Breast Cancer Chemo-immunotherapy through Liposomal Delivery of an Immunogenic Cell Death Stimulus Plus Interference in the IDO-1 Pathway

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Supporting Information

ABSTRACT: Immunotherapy provides the best approach to reduce the high mortality of metastatic breast cancer (BC). We demonstrate a chemo-immunotherapy approach, which utilizes a liposomal carrier to simultaneously trigger immunogenic cell death (ICD) as well as interfere in the regionally overexpressed immunosuppressive effect of indoleamine 2,3-dioxygenase (IDO-1) at the BC tumor site. The liposome was constructed by self-assembly of a phospholipid-conjugated prodrug, indoximod (IND), which inhibits the IDO-1 pathway, followed by the remote loading of the ICD-inducing chemo drug, doxorubicin (DOX). Intravenous injection of the encapsulated two-drug combination dramatically improved the pharmacokinetics and tumor drug concentrations of DOX and IND in an orthotopic 4T1 tumor model in syngeneic mice. Delivery of a threshold ICD stimulus resulted in the uptake of dying BC cells by dendritic cells, tumor antigen presentation and the activation/recruitment of naïve T-cells. The subsequent activation of perforin- and IFN-γ-releasing cytotoxic T-cells induced robust tumor cell killing at the primary as well as metastatic tumor sites. Immune phenotyping of the tumor tissues confirmed the recruitment of CD8+ cytotoxic T lymphocytes (CTLs), disappearance of Tregs, and an increase in CD8+/FOXP3+ T-cell ratios. Not only does the DOX/IND-Liposome provide a synergistic antitumor response that is superior to a DOX-only liposome, but it also demonstrated that the carrier could be effectively combined with PD-1 blocking antibodies to eradicate lung metastases. All considered, an innovative nano-enabled approach has been established to allow deliberate use of ICD to switch an immune deplete to an immune replete BC microenvironment, allowing further boosting of the response by coadministered IDO inhibitors or immune checkpoint blocking antibodies.

KEYWORDS: immunogenic cell death, indoleamine 2,3-dioxygenase, immune checkpoint, chemo-immunotherapy, dual-delivery liposome, doxorubicin, breast cancer

Although the treatment of localized breast cancer (BC) is highly successful with a 5-year survival rate of ~90%, metastatic breast cancer (MBC) is generally considered incurable with a high mortality rate, regardless of the use of radiation, chemotherapy, or estrogen blockers.1,2 In spite of the bleak picture for MBC, optimism has emerged with the advent of cancer immunotherapy, which utilizes the power of T-cell immunity to treat solid cancers, including BC. This is best exemplified by the use of immune checkpoint blocking antibodies, which have changed the treatment landscape for cancer, such as melanoma, renal cell carcinoma, and non-small cell lung cancer (NSCLC).3–5 However, BC is relatively resistant to treatment with checkpoint inhibitors,5 putatively because of its comparative immune deplete (“cold”) microenvironment, absence of tumor-infiltrating lymphocytes (TILs), and expression of poorly potent cytotoxic T lymphocytes (CTLs) for the initiation of tumor killing.5,7 These data are also in agreement with the observation that BC tumors tend to express a low burden of nonsynonymous DNA mutations, which serve as the tumor antigen neo-epitopes capable of inducing a robust T-cell response.8,9 This is also compatible with the notion that cancers with a high burden of...
nonsynonymous mutations are the most responsive to immune checkpoint inhibitors.\textsuperscript{10,11} In addition to its “cold” immune status, the strong immune suppressive micromilieu in the BC tumor site prevents effective T-cell priming.\textsuperscript{12,13} This includes a contribution from a number of immune suppressive mechanisms that are even more broadly oppressive than the checkpoint receptors being targeted by checkpoint blocking antibodies.\textsuperscript{6,7}

In addition to the utility of immune checkpoint inhibitors to initiate immunotherapy, chemotherapy can positively impact the immune system, leading to increased density of TILs at the BC tumor site, which correlates with improved disease prognosis.\textsuperscript{14--16} In this regard, Denkert \textit{et al.} demonstrated in a large BC study that the density of TILs predicts pathological complete responses (pCRs) to neoadjuvant chemotherapy, including during treatment with docetaxel, DOX, and cyclophosphamide.\textsuperscript{14} This is particularly evident in epidermal growth factor receptor 2-positive and triple negative (TNBC) disease.\textsuperscript{15} Moreover, neoadjuvant therapy with anthracycline drugs has demonstrated that an increase in the ratio of tumor-infiltrating CD8\textsuperscript{+} CTLs versus FOXP3\textsuperscript{+} regulatory T-cells correlates with the elimination of hyperploid BC cells in post-treatment biopsy specimens.\textsuperscript{17,18} Because the presence of activated CTLs is accompanied by IFN-\textgamma\ production, which controls PD-L1 at the site of immune responsive cancers,\textsuperscript{19} it is noteworthy that PD-1 or PD-L1 receptor blocking antibodies could elicit significant objective response rates (~20\%) in TNBC or HER2\textsuperscript{−}/ER\textsuperscript{−} breast cancer tumors expressing >1\% PD-L1 on the tumor cell surface.\textsuperscript{20}

In light of above observations, it is rational to ask whether the deliberate application of chemotherapeutic agents can reproducibly prime the immune response at the BC tumor site as a prelude to a practical approach for boosting immunotherapy to the immune checkpoint inhibitors. One possible approach is the use of chemo agents to induce immunogenic cell death (ICD) at the tumor site. ICD is a specialized form of tumor cell death that can be triggered by specific chemotherapeutic agents such as anthracyclines, taxanes, and oxaliplatin.\textsuperscript{21--23} ICD facilitates tumor antigen cross-presentation in dendritic cells as a result of calreticulin (CRT) expression on the dying tumor cell surface (Figure 1).\textsuperscript{21} CRT provides an “eat-me” signal for dendritic cell uptake via the CD91 receptor.\textsuperscript{24--27} Moreover, the delayed release of adjuvant stimuli, such as high mobility group box 1 protein (HMGB1; a TLR4 ligand) and ATP (a danger signal that activates the NLRP3 inflammasome), provides additional stimuli for dendritic cell maturation and the ability to present tumor antigens to naïve T-cells.\textsuperscript{21,22,28--31} ICD provides a deliberate means of triggering TIL recruitment prior to response boosting by additional immune modulators, including antibodies that bind immune checkpoint receptors or metabolic immune surveillance pathways that prevent effective T-cell priming.\textsuperscript{32} An important example is the indoleamine 2,3-dioxygenase (IDO-1) pathway that is overexpressed at the BC tumor site.\textsuperscript{13}

How can ICD be used to invoke an orchestrated anti-BC immune response? Doxorubicin (DOX), an anthracycline agent, is a potent ICD inducer in addition to serving as a first-
line chemotherapeutic drug for BC.\textsuperscript{26,34} It should be considered, however, that intravenous (IV) DOX administration is accompanied by off-target toxicity (e.g., of the heart and liver) and that the free drug has a relatively short circulatory half-life ($t_{1/2}$), which limits tumor drug uptake.\textsuperscript{35–37} This could explain the poor potency or failure of free DOX to induce ICD at the BC tumor site in our preliminary animal studies (see later). To improve the pharmacokinetics (PK) for clinical use, encapsulated DOX delivery (e.g., the PEGylated liposomal DOX formulation, Doxil) has been successfully employed for the treatment of AIDS-related Kaposi’s sarcoma, recurrent ovarian carcinoma, MBC, and multiple myeloma.\textsuperscript{38–40} Moreover, our own preliminary data indicate that DOX encapsulation by an in-house liposome can effectively induce an ICD response at the tumor site in an orthotopic animal model (see later). We have also demonstrated in an orthotopic pancreas cancer (PC) model that it is possible to provide an effective ICD stimulus by using a mesoporous silica nanoparticle (MSNP) for delivery of oxaliplatin, which as a free drug was incapable of triggering an immunogenic response \textit{in vivo}.\textsuperscript{22} All considered, these findings suggest that the use of a nanocarrier to improve the PK and tumor drug concentration of ICD-inducing chemotherapeutic agents could provide an effective means for initiating chemo-immunotherapy, which will be difficult to achieve on a reproducible basis by free drugs. Another potential advantage of using a nanocarrier is the ability to co-deliver synergistic drug combinations for improving treatment efficacy, as demonstrated in the PC tumor model, where contemporaneous delivery of 1-methyl-D-tryptophan (aka indoximod, IND) and oxaliplatin by the MSNP carrier triggered a synergistic immunotherapy response.\textsuperscript{22} Not only did the indoximod strongly synergize with oxaliplatin in calreticulin expression, but it also provided effective interference in the IDO-1 immune suppressive pathway, which is regionally overexpressed at the PC tumor site.\textsuperscript{22} The immunosuppressive effects of IDO-1 is due to its enzymatic conversion of tryptophan to kynurenine, resulting in tryptophan insufficiency, which interferes in the mTOR pathway or activation of the serine/threonine-protein kinase, GCN2 (general control nonderepressible), or kynurenine in excess, which activates the aryl hydrocarbon receptor (AhR) pathway.\textsuperscript{41,42} A possible basis for the synergy between ICD induction and interference in the IDO-1 pathway could be there closely linked paracrine relationship in the tumor microenvironment. Collectively, these effects interfere in activation of cytotoxic T cells, accumulation of Tregs and an overall immune suppressive outcome at the site of the regional tumor and draining lymph nodes. There is currently a strong

