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Endothelial Protective Monocyte Patrolling in Large Arteries Intensified by Western Diet and Atherosclerosis

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Rationale: **Nonclassical mouse monocyte (CX3CR1^{high}, Ly-6C^{low}) patrolling along the vessels of the microcirculation is critical for endothelial homeostasis and inflammation. Because of technical challenges, it is currently not established how patrolling occurs in large arteries.**

Objective: **This study was undertaken to elucidate the molecular, migratory, and functional phenotypes of patrolling monocytes in the high shear and pulsatile environment of large arteries in healthy, hyperlipidemic, and atherosclerotic conditions.**

Methods and Results: **Applying a new method for stable, long-term 2-photon intravital microscopy of unrestrained large arteries in live CX3CR1-GFP (green fluorescent protein) mice, we show that nonclassical monocytes patrol inside healthy carotid arteries at a velocity of 36 µm/min, 3× faster than in microvessels. The tracks are less straight but lead preferentially downstream. The number of patrolling monocytes is increased 9-fold by feeding wild-type mice a Western diet or by applying topical TLR7/8 (Toll-like receptor) agonists. A similar increase is seen in CX3CR1$^{+/GFP}$/apoE$^{-/-}$ mice on chow diet, with a further 2- to 3-fold increase on Western diet (22-fold over healthy). In plaque conditions, monocytes are readily captured onto the endothelium from free flow. Stable patrolling is unaffected in CX3CR1-deficient mice and involves the contribution of LFA-1 (lymphocyte-associated antigen 1) and α_4 integrins. The endothelial damage in atherosclerotic carotid arteries was assessed by electron microscopy and correlates with the number of intraluminal patrollers. Abolishing patrolling monocytes in Nr4a1$^{-/-}$ apoE$^{-/-}$ mice leads to pronounced endothelial apoptosis.**

Conclusions: **Arterial patrolling is a prominent new feature of nonclassical monocytes with unique molecular and kinetic properties. It is highly upregulated in hyperlipidemia and atherosclerosis in a CX3CR1-independent fashion and plays a potential role in endothelial protection. (*Circ Res*. 2017;120:1789-1799. DOI: 10.1161/CIRCRESAHA.117.310739.)**

Key Words: apoptosis ■ atherosclerosis ■ endothelium ■ homeostasis ■ inflammation

Intravascular interactions with the endothelium under shear conditions are a crucial feature of homeostatic and inflammatory immune cells to sense and react to local stimuli.[1] Such interactions include rolling, crawling, and patrolling, where patrolling is defined as long-distance, persistent migratory movement on the apical aspect of endothelial cells with and against the direction of flow and without immediate extravasation.[2] Patrolling was discovered in the microcirculation,[2] and all reports of patrolling to date concern microvessels in various organs.[3–5] Whereas capillaries and postcapillary venules can be readily imaged in vivo, arterial imaging poses technical difficulties, and, as a consequence, little is known about leukocyte–endothelial interactions in large arteries in situ. Patrolling in large arteries has not been described.

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Cells of the monocytic lineage show a vast repertoire of functions, and 2 individual subsets are characterized in detail. Classical (Ly-6C^{high} CX3CR1^{low}, CCR2^{high}) monocytes are highly responsive to inflammatory signals, invade inflamed tissues

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Novelty and Significance

What Is Known?

- The endothelium of microcirculatory vessels is patrolled by intraluminal nonclassical monocytes.
- Endothelial damage triggers accumulation of patrolling monocytes to initiate scavenging and repair processes.

What New Information Does This Article Contribute?

- Nonclassical monocytes patrol large arteries with different migratory and molecular properties compared with the microcirculation.
- Patrolling monocytes accumulate in hyperlipidemia and atherosclerosis, and their depletion aggravates endothelial damage.
- Therapeutic modulation of patrolling activity could be a novel way to reduce atherosclerosis.

Patrolling monocytes are crucial for the endothelial integrity of microcirculatory vessels. Their relevance in large atherosclerotic arteries had not been investigated. Here, we establish that carotid arteries are actively surveyed by nonclassical monocytes in healthy mice, suggesting that patrolling is an intraluminal surveillance mechanism throughout the entire vascular system. Arterial patrolling is markedly upregulated after TLR7 (Toll-like receptor) stimulation (viral danger signals), in hyperlipidemia and in atherosclerotic conditions. Monocyte migratory and molecular properties are different compared with the microcirculation, for example, in terms of adhesion and chemokine receptor requirements. The number of active patrolling monocytes correlates with the severity of endothelial damage during atherogenesis. Mice deficient in nonclassical monocytes show aggravated endothelial damage in atherosclerotic arteries. Together, these data establish the concept of arterial monocyte patrolling, define the migratory and molecular phenotype of patrolling monocytes, and suggest they have an endothelial protective role.

Nonstandard Abbreviations and Acronyms	
ILTIS	intravital live cell triggered imaging system
WD	Western diet

early, and can differentiate into macrophages.[6] In contrast, nonclassical monocytes (Ly-6C^{low}, CX3CR1^{high}, CCR2^{low}) exert endothelial surveillance by endovascular slow patrolling in search of tissue cues.[2,7] Mean patrolling velocity in the microcirculation is 12 μm/min (4–20 μm/min).[2] Motion patterns include waves, hairpins, and loops, with a confinement ratio (distance traveled divided by path length) of about 0.5 in venules.[2] Active patrolling depends on the $\alpha_L\beta_2$-integrin (LFA-1 [lymphocyte-associated antigen 1]).[2] Local TLR7 (Toll-like receptor)-dependent endothelial and tissue signals activate patrolling monocytes on the endothelium in a CX3CR1-dependent manner for scavenging of debris in collaboration with neutrophils.[3,8] Ly-6C^{high} monocytes are blood-borne precursors of patrollers,[9] and the transcription factor Nr4a1 controls the development and survival in the bone marrow.[10] Recently, it has been reported that the pulmonal microcirculation harbors a high amount of patrollers that are crucial in preventing pulmonal metastasis.[4]

In atherosclerosis, monocytes have gained long-standing attention as precursors of plaque phagocytes.[11,12] Both local macrophage proliferation[13] and steady recruitment of blood-borne monocytes (mostly Ly-6C^{high}) to plaque regions contribute to disease progression.[14–16] A hyperlipidemia-associated monocytosis is mostly fueled by classical monocytes,[17] but elevated nonclassical monocytes have also been reported.[18] The chemokine receptors CCR1 and CCR5 are thought to be involved in classical monocyte entry into atherosclerotic lesions,[16,18] where they can differentiate into CD11b$^+$CD11c$^+$ plaque macrophages and tip-dendritic cells with a proinflammatory phenotype.[11] Nr4a1 knockout mice lack nonclassical monocytes, show no patrolling in the microcirculation, and exhibit exacerbated atherosclerosis.[3,19,20] The latter finding is attributed to hyperinflammatory plaque macrophages.[20] The temporospatial fate of nonclassical monocytes in atherosclerosis remains unclear.

We recently developed an intravital live cell triggered imaging system (ILTIS) that allows stable in situ 2-photon microscopy imaging of unrestrained carotid arteries with intact adventitia.[21] Here, using ILTIS, we investigate the molecular and kinetic features of arterial monocyte patrolling in healthy, hyperlipidemic, and atherosclerotic conditions. We show that patrolling occurs under homeostatic conditions, is highly upregulated at early and late stages of atherogenesis, and potentially protects from endothelial apoptosis.

