

Immuno-PET imaging of CD38 expression in multiple myeloma using a ^{64}Cu -labeled nanobody

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Abstract

Background:

Accurate phenotyping of cancer lesions is critical for guiding targeted therapies. In multiple myeloma (MM), CD38 is a validated biomarker and a major therapeutic target. However, a companion diagnostic tool is still lacking. This study aimed to evaluate the potential of a copper-64-labeled anti-CD38 single-domain antibody (sdAb or nanobody), WF121, for immuno-PET (Positron Emission Tomography) imaging of CD38 expression *in vivo* in a preclinical model of MM.

The nanobody's binding affinity and specificity were assessed by flow cytometry using CD38⁺ MM1S and CD38⁻ U266 cell lines. Its internalization profile was evaluated by confocal microscopy. WF121 nanobody was conjugated to the NOTA (1,4,7-Triazacyclononane-1,4,7-triacetic acid) chelator through lysine residues and radiolabeled with copper-64.

In vivo PET imaging was performed in C.B-17/IcrHanHsd-Prkdc-scid mice bearing subcutaneous MM1S or U266 xenografts. MM1S-grafted mice underwent dynamic scans (0–2h post-injection) and static acquisitions at 1, 2 and 24h. U266-grafted mice were imaged at 1, 2 and 24h. *Ex vivo* biodistribution analyses were conducted at 2 h and 24 h for MM1S, and at 24 h for U266. Autoradiography was performed on excised tumors and kidneys for high-resolution localization of radiotracer uptake.

Results:

Flow cytometry showed selective binding of WF121 to CD38⁺ MM1S cells, with no binding to CD38⁻ U266 cells, while confocal microscopy revealed predominantly membrane-associated WF121 with partial internalization into the cytoplasm.

⁶⁴Cu-NOTA-WF121 demonstrated favorable *in vivo* behavior, with specific and high-contrast uptake in CD38⁺ MM1S tumors, low background uptake in CD38⁻ U266 models, and predominantly renal clearance. Autoradiography confirmed homogenous tumor uptake and highlighted WF121 accumulation pattern in the renal cortex.

Conclusion:

This study provides the first preclinical evidence that a ⁶⁴Cu-labeled anti-CD38 nanobody enables effective early and delayed immuno-PET imaging of CD38 expression in MM. These findings support the potential of nanobody-based radiotracers for non-invasive phenotyping and patient stratification in CD38 targeted therapies.

Background

Multiple myeloma (MM) is a hematological malignancy characterized by the abnormal proliferation of plasma cells within the bone marrow, leading to anemia as well as bone and renal complications. During first-line treatment or at relapse, nearly all patients receive repeated injections of Daratumumab, a monoclonal antibody targeting CD38 (1), a transmembrane molecule with extracellular enzymatic activity

that functions both as a receptor and an adhesion molecule (2). However, while some patients exhibit primary refractoriness, others develop resistance during therapy, a phenomenon partly attributed to the downregulation of CD38 expression on the surface of malignant plasma cells (3). In addition, a single course of Daratumumab costs several thousand USD (4) and treatment is often ($\geq 20\%$) associated with adverse effects, ranging from common events (e.g., infections, neutropenia, infusion-related reactions) to rarer but more severe complications, including severe allergic reactions, febrile neutropenia, hepatic disorders, and reactivation of latent viral infections (5). Assessing CD38 expression in malignant lesions could improve patient management and help reduce healthcare costs. Anti-CD38 immuno-PET represents a promising technique to guide clinical decision-making. It may provide essential information on CD38 expression across myeloma lesions and serve as a valuable companion diagnostic tool. Furthermore, CD38 immuno-PET may offer an advantage over ^{18}F -fluorodeoxyglucose (FDG) at early stages by providing a more specific identification of multiple myeloma lesions, given the lack of specificity of FDG and the existence of low FDG-avid results(6).

Performing immuno-PET directly with radiolabeled Daratumumab has been already done (7–9) but presents two major limitations. First, the pharmacokinetics of full-length antibodies, characterized by slow distribution and clearance, require patients to return several days after radiopharmaceutical injection to obtain images of sufficient quality, resulting in logistical constraints and increased costs. Second, the physiological biodistribution of monoclonal antibodies in the liver and spleen precludes accurate evaluation of lesions in these organs. The use of smaller CD38-targeting molecules may overcome these limitations. Among such molecules, single-domain antibodies (sdAb), also known as nanobodies, are of particular interest. Derived from camelid heavy-chain antibodies, nanobodies are small (12–15 kDa), easy to produce, resistant to extreme conditions such as pH and temperature variations, and low immunogenic (10). These properties enable rapid biodistribution, efficient tissue penetration, and facilitate radiolabeling. Moreover, nanobodies can be readily engineered to optimize radiolabeling strategies. Numerous preclinical studies, reviewed in (11), have highlighted their potential, demonstrating both rapid achievement of high tumor-to-background signal ratios and, conversely, high renal uptake. A first-in-human study with a gallium-68-labeled anti-CD38 nanobody showed rapid tumor uptake and excellent tumor-to-background contrast (12). However, a comparative study by Zeven et al. demonstrated that an anti-TIGIT nanobody radiolabeled with copper-64 yielded superior image quality compared with the same nanobody labeled with fluorine-18 or gallium-68 and may be more efficient for the detection of low-expression targets (13).

The present study aimed to investigate the use of copper-64 as a radionuclide for labeling an anti-CD38 nanobody (WF121) in a preclinical multiple myeloma model.

Methods

Nanobody production and purification

The sequence encoding the His-tagged WF121 nanobody (14) was cloned into an in-house Tac promoter plasmid. Purifications were performed by metal chelator affinity chromatography (Ni-IMAC, Thermo Fischer Scientific, Waltham, USA) and residual imidazole was removed by gel filtration (Sephadex G25). Nanobody quality was finally assessed by SDS-PAGE.

