High-Resolution Imaging of Intravascular Atherogenic Inflammation in Live Mice

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Rationale: The inflammatory processes that initiate and propagate atherosclerosis remain poorly understood, largely because defining the intravascular behavior of immune cells has been technically challenging. Respiratory and pulsatile movements have hampered in vivo visualization of leukocyte accumulation in athero-prone arteries at resolutions achieved in other tissues.

Objective: To establish and to validate a method that allows high-resolution imaging of inflammatory leukocytes and platelets within the carotid artery of atherosusceptible mice in vivo.

Methods and Results: We have devised a procedure to stabilize the mouse carotid artery mechanically without altering blood dynamics, which dramatically enhances temporal and spatial resolutions using high-speed intravital microscopy in multiple channels of fluorescence. By applying this methodology at different stages of disease progression in atherosusceptible mice, we first validated our approach by assessing the recruitment kinetics of various leukocyte subsets and platelets in athero-prone segments of the carotid artery. The high temporal and spatial resolution allowed the dissection of both the dynamic polarization of and the formation of subcellular domains within adhered leukocytes. We further demonstrate that the secondary capture of activated platelets on the plaque is predominantly mediated by neutrophils. Finally, we couple this procedure with triggered 2-photon microscopy to visualize the 3-dimensional movement of leukocytes in intimate contact with the arterial lumen.

Conclusions: The improved imaging of diseased arteries at subcellular resolution presented here should help resolve many outstanding questions in atherosclerosis and other arterial disorders. (Circ Res. 2014;114:770-779.)

Key Words: atherosclerosis ▪ blood platelets ▪ carotid arteries ▪ neutrophils

Atherosclerosis is a chronic inflammatory disease of large arteries and a major cause of morbimortality in Western societies.† Immune cells and platelets are considered important mediators of all phases of atherosclerosis, from the initial asymptomatic lesions to the establishment of complex vulnerable plaques that can rupture and provoke acute ischemic events (e.g., myocardial infarction or stroke) and associated disability and mortality.2,3 Different subsets of leukocytes accumulate in atherosclerotic lesions and contribute to the maturation and destabilization of the plaque.4,5 Particularly important are blood-borne monocytes, which accumulate in the injured arterial wall after interacting with dysfunctional endothelial cells and differentiate into macrophages, which ultimately propagate inflammation within the atherosclerotic plaque.5,6 Less abundant in the plaque, but also important mediators at specific stages of atherosclerosis, are neutrophils and B and T lymphocytes.5,5 On the luminal side of the plaque, neutrophils and platelets promote disease progression by releasing chemoattracting factors and other intracellular material.7–10 However, the absence of reliable tools for real-time visualization of leukocytes, platelets, or other thrombo-inflammatory mediators within affected arteries at high resolution severely limits our understanding of how intravascular inflammation drives atherogenesis.

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Multiple genetic models and immunohistological tools have been used to dissect key aspects of the etiopathogenesis of atherosclerosis, but they have not provided sufficient insight into the question of leukocyte accumulation and behavior at the endothelial...
interphase. Attempts to image leukocyte recruitment directly in athero-prone regions by applying epifluorescent or 2-photon laser scanning microscopy (TPLSM) in explanted arteries have yielded important mechanistic information on the processes that underlie disease progression, including a role of adhesion receptors in leukocyte recruitment,\(^\text{11}\) accumulation of activated platelets,\(^\text{8}\) or local presentation of antigens by dendritic cells to T lymphocytes.\(^\text{12}\)

Although these studies with explanted arteries dramatically improved image quality, they have failed to provide the complex rheological and immune context present in the arterial lumen of live animals. It is also noteworthy that application of real-time imaging in live mice to visualize atherogenic arteries (eg, aorta and carotid arteries) has been challenged by the respiratory and pulsatile movements of these vessels, which greatly limited the temporal and spatial resolution of live imaging and prevented analysis of the dynamics of leukocytes and platelets in the atherogenic environment, or the identification of subcellular structures.\(^\text{11,13–15}\)

Thus, key questions related to the recruitment of different leukocyte subsets, their activation and migration on and across the injured endothelium, and the formation of multicellular aggregates that remain to be characterized in the microvasculature of many tissues\(^\text{8,16,17}\) remain to be answered in the context of atherosclerosis.

Here, we sought to establish an easy to apply and reliable imaging methodology for in vivo visualization of dynamic processes during atherogenesis at subcellular and subsecond resolutions. We report a procedure to stabilize the carotid artery of live mice that dramatically reduces spontaneous vertical movements of the vessel generated by the heart cycle and respiration, without causing significant alterations of the physiological blood flow. Combined with high-speed multi-channel epifluorescence and TPLSM platforms, the method enables visualization of inflammatory phenomena within the lumen of the artery at high spatial and temporal resolutions in ≤4 dimensions. We have used this system to image intraluminal events in athero-prone apolipoprotein E–null mice (ApoE\(^{-/-}\)) at different stages of disease and combined it with reporter genes and fluorescent probes to track various subsets of inflammatory cells and subcellular structures in vivo.

### Methods

For detailed Materials and Methods, please see the Online Data Supplement.

### Mice

Mafa\(^{\text{apoE}^{-/-}}\) mice were generated by crossing ApoE\(^{-/-}\) (Charles River, Lyon, France) with Mafa mice, which express green fluorescent protein (GFP) under the control of the c-fms promoter.\(^\text{18}\) Alternatively, Lysm\(^{\text{GFP/ApoE}^{-/-}}\) mice\(^\text{19}\) were used to visualize neutrophils. All mice were in the C57BL/6J background and were housed in a pathogen-free barrier facility. For diet-induced atherosclerotic studies, 6- to 8-week-old male mice were fed an atherogenic high-fat diet (HFD: 10.8% total fat, 0.75% cholesterol; S8492-E0101, Stäffelf, Germany) for the indicated periods of time. All experimental protocols were approved by the local authorities for animal experimentation.

### Surgical Preparation and Stabilization of the Carotid Artery

Mice were anesthetized with a mixture of ketamine (Imalgene 1000; Merial, France) and medetomidine (Medeson; Urano, Spain; 50 and 0.5 mg/kg, respectively). Surgical preparation and stabilization of the carotid artery for imaging are described in Result section (Figure 1) and Online Data Supplement.

