Nanobodies Targeting Mouse/Human VCAM1 for the Nuclear Imaging of Atherosclerotic Lesions

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Rationale: A noninvasive tool allowing the detection of vulnerable atherosclerotic plaques is highly needed. By combining nanomolar affinities and fast blood clearance, nanobodies represent potential radiotracers for cardiovascular molecular imaging. Vascular cell adhesion molecule-1 (VCAM1) constitutes a relevant target for molecular imaging of atherosclerotic lesions.

Objective: We aimed to generate, radiolabel, and evaluate anti-VCAM1 nanobodies for noninvasive detection of atherosclerotic lesions.

Methods and Results: Ten anti-VCAM1 nanobodies were generated, radiolabeled with technetium-99m, and screened in vitro on mouse and human recombinant VCAM1 proteins and endothelial cells and in vivo in apolipoprotein E–deficient (ApoE−/−) mice. A nontargeting control nanobody was used in all experiments to demonstrate specificity. All nanobodies displayed nanomolar affinities for murine VCAM1. Flow cytometry analyses using human umbilical vein endothelial cells indicated murine and human VCAM1 cross-reactivity for 6 of 10 nanobodies. The lead compound cAbVCAM1-5 was cross-reactive for human VCAM1 and exhibited high lesion-to-control (4.95±0.85), lesion-to-heart (8.30±1.11), and lesion-to-blood ratios (4.32±0.48) (P<0.05 versus control C57Bl/6J mice). Aortic arch atherosclerotic lesions of ApoE−/− mice were successfully identified by single-photon emission computed tomography imaging. 99mTc-cAbVCAM1-5 binding specificity was demonstrated by in vivo competition experiments. Autoradiography and immunohistochemistry further confirmed cAbVCAM1-5 uptake in VCAM1-positive lesions.

Conclusions: The 99mTc-labeled, anti-VCAM1 nanobody cAbVCAM1-5 allowed noninvasive detection of VCAM1 expression and displayed mouse and human cross-reactivity. Therefore, this study demonstrates the potential of nanobodies as a new class of radiotracers for cardiovascular applications. The nanobody technology might evolve into an important research tool for targeted imaging of atherosclerotic lesions and has the potential for fast clinical translation. (Circ Res. 2012;110:927-937.)

Key Words: atherosclerosis ■ imaging ■ nanobody ■ nuclear medicine

Several radiotracers of various chemical natures have been evaluated thus far for nuclear imaging of atherosclerotic lesions, including lipoproteins, peptides, oligopeptides, antibodies, carbohydrates, antisense nucleotides, and nanoparticles. However, none of these radiotracers is currently used in routine clinical practice, mostly because of their inability to reach sufficient lesion-to-background ratios in vivo. Indeed, nuclear imaging of vulnerable plaques at the level of coronary arteries remains challenging, mostly because of the small volume of the lesions and their vicinity with the blood containing unbound circulating tracer. Thus, an ideal tracer should combine high affinity and specificity, good solubility and stability, and efficient radiolabeling with small size and fast blood clearance, so that high contrast images can be obtained shortly after

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The term nanobody is a trademark of Ablynx, Inc.

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The inflammatory process leading to the development of vulnerable atherosclerotic lesions is characterized by extensive recruitment of monocytes and lymphocytes into the arterial wall. Several endothelial adhesion molecules are implicated in the process of leukocyte rolling, firm adhesion, and transmigration, such as E- and P-selectins, vascular cell adhesion molecule-1 (VCAM1), and intercellular adhesion molecule-1 (ICAM1). VCAM1 is a receptor of the immunoglobulin family that binds to very late antigen-4 (VLA4) present on the surface of leukocytes. As active inflammation characterized by leukocyte infiltration is recognized as a major criterion for defining a vulnerable plaque, the adhesion molecule VCAM1 is a relevant molecular target for noninvasive detection of such lesions. Indeed, VCAM1 expression was observed at the level of the luminal endothelium as well as on neovessels of advanced lesions, on macrophages, and on activated smooth muscle cells. Therefore, molecular probes targeting VCAM1 have been evaluated by our group and others either for nuclear, magnetic resonance, fluorescent, or ultrasound in vivo imaging.

In the present study, our objectives were to generate and evaluate nanobody-based radiolabeled tracers for preclinical imaging of atherosclerotic plaques. Specifically, we describe (1) the generation and full in vitro characterization of cross-reactive mouse and human VCAM1-targeted nanobodies; (2) their $^{99m}$Tc-radiolabeling; and (3) their thorough assessment as tracers for noninvasive in vivo nuclear molecular imaging of atherosclerotic lesions in ApoE-deficient (ApoE$^{-/-}$) mice.

**Methods**

An expanded Methods section is available in the online-only Data Supplement.

**Nanobody Generation and Production**

VCAM1-targeting nanobodies were generated largely after published methods. Specifically, a dromedary was immunized with both mouse and human recombinant VCAM1 proteins (RnD Systems), blood lymphocytes were isolated, and RNA was purified. The variable domains of the heavy chain–only antibodies ($\text{V}_{\text{H}}$5 or nanobodies) were amplified using a 2-step RT-PCR method and cloned in frame with M13 bacteriophage gene 3. Nanobodies were phage-displayed and used in biopannings on immobilized immuno-gens. Crude bacterial extracts containing soluble nanobodies were used to select individual VCAM1 binders based on a positive signal in ELISA and in flow cytometry on tumor necrosis factor alpha (TNF$\alpha$)-stimulated bEND5 cells. After sequencing, selected anti-VCAM1 and irrelevant control cAbBeH10 nanobodies were produced as hexahistidine-tagged proteins in *Escherichia coli* and purified, as described previously.

**In Vitro Evaluation of Unlabeled Nanobodies**

**Cell Lines**

The mouse endothelial cell line bEND5 (ECACC) was cultured in supplemented DMEM medium and the human umbilical vein endothelial cells (HUVEC) in supplemented EndoGro basal medium (Millipore). VCAM1 expression was induced by stimulation with 10 ng/mL TNF$\alpha$ during 18 hours.

