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A novel small molecule TLR4 antagonist (IAXO-102) negatively regulates non-hematopoietic toll like receptor 4 signalling and inhibits aortic aneurysms development

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ABSTRACT

Objectives: The toll-like receptors (TLRs), including TLR4, have been shown to play a crucial role in vascular inflammatory diseases, such as atherosclerosis and aneurysm. The main goal of this study was to determine the potential of IAXO-102 (Innaxon, Tewkesbury), a novel small molecule TLR4 antagonist, to modulate non-hematopoietic TLR4 proinflammatory signalling and inhibit experimental abdominal aortic aneurysm (AAA) development.

Methods: Human umbilical vein endothelial cells (HUVEC) and Angiotensin II-induced experimental AAA development were our *in vitro* and *in vivo* models respectively. Western blotting, antibody array and ELISA approaches were used to explore the effect of IAXO-102 on TLR4 functional activity on two levels: modulation of TLR4-induced mitogen activated protein kinases (MAPK) and p65 NF-kB phosphorylation and expression of TLR4 dependent proinflammatory proteins.

Results: Following activation of TLR4, *in vitro/in vivo* data revealed that IAXO-102 inhibited MAPK and p65 NF-kB phosphorylation associated with down regulation of the expression of TLR4 and TLR4 dependent proinflammatory proteins. Furthermore, IAXO-102 decreased Angiotensin II-induced aortic expansion, rupture and incidence of AAA.

Conclusions: These results demonstrate the ability of IAXO-102 to negatively regulate TLR4 signalling and to inhibit experimental AAA development, suggesting the potential therapeutic use of this TLR4 antagonist for pharmacological intervention of AAA.

1. Introduction

The incidence of abdominal aortic aneurysm (AAA) is approximately 1–2% in men and 0.6–0.8% in women [1]. The natural history of most AAAs is to increase in size, increasing the risk of rupture. Aneurysm rupture is the thirteenth most common cause of sudden death in men over 65 years [1] Surgical repair, using endovascular stenting or open repair, both of which carry a significant risk of mortality, is the current treatment for this vascular disease. Discovery of drugs for treatment and stabilisation of AAA is a worthy challenge, with a significant commercial impact to relieve a significant global financial burden from the health services.

AAA is a complex vascular disease, involving the interaction of several fundamental biological processes. The early events in aneurysm formation, defined largely from animal models, involve inflammation as an initiating mechanism [2]. Toll-like receptors (TLRs) serve as pattern recognition receptors, within the immune system, and recognise exogenous ligands in response to inflammatory triggers. Among these receptors, TLR4 is known to be activated by Gram-negative bacteria. However, TLR4 is also activated in response to other non-bacterial ligands (known as ligands of sterile inflammation) such as heat shock proteins, fibronectins, small fragments of hyaluronan, and even saturated fatty acids and extracellular matrix components in response to cellular damage

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[3–5]. Besides the exogenous stimuli, endogenous host molecules, such as the oxidised form of low density lipoprotein (LDL), have also been shown to activate TLR4 [6]. TLR4 expression has been described in the vascular system (endothelial cells and smooth muscle cells) [7]. TLR4 has been reported to be implicated in inflammatory related cardiovascular diseases. Importantly, recent studies have demonstrated that deletion of the TLR4 gene protected against experimental atherosclerosis and aneurysm development or heart failure [8–10]. These findings strongly support the idea that regulation of TLR4, and its endogenous ligands, appears as a potential novel target for therapeutic control of aneurysms.

Manipulation of TLR4-mediated immune responses is a potential approach to treat a variety of inflammatory diseases. Over the last years several TLR4 antagonists have been evaluated in preclinical studies. However, only two drugs, E5564 (Eritoran, Eisai Inc) and TAK-242 (Takeda Biological) progressed to clinical trials for treatment of sepsis which have now been discontinued in different phases [11,12].

Recently a panel of synthetic glycolipids, named IAXO compounds, active as TLR4 antagonist [13] were developed by the research group headed by Professor Peri at the University of Milano Bicocca, Italy. Similarly to other cationic amphiphiles [14] IAXO glycolipids are thought to modulate TLR4 activation and signalling *in vitro* and *in vivo* by interfering selectively with the TLR4 co-receptors CD14 and MD-2 [15–17].

The main aim of this study was to investigate the potential of novel mimetic TLR4 antagonist, IAXO-102, for pharmacological intervention in experimental AAA. Our results determined the ability of IAXO-102 to negatively regulate a non-hematopoietic TLR4 signalling and inhibit experimental AAA, suggesting the potential therapeutic use of this TLR4 antagonist for treatment of degenerative vascular diseases.

2. Materials and methods

2.1. Materials

TLR4 antagonists IAXO-102 and LPS (*Salmonella minnesota* (Re) R595, TLRpure™) were kindly provided by Innaxon, Tewkesbury. For *in vitro* experiments IAXO-102 (10 mM) was reconstituted in ethanol. For *in vivo* experiments IAXO-102 was reconstituted in Lipodisq™, a biodegradable liposomal nano-disc formulation (Malvern Cosmeceutics, Malvern, UK) IAXO-102 in Lipodisq™ was formulated and prepared by Innaxon, Tewkesbury. FITC conjugated IAXO-102 was prepared by Professor Peri (University of Milan).

