Atherosclerosis 251 (2016) 445–453

Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

Lineage tracing of cells involved in atherosclerosis

Julián Albarrán-Juárez [a, *], Harmandeep Kaur [a], Myriam Grimm [a], Stefan Offermanns [a, b], Nina Wettschureck [a, b]

[a] Department of Pharmacology, Max Planck Institute for Heart and Lung Research, Bad Nauheim, Germany
[b] Medical Faculty, Goethe University Frankfurt, Germany

ARTICLE INFO

Article history:
Received 22 March 2016
Received in revised form
3 May 2016
Accepted 8 June 2016
Available online 11 June 2016

Keywords:
Atherosclerosis
Lineage tracing
Gene expression
Smooth muscle cell
Macrophage
Transdifferentiation

ABSTRACT

Background and aims: Despite the clinical importance of atherosclerosis, the origin of cells within atherosclerotic plaques is not fully understood. Due to the lack of a definitive lineage-tracing strategy, previous studies have provided controversial results about the origin of cells expressing smooth muscle and macrophage markers in atherosclerosis. We here aim to identify the origin of vascular smooth muscle (SM) cells and macrophages within atherosclerosis lesions.
Methods: We combined a genetic fate mapping approach with single cell expression analysis in a murine model of atherosclerosis.
Results: We found that 16% of CD68-positive plaque macrophage-like cells were derived from mature SM cells and not from myeloid sources, whereas 31% of αSMA-positive smooth muscle-like cells in plaques were not SM-derived. Further analysis at the single cell level showed that SM-derived CD68$^+$ cells expressed higher levels of inflammatory markers such as cyclooxygenase 2 (*Ptgs2*, $p = 0.02$), and vascular cell adhesion molecule (*Vcam1*, $p = 0.05$), as well as increased mRNA levels of genes related to matrix synthesis such as *Col1a2* ($p = 0.01$) and *Fn1* ($p = 0.04$), than non SM-derived CD68$^+$ cells.
Conclusions: These results demonstrate that smooth muscle cells within atherosclerotic lesions can switch to a macrophage-like phenotype characterized by higher expression of inflammatory and synthetic markers genes that may further contribute to plaque progression.

1. Introduction

Atherosclerosis, a chronic inflammatory disease of the arterial wall, is one of the most common causes of death worldwide due to the rupture of unstable plaques and associated acute thrombotic events [14]. Key players in atherosclerosis development are lipid-phagocytosing macrophages [11,15], but also dedifferentiated vascular smooth muscle cells (VSMCs), which are believed to migrate from the media to the intima, where they contribute to the propagation of the inflammatory response [3]. However, VSMC in the plaque may also have beneficial roles, since lesions that are prone to plaque rupture are associated with a reduced fraction of VSMCs relative to macrophages [23]. A number of studies also suggested that subgroups of VSMCs may transdifferentiate into macrophages and thereby contribute to plaque development [1,18], though these data remain controversial [22]. A current limitation in the field is that many of the markers used to identify cell types within a plaque, such as the macrophage/monocyte marker CD68 or smooth muscle (SM) cell marker SM α-actin (*Acta2*, also known as αSMA), are up- or down-regulated during cellular transdifferentiation and therefore only report the current state of a given cell, not its origin [8]. Therefore there is a substantial ambiguity about the origin of smooth muscle- and macrophage-positive marker cells within plaques contributing to atherosclerosis progression. The only way to circumvent these problems is the use of lineage tracing approaches in animals genetically engineered to express specific permanent markers in the cell population of interest. Using this technique in apolipoprotein E deficient mice (*ApoE−/−*), Feil et al. recently reported that SM-derived cells in the plaque express macrophage markers [7]. However, the low labelling efficiency in this study precluded a quantitative assessment of the overall contribution of these cells to atherosclerotic lesions [22]. Another recent lineage tracing study showed that VSMCs within atherosclerotic plaques could change phenotypes to become macrophage-like cells, thereby

* Corresponding author. Max Planck Institute for Heart and Lung Research, Ludwigstrasse 43, 61231, Bad Nauheim, Germany.
E-mail address: julian.albarran@mpi-bn.mpg.de (J. Albarrán-Juárez).

http://dx.doi.org/10.1016/j.atherosclerosis.2016.06.012

introducing a new way of plasticity of these cells [21]. We here combined a lineage tracing approach in two different reporter lines with a single cell expression analysis to define the proportion and functional relevance of VSMC transdifferentiation in atherosclerotic plaque cells.

2. Materials and methods

2.1. Mice

The Animal Ethics Committees of Karlsruhe and Darmstadt approved all mouse procedures (Protocol No. B2/1009). Generation of transgenic mouse lines on the C57BL6/N background was described previously: for smooth muscle myosin heavy chain transgenic mice (SMMHC-CreER^{T2}) [26] and for monocytes/macrophages (LysM-Cre) [5]. These mice were bred on the ApoE-deficient mouse line [17] and then crossed with the double fluorescent reporter line Rosa26$^{flox\text{-}mT\text{-}stop\text{-}flox\text{-}mG}$ (Jackson Lab, Stock 007576) reported by Ref. [16]. Mice were housed under a 12 h light-dark cycle with free access to food and water and under pathogen-free conditions. Cre recombinase was activated in male mice at 6–8 weeks of age with intraperitoneal injections of tamoxifen (1 mg/mouse, Sigma T5648), one per day for 5 consecutive days. Five days after the last tamoxifen injection, mice were fed a high fat diet for 16 weeks to accelerate development of atherosclerotic lesions. Diet contained 21% butter fat and 1.5% cholesterol (Ssniff® TD88137). Animals were euthanized by CO_2 under intraperitoneal anesthesia of ketamine (120 mg/kg, Pfizer, Germany) and xylazine (16 mg/kg, Bayer; Germany), and then perfused via the left ventricle with phosphate-buffered saline (PBS).

