Cell-specific pattern recognition receptor signaling in antibacterial defense
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Chapter 10

TLR4 inhibition impairs bacterial clearance in a therapeutic setting in murine abdominal sepsis

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Abstract

Objective and design: to investigate the therapeutic effect of E5564 (a clinically used TLR4 inhibitor) in murine abdominal sepsis elicited by intraperitoneal infection with a highly virulent *Escherichia coli* in the context of concurrent antibiotic therapy. Methods: Mice were infected with different doses (~2 x 10^4 - 2 x 10^6 CFU) of *E. coli O18:K1* and treated after 8 hours with ceftriaxone 20 mg/kg i.p. combined with either E5564 10mg/kg i.v. or vehicle. For survival studies this treatment was repeated every 12 hours. Bacterial loads and inflammatory parameters were determined after 20 hours in peritoneal lavage fluid, blood, liver and lung tissue. Plasma creatinin, AST, ALT and LDH were determined to assess organ injury. Results: E5564 impaired bacterial clearance under the antibiotic regime after infection with a low dose *E. coli* (1.7 x 10^4 CFU) while renal function was slightly preserved. No differences were observed in bacterial load and organ damage after infection with a tenfold higher (1.7 x 10^6 *E. coli*) bacterial dose. While treatment with E5564 slightly attenuated inflammatory markers provoked by the sublethal doses of 10^4–10^5 *E. coli* under the antibiotic regime, it did not affect lethality evoked by infection with 1.7 x 10^6 *E. coli*. Conclusions: The impact of TLR4 inhibition during abdominal sepsis by virulent *E. coli* bacteria is only beneficial at low infection grade at cost of bactericidal activity.
Introduction

Sepsis is a leading and increasing cause of death in non-coronary intensive care units (1). The abdomen is the second most common site of primary infection in sepsis (2). Sepsis is frequently caused by gram-negative pathogens, amongst which *Escherichia (E.) coli* is still one of the most frequently isolated organisms (2). Despite all efforts to improve therapeutic outcome, the mortality rate of sepsis remains as high as 20-40%.

The inflammatory response to pathogens is initiated by the recognition of bacterial structures by Pattern Recognition Receptors (PRRs) such as Toll-like receptors (TLRs). The proinflammatory host response is on the one hand essential to combat the infection adequately, but on the other hand can contribute to systemic inflammation and tissue injury, such as occurs during severe sepsis. TLR4 is central in the pathway that initiates and amplifies the inflammatory response since it not only recognizes exogenous microbial ligands but also endogenous ligands released from damaged tissues and dying cells collectively called alarmins (1, 3). Therefore, this pathway is regarded as a potential therapeutic target for sepsis. A highly potent synthetic inhibitor of TLR4, known as E5564 (Eritoran®) that binds to the essential co-receptor of LPS-signaling MD2 in a competitive way, was developed and shown to block LPS activity in vitro and in vivo (4). In 2011 the phase III multicenter “ACCESS” trial study, designed to study E5564 in patients with severe sepsis and a high mortality risk, was completed; although the results have not been published yet, E5564 was reported not to be effective in reducing 28-day mortality (5).

We here determine the effect of therapeutic administration of E5564 in a model of gram-negative sepsis in a clinically relevant setting, i.e. in the context of postponed treatment together with concomitant antibiotic therapy and observed that TLR4 is involved in bacterial clearance under these conditions.

Methods

Mice

Specific pathogen-free female C57BL/6 mice were obtained from Harlan (Horst, the Netherlands) (6-8). Mice were 10 weeks of age at the start of experiments. All experiments were approved by the Animal Care and Use Committee of the University of Amsterdam.

Experimental design

Peritonitis was induced as described (9) by intraperitoneal injection of different dosages of viable *E. coli* O18:K1 in 200 μl pyrogen free 0.9% NaCl (Baxter) as described. In the first experiment survival of mice was monitored after infection with increasing doses of *E. coli* (2 x 10^4 to 2 x 10^6 CFU per mouse) with or without i.p. antibiotic treatment with 20 mg/kg ceftriaxone (Bipharma, Almere, the Netherlands) or vehicle (200 μl sterile 0.9% NaCl) starting from 8 hours after infection, this was repeated every 12 hours (10-12). Ceftriaxone is used against Gram-positive and Gram-negative bacteria and commonly used for treatment of pneumonia, bacterial
meningitis, sepsis and other bacterial infections (13). In subsequent experiments mice received ceftriaxone i.p. and 10 mg/kg E5564 or vehicle (200 μl 0.9% NaCl) intravenously via the tail vein 8 hours after infection and survival was monitored or mice were sacrificed 20 hours after infection, and peritoneal lavage fluid (PLF), blood, liver and lung were harvested, processed and analyzed for CFU levels as described (9). We were advised on the treatment dose of E5564 by the supplier. In the survival studies treatments were repeated every 12 hours.

