1 **TITLE PAGE**

2 **Development and characterization of an $\alpha_v\beta_6$ -specific diabody and a disulfide-**
3 **stabilized $\alpha_v\beta_6$ -specific cys-diabody**

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1 **Key words:** Diabody, fluorine-18, copper-64, $\alpha_v\beta_6$ integrin, site-specific conjugation,
2 radiolabeling

3 **Abbreviated title:** Characterization of anti- $\alpha_v\beta_6$ antibody fragments

4

5 **ABSTRACT**

6 *Introduction:* This work describes the development and characterization of two antibody
7 fragments that specifically target the $\alpha_v\beta_6$ integrin, a non-covalent diabody and a
8 disulfide-stabilized cys-diabody. The diabodies were analyzed for their ability to bind
9 both immobilized and cell surface-bound $\alpha_v\beta_6$. Radiolabeling was done using non-site-
10 specific and site-specific conjugation approaches with *N*-succinimidyl 4-
11 [^{18}F]fluorobenzoate ([^{18}F]-SFB) and the bifunctional chelator 1,4,7-triazacyclononane-
12 triacetic acid maleimide (NOTA-maleimide) and [^{64}Cu], respectively. The affects of each
13 radiolabeling method on RCY, RCP, and immunoreactivity were analyzed for the [^{18}F]-
14 FB- $\alpha_v\beta_6$ diabody, [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, and the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody.

15 *Methods:* Diabodies were constructed from the variable domains of the humanized 6.3G9
16 anti- $\alpha_v\beta_6$ intact antibody. The anti- $\alpha_v\beta_6$ cys-diabody was engineered with C-terminal
17 cysteines to enable covalent dimerization and site-specific modification. Biochemical
18 characterization included SDS-PAGE, Western blot, and electrospray ionization to
19 confirm MW, and flow cytometry and ELISA experiments were used to determine
20 binding affinity and specificity to $\alpha_v\beta_6$. The diabodies were radiolabeled with [^{18}F]-SFB
21 and in addition, the anti- $\alpha_v\beta_6$ cys-diabody was also radiolabeled site-specifically using
22 NOTA-maleimide and [^{64}Cu]. Immunoreactivities were confirmed using *in vitro* cell
23 binding to DX3Puro β_6 ($\alpha_v\beta_6+$) and DX3Puro ($\alpha_v\beta_6-$) cell lines.

1 *Results:* The diabodies were purified from cell culture supernatants with purities >98%.
2 Subnanomolar binding affinity towards $\alpha_v\beta_6$ was confirmed by ELISA (diabody $IC_{50}=0.8$
3 nM, cys-diabody $IC_{50}=0.6$ nM) and flow cytometry revealed high specificity only to the
4 DX3Puro β_6 cell line for both diabodies. RCYs were $22.6\%\pm3.6\%$ for the [^{18}F]-FB- $\alpha_v\beta_6$
5 diabody, $8.3\pm1.7\%$ for the [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody and $43.5\pm5.5\%$ for the [^{64}Cu]-
6 NOTA- $\alpha_v\beta_6$ cys-diabody. *In vitro* cell binding assays revealed excellent specificity and
7 retention of immunoreactivity ([^{18}F]-FB- $\alpha_v\beta_6$ diabody= $58.7 \pm 6.7\%$, [^{18}F]-FB- $\alpha_v\beta_6$ cys-
8 diabody= $80.4\pm4.4\%$, [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody= $59.4\pm0.6\%$) regardless of the
9 radiolabeling method used.

10 *Conclusions:* Two novel diabodies with excellent binding affinity and specificity for the
11 $\alpha_v\beta_6$ integrin were developed. Radiolabeling of the diabodies with fluorine-18 ([^{18}F]) and
12 copper-64 ([^{64}Cu]) revealed advantages and disadvantages with regards to methodologies
13 and RCYs, however immunoreactivities were well preserved regardless of radiolabeling
14 approach.

15

16 **Abbreviations:** NOTA-maleimide: 1,4,7-triazacyclononane-1,4,7-triacetic acid
17 maleimide, Ni-NTA: nickel nitrilotriacetic acid, RCY: radiochemical yield, RCP:
18 radiochemical purity, mAbs: monoclonal antibodies, immuno-PET: immuno-positron
19 emission tomography, kDa: kilodaltons, V_H : variable heavy domain, V_L : variable light
20 domain, CEA: carcinoembryonic antigen, HER2: human epidermal growth factor
21 receptor 2, ALCAM: activated leukocyte cell adhesion molecule, PSCA: prostate stem
22 cell antigen, CDR: complementary determining region, scFv: single chain Fv, OSCC:
23 oral squamous cell carcinoma, MMP: matrix-metalloproteinase, ECM: extracellular

1 matrix, SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis, SEC:
2 size-exclusion chromatography, ELISA: enzyme-linked immunosorbant assay, HPLC:
3 high performance liquid chromatography, SFB: *N*-succinimidyl 4-fluorobenzoate, [¹⁸F]-
4 SFB: *N*-succinimidyl-4-[¹⁸F]-fluorobenzoate, iTLC: instant thin-layer chromatography

6 INTRODUCTION

7 The ability of monoclonal antibodies (mAbs) to recognize antigens with high
8 specificity has paved the way towards the discovery of a plethora of new biomarkers for
9 disease detection and treatment, particularly for cancer. The commercial success of
10 therapeutic mAbs has resulted in significant investment in research and development,
11 however the clinical translation of mAbs into molecular imaging agents has been met
12 with limited success. There are currently only four antibody-based diagnostic agents
13 approved by the FDA for cancer detection including [¹¹¹In]-satumomab pendetide
14 (OncoScint), [¹¹¹In]-capromab pendetide (ProtaScint), [^{99m}Tc]-nofetumomab nerpentan
15 (Verluma), and [^{99m}Tc]-arcitumomab (CEA-Scan), with only ProtaScint currently still on
16 the market in the U.S.[1] Recently, there has been a revival of interest in combining the
17 high specificity of mAbs with the sensitive quantitative imaging of positron emission
18 tomography (PET) to generate immuno-PET diagnostic imaging agents.[1-3]
19 Consolidation of these technologies has the potential to enable clinicians to quantify
20 antigen expression, assess therapeutic efficacy, and ultimately contribute to a more
21 personalized approach to the diagnosis and treatment of disease.[2]

22 The therapeutic impact that intact mAbs have made in the clinic is profound,
23 however the ability to rapidly generate high-contrast tumor images with intact antibodies

