

Expression of human TLR4/myeloid differentiation factor 2 directs an early innate immune response associated with modest increases in bacterial burden during *Coxiella burnetii* infection

Innate Immunity
2019, Vol. 25(7) 401–411
© The Author(s) 2019
Article reuse guidelines:
sagepub.com/journals-permissions
DOI: 10.1177/1753425919855420
journals.sagepub.com/home/ini


Amanda Robison¹, Deann T Snyder¹, Kelly Christensen¹, Emily Kimmel¹, Adeline M Hajjar², Mark A Jutila¹ and Jodi F Hedges¹ 

Abstract

Human TLR4 (hTLR4) and mouse TLR4 molecules respond differently to hypo-acylated LPS. The LPS of *Coxiella burnetii* is hypo-acylated and heavily glycosylated and causes a minimal response by human cells. Thus, we hypothesized that mice expressing hTLR4 molecules would be more susceptible to *C. burnetii* infection. Our results show that transgenic mice expressing hTLR4 and the human myeloid differentiation factor 2 (MD-2) adaptor protein (hTLR4/MD-2) respond similarly to wild type mice with respect to overall disease course. However, differences in bacterial burdens in tissues were noted, and lung pathology was increased in hTLR4/MD2 compared to wild type mice. Surprisingly, bone marrow chimera experiments indicated that hTLR4/MD-2 expression on non-hematopoietic cells, rather than the target cells for *C. burnetii* infection, accounted for increased bacterial burden. Early during infection, cytokines involved in myeloid cell recruitment were detected in the plasma of hTLR4/MD2 mice but not wild type mice. This restricted cytokine response was accompanied by neutrophil recruitment to the lung in hTLR4/MD2 mice. These data suggest that hTLR4/MD-2 alters early responses during *C. burnetii* infection. These early responses are precursors to later increased bacterial burdens and exacerbated pathology in the lung. Our data suggest an unexpected role for hTLR4/MD-2 in non-hematopoietic cells during *C. burnetii* infection.

Keywords

Chimera, *Coxiella burnetii*, cytokines, neutrophil infiltration, transgenic

Date Received: 8 February 2019; revised: 24 April 2019; accepted: 13 May 2019

Introduction

Coxiella burnetii is an intracellular Gram-negative bacterial pathogen of the lung and the cause of Q fever.^{1,2} Natural infection is usually through aerosol inhalation following exposure to infected animals or animal products. However, domestic infections are increasingly not associated with contact with animals.³ Acute infection presents with flu-like illness, including headache, neurological, gastrointestinal, endocrine, and renal symptoms.^{1,2} The infection can progress to a chronic state, associated with endocarditis and debilitating chronic fatigue.⁴ Although acute Q fever can, in some instances, be cleared naturally without treatment,

the prognosis is greatly improved upon administration of antibiotics. Recent outbreaks of Q fever and studies reporting under-diagnosis suggest that *C. burnetii* may

¹Department of Microbiology and Immunology, Montana State University, USA

²Department of Comparative Medicine, School of Medicine, University of Washington, USA

Corresponding author:

Jodi F Hedges, Department of Microbiology and Immunology, Montana State University, 2155 Analysis Dr., Bozeman, MT 59718, USA.
Email: jodi.hedges@gmail.com



be an emerging threat.^{3–5} There is no approved vaccine in the USA for *C. burnetii*. Thus, there is a need to develop new countermeasures against Q fever. Knowledge of the host response to the pathogen is essential and will facilitate the design of new therapeutic approaches to *C. burnetii* infection. Much of our current knowledge of innate immunity is derived from mouse models, which have known differences in innate protein expression and responses compared to humans and can therefore be a poor reflection of responses in people.⁶ Similarly, the mouse model for Q fever is not ideal because of the relative resistance of mice to *C. burnetii* challenge and differences in disease onset and outcome.⁷ Humans are highly susceptible to low-dose infection (~1–10 bacteria^{8,9}) and are even more susceptible than guinea pigs, which are considered the best experimental model.⁸ Humanized mice that reflect the human immune system can be used for such studies but are costlier. Also, such mice only reflect human cell responses in the hematopoietic compartment, excluding roles that may be unique to epithelial cells or other non-hematopoietic cells.

Permissive subsets of alveolar macrophages and recruited monocytes are believed to be the primary cellular targets of *C. burnetii* infection.¹⁰ In these cells, *C. burnetii* inhabits the macrophage phagolysosome that is extensively modified by bacterial proteins.¹¹ We determined that mouse TLR4, the receptor for LPS, contributes to protection from pulmonary *C. burnetii* infection, as TLR4-deficient mice are more susceptible to infection.¹² *C. burnetii* isolates responsible for disease are referred to as phase I *C. burnetii* and have an LPS with a heavily glycosylated O-Ag compared to the avirulent strain that arises after serial passage in mice. This glycosylation is the only difference between phase I and phase II strains and has a clear impact on virulence.¹³ The extensive glycosylation of virulent *C. burnetii* LPS is thought to shield it from recognition by cells, preventing an inflammatory response in human monocytes.^{14,15} The endotoxic effect of LPS lies in the lipid A molecule, and hexa-acylated lipid A, which occurs in *Escherichia coli*, is the most endotoxic form. All *Coxiella* lipid A molecules are tetra-acylated, which significantly diminishes their endotoxic capacity and inflammatory potential.^{13,16} This aspect of LPS signaling for *Coxiella* has not been a focus of study *in vivo* and may contribute to a dampened response in humans compared to mice. Specifically, hypo-acylated LPS triggers a non-inflammatory response in human cells but a robust inflammatory response in mouse cells.^{17,18} Notably, a dampened response of cells expressing human TLR4 (hTLR4) and myeloid differentiation factor 2 (MD-2) results in increased susceptibility to infection with *Yersinia pestis* expressing hypo-acylated LPS.¹⁸ Thus,

a major contributing factor to enhanced susceptibility to *C. burnetii* infection of humans compared to mice may be in how their TLR4 molecules respond to hypo-acylated LPS.

To compare human and mouse TLR4 responses to *C. burnetii* *in vivo*, we utilized a mouse strain that expresses hTLR4 and the adaptor protein MD-2, also of human origin.^{18,19} We compared infection in these hTLR4/MD-2 transgenic mice to wild type mice. Our results suggested that mice expressing the human molecules had slightly higher bacterial burdens compared to wild type mice. The reasons for the difference in bacterial burden were elusive, with few differences between mouse strains in cell populations or cytokine expression when the differences in bacterial detection were the greatest. Surprisingly, bone marrow chimera experiments demonstrated that the difference in TLR4 responses of the two strains may be specific to the non-hematopoietic compartment rather than the predicted monocyte/macrophages. Very early after infection, there was a strong yet restricted cytokine response detected in the blood, which was accompanied by an influx of neutrophils into the lung of hTLR4/MD-2 mice. This unexpected finding suggests a role for hTLR4 responses in cells that are not thought to be the primary target of infection *in vivo*.

