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Alginate hydrogel polymers enable efficient delivery of a vascular-targeted AAV vector into aortic tissue

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Gene therapeutic approaches to aortic diseases require efficient vectors and delivery systems for transduction of endothelial cells (ECs) and smooth muscle cells (SMCs). Here, we developed a novel strategy to efficiently deliver a previously described vascular-specific adeno-associated viral (AAV) vector to the abdominal aorta by application of alginate hydrogels. To efficiently transduce ECs and SMCs, we used AAV9 vectors with a modified capsid (AAV9SLR) encoding enhanced green fluorescent protein (EGFP), as wild-type AAV vectors do not transduce ECs and SMCs well. AAV9SLR vectors were embedded into a solution containing sodium alginate and polymerized into hydrogels. Gels were surgically implanted around the adventitia of the infrarenal abdominal aorta of adult mice. Three weeks after surgery, an almost complete transduction of both the endothelium and tunica media adjacent to the gel was demonstrated in tissue sections. Hydrogel-mediated delivery resulted in induction of neutralizing antibodies but did not cause inflammatory responses in serum or the aortic wall. To further determine the translational potential, aortic tissue from patients was embedded *ex vivo* into AAV9SLR-containing hydrogel, and efficient transduction could be confirmed. These findings demonstrate that alginate hydrogel harboring a vascular-targeting AAV9SLR vector allows efficient local transduction of the aortic wall.

INTRODUCTION

Cardiovascular disease represents a major cause of death in developed countries.[1] With progress in understanding the molecular mechanisms leading to vascular disease, it is possible to advance novel gene transfer approaches that potentially prevent, ameliorate, or even reverse the disease.[2] In comparison to classical curative strategies that require repeated administration of the drug, gene therapy provides a possible long-term correction of genes involved in disease progression.

Vascular gene transfer has been hampered by the lack of efficient vectors to deliver the gene of interest to endothelial cells (ECs) and smooth muscle cells (SMCs). Lentiviruses have shown high transduction efficiency in non-dividing cells, generating stable and long-term expression of the transgene. Targeted lentiviral vectors have been proven to specifically transduce liver endothelial cells upon systemic injection.[3] Additionally, lentivirus-based transgene overexpression was demonstrated in cultured human ECs and SMCs.[4] However, this approach raises major safety concerns, including induction of inflammatory response[5] and the possibility of random integration into genomic DNA of treated cells[6] that hinder clinical applications. Several strategies based on adenoviral vectors have been recently developed; however, strong activation of the immune response remains a critical drawback, leading to transient transduction and cell death.[7] On the other hand, adeno-associated virus (AAV) vectors are increasingly recognized as promising delivery systems for genes of interest because of a remarkable safety profile and long-term efficiency of transgene expression. In addition, different AAV serotypes have been already tested in clinical trials for a broad range of diseases such as Leber's congenital amaurosis, hemophilia, and spinal muscular atrophy, showing safety and remarkable efficacy.[8–10]

Conventional vascular gene transfer frequently requires systemic administration of the viral vector, lowering its concentration and transduction efficiency in the target area, such as particular regions in large arterial blood vessels like the aorta. Furthermore, systemic application of AAV vectors may also result in rare, but severe side effects.[11] Moreover, therapeutic vectors could result in undesired

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off-target effects in smaller vessels of different organs. Therefore, a more precise approach for vector delivery is required for clinical translation. Alginate hydrogels may offer a biocompatible and biodegradable material to effectively deliver therapeutic agents in a controlled manner in the organism.[12] Alginate hydrogels have previously been used as a delivery vehicle for "naked" DNA oligonucleotides,[13,14] synthetic mRNAs,[15] small interfering RNAs (siRNAs),[16] and even viral compounds such as lentiviral vectors and AAVs.[17]

Although AAV9 has been proven to successfully target muscle cells,[18] until now few serotypes have been demonstrated to transduce aortic SMCs, especially *in vivo*.[19–25] Therefore, we previously developed a vessel-directed AAV serotype 9 (AAV9SLR) that markedly improved transduction efficiency of ECs *in vitro* and *in situ*.[25,26]

Here we present a novel approach for efficient local delivery of this vascular-targeted AAV vector to the abdominal aorta of mice.

RESULTS

AAV9SLR does not transduce aortic cells after tail vein injection

We have previously characterized a novel, endothelium-specific AAV9 capsid variant (AAV9SLR) generated by *in vitro* selection of a random peptide library.[25] The resulting modification yielded in a dramatically increased transduction efficiency of both ECs and SMCs in cell culture experiments. To determine whether injection of AAV9SLR can transduce aortic cells *in vivo*, mice were systemically injected with 10^{12} virus particles (vp). Immunohistological analysis proves that 3 weeks after injection no positive signal could be detected in either CD31-positive ECs or SMCs in the tunica media (Figure 1A). In contrast, we could detect enhanced green fluorescent protein (EGFP)-positive cells in the liver (Figure 1B), revealing that transduction was primarily confined to non-desired organs. Quantification of viral genome copies confirmed transduction primarily in the liver, while almost no AAV9SLR vector was found in the aorta (Figure 1C). These data suggest that a local, targeted transduction method is required to successfully achieve transduction of aortic cells and prevent transduction of the liver.

AAV9SLR-containing hydrogels successfully transduce mouse and human SMCs *in vitro*

AAV-containing polymers were generated by directly embedding AAV9SLR vectors into alginate hydrogels. First, the presence of endotoxins in the alginate formulation was tested before polymerization in order to prevent potential side effects both *in vitro* and *in vivo*. As shown in Figure S1, endotoxins could not be detected in our samples. Next, to prove the release of viral vectors from the biomaterial, we incubated the AAV9SLR-containing polymer at 4°C for 24 h in PBS. The resulting supernatant was collected and subsequently added to primary murine SMCs. As shown in Figure 2A, we could detect ~80% EGFP-positive cells 3 days after transduction, suggesting that AAV9SLR remains active and stable after addition to the gels. In line with this, western blot analysis (Figure 2B) revealed EGFP expression under these conditions. Importantly, these results could be further confirmed in human cultured SMCs (Figure 2C).

