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Supplementary information

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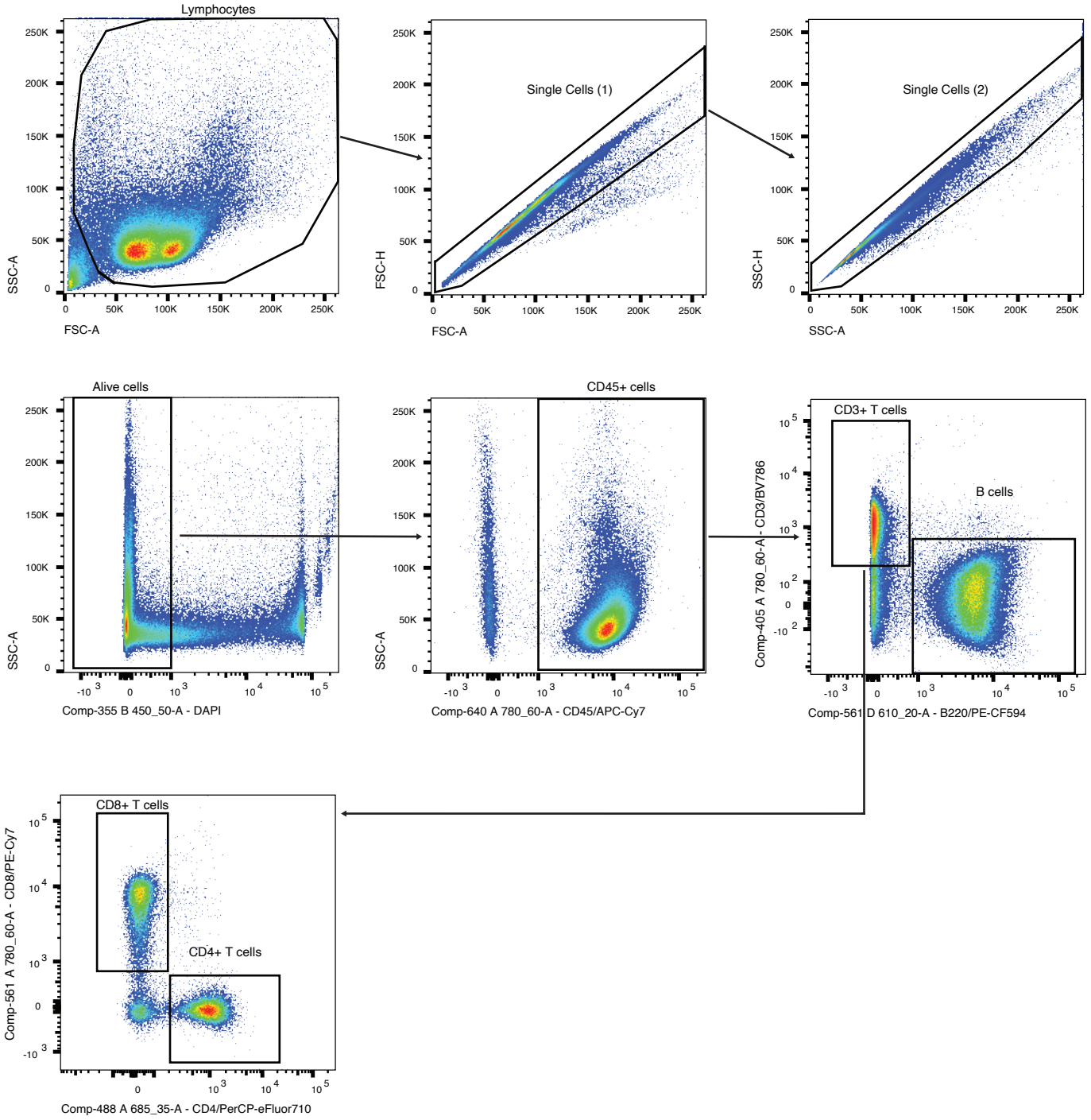
Antibody–peptide conjugates deliver covalent inhibitors blocking oncogenic cathepsins

In the format provided by the authors and unedited

Supplementary Figure



Supplementary Figure 1. Gating strategy used to determine the percentage of CD45+ cells, B cells, CD8+ T cells and CD4+ T cells by flow cytometry analysis of the splenocytes of treated vavP-Bcl2 mice (related to Figure 5g,h).