1 is often hindered by their large size (~150 kDa) and slow blood clearance properties.[4]
2 Diabodies represent an intermediate-sized (~55 kDa) genre of engineered antibody
3 fragments comprised of V_H and V_L domains connected by a short, flexible linker to
4 induce formation of the scFv dimer.[5-7] The diabody is the smallest antibody fragment
5 that retains the bivalent binding properties of the intact antibody. These engineered
6 fragments exhibit rapid tumor targeting and blood clearance, qualities that afford high-
7 contrast tumor images in as little as 2-4 hrs post-injection (p.i.).[2, 8] Imaging figure of
8 merit (IFOM) values have shown that when diabodies are radiolabeled with positron
9 emitters such as [¹⁸F] (t_{1/2}=109.7 mins), optimal imaging occurs at 2 h p.i.[9] This
10 concept was further examined with the T84.66 anti-carcinoembryonic antigen (CEA)
11 diabody, which produced high-contrast PET images of LS174T human colon carcinoma
12 xenografts in mice within 1 h after injection.[10]

13 Several studies have successfully demonstrated the conversion of anti-CEA, anti-
14 HER2, anti-PSCA, and anti-ALCAM diabodies into PET imaging agents through the
15 conjugation of fluorine-18 ([¹⁸F] t_{1/2}=109.7 mins) and copper-64 ([⁶⁴Cu] t_{1/2}=12.7 h) on
16 exposed lysine residues [10-13]. These methods rely on the chemical modification of
17 lysines using activated esters such as *N*-hydroxysuccinimide (NHS). This technique
18 allows random conjugation of the radiolabel to lysines but does not enable tight control
19 over the location of conjugation on the diabody. This is problematic when lysine residues
20 present within the CDRs become altered, which can potentially have adverse affects on
21 antigen recognition.

22 An alternative to the global introduction of the radiolabel is to perform site-specific
23 conjugation in which the radiolabel is introduced at specific locations on the diabody

1 distal to the CDR regions. Engineered cysteine residues have been appended onto the C-
2 terminus of anti-CEA, anti-HER2, and anti-ALCAM diabodies to generate covalently
3 bound dimers termed cys-diabodies.[13-15] The engineered disulfide bond creates a more
4 stable assembly between the two scFvs in the cys-diabody versus the non-covalent
5 diabody, and also provides a chemical group that after mild reduction can be used for
6 site-specific modification via thiol chemistry.[14] This concept has been successfully
7 applied to site-specifically radiolabel anti-HER2 and anti-ALCAM cys-diabodies with
8 [⁶⁴Cu] and yielded excellent preservation of immunoreactivity.[13, 16]

9 2-[¹⁸F]fluoro-2-deoxy-D-glucose ([¹⁸F]-FDG), approved by the FDA in 2004, is the
10 most commonly used radiotracer to detect tumors and monitor therapeutic response in
11 patients.[17] The quantification of glucose metabolism for tumor detection is not without
12 its limitations, however. The most prominent of these is the generation of false positives
13 due to [¹⁸F]-FDG uptake in inflamed tissue.[17] Therefore the need exists to develop
14 targeted molecular imaging agents that are disease-specific in order to achieve more
15 accurate diagnoses and enhance the development of improved therapies. The integrins are
16 a large family of cell surface receptors that have recently garnered increased scrutiny as
17 targets for molecular imaging.[18] Specifically, the $\alpha_v\beta_6$ integrin is expressed at nominal
18 levels in healthy epithelia, but in aggressive cancers such as oral squamous cell
19 carcinoma (OSCC), pancreatic, ovarian, and cervical cancer, this integrin is highly
20 overexpressed particularly at the leading edge of invasion.[19-22] This integrin has also
21 been implicated as a modulator of MMPs, enzymes that act to degrade and remodel the
22 ECM to promote tumor invasiveness and metastasis.[23] The activation of transforming
23 growth factor- β (TGF- β), a cytokine suspected of promoting tumor cell survival and

1 metastasis, is also mediated through $\alpha_v\beta_6$ binding to latency-activated peptide (LAP).[24]
2 Recent studies have shown that function-blocking antibodies against $\alpha_v\beta_6$ can
3 successfully inhibit tumor growth via blockade of $\alpha_v\beta_6$ -mediated TGF- β activation.[24,
4 25] Moreover, increased expression of the $\alpha_v\beta_6$ integrin has been correlated with
5 advancement in tumor grade and reduced survival, indicating that this integrin could
6 serve as a potential prognostic indicator and further solidifying evidence that $\alpha_v\beta_6$ may be
7 a valuable target for diagnosis and treatment.[22, 25, 26]

8 Research to develop $\alpha_v\beta_6$ -specific molecular imaging agents has resulted in the
9 characterization of peptides and antibody fragments as PET and SPECT imaging agents,
10 respectively.[27-29] Hausner et al. revealed rapid tumor targeting of the A20FMDFV2
11 peptide in $\alpha_v\beta_6$ -expressing tumors *in vivo* however the peptide was prone to rapid
12 degradation *in vivo*, which ultimately led to lower tumor uptake.[27] Efforts to improve
13 peptide stability have included pegylation and the development of cysteine-knot peptides
14 with improvements in radiotracer uptake observed in BxPC-3 ($\alpha_v\beta_6+$) tumor models.[28,
15 30] Kogelberg et al. successfully used CDR grafting to develop an anti- $\alpha_v\beta_6$ diabody that
16 when radiolabeled with [^{99m}Tc], enabled visualization of A375 β_6 ($\alpha_v\beta_6+$) tumors with
17 single photon emission tomography (SPECT).[29] Although SPECT imaging reveals a
18 plethora of information, arguably the biggest drawbacks of this modality are the lower
19 sensitivity and less accurate quantification when compared to PET.

20 Taking these recent advances into consideration, we describe the production and
21 characterization of two humanized diabody formats: a non-covalent anti- $\alpha_v\beta_6$ diabody
22 and a covalent disulfide-stabilized anti- $\alpha_v\beta_6$ cys-diabody designed for site-specific
23 radiolabeling (Fig. 1). The intact 6.3G9 antibody was selected as the basis for the

engineered diabodies because it exhibited superior binding affinity and function-blocking capabilities against $\alpha_v\beta_6$ over the commercially available 10D5 antibody (anti- $\alpha_v\beta_6$). Further, 6.3G9 was previously humanized whereas 10D5 is of murine origin, reducing the possibility of the diabodies eliciting an immune response.[24, 31] Biochemical characterization using SDS-PAGE, Western blot, electrospray ionization (ESI), and size-exclusion chromatography (SEC) confirmed MW and purity of each diabody. *In vitro* characterization was performed using competitive binding ELISA and flow cytometry to confirm binding affinity and specificity towards $\alpha_v\beta_6$, respectively. The diabodies were converted into PET imaging agents using two approaches. First, the diabody and cys-diabody were radiolabeled non-site-specifically on exposed lysine residues using [^{18}F]-SFB. Second, the cys-diabody was radiolabeled site-specifically using NOTA-mal and [^{64}Cu]. *In vitro* cell binding assays were separately conducted on the [^{18}F]-FB- $\alpha_v\beta_6$ diabody, the [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, and the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody and we demonstrated that regardless of the radiolabeling technique used, immunoreactivity was well preserved for each of these PET imaging agents. These results lay the groundwork for future *in vivo* evaluation of these PET imaging agents in a preclinical setting.