### Results

#### Preparation of the Carotid Artery for Stabilized Imaging

The bifurcation of the common left carotid artery is susceptible to atherosclerosis in humans and mice and is easily accessible with minor surgery. We therefore chose this region to develop a procedure that allowed vessel stabilization without significantly compromising blood flow for subsequent high-resolution in vivo imaging. Mice were anesthetized, placed in decubitus position, and the carotid artery exposed and carefully dissected.
without disrupting the surrounding muscular tissues or nerves (Figure 1A). To isolate the vessel from the respiratory motions of the thorax we placed a beveled flat metal piece under the vessel (Figure 1B). This bottom metallic support was attached to a rigid pole that was immobilized on a lateral holder. To reduce the pulsatile movements of the artery caused by the cardiac cycles, an image-grade round coverslip 12 mm in diameter was cut in half, placed over the artery and flexibly fixed to the bottom support using modeling clay (Figure 1C). This set up allowed us to restrain the z-motion of the arterial wall by gently pressing on the coverslip to obtain a separation of 400±50 µm between both supports. The stabilized region of the artery was then placed under the objective of a multichannel epifluorescence microscope with constant perfusion of warm saline buffer (Figure 1D). This simple preparation yielded a ≈1-mm long segment of the common carotid artery and the 2 branches of the bifurcation in which vertical displacement because of thoracic and pulsatile movements was dramatically reduced compared with nonstabilized carotid arteries (Figure 2A and 2B; Online Movie I). We quantified stabilization by determining the Pearson coefficient of the in-focus region of control and stabilized arteries that compared the fluorescent signal between consecutive frames captured at 5 Hz (ie, 5 frames/s). These analyses revealed higher Pearson coefficient in stabilized preparations (Figure 2C; Online Figure I). Although some lateral movement was still present in mechanically stabilized arteries, the use of epifluorescent illumination allowed continued imaging of the structures of interest over time and facilitated off-line analyses. Notably, the stability of the tissue was comparable with that obtained with the well-established cremaster muscle model (Online Figure IB and IC), which has been used extensively to study inflammation in the microvasculature.17,20

To assess whether the stabilizing procedure altered the hemodynamics of the carotid artery, we injected 1-µm fluorescent beads and measured velocities at low magnification. We found little perturbations in blood flow dynamics (Figure 2D; Online Movie II), with diastolic velocities of 4.4±0.6 cm/s that are consistent with those reported using ECG-gated MRI.21 For subsequent imaging of atherosclerotic regions within the living carotid artery, we chose regions proximal to the bifurcation, in areas where plaques developed over time under proatherogenic conditions (Figure 2E and 2F; Online Movie III). In these areas, the stabilization procedure allowed efficient imaging of defined intravascular structures even at high magnification (Online Movie IV). We thus set out to validate this method for visualization of atherogenic inflammation.

Leukocyte Dynamics in Atherogenic Regions

We first imaged the stabilized carotid artery at low magnification in atherosusceptible mice deficient in ApoE−/−. Animals

![Figure 2. Image stability and blood dynamics. A and B, Intravital microscopy of nonstabilized (A) and stabilized (B) carotid artery. The images show sequential images obtained under a 10× or 40× objectives at 5.7 and 3 Hz, respectively. Scale bar, 200 µm (10×) or 40 µm (40×). See also Online Movie I. C, Pearson colocalization coefficient from the 10× acquisitions of stabilized and nonstabilized arteries. D, Blood flow pattern within the stabilized carotid artery, obtained by imaging free-flowing fluorescent beads (red) at low magnification. Background autofluorescence of the arterial wall appears in green. Scale bars, 100 µm. See also Online Movie II. E, Intravital microscopy of the stabilized carotid artery presenting atheroma at its bifurcation level. High-fat diet (HFD)-fed Mafia:ApoE−/− (8 weeks) mouse was injected intravenously with rhodamine 6G to label luminal cells (ie, endothelial cells and leukocytes, red). Green fluorescent protein (GFP)-positive extravasated myeloid cells (green) accumulate at the bifurcation level of the carotid artery. Scale bars, 200 µm. See also Online Movie III. F, Three-dimensional (3D) reconstruction of whole-mounted carotid artery from HFD-fed Mafia:ApoE−/− mouse (6 weeks). Red signal shows autofluorescent arterial wall and green signal shows GFP-positive myeloid cells. GFP-positive extravasated cells accumulate at the bifurcation level of carotid artery. Scale bars, 200 µm.](https://circres.ahajournals.org/content/3/2/772)
were fed standard diet or challenged with an atherogenic HFD for 2 or 4 weeks and then intravenously injected with rhodamine 6G to label luminal cells and with fluorescent beads coated with anti–vascular cell adhesion molecule-1 antibodies to identify regions with dysfunctional endothelium (Figure 3A and 3B). The vascular cell adhesion molecule-1–coated beads, but not control rIgG-coated beads (Online Figure II), predominantly accumulated at the level of the bifurcation in fat-fed mice, and this correlated with large foci of rolling and adherent leukocytes in the same areas (Figure 3B). In contrast, leukocytes and vascular cell adhesion molecule-1 were both largely absent from areas outside the bifurcation in fat-fed mice or from the arteries of mice fed with standard chow (Figure 3A and 3B). Leukocyte recruitment was also largely absent from small arteriolar vessels of the cremasteric microcirculation of HFD-treated mice (Online Figure III). Thus, inflammation and the ensuing recruitment of leukocytes in the visualized carotid artery are specifically induced by the atherogenic diet, rather than by surgical manipulation.

To obtain high temporal and spatial resolutions (≤9.2 Hz and ≈0.6 μm), we next captured images using a 40× numerical aperture 1.0 objective in a single channel of fluorescence of the carotid artery of fat-fed ApoE−/− mice. Rhodamine labeling revealed high numbers of rolling leukocytes at the bifurcation as early as 2 weeks post HFD, which increased at 4 weeks (Figure 3C–3G; Online Movie V), whereas virtually no recruitment was seen under nonatherogenic standard conditions (Figure 3C, 3D, and 3G).

Luminal recruitment of leukocytes seemed to be the major way of recruitment during early atherosclerosis because at these stages we could not detect vasa vasora, contrary to...
their reported abundance in late-stage atheromata. The high temporal resolution of our acquisitions further allowed measurement of leukocyte rolling velocities, which decreased on fat feeding over time and inversely correlated with the number of rolling cells (Figure 3G and 3H).

**Identification of Leukocyte Subsets Recruited to Atherosclerotic Lesions**

Multiple leukocyte subsets contribute to atherosclerosis by triggering diverse innate and acquired immune processes. To assess the specific recruitment of various leukocyte subsets in the atherogenic artery, we took advantage of Mafia reporter mice, which allow easy identification of GFP+ circulating monocytes and neutrophils. Analysis of ApoE−/− mice expressing the Mafia transgene (Mafia:ApoE−/−) revealed a robust recruitment of myeloid cells as early as 10 days post HFD (Figure 4A), with elevated numbers of rolling and adherent GFP+ cells at the carotid bifurcation (Online Movie VI). In these experiments, many GFP+ cells were already present at the time of imaging, and we could estimate that 2.1% (9 of 430) of the myeloid cells that came rolling transitioned into firm arrest at the bifurcation. Interestingly, timestamp analyses revealed preferential areas of leukocyte rolling (Online Figure IVA), suggesting heterogeneous activation of endothelial cells within the same arterial regions.

To track the behavior of distinct leukocyte subsets simultaneously, we injected Mafia:ApoE−/− mice with fluorescently tagged antibodies against CD4, CD8, and Ly6G, using doses (0.4–1 μg/mouse) that do not significantly alter leukocyte behavior in vivo. We took advantage of the fast filter-change system of our intravital microscopy setup to acquire images in 3 channels of fluorescence for near-simultaneous identification of CD4/CD8+ T cells, Ly6G+ GFP+ neutrophils, and Ly6GNEG/LO GFP+ monocytes recruited to the carotid bifurcation after 2 and 6 weeks on HFD (Figure 4B). These analyses revealed increased numbers of rolling neutrophils over time, whereas that of monocytes was similar at both time points (Figure 4C).

