



Contents lists available at ScienceDirect

Atherosclerosis

journal homepage: www.elsevier.com/locate/atherosclerosis

TRIF adaptor signaling is important in abdominal aortic aneurysm formation



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ARTICLE INFO

Article history:

Received 10 September 2014

Received in revised form

27 April 2015

Accepted 9 June 2015

Available online 12 June 2015

Keywords:

Vascular disease

Inflammation

Toll-like receptor

Angiotensin II

ABSTRACT

Objective: Abdominal aortic aneurysm (AAA) is characterized by inflammation, loss of smooth muscle cells (SMCs), and degradation of the extracellular matrix in the vessel wall. Innate immune receptors such as Toll-like receptors (TLRs) were recently shown to regulate immunological processes leading to the formation and progression of atherosclerotic plaques as well as to other cardiovascular pathologies. Our aim was to investigate whether blockage of TLR signaling, under the control of TIR domain-containing adaptor protein including IFN- β (TRIF), could inhibit the inflammatory response and AAA development in mice.

Results: In human AAA, an increased TLR3 and TLR4 expression in association with macrophages and T lymphocytes was demonstrated with immunohistochemical analysis. Angiotensin (Ang) II-induced aneurysm formation was significantly reduced by 30% in *ApoE*^{-/-}*Trif*^{-/-} mice compared to *ApoE*^{-/-} mice. Morphologically, AngII-infused *ApoE*^{-/-}*Trif*^{-/-} mice had a more intact cellular and extracellular matrix while *ApoE*^{-/-} mice infused with AngII displayed an increased medial thickness associated with aortic dissection, thrombus formation, and a more disorganized vessel wall. Gene expression analysis of the abdominal aorta revealed a profound decrease of the inflammatory genes *CD68* ($P < 0.05$), *CD11b* ($P < 0.05$), and *TNF- α* ($P < 0.05$) and the protease gene *MMP-12* ($P < 0.01$) in *ApoE*^{-/-}*Trif*^{-/-} mice compared to *ApoE*^{-/-} mice infused with AngII.

Conclusion: Our results suggest that signaling through TRIF is important for the inflammatory response of AngII-induced AAA and that blockage of the TRIF pathway reduces vascular inflammation and protects against AAA formation.

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1. Introduction

Abdominal aortic aneurysm (AAA) is a common cardiovascular disease. AAA is associated with atherosclerosis but there are clear molecular differences between both diseases. Important histological features of AAA involve infiltration of cells such as macrophages and T lymphocytes – representing both innate and adaptive immune responses, respectively – into the aortic wall. These cells

favor AAA formation through production of various proinflammatory cytokines and matrix-degrading proteases resulting in increased smooth muscle cell (SMC) apoptosis, neovascularization, and degradation of the extracellular matrix [1–3].

Innate immune receptors such as Toll-like receptors (TLRs) were recently shown to regulate immunological processes leading to the formation and progression of atherosclerotic plaques as well as to other cardiovascular pathologies such as AAA [4–8]. TLRs are expressed on a number of cell types, including macrophages, mast cells, B lymphocytes, and dendritic cells, but are also found on endothelial cells and SMCs, and all of these cell types are present in AAA and contribute to the inflammatory response [6,9]. TLRs play an essential role in the activation of the innate immune response

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through detection of microbial structures such as lipopolysaccharides, flagellin, double-stranded RNA, and lipopeptides thereby providing the first line of defense against pathogens. In addition, TLRs also bind host-derived endogenous ligands released upon tissue damage and tissue remodeling [10], including fibronectin, hyaluronan fragments, heat shock protein, and heparin sulfate [11–13]. TLR signaling is controlled by cytoplasmic adaptor proteins, including myeloid differentiation factor-88 (MyD88), which is the universal adaptor for all TLRs (except for TLR3), and TRIF domain-containing adaptor protein including IFN- β (TRIF), which is the adaptor for TLR3 and TLR4. Triggering of the MyD88-dependent signaling cascade activates nuclear factor (NF)- κ B, and triggering of the TRIF-dependent pathway results in activation of both NF- κ B and interferon regulating factor (IRF)-3. Both the MyD88- and TRIF-dependent pathways result in induction of proinflammatory mediators such as tumor necrosis factor (TNF)- α , interleukin (IL)-1, IL-6, and interferon (IFN) γ [6,10–12,14].

Because aneurysms are characterized by both inflammation and tissue remodeling, the majority of the proposed endogenous TLR ligands could be present at various stages during disease progression. Recently, Owens and coworkers showed profound reduction of angiotensin (Ang) II-induced AAA and atherosclerosis in mice that were deficient in MyD88 or TLR4 [8]. Due to the ability of TRIF to regulate inflammatory-mediated activation of TNF- α and interleukins among other, inhibition of the TRIF signaling pathway would potentially inhibit these transcription factors and the subsequent production of a large cohort of inflammatory mediators. In the present study we sought to investigate the role of TRIF signaling in AngII-induced AAA formation.

2. Materials and methods

2.1. Human subjects

Patients undergoing elective surgery for infrarenal AAA ($n = 12$) after preoperative computed tomography showed an eccentric intraluminal thrombus were included in the study. Patients were included after informed, written and, signed consent from the Karolinska University Hospital, Stockholm, Sweden.

Control aortas were collected from organ donors without clinical or macroscopic signs of aortic atherosclerosis or aneurysm. Control ascending aorta samples for RNA studies and infrarenal control aorta samples for histology were collected from medicolegal autopsies performed in the Department of Forensic Medicine, University of Helsinki. The sections were immediately snap frozen in liquid nitrogen for RNA isolation or fixed in 4% formaldehyde for histology. The use of organ donor and autopsy tissues was approved by The National Authority for Medicolegal Affairs of Finland.