**Flow Cytometry**

TNF$\alpha$-stimulated (10$^5$) and unstimulated cells were incubated either with PE-labeled anti-VCAM1 monoclonal antibody (mAb) (anti-mouse from Abcam; anti-human from RnD Systems) or sequentially with 1 $\mu$g nanobody, 1 $\mu$g anti–His-tag mAb (Serotec), and 200 ng PE-labeled rat anti-mouse IgG1 (BD Biosciences). Binding was measured on a FACS Canto II analyzer (BD Biosciences) and data were analyzed with FlowJo software (TreeStar).

**Thermal Stability**

$T_m$ values (unfolding temperatures) were obtained on a J-715 spectropolarimeter (Jasco, Easton, MD), as previously described.

**Surface Plasmon Resonance–Based Affinity Evaluation**

Nanobodies affinity for recombinant human and mouse VCAM1 was determined by surface plasmon resonance (SPR) analysis on a Biacore 3000 apparatus. Recombinant mouse ICAM1 (RnD Systems) was used as a negative control. Recombinant proteins were immobilized on a CM5 sensor chip (Biacore) according to the manufacturer’s instructions. A 2-fold dilution series of nanobodies from 50–1 nmol/L were tested. Affinity constants were determined using a 1:1 standard association model fit (BIAevaluation software).

<table>
<thead>
<tr>
<th>Non-standard Abbreviations and Acronyms</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE$^{-/-}$</td>
<td>apolipoprotein E–deficient</td>
</tr>
<tr>
<td>CT</td>
<td>computed tomography</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3’diaminobenzidine</td>
</tr>
<tr>
<td>HER2</td>
<td>human epidermal growth factor receptor 2</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HUVEC</td>
<td>human umbilical vein endothelial cells</td>
</tr>
<tr>
<td>ICAM1</td>
<td>intercellular adhesion molecule-1</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin class G</td>
</tr>
<tr>
<td>$K_d$</td>
<td>equilibrium dissociation constant</td>
</tr>
<tr>
<td>mAb</td>
<td>monoclonal antibody</td>
</tr>
<tr>
<td>mVCAM1/hVCAM1</td>
<td>mouse/human vascular cell adhesion molecule-1</td>
</tr>
<tr>
<td>PET</td>
<td>positron emission tomography</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PE</td>
<td>phycoerythrin</td>
</tr>
<tr>
<td>ROI</td>
<td>region of interest</td>
</tr>
<tr>
<td>SPECT</td>
<td>single-photon emission computed tomography</td>
</tr>
<tr>
<td>SPR</td>
<td>surface plasmon resonance</td>
</tr>
<tr>
<td>$T_m$</td>
<td>unfolding temperature</td>
</tr>
<tr>
<td>TNF$\alpha$</td>
<td>tumor necrosis factor alpha</td>
</tr>
<tr>
<td>$V_{\text{H}}$</td>
<td>variable domain from conventional antibodies</td>
</tr>
<tr>
<td>$V_{\text{H}o}$</td>
<td>variable domain from heavy chain–only antibodies</td>
</tr>
<tr>
<td>VLA4</td>
<td>very late antigen-4</td>
</tr>
<tr>
<td>$^{99m}$Tc</td>
<td>technetium-99m</td>
</tr>
<tr>
<td>$%ID/g$</td>
<td>percent of injected dose per gram</td>
</tr>
<tr>
<td>$%ID/cm^3$</td>
<td>percent of injected dose per cubic centimeter</td>
</tr>
<tr>
<td>$%ID/TBV$</td>
<td>percent of injected dose in total blood volume</td>
</tr>
</tbody>
</table>
Epitope Competition Using SPR
SPR was used to determine which nanobodies compete for the same epitope. These procedures have been described in detail elsewhere.\(^5\)

Radiolabeling and High-Performance Liquid Chromatography Assessment of In Vitro and In Vivo Stability
Nanobodies were radiolabeled with \(^{99m}\text{Tc}\) using the tricarbonyl-method, as described elsewhere.\(^3\) Radiochemical purity was assessed immediately after labeling, after 6 hours at 20°C in phosphate-buffered saline (PBS) and in mouse blood 3 hours after injection. In the latter case, 100 \(\mu\)L of sampled whole blood was centrifuged and plasma was filtered using a Nanosep 10 kDa Omega Membrane. Radiochemical purity was determined by reverse-phase high-performance liquid chromatography (HPLC), using a C4 column eluted with a phosphate-buffered saline/trifluoroacetic acid gradient mobile phase. Radioactivity was monitored using a radiodetector (\(\gamma\)-RAM Model 4, LabLogic).

In Vitro Evaluation of \(^{99m}\text{Tc}\)-Labeled Nanobodies
Cells (250\(\times\)10\(^5\) bEND5) were plated in 24-well plates and stimulated 18 hours with 10 ng/mL TNF\(\alpha\). Five nanomoles per liter of each \(^{99m}\text{Tc}\)-nanobody was incubated in 0.5 mL PBS +1% human serum albumin for 1.5 hours at 37°C. Competition studies with a 500-fold excess of unlabeled nanobody were conducted to assess the specificity of the binding. After washing, bound \(^{99m}\text{Tc}\)-nanobody was collected and counted in a \(\gamma\)-counter (Canberra Packard). Nonspecific binding to the well was subtracted, and results were normalized to the TNF\(\alpha\)-negative condition.

Animal Model and Processing of Aortas
All animal experiments were approved by the Grenoble Research Center of the Army Health Services (CRSSA) committee. Female 35\(\pm\)2 week-old (mean\(\pm\)SD) ApoE\(^{-/-}\) and control C57Bl/6J mice were used (Charles River). ApoE\(^{-/-}\) mice (n=47) were fed a Western diet containing 0.25% cholesterol (Safe) for 18 weeks, whereas control mice (n=15) remained on a standard chow diet.

