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Chapter 5

Toll-like receptor 4 inhibitor TAK-242 treatment does not influence perfusion recovery in tissue ischemia

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Toll-like receptors (TLRs) are important in innate immune responses, which are crucial in collateral artery formation (arteriogenesis). TLR4−/− mice undergoing hind limb ischemia show decreased perfusion recovery accompanied by an impaired infiltration of inflammatory cells. TLR antagonists are currently developed and tested with the objective to inhibit acute exacerbation of organ damaging immune responses. However, systemic inhibition of innate immune responses may negatively influence arteriogenesis. In this study, we evaluated if TLR4 inhibition by a potent TLR4 inhibitor (TAK-242) would negatively influence perfusion recovery in a mouse model for arteriogenesis. Whole blood from human and mouse origin was stimulated with the TLR4 ligand lipopolysaccharide (LPS) following TAK-242 incubation. After stimulation, cellular TLR4 activation was measured using FACS and Tumor Necrosis Factor alpha (TNF-α) release was measured using ELISA. Next, the effect of TAK-242 was tested in a mouse model for arteriogenesis on perfusion recovery. TLR4 responses measured by TNF-α levels were inhibited by TAK-242 in human and mouse blood after long-term stimulation. TAK-242 attenuated TLR4 responses in vivo, but did not inhibit perfusion recovery in mice.

In conclusion, TAK-242 does not negatively influence perfusion recovery following hind limb ischemia despite its TLR4 inhibiting properties.
INTRODUCTION

Arterial occlusive diseases are a common cause of death. Patients suffering from peripheral artery disease (PAD) suffer from poor tissue perfusion due to local arterial occlusion in larger arteries, often caused by atherosclerosis. Adaptive collateral artery growth (arteriogenesis) is characterized by enlargement of pre-existing vascular anastomoses as a result of local arterial occlusion and subsequent increase in shear stress. However, arteriogenesis is often inadequate to prevent tissue ischemia in patients suffering from PAD.

Arteriogenesis is characterized as an inflammatory process, in which activation and local tissue infiltration of monocytes and T lymphocytes play an important role. Toll-like receptor (TLR) signaling is essential for perfusion recovery after arterial occlusion. TLRs are receptors of the innate immune system and expressed by all leukocytes, but also on several other vascular cell types, like endothelial cells and fibroblasts. The innate immune system is the first line of defense against exogenous pathogens, but also regulates the inflammatory responses in acute and chronic cardiovascular diseases in response to pathogen associated molecular patterns (PAMPS). Next to arteriogenesis, TLRs are involved in atherosclerosis and myocardial ischemia/reperfusion (I/R) injury.

Previously, we have shown that mice lacking TLR4 suffered from impaired perfusion recovery and less infiltration of monocytes in the local ischemic tissue. Furthermore, specifically the TLR4 expression on the bone marrow-derived cells and not the resident vascular cells explained the differences in arteriogenic responses after ischemia. Although this knockout model revealed that inborn deficiency of TLR4 is related to impaired perfusion recovery, it remains unclear whether in vivo administration of TLR4 antagonists in mice with normal TLR4 expression is able to negatively influence perfusion recovery after hind limb ischemia. TLR4 antagonists effectively inhibit TLR4 signaling, resulting in down toning of the immune response. Moreover, they have shown to work beneficial for inflammatory diseases. This includes treatment of elevated blood pressure in mice, injury following myocardial infarction in mice, prevention of experimental endotoxemia in guinea pigs, attenuation of inflammation after lung injury in mice and finally, reducing early stage atherosclerosis in diabetic ApoE-/- mice. One potent TLR4 inhibitor, ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl] cyclohex-1-ene-1-carboxylate (TAK-242), exclusively blocks TLR4 signaling by inhibiting ligand-induced intracellular signaling without inhibiting ligand binding to cells. Furthermore, TAK-242 strongly suppresses TLR4-mediated cytokine (e.g. Tumor Necrosis Factor (TNF)-α) and nitric oxide (NO) production by murine and human monocytes and macrophages. Sepsis and endotoxin mouse models have shown that TAK-242 treatment attenuated the TLR4 response, which resulted in lowering of cytokine production. Furthermore,
survival rates drastically increased after TAK-242 treatment following lipopolysaccharide (LPS) challenge. Based on the immune suppressive effects defined above, treatment with TAK-242 could result in unwanted side effects, such as impaired tissue perfusion during organ ischemia.