Chelators or fluorophores labeling

His-tagged WF121 was conjugated either to a bifunctional chelator (NCS-NOTA, Macrocyclics, Plano, USA) or to a fluorophore (NHS-dylight 650, Thermo Fischer Scientific, Waltham, USA), and Daratumumab (Darzalex, Janssen, USA) was conjugated to NHS-Alexa fluor 488 (Thermo Fischer Scientific). First, buffers of the nanobody and Daratumumab were exchanged to carbonate buffer (0.3M, pH 8.6). An excess of coupling reagent (20-fold molar excess for the nanobody and 5-fold molar excess for Daratumumab) was then added for 1h at room temperature. Excess unconjugated molecules were removed by filtration using Amicon Ultra-2 (Merck, Kenilworth, USA) for the nanobody, and PD-10 desalting column for Daratumumab. Finally, buffers of chelator- and fluorophore-conjugated molecules were exchanged for 0.1M metal-free sodium acetate (Alfa Aesar, Haverhill, MA, USA) or PBS, respectively.

Cell culture

MM1S and U266 MM cell lines (ATCC, Manassas, Virginia, USA) were cultured at 37°C, 5% CO₂, 95% humidity in RPMI 1640 medium containing 2 mM L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Thermo Fisher Scientific, Waltham, MA, USA), 10% heat-inactivated fetal calf serum (PAA Laboratories / GE Healthcare Europe GmbH). Mycoplasma testing (PlasmoTest™, Invivogen, Toulouse, France) was performed prior to engraftment.

Flow cytometry

A total of 200,000 cells were incubated 1h at 4°C with the indicated concentration of WF121-6His-NHS-Dylight 650 (WF121-DL650) or Daratumumab-NHS-AF488. Cells were washed three times in RPMI 1640 medium (Gibco, Saint Aubin, France) and analyzed using a FACSCanto II flow cytometer (BD biosciences, San Jose, USA). Data were analyzed using FlowJo Software (BD biosciences, San Jose, USA) and binding curves were fitted according to a one site-specific binding model using GraphPad PRISM 8.0 software.

Radiolabeling

⁶⁴Cu (T_{1/2} = 12.7 h, β⁺; 17.8%) was produced by the ARRONAX cyclotron (GIP ARRONAX, Saint-Herblain, France) using the ⁶⁴Ni (d, 2n)⁶⁴Cu reaction and delivered as [⁶⁴Cu]-CuCl₂ in HCl 0.1N.

To avoid a final purification step, the reaction was performed using a molar excess of chelators relative to metal ions. The reaction pH was increased to 6.5 by adding 2.5 M metal-free sodium acetate at a volume corresponding to one-tenth of the added $[^{64}\text{Cu}]\text{-}^{64}\text{CuCl}_2$ solution. The amount of $[^{64}\text{Cu}]\text{-CuCl}_2$ solution added to protein solution (prepared in 0.1M metal-free sodium acetate) was calculated based on the number of chelators per protein while accounting for variations in the specific activity of the copper source. After incubation for 30 min at 42°C, 1 mM EDTA solution was added to obtain a final concentration of 30 μM . Radiolabeling yield was assessed by instant thin layer chromatography on silica gel (ITLC-SG) using citrate buffer (0.1M pH 4.5) and analyzed using a Cyclone phosphorimager scanner (Perkin Elmer, Waltham, USA). Radiochemical purity was considered acceptable only when greater than 95%.

Quantification of the number of chelates per molecule

Based on the molecular masses of WF121 and chelators, the average number of chelators per molecule was determined by mass spectrometry analysis using a Synapt G2 HRMS Q-TOF mass spectrometer equipped with an electrospray ionization interface operating in positive mode, and coupled to an Acquity H-Class UPLC system equipped with a CSH Peptide C18 reversed-phase column (1.7 μm , 2.1 x 100 mm; Waters Corporation, Milford, MA, USA).

The number of chelators per nanobody was further confirmed by radiolabeling experiments. Copper was added at different molar equivalents relative to the protein (4, 2, and 1) under the same reaction conditions described above. Radiolabeling yields were determined by ITLC-SG. For example, a yield of 50% with 4 equivalents or 100% with 2 and 1 equivalents indicates the presence of two chelators per protein molecule.

Immunoreactivity

To determine the proportion of radiolabeled sdAb capable of binding CD38, 10 μL of CD38-coated magnetic beads (ACRO Biosystems, Newark, USA) were incubated with 1 pmol of ^{64}Cu -NOTA-WF121 for 15 minutes at room temperature. Tubes were then placed on a magnetic rack, and the supernatants were collected. Beads were washed with 100 μL of PBS containing 0.1% BSA returned to the magnetic rack. The wash supernatants were pooled with the initial supernatants and beads were resuspended in 200 μL of PBS containing 0.1% BSA. Radioactivity associated with beads and supernatants was measured using a gamma counter. Immunoreactivity was calculated as the ratio of bead-associated activity to total activity (beads + supernatant).

Internalization experiment

MM1S cells were seeded at 100,000 cells per well in 8-well IBltreat plates (Ibidi, Gräfelfing, Germany) with 400 μL of complete RPMI 1640 medium. The following day, culture medium was removed and WF-

121-6His-NHS-dylight 650 was added at 40 nM in pre-warmed complete medium. Hoechst 33342 (5µg/mL Merck, Kenilworth, USA) was added 15 minutes before the end of incubation. The supernatant was then removed, and cells were washed twice with 400 µL of PBS. Freshly prepared PBS with 2% paraformaldehyde (Electron Microscopy Sciences, Hatfield, USA) was subsequently added for 7 minutes, followed by replacement with PBS. Images were acquired using a confocal microscope (A1R LFOV FLIM, Nikon, MicroPICell core facility, Nantes).

Animal models

Mice were housed in the UTE animal facility of IRS-UN (Nantes University, license no. C-44-278) under conventional conditions. Animal experiments were approved by the “Comité d'éthique en expérimentation animale Pays de la Loire (CEEA – 006)” and the French Ministry of Research (APAFIS #34964). All procedures were conducted in accordance with institutional and national guidelines for animal care.

