Toll-Like Receptor 4 Is Involved in Human and Mouse Vein Graft Remodeling, and Local Gene Silencing Reduces Vein Graft Disease in Hypercholesterolemic APOE*3Leiden Mice

Jacco C. Karper, Margreet R. de Vries, Ben T. van den Brand, Imo E. Hoefer, Jens W. Fischer, J. Wouter Jukema, Hans W.M. Niessen, Paul H.A. Quax

Objective—The goal of this study was to explore the role of Toll-like receptor 4 (TLR4) in vein graft remodeling and disease.

Methods and Results—First, expression of TLR4 was analyzed in freshly isolated human saphenous veins (huSV), in freshly isolated huSV ex vivo perfused in an extracorporeal circulation, or in huSV used as coronary vein grafts. Marked induction of focal TLR4 expression was observed in perfused fresh huSV. Moreover, TLR4 was abundantly present in lesions in fresh huSV or in intimal hyperplasia in coronary vein grafts. Second, mouse venous bypass grafting was performed. In grafts of hypercholesterolemic APOE*3Leiden mice, increased TLR4 mRNA and protein was detected over time by reverse transcription–polymerase chain reaction and immunohistochemistry. Furthermore, the local presence of the endogenous TLR4 ligands heat shock protein 60, high-mobility group box 1, tenascin-C, and biglycan in the grafts was demonstrated. TLR4 deficiency in C3H-Tlr4dLPS-d (LPS indicates lipopolysaccharide) mice resulted in 48±12% less vein graft wall thickening (P=0.04) than in Balb/c controls. Moreover, local TLR4 gene silencing in hypercholesterolemic APOE*3Leiden mice using lentiviral short hairpin RNA against TLR4 administered perivascularly around vein grafts led to a 44±13% reduction of vessel wall thickening compared with controls (P=0.0059).

Conclusion—These results indicate that TLR4 is involved in vein graft remodeling and can be used as a local therapeutic target against vein graft disease. (Arterioscler Thromb Vasc Biol. 2011;31:00-00.)

Key Words: gene therapy ■ vascular biology ■ inflammation ■ Toll-like receptor ■ vein graft

Vein grafts are important conduits for revascularization during both coronary and peripheral bypass surgery, although vein grafts sometimes have poor long-term patency. Early graft failure is usually due to thrombosis, but long-term graft failure is caused by vein graft disease (VGD) induced by wall thickening due to intimal hyperplasia that is triggered by inflammation. Vein graft wall thickening is in part characterized by smooth muscle cell (SMC) proliferation, matrix turnover, and an influx of lipids and inflammatory cells.1,2

The innate immune system contains multiple receptors that recognize a broad variety of molecular structures. Toll-like receptors (TLRs) have a key role in driving inflammation regulating the innate response after binding pathogen-associated molecular patterns or damage-associated molecular patterns (DAMPs), respectively.1–7 TLR4 is a membrane-bound receptor located on a variety of immune and nonimmune cells, including macrophages, endothelial, and SMCs. Cell stress and tissue damage may cause a release of DAMPs that function as endogenous TLR4 ligands and lead to activation and upregulation of the expression of TLR4, resulting in a proinflammatory response.6,8–10 The role of TLR4 in vascular remodeling, especially arterial remodeling, has previously been demonstrated in various studies either directed at atherogenesis and plaque (de)stabilization or directed at the role of TLR4 in postinterventional arterial remodeling after angioplasty.11–16 However, the role of TLR4 and its endogenous ligands in remodeling of venous segments (eg, in vein graft remodeling) is still unknown. Furthermore, it has been suggested that unactivated arterial vascular smooth muscle cells (VSMC) do not express TLR4 and that TLRs may have a vessel-specific profile that may also vary because of changes in cellular activation, differentiation, and other local processes.17 Interestingly, nonactivated VSMC from huSV do express TL4.18
To modulate atherosclerotic plaque formation by interfering in the TLR4 pathway, systemic therapeutic interventions would be required. However, because of its important role in the host defense mechanism, such a systemic approach would be undesirable. In contrast, venous bypass grafts do permit local therapeutic interventions. From the point of therapeutic interventions in the TLR4 pathway to reduce vascular remodeling VGD is of more interest and has more potential. Local therapy against VGD can be done easily, for example, by topical inhibitor application to the adventitial layer, because adventitial cells contribute extensively to proliferation and migration of SMC and subsequent vein graft remodeling. Therefore, local gene transfer to silence TLR4 expression would be an interesting approach for therapy to improve graft survival, once the role of TLR4 in VGD has been established.