2.2. HUVEC

Human umbilical cord vein endothelial cells (HUVEC), purchased from Promocell (Heidelberg, Germany), were treated in accordance to the company's instructions. The cells were maintained at 37 °C at 5% constant atmospheric condition of CO_2 in endothelial cell growth medium 2 (Promocell) in 25 cm^2 flasks precoated with 1% attachment factor (Sigma UK). For all treatments, HUVEC were used between passages 3 and 5. HUVEC were pretreated with IAXO-102 (0–10 μM) for 1 h, then exposed to LPS (100 ng/ml) for 1 or 16 h.

2.3. Animal model

All animal experiments were approved by the local Animal Research Work Ethical Review Board at St George's, University of London. AAAs were induced in 6-month-old ApoE deficient/C57Bl6, as described previously [18]. Two *in-vivo* experiments were performed; animals were sacrificed at 3 days (protein), and at 28 days (aortic diameter). Two groups ($n = 10$) were infused with Angiotensin II (1 μg/min/kg), and the third ($n = 6$) was infused with saline. IAXO-102 was co-administered s.c. (3 mg/kg/day, 50 μl Lipodisq™) and the remaining two groups were co-administered s.c. with drug vehicle (50 μl Lipodisq™). At day 28, following Angiotensin II infusion, computer-assisted micrometry measurements defined AAAs by reference to normal aortic measurements, with an aneurysm being present when the maximum diameter of the aorta exceeded 50% of the normal diameter; all measurements were taken whilst the aorta remained *in-situ*. In the sham-operated group, the mean diameter of the suprarenal aorta was 0.86 mm, and an aneurysm was deemed to have formed when the maximum diameter of the suprarenal aorta exceeded 1.29 mm.

2.4. Tissue collection and processing

Termination of the experiments was scheduled at 3 days after insertion of the osmotic pumps. Animals were trans-cardially perfused, at physiological pressures, with phosphate buffered saline containing a cocktail of proteinase inhibitors (Sigma Aldrich, UK) for 10 min at 4 °C, after which the aortic tree was dissected at 4 °C, removing the loose connective tissue around the exterior of the artery. The thoracic, supra-renal and infra-renal aortic segments were harvested, frozen in liquid nitrogen, and stored at −80 °C until processed. Tissues were ground to a fine powder under liquid nitrogen and then lysed by sonication in a non-denaturing phosphate lysis buffer (20 mM sodium phosphate, 137 mM NaCl, 25 mM sodium β-glycerophosphate, 2 mM sodium pyrophosphate, 2 mM EDTA, 10% glycerol, 1% Triton X-100 and protease inhibitor cocktail; Sigma–Aldrich UK). Cell lysates were incubated on ice for 20 min and centrifuged for 20 min at 13,000 rpm at 4 °C. Supernatants were removed and the pellet of insoluble material discarded. Protein concentration, of the cell supernatant, was determined by the bicinchoninic acid method (Pierce, Rockford, IL).

2.5. Western blot analysis of protein expression and phosphorylation

Cell or tissue lysates (20 μg) were separated on a 10% SDS-PAGE gel and transferred to polyvinyl difluoride membranes (Bio-Rad, UK) and blocked using 5% (wt/vol) skimmed milk in Tris-buffered saline (TBS)/0.1% (vol/vol) Tween-20 for 1 h at room temperature. Blots were incubated overnight at 4 °C with primary antibodies: phospho-JNK, phospho-p38, phospho-ERK, phospho-p65 NF-kB and TLR4 from Cell Signalling Technology (NEB, Herts, UK) (1:1000 dilution in TBS, 1% milk). After washing in TBS/0.1% (vol/vol) Tween-20, blots were incubated with HRP-conjugated antibody at room temperature for 1 h in TBS/0.1% (vol/vol) Tween-20 and 5% milk. After the final wash, immunoreactivity was visualized using a chemiluminescent substrate ECL Plus (Healthcare, Bucks, UK). Densitometric analysis was performed using GS-800 Calibrated Densitometer (Bio-Rad UK). The level of cellular actin was used as a loading control.

2.6. Inflammation antibody array

Tissue protein lysates (20 μg) from supra-renal aortic segment were semi-quantitatively analysed on *Inflammation Antibody Arrays* (Ray-BioTech, containing 40 proinflammatory proteins) following manufacturer's instructions. Briefly, antibody array membranes were incubated with protein lysates and then incubated with antibody array biotinylated antibody. Finally the membranes were incubated with streptavidin HRP-conjugated antibody. Immunoreactivity was visualized using a chemiluminescent substrate.

Densitometric analysis was performed using GS-800 Calibrated Densitometer (Bio-Rad, UK).

