In Vivo Fluorescence-mediated Tomography Imaging Demonstrates Atorvastatin-mediated Reduction of Lesion Macrophages in ApoE-/- Mice

Jan Larmann, M.D., Ph.D., Tim Frenzel, M.D., Martina Schmitz, Ph.D.,
Anke Hahnenkamp, Ph.D., Philipp Demmer, M.Sc., Stephan Immenschuh, M.D.,
Uwe J.F. Tietge, M.D., Christoph Bremer, M.D., Gregor Theilmeier, M.D.,

ABSTRACT

Background: Macrophage recruitment into atherosclerotic plaques drives lesion progression, destabilization, and rupture. Chronic statin treatment reduces macrophage plaque content. Information on dynamics of macrophage recruitment would help assessing plaque vulnerability and guiding therapy. Techniques to image macrophage homing to vulnerable plaques in vivo are scarcely available. The authors tested if noninvasive fluorescence-mediated tomography (FMT) can assess plaque-stabilizing effects of short-term high-dose atorvastatin.

Methods: Macrophages from green-fluorescent-protein-transgenic mice were labeled with a near-infrared fluorescent dye and were injected IV in apolipoprotein E-deficient mice (n = 9) on Western diet 7 days after guidewire-injury of the carotid artery. FMT-scans, 2 and 7 days thereafter, quantified macrophage recruitment into carotid artery plaques. Atorvastatin was tested for macrophage adhesion, proliferation, and viability (n = 5 to 6) in vitro. Fourteen mice received atorvastatin or vehicle for 4 days after 16 weeks on Western diet. FMT assessed macrophage recruitment into aortic and innominate artery lesions. Means (±SD)% are reported.

Results: Double-labeled macrophages were recruited into carotid artery lesions. FMT resolved fluorescence projecting to vulnerable plaques in vivo. The results suggested that short-term statin treatment may produce favorable effects to stabilize atherosclerotic plaques and decrease cardiovascular risk.

What We Already Know about This Topic

- Statins have been shown to decrease perioperative cardiovascular events, but the mechanisms responsible for the beneficial effects of statins have been incompletely elucidated.

What This Article Tells Us That Is New

- Acute statin treatment decreased macrophage infiltration of atherosclerotic lesions, as visualized with in vivo fluorescence-mediated tomography, in mice. The results suggested that short-term statin treatment may produce favorable effects to stabilize atherosclerotic plaques and decrease cardiovascular risk.

Copyright © 2013, the American Society of Anesthesiologists, Inc. Lippincott Williams & Wilkins. Anesthesiology 2013; 119:129-41
recruitment demonstrated rapid plaque stabilization by 4-day atorvastatin treatment in apolipoprotein E-deficient mice.

Annually approximately 10% of the population in industrialized countries undergo major surgical procedures. It is estimated that more than a third of all Americans suffer from some kind of cardiovascular disease. In those patients with coronary artery disease vulnerable plaques, characterized by macrophage accumulation, can rupture due to perioperative stress and cause fatal perioperative myocardial infarction or stroke. Scores to predict perioperative cardiovascular events largely rely on clinical history and have moderate performance. Morbidity and mortality of perioperative cardiovascular events are high, preventive strategies are scarce, and their efficacy is debatable. Moreover, compared with the ambulatory setting, treatment options for perioperative events are rather limited.

During atherosclerosis, macrophages are recruited into the vessel wall. Macrophage content correlates with plaque vulnerability and plays a causative role for rupture of thin-cap fibroatheromas because macrophages can destabilize the fibrotic cap by secreting matrix-degrading enzymes.

3-hydroxy-3-methylglutaryl-coenzyme-A-reductase (HMG-CoA) reductase inhibitors, with their principal effect of lowering cholesterol in apoB-containing lipoproteins, are well-established for long-term plaque stabilization in ambulatory patients. Chronic statin treatment protects perioperative patients from cardiovascular events and reduces mortality, which is likely due to their effect on hyperlipidemia. More recently, it has been demonstrated that statin treatment as short as 30 days before surgery also prevents perioperative cardiovascular events.

It seems unlikely that a short-term lowering of lipid could have such strong effects on plaques. However, statins exert a multitude of pleiotropic effects, some of which are antiinflammatory and result in reduced macrophage accumulation.

In vivo data shedding light on short-term plaque stabilization as a potential mechanism that could explain how statins reduce perioperative cardiovascular events are not available, partly because of the lack of noninvasive diagnostic techniques to intrindividually (1) assess plaque extent and vulnerability, and (2) directly monitor the efficacy of plaque-stabilizing therapies.

Optical imaging of near-infrared fluorescent signatures offers high sensitivity due to excellent tissue penetration and low autofluorescence, facilitating detection of cellular and molecular events in vivo. Tomographic imaging approaches such as FMT aim at the in vivo acquisition of quantitative three-dimensional, deep tissue data sets of fluorescence signals.

