Nitric Oxide Prevents Aortic Neointimal Hyperplasia by Controlling Macrophage Polarization

Begoña Lavin, Monica Gómez, Oscar M. Pello, Borja Castejon, Maria J. Piedras, Marta Saura, Carlos Zaragoza

Objective—Nitric oxide synthase 3 (NOS3) prevents neointima hyperplasia by still unknown mechanisms. To demonstrate the significance of endothelial nitric oxide in the polarization of infiltrated macrophages through the expression of matrix metalloproteinase (MMP)-13 in neointima formation.

Approach and Results—After aortic endothelial denudation, NOS3 null mice show elevated neointima formation, detecting increased mobilization of LSK progenitor cells, and high ratios of M1 (proinflammatory) to M2 (resolving) macrophages, accompanied by high expression of interleukin-5, interleukin-6, MCP-1, VEGF, GM-CSF, interleukin-1β, and interferon-γ. In conditional c-Myc knockout mice, in which M2 polarization is defective, denuded aortas showed extensive wall thickening as well. Conditioned medium from NOS3-deficient endothelium induced extensive repolarization of M2 macrophages to an M1 phenotype, and vascular smooth muscle cells proliferated and migrated faster in conditioned medium from M1 macrophages. Among the different proteins participating in cell migration, MMP-13 was preferentially expressed by M1 macrophages. M1-mediated vascular smooth muscle cell migration was inhibited when macrophages were isolated from MMP-13–deficient mice, whereas exogenous administration of MMP-13 to vascular smooth muscle cell fully restored migration. Excess vessel wall thickening in mice lacking NOS3 was partially reversed by simultaneous deletion of MMP-13, indicating that NOS3 prevents neointimal hyperplasia by preventing MMP-13 activity. An excess of M1-polarized macrophages that coexpress MMP-13 was also detected in human carotid samples from endarterectomized patients.

Conclusions—These findings indicate that at least M1 macrophage-mediated expression of MMP-13 in NOS3 null mice induces neointima formation after vascular injury, suggesting that MMP-13 may represent a new promising target in vascular disease. (Arterioscler Thromb Vasc Biol. 2014;34:0000-0000.)

Key Word: matrix metalloproteinase 13

Vascular stents and percutaneous balloon angioplasty, despite their undoubted benefits, often have adverse effects, including restenosis of the vessel wall.12 Arterial wall restenosis is caused by mechanical disruption of the vascular endothelium, which, by unknown mechanisms, induces extensive smooth muscle and endothelial cell migration and proliferation, resulting in increased arterial wall thickness and a narrowing of the lumen.3,4 The increased cell migration, cell proliferation, and platelet aggregation that lead to neointima formation are triggered by nitric oxide (NO)–dependent endothelial dysfunction.5 The ability of endothelial NO synthase 3 (NOS3) to limit these events was demonstrated by viral and nonviral transfection in rabbits with NOS3, which significantly reduced neointima formation and endothelial regeneration, and by pharmacological inhibition in rats.6–11

The early response to arterial injury involves the recruitment, infiltration, and activation of M1 macrophages, which produce proinflammatory cytokines, chemokines, and base-ment membrane–degrading extracellular matrix metalloproteinases (MMPs), resulting in increased cell migration and proliferation. Polarization to M2 (resolving) macrophages induces the expression of several genes involved in wound healing and re-endothelialization. Differentiated macrophages are thought to be able to switch between the M1 and M2 phenotypes, a phenomenon known as macrophage plasticity. This would imply that cells that initially promote an inflammatory response can later have anti-inflammatory properties and suggest that controlling the signals that trigger macrophage polarization presents potential for the therapeutic regulation of neointima formation in injured vessels.12–15

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Macrophage inducible NOS (NOS2) drives the expression of several proinflammatory genes in M1 macrophages. Nitric oxide induces the expression and activity of collagenase MMP-13, which regulates proliferation and migration of several cell types. The effect of NO-mediated MMP expression on neointimal formation is largely unknown. Here we describe the contribution of macrophage polarization through MMP-13–mediated expression on neointimal formation in the absence of NOS3.

Materials and Methods
Materials and Methods are available in the online-only Supplement.

Results
Lack of NOS3 Promotes Neointima Formation
To explore the role of intimal derived endothelial NOS3 on neointimal formation, we used a nonwire surgical denudation procedure, to prevent destruction of smooth muscle cells from tunica media, and a wire denudation injury for validation purposes in aortas from wild-type (WT) and NOS3 null mice. For a 30-day period after endothelial denudation, aortas lacking NOS3 showed significant neointimal formation compared with WT aortas in both procedures (Figure 1A).