2.2. AngII-induced aneurysm model in mice

Trif^{-/-} mice were generated in the Akira laboratory (Department of Host Defense, Osaka University, Japan) and bred into the *ApoE*^{-/-} background (Center for Molecular Medicine, Karolinska Institute, Stockholm, Sweden). *ApoE*^{-/-} mice were obtained from Taconic and served as control mice. To induce AAA, mini-osmotic pumps (Model 1004, Alzet, CA, USA) were implanted subcutaneously into the right flank of *ApoE*^{-/-} *Trif*^{-/-} ($n = 11$) and *ApoE*^{-/-} ($n = 12$) mice to release AngII (1000 ng kg⁻¹ min⁻¹, cat.no.9525, Sigma Aldrich, St. Louis, USA) over the course of 28 days. A group of *ApoE*^{-/-} mice were infused with saline as controls ($n = 6$). One dose of analgesics, Buprenorphine (Temgesic, 10 μ g/mL), was given subcutaneously before surgery. Operated animals were monitored daily for any sign of pain or disability. Mice were fed a normal chow diet, and water was provided *ad libitum* throughout the whole study. After 28 days,

the mice were sacrificed, whole blood was drawn by jugular vein puncture, and the aortas were removed and fixated in 4% zinc formaldehyde for histology analysis or in RNAlater (Ambion, Austin, TX, USA) for gene expression analysis. The aortic diameter was measured as the adventitial diameter, and the definition of an aneurysm was set as a 1.5-fold enlargement of the vessel wall. Total serum cholesterol was measured using Infinity Total Cholesterol (Fisher Scientific). The investigation conforms to directive 2010/63/EU of the European Parliament.

2.3. Real-time reverse-transcription polymerase chain reaction (RT-PCR) analysis

Suprarenal aortas were homogenized with Tissue Lyser using safe-lock tubes with metal beads and trizol-chloroform. Total RNA from the aorta was isolated with the RNeasy Mini kit (Qiagen, Hilden, Germany) and reversely transcribed with random primers and Superscript III as described by the manufacturer (Invitrogen, Carlsbad, CA, USA). cDNA (1.0 ng) was amplified by RT-PCR reactions with TaqMan Universal PCR Mastermix (Applied Biosystems, Foster City, CA, USA) in 96-well fast plates on a 7500 Fast Real-time PCR Sequence Detector (Applied Biosystems). Samples were run in duplicate and semi-quantified against a standard curve. All probes were obtained from Applied Biosystems (Supplementary table 1) and results were normalized to values of human RPLP0 or murine TBP.

2.4. Immunohistology

Paraffin-embedded human abdominal aortas and mouse aortas were sectioned (5 μ m) and rehydrated in several changes of ethanol and Tissue-Clear® (Sakura Finetek, Leiden, The Netherlands). After antigen retrieval treatment using Diva decloaker 20 \times (Biacore Medical, Concord, CA, USA), endogenous peroxidase activity was quenched by treatment with 3% hydrogen peroxide for 5 min followed by incubation in 5% blocking bovine serum albumin solution. Sections were then incubated with primary antibodies against human; α -actin (Sigma Aldrich, Stockholm, Sweden, cat. no. A5228), von Willebrand factor (Dako, cat. no. A0082), CD68 (Leica Microsystems, cat. no. NCL-CD68-KP1), CD3 (Santa Cruz Biotechnology, cat. no. sc-20047), TLR3 (Sigma Aldrich, cat. no. sc-10740), and TLR4 (Santa Cruz Biotechnology, cat. no. sc-8694) and primary antibodies against mouse; CD68 (AbD Serotec, cat. no. MCA1957) and CD3 ϵ (Bioworld Technology, cat. no. BS3476) overnight at 4 °C. Isotype-matched immunoglobulin at the same concentration served as the negative control (Santa Cruz Biotechnologies, cat. no. NB810-56910). Thereafter, the sections were incubated with a secondary biotinylated goat anti-rabbit IgG, goat anti-mouse IgG, goat anti-rat IgG or horse anti-goat IgG (Dako) antibody. Avidin-biotin peroxidase complexes (Dako) were added followed by visualization with 3,3'-diaminobenzidine tetrahydrochloride (Dako). All sections were counterstained with Mayer's hematoxylin (Histolab Products, Göteborg, Sweden). Semi-quantification of CD68 and CD3 positive cells on representative areas of mice aortas was performed blinded.

2.5. Elastin staining

Paraffin-embedded aortas (5 μ m) were stained with Verhoeff's hematoxylin for 1 h, differentiated in 2% ferric chloride for 2 min, and counterstained with Van Gieson's stain for 5 min to identify elastic fibers in the aortic tissue. Quantification of elastin was performed blinded. A scoring system from 1 to 4 was used with 1 defined as intact elastin, 2 as low degradation of elastin, 3 as intermediate degradation of elastin, and 4 as high degradation of

elastin.

2.6. Masson Trichrome staining

Paraffin-embedded sections (5 μm) were incubated in Bouin's solution (Sigma Aldrich) overnight, counterstained with Weigert's hematoxylin (Histolab), and then stained with the Masson Trichrome kit (Sigma Aldrich) containing Biebrich's Scarlet-acid fuchsin for 15 min, phosphomolybdic-phosphotungstic acid solution for 10 min, and aniline blue for 5 min. Sections were finally washed in 1% acetic acid for 3 min. Collagen displays a blue staining and cytoplasm and muscle fibers are stained red.

2.7. Statistical analysis

The statistical analysis was performed with IBM SPSS Statistics (Version 22). All measurements are represented as median and SD. P-values < 0.05 are considered statistically significant. Two-group comparisons of the quantitative data were performed using the non-parametric Mann–Whitney Rank Sum test.