We previously developed a FMT-based method for noninvasive spatio-temporal quantitative imaging of macrophage recruitment to sites of inflammation. This technique was used here for noninvasive quantification of macrophage migration into vascular atheromas. Trafficking of near-infrared-labeled exogenous macrophages was quantified in two distinct apolipoprotein E-deficient (ApoE-/-) mouse models. First, we used guidewire-injury (GWI)-induced carotid artery plaques that can be imaged with limited background and without technical obstacles to establish FMT plaque imaging. Second, we investigated clinically more relevant diet-induced, spontaneous complex atherosclerotic plaques in the aorta and brachiocephalic artery. Effects of high-dosage atorvastatin on macrophage proliferation, viability, and adhesion to endothelial cells were tested in vitro. We went on to demonstrate that FMT can detect reduced macrophage recruitment in mice receiving atorvastatin for 4 days, emphasizing the potent short-term plaque-stabilizing effects of HMG-CoA reductase inhibitors.

Materials and Methods

Materials

Cell culture materials were purchased from PAA (Coelbe, Germany); 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide (DiR; Invitrogen, Karlsruhe, Germany) was dissolved in 99% ethanol to 10 mg/ml and stored at 4°C until use at 20 µg/ml. Unless otherwise noted, all other reagents are from Sigma, Munich, Germany.

Cell Harvesting and DiR-staining of Murine Macrophages

Peritoneal macrophages from enhanced green-fluorescent-protein–(eGFP) transgenic mice (eGFP-tg/C57/Bl6; Jackson Laboratories, Bar Harbor, ME) were harvested and labeled with the lipophilic near-infrared fluorescent dye DiR, as previously described. In brief, day-3 thioglycolate-elicited peritoneal macrophages were resuspended at 10^6/ml in Hanks Buffered Salt Solution, incubated with DiR for 15 min at room temperature, and washed twice in Hanks Buffered Salt Solution. Successful eGFP/DiR double labeling was proven by fluorescence microscopy (Eclipse TE300; Nikon, Düsseldorf, Germany) and cell viability was assessed in a flow cytometry assay (FACSA; Partec, Muenster, Germany) by propidium iodine staining (Sigma; 1 µl [0.1%]/1,000 µl cell suspension). These eGFP/DiR double-labeled cells were used for IV injections, and served as exogenous indicator macrophages, to study macrophage recruitment in vivo in two distinct mouse models for atherosclerotic lesion formation.

Oxidized Low-density Lipoproteins Preparation

Low-density lipoproteins (LDL) were prepared from freshly frozen plasma of healthy volunteers by density gradient ultracentrifugation, in the presence of 200 mU/ml butylated hydroxytoluene (Sigma, Taufkirchen, Germany), and 0.1% EDTA (Roth, Karlsruhe, Germany). LDL was dialyzed against iso-tonic sodium chloride solution, containing 0.1% EDTA and 20 µU butylated hydroxytoluene, in the dark at 4°C overnight, stored at 4°C under nitrogen atmosphere, and used within 72 h. Oxidized LDL (oxLDL) was prepared by the same procedure.
in the absence of EDTA and butylated hydroxytoluene. LDL oxidation was induced by the addition of 5 μM CuSO₄.

**Cell Culture**

To examine effects on macrophage adhesive function, *in vitro* endothelial cell adhesion assays were carried out. Immortalized murine endothelial cells (f.End5) were cultured in Dulbecco Modified Eagle Medium supplemented with 10% fetal bovine serum, glutamin, and penicillin/streptomycin. Confluent serum-starved f.End5 were activated for 12 h, with 10 μg/ml oxLDL or native LDL. oxLDL-activated endothelial cells were cultured in the presence of 1 μM atorvastatin or its vehicle dimethylsulfoxide (diluted 1:1,500 in Dulbecco Modified Eagle Medium). Peritoneal macrophages were subjected to 4 h of plastic adhesion to enrich macrophage concentration. macrophages were then allowed to adhere to endothelium on a rocking plate (20/min) for 30 min before decanting and two buffer washes. Adhering cells were counted in 15 preselected high power fields by a blinded investigator utilizing an IX81 fluorescence microscope (Olympus, Hamburg, Germany). Long-term statin treatment prevents macrophage proliferation. Also, effects on viability are controversial. Therefore, eGFP-macrophages were seeded in low-attachment six-well plates (Corning, Amsterdam, The Netherlands) at 5 x 10⁵ cells per well for viability studies. For proliferation experiments 2 x 10⁵ macrophages per well were used. Cells were cultured using Roswell Park Memorial Institute medium (Lonza, Verviers, Belgium) supplemented with 10% fetal bovine serum, glutamine, and penicillin/streptomycin. Macrophages were treated with 1 or 10 μM atorvastatin or vehicle. Twenty-five percent of the media was carefully replaced every second day. Propidium iodine staining and subsequent flow cytometry analysis were utilized to quantify viable cells at days 1, 2, 4, and 7. In separate experiments total cell number per well (trypan blue-negative cells) as a surrogate marker for proliferation was assessed at the same time points, using a Neubauer chamber.

