

Influence of caveolin-1 and endothelial nitric oxide synthase on adventitial inflammation in aortic transplants

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ABSTRACT

BACKGROUND Restenosis after endovascular interventions is a clinically relevant process that is directly associated with increased morbidity. Thereby, an increased migration and proliferation of vascular smooth muscle cells (VSMCs) is mainly responsible for recurrent lumen narrowing. Previously, we showed that caveolin-1 (Cav-1) and endothelial nitric oxide synthase (eNOS) were directly involved in neointimal proliferation.

AIMS In the current study, we investigated the impact of Cav-1 and eNOS on adventitial processes in a murine model.

METHODS Denuded aortas from C57Bl6n (wild-type [WT]), Cav-1^{-/-}, eNOS^{-/-}, and Cav-1^{-/-}/eNOS^{-/-} mice were transplanted into common carotid arteries of WT mice. The explantation was performed after 6 weeks, followed by Elastica van Gieson staining and immunohistochemistry.

RESULTS The Cav-1^{-/-} and the eNOS^{-/-} aortas showed an increase in the adventitial content of macrophages, whereas their combined knockout did not lead to additive effects. Differences were observed despite the same acceptor, suggesting the local origin of inflammatory cells. Furthermore, the WT transplants exhibited the highest content of vascular endothelial growth factor A (VEGF-A) despite the lowest macrophage content. In contrast, the knockout aortas showed a decreased content of VEGF-A as well as decreased expression of α -smooth muscle actin (α -SMA) in the tunica media, suggesting induced VSMC migration. Moreover, the WT aortas exhibited increased neovessel formation.

CONCLUSIONS Cav-1 and eNOS inhibit adventitial macrophage-derived inflammation and modulate its cellular function. The knockout of Cav-1 and eNOS leads to a decreased expression of VEGF-A, with decreased neovessel formation and increased migration of VSMCs, which promote a proatherogenic phenotype.

INTRODUCTION Restenosis is characterized by recurrent lumen narrowing after endovascular treatment such as a percutaneous coronary intervention (PCI) performed on an atherosclerotic plaque. Despite the use of modern drug-eluting stents, clinically relevant restenosis is still a major adverse event with an incidence of around 5%.¹ A PCI-derived injury could lead to new arterial wall thickening, causing a local vascular inflammation that leads to an intimal

hyperplasia of vascular smooth muscle cells (VSMCs)² smooth muscle migration/proliferation, extracellular matrix deposition, a process called neointimal proliferation.³ These VSMCs migrate mainly from the media, and exhibit phenotypic plasticity that enables their transdifferentiation into macrophage-like or fibroblast-like cells.^{4,5} In contrast, vascular inflammation can also promote a dilation of arteries resulting in coronary artery ectasia.⁶

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WHAT'S NEW?

Neointimal proliferation is crucial in the pathogenesis of myocardial reinfarction due to restenosis and is characterized by an increased migration and proliferation of vascular smooth muscle cells. Both adventitial inflammation and the interaction of caveolin-1 (Cav1) and endothelial nitric oxide synthase (eNOS) are known to influence neointimal proliferation. The current study showed that Cav-1 and eNOS further inhibit adventitial macrophage-derived inflammation and modulate cellular function in a murine model. The knockout of Cav-1, as well as eNOS, resulted in a decreased expression of vascular endothelial growth factor A and was accompanied by a decreased neovessel formation and increased vascular smooth muscle cell migration, both of which promote restenosis. These effects are caused by local vessel wall-derived cells and not by migrated blood-borne cells. The results clarify the importance of local mechanisms in the pathogenesis of restenosis and support a local vascular lesion-site therapy.

