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ORIGINAL RESEARCH

Adventitial Fibroblasts Release Interleukin 6 After Vascular Injury and Induce Smooth Muscle Cell Proliferation and Neointima Formation

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BACKGROUND: Vascular restenosis resulting from neointima formation significantly limits the efficacy of percutaneous interventional therapies compared with bypass surgery. The adventitial layer is involved in neointima formation, but the detailed pathophysiological interplay of the different cell types in this process is still unclear.

METHODS: We analyzed the correlation between adventitial and neointimal tissue size in human postmortem restenotic lesions after angioplasty. In porcine and mouse models of vascular injury, we examined early proliferation of fibroblasts and adventitial expansion. Using anti-CD45 antibodies, we identified recruited leukocytes as the source of fibroblast activation following vascular injury in mice. A time-course experiment on neointima formation demonstrated that adventitial activation precedes the proliferation of medial and neointimal smooth muscle cells (SMCs). To further investigate this process, we developed a mouse model enabling the surgical removal and transplantation of adventitial tissue.

RESULTS: We observed that activated adventitial fibroblasts release interleukin 6 and other cytokines, which strongly induce SMC proliferation and migration in vitro. In interleukin 6 knockout mice, supernatants from activated adventitia grafts failed to stimulate SMC proliferation and migration. Furthermore, transplantation of adventitial grafts from interleukin 6 knockout mice did not induce neointima formation. Cell fate tracking experiments using double transgenic reporter mice demonstrated that resident adventitial cells do not directly contribute to the neointimal cellular mass. Instead, medial SMCs were identified as the primary source of neointimal cells.

CONCLUSIONS: We show that the release of interleukin 6 by adventitial fibroblasts induces the subsequent proliferation and migration of medial SMC in the process of neointima formation. Thus, we propose a new paradigm for adventitial fibroblasts in this process as a paracrine inflammatory engine. Anti-inflammatory targeting of the vascular adventitia might thus be promising to limit neointima formation.

Key Words: adventitial fibroblasts ■ interleukin 6 ■ neointima formation ■ paracrine signaling ■ smooth muscle cells ■ vascular restenosis

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RESEARCH PERSPECTIVE

What Is New?

- This study introduces a novel pathophysiological concept of "outside-in" signaling in vascular remodeling, where leukocyte-induced activation of adventitial fibroblasts precedes and drives medial smooth muscle cell activation.
- Adventitial fibroblasts act as a paracrine inflammatory engine in neointima formation by releasing interleukin 6, which stimulates smooth muscle cell proliferation and migration.

What Question Should Be Addressed Next?

- Future research could evaluate the potential of stent-coating materials to deliver, for example, interleukin 6–targeting or anti-inflammatory agents to both the media and adventitia in large animal models.

Nonstandard Abbreviations and Acronyms

CANTOS	Canakinumab Anti-inflammatory Thrombosis Outcome Study
CCL	C-C motif chemokine ligand
COLCOT	Colchicine Cardiovascular Outcomes Trial
CoroCURE	Coronary Care Unit Registry
eGFP	enhanced green fluorescent protein
LoDoCo2	Colchicine in Patients With Chronic Coronary Disease trial
MCP-1	monocyte chemoattractant protein 1
Sca-1	stem cell antigen 1
SMC	smooth muscle cell
STAT3	signal transducer and activator of transcription 3

The development of in-stent-restenosis after percutaneous coronary intervention remains a major clinical burden. In several recent clinical trials involving patients with coronary heart disease, target lesion revascularization procedures due to neointima formation limit the efficacy of percutaneous coronary intervention compared with bypass surgery.[1]

The pathophysiological hallmarks of neointima formation are injury of endothelial cells, platelet and leukocyte recruitment to the arterial lesion site, as well as proliferation and migration of medial smooth muscle cells (SMCs). Following this proliferative phase, proteoglycan deposition and remodeling of the extracellular matrix, particularly by matrix metalloproteinases, induce reshaping of the vascular walls. Neointima formation is described as a general wound healing process following vascular injury. Enhanced or prolonged inflammation or increased SMC proliferation can result in excessive neointima formation with severe luminal stenosis. Our findings, consistent with those of others, demonstrate that bone marrow–derived cells do not directly contribute to the neointimal cellular mass and that neointimal cells have a local origin in the vessel wall.[2]

Vascular adventitial cells have been hypothesized to promote vascular remodeling processes.[3,4] In this context, fibroblasts from the adventitia have been shown to be activated and differentiated into myofibroblasts, which can be detected within the neointimal lesion.[5] Moreover, the detection of stem cell antigen 1 (Sca-1)$^+$ progenitor cells within the adventitia, capable of differentiating into smooth muscle–like cells in vitro, suggest that neointimal SMCs may originate directly from these progenitor cells.[6] Our group demonstrated that platelet-derived growth factor BB and sonic hedgehog are involved in activation of adventitial fibroblasts,[7] but evidence is lacking on the detailed mechanisms of adventitial contribution to vascular remodeling, as well as translational evidence to underpin this assumption. The interplay between inflammatory cells and the activation of adventitial fibroblasts, leading to SMC proliferation, is largely unknown.

In the current study, we show in a time course experiment on neointima formation a strong cellular proliferation in adventitial fibroblasts 7 days after injury, whereas proliferation of SMC typically starts later and is apparent 14 days after injury. Mechanistically, we identify leukocyte recruitment to induce activation of adventitial fibroblasts, which then produce interleukin 6 (IL-6) and induce the subsequent proliferation of SMCs. In contrast to previous publications, proliferating fibroblasts or adventitial progenitor cells do not significantly contribute to the neointimal cellular mass.

METHODS

The data that support the findings of this study are available from the corresponding author on reasonable request.

Human Samples

Human peri-stent tissue samples were obtained from the CoroCURE (Coronary Care Unit Registry) of the University Hospital of Aachen. The registry protocol was approved by the local ethics committee of the RWTH University of Aachen before initiation and complied with all ethical regulations (EK 062-13). Healthcare proxy representatives of all patients provided written

informed autopsy consent. For this prospective observational registry approach, patients at the coronary and intensive care unit of the RWTH University of Aachen who died of any cause following coronary intervention between January 2013 and November 2017 were included. Clinical information was acquired from hospital records. Catheterization laboratory reports were available from all patients. Processed samples provided to the authors were tagged with a code impeding the reidentification of patients according to the ethical requirements.

