Effects of Tumor Microenvironment Heterogeneity on Nanoparticle Disposition and Efficacy in Breast Cancer Tumor Models

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Abstract

Purpose: Tumor cells are surrounded by a complex microenvironment. The purpose of our study was to evaluate the role of heterogeneity of the tumor microenvironment in the variability of nanoparticle (NP) delivery and efficacy.

Experimental Designs: C3(1)-T-Antigen genetically engineered mouse model (C3-TAg) and T11/TP53Null orthotopic syngeneic murine transplant model (T11) representing human breast tumor subtypes basal-like and claudin-low, respectively, were evaluated. For the pharmacokinetic studies, non-liposomal doxorubicin (NL-doxo) or polyethylene glycol tagged (PEGylated) liposomal doxorubicin (PLD) was administered at 6 mg/kg i.v. x1. Area under the concentration versus time curve (AUC) of doxorubicin was calculated. Macrophages, collagen, and the amount of vasculature were assessed by IHC. Chemokines and cytokines were measured by multiplex immunochemistry. NL-doxo or PLD was administered at 6 mg/kg i.v. weekly x6 in efficacy studies. Analyses of intermediary tumor response and overall survival were performed.

Results: Plasma AUC of NL-doxo and PLD encapsulated and released doxorubicin was similar between two models. However, tumor sum total AUC of PLD was 2-fold greater in C3-TAg compared with T11 (P<0.05). T11 tumors showed significantly higher expression of CC chemokine ligand (CCL) 2 and VEGF-a, greater vascular quantity, and decreased expression of VEGF-c compared with C3-TAg (P<0.05). PLD was more efficacious compared with NL-doxo in both models.

Conclusion: The tumor microenvironment and/or tumor cell features of breast cancer affected NP tumor delivery and efficacy, but not the small-molecule drug. Our findings reveal the role of the tumor microenvironment in variability of NP delivery and therapeutic outcomes. Clin Cancer Res; 20(23); 6083–95. ©2014 AACR.

Introduction

There have been major advances in nanotechnology for targeted delivery of pharmacologic agents to sites of disease, such as cancer (1, 2). In oncology, nanoparticle (NP)-based therapies exploit the enhanced permeability and retention (EPR) effect caused by the unique vascular structure of solid tumors (i.e., hypervasculature and defective lymphatic drainage; ref. 3). The EPR effect is a key rationale that advances the use of NPs for treatment of cancer and renders the preferential delivery and accumulation of NPs to the tumor cells (3). This allows nanomedicines to have advantages over conventional medicines including prolonged...
Translational Relevance

Nanoparticle (NP)-based therapy exploits the enhanced permeability and retention effects of the abnormal tumor microenvironment and has proved to selectively target tumor cells and reduce the unwanted toxicities in normal cells. However, only few nanomedicines have been approved and used clinically due to limited impacts on overall survival. Heterogeneity of the tumor microenvironment has been observed within and between human tumor types, but its effects on the variable delivery and efficacy of nanomedicines have not been extensively investigated. In this study, we report that heterogeneous tumor microenvironment and/or tumor cell features correlated with significantly different tumor delivery and efficacy of polyethylene glycol tagged (PEGylated) liposomal doxorubicin, but not doxorubicin, between two intrinsic murine breast tumor subtypes. This finding implicates that profiling of the tumor and the microenvironment and selection of patients with tumors conducive to the NPs are required for the optimal delivery and therapeutic outcomes for NP-based therapy.

circulation, selective delivery of entrapped drug to tumor, and improved therapeutic index (4). However, there is limited data on the heterogeneity and factors affecting the EPR in preclinical tumor models and, especially, solid tumors in patients to correlate these factors with the highly variable clinical responses of NP-based therapies (5).

It has been shown that NPs have immunologic properties that may stimulate or suppress the immune system (5, 6). NPs can interact with immune components in blood, which influences the uptake and clearance by the mononuclear phagocyte system, and potentially biodistribution and delivery to the targeted site such as tumors (7, 8). Previously, we have reported that the variability in the pharmacokinetics (PK) and pharmacodynamics (PD) of nanomedicines, such as Doxil (PEGylated liposomal doxorubicin, PLD) and S-CKD602 (PEGylated liposome of CKD-602, a camptothecin analog), is associated with patient age, gender, and the function of circulating monocytes in plasma of patients with solid tumors (9–11). We also showed a bidirectional interaction between SCKD-602 and circulating monocytes in patients with solid tumors (12). However, these factors may not sufficiently explain the high variability in the PK and PD of NP-based therapies in patients with solid tumors.