Endothelial nitric oxide synthase (eNOS) is a 133-kDa homodimeric enzyme that is crucial for constitutive vascular nitric oxide (NO) synthesis,⁷ and is primarily expressed in caveolae, which are 50-to-100-nm invaginations of the cell membrane.⁸ Caveolin-1 (Cav-1), an integral transmembrane protein, structures these subcellular compartments and additionally acts as a tonic inhibitor of eNOS through its specific binding to the caveolin scaffolding domain.⁹ In the case of an increase in the intracellular calcium concentration, the proteins dissociate, which activates eNOS and subsequently increases the NO content.¹⁰ Apart from this physiological mechanism, there is another process called eNOS uncoupling, which leads to an elevated content of eNOS-derived reactive oxygen species (ROS).¹¹ The knockout of Cav-1, for example, is known to trigger eNOS uncoupling.^{12,13}

While Cav-1 and eNOS inhibit neointimal proliferation via direct or indirect pathways,^{14,15} ROS, resulting from eNOS uncoupling, increases it,¹⁶ rendering both proteins potential pathogenetic key effectors in restenosis. We previously discussed¹⁶ this hypothesis in an *in vivo* murine model of restenosis and showed that Cav-1^{-/-} increases intimal lesions, whereas the loss of eNOS acts in an ambivalent way depending on Cav-1 expression. Arteries of mice with Cav-1^{-/-} and eNOS^{-/-} (C/e^{-/-}) knockout did not develop a more atherogenic phenotype than the Cav-1^{-/-} aortas despite an increased intimal formation in the eNOS^{-/-} arteries, suggesting another pathogenic effector, like eNOS uncoupling, in the Cav-1^{-/-} mice (Supplementary material, *Figure S1A*). Furthermore, the neointimal proliferation was identified as being primarily responsible for intima formation. A relevant role of the bloodborne migrated cells was ruled out by using a sex-mismatch transplantation model. Only around 1% of migrated blood-borne cells, which must have a Y chromosome in this model, were detected by fluorescence *in situ* hybridization.¹⁷ Our previous study focused on

intimal processes; however, other compartments of the artery wall were shown to be relevant in atherogenesis.¹⁸ The media, for example, is the source of VSMCs.¹⁹ Tertiary lymphoid structures, which arise in the adventitia, produce cytokines, chemokines, and growth factors.²⁰ While interleukin 1 β , tumor necrosis factor α , or leukotriene E₄ amplify vascular inflammation, the vascular endothelial growth factor A (VEGF-A) influences neointimal proliferation and plays a key role in plaque neoangiogenesis.^{21,22}

However, the media- or adventitia-derived effects of Cav-1 or eNOS on restenosis are still not well understood. In the current study, we focused on this issue *in vivo* using a cervical aortic graft transplantation model.

METHODS Murine model Wild-type (WT) (C57Bl6n, Charles River Laboratories, Sulzfeld, Germany), eNOS^{-/-},²³ Cav-1^{-/-},²⁴ and specifically bred C/e^{-/-} mice were used in the current study. All parts of the study were approved by the local animal research ethics committee at Technische Universität Dresden and the Regional Council Dresden, and were performed in accordance with the institutional guidelines and the German animal welfare regulations (approval no. AZ 249168.111/201330).

At an average age of 10 weeks, the mice underwent a cervical aortic graft transplantation, whereby denudated aortas from female WT, Cav-1^{-/-}, eNOS^{-/-}, or C/e^{-/-} mice were interposed into the common carotid artery of male WT mice (Supplementary material, *Figure S1B*). All animals were adequately anesthetized during surgery, which has been described in detail previously.¹⁷

After the operation, all mice were fed a Western-type diet (ssniff EF R/M acc. TD88137 mod., crude fat 21.2%, sugar 33.2%) and subcutaneously received 0.1 mg/kg buprenorphine for analgesia. The explantation of the aortic grafts was performed 6 weeks after the transplantation, when intimal lesions reached a maximum. The *de novo* genesis of intima formation was proved through a time series analysis as well as an examination of the nontransplanted aortas.

Histology The tissue processing and staining protocols have been described previously.¹⁷ Elastica van Gieson (EvG) staining was performed, and the sections were digitalized for the determination of intima, media, and adventitia of the aorta. Neovessels were quantified by calculating their proportion in relation to the intima.