Materials and methods

Bacterial strain, mice, and in vivo infection

C. burnetii Nine Mile phase I (NMI) strain (RSA493) was kindly donated by Dr. Robert Heinzen (Rocky Mountain Laboratories, National Institutes of Health, Hamilton, MT). All use of *C. burnetii* NMI was done in a certified Biosafety Level 3 facility, approved for category B Select Agent use. For this study, we used hTLR4/MD-2 mice, on mouse TLR4^{-/-} and mouse MD-2^{-/-} background (Adeline M. Hajjar, University of Washington, Seattle, WA), generated as described elsewhere.¹⁸ All hTLR4/MD-2 mice were screened by PCR analyses of DNA derived from tail snips for expression of the hTLR4 gene and lack of mTLR4. Only those expressing hTLR4 were used. In these mice, hTLR4 was expressed at levels similar to mouse TLR4 in wild type animals in all tissues tested, and hTLR4/MD-2 mice responded similarly to wild type mice to hexa-acylated LPS from *Salmonella*.¹⁸ C57BL/6 wild type (The Jackson Laboratory, Bar Harbor, ME) and hTLR4/MD-2 transgenic mice were bred at the Animal Resources Center at Montana State University (Bozeman, MT). Since the human MD-2 gene is stably expressed from the Y chromosome, only male mice were used for these studies. Age-matched mice that were between 6 and

10 wk old in groups of five, in most experiments, were infected with 10–100,000 genome equivalents (GE) of phase I *C. burnetii* in PBS. Bacteria for each experiment were diluted in PBS from the same high-concentration, frozen glycerol stock to the experimental concentrations, following identical procedures each time. Following deep anesthesia with isoflurane, bacteria were delivered intratracheally (i.t.) 100 μ l/mouse using a 24-gauge gavage needle. Specifically, 50 μ l was instilled and allowed to be aspirated by a deep breath, followed by two instillations of 25 μ l each followed by deep breaths. Intratracheal delivery results in consistent and even distribution in the lung.²⁰ Uninfected controls were given PBS only. Infected mice were weighed daily. Mice were sacrificed with sodium pentobarbital anesthesia followed by exsanguination at varying intervals post infection. Type I IFN responses were enhanced by i.t. injection of recombinant universal type I IFN (200,000 IU/kg of body mass; R&D Systems, Minneapolis, MN). Treatments were on d 1 post infection and subsequently during infection on alternating days, as previously described.²¹ Alternatively, these responses were inhibited through use of anti-type I IFN receptor monoclonal Abs, as described elsewhere.²²

DNA extraction and PCR

Half of the spleen and approximately two thirds of the lung were processed for DNA extraction and qPCR, as previously described.^{12,23} The PCR reactions were performed in triplicate for each sample and control. *C. burnetii*-specific primers for the *rpos* target gene have been utilized in previous publications.²⁴ Results were compared to a standard curve generated using known amounts of *C. burnetii* DNA, and analyses were performed using GraphPad Prism v5.04 (GraphPad Software, Inc., San Diego, CA). The limits of detection by qPCR are a minimum of 10⁴ GE and a maximum of 10⁹ GE.

Bone marrow cell isolation

To isolate bone marrow cells, 6- to 12-wk-old donor mice were skinned, and the rear legs were removed at the hip joint. The legs were rinsed in PBS+gentamycin. The femur and tibia of each leg was flushed with 1–2 ml of ice-cold, sterile PBS using a 26-gauge needle and 3 ml syringe into a sterile petri dish. The marrow plug was disrupted to make a single cell suspension by gently drawing it up and down in the needle. Cells from donor mice were combined and filtered through 100 μ m mesh cones into a 50 ml centrifuge tube. The bone marrow cells were washed in ice-cold PBS and centrifuged at 170–245 g for 10 min. The supernatant

fluid was decanted, and the bone marrow cells were adjusted to approximately 5 \times 10⁷/ml, yielding 1 \times 10⁷ cells/200 μ l, which was delivered by i.v. tail injection.

Chimera methods

The chimera protocol was established using Ly5.1 mice ensuring lethal irradiation and complete engraftment. Six-wk-old recipient mice were lethally irradiated with 1000 cGy, administered in a split dose consisting of 500 cGy with a 4 h rest in between doses. Isolated bone marrow cells were administered within 1 h of the second radiation dose. Reconstituted mice were treated with the antibiotics sulfamethoxazole (480 μ g/ml) and trimethoprim (96 μ g/ml) in drinking water for the first 6 wk and then given regular water until and during infection. Mice were monitored daily, and there was no evidence of graft versus host disease. After 8–9 wk of reconstitution, mice were challenged i.t. with 1000 GE *C. burnetii*. Thus, mice used in the bone marrow chimera experiments were 14–18 wk old at the time of infection to allow for proper bone marrow engraftment.

Histology

The distal half of the spleen and the left pulmonary lobe, which is approximately one third of the lung, were collected from infected mice for histology. Tissues were fixed in 10% formalin, paraffin embedded, sectioned into 5 μ m sections, and stained with hematoxylin and eosin (H&E) following the manufacturer's instructions. Histological sections were stained for the presence of bacteria using immunofluorescence, as previously described.¹² Histological sections were scored based on a 0–3 scale (0: none; 1: mild; 2: moderate; 3: severe) for five separate parameters: infiltration across the parenchyma, bronchitis, peribronchitis, vasculitis, and perivasculitis. All images were acquired using a DS-Ri-1 camera (Nikon, Tokyo, Japan) mounted on a Nikon Eclipse 80i microscope.

Cytometric Bead Array

Cytokines in bronchoalveolar lavage (BAL) supernatant fluids and plasma were quantified using the BD Cytometric Bead Array (BD Biosciences, Franklin Lakes, NJ). Initial experiments assessed the cytokines in a 7-plex—G-CSF, GM-CSF, IFN- γ , IL-10, IL-12, IL-17, and IL-1 β —to assess BAL supernatant fluids and plasma collected at 6 d post infection. Subsequent experiments were expanded and refined to include in an 11-plex—G-CSF, IFN- γ , IL-10, IL-12, IL-17, IL-1 β , IL-2, IL-6, KC, TNF- α , and CD62L—to assess BAL supernatant fluids and plasma collected at 24 and 72 h

post infection. Finally, the Cytometric Bead Array was further refined as an 8-plex containing the cytokines G-CSF, IFN- γ , IL-12, IL-17, IL-6, KC, TNF- α , and CD62L because IL-10, GM-CSF, IL-1 β , and IL-2 were never detected. The 8-plex was used to detect cytokines at 16 and 40 h post infection. BAL fluids and plasma were minimally diluted, and the assay was performed according to the manufacturer's instructions. Data were analyzed using the BD FCAP ArrayTM Software, Microsoft Excel, and GraphPad Prism.

