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Appendix E1

Supplementary Methods 1: Labeling of Anti-HLA-DR Antibody with ^{160}Gadolinium

Antibody Labeling Protocol

Monoclonal, purified, antihuman HLA-DR antibody was purchased from BioLegend (LN3, Catalog#327002; San Diego/CA), delivered in a BSA and gelatin free phosphate-buffered solution containing 0.09% sodium azide (pH = 7.2), and fluorescein isothiocyanate (FITC)-conjugated antihuman HLA-DR antibody was purchased from Abcam (LN3, Catalog#ab1182; Cambridge/UK) and delivered in 0.5% BSA constituent 0.1% sodium azide buffer. The antibodies were labeled with ^{160}gadolinium (^{160}gad) using the commercially available MAXPAR X8 Antibody Labeling Kit (Fluidigm, San Francisco/CA) according to the manufacturer's instructions (Protocol PRD002v11) as previously described (18). Briefly, a polymer was preloaded with the ^{160}gad lanthanide and incubated at 37°C for 30–40 min. The buffer solution containing the antibody was then exchanged with 4mM TCEP-*R*-Buffer and also incubated at 37°C for 30 min to partially reduce the antibody. After incubation, the ^{160}gad-loaded polymer and the reduced antibody were purified with multiple washes using a 3kDa and 50kDa Filter (Millipore Sigma, Darmstadt/Germany) respectively. The retrieved solutions were then conjugated at 37°C for 90 min and multiple washing steps were performed afterward to remove nonconjugated particles. The percentage yield after ^{160}gad-labeling of the antibodies was determined by measuring absorbance at 280 nm and was determined to be 60%–75% at all labeling cycles. The ^{160}gad-conjugated antibodies were then supplemented with antibody stabilization buffer (Candor Bioscience GmbH, Wangen/Germany) and stored at 4°C (L.J.S, L.D., I.T.S.).

Cross-Validation of Successful Labeling Using Imaging Mass Cytometry and Immunofluorescence Microscopy

Labeling efficacy of anti-HLA-DR antibodies with ^{160}gad and constant unchanged binding sensitivity and specificity were confirmed on paraffin-embedded specimens. ^{160}Gad-labeled FITC-conjugated antibodies were used to compare spatial distributions of signal from ^{160}gad on imaging mass cytometry (IMC) and FITC on immunofluorescence microscopy on the same specimen slide.

The sample preparation necessary for IMC is identical to that for immunohistochemistry analyses (18). Formalin-fixed paraffin-embedded specimens from a tumor-bearing rabbit were stained with the ^{160}gad-labeled, FITC conjugated anti-HLA-DR antibody according to the manufacturer's protocol (PN400322 A3). A nuclei specific iridium label (Cell-ID Intercalator-Ir-125μM, Catalog#201192A, Fluidigm, San Francisco/CA) was used as a reference of known expression pattern. Briefly, the slides were dewaxed, hydrated in an ethanol dilution series, incubated in an antigen retrieval solution (EDTA 1.35 mM in deionized water, pH = 8.0), washed and blocked with bovine serum albumin (BSA), incubated with the antibody overnight at 4°C in a hydration chamber, and

counterstained with the intercalator. After labeling, the slides were rinsed with PBS and air-dried (L.J.S., L.D.).

IMC was executed to confirm successful labeling of ^{160}gad to the FITC conjugated anti-HLA-DR antibody and to determine spatial distribution of the antibody in the specimen. Regions for IMC ablation were determined on corresponding H&E slides and always contained tumor tissue, liver parenchyma as well as the peritumoral zone. IMC settings were selected as explained below.

