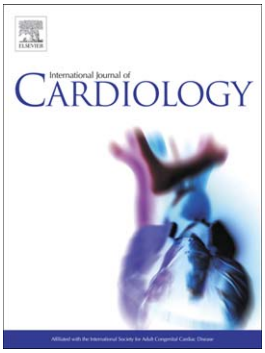

Systemic application of sirolimus prevents neointima formation not via a direct anti-proliferative effect but via its anti-inflammatory properties

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Systemic application of sirolimus prevents neointima formation not via a direct anti-proliferative effect but via its anti-inflammatory properties

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Keywords: neointima formation, sirolimus, re-endothelialization, inflammation, smooth muscle cells, progenitor cells.

Abstract

Background: Systemic treatment with sirolimus, as used for immunosuppression in transplant patients, results in markedly low rates of in-stent restenosis. Since the underlying mechanisms remain obscure, we aimed to determine the molecular and cellular effects of systemic sirolimus treatment on vascular remodeling processes.

Methods and Results: Systemic sirolimus treatment significantly reduced smooth muscle cell (SMC) proliferation 14 days after wire-induced injury and neointima formation 28 days after injury in C57BL/6 mice, while simultaneously impairing re-endothelialization. Interestingly, *in vitro,* sirolimus had no direct effect on the proliferation of SMC or endothelial cells (EC) at serum concentrations observed after systemic application. In contrast, sirolimus reduced the adhesion of leukocytes ($CD45^+$) and bone marrow-derived progenitor cells ($CD34^+$) to activated EC by down-regulating the adhesion molecules ICAM-1 and VCAM-1. In addition, sirolimus treatment also significantly reduced the upregulation of ICAM-1 and VCAM-1 and the recruitment of monocytic cells (MOMA-2^+) in neointimal lesions *in vivo*.

Conclusion: Our findings show that systemic sirolimus treatment effectively prevents SMC and EC proliferation *in vivo* without directly affecting these cells. Instead, sirolimus prevents neointima formation and re-endothelialization by attenuating the inflammatory response after injury with secondary effects on SMC and EC proliferation. Thus, despite a similar net effect, the mechanisms of systemic sirolimus treatment are largely different from the local effects achieved after application of sirolimus-eluting stents.

Keywords: neointima formation, sirolimus, re-endothelialization, inflammation, smooth muscle cells, progenitor cells.

Abbreviations

α-SMA	α-smooth muscle actin
BMPC	bone marrow-derived progenitor cells
BMTx	bone marrow transplantation
CFSE	carboxyfluorescein succinimidyl ester
DES	drug-eluting stents
EC	endothelial cells
ICAM-1	intercellular adhesion molecule 1
mTOR	mammalian target of rapamycin
PCI	percutaneous coronary intervention
PDGF-BB	platelet-derived growth factor-BB
Sca-1	stem cell antigen 1
SMC	smooth muscle cells
SM-like cells	smooth muscle-like cells
VCAM-1	vascular cell adhesion protein 1
VEGF	vascular endothelial growth factor

1. Introduction

Vascular proliferative diseases comprise atherosclerosis, restenosis following percutaneous coronary intervention (PCI), venous bypass graft failure, and transplant vasculopathy [1]. The pathophysiology is characterized by endothelial dysfunction or endothelial injury in the case of PCI and stenting, an inflammatory response to the injured vessel segments and proliferation of vascular smooth muscle cells (SMC). Therapeutic strategies addressing the excessive formation of a neointimal lesion as the cause of restenosis have predominantly been based on inhibiting the proliferation and migration of resident SMC [2]. Drug-eluting stents (DES) have successfully been introduced into clinical practice, and sirolimus or its derivates are currently the most frequently used substances incorporated in DES. Because local sirolimus inhibits endothelial recovery, it is necessary to prolong dual anti-platelet therapy after deployment of a DES, compared with the duration of dual anti-platelet therapy after the implantation of a bare-metal-stent (BMS) [3]. Even though the use of DES has strongly reduced the rates of restenosis, a relevant number of patients, especially those with diabetes mellitus or after treatment of vessels with small diameters, still develop a relevant narrowing of the treated lesions, requiring repeated revascularization [4, 5]. To selectively and efficiently optimize current treatment strategies it is therefore important to better understand the pathophysiology of neointima formation with its different aspects of the inflammatory response to injury, SMC proliferation and re-endothelialization.

The cellular and molecular mechanisms by which sirolimus exerts its clinical effect are incompletely understood. Sirolimus can directly prevent the proliferation of activated resident medial SMC after injury. More precisely, sirolimus induces cell cycle arrest in the late G1 phase by binding the cytosolic immunophilin FK506-binding protein 12 (FKBP12) and inhibiting the mammalian target of rapamycin (mTOR). The degradation of the cyclin-dependent kinase inhibitor p27kip is thereby prevented, resulting in cell cycle arrest in the G1 phase and

preventing the proliferation and migration of SMC [6]. However, a recent study has proposed that SMC express only minimal levels of FKBP12, so that very high local concentrations of sirolimus are necessary to achieve a direct anti-proliferative effect on vascular SMC and endothelial cells (EC) [7]. Since the inhibition of the inflammatory response to injury has become a recent focus in preventing neointima formation, the effect of sirolimus on other cell types is an intriguing question. In a mouse model of wire-induced injury, locally applied sirolimus, mimicking the release of sirolimus from a DES, was found to reduce bone marrow-derived progenitor cell (BMPC) accumulation and SMC proliferation within the lesion, resulting in diminished neointima formation. In this model, the local application of sirolimus also retarded the process of re-endothelialization [8].

Previous studies have suggested that a substantial number of BMPC accumulating in the neointimal lesion differentiate into “smooth muscle-like cells” (SM-like cells) and account for the neointimal cellular mass [9, 10]. Even though a significant differentiation of BMPC into genuine SMC in the process of neointima formation was challenged by our group and others, the paracrine effects of leukocytes and other BM-derived circulating cells within the neointima are certainly pivotal for the activation of local cells and subsequent lesion development [11, 12]. The markers of circulating cells exerting a high plasticity are still a matter of debate, but the most commonly used markers are among others stem cell antigen (Sca)-1 in mice and CD34 in humans [13]. It has been shown that grafted Sca-1^+ cells home into the intima of inflamed arteries and cause enhanced inflammation and neointima formation [14]. In contrast, circulating Sca-1^+ cell levels also correlate with endothelial recovery, so that these cells may play a dual role in restenosis by promoting both re-endothelialization and neointimal SMC proliferation [15]. In a previous clinical observation, angioplasty and stenting were shown to induce the expression of adhesion molecules on monocytic cells, and in-stent-restenosis was independently correlated with the upregulation of the leukocyte integrin Mac-1 [16]. Known ligands of Mac-1 are the intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1)

and blocking of ICAM-1 or VCAM-1 has been reported to prevent the accumulation of monocytic cells as well as the recruitment of BMPC [17]. All these data indicate that targeting the inflammatory response to injury represents an important approach in prevening clinical restenosis.

Sirolimus is an immunosuppressant drug that is widely used in patients following kidney or heart transplantation [18, 19]. In contrast to tacrolimus, sirolimus is not a calcineurin inhibitor but it has similar actions of suppressing the immune system. Of note, the main advantage of sirolimus compared with calcineurin inhibitors is its low nephrotoxicity [18]. In the clinical setting, systemic application of sirolimus (2 mg/day for 30 days, without a loading dose) has also shown efficacy for the prevention of in-stent restenosis with minor adverse events [20]. The mechanism underlying this inhibition, however, is not clear. Interestingly, a strong and significant reduction in in-stent-restenosis following PCI and BMS application was also seen in renal transplant patients receiving other immunosuppressive agents [21]. In the current study, we thus aimed to further evaluate the differential anti-inflammatory and anti-proliferative effects of sirolimus on vascular cells, and to dissect the mechanisms, which are responsible for the inhibition of neointima formation following systemic application of sirolimus.

