In Vivo Ultrasound-guided Focused Ultrasound Partial Thermal Ablation

System 1 (Custom in-house system): This system consisted of a 64 mm single-element 3.3 MHz transducer (Sonic Concepts) powered by a 400 W amplifier (E&I), orthogonally registered to an 8 MHz linear ultrasound imaging array (Siemens). Mice were acoustically coupled to the therapeutic and imaging ultrasound transducers via degassed water bath maintained at 37ºC. Sonication positioning was electronically driven via motorized 3D motion stage. The subcutaneous tumor was identified by B-mode ultrasound imaging, following which a grid pattern of sonications was painted onto the ultrasound-visible tumor (hypoechoic region) over a single plane of treatment. To reach thermally ablative temperatures, the transducer was operated at 3MHz in continuous wave mode, with each sonication carried out at 15W acoustic power for 10s. In total, two planes of sonication (separated by 2mm) were completed for each mouse, with grid points spaced 1mm apart.

System 2 (Theraclion Echopulse VTU): This system consisted of a 56 mm single-element 3 MHz transducer with 38 mm radius of curvature and embedded multi-frequency linear ultrasound imaging array generating a symmetrical imaging plane relative to VTU long axis. Mice were acoustically coupled to the therapeutic and imaging ultrasound transducers via degassed water bath maintained at 37ºC. Sonication positioning was electronically driven via motorized 3D motion stage. The subcutaneous tumor was identified by B-mode ultrasound imaging, following which thermally ablative sonications were applied to the ultrasound-visible tumor (hypoechoic region) at 30W (derated acoustic power) for 4s over a single plane of treatment.

Flow Cytometry

Tumors were incubated in collagenase/dispase (5 mg/mL; Sigma) and DNAse I (100ug/mL; Roche) at 37C for 1 hour followed by mechanical homogenization. The disaggregated tumors were filtered through 100 µm Nitex nylon mesh (Genesee) and applied to a concentration gradient (Lympholyte-M cell separation media; Cedarlane) for cell isolation.

Spleen and lymph nodes samples were mechanically homogenized and filtered through 100 µm Nitex nylon mesh then RBC lysed (Hybri-Max; Sigma).

To facilitate functional immune characterization, mice received an intravenous injection of Brefeldin A (250ug; Fisher) approximately 6 hours prior to tissue harvest. Samples were stained with viability dye (Live/Dead Aqua; Life Technologies), followed by 15min incubation with Fc block (anti-mouse functional grade CD16/32; Life Technologies). Surface staining was performed in FACS buffer + 2% normal mouse serum (Fisher) then permeabilized with either Cytofix/Cytoperm Kit (BD Biosciences) or Foxp3/Transcription Factor Staining Buffer Set (eBioscience/ThermoFisher), and stained for intracellular cytokines and transcription factors. The following antibodies were used across multiple panels for immune phenotyping from BioLegend - CD8a Brilliant Violet 650 (clone 53-6.7), CD279/PD-1 Brilliant Violet 605 (clone 29F.1A12), TIGIT/Vstm3 PE/Dazzle 594 (clone 1G9), CD11b Alexa Fluor 700 (clone M1/70), Ly-6C PE-Dazzle 594 or APC (clone HK1.4), Ly-6G Brilliant Violet 605 (clone 1A8), CD11c APC (clone N418), CD86 Brilliant Violet 650 (clone GL-1), CD155/PVR PE-Cy7 (clone TX56), TNF-α Brilliant Violet 421 (clone MP6-XT22), CD274/PD-L1/B7-H1 PE (clone 10F.9G2) - or eBioscience/ThermoFisher - CD4 eFluor 450 (clone RM4-5), CD44 AlexaFluor 700 or APC-eFluor 780 (clone IM7), CD335/NKp46 PE (clone 29A1.4), CD19 Super Bright 780 (clone 1D3), CD45.2 APC-eFluor 780 (clone 104), CD274/PD-L1/B7-H1 PerCP-eFluor710 (clone MIH5), F4/80 Super Bright 780 (clone BM8), Granzyme B PE-Cy7 (clone NGZB), IFN gamma APC (clone XMG1.2), FOXP3 PerCP-Cy5.5 (clone FJK-16s), CD279/PD-1 FITC (clone RMP1-30), CD45 Super Bright 702 (clone 30-F11). IL-12 PE (p40/70; clone C15.6) was purchased from BD Biosciences.

Overall Survival Criteria

The following humane endpoints were established for mice in survival studies: spontaneous death with validation of tumor involvement, euthanasia due to moribund appearance as a result of extensive tumor metastasis, or subcutaneous tumor outgrowth exceeding 20 mm diameter in any single dimension. The following endpoints were censored from survival data:

euthanasia due to tumor ulceration and non-tumor-related deaths. To reduce any bias in the processing of monitoring, all cages were labelled using alphanumeric codes that kept researchers, caretakers and veterinarians blinded from control and treatment groups throughout the course of survival studies.