Methods

Animals

CX3CR1-GFP (green fluorescent protein) reporter mice were provided by S. Jung (Weizmann Institute, Israel) and backcrossed onto C57BL/6J until a genetic purity of >99% was achieved (SNP analysis by Dartmouse, Dartmouth College). ApoE$^{-/-}$ mice were obtained from Jackson Laboratory (stock number: 002052). CX3CR1-GFP and apoE$^{-/-}$ mice were crossed, resulting in CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mice.[21] Homogenous expression of CX3CR1-GFP leads to a functional protein knockout.[22] In experiments comparing CX3CR1$^{+/GFP}$ and CX3CR1$^{GFP/GFP}$ genotypes, littermates were used. Nr4a1$^{-/-}$ apoE$^{-/-}$ mice were previously described.[20] All animal experiments were approved by the local animal ethics committee.

Intravital Live Cell Triggered Imaging System

Technical details of the ILTIS setup have been described before.[21] Preparation of the large arteries was done carefully with minimal handling to prevent surgery-related inflammation. The adventitia was not removed. Tissue was kept moist with phosphate-buffered saline at 35°C to 37°C. All imaging experiments were conducted on a Leica SP5 system using a water-dipping objective (Olympus XLUMPLFL 20X NA 0.95) and an objective heater. The system is composed of a DM6000 microscope, a Ti-Sapphire laser (Chameleon Ultra II, Coherent), tuned to 920 nm, a resonant scan head for fast scanning, and a Leica trigger box. Three NDD (nondescanned detector) detectors were used in most of the experiments with the following filter sets: 460/40 for second harmonics, 513/50 for GFP, and 640/40 for texas red. Videos were recorded in 512×256 pixel resolution (=455×227 μm) for 30 to 90 minutes. Under ketamine/xylazine anesthesia, the heart rate was typically at about 300 to 400 bpm. An Arduino-based circuit allowed image acquisition in triplets triggered by the signal of the pulse oxymeter attached to the thigh.

Cell Tracking and Data Analysis

After ILTIS postprocessing, which included frame selection and 2-dimensional registration using self-made plugins in ImageJ,[21] a final frame rate of 1 to 1.5 frames/s was achieved. In all movies analyzed, patrolling GFP+ cells were detected using an absolute intensity threshold and tracked using Imaris autoregressive motion algorithm (Bitplane). Only tracks with a duration longer than 90 s were considered patrolling cells. To denoise residual motion artifacts of imaging, cell tracks were smoothed using Matlab as described previously.[21] For quantification, patrolling monocytes were tracked for 17 minutes in each movie unless indicated otherwise. Fast cells moving at over 100 μm/min were considered rolling.

Further details are denoted in the Online Data Supplement.

Results

Nonclassical Monocytes Patrol Healthy Arteries

We used heterozygous CX3CR1$^{+/GFP}$ reporter mice[22] for intravital imaging. In these mice, nonclassical monocytes are brightly fluorescent, whereas classical monocytes are dimly fluorescent (Online Figure I). We confirmed ex vivo that the GFP^{dim} monocytes are not detectable using ILTIS (Online Figure II). Macrophages in the vessel wall are also GFP^{high} (Figure 1A); therefore, we used a blood tracer to clearly identify intraluminal monocytes (Figure 1A). In healthy 8-week-old mice on chow diet, we observed CX3CR1-GFP^{high} patrollers along the distal part of the external carotid artery (Figure 1A and 1B). The patrolling velocity averaged about 36 μm/min (0.5 μm/s) with a low confinement ratio (straightness; Figure 1C). Under these experimental conditions, most patrolling events lasted about 5 to 10 minutes until detachment (Figure 1C). To exclude a technical bias in measurements, we used the ILTIS technique on the ear microcirculation and obtained similar results to those previously published (Table 1).[2,3]

Arterial Patrolling Is Induced by TLR7/8 Agonists and Requires $\alpha_L\beta_2$ and α_4 Integrins

It has been reported that the local TLR7/8 agonist R848 increases the number of patrolling monocytes in venules because of endothelial activation.[3] Topical application of R848 on the carotid artery in healthy 8-week-old mice attracted CX3CR1-GFP^{high} patrollers in a time-dependent manner. Patrolling activity was increased over 2- and 10-fold after 3 and 5 hours incubation, respectively (Figure 2A and 2B). In contrast to the microcirculation,[3] LFA-1 blockade by intravenous antibodies induced detachment of only about 50% of CX3CR1-GFP^{high} patrollers, as assessed by intravital microscopy (Figure 2C). Additional blocking of the VLA-4 (very-late antigen 4) integrin (α_4 blockade) abolished most patrolling (Figure 2C). Although the directionality showed a flow bias (downstream) at baseline conditions, blocking LFA-1 or VLA-4 revealed a pan-directional patrolling pattern (Figure 2D). The net velocity decreased in response to R848 (Figure 2E). After LFA-1 and LFA-1/VLA-4 blockade, the motion characteristics of the remaining patrollers did not significantly change (Figure 2E). These results suggest that endothelial TLR7/8-dependent

