Transdifferentiation of Vascular Smooth Muscle Cells to Macrophage-Like Cells During Atherogenesis

Susanne Feil, Birgit Fehrenbacher, Robert Lukowski, Frank Essmann, Klaus Schulze-Osthoff, Martin Schaller, Robert Feil

Rationale: Atherosclerosis is a widespread and devastating disease, but the origins of cells within atherosclerotic plaques are not well defined.

Objective: To investigate the specific contribution of vascular smooth muscle cells (SMCs) to atherosclerotic plaque formation by genetic inducible fate mapping in mice.

Methods and Results: Vascular SMCs were genetically pulse-labeled using the tamoxifen-dependent Cre recombinase, CreER\(^{T2}\), expressed from the endogenous SM22\(\alpha\) locus combined with Cre-activatable reporter genes that were integrated into the ROSA26 locus. Mature SMCs in the arterial media were labeled by tamoxifen treatment of young apolipoprotein E–deficient mice before the development of atherosclerosis and then their fate was monitored in older atherosclerotic animals. We found that medial SMCs can undergo clonal expansion and convert to macrophage-like cells that have lost classic SMC marker expression and make up a major component of advanced atherosclerotic lesions.

Conclusions: This study provides strong in vivo evidence for smooth muscle-to-macrophage transdifferentiation and supports an important role of SMC plasticity in atherogenesis. Targeting this type of SMC phenotypic conversion might be a novel strategy for the treatment of atherosclerosis, as well as other diseases with a smooth muscle component. (Circ Res. 2014;115:662-667.)

Key Words: atherosclerosis • cell transdifferentiation • macrophages • muscle, smooth, vascular

Atherosclerosis leads to myocardial infarction and stroke and is the major cause of death in the Western world. It is a chronic inflammatory condition that results from complex interactions of modified lipoproteins and various cell types, including monocyte-derived macrophages and cells of the vessel wall.\(^1-3\) How each particular cell type contributes to the development of an atherosclerotic lesion is not completely understood.\(^4,5\) One unsolved issue is the role of mature smooth muscle cells (SMCs) that reside in the vascular media.\(^6,9\) It is generally accepted that vascular SMCs are not terminally differentiated and can undergo phenotypic transitions during lesion development. However, this concept is mainly based on in vitro results with cultured SMCs and conclusive in vivo studies that have assessed the contribution of SMC plasticity to the development of cells with alternative phenotypes within atherosclerotic plaques are lacking.\(^5,8\) Two previous studies found by immunostaining of human plaque sections that some intimal cells coexpressed markers of SMCs and macrophages, suggesting the existence of a SMC-macrophage chimeric cell type in human lesions.\(^10,11\) However, these experiments did not allow one to determine the origin of the chimeric cells (ie, whether they originated from SMCs, macrophages, or other cell types). Furthermore, these studies were based on the staining of plaque cells for the classic SMC marker, smooth muscle \(\alpha\)-actin (SMA), and did not allow the detection of potential SMC-derived plaque cells that have lost SMA expression. To evaluate the role of SMC plasticity in atherogenesis, there is a critical need for definitive in vivo SMC lineage tracing studies.\(^5,8\)

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Methods

Genetic inducible fate mapping of medial SMCs was performed in apolipoprotein E (ApoE)–deficient mice carrying a SMC-specific tamoxifen-activated Cre recombinase and Cre reporter alleles expressing \(\beta\)-galactosidase or fluorescent proteins. For details on experimental animals, detection of reporter gene expression, immunostaining, and electron microscopy, an expanded Methods Section is available in the Online Data Supplement.

