Time-Course Analysis on the Differentiation of Bone Marrow-Derived Progenitor Cells Into Smooth Muscle Cells During Neointima Formation

Jan-Marcus Daniel, Wiebke Bielenberg, Philipp Stieger, Soenke Weinert, Harald Tillmanns, Daniel G. Sedding

Objective—Bone marrow-derived progenitor cells have been implicated to contribute to neointima formation, but the time course and extent of their accumulation and differentiation into vascular cells and, most importantly, the long-term contribution of bone marrow-derived progenitor cells to the vascular lesion remain undefined.

Methods and Results—Wire-induced injury of the femoral artery was performed on chimeric C57BL/6 mice transplanted with bone marrow from transgenic mice expressing enhanced green fluorescence protein, and vessels were harvested at 3 days, 1, 2, 3, 4, 6, and 16 weeks after dilatation (n=8 animals per time point). Using high-resolution microscopy, we unexpectedly observed that the expression of smooth muscle cell or endothelial cell markers in enhanced green fluorescence protein positive cells was a very rare event. Indeed, most of the enhanced green fluorescence protein positive cells that accumulated during the acute inflammatory response were identified as monocytes/macrophages, and their number declined at later time points. In contrast, a substantial fraction of highly proliferative stem cell antigen-1 and CD34+ but enhanced green fluorescence protein negative and thus locally derived cells were detected in the adventitia.

Conclusion—These data provide evidence that the differentiation of bone marrow-derived progenitor cells into smooth muscle cell or endothelial cell lineages seems to be an exceedingly rare event. Moreover, the contribution of bone marrow-derived cells to the cellular compartment of the neointima is limited to a transient period of the inflammatory response.

Key Words: progenitor cells • smooth muscle cells • vascular remodeling • restenosis • inflammation
of the BM-derived cells accumulating in the neointimal lesion were identified as monocytes/macrophages that disappeared at later time points. In contrast, we found that a highly proliferative stem cell antigen (Sca)-1 and CD34 but enhanced green fluorescence protein (eGFP) negative fraction of cells is present in the adventitial layer, suggesting that perivascular progenitor cells may very likely represent an additional source of neointimal SMCs. Thus, even though there is no doubt that circulating cells are of major impact for the healing/remodeling process of injured vessels, neointimal SMCs seem to originate from local cells of the vessel wall, not from circulating (progenitor) cells.

### Methods

For an expanded Materials and Methods section, please see the supplemental data, available online at http://atvb.ahajournals.org. Key techniques involved the lethal irradiation of C57BL/6 mice followed by BM transplantation of eGFP-transgenic BM and a wire-induced injury model of the femoral artery as previously described. Before harvesting the dilated arteries at 3 days, 1, 2, 3, 4, 6, and 16 weeks after injury (n=8 mice per time point), the mice were perfused with 4% paraformaldehyde. Microscopy was performed with a DMRB fluorescent microscope (Leica) equipped with a PIFOC piezo-element driven Z-drive (Physik Instrumente). Deconvolution of 3D widefield epifluorescence z-stacks was done under the usage of adaptive blind deconvolution algorithm with AutoQuantX (Media Cybernetics, Inc.). Confocal imaging was performed using an Eclipse TE2000-E confocal LASER scanning microscope (Nikon). Postprocessing and image analyses were done with MetaMorph™ and ImageJ.

### Results

#### Fluorescence-Activated Cell Sorter Analysis of BM Chimeras

The percentage of hematopoietic chimerism after irradiation and BM transplantation was assessed by flow cytometry of peripheral blood mononuclear cells at 12 weeks after transplantation. The analysis revealed that 82 to 94% of circulating mononuclear cells were eGFP+ in the recipient mice after irradiation with 9.5 Gray (88.43±4.21%, n=6) (supplemental Figure I). As compared with nonpretreated littermates, the peripheral blood cell count of the irradiated and BM-transplanted mice was within the physiological range. As a further control, immunohistochemical staining revealed a very high percentage of eGFP+ cells in the spleen of the killed mice. These cells stained positive for the pan-leukocyte marker CD45 (data not shown).