Assays
Tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-10 and monocyte chemoattractant protein (MCP)-1 were measured by cytometric bead array (BD Biosciences, San Jose, CA). IL-1β, cytokine-induced neutrophil chemoattractant (KC), macrophage inflammatory protein (MIP)-2 and soluble E-selectin were measured by ELISA’s (R&D Systems, Minneapolis, MN). Creatinin, AST, ALT and LDH were measured by kits from Sigma (St. Louis, MO), using a Hittachi analyzer (Boehringer Mannheim, Mannheim, Germany).

Statistics
Data are expressed as Kaplan Meier plots (survival curves), medians with individual data points (bacterial loads) or means ± standard error of the mean. Comparison between groups was done by Mann Whitney U tests. p < 0.05 was considered statistically significant.

Results

**TLR4 inhibition does not affect survival, but impairs bacterial clearance during abdominal sepsis in the context of antibiotic treatment**

First, we aimed to study the effect of delayed combined treatment with antibiotics and TLR4 inhibition in a lethal model of abdominal sepsis caused by intraperitoneal infection with *E. coli*. To determine the infectious dose that was lethal despite antibiotic treatment, we first performed a pilot study in which mice were infected with increasing doses of *E. coli* and were treated with ceftriaxone or vehicle (n=5 per treatment group) after 8 hours and every subsequent 12 hours. Mice were followed until 48 hours (Figure 1). Ceftriaxone prevented mortality of mice after infection with $2 \times 10^4$ and $2 \times 10^5$ *E. coli*, but not the lethality provoked by $2 \times 10^6$ *E. coli*. Based on these pilot data, we infected two groups of 20 mice with $2 \times 10^6$ CFU and subjected them to antibiotic treatment with or without E5564 and scored survival rates. From 15 hours onward mortality occurred in both treatment groups and survival curves were not different after treatment with E5564 (median survival 26 hours in both groups; Figure 2A). To study the effect of E5564 on antibacterial defense and the inflammatory response in this setting of delayed antibiotic treatment, we performed separate experiments in which we infected mice with sublethal doses of $1.7 \times 10^4$ or $1.7 \times 10^5$ CFU and sacrificed them after 20 hours. After infection with $1.7 \times 10^4$ *E. coli*, cultures of PLF and blood showed no growth of bacteria in the vast
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Figure 1: Pilot survival study to establish lethality with different infectious inocula in the context of antibiotic treatment. Mice were inoculated with three different 10-fold increasing doses of E. coli intraperitoneally (i.p.) and treated after 8 hours with 20mg/kg ceftriaxone (open symbols) or vehicle i.p. (closed symbols) (n=5 per treatment group); treatment was repeated twice daily and mice were followed until 48 hours.

majority of mice irrespective of E5564 treatment (Figure 2B, C). In liver and lungs however, median bacterial loads were almost 10-fold higher in E5564 treated mice (P < 0.05 versus vehicle for liver and P < 0.01 versus vehicle for lung) (Figure 2D, E) indicating that E5564 decreased host defense in organ parenchyma. In contrast, in mice infected with a 10-fold higher dose (Figure 2B-E) of E. coli (1.7 x 10⁵) E5564 did not influence bacterial loads after infection with the higher dose (Figure 2B-E). Strikingly, bacterial loads in lungs of mice that were inoculated with 1.7 x 10⁴ CFU E. coli and treated with E5564 were higher than in the mice that received 1.7 x 10⁵ CFU E. coli treated with E5564. The latter indicates that the number of bacteria found in the organs is not just a function of the inoculated dose, but also depends on host defense. Indeed killing of the E. coli type O18:K1 bacterium, is dependent on cytokine activated macrophages (14) and thus we evaluated the host response.