Figure 2. Use of a vaccination approach to identify chemo agents that induce ICD in a BC model. Published consensus guidelines were used to identify effective ICD introducing chemotherapy agents by a combination of \textit{in vitro} 4T1 screening, followed by use of the dying tumor cells for a tumor vaccination procedure in syngeneic Balb/c mice.\textsuperscript{45} Multiparameter \textit{in vitro} screening showed that doxorubicin (DOX) and paclitaxel (PTX), but not cisplatin (CIS), induced surface expression of CRT on 4T1 cells in a dose-dependent fashion, as well as quantifiable HMGB1 and ATP release (Figure S1B–D). (A) Animal vaccination, using 2 rounds of subcutaneous (SC) injection of dying 4T1 cells 7 days apart, followed by SC injection of live cells on the contralateral side. Successful growth inhibition at the challenge site is suggestive of immune interference. (B) Spaghetti plots showing growth inhibition of the tumors in animals vaccinated by dying tumor cells treated with DOX and PTX, but not CIS or PBS ($n = 6$). Evidence for the involvement of the innate and cognitive immune systems in the vaccination experiment appears in Figure S1F–H.
Figure 3. Synthesis of the dual-delivery DOX/IND-Liposome. (A) Schematic to show that the carrier is synthesized by self-assembly of an IND prodrug to form a liposome, which is subsequently loaded with DOX. The synthesis commences by conjugating IND to a single chain phospholipid [1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (PL)] to derive the IND-PL prodrug, as previously described by us.22 The three-step synthesis process is schematically depicted in Figure S3. The prodrug is mixed with cholesterol and DSPE-PEG2K to form a lipid film for the construction of liposomes. (B) Schematic to outline the liposome synthesis steps. Briefly, the lipid film comprised of IND-PL, cholesterol, and DSPE-PEG2K, at a molar ratio of 70:25:5, was hydrated in a (NH₄)₂SO₄ solution, followed by sonication and removal of free (NH₄)₂SO₄. DOX was remotely loaded by using a proton gradient, as shown in the bottom panel. (NH₄)₂SO₄ dissociates into protons,
NH₄ and SO₄²⁻. DOX is a weak basic molecule that is capable of diffusing across the IND-PL lipid bilayer into the liposome, where it is converted to a (DOX-NH₃)₂SO₄ precipitate, incapable of back diffusion across the lipid bilayer. (C) Side-by-side comparison of DOX/IND-Liposome and Dox-NP for drug loading capacity, size, polydispersity, charge, and endotoxin levels (n = 3). (D) CryoEM pictures to show the morphological similarity between Dox-NP and IND-Liposome, including the presence of the drug precipitate.

interest inIDO-1 inhibitors for cancer treatment, including BC. Against this background, we set out to establish whether dual delivery of DOX and an indoximod (IND) prodrug by a liposome can initiate an anti-BC tumor immune response in an orthotopic tumor model. We constructed a phospholipid-conjugated IND prodrug that self-assembles into a lipid bilayer encapsulated nanovesicle or liposome. Following remote loading of DOX into the liposome through the use of a proton gradient, the innovative dual-delivery DOX/IND carrier was used to conduct PK, efficacy, and safety studies in a murine orthotopic model that resembles human triple negative breast cancer (TNBC). The ensuing innate and cognitive immune response dramatically reduced primary tumor volume, while also eliminating lung metastases. This treatment effect was further enhanced by treatment with an anti-PD-1 monoclonal antibody (mAb).

RESULTS

Doxorubicin Is an Effective ICD-Inducing Chemotherapy Agent in Breast Cancer. In addition to being considered as a first-line chemotherapeutic for breast cancer, the anthracycline drug, doxorubicin (DOX), is a potent inducer of ICD and therefore potentially useful to induce TILs in the BC tumor microenvironment. In order to provide proof-of-principle testing for the use of ICD in BC immunotherapy, consensus screening guidelines were used to compare the immunogenic effects of DOX with paclitaxel (PTX), cisplatin (CIS), and oxaliplatin (OX) in a 4T1 tumor model. Multiparameter cellular screening to assess cell surface expression of calreticulin (CRT) (cellular stress), ATP release (autophagy), and nuclear disintegration with HMGB1 release demonstrated that DOX, PTX, and OX are effective ICD inducers in 4T1 cells (Figure S1A–D). In contrast, CIS failed to induce the same ICD response parameters. In vivo confirmation of an ICD effect was provided by a vaccination approach in syngeneic Balb/c mice (Figure 2A). This requires subcutaneous injection of dying 4T1 cells exposed for 24 h to DOX (5 μM) or PTX (5 μM) in one flank of the animals on two occasions, 1 week apart. The animals were rechallenged by injection of live 4T1 cells in the contralateral flank 7 days later (Figure 2). This demonstrated that whereas vaccination with DOX- or PTX-treated cells significantly suppress tumor growth at the challenged site, CIS (100 μM) had no effect (Figure 2 and Figure S1E). In addition to the growth inhibitory effect of the vaccination procedure, we confirmed that the effect is immune mediated by demonstrating perforin and granzyme B-mediated cytotoxic T-cell killing at the tumor site, with increased expression of CD8⁺ T-cells and an increase in CD8/Treg ratios (Figure S1F–H).

Synthesis of a Liposomal Carrier for Dual Delivery of DOX and IND. In spite of the promising ex vivo ICD-inducing effects of DOX, our preliminary data demonstrate that IV administration of the free drug failed to induce CRT expression at the site of orthotopic 4T1 tumors (see Figure 9B). As it is known that the encapsulated DOX delivery in PE-glylated liposomes improves the PK and treatment efficacy in malignancies such as Kaposi’s sarcoma, ovarian carcinoma, and MBC, we asked whether it is possible to improve ICD at the BC tumor site through the use of a DOX-only liposome, Dox-NP (identical composition as Doxil for preclinical research use), as well as a dual-delivery liposomal carrier for DOX plus the IDO-1 inhibitor, IND. Contemporaneous targeting of IDO-1 is appropriate in light of its overexpression at the BC tumor site (including 4T1 orthotopic tumors, as shown in Figure S2) and synergy with ICD in a PC model. The first step toward constructing a dual-delivery carrier was the covalent conjugation of IND (1-methyl-p-troptophan or 1-p-MT) to 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphocholine (PL) to form an IND-PL produg, as schematically explained Figure 3A and Figure S3A. IND-PL self-assembles into a liposome (Figure 3A), which is constructed as outlined in Figure 3B. This requires mixing and suspension of IND-PL, cholesterol, and DSPE-PEG3k in an organic solvent, which is evaporated from the bottom of a round-bottom flask to form a uniform lipid film. Various molar ratios of IND-PL, cholesterol, and DSPE-PEG3k were tested to obtain optimal lipid bilayer stability and carrier size (Figure S4). Optimal results were obtained using a molar ratio of 75:20:5 for IND-PL, cholesterol, and DSPE-PEG3k respectively. To accomplish remote DOX loading, the protonating agent, (NH₄)₂SO₄ was introduced on top of the biofilm, followed by probe sonication and purification across a PD-10 column. The (NH₄)₂SO₄-loaded IND-Liposomes were subsequently incubated in a DOX-HCl solution to allow amphiphilic DOX to be imported across the liposomal membrane (Figure 3B, bottom panel). Drug protonation leads to the formation of an intraliposomal (DOX-NH₃)₂SO₄ precipitate, which is incapable of back-diffusion across the lipid bilayer. The combined synthesis process yielded a dual-delivery carrier with IND and DOX loading capacities (w/w) of 19.8 and 11.4%, respectively, as determined by UPLC-MS/MS. This is equivalent to an IND/DOX molar ratio of 4.3:1. Comprehensive liposome characterization demonstrated a carrier of 100 nm (DLS) size, a low polydispersity index, and a slight negative surface charge (Figure 3C). The liposome maintains its stability and size for up to one month in DI water, PBS, and 10% FBS-containing RPMI-1640 (Figure S4B). Noteworthy, ultrastructural viewing of the DOX/IND-Liposome by cryo electron microscopy showed a carrier that morphologically resembles Doxil as well as Dox-NP (Figure 3D), in addition to similarities in their drug loading capacities, size, and charge (Figure 3C). Both formulations had endotoxin levels of <0.1 EU/mL.

Dual-Delivery Liposome Improves the PK of Drug Delivery and Tumor Drug Concentrations in a 4T1 BC Model. The PK and drug biodistribution of the dual-delivery carrier was assessed in an orthotopic BC model. This model was established by injecting luciferase-transfected 4T1 cells into the right second mammary fat pad in Balb/c mice (Figure 4A). The animals develop rapidly growing primary breast
Figure 4. Pharmacokinetics (PK) of drug delivery by the DOX/IND-Liposome compared to Dox-NP in an orthotopic tumor model. (A) Syngeneic orthotopic model was established injecting luciferase-transfected 4T1 cells into the 2nd mammary fat pad of Balb/c mice (left). This is followed by the development of a primary tumor mass that can be viewed by IVIS imaging after 2 weeks. Animal sacrifice and collecting the tumors and organs confirmed the treatment effect on primary tumor mass as well as the presence of metastatic nodules in the lung. After 4 weeks, it was possible to visualize the large primary tumor mass and extensive lung metastases. (B) Drug dose calculations for
Figure 4. continued

the animal studies (first \( n = 2 \), then \( n = 6 \), refer to the Methods section): maximum tolerated dose (MTD) calculations were carried out for the DOX formulations shown, using a NCI protocol.\(^{35}\) (C) IVIS imaging of DOX fluorescence at the 4T1 orthotopic tumor site (\( n = 3 \)). Three animals in each group received free DOX, Dox-NP, and the DOX/IND-Liposome at 5 mg/kg DOX IV.\(^{35–37}\) The mice were sacrificed after 24 h for IVIS imaging. DOX fluorescence intensity was quantified by Living Image software. (D,E) PK and tissue drug distribution in 4T1 orthotopic tumor-bearing mice (\( n = 6 \)), receiving IV injection of free DOX, Dox-NP, and DOX/IND-Liposome at a DOX equivalent dose of 5 mg/kg. Panel D depicts the WinNonlin software calculation of the plasma concentration, expressed as the % of the injected DOX dose at the indicated time points (left panel). The corresponding tumor and tissue drug concentrations, expressed as % injected DOX dose/g tissue, appears in the right panel. The equivalent data for IND appears in panel E. Results are expressed as mean ± SD; \(*p < 0.01\) (ANOVA).

