The Janus face of *Bartonella quintana* recognition by Toll-like receptors (TLRs): a review

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ABSTRACT. *Bartonella quintana* (*B. quintana*) is a facultative, intracellular bacterium, which causes trench fever, chronic bacteraemia and bacillary angiomatosis. Little is known about the recognition of *B. quintana* by the innate immune system. In this review, we address the impact of Toll-like receptors (TLRs) on the recognition of *B. quintana* and the activation of the host defense. When experimental models using human mononuclear cells, transfected CHO cells, or TLR2$^{-/-}$ and TLR4$^{-/-}$ mice were used, differential effects of TLR2 and TLR4 have been observed. *B. quintana* micro-organisms stimulated cytokine production through TLR2-mediated signals, whereas no role for TLR4 in the recognition of this pathogen was observed. When single, water-phenol extraction was performed, *B. quintana* LPS, stimulated cytokine production in a TLR2-dependent manner. However, when double extraction was performed in order to generate highly purified LPS, *B. quintana* LPS entirely lost its capacity to stimulate cytokines, demonstrating that non-LPS components of *B. quintana* are responsible for the recognition through TLR2. Moreover, *B. quintana* LPS was shown to be a potent antagonist of Toll-like receptor 4 (TLR4). In conclusion, *B. quintana* is an inducer of cytokines through TLR2-, but not TLR4-, dependent mechanisms. This stimulation is induced by bacterial components other than lipopolysaccharide. *B. quintana* LPS is a naturally occurring antagonist of Toll-like receptor 4 (TLR4). In view of the role played by TLR4 in inflammation, *B. quintana* LPS may be useful as an anti-TLR4 agent with therapeutic potential in both infections and autoimmune inflammation.

Keywords: *B. quintana* LPS, TLR4, cytokine

Bartonellae are small, pleiomorphic, Gram-negative bacilli, which are transmitted by arthropods and produce a persistent bacteraemia in their reservoir host. The three *Bartonella* species reported as major pathogens for humans are *Bartonella quintana*, *Bartonella henselae*, and *Bartonella bacilliformis* [1]. Biological features of Bartonellae include a specific interaction with endothelial cells and erythrocytes [1]. Bartonellae are unique bacterial pathogens causing disease characterized by vascular, proliferative lesions in humans; these bacteria are well known modifiers of endothelial functions, resulting in cell invasion, suppression of apoptosis, and stimulation of proliferation [1].

B. quintana is transmitted by the human body louse, it is a facultative intracellular bacterium, and causes trench fever endocarditis and bacillary angiomatosis. The primary infection is associated with recurrent fever, headaches, and leg pain and is usually followed by chronic bacteraemia in 5-10% of homeless people, who may develop chronic endocarditis [2-5]. This is associated with a mortality higher than 12% when therapy is delayed [6].

The mechanisms leading to *B. quintana* persistence are unknown, but one defining characteristic of *Bartonella* bacteraemia is the absence of Gram-negative sepsis features such fever and septic shock sequelae. Therefore, systemic *Bartonella* infection is associated with an attenuation of the expected inflammatory response, which eventually leads to the persistence. It has been suggested that chronic *B. quintana* bacteraemia is linked to an oversecretion of IL-10 and an attenuation of the inflammatory response. The persistence of *B. quintana* inside erythrocytes may result from IL-10-mediated interference with the development of an adapted immune response [7], but the molecular mechanisms have not yet been identified.

On the other hand, proper activation of innate immunity is crucial for effective host defence. This is achieved by recognition of conserved structures of micro-organisms, called pathogen-associated molecular patterns (PAMPs) by pathogen recognition receptors (PRRs) on leukocytes, with subsequent stimulation of proinflammatory cytokine production. Recognition of Gram-negative lipopolysaccharide (LPS) by Toll-like receptor-4 (TLR4) and of

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bacterial lipoproteins by TLR2 are the most important recognition systems of Gram-negative bacteria. Lipopolysaccharide is a main component of the outer membrane of Gram-negative microorganisms, and the LPS from enteric bacteria (such as *Escherichia coli* or *Salmonella enterica*) is able to induce a strong proinflammatory response and sepsis syndrome [8-10].