Statistical analysis

Statistical analyses were performed using GraphPad Prism v5.04 (GraphPad Software, Inc.). The Kolmogorov–Smirnov test (with Dallal–Wilkinson–Liliefors *P* value) was used to confirm that the data formed a Gaussian distribution. Data were analyzed with Student's *t*-test when comparing only two groups, and one-way ANOVA with Bonferroni post test when comparing multiple groups.

Results

Comparison of hTLR4/MD-2 and wild type mice during *C. burnetii* infection

To determine the differences between mice that express hTLR4/MD-2 and wild type mice, both strains were infected with equivalent amounts of *C. burnetii*. Our prior studies indicated that delivery to the lung, the natural route of infection in humans, was necessary to assess the function of TLRs best.¹² Mice were infected i.t. with 10, 100, or 1000 GE of *C. burnetii* NMI and monitored for up to 30 d. There was no detection of bacterial genomes by qPCR (due to the limits of detection) in tissues from either mouse strain 3 d after i.t. infection with 1000 GE (data not shown). Bacteria were detected by immunofluorescence staining of histological sections 3 d post infection, only in the lung and no other tissues, but not at 1 d post infection. At 3 d post infection, there was no difference in immunofluorescent staining of *C. burnetii* between the two strains in the lung (data not shown). In the *C. burnetii* infection model in mice, mass loss is generally considered a proxy for greater inflammation in response to infection. All infected mice lost mass at about 6 d post infection then began to gain again at 9 d post infection. There were no consistent significant differences in mass changes between hTLR4/MD-2 and wild type mice (data not shown). This suggests that both strains mounted effective host immune responses, precluding survival studies as a measure of strain differences.

Splenomegaly is also a hallmark of *C. burnetii* infection. Figure 1a shows the spleen masses in mice infected with various doses of *C. burnetii* at 6, 9, or 30 d post infection. hTLR4/MD-2 mice generally had larger spleens at earlier intervals, or with lower doses of bacteria, and the difference was significant 6 d post infection with 100 GE *C. burnetii* and 9 d post infection with 10 GE. Similarly, at 6 d post infection, hTLR4/MD-2 mice had larger bacterial burdens in the spleen following infection with 100 and 1000 GE. By 9 d post infection, a significant difference in spleen bacterial burdens was noted for the lowest dose of bacteria (Figure 1b). We also measured bacterial burdens in the lung at these intervals. Figure 1c demonstrates that a burst of bacterial replication could be detected by 6 d post infection, but this was largely resolved by d 9, except following infection with 1000 GE. There were no differences in bacterial burdens in the lung at 6 d post infection, but at 9 and 30 d post infection with 1000 GE, mice expressing hTLR4/MD-2 had significantly greater bacterial burdens in the lungs. Thus, larger spleens and greater *C. burnetii* bacterial burdens could be detected in hTLR4/MD-2 mice compared to wild type animals, but these differences were not consistent in all comparisons and were dependent on the infectious dose and the interval post infection. The only dose that resulted in significant differences in bacterial burdens at both 6 d post infection (in the spleen) and 9 d post infection (in the lung) was 1000 GE, and so this dose was chosen for subsequent studies. Though hTLR4/MD-2 expression had a modest impact on *C. burnetii* burden, it did not render the mice overtly more susceptible to *C. burnetii* infection, contrary to our hypothesis and to findings with other bacteria with hypo-acetylated LPS.

We next assessed the importance of cellular compartment specificity for function of hTLR4/MD-2. Bone marrow chimera experiments were performed to determine if hTLR4/MD-2 expression was more important on hematopoietic or stromal/epithelial cells following infection with *C. burnetii*. We hypothesized that the function of hTLR4/MD-2 would primarily be found in macrophage/monocytes, since these cells are the accepted targets of infection. However, TLR4 is also expressed and responsive to LPS in stromal cells in the lung and other organs, specifically epithelial cells.²⁵ Following bone marrow cell transfers, mice were infected with 1000 GE/mouse for 9 d. Again, the differences in mass loss between the mouse groups were minimal (data not shown). At 9 d post infection, the chimera mice were euthanized, and bacterial burdens were determined in spleens and lungs by qPCR. Bacterial burden was greater in the spleens of mice that expressed hTLR4/MD-2 on non-hematopoietic cells (Figure 2). Furthermore, in this

