INNATE IMMUNE RESPONSES AGAINST *COXIELLA BURNETII* HUMAN VERSUS GOAT

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Innate immune responses against *Coxiella burnetii* human versus goat

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01

General introduction and outline of the thesis

The Gram-negative bacterium *Coxiella burnetii* is the causative agent of Q fever in both humans and animals. The world largest Q fever outbreak occurred in the Netherlands between 2007 and 2010, with more than 4.000 reported acute human Q fever cases. However, seroprevalence studies show that at least 40.000 individuals were infected with *C. burnetii*[1, 2]. The outbreak started in the spring of 2007 when six individuals with acute Q fever were admitted to the hospital with signs of pneumonia. At the end of 2007, a total of 168 acute Q fever cases were reported; a huge increase compared to the annual average of 30 human cases in the years before 2007. At first, the outbreak was thought to be an isolated incident, but the number of human cases increased to 1000 in 2008 and even to 2354 in 2009[3]. By then, evidence was found that persons living close to a dairy goat farm with recent abortion problems or individuals who had been in close contact with goat lambs were at higher risk to become infected[4, 5]. These findings pointed to dairy goat farms with Q fever-induced abortions as the main source of the Dutch human Q fever outbreak[6]. By the end of 2012, Dutch authorities declared the Q fever outbreak to be over, after about 60.000 pregnant goats had been culled and the remaining dairy goats had been given mandatory vaccination additional to hygienic measures, a transport ban and a breeding ban[5].

The magnitude of the outbreak led to many concerns and unanswered questions, and research was deemed necessary. In the past years, over 20 projects have been executed to expand the expertise and knowledge about Q fever. The research presented in this thesis focuses on the recognition of *C. burnetii* by the human and goat immune system. This introductory chapter gives an overview about the *C. burnetii* bacterium and Q fever infections, provides information about the immunology of Q fever infections and describes the aim and outline of the project in more detail.

Q fever infections

History of Q fever

Eighty years ago, in 1935, the Australian pathologist Edward Holbrook Derrick was the first to describe Q fever after an outbreak of febrile illness among abattoir workers in Brisbane, Queensland, Australia[7]. To avoid negative associations with either the cattle industry or Queensland, the name Query (Q) fever was chosen over 'abattoir fever' or 'Queensland rickettsial fever'[8]. Two years later, Frank Macfarlane Burnet and Mavis Freeman isolated the bacterium from one of Derrick's patients, and named it *Rickettsia burnetii*[9, 10]. At the same time, two American scientists, Herald Rea Cox and Gordon Davis isolated the same bacterium from a tick found at the Nine Mile Creek in Montana, USA[11]. In 1948, *R. burnetii* was classified into a new genus, *Coxiella*[12]. Even though the causative agent was now known, the 'temporary' given name Q fever remained.

Bacteriology

Coxiella burnetii is a small (0.3 - 1.0 µm) intracellular Gram-negative bacterium. Phylogenically, it is classified in the order of *Legionellales* and based on its 16S rRNA, it falls in the gamma group of the Proteobacteria[13]. *C. burnetii* can be cultured from animal and human samples using cell culture, embryonated eggs or under the more recently described axenic (host cell-free) culture conditions[14]. Due to the formation of a spore-like form, *C. burnetii* is highly resistant to harsh environmental conditions such as high temperature and ultraviolet light[15]. *C. burnetii* has two distinct morphological forms; a metabolically inactive extracellular small cell variant (SCV) that provides *C. burnetii* its resistant characteristics, and a large cell variant (LCV) which is the intracellular metabolically active form[16]. Its strong resistant character together with its extreme infectivity, classified *C. burnetii* as a group B bioterrorism agent by the Centers for Disease Control and Prevention in the USA[17]. The circular genome of *C. burnetii* is 1.995.275 base pairs long and comprises a facultative 33- to 42-kb plasmid. Four plasmid types have been described, QpH, QpRS, QpDG and QpDV, and some bacteria are plasmidless[18-20].

One of the major features of *C. burnetii* is the antigenic variation of its lipopolysaccharide (LPS), known as phase variation. In natural conditions, the virulent phase

I LPS is found, which corresponds with smooth LPS as found in the *Enterobacteriaceae* family[21, 22]. Unique for *C. burnetii* phase I LPS is the presence of galactosaminuronyl-α-glucosamide disaccharide, virenose and dihydrohydroxystreptose in the O-chain[23]. After multiple passages under laboratory conditions, modification of the LPS can occur, resulting in an antigenic shift to the non-virulent phase II LPS. This phase variation is due to chromosomal deletions involving a large group of LPS biosynthesis genes, arranged in the O-antigen cluster[24-26]. The LPS of *C. burnetii* is an important virulence factor, however in comparison with e.g. *Enterobacteriaceae* LPS the endotoxic activity of both *C. burnetii* phase I and phase II LPS is 100 to 1000 times less[27].

Epidemiology

C. burnetii can infect a wide range of insects and animals such as marine wildlife, ticks, stellar sea lions, cats, horses and birds[21, 28, 29], but domestic ruminants and their products are the main route for human exposure[18]. In most animals, infection with *C. burnetii* does not result in disease. Exceptions are goats and sheep where infection can result in late-term abortion. Mammals excrete the bacteria through their urine, feces, milk and birth products like placenta and amniotic fluid. These products contain high loads of *C. burnetii* and can persist in the environment for years[30]. Facilitated by windy, warm and dry weather, contaminated aerosols are able to travel up to 5km[30]. Analysis of commercial available cow milk and other milk products revealed a high prevalence of *C. burnetii* among dairy cattle population worldwide. Also, the genotypes of *C. burnetii* found in the consumer milk products were highly similar and could indicate a possible clonal spread of *C. burnetii* among the European dairy cattle population[31]. However, this genotype is only incidentally found in humans, suggesting that the risk of obtaining Q fever is much lower via exposure to infected cattle than infected small ruminants[31]. Infected goats and sheep experience abortions which result in large spread of *C. burnetii*, while cattle do not show this symptom. In 2012, a large review study was conducted in which among others the prevalence of *C. burnetii* in goats, sheep and cattle was investigated[32]. It revealed that in most outbreaks, infection of humans occurred via infected domestic small ruminants, i.e. goats or sheep. In contrast, no evidence was found of a major contribution of cattle in the history of Q fever in humans in the four studied countries (the Netherlands, Germany, Bulgaria and France)[32]. It has not been described that *C. burnetii* can be transmitted from human to human.

Differentiation of isolates is extremely important for both epidemiology and diagnosis of *C. burnetii*. When examined only by 16S RNA, *C. burnetii* isolates show considerable genetic homogeneity[33]. Restriction fragment length polymorphism and microarray whole genome comparison identified six distinct genomic groups (I to VI)[34, 35]. A useful tool for typing *C. burnetii* is multiple locus variable number tandem repeats analysis (MLVA). In the Dutch outbreak, MLVA typing of patient samples and material from farms with abortion problems due to *C. burnetii* revealed that one predominant genotype was responsible for clinical disease in humans and goats[36, 37]. Apparently, some *C. burnetii* isolates have a certain predilection to infect specific hosts only, explaining the low number of humans infected by *C. burnetii* from cattle. Overall, different *C. burnetii* isolates can have a diverse genetic background and they can have a different preference of hosts. It can be questioned whether these differences of *C. burnetii* isolates can result in the different clinical manifestations found in humans and goats upon infection.

Clinical manifestations

Goat

Observations in the field and during experimental infections clearly show *C. burnetii* as a cause of abortion and stillbirth, mainly at the end of the gestation. Despite of this serious clinical outcome, infection with *C. burnetii* does not result in any preceding clinical symptoms as seen in infected humans[38-41]. Metritis, inflammation of the endometrium of the uterus, can be present in dairy goat herds where Q fever abortions occurred. The offspring of *C. burnetii*-infected goats can have a low body weight and high mortality. Furthermore, nurturing of apparently healthy kids can be difficult due to digestive tract and respiratory disorders[42].

During an infection, the trophoblasts of the allantochorion are the target cells of *C. burnetii* and replication of the bacterium occurs in these cells[43]. During abortions of infected goats, up to 10^9 organisms per gram placenta tissue are excreted, leading to a massive spread into the environment[38]. In both aborting and non-aborting goats, *C. burnetii* DNA has been detected in milk, vaginal mucus and/or feces[39]. The shedding of bacteria can also occur in subsequent pregnancies[44].

Human

Primary infection (asymptomatic and acute Q fever)

In most of the infected individuals (60%), the clinical signs of Q fever infection are subclinical or very mild. The incubation period is approximately 20 days, and when the infected individual starts to develop symptoms it is referred to as acute Q fever. Acute Q fever usually presents itself as a self-limited flu-like syndrome which is characterized by fever, pulmonary symptoms, fatigue and headache. Often there are also signs of hepatitis[10, 21, 45]. Even though most acute Q fever infections resolve spontaneously and only a small minority (2%) of the patients need to be hospitalized, antibiotic treatment is recommended. In the Netherlands, doxycycline (200mg daily for 14 days) is described to reduce the duration of the symptoms in case an acute Q fever is suspected or confirmed[46, 47].

Chronic Q fever

Several months to years after the initial infection, chronic Q fever develops in 2-5% of the infected individuals[18]. Individuals with cardiac valve abnormalities or an aneurysm or vascular reconstruction have an increased risk to develop chronic Q fever[18]. Endocarditis and vascular infections (aortic aneurysm or vascular prosthesis infection) are frequently described manifestations. Other less common characterizations of chronic Q fever include lung infections, hepatitis and osteomyelitis[47]. It is problematic to diagnose chronic Q fever, as it is based on the presence of symptoms, risk factors, detection of *C. burnetii* in blood, serological test results and diagnostic imaging. Therefore, during the Dutch Q fever outbreak, the Dutch Q fever Consensus group proposed a guideline to categorize chronic Q fever patients into three categories: proven, probable and possible. The guideline on these chronic Q fever diagnostics were nicely depicted by Wegdam-Blans *et al*.[48]. Independent of the category, all chronic Q fever patients need frequent monitoring consisting of clinical and microbiological follow-ups every three months. Chronic Q fever patients are preferably treated with a combination of doxycyline and hydroxychloroquine for at least 18-24 months[47]. The mortality of chronic Q fever is 25%, and can reach up to 60% when left untreated[49]. Up to now it is unknown what factors determine the switch from a primary infection to chronic Q fever. Therefore, we are unable to predict which patients are likely to develop chronic Q fever and we are unable to prevent this. For this reason, the current practice is to follow all patients clinically for at least one year. Diagnosis of chronic Q fever is difficult and treatment is problematic due to the long duration of therapy which can be unsuccessful in the end.

Q fever fatigue syndrome

Up to 60% of the individuals with a past acute Q fever infection suffer from post-infection fatigue symptoms[6]. For example, a study among 85 patients from the Netherlands with acute Q fever showed that 52% of the individuals had persistent symptoms, in particular fatigue, at six months after disease onset. Furthermore, after one year, 25% still had complaints[50]. A second Dutch study among 515 acute Q fever patients revealed that 20% had severe fatigue and an impaired health status at 12–26 months of follow-up[51]. The fatigue following acute Q fever has been named Q fever fatigue syndrome (QFS)[52]. Based on the published Dutch algorithm, QFS can be diagnosed when there is a severe fatigue for at least six months after an identified acute Q fever infection. The fatigue must have been absent before the episode of acute Q fever and it must cause significant disabilities in the daily life of the patient. Also, at least, chronic Q fever or other causes of fatigue, like somatic or physical problems, need to be excluded[52]. At the moment a randomized placebo-control trial which compares cognitive behavioral therapy, long-term doxycycline and placebo therapy is ongoing and will fill the gap of evidenced-based recommendations for the treatment of QFS[53].

Immunology of Q fever infections

From the host point of view, the recognition of *C. burnetii* by the immune system plays an important role in the development of the infection. An important question is how the immune system recognizes *C. burnetii* in humans and goats. As described in the previous section, goats and humans display a diverse clinical outcome upon *C. burnetii* infection. Furthermore, there is a wide variety in the symptoms of humans infection. One of the research aims of this thesis is to examine the immune response against *C. burnetii* in humans and goats. More knowledge about the recognition of *C. burnetii* by important pattern recognition receptors (PRRs) and the induction of cytokine responses, could provide answers to the different clinical symptoms observed in goats and human, and among infected humans. In the next few paragraphs we briefly review what is known about the immunology in goats and human during Q fever infection.

The immune system

The immune system can be divided into the innate immune response versus the adaptive immune response. Innate immunity is an ancient form of host defense as it is also found in plants and insects, which becomes active in the first minutes to hours after exposure to micro-organisms[54]. In contrast to the innate immune response, the later responding adaptive immune response is highly specific to the invading pathogen and can provide long-lasting protection due to the formation of memory. The major components of the innate immune system include epithelial barriers, soluble molecules (complement proteins, cytokines, and natural antibodies), phagocytes (macrophages and neutrophils), and pattern recognition receptors (PRRs)[55]. The main cells of the adaptive immune system are lymphocytes (B- and T-cells), antigen-presenting cells and effector cells. Micro-organisms express unique pathogen-associated molecular patterns (PAMPs) on their surfaces which are recognized by PRRs of the host immune cells to activate host-defense mechanisms, such as phagocytosis and cytokine signaling[56]. Important PRRs involved in recognition of bacteria are Toll-like receptors (TLRs; TLR1, TLR2, TLR4, and TLR6) and Nucleotide- binding oligomerization domain receptor 1 and 2 (NOD1 and NOD2).

Part of the humoral innate immune system is the complement system, which can be activated via three main pathways: the classical, lectin and alternative pathway. The classical pathway is initiated when the specific immunoglobulins IgM or IgG form clusters after recognition of an antigen on the surface of the bacterium, and is therefore often referred to as antibody-dependent. The lectin pathway is activated when mannose-binding lectin (MBL) or ficolins bind to certain sugars on the surface of bacteria. The alternative pathway is initiated by many bacteria, viruses and fungi, by the activation of factor-D[57-59]. All three pathways eventually lead to the activation of complement factor-3 (C3). Subsequently C5 convertase is formed and cleaved into the anaphylatoxin C5a and C5b. In the end, this last fragment binds to C6, C7, C8 and C9, resulting in the formation of the terminal complement complex (TCC)[58, 60]. Formation of TCC leads to the induction of among others, pro-inflammatory cytokines and cell lysis.

Human

Antibodies

The antibody response against *C. burnetii* develops approximately seven to fifteen days after the symptom onset[21, 47]. Several techniques can be used to measure *C. burnetii* antibodies: indirect immunofluorescence assay (IFA), enzyme-linked immunosorbent assay (ELISA) and complement fixation test (CFT)[21]. During acute Q fever, IgM phase II antibodies will appear almost immediately, subsequently IgM phase I and IgG phase II will present simultaneously and finally IgG phase I will appear[61]. *C. burnetii* infection in humans leads to a transition of phase II antigens to phase I antigens; during an acute or recent infection anti-bodies against phase II are most present, while phase I antibodies predominate during chronic infections[62].

Complement system

Not many studies have focused on the role of complement activation by *C. burnetii*. Thirty years ago it was demonstrated that the virulent phase I LPS was serum resistant and poorly activated the complement system, while phase II LPS was killed by complement factors in human serum and activated the alternative pathway[63]. The complement system is important for antibody-mediated immunity against many bacterial pathogens, however of none of the three complement pathways play an essential role in antibody-mediated immunity to *C. burnetii*[64].

Pattern recognition receptors

TLR4 is mostly expressed by macrophages and dendritic cells and its main ligand is LPS from Gram-negative bacteria[65]. *C. burnetii* phase I possesses tetra-acylated LPS, which in other bacteria like *Rhodobacter sphaeroides* or the lpxL2 mutant of Neisseria meningitides H44/76 is found to be a very weak cytokine inducer and can even act as a TLR4 antagonist[66-68]. In humans there is no role of TLR4 in mediating *in vitro* cytokine production by PBMCs, however TLR4 knockout mice exhibited a defect in cytokine production and granuloma formation[69, 70].

TLR2 can be found on a large range of cells of the immune system, i.e. monocytes, macrophages and antigen presenting cells[71]. It binds many ligands, like bacterial lipoproteins, peptidoglycan from Gram-positive bacteria and lipoarabinomannan from the mycobacterial cell-wall[72]. Studies in mice, reporter cell-lines and human

PBMCs have demonstrated TLR2 as an important regulator of immune responses against *C. burnetii*[69, 73-75]. The TLR2 receptor is able to form heterodimers with either TLR1 or TLR6; TLR1/TLR2 heterodimers mainly recognize triacylated lipopeptides, whereas TLR2/TLR6 heterodimers recognize diacylated lipopeptides[76, 77]. A decade ago TLR10 was discovered, however its ligand and function remained unknown for a long period[78]. TLR10 shares a variety of agonists with TLR1 and, similar to TLR1, requires TLR2 for recognition[79]. More recently, the inhibitory effect of TLR10 on TLR2 derived immune responses was demonstrated[80].

In contrast with the discussed TLRs, which are located on the surface of cell membrane, the NOD-like receptors are expressed intracellular. NOD1 and NOD2 recognize the bacterial peptidoglycan structures g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP), respectively[81]. They are mainly expressed by macrophages, dendritic cells and epithelial cells[82]. NOD2 plays a role in the induction of a cytokine response induced by *C. burnetii* in both mice[69, 83].

Goat

Experimental *C. burnetii* infection in goats elicited a strong IgM phase II and IgG phase II humoral response, starting 2 to 3 weeks post intranasal inoculation. Antibodies against phase I arose at a later stage of the infection. During the first weeks of infection, cell-mediated immune responses were minimal as no systemic cytokine mRNA (i.e. IL-1β, TNF-α and IFN-γ) was measured and the outcome of IFN-γ Elispot on *C. burnetii* stimulated PBMCs were low[84]. Cell-mediated immune responses, like the up-regulation of TNF-α, IL-1β and IFN-γ, were first detected in the first week after parturition. However, these cell-mediated immune responses did not differ enough to distinguish between *C. burnetii*-infected and non-infected pregnant animals[84].

Differences between Q fever infections in humans and goats

In the Netherlands, the Q fever outbreak was concentrated around humans and goats. In the above paragraphs we already discussed the symptoms and immunology of Q fever infection in both species separately. Here, we emphasize more on the differences and similarities between Q fever infection in humans and goats. The first difference between *C. burnetii* infection in goats and humans is based on pregnancy. Most of the research is performed on pregnant goats, as abortion is one of the most important clinical presentations of Q fever infection. In pregnant woman, adverse pregnancy outcome has also been associated with Q fever infection[21]. However, no evidence was found for this adverse effect during the Dutch Q fever outbreak[85]. In both goats and humans, the initial Q fever infection is mainly asymptomatic. Goats do not show any clinical symptoms during infection until abortion occurs. In humans, 60% of the infected individuals do not suffer from any clinical symptoms and the remaining 40% develop acute Q fever which is often self-limiting. On the contrary, a majority of the infected goats eventually abort or have a still birth, while only 1-5% of the infected humans will develop the more severe chronic Q fever. In goats, we know that the thropoblasts in the allantochorion of the placenta are the primary target cells for *C. burnetii*. In contrast, in chronic Q fever patients the bacteria can be found surrounding the heart valve where it accumulates and forms septic matter. These differences in clinical outcome percentages may be explained by the initial recognition of *C. burnetii* by the hosts immune cells. It can be hypothesized that a decreased initial recognition of the Q fever bacterium leads to an attenuated innate immune response and evasion of the host defense against infection.

Objective and outline of this thesis

The aim of this thesis is to assess how *C. burnetii* is recognized and induces immune system responses in goats and humans, in order to determine whether insufficient immune recognition and response lead to persistence and abortion in goats and chronic disease in humans. The interplay between *C. burnetii* and the host immune system is an essential factor in the invasion and persistence of *C. burnetii*. Up to now, it is unclear how *C. burnetii* evades the immune system of goats and humans and is able to survive in these hosts. In order to elucidate how *C. burnetii* evades the immune system, more basic knowledge is needed about the innate immune response upon *C. burnetii* infection. However, investigations are hindered by several factors: In goats, the problem is that carriership is asympto-

matic and therefore hard to recognize, until a miscarriage ensues in pregnant goats. This could lead to a large reservoir of infected goats that are able to spread the disease. In humans, the problem is the development of chronic disease in a small proportion of individuals. These individuals need long term therapy and it cannot be predicted which patients develop chronic Q fever. The findings we present in this thesis concerning the immunological reaction to *C. burnetii* in humans and goats, could eventually lead to better diagnostic tests for humans and animals and treatments for patients with Q fever.

Chapters 2-6 presents the (innate) immune response of goat and human hosts against *Coxiella burnetii*. **Chapter 2** addresses the humoral part of the innate immune system. We examined which complement pathways are involved in the recognition of *C. burnetii* in humans and which pathway is necessary for a proper cytokine response in *C. burnetii*-stimulated human peripheral blood mononuclear cells (PBMCs).

Next to the humoral part of the innate immune system, the cellular recognition plays a central function in the first defense against micro-organisms. Especially Toll-like receptors (TLRs)1, 2, 4 and 6 and Nucleotide-binding oligomerization domain (NOD) like receptors 1 and 2 play an important role in the recognition of bacteria. **Chapter 3** describes whether these TLRs and NLRs are involved in the recognition of *C. burnetii in vitro.* **Chapter 4** presents the role of TLR10, a receptor which inhibits TLR2-induced cytokine responses, in *C. burnetii* induced cytokine responses.

Whether defects in recognition of *C. burnetii* by the innate immune system lead to the development of chronic Q fever was investigated by genetic analysis among 139 chronic Q fever patients and 220 controls. Within these individuals, specific single nucleotide polymorphisms (SNPs) in the genes coding for the pattern recognition receptors were identified and discussed in several chapters. **Chapter 2** shows the role of SNPs in mannose-binding lectin. **Chapter 4** examined whether genetic variation in TLR10 influences the probability to develop chronic Q fever after an initial infection. **Chapter 5** focuses on TLR1, 2, 4 and 6, NOD 1 and 2 and downstream pathways in relation to the development of chronic Q fever.

After studying the immune responses against *C. burnetii* in humans, **Chapter 6** focuses on the innate immune response of goats during an experimental *C. burnetii* infection.

Next to the hosts perspective, we also investigated the infection from the pathogen side. Does the origin of the *C. burnetii* bacterium determine infectivity and are there differences between *C. burnetii* strains that cause acute or chronic infections in humans? In **Chapter 7** a large collection of *C. burnetii* isolates with diverse mammalian origins ranging from human and goat to sheep and cattle, are studied in relation to the cytokine responses they elicit in human immune cells.

In the general discussion and summary (**Chapter 8**) the major findings of this thesis are presented and discussed in relation to the international literature. In addition, future perspectives and recommendations concerning the early detection of Q fever infections and treatment are provided.

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02

Activation of the complement system by *Coxiella burnetii*

SUBMITTED

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Abstract

Background: Knowledge of complement system activation by *Coxiella burnetii* is limited. We investigated how *C. burnetii* activates the complement system and how this influences cytokine production. In addition, we investigated whether polymorphisms in *MBL2* are associated with chronic Q fever development.

Methods: *C. burnetii*-stimulated plasma or PBMCs were incubated with anti-C2 (classical pathway), anti-MBL (MBL-pathway), anti-factor D (alternative pathway) or anti-C5 to measure the effect on terminal complement complex (TCC) formation and cytokine induction. Polymorphisms in *MBL2* were examined in 139 chronic Q fever patients and 220 controls.

Results: Anti-factor D inhibited complement activation by *C. burnetii*. In the presence of anti-*C. burnetii* antibodies, only anti-C2 and anti-factor D combined led to a decrease in TCC formation. Inhibition of C5a or factor D led to decreased IL-1β, TNF-α and IL-6 induction in *C. burnetii*-stimulated PBMCs. Conversely, anti-MBL reduced IFN-γ, IL-22 and IL-17 production significantly. MBL haplotypes were not different between chronic Q fever patients and controls.

Conclusion: *C. burnetii* activated the alternative pathway of complement in healthy controls, while the classical pathway was also involved in seropositive individuals. Production of monocyte-derived cytokines was mediated by C5a which was formed after activation of the alternative pathway by *C. burnetii*. MBL augmented the induction of T-cell-derived cytokines.

Introduction

Coxiella burnetii is the causative agent of Q fever, a disease that generally presents as an acute flu-like illness or pneumonia in approximately 40% of the infected individuals. A persistent chronic infection develops in 2-5% of the individuals with an initial infection, and is mainly characterized by endovascular infections or endocarditis[1].

Defense against micro-organisms is mediated by the early reactions of the innate immunity, later targeted and amplified by adaptive immune responses. Recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) is a key step in the activation of the cellular part of the innate immune system, whereas plasma zymogens belonging to the complement system, are pivotal in the humoral innate immune response. The complement system can be activated via three main pathways: the classical, lectin and alternative pathway. Activation of the complement system leads to pathogen opsonization by complement factor C4b and C3b, to formation of the terminal complement or membrane attack complex (TCC or MAC) and it produces anaphylatoxins C4a, C3a and C5a that activates cells, promote chemotaxis and stimulate cytokine production. The classical pathway is strongly initiated by IgM or IgG-complexes attached to the antigen, and therefore often referred to as antibody-dependent. The lectin pathway has a similar function as the classical pathway and is activated via mannose-binding lectin (MBL) and ficolins bound to repetitive sugar molecules on the pathogen. The alternative pathway, which accounts for up to 80-90% of the total complement activation, is activated by many bacteria, viruses and fungi but mainly functions as an amplification system of the classical and the lectin pathway. In brief, activation of each pathway eventually leads to activation of complement factor-3 (C3). Subsequently a C5 convertase is formed (C4b2a3b through the classical and lectin pathways or C3bBbC3b through the alternative pathway), and C5 is cleaved into C5b and the anaphylatoxin C5a. The first fragment eventually binds to C6, C7, C8 and several units of C9, leading to the formation of the TCC[2, 3].

Mannose-binding lectin, which is important for the activation of the lectin pathway, recognizes N-acetyl-D-glutosamine, mannose, N-acetyl-mannosamine, fructose

and glucose[4]. Six important single nucleotide polymorphisms (SNPs) are known within the gene coding for MBL (*MBL2*), which have a major effect on the MBL serum concentration and protein structure[5]. Three of these SNPs are located in exon 1 in codons 52 (Arg52Cys), 54 (Gly54Asp), and 57 (Gly57Glu) and are associated with MBL deficiency. The wild-type allele is named 'A', while the mutant alleles are called D, B and C respectively. In addition, three SNPs in the promoter region have an effect on the circulating MBL concentrations[6]. These SNPs are termed *'H/L', 'X/Y'* and *'P/Q',* and the four main promoter haplotypes described in different populations are *LXP, LYP, LYQ* and *HYP*[6, 7]. Due to a strong linkage disequilibrium, the six SNPs together are responsible for seven common haplotypes reported in Caucasian populations; HYPA, LYQA, and LYPA are associated with increased expression of circulating MBL, and LXPA, HYPD, LYPB, and LYQC are associated with deficiency of this protein[8, 9]. MBL deficiency is quite common, as approximately 5% of the population have no detectable MBL in their plasma and 35-40% of the individuals have low concentrations[10].

