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SHORT COMMUNICATION

# *Polymorphisms in Toll-like receptors-2 and -4 are not associated with disease manifestations in acute Q fever*

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*Coxiella burnetii is a macrophage-tropic, Gram-negative organism, which causes acute Q fever infection in humans. This zoonotic infection causes illness ranging from asymptomatic seroconversion to severe and protracted disease featuring hepatitis and pneumonia. Interactions between C. burnetii lipopolysaccharide (LPS) and host Toll-like receptors (TLR)-2 and -4 have been implicated in pathogen recognition, phagocytosis and signaling responses. Nonconservative single nucleotide polymorphisms in the coding regions of TLR-2 (Arg677Trp and Arg753Gln) and TLR-4 (Asp299Gly) have been found to correlate with mycobacterial infections and Gram-negative sepsis respectively. Associations between the TLR-2 and -4 polymorphisms, illness characteristics and immune response parameters were examined in subjects with acute Q fever (n = 85) and comparison subjects with viral infections (n = 162). No correlation was demonstrated between these polymorphisms and susceptibility to Q fever, illness severity or illness course.*
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## Introduction

Q fever is a zoonotic infection with a worldwide distribution.[1] The causative agent, *Coxiella burnetii,* is a strict intracellular pathogen with a lipopolysaccharide (LPS)-containing cell wall, which replicates within macrophage phagolysosomes. The infection is recognized predominantly in individuals working with cattle, sheep and goats. It typically manifests as a severe, febrile illness associated with prominent headache and profuse sweats, accompanied by muscle and joint pain. The acute illness is frequently complicated by hepatitis or pneumonia. A protracted clinical course marked by fatigue is common.[2] However, subclinical infections are at least four times more common than overt illness.

*C. burnetii* exists in two-phase variations (analogous to the rough-smooth variants of *Escherichia coli*). The phase I variant is infectious for mammals, while the phase II is avirulent. The pathogen recognition receptors, Toll-like receptors (TLR)-2 and -4 have been shown to be recruited to the macrophage phagosome, to differentiate between Gram-positive bacteria and yeast or Gram-negative bacteria, respectively, and to induce an appropriate inflammatory cytokine response for the type of pathogen present.[3,4] There is evidence for a role for both TLR-2 and -4 in *Coxiella*–macrophage interactions, including recognition, phagocytosis and intracellular survival.[5,6]

Functional polymorphisms in the human TLR-2 gene (Arg677Trp and Arg753Gln) have been associated with the mycobacterial infections, tuberculosis and leprosy,[7,8] and in the TLR-4 (Asp299Gly) gene with Gram-negative sepsis.[9] We examined these polymorphisms and their functional significance in 85 subjects with acute Q fever and 162 control subjects with unrelated viral infections.

## Results and discussion

Subject demographics are summarized in Table 1. The overrepresentation of males within the Q fever cohort reflects the exposure risk in male-dominated occupations (for example abattoir work, shearing), and was representative of the gender bias evident in national Q fever notifications.[10] Gender was not associated with severity of acute Q fever or illness course (data not shown). Subjects within the Ross River virus (RRV) and Epstein–Barr virus (EBV) cohorts who demonstrated previous exposure to Q fever (via serological testing) were excluded from the genotypic analysis.

All genotypes were distributed within Hardy–Weinberg equilibrium. None of the subjects carried the TLR-2 Arg677Trp (C2029T) polymorphism, or were homozygous for the TLR-2 Arg753Gln (G2258A) and TLR-4

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Asp299Gly (A896G) polymorphisms (Table 2). Frequencies of the polymorphic alleles in TLR-2 (Gln) and TLR-4 (Gly) were 2.4 and 7.1%, respectively, which are consistent with previous findings for TLR-2,[12] including with data from the International HapMap Consortium (3.3% for a population of Utah residents with ancestry from Northern and Western Europe),[13] whereas for TLR4 the frequency is higher than the 5.5% found in the HapMap population and lower than other findings.[12] There was no significant increase in the prevalence of the polymorphisms in the subjects with Q fever infection (Fisher's exact tests), indicating that these genetic variants are not associated with an increased risk of symptomatic Q fever. To assess the available power to detect a disease association of similar magnitude to previously published findings in comparable infectious diseases, with $\alpha$ set at 0.05 and our given sample size, we would have had 81% power to replicate the TLR2 association with pulmonary tuberculosis,[14] and 83% power to detect the association between the TLR4 single nucleotide polymorphisms (SNP) and Gram-negative sepsis.[15]

