Supplemental Data

Production and characterization of T4 and RT4

T4 is a novel sdAb targeting Trop2 we previously reported (*1*). The His-tagged T4 had an expression yield of 180.80 mg/L in Chinese Hamster Ovary cells. RT4 is a His-tag−free T4 derivative with an expression yield of 165.25 mg/L. The endotoxin level of the sdAbs was below 1EU/mg. The purities are determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and high-performance liquid chromatography (HPLC). The binding kinetics between the two sdAbs and the immobilized human Trop2 protein (TR2-H5223, ACRO Biosystems) were verified using the surface plasmon resonance (SPR) interaction test.

T3M-4 cells and cell-derived xenograft (CDX) tumor model

The T3M-4 cell line was acquired from MeisenCTCC in Zhejiang, China. The cells were cultured in RPMI 1640 medium from Shanghai BasalMedia Technologies Co., LTD., supplemented with 10% fetal bovine serum (FBS) from GE Healthcare in Chicago, IL, USA, and 1% penicillin/streptomycin from Invitrogen. The cell line was cultured in a humidified incubator at 37°C with 5% CO_2. Once the cells had grown sufficiently, equal proportions of matrigel matrix (Corning) and 5×10^6 tumor cells resuspended in sterile phosphate-buffered saline (PBS, HyClone) were mixed. The prepared tumor cells were implanted into the lateral abdomen of female nude mice aged between 4−6 weeks (GemPharmatech). Imaging experiments were performed when the tumor grew to a 6–8 mm diameter.

Conjugation, radiolabeling, and quality control

Preparation of labeling prerequisites: 20 mL of 0.05M $NaHCO_3$ solution (pH = 8.6) was used to

pre-equilibrate the PD-10 desalting columns (GE Healthcare). Then, 2 mg of sdAb (T4 or RT4) solution in the PBS was added to the PD-10 and replenished the volume to 2.5 mL with 0.05 M $NaHCO_3$ solution (pH = 8.6). 2.5 mL of solution was eluted using 0.05 M $NaHCO_3$ solution and collected. Weighed the certain molar amount of (±)-H_3RESCA-TFP (CAS number: 1919794-40-3), meeting (±)-H_3RESCA-TFP: sdAb = 12: 1 and dissolved it in 40 μL of dimethyl sulfoxide (DMSO). The (±)-H_3RESCA-TFP was added to the sdAb solution and mixed thoroughly. The reaction mixture was then placed on a shaker at room temperature for 2 hours. The reaction mixture was added to the equilibrated PD-10 column and eluted using 0.1 M CH_3COONH_4 solution (pH = 4.6). The eluate was collected and concentrated using Amicon Ultra Centrifugal Filters (10 kDa, Merck). The product RESCA-T4/RT4 was stored at four °C for subsequent labeling experiments. As for ^{18}F-labeling, the QMA column was sequentially activated using 5 mL of sterile water for injection, followed by 5 mL of air, 5 mL of 0.9% saline, and another 5 mL of air. The cyclotron passed 500 μL of water enriched with ^{18}F ions through the QMA column. The filtrate was discarded, and 500 μL of 0.9% saline was passed through the QMA column. The filtrate was collected, and the process was repeated twice. The activity was measured to be 462.5 MBq. Then, four μL of a two mM $AlCl_3$ solution was added, and the column was mixed thoroughly and allowed to stand for 5 minutes. At the end, 200 μg of RESCA-T4/RT4 was sequentially added, followed by mixing with 400 μL of 0.1 M CH_3COONH_4 solution. The reaction was carried out for 12 minutes at room temperature. Meanwhile, the PD-10 column was pre-equilibrated with 20 mL of 0.9% NaCl. The reaction solution was added to the PD-10 column and replenished to 2.5 mL using an elution solution. Then, 0.5 mL was added each time for elution in a total of 5 tubes, and the radioactivity

of the tracer was measured separately. [^{68}Ga]Ga-NOTA-T4 was prepared as before (*1*). We tested the final product's radiochemical purity (RCP) by instant thin-layer chromatography (iTLC; Eckert & Ziegler Radiopharma Inc.). The stability of [^{18}F]AlF-RESCA-T4 was tested at three-time points (30 min, one hour, and two hours) in 0.9% NaCl and 20% FBS.