Results

To track the fate of medial SMCs during atherogenesis in mice, we have used a Cre/lox-based genetic inducible fate mapping
system previously established by us.12,13 Experimental animals were ApoE-deficient (ApoE−/−)14 and carried transgenes encoding the tamoxifen-dependent CreER T2 recombinase integrated into the endogenous SM22α gene locus (SM22-CreERT2), which is selectively expressed in SMCs of adult mice,15 and the well-characterized ROSA26 Cre reporter allele (R26R), which on Cre-mediated recombination can express β-galactosidase in a wide array of cell types and tissues.16 The Cre-activated R26R allele is inherited to all progeny cells, so that the fate of the originally labeled cells can be followed by staining tissues with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) for β-galactosidase activity.

We pulse-labeled medial SMCs before the development of atherosclerosis by injecting 10-week-old mice for 5 consecutive days with tamoxifen. X-Gal staining of aortic sections 1 week after the last tamoxifen injection showed that this protocol resulted in sparse labeling of 11±1% SMCs in the aortic media (Figure 1A), allowing also for clonal analyses of cell fate. It was important to exclude ectopic labeling of non-SMCs that might have expressed the SM22α-driven CreERT2 recombinase during the labeling period and could potentially contribute to future plaque development, for instance, progenitor cells in the adventitia or bone marrow.6,17 However, our induction protocol did not result in labeling of cells in the adventitia (Figure 1A), and even after prolonged tamoxifen treatment for 10 days to increase the labeling efficiency, we did not detect any X-Gal–positive cells in the aortic adventitia and circulating blood cells (Figure 1B) or in bone marrow cells (Figure 1C, top). Moreover, virtually all X-Gal–positive cells also stained positive for the smooth muscle differentiation marker, smooth muscle myosin heavy chain (Figure 1D). The latter finding confirmed that we labeled mature differentiated...
SMCs in our model, but not presumptive vascular stem cells that might also reside in the vascular media and are smooth muscle myosin heavy chain negative.18

Next, we analyzed the fate of medial SMCs in 52-week-old ApoE−/− mice that were fed with a normal chow. X-Gal staining of atherosclerotic aortas revealed few labeled cells in the media and, interestingly, occasional large patches of labeled cells that were invariably located within atherosclerotic lesions and sometimes covered the entire intimal area of the plaque section (Figure 1E and 1F). These findings indicated that each continuous X-Gal–positive cell patch was derived by clonal expansion of an individual medial SMC that was originally labeled by tamoxifen treatment in the 10-week-old animal. Clonal SMC-derived plaque areas were observed in 19 of 22 analyzed mice (86%). Similar results were obtained in ApoE−/− mice that received a high-fat diet (20% fat, 1.5% cholesterol by weight) for 16 weeks after the last tamoxifen injection, with 8 of 12 analyzed mice (67%) showing SMC-derived cell patches within lesions. The existence of clonally grown SMC-derived lesional regions was further established using 2 alternative fluorescence-based Cre reporter alleles. The R26R-mT/mG reporter19 expresses membrane-tagged red and green fluorescent protein before and after Cre-mediated activation, respectively (Figure 1G). The R26R-Confetti multicolor Cre reporter20 expresses stochastically 1 of 4 fluorescent marker proteins generating nuclear green, cytoplasmic yellow, cytoplasmic red, or membrane-bound blue cells, which makes this reporter allele particularly useful for clonal tracing analysis of individually labeled SMCs (Figure 1H).

