Unraveling mechanisms of vascular remodeling in arteriovenous fistulas for hemodialysis

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Chapter 7

Deficiency of TLR4 homologue RP105 aggravates outward remodeling in a murine model of arteriovenous fistula failure

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Abstract

Arteriovenous access dysfunction is a major cause of morbidity for hemodialysis patients with a 1-year primary patency of only 60%. The pathophysiology of arteriovenous fistula (AVF) maturation failure is associated with inflammation, impaired outward remodeling (OR) and intimal hyperplasia. RP105 is a critical physiologic regulator of TLR4 signaling, and is expressed on numerous cell types. In the present study, we investigated the impact of differential RP105 expression on AVF maturation, and defined cell-specific effects of RP105 deficiency on macrophages and vascular smooth muscle cells (VSMCs). Overall, $RP105^{-/-}$ mice displayed a 26% decrease in venous OR. *The inflammatory response in $RP105^{-/-}$ mice* was characterized by an accumulation of the anti-inflammatory macrophage phenotype, a 76% decrease in pro-inflammatory macrophages, a 70% reduction in $CD3^+$ T-cells, and a 50% decrease in MMP-activity. *In vitro*, anti-inflammatory macrophages derived from $RP105^{-/-}$ mice were characterized by increased IL10 production, while the levels of MCP1 and IL6 secreted by $RP105^{-/-}$ pro-inflammatory macrophages were elevated, as compared to WT mice. VSMC content in $RP105^{-/-}$ AVFs was markedly decreased. *In vitro*, $RP105^{-/-}$ venous VSMCs proliferation was 50% lower, whereas arterial VSMCs displayed a 50% decrease in migration, relative to WT VSMCs. In conclusion, the marked decrease in venous OR in $RP105^{-/-}$ mice could potentially be the result of a shift in both macrophages and VSMCs towards a regenerative phenotype, identifying a novel relationship between inflammation and VSMC function in AVF maturation.

Introduction

The placement of an arteriovenous fistula (AVF) is currently regarded as the best available option for permanent vascular access in patients requiring chronic hemodialysis. For proper maturation of the AVFs, both a major increase in blood flow and venous diameter are required to allow adequate hemodialysis treatment. However, several clinical trials have shown that the 1-year primary patency rate of AVFs does not exceed 60%, illustrating the fact that the need for further improvement of this access conduit is vital[1,2]. AVF-related complications are frequently encountered shortly after AVF surgery, as 30-60% of the AVF fail to mature adequately to support dialysis therapy[3]. The exact mechanisms that lead to AVF maturation failure remain unknown, but both impaired outward remodeling (OR) and formation of intimal hyperplasia (IH) are regarded as primary contributors to this pathophysiology[4]. Recent studies have shown that the process of vascular adaptation after AVF creation is associated with an excessive inflammatory response[5-8] and proliferation and migration of arterial and venous vascular smooth muscle cells (VSMCs) towards the intima at the site of anastomosis[9-11]. In view of extensive adverse consequences resulting from AVF failure and the subsequent burden for hemodialysis patients, there is increasing emphasis on pathophysiological studies aimed to unravel the complex mechanisms underlying AVF failure. The latter is pivotal in efforts to identify novel molecular therapeutic targets that could potentially improve AVF patency.

Toll-like receptor 4 (TLR4) is a well-known sentry that induces a pro-inflammatory signaling cascade[12]. Its function is modulated not only by exogenous pathogens in the context of microbial infections[13], but also by several endogenous stimuli in inflammatory conditions such as atherosclerosis[14,15] or during vascular remodeling[16-19]. To initiate the TLR4 signaling cascade, activation of its adaptor molecule MD2 is required which is responsible for the recognition of bacterial lipopolysaccharide (LPS) on the cell surface[20]. Due to the hierarchical importance of TLR4 in the innate immune response and its ubiquitous function, the signaling activity of this protein is firmly regulated by several regulatory molecules. One such regulator is RP105 (radioprotective 105, also known as CD180), a cell surface protein expressed by numerous cell types, including inflammatory cells and VSMCs[21,22]. The structure of RP105 is evolutionarily similar to TLR4 and it associates with MD1, a MD2 homologue which promotes RP105 cell surface expression[23,24]. RP105–MD1 exerts dichotomous regulatory activities on TLR4-mediated LPS responses in a cell type-dependent fashion[25]. On B-cells, RP105-MD1 drives cellular proliferation and enhances B-cell-dependent inflammatory processes[26]. In contrast, in myeloid cells, RP105 acts as a natural antagonist of TLR4 signaling[27], while the functional role in VSMCs remains poorly understood. Previous studies from our group have demonstrated that RP105 deficiency results in decreased atherosclerotic lesion formation via alterations on pro-inflammatory B-cells[28] and a CCR2-dependent decrease in monocyte influx[29]. Strikingly, complete opposite effects were observed in a murine model of vein graft disease, where a 90% increase in graft lesion area was linked to a local increase in macrophage content and lesional levels of monocyte chemoattractant protein-1 (MCP1), expressed by VSMCs[21]. In a model of post-interventional vascular remodeling, artery cuff placement in RP105$^{-/-}$ mice resulted in increased neointima formation, which coincided with an increase in arterial VSMC proliferation *ex vivo*[22].