Porcine Coronary Artery Intervention Model

Porcine coronary arteries were provided by Boston Scientific Corporation (to T.J.H. and B.H.). Coronary interventions were performed at the MPI Research Inc. (study no. 1133-0101) accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication 85-23, revised 1996). Landrace-Duroc cross pigs were purchased from Bailey Terra Nova Farms (Schoolcraft, MI).

Porcine coronary artery intervention was performed as previously described.[8] Briefly, the animals were treated with aspirin (325 mg orally) and clopidogrel (75 mg orally) for 3 days before stent implantation and with nifedipine (30 mg orally) 1 day before stent implantation. On the day of stent implantation, general anesthesia was induced using tiletamine/zolazepam (8 mg/kg intramuscularly) and maintained by a semiclosed circuit of isoflurane (induction 5%, maintenance 2%–3%). Plain old balloon angioplasty was performed and bare-metal stents (12-mm length, 2.75- to 3.0-mm diameter; Express, Boston Scientific Corp.) were implanted in the left anterior descending, left circumflex, and right coronary arteries of male or female Landrace-Duroc cross pigs. The stents were, after initial placement, postdilated with a high-pressure noncompliant balloon as necessary to achieve a targeted ratio of stent to artery diameter of 1.1:1, as assessed by quantitative coronary angiography. The animals received aspirin (81 mg orally) and clopidogrel (75 mg orally) daily after stent implantation until the end of the study. After an implant duration of 7 days (±8 hours), the animals were euthanized by sodium pentobarbital solution administration followed by exsanguination and necropsy. The stented or ballooned arteries were perfusion-fixed with 10% neutral buffered formalin and embedded in paraffin.

Mice

All mouse experiments complied with the *Directive 2010/63/EU* of the European Parliament as well as with local ethical guidelines and had been approved by the Institutional Animal Care and Use Committee of Lower Saxony (LAVES).

Adult male C57BL/6J mice were purchased from Charles River (Sulzfeld, Germany), C57BL/6-Tg(CAG-EGFP)1Osb/J mice and IL6$^{-/-}$ mice (B6.129S2-Il6^{tm1Kopf}/J) were purchased from Jackson (Bar Harbor, ME). Myh11 creER(T2)$^{-/+}$ mTmG$^{-/+}$ double transgenic mice were generated by crossing smooth muscle myosin heavy chain (*Myh11*) creER(T2)$^{-/+}$ mice (on a C57BL/6 background) with mTmG reporter mice (B6.129(Cg)-Gt(ROSA)26Sor$^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J purchased from Jackson).[9]

Wire-Induced Injury of the Murine Femoral Artery

The dilation of the femoral artery was performed as previously described.[10,11] Briefly, C57BL/6J or Myh11 creER(T2)$^{-/+}$ mTmG$^{-/+}$ mice (23–25 g body weight) were anesthetized by a singular intraperitoneal injection of 100 mg/kg body weight ketamine hydrochloride (Anesketin, Albrecht) and 16 mg/kg body weight xylazine (Rompun 2%, Bayer Health Care AG) diluted in 0.9% sodium chloride. Adequate anesthesia was confirmed by the lack of tail-pinch–induced pain reflex. For the wire-induced injury model of the femoral artery, a straight spring wire (0.38 mm in diameter, Cook Medical Inc.) was advanced through the profunda femoris artery for 1 cm into the femoral artery and left in place for 1 minute. After withdrawal, the profunda femoris artery was ligated and reperfusion of the dilated femoral artery was confirmed.

At indicated time points after dilation, mice were euthanized by carbon dioxide inhalation and perfused with phosphate-buffered saline via the left ventricle. The femoral artery was carefully excised and either postprocessed and incubated in serum-free media for further in vitro experiments or embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B.V.) and snap-frozen.

Removal of the Femoral Adventitial Layer and Adventitial Transplantation

To remove the adventitial layer, the perivascular tissue was thoroughly detached following vascular injury and restoration of the blood flow. Subsequently, the femoral artery was carefully rubbed with surgical tweezers until it appeared transparent (Figure S1).

Adventitial grafts originated from the infrarenal aortas of donor mice. Donor mice were euthanized and perfused with phosphate buffered saline via the left ventricle. Following removal of the perivascular tissue, the infrarenal aorta was explanted and longitudinally opened. The adventitial tissue was separated from

the robust aortic medial layer and transplanted around the remaining medial layer of the dilated femoral artery (Figure S2).

Anti-CD45 Injection

In vivo depletion of white blood cells was conducted by single intraperitoneal injection of an anti-mouse CD45 antibody (100 μg, functional grade purified, #16-0451, eBioscience Inc.). A rat IgG2b κ isotype antibody served as control (purified, #14-4031, eBioscience Inc.). Leukocyte-depleted mice and control mice received an oral trimethoprim-sulfamethoxazole prophylaxis (240 mg/L drinking water, Cotrim K Saft, ratiopharm GmbH).

Murine Blood Samples

Blood (20 μL) was drawn daily by murine tail vein incision, diluted with 80 μL 0.9% sodium chloride, and white blood differential was determined by measurement with Sysmex XT-2000i (Sysmex Corporation).

Fate Mapping Studies

The origin of neointimal cells was investigated by using Myh11 creER(T2)$^{-/+}$ mTmG$^{-/+}$ double transgenic reporter mice treated with intraperitoneal tamoxifen injections (1 mg/d) once daily for 5 days. Fourteen days following the last injection, femoral artery dilation was performed as described above. The injured vessels were harvested 21 days following injury (Figures S3 and S4).

Morphometry

The whole murine femoral artery was cut into 6-μm serial sections, and 6 cross-sections from regular intervals throughout the artery were stained with Van Gieson staining. For morphometric analyses of murine and porcine samples, ImageJ version 1.48 software (National Institutes of Health) was used to measure the external elastic lamina, the internal elastic lamina, and the lumen circumference, as well as the medial and neointimal area and thickness. For the morphometric evaluation of the analysis, the dense compartment of the adventitia was considered and measured as a correlate for the adventitial tissue. To define the dense adventitia, we identified the region with high collagen fiber density and low cellularity, as indicated by the intense red Van Gieson staining. The transition to the loose adventitia was determined by a gradual decrease in staining intensity and a more open extracellular matrix structure (Figure S5).

Morphometric analysis of human samples was performed using NanoViewer Digital Pathology software NDP.view2 (Hamamatsu Photonics K.K.). Remnant parts of atherosclerotic lesion were left out of further morphometric analysis.