Seven-week-old female C.B-17/IcrHanHsd-Prkdc-scid mice (Envigo, Gannat, France) were subcutaneously grafted with 10^7 MM1S cells. Grafts were prepared using Matrigel® (Corning, NY, USA) according to the the manufacturer's instructions. Briefly, for each mouse, MM1S cells were detached using 0.05% trypsin-EDTA solution (Gibco, Saint Aubin, France), centrifuged, washed twice with PBS, and adjusted at 10^7 cells in 50 µL PBS. On ice and using pre-cooled materials, 50 µL of Matrigel® were added, and the mixture was injected subcutaneously into the right shoulder. Animals were imaged 20 days after engraftment.

For the U266 model, 8-week-old mice (one male and three females) were grafted subcutaneously with 10^7 U266 cells without Matrigel®. Tumor growth was slow and heterogeneous among mice. Imaging experiments were performed 65 days after engraftment.

Imaging sessions

All preclinical images were acquired at the multimodal imaging facility CIMA (Centre d'Imagerie Multimodale Appliquée, Nantes). Mice received approximately 9 MBq of ^{64}Cu radiolabeled protein by tail vein injection (8.6 to 10.8 µg of nanobody). Groups of three mice were placed in a thermoregulated imaging cell (Minerve, France), maintained under anesthesia with 2% isoflurane, and imaged using a PET-CT camera (IRIS, Inviscan SAS, Strasbourg, France). For sequential imaging, a CT scan was first performed (0.9 mA, 80 kV, 576 projections, 160 µm voxel size), followed by a 10-min PET acquisition. Images were reconstructed using the 3DOSEM-MC algorithm with eight subsets and eight iterations, including decay, random, and dead-time corrections. For dynamic imaging, animals were injected in close proximity to the TEP camera and transferred in the imaging cell within 5 minutes post injection of the radiolabeled sdAb. A 2-hour PET acquisition was then performed, followed by a 40-second CT scan. Images were reconstructed using the same protocol in consecutive 10-min frames. Image analysis was performed using OSIRIX software (Pixmeo, Geneva, Switzerland) and Imalytics Preclinical 3.1 software (GremseIT GmbH, Aachen, Germany). Tumor uptake was quantified as percentage injected dose per

milliliter (%ID/mL), while kidney activity was extracted and converted to %ID/g assuming a kidney mass of 200 mg.

Ex vivo biodistribution

Ex vivo biodistribution studies were performed immediately after the final imaging acquisition. Blood, tumor, skin, muscle, femur, flat bone, brain, lungs, heart, stomach, gut, spleen, liver and kidneys were collected, weighed and counted using a gamma-counter (Hidex Automatic Gamma Counter, Turku, Finland). Data are expressed as percentage injected dose per gram of tissue (%ID/g) and reported as mean values from three mice.

Digital autoradiography and HPS staining

Tumors and kidneys were embedded in optimal-cutting temperature compound (NEG-50, Thermo Fischer Scientific, Waltham, USA), then frozen for 1 min in a solution of 2-methylbutan at approximately -70°C . Ten- μm tissue sections were prepared using a cryomicrotome (CM3050 LEICA, Wetzlar, Germany), mounted on superfrost slides, and imaged by real-time autoradiography (BeaQuant®, Ai4R, Nantes, France) according to the manufacturer's instruction. Images were analyzed on the dedicated software Beamage® (Ai4R, Nantes, France). Adjacent sections were stained with Hematoxylin-Phoxine-Safran by the Cellular and Tissular Imaging Core Facility of Nantes University (MicroPICell) and scanned using a NanoZoomer system (Hamamatsu, Japan).

Statistical analysis

Statistical analyses were performed using GraphPad Prism version 8.00. Differences in nanobody uptake between the two models were assessed using a one-tailed nonparametric Mann–Whitney test.

Results

WF121 binding Specificity

Binding of anti-CD38 Daratumumab and WF121 was first assessed by flow cytometry on two human MM cell lines: CD38-positive MM1S cells and CD38-negative U266 cells (15). WF121 and Daratumumab were conjugated to fluorescent dyes through NHS-primary amine chemistry. The relative fluorescence data shown in Fig. 1 demonstrated consistent binding profiles for both Daratumumab and WF121 on MM1S (CD38⁺) and U266 (CD38⁻). WF121 exhibited an estimated K_d of approximately 10 nM, whereas Daratumumab exhibited an estimated K_d of 1 nM, consistent with previously published data (16).

Nanobody Uptake and Internalization

To further characterize WF121, confocal microscopy was performed to evaluate its internalization following binding to CD38 (Fig. 2). The nanobody was predominantly detected on the external cell membrane, although a small fraction was detectable within the cytoplasm as early as 15 min after

incubation with the cells. Internalization was observed in $77.8 \pm 10.8\%$ of cells at 15 min and in $69.0 \pm 6.1\%$ of cells at 60 min, with no significant difference between the two time points.

Conjugation and ^{64}Cu radiolabeling

Prior to radiolabeling, WF121 was conjugated to the metal chelator NOTA. Conjugation efficiency was assessed by mass spectrometry (Figs. 3A-B), and further confirmed by radiolabeling with ^{64}Cu . WF121 carried 0, 1 or 2 chelators per molecule with an average of 1.5 chelators per nanobody. Using thin-layer chromatography, radiolabeling yields were consistently above 95% (Fig. 3C). Immunoreactivity of radiolabeled WF121-NOTA measured using CD38-coated protein magnetic beads was $81.4 \pm 1\%$.

Anti-CD38 ^{64}Cu -NOTA-WF121 Dynamic PET Imaging

The *in vivo* properties of WF121 were assessed following intravenous injection of approximately 0.7 nmol of ^{64}Cu -radiolabeled nanobody into mice, followed by PET imaging. Because nanobodies exhibit rapid biodistribution kinetics, dynamic PET acquisition was performed on a first set of 3 animals from 5 to 125 min post-injection. Quantitative analysis revealed that $47.8 \pm 2.7\%$ of the total detected activity was localized in the kidneys during the first acquisition frame (5–15 min post-injection). This proportion increased to $63.5 \pm 2.4\%$ during the final frame (115–125 min post-injection). Owing to the rapid blood clearance of the nanobody, tumors were detectable from the earliest reconstructed frame (5–15 min) (Fig. 4).