In this study, we evaluated the effect of in vivo treatment with TAK-242 on perfusion recovery following tissue ischemia and thus examined if the compound would mimic TLR4 deficiency in a mouse model for arteriogenesis.

MATERIALS AND METHODS

Ethics statement
All animal experiments in the present study were approved by the university animal experimental committee of Leiden University Medical Center and Utrecht University following the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996).

Reagents
TAK-242 (ethyl (6R)-6-[N-(2-chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate) was synthesized and emulsified at Takeda Pharmaceutical Co. Ltd (Osaka, Japan). Lipopolysaccharide (LPS) (Escherichia coli 055:B5) was purchased from Sigma Aldrich (St Louis, MO, USA) and diluted in sterile phosphate buffered saline (PBS).

Whole blood stimulations
Whole blood from healthy volunteers and C57Bl/6J mice was collected in heparinized tubes. Blood was incubated with TAK-242 (0.3 mg/ml) for 30 minutes, followed by incubation with different LPS concentrations (0-500 ng/ml) at 37 °C. Dosage of TAK-242 was based on earlier studies. PBS stimulation served as a control. For CD11b and L-selectin expression, human and mouse whole blood were incubated with LPS in a 96-wells plate for 30 minutes at 37 °C followed by FACS analysis. For TNF-α expression measurements, human whole blood was incubated with LPS for 2 hours at 37 °C and mouse whole blood was incubated overnight (20 hours) at 37 °C. Whole blood was centrifuged at 300x g, for 5 minutes and plasma was stored for TNF-α measurements.

Flow cytometry
LPS stimulation on whole blood was followed by staining with FACS antibodies. The following antibodies were used: CD14 RPE-Cy5 (monocytes, Serotec), CD66b PE (neutrophils, Biolegend), CD11b PE-Cy7 (BD), CD62L ECD (L-selectin, Beckman Coulter). For mouse whole blood, the following antibodies were used: F4/80 Alexa-647 (monocytes,
eBioscience), Ly-6G PE (neutrophils, eBioscience), CD11b FITC (Bioconnect) and CD62L PE-Cy7 (Abcam).

**Enzyme-linked immunosorbent assay**

TNF-α levels in plasma from stimulated whole blood was quantified using sandwich ELISA kits according to manufacturer's instructions (BendermedSystems for human TNF-α; R&D Systems for mouse TNF-α).

**Animals, in vivo testing and operation procedures**

All procedures were performed on male C57BL/6J mice and started when the animals were at 10-12 weeks of age. For *in vivo* testing of TAK-242 in mice, TAK-242 was dissolved in poly ethylene glycol (PEG)-400 (1.2 mg/ml for i.m. injection). Dosage was based on earlier *in vivo* studies with TAK-24215, 16. Mice received a single dose of placebo (PBS) or TAK-242 either in the adductor muscle. Twenty four hours after i.m. injection whole blood was collected for LPS stimulation via tail vein cuts. Blood samples were incubated with different concentrations of LPS (0 ng/ml; 125 ng/ml; 250 ng/ml) for 20 hours at 37 °C. Whole blood was centrifuged and supernatant was taken off for TNF-α measurements.

For *in vivo* testing of TAK-242 effects during perfusion recovery, mice received TAK-242 dissolved in PEG-400 (5mg/ml) infused in Alzet osmotic micro-pumps (DURECT corp., model 1007D; delivery rate 0.5 ul/h, 7 days) and primed overnight at 37 °C according to manufacturer’s instructions. Delivery rate of TAK-242 was 3.0 mg/kg/day15, 16. Micro-pumps were placed subcutaneous (s.c.) between the scapulae, followed by unilateral double electrocoagulation of both femoral artery and iliac artery. Perfusion recovery was monitored using Laser-Doppler perfusion imaging (LDPI) (Moor Instruments, Devon, UK). LDPI measurements were performed before and after surgery and were continued until 28 days with intermediate measurements at day 3, 7, 10, 14 and day 21. The surgical procedure has been described in detail previously17. Perfusion recovery in the ischemic limb is expressed as a percentage of the contralateral non-ischemic limb perfusion. Blood was collected via a tail vein cut at day 3 and 7 and analyzed for TNF-α levels.