Interestingly, despite the progressive reduction in the relative amount of circulating T lymphocytes in fat-fed mice (Online Figure IVB and IVC), rolling of these cells was only detected after 6 weeks post HFD (Figure 4C), suggesting that activated T-cell subsets appear only at later stages of disease development. Analysis of the rolling dynamics of the different cell subsets revealed that neutrophils display high but similar rolling velocities at both time points, whereas the rolling velocity of monocytes was markedly reduced after 6 weeks on

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**Figure 4.** Simultaneous tracking of leukocyte subsets recruited to atherogenic lesions. Mafia:ApoE−/− mice were injected with fluorescently conjugated antibodies against CD4/CD8 and Ly6G before analysis. Arrows in A and B show the direction of flow. A, Representative images of myeloid leukocytes (green) rolling at the bifurcation of the carotid artery in mice fed standard chow or high-fat diet (HFD) for 10 days. Scale bars, 25 μm. See also Online Movie VI. B, Representative images of green fluorescent protein (GFP)+ myeloid cells, GFP+ Ly6G+ neutrophils, and GFPNEG/LO CD4/CD8+ T lymphocytes in the carotid bifurcation of mice fed HFD for 2 or 6 weeks. Images were acquired at 1.7 Hz under a 40× objective. Dashed lines delimit the lesion shoulder. Scale bars, 20 μm. C, Number of rolling neutrophils (red), monocytes (green), and T lymphocytes (blue). n=18 to 33 fields from 4 mice. Right graph shows rolling velocity of neutrophils (red) and monocytes (green). n=33 to 94 neutrophils and 9 to 35 monocytes from 4 mice. Lines show means±SEM, **P<0.01 and ***P<0.001.
HFD (Figure 4C). These observations reveal different kinetics of recruitment for monocytes, neutrophils, and T lymphocytes during atherogenesis and a more efficient (ie, slower rolling velocities) recruitment of monocytes as disease progresses.

**Intravascular Polarization and Crawling of Myeloid Leukocytes**

In the inflamed microcirculation, leukocyte recruitment proceeds through distinct phases. After rolling, leukocytes firmly adhere to the endothelium and this is followed by a rapid change in their morphology and redistribution of specific receptors to defined microdomains, a process known as polarization. In late stages, polarized neutrophils crawl on the endothelium in search of areas permissive for extravasation. To determine whether leukocyte polarization and crawling also occur in atherosclerotic vessels, we tracked the behavior of adhered myeloid GFP+ leukocytes at the carotid bifurcation of fat-fed Mafia:ApoE<sup>−/−</sup> mice. Before imaging, mice were injected with a fluorescently conjugated anti–L-selectin (CD62L) antibody to allow identification of the uropod in polarized leukocytes. Using this strategy, we identified a small fraction of GFP+ neutrophils that transitioned from rolling to arrest, underwent morphological polarization, and redistributed CD62L to the cell’s uropod (Figure 5A; Online Movie VII). The spatio-temporal resolution of the images allowed quantification of rolling and crawling velocities (Figure 5B and 5C), as well as tracking of CD62L redistribution in polarizing cells (Figure 5D and 5E). The CD62L-enriched uropod could be detected in many GFP+ cells crawling along the arterial endothelium, some of which moved against the direction of the flow, away from landmark structures present in the plaque (Figure 5B; Online Movie VII). We also observed that a fraction of crawling cells left tracks of CD62L-immunoreactive material on the endothelium (Online Figure V). These structures were not artifacts generated by the residual lateral motion of the preparation, as they were maintained over time with the same morphology (not shown). These observations demonstrate that leukocyte recruitment to atherosclerotic lesions follows the same fundamental phases described in the microvasculature and further reveal the feasibility of in vivo visualization of subcellular structures within inflamed large arteries.

**Accumulation of Activated Platelets in Atherosclerotic Lesions Requires Interaction With Recruited Myeloid Cells**

Activated platelets have been associated with the development of atherosclerosis. Binding of platelets to both myeloid and endothelial cells has been proposed to promote leukocyte recruitment through deposition of inflammatory chemokines. Thus, we next examined the mechanisms by which platelets are recruited to atherosclerotic lesions in vivo. To this end, we injected fat-fed Mafia:ApoE<sup>−/−</sup> mice with a fluorescently conjugated anti-CD41 antibody, which allows specific detection of platelets in vivo (Online Figure VI). Microscopic fields were divided into 3 categories: low adhesion (absence or low presence of adherent GFP+ myeloid cells), myeloid clusters (strong presence of singly adherent GFP+ cells and small autofluorescent structures), and lesion shoulder (presence of 3-dimensional [3D] structures and GFP+ cells). We found that few platelets bound to the endothelium in areas devoid of leukocytes (Online Movie VIII), and instead preferentially bound to adherent GFP+ myeloid leukocytes.

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**Figure 5.** Polarization and crawling of myeloid leukocytes recruited to atherogenic lesions. Mafia:ApoE<sup>−/−</sup> mice on high-fat diet for 3 weeks were injected with a fluorescently conjugated antibody to CD62L to allow in vivo visualization of myeloid cell polarization. **A**. Sequence showing CD62L distribution (white) on myeloid cells (green). The orange arrowhead points to leukocyte that arrests, polarizes, and completely redistributes CD62L to the uropod within 4 minutes. Another polarized cell is shown crawling against the blood flow (red arrowheads). In enlarged images of the framed regions, yellow dashed lines identify polarizing and crawling leukocytes, purple dashed line indicates nonpolarized state, and purple continued line indicates polarized state. Gray arrows show the direction of flow. Images were acquired at 2.5 Hz. Scale bars, 20 μm. See also Online Movie VII. **B**. Tracks of rolling and crawling leukocytes. Note that although all rolling cells follow the flow (indicated by the gray arrow), several crawling cells move against the flow. **C**. Distances and velocities of single cells rolling (black) or crawling (red) on the endothelium. **D**. Quantification of CD62L distribution along the axis of polarized or nonpolarized leukocytes (purple dashed and continued lines in **A**, respectively). **E**. Kinetics of CD62L redistribution in the polarizing cell shown in **A**. The graph shows the CD62L+ area expressed as a percentage of total leukocyte area over time.
present in the periphery of established plaques (lesion shoulders; Figure 6A; Online Movie IX). Depletion of circulating myeloid cells with an anti–Gr-1 (anti-Ly6G/C) antibody almost completely abolished platelet accumulation, as did specific depletion of neutrophils with an anti-Ly6G antibody (Figure 6B; Online Figure VII). These data indicate that the initial accumulation of neutrophils precedes and mediates platelet accumulation in the lumen of atherosclerotic arteries.

To determine the activation state of platelets that accumulated around arrested leukocytes and to further examine their interactions with myeloid leukocytes in vivo, we performed 4-channel imaging of GFP, Ly6G, and CD41 together with a fluorescently conjugated JON/A antibody (Online Movie X), which recognizes the active form of the $\alpha_{IIb}\beta_{3}$ integrin present in activated platelets. We confirmed that platelets bound to GFP+ cells (both Ly6G-positive and negative) and found that the activation epitope marked preferentially small thrombi associated with lesion shoulders (Figure 6C–6E; Online Movie X).

