MATERIALS AND METHODS

Synthesis of ^{64}Cu-LLP2A for Human PET Imaging. ^{64}Cu-LLP2A was synthesized according to the good manufacturing practice-compliant procedure in a chemistry hot cell. LLP2A-CB-TE1A1P (15 µg in 65 µL; 0.230 µg/µL) was transferred in a reaction vial containing 15 mCi (555 MBq) of Copper-64 (^{64}Cu) and 0.5 M ammonium acetate (pH 6.5) as the radiolabeling buffer. The reaction vial was incubated at 70°C for 30 min. After the incubation, the reaction vial was removed from the heating block and allowed to cool for 5 min in a lead container. At this stage, 0.2 µg/µL gentisic acid in 0.9% sodium chloride solution was added into the reaction vial. The set-up for ^{64}Cu radiolabeling of LLP2A in a hot cell consisted of the reaction vessel, final product vial (FPV), sterilizing filters, PEEK needles and C-flex tubing. Two small holes were made in the septum of the reaction vessel with an 18G needle. The PEEK needle and C-flex tubing was attached to the connector using nylon male and female tube fittings. The PEEK needle was inserted into the reaction vessel through one of the holes created by the 18G needle, and the other end of the C-flex tubing was connected to the sterilizing filter attached to the FPV. 50mL sterile syringe and a separate C-flex tubing was attached to different ends of the 3-way valve. Another PEEK needle was attached to a connector on one end and 1mL sterile syringe on the other end. This PEEK needle was inserted into the reaction vessel through the second hole created by the 18G needle. The 1 mL syringe was replaced by the C-flex tubing attached to the 3-way valve after adding ^{64}Cu into the reaction vessel. ^{64}Cu-LLP2A was transferred to the FPV through the sterilizing filter. A schematic of the set-up is shown in the Supplemental Figure 1.

Pre-release and Post-release Quality Control (QC) Specifications. ^{64}Cu-LLP2A was released for clinical use after it met all pre-release QC specifications. Pre-release testing involved specific activity, radionuclidic identity, filter membrane integrity, visual appearance, pH, radiochemical purity, and bacterial endotoxin tests. The radiochemical purity was evaluated by radio-high performance liquid chromatography. Post-release evaluations included sterility testing and cell binding assays that was performed after radiotracer release for patient injections (Supplemental Figure 2).

Cell Uptake Assay. Cell uptake assay was performed as previously described to calculate the percentage specific uptake of ^{64}Cu-LLP2A in the VLA4 positive myeloma cell line, 5TGM1 *(24).* Briefly, 5TGM1 murine myeloma cells were grown in IMDM media and were allowed to reach confluency. The cells were then harvested, washed, and re-suspended in 1x phosphate buffered saline (PBS) in acid washed eppendorf tubes. A solution of ^{64}Cu-LLP2A in ammonium acetate buffer (0.1 nM) was added to all the tubes. A 200-fold excess of non-radiolabeled LLP2A precursor as a block was added to a separate cohort of eppendorf tubes. All of the reaction tubes (block and non-block) were incubated for 1 h at 37°C, with slight shaking. After the incubation, samples were centrifuged at 1,500 rpm for 3 min, and the supernatant was removed by aspiration. Cells were washed twice with ice-cold 1x PBS and the radioactivity in the remaining pellets were measured using a gamma counter.

Toxicity Studies in Mice. The toxicity of [^{Nat}Cu]-labeled LLP2A (Cu-LLP2A) was performed in collaboration with Seventh Wave, Maryland Heights, MO. Cu-LLP2A was administered once on day 1 in CD-1 IGS mice (n=15 male and n=15 female, respectively). 24 h later (day 2), a group of these mice (n=10/sex/group) was sacrificed and necropsied.

Another set of mice (n=5/sex/group) was observed until day 15 and then euthanized. Mice for each time point were divided into two groups – group 1 received vehicle (8% ammonium acetate in water), and group 2 received 0.0103 mg/mouse Cu-LLP2A (prepared with 8% ammonium acetate in water), *via* tail vein at a dose volume of 0.125 mL/mouse. Mice were observed daily and their body weight was recorded daily. Blood samples from these mice were collected on day 2 and day 15, respectively, before euthanizing them for necropsy. The blood samples were used to evaluate hematology and clinical chemistry endpoints. Different organs were collected, weighed, and fixed for microscopic evaluation.

Clinical Studies

PET Imaging Procedures. Continued from the main file: Total urine volume and urine radioactivity concentration was measured before each imaging session following administration of the radiotracer. Urine was also collected and counted for radioactivity at several time points for 24 h following injection.