#### Time-Course Analysis on NI Formation

Following wire-induced dilatation of the femoral artery, the size of the NI increased over a time period of 4 weeks (NI/media ratio of 2.13±0.26), whereas the ratio of eGFP+ cells/all neointimal cells constantly decreased toward later time points (Figure 1). As previously described, the wire-induced dilatation of the femoral artery caused a complete disruption of the endothelial layer and a substantial loss of medial SMCs. Platelets were the first cells adhering to the injured luminal surface, followed by the recruitment of leukocytes that peaked at 2 weeks after injury (supplemental Figure II). At this time point, the eGFP+ cells accounted for 68.95±2.74% of all neointimal cells. Subsequently, medial cells showed high proliferative indices, as determined by proliferating cell nuclear antigen staining (Figure 2). A broad accumulation of α-SMA-expressing cells in the neointimal lesion was observed at 3 to 4 weeks after dilatation. Interestingly, the neointimal lesion development in nonirradiated littermates occurred earlier with a peak in leukocyte recruitment at 1 week and a SMC accumulation already at 2 weeks after dilatation (supplemental Figure III). The neointimal size

![Figure 1. Time-course analysis on NI formation in chimeric mice. A, Hematoxylin and eosin staining (upper) and costaining for α-SMA and eGFP (lower). B, The NI size was calculated as NI/media ratio (means±SD, n=8 mice). C, The numbers of eGFP+ cells and total cells were counted in the complete neointimal lesion of each cross-section (6 cross-sections throughout the lesion area; n=8 arteries per time point).](http://atvb.ahajournals.org/)

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was dose-dependently attenuated in irradiated and BM-transplanted mice (supplemental Figure III), but this effect was independent on the use of wild-type BM or eGFP-transgenic BM (data not shown). Importantly, the fractions of eGFP\(^+\)-mononuclear cells in mice irradiated with 10.5 Gray (89.74±5.09\%) revealed no significant differences compared with mice irradiated with 9.5 Gray (88.43±4.21\%), and we detected abundant staining of BM-derived cells in the adventitia/perivascular tissue.

Neointimal SMCs exhibited a dedifferentiated phenotype with relatively low levels of \(\alpha\)-SMA at 4 weeks after injury. At later time points, a more differentiated population of neointimal SMCs was restored, as shown by the strong expression of \(\alpha\)-SMA, SM-MHC, and calponin. In contrast, the number of eGFP\(^+\) cells in the neointimal lesion further declined and accounted for only 2.16±0.78\% of all neointimal cells at 16 weeks after injury (Figure 2).

**Differentiation of BMPCs Into Vascular SMCs**

Following wire-induced injury, the complete lesion range of each artery was carefully analyzed for the coexpression of \(\alpha\)-SMA, SM-MHC, or calponin in eGFP\(^+\) cells, in order to identify SMCs derived from BMPCs (n=6 cross-sections throughout the dilated area of each artery; n=8 mice per time point). Deconvolution analysis of high-resolution z-axis image stacks revealed that 0.9±2.1\% of all eGFP\(^+\) cells coexpressed \(\alpha\)-SMA at 4 weeks after injury (Figure 3A and 3B and supplemental Figure IV). However, 3D reconstructions of z-stacks obtained by confocal microscopy revealed that the expression of \(\alpha\)-SMA was only very faint in BM-derived cells (see supplemental video). Therefore, the expression of \(\alpha\)-SMA in BM-derived cells was found to be a very rare event during NI formation despite the fact that high numbers of eGFP\(^+\) cells were present in the neointimal lesion at 4 weeks after injury. Importantly, we could not detect
Further Characterization of eGFP⁺-Cells During NI Formation
The absolute number of eGFP⁺ cells in the NI, as well as the eGFP⁺ cells/all neointimal cells ratio, continuously declined at later time points after injury due to both a loss of eGFP⁺ cells and increasing numbers of locally derived, proliferating SMCs in the NI (Figure 4). To further characterize the accumulating eGFP⁺ cells, we performed immunohistochemical staining with different leukocyte markers. During the first weeks after injury, nearly all accumulating cells were positive for the pan-leukocyte marker CD45 (data not shown). The fraction of monocytes/macrophages (MoMa-2-expressing cells) of all eGFP⁺ cells in the NI increased from 0.31 ± 0.04 at 3 days to 0.88 ± 0.11 at 16 weeks after dilatation, so that the neointimal eGFP⁺ cells at late time points were predominantly identified as macrophages (Figure 5B). Importantly, the absolute number of macrophages in the neointimal lesion