MATERIALS AND METHODS

Cell lines and reagents

The 293-F cell line (Invitrogen) was maintained as described by the vendor. Stable cell lines expressing the diabodies were cultured in selective growth media with DMEM, 10% FBS, 1% NEAA, 1% PSG, and 300 $\mu\text{g}/\text{mL}$ Zeocin (Invitrogen). Production media consisted of Opti-MEM (Invitrogen) supplemented with 1% NEAA, 1% PSG, 100 $\mu\text{g}/\text{mL}$

Zeocin. The DX3Puro and DX3Puro β_6 cell lines (a generous gift from Dr J.F. Marshall, Barts Cancer Institute) were cultured as previously described.[32] All cell lines were cultured at 37°C in a humidified incubator with 5% CO₂. All chemicals were of reagent grade and used without further purification.

Design and construction of anti- $\alpha_v\beta_6$ diabody and anti- $\alpha_v\beta_6$ cys-diabody

To generate the anti- $\alpha_v\beta_6$ diabody, the sequences from the variable domains of the intact humanized hu6.3G9 antibody were separated by a three amino acid linker (G₂S) and appended with a 6x-His tag (Fig. 1).[24] The expression plasmid pSecTag-2A (Invitrogen) was mutated to include the AgeI restriction site using the Quick Change Site-Directed Mutagenesis kit (Agilent) and primers JW51-For and JW52-Rev (Table 1) to create pSecTag-2A-AgeI. The pSecTag-2A-AgeI plasmid was digested with AgeI and EcoRI and ligated with the sequence encoding the diabody. Assembly of the new plasmid pSecTag-2A-B6DiaR-G2S was verified by restriction digestion and sequencing.

The anti- $\alpha_v\beta_6$ cys-diabody was generated using mutagenic polymerase chain reaction (PCR) primers JW57-For and JW58-Rev (Table 1). Two glycine residues and a cysteine residue (GGC) were inserted between the 6x-His tag of the original anti- $\alpha_v\beta_6$ diabody in the pSecTag-2A-B6DiaR-G2S template (Fig. 1). The remaining original features from the anti- $\alpha_v\beta_6$ diabody were left unchanged. DNA sequencing was used to verify insertion of the GGC residues, creating plasmid pSecTag-2A-B6DiaR-G2S-Cys. Plasmid DNA was purified using a plasmid plus midi kit (Qiagen).

Generation of stable cell lines

Approximately 1×10^6 293-F cells were transfected with the pSecTag-B6DiaR-G2S or pSecTag-B6DiaR-G2S-Cys plasmid (4 μ g) using Lipofectamine LTX (Invitrogen) and grown under selective conditions for 3-4 weeks. Expression from stable mixed pools was analyzed by Western blotting. Diabodies were detected with the Penta-His HRP antibody (1:2000 in blocking buffer, Qiagen) and membranes were developed using peroxidase chemiluminescent substrate (Cell Signaling). Stable lines were expanded into triple flasks for production and supernatants were harvested, sterile filtered (0.2 μ m), and stored at 4°C until purification.

Purification of anti- $\alpha_v\beta_6$ diabodies

Nickel nitrilotriacetic acid (Ni-NTA, Thermo Scientific) resin (2 mL) was equilibrated in binding buffer (0.1 M HEPES, 0.5 M NaCl, 50 mM imidazole, pH 7.1) and transferred to filtered supernatants (2-3 L). Binding was performed overnight at 4°C. Ni-NTA resin was transferred to a 10 mL fritted column, washed with binding buffer (10 mL), and eluted with elution buffer (0.1 M HEPES, 0.5 M NaCl, 400 mM imidazole, pH 7.1). Eluted fractions were electrophoresed on a 4-20% precast gel (Bio-Rad) and stained with PageBlue (Fermentas). Fractions containing purified diabody or cys-diabody were pooled and dialyzed with dialysis buffer (0.1M HEPES, 0.5M NaCl, 0.1M ethylenediaminetetraacetic acid (EDTA), pH 7.1) followed by storage buffer (0.1M HEPES, 0.5M NaCl, 10% glycerol, pH 7.1) using Slide-A-Lyzer G2 dialysis cassettes (10 kDa MWCO, Thermo). Diabodies were concentrated with centrifugal filters (10 kDa MWCO, Millipore) and concentrations were measured via A_{280} absorbance using a

Nanodrop 2000c (Thermo Scientific). The molecular weights and extinction coefficients were calculated using the ExPASy ProtParam webtool (www.expasy.org).

SDS-PAGE and size-exclusion chromatography (SEC)

Purities of the concentrated diabodies and cys-diabodies were determined using reducing and non-reducing SDS-PAGE and SEC. Purified samples were electrophoresed as described during purification. SEC was performed isocratically in 0.1 M sodium phosphate buffer, pH 6.8 at a flow rate of 0.5 mL/min and using a Superdex 75 HR 10/300 column (GE Healthcare). Absorbance was monitored at 280_{nm} and retention times were compared to molecular weight standards of bovine serum albumin (BSA) and carbonic anhydrase.

Electrospray ionization (ESI-MS)

Samples (10 µL) were injected onto a C8 protein column (Aeris) and eluted with ballistic gradient (12 min) with desalting step (2 min). Data was acquired in positive ion profile mode using the ion trap mass analyzer (LTQ Orbitrap XL, Thermo) scanning from 500-1500 m/z. Data was deconvoluted with MagTran software using standard settings to obtain parent masses.[33]

Competitive binding enzyme-linked immunosorbant assay (ELISA)

The P2W7 antibody (anti- α_v , 5 µg/mL, Abcam) was plated into a 96-well plate (50 µL/well) and incubated at 37°C for 1 h. Wells were washed with PBS (3×), blocked with blocking buffer (PBS, 5% bovine serum albumin (BSA), 1% Tween 20) for 3 hrs, then