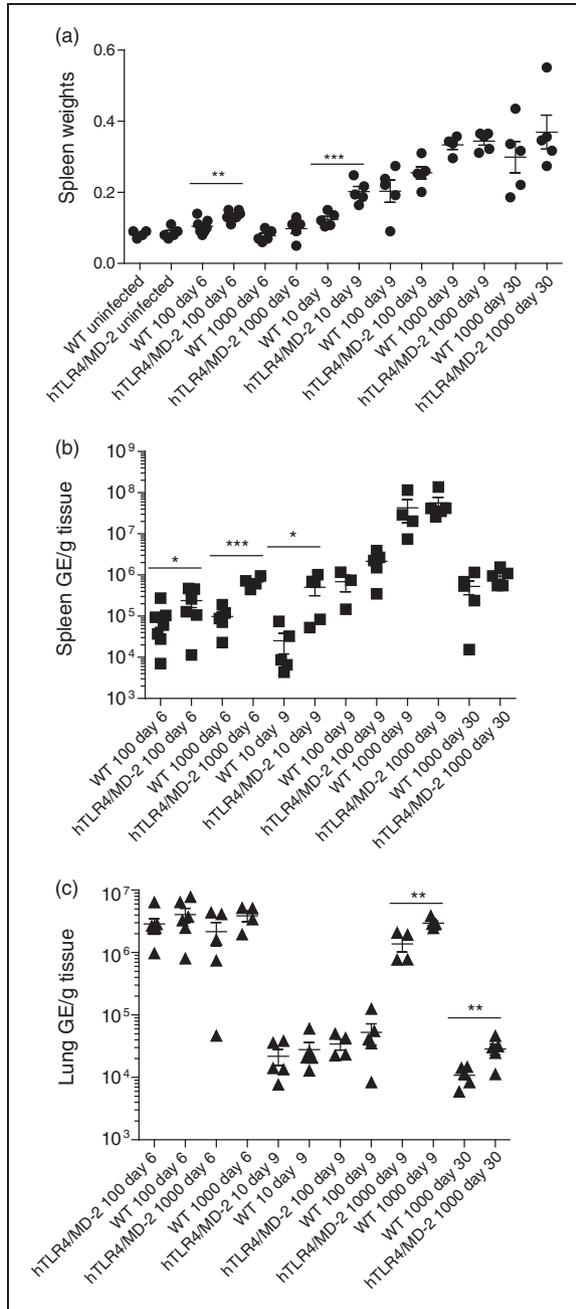


Figure 1. Mice expressing human TLR4 (hTLR4)/myeloid differentiation factor 2 (MD-2) have larger spleens and higher spleen *C. burnetii* burdens early and with lower infectious doses and higher lung bacterial burdens later after higher dose infection. Wild type and hTLR4/MD-2 transgenic mice were infected intratracheally (i. t.) with 10, 100, or 1000 genome equivalents (GE) of *C. burnetii* Nine Mile phase I (NMI) and sacrificed 6, 9, or 30 d post infection. (a) Spleens were weighed as a marker of disease severity. Bacterial burdens in spleens (b) and lungs (c) were assessed by qPCR. Graphs contain data from experiments conducted at different times. All graphs represent the average of at least four mice per group, with standard error shown. These data are representative of between two and four repeat experiments. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ as measured in pairwise comparisons of two groups using Student's *t*-test.

experiment, there was a significant difference in spleen bacterial burdens between wild type mice that received wild type bone marrow and hTLR4/MD-2 mice that received hTLR4/MD-2 bone marrow. These control groups are essentially representative of wild type and hTLR4/MD-2 mice in the initial experiments shown in Figure 1. However, the experimental conditions were quite different. The groups shown in Figure 2 included all the procedures involved in the bone marrow transplantation, such as radiation and antibiotic treatments, which may have pre-disposed the hTLR4/MD-2 mice to even higher susceptibility to infection. Nonetheless, this difference supports conclusions from our initial experiments on bacterial burden in hTLR4/MD2 versus wild type mice. There were similar trends in lung bacterial burdens between the chimeric mice, but variation within groups precluded significance. Importantly, these chimera results suggest that expression of hTLR4/MD-2 on non-hematopoietic cells accounts for the greater susceptibility to *C. burnetii* infection. Of note, histological staining for *C. burnetii* in lung and spleen tissues presented no evidence of increased infection of stromal cells in hTLR4/MD-2 mice (data not shown). Surprisingly, hTLR4 expression on hematopoietic cells was of minimal consequence, contrary to our expectations. These data suggest that expression of hTLR4 in stromal and/or epithelial compartments may direct increased susceptibility in hTLR4/MD-2 mice.

We also assessed histological differences by H&E staining between the two mouse strains at various intervals post infection with 1000 GE *C. burnetii*. Mice expressing hTLR4/MD-2 had significantly increased cellular infiltration in the lung compared to wild type mice 9 d post infection (Figure 3). Other differences in lung pathology were apparent at 9, 30, and 60 d post infection. Mice expressing hTLR4/MD-2 had increased infiltration throughout the lung parenchyma, and significantly increased bronchial and vascular inflammation and peribronchial and perivascular inflammation at all intervals post infection compared to wild type mice (Figure 3). Histological differences in the spleens were not apparent (data not shown). These histology findings show that even though body or spleen masses were not different, hTLR4/MD-2 mice experienced greater *C. burnetii*-induced lung pathology compared to wild type mice.

TLR4 signaling can lead to type I IFN production. Thus, we tested whether type I IFN could account for the different phenotype in hTLR4/MD-2 mice. In other studies, we showed that type I IFN responses could hinder or enhance host clearance of *C. burnetii* in a tissue-specific fashion.²¹ hTLR4/MD-2 and wild type mice were compared in experiments in which type I IFN responses were either enhanced through injection

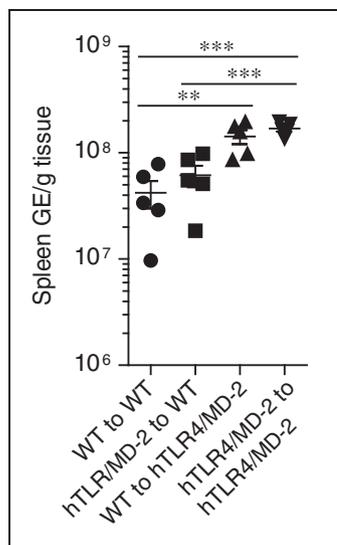


Figure 2. Chimeric mice demonstrate that non-hematopoietic cells direct enhanced susceptibility in hTLR4/MD-2 mice. The mice expressing hTLR4/MD-2 on stromal/epithelial cells had greater bacterial burdens in the spleens compared to wild type mice, regardless of the type of hematopoietic cells they received. These data are representative of two repeat experiments. ** $P < 0.01$ and *** $P < 0.001$ as measured by one-way ANOVA with Bonferroni's multiple comparison test, Error bars represent standard error.

of recombinant type I IFN, as previously described,²¹ or inhibited through the use of anti-type I IFN receptor mAbs.²² The results showed that wild type and hTLR4/MD-2 mice responded similarly to these treatments following infection with *C. burnetii*. Thus, a differential type I IFN response between the two strains did not account for the difference in phenotype (data not shown).

Early responses to *C. burnetii* infection

We measured cytokines in the BAL and plasma at various intervals post infection. We expected that the largest difference in innate cytokine detection would occur at the intervals at which the most prominent differences in bacterial load occurred. However, we only detected significant differences in G-CSF and KC (CXCL1) in the plasma at very early intervals following infection. Mice were infected with 1000 GE and assessed at 24 and 72 h post infection, or were infected with 100,000 GE *C. burnetii* and assessed at 16 and 40 h post infection. The higher dose was used to ensure that all lung cells were infected in concert. In both experiments, at the early interval post infection, hTLR4/MD-2 mice had significantly more KC (Figure 4a and b) and nearly significant or significantly more G-CSF (Figure 4c and d, respectively) in plasma than did wild type mice. By 40 and 72 h post infection, these

differences were no longer apparent. These differences were only detected in plasma. Such differences in cytokines were not detected in BAL fluids at any interval, even though bacteria were detectable in the lung tissue (by immunofluorescence) at 72 h post infection (data not shown). Figure 4e shows that the difference in G-CSF between the strains was not present 6 d post infection. Notably, at 6 d post infection, expression of all seven assessed cytokines in the plasma of both mouse strains was substantially lower, and there were no differences between the strains for any cytokines. Thus, even though the Cytometric Bead Array used at 6 d post infection did not include KC, we expect that expression of KC is also lower and similar between strains at this interval, consistent with the other cytokines. Even lacking this datum, altogether these results suggest that hTLR4/MD-2 may direct an early event, resulting in expression of G-CSF and KC in the blood soon after infection that quickly diminishes.

