Supplemental Materials and Methods

Cell lines and tumor models

NCr-*nu/nu* (nude) mice were originally obtained from the National Cancer Institute and BALB/c, C57BL/6 and *NOS2*$^{-/-}$ mice were originally obtained from Jackson Laboratories. All mice were bred and maintained in Cox-7 gnotobiotic animal facility of Edwin L. Steele Laboratory. BALB/c mice were used for MFP windows for technical reasons; windows are easier to establish, have less inflammation, and last longer in BALB/c than in nude.

4T1 cells (ATCC) were cultured in high-glucose DMEM (ATCC) with 10% FBS and 1% MEM non-essential amino acids (Invitrogen). E0771 cells (obtained from Roswell Park Cancer Institute, Buffalo, NY) were cultured in DMEM with 10% Serum Supreme (Lonza) and 1% HEPES buffer (Invitrogen). Cell lines were authenticated in 2013 by IDEXX laboratories (IDEXX RADIL Case # 14116-2013; Sample 4T1: The genetic profile 1 is identical to the genetic profile established for the 4T1 (ATCC# CRL-2539) cell line; Sample E0771: The genetic profile for this cell line contains extra alleles at 12 markers and different allele sizes at 5 markers. In addition, there are more than 2 alleles at 3 markers. The genetic profile for this cell line is not consistent with that of the C57BL/6 inbred mouse strain and is instead more consistent with having been derived from a mouse with a mixed/stock genetic background. Re-testing of this cell line after another 5-10 passages (IDEXX RADIL Case # 15518-2013) clarified that the genetic profile is intrinsic to this cell line but not likely cross-contaminated with another mouse cell line).

For local radiation treatment non-tumor areas were protected by positioning tumors within a 15 mm aperture of a 6 mm thick lead collimator placed over the mouse.

Tumor growth

Tumor size was assessed every 48 hours using calipers. Tumor volume was calculated as: $4/3 \times \pi \times (\text{long axis}/2) \times (\text{short axis}/2)^2$. The time (days) taken for tumors to reach 800 mm^3 was used for the comparison of tumor growth and assessment of therapeutic efficacy. The expected additive effect of monotherapies was calculated as previously performed (1). For tumor growth studies

presented in Figure 2, n = 10 per group for the 4T1 tumor model and 7 per group for the E0771 model. For tumor growth curves presented in Figure 6, n = 5-6 for the 4T1 tumor model and 4 to 6 in the E0771 tumor model. For analysis of time to reach 800 mm^3 n = 4-7 except for the radiation plus 1400W group for which only 3 animals survived with tumors to 800 mm^3 (in some cases deterioration of animals health necessitated sacrifice prior to tumors reaching 800 mm^3).

Tissue collection

Tumor tissue was collected at 4, 6, and 10 days after the initiation of treatment and either snap frozen in liquid nitrogen before mounting in OCT (Tissue-Tek) or fixed in 10% neutral buffered formalin (NBF) for paraffin embedding. Day 4 tumors were injected (retro-orbital) with Hoechst 33342 (Sigma) 2 min prior to euthanasia. Day 6 tumors were snap frozen for protein extraction for Western blot and protein arrays.

Immunofluorescence and histological staining

Frozen 20-μm sections were post-fixed in acetone and blocked for 1 hour with 3% bovine serum albumin (Jackson ImmunoResearch) except for mouse on mouse studies (MOM kit following manufacturer's instructions, VectorLaboratories). Primary antibodies were applied overnight at 4°C. Secondary antibodies included: Cy3/Alexafluor-488, Cy5/Alexafluor-647, and Dylight-649 (all Jackson ImmunoResearch). DAPI or SYTOX were used as nuclear markers.

Formalin-fixed paraffin embedded tumor sections (5 μm) were sent to MGH Histopathology Research Core for Masson's trichrome staining and hematoxylin and eosin (H&E). For PCNA and Apoptag staining, endogenous peroxidases were blocked with 3% hydrogen peroxide before staining. Apoptag (Millipore, ApopTag® Peroxidase In Situ Apoptosis Detection Kit, #S7100) was performed following manufacturer instructions and developed with DAB before counterstaining with hematoxylin. PCNA (DAKO, #M0879) staining required antigen retrieval (DAKO regular target retrieval solution) at

97°C for 10 minutes and MOM block (VectorLaboratories) followed by incubation with DAKO Mouse EnVision Polymer and DAB development.