We next aimed to characterize the time course of vector release. For this purpose, cells were cultured with 10^{12} vp AAV9SLR-containing hydrogel in a Transwell system, with the biomaterial placed on the upper chamber. As shown in Figure 2D, viral genomes could be detected in human SMCs as early as 6 h after incubation, proving successful release and transduction of cells *in vitro*.

Hydrogel implantation into mice allows AAV release and vascular cell transduction

We further tested whether the alginate polymer is suitable for AAV9SLR delivery into the vasculature after implantation into mice. For this purpose, we performed infrarenal implantation of AAV9SLR-containing hydrogel into C57BL/6J mice and analyzed EGFP expression 5 days (early transduction) and 3 weeks (late transduction) after surgery. No hydrogel residuals were observed on visual inspection, neither after 5 days nor after 3 weeks in the area of implantation when organs were harvested. As shown in Figures 3A–3C, immunofluorescence analyses revealed successful transgene expression in 85% ± 7% CD31-positive ECs, calculated as percentage of colocalization of the two fluorescent signals, as well as 93% ± 3% area of the middle coat (tunica media) of treated grafts, while control tissue presented with no immunoreactivity. Moreover, we could demonstrate the presence of AAV vector genomes in DNA extracts of the aortic region treated with AAV9SLR-containing hydrogel at both time points (Figure 3D). We next quantified vector genome copies in non-target organs (kidney, liver, spleen, brain, intestine, serum, and untreated thoracic aorta) in a separate experimental cohort to assess potential off-target transduction (Figure 3E). Importantly, we could detect the transgene in aortic tissue where the AAV9SLR-containing hydrogel was originally applied but not in untreated aortae or in non-targeted organs. These results show that alginate hydrogel can efficiently and specifically deliver AAV9SLR to the vasculature *in vivo*. Importantly, our results prove the presence of the vector in the aortic wall as early as 5 days after implantation, indicating a fast release of AAV9SLR from hydrogels.

Alginate hydrogel does not cause systemic or local inflammation after implantation

To ensure that implantation of the hydrogel does not activate a pro-inflammatory response, we measured the levels of interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) in serum isolated from mice undergoing surgery. As demonstrated in Figures 4A and 4B, ELISA analyses did not reveal any significant changes in the level of the pro-inflammatory cytokines IL-6 and TNF-α in all groups at the early and late time points.

Moreover, immunofluorescence analysis for Mac2 and CD4 as specific markers of macrophages and T cells, respectively, did not reveal the presence of these pro-inflammatory cells in the treated aortae (Figure 4C, positive control shown in Figure S1). Similarly, images of hematoxylin and eosin-stained aortic tissue did not evidence any structural differences caused by hydrogel implantation (Figure 4D).