2.2. Histological analyses

Upon sacrifice, aortic arch arteries were carefully dissected and fixed in cold 4% paraformaldehyde (PFA) for 1 h on ice. Following fixation, vessels were washed with cold PBS several times, embedded in O.C.T. tissue freezing medium (Sakura®, The Netherlands) and stored at −80 °C before sectioning. For immunofluorescence analyses, arteries were sectioned (10 μm) and fixed with ice-cold acetone for 10 min, O.C.T. tissue freezing medium was subsequently removed from the sections by washing three times (5 min each) with PBS. Slides were then blocked in 5% normal goat serum (Thermo Fisher, PCN5000), in PBS with 0.1% Triton X-100 (Sigma, Munich; Germany) at room temperature for 30 min. Blocked sections were probed overnight at 4 °C in dark with the following antibodies: rat anti-mouse CD68 (dilution 1:100; Serotec, MCA195, clone FA-11), mouse anti-αSMA (dilution 1:100; Abcam, ab125057-biotin, clone 1A4), and rabbit anti-mouse SMMHC (dilution 1:100; Abcam, ab53219). After primary antibody incubation, sections were rinsed three times for 5 min with PBS and incubated with appropriate secondary antibodies in dark for 1 h at room temperature: streptavidin (Alexa Flour 647; S32357), goat anti-rat IgG (Cy5 conjugate; A10525) or goat anti-rabbit IgG (Cy5 conjugate; A10523) from Life Technologies, (1:200 dilutions). Cell nuclei were then labeled with DAPI (Invitrogen, D3571) for 10 min in the dark (5 μg/ml dilution 1:1000). Sections were thoroughly washed with PBS, air dried and mounted with FluoroMount (Sigma, F4680).

2.3. Confocal images acquisition and analysis

Images were acquired with a SP5 Leica (Mannheim, Germany) confocal microscope. Sequential 1 μm optical sections were acquired with a 40× oil immersion objective at 405 (DAPI), 488 (EGFP), 561 (Tomato) and 633 nm (immunostaining) wavelengths, and contrast was enhanced using Leica software. A series of three to five *z*-stack images (*z*-wide) were acquired from each microscope field view from different regions of the lesion such as shoulder, fibrous cap and media. Examination of each plane of the *z*-stack was processed, reconstructed and quantified using Fiji software [19], using maximum intensity projections. Immunofluorescence staining was only quantified if single cell nuclei (DAPI signal) were associated with either EGFP or Tomato signal. Merged signals and split channels were performed to delineate the signal at single cell resolution. Quantification was based on the analysis of 3 individual *z*-stacks per section, total of 5–6 sections per area or atherosclerotic lesion, 4–5 lesions per vessel ($n = 6$ mice per genotype).

2.4. Single cell suspension from peritoneal cavity, bone marrow and blood

Resident peritoneal macrophages were isolated as previously described [9] briefly cells were collected by a peritoneal lavage with ice-cold PBS supplemented with 2 mM EDTA (Sigma). Cells were spun down for 10 min at 400x *g* at 4 °C and resuspended at 1×10^6/ml in PBS with 2 mM EDTA and 1% BSA, pH 7,4. Bone marrow-derived cells were collected by flushing both femur and tibia with ice-cold PBS with 2 mM EDTA and 1% BSA. Bone marrow was resuspended in the same buffer and by using a 70 μm cell strainer a single cell solution was obtained. 200 μl of heparinized blood were incubated in 1 ml Red blood cell lysis buffer (Sigma) for 3 min at room temperature. The lysed blood was centrifuged for 5 min at 350x *g* and the pelleted were resuspended in PBS with 2 mM EDTA and 1% BSA at a concentration of 1×10^6/ml. Cells were then stained for flow cytometry.

2.5. Flow cytometry

The following mAbs from eBioscience (except where another vendor is noted) were used for staining: PE-Cy7-conjugated mAbs to CD45R/B220 (103221, BioLegend), CD8a (25-0081) and Ly6C (128017, BioLegend); APC-conjugated mAbs to CD4 (17-0042-81) and Ly6G (127613, BioLegend); eFluor450-conjugated mAbs to CD45 (48-0451-80) and CD11b (48-0112-82) and PerCpCy5.5-conjugated mAb to F4/80 (45-4801-82). Conjugated isotype-matched control mAbs were obtained from eBioscience, BioLegend and BD. Cells were acquired on a Canto II flow cytometer (BD, Biosciences) and analyses were performed using Diva software (BD, Biosciences).

2.6. Microfluidic capture of single cell and gene expression analysis

Aortae were isolated and all connective tissue was carefully removed under the microscope. Then, arteries were cut longitudinally and opened facing upward. Aortic atherosclerotic plaques were carefully collected from SM reporter mice ($n = 8$) with Dumont forceps (11251-35, F.S.T) and directly placed on digestion mix containing: collagenase II (2 mg/ml), elastase-I (0.04 mg/ml) and DNase1 (5 U/ml) at 37 °C for 60 min while shaking (all from Worthington). Digested plaques were filtered through 40 μm mesh and washed with PBS. Cells were sorted by forward and sideward scatter and loaded onto a microfluidic-C_1 Single-Cell Auto Prep System (Fluidigm) for RNA isolation and cDNA amplification. Quantitative PCR was performed on harvested cDNA at single cell level using 96.96 Dynamic Array IFC with a