**Animals**

Animal studies were approved by the animal care committee of the University of Muenster (Muenster, Germany). Animals were handled according to the Guide for the Care and Use of Laboratory Animals published by the National Academy of Science. ApoE⁻/⁻ mice (Jackson Laboratories) were fed a Western diet containing 1.25% cholesterol (Nr. 1324; Altromin, Lage, Germany). Nine mice were subjected to accelerated carotid artery atherosclerosis induced by GWI. Animals underwent GWI-induced endothelial denudation after 3 weeks on diet by introducing a 21-gauge guide wire (Arrow International, Reading, PA) thrice from the left external carotid artery into the common carotid artery, as previously described. The right carotid artery was likewise dissected but not treated with GWI to serve as sham-control. We used mice that were only anesthetized and shaved (n = 4), to control for macrophage recruitment caused by the surgical trauma. Seven days after GWI of the left carotid artery, mice received 10⁷ eGFP/Dir indicator macrophages in 300 μl Hanks Buffered Salt Solution IV for optical imaging studies, as outlined below.

Subsequently, a second, nonsurgical model, which closely resembles clinically relevant atherosclerotic disease, was implemented to confirm findings for macrophage recruitment and test therapeutic interventions. Complex atherosclerotic lesions in the brachiocephalic artery and the aortic sinus were induced by feeding a high-cholesterol diet. Kinetics of macrophage recruitment in this model were compared with nonatherosclerotic C57Bl6 mice on normal chow (n = 4). As a proof-of-concept intervention that relates to clinical practice, a group of 14 ApoE⁻/⁻ mice received the cholesterol-rich diet for 16 weeks and subsequently, treated for 4 days with vehicle or 80 mg/kg body weight atorvastatin (Pfizer, Karlsruhe, Germany). In mice, this dosage can effectively lower cholesterol and increase high-density lipoprotein levels, when given for several weeks. To account for the relatively short plasma half-life of atorvastatin observed in mice, and to limit gavage volume, atorvastatin was administered in two dosages of 40 mg/kg body weight via an 18-gauge gastric tube. The diet was continued until the end of the respective experiment in all animals. Mice were sacrificed by an overdose of pentobarbital, and a 24-gauge catheter was placed in the left ventricle for perfusion at physiological pressure (70 mmHg) with saline (0.9%). Carotid arteries, aortic arch, and brachiocephalic artery were excised and embedded for *ex vivo* analysis.

**In vivo FMT**

For FMT measurements, mice were anesthetized with 60 μg/g body weight pentobarbital intraperitoneally. Animals were shaved and placed in the small-animal FMT system (VisEn Medical Inc., Woburn, MA), as described in detail elsewhere. Mice were situated in index-matching fluid warmed to 37°C, which is used to improve sensor contact and signal recovery during FMT scans. The excitation wavelength was tuned to 745 nm operating in the range of 5 to 150 mW. Optical signals were detected by a thermoelectrically-cooled charge-coupled device camera, using appropriate band pass filters (755 nm for excitation and 770 nm for emission measurements). After GWI, animals were scanned with the FMT system at 2 and 7 days after IV injection of indicator macrophages. Sequential coronal images of the cervical region were collected throughout the three-dimensional region of interest (ROI) at the site of plaque formation and the contralateral control. For quantitative analysis of eGFP/Dir macrophages recruited into carotid artery lesions, fluorochrome concentrations were measured and were reported as mean fluorescence intensity (nm) ± SEM. To assess effects of acute atorvastatin treatment on *de novo* macrophage recruitment into established complex brachiocephalic artery and aortic arch lesions, FMT baseline scans were performed in mice after 16 weeks.
on Western diet and 2 days postinjection of 10^7 eGFP/DiR macrophages. Afterwards, atorvastatin treatment was initiated. Subsequent FMT scans for the assessment of alterations in macrophage recruitment were performed on days 2 and 4. A ROI was placed in the thoracic region covering aortic sinus, aortic arch, and brachiocephalic artery. Fluorochrome concentrations in the ROI were measured and were reported as percent of pretreatment baseline values (% ± SEM for each group.

**Ex vivo Fluorescence-reflectance Imaging (FRI)**

Injured and sham-operated carotid arteries were placed on the multichannel fluorescence-reflectance small-animal imager In-Vivo FX Pro Imaging System (Kodak Molecular Imaging Systems, New Haven, CT). The system is equipped to provide multichannel fluorescence imaging. A 150 W halogen illuminator provides fluorescence. Appropriate filters for DiR imaging were selected (excitation 720 ± 18 nm and emission 790 ± 17.5 nm). Fluorescence is captured with a 4 million pixel-cooled charge-coupled device camera equipped with a ×10 zoom lens. Image acquisition times were 3 s per animal. ROIs were selected and analyzed with the Kodak MI 4.0 software. Near-infrared fluorescence data were collected as mean peak fluorescence intensities from manually drawn ROIs and background. The target-to-background ratio was calculated (target-to-background-ratio = mean peak fluorescence intensities_{plaque}/mean peak fluorescence intensities_{background}).