Preclinical small animal imaging and ROI analysis

Tracers (4.12 ± 0.18 MBq of [^{18}F]AlF-RESCA-T4 containing 1.78 ± 0.08 μg of T4, 5.71 ± 0.35 MBq of [^{18}F]AlF-RESCA-RT4 containing 2.47 ± 0.15 μg of RT4, and 4.05 ± 0.31 MBq of [^{68}Ga]Ga-NOTA-T4 containing 9.39 ± 0.71 μg of T4) were injected into the lateral tail veins of the tumor-bearing mice. MicroPET/CT imaging was completed at specific time points (45 min or 2.5 h) after the tracer injection. The mice were anesthetized with isoflurane for 15 min before scanning and then scanned for 10 min. The T4 and RT4 blocked groups were co-injected with 400 μg of unlabeled T4 or RT4 possessing the same activity. Data were collected following the completion of imaging at the designated time points. The images were reconstructed using a nonscatter-corrected 3D-ordered subset expectation optimization/maximum a posteriori (OSEM3D/MAP) algorithm. Data was analyzed using OsiriX Lite software (Pixmeo SARL) and Inveon Research Workplace (Siemens Preclinical Solutions). The region of interest (ROI) of the tumor and major organs, including the heart, lung, liver, kidney, bone, and muscle, were outlined using the software, and quantitative values were presented in terms of the percentage of injected dose per gram of tissue (%ID/g).

***Ex vivo* biodistribution and immunohistochemistry**

Mice were euthanized after the immunoPET/CT imaging. The tumor and major organs or tissues

were collected and weighed, including blood, skin, muscle, bone, heart, lung, liver, kidney, spleen, pancreas, stomach, intestine, and brain. Then, the tissues were counted using an automated gamma counter (PerkinElmer, WIZARD2 2470). Tumors and surgically collected tissues were fixed, and immunohistochemistry was stained with Trop2 antibody (sc-376746, Santa Cruz Biotechnology) based on the standard protocols.

Pilot clinical PET/CT imaging in patients

The Institutional Review Board of Huashan Hospital, Fudan University, provided ethical permission for the clinical trial included in the study (2023-1017). The research was documented as an upcoming clinical trial (ClinicalTrials.gov Identifier: NCT06203574). The WMA Declaration of Helsinki principles and the Department of Health and Human Services Belmont Report were both followed by clinical imaging studies. The inclusion criteria were as follows: Being between 18 and 80 years of age and of either sex; presented with suspected solid tumors; imaging revealed suspicious lymph nodes or distant metastases; informed consent must be signed in writing by the subject or their legal guardian or caregiver; willingness and ability to cooperate with all programmers of this study. The exclusion criteria were as follows: severe hepatic and renal insufficiency; renal function: serum creatinine more than or equal to the upper limit of the normal range; liver function: bilirubin, glutamic pyruvic transaminase or glutamic oxaloacetic transaminase more than or equal to the upper limit of the normal range. All participants signed a consent form and were informed that this was a diagnostic clinical experiment. The participants were aware of their group assignment because the study was not blinded. No additional special preparation was required for the [^{18}F]AlF-RESCA-T4 PET/CT examination. The patients received

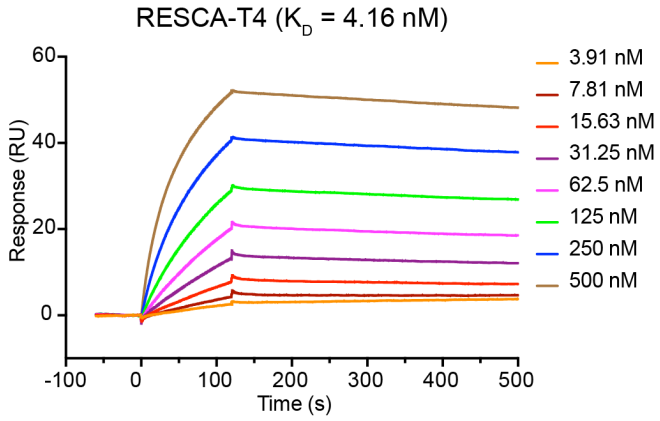
an average dose of [^{18}F]AlF-RESCA-T4 of 157.87 ± 11.30 MBq (n = 3). The occurrence of adverse reactions within 4 hours of the injection of [^{18}F]AlF-RESCA-T4 was monitored. Static PET/CT imaging was conducted 45 minutes after injection using a PET/CT scanner (uMI510, United Imaging, Shanghai, China). A series of [^{18}F]AlF-RESCA-T4 PET/CT scans were conducted, commencing at the cranial region and extending to the upper thigh. One patient underwent a conventional ^{18}F-FDG PET/CT scan. The acquired data were subsequently transferred to a dedicated workstation (United Imaging, Shanghai, China) for analysis. The standard ordered subset expectation maximization (OSEM) algorithm was employed for image reconstruction. For quantitative assessment, the maximum standardized uptake value (SUVmax) was used to quantify the uptake of radiopharmaceuticals by the lesion.