Supplementary Table 1. X-ray data collection and refinement statistics.

	Cathepsin S mutant + NNPI-C10	Cathepsin S WT + NNPI-C10
Data collection		
Space group	*P* 42 21 2	*P* 21 21 2
a, b, c (Å)	87.71, 87.71, 69.44	44.63, 129.22, 37.67
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	43.85-1.73 (1.82-1.73)	42.19-1.56 (1.64-1.56)
R_{meas}	0.12 (2.71)	0.143 (1.46)
Mean I/σI	13.1 (0.74)	8.67 (1.31)
Completeness (%)	99.9 (99.3)	99.8 (99.7)
Multiplicity	8.5 (8.1)	5.7 (5.4)
CC1/2	0.99 (0.324)	0.997 (0.665)
Refinement		
Resolution (Å)	43.85-1.73	42.19-1.56
Total reflections	460653	344424
R_{work}[#]	0.1761 (0.3881)	0.1708 (0.3365)
R_{free}[#]	0.2059 (0.4062)	0.2251 (0.3663)
Number of non-hydrogen atoms	1928	1946
macromolecules	1781	1775
ligands	82	61
solvent	65	110
protein residues	226	226
RMS deviations (bonds)[#]	0.008	0.006
RMS deviations (angles)[#]	0.90	0.81
Ramachandran favored (%)[#]	97.73	97.73
Ramachandran allowed (%)[#]	2.27	2.13
Ramachandran outliers (%)[#]	0	0
Rotatmer outliers (%)[#]	1.59	2.13
Clashscore[#]	4.10	4.00
Average B-factor[#]	38.85	27.96
macromolecules	38.08	26.79
ligands	55.38	59.07
solvent	39.11	29.56
PDB	**8PI3**	**8RND**

[#]as defined by phenix.table_one and phenix.model_vs_data

Supplementary Methods

Proteins expression and purification

CTSS

Cathepsin S (CTSS) production and purification was performed as previously reported[1]. The lysosomal localization signal (amino acids 17 to 331) was removed from the *CTSS* gene sequence by amplifying it from pLVX plasmids expressing WT or Y132D CTSS using the following primers:

CTSS_pHLsec_fw: GCTGAAACCGGTCAGTTGCATAAAGATCCTAC,

CTSS_pHLsec_rev: GTGCTTGGTACCGATTTCTGGGT AAGAGGGAAAGC.

AgeI and Kpn1 restriction enzymes (NEB, cat #R3552S and #R3142S) were used to digest the PCR products obtained, which were then cloned in pHLsec plasmids having a His-tag sequence at the C-terminal of the MCS and a secretion signal at the N-terminal of the MCS . HEK293 cells were transfected with the pHLsec-CTSS plasmids, using PEI (polyethyleneimine) in presence of VPA (valproic acid). Proteins were purified from the supernatant using a 5 ml His-Trap FF column on an AKTA pure system (GE healthcare). A 300 mM imidazole solution was used to elute bound proteins. Size exclusion chromatography using a HiLoad 16/600 Superdex 75 pg column (GE Healthcare) and PBS as mobile phase was performed to further purify the concentrated proteins. The proteins were then quantified by Nanodropand stored at -80°C, after dilution in PBS and aliquoting.

Antibodies

For antibody production, transient transfection of ExpiCHO or HEK293 cells with the light chain and heavy chain encoding plasmids was performed in ProCHO5 + 2%

DMSO. After 6 days, the filtered supernatant was incubated with Mab select beads (Amersham BioScience, cat #17-5199-03) and left rotating overnight at 4°C. The beads were then loaded onto a disposable column and washed thoroughly with 20 CV of PBS. Elution was performed with 5 CV of Glycine 0.1 M pH 3, buffered with Tris 1.5 M pH 8. Next, dialysis in PBS 1x was performed, and then the antibody was concentrated with an Amicon 30 kDa column. Subsequently, it was loaded on a Superdex 200 HiLoad 16/600 (1 x 5 ml), run in PBS, and peak fractions were pooled together. The antibody was then sterile-filtered through a 0.22 µm mesh.

