Chapter 6

Preclinical T cell imaging with positron emission tomography using CD4 and CD8 targeting $^{89}$Zr-F(ab$'$)$_2$ fragments

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ABSTRACT

Rationale: Despite clinical efficacy, not all patients respond to immunotherapy. Therefore, novel immunotherapy approaches are developed. In the immune response CD4+ T and CD8+ T-cells play an important role. To get more insight into their presence, we studied CD4+ and CD8+ T cell distribution with zirconium-89 (\(^{89}\)Zr) positron emission tomography (PET) in healthy and syngeneic tumor-bearing mice, without and following an actinium-225 labeled antibody (\(^{225}\)Ac-mAb) radionuclide therapy.

Methods: At day 0, 30 µg \(^{89}\)Zr-CD4, \(^{89}\)Zr-CD8, or nonspecific \(^{89}\)Zr-control F(ab'){\(_2\)} tracer was administered to DBA/2 mice who underwent PET 24 h post-injection (pi). Day 7, mice received a second tracer injection followed by PET at 24 h. At both time points, also ex vivo biodistribution analysis was performed. Mice bearing syngeneic KLN205 squamous cell lung cancer received 250 kBq/kg, 0.75 mg/kg \(^{225}\)Ac-mAb or vehicle. On day 4, \(^{89}\)Zr-CD4 was injected, followed by PET at 24 h, and ex vivo biodistribution. PET tracer tissue uptake was expressed as mean standardized uptake value (SUV\(_{\text{mean}}\)). Autoradiography and immunohistochemistry were performed on lymph nodes.

Results: In healthy mice, \(^{89}\)Zr-CD4 and \(^{89}\)Zr-CD8 PET showed respective SUV\(_{\text{mean}}\) of 2.7 ± 0.3 and 1.3 ± 0.4 in spleen, 2.2 ± 0.3 and 1.1 ± 0.1 in mesenteric lymph nodes, and 1.6 ± 0.1 and 0.9 ± 0.1 in cervical lymph nodes, at 24 h. \(^{89}\)Zr-CD4 and \(^{89}\)Zr-CD8 showed higher uptake in these lymphoid tissues than \(^{89}\)Zr-control, which was only visible in kidneys (SUV\(_{\text{mean}}\) 5.8 ± 0.4). Murine antibody formation precluded repeat CD8 tracer administration. Tissue distribution did not differ after the first and second \(^{89}\)Zr-CD4 injections. Autoradiography of lymph node analyses confirmed \(^{89}\)Zr-CD4 uptake at sites of immunohistochemical CD4 expression. \(^{225}\)Ac-mAb treatment did not affect \(^{89}\)Zr-CD4 uptake in tumors and lymphoid tissues.

Conclusion: \(^{89}\)Zr-CD4 and \(^{89}\)Zr-CD8 PET showed higher uptake in spleen and lymph nodes than \(^{89}\)Zr-control PET. \(^{89}\)Zr-CD4 biodistribution was reproducible and reflected CD4+ T cell presence in healthy and tumor-bearing mice. \(^{225}\)Ac-mAb did not affect CD4+ T cell uptake in the tumor.
CD4 and CD8 T cell PET imaging

BACKGROUND

Immunotherapy induces responses and increases survival in patients with several cancer types by blocking tumor immune escape mechanisms. However, only not all patients respond to this treatment (1). Insight into immune cell whole-body distribution might provide valuable information to distinguish upfront those that may respond. Furthermore, when the early effect of immunotherapy could be studied non-invasively, treatment can potentially be modified. Molecular imaging of immune cell markers is a potential tool to provide insight into the tumor- and whole-body immune status (2). CD8+ T cell-induced tumor cell kill is explored in patients as a molecular imaging read-out for response to immune checkpoint inhibitors (3, 4). Next to CD8+ cells, CD4+ T cells are essential partners in the antitumor immune response. Activation and expansion of CD8+ T cells rely on CD4+ T cells' helper function in the tumor-specific antigen presentation by dendritic cells (5-7). Moreover, CD4+ T cells display antitumor cytotoxicity, independent of CD8+ T cells (8-10). A molecular imaging study with an F(ab')2 tracer showed that CD4+ T cell levels increased in tumors of mice that responded to immune checkpoint inhibition (11). Targeted radionuclide therapy, such as beta-emitter lutetium-177 labeled tumor-targeting moieties, shows antitumor activity in patients with neuroendocrine tumors and prostate cancer (12). Less far-traveling, more potent alpha particle emitters, such as actinium-225 (225Ac) radiopharmaceuticals, are currently tested in clinical trials (NCT04597411, NCT04644770, NCT05219500, NCT03746431). Radiation-induced tumor cell death could lead to the activation of tumor-specific T cells, triggering an antitumor immune response (13-16). Still, radiation can also downregulate or kill immune cells, leading to tumor immune escape (17). Non-invasive whole-body insight into the immune status could lead to a better understanding. We aimed to investigate T cell imaging with positron emission tomography (PET) using CD4+ and CD8+ T cell targeting zirconium-89 (89Zr) labeled F(ab')2 fragments. In healthy mice, we tested the reproducibility of T cell PET imaging in the same animals. In syngeneic lung tumor-bearing mice, we studied the potential radionuclide-induced antitumor immune response induced by a 225Ac labeled tumor-targeting monoclonal antibody.