2.7. ELISA

Human MCP-1 and IL-8 and mouse MIP-1γ production were measured in cell and tissue lysates (20 μg protein) using ELISA kits (Ray-Bio Tech, USA) following the manufacturer's instructions. At the final stage absorbance was measured at 450 nm using a microplatereader SpectraMax-340 (Molecular Devices, USA). Protein concentration was determined using SoftMax Pro5 software package (Molecular Devices, USA).

2.8. Statistical analysis

Data are reported as mean ± SD and analysed with one-way Anova followed by the *Bonferroni post-hoc test* for multiple comparisons using GraphPad Prism version 4.0. A value of $P < 0.05$ was considered significant.

3. Results

3.1. IAXO-102 negatively regulates TLR4 signalling in HUVEC

Changes in the functional activity of vascular endothelial cells have been shown to play a critical role in vascular inflammatory based diseases [19]. To test the ability of IAXO-102 to modulate *in vitro* TLR4 signalling pathways we utilised HUVEC as an *in vitro* model. To determine the effect of IAXO-102 on TLR4 signalling in HUVEC we analysed two readouts: activation of MAPK/NF-kB as a second messengers in TLR4 signalling and production of TLR4 dependent proinflammatory proteins.

Initially, we evaluated the effect of IAXO-102 on HUVEC viability. HUVEC cells were exposed to different concentrations of IAXO-102 (0–20 μM) in the presence or absence of LPS (100 ng/ml) for up to 24 h. Results from MTT assay demonstrated that IAXO-102 did not affect cell viability at concentrations up to 10 μM, however, IAXO-102 at concentration of 20 μM reduced significantly HUVEC viability (Fig. 1 Supplement). Based on these data we have used IAXO-102 up to 10 μM in the next series of experiments.

To investigate the effect IAXO-102 of MAPK/NF-kB activation in TLR4 signalling, HUVEC were pre-treated with IAXO-102 (1–10 μM) for 1 h and then exposed to LPS (100 ng/ml) for additional 1 h. Western blot data revealed that JNK, p38, ERK and p65 NF-kB phosphorylation was elevated in response to LPS in HUVEC (Fig. 1 and Fig. 2 Supplement). Pre-treatment of HUVEC with IAXO-102 clearly demonstrated the ability of this small molecule to significantly inhibit LPS-stimulated MAPK/p65nF-kB phosphorylation. Importantly, the blocking effect of IAXO-102 was evident at concentrations of 10 μM, and to lesser extent at concentration of 1 μM (Fig. 2 Supplement).

Having shown that IAXO-102 negatively regulated activation of TLR4 second messengers MAPK/p65NF-kB in TLR4 signalling, we next explored whether this small molecule could have an impact on LPS-driven production of pro-inflammatory proteins in HUVEC. Cells were pretreated with IAXO-102 (10 μM) for 1 h and then exposed to LPS (100 ng/ml) for additional 16 h. Cell lysates were analysed on human *Inflammation Antibody Array* (containing 40 proinflammatory proteins). The semi-quantitate analysis demonstrated that LPS upregulated 20/40 proinflammatory proteins. In contrast, IAXO-102 pretreatment inhibited, to different extent the expression of 17/20 LPS driven proinflammatory proteins (Table 1 Supplement). MCP-1 and IL-8 are previously reported proinflammatory proteins which are produced in vascular endothelial cells in response to LPS and other inflammatory triggers [19]. They are both immune chemotactic factors and extravasation of immune cells is an early event in the generation of lesions in this model. Therefore, we validated the negative effect of IAXO-102 on MCP-1 and IL-8 expressions by ELISA. Again, IAXO significantly blocked LPS driven MCP-1 and IL-8 production in HUVEC (Fig. 2).

These data demonstrate that IAXO-102 is a negative regulator of TLR4 signalling in HUVEC.

3.2. IAXO-102 downregulates TLR4 signalling in mouse aorta

Regression of experimental AAA by deletion of TLR4 was previously demonstrated, suggesting that TLR4 may represent a novel therapeutic target to aneurysms treatment [8]. To test the potency of IAXO-102 to modulate *in vivo* TLR4 signalling pathways we utilised the Angiotensin II-driven experimental aneurysm development as an *in vivo* model. Our previous studies demonstrated that infusion of Angiotensin II in Apo E deficient mice induced the inflammatory process which was associated with an increase of TLR4 expression and production of proinflammatory proteins at 72 h in mouse aorta [20].

Initially, we performed pharmacokinetic study to investigate the distribution and clearance of IAXO-102-FITC (1 and 3 mg/kg) following s.c. injection of IAXO-102-FITC in Apo-E deficient C57BL/6 mice. Results demonstrated that IAXO-102-FITC was distributed in different compartments of the animals and the signal was stable up to 24 h of 1 mg/kg and up to 72 h of 3 mg/kg (data are not shown). Analysis of target organs following 24 h injection showed that IAXO-102-FITC was present in the bladder, small and large intestine, to some extent, in kidney or liver (data are not shown). In addition, FACS analysis of the blood taken from animals demonstrated that IAXO-102-FITC was bound mainly to monocytes but not to lymphocytes or granulocytes (Fig. 3 Supplement).

