Engineered antibody fragments for immuno-PET imaging of endogenous CD8\(^+\) T cells in vivo

Richard Tavaré\(^a\)*, Melissa N. McCracken\(^b\), Kirstin A. Zettlitz\(^a\), Scott M. Knowles\(^a\), Felix B. Salazar\(^a\), Tove Olafsen\(^a\), Owen N. Witte\(^c,d,e,1\), and Anna M. Wu\(^a,1\)

*Crump Institute for Molecular Imaging and \(^b\)Department of Molecular and Medical Pharmacology, David Geffen School of Medicine, \(^c\)Howard Hughes Medical Institute, \(^d\)Department of Microbiology, Immunology, and Molecular Genetics, and \(^e\)Eli and Edythe Broad Center of Regenerative Medicine and Stem Cell Research, University of California, Los Angeles, CA 90095

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The noninvasive detection and quantification of CD8\(^+\) T cells in vivo are important for both the detection and staging of CD8\(^+\) lymphomas and for the monitoring of successful cancer immunotherapies, such as adoptive cell transfer and antibody-based immunotherapeutics. Here, antibody fragments are constructed to target murine CD8 to obtain rapid, high-contrast immuno-positron emission tomography (immuno-PET) images for the detection of CD8 expression in vivo. The variable regions of two anti-murine CD8-depleting antibodies (clones 2.43 and YTS169.4.2.1) were sequenced and reformatted into minibody (Mb) fragments (scFv-C\(_2\)). After production and purification, the Mbs retained their antigen specificity and bound primary CD8\(^+\) T cells from the thymus, spleen, lymph nodes, and peripheral blood. Importantly, engineering of the parental antibodies into Mbs abolished the ability to deplete CD8\(^+\) T cells in vivo. The Mbs were subsequently conjugated to 52-(4-isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid for \(^{64}\)Cu radiolabeling. The radiotracers were injected i.v. into antigen-positive, antigen-negative, immunodeficient, antigen-blocked, and antigen-depleted mice to evaluate specificity of uptake in lymphoid tissues by immuno-PET imaging and ex vivo biodistribution. Both \(^{64}\)Cu-radiolabeled Mbs produced high-contrast immuno-PET images 4 h postinjection and showed specific uptake in the spleen and lymph nodes of antigen-positive mice.

The rapid increase of therapeutic antibodies approved by the US Food and Drug Administration (FDA) and those currently in phase I–III clinical trials for oncological, autoimmune, and inflammatory diseases, among other conditions, has benefited from advances in antibody engineering, protein conjugation chemistry, and biomarker identification (1–3). Concurrently, immuno-PET imaging agents based on intact antibodies have shown promise both preclinically and clinically for the detection of cancer in vivo (4). Noninvasive detection of specific biomarkers of disease can provide crucial information for diagnosis, prognosis, response to therapy, dosage for radioimmunotherapy, and targeted therapy selection.

Although much progress has been made in the immuno-PET detection of oncological markers (4), the noninvasive monitoring of immune cells in the fields of oncology, autoimmunity, and infection remains challenging. Practiced methods for lymphocyte detection include isolation of cells from the peripheral blood or, less commonly, the tissue of interest. However, the invasive tissue sampling methods are prone to error and do not provide dynamic information that reflects the number, location, and movement of lymphoid cells. Therefore, problems still exist for the evaluation of immunotherapy protocols due to the lack of effective methods to monitor the extent and duration of the therapy.

Current methods to monitor immune cells noninvasively using emission tomography include direct cell labeling, reporter genes, small-molecule PET tracers, and radiolabeled intact antibodies. The ex vivo direct labeling of immune cells with PET or single-photon emission computed tomography probes before subsequent reinjection and imaging has enabled in vivo trafficking of lymphocytes (5, 6). However, this method has inherent limitations, such as radioisotope t\(_{1/2}\) and cell division in vivo that lead to probe dilution. Reporter gene imaging, whereby cells are transfected with a PET reporter gene that encodes a protein specifically targeted via a radiolabeled reporter probe (7, 8), has been used to image adoptive cell transfer of transduced T-cell receptor-engineered lymphocytes (9). Reporter gene imaging allows for longitudinal tracking of cells but relies on the ex vivo transfection of cells and, for clinical translation, the development of nonimmunogenic PET reporter proteins (8).