Tumors that can be detected by IVIS imaging within 2 weeks. This was followed by tumor metastasis to the lung within 30 days postimplantation.\(^{48}\) In order to determine a relevant and safe DOX dose for in vivo use, we first assessed the maximum tolerated dose (MTD) of free DOX, the DOX/IND-Liposome, and Dox-NP by a National Cancer Institute protocol, as described in the Methods section.\(^{49}\) The MTD in Balb/c mice was calculated as 8, 15, and 15 mg/kg for DOX, Dox-NP, and DOX/IND-Liposome, respectively. As Doxil is usually injected as an IV dose of 50 mg/m\(^2\) in patients,\(^{50}\) we used this as the basis to calculate an IV animal dose of 5 mg/kg of DOX in tumor-bearing mice, using dose conversion data.\(^{35–37}\)

As DOX is a fluorescent drug substance, IVIS imaging was used in the first experiment to monitor the drug fluorescence intensity following a single IV dose of free drug, Dox-NP or the dual-delivery carrier at a DOX equivalent dose of 5 mg/kg (\( n = 3 \)) (Figure 4C). Twenty-four hours postinjection, major organs were collected and DOX fluorescence was quantified by Living Image software (PerkinElmer, version 4.5), using an excitation filter of 500 nm and a DsRed emission filter. Compared to the weak fluorescence intensity of free DOX at the tumor site, there was an approximate ~10-fold increase in fluorescence intensity in mice injected with the DOX/IND-Liposome or Dox-NP (Figure 4C). This was followed by a comprehensive PK study, in which orthotopic tumor-bearing mice (\( n = 6 \)) were IV injected with free DOX, Dox-NP, and the DOX/IND-Liposome to deliver a DOX equivalent dose of 5 mg/kg. Blood was withdrawn at predetermined time points (0.083, 0.25, 0.5, 1, 2.5, 8, 24, and 48 h) and the plasma used for quantitation of the IND and DOX content by UPLC-MS/MS. Data calculation with WinNonlin software demonstrated a significant increase in the plasma 1/2 and intratumoral levels of both encapsulated DOX preparations compared to the free drug (Figure 4D). Whereas free DOX was rapidly eliminated from the circulation (t\(_{1/2}\) of <0.083 h) the circulatory t\(_{1/2}\) was increased to ~3 h for Dox-NP and the DOX/IND-Liposome. Drug-dose calculation as a % of the total injected dose, demonstrated that up to ~10 wt % (dose/g tissue) of the encapsulated DOX could be seen to distribute to the 4T1 tumor site by 48 h, as compared to ~0.6% for free DOX (Figure 4D). There was also a significant improvement in the circulatory t\(_{1/2}\) of free IND as a result of liposomal encapsulation (Figure 4D). UPLC-MS/MS measurement of the IND content at the tumor site confirmed an increase from 0.6 to 9.6 wt % for free versus encapsulated drug delivery (Figure 4E).

Effective Tumor Growth Reduction and Inhibition of Metastases by the DOX/IND-Liposome. A comprehensive study was performed to compare the effect of free DOX with the effect of encapsulated DOX on tumor growth in the orthotopic 4T1 model. In addition, we also compared the result to treatment with the checkpoint blocking antibody, anti-PD-1, as well as combining anti-PD-1 with the dual-delivery liposome. The comprehensive array of treatment groups used in a single large experiment is shown in Table 1.

| Treatment groups | Fig. 5 | Fig. 6 | Fig. 7 |
|------------------|-------|-------|-------|
| 1. Saline        | √     |       |       |
| 2. DOX           |       |       |       |
| 3. Dox-NP        |       |       |       |
| 4. IND-Liposome  |       |       |       |
| 5. DOX + IND-Liposome |   |       |       |
| 6. DOX + anti PD-1 |   |       |       |
| 7. DOX/IND-Liposome |   |       |       |
| 8. DOX/IND-Liposome + anti PD-1 |   |       |       |
| 9. DOX/IND-Liposome + anti CD8 |   |       |       |

“We used nine groups of animals (each including nine mice) to perform a comparative efficacy study in the 4T1 model. For ease of description and to prevent data crowding, the data are displayed for three comparative groupings as outlined in Figures 5–7. Figure 5 addresses the synergy of DOX and IND-PL co-delivery in comparison to Dox-NP. Figure 6 shows the effect of combining the anti-PD-1 antibody with DOX or the dual-delivery liposome, and Figure 7 investigates the effect of anti-CD8 antibody on the immune response to the DOX/IND-Liposome. The experiment was repeated once, in which data were displayed to show animal survival.

For ease of data description, we will provide the data analysis as three groupings, which correspond to Figures 5–7. The first data set, outlined in Figure 5A–E compares 1, saline; 2, free DOX; 3, Dox-NP; 4, IND-Liposome (non-DOX-loaded, self-assembled nanovesicle); 5, DOX + IND-Liposome; and 7, DOX/IND-Liposome. The second data set, outlined in Figure 6A–F, compares 1, saline; 6, DOX + anti PD-1; 7, DOX/IND-Liposome; and 8, DOX/IND-Liposome + anti PD-1. The third data set, discussed in Figure 7A–D, compares 1, saline; 7, DOX/IND-Liposome, with or without the injection of an anti-CD8 monoclonal antibody, to determine the effect of CD8 depletion on the immune response. The dose equivalents of DOX and IND were 5 and 8.7 mg/kg based on an optimal construction DOX/IND-Liposome (i.e., ratio 4, Figure S4), respectively, using a total of three injections 3 days apart (Figure 5A). The results in the first comparative grouping demonstrated that the DOX/IND-Liposome was superior to free DOX and
DOX or IND. The inhibition of tumor growth by the DOX/IND-Liposome is significant compared to Dox-NP (*p < 0.05) and other controls (**p < 0.01, ANOVA). (B,C) Representative tumor images and average tumor weights after animal sacrifice on day 22. (D) Representative IVIS imaging and quantification of bioluminescence intensity of lung metastases (*p < 0.05; **p < 0.01, ANOVA). (E) Kaplan–Meier analysis to show that the DOX/IND-Liposome dramatically prolongs animal survival (n = 9, *p < 0.01, Log-rank Mantel-Cox test) in a separate experiment.

Dox-NP (p < 0.05) in shrinking primary tumor size (Figure 5A-C). Moreover, IND-PL enhanced the reduction in tumor size when co-delivered with DOX, but had no effect by itself or when combined with free DOX. Noteworthy, Dox-NP had a statistically significant effect (p < 0.01) compared to that with treatment groups, other than the dual-delivery liposome. Evidence that the reduction in tumor size by encapsulated DOX is due to an immunogenic effect with innate and cognate immune features, is discussed in Figures 8 and 9. In order to determine what effect treatment had on metastatic spread to the lung,48 mice were sacrificed on day 22, and the lungs were harvested for ex vivo IVIS imaging (Figure 5D). Quantitative expression of the imaging intensity demonstrated a highly significant reduction in metastatic spread in response to encapsulated co-delivery of DOX and IND compared to DOX only (Dox-NP). However, Dox-NP did exert a statistically significant effect compared to other treatment groups. A parallel survival study was performed, using nine animals in each group. Kaplan–Meier plots confirmed that the dual-delivery liposome resulted in a significant survival benefit, including in comparison to Dox-NP (Figure 5E).

Antibodies that block the PD-1 and CTLA-4 receptors have been used with great success in soliciting of anticancer immunity in melanoma, NSCLC, and renal carcinoma.51,52 In contrast, the effect of immune checkpoint inhibitors in BC has been disappointing, possibly due to the immune deplete microenvironment in BC, where the absence of TILs as well as the failure to express PD-1 or its ligand may preclude an immune response to checkpoint blocking antibodies.12,13 As ICD may trigger an immune response that can be boosted by treatment with anti-PD-1, an antibody from the anti-PD clone, RMP1-14 (BioXcell), was administered intraperitoneally (IP) at 100 μg/mouse on day 8, 11, and 14. Immunohistochemistry (IHC) confirmed PD-1 expression at the 4T1 BC tumor site (Figure 6A). Assessment of tumor size showed a significant reduction of tumor volume in the animals receiving combined treatment with the DOX/IND-Liposome plus the anti-PD-1 antibody (Figure 6B–D). In addition, there was a total disappearance of lung metastases using the combination therapy (Figure 6E). Strikingly, the addition of the antibody was also responsible for significant further improvement in survival outcome, as demonstrated by Kaplan–Meier analysis (Figure 6F).

DOX/IND-Liposome Induces Synergistic and Effective Innate and Adaptive Anti-BC Immune Responses in an Orthotopic Model. In order to demonstrate the involvement of the immune response in the growth inhibitory effects of the dual-delivery liposome at the primary tumor site, we asked whether the depletion of CD8+ T-cells will affect treatment outcome. An anti-CD8 monoclonal antibody was IP administered 3 days prior to the first treatment with the
Figure 6. Anti-PD-1 coadministration with the DOX/IND-Liposome augments growth inhibition and eradication of lung metastases. Green arrows represent the treatment time points including DOX formulations and/or anti PD-1. (A) IHC staining showing pronounced PD-1 expression in the 4T1 BC tissue. (B) Tumor growth was assessed as in Figure 5, demonstrating that the addition of anti-PD-1 (injected IP at 100 μg/mouse on days 8, 11, and 14) exerted additional growth inhibitory effects (n = 9, **p < 0.05 ANOVA). (C,D) Representative tumor images and tumor weights for the treatment groups. (E) Representative IVIS images and quantitative data to show the complete disappearance of lung metastases in animals receiving coadministration of anti-PD-1. (F) Kaplan–Meier analysis to show prolonged animal survival by anti-PD-1 administration (***p < 0.05, Log-rank Mantel-Cox test) in a separate experiment (n = 9).
Figure 7. Anti-CD8 monoclonal antibody interferes in the antitumor efficacy of the DOX/IND-Liposome. In order to demonstrate the critical role of cytotoxic CD8+ T-lymphocytes in antitumor immunity, anti-CD8 monoclonal was IP injected in treatment group 9, 3 days prior to the first drug administration and repeated every 2–3 days until the termination of the study. (A) Comparative tumor growth inhibition as described in Figure 5 ($n=9$). (B) IVIS imaging data align with the growth inhibitory effects. (C) Representative ex vivo IVIS imaging, with quantification of luciferase expression, to show interference of anti-CD8 on lung metastatic spread. (D) Kaplan–Meier analysis to show that CD8 depletion dramatically reduces animal survival. Results are expressed as mean ± SD ($n=9$, **$p<0.01$, ANOVA).
liposome, and repeated every 2−3 days (group 9, Table 1). When compared to the response in group 7, which was treated with the DOX/IND-Liposome alone, we observed that the depletion of CD8+ T-cells (confirmed by IHC in Figure 8B) could dramatically interfere in the growth inhibitory effects of the liposome at the primary tumor site as well as the disappearance of metastases (Figure 7A−C). Anti-CD8 administration also interfered in survival outcome (Figure 7D). These findings are indicative of the generation of a CTL mediating the anti-BC immune response. Anti-CD8 administration also significantly reduced the immune response to Dox-NP as well as the DOX/IND-Liposome in Figure S6.