In the context of both AVF maturation and failure, numerous cell types are involved including inflammatory cells and VSMCs from both the feeding artery[10,30] and local venous wall[31], making it a unique model to unravel specific functional consequences of RP105 on remodeling in AVF. In the present study, we aimed to elucidate the role of RP105 on AVF maturation in a murine model of AVF failure by assessing cell type-specific effects of RP105 deficiency, on macrophage polarization and VSMC behavior.

Results

RP105 deficiency influence AVF maturation

To investigate how differential expression of RP105 could influence AVF maturation, we evaluated AVF material two weeks after creating in wild-type (WT) and RP105$^{-/-}$ mice using morphometric and immunohistochemical approaches. RP105$^{-/-}$ mice showed a 26% smaller circumference of the external jugular vein compared to WT mice (P=0.03) (Figure 1a), indicating that RP105deficiency impacts outward remodeling. As shown in Figure 1b, diminished RP105 expression did not influence IH in the venous outflow tract of the AVF. Importantly, immunohistochemical staining revealed that the vast majority of intimal cells are αSMA^{+} in both WT and RP105$^{-/-}$ mice (Figure 1b).

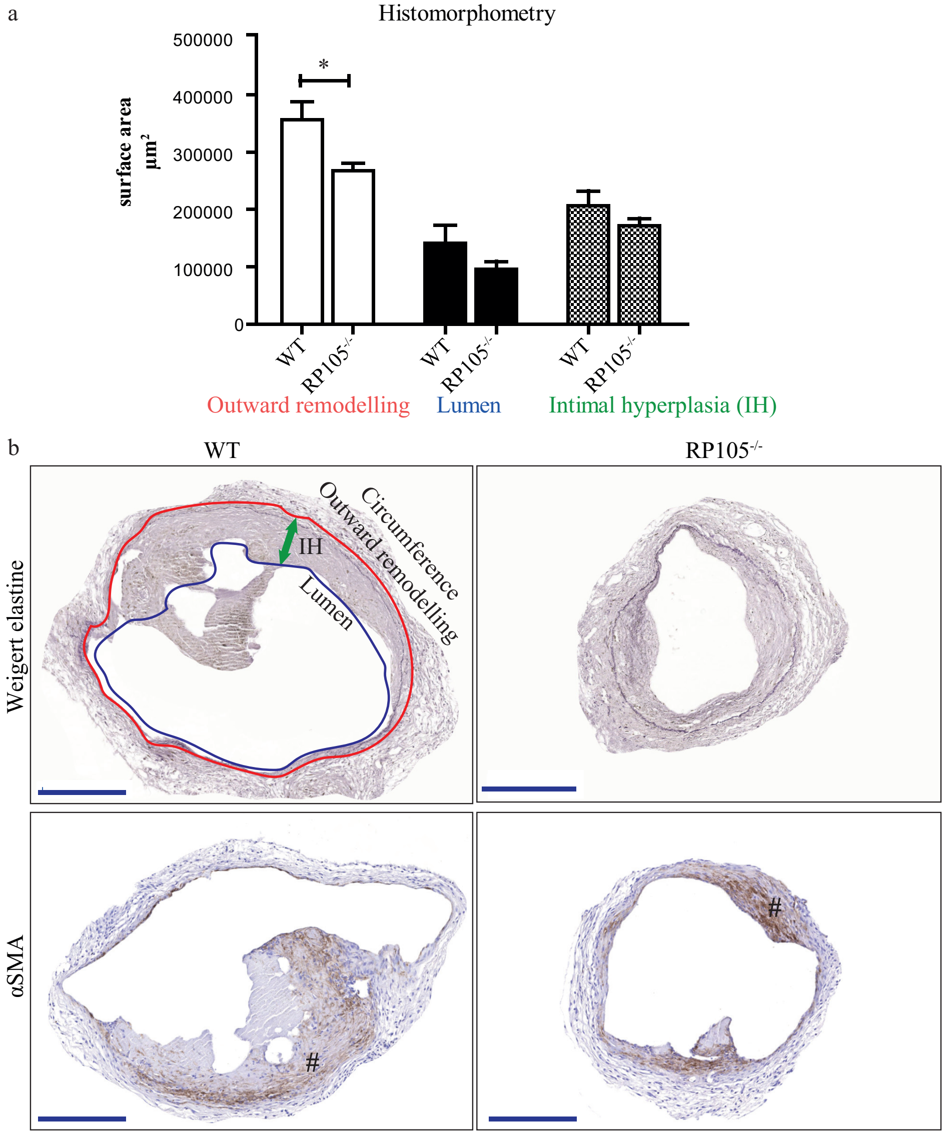

Figure 1. Effect of RP105 deficiency on AVF maturation *in vivo*. (a) Quantification of morphometric parameters. Decrease in vessel circumference (outward remodeling) in RP105 deficient mice was observed 14 days after AVF creation, as compared to WT. Lumen and intimal hyperplasia did not differ between RP105$^{-/-}$ and WT mice. (b) Histological staining of venous outflow tract 14 days after surgery. Weigert elastin staining was used to determine histomorphometrical parameters of the vessel. Circumference (internal elastic lamina area) was used to quantify outward remodeling (red line). Intimal hyperplasia (green arrow) measured as a difference between luminal area (blue line) and vessel circumference. αSMA staining shows area of intimal hyperplasia 14 days after AVF creation. (#) intimal hyperplasia; (*) P<0.05; n=11 per group. Bar=200 µm: 100x magnification.