Organ Time-Integrated Activity and Radiation Dose. The TIA of the urine excreted activity was then calculated from the TIA of the total urine content minus the bladder content TIA. The remainder of the body TIA was finally computed from the difference between the maximum TIA (Cu-64 half-life of 12.7 h/ln(2) minus the measured organ TIAs and urine excreted TIA. Organ radiation dose were then computed for the adult male or adult female models using the computer software OLINDA/EXM 1.1.

Blood Metabolism Study. To determine the stability ^{64}Cu-LLP2A and measure the metabolites of ^{64}Cu-LLP2A in human samples, whole blood samples were collected and

analyzed by analytical radio-high performance liquid chromatography. Briefly, whole blood samples were collected from all study participants. Samples were collected at 1-2 h post injection of the radiotracer in EDTA tubes. Prior to the analysis, the blood samples were centrifuged for 5 min @5000 g, followed by the separation of plasma. The radioactivity was measured in the whole sample (~1 mL) as well as in post centrifugation plasma samples. The plasma sample was diluted with milliQ water (1:1 ratio) and passed through a 0.22 μm filter before injecting into the radio-HPLC. 10 μL of the sample was injected into the HPLC and radioactive fractions were collected over a period of 10 min (0.5 mL/30 sec). The radioactivity from the collected HPLC fractions was measured using the gamma counter.

Flow Cytometry Study. Two of the three patients with MM also agreed to provide blood and bone marrow (BM) samples for an institutional banking study of plasma cell dyscrasias. For this study, sample from one of the MM patient was analyzed. The CD138 positive and negative populations BM were sorted by autoMACS pro separator using human CD138 microbeads and indirect human CD34 microbead kit (Miltenyi Biotec, Germany), respectively. Briefly, CD138 + cells from the BM of patients were purified by immunomagnetic selection using an automacs device. Purified cells were > 97% for CD38 + CD138 + plasma cells. LLP2A-Cy5 synthesis and flow cytometry were performed as previously described *(19).* Briefly, cryopreserved human peripheral blood and BM mononuclear cells were thawed, washed in phosphate-buffered saline (PBS) and stained for 15 minutes at room temperature with a LIVE/DEAD Fixable Blue Dead Cell Stain kit (Invitrogen, Carlsbad, CA). Cells were washed in Hanks Balanced Salt Solution (HBSS) containing Ca2+, Mg2+ and 0.1% BSA and incubated for 10 min at room temperature

with human Fc Block (BD Biosciences; San Jose, CA). Samples were then incubated for 30 min at room temperature with pre-titrated saturating dilutions of the following fluorochrome-labeled antibodies (clone and source designated in parenthesis): CD45-BUV395 (HI30; BD), CD19-BUV496 (SJ25C1; BD), CD33-BUV737 (P67.6; BD), CD138-BV421 (MI15; BD), CD3-VioBlue (BW264/56; Miltenyi), CD16-BV510 (3G8; BioLegend), CD56-BV711 (NCAM16.2; BD), HLADR-BV785 (L243; BioLegend), CD29-VioBright 515 (REA1060; Miltenyi), CD49d-PE (9F10; BD), and CD11c-PE-Cy7 (B-ly6; BD). Fluorescence minus one controls were used to assess background fluorescence intensity and set gates for negative populations. Samples were analyzed on a ZE5 (Bio-Rad, Hercules, CA) flow cytometer. Single stain compensation controls were obtained using UltraComp eBeads (Thermo Fisher Scientific) and data were analyzed using FCS Express (DeNovo Software, Pasadena, CA).

