AMERICAN SOCIETY FOR MICROBIOLOGY | Infection and Immunity®

BACTERIAL INFECTIONS

Viable *Coxiella burnetii* Induces Differential Cytokine Responses in Chronic Q Fever Patients Compared to Heat-Killed *Coxiella burnetii*

Anne F. M. Jansen,[a,b] Annemieke Dinkla,[c,d] Hendrik-Jan Roest,[c,d] Chantal P. Bleeker-Rovers,[a,b] Teske Schoffelen,[a,b] Leo A. B. Joosten,[a,b] Peter C. Wever,[e] Marcel van Deuren,[a,b] Ad P. Koets[c,d]

[a]Department of Internal Medicine, Radboud Center for Infectious Diseases (RCI), Radboud University Medical Center, Nijmegen, the Netherlands

[b]Radboud Expert Centre for Q Fever, Radboud University Medical Center, Nijmegen, the Netherlands

[c]Department of Bacteriology and Epidemiology, Wageningen Bioveterinary Research, Lelystad, the Netherlands

[d]Department of Farm Animal Health, Faculty of Veterinary Medicine, Utrecht University, Utrecht, the Netherlands

[e]Department of Medical Microbiology and Infection Control, Jeroen Bosch Hospital, 's-Hertogenbosch, the Netherlands

ABSTRACT Cytokine responses of chronic Q fever patients to the intracellular bacterium *Coxiella burnetii* have mostly been studied using *ex vivo* stimulation of immune cells with heat-killed *C. burnetii* due to the extensive measures needed to work with viable biosafety level 3 agents. Whether research with heat-killed *C. burnetii* can be translated to immune responses to viable *C. burnetii* is imperative for the interpretation of previous and future studies with heat-killed *C. burnetii*. Peripheral blood mononuclear cells (PBMCs) of chronic Q fever patients ($n = 10$) and healthy controls ($n = 10$) were stimulated with heat-killed or viable *C. burnetii* of two strains, Nine Mile and the Dutch outbreak strain 3262, for 24 h, 48 h, and 7 days in the absence or presence of serum containing anti-*C. burnetii* antibodies. When stimulated with viable *C. burnetii*, PBMCs of chronic Q fever patients and controls produced fewer proinflammatory cytokines (interleukin-6 [IL-6], tumor necrosis factor alpha, and IL-1β) after 24 h than after stimulation with heat-killed *C. burnetii*. In the presence of Q fever seronegative serum, IL-10 production was higher after stimulation with viable rather than heat-killed *C. burnetii*; however, when incubating with anti-*C. burnetii* antibody serum, the effect on IL-10 production was reduced. Levels of adaptive, merely T-cell-derived cytokine (gamma interferon, IL-17, and IL-22) and CXCL9 production were not different between heat-killed and viable *C. burnetii* stimulatory conditions. Results from previous and future research with heat-killed *C. burnetii* should be interpreted with caution for innate cytokines, but heat-killed *C. burnetii*-induced adaptive cytokine production is representative of stimulation with viable bacteria.

KEYWORDS chronic Q fever, *Coxiella burnetii*, cytokines, immune response

Coxiella burnetii is the causative microorganism of Q fever, a highly infectious zoonosis that is prevalent worldwide. Infection is contracted by inhalation of contaminated aerosols. After inhalation and infection, the obligate intracellular growing *C. burnetii* invades the alveolar macrophages. Replication and outgrowth of *Coxiella* take place in a peculiar compartment called the *Coxiella*-containing vacuole (CCV) within macrophages or monocytes (1). Unlike other intracellular bacteria, *C. burnetii* does not try to escape phagolysosomes but instead uses the acidic compartment to activate an important virulence factor,

Received 4 May 2018 **Returned for modification** 30 May 2018 **Accepted** 17 July 2018

Accepted manuscript posted online 23 July 2018

Citation Jansen AFM, Dinkla A, Roest H-J, Bleeker-Rovers CP, Schoffelen T, Joosten LAB, Wever PC, van Deuren M, Koets AP. 2018. Viable *Coxiella burnetii* induces differential cytokine responses in chronic Q fever patients compared to heat-killed *Coxiella burnetii*. Infect Immun 86:e00333-18. https://doi.org/10.1128/IAI.00333-18.

Editor Craig R. Roy, Yale University School of Medicine

Address correspondence to Anne F. M. Jansen, Anne.FM.Jansen@radboudumc.nl, or Marcel van Deuren, Marcel.vanDeuren@radboudumc.nl.

the type IV secretion system (T4SS), through which it modulates host cell metabolism and gene expression to enhance its CCV survival (2).

In humans, acute Q fever is most often associated with a relatively mild flu-like disease or, in some cases, pneumonia or hepatitis. Initial infection occurs asymptomatically in 60% of infections. In a minority of infected individuals, however, initial symptomatic or asymptomatic infection persists, often manifesting as an endocarditis or infection of an aneurysm or vascular prosthesis (1). During such persistent infection, also referred to as chronic Q fever or persistent focalized Q fever infection (3), *C. burnetii* is not eradicated by the host immune response. Individuals with heart valve defects, aneurysms, or vascular prostheses are susceptible to develop a persistent infection (4). During the major Q fever epidemic in the Netherlands, approximately 30% of individuals with known aorto-iliac disease and serological evidence of Q fever infection developed chronic Q fever (5). Therefore, an additional risk factor resulting in a defective systemic or local immune response is likely to be required for the development of chronic Q fever.

To evaluate the systemic immune response in chronic Q fever patients, various studies examined the cytokine and chemokine response to heat-killed *C. burnetii*. Nearly all of these studies showed a robust innate cytokine response in chronic Q fever patients, with a strikingly more profound production of interferon gamma (IFN-γ) than in Q fever seronegative controls who lacked IFN-γ response (6–9). Various studies also showed a higher production of anti-inflammatory interleukin-10 (IL-10) in chronic Q fever patients (10–13).

Due to its highly infectious nature, *C. burnetii* is classified as a biosafety level 3 (BSL3) agent (14). Therefore, nearly all stimulation studies were performed with heat-killed *C. burnetii* or with the avirulent phase II organisms to prevent researchers from exposure and to circumvent complicated and expensive biosafety procedures. Whether results obtained in experiments with heat-killed bacteria can be translated to the immune response to viable *C. burnetii*, armed with tools to influence host cell metabolism, has not been evaluated.