Research has mainly focused on the role of TLRs and NLRs in the recognition of *C. burnetii* and the development of acute or chronic Q fever infections[11-16]. To our knowledge only a few articles discuss the role of complement activation during *C. burnetii* infection[17, 18]. We aimed to identify which complement pathway is activated by *C. burnetii* Nine Mile phase I in healthy individuals and in chronic Q fever patients. We examined which complement pathway is involved in *C. burnetii* induced cytokine production. Finally, we examined whether the presence of SNPs in the MBL gene leads to a higher susceptibility to develop chronic Q fever.

Material and Methods

Coxiella burnetii

Coxiella burnetii Nine Mile phase I was a kind gift from the Bundeswehr Institute for Microbiology (Munich, Germany) and cultured at the Central Veterinary Institute. Lipopolysaccharide (LPS) phase determination was performed by SDS-PAGE and silver staining, using purified phase I (RSA493) and phase II (RSA439) *C. burnetii* NM LPS (kindly provided by R. Toman) as controls[19, 20]. The concentration was determined using Taqman (Quanta BioSciences) real-time PCR as described previously[21]. Heat killed *C. burnetii* cultures were prepared by heating the cultures for 30 minutes at 99°C.

Inhibitory anti-complement antibodies

Mouse immunoglobin G1 (IgG1) monoclonal antibodies against human C2 (clone 175-62), factor D (fD) (clone 166-32), C5 (clone 137-30), C5a (clone 137-26) and the isotype matched control (clone G3-519) were a kind gift from Michael Fung (Tanox Inc, Texas, USA). Purified mouse IgG1 monoclonal antibody to human mannose-binding lectin (MBL) (clone 3B6) was purchased from the Antibodyshop (BioPorto Diagnostics A/S, Gentofte, Denmark). All antibodies were used in a concentration of 25 µg/ml.

Preparation of plasma samples for complement activation

Polypropene tubes of 5 ml were hand-filled with 100 µl of [3.3 mg/ml] lepirudin (Redswan, Utrecht, the Netherlands). Blood was drawn from the individuals and centrifuged for 10 minutes at 1700rpm at 4°C. Subsequently, plasma was taken and incubated for 30 minutes with either phosphate buffered saline (PBS), anti-C2, anti-fD, anti-MBL, anti-C5 or isotype control in combination with *C. burnetii* Nine Mile $1x10^7$ or PBS as control. Complement activation was stopped by adding 250mM ethylenediaminetetraacetic acid (EDTA). Samples were stored at -20°C until TCC activation was measured by enzyme linked immunosorbent assay (ELISA).

Serum samples

Serum from healthy seronegative (anti-*C. burnetii* phase I IgG [IgG1] <16) persons was used in the peripheral blood mononuclear cells (PBMCs) experiments. The complement in this serum was inactivated by 30 minutes incubation at 56°C. In some TCC experiments, seropositive serum was used of one patient with a past Q fever infection (IgG1/IgG2 256/512, IgM1/IgM2 128/32) and two chronic Q fever patients (IgG1/IgG2 >4096, IgM1/IgM2 negative).

Study population

Blood was drawn from chronic Q fever patients and controls. The patients were

diagnosed with a probable or proven chronic Q fever, as defined by the Dutch consensus on chronic Q fever[22]. The control group consisted of individuals from the same area also with valvular or vascular abnormalities and serological evidence of an initial *C. burnetii* infection, but without symptoms or serological evidence of chronic Q fever. In Table 1 the demographic and clinical characteristics, retrieved from the patients' and controls' medical records, are summarized. A more detailed description of the background and recruitment of the patients and controls is described by Schoffelen *et al.*[12]. The study was approved by the Ethical Committee of Radboud university medical center, Nijmegen, The Netherlands. Subjects were enrolled after providing written informed consent. Institutional Review Boards of participating hospitals approved the inclusion of patients and controls in this study. All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

PBMC isolation

Buffy coats from healthy donors were obtained from Sanquin (Nijmegen, the Netherlands). Peripheral blood mononuclear cells (PBMCs) were isolated according to standard protocols with minor modifications as described previously[23]. The cells were counted in a ZH Coulter Counter (Beckman Coulter) and adjusted to $5x10^6$ cells/ml in RPMI. A volume of 100 µl was added to each well of a round-bottom 96 well plate (Costar, Corning, The Netherlands). After 24 hours and 7 days, the supernatants were harvested and stored at -20°C until further analysis.

Genomic DNA isolation and single nucleotide polymorphism analysis

DNA from the patients and controls was isolated using the Gentra Pure Gene Blood kit (buffy coats and whole blood) (Qiagen, USA) or the Isohelix isolation kit for buccal swabs (Isohelix, UK), according to the manufacturer's protocols. Four SNPs in the *MBL2* gene (rs11003125, rs7096206, rs5030737 and rs1800451) were genotyped with a Sequenom mass-spectrometry genotyping platform. The fifth SNP in *MBL2* (rs1800450) was analyzed using a pre-designed TaqMan® SNP genotyping assay (Applied Biosystems, USA). Before genotyping of the fifth SNP, DNA isolated from heparin-anticoagulated whole blood was treated with heparinase I (Sigma-Aldrich, USA) as described previously[24]. Details of the SNPs can be found in Table 2.

Table 1: Characteristics of the chronic Q fever patients and controls for genetic analysis

	Chronic Q fever Cohort (n=139)	Control Cohort (n=220)	P-value[a]
Median age, y (IQR)	70.0 (63.0-75.6)	67.6 (58.3-74.3)	0.02
Male sex (%)	114 (82)	169 (77)	0.29
Classification of chronic Q fever			
Proven (%)	92 (66.2)	0	
Probable (%)	47 (33.8)	0	
Cardiovascular risk factor for chronic Q fever[b]			
Vascular aneurysm / prosthesis (%)	95 (68.3)	129 (58.6)	0.07
Valvular defect / prosthesis (%)	39 (28.1)	111 (50.5)	<0.001
Immunocompromised state[c] (%)	21 (15.1)	14 (6.4)	0.01
Serology (Median, IQR)			
C. burnetii phase I IgG	4096 (2048-16384)		
C. burnetii phase II IgG	4096 (2048-16384)		

Abbreviations: IQR, interquartile range.

[a] Fisher exact test for categorical variables and Mann-Whitney for continuous variables.

[b] Both vascular and valvular risk conditions in 9 case-patients and 22 controls.

[c] Case-patients: Auto-immune disease with immunosuppressive drugs n=14; renal insufficiency n=1; renal transplantation n=1; malignancy n=3; prednis(ol)one use not specified n=1. Controls: Autoimmune disease with immunosuppressive drugs n=5; renal transplantation n=2; malignancy n=4; prednis(ol)on use not specified n=4.

Table 2: *MBL2* SNPs analysed in the study

SNP ID	nucleotide change	amino acid change	alleles	effect	genotyping assay
rs11003125	c.-618G>C		H and L	MBL serum concentration	Sequenom
rs7096206	c.-289G>C		Y and X	MBL serum concentration	Sequenom
rs5030737	c.154C>T	p.Arg52Cys (D52C)	A and D	MBL deficiency	Sequenom
rs1800451	c.170G>A	p.Gly57Glu (G57E)	A and C	MBL deficiency	Sequenom
rs1800450	c.161G>A	p.Gly54Asp (G54D)	A and B	MBL deficiency	Taqman

Cytokine and TCC measurement

Cytokine production was measured using ELISA according to the respective manufacturers' protocols. ELISA kits were purchased from Sanquin (PeliKine Compact, Amsterdam, the Netherlands) for interleukin 6 (IL-6), IL-10, and IFN-γ, and from R&D Systems (Minneapolis) for tumor necrosis factor alpha (TNF-α), IL-1β, IL-17 and IL-22. Measurement of TCC was done as previously described with minor modifications[25]. Maxi-sorp plates (Nunc) were coated overnight with antiE1195B. As a secondary antibody, biotin-labeled anti-C6, was used. Adsorption was measured at 450 nm using a Bio-Rad multiplate reader (Bio-Rad Laboratories, Veenendaal, the Netherlands).

Statistics

The presence of Hardy-Weinberg equilibrium (HWE) for all five SNPs in *MBL2* in healthy controls was analyzed using a web-based HWE calculator[26]. The association between susceptibility to chronic Q fever and a SNP was investigated by means of univariate logistic regression models (Fisher's exact test) with IBM SPSS 18 software (IBM Corp., USA). SNPs were evaluated using a dominant and recessive model analyses, for which odds ratios (OR) including 95% confidence intervals (CI) were reported. Differences between experimental groups were tested using either the Mann-Whitney *U* test or the Wilcoxon matched-pairs signed rank test, as mentioned in the Figure legends. The data are expressed as mean ± standard error of the mean (SEM) unless indicated otherwise. GraphPad Prism 5 software was used. Differences with a p-value <0.05 were considered statistically significant.

Results

The alternative complement pathway is activated by *Coxiella burnetii* in healthy individuals

Plasma derived from lepirudin-anticoagulated blood from healthy individuals was incubated for 30 minutes with *C. burnetii* NM in the presence of monoclonal antibodies blocking C2 (classical pathway), MBL (lectin pathway), factor D (alternative pathway) or C5 (the final common pathway). TCC formation was used as a read out of final common pathway activation. Figure 1 shows that TCC formation was significantly reduced in the presence of anti-factor D compared to the isotype control. The same trend was observed after inhibition of C5. In contrast, the addition of anti-C2 or anti-MBL did not lead to a reduced TCC formation. Untreated plasma was capable of minor spontaneous formation of complement (Figure 1, striped bar).

Antibodies against *Coxiella burnetii* in serum of chronic Q fever patients activate the classical complement pathway

To investigate the role of *C. burnetii* specific antibodies in complement activation, serum from either *C. burnetii*-seronegative individuals or seropositive serum from individuals with a past or chronic Q fever infection (anti-*C. burnetii* phase I and II IgG ≥ 256) was incubated with plasma derived from lepirudin-anticoagulated whole blood from healthy individuals. In the plasma samples incubated with seronegative serum, TCC formation was strictly dependent on the alternative (factor D) pathway activation. In plasma incubated with anti-*C. burnetii* seropositive serum, inhibition of the alternative pathway (factor D) activation led to a significant but

incomplete decrease in TCC formation, whereas inhibition of the classical pathway (anti-C2) did not lead to a significant decline in TCC formation. Interestingly, inhibition of both classical and alternative pathways, by using anti-C2 and anti-factor D combined, led to a decrease in complement activation similar to inhibition of C5 (Figure 2A). Thus, in *C. burnetii*-seronegative individuals the complement system was activated via the alternative pathway, while in individuals with positive anti-*C. burnetii* serology both the classical and the alternative pathway were involved. The results shown in Figure 2A suggested that addition of seropositive serum to healthy plasma resulted in a higher TCC formation in general. This observation was confirmed by stimulating plasma from healthy individuals and plasma from chronic Q fever patients with *C. burnetii* NM. The TCC formation in *C. burnetii*-stimulated plasma of chronic Q fever patients was significantly higher than in plasma of healthy individuals (Figure 2B).

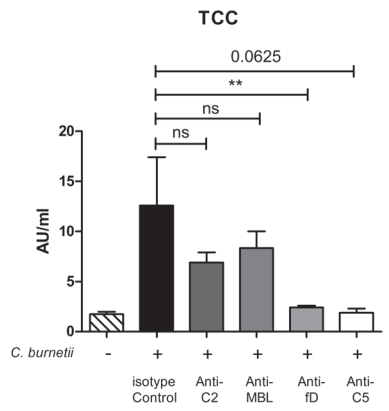
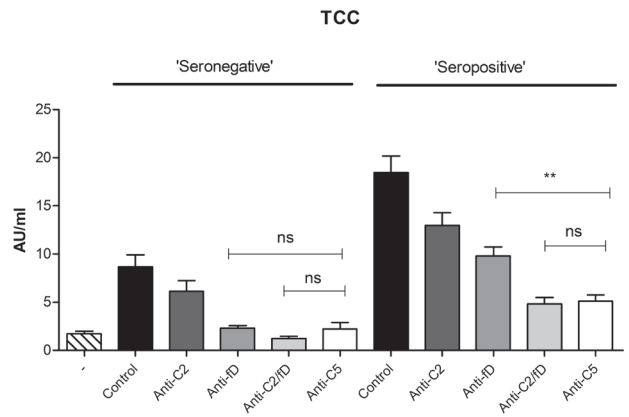


Figure 1: TCC activation by *C. burnetii* is dependent on the alternative pathway

Plasma-derived from lepirudin-anticoagulated whole blood of healthy individuals (seronegative; anti-*C. burnetii* phase 1 IgG<16) was stimulated with heat inactivated *C. burnetii* NM 1x10^7/ml in the presence of isotype control, anti-C2, anti-MBL, anti-fD or anti-C5. After 30 minutes incubation, complement activation was stopped by adding 250 mM EDTA, and TCC activation was measured by ELISA. The striped bar shows the result of non-stimulated plasma. Mean ± SEM of three to four separate experiments, giving n=5 (anti-C5) or n=8, are presented. **p<0.01, Wilcoxon matched-pairs U-test signed rank test.

A



B

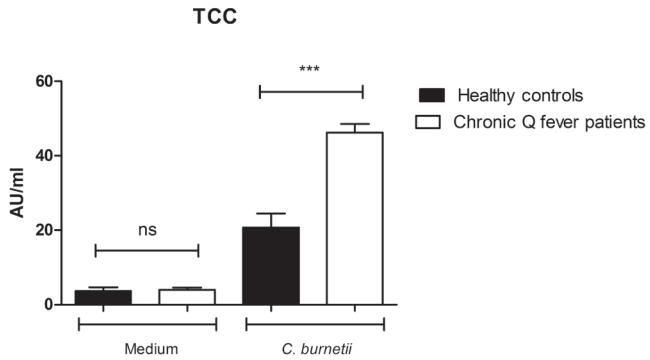


Figure 2: The classical pathway is activated by *C. burnetii* in the presence of *C. burnetii* seropositive serum

A) Plasma derived from lepirudin-anticoagulated whole blood of healthy individuals stimulated with *C. burnetii* Nine Mile 1x10^7/ml in the presence of isotype control, anti-C2, anti-MBL anti-fD or anti-C5. Either seronegative serum (IgG1 < 16) or serum from seropositive past/chronic Q fever patients (IgG1 ≥ 256) was added. The striped bar shows the result of non-stimulated plasma. n=4-6

B) Plasma derived from lepirudin-anticoagulated whole blood of healthy individuals (n=8) and chronic Q fever patients (n=14) stimulated with PBS or *C. burnetii* Nine Mile 1x10^7/ml. After 30 min incubation, complement activation was stopped with by adding 250 mM EDTA and TCC activation was measured by ELISA. Mean ± SEM are presented, **p<0.01, ***p<0.001, Wilcoxon matched-pairs U-test signed rank test.

Coxiella burnetii-induced cytokine production is partly dependent on heat-labile factors in serum

The influence of complement factors in serum on the cytokine production of *C. burnetii*-stimulated PBMCs of healthy individuals was examined. The amount of cytokines produced by PBMCs after *C. burnetii* stimulation in the absence of serum did not differ much from unstimulated PBMCs. In the presence of serum, a solid production of IL-1β, TNF-α and IL-6 was observed (Figure 3). After inactivating the serum by 30 minutes heating at 56°C, IL-1β and IL-6 production by *C. burnetii*-stimulated PBMCs was significantly, but not completely decreased (Figure 3).

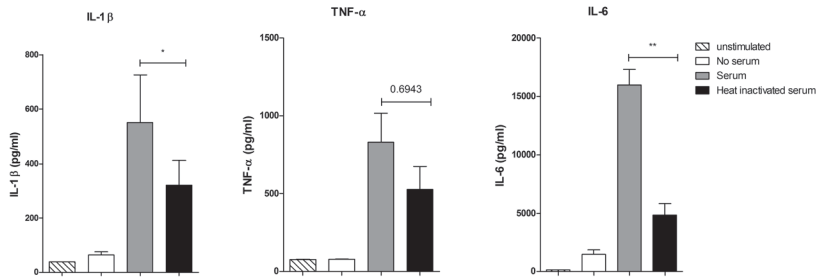


Figure 3: Cytokine production by _C. burnetii_-stimulated PBMCs is partly dependent on complement factors

PBMCs stimulated with *C. burnetii* Nine Mile 1 x10^6 in the absence of serum, in the presence of 10% serum or 10% heat-inactivated serum. Striped bars show cytokine production without stimulation, in the presence of 10% serum. IL-β, TNF-α and IL-6 were measured in the supernatant by ELISA after 24h incubation. Mean ± SEM are presented of four separate experiments, giving n=8, *p<0.05; **p<0.01, Mann-Whitney U-test.

Monocyte-derived cytokine production after _Coxiella burnetii_ stimulation is dependent on C5a and mediated by the alternative pathway, while T-cell-derived cytokine production involves mannose binding lectin

The finding that *C. burnetii*-induced cytokine production by PBMCs is decreased by inactivation of the serum and thus likely partly dependent on the activation of complement, raised the question which complement pathway is involved in cytokine induction by *C. burnetii*. After 24h stimulation of PBMCs from healthy individuals with *C. burnetii* in the presence of anti-C2, anti-MBL, anti-factor D and anti-C5a, monocyte-derived cytokines (IL-β, TNF-α and IL-6) were measured. Inhibition of the alternative pathway by blocking factor D and blocking the anaphylatoxin C5a resulted in significantly lower IL-1β and IL-6 production and the same trend, although not significant, was observed for TNF-α (Figure 4A). Inhibition of the classical pathway and lectin pathway by respectively anti-C2 and anti-MBL did not result in lower cytokine responses from *C. burnetii* stimulated PBMCs.

We also investigated the role of the complement pathways in the production of T-cell-derived cytokines, IL-17, IL-22 and IFN-γ, after 7 days of *C. burnetii* stimulation. Heat inactivation of complement factors in serum led to a lower IL-17, IL-22 and IFN-γ response, although not as much as compared to IL-1β, TNF-α and IL-6 (Figure 4A/B). The most surprising finding was the significant decrease in IL-17, IL-22 and IFN-γ production when MBL was inhibited (Figure 4B). In contrast to what was found for IL-1β, TNF-α and IL-6, inhibition of factor D did not result in lower IL-17, IL-22 and IFN-γ production (Figure 4B). In both monocyte-derived and T-cell-derived cytokines, inhibition of the classical pathway did not lead to lower cytokine production by stimulated PBMCs (Figure 4A/B). The role of C5a is less straightforward, it seems that inhibition of C5a results in less IL-22 and IFN-γ, but has no effect on IL-17 production.

Polymorphisms in the *MBL2* gene do not lead to a higher susceptibility to develop chronic Q fever

Recent findings by Schoffelen *et al.* indicated that chronic Q fever patients are able to produce large amounts of IFN-γ upon *C. burnetii* stimulation[27]. As our study revealed that in the absence of MBL, the IFN-γ response against *C. burnetii* was reduced, we were intrigued to examine the role of genetic variations in the *MBL2* gene in the development of chronic Q fever. DNA was collected from 139 chronic Q fever patients (both probable and proven). Individuals from the same area with valvular or vascular abnormalities predisposing to chronic Q fever were collected as controls (n=220). The controls had serological evidence of a previous *C. burnetii* infection, but did not develop chronic Q fever. The distribution of all five SNPs did not differ between the chronic Q fever patients and the matched controls (data not shown).To investigate the haplotype distribution among the chronic patients group and controls, a distinction was made between medium and high producers based on their X/Y and L/H genotype (rs7096206 and rs11003125 respectively). Subsequently, low MBL producers were separated based on mutations in the MBL gene caused by the presence of D, B or C. These mutations are dominant and result in low MBL production independent of the presence of either X,Y,L or H. In the controls, 41.9% of the individuals bear one of the mutant alleles (D, C or B) causing low MBL levels, this percentage equals that of chronic Q fever patients (41.7%) with a MBL deficient genotype (p=0.53). Also, the percentage of medium and high MBL producers did not differ between the groups (Table 3).

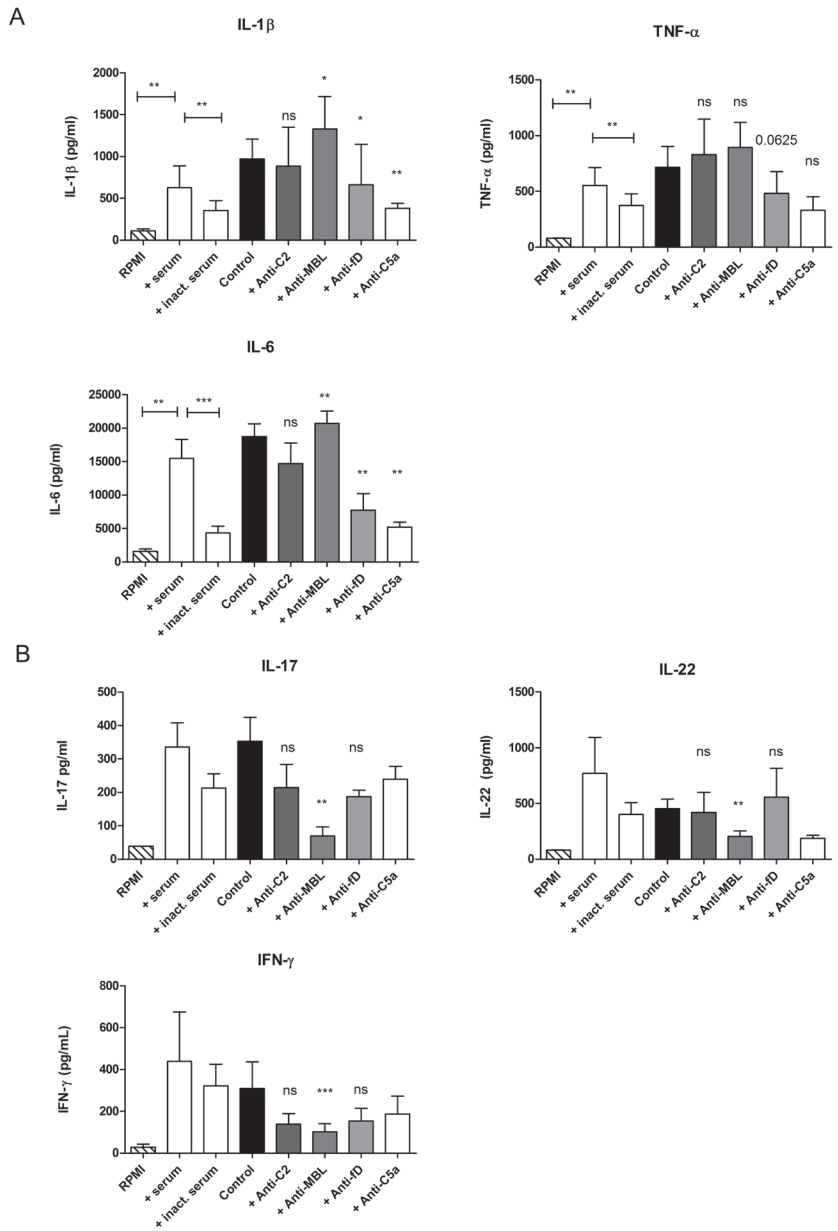


Figure 4: Cytokine production is mediated by the alternative pathway

PBMCs stimulated with *C. burnetii* Nine Mile 1x10^6/ml in the absence of serum, in the presence of 10% serum, heat-inactivated serum or serum that was one hour pretreated with isotype control, anti-C2, anti-MBL, anti-fD or anti-C5a. Striped bars show cytokine production without stimulation, in the presence of 10% serum.

A) IL-1β, TNF-α and IL-6 were measured in the supernatant by ELISA after 24h incubation.

B) IL-17, IL-22 and IFN-γ were measured after 7 days incubation. Mean values with SEM of two to four separate experiments, giving n=4 (anti-C5a), n=6-8 (all others), ns = not significant *p<0.05; **p<0.01, Wilcoxon matched-pairs signed rank test.

Table 3: Distribution of MBL haplotypes in patients with chronic Q fever and controls

	Low MBL serum haplotypes[a] (presence of either D,B or C) **n (%)**	**Medium MBL serum haplotypes**[a] (LX/LX, LY/LX, HX/LX, HY/HX) **n (%)**	**High MBL serum haplotypes**[a] (LY/LY, HY/LY, HH/HX, HY/HX, HY/HY) **n (%)**
Patients	55 (41.7)	61 (46.2)	16 (12.2)
Controls	83 (41.9)	90 (45.5)	25 (12.6)

[a] MBL serum haplotypes as described by de Messias-Reason *et al.*[9].

Discussion

In this study we showed that in healthy individuals the *C. burnetii* activates the alternative pathway of the complement system. In serum of Q fever patients, both the classical and alternative pathway were activated. Furthermore, the induction of monocyte-derived cytokines (IL-1β, TNF-α and IL-6) is mediated by the anaphylatoxin C5a which is formed after activation of the alternative pathway by *C. burnetii*. An unexpected finding was the role of MBL, which did not influence complement activation by *C. burnetii*, but did have a striking effect on IFN-γ, IL-22 and IL-17 production. As previous studies revealed a high IFN-γ production by chronic Q fever patients upon *C. burnetii* stimulation, the influence of genetic variants in the *MBL2* gene on predisposition to chronic Q fever was examined. However, the distribution of high and low MBL serum haplotypes did not differ between the chronic Q fever patients and the control individuals and thus in patients the MBL concentration is not decisive in the progression towards chronic Q fever.

In general, Gram-negative bacteria can activate individual pathways or a combination of any: *Chlamydia trachomatis* for example activates the alternative pathway[28], *Bordetella persussis* activates the classical pathway[29], and *Neisseria meningitidis* activates both the alternative and the lectin pathway[2]. Our study revealed that in *C. burnetii*-naive individuals, only the alternative pathway was activated by *C. burnetii*. As can be expected, activation of both the alternative and the classical pathway was observed in the presence of anti-*C. burnetii* seropositive serum, considering that the classical pathway is activated by immunoglobulins which are recognized by C1q. In 1988, two years before the lectin pathway was discovered, Vishwanath *et al.* concluded that *C. burnetii* phase I (smooth) LPS poorly activated complement, while our findings suggested that *C. burnetii* NM phase I activates complement via the alternative pathway[18]. Several conditions can explain the difference between these observations. First, the *C. burnetii* isolates used in Vishwanaths study were passed in guinea pigs and (cell cultures of) chicken embryos, while the *C. burnetii* Nine Mile isolate in our study was passed in mice and cultured in BGM cells[30]. Differences in these passage methods could influence the expression of surface molecules of the isolates, and thereby have an effect on the complement activation. Secondly, different techniques were used

to detect complement activation. While we made use of enzyme-linked immunosorbent assay (ELISA), Vishwanath *et al.* measured complement haemolytic activity[18].

C. burnetii NM expresses sugars, such as mannose and N-acetyl-D-glucosamine, which can be recognized by MBL. Mannose (D-mannose) can be found in both the O-specific chain and the core region, while N-acetyl-D-glucosamine can only be found in the O-specific chain of phase I LPS[31, 32]. Despite of multiple passages of the *C. burnetii* NM strain used in our study, silverstaining of the LPS indicated that it was still in phase I. Based on the presence of these sugars, we hypothesized that MBL is able to recognize *C. burnetii*. However, we did not find evidence that the lectin pathway plays a role in complement activation by *C. burnetii*.