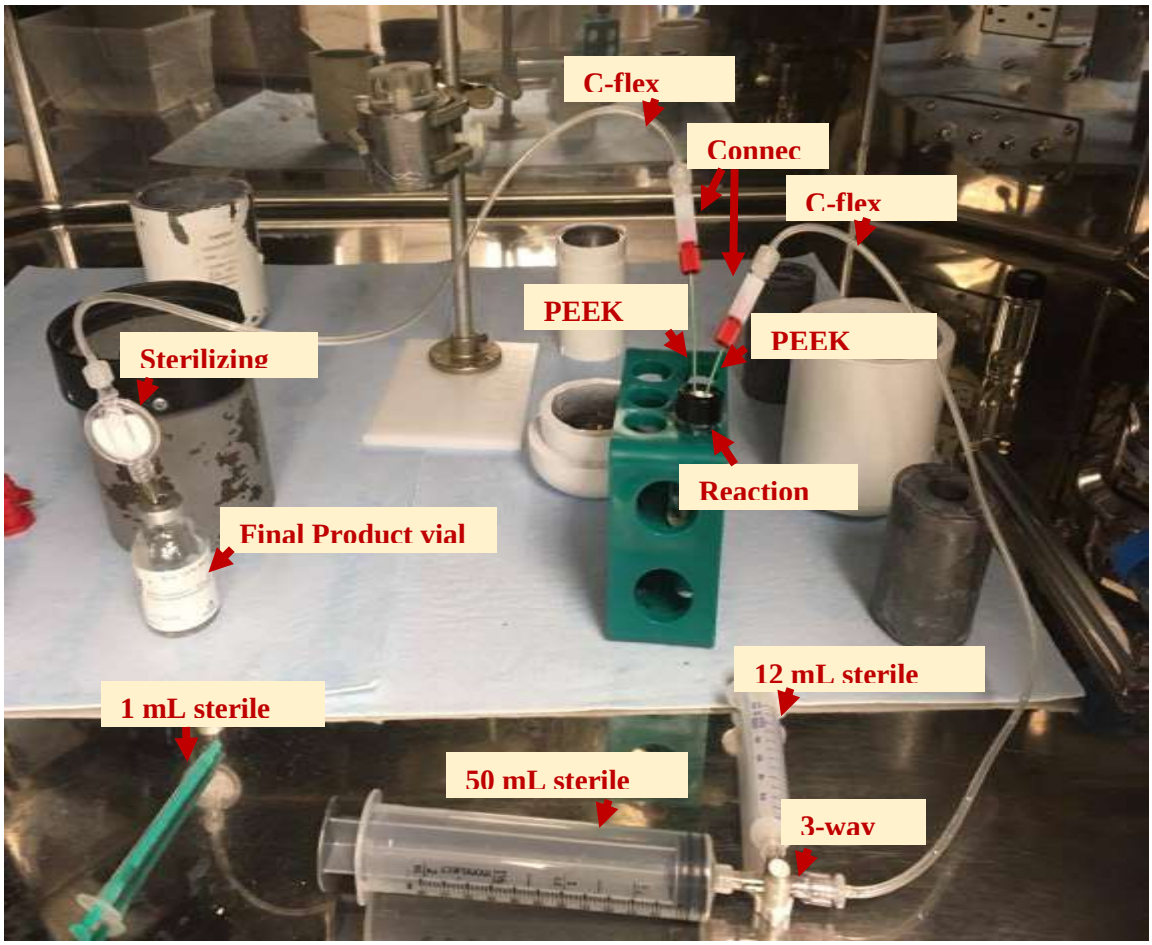
RESULTS

Animal toxicity studies. There was no test compound (Cu-LLP2A) related effect on body weight observed following one IV dose administration. There were also no definitive related changes in clinical observations, pathology, clinical chemistry and hematology parameters. Some minor differences in individual or group mean hematology and clinical chemistry values in male/female mice given Cu-LLP2A compared to control mice were insignificant, within the observed normal range for mice of this strain and age, were single in occurrence or were not dose dependent, and, therefore, were not considered Cu-LLP2A related. There were no macroscopic observations in animals administered with Cu-LLP2A. The only gross lesions documented were in control mice that consisted of a focal, unilateral ovarian cyst, and an abnormally shaped left kidney. Based on the normal

clinical observations coupled with no definitive Cu-LLP2A-related effects on body weight, clinical pathology parameters, organ weight values, and microscopic findings, a single IV injection of 0.0103 mg/mouse of Cu-LLP2A was well tolerated in male and female CD-1 IGS mice and was considered NOEL (no observable effect level) (Supplemental Figure 4).

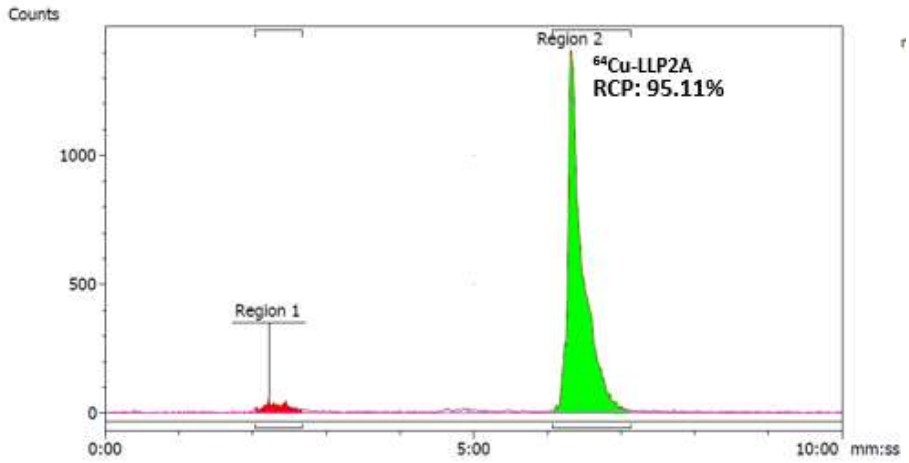
SUPPLEMENTAL FIGURES.