**Morphometric Analyses**

Carotid and brachiocephalic arteries were cryoembedded and stored at −80°C. 7-µm serial sections were stained with 4′,6-diamidino-2-phenylindol (1 µg/ml, 5 min) and photomicrographs of the eGFP, DiR, and 4′,6-diamidino-2-phenylindol signatures were taken, using a charge-coupled device camera equipped with a near-infrared emission filter (AHF, Tübingen, Germany). eGFP/DiR cells were counted on sections every 70 µm throughout the plaque. The total amount of calculated cells per lesion is displayed. Adjacent sections were stained with hematoxylin and eosin. Lesion size, quantified on hematoxylin and eosin stainings, is expressed as absolute area (mm²). Sections prepared for immunohistochemistry were stained with 4′,6-diamidino-2-phenylindol (1 µg/ml, 5 min) and stored at −80°C. 7-ultracentrifugation. The cholesterol content within different lipoprotein subclasses from individual plasma samples was determined after sequential tabletop ultracentrifugation, as published.43 Cholesterol concentrations within each fraction were measured, as detailed above.

**Lipoprotein Profiles**

Plasma samples were collected from the infrarenal aorta and total cholesterol, triglycerides (Roche Molecular Biochemicals, Mannheim, Germany), and phospholipids (Wako, Neuss, Germany) were determined enzymatically, using commercially available kits. The cholesterol content within different lipoprotein subclasses from individual plasma samples was determined after sequential tabletop ultracentrifugation, as published.43 Cholesterol concentrations within each fraction were measured, as detailed above.

**Statistical Methods**

Data were analyzed using GraphPad Prism 5.0b (GraphPad Software, San Diego, CA). Kolmogorov-Smirnov test was used to test for Gaussian distribution. Unpaired Student’s t test was used for comparisons between two groups if values showed Gaussian distribution. Mann–Whitney U test was used if values did not follow Gaussian distribution. All hypotheses tested were two-tailed. After proving Gaussian distribution, One-way ANOVA followed by Bonferroni multiple comparisons test was used for multiple group comparisons. Kruskal–Wallis test with Dunn posttest were used for multiple comparisons, when data did not follow Gaussian distribution. Two-way ANOVA followed by Bonferroni test was used to compare effects of different atorvastatin concentrations to vehicle over time in *in vitro* assays. GraphPad Prism 6.0 (GraphPad Software) was used to calculate exact P values if multiple group comparisons did not reveal statistical significant differences between groups. Data are presented as mean ± SD. P value less than 0.05 was considered significant.

**Results**

**Recruitment of Exogenous eGFP/DiR-labeled Macrophages to Carotid Artery Lesions Can Be Visualized by FMT-Imaging**

The left carotid artery GWI model allows the study of rapidly developing atherosclerotic plaques in ApoE−/− mice. Compared with other models, GWI-induced plaques comprise an excess of macrophages.44 In our hands, macrophage-rich lesions developed within 2 weeks after GWI of the left carotid artery (fig. 1A), allowing to study of macrophage recruitment during a defined period of time. Thioglycollate-elicited peritoneal macrophages carrying the eGFP/DiR double label were identified by fluorescence microscopy in atherosclerotic lesions of the left carotid artery 7 days after
IV injection (fig. 1B), indicating effective recruitment during GWI-induced atheroma formation.

In the GWI model, the ROI is distant from other organs, in which circulating macrophages may accumulate and each animal’s right carotid artery can serve as a control. Thus, we used this model to test whether FMT can principally detect macrophage recruitment in vivo. FMT reconstructions of three-dimensional data sets in frontal, sagittal, and transversal slices uncovered recruitment of IV-injected exogenous DiR-macrophages into vascular atheromas in vivo (fig. 2, A–D). The recorded near-infrared fluorescence signal in the cervical region of mice, which received DiR-macrophages but had no GWI (control) was low, did not show differences between left and right carotid artery, and did not change over time (fig. 2E). GWI of the left carotid artery resulted in a statistically significant increase of macrophage accumulation 2 and 7 days postmacrophage injection compared with sham-operated contralateral carotids (fig. 2F). The near-infrared fluorescence signal intensity did not change between days 2 and 7. To further confirm that the near-infrared fluorescence signals corresponded to macrophages located inside carotid artery atheromas, we subjected explanted carotid arteries to two-dimensional FRI.

**FRI Detects DiR Macrophages Inside Atheromas**

Carotid arteries were excised, surrounding tissue was removed, and vessels were imaged ex vivo by two-dimensional FRI (fig. 3A). The near-infrared fluorescence signal in carotid arteries from control animals was low, even 7 days postmacrophage injection (fig. 3B), indicating that macrophage accumulation in carotids can be neglected when the endothelium is not denuded by GWI. The target-to-background ratio in control carotids was close to 1, and comparable with values measured in sham-operated animals, demonstrating that spontaneous carotid plaques do not develop at this age or duration of diet. Finally, FRI confirmed increased recruitment of DiR-labeled macrophages into carotid arteries subjected to GWI compared with sham animals at day 7 postsurgery (fig. 3C), demonstrating that FMT reliably detects macrophage trafficking into rapidly developing small-vascular plaques in vivo. As spontaneous development of atherosclerosis is more relevant for the translation to clinical applications, and for studying the influence of therapeutic interventions on macrophage recruitment as a potential plaque-stabilizing mechanism, we also explored FMT-imaging in a mouse model of diet-induced spontaneous atherosclerosis.