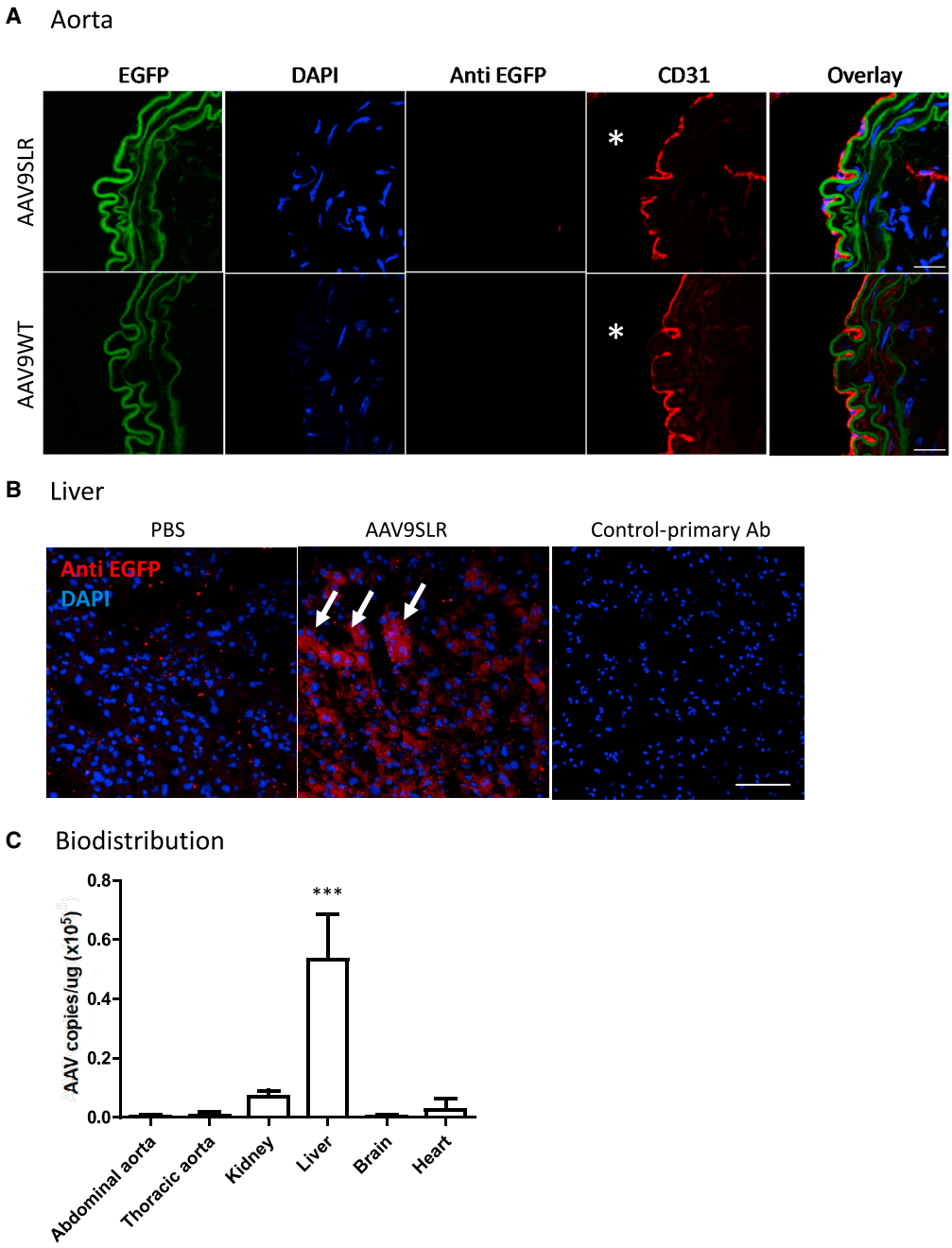


Figure 1. Systemic injection of AAV9SLR does not transduce murine aortic tissue

(A) Illustrative images of aortic cryosections subjected to EGFP immunohistochemistry (purple). Endothelial cells were labeled with a CD31 antibody (red). The lumen is marked with an asterisk. Scale bar represents 12.5 μm. (B) Representative immunohistochemistry images proving liver transduction following systemic AAV9SLR injection. EGFP positive cells are shown in red (marked with arrows), while DAPI (blue) was used as a nuclear marker. (n = 3 mice/group). Sections incubated without primary antibody served as negative controls. Scale bar represents 25 μm. (C) Statistical quantification of viral genome copies in organs harvested from mice subjected to tail vein injection of AAV9SLR (n = 3, ***$p < 0.001$ to all other treatment groups).

In summary, this suggests that implantation of the AAV9SLR-containing hydrogel does not trigger activation of a pro-inflammatory response either 5 days or 3 weeks after surgery.

Another parameter evidencing the safety of biomaterial implantation is the absence of endotoxins prior to implantation *in vivo*. Importantly, we could prove the absence of lipopolysaccharides, suggesting

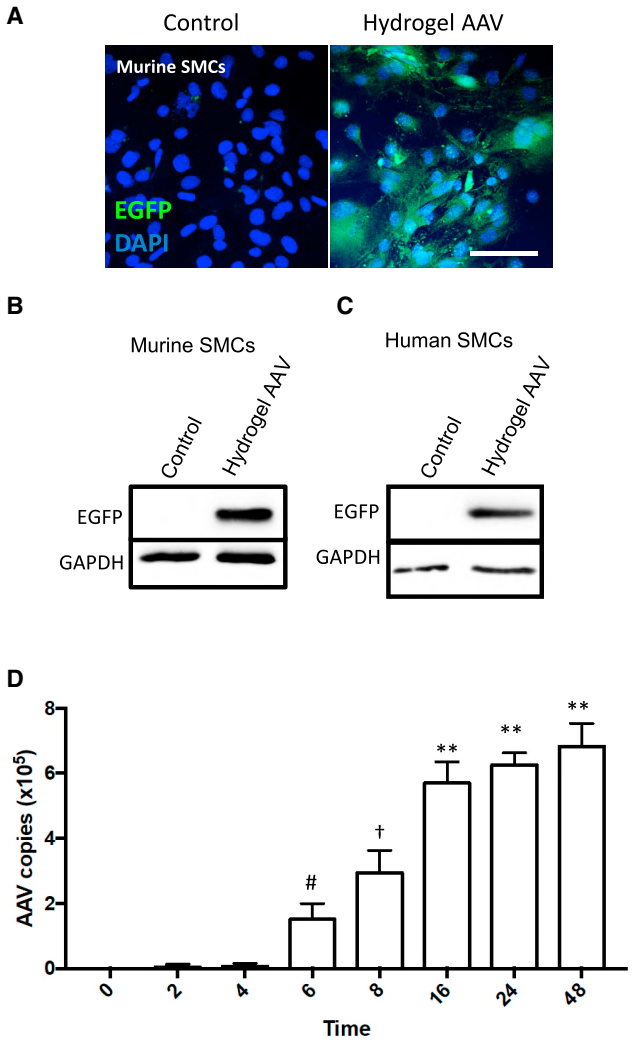


Figure 2. AAV9SLR-containing alginate hydrogel transduces primary aortic murine and human SMCs *in vitro*

(A) Representative images of SMCs 3 days after transduction. Successfully transduced EGFP-positive cells were visualized on the green channel, and nuclei were stained with DAPI (blue). Scale bar represents 50 μm. (B) EGFP levels were additionally analyzed in SMC lysates by western blot 3 days after transduction. Experiments were repeated independently 4 times. (C) Representative image showing western blot analysis of EGFP expression in SMC lysates 3 days after transduction. Immunodetection of GAPDH served as a loading control. (D) The time course of transduction was analyzed *in vitro* by quantitating AAV vector genomes in human SMC at different hours after incubation with AAV9SLR-loaded hydrogels (n = 4 per time point). ***p < 0.001.

that our preparation presents with an excellent safety profile (Figure S2).

Alginate hydrogel-mediated delivery does not prevent induction of neutralizing antibodies against AAV9SLR

We next analyzed whether hydrogel-supported delivery of AAV9SLR would reduce the amount of developed neutralizing antibodies (nAbs) compared with systemic delivery 5 days after gene transfer. Whereas systemic delivery of AAV9SLR resulted in high nAb titers (between 1/1,024 and 1/2,048), nAb response to AAV9SLR hydrogel was between 1/64 and 1/2,048, indicating that there is no clear effect on reducing humoral immunogenicity despite decreased nAb titers in individual mice. Hydrogel without AAV did not result in nAb titers above the detection limit of 1/16 in our assay (Figure S3).

Human aortic tissue is transduced by hydrogel containing AAV9SLR viral particles

To further assess transduction efficiency directly in human vascular tissue, biopsies from aortic aneurysms were embedded into AAV9SLR-containing alginate hydrogels. Immunofluorescence analysis using an anti-EGFP antibody revealed a EGFP signal in 79 ± 4% of the area within the middle coat (tunica media) (Figures 5A and 5B). Moreover, western blot experiments using lysates isolated from human aortic tissue also revealed significant transduction under these conditions (Figure 5C), with an efficiency (in vector genomes per μg DNA) similar to that of the murine aortae *in vivo* (Figure 5D).