Immunohistochemistry The α -smooth muscle actin (α -SMA) was stained for detection of VSMCs, whereas galectin-3 was used as a marker for monocytes and macrophages. Both staining protocols have been described

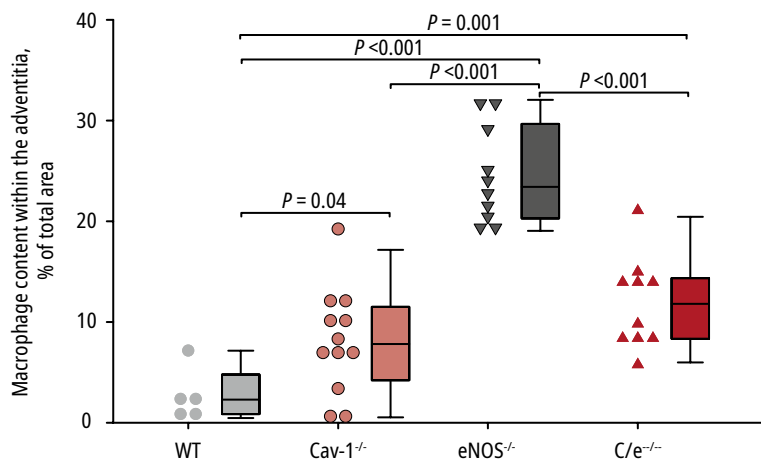


FIGURE 1 A modified whisker plot showing adventitial macrophage content: galectin-3 was used as a marker for monocytes and macrophages. Endothelial nitric oxide synthase knockout (eNOS^{-/-}) transplants (n = 10) showed an increased macrophage content, which was decreased by additional knockout of caveolin-1 (Cav-1; n = 10). Furthermore, an increased macrophage-derived inflammation was detected in Cav-1^{-/-} aortas (n = 12) as compared with the wild-type transplants (n = 5). Lower and upper ends of the boxes indicate the first and third quartiles. The horizontal bar inside the boxes indicates the median. The whiskers indicate values within 1.5 × interquartile range. Each circle and triangle indicates a single value. This is valid for all figures.

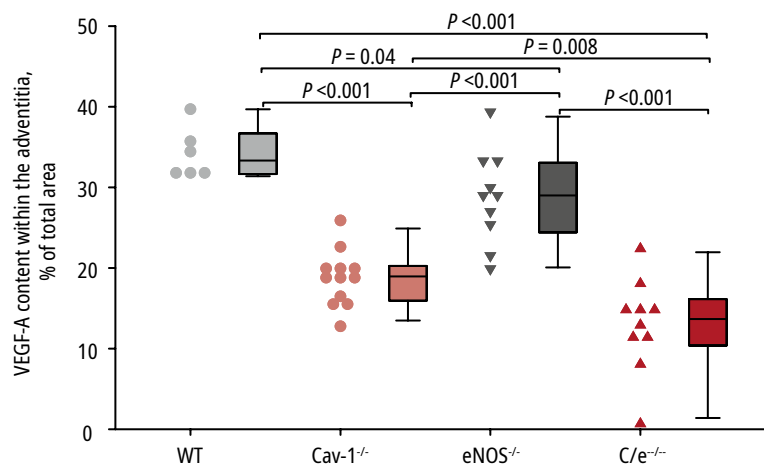


FIGURE 2 A modified whisker plot showing adventitial vascular endothelial growth factor A (VEGF-A) expression: notwithstanding an increased galectin-3 expression, the caveolin-1 (Cav-1^{-/-}; n = 12), endothelial nitric oxide synthase (eNOS^{-/-}; n = 10), and C/e^{-/-} (n = 10) knockout transplants showed a decreased adventitial VEGF-A expression compared with the wild-type arteries (n = 6).

previously,²⁵ and served as the basis for detecting VEGF-A and cluster of differentiation (CD) 3, a T cell marker. Recombinant rabbit polyclonal antibodies were used for both stainings (anti-VEGF-A, DP3520P, Acris Antibodies GmbH, Herford, Germany; anti-CD3, A 0452, Dako, Hamburg, Germany). For the negative control, the primary antibody was changed against purified serum proteins from nonimmunized rabbits (ChromPure Rabbit IgG, Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, United States, 1:2000 dilution). Counterstaining was performed with Mayer hemalum.