FRET assays

Human and murine cathepsin S (CTSS)

For FRET experiments, human CTSS-His WT and Y132D, as well as murine CTSS-His WT were diluted to 10 µg/ml in Assay buffer (50 mM NaOAc, 5 mM DTT, 250 mM NaCl), at pH 4.5 and incubated at 37°C for 90 minutes to allow auto-activation. The proteins were then diluted to 1 µg/ml in Assay buffer and plated in low binding 96-well plates (Corning, cat #3474) (50 µl/well). Small molecules or Non-Natural Peptide inhibitors (NNPI) were added at the indicated concentration and incubated with cathepsin S for 30 minutes. The substrate peptide DNP-KLRMKLPKP-MCA was produced by the PPCF Facility (UNIL) and diluted to 40 µM in Assay Buffer, then added to the activated CTSS (50 µl/well). A substrate blank containing 50 µl of Assay Buffer and 50 µl of 40 µM substrate peptide without any CTSS was included. MCA fluorescent emission was measured with SpectraMax iD3 (Molecular Devices) in kinetic mode for 120 minutes (excitation: 330 nm, emission: 390 nm).

Cathepsin B (CTSB)

Commercially available human CTSB purified protein (R&D systems, cat #953-CY-010) was activated and used following the protocol suggested by the manufacturer. A different specific substrate was used, and the final concentrations of enzyme and substrate were adapted to improve signal detection. In brief, CTSB was diluted to 10 µg/ml in Activation Buffer (25 mM MES, 5 mM DTT, pH 5.0) and incubated at room temperature for 15 minutes. After activation, CTSB was diluted to 1 µg/ml in Assay Buffer (25 mM MES, pH 5.0). The substrate peptide DNP-KPPKPVSD-MCA was produced by the PPCF Facility (UNIL) and diluted to 40 µM in Assay buffer. Next, 50 µl of the 1 µg/ml CTSB was loaded in a 96-well plate, inhibitors to be tested were added to the enzyme and incubated for 30 minutes, and then the reaction was started by adding 50 µl of 40 µM substrate. A substrate blank containing 50 µl of Assay Buffer and 50 µl of 40 µM substrate without any CTSB was included. Fluorescent emission was measured with SpectraMax iD3 (Molecular Devices) in kinetic mode for 120 minutes (excitation: 330 nm, emission: 390 nm).

Cathepsin H (CTSH)

Commercially available human CTSH purified protein (R&D systems, cat #7516-CY) was activated and used following the protocol suggested by the manufacturer. A different specific substrate was used, and the final concentrations of enzyme and substrate were adapted to improve signal detection. In brief, CTSH was diluted to 200 µg/ml in Activation Buffer (50 mM MES, 10 mM CaCl2, 150 mM NaCl, pH 6.5), while thermolysin (R&D systems, cat #3097-ZN) was diluted to 100 µg/ml in Activation Buffer. Equal volumes of CTSH and thermolysin were mixed and then incubated at room temperature for 3 hours. The reaction was stopped by adding an equal volume of 2 mM Phosphoramidon (Tocris, cat #6333) in Assay Buffer (50 mM MES, pH 6.5)

to the reaction mixture, and incubating at room temperature for 10 minutes. Next, an equal volume of Assay Buffer containing 20 mM DTT was added, and the mixture was incubated at room temperature for 5 minutes. After activation, CTSH was diluted to 1 µg/ml in Assay Buffer. The substrate peptide DNP-KLRMKLPKD-MCA was produced by the PPCF Facility (UNIL) and diluted to 40 µM in Assay buffer. Next, 50 µl of the 1 µg/ml CTSH was loaded in a 96-well plate, inhibitors to be tested were added to the enzyme and incubated for 30 minutes, and then the reaction was started by adding 50 µl of 40 µM substrate. A substrate blank containing 50 µl of Assay Buffer and 50 µl of 40 µM substrate without any CTSH was included. Fluorescent emission was measured with SpectraMax iD3 (Molecular Devices) in kinetic mode for 120 minutes (excitation: 330 nm, emission: 390 nm).