In the current study, we focus on the role of TLR4 in vein graft remodeling by illustrating the presence and upregulation of TLR4 and its endogenous ligands in both human and murine venous segments used for vein grafting. Moreover, a causal role of TLR4 was studied by performing vein grafting in TLR4-deficient mice (C3H-Tlr4LPS-d mice; LPS indicates lipopolysaccharide) or by local TLR4 gene silencing in murine vein graft using a lentiviral short hairpin RNA (shRNA) construct against TLR4. The latter study was performed in hypercholesterolemic APOE*3Leiden mice to mimic the situation of vein grafting in hypercholesterolemic patients as closely as possible.

Materials and Methods

For a detailed description of all materials and methods used, see the supplemental material, available online at http://atvb.ahajournals.org.

In brief, immunohistochemistry for TLR4 was performed on paraffin-embedded sections of human saphenous vein (huSV) segments, freshly isolated, freshly isolated and ex vivo perfused in an extracorporeal circulation for 4 hours, or used for at least 5 years as coronary vein grafts. Also, murine vein graft segments were analyzed for TLR4 expression and for its endogenous ligands heat shock protein 60 (HSP60), high-mobility group box 1 (HMGB1), tenascin-C, and biglycan.

Lentiviral shRNA vectors against murine TLR4 were established based on the Mission library (Sigma-Aldrich).

Vein grafting in mice was performed by placing a venous interposition (vena cava) in the carotid artery of either Balb/c mice, TLR4-deficient (C3H-Tlr4LPS-d) mice, or hypercholesterolemic APOE*3Leiden mice. In the APOE*3Leiden mice, grafts were treated locally with the lentiviral short hairpin TLR4 (shTLR4) construct.
Results

TLR4 Presence in huSV and Coronary Vein Grafts

Human vein graft was obtained in the operating room during coronary artery bypass grafting procedures. One segment was directly taken for histological and immunohistochemical examination, and the remaining segment was placed in a perfusion circuit that was connected to the heart-lung machine. Samples were perfused with autologous whole blood, with a pressure of 60 mm Hg (nonpulsatile flow). After 4 hours of perfusion, segments were taken for histological and immunohistochemical examination. Furthermore, vein grafts that served as coronary arterial bypass grafts for more than 6 years were derived at autopsy. To study the presence of TLR4 during remodeling in huSV, TLR4 expression was analyzed in the freshly isolated huSV, in preexisting intimal lesions within these segments and the coronary vein grafts. In the fresh huSV, TLR4 expression could be detected, especially in the circular SMC layer (Figure 1A). Also, the endothelial layer and adventitial vessels stained positive for TLR4 (not shown). Some of the venous segments contained spontaneous lesions, indicated by intimal hyperplasia formation. These lesions represent a focal area of spontaneous remodeling and demonstrated a marked presence of TLR4 (Figure 1B). In addition, a profound TLR4 expression was observed in severely remodeled coronary vein grafts with a saphenous vein origin (Figure 1C). These grafts were obtained at autopsy. They had been in situ for more than 5 years and are representative sections of remodeled vein grafts.

Focal TLR4 Expression After Graft Perfusion

In vein grafting, early damage to the vessel wall is initiated immediately by surgical manipulation and after transplantation because of increased shear stress and wall tension initiated by increased perfusion pressure. Nonperfused fresh huSV showed few TLR4-positive cells in the subendothelial longitudinal muscle layer (Figure 2A). Parallel sections of the same fresh huSV were subjected to 4 hours of perfusion with autologous blood on arterial pressure. These sections showed an impressive focal increase of TLR4 expression within the longitudinal layer (Figure 2B).

Figure 3. TLR4 expression on murine vein grafts of hypercholesterolemic APOE*3Leiden mice was detected by immunohistochemistry and reverse transcription–polymerase chain reaction. Analysis over time was done on grafts harvested at t=1 (A), 3 (B), 7 (C), and 14 (D) days after surgery. Rabbit IgG isotype control (E). Arrows indicate positive TLR4 staining. Reverse transcription–polymerase chain reaction of TLR4 (F) CD68 (G), and α-smooth muscle actin (H) mRNA was performed at t=0 hours, 6 hours, and 1, 3, 7, and 28 days after surgery. A Student t test was used for statistical analysis; *P<0.05 compared with t=0.
TLR4 Expression in Murine Vein Grafts During Remodeling