3. Results

3.1. Expression of TLR3, TLR4 and TRIF in human AAA

Immunohistochemical analysis of human aortas with AAA (Fig. 1) demonstrated a high expression of TLR3 and TLR4 (Fig. 1B and C). The expression was to some extent associated in areas of SMCs

and endothelial cells (data not shown) but more frequently expressed in the same areas as macrophages and T lymphocytes (Fig. 1D–E). Non-aneurysmal control aortas only expressed TLR3 and TLR4 weakly in media but not adventitia layer of the aorta (Fig. 1F–G). The gene-expression profile showed twice as high expression of *TLR4* ($P < 0.001$), but there were no differences in *TLR3* or *Trif* within the aneurysmal wall compared to non-aneurysmal control aortas (Fig. 2).

3.2. TRIF deficiency reduces AngII-induced AAA in mice

To determine whether disrupted TRIF signaling could influence AAA formation, *ApoE*^{-/-} mice deficient in TRIF were infused with AngII for 28 days. Histological features of the aneurysmal *ApoE*^{-/-} mice aortas infused with AngII revealed a markedly expanded aortic mass and disorganized adventitial tissue compared to saline-infused *ApoE*^{-/-} control aortas when stained with Masson Trichrome (Fig. 3A and B). The presence of a thrombus was also identified in the adventitial regions in some of the aneurysmal aortas (Fig. 3B). However, aortas from *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII (Fig. 3C) were similar to the saline-infused control aortas and displayed a more organized vessel wall and did not have any thrombotic material or aortic masses compared with aortas from aneurysmal *ApoE*^{-/-} mice.

An aorta was classified as an AAA when the adventitial aortic diameter was increased by at least 50% compared to saline-infused control mouse aortas (0.46 ± 0.05 mm). This corresponds to an enlargement by > 0.68 mm. The absence of TRIF resulted in a 30%

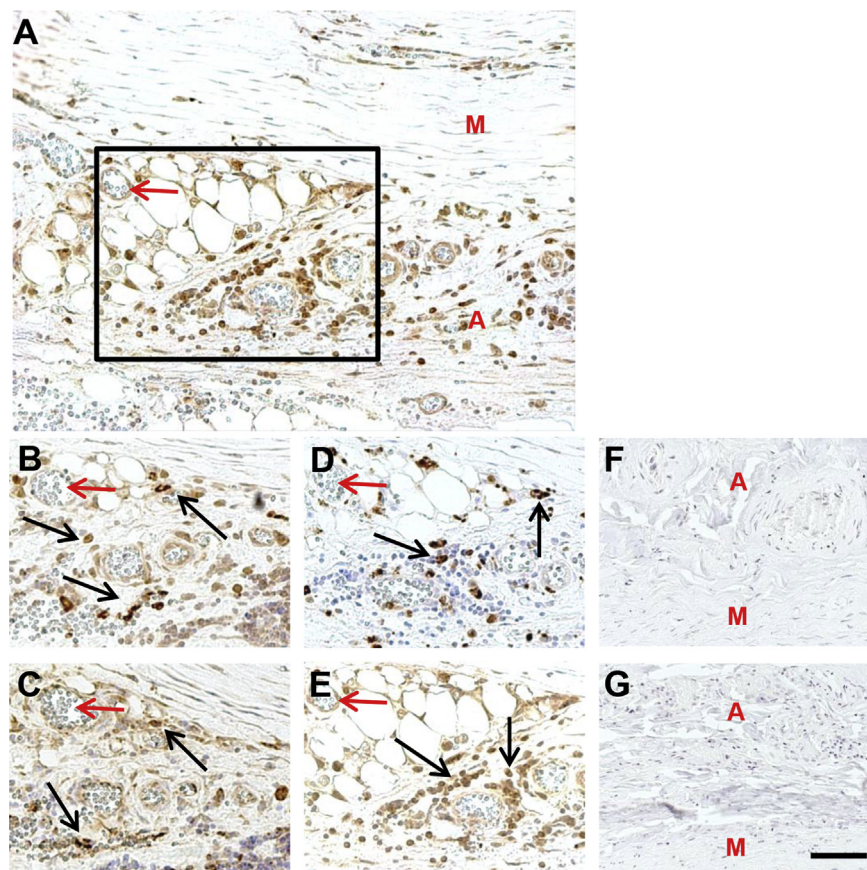


Fig. 1. Immunohistochemical staining of human AAA and control aorta. Overview of aneurysmal vessel with CD3 positive staining (A) including a square indicating magnified area. Histological staining of TLR-3 (B), TLR-4 (C), macrophage marker CD68 (D) and T lymphocyte marker CD3 (E) in human AAA and TLR3 (F) and TLR4 (G) in non-aneurysmal control aorta. Black arrows indicate positive staining and red arrows indicate blood vessel from the same area. A indicates adventitia and M indicates media. Scale bar represents 50 μm .

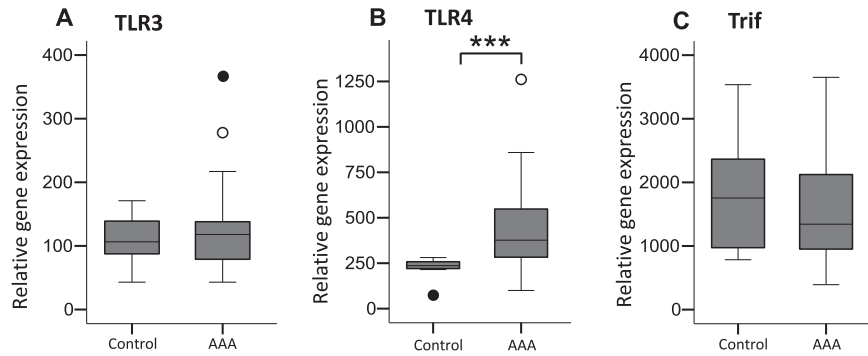


Fig. 2. Gene expression of *TLR3* (A), *TLR4* (B) and the adaptor protein *Trif* (C) from human non-aneurysmal control aortas and AAA samples as determined by real-time PCR. *** $P < 0.001$. "○" Outlier greater than 1.5 times the interquartile range. "●" Outlier greater than 3 times the interquartile range.

