mice (Figure 3, C and D). These results are consistent with prior studies that suggested that certain, but not all, chemotherapies can effectively induce immunological cell death [18].

Although the combination of Doxil with anti–PD-1 and anti–CTLA-4 antibodies showed strong antitumor effects in the CT26 model (Figure 1), the limitations of this experiment were that it was a preventative study and that suboptimal doses of anti–PD-1 and anti–CTLA-4 antibodies were used. As free doxorubicin was inactive against established CT26 tumors, Doxil was chosen to investigate combinations with immunotherapy in this setting. CT26 tumor-bearing mice were treated with Doxil alone or in combination with anti–CTLA-4, anti–PD-1, or anti–PD-L1, as well as a mouse OX40 ligand fusion protein (OX40L FP) or a mouse GITR ligand fusion protein (GITRL FP) all at maximally efficacious doses once tumors were around 200 to 300 mm^3 (Figure 4). Prior studies demonstrated that higher doses of these mouse-reactive immunotherapeutics did not result in more antitumor activity at these established tumor volumes (data not shown). Doxil treatment resulted in a temporary control of tumor growth, followed by rapid regrowth of tumors, and only one CR (Figure 4B). Treatment with OX40L FP, anti–PD-1, anti–PD-L1, and anti–CTLA-4 antibodies demonstrated low to moderate activity (Figure 4, C–F), with a few complete responders in each group. Treatment of mice with GITRL FP in these mice was moderately potent, with 6/12 mice achieving CR and 2/12 achieving stable disease (Figure 4G). The combination of Doxil with OX40L FP was additive and increased the time to tumor progression compared with single-agent therapy (Figure 4H). Strikingly, Doxil in combination with anti–PD-1, anti–PD-L1, and anti–CTLA-4 produced a synergistic increase in the number of complete responders, with 11/12, 9/12, and 8/12, respectively (Figure 4, I–K). These results were consistent with the synergy observed with the combination of Doxil with checkpoint inhibitors in the preventative study (Figure 1). Equally compelling was the observation that the combination of Doxil with a mouse GITRL fusion protein was also synergistic, with all 12/12 mice achieving CR (Figure 4L). These experiments demonstrated that Doxil in combination with checkpoint blockade antibodies produced dramatic increases in antitumor responses, as well as with a GITRL fusion protein, even in established-tumor settings at maximally efficacious doses. This was reflected also in Kaplan-Meier survival plots which demonstrated that all mice treated with the combination of Doxil with anti–PD-1, PD-L1, or CTLA-4 antibodies survived longer than either single agent alone. In addition, mice treated with Doxil and GITRL FP survived longer than mice treated with Doxil alone (Supplemental Figure 2).

As the CT26 tumor model is highly sensitive to immunotherapies, we determined whether Doxil could enhance the activity of immunotherapies in a potentially less sensitive model, MCA205. This
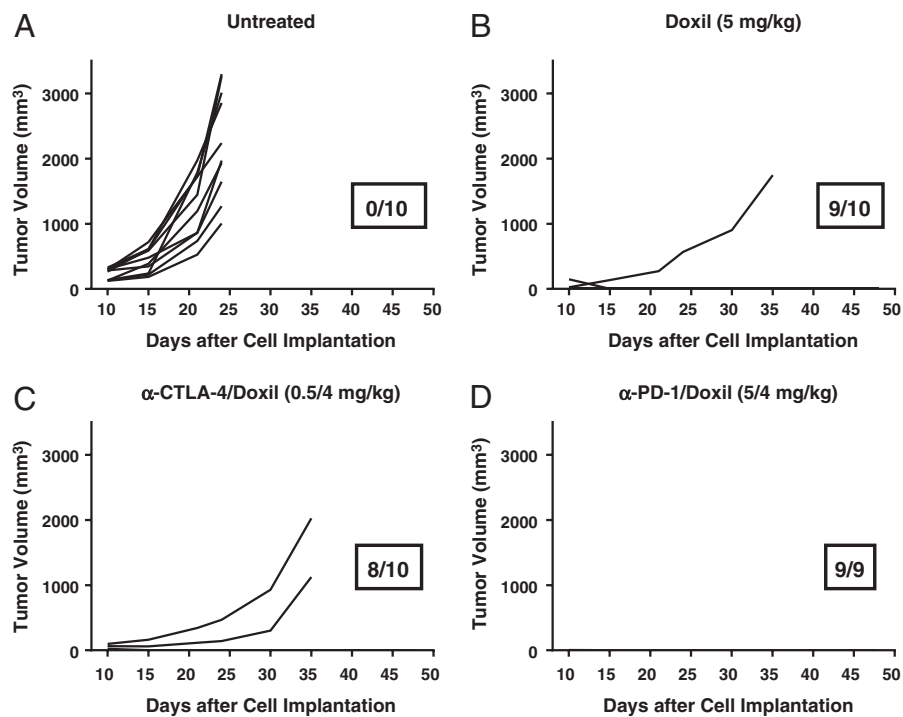
Figure 2. Mice achieving CR with Doxil alone or in combination with anti–CTLA-4 or anti–PD-1 antibodies resisted tumor rechallenge. (A) Ten naive Balb/C mice and mice that achieved CR by (B) by Doxil treatment, (C) α-CTLA-4 + Doxil treatment, and (D) α-PD-1 + Doxil treatment were rechallenged with CT26 cells, and tumor growth was monitored. The numbers indicate the number of mice that rejected tumors out of the total number of mice in the group.