Statistical analysis

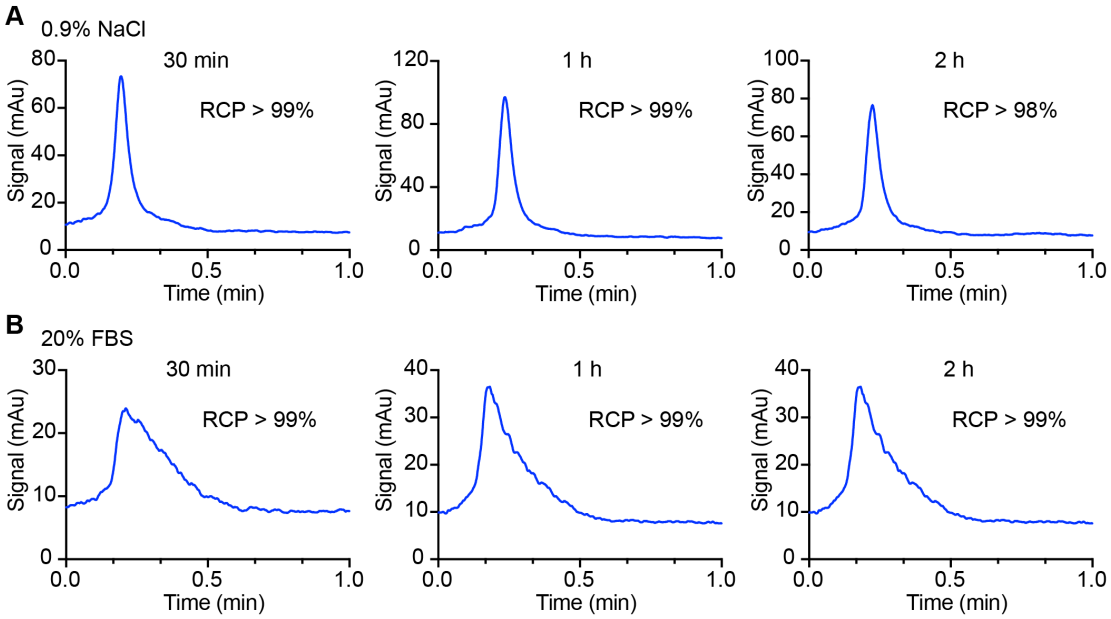
Statistical analysis was performed using Prism software (Version 10.0, GraphPad Software) and presented as means ± standard deviation (SD). Significance was determined using the two-tailed unpaired *t*-test and two-way analysis of variance (ANOVA). The differences between the two groups were considered significant when $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$ were present.

Data availability

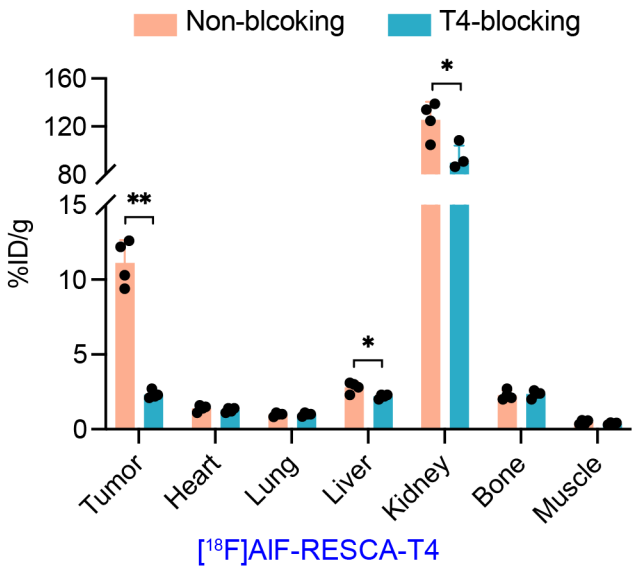
The data generated in this study are available upon request from the corresponding author.



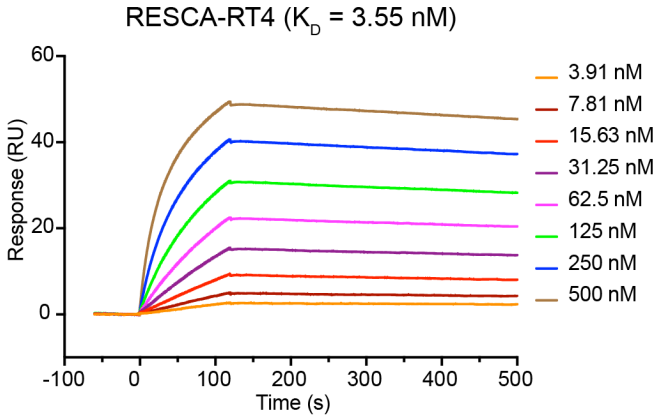
Supplemental Figure 1. SPR test result of RESCA-T4 interacting with recombinant human Trop2 protein.