Cathepsin K (CTSK) and cathepsin L (CTSL)

Commercially available human CTSK (Sigma-Aldrich, cat #219461-25UG) and CTSL purified proteins (R&D systems, cat #952-CY-010) were activated and used for the assay following the protocol suggested by the manufacturer for CTSL. In brief, CTSK or CTSL was diluted to 20 µg/ml or 40 µg/ml respectively, in Assay Buffer (50 mM MES, 5 mM DTT, 1 mM EDTA, 0.005% (w/v) Brij-35, pH 6.0), and incubated on ice for 15 minutes. After activation, CTSK or CTSL was diluted to 0.02 µg/ml in Assay Buffer. The substrate peptide Z-Leu-Arg-AMC (R&D systems, cat #ES008) was then diluted to 20 µM in Assay buffer (40 µM for CTSL). Next, 50 µl of the enzyme solution was loaded in a 96-well plate, inhibitors to be tested were added to the enzyme and incubated for 30 minutes, and then the reaction was started by adding 50 µl of the fluorogenic substrate. A substrate blank containing 50 µl of Assay Buffer and 50 µl of substrate without any cathepsin was included. Fluorescent emission was measured

with SpectraMax iD3 (Molecular Devices) in kinetic mode for 60 minutes (excitation: 380 nm, emission: 460 nm).

Cathepsin Z (CTSZ)

Commercially available human CTSZ purified protein (R&D systems, cat #934-CY) was activated and used following the protocol suggested by the manufacturer. In brief, CTSZ was diluted to 10 µg/ml in Assay Buffer (25 mM Sodium Acetate, 5 mM DTT, pH 3.5) and incubated at room temperature for 5 minutes. After activation, CTSZ was further diluted to 0.4 µg/ml in Assay Buffer. The substrate peptide Mca-RPPGFSAFK(Dnp)-OH (R&D systems, cat #ES005) and diluted to 20 µM in Assay buffer. Next, 50 µl of the 0.4 ug/ml CTSZ was loaded in a 96-well plate, inhibitors to be tested were added to the enzyme and incubated for 30 minutes, and then the reaction was started by adding 50 µl of 20 µM substrate. A substrate blank containing 50 µl of Assay Buffer and 50 µl of 20 µM substrate without any CTSZ was included. Fluorescent emission was measured with SpectraMax iD3 (Molecular Devices) in kinetic mode for 60 minutes (excitation: 320 nm, emission: 405 nm).

Cathepsin C (CTSC)

Commercially available human CTSC purified protein (R&D systems, cat #1071-CY) was activated and used following the protocol suggested by the manufacturer. In brief, CTSC was diluted to 200 µg/ml in Activation Buffer (25 mM MES, 5 mM DTT, pH 6.0) and mixed 1:1 to CTSL previously diluted to 40 µg/ml in Activation buffer. The mixture was incubated at room temperature for 1 hour. After activation, CTSC was diluted to 0.5 µg/ml in Assay Buffer (25 mM MES, 50 mM NaCl, 5 mM DTT, pH 6.0). The substrate Gly-Arg-AMC (Bachem, Catalog # I-1215) was diluted to 20 µM in Assay buffer. Next, 50 µl of the 0.5 µg/ml CTSC was loaded in a 96-well plate, inhibitors to

be tested were added to the enzyme and incubated for 30 minutes, and then the reaction was started by adding 50 µl of 20 µM substrate. A substrate blank containing 50 µl of Assay Buffer and 50 µl of 20 µM substrate without any cathepsin was included. Fluorescent emission was measured with SpectraMax iD3 (Molecular Devices) in kinetic mode for 60 minutes (excitation: 380 nm, emission: 460 nm).