Solid tumors are characterized by a complex and unique microenvironment that consists of infiltrating immune cells such as tumor-associated macrophages (TAM), a variety of growth factors, chemokines and cytokines, dense interstitial matrix, and the abnormal blood and lymphatic vascular structure (13, 14). These factors interplay with the tumor cells to modify the tumor microenvironment and promote tumor progression (13, 14). It has been reported that there is intra- and intertumor variability in the tumor cells and the microenvironment that results in the heterogeneity of molecular, pathologic, and clinical features of each tumor type (15–17). Studies have revealed that normal vascular architecture and dense collagen matrix can act as environmental barriers hindering the delivery of NPs and result in suboptimal clinical outcomes (17). Previously, we have also reported that increased tumor delivery and release of CKD-602 from S-CKD602 in SKOV-3 ovarian xenografts compared with A375 melanoma xenografts correlated with increased expression of CD11c-positive dendritic cells (DC) in the tumors, suggesting that the variability in NP tumor disposition may be associated with the phagocytic cells (i.e., DC and TAMs; ref. 18). Given the heterogeneity of the tumor microenvironment between and within human cancer types and its potential impacts on the tumor delivery and therapeutic outcomes of NP-based therapy, it is imperative to evaluate the roles of the microenvironment factors and their interaction with NPs in a systemic manner for the optimal delivery and efficacy of nanomedicines (5, 17).

Genetically engineered mouse models (GEMM) and orthotopic syngeneic murine transplant (OST) have proven to be valuable experimental models for discovery of biomarkers and prediction of chemotherapy responses in human cancers (19, 20). RNA expression profiling of 13 distinct GEMMs of breast cancer identified mouse models that faithfully represent human intrinsic breast tumor subtypes, including Basal-like [C3(1)-T-Antigen (C3-TAg); ref. 21] and Claudin-low [T11/TP53−/− (T11); ref. 22]. No single claudin-low GEMM was found, but orthotopic syngeneic transplantable tumors from a BALB/c TP53 Null mouse was shown to faithfully recapitulate the human claudin-low expression phenotype (22). Basal-like subtype (C3-TAg) is characterized by high expression of basal gene expression features and the proliferation signature (19, 21). The majority of these tumors are clinically estrogen receptor negative, progesterone receptor negative, and HER2-negative (triple negative breast cancer), which leads to lack of validated biologic targets and poor responses to current chemotherapies (19, 21). The majority of claudin-low T11 tumors are also triple-negative breast tumors with poor prognosis but were shown to have lower pathologic complete response rate to anthracycline/taxane-based chemotherapies compared with basal-like tumors (23). Claudin-low T11 tumors exhibited distinguishing gene expression characteristics (i.e., more enriched in immune system responses and endothelial cell–like signature) as well as histologic phenotypes (i.e., endothelial/tube-like morphology and high vascular permeability) compared with basal-like C3-TAg tumors (23, 24).