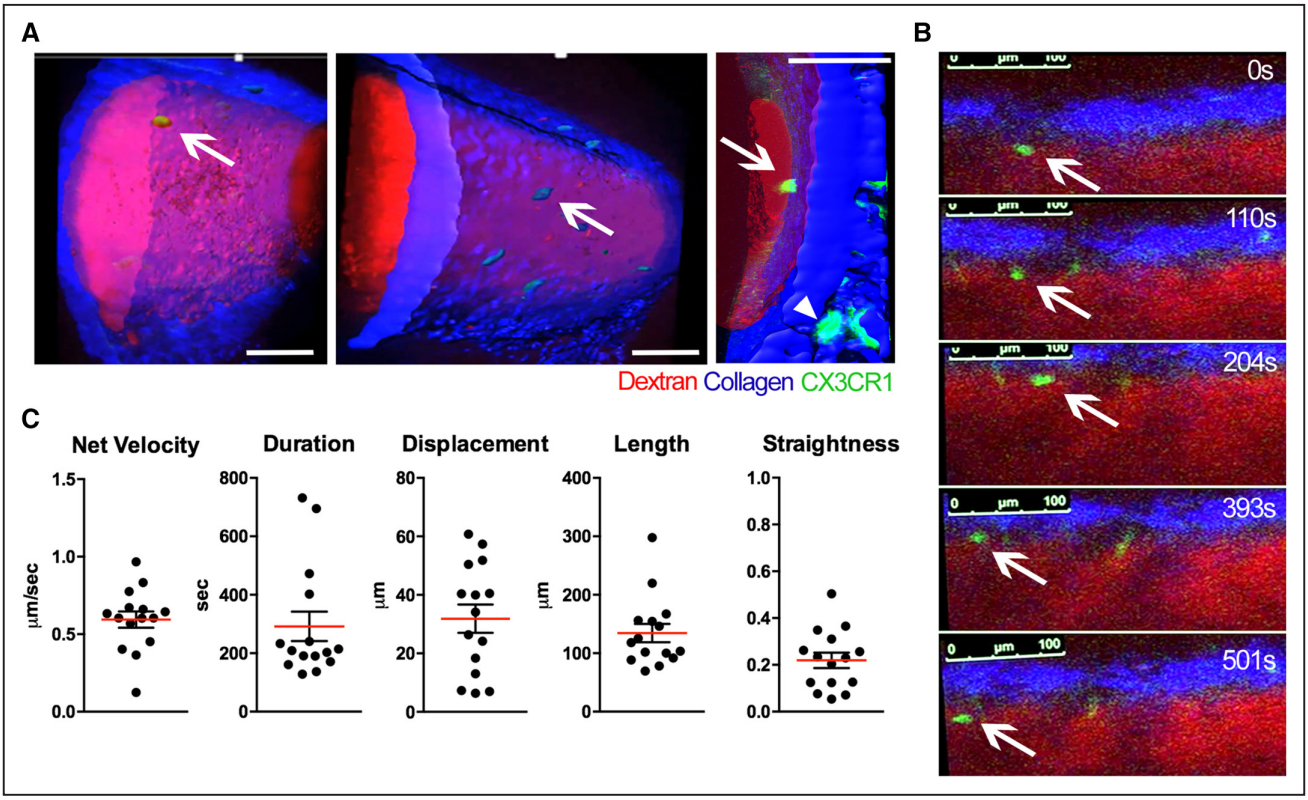

Figure 1. Nonclassical monocytes patrol healthy arteries. A, Three-dimensional (3D) reconstruction showing a CX3CR1-GFP^{high} (green fluorescent protein) monocyte (arrow) patrolling in the lumen of the external carotid artery of a 3-mo-old CX3CR1$^{+/GFP}$ reporter mouse. The image was acquired in a nonbeating artery in situ shortly after an intravital recording. Few tissue-resident CX3CR1-GFP^{high} macrophages are visible in the media/adventitia (**right**, triangle). Scale bar=50 μm. **B**, Time-lapse images of a patrolling monocyte (arrow) moving along the endothelium of the carotid artery in a live CX3CR1$^{+/GFP}$ mouse at a heart rate of 380 bpm. Blood flow from right to left. Scale bar=50 μm. **C**, Kinetic features of monocytes patrolling healthy arteries. Each dot represents 1 cell. Mean and SEM shown for 15 patrollers in 5 animals.

Table 1. Using ILTIS Microscopy, Monocyte Patrolling in CX3CR1$^{+/GFP}$ Mice Was Analyzed in Both the Ear Microcirculation and the Carotid Artery, and Motion Characteristics Were Determined

	Carotid Artery	Ear Microcirculation	Ear Microcirculation[2]
Microscopy technique	ILTIS	ILTIS (10 stack in Z)	Confocal (10 stack in Z)
Duration, min	4.7±3.1	14±3	range 9–12
Length, μm	134±58	249±83	≈220
Displacement, μm	31±18	162±20	≈100
Confinement ratio	0.22±0.13	0.63±0.13	≈0.5
Velocity, μm/min	36±12	17±5	12

Data shown as mean±SD n=5/15 (carotid) and 2/9 (ear) animals/cells. Data derived from Auffray et al[2] were added as independent reference. GFP indicates green fluorescent protein; and ILTIS, intravital live cell triggered imaging system.

signals effectively trigger patrolling in arteries in an LFA-1- and integrin α_4-dependent manner.

Increased Patrolling in Hyperlipidemia Independent of CX3CR1

Next, we investigated the impact of hyperlipidemia on patrolling activity. In CX3CR1$^{+/GFP}$ apoE$^{+/+}$ mice on Western diet (WD) for 4 weeks, we observed a 9-fold increase in CX3CR1-GFP^{high} patrolling compared with baseline (Figure 3A; Online Movie I). Comparably, in CX3CR1-GFP^{high} apoE$^{-/-}$ mice on chow diet (aged 4–6 months), an 8-fold increase was detected (Figure 3A and 3B). Similar to R848 induction, patrolling could be blocked by LFA-1 and VLA-4 antibody inhibition (Figure 3C). Migration analysis over 45 minutes shows that some areas of the endothelium were preferentially frequented in the carotid artery (Figure 3D; Online Movie II). The overall directionality of their endothelial migration was downstream (Figure 3E and 3F). Both models of hyperlipidemia showed no significant differences in patrolling microkinetics (Figure 3G).

Homozygous expression of CX3CR1-GFP leads to a functional protein knockout that allows to study monocyte behavior in CX3CR1-deficient conditions.[2,22] In these mice (CX3CR1$^{GFP/GFP}$ apoE$^{-/-}$ on chow diet), the number of patrolling monocytes and the migration pattern was similar to heterozygous CX3CR1$^{+/GFP}$ apoeE$^{-/-}$ mice, suggesting that patrolling in large arteries occurs independently of CX3CR1 (Online Figure III).

Massive Patrolling in Atherosclerotic Arteries

In CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mice fed WD for 6 weeks, arterial plaques were present in the carotid artery (Figure 4A and 4B).[21] High and low GFP expression of nonclassical and classical monocytes, respectively, was confirmed in these mice using flow cytometry (Online Figure I). NK- and T-cell subsets are known to be GFP positive in CX3CR1-GFP mice.[22] Therefore, we tested whether GFP is an adequate marker to identify patrolling monocytes in arteries with active plaques. Atherosclerotic aortas, including carotid arteries, were explanted, flushed, and the flow through analyzed by flow cytometry. This experiment confirmed that the majority (about two thirds) of intraluminal GFP^{high} cells in atherosclerotic arteries are patrolling monocytes (Online Figure IV).

In these atherogenic conditions, we observed many GFP^{+} macrophages in the plaque (Figure 4A), whereas patrolling monocytes could be identified in the lumen in contact with the plasma tracer (Figure 4B). Of all interacting intravascular CX3CR1-GFP–positive cells, about 60% to 70% were patrolling, and about 30% of these showed jerky patrolling with intermittent rolling (Figure 4C). We observed a strong increase in patrolling activity, about 22-fold over healthy conditions, and about 2- to 3-fold over apoE$^{-/-}$ on chow diet (Figure 4D). The patrolling phenotype differed between plaque-close and plaque-distant areas. Patrolling monocytes in plaque proximity showed a lower velocity, longer duration, and a lower confinement ratio (Figure 4E).

The time resolution of ILTIS is about 1 frame/s (after frame selection), which allows us to investigate kinetic events before and during patrolling. Many GFP^{+} cells attached to the endothelium from free flow (Online Movies III and IV). Frame-by-frame analysis revealed that short rolling interactions often precede patrolling (Figure 5A and 5B). Intermittent jumps (short spikes in velocity, ie, rolling) during patrolling were also evident (Figure 5A; Online Movies III and IV).

Arterial Patrolling Is Associated With Endothelial Damage

We analyzed the endothelium of the carotid artery in hyperlipidemic and atherosclerotic conditions using transmission electron microscopy. Morphological signs of endothelial damage included loss of electrodensity, vacuolization (incipient signs), cytoplasmic edema, chromatin fragmentation, nuclear condensation, enucleation, cell shedding, and denudation (severe signs). The endothelial cell layer in control mice on chow diet was healthy (Figure 6A and 6B; Online Figure V). WD and apoE knockout increased the extent of damaged endothelial cells per section, and apoE$^{-/-}$ mice fed WD showed a further increase (Figure 6A and 6B; Online Figure V). The extent of endothelial cell damage significantly correlated with the number of patrolling monocytes we observed in these conditions using ILTIS (Figure 6C), suggesting a response to local damage.