These analyses indicate that activated platelets are dynamically recruited to the developing atheroma and that they do so by interacting with previously adhered monocytes and neutrophils.

**Visualization of Myeloid Cell Dynamics in 3D Over Time**

As a proof of principle, we also visualized myeloid cell dynamics using TPLSM, an optical imaging method with superior depth discrimination enabling multidimensional imaging but also suffering severely from the impact of motion of a sample. We applied TPLSM to visualize myeloid cells recruitment in fat-fed Lysmegfp/egfpApoE–/– mice. Even if application of the stabilizer tool strongly reduced the impact of both cardiac and respiratory movement, additional triggering on the respiratory cycle further limited the impact of respiratory movement artifacts, thereby yielding better image quality and stability. This synchronization ultimately allowed us to acquire multidimensional datasets over prolonged times (Figure 7A–7D; Online Movies XI and XII). Individual

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**Figure 6. Platelet recruitment to atherosclerotic lesions.** Mfia:ApoE–/– mice were fed high-fat diet for 1 month and injected with a fluorescently conjugated antibody against CD41, and images were acquired during at least 40 s. Representative image panels show myeloid leukocytes (green) and platelets (red). White arrowheads indicate platelets. Scale bars, 20 μm (A and B). A, Representative images from regions devoid of leukocytes (left), regions with clusters of green fluorescent protein (GFP)+ myeloid cells (middle), and in lesion shoulders (right). See also Online Movies VIII and IX. Graph shows quantification of adhered platelets in different regions. n=7 to 12 fields from 4 to 7 mice. Bars show mean±SEM. ***P<0.001. B, Representative images of lesion shoulders from control mice (left), Ly6G-depleted mice (middle), and Ly6G/C-depleted mice (right). Graph shows quantification of adhered platelets in depleted groups respective to the rlgG-injected group. n=10 to 21 fields from 3 mice. Bars show mean±SEM. ***P<0.001. C, Mice prepared as in A were additionally injected with fluorescently conjugated antibodies recognizing the activated $\beta_{3}$-integrin (clone JON/A) and Ly6G before imaging. Images were acquired in 4 channels of fluorescence at 1 Hz under a 10× objective. Scale bar, 100 μm. D, Patched-composition of the region shown in C, with images acquired using a 40× objective. Scale bar, 20 μm. E, Enlarged merged image of the region shown in D, showing also single fluorescence channels (right). The $\beta_{3}$-activation epitope associates with CD41+ thrombi (orange arrowhead) that seem associated with GFP+ myeloid cells in lesion shoulders. GFP+ Ly6G-positive neutrophils (blue arrowhead) and GFP+ Ly6G-negative monocytes can be identified. The dashed line delimits the contour of the lesion in this arterial segment, also shown in C. Scale bar, 20 μm.
myeloid cell movement along the arterial lumen could be studied in a relatively large field of view (450×450 μm; Figure 7B; Online Movie XI), in which we noted rapid changes in the shape of polarized cells as well as changes in their directionality of crawling. Intravenous application of 150 kDa tetramethylrhodamine isothiocyanate-dextran allowed for better discrimination between lumen and vessel wall (Figure 7D; Online Movie XIII), revealing that the cells were firmly adherent to the arterial wall, flattened and migrated in clusters along the lumen (Figure 7B). Moreover, image processing enabled volume rendering and provided a spatial context of the leukocyte, the vessel wall, and the lumen (Figure 7C and 7E); it also provided detailed kinetics in 3D of single and clustered leukocytes (Figure 7F; Online Movie XIII). These TPLSM analyses demonstrate the feasibility of high-resolution imaging of inflammatory cells in ≤4 dimensions within atherosclerotic arteries in vivo.

Discussion

In this study, we have developed a surgical procedure that stabilizes the carotid artery without significant alterations in flow dynamics that enabled high-resolution imaging of atherosclerotic lesions in live mice. We have applied wide-field fluorescence and TPSLM microscopy to the stabilized artery at different stages of atherogenesis. We show for the first time the feasibility of subsecond and submicron imaging of atherogenic inflammation in the presence of physiological flow, which allowed us to make several new observations: first, we dissect the kinetics and dynamics of recruitment of various subpopulations of inflammatory leukocytes and show that this process is an early event during atherogenesis. Second, we identify the presence of activated platelets in established atherosclerotic plaques and show that platelet incorporation into the injured wall largely relies on the presence of neutrophils. Finally, we show that the degree of arterial stabilization is sufficient for 2-photon microscopy–driven multidimensional imaging of leukocyte–vessel wall interactions.

Important for these imaging analyses of inflammatory cell behavior during atherosclerosis was the use of different myeloid GFP-based reporter models in an ApoE−/− background. We have shown that the use of these reporter mice in combination with fluorescent probes allows efficient visualization of myeloid leukocytes, subcellular structures, and platelet dynamics. Other molecules and cells of interest during atherosclerosis can be

Figure 7. Three-dimensional (3D) imaging of atherogenic arteries over time. Two-photon microscopic imaging of the stabilized carotid artery of LysmEGFP/ApoE−/− mice presented over time as 2D using extended depth of field projections, where the total Z information of each z-stack is projected in 1 XY image representing a total thickness of ~20 μm (B and D) or as 3D reconstructions (C, E, and F). A, Overview of recording sites within the carotid artery: the yellow box identifies regions imaged in B and C; the purple box identifies the area imaged in D, E, and F. B, Time series of z-stacks projected in 2 dimensions revealing myeloid cells (green) rolling (yellow arrow heads) and crawling (red box) on the vascular wall (blue, collagen fibers; gray, autofluorescence of the extracellular matrix). See also Online Movie XI. The shown extended depth of field projections are derived from z-stacks with a total thickness of 24 μm (step size=2 μm; total time window, 250 s). Scale bar, 75 μm. C, Three-dimensional reconstruction of an XYZ stack of the data set in B, where the viewing angle was altered to mimic a transversal view on the vessel wall (blue–green) and the tracked adherent cell (green, red box). D, Extended depth of field projection demonstrating myeloid cell crawling in close contact with the vessel wall in more detail (scale bar, 25 μm). Tetramethylrhodamine isothiocyanate (TRITC)-dextran (orange) was used to label the blood stream. Total z-depth of the extended depth of field projection is ~18 μm (step size=2 μm; total time window, 450 s; Online Movie XIII). E, Three-dimensional reconstruction of an XYZ stack of the same data set, where the viewing angle was altered to mimic a transversal view on the vessel wall and adherent cells (green), collagen in the tunica adventitia (blue), and the lumen (red). Cells are crawling directly on the vessel wall because no dextran was detectable between myeloid cells and the wall. F, Tracking of myeloid cells (depicted in the red box) over time using 3D isosurface rendering derived from z-stacks over time (see also Online Movie XIII). Scale bar, 25 μm. Gray arrows (A, B, D, and F) represent the direction of blood flow. ECM indicates extracellular matrix.
visualized with this technology using multiple fluorescent probes that are commercially available (eg, antibodies or fluorogenic substrates), as well as other reporter murine strains. For example, it should be possible to examine the specific recruitment and behavior of regulatory and interleukin 17–producing T cells, patrolling or inflammatory populations of monocytes, neutrophils or platelets during atherosclerosis using existing reporter mice.11–14