We monitored the effect of IAXO-102 in Lipodisq™ carrier on TLR4 signalling *in vivo* on two levels: activation of MAPK/p65NF-kB and production of proinflammatory proteins in three regions of the mouse aorta at day 3 of Angiotensin II infusion. Our results demonstrated that JNK, ERK and p65 NF-kB phosphorylation was significantly elevated in three regions (thoracic, supra-renal and infra-renal) of mouse aortas following Angiotensin II infusion (Fig. 3). In comparison with our previous published observations [20.21] we found some differences in the JNK/ERK phosphorylation profile of different aortic segments that might be affected by the nature of carrier and time of administration. Western blot analysis clearly demonstrated the ability of IAXO-102 to significantly inhibit Angiotensin II-stimulated JNK/ERK/p65NF-kB phosphorylation (Fig. 3). Furthermore, the blocking effect of IAXO-102 was evident in all three regions of mouse aortas.

We have previously reported that TLR4 receptor was dramatically up-regulated in mouse aorta peaking 3 fold at 72 h following Angiotensin II infusion [20]. Importantly, TLR4 up-regulation was evident only in supra-renal but not in thoracic and infra-renal aortic regions. Furthermore, immunoblotting data revealed that TLR4 expression was significantly down-regulated following co-treatment with IAXO-102, suggesting that this site-specific effect could be associated with modulation of experimental AAA formation (Fig. 4B).

We have previously documented that infusion of Angiotensin II-induced production of a number of proinflammatory proteins in the early stages of AAA development [20,21]. Having shown that IAXO-102 could negatively regulate the second messengers' activation in TLR4 signalling and TLR4 expression, we next explored whether this modulating effect could have an impact on Angiotensin II-driven production of pro-inflammatory proteins in the initiation stage of experimental aneurysm. Mouse tissue lysates were analysed on a human *Inflammation Antibody Array* (containing 40

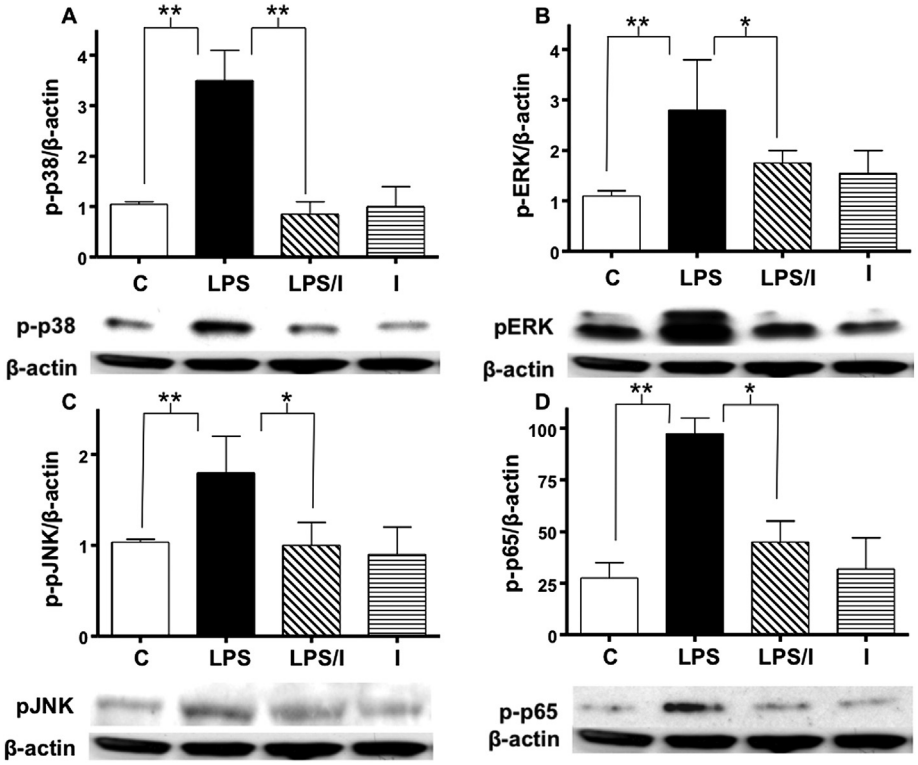

Fig. 1. IAXO-102 inhibits LPS-induced MAPK/p65 NF-kB signalling in HUVEC. Cells were pre-incubated with IAXO-102 (I) (10 μM) for 1 h and then exposed to LPS (100 ng/ml) up to 60 min. Cell lysates were analysed for p38 (A), ERK (B), JNK (C) and p65 NF-kB (D) phosphorylation using immunoblotting analysis. Actin was used as a loading control. Data are mean ± SD, $n = 4$ at each data point. $p^{*}<0.05$ and $p^{**}<0.01$ [Anova].

proinflammatory proteins). This semi-quantitative analysis demonstrated that Angiotensin II infusion upregulated 22/40 proinflammatory proteins. In contrast, IAXO-102 pretreatment inhibited, to varying extent, the expression of 14/22 Angiotensin II driven proinflammatory proteins (Table 2 Supplement). Next, we validated the blocking effect of IAXO-102 on MIP-1γ expression by ELISA. MIP-1γ production was significantly elevated in all three regions of mouse aorta. Again, IAXO-102 treatment significantly inhibited production of MIP-1γ in all three regions of the mouse aorta (Fig. 4A).

