Ultrasound Molecular Imaging of Atherosclerosis With Nanobodies
Translatable Microbubble Targeting Murine and Human VCAM (Vascular Cell Adhesion Molecule) 1

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OBJECTIVE: Contrast-enhanced ultrasound molecular imaging (CEUMI) of endothelial expression of VCAM (vascular cell adhesion molecule)-1 could improve risk stratification for atherosclerosis. The microbubble contrast agents developed for preclinical studies are not suitable for clinical translation. Our aim was to characterize and validate a microbubble contrast agent using a clinically translatable single-variable domain immunoglobulin (nanobody) ligand.

APPROACH AND RESULTS: Microbubble with a nanobody targeting VCAM-1 (MB_{cAbVcam1-5}) and microbubble with a control nanobody (MB_{VHH2E7}) were prepared and characterized in vitro. Attachment efficiency to VCAM-1 under continuous and pulsatile flow was investigated using activated murine endothelial cells. In vivo CEUMI of the aorta was performed in atherosclerotic double knockout and wild-type mice after injection of MB_{cAbVcam1-5} and MB_{VHH2E7}. Ex vivo CEUMI of human endarterectomy specimens was performed in a closed-loop circulation model. The surface density of the nanobody ligand was 3.5×10^5 per microbubble. Compared with MB_{VHH2E7}, MB_{cAbVcam1-5} showed increased attachment under continuous flow with increasing shear stress of 1-8 dynes/cm² while under pulsatile flow attachment occurred at higher shear stress. CEUMI in double knockout mice showed signal enhancement for MB_{cAbVcam1-5} in early (P=0.0003 versus MB_{VHH2E7}) and late atherosclerosis (P=0.007 versus MB_{VHH2E7}); in wild-type mice, there were no differences between MB_{cAbVcam1-5} and MB_{VHH2E7}. CEUMI in human endarterectomy specimens showed a 100% increase in signal for MB_{cAbVcam1-5} versus MB_{VHH2E7} (20.6±2.77 versus 9.6±14.7, P=0.0156).

CONCLUSIONS: CEUMI of the expression of VCAM-1 is feasible in murine models of atherosclerosis and on human tissue using a clinically translatable microbubble bearing a VCAM-1 targeted nanobody.

VISUAL OVERVIEW: An online visual overview is available for this article.

Key Words: atherosclerosis ■ inflammation ■ microbubble ■ molecular imaging ■ ultrasonography
development, and plays a role in plaque initiation and progression. Therefore, imaging techniques that detect the expression of VCAM-1 could be used to assess an individual's risk for future atherosclerosis-related events. In recent years, the feasibility of contrast-enhanced ultrasound molecular imaging (CEUMI) for detecting the expression of VCAM-1 using full-size antibodies has been shown in animal models. However, no clinical studies using this technique have been performed thus far. For clinical translation, biotin-streptavidin linking of ligands to the microbubble used in the aforementioned studies will need to be replaced as it could potentially lead to binding of endogenous biotin, and, more importantly, regarding full-size antibody ligands, there is concern about the use in humans both in terms of safety and costs.

Nanobodies or single domain antibodies are antibody fragments consisting of a single monomeric variable antibody domain derived from heavy-chain-only antibodies that are by nature present in cameldids. Nanobodies are the smallest possible (10 to 15 kDa) antibody-derived polypeptide structure that binds to a specific antigen. Nanobodies possess advantages over conventional antibodies for clinical applications. They lack an Fc region and therefore do not induce complement-triggered cytotoxicity nor bind to Fc receptors on immune and other type of cells. In addition, there is homology between camelid and human antibody heavy chains, which makes humanization of nanobodies unproblematic. In preclinical studies, nanobodies targeted to VCAM-1 have already been used for imaging of atherosclerotic lesions using single photon emission computed tomography or positron emission tomography imaging, and a GMP-produced variant is on track for testing in a phase I nuclear imaging study in patients. VCAM-1 targeting with nanobodies attached to microbubbles using biotin-streptavidin bridging has been recently accomplished in a mouse tumor model. In the current study, we, therefore, studied the use of a microbubble with maleimide-thiol conjugation of an anti–VCAM-1 nanobody to detect VCAM-1 expression in a mouse model of atherosclerosis and ex vivo in human endarterectomy specimens using noninvasive ultrasound imaging.