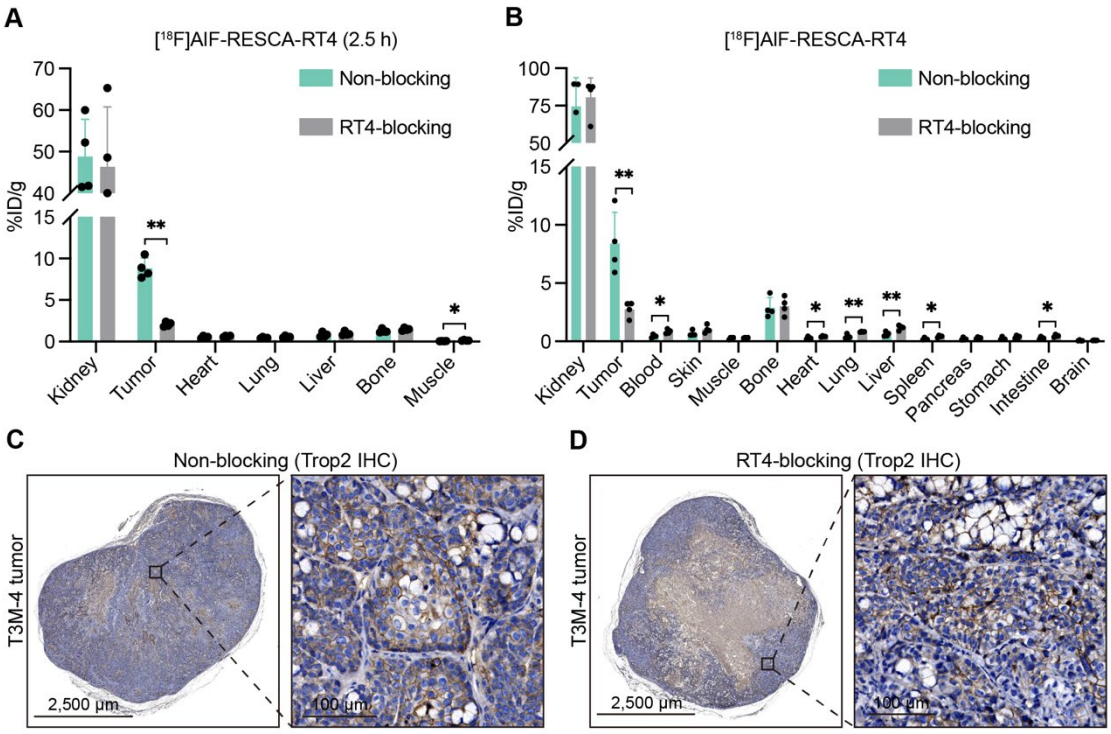
Supplemental Figure 2. The stability of [^{18}F]AlF-RESCA-T4 at 30 min, one hour, and two hours in 0.9% NaCl and 20% FBS.



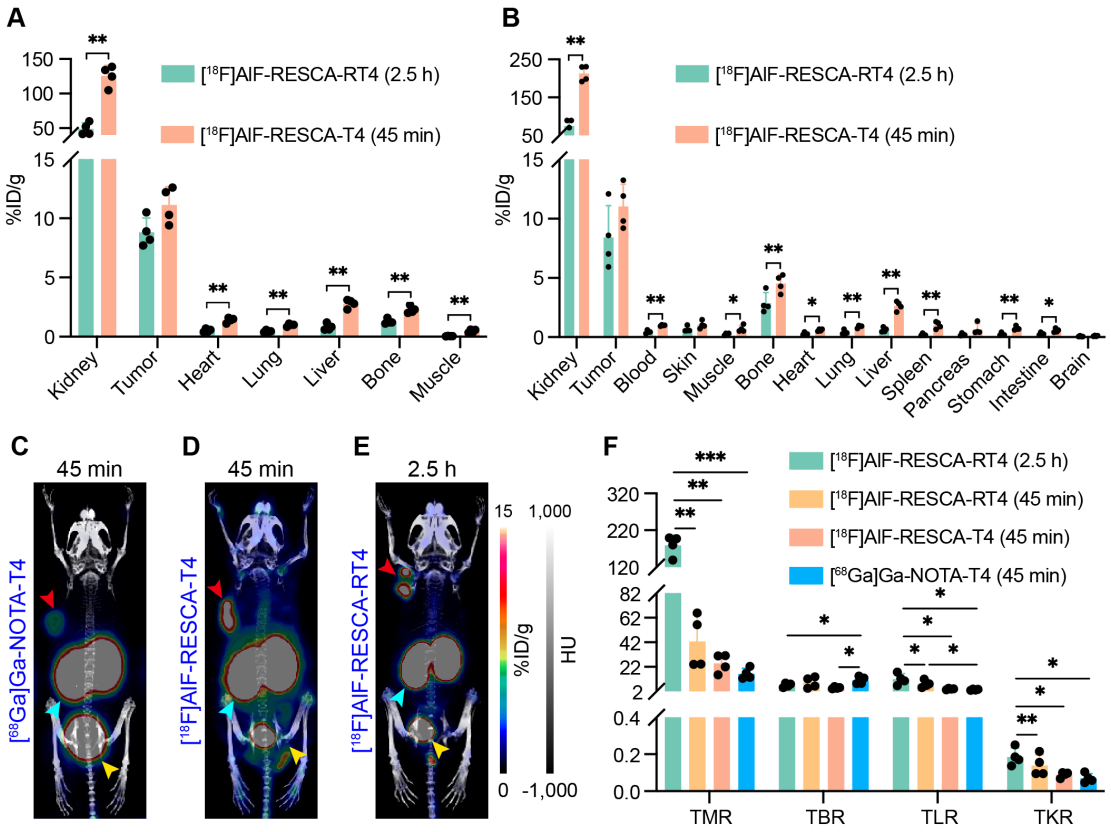
Supplemental Figure 3. ROI analysis of the non-blocking and T4-blocking groups after [^{18}F]AlF-RESCA-T4 immunoPET/CT imaging. *$P < 0.05$, **$P < 0.01$.