Therefore, in the present study, we compare the cytokine-inducing capacities of strains of heat-killed and viable *C. burnetii* in peripheral blood mononuclear cell (PBMC) cultures of chronic Q fever patients and healthy controls.

RESULTS

Viable *C. burnetii* induces few cytokines in PBMCs of Q fever naive controls in the presence of Q fever naive serum. In a small study ($n = 4$) aimed to justify the final experimental setup, the effect of heat-killed and viable bacteria on cytokine production of PBMCs of Q fever naive controls in the presence of Q fever naive serum was examined. To this end, cells were stimulated with two *C. burnetii* strains, either viable or heat-killed Nine Mile or 3262 strain. It appeared that the production of tumor necrosis factor alpha (TNF-α), IL-1β, and IL-6, mostly secreted by monocytes, was lower after stimulation with viable *C. burnetii* than with heat-killed *C. burnetii* (see Fig. S1 in the supplemental material). Generally, the Nine Mile and 3262 strains induced similar effects, although not for every cytokine. Levels of IL-10 production induced by the 3262 strain were similar for viable and heat-killed bacteria, but the live Nine Mile strain showed a higher cytokine response than the heat-killed bacteria. Due to the small sample size, results are not statistically significant.

The levels of cytokines IFN-γ, IL-17, and IL-22, mostly secreted by T cells and, therefore, belonging to the adaptive immune response, were determined in the supernatants after 48 h and 7 days. Stimulation with viable and heat-killed bacteria from either of the strains led to similar cytokine production (Fig. S2). Cytokine concentration in the unstimulated cells remained below the detection limit. However, IL-17 production seemed to be higher after stimulation with viable than heat-killed bacteria. Due to the small sample size, results are not statistically significant.

The effect of anti-*C. burnetii* IgG phase I antibodies in the *C. burnetii* stimulation experiments was determined with PBMCs from four chronic Q fever patients and four healthy controls. The controls and patients responded similarly to the serum of a chronic Q fever patient with high anti-*C. burnetii* antibody titers (1:4,096) (Fig. 1).

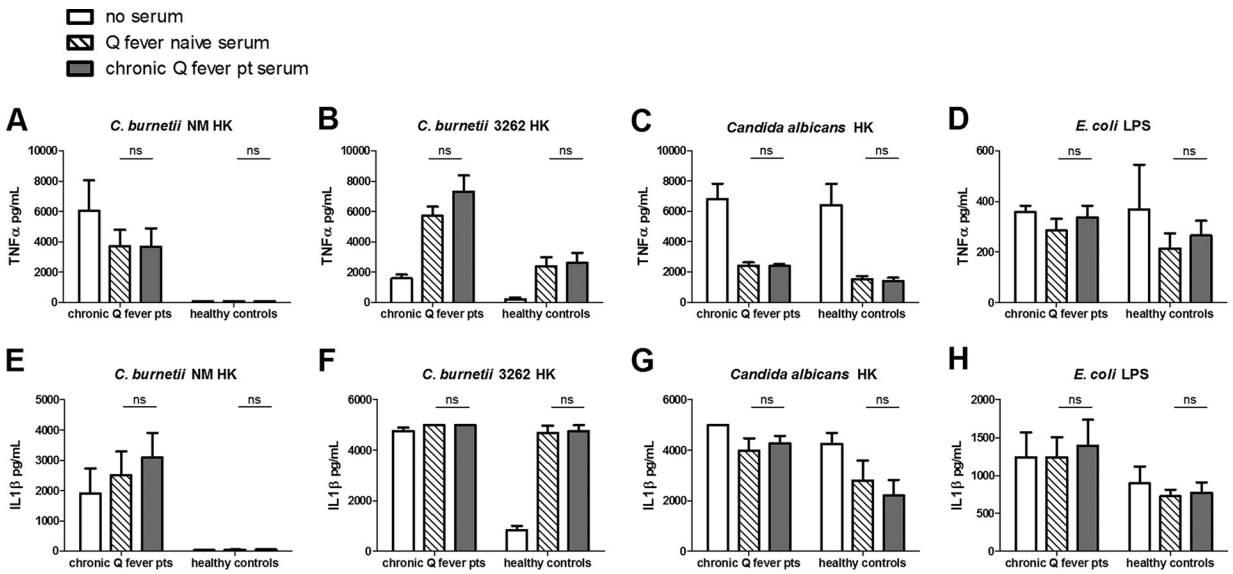

FIG 1 (A to H) TNF-α and IL-1β production of peripheral blood mononuclear cells after 24 h of stimulation with heat-killed *C. burnetii* NM (10^6 bacteria/ml) (A and E), *C. burnetii* 3262 (10^6 bacteria/ml) (B and F), *Candida albicans* conidiae (10^5/ml) (C and G), and *Escherichia coli* LPS (10 ng/ml) (D and H). Cells (5×10^5) of chronic Q fever patients or healthy controls were incubated without serum, with Q fever naive serum, or with serum from a chronic Q fever patient with high IgG phase I titers. Abbreviations: HK, heat-killed; NM, Nine Mile strain; ns, nonsignificant; pts, patients. Q fever naive and Q fever serum conditions were compared with the Mann-Whitney test.

Viable *C. burnetii* induces less innate cytokine production than heat-killed *C. burnetii* in PBMCs of chronic Q fever patients and Q fever naive controls in the presence of serum containing Q fever antibodies. PBMCs of 10 chronic Q fever patients and 10 healthy Q fever naive controls were stimulated with viable or heat-