Here, we used the genomically validated basal-like C3-TAg and claudin-low T11 murine breast tumor models to test our hypothesis that the heterogeneity of the tumor cells and the tumor microenvironment between breast tumor subtypes affect the tumor delivery and therapeutic outcomes of NP. We evaluated PLD (Doxil), which has been used for treatment of metastatic breast cancer and nonliposomal doxorubicin (NL-doxo; Adriamycin) as a
Tumors derived from BALB/c TP53 the tumor size was met (antigen strain of FVB/n carrying a transgene for C3(1)SV40 T-University of North Carolina at Chapel Hill's Institutional 0 to t (AUC0–t) was calculated using the linear up and log The area under the concentration versus time curve from were performed using Phoenix v.6.2 (Pharsight Corp.). Analyses of NL-doxo and PLD in plasma, tumor, and tissues fluorescence (HPLC-FL) assay (18, 26). Noncompartmental PK studies in plasma, tissues, and tumor Doxorubicin concentration was determined using an exiting high-performance liquid chromatography–fluorescence (HPLC-FL) assay (18, 26). Noncompartmental PK analyses of NL-doxo and PLD in plasma, tumor, and tissues were performed using Phoenix v.6.2 (Pharsight Corp.). The area under the concentration versus time curve from 0 to t (AUC0–Ct) was calculated using the linear up and log down rule. IHC for F4/80 (TAM), collagen IV, and CD31 (endothelial cells) was performed as well (27). Further information is described in Supplementary Data. Morphometric quantitation of macrophages, collagen, and microvessel density Whole tumor images were captured using an Aperio slide scanner at the UNC Translational Pathology Laboratory. F4/80+ macrophages were classified into three groups: (i) peritumoral/peripheral—capsule, (ii) intratumoral—viable, and (iii) intratumoral—necrotic (Supplementary Fig. S1; refs. 28, 29). For scoring collagen IV semiquantitatively, H-scores were generated by the Aperio color deconvolution methods. Microvessel density (MVD) was calculated by number of vessels divided by stained areas (mm²) using the Definiens Tissue Studio software (30). The physiologic state of the vasculature, including the size and open lumen status, was assessed in representative vascularized areas within tumor (Supplementary Fig. S2). Collagen and MVD were assessed in capsule and viable tumor only. Further information is described in Supplementary Data. Multiplex-bead array assay Mouse Cytokine/Chemokine Magnetic Bead Panel was purchased from Milipore. The 96-well plate kit was customized to measure CC chemokine ligand (CCL) 2, also known as monocyte chemotactic protein-1 (MCP-1), and CC chemokine ligand (CCL) 5, also known as regulated upon activation, normally T-cell expressed and secreted (RANTES), in plasma and tumor and VEGF-a and VEGF-c in tumor (14, 30). Further information is described in Supplementary Data. Efficacy studies Once tumor mass reached 60 to 100 mm³, mice were randomized into treatment groups. NL-doxo and PLD were administered at 6 mg/kg x1 weekly for 6 weeks. Tumors were measured using calipers daily, and tumor volume was calculated as (Volume = [(width)² x length]/2). Treatment outcome was assessed as intermediary tumor volume and tumor growth inhibition (TGI%; ref. 31). TGI was calculated from the following formula: TGI (%) = (1 – T/C) x 100, where T indicates the mean final tumor volume (mm³) of the treatment group and C indicates the mean final tumor volume (mm³) of the control group (31). Survival was monitored individually, and mice were euthanized for tumor ulceration, tumor size of 2 cm in any dimension with single tumor, or 1.3 cm in any dimension with multiple tumors. Statistical analysis Statistical analyses were carried out using SAS v.9.2 and Prism5 software (GraphPad Software, Inc.). Equality of AUC between C3-TAg and T11 models was tested with Nedelman’s modification of the Bailer method for sparse samples, using a two-sample t test (32). Analysis of covariance (ANCOVA) with baseline tumor volume as covariate was performed followed by adjustment for comparator (25). Furthermore, we examined the dynamic modulation of the tumor physiology, such as TAMs, collagen, tumor vasculature, and chemokines, after treatment with each agent in these mouse models. Materials and Methods Treatments PLD was purchased from FormuMax Scientific (Supplementary Table S1) and diluted with 5% dextrose to 1.2 mg/mL before injection. NL-doxo was purchased from Sigma Aldrich and diluted with 0.9% NaCl to 1.2 mg/mL before injection. Animal models In vivo experiments were performed with the approval of University of North Carolina at Chapel Hill’s Institutional Animal Care and Use Committee (IACUC). GEMMs of strain of FVB/n carrying a transgene for C3(1)SV40 T-antigen (C3-TAg) were bred in-house and observed until the tumor size was met (>0.5 cm) in any dimension (21). Tumors derived from BALB/c TP53-/- orthotopic mammary gland transplant line (T11) was transplanted into the inguinal mammary fat pad of 12-week-old wild-type BALB/c mice (The Jackson Laboratory; strain 000651; ref. 19). Mice were housed in the UNC Lineberger Comprehensive Cancer Center’s Mouse Phase I Unit (MP1U) and observed for tumors as per the standard practice (22). Mice were randomized to treatment cohorts, and therapy began once a tumor reached 60 to 100 mm³. PK studies in plasma, tissues, and tumor NL-doxo and PLD were administered at 6 mg/kg (doxorubicin equivalent) i.v. x1 via a tail vein. Mice (n = 3) were euthanized before and at 0.083, 0.5, 1, 3, 6, 24, 48, 72, and 96 hours after administration of each drug. Each blood sample was processed to evaluate encapsulated and released doxorubicin in plasma as described previously (18, 26). In the same mice, liver, spleen, lung, and tumors were collected, preserved by snap freezing, and stored at –80°C. To measure the sum total (encapsulated and released) doxorubicin in tumors and tissue samples, samples were thawed on ice, weighed, and homogenized with pH 7.4 PBS buffer (1 g tissue: 3 mL PBS) using a Precellys (13-RD000) 24 Bead Mill Homogenizer (Omni International, Inc.). Doxorubicin concentration was determined using an existing high-performance liquid chromatography–fluorescence (HPLC-FL) assay (18, 26). Noncompartmental PK analyses of NL-doxo and PLD in plasma, tumor, and tissues were performed using Phoenix v.6.2 (Pharsight Corp.). The area under the concentration versus time curve from 0 to t (AUC0–t) was calculated using the linear up and log down rule. IHC and digital imaging Tumor samples were collected at necropsy, fixed overnight in 10% neutral-buffered formalin, processed routinely, and stained with hematoxylin and eosin (H&E).
multiple comparisons using the Holm test to test intermediary tumor volume among treatment groups (33). Kaplan–Meier (KM) survival curves were plotted, and the difference in overall survival between the groups was analyzed by the log-rank test. P value of less than 0.05 was considered statistically significant. All statistical tests were two sided.