Nr4a1-deficient mice have been shown to lack most patrolling monocytes in the blood.[10] These mice on an apoE$^{-/-}$ background consuming WD develop aggravated atherosclerosis.[20] We first asked whether patrolling monocytes are indeed depleted locally from the endothelium of atherosclerotic arteries. By flushing explanted aortas (Figure 7A), we confirmed fewer monocytes on the endothelium, and the remainders were classical monocytes (Figure 7B). In transmission electron microscopy analyses, the carotid endothelium of Nr4a1-deficient mice showed aggravated endothelial damage (more lesions per section; Figure 7C and 7D; Online Figure V). Compared with Nr4a1$^{+/+}$ apoE$^{-/-}$ controls, the lesions preferentially comprised multiple severe signs of cell death. A TUNEL apoptosis assay confirmed a higher number of damaged endothelial cells in Nr4a1-deficient mice (Online Figure VI).

Discussion

We show that patrolling monocytes monitor the endothelium of healthy carotid arteries. Net velocity of arterial crawling is

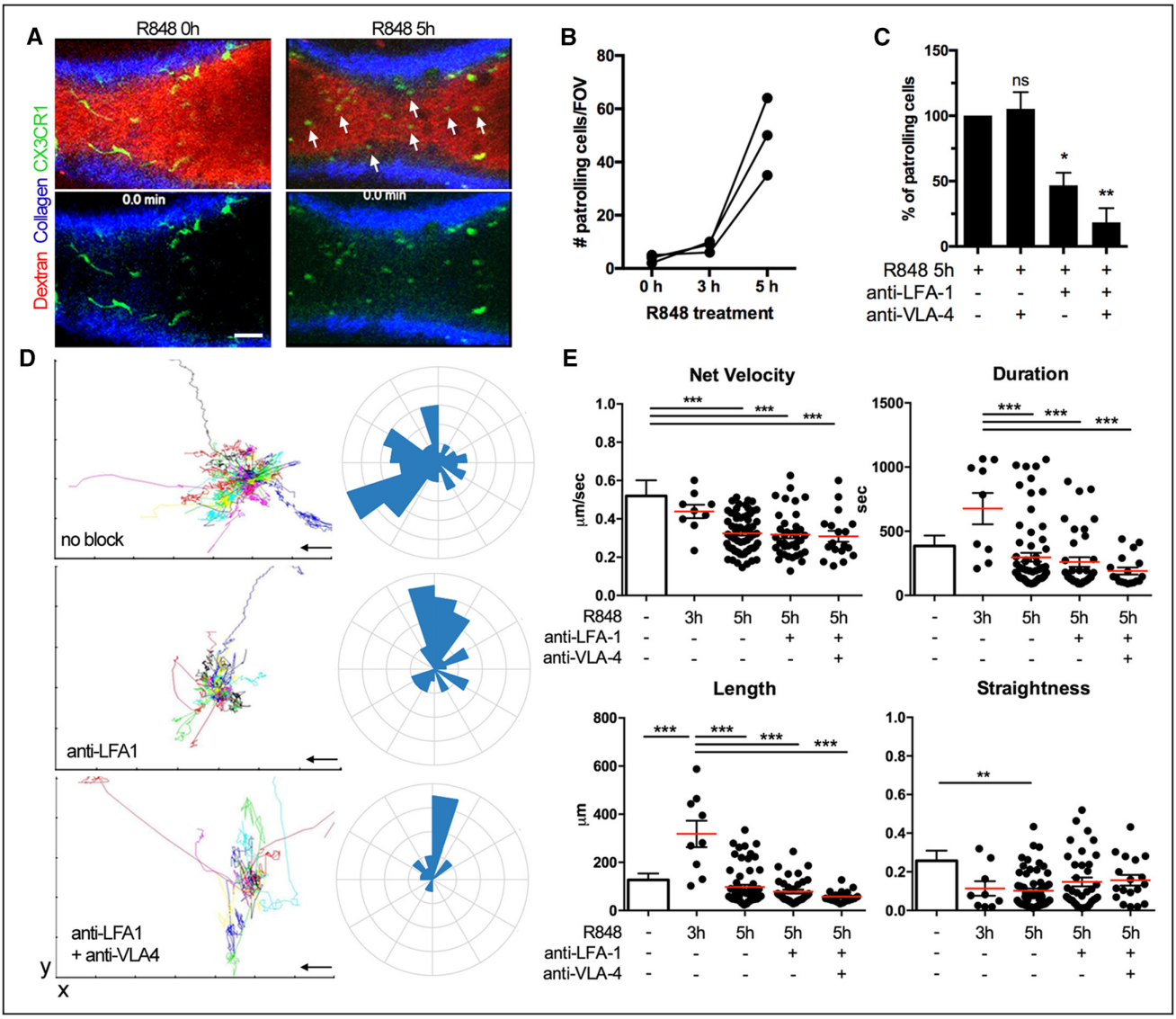

Figure 2. Arterial patrolling induced by the TLR7/8 (Toll-like receptor) agonist R848. A, Patrollers in the carotid artery imaged before and after topical application of the TLR7/8 agonist R848. The red channel is omitted in the **bottom** row. Scale bar=50 µm. Arrows indicate patrolling CX3CR1-GFP^{high} (green fluorescent protein) cells. **B**, Quantification of patrolling CX3CR1-GFP^{high} cells in the carotid artery after R848 stimulation assessed by intravital microscopy. The time course in 3 animals is shown. **C**, Impact of function blocking anti-LFA-1 (lymphocyte-associated antigen 1) and anti-VLA-4 (very-late antigen 4) antibodies on patrolling. After 5-h R848 treatment, antibodies were injected intravenously, and the reduction of numbers was expressed relative to untreated. One-way ANOVA with Tukey multiple comparison. *P<0.05, **P<0.01, n=3 animals. Mean±SEM shown. **D**, Tracks are shown as spider plots after aligning the starting positions for untreated, LFA-1, and LFA-1+VLA-4 inhibited patrollers. Rose plots visualize the overall directionality. Tracks are randomly colored. The arrow indicates the blood flow. **E**, Patrolling characteristics in R848- and antibody-treated conditions compared with baseline data shown in Figure 1. Every dot represents 1 cell. Data shown as mean±SEM of 3 independent experiments. One-way ANOVA corrected for multiple comparisons (Tukey). *P<0.05, ** P<0.01, *** P<0.001.

about 3× as fast as in the microcirculation (Table 1), and tracks preferentially lead downstream (with the blood flow), despite a low confinement ratio. Endothelial stimulation via TLR7/8 agonists, hyperlipidemia, and atherosclerosis triggers the accumulation of patrollers, with the latter condition having a 22-fold increase over baseline. Patrollers are readily captured from free flow, show short- and long-term interactions, and can alternate patrolling with rolling. Sequential blocking of LFA-1/VLA-4, but not VLA-4 alone, triggers detachment of arterial patrollers. The number of patrolling monocytes in situ correlates with the local endothelial damage, and patroller-deficient Nr4a1$^{-/-}$ mice show increased signs of endothelial cell death. These data establish the concept of arterial Ly-6C^{low} monocyte patrolling, lay out the fundamental migratory and molecular phenotype, and suggest an endothelial protective role.