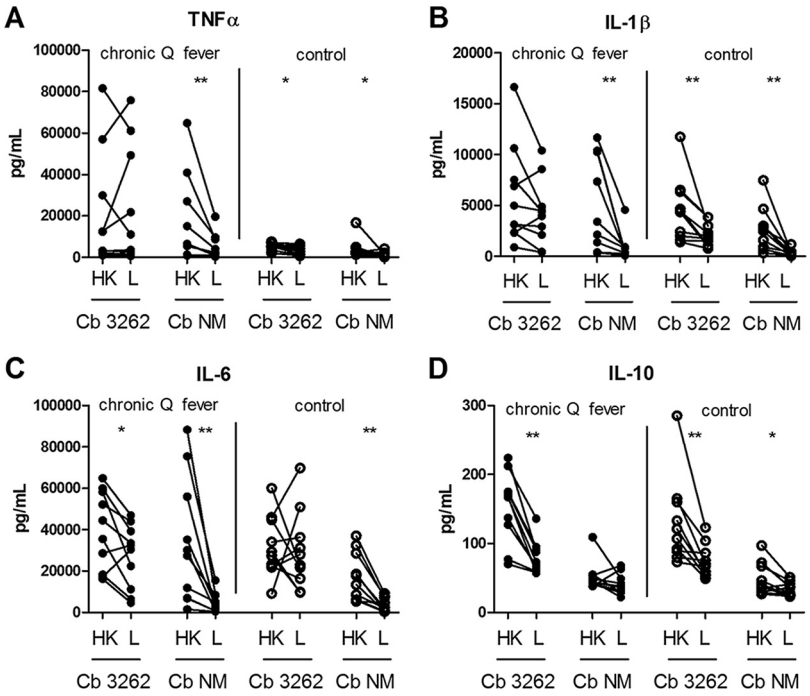

FIG 2 (A to D) Innate cytokine production after heat-killed (HK) and viable (L) *Coxiella burnetii* stimulation in peripheral blood mononuclear cells of chronic Q fever patients ($n = 10$) and healthy Q fever naive controls ($n = 10$). The Dutch outbreak strain 3262 (Cb 3262) and the Nine Mile strain (Cb NM) were used to stimulate the cells with 2×10^6 bacteria per condition. Cells (5×10^5) were incubated in the presence of serum containing anti-*C. burnetii* antibodies for 24 h, after which IL-6, IL-1β, and TNF-α were measured. IL-10 production was measured after 48 h. *, $P < 0.05$; **, $P < 0.01$. Nonsignificant results are not indicated by a symbol, and results were analyzed with the Wilcoxon signed-rank test.

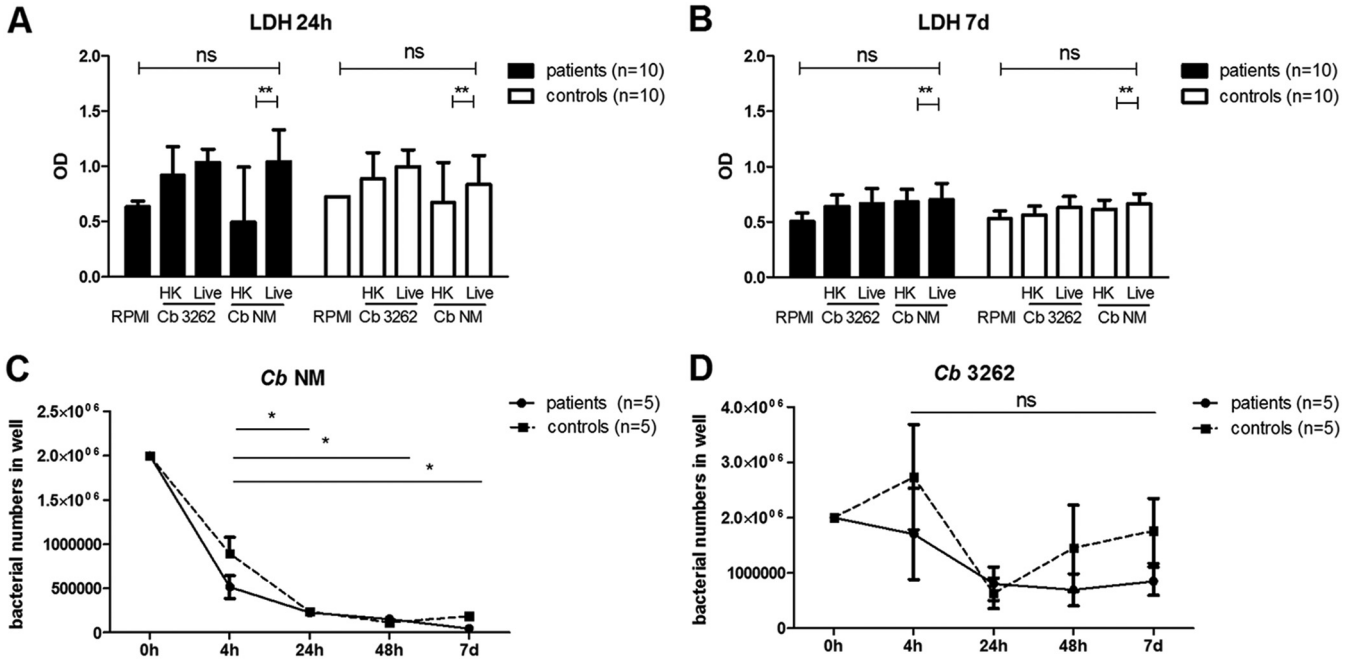

FIG 3 (A and B) Lactate dehydrogenase (LDH) assay to assess necrotic cell death after 24 h (A) or 7 days (B) of stimulation with heat-killed (HK) and live *C. burnetii* Nine Mile (NM) strain or 3262 strain. There was no difference in cell death compared to that for the medium control. (C and D) *C. burnetii* genomic copies between start of experiment and 7 days. Cb NM copy numbers significantly decreased during cell culture, while *C. burnetii* 3262 did not significantly decrease. *, $P < 0.05$; **, $P < 0.01$; ns, nonsignificant. Results were analyzed with the Wilcoxon signed-rank test (A and B) and one-way ANOVA with Bonferroni's multiple-comparison test (C and D).

killed *C. burnetii*. Cells of chronic Q fever patients were used in the experiments, because it is unknown whether these patients, who are assumed to have a defective immune response to *C. burnetii*, respond to viable bacteria in a manner similar to that of healthy individuals. To mimic the *in vivo* situation, the cells were incubated with serum from a patient with chronic Q fever containing high titers of anti-*C. burnetii* IgG phase I. Two strains of *C. burnetii* were used, the laboratory strain Nine Mile and the predominant strain during the Q fever outbreak in the Netherlands (*C. burnetii* 3262) that infected approximately 40,000 individuals.

The innate cytokines TNF-α, IL-1β, and IL-6 were measured after 24 h of stimulation (Fig. 2). Cytokine production in unstimulated cells remained below the detection limit of the tests.