Direct visualization of exposed carotid arteries at low magnification previously allowed analysis of leukocyte rolling15 or identification of cellular clusters, but visualization at higher resolution or in 3D was prevented by the pulsatile motion of the arterial wall. Synchronization of acquisition with arterial wall motion allowed, nonetheless, an increase in spatial resolution in the z axis at the cost of temporal resolution.15 In contrast, other studies in which the artery was fully immobilized allowed imaging of the arterial wall at high spatial resolution but completely abrogated the physiological blood flow and could not be used to study the dynamic inflammatory events that occurred intravascularly.35 Here, by stabilizing the vessel in the vertical plane and using epifluorescent illumination, we tracked dynamic events at high resolution, despite the persistence of residual XY movements. This approach yielded a high temporal resolution in several channels of fluorescence and thus the possibility to track the dynamics of various cells populations in the arterial wall. Using subset-specific markers, we could estimate rolling frequencies and velocities for monocytes, neutrophils, and T lymphocytes within the same vessels. Notably, although the use of epifluorescence and the large size of the vessel limits imaging to the upper side of the artery and may exclude other areas of interest (eg, the branching point at the bifurcation fork), our data demonstrate the feasibility and utility of the approach to investigate thrombo-inflammatory events in areas where leukocytes accumulate and plaques form.

Another limitation of the method is the persistence of lateral movements of the vessel because of the compression of the artery against the stabilizing surfaces and residual respiratory movement; nonetheless, we show the stabilization to be sufficient for 2-photon imaging when combined with respiration-triggered acquisition. This improvement allowed, for the first time, detailed analyses in 3D of the intravascular lesion over time and revealed clusters of neutrophils crawling in intimate contact with the endothelium. Caution should also be exerted with the choice of anesthetics used for mouse preparation, as some mixtures may result in alterations in global hemodynamics36 and may introduce potential alterations in the behavior of inflammatory leukocytes and platelets in the atherogenic artery.

Using these new tools, we show that myeloid cell recruitment to the injured arterial wall occurs early during atherogenesis. Whereas the role of monocytes and macrophages is widely accepted, neutrophils have only been recognized recently to participate in disease progression.38 The observation that platelet recruitment is favored by neutrophils already present in the atherosclerotic lumen reconciles the established roles of platelet-derived factors with the proatherogenic functions of neutrophils in the early stages of the disease.13 We also demonstrate that leukocyte recruitment in injured areas occurs through the same fundamental steps reported in the microvasculature,4 with cells transitioning from rolling to firm arrest, with subsequent polarization and crawling on the endothelium. Interestingly, interactions between platelets and polarized neutrophils have been associated to other forms of acute vascular inflammation77 and may underlie the initial stages of endothelial damage associated with atherosclerosis. Altogether, these findings illustrate the potential of this approach to study relevant thrombo-inflammatory events that may occur during atherogenesis, including the recruitment of microparticles,28 formation of thrombi,29 or the deposition of DNA-based structures from activated neutrophils.9 Identification of subcellular domains on leukocytes and small luminal CD62L +–debris suggests that the method should be also useful to study the role of small biological structures (eg, lipid deposits, cellular microdomains, or apoptotic bodies) in atherosclerosis. We propose that this approach will be instrumental for the study of the thrombo-inflammatory phenomena that underlie atherosclerosis and other forms of arterial disease.

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Disclosures

None.

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Supplemental Material

High-resolution imaging of intravascular atherogenic inflammation in live mice

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Online Methods

Mice. Mafia: ApoE\(^{-/-}\) mice were generated by crossing ApoE\(^{-/-}\) (Charles River, Lyon, France) with Mafia mice, which express GFP under the control of the c-fms promoter.\(^1\) Alternatively, Lysmegfp/egfpApoE\(^{-/-}\) mice\(^2\) were used to visualize myeloid cells. All mice were in the C57BL/6J background and were housed in a pathogen-free barrier facility. For diet-induced atherosclerotic studies, 6 to 8 week-old male mice were fed an atherogenic high-fat diet (HFD: 10.8% total fat, 0.75% cholesterol, S8492-E010, Ssniff, Germany) for the indicated periods of time. All experimental protocols were approved by the local authorities for animal experimentation.

Surgical preparation and stabilization of the carotid artery. Mice were anesthetized with a mixture of ketamine (Imalgene 1000; Merial, France) and medetomidine (Medeson; Urano, Spain) (50 mg/kg and 0.5 mg/kg, respectively). The neck was shaved, and mice were immobilized in decubitus position. The right carotid artery was exposed and carefully dissected from the surrounding tissues as described.\(^3\) In short, sutures were used to maintain the salivary gland and adjacent muscles (omohyoid muscle, mastoid part of the sternocphalic muscle and the sternothyroid muscle) away from the artery (Figure 1A), and the right vague nerve was carefully separated from the artery. Throughout the procedure, warm saline was applied to the tissues. A flat metal piece with a beveled edge (back end of a surgical blade) was placed under the vessel, while the other side of the piece was fixed to a lateral holder (Figure 1B). Finally, an image-grade round coverslip (12 mm in diameter) cut in half was placed over the artery (0.5 mm diameter) and flexibly fixed to the bottom support using water insoluble modeling clay (Figure 1C). Gentle pressing on the coverslip led to a separation of approximately 400±50 μm between both supports, which visually restrained the vertical pulsatile movements without significantly compressing the artery. The stabilized region of the artery was then placed under the water-dipping objective of a multichannel epifluorescence microscope or a two-photon microscope equipped with a resonance scanner (Figure 1D). The humidity and temperature of the tissue were maintained through constant dripping of saline at 37ºC delivered onto the objective through a water-jacketed heating coil (Radnoti, Monrovia, CA).

Whole mount confocal laser scanning microscopy. Carotid arteries from Mafia: ApoE\(^{-/-}\) mice fed high fat diet for 6 weeks were recovered and fixed in PFA 2% overnight. Whole arteries were mounted under a coverslip and images were captured using a Leica TCS-SP5
confocal scanning laser unit attached to an inverted epifluorescence microscope (DMI6000B) fitted with a 20X/0.75 NA multi-immersion objective. 3D reconstruction of arterial wall and atheroma plaque were performed using IMARIS software (Bitplane, Zurich, Switzerland).

Intravital microscopy systems.
The intravital microscopy system was built by 3i (Intelligent Imaging Innovations, Denver, CO) upon an AXIO Examiner Z.1 work station (Zeiss, Oberkochen, Germany) mounted on a 3-Dimensional Motorized Stage (Sutter Instrument, Novato, CA) for fast control of the focal plane and precise computer-controlled lateral movement between XY positions. The microscope was equipped with a CoolLED pE widefield fluorescence LED light source system (CoolLED Ltd. UK), and a quad pass filter cube was used with a Semrock Di01-R405/488/561/635 dichroic and FF01-446/523/600/677 emitter. We used plan-APOCHROMAT 40X NA1.0 and N-ACHROPLAN 10X NA0.3 water-immersion objectives (Zeiss). Images were captured with CoolSnap HQ² camera (Photometrics, Tucson, AZ). The SlideBook software 5.0 (Intelligent Imaging Innovations) was run on a Dell Precision T7500 computer system (Dell Inc., Round Rock, TX) to coordinate image acquisition and for offline data analysis. In some single-channel experiments we also used a Leica DM6000-FS using an Apo 63X NA 0.9 water-immersion objective, equipped with a DFC350-FX camera and the LAS-AF software for acquisition and image processing.