Little is known about the host recognition of Bartonella spp. *B. henselae* LPS is a very weak inducer of cytokines through a TLR4-dependent mechanism, while being recognized by TLR2 [11]. *B. henselae* LPS has an atypical lipid A, featuring a 2, 3-diamino-2, 3-dideoxy-glucose disaccharide bisphosphate backbone, penta-acetylated with a set of fatty acids, which includes one 25-hydroxyhexacosanoic or 27-hydroxyoctacosanoic acid [11]. It is likely that *B. quintana* LPS has a similar structure and this might explain its poor capacity to induce proinflammatory mediators, and the weak endotoxic activity of *B. quintana* LPS *in vivo*, as we previously demonstrated [12].

LPS extracted from *B. quintana* Oklahoma strain showed a molecular weight of approximately 5,000 Da, very similar to the "deep rough" chemotype. In human leucocytes or in endothelial cells, as well as in a rat model, *B. quintana* LPS was not able to induce significant levels of blood TNFα. Moreover, *B. quintana* LPS induced an increase in the white blood cell count without a substantial change in heart rate, hematocrit, platelet count or blood pressure [12-14].

The unusual absence of obvious clinical signs of sepsis, in patients with *B. quintana* bacteraemia increased our interest in *Bartonella*-induced disorders. Therefore, we investigated the biological activities of *B. quintana* LPS in terms of induction of proinflammatory cytokines and interaction with Toll-like receptors.

THE ROLE OF TLR2 AND TLR4 IN THE RECOGNITION OF *B. QUINTANA*

In our study on the interaction between *B. quintana* and TLRs, we decided to stimulate cells with two types of preparations from *B. quintana*. First, we used heat-killed *B. quintana*, in order to avoid confounding factors associated with bacterial replication, metabolic changes and heat-labile factors (*e.g.* enzymes, antigenic protein variations). LPS was used as the most important and abundant virulence factor of the outer membrane of these Gram-negative bacteria, and because it should be expected that LPS/host cell receptor interactions play a pivotal role in host defence against *B. quintana*. In this respect, crude LPS preparations, as used in an early series of experiments, closely resemble the blebs produced by this bacterium during the infectious process [4], as well as the natural state of endotoxin released during complement- or antibiotic-induced bacterial lysis [15].

Heat-killed *B. quintana* stimulated TNF-α and IL-10 production in a dose-dependent manner. However, the release of heat-killed *B. quintana*-stimulated cytokines by PBMC was TLR4-independent; indeed, it was not influenced by preincubation with an anti-TLR4 antibody *(figure 1)*. A substantial response was evoked by heat-killed *B. quintana* in TLR2-transfected 3E10/TLR2 cells, whereas both control 3E10 and TLR4-transfected 3E10/TLR4 showed no response to the same stimulus. The stimulation of 3E10/TLR2 cells by increasing concentrations of crude *B. quintana* LPS, showed a progressive increase in the response, whereas TLR4-transfected cells did not respond to such stimulation.

These data demonstrate the involvement of TLR2, but not of TLR4, in the recognition of *B. quintana,* and are in line with two recent studies demonstrating TLR2-dependent/TLR4-independent cytokine stimulation by *B. henselae*

