Flow Perturbation Mediates Neutrophil Recruitment and Potentiates Endothelial Injury via TLR2 in Mice
Implications for Superficial Erosion

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Rationale: Superficial erosion currently causes up to a third of acute coronary syndromes; yet, we lack understanding of its mechanisms. Thrombi because of superficial intimal erosion characteristically complicate matrix-rich atheromata in regions of flow perturbation.

Objective: This study tested in vivo the involvement of disturbed flow and of neutrophils, hyaluronan, and Toll-like receptor 2 ligation in superficial intimal injury, a process implicated in superficial erosion.

Methods and Results: In mouse carotid arteries with established intimal lesions tailored to resemble the substrate of human eroded plaques, acute flow perturbation promoted downstream endothelial cell activation, neutrophil accumulation, endothelial cell death and desquamation, and mural thrombosis. Neutrophil loss-of-function limited these findings. Toll-like receptor 2 agonism activated luminal endothelial cells, and deficiency of this innate immune receptor decreased intimal neutrophil adherence in regions of local flow disturbance, reducing endothelial cell injury and local thrombosis (P<0.05).

Conclusions: These results implicate flow disturbance, neutrophils, and Toll-like receptor 2 signaling as mechanisms that contribute to superficial erosion, a cause of acute coronary syndrome of likely growing importance in the statin era. (Circ Res. 2017;121:31-42. DOI: 10.1161/CIRCRESAHA.117.310694.)

Key Words: acute coronary syndromes • disturbed flow • endothelium • neutrophils • superficial erosion

Rupture of an atheromatous plaque with a thin fibrous cap has received much attention as a cause of acute coronary syndrome (ACS).1,2 Abundant human and experimental findings indicate that lipid lowering, in particular statin treatment, mitigates this mechanism of plaque disruption.3 Current clinical data show a shift in the characteristics of plaques associated with rupture (lipid and macrophage rich), concomitant with increased statin use, smoking cessation, and other reductions in risk factors. Human atheromata today contain less lipid and fewer macrophages than just a decade ago.4,5 But even with the best current medical and interventional therapy, the residual burden of recurrent events post-ACS remains unacceptable.6 Indeed, ruptured thin-capped atheromata may now cause fewer ACS,7 and superficial erosion appears on the rise. Yet, mechanisms involved in superficial erosion have received scant attention and remain a major knowledge gap.

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Thrombi associated with superficial erosion generally overlie fibrous rather than lipid-rich plaques. In contrast to plaques with ruptured fibrous caps, eroded plaques contain few macrophages but abundant smooth muscle cells (SMCs). In stark contrast with collagen-poor ruptured plaques, eroded atheromata characteristically contain abundant type III collagen,8,9 glycosaminoglycans, and proteoglycans.10 Eroded plaques may localize preferentially in regions of low shear stress and exhibit impaired endothelial antithrombotic and atheroprotective functions11–13 and loss of endothelial cell (EC).14,15 Markers of EC apoptosis increase downstream of obstructive atherosclerotic plaques in humans, sites of disturbed flow.16 Oscillatory wall shear stress (WSS) favors EC...
## Novelty and Significance

### What Is Known?
- Superficial erosion involves discontinuity in the intimal endothelium and thrombus formation without plaque rupture.
- Erosion-prone plaques have specific features and associate with the presence of neutrophils.

### What New Information Does This Article Contribute?
- Validation of an in vivo approach to recapitulate aspects of superficial erosion in mice to permit mechanistic explorations.
- A demonstration that neutrophils contribute critically to arterial endothelial cell injury in regions of disturbed arterial flow.
- Establishing a role for endothelial Toll-like receptor 2 in local neutrophil recruitment.

### Postmortem pathological studies

Postmortem pathological studies demonstrated the loss of endothelial cells at sites of superficial erosion of the culprit lesions of acute coronary syndromes; yet, the mechanisms that drive this process remain elusive. We developed a new in vivo approach in mice that involves creating a chronic intimal lesion that recapitulates certain features associated with superficial erosion, followed by introduction of flow disturbance. We found that neutrophils selectively accumulate in the lumen of carotid arteries under these circumstances. This neutrophil recruitment locally disrupted the endothelial layer. Toll-like receptor 2 loss of function in arterial but not in bone marrow–derived cells blunted these effects. These findings illustrate the utility of a novel experimental tool to study the mechanisms of arterial thrombi in the absence of plaque rupture.

### Methods

See Methods in the Online Data Supplement.

### Results

Thrombi caused by superficial erosion typically complicate plaques rich in HA, collagen, and SMC, but with scant macrophages, often located at sites of human coronary arteries surrounding stenoses, regions of disturbed flow. We sought clues regarding the mechanisms which might participate in endothelial loss in humans through study of atheromata harvested at carotid artery surgery. We used morphological criteria to classify different categories of plaques from specimens in our collection with well-preserved intimal surfaces (Figure 1). A group of plaques with a fibrous SMC-rich and macrophage-poor appearance was further subdivided into those with an erosion-prone morphology (high apoptotic EC content, n=7) or a stable fibrous morphology (low apoptotic EC content, n=10). A third group of fibrous plaques harbored a nonobstructive mural thrombus (eroded morphology, n=8; Online Figure IA). We compared these fibrous lesions to a group of atheromata with features associated with fibrous cap rupture: few SMC, many macrophages, and large lipid cores (n=11). Fibrous plaques with many apoptotic EC or those with mural thrombi displayed much greater accumulation of neutrophils near the luminal endothelium than those with few apoptotic EC (stable fibrous) or the thin-capped lipid-and macrophage-laden lesions (Figure 1A through 1D). Mural thrombi contained abundant neutrophils (CD66b+ and neutrophil elastase [NE]+; Figure 1C and 1D) as previously described. 

### Nonstandard Abbreviations and Acronyms

<table>
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<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tr>
<td>ACS</td>
<td>acute coronary syndrome</td>
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<tr>
<td>ApoE</td>
<td>apolipoprotein E</td>
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<tr>
<td>CC</td>
<td>constrictive cuff</td>
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<tr>
<td>CXCL</td>
<td>chemokine (C-X-C motif) ligand</td>
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<tr>
<td>EC</td>
<td>endothelial cell</td>
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<tr>
<td>HA</td>
<td>hyaluronic acid/hyaluronan</td>
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<td>LCCA</td>
<td>left common carotid artery</td>
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<td>MPO</td>
<td>myeloperoxidase</td>
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<tr>
<td>NC</td>
<td>Nonconstrictive cuff</td>
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<tr>
<td>NETs</td>
<td>neutrophil extracellular traps</td>
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<td>Pam3</td>
<td>Pam3CSK4</td>
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<tr>
<td>SMC</td>
<td>smooth muscle cell</td>
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<td>TLR2</td>
<td>Toll-like receptor 2</td>
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<td>VCAM-1</td>
<td>vascular cell adhesion molecule 1</td>
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<td>WSS</td>
<td>wall shear stress</td>
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Thus, flow perturbation may comprise a first hit leading to chronic endothelial activation, propensity to slough, and impaired ability to repair small intimal breaches, setting the stage for an enhanced response to a second hit, such as local recruitment of neutrophils, able to augment EC apoptosis and propagate desquamation. Such unpaired rents in the endothelial monolayer could then trigger platelet entrapment, activation, and thrombin generation, fostering clot formation. These hypotheses, derived from in vitro observations, require exploration in vivo because cell culture experiments cannot fully mimic either the flow conditions or the abnormal intimal substrate associated with human superficial erosion.
the fibrous plaques, regions of high neutrophil content colocalized with disruption of the EC monolayer (Figure 1B and 1C). In contrast, thin-capped atheromatous lesions generally had an intact endothelial lining and contained few if any neutrophils. Inspection of the deeper portions of plaques served as control for neutrophil content (Figure 1E). Further studies localized neutrophils in representative human atheromata with characteristics of superficial erosion. Double staining for myeloperoxidase (MPO) and CD61 showed neutrophils (MPO+) adjacent to but separate from platelets (CD61+). MPO/NE staining colocalized MPO with NE (Online Figure IB). Thus, neutrophil accumulation seems much more abundant in human lesions with erosion-prone characteristics than in those with features associated with fibrous cap rupture. Immunofluorescent staining showed a preferential accumulation of TUNEL+ cells in the intima, often colocalizing with CD31+ ECs, in erosion-prone plaques as compared with rupture-prone plaques (Figure 1F; Online Figure IC). Apoptotic ECs localized preferentially in the vicinity of NE+ neutrophils (Online Figure IC).