Our data show that systemic sirolimus treatment effectively reduces SMC proliferation and neointima formation. However, serum levels achieved after systemic application of sirolimus were not sufficient to directly affect SMC or EC proliferation *in vitro*. Consistently, dose-finding experiments revealed that high concentrations of sirolimus were necessary to directly prevent SMC or EC proliferation, which probably can only be achieved by local drug application directly into the tissue, as with the use of DES. In contrast, EC activation and adhesion molecule expression (ICAM-1 and VCAM-1), as well as subsequent leukocyte adhesion, is prevented by serum levels achieved after systemic application of sirolimus *in vitro*. In accordance with these results, we found a significantly reduced recruitment and accumulation of circulating BM-derived

cells to injured vessel segments after systemic treatment with sirolimus. Subsequently, the proliferation of local SMC, as well as endothelial regeneration and re-endothelialization, were significantly impaired. These data provide evidence that –despite a similar net effect of local or systemic sirolimus application on neointima formation- completely different molecular and cellular mechanisms are responsible for this effect. Moreover, our data further underscore the pivotal role of the early inflammatory response for later activation and proliferation of local SMC as well as for EC regeneration and neointima formation.

2. **Methods**

2.1. Animals

All procedures involving experimental animals were approved by the institutional committee for animal research of Giessen University (GI 20/10 Nr. 40/2002) and complied with the directive 2010/63/EU of the European Parliament. All animals received humane care according to the institution's guidelines. All experiments were performed on at least 8-week-old adult male C57BL/6 mice purchased from Charles River (Sulzfeld, Germany). Enhanced green fluorescence protein (eGFP)-transgenic mice on a C57BL/6 mouse background (*C57BL/6-Tg(CAG-EGFP)1Osb/J*) were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Sample sizes for *in vivo* experiments were 5-10 for each group depending on the difference between means of interest. Calculation of sample sizes was performed by the Institute of Biometrics of the Hannover Medical School (No. 14.0082) and animals were randomly assigned to any group.

2.2. Irradiation and bone marrow transplantation (BMTx)

C57BL/6 mice were irradiated with a dose of 9.5 Gy and BMTx was performed on the same day with ~5 x 10^6 cells derived from eGFP-transgenic littermates ($eGFP^+$-BM) by tail vein injection as

previously described [11]. Enrofloxacin (Baytril®, Bayer, Leverkusen, Germany) was administered to the drinking water for 2 weeks after BMTx. At 12 weeks after transplantation, flow cytometry analysis (FACS Scan, Becton Dickinson, Franklin Lakes, NJ, USA) of blood samples was performed to monitor the success of BMTx ($eGFP^+$-cells per total mononuclear cells). In addition, we analyzed peripheral blood samples for leukocyte subpopulations and compared the proportion of leukocyte subpopulations between transplanted and non-transplanted mice by flow cytometry.

2.3. Wire-induced injury of the femoral artery

The dilation of the left femoral artery was performed as described previously [11]. In brief, male C57BL/6 mice were anaesthetized using 100 mg ketamine hydrochloride/kg body weight (Anesketin, Albrecht, Aulendorf, Germany) and 16 mg xylazine hydrochloride/kg body weight (Rompun® 2%, Bayer, Leverkusen, Germany). The femoral artery was dilated using a straight spring wire (0.38 mm in diameter; Cook, Bloomington, IN, USA) that was inserted approximately 10 mm toward the iliac artery. Post-interventional analgesic therapy was performed by intraperitoneal (i.p.) administration of 0.1 mg buprenorphine/kg body weight/day for 3 days. Blood was drawn from the right ventricle and the vessels were harvested at the indicated time points. The arteries were fixed in 4% paraformaldehyde (PFA) and embedded in Tissue Tek OCT embedding medium (Sakura Finetek Europe B. V., Zoeterwoude, The Netherlands). All arteries were snap-frozen and stored at -80 °C until sectioning.

2.4. Administration of sirolimus

Sirolimus was purchased from Sigma-Aldrich (Munich, Germany) and dissolved in 0.2% sodium carboxymethylcellulose, 0.25 % polysorbate-80 in water. Sirolimus was administered by daily i.p. injection until harvesting of the vessels at a dosage of 2mg/kg body weight/day. The dose of sirolimus was based on the literature with respect to its use as an immunosuppressant in mice

[22, 23]. This dose results in serum levels comparable to sirolimus serum levels in transplant patients [24]. The vehicle used in control groups was the solvent for sirolimus.

2.5. Morphometry

The dilated part of the femoral artery was excised, embedded in Tissue-Tek O.C.T. (VWR, Hannover, Germany), snap frozen and cut into 6-µm serial sections. 3 cross-sections each at 750 µm intervals over a length of at least 4.5 mm throughout the excised artery segment were stained with van Gieson staining. For morphometric analyses, ImageJ 1.48 software (National Institutes of Health, Bethesda, MD, USA) was used to measure the external elastic lamina, internal elastic lamina, and lumen circumference, as well as medial and neointimal area. The intima-to-media (I/M-ratio) was calculated from neointimal area divided by medial area. Luminal stenosis was calculated as percent stenosis = [1 − (AL/AN)] × 100 (AL = luminal area, and AN = area of the normal artery defined as the area surrounded by internal elastic lamina).

2.6. Immunohistochemistry

Femoral artery cross-sections were incubated with antibodies recognizing α-smooth muscle actin (α-SMA, Sigma-Aldrich), calponin (Abcam, Cambridge, UK), Ki-67 (Abcam), von Willebrand factor (vWF, Dako, Glostup, Denmark), monocyte + macrophage antibody (MOMA)-2 (Serotec, Oxford, UK), Sca-1/ Ly-6A/E (R&D Systems, Minneapolis, MN, USA), ICAM-1 and VCAM-1 (Santa Cruz Biotechnology, Dallas, TX, USA), and CD34 (BD Pharmingen, Franklin Lakes, NJ, USA). Ensuing incubations were carried out with Cy5- or Cy3-coupled secondary antibodies (Molecular Probes, Eugene, OR, USA) and counterstained with nuclear 4.6-diamidino-2-phenylindole (DAPI) (Linaris, Wertheim, Germany). Monoclonal antibodies to α-SMA were labelled directly with Cy3. For negative controls, the primary antibody was substituted by an appropriate species- and isotype-matched control antibody (Santa Cruz Biotechnology). Function-blocking mouse anti-human ICAM-1 (cloneP2A4) and VCAM-1 (clone P1B8) antibodies

were obtained from Chemicon International (Hempshire, UK). Semi-quantitative analysis of immunohistochemistry was performed using a visual scale ranging from 1 to 4, indicating very low staining for 1 and very strong staining for 4.

2.7. Microscopy

Tissue samples were analyzed using bright field or immunofluorescence microscopy (DMRB, Leica, Wetzlar, Germany) equipped with appropriate filter blocks. For deconvolution analysis of *z*-axis image stacks, a piezo stage scanner (PI, Karlsruhe, Germany) was used together with three-dimensional image processing software (Autoquant Deblur 9.3; Media Cybernetics, Rockville, MD, USA). In addition, confocal microscopy was performed on a subset of slides (Eclipse TE2000-E, Nikon, Tokyo, Japan).

2.8. Assessment of re-endothelialization

To measure the re-endothelialized area, wild-type animals were perfused *in vivo* with Evans blue dye (Sigma-Aldrich) 0, 7 and 14 days after injury, as described previously [25]. Briefly, 50 µL of 5% Evans blue diluted with saline were injected into the tail vein 10 min before the animals were sacrificed, followed by fixation via perfusion with 4% paraformaldehyde (PFA) for 5 minutes. Blood, saline, and fixative were removed through an incision in the right atrium. Pictures of *en face* prepared injured arteries were taken and re-endothelialization was assessed. The re-endothelialized area was calculated as difference between the non-stained and blue-stained area of the injured vessel segment by computer-assisted morphometric analysis (ImageJ 1.48 software) and presented as percentage of re-endothelialization.