Figure 3. Rare differentiation of BMPCs into vascular cells. Costaining for α-SMA and eGFP at 4 weeks after dilatation analyzed with confocal microscopy. A and B, Despite an abundant staining of eGFP⁺ cells in the NI, hardly any cells coexpressed α-SMA in confocal imaging analysis, shown here as maximum intensity projections of the analyzed volumes. Costaining for CD31 and eGFP at 6 weeks after dilatation analyzed with widefield epifluorescent microscopy and subsequent deconvolution. C and D, Although exceedingly rare, BM-derived endothelial-like cells could be detected (arrowhead; C). DAPI, 4’,6-diamidino-2-phenylindole.

Figure 4. Time-course analysis on the neointimal cellular mass. The graph illustrates an increase in eGFP⁺ cells up to 14 days after injury and then their constant decline, so that only very few eGFP⁺ cells were present in the neointimal lesion at 16 weeks after dilatation. In contrast, the number of eGFP negative and thus locally derived neointimal SMCs started to accumulate later but largely contributed to the cellular mass of the NI in the long term. The number of proliferating cell nuclear antigen positive cells increased in parallel to the number of resident cells expressing α-SMA. The expression of α-SMA in eGFP⁺ cells accounted for 0.9±2.1% of eGFP⁺ cells at 3 to 4 weeks after injury and could not be detected at other time points. Overall, n=6 cross-sections throughout the dilated area of each artery (n=8 animals per time point) were analyzed. DAPI, 4’,6-diamidino-2-phenylindole; PCNA, proliferating cell nuclear antigen.

Figure 5. Further characterization of eGFP⁺ cells. A, Costaining for MoMa-2 and eGFP. Monocytes/macrophages accounted for the major fraction of eGFP⁺ cells in the NI. B, Remaining eGFP⁺ cells at late time points were predominantly identified as monocytes/macrophages. C, At time points later than 2 weeks after injury, the absolute number of MoMa-2⁺ cells constantly declined in parallel to the total number of eGFP⁺ cells. DAPI, 4’,6-diamidino-2-phenylindole.
declined from 97.88±9.6 macrophages at 2 weeks to 5.13±1.89 macrophages at 16 weeks after injury (Figure 5C). This decline correlated with the decrease of all neointimal eGFP cells, respectively (Figure 4). Indeed, the inflammatory cells were not primarily targeted to the NI per se, because the majority of eGFP cells was detected in the perivascular tissue. Interestingly, large portions of the medial layer lacking SMCs were also impregnated with eGFP cells that did not coexpress α-SMA (Figure 2 and supplemental Figure II). Moreover, there often seemed to be completely distinct regions of either BM-derived macrophages or locally derived vascular SMCs as shown by staining for CD68 (monocytes/macrophages) and α-SMA in 2 adjacent slides of the same vessel (supplemental Figure VI). Thus, BM-derived cells can be considered to contribute to vascular remodeling by paracrine actions rather than being a relevant source of subsequently differentiated vascular cells.