Anti-CD38 ^{64}Cu -NOTA-WF121 sequential PET imaging

Based on the dynamic imaging data, sequential PET imaging was subsequently performed at 1, 2, and 24 h after intravenous injection of ^{64}Cu -NOTA-WF121 in mice bearing subcutaneous MM1S or U266 grafts (Fig. 5). In the MM1S model, renal uptake values were: 174.7 ± 6.8 , 185.7 ± 19.4 , $25.8 \pm 5.3\%$ ID/g at 1, 2, and 24 h post-injection, respectively. As expected, tumor uptakes was consistently higher in the MM1S model at all time points with values of 3.42 ± 0.65 , 4.06 ± 0.72 , $2.74 \pm 0.38\%$ ID/mL at 1, 2, and 24 h, respectively, compared with 1.22 ± 0.24 , 1.01 ± 0.2 , 0.64 ± 0.07 in the U266 model. The MM1S-to-U266 tumor uptake ratios were 2.9, 4.0, and 4.3 at 1, 2, and 24 h post-injection, respectively.

Ex vivo biodistribution

To validate PET-derived uptake measurements, ex vivo biodistribution studies were performed at two time points following injection of ^{64}Cu -NOTA-WF121 in the CD38⁺ MM1S model (Fig. 6A). Results, expressed as percentage of injected dose per gram (ID/g), To validate PET-derived uptake measurements, ex vivo biodistribution studies were performed at two time points following injection. At 2 h post-injection, renal uptake reached 316% ID/g, corresponding to 61% of the injected dose, and decreased to 31.8% ID/g at 24 h (8.1% of the ID). After correction for radioactive decay, renal clearance of

the nanobody between 2 and 24 h was 66%, indicating efficient renal elimination and no sustained kidney retention.

Tumor uptake in MM1S grafts decreased from 3.77 ± 0.25 to $1.09 \pm 0.19\%$ ID/g between 2 and 24 h (Fig. 6A), with 89% of the decrease attributable to radioactive decay. The results shown in Fig. 6B further confirmed the higher uptake in MM1S tumor, whereas U266 tumors exhibited a mean uptake of only $0.18 \pm 0.16\%$ ID/g at 24 h post-injection. The MM1S-to-U266 tumor uptake ratio was 5.58, consistent with the ratio derived from PET imaging (4.5).

Autoradiography and Histology

To further investigate the tissue distribution of WF121 beyond its expected renal clearance, *ex vivo* autoradiography and histological analyses were performed on kidney sections. PET imaging and quantitative autoradiography demonstrated that ^{64}Cu -NOTA-WF121 was predominantly localized within the renal cortex at 2 h post-injection (Fig. 7A). Autoradiography analyses of tumor sections confirmed the PET imaging findings. As shown in Fig. 7B, radioactive signal was detected throughout the tumor and appeared more pronounced at the tumor periphery. Histological analysis revealed a dense vascular network within the tumor, while macroscopic examination showed accumulation of blood in the peritumoral region.

Discussion

In MM, targeted therapies using monoclonal antibodies, bispecific antibodies or CAR-T cells directed against CD38 antigen have expanded considerably in recent years, leading to major improvements in patient outcomes (17). However, their clinical use could be further optimized by selecting patients who are most likely to benefit from these therapies, thereby avoiding unnecessary adverse effects and reducing healthcare costs.

Immuno-PET has emerged as a highly relevant imaging modality, providing valuable information for patient selection through comprehensive and non-invasive mapping of antigen expression across all tumor lesions. Although not yet implemented in routine clinical practice, this approach has already been evaluated in several clinical studies, including breast cancer patients imaged with ^{68}Ga -Her2 nanobodies (18) or ^{18}F -Her2 affibodies (19), as well as patients with various tumor types imaged using ^{89}Zr -atezolizumab, an anti-PD-L1 antibody (20). In MM, a first-in-human study investigated the potential of ^{68}Ga -TOHP-CD3813, an anti-CD38 nanobody, and reported encouraging results in terms of tumor detection (12).

A systematic and longitudinal follow-up using CD38 immuno-PET could provide substantial clinical benefits by offering valuable insights into disease progression and treatment response, while potentially outperforming FDG PET, whose uptake remains non-specific. However, considering the high proportion

of favorable responders to first-line Daratumumab therapy and the substantial costs associated with routine imaging, CD38 immuno-PET may be particularly relevant in relapsed or refractory settings.

From an imaging perspective, the present study demonstrated the strong potential of nanobodies for rapid acquisition of high-contrast images and homogeneous intratumoral signal distribution. Dynamic imaging confirmed the rapid blood clearance previously described for nanobodies, together with high uptake in CD38⁺ tumors, with the optimal tumor-to-blood ratio reached only 75 min post-injection. Tumor uptake values derived from biodistribution studies were comparable to those previously reported by Wang et al. using [68Ga]Ga-NOTANb1053 in the same MM1S subcutaneous model (21). Indeed, ⁶⁴Cu-NOTA-WF121 showed a tumor uptake at 2h of 3.77%ID/g at 2 h post-injection, compared with 3.02%ID/g at 1 h for [68Ga]Ga-NOTANb1053.

In the CD38⁻ U266 model, we observed a low but detectable signal, approximately fourfold lower than that observed in the MM1S model, despite the absence of detectable CD38 expression by both flow cytometry (Fig. 1) and immunofluorescence (12). This residual uptake may be explained by the enhanced permeability and retention (EPR) effect, whereby molecules extravasate through permeable tumor vasculature and accumulate within tumor tissues (22, 23). Future clinical studies will be required to define uptake thresholds above which lesions are likely to respond to CD38-targeted therapies. Such thresholds could ultimately guide patient selection, optimize treatment planning, and improve therapeutic outcomes. Besides tumor uptake, and similarly to other small targeting molecules, WF121 was primarily eliminated through the kidneys. Autoradiography refined the PET findings by demonstrating strong signal localization within the renal cortex. Biodistribution data corrected for radioactive decay showed that the proportion of nanobody-associated activity in the kidneys decreased from 68% to 30% between 2 and 24 h post-injection, illustrating efficient renal clearance and indicating the absence of prolonged renal retention. This elimination profile is characteristic of radiolabeled nanobodies and could potentially be further improved by co-injection of Gelofusine® and lysine, which reduce tubular reabsorption mediated by megalin and cubilin receptors (24, 25).