We showed that monocyte-derived cytokine production by *C. burnetii* in healthy individuals partly depends on complement factors, in particular factor D. The immune response against several other micro-organisms also depends on serum factors to mount a proper cytokine response, i.e. *Candida albicans* and *Streptococcus pyogenes*[33, 34]. Besides complement components, serum contains other elements such as albumin, α-, β- and γ- globulins and hormones which can contribute to the cytokine production by micro-organisms[35]. Furthermore, the presence of the soluble serum factor LBP (LPS-binding protein), can significantly enhance the PBMCs response to LPS via CD14[36]. It can be hypothesized that these factors play a role in cytokine induction by *C. burnetii* as well, explaining the partial decrease in cytokine production after heat-inactivation of the serum. Several studies demonstrated that MBL could play a role in the regulation of monocyte-derived cytokine inflammation[37-40]. However, we did not observe any effect of MBL on the *C. burnetii*-induced production of IL-1β, TNF-α or IL-6 production by PBMCs after 24h. In contrast, MBL did have an effect on the production of IL-17, IL-22 and IFN-γ, which are primary T-cell-derived cytokines (and also NK-cell-derived in case of IFN-γ). These findings could imply that, MBL influences the recognition of *C. burnetii* antigens to T-cells, independent of complement system activation.

Based on the high amount of IFN-γ production by PBMCs of chronic Q fever patients in response to *C. burnetii*[41], and our finding that MBL is involved in *C. burnetii*-induced IFN-γ production, we hypothesized that genetic variability in *MBL2*, might be associated with the development of chronic Q fever. However, our analysis showed that the distribution of the *MBL2* haplotypes did not differ between chronic Q fever patients and controls. Thus, the presence of SNPs in *MBL2* and the corresponding MBL serum levels, are not associated with chronic Q fever.

In conclusion, this study shows that the complement system is activated by *C. burnetii*, involving both the alternative and classical pathway. Despite the involvement of MBL in *C. burnetii*-induced IFN-γ production, no association was found between polymorphisms in the *MBL2* gene - directly related to MBL serum levels - and the likelihood to develop chronic Q fever. The role of MBL in *C. burnetii* phagocytosis and infection would be interesting to address in future studies.

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03

Recognition of *Coxiella burnetii* by Toll-like receptors and nucleotide-binding oligomerization domain–like receptors

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Abstract

Infection with *Coxiella burnetii* can lead to acute and chronic Q fever. Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR6, Nucleotide-binding oligomerization domain receptor 1 (NOD1), NOD2, and the mitogen-activated protein kinases are central in the innate immune response against microorganisms, but little is known about their role in the recognition of *C. burnetii* in humans.

Human peripheral blood mononuclear cells (PBMCs) were stimulated with *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262. TLRs were inhibited using specific antibodies or antagonists. Additionally, the influence of human polymorphisms in TLRs and Nod-like receptors (NLRs) on *C. burnetii*–induced cytokine production was assessed.

Inhibition of TLR2, p38, JNK, and ERK led to decreased cytokine responses in *C. burnetii*–stimulated human PBMCs. Humans with polymorphisms in TLR1 and NOD2 had reduced cytokine production, compared with humans with wild-type genotypes, after stimulation. Interestingly, polymorphisms in TLR6 led to decreased cytokine production after *C. burnetii* 3262 stimulation but not after *C. burnetii* Nine Mile stimulation.

The TLR1/TLR2 heterodimer and NOD2 are important recognition receptors for the induction of cytokine responses against *C. burnetii* in humans. Furthermore, an interesting finding was the divergent recognition of *C. burnetii* Nine Mile and *C. burnetii* 3262.

Introduction

Coxiella burnetii is the causative agent of Q fever which can lead to either acute or chronic infection in humans. Acute Q fever is associated with inflammation, hepatitis and pneumonia. Chronic Q fever, defined as a cardiovascular chronic infection, develops over several months to years following an initial infection and is characterized primarily by endocarditis or endovascular infection[1]. Even though only a small proportion (2-5%) of the infected individuals develops chronic Q fever, the burden for patients is significant, as long-term antimicrobial drug treatment is necessary and mortality can reach to more than 60% when left untreated[1, 2]. In the Netherlands, one specific *C. burnetii* genotype, referred to as *C. burnetii* 3262, dominated the Q fever outbreak, which resulted in several thousands of cases and more than 250 chronically infected patients[3].

The innate immune system provides the first line of host defense against micro-organisms. Unique pathogen associated molecular patterns (PAMPs) on the surfaces of micro-organisms are recognized by the pattern recognition receptors (PRRs) of the host immune cells in order to activate host defense mechanisms such as phagocytosis and cytokine signaling[4]. The main PRRs involved in recognition of bacteria are Toll-like receptors (TLRs) (TLR1, TLR2, TLR4 and TLR6) and the Nucleo-tide-binding oligomerization domain (NOD) receptors NOD1 and NOD2. Previous studies in mice and reporter cell lines have demonstrated TLR2 as an important regulator of immune responses against *C. burnetii*[5-7]. The TLR2 receptor is able to form heterodimers with either TLR1 or TLR6; TLR1/TLR2 heterodimers mainly recognize triacylated lipopeptides, whereas TLR2/TLR6 heterodimers recognize diacylated lipopeptides[8, 9]. Polymorphisms in TLR1 and TLR6 have been associated with increased susceptibility to candidemia, invasive aspergillosis, impaired mycobacterial signaling and innate immune responses in sepsis[10-13]. NOD1 and NOD2 recognize bacterial peptidoglycan structures, respectively g-D-glutamyl-meso-diaminopimelic acid (iE-DAP) and muramyl dipeptide (MDP)[14]. They are associated with the recognition of a broad range of bacteria and interact with TLR2 through the Receptor-Interacting Protein (RIP)-Like Interacting Caspase-Like Apoptosis Regulatory Protein Kinase (RICK) as the central signaling molecule[15].

An unanswered question is how *C. burnetii* is initially recognized by the immune system in humans, and how *C. burnetii* evades these mechanisms leading to acute and chronic Q fever in patients. Previously, it has been shown that the defective immune response that accompanies chronic Q fever is associated with the failure of *C. burnetii* to induce lymphocyte proliferation, the absence of granulomas and cytokine dysfunction[1, 16]. However, our group recently showed that chronic Q fever patients have high T-cell derived IFN-γ production upon contact with *C. burnetii*[17]. This finding may indicate that a defective innate immune recognition of *C. burnetii* and subsequent induction of appropriate cytokines are important for the pathogenesis of chronic Q fever and may not be due to the failure to activate lymphocytes. To investigate this hypothesis, more fundamental knowledge is needed on the PRRs involved in sensing *C. burnetii*. Therefore, this study investigates the role of TLR1, TLR2, TLR4, TLR6, NOD1, NOD2 and the downstream MAP-Kinases p38, JNK and ERK in the recognition of two *C. burnetii* phase I strains, the reference strain *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262. The study will provide a better insight in the recognition of *C. burnetii* by PRRs in general and could be used in further investigations on immunological causes of chronic Q fever infection.

Material and Methods

Coxiella burnetii strains

Two *C. burnetii* strains were used: *C. burnetii* Nine Mile RSA493 (NM) phase I (a kind gift from the Bundeswehr Institute for Microbiology (Munich, Germany)) and *C. burnetii* X09003262 (3262) phase I, isolated from the placenta of an aborted goat in the Netherlands[3]. Both strains were cultured on Buffalo Green Monkey cells and the numbers of Coxiella DNA copies was determined using Taqman real-time PCR as described previously[18]. LPS phase determination was performed by SDS-PAGE and silver staining, using purified phase I (RSA493) and phase II (RSA439) *C. burnetii* NM LPS (kindly provided by R. Toman) as controls[19, 20]. Both *C. burnetii* strains were inactivated by heating for 30 minutes at 99°C.

Study population

PBMCs were derived from buffy coats of blood donors (Sanquin, the Netherlands) and from whole blood of volunteers (SNP analyses). All were healthy Dutch volunteers of European descent. Donor blood was not serologically screened for *C. burnetii* infection and the individuals were not immunized[21]. In the SNP experiment with *C. burnetii* 3262, cells were isolated from n=85 volunteers (23-73 years old, 23% females and 77% males). In the experiment with *C. burnetii* NM, cells were isolated from n=123 volunteers (22-70 years old, 16% females and 84% males). The NOD2 deficient individuals bear the homozygous NOD2 3020insC polymorphism. The 3020insC polymorphism was analyzed as described previously[22]. Venous blood was drawn after informed consent was obtained. The experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Isolation of human peripheral blood mononuclear cells and stimulation

Peripheral blood mononuclear cells (PBMCs) from healthy and NOD2 deficient individuals were isolated as described previously[23]. A volume of 100 µl containing $5x10^5$ cells was added to a round-bottom 96-well plate (Corning, The Netherlands) and incubated with *C. burnetii* NM or *C. burnetii* 3262 (ranging from $1x10^6$- $1x10^7$/ml) in the presence of 10% human serum. After 24 hours, supernatants were harvested and stored at -20°C. In some experiments, PBMCs were pre-incubated for 60 minutes with antagonizing or inhibiting agents. A monoclonal antibody for blocking TLR2 (clone TL2.1) and mouse IgG2a isotype were used at a concentration of 5µg/ml (InvivoGen, France). Highly purified *Bartonella quintana* LPS (100ng/ml) was used as a TLR4 antagonist[24]. P38 was inhibited using 1mMol/L p38 inhibitor SB202190 (Sigma-Aldrich, the Netherlands)[25]. JNK and ERK1/2 were inhibited by respectively 20µMol/L JNK SP600125 (AG Scientific, USA) and 10µMol/L MEK U0126 (Promega, USA).

Cytokine measurement

Cytokine production was measured using enzyme-linked immunosorbent assay (ELISA). The following kits were used: interleukin 6 (IL-6) (Sanquin, the Netherlands), tumor necrosis factor-α (TNF-α), IL-1β, (R&D Systems, USA) and mouse IL-6 (Invitrogen, USA). Absorption was measured at 450 nm (Bio-Rad Laboratories, the Netherlands).

Genomic DNA isolation and single nucleotide polymorphism analysis

DNA was isolated using the Gentra Pure Gene Blood kit (Qiagen, USA) according to the manufacturer's protocol for whole blood. Polymorphisms were identified using a pre-designed TaqMan® SNP genotyping assay (Applied Biosystems, USA) or Illumina Immunochip. The following SNPs were analyzed: *TLR1* (rs5743611, rs4833095, rs5743618), *TLR2* (rs5743704), *TLR4* D299G/T399I (rs4986790/ rs4986791), *TLR6* P249S (rs5743810), *NOD2* (rs2066842, rs5743293, rs2066845, rs2066844, rs9302752, rs7194886, rs8057341, rs751271, rs3135499) and *NOD1* insertion/deletion polymorphism *NOD1* +32656 (Table 1).

Animals

TLR1, *TLR2* and *TLR6* knockout mice were kindly provided by Prof. S. Akira (Department of Host Defense, Osaka University, Osaka, Japan) and are fully backcrossed to C57BL/6 background. Age and gender matched control C57BL/6 mice were obtained from Charles River Wiga (Sulzfeld, Germany). Mice were housed in filter-top cages, and water and food were provided *ad libitum*. Wild type, *NOD1*, *NOD2* and *NOD1/2* knockout mice were bred and maintained in the St. Jude Children's Research Hospital, Memphis, TN, USA. Bone marrow derived macrophages (BMDMs) were used in all experiments. After dissecting of the mouse legs, the bone marrow was flushed out using sterile PBS. Differentiation into macrophages occurred in 7 days at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12 (Gibco, Invitrogen, USA) supplemented with 30% L929 medium, 10% heat-inactivated filtered foetal bovine serum (Invitrogen, USA), 1% non essential amino acids and 1% 100 U/mL penicillin and 100 mg/mL streptomycin. At day 6, the BMDMs were counted and added to either a 24-wells plate in a concentration of $1x10^6$ cells/ml or a 96-wells plate in a concentration of $1x10^5$ cells/ml. After 24h of resting at 37°C, the cells were stimulated with medium, *C. burnetii* NM $1x10^6$/ml, *C. burnetii* 3262 $1x10^6$-$1x10^7$/ml, Pam3Cys 10µg/ml and *E. coli* LPS 10µg/ml.
The experiments were approved by the Ethics Committee on Animal Experiments of the Radboud University Medical Center, and protocols were approved by the St. Jude Children's Research Hospital's Committee on the Use and Care of Animals.

Statistical analysis

The data are expressed as mean ± SEM unless indicated otherwise. Differences between experimental groups were tested using the Mann-Whitney *U* test. In experiments using antagonists or inhibitors, the Wilcoxon matched-pairs signed rank test was used. GraphPad Prism 5 software was used. Differences with a p-value <0.05 were considered statistically significant.

Table 1: Single-Nucleotide Polymorphisms (SNPs) Used in the Study

Gene	SNP ID	Mutation	Nucleotide change*	AA Change	TaqMan Assay ID
TLR1	rs4833095	Missense	C > T	S248N	C__44103606_10
TLR1	rs5743611	Missense	G > C	R80T	C__27855269_10
TLR1	rs5743618		G > T	S602I	Hs00248869_CE
TLR2	rs5743704	Missense	C > A	P631H	C__25607736_10
TLR4	rs4986790	Missense	A > G	D299G	C__11722238_20
TLR4	rs4986791	Missense	C > T	T399I	C__11722237_20
TLR6	rs5743810	Missense	C > T	P249S	C___1180648_20
NOD1	rs6958571#			+32656	
NOD2	rs2066842	Missense	C > T	P268S	C__11717470_20
NOD2	rs2066847	Frameshift		1007finsC	
NOD2	rs2066845	Missense	C > G	G908R	C__11717466_20
NOD2	rs2066844	Missense	C > T	R702W	C__11717468_20
NOD2	rs9302752		C > T		Genotyped using the Illumina Immunochip platform, as described by Smeekens *et al.* [39].
NOD2	rs7194886		C > T		
NOD2	rs8057341		A > G		
NOD2	rs751271		G > T		
NOD2	rs3135499		A > C		

* The first nucleotide (and corresponding AA) is the ancestral nucleotide and therefore is considered the wild-type allele.

\# This is the SNP Database reference for wa t/g single base pair substitution at the same position without the accompanying insertion/deletion.

Results

Role for TLR2, but not TLR4, in *C. burnetii* induced cytokine response in humans

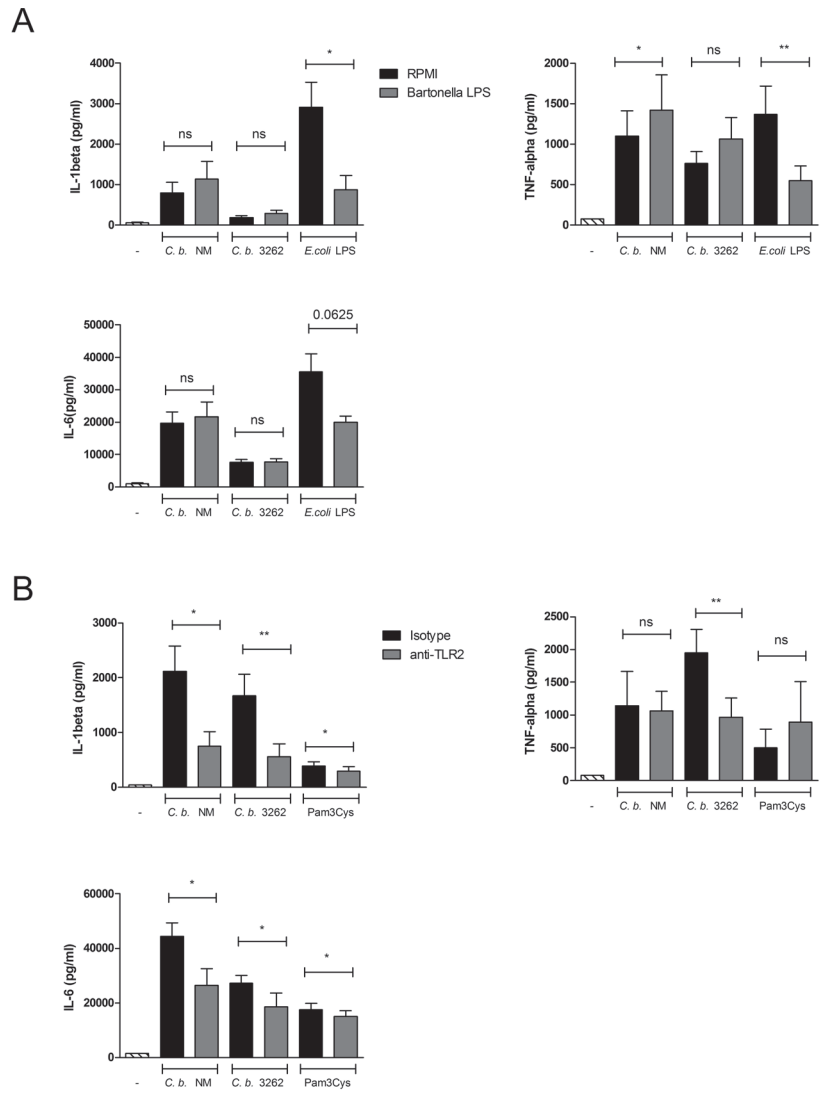
TLR4 is an important receptor for lipopolysaccharide (LPS). As *C. burnetii* phase I possesses tetracyclated LPS[26], it can be hypothesized that TLR4 could play a role in cytokine production by *C. burnetii*. Upon TLR4 blockade by the TLR4 antagonist *Bartonella* LPS, the IL-1β, IL-6 and TNF-α responses of PBMCs after *E. coli* LPS stimulation are effectively inhibited. However, this potent TLR4 antagonist was unable to inhibit *C. burnetii* NM and *C. burnetii* 3262 induced cytokine responses in PBMCs (Figure 1A).

PBMCs pre-incubated with an antagonistic antibody directed against TLR2 produced less IL-1β and IL-6 after encounter with *C. burnetii* NM and *C. burnetii* 3262 (Figure 1B). TNF-α levels were decreased after stimulation with *C. burnetii* 3262; conversely, this was not observed after stimulation with *C. burnetii* NM and the TLR2 ligand Pam3Cys (Figure 1B). Additionally, the role of TLR4 and TLR2 in *C. burnetii* recognition was investigated using PBMCs from volunteers with or without single nucleotide polymorphisms (SNPs) in these genes. Polymorphisms in TLR4 (D299G and T399I) did not result in decreased cytokine responses (Figure 1C). PBMCs from volunteers homozygous for a SNP in TLR2 (P631H) showed significantly decreased IL-1β responses after stimulation with *C. burnetii* 3262 only (Figure 1D). An explanation can be that the location of the TLR2 SNP is more important for the binding of *C. burnetii* 3262 than for *C. burnetii* NM or Pam3Cys.

Finally, we investigated the role of TLR2 in the cytokine induction by *C. burnetii* in murine BMDMs. BMDMs derived from TLR2 knockout mice had reduced IL-6 production compared to the wild type mice after stimulation with *C. burnetii* NM and *C. burnetii* 3262 (Figure 1E).

Cytokine induction by *C. burnetii* NM stimulation of human PBMCs depends on TLR1, while *C. burnetii* 3262 recognition is mediated by both TLR1 and TLR6

The finding that TLR2 plays an important role in the recognition of *C. burnetii* led to the question whether this response is mainly mediated by TLR1/TLR2 or TLR2/TLR6 heterodimers[27].



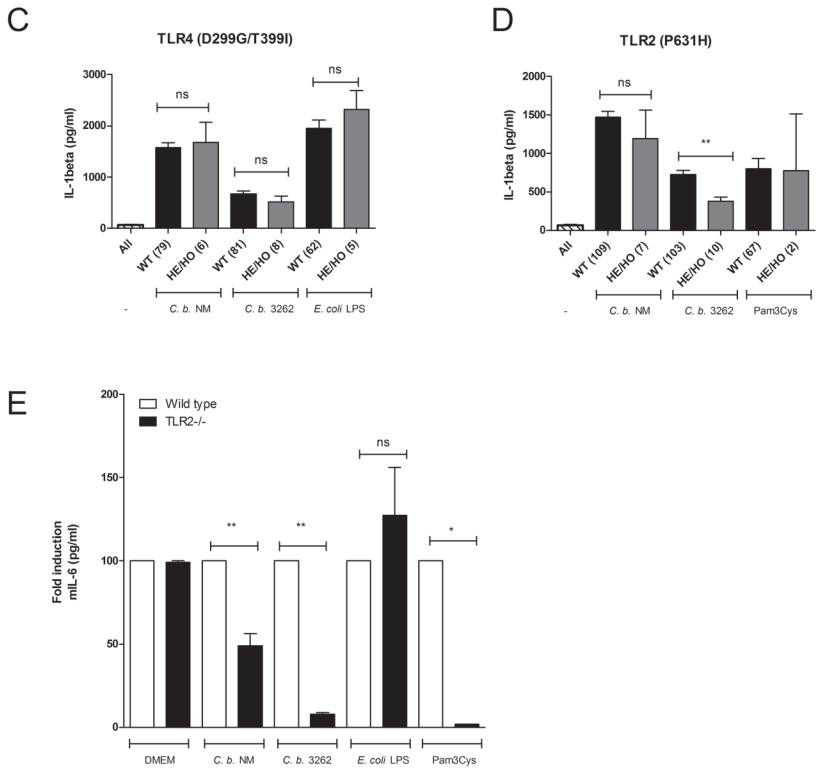


Figure 1: Recognition of *C. burnetii* by TLR2, but not by TLR4, in human and mice

1A/B) PBMCs were pre-treated with (Figure 1A) medium or *Bartonella* LPS 100µg/ml or (Figure 1B) isotype control IgG2B or anti-TLR2 for one hour and subsequently stimulated with RPMI (striped bar), *C. burnetii* NM 1x10^6/ml, *C. burnetii* 3262 1x10^6/ml, *E. coli* LPS 10ng/ml or Pam3Cys 10µg/ml in the presence of serum, n=5-9. Mean values ± SEM are shown, *p<0.05, ** p<0.01 Wilcoxon matched-pairs signed rank test two-tailed.

1C-D) PBMCs of healthy individuals genotyped for the TLR4 D299G/T399I SNP (Figure 1C) and TLR2 P631H (Figure 1D) were stimulated with RPMI (striped bar), *C. burnetii* NM 3x10^6/ml and *C. burnetii* 3262 3x10^6/ml in the presence of serum. Data are mean values with SEM *p<0.05, ** p<0.01 Mann-Whitney *U* test. IL-1β, TNF-α and IL-6 were measured in the supernatant by ELISA after 24h incubation.

1E) Bone marrow derived macrophages from wild type and TLR2 knockout mice were stimulated with DMEM, *C. burnetii* NM 1x10^6/ml, *C. burnetii* 3262 1x10^6/ml, Pam3Cys 10µg/ml and *E. coli* LPS 10µg/ml. mIL-6 was measured after 24h incubation using ELISA. Data are combined results of 2 separate experiments containing three mice in each group (Pam3Cys stimulation one experiment only). The mIL-6 production of the wild type mice is set on 100%.

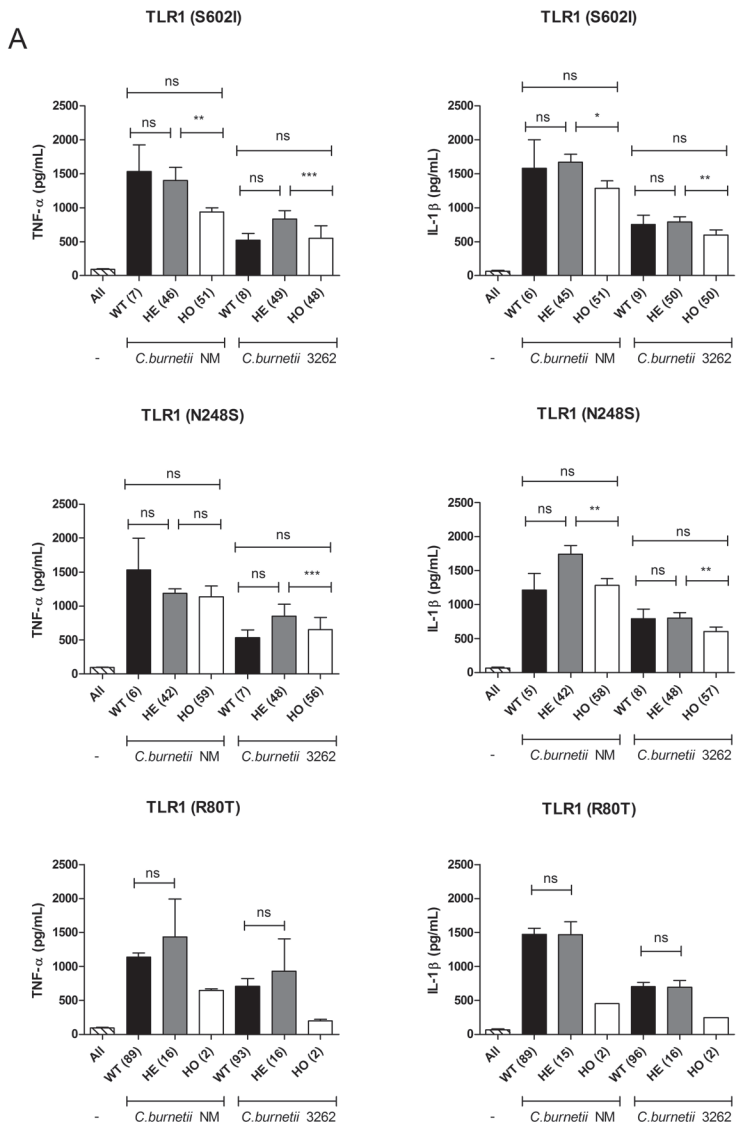
PBMCs of individuals bearing the homozygous mutant variant of TLR1 (S609I) and TLR1 (N248S) produced significantly less IL-1β and TNF-α after stimulation with *C. burnetii* NM and *C. burnetii* 3262 than individuals who are heterozygous for these particular SNPs or bear the wild type variant (Figure 2A). The same trend was observed for the TLR1 (R80T) SNP, of which only a few individuals in the cohort carry the homozygous variant. Decreased cytokine responses were also observed in these individuals after stimulation with the purified TLR2 ligand Pam3Cys (data not shown). Among the individuals carrying the TLR6 (P249S) SNP, the cytokine responses induced by the two *C. burnetii* strains differed from each other. *C. burnetii* NM stimulation did not result in decreased IL-1β and TNF-α production, while stimulation with *C. burnetii* 3262 revealed a significantly lower IL-1β and TNF-α production compared with individuals who are heterozygous or bear the wild type variant (Figure 2B). However, stimulation with fibroblast stimulating ligand-1 (FSL-1), a TLR2/6 agonist, did not result in lowered cytokine responses (data not shown).

In mice, BMDMs derived from TLR1 knockout mice produced less IL-6 after stimulation with *C. burnetii* NM, while this was not observed after *C. burnetii* 3262 stimulation. On the other hand, IL-6 production of BMDMs from TLR6 knockout mice was strongly diminished after stimulation with both *C. burnetii* NM and *C. burnetii* 3262 (Figure 2C).

The role of NOD2 in *C. burnetii* induced cytokine response

NLRs are involved in initiating an immune response against micro-organisms through recognition of bacterial peptidoglycan. As shown in Figure 3A, PBMCs of individuals heterozygous and homozygous for the NOD1 insertion-deletion +32656 polymorphism display similar IL-1β and TNF-α production after *C. burnetii* NM and *C. burnetii* 3262 stimulation as individuals bearing the wild type genotype.