Possible Role for Perivascular Stem Cells During NI Formation

Because neointimal cells seem to be derived primarily from local cells of the vessel wall, we further analyzed the role of non-BM-derived perivascular progenitor cells during NI formation. In nondilated arteries, 42.84% of adventitial cells were identified as Sca-1+ cells, whereas hardly any eGFP+ cells could be detected within the vessel wall. The fraction of resident Sca-1+ cells did virtually not change at 1 and 2 weeks after injury, but we observed eGFP+ cells in the adventitia that also expressed Sca-1 at these time points. At 3 and 4 weeks after dilation, we detected the highest rates of proliferating cells within the adventitia and also found increased absolute numbers of adventitial Sca-1+ cells. At 4 weeks after injury, the absolute number of Sca-1+ cells increased from 47.86±7.18 cells/slide in noninjured arteries to 158.5±16,656 cells/slide, and the fraction of proliferating adventitial cells rose to 14.94% of all adventitial cells (Figure 6 and supplemental Figure VII). Importantly, we observed singular Sca-1+ cells and CD34+ cells migrating into the NI at 3 and 4 weeks after dilation (Figure 6E). Otherwise, the expression of Sca-1 was restricted to cells within the adventitia.

Impact of Fixation and Image Acquisition Techniques for the Detection of Differentiated BMPCs

Because the endogenous eGFP signal was already very strong in the transplanted cells, an anti-eGFP antibody only showed a moderate enhancement of the signal (data not shown). However, such antibodies have been used to generate previously published data.3 Immediate perfusion and subsequent fixation with 4% paraformaldehyde were indispensable for maintaining the cell-specific eGFP signal. In contrast to direct fixation, we observed a diffusion of the tracer molecule throughout artery cross-sections. This unspecific staining was further enhanced by the use of an anti-eGFP antibody (supplemental Figure VIIIA). Moreover, the use of inadequate filter blocks with overlapping emission wavelengths revealed false-positive results. In areas with strong red fluorescence (staining for α-SMA), signals of this staining were also detected in the green channel, where eGFP signals were assessed (supplemental Figure VIIIIB). These results indicate that proper fixation, staining, and acquisition techniques are mandatory for the accurate assessment of the contribution of BM-derived cells in this model and might explain the confusing results in some previous reports.

Discussion

Inhibiting SMC proliferation is a highly effective way to prevent luminal stenosis due to neointimal lesion formation. Drug-eluting stents antagonize the stenotic process of NI formation and have been shown to reduce the rate of restenosis after percutaneous coronary intervention, but delayed reendothelialization has increased the risk of in-stent thrombosis and defines the requirement for prolonged anti-
platelet therapy. Thus, elucidating the exact pathophysiolo-
gical mechanisms and especially the origin of vascular cells
that contribute to neointimal lesion formation is essential to
optimize future invasive therapeutic strategies. The results of
the present study provide evidence that the definite differenti-
tation of BMPCs cells into SMCs or ECs is only an exceedingly rare event, i.e., the exception rather than the rule.
Moreover, most of the BM-derived cells found in the NI were
monocytes/macrophages, and there was no apparent substantial
long-term contribution of these cells to the cellular mass of
the NI.

Because we only found solitary α-SMA-expressing BM-
derived cells in the neointimal lesion, our results are in direct
contrast to previous reports claiming a BM origin for nearly
half of the vascular SMCs as well as a substantial fraction of
ECs. However, the suggested differentiation of BMPCs into
vascular cells during atherosclerosis or NI formation has always been a matter of debate. One reason for some of the
controversy is likely to be related to methodological problems
and “false-positive” staining. Our results highlight the critical
fact that only high-resolution microscopy as used in this study
can distinguish between a genuine double positive staining
and “pseudo” marker colocalization due to the superimposi-
tion of vascular cells with adjacent BM-derivd cells. More-
over, the failure to immediately and adequately fix tissues
results in the diffusion of the eGFP tracer molecule to adjacent
cells. This phenomenon may then be easily misinter-
preted as a faint eGFP signal in locally derived SMCs that
in fact do not express eGFP.