In another approach, small-molecule PET probes targeting metabolic pathways, including \(^{[18]}\)F-fluorodeoxyglucose (\(^{[18]}\)FDG), \(^{[18]}\)F-fluorothymidine (\(^{[18]}\)FLT), and \(^{[18]}\)F-1-(2-deoxy-2-fluoro-arabinofuranosyl)cytosine (\(^{[18]}\)FAC), all have the potential to monitor diverse cell types of both innate and adaptive immunity noninvasively (reviewed in ref. 10). Clinically, \(^{[18]}\)F-FDG-PET has been used to evaluate inflammation in a range of diseases. However, in the context of immune cell detection in oncology, false-positive signals can arise from the utilization of glycolysis in both cancerous and immune cells, both innate and adaptive, in the tumor itself or in the draining lymph nodes (11, 12). \(^{[18]}\)F-FLT-PET accumulates in highly proliferative tissues, and most research has been focused on cancer detection. \(^{[18]}\)F FLT-PET suffers from high uptake in proliferating bone marrow, therefore limiting detection of lesions in bone. However, \(^{[18]}\)F FLT-PET was used clinically to detect cytotoxic T-lymphocyte antigen-4 blockade-induced cell replication in the spleens of patients with melanoma (13) and, more recently, to detect antigen-specific immune responses in patients with melanoma who have lymph node metastases using dendritic cell therapy (14). \(^{[18]}\)F-FAC-PET can distinguish between innate and adaptive immune cells due to the up-regulation of deoxycytidine kinase in proliferating T cells; however, the uptake in a Moloney murine sarcoma virus/murine leukemia virus complex-induced sarcoma model was limited to proliferating T cells in the draining lymph nodes and spleen (15). Therefore, like the other metabolic tracers FDG and FLT, FAC uptake due to activation-induced T-cell

**Significance**

**Anti-CD8 immuno-PET imaging agents provide the potential to monitor the localization, migration, and expansion of CD8-expressing cells noninvasively in vivo. Shown here is the successful generation of functional anti-CD8 imaging agents based on engineered antibodies for use in a variety of preclinical disease and immunotherapeutic models.**

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Conflict of interest statement: A.M.W. and T.O. have a financial interest in ImaginAb, Inc. T.O. is an employee of ImaginAb, Inc.

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1To whom correspondence should be addressed. E-mail: awu@mednet.ucla.edu.

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proliferation is restricted to the draining lymph nodes and was unable to image tumor T-cell infiltration (15). This makes immuno-PET imaging using antibody fragments targeting specific immune cell antigens (i.e., CD8 expressed on cytotoxic T cells and CD4 expressed on helper T cells) potentially critical for immunotherapeutic apnea diagnosis, because the expression of CD8 is present on all cytotoxic T cells and binding is not proliferation-dependent.

Intact antibodies have relatively long serum t1/2S (1-3 wk) compared with their engineered counterparts, such as the diabody αβ expressed as either the homodimer CD8 cytotoxic T cells and a subset of dendritic cells. Functional CD8 is a glycoprotein expressed mainly on a subset of T cells known as cytotoxic T cells and binding is not proliferation-dependent.

In this study, the parental antibodies from the hybridomas YTS 169.4.2.1 (YTS169) and 2.43 were engineered into Mb fragments (Fig. 1A). Both the YTS169 and 2.43 antibodies bind mCD8α (Lyt2). However, they differ in that the YTS169 antibody binds both Lyt2.1 and Lyt2.2, whereas the 2.43 antibody binds an epitope that is Lyt2.2-specific (Fig. 1B). These newly engineered Mbs retained their antigen specificity, as shown by flow cytometry and 125I immuno-PET imaging. Most importantly, both the 2.43 and YTS169 Mbs produce high-contrast immuno-PET images of CD8+ lymphoid organs at only 4 h p.i. This report details successful antibody fragment-based immuno-PET detection of CD8 expression in vivo.