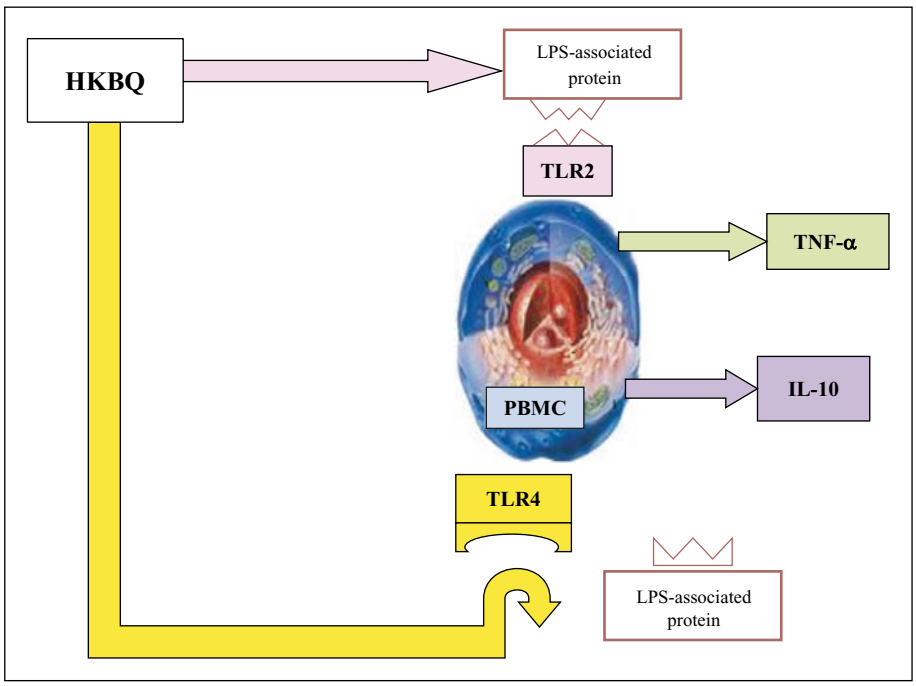

Figure 1
Production of TNF-α and IL-10 by PBMCs after stimulation with heat-killed *B. quintana.* Anti-TLR4 antibody did not influence cytokines production; therefore this feature strongly suggested an interaction between TLR2 and LPS-associated protein.

[16, 17]. In addition, Zahringer *et al*. reported TLR2-dependent induction of cytokines by crude *B. henselae* LPS preparations, and very low TLR4-inducing effects by highly purified preparations of *B. henselae* LPS [11].

The reduced role of LPS-TLR4 interaction in the induction of an immune response to *B. quintana* is not uncommon to *Bartonella* spp. Previous reports indicated that other systems such as the proteinaceous VirB type IV secretion system [8], OMPs [18] and BadA [19], rather than LPS, are responsible for the stimulation of cytokines by *B. henselae*.

Heat-killed *B. quintana* induced greater IL-10 release than *E. coli* LPS, especially relatively to the amounts of the proinflammatory cytokines TNFα and IL-6 induced by the same stimuli. This finding is consistent with the increased IL-10 concentrations found in *B. quintana*-infected patients during bacteraemia [7], and might explain the paucity of symptoms in these patients. Our findings are strengthened by a recent report showing that *B. henselae* induces a more tolerigenic phenotype in human dendritic cells, characterized by high IL-10 and low IL-12p70 production [17]. These effects could have been explained by the specific stimulation of TLR2-dependent pathways by *Bartonella spp.*: TLR2 has been demonstrated to induce anti-inflammatory effects such as IL-10 production [20, 21], a tolerigenic DC phenotype [22], and T-regulatory cell proliferation [23].

The skewing of *B. quintana* stimulation towards a more anti-inflammatory cytokine profile may therefore represent an important mechanism for evading the host defence mechanisms. In this respect, it is also interesting to mention that flagellin from *B. bacilliformis*, another member of the *Bartonella* family, is not recognized by TLR5 [24]. Therefore, it is apparent that members of the *Bartonella spp.* have evolved mechanisms to evade two of the most important recognition mechanisms of Gram-negative bacteria, recognition of LPS by TLR4, and recognition of flagellin by TLR5. On the one hand, this may contribute to the survival of *Bartonella* in the host and the chronic nature of *Bartonella* infections, and on the other hand this could explain the absence of symptoms in patients with *Bartonella* bacteraemia.

Recent data suggest that escaping TLR4-dependent responses could play a role in the virulence of Gram-negative bacteria [15]. Other bacterial pathogens, such as *Helicobacter pylori, Legionella pneumophila, Porphyromonas gingivalis or Leptospira interrogans*, [8, 25-27], interact with TLR2 rather than with TLR4. In a separate series of experiments, we studied stimulation of macrophages from TLR2- and TLR4-deficient mice. Macrophages from TLR2$^{-/-}$, but not TLR4$^{-/-}$, mice released significantly lower amounts of TNF-α and IL-6 when challenged with heat-killed *B. quintana* cells or with *B. quintana* crude LPS, in comparison with the wild-type mouse strain (C57BL/6J) macrophages *(figure 2 A, B)*. Heat-killed *B. quintana* and *B. quintana* crude LPS also stimulated IL-10 production, but this was not different between macrophages isolated from TLR2$^{-/-}$ and control mice (*figure 2*C). Therefore, it was TLR2$^{-/-}$, but not TLR4$^{-/-}$, mice that had a defective cytokine response after stimulation with *B. quintana*.