Biodistribution
Each anti-VCAM1 nanobody was evaluated in 3 ApoE\(^{-/-}\) mice except \(^{99m}\text{Tc}\)-cAbVCAM1-5 (n=6), which was also further evaluated in control C57Bl/6J mice (n=4). \(^{99m}\text{Tc}\)-cAbBcl10 was evaluated as a negative control in both ApoE\(^{-/-}\) (n=4) and control mice (n=5). Two hours after \(^{99m}\text{Tc}\)-radiolabeled nanobody administration (67.4 MBq i.v.), single-photon emission computed tomography/computed tomography (SPECT/CT) acquisition was performed in all animals (nanoSPECT, Bioscan, see below). Mice were then euthanized and aortas were cut into 12 segments. A lesion-extension index was attributed to each segment as shown in Online Figure I: (−) no lesion (control segments), (+) lesion covering up to 50% of the arterial segment length, (+) lesions covering >50% of the arterial segment length, and (++) lesions extending over the whole segment length. Biodistribution results were expressed as a percent of injected dose per gram of tissue (%ID/g). Aortic lesion and control uptakes were defined as the average uptake in all segments ranked (++++) or (−−−), respectively. Lesion-to-control, lesion-to-blood, and lesion-to-heart ratios were also determined. Adjacent 20-\(\mu\)m and 8-\(\mu\)m-thick cryosections were obtained from all 12 aortic segments for microautoradiography imaging (BASS-5000, Fujifilm) and immunohistochemical VCAM1 staining, respectively.

Competition
Biodistribution of \(^{99m}\text{Tc}\)-cAbVCAM1-5 in ApoE\(^{-/-}\) mice was assessed by in vivo SPECT/CT imaging and ex vivo y-well counting with (n=6) or without (n=4) coinciding a 100-fold excess of unlabeled competitor nanobody cAbVCAM1-1. Results are expressed in %ID/g.

Pharmacodynamics
A subgroup of C57Bl/6J mice was used to evaluate \(^{99m}\text{Tc}\)-cAbVCAM1-5 pharmacodynamics in major organs using dynamic SPECT/CT imaging from 0–180 minutes after injection (86.1\(\pm\)28.0 MBq) (n=3). Results are expressed as %ID/cm\(^3\).

Blood Kinetics
\(^{99m}\text{Tc}\)-cAbVCAM1-5 blood clearance was assessed in C57Bl/6J mice (n=3) by collecting blood samples at several time points after injection. Results are expressed as %ID in total blood volume (%ID/TBV).

Immunohistochemistry
Primary anti-VCAM1 antibody (Santa-Cruz Biotechnology) was applied overnight at 4°C, biotinylated secondary antibody (Jackson ImmunoResearch) was incubated for 1 hour at 20°C, and DAB was used as the chromogen. Staining specificity was assessed by omitting the primary antibody. In a subset of ApoE\(^{-/-}\) and control mice, VCAM1 immunostaining was also performed on heart, muscle, salivary gland, liver, bone marrow, lymph node, spleen, and thymus.

SPECT/CT Imaging
Two hours after intravenous injection, anesthetized animals were placed in a temperature-controlled bed and whole-body SPECT/CT acquisitions were performed from 2–3 hours after injection (nano-SPECT, Bioscan). CT and SPECT acquisitions were reconstructed, fused, and quantified using dedicated software (In VivoScope). SPECT scale was normalized to %ID/cm\(^2\) to allow direct visual comparison between animals. Regions of interest (ROIs) were drawn at the level of the aortic arch and left ventricle cavity for determination of arch-to-blood ratio.

Autoradiography
For each animal, autoradiographic images were obtained after overnight exposure of 3 sets of 20-\(\mu\)m-thick slices obtained at distinct levels of the 12 aortic segments. Images were quantified using dedicated software (Image Gauge, Fujifilm). ROIs were drawn around atherosclerotic lesions and control VCAM1-negative aortic wall. Results were corrected from background and expressed as average lesion-to-control ratios.

Statistical Analysis
All results are presented as mean\(\pm\)SEM. Nonparametric Mann-Whitney U, Wilcoxon, and Spearman tests were used to compare unpaired datasets, paired datasets, and correlations between aortic uptake and lesion extension, respectively. Differences were considered significant for \(P<0.05\).

Results
Generation of Anti-VCAM1 Nanobodies
To make future clinical translation possible, we aimed at developing nanobodies cross-reactive for mouse and human VCAM1. Nanobodies were therefore generated by immunizing a dromedary with both mouse and human VCAM1 recombinant proteins followed by biopannings of the resulting phage-displayed immune nanobody library. Crude bacterial extracts containing individual nanobodies were screened by ELISA for binding to VCAM1 recombinant proteins and in flow cytometry to bind to VCAM1-expressing bEND5 cells (data not shown).

On sequencing, 31 different anti-VCAM1 nanobodies were identified that could be grouped into 12 families, based on similar sequences in antigen-binding loops. Six nanobody families were mouse VCAM1 (mVCAM1)-specific and 6 families bound to both mouse and human VCAM1 (hVCAM1). Based on ELISA and flow cytometry signals of
Vaccine candidates could be grouped into 3 epitope-targeting categories: cAbBcII10 did not bind to VCAM1 in SPR studies. Based on SPR anti-VCAM1 nanobody, and the control nanobody cAb-VCAM1-10) were selected for further studies. Nanobody production yield ranged from 0.8–10.5 mg/L bacterial culture crude extracts, 10 nanobodies (called cAbVCAM-1 to cAbVCAM-10) were used as a nontargeting control nanobody in further compar-

