Noninvasive imaging of immune responses

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At their margins, tumors often contain neutrophils, dendritic cells, and activated macrophages, which express class II MHC and CD11b products. The interplay between stromal cells, tumor cells, and migratory cells such as lymphocytes creates opportunities for noninvasive imaging of immune responses. We developed alpaca-derived antibody fragments specific for mouse class II MHC and CD11b products, expressed on the surface of a variety of myeloid cells. We validated these reagents by flow cytometry and two-photon microscopy to obtain images at cellular resolution. To enable noninvasive imaging of the targeted cell populations, we developed a method to site-specifically label VHHs [the variable domain (VH) of a cameld heavy-chain only antibody] with 18F or 64Cu. Radiolabeled VHHs rapidly cleared the circulation (t1/2 ≈ 20 min) and clearly visualized lymphoid organs. We used VHHs to explore the possibility of imaging inflammation in both xenogeneic and syngeneic tumor models, which resulted in detection of tumors with remarkable specificity. We also imaged the infiltration of myeloid cells upon injection of complete Freund’s adjuvant. Both anti-class II MHC and anti-CD11b VHHs detected inflammation with excellent specificity. Given the ease of manufacture and labeling of VHHs, we believe that this method could transform the manner in which antigen responses and/or infectious events may be tracked.

PET imaging | non-invasive imaging | inflammation | cancer | camelid single domain antibodies

Myeloid cells, such as neutrophils, dendritic cells, and activated macrophages, express CD11b and/or class II MHC products and are often present at the tumor margin (1, 2). Macrophages, depending on their functional properties (M1 or M2 type) (3), help establish a (micro) environment either detrimental or favorable to tumor growth (3, 4). The ability to image myeloid cells’ presence is thus of diagnostic relevance and may be an approach more generally applicable than detection of tumor cells via unique tumor-specific markers. Current practice either relies on sampling of peripheral blood to enumerate and characterize these cells in the circulation or requires more invasive procedures, such as histological analysis of biopsies or surgical resection specimens. Noninvasive detection of sites of an ongoing inflammatory or immune response might provide clues to the existence and location of tumor foci, which a biopsy could then confirm. PET tracers, such as 18F-2-deoxyfluoroglucose (FDG), visualize differences in metabolic activity that accompany inflammation but lack the discriminatory power that antibodies or their fragments might afford (5). To detect inflammatory cells by recognition of surface markers, we developed imaging tools to track the behavior of class II MHC+ and CD11b+ cells in mice.

Several obstacles stand in the way of using full-sized antibodies for in vivo imaging. First, installation of an appropriate fluorescent or radioactive label onto the relevant antibodies without inflicting damage to the antibody itself is at times demanding. Second, the short half-lives of isotopes most suitable for PET imaging (18F, t1/2 = 110 min; 68Ga, t1/2 = 68 min; 64Cu, t1/2 = 12 h) require rapid generation and purification of the desired adduct, which is often impractical. Third, tissue penetration of a full-sized antibody is poor. Finally, a labeled, full-sized antibody persists in the circulation because of its comparatively long circulating half-life. Other scaffolds, such as proteins based on fibronectin type III domains (FN3) and small molecules that serve as receptor-specific ligands, have also been used for PET imaging (6, 7) but do not provide information about immune responses (6, 7). Camelid-derived single-domain VHHs [the variable domain (VH) of a camelid heavy-chain only antibody] (8) present several advantages and workarounds that address these obstacles. VHHs are smaller (~15 kDa) than immunoglobulins (~150 kDa), “diabody” antibody derivatives (~60 kDa) (9), Fab fragments (~50 kDa), or ScFvs (~25 kDa), and they lack an Fc region. Single-domain VHHs of desired specificity can be readily generated, isolated, and characterized by their amino acid sequence using standard procedures (10). Approaches to modify (11) and humanize (12) these camelid VHHs exist, and early-phase clinical testing is underway (13).