was based on prior studies which showed poor response in this model to anti–PD-L1 and anti–PD-1 antibodies, although MCA205 expressed marginally higher levels of PD-L1 compared with CT26 (data not shown and Supplemental Figure 3). Doxil, anti–PD-1, anti–PD-L1, and anti–CTLA-4 antibodies as well as OX40L FP and GITRL FP were dosed at maximally efficacious doses in established MCA205 tumors starting at a tumor volume between 100 and 150 mm^3 (Figure 5). In this model, Doxil temporarily controlled tumor growth; however, the majority of the tumors regrew (Figure 5B). One mouse did achieve a CR in the Doxil group. In contrast to the CT26 model, anti–PD-1, anti–PD-L1, OX40L FP, and GITRL FP were minimally active in this model, with some delay in tumor progression but only one complete responder in the OX40L FP and GITRL FP groups (Figure 5, C–E and G). Treatment with an anti–CTLA-4 antibody produced modest activity with 8/12 mice achieving CR (Figure 5F). For the combination treatments, combining Doxil with OX40L FP or GITRL FP agonists did not delay tumor growth significantly more than Doxil alone and also did not provide a significant increase in complete responders (Figure 5, H and L). In contrast, Doxil in combination with antibodies to checkpoint inhibitors PD-1, PD-L1, and CTLA-4 produced striking responses with 9/12, 12/12, and 12/12 mice achieving CR, respectively (Figure 5, I–K). These results demonstrate that, in this model, combining checkpoint inhibition with Doxil produced synergistic effects. Supplementary Figure 4 demonstrates increased survival in mice treated with Doxil + anti–PD-1, anti–PD-L1, and anti–CTLA-4 antibodies compared with Doxil treatment in this study.

Based on these results, a pharmacodynamic study was performed to elucidate any effects of Doxil on immune cell populations *in vivo*. Because the synergistic effects of Doxil were greatest when combined with checkpoint inhibition and were similar