This study aimed to explore experimentally in vivo the mechanisms that underlie these findings in human plaques related to erosion versus rupture. To this end, we first tailored arteries to harbor matrix-rich fibrous intimas by injury followed by healing. We then subjected these arteries with preformed fibrous intimal lesions to flow perturbation induced by stenosis. The initial injury impaired EC permeability at day 0, and the EC layer recovered after a 4-week healing period (Figure 2A; Online Figure IIA), with local neointima formation (Figure 2A, right panel; Online Figure IIB). The intima/wall (media+adventitia) ratio increased significantly postinjury...
compared with uninjured left common carotid arteries (LCCA; Online Figure IIB and IIC; \(P<0.01\)), but the media-to-wall ratio did not change significantly (\(P=0.2\)). Postinjury, LCCAs developed an SMC-rich neointima, with few macrophages, lacking a lipid core, and rich in HA (Figure 2A). This approach replicates key features of human plaques associated with superficial erosion (9) (Figure 1). Postinjury, arterial extracts contained significantly more mRNA encoding type I and type III collagen precursors and HA synthase 2 and decreased type I hyaluronidase mRNA, compatible with a slant toward HA accumulation (Figure 2B). E-selectin (\(P<0.001\)) and VCAM-1 (\(P<0.0001\)) expression remained elevated 4 weeks after injury, indicating persistent endothelial activation at these sites, as we previously observed in injured arteries in rabbits (Figure 2C; Online Figure IID). (13) Human saphenous vein ECs cultured on a gelatin substrate supplemented with various molecular weight fractions of HA showed increased EC responses to a 5 kDa fraction of HA (Figure 2D). Furthermore, human saphenous vein ECs cultured on a 5-kDa HA-enriched coating showed concentration-dependent activation (Figure 2E). Thus, regional electric injury induces SMC and HA-rich intimal hyperplasia, and exposure of the basal surface of EC to low molecular weight HA can chronically activate EC.

**In Vivo Flow Perturbation**

Placement of cone-shaped polyethylene cuffs around the LCCA in mice and secured by a circumferential suture. Effect of nonconstrictive control cuff (G) or constrictive cuff placement on flow dynamics in carotid arteries (H). Doppler-coupled ultrasonography showing velocity patterns along the downstream flow (left, arrow) and the velocity peak downstream of the cuff (right, arrow) measured at 1.5 mm downstream of the cuff (arrowhead). Computational fluid dynamics using finite volume analysis verified the extent of flow perturbation and recirculation. Longitudinal cross-sectional simulation of the effect of a nonconstrictive cuff (G) or a constrictive cuff (H) showing a normalized axial velocity contour with superimposed velocity vectors (G and H). CC indicates constrictive cuff; ECA, external carotid artery; ICA, internal carotid artery; LCCA, left common carotid artery; NC, nonconstrictive cuff; ni, neointima; and RCCA, right common carotid artery.
Further downstream (Online Figure IIG). At peak systole, the LCCA with NC presented a low evenly distributed WSS profile (Online Figure IIH), whereas a high variability in the WSS distribution accompanied the CC placement, with increasing values observed along the cuff and lowest values immediately downstream (Online Figure III). Time-averaged WSS plots obtained at sequential locations (Online Figure IIIJ) showed no significant changes along the carotid in NC condition or proximal to the CC (site 1). Distal to the CC, mural WSS greatly increased at the narrowest cuff site (site 2), became negative with increased amplitude slightly downstream from cuff (site 3), and oscillated from positive to negative values further downstream from the cuff (site 4). These data show that flow restriction in carotid arteries produces oscillatory shear stress downstream, permitting controlled and characterized experimental manipulation of local hydrodynamics in normal arteries or those with tailored fibrous intimal hyperplasia.

Flow Perturbation Promotes Neutrophil Recruitment and Thrombus Formation in Previously Injured Arteries

Mice underwent placement of CC (n=45) or a NC (n=15) on either a normal or previously injured LCCA (Figure 3A and 3B; normal, n=19; post injury, n=52). Control animals underwent a sham procedure (n=11). Neither NC placement nor sham procedure altered significantly intimal leukocyte accumulation downstream, demonstrating that neither the cuff material nor the surgical manipulations evoked acute inflammation. Neutrophils (Ly6G$^+$ cells) did, however, adhere to the arterial intima distal to the CC, much more in previously injured than in normal LCCA (Figure 3B; $P<0.001$). Further experiments extended the histological observations by flow cytometric analysis of luminal eluates of downstream arterial segments from previously injured arteries: segments of arteries experiencing flow perturbation had significantly increased CD45$^+$Ly6G$^+$ cells (CC; Figure 3C and 3D; $P<0.001$). Ly6C$^+$ monocytes also increased in the CC group, albeit 10-fold less than did neutrophils, illustrating the order-of-magnitude preponderance of neutrophils in this acute response (Figure 3C and 3D; $P<0.05$). Ly6G$^+$ cells lined sites of flow disturbance in LCCA (Figure 3E, downstream; Online Figure IVC and IVE). In contrast, upstream areas had few leukocytes. LCCA subjected to more prolonged flow perturbation (6 hour) still exhibited substantial Ly6G$^+$ cell accumulation, although the cell number declined 2-fold compared with that at 1 hour (Online Figure IV; $P<0.001$). Flow disturbance caused Ly6G$^+$ cell accumulation for up to 3 weeks (data not shown), compatible with chronic intimal disturbance. The adherent cells

**Figure 3.** Flow perturbation promotes neutrophil recruitment and thrombus formation. **A**, Experimental protocol and time points studied. **B**, Ly6G immunohistochemical staining of LCCA downstream segments shows the accumulation of neutrophils 1 h after sham procedure (Sham; upper, n=9), placement of nonconstrictive cuff (NC; intermediate, n=14) or constrictive cuff (CC; lower, n=46) either on normal (center) or post-injured arteries (right). Uninjured upstream segments are shown on the left. The graph shows assessment of the number of adherent neutrophils to the intima. Scale bars: 30 μm. **C**, Flow cytometry performed on post-injured arteries after enzymatic digestion 1 h after sham procedure (top), placement of a NC (middle), or a CC (bottom). n=3 per group. Viable neutrophils were identified as Lin$^{-}$CD45$^+$CD11b$^+$Ly6G$^+$ and viable Ly6Chigh monocytes were identified as CD45$^+$CD11b$^+$Ly6C$^+$. **D**, Flow cytometric quantification of neutrophils and Ly6Chigh monocyte. *P<0.05, **P<0.001. **E**, Representative en face Ly6G immunofluorescent staining performed on previously injured LCCA subjected to flow perturbation (n=3). Segment downstream from the cuff (CC) is shown in higher magnification. **F**, Electron microscopic images show the intima of previously injured arteries after sham procedure (top) or CC placement (bottom). Scale bars, 2.5 nm. Immunofluorescent staining for fibrinogen (G) or Carstairs’ staining (H) performed on a selection of postinjured LCCA that have formed intraluminal thrombus 1 h after flow perturbation. Scale bars, 100 μm. **I**, Immunoblotting showing elevated fibrinogen downstream of CC. Normalization, β-actin. **J**, Quantification by ELISA of circulating D-dimer concentrations in mice subjected to sham (n=4) vs flow perturbation (CC, n=19). Data are expressed as mean±SEM. Mann–Whitney U test. LCCA indicates left common carotid artery.
exhibited ultrastructural characteristics of neutrophils with segmented nuclei and plentiful cytoplasmic granules (Figure 3F). The CC groups displayed increased thrombus formation (Table II; P<0.05 and P<0.01) and fibrin-rich intraluminal thrombi in LCCAs after 1 hour of flow perturbation (Figure 3G and 3H). Arterial segments subjected to flow perturbation accumulated intimal fibrinogen (Figure 3I). Moreover, circulating D-dimer, a product of fibrin degradation, increased in the serum of mice from CC group after 1 hour of flow disturbance (Figure 3J). Finally, en face observation of the LCCA showed thrombus formation downstream of stenoses (Online Figure IVD). These results demonstrate that flow perturbation provokes local neutrophil and thrombus accumulation in arteries with fibrous intimal hyperplasia.