Results

Anti-Mouse Mouse Class II MHC and Anti-Mouse CD11b Stain Secondary Lymphoid Organs. Two VHHs, VH7 (anti-mouse class II MHC) and VH1DC13 (anti-mouse CD11b) were generated following standard procedures (SI Materials and Methods) (10). To test whether anti-class II MHC and anti-CD11b–specific VHHs could be used for in vivo labeling of class II MHC- and CD11b-expressing cells, we installed a fluorophore on VH7 and

Significance

Tumors are often surrounded and invaded by bone marrow-derived cells. Imaging the infiltration of such immune cells into tumors may therefore be an attractive means of detecting tumors or of tracking the response to anticancer therapy. We show that it is possible to detect these cells noninvasively by positron emission tomography (PET) via the surface markers displayed by them. The ability to monitor the immune response in the course of therapy will enable early determination of the efficacy of treatment and will inform decisions as to whether treatment should be stopped or continued. Noninvasive monitoring could therefore change how therapies are applied and assessed, to the benefit of many patients.

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VHHDC13 in a sortase-catalyzed reaction and used this preparation for two-photon microscopy and flow cytometry (14). We have previously established that VHH7 recognizes murine I-A\textsuperscript+ Mel-Juso cells maintained in culture. We injected 20 \( \mu \)g of Texas Red-labeled VHH7 i.v. into class II MHC-eGFP (15) or class II MHC-deficient mice (16). After 90 min, we explanted lymph nodes and spleen to subject them to two-photon microscopy. The class II MHC-eGFP marker enables visualization of all class II MHC\textsuperscript+ cells without the aid of an antibody, and therefore the efficacy of in vivo immunostaining with VHH7 can be accurately assessed. Two-photon imaging of class II MHC-eGFP mice showed perfect concordance with Texas Red-VHH7–stained cells (Fig. 1A and B). Class II MHC\textsuperscript+/− mice showed no specific staining (Fig. 1C). In the absence of injected Texas Red-VHH7, no staining was apparent in the corresponding channel (Fig. L4 and Fig. S1). These results establish specificity of staining and show that, at 90 min postinjection, VHH7 achieved excellent penetration of spleen and lymph nodes. We similarly labeled VHHDC13, which recognizes CD11b, a marker for neutrophils, macrophages, and dendritic cells. The molecular target of VHHDC13 was identified by mass spectrometry of immunoprecipitates prepared with immobilized VHHDC13. VHHDC13 specifically binds to CD11b\textsuperscript+ cells in lymph nodes and spleen (Fig. 1E1 and E2 and Fig. S1). To further confirm the two-photon experiments, WT, MHC-II-deficient, or CD11b-deficient mice were injected with 2 or 20 \( \mu \)g of VHH7 or VHHDC13-Alexa 647. After 90 min, spleen and brachial lymph nodes were analyzed by flow cytometry after staining with fluorophore-conjugated antibodies to CD3 and CD19, for VHH7, and to CD11b, for DC13. Results clearly confirmed the specificity of the VHHs for their corresponding targets (Fig. 1 G and H). In vivo staining with labeled VHHs obviates the need for fluorescent-protein fusions as the target for multiphoton imaging. A 90-min delay between administration and imaging is sufficient to obtain adequate staining with VHHs in spleen and lymph nodes. Although two-photon microscopy affords cellular resolution and can be used intravitally, it is nonetheless a mostly invasive method. We therefore explored the possibility of noninvasive imaging by positron emission tomography (PET).

**Site-Specific \( ^{18} \text{F} \) or \( ^{64} \text{Cu} \) Labeling of Single-Domain Antibodies (VHHs) Using Sortase.** Antibody-labeling strategies for PET often revolve around modification with metal chelators to enable installation of radioisotopes such as \( ^{64} \text{Cu}, ^{68} \text{Ga}, \) or \( ^{89} \text{Zr} \) (17–19). Although the
creation of fluorine–carbon bonds remains a synthetic challenge (20, 21), its use has advantages over 68Ga, 64Cu, 89Zr, and 124I: lower energy positron emission, shorter half-life, reduced cost, and wider availability. 18F-PET imaging is used both for diagnostic purposes and to monitor therapeutic efficacy, but these applications rely on agents that report on metabolic activity, such as FDG or labeled precursors to nucleic acids (22–25). The feasibility of noninvasive imaging of lymphocytes using an antibody fragment was demonstrated only recently, using a ~60-kDa 65Cu-labeled anti-CD5 antibody fragment (26). The half-life (t1/2 ≈ 110 min) of 18F requires it to be used shortly after installation. We therefore developed a simple two-step process (Fig. 2) for 18F labeling of VHHS bearing a sortase-recognition motif, using a combination of a click reaction and a sortase-catalyzed modification. This method is robust, reproducible, and site-specific, without compromising the VH1 antigen-binding site. This technique is further applicable to any other suitably modified biological entity (11).