In contrast, PBMCs from NOD2 deficient individuals, produced significantly less IL-1β and IL-6 than PBMCs from healthy controls after *C. burnetii* 3262 stimulation (Figure 3B). The same trend was observed after *C. burnetii* NM stimulation (Figure 3B) and for TNF-α production (not shown). The effect of nine NOD2 SNPs was further analyzed. Stimulation with *C. burnetii* NM resulted in decreased, yet not significant, TNF-α production in six out of these nine analyzed NOD2 SNPs, while four of these SNPs modulated stimulation with *C. burnetii* 3262, resulting in



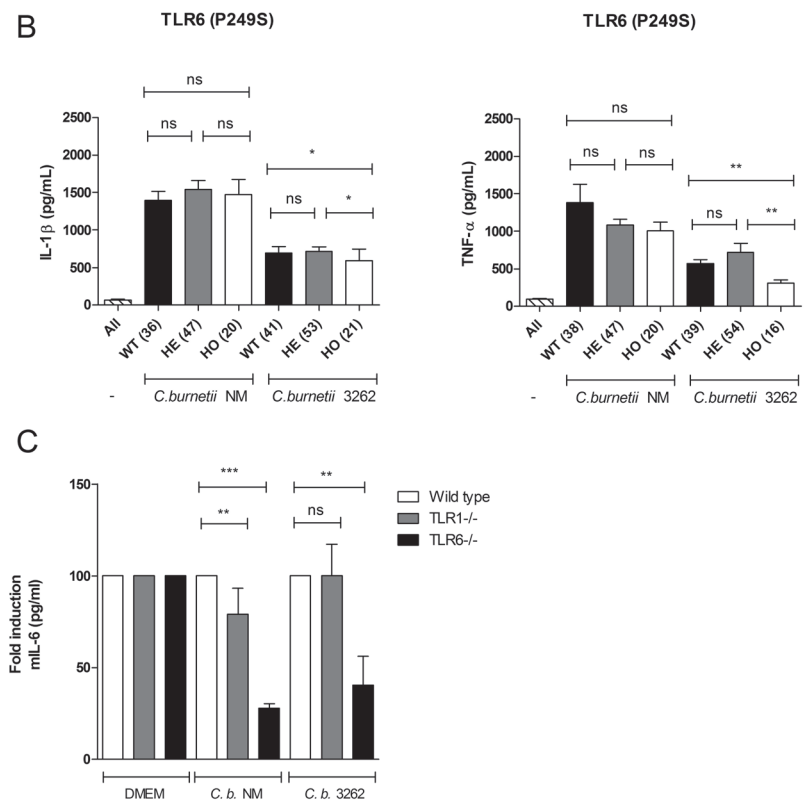
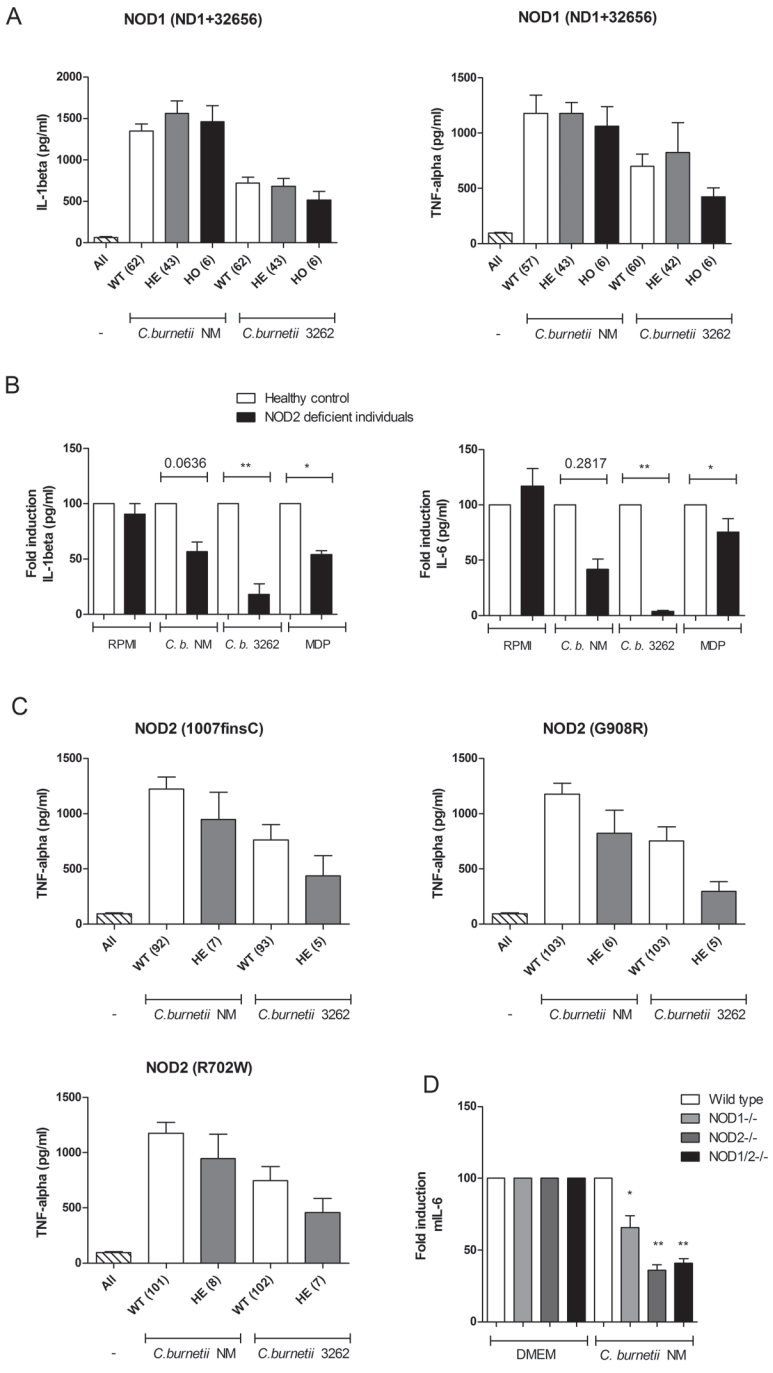


Figure 2: Recognition of *C. burnetii* by TLR1 and TLR6 in human and mice

2A-B) PBMCs of healthy individuals genotyped for the TLR1 S602I, TLR1 N248S and TLR1 R80T SNP (Figure 2A) and TLR6 P249S SNP (Figure 2B) were stimulated with RPMI (striped bar), *C. burnetii* NM $3x10^6$/ml and *C. burnetii* 3262 $3x10^6$/ml in the presence of serum. IL-1β and TNF-α were measured in the supernatant by ELISA after 24h incubation. *p<0.05, ** p<0.01, *** p<0.001 Mann-Whitney *U* test.

2C) Bone marrow derived macrophages of wild type, TLR1 knockout and TLR6 knockout mice were stimulated with medium only, *C. burnetii* Nine Mile $1x10^6$/ml and *C. burnetii* 3262 $1x10^6$-$1x10^7$/ml. After 24h stimulation, mouse IL-6 was measured by ELISA. White bars represent the wild type mice, grey bars represent the TLR1 knockout mice and the black bars represent the TLR6 knockout mice. Experiments were performed in duplicates (3-4 mice in each group), mean± SEM are depicted. The IL-6 production of the wild type mice is set on 100%. *p<0.05, ** p<0.01, Mann-Whitney *U* test.



lower TNF-α and IL-1β production. Figure 3C shows these TNF-α responses of the NOD2 1007finsC, NOD2 G908R and NOD2 R702W SNPs after *C. burnetii* NM and *C. burnetii* 3262 stimulation.

In mice, *C. burnetii* NM stimulation of BMDMs led to a 35% reduction of IL-6 production in NOD1 knockout mice compared to wild type, while in NOD2 knockout and double knockouts the IL-6 production was reduced more than 50% (Figure 3D). Unfortunately, stimulation of the BMDMs with *C. burnetii* 3262 resulted in low production of IL-6 and was not useful to compare the different groups (data not shown).

Figure 3: NOD2 is involved in the recognition of *C. burnetii,* while NOD1 is not

3A) PBMCs of healthy volunteers genotyped for the NOD1 SNP ND1 +32656 were stimulated with RPMI (striped bar), *C. burnetii* NM 3x10^6/ml and *C. burnetii* 3262 3x10^6/ml.

3B) PBMCs of individuals with a homozygous NOD2 3020insC mutation and PBMCs of healthy controls were stimulated with RPMI, *C. burnetii* NM 1-2x10^6/ml, *C. burnetii* 3262 1-2x10^6/ml and muramyl dipeptide (MDP) 5μg/ml. The IL-1β and IL-6 production of the healthy controls is set on 100%.

3C) PBMCs of healthy volunteers genotyped for the NOD2 SNPs 1007finsC, NOD2 G908R and NOD2 R702W were stimulated with RPMI (striped bar) *C. burnetii* NM 3x10^6/ml and *C. burnetii* 3262 3x10^6/ml. In all experiments, PBMCs were stimulated in the presence of human serum. IL-1β, IL-6 and TNF-α were measured in the supernatant by ELISA after 24h incubation. * p<0.05, ** p<0.01, Mann-Whitney *U* test.

3D) Bone marrow derived macrophages of wild type, NOD1 knockout, NOD2 knockout and NOD1/2 knockout mice were stimulated with medium only and *C. burnetii* Nine Mile 1x10^7/ml. After 24h stimulation, mouse IL-6 was measured by ELISA. White bars represent the wild type mice, light grey bars represent the NOD1 knockout mice, dark grey represent the NOD2 knockout mice and the black bars represent the NOD1/2 knockout mice. Experiments were performed in duplicates (n=2-3), mean± SEM are depicted. The IL-6 production of the wild type mice is set on 100%. *p<0.05, ** p<0.01, Mann-Whitney *U* test.

P38, JNK and ERK are downstream mediators of *C. burnetii*-induced cytokine production

MAPKs like p38, JNK and ERK have been proven to be downstream of TLRs and their activation eventually lead to cytokine production, proliferation, and apoptosis[28].

Here we confirm this function of MAPKs upon *C. burnetii* stimulation as inhibition of p38 and ERK led to significant decreased IL-1β, TNF-α and IL-6 production in PBMCs (Figure 4). PBMCs pre-incubated with a specific JNK inhibitor also produced less IL-1β and TNF-α after *C. burnetii* stimulation, however IL-6 production was not altered in comparison to the DMSO control (Figure 4).

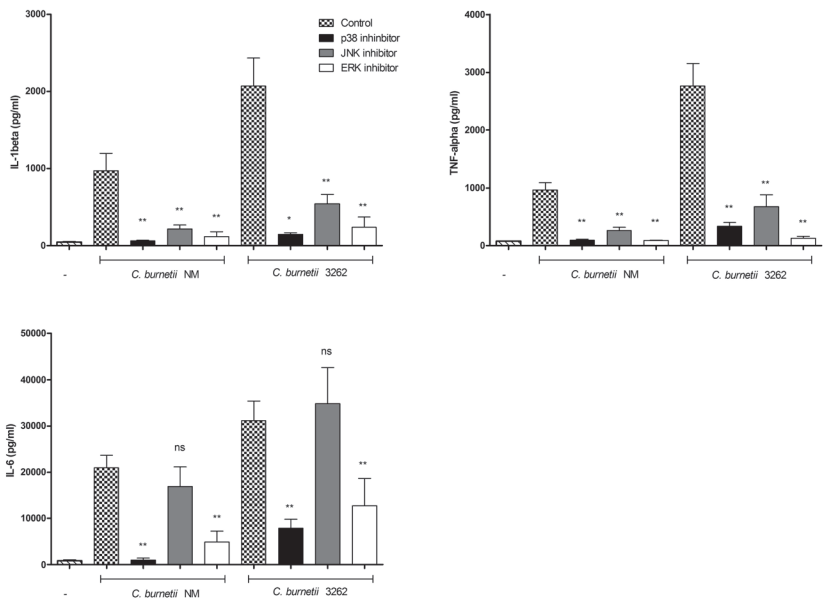


Figure 4: Involvement of MAPKs in *C. burnetii*-induced cytokine production

PBMCs of eight healthy volunteers were pre-treated with DMSO (control), p38 inhibitor, JNK inhibitor or ERK inhibitor for one hour and subsequently stimulated with RPMI (striped bar), *C. burnetii* NM 1x10^6/ml and *C. burnetii* 3262 1x10^6/ml, in the presence of human serum. IL-1β, TNF-α and IL-6 were measured in the supernatant by ELISA after 24h incubation. Mean values ± SEM of three separate experiments are shown. *p<0.05, ** p<0.01, Wilcoxon matched-pairs signed rank test two-tailed.

Discussion

In the present study we investigated the role of TLRs and NLRs in the recognition of *C. burnetii*. Using different approaches, we found clear evidence for involvement of TLR1, TLR2 and NOD2 but not for TLR4 and NOD1. An additional important finding was the divergent recognition of *C. burnetii* NM and the Dutch outbreak isolate *C. burnetii* 3262. *C. burnetii* NM was mediated by TLR1/TLR2 only, while *C. burnetii* 3262 was recognized by both the TLR1/TLR2 and TLR2/TLR6 heterodimers. Interestingly, recognition of these two strains also differed with respect to the role of TLR6 in men and mice.

Our results revealed no important role of TLR4 in mediating in-vitro cytokine production by human PBMCs after *C. burnetii* encounter. Although Honstrette *et al.*[29] reported that TLR4 knockout mice exhibited a defect in cytokines production and granuloma formation, our observation that *C. burnetii*-induced cytokine production in humans is not TLR4 mediated is not surprising. As reported before, LPS with a tetra-acylated lipid A component as expressed by bacteria such as *Rhodobacter sphaeroides* or the lpxL2 mutant of *Neisseria meningitidis* H44/76, is found to be a very weak cytokine inducer and even act as TLR4 antagonists[30, 31]. Accordingly, an antagonistic effect of *C. burnetii* phase I lipid A has been demonstrated[7].

We observed an important role of TLR2 in *C. burnetii* recognition in mice and, moreover, our study revealed that TLR2 is also important for *C. burnetii* recognition by human PBMCs. *C. burnetii* NM and *C. burnetii* 3262 are both able to induce cytokine production via TLR2 signaling, which suggests that both strains comprise PAMPs which can activate TLR2. *C. burnetii* phase II activation of TLR2 has previously been reported by Zamboni *et al.*[7]. They showed that macrophages from TLR2 knockout mice were highly permissive to *C. burnetii* growth compared to either TLR4 knockout or wild type mice. Furthermore, they found that macrophages from TLR2 knockout mice produced lower levels of TNF-α and IL-12[7]. While Zamboni *et al.* used the avirulent *C. burnetii* phase II, we used the virulent *C. burnetii* phase I which is the infective phase that is found in the environment.

We additionally investigated the role of TLR1 and TLR6 in cytokine induction by *C. burnetii*, because TLR2 forms heterodimers with either TLR1 or TLR6. We addressed the issue by performing experiments with PBMCs from individuals with known SNPs in TLR1 and TLR6. We found that individuals carrying polymorphisms in TLR1 showed a diminished cytokine response against *C. burnetii* NM and *C. burnetii* 3262. Our findings suggest that the TLR1/TLR2 heterodimer plays a role in *C. burnetii* recognition. BMDMs of TLR1 knockout mice stimulated with *C. burnetii* NM have a reduced IL-6 production compared to wild type mice. Conversely, stimulation with *C. burnetii* 3262 did not lead to lower cytokine production in TLR1 knockout BMDMs.

The role of TLR6 in the induction of a cytokine response against *C. burnetii* is less clear. Our results in human PBMCs suggest that *C. burnetii* 3262 is recognized by TLR6, while this is not the case for *C. burnetii* NM. This can be explained by a different binding site of *C. burnetii* 3262 to TLR6 compared to *C. burnetii* NM and FSL-1. In mice, it seemed that TLR6 plays a major role in the cytokine induction by *C. burnetii*, for both the reference strain as well as the Dutch outbreak isolate. We observed a different role for TLR1 and TLR6 in mice and humans in our experiments, showing that innate immune recognition of *C. burnetii* can differ between mice and men. However, much of the so far published data on innate immune recognition of *C. burnetii* is derived from murine experiments. We can conclude that assumptions on immune recognition of *C. burnetii* in humans based on murine studies alone, should be made carefully[32, 33].

The shared intracellular localisation of the NOD2 receptor and *C. burnetii* together with the confirmed role of TLR2 in the recognition of *C. burnetii* made the investigation of NOD2 of interest. Our study in humans revealed that NOD2 plays a major role in cytokine induction upon *C. burnetii* encounter, as individuals bearing the NOD2 3020insC mutation (causing a loss of function of the NOD2 gene[22]) have significant lower cytokine responses. This finding was further strengthened by our observation in individuals bearing SNPs in NOD2. The important role for both TLR2 and NOD2 signaling in the induction of cytokines has been demonstrated for other bacteria, for example *B. burgdorferi* and *Mycobacterium paratuberculosis*[23, 34]. So far, only one other research group has investigated the role of NOD2 in *C. burnetii* infection[35]. They concluded that NOD2 is not essential for the control of *C. burnetii* infection in mice, although they observed lower TNF-α and MCP-1 mRNA responses in *C. burnetii* stimulated BMDMs of NOD2 knockout mice compared to wild type mice. Our results showed lower IL-6 responses in *C. burnetii* stimulated BMDMs of NOD1 knockout mice compared to wild type mice, but even a larger decrease was observed in NOD2 knockout mice. As the IL-6 response was not further decreased in the NOD1/2 knockout mice, we suggest that the role of NOD2 is more important.

The strain *C. burnetii* 3262 was the predominant genotype found during the Q fever outbreak in the Netherlands. This single genotype of *C. burnetii* caused the largest outbreak of Q fever ever reported world-wide. Besides increased exposition of humans to this pathogen due to intensive goat-herding, it may be hypothesized that this strain is less effectively recognized by the human immune system and is therefore more virulent than other strains. However, our study showed that, similar to *C. burnetii* NM, *C. burnetii* 3262 in humans was recognized by TLR1, TLR2 and NOD2, and induced a robust pro-inflammatory cytokine response.

Interestingly, we observed a possible role for TLR6 in cytokine induction by *C. burnetii* 3262, while this was not seen for *C. burnetii* NM. It can be questioned whether extended activation of the immune response is beneficial for the infected individual, as enhanced uptake of *C. burnetii* by monocytes and macrophages can favor the intracellular replication of *C. burnetii*, leading to a possible infection. However, it has to be further investigated whether the recognition of *C. burnetii* 3262 by TLR6 can lead to increased virulence.

It is not completely clear why chronic Q fever only develops in a small proportion of infected individuals. Although patient-related factors such as the presence of valvular pathology or atherosclerotic vascular disease are a contributing factor, polymorphisms in PRR genes may also predispose to the development of chronic Q fever. Studies performed in our group show associations between polymorphisms in TLR1, TLR2 and TLR6 with increased susceptibility to complicated skin and skin structure infections[36]. In addition, others observed associations concerning the TLR6 SNP with malaria and invasive aspergillosis and overrepresentation of TLR2 in tuberculosis patients[13, 37, 38]. Therefore, it would be interesting for future studies to investigate whether polymorphisms in TLR1, TLR2, TLR6 and NOD2 are retrieved more often in chronic Q fever patients, and if they can be related to disease severity after *C. burnetii* infection or to specific *C. burnetii* strains.

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04

Genetic variation in TLR10 is not associated with chronic Q fever, despite the inhibitory effect of TLR10 on *Coxiella burnetii*-induced cytokines *in vitro*

CYTOKINE

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Abstract

Coxiella burnetii, the causative agent of Q fever, is recognized by TLR2. TLR10 can act as an inhibitory receptor on TLR2-derived immune responses. Therefore, we investigated the role of TLR10 on *C. burnetii*-induced cytokine production and assessed whether genetic polymorphisms in *TLR10* influences the development of chronic Q fever. HEK293 cells, transfected with TLR2, TLR10 or TLR2/TLR10, and human peripheral blood mononuclear cells (PBMCs) in the presence of anti-TLR10, were stimulated with *C. burnetii*. In both assays, the absence of TLR10 resulted in increased cytokine responses after *C. burnetii* stimulation. In addition, the effect of single nucleotide polymorphisms (SNPs) in *TLR10* was examined in healthy volunteers whose PBMCs were stimulated with *C. burnetii* Nine Mile or the Dutch outbreak isolate *C. burnetii* 3262. Individuals bearing SNPs in *TLR10* displayed increased cytokine production upon *C. burnetii* 3262 stimulation. Furthermore, 139 chronic Q fever patients and 220 controls were genotyped for *TLR10* N241H, I775V and I369L. None of these polymorphisms were associated with increased susceptibility to chronic Q fever. In conclusion, TLR10 has an inhibitory effect on *in vitro* cytokine production by *C. burnetii*, but the presence of *TLR10* polymorphisms does not lead to an increased risk of developing chronic Q fever.

Introduction

Coxiella burnetii is a Gram-negative intracellular bacterium that can cause either acute and/or chronic Q fever infection in humans. Whereas the majority of infected individuals develops no symptoms, approximately 40% of the infected individuals presents with acute symptoms, usually pneumonia or hepatitis. Several months to years following initial infection, chronic Q fever develops in 1-5% of the individuals, which is characterized primarily by endocarditis or endovascular infection[1]. Despite intensive research in the past years, the underlying cause of why only a minority of the individuals with an initial infection develops chronic Q fever remains unknown. A possible explanation is insufficient early recognition of the bacterium by the innate immune system, which can lead to a deficient elimination of *C. burnetii* and a reduced initiation of adaptive immune responses.

Recognition of invading micro-organisms by the host immune cells starts with the activation of the innate immune system. Pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) expressed among others on the surface of micro-organisms. These PRRs comprise among others the Toll-like receptor (TLR) family, which consists of ten members in humans, and are able to recognize a large panel of ligands. In a recent study we examined the role of TLR1, TLR2 and TLR6 in the recognition of *C. burnetii*. We observed that TLR2 plays an essential role and revealed that both *C. burnetii* Nine Mile (NM) and the Dutch outbreak isolate *C. burnetii* 3262 are recognized by the TLR1/TLR2 heterodimers, while TLR2/TLR6 was also involved in recognizing *C. burnetii* 3262[2].
TLR10 was discovered in 2001 by Chuang *et al.* revealing large homology with TLR1 and TLR6, sharing respectively 50% and 49% of the overall amino acid sequence[3]. For a long time TLR10 was considered an orphan receptor as the ligand and function was unknown[3]. In 2010, Guan *et al.* reported that TLR10 shares a variety of agonists with TLR1 and, similar to TLR1, requires TLR2 for recognition[4]. Recently, we demonstrated the inhibitory effect of TLR10 on TLR2 derived immune responses[5]. This inhibitory effect of TLR10 on TLR2 derived immune responses and its ability to form heterodimers with TLR2, led us to examine the role of TLR10 in *C. burnetii*-induced cytokine responses and to investigate whether genetic variation in *TLR10* influences the probability to develop chronic Q fever after an initial infection.

Material and methods

Coxiella burnetii

C. burnetii Nine Mile (NM) RSA493 phase I (a kind gift from the Bundeswehr Institute for Microbiology (Munich, Germany)) and *C. burnetii* X09003262 (3262) phase I were used. *C. burnetii* 3262 was isolated from the placenta of a goat that aborted in the Netherlands during the Q fever outbreak[6]. Both strains were cultured on Buffalo Green Monkey cells and the numbers of *C. burnetii* DNA copies was determined using Taqman real-time PCR as described previously[7]. Both *C. burnetii* strains were heat-inactivated for 30 minutes at 99°C.

HEK293 cells

Transfection of HEK293 cells with human *TLR2*, *TLR10* and both *TLR2*/*TLR10* was performed as previously described and confirmed by RT-PCR and flowcytometry[5, 8]. All HEK293 cells were cultured under selection in DMEM F12 medium (Gibco, Life Technologies, USA) supplemented with 100 U/ml Penicillin (Invitrogen, USA), 100 µg/ml Streptomycin (Invitrogen, USA) and 7.5% non-heat inactivated Fetal Bovine Serum (HyClone, USA) at 37°C and 5% CO_2. $1x10^6$ cells were added to a 96-wells plate and stimulated with either medium (DMEM), Pam3Cys 1 µg/ml (EMC Microcollections, Germany), *Escherichia coli* LPS 10ng/ml (Sigma-Aldrich, USA) or *C. burnetii* NM $1x10^7$/ml. After 24h stimulation, the supernatants were collected and IL-8 was measured using enzyme-linked immunosorbent assay (ELISA) (Sanquin, The Netherlands).

Study population

Healthy volunteers

PBMCs were derived from buffy coats of blood donors (Sanquin, the Netherlands). DNA for SNP analysis was derived from whole blood of volunteers. The latter were healthy Dutch volunteers of Western European descent. Donor blood was not screened for *C. burnetii* antibodies and the individuals were not vaccinated for Q fever[9]. In the SNP experiment with *C. burnetii* 3262, cells were isolated from 85 volunteers (23-73 years old, 23% females and 77% males). In the experiment with *C. burnetii* NM, cells were isolated from 123 volunteers (22-70 years old, 16% females and 84% males). Venous blood was drawn after informed consent was obtained.

Patient and control group

Blood was drawn from patients with probable or proven chronic Q fever, as defined by the Dutch consensus guideline on chronic Q fever[10]. The control group consisted of individuals from the same area with valvular or vascular abnormalities predisposing to chronic Q fever, who had serological evidence of exposition to *C. burnetii*, without evidence of chronic Q fever. Demographic and clinical characteristics were retrieved from medical records and summarized in Table 1. A more detailed description of the background and recruitment of the patients and controls is described by Schoffelen *et al.*[11]. The study was approved by the Ethical Committee of Radboud University Medical Center, Nijmegen, The Netherlands. Subjects were enrolled after providing written informed consent. Institutional Review Boards of participating hospitals approved the inclusion of patients and controls in this study. All experiments were conducted according to the principles expressed in the Declaration of Helsinki.

Isolation of human peripheral blood mononuclear cells, stimulation and TLR10 inhibition

PBMCs from healthy individuals were isolated according to standard protocols (Invitrogen, USA) as described previously[12]. $5x10^5$ cells were added to a round-bottom 96-well plate (Corning, The Netherlands) and incubated with medium (RPMI), *C. burnetii* NM or *C. burnetii* 3262 in the presence of 10% human serum. After 24 hours, supernatants were harvested and stored at -20°C. In some experiments, PBMCs were pre-incubated for 60 minutes with neutralizing anti-TLR10 antibody (Abcam, UK, Clone 3C10C5) or mouse IgG1κ monoclonal antibody (Clone 11711, R&D Systems, USA) as isotype control.

Cytokine measurements

Cytokine production was measured in the supernatants using ELISA according to the manufacturer's protocols. The following kits were used: IL-6, IL-8 (Sanquin, the Netherlands), TNF-α, IL-1β, IL-1RA (R&D Systems, USA). Absorption was measured at 450 nm (Bio-Rad Laboratories, the Netherlands).