To exclude potential pitfalls of our cell tracing strategy, several additional control experiments were performed. Plaque sections prepared from X-Gal stained aortas of nontamoxifen-treated mice did not show blue-stained cells (Online Figure IA), indicating that our labeling system was not leaky in the absence of tamoxifen and that the X-Gal staining observed in tamoxifen-treated animals (Figure 1A–1F) was specific. The specificity of signals that were detected in whole mount preparations of the tamoxifen-induced fluorescence-based Cre reporter mice (Figure 1G and 1H) was controlled by comparison with tissues from nontamoxifen-treated mice (Online Figure IB) and by fluorescence microscopy of thin sections prepared from whole mounts of tamoxifen-treated mice (Online Figure IC). Furthermore, immunostaining of aortic sections showed that cells in the aortic media did not express MAC-2 (also known as Galectin-3 or LGALS3), which is expressed in macrophages (Online Figure ID). Finally, cells from aorta, plaque, peripheral blood, and spleen of R26R-mT/mG reporter mice were analyzed by flow cytometry (Online Figure II). We did not detect green fluorescent–labeled cells in tissues from nontamoxifen-treated mice. Tamoxifen treatment induced the appearance of green fluorescent cells in aorta and in plaque but not in peripheral blood or spleen (Online Figure IIA–IID). In particular, we did not detect labeled Gr-1+ neutrophils or monocytes (Online Figure IIE–IIG). These findings confirmed that our inducible fate mapping system was not leaky and that the green fluorescence observed in whole mounts, sections, and cells of plaques from tamoxifen-induced R26R-mT/mG mice (Figure 1G; Online Figures IC and IIB) was specific and not because of autofluorescence. Importantly, our extensive control experiments did not reveal any evidence that myeloid cells in the blood and spleen were ectopically labeled during the tamoxifen pulse. This finding is also in agreement with a previous report, showing that the SM22-CreER21 transgene used in our study is not expressed in hematopoietic stem cells of adult mice.21 Formally, it might be conceivable that the X-Gal–positive or green fluorescent lesional cells originated as monocyte-derived macrophages that had phagocytized labeled SMCs. However, this possibility is highly unlikely for ≥2 reasons. First, the upregulation of macrophage markers is also observed in cultured SMCs that presumably do not contain monocytes/macrophages.22 Second, the biological activity of the reporter proteins that is required for detection of the labeled cells (ie, enzymatic β-galactosidase activity or green fluorescence) would most likely not be preserved after phagocytosis of the SMC.

Immunostaining of X-Gal–stained plaque sections showed that, as expected, cells in the media and fibrous cap strongly expressed SMA (Figure 2A and 2B). In contrast, the SMC-derived cells in the intima were either not stained for SMA or expressed it at a much lower level than SMCs in the media or fibrous cap (Figure 2B). Weak staining for SMA was detected in 39±5% of X-Gal–positive intimal cells. Thus, it is likely that the SMC-derived intimal cells went undetected in previous studies that analyzed the contribution of SMCs to lesion development by immunostaining for SMA. Interestingly, the majority of the SMC-derived cells in the intima stained positive for 2 markers that are commonly used to detect macrophages in plaque sections, MAC-2 (62±4% of X-Gal–positive intimal cells; Figure 2C) and CD68 (54±4% of X-Gal–positive intimal cells; Figure 2D). A macrophage-like function of the SMC-derived plaque cells was further supported by positive staining for oxidized low-density lipoprotein (81±2% of X-Gal–positive intimal cells; Figure 2E), as well as by electron microscopic studies, which showed a foam cell-like morphology of the X-Gal–positive intimal cells (Figure 2F, middle). However, the SMC-derived cells contained significantly less lipid droplets than X-Gal–negative classical foam cells (Figure 2F, lower; 7±1 droplets/cell; n=48 cells versus 29±3 droplets/cell; n=26 cells; P<0.001).