Results
Plasma, tissue, and tumor disposition of NL-doxo and PLD
To predict plasma, tissue, and tumor disposition of NL-doxo and PLD in human basal-like and claudin-low breast tumor subtypes, we performed the PK studies of NL-doxo and PLD in their murine counterparts, C3-TAg and T11 models, respectively. Plasma, tissues, and tumor doxorubicin concentration versus time profiles after administration of NL-doxo or PLD at 6 mg/kg i.v. x1 are presented in Fig. 1. AUC from 0 to 96 hours, a measured index of the total doxorubicin exposure over time, was calculated by noncompartmental PK analysis. PK parameters for both treatments are presented in Supplementary Table S2.

The doxorubicin AUC0–96h in plasma, tissues, and tumors after NL-doxo administration was similar between two models (P > 0.05; Fig. 1A, C–F). In PLD-treated mice, both plasma encapsulated (the drug within the liposomal carrier) and released (active-drug released from the liposomal carrier) doxorubicin AUC0–96h were similar in the

Figure 1. Concentration versus time profiles of doxorubicin after administration of PLD or NL-doxo at 6 mg/kg i.v. x1 via tail vein in plasma (A and B), tumor (C), liver (D), spleen (E), and lung (F) in basal-like C3-TAg and claudin-low T11 breast tumor models. Samples (n = 3 mice at each time point) were obtained at 0.083, 0.5, 1, 3, 6, 24, 48, 72, and 96 hours following PLD or NL-doxo administration. Encapsulated and released doxorubicin after administration of PLD in plasma (B) and sum total (encapsulated and released) doxorubicin in tumor and tissues (C–F) are presented. Each time point is represented as the mean ± SD. * P < 0.05 (AUC0–96h in the C3-TAg model vs. AUC0–96h in the T11 model). Equality of AUC was tested using Nedelman’s modification of the Bailer method for sparse samples, using a two-sample test (32). LLOQ for encapsulated doxorubicin: 300 ng/mL, released doxorubicin: 10 ng/mL, and sum total doxorubicin in tissue: 10 ng/g. LLOQ, lower limit of quantification.
two models (Fig. 1B). However, after PLD administration, the sum total (encapsulated + released) doxorubicin AUC0–96 h in tumors was 2-fold greater in C3-TAg compared with T11 (480 ± 71 vs. 210 ± 30 µg·h/g, respectively; P = 0.012; Fig. 1C and Supplementary Table S2). The doxorubicin accumulation in the liver was 1.5-fold higher in T11 compared with C3-TAg after PLD administration (687 ± 61 vs. 438 ± 18 µg·h/g, respectively; P = 0.002; Fig. 1D). The doxorubicin accumulation in the spleen and the lung was similar between C3-TAg and T11 after NL-doxo and PLD administration (Fig. 1E and F). These data suggest that heterogeneity of the tumor microenvironment and/or tumor cell features between C3-TAg and T11 models affected PLD tumor delivery and distribution to the liver, but not NL-doxo.

**TAMs**

To evaluate the effects of TAMs on the delivery of NL-doxo and PLD and characterize the interaction with these drugs, C3-TAg tumors and T11 tumors from the PK studies were stained for F4/80 (Fig. 2). At baseline, most TAMs were located in the hypoxic necrotic area and the periphery (capsule) with lower numbers present in viable tumor in both models (Supplementary Table S3). There was no significant difference in the baseline level of TAMs in all subregions between two models.