MATERIALS AND METHODS

Preparation and characterization of the 89Zr-DFO-F(ab')2 tracers and 225Ac-mAb

F(ab')2 fragments were generated from rat-anti-mouse antibodies targeting CD4, and CD8α, and an isotype control antibody (BioXCell: CD4 clone #BE0003-1; CD8α clone # BE0117; IgG2b #BE0090). Antibodies were cleaved at the hinge with a FabBRICATOR kit
(Genovis # A0-FR1-050) as described earlier (11). The F(ab’)2 fragments were subsequently purified with preparative ultra-performance liquid chromatography eluting with 170 mM ammonium acetate buffer, 300 mM NaCl, 2.5 mM DTPA, and 10% DMSO, pH 7.0, at 0.7 mL/min (Yarra 3 µm SEC-2000, LC Column 300 x 7.8 m, Ea, Phenomenex #00H-4512-K0). Buffer exchange was performed with a centrifugal concentrator (Vivaspin, cut-off 30Kd) to 25 mM sodium bicarbonate buffer, pH 9. Then, p-SCN-Bn-desferrioxamine (DFO, Macrocyclics) conjugated to the F(ab’)2 for 1 h at 37 °C, at a 1 to 10 molar ratio. The F(ab’)2-DFO conjugates were radiolabeled with 89Zr-oxalate (Perkin Elmer) in 1.0 M HEPES, pH 7.4, 3 MBq per 30 µg protein, for 1 h at 37 °C, resulting in 89Zr-CD4, 89Zr-CD8 and 89Zr-control. The tracer solutions were diluted with 0.9% NaCl to a concentration of 30 µg protein per 150 µL injection volume. 89Zr-tracers were characterized for monomer purity with size-exclusion liquid chromatography (conditions as above). Radiochemical purity was determined with an immunoprecipitation assay using trichloroacetic acid (TCA). The affinity of the PET tracers for recombinant mouse CD4 His-tag (R&D #10476-CD) and recombinant mouse CD8 alpha Protein CF (R&D #9135-CD) were measured with a competitive binding assay. For 89Zr-CD4 or 89Zr-CD8, their respective intact antibodies served as competitors, and binding was compared with the unspecific 89Zr-control at 6.7 nM. TCA and binding assays are described previously (18). The therapeutic antibody (target not disclosed) was generated by radiolabeling mAb-NCS-2-macropa conjugate with 225Ac in 0.1 M sodium acetate buffer pH 5.5 at room temperature for 60 min. A 0.75 mg/kg, 250 kBq/kg dose was injected in a 100 µL volume. Radiochemical purity was determined with instant-thin-layer-chromatography, strip with silica gel, with 0.1 M citrate buffer pH 5.5 as mobile phase.

Cell line

The KLN205 murine squamous cell lung cancer cell line was obtained from American Type Culture Collection. Cells were cultured in DMEM high glucose medium, with 10% fetal calf serum, 1 mM sodium pyruvate, and non-essential amino acids and incubated at 37 °C with 5% CO2 in a humidified incubator. The medium was refreshed twice a week. The cells were mycoplasma-free. Tumor target expression was determined with fluorescence-activated cell sorting. KLN205 cells expressed low 225Ac-mAb target levels, with 1.7-fold higher fluorescent counts than a non-expressing cell line (Suppl. Fig. 1).