As the findings presented above indicate an increase of TLR4 presence during huSV remodeling, TLR4 presence on the protein and mRNA level was analyzed during remodeling in mice. Therefore, a murine vein graft model was used that represents human vein graft thickening and accelerated atherosclerosis. TLR4 protein presence was studied by immunohistochemistry during progression of remodeling over time in hypercholesterolemic APOE*3Leiden mice. A marked expression of TLR4 was detectable in these vein grafts. In the early remodeling phase, when the graft was only 1 or 2 cell layers thick, TLR4-positive spots were observed (Figure 3A and 3B). At t=7 and t=14 days, vessel wall thickness has increased markedly. Within these segments, focal areas of intense TLR4 protein expression could be detected (Figure 3C and 3D). Rabbit IgG isotype control showed no staining (Figure 3E). Expression of TLR4 mRNA during remodeling was detected by reverse transcription–polymerase chain reaction in hypercholesterolemic APOE*3Leiden mice over time at t=0 hours, 6 hours, and 1, 3, 7, and 28 days after graft placement. A rapid increase in TLR4 mRNA could be detected in the early remodeling phase. During intermediate and late-phase remodeling, TLR4 mRNA remained upregulated (Figure 3F) (t=0 versus 6 hours, P=0.03; t=0 versus 1 day, P=0.0039; t=0 versus 3 days, P=0.02; t=0 versus 7 days, P=0.1; t=0 versus 28 days, P=0.09). The increase of TLR4 expression may be partly related to an influx of 68+ cells (t=0 versus 6 hours, P=0.6; t=0 versus 1 day, P=0.0037; t=0 versus 3 days, P=0.0026; t=0 versus 7 days, P=0.0032; t=0 versus 28 days, P=0.0003) during remodeling and proliferation/migration of SMC (t=0 versus 6 hours, P=0.07; t=0 versus 1 day, P=0.0007; t=0 versus 3 days, P=0.17; t=0 versus 7 days, P=0.02; t=0 versus 28 days, P=0.006; Figure 3G and 3H).

Presence of Endogenous TLR4 Ligands in Murine Vein Grafts

Remodeled grafts have been subjected to proliferation, migration, and turnover of cells and matrix components. These processes may cause an upregulation of DAMPs that can be recognized by TLR4. We localized several of these DAMPS in vein grafts of hypercholesterolemic APOE*3Leiden mice harvested at t=14 days. Proteins in response to cellular stress, such as HSP60 and high-mobility group box 1 (HMGB1), are known TLR4 ligands. The presence of HSP60 was detected, especially in the subendothelial layer of the thickened vessel wall (Figure 4A). HMGB1, normally at rest inside the nucleus, was now detected in the cytoplasm, where it is known to act in a cytokine-like way (Figure 4B). In addition, matrix components that are expressed during matrix turnover were studied. The glycoprotein tenasin-C was demonstrated, especially at the matrix-rich adventitial site of the graft but also near the luminal site in matrix-containing areas (Figure 4C). These endogenous ligands could potentially colocalize with TLR4 because they were present in the lesion site and in areas that also express TLR4 at the same time point (Figures 3D, 4A, 4B, and 4C). Furthermore, mRNA expression of the matrix component biglycan, which may function as a TLR4 ligand, was studied over time, and a more than 10-fold increase of mRNA was detectable after 7 days. This increase in biglycan expression remained upregulated during the remodeling process (t=0 versus 6 hours, P=0.25; t=0 versus 1 day, P=0.017; t=0 versus 3 days, P=0.21; t=0 versus 7 days, P=0.004; t=0 versus 28 days, P=0.021; Figure 4D).

Functional Role of TLR4 in Murine Vein Graft Remodeling

To explore whether TLR4 has a functional role in vein graft thickening, an essential step in VGD leading to vein graft failure, a venous interposition was placed in the carotid artery of Balb/c (n=8) and TLR4-deficient (C3H-Tlr4<sup>LPs-d</sup>) mice (n=7). C3H-Tlr4<sup>LPs-d</sup> mice showed 48±12% (0.264±0.06 versus 0.136±0.02 mm<sup>2</sup>, P=0.04) less wall thickening than Balb/c controls (Figure 5A). Graft patency was kept by compensatory outward remodeling in the Balb/c mice, indicated by a significantly larger total vessel wall area (0.60±0.03 versus 0.42±0.05 mm<sup>2</sup>, P=0.01) (Figure 5B).
thereby creating only a small difference in the ratio of lumen to total vessel wall area (Supplemental Figure I). Furthermore, the reduced vessel wall thickening correlated with a reduced α-SMC-actin positive area in the C3H-Tlr4<sup>ps-d</sup> mice (0.022±0.003 versus 0.047±0.009 mm<sup>2</sup>, P=0.035) (Figure 5C).