Discussion

On the basis of these in vivo findings, we propose that mature medial SMCs can undergo clonal expansion and transdifferentiate to macrophage-like cells during the development of atherosclerotic lesions. This model is consistent with reports showing that (1) cultured mouse and human aortic SMCs can gain a macrophage-like phenotype in vitro that is characterized by decreased expression of SMC markers, upregulation of macrophage markers, and increased phagocytic activity,22,23 (2) a large fraction of intimal MAC-2–positive cells in murine plaques is not derived from bone marrow progenitors,12,24 and (3) many cells within human atherosclerotic lesions express both SMA and CD68.10,11 Our observation that not all X-Gal–stained cells in the intima expressed macrophage markers (Figure 2C and 2D) is consistent with the SMC-to-macrophage transdifferentiation process. Alternatively,
these cells could represent other SMC-derived cell types, such as chondrocyte-like cells.25,26 Because of the fact that only few medial SMCs were initially genetically labeled in the 10-week-old animal, we cannot draw conclusions about the origin of plaque cells that were not stained positive with X-Gal. Considering our sparse labeling strategy and the high proportion of macrophage-like X-Gal–positive cells in the stained plaques, it seems that SMC-derived macrophages, in addition to conventional monocyte-derived macrophages, represent a significant component of advanced atherosclerotic lesions in ApoE-deficient mice. In this context, it is interesting to note that Robbins et al27 have recently reported that monocyte recruitment cannot fully account for lesional macrophage accumulation in established atherosclerosis. It is possible that the plaque macrophages detected by Robbins et al27 were derived from few bone marrow monocytes that proliferated after recruitment to the lesion. However, alternative mechanisms should not be excluded, for instance, that certain plaque macrophages/macrophage-like cells originate and are maintained independently of monocyte input. Indeed, recent evidence shows that monocytes do not substantially contribute to most tissue macrophage populations in the steady state or during certain types of inflammation.28

To characterize the SMC-derived plaque macrophages further, we compared the expression of selected proteins in nonatherosclerotic aorta versus cultured aortic SMCs, which undergo phenotypic changes during in vitro culture that might also occur in vivo during SMC-to-macrophage transdifferentiation.29 Indeed, in line with previous in vitro results30 and our in vivo data, Western blot analyses demonstrated downregulation of SMA and smooth muscle myosin heavy chain and upregulation of MAC-2 and CD68 in cultured SMCs when compared with nonatherosclerotic aorta (Figure 3A). Other proteins that we found to be upregulated in cultured SMCs (Figure 3B–3E, left) were proliferating cell nuclear antigen (PCNA), heme oxygenase-1 (HO-1), thrombospondin-1 (Tsp-1), and hypoxia-inducible factor-1α (HIF1α) to be upregulated in cultured SMCs (c) vs normal aorta (a). Subsequently, X-Gal–stained plaque sections of 52-week-old ApoE−/− mice were stained with the respective antibodies. Left, Overviews of the plaques; middle and right, higher powered magnifications. Right, The boxed regions in the respective middle panels. Scale bars, 100 μm (left and middle) and 10 μm (right). The Western blots are representative of ≥3 independent experiments. FC indicates fibrous cap; I, intima; and M, media.
nuclear antigen, heme oxygenase-1, thrombospondin-1, and hypoxia-inducible factor-1α, all of which are associated with cell growth and stress. Importantly, these proteins were also expressed in the SMC-derived intimal plaque cells in vivo (Figure 3B–3E). Proliferating cell nuclear antigen, heme oxygenase-1, thrombospondin-1, and hypoxia-inducible factor-1α were detected in 24±3%, 60±3%, 69±6%, and 86±3% of X-Gal–positive intimal cells, respectively. However, the SMC-derived cells did not stain for markers of M1 or M2 macrophages, inducible nitric oxide synthase or arginase-1, respectively (data not shown). Intriguingly, heme oxygenase-1 and thrombospondin-1 have been recently described as markers for the Mox macrophage subtype30 that, consistent with our electron microscopy data (Figure 2F), has a lower phagocytic capacity than M1 or M2 macrophages. Mox macrophages develop in response to oxidative tissue damage and may play an important role in atherosclerosis.7,30 Additional studies are required to understand the mechanism of this type of SMC phenotypic modulation fully, as well as its function and pathological relevance of SMC-derived versus monocyte-derived plaque macrophages. Indeed, combined gene targeting and cell tracking indicates that it is possible to modulate atherogenesis by targeting SMC-to-macrophage transdifferentiation.12,13 It will be interesting to perform similar inducible fate mapping experiments with monocyte-derived plaque cells. Then, both SMC-derived and monocyte-derived intimal cells could be isolated from plaques and comparatively characterized, for instance, by thorough gene expression profiling. Taken together, this study highlights the heterogeneity of both SMCs and macrophages in atherosclerotic lesions and provides strong in vivo evidence for a major role of SMC plasticity in atherosclerosis. According to our new model, mature SMCs that are present in the media of the nonatherosclerotic vessel wall possess the potential to become activated during plaque development and transdifferentiate to macrophage-like cells with a Mox phenotype that reside within the lesion. The SMC-derived plaque macrophages have a clonal origin in the vascular media and can make up a major fraction of the intimal cells. These findings indicate that previous studies that were based on immunostaining of plaque cells for smooth muscle markers have vastly underestimated the role of SMC plasticity in atherosclerosis and, because SMCs are present in almost every tissue, possibly in many other diseases, including hypertension, lung injury, liver injury, and cancer. Targeting SMC-to-macrophage transdifferentiation could be a novel therapeutic strategy to treat these diseases.