**Results**

**Sequencing Variable Regions of Parental Rat Anti-murine CD8 Antibodies.** RT-PCR was repeated until at least two individual experiments produced three replicates of the same sequence for the VH and VL domains for each hybridoma for sequence assurance. For further sequence validation of hybridoma 2.43, the VH and VL from RT-PCR sequences were confirmed with tryptic digest mass spectrometry (MS) of the parental antibody. VH amino acid coverage was 35% (41 of 117), including the complete complementarity-determining region 1 (CDR1) and half of CDR2, and VL amino acid coverage was 62% (66 of 107), including both CDR2 and CDR3. For YTS169, MS sequence verification was performed.

**Production and Characterization of Mb Fragments.** Mb purification from NS0 supernatant was performed using nickel-nitrilotriacetic acid (Ni-NTA) columns and imidazole elution (Fig. S1A). SDS/PAGE showed that the Mb eluted between 25 and 45 min (Fig. S1B). The yields of the 2.43 and YTS169 Mbs were 6.6 and 8.9 mg/L, respectively. Purified protein was then analyzed on Superdex 200 size exclusion chromatography (SEC) and compared with reference standards to confirm assembly, purity, and dimerization (Fig. S1C). The 2.43 and YTS169 Mbs are purified as 81% or 23% 80-kDa dimers, respectively, as calculated by peak areas, with the remaining 19% and 77% eluting as higher molecular-weight multimers.

In this report, we develop two anti-murine CD8 Mbs (Fig. 1A) for the detection of CD8 expression. Murine CD8 is a cell surface glycoprotein expressed mainly on a subset of T cells known as cytotoxic T cells and a subset of dendritic cells. Functional CD8 is expressed as either the homodimer CD8αβ or the heterodimer CD8αβ of the two isoforms of CD8, αβ and βα. Mice have two allelic forms of CD8, Lyt2.1 and Lyt2.2, which are restricted to certain mouse strains. Lyt2.1, for example, is expressed in the mouse strains CBA, AKR, C3H, and DBA, whereas Lyt2.2 is expressed in the mouse strains BALB/c and C57BL/6 (B6). The difference between Lyt2.1 and Lyt2.2 is a methionine (Lyt2.2)-to-valine (Lyt2.1) substitution at residue 78 of the mature CD8α (Fig. 1B).

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Linker. The soluble antigen was purified using Ni-NTA affinity binding of the 2.43 and YTS169 Mbs to the purified sCD8 αβ C3H (Lyt2.1) primary cells from various organs of the antigen-negative Lyt2.1 C3H mice. The 2.43 Mb shows comparable binding to cells isolated from antigen-positive Lyt2.2 B/6 mice and does not bind CD8 in (Fig. 2). The 2.43 Mb was conjugated to FITC at a 1.4:1 ratio demonstrating further the epitope specificity of the 2.43-Mb construct, the 2.43 Mb was conjugated to FITC at a 1.4:1 ratio of fluorescein/Mb. Single-cell suspensions from the peripheral blood, thymus, spleen, and lymph nodes for staining with anti-CD4-PE and FITC-conjugated 2.43 Mb.

Flow cytometry confirmed epitope specificity of the Mb fragments. The murine CD8α T-cell lymphoma lines BW5/742 (Lyt2.2,2) and TK-1 (Lyt2.1,1) were stained with either 2.43 or YTS169 Mb, followed by anti-mouse IgG2a-phycocerythrin (Fig. S1D). To demonstrate further the epitope specificity of the 2.43-Mb construct, the 2.43 Mb was conjugated to FITC at a 1:4:1 ratio of fluorescein/Mb. Single-cell suspensions from the peripheral blood, thymus, spleen, and lymph nodes of B/6 (Lyt2.2,2) or C3H (Lyt2.1,1) mice were stained with either the FITC-2.43 Mb or a commercial FITC–anti-CD8 antibody and anti-CD4 (Fig. 2). The 2.43 Mb shows comparable binding to cells isolated from antigen-negative Lyt2.2 B/6 mice and does not bind CD8 in primary cells from various organs of the antigen-negative Lyt2.1 C3H mice.

To determine the affinity of both the 2.43 and YTS169 Mb, a recombinant soluble CD8αβ (sCD8αβ) heterodimer fusion protein was constructed by removing the transmembrane domains of both CD8α and CD8β and fusing them with a 29-aa α-helical linker. The soluble antigen was purified using Ni-NTA affinity chromatography, followed by SEC (Fig. S2). Solution-phase binding of the 2.43 and YTS169 Mbs to the purified sCD8αβ antigen was first confirmed by SEC. Briefly, equimolar amounts of soluble antigen and the Mb in question were incubated for 5 min in PBS before SEC analysis. All Mb peaks eluted 2.8–3 min earlier in the presence of sCD8αβ, confirming Mb and antigen-bound complexes of a larger size. Additionally, the Mb multimer detected by size exclusion eluted 2.5–2.8 min earlier (Fig. S3).