These data demonstrate that IAXO-102 treatment is a negative regulator of Angiotensin II driven inflammation and TLR4 signalling is the target by which IAXO-102 exerts its effect.

3.3. IAXO-102 inhibits the rupture, incidence and development of experimental AAA

In our experimental model Angiotensin II infusion produced mainly suprarenal AAA which was measured by computer-assisted micrometry four weeks following Angiotensin II infusion. In addition, two mice from Angiotensin II only treated group developed a thoracic aneurysm along with suprarenal AAA. Micrometry data clearly demonstrated that co-treatment with IAXO-102 (3 mg/kg/day) significantly retarded Angiotensin II-induced increase in aortic diameter (Fig. 5A). Aortic diameter in the IAXO-102 group was significantly smaller (1.055 ± 0.081 mm) than those in the positive control group (1.865 ± 0.501) at four weeks following Angiotensin II infusion. (Fig. 5 and Table 1). Furthermore, 30% of animals died of aortic rupture and 86% of them developed AAA in the positive

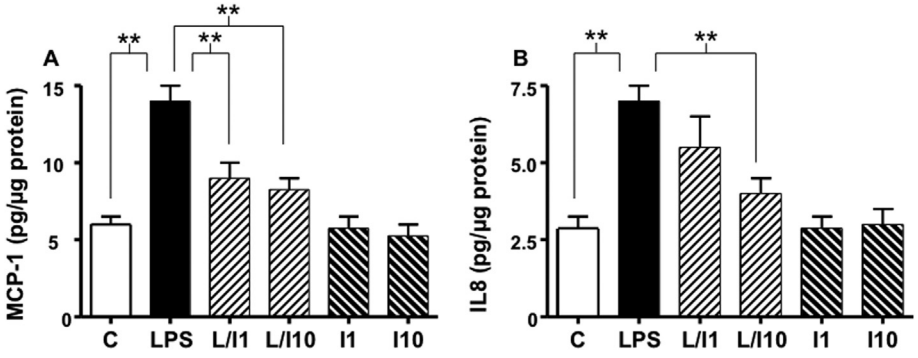

Fig. 2. IAXO-102 down regulates LPS driven MCP-1 and IL-8 production in HUVEC. Cells were pre-incubated with IAXO-102 (I) (1–10 μM) for 1 h and then exposed to LPS (100 ng/ml) up to 24 h. Cell lysates were analysed for MCP-1and IL-8 expression using ELISA analysis. Data are mean ± SD, $n = 4$ at each data point. $p^{**}<0.01$ [Anova].