The use of cells from different species in this study rules out species-specific effects in the recognition of *B. quintana*. Earlier investigations have suggested that many lipid A analogues, as well as LPS from different bacteria, may produce different effects and use diverse signalling mechanisms depending on the animal species studied [28]. Our experiments demonstrate that this is not the case for heat-killed B. *quintana* and *B. quintana* LPS.

B. QUINTANA LPS IS A TLR4 ANTAGONIST

Previous studies have raised concerns as to whether some of the biological effects of LPS preparations are due to small amounts of LPS-associated proteins [29]. To address this issue, a double extraction procedure was carried out on *B. quintana* LPS in order to remove trace proteins, which have been reported to be present as LPS-associated proteins in some preparations [30]. LPS was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stained for protein by the enhanced colloidal gold procedure [31]. The stain was able to detect 100 pg of protein, employing bovine serum albumin as a standard. The colloidal gold procedure revealed the absence of any protein contaminants in LPS samples after purification. Double-extracted *B. quintana* LPS did not stimulate the production of TNFα, IL-1β, IL-8 or IL-6 in human PBMCs [32].

Similarly, the repurified *B. quintana* LPS completely lost its capacity to stimulate release of cytokines from murine macrophages, demonstrating that the induction of cytokines by the crude *B. quintana* LPS, purified in a one-step procedure, was due to TLR2-mediated signals induced by LPS-associated proteins. These results demonstrated that *B. quintana* LPS was not able to induce cytokine production, and suggested that it is devoid of direct biological activity.

We have hypothesized that B. *quintana* LPS, similar to other lipopolysaccharides with no agonist activity, could function as a TLR4 antagonist *(figure 3)*. Human PBMCs, preincubated with various concentrations of *B. quintana* LPS, were triggered with *E. coli* LPS. *B. quintana* LPS completely abolished *E. coli* LPS-induced TNF-α release, and inhibited the transcription of the proinflammatory cytokines TNF-α, IL-1β and IL-6 [32]. These results demonstrated the blocking effects on TLR4 by *B. quintana* LPS as further confirmed in CHO cells transfected with TLR4. In addition, we performed oligonucleotide genome array (Affymetrix) analysis to test the influence of *B. quintana* LPS on gene expression, on its own and in combination with *E. coli* LPS. *B. quintana* LPS totally inhibited the effect of *E. coli* LPS, while it did not have any effect on its own. While the *E. coli* LPS induced upregulation of 679 genes, *B. quintana* LPS - TLR4 interaction did not cause gene transcription, as demonstrated by the lack of gene regulation. Moreover, *B. quintana* LPS completely blocked the transcription of the genes induced by *E. coli* LPS [32].

It has been reported that LPS from other bacteria also possesses antagonistic effects on TLR4 [33, 34]. In a previous study dealing with the importance of the lipid A shape in determining the interaction of lipopolysaccharides with Toll-like receptors, the great differences between conical or cylindrical LPS structures have been underlined. These are determined by the presence of hexa-acylated or tetra-acylated lipid A. The TLR4 antagonistic activity of *B. quintana* LPS is probably caused by the cylindrical conformation of its lipid A due to the presence of a long-chain fatty acid, a feature that is shared by *B.*