Supplemental Figure 1. Experimental set up in the chemistry hot cell for radiolabeling the precursor (LLP2A) with copper-64 (^{64}Cu).



Supplemental Figure 2. (A) Representative analytical radio-HPLC chromatogram of ^{64}Cu-LLP2A showing radiochemical purity. **(B)** Pre- and post- release quality control specifications.

(A)

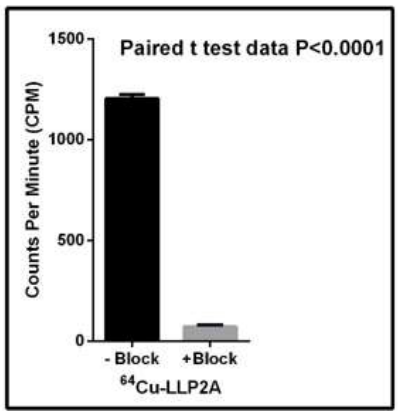


(B)

Test	Acceptance criteria
pH	5.0-6.5
Radiochemical purity by HPLC	≥ 90%
Specific activity (at EOS)	≥ 600 µCi/µg
Radionuclide purity (annually)	≥ 99.0%
Endotoxin assay	Negative

Supplemental Figure 3. (A) Representative cell study : Percentage cell uptake of ^{64}Cu-LLP2A in 5TGM-GFP (murine myeloma) cells at 37°C in the presence and absence of excess blocking dose of unlabeled precursor (LLP2A). **(B)** Table showing values of % blocking for each assay performed during the radiolabeling validations and for clinical studies with study participants.

(A)



% Blocking: 94
100-(74/1205)*100

Raw Gamma Counts (CPM)

- Block	+Block
1216.0	65.0
1183.0	75.0
1216.0	82.0

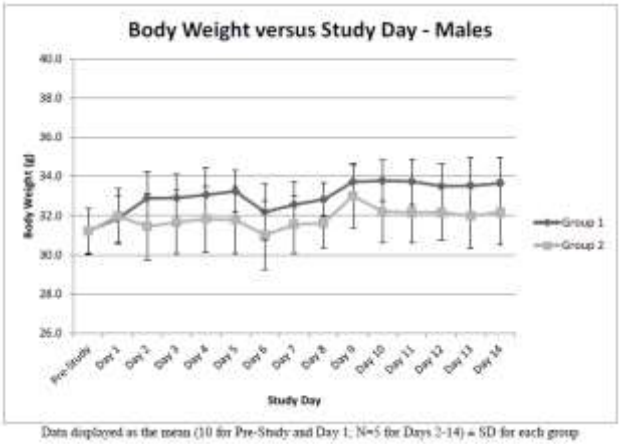
	Average	SD
64-Cu-LLP2A	1205	19
64-Cu-LLP2A + Block	74	9

(B)

Run	% Blocking
1	94.0
2	72.8
3	80.3
4	80.4
5	97.1
6	70.1
7	92.2
8	97.8
9	70.2
10	93.2
11	85.0
12	83.0
13	77.3

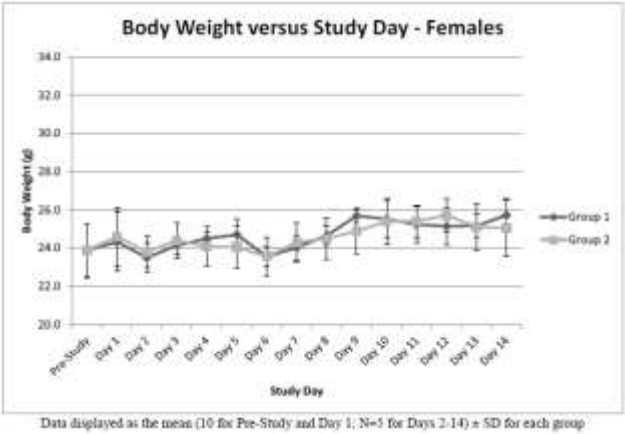
Supplemental Figure 4. Changes in body weights over time to evaluate toxicity of ^{64}Cu-LLP2A in **(A)** male and **(B)** female CD-1 IGS mice. **(C-D)** Summary of clinical observations determining the toxicity of ^{nat}Cu-LLP2A in male and female CD-1 IGS mice.