TAK-242 was also tested *in vivo* via a single i.m. injection in the right adductor muscle followed by unilateral femoral artery ligation. Mice underwent unilateral femoral artery ligation and received follow-up of perfusion recovery using LDPI at day 4 and 7 and were terminated at day 7. The surgical procedure has been described in detail previously18.
Statistical analysis

SPSS version 20.0 (Chicago, IL, USA) was used for statistical analyses. Comparisons between means were analyzed with an independent T test. Differences between means with p-values <0.05 were regarded as statistically significant. Data are expressed as mean ± SEM.

RESULTS

Ex vivo TAK-242 effects on TLR4-induced cell activation

Heparinized human whole blood from a healthy volunteer and whole blood from a C57BL/6J mouse was stimulated with different LPS concentrations with or without pre-incubation of TAK-242. With FACS analysis, expression of CD11b and L-selectin on monocytes and neutrophils were analyzed.

As expected, monocytic CD11b expression was upregulated and L-selectin was shed with concomitant downregulated expression following LPS stimulation of human whole blood. TAK-242 pre-incubation inhibited this LPS-induced activation. TLR4 inhibition by TAK-242 appeared more effective following administration of the lower LPS concentrations (0.01 ng/ml to 1 ng/ml) compared with higher LPS concentrations (10 ng/ml and 100 ng/ml) (Figure 1A). Neutrophils revealed similar patterns where TAK-242 affected CD11b and L-selectin expression at higher LPS concentrations as well (Figure 1B). In mice, hardly any inhibitory effect was observed of TLR4 activation by TAK-242 on cell-based activation markers (Figure 2A-B).

Levels of TNF-α were measured in plasma from heparinized whole blood both from a healthy donor as well as C57BL/6J mice at baseline following LPS stimulation. A dose-dependent TNF-α production could be observed after LPS stimulation. Pre-incubation of TAK-242 resulted in dramatic decrease of TNF-α production in human (100% decrease, Figure 3A) as well as mouse whole blood (88-91% decrease, Figure 3B). For human blood, TNF-α levels after TAK-242 incubation and subsequent LPS stimulation were below the detection limit.

In vivo TAK-242 effects on perfusion recovery after slow and systemic release

After confirming the TLR4 inhibitory effects of TAK-242 in vitro, we assessed the effect of TAK-242 in vivo. TAK-242 was administered via Alzet micro-pumps for continuous, slow and systemic release subcutaneously in mice followed by unilateral femoral artery ligation. Perfusion recovery was measured over 28 days following surgery. No significant differences in perfusion recovery were observed at all measured time points between TAK-242 treatment and control. All mice showed full recovery after 7 days
To test the inhibitory effects of TAK-242 on TLR4 stimulation after micro-pump administration, whole blood was collected at day 3 and 7 after surgery. At day 3, ex vivo whole blood stimulation with LPS after TAK-242 administration resulted in higher TNF-α levels compared to LPS stimulation only (control mice). However, no statistical significant differences could be observed (LPS 1 ng/ml, p=0.81; LPS 10 ng/ml, p=0.92; LPS 100 ng/ml, p=0.11). At day 7, TAK-242 administration inhibited TNF-α levels after LPS stimulation, whereas control mice maintained TNF-α levels compared to day 3 (LPS 100 ng/ml, p=0.03) (Figure 4B).

**Figure 1.** CD11b and L-selectin expression on human monocytes and neutrophils after TLR4 inhibition by TAK-242. Whole blood was collected from a healthy volunteer (data from n=1). Blood was pre-incubated with PBS (control) or TAK-242 (30 minutes), followed by LPS stimulation for 30 minutes. FACS was used to analyze CD11b and L-selectin expression on human monocytes (A) and neutrophils (B). Dotted line represents mean fluorescence intensity of unstimulated sample (PBS) and is used as a reference.
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Intramuscular administration of TAK-242 in vivo

Since continuous and slow release of TAK-242 via micro-pumps did not inhibit perfusion recovery after hind limb ischemia, to test whether the route of application was responsible for the observed results, the compound was tested in vivo using intramuscular (i.m.) injection. Control mice received PBS injection. Blood was collected 24 hours after injection and was stimulated for 20 hours with three different concentrations of LPS (0 ng/ml, 125 ng/ml, 500 ng/ml). After i.m. administration, TAK-242 treatment resulted in a 60% to 65% decrease in TNF-α production compared to control (PBS) treatment 24 hours after administration. These differences were not statistically significant (control vs TAK-242; LPS 125 ng/ml, p=0.11; LPS 500 ng/ml, p=0.15) (Figure 5).