The 18F radionuclide was conjugated to the VHHS via a tetrazine (Tz)/transcyclooctene (TCO) reverse-electron demand Diels–Alder cycloaddition. The TCO-tetrazine reaction is the fastest known bioorthogonal reaction to date, with an estimated second-order rate constant of 210–26,000 M−2 s−1 (27–29). We synthesized the requisite sortase nucleophile, GGG-tetrazine, which participates in sortase reactions to modify the VH (Fig. 2A). 18F radiolabeling of tosyl-transcyclooctene (TCO) was achieved with 18F-F/K222/K2CO3 for ~10 min at 90 °C to produce 18F-TCO (30) (>75% yield, decay-corrected) and purified by HPLC (Fig. 2B). 18F-TCO was then added to the Tz-modified VH, and the reaction was allowed to proceed for ~20 min at pH 7. Thus, using 18F-TCO, we readily produced >5 mCi of 18F-labeled VH7 in two steps (Fig. 2C). The 18F-labeled VH was purified by size-exclusion chromatography in PBS, providing a radiolabeled VH solution ready for injection. Labeling experiments with 18F-TCO yielded 18F-VH7 in 61 ± 9% decay-corrected radiochemical yield.

Fig. 2. (A–E) Site-specific 18F or 18O labeling of single-domain antibodies (VHHs) using sortase. (A) A single-domain antibody fragment (VHH) equipped at its C terminus with the LPXTG sortase recognition motif followed by a His tag, is incubated with sortase A, which cleaves the threonine-glycine bond to yield the reactive thioaldehyde intermediate. Addition of a peptide with N-terminal glycine residues and a functional moiety of choice enables site-specific modification of the VHH. We thus modified a VHH with a Gly46-tetrazine (Tz), as confirmed by LC–MS (liquid chromatography–mass spectrometry) (D, VH7-Tz). (B) A tosyl-TCO and 18F-K222/K2CO3 were combined to produce 18F-TCO. (C) 18F-TCO was added to the Tz-modified VH, and, after ~20 min, the labeled VH was retrieved by rapid size-exclusion chromatography. (E) The sortase reaction was used to install a NOTA functionality at the C terminus of a VH followed by addition of 64Cu and 18F-VH7 to produce 64Cu-VHH/(18)F-VH7. (F–M) PET-CT images of class II MHC−/− mice (18F-VH7) and 18F-VH7 detects secondary lymphoid organs. (F and G) PET-CT images of class II MHC−/− (G) and C57BL/6 (F) mice 2 h postinjection of 18F-VH7; numbers indicate (i) lymph nodes (numbers 1, 2, 3, 4, and 7), (ii) thymus (number 5), and (iii) spleen (number 6). (H and I) Coronal PET-CT images of C57BL/6 mouse image with 18F-VH7, moving from anterior to posterior. In H and I, different sets of lymph nodes and thymus are visible. (K and L) Coronal PET-CT images of an MHCII−/− mouse image with 18F-VH7, moving from anterior to posterior. In neither K nor L, lymph nodes or thymus is visible. (M) PET-CT as maximum intensity projections of all slices for a C57BL/6 and a class II MHC−/− mouse 2 h postinjection of 18F-VH7. In green, accumulation of 18F-VH7 in lymph nodes and thymus. PET scale bars have arbitrary units.) See Movies S1A, S1B, S2A, and S2B for 3D visualization of secondary lymphoid organs. (N and O) PET signals in vivo and postmortem biodistribution in all organs.