Genomic DNA isolation and single nucleotide polymorphism analysis

DNA from the healthy volunteers (buffy coats), patients and controls (buffy coats and whole blood) was isolated using the Gentra Pure Gene Blood kit (Qiagen, USA)

according to the manufacturer's protocol. A part of the DNA samples from patients and controls were isolated from buccal swabs, as described by the manufacturer's protocol (Isohelix, UK). DNA isolated from heparin derived whole blood was treated with heparinase I (Sigma-Aldrich, USA) as described previously[13].

Table 1: Baseline characteristics of the chronic Q fever patients and controls

	Chronic Q fever Cohort (n=139)	Control Cohort (n=220)	P-value[a]
Median age, y (IQR)	70.0 (63.0-75.6)	67.6 (58.3-74.3)	0.02
Male sex (%)	114 (82)	169 (77)	0.29
Classification of chronic Q fever			
Proven (%)	92 (66.2)	0	
Probable (%)	47 (33.8)	0	
Cardiovascular risk factor for chronic Q fever[b]			
Vascular aneurysm / prosthesis (%)	95 (68.3)	129 (58.6)	0.07
Valvular defect / prosthesis (%)	39 (28.1)	111 (50.5)	<0.001
Immunocompromised state[c] (%)	21 (15.1)	14 (6.4)	0.01

Abbreviations: IQR, interquartile range

[a] Fisher exact test for categorical variables and Mann-Whitney *U* test for continuous variables.

[b] Both vascular and valvular risk conditions in 9 chronic Q fever patients and 22 controls.

[c] Chronic Q fever patients: Auto-immune disease with immunosuppressive drugs n=14; renal insufficiency n=1; renal transplantation n=1; malignancy n=3; prednis(ol)one use n=1. Controls: Autoimmune disease with immunosuppressive drugs n=5; renal transplantation n=2; malignancy n=4; prednis(ol)on use n=4.

Polymorphisms were identified using a pre-designed TaqMan® SNP genotyping assay (Applied Biosystems, USA). The following SNPs in *TLR10* were analyzed in the healthy control group; N241H (rs11096957), M326T (rs11466653), I775V (rs4129009) and I369L (rs11096955). In the patient and control study, only N241H, I775V and I369L were examined. Details of the SNPs can be found in Table 2.

Statistics

The presence of Hardy-Weinberg equilibrium (HWE) for *TLR10* N241H, I775V and I369L in healthy controls was analyzed using a web-based HWE calculator[14]. The association between susceptibility to chronic Q fever and a SNP was investigated by means of Fisher's exact test and univariate logistic regression models with IBM SPSS 18 software (IBM Corp., USA). SNPs were evaluated using a dominant and recessive model analyses, for which odds ratios (OR) including 95% confidence intervals (CI) were reported. Differences between experimental groups (HEK293 cells and healthy individuals) were tested using the Mann-Whitney *U* test. In experiments using anti-TLR10, the Wilcoxon matched-pairs signed rank test was used. The data are expressed as mean ± SEM unless indicated otherwise. GraphPad Prism 5 software was used. Differences with a *p*-value <0.05 were considered statistically significant.

Table 2: Single nucleotide polymorphisms analyzed in the study

Gene	SNP ID	Mutation	Nucleotide change*	AA Change	TaqMan Assay ID
TLR10	rs11096957	Missense	A > C	N241H	C____309088_10
TLR10	rs4129009	Missense	A > C	I775V	C____309084_10
TLR10	rs11096955	Missense	A > C	I369L	C____309086_10
TLR10	rs11466653	Missense	T > C	M326T	C__25643406_10

Abbreviations: AA, amino acid

* The first nucleotide (and corresponding AA) is the ancestral nucleotide and therefore is considered the wild-type allele

Results

Co-transfection of HEK293 cells with TLR2 and TLR10 decreases IL-8 production after *C. burnetii* stimulation

To investigate the effect of TLR10 on the recognition of *C. burnetii*, HEK293 cells transfected with human *TLR2*, *TLR10* or both *TLR2* and *TLR10* were stimulated with the reference strain *C. burnetii* NM.

Figure 1 shows that stimulation of HEK293 cells expressing human *TLR10* with *C. burnetii* NM 1x10^7/ml resulted in 2-fold (p=0.015) increased IL-8 production compared to stimulated non-transfected HEK293 cells. In contrast, the IL-8 production increased 7-fold (p=0.002) in *C. burnetii* NM stimulated HEK293 cells transfected with human *TLR2*. Co-transfection with both *TLR2* and *TLR10* resulted in significant reduction of IL-8, indicating an inhibitory effect of *TLR10* on *C. burnetii*-induced IL-8 production by HEK293 cells. Similar results were observed after stimulation with the TLR2 ligand Pam3Cys, whereas no differences were observed after stimulation with the TLR4 ligand *E. coli* LPS (Figure 1).

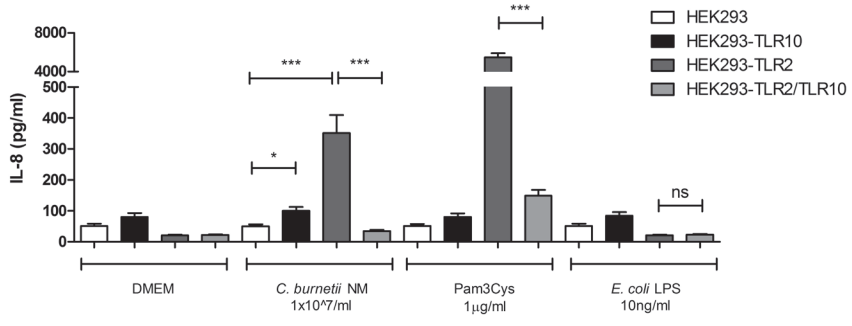


Figure 1: TLR10 inhibits IL-8 production by *Coxiella burnetii*-stimulated HEK293 cells

HEK293 cells and HEK293 cells expressing human TLR2, TLR10 and TLR2/10 were stimulated with medium (DMEM), *C. burnetii* NM 1x107/ml, Pam3Cys 1µg/ml or E. coli LPS 10ng/ml. After 24 incubation, IL-8 was measured in the supernatant by ELISA. Data are mean values with SEM, n=8, * p<0.05, ***p<0.001, Mann-Whitney *U* test.

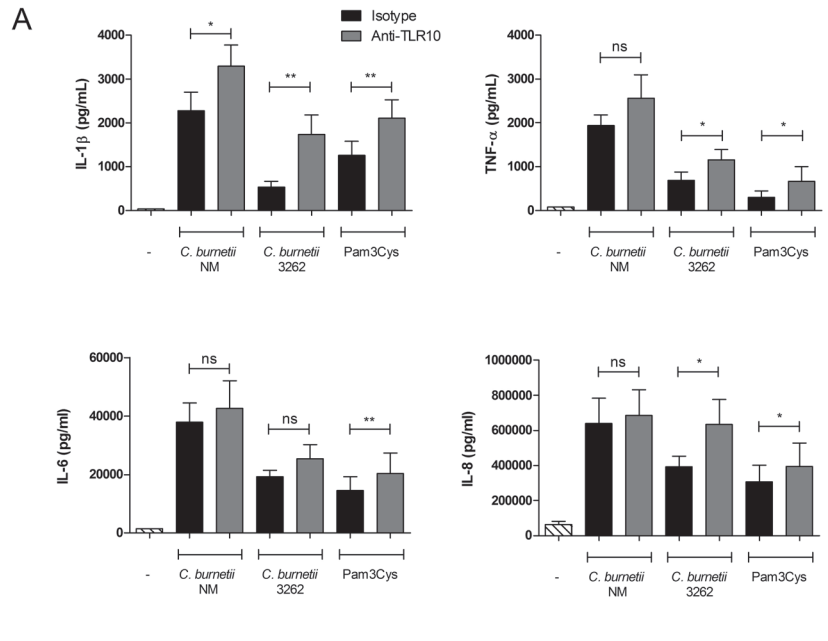


Figure 2: Inhibition of TLR10 in PBMCs leads to increased cytokine responses after *C. burnetii* stimulation

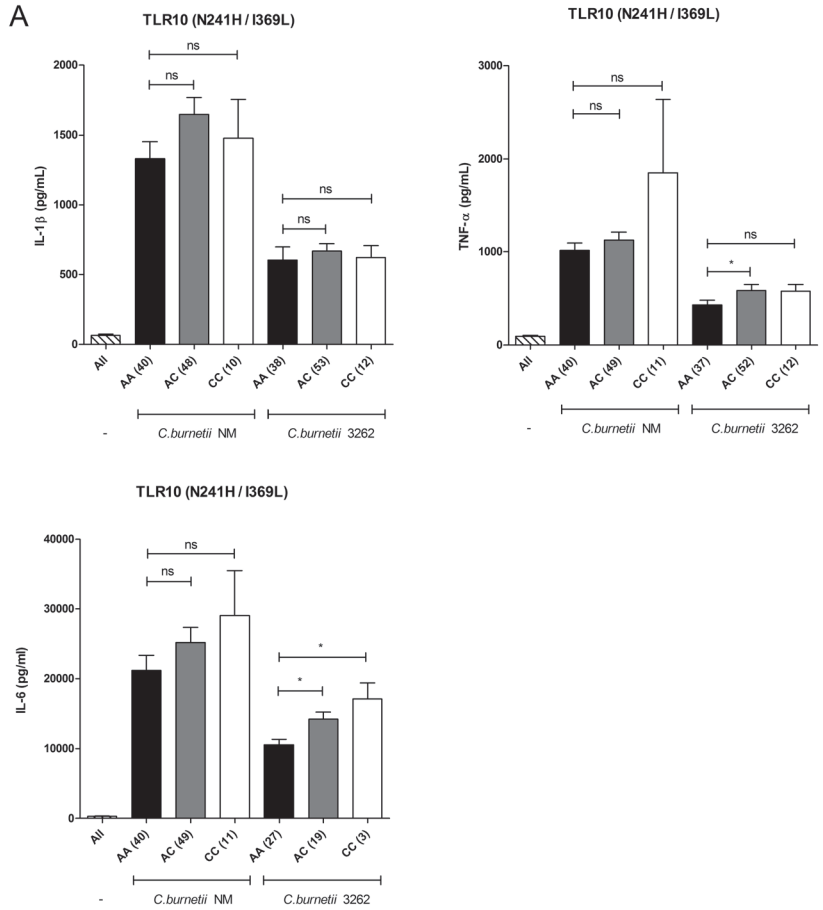
PBMCs of healthy volunteers were incubated with IgG1 isotype control or anti-TLR10 for 1h and subsequently stimulated with either medium control (RPMI, striped bar), *C. burnetii* NM 1x10^6/ml or *C. burnetii* 3262 1x106/ml or Pam3Cys 10µg/ml. After 24h incubation, IL-β, TNF-α, IL-6, IL-8 **(2A)** and IL-1RA **(2B)** were measured in the supernatant by ELISA. Data are presented as mean values with SEM, n=9 from 3 experiments, *p<0.05, ** p<0.01, Mann-Whitney *U* test.

Inhibition of TLR10 leads to increased cytokine responses in *C. burnetii* stimulated human PBMCs

The inhibitory effect of TLR10 on *C. burnetii*-induced cytokine production was further investigated by stimulation of human PBMCs with *C. burnetii* NM and *C. burnetii* 3262 in the presence or absence of TLR10 neutralizing antibodies. When PBMCs were pre-incubated with anti-TLR10 and stimulated with *C. burnetii* 3262, a significantly increased production of IL-1β, TNF-α and IL-8 was observed, and a similar trend was seen for IL-6 (Figure 2A). Stimulation with *C. burnetii* NM resulted in a significantly increased IL-1β production in the presence of anti-TLR10. The same, though not significant, trend was observed for the production of TNF-α, IL-6 and IL-8 (Figure 2A). Oosting *et al.*[5], hypothesized that the increased pro-inflammatory response could be a reduced production of the anti-inflammatory cytokine IL-1RA. Therefore, the IL-1RA production was measured in PBMCs stimulated with both *C. burnetii* strains in the presence of anti-TLR10. Figure 2B shows that, similar to the other cytokines measured, the IL-1RA production is significantly increased when the function of TLR10 is inhibited.

PBMCs from healthy individuals with single nucleotide polymorphisms in *TLR10* produce more cytokines upon *C. burnetii* 3262 stimulation

The effect of genetic variation in *TLR10* on *C. burnetii* induced cytokine production by PBMCs was examined in healthy individuals, genotyped for three polymorphisms in *TLR10*; N241H (rs11096957), I369L (rs11096955), I775V (rs4129009) and M326T (rs11466653). Hereto, their PBMCs were stimulated with *C. burnetii* NM and *C. burnetii* 3262 for 24h. The polymorphisms I369L and N241H were in complete linkage in the cohort of healthy individuals and therefore the results were combined. Stimulation with *C. burnetii* NM resulted in an increased IL-1β, TNF-α and IL-6 production. However no differences in cytokine expression were found between the different genotypes of *TLR10* N241H/I369L (Figure 3A) and I775V (Figure 3B). In contrast, heterozygosity for *TLR10* N241H/I369L and *TLR10* I775V led to increased TNF-α and IL-6 production after stimulation with *C. burnetii* 3262 (Figure 3A and 3B). PBMCs derived from individuals heterozygous for the *TLR10* M326T polymorphism did not display higher cytokine levels upon stimulation with either *C. burnetii* NM or *C. burnetii* 3262 (data not shown).



B

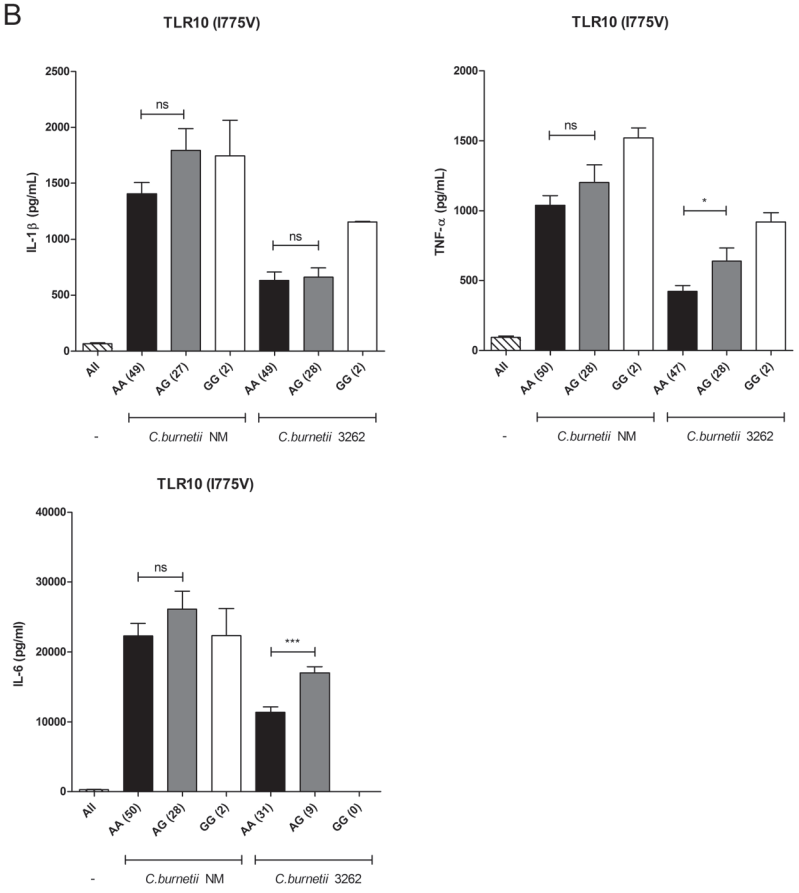


Figure 3: SNPs in *TLR10* lead to increased cytokine responses after *C. burnetii* 3262 stimulation
PBMCs of healthy individuals genotyped for *TLR10* N241H/I369L **(3A)** and *TLR10* I775V **(3B)** were stimulated with medium (RPMI, striped bar), *C. burnetii* NM 3x10⁶/ml or *C. burnetii* 3262 3x10⁶/ml in the presence of serum. Cytokines were measured in the supernatant by ELISA after 24h incubation. The numbers between brackets behind the genotypes indicate the number of individuals analyzed. Data are presented as mean values with SEM, *p<0.05, ** p<0.01, *** p<0.001, Mann-Whitney *U* test.

Polymorphisms in *TLR10* are not associated with chronic Q fever

To assess the role of TLR10 polymorphisms N241H, I369L and I775V in the susceptibility to chronic Q fever, DNA samples of 139 chronic Q fever patients and 220 controls were analyzed. The controls are individuals from the same area with risk factors for chronic Q fever (valvular or vascular abnormalities) and serological evidence of *C. burnetii* exposure (anti-*C. burnetii* phase II IgG antibodies ≥1:32) without evidence of chronic Q fever. In case of *TLR10* N241H >93% of all subjects were successfully genotyped, for *TLR10* I775V the percentage reached >95%, and 86% in case of *TLR10* I369L. All polymorphisms were in HWE in the control group. No significant differences in genotype distribution were found between the chronic Q fever cohort and the control cohort, the *P* values for N241H, I369L and I775V after the Fisher's exact test were respectively 0.39, 0.56 and 0.30 (Table 3). Also, univariate logistic regression analysis in both the dominant as recessive model revealed that *TLR10* polymorphism N241H, I369L and I775V were not associated with susceptibility to chronic Q fever (Table 3).

Table 3: Distribution of TLR10 genotypes in patients with chronic Q fever and controls

Polymorphism	Genotype distribution			*p* value[a]	Dominant model analysis[b]		Recessive model analysis[b]	
	No. (%)	No. (%)	No. (%)		*p* value	OR (95% CI)	*p* value	OR (95% CI)
***TLR10* (N241H)**	AA	AC	CC	0.39	0.54	0.87 (0.56-1.36)	0.74	0.90 (0.48-1.69)
Patients	57 (42.5)	59 (44.0)	18 (13.4)					
Controls	80 (39.2)	94 (46.1)	30 (14.7)					
***TLR10* (I775V)**	AA	AC	CC	0.30	0.62	0.89 (0.56-1.40)	0.94	0.95 (0.22-4.02)
Patients	91 (67.4)	41 (30.4)	3 (2.2)					
Controls	138 (64.8)	70 (32.9)	5 (2.3)					
***TLR10* (I369L)**	AA	AC	CC	0.56	0.48	0.83 (0.52-1.30)	0.87	0.89 (0.46-1.74)
Patients	54 (43.5)	54 (43.5)	16 (12.9)					
Controls	74 (39.4)	87 (46.3)	27 (14.4)					

Abbreviations: AA, amino acid

[a] Fisher's exact test

[b] Logistic regression

Discussion

In the present study, we examined the role of TLR10 on *C. burnetii*-induced cytokine production and investigated whether genetic variants in *TLR10* were associated with development of chronic Q fever. The results demonstrate that TLR10 inhibits cytokine induction of *C. burnetii* mediated by TLR2. In addition, individuals with polymorphisms *TLR10* N241H/I369L or I775V revealed higher cytokine production after PBMC stimulation with the Dutch outbreak isolate *C. burnetii* 3262. Despite these observed effects of TLR10 on the production of cytokines *in vitro*, polymorphisms in *TLR10* were not associated with the development of chronic Q fever.

While the function and ligands of TLR1, TLR2 and TLR6 have been studied extensively, less is known about the role of TLR10 in general and in *C. burnetii* recognition. Similar to most TLRs, TLR10 signals via the myeloid differentiation primary response gene 88 (MyD88) adaptor protein, it however does not evoke common TLR-induced signaling including NF-κB and IL-8 driven pathways[4, 5]. TLR10 is located in a gene cluster on chromosome 4 with TLR1, TLR2 and TLR6 and studies suggest that TLR10 is able to form heterodimers with TLR2 as well as TLR1 and TLR6[4, 15, 16]. More recently, it was demonstrated that TLR10 acts as an anti-inflammatory receptor, suppressing TLR2 driven cytokine production[5]. Our results strengthened our finding that *C. burnetii* signals via TLR2[2] and *C. burnetii* can be added to the broad range of TLR2 micro-organisms, including *Borrelia burgdorferi, Yersinia pestis* and *Bacteroides fragilis*[5, 8, 17] in which inhibitory effects of TLR10 on cytokine induction has been observed.

C. burnetii 3262 stimulated PBMCs showed an enhanced cytokine production in individuals bearing genetic variation in *TLR10* N241H/I369L and I775V. We investigated this in *C. burnetii* induced cytokine production by PBMCs from healthy volunteers having these polymorphisms. This finding was also observed in response to the TLR1/2 ligand Pam3Cys, *Borrelia burgdorferi, Yersinia pestis* and *Bacteroides fragilis*[5, 8, 17]. In contrast, cytokine production by PBMCs from individuals carrying polymorphisms in *TLR10* N241H/I369L or I775V did not differ after stimulation with *C. burnetii* NM compared to individuals without these SNPs. The results presented here could suggest a possible different role for TLR10 in sensing the laboratory strain *C. burnetii* NM compared to the Dutch outbreak isolate *C. burnetii* 3626. Recently, we reported divergent recognition of these two strains by the TLR1/TLR2 and TLR2/TLR6 heterodimers[2]. *C. burnetii* 3262 is recognized by both TLR1/TLR2 and TLR2/TLR6 heterodimers, while the sensing of *C. burnetii* NM only involves the TLR1/TLR2 heterodimer[2]. TLR10 is closely related to both TLR1 and TLR6, and experiments have shown that the inhibitory effects of TLR10 are mediated via interaction with TLR2 and possibly TLR1 and TLR6[4, 18]. From our results it can be hypothesized that the suppressive function of TLR10 on *C. burnetii*-induced cytokines is more likely to be associated with TLR6 than TLR1, as the presence of genetic variants in *TLR10* had more effect on cytokine production by PBMCs stimulated with *C. burnetii* 3262 than *C. burnetii* NM. A second explanation can be the presence of a different binding site of the two *C. burnetii* strains to the TLR10 receptor. In case of *C. burnetii* NM, the presence of polymorphisms in *TLR10* does not influence cytokine production by PBMCs, while cytokine production derived by *C. burnetii* 3262 stimulation was suppressed by the presence of the common genotypes of the *TLR10* polymorphisms. Based on these findings it can be hypothesized that the examined polymorphisms in *TLR10* do not influence the binding of *C. burnetii* NM to TLR10, while this can be presumed for *C. burnetii* 3262. This also suggests a difference between *C. burnetii* strains, that might influence the susceptibility of individuals to different *C. burnetii* strains.

Recently, the genetic variation in pattern recognition receptors in chronic Q fever patients and controls was investigated. The presence of single nucleotide polymorphisms in *TLR1* (R80T, rs5743611), *NOD2* (1007fsX1, rs2066847) and *MyD88* (-938C>A, rs4988453) was differently distributed among chronic Q fever patients compared to control individuals, indicating that these genes are associated with development of chronic Q fever[11]. Although *in vitro* human and mice studies showed a significant role for TLR2 in *C. burnetii* recognition, no evidence was found that particular SNPs in *TLR2* led to increased susceptibility to either acute or chronic Q fever[2, 11, 19-21]. Barreiro *et al.* reported that *TLR10* is under selective pressure in European countries and that polymorphisms in *TLR10* are able to influence its function[22]. Indeed, polymorphism in *TLR10* I775V and N241H are both involved in the susceptibility to prostate cancer, Crohn's disease and complicated skin and skin structure infections[17, 23-25]. Based on our in-vitro findings and the association of TLR10 polymorphisms with several diseases, we studied

the genetic variation within *TLR10* in chronic Q fever patients and controls. To examine this, 139 chronic Q fever patients and 220 controls (individuals with same risk factors and a previous Q fever infection) were genotyped for *TLR10* N241H, I369L and I775V. No significant differences in genotype distribution of *TLR10* N241H and *TLR10* I775V were observed between chronic Q fever patients and controls, demonstrating that these polymorphisms in *TLR10* do not influence the risk to develop chronic Q fever. *TLR10* I369L is located in the region of the receptor dimer interface of the TLR2/TLR10 heterodimer[4]. It can therefore be assumed that this SNP has a more potential role on TLR10 than the other SNPs examined. However, *TLR10* I369L was also not associated with the development of chronic Q fever.

In the Dutch Q fever outbreak, *C. burnetii* 3262 was the predominant genotype responsible for clinical disease in humans[6, 26]. We therefore assume that the chronic Q fever patients in this study are infected with this strain. Even though our in-vitro study showed a larger effect of TLR10 on cytokine production by *C. burnetii* 3262 than *C. burnetii* NM, infection with *C. burnetii* 3262 and genetic variations in *TLR10* are not associated with a tendency to develop chronic Q fever.
In the present study, we only compared the genetic variation of *TLR10* within chronic Q fever patients and individuals who have had an initial infection, but did not develop chronic Q fever. Therefore, whether TLR10 has a role in development of symptoms or severity in acute Q fever was not studied.

In conclusion, the present study demonstrates that TLR10 suppresses the cytokine production of human mononuclear cells after encounter with *C. burnetii*. Despite the evident inhibitory effect of TLR10 on *C. burnetii*-induced cytokine production, the observed functional consequences of polymorphisms in *TLR10* in healthy individuals are only observed upon *C. burnetii* 3262 stimulation and are not linked to the development of chronic Q fever. In addition, the present study confirms the important role of TLR2 in the recognition of *C. burnetii*.

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Genetic variation in pattern recognition receptors and adaptor proteins associated with development of chronic Q fever

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Abstract

Q fever is an infection caused by *Coxiella burnetii*. Persistent infection (chronic Q fever) develops in 1-5% of patients. We hypothesize that inefficient recognition of *C. burnetii* and/or activation of host-defense in individuals carrying genetic variants in pattern recognition receptors or adaptors would result in an increased likelihood to develop chronic Q fever.

Twenty-four single nucleotide polymorphisms (SNPs) in genes encoding TLRs, NOD-like receptor-2, αvβ3-integrin, CR3, and adaptors MyD88 and TIRAP were genotyped in 139 chronic Q fever patients and in 220 control individuals with cardiovascular risk-factors and previous exposure to *C. burnetii*. Associations between these SNPs and chronic Q fever were assessed by univariate logistic regression models. Cytokine production in whole blood stimulation assays was correlated to relevant genotypes.

Polymorphisms in *TLR1*(R80T), *NOD2*(1007fsX1) and *MYD88*(-938C>A) were associated with chronic Q fever. No association was observed for polymorphisms in *TLR2,TLR4,TLR6,TLR8,ITGAV,ITGB3,ITGAM* and *TIRAP*. No correction for multiple testing was performed since only genes with a known role in initial recognition of *C. burnetii* were included. In the whole-blood assays, individuals carrying the TLR1 80R-allele showed increased IL-10 production upon *C. burnetii* exposure.

Polymorphisms in *TLR1*(R80T), *NOD2*(L1007fsX1) and *MYD88*(-938C>A) are associated with predisposition to development of chronic Q fever. For *TLR1*, increased IL-10 responses to *C. burnetii* in individuals carrying the risk allele may contribute to the increased risk of chronic Q fever.