Surface plasmon resonance kinetic analysis was performed using Biacore 3000 (Precision Antibody, Inc.) with immobilized Mb and soluble monomeric sCD8αβ. The equilibrium constants (Kd) for 2.43 and YTS169 were 74 and 53 nM, respectively. A full analysis is provided in Fig. S4.

**In Vivo Depletion.** Single-cell suspensions from the spleen, peripheral blood, thymus, and lymph nodes of WT B/6 mice, B/6 mice treated with a CD8-depleting antibody, or B/6 mice treated with the 2.43 Mb were analyzed by flow cytometry for effective CD8 depletion (Fig. 3). Mice treated with the depleting antibody showed >95% loss of CD8+ leukocytes, whereas mice treated with the 2.43 Mb did not show a reduction in CD8+ leukocytes.

**NOTA Conjugation and Radiolabeling.** Following conjugation of both 2.43 and YTS169 Mb to S-2-(4-Isothiocyanatobenzyl)-1,4,7-triazacyclononane-1,4,7-triacetic acid (p-SCN-Bn-NOTA) and 64Cu radiolabeling, 64Cu incorporation was consistently >80% and the radiochemical purity was >98% after spin column purification (n = 10 radiolabelings). The immunoreactive fraction of the 64Cu-NOTA Mbs ranged from 65 to 75%. The specific activity was between 290 and 370 MBq/mg (8–10 μCi/mg), and mice were injected with 2.6–2.9 MBq (70–80 μCi) i.v.

**Immuno-PET and ex Vivo Biodistribution.** Due to the specificity for Lyt2.2, WT B/6 (Lyt2.2,2) mice were initially imaged with 64Cu-NOTA-2.43 Mb (Fig. 4). High-contrast immuno-PET images showed a high percent-injected dose per gram of tissue (%ID/g) uptake in the spleen, lymph nodes, and liver of the antigen-positive B/6 mice, and ex vivo biodistribution confirmed uptake of 75 ± 8.5%ID/g, 27 ± 7.9%ID/g, and 57 ± 11%ID/g, respectively (Table 1). When injected into antigen-negative Lyt2.1 C3H mice, the 64Cu-NOTA-2.43 Mb showed similar %ID/g uptake in the liver and fivefold reduced uptake in the spleen (15 ± 2.3%ID/g) and lymph nodes (2.7 ± 0.71%ID/g) compared with the B/6 mice (Fig. S4 and Table 1). The average %ID/g blood after only 4 h in B/6 and C3H mice was 0.90 ± 0.14%ID/g and 1.3 ± 0.10%ID/g, respectively.

To confirm the radiotracer uptake of 64Cu-NOTA-2.43 Mb in antigen-negative C3H mice, the 64Cu-NOTA-2.43 Mb was injected into immunodeficient NOD.Cg-Prkdcsix1Igαtm1Wjl/SzJ (NSG) mice that lack mature T cells, B cells, and natural killer cells. Immuno-PET images and ex vivo biodistribution in NSG mice were very similar to those of the 64Cu-NOTA-2.43 Mb in
antigen-negative C3H mice, confirming the high liver uptake as unspecific hepatic clearance of the radiolabeled Mb (Fig. 5A and Table 1). For the YTS169 Mb, the radiolabeling, specific activity, and immunoreactive fraction were similar to those of the $^{64}$Cu-NOTA-2.43 Mb. The immuno-PET imaging and ex vivo biodistributions in WT B/6 mice using the $^{64}$Cu-NOTA-YTS169 Mb were similar to those of $^{64}$Cu-NOTA-2.43 Mb in B/6 mice (Fig. 5B and Table 2). Interestingly, the %ID/g in the liver and spleen of the $^{64}$Cu-NOTA-YTS169 Mb in C3H mice is reduced by 29% and 48%, respectively, compared with B/6 mice (Fig. 5B and Table 2).