In order to corroborate the above findings, extensive further immune phenotyping was performed on tumors harvested from the treatment groups in Table 1, using IHC staining and multiparameter flow cytometry (Figures 8 and 9). Flow cytometry showed that CD8/Treg ratios were markedly increased in DOX/IND-Liposome-treated mice compared to Dox-NP, which in turn, showed significant differences from other treatment groups (Figure 8A). The CD8/Treg ratio in group 7 was significantly enhanced by treatment with anti-PD-1, whereas the administration of anti-CD8 essentially restored the ratio to values seen in the saline treatment group (Figure 8A). These treatment effects were further corroborated by IHC staining for CD8, which clearly demonstrate the appearance of CTLs in response to DOX and IND co-delivery; the numbers were further boosted by the addition of anti-PD-1 (Figure 8B). A diagonally opposite trend was seen during IHC staining for FOXP3 expression, which showed disappearance of Tregs (Figure S5C). The effective induction of a cognate immune response was further corroborated by flow cytometry and IHC results looking at the local production or expression of IFN-γ, granzyme B, perforin, activated caspase 3 and IL12p70 (Figure 8C,D and Figure S5D,E).

Apart from the impact on adaptive immunity, we also looked at innate parameters that reflect the induction of ICD (Figure 9A,B and Figure S5F,H). This was demonstrated by the dramatic increase in CRT expression as well as the levels of its counter receptor, CD91, on DCs in tumor tissue of animals treated by the dual-delivery liposome (Figure 9A,B). Moreover, the response was further enhanced or reduced by treatment with anti-PD-1 or anti-CD8 monoclonal antibodies, respectively, as described above. Similar responses were seen for biomarkers that reflect dendritic cell activation, including the integrin receptor, CD103 (Figure 9A), that is used by DCs to facilitate the CD8+ T-cell development and activation, as well as CD80 and CD86 (Figure S5H). We also obtained evidence for increased expression of microtubule-associated protein 1A/1B-like chain three (LC-3), which is involved in autophagy and responsible for ATP release during the ICD response (Figure S5G). All considered, these results

Figure 8. Immune phenotyping to demonstrate the effect of the DOX/IND-Liposome on initiating adaptive anti-BC immunity. Tumors were harvested from the different animal groups depicted in Table 1 to perform IHC staining and flow cytometry. (A) Multicolor flow cytometry analysis to show the impact on CD8/Treg ratios (n = 9). (B) IHC staining for CD8 expression in tumor tissue sections. (C) Flow cytometry analysis of T-cell IFN-γ expression in a CD45−CD3−CD8− gated cell population; granzyme B+ expression in a CD45−CD3−CD8− gated cell population; (D) IHC staining for perforin expression.

Figure 8. continued

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demonstrate synergy between DOX and IND in generating a robust innate immune response at the tumor site.

As it was not logistically possible to include additional treatment groups in the experiment in Table 1, it was necessary to perform an additional study to assess the comparative effect of anti-PD-1 on the response to Dox-NP (Figure S6). This experiment compared the effect of Dox-NP + anti-PD-1 to treatment with saline, anti-PD-1, Dox-NP, the DOX/IND-Liposome, and DOX/IND + anti-PD-1. We also compared the outcome to the use of anti-CD8 in the Dox-NP and the DOX/IND-Liposome groups (Figure S6). The data demonstrate that whereas both the DOX/IND-Liposome and Dox-NP groups benefited from anti-PD-1 administration, the outcome was significantly enhanced in the former treatment group after the first three 3 IV injections (day 17) (Figure S6A,B). The magnitude of this difference was further boosted by the addition of 2 further IV administrations (up to day 24). The use of flow cytometry to perform immunophenotyping of cells harvested from the tumor site confirmed that the increase in the CD8/Treg ratios was significantly higher during anti-PD-1 co-treatment in the DOX/IND-Liposome compared to the Dox-NP group (Figure S6D). There was no obvious weight loss or change in AST and ALT enzyme levels in response to any of the treatments (Figure S6C,E). In addition, anti-CD8 administration also significantly reduced the immune response to Dox-NP as well as the DOX/IND-Liposome in Figure S6.

It was possible to discern the effective inhibitory effect of IND-PL on the IDO-1 metabolic pathway at the tumor site by conducting Western blotting to show the phosphorylation status of S6 kinase (Figure 9C, left panel). Activated P-S6 kinase plays a role in the mTOR pathway to reverse the immune suppressive effects of IDO-1 (Figure S2). This corroborates similar effects in 4T1 cells, in which IND-PL dramatically increased the intracellular retention of IND, including an exaggerated effect on S6 kinase phosphorylation (Figure S3B,C). We also observed in assessing IL-6 mRNA expression at the tumor site, that the message abundance was significantly decreased in animals receiving either one of the three IND-containing carriers (Figure 9C, right panel). IL-6 is involved in sustaining IDO-1 expression by an integrated IL-6/STAT3/AHR autocrine loop, which is disrupted by IND.22,42 A similar IND-PL effect was also seen in 4T1 cells, where the produg dose-dependently interfered in IL-6 release in the cellular supernatant (Figure S3C).

**Encapsulated DOX Delivery Improves Drug Safety.**

One of the major advantages of the PEGylated liposomal DOX formulation has been the improvement of DOX safety. This was confirmed by assessing biomarkers of cardiac, liver, and kidney toxicity in animals that were treated in 4T1 orthotopic efficacy study. The results show that the co-delivery liposome is associated with normalization of the increased troponin I, creatine kinase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine levels in response to free DOX, similar to Dox-NP (Figure 10).

**DISCUSSION**

We demonstrate a chemo-immunotherapy approach for breast cancer, using a dual-drug delivery liposomal carrier that introduces an immunogenic cell death stimulus while, at the same time also interfering in the regionally overexpressed IDO-1 pathway that prevents effective T-cell priming. Through innovative design of an IND-PL prodrug that self-assembles into a liposome that can be remotely loaded with DOX, we were able to develop a dual-delivery carrier that could improve the pharmacokinetics of both drugs at the tumor site. Their combined action was to reduce the primary tumor growth as well as interfere in tumor metastases in a syngeneic mouse.
The use of chemotherapy to initiate immunotherapy is a logical choice in light of emerging clinical data indicating that neoadjuvant chemotherapy with DOX, docetaxel, cyclophosphamide and taxanes is capable of inducing pathological complete responses due to recruitment of TILs in BC patients.\(^{12,15,53}\) In addition, there are also indications that neoadjuvant chemotherapy may have a survival effect compared to adjuvant chemotherapy or surgery in patients with colon or urothelial cancer.\(^{54,55}\) It is interesting, therefore, that a number of chemotherapeutics, such as the anthracyclines (DOX, daunorubicin, and mitoxantrone), oxaliplatin, cyclophosphamide, and paclitaxel, can induce a programmed cell death response in tumor cells that is immunogenic in nature.\(^{22,28,29,56}\) This ICD response includes the translocation of CRT, which is normally expressed in the endoplasmic reticulum, to the cancer cell surface, where it provides an “eat-me” signal for dendritic cell uptake.\(^{36,57}\) ICD is also accompanied by the release of the alarmin, HMGB1, at a more advanced stage of cell death, with the ability to induce dendritic cell maturation and tumor antigen presentation through binding to TLR-4.\(^{57}\) Moreover, ICD also involves autophagy, which leads to the release of ATP.\(^{58,59}\) ATP serves as a danger signal that triggers a purinergic dendritic cell receptor which leads to assembly of the NLRP3 inflammasome and IL-1β production.\(^{60-63}\)

Although the ICD concept dates back more than a decade, the deliberate use of chemo-immunotherapy has only been pursued in a limited number of animal studies, including in murine lung and colon cancer models.\(^{44,64}\) It is also possible to induce ICD through the use of physicochemical stimuli, such as ionizing radiation or photodynamic therapy.\(^{65}\) Here, we demonstrate the use of a liposomal carrier to induce ICD at an orthotopic BC tumor site, an outcome that is not accomplishable with free DOX (Figure 9B). We ascribe the tumor-associated ICD effect of the nanocarrier.\(^{39}\) Our animal PK data of Dox-NP (Figure 4) agrees with the highly significant improvement in the PK and tumor levels of DOX in clinical Doxil treatment studies.\(^{39,66-68}\) For instance, the clinical PK studies demonstrate an initial DOX distribution phase with a t\(_{1/2}\) of 1−3 h, followed by a second distribution phase with a t\(_{1/2}\) of 20−90 h.\(^{39,66-68}\) Moreover, the “area under the curve” for Doxil delivery in human studies is ~300-fold bigger than the free drug, whereas the clearance and volume of distribution are reduced by factors of 250- and 60-fold, respectively.\(^{39,66-68}\) Although Dox-NP was effective in inducing CRT expression and initiating an effective BC tumor immune response in our animal studies, the efficacy of the response and survival outcome was significantly less than the impact of the DOX/IND-Liposome (Figures 5−9), even in the presence of anti-PD-1. As there was no difference between Dox-NP and the dual-delivery liposome in terms of the PK and intratumor concentrations of DOX, we ascribe the improved tumor growth and metastasis inhibition by the DOX/IND-Liposome to the action of co-delivered IND (Figures 5A,D). Moreover, the PK of IND-PL was also dramatically improved by encapsulated delivery (Figure 4E), with significant accentuation of CD8+ CTL recruitment (Figure 8B), disappearance of Tregs (Figure SSC), and increase in the CD8/Treg ratios compared to Dox-NP (Figure 8A). These findings are
congruent with the demonstration that encapsulated IND-PL delivery effectively interfered IL-6 mRNA expression (Figure 9C, right) in parallel with phosphorylated S6 kinase activation (Figure 9C, left). These response outcomes are indicative of interference in the metabolic action of IDO-1, which is overexpressed at the BC tumor site (Figure S2). Cellular data further confirm that the increased uptake and retention of IND-PL exerts a stronger effect on P-S6 kinase and IL-6 production compared to the free IND (Figure S3B). A report was recently published describing the use of a polymeric carrier for the co-delivery of DOX and NLG919 (another IDO inhibitor). This study demonstrated a redox-responsive immunostimulatory polymeric prodrug micellar carrier for co-delivery of DOX and NLG919 with synergistic antitumor efficacy. However, there was no attempt to study the impact of the treatment on ICD, and this inhibitor (aka GDC-0919) also performed less favorably in two Phase 1 studies.70,71 In contrast, there are currently a total of 17 indoximod clinical studies, according to the NIH database (clinicaltrials.gov).