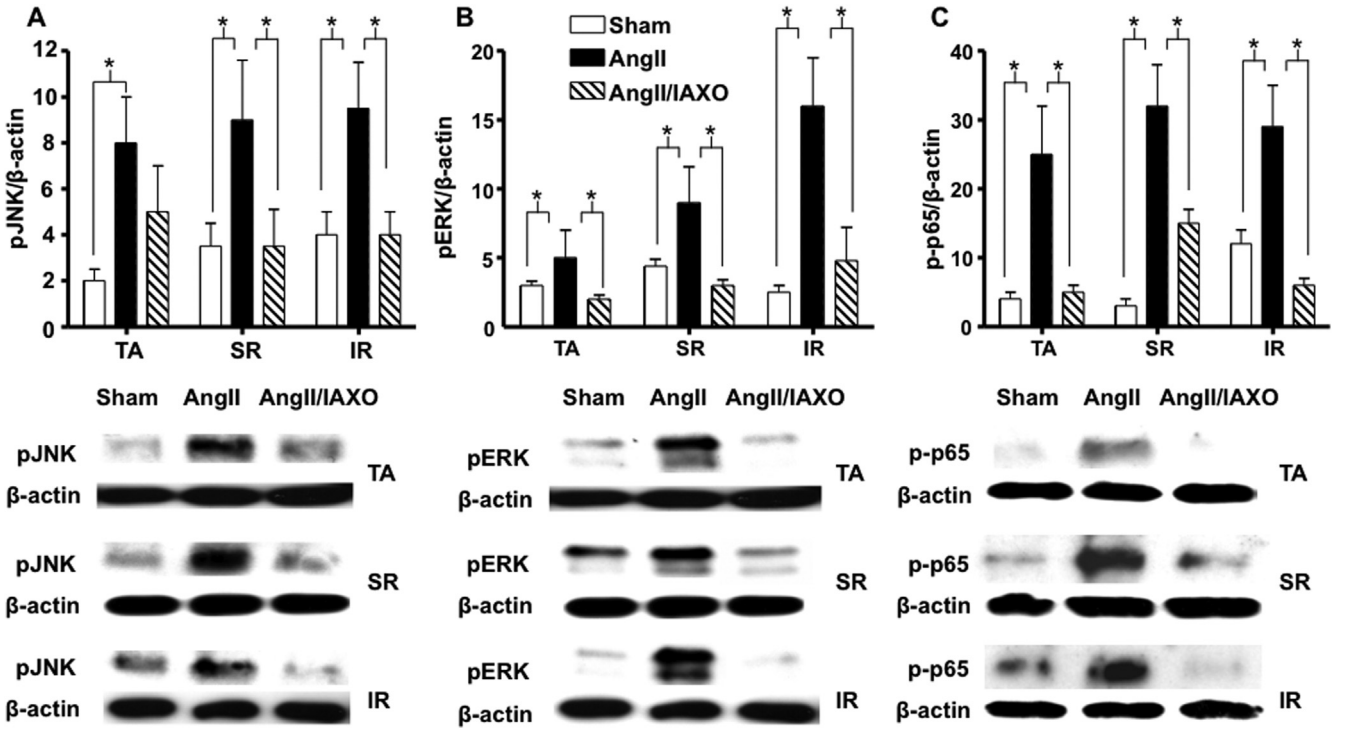

Fig. 3. IAXO-102 inhibits Angiotensin II-induced JNK, ERK and p65 NF-kB phosphorylation in the initiation stage of experimental AAA. Mice were divided into three groups: sham control negative group, Angiotensin II group, Angiotensin II/IAXO-102 co-treated group. IAXO-102 (3 mg/kg/day in 50 μl Lipodisq™) was administered s.c. up to 72 h. Tissue samples from thoracic aorta (TA); supra-renal aorta (SR) and infra-renal aorta (IR) were prepared at 72 h, and soluble proteins were analysed for JNK phosphorylation (A), ERK phosphorylation (B) and p-65 NF-kB phosphorylation (C) using immunoblotting analysis. Actin was used as a loading control. Data are mean ± SD, $n = 4$ at each data point. $p^*<0.05$ [Anova].

Angiotensin II infused group (Table 1). In contrast, IAXO-102 completely abolished rupture, reduced aneurysm formation to the level of 30% (Table 1) and increased mice survival (Fig. 5B). All these data strongly support the clinical effect of IAXO-102 on reducing aneurysm development and preventing aneurysm rupture.

4. Discussion

Although, primarily through cessation of smoking, the incidence of AAAs are globally less, there remain a large number of AAAs which will require eventually repairing by the costly, high risk, surgical process. Currently, management of modifiable risk factors and surgical repair are the only redress for AAAs. The development of a drug treatment for AAA remains a worthy goal with a great commercial impact.

We show that a small molecule antagonist of TLR4 (IAXO-102) has the potential to inhibit experimental AAAs, through a mechanism associated with its ability to prevent vascular inflammation.

Inflammation has been documented as an early and critical event in aneurysm formation. In this regard, a number of studies have shown the essential role of TLR4 in several cardiovascular pathologies, suggesting the benefits of modulating TLR4 signalling pathways [22]. Pharmacological intervention using TLR4

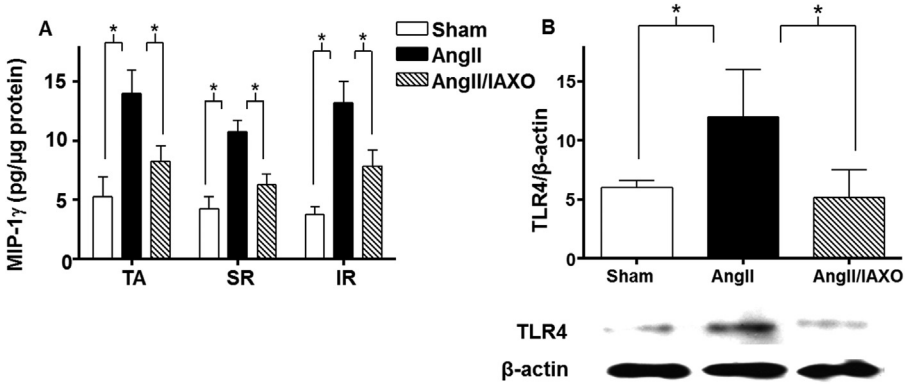

Fig. 4. IAXO-102 down regulates Angiotensin II-induced MIP-1γ expression and TLR4 expression in the initiation stage of experimental AAA. Mice were divided into three groups: sham control negative group (S), Angiotensin II group (A), Angiotensin II/IAXO-102 co-treated group (A/I). IAXO-102 (3 mg/kg/day in 50 μl Lipodisq™) was administered s.c. up to 72 h. Tissue samples from thoracic aorta (TA), supra-renal aorta (SR) and infra-renal aorta (IR) were prepared at 72 h, and soluble proteins were analysed for mouse MIP-1γ expression (TA, SR and IR) using ELISA (A) and TLR4 expression (SR) (B). Data are mean ± SD, $n = 4$ at each data point. $p^*<0.05$ [Anova].