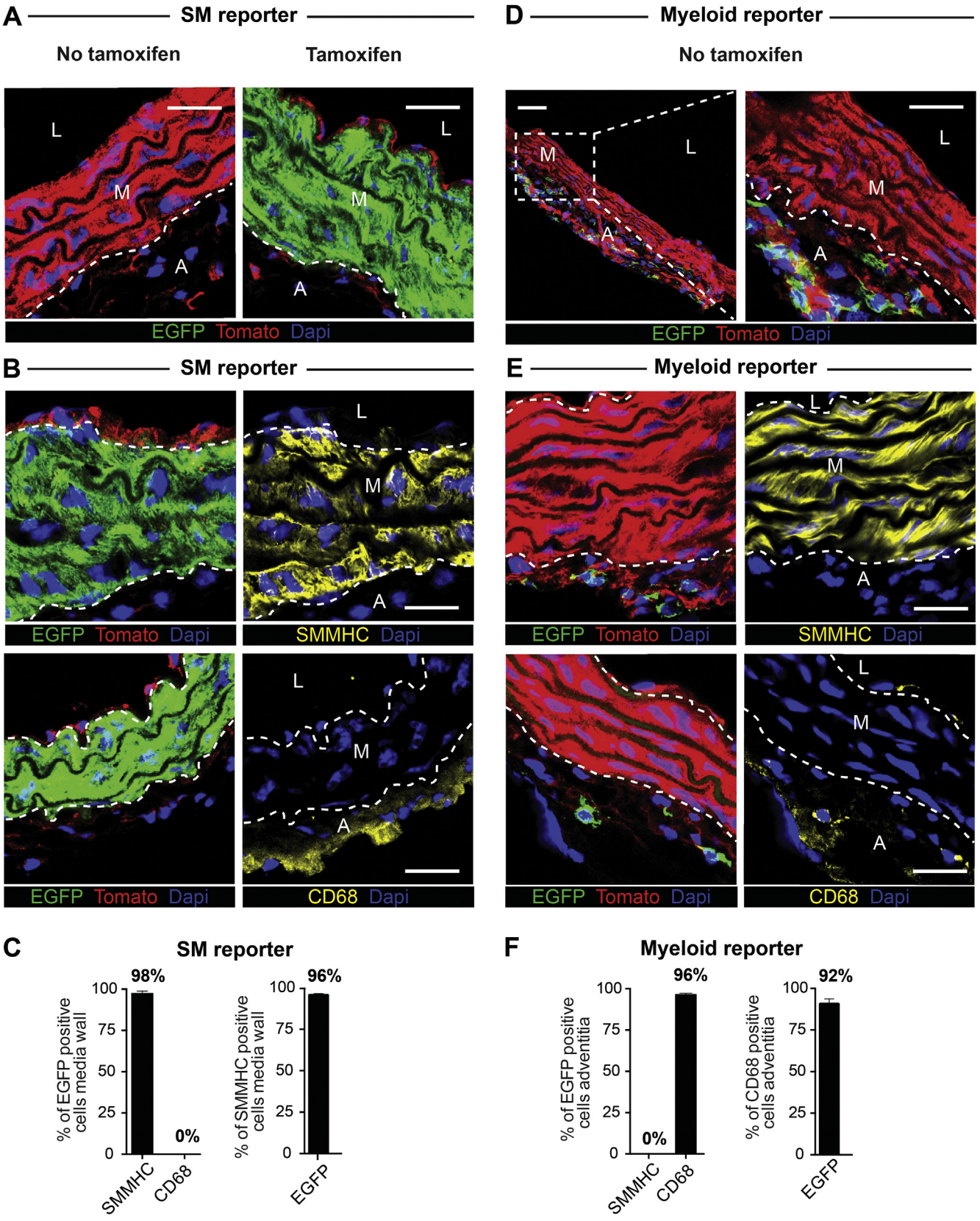

Fig. 1. Characterization of lineage tracing models. Double fluorescent (Rosa26$^{flox\text{-}mT\text{-}stop\text{-}flox\text{-}mG}$) reporter mice were crossed with atheroprone ApoE deficient mice and two cell-specific Cre transgenic lines: the tamoxifen-inducible SMMHC-CreER^{T2} line, to label vascular smooth muscle cells (SM reporter), and the constitutive LysM-Cre, to label monocytes-derived macrophages (myeloid reporter). (A) Representative aortic sections from SM reporter mice treated with vehicle (no tamoxifen) and after tamoxifen treatment. (B) Representative immunofluorescence staining for SMMHC and CD68 in aortic sections from SM reporter mice five days after tamoxifen treatment. (C) Percentages of EGFP-positive cells that expressed SMMHC or CD68 (left) and of SMMHC-positive cells that were EGFP^{+} (right) in the media of SM reporter mice. (D) Representative aortic image from myeloid reporter mice. (E) Representative staining for SMMHC and CD68 in aortic sections from myeloid reporter mice. (F) Percentages of EGFP-positive cells that expressed SMMHC or CD68 (left) and of CD68-positive cells that were EGFP^{+} (right) in the adventitia from myeloid reporter mice. Error bars show mean ± s.e.m. Scale bars 50 μm. L: lumen; M: media wall; A: Adventitia. Quantification was based on the analysis of 3 individual *z*-stacks per section, total of 5–6 sections per region of interest, 4–5 regions per vessel ($n = 6$ mice per genotype).