RP105 deficiency leads to reduced VSMC content in AVF lesions

Given this VSMC enrichment in the intimal region of mature AVF, we sought to determine the proliferation capacity of these cells immunohistochemically. For this, we quantitated the amount of proliferating αSMA$^+$/Ki67$^+$ cells in AVF sections. These studies revealed a 31% decrease in αSMA$^+$/Ki67$^+$ VSMCs in RP105$^{-/-}$ mice, as compared to WT mice (Figure 2a). Since we cannot discriminate between arterial and venous VSMCs *in vivo*, this borderline significance in the number of αSMA$^+$/Ki67$^+$ VSMCs (P=0.07) might be relevant (Figure 2b).

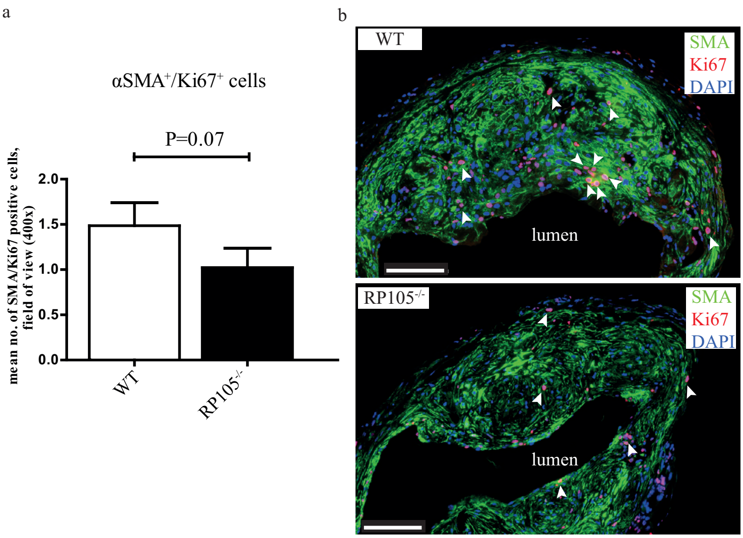

Figure 2. Effect of RP105 deficiency on VSMC proliferation *in vivo*. Quantification (a) and immunofluorescent staining (b) of αSMA+/Ki67+ cells (white arrows) revealed reduction in number of proliferating VSMCs in AVF lesions of RP105$^{-/-}$ mice compared to WT 14 days after AVF surgery. n=11 per group. Bar=100 μm.

Diminution of RP105 differently affects arterial and venous VSMC function

To further dissect the contribution of arterial and venous VSMCs to AVF maturation and failure, we elected to study the consequences of differential RP105 expression in arterial and venous VSMCs *ex vivo*. For this, we cultured explant material from the carotid artery and vena cava of WT and RP105$^{-/-}$ mice for 2 weeks. Morphologically, we observed that arterial VSMCs possessed an elongated phenotype, whereas venous cells had a more stellate appearance (Figure 3a). This phenotypic difference was maintained throughout cell culture, and the vascular origin of the cells was confirmed by assessing EphB4 expression levels, an established embryological marker of venous origin, which was increased in cultured venous VSMCs up to 2 weeks after isolation (Figure 3b).

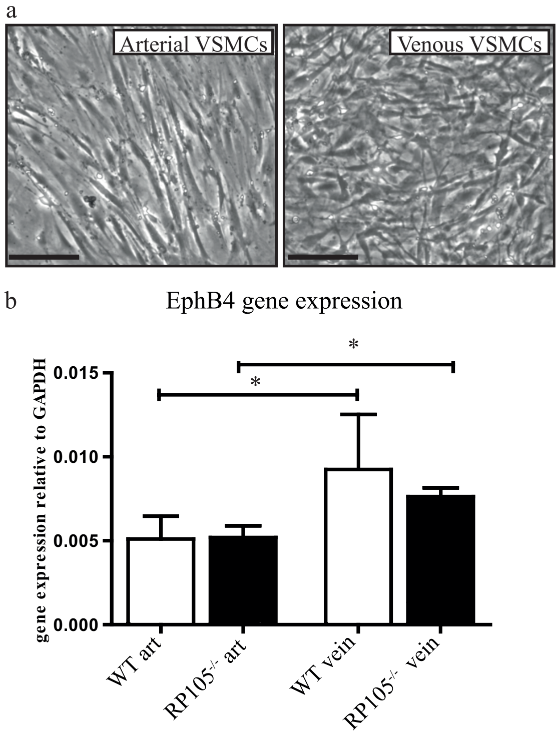

Figure 3. Proliferation and migration of arterial and venous SMCs *in vitro*. (a) A reduction in the proliferative rate was limited to SMCs isolated from RP105$^{-/-}$ veins. (b) Decrease in migration of SMCs isolated from RP105$^{-/-}$ mice was restricted to arterial cells only. Cells were maintained in culture for 14 days. Proliferation and migration were measured over 16 h time period. (*) P<0.05; n=3.

Next, we further determine expression levels of RP105 associating molecules. Interestingly, RT-PCR analysis of RP105 and its accessory molecule MD1 by WT VSMCs also revealed a striking > 100-fold increase in gene expression on venous VSMCs compared to arterial VSMCs (Figure 4a,b). Furthermore, mRNA expression levels of the inflammatory marker TLR4 was also elevated by 48% in venous cells, as compared to arterial VSMCs (Figure 4c). Expression of the TLR4 accessory molecule MD2 did not differ between WT and RP105$^{-/-}$ mice arterial and venous VSMCs (Supplementary Figure 1).