18F-VH7 Detects Secondary Lymphoid Organs. Administration of 200 μCi/10 μg 18F-VH7 in vivo in C57BL/6 WT mice (class II MHC-sufficient) allowed us to visualize lymph nodes, spleen, and thymus with a high degree of specificity and with excellent signal-to-noise ratios (Fig. 2 G–J). No nonspecific staining was seen in lymphoid tissues when using 18F-VH7 in class II MHC−/− mice (B6.129S2-H2<klAb1-Ea-i>) (Fig. 2 F and K–M and Movies S14, S1B, S2A, and S2B). Biodistribution and circulatory half-life measurements of 18F-VH7 in WT mice showed a t1/2 of ~20 min (Fig. S2).

18F-VH7 and 18F-VHDC13 Detect Inflammation. We next used 18F-VH7 and DC13 to image the behavior of class II MHC+ host cells in a xenograft tumor model. Neither the distribution of class II MHC or CD11b+ cells at steady state nor changes in their distribution and location over time have been specifically imaged noninvasively using 18F-labeled tracers. In many tumor models, the margins of the tumor contain macrophages, which, when activated, express class II MHC and CD11b.

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positive for VHH7 and DC13 (Fig. 3 H and I). Postmortem histology of the tumor further verified the presence of inflammatory cells in around the tumor, with clearly visible infiltrating macrophages and neutrophils (Fig. 3L). We have thus demonstrated noninvasive imaging of class II MHC\(^+\) cells and CD11b\(^+\) cells, both in normal and in tumor-bearing mice.

**18F-VHH Detects Class II MHC\(^+\) Cells Associated with Small Tumors at Early Stages.** Is it possible to image small tumors also at earlier stages of growth? To address this question, we imaged NOD/SCID mice xenografted with 5 x 10\(^6\) Mel-Juso human melanoma cells at 6 d, 20 d, and 27 d postinjection. We detected inflammation at the site of the malignant growth at the earliest time point after injection, a time when the incipient tumors are neither visible nor detectable by palpation (Fig. 3E). FACS analysis on cell suspensions prepared from the excised tumors again confirmed the presence of tumor-infiltrating class II MHC\(^+\) cells (Fig. 3H). It may thus be worthwhile to explore early-stage detection of diseases characterized by an inflammatory signature, such as multiple sclerosis, diabetes, or infections (5).

The longer half-life of \(^{64}\text{Cu}\) (12.7 h) relative to \(^{18}\text{F}\) (110 min) in principle allows a more extended imaging period to establish tissue penetration and dwell time of VHHS on their targets. We developed a NOTA (Gly) sortase nucleophile to enable site-specific labeling of VHHS (Fig. 2D and Fig. S3) via this high-affinity copper-chelating agent (\(K_{\text{ass}}\) for Cu\(^{2+}\) of \(\sim10^{12}\)) (32). The [Cu\(^{2+}\)-NOTA] complex is kinetically inert, with minimal metal exchange when exposed to other metals present in body (33). We produced site-specifically labeled [\(\left[{\text{Cu}}^{2+}\right]\)-NOTA]-VHHS in high radioactive yield (\(\sim90\%\) decay corrected) and used \(^{64}\text{Cu}\)-VHH7 to image a C57BL/6 WT mouse at 4 h, 8 h, and 24 h postinjection. VHH7 stayed on its target (secondary lymphoid organs) even after 24 h (Fig. S4) but produced images with an inferior signal-to-noise ratio compared with \(^{18}\text{F}\)-VHH7 (compare Fig. 2 with Fig. S4).

\(^{18}\text{F}\)-VHH7 and \(^{18}\text{F}\)-VHHDC13 Detect Infiltration of Immune Cells in B16 Melanoma Tumor-Bearing Mice in a Syngeneic Setting. Next, we explored B16 melanoma-bearing mice and found that here, too, we could readily image the presence of the primary tumor via the presence of CD11b\(^+\) and class II MHC\(^+\) cells, without a need for melanoma-specific markers (Fig. 4 A and B and Movies S5A, S5B, S5C, S6A, and S6B). We also performed imaging experiments on B16 melanoma-bearing mice using \(^{18}\text{F}\)-2-fluorodeoxyglucose (\(^{18}\text{F}\)-FDG), a standard means of staging tumor patients by PET. \(^{18}\text{F}\)-FDG revealed the presence of the tumor but produced images of inferior specificity compared with the use of \(^{18}\text{F}\)-VHHS (Fig. 4C and Movie S7).