Mass spectrometry analysis of APICs

An Orbitrap Q-Exactive HF instrument with BioPharma option (Thermo Fisher Scientific, Waltham, MA, USA) operating in the high mass range was used for the mass spectrometry analysis of APICs. Protein Deconvolution Software (Thermo Fisher Scientific, Waltham, MA, USA) was utilized to deconvolve the mass spectrometry spectra. In addition, ultra-performance liquid chromatography was performed. Separation was performed on a MAbPAC SEC-1 column, 5 µm, 300 Å, 4 x 150 mm (Thermo Fisher Scientific, Waltham, MA, USA) with ammonium acetate 50 mM pH 7.0 at 0.3 ml/min as mobile phase. Since the mass of the unconjugated antibody was known, by observing the main peaks of APIC, an average number of NNPI molecules linked to the antibody was calculated.

Western Blot to test APIC binding to CTSS

After activating CTSS as described above, by diluting it in activation buffer to a concentration of 75 µg/ml (2 µM), the enzyme was incubated with a 3.3x molar excess of APIC for 30 min at RT. Unconjugated antibody and NNPI-C10 were mixed with CTSS as negative control. As additional control, CTSS was pre-incubated with E64 (150 µM) before mixing it with CTSS. After addition of reducing loading buffer, all samples were then heated for 5 min at 95°C and run on a SDS-PAGE gel (4-15% Mini-

PROTEAN TGX Stain-Free Protein Gel, #4568084). Proteins were then transferred to a nitrocellulose membrane by semi-dry transfer, for 37 min at 20 V. After blocking in 5% milk (Applichem, cat #A0830) in PBS-0.1%Tween (Fisher scientific, cat #BP337), the membrane was incubated at 4 °C overnight in 5% milk in PBS-Tween with an anti-his-Tag primary antibody (Cell Signaling, cat #D3I10, diluted 1:1000).

The membrane was then washed for 30 minutes and incubated with a fluorescent goat anti-Rabbit secondary IgG antibody (LI-COR, cat #926-32211, diluted 1:10'000). Image acquisition was performed with LI-COR Odyssey CLx.

X-ray analysis of CTSS crystal structure

Crystallization of human CTSS WT and Y132D alone or with C10

Crystals were prepared by the sitting-drop vapor diffusion method. To study the structure of the complex CTSS-NNPI-C10, the enzyme at 240 µM was mixed with NNPI-C10 at a final concentration of 2 mM. 200 nL of protein and 200 nL of reservoir were combined in an MRC SD2 crystallization plate using a Mosquito crystallization robot. Crystals were discovered after 2-4 weeks equilibration against a reservoir solution consisting of 0.2 M ammonium sulfate, 0.01 M Cadmium chloride hemi(pentahydrate), 0.1 M Pipes pH 7, 15% PEG Smear Broad, 10% Ethylene glycol, at 277K.

Data collection and structure refinement

Crystals were cryopreserved by briefly soaking in reservoir solution overlain with Fomblin-Y and supplemented with 30% sucrose. Crystals were flash cooled by direct immersion in liquid nitrogen. A diffraction data set was collected at the Se-peak wavelength (0.979420 Å) to 1.9 Å resolution at beamline 23-ID-B, at the Advanced

Photon Source. The data were processed with XDS. Crystals belonged to the space group P42212 with cell dimensions a = 87.7 Å, b = 87.7 Å, c =69 Å. Matthews calculations predicted the asymmetric unit of the crystal to contain one molecule, with a solvent content of ~66.5%. The structure was solved by a combination of Se-SAD phasing in Phenix and molecular replacement with PHASER. Refinement was performed with Phenix. Iterative cycles of model building with Coot and restrained refinement were performed. The final model contains one molecule in the asymmetric unit with all residues and no Ramachandran outliers. Statistics from data processing and model refinement are shown in **Supplementary Table 1**. The atomic models have been deposited to the Protein Data Bank (PDB, https://www.wwpdb.org/) under accession codes 8PI3 and 8RND. Figures were prepared using PyMOL (The PyMOL Molecular Graphics System, Schrödinger, LLC.).

Generation of Syngeneic Cell Lines by CRISPR/Cas9

CTSS KO cell lines were obtained using the plasmids pSPCas9(BB)-2A-GFP (PX458) (Addgene, cat #48138) expressing GFP, Cas9 and a gene-specific gRNA, which was cloned using the BbsI (NEB, cat #R0539) or BsmBI (NEB, cat #R0580) restriction sites. Raji and WSU-DLCL2 cells were electroporated with the CRISPR plasmid using the NEPA21 electroporator (Nepagene) and single-cell cloning of GFP+ cells was performed by FACS 48 h after electroporation. 10-20 days after sorting, clones negative for human CTSS were screened by Western blot.