2.9. Cell Culture, in vitro assays and western blotting

Human coronary artery SMC, human coronary artery EC as well as $CD34^+$ cells and $CD45^+$ cells derived from G-CSF-mobilized peripheral blood cells by immunomagnetic separation were all purchased from Cambrex (Verviers, Belgium). Vascular cells between passages 3 and 6 were used for all experiments and cells were cultured in optimized growth medium (Cambrex) according to the supplier's protocols. Serum from mice treated with sirolimus or vehicle control for 14 days was obtained 2 h after the last i.p. injection of sirolimus. Platelet-derived growth factor (PDGF)-BB and vascular endothelial growth factor (VEGF) were purchased from Sigma-Aldrich. Quantification of cell proliferation was assessed using a BrdU-based Cell Proliferation ELISA assay according to the manufacturer's protocol (Cat. 11 647 229 001, Roche Applied Science, Mannheim, Germany). $CD34^+$ cells and $CD45^+$ cells were used for the adhesion assay on activated human coronary artery EC. Activation of EC was performed by adding TNF-α (10 ng/mL, Sigma-Aldrich) for 8 hours in the presence or absence of sirolimus in the concentrations indicated or in the presence or absence of blocking antibodies against ICAM-1 or VCAM-1. $CD34^+$ cells and $CD45^+$ cells were labeled with carboxyfluorescein succinimidyl ester (CFSE, 5 μM), and identical cell numbers were placed onto culture dishes containing the activated EC. After 60 minutes of co-culture at 37°C, non-adherent cells were removed by washing and adherent $CD45^+$ cells or $CD34^+$ cells were evaluated by counting labeled cells in three fields of view per condition. To analyze changes in protein expression, EC were cultured for 8 hours in the absence or presence of TNF-α (10 ng/mL) and with or without sirolimus (1ng/mL). Western Blotting was performed as previously described [26]. All *in vitro* experiments were performed at least in triplicates of independent experiments (n = 3-4 in each experiment).

2.10. Statistical analysis

Data were stored and analyzed on personal computers using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) and GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). The data are presented as the mean±SD. The data were compared using analysis of

variance (ANOVA), with Fisher's corrected t-test as the post-hoc analysis. Depending on the number of comparisons, ANOVA was followed by pair-wise multi-comparison using the Tukey method (comparison of 6 or more groups). $P<0.05$ was considered statistically significant in all comparisons.

3. **Results**

3.1. Systemic sirolimus treatment prevents SMC proliferation and neointima formation

To assess SMC proliferation *in vivo*, we performed Ki-67 and α-SMA staining in cross-sections of neointimal lesions. Systemic sirolimus treatment with 2 mg / Kg / day significantly prevented the proliferation of medial and neointimal SMC compared with the control group 14 days after injury (0.15 ± 0.01 vs. 0.08 ± 0.01, n = 5, $^{**}p = 0.002$, Fig. 1A + B).

Systemic sirolimus treatment (2 mg / Kg / day) also significantly reduced neointima formation and luminal stenosis 28 days after wire-induced injury (intima/media ratio: 0.31 ± 0.18 vs. 1.01 ± 0.36, n = 5 for 28 days after injury, $^{***}p < 0.001$, Fig. 1C, D, and luminal stenosis 48.84 ± 20.43% vs. 16.44 ± 11.25%, n = 5, $^{**}p = 0.001$, Fig. 1E). Systemic sirolimus treatment using a reduced dose (1mg/Kg) resulted in a clear trend towards reducing neointima formation but just missed statistical significance (0.62 ± 0.14 vs. 0.90 ± 0.28, n = 5, $p = 0.08$, Supplemental figure 1).

3.2. Sirolimus retards re-endothelialization following vascular injury

Because the effects of sirolimus are not cell-type specific, we assessed the effect of systemic sirolimus treatment on endothelial recovery after vascular injury. Re-endothelialization of denuded femoral arteries was quantified by en face microscopy of harvested vessels following Evans blue-staining. Systemic daily sirolimus treatment significantly retarded re-endothelialization compared with that in control mice 14 days after vascular injury (54.6 ± 12.5

vs. 78.6 ± 7.8%, n = 4, *p = 0.017, Fig. 2A, B). At 28 days after injury, the vessels of sirolimus-treated and control mice showed almost complete endothelial recovery (sirolimus: 96.5 ± 2.2%, control: 97.6 ± 1.7%, n = 4, p = 0.461, Fig. 2A, B). These data indicate that by continuous administration of sirolimus, re-endothelialization was retarded but completed after a longer time period.

3.3. Sirolimus inhibits the accumulation of all bone marrow-derived cells and monocytes within the neointima

To assess the contribution of all bone marrow-derived cells to the neointimal lesion, bone marrow transplantation (BMTx) of eGFP transgenic mice was performed into wild-type mice. The percentage of hematopoietic chimerism after irradiation and BMTx was assessed by flow cytometry of peripheral blood mononuclear cells 12 weeks after transplantation as previously described (88.43% ± 4.21, n = 6) [11]. At 12 weeks after BMTx, we performed dilation of the femoral artery. Following daily systemic treatment with sirolimus, we found a significant reduction in the relative number of $eGFP^+$ cells / all cells ($DAPI^+$) within the neointimal lesion compared with that in control vessels 14 and 28 days after injury (0.309 ± 0.294 vs. 0.090 ± 0.057 on day 14 and 0.411 ± 0.192 vs. 0.067 ± 0.093 on day 28, n = 10, *p = 0.043 and ***p < 0.001; Fig. 3A, B). To further characterize the population of BM-derived ($eGFP^+$) cells following injury, tissue sections were analyzed for the expression of MOMA-2, a marker for monocytes/ macrophages, within the neointimal and the medial layer. At 14 days after injury, sirolimus treatment resulted in a trend towards a reduced relative number of MOMA-2^+eGFP^+ cells / all $eGFP^+$ cells (0.079 ± 0.146 vs. 0.025 ± 0.056, n = 8, p = 0.458). At 28 days after injury, we detected a significant reduction in the relative number of MOMA-2^+eGFP^+ cells / all $eGFP^+$ cells in the neointima following systemic sirolimus treatment compared with that in control vessels (0.201 ± 0.098 vs. 0.062 ± 0.121, n = 8, *p = 0.040, Fig. 3C, D). These results indicate that systemic sirolimus treatment in general prevents the accumulation of BM-derived circulating cells and monocytes after vascular injury.

3.4. Accumulation and differentiation of BMPC into bona fide vascular cell types is an extremely rare event.

To assess the effect of systemic sirolimus treatment on the accumulation of BMPC within the lesions following vascular injury, we performed immunohistochemistry of mice after BMTx with $eGFP^+$ BM for Sca-1, calponin and vWF. In the sirolimus treated group as well as in the control group, we found only a few eGFP/Sca-1 double-positive cells 14 days after injury (< 0.5%, Fig. 4A). Moreover, only a few BM-derived cells expressed markers for differentiated SMC (calponin, Fig. 4B) or EC (vWF, Fig. 4C). The number of double-positive cells was very low (< 0.1%) 28 days after injury in both groups; therefore, no significant differences could be detected. In contrast, a peripheral blood count after treatment with sirolimus did not show significant differences in leukocyte, erythrocyte, or platelet numbers following BMTx with $eGFP^+$ bone marrow (Supplemental table 1).

3.5. Sirolimus prevents proliferation of SMC and EC in vitro only at high concentrations

Since neointima formation is dependent on the proliferation of SMC and is reciprocally correlated with EC proliferation, we assessed the direct effects of sirolimus on the proliferation of these vascular cell types *in vitro*. Importantly, sirolimus only inhibited the proliferation of SMC in response to growth medium at concentrations as high as 20 ng/mL; there were no significant effects at lower concentrations (n = 4, $^{**}p = 0.009$, Fig. 5A). Likewise, sirolimus only prevented proliferation of EC in a dose-dependent manner at high concentrations (n = 4, $^{*}p = 0.022$, $^{***}p < 0.001$, Fig. 5B). These data suggest that the anti-proliferative effect of systemic sirolimus treatment on SMC and EC may rather be an indirect effect, since systemic application of sirolimus does not result in serum concentrations as high as needed to directly prevent SMC and EC proliferation.