In chronic Q fever patients, TNF-α production of viable *C. burnetii* 3262-stimulated cells was not different from the TNF-α production induced by heat-killed *C. burnetii*. However, viable *C. burnetii* Nine Mile induced less TNF-α production than heat-killed *C. burnetii* Nine Mile ($P < 0.01$). In controls, both *C. burnetii* strains showed the same effect, viable *C. burnetii* induced less TNF-α production than heat-killed ($P < 0.01$). Chronic Q fever patients showed high variability in cytokine production, and the cytokine responses were not significantly different from those of healthy controls.

The IL-1β production induced by viable *C. burnetii* 3262 in chronic Q fever patients was similar to that of heat-killed *C. burnetii* 3262, in contrast to the lower cytokine production induced by viable *C. burnetii* NM than heat-killed *C. burnetii* NM ($P < 0.01$). In controls, for both strains the viable *C. burnetii* condition led to less cytokine production ($P < 0.01$). As for TNF-α, IL-1β production by PBMCs of patients was highly variable, and there was no difference with IL-1β production by PBMCs of healthy controls.

Chronic Q fever patients and controls readily produced the proinflammatory cytokine IL-6. In chronic Q fever patients, for both *C. burnetii* strains, viable *C. burnetii* induced less IL-6 than heat-killed *C. burnetii* (*C. burnetii* 3262, $P < 0.05$; *C. burnetii* Nine Mile, $P < 0.01$). For the *C. burnetii* 3262 strain, healthy controls showed similar levels of

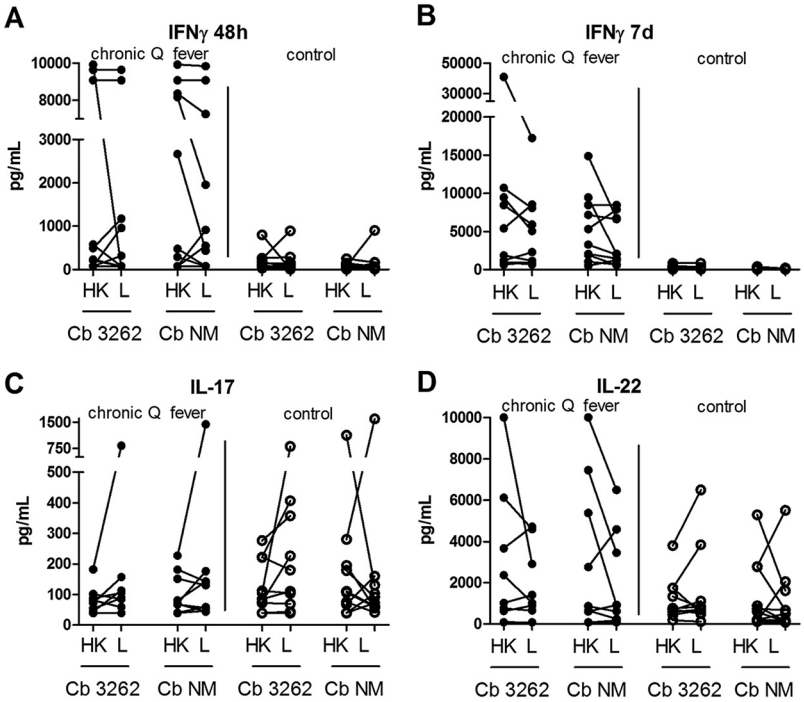

FIG 4 (A to D) Adaptive cytokine production after heat-killed (HK) and viable (L) *Coxiella burnetii* stimulation in peripheral blood mononuclear cells of chronic Q fever patients ($n = 10$) and healthy Q fever naive controls ($n = 10$). The Dutch outbreak strain 3262 (Cb 3262) and the Nine Mile strain (Cb NM) were used to stimulate the cells with 2×10^6 bacteria per condition. Cells (5×10^5) were incubated in the presence of serum containing anti-*C. burnetii* antibodies for 48 h and 7 days, after which IFN-γ production was measured. IL-17 and IL-22 production was measured after 7 days. Nonsignificant results are not indicated by a symbol, and results were analyzed with the Wilcoxon signed-rank test.

IL-6 production under the viable and heat-killed conditions. With regard to the Nine Mile strain, viable *C. burnetii* induced less IL-6 than heat-killed *C. burnetii* ($P < 0.01$). IL-6 production was not different between chronic Q fever patients or controls in any of the stimulations.

The anti-inflammatory cytokine IL-10 was measured after 48 h. In chronic Q fever patients, viable *C. burnetii* 3262 induced less IL-10 than heat-killed *C. burnetii* ($P < 0.01$), although this was not the case for the Nine Mile strain. PBMCs of controls produced less IL-10 after viable *C. burnetii* 3262 and Nine Mile strain stimulation than after heat-killed *C. burnetii* stimulation ($P < 0.01$ and $P < 0.05$, respectively).

Bacterial replication, assessed with quantitative PCR (qPCR), did not take place within the first 48 h of incubation (Fig. 3). Cell lysis, measured by lactate dehydrogenase (LDH) release, did not differ between unstimulated and stimulated conditions after 24 h (Fig. 3).

Lower innate cytokine production induced by viable bacteria does not lead to reduced adaptive cytokine production. After 7 days, IFN-γ, IL-17, and IL-22 production was determined in the supernatants, and IFN-γ production was also measured after 48 h (Fig. 4). IL-10 production was also measured after 7 days but was undetectable under all conditions.