Immunohistochemistry

Fresh-frozen femoral artery cross-sections were incubated with antibodies recognizing α-SMA (C6198, Sigma-Aldrich Co. LLC; 1:200), Ki-67 (ab15580, Abcam plc; 1:200), CD45 (ab25350, Abcam plc; 1:200), platelet-derived growth factor receptor α (ab65258, Abcam plc; 1:200), vimentin (ab8978, Abcam plc; 1:200), periostin (ab14041, Abcam plc; 1:200), or IL-6Rα (sc-374 259, Santa Cruz Biotechnology Inc.). For antigen retrieval, samples were incubated at 95 °C for 10 minutes before incubation with primary antibodies. Ensuing incubations were performed with Alexa 488– or Alexa 594–coupled secondary antibodies (LifeTechnologies) and counterstained with nuclear 4.6-diamidino-2-phenylindole (Immunoselect Antifading Mounting Medium DAPI, Dianova GmbH). Monoclonal antibodies to α-smooth muscle actin were labeled directly with Cy3. Monoclonal antibodies to CD45 were labeled directly with Cy5. Monoclonal antibodies to IL-6Rα were labeled directly with FITC.

Porcine coronary arteries were deparaffinized and incubated with antibodies recognizing Ki-67 (ab15580, Abcam plc; 1:50). Ensuing incubations were peformed with a biotin-labeled secondary antibody (Thermo Fisher Scientfic), peroxidase-labeled streptavidin (Vector Laboratories Inc.), and 3,3′-diaminobenzidine-tetrahydrochloride (Thermo Fisher Scientific). Subsequent staining was performed with hematoxylin–eosin.

Negative controls for all stainings were conducted by substituting the primary antibody through an appropriate species- and isotype-matched control antibody (Santa Cruz Biotechnology Inc.).

Microscopy

Tissue samples were analyzed using bright-field and immunofluorescence microscopy (Eclipse TE2000-S, Nikon Instruments Europe B.V.) equipped with appropriate filter blocks and image processing software (NIS Elements AR 4.20.01, Nikon Instruments Europe B.V.).

Primary Cell Culture

Human coronary artery SMCs were purchased from Lonza. Cells between passages 3 and 6 were used for all experiments and cultured in optimized growth media according to the supplier's protocols.

Functional In Vitro Assay

Recombinant carrier-free C-C motif chemokine ligand (CCL) 5, CCL11, and soluble tumor necrosis factor receptor I were purchased from BioLegend, recombinant CCL9 was purchased from antibodies-online.com, and recombinant IL-6 was purchased from PreProTech GmbH.

Tissue samples (native aortic adventitia, adventitial graft, and remaining media/neointima, as well as wild-type adventitial graft and IL6$^{-/-}$ adventitial graft) were harvested 14 days following femoral artery dilation and adventitia transplantation and incubated in 1.4 mL of serum-free media, respectively, for 24 hours at 37 °C.

Human coronary artery SMCs were serum-starved for 24 hours, followed by a 24-hour incubation period with the respective conditioned medium or cytokine. Quantification of cell proliferation was assessed using a BrdU-based Cell Proliferation ELISA according to the manufacturer's protocol (Cat. 11647229001, Roche Applied Science). Cell migration was determined by a scratch wound assay during an incubation period of 24 hours documented by using CytoSMART live cell imaging systems (Lonza). Cell tracks, migration directionality (defined as the quotient of migration distance and migration length), and migration velocity were determined using the MTrack2 plugin for ImageJ version 1.49.

Cytokine content in the conditioned media from the same experiments was detected using a murine cytokine antibody array according to the manufacturer's instructions (ab133995, Abcam plc) followed by densometric analysis (ImageJ version 1.49).

Statistical Analysis

Data were stored and analyzed on personal computers using Microsoft Excel 2010 (Microsoft Corporation) and GraphPad Prism 6.01 (GraphPad Software Inc.). As part of the statistical workflow, data were assessed for normality (D'Agostino-Pearson omnibus test or Shapiro–Wilk test, depending on sample size) and homogeneity of variances (*F* test for 2-group comparisons and Brown-Forsythe test for multiple-group comparisons). As all data sets met the assumptions for parametric testing, statistical comparisons were performed using a 2-sided unpaired Student *t* test, repeated measures 1-way ANOVA, or 2-way ANOVA (see figure legends for the respective tests applied). The linear relationship between study groups was measured by the Pearson correlation coefficient (see figure legends for respective tests applied). All data are represented as mean ± SEM. A P value <0.05 was considered statistically significant for all comparisons. All mice were randomly assigned to different experimental groups. The investigators were not blinded to mice allocation during experiments and analysis.

RESULTS

Correlation Between the Size of the Adventitia and the Neointimal Lesion

To gain clinically relevant insight into the importance of the adventitia in the process of neointima formation, we initially performed morphometric analysis of 25 human postmortal peri-stent samples from the CoroCURE registry and measured adventitial and neointimal thickness and area size (see workflow Figure 1A). The median patient age at coronary stent implantation was 70.8 years (range, 39.6–86.0 years), the median duration from stent placement until patient death was 3.3 years (range, 1 day–13.9 years). Both luminal restenosis and adventitial size increased over time after angioplasty (Figure 1B). Both total adventitial area (Figure 1D) and adventitial thickness (Figure 1E) significantly correlated with neointimal size at the corresponding sites (Figure 1C through 1E). Consistently, we observed the same significant correlations in a mouse model of neointima formation 21 days following wire-induced injury of the femoral artery (Figure 1F through 1H). At sites of an activated and increased adventitial lesion, we also found enhanced neointimal lesions. In contrast, areas without activation and growth of the adventitia did not show a substantial neointima formation.

Proliferation of Adventitial Fibroblasts Precede SMC Proliferation in a Time Course Experiment on Neointima Formation

In a mouse model of wire-induced neointima formation, we analyzed cellular proliferation as well as the size of the different vascular layers 7, 14, and 21 days after injury. The adventitial cellular compartment dynamically increased in size before the beginning of neointimal thickening (Figure 2A and 2B). The numbers of proliferating cells within the adventitia by far exceeded the numbers of proliferating intimal cells throughout the whole time course following injury. Importantly, adventitial cell proliferation peaked as early as 7 days, whereas the peak of medial and intimal cell proliferation followed at 14 days after injury (Figure 2A and 2C). Upon further immunohistochemical characterization, we demonstrated that adventitial proliferating cells are neither Sca-1$^+$ local niche progenitor cells nor CD45$^+$ inflammatory cells. Instead, the cells were characterized by the fibroblast markers platelet-derived growth factor receptor α, periostin, and vimentin (day 7 after injury, Figure S6). In order to analyze the effects of leukocytes on the activation of the adventitia, we depleted leukocytes in mice by injection of anti-CD45 antibodies. A single-dose injection resulted in a significant depletion of CD45$^+$ leukocytes in a white blood cell count over time. Whereas neutrophils and lymphocytes were significantly depleted for 7 days, the monocyte count showed a significant reduction at 5 days but not at 7 days after injection. When these

leukocyte-depleted mice were subjected to wire-induced arterial injury, we prevented leukocyte accumulation within the adventitia as well as adventitial cell proliferation, indicating that invading leukocytes trigger early proliferation and expansion of adventitial fibroblasts (Figure S7).