Supplementary Methods 2: Imaging Mass Cytometry

IMC allows the detection of metal-labeled antibodies on solid tissue samples with high spatial resolution (18). IMC was performed with the Hyperion Imaging System coupled to a Helios Mass Cytometer (Fluidigm, San Francisco/CA) equipped with an argon-based laser. For noise determination and to ensure that the metal labeling did not interfere with their target antigen specificity, all antibodies were validated with the IMC platform by comparing metal labeled antibody to unlabeled antibody as positive and negative controls (18). Automated ablation of selected histospots was conducted in a rastering pattern at a frequency of 200 Hz with a laser power of 5 dB. Laser steps were 1μm in both X and Y axes. The detection limit for IMC was calculated for signals in an overall exposure time identical to the integration time for one pixel in a single laser shot (18). Raw data were visualized and analyzed to study the distribution and intensity at a pixel level (1 μm^2) using the MCD Viewer (v1.0.560.6; Fluidigm, San Francisco/CA). To define the gadolinium signal from background, the frequency distribution of pixel intensity was assessed where the ^{160}gad-labeled antibody showed a recognizable, specific pattern of staining that reproduced the staining patterns and the percentage of cells expressing the given marker as determined by immunohistochemistry. Signal distribution was evaluated in three regions (tumor, peritumoral rim, liver) defined on bright field microscopy of H&E slides. Additionally, the spleen served as a positive control for the HLA-DR staining. For illustration purposes, only the highest expressing pixels above the 95th percentile threshold were retained as previously described (34). Samples were prepared by L.J.S., L.D., I.T.S. and analysis and interpretation were performed by L.J.S., R.M., L.L., R.B., and J.C.

Supplementary Methods 3: Histologic Analysis

Tissue Collection and Processing

Immediately after the terminal MRI, all animals were sacrificed by intravenous injection of euthasol (0.5 mL/kg). Necropsy was performed and tumor, surrounding and contralateral liver, spleen, and kidney were immediately harvested, sectioned in slices of 3–5 mm, fixed in 10% buffered formalin overnight and paraffin-embedded for radiologic-histopathological correlation (L.J.S., 6 years; I.T.S., 2 years; L.D., 1 year of experience in animal experiments).

Immunohistochemistry

The tissue was cut into 2 μm slices and processed as previously described (35). First, hematoxylin/eosin (H&E) stain according to standard protocols was used for general histopathology evaluation and quantification of tumor viability and necrosis. In addition, immunohistochemistry was performed evaluating for HLA-DR (LN-3; Catalog#ab166777, Cambridge/UK; 1:50) and CD11b (M1/70, Catalog#ab8878, Cambridge/UK; 1:500 in PBS).

In rabbits that received superparamagnetic iron oxide nanoparticles (SPIONs), Prussian Blue staining was conducted according to the Ferrocyanide method to detect iron and its colocalization with macrophages (21).

Histology samples were digitalized and visualized at up to 20× magnification using Aperio ImageScope software (v12.3; Leica Biosystems Imaging; Vista/CA). Five random high power fields were analyzed using a length measurement tool in Aperio to quantify peritumoral rim thickness and compare histologic to imaging findings (19).

Immunofluorescence Microscopy

Immunofluorescence microscopy was conducted using a Confocal SP5 Microscope (Leica Microsystems, Heidelberg/Germany) at 10× magnification (Objective HC PL FLUOTAR 10× x 0.3, dry) using the HyD Detector at room temperature. The acquired images were studied with Leica Application Suite Advanced Fluorescence Lite 2.0.0 (Leica, Heidelberg/Germany) (L.J.S., L.D.).

First, paraffin-embedded samples from VX2 tumors stained with the ^{160}gad-labeled FITC-conjugated anti-HLA-DR antibody were investigated for occurrence of FITC fluorescence signal (wavelength 520 nm). The acquired signal was compared with the IMC signal to confirm successful labeling of the FITC-conjugated antibody with ^{160}gad.

In rabbits receiving SPIONs, the presence and localization of rhodamine-conjugated SPIONs was confirmed on paraffin-embedded unstained tumor tissue slides for rhodamine (wavelength 561–587 nm) to confirm MRI contrast from SPIONs (L.J.S., L.D.). Excitation and emission wavelengths were those recommended by the conjugate manufacturers.

Supplementary Methods 4: Detailed MRI Protocol

All animals underwent baseline imaging using multiparametric MRI performed on a human-size 3T MRI unit (Magnetom Prisma; Siemens, Erlangen, Germany) using a 15-channel knee coil (L.J.S., L.D., I.T.S., D.C., L.A.). The multiparametric MRI protocol included respiratory-gated T2-weighted spin echo-images and noncontrast and IV contrast-enhanced T1-weighted Dixon images. Tumor enhancement was assessed on dynamic IV contrast-enhanced T1-weighted images using a 3D Volumetric Interpolated Breath-hold Examination (VIBE) sequence with CAIPIRINHA (2 × 2) parallel imaging. Scan parameters were: TR/TE/O = 3.45 ms/1.28 ms/9°, matrix 192 × 100, 6/8 partial Fourier, bandwidth 500 Hz/pix, field of view (FOV) = 200 × 120 mm^2, 25–32 slices, 2–3s/volume,1 × 2 × 2.5 mm^3. Eighty multislice volumes were acquired repeatedly before, during and after the bolus injection of 0.1 mmol/kg intravenous macrocyclic gadolinium (Dotarem; Guerbet, Bloomington/IN). Follow-up imaging consisted of the same imaging protocol as baseline imaging but without contrast-enhanced (Dotarem) MRI.