3.6. Sirolimus reduces expression of ICAM-1 and VCAM-1 in activated EC and prevents adhesion of BMPC and monocytes at low concentrations in vitro

Recruitment of BM-derived cells to the site of vascular injury is dependent on various cellular adhesion molecules. ICAM-1 and VCAM-1 are pivotal molecules in this process. Therefore, we quantified the effects of sirolimus on ICAM and VCAM expression in activated EC and performed cell adhesion experiments on activated EC using $CD45^+$ leukocytes and BMPC. Treatment with a low dose sirolimus (1 ng/mL) significantly reduced the expression of ICAM-1 and VCAM-1 in EC after activation with TNF-α, as detected by western blotting (n = 3, Fig. 6A). To address the direct effects of sirolimus on the adhesion of leukocytes and BMPC, human $CD45^+$ cells and purified $CD34^+$ cells were allowed to adhere to TNF-α-stimulated EC, and the effect of sirolimus and neutralizing antibodies to ICAM-1 and VCAM-1 on cell adhesion was assessed. Sirolimus dose-dependently attenuated the adhesion of leukocytes ($CD45^+$) to stimulated EC (sirolimus 20 nM: 32 ± 8, sirolimus 1 nM: 57 ± 10, control: 84 ± 22 cells/high power field, n = 4, ***$p < 0.001$, Fig. 6B). Moreover, we found a dose-dependent inhibition of sirolimus of the adhesion of $CD34^+$ cells to stimulated EC (sirolimus 20 nM: 13 ± 8, sirolimus 1 nM: 36 ± 12, control: 78 ± 16 cells/high power field, n = 4, **$p = 0.003$, Fig. 6C). Preincubation of EC with neutralizing antibodies to ICAM-1 (40 µg/mL) or VCAM-1 (40 µg/mL) was effective in reducing adhesion of $CD45^+$ cell to activated EC (ICAM-1: 48 ± 11, VCAM-1: 41 ± 7, control IgG: 85 ± 13 cells/high power field, n = 4, ***$p < 0.001$, Fig. 6D). Interestingly, preincubation of EC with neutralizing antibodies indicated that only neutralizing antibodies to ICAM-1 but not to VCAM-1 significantly reduced $CD34^+$ cell adhesion (ICAM-1: 29 ± 6, VCAM-1: 57 ± 18, control IgG: 83 ± 13 cells/high power field, n = 4, *$p = 0.022$, Fig. 6E). These data indicate that sirolimus directly prevents the expression of ICAM-1 and VCAM-1 and thus the adhesion of leukocytes and BMPC to the site of injury.

3.7. *Sirolimus concentrations in mouse serum achieved after systemic application do not prevent the proliferation of SMC or EC but prevent the expression of ICAM-1 and VCAM-1 and the adhesion of leukocytes and BMPC in vitro.*

To determine whether serum levels of sirolimus in mice treated systemically with sirolimus can directly prevent vascular cell proliferation, sirolimus (2 mg/kg body weight, i.p.) or vehicle control was applied daily for 14 days and serum was isolated from treated mice 2 hours after the last administration. SMC were incubated for 24 h with serum obtained from mice treated with sirolimus or with vehicle control in the presence or absence of PDGF-BB. Interestingly, incubation of SMC or EC with serum obtained from sirolimus treated mice did not affect SMC or EC proliferation, as determined by BrdU incorporation (n = 4, $p = 0.328$ w/o PDGF and $p = 0.998$ w/ PDGF in SMC and $p = 0.716$ w/o PDGF and $p = 0.796$ w/ PDGF in EC, Fig. 7A and B).
In contrast, incubation of EC with serum obtained from sirolimus-treated mice attenuated the adhesion of leukocytes ($CD45^+$) to stimulated EC (vehicle treated serum: 85.89 ± 12.76, sirolimus-treated serum: 46.00 ± 11.94 cells/high power field, n = 4, ***p < 0.001, Fig. 7C). Moreover, we found a significant inhibition of adhesion of $CD34^+$ cells to stimulated EC after incubation with serum obtained from sirolimus-treated mice as compared with EC incubated with serum from vehicle-treated mice (vehicle-treated serum: 36.89 ± 11.84, sirolimus-treated serum: 17.78 ± 3.99 cells/high power field, n = 4, ***$p < 0.001$, Fig. 7D).

3.8. *Sirolimus prevents adhesion molecule expression after vascular injury*

To assess the effects of systemic sirolimus treatment on the expression of ICAM-1 and VCAM-1 *in vivo*, we analyzed the expression of these cellular adhesion molecules following vascular injury. Treatment with sirolimus resulted in a significantly reduced expression of ICAM-1 and VCAM-1 14 days after vascular injury, indicating that this effect may contribute to the decreased accumulation of BM-derived cells within the developing neointima (ICAM-1: 0.8 ± 0.3 vs. 2.3 ± 0.4, n = 4, *$p = 0.013$, VCAM-1: 1.2 ± 0.2 vs. 2.8 ± 0.3, n = 4, **$p = 0.005$, Fig. 8A,B).

4. Discussion

Sirolimus and its derivates are currently widely used as anti-proliferative substances coated on DES. Systemic application of sirolimus is also effective in reducing in-stent-restenosis, but the mechanisms remain poorly understood [20]. Importantly, sirolimus is widely used as an immunosuppressive drug for the treatment of auto-immune diseases or after organ transplantation, especially following heart or kidney transplantation. These patients have a high risk of developing symptomatic coronary artery disease and often require PCI and/or coronary stent implantation. Therefore, we investigated the effects of systemic sirolimus application on neointima formation in a mouse model of vascular injury, and aimed to further elucidate the molecular and cellular mechanisms underlying the protective effect of sirolimus under these conditions. The mouse model used in this study is widely accepted for studies of post-angioplasty restenosis, because it closely resembles the angioplasty procedure that injures both endothelium and vessel wall [27]. Sirolimus was administered i.p. daily in a dose of 2 mg / kg body weight, which results in serum levels comparable so sirolimus serum levels in transplant patients [28, 29]. Consistent with clinical observations, we found a significant reduction in neointima formation but, importantly, also in endothelial recovery after injury. Further, we demonstrated that the mechanisms of systemic sirolimus treatment are quite distinct from the effects of a local release of sirolimus from DES. Since a direct anti-proliferative effect in SMC and EC can only be observed after administration of high concentrations of sirolimus, as achieved by a local release into the vessel wall, the lower circulating drug levels after systemic sirolimus treatment exert anti-inflammatory properties, not direct anti-proliferative properties; thus, sirolimus only indirectly inhibits SMC and EC proliferation by preventing the inflammatory response and the release of growth factors and cytokines by the recruited leukocytes.

4.1. Mechanisms and cellular cross-talk during neointima formation

Vascular SMC from the medial layer are the predominant cell type within neointimal lesions. The inhibition of SMC proliferation and migration has thus been the primary therapeutic approach in recent years [13]. Other therapeutic strategies aim to attenuate the inflammatory response or to accelerate endothelial recovery following injury [2]. Importantly, inflammation and endothelial recovery are strongly related to each other, since leukocyte-derived growth factors and cytokines do not only stimulate the proliferation of SMC but also EC. On the other hand, stimulation of re-endothelialization has been shown to be effective in reducing the inflammatory response to injury, and also in directly preventing SMC proliferation, due to the release of nitric oxide in the course of neointimal lesion formation [25]. Moreover, inflammation and SMC proliferation are directly linked and a reduced inflammatory response results in reduced SMC proliferation and neointima formation.[1]

4.2. BMPC in neointima formation

The design of the study using BMTx of $eGFP^+$ bone marrow cells enabled us to investigate the extent of the inflammatory response, as well as the accumulation and possible differentiation of circulating BMPC in the neointimal lesions. We therefore aimed to first determine the total number of cells recruited from the circulation, followed by a more detailed analysis of the cellular subtypes and their relative numbers after sirolimus treatment. Tracking all circulating and recruited cells from the circulation is mandatory for such an approach. Thus, we used a model of BMTx, in which -after the reconstitution of the transplanted BM- the vast majority of all circulating cells were eGFP-labeled (88.43% ± 4.21 $eGFP^+$, n=6) and could be tracked over time [11]. Using specific antibodies for additional cell markers, we were able to differentially determine the numbers of resident vs. recruited cells expressing SMC or EC markers or markers of cells with high plasticity.