DISCUSSION

In the present study, we investigated a novel local delivery approach for vascular-targeted AAV vectors into the wall of the infrarenal aorta by placing an AAV9SLR-containing hydrogel around this vessel. This strategy enabled transgene expression in the endothelial layer and the middle coat (tunica media) and was not accompanied by activation of a significant local or systemic immune response.

Gene delivery to the vasculature could become a valuable strategy for improving or reversing endothelial cell function or ameliorating the pro-inflammatory environment associated with vascular disease progression. Since the first reported vascular gene transfer using a retroviral vector system,[27] retargeted AAV vectors have been developed with considerably improved transduction efficiency of ECs or SMCs.[19,21–25] Nevertheless, the development of vascular gene therapy approaches, in particular of diseases of the aorta or large peripheral arteries, has been still limited by the overall low gene transfer efficiency of vessels *in vivo*.[28] We previously characterized a capsid modification in AAV9 capsid that dramatically enhances transduction efficiency and specificity of vascular ECs and SMCs *in vitro*.[25] However, when systemically injected in mice, AAV9SLR did not lead to transduction of aortic tissue. This could presumably be due to the high blood flow rates in large vessels such as aorta, which do not allow a sufficient time of incubation of target tissue with the vector.[29] Furthermore, because of the integrity of tight junctions and the endothelial barrier in healthy mice, AAVs cannot reach SMCs in the tunica media, which are critical target cells in various vascular diseases. Hence, we have recently established successful transduction of explanted murine aortae with the vascular-targeted AAV9SLR by *ex vivo* immersion into the vector solution and reimplantation into recipient mice,[26] indicating that high local concentrations of this vector facilitate transduction. To further facilitate this method, we aimed to deliver AAV9SLR into vascular tissue by implantation of vector-loaded alginate hydrogels.

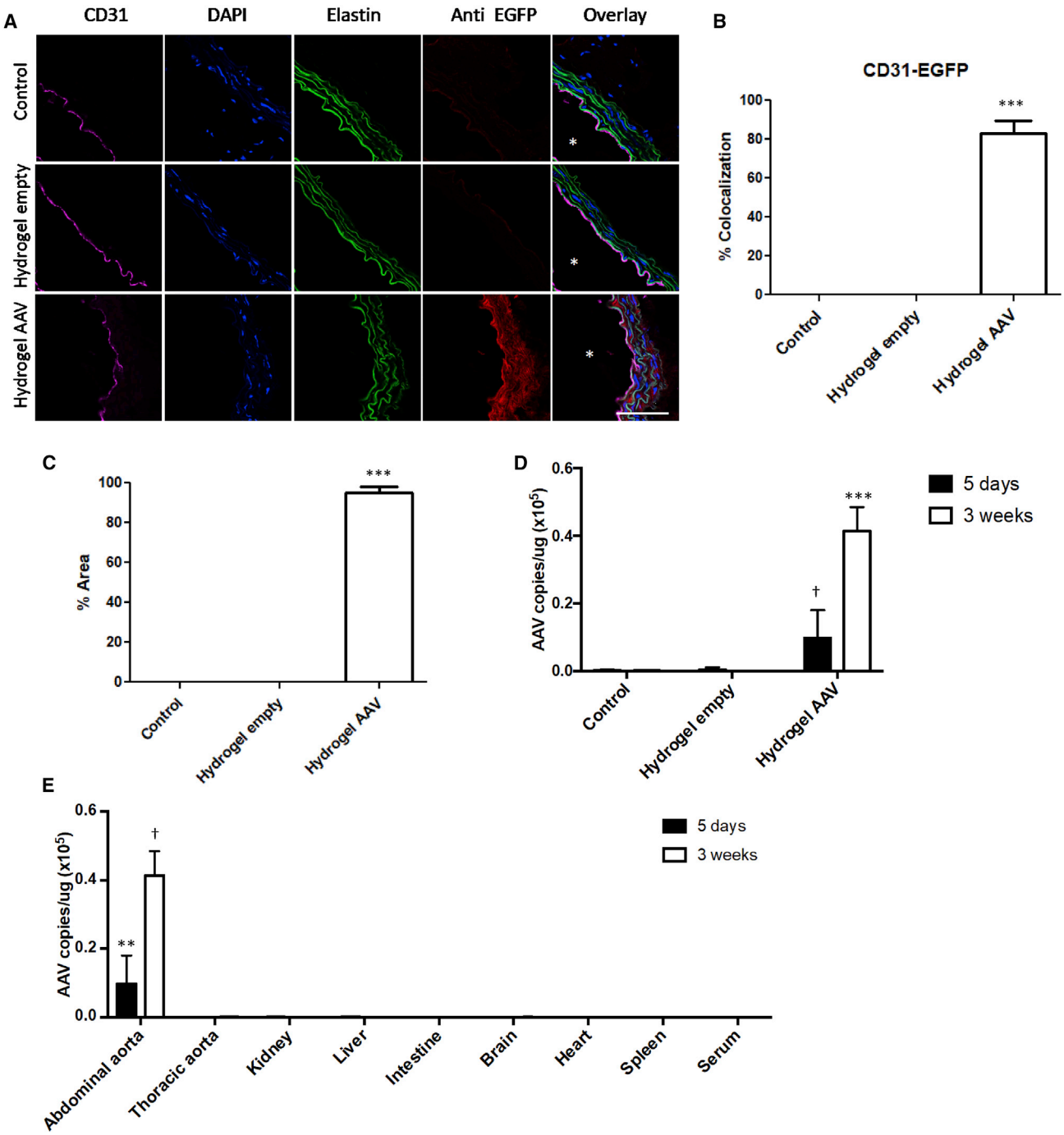


Figure 3. AAV9SLR-containing hydrogel efficiently and specifically transduces aortic grafts *in vivo* after infrarenal implantation into mice