between anti–PD-1, anti–PD-L1, and anti–CTLA-4, we chose to combine Doxil with one of these agents in the MCA205 model. MCA205 tumor-bearing mice were treated with Doxil, anti–PD-L1, or the combination, and tumors and blood were harvested at the end of treatment. In these mice, Doxil increased the percent of CD8$^+$ T cells in the blood, and the combination of Doxil and PD-L1 produced a significant increase in the percent of CD8$^+$ T cells in the tumor (Figure 6, A and B). Doxil also significantly decreased the amount of tumor-infiltrating Tregs in the tumor, which seemed to be further augmented by the combination of Doxil and anti–PD-L1 (Figure 6C). To examine the cause of the T-cell changes, we investigated the phenotype of the myeloid compartment in blood and tumor. In both the blood and tumor, Doxil and Doxil + anti–PD-L1, but not anti–PD-L1 alone, induced the expression of the costimulatory molecule CD80 on CD45$^+$CD11c$^+$MHCII^{hi} cells, which represent mature dendritic cells (Figure 6, D and E). The level of CD80 expression tended to be higher in the combination group compared with Doxil alone. At the same time, Doxil treatment also increased the percent of CD45$^+$CD11c$^+$MHCII^{hi} cells in the blood, which was further significantly increased when combined with anti–PD-L1 (Figure 6F). This demonstrates that Doxil can not only increase the level of CD80 on mature dendritic cells but also induce expansion of these cells. Interestingly, the effect of Doxil-induced upregulation of CD80 was also observed on CD45$^+$CD11b$^+$Ly6c$^+$ and CD45$^+$CD11b$^+$Ly6G$^+$ myeloid cells within the tumor (Figure 6, G and H). The flow cytometric gating paths for the T cells and myeloid cells are shown in Supplemental Figures 5 and 6, respectively. In summary, these results demonstrated that, *in vivo*, Doxil decreases tumor Tregs, induces CD8$^+$ T-cell expansion, and upregulates CD80 expression in mature DCs as well as myeloid cells in tumors. These findings are consistent with and may provide an explanation for the
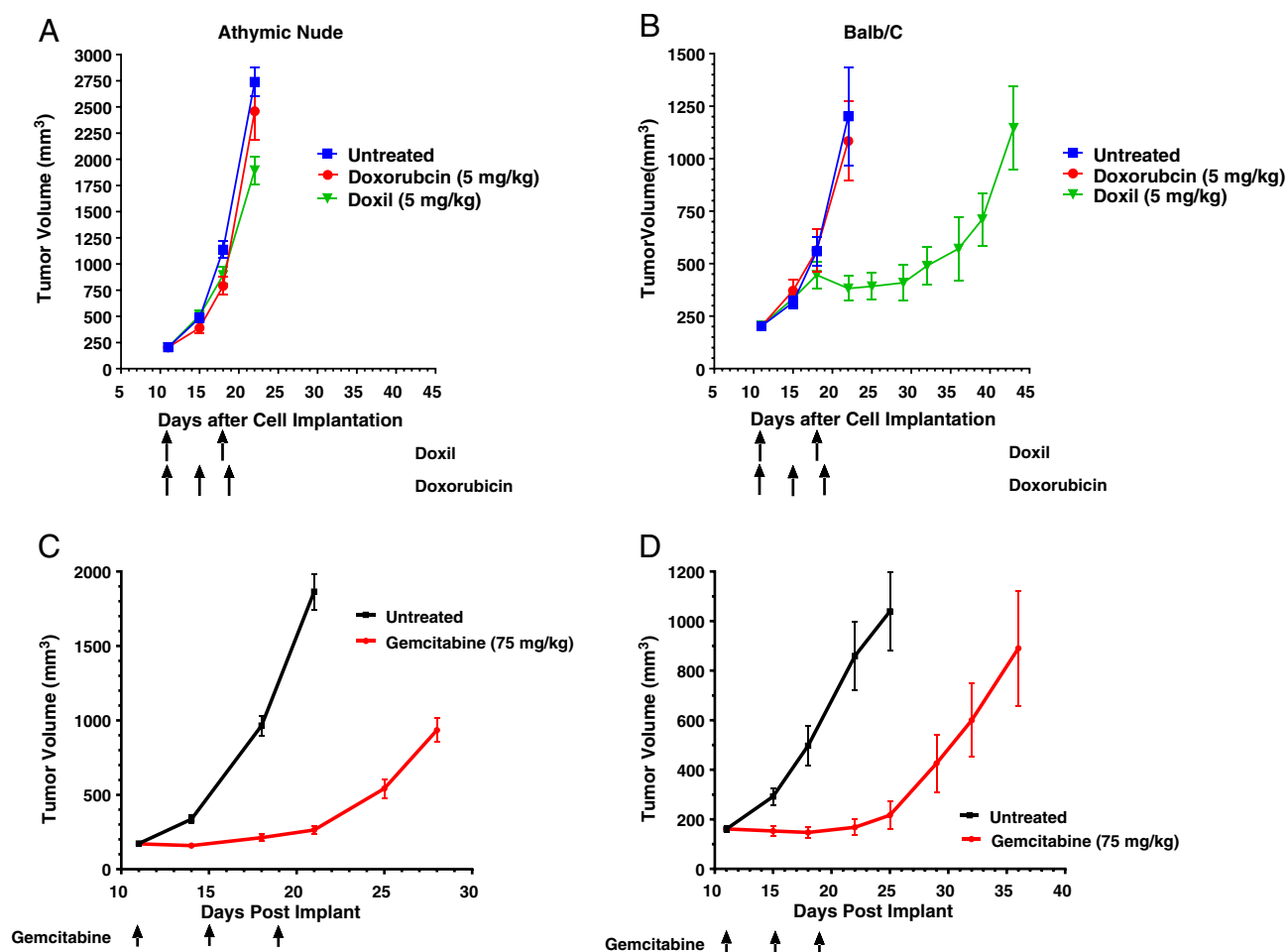
Figure 3. A functional immune system increases Doxil activity *in vivo*. CT26 tumor-bearing nude mice (A, C) or CT26 tumor-bearing Balb/C mice (B, D) were dosed with Doxil or doxorubicin (A and B) or gemcitabine (C and D), and tumor growth was monitored. Arrows indicate dose administration.

profound antitumor effects that Doxil had in combination with anti–PD-L1 and potentially the other mediators of checkpoint blockade observed in this study as well.