Although it is clear that the dual-delivery carrier yields superior antitumor immune effects compared to Dox-NP, it is uncertain to what extent the improvement is due to the harmonized PK of the drugs, the regional delivery of IND to the site where IDO-1 is overexpressed or a synergistic mechanism of action (Figures 5–7). A case for synergy can be made insofar as the generation of INF-γ production by ICD-induced cytotoxic T-cells can induce additional IDO-1 expression and PD-1 ligand expression in a paracrine fashion. This will create a paradoxical increase in the metabolic and immune checkpoint receptor-mediated immune suppression at the same site in the tumor microenvironment where ICD is induced. Thus, inhibition of IDO-1 activity at the same site as ICD induction may be necessary for the effects of ICD to be propagated. Even though neoadjuvant chemotherapy is capable of improving TIL recruitment with a complete pathological response the BC tumor site, it is difficult to predict in advance which patients will respond. We propose that the improved delivery of the ICD stimulus by a nanocarrier provides a more predictable approach for the implementation of chemio-immunotherapy in terms of biodistribution, and dosimetry considerations. It is also important to consider that even though ICD-inducing drugs (such as DOX) may induce immunogenic effects at the tumor site, the free drug could exert immunosuppressive effects at systemic level to counter the local outcome. In addition to the use of the DOX/IND nanocarrier, it would also be possible to develop custom-design nanocarriers that deliver taxanes (e.g., paclitaxel, docetaxel) or cyclophosphamide to initiate ICD responses for cancer immunotherapy.45 It is also possible to include small molecule inhibitors of the IDO-1 pathway other than IND, including epacadostat.27–74

Aside from the ability of the DOX/IND-Liposome to serve as a standalone immunotherapy platform for BC, we demonstrate that the antitumor response of the nano-enabled platform can be enhanced by combining the liposome with an anti-PD-1 antibody (Figure 6). This finding could be of significance to a wide range of immune checkpoint inhibitors, which even under the best treatment circumstances (e.g., melanoma and NSCLC), yield response rates of 20–40% only.73–79 Although the exact explanation for the limited response rate is being researched, the current view is that responsiveness to immune checkpoint inhibitors is dependent on the presence of TILs that express immune checkpoint receptors.11,80–83 Moreover, there appears to be a requirement for a high burden of nonsynonymous DNA mutations at the cancer site to develop a hot immune microenvironment.10,11 These mutations give rise to potent neo-epitopes that are presented to T-cells by class I major histocompatibility complex on antigen-presenting dendritic cells.84–86 Although putatively this leads to robust activation of naïve T-cells and the recruitment of TILs,87,88 it is difficult to predict in a population of patients who the responders will be. We propose that the guesswork could be diminished by nanocarriers that introduce ICD stimuli in a predictable manner. Moreover, our study show that interference in the IDO-1 pathway is nonredundant with the effect of the PD-1 checkpoint receptor, allowing the DOX/IND-Liposome to be combined with anti-PD-1, which further boosts the immune response (Figure 6). We propose that the same treatment effect will also be accomplishable with additional immune checkpoint blocking antibodies, and that a combinatorial approach could also benefit other “cold” solid tumors.

An interesting question regarding the use of ICD to improve immunotherapy is the identity of the tumor-associated antigens that are presented to the cognitive immune system. Theoretically, ICD should allow the presentation of mutational as well as nonmutational tumor antigens, which could expand the clonal diversity of the T-cell response. Antigen proof-reading by the T-cell antigen receptors (TCR) could allow the selection of T cells that could mount an effective immune response to nonmutational antigens. Moreover, the expanded repertoire of responding T cells could improve T-cell memory, which is important for a durable immune response that controls disease resurgence and development of metastases.86,89–92 The robustness of the TCR activation response could also have a bearing on the severe immunological side effects that develop as a result of interference in the regulation of activated T cells by checkpoint receptors.73–96 One possibility is that ICD could lead to a lesser tendency toward an over-reactive immune response by involving less potent nonmutational antigens.97,98 This possibility will be investigated in future studies. In addition to the possibility of reducing immunological side effects, the encapsulated delivery of DOX by the dual-delivery liposome was equally effective to Dox-NP in protecting against cardiac, liver, and renal side effects (Figure 10).

In studying a combination of therapies, it is difficult to include a comprehensive series of controls in every experiment due to the logistics and limitation of the number of animals that can be included in a single study. This was illustrated by the necessity to perform a separate experiment (from the data shown in Figures 5–7) to assess the combination of Dox-NP with anti-PD-1 (Figure S6). It was also not possible to independently assess the combination of Dox-NP with IND (either as a free drug or as a separate liposomal form). However, we did demonstrate previously that the combination of chemotherapy (free and encapsulated) with separately administered IND (free and encapsulated) did not achieve the same efficacy as combining the chemotherapy with IND-PL in a single nanocarrier (in pancreas cancer).27

CONCLUSION

In summary, we developed a liposomal chemo-immunotherapy approach for use in BC. The liposome delivers DOX to provide an ICD stimulus as well as an IND prodrug to interfere in the metabolic immunosuppressive effects of the IDO-1
pathway in the tumor microenvironment. IV injection of the DOX/IND-Liposome favorably improved the PK and tumor uptake of both drugs at the tumor site of a syngeneic 4T1 orthotopic BC model. Compared to the DOX-only liposome, Dox-NP, the dual-delivery carrier significantly enhanced the anti-BC immune response at the primary as well as metastatic tumor sites. The response was further augmented by the addition of an anti-PD-1 monoclonal antibody, demonstrating that the potential use of ICD to generate a “hot” or immune replete BC tumor microenvironment to increase the number of responders in immunotherapy studies that use IDO inhibitors or immune checkpoint blocking antibodies. Although the concept of chemotherapy-induced ICD has been described in the literature for a number of years, this concept has not been deliberately pursued as a practical and reproducible immunotherapy principle that can be executed by a FDA-approved drug carrier. Our preclinical data clearly demonstrate the key benefit of dual delivery for an immunological perspective in comparison to free drugs or Dox-NP. This has obvious significance for possible clinical translation in human breast cancer where the use of liposomal carriers has already been approved, without the need to develop a new delivery platform from inception. Our demonstration that such a liposome can be made from a self-assembling, lipid-conjugated IND prodruk also provides significant innovation in how a synergistic drug combination can be accomplished. In summary, we report the innovative use of the triad of liposomal properties that provide: (1) effective co-packaging of an IND prodruk with DOX through self-assembly and drug import; (2) effective regional buildup of DOX and IND at the tumor site, sufficient for concurrent ICD induction and immunological modulation; and (3) a combined in vivo effect, providing interference in primary tumor growth and elimination of tumor metastases.

METHODS

Use of 4T1 Cells To Establish an Orthotopic Tumor Model in Syngeneic Balb/c Mice. The 4T1 cell line was obtained from ATCC and was cultured in complete DMEM, containing 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 mM l-glutamine at 37 °C in a CO₂ incubator. 4T1 represents an aggressive phenotype that is representative of human triple-negative BC. In order to visualize tumor growth by bioluminescence imaging, 4T1 cells were stably transfected with a luciferase expressing lentivirus in the vector core facility at UCLA. A total of 1 × 10⁶ cells in 30 μL DMEM/matrigel, 1/1, v/v, were injected into the right second mammary fat pad of female Balb/c mice (Charles Rivers), 6–8 weeks old (Figure 3). The orthotopic growing tumor, which mimics stage IV breast cancer in humans, leads to the development of lung metastases after 3–4 weeks. The mice were housed under pathogen-free conditions, and all animal experiments were approved by the UCLA Animal Research Committee.

Confirmation of Immunogenic Cell Death (ICD) by Cellular Biomarkers. A total of 1 × 10⁶ 4T1 cells were plated in 24-well plates overnight in complete DMEM medium. The culture medium was replenished with cisplatin (CIS), doxorubicin (DOX), paclitaxel (PTX), and oxaliplatin (OX) at the indicated concentrations for 24 h. One hundred microliters of supernatant from each well was obtained and homogenized in RIPA buffer. The lysates at 12000 rpm for 10 min, equal amounts of protein in the supernatants were loaded onto a 10–20% Tris-glycine gel (Novex gel, Invitrogen) and then transferred to a PVDF membrane. The membrane was blocked with 5% BSA/TBST, before incubation with primary and HRP-conjugated secondary antibodies that recognize S6K, P-S6K, and GAPDH. The blots were developed by soaking in ECL substrate (Thermo Scientific). The intensity of each protein band on the film was quantified by Image J software.

To measure IL-6 mRNA expression by PCR, total RNA was extracted using TRIzol reagent (Invitrogen), treated with DNase I (Ambion), and reverse transcribed using iScript cDNA synthesis kit (BioRad). Quantitative RT-PCR (qPCR) was performed using iQ and SYBR Green detection kits (Bio-Rad, Hercules, CA). Primers were TCC ACG ATT TCC CAG AGA AC (forward); AGT TGC CTT CTT GGG ACT GA (reverse) (Invitrogen). PCR was conducted using a 3 min step at 95 °C, followed by 40 cycles at 94 °C for 15 s, 60 °C for 30 s, and 72 °C for 30 s.

ICD Vaccination Screening in Balb/c Mice. The procedure and experimental timeline are delineated in Figure 2. 4T1 cells were treated with PBS, 100 μM CIS, 5 μM DOX, and 5 μM PTX for 24 h. Cell suspensions were collected to confirm CRT surface expression by flow cytometry. Subsequently, 1 × 10⁶ dying 4T1 cells in 0.1 mL of DMEM were administered on two occasions into the right flank of female Balb/c mice (n = 6), 1 week apart. The same mice received SC injection of healthy 1 × 10⁶ 4T1 cells in 0.1 mL of DMEM/Matrigel, 1/1, v/v in the left flank, 1 week later. Tumor size was closely monitored every 3–4 days by a digital caliper, using the formula: size = width x length x thickness. Mice were sacrificed on day 19, and the tumors were collected for flow cytometry and IHC analysis. The statistical difference between groups was calculated using two-way analysis of variance (SPSS software).