An elegant study by Chèvre et al[23] provided insight into neutrophil interactions in atherosclerotic carotid arteries by applying mechanical stabilization of the vessel. In our study, a minimum video acquisition time of about ≥20 minutes was necessary to effectively describe slow monocyte patrolling. As vascular mechanical forces, including pulsatile flow, have been shown to impact on cellular responses,[24,25] long-term acquisition data with mechanical stabilization could be flawed. ILTIS microscopy does not require any physical contact with

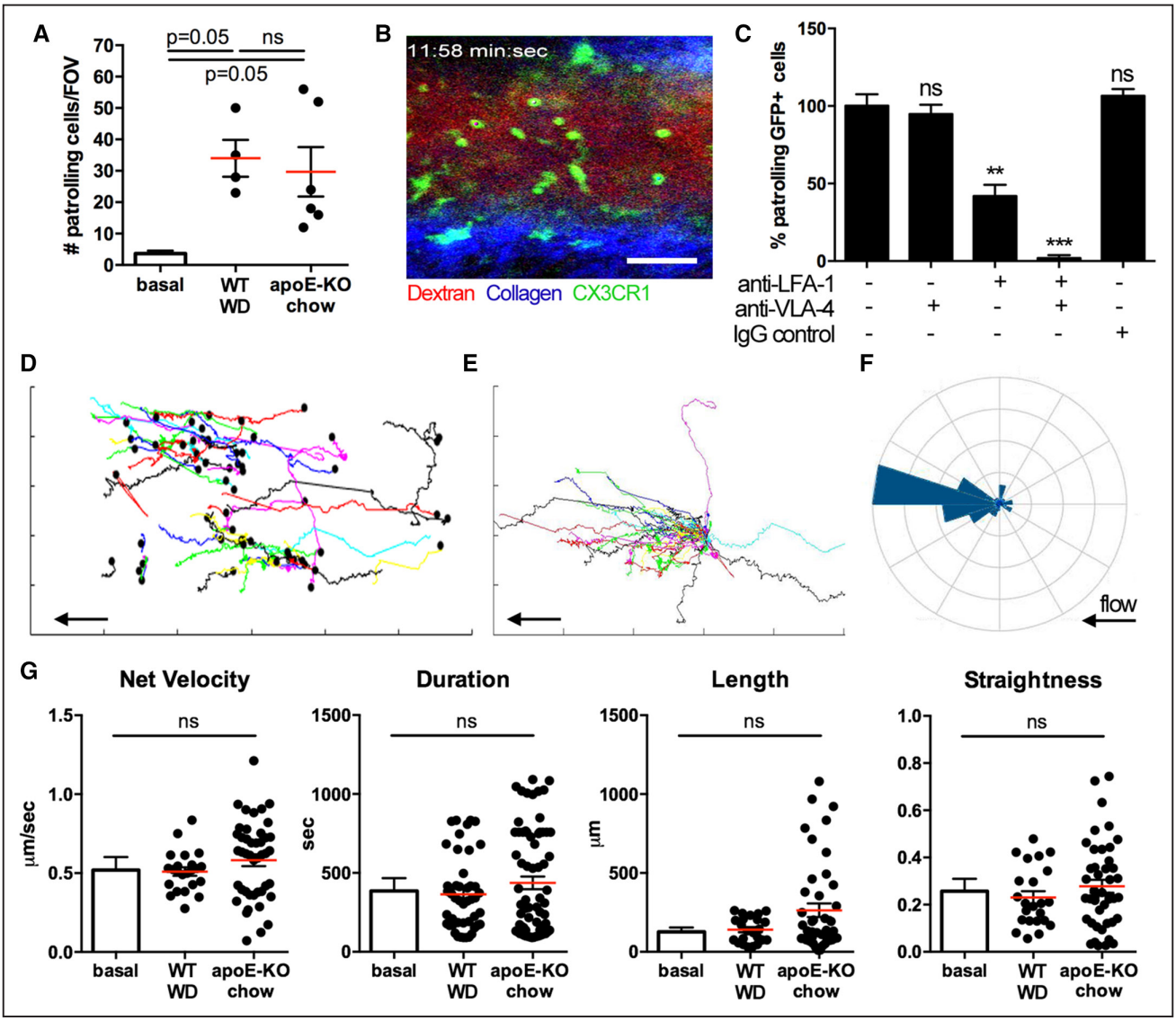

Figure 3. Hyperlipidemia intensifies monocyte patrolling in arteries. A, The number of patrollers in the carotid artery was determined by intravital microscopy in CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mice on chow diet (6 mo old) and CX3CR1$^{+/GFP}$ mice on 4 wk Western diet (WD, 3 mo old) compared with baseline as shown in Figure 1. Every dot represents 1 animal. Data as mean±SEM. One-way ANOVA with Tukey correction for multiple comparison. **B**, Representative image of an intravital recording. Scale bar=50 µm. **C**, Blocking effect of anti-LFA-1 (lymphocyte-associated antigen 1) and anti-VLA-4 (very-late antigen 4) antibodies on patrolling. One-way ANOVA with Tukey multiple comparison. n=3 animals. ***P*<0.01, *** *P*<0.001. **D**–**F**, Analysis of all patrollers in a CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mouse on chow diet in a 45-min recording shown in **B**. Patrolling tracks are graphed with absolute (**D**) or relative coordinates (**E**). The arrow indicates the direction of the blood flow. The black dot marks the starting position. A rose plot (**F**) shows the directionality of patrolling (start vs end position). **G**, Kinetic features compared with baseline condition as shown in Figure 1. Data as mean±SEM from 4 (WD) and 6 (apoE$^{-/-}$) animals. Every dot represents 1 patroller. One-way ANOVA with Tukey multiple comparison.

the artery during preparation or imaging.[21] Therefore, this technique is ideally suited for the purpose of this study.

Wall shear stress is low in venules, intermediate in large arteries, and high in small precapillary arterioles.[25] Unlike patrolling in arterioles and venules of the mesentery and dermis,[2,3] we find a higher velocity, a lower confinement ratio, and no typical hairpin and loop patterns in the carotid artery. It has been shown that T effector cells tend to crawl upstream (against the flow) under high shear conditions.[26] In contrast, the overall directionality of patrollers in the carotid artery was downstream (with the blood flow). This excludes the possibility of retrograde migration from the microcirculation. We observed a significant number of blood-borne monocytes attaching from free flow in atherosclerotic conditions. This was often preceded or interrupted by a short rolling step which suggests a cascade-like fashion of endothelial interactions to slow down the cell in medium/high shear conditions.