Importantly, for potential translation of the results to the human setting, we confirmed the short-term proliferative

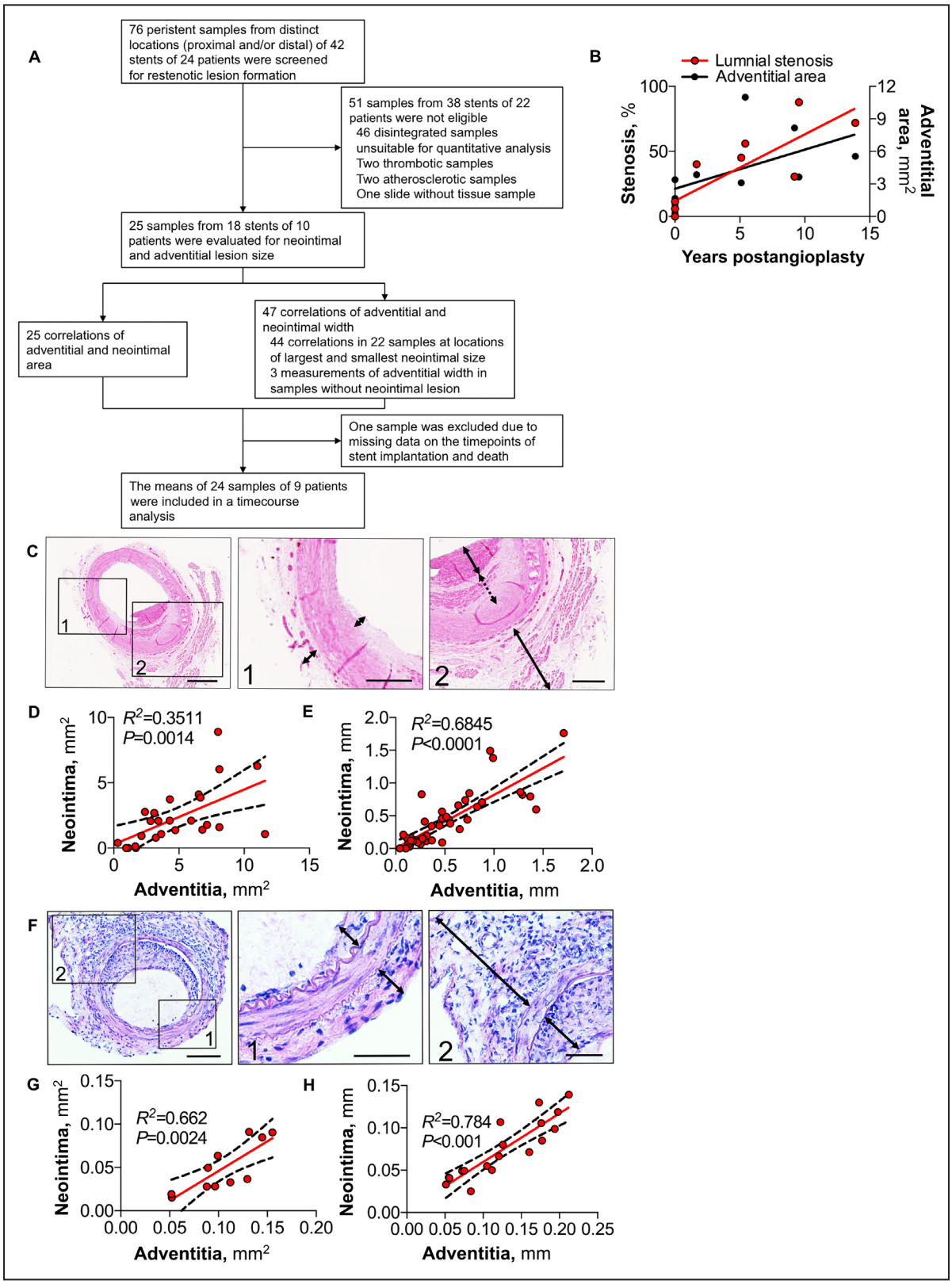

Figure 1. Restenotic adventitial and neointimal growth correlate with each other.
A, Patient selection workflow resulting in 24 analyzed peri-stent samples. **B**, Both the luminal stenosis and adventitial areas increase over time postangioplasty. **C**, Representative pictures of adventitial and neointimal thickness assessment in human peri-stent samples. Inlay 1 presents a representative location of the smallest neointimal size, as compared with inlay 2, showing location of the largest intimal size. Arrows indicate adventitia and neointima in both inlays. Dotted arrow indicates the underlying atherosclerotic lesion not included in the analysis. Scalebars: 1 mm, inlays: 500 μm. **D**, Adventitial size and (**E**) adventitial thickness correlate with neointima area at corresponding sites (determined by Pearson correlation analysis). Similarly, (**F** through **H**) show representative pictures of adventitial and neointimal thickness assessment in murine wire–induced injury model samples (21 days after vascular injury, scalebars: 200 μm, inlays: 100 μm [1] and 50 μm) and a positive correlation between (**G**) adventitial size and (**H**) adventitial thickness, with neointima area at corresponding sites, according to Pearson correlation analysis.

response following angioplasty in 2 established porcine percutaneous coronary intervention models. Here, we observed a higher number of proliferating cells in the adventitia compared with the media at 7 days following balloon angioplasty or implantation of uncoated bare-metal stents in pig coronary arteries (Figure S8).