Introduction

Q fever is an infection with the intracellular Gram-negative bacterium *Coxiella burnetii*. Although most symptomatic individuals experience only mild flu-like symptoms or pneumonia (acute Q fever), some patients develop a persistent infection with severe complications including endocarditis and vascular (prosthesis) infection (chronic Q fever). Persistent infection occurs in overall 1-5% of *C. burnetii*-infected subjects and develops insidiously, which contributes to its late diagnosis and high mortality. It is well established that the main risk factors for chronic infection are pre-existing abnormalities of cardiac valves (including valvular prosthesis), vascular aneurysms, vascular prosthesis and immunosuppression[1, 2]. However, in a large outbreak in the Netherlands from 2007 to 2010, only a minority of patients with these risk factors developed chronic Q fever after (serological evidence of) infection with *C. burnetii*[3, 4]. Inefficient early recognition of the bacterium by the innate immune system followed by incomplete eradication and/or inadequate initiation of adaptive immune responses may be a contributing factor in the development of chronic Q fever.

The innate immune system provides the first barrier against *C. burnetii* infection. In general, pattern recognition receptors (PRRs) on innate immune cells recognize molecular moieties on the surface of microorganisms. Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain proteins (NODs) are the main PRRs involved in the recognition of bacteria. TLRs interact with several adaptor proteins, including myeloid differentiation primary response gene 88 (MyD88) and Toll interleukin-1 receptor domain-containing adaptor protein (TIRAP), to activate transcription factors, leading to production of proinflammatory cytokines, activation of antimicrobial mechanisms and subsequent initiation of adaptive immune responses. MyD88 is also required for the production of interleukin (IL)-1, IL-18 and IL-33, which emphasizes its importance for host defense against microorganisms. We recently demonstrated that *C. burnetii*-induced cytokine production by human innate immune cells is mediated through recognition by the heterodimer TLR1/TLR2 on the cell membrane and by the cytoplasmic NOD2[5]. TLR6 appears to be involved specifically in the recognition of the *C. burnetii* strain 3262 that was isolated during the outbreak in the Netherlands[5]. Microorganisms such as *C. burnetii* use phago-

cytosis as an efficient mechanism of entry into monocytes/macrophages where they can survive intracellularly in a parasitophorous vacuole (PV)[6]. An important role in *C. burnetii* uptake has been described for leucocyte response integrin ($\alpha^v\beta^3$ integrin) and complement receptor 3 (CR3 [$\alpha M\beta^2$, CD11b/CD18]). Virulent phase I *C. burnetii* uptake is mediated by $\alpha^v\beta^3$ integrin, while phagocytosis of avirulent phase II *C. burnetii* also involves CR3[7, 8].

Genetic variants in immune cell receptors such as TLRs and NODs and their adaptors have been associated with increased susceptibility to bacterial infections[9-16]. Thus, it is tempting to speculate that inefficient recognition of *C. burnetii* and activation of host defense in certain individuals carrying genetic variants in these receptors or adaptors, would result in an ineffective early clearance of the bacterium and an increased likelihood to develop chronic Q fever. No study so far has been able to investigate this, due to the low prevalence of the disease. The recent Q fever outbreak in the Netherlands led to over 4000 reported acute Q fever cases and more than 250 cases of chronic Q fever[17], which offered an unique opportunity to study this.

We hypothesized that certain polymorphisms in the host's PRR genes or in genes encoding their signaling adaptor proteins influence the risk of persistent *C. burnetii* infection and hence the development of chronic Q fever. To test this hypothesis, we analyzed the prevalence of specific single nucleotide polymorphisms (SNPs) in 11 candidate genes – *TLR1,TLR2,TLR4,TLR6, TLR8,MYD88,TIRAP,NOD2,ITGAV,ITGB3* and *ITGAM (CD11b)* – in a cohort of 139 chronic Q fever patients and a control cohort of 220 individuals, living in the same area and with valvular or vascular predisposing factors for chronic Q fever, who had contracted *C. burnetii* (based on positive serology) but did not develop chronic Q fever. The genetic study was complemented with functional assays investigating the effect of the specific polymorphisms on the *in vitro* cytokine production in whole blood incubated with *C. burnetii*.

Material and Methods

Ethics statement

The study was approved by the Ethical Committee of Radboud university medical center, Nijmegen, The Netherlands. Subjects were enrolled after providing written informed consent (or waiver when deceased [n=5], as approved by the Ethical Committee). Institutional Review Boards of participating hospitals approved the inclusion of patients and controls in this study. The study has been performed in accordance with the Declaration of Helsinki.

Subjects

All patients with probable or proven chronic Q fever, as defined by the Dutch consensus on chronic Q fever (Table 1)[18], who visited the outpatient clinic at the Departments of Internal Medicine of the participating hospitals, were asked to participate in the study. The recruitment of patients took place between July 2011 and July 2014 at Radboud university medical center (Nijmegen), Canisius-Wilhelmina Hospital (Nijmegen), Catharina Hospital (Eindhoven), Elkerliek Hospital (Helmond), Atrium Medical Center (Heerlen), Elisabeth Hospital (Tilburg), Bernhoven Hospital (Oss) and Jeroen Bosch Hospital ('s-Hertogenbosch). Demographic and clinical characteristics (classification based on serological titers and imaging results, cardiovascular risk factors, immunosuppressive co-morbidity or medication) were retrieved from the patients' medical records. The population-matched control group consisted of individuals from the same area with valvular or vascular abnormalities predisposing to chronic Q fever, who had serological evidence of exposition to *C. burnetii* (anti-*C. burnetii* phase II IgG antibodies ≥1:32) without clinical symptomatology or serological evidence of chronic Q fever. These individuals were recruited in the Q fever pre-vaccination screening campaign January-April 2011 [19, 20] and in a Q fever screening of patients with vascular risk factors living in the Q fever outbreak area[21].

Genotyping

From patients who came to the outpatient clinic or to the Q fever pre-vaccination screening, venous blood was drawn and stored at -80°C until use. DNA was isolated from these blood samples using standard methods[22]. Other participants, both

Table 1: Classification chronic Q fever according to the Dutch consensus guidelines

Classification	Criteria
Proven chronic Q fever	1. Positive *C.burnetii* PCR in blood or tissue[a] OR 2. IFA titer of ≥ 1:800 or 1:1,024 for *C.burnetii* phase I IgG[b] AND – definite endocarditis according to the modified Duke criteria [50] OR – proven large vessel or prosthetic infection by imaging studies (FDG-PET, CT, MRI or AUS)
Probable chronic Q fever	IFA titre of ≥ 1:800 or 1:1024 for *C.burnetii* phase I IgG[b] AND One or more of the following criteria: – Valvulopathy not meeting the major criteria of the modified Duke criteria [50] – Known aneurysm and/or vascular or cardiac valve prosthesis without signs of infection by means of TEE/TTE, FDG-PET, CT, MRI or abdominal ultrasound – Suspected osteomyelitis, pericarditis, or hepatitis as manifestation of chronic Q fever – Pregnancy – Symptoms and signs of chronic infection, such as fever, weight loss and night sweats, hepato-splenomegaly, persistent raised ESR and CRP – Granulomatous tissue inflammation, proven by histological examination – Immunocompromised state
Probable chronic Q fever	IFA titer of ≥ 1:800 or 1:1024 for *C.burnetii* phase I IgG[b] without manifestations meeting the criteria for proven or probable chronic Q fever

Abbreviations: IFA, immunofluorescence assay; FDG-PET, fluorodeoxyglucose positron emission tomography; MRI, magnetic resonance imaging; CT, computer tomography; TEE, transesophageal echocardiography; TTE, Transthoracic echocardiography. AUS, abdominal ultrasound.

[a] In absence of acute infection.

[b] Cutoff is dependent on the IFA technique used (≥1:800 for a technique developed in house and ≥1:1024 for a commercial IFA technique).

patients and controls, received a buccal swab kit (Isohelix, Cell Projects Ltd., Harrietsham, Kent, UK) to obtain epithelial cells for DNA isolation. DNA was isolated using a buccal DNA isolation kit (Isohelix), according to the manufacturer's protocol. SNPs were selected based on known functional effects on protein function or gene expression, published associations with human diseases and/or haploview data. In total, 25 SNPs in *TLR1,TLR2,TLR4,TLR6, TLR8,TIRAP,MYD88,NOD2,ITGAV,ITGB3* and *ITGAM* were genotyped with a Sequenom mass-spectrometry genotyping platform. Quality control was performed by duplicating 5% of the samples within and across plates, by the incorporation of positive and negative control samples and by sequencing samples to verify the various genotypes.

In-vitro whole blood stimulation and cytokine measurements

In a subgroup of the control subjects – those who participated in the Q fever pre-vaccination screening study as described earlier[23] – whole blood assays were performed and *C. burnetii*-induced cytokines were measured (n=93). In short, venous blood was drawn into 5 ml endotoxin-free lithium-heparin tubes (Vacutainer, BD Biosciences) and samples were processed within 12 hours. Blood was aliquoted in separate tubes and incubated at 37°C for 24 hours with heat-inactivated (30 min at 99°C) *C. burnetii* Nine Mile (NM) RSA493 phase I [24](10^7 bacteria/ml), mitogen (positive control) or without stimulus (negative control) as previously described[23]. After incubation, blood cultures were centrifuged at 4656 g for 10 minutes and supernatants were collected. Supernatants were stored at -20ºC until cytokine concentrations – including IL-1β, tumor-necrosis factor (TNF), IL-2, IL-6 and IL-10 – were measured by a commercially available magnetic beads multiplex assay according to manufacturer's instructions (Merck Millipore, Billerica, MA, USA).

Statistical analysis

Presence of Hardy-Weinberg equilibrium (HWE) was analysed for all 24 SNPs separately in the control cohort[25]. For the two TLR8 SNPs on the X-chromosome, only the female subjects were included in the HWE analysis and relative allele frequencies were subsequently compared between female and male subjects. The difference in genotype frequencies between the patients and the control group were analyzed by means of a gene dosage model, with Fisher's exact test to test for significance. Subsequent dominant and recessive model analysis was

performed by means of univariate logistic regression, for which odds ratios (ORs) and their 95% confidence intervals (95% CI) were reported. Because the choice of the genetic variants was based exclusively on gene products with an established or suspected role in *C. burnetii* recognition, rather than exploratory, no correction for multiple testing was performed. Statistical analyses were carried out with the IBM SPSS software (version 20). Cytokine concentrations in the *in vitro* whole blood stimulation assays were stratified according to genotype and compared using Mann-Whitney *U*-tests, using GraphPad Prism (GraphPad software Inc., version 5). Overall, statistical tests were two-sided and a *P*-value below 0.05 was considered statistically significant.

Results

Baseline characteristics

In total, 139 (92 proven and 47 probable) chronic Q fever patients and 220 control subjects without chronic Q fever but with serological evidence of *C. burnetii* exposure and a risk factor for chronic Q fever were included in the study. Demographic and clinical characteristics of patients and control subjects are summarized in Table 2. Patients were slightly older than controls (2.4 yrs difference in median age). In both groups, vascular risk factors were more prevalent than cardiac valvular risk factors. Valvular risk factors were, however, more prevalent in the control subjects than in the patients. Chronic Q fever patients were more often immunocompromised than the control subjects.

SNPs in MYD88, NOD2 and TLR1 are associated with chronic Q fever

Genotyping of patients and controls was successful for all polymorphisms in genes encoding TLRs, NOD2, MyD88, TIRAP, $\alpha^{v}\beta^{3}$ integrin and CR3 presented in Table 3. For each polymorphism, >94% of the subjects were genotyped. All SNPs were in Hardy-Weinberg equilibrium in the control group, except for *ITGB3* rs4642 (x^2=6.18). This SNP was excluded from further analysis.

In the analysis of all chronic Q fever patients, a significantly different distribution of one of the three *NOD2* polymorphisms and one of the two *MYD88* polymorphisms was revealed in the dominant model: *NOD2* L1007fsX1 (P = 0.02; OR, 3.34 [95% CI, 1.22-9.14]) and *MYD88* -938C>A (*P* = 0.04; OR, 2.15 [95% CI, 1.05-4.39]) (Table 4). In comparison to controls, patients were more often heterozygous for the allelic variant of these SNPs.

Table 2: Baseline patient characteristics of the chronic Q fever patients and controls

	Chronic Q fever Cohort (n=139)	Control Cohort (n=220)	P-value[a]
Median age, y (IQR)	70.0 (63.0-75.6)	67.6 (58.3-74.3)	0.02
Male sex (%)	114 (82)	169 (77)	0.29
Classification of chronic Q fever			
Proven (%)	92 (66.2)	0	
Probable (%)	47 (33.8)	0	
Cardiovascular risk factor for chronic Q fever[b]			
Vascular aneurysm / prosthesis (%)	95 (68.3)	129 (58.6)	0.07
Valvular defect / prosthesis (%)	39 (28.1)	111 (50.5)	<0.001
Immunocompromised state[c] (%)	21 (15.1)	14 (6.4)	0.01

Abbreviations: IQR, interquartile range

[a] Fisher exact test for categorical variables and Mann-Whitney for continuous variables.

[b] Both vascular and valvular risk conditions in 9 case-patients and 22 controls.

[c] Case-patients: Auto-immune disease with immunosuppressive drugs n=14; renal insufficiency n=1; renal transplantation n=1; malignancy n=3; prednis(ol)one use not specified n=1. Controls: Auto-immune disease with immunosuppressive drugs n=5; renal transplantation n=2; malignancy n=4; prednis(ol)on use not specified n=4.

Table 3: Genotyped SNPs in genes encoding immune receptors and adaptor molecules

Gene	SNP ID	Gene Region	Nucleotide change[a]	Amino Acid Change
TLR1	rs4833095	Exon 4	T > C	S248N
	rs5743611	Exon 4	G > C	R80T
TLR2	rs111200466	5'UTR	ins > del	
	rs5743704	Exon 3	C > A	P631H
	rs5743708	Exon 3	G > A	R753Q
TLR4	rs4986790	Exon 3	A > G	D299G
	rs1927911	Intron 1	C > T	
TLR6	rs1039559	Intron 1	T > C	
	rs5743818	Exon 2	T > G	Synonymous (A644A)
TLR8	rs3747414	Exon 3	C > A	Synonymous (I751I)
	rs3764880	Exon 1	A > G	M1V affects protein start
NOD2	rs1077861	Intron 10	A > T	
	rs2066847	Exon 11	– > C	frameshift and stopcodon (L1007fsX1)
	rs2066844	Exon 4	C > T	R702W
MYD88	rs4988453	Promoter	C > A	
	rs6853	3' UTR	A > G	
TIRAP	rs8177348	Intron 1	C > T	
	rs8177374	Exon 5	C > T	S180L
ITGAV	rs3738919	Intron 17	C > A	
	rs9333288	Intron 3	A > G	
ITGB3	rs3809865	3' UTR	A > T	
	rs4642	Exon 10	G > A	Synonymous (E511E)
ITGAM	rs1143679	Exon 3	G > A	R77H
	rs1143678	Exon 30	C > T	P1146S

Abbreviations: SNP, single nucleotide polymorphism; ID, identification number

[a] The first nucleotide is the most common nucleotide.

When only proven chronic Q fever patients were considered, *TLR1* R80T was significantly differently distributed among patients and controls in the dominant model (*P* = 0.034, OR, 0.48 [95% CI, 0.24-0.95) (Table 5), while it was marginally significant (*P* = 0.08, OR, 0.61 [95% CI, 0.35-1.06) when all patients (both proven and probable) were included. Patients were less often heterozygous or homozygous for the allelic variant of this SNP. The distributions of *NOD2* L1007fsX1 and *MYD88* -938C> showed a trend toward significance among patients and controls (*P* = 0.065 and *P* = 0.091, respectively) when only proven chronic Q fever patients were included in the analysis.

No associations were observed between polymorphisms in *TLR2,TLR4,TLR6,TIRAP,ITGAV, ITGB3,ITGAM* and development of chronic Q fever.

TLR1 SNP R80T affects *in vitro* IL-10 production by *C. burnetii*-stimulated whole blood

Functional consequences of *TLR1* R80T, *NOD2* L1007fsX1 and *MYD88* -938C>A were studied by *C. burnetii*-stimulation of whole blood obtained from 93 control subjects with different *TLR1* R80T, *NOD2* L1007fsX1 and *MYD88* -938C>A genotypes. Cytokine production of IL-1β, TNF, IL-2, IL-6 and IL-10 was measured after 24 hours of stimulation. Decreased IL-10 production was observed upon stimulation with *C. burnetii* by blood cells with *TLR1* 80T containing genotypes, which is associated with lower susceptibility to chronic Q fever. Pro-inflammatory cytokine production, including IL-1β, IL-6, TNF and IL-2, was not different among *TLR1* R80T genotypes (Figure 1).

IL-1β, TNF and IL-10 responses to *C. burnetii* in relation to *NOD2* L1007fsX1 and *MYD88* -938C>A polymorphisms revealed no significant differences (Figure 2). IL-6 and IL-2 are not shown (*P*-value of 0.78 and 0.58 for IL-6 and IL-2 respectively, when stratified by genotype *NOD2* 1007fsX1; *P*-value of 0.51 and 0.52 for IL-6 and IL-2 when stratified by genotype *MYD88* -938C>A).

Table 4: Associations of polymorphisms in immune receptors and adaptors genes and susceptibility to chronic Q fever

Polymorphism	Genotype distribution				Dominant model analysis[b]		Recessive model analysis	
				p value[a]	*p* value[b]	OR (95% CI)[b]	*p* value[b]	OR (95% CI)[b]
***TLR1* rs4833095**	TT	CT	CC	.60	.40	1.21 (0.78-1.87)	.48	1.42 (0.53-3.77)
controls (%)	121 (57.6)	80 (38.1)	9 (4.29)					
patients (%)	71 (53.0)	55 (41.0)	8 (5.97)					
***TLR1* rs5743611**	GG	GC	CC	.21	.08	0.61 (0.35-1.06)	.77	0.78 (0.14-4.30)
controls (%)	158 (75.6)	47 (22.5)	4 (1.91)					
patients (%)	112 (83.6)	20 (14.9)	2 (1.49)					
***TLR2* rs111200466**	ins/ins	ins/del	del/del	.48	.67	0.90 (0.56-1.46)	.36	1.64 (0.56-4.79)
controls (%)	145 (69.4)	57 (27.3)	7 (3.35)					
patients (%)	93 (71.5)	30 (23.1)	7 (5.38)					
***TLR2* rs5743704**	CC	CA	AA	.69	.67	1.19 (0.54-2.59)	na	
controls (%)	193 (92.3)	16 (7.66)	0					
patients (%)	122 (91.0)	12 (8.96)	0					
***TLR2* rs5743708**	GG	GA	AA	.89	.51	1.34 (0.56-3.18)	.75	1.58 (0.10-25.46)
controls (%)	207 (94.5)	11 (5.02)	1 (0.46)					
patients (%)	129 (92.8)	9 (6.47)	1 (0.72)					
***TLR4* rs4986790**	AA	AG	GG	.19	.36	0.75 (0.41-1.38)	1.00	2.55E9 (0.0-∞)
controls (%)	174 (82.9)	36 (17.1)	0					
patients (%)	116 (86.6)	17 (12.7)	1 (0.75)					
***TLR4* rs1927911**	CC	TC	TT	.48	.25	1.30 (0.83-2.01)	.90	1.06 (0.46-2.43)
controls (%)	130 (61.9)	65 (31.0)	15 (7.14)					
patients (%)	74 (55.6)	49 (36.8)	10 (7.52)					
***TLR6* rs1039559**	TT	TC	CC	.81	.50	1.18 (0.72-1.94)	.90	1.03 (0.60-1.78)
controls (%)	60 (28.7)	108 (51.7)	41 (19.6)					
patients (%)	34 (25.4)	73 (54,5)	27 (20.1)					

Table 4: Continued

Polymorphism	Genotype distribution				Dominant model analysis[b]		Recessive model analysis	
				p value[a]	*p* value[b]	OR (95% CI)[b]	*p* value[b]	OR (95% CI)[b]
***TLR6* rs5743818**	TT	TG	GG	.34	.19	0.75 (0.48-1.16)	.24	0.62 (0.27-1.38)
controls (%)	104 (49.5)	84 (40.0)	22 (10.5)					
patients (%)	76 (56.7)	49 (36.6)	9 (6.72)					
***TLR8* rs3747414[c]**	allele C	allele A		.74				
controls (%)	276 (66.3)	140 (33.7)						
patients (%)	173 (65.0)	93 (35.0)						
***TLR8* rs3764880[c]**	allele A	allele G		.15				
controls (%)	319 (77.1)	95 (22.9)						
patients (%)	193 (72.0)	75 (28.0)						
***NOD2* rs1077861**	AA	TA	TT	.89	.74	1.08 (0.70-1.67)	.64	1.18 (0.58-2.40)
controls (%)	97 (46.6)	91 (43.8)	20 (9.62)					
patients (%)	60 (44.8)	59 (44.0)	15 (11.2)					
***NOD2* rs2066847**	-/-	-/C	C/C	.02	.02	3.34 (1.22-9.14)	na	
controls (%)	204 (97.1)	6 (2.86)	0					
patients (%)	122 (91.0)	12 (8.96)	0					
***NOD2* rs2066844**	CC	CT	TT	.50	.48	1.27 (0.66-2.45)	na	
controls (%)	196 (89.5)	23 (10.5)	0					
patients (%)	121 (87.1)	18 (12.9)	0					
***MYD88* rs4988453**	CC	CA	AA	.04	.04	2.15 (1.05-4.39)	na	
controls (%)	195 (92.9)	15 (7.14)	0					
patients (%)	115 (85.8)	19 (14.2)	0					
***MYD88* rs6853**	AA	GA	GG	.52	.30	1.29 (0.80-2.07)	.78	0.78 (0.14-4.32)
controls (%)	155 (73.8)	51 (24.3)	4 (1.90)					
patients (%)	92 (68.7)	40 (29.9)	2 (1.49)					

Table 4: Continued

Polymorphism	Genotype distribution				Dominant model analysis[b]		Recessive model analysis	
				p value[a]	*p* value[b]	OR (95% CI)[b]	*p* value[b]	OR (95% CI)[b]
***TIRAP* rs8177348**	CC	CT	TT	.23	.26	1.29 (0.83-2.01)	.12	1.93 (0.84-4.45)
controls (%)	128 (61.8)	68 (32.9)	11 (5.31)					
patients (%)	74 (55.6)	46 (34.6)	13 (9.77)					
***TIRAP* rs8177374**	CC	CT	TT	.77	.76	0.93 (0.58-1.50)	.58	1.57 (0.31-7.91)
controls (%)	156 (71.9)	58 (26.7)	3 (1.38)					
patients (%)	102 (73.4)	34 (24.5)	3 (2.16)					
***ITGAV* rs3738919**	CC	CA	AA	.70	.54	0.87 (0.56-1.36)	.69	1.14 (0.60-2.19)
controls (%)	82 (39.2)	102 (48.8)	25 (12.0)					
patients (%)	57 (42.5)	59 (44.0)	18 (13.4)					
***ITGAV* rs9333288**	AA	AG	GG	.88	.70	1.09 (0.71-1.67)	.81	0.90 (0.37-2.20)
controls (%)	123 (56.4)	81 (37.2)	14 (6.42)					
patients (%)	75 (54.3)	55 (39.9)	8 (5.80)					
***ITGB3* rs3809865**	AA	TA	TT	.17	.07	0.67 (0.44-1.03)	.27	0.68 (0.34-1.35)
controls (%)	86 (39.4)	103 (47.2)	29 (13.3)					
patients (%)	68 (49.3)	57 (41.3)	13 (9.42)					
***ITGAM* rs1143679**	GG	GA	AA	.68	.65	0.88 (0.49-1.56)	.57	1.60 (0.32-8.04)
controls (%)	180 (82.2)	36 (16.4)	3 (1.37)					
patients (%)	116 (84.1)	19 (13.8)	3 (2.17)					
***ITGAM* rs1143678**	CC	CT	TT	.51	.49	1.19 (0.73-1.95)	.27	1.87 (0.62-5.70)
controls (%)	159 (75.7)	45 (21.4)	6 (2.86)					
patients (%)	97 (72.4)	30 (22.4)	7 (5.22)					

Abbreviations: na, not applicable

[a] Fisher's exact test

[b] Logistic regression

[c] X-chromosomal

Table 5: Distribution of *TLR1* (R80T), *NOD2* (L1007fsX1) and *MYD88* (-938C>A) genotypes in proven chronic Q fever patients (n=92) compared to controls (n=220)

Polymorphism	Genotype distribution				Dominant model analysis[b]		Recessive model analysis	
				p value[a]	*p* value[b]	OR (95% CI)[b]	*p* value[b]	OR (95% CI)[b]
***TLR1* rs5743611**	GG	GC	CC	.047	.034	0.48 (0.24-0.95)	.86	1.17 (0.21-6.48)
controls (%)	158 (75.6)	47 (22.5)	4 (1.91)					
patients (%)	78 (86.7)	10 (11.1)	2 (2.22)					
***NOD2* rs2066847**	-/-	-/C	C/C					
controls (%)	204 (97.1)	6 (2.86)	0	.007	.007	2.87 (0.94-8.79)	na	na
patients (%)	83 (92.2)	7 (7.78)	0					
***MYD88* rs4988453**	CC	CA	AA	.121	.091	2.00 (0.89-4.5)	na	na
controls (%)	195 (92.9)	15 (7.14)	0					
patients (%)	78 (86.7)	12 (13.3)	0					

Abbreviations: na, not applicable

[a] Fisher's exact test

[b] Logistic regression

[c] X-chromosomal

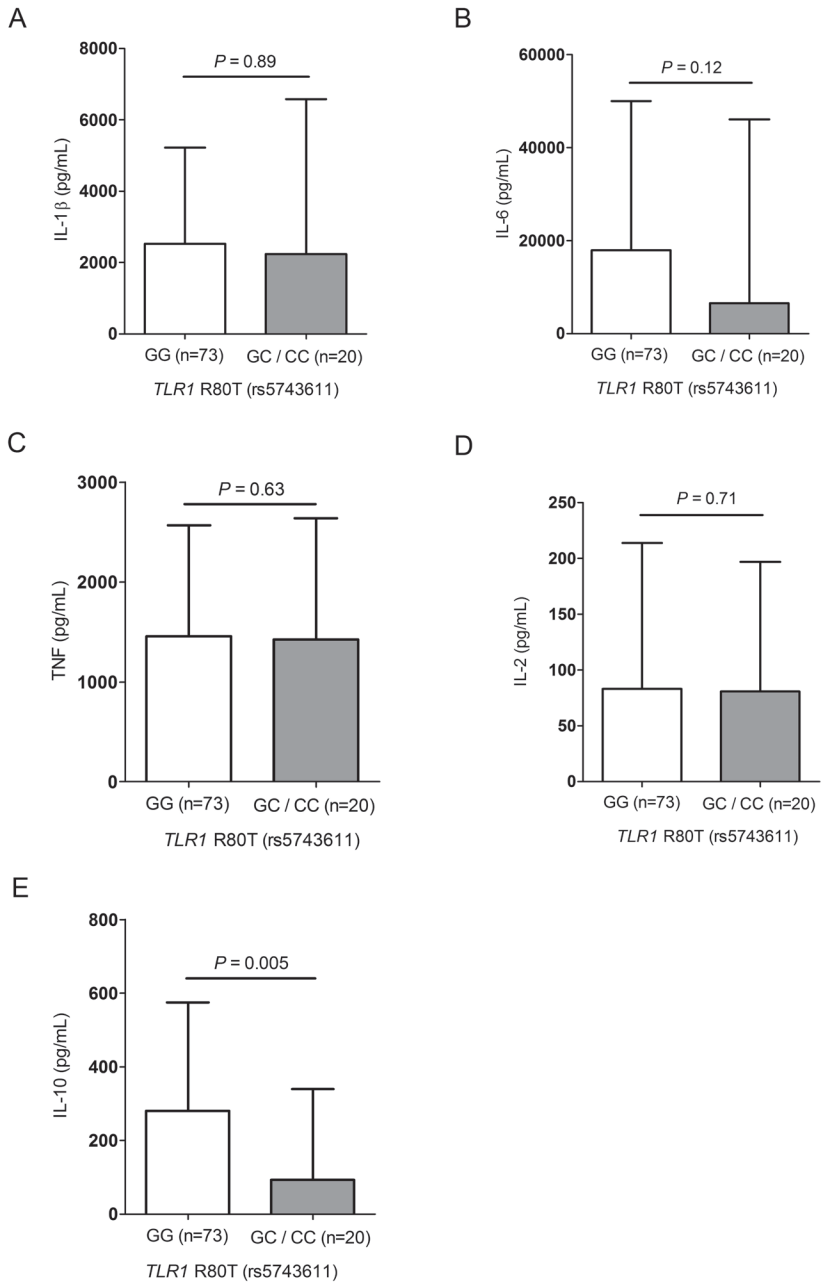

Figure 1: Association between *TLR1* R80T genotypes and cytokine production after 24 hours whole blood stimulation with *C. burnetii*

IL-1β, interleukin 1β; IL-2, interleukin 2; IL-6, interleukin 6; IL-10, interleukin 10; TNF, tumor necrosis factor. Stimulation was performed for 24 hours with heat-killed *C. burnetii* Nine Mile phase I (RSA 493) (bacteria count, 1×10^7/mL). Data are presented as medians and interquartile ranges, and groups were compared with the Mann-Whitney *U* test.