Successful Detection of Infiltrated Myeloid Cells Using \(^{18}\text{F}\)-Labeled VHHD13 upon Injection of Complete Freund’s Adjuvant. Finally we examined inflammation in response to administration of complete Freund’s adjuvant (CFA). Administration of CFA leads to the influx and accumulation of myeloid cells at the site of the injection and draining lymph nodes within 24 h. When we injected animals with CFA into the left front paw and imaged them 24 h later with \(^{18}\text{F}\)- or \(^{64}\text{Cu}\)-labeled VHH7, inflammation was readily apparent, with VHHD13 showing a stronger signal than VHH7 in the inflamed region (Fig. 4 D and E, and Movies S8A, S8B, S9, S10, and S11). \(^{18}\text{F}\)-2-fluorodeoxyglucose (FDG) was used for comparison and failed to detect inflammation evoked by CFA (Fig. 4F and Movies S12A, S12B, and S12C). Accumulation of \(^{18}\text{F}\)-VHHD13 is consistent with the massive influx of CD11b\(^+\) neutrophils generally observed at the site of injection 24 h after administration of CFA.

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accumulation of myeloid cells is a general phenomenon, as suggested by the available evidence (2, 34). With the availability of anti-human VHHs, the ability to monitor the presence or absence of neutrophils and activated macrophages as an indicator of inflammation should be transposable to a clinical setting as a diagnostic tool. The potential immunogenicity of VHHs may limit the use of VHH-based PET to a clinical setting as a diagnostic tool. The approach described here obviates the need for the development of single-domain antibodies (VHHs), if indeed such in the application of antibodies that target immunological checkpoints, there is an urgent need to monitor the progression of such immunotherapies. The advantages of the small size of VHHs in conjunction with PET-compatible isotopes, such as 18F, should enable further refinement of immuno-PET in a clinical setting. Although the present results are geared toward the detection of neutrophils and antigen-presenting cells, an important future goal will be to extend this approach to T cell-specific markers.

Discussion

Using a combination of single domain antibodies (VHHs), we showed that it is possible to track immune responses with excellent specificity. CD11b+ or class II MHC+ cells can serve as sentinels to detect the presence of the cancerous tissue they surround or invade. Although class II MHC and CD11b are expressed on many activated myeloid cells, such as macrophages and dendritic cells, the observed signal strength for imaging may vary with the extent of activation, and not all tumors may recruit myeloid cells equally efficiently. The approach described here obviates the need for the generation of panels of tumor-specific antibodies, if indeed such

Materials and Methods

Synthesis of (Gly)^3-Tetrazine.

The tetrapeptide GGGC was synthesized by standard solid-phase peptide synthesis. Maleimide-tetrazine (ClickChemistryTools) was dissolved in 0.1 M phosphate buffer (PB), pH 7. The tetrapeptide GGGC was added and left to stir at room temperature for 3 h until TLC (1:1 Hex:EtOAc vol/vol) indicated near-complete conversion to the product. The solution was filtered and purified by reverse-phase HPLC with a semipreparative column (C18 column, Gemini, 5 μm, 10 × 250 mm; Phenomenex) at a flow rate of 5.0 mL/min: solvent A, 0.1% TFA in H2O; solvent B, 0.1% TFA in CH3CN. (Gly)^3-Tetrazine eluted at 20–35% (vol/vol) solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for C2H20H41O13S[M+H]+ was 892.362, found 892.370.