For multidimensional and multichannel imaging, we used an upright Leica SP5II MP TPLSM (Leica Mannheim, Germany) equipped with resonance scanner, a HCX Apo 20 X NA 1.00 water dipping objective and a pre-chirped, pulsed Ti:Sa laser (MaiTai DeepSee HP, Spectra Physics, USA) tuned to 820 nm for two-photon fluorescence excitation. When needed optical zoom was applied to further enhance spatial resolution. Emitted fluorescent signals were detected by three internal Hybrid detectors (HYD) tuned for the corresponding wavelengths using an acousto-optical beam splitter (HYD1; 390-465 nm for autofluorescence of elastin and second harmonics generation of collagen, HYD2; 500-538 nm for GFP and autofluorescence of elastin, HYD3; 570-612 nm for Tritc-Dextran). Single images (512×512 pixels) were recorded in resonance mode in the xy-plane and subsequently collected at successive depths using a Leica galvo-z-drive at 40 Hz (distance between the xy-planes was 0.6-4 μm) and line averaging (3-4 x). The resulting acquisition rate achieved was 7.5-10 Hz. All single images (of XYZT series) were acquired with the aid of respiration triggering to further reduce the movement artifacts caused by respiration. Dependent on the settings, triggered resonance acquisition resulted in an overall temporal
resolution of 1-3 Hz and a spatial resolution of \( \text{XY} \approx 1.5-2.5 \text{ µm}, \text{Z} \approx 2.0-5.0 \text{ µm} \). Triggering was performed using a small animal trigger unit (Rapid Biomedical, Würzburg, Germany) as described previously.\(^4\) TPLSM data were processed and analyzed with LAS AF 3.0 (Leica), Imagepro Analyzer 3D (version 7.0, Media Cybernetics, USA), and ImageJ 1.47v. Image processing consisted mainly of merging of successive image series, noise reduction performed by 3D Gaussian filtering (\( n=1 \); size 3x3 or 5x5; strength 5-8), and generation of maximum intensity extended depth of field projections and 3D (isosurface) reconstructions.

**Antibodies.** Fluorescently-conjugated anti-mouse CD4 (clone H129.19), CD8a (clone 53-6.7), CD62L (MEL-14), CD41 (clone MWReg30) were purchased from eBiosciences (San Diego, CA) and CD49b (clone HMa2) was purchased from BD Biosciences Pharmingen (San Diego, CA). The JON/A antibody specific for the active form of mouse \( \alpha \text{IIb} \beta 3 \) integrin was from Emfret Analytics (Eibelstadt, Germany). The anti-Ly-6G antibody (clone 1A8; BioXcell, Lebanon, NH) was labeled using Dylight 405 and Dylight 650 Antibody Labeling Kits (ThermoScientific; Rockford, IL) following the manufacturer’s instructions. For neutrophils and monocytes depletion, the anti-Ly-6G antibody (clone 1A8; BioXcell) and anti-Ly-6G/C antibody (clone GR-1; BioXcell, Lebanon, NH) were injected intra-peritoneally for two days (50 µg / mouse / day). For controls, we injected rat IgG (rIgG, Sigma).

**Intravital imaging and analyses.**

**Detection of VCAM-1 and rhodamine-labeled cells.** For luminal detection of VCAM-1, 40 µL of green- Neutravidin-labeled microspheres (Ex/Em 505 / 515, 1 µm in diameter; Molecular Probes) were coupled to 10 µg of biotinylated-anti CD106 antibody (eBioscience). Following manufacturer instructions, beads and antibodies were incubated for 1 hour at room temperature, washed twice and subsequently intravenously injected in ApoE\(^{-/-}\) mice right before imaging. As a control, microspheres conjugated with non-specific rat IgG (Sigma) or microspheres alone were injected and did not bind to the atherosclerotic areas. Luminal cells of the carotid artery were labeled by injecting 50 µg of rhodamine 6G (Sigma). Images were acquired under a using a 10x NA 0.3 objective, with 4 x 4 binning in the FITC and Cy3 channels, using a CoolSnap camera. In some experiments, rhodamine-labeled leukocytes were imaged using a 40x NA 1.0 objective. Single-channel acquisitions were performed at speeds of up to 9 Hz.
Labeling of leukocyte subsets and rolling measurements. Circulating leukocytes were labeled in vivo by intravenous injection of phycoerythrin (PE)-conjugated anti-CD4 (0.4 µg/mouse), PE-conjugated anti-CD8 (0.4 µg/mouse) and Dy650-conjugated anti Ly6G (1 µg/mouse) antibodies into mice prepared for imaging as described above. Images were captured using a 40x NA 1.0 objective and the CoolSnap camera with 4 x 4 binning in FITC, Cy3 and Cy5 channels (1.7Hz). The number of rolling cells was determined by counting the number of leukocytes crossing an imaginary line perpendicular to the vessel during at least 30 seconds. Several fields (typically 5) were imaged from the carotid bifurcation, or from the common carotid artery, and each data point represents one field. Although the appearance of plaques in the carotid artery is heterogeneous, for visualization we chose areas prone to plaque development (see Figure 1E-F). Data were normalized to the number of rolling cells per minute. Rolling velocity (in μm/s) was determined by measuring time and distances travelled (for an average of 70 µm) by individual leukocytes using a digital caliper with the Slidebook software, (Intelligent Imaging Innovations), and is shown as μm/s. Five to ten leukocytes were analyzed per field and each data point represents one leukocyte.

Leukocyte polarization and crawling. Mafia:ApoE<sup>−/−</sup> mice were injected with an allophycocyanin (APC)-conjugated anti-CD62L antibody (0.5 µg/mouse). Images were captured using the CoolSnap camera with 2 x 2 binning in the FITC and Cy5 channels at 2.5Hz. For offline analyses of CD62L redistribution, a longitudinal line was defined across the middle axis of selected GFP+ cells and fluorescence intensity in the Cy5/APC channel was measured along the line using the Metamorph software (Molecular Devices, Sunnyvale, CA). To track polarization over time, the CD62L+ area associated to a given cell was measured at various time points and normalized as a percentage of the cell area. For offline analysis of leukocyte crawling and rolling, 33 cells were tracked in the CD62L/Cy5 channel using the ImageJ software (National Institutes of Health, USA), and directionality, distance and velocity of each cell were calculated using built-in algorithms or plug-ins.