Figure 2. CD11b and L-selectin expression on murine monocytes and granulocytes after TLR4 inhibition by TAK-242. Whole blood was collected from a wild type (C57BL/6J) mouse (data from n=1). Blood was pre-incubated with PBS (control) or TAK-242 (30 minutes), followed by LPS stimulation for 2 hours. FACS was used to analyze CD11b and L-selectin expression on murine monocytes (A) and granulocytes (B). Dotted line represents mean fluorescence intensity of unstimulated sample (PBS) and is used as a reference.
In vivo TAK-242 effects on perfusion recovery after intramuscular injection

Since mice show a biologically active response to TAK-242 treatment via i.m. injection, we also tested this route of administration combined with unilateral femoral artery ligation. Again, mice receiving a PBS injection served as a control. Perfusion recovery was measured over 7 days after operation. No effect of TAK-242 administration could be observed on perfusion recovery, since both groups recovered equally over 4 and 7 days after ligation (Figure 6).

Figure 3. TNF-α expression in human and mouse whole blood after TLR4 inhibition by TAK-242. Whole blood was collected from healthy donors (n=3) (A) and wild type (C57BL/6J) mice (n=4) (B). Blood was pre-incubated with PBS (control; white bars) or TAK-242 (black bars) for 30 minutes, followed by LPS stimulation for 2 hours (healthy donor) or 20 hours (mouse). Data are presented as mean ± SEM.
In arteriogenesis, immune responses and subsequent local influx of inflammatory cells around developing collateral arteries are crucial for restoring poor tissue perfusion. TLR4 has been identified as a target candidate for stimulating *in vivo* arteriogenesis\(^1\)\(^{19}\).

**DISCUSSION**

In arteriogenesis, immune responses and subsequent local influx of inflammatory cells around developing collateral arteries are crucial for restoring poor tissue perfusion. TLR4 has been identified as a target candidate for stimulating *in vivo* arteriogenesis\(^1\)\(^{19}\).
Different compounds have been tested to block TLR activation to analyze the effects on inflammatory diseases. For example, treatment with a specific humanized TLR2 antibody reduced myocardial I/R injury in pigs\textsuperscript{5} and was able to provide protection from I/R injury in a model for kidney transplantation in mice\textsuperscript{20}. Next to TLR2 inhibitors, also TLR4 inhibitors have been extensively studied in infectious diseases as described earlier with different approaches for administration.
In this study, we investigated whether \textit{in vivo} administration of a TLR4 antagonizing compound would negatively influence perfusion recovery after hind limb ischemia in mice. We hypothesized that systemic \textit{in vivo} administration of TAK-242 could inhibit perfusion recovery after hind limb ischemia, which could be considered as a negative side effect of TLR4 inhibition.

First, we observed that TAK-242 was able to inhibit TLR4 activation measured as CD11b and L-selectin expression on human monocytes and neutrophils \textit{in vitro}. However, this effect could not be observed in murine blood cells as was analyzed by FACS. In contrast, in both human and murine whole blood, inhibition of LPS induced TNF-\(\alpha\) production was observed. Previously, it has been demonstrated that TNF-\(\alpha\) can exert an accelerating role by positively modulating arteriogenesis in mice and rabbits\textsuperscript{18, 21, 22}. Therefore, measuring TNF-\(\alpha\) levels after LPS stimulation is not just reflecting TLR4 activation, but it also reveals increased expression of an inflammatory parameter that can stimulate arteriogenesis. However, one should keep in mind that effects of TAK-242 on human and murine cells are difficult to compare, since TAK-242 metabolites are known to have differences in pharmacokinetics in various species\textsuperscript{23}.