(A) Representative images demonstrating EGFP expression in aortic tissue 3 weeks after surgery. EGFP was detected with a specific antibody (red). Elastin autofluorescence is shown in green, and DAPI (blue) was used as a nuclear counterstain. Asterisks mark the lumen. Scale bar represents 50 μm (n = 5 mice/group, 3 images analyzed/mouse). (B) Statistical quantification of CD31-EGFP colocalization pattern in stained aortic grafts (***$p < 0.001$, one-way ANOVA with Tukey post hoc test, n = 5, 10 images analyzed per group). (C) Analysis of EGFP-positive area within the aortic middle coat (tunica media) (***$p < 0.001$, one-way ANOVA with Tukey post hoc test, n = 5, 10 images analyzed per group). (D) Quantitation of viral genome copies in transduced aortic grafts harvested after 5 days or 3 weeks (n = 5, †$p < 0.01$ to hydrogel AAV 3 weeks and $p < 0.001$ to all other experimental groups, ***$p < 0.001$ to all other treatment groups). (E) Quantitation of EGFP mRNA expression in representative organs (n = 5/organ, **$p < 0.01$ to abdominal aorta 3 weeks and $p < 0.001$ to the other experimental groups, †$p < 0.001$ to all experimental groups, one-way ANOVA with Tukey post hoc test).

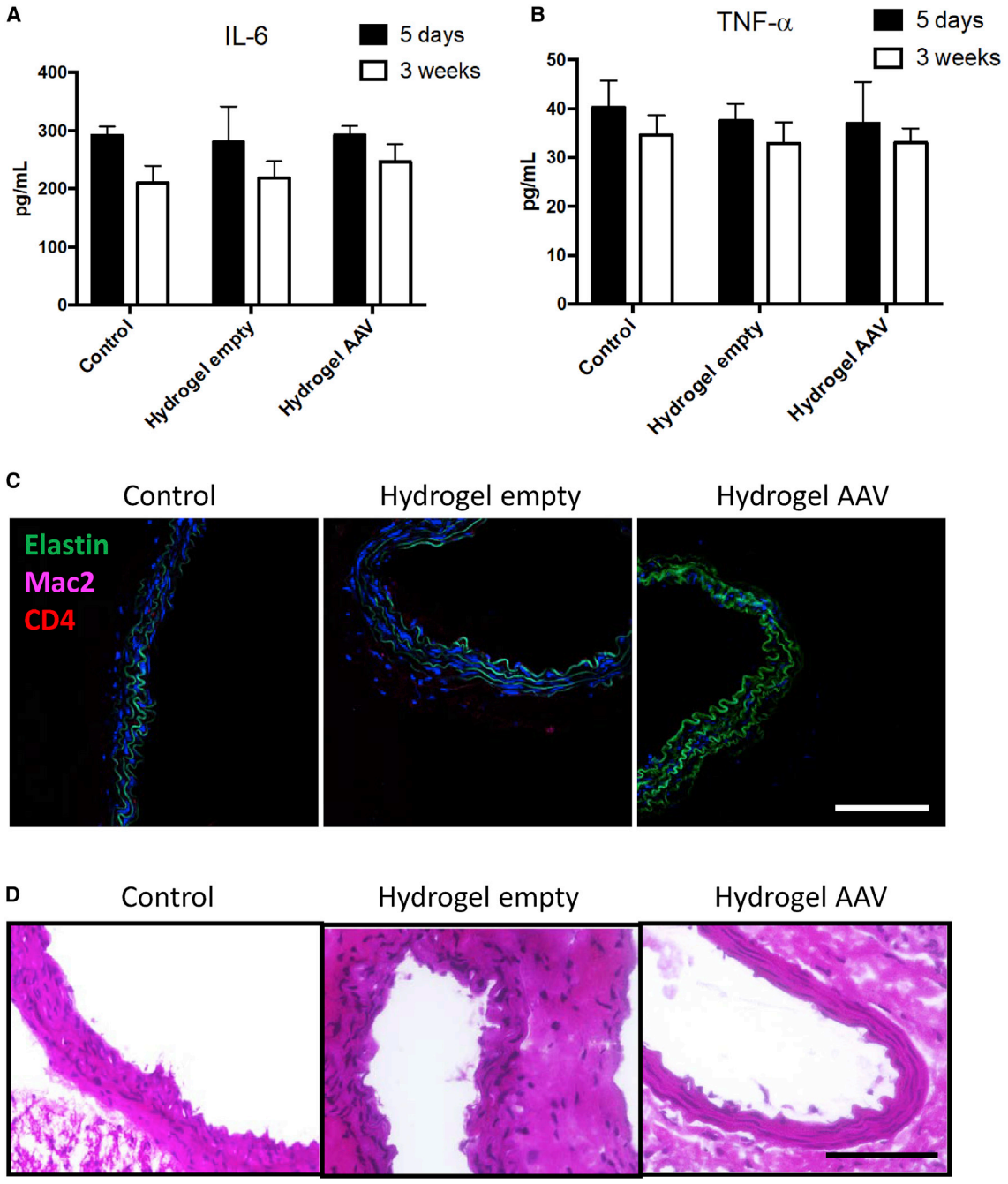


Figure 4. Alginate hydrogel does not cause a vascular or systemic inflammatory response in treated mice after implantation

(A and B) Statistical quantification of pro-inflammatory markers (A) IL-6 and (B) TNF-α in serum after surgery (n = 5 mice/group). (C) Representative images showing CD4- and Mac2-specific immunofluorescence in aortic sections of the respective treatment groups. Note the absence of pro-inflammatory cells in the vessel wall after implantation of the biomaterial. (D) Illustrative hematoxylin and eosin stainings of aortic tissue of the depicted treatment groups. Scale bars represent 12.5 μm (n = 5 mice/group).