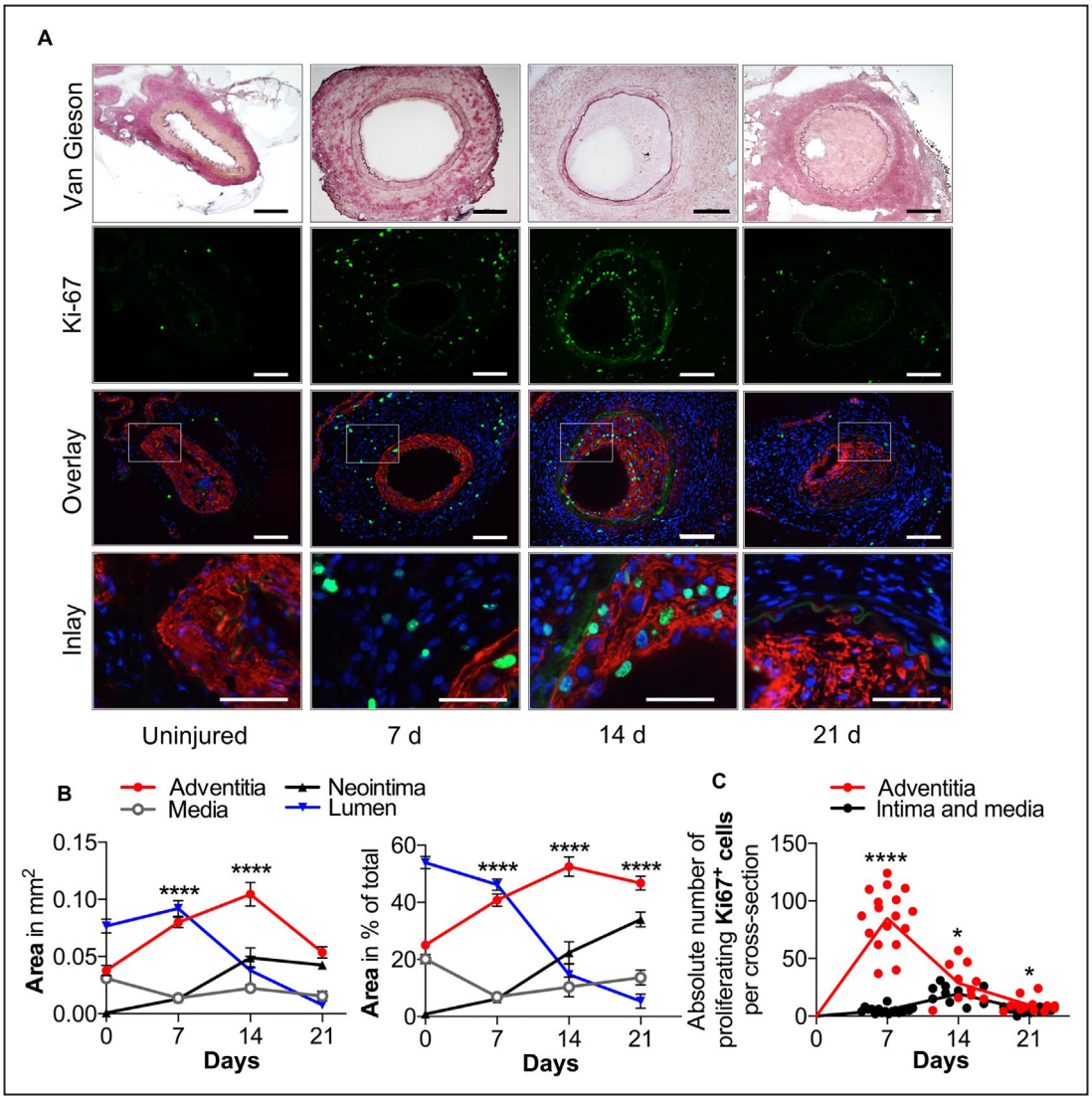

Figure 2. Adventitial proliferation and growth precedes medial proliferation and growth upon vascular injury.
A, Representative pictures of Van Gieson and Ki-67 staining of uninjured as well as injured femoral arteries, 7, 14, and 21 days after injury. Overlays and inlays are presented. Scalebars: 200 μm, inlays: 100 μm. **B**, Adventitial thickening was assessed by planimetric analysis after Van Gieson staining of samples from the same experiment. ****P<0.0001 compared with 0 days, presented as mean±SEM, repeated-measures 2-way ANOVA. **C**, Ki-67⁺ cells are mainly located in the adventitial region 7 days after injury, n=17 (7 days), n=10 (14 days), and n=13 (21 days), *P<0.05, ****P<0.0001 compared with intima and media; repeated-measures 1-way ANOVA.

Surgical Removal of the Adventitial Layer Nearly Completely Impedes Neointima Formation

Supporting the hypothesis on the contribution of adventitial cells to neointima formation,[7,12] we established a new surgical model of removal of the adventitial layer following wire-induced arterial injury. Removal of the adventitia resulted in an almost complete absence of neointimal lesion formation in C57BL/6 mice (Figure 3A and 3B, Figures S1 and S9). Next, we performed adventitial reconstitution by (re-)transplanting/wrapping adventitial tissue from the aorta of a different donor C57BL/6 mouse around the previously stripped artery following wire-induced injury. This isograft transplantation model restored neointimal lesion formation 21 days after injury (Figure 3C and 3D, Figures S2 and S10).

In order to investigate the amount of adventitia-derived cells within the neointimal lesion, we used the