Flow-Mediated Neutrophil Recruitment Promotes Endothelial Injury

Sites of fatal superficial erosion complicating human atheromata show EC loss and neutrophil accumulation.28 We, therefore, tested here the hypothesis that neutrophils contribute critically to loss of intimal EC in circumstances implicated in erosion. We examined the effect of 6 hours of continuous flow perturbation on luminal endothelium in arteries with fibrous thickened intimas (Figure 4A). In comparison to controls, in the CC group, VCAM-1 and E-selectin protein increased in extracts of downstream arterial segments (Figure 4B). Flow perturbation also promoted disturbed EC barrier function (Figure 4C; P<0.01) and apoptosis (Figure 4D). Further immunostaining using an anticleaved-caspase-3 antibody buttressed these findings (Online Figure IXB). Locally, ECs showed ultrastructural features of dying cells, including blebbing and vacuolization (Figure 4E). Flow perturbation significantly decreased endothelial continuity (P<0.05), altered EC morphology, and led to EC desquamation (Figure 4F and 4H; P<0.05). Discontinuity in the intimal endothelium correlated with the number of firmly adherent neutrophils (Figure 4G). Numerous neutrophils also congregated with activated EC or colocalized with patches of intimal denudation (Figure 4I).

Further experiments assessed the causal relationship between neutrophil presence and endothelial dysfunction/loss (Figure 4J). Anti-Ly6G blocking antibody treatment 24 hours before cuffing induced neutropenia (N=11). Alternatively, administration of an anti-LFA-1 (lymphocyte function-associated antigen 1) blocking antibody 1 hour before the experiment aimed to reduce neutrophil recruitment. Either treatment abolished the recruitment of Ly6G+ neutrophils in the LCCA after 1 or 6 hours of flow perturbation (Figure 4K). After 6 hours of flow perturbation, either antineutrophil treatment likewise limited endothelial permeability in the arterial segments subjected to disturbed flow (Figure 4L), decreased the number of luminal CD31+TUNEL+ apoptotic EC (Figure 4M; P<0.05), and preserved endothelial continuity (Figure 4N; P<0.05). These results identify neutrophils as effectors of EC injury, death, and detachment under circumstances associated with thrombosis because of superficial erosion.

Endogenous TLR2 Activates EC and Promotes Neutrophil Chemoattraction

Further experiments tested the hypothesis that TLR2 participates in EC activation and potentiates neutrophil chemoattraction or leukocyte adhesion in arteries that share features of eroded human atheromata. Human saphenous vein ECs incubated with the TLR2 agonist Pam3CSK4 (Pam3) or with vehicle showed time-dependent increases in VCAM-1, ICAM-1 (intercellular adhesion molecule 1), E-selectin, and interleukin-8 (Figure 5A). Six hours after Pam3 or vehicle administration intraperitoneally to apolipoprotein E-deficient (Apoe−/−) or Apoe−/−Tlr2−/− mice, luminal eluates, and extracts of the remaining aorta wall (intima excluded) underwent RNA isolation and quantitative polymerase chain reaction analysis (Figure 5B). Arterial luminal eluates from Pam3-treated Apoe−/− mice showed significantly increased Vcam-1, E-selectin, chemokine (C-X-C motif) ligand (Cxcl)-1, Cxcl-2, and Cxcl-5 mRNAs (Figure 5C). Aortic tunica media extracts from Pam3-treated Apoe−/− mice had increased concentrations of the chemoattractants Cxcl-1, Cxcl-2, and Cxcl-5 mRNAs but not messages that encode the endothelial adhesion molecules Vcam-1 or E-selectin. Pam3 did not exert these actions in Apoe−/−Tlr2−/− animals, indicating that these arterial responses to Pam3 depend on TLR2. The Bax/Bcl2 mRNA ratio also increased in these intimal extracts, implicating TLR2 in endothelial apoptosis in vivo (Figure 5D). Pam3 administration augmented both VCAM-1 and E-selectin protein in aortas isolated from Apoe−/− mice, but not in Apoe−/−Tlr2−/− animals (Figure 5E), and selectively activated luminal EC in vivo (Figure 5F). Circulating CXCL-1 also increased in Pam3-treated Apoe−/− but not in Apoe−/−Tlr2−/− animals (Figure 5G). Together, these results indicate that TLR2 activation leads to the overexpression of neutrophil chemoattractants by both EC and mesenchymal cells, while it increases EC expression of leukocyte adhesion molecules and augments luminal EC apoptosis.