Among the subjects with Q fever, the influence of each polymorphism on the severity of the acute illness (reflected by the symptom score) and duration of illness (reflected by the number of days with ongoing symptoms) was examined (Table 3). No significant differences were found. With our sample size of at least $n = 85$ per group, and $\alpha$ set at 0.05, we had 80% power to confidently establish statistical equivalence for obtained group differences of $\pm 2$ points on the SOMA scale. The obtained group differences in severity for both polymorphisms (TLR2: 0.3; TLR4: 1.9) fall within this range.[16]

Permissiveness to phagocytosis and replication of the organism within macrophages, which are the target cells for *C. burnetii* infection, is likely to correlate with the likelihood of symptomatic disease. In this study, polymorphisms in the TLR-2 and -4 genes showed no association with susceptibility to symptomatic Q fever. Evidence obtained in mice suggests that TLR-2 is required for prevention of large replicative vacuole (LRV) formation in macrophages, and for control of *Coxiella* replication within the LRV.[6] Similarly, the presence of TLR-4 enhanced phagocytosis of *C. burnetii* in murine macrophages,[5] and the absence of TLR-4 resulted in delayed kinetics of antibody production in *C. burnetii* infection in knockout mice.[6] Thus, it is somewhat surprising that the putative functional polymorphisms in TLR-2 and -4 did not affect susceptibility to acute Q fever in humans. It is reasonable to conclude that the polymorphic variants of TLR-2, which result in amino-acid substitutions in the intracellular domain of the

**Table 1** Study participants

| *Cohort* | *No. of subjects* | *Mean age (range)* | *% male* |
|---|---|---|---|
| Q fever | 85 | 39 (15–73) | 84 |
| RRV | 84 | 41 (17–77) | 56 |
| EBV | 83 | 25 (16–59) | 35 |

Subjects were enrolled in the Dubbo Infection Outcomes Study, which is a prospective cohort study based in western New South Wales, Australia examining the determinants of illness severity and outcome in acute Q fever, or infection due to Epstein–Barr virus (EBV) infection (infectious mononucleosis) or Ross River virus (RRV; epidemic polyarthritis).[2] Written, informed consent was obtained prior to enrolment. Human research ethics approvals were provided by the appropriate institutional review boards. Subjects with EBV and RRV (excluding those with previous Q fever infection by standard diagnostic serology) were used as control subjects for the genotypic analysis.

**Table 3** Comparison of acute illness severity and duration by TLR-2 and TLR-4 genotype

| | *TLR-2 Arg753Gln* | | *TLR-4 Asp299Gly* | |
|---|---|---|---|---|
| | *Arg/Arg* | *Arg/Gln* | *Asp/Asp* | *Asp/Gly* |
| Severity of acute illness[a] | 5.7 (3.2) | 6.0 (4.0) | 5.3 (3.1) | 7.2 (2.9) |
| Illness duration[b] | 106 (112) | 88 (91) | 104 (113) | 109 (101) |

Abbreviation: TLR, Toll-like receptor.
At enrolment, demographic variables and a clinical history were recorded. Self-report measures of symptom severity and functional impairment were recorded at regular intervals over 12 months as described.[2] A severity index of the acute illness was based on the score in the SOMA subscale of the SPHERE questionnaire, which has been shown to correlate with significant disability due to illness and the need for medical care in this cohort. The duration of illness was designated as the number of days from onset to resolution of significant illness on the SOMA subscale (reflected by a score of three or more out of the possible 12 on the scale).
[a]Mean values on the SOMA scale with standard deviations (within parentheses) for illness severity (possible range 0–12).
[b]Mean illness duration (days SOMA score $>3$) with standard deviations (within parentheses).