IFN-γ is crucial in the defense against intracellular pathogens and known to be highly produced upon stimulation with heat-killed *C. burnetii* by chronic Q fever patients, in contrast to healthy controls (6, 7). In contrast to the mostly innate cytokines, IFN-γ production was not influenced by *C. burnetii* viability in chronic Q fever patients. Controls also showed no differences in cytokine production between viable and heat-killed *C. burnetii* conditions. At both time points and regardless of the viability of the bacteria, IFN-γ production was higher in chronic Q fever patients after stimulation with *C. burnetii* 3262 (viable; median, 638 pg/ml; range, 78 to 46,733) than in controls

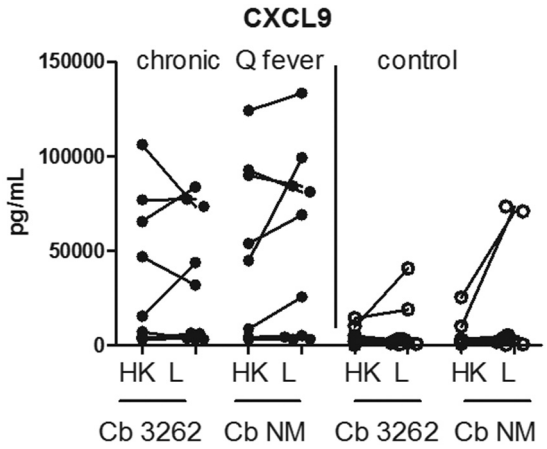

FIG 5 CXCL9 production after heat-killed (HK) and viable (L) *Coxiella burnetii* stimulation in peripheral blood mononuclear cells of chronic Q fever patients ($n = 10$) and healthy Q fever naive controls ($n = 10$). The Dutch outbreak strain 3262 (Cb 3262) and the Nine Mile strain (Cb NM) were used to stimulate the cells with 2×10^6 bacteria per condition. Nonsignificant results are not indicated by a symbol, and results were analyzed with the Wilcoxon signed-rank test.

(median, 77 pg/ml; range, 8 to 890 pg/ml; $P < 0.01$) or with *C. burnetii* Nine Mile (viable patients, median, 728 pg/ml; range, 78 to 41,295 pg/ml; controls, median, 59 pg/ml; range, 8 to 900 pg/ml). In chronic Q fever patients and controls, IL-17 and IL-22 production also did not differ between heat-killed and viable *C. burnetii*, which was apparent in both strains. There was no difference between chronic Q fever patients and controls regarding IL-17 and IL-22 production.

After 7 days, bacterial numbers did not exceed the number of bacteria used to initially stimulate the cells (Fig. 3). There was no difference in cell death, assessed by LDH release and trypan blue staining, between the unstimulated and stimulated conditions after 7 days of culture (Fig. 3).

Chemokine CXCL9 is not differentially produced after incubation with viable or heat-killed *Coxiella*. CXCL9, also known as monokine induced by interferon gamma (MIG), is a chemokine that is primarily induced by IFN-γ. It predominantly attracts T cells and thereby plays a role in orchestrating the T-helper 1 cell response (15). We previously reported that CXCL9 is scarcely produced after heat-killed *C. burnetii* stimulation of PBMCs of healthy controls, but evidently it is produced by cells taken from chronic Q fever patients (16). Apart from altered cytokine production, dysfunctional chemokine production may prevent adequate influx of T effector cells, affecting the adaptive immune response. Therefore, we determined the CXCL9 concentration after 24 h of incubation (Fig. 5). There was no significant difference in CXCL9 concentration after live or heat-killed *C. burnetii* incubation.

DISCUSSION

In the present study, we showed that viable *C. burnetii* induces fewer of the innate cytokines TNF-α, IL-1β, IL-6, and IL-10 than heat-killed bacteria but equal amounts of T-cell-derived (adaptive) cytokines IFN-γ, IL-17, and IL-22 and the IFN-γ-dependent chemokine CXCL9. Hence, data obtained in studies evaluating innate cytokine production induced by heat-killed *C. burnetii* cannot be extrapolated to the real-life situation where infection is induced by viable *C. burnetii*. Regarding the interpretation of previous research with heat-killed *C. burnetii*, the *in vivo* immune activation due to viable bacteria has likely been overestimated. On the other hand, measurements of the adaptive cytokine production, such as that with an IFN-γ assay (17), reflect the response to viable bacteria appropriately. Consistent with previous studies performed by our research group (6, 7, 16), heat-killed *C. burnetii* induced higher IFN-γ and CXCL9 production in chronic Q fever patients than in controls, which could now also be confirmed for viable *C. burnetii*.

Multiple theories may explain the attenuated cytokine production after incubation with viable bacteria compared to that of heat-killed bacteria. It is conceivable that heat killing increases the release and exposure of bacterial pathogen-associated molecular patterns (PAMPs) and thereby affects signaling by PAMP receptors. The uptake, internalization, and phagosomal pathway of viable and dead bacteria are less likely to influence cytokine production, because viable and dead bacteria are both taken up passively via actin-dependent phagocytosis, and the maturation of the CCV occurs irrespective of *C. burnetii* replication or protein synthesis (18, 19). However, once they arrive in the CCV, viable bacteria are likely to influence the host cell due to the type IV secretion system (T4SS), which can deliver bacterial effector proteins into the cytosol of the host. For example, *C. burnetii* is able to inhibit apoptosis, promote fusion of the CCV with autophagosomes, and modulate host gene expression, including genes of the immune response. Reportedly the genes *IL-8*, *CCL2*, *CXCL1*, and *SPP1*, which control cell trafficking and IL-12 production, are actively downregulated by *C. burnetii* (20). Thus, several probable mechanisms may explain the lower proinflammatory innate cytokine induction after stimulation with viable rather than nonviable *C. burnetii*.

In addition to the observed differences in cytokine induction by viable and heat-killed *C. burnetii*, we noticed a trend toward higher anti-inflammatory IL-10 production after stimulation with viable bacteria in the presence of Q fever naive serum. If this observation can be validated, it supports the assumption that the induction of IL-10 early in the course of the disease is one of the immune evasion strategies of (viable) *C. burnetii*. Similar to our observation, *Mycobacterium tuberculosis* has been reported to enhance IL-10 production and downregulate proinflammatory cytokines (21). Differences between strains in the induction of IL-10 may also be one of the virulence factors of *C. burnetii*. In a mouse model of Q fever, the Nine Mile strain appeared to be more virulent than the 3262 strain (22). Notably, in the presence of *C. burnetii* IgG phase I antibodies, this shift toward higher IL-10 production was absent. In Q fever patients, phase I antibodies appear during the transition to chronic infection. The role of anti-*C. burnetii* IgG antibodies has remained controversial. It was shown that opsonization of *C. burnetii* favors replication in human-derived monocytes, while unopsonized bacteria survive poorly (23). However, Fcγ receptor (FcγR)-mediated phagocytosis by dendritic cells induces cytokine production, whereas non-antibody-containing serum with *C. burnetii* does not induce changes in dendritic cells (24). In mice, FcγR- or complement-mediated phagocytosis does not play a role *in vivo* (24). If the observed trend of higher IL-10 production after stimulation with viable bacteria can be validated, our results suggest that in the chronic phase of the disease, *C. burnetii* IgG phase I antibodies prevent a possible deleterious overproduction of IL-10 and thus may be considered in part protective.