Nonstandard Abbreviations and Acronyms

| Abbreviation | Definition |
|--------------|------------|
| CEUMI        | contrast-enhanced ultrasound molecular imaging |
| DKO          | double knockout |
| MBAbVcam1-5  | microbubble with a nanobody targeting VCAM-1 |
| MBVHH2E7     | microbubble with a control nanobody |
| VCAM-1       | vascular cell adhesion molecule 1 |
| WT           | wild type |

Highlights

- Inflammation plays a key role in initiation and progression of atherosclerosis.
- Noninvasive detection of vascular inflammation could improve risk stratification.
- Ultrasound with a microbubble carrying anti–VCAM (vascular cell adhesion molecule)-1 nanobody detects murine vascular inflammation.
- This clinically translatable microbubble also detects VCAM-1 on human plaques.

MATERIAL AND METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Study Design

Microbubbles carrying VCAM-1 targeted nanobodies were characterized using in vitro fluorescence and flow-chamber studies. All animal experiments were performed in accordance with Swiss Federal Legislation and approved by the local Animal Care and Use Committee of the University Hospital of Basel and the ethics committee of the Veterinary Office of the Canton of Basel.

Thirty-two male mice (B6;129S-Ldlrtm1Her Apobtm2Sgy/J) referred to as double knockout (DKO) mice, underwent molecular imaging either at the age of 10 weeks (n=15) or 40 weeks (n=17). These mice are deficient in low-density lipoprotein receptor and apo B48. Deficiency in apo B100 is accomplished by introduction of a C-terminal cysteine thiol for covalent conjugation to a microbubble used in the aforementioned studies will need to be replaced as it could potentially lead to binding of endogenous biotin, and, more importantly, regarding full-size antibody ligands, there is concern about the use in humans both in terms of safety and costs.

All subjects gave written informed consent for participation.

Ligand and Microbubble Preparation

Nanobody with cross-reactivity for mouse and human VCAM-1 (cAbVcam1-5; QVQ, Utrecht, the Netherlands) and an iso-type control nanobody (VHH2E7) were modified to carry a C-terminal cysteine thiol for covalent conjugation to a maleimide anchor on the surface of lipid-shelled decafluorobutane microbubbles. The binding specificity of the nanobody to VCAM-1 has already been shown by in vivo competitive experiments, autoradiography, and immunohistochemistry.
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previously described. For flow-chamber studies, microbubbles (KB.7) were then prepared using biotin-streptavidin linkage as the biotinylated full-size monoclonal antibody to VCAM-1 (M/K2.7), previously used for molecular imaging. Microbubbles (MBmAbVcam1) carrying a biotin moiety instead of maleimide (1,2-distearoyl-sn-glycero-3-phosphoethanolaminecholine-N-[amino(polyethylene glycol)-3400]maleimide (0.14 mg/mL; Creative PEGWorks, Winston Salem) in a glass vial. The microbubbles were washed by flotation-centrifugation to remove lipids not incorporated into the microbubble shell. Targeted (microbubble with a nanobody targeting VCAM-1 [MBcAbVcam1-5]) and control microbubbles (microbubble with a control nanobody [MBVHH2E7]) were prepared by adding reduced cAbVcam1-5 and VH2E7 nanobodies respectively to the maleimide-functionized microbubbles. After incubation for 120 minutes, nonbound nanobodies were removed by flotation-centrifugation. For a comparison of the relative performance of a microbubble bearing a monoclonal antibody to VCAM-1 (M/K2.7), previously used for molecular imaging versus the microbubble carrying the fluorochrome labeled cAbVcam1-5. Microbubble concentration and size were measured by electrozone sensing (Multisizer III, Beckman- Coulter).

Nanobody Density on the Microbubble Surface
For assessing the amount of nanobody (cAbVcam1-5) required to saturate maleimide binding sites, microbubbles were incubated with increasing amounts of fluorescently labeled cAbVcam1-5 (1, 10, 20, 60, 100, 140 µg per 1×10⁶ microbubbles). After washing, the amount of fluorescence retained on the microbubble surface was measured with Accuri C6 flow cytometer (BD Biosciences). To quantify the site density of nanobodies on the microbubble surface after conjugation to maleimide, cAbVcam1-5 was fluorescently labeled using N-Hydroxysuccinimide-fluorescein (Thermo Fisher Scientific). This procedure resulted in a ratio of 2 fluorescein molecules per nanobody. Obtained using fluorescently labeled cAbVcam1-5 were destroyed by application of pressure. The concentration of fluorescein—cAbVcam1-5 was then measured using a Synergy H1 microplate reader (Biotek) and compared with a reference standard of known concentrations of fluorescently labeled cAbVcam1-5. Microbubble concentration and size measurements obtained before destruction were used to calculate ligand surface density and number of ligands conjugated per microbubble.