1 washed. Recombinant $\alpha_v\beta_6$ integrin (or $\alpha_v\beta_3$ or $\alpha_v\beta_5$, 3 $\mu\text{g/mL}$, R&D Systems) in
2 conjugate buffer (20 mM Tris, 1 mM MnCl_2 , 150 mM NaCl, 0.1% Tween, 1% BSA) was
3 added to the wells (50 $\mu\text{L/well}$) for 1 h and washed with wash buffer (20 mM Tris, 1 mM
4 MnCl_2 , 150 mM NaCl, 0.1% Tween, 3 $\times$). Serial dilutions of the diabody or cys-diabody
5 (1 μM – 0.5 pM in conjugate buffer) and biotinylated fibronectin (for $\alpha_v\beta_6$ ELISA;
6 biotinylated vitronectin was used for $\alpha_v\beta_3$ and $\alpha_v\beta_5$ ELISAs) (50 $\mu\text{L/well}$, Sigma) were
7 incubated for 1 h and washed with wash buffer (3 $\times$). As a comparison, the $\alpha_v\beta_6$ -specific
8 peptide [^{19}F]-FBA-PEG₂₈-A20FMDV2 was serially diluted (1 μM – 1 pM) and competed
9 against fibronectin for 1 h. Extravidin HRP conjugate (50 $\mu\text{L/well}$, Sigma Aldrich)
10 was added to each well for 1 h followed by washing with wash buffer (3 $\times$). Detection of
11 extravidin HRP was performed using 3,3',5,5'-tetramethylbenzidine (TMB, Dako)
12 substrate (50 $\mu\text{L/well}$) for 10-15 mins. The reaction was terminated with 1N sulfuric acid
13 (50 $\mu\text{L/well}$) and absorbance at 450_{nm} was measured in a plate reader (Multiscan Ascent,
14 Thermo Scientific). Wells containing biotinylated fibronectin served as the positive
15 control and wells containing no P2W7 and no diabody (or cys-diabody) as well as no
16 biotinylated fibronectin and no diabody (or cys-diabody) were used as negative controls.

18 Flow cytometry

19 Cell suspensions (3×10^5 cells/mL, 50 μL each) of DX3Puro and DX3Puro β_6 were
20 transferred to microcentrifuge tubes. The anti- $\alpha_v\beta_6$ antibody, (10D5, Millipore), IgG2a
21 isotype control (Biolegend), anti- $\alpha_v\beta_6$ diabody, and anti- $\alpha_v\beta_6$ cys-diabody were diluted to
22 10 $\mu\text{g/mL}$ in serum-free DMEM and incubated separately (50 μL each) with each cell
23 suspension for 1 h at 4°C. Cells were washed with wash buffer (3 $\times$) and the 10D5 and

1 IgG2a antibodies were detected with AlexaFluor488 goat anti-mouse secondary antibody
2 (10 µg/mL, Invitrogen) for 30 mins at 4°C. The diabody and cys-diabody were detected
3 with the Penta-His AlexaFluor488 conjugate (10 µg/mL, Qiagen). Fluorescent signal was
4 acquired with a FC500 FACS instrument (Beckman Coulter). Data was analyzed using
5 the FlowJo software package (Flowjo, LLC).

6 7 **[¹⁸F]-SFB radiolabeling of anti- $\alpha_v\beta_6$ diabodies**

8 Automated synthesis of [¹⁸F]-SFB was conducted on a GE Tracerlab FX
9 radiosynthesis module as previously described with the addition of high performance
10 liquid chromatography (HPLC) purification (Scheme 1A).[34] Purified [¹⁸F]-SFB was
11 trapped on a preconditioned C-18 SepPak (Waters, preconditioned with 10 mL H₂O, 10
12 mL EtOH), washed with 10 mL of H₂O, and eluted with anhydrous DCM (1 mL).
13 Aliquots of eluted [¹⁸F]-SFB (1.85-2.22 GBq) were dried under a stream of nitrogen in
14 microcentrifuge tubes. Diabodies (200-300 µg) were diluted with borate buffer (final
15 concentration 50 mM, pH 8.7) and incubated with dried [¹⁸F]-SFB at 37°C for 10 mins
16 (Scheme 1B). The [¹⁸F]-SFB radiolabeled diabodies were purified on a BioSpin P-30
17 column (40 kDa MWCO, Bio-Rad) pre-equilibrated with phosphate-buffered saline.
18 Radiochemical yields (RCY) were calculated based on dose calibrator measurements
19 (CRC-25R, Capintec) of eluted [¹⁸F]-SFB radiolabeled diabodies divided by the total
20 radioactivity applied to the spin columns. Radiochemical purities (RCP) were confirmed
21 via isocratic SEC (0.1M NaPO₃, pH 6.8, 0.5 mL/min) connected in series to a
22 photomultiplier tube (PMT).

1 Site-specific radiolabeling of anti- $\alpha_v\beta_6$ cys-diabody with [^{64}Cu]

2 The anti- $\alpha_v\beta_6$ cys-diabody (3.45 mg/mL, 600 μL) in Chelex-treated (Bio-Rad) 0.1
3 M HEPES, 0.5 M NaCl, 10% glycerol was reduced with tris(2-carboxyethyl)phosphine
4 (TCEP, 25 mM final conc., Pierce). The reaction was mixed and allowed to proceed at
5 4°C for 30 mins. NOTA-maleimide (1.36 mg, 2 μmol , Chematech) was dissolved in
6 DMSO (30 μL) and added directly to reduced cys-diabodies (Scheme 2A). The molar
7 ratio of NOTA-maleimide to diabody was 50:1. The conjugation reaction proceeded for 5
8 hrs at 4°C with occasional mixing. Excess chelator was removed with a PD-10 column
9 (GE Life Sciences) pre-equilibrated with Chelex-treated storage buffer. Fractions
10 containing NOTA-conjugated cys-diabody were pooled and reconcentrated using
11 centrifugal filters (Amicon, 10K MWCO). Final concentrations were determined by A_{280}
12 absorbance on a Nanodrop 2000c.

13 The [^{64}Cu] was obtained from Washington University School of Medicine (St.
14 Louis, MO) in the form of [^{64}Cu]Cl₂ (111-296 GBq) in 0.5 M HCl (5-10 μL) and was
15 subsequently diluted in Chelex-treated 0.1 M NH₄OAc, pH 7. The NOTA- $\alpha_v\beta_6$ cys-
16 diabody (1.7 mg/mL, 40 μL) was buffer-exchanged into Chelex-treated 0.1 M NH₄OAc,
17 pH 7 using a spin column and added directly to [^{64}Cu]OAc (37-111 GBq, 4-12 μL). The
18 reaction was incubated at 40°C for 1 h (Scheme 2B). RCY of the crude reaction was
19 determined using antibody instant thin layer chromatography strips (iTLC, Biodex) in
20 mobile phase (PBS, 90 mM EDTA, pH 7) followed by analysis on a radio-TLC imager
21 (AR-2000, Bioscan). The crude reaction was challenged with 0.1 M NH₄OAc, 5 mM
22 EDTA, pH 7 then purified on a spin column. Final RCY was determined by dividing the
23 radioactivity eluted from the spin column by the initial amount of radioactivity applied to

the spin column. Radiochemical purity of the purified [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody was confirmed by radio-SEC as described above.