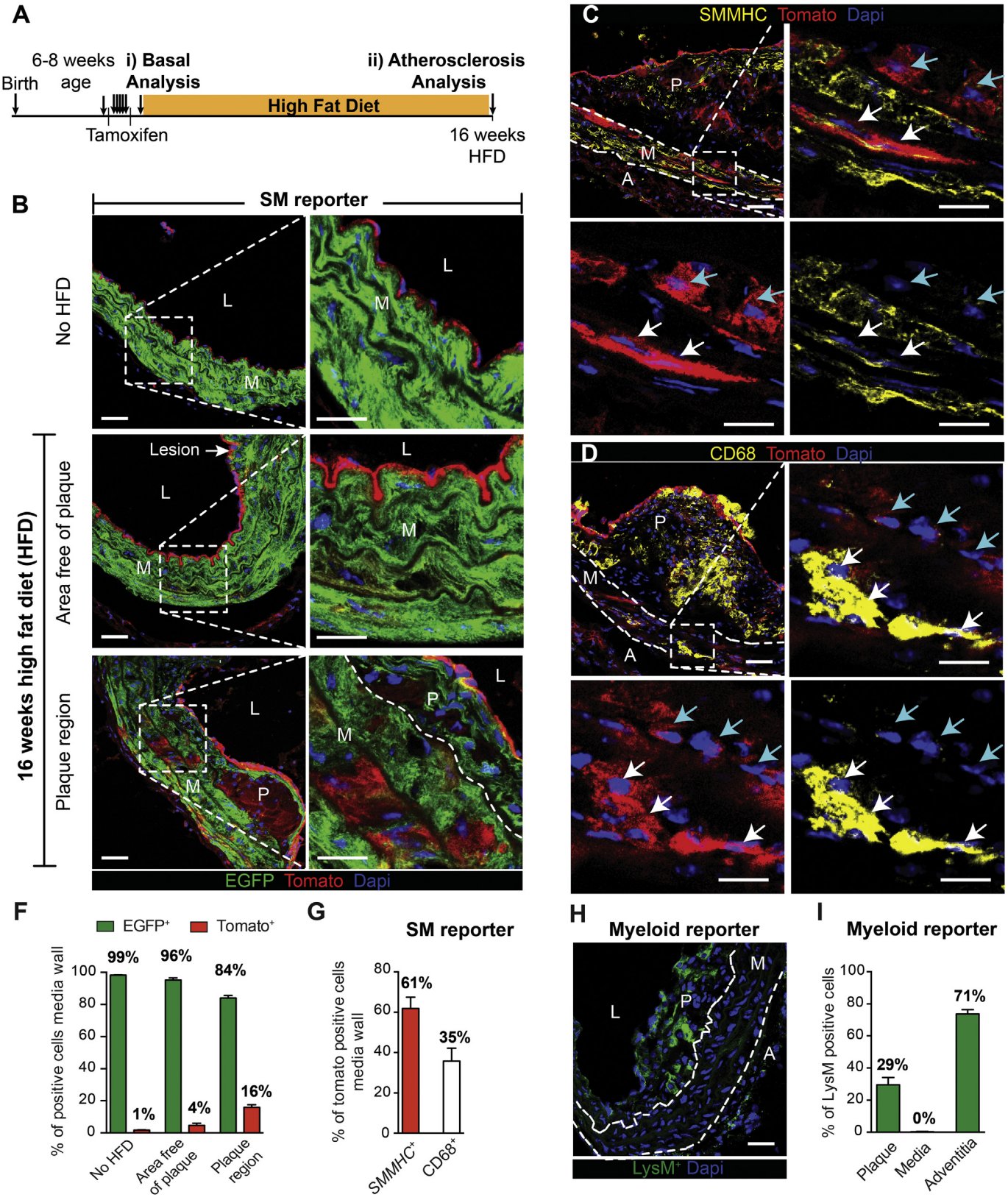

Fig. 2. Outline of the study and characterization of non SM-derived cells in the atherosclerotic media. (A) A schematic diagram showing the outline of the study. SM reporter mice received a daily injection of tamoxifen for five days at the age of 6–8 weeks. Arteries from SM and myeloid reporter lines were analyzed at different time points: i) one week after tamoxifen or vehicle treatment for basal analysis and ii) after 16 weeks of high fat diet (HFD). (B) Representative cross-sections of arteries from SM reporter mice at different time points: at basal conditions in the absence of HFD (top), after 16 weeks of HFD in areas devoid of atherosclerotic plaque (middle) and in areas with atherosclerosis (bottom). (C, and D) Representative aortic cross-sections from SM reporter mice after 16 weeks of HFD co-stained for smooth muscle myosin heavy chain (SMMHC) (C) and CD68 (D). White arrows on magnifications indicate Tomato$^+$ and SMMHC$^+$ or Tomato$^+$ and CD68$^+$ double positive cells in the media, cyan arrows indicate Tomato$^+$ cells that do not express either SMMHC or CD68. (F) Percentages of EGFP$^+$ and Tomato$^+$ cells in the aortic media wall from SM reporter mice at different time points, as described in B. (G) Percentage of non-SM derived medial cells (Tomato$^+$) that expressed

BioMark system (Fluidigm) using Sso-Fast EvaGreen Supermix low ROX (BioRad, Hercules, CA, USA) and Delta Gene primer assays. Only single cell cDNAs negative for lineage markers *Cdh5*, CD4, CD8, *or* CD19 and positive for *Htrp* ($C_t < 20$) were included into statistical analyses. Because C1 cDNA samples are potentially contaminated with genomic DNA, intron-spanning primer design was used for all genes. For EGFP no intron-spanning design was possible and a weak background amplification ($C_t > 20$) was observed even in the absence of Cre, most likely due to genomic DNA amplification. For EGFP analysis therefore only cells with $C_t < 20$ (genomic background) were evaluated. The limit of detection for the BioMark HD System has been estimated to be at a C_t value of 24 cycles (Limit of detection (LoD) C_t); all sample C_t values were therefore subtracted from the (LoD) C_t using the formula: gene expression $= 2^{(\text{Limit of Detection(LoD) Ct} - \text{measured Ct})}$, as previously described [12]. Primers used for single-cell RT-PCR are listed in Supplementary Table 1.

2.7. Statistical analysis

All results are presented as mean ± s.e.m. Statistical analysis was performed using GraphPad Prism 5 software (San Diego, USA). Statistical comparison of two groups was performed using 2-tailed Student's *t*-test with *p*-values ≤ 0.05 considered statistically significant. "*n*" refers to the number of independent experiments or mice per group.

3. Experimental results

To specifically label and lineage trace cells contributing to the development of atherosclerotic lesions, we crossed atherosclerosis-prone *ApoE*−/− mice with a double fluorescent ($Rosa26^{flox\text{-}mT\text{-}stop\text{-}flox\text{-}mG}$) reporter mouse and two different cell type specific Cre lines: the tamoxifen-inducible SMMHC-$CreER^{T2}$ line for VSMCs (SM reporter) and the constitutive LysM-Cre line for neutrophils and monocyte-derived macrophages (myeloid reporter) (Fig. 1A,D). In this reporter strain, the red fluorescent protein tomato is ubiquitously expressed at baseline and switches to enhanced green fluorescent protein ($EGFP^+$) in all cells that have undergone Cre-mediated recombination as well as their progeny.

Five days after the last tamoxifen injection aortic sections from SM reporter mice showed a homogeneous $EGFP^+$ expression in the media wall, but not in the adventitia or the endothelial layer (Fig. 1A). Immunostaining with antibodies to smooth muscle marker myosin heavy chain (SMMHC) and macrophage/monocyte marker CD68 showed that 98% of $EGFP^+$ cells were SMMHC-positive, and that 96% of $SMMHC^+$ cells expressed EGFP. Furthermore, we did not detect EGFP expression in $CD68^+$ cells (Fig. 1B,C). In addition, flow cytometry analyses of various leukocyte populations from blood or bone marrow from SM reporter mice did not show significant $EGFP^+$ expression (Supplementary Table 2). These data show that SM reporter mice specifically labeled mature differentiated VSMCs.

As lysozyme M promoter activity in myeloid reporter mice is maturation stage-dependent [6], we evaluated the degree of recombination in aortae from myeloid reporter mice at the steady stage. In aortic sections of myeloid reporter mice, $EGFP^+$ expression was restricted to the adventitia (Fig. 1D). In detail, 96% of EGFP-expressing cells were CD68-positive cells, but none were SMMHC-positive (Fig. 1E,F). Among the $CD68^+$ cells of the adventitia, 92% were EGFP positive (Fig. 1F). Flow cytometry analysis showed that recombination in other myeloid populations varied depending on the maturation stage between 30 and 98%, while expression in lymphocytes was negligible (Supplementary Table 2).

To study the origin of atherosclerotic plaque cells both mouse lines were kept for 16 weeks on a western style high fat diet (HFD) and then atherosclerotic aortae were subjected to immunohistochemical analyses (Fig. 2A). Furthermore, single cell gene expression analyses were performed with atherosclerotic plaque cells from SM reporter mice.