We calculated the intima, intima/media, lumen, total vessel areas, and the amount of smooth muscle cells at the time points indicated finding significant differences between WT and NOS3 null aortas at 15 and 30 days after denudation, revealing an impaired remodeling of NOS3 knockout mice. Endothelial regeneration was also defective in these mice (Figure 1A, right). The protective effect of NOS3 was further verified in this model by intraperitoneal injection of the NO donor sodium nitroprusside into NOS3 null mice, showing...
significant regression of the neointimal layer (Figure 1B), whereas in WT mice injection of the NOS inhibitor L-NAME increased intima/media thickness, mimicking the effect of NOS3 gene deletion (Figure 1C). Sodium nitroprusside and L-NAME had no effect in endothelial denuded WT and NOS3 null mice, respectively (Figure 1B and 1C).

Transplantation of NOS3 bone marrow into irradiated WT animals had no effect on neointima formation (Figure 1B in the online-only Data Supplement), whereas transplantation of WT bone marrow into NOS3-deficient mice significantly reduced wall thickness compared with NOS3 null mice transplanted with NOS3 null bone marrow and with non irradiated/bone marrow–transplanted NOS3 null mice (Figure IC in the online-only Data Supplement), indicating that bone marrow from NOS3-expressing mice may participate in the prevention of neointimal formation in response to endothelial injury.

To explain why bone marrow from WT mice had such impact on NOS3 denuded aortas, we analyzed bone marrow LSK (multipotent hematopoietic progenitor cells) and myeloid progenitor cells from WT and NOS3 null animals, finding no differences in cell content between strains in basal conditions (Figure ID in the online-only Data Supplement). However, in response to injury, at 7 days after endothelial denudation, NOS3 knockout mice contain twice LSK progenitor cells with respect to their WT counterparts in circulating blood (Figure 1E in the online-only Data Supplement, left), indicating that in the absence of NOS3 more progenitor cells are mobilized, and thereby NOS3 may prevent neointimal formation by controlling the migration of bone marrow–derived cells in response to endothelial denudation (Figure 1; Figure IC in the online-only Data Supplement). Nevertheless, no differences were detected in myeloid progenitor cell mobilization between mice (Figure 1E in the online-only Data Supplement, right), suggesting a key role of lymphoid cell mobilization in NOS3 null mice.

**Lack of NOS3 Induces Accumulation of M1 Proinflammatory Macrophages After Denudation**

Aortic denudation provoked an inflammatory response characterized by leukocyte infiltration into the vessels. No differences in CD68+ cell content was observed in both strains, although in the absence of NOS3, there is an imbalance M1>M2 of pro-inflammatory macrophages per lesional area (CD68+/inducible NOS+), whereas in WT aortas extensive M2 infiltrated macrophages at 14 days after injury (CD68+/MRC1+) were detected, thus contributing to the shortening of inflammation (Figure 2A; Figure IIA in the online-only Data Supplement).

This was also evidenced in mice conditionally deficient for macrophage specific c-Myc expression (Figure 2B), which blocks their polarization to the M2 phenotype,

proinflammatory versus resolving macrophages at the injured area. To investigate the role of NOS3 deficiency in the accumulation proinflammatory macrophages, we first measured 20 cytokines in plasma collected from NOS3-WT and NOS3 null mice after aortic denudation at the times indicated (Figure 3A). Increased levels of interleukin-5, interleukin-6 (IL-6), and MCP-1 were detected in the first 24 hours after denudation. By contrast, the levels of monocyte chemotractant MCSF and CCL5 proteins remained unchanged (Figure III in the online-only Data Supplement). To address whether vascular endothelial cells are responsible of such profile, we analyzed cell culture supernatants collected from wounded NOS3-WT and NOS3 null murine aortic endothelial cell monolayers. In these

![Image](http://atvb.ahajournals.org/)

