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Lineage tracing of cells involved in atherosclerosis

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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

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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