However, the time profile of TAMs over 96 hours was distinguished between after NL-doxo and PLD administration (Fig. 3). The changes in the TAM infiltration after NL-doxo administration were inconsistent between C3-TAg and T11 models (Fig. 3A and C). In contrast, after PLD, a nadir occurred at 24 hours followed by TAM infiltration in both models (Fig. 3B and D). The percent decrease at nadir (24 hours) in TAMs localized in the viable tumors was greater in T11 compared with C3-TAg (37.2% vs. 6.6%, P > 0.05; Fig. 3B and D). The AUC0–96 h of F4/80 H-score, an indicator of total influx of TAMs, was similar between C3-TAg and T11 models after administration of NL-doxo and PLD (Supplementary Table S3).

**CCL2 and CCL5 in tumors and plasma**

CCL2 and CCL5 are overexpressed in human and murine breast tumors and play a central role in mobilization of monocytes into tumors and differentiation into TAMs (14, 34). To evaluate whether these chemokines are associated with tumor delivery of PLD or NL-doxo in vivo, we measured intratumoral concentrations of CCL2 and CCL5...
Baseline CCL2 concentration was 5-fold higher in T11 tumors than C3-TAg tumors \([18 \pm 1.5 \text{ vs. } 3.8 \pm 0.5 \text{ ng/g: mean } \pm \text{ SEM (n = 4 for each model), respectively; } P < 0.0001; \text{ Fig. 4A})\]. CCL2 concentration in tumors increased over 96 hours to a greater extent after PLD compared with NL-doxo in both models (Fig. 4A). Interestingly, it was noted that the time profile of CCL2 in tumors resembled the time course of TAMs after NL-doxo and PLD in T11 (Figs. 3C, D, and 4A). In plasma, baseline CCL2 concentrations were 2-fold higher in T11 compared with C3-TAg (148 ± 49 vs. 74 ± 8 ng/mL, respectively; \(P = 0.19\); Fig. 4B). Plasma CCL2 concentration was significantly increased at 96 hours after PLD in the C3-TAg model \((P = 0.023)\), but little was changed in the T11 model (Fig. 4B).

In contrast to CCL2, there was no difference in baseline CCL5 tumor concentrations between two models \((0.5 \pm 0.1 \text{ vs. } 0.4 \pm 0.1 \text{ ng/g: mean } \pm \text{ SEM; Fig. 4C})\). After PLD administration, there was a noticeable increase in CCL5 tumor concentrations at 96 hours in the T11 model \((P = 0.002)\), but a high variability was observed at 96 hours in the C3-TAg model \((P = 0.24; \text{ Fig. 4C})\). In plasma, baseline CCL5 concentrations were similar between two models. Little change was observed in plasma CCL5 concentration after PLD or NL-doxo administration in both models (Fig. 4D).

Next, we assessed the total amount of chemokine produced in tumors after PLD or NL-doxo administration by calculating the AUC from 0 to 96 hours, a measured index of the total amount of a chemokine produced over time. CCL2 AUC\(_{0-96h}\) was significantly greater in T11 tumors compared with C3-TAg tumors after PLD \((707 \pm 119 \text{ vs. } 2,332 \pm 235 \text{ ng h/g: mean } \pm \text{ SEM; } P = 0.0008)\) and NL-doxo administration \((406 \pm 76 \text{ vs. } 1,505 \pm 218 \text{ ng h/g; } P = 0.003; \text{ Fig. 4A})\). However, there was no difference in plasma CCL2 AUC\(_{0-96h}\) plasma CCL5 AUC\(_{0-96h}\) and tumor CCL5 AUC\(_{0-96h}\) between two models (Fig. 4B–D). Together, these data indicate that there was an inverse relationship between the levels of intratumoral CCL2 expression and tumor delivery of PLD. Moreover, PLD showed to induce CCL2 expression in tumors to a greater extent than NL-doxo, and the increase was more pronounced in the T11 model compared with the C3-TAg model.

Collagen and the vasculature in tumors

To assess the effects of tumor physiology on the tumor delivery of NL-doxo and PLD, tumors at baseline were stained for extracellular collagen matrix (collagen IV) and the quantity and physiologic state of the vasculature (CD31; Fig. 2). We also evaluated the changes from baseline at 96 hours after NL-doxo or PLD administration to characterize the interaction between the drugs and these factors.
The H-score of collagen at baseline was 1.5-fold greater in C3-TAg than T11 (82 ± 13 vs. 53 ± 24, respectively; P > 0.05). Collagen expression tended to modestly increase after administration of NL-doxo and PLD in both models except in T11 tumors treated with NL-doxo (P > 0.05; Supplementary Fig. S3).