**Figure 2.** Mice lacking nitric oxide synthase 3 (NOS3) show increased levels of proinflammatory M1 macrophages after aortic endothelial denudation. **A,** Wild-type (WT) and NOS3 knockout (KO) aortic CD68+/MRC1− (M1) and CD68+/MRC1+ (M2) infiltrated macrophages at 7 and 15 days after endothelial denudation. Representative images show en face confocal microscopic detection of CD68+ (fluorescein isothiocyanate, green), MRC1+ (Cy3, red), and nuclei (Hoecht, blue). Data were graphically represented as percentage of lesional area, (n=6 mice/group per day by triplicate; mean±SD, *P<0.05 M2 WT 14 vs M1 NOS3 14, **P<0.05 M2 WT 14 vs M2 NOS3 14. **B,** Bone marrow macrophages from WT and c-Myc conditional null mice were polarized to M1 and M2 macrophages, and the levels of c-myc mRNA were evaluated by quantitative reverse transcription polymerase chain reaction (n=6 mice/group by triplicate, mean±SD, *P<0.05 M2 WT vs M2 c-Myc KO). **C,** Intima/media area of aortas collected from WT and c-Myc conditional null mice at 0, 15, and 30 days after endothelial denudation (n=6 mice/group by triplicate; mean±SD, *P<0.05 WT 30 vs M1 c-Myc).
conditions, we also observed significant levels of proinflammatory cytokines and chemokines including IL-6, GM-CSF, VEGF, interferon-γ, and interleukin-1β in NOS3 null murine aortic endothelial cell (Figure 3B).

To assess whether NOS3 deficiency may also induce M2 repolarization to an M1 phenotype, we cultured WT M1- and M2-polarized bone marrow–derived macrophages in conditioned medium from endothelial cells isolated from WT or NOS3 null mouse aortas. Conditioned medium from NOS3 null murine aortic endothelial cell induced WT M2-polarized macrophages to express high levels of inducible NOS (Figure 3C, top), a classical marker of M1 proinflammatory activated macrophages (Figure 3C, bottom), together with marked levels of proinflammatory cytokine IL-6, compared with the levels found in M2 macrophages incubated with conditioned medium from WT murine aortic endothelial cell, as detected by immunoblot (Figure 3D).

Taken together, lack of NOS3 induces proinflammatory macrophage accumulation by inducing M1 polarization and repolarization of M2 macrophages to the M1 phenotype.

M2-Polarized Macrophages Slow Vascular Smooth Muscle Cell Proliferation and Migration in the Absence of NOS3

Vascular smooth muscle cell (VSMC) proliferation and migration are key steps during neointimal formation. To test whether proinflammatory or resolving macrophages may have an effect on these processes, we incubated VSMC with conditioned medium from M1- and M2-polarized macrophages, finding that cells proliferate more and migrate faster with conditioned medium from M1 respect to M2 macrophages (Figure 4A and 4B), and confirmed by exogenous administration the NO donor sodium nitroprusside to M2-polarized macrophages (Figure 4C). Interestingly, M1 and exogenous M2 macrophages incubated with sodium nitroprusside express high levels of MMP-13 mRNA and secreted MMP-13, a protein implicated in migration of different cell types, compared with polarized M2 macrophages (Figure 4D). To test whether macrophage-mediated MMP-13 may regulate VSMC migration, we found no significant differences when macrophages were isolated from MMP-13 null mice (Figure 5B), whereas exogenous administration of MMP-13 restored the migratory phenotype (Figure 5C). Taken together, M1 macrophage-mediated MMP-13 expression and activity induce VSMC migration in the absence of NOS3, shedding light about the mechanism by which NOS3 prevents neointimal formation.

MMP-13 Expression Regulates Neointima Hyperplasia in NOS3-Injured Vessels

To test whether MMP-13 plays a role in the absence of NOS3, we generated a double NOS3/MMP-13 null mouse strain (Figure 6A). Although the absence of MMP-13 in NOS3-expressing vessels (MMP-13 null mice) has no effect on neointima formation, lacking of MMP-13 in the NOS3 null background (double NOS3/MMP-13 null mice) prevented the aortic wall thickening seen in mice singly deficient for NOS3 30 days after endothelial aortic denudation (Figure 6B).

Transplantation of MMP-13–deficient bone marrow (either from MMP-13 null mice or double MMP-13/NOS3 knockout mice) into irradiated NOS3 null animals significantly reduced
infiltrates, as result of significant accumulation of several M1 proinflammatory versus M2 resolving macrophage formation by promoting inflammation through an imbalance of NOS3, aortic endothelial disruption increases neointimal prevention of arterial neointimal hyperplasia. In the absence of NOS3, MMP-13 was barely detected (Figure 6C), indicating that MMP-13–expressing bone marrow–derived cells may regulate neointimal formation in NOS3 null mice.

Consistent with these findings, we detected high levels of MMP-13 in cross sections of human aortic endarterectomies, as detected by confocal microscopy, and extensive neointimal hyperplasia, whereas in healthy human arteries, MMP-13 was barely detected (Figure 6D). A proposed mechanism is shown in Figure IV in the online-only Data Supplement.