Serum stability

Serum stabilities of the anti- $\alpha_v\beta_6$ diabodies and cys-diabodies (200 μg each) were analyzed by separate incubations in mouse serum (300 μL) at 37°C . At 1, 2, 4, and 6 h post-incubation, sample aliquots were removed and snap frozen in liquid nitrogen until analysis. Thawed samples were analyzed for degradation by SDS-PAGE and Western blot as described above. In addition, the radiolabeled diabodies were also subjected to separate incubations in mouse serum (500 μL) at 37°C . Stabilities of each radiotracer were analyzed by SDS-PAGE, autoradiography, and instant TLC. Briefly, serum aliquots of [^{18}F]-SFB-radiolabeled diabodies were removed at 1, 2, and 4 h post-incubation, while aliquots of the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody were removed at 4, 12, and 24 h. Samples were frozen on dry ice until analysis by SDS-PAGE. Samples were electrophoresed on 4-20% precast gels then placed on a phosphor imaging screen to develop overnight. Phosphor screens were read the following day at 50 μm resolution using a phosphor imager (Storm 860, Molecular Devices). In addition to autoradiography, radiotracer samples from each time point were analyzed using antibody instant TLC strips in mobile phase (for [^{18}F]-FB- $\alpha_v\beta_6$ diabodies: PBS, pH 7.4; for [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody: PBS, 90 mM EDTA, pH 7) followed by analysis on the radio-TLC imager.

Immunoreactivity

Microcentrifuge tubes were blocked with blocking buffer (5% BSA in PBS) for 15

mins then washed with serum-free DMEM (2×). Cells suspensions (75×10^6 cells/mL, 50 μ L) of either DX3Puro or DX3Puro β_6 in serum-free DMEM were combined with [^{18}F]-FB- $\alpha_v\beta_6$ diabody, [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, or [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody (50 μ L, 7.4 KBq, 4.5 μ g of each), and incubated at room temperature for 1 h (n=4/cell line/radiotracer). Cells were pelleted by centrifugation and washed with serum-free DMEM (3×). Radioactivity in the pellets was compared to that present in the supernatant fractions via gamma counter (Wizard, Perkin Elmer). Immunoreactivity was calculated by dividing the radioactivity in the pellets by the total radioactivity in the supernatant and pellet fractions, respectively.

RESULTS

Expression and purification

Expression yields from 293-F stable mixed pools were approximately 3 mg/L and 1-2 mg/L for the diabody and cys-diabody, respectively following Ni-NTA purification. Under reducing conditions, SDS-PAGE revealed a single band present at ~25 kDa corresponding to the expected size for the reduced monomeric species of the diabody with a purity >95% (Fig. 2A). Reduced SDS-PAGE conditions for the cys-diabody yielded a single band at ~25 kDa representing the monomeric species while non-reducing conditions yielded a prominent band at ~50 kDa confirming formation of the covalently bound dimer (Fig. 2C). SEC further confirmed the high purity of each diabody following Ni-NTA chromatography (diabody T_R =23.2 mins, Fig. 2B; cys-diabody T_R =23.3 mins, Fig. 2D). Interestingly, SEC showed that both the diabody and cys-diabody eluted with a T_R later than that of the carbonic anhydrase standard, which indicated smaller, more

1 compact folding structures.

2

3 **Electrospray ionization**

4 Electrospray ionization was used to further confirm molecular weight of the
5 diabody and cys-diabody. After injection deconvolution of the raw spectra data, a clear
6 peak corresponding to the monomeric species of the diabody was visible at 25,836 kDa
7 (calc: 25,840, Fig. 3A). Similar results were obtained for the cys-diabody after injection
8 with a deconvoluted peak present at 52,084 kDa which further confirmed the covalent
9 formation of the cys-diabody (calc: 52,098 Da, Fig. 3B).

10

11 **Enzyme-linked immunosorbant assay (ELISA)**

12 Figure 4 demonstrates the binding affinity of the anti- $\alpha_v\beta_6$ diabody and anti- $\alpha_v\beta_6$
13 cys-diabody following competition with biotinylated fibronectin or vitronectin against
14 immobilized $\alpha_v\beta_6$, $\alpha_v\beta_3$, and $\alpha_v\beta_5$ integrin (n=3 each). Sigmoidal curves were fitted to the
15 absorbance data (540_{nm}) for each diabody using GraphPad Prism and IC₅₀ values were
16 calculated to determine the concentration of each diabody needed to displace 50% of
17 bound fibronectin or vitronectin. Potent binding was observed for both the diabody and
18 cys-diabody towards the $\alpha_v\beta_6$ integrin, which produced IC₅₀ values of 0.8 nM (diabody)
19 and 0.6 nM (cys-diabody), respectively. IC₅₀ values were >100 uM for each diabody
20 against $\alpha_v\beta_3$, and $\alpha_v\beta_5$, confirming specificity towards the $\alpha_v\beta_6$ integrin.

21

22 **Flow cytometry**

23 Using the paired engineered cell lines DX3Puro β_6 ($\alpha_v\beta_6^+$) and DX3Puro ($\alpha_v\beta_6^-$),

flow cytometry was performed using the diabody and cys-diabody as primary detectors of the $\alpha_v\beta_6$ integrin (Fig. 5). As expected, the IgG2a isotype control antibody yielded no shift in fluorescent signal on either cell line. The positive control 10D5 antibody ($\alpha_v\beta_6$ -specific) produced a distinct shift in fluorescent signal only on the DX3Puro β_6 cell line, confirming specificity to $\alpha_v\beta_6$. Similarly, the diabody and cys-diabody revealed highly selective binding only to the DX3Puro β_6 cell line, which produced fluorescent shifts comparable to that of the positive control 10D5 intact antibody.

Radiolabeling anti- $\alpha_v\beta_6$ diabodies with [^{18}F]-SFB

Automated synthesis of [^{18}F]-SFB resulted in a decay-corrected radiochemical yield (RCY) of $36.1\pm 2.3\%$ with an overall synthesis time of 63.3 ± 0.96 mins (n=8) from end of bombardment. Radiochemical purity of [^{18}F]-SFB was $>98\%$ as determined from analytical radio-HPLC. Due to the isoelectric point of the anti- $\alpha_v\beta_6$ diabodies (pI=8.4) radiolabeling with HPLC-purified [^{18}F]-SFB was accomplished within 10 mins in efforts to avoid precipitation at the basic pH necessary for radiolabeling. Despite this, RCYs of $22.6\pm 3.6\%$ and $8.3\pm 1.7\%$ (n=12 each) were obtained for the [^{18}F]-FB- $\alpha_v\beta_6$ diabody and [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, respectively. Since separation of unlabeled diabodies from the [^{18}F]-labeled diabodies is challenging, the effective specific activity (ESA) was calculated by dividing the original radioactivity in the crude reaction by the total mass of diabody in each reaction.[12] This value was corrected by multiplying the ESA by the final radiochemical yield of the [^{18}F]-FB- $\alpha_v\beta_6$ diabody (or [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody). ESAs were calculated as approximately 20.4 GBq/ μmol for the [^{18}F]-FB- $\alpha_v\beta_6$ diabody and 14.8 GBq/ μmol for the [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody. Spin-column purification

1 successfully separated unreacted [^{18}F]-SFB from the radiolabeled diabodies and resulted
2 in excellent radiochemical purities >98% as determined from size-exclusion
3 chromatography, with no additional purification required (Fig. 6A and 6B).