Analysis of vessels of the SM reporter line showed that in the absence of HFD treatment as well as in plaque-free vessel regions of HFD-treated mice, almost all cells in the media wall were EGFP-positive, i.e., mature smooth muscle cells or derivatives thereof (Fig. 2B, top and middle, 2F). Interestingly, in media regions directly underlying atherosclerotic plaques an increased proportion of EGFP-negative, non SM-derived cells, was observed (Fig. 2B, bottom, 2F). 61% of these non-SM-derived medial cells were SMMHC-positive (Fig. 2C,G), while 35% expressed CD68 (Fig. 2D,G) or neither marker. To determine whether these non SM-derived cells were of myeloid origin, we evaluated EGFP expression in the media of myeloid reporter mice (Fig. 2H). We did not detect any myeloid-derived cells in the plaque-adjacent media of myeloid reporter mice (Fig. 2H,I), indicating that these EGFP-negative cells that we observed in the media of SM reporter mice are not derived from aortal macrophages and do not express LysM. We therefore conclude that cell populations other than SM-derived cells or LysM-positive cells contribute to remodeling processes in the atherosclerotic media.

We then investigated the origin of αSMA^+ smooth muscle-like cells within the plaque. It is in this context interesting to note that in SM reporter mice more than 70% of $EGFP^+$ (SM-derived) cells within plaques had lost their αSMA expression, indicating a massive dedifferentiation (Fig. 3A). While αSMA-negative SM-derived cells were mainly localized in central plaque areas, αSMA^+ SM-derived cells were mostly found in the fibrous cap (Fig. 3A), where they have been shown to form an atheroprotective layer associated with plaque stability [10,24]. Our data show that the majority of αSMA^+ cells found in plaques were $EGFP^+$ and therefore SM-derived. Interestingly, approximately 31% of αSMA^+ cells in plaques were EGFP-negative, showing that non SM-derived cells up-regulate αSMA under atherosclerotic conditions (Fig. 3A,C). Indeed, in atherosclerotic plaques from myeloid reporter mice 17% of αSMA^+ cells co-expressed EGFP, indicating they were myeloid cell-derived (Fig. 3B,C). To investigate whether SM-derived and non-SM-derived αSMA-positive cells show functional differences, we performed single cell expression analyses of atherosclerotic plaque cells from SM reporter mice. We found that SM-derived αSMA^+ plaque cells tended not only to express higher mRNA levels for SM differentiation markers such as αSMA (*Acta2*), transgelin *(Tagln)* or smoothelin *(Smtn)* than non SM-derived αSMA^+ cells (Fig. 3D), but also to express more pro-inflammatory marker genes and growth factors such as cyclooxygenase 2 (*Ptgs2*), chemokine ligand 10 (*Cxcl10*), vascular cell adhesion molecule (*Vcam1*), or isoforms of transforming growth factor β (*Tgfb*) (Fig. 3E).

SMMHC or CD68 markers. (H) Representative confocal image showing EGFP expression in atherosclerotic lesions from myeloid reporter mice. (I) Percentages of EGFP-positive cells in aortic plaque, media and adventitia from myeloid reporter mice. Error bars show mean ± s.e.m. Scale bar: 50 μm. Boxed regions are shown at higher magnification (scale bar: 25 μm). L: lumen; M: media wall; P: plaque; A: Adventitia. Quantification was based on the analysis of 3 individual *z*-stacks per section, total of 5–6 sections per area or atherosclerotic lesion, 4–5 lesions per vessel ($n = 6$ mice per genotype).