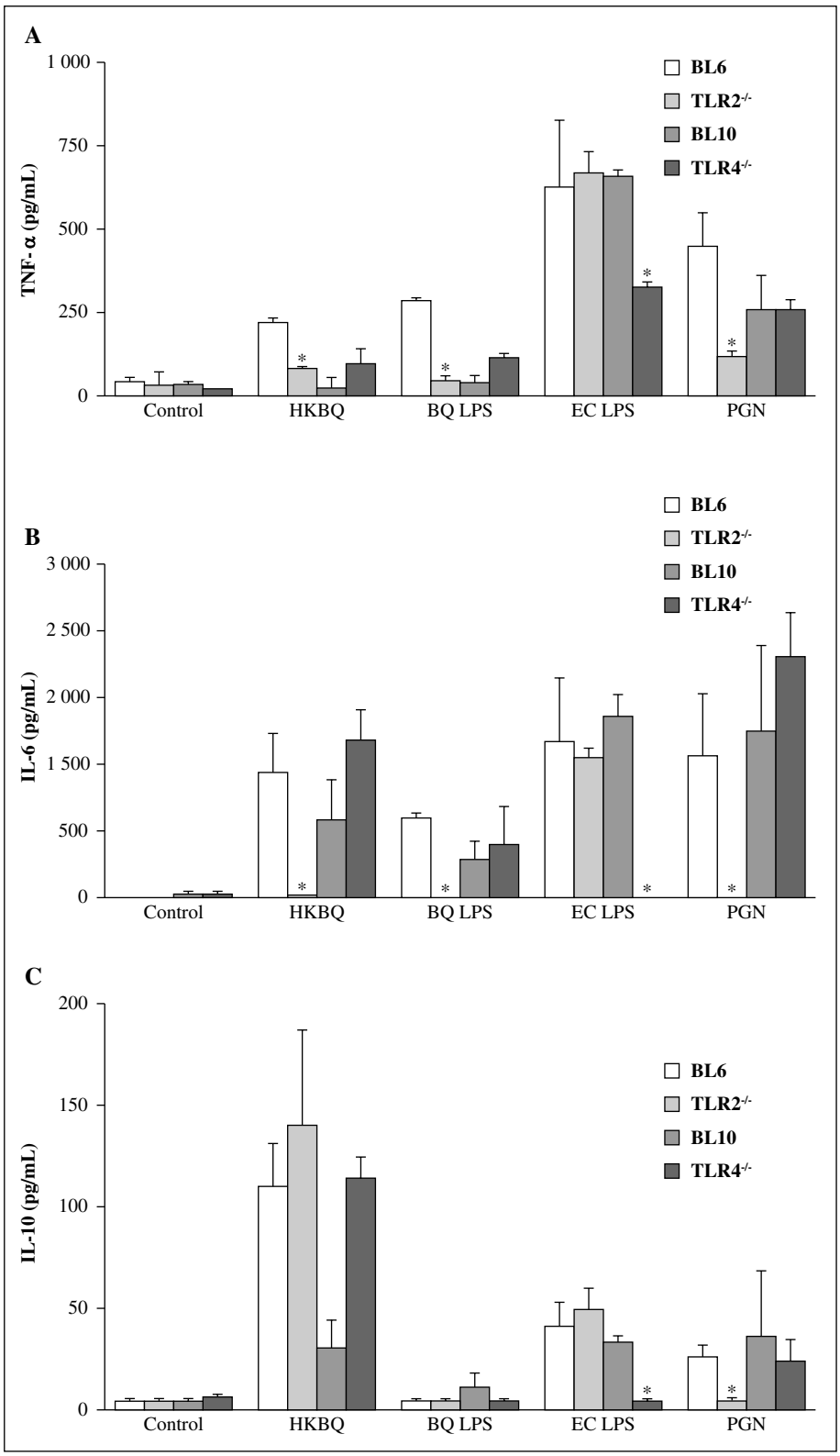

Figure 2

Production of TNF-α (**A**), IL-6 (**B**) and IL-10 (**C**) by macrophages of TLR2$^{-/-}$ mice and its corresponding parental strain C57BL/6J (BL6) and from TLR4$^{-/-}$ and its parental strain C57BL/10J (BL10). Peritoneal macrophages, were stimulated by heat-killed *B. quintana* cells (HKBQ), 10^7 CFU/mL), *B. quintana* crude LPS (BQ LPS, 1 000 ng/mL), repurified *E. coli* LPS (ECLPS, 10 ng/mL), *S. aureus* peptidoglycan (PGN, 0.1 µg/mL), and cell culture medium (control) for 24 h at 37°C. Cytokines were measured by specific RIA.
Data are expressed as means + SEM. * $p < 0.05$ *versus* corresponding parental mouse strain by the Mann-Whitney *U* test.

henselae LPS [11]. This feature has been also described in other LPS types with TLR4 antagonistic properties, such as LPS from *Helicobacter pylori* and *Rhodobacter capsulatus* [25, 34].