**TLR4 Silencing In Vitro**

To create a tool for local TLR4 gene silencing, 5 lentiviral-based shRNAs were produced and validated by measurement of murine TLR4/MD2 expression on CHO cells by fluorescence-activated cell sorting analysis, and the best vector was selected (not shown). The selected vector showed a dose-dependent downregulation of TLR4 protein expression (Supplemental Figure II). Murine 3T3 fibroblasts were then transduced by selected lenti-shTLR4 (TRCN0000065787), lenti-control, or PBS and subsequently stimulated with different proinflammatory stimuli to obtain insight on its specificity in reducing TLR4 induced nuclear factor-κB (NF-κB) activation. Lenti-shTLR4 gave a significant reduction in NF-κB activation after administration of TLR4 ligand LPS (P<0.01). Application of Pam3Cys, tumor necrosis factor-α, or interleukin-1β had no different effect on NF-κB activation compared with the controls (Supplemental Figure III).

**Local TLR4 Silencing in Hypercholesterolemic APOE<sup>*</sup>3Leiden Mice**

To validate the potential of TLR4 as a local therapeutic in VGD, a study was pursued using local TLR4 gene silencing to diminish VGD in APOE<sup>*</sup>3Leiden mice. These mice, when fed a high-cholesterolemic diet, are well known to develop massive VGD because of neointima formation and accelerated atherosclerosis. After graft placement in APOE<sup>*</sup>3Leiden mice pluronic gel with PBS (n=8), lenti-control (n=9) or lenti-shTLR4 (n=7) was lubricated around the graft. Mice were fed a Western-type diet for the total duration of the experiment, starting 3 weeks before surgery. No significant differences between groups in cholesterol level (PBS 9.6±0.9 mmol/L, lenti-control 9.7±1.0 mmol/L, and lenti-shTLR4 10.2±0.6 mmol/L) or body weight were observed (not shown).

Local gene silencing of TLR4 led to a 44±13% reduction of vessel wall thickening in the graft segment (PBS, 0.40±0.04 mm<sup>2</sup>; lenti-control, 0.42±0.04 mm<sup>2</sup>; lenti-shTLR4, 0.23±0.03 mm<sup>2</sup>) (PBS versus lenti-shTLR4, P=0.0059; lenti-control versus lenti-shTLR4, P=0.0052; PBS versus lenti-control, P=0.96). A difference in total vessel wall area was found only between lenti-control and lenti-shTLR4. In addition, a beneficial lumen/total cross-section ratio showed a beneficial outcome for the lenti-shTLR4-treated group (PBS, 0.45±0.05 mm<sup>2</sup>; lenti-control, 0.54±0.04 mm<sup>2</sup>; lenti-shTLR4, 0.70±0.03 mm<sup>2</sup>) (PBS versus lenti-shTLR4, P=0.0012; lenti-control versus lenti-shTLR4, P=0.0079; PBS versus lenti-control, P=0.14) (Figure 6A to 6D). Furthermore, although quantitative interpretation of immunohistochemistry has to be done with extreme caution, treatment with lenti-shTLR4 gave a reduction in focal TLR4 expression in the graft (Figure 6E). After TLR4 silencing in the vein grafts of APOE<sup>*</sup>3Leiden mice, the areas positive for macrophages (MAC3-positive area) and SMC (α-SMC-actin-positive area) were reduced by 54% and 61%, respectively, compared with the PBS group. (Details are given in Supplemental Table I.)

**Discussion**

The present study describes the role of TLR4 in vein graft remodeling in humans and mice with a profound presence of TLR4, endogenous TLR4 ligands during remodeling, and the therapeutic potential for local TLR4 silencing. Abundant TLR4 presence was noticed in freshly isolated huSV. In addition, in remodeled areas of fresh huSV, in fresh huSV after reperfusion, and in coronary vein grafts derived at...
autopsy, an upregulation of TLR4 protein was observed. In murine vein grafts of hypercholesterolemic APOE*3Leiden mice, TLR4 expression is present over time at the protein and mRNA levels, and a series of endogenous TLR4 ligands, HSP60, HMGB1, tenascin C, and biglycan was expressed. Furthermore, TLR4-deficient mice show a significant reduction in vein graft thickening and less outward remodeling after vein grafting. Moreover, local gene silencing of TLR4 gives a clear reduction in vein graft thickening and a beneficial lumen/total cross-section area ratio.