A note of caution is due here, because all patients used antimicrobial drugs, which are known to influence cytokine production through inhibited transcription (25) and may also affect bacterial elimination. It should also be noted that patients were recruited multiple months after they started antibiotic treatment. Furthermore, the viable bacterial inoculum inevitably contained a small percentage of dead bacteria, which could have affected the cytokine production. The percentage of dead bacteria under the viable stimulatory condition is unknown; however, *C. burnetii* is very resilient and can resist many chemical compounds, and so large percentages of dead *C. burnetii* bacteria were unlikely.

Conclusions. Heat-killed *C. burnetii* is frequently used as an alternative for viable *C. burnetii* due to the requirement of dedicated facilities. However, innate proinflammatory cytokine production induced by viable bacteria are lower than that by heat-killed *C. burnetii*. Levels of adaptive cytokine production are comparable between induction by viable and heat-killed *C. burnetii* in humans. The results of this study can be used to interpret previous and future research with heat-killed *C. burnetii* regarding *C. burnetii*-specific cytokine production in healthy individuals and chronic Q fever patients.

MATERIALS AND METHODS

Subjects. Healthy controls ($n = 10$) were hospital personnel, all seronegative for Q fever, as tested by immunofluorescence assay (IFA). Chronic Q fever patients ($n = 10$) were recruited from the outpatient

clinic of the Radboud University Medical Center Nijmegen. Patients were diagnosed according to the Dutch Consensus Guidelines on chronic Q fever diagnosis (26); this guideline categorizes diagnosis as proven, probable, or possible chronic infection and is based on serology, PCR, clinical symptoms, risk factors, and diagnostic imaging. All patients fulfilled the criteria of proven chronic Q fever and were being treated with antibiotics, mostly doxycycline, and hydroxychloroquine at the time of blood drawing. Patient characteristics are shown in Table S1 in the supplemental material. Serum with anti-*C. burnetii* IgG phase I antibodies was drawn from a chronic Q fever patient with high IgG phase I titers (1:4,096) determined by IFA. Q fever naive serum was drawn from a healthy control who participated in the study.

Bacteria. *C. burnetii* strains were cultured on Buffalo green monkey cells at Wageningen Bioveterinary Research (former Central Veterinary Institute), the Netherlands, as described previously (27). Two strains were used, the frequently used Nine Mile (RSA 693) strain and the most prominent Dutch outbreak strain, 3262, isolated in 2009 (28). The Nine Mile strain is a laboratory strain isolated from a tick and is generally used for research purposes (29). The *C. burnetii* 3262 strain had been isolated from a goat during the Dutch epidemic and is pathogenic to humans.

Bacterial DNA, measured to quantify the amount of bacteria, was isolated using a DNA isolation kit (DNeasy blood and tissue kit; Qiagen, the Netherlands). Quantification by qPCR was done as described by Roest et al. (28). In brief, a single-copy gene encoding a *C. burnetii* specific hypothetical protein (GenBank accession number AY502846) was targeted using the forward primer 5′-ATAGCGCCAATCGA AATGGT-3′, the reverse primer 5′-CTTGAATACCCATCCCGAAGTC-3′, and the NED-labeled probe 5′-CCC AGTAGGGCAGAAGACGTTCCCC-3′. To quantify the bacterial numbers, the commercially available *C. burnetii* standard positive-control dilution series was used (Adiavet Cox, Adiagene, Marcy l' Etoile, France). qPCR was performed on a 7500 Fast real-time PCR system (Applied Biosystems, USA). After determination of the amount of both *Coxiella* batches, they were brought to a concentration of 4×10^7 bacteria/ml. The first half was heat killed at 99°C for 30 min (30) while the second half remained viable. Both were aliquoted and frozen at −80°C until the experiment, which was performed within 3 months. Determination of *C. burnetii* gene copies at specific time points (4 h, 24 h, 48 h, and 7 days) after stimulation experiments in frozen cell pellets was executed by following the same DNA isolation and qPCR protocol as that described above.

PBMC isolation and stimulation. PBMCs from all individuals were isolated according to a standardized protocol, described in detail by Oosting et al. (31). In short, cells were separated by density centrifugation with Ficoll Paque (GE Healthycare, Zeist, the Netherlands), taken from the interphase layer, and washed twice in cold phosphate-buffered saline. The cells were counted with a Z1 Coulter particle counter (Beckman Coulter, Woerden, the Netherlands) and brought to a concentration of 5×10^6 cells/ml in RPMI 1640 (Life Technologies/Invitrogen, Breda, the Netherlands) supplemented with 2 mM GlutaMAX and 1 mM pyruvate without antibiotics. Cells were subsequently transported on ice to the BSL3 facility. In a round-bottom 96-well plate (Corning, New York, USA), 5×10^5 cells were incubated with 10% human serum and either Q fever naive serum or serum containing IgG phase I and II antibodies (original IFA titers of 1:4,096). Subsequently, the cells were stimulated with either 2×10^6 heat-killed or viable *C. burnetii* Nine Mile bacteria and with 2×10^6 heat-killed or viable *C. burnetii* 3262 bacteria. The infection index, i.e., the ratio of bacteria to PBMCs, was 4:1 and the estimated number of bacteria per monocyte was 20:1, identical to that used in earlier studies on the measurements of cytokine production in chronic Q fever patients and healthy controls (6, 32). Positive and negative controls were heat-killed *Candida albicans* conidia (10^5/ml) and culture medium, respectively. After 24 h, 48 h, and 7 days, the supernatants were harvested after centrifugation and stored at −80°C. Cell death at 24 h and 7 days was assessed by measuring lactate dehydrogenase (LDH) in the supernatants using Cytotox96 nonradioactive cytotoxicity assay (Promega Corporation, Madison, WI, USA) and additionally at 7 days by staining with trypan blue and subsequent counting by microscopy. The remaining cell pellets were frozen at −80°C to assess *C. burnetii* replication during the experiments.