Flow-Chamber Experiments
Parallel plate flow-chamber adhesion assays using activated bEnd.3 (ATCC CRL2299) murine endothelial cells expressing VCAM-1 were performed. Fibronectin coated (45µg/mL) 35x10 mm culture dishes (Corning) were seeded with bEnd.3 murine endothelial cells. Cells were grown to confluence in DMEM (ATCC20-2002) containing 10% FBS and 1% penicillin/streptomycin. The bEnd.3 endothelial cells were activated with tumor necrosis factor-alpha (50 ng/mL) 240 min before flow-chamber experiments. The culture plates were mounted in an inverted position in a parallel plate flow chamber (Glycotech, Inc) with a 0.254 mm gasket thickness and a 2.5 mm channel width. MBcAbVcam1-5 or MBVHH2E7 (3×10⁶ /mL) were drawn through the flow chamber using a syringe pump (Genie plus, Kent Scientific). The speed of the syringe pump was set to obtain wall shear stresses of 1, 2, 4, and 8 dynes/cm² for adhesion studies. Binding of fluorescently labeled microbubbles was quantified on 20 randomly selected visual fields using a fluorescent microscope (Olympus BX51WI) and a ×40 magnification. In separate experiments on separate culture plates, binding of MBcAbVcam1-5 versus MBVHH2E7, microbubbles was assessed at 2 dynes/cm². Because aortic flow is pulsatile, adhesion at the highest shear rate of 8 dynes/cm² was also assessed after transient (5 s) reductions of shear to <0.5 dynes/cm². After maximal attachment of MBcAbVcam1-5 at lowest shear stress of 0 to 0.5 dynes/cm², the detachment characteristics of the microbubbles were determined by sequentially increasing the shear stress by 5 dynes/cm² up to a maximum of 43 dynes/cm².

Animal Instrumentation
For echocardiography and CEUMI, mice were anesthetized with inhaled isoflurane (1% to 1.5% in room air for maintenance), and body temperature was maintained at 37°C with a heating pad. The chest was deaired. For microbubble injections, the right internal jugular vein was cannulated (PE 50 tubing), and the animals were transferred onto a temperature-controlled imaging stage (Vevo Imaging Station). The heart rate was monitored.

Echocardiography
High-frequency (40 MHz, MS 550D transducer) ultrasound imaging (Vevo 2100, VisualSonics, Inc) was performed to assess the left ventricular ejection fraction, as well as aortic arch diameter and flow velocity. M-Mode images of the left ventricle in the parasternal short-axis plane at the midpapillary muscle level were used to measure left ventricular internal diameters at end-diastole and end-systole. Left ventricular ejection fraction was calculated using the cube formula (left ventricular ejection fraction (%)=100×[(left ventricular internal diameters at end-diastole− left ventricular internal diameters at end-systole) / left ventricular internal diameters at end-diastole]). Parasternal long-axis images of the aortic arch were used to measure internal diameter and the centerline peak systolic velocity in the aortic arch just distal to the brachiocephalic artery was measured by pulsed-wave doppler.

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CEUMI in Murine Atherosclerosis

CEUMI (Sequoia Acuson C512; Siemens Medical Systems) was done with a high-frequency linear-array probe (15L8) secured in place by a railed gantry system. The ascending aorta of the mice, including the sinus of Valsalva, and the takeoff of the brachiocephalic artery was imaged in a long-axis plane from a right parasternal window. Using a combination of power modulation and pulse inversion (Contrast Pulse Sequence), the contrast microbubbles were imaged at a transmission frequency of 7 MHz and a dynamic range of 50 dB. The gain settings were adjusted to levels just below noise speckle and maintained constant. MB<sub>AntiVan1-5</sub> and MB<sub>VHH2E7</sub> (1×10<sup>6</sup> microbubbles per injection) were injected intravenously in random order. Ultrasound imaging was paused from the beginning of microbubble injection for 8 minutes. Imaging was then resumed at a mechanical index of 0.87. The first acquired image frame was used to derive the total amount of microbubbles present within the aorta. The microbubbles in the ultrasound beam were then destroyed using several (>10) image frames at a mechanical index of 0.87. Several image frames (n=3) at a long-pulsing interval (10 s) were then captured to measure signal from freely circulating microbubbles. The video intensities were log-linear converted, and frames representing freely circulating microbubbles were averaged and subtracted digitally from the first image to derive signal from attached microbubbles alone. Contrast intensity was measured from a region of interest encompassing the sinus of Valsalva, the ascending aorta, and extending into the origin of the brachiocephalic artery. The selection of the region of interest was guided by fundamental frequency anatomic images of the ascending aorta and the aortic arch acquired at 14 MHz at the end of each individual imaging sequence.