Testing the hypothesis that TLR2 of arterial origin participates in the recruitment of neutrophils used chimeric mice constructed by transplantation of either Apoe−/− or Apoe−/−Tlr2−/− bone marrow to lethally irradiated Apoe−/− or Apoe−/−Tlr2−/− recipients. Mice from each of the 4 groups, thus, generated (Figure 5H) received Pam3 or vehicle and underwent analysis after 6 hours (Figure 5I). Eluates from aortic luminal extracts furnished RNA for quantitative polymerase chain reaction analysis (Figure 5I). As expected, Pam3 injection strongly activated luminal cells in mice expressing TLR2 in both intrinsic arterial and bone marrow–derived cells (group 1; Figure 5J) but not in Tlr2−/− mice (group 4). Animals lacking TLR2 in bone marrow–derived cells alone (group 3) retained responsiveness to Pam3, to an extent comparable with mice from group 1. In contrast, mice lacking TLR2 in intrinsic arterial cells, but reconstituted with bone marrow from TLR2-sufficient animals (group 2), showed a much lower activation than group 1 mice. While Pam3 increased circulating CXCL-1 in groups 1, 2, and 3, group 2 showed significantly lower blood concentration of CXCL-1 compared with groups 1 and 3 (Figure 5K; P<0.001). Pretreatment with Pam3 strongly increased TLR2 expression in luminal ECs (Online Figure VIII). Immunofluorescent colocalization by confocal microscopy reveals that ECs express most arterial TLR2, although adherent neutrophils and some SMCs also show limited positivity. A 3-dimensional
Figure 4. Flow-mediated neutrophil recruitment potentiates endothelial cell dysfunction and loss. A, Experimental protocol and time points studied: left common carotid artery (LCCA) were subjected to injury and 4 weeks later to 6 h of flow perturbation (constrictive cuff [CC]). Sham, nonconstrictive cuff (NC), or CC arteries probed for the expression of vascular cell adhesion molecule 1 (VCAM-1) and E-selectin by Western blot (B) or for Evans blue extravasation (C). Normalization, β-actin. D, Immunofluorescent staining for endothelium (CD31), apoptosis (TUNEL), and DNA (DAPI) in cross sections of Sham, NC, or CC arteries. Each image shows higher magnification on the right. Arrows show the presence of luminal apoptotic endothelial cells (ECs). E, Electron microscopy image showing ECs subjected to flow perturbation in previously injured arteries. Scale bars, 2 μm. F, Quantification of endothelial continuity. G, Negative correlation between endothelial continuity and the number of adherent Ly6G+ neutrophils (P<0.0001, R²=0.29). Immunohistochemical staining for CD31 (top) or en face visualization in immunofluorescence of EC (CD31) and DNA (DAPI; bottom) in downstream arteries after sham procedure (left, n=3), placement of a nonconstrictive cuff (middle, n=4), or constrictive cuff (right, n= 8). Scale bars, 20 μm. Arrows show patches of EC denudation. Semiquantitative assessment of endothelial continuity cross sections CD31 immunohistochemistry is shown in the graph (right). I, Immunofluorescent stainings for E-selectin, EC (CD31), and DNA (DAPI) show EC undergoing detachment (arrow), in contact with neutrophils (arrowhead) in arteries subjected to flow perturbation (CC). J, Experimental protocol and time points showing LCCA subjected to injury, followed 4 weeks later by systemic injections of neutralizing Ly6G antibody, LFA-1 (lymphocyte function-associated antigen 1) antibody, or vehicle before flow perturbation (CC). K, Ly6G staining in immunohistochemistry shows neutrophil recruitment after 1 h of flow perturbation in LCCA subjected to flow in groups receiving vehicle, anti-Ly6G, or anti-LFA-1. The graph shows quantification of adherent neutrophils in each group, after either 1 or 6 h of flow perturbation. L, LCCA from group vehicle, anti-Ly6G, and anti-LFA-1 probed for (Continued)
Figure 4 Continued. endothelial permeability using Evans blue intravital staining after 6 h of flow perturbation. M, Immunofluorescent staining for endothelium (CD31, red), early apoptosis (green), and DNA (DAPI, blue) shows luminal endothelial apoptotic cells (arrows) in group vehicle, anti-Ly6G, or anti-LFA-1 after 6 h of flow perturbation. N, Immunofluorescent staining for endothelium (CD31, red), elastin autofluorescence (green), and DNA (DAPI, blue) shows endothelial continuity in group vehicle, anti-Ly6G, or anti-LFA-1 after 6 h of flow perturbation. Data are expressed as means±SEM. *P<0.05, **P<0.01, ***P<0.001, and ****P<0.0001, Mann–Whitney U test. bm indicates basement membrane; ec, endothelial cell; iel, internal elastic laminae; smc, smooth muscle cell; and v, vacuole.

reconstruction revealed that TLR2 localizes mainly on the basal surface of ECs in contact with the basement membrane (Online Movie I). In contrast, the apical surface of EC did not contain TLR2. These results demonstrate that TLR2 expressed by intrinsic arterial cells participates prominently in intimal cell activation and supports the hypothesis that endothelial TLR2 promotes neutrophil recruitment in the context of superficial erosion.

TLR2 Participates in Neutrophil Recruitment, EC Death, and Dysfunction in Arteries With Fibrous Intimal Hyperplasia in Response to Flow Disturbance

Areas of disturbed arterial flow in mouse and human atheromata exhibit overexpression of TLR2. These observations and the ability of TLR2 ligation to activate ECs suggested the hypothesis that TLR2 participates in the recruitment of intimal...
neutrophils. Eight-week-old $\text{Apoe}^{-/-}$ or $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ mice first underwent LCCA injury (Figure 6A). LCCA with fibrous intimal thickening in $\text{Apoe}^{-/-}$ versus $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ showed no significant differences in internal or external diameter or in the intima-to-wall ratio ($P=0.2$; Figure 6B and 6C). Tlr2 deficiency in normal or previously injured arteries did not affect the

**Figure 6.** Flow perturbation promotes neutrophil recruitment through TLR2. **A**, Experimental timeline. **B**, Hematoxilin and eosin (H&E) staining shows neointima formation after left common carotid artery (LCCA) injury in $\text{Apoe}^{-/-}$ and $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ mice. The star indicates the lumen. **C**, Morphometric analysis showing measurement of internal (top, left) and external diameters (bottom, left) and the ratio intima/wall (top, right) and media/wall (bottom, right). **D**, Experimental protocol involving LCCA injury followed by flow perturbation (CC) in $\text{Apoe}^{-/-}$ and $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ mice. **E**, Ly6G immunohistochemistry staining of LCCA cross-sections showing the recruitment of neutrophils 1 or 6 h after flow perturbation in $\text{Apoe}^{-/-}$ and $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ mice. The graph shows the semiquantitative assessment of adherent neutrophils to the intima after 6 h of flow perturbation. Scale bar: 60 $\mu$m. **F**, CD31 immunohistochemistry staining shows endothelium. The graph (right) shows assessment of endothelial continuity. **G**, LCCA isolated from $\text{Apoe}^{-/-}$ and $\text{Apoe}^{-/-}\text{Tlr2}^{-/-}$ mice were probed for endothelial permeability using Evans blue intravital staining after 6 h of flow perturbation. **H**, Summary diagram of the main findings of this study. Data are expressed as mean±SEM. *$P<0.05$, **$P<0.01$*. Mann–Whitney U test. a indicates adventitia; CC, constrictive cuff; IEL, internal elastic lamina; Lmw-HA, low molecular weight–hyaluronan; m, media; MPO, myeloperoxidase; ni, neointima; NE, neutrophil elastase; NETs, neutrophil extracellular traps; NF-κB, nuclear factor-κB; ROS, reactive oxygen species; SMC, smooth muscle cell; and TLR2, Toll-like receptor 2.
arterial expression of the atherothrombosis-related genes evaluated (Online Figure VC). Other mice underwent LCCA injury followed 4 weeks later by flow perturbation (Online Figure VD). While local neutrophil number did not differ between groups after 1 hour (Online Figure VD), after 6 hours, the distal arterial segments of Apoe−/− Tlr2−/− contained significantly fewer Ly6G+ neutrophils than Apoe−/− (Figure 6E; P<0.01). Furthermore, Apoe−/− Tlr2−/− mice showed less disruption in endothelial continuity in arterial segments subjected to flow perturbation (Figure 6F), as well as decreased permeability (Figure 6G). Apoe−/− Tlr2−/− mice had reduced local thrombus formation after 1 or 6 hours of flow perturbation (Online Table II) and diminished circulating D-dimer concentrations after 6 hours of flow perturbation (Online Figure III). These results support the participation of TLR2 in the recruitment of neutrophils, in EC dysfunction/loss, and in subsequent thrombosis in the context of superficial erosion (Figure 6H).

**Discussion**

Superficial erosion causes arterial thrombosis, and hence ACS, without plaque rupture, as disclosed by postmortem examination and optical coherence tomographic imaging in intact patients. Studies of human autopsy specimens do not permit dissection of the mechanisms that underlie this modus of thrombotic complication of atherosclerosis. This study extends in vivo our prior in vitro observations that implicated TLR2 signaling, engagement of this innate immune receptor by HA or other ligands, and the participation of neutrophils in aspects of superficial erosion.