Cytokine measurements. Cytokine concentration was measured at different time points in the PBMC culture supernatants using enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions. After 24 h, the concentration of TNF-α, IL-6, IL-1β, and CXCL9 (R&D Systems, Minneapolis, MN, USA) was measured. After 48 h, the concentrations of IL-10 and IFN-γ (Sanquin, Amsterdam, the Netherlands) were determined. After 7 days, the concentrations of IL-10, IFN-γ, IL-17, and IL-22 (R&D Systems) were measured.

Statistical analysis. All data were analyzed using GraphPad Prism 5.0. Medians and interquartile ranges were calculated for cytokine production. Differences between cytokine production induced by heat-killed and viable bacteria were analyzed with a Wilcoxon signed-rank test. Differences in number of bacteria were analyzed by means of one-way analysis of variance (ANOVA) with Bonferroni *post hoc* tests. A P value below 0.05 was considered statistically significant.

Ethical consideration. This study was approved by the local institutional review board (NL35784.091.11; CMO Arnhem-Nijmegen). All subjects provided written informed consent.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00333-18.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.
SUPPLEMENTAL FILE 2, PDF file, 0.2 MB.
SUPPLEMENTAL FILE 3, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

We thank Michiel Kroese, Betty van Gelderen, and Marianne Vahl for their assistance with the experiments and Dimitrios Frangoulidis, Bundeswehr Institute of Microbiology, for providing the *C. burnetii* Nine Mile strain.

A.J., T.S., and A.D. performed the experiments; P.C.W. performed additional experiments; M.V.D., A.K., H.J.K., and L.A.B.J. designed the study; C.B. collected patient material; A.F.M.J. drafted the manuscript; all authors reviewed and approved the final manuscript.

This study was funded by the Q-Support Foundation. The funders had no role in study design, experiments, or drafting of the manuscript.

We have no conflicts of interest to declare.