(A)



Data displayed as the mean (10 for Pre-Study and Day 1; N=5 for Days 2-14) ± SD for each group

(B)



Data displayed as the mean (10 for Pre-Study and Day 1; N=5 for Days 2-14) ± SD for each group

(C)

Study Day	Observation	Group 1	Group 2
Day 1 5/18/2017	**Number of Animals Observed**	15	15
	No Abnormal Findings	15	15
Day 2 5/19/2017	**Number of Animals Observed**	15	15
	No Abnormal Findings	15	15
Day 3[a] 5/20/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 4 5/21/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 5 5/22/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 6 5/23/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 7 5/24/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 8 5/25/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 9 5/26/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 10 5/27/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 11 5/28/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 12 5/29/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 13 5/30/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 14 5/31/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 15 6/1/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5

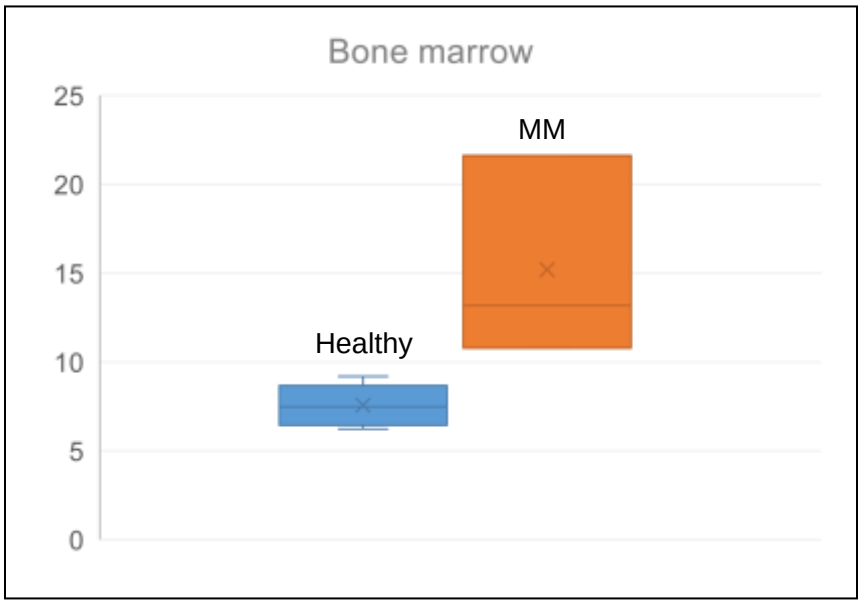
[a] Animals xM001-xM010 were sacrificed on Day 2.

(D)

Study Day	Observation	Group 1	Group 2
Day 1 5/18/2017	**Number of Animals Observed**	15	15
	No Abnormal Findings	15	15
Day 2 5/19/2017	**Number of Animals Observed**	15	15
	No Abnormal Findings	15	15
Day 3[a] 5/20/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 4 5/21/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 5 5/22/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 6 5/23/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 7 5/24/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 8 5/25/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 9 5/26/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 10 5/27/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 11 5/28/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 12 5/29/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 13 5/30/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 14 5/31/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5
Day 15 6/1/2017	**Number of Animals Observed**	5	5
	No Abnormal Findings	5	5

[a] Animals xF001-xF010 were sacrificed on Day 2.

Supplemental Figure 5. BM SUV was measured as the average of lumbar vertebra 3,4 and 5 for most patients. For some patients, the marrow uptake was non-uniform in those regions, in which case marrow uptake in the lumbar 1 or 2, or thoracic 11-12 was used. Data were taken at the second imaging time point and ranged from 1 to 4 h post injection. *Levene's test (equal variances not assumed), one tailed, p = 0.072 (Borderline statistically significant for n = 9).



Healthy	Time (h)	Location		MM	Time	Location
6.52	1.99	L345		10.79	1.54	L345
8.5	1.48	L234		13.18	2.00	L234
6.23	3.00	L345		21.62	4.00	T11-12, L1
6.91	3.92	L234				
8.07	3.88	L345				
9.195	3.52	L345				

Supplemental Figure 6. Comprehensive safety evaluations were performed on all study participants.