Synthesis of the DOX/IND-Liposome. The stepwise synthesis protocol is outlined in Figure 3B. The process begins with the synthesis of the IND prodruk, which requires IND conjugation to a phospholipid (PL), as described by us. This involves three steps:
(i) protection of the amine group on IND by coupling to di-tert-butyl dicarbonate (Boc anhydride); (ii) conjugating the phospholipid−OH to the IND−COOH by an esterification reaction; (iii) removal of Boc to yield the IND-PL prodrg. Successful synthesis was verified by ESI-MS (Figure S3A). The liposome was constructed by preparing 50 mg of cholesterol, and 5% DSPE-PEG2k (of a lipid mixture in chloroform, containing 75% IND-PL, 20% cholesterol, and 5% DSPE-PEG2k, i.e., molar ratio of 15:4:1). An optimal ratio was determined by experimentation with different lipid mixtures (Figure S5). The chloroform solution was added to the bottom of a 50 mL round-bottom glass flask, followed by rotary evaporation of the solvent. This formed a thin lipid biofilm, which was further dried under a vacuum overnight. The film was subsequently hydrated in 2 mL of (NH₄)₂SO₄ (123 mM) before probe sonication for 30 min, using a 20/15 s on/off cycle at a power output of 32.5 W. The free (NH₄)₂SO₄ was removed over a PD-10 column (Sephax G-25, GE Healthcare) using PBS elution. To obtain uniformly sized liposomes, the suspension was extruded 15 times through a mini-extruder (Avanti Polar Lipids), using a polycarbonate membrane (Avanti Polar Lipids) with 100 nm pore size at 80 °C. To achieve remote DOX loading, the (NH₄)₂SO₄/IND-Liposome solution was incubated with 10 mg/mL DOX for 30 min at 65 °C. A further round of purification was carried out over a PD-10 column to obtain DOX/IND-Liposomes. The liposomes were comprehensively characterized for size, ζ-potential, loading capacity, morphology, and endotoxin level using DLS, UPLC-MS/MS, cryoEM, and the chromogenic LAL assay, respectively. DOX loading capacity was calculated using the reported formula shown below:

\[ \text{weight of encapsulated DOX} = \frac{\text{weight of liposome} + \text{weight of encapsulated DOX}}{100%} \]

An optimal batch was considered as liposomes with an average size ~100 nm, slightly negative charge and stability for at least one month at 4 °C. The liposomes were stored at 4 °C in the dark until use.

**Calculation of Maximum Tolerated Dose (MTD).** The MTDs for free DOX, the DOX/IND-Liposome, and Dox-NP were determined by a National Cancer Institute protocol. The protocol commences with two Balb/c mice receiving IV administration of a C1 dose equivalent of 2.5 mg/kg DOX. This is followed by incremental drug administration, using a dose escalation factor of 1.08 until the death of the animals in <24 h (aka the Cn dose). Subsequently, the Cn-1 dose was used with a 1.15 escalation factor (n = 2) to reach the MTD, which is characterized by the absence of morbidity or mortality. MTD was further corroborated in 6 healthy mice, which received IV injection at the calculated dose. These animals were closely monitored for 15 days, with the stipulation of <15% weight loss without morbidity and mortality.

**Remission of the Pharmacokinetics (PK) and Biodistribution of Free and Encapsulated Drugs.** The experiment was carried out in 4T1 orthotopic tumor bearing mice. To assess the biodistribution of free DOX, Dox-NP, and DOX/IND-Liposome, these formulations were injected IV, at a dose equivalent of 5 mg/kg DOX (n = 3). Twenty-four hours post-administration, mice were sacrificed and ex vivo DOX fluorescence images were obtained for tumor tissue, heart, liver, spleen, lung, and kidneys, using an IVIS imager (excitation filter = 500 nm; emission filter = DsRed). Tissue autofluorescence was established at t = 0, before the drug injections. DOX fluorescence intensity was obtained by Living Image software (PerkinElmer, version 4.5). In the performance of the PK study, tumor-bearing mice (n = 6) received a single IV injection of free DOX, Dox-NP, and the DOX/IND-Liposome at a DOX dose equivalent of 5 mg/kg. At 0.0833, 0.25, 0.5, 1, 2.5, 8, 24, and 48 h post-IV injection, 50 μL of blood was drawn to obtain plasma for assessment of INDO and DOX concentrations by UPLC-MS/MS analysis. In a separate study, tumor tissues, heart, liver, spleen, lung, and kidneys were harvested 24 h post-IV administration and digested for 48 h in methanol to determine INDO and DOX levels by UPLC-MS/MS analysis. To quantify DOX, the following UPLC-MS/MS conditions were used: C18 Column (130 Å, 1.7 μm, 2.1 mm × 50 mm), connected to Waters LCT Premier with ACQUITY UPLC and Auto sampler; gradient elution sequence: (i) 0−4 min, 95% water +5% acetonitrile; (ii) 4−6.5 min, 5% water + 95% acetonitrile; and (iii) 6.5−10 min, 95% water + 5% acetonitrile. The flow rate was 0.4 L/min. The t₁/₂ of DOX formulations was calculated for display as a noncompartmental as well as a two-compartmental model, using WinNolin software.

**Therapeutic Efficacy Studies Using the DOX/IND-Liposome.** These experiments were carried out by injecting luciferase-expressing 4T1 cells in the mammary pad, as described above. The animals were randomly assigned to nine groups (nine animals each) at this stage when the tumor sizes approached 100−150 mm³ (~day 8 postimplantation). One group of animals (group 7) received IV injection with the DOX/IND-Liposome to deliver a dose of 5 mg/kg DOX and 8.7 mg/kg IND on days 8, 11, and 14 (Table 1). Other treatment groups included animals receiving saline or free DOX, Dox-NP, the IND-Liposome only, or free DOX + IND-Liposome at similar doses and administration frequency. We also tested whether intraperitoneal (IP) injection of an antimurine PD-1 monoclonal antibody (mAb clone: RMP1−14, BioXcell) on days 8, 11, and 14 will impact the treatment response to free DOX or the DOX/IND-Liposome. In order to demonstrate the role of cytotoxic T-cells in the antitumor response, we also assessed the effect of IP administration of 200 μg of an anti-CD8α mAb (clone 53−6.72, 200 μg/mouse, BioXcell) in animals receiving injection with the DOX/IND-Liposome. Treatment with the mAb commenced 3 days prior to the first IV injection of the DOX/IND-Liposome, and was repeated every 2−3 days until the end of the study. CD8 T-cell depletion was confirmed by immunohistochemistry (IHC).

Tumor development was carefully monitored by a digital caliper on days 8, 11, 14, 17, and 22. Additionally, we visualized the tumor burden by IVIS imaging, following injection of 75 mg/kg D-Luciferin IP. We also assessed the tumor weight after tissue harvesting from euthanized animals. This included the collection of lung tissue for ex vivo imaging of metastatic spread, which was quantified by bioluminescence intensity in the region of interest, using Living Image software (PerkinElmer, version 4.5). The tumor tissues were also subdivided for performance of flow cytometry, IHC analysis, Western blotting and RT-PCR for IL-6 mRNA. Blood was withdrawn for the measurement of cardiac enzymes (troponin I and creatine kinase), liver enzymes (ALT and AST), and creatinine levels (renal function). In order to determine survival outcome, the animal efficacy study was repeated in a separate study, using the same treatment groups (n = 9) and procedures. The survival data were displayed as Kaplan−Meier plots. The criteria for animal sacrifice during Kaplan−Meier analysis included animal death or moribund status. Moribund status, as defined by our approved animal protocol, required 15% body weight loss, lack of stool production, and severe dehydration.

**Immunohistochemistry.** In order to visualize the phenotypic changes in the immune system of the treated groups, IHC analysis of tumor slices was undertaken to visualize CD6, FOXP3, C3, CD91, IL1p70, activated caspase 3 (CC-3), LC-3, and IOD-1 expression, as previously described by us. Tumor chunks were fixed in 10% formalin, paraffin embedded, and sliced into 4 μm sections, which were mounted on glass slides in the UCLA Jonsson Comprehensive Cancer Center Translational Pathology Core Laboratory. The slides were deparaffinized and incubated in 3% methanol−hydrogen peroxide, prior to immersing in 1 mM sodium citrate (pH 6) or 10 mM EDTA (pH 8) at 95 °C in Decloaking NxGen Chambers (Biocare Medical, DC2012). Following rinsing in PBS containing 0.05% Tween-20 (PBST), the slides were overlaid with the different primary antibodies for 1 h, followed by addition of the corresponding HRP-conjugated secondary antibodies at room temperature for 30 min. For visualization of different immune cells, the slides were incubated with V龛 Fast Red Chromogen Kit 2 (Biocare Medical, FR605) or DAB (3,3’-diaminobenzidine). After being rinsed in tap water, the slides were counterstained by Harris’ hematoxylin, dehydrated in ethanol, and mounted with media prior to scanning in an Aperio AT Turbo digital pathology scanner (Leica Biosystems).
For PD-1 staining, slides were baked in 65 °C oven for 1 h and were then deparaffinized in xylene and rehydrated through graded ethanol to water. Heat-induced epitope retrieval was performed in a pressure cooker with high pH buffer (Leica Bond ER2 retrieval solution). Slides were cooled and washed with Leica Bond wash buffer and loaded onto Shandon Sequenza staining system. Leica Bond protein block was applied to slides for 5 min, and then primary anti-mouse PD-1 antibody was applied and incubated in refrigerator overnight. In the morning, slides were rinsed in Bond Wash buffer, and the remaining detection steps were performed on the Leica Bond III using bond refine detection reagents (anti-rabbit polymer, H2O2, quenching, DAB, and hematoxylin). Slides were then dehydrated through ethanol to xylene and coverslipped, and subsequently scanned by Aperio AT Turbo digital pathology scanner (Leica Biosystems). The slides were read by a veterinary pathologist. The following reagents were used. Primary antibodies: anti-CD3 (4093, BioLegend) for 20 min. Multiparameter staining was performed by utilizing di-Fluor 647 (ab195568, 1/250) were from Abcam. CD3-APC-APC (505810, 1/100) were from BioLegend. LRP1 (CD91)-Alexa Fluor 647 (ab557659, 1/100), and CD11c-V450 (560521, 1/100) were from BD Biosciences. To block nonspecific binding, cell suspensions were incubated with FcBlock (TruStain fcX anti-mouse CD16/32, clone 93, BioLegend) for 20 min. Multiparameter staining was performed by utilizing di-Fluor 647 (ab195568, 1/250) were from Abcam. Anti-mouse antibodies sources are as follows: CD45-V450 (#561095, 1/100), CD4-Alexa Fluor 488 (#14-0808, 1/100), and anti-FOXP3 (#13-5773, 1/200) were from eBioscience; anti-CRT (ab2907, 1/50), anti-LRP1(CD91) (ab92544, 1/50), antiperforin (ab16074, 1/100), and anti-PD-1 (ab21421, 1/1000) were from Abcam; anticleaved caspase 3 antibody (#9664, 1/200) was from Cell Signaling; anti-IFN-γ (NBPI-19761, 1/200) and anti-IL12p70 (NBPI-85564, 1/100) were from Novus Biologicals; anti-NC-3 (0231-100/LC3-5F10, 1/100) was from Nanotools; anti-IDO (#122402, 1/100) was from BioLegend. Secondary antibodies: Biomarkers were detected by a HRP-labeled polymeric anti-rabbit antibody (Dako, K4003), with the exception of CD91, which were visualized by a MACH2 rabbit AP-polymorphic antibody (Biocare Medical, RALP525).