Differential counts of cell populations in the BAL fluids were also assessed to determine a phenotypic difference in cell recruitment to the lungs between the mouse strains. While there were no differences in BAL cell differentials at 6 d (data not shown) and 9 d post infection, a difference was detected at 16 h post infection (Figure 5). At this early interval, it was visually apparent on cytopsin slides that a large percentage of neutrophils was present in the lungs of most hTLR4/MD-2 mice that was not represented in wild type lungs (Figure 5a). Differential counts of cell types represented on cytopsin slides were graphed for the intervals 16 h, 40 h, and 9 d post infection. There were more neutrophils in most hTLR4/MD-2 mice at 16 h post infection, an effect that was resolved by 40 h post infection, and neutrophils were increased in both strains equally by 9 d post infection (Figure 5b). There appeared to be slightly more macrophages in BALs from wild type mice at all intervals (Figure 5c). However, variability between individual mice precluded significance. These findings suggest that a very early event resulted in accumulation of limited cytokines in the plasma, presumably produced by the infected lung tissue. Early neutrophil recruitment into the lung may possibly contribute to the enhanced pathology later in infection detected by histology.

Discussion

These data show that mice expressing hTLR4/MD-2 are slightly more susceptible to *C. burnetii* infection at some intervals than those expressing mouse TLR4. However, the overall response to infection is similar in both strains of mice, confirming that the wild type mouse model of *C. burnetii* infection is generally predictive of disease course in terms of TLR4 function.

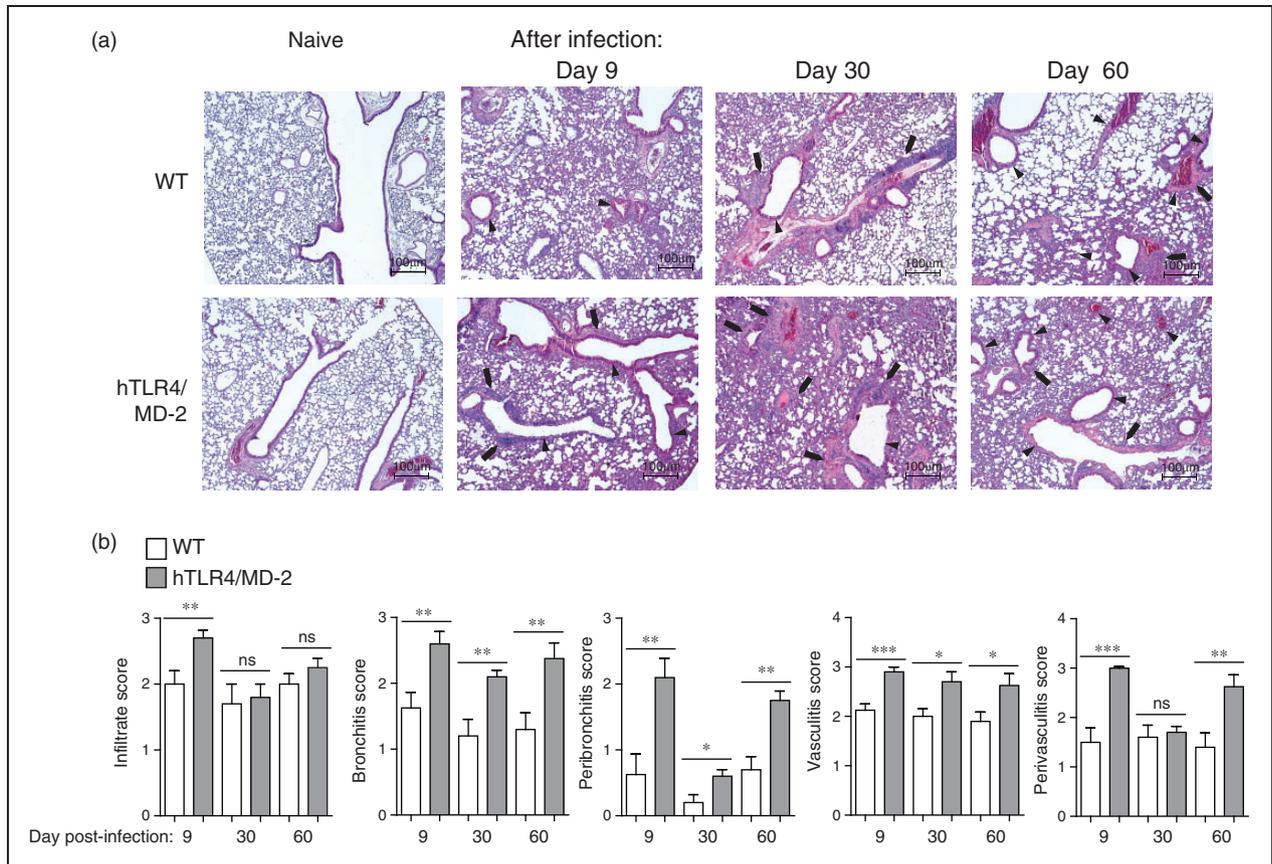


Figure 3. Mice expressing hTLR4/MD-2 had increased cellular infiltration and pathology in the lung compared to wild type mice. Wild type and hTLR4/MD-2 mice were infected i.t. with 1000 GE of *C. burnetii* NMI for 9, 30, and 60 d. Lung and spleen tissue sections were stained with hematoxylin and eosin. (a) At all three intervals, hTLR4/MD-2 lung sections show increased infiltration throughout the parenchyma, increased bronchial and vascular inflammation (arrows), and moderate peribronchial and perivascular inflammation (arrowheads) compared to wild type mice. (b) The sections were scored for five parameters on a 0–3 scale, and the average score per group at each interval was graphed. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, as measured in pairwise comparisons of two groups using Student's *t*-test, Error bars represent standard error.