Participant ID	Safety Evaluations
MMDN01	Y
MMDN02	Y
MMDN03	Y
MMDN04	Y
MMDN05	Y[#]
MMDN06	Y
MMDM01	Y
MMDM02	Y
MMDM03	Y

Safety Evaluations: Safety Labs at least 30 min prior to injection and at study discharge: CBC with differential and CMP.

Safety Evaluations: Vital Sign Measurements-Blood pressure, heart rate, breathing rate, temperature at least 30 min prior to injection at least 60 min post injection and study discharge.

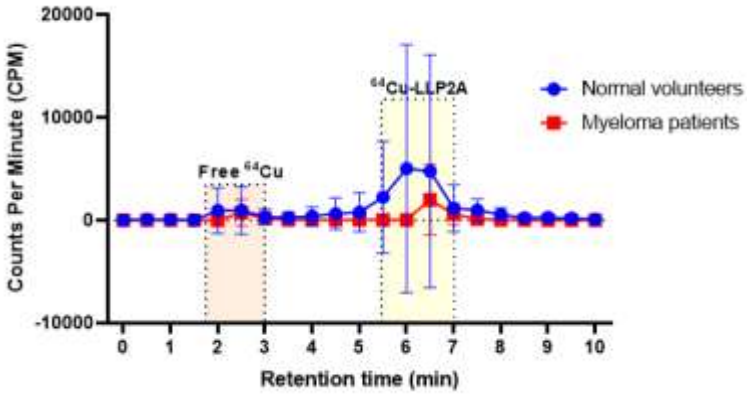
Safety Evaluations: 12 lead ECG at least 30 min prior to injection, at least 60 min post injection and study discharge.

Safety Evaluations: Baseline assessment for adverse events, follow up AE assessments immediately post injection, after each scan, study discharge (in person) + 24-72 h post injection.

[#]Safety Evaluations: Additional telephone follow up at 122 h post injection due to change in discharge labs + additional safety labs drawn at 7 d post injection - AE not related to study drug or imaging. Attributed to alcohol consumption and protein powder taken in excess at recent workouts.

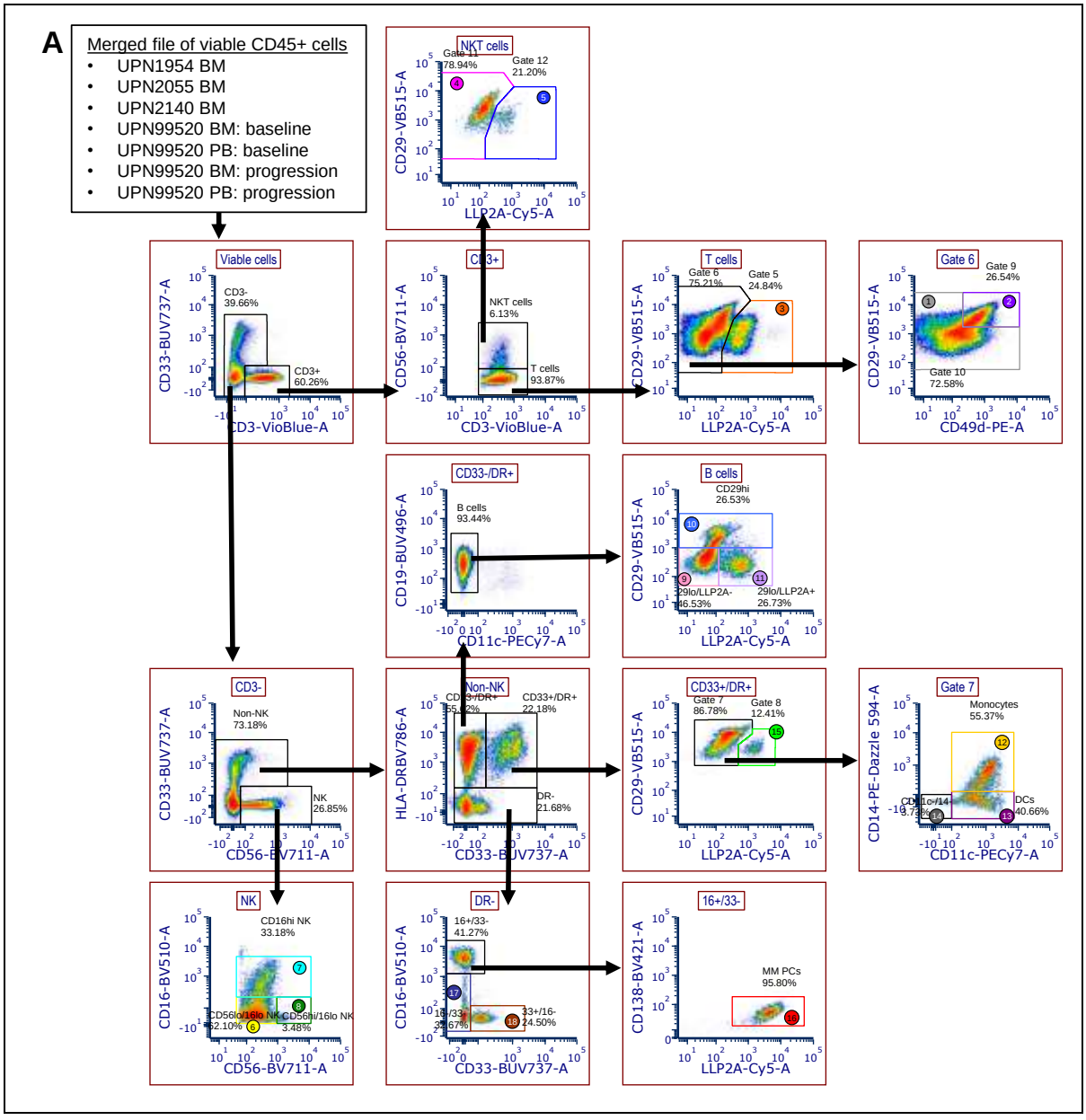
Supplemental Figure 7.