ApoE knockout mice on chow or WD develop an 8- or 22-fold increase in serum levels of very-low-density lipoprotein/intermediate-density lipoprotein, respectively, when compared with control mice.[27] It has been reported that elevated lipids, such as β-very-low-density lipoprotein and low-density lipoprotein, promote adhesion of monocytes to

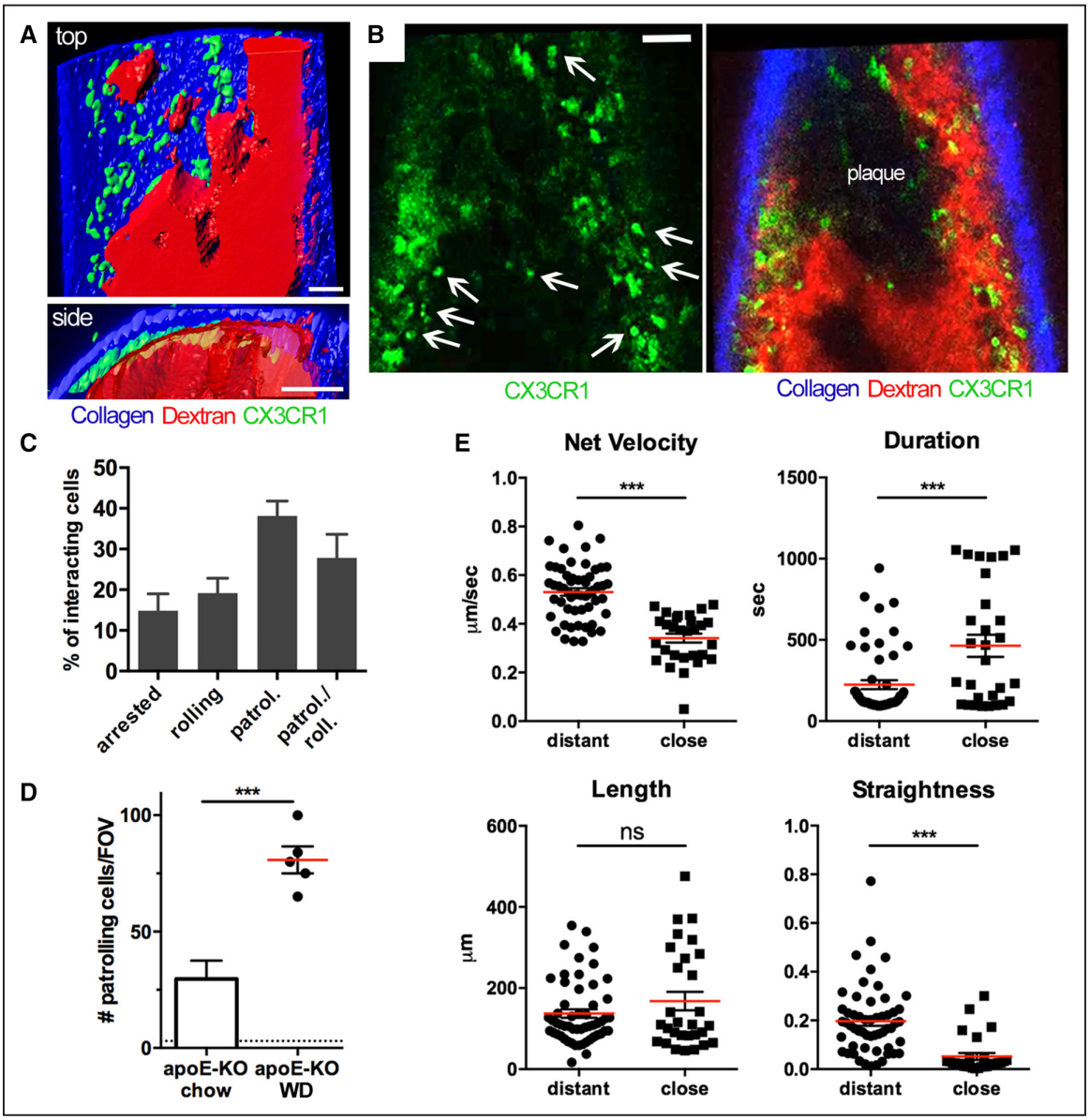

Figure 4. Massive patrolling in arteries with atherosclerotic plaque. A, Three-dimensional (3D) reconstruction in top down (**top**) and axial (**bottom**) view and (**B**) representative frames from an intravital 2D time-lapse sequence of the carotid artery of a CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mouse fed Western diet (WD) for 6 wk. The plasma tracer (red) marks nonplaque regions. Intravascular CX3CR1-GFP^{high} (green fluorescent protein) patrollers are marked by arrows. CX3CR1-GFP^{high} macrophages are located in the vessel wall. The 3D image was recorded in a nonbeating artery. All scale bars=50 µm. **C**, Relative fraction of migration phenotypes of intravascular cells in CX3CR1$^{+/GFP}$ apoE$^{-/-}$ mice fed WD for 6 wk. Patrolling/rolling indicates alternating patterns during video acquisition. n=6 animals. Data as mean±SEM. One-way ANOVA corrected for multiple comparisons (Tukey). *P<0.05, **P<0.01. **D**, Numbers of patrollers observed in intravital microscopy. Every symbol represents 1 animal. The bar shows data from Figure 3 as reference, and a dotted line indicates baseline (as shown in Figure 1). Unpaired t test. ***P<0.001. **E**, Kinetic features of patrollers distant and close to plaque areas. Data shown as mean±SEM. n=6 (chow) and 5 (WD) animals per condition. Every symbol represents 1 cell. Unpaired t test. ***P<0.001.

the endothelium.[28–30] Endothelial activation occurs early in atherosclerosis,[24] and metabolite-related endogenous danger signals precede monocyte infiltration.[31] Our intravital imaging data confirm early nonclassical monocyte accumulation in the carotid artery in mild hyperlipidemia. We also show that the TLR7 agonist R848 locally attracts patrolling monocytes, pointing to a conserved pathway in large arteries and the microcirculation.[3] A previous study suggested that TLR7 inactivation results in aggravated atherosclerosis.[32] Therefore, it could be hypothesized that TLR7 activation in damaged endothelium during atherogenesis is required to mount a protective response by patrolling monocytes.

In the microcirculation, R848-mediated accumulation of patrolling monocytes is CX3CR1 dependent. Notably, few patrollers also remained in CX3CR1-deficient mice. In contrast, massive patrolling in atherosclerosis is independent of CX3CR1, suggesting that the CX3CR1-independent fraction expanded. We found a requirement of the integrins LFA-1 ($\alpha_L\beta_2$) and VLA-4 ($\alpha_4\beta_1$). Whereas only LFA-1 is relevant in microcirculatory patrolling,[2,3] VLA-4 was recently