Another explanation might be that infiltrating macrophages
can indeed temporarily express α-SMA in an inflammatory
environment. These cells resemble an intermediate pheno-
type of the macrophage at the interim state between late
inflammation and scar formation and thus lack markers for
highly differentiated SMCs. These intermediate phenotype
macrophages may also possibly have confounded the data of
previous studies. This hypothesis is most convincingly sup-
ported by the inability of these studies as well as our study
to detect more specific markers for differentiated SMCs, such as
SM-MHC or calponin in BM-derived cells in neointimal or
atherosclerotic lesions. Especially, cross-immunoreactivity
of antibodies detecting SM-MHC with nonmuscle MHC is an
issue of concern. Thus, we very carefully assessed the
specificity of the used SM-MHC antibody (supplemental
Figure V). With the use of this antibody, however, we were
not able to detect SM-MHC expression in BM-derived cells at
any time point. Indeed, in a recent review, Tanaka and Sata13
speculated that the BM-derived cells expressing α-SMA that were
described and quantified in their initial observations might represent “SMC-like macrophages” rather than differentiated SMCs. However, there was still a lack of conclusive data supporting this hypothesis. Although a study of an atherosclerotic mouse model has already questioned the BM-derived origin of SMCs in atherosclerosis, it has been claimed that the accumulation of differentiated BMPCs correlates with the severity of the injury model. Consequently, even though a differentiation of BM cells into SMC is missing during the chronic process of atherosclerosis develop-
ment, a substantial contribution of BMPCs to neointimal
lesion formation may exist following the acute denudation
and dilatation by wire-induced injury, which resembles the acute severe injury during percutaneous coronary interven-
tion. However, our data now provide the first real evidence of
a missing long-term contribution and terminal differentiation
of BM-derived cells into SMCs in neointimal lesions beyond
the state of the inflammatory response. Moreover, also an exceedingly rare differentiation of BM-derived cells into ECs
was observed. These cells may not represent genuine ECs but
rather CD31-expressing “endothelial-like” myeloid cells, be-
cause cells of myeloid origin have also been shown to express
CD31. We were not able to detect further EC-specific markers like von Willebrand factor in BM-derived cells.

In fact, a growing body of evidence confirms the lack of
developmental plasticity of adult BMPCs. Circulating en-
dotheilial progenitor cells were shown to mainly represent
monocytic cell lineages that do not have the ability to sub-
stantially “transdifferentiate” into genuine vascular cells
in vivo. This was confirmed by the inability of BMPCs to
differentiate into ECs in a time-course experiment of tumor-
induced angiogenesis. In addition, there was no evidence
for BM-derived renewal of the endothelium in a mouse model
of atherosclerosis. Because we now demonstrate that this is
also true for vascular cells after wire-induced injury of the
mouse femoral artery, the properties of BMPCs to accelerate
vascular SMC or EC proliferation seem to be rather attributed
to their paracrine effects.

This hypothesis is also supported by the results of recent
clinical trials that assessed the effects of injecting BMPCs
into coronary arteries of patients with acute myocardial
infarction following percutaneous coronary intervention. Im-
portantly, this treatment did not aggravate the development
of restenosis, thus indirectly implying that there is not a
substantial differentiation of BM-derived cells into functional
vascular SMCs in humans. Indeed, a clinical trial on NI
formation in human cardiac allograft vasculopathy recently
confirmed the exclusive local origin of neointimal SMCs. In
accordance with the data from our animal model, the authors
did not find any long-term contribution of BMPCs to neoin-
timal lesions.