Flow Cytometry. Multi parameter staining for cell suspensions was performed as published previously. Briefly, tumors collected during the vaccination and orthotopic engraftment studies were cut into small pieces, followed by digestion in collagenase type I (0.5 mg/mL, Worthington Biol Corporation) in a benchtop incubating shaker (MaxQ Digital 4450, Thermo Scientific) for 1 h at 37 °C. The digested tissues were meshed twice through a 70-μM cell strainer, and the cell pellets suspended in 5 mL of Ack lysis buffer (Gibco) at 4 °C for 5 min to lyse red blood cells. After centrifugation at 1500 rpm for 5 min, the single cell suspensions were washed twice with cold PBS twice and then resuspended in staining buffer (545656, BD Biosciences). To block nonspecific binding, cell suspensions were incubated with FcBlock (TruStain fcX anti-mouse CD16/32, clone 93, BioLegend) for 20 min. Multi parameter staining was performed by utilizing different combinations of fluorochrome-conjugated antibodies for 40 min at 4 °C. Dead cells were excluded by 7- aminoaincinomycin D (7AAD, Sigma) staining. Doublet cells were excluded based on their forward and side scatter characteristics. The following immune cell subpopulations were investigated, using multichannel gating: (i) CD8+ T cells (CD45+CD3+CD8+CD25+), (ii) Tregs (CD45+CD3+CD4+FOXP3+), (iii) IFN-γ+ T cells (CD45+CD3+CD8+IFN-γ+), (iv) granzyme B+ T cells (CD45+CD3+CD8+granzyme B+), (v) CD91+ DC-like cells (CD45+CD11b+CD11c+CD91+), (vi) CD80+/CD86+ DCs (CD45+CD11c+CD80+CD86+), and (vii) CD103+ DCs (CD45+CD11b+CD11c+CD103+). Anti-mouse antibodies sources are as follows: CD45-V450 (#560501, 1/100), CD45-APC-Cy7 (#557665, 1/100), CD4-Alexa Fluor 488 (#557667, 1/100), FOXP3-PE (#563101, 1/100), CD8α-PE (#561095, 1/100), CD11b-PE (#553311, 1/100), and CD11c-VE450 (#560521, 1/100) were from BD Biosciences; CD103-Alexa Fluor 647 (#121410, 1/250) and IFN-γ-APC (505810, 1/100) were from BioLegend. LRP1 (CD91)-Alexa Fluor 647 (ab195568, 1/250) were from Abcam. CD3-APC-eFluor780 (#47-0032-82, 1/100), CD25-APC (#17-0251-82, 1/100) and granzyme B-eFluor 660 (50-8898-82, 1/100) were from eBioscience. For intracellular staining of FOXP3, IFN-γ, and granzyme B cells, they were fixed and permeabilized using a Staining Buffer Set (00-5523-00, eBioscience) followed by PBS washing prior to conducting flow cytometry in a LSRII (BD Biosciences). The data were plotted as a change in the normalized ratio in the experimental versus the control sample by FlowJo software (Tree Star).

Statistical Analysis. Differences among groups were estimated by the analysis of variance (ANOVA); Kaplan–Meier survival curves were compared using the Log-rank Mantel-Cox test (version 23, SPSS). Results were presented as mean ± standard deviation (SD), representing at least three independent experiments. Statistical significance was set at *p < 0.05; **p < 0.01; ***p < 0.001, as indicated in the figure legends.

ASSOCIATED CONTENT

# Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsnano.8b05189.

Additional figures, table, and results as described in the text (PDF)

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**Notes**
The authors declare the following competing financial interest(s): Andre E. Nel and Huan Meng are co-founders and equity holders in Westwood Biosciences Inc. The remaining authors declare no conflict of interest.

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**REFERENCES**
(1) Edwards, B. K.; Ward, E.; Kohler, B. A.; Eheeman, C.; Zauber, A. G.; Anderson, R. N.; Jamal, A.; Schymura, M. J.; Lantsorp-Vogelaar, I.; Seeff, L. C.; van Ballegooijen, M. C.; Goede, S. L.; Ries, L. A. G. Annual Report to the Nation on the Status of Cancer, 1975–2014. J. Womens Health 2016, 25, 544–573.
(2) Rashid, O. M.; Takabe, K. Does Removal of the Primary Tumor in Metastatic Breast Cancer Improve Survival? J. Womens Health 2010, 116, 544–573.
(3) Pardoll, D. M. The Blockade of Immune Checkpoints in Cancer Immunotherapy. Nat. Rev. Cancer 2012, 12, 252–264.
(4) Sharma, P.; Allison, J. P. The Future of Immune Checkpoint Therapy. Science 2015, 348, 56–61.
(5) Sharma, P.; Wagner, K.; Wolchok, J. D.; Allison, J. P. Novel Cancer Immunotherapy Agents with Survival Benefit: Recent Successes and Next steps. *Rev. Cancer 2011, 11*, 805−812.

(6) Vanderheide, R. H.; Dombek, S. M.; Clark, A. S. Immunotherapy for Breast Cancer: What Are We Missing? *Clin. Cancer Res. 2017, 23*, 2640−2646.

(7) Nagarsheth, N.; Wicha, M. S.; Zou, W. Chemokines in the Cancer Microenvironment and Their Relation in Cancer Immunotherapy. *Nat. Rev. Immunol. 2017, 17*, 559−572.

(8) Stephens, P. J.; Tarpey, P. S.; Davies, H.; Van Loon, P.; Greenman, C.; Wedge, D. C.; Nik-Zainal, S.; Martin, S.; Varela, I.; Bignell, G. R.; Yates, L. R.; Sturmski, M.; Hagemann, I. S.; Miller, C. A.; Ellis, M. J.; Mardis, E. R.; Hansen, T.; Fleming, T. P.; et al. Breast Cancer Neoantigens Can Induce CD8(+ ) T-Cell Responses and Antitumor Immunity. *Cancer Immunol. Immunother. 2015, 5*, S16−523.

(9) Chauvin, F.; Sartore-Bianchi, A.; Chauffert, B.; Fumoleau, P.; Ghiringhelli, F. Natural and Therapy-Induced Immunosurveillance in Breast Cancer. *Annu. Rev. Immunol. 2016, 33*, 1128−1149.

(10) Di Lorenzo, A.; Canedon, C.; Paccioni, V.; Gatta, G.; Rinaldi, S.; Levi, D.; Fedorov, A.; Viale, G.; Montina, I.; et al. Cancer Neoantigens Can Induce CD8(+) T-Cell Responses and Antitumor Immunity. *Cancer Immunol. Immunother. 2015, 5*, S16−523.

(11) Colli, L. M.; Machiela, M. J.; Myers, T. A.; Jessop, L.; Yu, K.; Chirghead, S.; Jaffe, E. L.; et al. Breast Cancer Neoantigens Can Induce CD8(+) T-Cell Responses and Antitumor Immunity. *Cancer Immunol. Immunother. 2015, 5*, S16−523.

(12) Dominguez-Calderon, L.; Collins, C. D.; Ramirez-Valverde, S.; Dominguez, F.; Wixom, B.; et al. Breast Cancer Neoantigens Can Induce CD8(+) T-Cell Responses and Antitumor Immunity. *Cancer Immunol. Immunother. 2015, 5*, S16−523.

(13) Ghiringhelli, F.; Raviola, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(14) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(15) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(16) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(17) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(18) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.

(19) Ghiringhelli, F.; Vicenzi, E.; Zitvogel, L.; Kroemer, G. Autophagy-Dependent Immune Activation Drives Regression of Triple-Negative Breast Cancer. *Nat. Med. 2010, 16*, 1128−1138.
(69) Sun, J. J.; Chen, Y. C.; Huang, Y. X.; Zhao, W. C.; Liu, Y. H.; Venkataramanan, R.; Lu, B. F.; Li, S. Programmable Co-delivery of the Immune Checkpoint Inhibitor NLS919 and Chemotherapeutic Doxorubicin via a Redox-Responsive Immunomodulatory Polymeric Prodrug Carrier. *Acta Pharmacol. Sin.* 2017, 38, 823–834.

(70) National Library of Medicine (US). Identifier NCT02048709. Indoleamine 2,3-Dioxygenase (IDO) Inhibitor in Advanced Solid Tumors. Jan 29, 2014–Feb 6, 2017. Available from https://clinicaltrials.gov/ct2/show/NCT02048709.

(71) National Library of Medicine (US). Identifier NCT02471846. A Study of GDC-0919 and Atezolizumab Combination Treatment in Participants with Locally Advanced or Metastatic Solid Tumors. June 15, 2015–July 30, 2018. Available from https://clinicaltrials.gov/ct2/show/NCT02471846.