Nowadays, TLR4 is considered to be an important factor in inflammatory-mediated diseases. VGD is strongly mediated by remodeling initiated by wall stress, cell damage, and inflammation. The level of TLR4 expression may vary among different vessel specimens and is dependent on activation and local environmental changes, such as wall stress, cell damage, and inflammation. Furthermore, these differences may also be relevant for the presence and levels of endogenous TLR4 ligands. Therefore, it is of major importance to evaluate TLR4 expression, function, and therapeutic potential in a VGD-related setting. Here, we report the presence of TLR4 on the circular muscle layer of fresh huSV and a rapid increase of focal TLR4 expression within 4 hours of reperfusion in an extracorporeal circulation set up with arterial pressure on sections of fresh huSV. Increased TLR4 expression may be initiated by damage-inducing processes, such as ischemia/reperfusion and increased wall tension/shear stress due to pressure and flow disturbances. In addition, the initiated damage to the vessel contributes, most likely by inducing the release of endogenous ligands, to the proliferation and migration of VSMC and the influx of inflammatory cells, such as macrophages, resulting in thickening of the vessel wall. These cells, which play an important role in vascular remodeling, have been shown to express TLR4. In support of this, we noticed an abundant TLR4 presence within the thickened vessel wall of severely

Figure 6. Vein graft remodeling after local TLR4 silencing in hypercholesterolemic APOE*3Leiden mice (PBS, n=8; lenti-control, n=9; lenti-shTLR4, n=7). Areas of vein graft sections were quantified by using 6 sequential sections per vein graft vein graft of each mouse. A mean of these 6 sections was used as the outcome of vein graft remodeling per mouse. Outcomes of analysis are expressed in millimeters squared (mean ± SEM). Vein graft wall thickening was reduced in the lenti-shTLR4 treated group compared with the controls (A). Total vessel wall area was smaller in the lenti-shTLR4 group compared with the lenti-control (B). A beneficial lumen/total cross-section area ratio was observed in the lenti-shTLR4 treated group (C). Shown are representative HPS-stained sections of focal vein graft wall thickening (D). Focal TLR4 expression is shown in vein grafts locally treated with PBS (left), lenti-control (middle), and lenti-shTLR4 (right) (E). *P<0.01. Statistical analysis was performed with a 1-way ANOVA.
remodeled areas in vein grafts of human and mouse origin. Previously, localization of TLR4 has been shown in remodeled arteries during atherogenesis and after angioplasty, and the presence of TLR4 ligands may alter its expression.3,4,5,6,7,8

The discovery that these TLR4 ligands can be of endogenous origin further emphasizes the importance of TLR4 in inflammatory-mediated diseases.9 The endogenous ligands, most often referred as DAMPs, are most often danger signals or degradation products of matrix component that become available in response to injury. Bypass surgery itself and the subject of the transplanted vessel to hypoxia and increased blood pressure can cause a release of powerful inducers of cellular stress and tissue damage. The observed presence of HSP60, extracellular high mobility group box 1 (HMGB1), tenascin-C, and upregulation of biglycan mRNA during remodeling is a good example of this. Previously, Hochleitner et al described the release of HSP60 after shear stress,10 and others showed the capability of HSP60 to bind directly to TLR4, thereby initiating proliferation of VSMC.11 Cellular stress may also cause passive release of HMGB1 by a variety of cell types, including endothelium and monocytes/macrophages, and acts as a cytokine capable of binding to TLR4, thereby initiating a proinflammatory response.12,13,14 Matrix turnover importantly mediates vein graft remodeling. The extracellular matrix glycoprotein tenascin-C is related to tissue repair and injury. Normally, tenascin-C is not present in huSV, but after graft placement, its expression is significantly increased in huSV grafts.15 If present, it is capable of promoting SMC migration16 and growth17 and affecting important genes in vascular remodeling. Recently, tenascin-C was found to be an endogenous TLR4 ligand without the need for CD14 or MD-2 as accessory molecules for signal transduction, and therefore tenascin-C may act in vein grafts also via TLR4 signaling.18 The matrix component biglycan is communoprecipitated with TLR4, and by signaling via TLR4 on macrophages it has a proinflammatory effect. Moreover, it enhances SMC proliferation.19,20 The observed increase in biglycan expression over time in the vein graft points toward an increased TLR4 signaling in murine vein grafts.21,22

The reduced vein graft wall thickening in the C3H-Tlr4-/- mice by reducing total SMC amount demonstrates that not only are TLR4 and its endogenous ligands expressed in the remodeling vein graft segments but TLR4 is also causally involved in vein graft remodeling.