The implications of our results lead us back to the original
hypothesis of a local origin of vascular SMCs in NI forma-
tion. Many years ago, the groups of Benditt and Schwartz
provided evidence for a monoclonal origin of SMCs in
atherosclerotic lesions. This key finding has recently been
complemented by the identification of ABCG-2-expressing
cells in the medial layer of the mouse aorta and femoral
artery, suggesting a particular involvement of this develop-
mental subpopulation of medial SMCs. We now demon-
strate that a Sca-1− and CD34− but eGFP negative fraction of
cells within the adventitial layer exerts high proliferative
indices during NI formation and very likely represents an
additional source of neointimal SMCs. In vitro experiments
from Passman et al indicate that 30 to 50% of adventitial
Sca-1+ cells can differentiate into smooth muscle-like cells and
thus lose Sca-1 from their cell surface, whereas other
adventitial Sca-1+ cells self-renew at the same time. Al-
though we confirm these data in our in vivo model, we do not,
in fact, know whether these cells represent genuine progenitor
cells and thus have the ability to account for highly differentiated SMCs. However, the high proliferative indices of adventitial cells observed in our time-course experiment of NI formation, as well as recent data from a transplant atherosclerosis model, certainly support this possibility. Further research is needed to clarify the contribution of perivascular progenitor cells compared with proliferating medial SMCs or medial progenitor cells in the development of a neointimal lesion.

In conclusion, we clearly show that BM-derived cells do not account for highly differentiated vascular SMCs detected during NI formation. Instead, the very rare numbers of BM-derived cells that faintly and temporarily express α-SMA in the neointimal lesions very likely represent SMC-like macrophages in an interim state. Consequently, these cells do not contribute to a completely restructured vessel in the long term but are only transiently present in the NI at time points that are correlated with a pronounced inflammatory response. Nevertheless, targeting these cells might interfere with the inflammatory response and thus might represent an interesting therapeutic strategy to prevent the activation of local cells and thus NI formation. These findings newly define the role of BM-derived cells for the development of neointimal lesions after vascular injury and substantially add to the current understanding of the pathogenesis of NI formation and restenosis as its clinical correlate.

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Disclosures

None.

References

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Materials and Methods

Animals: All procedures concerning animal experiments were in accordance with local ethical guidelines. The experiments had been approved by the institutional committee for animal research at Giessen University and complied with the Guide for the Care and Use of Laboratory Animals (NIH publication No. 86-23, revised 1985).

Experiments were performed on adult male C57/BL6 mice purchased from Charles River (Sulzfeld, Germany). Enhanced green fluorescence protein (eGFP)-transgenic mice, backcrossed more than 10 times to C57/BL6 mice, were kindly provided by Dr. R. Voswinckel, Max-Planck-Institute, Bad Nauheim, Germany.

Irradiation and bone marrow transplantation (BMTx): C57/BL6 mice were lethally irradiated with a dose of 9.5 Gy. On the same day, ~5 x 10^6 cells derived from eGFP-transgenic littermates (eGFP^+-BM) were transplanted by tail vein injection as previously described.\(^1\) In brief, the eGFP-transgenic mice were euthanized and the BM of the femur and the tibia was rinsed out with RMPI Medium 1640 containing 1 % fetal calf serum (FCS) (Invitrogen, Karsruhe, Germany), 100 U/ml penicillin and 1000 U/ml streptomycin (Sigma-Aldrich, Munich, Germany). This suspension was filtered through a nylon sieve (20µm, Becton Dickinson, Franklin Lakes, NJ, USA). An erythrolysis was performed using erythrocyte lysis buffer (Biolegend, San Diego, CA, USA), and the mononuclear cells were counted using a hemocytometer prior tail vein injection. Enrofloxacin (Baytril®, Bayer, Leverkusen, Germany) was administered to the drinking water for 2 weeks after BMTx. Additionally, C57BL/6 were irradiated with 10.5 Gy (n=6), and BMTx with eGFP^+-BM was performed according to the same protocol. As a negative control, WT-BM-cells were transplanted into WT-mice after irradiation with 10.5 Gy (n=6). Non-irradiated mice were used as further controls (n=6). At 12 weeks after transplantation, flow cytometry analysis (FACS Scan, Becton Dickinson) of blood
samples was performed to monitor the success of BMTx (eGFP\(^{-}\)-cells per total mononuclear cells).