Discussion

In the present study, we demonstrate that the combination of doxorubicin or Doxil with cancer immunotherapies induces a potent increase in antitumor efficacy in preclinical models. In a CT26 preventative mouse tumor model, a synergistic antitumor effect was observed when doxorubicin or Doxil was combined with anti–PD-1 or anti–CTLA-4 antibodies. In addition to the CT26 preventative model, synergy of cancer immunotherapies with Doxil was also observed in a CT26 established-tumor model in combination with anti–PD-1, PD-L1, and CTLA-4, as well as with a GITRL ligand fusion protein. Strong synergy was also observed with Doxil and checkpoint inhibition in the MCA205 model. Although additional tumor models should be examined, these data suggest that Doxil may act as a broad booster of antitumor activity when combined with immunotherapy.

The immunomodulatory effects of doxorubicin have been well described *in vitro* and in mouse models; however, these effects had not been reported for Doxil [14,15,17–19,25–27]. Based on these findings, Doxil was compared with doxorubicin for its ability to

promote immunomodulatory effects in established CT26 tumors. It was found that Doxil, but not doxorubicin, was able to slow CT26 tumor growth in immunocompetent mice but not in athymic nude mice, suggesting a role for adaptive immunity in the activity of Doxil. This finding is consistent with a recent report that described a role for T cells in the activity of doxorubicin in syngeneic models, and that we observed a lack of activity with Doxil in T-cell–deficient animals suggests that T cells are likely important for Doxil activity *in vivo* as well [14]. Mice that achieved CR with Doxil treatment in our study rejected tumors upon