Contrast-Enhanced Ultrasound in Human Endarterectomy Specimens

Endarterectomy specimens were obtained directly from the operating theater, stored in NaCl 0.9% at 4°C, and the imaging procedures were performed within 2 hours. The tissue was cut inside a polyethylene tubing (inner diameter 10 mm) with the endothelial layer facing inside and immobilized using reoxel adhesive (San Diego Plastics) applied on the abluminal surface resulting in a lumen diameter from 5.5 to 8 mm depending on the thickness of the endarterectomy specimen. The tubing was immersed into a water bath (37°C) and connected to a closed-circuit filled with pooled human serum (6 donors, blood group AB) obtained from the local blood bank. Inlet and outlet ports in the circuit were used for microbubble administration and removal. A peristatic pump (Masterflex, easy load II) was used to generate flow within the circuit (total circuit volume 25 mL, shear stress ≈3–9 dynes/cm<sup>2</sup>). A 15L8 probe immersed into the water bath was used to acquire B-mode images (14MHz) of the endarterectomy specimens (Figure I in the online-only Data Supplement for experimental setup). Boluses of 2.5×10<sup>7</sup> MB<sub>AntiVan1-5</sub> and MB<sub>VHH2E7</sub> were injected into the circuit in random order. Seven minutes after microbubble injection, freely circulating microbubbles were reduced by replacing the fluid volume in the circuit by injection of 25 mL of serum. Microbubble signal from attached and remaining circulating microbubbles was recorded using CPS imaging at 7 MHz with a mechanical index of 0.87. Attached microbubbles were destroyed with >10 frames (mechanical index of 0.87).

Signal from remaining circulating microbubbles was recorded at a 10-s pulsing interval. Signal from attached microbubbles was derived by subtracting 3 averaged postdestruction frames from the first image frame before microbubble destruction. Targeted signal was measured from a region of interest encompassing the tissue-fluid interface derived from 14MHz fundamental frequency images obtained at the end of each sequence.

Immunohistology

For excision of the thoracic aorta, a limited thoracotomy was performed under deep sedation. The blood volume was removed with direct cardiac puncture, and the aorta was perfused with 10% formalin. Connective tissue and fat were removed from the thoracic aorta and the ascending portion, and arch, including the takeoff of the arch vessels, was excised. Immersion fixation of the tissue in 4% paraformaldehyde was performed overnight. Tissues were subsequently embedded in paraffin and cut into 5-µm sections. Immunohistochemistry was performed to qualitatively assess the endothelial expression of VCAM-1 by using a rabbit monoclonal antibody to mouse VCAM-1 (ab134047; Abcam) or a matched isotype IgG control (ab172730; Abcam, Figure III in the online-only Data Supplement) at a 1.4 µg/mL concentration, and a goat anti-rabbit IgG-horseradish peroxidase as a secondary antibody (PO448, Dako). Before insertion of endarterectomy specimen into the closed-circuit, part of each tissue was set aside and snap-frozen in optimal cutting temperature medium by immersion in liquid nitrogen. Ten micrometer sections were stained with Masson trichrome and adjacent sections incubated with a rabbit polyclonal to human VCAM-1 (ab106777; Abcam) or a matched isotype IgG control (011-000-003, Jackson ImmunoResearch, Figure IV in the online-only Data Supplement) at a 5 µg/mL concentration and a goat anti-rabbit IgG-Alexa Fluor 546 as a secondary antibody (A-11010, Thermo Fisher Scientific) plus DAPI (4',6-diamidino-2-phenylindole dihydrochloride; D1306, Thermo Fisher Scientific). Microscopy imaging was performed with a Nikon Ti microscope.

Statistical Analysis

Data are expressed either as medians and 25th to 75th percentile or as mean±SD, as appropriate. For comparison of MB<sub>AntiVan1-5</sub> versus MB<sub>VHH2E7</sub> attachment in the flow-chamber experiments with continuous flow conditions, a Mann-Whitney U test was used. For evaluating the microbubble detachment characteristics and attachment under pulsatile flow across multiple measurements, Friedman ANOVA with Dunn multiple comparison post hoc test was used. For multiple comparison of CEUMI signals from MB<sub>AntiVan1-5</sub> and MB<sub>VHH2E7</sub> both within and between animal groups, a Kruskal-Wallis ANOVA with Dunn multiple comparison post hoc test was used. Microbubble signals from human endarterectomy specimens were compared using a Wilcoxon matched pairs signed rank test. Data were analyzed on GraphPad Prism (version 7). A P<0.05 (2-sided) was considered statistically significant.