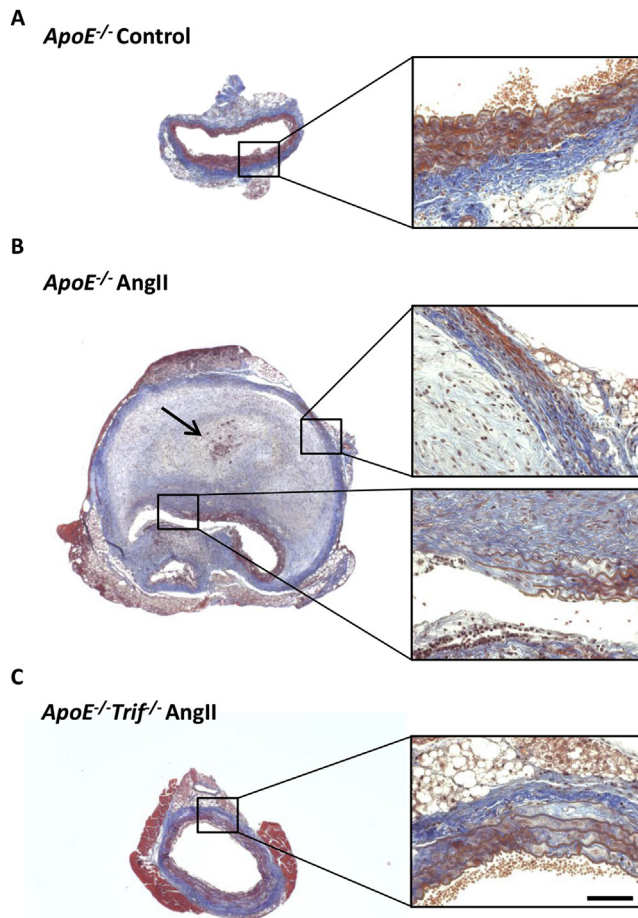


Fig. 3. Histological staining with Masson Trichrome of saline-infused *ApoE*^{-/-} control mouse aorta (A), AngII-infused *ApoE*^{-/-} mouse aorta whereas a blood clot is present (arrow) (B) and AngII-infused *ApoE*^{-/-}*Trif*^{-/-} mouse aorta (C). Scale bar = 50 μ m.

reduced adventitial diameter (0.57 ± 0.17 mm) compared with AngII-infused *ApoE*^{-/-} mice (0.78 ± 0.26 mm) ($P < 0.05$; Fig. 4A). After AngII infusion, 55% of the *ApoE*^{-/-} mice developed an aneurysm but only 9% of the *ApoE*^{-/-}*Trif*^{-/-} mice developed such lesions (Fig. 4B).

Histological staining and scoring of elastin in aorta media demonstrated a variation in the degree of elastin degradation in all

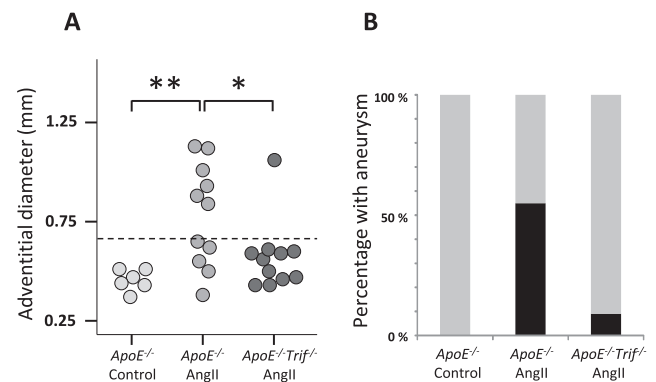


Fig. 4. Adventitial aorta diameter (A) and the percentage of mice with aneurysm (B) in saline-infused *ApoE*^{-/-} mice and AngII-infused *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice. * $P < 0.05$, ** $P < 0.01$. Grey bar represent no aneurysm and black bar represent aneurysm.

ApoE^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII compared to saline-infused control aortas when stained with Verhoeff's van Gieson (Supplementary figure 1). There were no differences in elastin degradation between *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII.

No difference in total serum cholesterol between AngII-infused *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} and saline-infused *ApoE*^{-/-} mice was detected (Supplementary table II).

3.3. Mice deficient in TRIF display an anti-inflammatory profile through regulation of the cellular content

To further assess the outcome of TRIF deficiency on the inflammatory response present during aneurysm formation, several genes involved in the process were examined in the aortas from all AngII-infused and saline-infused mice. Compared with saline-infused mice, mRNA expression in the cellular contents showed an increase in the leukocyte marker *CD11b* and the macrophage marker *CD68* in both groups of AngII-infused mice. However, the expression of *CD11b* and *CD68* were significantly lower by 41% ($P < 0.05$) and 40% ($P < 0.05$), respectively, in the aortas of *ApoE*^{-/-}*Trif*^{-/-} mice compared to *ApoE*^{-/-} mice infused with AngII (Fig. 5A and B). The T lymphocyte marker *CD3e* showed a tendency of a reduction (67%) in gene expression – to levels equal to saline-infused control aortas – in *ApoE*^{-/-}*Trif*^{-/-} mice compared with aortas from *ApoE*^{-/-} AngII-infused mice ($P = 0.14$; Fig. 5C). Gene expression analysis of chemokine (C–C motif) ligand 2 (*CCL2*) and *CCL5* showed no significant difference in gene expression between