rechallenge, demonstrating that Doxil induces a memory T-cell response. It should be noted that the general feature of chemotherapy having increased *in vivo* antitumor activity in immunocompetent mice is not a property of all drugs. Indeed, gemcitabine had equivalent antitumor activity in both immunocompetent and immunodeficient mice. This finding is in apparent contrast to a prior report that demonstrated less activity of gemcitabine in immunodeficient compared with immunocompetent mice, although not using the CT26 model as performed in our study [28]. This suggests that there must be differences between tumor models and their sensitivity to gemcitabine in mice that are T-cell deficient. This also suggests that, for some models, both direct cytotoxicity and immunomodulatory effects may be contributing to antitumor activity.
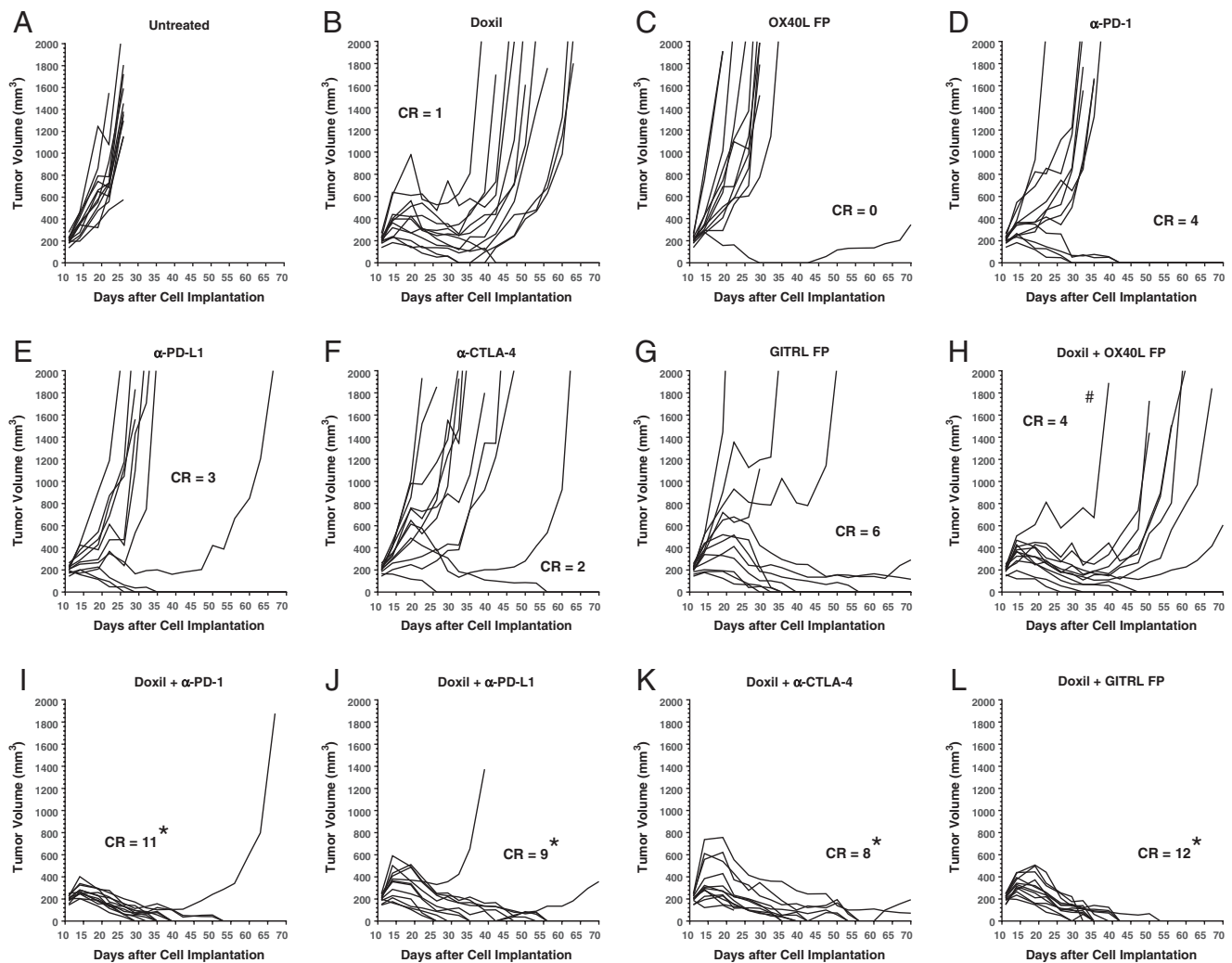
Figure 4. Synergistic antitumor responses of Doxil in combination with multiple immunotherapies in an established CT26 tumor model. Balb/C mice bearing established (~200-300 mm^3) CT26 tumors were randomized by tumor volume and treated with maximally efficacious doses of Doxil (5 mg/kg, days 11 and 19), OX40L FP (2.5 mg/kg; days 14 and 19), α-PD-1 (20 mg/kg; days 11, 14, 19, and 22), α-PD-L1, (30 mg/kg; days 11, 14, 19, and 22), α-CTLA-4 (20 mg/kg; days 14, 19, 22, and 26), and GITRL FP (5 mg/kg; days 14, 19, 22, 26, 29, and 32). The groups were as follows: (A) untreated, (B) Doxil, (C) OX40FP, (D) α-PD-1, (E) α-PD-L1, (F) α-CTLA-4, (G) GITRL FP, (H) Doxil + OX40L FP, (I) Doxil + α-PD-1, (J) Doxil + α-PD-L1, (K) Doxil + α-CTLA-4, and (L) Doxil + GITRL FP. The CR number indicates the number of mice that achieved CR out of 12. #$P = .056$; *$P < .008$, Bliss independence test.