Finally, the $^{64}$Cu-NOTA-2.43 Mb was injected into B/6 mice that were blocked with coinjection of 80 μg (4 mg/kg) cold 2.43 Mb or had received anti-CD8 antibody depletion therapy (16 mg/kg for three consecutive days). Immuno-PET images and ex vivo biodistribution acquired 4 h p.i. of antigen-blocked and antigen-depleted mice (Fig. 6B and C and Table 2) showed similar uptake in the spleen (18 ± 1.9%ID/g and 15 ± 1.0%ID/g, respectively) and lymph nodes (5.0 ± 1.3%ID/g and 4.5 ± 2.7%ID/g, respectively) relative to the antigen-negative C3H and NSG mice. CD8 depletion was confirmed using flow cytometry as described above.

**Discussion**

The two anti-CD8 Mbs developed here for $^{64}$Cu immuno-PET imaging of CD8 expression retain their respective antigen specificities following engineering to the Mb format. Both produced at high yields in mammalian cell culture and could be purified using one-step immobilized metal affinity purification. In vivo studies demonstrated that both Mbs target the spleen and lymph nodes of antigen-positive mice. The lymph node uptake determined from the PET images is lower than the ex vivo biodistribution values due to the partial volume effect encountered when imaging small objects near or below the spatial resolution of the PET scanner (1.5 mm for the Inveon scanner). It has been calculated that a naive lymph node contains ~$10^6$ cells (18) and that 7–12% of the leukocytes in the lymph node are CD8$^+$.

This would suggest that we are able detect an estimated 70,000–120,000 CD8$^+$ cells in a mouse lymph node. We have also demonstrated that our engineered antibody fragments lacking the full Fc domain do not deplete CD8$^+$ T cells in vivo, a critical feature in the development of a biologically inert imaging agent targeting immune cells. Mbs lacking the C$_\gamma2$ domain do not have effector functions because they cannot bind Fc$\gamma$ receptors. However, further studies need to be performed to see if CD8 cross-linking due to the bivalent nature of the Mb activates CD8$^+$ T cells in vivo.

**Table 1. Ex vivo biodistribution analysis of $^{64}$Cu-NOTA-2.43 Mb 4 h p.i. in Lyt2.2$^{+}$ B/6 mice, Lyt2.1$^{+}$ C3H mice, NSG SCID mice, antigen-blocked B/6 mice, and antigen-depleted B/6 mice**

