**Supplementary Materials and Methods**

**Immunohistochemistry (IHC) and Digital Imaging**. Rat monoclonal antibody against F4/80 and rabbit polyclonal antibodies against CD31 and collagen IV (Col IV) were from eBioscience (San Diego, CA), Abcam (Cambridge, MA), and EMD Millipore (Billerica, MA), respectively. IHC for F4/80 (murine macrophage marker), collagen IV, and CD31 (marker for endothelial cells) was performed on an automated Bond II immunostainer (Leica Microsystems, Norwell, MA). Tumor slides were dewaxed in Bond Dewax solution (AR9222) and hydrated in Bond Wash solution (AR9590). Antigen retrieval for F4/80 was done for 20 min and for 30 min for CD31 and Col IV in Bond-Epitope Retrieval solution1 pH-6.0 (AR9961). Slides were incubated for 45 min with F4/80 (1:100), for 1h with CD31 (1:200), and for 30min with Col IV (1:200). Stained slides were dehydrated and cover-slipped. Detection of the antibodies was performed using the Bond Polymer Refine Detection System without post primary step (DS9800). Positive and negative controls (no primary antibody) were included for each antibody. Stained slides were digitally imaged at an apparent 20X magnification using the Aperio ScanScope XT (Aperio Technologies, Vista, CA). The Aperio ImageScope positive pen tool was used to create the different annotation layers (capsule, viable, and necrotic areas) for each tumor section under the guidance of board-certified veterinary pathologist (A.B.R.) in a blinded manner.

**Morphometric Quantitation of Macrophages and Collagen.** For semi-quantification of F4/80 immunoreactivity, H-scores were generated by The Aperio Membrane v9 algorithm. Staining intensity was graded as undetectable (1), weak (2), medium (3), or strong (4), and the percentage of positive cells per each intensity level was evaluated. The intensity score and the percentage of positive cells were then multiplied to give an H-score (possible range, 0-400) within each annotation layer (27, 28).

For scoring collagen IV semi-quantitatively, H-scores were generated by the Aperio color deconvolution methods. Staining intensity was graded as weak (1), moderate (2), and strong (3), and % of positive pixel per each intensity level was evaluated to calculate H-score (0-300) (27).

**Multiplex-bead Array Assay**. The 96-well plate kits (Miliplex®) were customized to measure chemokine CCL2 and CCL5 in plasma and tumor and VEGF-a and VEGF-c in tumor. Tumor tissues were weighed and homogenized with pH 7.4 PBS buffer spiked with a protein inhibitor cocktail (Calbiochem, MA) in 1:3 (tumor weight: PBS volume) using a Precellys (13-RD000) 24 bead mill homogenizer (Omni International, Inc.). Supernatant was extracted and used for assay. Standards were made using Assay Buffer provided with the kit. Standards and QC controls were placed to appropriate wells and samples diluted with Assay Buffer were placed to designated sample wells. The resulting raw data were collected using Luminex-100 system (Luminex, Austin, TX) and the concentration was corrected based on dilution factor used during procedures.

**Table S1. Product information from FormuMax Scientific, Inc.**

|  |  |
| --- | --- |
| Description | DoxovesTM-Liposomal Doxorubicin HCl |
| Catalogue # | F30204B-D |
| Lipid composition | HSPC/CHOL/Mpeg2000-dspe (56.3:38.4:5.3 mol%) |
| Active | Doxorubicin HCl |
| Analytical data | |
| Lipid concentration | 40.5 ± 0.5 Mm (29.7 ± 0.4 mg/ml) (Stewart assay) |
| Drug concentration | 4.00 ± 0.07 mg/ml (UV) |
| Free drug concentration | 0.02 mg/ml (filtration/UV) |
| Drug encapsulation efficiency | > 99.0%  (calculated from free drug and total drug concentration) |
| Hydration solution (battery) | 250 mM ammonium sulfate |
| External buffer solution | 10 wt% sucrose, 10 mM histidine pH6.5 |
| Particle size (ZetaPALS) | Mean diameter: 79.1 ± 0.5 nm; Half-width:20.5 ± 1.0 nm, Polydispersity: 0.07 ± 0.01 |
| Zeta potential (ZetaPALS) | -36.1 ± 1.0 mV(measured in 1 mM NaCl) |
| Form/color | Translucent, red and free flow liposomal dispersion, no visible particles/aggregates |
| Stability | Product is sterile filtered and filled in autoclaved vials |

a: HSPC: fully hydrogenated phosphatidylcholine

b: CHOL: cholesterol,

c: mPEG2000-DSPE: 1,2-distearoyl-sn-glycero-3-phophoethanolamine-N-methoxy(polyethylene glycol)-2000]