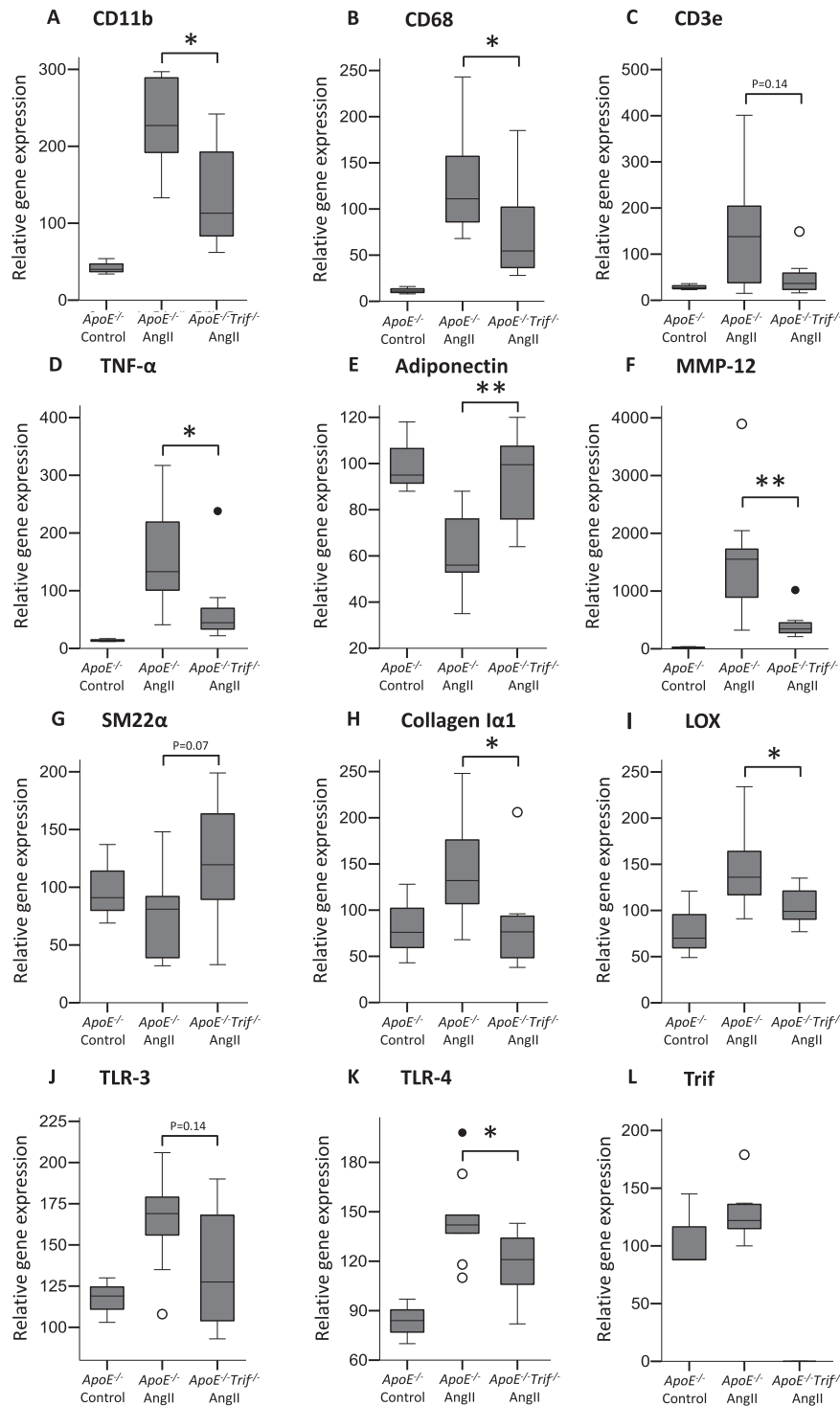


Fig. 5. Gene expression of *CD11b* (A), *CD68* (B), *CD3e* (C), *TNF-α* (D), *adiponectin* (E), *MMP-12* (F), *SM22α* (G), *collagen Iα1* (H), *lysyl oxidase (LOX)* (I), *TLR3* (J), *TLR4*, (K) and *Trif* (L) in saline-infused *ApoE*^{-/-} mice and AngII-infused *ApoE*^{-/-} and *ApoE*^{-/-}*Trif*^{-/-} mice. *P < 0.05, **P < 0.01. "○" Outlier greater than 1.5 times the interquartile range. "●" Outlier greater than 3 times the interquartile range.

AngII-infused *ApoE*^{-/-} mice and *ApoE*^{-/-}*Trif*^{-/-} mice (Supplementary Fig. 2A–B). Infiltration of CD68 positive macrophages and CD3 positive T lymphocytes was further validated with immunohistochemical staining where we found a higher infiltration of predominantly macrophages in AngII-infused *ApoE*^{-/-} mice as compared to *ApoE*^{-/-}*Trif*^{-/-} mice. Infiltration of macrophages was mainly localized to the adventitia of the AngII-infused *ApoE*^{-/-}

mice while absent in AngII-infused *ApoE*^{-/-}*Trif*^{-/-} mice (Supplementary Fig. 3A–C). The expression of T lymphocytes was found in both saline-infused and AngII-infused mouse aortas. In *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII, T lymphocytes were localized to the media while in *ApoE*^{-/-} mice the expression was found both in media and adventitia (Supplementary Fig. 3D–F). Histological staining and semi-quantification of CD68 positive cells

and CD3 positive cells confirmed a higher infiltration of cells ($P < 0.01$ and $P < 0.05$) in aortas of AngII-infused $ApoE^{-/-}$ mice as compared with $ApoE^{-/-}Trif^{-/-}$ mice infused with AngII (Supplementary Fig. 3 G–H).