4 5 **Site-specific radiolabeling of anti- $\alpha_v\beta_6$ cys-diabody with [^{64}Cu]**

6 The cys-diabody was reduced and conjugated to the bifunctional chelator NOTA-
7 maleimide to enable radiolabeling with [^{64}Cu]. Radiolabeling with [^{64}Cu] was
8 accomplished with crude radiochemical yields >93% by iTLC (n=6, Fig. 7A).
9 Optimization of the radiolabeling conditions revealed that reactions with specific
10 activities in the range of 46.3 GBq/ μmol produced the highest crude RCYs as determined
11 by iTLC. Final radiolabeling yields of the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody were
12 43.5 $\pm$ 5.5% (n=6) after spin-column purification with RCP>98% as determined by radio
13 size-exclusion chromatography (Fig. 7B). Effective specific activities of the final purified
14 [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody were approximately 19.2 GBq/ μmol .

15 16 **Serum stability**

17 Serum stability assays were used to evaluate the stability of the anti- $\alpha_v\beta_6$ diabodies
18 and cys-diabodies over a 6 h incubation period at 37°C. Western blot analysis of the
19 samples revealed single bands present at the expected MW of ~25 kDa for the diabody,
20 with no detectable aggregation or degradation of the fragment at t=1 h, 2 h, 4 h, and 6 h.
21 (Fig. 8A, lanes 1-4). The cys-diabody revealed two distinct bands at t=1 h, 2 h, 4 h, and 6
22 h, one which corresponds to the expected molecular weight of the monomer at ~25 kDa
23 and a second band at ~50 kDa representing the covalently bound dimer (Fig. 8A, lanes 5-

8). In comparison to previous Western blots in which the cys-diabody had not been exposed to serum (using 0.1M HEPES, 0.5M NaCl, pH 7.1 as control buffer), the cys-diabody formed a stable covalent dimer with an intense band at ~50 kDa and only a slight band present at ~25 kDa. After serum incubations however, we observed that the majority of the cys-diabody had shifted to ~25 kDa representing the non-covalent dimer, with only a slight band present at ~50 kDa. No other bands were observed for the cys-diabody to indicate aggregation or fragmentation at any time point.

Autoradiography and instant TLC analysis of the radiolabeled diabodies showed good stability of each radiotracer throughout each time point. After 4 and 24 h incubation in serum, the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody remained approximately 96% and 91% intact, respectively (Fig. 8B). The [^{18}F]-FB- $\alpha_v\beta_6$ diabody and [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody showed slightly less stability at the 4 h time point with approximately 86% and 81% remaining intact, respectively (Fig. 8C).

Immunoreactivity

Figure 9 illustrates the results of cell binding experiments with DX3Puro β_6 and DX3Puro cell lines with the [^{18}F]-FB- $\alpha_v\beta_6$ diabody, [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, and [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody. Specificity and immunoreactivity were well preserved for the [^{18}F]-SFB radiolabeled diabodies as demonstrated by the percentage of total radioactivity remaining bound to the DX3Puro β_6 cell line ([^{18}F]-FB- $\alpha_v\beta_6$ diabody=58.7 $\pm$ 6.7%, [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody=80.4 $\pm$ 4.4%). Conversely, only 2.5 $\pm$ 0.1% and 2.6 $\pm$ 0.1% of the total radioactivities bound to the DX3Puro cell line for the [^{18}F]-FB- $\alpha_v\beta_6$ diabody and [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody, respectively (n=4/cell line).

1 Similar results were observed for the site-specifically radiolabeled [^{64}Cu]-NOTA- $\alpha_v\beta_6$
2 cys-diabody. Immunoreactivity and selectivity was well-preserved with $59.4\pm 0.6\%$ of the
3 total radioactivity remaining bound to the DX3Puro β_6 cell line, whereas only $1.8\pm 0.5\%$
4 remained bound to the DX3Puro cell line.

6 **DISCUSSION**

7 This work describes the design and characterization of two engineered antibody
8 fragments specific for the $\alpha_v\beta_6$ integrin: a non-covalent anti- $\alpha_v\beta_6$ diabody and a disulfide-
9 stabilized anti- $\alpha_v\beta_6$ cys-diabody. The diabody format was chosen because it is the
10 smallest antibody fragment that retains the potent bivalent binding of the intact mAb and
11 offers increased tumor uptake over the monovalent single-chain Fv (scFv) and traditional
12 peptide-based targeting reagents containing a single binding motif.[7, 8, 35] The
13 molecular weight of the diabody (~55 kDa) minimizes first-pass clearance from
14 circulation and results in a decreased clearance rate when compared to smaller scFvs (~25
15 kDa) and peptides (<5 kDa), which are prone to rapid clearance in as little as one
16 hour.[36] Consequently, the inherent features of the diabody have enabled it to emerge as
17 an attractive format for the development of targeted molecular imaging agents. To this
18 end, our goal was to radiolabel these engineered antibody fragments using non-site-
19 specific and site-specific radiolabeling approaches with [^{18}F] and [^{64}Cu], respectively,
20 and determine the effects of each radiolabeling approach on binding affinity and
21 specificity towards the $\alpha_v\beta_6$ integrin.