Finally, the percentage of positively stained area in the media or the adventitia was evaluated by using RGB color space deconvolution (Supplementary material, Figure S2).

Statistical analysis Data are presented as mean (SE). The statistical testing between the WT, eNOS^{-/-}, Cav-1^{-/-}, and C/e^{-/-} transplants was performed using the 1-way analysis of variance (ANOVA) followed by the Fisher least significant difference post-hoc test. The global type I error level was set at 0.05.

RESULTS Increased adventitial macrophages in knockout transplants

Caveolin-1 and eNOS have an atheroprotective function through a decrease in neointimal proliferation in murine restenosis, as shown previously.¹⁷ However, these investigations mainly focused on intimal issues. In the current study, we examined the other compartments of the artery wall, especially with regard to inflammatory processes.

A significant increase in adventitial macrophages was observed in the knockout transplants 6 weeks after transplantation (Figure 1 and Supplementary material, Figure S3). The preceding data from the intima did not indicate any significant differences. The eNOS^{-/-} aortas showed the highest content of macrophages in the adventitia (mean [SE], 24.5% [1.5%]; n = 10), as detected by galectin-3 staining. The additional knockout of Cav-1 in the C/e^{-/-} transplants led to a decrease in macrophage-derived inflammation (mean [SE], 11.8% [1.4%]; n = 10), indicative of Cav-1-dependent eNOS effects. However, Cav-1 also seems to influence adventitial macrophage content as it was increased in Cav-1^{-/-} transplants (mean [SE], 8.1% [1.5%]; n = 12) compared with WT artery grafts (mean [SE], 2.7% [1.2%]; n = 5). No differences in the adventitial cross-sectional area were observed between the different transplants.

Adventitial VEGF-A content

Macrophages are mainly responsible for the adventitial production of growth factors such as VEGF-A. Immunohistochemistry was performed to determine the expression of VEGF-A in the adventitia. The highest content of VEGF-A was found in the WT transplants (mean [SE], 34.2% [1.3%]; n = 6), whereas the eNOS^{-/-} aortas, which showed the highest number of adventitial macrophages, had lower levels of VEGF-A (mean [SE], 28.8% [1.8%]; n = 10) (Figure 2 and Supplementary material, Figure S4). Furthermore, a significant decrease in the VEGF-A-positive area was detected in the Cav-1^{-/-} arteries (18.7% [1.0%], n = 12) as well as in the C/e^{-/-} (mean [SE], 12.9% [1.8%]; n = 10) aortic grafts. No significant changes in VEGF-A content were detected within the intima (data not shown).

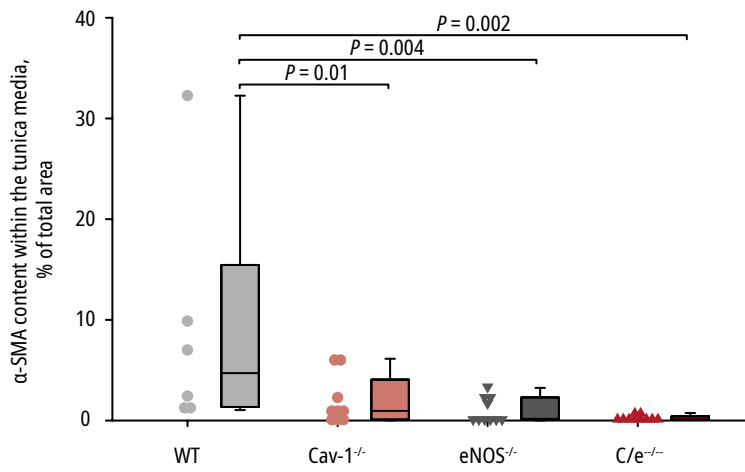


FIGURE 3 A modified whisker plot showing expression of α -smooth muscle actin (α -SMA) in the tunica media: significantly decreased expression of α -SMA in the cavelin-1 (Cav-1^{-/-}; n = 9), endothelial nitric oxide synthase (eNOS^{-/-}; n = 10), and C/e^{-/-} (n = 10) transplants compared with the wild-type aortas (n = 6).