Animal studies

Animal experiments were approved by the ethical board for animal experiments in the Netherlands. To investigate reproducible T cell monitoring (e.g., before and after treatment), healthy DBA/2 female mice underwent tracer injection and PET scans at two time points with 7 days in between. Therefore, at day 0, n=6 mice received 30 µg, 3 MBq, of 89Zr-CD4, 89Zr-CD8, or 89Zr-control intravenously (iv). At 24 h, all mice underwent imaging (PET 1). Three mice were sacrificed afterwards, for ex vivo biodistribution analysis...
CD4 and CD8 T cell PET imaging

(Fig. 3A, Suppl. Fig. 4A). To study the $^{89}\text{Zr-F(ab')}_2$ fragments distribution in time, the other three mice per group were also scanned at 4 h and 48 h post-injection (pi). On day 7, they received a second tracer injection and were imaged again at 24 h pi (PET 2), followed by ex vivo analyses. PET 1 and PET 2 were statistically analyzed for similarity. To study the effect of a single $^{225}\text{Ac-mAb}$ dose on CD4$^+$ T cells in a syngeneic tumor model, mice received a subcutaneous injection of $0.8*10^6$ KLN205 cells in the right shoulder on day -14. The tumor size inclusion criterion for PET was $> 200$ mm$^3$. Mice received 250 kBq/kg, 0.75 mg/kg $^{225}\text{Ac-mAb}$ targeting tumor cells or vehicle (100 µL, 0.1 M sodium acetate buffer) iv at day 0 followed by $^{89}\text{Zr-CD4}$ iv at day 4, and PET scans 24 h, 48 h, and 72 h after tracer injection (Fig. 5A). Four mice per group were sacrificed at 24 h and 72 h to perform ex vivo biodistribution analysis. After each sacrifice, tissues were weighed, and radioactivity was measured in a calibrated Wizard gamma counter (PerkinElmer). Uptake is expressed as the percentage injected dose per gram (%ID/g). Scans were obtained with a Focus 220 PET scanner (CTI Siemens). PET data was reconstructed and corrected for decay, random coincidences, scatter, and attenuation. PET tracer uptake was quantified with AMIDE 1.0.4 software and expressed as the mean standardized uptake value (SUV$_{\text{mean}}$).

Analysis of plasma, lymph nodes, and tumor

Tracer integrity of $^{89}\text{Zr-CD4}$ and $^{89}\text{Zr-control}$ in plasma of the mice was studied by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Mini-Protean® TGX™ precast protein gels 4-15% (BioRad) were loaded with 80 µg plasma protein. The control sample contained the tracer stored for 7 days at 2-8 °C. Gels ran for 30-45 min at 100 V. Formalin-fixed lymph node tissues were paraffin-embedded and sliced into 4 µm sections. Autoradiography of gels and lymph node sections was assessed by overnight exposure to a multipurpose phosphor plate (Perkin Elmer) at -20 °C and captured with Cyclone® phosphor imager (Perkin Elmer). All tissues were stained with hematoxylin-eosin. Tumor sections were stained for DNA damage marker gamma histone 2aX ($\gamma$H2aX), as described previously (18), using 0.5 µg/mL primary antibody (Millipore, #05-636). The tumor draining right axillary lymph nodes might play an important role in T cell recruitment towards the tumor implanted on the right shoulder. Therefore, $^{89}\text{Zr-CD4}$ uptake and CD4 and CD8 immunofluorescence were investigated, comparing left- (control) and right axillary lymph nodes. Tumors and axillary lymph nodes sections were stained for CD4 and CD8 with immunofluorescence, using 1.0 µg/mL anti-CD4 primary antibody (CD4, BD, #550278) and 0.5 µg/mL anti-CD8 primary antibody (CD8, AbD Serotec, #MCA1768) and subsequently incubated with a secondary antibody and fluorescent marker (Vector #MP-7444; CD8 Opal 570, red; Opal 520, green; DAPI nucleus staining, blue). The presence of murine antibodies in plasma, harvested 24 h after the second injection of $^{89}\text{Zr-CD8}$ and -CD4, was studied with an enzyme-linked immunosorbent assay. Plates were coated with the intact CD4- and CD8 antibodies, plasma was added, and a rabbit anti-mouse IgG HRP (#315-035-047, Jackson) was used for detection.
Statistical analysis

Similarity between the two groups was analyzed using a Mann-Whitney U test. Data are presented as single data points ± standard deviation. All statistical tests were performed in GraphPad Prism 9.3.1, and $P$-values < 0.05 were considered significant.