**Table S2. Doxorubicin AUC0-96h in plasma, tissues, and tumor in the C3-TAg model and the T11 model after PLD or NL-doxo administration at 6 mg/kg I.V. x 1 via tail vein**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| Model | C3-TAg | | |  | T11 | | |
| Drug | PLD | | NL-doxo | PLD | | NL-doxo |
| Plasma | Encapsulated | Released | 0.56 ± 0.03 | Encapsulated | Released | 0.87 ± 0.06 |
| 1,610 ± 111 | 31 ± 3 | 1,449 ± 57 | 27 ± 1.6 |
| Tumor | Sum total doxorubicin | | Doxorubicin | Sum total doxorubicin | | Doxorubicin |
| 480 ± 71\* | | 57 ± 10 | 210 ± 30\* | | 61 ± 12 |
| Liver | Sum total doxorubicin | | Doxorubicin | Sum total doxorubicin | | Doxorubicin |
| 438 ± 18\* | | 199 ± 17 | 687 ± 61\* | | 247 ± 15 |
| Spleen | Sum total doxorubicin | | Doxorubicin | Sum total doxorubicin | | Doxorubicin |
| 153 ± 35 | | 145 ± 32 | 306 ± 86 | | 111 ± 17 |
| Lung | Sum total doxorubicin | | Doxorubicin | Sum total doxorubicin | | Doxorubicin |
| 255 ± 16 | | 133 ± 7 | 248 ± 19 | | 208 ± 21 |

NOTE: AUC0-t of NL-doxo and PLD was calculated by noncompartmental analysis using Phoenix v.6.2. Data are presented as mean ± standard error of the mean (SEM).

a:\*,*P* <0.05 (C3-TAg vs. T11). *P*-values were calculated using Nedelman’s modification of the Bailer method for sparse samples, using a two-sample test (32).

b: NL-doxo: NL-doxorubicin.

c: AUC0-96h (µg·h/mL and µg·h/g for plasma and tissues, respectively) = area-under the concentration versus time curve from 0 h to 96 h.

**Table S3. Baseline and total influx of F4/80+ TAMs in the C3-TAg model and the T11 model after PLD or NL-doxo administration at 6 mg/kg I.V. x 1 via tail vein**

|  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- |
| **Model** | **C3-TAg** | | | **T11** | | |
| **Regions Of Interest**  **(ROI)** | **Baseline** | **AUC0-96h**  **post PLD** | **AUC0-96h**  **post NL-doxo** | **Baseline** | **AUC0-96h**  **post PLD** | **AUC0-96h**  **post NL-doxo** |
| Capsule | 191 ± 4.3 | 16,356 ± 703 | 16,473 ± 604 | 166 ± 95.6 | 17,304 ± 274 | 15,855 ± 1,099 |
| Viable | 61 ± 9.2 | 9,855 ± 1182 | 8,605 ± 951 | 90 ± 21.9 | 12,821 ± 428 | 10,185 ± 1,679 |
| Necrotic | 199 ± 3.3 | 18,764 ± 645 | 18,946 ± 562 | 189 ± 8.1 | 17,656 ± 1,098 | 15,664 ± 1,518 |

NOTE: TAMs were measured by F4/80 in tumor from individual mouse via IHC. The expression of F4/80 at baseline and AUC0-96h of F4/80, an indicator of total influx of macrophages, after PLD or NL-doxo administration was represented as mean ± SEM of three to four mice. AUC0-96h was calculated by non-compartmental analysis using Phoenix v.6.2.

a: AUC0-96h = area-under the H-score of F4/80 versus time curve from 0 h to 96 h.

**Supplementary Figure Legends**

**Supplementary Fig. S1.** Identification of subregions in the tumor for classification of F4/80-positive tumor-associated macrophages (TAM). TAMs were visualized by staining fixed sections of C3-TAg and T11 tumors using antibodies that specifically recognize the murine macrophage marker F4/80. Whole tumor images were captured using an Aperio slide scanner and the Aperio ImageScope positive pen tool was used to create the different annotation layers depending on subregions in the tumor (e.g., capsule, viable, and necrotic tumor) for each tumor section under the guidance of board-certified veterinary pathologist in a blinded manner. (A) Digital image of a F4/80-stained T11 tumor section with annotation layers for viable and capsule areas (green) and necrotic areas (blue). (B) Digital image of the same F4/80-stained T11 tumor section with the necrotic areas analyzed by the Aperio Membrane v9 algorithm. The same optimized algorithm was run separately on each annotation layer.

**Supplementary Fig. S2.** The lumen analysis in representative vascularized areas in the T11 tumor. (A) Blood vessels consisting of endothelial cells are detected according to CD31 stain (brown stain) using the Definiens vessel algorithm (Munich, Germany). (B) Digital image of the blood vessels with the lumen (green) detected. The algorithm takes a step-wise approach to identify lumens and connect the endothelial cells around the lumen together to identify a single vessel (purple).

**Supplementary Fig. S3.** Profiling of collagen in basal-like C3-TAg model and claudin-low T11 model at baseline and at 96 h after administration of PLD or NL-doxo at 6 mg/kg I.V. x 1 via tail vein. Data are presented as mean ± SEM of collagen H-score (n=3 or 4) in (A) the C3-TAg and (B) the T11 tumors. The baseline collagen amount was similar between the two models. There was no significant change at 96 h after NL-doxo or PLD administration in both models. Collagen in the capsule and viable tumor were assessed for analysis.

**Supplementary Fig. S4.** Size distribution of baseline blood vessels in the C3-TAg tumors and the T11 tumors. Most of the vessels identified were small (~87%), followed by medium (12%) and large (1%) in the both tumors. The vascular size was defined as small < 40 µm2, medium 40 µm2 ≤ and < 400 µm2, and large ≥ 400 µm2. Five most vascularized areas within the tumors (‘hotspot’/0.74 mm2) were chosen for evaluation of the size distribution. Each of these five areas was analyzed and the mean was calculated per slide. Data are presented as mean ± SEM (n= 3 or 4).