### Table 1. Comparison Among 10 Evaluated Anti-VCAM1 Nanobodies

<table>
<thead>
<tr>
<th>Radiotracer</th>
<th>%ID/g Lesion</th>
<th>Lesion: Control</th>
<th>Lesion: Blood</th>
<th>Lesion: Heart</th>
<th>K_d mVCAM1, nmol/L</th>
<th>K_d hVCAM1, nmol/L</th>
<th>Production Yield, mg/L</th>
<th>T_m, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>cAbVCAM1-1</td>
<td>0.87±0.08 #9</td>
<td>2.15±0.20 #9</td>
<td>0.74±0.10* #10</td>
<td>2.65±0.23 #9</td>
<td>8.3±1.2 #7</td>
<td>12.4±0.5 #5</td>
<td>2.0 #7</td>
<td>72.3±0.1 #2</td>
</tr>
<tr>
<td>cAbVCAM2-1</td>
<td>2.15±0.29* #6</td>
<td>2.90±0.45 #6</td>
<td>3.37±0.32* #5</td>
<td>5.55±0.58 #7</td>
<td>0.3±0.0 #2</td>
<td>Not cross-reactive</td>
<td>5.0 #5</td>
<td>62.3±0.1 #6</td>
</tr>
<tr>
<td>cAbVCAM1-3</td>
<td>2.95±0.16* #2</td>
<td>4.07±0.56 #3</td>
<td>5.06±0.39* #1</td>
<td>7.40±0.91* #3</td>
<td>2.4±0.1 #5</td>
<td>9.1±0.9 #4</td>
<td>6.8 #3</td>
<td>59.7±0.1 #9</td>
</tr>
<tr>
<td>cAbVCAM1-4</td>
<td>2.21±0.59* #5</td>
<td>3.20±0.74 #5</td>
<td>8.41±0.29 #9</td>
<td>1.96±0.56 #10</td>
<td>0.2±0.0 #1</td>
<td>Not cross-reactive</td>
<td>3.8 #3</td>
<td>59.4±0.1 #10</td>
</tr>
<tr>
<td>cAbVCAM1-5</td>
<td>2.53±0.08* #3</td>
<td>4.95±0.85* #1</td>
<td>4.32±0.48* #2</td>
<td>8.30±1.11* #1</td>
<td>2.0±0.0 #4</td>
<td>6.5±0.7 #3</td>
<td>10.5 #1</td>
<td>&gt;87 #1</td>
</tr>
<tr>
<td>cAbVCAM1-6</td>
<td>0.73±0.08 #10</td>
<td>4.57±0.93* #2</td>
<td>1.85±0.37 #8</td>
<td>4.98±0.75 #8</td>
<td>5.2±0.6 #6</td>
<td>Not cross-reactive</td>
<td>3.0 #6</td>
<td>72.0±0.1 #3</td>
</tr>
<tr>
<td>cAbVCAM1-7</td>
<td>1.27±0.25* #8</td>
<td>2.88±0.65 #7</td>
<td>4.02±1.05* #3</td>
<td>5.98±0.96 #4</td>
<td>26.6±1.2 #9</td>
<td>Not cross-reactive</td>
<td>6.9 #2</td>
<td>60.9±0.3 #8</td>
</tr>
<tr>
<td>cAbVCAM1-8</td>
<td>2.48±0.46* #4</td>
<td>1.40±0.10 #10</td>
<td>3.66±0.10* #4</td>
<td>7.71±0.38* #2</td>
<td>13.2±0.3 #8</td>
<td>1.4±0.5 #1</td>
<td>1.5 #8</td>
<td>61.5±0.1 #7</td>
</tr>
<tr>
<td>cAbVCAM1-9</td>
<td>2.99±0.07* #1</td>
<td>2.19±0.60 #8</td>
<td>2.51±0.03* #6</td>
<td>5.69±0.36* #6</td>
<td>0.9±0.2 #3</td>
<td>5.3±0.7 #2</td>
<td>0.9 #9</td>
<td>66.8±0.2 #4</td>
</tr>
<tr>
<td>cAbVCAM1-10</td>
<td>1.93±0.14* #7</td>
<td>3.47±0.67* #4</td>
<td>2.01±0.14* #7</td>
<td>5.76±0.56* #5</td>
<td>45.7±2.0 #10</td>
<td>18.4±7.0 #6</td>
<td>0.8 #10</td>
<td>63.4±0.2 #5</td>
</tr>
<tr>
<td>cAbBcII10</td>
<td>0.68±0.06</td>
<td>1.66±0.28</td>
<td>1.57±0.09</td>
<td>4.00±0.14</td>
<td>ND</td>
<td>ND</td>
<td>5.0</td>
<td>77.5±0.2</td>
</tr>
</tbody>
</table>

ND indicates not detectable.

Mean±SEM values and rank (#) are given for parameters obtained either ex vivo by gamma-well counting (%ID/g lesion, lesion-to-control ratio, lesion-to-blood ratio, lesion-to-heart ratio) or in vitro (K_d for mVCAM1 or hVCAM1, production yield and T_m). cAbVCAM1-5 is the most potent candidate, based on these criteria.

*P<0.05 versus cAbBcII10.

**In Vitro Characterizations**

Flow cytometry analysis of mouse bEND5 and human HUVEC endothelial cells stained with an anti-VCAM1 antibody showed low basal VCAM1 expression that was strongly elevated on TNFα treatment (Figure 1A and 1B). Under these conditions, all 10 selected nanobodies interacted with mVCAM1 on stimulated bEND5 cells (Figure 1A). Among them, 6 were found to be cross-reactive for hVCAM1 expressed on stimulated HUVECs (Figure 1B). As demonstrated by SPR analyses summarized in Table 1 and as exemplified in Figure 1C, all selected nanobodies bound to mVCAM1 with high affinities ranging from 0.2–45.7 nmol/L. Moreover, in accordance with that observed by flow cytometry, 6 nanobodies were found cross-reactive for hVCAM1 with affinities remaining in the nanomolar range (Table 1). No binding to the related adhesion receptor ICAM1 was observed for any anti-VCAM1 nanobody, and the control nanobody cAbBcII10 did not bind to VCAM1 in SPR studies. Based on SPR competition studies (Online Figure II), cAbVCAM1 nanobodies could be grouped into 3 epitope-targeting categories: cAbVCAM1-1/5, cAbVCAM1-2/3/6/7/9/10, and cAbVCAM1-4/8. All nanobodies exhibited high thermal stability as demonstrated by unfolding temperatures ranging from 59.4°C to >87°C (Table 1).