Functionally, venous VSMCs derived from RP105$^{-/-}$ mice displayed a 50% reduction in their rate of proliferation, relative to VSMCs derived from WT mice, while arterial VSMCs proliferation was unaltered (Figure 5a). In contrast, migratory capacity was reduced by 50% over a 16h time period in arterial VSMCs derived from RP105$^{-/-}$, venous SMCs showed no difference in migration between WT and RP105$^{-/-}$ mice (Figure 5b).

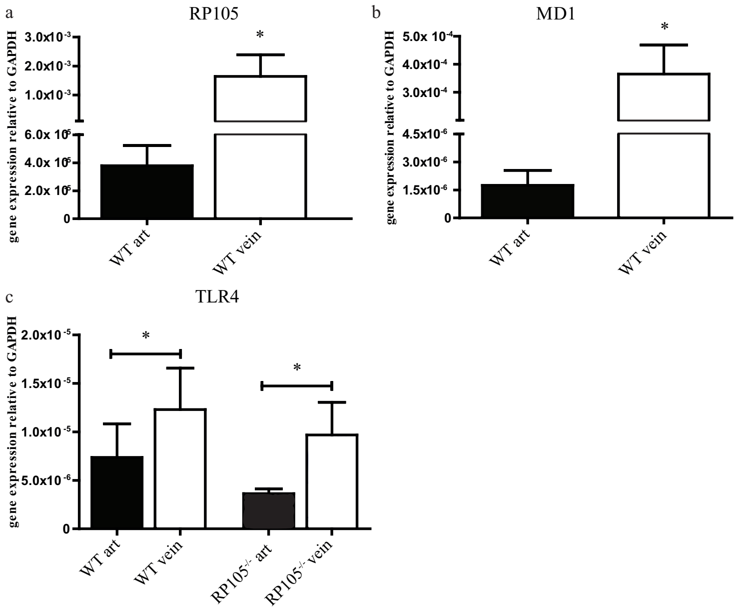

Figure 4. Difference in mRNA expression levels between arterial and venous SMCs *in vitro*. (a) RP105, (b) MD1, (c) TLR4. The relative expression normalized to GAPDH. (*)P<0.05; n=3.

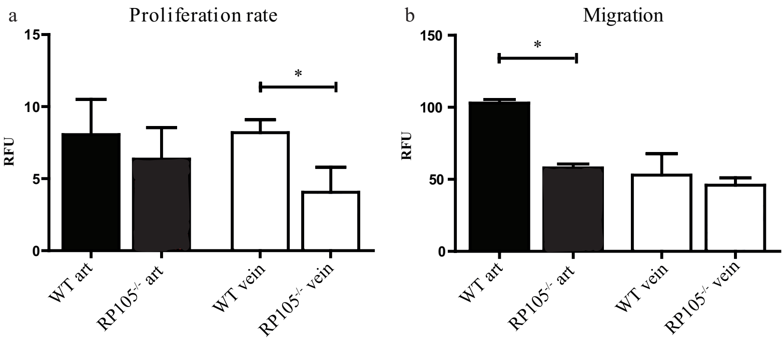

Figure 5. *In vitro* cultured arterial and venous SMCs. (a) Microscopic representation of morphological difference between cultured arterial and venous SMCs. Bar=100μm, x200 magnification. (b) Stable increase in EphB4 mRNA levels was detected in venous SMCs isolated from WT and $RP105^{-/-}$ mice. Cells were maintained in culture for 14 days. (*) P<0.05; n=3.

RP105 deficiency impacts the inflammatory status of AVF infiltrating cells

To gain insight into the consequences of differential expression of RP105 on the inflammatory response to injury *in vivo*, we evaluated the inflammatory cell composition of AVFs in RP105$^{-/-}$ and WT mice. Analysis of AVF material 2 weeks after surgery revealed a 76% reduction in MAC3^{+}/CCR2^{+} pro-inflammatory macrophages cell number in the venous lesions of RP105$^{-/-}$ mice. Furthermore, the number of infiltrating MAC3^{+}/CD206^{+} anti-inflammatory macrophages was increased by 35%, as compared to WT mice (Figure 6a). The number of CD3^{+} T-lymphocytes in RP105$^{-/-}$ mice was decreased by 70% (Figure 6b). Interestingly, we observed an enrichment of these inflammatory cells in the adventitial layer of the vessel (Figure 6a, b). No changes between RP105$^{-/-}$ and WT mice were found in the number of MCP1^{+} cells in the AVF lesions at 2 weeks after surgery (Supplementary Figure 2).

Notably, the distribution of the total population of macrophages in RP105$^{-/-}$ 2 weeks after AVF creation was skewed towards a tissue repair, or regenerative state. More than 90% of all MAC3^{+} cells were CD206^{+}, a cell surface protein that defines the anti-inflammatory macrophage phenotype, whereas but 6% of these MAC3^{+} macrophages were found to express CCR2, the pro-inflammatory macrophage marker.