The finding that systemic administration of Doxil to tumor-bearing mice can produce immunomodulatory effects is significant. Previous studies have shown this effect with local administration of doxorubicin only [18,27,29]. Our data suggest that there is no inherent difference in the ability of Doxil and doxorubicin to synergize with immunotherapy; however, the data do suggest that, at least in preclinical models, there may be a concentration threshold of doxorubicin within the tumor that is required to induce these effects. In contrast to Doxil, doxorubicin was ineffective in slowing CT26 tumor growth in an established-tumor setting in immunocompetent animals. This suggests that this concentration threshold is surpassed by Doxil but not by free doxorubicin. This is likely due to fact that, as a nanoparticle, Doxil delivers much higher concentrations of doxorubicin to tumors compared with free doxorubicin due to its extended circulation time [30]. Thus, although Doxil may be more active preclinically than doxorubicin, this is not to suggest that doxorubicin may not be able to exert these effects clinically.

In both CT26 and MCA205 syngeneic models, the combination of Doxil with anti–PD-1, PD-L1, or anti–CTLA-4 antibodies generally produced the strongest antitumor response, although Doxil combined with a novel mouse GITR ligand fusion protein led to CRs in 100% of the mice in the CT26 model. The CT26 model was generally more sensitive to these immunotherapies compared with MCA205, although interestingly this cell line had less expression of PD-L1 (Supplemental Figure 3). This being stated, it is difficult to determine whether PD-L1 expression may predict sensitivity to a combination regimen that includes Doxil, as this study only examined two syngeneic models. One potential explanation for the increased activity of Doxil in combination with inhibitors of checkpoint blockade, for example with anti–PD-L1, may be due to upregulation of the target itself following doxorubicin treatment. Sistigu et al. have recently shown that treatment of MCA205 tumors with doxorubicin increased PD-L1 transcript levels [25]. A Doxil-induced expression of PD-L1 may explain the higher sensitivity
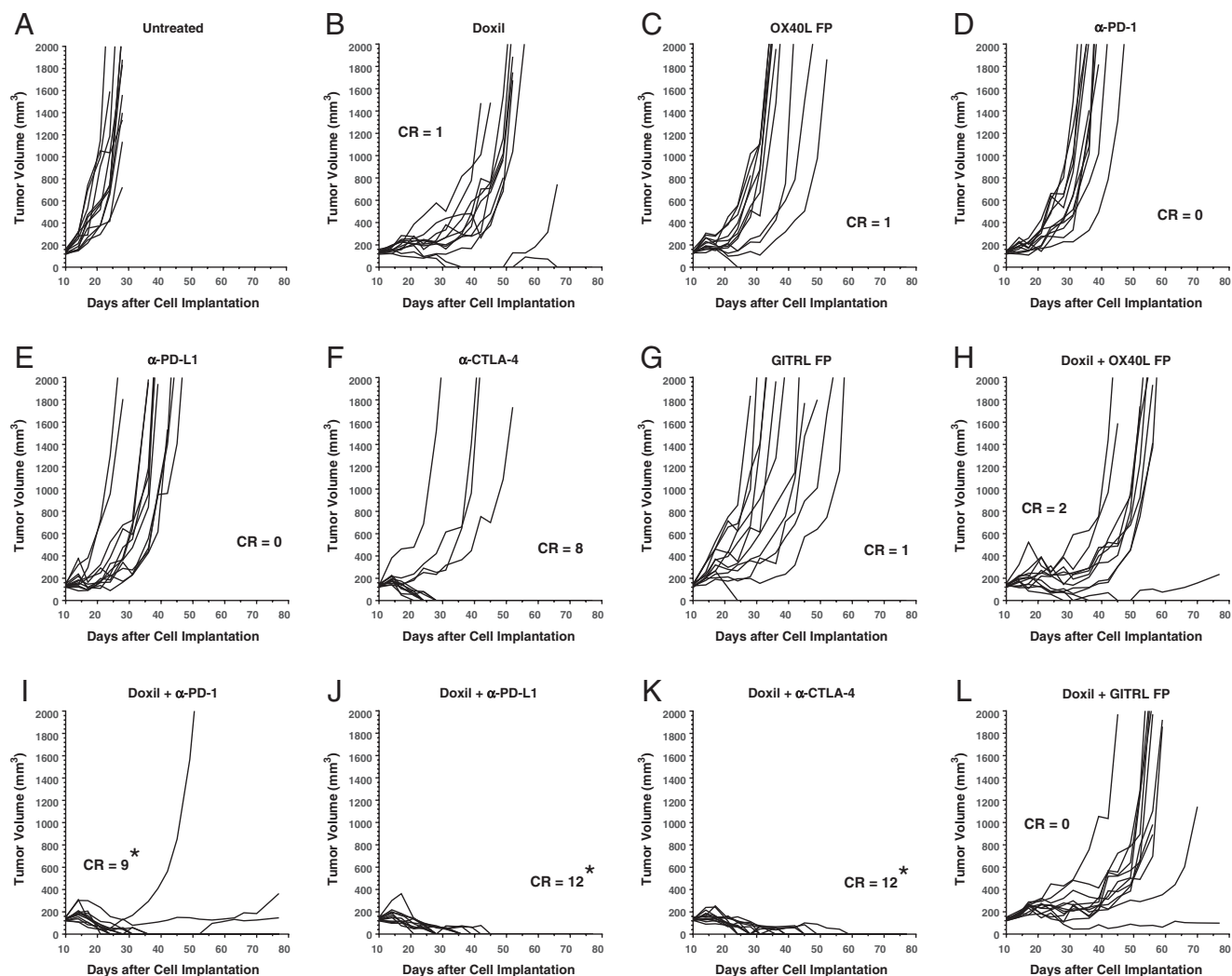
Figure 5. Synergistic antitumor responses of Doxil in combination with PD-1, PD-L1, and CTLA-4 antibodies in the MCA205 syngeneic model. C57BL/6 mice bearing established ($\sim$100-150 mm^3) MCA205 tumors were randomized by tumor volume and treated with maximally efficacious doses of Doxil (5 mg/kg; days 10, 17, and 24), OX40L FP (20 mg/kg; days 10 and 14), α-PD-1 (10 mg/kg; days 10, 14, 17, and 21), α-PD-L1 (20 mg/kg; days 10, 14, 17, and 21), α-CTLA-4 (10 mg/kg; days 10, 14, 17, and 21), and GITRL FP (5 mg/kg; days 10, 14, 17, 21, 24, and 28). The groups were as follows: (A) untreated, (B) Doxil, (C) OX40L FP, (D) α-PD-1, (E) α-PD-L1, (F) α-CTLA-4, (G) GITRL FP, (H) Doxil + OX40L FP, (I) Doxil + α-PD-1, (J) Doxil + α-PD-L1, (K) Doxil + α-CTLA-4, and (L) Doxil + GITRL FP. The CR number indicates the number of mice that achieved CR out of 12. *P < .008, Bliss independence test.