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Disclosures

None.

References


What Is Known?

- Atherosclerosis leads to myocardial infarction and stroke and is the major cause of death in the Western world.
- Atherosclerosis is a chronic inflammatory disease that arises from an interaction of modified lipoproteins and various cell types, including monocyte-derived macrophages from the blood and smooth muscle cells (SMCs) in the vessel wall.
- It is unclear how each particular cell type contributes to the development of atherosclerotic lesions.

What New Information Does This Article Contribute?

- During the development of atherosclerotic plaques, vascular SMCs can expand clonally and transdifferentiate to macrophage-like cells that reside within the lesions.
- Previous studies have underestimated the plasticity of SMCs and the heterogeneity of macrophage populations in atherosclerotic lesions.

The origins of cells that contribute to the development of atherosclerotic plaques are not well understood. One highly controversial issue is the contribution of vascular SMCs to plaque growth. Here, we performed lineage tracing experiments in mice, in which we have genetically labeled mature SMCs in the vessel wall of young mice before the onset of the disease and then monitored their fate in older atherosclerotic animals. We found that SMCs in the arterial wall can undergo clonal expansion during disease progression, and that they can transdifferentiate into macrophage-like cells that have lost the classical SMC marker, α-smooth muscle actin. The SMC-derived macrophages make up a major component of advanced atherosclerotic lesions. These findings indicate that previous studies that were based on immunostaining of plaque cells for smooth muscle and macrophage markers have vastly underestimated the role of SMCs and overestimated the role of monocyte-derived macrophages in atherosclerosis. Targeting SMC-to-macrophage transdifferentiation could be a novel therapeutic strategy to treat atherosclerosis.
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SUPPLEMENTAL MATERIAL

Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis

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Methods

Experimental Animals. Mice carrying the SM22-CreERT2 knock-in allele and the ROSA26 LacZ Cre reporter (R26R) allele were generated on an ApoE-deficient C57BL/6 genetic background by crossing the parental lines. As alternatives to the R26R LacZ Cre reporter, mice carrying fluorescence-based Cre reporter alleles were used, i.e. the R26R-mT/mG reporter strain or the R26R-Confetti multicolor Cre reporter strain, and mated with ApoE-/- and SM22-CreERT2 mice to generate experimental animals. Mice were housed with ad libitum access to food and water with a 12-h light/dark cycle. To induce Cre-mediated activation of the R26R reporter allele and cell labeling, 10-week-old mice were injected intraperitoneally with 1 mg tamoxifen (Sigma) for 5 or 10 consecutive days. Experiments had been approved by the committee on animal care and welfare of the local government.