The current results indicate the presence and even causal involvement of TLR4 and the upregulation of multiple endogenous ligands in VGD; therefore, several new targets can be proposed for therapeutic interference. Long-term systemic antiinflammatory treatment against TLR4 would be difficult and undesirable because of the expected adverse side effects. Furthermore, targeting of a single DAMP would be not a good option because multiple DAMPs are upregulated and are able to trigger TLR4 signaling involved in vascular remodeling, making it difficult to select them as therapeutic targets individually. Local RNA interference for TLR4 may overcome this problem. shRNA constructs are able to inhibit gene translation into protein, thereby initiating effective gene silencing.39,40

Previously, our group showed that local graft transduction using a lentivirus can be performed efficiently in vivo.41 In support, others have shown encouraging results for the applicability of local gene therapy.42,43

Therefore, local lentiviral infection with the selected TLR4 shRNA was performed in vein grafts positioned in the carotid artery of hypercholesterolemic APOE*3Leiden mice. The specificity of the shRNA construct used was demonstrated by its capacity to decrease murine TLR4 expression on transformed CHO cells and to reduce NF-kB activation in murine fibroblasts after LPS activation. NF-kB activation after challenging the cells with stimuli other than the TLR4 ligand LPS, such as Pam3Cys, tumor necrosis factor-α, or interleukin-1β, was not affected, demonstrating the specificity of the construct.

Morphometric analysis after silencing of TLR4 demonstrated a reduction in vessel wall thickening, which was supported by a focal decrease in TLR4 expression and a reduction in the total number of macrophages in the lesions. As in the TLR4-deficient mice, reduction of total SMC in the lenti-shTLR4 treated group correlated with the decrease in vessel wall thickening. Furthermore, local TLR4 silencing increases the ratio of lumen versus total cross-sectional area. This indicates a beneficial effect of local TLR4 silencing on vein graft remodeling, with effects on graft patency in the long term.

This also might have significance for the treatment of patients because vein grafts can readily be treated locally ex vivo during surgery, thus presenting a unique opportunity for gene transfer or gene inhibition to alter the remodeling response.

In summary, upregulation of TLR4 expression (in both human and mouse), as well as its endogenous ligands, in vein grafts during graft remodeling and the reduced vein graft wall thickening in TLR4-deficient C3H-Tlr4-/- mice point out the important involvement of TLR4 and its endogenous ligands in VGD. Furthermore, the reduction of vein graft vessel wall thickening in hypercholesterolemic APOE*3Leiden mice after local lentiviral shRNA-mediated interference in the TLR4 pathway indicate the potential of TLR4 as a local therapeutic target to improve vein graft survival.

Disclosures
None.

References


Toll-Like Receptor 4 Is Involved in Human and Mouse Vein Graft Remodeling, and Local Gene Silencing Reduces Vein Graft Disease in Hypercholesterolemic APOE*3Leiden Mice
Jacco C. Karper, Margreet R. de Vries, Ben T. van den Brand, Imo E. Hoefer, Jens W. Fischer, J. Wouter Jukema, Hans W.M. Niessen and Paul H.A. Quax

Arterioscler Thromb Vasc Biol. published online February 17, 2011;
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2011 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://atvb.ahajournals.org/content/early/2011/02/17/ATVBAHA.111.223271

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2011/02/17/ATVBAHA.111.223271.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
**Supplement Material**

**Materials and Methods**

**Mice**

All animal experiments were approved by the animal welfare committee of our institute. 10 week old male Balb/c, C3H-\(Tlr4^{LPS-d}\) and APOE*3Leiden mice, bred in our laboratory, were used. C3H-\(Tlr4^{LPS-d}\) mice have a Balb/c background but carry a mutation that causes a deficiency in TLR4 signaling.

APOE*3Leiden were fed a western-type diet (Arie Blok, The Netherlands), starting 3 weeks before surgery and continued during the experiment to develop a diet dependent hypercholesterolemia.