An important consideration when developing a companion diagnostic is the epitope recognized by the imaging vector, which may be identical to or distinct from that targeted by the therapeutic agent. The potential emergence of CD38 variants following repeated cycles of Daratumumab therapy suggests that imaging vectors recognizing the same epitope as the therapeutic antibody may be preferable for specifically identifying targetable malignant cells (26). This strategy was implemented in the present study using WF121 and has previously been reported by Wang et al. using the MU1053 nanobody. In contrast, the radiotracer ⁶⁸Ga-TOHP-CD3813 described by Huang and colleagues does not compete with Daratumumab, which may limit its value as a companion diagnostic in this therapeutic setting (12).

Radionuclide selection also represents a critical determinant of image quality. Fluorine-18 exhibits an almost ideal positron branching ratio (96.8%) and has demonstrated excellent performance when coupled to nanobodies (27). However, fluorine chemistry is challenging and requires careful optimization. However, fluorine chemistry remains technically challenging and requires careful

optimization. Gallium-68 also exhibits a high positron branching ratio (89%) and has proven effective in nanobody-based imaging (12). Although copper-64 has a lower positron branching ratio (18%), it offers the major advantage of a longer physical half-life (12.7 h), compared with 1.8 h and 1.1 h for fluorine-18 and gallium-68, respectively. In addition, copper-64 enables delayed imaging when necessary, potentially providing complementary information or facilitating lesion detection near the kidneys after renal clearance of the tracer. Finally, Zeven et al. demonstrated that copper-64 yielded superior tumor uptake and improved image contrast compared with fluorine-18 and gallium-68 when coupled to small targeting molecules (13).

Conclusions

The present study demonstrates the strong potential of the ^{64}Cu -NOTA-WF121 nanobody for rapid and high-contrast detection of CD38 + tumor lesions. These findings reinforce previous reports highlighting the value of nanobodies as PET imaging agents and, for the first time, demonstrate the feasibility and promise of combining an anti-CD38 nanobody with copper-64 for immuno-PET imaging in multiple myeloma.

Abbreviations

- %ID/g: percentage injected dose per gram
- %ID/mL: percentage injected dose per milliliter
- CAR-T: chimeric antigen receptor T-cell therapy
- CT: computed tomography
- EPR: enhanced permeability and retention
- FDG: 18F-fluorodeoxyglucose
- MM: multiple myeloma
- NCS: N-chlorosuccinimide
- NHS: N-hydroxysuccinimide
- NOTA: 1,4,7-Triazacyclononane-1,4,7-triacetic acid
- PET: positron emission tomography
- sdAb: single-domain antibody
- TIGIT: T-cell immunoreceptor with immunoglobulin and immunoreceptor tyrosine-based inhibitory motif domain
- TOHP: tris(hydroxypyridinone)

Declarations

Ethics approval and consent to participate:

The study was performed in accordance with institutional guidelines for animal experimentation and was approved by the French Ministry of Research (APAFIS #34964). All experiments were conducted in compliance with the ARRIVE guidelines.

Consent for publication:

Not applicable.

Availability of data and material:

The datasets generated and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests:

The authors declare that they have no competing interests

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Study design: SG, TP, NB, MC, JG. Nanobody production, labeling and controls: SG, TP, PLS, VN. *In vitro* experiments: SG, VN, JF. Animal model conception: SG, SML. Acquisition of data: SG, TP, PLS, VN, SML, JF. Analysis and interpretation SG, TP, PLS. Drafting of the manuscript: SG. Critical revision: NB, CB, JG, MC. Funding acquisition: CB, MC, JG.