RESULTS

Characterization of Targeted Microbubbles

The mean size of microbubbles used for characterization was 2.88±1.61 µm, the mean concentration 8.5×10<sup>7</sup>
microbubbles/mL. Flow-cytometry analysis demonstrated the optimal conjugation saturation of the microbubble surface with nanobodies (Figure 1A) to be reached at an incubation with 140 µg nanobody per 1×10⁸ microbubbles. At this concentration, the fluorescent spectroscopy calculations revealed 3.5×10⁵ cAbVcam1-5 molecules coupled to the microbubble surface which corresponded to ≈1 cAb-Vcam1-5 molecule per 74 nm² (8.6×8.6 nm) or 1.3×10⁴ cAbVcam1-5 molecules per µm² of the microbubble surface (Figure 1B) and a calculated coupling yield of 0.7%.

In Vitro Retention of Targeted Microbubbles to Mouse Endothelial Cells Under Flow Conditions

Compared with MB_{VHH2E7}, MB_{cAbVcam1-5} showed increased retention (Figure 2A) on activated murine endothelial cells expressing VCAM-1 under continuous flow conditions at different shear rates of 1 (P<0.005), 2 (P<0.005), 4 (P<0.05), and 8 (P<0.005) dynes/cm², with a gradual reduction of microbubble retention with increasing shear rate. Sequential brief reductions in shear rate resulted in a stepwise increase (P<0.0001) of the attachment of MB_{cAbVcam1-5} (Figure 2B). After adhesion at low shear stress, MB_{cAbVcam1-5} showed minimal detachment up to a shear rate of 25 dynes/cm², with increased detachment at 30 dynes/cm², and significant detachment compared with baseline reached after 35 dynes/cm² (P<0.05 versus baseline; Figure 2C). When compared with microbubbles carrying a monoclonal antibody to VCAM-1 (MB_{mAbVcam1}), retention of MB_{cAbVcam1-5} tended to be less; however, this difference was statistically nonsignificant (Figure 2 in the online-only Data Supplement).

In Vivo CEUMI of VCAM-1 Expression in the Mouse Aorta

Body weight and heart rate were not different between DKO and WT mice at age 10 and 40 weeks. Left ventricular ejection fraction was in the normal range in both DKO and WT mice at 10 and 40 weeks but was significantly lower by 7 to 8 percentage points in DKO mice. At 40 weeks of age, internal aortic diameters and peak aortic flow velocity were increased in DKO mice (Table). Microbubble preparations used for in vivo CEUMI had mean sizes of 2.63±1.34 µm for MB_{cAbVcam1-5} and 2.44±1.24 µm for MB_{VHH2E7} (P=0.2 for comparison of the 2 mean sizes); microbubble concentrations were 2.7×10⁸ and 2.5×10⁸ microbubble/mL, respectively. CEUMI showed selective signal enhancement for MB_{cAbVcam1-5} in DKO mice only, both in very early stages of atherosclerosis at age 10 weeks and in established atherosclerosis at age 40 weeks. Signal enhancement was

![Figure 1. Characterization of nanobody-targeted microbubbles.
A, Optimal conjugation concentration: Fluorescence intensity based on flow cytometry after incubation of 1×10⁸ microbubbles (MBs) at increasing concentrations (µg) of cAbVCAM1-5. Binding saturation was achieved at 140 µg. B, MB with a nanobody targeting VCAM-1 (MB_{cAbVcam1-5}) surface density. VCAM indicates vascular cell adhesion molecule.](image-url)
approximately 3-fold increased over MB$_{VHH2E7}$ control signal at 10 weeks, whereas at 40 weeks this ratio was ≈2-fold. In WT mice, there were no differences in signal between MB$_{cAbVcam1-5}$ and MB$_{VHH2E7}$ both at 10 and 40 weeks, but the signal tended to increase for both microbubbles at 40 weeks (Figures 3 and 4).