**Atorvastatin Prevents Macrophage Adhesion to oxLDL-activated Endothelial Cells In Vitro**

oxLDL activation of endothelial cells promoted macrophage adhesion to the endothelium, whereas atorvastatin was capable to prevent this effect (fig. 4A). In culture, the amount of nonviable macrophages increased over time. At day 4, approximately 85% of cells were still viable. At day 7, we found approximately 74% of macrophages to be negative for propidium iodine, with no difference between groups (fig. 4B). As viability was not affected by either dosage of atorvastatin, the number of cells in culture could be used as a surrogate marker for proliferation. The overall number of cells per well increased over time. After 4 days, we found a slightly lower cell count for both the atorvastatin groups when compared with vehicle. However, this difference became statistically significant only at day 7 (fig. 4C).

**Short-term High-dosage Atorvastatin Rapidly Reduces Macrophage Content in a Mouse Model with Advanced Atherosclerotic Lesions**

Macrophages are causative for plaque destabilization. Chronic statin treatment promotes plaque-stabilizing effects and prevents perioperative myocardial infarction when given for 4 weeks. Turnover of macrophages in advanced atherosclerotic lesions is continuous and a rather rapid process. Therefore, to prove the principal ability of FMT to be useful for surveillance of therapy,
we examined whether reduction in macrophage recruitment could account for the lesion stabilization. To that end, we used a 4-day high-dose statin treatment and investigated if this was sufficient to convey lesion-stabilizing effects in a mouse model of spontaneous, diet-induced atherosclerosis. Within 16 weeks of high-cholesterol diet, ApoE-/- mice developed complex atherosclerotic plaques in the brachiocephalic artery (fig. 5A). As expected, total lesion size did not change in mice that were treated with 40 mg/kg atorvastatin, twice daily, for 4 days compared with vehicle-treated controls (fig. 5B). However, the CD68-positive lesion area (fig. 5C) assessed as a measure of plaque vulnerability, was reduced (fig. 5D). To address the question whether the observed difference in overall macrophage content is the result of a statin-promoted reduction of continuous macrophage recruitment, we quantified trafficking of eGFP/DiR double-labeled macrophages into atherosclerotic lesions in mice receiving atorvastatin (fig. 5E). Less exogenous eGFP/DiR-labeled cells were recruited into atherosclerotic plaques of the brachiocephalic artery in the statin group compared with controls (fig. 5F), indicating that statins reduce macrophage content by decreasing the constant recruitment and influx of additional macrophages into the lesion. The number of proliferating (fig. 5, G and H) or apoptotic (fig. 5, I and J) macrophages in atherosclerotic lesions was unaffected by short-term, high-dosage atorvastatin, as determined by immunohistochemistry. Within the 4-day treatment course, we detected a trend toward a reduction of total plasma cholesterol ($P = 0.07$) in statin-treated animals compared with control animals. Triacylglycerides, phospholipids, and high-density lipoprotein were not affected in atorvastatin versus control mice. Also, low-density lipoprotein and very-low-density lipoprotein the main atherogenic lipoproteins in rodents did not differ in a statistically significant manner between the two groups (table 1).

Fig. 2. Fluorescence-mediated tomography (FMT) detects a near-infrared fluorescence signal derived from exogenous enhanced green-fluorescent-protein (eGFP)/1,1'-dioctadecyl-3,3',3''-tetramethylindocarbocyanine iodide (DiR) macrophages recruited into carotid artery plaques of apolipoprotein E-deficient (ApoE -/-) mice. (A–D) FMT detected a strong near-infrared fluorescence signal in the left carotid artery that was subjected to guidewire injury (GWI). Near-infrared fluorescence was absent in the right control vessel. Coronal, sagittal, and transverse sections of a mouse, 2 weeks after GWI and 7 days after injection of eGFP/DiR-labeled macrophages, are displayed. Note the absence of fluorescence at the contralateral control-side (*). The color coding represents the concentration of DiR. (E) In sham-operated animals (n = 4), FMT signals were similar between left and right carotid artery or between day 2 and day 7. (F) After GWI the FMT signal was increased after 2 days (n = 9) and remained elevated on day 7 (n = 8), with a significant difference between right (sham) and left (GWI) carotid artery ($P < 0.05$). One animal died before FMT analysis on day 7. contr. = control; L = left; nm = nanomolar; R = right.
PERIOPERATIVE MEDICINE

Larmann et al.

FMT Uncovers the Potential of Atorvastatin to Suspend Continuous Macrophage Recruitment into Atherosclerotic Lesions

Kinetics of lesion progression in this model of diet-induced spontaneous atherosclerosis were expected to be slower than in the GWI-induced model of accelerated lesion formation. However, as FMT had already detected the maximum macrophage recruitment in the first model early after GWI, we decided to perform baseline FMT scans also in the second model 2 days postinjection of eGFP/DiR macrophages, to quantify the near-infrared fluorescent DiR signal in a ROI covering aortic sinus, aortic arch, and brachiocephalic artery. Follow-up FMT scans on treatment days 2 and 4 (fig. 6) detected constant recruitment of additional macrophages into atherosclerotic plaques. In nonatherosclerotic C57Bl6 mice on normal chow, the FMT signal remained low (fig. 6B). Subsequently, we assessed the DiR signal derived from infiltrating macrophages in atorvastatin-treated and control mice. The near-infrared fluorescent value on day 4, expressed as percentage of baseline fluorescence, was lower when animals received atorvastatin (fig. 6, C and D).