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Figures

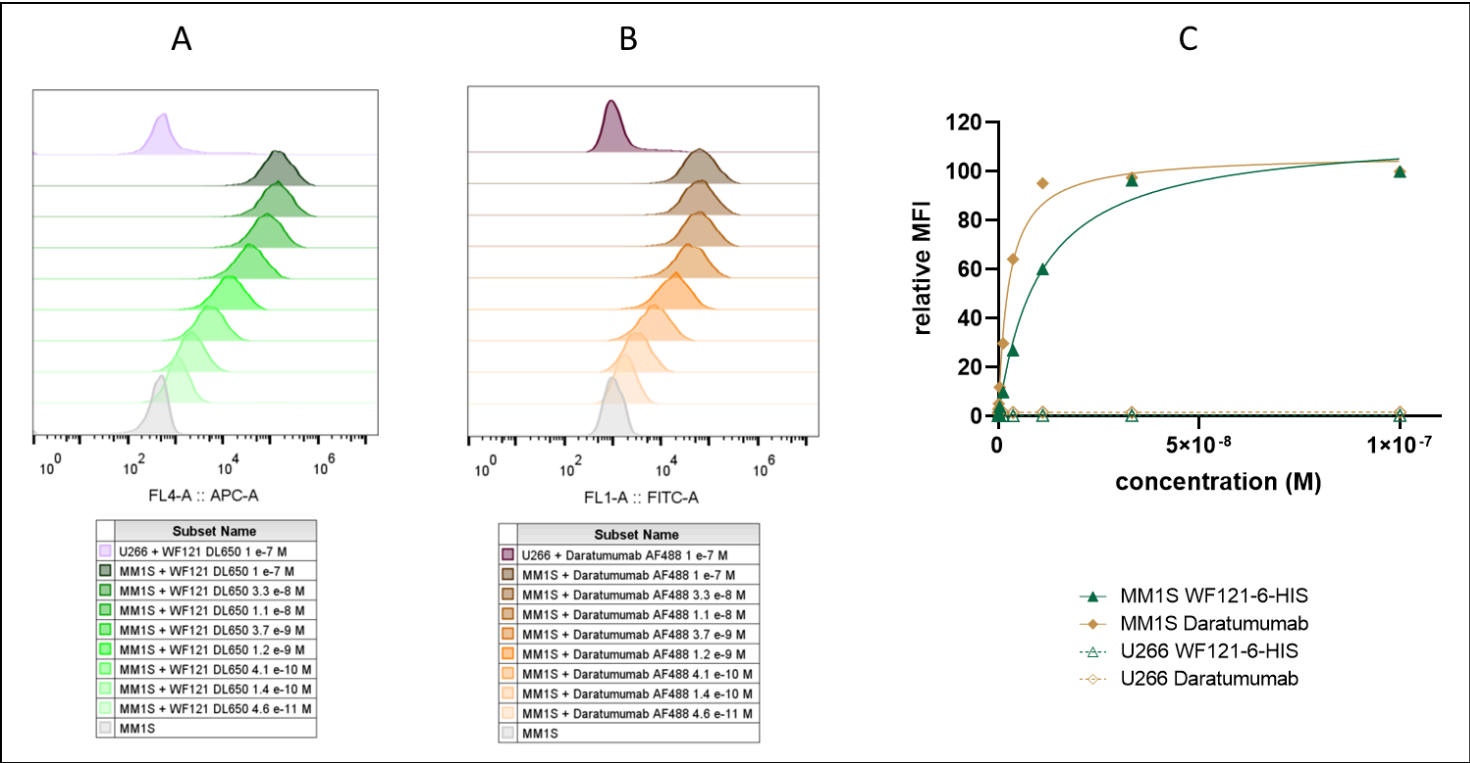


Figure 1

The WF121 nanobody efficiently binds CD38-expressing tumor cells. A) Flow cytometry histograms of WF121 DL650 binding to CD38⁺ MM1S and CD38⁻ U266 cells. B) Flow cytometry histograms of Daratumumab AF488 binding to CD38⁺ MM1S and CD38⁻ U266 cells. Cells were incubated with

fluorescent conjugates at concentrations ranging from 4.6×10^{-11} to 1×10^{-7} M. C) Relative median fluorescence intensity (RMFI) as a function of antibody concentration for WF121 and Daratumumab.

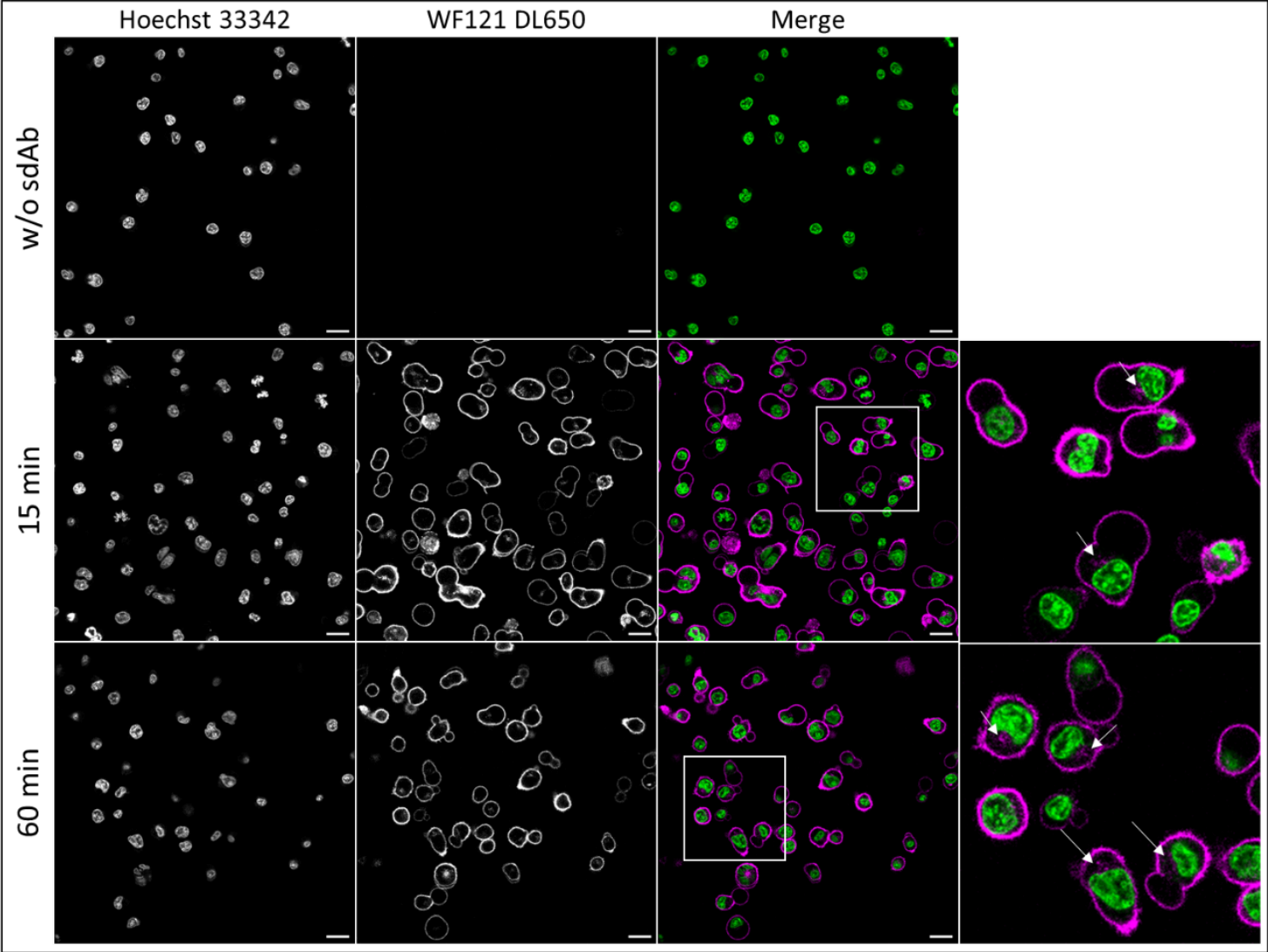


Figure 2

Partial internalization of the WF121 nanobody following CD38 binding. Confocal microscopy images showing partial internalization (white arrows) of WF121–DL650 in MM1S cells after 15 and 60 min of incubation. Cell nuclei were stained with Hoechst 33342. The last column displays magnified views of the regions outlined in the merged images.