After 99mTc-radiolabeling and purification steps, radiochemical purities were >95% for all nanobodies. 99mTc-labeling did not affect VCAM1 recognition for most binders as demonstrated by the in vitro binding assay on bEND5 cells (Figure 1D): besides cAbVCAM1-8, binding on VCAM1-positive, TNFα-stimulated cells was significantly higher than on unstimulated cells. Moreover, binding on TNFα-stimulated cells was successfully inhibited by competition with an excess of unlabeled nanobody and binding of the negative control cAbBcII10 to either stimulated or unstimulated cells was negligible, thereby demonstrating specificity.

**Immunochemistry and Biodistribution Analyses**

As depicted in Figure 2, VCAM1 constitutive expression was observed in lymphoid tissues (ie, bone marrow, lymph node, spleen, and thymus) in both C57Bl/6J control and hypercholesterolemic ApoE−/− mice, whereas no VCAM1 expression was found in heart, muscle, and salivary gland. VCAM1 staining was also found in ApoE−/− mice liver. Moreover, strong VCAM1 staining was also observed within atherosclerotic lesions at the level of the luminal endothelium as well as inside the atherosclerotic plaque but not in the aorta of control C57Bl/6J mice.

Biodistributions of 99mTc-labeled nanobodies in ApoE−/− mice are summarized in Online Table I. All nanobodies, including control cAbBcII10, exhibited high kidney uptake ranging from 97±16 to 315±33%ID/g and high activities in the bladder. As expected, 99mTc-cAbVCAM1 uptakes in VCAM1-positive tissues were higher than that of the nontargeting control 99mTc-cAbBcII10, a difference which reached statistical significance for 99mTc-cAbVCAM1-3 (spleen and thymus), 99mTc-cAbVCAM1-4/5 (spleen, thymus, liver and bone marrow), and 99mTc-cAbVCAM1-9 (thymus and liver), and 99mTc-cAbVCAM1-1/8/10 (liver) (P<0.05 versus 99mTc-cAbBcII10). With the exception of the lung (mean uptake of 2.5±0.8%ID/g), uptake was lower than 2%ID/g in other investigated tissues, including the blood and myocardium.

Aortas from hypercholesterolemic ApoE−/− mice injected with 99mTc-labeled nanobodies were segmented and samples were macroscopically scored according to relative lesion content. As shown in Table 1, uptake in atherosclerotic lesions was greater than 2%ID/g for 6 of 10 cAbVCAM1, with a maximum value of 2.99±0.07%ID/g for 99mTc-cAbVCAM1-9 (P<0.05 versus 99mTc-cAbBcII10), whereas...
the lowest uptake was noted for the nontargeting control 99mTc-cAbBcII10.

Lesion-to-control, lesion-to-blood, and lesion-to-heart ratios were determined from biodistribution data (Table 1). Lesion-to-control ratios were $\frac{1}{H11022}$ for all VCAM1-specific nanobodies with the exception of 99mTc-cAbVCAM1-8, with a maximum ratio of 4.95 $\pm 0.85$ for 99mTc-cAbVCAM1-5 ($P < 0.05$ versus 99mTc-cAbBcII10). Lesion-to-blood ratio was $\frac{1}{H11022}$ for 9 of 10 99mTc-cAbVCAM1 nanobodies, with a maximum ratio of 5.06 $\pm 0.39$ for 99mTc-cAbVCAM1-3 ($P < 0.05$ versus 99mTc-cAbBcII10). Finally, lesion-to-heart ratio was $\frac{1}{H11022}$ for all nanobodies, with a maximum value of 8.30 $\pm 1.11$ for 99mTc-cAbVCAM1-5 ($P < 0.05$ versus 99mTc-cAbBcII10).

**Further Evaluations of the Lead Nanobody cAbVCAM1-5**

Based on selection criteria summarized in Table 1, cAbVCAM1-5 was selected among the 10 evaluated anti-VCAM1 nanobodies for further investigations and compared with nontargeting control 99mTc-cAbBcII10.

**Stability**

As demonstrated by HPLC, 99mTc-cAbVCAM1-5 was stable in vitro for up to 6 hours after radiolabeling, as well as in vivo in the blood at 3 hours after injection, after completion of SPECT imaging (Figure 3A through 3C).

**Biodistribution**

99mTc-cAbVCAM1-5 rapidly cleared from the circulation and background tissues (Online Figure III) and uptake in kidneys, bladder, and VCAM1-positive lymphoid tissues was clearly identifiable in vivo SPECT images from control C57Bl/6J mice, whereas only the kidneys and bladder were visible after the injection of the nontargeting control 99mTc-cAbBcII10 (Figure 3D and 3E). 99mTc-cAbVCAM1-5 uptake in lymphoid tissues was further confirmed ex vivo by biodistribution analyses (Table 2). Indeed, 99mTc-cAbVCAM1-5 uptake represented 7.4 $\pm 0.2$, 1.5 $\pm 0.1$ and 7.9 $\pm 2.0$%ID/g in spleen, thymus, and bone marrow of control mice, respectively ($P < 0.05$ versus nontargeting control 99mTc-cAbBcII10). Furthermore, 99mTc-cAbVCAM1-5 biodistribution in ApoE$^{-/-}$ mice major organs was similar to that observed in C57Bl/6J mice (Table 2, $P=NS$).
Uptake in Atherosclerotic Lesions

In ApoE−/− mice, 99mTc-cAbVCAM1-5 aortic uptake correlated with the lesion-extension index. Indeed, 99mTc-cAbVCAM1-5 uptake in individual aortic segments increased together with the relative volume of the atherosclerotic lesion, whereas no such gradient was observed for 99mTc-cAbBcII10 (Spearman ρ = 0.894; P < 0.0001; Figure 4A). 99mTc-cAbVCAM1-5 uptake in the aorta was further characterized using autoradiography of sections. As shown in Figure 4B and Online Figure IV, 99mTc-cAbVCAM1-5 accumulated within VCAM1-positive atherosclerotic lesions, resulting in a lesion-to-control ratio of 8.7 ± 0.8 (P < 0.05 versus cAbBcII10).