Platelet recruitment. To track platelet behavior, we injected Dy650-conjugated anti Ly6G (1 µg/mouse), PE-conjugated anti αIIbβ3 (JON/A; 1 µg/mouse), PE-conjugated anti CD49b (1.6 µg/mouse) and APC-conjugated anti-CD41 (0.4µg/mouse) antibodies into Mafia:ApoE<sup>−/−</sup> mice right before imaging. Images were captured using the CoolSnap camera with 4 x 4 binning in DAPI, FITC, Cy3 and Cy5 channels at 1Hz, or in FITC and Cy5 channels at 2.5Hz. For quantification of platelet adhesion, fields were divided in three categories: “Low adhesion” (absence or very low presence of adherent GFP+ myeloid cells),...
“Myeloid clusters” (strong presence of singly adherent GFP+ cells and small autofluorescent structures), and “Lesion shoulder” (presence of three dimensional GFP+ myeloid structures). Activated platelets were defined as CD41+ JON/A+ events. Images were acquired for 1 to 2 minutes, and adherent CD41+ platelets were counted for each field. For plaque reconstruction, images acquired in various regions of atherosclerotic plaques, were manually assembled into a single image. For neutrophil- and monocyte-depletion experiments, anti-Ly6G and anti-Ly6G/C antibodies were injected intraperitoneally (50 µg/mouse/day) for 2 days before imaging. In control groups, rat IgG (Sigma) or saline were injected with identical results. Results are expressed as a percent of the rIgG group.

Intravital microscopy of the cremaster muscle. Mice were anesthetized as indicated above. The cremaster muscle was carefully exteriorized, opened with cautery, and secured across a Plexiglas platform with sutures. Exposed tissues were continuously superfused with endotoxin-free saline warmed to 37°C, and imaging and analyses were performed as reported.6

Four dimensional (4D) imaging of cell recruitment and crawling. Prior to TPLSM imaging, Lysm<sup>egr/egr</sup>ApoE<sup>−/−</sup> mice with fluorescent myeloid cells on HFD for 4 weeks were administered with 150 kDa TRITC- Dextran (Sigma; 2.5µg/100µl) via a jugular vein catheter for discrimination between luminal space and the vessel wall. Imaging was performed in naturally respirated mice at 37°C (using a climate chamber), HBSS solution (Gibco) was constantly perfused in order to maintain the water column between objective and artery. XYZT-stacks were recorded in the mid-section of the stabilized common carotid artery. Acquisition was controlled for each individual optical frame by respiration triggering. The acquisition rate of 7.5-10Hz was sufficiently fast to enable recording of each individual frame in between respiratory cycles (thereby avoiding motion artifacts).

Quantification of arterial stability. To estimate the Pearson’s colocalization coefficient of autofluorescent structures in the vessel wall of non-stabilized and stabilized carotid arteries, we acquired image sequences at 5Hz (5 frames per second; for 10x magnification). One focus plane was used as reference, and Pearson’s colocalization coefficient was calculated for each frame of a 10 seconds sequence using the Imaris software (Bitplane, Zurich, Switzerland). Graphs represent variations of Pearson’s coefficient in colocalized volume over time.
Blood flow analysis. Fluorescent beads (red fluospheres of 1µm diameter, (Ex/Em): (580/605), Invitrogen) were injected intravenously into mice prepared for imaging of the carotid artery. The velocity of movement of free-flowing beads in diastole was evaluated by off-line analysis. For visualization of flow patterns, images were acquired with exposure times of 50 ms.

Flow cytometry. Blood samples taken from mice at different times of diet were lysed to eliminate red blood cells, and leukocytes were washed twice in PBS and incubated 20 min on ice with Dy650-anti-Ly6G, PE-anti-CD8 and PE-anti-CD4. Cells were washed in PBS and samples analyzed with an LSRFortessa analyzer (BD Bioscience, San Diego, CA). Data were analyzed using the DIVA software (BD Bioscience). Leukocyte subsets were identified as follows: Neutrophils, GFP+ Ly6G+; monocytes, GFP+ Ly6GNEG; and T-lymphocytes, GFPNEG;Ly6GNEG CD4/CD8+.

Statistical analyses. Comparison of rolling cell numbers was performed using one-way ANOVA with Turkey’s multiple comparison tests for time course comparisons, or two-way ANOVA with Bonferroni correction for multigroup comparisons. The correlation of rolling numbers and velocities was assessed by linear regression tests with 95% of confidence interval. Platelet adhesion was analyzed using one-way ANOVA and Turkey’s multiple comparison test to compare all groups with each other. For flow cytometric experiments, we used two-way ANOVA with Bonferroni correction. The GraphPad Prism 5 software was used for all statistical analyses. P values below 0.05 were deemed significant.

References


Online Figures

Online Figure I. Stability of the carotid artery.

(A) Image sequence of the carotid artery with or without stabilization from a movie captured at 3 Hz. The signal (green) corresponds to autofluorescence of the arterial wall. White dashed lines delimitate reference structures in the initial image, which remain fixed only in the stabilized preparation. Scale bars, 40 μm. (B) Image sequence of an arteriole in the stabilized cremaster muscle of a Mafia:ApoE<sup>−/−</sup> mouse, from a movie captured at 3 Hz. Scale bar, 40μm. (C) Graphical representation of Pearson's co-localization coefficient, which has a value of 1 when total colocalization occurs. Correlative images acquired under a 40x objective were aligned with a selected focus plane. Pearson's coefficient of fluorescent vessel wall structures was calculated for each time point.
Online Figure II. Anti-VCAM-1 coated beads bind specifically to the carotid bifurcation

Fluorescent beads (red) conjugated to control rat IgG (rlgG) do not bind to atherogenic regions of the carotid artery (left panel) in fat-fed ApoE−/− mice, whereas beads conjugated to anti-VCAM-1 antibody accumulate at bifurcation areas (right panel). Scale bar: 200µm.
Online Figure III. Absence of leukocyte recruitment in arterioles of the microcirculation during atherogenesis.

The cremaster muscle of ApoE−/− mice fed HFD for 3 weeks was analyzed by intravital microscopy. (A) Representative image of an arteriole with a single adherent leukocyte (small arrow). The large arrow shows the direction of flow. Scale bar, 20μm. (B) Quantification of the number of rolling and adherent cells in cremasteric arterioles. n=15 arterioles from 3 mice.
Online Figure IV. Tracking leukocyte subsets in atherosclerotic mice.

(A) Representative image showing the tracks left by rolling leukocytes during 20s on the lumen of the carotid bifurcation of a fat-fed Mafia:ApoE^{−/−} mouse. Dashed lines highlight a region of preferential rolling in this experiment. (B) Flow cytometry plots of blood leukocytes from Mafia:ApoE^{−/−} mice before and after 2 or 8 weeks on HFD. Leukocytes subsets were labeled with antibodies against Ly6G, CD4 and CD8. (C) Frequency of GFP+ Ly6G^+ neutrophils, GFP+ Ly6G^<sup>NEG</sup> monocytes and GFP^<sup>NEG</sup> CD4/CD8^+ T lymphocytes in the blood of the mice shown in (B). Data represent mean ± s.e.m. from n=4 mice. *P<0.05, **P<0.01, ***P<0.001; ns, not significant.
Online Figure V. Visualization of CD62L-immunoreactive membrane fragments.