The baseline vascular quantity measured by MVD score was significantly greater in T11 tumors compared with C3-TAg tumors (933 ± 65 vs. 691 ± 67: mean MVD ± SEM, respectively; P = 0.04; Fig. 5A). Most of the blood vessels identified were small (~87%), followed by medium (12%) and large (1%) in both models (Supplementary Fig. S4). In addition, a significantly greater number of blood vessels in T11 tumors were shown to have open lumen compared with C3-TAg tumors (P = 0.01), indicating hyperperfusion and hyperpermeability of the vasculature in T11 tumors (Fig. 5B). After administration of NL-doxo or PLD, the vascular quantity in C3-TAg tumors did not change over time (Fig. 5C); however, there was a 31% decrease in MVD score from baseline to 96 hours after PLD in T11 tumors (933 ± 65 at 0 hours vs. 555 ± 69 at 96 hours: mean MVD ± SEM; P > 0.05; Fig. 5D).

VEGFs in tumors

VEGF-a and VEGF-c are known for their central role in angiogenesis and lymphangiogenesis in the tumor microenvironment, respectively (30). As these growth factors play a crucial role in determining vascular permeability, lymphatic drainage of fluid from tumors, and EPR effects, intratumoral VEGF-a and VEGF-c were measured to examine the impacts on transvascular transport of NL-doxo and PLD. At baseline, VEGF-a was approximately 7-fold higher in T11 tumors than C3-TAg tumors (11 ± 1.5 vs. 1.6 ng/g; mean ± SEM; P = 0.003; Fig. 5E). VEGF-c was 2-fold higher in C3-TAg tumors compared with T11 tumors at baseline (2.3 ± 0.4 vs. 1.1 ± 0.1 ng/g; P = 0.03; Fig. 5F).

To explore the effects of PLD and NL-doxo on angiogenesis in different breast tumor subtypes, we evaluated the modulation of these proangiogenic factors over time after each drug treatment. Surprisingly, the effects of PLD and
NL-doxo on expression of VEGF-a and VEGF-c appeared to vary with breast tumor subtypes. After PLD administration, VEGF-a in T11 tumors was steadily decreased in T11 but increased in C3-TAg tumors \( (P = 0.02; \text{Fig. 5E}) \). This finding was consistent with the changes seen in the MVD score after PLD administration (Fig. 4E and F). PLD significantly reduced VEGF-c in C3-TAg tumors \( (P = 0.02) \), but elevated it in T11 tumors \( (P = 0.05; \text{Fig. 5F}) \). NL-doxo also...
altered the expression of VEGF-a and VEGF-c in both models, but to a lesser extent compared with PLD (Fig. 5E and F). These results suggest that T11 tumors exhibit hypervascularization and impaired lymphatic functions compared with C3-TAg tumors as demonstrated by higher VEGF-a and lower VEGF-c, which may hamper the tumor delivery of PLD (13, 17). In addition, PLD demonstrated antiangiogenic effects and prolymphangiogenic effects on T11 tumors, which may return the vasculature to more normal phenotype over 96 hours, but not on C3-TAg tumors (17, 35).

Efficacy
To evaluate the effectiveness of PLD or NL-doxo in C3-TAg and T11 breast tumors, we performed the efficacy studies using no treatment (NT), NL-doxo, and PLD. With a null hypothesis of equal tumor growth with a chemotherapeutic agent in two groups of murine breast tumor models, 15 mice per group give 80% power to detect a difference of 1.06 SD at two-sided \( \alpha \) level of 0.05 based on error estimates from previous study results (20). We tested 20 mice per treatment group for each mouse model to have an appropriate power to detect a difference in responses.

Intermediary tumor volume at 21 days for the C3-TAg model and at 14 days for the T11 model was used to quantify the therapeutic responses. The 21-day and 14-day response for each model was chosen as the primary response endpoint based on the fact that 50% of the untreated animals did not survive past 21 days for the C3-TAg model and 14 days for the T11 model. The median and range of survival days of untreated C3-TAg and T11 mice were 20 (12–47) and 15 (14–20), respectively (20).

Mean tumor growth is presented in Fig. 6A and B. Mean tumor volume comparison indicated that PLD was more efficacious at inhibiting the growth of both breast tumors compared with no treatment or NL-doxo (\( P = 0.013 \) and \( P < 0.0003 \), respectively). TG16% of NL-doxo and PLD was 34.6 and 56.8 in the C3-TAg model, and 29.6 and 76.3 in the T11 model, respectively, which indicates that T11 tumors may be slightly more responsive to PLD compared with C3-TAg tumors (Fig. 5C and D). KM plots are presented in Fig. 6E and F. PLD significantly prolonged the survival of C3-TAg models (\( P < 0.0001 \)), but modestly in T11 models (\( P = 0.083 \)) compared with no treatment and NL-doxo. However, it should be noted that T11 tumors treated with PLD became ulcerative in 18 days after treatment and were terminated in accordance to IACUC guidelines as a humane endpoint for the study. Ulceration is a lesion typified by a necrosis of tissues, which may reflect responses of T11 tumors to PLD (36). Thus, the results of overall survival for T11 mice treated with PLD may not reflect the accurate survival outcomes.