Effect of TLR4 inhibition on inflammatory response

To evaluate the effect of delayed TLR4 inhibition on the inflammatory response we determined levels of proinflammatory cytokines (TNF-α, IL-6, IL-1β), an anti-inflammatory cytokine (IL-10) and chemokines (KC, MIP-2, MCP-1, LIX) at the primary site of infection and in the circulation. Although trends were observed, E5564 did not significantly downregulate cytokine or chemokine levels in PLF (relative to treatment with ceftriaxone plus vehicle; Table 1). Moreover, there were no differences in total cell numbers and composition of the recruited cells in PLF between treatment groups (data not shown). Plasma levels of cytokines and chemokines tended to be lower in both E5564 treated groups, which was significant for MCP-1 (p < 0.05 and p < 0.01 versus vehicle treated groups). As a read-out for endothelial cell activation we determined the plasma levels of soluble E-selectin. TLR4 inhibition was associated with lower plasma soluble E-selectin concentrations after infection with the higher E. coli dose (p < 0.01 versus vehicle).
Figure 2: TLR4 inhibition impairs bacterial clearance during abdominal sepsis in the therapeutic setting with concurrent antibiotic treatment. Mice were inoculated with *E. coli* intraperitoneally (i.p.) and treated after 8 hours with 20mg/kg ceftriaxone i.p. and 10 mg/kg E5564 (open rounds) or vehicle (closed rounds) intravenously; treatment was repeated twice daily during survival study. Survival from mice infected with 2 x 10^6 CFU *E. coli* (n=20 mice per treatment group) (A). Bacterial loads in peritoneal lavage fluid (PLF) (B), blood (C), liver (D) and lung (E) 20 hours after infection with 1.7 x 10^4 (n=8 per treatment group) or 1.7 x 10^5 CFU *E. coli* (n=8 per treatment group). Each symbol represents an individual mouse, with horizontal lines showing medians. ** *p* < 0.01 versus vehicle treated group that was infected with the same dose of bacteria, determined with Mann-Whitney U test.

**Effect of TLR4 inhibition on tissue injury**

E5564 preserved renal function in mice infected with the lower *E. coli* dose as reflected by lower plasma levels of creatinin (Figure 3A, *p* < 0.05 versus vehicle). E5564 did not impact on hepatocellular injury as evaluated by the plasma concentrations of ALT and AST, nor did it attenuate cellular injury in general as indicated by plasma LDH (Figure 3B-D).
Table 1: Effect of TLR4 inhibition on peritoneal and plasma levels of cytokines and chemokines.

<table>
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<tr>
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<th>1.7 x 10^4 CFU</th>
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<tr>
<td></td>
<td>Ceftriaxone +</td>
<td>Ceftriaxone +</td>
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<tr>
<td></td>
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<tr>
<td>PLF</td>
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<td>IL-1β</td>
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<tr>
<td>KC</td>
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<tr>
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<td>IL-6</td>
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<tr>
<td>MCP-1</td>
<td>3940 ± 47</td>
<td>52 ± 11**</td>
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<td>E-selectin</td>
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Mice were inoculated with 1.7 x 10^4 (n = 8 per treatment group) or 1.7 x 10^5 CFU (n = 8 per treatment group) *E. coli* intraperitoneally (i.p.) and treated after 8 hours with 20mg/kg ceftriaxone i.p. and 10 mg/kg E5564 or vehicle intravenously. Data are means ± standard error of the mean. Cytokines and chemokines levels are in pg/mL; soluble E-selectin levels in ng/mL. PLF = peritoneal lavage fluid. *p < 0.05, **p < 0.01 versus vehicle treated group that was infected with the same dose of bacteria, determined with Mann-Whitney U test. Bd= below detection level.
Figure 3: TLR4 inhibition preserves renal function during abdominal sepsis without influencing (hepato)cellular injury. Mice were inoculated with 1.7 x 10^4 (n = 8 per treatment group) or 1.7 x 10^5 CFU (n = 8 per treatment group) E. coli intraperitoneally (i.p.) and treated after 8 hours with 20mg/kg ceftriaxone i.p. and 10 mg/kg E5564 or vehicle intravenously. Plasma levels (20 hours post infection) of creatinin (A), ALT (B), AST (C) and LDH (D). Bars represent mean ± standard error of the mean. * p < 0.05 versus vehicle treated group that was infected with the same dose of bacteria, determined with Mann-Whitney U test.

Discussion

Several adjunctive therapies to improve sepsis outcome have been evaluated in clinical trials during the last decades, almost invariably yielding negative results (15). Anti-TLR4 therapy was designed as an additional therapy to attenuate excessive inflammation caused by high bacterial loads and the release of endogenous “danger molecules”. On the other hand, TLR4 is important for bacterial clearance of several gram-negative pathogens (9, 16, 17). We here studied the effects of TLR4 inhibition in a murine model of abdominal sepsis caused by E. coli and revealed part of the dual function of TLR4: after infection with a relatively low bacterial inoculum E5564 treatment impaired bacterial clearance in the presence of concurrent antibiotic therapy but at the same time attenuated the cytokine response and preserved renal function.