<table>
<thead>
<tr>
<th>Organ</th>
<th>WT B/6 (n = 6)</th>
<th>WT C3H (n = 3)</th>
<th>NSG (n = 3)</th>
<th>B/6 + block (n = 3)</th>
<th>B/6 + depletion (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.90 ± 0.14</td>
<td>1.3 ± 0.10**</td>
<td>0.89 ± 0.13</td>
<td>2.1 ± 0.31***</td>
<td>1.9 ± 0.10***</td>
</tr>
<tr>
<td>Axillary lymph nodes</td>
<td>27 ± 7.9</td>
<td>2.7 ± 0.71**</td>
<td>N/A</td>
<td>5.0 ± 1.3**</td>
<td>4.5 ± 2.7**</td>
</tr>
<tr>
<td>Spleen</td>
<td>75 ± 8.5</td>
<td>15 ± 2.33***</td>
<td>13 ± 3.9***</td>
<td>18 ± 1.93***</td>
<td>15 ± 1.03***</td>
</tr>
<tr>
<td>Stomach</td>
<td>1.1 ± 0.42</td>
<td>1.1 ± 0.11</td>
<td>0.43 ± 0.11*</td>
<td>1.0 ± 0.09</td>
<td>1.6 ± 0.92</td>
</tr>
<tr>
<td>Intestines</td>
<td>3.8 ± 0.58</td>
<td>3.2 ± 0.17</td>
<td>1.1 ± 0.04***</td>
<td>4.3 ± 0.47</td>
<td>3.4 ± 0.73</td>
</tr>
<tr>
<td>Liver</td>
<td>57 ± 11</td>
<td>47 ± 1.6</td>
<td>38 ± 1.0*</td>
<td>71 ± 1.2</td>
<td>59 ± 6.5</td>
</tr>
<tr>
<td>Kidneys</td>
<td>5.6 ± 0.72</td>
<td>5.8 ± 0.72</td>
<td>3.8 ± 0.26***</td>
<td>7.0 ± 0.31*</td>
<td>6.4 ± 0.50</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.89 ± 0.63</td>
<td>0.46 ± 0.05</td>
<td>2.2 ± 0.89*</td>
<td>1.8 ± 0.42</td>
<td>1.1 ± 0.46</td>
</tr>
<tr>
<td>Heart</td>
<td>1.6 ± 0.22</td>
<td>2.3 ± 0.09**</td>
<td>1.3 ± 0.15</td>
<td>3.1 ± 0.36***</td>
<td>2.7 ± 0.19***</td>
</tr>
<tr>
<td>Lungs</td>
<td>3.3 ± 1.3</td>
<td>2.3 ± 0.61</td>
<td>1.2 ± 0.11</td>
<td>3.1 ± 0.05</td>
<td>2.4 ± 0.48</td>
</tr>
<tr>
<td>Muscle</td>
<td>0.16 ± 0.03</td>
<td>0.15 ± 0.01***</td>
<td>0.15 ± 0.03</td>
<td>0.42 ± 0.06***</td>
<td>0.3 ± 0.06***</td>
</tr>
<tr>
<td>Bone</td>
<td>8.2 ± 2.5</td>
<td>4.0 ± 0.34*</td>
<td>3.6 ± 0.49*</td>
<td>9.2 ± 0.7</td>
<td>9.0 ± 1.5</td>
</tr>
<tr>
<td>Carcass</td>
<td>0.91 ± 0.15</td>
<td>0.83 ± 0.04</td>
<td>0.53 ± 0.03*</td>
<td>1.4 ± 0.12**</td>
<td>1.3 ± 0.13**</td>
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Values are represented as mean ± SD. *P < 0.05; **P < 0.005; ***P < 0.0005. N/A, not applicable.
naturally expressed CD8 antigen throughout the body, termed the antigen sink. This results in rapid accumulation of the radiotracer in organs outside of the blood but makes imaging nonantigen sink organs (i.e., a tumor) difficult. The rapid biological $t_{1/2}$ of these novel Mbs, however, is well matched to the intermediate physical $t_{1/2}$ of $^{64}$Cu (12.7 h) immuno-PET radio-nuclide compared with other positron emitters, including $^{18}$F (1.8 h), $^{124}$I (100.2 h), and $^{89}$Zr (78.4 h).

Recent studies imaging CD20 in a human CD20-expressing transgenic mouse model using $^{64}$Cu- or $^{89}$Zr-radiolabeled rituximab mimic the targeting ability of the CD8 Mbs in vivo because the abundance and location of CD8 and CD20 antigen expression are similar in vivo (20, 21). Unlike the work presented here, the radiolabeled rituximab is still biologically active and not ideal for imaging studies. Engineering rituximab to other antibody formats could decrease the Fc-dependent biological activity of the imaging radiopharmaceutical (22, 23). In the context of tumor targeting, this antigen sink has been overcome by blocking endogenous target with cold antibody either during (bolus injection) or before (predosing/blocking injection) administration of the radiotracer. Blocking studies were performed in the human CD20 transgenic model that resulted in an increased radiotracer blood $t_{1/2}$ that could greatly influence the ability to target CD20+ B-cell lymphomas in vivo. In fact, the FDA-approved radioimmunotherapeutic Zevalin, a $^{90}$Y-radiolabeled anti-CD20 antibody, requires a predose of cold rituximab to block accumulation of the $^{90}$Y radioimmunotherapeutic in the spleen and to increase targeting of lymphoma cells (24).

The technique of bolus or predosing injections has proven important for targets other than immunological cell surface molecules where antigen sinks exist. Bolus injections were used recently, for example, in an $^{89}$Zr-radiolabeled trastuzumab immuno-PET study that required high doses for reliable targeting in patients due to shed extracellular domain of HER2 in the plasma (25). Also, both $^{111}$In-radiolabeled anti-EGF receptor and anti-VEGF receptor antibodies demonstrated high lung and/or liver uptake that could be reduced and tumor uptake enhanced when higher protein doses were injected (26, 27). Furthermore, the concept of blocking the antigen sink has repercussions in the field of therapeutic antibody–drug conjugates. For example, predosing injections were used to block the antigen sink of tomoregulin, or TENB2, a transmembrane protein overexpressed in prostate tumors, to increase the therapeutic index of the monomethyl auristatin E-conjugated anti-TENB2 antibody (28). This study also highlights the fine balance between efficient blocking of the antigen sink vs. displacing the tumor uptake. The ability of these anti-CD8 Mb fragments to image either CD8+ lymphomas or tumor-infiltrating CD8+ T cells, for example, might rely on efficiently blocking the antigen sink for consistent targeting.