In accordance with prior reports, our data confirmed that circulating BMPC can temporarily accumulate within the lesion and express rather unspecific SMC marker genes, such as α-SMA. However, α-SMA is also expressed by monocytes/ macrophages in a certain interim state; therefore, these cells do not necessarily represent differentiated genuine SMC [13]. Nonetheless, highly active and plastic BMPC recruited from the circulation may be important modulators of the local response to injury by the paracrine effects of these cells on the surrounding resident cells. These effects have predominantly been documented for the processes of angiogenesis and arteriogenesis with effects on EC, but also in atherosclerosis and neointima formation [30]. In accordance with our results, only a few BM-derived SM-like cells expressing SMA could be detected within atherosclerotic plaques in these studies. Nevertheless, using genetic mouse models, these cells were found to secrete various pro-inflammatory cytokines and mitogens, thereby promoting the proliferation of adjacent SMC and progression of the atherosclerotic lesion [31]. Since our *in vitro* data demonstrate that systemic application of sirolimus potently prevents not only the adhesion of inflammatory ($CD45^+$) cells but also of circulating BMPC ($CD34^+$), this combined action may contribute to the indirect anti-proliferative effect of sirolimus on local SMC and the prevention of neointima formation. However, further studies are required to precisely determine the relative importance of leukocytes vs. BMPC on the paracrine activation of resident cells.

4.3. Sirolimus attenuates the recruitment of circulating cells by down-regulation of the adhesion molecules ICAM-1 and VCAM-1

Our *in vitro* data showed that sirolimus has a direct inhibitory effect on the adhesion of leukocytes and BMPC to TNF-α-activated EC. Leukocyte recruitment is strongly dependent on the expression of both ICAM-1 and VCAM-1 in activated EC and inhibition of these molecules can effectively prevent the inflammatory response and vascular lesion formation [32]. We also showed that sirolimus prevents the up-regulation of these adhesion molecules in EC. In fact, a

recent study confirmed that inhibition of the mTOR signaling complex mTORC2 by sirolimus can prevent the TNF-α-mediated induction of VCAM-1 in EC [33]. However, numerous other adhesion molecules are involved in the complex cascade of circulating cell recruitment, and may be influenced, as well. Thus, preventing the expression of ICAM-1 and VCAM-1 may represent only one aspect of the anti-adhesive properties of sirolimus, and other mechanisms very likely contribute to the potent *in vivo* effects observed in our study.

4.4. Systemic application of sirolimus only indirectly reduces SMC proliferation

The effect of sirolimus derived from DES on neointima formation is mainly explained by a local inhibition of the proliferation of resident SMC and EC. Importantly, our data show that sirolimus only prevents SMC proliferation at relatively high concentrations of 20 ng/mL. This can be explained by the recent finding that vascular SMC only express low levels of FKBP12, which is the cytosolic target of sirolimus for inhibiting mTOR [7]. Accordingly, local tissue concentrations of sirolimus are high following the implantation of a DES and thus exert direct anti-proliferative effects. Despite the high local concentrations, the wide therapeutic range of sirolimus or its derivates explains the lack of toxic or apoptotic effects on cells of the vessel wall. Even though high local tissue concentrations are achieved, systemic concentration of sirolimus has not been reported to exceed concentrations of 4 ng/mL directly after DES implantation and is generally below detection levels at later time points [34]. In contrast, systemic application of sirolimus, e.g. following organ transplantation, is adapted to achieve serum concentrations of 4 to 12 ng/mL depending on the patient characteristics and the other drugs in the immunosuppressive regimen, and vascular tissue concentrations are not reported to exceed these serum concentration levels [35]. Therefore, only sirolimus derived from DES may have direct effects on SMC proliferation at concentrations as high as 20 ng/mL or more. In contrast, systemic application of sirolimus attenuates the recruitment of leukocytes and BMPC and thus indirectly attenuates SMC

proliferation within the neointimal lesions, because SMC proliferation is largely triggered and driven by cytokines and growth factors released from infiltrating inflammatory cells after injury.

4.5. Systemic application of sirolimus prevents endothelial activation and attenuates re-endothelialization following injury

Endothelial recovery is a key aspect for the prevention or limitation of neointima formation. Since sirolimus released from DES retards endothelial recovery after PCI, dual anti-platelet therapy should be prolonged in order to prevent in-stent-thrombosis. We showed in our study that re-endothelialization is also impaired following systemic application of sirolimus after wire-induced injury in mice. According to our *in vitro* results, sirolimus only reduces proliferation of EC at higher concentrations of sirolimus, and serum from mice receiving systemic application of sirolimus did not prevent EC proliferation, so that a direct inhibitory effect of sirolimus on endothelial proliferation after systemic application of sirolimus at the concentrations used can be excluded. In contrast, serum isolated from mice after systemic sirolimus treatment attenuated the TNF-α-induced up-regulation of endothelial adhesion molecules, indicating that sirolimus exerts different effects on EC in a concentration-dependent manner.

It is well-established that endothelial recovery is very much dependent on the recruitment of leukocytes and BMPC. In accordance with these findings, a recent clinical trial demonstrated enhanced endothelial healing, when sirolimus-eluting DES were coated with CD34-recognizing antibodies on the luminal side, which captured and recruited circulating BMPC [30, 36]. Our *in vitro* and *in vivo* data demonstrate that systemic treatment with sirolimus prevents the recruitment and the accumulation of leukocytes and BMPC. Therefore, the reduced re-endothelialization observed in our *in vivo* experiments after systemic sirolimus treatment is very likely due to a reduced expression of adhesion molecules and subsequently reduced recruitment of leukocytes and BMPC, which under normal conditions would support endothelial regeneration by the local secretion of growth factors and cytokines. However, BMPC levels are also positively

correlated with restenosis, indicating that there is no specific effect of BMPC on re-endothelialization alone [15, 37].

4.6. Effects of sirolimus on the function and differentiation of BMPC

In a previous report using a model of wire-induced injury in mice, the local application of sirolimus around the injured vessel segment resulted in a reduced accumulation and differentiation of BMPC into SM-like cells within neointimal lesions [8]. In accordance, our *in vitro* data show that serum of mice treated systemically with sirolimus also directly attenuated adhesion of $CD34^+$ BMPC to activated EC. However, even though our *in vivo* experiments using a systemic sirolimus application were designed to detect differences in the differentiation capacity of BMPC within neointimal lesions, the numbers of $eGFP^+$ cells expressing Sca-1 or vascular cell markers were very low and double-positive cells could only be observed occasionally, even in control animals. Consequently, there was no statistical difference in the few $eGFP^+$ cells expressing SMC markers (calponin) or EC markers (vWF) between the sirolimus-treated group and the control group 14 or 28 days after injury.

The differing results in our study can be explained by the fact that novel, more specific labeling techniques and antibodies as well as 3D confocal laser scanning microscopy techniques were used that enable the identification of false positive results, as described previously by us and others [11, 12].

Nevertheless, our *in vitro* findings on the inhibition of BMPC adhesion by sirolimus and the strongly reduced numbers of all BM-derived cells within the neointimal lesion also suggest that also BMPC recruitment may be reduced following treatment with sirolimus *in vivo*, even though our data 14 days and 28 days after injury do not provide clear evidence because of the low numbers of BMPC detected within neointimal lesions. Thus, the role of BMPC in the sirolimus-mediated effects remains largely elusive.