**Mouse femoral artery injury model of neointimal hyperplasia:** The mice were anesthetized by a singular intramuscular injection of \(~3\) mg ketamine (Inresa, Freiburg, Germany) and \(~2.5\) \(\mu\)g xylazine (Rompun\(^{\circledR}\), Bayer, Leverkusen, Germany) diluted in 0.9% sodium chloride solution into the right hind limb. Subsequently, \(~5\) \(\mu\)g atropine (Fresenius Kabi, Bad Homburg, Germany) was injected into the contralateral limb to antagonize the vagal effects of the anesthesia.

For the injury model of the femoral artery, a straight spring wire (0.38 mm in diameter, Cook, Bloomington, IN, USA) was used as previously described. The spring wire was inserted into the profunda femoris artery, pushed forward for \(~10\) mm toward the iliac artery and left in place for 1 minute to dilate the artery. The blood flow was reconstituted after ligation of the profunda femoris branch. The mice were killed at 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 6 weeks, and 16 weeks after dilatation (n=8 per time point) by an overdose of isoflurane (Baxter, Unterschleissheim, Germany) and perfused with 4% para-formaldehyde (PFA) (Carl Roth, Karlsruhe, Germany) for 5 min. The femoral artery was carefully excised, postfixed in 4% PFA for 1h, and embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B.V., Zoeterwoude, The Netherlands). Afterwards, the arteries were snap-frozen and stored at \(-80^\circ\)C until sectioning.

**Morphometry:** The whole artery was cut in 6 \(\mu\)m serial sections and 6 cross-sections from regular intervals throughout the artery were stained with hematoxylin-eosin. For morphometric analyses, Metamorph Imaging 7.0 software (Molecular Devices, Downingtown, PA, USA) was used to measure external elastic lamina, internal elastic lamina, and lumen circumference, as well as medial and neointimal area.
**Immunohistochemical staining:** After fixation and rehydration, the slides were pre-incubated with 10% normal goat serum (Zymed® Laboratories Inc., San Francisco, CA, USA) and then incubated with antibodies against α-SMA (Sigma-Aldrich, Munich, Germany), SM22α (Abcam, Cambridge, UK), SM-MHC-1 (Kamiya Biomedical Company, Seattle, WA, USA), calponin-1 (Epitomics, Burlingame, CA, USA), CD31 (BD Pharmsingen, Franklin Lakes, NJ, USA), CD45 (BD Pharmsingen), CD68 (Serotec, Oxford, UK), MoMa-2 (Serotec), CD41 (BD Pharmsingen), CD34 (eBioscience, San Diego, CA, USA and BD Pharmsingen), Sca-1/ Ly-6A/E (R&D Systems, Mineapolis, MN, USA and BD Pharmsingen) separately or together with a rabbit antibody against eGFP (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Ensuing incubations were carried out with Cy5- or Cy3-coupled secondary antibodies (Molecular Probes, Eugene, Oregon, USA) and counterstained with nuclear 4,6-diamidino-2-phenylindole (DAPI) (Linaris, Wertheim, Germany). Monoclonal antibodies to α-SMA were labelled directly with Cy3, respectively. Staining for proliferating cell nuclear antigen (PCNA) was performed by using a PCNA staining kit (Invitrogen). Negative controls were conducted by substituting the primary antibody through an appropriate species- and isotype-matched control antibody (Santa Cruz).