Discussion

Noninvasive FMT optical imaging was used for spatio-temporal resolution of macrophage trafficking to atherosclerotic plaques. Using FMT, we quantified macrophage infiltration and characterized an atorvastatin-mediated decrease in continuous macrophage recruitment to advanced atherosclerotic lesions in ApoE−/−mice. The prevention of additional macrophage infiltration during 4 days of statin administration led to a profound reduction in overall macrophage content, indicating rapid plaque stabilization promoted by short-term, high-dose atorvastatin treatment.

Relevance of Noninvasive Imaging of Plaque Phenotype

Current techniques for carotid plaque imaging fall short of providing functional or molecular information on plaque microenvironment or its inflammatory phenotype, which would be critical for the assessment and prediction of plaque rupture in a given lesion.46,47 It would be of major importance to make techniques available that allow detection and localization of plaques that are prone to rupture. Particularly, preoperative patients would benefit from the ability to assess the degree of vulnerability and the risk of rupture before surgical procedures. If plaque-stabilizing therapies were available, one would, in addition, demand that therapeutic success or failure of these therapies be monitored.

The amount of macrophages that accumulate in the vessel wall is not necessarily a surrogate parameter for the current inflammatory activity of a particular lesion. Mere information on the number of macrophages, lacks dynamic information and does not predict the vulnerability of the plaque. Dynamic information reflecting inflammatory cell recruitment and its intensity are derived from macrophage-trafficking assays because they detect the rate of additional macrophage uptake. Such applications have thus far been restricted to postmortem analysis, in which macrophages were labeled and their recruitment to the lesion was quantitatively detected by fluorescence microscopy.48–52 New optical imaging technologies pave the way to transfer these cell-homing methodologies into noninvasive in vivo settings. They permit the detection of highly diluted labels, and thus, lower amounts of labeled cells.50,53,54 Finally, the availability of quantitative tomographic imaging
techniques is another major advancement for the in vivo application because even deep-seated fluorescent signatures can be localized and quantified.55

**FMT Detects Macrophage Recruitment to Atherosclerotic Lesions**

We demonstrate, in a model of accelerated atherosclerotic lesion formation with rapid macrophage recruitment to a GWI-denuded carotid artery, that injection of NIRF-labeled indicator macrophages leads to detectable accumulation of indicator cells in the plaque. The fluorescent dye was solely confined to viable cells that carried the double–label-excluding dye transfer as the mechanism of signal accumulation. The injected macrophages were functionally intact with respect to adhesion and transmigration, as we have shown previously in in vitro assays.30

**FMT Detects Macrophage Recruitment to Spontaneous Atherosclerosis**

We have shown along with others that results from macrophage-trafficking assays are directly related to speed and intensity of macrophage recruitment and that they are sensitive to therapeutic interventions.50,56 Moreover, homing of macrophages to plaques is independent of plaque size and age and is directly related to the rate of macrophage recruitment.50 The influx rate of macrophages into the vessel wall here conceivably represents the most direct measure for plaque destabilization.56

Therefore, we set out to detect macrophage recruitment in a chronic but clinically more relevant model of spontaneously developing atherosclerosis. FMT was used to quantitatively image recruitment of IV-injected indicator macrophages to aorta and brachiocephalic artery. The signal increased until day 4 and then leveled off. Thus, imaging of macrophage influx into spontaneously developing lesions is also feasible.

**Statins Reduce Macrophage Content of Atherosclerotic Plaques within 96 Hours**

Chronic but also short-term HMG-CoA reductase inhibitor treatment reduces macrophage plaque burden, thus improving plaque stability.13,14,16 These effects have been shown to be also effective during the perioperative phase,19–21 and current guidelines suggest that statin use is to be advised for patients undergoing major vascular surgery with or without clinical risk factors.57 We treated mice for 4 days with 80 mg/kg body weight atorvastatin. In clinical studies, the mechanism of the rapid statin effect is not addressed, but antiinflammatory effects have been suggested to be responsible. Lowering of LDL and very-low-density lipoprotein plasma cholesterol and/or increasing high-density lipoprotein cholesterol is the proposed mechanism by which chronic HMG-CoA reductase inhibitor treatment improves endothelial function, slows the progression of atherosclerosis,58 and reduces the rate of cardiovascular events and mortality.13 The contribution of pleiotropic effects to perioperative cardiovascular risk reduction is controversial.22,59 Our data indicate that a lipoprotein-independent mechanism likely accounts for the observed profound reduction in macrophage content and change toward more stable atherosclerotic lesions in mice receiving atorvastatin.

Our in vitro studies demonstrate that atorvastatin can rapidly affect macrophage adhesion to the endothelium.