Analytical radio-high-performance liquid chromatography (HPLC) chromatogram of the ^{64}Cu-LLP2A plasma sample fractions, 1h post injection of ^{64}Cu-LLP2A in healthy and MM participants.

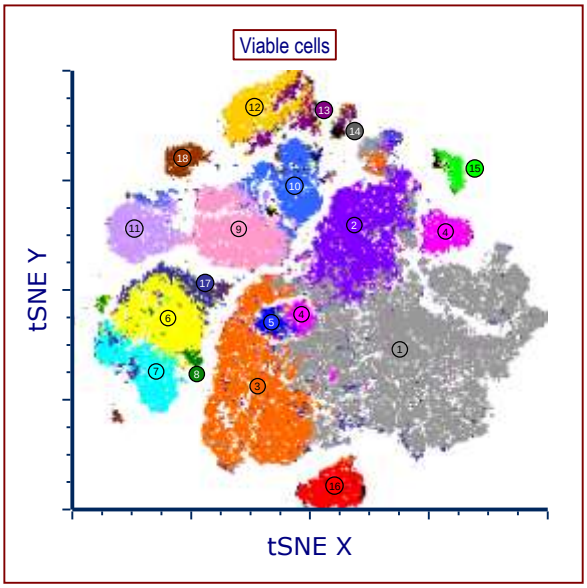


Normal volunteers (n=6)
Myeloma patients (n=3)
Retention time of ^{64}Cu-LLP2A 5.5-7 min

Supplemental Figure 8. Flow cytometry gating strategy and t-SNE (t-distributed stochastic neighbor embedding) plot. (A) Gating strategy used to define eighteen hematopoietic cell subsets in a merged flow cytometry file of BM and/or peripheral blood (PB) mononuclear cells from healthy donors (UPNs 1954, 2055, 2140) and a patient (UPN99520, MMDM02) with MM at baseline and after disease progression four months later. **(B)** tSNE projection of merged flow cytometry file showing eighteen hematopoietic populations based on defined cell surface markers described in panel **A**.