Previous studies have shown that anti-TLR4 directed therapy can be protective during rodent peritonitis (5, 17-21). In the model of colon ascendens stent peritonitis anti-TLR4 was protective if initiated immediately upon the intervention (18). In two separate reports, antibodies directed against TLR4 reduced mortality from E. coli peritonitis if administered prophylactically in infection models with high doses of less virulent bacteria and antibiotics (19, 20) or therapeutically in a postponed treatment.
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setting in combination with TLR2 inhibition (19, 20). This suggests that excessive TLR4 mediated hyperinflammation is important in causing lethality during bacterial peritonitis. We recently reported an impaired antibacterial defense in TLR4 deficient mice, especially in early stage *E. coli* peritonitis by infection with a relatively low amount of highly virulent bacteria (9). In the present study we established by use of the MD2/TLR4 inhibitor E5564 (22, 23) that TLR4 is important for antibacterial defense even during progressive infection after infection with a relatively low dose of bacteria (i.e. when inhibited 8 hours after infection) and in the presence of antibiotic treatment. TLR4 inhibition did not impact on bacterial loads after high dose infection, suggesting that in this condition after 8 hours the time window during which TLR4 contributes to antibacterial defense has passed or becomes redundant. The differential effect of anti-TLR4 treatment on antibacterial defense after infection with increasing doses might be explained by the different amounts of LPS that induce the production of cytokines, which in turn, initiate macrophages to kill *E. coli* (14). Indeed mice infected with the high dose *E. coli* displayed high cytokine/chemokine levels at the primary (peritoneal) site of infection irrespective of TLR4 inhibition (table 1). It is noteworthy that the irritants from the peritoneal cavity are drained by the thoracic duct via the subclavial vein in the lung circulation. Potentially peritoneal originated mediators aid in bacterial control in the lung and are involved in our observation that the bacterial load is actually lower in the lungs of 1.7 x 10^5 infected E5564 treated mice compared to E5564 treated mice infected with 1.7 x 10^4 *E. coli* (Figure 2E). Moreover, this could be due to the low peritoneal as well as low systemic cytokine/chemokine levels in the 1.7 x 10^4 infected E5564 treated group (table 1). Alternatively, E5564 treatment could impair phagocytosis, since the phagocytic capacity of macrophages has been reported to be enhanced by TLR4 stimulation and MD-2 was reported to function as an opsonin (24, 25). Overall the results indicate that antibiotic control of established *E. coli* infection in the organs falls short when TLR4 is inhibited.

Postponed TLR4 inhibition attenuated the systemic levels of cytokines to some extent and partially preserved renal function. This is in line with recent reports that TLR4 is involved in the pathophysiology of sepsis induced acute kidney injury (26, 27). In accordance, E5564 was reported to be protective in a model of renal ischemia-reperfusion injury (28). Moreover, E5564 treated mice showed lower circulating levels of soluble E-selectin, indicating that TLR4 is involved in endothelial cell activation during progressive abdominal sepsis. Discrepancies between the previous reports on the role of TLR4 during murine peritonitis (18-20) and our earlier (9) and current reports may be largely due to differences in the models: we administered relatively low doses of a highly pathogenic *E. coli* O18:K1 strain that induces 80-100% lethality if not treated (9), while in the previous studies 1000-100,000x higher doses of less virulent *E. coli* were administered (18-20). Also, we used a MD2/TLR4 inhibitor which binds only to MD2, and it is under debate whether alarmin signalling by TLR4 is MD2 dependent (29). Furthermore, in here we used the antibiotic ceftriaxone that is reported to have an adequate penetration in lung tissue and abdominal organs (30, 31) and showed that although blood and peritoneal cavity indeed became virtually sterile, *E. coli* may persist in organs such
as the liver and the lungs during the initial phase of TLR4 treatment. It is clear that at high bacterial loads in addition to TLR4 other TLRs such as TLR2 are important in the recognition of E. coli (9, 20, 32), while for the initial detection of a lower dose of (pathogenic) bacteria TLR4 is most important as our group demonstrated in vivo and in vitro (9). Specifically, TLR2 can be activated by Peptidoglycan-associated lipoprotein (PAL), a constituent of the outer membrane of E. coli (33).

Conclusions

This study illustrates that therapeutic targeting of TLR4 signalling can potentially interfere with bacterial clearance even in the context of antibiotic therapy, but on the other hand can help limit some aspects of tissue injury depending on the infectious dose and kinetics of the infection model.

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