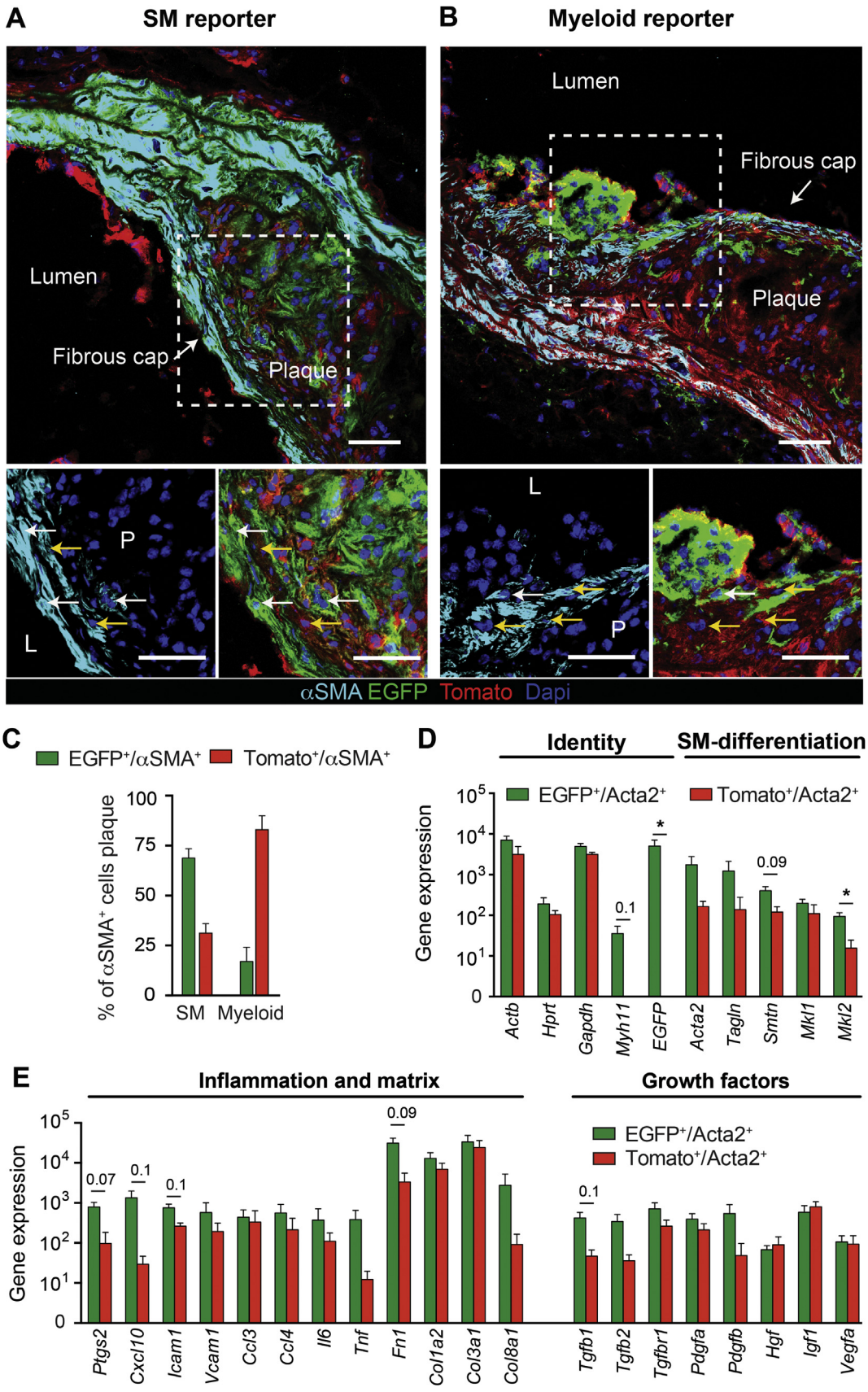

Fig. 3. Lineage tracing and expression profile of smooth muscle positive-marker cells in atherosclerotic plaques. (A and B) show representative images of αSMA-stained aortic sections from SM reporter mice (A) and myeloid reporter mice (B). Boxed regions show higher magnifications, white arrows indicate EGFP$^+$ cells that were αSMA$^+$ while yellow arrows show Tomato$^+$ cells that were αSMA$^+$. (C) Percentages of αSMA$^+$ cells that were EGFP$^+$ or Tomato$^+$ in atherosclerotic plaques from SM and myeloid reporter mice. (D and E) Gene expression profile of EGFP-positive (SM-derived) and EGFP-negative (non SM-derived) Acta2$^+$ cells in atherosclerotic plaques from SM reporter mice. Expression data are calculated as follows: gene expression $= 2^{(\text{Limit of Detection(LoD) Ct} - \text{measured Ct})}$, LoD C_t was set to 24. Error bars show mean ± s.e.m. Comparisons between groups were performed using 2-tailed *t*-test. *, $p \leq 0.05$. Scale bar: 50 μm. Boxed regions scale bar: 25 μm. L: lumen; M: media wall; P: plaque; A: Adventitia. Quantification of immunostainings was based on the analysis of 3 individual *z*-stacks per section, total of 5–6 sections per atherosclerotic lesion, 4–5 lesions per vessel ($n = 6$ mice per genotype). For single cell analysis, aortic atherosclerotic plaque was pooled together from vessels of 8 SM reporter mice.