of tumors to this combination and perhaps less activity in combination with OX40L FP. The combination of Doxil with either anti–PD-1 or anti–PD-L1 greatly increased complete responder rates in both models, further emphasizing the importance of the PD-1/PD-L1 axis in the response to Doxil. It remains to be determined whether the combination of Doxil with anti–PD-1 and anti–PD-L1 is also potent in other syngeneic models, and this should be explored.

Previous studies have highlighted the importance of dose scheduling to achieve maximal antitumor effects in preclinical and clinical studies of immunotherapy combinations with chemotherapy [31,32]. In our studies, two different dose schedules were investigated. In separate studies, Doxil and doxorubicin were dosed both before (7 days) and concurrent with immunotherapy, with no apparent differences in antitumor response when combined with immunotherapies. This suggests that the cell death induced by doxorubicin generates an immunogenic effect lasting at least a week in

mice, possibly via the release of tumor antigens and thus enhancing the effects of antigen-presenting cells and T-cell priming. However, it remains to be determined whether the duration between doxorubicin administration and immunotherapy treatment could be extended even longer.

Investigations into the mechanism as to how Doxil was functioning *in vivo* demonstrated a key role for Doxil in the induction of CD80 expression on dendritic cells. Doxil also increased the percent of $CD45^+CD11c^+MHCII^{hi}$ dendritic cells in the blood. In addition, upregulation of CD80 tended to be higher when Doxil was combined with anti–PD-L1. These data provide evidence that Doxil activates dendritic cells and results in increased antigen presentation. Interestingly, we also found that Doxil increased CD80 expression on granulocytic and monocytic myeloid cells. This finding is in apparent contrast to a report that showed that CD80 expression on granulocytic myeloid-derived suppressor cells had immunosuppressive
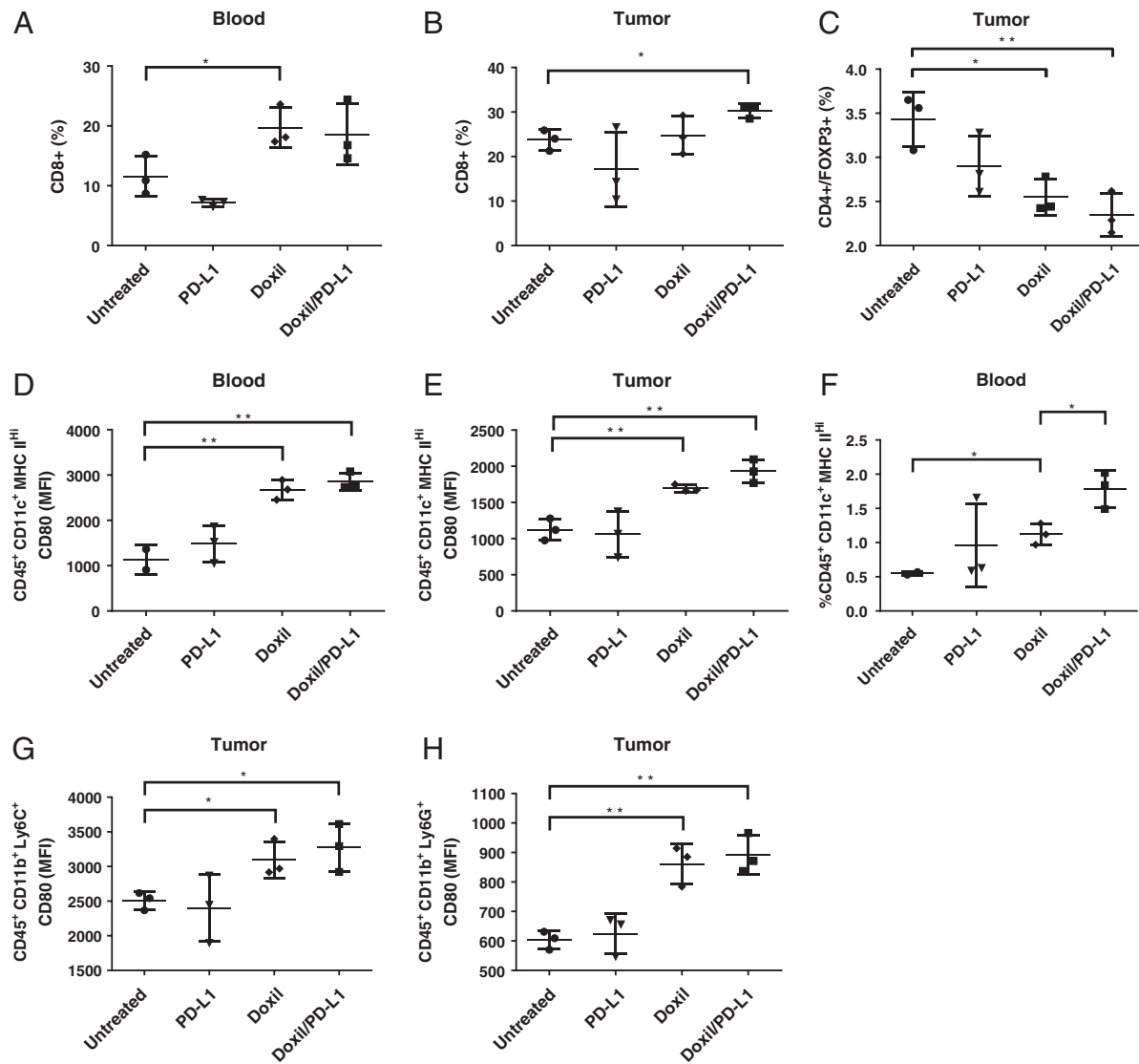
Figure 6. Immunomodulatory functions of Doxil *in vivo*. C57BL/6 mice bearing MCA205 tumors were dosed with α-PD-L1, Doxil, or the combination with three mice per group, and immunophenotyping was performed on blood and tumor. (A) The percent of $CD8^+$ T cells in the blood and (B) in the tumor. (C) The percent of $CD4^+/FoxP3^+$ cells in the tumor. (D) The expression of CD80 in $CD45^+CD11c^+MHCII^{hi}$ cells in the blood and (E) in the tumor. (F) The percent of $CD45^+CD11c^+MHCII^{hi}$ cells was increased in the blood in Doxil-treated animals, which was further augmented by the addition of α-PD-L1. (G) Expression of CD80 in tumor-isolated $CD45^+CD11b^+Ly6C^+$ cells as well as (H) tumor-isolated $CD45^+CD11b^+Ly6G^+$ cells. *$P < .05$, **$P < .01$ (unpaired two-tailed Student's *t* test).