4.7. Clinical implications of basic research

Our data show that systemic application of sirolimus prevents post-angioplasty neointima formation and vessel narrowing by inhibiting the inflammatory response to injury and possibly the recruitment of BMPC to injured vessel segments. Importantly, systemic sirolimus treatment also impairs endothelial regeneration after angioplasty. This information is of particular importance for patients after organ transplantation, especially heart or kidney transplant recipients who are treated systemically with sirolimus, since these patients are at high risk of developing coronary artery disease and of receiving PCI. Under these conditions, systemic sirolimus treatment may support the effect of locally released sirolimus derivatives from DES to prevent restenosis of the dilated and stented vessel segments, which may beneficially influence the outcome of coronary interventions in these patients. On the other hand, our data indicate that re-endothelialization after coronary interventions may be severely impaired in patients receiving systemic sirolimus treatment. Thus, coronary interventions require a subsequent prolonged anti-platelet therapy because of the high thrombogenicity of non re-endothelialized vessel or stent segments. According to the data from this study, extreme caution should be paid to current approvals of new generation DES to reduce dual anti-platelet therapy time to 3 months only, since safety data for this approval have not been obtained in patients systemically treated with sirolimus or in organ transplant patients. The data from this study clearly indicate the need for further clinical trials to determine the optimal duration of anti-platelet therapy after coronary interventions in patients under systemic sirolimus treatment.

4.8. Study limitations

Despite the use of human cells in our *in vitro* experiments to best simulate the responses to sirolimus in human coronary arteries, the *in vivo* experiments of this study were performed in mice. Further studies in larger animals or in humans would be required to confirm these results.

Additionally, to determine the impact of systemic sirolimus treatment on the inflammatory response and the adhesion and recruitment of inflammatory cells, we aimed to also determine the effect on circulating BMPC. For this purpose, we decided to use a BMTx model. However, the number of BMPC expressing Sca-1 or vascular cell markers was found to be very low in neointimal lesions, so we could not detect a significant effect of systemic sirolimus treatment on BMPC recruitment or differentiation after injury. Even though these data are consistent with recent reports investigating the role of BMPC in atherosclerotic lesions [10, 24], the initial BMTx may affect the number and functional capacity of circulating BMPC, so further studies in different animal models or humans are required to elucidate the impact of systemic sirolimus treatment on the function of BMPC during neointima formation. Due to the complex and directly linked interplay of injury-induced inflammation and SMC proliferation, mechanistic data on the selective action of distict sirolimus doses on either inflammation or SMC proliferation can only be obtained *ex vivo*. Even though it's very likely that these data reflect the processes *in vivo*, a direct proof has to be obtained.

5. **Conclusions**

The data presented in this manuscript challenge the current assumption that systemic treatment with sirolimus directly affects the proliferation of SMC or EC and thereby influences vascular lesion formation. Significantly higher concentrations than those usually achieved by systemic treatment seem necessary to directly prevent SMC or EC proliferation. Moreover, serum isolated from sirolimus-treated mice did not exert direct anti-proliferative properties on vascular cells *in vitro*. In contrast, sirolimus at (lower) concentrations usually achieved by systemic application potently prevented the recruitment of circulating inflammatory cells to the injured vessel segment. Therefore, the reduced proliferation of EC and SMC after systemic treatment with sirolimus is most likely an indirect effect of the reduced inflammatory response. These novel

findings extend our understanding of the mechanisms responsible for the effects of systemic sirolimus treatment on vascular remodeling processes. Moreover, as a clinical implication, our data strongly suggest that dual anti-platelet therapy after PCI should be extended in patients receiving systemic sirolimus treatment because of delayed endothelial recovery.

Author contributions

Conception and design of the study: JMD, JD, HB, RBD, DGS. Acquisition of data: JMD, JD, HB, DGS. Analysis and interpretation of data: JMD, JD, HB, JB, RBD, DGS. Drafting the article or revising it critically for important intellectual content: JMD, JD, HB, JB, RBD, DGS. Final approval of the version to be submitted: JMD, JD, HB, JB, RBD, DGS.

Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Sirolimus prevents SMC proliferation and neointima formation after wire-induced injury. A, Representative cross-sections of mouse femoral arteries 14 days after injury treated with vehicle (n=6 mice) or sirolimus (n=5 mice) were stained for Ki-67 and α-SMA (scale bar: 100 µm). B, Proliferation of neointimal and medial cells was quantified by counting $Ki67^+$ cells at 14 days after vascular injury within the medial and neointimal layer (n = 5 per group, $^{**}p = 0.002$). C, Representative cross-sections of mouse femoral arteries 28 days after wire-induced injury treated with vehicle (n=7 mice) or sirolimus (n=9 mice) were stained for van Gieson (scale bar: 100 µm). D, Morphometric quantification of vehicle-treated (black bars) and sirolimus-treated (white bars) lesions was performed 28 days after injury. The intima-to-media (I/M-ratio) was calculated from the neointimal area divided by the medial area ($^{***}p < 0.001$). E, Luminal stenosis was calculated as percent stenosis = $[1 - (A_L/A_N)] \times 100$, A_L = luminal area, and A_N = area of the normal artery defined as the area surrounded by internal elastic lamina ($^{***}p = 0.001$).

Fig. 2. Sirolimus impairs re-endothelialization after vascular injury. A, Representative images of mouse femoral arteries 14 days (left) and 28 days (right) after wire-induced injury treated with vehicle or sirolimus. Evans blue staining identifies segments of each artery that have not recovered functionally intact endothelium. B, Quantification of re-endothelialized area assessed

by Evans blue dye staining of whole-mounted femoral arteries 14 days and 28 days after injury (n= 4, *p = 0.017, n.s. = not significant (p = 0,461)).

Fig. 3. Sirolimus prevents the accumulation of BM-derived cells in the neointima. A, Representative cross-sections of mouse femoral arteries 14 days and 28 days after injury treated with vehicle or sirolimus were stained for DAPI (blue) to localize nuclei and α-SMA (red). The eGFP signal represents the endogenous signal (green) without additional antibody staining, (scale bar: 100 µm). B, Cells of the neointima and media expressing both eGFP (green) and DAPI were correlated to the total cell count, and the relative number of $eGFP^+$ (BM-derived) cells in the neointima and media was quantified (*p = 0.043, ***p < 0.001). C, Representative cross-sections of mouse femoral arteries 14 days and 28 days after injury treated with vehicle or sirolimus were stained for MOMA-2 (red) to localize monocytes / macrophages (scale bars: 100 µm and 25 µm (40x)). D, Cells of the neointima and media expressing both MOMA-2 (red) and eGFP (green) were correlated to the total cell count of all $eGFP^+$ cells and the relative number of monocytes / macrophages in the neointima and media was quantified (*p = 0.040).

Fig. 4. Accumulation and differentiation of BMPC into *bona fide* vascular cell types is an extremely rare event. A, Representative cross-sections of mouse femoral arteries 14 days after injury treated with vehicle or sirolimus were stained for DAPI (blue) and Sca-1 (red) to identify BMPC. Cells of the neointima and media expressing both Sca-1 (red) and eGFP (green) indicate BMPC (arrowhead) (scale bar: 50 µm). B,C Representative cross-sections of mouse femoral arteries 28 days after injury treated with vehicle or sirolimus were stained for calponin (red) to detect BM-derived SMC, or vWF (red) to detect BM-derived EC (scale bars 50 µm).

Fig.5. Sirolimus only prevents proliferation of SMC and EC at high concentrations in a dose-dependent manner. A, SMC were grown in the presence or absence of growth medium and the indicated concentrations of sirolimus. Incorporation of BrdU was determined after 24 h (n = 4,

**p = 0.009). B, EC were grown in the presence or absence of growth medium and the indicated concentrations of sirolimus. Incorporation of BrdU was determined after 24 h (n = 4, *p = 0.022, ***p < 0.001).