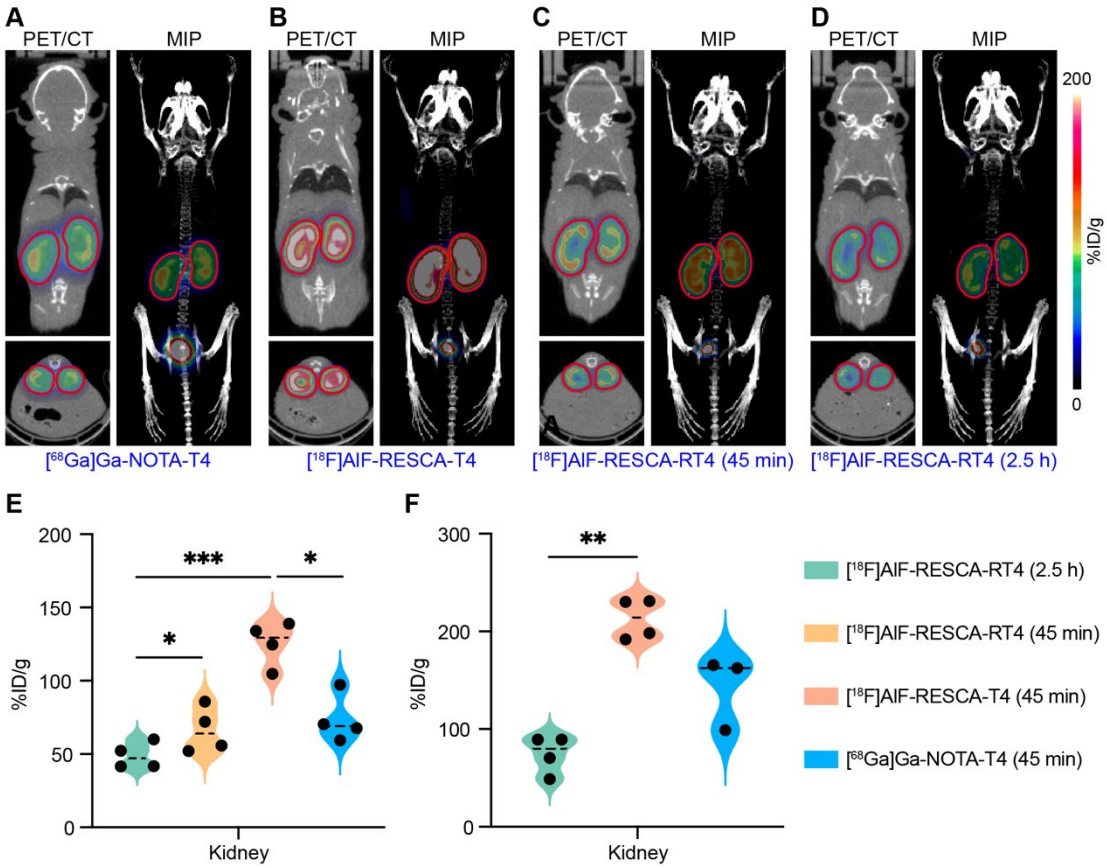
Supplemental Figure 4. SPR test results of RESCA-RT4 interacting with recombinant human Trop2 protein.