Histology of 40 weeks old DKO mice (Figure 5A) showed large atherosclerotic plaques and abundant expression of VCAM-1 both on the endothelial surface and in plaque macrophages compared with normal histology and low endothelial staining for VCAM-1 in WT mice (Figure 5B). At 10 weeks, DKO mice (Figure 5C) showed a moderate increase in endothelial expression of VCAM-1 in comparison to WT mice (Figure 5D).

**Figure 2.** In vitro attachment efficiency of microbubbles (MBs) to TNF-α (tumor necrosis factor-alpha) activated bEnd.3 mouse endothelial cells. 
A, Continuous flow conditions: MB with a nanobody targeting VCAM (vascular cell adhesion molecule)-1 (MB$_{cAbVcam1-5}$) vs MB with a control nanobody (MB$_{VHH2E7}$) at shear rates of 1, 2, 4, and 8 dynes/cm$^2$ (*$P<0.05$, **$P<0.005$; n=7). B, Pulsatile flow conditions: adhesion of MB$_{cAbVcam1-5}$ vs MB$_{VHH2E7}$ after transient (0.5 s) reductions (P1, P2, P3, and P4) of shear to <0.5 dynes/cm$^2$, (n=7). C, % Retention at high-shear stress: After attachment of MB$_{cAbVcam1-5}$ at shear stress of 0 to 0.5 dynes/cm$^2$, the detachment characteristics were determined by sequentially increasing the shear stress by 5 dynes/cm$^2$ up to a maximum of 43 dynes/ cm$^2$, (*$P<0.05$, **$P<0.01$, ***$P<0.001$ vs baseline; n=5). Data for (A), (B), and (C) are median value (horizontal line) and range of values (whiskers). For (A) and (C), the boxes represent 25% to 75% percentiles. D, Representative fluorescent microscopic images of a single optical field (×40) showing MB$_{cAbVcam1-5}$ (1) and MB$_{VHH2E7}$ (2) retention at 1 dynes/cm$^2$. Scale bar=20 µm.

**Table.** Physiological and Echocardiographic Data

|                | 10 wk | 40 wk | 10 wk | 40 wk |
|----------------|-------|-------|-------|-------|
|                | WT Mice | DKO Mice | WT Mice | DKO Mice |
| Body weight, g | 26.8±1.5 | 27.6±1.1 | 32.1±2.9 | 32.5±1.8 |
| Heart rate, bpm | 485±39 | 476±37 | 489±33 | 477±36 |
| Aortic PSV, m/s | 1.3±0.3 | 1.5±0.2 | 1.3±0.3 | 1.8±0.4* |
| Aortic ID, mm   | 1.44±0.1 | 1.50±0.1 | 1.57±0.1 | 1.72±0.1† |
| LVEF, %         | 72±6 | 65±8# | 70±5 | 62±6* |

Values are means± SD. DKO indicates double knockout; ID, internal diameter; LVEF, left ventricular ejection fraction; PSV, peak systolic velocity; and WT, wild-type.

* $P<0.005$ vs 40-wk WT mice.
† $P<0.0001$ vs 40-wk WT mice.
‡ $P<0.05$ vs 10-wk WT mice.

**Ex Vivo CEUMI of VCAM-1 Expression in Endarterectomy Specimens**

Imaging was performed in a total of 7 specimens: 5 from carotid endarterectomy and 2 from femoral endarterectomy. Overall, there was a statistically significant 100% signal increase, $P=0.0156$ for MB$_{cAbVcam1-5}$ as compared to MB$_{VHH2E7}$, from microbubble attachment to the endothelial surface of the human endarterectomy specimens.
In all specimens, retained microbubbles could be appreciated visually as a linear contrast enhancement on the endothelium (Figure 6B). There were large differences in signal obtained between specimens; however, as shown in Table I in the online-only Data Supplement, in every single tissue, signal for MBcAbVcam1-5 was larger compared to MBVHH2E7. Immunohistology of the endarterectomy specimens showed atherosclerotic plaques and expression of VCAM-1 both on the endothelial surface as well as in plaque macrophages (Figure 6C).

**DISCUSSION**

In this study, we have characterized a microbubble contrast agent targeted to VCAM-1 with a single-variable domain immunoglobulin (nanobody) that is feasible for human use. We show that this microbubble detects the expression of VCAM-1 in murine models of both early and established atherosclerosis. In addition, we show that the same agent can be used to detect the expression of VCAM-1 on human endarterectomy specimens with ultrasound imaging (Figure 6).