Discussion

We show for the first time a MMP as a target of NOS3 in the prevention of arterial neointimal hyperplasia. In the absence of NOS3, aortic endothelial disruption increases neointimal formation by promoting inflammation through an imbalance of M1 proinflammatory versus M2 resolving macrophage infiltrates, as result of significant accumulation of several proinflammatory cytokines in plasma and in endothelial cells from NOS3-deficient mice. M1 macrophages expressing MMP-13 induce VSMC migration and proliferation, which are inhibited by M1 macrophages from MMP-13 null mice and reversed by exogenous replacement of the missing protease. Furthermore, in double NOS3/MMP-13–deficient animals, lack of MMP-13 was sufficient to prevent NOS3 null-dependent neointimal formation. These data, together with the detection of extensive MMP-13 and M1 macrophage colocalization in human carotid endarterectomies, suggest MMP-13 as a new target to prevent human neointima hyperplasia.

NOS3 prevents neointimal hyperplasia by limiting smooth muscle cell proliferation through as-yet undefined mechanisms. Arterial wall thickness is reduced in animal models of vascular injury by experimental approaches, including NO delivery by exogenous systemic or local administration of NO donors, or by viral-mediated transfection with NOS3. However, no preclinical findings have been successfully implemented to date. Recent contributions elegantly describe the effect of NO in the ubiquitin–proteasome system and the inhibition of the PDGF-surviving pathway during flow-dependent vascular remodeling. Our work is the first to describe the role of NOS3 in the resolution of the immune response elicited in the vessel as result of endothelial denudation, identifying MMP-13 as a new target.

After endothelial denudation, monocytes infiltrate in response to chemokines produced by a variety of cells, including immune and endothelial cells. M-CSF induces recruitment and differentiation of circulating monocytes into macrophages. The relevance of NOS3 to macrophage polarization has not been described to date. We found high levels of MCP-1 but not MCSF or CCL5 in plasma from NOS3 null mice, which may explain why monocyte recruitment is not affected by the absence of NOS3 (Figure 2A; total WT versus total knockout). However, the extensive expression of GM-CSF and interferon-γ in the absence of NOS3 may explain the increased M1 proinflammatory macrophage polarization in these mice.

The levels of IL-6 detected in vascular endothelium and plasma from NOS3-deficient denuded aortas are of significant interest. During the past decade, a large number of studies have demonstrated the importance of IL-6 in the progression of several inflammatory cardiovascular complications including unstable angina, coronary artery disease, and myocardial infarction. Coronary artery atherosclerosis is characterized by infiltration of activated macrophages, which generate high levels of IL-6. IL-6 is associated with increased mortality and also it serves to identify patients who may benefit most from early invasive strategies. Furthermore, heart transplanted patients treated with simvastatin have low levels of IL-6 and improved levels of endothelial function. In this regard, inhibition of NO-mediated endothelial cell–driven relaxation is also strongly associated with increased levels of IL-6. The levels of IL-6 found in denuded aortas from NOS3 null mice further support the importance of IL-6 in the progression of neointimal formation.

MMPs play an important role in the proliferation and migration of several cell types, but the impact of NOS3-mediated
MMP regulation of neointimal hyperplasia is yet unknown. Here, we found that MMP-13 expression is increased in M1 and NO-treated M2 macrophages and induces migration of VSMC. MMP-13 null M1 macrophages by contrast had no effect on cell migration, unless exogenous MMP-13 is administered, pointing MMP-13 as a target of M1 macrophage-derived VSMC migration.

Macrophage expression of MMP-13 can be stimulated by proinflammatory cytokines, including endothelial IL-6, which is strongly upregulated in the absence of NOS3, thereby

**Figure 5.** M1-mediated vascular smooth muscle cell (VSMC) migration depends on macrophage-dependent matrix metalloproteinase (MMP)-13 expression. A, Expression of MMP-13 in polarized macrophages. Immunoblot detection of MMP-13 (top), gelatin zymography (middle) from macrophage cell culture supernatants (top), and macrophage MMP-13 mRNA detection (lower graph) 24 hours after polarization into M1, M2, or into M2 macrophages and incubated with the nitric oxide donor sodium nitroprusside (SNP; n=3 by triplicate; mean±SD; *P<0.05 M2 vs M2+SNP; **P<0.05 M1 vs M2). B, VSMC migration in the presence of polarized M1, M2, and M2 +SNP macrophage supernatants isolated from MMP-13 null mice (n=3 by triplicate; mean±SD). C, VSMC migration as in B, in which cell monolayers were also incubated with exogenous recombinant active MMP-13 (n=3 by triplicate; mean±SD; *P<0.05 MMP-13 KO M2 4 hours vs MMP-13 KO M2+MMP-13 4 hours; **P<0.05 MMP-13 KO M2 6 hours vs MMP-13 KO M2+MMP-13 6 hours).