After SPECT/CT imaging, 99mTc-cAbVCAM1-5 uptake was readily visualized on atherosclerotic lesions from the aortic arch of hypercholesterolemic ApoE−/− mice, whereas no tracer uptake was observed at the same location in control C57Bl/6J animals or with the nontargeting control 99mTc-cAbBcII10 in either mouse strain (Figure 5A). As a result, the 99mTc-cAbVCAM1-5 aortic arch-to-blood ratio from ApoE−/− mice was significantly higher than that observed in C57Bl/6J animals or than that obtained after injection of the nontargeting control nanobody (P < 0.05) (Figure 5B). Finally, coinjection with an excess of unlabeled cAbVCAM1-1, a nanobody recognizing the same VCAM1-epitope as
cAbVCAM1-5 (Online Figure II, B), resulted in significant decrease in $^{99m}$Tc-cAbVCAM1-5 uptake in liver, lymphoid tissues, and atherosclerotic lesions, thereby demonstrating specificity of the signals (Figure 6).

**Discussion**

This study was designed to generate nanobodies recognizing both mouse and human VCAM1 homologues, since such cross-reactive binders would be suitable for translation into both mouse and human VCAM1 homologues, since such specificity of the signals (Figure 6).

$^{99m}$Tc-cAbVCAM1-1, lesion-to-control, lesion-to-blood, and lesion-to-heart ratios were all $>1$, with mean ratios of 3.2, 2.9, and 4, respectively. $^{99m}$Tc-cAbVCAM1-5 was taken up at the level of the bladder (Bl) and kidneys (Kd) as well as in the lymphoid tissues: lymph nodes (LN), bone marrow (BM), thymus (Tm), and spleen (Sp).

**Table 2. Ex Vivo Biodistribution of $^{99m}$Tc-Labeled cAbBcII10 and cAbVCAM1-5 Nanobodies 3 Hours After Injection in C57Bl/6J and ApoE$^{-/-}$ Mice**

<table>
<thead>
<tr>
<th></th>
<th>C57Bl/6J</th>
<th>ApoE$^{-/-}$</th>
<th>C57Bl/6J</th>
<th>ApoE$^{-/-}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood</td>
<td>0.4±0.0</td>
<td>0.4±0.0</td>
<td>0.5±0.1</td>
<td>0.6±0.1</td>
</tr>
<tr>
<td>Heart</td>
<td>0.1±0.0</td>
<td>0.2±0.0</td>
<td>0.2±0.0*</td>
<td>0.3±0.1*</td>
</tr>
<tr>
<td>Lung</td>
<td>0.8±0.2</td>
<td>1.0±0.1</td>
<td>1.7±0.2*</td>
<td>2.3±0.3*</td>
</tr>
<tr>
<td>Liver</td>
<td>1.0±0.1</td>
<td>0.6±0.1†</td>
<td>1.4±0.2</td>
<td>1.8±0.3*</td>
</tr>
<tr>
<td>SM</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
<td>0.1±0.0</td>
</tr>
<tr>
<td>SG</td>
<td>0.3±0.0</td>
<td>0.2±0.0</td>
<td>0.5±0.0*</td>
<td>0.5±0.1*</td>
</tr>
<tr>
<td>Thyroid</td>
<td>0.5±0.1</td>
<td>0.4±0.1</td>
<td>0.7±0.1</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>Stomach</td>
<td>0.4±0.0</td>
<td>0.4±0.0</td>
<td>0.5±0.0*</td>
<td>0.6±0.1*</td>
</tr>
<tr>
<td>Bile</td>
<td>0.5±0.1</td>
<td>0.4±0.1</td>
<td>0.3±0.0</td>
<td>0.7±0.2</td>
</tr>
<tr>
<td>Kidney</td>
<td>350±16</td>
<td>267±14†</td>
<td>287±43</td>
<td>222±12</td>
</tr>
<tr>
<td>Spleen</td>
<td>0.3±0.0</td>
<td>0.4±0.0</td>
<td>7.4±0.2*</td>
<td>9.2±1.0*</td>
</tr>
<tr>
<td>Thymus</td>
<td>0.1±0.0</td>
<td>0.2±0.0</td>
<td>1.5±0.1*</td>
<td>1.7±0.1*</td>
</tr>
<tr>
<td>BM</td>
<td>0.4±0.0</td>
<td>1.0±0.7</td>
<td>7.9±2.0*</td>
<td>10.7±2.9*</td>
</tr>
</tbody>
</table>

SM indicates skeletal muscle; SG, salivary glands; BM, bone marrow. Results are expressed as mean±SEM.

*P<0.05 versus strain-matched cAbBcII10.
†P<0.05 versus C57Bl/6J.

**Figure 3. $^{99m}$Tc-cAbVCAM1-5 HPLC profiles indicating that this nanobody was stable in vitro at 0 (A) and 6 hours (B) after radiolabeling, as well as in vivo in the blood 3 hours after injection (C). Representative in vivo SPECT/CT whole-body maximum intensity projections images (MIP) of control $^{99m}$Tc-cAbBcII10 (D) and $^{99m}$Tc-cAbVCAM1-5 (E) nanobodies obtained 2–3 hours after intravenous injection in C57Bl/6J mice (n=5 and 4, respectively). $^{99m}$Tc-cAbVCAM1-5 was taking up at the level of the bladder (Bl) and kidneys (Kd) as well as in the lymphoid tissues: lymph nodes (LN), bone marrow (BM), thymus (Tm), and spleen (Sp).**

$^{99m}$Tc-cAbVCAM1-8. As expected, due to their small size, nanobodies exhibited fast blood clearance in vivo, resulting in a mean circulating activity of 0.8%ID/g at 3 hours after injection in ApoE$^{-/-}$ mice (range, 0.3–1.5%ID/g). In addition, myocardial background activity was also minimal (mean, 0.4%ID/g; range, 0.3–1.5). These biodistribution kinetics are in accordance with those previously obtained using anti–epidermal growth factor receptor and anti-HER2 $^{99m}$Tc-labeled nanobodies. More importantly, cAbVCAM1 nanobody uptake in aortic atherosclerotic lesions was higher than that of a nontargeting control nanobody cAbBcII10, and this difference reached statistical significance for 8 of the 10 evaluated nanobodies. Consequently, with the exception of cAbVCAM1-1, lesion-to-control, lesion-to-blood, and lesion-to-heart ratios were all $>1$, with mean ratios of 3.2, 2.9, and 5.6, respectively.