Nanobodies possess several advantages over full-size antibodies for human use. This includes easy large-scale production in prokaryotic and eukaryotic hosts, lack of Fc, amenability for site-directed immobilization, and a high degree of sequence homology (>80%) with human variable heavy chain region antibodies. In fact, caplacizumab, a nanobody targeting von Willebrand factor, has recently received clinical approval for treatment of adults

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**Figure 3.** Background-subtracted contrast-enhanced ultrasound molecular imaging signal intensity from the aortic arch after injection of microbubble (MB) with a nanobody targeting VCAM (vascular cell adhesion molecule)-1 (MBcAbVcam1-5) and MB with a control nanobody (MBVHH2E7) MBs in double knockout (DKO) and wild-type (WT) mice at (A) age 10 wk (n=15 each) and (B) age 40 wk (n=17 each).

Data show median values (horizontal line), 25% to 75% percentiles (box), and range of values (whiskers). Signal enhancement for VCAM-1 between DKO MBcAbVcam1-5 and DKO MBVHH2E7 group at age 10 ($\#P<0.001$) and 40 wk ($\dagger P<0.005$); DKO MBcAbVcam1-5 and WT MBcAbVcam1-5 group at age 10 ($\dagger P<0.005$) and 40 wk ($\dagger P<0.05$). AU indicates acoustic intensity.

**Figure 4.** Representative color-coded contrast-enhanced ultrasound (CEU) molecular imaging images (after correction of signal from freely circulating microbubbles) overlaid on anatomic images from the aortic arch after injection of microbubble with a nanobody targeting VCAM (vascular cell adhesion molecule)-1 (MBcAbVcam1-5; top row) and microbubble with a control nanobody (MBVHH2E7; bottom row) microbubbles in a 10-week and 40-week-old double knockout (DKO) and wild-type (WT) mouse.

The color scale (with acoustic intensity values in white) for the CEU images is shown at the bottom of each frame.
experiencing an episode of autoimmune thrombotic thrombocytopenic purpura. Successful in vivo targeting of nanobody-contrast agents to VCAM-1 has been shown either in low-shear stress conditions or using small molecule tracers subject to less drag forces compared with microbubbles. Targeting of microbubbles to disease markers in large arteries needs to rely on high targeting efficiency with fast on-rates and low off-rates of the ligand used. We demonstrate that using maleimide coupling, $3.5 \times 10^5$ nanobodies are attached to a microbubble, which is comparable to other conjugation strategies, and well above thresholds that negatively impact targeting efficiency. In our flow-chamber experiments, retention of microbubbles decreased as continuous shear stress increased. However, brief reductions in shear stress resulted in sequential increases in targeted microbubble retention (fast on-rate), and firmly attached microbubbles were able to withstand shear forces up to 25 to 30 dynes/cm$^2$ that are within the range of peak wall shear stress values encountered in large human arteries (low off-rate), indicating that the cAbVcam1-5 nanobody is feasible for VCAM-1 targeting in pulsatile high shear stress. This translated to robust signal from MB$cAbVcam1-5$ when noninvasively imaging the aorta in mouse models of early and late atherosclerosis, while signal was no different from control in WT animals. Interestingly, the targeted signal to control signal ratio was higher in early versus late atherosclerosis, which could potentially be explained by higher background signal from nonspecific attachment of MB$VHH2E7$ to leukocytes.

Molecular imaging of VCAM-1 in humans could cover several unmet clinical needs. This includes risk assessment in individuals that are classified as having an intermediate risk by traditional risk markers and assessment of plaque vulnerability in patients with established atherosclerotic disease. The rationale for selecting VCAM-1 for

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**Figure 5.** Representative images of VCAM (vascular cell adhesion molecule)-1 staining by immunohistochemistry of the thoracic aorta.