Primary antibodies dilutions and sources are as follows: rabbit anti-MMP14 (Abcam, ab53712, 1:200), hamster anti-CD31 (endothelial cell marker; Millipore, MAB1398Z, 1:200); rabbit anti-NG2 (pericyte marker; Chemicon, AB5320, 1:1000); rabbit anti-Collagen IV (basement membrane marker; Millipore, AB7569, 1:1000),mouse anti- iNOS-FITC (BD Biosciences, 610330, 1:100), rabbit anti-CA9 (hypoxia marker; Abcam, ab15086, 1:3000), rabbit anti-HSP90 (Cell Signaling, 4874S, 1:100), SMAD2/3 (Cell Signaling, 3102S, 1:100), rat anti-F4/80-Cy5 (macrophage marker; AB Serotec, MCA497BB, 1:50), rat anti-MRC1 (M2 marker; Serotec, MCA2235, 1:50), rabbit anti-Granzyme B (cytotoxic activity marker; Abcam, ab4059, 1:200), rabbit anti-NK1.1-Cy3 (NK cell marker; Bioss, bs-4682R), mouse anti-PCNA (proliferation marker; DAKO, M0879), and Apoptag (apoptosis marker; Millipore, S7100). Rabbit anti-TGFβ (Cell Signaling, 3711S, 1:1000) was used for Western blot analysis (described below). All rabbit anti-bodies were polyclonal, while rat and mouse antibodies were monoclonal.

Optical frequency-domain imaging (OFDI)

BALB/c mice (n = 7) bearing MFP windows and 4T1 tumors were assessed on day 4. Approximately 200 to 2500 vessels over a tumor section 7.8 mm by ~700-900 μm were assessed using an in-house MATLAB algorithm; providing data on individual vessel diameters, lengths, and tortuosity (2). Vessel tortuosity was calculated as the actual vessel length divided by the chord length (the minimum path between two points on a vessel). In addition to mean values, the advantage of OFDI is that frequency histograms can be generated for hundreds to thousands of individual vessel diameters and tortuosity values from an entire tumor section.

Western blotting and Antibody arrays

Approximately 50 μg of protein from tumor or cell lysate were loaded for 10% SDS-PAGE and

transferred to nitrocellulose membranes. Nonspecific binding was blocked by 5% non-fat milk for 1 hour. Membranes were incubated overnight at 4°C with primary antibodies and were followed by anti-rabbit HRP secondary (Cell Signaling, 1:1000). Membranes were developed with either high-sensitivity Pierce ECL 2 Western Blotting Substrate (Thermo Scientific, iNOS) or low sensitivity Amersham ECL Western Blotting Analysis System (GE Healthcare, MMP-14) and exposed to film. Membranes were stripped and probed for β-actin (Sigma, A5441, 1:5000) to provide a loading control.

RayBiotech mouse inflammatory array (AAM-INF-1), which holds 39 key cytokines and chemokines, was used to assess general changes in inflammatory conditions in the tumor microenvironment. Whole tumor lysates were used and 250 μg of protein from 4 tumors (2 DX-2400 treated and 2 CT treated tumors) were applied on 4 membranes (with internal duplicates on each membrane). Manufacture-suggested protocols were followed and membranes were exposed to film and analyzed similar to Western blots.

Image analysis

Quantification of stained area was performed using an in-house MATLAB segmentation algorithm or ImageJ for cell counts and the number of perfused vessels per ten high power (40X or 20X) fields of view (FoV). The area of necrosis was measured on merged 20X magnification images of H & E stained tumor sections using Image J (3) to outline the tumor and necrotic areas. Relative densitometry of Western blots and the antibody array was determined using ImageJ with normalization to the loading control.