**Uptake in VCAM1-Positive Tissues**

In addition to the expected uptake in atherosclerotic lesions, most cAbVCAM1 nanobodies were taken up by lymphoid tissues in both normal and hypercholesterolemic mice, as demonstrated by biodistribution and in vivo SPECT imaging experiments. More specifically, the 5 cAbVCAM1 nanobodies presenting with the highest affinities for mVCAM1 (cAbVCAM1-2/3/4/5/9, all $K_D$ <2.5 mmol/L) exhibited the highest uptakes in the spleen and bone marrow. Corresponding mVCAM1 constitutive expression was observed by immunohistochemistry in spleen, bone marrow, lymph nodes, and thymus. Therefore, cAbVCAM1 binding to lymphoid...
tissues was likely due to specific VCAM1 binding in vivo. VCAM1 constitutive expression in lymphoid tissues has been previously reported by others, either in mouse bone marrow, spleen, lymph nodes, and thymus or in human thymus, bone marrow, and fetal or activated spleen. Furthermore, specific uptake of radiolabeled anti-VCAM1 antibodies in mouse lymphoid organs has also been demonstrated previously. Finally, an increase in the liver activity of several cAbVCAM1 nanobodies was observed, in accordance with the hepatic expression of VCAM1 that was evidenced in ApoE-/- animals in the present study and elsewhere.

Selection of Lead Compound

Based on the parameters summarized in Table 1, cAbVCAM1-5 was selected as the lead compound among 10 evaluated anti-VCAM1 nanobodies. Indeed, cAbVCAM1-5 exhibited the highest lesion-to-control and lesion-to-heart ratios, as well as a high lesion-to-blood ratio. In addition, cAbVCAM1-5 was cross-reactive with hVCAM1 with nanomolar affinities for both mVCAM1 and hVCAM1 as demonstrated by SPR and flow cytometry experiments, a highly relevant result when considering the future potential clinical evaluation of cAbVCAM1-5. Finally, cAbVCAM1-5 also displayed the highest heat resistance and production yield. The absence of lysine residue in the antigen-binding regions was also a criterion for cAbVCAM1-5 selection, since the presence of lysine could be a potential hurdle for future studies requesting coupling chemistry via amino residues, such as for fluorescent or radiolabeling for positron emission tomography (PET) imaging.

cAbVCAM1-5 In Vivo Imaging

99mTc-cAbVCAM1-5 was stable in vitro for up to 6 hours after radiolabeling as well as in vivo in mouse blood as demonstrated by HPLC, thereby allowing SPECT/CT imaging at 2–3 hours after injection. At this time point, atherosclerotic lesions located within the aortic arch of ApoE-/- mice were successfully identified by SPECT/CT imaging, with low myocardial and blood background activities. In vivo blocking experiments demonstrated the specificity of the uptake in VCAM1-expressing tissues. Autoradiography and immunohistochemistry further confirmed that 99mTc-cAbVCAM1-5 aortic uptake was focalized in VCAM1-positive atherosclerotic lesions. 99mTc-cAbVCAM1-5 is therefore a suitable radiotracer for the noninvasive in vivo imaging of inflammatory processes occurring in atherosclerotic lesions.

Comparison With Other Radiotracers

Other antibody-derived radiotracers have been evaluated recently for the imaging of vulnerable atherosclerotic plaques using SPECT. However, the slow blood clearance of full-sized antibodies resulted in suboptimal target-to-background ratios, therefore emphasizing the need to use antibody fragments (Fab, scFv) or engineered variants. Among the other radiotracers previously evaluated for SPECT or PET imaging of atherosclerotic lesions, 18FDG exhibited an elevated uptake in macrophages, thereby allowing in vivo imaging of carotid lesions in humans. However, due to high myocardial background, imaging of coronary lesions remains extremely challenging despite the potential use of a specific diet aimed at lowering myocardial uptake. Similarly, in a mouse model of atherosclerosis, Laitinen et al found that 18FDG myocardial uptake was 18.13 ± 10.59%ID/g in comparison to 0.41 ± 0.16%ID/g in atherosclerotic lesions at 1 hour after injection. 18F-4V, a VCAM1-targeting peptide-based tracer, has recently been evaluated for PET imaging of vulnerable lesions in mice. Interestingly and similarly to that observed in the present study, 18F-4V uptake was elevated in VCAM1-expressing tissues such as the lymph nodes and spleen and to a lower extent in the thymus (3.7 ± 0.3, 2.1 ± 0.6, and 0.9 ± 0.3%ID/g at 4 hours after injection, respectively); however, the potential specificity of this binding was not further discussed by the authors. Advantages of the 99mTc-labeled nanobody cAbVCAM1-5 over 18F-4V include a more than 40-fold higher affinity for mVCAM1, since the IC50 of 18F-4V was 86.6 nmol/L, whereas cAbVCAM1-5 kD was 2.0 ± 0.0 nmol/L. Of note, 18F-4V affinity for hVCAM-1 has not been reported yet.

99mTc-cAbVCAM1-5 also exhibited a lower uptake at 3 hours
after injection than \(^{18}\text{F}\)-F-4V at 4 hours after injection in background tissues such as the blood (0.5 ± 0.1 versus 1.5 ± 0.4%ID/g), myocardium (0.2 ± 0.0 versus 0.6 ± 0.2%ID/g), and control aorta (0.6 ± 0.1 versus 1.3 ± 0.4%ID/g), resulting in a more favorable lesion-to-control ratio in ApoE\(^{-/-}\) mice (4.95 ± 0.85 versus 3.12).

Limitations of Nanobody Methodology and Clinical Translatability

Immunization of a camelid with a target protein of interest probably remains a necessary step because nanobodies from naive or synthetic libraries are mostly of lower affinity. In addition, whereas the obtention of recombinant proteins corresponding to large extracellular domains of type I or II transmembrane receptors can be easily achieved, as is the case for VCAM1, the same does not hold for more complex structures such as heterodimeric receptors or receptors spanning the cellular membrane multiple times.