All mice received water and food ad libitum. One week before surgery cholesterol levels in serum were determined (Roche Diagnostics, The Netherlands).

**Production of lentiviral shRNA against TLR4**

Out of the “Mission Library” (Sigma Aldrich, The Netherlands) 5 sequence-verified short hairpin RNA (shRNA) lentiviral-plasmids against TLR4 were isolated and produced. Lentiviral packaging vectors (pCMV-VSVG, pMDLg-RREgag/pol, pRSV-REV) and transfer vector (pLKO.1-puro Vector) were co-transfected in HEK293T cells using calcium-phosphate precipitation method. Lentiviral particles were concentrated using sucrose cushion centrifugation (30,000rpm, 2h). Quantification of lentiviral titer was done by p24 ELISA (ZeptoMetrix, USA).

The vectors encoded the shRNAs sequences against TLR4 (lenti-shTLR4) as indicated:
In vitro targeting of murine TLR4 expression

CHO cells expressing murine TLR4/MD2 and control cells (kindly provided by Dr. A. Garritsen, Merck Sharp Dome, The Netherlands) were transduced with the 5 different lentiviral based shRNAs in a 0.1:1, 0.5:1, 1:1 and 2:1 ratio (lentiviral particles/cells) in DMEM (Invitrogen, The Netherlands) containing 0.8ug/ml polybrene (Sigma Aldrich, The Netherlands) with 5% Fetal Calf Serum. After overnight transduction medium was replaced by fresh medium. Subsequent TLR4 expression was measured by FACS analysis with a TLR4-MD2 FITC antibody (HyCult biotechnology, The Netherlands) after 5d.

Transduction of murine 3T3 fibroblasts:

NIH 3T3 mouse fibroblasts stably transfected with a 5xFκB-Luciferase vector as described 1. After overnight adherence, cells were transduced with, lenti-control (lentivirus-GFP) or lenti-shTLR4 (TRCN0000065787) per well (MOI of 7.5) or PBS. Medium was discarded after 4h transduction and replaced by fresh medium. Cells were cultured for 5d followed by stimulation with LPS, TNFα, Pam3Cys, or IL1β. After 6h stimulation, cells were washed with 0.9% NaCl and lysed for 15min on ice. Bright-Glo was added and luminescence was measured on the Lumistar Optima.
**Murine vein graft model**

In the mouse carotid artery a venous interposition was placed as described previously\(^2\). Caval veins of donor mice were placed as vein grafts in the right carotid artery of recipient mice.

**Lentiviral transduction of the vein graft in situ**

Before wound closure, in the APOE*3Leiden mice, 150\(\mu\)l 20% pluronic gel (Sigma Aldrich, The Netherlands) containing 1.5\(\times\)10\(^6\) lentiviral particles (P24 Elisa, ZeptoMetrix, USA) and 0.8\(\mu\)g/ml DEAE (Sigma Aldrich, The Netherlands) was lubricated around the graft.

**Vein graft thickening quantification**

Mice were sacrificed 28d (or as stated otherwise) after surgery for histological analysis. Vein graft segments were harvested after perfusion fixation with 4% formaldehyde, fixated overnight and paraffin-embedded using an automated tissue processor (Leica, Germany). Cross-sections were made throughout the embedded vein grafts. Six representative sections per vessel segment were stained with Haematoxylin-Phloxine-Saffron (HPS) for histological and morphometric analysis (Leica, Germany). Vein graft thickening was defined as the area between lumen and adventitia and determined by subtracting the luminal area from the total vessel wall area.

**Human vein graft tissue**

Veins were surplus segments of huSVs that were collected in the operating room from patients undergoing coronary artery bypass grafting (CABG). Vessel specimens were
freshly collected under sterile conditions. One part of the segment was directly fixated for histopathological examination and another part was perfused on an extracorporeal perfusion circuit connected to the heart-lung machine during the CABG procedure with autologous blood and under arterial pressure (60mmHg) as described by Stooker\(^3\). The study was approved by the local ethics committee. Patients were included in the study after providing informed consent. Non-perfused fresh huSV segments (n=6 patients) and parallel fresh huSV segments that were perfused for 4h respectively (n=6 patients) were analyzed huSV segments that have served as coronary bypass for more than 5yrs (n=5 patients) were derived at autopsy. Use of patient material after completion of the diagnostic process is part of the patient contract in the VU University Medical Centre.