Synthesis of (Gly)^3-NOTA. Maleimide-NOTA (Macromolecules) was dissolved in 0.1 M phosphate buffer (PB), pH 7. The tetrapeptide GGGC was added and left to stir at room temperature for 3 h until TLC (1:1 Hex:EtOAc vol/vol) indicated almost complete conversion to the product. The solution was filtered and purified by reverse-phase HPLC with a semipreparative column (C18 column, Gemini, 5 μm, 10 × 250 mm; Phenomenex) at a flow rate of 5.0 mL/min: solvent A, 0.1% TFA in H2O; solvent B, 0.1% TFA in CH3CN. (Gly)^3-NOTA eluted at 20–35% (vol/vol) solvent B. Fractions containing pure product were collected and lyophilized. LC-MS calculated for C2H20H41O13S[M+H]+ was 892.362, found 892.370.
Enzymatic Incorporation of Substrates into Proteins Using Sortase. The penta-
mutant sortase A, with an improved Kcat was used (35). Reaction mixtures (1 mL) contained Tris-HCl (50 mM, pH 7.5), CaCl2 (10 mM), NaCl (150 mM), triglycerine-
containing probe (500 μM), LPETG-containing probe (100 μM), and sortase (5 μM) (11, 36). After incubation at 4 °C with agitation for 2 h, reaction products were analyzed by LC-MS, with yields generally >90%. When the yield was below 90%, the reaction was allowed to proceed for an additional 2 h, with addition of sortase to 10 μM and triglycerine-containing probe to 750 μM. Ni-NTA beads were added to the reaction mixture with agitation for 5 min at 25 °C, followed by centrifugation to remove sortase and any remaining unreacted Histagged substrate. The final product—either the tetrzine-labeled protein, NOTA-
labeled protein, or fluorophore-labeled protein—was purified by size-exclusion chromatography in PBS or Tris-HCl (50 mM, pH 7.5). The labeled protein was stored at −20 °C with 5% (vol/vol) glycerol and was stable for up to 3 mo.

Synthesis and Characterization of 18F-VHHs. In a typical reaction, a 1.5-mL centrifuge tube was loaded with VHH7-Tz in 1× PBS (40 μL, 150 μM), 1× PBS (200 μL), and 18F-TcO in DMSO [4.0 μCi (148.0 MBq), 100 μL]. The tube was sealed and shaken at room temperature for 20 min. The mixture was analyzed by radio-TLC (instant thin layer chromatography (ITLC), 100% MeCN, Rf 18F-TcO = 0.9, Rf 18F-VHH7 = 0.0) showing 90% conversion to 18F-VHH7. The reaction mixture was loaded onto a PD-10 size-exclusion cartridge (GE Healthcare), and elution with 1× PBS provided 2.3 μCi (85.1 MBq) of 18F-VHH7 in 75.8% decay-corrected radiochemical yield. Starting with 5.3 μCi (196.1 MBq) 18F-TcO, 18F-VHHDC13 was prepared following the same procedure as described for 18F-VHH7 to give 2.8 μCi (103.6 MBq) after size-exclusion chromatography, a 93.1% decay-corrected radiochemical yield.

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Synthesis and Characterization of 64Cu-VHHs. In a typical reaction, a 1.5-mL centrifuge tube was loaded with VHH7-NOTA (400 μL, 20 μM in 200 mM NH4OH buffer [pH 6.5]) and 64CuCl2 (5.7 μCi, 210.8 MBq) in 200 mM NH4OH buffer (75 μL, pH 6.5). The tube was sealed and shaken at 37 °C for 20 min. The mixture was analyzed by radio-TLC (ITLC, 50 mM EDTA, pH 7, Rf 64CuEDTA = 1.0, Rf 64Cu-VHH7 = 0.0) showing 98% conversion to 64Cu-
VHH7. At this time, the mixture was loaded onto a PD-10 size-exclusion cartridge, and elution with 1× PBS provided 5.2 μCi (192.4 MBq) of 64Cu-VHH7 in 94.2% decay-corrected radiochemical yield. Starting with 3.5 mCi (129.5 MBq) 64CuCl2, 64Cu-VHHDC13 was prepared following the same procedure as described for 64Cu-VHH7 to give 3.1 μCi (114.7 MBq) after size-exclusion chromatography, a 92.3% decay-corrected radiochemical yield. Before injection, both 64Cu-VHH7 and 64Cu-VHHDC13 were analyzed by radio-TLC (ITLC, 50 mM EDTA, pH 7, Rf 64CuEDTA = 1.0, Rf 64Cu-VHH7 and 64Cu-VHHDC13 = 0.0) and were found to have 99.6% and 99.8% radiochemical purity, respectively.

PET-CT Imaging. All procedures and animal protocols were approved by the Massachusetts General Hospital subcommittee on research animal care.

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