**Figure 6.** Lack of matrix metalloproteinase (MMP)-13 prevents neointimal thickening of nitric oxide synthase 3 (NOS3) null mice. A, DNA electrophoresis from wild-type (WT) and double NOS3- and MMP-13-deficient mice, showing the different allelic combinations as indicated. B, Aortic intima/media thickness from WT, MMP-13 null mice, NOS3 null mice, and NOS3/MMP-13 null mice 15 and 30 days after endothelial denudation (n=10 mice/group per day by triplicate; mean±SD; *P<0.05 NOS3 null 30 vs NOS3/MMP-13 null 30). C, Aortic intima/media thickness from a 30-day injured and transplanted NOS3-deficient mouse with bone marrow from MMP-13 null or double MMP-13/NOS3 null mice (n=6 mice/group per day by triplicate; mean±SD; P<0.05 NOS3 healthy control [open bar] vs NOS3 knockout [KO] denuded 30 days; **P<0.05 NOS3 KO denuded 30 days vs NOS3 KO irradiated/BM MMP-13 KO; ***P<0.05 NOS3 KO denuded 30 days vs NOS3 KO irradiated/BM MMP-13/NOS3 KO). D, Immunohistochemical detection of MMP-13 in cross sections of carotid samples from endarterectomized patients and healthy mammary arteries. The white box corresponds to the carotid magnified region as also shown. Scale bars, 50 μm. **Bottom**, Confocal microscopy detection of CD68 (green, fluorescein isothiocyanate) and NOS2 (red Cy3) in the same carotid section as above. Colocalization of both signals is represented in yellow (merged). Arrows indicate colocalization of macrophage infiltrates and MMP-13.
promoting proinflammatory macrophage accumulation and increased macrophage-mediated MMP-13 expression, as detected, and as described in osteoblasts, fibroblasts, and cancer cells. High levels of MMP-13 were detected in carotid M1 infiltrated macrophages from patients undergoing carotid endarterectomy, with extensive neointimal hyperplasia. To this regard, in patients undergoing percutaneous transcutaneous coronary angioplasty, neointima hyperplasia might be reduced by promoting M1 proinflammatory macrophage polarization to M2 resolving macrophages, in which expression of MMP-13 may play a pivotal role.

Lack of MMP-13 neither induce neointimal hyperplasia in mice expressing NOS3 nor promotes M1 macrophage-mediated VSMC migration, whereas increased aortic thickness shown in the absence of NOS3 is significantly reduced in double NOS3/MMP-13–deficient mice. Taking all these findings together, we propose that NO may prevent neointimal formation in mice by at least targeting MMP-13.

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Disclosures
None.

References


**Significance**

Arterial wall restenosis may be caused by mechanical disruption of the vascular endothelium, inducing extensive smooth muscle and endothelial cell migration and proliferation, resulting in increased arterial wall thickness and a narrowing of the lumen. We show the impact that arterial endothelium expressing nitric oxide has in the modulation of the inflammatory response, elicited as result of endothelial disruption, in which endothelial nitric oxide synthase 3 plays a critical role. Nitric oxide synthase 3 regulates macrophage polarization by preventing accumulation of proinflammatory macrophages, thereby contributing to the resolution of inflammation, and limiting restenosis. The significance of this finding is based in the effect on proinflammatory macrophage-derived matrix metalloproteinase-13 stimulation of smooth muscle cell proliferation and migration in the absence of nitric oxide synthase 3, proposing matrix metalloproteinase-13 as a novel target in the prevention of restenosis.
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Vascular endothelium regulates inflammation by preventing accumulation of proinflammatory macrophages through NOS3-dependent expression of MMP-13.

Materials and Methods

Reagents.

General cell culture supplies were from BD Biosciences (Spain), calf serum was from BioWhittaker (Verviers, Belgium), BD cell recovery solution (MatriSperse), GFR Matrigel solution were from Becton Dickinson and cell culture gelatin, trypsin and antibiotics, IFNy, IL-4, LPS, Hematoxillin and Eosin, CFSE were from Sigma (Spain). Primary antibodies were obtained as follows: anti-MMP13 from Calbiochem (CN biosciences, UK), goat anti-human-MMP-13, goat anti-MRC1, rabbit anti-inducible NO synthase (NOS2), and goat anti-IL6 were from Santa Cruz Biotechnology (Santa Cruz, CA), mouse anti-Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) from Chemicon (Millipore, Iberica, Spain), rat anti-CD68 from Serotec, Rabbit anti-c-Myc from Millipore. Mouse Cytokine 20 plex panel was from Invitrogen. TRIzol was from Invitrogen. miRNeasy mini kit was from Quiagen. High Capacity cDNA Achieve kit was from Applied Biosystems. L-NAME, 1400W and DETA-NO were from Enzo Life Sciences. Liquid DAB+ Substrate Chromogen System was from Dako. Collagenase was from Worthington Biochemical Corporation (Lakewood, NJ).