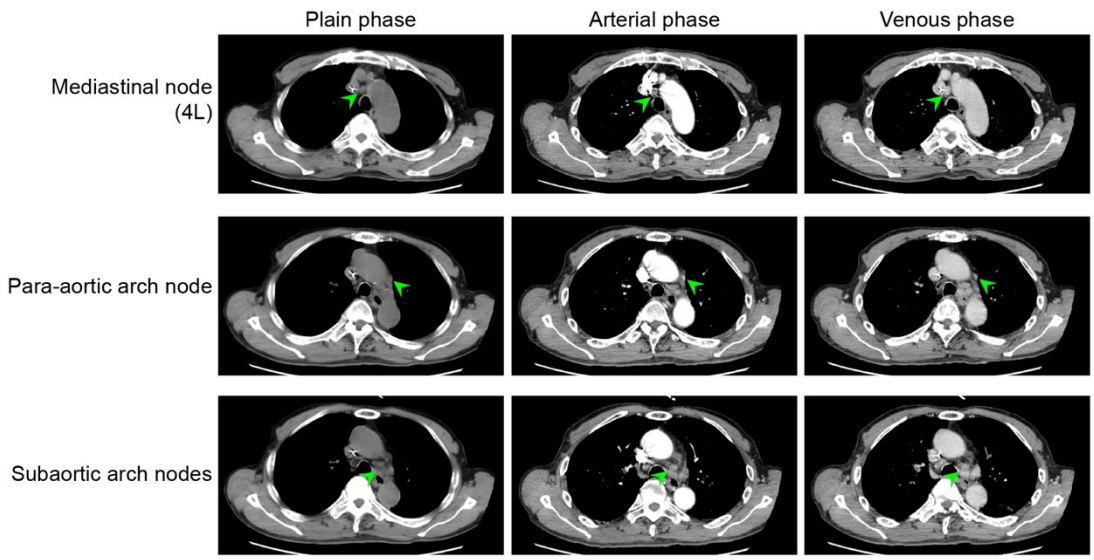
Supplemental Figure 5. [^{18}F]AlF-RESCA-RT4 bound specifically with Trop2. (A) Comparison of the ROI results of the non-blocking group with the RT4-blocking group at 2.5 h p.i. (B) Biodistribution data of two groups after [^{18}F]AlF-RESCA-RT4 immunoPET/CT imaging. (C and D) Immunohistochemistry staining of the fixed tumor tissues using Trop2 antibody. *P < 0.05, **P < 0.01.



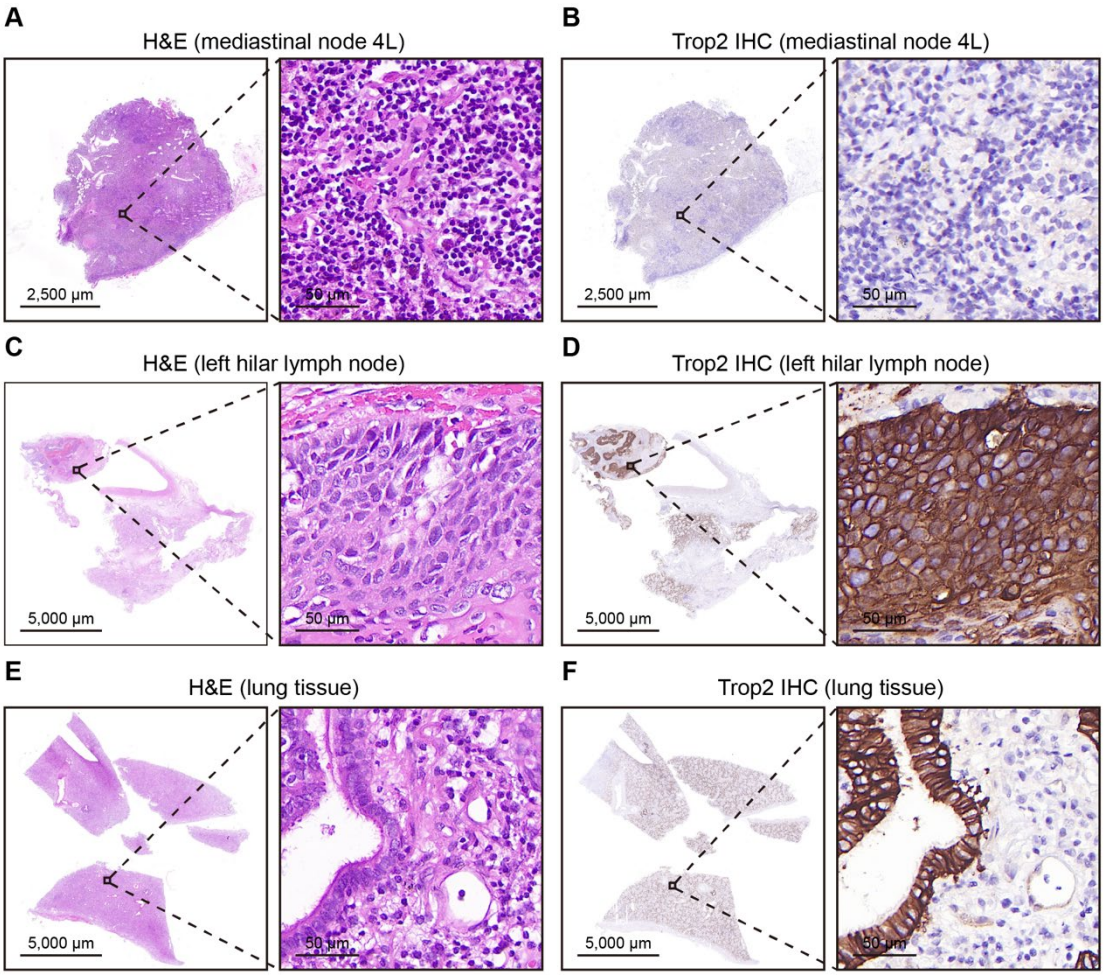
Supplemental Figure 6. Comparison of tumor uptake and pharmacokinetics of three tracers. (A and B) Comparison of ROI analysis (A) and biodistribution (B) results for [^{18}F]AlF-RESCA-RT4 and [^{18}F]AlF-RESCA-T4. (C–E) MIP images of three probes ([^{68}Ga]Ga-NOTA-T4, [^{18}F]AlF-RESCA-T4, and [^{18}F]AlF-RESCA-RT4) at 45 min or 2.5 h p.i. (F) Comparison of the tumor-to-organ ratios of the three tracers. Tumor-to-muscle ratio (TMR), tumor-to-bone ratio (TBR), tumor-to-liver ratio (TLR), and tumor-to-kidney ratio (TKR). Red arrowheads: tumors, blue arrowheads: kidneys, and yellow arrowheads: bladders. *$P < 0.05$, **$P < 0.01$.