RESULTS

Characterization $^{89}$Zr-DFO-F(ab’)$_2$ tracers

The intact murine CD4- and CD8-targeting antibodies and an unspecific control antibody (150 kDa) were successfully cleaved at the hinge region and purified, generating the F(ab’)$_2$ fragments (110 kDa; Fig. 1A). Quality control of radiolabeled DFO-F(ab’)$_2$ conjugates $^{89}$Zr-CD4, $^{89}$Zr-CD8, and $^{89}$Zr-control revealed respective monomer purity of 96.2%, 92.0%, and 92.9% and radiochemical purity of 99.5%, 99.2%, and 99.3%. CD4- and CD8 binding affinities were preserved (83.8% and 89.1%) and specificity absence of $^{89}$Zr-control was confirmed (Fig. 1B, C).

$^{89}$Zr-CD4 and $^{89}$Zr-CD8 PET in healthy mice

Both tracers showed fast distribution from blood to tissues. Over the course of 48 h, the blood pool levels dropped from respectively SUV$_{\text{mean}}$ 2.2 ± 0.1 and 2.8 ± 0.5 at 4 h to SUV$_{\text{mean}}$ of 0.3 ± 0.0 and 0.4 ± 0.1 at 48 h (Fig. 2A-C). The $^{89}$Zr-CD8 kidney SUV$_{\text{mean}}$ increased strongly to 6.9 ± 0.6 at 48 h, 2.0-fold higher than $^{89}$Zr-CD4 (3.4 ± 0.2). At 24 h, the SUV$_{\text{mean}}$ of $^{89}$Zr-CD4 spleen uptake was 2.7 ± 0.3. In mesenteric lymph nodes uptake was 2.2 ± 0.3 in the cervical lymph nodes 1.6 ± 0.1. The SUV$_{\text{mean}}$ for $^{89}$Zr-CD8 in the same tissues was at least 1.9-fold lower, namely, in spleen 1.3 ± 0.4; in mesenteric lymph nodes 1.1 ± 0.1; and in cervical lymph nodes 0.9 ± 0.1. $^{89}$Zr-CD4 and $^{89}$Zr-CD8 showed higher uptake in these lymphoid tissues than $^{89}$Zr-control at 24 h, which was only present in kidneys (SUV$_{\text{mean}}$ 5.8 ± 0.4, Fig 2D). As at 24 h the blood level of the tracers was very low, this was considered the optimal moment for further experiments.
Figure 1. Characterization of $^{89}$Zr-DFO-F(ab’)$_2$ tracers

A Size exclusion high-performance liquid chromatography of precursor intact antibody (1), cleaved in F(ab’)$_2$ and Fc intermediates (2), purified F(ab’)$_2$ (3), and conjugated, radio-labeled $^{89}$Zr-DFO-F(ab’)$_2$ (4) (data shown for CD4 tracer)

B Competitive binding of $^{89}$Zr-DFO-F(ab’)$_2$ to CD4 (left) and CD8 (right), respectively, against intact antibody

C Summary quality control parameters of $^{89}$Zr-DFO-F(ab’)$_2$ radiolabeled PET tracers, *280 nm main peak from DFO-F(ab’)$_2$, not $^{89}$Zr-DFO-F(ab’)$_2$. Ctrl, control; DFO, deferoxamine; NA, not assessed; RAD, radioactivity detection; RCP, radiochemical purity.
Figure 2. $^{89}$Zr-CD4 and $^{89}$Zr-CD8 PET in healthy mice. A study outline of $^{89}$Zr-F(ab’)$_2$ PET imaging B coronal planar projections of $^{89}$Zr-CD4 PET (upper panel) and $^{89}$Zr-CD8 PET (lower panel) in healthy DBA/2 mice (n=3) at 4 h, 24 h, 48 h quantified, as mean standardized uptake value D compared to $^{89}$Zr-control at 24 h. Statistical differences ($P$-values < 0.05) are indicated with a star for $^{89}$Zr-CD4 and $^{89}$Zr-CD8 compared to control. B, blood C(LN), cervical lymph nodes; K, kidney; L, liver; M(LN): mesenteric lymph nodes; PET: positron emission tomography; S: spleen; SUV: standardized uptake value.
Repeated $^{89}$Zr-CD4 and $^{89}$Zr-CD8 PET in healthy mice