Discussion
Heterogeneity in tumor cells and/or the tumor microenvironment observed within and between tumor types is suggested to be a contributing factor to inefficient transport of nanomedicines to tumors, but there are limited preclinical and clinical data to understand the mechanisms and correlate the varying clinical responses to nanomedicines (5, 9, 11).

We used genomically validated murine breast tumor models and demonstrated that tumor delivery of PLD was significantly greater in the basal-like C3-TAg model compared with the claudin-low T11 model (\( P = 0.012 \)), whereas the difference in tumor delivery was not seen with NL-doxo. In addition, claudin-low T11 tumors were more responsive to PLD compared with basal-like C3-TAg tumors. To evaluate which tumor-associated factors may contribute to the variable tumor delivery and efficacy of PLD in breast tumor subtypes, we assessed the physiologic factors that have shown to be associated with NP transportation into and within tumors, including TAMs, collagen, and blood and lymphatic vasculature as well as chemokines (13, 14, 17).

TAMs are major leukocytes recruited into murine and human tumors by chemotactants and educated by the tumor microenvironment to promote tumor progression (13, 29). Macrophages have been shown to be involved in phagocytosis and clearance of NPs (7, 8, 10, 11) and to promote delivery of NPs into tumors serving as a “Trojan Horse” (37). On the basis of the evidence, we evaluated TAMs in association with tumor delivery and efficacy of PLD compared with NL-doxo in two breast tumor subtypes. The baseline levels of TAMs were similar between two models, which indicates that the level of TAMs may not account for the variable tumor delivery of PLD in these breast tumor subtypes (38, 39). However, the different changes of TAMs over time after NL-doxo or PLD in two models implicate the drug- and tumor-dependent interaction between TAMs and drugs. Consistent with the data generated in studies by our group and others, our finding indicates that uptake of PLD by TAMs may lead to cytotoxicity and cause a temporal decrease in the number of TAMs (12, 40). Further experiments using microscopy-based approaches will help confirm PLD uptake by TAMs and subsequent cytotoxic effects on the cells.

Recruitment of TAMs to solid tumors is regulated by chemokines, in particular CCL2, secreted by both malignant and stromal cells (14, 41). Hence, the system of CCL2 and its major receptor CCR2 has been shown to promote tumor cell survival and motility (42), metastasis (43), and angiogenesis (41). In addition, induction of CCL2 expression and CCL2-mediated monocyte/myeloid cell recruitment have shown to mediate therapeutic anticaner immune response elicited by immunogenic chemotherapy (i.e., doxorubicin; ref. 44) and counteract the antitumor effects of vascular-targeted therapies (i.e., VEGF inhibitor) as a compensatory mechanism (45, 46). Consistent with these observations, the expression of CCL2 in both C3-TAg and T11 tumors was increased after NL-doxo and, to a greater extent, after PLD, suggesting that PLD is more immunogenic compared with NL-doxo (6, 7). In addition, in T11 tumors where PLD...
exhibited antiangiogenic effects, PLD-mediated induction of CCL2 expression was significantly greater compared with C3-TAg tumors (45, 46).

In addition to cancer, CCL2 is also well studied for its chemotactic effects, activation of stellate cells, and angiogenesis in acute and chronic liver inflammation (47). Given that cancer can be considered as a chronic inflammatory disease, it is possible that elevated plasma CCL2 may serve as an inflammatory stimulus to monocytes and influence the migration and infiltration of macrophages to liver, spleen, and other organs (48). NP transport may be enhanced via increased angiogenesis, which influences the uptake by macrophages in the affected organs (8, 39, 47, 48). This may explain the greater accumulation of PLD in the liver and spleen from the T11 model compared with the C3-TAg model. However, to confirm this possibility, further experiments to assess hepatic blood vessels and the function of macrophages in these organs before and after PLD administration are needed.

The tumor interstitial spaces comprise a densely interconnected network of collagen fibers that interact with proteoglycans and glycosaminoglycans (17). In our studies, the baseline level of collagen was similar between two breast tumor subtypes, and the changes in collagen after NL-doxo or PLD administration were not noticeable. These data suggest...
that collagen matrix may not account for different tumor delivery of PLD between two murine breast tumor models. Further experiments are warranted to validate the effects of interstitial collagen matrix on the transport of PLD in tumors and interaction between collagen and PLD.