As shown here with local delivery using AAV9SLR-loaded hydrogel polymers, EGFP expression is limited to the infrarenal aorta, whereas non-target organs remain non-transduced during the procedure. Thus, the hydrogel approach seems to be superior to intraluminal application by simple intravenous injection because of its increased local AAV concentration and extended contact time with the target tissue. The absence of hepatic transduction suggests low systemic availability of AAV9SLR with hydrogel-mediated delivery.

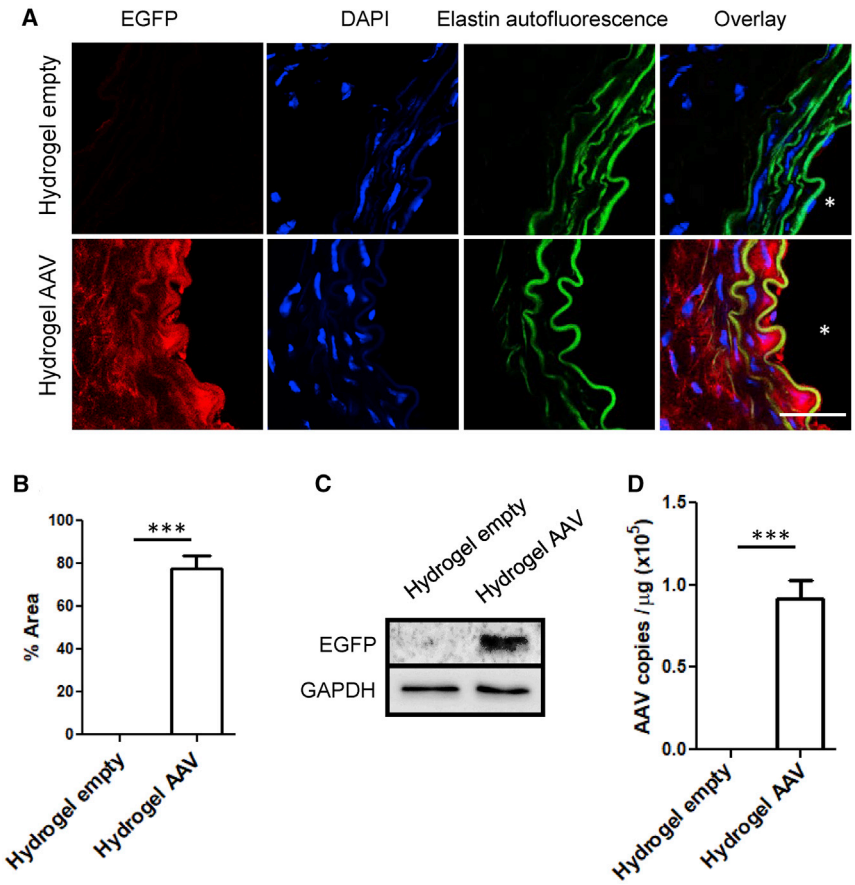


Figure 5. AAV9SLR-containing hydrogel successfully transduces human aortic tissue *in situ*

(A) Representative images of EGFP-specific immunofluorescence (red) 3 days after *in situ* tissue transduction. Elastin autofluorescence was detected on the green channel, while nuclei were stained with DAPI (blue). Scale bar represents 12.5 μm. Asterisks mark the lumen. (B) Analysis of percentage of area of transduced, EGFP-positive cells in human aortic sections. (C) Analysis of EGFP expression 3 days after treatment by western blot. (D) Quantitation of viral genome copies in transduced tissue (*$p < 0.05$, Mann-Whitney U test, n = 5).

Interestingly, hydrogel implantation also resulted in the successful transduction of medial and adventitial aortic cells. In contrast, previous local delivery of viral vectors to blood vessels using intraluminal application systems such as the Dispatch catheter[30] or stents[31] led to high transduction efficiency of ECs, whereas transgene expression in SMCs and adventitial fibroblasts remained low. Nonetheless, accumulating evidence suggests that these cells are major players in pathophysiological vessel remodeling,[32,33] and hence gene delivery to the outer layers of blood vessels might be of advantage.

A critical factor for long-term biocompatibility and functionality of implanted materials is the induction of a pro-inflammatory immune reaction.[34] The *in vivo* host response is characterized by infiltration of activated macrophages, neutrophils, and T cells following implantation,[35–37] potentially leading to biomaterial loss by increased formation of reactive oxygen species and secretion of degrading enzymes. In line with other studies,[38] the developed AAV-containing alginate hydrogel did not induce a significant elevation in serum levels of the pro-inflammatory cytokines IL-6 and TNF-α at early or late time points. Moreover, we could not detect the presence of pro-inflammatory cells in treated aortic grafts, indicating a high biocompatibility of the hydrogel polymer. Additionally, we measured the levels of endotoxin in our alginate preparation before implantation, due to the fact that most commonly used biomaterials present with high affinity to endotoxins, which could induce a strong pro-inflammatory host response and tissue damage in the long term.[39] Importantly, we could not detect endotoxin contaminations in our hydrogels, indicating the absence of potential toxicity in our preparations.

Cellular and humoral immunity induced by AAV vectors play an increasing role in clinical translation.[40,41] As analysis of cellular immunity against AAV capsids in mice is not predictive for patients,[40] we focused on measuring nAbs in serum samples of mice. Interestingly, both AAV9SLR hydrogel implantation and high-titer systemic injections of AAV9SLR resulted in induction of high nAb titers, indicating that humoral immunity is not reduced by hydrogel-supported delivery. Further studies are necessary to investigate whether hydrogel-mediated gene transfer may even shield AAVs from existing nAbs.