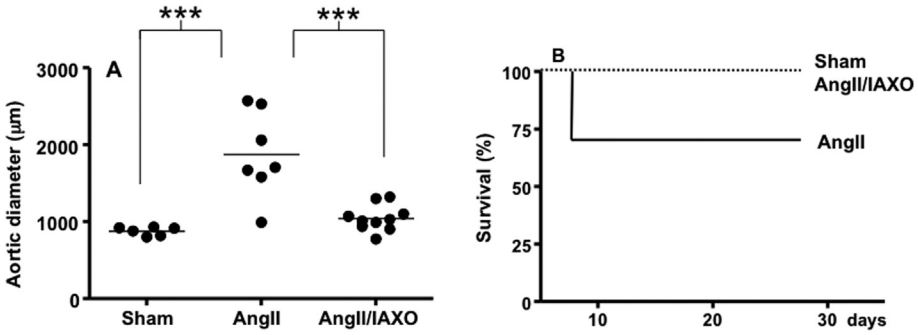

Fig. 5. IAXO-102 inhibits Angiotensin II-induced development, incidence and rupture of experimental AAA and increases mice survival. Twenty six 6 month old male Apo-E deficient C57BL/6 mice were divided into three groups: sham control negative group, Angiotensin II group, Angiotensin II/IAXO-102 co-treated group. All animals had osmotic pumps implanted at time 0. The sham group (n = 6) had pumps delivering saline whereas the other two groups (n = 10) had pumps delivering Ang II (1 μg/min/kg). The Angiotensin II/IAXO-102 co-treated group received s.c. injections of IAXO-102 (3 mg/kg/day in 50 μl Lipodisq™) whereas the other two groups received daily s.c. injections of vehicle (Lipodisq™). Animals were euthanized at day 28 after pump implementation and the maximal aortic diameter was measured by computer micrometry. Data are mean ± SD, *n* = as indicated. p***<0.001 [Anova].

Table 1
IAXO-102 inhibits expansion, incidence and rupture of experimental AAA.

Groups	Mice number	Early rupture (%)	AAA incidence (%)	Aortic diameter (mm ± SD)
Sham	6	0	0	0.86 ± 0.08
Ang II	10	30	86	1.86 ± 0.56
Ang II/IAXO-102	10	0	30	1.05 ± 0.18

antagonists has been a challenging approach for the last two decades, however they failed in different stages of clinical trials and generation of new TLR4 modulators is of great interest [11,12].

We, and other groups, have found that early stages of experimental AAA development are associated with production of proinflammatory proteins in the experimental model of Angiotensin II infusion in hypercholesterolemic mouse [20,23]. TLR4 signalling plays an essential role in propagation of inflammation and mediates production of large proportion of these proinflammatory proteins. Recently, it was reported that TLR4 deficiency attenuated AAA and atherosclerosis development, suggesting that TLR4 signalling is fundamental in these vascular pathologies [8]. Intriguingly, TLR4 signalling exerted effects through non-hematopoietic cell types, suggesting that vascular cells might use TLR4 signalling network in response to inflammatory environment [8].

We examined the potential of IAXO-102 to modulate *in vivo* TLR4 signalling utilising a well-established model of Angiotensin II driven AAA in hypercholesterolemic Apo E deficient mouse. Using nano-carriers, Lipodisq™ we were able to deliver the required concentration of IAXO-102 which has poor solubility in organic solvents. A similar nano-particle approaches to drug delivery has been used for administration of anticancer compounds, such as Doxorubicin for targeted delivery [24]. Pharmacokinetic studies using IAXO-102-FITC demonstrated that the dose of 3 mg/kg was sufficient to produce stable signal and drug distribution among several organs. In addition, blood sample analysis revealed that IAXO-102 in predominantly bound to monocytes but not to lymphocytes or granulocytes. Data from the literature have shown a differential TLR4 or CD14 expression among different types of immune cells including lymphocytes and granulocytes [25]. In our hands, we found that TLR4/CD14 are expressed in vascular cells from human or mouse origin (data are not shown). Since IAXO-102 has been shown to interfere with TLR4/CD14 complex, it is possible that IAXO-102 requires the presence of this complex for modulation of TLR4 signalling in different types of immune and non-immune cells [26].

It has been well established that Angiotensin II infusion induced a site specific AAA formation in suprarenal region of the mouse aorta [27]. In this study, we examined not only the supra-renal region of mouse aorta, but also the thoracic and infra-renal regions. Comparative analysis of signal events in those three aortic regions are a useful tool to identify site specific targets or signalling events for IAXO-102 action.