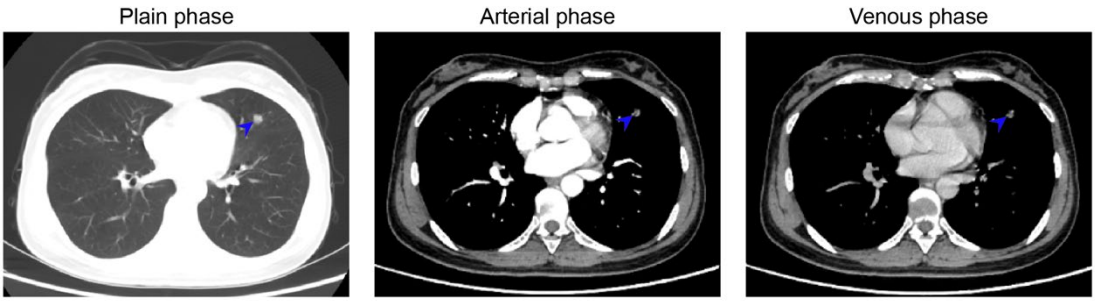
Supplemental Figure 7. Comparison of renal radioactivity accumulation with three tracers. (A–D) The PET/CT and maximum intensity projection (MIP) images of three tracers ([^{68}Ga]Ga-NOTA-T4, [^{18}F]AlF-RESCA-T4, and [^{18}F]AlF-RESCA-RT4). Red outlines showed the kidneys. (E and F) Quantitative results of renal radioactivity accumulation in ROI and biodistribution results for three probes. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$.



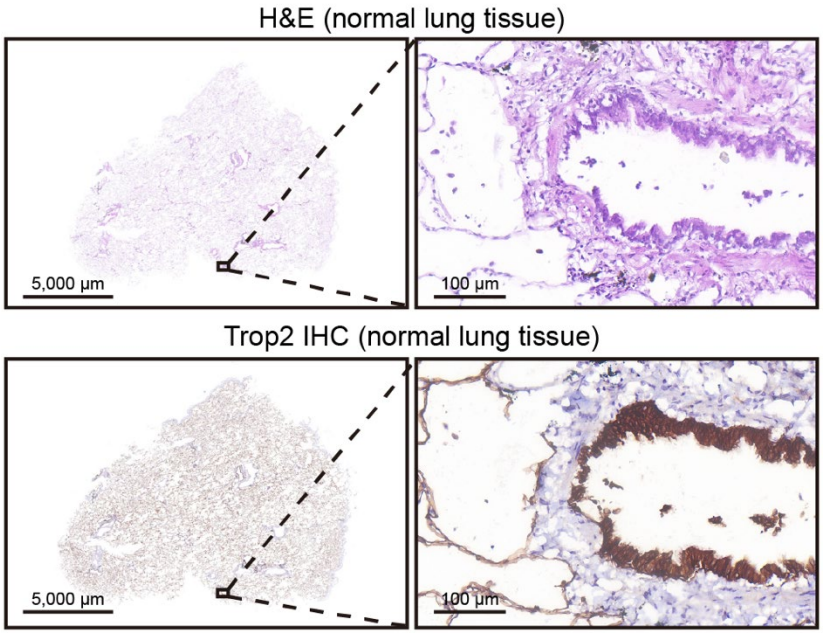
Supplemental Figure 8. Preoperative enhanced chest CT image of Patient #1. Images show enlarged mediastinal node (4L), para-aortic arch node and subaortic arch nodes in plain phase, arterial phase, and venous phase.