The rapid clearance of the anti-CD8 Mbs could also be due to the presence of multimers, causing increased liver uptake at early time points. At 4 h p.i., the CD8 Mbs have $\sim$60–70% ID/g in the liver, compared with other $^{64}$Cu-radiolabeled Mbs that range from 15 to 32.4% ID/g at 4–5 h p.i. (19, 29). Nonspecific liver uptake and retention occur when using $^{64}$Cu due to the transchelation of copper to enzymes in the liver (30). However, an Mb dimer ($\sim$160 kDa) is similar in size to an intact antibody but lacks the full Fc domain that allows for neonatal Fc receptor recycling. When injected with $^{64}$Cu-NOTA-2.43 Mb, both B/6 and C3H mice showed decreased uptake in lymph nodes, spleen, and blood compared with the $^{64}$Cu-NOTA-2.43 Mb. This could be attributed to the higher amount of multimer in the YT$^{169}$Mb than in the 2.43 Mb, as shown by SEC, accelerating hepatic clearance and resulting in a decreased blood $t_{1/2}$ and lower ability to target lymph nodes.
should be noted that the Mb multimers of both YTS169 and 2.43 importantly retain their ability to bind sCD80F, as shown by SEC. Potential aggregation/dimerization due to inter-\(V_\text{H}\)-\(V_\text{L}\) binding can be reduced, for example, by engineering two cysteines that stabilize the interaction between \(V_\text{H}\) and \(V_\text{L}\), among other methods (31, 32).

The \(^{64}\text{Cu}-\text{NOTA}-\text{YTS169}\) Mb that binds both Lyt2.1 and Lyt2.2 expressed in different mouse strains showed varying uptake in the spleens and livers of B6 and C57 mice. In this study, however, the C3H mice were 20 wk old and the B6 mice were 8 wk old, with average spleen and liver weights of either 96 ± 9 mg and 883 ± 43 mg or 54 ± 3 mg and 653 ± 10 mg, respectively. Therefore, the weights of the organs greatly affect the %ID/g, and the actual %ID per organ 4 h.p.i. is similar in the spleen and liver (Table 2). This highlights an important fact that %ID/g values are not consistent for every experiment but are very reproducible within groups of mice of the same age and weight.

The development of these anti-CD8 immuno-PET radiotracers will be beneficial for studying a host of preclinical disease models, including, but not limited to, lymphoma detection and tumor T-cell infiltration. Preclinical immunotherapy models enhancing the dynamic function and proliferation of cytotoxic T cells could potentially be monitored noninvasively in vivo using immuno-PET. Furthermore, the lessons learned in a preclinical setting will prove beneficial for the development and translation of anti-human CD8 antibody fragments for immuno-PET imaging in the clinic.

Conclusion

Described here is the successful development of functional CD8 imaging agents based on engineered antibodies for immuno-PET imaging in a variety of preclinical disease and immunotherapeutic models. Two allele-specific Mb fragments were produced, characterized, radiolabeled with \(^{64}\text{Cu}\), and used in micro-PET imaging to quantify uptake in lymphoid organs in WT mice in vivo. Examining antibody-based immuno-PET imaging of mouse CD8 expression is useful not only for immuno-PET imaging of preclinical models of CD8-based immunotherapy, but it has further implications for the development of an anti-human CD8 antibody or fragment-based immuno-PET imaging agent that is transplantable to the clinic.

Materials and Methods

Animal studies were approved by the University of California, Los Angeles (UCLA) Chancellor’s Animal Research Committee. Mice were purchased from Jackson Laboratory. Detailed information on animals, cloning, Mb and recombinant antigen design, protein expression and purification, protein conjugation, flow cytometry, depletion assays, radiolabeling, immuno-PET imaging, biodistribution, and data analysis can be found in SI Materials and Methods.

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