When produced as recombinant proteins, evident questions arise regarding immunogenicity, toxicity, and safety when nanobody-based tracers are designed for clinical translation. In this regard, it should be noted that several nanobodies, including an anti–von Willebrand Factor nanobody for prevention of the acute coronary syndromes, were already clinically evaluated in phase Ia, Ib, and II clinical trials without demonstrating adverse events and detectable immunogenicity at repeatedly administered therapeutic doses far above the single injected dose that will be used for diagnostic purposes as described in the present study.\(^{33}\) Also, we are currently running a “first-in man” phase I clinical study with a \(^{68}\text{Ga}\)-labeled anti-HER2 nanobody\(^{5}\) for PET imaging of breast cancer patients, in which efficacy, safety, and dosimetry will be the monitored parameters.
Conclusions and Perspectives

Unlike anatomic imaging methodologies, which are confronted with strong resolution requirements to distinguish distinct plaque components for the identification of vulnerable lesions based on the size of the necrotic core or the thickness of the fibrous cap, the challenges associated with atherosclerosis molecular imaging are strongly related to the sensitivity of the detection systems. Nuclear imaging presents an exquisite, femtomolar sensitivity that is well suited for the molecular imaging of atherosclerotic lesions. This study evaluated for the first time the potential of nanobodies as radiotracers dedicated to nuclear cardiology. When taken into perspective with previously published results related to tumor imaging, our results confirm that nanobodies constitute a promising new class of radiotracers with great potential for noninvasive imaging.

In the present study, 10 anti-VCAM1 nanobodies were evaluated. Procedures were carefully designed to generate nanobodies cross-reactive for hVCAM1 protein.99mTc-cAbVCAM1-5 was selected as the most potent candidate for the development of a new radiopharmaceutical for noninvasive imaging of vulnerable atherosclerotic lesions. In ApoE−/− atherosclerotic mice, VCAM1-positive lesions were successfully identified by SPECT/CT imaging using the human and mouse cross-reactive 99mTc-cAbVCAM1-5, thereby demonstrating a strong potential for clinical translation.

In addition to high production yield, high stability, and fast blood clearance, nanobodies engineering offers a number of advantages. Specifically, successful radiolabeling of nanobodies with a positron emitter was recently described,2 as well as labeling of nanobodies with near infrared dyes34 and coupling to microbubbles35 or Gadolinium vesicles,36 hereby allowing the use of SPECT, PET, optical, ultrasound, or MRI systems with nanobody-based tracers. In addition, humanized and bivalent nanobodies can easily be generated.4,37 In particular, further studies will be conducted to evaluate the performances of bivalent cAbVCAM1 constructs targeted at 2 distinct epitopes as well as the potential of 68Ga- or 18F-labeled cAbVCAM1-5 for PET imaging of atherosclerotic lesions.

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References
Nanobodies constitute a promising new class of radiotracers for molecular imaging of vulnerable plaques. A specific nanobody, cAbVCAM1-5, was selected because it binds in vitro and in vivo with high affinities and specificities to both mouse/human cross-reactive VCAM1-positive plaques in a mouse atherosclerosis model and human VCAM1-expressing cells. Following technetium-99m labeling, cAbVCAM1-5 successfully identified VCAM1-positive plaques in a mouse atherosclerosis model by noninvasive single photon emission computed tomographic (SPECT) imaging, thereby demonstrating promising characteristics for clinical translation.

**Novelty and Significance**

**What Is Known?**
- No clinical tool is available for the noninvasive detection of vulnerable atherosclerotic plaques.
- VCAM1 is a well-recognized biomarker of inflamed lesions, potentially allowing noninvasive detection via molecular imaging, providing a robust VCAM1-specific tracer is used.

**What New Information Does This Article Contribute?**
- A specific nanobody, cAbVCAM1-5, was selected because it binds in vitro and in vivo with high affinities and specificities to both mouse and human VCAM1-expressing cells.
- Following technetium-99m labeling, cAbVCAM1-5 successfully identified VCAM1-positive plaques in a mouse atherosclerosis model by noninvasive single photon emission computed tomographic (SPECT) imaging, thereby demonstrating promising characteristics for clinical translation.
- Nanobodies constitute a promising new class of radiotracers for cardiovascular molecular imaging.

Rupture of vulnerable atherosclerotic plaques is responsible for the majority of coronary events. However, no clinical tool is available for the detection of such lesions. Active inflammation is a major criterion distinguishing vulnerable from stable plaques. VCAM1 is a well-recognized biomarker of inflamed lesions, potentially allowing noninvasive detection via molecular imaging, providing a robust VCAM1-specific tracer is used. Nanobodies represent the smallest antibody-derived fragment that can be generated to bind to virtually any protein of interest. Nanobodies are a potential new class of radiotracers with rapid clearance and nanomolar affinities. In the present study, several mouse/human cross-reactive anti-VCAM1 nanobodies were generated and radiolabeled with 99mTc-technetium and were shown to bind in vitro and in vivo to VCAM1-positive cells with high affinities and specificities. cAbVCAM1-5 was selected as the most potent candidate. VCAM1-positive atherosclerotic lesions were successfully identified in APOE-deficient mice after SPECT imaging with 99mTc-cAbVCAM1-5. Therefore, this study constitutes the first application of the nanobody technology to the field of cardiovascular imaging. This new class of radiotracers might evolve into an important research tool for targeted imaging of atherosclerotic lesions and has the potential for early clinical translation.
Nanobodies Targeting Mouse/Human VCAM1 for the Nuclear Imaging of Atherosclerotic Lesions
Alexis Broisat, Sophie Hernot, Jakub Toczek, Jens De Vos, Laurent M. Riou, Sandrine Martin, Mitra Ahmadi, Nicole Thielens, Ulrich Wernery, Vicky Caveliers, Serge Muyldermans, Tony Lahoutte, Daniel Fagret, Catherine Ghezzi and Nick Devoogdt

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