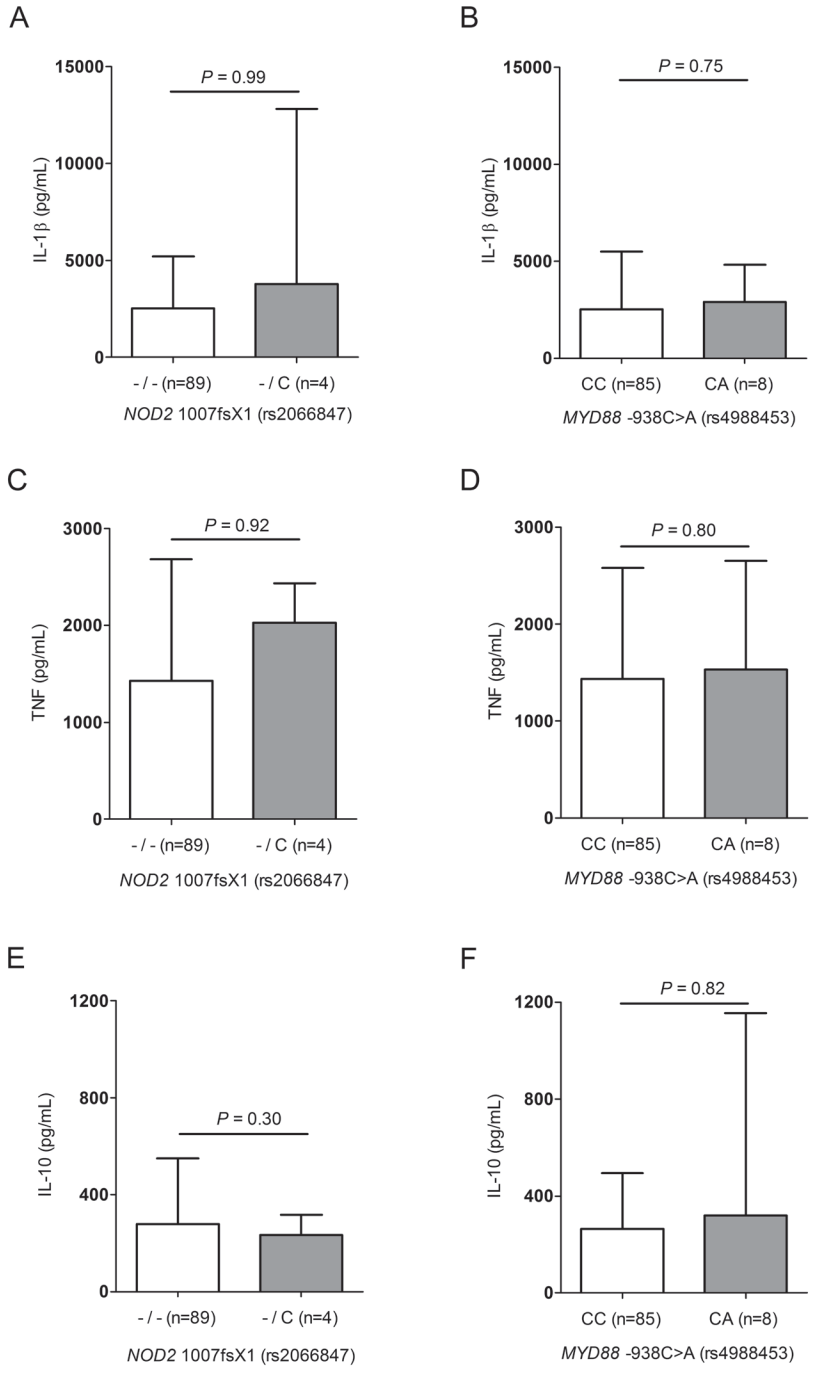

Figure 2: Association between *NOD2* and *MYD88* genotypes and cytokine production after 24 hours whole blood stimulation with *C. burnetii*

Subjects were stratified by genotype *NOD2* 1007fsX1 **(A, C, E)** and *MYD88* -938C>A **(B, D, F)**. IL-1β **(A, B)**, TNF **(C, D)** and IL-10 **(E, F)** cytokine productions are shown. Stimulation was performed for 24 hours with heat-killed *C. burnetii* Nine Mile phase I (RSA 493) (1x 10^7 bacteria/ml). Data are presented as medians ± interquartile range. Groups were compared by Mann-Whitney *U*-test.

Discussion

In the present study we investigated whether genetic variants in pattern recognition receptors and adaptor proteins were associated with chronic Q fever in *C. burnetii*-infected individuals with vascular or valvular risk factors. Our results showed that *TLR1* R80T (rs5743611), *NOD2* L1007fsX1 (rs2066847) and *MYD88* -938C>A (rs4988453) are linked to the development of chronic Q fever. In addition, blood cells from individuals carrying the *TLR1* 80T variant that led to decreased susceptibility to chronic Q fever, showed lower IL-10 production upon *C. burnetii* stimulation. No influence of *TLR2, TLR4, TLR6, TLR8, TIRAP, ITGAV, ITGB3* and *ITGAM* SNPs on the susceptibility to chronic Q fever was observed.

To our knowledge, this is the first genetic study that investigates the role of SNPs in PRR and adaptor molecule genes in development of chronic Q fever, the most severe complication of *C. burnetii* infection. Our study comprises the largest chronic Q fever cohort ever described. Previous studies on genetic predisposition to Q fever concerned either small cohorts of patients or focused on other clinical Q fever manifestations (acute Q fever or Q fever fatigue syndrome). Everett *et al.* studied genetic susceptibility to acute Q fever by focusing on *TLR2* and *TLR4* SNPs, for which they demonstrated no correlation in 89 acute Q fever patients and 162 controls[26]. Helbig and colleagues investigated association between genetic variants in *HLA, IFNG, IL10* and *TNFR* genes and chronic Q fever or post-Q fever fatigue in 22 and 31 patients, respectively[27]. They described significant differences in the frequencies of *IL10* promoter microsatellites in chronic Q fever compared to a control panel of 181 individuals without Q fever exposure. Vollmer-Conna and colleagues demonstrated that functional polymorphisms in *IFNG* and *IL10* affect disease severity and duration after serologically-confirmed acute Q fever[28].

We focused on polymorphisms in immune genes involved in the initial recognition of *C. burnetii* based on the assumption that chronic Q fever will develop only after inefficient early recognition resulting in inadequate early clearance of the bacterium. Previous studies have shown that chronic Q fever patients exhibit a different cytokine response to *C. burnetii*[29-31]. It is, however, not clear whether this different response is specific for the myeloid cells of these individuals or a result of persistent presence of the bacteria in monocytes/macrophages. Deciphering genetic polymorphisms in immune genes of the innate response to *C. burnetii* may give more insight in the mechanism.

When performing genetic association studies, the choice of the control group is of pivotal importance. We deliberately chose a control cohort of subjects living in the same Q fever endemic area, with past *C. burnetii* infection (as shown by positive anti-*C. burnetii* serology), as well as cardiovascular risk factors for chronic Q fever, who did not develop chronic Q fever. When observing a different distribution of genetic polymorphisms between patients and these controls, it indicates that these polymorphisms modify the risk for developing chronic Q fever, and not for contracting *C. burnetii* infection or any underlying predisposing factors. The control cohort, however, was somewhat younger, had significantly more often valvular risk factors and less often immunocompromised conditions than the patient cohort.

Notably, antimicrobial drug treatment at the time of acute Q fever might decrease the risk of chronic Q fever. Although information on previous treatment for acute Q fever was not obtained from the controls in this study, we assume that the majority of these individuals did not receive antibiotic treatment for acute Q fever since they participated in screening programs to detect previous *C. burnetii* infection.

The patient cohort included patients with proven or probable diagnosis of chronic Q fever, while possible cases were left out. Since diagnosis of chronic Q fever is often difficult – because direct detection of the bacterium is not possible in many cases – a probable chronic Q fever diagnosis is based on indirect evidence of persistent *C. burnetii* infection. These patients also receive long-term antibiotic treatment, and clinicians use identical treatment and follow-up schemes as for proven chronic Q fever patients, but a part of these patients may have no persistent infection. We, however, also performed an analysis in which only the subset of patients with proven chronic Q fever were included. The latter increased the level of diagnostic certainty of chronic Q fever in the case-patients, but had decreased power to detect associations.

We identified a significant association between the *TLR1* R80T polymorphism and the risk for chronic Q fever. This *TLR1* SNP is previously described to be a risk factor

for invasive aspergillosis after hematopoietic stem cell transplantation[16], candidemia[32] and inflammatory bowel disease[33]. *TLR1* S248N, which is not linked to *TLR1* R80T, did not show association with development of chronic Q fever. We studied the functional consequences of the *TLR1* R80T polymorphism in a subset of controls. Presence of *TLR1* 80T allele resulted in significantly lower production of the anti-inflammatory cytokine IL-10, but did not affect TNF, IL-1β or other pro-inflammatory cytokines, as found before[5]. The *TLR1* 80T allele was present significantly less frequent in the proven Q fever patient cohort compared to the control cohort. This suggests that having lower IL-10 production upon contact with *C. burnetii* may protect against chronic Q fever. This finding is in line with the role of the anti-inflammatory cytokine IL-10 in the development of human chronic Q fever that has been extensively reported[29, 34-36]. In short, IL-10 induces *C. burnetii* replication in naive monocytes[34]. In addition, IL-10 production in unstimulated peripheral blood mononuclear cells was increased after *C. burnetii* infection in individuals who subsequently developed chronic endocarditis in comparison to individuals who did not develop endocarditis[35]. Moreover, transgenic mice that constitutively over-express IL-10 is the only efficient model for chronic Q fever so far[37].

NOD2 is a cytoplasmic receptor involved in bacterial peptidoglycan sensing. The intracellular cytoplasmic localization of NOD2 and the survival of *C. burnetii* in the intracellular PV may lead to ongoing interaction between them. The active secretion of bacterial proteins by *C. burnetii* through the PV membrane, demonstrated to occur by a type IV secretion apparatus[38], may play a role in this process. Genetic variation in *NOD2* has previously been linked to auto-inflammatory diseases such as Crohn's disease and Blau syndrome[39]. In addition, association with susceptibility to tuberculosis and leprosy has been described[40, 41]. The *NOD2* L1007fsX1 SNP, leading to a frameshift and a premature stopcodon, has a large effect on the protein. Its association with Crohn's disease has been extensively described[42, 43]. We previously showed that *C. burnetii*-induced cytokine production by human mononuclear cells is mediated through NOD2, with NOD2-deficient individuals having strongly decreased IL-1β and IL-6 responses[5]. In the present study, we were unable to show a significant effect on *C. burnetii*-induced cytokine responses when we stratified for the polymorphism *NOD2* L1007fsX1. This is most likely due to the rare occurrence of heterozygotes for the allelic variant and absence of homozygotes in this group.

The role of the adaptor molecule MyD88 in *C. burnetii* infection has not been studied previously. We found that *MYD88* -938C>A was significantly associated with susceptibility to chronic Q fever. In general, MyD88 plays a central role in innate immune responses, being downstream of all TLRs (except for TLR3) and the IL-1 receptor. MyD88 deficiency leads to recurrent infections with pyogenic bacteria in early childhood but seems redundant for most other infections[15]. The *MYD88* -938C>A SNP has been shown to decrease promoter activity[44] and was found to be associated with development of sarcoidosis[45].

TLR2 is a receptor for bacterial lipopeptides, which are either recognized by TLR2/TLR1 or TLR2/TLR6 heterodimers. Although multiple studies have shown that TLR2 is involved in the host's immune response against *C. burnetii*[5, 46-48], we found no association between the TLR2 SNPs included in this study and development of chronic Q fever. This could indicate that either TLR2 has no prominent role in elimination of *C. burnetii* or the consequences of the genetic variants for the function of the protein in *C. burnetii* defense are limited. We did not observe any association of chronic Q fever with *TLR4, TLR6, TLR8, TIRAP* polymorphisms either. Genetic polymorphisms in the genes for the receptors $\alpha^{v}\beta^{3}$ integrin and CR3, which are described to be involved in *C. burnetii* uptake and TNF production[7, 49], were also not significantly different distributed among chronic Q fever patients compared to the control cohort.

Our study has some limitations that need to be taken into account. Although this is the largest cohort of chronic Q fever patients ever described, it is still relatively small for a study of genetic association of an infectious disease. We tried to overcome this limitation by the use of a matched control group with respect to gender, age, presence of cardiovascular risk factors and exposition to the same virulent *C. burnetii* strain. Another important consideration is that no correction for multiple testing was performed. This was decided because candidate genes with a known or suspected role in *C. burnetii* recognition were investigated instead of randomly selected genes. It has to be taken into account that, when applying correction for multiple testing, statistical significance of the *NOD2*, *MYD88* and *TLR1* SNPs association with susceptibility to chronic Q fever is lost. To confirm the findings of the current study, these need to be replicated in another cohort of chronic Q fever patients.

In conclusion, the current study suggests an association between *TLR1, NOD2* and *MYD88* polymorphisms and the risk to develop chronic Q fever after infection with *C. burnetii*. Interestingly, we found that the protective *TLR1* 80T allele was associated with decreased *C. burnetii*-induced IL-10 production. Further research is warranted to elucidate the exact role of these receptors and adaptor molecule in host defense against *C. burnetii* in humans, which could be used for strategies of risk assessment, prophylactic treatment or targeted therapy of chronic Q fever.

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The effect of *C. burnetii* infection on the cytokine response of PBMCs from pregnant goats

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Abstract

In humans, infection with *Coxiella burnetii*, the causative agent of Q fever, leads to acute or chronic infection, both associated with specific clinical symptoms. In contrast, no symptoms are observed in goats during *C. burnetii* infection, although infection of the placenta eventually leads to premature delivery, stillbirth and abortion. It is unknown whether these differences in clinical outcome are due to the early immune responses of the goats. Therefore, peripheral blood mononuclear cells (PBMCs) were isolated from pregnant goats. In total, 17 goats were included in the study. Six goats remained naive, while eleven goats were infected with *C. burnetii*. Toll-like receptor (TLR) and cytokine mRNA expression were measured after *in vitro* stimulation with heat-killed *C. burnetii* at different time points (prior infection, day 7, 35 and 56 after infection). In naive goats an increased expression of interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-10 and interferon (IFN)-γ mRNA upon *C. burnetii* stimulation was detected. In addition, TLR2 expression was strongly up-regulated. In goats infected with *C. burnetii*, PBMCs re-stimulated *in vitro* with *C. burnetii*, expressed significantly more TNF-α mRNA and IFN-γ mRNA compared to naive goats. In contrast, IL-10 mRNA production capacity was down-regulated during *C. burnetii* infection. Interestingly, at day 7 after inoculation a decreased IFN-γ protein level was observed in stimulated leukocytes in whole blood from infected goats, whereas at other time-points increased production of IFN-γ protein was seen. Our study shows that goats initiate a robust pro-inflammatory immune response against *C. burnetii in vitro*. Furthermore, PBMCs from *C. burnetii* infected goats show augmented pro-inflammatory cytokine responses compared to PBMCs from non-infected goats. However, despite this pro-inflammatory response, goats are not capable of clearing the *C. burnetii* infection.

Introduction

Q fever is a zoonosis caused by the intracellular Gram-negative bacterium *Coxiella burnetii*. In humans, infection may remain unnoticed in approximately half of the infected individuals and causes disease, a short flu-like acute febrile disease, pneumonia or hepatitis, in the other half[1]. After infection, either unrecognized or symptomatic, 1 to 5% may develop chronic Q fever months to years later, which presents primarily as endocarditis or vascular infection and may be lethal when left untreated[1]. Infected goats, sheep and cattle are the main reservoir for human infections, these animals can excrete high numbers of *C. burnetii* into the environment during parturition[2, 3].

In contrast to human infections, infection in goats by *C. burnetii* does not lead to recognizable systemic disease in non-pregnant animals, while in pregnant animals infection will lead only to abortion, stillbirth or premature delivery explained by the high bacterial numbers in the placenta and severe local inflammation[4]. Although clinical symptoms are absent in goats, two weeks after infection a clear specific humoral immune response occurs with the appearance of IgM and IgG anti-Phase 2 *C. burnetii* antibodies[3]. So far little is known about the early immune response in goats and the differences in the immune response to *C. burnetii* in man and goat.

Toll-like receptors (TLRs) are assumed to be the principal receptors involved in the early recognition and signalling of *C. burnetii* by innate immune cells. Upon recognition, peripheral blood mononuclear cells (PBMCs) derived from healthy humans produce various cytokines including interleukin-1beta (IL-1β), tumour necrosis factor-alpha (TNF-α) and interleukin-10 (IL-10), but no interferon gamma (IFN-γ) [5-7]. After infection, stimulation of human PBMCs with *C. burnetii* also leads to up-regulation of IL-1β[6, 8-10]. In patients with chronic Q-fever, the anti-inflammatory cytokine IL-10 is up-regulated[7, 11].

It is generally assumed that the type and degree of the early innate immune response relates to the severity of early disease manifestations and determines the early outgrowth of the infection[12]. Therefore it may be hypothesized that this response is different in goats which do not develop disease after infection as

compared to humans. To test this hypothesis we evaluated the *C. burnetii* induced TLR and cytokine responses of PBMCs derived from naive and from infected pregnant goats in the last months of pregnancy. Furthermore, Roest *et al.* recently examined the circulating cytokine levels in naive and *C. burnetii* infected goats. They observed an absence of a systemic cytokine mRNA response and hypothesized that PBMCs might not have been in contact with *C. burnetii* during infection. To clarify this we focus on PBMCs and their ability to induce an immune response against heat-killed *C. burnetii*.

Material and Methods

Animals, Infection and Experimental Design

Seventeen pregnant healthy Alpine yearling goats were included in the study. The experimental setup has been published in detail by Roest *et al.*[3]. In short, on day 76 of the pregnancy eleven goats were intranasally inoculated with 1ml of $1x10^6$/ml *C. burnetii* X09003262-001 (*C. burnetii* 3262) in culture medium. As a negative control, six goats were inoculated with 1 ml of culture medium only. Serum samples were taken weekly to measure antibody titers against *C. burnetii* (LSIVET RUMINANT milk/serum Q-fever ELISA kit, LSI, France). EDTA and heparin blood samples for stimulation experiments were taken from each goat; at day 0 (before inoculation) and at day 7, 35 and 56. To prove successful *C. burnetii* infection of the infected goats, the placenta was sampled after parturition from each goat for immunohistochemistry (IHC) and vaginal mucus was sampled for detection of *C. burnetii* DNA by PCR.

Ethics Statement

All animal experiments were approved by the Animal Experiment Commission of the Central Veterinary Institute, part of Wageningen UR, in accordance with the Dutch regulations on animal experimentation (registration number 2011111.c). Everything possible was done to minimise animal suffering. Humane endpoints were defined in advance. Whenever these endpoints were reached, animals were euthanized.

***Coxiella burnetii* strains**

C. burnetii 3262 was used as infectious organism. This strain was isolated from the placenta from a goat that aborted on a farm during the Q fever outbreak in the Netherlands[13]. The strain was genotyped as CbNL01, which was the main *C. burnetii* genotype during the Dutch Q fever outbreak[13]. Ex vivo stimulation experiments were performed with the heat-killed *C. burnetii* Nine Mile (NM) reference strain. Both *C. burnetii* NM and *C. burnetii* 3262 were cultured at the Central Veterinary Institute as described previously[3]. The strains represent LPS phase I. LPS phase determination was performed by SDS-PAGE and silver staining, using purified phase I (RSA493) and phase II (RSA439) *C. burnetii* NM LPS (kindly provided by R. Toman) as controls[14, 15]. The number of *Coxiella* DNA copies was determined using Taqman (Quanta BioSciences, USA) real-time PCR as mentioned earlier[4]. MLVA typing was performed by using 12 of the 17 loci described by Arricau-Bouvery *et al.*[16]. A second series of stimulation experiments was simultaneously performed also with three heat-killed isolates, *C. burnetii* 14160-001, *C. burnetii* 3345937 and *C. burnetii* 3262. Details of all strains are mentioned in Table 1. Bacteria were killed by heating for 30 minutes at 99°C.

Isolation and stimulation of peripheral blood mononuclear cells

The PBMC fraction was obtained by density centrifugation of EDTA blood using a Ficoll-Paque gradient (Pharmacia Biotech, USA) or Leucosep tubes prefilled with Ficoll-Paque (Greiner, the Netherlands). After washing, the cells were re-suspended in RPMI 1640 Dutch modified (Gibco Invitrogen, USA) supplemented with 50 mg/L gentamycin (Centrafarm, the Netherlands), 2 mM L-glutamine, and 1 mM pyruvate (Gibco Invitrogen, USA). A volume of 100 µl containing $5x10^5$ mononuclear cells was added to round-bottom 96-well plates (Costar, Corning, The Netherlands) and incubated at 37°C and 5% CO2 for both 4h and 24h with either 50 µl RPMI (medium control), *C. burnetii* NM $1x10^7$/ml, *C. burnetii* 3262 $1x10^6$/ml, *C. burnetii* 3345937 $3.2x10^4$/ml, *C. burnetii* 14160-001 $1x10^7$/ml and *E. coli* LPS 10ng/ml in the presence of 50 µl 5% naive goat serum (in house). Following incubation, the supernatant was harvested and stored at -20°C. Subsequently Trizol (Invitrogen, USA) was added to cell pellet and stored at -80°C till further RNA isolation.

Table 1: List of *C. burnetii* isolates used in this study

Name	Country of origin	Source	LPS Phase	MLVA marker and number of repeats											
				01	03	20	21	22	28	24	30	31	34	27	36
C. burnetii 3262	The Netherlands	Goat placenta	I	4	7	19	6	6	3	11	5	3	7	3	13
C. burnetii 3345937	The Netherlands	Human heart valve	I	4	7	19	6	6	3	11	5	3	7	3	13
C. burnetii 14160-001	The Netherlands	Goat placenta	I	3	6	15	6	6	7	13	6	3	9	2	4
C. burnetii Nine Mile	USA	Tick	I	4	7	15	6	6	6	27	6	5	5	4	4

Table 2: Primer sequences for TLR1, TLR2, TLR4 and TLR6

Name	Primer sequence 5'-3' Forward/reverse	Accession no.	Annealing Temperature
TLR1	GGGTTGAGTGCCACACAGTTACCCCATAAGTATCTCCTAAGACCAATAAAA	HQ263209.1	60°C
TLR2	ACTGGGTGGAGAACCTCATGGTCATTTGCCAGGGACGAAGTC	HQ263214.1	60°C
TLR4	TCTGCCTTCACTACAGGGACTTTATGGCTCTTGTGGAAACCTTCCT	HM627213.2	60°C
TLR6	TCTCAAGCATTTAGACCTCTCATTCACTGGGTCAAGTTGCCAAATTC	HQ263211.1	60°C

mRNA expression of Toll-like receptors and cytokines

RNA was isolated from the Trizol samples using the manufacturer's guidelines. In the end, the pellet was re-suspended in 25 µl RNAse free water (Sigma–Aldrich, USA) with 0.5µl RNAseOUT Recombinant ribonuclease Inhibitor (Invitrogen, USA). Further purification of the samples was performed with the DNA-free kit (Ambion, USA) and the concentration of the RNA samples was determined using the Nanodrop (ND1000, NanoDrop Technologies, Inc, USA). Reverse transcription was performed using random primers and SuperScript III Reverse Transcriptase (Invitrogen, USA). PCR on the cytokines mRNA (TNF-α, IL-1β, IL-10 and IFN-γ) was performed using specific goat primers[3] and SYBR Green PCR Master Mix (Applied Biosystems, USA) in an ABI 7500 Real-time PCR system (PE Applied Biosystems, USA). Results were normalized according to the Succinate dehydrogenase complex subunit A (SDHA) gene of the same sample (Bos Taurus, Primerdesign Ltd, United Kingdom). Data was not corrected for the medium control, hereby the influences of the pregnancy on the cytokine mRNA expression remain visible. The primer sets for TLR1, TLR2, TLR4 and TLR6 were designed using PrimerExpress (Invitrogen, USA) (Table 2). The Livak method (2^ΔΔCt) was used to represent the TLR expression data.

Whole blood stimulation

Undiluted heparin blood (500 µl) was stimulated with either nothing or 25 µl heat-killed *C. burnetii* NM 1x10^7/ml. As control, samples were stimulated with concanavalin A (ConA) and/or phytohemagglutinine (PHA). After 24h incubation at 37°C, the samples were centrifuged and the supernatant was stored at -20°C till further investigation. The production of IFN-γ protein by the leukocytes in the whole blood samples was analysed with the Bovigam IFN-γ test kit for cattle (Prionics, Schileren-Zurich, Switzerland) which was evaluated for goats. ELISA results were obtained as Optical Density determined at 450nm (OD450) with a 635nm reference filter (EL 808 Ultra micro plate reader, Bio-tek instruments, USA). Recombinant goat IFN-γ was purchased from Cusabio to invest to proportion between the measured OD values and the IFN-γ concentration. Hereto, a dilution series of IFN-γ was made with a maximum concentration of 25 ng/ml. The IFN-γ concentration and the corresponding OD values are proportional. Because the Bovigam IFN-γ test kit is able to measure goat IFN-γ in a dose-dependent manner, we decided to use this kit in our study.