Another indicator of the safety of our approach is the absence of hydrogel residuals on visual inspection before harvest of the aortae 5 days and 3 weeks after hydrogel implantation. Although histological sections of the aorta did not reveal any inflammation or hydrogel residuals at the adventitial layer, incomplete biodegradation of the hydrogel cannot be completely ruled out, considering that particles too small for visual inspection might have been lost when the tissue was processed. As hydrogels are placed around the external side of the aorta (adventitia), we have not observed and do not expect any occlusion of the vessel lumen. Targeted delivery, absence of inflammation, and lack of hydrogel residues at least on visual inspection justify further studies investigating hydrogel-mediated AAV9SLR delivery to small and more vulnerable areas such as the aortic root, coronary arteries, or even atherosclerotic lesions, as AAV9SLR transduces proliferating EC and SMC *in vitro*.[25] Further studies are necessary to define the minimal dose of AAV9SLR in hydrogels sufficient for efficient vascular transduction as well as to rule out transduction of other retroperitoneal or mediastinal vessels adjacent to the hydrogel implantation site.

Our pre-clinical study can be further expanded into translational approaches, based on overexpression of disease-relevant genes specifically into the aortic wall in various settings of vascular diseases. For example, various candidate genes have been identified to play major roles in aortic aneurysm progression, and delivery of therapeutic targets into the aortic tissue was proven to be an effective strategy to stop or reverse disease development.[42] Similarly, hydrogel-mediated transduction of carotid arteries with therapeutic transgenes could constitute a feasible gene therapy approach for atherosclerosis.[43] In summary, we describe a novel gene transfer approach to aortic tissue of mice using alginate hydrogel-mediated delivery of a retargeted AAV vector. Future studies are required to assess the feasibility and efficacy of therapeutic gene overexpression in specific vascular diseases via this route of administration. In the long run, this strategy needs to be further validated in a large-animal model to evaluate its potential for further translational approaches.

MATERIALS AND METHODS

Preparation and infrarenal implantation of hydrogels

Alginate hydrogels were prepared as previously described.[17] In brief, 2% sodium alginate solution (Sigma-Aldrich, Munich, Germany, catalog no. PHR1471-1G, lot no. LRAB5524) was prepared in sterile endotoxin-free water under continuous stirring at 37°C for 1 h until no traces of undissolved compound could be detected. Next, AAV9SLR-EGFP was added to a concentration of 10^{12} vp/mL and cast in molds with a thickness of 2 mm and area of 7 × 7 mm. $CaCO_3$ (final concentration 70 mM) and 35 mM glucono deltalactone was used as a polymerizing reagent. Hydrogels were stored at 4°C for a maximum of 24 h until surgery.

All procedures involving the use and care of animals were performed according to Directive 2010/63/EU of the European Parliament and the German animal protection code. Permission was granted by local authorities (G 52/17, Regional Council Karlsruhe, Germany). C57BL/6J mice (5 mice/group) were anesthetized by inhalation of a 5% isoflurane-oxygen mixture and intraperitoneal injection of Temgesic (0.3 mg/mL buprenorphine; 2 mg/100 g body weight). The fur was removed, and the skin was carefully disinfected. Afterward, median laparotomy was performed, and the small and large intestine were mobilized to expose the abdominal vessels. The infrarenal abdominal aorta was carefully separated from the inferior vena cava between the renal branches of the aorta and its branching into the iliac vessels. Next, the alginate gel (7 × 7 mm, 2 mm thickness) was carefully wrapped around the infrarenal aorta ~5–8 mm below the renal branches. Finally, the laparotomy was closed with a layered suture. Sham-operated animals only received a median laparotomy, and the small and large intestine were mobilized. Thereafter the laparotomy was closed with a layered suture. As further controls, adult mice (7–8 weeks) were intravenously injected with AAV9-EGFP or AAV9SLR-EGFP into the tail vein at a dose of 10^{12} genomic particles. Mice were euthanized after 5 days or 3 weeks. After visual inspection for hydrogel residuals, aortae were harvested and embedded into OCT (optimal cutting temperature) medium (Tissue Tek, Leica Biosystems, Nußloch, Germany). In addition, non-target organs such as thoracic aorta, kidney, liver, intestine, brain, heart, and spleen were harvested. Blood was collected and centrifuged at 1,000 × *g* for 10 min at 4°C. The resulting supernatant (serum) was frozen and stored at −80°C. A list of the numbers of animals used in our study is presented in Table S1.

Isolation of primary murine and human SMCs

Vascular tissue was collected from patients undergoing surgical treatment at the University Hospital Schleswig-Holstein, Kiel, Germany (permission no. D495/19). Human and murine SMCs were isolated as previously described, with slight modifications.[44] In brief, aortae were rinsed with sterile PBS, intima was excised, and tunica media was cut into fine pieces of 1–2 mm^2. The tissue was further incubated with a solution containing 0.1% collagenase type 1 (Worthington, Columbus, OH, USA) at 37°C for 3 h under continuous shaking. The resulting cell suspension was filtered, centrifuged, and cultured at 37°C, 5% CO_2. Cells were routinely analyzed for the SMC marker smooth muscle actin (SMA) to ensure high purity of the primary cell culture. Cultured SMCs were used up to passage 5 in subsequent experiments.

Transduction of human aortic tissue

Human aortic tissue was cut into 5 mm^2 pieces and placed into a 2% alginate solution containing AAV9SLR-EGFP (10^{12} vp/mL) to a final volume of 10 × 5 × 5 mm. Afterward, aortae were immersed into a solution containing 70 mM $CaCO_3$ to ensure polymerization of the hydrogel. Aortic tissue was next cultured for 3 days in complete Ham′s medium containing 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific, Darmstadt, Germany). Experiments were performed 4 times, with triplicates used for each repetition.

AAV production

AAV9-EGFP and AAV9SLR-EGFP vectors were generated as previously described with a 3-plasmid transduction system with p5E18-VD-2/9 for wild-type AAV9 or p5E18-VD-2/9-*Sfi*I1759(SLRSPPS) for the vascular-targeted AAV9 capsid variant and pdsAAV-CMV-EGFP for the self-complementary CMV-EGFP vector genome.[25] Vector purification and titration was performed as reported previously.[45]

In vitro transduction of SMCs

SMCs were cultured in 12-well plates until reaching 80% confluency. Next, AAV9SLR-containing hydrogels were added on a Transwell insert containing a permeable membrane with a pore size of 0.2 µm. Cells were harvested at various time points, including 2, 4, 6, 8, 16, 24, and 48 h. Experiments were performed three times, using triplicates for each experimental group.