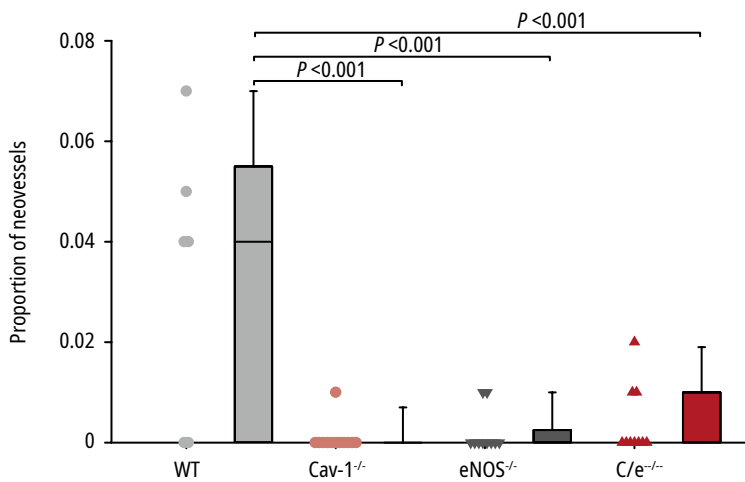


FIGURE 4 A modified whisker plot showing proportion of intimal neovessels: according to the elevated content of vascular endothelial growth factor A, the wild-type aortas showed a significantly increased proportion of neovessels (n = 6–12).

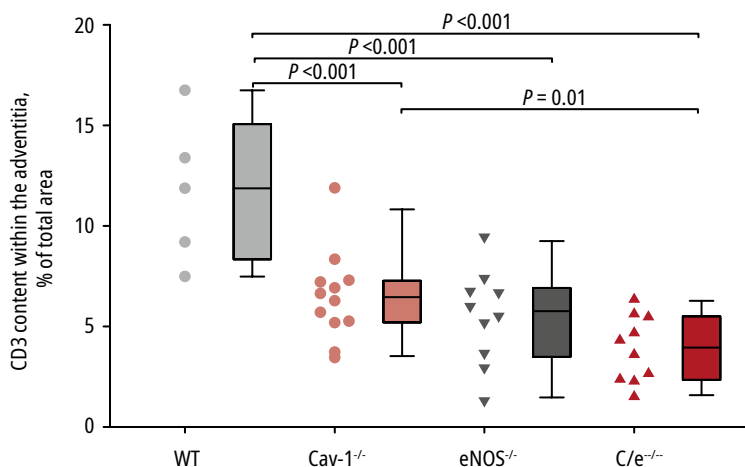


FIGURE 5 A modified whisker plot showing adventitial cluster of differentiation 3 (CD3) expression: adventitial T cells were significantly increased in wild-type transplants (n = 5–12).

Highest α -smooth muscle actin contents in the tunica media of wild-type transplants

It is known that VEGF-A modulates VSMC migration and proliferation in restenosis. Accordingly, the Cav-1^{-/-}, eNOS^{-/-}, and C/e^{-/-} transplants exhibited a significant increase in intimal α -SMA and proliferating cell nuclear antigen levels that corresponded to the decrease in the adventitial VEGF-A content.¹⁷ Furthermore, α -SMA had a significantly higher expression in the tunica media of the WT transplants (mean [SE], 9.0% [4.9%]; n = 6) than in the knockout transplants (minimum, C/e^{-/-} 0.2% [0.1%]; n = 10; maximum, Cav-1^{-/-} 1.9% [0.8%]; n = 9), suggesting a decrease in intimal VSMC migration (FIGURE 3 and Supplementary material, Figure S5).