**Immunohistochemistry**

Paraffin embedded sections (5µm) were deparaffinized in xylene. Peroxidase activity was blocked by incubation in 0.3% (v/v) H\(_2\)O\(_2\) in methanol for 20min. Antigen retrieval was performed and tissue sections were pre-incubated with 5% bovine serum albumin (BSA), followed by incubation with detecting antibody.

TLR4 presence was stained with TLR4 antibody (sc-10741 rabbit anti-human, Santa Cruz, United States)\(^4\), HSP60 (rabbit anti-mouse, Abcam, UK), HMGB1 (rabbit anti-mouse Abcam, UK), Tenascin C (rabbit anti-mouse, Millipore, USA), SMCs with \(\alpha\)-smooth muscle cell actin staining (Roche Applied Biosciences, Germany) and macrophages with MAC3ab (BD Pharmigen, USA).

After washing in PBS, sections were incubated for 1h with a secondary antibody (Donkey anti-Rabbit, GE Healthcare, USA), washed in PBS, incubated for 1h with AB complex
(Vector laboratories, The Netherlands) and visualized with Novared (Vector laboratories) or DAB (Dako, Denmark). Slides were counterstained with haematoxylin. Immunopositive areas of SMC and macrophages were calculated as percentage of total vein graft area in cross-sections by morphometry (Leica, Germany). To increase the TLR4 detection after AB complex slides were incubated for 10 minutes with biotinylated thyramids and again 1h with ABcomplex prior to visualization with Novared.

As control parallel sections were incubated with 1% PBS/BSA alone or with Rabbit IgG isotype without adding detecting antibodies. Controls were all negative (not shown).

**RT-PCR:**

Total RNA was isolated using Tri-Reagent (Sigma-Aldrich) according to the manufacturer's protocol. The expression levels of TLR4 and Biglycan were analyzed by RT-PCR (real time polymerase chain reaction). The relative mRNA expression levels were determined by using GAPDH as house keeping gene and the $2^{-\Delta\Delta C(T)}$ method. Values were expressed as fold of respective controls.

**Statistical analysis**

Values are presented as mean±standard error of the mean (SEM). Statistical significance was calculated in SPSS for Windows-17.0. Differences between groups were determined using a non parametric Mann-Whitney and One-way ANOVA (Kruskal-Wallis, non-parametric Dunn’s Multiple Comparison Test) tests. A Student’s t-test was used for statistical analysis of in vitro assays. Probability values of less than 0.05 were considered statistically significant.
Acknowledgements:

This work was performed within the framework of Dutch Top Institute Pharma, project D1-101.

Legend online supplement

Figure I:

Ratio lumen/total vessel wall area of vein grafts of balb/c (n=8) and TLR4 deficient (C3H-\textit{T}lr4^{LPS-d}) mice (n=7).

Figure II:

In vitro analysis of lenti-shTLR4 silencing. Murine TLR4 expression on CHO cells was analyzed after a dose dependent transduction with lenti-shTLR4. Positive control (A). Dose dependent transduction of lenti-shTLR4 in a 1:0.1 (B) 1:0.5 (C) 1:1 (D) 1:2 lentiviral particles/cells ratio showed a profound dose dependent reduction of TLR4 expression (E).

Figure III:

Murine 3T3 fibroblast were transduced with PBS, lenti-control or lenti-shTLR4 and subsequently stimulated with LPS, 100ng/ml; Pam3Cys, 10\mu g/ml; TNF-\alpha, 10ng/ml; IL-1\beta, 10ng/ml; Non Stimulated. NFkB activation due to LPS stimulation was significantly reduced in the lenti-shTLR4 transduced fibroblasts (F), A students t-test was used for statistical analysis * = P<0.01.
Tabel I:

<table>
<thead>
<tr>
<th></th>
<th>PBS</th>
<th>Lenti-control</th>
<th>Lenti-shTLR4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Macrophages</td>
<td>406±53μm² *</td>
<td>446±87μm²</td>
<td>187±64μm²</td>
</tr>
<tr>
<td>SMC</td>
<td>1289±120μm² *</td>
<td>515±169μm²</td>
<td>511±63μm²</td>
</tr>
</tbody>
</table>

Macrophages: PBS vs. lenti-shTLR4 p=0.037, lenti-control vs. lenti-shTLR4 p=0.054, PBS vs. lenti-control p=1.00. SMC: PBS vs. lenti-shTLR4 p=0.00006, lenti-control vs. lenti-shTLR4 p=0.53, PBS vs. lenti-control p=0.0059.

References