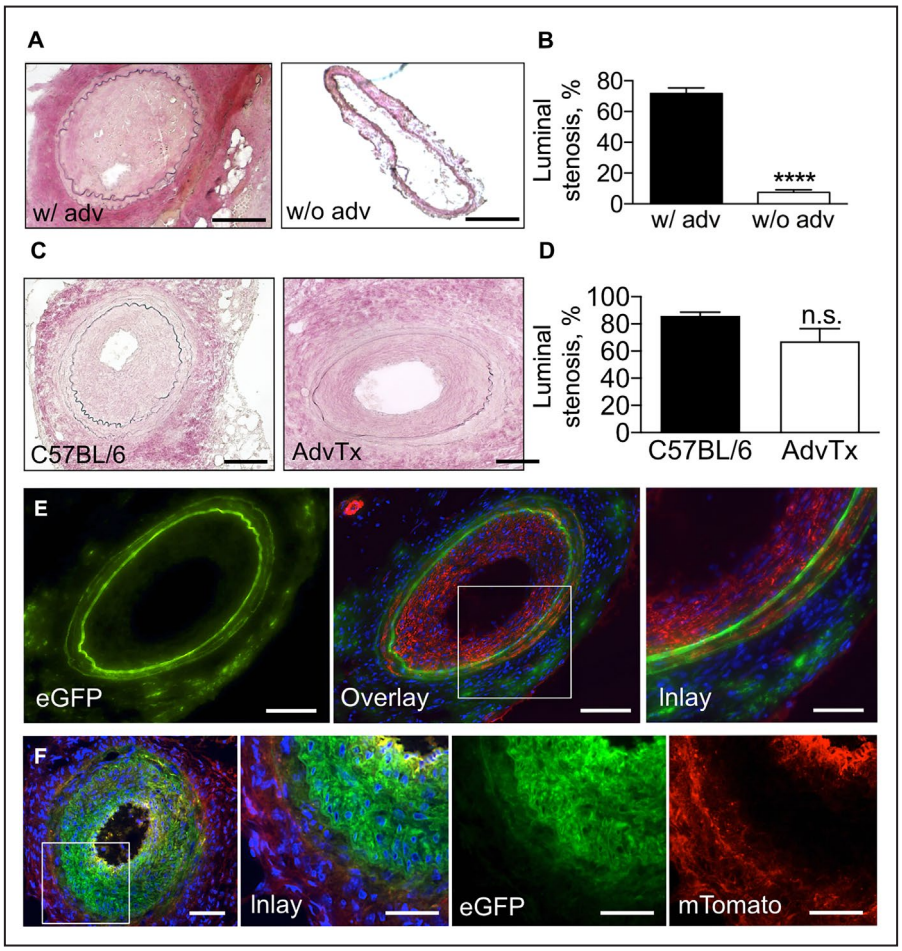

Figure 3. Adventitial activation is critical for neointima formation, but neointimal cells derive from the medial layer and not from the adventitia.
A and **B**, Morphometric analysis of neointimal lesion formation 21 days following vascular injury and removal of the adventitial layer with or without adventitial tissue removal. n=5, scalebar 200 μm, ****P<0.0001, presented as mean±SEM, 2-sided unpaired Student t test. **C** and **D**, Van Gieson staining and morphometric analysis 21 days following vascular injury in C57BL/6 mice with and without transplantation of the adventitial layer. n=6, scalebar 200 μm, presented as mean±SEM, 2-sided unpaired Student t test. **E**, 21 days following vascular injury and transplantation of ubiquitously eGFP-expressing adventitial layers from *C57BL/6-Tg(CAG-EGFP)1Osb/J* donor mice, immunofluorescence microscopy was performed. Blue: DAPI, green: eGFP, red: α-smooth muscle actin. n=6, scalebars 200 μm, inlay: 100 μm. None of the mice studied show leakage/trafficking of adventitial (green) cells to the media/neointima. **F**, 21 days following vascular injury of double transgenic reporter mice, fluorescence microscopy was performed to detect differentiated SMCs before surgery (medially derived, green) and remaining cells (red). n=8, scalebars 200 μm, inlay: 100 μm. AdvTx indicates adventitial layer; eGFP, enhanced green fluorescent protein; SMC, smooth muscle cell; and Tx, transplantation.

isograft transplantation model for cell fate tracking experiments. Therefore, we transplanted genetically labeled enhanced green fluorescent protein (eGFP)$^+$ adventitial grafts of *C57BL/6-Tg(CAG-EGFP)1Osb/J* mice into C57BL/6 wild type after wire-induced arterial injury. Costaining for eGFP and the SMC marker α-smooth muscle actin in excised lesions did not reveal any relevant occurrence, indicating that migration of adventitia-derived cells (eGFP$^+$ cells) does not substantially contribute to the neointimal cellular mass (Figure 3E).

Neointimal SMC Originate From Proliferating Medial SMC

To investigate the origin of neointimal SMCs we used inducible *Myh11creER(T2)*$^{-/+}$*mTmG*$^{-/+}$ dual-reporter transgenic mice. These mice express mTomato (red) in all cells. Treatment with tamoxifen induces SMC-specific Cre-activation under the control of Myh11 (smooth muscle myosin heavy chain), resulting in expression of eGFP (green) instead of mTomato only in differentiated SMCs. After 20 days of tamoxifen treatment and thus induction of eGFP only in differentiated SMC, the mice were subjected to wire-induced injury of the femoral artery and the neointimal lesions were analyzed 21 days after injury (Figures S3 and S4). We found that the majority of neointimal cells expressed eGFP, pointing towards their origin from medial SMCs. In contrast, non–SMC-derived cells (mTomato$^+$) such as advential fibroblast, resident progenitor cells, or bone marrow–derived cells rarely populated neointimal lesions (Figure 3F). These data provide compelling evidence that the most neointimal SMCs originate from previously differentiated SMCs of the medial layer rather than from bone marrow–derived cells or cells of the adventitial compartment.

Adventitial Grafts Secrete IL-6, Which Stimulates SMC Proliferation and Migration

In order to analyze paracrine effects of ex vivo adventitial grafts on SMC function in vitro, we again performed our established isograft transplantation model following wire-induced arterial injury. At 14 days after dilation of the artery and transplantation of the adventitia, the adventitial isografts were harvested and incubated in serum-free medium (Figure 4A). This “activated” adventitial graft conditioned medium potently stimulated human coronary artery SMC proliferation (Figure 4B) and SMC migration (Figure 4C) in vitro compared with “nonactivated” adventitial grafts of mice without wire-induced arterial injury. In contrast to migration directionality, the cell velocity of SMCs in a scratch wound assay was not different between the 2 groups (Figure S11, Videos S1 through S4). Proteomic evaluation of activated adventitial graft–conditioned medium identified 4 different cytokines and a soluble cytokine receptor, which were significantly and exclusively upregulated in the supernatants of the “activated” adventitial grafts compared with nonactivated grafts (Table S1). These factors include the cytokines IL-6, CCL5 (RANTES), CCL9, CCL11, and soluble tumor necrosis factor receptor 1. Of these factors, IL-6 had the strongest impact on SMC proliferation and migration in vitro (Figure S12), indicating that IL-6 from the adventitia plays a key role in the activation of medial SMCs.

Adventitial Activation and Neointima Formation Are Reduced in IL-6–Deficient Mice

We next analyzed the effects of adventitial activation on neointima formation in IL-6–deficient mice. Supernatants of activated adventitial grafts obtained from IL-6–deficient mice induced significantly lower SMC proliferation and migration responses compared with activated wild-type grafts in vitro (Figure 4D and 4E, Figure S13). Moreover, neointima formation was significantly reduced following transplantation of adventitial grafts from IL-6–deficient mice as compared with grafts from wild-type mice (Figure 4F through 4J). These data indicate that among the cytokines detected in the supernatant of activated adventitial grafts, especially IL-6 secreted from adventitial fibroblasts at early time points following vascular injury, strongly promote SMC proliferation and migration, revealing a paracrine mechanism mediating the adventitia-induced neointima formation.