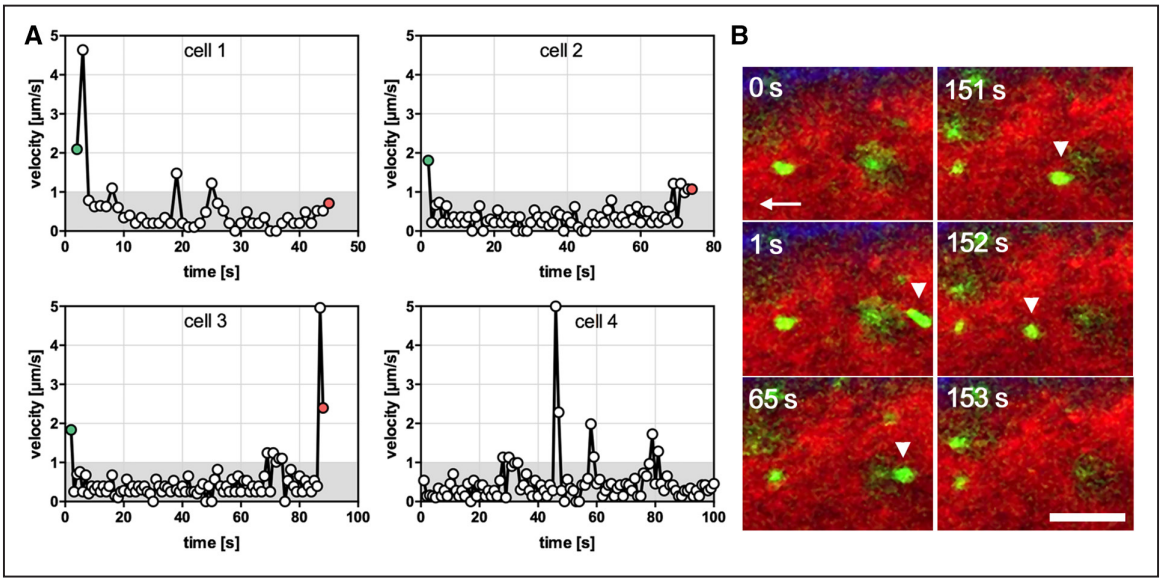

Figure 5. Microkinetics and primary monocyte capture in atherosclerotic arteries. A, Velocity profiles of 4 individual cells patrolling in atherosclerotic vessels over time (time resolution 1 frame/s). Cells 1 to 3 are captured from free flow and show short-term patrolling (< 90 s), whereas cell 4 continues patrolling with an intermittent jump. Green dots indicate capture of a blood-borne cell and red dots detachment. Grayed areas indicate that velocities <1 µm/s cannot be highly resolved. **B**, Imaging data of a CX3CR1-GFP^{high} (green fluorescent protein) patroller captured from free flow, followed by arrest, patrolling, and a short rolling phase before detachment. Scale bar=50 µm. See also Online Movies III and IV.

implicated in patrolling of renal glomeruli.[5] Furthermore, as shown in both R848-triggered and hyperlipidemic conditions, about 50% of patrollers detach on LFA-1 blockade alone, but many patrollers also require the sequential blockade of α_4 integrins to detach. This finding raises 2 main questions: first, do 2 CX3CR1-independent subsets of patrollers exist in atherosclerosis, where one is strictly LFA-1–dependent and another one can also patrol via α_4 integrins? Second, how do changes of the atherosclerotic endothelium such as VCAM-1 upregulation[33,34] affect patroller

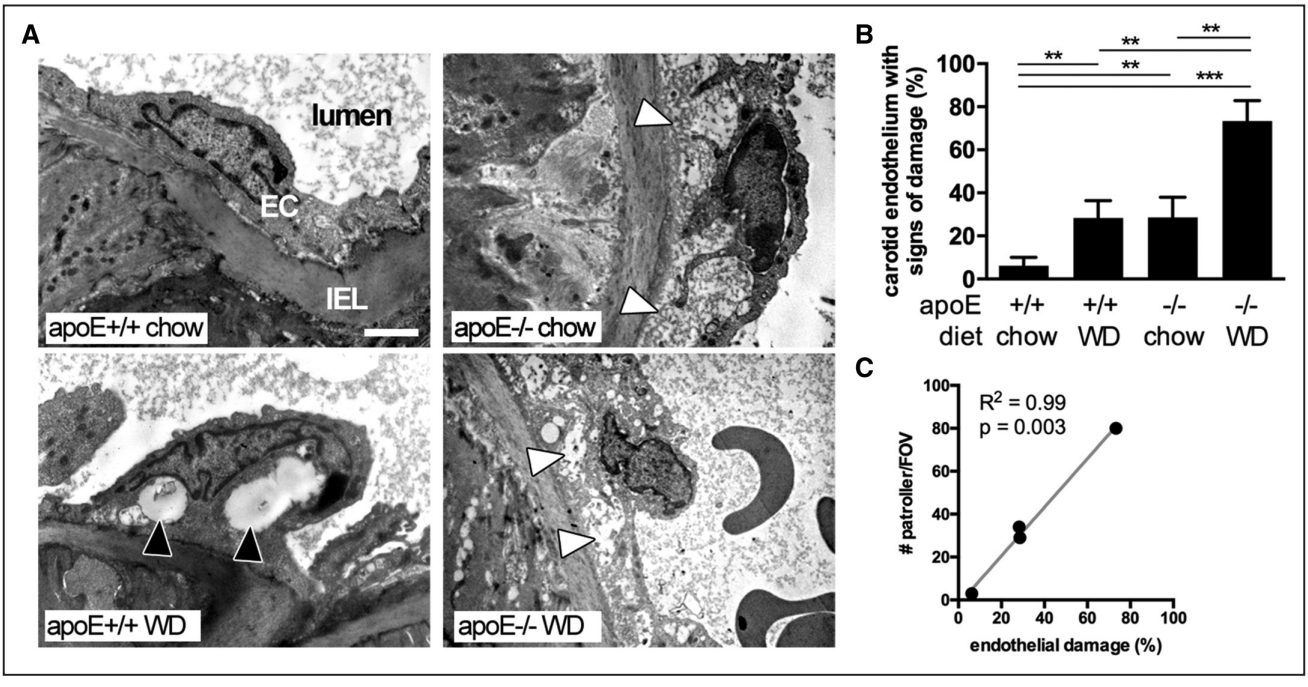

Figure 6. Correlation of monocyte patrolling and endothelial damage during atherogenesis. A and **B**, Morphological signs of endothelial cell damage were assessed in control and apoE$^{-/-}$ mice with chow or Western diet (WD, 6 wk) using transmission electron microscopy of carotid arteries. White and black arrows indicate cytoplasmatic edema and vacuolization, respectively. More images shown in the Online Figure V. **B**, Quantification of the extent of the endothelial damage, n=2/3/2/5 animals (for each condition indicated) with 12 to 15 sections each. One-way ANOVA with Tukey multiple comparison. ***P*<0.01, ****P*<0.001. **C**, Linear regression of endothelial cell damage as shown in (**B**) and active patrolling monocytes (per field of view) observed in situ by intravital microscopy. Pearson correlation, R^2, and *P* value indicated. EC indicates endothelial cell layer; and IEL, internal elastic lamina.