Among several processes that may contribute to superficial erosion, flow perturbation can promote endothelial dysfunction and death. Yet, areas subjected solely to flow perturbation seldom develop superficial erosion, suggesting that flow disturbance alone does not suffice to trigger thrombosis. We, therefore, recently proposed a 2-hit schema for the pathogenesis of superficial erosion, a mechanism of coronary thrombosis apparently on the rise.6,28 Plaques that have precipitated thrombi because of erosion differ distinctly from the so-called rupture-prone plaque. These differences suggest striking divergences in the pathological mechanisms and support the concept that plaque composition influences the mode of thrombotic complication. This in vivo study used an experimental approach designed to mimic certain characteristics of plaques that have caused thrombosis because of superficial erosion. We created in mouse arteries expanded intimas enriched with SMCs and a glycosaminoglycan-rich extracellular matrix with few inflammatory cells, which recapitulates some key features of the substrate associated with eroded plaques in humans.8,9 We used ApoE-deficient mice consuming a chow rather than atherogenic diet to avoid producing plaques overloaded with lipids and macrophage foam cells, characteristics of ruptured rather than eroded lesions.25 The use of electric injury to stimulate intimal expansion proved more reproducible in extensive pilot experiments than did endothelial intervention. The resultant lesions accumulated HA and exhibited altered HA turnover, as indicated by reciprocal changes in the concentrations of mRNAs that encode enzymes involved in HA production and degradation. Heightened expression of E-selectin and VCAM-1 in these arteries indicated sustained chronic EC activation, findings concordant with our prior work on injured rabbit arteries.33 Other studies have shown HA accumulation during neointimal hyperplasia in association with the migration and the proliferation of SMCs and that intimal HA may accentuate atherosclerosis.35,36 In vitro, a culture substrate enriched with low molecular weight HA caused low-level activation of human ECs, consistent with the notion that intimal HA could pave the way for thrombotic complication because of superficial erosion. Prior in vitro studies have seldom aimed to replicate the hydrodynamic conditions that prevail downstream of arterial stenosis in humans. The current in vivo findings show that flow disturbance sets the stage for thrombosis in arteries with fibrous intimal thickening.

Disturbed flow and low shear stress can activate arterial ECs through nuclear factor-κB and also rapidly augment P-selectin and interleukin-8 elaboration nontranscriptionally by triggering their translocation to the EC luminal surface.39 Experimental stenosis in veins promotes the accumulation of neutrophils that participate in P-selectin-dependent initiation/amplification of thrombosis.40 Here, arterial ECs exposed to flow perturbation displayed markers of activation and apoptosis acutely and developed patches of endothelial desquamation. Oscillatory shear stress can induce EC apoptosis through various pathways, including p53 and protein kinase C-ζ.18,41 Apoptosis could in turn exacerbate EC detachment and promote thrombosis.

Substantial neutrophil accumulation distal to stenosis occurred in the arteries with fibrous intimal expansion. Interruption of neutrophil trafficking protected EC from activation, apoptosis, and detachment. These observations support the local recruitment of neutrophils in eroded plaques and their role in extending injury,38,42 whether or not these leukocytes participate in plaque formation.43 Neutrophil arrival at sites of early erosion could, thus, amplify and propagate conditions that promote local thrombosis, in accord with our proposed 2-hit scheme. Granulocytes can harm EC in many ways,44 including by producing proteinases that sever the tethers of the ECs to the basement membrane favoring desquamation.45 Neutrophils strongly activate cultured ECs, disturb their ability to adhere to the basement membrane, and disturb EC morphology.46,44 NE can degrade basement membrane constituents,46 enhance EC injury,37 and favor anoikis and apoptosis,48 and concentrations of this enzyme increase in patients with myocardial infarction.49 Neutrophils also contain abundant MPO, an enzyme that produces hypochlorous acid, an inducer of EC apoptosis and tissue factor production.50 While a subset of recruited cells after flow perturbation express MPO in this study, MPO does not necessarily colocalize with Ly6G+ neutrophils. Rather, in mice, macrophages contain considerable MPO, an example of the discrepancy between murine and human innate immunity.

Dying granulocytes also generate NETs, structures that can induce EC death and dysfunction.50,51 NETs could also promote EC detachment as they contain matrix metalloproteinase-9, a metalloproteinase involved in the degradation of the basement membrane type IV collagen and are able to activate endothelial pro-matrix metalloproteinase-2.51 We previously reported the presence of NETs and NE at the surface of human plaques resembling those implicated in superficial
erosion.28 NETs contain tissue factor procoagulant and can further furnish scaffolds for coronary thrombi in culprit lesions of ACS.52,53

This study demonstrates in vivo that TLR2 participates in EC activation and amplifies the recruitment of neutrophils after flow perturbation. ECs express TLR2 that participates in experimental atherosclerosis. TLR2 also localizes in human atheromata with characteristics of those that have caused thrombosis because of erosion.29 Previous studies showed that low molecular weight fractions of HA can activate TLR2 signaling through a nuclear factor-xB–dependent pathway.27 Thus, HA could act as an endogenous agonist of TLR2 and contribute to neutrophil recruitment in the setting of disturbed flow, as supported by the present findings. The use of chimeric mice indicates that TLR2 expressed by intrinsic arterial cells rather than leukocytes mediates the release of neutrophil chemottractants and the production of endothelial adhesion molecules. In addition to TLR2, HA can also bind various receptors, including CD44 or ICAM-1. Flow perturbation could also activate alternative signaling (ie, by integrins) that may synergize with constitutive TLR2 signaling driven by HA binding. Defining the roles of these and other potential pathways will require further study.

The lack of experimental tools in vivo for studying superficial erosion has constituted a considerable hindrance and contributes to the knowledge gap in the field. The experimental approach used here permitted us to test hypotheses in vivo regarding the pathogenesis of superficial erosion that emerged from in vitro or descriptive observations made by our laboratory and others. This in vivo approach permitted testing of focused mechanistic hypotheses related to the pathophysiology of superficial erosion. The short time course of the experiments presented here represents one of several limitations to the ready extrapolation of our results to a human disease that plays out over decades. Yet, these findings furnish some early and novel insights into the mechanisms of superficial erosion, a major gap area in our understanding of the thrombotic complications of human atherosclerosis. This quest has become increasingly clinically compelling because current therapies have made substantial inroads in reducing plaque rupture and because superficial erosion has emerged as a considerable continuing contributor to residual risk in the current era.5,34,35

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Disclosures

None.

References


Flow Perturbation Mediates Neutrophil Recruitment and Potentiates Endothelial Injury via TLR2 in Mice: Implications for Superficial Erosion
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SUPPLEMENTAL MATERIAL

Material and Methods

*Human atheromata selection:* Human specimens of carotid plaques were obtained after endarterectomy (n = 146) by protocols approved by the Human Investigation Review Committee at the Brigham and Women's Hospital. We used morphologic criteria to categorize plaques as “erosion prone” (SMC-rich, macrophage-poor, high apoptotic luminal EC content; 15.3 ± 4.8 TUNEL+ EC per field, n=7), “stable fibrous” (SMC-rich, macrophage-poor, low apoptotic luminal EC content; 3 ± 0.6 TUNEL+ EC per field, n=10, p=0.0014), or “eroded” (SMC-rich, macrophage-poor, non-obstructive mural thrombus, n=8). Atheromata with features associated with fibrous cap rupture (few SMC, many macrophages, large lipid core, n=11) was used as a control group. Damage tissues (n=49), calcified nodules or other morphologies (n=40) were excluded from the study. Two investigators independently performed the analysis and classification of the tissue specimens.