FUTURE PERSPECTIVES

TLRs have been proposed to be a potential therapeutic target in several autoimmune diseases, including rheuma-

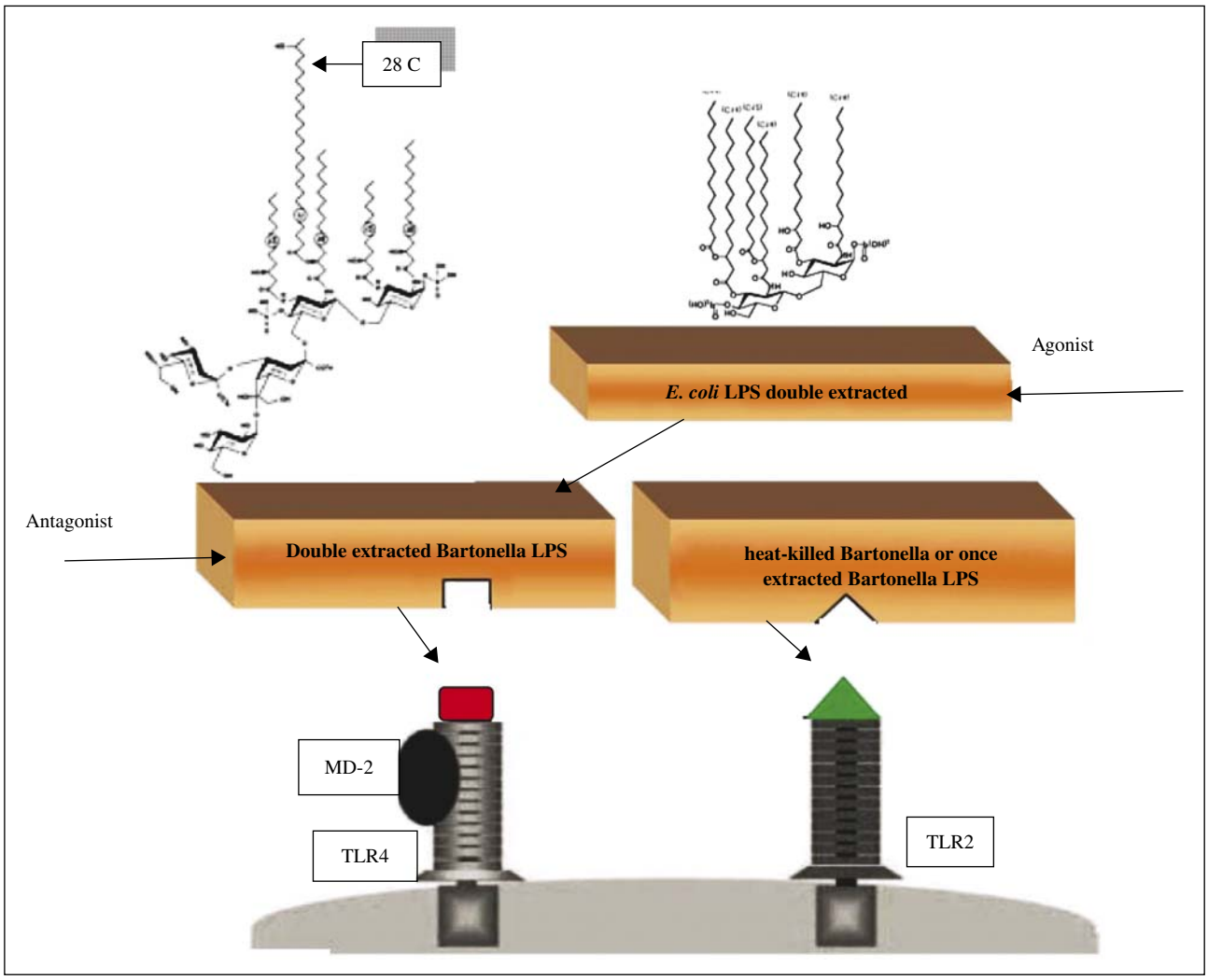

Figure 3
Proposed model involving the blocking effect on TLR4 of the double-extracted *B. quintana* LPS, and the single extraction of LPS from heat-killed *B. quintana* agonist that exerts its activity through TLR2.

toid arthritis [35]. In a recent study, Abdollahi-Roodsaz *et al*. demonstrated that inhibition of TLR4, using highly purified lipopolysaccharide from *B. quintana* as a naturally occurring TLR4 antagonist, suppresses the severity of experimental arthritis and results in lower IL-1β expression in arthritic joints [36]. Because TLR4 proinflammatory signals are involved in a variety of pathological inflammatory reactions, the use of the TLR4 antagonistic properties of *B. quintana* LPS may confirm its potential therapeutic value. These studies have also contributed to the increased molecular knowledge concerning the interaction between bacterial pathogens and TLR signalling.

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