Mafia:ApoE−/− mice fed for 3 weeks with HFD were injected with a fluorescently-conjugated anti-CD62L antibody. The left micrograph shows polarized GFP+ CD62L+ cells. The enlarged image in the middle shows one polarized GFP+ cell leaving a CD62L+ track (yellow arrowhead), as well as dispersed CD62L+ debris (white arrowheads) on the endothelium. Scale bars, 10 μm.
Online Figure VI. CD49b colocalizes with CD41 in platelets.

Mafia:ApoE<sup>−/−</sup> mice were fed HFD for one month and injected with an APC-conjugated antibody against CD41 and PE-conjugated antibody against CD49b before imaging. Representative images panels show myeloid leukocytes (green) interacting with platelets which are CD41 and CD49b positive (red/yellow respectively). Scale bars, 10μm.
**Online Figure VII. Blood populations after αLy6G and αLy6G/C depletion**

(A) Neutrophil (PMN) depletion with Ly6G (1A8) does not affect monocyte (MON) number. A group of 9 mice was injected with Ly6G antibody (clone 1A8, red bars) or control rIgG (grey bars) (both at 50 µg/day for 2 days, i.p.). (B, C) Blood populations after αLy6G and αLy6G/C depletion. Mafia:ApoE−/− mice fed for 1 month with HFD were injected with anti-Ly6G (B) or anti-Ly6G/C (C) antibodies for two days. Histograms show hematological analysis performed using Abacus Junior (Diatron). Absolute number of total white blood cells (WBC), Lymphocytes (LYM), Monocytes (MON), granulocytes (GRA) and platelets (PLT) are shown. Grey bars show blood counts before depletion, red bars show blood counts after depletion. * p<0.05, ** p<0.01, *** p<0.001
### Online Movie Captions

| Online Movie I | Real-time imaging of a non-stabilized (left video) and stabilized (right video) carotid artery in ApoE\(^{-/-}\) mice. Green signal is autofluorescence. Strong lateral and vertical movements can be appreciated in the left video. After stabilization, vertical movements are markedly reduced and only some lateral movements can be appreciated. Scale bar, 100 μm. |
| Online Movie II | Free-flowing fluorescent beads (red) on a stabilized carotid artery (green autofluorescence). Red tracks show the flow pattern of the beads, corresponding to their displacement during 50 ms. Movie-rate has been accelerated 3 times. Scale bar, 100 μm. |
| Online Movie III | GFP Myeloid cells (green) accumulate to form atheroma plaque at the level of the carotid bifurcation in Mafia: ApoE\(^{-/-}\) mice fed a high-fat diet for 2 months. Rhodamine 6G (red) was injected to label luminal cells. Movie rate has been accelerated 3 times. Scale bar, 100 μm. |
| Online Movie IV | High magnification (40X objective) imaging of a non-stabilized (left) and stabilized (right) carotid artery in ApoE\(^{-/-}\) mouse fed a high-fat diet for 4 weeks. An anti-CD11b antibody was injected to label luminal myeloid cells (blue) and an anti-CD41 to label platelets (red). Strong lateral and vertical movements can be appreciated in the left video. Vertical movements are strongly reduced and only some lateral movements can be appreciated after stabilization, allowing better imaging of bound leukocytes and platelets. Movie rate has been accelerated 3 times. Scale bar, 10 μm. |
| Online Movie V | Rhodamine-labeled leukocytes rolling or adhered on the endothelium of a stabilized carotid artery of an ApoE\(^{-/-}\) mouse fed a high-fat diet for 4 weeks. Individual endothelial cells can also be visualized. Movie-rate has been accelerated 3 times. Scale bar, 10 μm. |
| Online Movie VI | GFP-positive myeloid cells rolling, adhered or extravasated in a stabilized carotid artery of a Mafia: ApoE\(^{-/-}\) mouse fed high-fat diet for 10 days. Movie-rate has been accelerated 3 times. Scale bar, 10 μm. |
| Online Movie VII | Polarization and crawling of leukocytes on the atherogenic endothelium of a Mafia: ApoE\(^{-/-}\) mouse fed a high-fat diet for 3 weeks. One GFP+ cell arrives to this area, adheres and polarizes to form an CD62L+ uropod (white). Another GFP+ cell with a pre-formed CD62L+ uropod crawls against the flow. Movie has been accelerated 40 times. Scale bar, 10 μm. |
| Online Movie VIII | CD41-positive platelets (red) are not recruited to areas devoid of GFP+ myeloid leukocytes in the carotid artery of a Mafia: ApoE\(^{-/-}\) mouse fed a high-fat diet for 4 weeks. Movie-rate has been accelerated 5 times. Scale bar, 10 μm. |
**Online Movie IX**
Recruitment of CD41+ platelets (red) to lesions containing GFP+ myeloid cell clusters in the carotid artery of a Mafia: ApoE<sup>−/−</sup> mouse fed a high-fat diet for 4 weeks. Movie shows 2 different fields. Movie-rate has been accelerated 4 and 5 times. Scale bar, 10 μm.

**Online Movie X**
Four channel imaging of the luminal atheroma. Mafia: ApoE<sup>−/−</sup> mice were fed a high-fat diet for 4 weeks and injected with different fluorescently-labeled antibodies before imaging. High-speed acquisition allows identification of neutrophils (anti-Ly6G-Dy405, purple); myeloid leukocytes (GFP+, green); activated platelets (JON/A-PE, yellow) and total platelets (CD41, red); Platelets appear bound in regions containing GFP+ cells. Only the larger platelet cluster shows activation (JON/A+). The Movie shows 4 individual channels (from left to right: Ly6G-Dy405, myeloid cells-GFP, JON/A-PE, CD41-APC). The far-right video shows the merged channels. Movie-rate has been accelerated 10 times. Scale bar, 10 μm.

**Online Movie XI**
Two-photon imaging of the stabilized carotid artery of Lysm<sup>mgpl<sup>egfp</sup>ApoE<sup>−/−</sup> mice showing GFP+ myeloid cells crawling on the arterial wall at low magnification. Images are presented as 2D extended depth-of-field pictures using maximum intensity projection. Green are myeloid cells, blue shows collagen-derived second harmonic generation in the tunica adventitia, grey represents autofluorescence of the extracellular matrix in the tunica media; and red is 150kDa TRITC-Dextran in the circulating blood, thus demarcating the lumen. Scale bar, 75 μm.

**Online Movie XII**
Two-photon imaging of the stabilized carotid artery of Lysm<sup>mgpl<sup>egfp</sup>ApoE<sup>−/−</sup> mice showing GFP+ myeloid cells crawling on the arterial wall at high magnification. Images are presented as 2D extended depth-of-field pictures using maximum intensity projection. Green are myeloid cells, blue shows collagen-derived second harmonic generation in the tunica adventitia, grey represents autofluorescence of the extracellular matrix in the tunica media; and red is 150kDa TRITC-Dextran in the circulating blood, thus demarcating the lumen. Scale bar, 25 μm.

**Online Movie XIII**
Two-photon imaging of leukocytes crawling on the stabilized carotid artery of Lysm<sup>mgpl<sup>egfp</sup>ApoE<sup>−/−</sup> mice. Images are presented as as 3D reconstructions. Movie shows cells isolated from the XYZT series in Sup. Mov. 12 by selecting the GFP emission channel which is subsequently projected in 3D using isosurface rendering. Scale bar, 25μm.