22 Construction of the anti- $\alpha_v\beta_6$ -diabodies was based on the variable domains of the
23 humanized 6.3G9 intact antibody.[24] Utilizing the humanized antibody platform will be

1 highly beneficial with regards to clinical translation as it should virtually eliminate the
2 human anti-mouse antibody (HAMA) response observed from antibodies of murine
3 origin.[37] A flexible GGS linker was used to separate the variable domains of the
4 diabodies to promote dimerization and the anti- $\alpha_v\beta_6$ cys-diabody was additionally
5 engineered with a C-terminal cysteine to generate a covalent dimer and provide a location
6 for site-specific conjugation. The diabodies were expressed from stable-transfected 293-F
7 cell lines and the exposed 6x-His purification tags enabled high purities to be obtained
8 following Ni-NTA column chromatography. We observed approximately 50% lower
9 expression yields from the anti- $\alpha_v\beta_6$ cys-diabody when compared to the parental anti- $\alpha_v\beta_6$
10 diabody, which might be attributable to the presence of unpaired cysteine residues.
11 Olafsen et al. reported similar results during production of the anti-CEA cys-diabody and
12 Alberini et al. suggested that unpaired cysteines present during IgM expression hinder
13 transport through the endoplasmic reticulum and promote misfolding and aggregation.[14,
14 38] Our SDS-PAGE and Western blot data further support this hypothesis since we
15 observed a weak band present at ~25 kDa for the cys-diabody under non-reducing
16 conditions, indicating that not all disulfide bonds were completely formed. The anti- $\alpha_v\beta_6$
17 diabodies demonstrated potent sub-nanomolar binding affinity and selectivity towards
18 $\alpha_v\beta_6$ with excellent serum stability, qualities that are highly desirable for *in vivo*
19 translation where specificity and structural integrity of the radiotracer are paramount.

20 Two radiolabeling approaches, with [^{18}F] and [^{64}Cu], were used to convert the
21 diabodies into molecular imaging agents. First, a non-site-specific method was employed
22 using the activated ester [^{18}F]-SFB conjugated to ϵ -amines of exposed lysine residues on
23 the anti- $\alpha_v\beta_6$ diabody and the anti- $\alpha_v\beta_6$ cys-diabody. Overall RCY yields exceeded those

1 of anti-CEA and anti-HER2 diabodies previously radiolabeled manually with [^{18}F]-SFB
2 (2-8%), but closely resembled those reported after optimization with microfluidics
3 (~25%). [10-12] We surmise that RCYs could be improved but is most likely limited by
4 several factors derived from the inherent properties of the diabodies, as well as the [^{18}F]-
5 SFB radiolabel itself. The isoelectric point for the anti- $\alpha_v\beta_6$ diabodies is 8.4, and in this
6 case, it was difficult to maintain diabody solubility in the narrow pH range required for
7 conjugation with an activated ester (pH 8.6-8.8). To minimize exposure of the diabodies
8 at this pH and reduce precipitation, shorter incubation times with [^{18}F]-SFB were utilized
9 that could have prevented higher RCYs from being obtained. In addition, the chemical
10 properties of [^{18}F]-SFB require a basic pH to enable activation of the ester, but it is
11 increasingly susceptible to hydrolysis as the pH increases. Therefore, a balance must be
12 struck in which the pH is high enough to allow reactivity while simultaneously staving
13 off hydrolysis.[39] Using a microfluidic device to optimize [^{18}F]-SFB conjugation to A2
14 diabodies, Liu *et al.* revealed that a narrow pH range (8.6-8.8) exists in which [^{18}F]-SFB
15 remains reactive enough to form an amide bond without hydrolysis to the unreactive 4-
16 [^{18}F]fluorobenzoic acid ([^{18}F]-FBA).[12] The authors further demonstrated that the
17 stability of [^{18}F]-SFB drastically decreases to ~30% at pH 8.7 in only 10 mins. Future
18 optimization of the [^{18}F]-SFB radiolabeling conditions for these diabodies, particularly
19 with microfluidics, could prove highly beneficial to improving the RCYs and are
20 currently under investigation.

21 The second radiolabeling approach with [^{64}Cu] relies on the premise of site-
22 specific modification of the anti- $\alpha_v\beta_6$ cys-diabody following mild reduction of the
23 engineered disulfide bond. The NOTA-maleimide bifunctional chelator was chosen due

1 to previous studies demonstrating higher radiolabeling efficiency and excellent stability
2 of NOTA compared to 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraaceticacid (DOTA)
3 and macrobicyclic hexamine (sarcophagine) derivatives.[40, 41] Also, a range of
4 radioisotopes have been successfully captured with NOTA including [^{68}Ga] ($t_{1/2}$ =271
5 days), [^{111}In] ($t_{1/2}$ =2.8 days), and [^{18}F] ($t_{1/2}$ =109.7 mins), thus enabling flexibility in terms
6 of choice of radioisotope and imaging modality.[42-44] The introduction of engineered
7 cysteine residues enabled the use of maleimide chemistry and eliminated the pH and
8 reaction time constraints observed with [^{18}F]-SFB. Conjugation of NOTA-maleimide
9 following mild reduction of the engineered thiols could be performed in molar excess,
10 ensuring complete conjugation of the reduced thiols. The formation of Cu(II)
11 coordination complexes is well established at pH 6-8, which eliminated the difficulties
12 associated with [^{18}F]-SFB radiolabeling at pH 8.6-8.8, and enabled increased reaction
13 times to be employed.[45] Consequently, we observed a marked increase in RCY for the
14 [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody ($43.5\pm 5.5\%$) compared to the [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody
15 ($8.3\pm 1.7\%$).

16 To determine the effects of each radiolabeling technique on diabody
17 immunoreactivity, we subjected the [^{18}F]-FB- $\alpha_v\beta_6$ diabody, the [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody,
18 and the [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody to separate cell binding assays. Incubation of
19 each radiolabeled diabody with the engineered cell lines DX3Puro and DX3Puro β_6
20 revealed excellent retention of immunoreactivities regardless of which radiolabeling
21 technique was used. Recent literature has demonstrated that [^{18}F]-SFB-labeled anti-PSCA
22 and anti-CEA diabodies maintained immunoreactivities in the range of 57-59%. Olafsen
23 et al. obtained similar immunoreactivity results in the range of 52-59% for the [^{18}F]-FB-

1 HER2 cys-diabody, with results dependent on spin-column versus SEC purification. Of
2 notable interest was the higher immunoreactivity observed from the covalently bound
3 [^{18}F]-FB- $\alpha_v\beta_6$ cys-diabody ($80.4\pm 4.4\%$). The covalent dimerization of the cys-diabody is
4 likely to constrict conformation of the protein, a hypothesis that could be supported by
5 both the shift in SEC retention time as well as the decreased RCY for the [^{18}F]-FB- $\alpha_v\beta_6$
6 cys-diabody. Although RCYs may have suffered as a result of conformational-induced
7 changes to solvent accessibility, it may have proven favorable in the context of antigen
8 binding on a highly dynamic cell surface. The resultant changes in conformation on the
9 cys-diabody could alter the bivalent binding interactions to $\alpha_v\beta_6$ and contribute to the
10 overall increase in immunoreactivity that we observed.