The primers used for gRNA cloning were the following:

hCTSS-gRNA-For: CACCGTTGCATAAAGATCCTACCC

hCTSS- gRNA-Rev: AAACGGGTAGGATCTTTATGCAAC

Biodistribution and PET/SPECT imaging

p-SCN-Bn-NOTA conjugation

αCD22 and αCD79 APICs were conjugated with p-SCN-Bn-NOTA (Macrocyclics, Plano, Texas, USA; M_W: 559.9 Da). Prior to the coupling procedure, the antibodies were conditioned at 5 mg/ml in carbonate buffer 0.2 M pH 9.0 by ultrafiltration (Amicon Ultra, 0.5 ml, 50 kDa, Merck). A solution of p-SCN-Bn-NOTA was prepared at 1 equivalent per µl in 0.2 M pH 9 carbonate buffer containing 10% DMSO (V/V). Twenty equivalents of p-SCN-Bn-NOTA solution was added to 1 mg of antibody. Antibody coupling solution was incubated for 1 h at 37 °C and the conjugated antibodies were washed by four ultrafiltrations using PBS pH 7.4 before. Chemical purity of the conjugates was controlled by high-pressure liquid chromatography (HPLC). Conjugated antibodies were subsequently stored between 2-8°C.

Mass spectrometry analysis

Mass spectrometry analysis of NOTA-conjugated APICs was performed as described above for APICs. Knowing the mass of the APIC and the average mass distribution of the NOTA-conjugated APIC, the average number of chelators bound to the APIC was calculated.

Radiolabeling

Radiolabelings with 111In ([^{111}In]indium chloride in 0.02 M HCl solution, B.E:Imaging, Switzerland) of NOTA-conjugated αCD22 and αCD79 APICs were performed in parallel. Prior radiolabeling the antibodies were conditioned in 0.4 M pH 4.6 by ultracentrifugation. NOTA-αCD22 and NOTA-αCD79 were added to 111In in sodium acetate buffer 0.4 M pH 5.6 for 1 hour incubation time at 37 °C. Radiolabeling with

64Cu ([^{64}Cu]copper chloride in 0.1 M HCl solution, ARRONAX, Saint Herblain, France): αCD79 APIC antibody conditioned in sodium acetate buffer 0.4 M pH 4.6 was added to the 64Cu in sodium acetate 2.5 M buffer and incubated at 42 °C for 30 minutes. Free 64Cu was then quenched with an excess of EDTA 1 mM. For both radiolabelings, the radiochemical purity was assessed by instant thin layer chromatography (iTLC) and by radio-HPLC.

Radio-HPLC

HPLC analyses were performed as previously reported[2]. Briefly, a Shimadzu System (Prominence Liquid Chromatograph, Shimadzu Europe, Duisburg, Germany) coupled to a GabiStar detector (Raytest, Straubenhard, Germany) was used and compounds were separated with a size exclusion column, Xbridge protein BEH 200 Å SEC 3.5 µm, dimension 7.8 × 300 mm (Waters, Baden-Dättwil, Switzerland). The samples were eluted using phosphate buffer pH 6.8 (1 ml/min) as mobile phase and the process was monitored via absorbance at 220/280 nm or γ detection.

iTLC

iTLC analysis were performed using dried iTLC-SG Glass microfiber chromatography paper impregnated with silica gel (Agilent Technologies, Folsom, CA 95630). Citrate buffer 0.1 M pH 5 was used as mobile phase. Radiochromatogram profiles were obtained with a Scan-RAM TLC scanner (LabLogic, Sheffield, United Kingdom). 111In or 64Cu radiolabeled APICs remained at Rf = 0 while the unbound 111In or 64Cu migrated with the solvent front.