DISCUSSION

The current study provides evidence that adventitial fibroblasts play a major role in vascular proliferative diseases by secreting IL-6 and other cytokines but not by a direct contribution to the neointimal cellular mass. Based on our results, we propose that our current understanding of the initial mechanisms triggering neointima formation after vascular injury needs to be revised: recruitment of leukocytes to the arterial injury site induce adventitial fibroblast activation and proliferation. Functioning like an inflammatory engine, these activated fibroblasts produce large amounts of cytokines and growth factors, especially IL-6 (phase I). Excess levels of IL-6 released from adventitial fibroblasts, in concert with other cytokines such as CCL5, potently boost the proliferation of a subset of SMC in the injured medial layer (phase II), subsequently resulting in migration of locally derived medial SMCs and intimal hyperplasia (phase III; Figure S14).

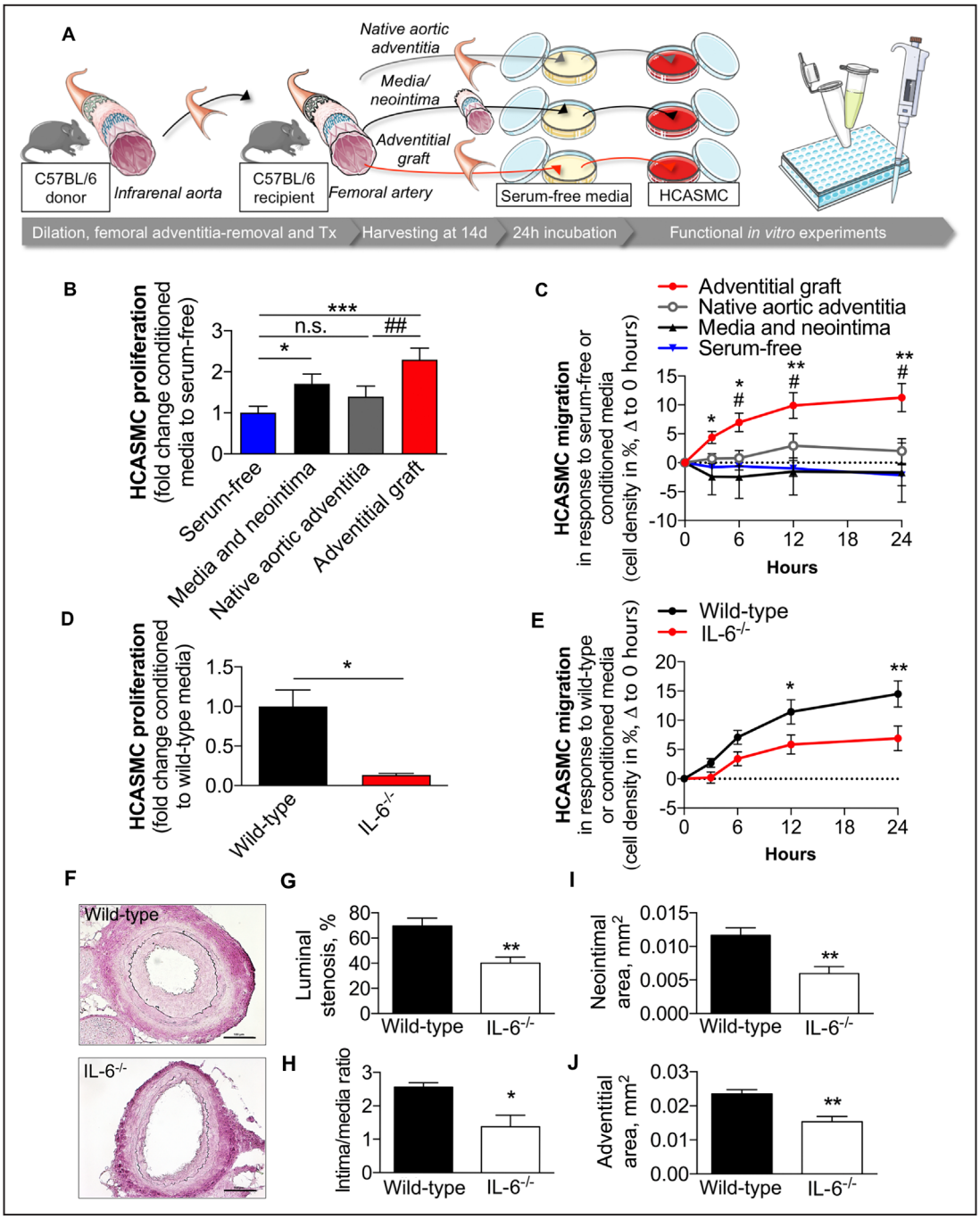

Figure 4. Adventitial IL-6 release is critical for neointima formation.
A, Vascular injury of C57BL/6 mice was followed by adventitia transplantation from donor mice (C57BL/6 or IL-6$^{-/-}$ background). Tissue samples were harvested 14 days following transplantation and incubated in serum-free media for 24 hours. Respective conditioned media were used for functional in vitro experiments on HCASMCs. **B**, HCASMC proliferation and (**C**) migration are increased by supplementation of conditioned media derived from adventitial grafts. n=5, **P*<0.05, ***P*<0.01, and ****P*<0.001 compared with serum-free control. $^{\#}P$<0.05 and $^{\#\#}P$<0.01 compared with native aortic adventitia, presented as mean±SEM, repeated-measures 1-way ANOVA (**B**) and 2-way ANOVA (**C**). **D**, Using the same 4a setup, IL-6$^{-/-}$ adventitial grafts significantly reduced HCASMC proliferation and (**E**) migration as compared with transplanted wild-type adventitial grafts in vitro. n=5, **P*<0.05, ***P*<0.01, compared with wild-type mice, presented as mean±SEM, 2-sided unpaired Student *t* test. **F,** Representative pictures of Van Gieson staining of neointima formation 21 days after wire-injured mice, transplanted with wild-type or IL-6$^{-/-}$ infrarenal aorta adventitial grafts, scalebars 200 μm. **G** through **J**, Morphometric analysis of neointimal lesion formation parameters 21 days following vascular injury and transplantation of the adventitia of C57BL/6 donor mice or IL-6$^{-/-}$ donor mice. n=5, **P*<0.05, ***P*<0.01, presented as mean±SEM, 2-sided unpaired Student *t* test. HCASMC indicates human coronary artery smooth muscle cell; IL-6, interleukin 6; and n.s., not significant.