Macrophage-mediated cytokine production is affected by RP105 expression levels

Having identified that AVFs in RP105$^{-/-}$ mice are enriched for anti-inflammatory macrophages, we subsequently isolated bone marrow from WT and RP105$^{-/-}$ mice and polarized bone marrow-derived macrophages towards either pro- or anti-inflammatory phenotypes with LPS/IFN-gamma or IL4/IL13 treatment for 24h, respectively. We observed an augmented inflammatory response by pro-inflammatory macrophages derived from RP105$^{-/-}$ mice as evidenced by a 40% increase in MCP1 secretion and a 73% up regulation in IL6 production, as compared to macrophages obtained from WT mice (Figure 7a). Macrophages that were isolated from RP105$^{-/-}$ mice and driven towards the anti-inflammatory phenotype exhibited a 72% increase in anti-inflammatory cytokine IL10 production as compared to WT macrophages (Figure 7b).

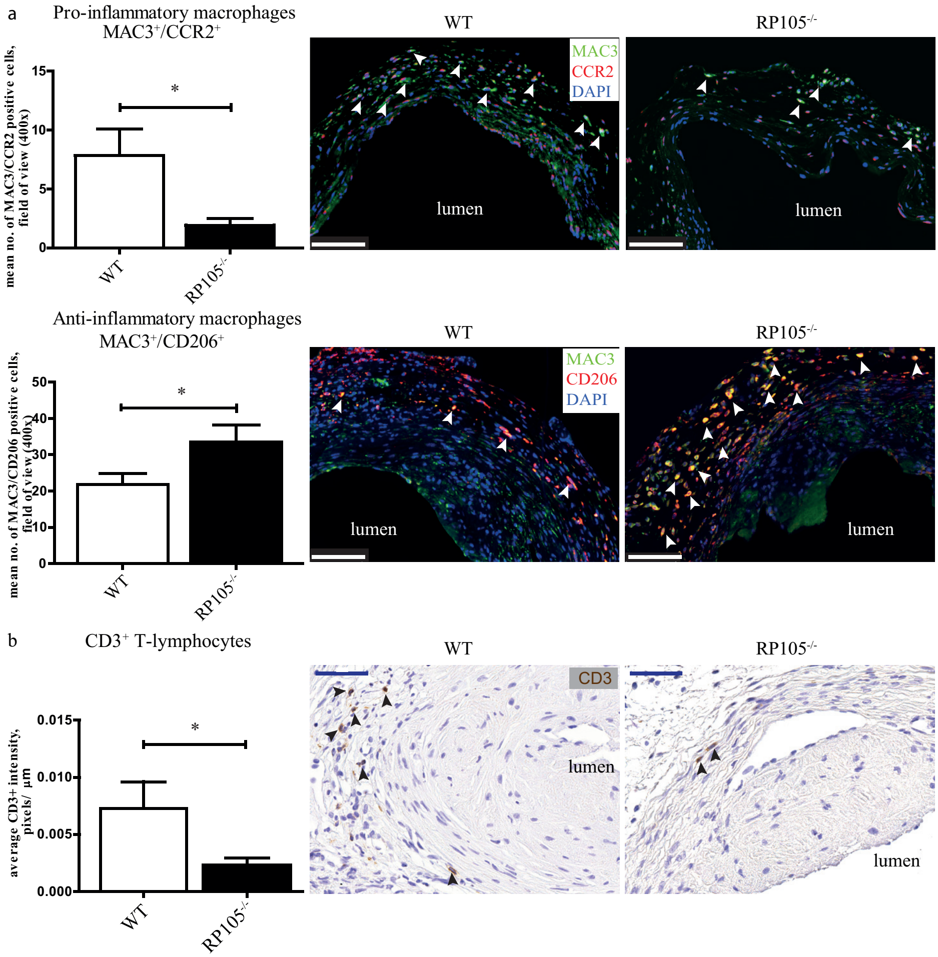

Figure 6. Effects of RP105 deficiency on inflammatory response *in vivo*. Quantification and immunohistochemical staining of (a) MAC3+/CCR2+ macrophages and MAC3+/CD206+ macrophages (white arrows) and (b) CD3+ T-lymphocytes (black arrows) in AVF lesions 14 days after surgery. Decrease in cell number of pro-inflammatory macrophages and upregulation of anti-inflammatory macrophages upon RP105 deletion was observed. Bar=100 µm. Number of CD3+ T-lymphocytes was reduced in RP105$^{-/-}$ as compared to WT. Bar=50 µm: 400x magnification. (*) P<0.05; n=11 per group.

MMP activity is decreased in AVF lesions of RP105 deficient mice

Matrix metalloproteinases (MMPs) are known for the role they play in extracellular matrix (ECM) remodeling, such as collagen and elastin. MMP-mediated degradation of the ECM is critically involved in vascular remodeling following AVF placement and during AVF maturation[32]. We assessed MMP activity in the lesions using *in vivo* near-infrared fluorescent imaging. We observed a two-fold reduction (6.3±1.6 WT vs. 2.9±0.2 RP105$^{-/-}$ AU) in fluorescence intensity indicating reduced *in vivo* MMP activity in RP105$^{-/-}$ mice as compared to WT (Figure 8).