11 From a clinical perspective, construction of $\alpha_v\beta_6$ -specific molecular imaging agents
12 could have significant implications for disease localization, staging, and evaluating
13 treatment response. By combining the specificity of the anti- $\alpha_v\beta_6$ diabodies with short-
14 lived positron-emitting radioisotopes, quantitative analyses of $\alpha_v\beta_6$ -positive disease
15 progression become feasible with the high resolution and sensitivity of PET. The *in vitro*
16 characterization presented herein demonstrates the feasibility of applying both random
17 and site-specific radiolabeling methods to anti- $\alpha_v\beta_6$ diabodies without detriment to
18 immunoreactivity, but more importantly, it highlights the potential application of these
19 novel radiotracers to specifically target the $\alpha_v\beta_6$ integrin *in vivo*. We highlighted factors
20 that could be critical during *in vivo* translation of the anti- $\alpha_v\beta_6$ diabodies including the
21 choice of conjugation chemistry (activated ester versus maleimide) and reaction
22 conditions and successfully laid the groundwork for the *in vivo* evaluation of these PET
23 imaging agents in the future. Ultimately, *in vivo* translation will require careful

1 consideration of these parameters and their effects on diabody pharmacokinetics, tumor
2 targeting, and biodistribution. For example, diabodies radiolabeled with [^{18}F] typically
3 yield much lower hepatic uptake when compared to their [^{64}Cu]-labeled counterparts, in
4 part because of the lack of transchelation through [^{64}Cu]-binding proteins present in the
5 liver.[45] Despite this issue, the residualizing nature of the [^{64}Cu] radiometal make it
6 attractive for antibody fragments that are internalized upon antigen binding.[17] A
7 comprehensive investigation of these variables and how they contribute to *in vivo* tumor
8 targeting and pharmacokinetics will be required as we gauge the translational likelihood
9 of these novel PET imaging agents.

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19 Disclosure of Potential Conflicts of Interest

20 The authors have no potential conflicts of interest to declare.

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Summary of Figures, Tables, and Schemes

Development and characterization of an $\alpha_v\beta_6$ -specific diabody and a disulfide-stabilized $\alpha_v\beta_6$ -specific cys-diabody

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Diabody

Cys-diabody

Figure 1. Construction of the anti- $\alpha_v\beta_6$ diabody and the anti- $\alpha_v\beta_6$ cys-diabody from the intact humanized 6.3G9 anti- $\alpha_v\beta_6$ antibody.

Figure 2. SDS-PAGE and size-exclusion chromatograms of Ni-NTA purified anti- $\alpha_v\beta_6$ diabody and anti- $\alpha_v\beta_6$ cys-diabody. A. Anti- $\alpha_v\beta_6$ diabody (reduced). B. Anti- $\alpha_v\beta_6$ diabody (blue, $T_R=23.2$ mins) overlaid with BSA and carbonic anhydrase standards (black). C. Anti- $\alpha_v\beta_6$ cys-diabody (reduced and non-reduced). D. Anti- $\alpha_v\beta_6$ cys-diabody (blue, $T_R=23.3$ mins) overlaid with BSA and carbonic anhydrase standards (black). Size-exclusion eluent: 0.1M NaPO₃, pH 6.8, flow rate: 0.5 mL/min.

A.

B.

Figure 3. Electrospray ionization of diabody and cys-diabody A. Deconvolution of raw spectrum data reveals mass peak corresponding to monomeric species of diabody at 25,834 Da (calc: 25,840 Da). B. Deconvolution of raw spectrum data for cys-diabody demonstrates covalent formation of cys-diabody with molecular weight of 52,099 Da (calc: 52,098 Da).

Figure 4. Determination of binding affinity (IC_{50}) by competitive binding ELISA for the anti- $\alpha_v\beta_6$ diabody (blue) and anti- $\alpha_v\beta_6$ cys-diabody (black) following separate incubations with fibronectin against immobilized $\alpha_v\beta_6$ integrin. Each data point represents mean of triplicate experiments and errors bars indicate standard deviation.

Figure 5. Flow cytometry on DX3Puro β_6 and DX3Puro cell lines. Incubation with IgG2a isotype control, 10D5 positive control, diabody and cys-diabody on the DX3Puro cell line revealed no shift in fluorescence. Incubation with the 10D5 antibody, diabody, and cys-diabody revealed highly specific binding to the DX3Puro β_6 cell lines and produced nearly identical fluorescent shifts. No fluorescent shift was observed for the IgG2a isotype control on the DX3Puro β_6 cell line.

Figure 6. Serum stability analysis of diabody and cys-diabody by Western blot. Lanes 1-4: Diabody after incubation in serum at 1, 2, 4, and 6 h. Lanes 5-8: Cys-diabody after incubation in serum at 1, 2, 4, and 6 h. Detection of diabodies was done using the Penta-His-HRP secondary antibody.

Figure 7. Radio size-exclusion chromatograms of purified $[^{18}\text{F}]$ -SFB-radiolabeled diabodies. A. $[^{18}\text{F}]$ -FB- $\alpha_v\beta_6$ diabody B. $[^{18}\text{F}]$ -FB- $\alpha_v\beta_6$ cys-diabody. Black=PMT, Red= A_{280} , Blue= A_{254} .

Figure 8. A. Instant thin-layer chromatogram from crude radiolabeling reaction of NOTA- $\alpha_v\beta_6$ cys-diabody with [^{64}Cu]. B. Radio size-exclusion chromatogram of spin-column purified [^{64}Cu]-NOTA- $\alpha_v\beta_6$ cys-diabody.

Figure 9. Immunoreactivities of [¹⁸F]-FB-α_vβ₆ diabody, [¹⁸F]-FB-α_vβ₆ cys-diabody, and [⁶⁴Cu]-NOTA-α_vβ₆ cys-diabody following incubation with DX3Puro and DX3Puroβ₆ cell lines. Data represents mean of quadruplicate experiments and errors bars indicate standard deviation.

Scheme 1. A. Schematic representation of $[^{18}F]$ -SFB synthesis. B. Radiolabeling of diabody and cys-diabody with $[^{18}F]$ -SFB on ϵ -amines of exposed lysine residues.

Scheme 2. A. Site-specific conjugation of NOTA-maleimide to reduced anti- $\alpha_v\beta_6$ cys-diabody. B. Site-specific radiolabeling of NOTA- $\alpha_v\beta_6$ cys-diabody with [^{64}Cu].

Primer name	Sequence
JW51-For	5'-accggtgaggtgcagctggtggagagc-3'
JW52-Rev	5'-gaattctcagtgggtgggtgggtgggtg-3'
JW57-For	5'-caaggtggagatcaagggcggctgccaccaccaccacc-3'
JW58-Rev	5'-ggtggtgggtgggtgggtggcagccgcccttgatctccaccttg-3'

Table 1. DNA primers used during the construction of pSecTag-2A-B6DiaR-G2S and pSecTag-2A-B6DiaR-G2S-Cys plasmids.