Fig. 6. Sirolimus prevents adhesion of leukocytes and BMPC to activated EC *in vitro* by down-regulation of ICAM-1 and VCAM-1. A, EC were cultured for 8 hours in the absence or presence of TNFα and with sirolimus (1 ng/mL) or vehicle control. Expression of ICAM-1 or VCAM-1 was determined by Western Blotting in lysates from EC. Representative blots of three independent experiments are shown. B,C, EC were cultured in the absence or presence of TNFα and treated with sirolimus at the given concentrations or the control buffer. $CD45^+$ cells (B) or $CD34^+$ cells (C) were labeled with CFSE (5 µM) prior to co-culture in adhesion assays on EC. After 60 minutes of co-culture, nonadherent cells were removed by washing and adherent $CD45^+$ cells or $CD34^+$ cells were evaluated by counting labeled cells in three fields of view per condition (n = 4, ** p = 0.003, ***p < 0.001). D,E, The adhesion assay was performed under the same conditions, but instead of sirolimus treatment blocking antibodies against ICAM-1 (D) or VCAM-1 (E) were used, and adhering cells were quantified (n = 4, *p = 0.022, ***p < 0.001).

Fig. 7. Systemic concentration levels of sirolimus do not prevent SMC or EC proliferation but prevent adhesion of leukocytes and BMPC to activated EC *in vitro*. A, SMC were incubated for 24 h with serum obtained from mice treated with sirolimus (2 mg/kg body weight/day i.p.) or with vehicle control for 14 days in the presence or absence of PDGF-BB (10 ng/mL). Incorporation of BrdU was determined after 24 h (n = 4, n.s. = not significant (p = 0.716)). B, EC were incubated for 24 h with serum obtained from mice treated with sirolimus (2 mg/kg body weight/day i.p.) or with vehicle control for 14 days in the presence or absence of VEGF (20 ng/ml). Incorporation of BrdU was determined after 24 h (n = 4, n.s. = not significant (p = 0796)). C, EC were incubated with serum obtained from mice treated with sirolimus (2 mg/kg body weight/day i.p.) or with vehicle control in the presence or absence of TNFα. $CD45^+$ cells (C) or $CD34^+$ cells (D) were

labeled with CFSE (5 μM) prior to co-culture in adhesion assays on EC. After 60 minutes of co-culture, nonadherent cells were removed by washing and adherent $CD45^+$ cells or $CD34^+$ cells were evaluated by counting labeled cells in three fields of view per condition (n = 4, ***$p < 0.001$, ***$p < 0.001$).

Fig. 8. Sirolimus prevents ICAM-1 and VCAM-1 adhesion molecule expression *in vivo*. A, Representative cross-sections of mouse femoral arteries 14 days after injury treated with vehicle or sirolimus (2 mg/kg body weight/day i.p.) were stained for ICAM-1 or VCAM-1 (scale bar: 50 μm). B, Quantification of ICAM-1 and VCAM-1 expression of mouse femoral arteries was performed using a visual scale ranging from 1-4 (n = 4, *$p = 0.013$, **$p = 0.005$).