Differences in bacterial burdens in the spleens between the strains were greater at 6 d post infection and at 9 d with low-dose infection. Differences in bacterial burdens in the lungs could be detected after longer infection intervals (9 and 30 d) after infection with higher doses. Differences in bacterial burdens were not dramatic enough to lead to significant differences in morbidity and/or moribundity. We also assessed infection with 10^4 and 10^5 bacteria per mouse, which are unrealistically high for modeling natural *C. burnetii* infection. At these doses, there were also no differences in bacterial burdens between strains (data not shown). Based on inflammation pathology, hTLR4/MD-2 mice had an increased cellular response to infection with *C. burnetii* NMI in the lung. The cellular infiltrate consisted of monocytes, macrophages, and neutrophils in both strains. However, there was greater infiltration and pathology in mice expressing hTLR4/MD-2. A similar observation was previously noted in mice

deficient in TLR signaling.¹² This increased infiltration may provide more cells as targets for infection or may be a secondary effect caused by slightly greater bacterial replication.

Few differences in cytokine expression and cellular subsets were detected at the times when the differences in bacterial burden were greatest. Rather, when we assessed cytokines in the plasma and cellular influx into the lung at very early intervals, significant differences were detected. We expected that early events specific to hTLR4/MD-2 mice, if any, would be suggestive of a dampened innate immune response that would render these mice more susceptible to infection. The results, however, suggested an enhanced, yet limited, early inflammatory response of hTLR4/MD-2 mice compared to wild type animals. hTLR4/MD-2 mice accumulated G-CSF and KC in the blood early in infection, accompanied by neutrophil influx into the lung that quickly resolved. However, it was a prelude

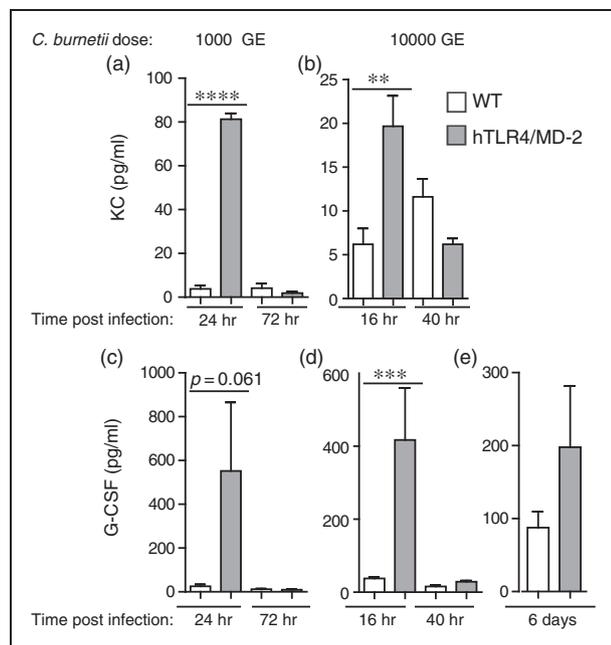


Figure 4. Differences in plasma cytokine concentrations between the two strains were detected early following *C. burnetii* infection. Significantly more (a and b) CXCL1 (KC) and (c and d) G-CSF was detected in plasma from mice expressing hTLR4/MD-2 compared to wild type mice early after infection (16 or 24 h) with 1000 or 10,000 GE *C. burnetii*, respectively. (e) By 6 d post infection, in contrast, the detection of G-CSF was lower, and levels were not significantly different between the two strains. All graphs represent the average of at least four mice per group with standard deviation shown. ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ as measured in pairwise comparisons of two groups using Student's t-test.

to greater pathology later in infection. The results from the chimeric mice suggested that cells responsible for the subtle difference in susceptibility between hTLR4/MD-2 and wild type mice reside in the non-hematopoietic compartment. The likely source for early KC and G-CSF is the epithelial, endothelial, or stromal cells of the lung, since this is the only tissue that has had contact with the bacteria at this early interval. Notably, each of these non-hematopoietic cell subsets expresses TLR4, and KC and G-CSF are known downstream effects of stromal cell signaling in the lung.^{25,26} In addition, epithelial cell-derived cytokine-mediated down-regulation of macrophage function may play a role in persistence.^{27,28} The cytokine response in hTLR4/MD-2 lungs is best described as an enhanced early innate response, contrary to our prediction of a dampened response in these mice. The early neutrophil influx and cytokine detection was only tested following higher dose infections. This was done to synchronize the innate response for better detection. It may be necessary to determine if these events also occur following lower dose infection, but in this case,

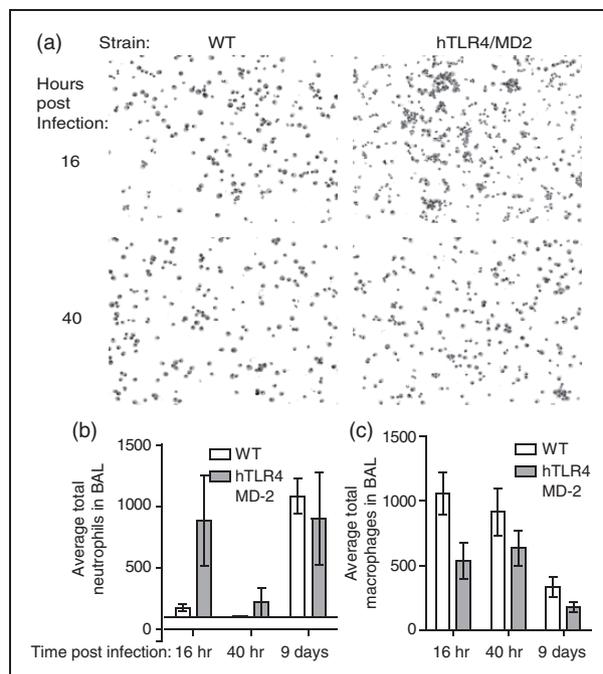


Figure 5. Early neutrophil influx to the lungs of hTLR4/MD-2 mice. (a) Representative bronchoalveolar lavage (BAL) cytopsin images show that at 16 h post infection with 10^5 GE *C. burnetii*, mice expressing hTLR4/MD-2 had a notable influx of neutrophils that was not observed in wild type mice and was largely resolved by 40 h post infection. (b) The number of neutrophils was greater but not significantly different at 16 h post infection. This trend was not apparent at later intervals (at least three mice per group). (c) The number of neutrophils at 16 h post infection was not associated with a substantial decrease in macrophages in the BAL. There were no significant differences in BAL macrophages at any interval. Error bars represent standard error.

the events may be somewhat drawn out and may require more involved assessment of various intervals post infection.