TAK-242 has been described extensively as a specific inhibitor of TLR4 signaling \textit{in vitro} using murine peritoneal macrophages for cytokine read-out after LPS stimulation\textsuperscript{24}. Zhou \textit{et al} also showed that TAK-242 inhibits all downstream effects of TLR4 activation in THP-1 cells, a human monocytic cell line often used for TLR4 signaling research\textsuperscript{25}. From these and our research we can state that TAK-242 is biologically active in inhibiting TLR4 responses in both species. Unfortunately, the authors did not mention duration of TAK-242 pre-incubation, since this is crucial for the regulation of CD11b and L-selectin expression, as well as TNF-\(\alpha\) production. Also, Takashima \textit{et al}\textsuperscript{14} described the inhibitory effects of TAK-242 on TLR4 activation in a human monocytic cell line, but it was not described if TAK-242 was incubated prior to or simultaneously with LPS stimulation. Our results may seem contradictory, however, long-term effects of TAK-242 on TLR4 stimulation is reflected by TNF-\(\alpha\) release, whereas the changes in CD11b and L-selectin expression can already be normalized due to tolerance induction long after TLR4 stimulation.

TAK-242 was delivered \textit{in vivo} by systemic release of TAK-242 in osmotic micro-pumps to achieve systemic TLR4 inhibition in combination with unilateral iliac and femoral artery ligation. However, inhibition of perfusion recovery was absent. This raised the question whether TAK-242 was able to inhibit TLR4 activation \textit{in vivo} in mice by systemic delivery via osmotic micro-pumps. At day 3, we did not observe an effect of TAK-242 treatment regarding TNF-\(\alpha\) responses. Moreover, TAK-242 treatment even led to a higher response in compared to PBS treatment as a result of the placement of osmotic micro-pumps. Therefore, the effect of TAK-242 treatment on TLR4 stimulation was overruled by the subcutaneous pump placement observed on day 3, since control
mice did not receive a pump. However, at a later time point (day 7), the TNF-α response in control mice was still high, whereas TAK-242 treatment was able to induce TLR4 inhibition, measured as lower TNF-α levels. Although TLR4 inhibition was eventually achieved by TAK-242, this delayed effect can explain why perfusion recovery was not inhibited compared to the control group. Previously, TNF-α levels have been used as a measure of TLR responsiveness, but we did not study the association between in vivo TNF-α levels and arteriogenesis in this study. Therefore, our results do not contradict the current view that TNF-α release induces arteriogenesis.

Since the subcutaneous delivery of TAK-242 did not affect perfusion recovery, we hypothesized that local administration of TAK-242 might be able to inhibit perfusion recovery. First, we tested in vivo TAK-242 administration via i.m. injection and measured the systemic TNF-α levels. These results show that in vivo i.m. administration of TAK-242 in mice resulted in systemic TLR4 inhibition 24 hours after injection. Based on this, we hypothesized that i.m. injection of TAK-242 could inhibit TLR4 response and thereby inhibit perfusion recovery. However, we could not observe any effects on perfusion recovery after TAK-242 treatment.

It still remains unclear why pharmaceutical inhibition of TLR4 by TAK-242 did not have an effect on perfusion recovery, whilst knockout of TLR4 in mice did. One major difference between the two set-ups is the complete lack of TLR4 on all cells in the TLR4−/− mice. Wild type and TLR4−/− mice are not only different in their genotype. Also responsive phenotypical changes occur as a result of TLR4 deletion, due to adaption of, for instance, immunological components (i.e. differences in baseline characteristics), which might explain the differences in immunological responses. Therefore, comparing acute pharmaceutical intervention in wild type mice to TLR4−/− mice remains difficult.

**Limitations**

In this study, we did not investigate the dose-dependency of TAK-242 in our model for arteriogenesis. This would be useful to assess the optimal effect of TAK-242 on perfusion recovery after hind limb ischemia. Furthermore, the extrapolation of animal models to a human setting merits careful consideration, since there appears to be a wide range of species-dependent variation in the effect of TAK-242. Therefore, the results from this mouse study need to be carefully interpreted, before any conclusions can be drawn regarding the effects of TAK-242 in a clinical setting.

**Conclusion**

TAK-242 has inhibitory effects on TNF-α production after TLR4 stimulation both in vitro as well as in vivo in mice. However, this inhibition may only exert a short-term effect. Although TLR4 inhibition as recently been proven to inhibit atherosclerotic lesion de-
velopment in mice\textsuperscript{11}, we observed that TAK-242 treatment had no negative side effects on long-term perfusion recovery after hind limb ischemia in mice.

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REFERENCE LIST