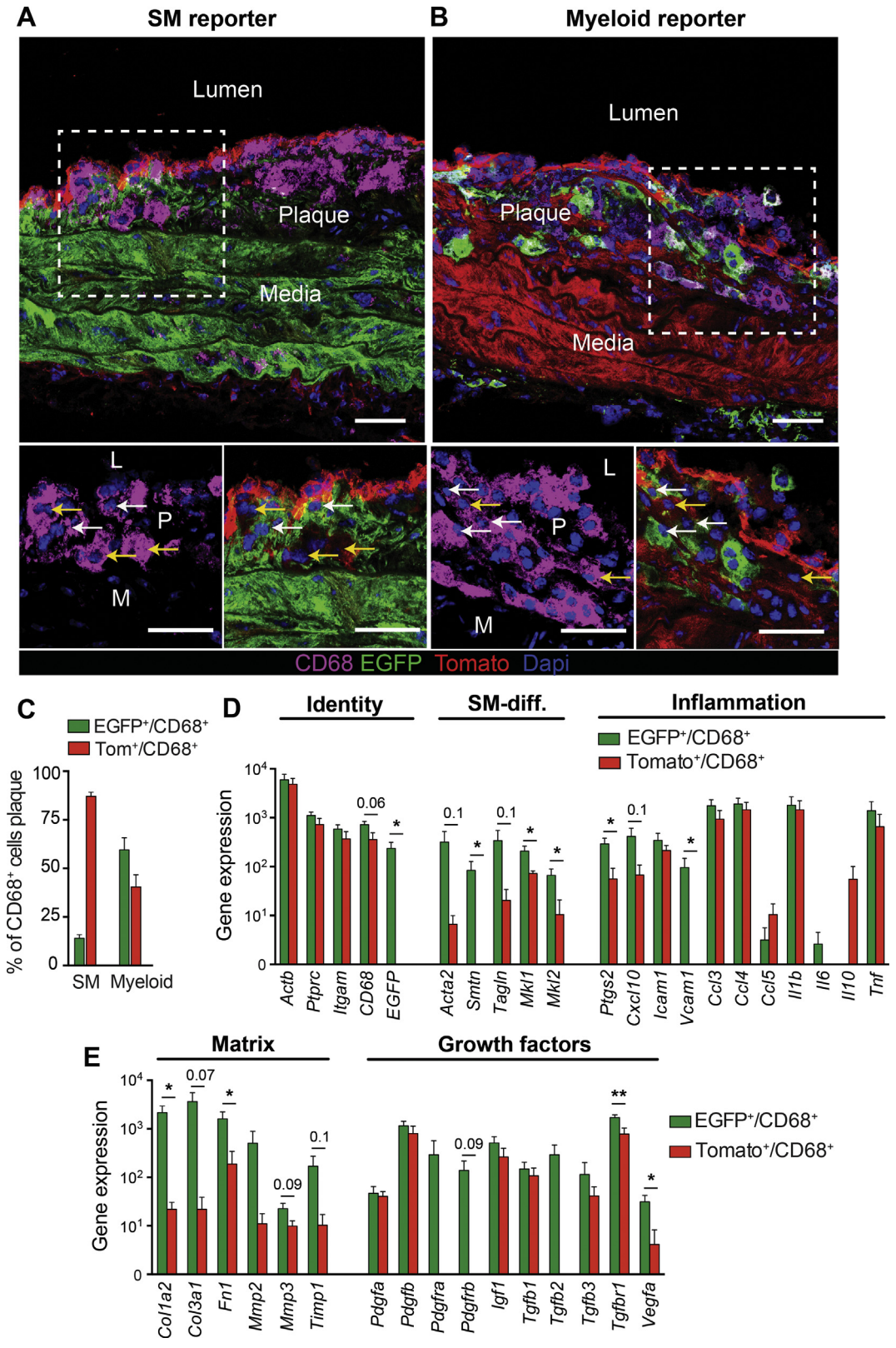

Fig. 4. Lineage tracing and expression profile of macrophage positive-marker cells in atherosclerotic plaques. (A and B) show representative immunofluorescence staining for CD68 in aortic atherosclerotic lesions from SM reporter mice (A) and from myeloid reporter mice (B). Boxed regions show higher magnifications, white arrows indicate EGFP$^+$ cells that were CD68$^+$ while yellow arrows show Tomato$^+$ cells that were CD68$^+$. (C) Percentages of total CD68$^+$ cells that were EGFP$^+$ or Tomato$^+$ in atherosclerotic plaques from SM and myeloid reporter lines. (D and E) Gene expression profiles of EGFP-positive (SM-derived) and EGFP-negative (non SM-derived) CD68$^+$ cells in plaques from SM reporter mice. Expression data are calculated as follows: gene expression $= 2^{(\text{Limit of Detection(LoD) Ct} - \text{measured Ct})}$, LoD C_t was set to 24. Error bars show mean ± s.e.m. Comparisons between groups were performed using 2-tailed *t*-test. *, $p \leq 0.05$; **, $p \leq 0.01$. Scale bars 50 μm. Boxed regions are shown at higher magnification (scale bar: 25 μm). HFD: high fat diet; L: lumen; M: media wall; P: plaque. Quantification of immunostainings was based on the analysis of 3 individual *z*-stacks per section, total of 5–6 sections per atherosclerotic lesion, 4–5 lesions per vessel ($n = 6$ mice per genotype). For single cell analysis, aortic atherosclerotic plaque was pooled together from vessels of 8 SM reporter mice.

To determine the origin of macrophage marker-positive cells in atherosclerotic plaques, we used CD68 staining. Much to our surprise we found in the SM reporter mice that 16% of CD68$^+$ cells within plaques were EGFP$^+$ and therefore originated from medial smooth muscle cells rather than from myeloid cells (Fig. 4A,C). In atherosclerotic plaques from myeloid reporter mice, 60% of CD68$^+$ cells were EGFP-positive and therefore most likely myeloid-derived macrophages or CD68$^+$ neutrophils [2] (Fig. 4B,C). 40% of CD68$^+$ cells were EGFP-negative indicating that they were non myeloid-derived. To further characterize the differences between non SM-derived CD68$^+$ cells (Tomato$^+$/CD68$^+$) and SM-derived CD68$^+$ cells (EGFP$^+$/CD68$^+$), we again performed single cell expression analysis by RT-PCR. We found that SM-derived CD68$^+$ cells (EGFP$^+$/CD68$^+$) expressed not only higher levels of smooth muscle marker genes *Acta2*, *Smtn*, and *Tagln*, but also showed increased expression of various pro-inflammatory markers (Fig. 4D). In addition, they showed increased mRNA levels of genes related to matrix synthesis and remodeling as well as growth factors (Fig. 4E). The only gene that showed a trend to higher expression in non-SM-derived cells was *IL10*, a cytokine with known anti-inflammatory properties [13]. These findings suggest that SM-derived CD68$^+$ cells more strongly upregulate genes related to matrix remodeling and inflammation in plaques than their non SM-derived counterparts.

4. Discussion

In this study we provide a quantitative analysis of VSMCs transdifferentiation in murine atherosclerotic plaques and describe the transcriptional differences between SM-derived and non SM-derived cell types. Much uncertainty in the field is due to the use of immunohistochemical markers for VSMCs that are well suited to report the current state of a given cell, but not its origin [8]. Typical markers of SM differentiation such as αSMA or transgelin, for example, are rapidly down-regulated upon smooth muscle dedifferentiation [25]; in line with this we found that 70% of SM-derived plaque cells had lost their αSMA expression. Furthermore, αSMA was up-regulated in various non SM-derived cells, for example in 17% of myeloid-derived cells. Similar observations were made in Y chromosome lineage tracing studies in humans who had undergone cross-gender bone marrow transplantation where myeloid-derived cells express αSMA in advanced coronary artery lesions [4]. Based on these data we conclude that the presence or absence of SM markers such as αSMA does not allow to draw conclusions about the origin of a given plaque cell.

One of the major unresolved questions in the field is whether plaque macrophages can give rise non-myeloid precursor cells, and if so, how these cells can be distinguish from their myeloid-derived counterparts [22]. Our lineage-tracing experiments in myeloid reporter mice showed that 40% of all CD68-positive plaque cells were EGFP-negative, indicating that the LysM promoter was never active in these cells. The activity of the LysM promoter in myeloid cells is known to be maturation stage-dependent [5,6], and our own analyses indicated that fully mature macrophages show recombination frequencies of 95–98% in the myeloid reporter line. We therefore hypothesize that these 40% EGFP-negative, CD68-positive cells in the myeloid reporter are largely, though maybe not exclusively, derived from other than myeloid cell populations. Potential candidates for this population are for example specific dendritic cells subsets, which have been shown to express CD68, but not LysM [20]. In addition, our analyses in SM reporter mice provide clear evidence for a role of VSMC transdifferentiation [1,7,21]: 16% of CD68$^+$ cells in plaques are not of myeloid origin, but are SM-derived cells. Our single cell expression analysis data also clearly show that up-regulation of CD68 is not the only feature of SM to macrophage transdifferentiation, since SM-derived CD68-positive cells expressed similar levels of leukocyte and macrophage markers such as CD45 (*Ptprc*) and CD11b (*Itgam*) as their non SM-derived counterparts. Interestingly, SM-derived CD68$^+$ cells expressed higher mRNA levels of pro-inflammatory and matrix synthesis/remodeling genes than CD68$^+$ cells of myeloid origin, suggesting they represent a more aggressive population of plaque macrophages.

Taken together, our results illustrate that VSMCs and macrophages within atherosclerotic lesions possess a high degree of plasticity with regard to the expression of markers of other cell lineages. This suggests a considerable amount of trans-differentiation occurs *in vivo*, both from mature smooth muscle cells to macrophage-like cells and from myeloid cells to smooth muscle-like cells.

Conflict of interest

The authors declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

Financial support

This project was supported by the Transregional Collaborative Research Center 23 (SFB/TR23) of the German Research Foundation Project C7.

Acknowledgments

We thank Karin Jäcklein, Ulrike Schlapp, and Claudia Ullmann for their expert technical assistance.

Appendix B. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2016.06.012.