A, Double knockout (DKO) mouse (age 40 wk) showing large atherosclerotic plaques and abundant expression of VCAM-1 both on the luminal endothelial surface as well as in plaque macrophages; (B) wild-type (WT) mouse (age 40 wk) showing normal histology and minimal endothelial staining for VCAM-1; (C) 10-week-old DKO mouse showing minimal atherosclerotic changes and moderate endothelial expression of VCAM-1 as compared to WT mouse (D).
detection and risk stratification during the initial disease process is based on evidence from preclinical studies implicating VCAM-1 in very early endothelial inflammatory activation and contribution to leukocyte recruitment to the vascular wall. As a consequence, tracers for imaging the expression of VCAM-1 have been developed for magnetic resonance and nuclear imaging, as well as for ultrasound. The novelty reported in our study is the use of an ultrasound contrast agent for VCAM-1 imaging that is fully clinically translatable which has been validated for use in high-shear conditions, and has been tested on human tissue under flow conditions. Using endarterectomy specimens, we show that MBcAbVcam1-5 can be used to detect the expression of VCAM-1 present on plaques from human tissue under continuous shear conditions that are well above diastolic wall shear stress values of around 6 dynes/cm² and close to the peak systolic shear stress reported in one study, whereas another study reported higher values for peak systolic shear stress. The results from our flow-chamber studies with incremental attachment during pulsatile flow and retention up to shear forces of 25 to 30 dynes/cm² together with successful targeting of VCAM-1 in the murine aorta indicate that in vivo imaging of VCAM-1 expression in humans will be feasible. However, the incremental value of molecular imaging will depend on whether inflammatory activity in a reference vessel correlates with atherosclerotic burden in vessels of interest, such as the coronary arteries. Data from large animal models show that the endothelial expression of VCAM-1 in carotid arteries correlates with plaque burden in the coronary arteries and that VCAM-1 in that respect...
outperforms measurement of carotid intima-media thickness or traditional risk factors, such as blood glucose and cholesterol values. Cross-sectional studies in humans have shown that soluble VCAM-1 measured in the serum predicts the presence of coronary artery disease in symptomatic patients referred for coronary angiography, and that values for soluble VCAM-1 are particularly elevated in patients with high levels of pro-inflammatory low-density lipoprotein triglycerides.

Another clinical area where noninvasive assessment of VCAM-1 expression could be valuable is in assessment of plaque vulnerability. According to current clinical guidelines, treatment of carotid artery stenosis with endarterectomy is recommended in patients with recent focal neurological symptoms and in asymptomatic patients with >70% stenosis. The benefit of surgery is, however, much reduced in asymptomatic patients where carotid artery stenosis carries an annual stroke risk of only 0.5% to 1%. Further risk stratification in this population could be of value. In that respect, recent data show increased endothelial levels of VCAM-1 on histology from high-risk versus low-risk carotid endarterectomy specimens, suggesting that noninvasive imaging of VCAM-1 expression may add incremental prognostic value in patients with asymptomatic carotid artery stenosis. In addition, intraplaque neovessels and neovessels inflammation are markers of plaque vulnerability and may contribute to increased signal upon in vivo imaging.

Several limitations of our study deserve mentioning. First, the coupling yield of the cAbVcam1-5 nanobody to the microbubbles was very low, and thus a large amount of the nanobody was lost during microbubble preparation. However, by using flow-cytometry data (Figure 1) to select an incubation with 140 µg per 1×10^8 microbubble, we traded a lower coupling yield for a somewhat higher surface density. Figure 1 shows that using lower amounts of nanobody for incubation would not have dramatically decreased saturation, but would have resulted in a better coupling yield. Also, despite >90% desalting of the nanobody after the reduction step, remaining 2-mercaptoethylamine could have had an impact on the coupling yield. Thus, for translation purposes, microbubble preparation protocols that minimize loss of nanobody while retaining targeting efficiency will have to be developed. Second, the animal model that we used is representative for early and late stages of stable atherosclerotic disease. However, vulnerable plaques do not develop in this animal model, and therefore, studies in suitable animal models will be necessary. Third, we evaluated the targeting efficiency of MB_cAbVcam1-5 versus MB_VHH2E7 in WT animals. In the closed-loop model, we used pooled human serum to closely mimic in vivo conditions; however, limited availability of serum allowed for replacing the serum inside the system only once. Thus, transient attachment of microbubbles to the conduit tubing resulted in a relatively large concentration of remaining circulating microbubbles, which may have mitigated the targeted signal enhancement to some degree. Last, the technical setup of the closed-loop cardiovascular circulation model for CEUMI on human endarterectomy specimens did not allow for creation of pulsatile flow. However, the continuous shear stress of 3 to 9 dynes/cm^2 used in the model is well above reported diastolic shear stress values in human carotid arteries and we would, therefore, not expect a negative effect on targeting efficiency in vivo.

In summary, we have characterized a targeted contrast agent using a nanobody with maleimide covalent binding to the microbubble surface for detection of VCAM-1 in large arteries. Using this contrast agent, vascular inflammation during early and late stages of stable atherosclerosis can be diagnosed with noninvasive imaging in animal models. Importantly, we show that this contrast agent allows detection of VCAM-1 on human tissue with ultrasound imaging. These findings pave the way for clinical translation of CEUMI to detect early pathophysiological changes and improve risk stratification for atherosclerotic complications.

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