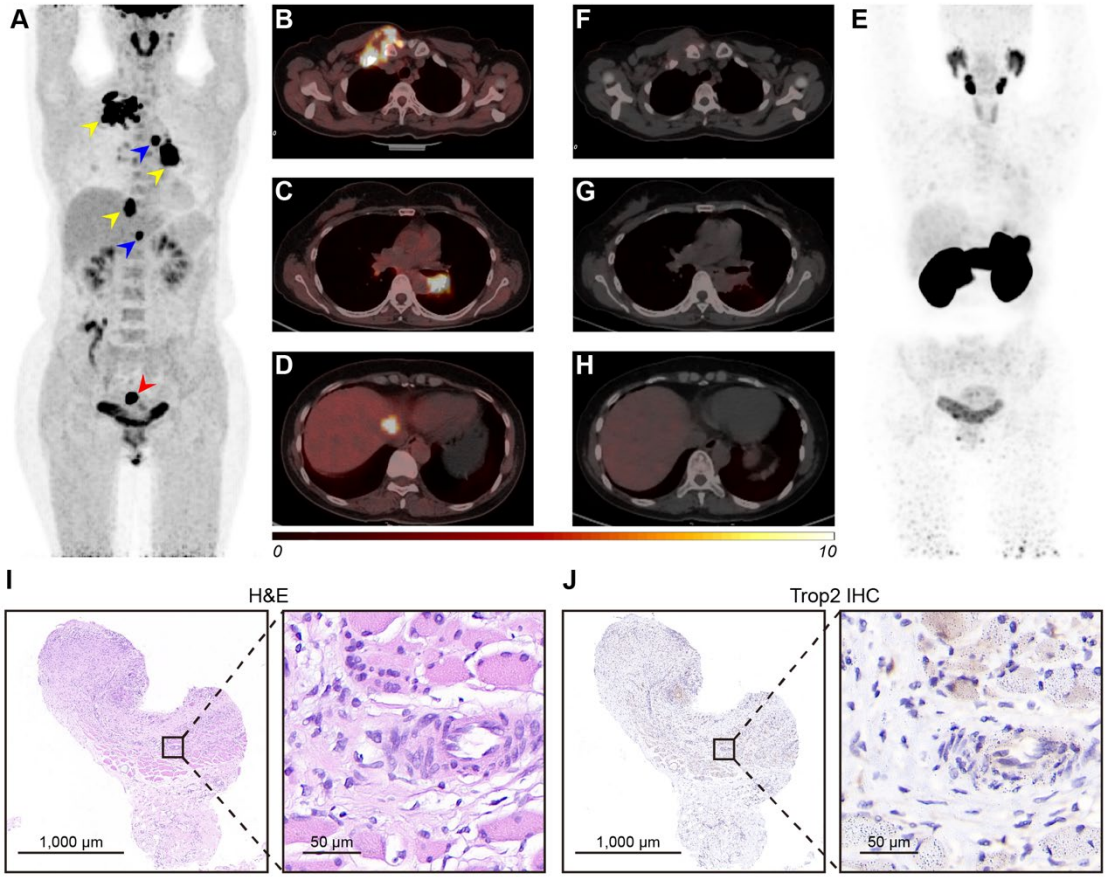
Supplemental Figure 9. H&E and Trop2 immunohistochemistry staining results of mediastinal node (4L) left hilar lymph node and lung tissue of Patient #1.



Supplemental Figure 10. Preoperative enhanced chest CT findings in patient #2. The blue arrowheads show the nodule in the left lung.



Supplemental Figure 11. H&E and Trop2 immunohistochemistry staining results of normal lung tissue from Patient #2.



Supplemental Figure 12. ^{18}F-FDG PET/CT and [^{18}F]AlF-RESCA-T4 immunoPET/CT imaging in a patient with tuberculosis. (A) MIP and (B−D) fusion images of ^{18}F-FDG PET/CT of the patient. Yellow arrowheads show three major masses in fusion images. Mediastinum and retroperitoneal lymph nodes are indicated by blue arrowheads, and a uterine nodule by a red arrowhead. (E) MIP image and (F−G) fusion images of [^{18}F]AlF-RESCA-T4 immunoPET/CT. (I) H&E and (J) Trop2 staining of the biopsied right front chest wall mass show epithelioid cells and Langhans giant cells with negative Trop2 staining.

References:

1. Huang W, Zhang Y, Cao M, et al. ImmunoPET imaging of Trop2 in patients with solid tumours. *EMBO Mol Med.* 2024;16:1143-1161.