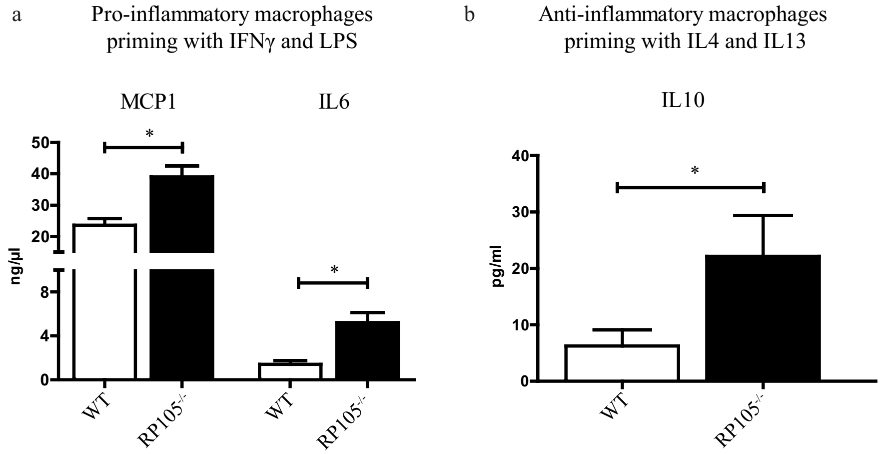

Figure 7. Effect of RP105 deficiency on macrophage function. (a) Bone marrow-derived macrophages from RP105$^{-/-}$ primed towards pro-inflammatory phenotype secrete increased levels of MCP1 and IL6 as compared to WT control mice. (b) Anti-inflammatory macrophages from RP105$^{-/-}$ secrete increased levels of anti-inflammatory cytokine IL10. (*) P<0.05; n=3.

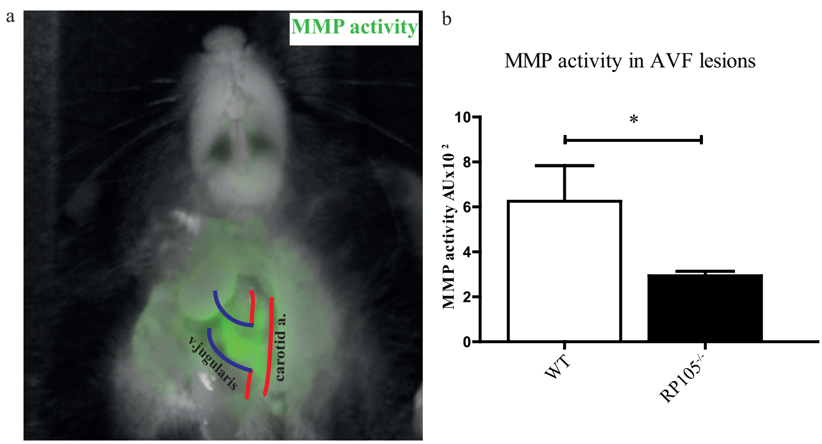

Figure 8. In vivo near-infrared biofluorescent imaging and quantitative analysis of MMP activity. (a) Quantitative analysis of fluorescent intensity revealed a decrease in MMP activity in RP105$^{-/-}$ mice, as compared to WT. (b) Visual representation of near-infrared signal from active MMPs. Accumulation of green color can be seen in the anastomotic region 24 h after injection of MMPSense™ 680 probe. (*) P<0.05; n=4 per group.

Discussion

In this study, we addressed the specific role of TLR4 homologue RP105 in vascular remodeling, inflammation and VSMCs function in a murine model of AVF failure. The process of AVF maturation is complex and integrates several cellular responses, including the infiltration of inflammatory cells shortly after AVF surgery[5,33]. In addition, VSMCs play a pivotal role in AVF maturation as they contribute to thickening of the venous vessel wall and the concurrent outward remodeling. Here, we clearly show that RP105 deficiency affects the inflammatory and VSMC-mediated response to injury during the course of AVF maturation, accumulating in an impaired outward remodeling 14 days after the placement of an AVF.

Contribution of VMSCs proliferation to AVF maturation in RP105 deficient mice

A vital aspect of AVF maturation involves the outward remodeling response, a vessel widening process that is tightly coupled with VSMC proliferation. While VSMC proliferation in IH is generally considered to be detrimental, the process is beneficial for vascular adaptation in AVF, especially in the early phase of AVF maturation. To this end, the reduction in venous outward remodeling in $RP105^{-/-}$ mice, coupled with a reduction in proliferating venous SMC within AVFs and ex vivo, suggests that inhibiting VSMCs proliferation (and migration) could be detrimental for long-term AVF maturation.

Distinct *in vitro* effects of RP105 deficiency on arterial and venous VSMCs

A striking observation in our studies was that RP105 diminution differentially affected arterial and venous SMCs, as evidenced by RP105-specific effects on venous proliferation and arterial migration. The endogenous expression levels of RP105 in arterial and venous SMCs support this finding, along with differential expression profiles of associating TLR4-family members (including TLR4 and MD-1). Collectively, these findings suggest that the susceptibility for inflammatory stimuli could potentially differ between arterial and venous VSMCs. Importantly, our studies support the notion that numerous cell sources are involved in venous IH in AVF (including resident venous cells, infiltrating arterial cells, and circulating bone marrow-derived cells[7,34-37]). Furthermore, our studies illustrate the need for continued investigation of the phenotypic properties and functional characteristics of VSMCs in AVFs, in particular due to the contrasting lineage tracing studies detailing a predominance of arterial SMCs10 versus venous SMCs[31] in venous IH following AVF placement.