Animals.

Animals were housed in our animal facilities in isolated rooms. All animal procedures were approved by the CNIC Research Ethics Committee (Certificate PA-227/2), and conformed to EU Directive 86/609/EEC and Recommendation 2007/526/EC regarding the protection of animals used for experimental and other scientific purposes, enacted under Spanish law 1201/2005. Wild-type C57BL/6 mice and C57BL/6 NOS3-null mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 MMP13-null mice were kindly donated by Dr. Carlos López Otín (University of Oviedo, Spain). Wild-type C57BL/6 c-Myc fl/fl LysMcre/+ mice (Mφ-c-mycKO mice) were from Dr. Pello. C57BL/6 MMP13/NOS3 double-null mice were generated in our laboratory by crossing C57BL/6 NOS3-null mice C57BL/6 MMP13-null mice. The second generation was genotyped by PCR with the following primers:

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<th>WT-PCR Reverse</th>
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Double deficient animals were backcrossed for 10 generations.

Cells

Murine aortic endothelial cells were grown on gelatin as described previously. Bone marrow-derived macrophages (BMDMs) were obtained by flushing mouse tibiae and femurs with ice-cold PBS and passing the suspension through a cell strainer (70µm cut-off). Cells (7x10^6) were seeded in 100x20mm non-treated cell culture plates with 10 ml RPMI 1640 supplemented with 10% L929-cell conditioning medium, as a source of macrophage colony-stimulating factor (M-CSF) (American Type Culture Collection CCL-1TM). Cultures were incubated at 37°C, 5% CO2 for 7 days to obtain 95%-pure CD11+ BMDMs. Macrophages were cultured with IFNy and LPS for 24 hours to obtain M1 Macrophages, and with IL-4 for 24 hours to obtain M2 Macrophages as previously described.

Murine bone marrow myeloid (Lin-, Sca1+, c-Kit-), and LSK progenitor cells (Lin-, Sca1+, c-Kit+), were detected by flow cytometry with a FACSCanto flow cytometer and FACSDiva software (BD Biosciences). Viable cells were identified by propidium iodide exclusion, and single cells were discerned with a stringent multiparametric gating strategy based on FSC and SSC (pulse width and height), and sorted on a FACSaria flow cytometer (BD Biosciences). The following rat-anti-mouse specific antibodies were used:
Vascular endothelium regulates inflammation by preventing accumulation of proinflammatory macrophages through NOS3-dependent expression of MMP-13.

Lin-cell were detected by using a "Biotin mouse lineage" cocktail from BD Pharmingen; specific anti-Sc1-FITC, and anti c-Kit-PE-Cy7 from eBiosciences. Vascular Smooth Muscle Cells (VSMC) were obtained from mouse aortas. Aortic rings were cut into 30-μm-thick segments and incubated with collagenase for 20 minutes at 37°C, 5% CO2. The adventitia layer was removed, and rings were incubated with shaking for 2h at 37°C. After that, aortic rings were centrifuged and rinsed twice with RPMI medium. Aortic rings were then seeded in 6-well plates.

**Human Arterial Specimens**

Human carotid endarterectomy specimens were obtained from 25 patients (21 men: mean age, 75±5 years; 4 women: mean age, 71±8 years) with >70% carotid stenosis as demonstrated by digital subtraction angiography and Doppler ultrasonography. Non-atherosclerotic internal mammary artery specimens (controls) were obtained from 10 male patients (mean age, 65±5 years) undergoing coronary artery bypass surgery. All samples were obtained as surgical residues in accordance with local ethics committee regulations, and patients gave informed consent.