Western blot analysis

Western blot analyses were performed according to standard protocols. A 30-µg aliquot of protein was loaded into each well. Antibodies against GFP (Cell Signaling, 2956T, 1:1,000) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Sigma-Aldrich, G5262, 1:10,000) were used here.

Immunofluorescence analysis

Immunohistochemistry was performed according to standard protocols. In brief, 5 μm thin sections were fixed with 4% paraformaldehyde (PFA) for 5 min and incubated for 2 h with 2% BSA solution containing 0.02% Triton X-100 to block non-specific binding of antibodies. Primary antibodies against EGFP and CD4 (Cell Signaling, Frankfurt am Main, Germany, catalog nos. 2956S and 25229S, respectively, dilution 1:500), Mac 2 (Dianova, Hamburg, Germany), and CD31 (Santa Cruz, Heidelberg, Germany, catalog no. sc-376764, dilution 1:400) were diluted in blocking buffer and incubated overnight in a humidified atmosphere. Corresponding secondary antibodies (Thermo Fisher Scientific, dilution 1:400) were incubated for 1 h. Sections were stained with DAPI as a nuclear marker. Images were taken with a confocal microscope (LSM800, Zeiss, Oberkochen, Germany) and analyzed with ImageJ (Fiji version 1.51p, National Institutes of Health, Bethesda, MD, USA). Colocalization analyses were performed with the Coloc 2 plugin. Transduced cells in the media were selected by threshold method. Specifically, images were pre-processed to extract positive signal by applying a threshold. Medial area was calculated by manually selecting the region of interest.

Immunocytochemistry

After transduction, primary SMCs were cultured on glass coverslips until they reached 80% confluency. Afterward, cells were incubated with a solution containing 4% PFA and subsequently incubated with a blocking buffer for 2 h. Next, cells were incubated with an anti-SMA antibody (Abcam, Cambridge, UK, catalog no. ab202368, dilution 1:300) for a further 2 h. Imaging was performed with a confocal microscope (Zeiss, Oberkochen, Germany).

Enzyme-linked immunosorbent assay

TNF-α and IL-6 levels in mouse serum were measured by ELISA performed according to the manufacturer's protocol (BioLegend, San Diego, CA, USA). Samples were diluted 1:4 before analysis.

Measurement of neutralizing antibodies

Quantitation of nAbs was performed according to a previously established protocol.[46] Serum was collected when mice were euthanized 5 days after gene transfer (hydrogel implantation or i.v. injection). PBS-injected mice served as controls. 2.5×10^5 HEK293 cells were seeded in each well of a 96 well plate in 50 μL of DMEM with 10% heat-inactivated FBS (complete medium). Cells were further incubated for 4–5 h at 37°C, 5% CO_2. Serum samples were diluted to 1/16 with complete DMEM. Serial dilutions with concentrations ranging from 1/32 to 1/2,048 were performed in complete medium and applied to HEK cells. Complete medium without serum was used as positive control. Non-infected HEK293 cells served as negative control. Next, 1,000 vg/cell of AAV9SLR-CMV-luciferase was mixed with 40 μL of culture medium and incubated with the serum dilutions for 30 min at 37°C, 5% CO_2. Vector-containing serum mix was added carefully on cells in triplicate. After incubation for 48 h at 37°C, 5% CO_2, luciferase was detected with the Bright-Glo Luciferase Assay System (Promega). Percentage of transduction efficiency to non-serum control was calculated according to the formula (average of relative luciferase units (RLU) of sample − average of RLU of negative control)/(average of RLU of positive control − average of RLU of negative control) × 100.

Total DNA isolation and quantitative PCR

Total DNA was extracted from murine or human tissue with the DNeasy Blood & Tissue Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Approximately 5 mg of tissue was used for each sample. Samples were processed as duplicates for each organ except for the target section of the infrarenal aorta where the hydrogel was previously wrapped around. A 25-ng aliquot of DNA was used in each well. Subsequently, quantitative PCR (qPCR) was performed to verify EGFP expression in aortic DNA samples on a CFX96 detection system (Bio-Rad, Munich, Germany) with SensiFAST Green (Bioline, Luckenwalde, Germany). Primer sequences (Eurofins Genomic, Luxembourg) used were as follows: EGFP forward 5′-AGTCCGCCCTGAGCAAAGA-3′, EGFP reverse 5′-TCC AGCAGGACCATGTGATC-3′.

Measurement of endotoxin levels in alginate preparations

Endotoxin presence was assessed with the Pierce Chromogenic Endotoxin Quant Kit (Thermo Fisher Scientific) according to the instructions provided by the manufacturer. Fifty microliters of 2% sodium alginate was used per well.

Statistical analysis

Data are presented as means ± SD and were analyzed for statistical significance with the Mann-Whitney U test for comparing two groups and one-way analysis of variance (ANOVA) with Tukey post hoc test for three or more groups. Graphs were generated with Prism version 5 software. $p < 0.05$ was considered as significant throughout the study.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtm.2021.02.017.

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AUTHOR CONTRIBUTIONS

A.R., N.F., and O.J.M. initially conceived the project. A.R., D.I.B., C.B., S.H., L.K., J.S., and M.Z. performed the experiments under the supervision of A.H.W., M.H., D.F., R.A., G.L., N.F., and O.J.M. C.B. and S.H. produced AAVs, A.R., D.I.B., and T.P. performed the *in vitro* studies, L.K., J.S., and M.Z. operated on and euthanized mice, and A.R. and D.I.B. carried out histological analyses. A.R., C.B., and D.I.B. analyzed data. A.R., M.Z., and O.J.M. wrote the manuscript with input from all authors. All authors approved the final manuscript.

DECLARATION OF INTERESTS

O.J.M. has previously filed a patent application on the vascular-targeted AAV9SLR capsid variant. The other authors declare no competing interests.

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