KC is primarily known for recruiting neutrophils, but can also promote monocyte trafficking.²⁹ G-CSF is a cytokine primarily associated with neutrophil development and stimulation. G-CSF and KC direct myeloid cell responses, reflected by increased bone marrow output and recruitment into tissues.^{29,30} This recruitment event may supply more monocyte/macrophages to the tissue that could then be infected with *C. burnetii*. Neutrophils have been assessed in the context of *C. burnetii* infection. In SCID mice, neutrophil influx was not substantial until 7 d post infection.³¹ Furthermore, *in vitro* data suggested that *C. burnetii* NMI could infect neutrophils and “hide” there until subsequently infecting macrophages. Thus, neutrophils may have the capacity to enhance infection.³¹ On the other hand, there is also evidence that neutrophils are beneficial and necessary for protection against *C. burnetii* infection.^{31,32} Neutrophils were chronically

depleted by treating mice on alternating days throughout infection with the monoclonal Ab RB6-8C5. Mice lacking neutrophils had more dramatic mass loss and increased bacterial burden compared to isotype-treated controls, indicating that neutrophils enhanced protection in this case.³² Thus, it is not clear whether this early neutrophil surge observed in hTLR4/MD-2 mice would protect against or enhance infection with *C. burnetii*. In an attempt to answer this question, we determined that injecting mice once with 20 µg RB6-8C5 could selectively block short-term neutrophil recruitment to the lung. When this treatment was applied to hTLR4/MD-2 and wild type mice, the differences in *C. burnetii* bacterial burdens were not altered (data not shown). The early events we have characterized were not detected in other mouse studies, suggesting that our findings may be observable only when hTLR4/MD-2 is present.

There is some precedence for a species difference in gene expression induced downstream of TLR4. Schroder et al. compared the response of human and mouse macrophages to *Salmonella* LPS, and measured transcript expression at 2, 6, and 24 h post stimulation.³³ They found that the genes robustly expressed early by human macrophages included two chemokines and G-CSF. Starting at 2 and 6 h after stimulation, human macrophages exhibited 50-fold induction of CCL20 and CXCL13, respectively. In contrast, mouse macrophages did not express these chemokines at any of the intervals assessed. Human macrophages also strongly induced G-CSF (also known as CSF-3) transcripts at 2 h after stimulation, whereas mouse macrophages only expressed G-CSF after 6 h of stimulation.³³ Thus, hTLR4 may direct an altered/enhanced early G-CSF expression, regardless of the acylation of the stimulating LPS.

Other studies have shown that the expression of MyD88 displays a role comparable to that of hTLR4/MD-2 in lung parenchymal (non-bone marrow) cells during infection with *Pseudomonas*.³⁴ In this case, MyD88 expressed on non-bone marrow cells directed early expression of chemokines (CXCL1 and MIP-2), leading to attraction of critical immune cells that contribute to protection from infection. Similarly, the expression of hTLR4/MD-2 on non-hematopoietic cells may change innate responses of epithelial, endothelial, or stromal cells, affecting mechanisms leading to enhanced innate responses. In contrast, using similar chimera experiments, we determined that during *C. burnetii* infection, the roles for mouse TLR2¹² and MyD88 (data not shown) were primarily dependent on hematopoietic cells for limiting bacteria growth at 9 d post infection. Further study utilizing the chimera models described herein is necessary to determine the response of hTLR4/MD2 to *C. burnetii* infection by non-hematopoietic cells.

Since *C. burnetii* does not induce a strong inflammatory response from human cells,^{14,35} our study may reinforce that this dampened effect is due to the excessive glycosylation on the O-Ag and not because of hypo-acylation. In other words, the heavy glycosylation on the O-Ag of virulent *C. burnetii* is more important than the acylation of the lipid A for contribution to the dampened inflammatory effect in humans *in vivo*.

Mounting evidence suggests that the genotype of specific innate immune genes including TLR4 can direct the composition of the microbiota, which in turn can alter susceptibility to infection.³⁶ Even though our mouse strains are held in the same animal facility and would be expected to reflect similar microbiomes, differences in TLR4 responses may alter the composition of the microbiome in hTLR4/MD-2 compared to wild type mice. An altered microbiome directed by the expression of hTLR4/MD-2 may explain altered susceptibility to *C. burnetii* infection of these mice and possibly of humans or other animals as well. However, additional comprehensive studies would clearly be required to confirm this theory.

In summary, we have demonstrated that hTLR4/MD-2-expressing mice are slightly more susceptible to infection with *C. burnetii*. This strain harbored slightly greater bacterial burdens, but this was dependent on the tissue assessed and the interval post infection and had enhanced lung pathology later in infection. Unexpectedly, the basis for increased susceptibility lies within a non-bone marrow-derived compartment, which is the focus of ongoing studies. The hTLR4/MD-2 mice displayed a robust innate response very early following infection that was not apparent in wild type mice. Though hTLR4/MD-2 mice do not represent a dramatically improved model for Q fever, the use of these mice may provide some new insights into host responses against *C. burnetii*. The early neutrophil response we noted in these mice may affect the outcomes of infection with other pathogens. Furthermore, the hTLR4/MD-2 mice are likely to be critical to determining how agonist recognition by hTLR4 might help shape the microbiome or downstream adaptive responses in vaccine or adjuvant studies.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The author(s) disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: Research reported in this publication was supported by the National Institutes of Health under award numbers

NIH COBRE (P20 RR020185), R21AI117441 (M.A.J.), and R21OD017233 (A.M.H.), with partial funding through NIH R21AI119772, NIH-NCCAM (PO1AT0004986), USDA NIFA (2014-67016-21552), M.J. Murdock Charitable Trust and The Montana State University Agricultural Experimental Station/NIFA.