RP105 targets both the pro-inflammatory and repair associated arms of the immune system

Increased expression of the pro-inflammatory mediators IL6, TNF and MCP1 are associated with AVF failure[8,38,39], while the reduction of anti-inflammatory molecule heme oxygenase-1 (HO-1) is linked to AVF failure[40-42]. In our study, we demonstrated that the polarization of macrophages isolated from the bone marrow of RP105$^{-/-}$ mice towards the pro- or anti-inflammatory phenotypes appears to remove a regulatory repressor, as both phenotypes displayed an up regulation of signature cytokines being produced. The augmentation of pro-inflammatory cytokine production in RP105$^{-/-}$ macrophages is in keeping with RP105 being an antagonist of pro-inflammatory TLR4 signaling[25,27,43], while the spike in IL10 production is supported by recent reports that low grade inflammation triggers bone marrow-derived macrophages to generate anti-inflammatory cytokines in a TLR4 dependent fashion[44].

AVF placement in RP105$^{-/-}$ mice yielded decreased MAC3^{+}/CCR2^{+} macrophages and CD3^{+} T-lymphocytes. A noteworthy observation two weeks after AVF placement in RP105$^{-/-}$ mice was the attenuation of vessel wall MMP activity. While the type of vascular injury impacts the degree by which MMPs remodel the vascular wall, these factors also play a role in determining which MMPs are activated[45], and could differ between arterial and venous segments. Castier *et al.* reported that increased MMP-9 activity coincided with increased OR in the arterial segment of the AVF[46], while Nieves Torres *et al.* demonstrated that MMP inhibition enhanced venous OR in AVF[47]. Our studies contradict this finding, and suggest instead that decreased MMP limits venous OR in maturating AVFs.

During the process of vascular remodeling the initial pro-inflammatory reaction is gradually changing towards resolution of inflammation characterized by accumulation of anti-inflammatory cells[48-51]. The specific dynamics with regard to pro-/anti- inflammatory response in the context of AVF maturation is still unknown. In our murine model, RP105 deficiency caused significant increase in MAC3^{+}/CD206^{+} anti-inflammatory macrophages in the venous lesions of AVF, compared to controls. Overall prevalence in anti-inflammatory population (93.7%), compared to 6.3% of pro-inflammatory macrophages at 2 weeks after AVF creation might suggest either that in the current model pro-inflammatory response is completed at earlier time points or that anti-inflammatory macrophages play a dominant role in the tissue response in murine AVF. Thus, despite the increased production of both pro- and anti-inflammatory cytokines by macrophages *in vitro*, the effect of RP105 deletion on anti-inflammatory macrophages was dominant in the venous lesions of murine AVF.

Study limitations

Current study is performed in mice, which do not precisely mimic the human inflammatory response to injury; however this model remains highly useful for studying the vital pathophysiological aspects of AVF maturation and failure. Another limitation is the absence of uremia, given that a recent *in vivo* study by Kang et al. demonstrated that fistula maturation in mice is affected by CKD[42]. Here, the chronic accumulation of waste products and uremic toxins in the blood impacted AVF flow, resulting in increased venous wall thickness and thrombus formation. Also, future studies should include flow measurements, as the rate of blood flow is critical functional parameter of AVF.

In conclusion, our study demonstrates the complex role of RP105 in VSMCs and macrophages in a murine model of AVF. The design and implementation of therapeutic strategies targeting the TLR4/RP105 axis to prevent AVF failure must include cell specific targeting approaches and be temporally controlled.

Material and Methods

Animals

Murine model of AVF failure

This study was performed in compliance with Dutch government guidelines and the Directive 2010/63/EU of the European Parliament. All animal experiments were approved by the Institutional Committee for Animal Welfare of Leiden University Medical Center. RP105$^{-/-}$ mice (C57BL/6 background) were obtained from the local animal breeding facility, WT C57BL/6 mice were obtained from Charles River. Adult male mice aged 10-11 weeks were used for the experiments. AVF were created in an end-to-side manner between the dorsomedial branch of the external jugular vein and the common carotid artery as previously described[5,52] (Supplementary Methods S1). The mice were euthanized at 2 weeks after AVF surgery.

In vivo near-infrared MMPs assay

In vivo MMP activity of endogenous MMP-2, -3, -9, -12 and -13 was assessed by injecting fluorescent imaging agent MMPSense™ 680 from PerkinElmer's (Waltham, MA, USA) which is activated in the presence of active MMPs[53]. First, AVF was created as described above (n=4 per group). 14 days later mice were anesthetized under isoflurane and 4 nmol of MMPSense™ 680 probe were injected into the tail vein. 24 hours later, mice were placed under anesthesia, AVF was dissected and mice were scanned using the Optix MX2 optical imaging system. Excitation was performed with a 670-nm pulsing laser, and emission was detected with a 693-

nm long-pass filter. Lifetime analysis was used to confirm the specificity of MMP-activated probes. Fluorescence intensities and fluorescence lifetime were expressed in pseudo colors and projected on the bright field grayscale image of the mouse. Quantification of the fluorescent intensity was performed using the Optiview 2.2 software as described previously[54].

Tissue harvesting and processing

14 days after surgery, the mice were anesthetized using isoflurane whereupon the AVF was dissected. After a thoracotomy, the inferior vena cava was transsected followed by a mild pressure perfusion fixation with 4% formalin through an intracardiac perfusion. The tissue was embedded in paraffin and 5 µm-thick sections of the venous outflow tract were made perpendicular to the vein with an interval of 150 µm.