We further observed increased mRNA expression of the pro-inflammatory marker $TNF-\alpha$ in AngII-infused $ApoE^{-/-}$ mice. The levels of $TNF-\alpha$ were 130% higher compared to $ApoE^{-/-}Trif^{-/-}$ mice ($P < 0.05$; Fig. 5D). We also examined the expression of the anti-inflammatory adipokine, adiponectin, and found a significant down regulation by 33% ($P < 0.01$) in the aortas from $ApoE^{-/-}$ mice infused with AngII as compared with $ApoE^{-/-}Trif^{-/-}$ mice, which were similar to the saline-infused control aortas (Fig. 5E). We also performed analysis of the anti-inflammatory factors TGF- β and IL-10. TGF- β was expressed low in control aortas and significantly higher in AngII-infused $ApoE^{-/-}$ mice and $ApoE^{-/-}Trif^{-/-}$ mice. However, TGF- β was significantly higher ($P < 0.05$; Supplementary figure 2C) in aortas from AngII-infused $ApoE^{-/-}$ mice compared to aortas from $ApoE^{-/-}Trif^{-/-}$ mice, which resembled an expression more towards the control. A difference in gene expression of IL-10 was not found between AngII-infused $ApoE^{-/-}$ mice and $ApoE^{-/-}Trif^{-/-}$ mice (Supplementary figure 2D). Another gene of interest was IFN- β . Gene expression analysis demonstrated no differences between saline-infused $ApoE^{-/-}$ mice or AngII-infused $ApoE^{-/-}$ mice and $ApoE^{-/-}Trif^{-/-}$ mice.

To determine the contribution of TRIF deficiency on matrix-degrading proteases, gene expression analysis were performed on relevant matrix degrading proteases in AAA formation (Supplementary table 1). Interestingly macrophage elastase MMP-12 in the aortas from $ApoE^{-/-}Trif^{-/-}$ versus $ApoE^{-/-}$ mice infused with AngII was the only protease that showed a significant reduced expression by 72% ($P < 0.01$, Fig. 5F).

In $ApoE^{-/-}$ mice infused with AngII a 38% ($P = 0.07$) decreased expression of the SMC marker $SM22\alpha$ was observed compared to aortas from $ApoE^{-/-}Trif^{-/-}$ mice (Fig. 5G). Further, as compared with $ApoE^{-/-}Trif^{-/-}$ mice, aortas from $ApoE^{-/-}$ mice infused with AngII exhibited a significant increase in the synthesis of pro-collagen I α 1 by 74% ($P < 0.05$) as well as lysyl oxidase (LOX) by 37% ($P < 0.05$), a key enzyme involved in post translational modification and maturation of collagens (Fig. 5H–I).

TRIF mediates signaling from TLR3 and TLR4, therefore gene expression analysis was performed on these receptors. In the aortas from $ApoE^{-/-}$ mice infused with AngII, the mRNA levels of $TLR3$ and $TLR4$ were increased by 22% ($P = 0.14$) and 23% ($P < 0.05$), respectively, compared to $ApoE^{-/-}Trif^{-/-}$ mice (Fig. 5J–K). Gene expression was also performed on $Trif$ in order to assess the absence of the gene in $ApoE^{-/-}Trif^{-/-}$ mice. $ApoE^{-/-}$ infused with AngII expressed the $Trif$ gene to a similar extent as saline-infused control mice. Mice deficient in TRIF completely lacked $Trif$ gene expression (Fig. 5L).

4. Discussion

TLRs play a key role as initiators of both the innate and adaptive immune systems and are important in many inflammatory diseases. Here we demonstrate that genetic deficiency of TRIF in mice with an $ApoE^{-/-}$ background has an anti-inflammatory effect and inhibits AngII-induced AAA formation. TRIF deficiency was associated with a more stable aorta together with reduced macrophage and leukocyte content and markedly decreased expression of the $TNF-\alpha$ cytokine and the MMP-12 protease.

MyD88 is thought to transduce signaling of all TLRs (except TLR3), and it is a well-described adaptor protein in cardiovascular research that has been shown to be important in the development of cardiovascular disease [15]. It has previously been shown that atherosclerosis-prone hypercholesterolemic $ApoE^{-/-}$ mice with a

genetic deficiency of either TLR4 or MyD88 had reduced aortic atherosclerosis [8]. These mice had reduced lipid and macrophage accumulation and markedly decreased expression of the pro-inflammatory cytokine monocyte chemoattractant protein 1 (MCP-1). In $ApoE^{-/-}MyD88^{-/-}$ double knockout mice, decreased levels of the pro-inflammatory enzyme cyclooxygenase-2 (COX-2) and cytokine IL-12p40 were observed compared to $ApoE^{-/-}TLR4^{-/-}$ double knockout mice. This indicates an important role for TLR4 and MyD88 signaling in atherosclerosis-prone hypercholesterolemic mouse models [16]. A recent study performed by Owens and co-workers reported that whole-body deficiency of MyD88 had a profound effect on the reduction of AngII-induced AAA and atherosclerosis. Deficiency of MyD88 in bone marrow-derived cells also resulted in reduced AAA and atherosclerosis implying that the effects of MyD88 on AAA and atherosclerosis are mediated by hematopoietic cells. Whole-body deficiency of TLR4 had a similar ability to reduce AngII-induced vascular pathologies, although bone marrow-deletion of TLR4 clearly demonstrated that these effects were unrelated to MyD88-mediated signaling in hematopoietic cells [8].

In the past years, attention has been brought to TRIF, which is also a cytoplasmic adaptor protein and is important for transduction of TLR3 and TLR4 signaling. TRIF and its association to TLR3 and TLR4 have been studied in atherosclerosis [17]. Recently Lundberg et al. demonstrated that hematopoietic deficiency of TRIF in $LDLR^{-/-}$ mice led to a 40% reduction in lesion size compared to control mice. These effects were accompanied by reduced infiltration of macrophages and T lymphocytes together with lower expression of pro-inflammatory $TNF-\alpha$, IFN- γ , and IL-6 as well as CCL2, CCL5, and chemokine (C-X-C motif) ligand 10 (CXCL10) all of which are important for the recruitment of macrophages and T lymphocytes in atherosclerosis [17]. However, the role of TRIF in AAA formation is currently unknown.