---

**Fig. 4.** Atorvastatin prevents oxidized low-density lipoprotein (oxLDL)-induced macrophage adhesion to endothelial cells. (A) Atorvastatin coinubcation of oxLDL-activated endothelial cells prevented increased macrophage adhesion in an in vitro assay (n = 6; P < 0.01). (B) Characterization of viability by propidium iodine revealed that while viability decreased over time († P < 0.001; n = 5) neither dosage of atorvastatin affected this process (vehicle vs. 1 nm atorvastatin, ‡ P > 0.99, n = 5; vehicle vs. 10 nm atorvastatin, †† P > 0.99, n = 5). (C) Within 4 days of culture the amount of macrophages per well increased due to proliferation (day 4 vs. day 0; # P < 0.001; n = 5). However, the effect of atorvastatin on proliferation only became obvious at day 7 (vehicle vs. atorvastatin; *P < 0.02; n = 5) whereas no relevant difference was observed until day 4. nLDL = native low-density lipoprotein; nm = nanomolar.
Fig. 5. Atorvastatin reduces macrophage content in brachiocephalic artery lesions. (A) A high-cholesterol diet was fed to 14 apolipoprotein E-deficient (ApoE−/−)-mice for 16 weeks to induce complex atherosclerotic plaques in the brachiocephalic artery. (B) Short-term, high-dose atorvastatin treatment did not affect lesion size measured on cross-sections stained for hematoxilin and eosin (H&E; P = 0.69). (C) Lesion area that stained positive for the macrophage-specific marker CD68 was reduced (D) in mice treated with atorvastatin for 4 days (P < 0.05). (E) 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindotricarbocyanine iodide (DiR)-labeled enhanced green-fluorescent-protein (eGFP)-transgenic macrophages were injected intravenously before initiation of statin treatment and detected by fluorescence microscopy in atherosclerotic plaques on day 4. (F) Significantly less of those exogenous indicator cells were found in atherosclerotic plaques of atorvastatin-treated mice compared with vehicle controls (P < 0.05). (G) Double immunohistochemistry for CD68 (green) and proliferating cell nuclear antigen (PCNA; red) revealed (H) no differences for proliferating macrophages inside atherosclerotic lesions for vehicle versus atorvastatin-treated animals. (I and J) No differences between both groups were seen for apoptotic (cleaved caspase 3 [cC3; red]) macrophages (CD68 [green]) in plaques. 4′,6-Diamidin-2-phenylindol (DAPI; blue) depicts nuclei; scale bar: 40 µm. n.s. = not significant.
### Table 1. High-dosage, Short-term Atorvastatin Did Not Significantly Change the Lipoprotein Profile in ApoE<sup>-/-</sup> Mice on High-cholesterol Diet

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>Vehicle</th>
<th>Atorvastatin</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cholesterol, mg/dl</td>
<td>504.4 ± 154.1</td>
<td>371.2 ± 79.2</td>
<td>0.07</td>
</tr>
<tr>
<td>VLDL cholesterol, mg/dl</td>
<td>313.7 ± 128.8</td>
<td>187.2 ± 68.9</td>
<td>0.07</td>
</tr>
<tr>
<td>LDL cholesterol, mg/dl</td>
<td>140.1 ± 26.4</td>
<td>130.4 ± 10.4</td>
<td>0.57</td>
</tr>
<tr>
<td>HDL cholesterol, mg/dl</td>
<td>50.6 ± 7.0</td>
<td>53.8 ± 8.8</td>
<td>0.49</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
<td>75.1 ± 24.2</td>
<td>71.00 ± 21.8</td>
<td>0.68</td>
</tr>
<tr>
<td>Phospholipids, mg/dl</td>
<td>310.1 ± 88.9</td>
<td>319.4 ± 66.0</td>
<td>0.85</td>
</tr>
</tbody>
</table>

Mice treated with high-dosage atorvastatin for 4 days did not show significant differences in their lipoprotein profile when compared with controls. However, a trend ($P = 0.07$) toward lower total cholesterol and VLDL levels was observed in atorvastatin-treated animals. ApoE<sup>-/-</sup> = apolipoprotein E-deficient; HDL = high-density lipoprotein; LDL = low-density lipoprotein; VLDL = very low-density lipoprotein cholesterol.