X-Gal Staining and Immunohistochemistry. Aortas or bone marrow cells harvested from femurs were fixed for 30 min at room temperature in PBS containing 2% formaldehyde and 0.2% glutaraldehyde, washed twice in PBS, and incubated in X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside) staining solution (1 mg/mL X-Gal, 2 mM MgCl₂, 5 mM K₃Fe(CN)₆, 5 mM K₄Fe(CN)₆ in PBS, pH 7.4) overnight at room temperature. X-Gal stained aortas were either cryosectioned at 6 µm or embedded in paraffin and cut at 6 µm. For immunohistochemistry, antigen retrieval was accomplished by microwaving slides in 10 mM citric acid, pH 6.0 for 1 min. X-Gal stained paraffin sections were co-stained with antisera against smooth muscle α-actin (SMA) (Sigma, A2547), MAC-2 (Cedarlane, CL8942AP), CD68 (Serotec, MCA1957T), oxidized low-density lipoprotein (oxLDL) (Merck, 428033), proliferating cell nuclear antigen (PCNA) (Santa Cruz Biotechnology, sc-7907), heme oxygenase-1 (HO-1) (Santa Cruz Biotechnology, sc-10789), thrombospondin-1 (Tsp-1) (Lab Vision, MS-421-P0), hypoxia-inducible factor-1α (HIF-1α) (Novus Biologicals, NB 100-479), inducible NO synthase (iNOS) (Santa Cruz Biotechnology, sc-651), and Arginase-1 (Arg-1) (Santa Cruz Biotechnology, sc-20150; LifeSpan BioSciences, LS-C11252). X-Gal stained cryosections were co-stained with an antiserum against smooth muscle myosin heavy chain (SM-MHC) (Abcam, ab53219). For detection of primary antibodies, we used the avidin-biotin method with diaminobenzidine as a chromogen (Vector Laboratories). For detection of SMA, we used the Vector® M.O.M. Basic Kit (Vector Laboratories). Control sections were processed in the absence of primary antibodies. Sections were mounted in 80% glycerol containing 1 µg/mL Hoechst 33258 (Sigma) to visualize cell nuclei. Labeling efficiency was determined as X-Gal positive cells (blue) per total number of cells (Hoechst stained nuclei). For semi-quantitative analysis of lesional SMC-derived cells, X-Gal positive cells (blue) and X-Gal positive cells (blue) that co-stained for SMA, MAC-2, CD68, oxLDL, PCNA, HO-1, Tsp-1, or HIF-1α (brown) were counted on serial plaque sections (n = 5-15) in equivalent regions within each lesion. Data are presented as mean ± SEM.

Electron Microscopy. X-Gal stained plaques were post-fixed in 1.5% osmium tetroxide for 1 h. After following standard methods, blocks were embedded in glycol methacrylate and cut using an ultra microtome (Ultracut, Reichert). Ultra-thin sections (30 nm) were mounted on copper grids and analyzed using a Zeiss LIBRA 120 transmission electron microscope (Carl Zeiss) operating at 120 kV. Electron-dense X-Gal precipitates are typically localized at membranous compartments, particularly around the nuclear envelope. For semi-quantitative analysis of lipid droplet content, lipid droplets were counted on the micrographs of cells that showed a nuclear membrane. This allowed us to identify X-Gal positive and negative cells and to standardize the cellular region that was analyzed. Data are presented as mean ± SEM.
**Western Blot Analysis.** Three to six aortas were isolated from 4- to 8-week-old wild-type mice on a C57BL/6 genetic background. Aortas were carefully freed from perivascular/adventitial tissue, minced with scissors and pooled. Then, one half each of the tissue was immediately homogenized in lysis buffer (21 mM Tris-Cl, pH 8.3, 0.7% SDS, 0.2 mM phenylmethylsulfonyl fluoride) using a FastPrep homogenizer with lysing matrix A (MP Biomedicals) or used to establish a primary smooth muscle cell (SMC) culture by enzymatic digestion as previously described. After 6 days of primary culture in DMEM supplemented with 10% FCS, cells were extracted in lysis buffer. The lysates of aortas and cultured aortic SMCs were analyzed by Western blotting with the antibodies that were also used for immunohistochemistry (see above) except for HIF-1α, which was detected with another antibody (Novus Biologicals, NB 100-449). SDS gels were loaded with 15 µg protein and equal loading of lanes was confirmed by Coomassie staining.