Several evidences from the literature, including our data, highlight the role of MAPK in AAA development [20,21,28]. All three subclasses of MAPK have been shown to be activated in Angiotensin II-induced AAA formation. JNK has been shown as important target for AAA treatment demonstrating that JNK inhibitor markedly reduces aneurysmal size [29]. Previously, we have identified JNK as a non-site specific target by which Rosiglitazone inhibited inflammatory responses and AAA development in this experimental model [20]. Similarly, in this study IAXO-102 efficiently inhibited JNK/ERK phosphorylation in all three origins of mouse aorta. Notably, this small molecule was able to block a suprarenal site of Angiotensin II-induced upregulation of TLR4. IAXO-102 was originally generated as a small molecule to block TLR4/CD14 interactions [26]. It is possible therefore, by blocking formation of TLR4/CD14 complex IAXO-102 to stimulate internalization and degradation of TLR4 rather to have an impact on TLR4 gene expression. Further studies are needed to clarify this issue.

Similarly to Rosiglitazone in this model, IAXO-102 negatively regulated 50% of mouse TLR4 dependent proinflammatory proteins. Validation analysis demonstrated that IAXO-102 downregulated MIP-1γ in all three regions of mouse aorta, suggesting that this effect is not site specific. Activation of NF-kB signalling plays a critical role in production of TLR4 dependent proteins. In this study, IAXO-102 administration inactivated NF-kB pathways supporting its potential application for treatment of vascular inflammatory based diseases.

These results clearly demonstrated the ability of IAXO-102 to block the progression of inflammation by inhibiting the important TLR4 dependent proinflammatory mediators in the mouse aorta.

Although the protein sequence of TLR4 is conserved across

species, they differ considerably with regard to their function, for example with regard to ligand discrimination or adaptor selection between human and mouse TLR4 [30]. Our data strongly confirm the ability of IAXO-102 to block both human and mouse TLR4 signalling pathways. In contrast, TLR4 blocking antibodies can inhibit TLR4 from specific species which make them unsuitable to be used in both animal and human model systems. In support, our screening analysis of several anti-human monoclonal TLR4 blocking antibodies demonstrated their ability to affect human but not mouse TLR4 (data are not shown). The fact that IAXO-102 was initially designed to block TLR4/CD14 interactions may explain its broad biochemical properties [26].

Furthermore, this study also shows the potential of IAXO-102 to modulate TLR4 signalling in response to distinct TLR4 ligands. In our *in vitro* cell system (HUVEC) IAXO-102 blocked TLR4 in response to well-known TLR4 specific agonist LPS. Data from the literature indicates that the elevated level of cholesterol is a critical factor for Angiotensin II induced experimental aneurysm, as wild type mice do not develop aneurysms when infused with Angiotensin II [31,32]. It has been demonstrated that artificial Angiotensin II infusion triggers 'sterile' inflammation, the process that is negatively regulated by TGF-β signalling which controls overproduction of proinflammatory proteins, excessive macrophage activation, inhibition of matrix degradation and preservation of smooth muscle cells survival in a wild type mice [33]. Furthermore, TGF-β signalling has been reported to mediate the anti-inflammatory modulation of TLRs including TLR4 [34].

Angiotensin II infusion triggers generation of reactive oxygen species leading to formation of oxidised LDL in hyperlipidemic mice [27]. In this relation we suggest that this small molecule IAXO-102 inhibited Angiotensin II-induced TLR4 signalling activated by ligands of 'sterile' inflammation such as oxidised LDL or small fragments of hyaluronan. In support to this notion, recent data have demonstrated that oxidised LDL activated TLR4 signalling in vascular endothelial cells [35]. In addition, a small fragments of hyaluronan have the potential to activate TLR4 pathways in vascular endothelium [36]. Furthermore, we found that IAXO-102 partially downregulated TLR4 signalling in response to minimally-oxidised LDL and small fragments of hyaluronan in HUVEC [unpublished data]. This is an important issue because different TLR4 ligands determine a specific signature in production of TLR4 dependent proteins with various outcomes. On the other hand, it is believed that TLR4 activation by 'sterile' ligands from non-bacterial origin are likely to contribute to development of vascular diseases including aneurysms [37].

Importantly, we have demonstrated that IAXO-102 exerted a protective effect on aneurysm formation, incidence and rupture suggesting the clinical relevance of using this compound for treatment of AAAs. To date, Shang and colleagues have demonstrated that Tanshinon IIA, a lipophilic component isolated from *Salvia multiorrhiza*, inhibits elastase-induced AAA through TLR4/MyD88 signalling, suggesting that TLR4 is an important pharmacological target for treatment of AAA [38].

In conclusion, the results from this study have demonstrated for the first time that a novel mimetic IAXO-102, the TLR4 antagonist, is effective in blocking vascular inflammation leading to inhibition of experimental AAA development. While further studies are required to investigate the modulating effect of IAXO-102 on other processes (cell death, remodelling of extracellular matrix or neoangiogenesis) associated with this complex vascular disease the findings from this study suggest that IAXO-102 is a potential new drug for pharmacological intervention of aneurysms.

Conflicts of interest

FN is founder of and holds shares in Innaxon.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.atherosclerosis.2015.08.010.

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