Fig. 6. Fluorescence-mediated-tomography (FMT) reliably quantifies 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine iodide (DiR)/enhanced green-fluorescent-protein (eGFP) macrophage recruitment and uncovers atorvastatin-promoted rapid plaque stabilization. (A) In the diet-induced spontaneous atherosclerosis model, FMT detected a near-infrared fluorescent signal in a region of interest covering aortic sinus, aortic arch, and brachiocephalic artery, 2 days after intravenous injection of eGFP/DiR macrophages (day 0). (B) The DiR signal indicating macrophage recruitment in the region covering several atherosclerosis-prone vessels increased over time and was significantly higher on day 4 compared with baseline day 0 (day 4 vs. day 0, *$P < 0.05$). In atherosclerosis-free C57Bl6 mice on normal chow, no significant difference was observed ($n = 4$). (C) For 14 mice, treated with atorvastatin or vehicle, the DiR-macrophage fluorescence was quantified on treatment day 4 as percentage of day 0 baseline fluorescence. (D) In statin-treated animals the FMT signal on day 4 remained low and was significantly reduced compared with vehicle-treated mice ($P < 0.05$) indicating that high-dosage, short-term atorvastatin prevented the recruitment of exogenous eGFP/DiR macrophages into atherosclerotic lesions. ApoE<sup>-/-</sup> = apolipoprotein E-deficient; d = day; L = left; nm = nano molar; R = right.
Long-term statin incubation prevents macrophage proliferation\textsuperscript{33} whereas effects on mononuclear cell viability are heterogeneous and depend on cell type, species, and the specific drug used.\textsuperscript{35} However, we could not find relevant effects of even high dosages of atorvastatin on proliferation or viability of peritoneal macrophages during the first 4 days of atorvastatin incubation. Concentrations of 1 and 10 µM atorvastatin were chosen for the \textit{in vitro} experiments because they correspond to mean atorvastatin levels found in rodents after high-dose atorvastatin treatment.\textsuperscript{41,60} Using FMT, we demonstrate that a very brief statin treatment rapidly abrogates the continuous trafficking of macrophages into plaques. The overall macrophage content was reduced within 4 days of statin treatment, demonstrating that loss of signal in FMT studies was consistent with the postmortem histological findings, and that FMT was able to detect this change non-invasively in live animals.

Limitations
Mouse atherosclerosis develops faster compared with human disease. Therefore, it is difficult to estimate which duration of statin treatment in humans would be equivalent to the 4 days of atorvastatin therapy that were used in this study. We did not observe statistically significant changes in lipoprotein profiles. Clinical trials that have used statins to prevent cardiovascular complications by a 30-day statin treatment have described a clinically significant lowering of total and LDL cholesterol.\textsuperscript{19,21} It is therefore, possible that our regimen resembles an even shorter statin treatment. For our present study we tested a high-dose statin regimen, which we thought would have the highest potential to promote rapid effects on plaque stability. We chose lipophilic atorvastatin over hydrophilic HMG-CoA reductase inhibitors because atorvastatin had been shown to beneficially affect rodent lipoprotein profiles. Clinical trials that have used statins to prevent cardiovascular complications by a 30-day statin treatment have described a clinically significant lowering of total and LDL cholesterol.\textsuperscript{19,21} It is therefore, possible that our regimen resembles an even shorter statin treatment. For our present study we tested a high-dose statin regimen, which we thought would have the highest potential to promote rapid effects on plaque stability. We chose lipophilic atorvastatin over hydrophilic HMG-CoA reductase inhibitors because atorvastatin had been shown to beneficially affect rodent lipoprotein profiles\textsuperscript{38-40} and was also effective in clinical studies of perioperative protection from cardiovascular events.\textsuperscript{20} The question whether the observed effects persist at lower statin dosages or with lipophilic statins will stimulate our future research. Peritoneal macrophages were used because they resemble peripheral blood mononuclear cells with regard to their adhesive and migratory function.\textsuperscript{50} For future clinical use of this technology, autologous peripheral blood monocytes are the likely candidates that bear the potential to be used in human trials and diagnostic assays.

Because FRI can easily be miniaturized and integrated, e.g., into catheter-based systems,\textsuperscript{61,62} or hand-held optical devices, a clinical translation of the technique can be briefly envisioned and may be applicable for noninvasive assessment of atherosclerotic lesions in supraaortic vessel segments.\textsuperscript{61}

Conclusion
We demonstrate that optical imaging techniques can readily and efficiently be utilized for tracking of near-infrared-labeled macrophages in mouse models of atherosclerosis with high relevance to human disease. Using these techniques, we demonstrate that atorvastatin reduces recruitment of macrophages to existing lesions. This could conceivably be a major mechanism, by which brief preoperative statin treatment contributes to a more stable lesion phenotype, and reduces major adverse cardiac events in a time frame relevant for preoperative evaluation, and cardiac risk optimization for a large cohort of elective surgical patients.

The authors thank and acknowledge the assistance of Kerstin Reher, B.Sc., Silke Ecklebe, B.Sc., Christine Ritter, B.Sc. (Technicians, Department of Anesthesiology and Intensive Care Medicine, Hannover Medical School, Hannover, Germany), Annelie Ahle, B.Sc., Stefan Lütke Enking, B.Sc., Ulrike Niehues, B.Sc., Dirk Reinhardt B.Sc., (Technicians, Institute for Anatomy, University of Muenster, Muenster, Germany), and Wiebke Gottschlich, B.Sc. (Technician, Institute for Clinical Radiology, University of Muenster).

References

10. Lendon CL, Davies MJ, Born GV, Richardson PD: Atherosclerotic plaque caps are locally weakened when macrophages density is increased. Atherosclerosis 1991; 87:87–90
In Vivo Imaging of Plaque Stabilization by Statins


43. Tietge UJ, Maugeais C, Cain W, Grass D, Glick JM, de Beer FC, Rader DJ: Overexpression of secretory phospholipase A2 causes rapid catabolism and altered tissue uptake of high density lipoprotein cholesterol ester and apolipoprotein A-I. J Biol Chem 2000; 275:17778–86