**Endothelial denudation in mice**

Mice (8 to 12 weeks) were anesthetized by i.p. injection of ketamine/xylacinne (100mg/kg)/xylacine (5mg/kg), and a vertical laparotomy was practiced. With the help of a surgical stereomicroscope (Leica), the abdominal aorta was isolated and below the renal arteries and above iliac arteries, the abdominal aorta was temporary occluded, creating an aortotomy with a 30 gauge-needle. The aortas were infused three times under pressure (100 mmHg) with saline buffer to remove the endothelial cells. The aortotomy was then repaired, occlusion was eliminated, and blood flow restoration was visualized. Endothelial denudated animals and sham operated controls were closed, housed, and subjected to further experimentation. For histologic analysis paraffin embedded tissue was used to perform at least 10 cross sections (4 μm thick) at every 100 μm intervals throughout the selected portion of the abdominal aorta.

The effectiveness of the denudation protocol was evaluated by counting endothelial cells at the same time, 15 days and 30 days after the procedure, by performing HE immune staining on crossed sections covering the full abdominal aortic segment selected for denudation, with anti ICAM-2 antibody (Supplemental Figure 1).

**Morphometry**

Morphometric analysis was performed in 12 cross HE stained sections/mice to measure the areas of tunica media, tunica intima and luminal area, by using the Motic Images Plus software. Intima/media thickness was calculated as the external elastic lamina area subtracted from the lumen area, whereas neointimal formation was calculated as the area covered by the internal elastic lamina minus the lumen area.

**RNA isolation and quantitative real-time PCR (qPCR).**

Total RNA from tissue and cell extracts was extracted with TRIzol as previously described 1. First-strand cDNA was synthesized starting from 1μg of total RNA in a 20 μl reaction mixture using the High Capacity cDNA Achieve kit, as recommended by the manufacturer instructions. The following primers for qPCR were the following:

Arg1-Forward: CAGAAGAATGGAAAGAGTCAG
Arg1-Reverse: CAGATATGCAGGGAGTCACC
NOS2-Forward: TGCATGGACCAGTATAAGGCA
NOS2-Reverse: GCTTCTGGTCGATGTCATGAG

Three replicates were performed for each experimental condition, and differences were assessed with a two-tailed Student’s t test. Results were normalized using housekeeping gene GAPDH, and the ∆∆ cycle threshold method, and are expressed in arbitrary units.
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Confocal Microscopy
Proteins were detected by confocal microscopy in paraffin-embedded tissue sections as previously described.

En-face immunohistochemical detection of Macrophages.
Detection of M1 and M2 Macrophages were carried out in abdominal aortas ex vivo. Aortas were extracted from mice, sectioned in two parts; sagittal cuts were performed and incubated with antibodies to CD68 (broad), NOS2 (M1), or MRC1 (M2). The rings were then washed three times, and the aorta was visualized in en face preparations by confocal microscope. Fluorescent images were captured at ×10 magnification. The intensity of the signals corresponding to CD68, NOS2 and MRC1 staining were analyzed using the Imaris x64 7.3.1 (Bitplane, Zurich, Switzerland) imaging software, in which at least five full fields per vessel were analyzed. Fluorescence positive cells were counted in 1mm² sections.

Immunohistochemistry
Paraffin-embedded tissue from mouse and human biopsies were sliced 4µm thick. Vessel morphology was monitored by H&E staining, and visualized by bright field microscopy. In human biopsies, MMP-13, NOS2, and NOS3 aortic sections were incubated with normal serum, washed 5 minutes with PBS, and then incubated for 1 hour with the corresponding primary antibody in humidity chambers. Controls were also incubated with nonimmune serum. Slices were washed three times with PBS and then incubated with horseradish peroxidase-conjugated secondary antibodies, and immunoreactive complexes were detected with alkaline phosphatase substrate.

Migration and Proliferation assays
BMDMs were polarized to M1 or M2 Macrophages as described above. Growing medium was then discarded, culture plates were rinsed twice with PBS, and fresh medium was added for 24h to obtain macrophage-conditioned medium. For the migration assays, vascular smooth muscle cells (VSMC) grown in 24-well plates, were cut with a scalpel blade across the confluent cell monolayers, and incubated with control medium, M1 or M2 conditioned medium over time. Cell migration was monitored by time-lapse photomicroscopy and quantified with the ImageJ software. For proliferation assays, VSMC were labeled with 5 µmol/L CFSE for 5 minutes and then cultured in Macrophage-conditioned medium. VSMC proliferation was measured as the reduction of CFSE fluorescence intensity after 24h and 48h using FACSCanto flow cytometer (BD Biosciences).

Multi-plex assays
Multi-plex analysis was performed with blood serum or with cultured supernatants, according to the manufacturer instructions. To prepare mouse serum, peripheral blood was incubated 30 min at 37°C, and then incubated at 4°C for 2 hours. Blood was centrifuged at 10000 x g for 15 minutes at 4°C. On the other hand, cultured media collected from 6-well plate growing cells were discarded, culture plates were rinsed twice with PBS, and fresh medium was added at different time points to obtain MAEC-conditioning medium, which will be used for the assay.