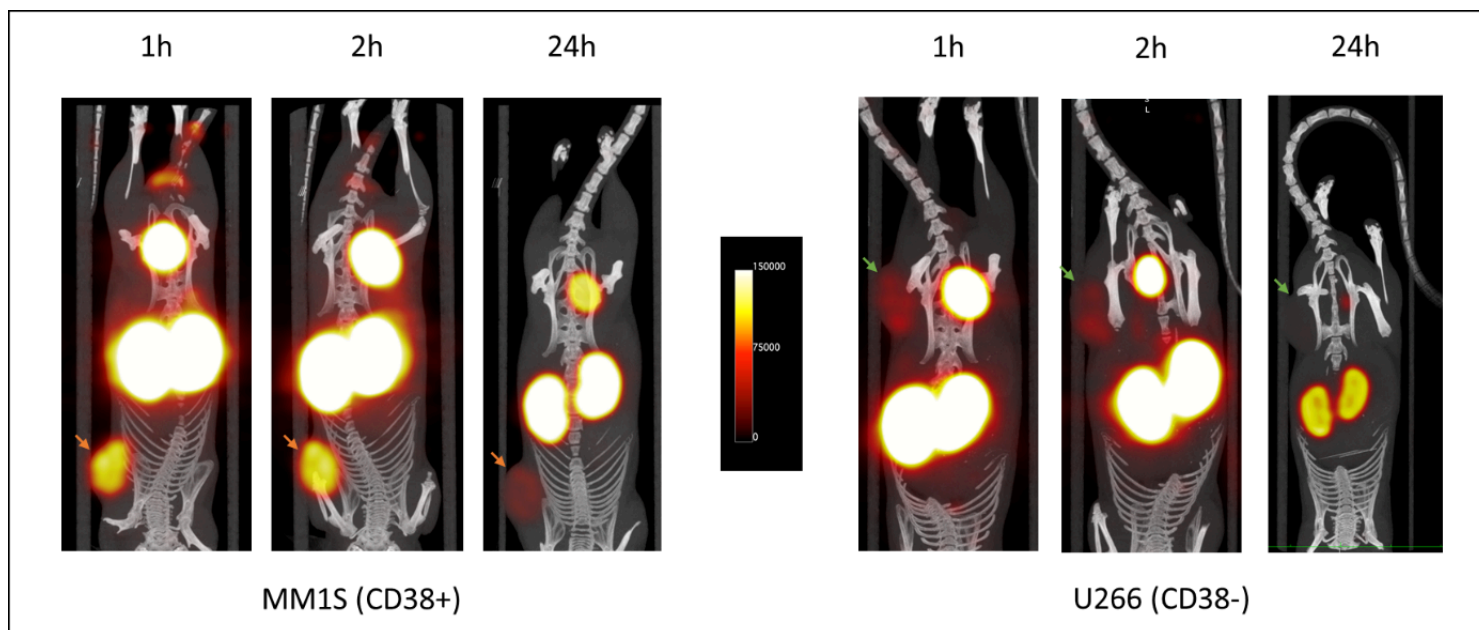


Figure 5

Sequential PET imaging of tumor-bearing mice injected with radiolabeled WF121. MIP PET/MIP CT acquisitions obtained following injection of ^{64}Cu -NOTA-WF121 in (A) MM1S (CD38⁺) subcutaneous model, and (B) U266 (CD38⁻) subcutaneous xenograft models. Tumors are indicated by arrows. PET scale: 0 to 150 kBq/mL.