Murine xenograft model

Experiments were conducted in compliance with the cantonal authorization VD2993 and the guidelines of the Institution. Xenograft Raji tumors were established by subcutaneous injection of 2.5x10e6 Raji cells in the right shoulder of 6-10-week-old female NOD scid gamma mice (bred and housed at the Epalinges Animal Facility, Lausanne University, Switzerland). After 18 days of tumors growth animal were prepared for biodistribution and PET/SPECT/CT studies. Tumors never exceeded the maximal size permitted by the corresponding animal license (listed above), i.e. 1000 mm3.

Biodistribution

For radiolabeling with 111In, each mouse received 25 kBq of [111In]In-αCD22 or [111In]In-αCD79. Alternatively for radiolabeling with 64Cu, each mouse received 5 MBq of [64Cu]Cu-αCD79 containing in total 50 µg of APIC. Each radiotracer was administered through intraperitoneal injection, without anesthesia, and sterile filtration. For blocking experiments, cold APIC was co-administrated in excess (1 mg).

The animals injected with [111In]In-αCD22 or [111In]In-αCD79 were sacrificed 24 hours post injections; while animals that received [64Cu]Cu-αCD79 were sacrificed either 24 or 48 hours after the injection. Animals were euthanized by CO2 inhalation and exsanguination. Blood was collected, organs and tumors were excised, weighed, and counted with a gamma counter (Wizard 3”, Perkin Elmer, Waltham, Massachusetts). Results were expressed as the percentage of injected dose (ID) per gram of tissue (%ID/g).

PET/SPECT/CT imaging

For radiolabeling with 111In (SPECT imaging), mice received 50 MBq of [111In]In-αCD22 APIC or [111In]In-αCD79 APIC by intraperitoneal injection. For radiolabeling with 64Cu (PET imaging), mice received 25 Mbq of [64Cu]Cu-αCD79 APIC (for a total of 50 ug antibody) by intraperitoneal injection. Blocking experiments were done by co-injecting an excess of cold APIC (1 mg). Mice were anesthetized by inhalation of 1.5% isoflurane/O2 and placed on a heated bed. All imagings were acquired using a small-animal PET/SPECT/CT (Albira Si, Bruker Biospin Corporation, Woodbridge, Connecticut, USA). For SPECT imaging, 20 minutes static images were acquired at 2, 24, 48, and 72 hours after injection of the radiolabeled APIC. A 80 mm transversal field-of-view was chosen and a single pinhole collimator was used. For PET imaging, 10 minutes static images were acquired at 2, 24 and 48 hours post injection. Immediately after the SPECT and PET acquisitions, a CT was done for anatomical reference (400 µA intensity and 35 kV voltage, 600 projection). Acquisitions were reconstructed with the following algorithms: OSEM for SPECT, MLEM for PET and FBP for CT, using the software provided with the PET/SPETC/CT. Image preparation and analysis was done using the PMOD software (PMOD technologies, version 3.709, Zurich, Switzerland).

Western Blot analysis of cell lysates

Proteins were extracted using RIPA buffer supplemented with protease inhibitors (Sigma Aldrich, cat #11836153001) and phosphatase inhibitors (Roche, cat #04906837001) and quantified with the Bradford reagent (Bio-Rad, cat #5000006). 20-30 mg of proteins were resolved on SDS-PAGE and transferred at 20V for 20-30 minutes to Immobilon-P-membranes (Millipore). After blocking in 5% milk (Applichem,

cat #A0830) in PBS-0.1%Tween (Fisher scientific, cat #BP337), membranes were incubated overnight in 5% milk in PBS-Tween with the following primary antibodies: goat anti-human Cathepsin S (Bio-Techne, cat #AF1183, diluted 1:2'000), rabbit anti-human Cathepsin B (D1C7Y) XP Rabbit mAb (Cell Signaling, cat #31718), anti-His-Tag (D3I1O) XP rabbit mAb (Cell Signaling, cat #12698, diluted 1:1'000), mouse anti-human alpha-Tubulin (clone B-5-1-2, Sigma Aldrich, cat #T6074, diluted 1:10'000). Membranes were then washed for 30 minutes and incubated with the LI-COR fluorescent secondary antibodies diluted 1:10'000, *i.e.* IRDye 800CW goat anti-Rabbit IgG (LI-COR, cat #926-32211), IRDye 680RD goat anti-Mouse IgG (LI-COR, cat #926-68070), or with HRP-conjugated antibodies diluted 1:2'500, *i.e.* goat anti-rabbit antibody (H+L) (Merck Millipore, cat #AP307P), goat anti-mouse antibody (H+L) (Merck Millipore, cat #AP308P), rabbit anti-goat IgG (H+L) (ThermoFisher scientific, cat #31402). Image acquisition was performed with an Odyssey CLx Imager (LI-COR) for fluorescent antibodies or using WesternBright Sirius HRP Substrate (Advansta, cat. #K-12043-D10) for chemiluminescent antibodies.