Repeated PET of $^{89}$Zr-CD4, $^{89}$Zr-CD8, and $^{89}$Zr-control biodistribution was tested in the same animal with a 7-day interval (Fig 3A, Supp. Fig. 4A). $^{89}$Zr-CD4 distributed highly similarly at both scans, as did $^{89}$Zr-control (Fig. 3B, C). Ex vivo analysis confirmed similar $^{89}$Zr-CD4 distribution at the two time points. The spleen contained 61.0% ± 4.5% ID/g $^{89}$Zr-CD4, and 62.4% ± 3.3% ID/g, respectively. In mesenteric lymph nodes $^{89}$Zr-CD4 uptake was 67.4% ± 17.1% ID/g the first time and 26.9% ± 19.3% ID/g the second time. The 2-fold lower uptake after repeated PET can be explained by histological analysis, which showed fat and muscle tissue adjacent to the harvested mesenteric lymph nodes, affecting tissue weight (Fig. 3D, Suppl. Fig.2). $^{89}$Zr-CD8 was tested in a similar design as $^{89}$Zr-CD4, yet it revealed a completely different distribution after the second injection. The tracer accumulated...
namely mainly in the liver. This was confirmed ex vivo, by an $^{89}$Zr-CD8 liver uptake of 16.5 ± 1.1 % after the first-, and 37.0 ± 4.3% ID/g after the second injection (Supp. Fig. 4B, C). Blood levels were 7.6 ± 0.3% ID/g after the first injection and 0.3 ± 0.1% ID/g after the second injection. Mice receiving the first and second $^{89}$Zr-CD4 or $^{89}$Zr-CD8 administration showed 1.4- and 2.4-fold increase in murine antibody presence, compared to a negative control (Supp. Fig. 5).

 Autoradiography of lymph nodes and plasma
 Autoradiography showed higher $^{89}$Zr-CD4 presence in the lymph nodes than the $^{89}$Zr-control (Fig. 4 upper vs lower panel). Lymph node histology shows that the presence of immune cells matched with $^{89}$Zr-CD4 hot spots, whereas $^{89}$Zr-CD4 was absent in fat, muscle fiber, and gland cells (Fig. 4, Suppl. Fig 3). This indicates specific $^{89}$Zr-CD4 uptake by the CD4+ T cell-rich lymph nodes. $^{89}$Zr-DFO-F(ab’)2 tracers in plasma disintegrated at 24 h to separate
single $^{89}$Zr-DFO-Fab fragments, this was more pronounced for the CD4 and CD8 tracer than for the control (Suppl. Fig. 4). The remaining radioactivity per mouse before the second tracer injection on day 7 was around 0.3 MBq. Prolonging the time between injections for additional radionuclide clearance and decay would result in too large tumors. Thus, a pretreatment scan was omitted in the next experiments, and $^{89}$Zr-CD4 PET was only performed after the $^{225}$Ac-mAb treatment.

**Figure 5.** $^{89}$Zr-CD4 PET in tumor-bearing mice that received $^{225}$Ac-mAb. A study outline of $^{89}$Zr-CD4 PET in KLN205 tumor-bearing mice that received $^{225}$Ac-mAb or vehicle B maximum intensity projections of $^{89}$Zr-CD4 PET imaging in $^{225}$Ac-mAb-treated mice versus vehicle-treated mice 24 h, after tracer injection and C quantification of tumor, cervical-, and mesenteric lymph nodes, blood, spleen, and kidneys, expressed as mean standardized uptake value. D Ex vivo biodistribution at 24 h after tracer injection expressed as % injected dose per gram. ALN, axillary lymph nodes C(LN), cervical lymph nodes; K, kidney; L, liver; M(LN), mesenteric lymph nodes; PET: positron emission tomography; S, spleen; SUV, standardized uptake value; T, tumor.
Figure 6. CD4 and CD8 in axillary lymph nodes of tumor-bearing mice that received $^{225}$Ac-mAb. A immunofluorescence in paraffin-embedded axillary lymph node 4 µm slices of CD4 (green) and CD8 (red) and nuclei (DAPI, blue). B quantification of CD4 and CD8 immunofluorescence expressed as area percentage.