Although many risk factors are known for AAA, including smoking, atherosclerosis, inflammation, male gender, and age, the etiology of human AAA is still not well understood. To gain an insight into the mechanism of AAA, several animal models have been developed [18]. One of the more widely used animal models is infusion of AngII that results in many features similar to the human disease, including luminal dilatation, leukocyte infiltration, extracellular matrix deformation, and thrombus formation [19]. In the present study we demonstrated that $ApoE^{-/-}$ mice infused with AngII displayed a large aneurysm with a disorganized vessel morphology associated with pronounced adventitial remodeling – including thrombus formation in some of the vessels – as previously described in the literature [20]. We also demonstrated that deficiency in TRIF was protective against AngII-induced aneurysm formation. These $ApoE^{-/-}Trif^{-/-}$ mice exhibited a more organized vessel wall, lacked thrombotic material, and had an aortic mass similar to the saline-infused control aortas compared to $ApoE^{-/-}$ mice infused with AngII. However these mice still exhibited elastin degradation similar to $ApoE^{-/-}$ mice infused with AngII. Expression of the SMC markers $SM22\alpha$ indicated a tendency towards a significant increase in $ApoE^{-/-}Trif^{-/-}$ mice compared with $ApoE^{-/-}$ mice infused with AngII, suggesting that TRIF might play an important role in reducing SMC apoptosis or increasing proliferation. SMCs are one of the major producers of matrix collagen. In aneurysm, increased collagen synthesis occurs as a response to matrix degradation and expansion of the vessel wall in the initial/early phase of aneurysm disease. We demonstrated that $ApoE^{-/-}$ mice infused with AngII had a high synthesis of pro-collagen I α 1 as well as LOX, a key enzyme involved in post translational modification and maturation of collagens, as a response to aneurysmal formation. $ApoE^{-/-}Trif^{-/-}$ mice however, lacking aneurysm formation, displayed synthesis of pro-collagen I to similar expression as saline-

infused *ApoE*^{-/-} mice.

One of the early key events in the progression of AngII-induced AAA is infiltration of macrophages predominantly localized to the adventitia whereas in later stages infiltration of other leukocytes such as T and B lymphocytes can be detected [21,22]. We found reduced levels of leukocytes, including macrophages and T lymphocytes, in the aortic wall in *ApoE*^{-/-}*Trif*^{-/-} mice together with decreased levels of the pro-inflammatory cytokine TNF- α – important for the initiation of inflammation – as well as the macrophage protease MMP-12. These mice also show increased levels of adiponectin, a protein known to exert anti-inflammatory effects in the aorta and to attenuate aneurysm formation [23,24]. The effects observed within the vessel wall of *ApoE*^{-/-}*Trif*^{-/-} mice might be both haematopoietic effects and influence of the vascular cells. These results suggest that TRIF signaling increases aneurysm formation by promoting the infiltration of immune cells such as macrophages and T lymphocytes into the aortic wall. Of all AngII-infused *ApoE*^{-/-} mice, 55% displayed an aneurysm formation according to the definition provided above. However, all AngII-infused *ApoE*^{-/-} mice had similar increases in inflammatory responses in their aortas. Of all *ApoE*^{-/-}*Trif*^{-/-} mice infused with AngII only one mouse developed an aneurysm. This aorta displayed an inflammatory response to the same extent as *ApoE*^{-/-} mice infused with AngII which can be observed as an outlier in several figures. This outlier affected the outcome of several genes, among these the macrophage protease cathepsin K, CCL2 and SM22 α . If removing this outlier these genes are significantly different between *ApoE*^{-/-}*Trif*^{-/-} mice and *ApoE*^{-/-} mice infused with AngII.

TLRs play a key role in the innate immune response and are involved in many inflammatory diseases, including atherosclerosis [10]. During aneurysmal progression, fragments from the extracellular matrix such as fibronectin and hyaluronan, among others, are released due to tissue damage and matrix remodeling. These components are known to trigger the TLRs and thereby lead to NF- κ B activation. In the present study, we identified increased TLR3 and TLR4 expression in human AAA in association with macrophages and T lymphocytes and to some extent with endothelial cells and SMCs. Similar to mice lacking TRIF, Lundberg et al. also demonstrated that TLR3 deficiency in hematopoietic cells in *LDLR*^{-/-} mice resulted in reduced atherosclerotic plaques with markedly decreased levels of macrophages and T lymphocytes suggesting that TRIF mediates its proatherogenic role downstream of TLR3 [17]. In line with these data, Zimmer et al. demonstrated that TLR3 enhanced atherosclerotic plaque formation in *ApoE*^{-/-} mice [25]. We further investigated the presence of TLR3 and TLR4 in aortas from experimentally induced AAA in mice and found an up regulation of TLR4 in the *ApoE*^{-/-} mice infused with AngII. However, deficiency of the adaptor protein TRIF reduced the expression of TLR4, and this might be explained by impaired recruitment of inflammatory cells to the aortic tissue. These data suggest that signaling through TRIF promotes aneurysmal progression and formation in *ApoE*^{-/-} mice infused with AngII and that reduced aneurysmal formation in *ApoE*^{-/-} mice deficient in TRIF is associated with reduced vascular inflammation.

5. Conclusion

TRIF is an essential adaptor protein in the TLR signaling pathway that triggers the inflammatory response in the aortic wall and is important in the initiation and progression of AAA. In this study we show that mice deficient in TRIF have reduced inflammation; reduced levels of macrophages, T lymphocytes, and pro-inflammatory TNF- α and proteases; and a more organized aortic morphology. Taken together, our results suggest that TRIF affects the inflammatory response of AngII-induced AAA and that blockage

of the TRIF pathway reduces vascular inflammation and protects against AAA formation.

Disclosures

No disclosures.

Acknowledgments

We thank M.D, PhD Shizuo Akira (Department of Host Defense, Osaka University, Japan) for kindly providing TRIF-deficient mice. This work is supported by the Swedish Research Council (K2013-99X-22231-01-5).

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.atherosclerosis.2015.06.014>.