ORCID iD

Jodi F Hedges  <https://orcid.org/0000-0002-7461-3972>

References

1. Marrie TJ. *Coxiella burnetii* pneumonia. *Eur Respir J* 2003; 21: 713–719.
2. Parker NR, Barralet JH and Bell AM. Q fever. *Lancet* 2006; 367: 679–688.
3. Dahlgren FS, McQuiston JH, Massung RF, et al. Q fever in the United States: summary of case reports from two national surveillance systems, 2000–2012. *Am J Trop Med Hyg* 2015; 92: 247–255.
4. Morroy G, Peters JB, van Nieuwenhof M, et al. The health status of Q-fever patients after long-term follow-up. *BMC Infect Dis* 2011; 11: 97.
5. Dahlgren FS, Haberling DL and McQuiston JH. Q fever is underestimated in the United States: a comparison of fatal Q fever cases from two national reporting systems. *Am J Trop Med Hyg* 2015; 92: 244–246.
6. Seok J, Warren HS, Cuenca AG, et al. Genomic responses in mouse models poorly mimic human inflammatory diseases. *Proc Natl Acad Sci U S A* 2013; 110: 3507–3512.
7. Russell-Lodrigue KE, Zhang GQ, McMurray DN, et al. Clinical and pathologic changes in a guinea pig aerosol challenge model of acute Q fever. *Infect Immun* 2006; 74: 6085–6091.
8. Brooke RJ, Kretzschmar ME, Mutters NT, et al. Human dose response relation for airborne exposure to *Coxiella burnetii*. *BMC Infect Dis* 2013; 13: 488.
9. Tigertt WD, Benenson AS and Gochenour WS. Airborne Q fever. *Bacteriol Rev* 1961; 25: 285–293.
10. Calverley M, Erickson S, Read AJ, et al. Resident alveolar macrophages are susceptible to and permissive of *Coxiella burnetii* infection. *PLoS One* 2012; 7: e51941.
11. Heinzen RA, Scidmore MA, Rockey DD, et al. Differential interaction with endocytic and exocytic pathways distinguish parasitophorous vacuoles of *Coxiella burnetii* and *Chlamydia trachomatis*. *Infect Immun* 1996; 64: 796–809.
12. Ramstead AG, Robison A, Blackwell A, et al. Roles of TLR2, TLR4, and MyD88 during pulmonary *Coxiella burnetii* infection. *Infect Immun* 2016; 84: 940–949.
13. Narasaki CT and Toman R. Lipopolysaccharide of *Coxiella burnetii*. In: Toman R, Heinzen RA, Samuel JE and Mege JL (eds) *Coxiella burnetii: recent advances and new perspectives in research of the Q fever bacterium*. Dordrecht: Springer Netherlands, 2012. pp.65–90.
14. Shannon JG, Howe D and Heinzen RA. Virulent *Coxiella burnetii* does not activate human dendritic cells: role of lipopolysaccharide as a shielding molecule. *Proc Natl Acad Sci U S A* 2005; 102: 8722–8727.
15. Barry AO, Boucherit N, Mottola G, et al. Impaired stimulation of p38 α -MAPK/Vps41-HOPS by LPS from pathogenic *Coxiella burnetii* prevents trafficking to microbicidal phagolysosomes. *Cell Host Microbe* 2012; 12: 751–763.
16. Toman R, Hussein A, Palkovic P, et al. Structural properties of lipopolysaccharides from *Coxiella burnetii* strains Henzlerling and S. *Ann New York Acad Sci* 2003; 990: 563–567.
17. Freudenberg MA, Tchaptchet S, Keck S, et al. Lipopolysaccharide sensing an important factor in the innate immune response to Gram-negative bacterial infections: benefits and hazards of LPS hypersensitivity. *Immunobiology* 2008; 213: 193–203.
18. Hajjar AM, Ernst RK, Fortuno ES, et al. Humanized TLR4/MD-2 mice reveal LPS recognition differentially impacts susceptibility to *Yersinia pestis* and *Salmonella enterica*. *PLoS Pathog* 2012; 8: e1002963.
19. Hajjar AM, Ernst RK, Tsai JH, et al. Human Toll-like receptor 4 recognizes host-specific LPS modifications. *Nat Immunol* 2002; 3: 354–359.
20. Driscoll KE, Costa DL, Hatch G, et al. Intratracheal instillation as an exposure technique for the evaluation of respiratory tract toxicity: uses and limitations. *Toxicol Sci* 2000; 55: 24–35.
21. Hedges JF, Robison A, Kimmel E, et al. Type I interferon counters or promotes *Coxiella burnetii* replication dependent on tissue. *Infect Immun* 2016; 84: 1815–1825.
22. Shahangian A, Chow EK, Tian X, et al. Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. *J Clin Invest* 2009; 119: 1910–1920.
23. Read AJ, Erickson S and Harmsen AG. Role of CD4+ and CD8+ T cells in clearance of primary pulmonary infection with *Coxiella burnetii*. *Infect Immun* 2010; 78: 3019–3026.
24. Coleman SA, Fischer ER, Howe D, et al. Temporal analysis of *Coxiella burnetii* morphological differentiation. *J Bacteriol* 2004; 186: 7344–7352.
25. Perros F, Lambrecht BN and Hammad H. TLR4 signalling in pulmonary stromal cells is critical for inflammation and immunity in the airways. *Respir Res* 2011; 12: 125.
26. Guillot L, Medjane S, Le-Barillec K, et al. Response of human pulmonary epithelial cells to lipopolysaccharide involves toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. *J Biol Chem* 2004; 279: 2712–2718.
27. Capo C, Zugun F, Stein A, et al. Upregulation of tumor necrosis factor alpha and interleukin-1 beta in Q fever endocarditis. *Infect Immun* 1996; 64: 1638–1642.
28. Honstetter A, Ghigo E, Moynault A, et al. Lipopolysaccharide from *Coxiella burnetii* is involved in bacterial phagocytosis, filamentous actin reorganization, and inflammatory responses through toll-like receptor 4. *J Immunol* 2004; 172: 3695–3703.
29. Vries MHM, Wagenaar A, Verbruggen SEL, et al. CXCL1 promotes arteriogenesis through enhanced

- monocyte recruitment into the peri-collateral space. *Angiogenesis* 2015; 18: 163–171.
30. Lieschke GJ, Grail D, Hodgson G, et al. Mice lacking granulocyte colony-stimulating factor have chronic neutropenia, granulocyte and macrophage progenitor cell deficiency, and impaired neutrophil mobilization. *Blood* 1994; 84: 1737.
 31. Elliott A, Peng Y and Zhang G. *Coxiella burnetii* interaction with neutrophils and macrophages *in vitro* and in SCID mice following aerosol infection. *Infect Immun* 2013; 81: 4604–4614.
 32. Elliott A, Schoenlaub L, Freches D, et al. Neutrophils play an important role in protective immunity against *Coxiella burnetii* infection. *Infect Immun* 2015; 83: 3104–3113.
 33. Schroder K, Irvine KM, Taylor MS, et al. Conservation and divergence in toll-like receptor 4-regulated gene expression in primary human versus mouse macrophages. *Proc Natl Acad Sci U S A* 2012; 109: E944–E953.
 34. Hajjar AM, Harowicz H, Liggitt HD, et al. An essential role for non-bone marrow-derived cells in control of *Pseudomonas aeruginosa* pneumonia. *Am J Respir Cell Mol Biol* 2005; 33: 470–475.
 35. Shannon JG and Heinzen RA. Infection of human monocyte-derived macrophages with *Coxiella burnetii*. *Methods Mol Biol* 2008; 431: 189–200.
 36. Olivares M, Laparra JM and Sanz Y. Host genotype, intestinal microbiota and inflammatory disorders. *Br J Nutr* 2013; 109: S76–S80.