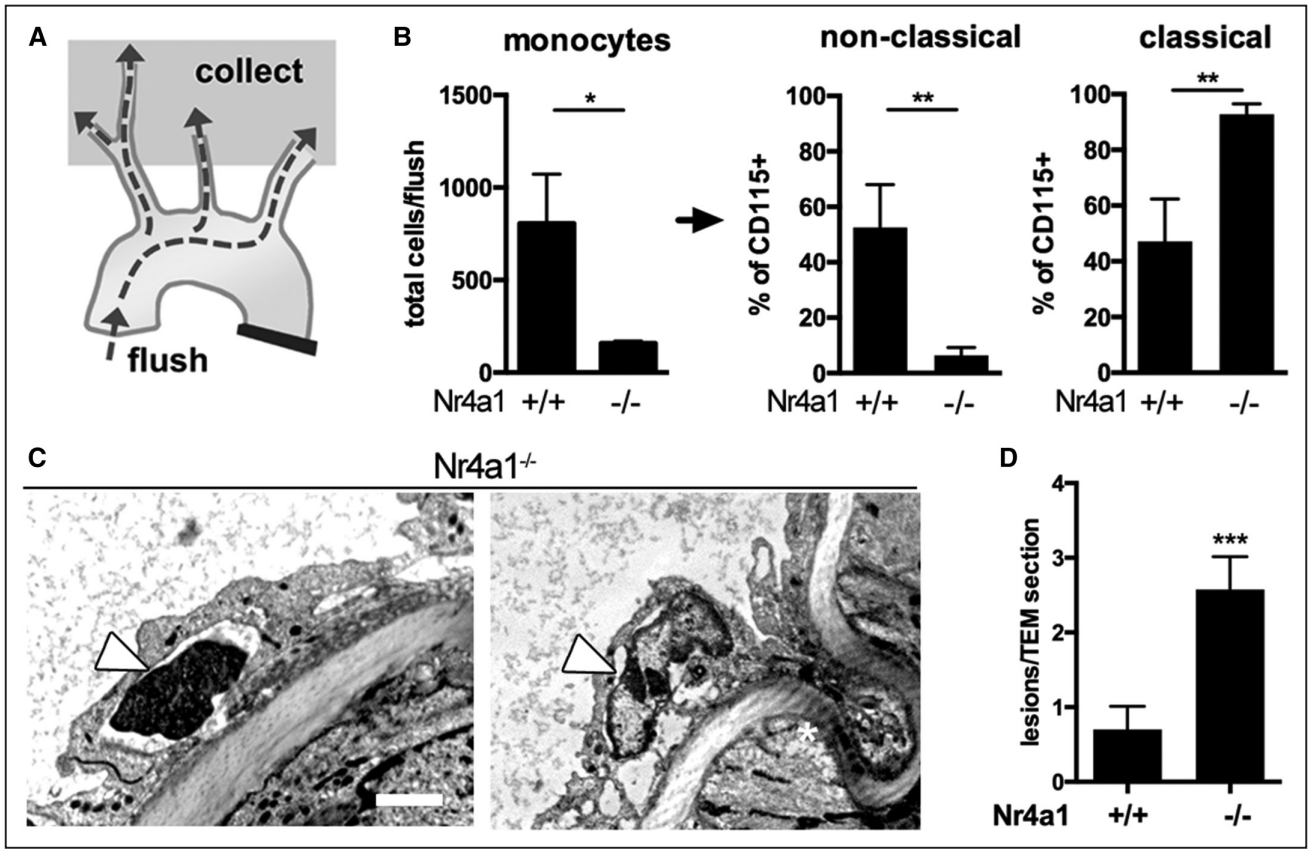

Figure 7. Enhanced endothelial damage in atherosclerotic arteries of patroller-deficient Nr4a1$^{-/-}$ mice. A and **B**, Explanted aortas of apoE$^{-/-}$ Nr4a1 knockout or control mice fed 6 wk Western diet were flushed, and the flow through was analyzed by flow cytometry. n=3 per group. Unpaired *t* test. **P*<0.05, ***P*<0.01. **C**, Transmission electron micrographs of apoE$^{-/-}$ Nr4a1$^{-/-}$ carotid arteries. Nuclear condensation (**left**) and chromatin fragmentation (**right**) are depicted as signs of severe cell damage. More images shown in the Online Figure V. **D**, Quantification of the severity of endothelial cell death (number of lesions per section as defined in the Method). n=3 animals with 12 to 15 sections each. Data shown as mean±SEM. Unpaired *t* test. ****P*<0.001.

recruitment? In this line, our data show that the plaque shoulder mediates different patrolling kinetics compared with plaque-distant sites. Interestingly, similar motion characteristics were found in R848-treated arteries, that is, very low confinement ratios and velocities (Table 2). Finally, it also needs to be considered that we found around one third of GFP^{high} cells in atherosclerotic CX3CR1-GFP mice to be nonmonocytes. Further data are required to elucidate the interplay of patrolling monocytes and endothelial cues in atherosclerotic conditions.

Table 2. Synopsis of All Parameters Acquired by Intravital Live Cell Triggered Imaging System

						apoE$^{-/-}$+WD	
	Unit	Basal	R848	WD	apoE$^{-/-}$	Distant	Close
Patroller	n/FOV	3±2	29±18	34±11	29±19	80±12	
	fold	1	7.9±4.9	9.2±3.2	8.1±5.2	22.1±3.5	
Velocity	μm/s	0.60±0.20	0.33±0.10	0.50±0.12	0.58±0.24	0.53±0.11	0.34±0.10
	μm/min	36±12	19±6	30±7	34±14	32±7	20±6
Duration	s	284±190	343±309	364±236	437±330	224±204	463±365
Length	μm	134±58	124±116	140±79	263±287	137±78	167±22
Confinement		0.22±0.13	0.10±0.09	0.23±0.12	0.27±0.18	0.20±0.14	0.05±0.07
Displacement	μm	31±18	12±22	30±20	68±79	20±18	15±17
		n=15	n=64	n=23	n=44	n=55	n=29

All data were obtained in 17 min long videos with an FOV of 455×227 μm. Data shown as mean±SD. R848=5 h topical application at 1 mg/mL. WD=C57Bl/6J mice on 4 wk Western diet. apoE$^{-/-}$=4- to 6-mo-old mice on chow diet. apoE$^{-/-}$+WD=fed WD for 6 wk. Plaque distant and close patrollers were separately analyzed. FOV indicates field of view; and WD, Western diet.

In line with a published body of evidence,[2–4,35] we posit that the increased endothelial damage in atherosclerotic Nr4a1 knockout mice and the correlation of patrolling intensity with endothelial apoptosis are related to the missing housekeeping function of patrolling monocytes. However, the deletion of Nr4a1 has pleiotropic effects, and alternative interpretations of these findings are possible. A hyperinflammatory phenotype of plaque macrophages has been described,[19,20] which could affect the microenvironment in a detrimental way. Second, the lack of peripheral patrolling could have indirect effects on plaque patrolling. Third, a local imbalance of monocyte subsets or other leukocytes in situ could adversely affect endothelial fitness of Nr4a1-deficient mice. Future monocyte studies will be greatly improved by using a new enhancer knockout mouse with specific effects on patrollers but not macrophages.[36]

We conclude that large arteries are monitored by patrolling monocytes at steady state, with strong upregulation in hyperlipidemia and atherosclerosis as a potential mechanism to maintain endothelial homeostasis.

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A. Quintar and K. Buscher performed most experiments. A. Quintar, K. Buscher and S. McArdle analyzed the data. K. Buscher, D. Wolf, E. Ehinger, and M. Vassallo did flow cytometry experiments. A. Marki did additional intravital microscopy experiments. S. McArdle and Z. Mikulski established and maintained the intravital live cell triggered imaging system (ILTIS) microscopy setup and provided training. J. Miller maintained the mouse colony. A. Quintar performed electron microscopy. K. Buscher and K. Ley designed the study and wrote the article.

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Disclosures

None.

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