**Table 2** Observed TLR-2 and TLR-4 genotype frequencies in Q fever and control cohorts

| | *TLR-2 Arg753Gln (rs5743708)* | | | *TLR-4 Asp299Gly (rs4986790)* | | |
|---|---|---|---|---|---|---|
| | *Arg/Arg* | *Arg/Gln* | *Gln/Gln* | *Asp/Asp* | *Asp/Gly* | *Gly/Gly* |
| Q fever | 82 (80.9) | 3 (4.1) | 0 (0.1) | 71 (72.9) | 14 (12.1) | 0 (0.4) |
| RRV | 78 (78.0) | 4 (3.9) | 0 (0.0) | 71 (72.0) | 13 (12.0) | 0 (0.4) |
| EBV | 75 (76.1) | 5 (3.9) | 0 (0.0) | 74 (71.1) | 9 (11.9) | 0 (0.4) |

Abbreviations: EBV, Epstein–Barr virus; RRV, Ross River virus; TLR, Toll-like receptor.
The presence of polymorphic variants in TLR-2 and TLR-4 were examined in Q fever subjects and compared to those in subjects with unrelated viral infections. Genotype at each polymorphic site was determined via methods published elsewhere[11,12] on genomic, PBMC-derived DNA. Expected frequencies (within parentheses) are based on allelic frequencies across the entire cohort.

TLR-2 protein, do not affect phagocytosis of *C. burnetii* by human macrophages. Similarly, the polymorphic variation in TLR-4, which results in an extracellular amino-acid change, also does not appear to affect phagocytosis of the organism.

We have previously shown that the severity of the acute Q fever illness correlates with *ex vivo* production of the proinflammatory cytokines, interleukin (IL)-1β and IL-6,[17] and the TLR-4$^{-/-}$ genotype has been associated with decreased production *in vitro* of interferon (IFN)-γ, tumor necrosis factor (TNF)-α and IL-10 by mouse splenocytes and macrophages in response to *C. burnetii*.[5] Accordingly, peripheral blood samples from a subset of subjects with acute Q fever were studied further to investigate the functional significance of the TLR-4 Asp299Gly polymorphism by assessment of either spontaneous *ex vivo* or LPS-induced cytokine production. No significant differences were observed in the cytokine production by peripheral blood mononuclear cells (PBMC) from wild type ($n = 47$) in comparison to heterozygote ($n = 11$) subjects (Figure 1).

TLR-2$^{-/-}$ mouse macrophages were found to produce less TNF-α and IL-12 than wild type or TLR-4$^{-/-}$ macrophages in response to *C. burnetii* infection.[6] However, no association was found between TLR-2 or -4 genotype and severity of disease in this cohort, suggesting that the associated amino-acid substitutions, do not contribute to the signaling pathways involved in cytokine production in response to *C. burnetii*.

It remains somewhat unresolved whether the SNPs in TLR-2 and -4 are in fact functional. The Arg753Gln polymorphism in TLR-2 has been found in approximately 3–10% of Caucasian populations, and has been associated with reduced responsiveness to bacterial lipopeptides as determined by downstream activation of transcription factor, nuclear factor-κB.[18] While macrophages from homozygotes with the Arg753Gln polymorphism have been shown to lose lipoteichoic acid responsiveness, heterozygotes showed no loss of function, suggesting a single wild-type copy may be sufficient for normal TLR-2 function.[19] This is consistent with the findings in our study, in which no homozygous individuals at this allele were identified, and no differences in severity or duration of acute Q fever were found between carriers and wild-type individuals (Table 3).

The Asp299Gly polymorphism in TLR-4 has been associated with susceptibility to Gram-negative septic shock,[9] and with reduced induction of transcription factors in response to LPS.[20] In the data reported here, no association with this polymorphism and susceptibility to symptomatic Q fever, or the outcomes of the Q fever illness, were documented. In addition, the reported functional significance of this variant with altered patterns of proinflammatory cytokine production was not found in the heterozygotes studied here. This finding is consistent with the report that carriers of the Asp299Gly polymorphism did not differ in endotoxin sensitivity with regard to cytokine production or mitogen-activated protein kinase activity in PBMC,[21] although other reports document that TLR-4 Asp299Gly heterozygotes did have altered cytokine production in other cell types, for instance in response to *Porphyromonas gingivalis* in epithelial cells.[22]