$^{89}$Zr-CD4 PET in tumor-bearing mice that received $^{225}$Ac-mAb

$^{89}$Zr-CD4 PET was lastly tested in KLN205 tumor-bearing mice treated with $^{225}$Ac-mAb or vehicle iv (Fig. 5A). At day 5 $^{89}$Zr-CD4 tumor SUV$_{\text{mean}}$ was 1.0 ± 0.3 at 24 h in treated mice. Uptake did not differ from tumor uptake in vehicle-treated mice (Fig. 5B, C). Ex vivo data confirmed similar $^{89}$Zr-CD4 tumor presence in $^{225}$Ac-mAb- vs vehicle-treated mice, namely, 6.6% ± 3.8% vs 5.0% ID/g ± 1.1% ID/g. Ex vivo $^{89}$Zr-CD4 uptake in mesenteric-, cervical- and axillary lymph nodes did also not differ between the $^{225}$Ac-mAb and vehicle treatment group. Some mice were additionally imaged 48 h and 72 h after tracer injection. This also showed no relevant changes of $^{89}$Zr-CD4 distribution (Suppl. Fig 7). Tumor analysis of day 5 after $^{225}$Ac-mAb administration showed no change in the DNA damage marker $\gamma$H2aX or CD8 and CD4 expression (Suppl. Fig. 8). Looking further into T cell immunofluorescence in the axillary lymph nodes, a higher CD8$^+$ T cells signal was observed in the right- compared to the left side, of mice that received $^{225}$Ac-mAb vs vehicle. CD4 immunofluorescence did not differ between the right and left axillary lymph nodes (Fig. 7).
DISCUSSION

This study showed in healthy mice with $^{89}$Zr-CD4 and $^{89}$Zr-CD8 F(ab$'$)$_2$ PET imaging, specific uptake in the spleen and in mesenteric-, cervical- and axillary lymph nodes. Ex vivo autoradiography matched with the presence of immune cells with hematoxylin-eosin analysis. A second administration 7 days later of $^{89}$Zr-CD4 results were reproducible with a similar biodistribution pattern. For the $^{89}$Zr-CD8 tracer increased murine antibody blood levels precluded the repeat imaging. In KLN205 tumor-bearing mice $^{89}$Zr-CD4 tumor uptake occurred, which was not affected by $^{225}$Ac-mAb treatment. CD4 received for a long time less attention than CD8. It now emerges as a valuable T cell subset to study in oncology and autoimmune diseases. Here we demonstrated that we can reliably image CD4$^+$ T-cells. We produced the F(ab$'$)$_2$ fragments tracers with high purity and preserved binding capacity. Uptake matched with T-cell presence in contrast to uptake of the control antibody. And after a second tracer injection the biodistribution is similar to the first. The observed specific uptake in the spleen, lymph nodes, and tumor matches the literature (20). Moreover, our data revealed higher $^{89}$Zr-CD4 than $^{89}$Zr-CD8 uptake in the spleen and lymph nodes. In healthy mammals, CD4/CD8 T cell ratios are reported to vary between 1.5-2.5 (24, 25). The substantial kidney accumulation that occurred is most likely caused by in vivo breakage into single Fabs of 55 kDa, indicating hinge region instability. This may have reduced the half-life. A more stable tracer in which this region is fortified might circumvent this. Fab-fragment tracers can have several advantages over intact full-length monoclonal antibodies or CD4$^+$ T cell targeting constructs, including minibody, diabody, scFv, and single-domain fragments used in other studies (20-23). F(ab$'$)$_2$ lack the Fc part that could potentially interfere with T cell function or cause T cell depletion. And moreover, the molecular weight is lower; the clearance is faster, and therefore, repeat imaging is possible in a relatively shorter timeframe. Compared to smaller compounds, the 110 kDa F(ab$'$)$_2$ fragments allow for considerable tracer accumulation in tumor and immune-related tissues. $^{89}$Zr and $^{64}$Cu are suitable isotopes for radiolabeling. A PET tracer targeting human CD4, when proven nontoxic, might be used in the clinic to get insight into patients' whole-body immune status before, during, and after treatment with immune-activating anticancer therapies. The effect of radiation on immune cells is thoroughly studied in mice (17, 26-30). In one study CD4 T cells increased after low-dose total body irradiation (27). We used the $^{89}$Zr-CD4 tracer to study the effect of targeted radionuclide therapy with a new $^{225}$Ac radiolabeled tumor-targeting antibody on CD4 T cells. KLN205 tumor cells (31) express low tumor cell target levels for the $^{225}$Ac-mAb. In KLN205 tumor bearing mice. An intermediate treatment dose of 250 kBq/kg and scans 5-7 days post-treatment, the CD4 presence was unchanged. Tracers that can in vivo track CD4 and CD8 positive T cells whole body over time might help to provide more insight into the biology of T cell infiltration upon specific treatment. In conclusion, we robustly show specific whole body CD4 and CD8 cell imaging, and for CD4 we could also perform repeat imaging. $^{225}$Ac-mAb did not affect
CD4 T cell distribution after 5 to 7 days. Our data encourages further CD4 and CD8 imaging investigation for in vivo T cell tracking.