*Animals:* C57B6 Apolipoprotein-E deficient mice (*Apoe*−/−) and Toll-like receptor 2 deficient mice (TLR2−/−) male mice (Jackson Laboratories, USA), 8–12 weeks of age, were housed in the Harvard Medical School Facilities at the New Research Building (Boston, MA, USA) and consumed a normal chow diet with water ad libitum. Mice were certified free of common pathogens by the suppliers and were monitored by the Harvard Medical Area Standing Committee on Animals. Double compound *Apoe*−/−TLR2−/− were generated in house. Genotyping for both *Apoe−/-* and *Apoe−/-*TLR2 mice was performed routinely by tail biopsies (*Supplementary Fig. IIIIf*). Sample size was determined according to pilot experiments.

*Arterial injury:* The protocol described below resulted from optimization by extensive pilot experiments. Eight-week-old *Apoe*−/− mice underwent electrical injury of the left common carotid artery (LCCA) as previously described¹ to produce an intimal lesion rich in smooth muscle cells and in proteoglycan and glycosaminoglycans including hyaluronan (*Fig. 1*). Briefly, the left common carotid artery (LCCA, 500 ± 20 μm diameter) was exposed by an incision on the ventral side of the neck. LCCA was injured by electric current using a bipolar microcoagulator (Erbe ICC 200, USA) delivered through the tips of microforceps positioned perpendicularly to the longitudinal axis of the artery. A uniform injury was induced by applying a current pulse two times every millimeter from beneath the LCCA bifurcation. The total injured distance encompassed approximately 2 mm, depending on the anatomical
variability of the artery. A current pulse of 3W was delivered by the microcoagulator for 4s each time. Such pulses resulted in reproducible neointima formation (Fig. 1b,c), whose features mimicked the morphologic and the biochemical characteristics of human atheromata that have produced thrombosis to superficial erosion \(^2,^3\). Left common carotid arteries served as uninjured controls.

*In vivo flow perturbation:* Four weeks after arterial injury (12-week-old mice), the intima had healed and re-endothelialization was complete (Fig. 1a). Local flow perturbation was induced either in normal LCCA or in the previously injured LCCA. This procedure used polyethylene cuffs custom manufactured to our specifications by 3D-stereolithography printing (Proto Labs, USA) that form a cone-shaped lumen and produce vortices downstream when placed and fixed with a 7-0 Ethilon nylon suture (Ethicon) around the artery (Fig. 2). The non-constrictive proximal internal diameter (500 µm) of the cuff decreases gradually to become constrictive at its end (distal internal diameters: 250 µm). Blood flow in both the pre- and post-stenotic region was monitored during cuff placement and its perturbation was assessed by Doppler-coupled ultrasonography (VisualSonics 2100). Animals that underwent a sham procedure (with no cuff placement) and those that had placement of a non-constrictive device served as controls for the surgical manipulations and for adventitial injury and inflammation.

*Computational fluid dynamics: Geometry, Mesh Construction, and Simulation Set-up:* Computational fluid dynamics using finite volume analysis verified the extent of flow perturbation, recirculation, and the presence of oscillatory shear stress distal to the constrictive cuff. Using previously injured vessels, the LCCA geometry with or without cuff placement was virtually constructed using Ansys DesignModeler (Ansys Inc., Canonsburg, USA). Each mesh was built with 1.3x10^6 elements and 2.7x10^5 nodes and imported into the CFX CFD solver (CFX version 15.0, Ansys Inc., Canonsburg, USA). At the inlet, a normalized transient velocity waveform boundary condition was prescribed, representing the vessel flow velocity measured by Doppler-coupled ultrasonography. The outlet was set to zero relative-pressure, and no-slip conditions were imposed at the vessel walls. All simulations utilized a Newtonian, viscous model, with laminar flow conditions with the following fluid material properties: density = 1.025g/cm3 and dynamic viscosity = 3.5 cp (Windberger et al. 2003). A convergence criteria of 1x10^-9 was reached for the momentum and continuity equations. Simulations were conducted in a Hewlett Packard workstation with intel(R) Xeon(R) CPU, x5550@ 3.67GHz (6 processors) with 32.0 GB installed memory and
64-bit windows 8 operating system. Data post-processing was performed through Tecplot (Tecplot Inc., Bellevue, USA).

**In vivo TLR2 activation:** 8-10-week-old mice received a potent TLR2 agonist, Pam3CSK4 (1mg/kg i.p., InvivoGen), a synthetic endotoxin-free triacylated lipopeptide that mimics the acylated amino terminus of bacterial LPS. After 6h, injected mice were euthanized and their arteries were used for further QPCR or western blot experiments.

**Biological analysis:** Flow perturbation was conducted for 1 or 6 hours and mice were euthanized by CO₂ inhalation and bilateral thoracotomy. Peripheral blood sampling was performed and mice were perfused with cold PBS using intracardiac perfusion, then fixed with 4% paraformaldehyde. Distal and proximal LCCA samples were collected and embedded in optimal cutting temperature medium (OCT). Six µm cryo-tome sections were prepared and stained for immunohistochemistry/immunofluorescence investigation.

**Immunohistochemistry / Immunofluorescence:** Immunostaining on frozen cross sections was performed using rat monoclonal anti-mouse Ly6G (Santa Cruz Biotechnologies) and rat monoclonal anti-mouse CD31 (BD Pharmingen). Vessels showing total occlusion or aneurysmal features were excluded from the analysis. This criterion was pre-established. After incubation with a biotin-conjugated anti-rat antibody (Vector Laboratories), immunostaining was amplified using peroxidase-conjugated streptavidin complexes (Vector Laboratories) and peroxidase was detected using AEC (Vector Laboratories) substrate. Sections were lightly counterstained with hematoxylin, mounted in gelatin glycerol and examined with a bright field microscope (Nikon Optiphot-2 equipped with a Nikon digital camera DXM 1200F). For double immunostaining study, cross sections were incubated with primary antibodies followed by the incubation with a fluorophore-coupled anti-species antibody (Life Technologies), stained with DAPI, and mounted with a fluorescent mounting medium (DAKO). Slides were kept in the dark at 4°C. **For thrombus formation, Carstairs staining (Electron Microscopy Sciences, USA) was performed according to manufacturer’s protocol. The fibrinogen immunostaining used a goat anti-fibrinogen antibody (SantaCruz) and was performed on serial sections of carotid sections subjected to flow perturbation after 1 and 6 hours.**

**Endothelial permeability:** Endothelial permeability was investigated by monitoring extravasation of Evan's Blue dye (EBD). The blue staining of EBD-albumin conjugate was evident in LCCA sections subjected to injury (**Fig. 1**) and/or flow perturbation (**Fig. 4**). Mice were injected after electric injury or 6 hours after flow perturbation, via retro-orbital injection.
of 50 µL of 7% EBD (Sigma). After 10 min, mice were euthanized by CO\textsubscript{2} inhalation, perfused by left ventricular apical cannulation with PBS at 4°C supplemented with heparin 10U/ml, and were then fixed with a perfusion of 10% formalin. LCCA was embedded in OCT and kept at -80°C for histology. Whole LCCA was carefully harvested, opened longitudinally from the aortic arch to the bifurcation and stabilized on glass with hardening mounting medium (Vector, Vecta Mount H-5000). In some mice, the LCCA served as a control. Arteries were examined by bright field imaging.

**Neutropenia and neutralization of neutrophil recruitment:** Circulating neutropenia was induced 24 hours before experiments using a single injection i.v of anti-Ly6G blocking monoclonal antibody (300µg, Clone RB6-8C5, BioXCell) in a total of 300µl in sterile saline as diluent. Neutralization of neutrophil recruitment was performed with a single injection i.v of anti-LFA-1 blocking monoclonal antibody 1h before experiments (150µg, Clone I21/7, ThermoScientific). Control animals were injected with saline 1h before experiment.