In our immunohistochemical characterization, we show that the adventitial proliferating cells are neither CD45$^+$ inflammatory cells nor Sca-1$^+$ local niche progenitor cells, in contrast to earlier reports.[13] In fact, the proliferating cells stain positive for typical fibroblast markers, even though none of these markers can be described as fibroblast-specific. Interestingly, proliferation starts within the adventitia, indicating an "outside-in" signaling in neointima formation. Using a systemic application of an anti-CD45 antibody, we show that the inflammatory response triggers this early boost of adventitial fibroblast proliferation upon vascular injury. Large animal models of angioplasty confirm this early activation of the adventitia.[5,12,14,15] Our results from a porcine model of angioplasty and postmortem human data show that the adventitial area is correlated to luminal stenosis. Results on the direct contribution of adventitial fibroblasts to the neointimal lesion from porcine models of angioplasty are currently contradictory.[5,16] Whereas we provide data that adventitial cells do not contribute to the neointimal cellular mass in mice, the origin of neointimal SMCs in larger animals has not yet been determined because of methodological limitations.

Mechanistically, we provide evidence that IL-6 is a key factor for early adventitial activation. Other studies have demonstrated that IL-6 is an important factor for neointima formation in mice.[17] IL-6 is produced in leukocytes, fibroblasts, and SMCs, indicating that IL-6 has a substantial effect on all phases of neointima formation.[18] In our in vitro experiments, IL-6–conditioned media stimulated the proliferation and migration of SMC, which likely represent dedifferentiated SMC-like cells, as is typical for in vitro cultures. Secretion of IL-6 by inflammatory cells and fibroblasts leads to activation of surrounding fibroblasts, which, in turn, secrete more IL-6 and amplify the inflammatory response.[19] This IL-6–dependent activation of fibroblasts is relevant to cardiac fibrosis, whereas the activation of adventitial fibroblasts in the process of neointima formation by IL-6 has not been previously described.[20,21] The signal transducer and activator of transcription 3 (STAT3) signaling pathway is a major downstream target of IL-6. Our data show that STAT3 inhibition prevents SMC activation in vitro and neointima formation in vivo, indicating that the IL-6/STAT3 axis is important for the interplay of fibroblasts and SMCs in the process of neointima formation. Furthermore, one can speculate whether, analogous to observations from a mouse model of aortic dissection, a stretch-induced early fibroblastic expression of monocyte chemoattractant protein-1 (MCP-1) as well as IL-6/MCP-1 interplay could precede leukocyte recruitment into the adventitia.[22] However, our proteomic evaluation of activated adventitial graft–conditioned medium showed no statistically significant change in MCP-1 expression.

The chemokine CCL5 is produced by macrophages or fibroblasts and is also released by activated platelets. CCL5 enhances the inflammatory response in vascular proliferative diseases, and CCL5 deficiency has been shown to prevent neointima formation.[23] Our in vitro data indicate that infiltrating macrophages and activated fibroblasts produce CCL5 within the activated adventitia and thus sustain the inflammatory response to injury. Data from double knockout mice of CCL5 and its receptor CCR5 show that CCL5 also induces SMC activation in addition to its proinflammatory properties.[24] Therefore, activated fibroblasts secrete various proinflammatory and proliferation-promoting factors, of which IL-6 and CCL5 are among the most important.

Previous studies hypothesized a direct cellular contribution of adventitial (progenitor) cells to the neointimal lesion.[3,4,25,26] However, the current lack of suitable preclinical models has so far precluded investigators from providing robust evidence.[4,25] Our investigation provides evidence that adventitia-derived fibroblasts or resident progenitor cells do not contribute to the neointimal cellular mass to a relevant extent. Here, we established for the first time a murine model of selective removal/stripping of the adventitial tissue following vascular injury. This approach was specifically designed to address the limitations of existing genetic tools for selectively targeting adventitial fibroblasts. In addition, we confirmed the SMC origin of neointima cells using genetic lineage tracing. While our results strongly indicate that adventitial cells do not significantly contribute to the neointimal SMC population, we cannot entirely exclude the possibility that some adventitial cell progeny, which might not express eGFP, could be present within the neointima. It is known that only a small proportion of medial SMCs undergoes clonal expansion after vascular injury and contributes to the neointimal cellular mass.[27] Our own studies in neointima formation support this hypothesis by showing clusters of proliferating SMCs.[2]

Although atherosclerosis is a systemic disease, it is noteworthy that arteries such as the mammary and radial arteries are only rarely affected.[28] These clinical observations as well as basic research support additional outside-in signaling in the process of vascular proliferative diseases. According to this paradigm, vascular inflammation activates resident adventitial fibroblasts, which, in turn, sustain the inflammatory response and induce luminal stenosis and the formation of vasa vasorum.[29] In fact, the observation of an activated adventitia in atherosclerotic lesions was made 50 years ago, but the pathophysiology role of the adventitia in vascular proliferative diseases remains poorly understood. In the current study, we provide data on the role of the adventitia in neointima formation, but data on atherosclerosis are pending and may change our view of proliferative vascular disease in the future.

Adventitial inflammation has been implicated in negative vascular remodeling processes.[30,31] As such, inflammation has been investigated as a therapeutic target, with first results of IL-1β blockade in CANTOS (Canakinumab Anti-inflammatory Thrombosis Outcome Study), anti-inflammatory medication colchicine in COLCOT (Colchicine Cardiovascular Outcomes Trial), and the LoDoCo2 (Colchicine in Patients with Chronic Coronary Disease) trial.[32–34] These trials have shown a better outcome of anti-inflammatory medication in the clinical setting. Importantly for our study results, lowering of IL-6 was observed in patient serum treated with canakinumab or colchicine.[32,35,36] However, the source of the higher IL-6 serum levels in the above-mentioned trial has not been determined.

CONCLUSIONS

The results presented here provide a new mechanistic insight into the pathophysiology of vascular remodeling. We show early activation of adventitial fibroblasts to be a prerequisite for neointima formation. In particular, IL-6 released by adventitial fibroblasts contributes to activation of medial SMCs, which promote neointima formation. With these results, we provide evidence for the importance of adventitial fibroblasts on neointima formation and possibly on the development of other vascular proliferative diseases such as atherosclerosis and transplant vasculopathy.

ARTICLE INFORMATION

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Disclosures

None.

Supplemental Material

Table S1
Figures S1–Figure S14
Videos S1–S4

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