TLR-mediated responses are known to be critical for modulating adaptive immune responses. Signaling via TLR activates dendritic cell maturation, resulting in induction of helper T cell type 1 responses, and signaling via TLR on B cells enhances the formation of germinal center B cells and differentiation into antibody-producing plasma cells.[23] Consistent with this data, specific antibody production in TLR-4$^{-/-}$ mice showed delayed kinetics in response to *C. burnetii* infection.[5] However in the study reported here, no correlation was found between the level of Q fever-specific immunoglobulin G (IgG) production at 6 weeks or 12 months post-enrolment, and the presence of either TLR-2 Arg753Gln or TLR-4 Asp299Gly polymorphic alleles ($n = 75$), using the optical density to cutoff ratio in the enzyme-linked immunosorbent assay (ELISA) assay measuring anti-*C.*

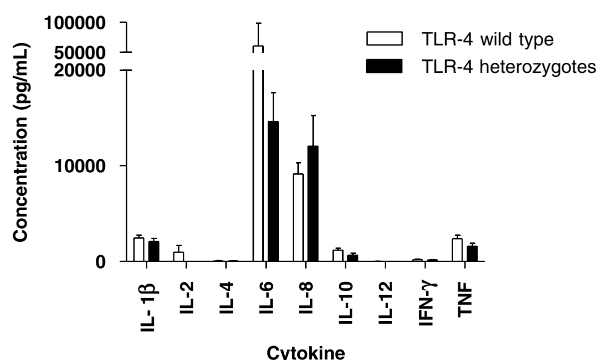


**Figure 1** Cytokine production by LPS-stimulated PBMC. *Ex vivo* cytokine production was assessed as previously described,[14] by overnight incubation of PBMC with or without *Salmonella typhimurium* LPS (10 ng ml$^{-1}$) before harvesting the supernatants and measurement of cytokine production (interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10 IL-12, interferon (IFN)-γ, tumor necrosis factor (TNF)) using a multiplex bead-based immunoassay (Bioplex, BioRad, Hercules, CA, USA). Sample collection and handling, as well as stimulation assays were conducted under endotoxin-minimized conditions. Data are presented as means for subjects of each Toll-like receptor (TLR)-4 genotype after background values obtained without stimulus were subtracted; bars represent standard error values. No statistically significant differences were found between subjects who were TLR-4 wild type ($n = 49$) and heterozygous ($n = 11$) for any of the cytokines measured (Mann–Whitney *U*-test).

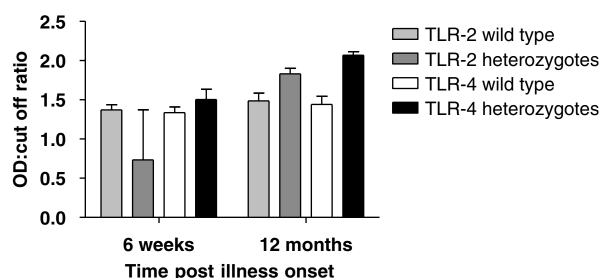


**Figure 2** Q fever-specific IgG production by Toll-like receptors (TLR)-2 and -4 genotype. For comparison of the humoral responses to *C. burnetii* by genotype, IgG antibodies against combined phase I and phase II antigens was assayed by ELISA (PanBio, Brisbane, Australia) in serum samples collected at 6 weeks and 12 months postenrolment. Subjects' results were grouped according to presence or absence of the TLR-2 Arg753Gln or TLR-4 Asp299Gly polymorphisms. Results are presented as mean optical density to cutoff ratio. Bars represent standard error values. No statistically significant differences were found in Q fever-specific IgG levels across different TLR genotypes (Mann–Whitney *U*-test).

*burnetii* IgG antibodies, was used as a surrogate for antibody titer (Figure 2).

Overall, these findings indicate that polymorphisms in the TLR genes do not contribute to the pathogenesis of disease in acute Q fever infection. Further studies in human subjects are required to verify the putative role of TLR-2 and -4 in *Coxiella*–macrophage interactions.

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## Conflict of Interest

The authors state that they have no commercial or other association which would pose a conflict of interest in this work.

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