References

- [1] K. Shimizu, R.N. Mitchell, P. Libby, Inflammation and cellular immune responses in abdominal aortic aneurysms, *Arterioscler. Thromb. Vasc. Biol.* 26 (5) (2006) 987–994.
- [2] J.B. Michel, J.L. Martin-Ventura, J. Egido, et al., Novel aspects of the pathogenesis of aneurysms of the abdominal aorta in humans, *Cardiovasc. Res.* 90 (1) (2011) 18–27.
- [3] A. Lopez-Candales, D.R. Holmes, S. Liao, M.J. Scott, S.A. Wickline, R.W. Thompson, Decreased vascular smooth muscle cell density in medial degeneration of human abdominal aortic aneurysms, *Am. J. Pathol.* 150 (3) (1997) 993–1007.
- [4] A. Katsargyris, C. Klonaris, E. Bastounis, S. Theocharis, Toll-like receptor modulation: a novel therapeutic strategy in cardiovascular disease? *Expert Opin. Ther. Targets* 12 (11) (2008) 1329–1346.
- [5] K.S. Michelsen, T.M. Doherty, P.K. Shah, M. Arditi, TLR signaling: an emerging bridge from innate immunity to atherogenesis, *J. Immunol.* 173 (10) (2004) 5901–5907.
- [6] A.M. Lundberg, G.K. Hansson, Innate immune signals in atherosclerosis, *Clin. Immunol.* 134 (1) (2010) 5–24.
- [7] K. Edfeldt, J. Swedenborg, G.K. Hansson, Z.Q. Yan, Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation, *Circulation* 105 (10) (2002) 1158–1161.
- [8] A.P. Owens 3rd, D.L. Rateri, D.A. Howatt, et al., MyD88 deficiency attenuates angiotensin II-induced abdominal aortic aneurysm formation independent of signaling through toll-like receptors 2 and 4, *Arterioscler. Thromb. Vasc. Biol.* 31 (12) (2011) 2813–2819.
- [9] O. Pryshchep, W. Ma-Krupa, B.R. Younge, J.J. Goronzy, C.M. Weyand, Vessel-specific toll-like receptor profiles in human medium and large arteries, *Circulation* 118 (12) (2008) 1276–1284.
- [10] S. Frantz, G. Ertl, J. Bauersachs, Mechanisms of disease: toll-like receptors in cardiovascular disease, *Nat. Clin. Pract. Cardiovasc. Med.* 4 (8) (2007) 444–454.
- [11] S.A. Arancibia, C.J. Beltran, I.M. Aguirre, et al., Toll-like receptors are key participants in innate immune responses, *Biol. Res.* 40 (2) (2007) 97–112.
- [12] S. Akira, K. Takeda, Toll-like receptor signalling, *Nat. Rev. Immunol.* 4 (7) (2004) 499–511.
- [13] A.F. McGettrick, L.A. O'Neill, Localisation and trafficking of toll-like receptors: an important mode of regulation, *Curr. Opin. Immunol.* 22 (1) (2010) 20–27.
- [14] H.S. Youn, J.Y. Lee, S.I. Saitoh, et al., Suppression of MyD88- and TRIF-dependent signaling pathways of toll-like receptor by (-)-epigallocatechin-3-gallate, a polyphenol component of green tea, *Biochem. Pharmacol.* 72 (7) (2006) 850–859.
- [15] H. Bjorkbacka, V.V. Kunjathoor, K.J. Moore, et al., Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways, *Nat. Med.* 10 (4) (2004) 416–421.
- [16] K.S. Michelsen, M.H. Wong, P.K. Shah, et al., Lack of toll-like receptor 4 or myeloid differentiation factor 88 reduces atherosclerosis and alters plaque phenotype in mice deficient in apolipoprotein E, *Proc. Natl. Acad. Sci. USA* 101 (29) (2004) 10679–10684.
- [17] A.M. Lundberg, D.F. Ketelhuth, M.E. Johansson, et al., Toll-like receptor 3 and 4 signalling through the TRIF and TRAM adaptors in haematopoietic cells promotes atherosclerosis, *Cardiovasc. Res.* 99 (2) (2013) 364–373.
- [18] A. Daugherty, L.A. Cassis, Mouse models of abdominal aortic aneurysms, *Arterioscler. Thromb. Vasc. Biol.* 24 (3) (2004) 429–434.
- [19] A. Daugherty, M.W. Manning, L.A. Cassis, Angiotensin II promotes atherosclerotic lesions and aneurysms in apolipoprotein E-deficient mice, *J. Clin. Investig.* 105 (11) (2000) 1605–1612.
- [20] M.W. Manning, L.A. Cassi, J. Huang, S.J. Szilvassy, A. Daugherty, Abdominal

- aortic aneurysms: fresh insights from a novel animal model of the disease, *Vasc. Med.* 7 (1) (2002) 45–54.
- [21] D.L. Rateri, D.A. Howatt, J.J. Moorleggen, R. Charnigo, L.A. Cassis, A. Daugherty, Prolonged infusion of angiotensin II in apoE(-/-) mice promotes macrophage recruitment with continued expansion of abdominal aortic aneurysm, *Am. J. Pathol.* 179 (3) (2011) 1542–1548.
- [22] K. Saraff, F. Babamusta, L.A. Cassis, A. Daugherty, Aortic dissection precedes formation of aneurysms and atherosclerosis in angiotensin II-infused, apolipoprotein E-deficient mice, *Arterioscler. Thromb. Vasc. Biol.* 23 (9) (2003) 1621–1626.
- [23] N. Ouchi, J.L. Parker, J.J. Lugus, K. Walsh, Adipokines in inflammation and metabolic disease, *Nat. Rev. Immunol.* 11 (2) (2011) 85–97.
- [24] S. Yoshida, J.J. Fuster, K. Walsh, Adiponectin attenuates abdominal aortic aneurysm formation in hyperlipidemic mice, *Atherosclerosis* 235 (2) (2014) 339–346.
- [25] S. Zimmer, M. Steinmetz, T. Asdonk, et al., Activation of endothelial toll-like receptor 3 impairs endothelial function, *Circ. Res.* 108 (11) (2011) 1358–1366.