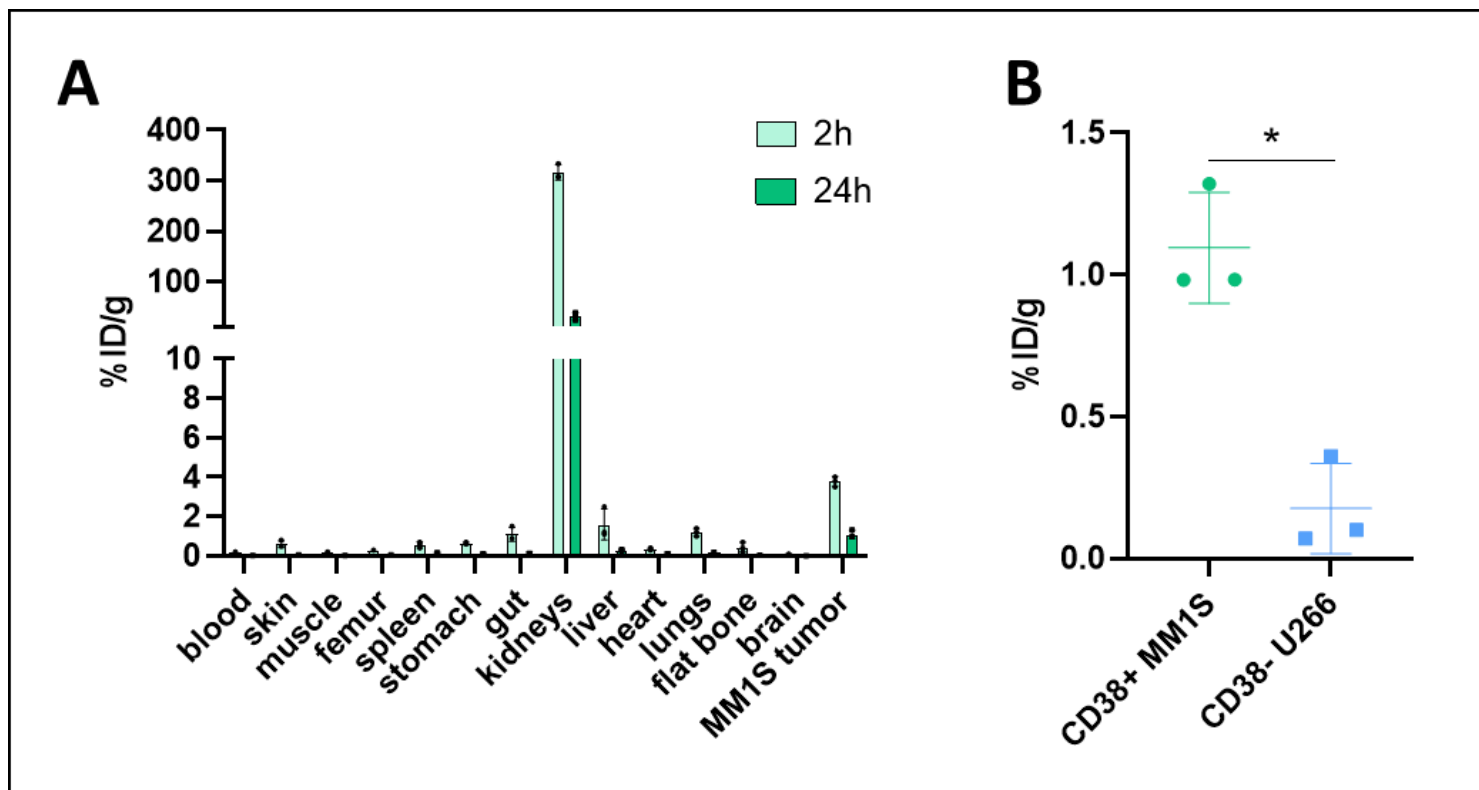


Figure 6

Ex vivo biodistribution of ^{64}Cu -NOTA-WF121 (without decay correction). (A) Biodistribution in MM1S-bearing mice (n = 3) at 2 and 24 h post-injection. (B) Tumor uptake in CD38⁺ MM1S and CD38⁻ U266 xenografts at 24h post-injection (n=3).

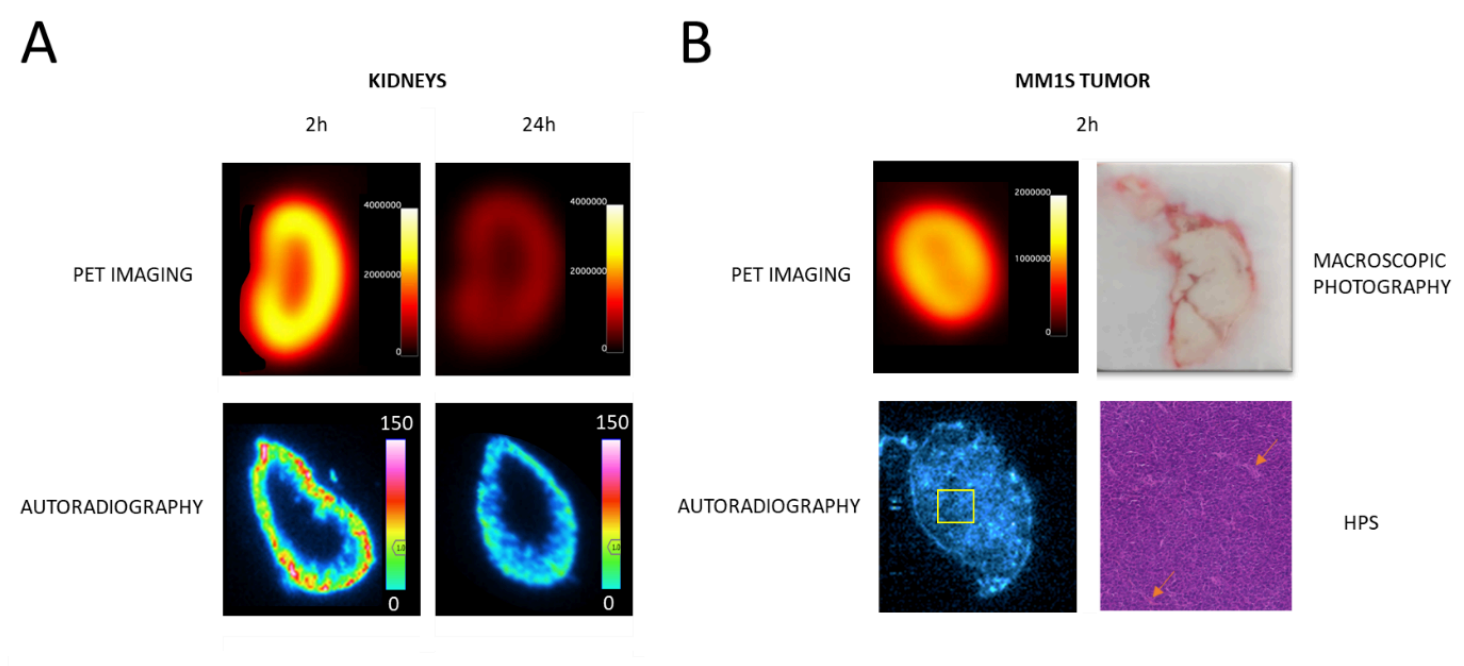


Figure 7

Multimodal imaging acquisitions. (A) Kidney sections extracted from PET imaging and representative digital autoradiography acquisitions performed at 2 h and 24 h post-injection of ^{64}Cu -NOTA-WF121. (B) MM1S tumor sections extracted from PET imaging, representative digital autoradiography acquisition, macroscopic photograph, and histological analysis (Haematoxylin–Phloxin–Saffron staining) performed at 2 h post-injection of ^{64}Cu -NOTA-WF121. The histological image corresponds to the area delineated by the yellow rectangle on the autoradiography image. Orange arrows indicate large blood vessels.