**Electron microscopy:** Samples (injured LCCA subjected to flow after 1 or 6h, or injured LCCA with no flow, n=3) were isolated and immediately placed in fixative solution (Formaldehyde/Glutaraldehyde, 2.5% each in 0.1M sodium cacodylate buffer, pH 7.4) overnight. Ultrathin sections of 70 to 80 nm were cut on a Reichert Ultracut-S, stained with 2% uranyl acetate and viewed using a JEOL 1200EX transmission electron microscope equipped with an AMT 2k CCD camera.

**En face microscopy:** The mouse vasculature was fixed with a perfusion of cold 4% PFA. The carotids (n=3 per group) were dissected and longitudinally opened on a sylgard dish and extensively fixed in 4% PFA for 48-72h. Fixation was quenched with 100 mM glycine (pH 7.4), and carotids were washed extensively in PBS. Arteries were permeabilized and treated with a blocking buffer containing 5% NGS, 1%BSA and 0.05% Triton X-100 during 2 h at room temperature. Primary antibodies were diluted in blocking buffer and incubated with carotid artery specimens for 48 h at 4°C. Tissues were washed in PBS, then washed in blocking buffer for 2 h. The carotids were incubated 1 h at room temperature, with fluorophore-conjugated antibodies diluted in blocking buffer. Finally, the tissues were washed in blocking buffer for 1 h at room temperature, then washed in PBS. Primary antibodies used included: CD31 (BD Pharmingen), Ly6G (Santa Cruz Biotechnologies), citrullinated Histone H3 (Abcam), and Myeloperoxidase (Abcam). Secondary antibodies (Life Technologies) were all conjugated with Alexa-Fluor 488 or 555. All preparations were mounted with Vectashield
HardSet Antifade Mounting Medium containing DAPI for DNA staining (Vector Laboratories) and stored in the dark at room temperature.

**Bone marrow transplantation:** Apoe\(^{−/−}\) or Apoe\(^{−/−}\)Tlr2\(^{−/−}\) male C57B6 mice served as recipients for transplantation (6-8 week old). Recipient mice received 1,050 rads of split-dosed lethal irradiation (5.25 Gy, twice) 4 h apart to reduce gastrointestinal toxicity. Recipient mice received antibiotics (sulfamethoxazole and trimethoprim, HiTech Parmacal) until the end of the experiment. Total bone marrow cells from Apoe\(^{−/−}\) or Apoe\(^{−/−}\)Tlr2\(^{−/−}\) donor mice were prepared from the femurs and tibias collected aseptically in sterile RPMI 1640 medium supplemented with 2% FBS, 10 U/ml heparin, penicillin and streptomycin. Bones were punched with a needle at both of their extremities and centrifuged using a mounting of modified sterile tubes (**Supplementary Fig. Vb**). Cells were resuspended and washed twice in sterile serum-free RPMI 1640 medium supplemented with 20mM HEPES, penicillin and streptomycin at pH 7.4. After counting, 5 million total bone marrow cells were injected i.v into each recipient mouse (6-week-old) for reconstitution. Mice with no bone marrow reconstitution served as sentinels. Mice were monitored until the final experiments, and survival curves were established for each group (**Supplementary Fig. Va**).

**RNA isolation:** For EC peeling, carotids were isolated, rinsed with cold PBS, and flushed with 1ml of Trizol reagent (ThermoFisher Scientific) using syringe 27 gauge (BD). After chloroform addition, total RNA extraction was performed using PureLink Micro kit (Life Technologies) according to manufacturer’s instructions. For whole arteries, carotid samples or aortas were ground to a fine powder in liquid nitrogen and total RNA was extracted in Trizol reagent and PureLink RNA Micro kit. The purity and concentration of total extracted RNA was evaluated using a spectrophotometer (NanoDrop). The RNA was reverse transcribed using the SuperScript II reverse transcriptase (Invitrogen). The resulting cDNA mixture was stored at -80°C until further use.

**Quantitative PCR:** The mRNA levels of 15 candidate genes (**Supplementary Table 1**) were determined by quantitative real-time-polymerase chain reaction (QRT-PCR) using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad) using SsoAdvanced Universal SYBR Green Supermix (Bio-Rad). Amounts of mRNA of interest were normalized to that of βActin (\(\Delta\text{Ct} = \text{Ct gene of interest} - \text{Ct βActin}\)). Analysis was performed using the 2(-\(\Delta\text{Ct}\)) or 2(-\(\Delta\Delta\text{Ct}\)) method.

**Immunoblotting:** Samples were macerated on ice and lysates were prepared with ice-cold RIPA buffer (Boston BioProducts, USA) supplemented with proteases inhibitors (Complete
Mini, Roche). Lysates were separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. The membranes were probed overnight with primary antibodies and anti-β-actin antibody at 4°C. After incubation with horseradish peroxidase-linked secondary antibodies (ThermoFisher Scientific), immunoreactive proteins were visualized with an ultra-sensitive enhanced chemiluminescent substrate (ThermoFisher Scientific, USA).

**ELISA:** Peripheral blood was collected and levels of circulating fibrin byproduct D-dimer and neutrophil chemoattractant KC were evaluated using commercially available ELISA kits (NeoBiolab and Promokine, respectively).

**Flow cytometry:** After harvesting, carotid arteries were gently washed with cold PBS solution supplemented with 5% BSA and minced with surgical scissors. Single-cell suspension was obtained through enzymatic digestion in collagenase I (450 U/ml), collagenase XI (125 U/ml), DNase I (60 U/ml), and hyaluronidase (60 U/ml) (Sigma-Aldrich, St. Louis, MO, USA) at 37°C on a shaker set at 750 rpm for 1 hour. Samples were filtered with a 40 µm cell strainer and stained with the following anti-mouse biotinylated antibodies (1:600 dilution) against lineage (Lin) markers: B220 (Biolegend, clone RA3-6B2), NK1.1 (Biolegend, clone PK136), Ter119 (Biolegend, clone TER-119), CD90 (Biolegend, clone 53-2.1), IL7Rα (Biolegend, clone A7R34), CD4 (Biolegend, clone GK1.5) and CD8 (BD Biolegend, clone 53-6.7). After the lineage staining, single-cell suspension was stained with the following antibodies (1:600 dilution): Brilliant Violet 711 anti-mouse CD45 (Biolegend, clone A20), PE/Cy7 anti-mouse F4/80 (Biolegend, clone BM8), PE/Cy5 anti-mouse Ly6G (Biolegend, clone 1A8), Brilliant Violet 605 anti-mouse Ly6C (Biolegend, clone HK1.4), APC anti-mouse/human CD11b (Biolegend, clone M1/70), and Brilliant Violet 510 Streptavidin (Biolegend). Viability was assessed using the Fixable Viability Dye eFluor 780 (eBioscience). Live neutrophils were identified as Lin-CD45+CD11b+LY6G+.

For the quality control of the chimeric mice 4 weeks after bone marrow reconstitution, 100 µl of blood was collected through eye bleeding. Samples were incubated with RBCs lysis buffer (Biolegend) for 2 minutes at room temperature and stained with biotin anti-mouse CD90 (Biolegend, clone 30-H12). Samples were later incubated with the following anti-mouse antibodies: FITC LY6C (Biolegend, clone HK1.4), CD115 PERCP (Biolegend, clone AFS98), CD19 PE/Cy7 (Biolegend, clone 6D5), APC LY6G (Biolegend, clone 1A8), APC/Cy7 CD11b (Biolegend, clone M1/70), PE TLR2 (Biolegend, clone CB225), and Brilliant Violet 605 Streptavidin (Biolegend, clone). Neutrophils were identified as CD90-CD19-CD115-CD11b+LY6G+ cells. Bone marrow chimerism was assessed as percentage of
Material and Methods

TLR2+ or - neutrophils. We acquired data on an LSRII flow cytometer (BD Bioscience) with FACSDiva software (BD Bioscience). Experimental data were analyzed using FlowJo software (Tree Star Inc.).