Bone marrow transplant
Mice (4 weeks) were irradiated twice with 6.5 Gy (13 Gy in total) dosages, waiting 3h between irradiation. One day after irradiation, bone marrow from the donor source was transplanted by injecting 30x10⁶ cells. Animals were recovered for 8 weeks, and after that, they were subjected to endothelial denudation as previously described.

Statistical analysis
Results are expressed as mean±SD and analyzed using the GraphPad-Prism software package (GraphPad Software, LaJolla, Ca). Unless otherwise specified, assays were repeated at least three times, and the differences were considered statistically significant at p< 0,05, as determined by unpaired 2-sided Student’s test (assays with
Vascular endothelium regulates inflammation by preventing accumulation of proinflammatory macrophages through NOS3-dependent expression of MMP-13.

two experimental conditions) or two-way ANOVA followed by Bonferroni’s test (assays with more than two experimental conditions).

References


Supplemental Figure I. The absence of NOS3 increases LSK progenitor cells in response to aortic endothelial denudation. (A) Confocal microscopy detection of ICAM-2 in aortic rings of control (Upper) and denuded WT mice. L: lumen. Scale bars 50 µm. WT (B) and NOS3-deficient mice (C) underwent aortic denudation, and bone marrow from NOS3 null (A) or WT (B) mice was transplanted. As indicated, bone marrow from WT mice and NOS3-null animals were also transplanted into WT mice and NOS3-null mice, as controls (n=6 mice/group/day by triplicate. A. Mean ± SD * p<0.05 control vs WT 30; ** p<0.05 control vs WT 30/BM WT; *** p<0.05 control vs WT 30/BM NOS3 KO). B. * p<0.05 control vs NOS3 KO 30; **p<0.05 control vs NOS3 KO 30/BM NOS3 KO; *** p<0.05 control vs NOS3 KO 30/BM WT; **** p<0.05 NOS3 KO 30 vs NOS3 KO 30/BM WT; ***** p<0.05 NOS3 KO 30/BM NOS3 KO vs NOS3 KO 30/BM WT). D. Progenitor LSK (left) and myeloid cells (right) from WT and NOS3 KO healthy mice. E. Progenitor LSK (left) and myeloid cells (right) collected from WT and NOS3 injured mice at the times indicated after endotelial denudation(n=6 mice/group/day by triplicate. Mean ± SD * p<0.05 WT 7 vs NOS3 KO 7).
Supplemental Figure II. Mice lacking NOS3 show increased levels of pro-inflammatory M1 macrophages after aortic endothelial denudation. A. WT and NOS3 KO aortic CD68+/iNOS+ (M1) and CD68+/iNOS- (M2) infiltrated macrophages at 7 and 15 days after endothelial denudation. Representative images show "en face" confocal microscopy detection of CD68+ (FITC, green) iNOS+(Cy3, red), and nuclei (Hoechst, blue). Data was graphically represented as percentage of lesional area, (n=6 mice/group/day by triplicate; mean ± SD, * p<0.05 M1 WT 14 vs M1 NOS3 14, ** p<0.05 M2 WT 14 vs M2 NOS3 14. B. WT and c-Myc KO aortic CD68+/iNOS+ infiltrated macrophages 15 days after endothelial denudation. Data was graphically represented as percentage of lesional area, (n=6 mice/group/day by triplicate; mean ± SD, * p<0.05 WT 14 vs c-Myc14.
Supplemental Figure III. Monocyte recruitment MCSF and CCL5 (RANTES) levels in WT and NOS3 KO mice after endothelial polarization. Plasma levels of MCSF (A) and CCL5 (B) proteins isolated from WT and NOS3 null mice after aortic endothelial denudation at the times indicated (n=3 mice/group/day by triplicate, mean ± SD).
Supplemental Figure IV

NOS3 WT

NOS3 KO

M1, NOS2+, MMP-13+ macrophages

M2, NOS2-, MMP-13- macrophages

L: Lumen

Endothelial cell

Smooth muscle cell

Endothelial Denudation

Macrophage Recruitment

Endothelial regeneration

Inflammation resolution

Neointimal

1

2

3

4

L: Lumen

Endothelial cell

Smooth muscle cell
### Supplemental Figure V

Plasma levels of pro-inflammatory cytokines isolated from WT and NOS3 null mice after aortic endothelial denudation at the times indicated.