**Microscopy:** Tissue samples were analyzed with a DMRB fluorescent microscope (Leica, Wetzlar, Germany) equipped with a PIFOC piezo-element driven Z-drive (Physik Instrumente, Jena, Germany). Acquisition and device control was preformed by MetaMorph™ (Molecular Devices). Deconvolution of three dimensional widefield epi-fluorescence z-stacks was done under the usage of adaptive blind deconvolution algorithm with AutoQuantX (Media Cybernetics Inc, Bethesda, MD, USA). Single stained tissue samples were used to exclude crosstalk and bleed through of fluorochromes. Widefield epi-fluorescence imaging results were conformed by confocal imaging with the Eclipse TE2000-E confocal LASER scanning microscope (Nikon, Tokyo, Japan). Post processing and image analyses were done with MetaMorph™ and ImageJ. (Bolte, S. und Cordelieres, F. P. 2006; Rasband, W. S. 1997)
Statistical analysis: Data were stored and analyzed on personal computers using Excel 2007 (Microsoft) and Sigma Stat 2.03 (Systat, Erkrath, Germany). Data between the study groups were analyzed by 1-way ANOVA followed by pair wise multi comparison using the Holm-Sidak method. All data are represented as mean ± standard deviation (SD). A probability value <0.05 was considered statistically significant for all comparisons.


Supplemental Figure I: Methodological background: At 12 weeks after BMTx, flow cytometry was performed and revealed a fraction of 88.43% ± 4.21 GFP⁺-mononuclear cells. The mice were subjected to dilatation of the femoral artery (n=8 mice per time point), and the vessels were harvested at the dates indicated in the scheme.
Online Figure II: Contribution of eGFP⁺-cells to NI formation: Co-staining for α-SMA and eGFP (A-F) at 3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, and 16 weeks after injury: Initial apoptosis of medial SMCs, adhesion of eGFP⁺-leukocytes, accumulation of resident SMCs in the NI, and a decline of the total number of eGFP⁺-cells were observed.
Supplemental Figure III: Earlier NI development in non-pretreated mice: A, Staining for MoMa-2: At 1 week after injury, the accumulation of monocytes/macrophages was increased in non-irradiated arteries compared to the arteries of irradiated mice. B, Staining for α-SMA: At 2 weeks after injury, many SMCs were already present in the NI. C, Irradiation inhibited the NI/media ratio in a dose dependent manner (means ± SD, **P<0.001, n=6).
Supplemental Figure IV: Further characterization of eGFP and α-SMA double positive cells: A, (average) projection of a confocal image representing a 212 x 212 x 6 µm volume. B, confocal planes in relative Z position of 1 µm in single channel or merge of the region of interest (ROI) indicated in A. C, (average) projection of the ROI volume. Line is marking the pixels which were used for the Z-projections. D, Z-projection along the line in C in merged channels or single channels.
Supplemental Figure V: Staining with the Kamiya MC-352 antibody: The rat IgG2A monoclonal antibody directed against smooth muscle-myosin heavy chain (SM-MHC)-1 was tested on different mouse tissues and specifically stained vascular smooth muscle cells of arteries, arterioles or veins.
Supplemental Figure VI: Distinct regions of α-SMA and CD68 expressing cells:
A, Co-staining for α-SMA and eGFP: At 6 weeks after dilatation, there was still an abundant staining of eGFP⁺-cells in a single vessel, but the staining of α-SMA did not at all overlap with BM-derived cells. B, Co-staining for CD68 and eGFP: In contrast, the eGFP⁺-cells in an adjacent cross-section of the same vessel showed large immunoreactivity to CD68, a specific marker for monocytes/macrophages.
Supplemental Figure VII: Time course analysis on adventitial cells during NI formation: The first increase in the absolute numbers of adventitial cells was due to the inflammatory response to the injury and the accumulation of eGFP⁺-cells. At 3 and 4 weeks after injury, cells in the adventitia exerted high proliferative indices and the number of Sca-1⁺-cells increased.
Supplemental Figure VIII: Possible sources of false positive results: A, B Co-staining for α-SMA and eGFP: Without immediate fixation of the artery with PFA, the tracer molecule diffused from the genuine eGFP⁺-cells all over the tissue (A). The use of inadequate filter blocks also revealed false positive results (B).
Online Figure 9: Negative controls in immunohistochemistry: Negative controls were conducted by substituting the primary antibody through an appropriate species- and isotype-matched control antibody.