A mosaic image of the entire stained tumor cross-section (20X) and/or ¼ of the tumor area (60X) was generating using multi-area time-lapse confocal imaging (Olympus FV1000 microscope). Quantification per area DAPI of total iNOS, CA9 (hypoxia), Hoechst (perfusion), and CD31 (endothelial cells) as well as NG2 (pericyte) and COL IV (basement membrane [BM]) coverage (colocalization with CD31) was performed using an in-house MATLAB segmentation algorithm on 20X tumor mosaics, while macrophage and NK cell staining was assessed using 60X mosaic images. Granzyme colocalization with F4/80 was counted manually with imageJ per merged 60X FoVs as was

perfusion, assessed by counting the number of perfused vessels (those containing red blood cells) per ten high power (40X) fields on trichrome stained tumor sections. Similarly, a mitotic index was established by counting the number of mitotic figures per high power FoV. PCNA was also assessed using imageJ but the automated cell counter was used (images were converted to 8 bit grey scale and thresholded to only show PCNA positive nuclei). Apoptag was assessed by manual counts of positive cells per high power FoV in imageJ. In all cases were FoV were assessed rather than the whole tumor section, at least 1/3rd of large tumors were covered while over ½ of small tumors were covered (this typically required between 5 and 20 images depending on the size of the tumor). For necrosis, the area of necrosis, clearly visible on H&E stained sections, was outlined in imageJ and then the entire tumor area was outlined. The percent necrosis was then calculated based on the areas generated.

Necrosis analysis n = 8 CT and n = 9 DX. PCNA, Apoptag, CD31 and NG2 staining n= 4 - 7 per group for 4T1 samples. 4T1 HSP90+iNOS staining n = 7 per group. For all other staining, n = 8 - 10 per group for 4T1 samples and n= 6 per group for E0771 samples.

Flow cytometry

BALB/c -4T1 tumors (n= 6 CT and n = 5 DX) were perfused with phosphate buffered saline at physiologic pressure before collection, mincing, and digestion (DMEM with 1.5mg/mL collagenase type 1A and hyaluronidase and 0.4mg/mL DNase). The M1 ($CD45^+CD11b^+F4/80^+CD11c^+MRC1^-$) macrophage population was identified from total macrophages ($CD45^+CD11b^+F4/80^+$). 7AAD was used to assess cell viability. Fluorochrome-conjugated, isotype-matched IgGs were used as controls. Data were acquired on a LSRII flow cytometer (Becton Dickinson) and analyzed with FACSDiva software.

In Situ Zymography

Cryostat tumor sections (n = 7 DX and n = 10 CT) were air-dried for 10 minutes, fixed with acetone for 5 minutes and treated for 30 minutes with aprotinin (2 µg/ml in PBS, Sigma). A solution of 0.5% agarose/PBS was combined with DQ-collagen type-I (1.0 mg/ml, Invitrogen) in a 10:1 ratio. The

combined mixture (40 µl) was applied to tumor sections and covered with a coverslip. After 5 minutes at room temperature for agarose gelation, the slides were incubated at 37°C for 60 minutes. Cell nuclei stained with DAPI and unquenched FITC from MMP digestion of DQ-collagen were imaged on a confocal microscope (Olympus FV1000 microscope). Image acquisition was performed maintaining the same laser power, gain, and offset settings. Fluorescence intensity quantifications were performed by ImageJ (NIH).

Collagen Invasion assay

The collagen invasion assay was performed using standard methodologies (4, 5). Transwells (8 µm pore diameter; BD Biosciences) were coated with rat-tail type-I collagen gel layer (70 µl at 1.6 mg/ml) and 4T1 and E0771 cells seeded at 105 cells per well in serum-free DMEM (Lonza). DMEM containing 10% FCS was used as an attractant in the lower chamber. Cells were allowed to adhere for 1 hour prior to the addition of the indicated quantities (0.2-100 µg/ml) of DX2400 (DX) or control (IgG). Following 48 hours, the cells on the bottom of the coated transwell were photographed by a 20 x magnification objective and counted as cells per field (5 fields per well) by automatic selection of cells (ImageJ). Three replicates were performed.

Viability Assay

Cell Titer-GLO was used to assess 4T1 and E0771 cell viability after treatment with DX2400 (DX) at indicated concentrations (0.1-50 µg/ml) or control IgG (at 10 µg/ml). Briefly, 3,000 cells were seeded in a 96-well plate in cell culture media supplemented with 10% FBS. After 24 hours the medium was removed, the cells were washed with 1 X PBS and 100 µl of cell culture medium containing IgG or DX2400 (with or without FBS) was added. At 24 h, 48 h and 72 h post treatment, cell growth was measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega). Five replicates were performed.

References

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