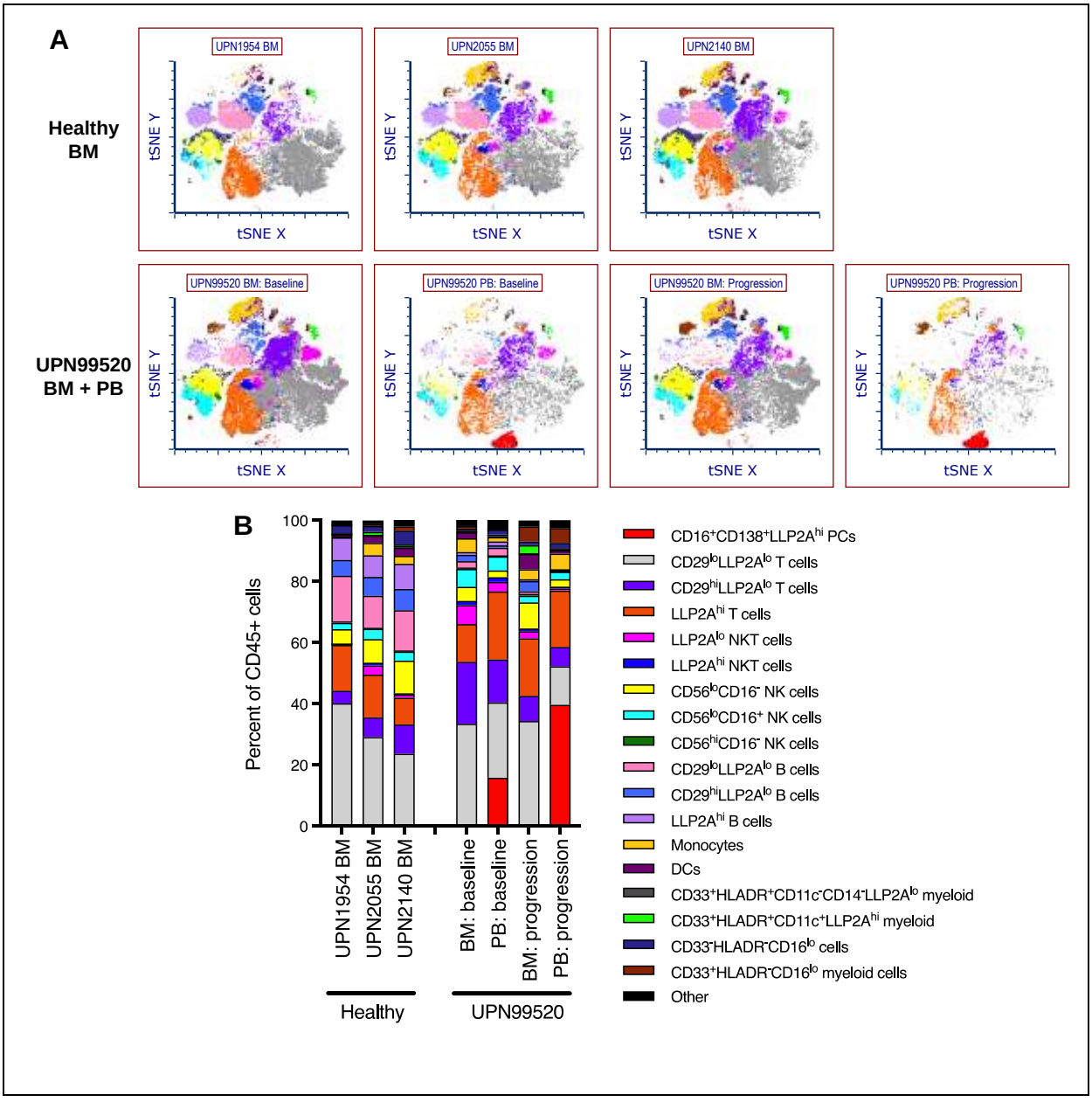


B



- $CD29^{lo}LLP2A^{lo}$ T cells 1
- $CD29^{hi}LLP2A^{lo}$ T cells 2
- $LLP2A^{hi}$ T cells 3
- $LLP2A^{lo}$ NKT cells 4
- $LLP2A^{hi}$ NKT cells 5
- $CD56^{lo}CD16^{-}$ NK cells 6
- $CD56^{lo}CD16^{+}$ NK cells 7
- $CD56^{hi}CD16^{-}$ NK cells 8
- $CD29^{lo}LLP2A^{lo}$ B cells 9
- $CD29^{hi}LLP2A^{lo}$ B cells 10
- $LLP2A^{hi}$ B cells 11
- Monocytes 12
- DCs 13
- $CD33^{+}HLADR^{+}CD11c^{-}CD14^{-}LLP2A^{lo}$ myeloid 14
- $CD33^{+}HLADR^{+}CD11c^{+}LLP2A^{hi}$ myeloid 15
- $CD16^{+}CD138^{+}LLP2A^{hi}$ PCs 16
- $CD33^{-}HLADR^{-}CD16^{lo}$ cells 17
- $CD33^{+}HLADR^{-}CD16^{lo}$ myeloid cells 18
- Other

Supplemental Figure 9. Individual t-SNE representations and hematopoietic cell subset distributions. (A) Individual t-SNE representations for each sample in the merged flow cytometry file presented in Supplemental Figure 6. **(B)** Quantification of data in **A**; each defined population is shown as a percentage of the total CD45+ cells.



Supplemental Figure 10. Heatmaps of parameters included in the t-SNE analysis.