(72) Beatty, G. L.; O’Dwyer, P. J.; Clark, J.; Shi, J. G.; Bowman, K. J.; Scherle, P. A.; Newton, R. C.; Schaub, R.; Maleksi, J.; Leopold, L.; Gajewski, T. F. First-in-Human Phase I Study of the Oral Inhibitor of Indoleamine 2,3-Dioxygenase-1 Epacadostat (INCB024360) in Patients with Advanced Solid Malignancies. *Clin. Cancer Res.* 2017, 23, 3269–3276.

(73) Dhiman, V.; Giri, K. K.; S. P.; Zainuddin, M.; Rajagopal, S.; Mullangi, R. Determination of Epacadostat, a Novel IDO1 Inhibitor in Mouse Plasma by LC-MS/MS and Its Application to a Pharmacokinetic Study in Mice. *Biomed. Chromatogr.* 2017, 31, e3794.

(74) Yue, E. W.; Sparks, R.; Polam, P.; Modi, D.; Douty, B.; Wayland, B.; Glass, B.; Takvorian, A.; Glenn, J.; Zhui, W.; Bower, M.; Liu, X.; Leffet, L.; Wang, Q.; Bowman, K. J.; Hansbury, M. J.; Wei, M.; Li, Y.; Wynn, R.; Burn, T. C.; et al. INCB024360 (Epacadostat), a Highly Potent and Selective Indoleamine-2,3-Dioxygenase 1 (IDO1) Inhibitor for Immuno-oncology. *ACS Med. Chem. Lett.* 2017, 8, 486–491.

(75) Dang, T. O.; Ogunniyi, A.; Barbee, M. S.; Drilon, A. Pembrolizumab for the Treatment of PD-L1 Positive Advanced or Metastatic Non-Small Cell Lung Cancer. *Expert Rev. Anticancer Ther.* 2016, 16, 13–20.

(76) Emens, L. A.; Asciento, P. A.; Darcy, P. K.; Demaria, S.; Eggermont, A. M. M.; Redmond, W. L.; Seliger, B.; Marincola, F. M. Cancer Immunotherapy: Opportunities and Challenges in the Rapidly Evolving Clinical Landscape. *Eur. J. Cancer* 2017, 81, 116–129.

(77) Harris, S. J.; Brown, J.; Lopes, J.; Yap, T. A.; et al. Immuno-Oncology Combinations: Raising the Tail of the Survival Curve. *Cancer Biol. Med.* 2016, 13, 171–193.

(78) Jacquelt, N.; Roberti, M. P.; Enot, D. P.; Lu, J. F.; Tjulandin, S.; Ternes, N.; Jégou, S.; Woods, D. M.; Sodre, A. L.; Hansen, M.; Hollmann, T. J.; Bruggeman, C.; Kannan, K.; Li, Y.; Elipenahli, C.; Liu, C.; Habison, C. T.; Wang, L.; Ribas, A.; Bolis, A.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* 2014, 371, 2189–2199.

(79) Wang, T. L.; Lenschow, D. J.; Bakker, C. Y.; Linsley, P. S.; Freeman, G. J.; Green, J. M.; Thompson, C. B.; Bluestone, J. A. CTLA-4 Can Function as a Negative Regulator of T Cell Activation. *Immunity* 1999, 1, 405–413.

(80) Nishimura, H.; Nose, M.; Hiai, H.; Minato, N.; Honjo, T. Development of Lupus-like Autoimmune Diseases by Disruption of CTLA-4. *Cell* 1994, 77, 405–413.

(81) Makarov, V.; Havel, J. J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T. S.; Hollmann, T. J.; Bruggeman, C.; Kannan, K.; Li, Y.; Elipenahli, C.; Liu, C.; Habison, C. T.; Wang, L.; Ribas, A.; Bolis, A.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* 2014, 371, 2189–2199.

(82) Wang, T. L.; Lenschow, D. J.; Bakker, C. Y.; Linsley, P. S.; Freeman, G. J.; Green, J. M.; Thompson, C. B.; Bluestone, J. A. CTLA-4 Can Function as a Negative Regulator of T Cell Activation. *Immunity* 1999, 1, 405–413.

(83) Nishimura, H.; Nose, M.; Hiai, H.; Minato, N.; Honjo, T. Development of Lupus-like Autoimmune Diseases by Disruption of the PD-1 Gene Encoding an ITIM Motif-Carrying Immunoreceptor. *Immunity* 1999, 11, 141–151.

(84) Makarov, V.; Havel, J. J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T. S.; Hollmann, T. J.; Bruggeman, C.; Kannan, K.; Li, Y.; Elipenahli, C.; Liu, C.; Habison, C. T.; Wang, L.; Ribas, A.; Bolis, A.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* 2014, 371, 2189–2199.

(85) Wang, T. L.; Lenschow, D. J.; Bakker, C. Y.; Linsley, P. S.; Freeman, G. J.; Green, J. M.; Thompson, C. B.; Bluestone, J. A. CTLA-4 Can Function as a Negative Regulator of T Cell Activation. *Immunity* 1999, 1, 405–413.

(86) Rizvi, N. A.; Hellmann, M. D.; Snyder, A.; Kvistborg, P.; Makarov, V.; Havel, J. J.; Lee, W.; Yuan, J.; Wong, P.; Ho, T. S.; Miller, M. L.; Rekhtman, N.; Moreira, A. L.; Ibrahim, F.; Bruggeman, C.; Gasmi, B.; Zappasodi, R.; Maeda, Y.; Sander, C.; Caron, E. B.; et al. Cancer Immunology. Mutational Landscape Determines Sensitivity to PD-1 Blockade in Non-Small Cell Lung Cancer. *Science* 2015, 348, 124, 128–128.

(87) Colmar, J. D.; Philip, R. MHC Class I Antigen Presentation and Implications for Developing a New Generation of Therapeutic Vaccines. *Ther. Adv. Vaccines* 2014, 2, 77–89.

(88) Palucka, K.; Banchereau, J. Dendritic-Cell-Based Therapeutic Cancer Vaccines. *Immunol. Rev.* 2013, 35, 38–48.

(89) McGranahan, N.; Furness, A. J.; Rosenthal, R.; Ramskov, S.; Lyngaa, R.; Saini, S. K.; Jamal-Hanjani, M.; Wilson, G. A.; Birikbak, N. J.; Hiley, C. T.; Watkins, T. B.; Shafi, S.; Murugaesu, N.; Mitter, R.; Akarca, A. U.; Linares, J.; Marafioti, T.; Henry, J. Y.; Van Allen, E. M.; Miao, D.; et al. Clonal Neoantigens Elicit T Cell Immunoreactivity and Sensitivity to Immune Checkpoint Blockade. *Science* 2016, 351, 1463–1469.

(90) Pearce, E. L.; Walsh, M. C.; Cezas, P. J.; Harms, M. G.; Shen, H.; Wang, L. S.; Jones, R. G.; Choi, Y. Enhancing CD8 T-Cell Memory by Modulating Fatty Acid Metabolism. *Nature* 2009, 460, 103–107.

(91) Snyder, A.; Makarov, V.; Merghoub, T.; Yuan, J.; Zaretsky, J. M.; Desrichard, A.; Walsh, L. A.; Postow, M. A.; Wong, P.; Ho, T. S.; Hollmann, T. J.; Bruggeman, C.; Kannan, K.; Li, Y.; Elipenahli, C.; Liu, C.; Habison, C. T.; Wang, L.; Ribas, A.; Wolchok, J. D.; et al. Genetic Basis for Clinical Response to CTLA-4 Blockade in Melanoma. *N. Engl. J. Med.* 2014, 371, 2189–2199.

(92) Galukande, M.; Krishnan, S.; Asea, A. A Mouse Model for Triple-Negative Breast Cancer Tumor-Initiating Cells (TNBC-TICs) Exhibits Similar Aggressive Phenotype to the Human Disease. *BMC Cancer* 2012, 12, 120.
(100) Metz, R.; Rust, S.; Duhadaway, J. B.; Mautino, M. R.; Munn, D. H.; Vahanian, N. N.; Link, C. J.; Prendergast, G. C. IDO Inhibits a Tryptophan Sufficiency Signal that Stimulates mTOR: a Novel IDO Effector Pathway Targeted by D-1-Methyl-Tryptophan. *Oncoimmunology* 2012, 1, 1460–1468.

(101) Lu, J.; Huang, Y.; Zhao, W.; Marquez, R. T.; Meng, X.; Li, J.; Gao, X.; Venkataramanan, R.; Wang, Z.; Li, S. PEG-Derivatized Embelin as a Nanomicellar Carrier for Delivery of Paclitaxel to Breast and Prostate Cancers. *Biomaterials* 2013, 34, 1591–1600.

(102) Huang, Y.; Lu, J.; Gao, X.; Li, J.; Zhao, W.; Sun, M.; Stolz, D. B.; Venkataramanan, R.; Rohan, L. C.; Li, S. PEG-Derivatized Embelin as a Dual Functional Carrier for the Delivery of Paclitaxel. *Bioconjugate Chem.* 2012, 23, 1443–1451.

(103) Mazzucchelli, S.; Ravelli, A.; Gigli, F.; Minoli, M.; Corsi, F.; Ciuffreda, P.; Ottria, R. LC-MS/MS Method Development for Quantification of Doxorubicin and Its Metabolite 13-Hydroxy Doxorubicin in Mice Biological Matrices: Application to a Pharmacaco-Delivery Study. *Biomol. Chromatogr.* 2017, 31, e3863.

(104) Joerger, M.; Huitema, A. D.; Meenhorst, P. L.; Schellens, J. H.; Beijnen, J. H. Pharmacokinetics of Low-Dose Doxorubicin and Metabolites in Patients with AIDS-Related Kaposi Sarcoma. *Cancer Chemother. Pharmacol.* 2005, 55, 488–496.

(105) Lim, Y.-W.; Goh, B.-C.; Wang, L.-Z.; Tan, S.-H.; Chuah, B.; Lim, S.-E.; Iau, P.; Buhari, S.; Chan, C.-W.; Sukri, N.; et al. Pharmacokinetics and Pharmacodynamics of Docetaxel with or Without Ketoconazole Modulation in Chemonaive Breast Cancer Patients. *Ann. Oncol.* 2010, 21, 2175–2182.