Increased proportion of neovessels in wild-type grafts

It is known that VEGF-A affects substantial processes in restenosis, such as the development of collaterals, angiogenesis, and vasculogenesis. In contrast, angiogenesis in the intimal formation with resulting neovessels is mainly responsible for lesion instability. The EvG staining in the current study revealed that the WT transplants showed a significant increase in the proportion of neovessels in the intima (mean [SE], 3.42% [1.17%]; n = 6) compared with the knockout aortas (minimum, Cav-1^{-/-} 0.12% [0.09%], n = 12; maximum, C/e^{-/-} 0.34% [0.17%], n = 10) (FIGURE 4 and Supplementary material, Figure S6).

Increased adventitial T cells in wild-type transplants

T cells are known to influence the development of vascular lesions due to the production of cytokines or induction of selective cell death. In our model, WT transplants showed a significant increase in adventitial T cell levels (mean [SE], 11.7% [3.6%]; n = 5) compared with Cav-1^{-/-} (6.5% [2.2%], n = 12) and eNOS^{-/-} arteries (mean [SE], 5.5% [2.4%]; n = 10). The lowest T cell count was found in the C/e^{-/-} aortas (mean [SE], 3.9% [1.7%]; n = 10) (FIGURE 5 and Supplementary material, Figure S7).

DISCUSSION

Previously, we showed (both in vitro and in vivo) an increase in intima formation in the absence of Cav-1 and eNOS due to an increase in neointimal proliferation.^{12,17,26} The main findings of the present study clarify the impact of adventitial processes in the proatherogenic phenotype of these knockouts. Thereby, an increased macrophage-derived inflammation in the Cav-1^{-/-}, C/e^{-/-}, and especially in the eNOS^{-/-} transplants plays an important role. These macrophages were derived from local sources rather than other.¹⁷ Furthermore, the cellular functioning is relevantly affected by the loss of eNOS and, particularly, Cav-1. The knockout transplants showed a significantly

decreased expression of VEGF-A despite an elevated content of macrophages, which are mainly responsible for the adventitial production of this growth factor. Besides the promotion of neointimal proliferation, reduced levels of adventitial VEGF-A were associated with an elevated migration of VSMCs from the tunica media to the intima. On the contrary, the increased VEGF-A level in WT aortas was accompanied by an increased proportion of neovessels.

Several studies have revealed the relevance of intimal processes like migration and proliferation of VSMCs in the pathogenesis of restenosis due to Cav-1 and eNOS expression.^{15,27} Interestingly, eNOS function is highly dependent on Cav-1 expression. Loss of Cav-1 resulted in eNOS uncoupling with an enhanced production of proatherogenic ROS¹² and intimal formation due to increased neointimal proliferation, whereas the double knockout transplants had a decreased number of vascular lesions.¹⁷

Here, we examined the adventitial macrophage-derived inflammation. The eNOS^{-/-} transplants had the highest content of macrophages, caused by the loss of constitutive NO production, which is associated with an increased expression of adhesion molecules (CD54, CD106), integrins (CD11/CD18), and chemotactic proteins such as monocyte chemoattractant protein 1.^{28,29} In contrast, the Cav-1^{-/-} aortas exhibited increased contents of NO and ROS.¹² NO inhibits macrophage migration, whereas ROS increases it.³⁰ In the current study, the NO effect might have been prevalent in the Cav-1^{-/-} arteries. In contrast, reduction of increased adventitial macrophages in C/e^{-/-} compared with eNOS^{-/-} grafts suggests an eNOS-dependent effect on Cav-1. In addition, Cav-1 is known to modulate macrophage differentiation and polarization, influencing its function.³¹⁻³³

For a long time, macrophages were known as cells that primarily migrate from blood to a lesion-site,³⁴ but an increasing number of studies have strengthened the importance of local macrophages.^{35,36} However, macrophage migration might vary according to the genotype of the animal model. In this study, we detected significant differences in adventitial macrophage contents between the different aortic grafts, despite a transplantation procedure that always used the same recipient background, WT mice. In contrast, there was no significant difference in intimal macrophages.¹⁷