References

[1] S. Allahverdian, A.C. Chehroudi, B.M. McManus, T. Abraham, G.A. Francis, Contribution of intimal smooth muscle cells to cholesterol accumulation and macrophage-like cells in human atherosclerosis, Circulation 129 (2014) 1551–1559.
[2] A. Amanzada, I.A. Malik, M. Blaschke, S. Khan, H. Rahman, G. Ramadori, F. Moriconi, Identification of CD68(+) neutrophil granulocytes in in vitro model of acute inflammation and inflammatory bowel disease, Int. J. Clin. Exp. Pathol. 6 (2013) 561–570.
[3] M.R. Bennett, S. Sinha, G.K. Owens, Vascular smooth muscle cells in atherosclerosis, Circ. Res. 118 (2016) 692–702.
[4] N.M. Caplice, T.J. Bunch, P.G. Stalboerger, S. Wang, D. Simper, D.V. Miller, S.J. Russell, M.R. Litzow, W.D. Edwards, Smooth muscle cells in human coronary atherosclerosis can originate from cells administered at marrow transplantation, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 4754–4759.
[5] B.E. Clausen, C. Burkhardt, W. Reith, R. Renkawitz, I. Forster, Conditional gene targeting in macrophages and granulocytes using LysMcre mice, Transgenic Res. 8 (1999) 265–277.
[6] M. Cross, I. Mangelsdorf, A. Wedel, R. Renkawitz, Mouse lysozyme M gene: isolation, characterization, and expression studies, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 6232–6236.
[7] S. Feil, B. Fehrenbacher, R. Lukowski, F. Essmann, K. Schulze-Osthoff, M. Schaller, R. Feil, Transdifferentiation of vascular smooth muscle cells to macrophage-like cells during atherogenesis, Circ. Res. 115 (2014) 662–667.
[8] D. Gomez, G.K. Owens, Smooth muscle cell phenotypic switching in atherosclerosis, Cardiovasc. Res. 95 (2012) 156–164.
[9] M. Grimm, D. Tischner, K. Troidl, J. Albarran Juarez, K.K. Sivaraj, N. Ferreiros Bouzas, G. Geisslinger, C.J. Binder, N. Wettschureck, S1P2/G12/13 signaling negatively regulates macrophage activation and indirectly shapes the atheroprotective B1-cell population, Arterioscler. Thromb. Vasc. Biol. 36 (2016) 37–48.
[10] R.T. Lee, P. Libby, The unstable atheroma, Arterioscler. Thromb. Vasc. Biol. 17 (1997) 1859–1867.
[11] K. Ley, Y.I. Miller, C.C. Hedrick, Monocyte and macrophage dynamics during atherogenesis, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 1506–1516.
[12] K.J. Livak, Q.F. Wills, A.J. Tipping, K. Datta, R. Mittal, A.J. Goldson, D.W. Sexton,

C.C. Holmes, Methods for qPCR gene expression profiling applied to 1440 lymphoblastoid single cells, Methods 59 (2013) 71–79.
[13] Z. Mallat, S. Besnard, M. Duriez, V. Deleuze, F. Emmanuel, M.F. Bureau, F. Soubrier, B. Esposito, H. Duez, C. Fievet, B. Staels, N. Duverger, D. Scherman, A. Tedgui, Protective role of interleukin-10 in atherosclerosis, Circ. Res. 85 (1999) e17–24.
[14] K.J. Moore, I. Tabas, Macrophages in the pathogenesis of atherosclerosis, Cell 145 (2011) 341–355.
[15] K.J. Moore, F.J. Sheedy, E.A. Fisher, Macrophages in atherosclerosis: a dynamic balance, Nat. Rev. Immunol. 13 (2013) 709–721.
[16] M.D. Muzumdar, B. Tasic, K. Miyamichi, L. Li, L. Luo, A global double-fluorescent Cre reporter mouse, Genesis 45 (2007) 593–605.
[17] J.A. Piedrahita, S.H. Zhang, J.R. Hagaman, P.M. Oliver, N. Maeda, Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 4471–4475.
[18] J.X. Rong, M. Shapiro, E. Trogan, E.A. Fisher, Transdifferentiation of mouse aortic smooth muscle cells to a macrophage-like state after cholesterol loading, Proc. Natl. Acad. Sci. U. S. A. 100 (2003) 13531–13536.
[19] J. Schindelin, I. Arganda-Carreras, E. Frise, V. Kaynig, M. Longair, T. Pietzsch, S. Preibisch, C. Rueden, S. Saalfeld, B. Schmid, J.Y. Tinevez, D.J. White, V. Hartenstein, K. Eliceiri, P. Tomancak, A. Cardona, Fiji: an open-source platform for biological-image analysis, Nat. Methods 9 (2012) 676–682.
[20] H.A. Schreiber, J. Loschko, R.A. Karssemeijer, A. Escolano, M.M. Meredith, D. Mucida, P. Guermonprez, M.C. Nussenzweig, Intestinal monocytes and macrophages are required for T cell polarization in response to Citrobacter rodentium, J. Exp. Med. 210 (2013) 2025–2039.
[21] L.S. Shankman, D. Gomez, O.A. Cherepanova, M. Salmon, G.F. Alencar, R.M. Haskins, P. Swiatlowska, A.A. Newman, E.S. Greene, A.C. Straub, B. Isakson, G.J. Randolph, G.K. Owens, KLF4-dependent phenotypic modulation of smooth muscle cells has a key role in atherosclerotic plaque pathogenesis, Nat. Med. 21 (2015) 628–637.
[22] F.K. Swirski, M. Nahrendorf, Do vascular smooth muscle cells differentiate to macrophages in atherosclerotic lesions? Circ. Res. 115 (2014) 605–606.
[23] I. Tabas, G. Garcia-Cardena, G.K. Owens, Recent insights into the cellular biology of atherosclerosis, J. Cell Biol. 209 (2015) 13–22.
[24] R. Virmani, F.D. Kolodgie, A.P. Burke, A. Farb, S.M. Schwartz, Lessons from sudden coronary death: a comprehensive morphological classification scheme for atherosclerotic lesions, Arterioscler. Thromb. Vasc. Biol. 20 (2000) 1262–1275.
[25] B.R. Wamhoff, M.H. Hoofnagle, A. Burns, S. Sinha, O.G. McDonald, G.K. Owens, A G/C element mediates repression of the SM22alpha promoter within phenotypically modulated smooth muscle cells in experimental atherosclerosis, Circ. Res. 95 (2004) 981–988.
[26] A. Wirth, Z. Benyo, M. Lukasova, B. Leutgeb, N. Wettschureck, S. Gorbey, P. Orsy, B. Horvath, C. Maser-Gluth, E. Greiner, B. Lemmer, G. Schutz, J.S. Gutkind, S. Offermanns, G12-G13-LARG-mediated signaling in vascular smooth muscle is required for salt-induced hypertension, Nat. Med. 14 (2008) 64–68.