Morphometric and histological analysis

Morphometric analysis was performed on Weigert's elastin stained sections using ImageJ software. Vessel circumference as a parameter displaying the process of outward remodeling was determined by measuring the length of the internal elastic lamina (IEL). The intimal hyperplasia was calculated by subtracting the luminal area from the area within the IEL.
Immunohistochemical staining was performed for macrophages (MAC3, 1:200, BD-Pharmingen, SanDiego, USA) in a combination with CCR2 for pro-inflammatory phenotype (1:400, Abcam, Cambrigde, UK) or CD206 (1:1000, Abcam) for anti-inflammatory phenotype, T-lymphocytes (CD3, 1:300, Abcam) and VSMCs (αSMA, 1:1000, Dako, Glostrup, Denmark) in a combination with Ki67 (1:200, Abcam) to detect proliferating cells. For the immunohistochemical analysis of the MAC3/CD206, MAC3/CCR2 and αSMA/Ki67 staining, the number of positive cells was counted in 3 random fields of view using a 400x magnification from which the mean was calculated. Quantification of $CD3^+$ cells was performed with ImageJ software by calculating % DAB positive area from the total vessel area. All immunohistochemical quantifications were performed on the first 3 venous sections starting from the anastomosis per AVF. Slides were digitized using an automated microscopic scanner (Panoramic digital MIDI, 3DHISTECH, Hungary). Results are expressed as mean±standard error of the mean.

Cell culture

Vascular smooth muscle cells

Primary arterial and venous vascular smooth muscle cells were isolated from murine carotid artery and vena cava of C57Bl/6 and $RP105^{-/-}$ mice (n=3 per group) respectively. Connective tissues were removed and vessels cut open. Endothelial monolayer was detached by gentle scraping with sterile surgical forceps. The carotid artery and caval vein were dissected into small pieces and plated onto Petri dish 100 mm or 60 mm diameter coated with 0.1 mg/ml fibronectine. After 14 days of culture with DMEM medium supplemented with 20% FCS, 2 mmol/l l-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin, cells were trypsinized and re-plated onto 6 or 12 well plates and left for 7 days in culture. Upon enrichment in 80-90% confluence VSMCs were trypsinized and seeded at required density for further functional assays.

Macrophages

Macrophages were derived from bone marrow by flushing tibia and femur of healthy C57Bl/6 or RP105$^{-/-}$ mice (n=3 per group) and seeded at a density of 500.000 cells/well in 6-wells plates. Cells were cultured for 7 days in RPMI GlutaMax (Gibco) supplemented with 100 U/ml penicillin/streptavidin, 25% Fetal Calf Serum (FCS) and 20 mg/ml M-CSF (Myltec Biotechnologies) as described previously[6]. On day 7, cells were stimulated either with LPS (100 ng/ml) and IFN-gamma (10 ng/ml) to differentiate them towards pro-inflammatory phenotype or with IL4 (10 ng/ml) and IL13 (10 ng/ml) (all from Preprotech) for anti-inflammatory phenotype. After 24 hours the supernatants were collected for ELISA assays and cells were lysed with Trizol reagent (Invitrogen, Carlsbad, CA, USA) for RNA isolation.

VSMC proliferation assay

Murine VSMCs, explanted from aortas and veins of control or RP105$^{-/-}$ mice, were subsequently cultured as described above, and proliferation was measured using neutral red cell proliferation and cytotoxicity assay kit from **Boster Bio (Pleasanton, CA, USA)** according to the manufacturer protocol (Supplementary Methods S2).

VSMC migration assay

Primary arterial and venous VSMCs from control and RP105$^{-/-}$ mice were grown to confluence and then made quiescent in cultured medium supplemented with 1% FCS for 24 hours. Cells were detached from the surface using Accutase Cell Detachement Solution (Innovative Cell Technologies, Inc., San Diego, CA, USA) and suspended at a concentration of 100.000 cells/ml in culture medium supplemented with 1% FCS. Migration was assayed with a polycarbonate membrane inserts having 8 µm-pores in 24-well chemotaxis chambers using commercial CytoSelect Cell Migration Assay Kit (Cell Biolabs, Inc., San Diego, CA, USA) over 16 hours towards the 20% FCS gradient. All migratory cells were lysed and labeled with fluorescent dye (CyQuant GR). Quantification was performed on a fluorescence plate reader at 480nm/520nm.

ELISA assays

ELISA assays for MCP1, IL6 and IL10 production were performed with cell free supernatant collected from bone marrow-derived macrophages after 24 hours polarization towards pro- or anti-inflammatory phenotype using commercial available kits following the instructions of the manufacturer (BD Biosciences, San Jose, CA, USA: MCP1- Catalog No 555260; IL6- Catalog No 555240, IL10- Catalog No 555252).

RT-PCR

Total RNA was extracted from the macrophages and VSMCs using Trizol reagent (Invitrogen) according to the manufacturer's protocol. RNA was reverse transcribed using a 5-minute 65°C incubation of 1 μg total RNA with deoxyribonucleotide triphosphates (Invitrogen) and random primers (Invitrogen). c-DNA was synthesized using an M-MLV First-Strand Synthesis system (Invitrogen), and used for quantitative analysis of mouse genes (Supplementary Table 1) with an SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA). The relative mRNA expression levels were determined by normalization to murine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) using $2[-\Delta\Delta C(T)]$ method.

Statistical analysis

Results are expressed as mean±SEM and considered statistically significant for $p<0.05$; t tests and Mann-Whitney tests for parametric and nonparametric data, respectively, were used as appropriate. All *in vitro* experiments were performed in biological n=3 in experimental triplicates.

Acknowledgements

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