functions, which were dependent on the presence of Tregs [33]. One potential explanation for this is that, in our study, Doxil alone significantly reduced the percentage of Tregs in the tumor, which was further decreased when combined with anti–PD-L1. Therefore, depletion of Tregs may allow relief of inhibitory functions of CD80 on these cells and other myeloid cell populations. It is possible that these cells possess more of an antigen-presenting phenotype due to the upregulation of CD80 and the correlation of this with antitumor activity. In a finding that is consistent with the observed increased CD80 expression on dendritic cells, Doxil was shown to increase the percent of $CD8^+$ cells in the blood and, in combination with anti–PD-L1, to increase the percent of $CD8^+$ cells in the tumor. In summary, these results likely explain the striking antitumor activity of Doxil in combination with anti–PD-L1 and likely with the other mediators of checkpoint blockade.

The combination of doxorubicin with immunotherapy has been conducted previously, notably in combination with IL-2 or IL-18 in preclinical animal models as well as in human clinical trials [34–36]. Increases in survival were observed in the preclinical studies which offer promise for clinical successes; however, IL-2 therapy is associated with significant side effects and requires careful management [37]. A recent report also investigated localized treatment of microparticle formulations of doxorubicin in combination with anti-OX40 and CTLA-4 antibodies in mice [29]. Our study is the first to demonstrate the potential of Doxil to be combined with cancer immunotherapies such as T-cell checkpoint blockers or TNFR-family agonists, including a novel GITR ligand fusion protein. Based on the potent combination effect with Doxil seen in these studies, lower doses of certain immunotherapies, such as anti–CTLA-4 antibodies, could be used in a combination strategy to alleviate some of the

toxicities that have been observed in clinical trials [38]. In summary, our findings give strong preclinical rationale for clinical evaluation of Doxil combinations with these and similar classes of cancer immunotherapies.

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