Macrophage dysfunction due to loss of Cav-1 or eNOS might be the reason for a decrease in adventitial VEGF-A expression despite an enhanced cellular content. The loss of constitutive NO production in eNOS^{-/-} transplants might be responsible for the lower expression of VEGF-A, due to an increased degradation of hypoxia-inducible factor 1 α (HIF1 α), which is a prominent

transcription factor in VEGF-A regulation.³⁷ Furthermore, NO stabilizes HIF1 α through S-nitrosylation.³⁸ The Cav-1 transplants, which revealed the highest NO concentration,³⁹ challenged this hypothesis because of the decrease in VEGF-A expression. However, these arteries developed the most distinct intima formation, resulting in a local hypoxia with different regulatory mechanisms. Under these conditions, NO activates the degradation of HIF1 α leading to a lower VEGF-A content.⁴⁰ Furthermore, ROS, which are collaterally enhanced in Cav-1^{-/-} aortas, activate VEGF-A transcription, and might be the reason for increased VEGF-A expression in Cav-1^{-/-} transplants compared with C/e^{-/-} aortas.⁴¹

The growth factor is known for inhibiting VSMC proliferation directly via the mitogen-activated protein kinase pathway.⁴² Accordingly, we previously showed an increased neointimal proliferation in Cav-1^{-/-}, eNOS^{-/-}, and C/e^{-/-} transplants, which exhibited decreased VEGF-A expression.¹⁷ In the current study, we showed an increase in the content of α -SMA in the media of WT transplants, suggesting a decreased migration of VSMCs due to an elevated VEGF-A level. However, some studies have described an increase in migration, but not proliferation of VSMCs resulting from downregulation of the VEGF receptors flk-1 and flt-1 during the VSMC phenotypic switch from contractile to synthetic phenotypes.^{43,44} Therefore, the different results regarding the migration might be due to differences in VSMC phenotypes, as the synthetic phenotype predominates in vitro. However, decreased vascular lesions have also been ascribed to Cav-1 deficiency, but these studies always use a murine apolipoprotein E^{-/-} or low-density lipoprotein^{-/-} background.^{45,46} Both models are known for vascular lesions with an extremely high influx of lipids. In contrast, lesions induced by cervical aortic graft transplantation are characterized by intimal proliferation of VSMCs comparable to restenosis after PCI.

Angiogenesis is one of the most important effects of VEGF.⁴⁷ In the current study, an increased proportion of neovessels revealed by the EvG staining coincided with elevated VEGF-A levels in the WT transplants. These intralésional neovascularizations are known for destabilizing intimal formation.⁴⁸ However, the WT aorta developed the smallest vascular lesions. Further studies are warranted to assess whether the neovessels had an atheroprotective function in this model due to better oxygenation of the intima formation, or whether its destabilizing function occurred later in time in association with larger lesions.

Adventitial inflammation due to T cells was pronounced in WT transplants. Most T cell subpopulations are associated with an increase in the number of vascular lesions.²⁰ However,

regulatory T cells pre-eminently show an atheroprotective function.⁴⁹ The CD3 staining was not able to differentiate between the different T cell subpopulations. Further studies are needed to prove the hypothesis of a prevalence of regulatory T cells in our model.

In summary, Cav-1 and eNOS have an inhibiting function in adventitial macrophage-derived inflammation. Whereas the anti-inflammatory effect of the eNOS is NO derived, the loss of Cav-1 results in an increased production of pro-inflammatory ROS due to eNOS uncoupling. Furthermore, Cav-1 and eNOS are crucial for macrophage functioning. Their loss results in a decreased VEGF-A expression that directly affects the proliferation and migration of VSMCs as well as neovessel formation.

SUPPLEMENTARY MATERIAL

Supplementary material is available at www.mp.pl/kardiologiapolska.

ARTICLE INFORMATION

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CONFLICT OF INTEREST None declared.

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