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REFERENCES


SUPPLEMENTAL MATERIAL

Supplemental Figure 1. Target expression KLN205 murine lung tumor cell line. Tumor target expression was determined with fluorescence-activated cell sorting. The count of the KLN205 negative control was 603 and for an isotype control 574.

Supplemental Figure 2. Mesenteric lymph nodes with additional tissue. Hematoxylin-eosin of paraffin-embedded mesenteric lymph node 4 µm slices, including additional fat-, and gland-, tissue. Weight in mg and 89Zr-CD4 uptake in %ID/g given for each tissue. The mesenteric lymph nodes of the third mouse of PET 1 are included in figure 4: 19.0 mg, 57.5 % ID/g. %ID/g, percentage injected dose per gram, HE, Hematoxylin-eosin; LN, lymph node; PET, positron emission tomography.
Supplemental Figure 3. CD4 immunofluorescence in the axillary lymph node. Immunofluorescence in paraffin-embedded axillary lymph node 4 µm slices of CD4 (green) and nuclei (DAPI, blue).

Supplemental Figure 4. Repeated $^{89}$Zr-CD8 PET in healthy mice. A study outline of $^{89}$Zr-CD8 PET imaging B maximum intensity projections of $^{89}$Zr-CD8 PET 1 at 24 h and 48 h vs. $^{89}$Zr-CD8 PET 2 at 24 h in healthy DBA/2 mice, and C Ex vivo biodistribution in healthy DBA/2 mice at 24 h after tracer injection expressed as % injected dose per gram. Statistical differences (P-values < 0.05) are indicated with a star. ALN, axillary lymph nodes; CLN, cervical lymph nodes; ctrl, control; K, kidneys; L, liver; MLN: mesenteric lymph nodes; PET, positron emission tomography.
Supplemental Figure 5. Tracer uptake in the liver and ADA-formation. A quantification of $^{89}$Zr-CD8 and $^{89}$Zr-CD4 liver uptake at 24 h (PET 1), and following a second injection at day 7, after 24 h (PET 2), expressed as mean standardized uptake value. B murine antibody levels in plasma samples 24 h after the second injection, expressed as absorbance fold over negative control assessed with enzyme-linked immunosorbent assay. Statistical differences ($P$-values < 0.05) are indicated with a star. PET, positron emission tomography; SUV, standardized uptake value.

Supplemental Figure 6. Tracer integrity of $^{89}$Zr-DFO-F(ab')$_2$ in plasma. Autoradiography of a sodium dodecyl sulfate gel, demonstrating $^{89}$Zr-DFO-F(ab')$_2$ integrity in plasma, compared to a reference sample from the injection solution. R, reference.
Supplemental Figure 7. $^{89}$Zr-CD4 PET in tumor-bearing mice that received $^{225}$Ac-mAb. A study outline of $^{89}$Zr-CD4 PET in KLN205 tumor-bearing mice that received $^{225}$Ac-mAb or vehicle B maximum intensity projections of $^{89}$Zr-CD4 PET imaging in $^{225}$Ac-mAb-treated mice versus vehicle-treated mice 24 h, 48 h, and 72 h after tracer injection and C quantification of tumor, cervical-, and mesenteric lymph nodes, blood, spleen, and kidneys. D Ex vivo biodistribution at 72 h after tracer injection expressed as % injected dose per gram. ALN, axillary lymph nodes; C(LN), cervical lymph nodes; K, kidney; L, liver; M(LN), mesenteric lymph nodes; PET: positron emission tomography; S, spleen; SUV, standardized uptake value; T, tumor.
Supplemental Figure 8. DNA damage and T cells in tumors of mice that received $^{225}$Ac-mAb. A γH2AX immunohistochemistry and B CD4 (green), CD8 (red) immunofluorescence of KLN205 tumors, harvested from DBA/2 mice that received $^{225}$Ac-mAb (left) or vehicle (right). Representative samples are shown.