Group A: T1-weighted MRI of ^{160}gad-labeled Antibodies

Contrast-enhanced imaging with ^{160}gad-conjugated antibodies was performed using 2D distortion-corrected T1-weighted Dixon images. Scan parameters were TR/TE1/TE2 = 5.19 ms/2.46 ms/3.69 ms, slice thickness 2.5 mm, FOV read 200 mm, FOV phase 59.4%, voxel size = 0.5 × 0.5 × 2.5 mm, 3 averages, 2 min acquisition. The peritumoral rim on MRI was determined on postcontrast imaging by overlaying the tumor mask outlined on the baseline images onto the postcontrast imaging. Peritumoral gadolinium-signal on MRI was then quantified by two

radiologists with 5 (L.J.S.) and 7 years of experience (J.C.) in abdominal MRI. Briefly, using the standard length measurement tool in RadiAnt DICOM Viewer (v4.6.9, Medixant, Poznan/Poland), the rim enhancement diameter (rim thickness) was measured in 5 random locations on the DICOM image with the largest tumor diameter in the axial plane, as previously described elsewhere (19).

Group B: T2-weighted MRI of SPIONs

Scan parameters for SPION imaging were respiratory-gated (expiration phase, trigger delay = 0s, threshold = 20%) distortion-corrected T2-weighted spin-echo TR/TE = 1000 ms/78 msec with fat-suppression by SPAIR, slice thickness 3 mm flip angle = 160°, FOV read 200 ms, FOV phase = 65.6%, voxel size = 0.8 × 0.8 × 3.0 mm, GRAPPA-factor of 2, acquisition window = 1000 ms, 2 averages. 3min acquisition. To quantify contrast alterations by SPIONs, the same method used for gadolinium-signal assessment was applied on post-SPION T2-weighted MRI to quantify the diameter of hypoenhancing signal in the peritumoral rim.

Group C: pHe-Mapping Using Biosensor Imaging of Redundant Deviation in Shifts

Animals in *Group C* underwent pHe-specific MR spectroscopy at the follow-up MRI using biosensor imaging of redundant deviation in shifts (BIRDS). BIRDS with the use of the paramagnetically active contrast agent $TmDOTP^{5-}$ (Thulium (III) 1,4,7,10-Tetraazacyclododecane-1,4,7,10-tetra; Macrocyclics, Pano/TX) allows for determination of tissue pHe in the tumor and liver by measuring the chemical shift of the pHe-sensitive resonances H2, H3, and H6 (17). Briefly, 15 mL of 0.5 mmol/kg $TmDOTP^{5-}$ was infused at a rate of 0.5 mL/min for ∼30 min. The BIRDS data were acquired using a 3D chemical shift imaging sequence with a FOV = 20 × 20 × 25 cm^3, 2197 rectangular encoding steps, 20 averages, TR = 8 msec (limited by specific absorption rate), 6 min acquisition, and reconstructed to 25 × 25 × 25 mm with a voxel resolution of 8 × 8 × 10 mm. Excitation was achieved using a dual-band 640 µs Shinnar-Le Roux radiofrequency pulse which selectively excited the peaks of interest on either side of water. T1-VIBE images for registration were obtained using a FOV = 20 × 20 cm, 384 × 384 matrix, 60 slices of 2.5 mm thickness, TR/TE = 5.2 ms/2.5 msec (17).

The $TmDOTP^{5-}$ resonances were overlaid on the T1-VIBE for correct localization. The tumor was identified on the T1-VIBE and the pHe was assessed for voxels inside the whole tumor, at the tumor edge defined as the peritumoral zone, and outside the tumor in the liver parenchyma. The pHe for each voxel was calculated using Matlab (vR2020a 9.8.0; MathWorks, Natick/MA) from the chemical shifts of H2, H3 and H6 of $TmDOTP^5$ (data processing and analysis: D.C., L.A.; interpretation: L.J.S., D.C., F.H., L.A., J.C.).