T cell assay

Cell Purification

HA-specific TCR CD8+ T cells were isolated from the spleen of CL4 TCR transgenic mice (8-12 weeks old). The spleen was smashed, the cells were washed in PBS, resuspended in RBC lysis buffer (Miltenyi Biotec, cat #130-094-183) for 4 minutes at room temperature to lyse red blood cells, PBS was added to stop lysis and cells were filtered through a 40 µm filter (Falcon, #352350) to generate a single cell suspension. Single cell suspension was then processed through a negative mouse CD8 T cell (Miltenyi Biotec, cat #130-104-075) isolation kit following the manufacturer's instructions.

Co-culture

A20-HA lymphoma cells WT, Ctss KO, or B2m KO, expressing HA (hemagglutinin) as antigen were pre-treated with the indicated compounds for 16 hours and then co-incubated for 72 h with CellTrace Violet-stained (Thermo Fisher, cat #C34557) CD8+ T cells isolated from CL4-HA transgenic animals, in presence or absence of treatment. At the end of the lymphoma cells/T cells co-culture, cells were collected, stained with DAPI viability dye (Thermo Fisher, cat #D1306) and analyzed for the proliferation of T cells by quantifying CellTrace Violet proliferation peaks, using a LSR Fortessa flow cytometer (BD Biosciences). The absolute number of A20 lymphoma cells in each sample was also assessed through the use of CountBright Absolute Counting Beads (Thermo Fisher, cat #C36950), by comparing the ratio of bead events to cell events for each sample. The Proliferation score was computed as a weighted sum of the percentages of CD8 T cells identified in different proliferation peaks: activated T cells in the first peak (highest fluorescence of Cell trace Violet) were given a weight of 1, T cells in the second peak were given a weight of 2, and so on until the last proliferation peak (corresponding to the T cells that had proliferated the most).

Transwell invasion assay

Transwell invasion assay was performed using 12-well plate cell culture inserts (Corning, cat #353182). For each insert, 35 µl of matrigel (Corning, cat #356234) were mixed to 65 µl of PBS on ice, and then the mixture was added to the insert, which was then incubated for 1 hour at 37 °C to allow gel formation. Cells were harvested and resuspended in serum-free medium. 1×10e5 cells were added into the upper chamber, RPMI medium containing 10% FBS was added into the bottom chamber and served as a chemoattractant. The APIC or control compounds to be tested were added

exclusively to the upper chamber. After incubation for 48 hours at 37 °C in 5% CO2, remaining matrigel and cells in the upper chamber were removed, and then the cells that invaded to the lower surface of the membrane were fixed with 100% methanol (15 minutes at room temperature). Each insert was then moved to a spare well to air-dry, and then the remaining cells on the upper side of the membrane were wiped out with a cotton swab. The cells on the bottom side were stained with a 0.5% crystal violet solution (Acros Organics, cat #548-62-9) and counted under a microscope (Leica Dmi1).

Supplementary References

1. Dheilly, E. *et al.* Cathepsin S Regulates Antigen Processing and T Cell Activity in Non-Hodgkin Lymphoma. *Cancer Cell* **37**, 674-689.e12 (2020).
2. Delage, J. A. *et al.* 177Lu radiolabeling and preclinical theranostic study of 1C1m-Fc: an anti-TEM-1 scFv-Fc fusion protein in soft tissue sarcoma. *EJNMMI Res.* **10**, 98 (2020).