REFERENCES

1. Raoult D, Marrie T, Mege J. 2005. Natural history and pathophysiology of Q fever. Lancet Infect Dis 5:219–226. https://doi.org/10.1016/S1473-3099(05)70052-9.
2. Winchell CG, Graham JG, Kurten RC, Voth DE. 2014. Coxiella burnetii type IV secretion-dependent recruitment of macrophage autophagosomes. Infect Immun 82:2229–2238. https://doi.org/10.1128/IAI.01236-13.
3. Million M, Raoult D. 2017. No such thing as chronic Q fever. Emerg Infect Dis 23:856–857. https://doi.org/10.3201/eid2305.161538.
4. Fenollar F, Fournier PE, Carrieri MP, Habib G, Messana T, Raoult D. 2001. Risks factors and prevention of Q fever endocarditis. Clin Infect Dis 33:312–316. https://doi.org/10.1086/321889.
5. Hagenaars JC, Wever PC, van Petersen AS, Lestrade PJ, de Jager-Leclercq MG, Hermans MH, Moll FL, Koning OH, Renders NH. 2014. Estimated prevalence of chronic Q fever among Coxiella burnetii seropositive patients with an abdominal aortic/iliac aneurysm or aorto-iliac reconstruction after a large Dutch Q fever outbreak. J Infect 69:154–160. https://doi.org/10.1016/j.jinf.2014.03.009.
6. Schoffelen T, Sprong T, Bleeker-Rovers CP, Wegdam-Blans MC, Ammerdorffer A, Pronk MJ, Soethoudt YE, van Kasteren ME, Herremans T, Bijlmer HA, Netea MG, van der Meer JW, Joosten LA, van Deuren M. 2014. A combination of interferon-gamma and interleukin-2 production by Coxiella burnetii-stimulated circulating cells discriminates between chronic Q fever and past Q fever. Clin Microbiol Infect 20:642–650. https://doi.org/10.1111/1469-0691.12423.
7. Schoffelen T, Textoris J, Bleeker-Rovers CP, Ben Amara A, van der Meer JW, Netea MG, Mege JL, van Deuren M, van de Vosse E. 2017. Intact interferon-gamma response against Coxiella burnetii by peripheral blood mononuclear cells in chronic Q fever. Clin Microbiol Infect 23:209–215. https://doi.org/10.1016/j.cmi.2016.11.008.
8. Schoffelen T, Wegdam-Blans MC, Ammerdorffer A, Pronk MJ, Soethoudt YE, Netea MG, van der Meer JW, Bleeker-Rovers CP, van Deuren M. 2015. Specific in vitro interferon-gamma and IL-2 production as biomarkers during treatment of chronic Q fever. Front Microbiol 6:93. https://doi.org/10.3389/fmicb.2015.00093.
9. Ka MB, Mezouar S, Ben Amara A, Raoult D, Ghigo E, Olive D, Mege JL. 2016. Coxiella burnetii induces inflammatory interferon-like signature in plasmacytoid dendritic cells: a new feature of immune response in Q fever. Front Cell Infect Microbiol 6:70. https://doi.org/10.3389/fcimb.2016.00070.
10. Capo C, Zaffran Y, Zugun F, Houpikian P, Raoult D, Mege JL. 1996. Production of interleukin-10 and transforming growth factor beta by peripheral blood mononuclear cells in Q fever endocarditis. Infect Immun 64:4143–4147.
11. Ghigo E, Honstettre A, Capo C, Gorvel JP, Raoult D, Mege JL. 2004. Link between impaired maturation of phagosomes and defective Coxiella burnetii killing in patients with chronic Q fever. J Infect Dis 190:1767–1772. https://doi.org/10.1086/425041.
12. Honstettre A, Imbert G, Ghigo E, Gouriet F, Capo C, Raoult D, Mege JL. 2003. Dysregulation of cytokines in acute Q fever: role of interleukin-10 and tumor necrosis factor in chronic evolution of Q fever. J Infect Dis 187:956–962. https://doi.org/10.1086/368129.
13. Ka MB, Gondois-Rey F, Capo C, Textoris J, Million M, Raoult D, Olive D, Mege JL. 2014. Imbalance of circulating monocyte subsets and PD-1 dysregulation in Q fever endocarditis: the role of IL-10 in PD-1 modulation. PLoS One 9:e107533. https://doi.org/10.1371/journal.pone.0107533.
14. Madariaga MG, Rezai K, Trenholme GM, Weinstein RA. 2003. Q fever: a biological weapon in your backyard. Lancet Infect Dis 3:709–721. https://doi.org/10.1016/S1473-3099(03)00804-1.
15. Van Raemdonck K, Van den Steen PE, Liekens S, Van Damme J, Struyf S. 2015. CXCR3 ligands in disease and therapy. Cytokine Growth Factor Rev 26:311–327. https://doi.org/10.1016/j.cytogfr.2014.11.009.
16. Jansen AFM, Schoffelen T, Textoris J, Mege JL, Nabuurs-Franssen M, Raijmakers RPH, Netea MG, Joosten LAB, Bleeker-Rovers CP, van Deuren M. 2017. CXCL9, a promising biomarker in the diagnosis of chronic Q fever. BMC Infect Dis 17:556. https://doi.org/10.1186/s12879-017-2656-6.
17. Schoffelen T, Joosten LA, Herremans T, de Haan AF, Ammerdorffer A, Rumke HC, Wijkmans CJ, Roest HI, Netea MG, van der Meer JW, Sprong T, van Deuren M. 2013. Specific interferon gamma detection for the diagnosis of previous Q fever. Clin Infect Dis 56:1742–1751. https://doi.org/10.1093/cid/cit129.
18. Tujulin E, Macellaro A, Lilliehook B, Norlander L. 1998. Effect of endocytosis inhibitors on Coxiella burnetii interaction with host cells. Acta Virol 42:125–131.
19. Howe D, Melnicakova J, Barak I, Heinzen RA. 2003. Maturation of the Coxiella burnetii parasitophorous vacuole requires bacterial protein synthesis but not replication. Cell Microbiol 5:469–480. https://doi.org/10.1046/j.1462-5822.2003.00293.x.
20. Mahapatra S, Ayoubi P, Shaw EI. 2010. Coxiella burnetii Nine Mile II proteins modulate gene expression of monocytic host cells during infection. BMC Microbiol 10:244. https://doi.org/10.1186/1471-2180-10-244.
21. Tiwari BM, Kannan N, Vemu L, Raghunand TR. 2012. The Mycobacterium tuberculosis PE proteins Rv0285 and Rv1386 modulate innate immunity and mediate bacillary survival in macrophages. PLoS One 7:e51686. https://doi.org/10.1371/journal.pone.0051686.
22. Kuley R, Smith HE, Frangoulidis D, Smits MA, Jan Roest HI, Bossers A. 2015. Cell-free propagation of Coxiella burnetii does not affect its relative virulence. PLoS One 10:e0121661. https://doi.org/10.1371/journal.pone.0121661.
23. Desnues B, Imbert G, Raoult D, Mege JL, Ghigo E. 2009. Role of specific antibodies in Coxiella burnetii infection of macrophages. Clin Microbiol Infect 15(Suppl 2):S161–S162. https://doi.org/10.1111/j.1469-0691.2008.02208.x.
24. Shannon JG, Cockrell DC, Takahashi K, Stahl GL, Heinzen RA. 2009. Antibody-mediated immunity to the obligate intracellular bacterial pathogen Coxiella burnetii is Fc receptor- and complement-independent. BMC Immunol 10:26. https://doi.org/10.1186/1471-2172-10-26.
25. Bostanci N, Akgul B, Tsakanika V, Allaker RP, Hughes FJ, McKay IJ. 2011. Effects of low-dose doxycycline on cytokine secretion in human monocytes stimulated with Aggregatibacter actinomycetemcomitans. Cytokine 56:656–661. https://doi.org/10.1016/j.cyto.2011.08.039.
26. Wegdam-Blans MC, Kampschreur LM, Delsing CE, Bleeker-Rovers CP, Sprong T, van Kasteren ME, Notermans DW, Renders NH, Bijlmer HA, Lestrade PJ, Koopmans MP, Nabuurs-Franssen MH, Oosterheert JJ, Dutch Q Fever Consensus Group. 2012. Chronic Q fever: review of the literature and a proposal of new diagnostic criteria. J Infect 64:247–259. https://doi.org/10.1016/j.jinf.2011.12.014.
27. Roest HI, Post J, van Gelderen B, van Zijderveld FG, Rebel JM. 2013. Q fever in pregnant goats: humoral and cellular immune responses. Vet Res 44:67. https://doi.org/10.1186/1297-9716-44-67.
28. Roest H-J, van Gelderen B, Dinkla A, Frangoulidis D, van Zijderveld F, Rebel J, van Keulen L. 2012. Q fever in pregnant goats: pathogenesis and

excretion of Coxiella burnetii. PLoS One 7:e48949. https://doi.org/10.1371/journal.pone.0048949.
29. Seshadri R, Paulsen IT, Eisen JA, Read TD, Nelson KE, Nelson WC, Ward NL, Tettelin H, Davidsen TM, Beanan MJ, Deboy RT, Daugherty SC, Brinkac LM, Madupu R, Dodson RJ, Khouri HM, Lee KH, Carty HA, Scanlan D, Heinzen RA, Thompson HA, Samuel JE, Fraser CM, Heidelberg JF. 2003. Complete genome sequence of the Q-fever pathogen Coxiella burnetii. Proc Natl Acad Sci U S A 100:5455–5460. https://doi.org/10.1073/pnas.0931379100.
30. Samuel JE, Hendrix LR. 2009. Laboratory maintenance of Coxiella burnetii. Curr Protoc Microbiol Chapter 6:Unit 6C.1.
31. Oosting M, Berende A, Sturm P, Ter Hofstede HJ, de Jong DJ, Kanneganti TD, van der Meer JW, Kullberg BJ, Netea MG, Joosten LA. 2010. Recognition of Borrelia burgdorferi by NOD2 is central for the induction of an inflammatory reaction. J Infect Dis 201:1849–1858. https://doi.org/10.1086/652871.
32. Ammerdorffer A, Schoffelen T, Gresnigt MS, Oosting M, den Brok MH, Abdollahi-Roodsaz S, Kanneganti TD, de Jong DJ, van Deuren M, Roest HJ, Rebel JM, Netea MG, Joosten LA, Sprong T. 2014. Recognition of Coxiella burnetii by Toll-like receptors and nucleotide-binding oligomerization domain-like receptors. J Infect Dis 211:978–987. https://doi.org/10.1093/infdis/jiu526.