Figure 1

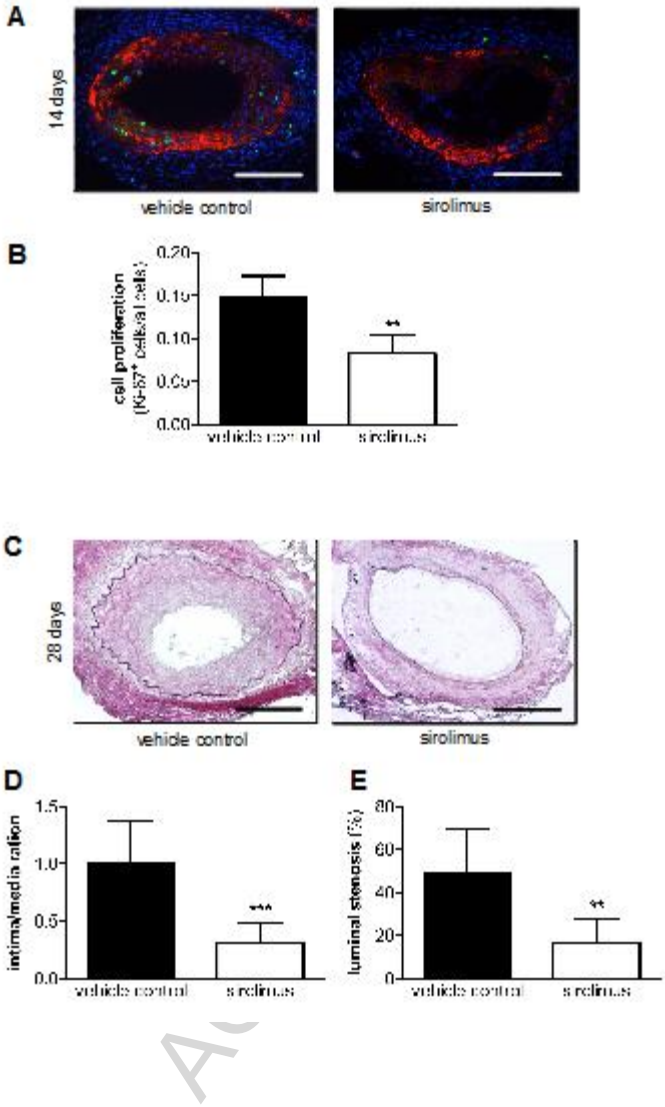

Figure 2

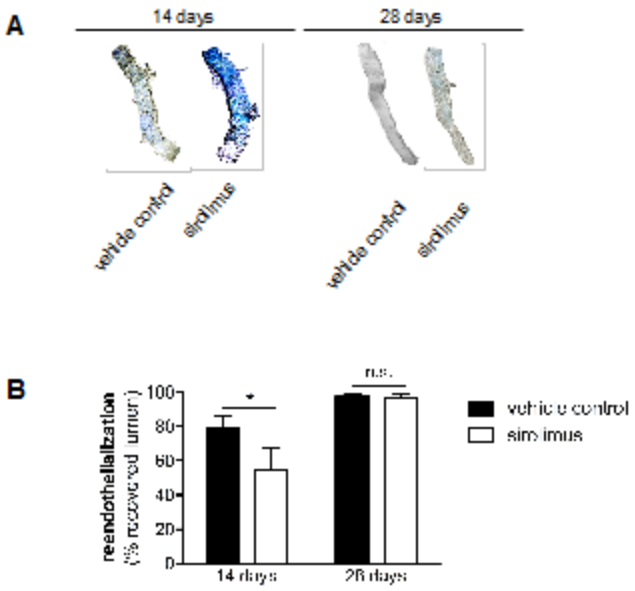

Figure 3

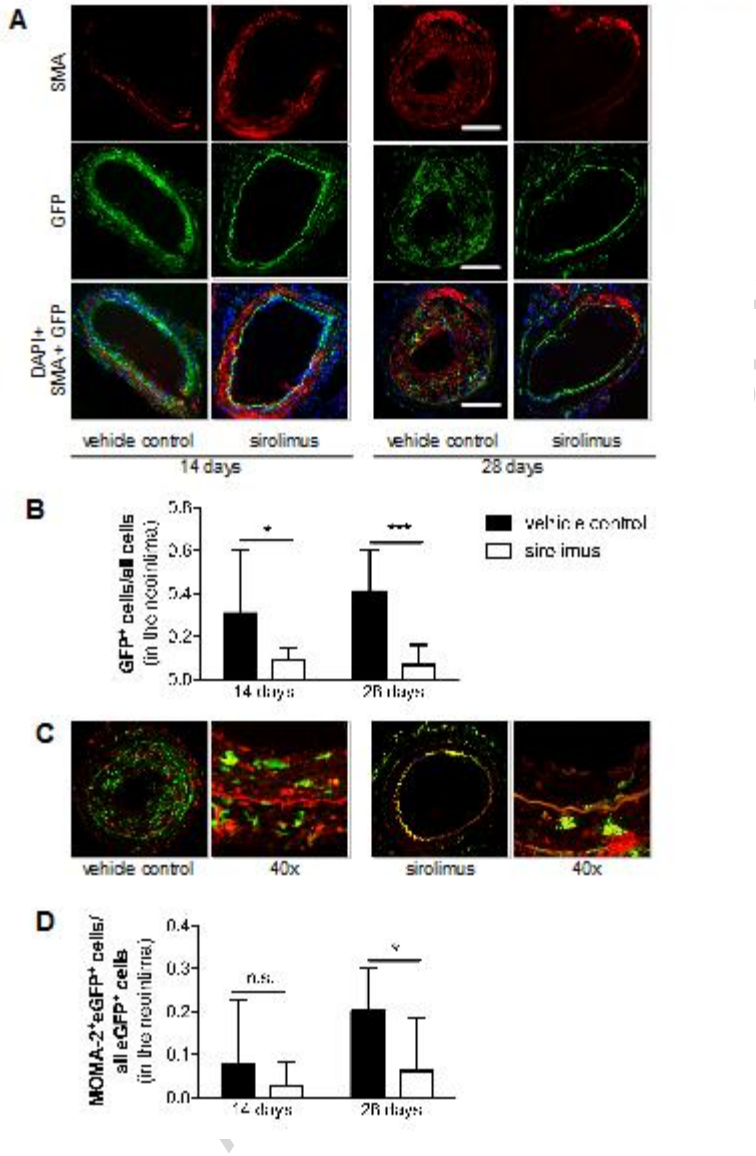

Figure 4

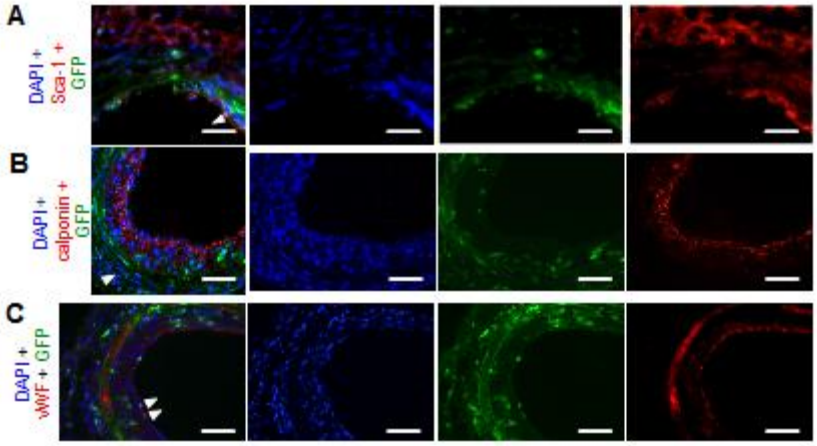

Figure 5

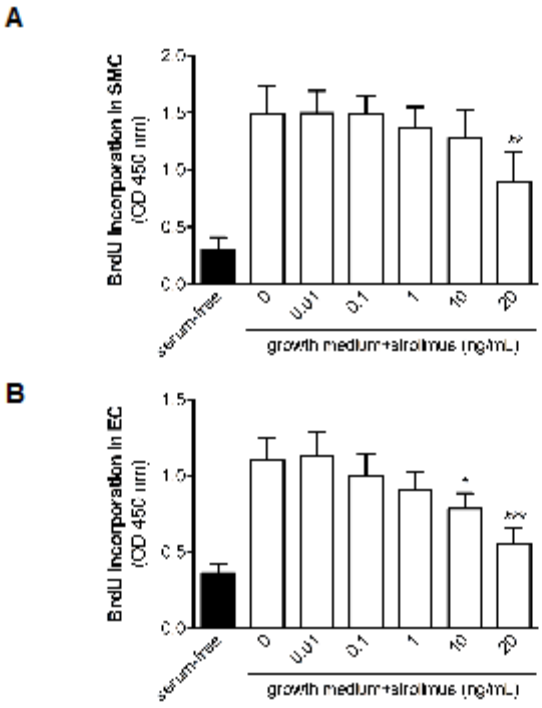

Figure 6

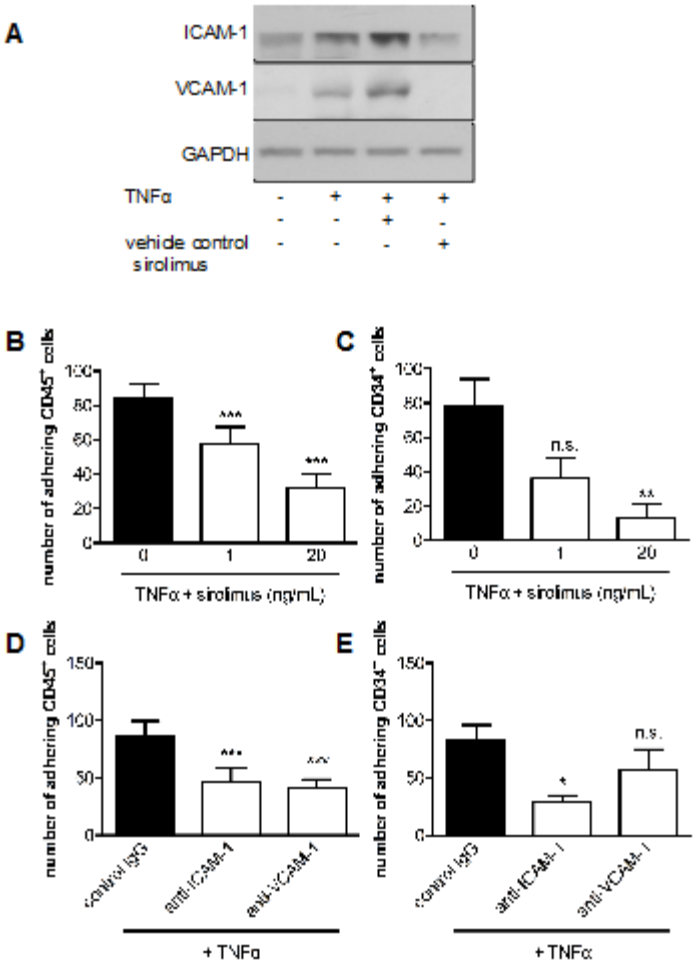

Figure 7

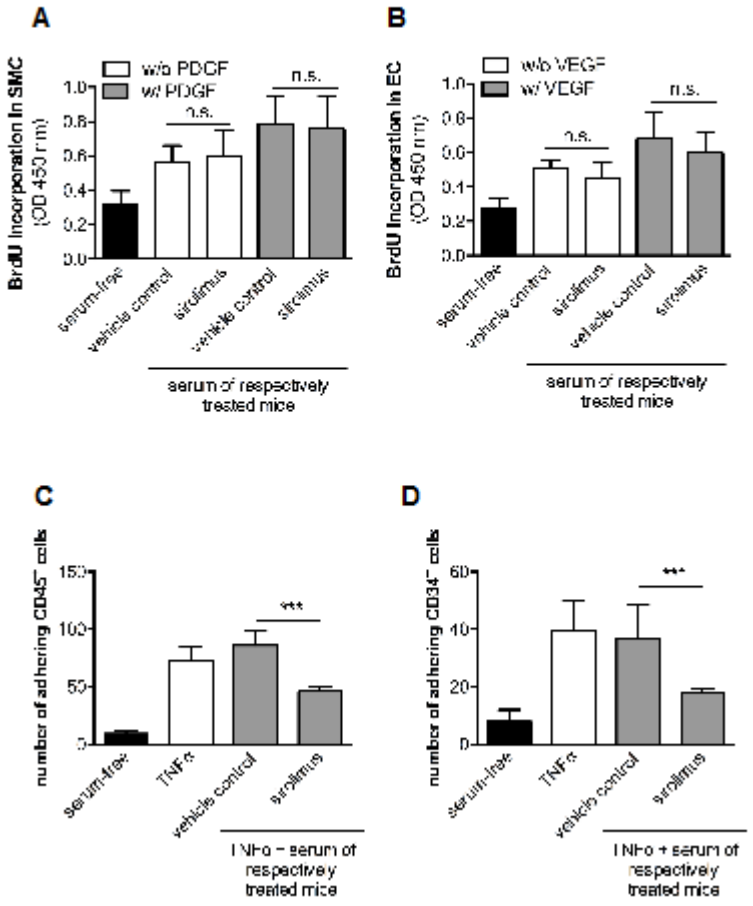

Figure 8