**Flow Cytometry.** Aortas were freed from perivascular/adventitial tissue and cut open longitudinally. Then the visible plaques were carefully removed with forceps. The plaque tissue and the residual aorta were enzymatically digested separately as previously described. Peripheral blood was collected by cardiac puncture using a 5% EDTA solution in the syringe as an anticoagulant. Spleens were homogenized through a 40-µm nylon mesh. Cells were washed with PBS and fixed in 0.5% paraformaldehyde in PBS on ice before analysis on a LSR II flow cytometer (BD Bioscience). 100,000-130,000 events were collected for each sample with the exception of plaque cells, for which 40,000 events were counted. For the analysis of specific subsets of blood cells, erythrocyte lysis was performed and the remaining unfixed cells were stained with an anti-mouse Ly-6G (Gr-1) Alexa Fluor® 700 antibody (eBiosience, 56-5931). 1,200-3,000 events were counted. Data were analyzed with FlowJo v0.7 (Tree Star, Inc).

**Supplementary References**

Online Figures

A

![Image of aortic plaque stained with X-Gal and Hoechst](image)

B

![Image of whole mount preparations of atherosclerotic aortas](image)

C

![Image of cryosections of the whole mount plaque preparation](image)

D

![Image of immunohistochemical staining](image)

Online Figure I. Control experiments regarding the cell tracing strategy. A, Section of an X-Gal stained aortic plaque (left) from a non-tamoxifen treated 52-week-old ApoE<sup>−/−</sup> mouse fed with a normal chow and carrying the SM22-CreERT<sup>2</sup> and R26R transgenes. Cell nuclei were stained with Hoechst 33258 (right). Scale bars, 100 µm. B, Whole mount preparations of atherosclerotic aortas cut open longitudinally. The aortas were isolated from 52-week-old ApoE<sup>−/−</sup> mice fed with a normal chow. The middle aorta was from an ApoE<sup>−/−</sup> mouse without additional transgenes (ApoE<sup>−/−</sup>); the upper and lower aortas were from ApoE<sup>−/−</sup> mice that also carried the SM22-CreERT<sup>2</sup> and R26R-mT/mG transgenes and were either pulse-labeled by tamoxifen treatment of 10-week-old mice for 5 consecutive days (upper, +Tam) or non-treated (lower, -Tam). Note that the upper aorta shows green fluorescent labeled regions that can be clearly distinguished from the background fluorescence displayed by the middle aorta and also from the red fluorescence of the non-recombined reporter displayed by the lower aorta. Scale bar, 1 mm. C, 10-µm cryosections of the whole mount plaque preparation shown in Figure 1G. Note that green fluorescent labeled cells can be detected in the media and intima of the plaque. D, Immunohistochemical staining of an aortic section of a 12-week-old wild-type mouse with an antibody against MAC-2. Scale bars, 100 µm. A, adventitia; FC, fibrous cap; I, intima; L, lumen; M, media.
Online Figure II. Analysis of labeled cells by flow cytometry. Cells were isolated from 52-week-old ApoE−/− mice that were fed a normal chow and carried the SM22-CreER<sup>T2</sup> and R26R-mT/mG transgenes. Animals have been either pulse-labeled by tamoxifen treatment for 5 consecutive days at an age of 10 weeks (grey, +Tam) or left non-treated (white, -Tam). GFP fluorescence (indicative of Cre activity/recombination) was analyzed in cells from **A**, aorta, **B**, plaque, **C**, peripheral blood, and **D**, spleen. **E-G**, Analysis of GFP fluorescence in neutrophils and monocytes of peripheral blood. **E**, Forward scatter (FSC) and side scatter (FSC) plots with gates used for neutrophils and monocytes/lymphocytes. GFP fluorescence was monitored **F**, in Gr-1<sup>+</sup> cells in the neutrophil gate (R1) and **G**, in Gr-1<sup>+</sup> cells in the monocyte/lymphocyte gate (R2).