Cell culture: Human saphenous vein EC (HSVEC) were isolated from saphenous veins enzymatically, and cultured on 1% gelatin-coated dishes in Medium 199 (Lonza) supplemented with 20% fetal bovine serum (FBS), penicillin/streptomycin, L-glutamine, heparin, and 0.05mg/ml ECGS (Alfa Aesar) between passages 2 and 5. After reaching confluence, HSVECs were deprived with OptiMEM medium (ThermoFisher) supplemented with 3% FBS for 6 to 12 hours before activation by TLR2 agonists Pam3csk4 (Pam3, 2 µg/ml) for the indicated times (Invivogen). Alternatively, EC were seeded on a HA-supplemented gelatin coating with selected molecular weight (HA, Lifecore Biomedical). Cell lysates were prepared after 48 hours for RNA isolation. Cultured HSVECs were previously authenticated by morphology, and the assessment of the expression of von Willebrand Factor by immunofluorescence and qPCR.

Acquisition and morphometric analysis: In situ images were captured digitally using a BX61WI microscope coupled with a Fluoview FV1000 confocal unit (Olympus) equipped with an Olympus DP72 camera and running Fluoview 10-ASW Software (Olympus) for confocal microscopy or a Nikon Optiphot-2 equipped with a Nikon digital camera DXM 1200F for bright field microscopy. Macroscopic images were taken using the confocal apparatus and Cell Sens software (Olympus). The number of Ly6G-positive cells, CD31-positive cells, CD31+TUNEL+ cells, endothelium continuity, and Evans blue coverage were quantified after an immunostaining computer-assisted method (Image-Pro Plus, Media Cybernetics) and using a double-blind randomized approach.

Statistics: Animals were randomly allocated to treatment or control groups. Investigator was blinded to the group allocation during the experiment. Data were analyzed to assess normality of distribution. A non-parametric test was used for skewed data. For normally-distributed variables, data were expressed as mean ± SEM and analyzed by the t-test, Mann-Whitney or Kruskal–Wallis one-way analysis of variance and Dunn’s post (GraphPad Prism, USA). Differences were considered statistically significant at the p<0.05 level (unadjusted p values). Correlation was estimated with Pearson product-moment.
References


**Figure I: Characterization of human endarterectomy samples.** (A) Screening of the sample was performed after classifying plaques as SMC-rich, macrophage-poor, without lipid cores (n=25) or macrophage-rich, SMC-poor with the presence of a lipid core (n=11). The first group was divided into “Erosion-prone” fibrous plaque (high apoptotic EC content, n=7), “stable” fibrous plaque (low apoptotic EC content, n=10) or “Eroded” fibrous plaques (presence of a non-obstructive mural thrombus, n=8). The second group (yellow) classified the thin-capped atheromatous lesions. (B) Immunofluorescent labeling of neutrophils, platelets, myeloperoxidase, and neutrophil elastase in a human plaque with erosion-prone characteristics obtained at carotid endarterectomy. (C) Immunostaining showing luminal TUNEL positive cells in contiguity with neutrophil elastase-bearing cells in a human plaque with erosion-prone characteristics obtained at endarterectomy.
**Figure II Creation of an experimental substrate for probing superficial erosion in mice.** (A) Experimental protocol showing carotid electric injury (yellow segment) performed at day 0 and followed after 4 weeks by completed endothelial reconstitution assessed by an Evans blue staining. The arrow represents flow orientation. (B) H&E staining shows the formation of a neointima in carotid subjected to injury. ni: intima, m: media, a: adventitia, dashed line: internal elastic laminae. Scale bars, 200 µm. (C) Quantification of the ratio intima/wall (left) and the ratio media/wall (right) in normal arteries vs previously injured arteries. **p<0.01, ns, non-significant. QPCR analysis was performed on normal vs previously injured carotid for the expression of endothelial adhesion molecules (D). Data are expressed in 2^-ΔCt to β-actin. Each dot represents data from one animal. (E) Placement of a cone-shape polyethylene cuff around the RCCA in mice and secured by a circumferential suture. Non-constrictive control cuff (F) or constrictive cuff (G) effect on blood flow perturbation in carotid arteries. Doppler-coupled ultrasonography showing velocity patterns along the downstream flow (left, arrow) and the velocity peak downstream of the cuff (right, arrow) measured at 1.5 mm downstream of the cuff (arrowhead). Computational fluid dynamics using finite volume analysis verified the extent of flow perturbation, recirculation, and the presence of oscillatory shear stress distal to the constrictive cuff. Longitudinal cross-sectional simulation of the effect of a non-constrictive cuff (F,H) or a constrictive cuff (G,I) showing a normalized axial velocity contour with superimposed velocity vectors (F,G) or showing a normalized shear stress contour (H,I). (J) Time averaged wall shear stress plots measured after the placement of non-constrictive (blue line) vs constrictive cuff (red line) at identical locations: (1) upstream from the cuff, (2) narrowest cuff point, (3) slightly downstream from the cuff, (4) further downstream from the cuff. RCCA: right common carotid artery, LCCA: left common carotid artery, BCA: Brachiocephalic artery, ECA: external carotid artery, ICA: internal carotid artery, NC: non-constrictive cuff, CC: constrictive cuff. Data are expressed in Dynes/cm².
Figure III Gene expression in carotids after electric injury. mRNA expression of Hyaluronan Synthase 1, Hyalluronidase 2, Interecellular adhesion molecule 1 (ICAM-1) and Toll-like recepteur 2 (TLR2).
Figure IV Recruited neutrophils during flow perturbation and localization of thrombus. (A) Colocalization in immunofluorescence and en face visualization show the expression of MPO by Ly6G$^+$ and Ly6G$^-$ cells. Quantification of downstream firmly adherent Ly6G$^+$ cells 1 and 6h after flow perturbation, ***p<0.001. (B) Ly6G staining on a longitudinal section showing the site of maximal stenosis, devoid of neutrophil, and neutrophil accumulation downstream. (C) Localization of thrombus formation in the mouse carotid artery after flow perturbation. The carotid was opened for en face visualization of the intima. Cuff position is notified with the black arrows. The blue indicates EC permeability to Evans Blue dye, whereas the brown color shows accumulation of erythrocyte-enriched fresh thrombus. (D) En face immunofluorescent observation of adherent neutrophils in the mouse carotid after flow perturbation using the anti-Ly6G clone 1A8 antibody. LCCA: left common carotid artery.
Figure V Effect of Tlr2 knock out on weight, arterial composition and neutrophil recruitment.
Figure VI Effect of a systemic injection of two different TLR2 agonists on the levels of circulating CXCR-1
Figure VII Assessment of the bone marrow chimerism
Figure VIII Expression of TLR2 by ECs in the mouse carotid. Immunofluorescence images show the over-expression of TLR2 by EC (CD31) in Pam3-treated mice (upper panel). 3D reconstitution of the 10µm thick section showing a basal localization of TLR2 expressed by luminal ECs (lower panel).
Figure IX Immunofluorescence and immunohistochemistry assessment of apoptosis. (A) Immunofluorescent staining TUNEL/CD31 and cleaved-caspase 3 staining in mouse carotid arteries subjected to disturbed flow. The negative control shows a normal mouse carotid artery without TUNEL staining, showing green autofluorescence only (mainly elastin). (B) Immunofluorescence and immunohistochemistry of cleaved-caspase 3/CD31.
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## Supplementary Table II: Comparison between carotid thrombus occlusion between ApoE-/- mice and ApoE-/-TLR2-/- mice

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<table>
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