The ratio of tumor to plasma $AUC_{0-\infty}$ b. of PLD in C3-TAg and T11 models was 0.30 and 0.15, respectively, which suggests that the efficiency of transvascular transportation of PLD into tumor is 2-fold higher in the C3-TAg model. These findings led us to measure the amount and physiologic state of the vasculature and pro-angiogenic factors for blood microvascular endothelial cells (VEGF-a) and lymphatic endothelial cells (VEGF-c) in these models. Interestingly, claudin-low T11 tumors exhibit features of hypervascularization and inefficient lymphatic networks, which may increase interstitial fluid pressure (17) and hamper the transvascular transport of PLD (17, 49). Consistent with our findings, RNA expression profiling of more than 3,000 human breast tumors showed that claudin-low tumors had the highest vasculature signature expression compared with any of the other breast tumor subtypes (24). In addition, claudin-low tumor cell lines exhibited endothelial cell–like tube morphology with high vascular permeability compared with other breast tumor subtypes (24).

The interactions between PLD and the tumor vasculature were different between two breast tumor subtypes. PLD exhibited normalizing effects on the blood and lymphatic vessels over 96 hours and decreased the vascular density (MVD score) by 30% in claudin-low T11 tumors; however, no such changes were observed in basal-like C3-TAg tumors. Consistent with our finding, it has been reported that Doxil treatment exerted strong tumor growth inhibitory effects in B16.F10 melanoma–bearing mice as well as strongly reduced the intratumoral level of VEGF-a, which is produced in high amounts by melanoma cells (38). After clodronate-containing long circulating liposome (LCL), known for its TAM-suppressive effects, a significantly strong additional antitumor effect was observed after Doxil administration compared with that induced by clodronate-LCL; however, no additional strong reducing effect on VEGF-a was reported (38). This indicated that Doxil mainly acts via direct cytotoxic effects on tumor cells with slightly suppressive effects on TAM-mediated angiogenesis (38). On the basis of this observation, T11 tumor–specific VEGF-a suppressing effect of PLD may be associated with greater responsiveness of T11 tumors to PLD. In vitro studies assessing $IC_{50}$ of PLD in human basal-like cells and claudin-low cells would further confirm the different sensitivity to PLD between two breast tumor types.

The fact that T11 is an OST model compared with C3-TAg GEMM may play a role as confounding factor in our study despite the conserved gene expression features between murine T11 OST tumors and human claudin-low tumors (19, 23, 24). However, the clinical relevance of our results from T11 OST models can be justified on the basis of the evidence showing that gene expression signatures derived from chemotherapy-treated T11 models successfully predicted the pathologic complete response to anthracycline/taxane therapy in human patients with breast cancer (20, 23). The background difference between two murine models may confound the breast tumor subtype-specific difference in PLD delivery, and further studies are warranted to evaluate potential effects. Our findings may not be applicable to other NP platforms with different targeting strategies and/or surface characteristics (i.e., passive targeting vs. active targeting, lipid vs. polymeric; refs. 1, 2, 4, 17). Jain and colleagues reported that vessel normalization with antiangiogenic therapies improved the delivery and effectiveness of small NP (diameter, 12 nm) but not large NP (125 nm), emphasizing the importance of optimization of both NP and the tumor microenvironment (50). Thus, it is critical to profile the tumor microenvironment within and between tumor types and select patients with tumors that are likely to respond to NP-based therapies (5, 17).

Studies have reported that breast tumor classification based on gene expression profiling adds significant prognostic and predictive information to standard parameters (i.e., hormone receptor status, tumor size and grade, and node status, etc.) for patients with breast cancer (16, 19–24). Intrinsic human and murine breast tumor subtypes have shown different sensitivities to chemotherapies, including neoadjuvant anthracycline/taxane-based treatment (19, 20, 23). In line with that, we demonstrated that the biologic heterogeneity of breast tumors may affect the delivery and efficacy of NP-based therapies in murine models, and not all breast tumors may be uniformly conducive to NP therapies. Our findings suggest that PLD and other NP-based therapies for breast cancer need to be evaluated with respect to the heterogeneity of breast tumors in the retrospective and prospective studies.

Disclosure of Potential Conflicts of Interest

C.M. Perou is an employee of and has ownership interest (including patents) in Bioclassifier LLC, and University Genomics. No potential conflicts of interest were disclosed by the other authors.

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