Statistical analysis

Statistics regarding mRNA expression and cytokine production were performed using GraphPad Prism 5 software (Graphpad). Differences between experimental groups were tested using the Mann-Whitney *U* test, and differences with a p-value ≤0.05 were considered statistically significant. Samples with values lower or higher than two times the standard deviation were defined as outliers and excluded from the statistical analysis. These outliers are indicated in the figures as an open circle. In case the value of the outlier exceeded the y-axis of the graph, its value is mentioned. The PCR efficiency was determined for all TLRs and SDHA using a standard curve generated with different dilutions of a mixture of the cDNA of all samples.

Results

Experimental *C. burnetii* infection in goats

The experimental infection of the goats with *C. burnetii* 3262 was successful in all infected goats as shown by a positive IHC of the cotyledon with adjacent allantochorion of the placenta and by positive PCR of vaginal swabs. All naive goats remained negative in both tests. The IHC pictures of a *C. burnetii* positive and negative cotyledon can be found in the article of Roest *et al.*[4] in which the goats from experiment III resemble the goats in our study. Three infected goats gave birth to dead lambs, the lambs of the remaining six infected goats were alive. The offspring of the naive goats were all alive and healthy.

The weekly antibody response of all goats against *C. burnetii* during the infection period is shown in Figure 1. The antibody response in the infected goats increased strongly 21 days after infection, whereas the antibody response of the naive goats did not alter. The average pregnancy of the infected goats was 143 days (range 128-146 days), significantly shorter (p<0.001) than the 153 days (range 149-157 days) of the naive goats.

TLR up-regulation and cytokine responses in *C. burnetii* stimulated PBMCs from all goats at day 0

Stimulation of PBMCs from all pregnant goats at day 0 (prior to infection) with *C. burnetii* NM for 4 hours resulted in a slightly lower mRNA expression of TLR4, the mRNA expression of TLR1 and TLR6 was slightly up-regulated, and the expression of TLR2 mRNA was strongly up-regulated in comparison with unstimulated PBMCs (Figure 2A). In general, a fold induction of factor 2 indicates biological relevance. Despite the significantly increased TLR1, TLR2 and TLR6 mRNA expression after *C. burnetii* stimulation, the fold induction remained below factor 2. The mRNA expression of IL-1β and IFN-γ was up-regulated after stimulation of PBMCs from naive goats with the positive control *E. coli* LPS (data not shown), showing the reliability of the experimental approach to measure cytokine mRNA expression in goat PBMCs. Stimulation of goat PBMCs with *C. burnetii* NM resulted in an increased mRNA expression of IL-1β and the same trend was observed for TNF-α (Figure 2B).

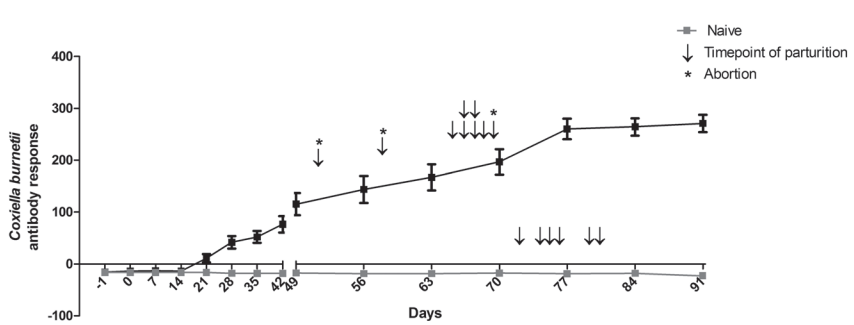


Figure 1: Successful experimental infection of goats with *Coxiella burnetii* 3262

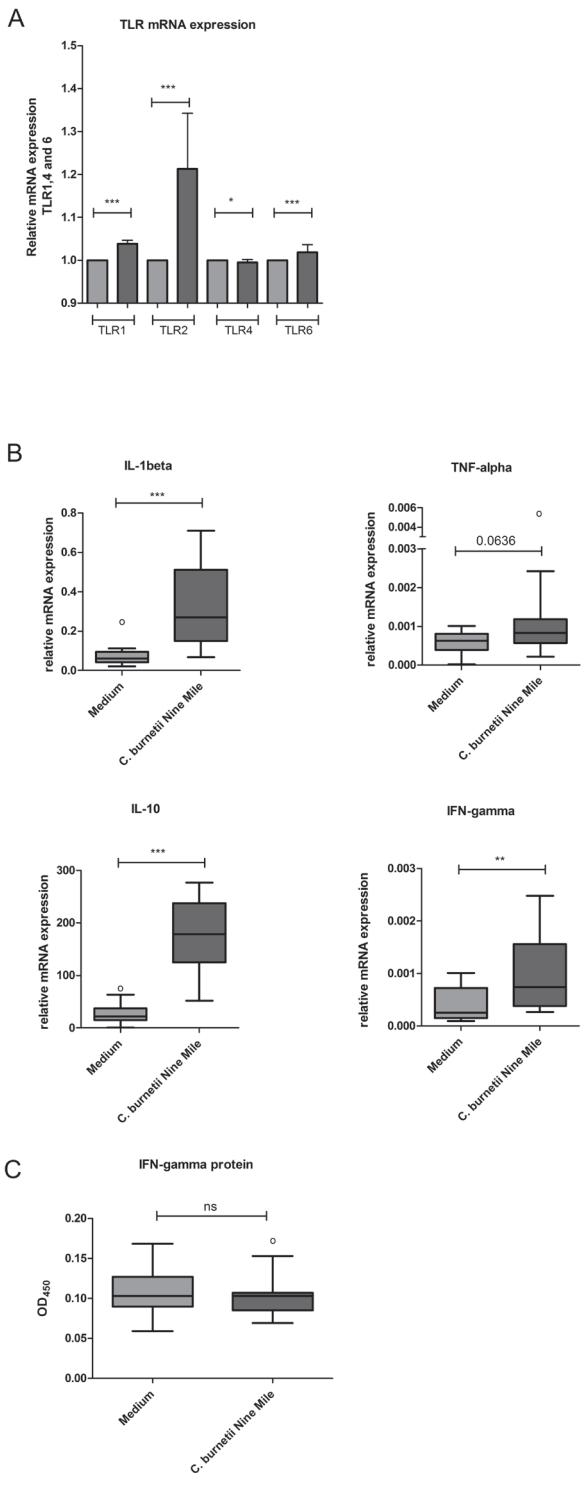
Eleven out of seventeen goats were intranasally infected with *C. burnetii* 3262. The remaining six were kept naive. During the infection, antibodies against *C. burnetii* were measured weekly in the serum of all goats using the LSIVET RUMINANT milk/serum Q-fever ELISA kit. Grey squares represent naive goats, black squares represent infected goats. Median values with SEM are shown. Parturition is indicated for each individual goat using an arrow. Abortion, defined as birth of dead lambs, is indicated with an asterisk. The final two remaining pregnant infected goats were culled for other purposes, and therefore not included in this graph.

In PBMCs stimulated for 24 hours, the expression of the anti-inflammatory cytokine IL-10 strongly increased. Furthermore, the mRNA expression of the T-cell or NK-cell derived cytokine IFN-γ also showed an increase (Figure 2B), although this did not lead to increased IFN-γ protein concentrations in the supernatants of these cells (Figure 2C).

PBMC stimulation experiments from naive and infected pregnant goats during the course of pregnancy

The course of the cytokine mRNA expression of PBMCs was investigated in unstimulated PBMCs, derived from naive and infected goats during the study period of 56 days (Figure 3A). IL-1β mRNA was not up-regulated in PBMCs from both naive and infected pregnant goats. At day 56 of the study TNF-α mRNA expression was increased in PBMCs derived from both naive and infected goats compared to TNF-α mRNA expression at the start of the study. In the infected goats this increase of TNF-α mRNA was significantly higher than in non-infected goats. In contrast, the IL-10 mRNA expression at day 56 was increased in the PBMCs from non-infected goats, while this increase was not observed in the PBMCs of infected goats. Both IFN-γ mRNA and IFN-γ protein levels were not up-regulated in respectively unstimulated PBMCs and unstimulated whole blood from both naive and infected pregnant goats during the study period (Figure 3A and 3C).

To investigate whether stimulation with *C. burnetii* resulted in different TLR up-regulation and cytokine responses of PBMCs derived from naive or infected goats we stimulated these cells *in vitro* with *C. burnetii* NM. The mRNA expression of TLR1, TLR2, TLR4 and TLR6 of *C. burnetii* NM stimulated PBMCs derived from naive and infected goats at day 35 equalled the expression as prior infection (data not shown). The expression of IL-1β and TNF-α mRNA of the infected goats was significantly higher at day 56 of the study than at the start of the study (Figure 3B). In contrast, *C. burnetii* stimulated PBMCs from naive goats did not show an increased IL-1β and IFN-γ mRNA expression. The expression of TNF-α mRNA at day 56 of the study was significantly higher in the infected goats compared to the naive goats. Compared to day zero, IL-10 mRNA expression increased within the 56 days of the study in the naive goats. In contrast, no significant difference in IL-10 mRNA expression between day zero and day 56 was observed in the infected goats. In addition, the expression of IL-10 mRNA was lower in the infected goats compared



with the naive goats at day 7. The infected goats expressed more IFN-γ mRNA after PBMC stimulation starting from day 7 onwards (Figure 3B). Furthermore, at day 56 we found that in infected goats IFN-γ protein in the supernatants was higher compared to naive goats (Figure 3D) and unstimulated PBMCs ($P<0.0017$). At other time points, IFN-γ protein in the *C. burnetii* stimulated samples did not exceed that of the unstimulated samples, but the IFN-γ protein level was significantly lower in the PBMCs obtained of infected goats compared to PBMCs of naive goats at day 7 and higher at day 35. The increased IFN-γ mRNA expression found in the infected goats at day 35 and 56 after *C. burnetii* NM stimulation was not observed when PBMCs of these infected goats were stimulated with *E. coli* LPS (Figure 3E).

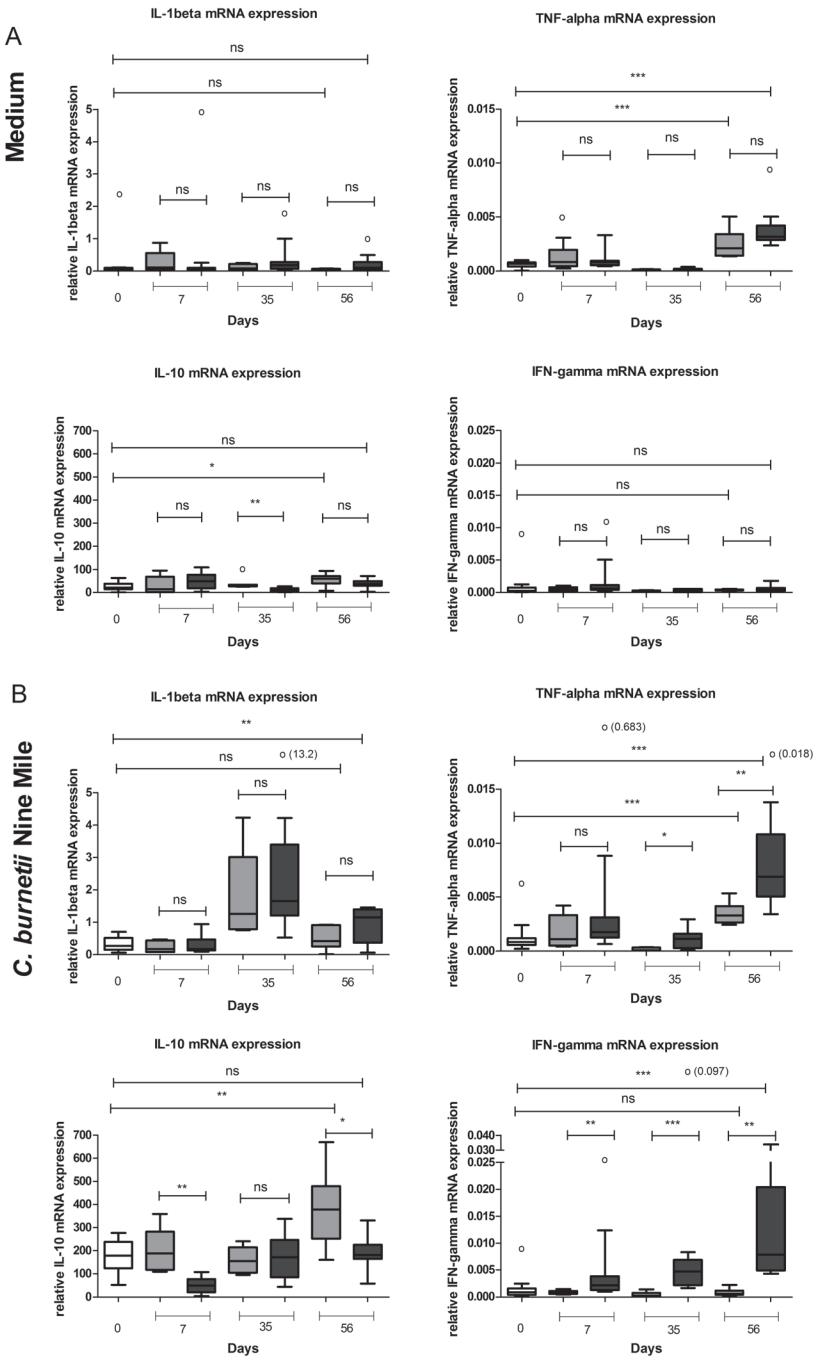
Whole blood stimulation experiments from naive and infected pregnant goats during the course of pregnancy

The role of whole blood components on the early immune response against *C. burnetii* was investigated by stimulation of whole blood from naive and infected goats with nil or *C. burnetii* NM. In non-stimulated blood from both naive and infected goats, IFN-γ production did not significantly alter at day 7 and day 35 of the study. However, at day 56, IFN-γ concentration increased in both naive as infected goats (Figure 4A). Figure 4B shows that *in vitro* stimulation of whole blood with *C. burnetii* NM does not lead to changes in the IFN-γ production in naive goats during the 56 days. In whole blood of the infected goats, the IFN-γ protein level is decreased at day 7 which was not observed in the naive goats. At day 35 of the study, the IFN-γ level is significantly higher in whole blood of infected goats compared to naive goats after stimulation with *C. burnetii* NM.

Figure 2: TLR up-regulation and cytokine responses of *C. burnetii* stimulated PBMCs from naive goats

2A/B) PBMCs were incubated 4h (TLRs, IL-1β, TNF-α) or 24h (IL-10, IFN-γ) with either medium (grey bars) or heat killed *C. burnetii* NM $1x10^7$/ml (dark grey bars). mRNA expression of TLR1, 2, 4, 6 and IL-1β, TNF-α, IL-10 and IFN-γ was determined using qPCR.

2C) IFN-γ protein was measured in the supernatant of PBMCs stimulated for 24h with nil or *C. burnetii* NM $1x10^7$/ml. IFN-γ was measured using the Bovigam Elisa kit. Box and Whisker plots are shown. Outliers are indicated as open circles. NS not significant; *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney *U*-test.



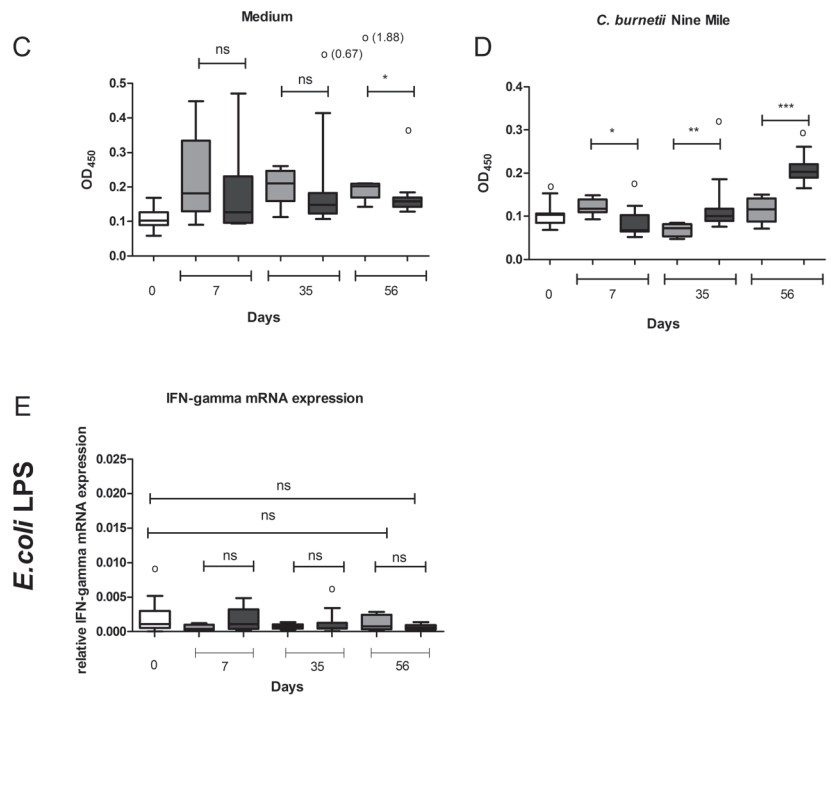


Figure 3: Cytokine profile of *C. burnetii* stimulated PBMCs from naive and *C. burnetii* infected goats

3A/B) EDTA blood was sampled at day 7, 35 and 56 of the study from six naive goats (grey bars) and eleven infected goats (dark grey bars). The white bars show the mRNA expression at day zero. PBMCs were isolated and incubated for 4h or 24h with either medium **(3A)**, heat killed *C. burnetii* NM 1x10^7/ml **(3B)** or *E. coli* LPS 10ng/ml **(3E)**. mRNA expression of TNF-α, IL-1β, IL-10 and IFN-γ was determined using qPCR.

3C/D) IFN-γ protein was measured in the supernatant of PBMCs stimulated for 24h with nil **(3C)** or *C. burnetii* NM 1x10^7/ml **(3D)**. IFN-γ was measured using the Bovigam Elisa kit. Grey bars represent six naive goats, dark grey bars represent eleven infected goats. Box and Whisker plots are shown. Outliers are indicated as open circles. NS; not significant *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney *U*-test.

In vitro stimulation of PBMCs from naive and infected pregnant goats with different *C. burnetii* strains

PBMCs from naive and infected goats were stimulated *in vitro* with four different *C. burnetii* strains to investigate whether all strains were able to induce up-regulation of cytokine mRNA levels. At day zero, only *C. burnetii* 14160-001 induced significantly more TNF-α mRNA compared to unstimulated PBMCs. The IFN-γ mRNA levels increased after PBMC stimulation with *C. burnetii* NM, *C. burnetii* 3262 and *C. burnetii* 14160-001. *C. burnetii* 3345937 stimulation did not induce either TNF-α and IFN-γ mRNA (Figure 5A). At day 35, PBMCs re-stimulated with all strains, except *C. burnetii* 3262, showed a trend towards an increased TNF-α mRNA expression in PBMCs of infected goats than PBMCs of naive goats. IFN-γ mRNA expression was significantly increased in PBMCs from infected goats after encounter with all four *C. burnetii* strains compared to naive goats (Figure 5B).

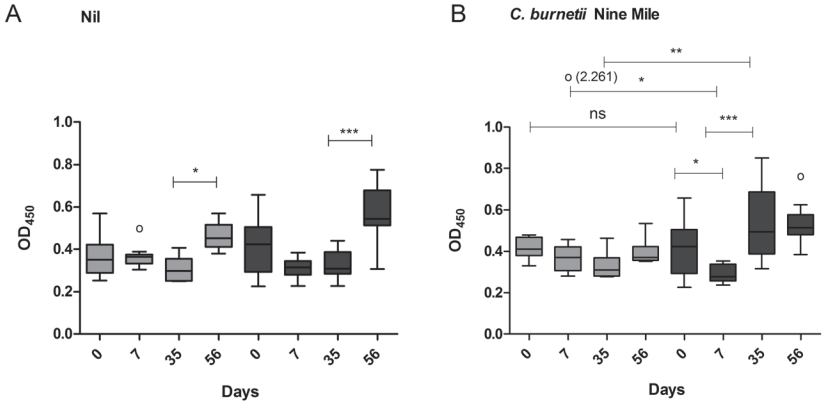


Figure 4: Increased Interferon-γ production in *C. burnetii* infected goats after stimulation with *C. burnetii*

Stimulation of undiluted heparin blood with nil **(4A)** or heat killed *C. burnetii* NM 1x10^7/ml **(4B)**. Grey bars represent six naive goats, dark grey bars represent eleven infected goats. Blood was sampled at day zero and at day 7, 35 and 56 of the study. IFN-γ was measured after 24h incubation using the Bovigam IFN-γ ELISA kit. Box and Whisker plots are shown. Outliers are indicated as open circles. NS; not significant *p<0.05; **p<0.01; ***p<0.001, Mann-Whitney U-test.

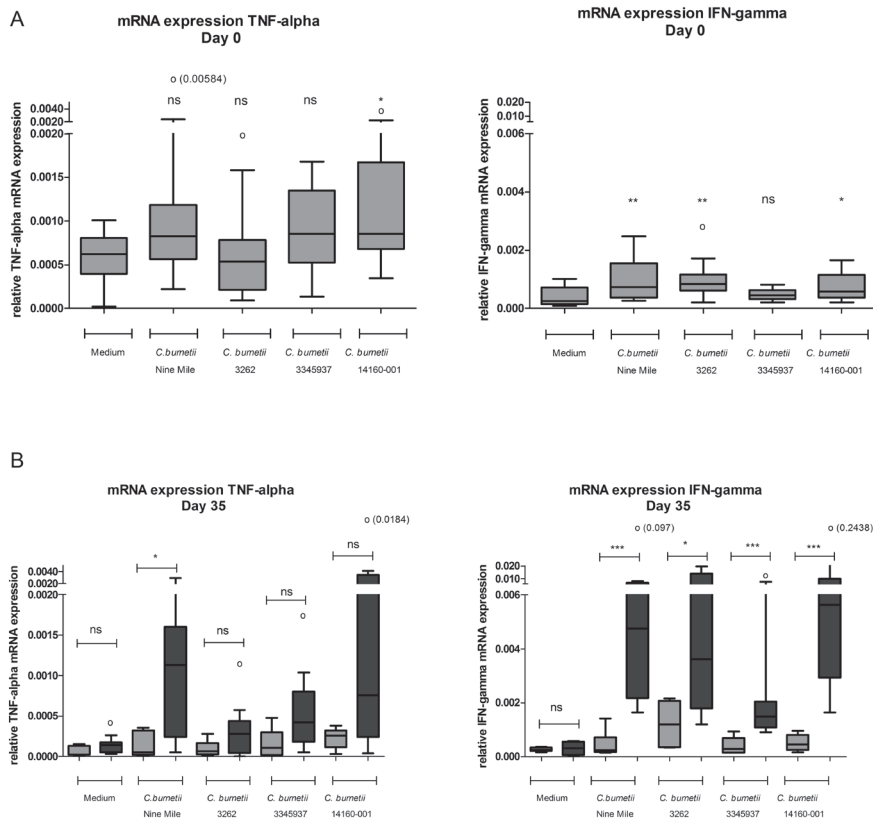


Figure 5: Stimulation of *C. burnetii* infected PBMCs results in an increased cytokine expression

EDTA blood was sampled at day zero **(5A)** and day 35 **(5B)** of the study. Peripheral blood mononuclear cells were isolated and incubated for 4h or 24h with either medium, heat killed *C. burnetii* NM 1x10^7/ml, heat killed *C. burnetii* 3262 1x10^6/ml, heat killed *C. burnetii* 3345937 3.2x10^4/ml, heat killed *C. burnetii* 14160-001 1x10^7/ml. mRNA expression of TNF-α and IFN-γ was determined using qPCR. Grey bars represent six naive goats, dark grey bars represent eleven infected goats. Box and Whisker plots are shown. Outliers are indicated as open circles. NS not significant; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001, Mann-Whitney *U*-test.

Discussion

PBMCs from pregnant goats have a clear cytokine response upon contact with *C. burnetii*. This response is observed both in goats without any previous contact with the bacterium as well as in goats who where recently infected. This latter observation is interesting because infections of pregnant goats do not lead to systemic disease of the animal except for stillbirth of the lamb. In PBMCs of healthy pregnant goats we observed that the mRNA expression of IL1-β, TNF-α, IL-10 and IFN-γ were up-regulated after *C. burnetii* stimulation. These findings correspond with other studies in which increased TNF-α protein levels were found in human monocyte-derived dendritic cells stimulated with the *C. burnetii* outer membrane protein 1, and THP-1 cells which showed increased TNF-α and IFN-γ expression after *C. burnetii* stimulation[17, 18]. Recently, Graham *et al.*[19] demonstrated that stimulation of human alveolar macrophages with *C. burnetii* skewed towards a Th1 response. Increased TNF-α and IFN-γ protein levels were also found in serum from mice challenged with *C. burnetii*, and TNF-α mRNA expression increased in mice of which bone marrow derived macrophages were stimulated with *C. burnetii*[20, 21]. Thus, our results show that PBMCs of goats do not differ from humans and mice in their augmented TNF-α and IFN-γ mRNA response after *in vitro* stimulation with *C. burnetii*. During *C. burnetii* infection increased IFN-γ protein was measured in *C. burnetii* stimulated whole blood of goats which corresponds with the higher IFN-γ protein levels found in infected mice and chronic Q fever patients[20, 22].

The expression of IL-1β, IL-10 and IFN-γ mRNA was not up-regulated in the PBMCs of pregnant goats who were infected with *C. burnetii*. The observed up-regulation of TNF-α mRNA expression in PBMCs derived from infected goats may be seen as a consequence of pregnancy because this pattern was also found in PBMCs from naive goats. A possible explanation for the absence of an increased cytokine mRNA expression is that the non re-stimulated PBMCs did not respond to a nasal infection of *C. burnetii*. One of the reasons can be that in goats the *C. burnetii* infection is localised specifically in the placenta, and therefore no persistent systemic mRNA up-regulation was observed in the PBMCs from infected goats. Ben Amara *et al.*[23] indeed found that *C. burnetii* replicates *in vitro* in placental derived trophoblasts and induces an up-regulation of cytokine mRNA. Therefore, they assumed that the specific inflammatory environment in the placenta, skewed towards tolerance and anti-inflammatory responses, permits persistence of *C. burnetii*. This specific compartmentalized infection is also observed in chronic Q fever patients, as in these persons *C. burnetii* is mainly localised in the heart valve and vessels[1].

At day 56 of our study TNF-α mRNA expression was increased in all goats compared to the initial level, but a significantly higher TNF-α mRNA expression was seen in the infected goats. This suggests that, besides the influence of the pregnancy, infection with *C. burnetii* also increases expression of TNF-α mRNA. We also showed a clear increase in IL-1β and IFN-γ mRNA expression in the *C. burnetii* NM stimulated PBMCs derived from infected goats from day zero till day 56 of the study. However, at day 56, only IFN-γ mRNA expression was significantly higher in the PBMCs from infected goats. The fold induction (*C. burnetii* stimulated PBMCs divided by unstimulated PBMCs) of naive and infected goats at day 56 was respectively 1.96 and 24.21, indicating the large effect on IFN-γ mRNA expression of *C. burnetii* stimulated PBMCs from infected goats.

The fold induction at day 56 in *C. burnetii* stimulated PBMCs from naive and goats was respectively 1.96 and 24.21. Besides the observed mRNA up-regulation, IFN-γ protein levels were increased in *C. burnetii* stimulated PBMCs and whole blood from infected goats compared to naive goats. Thus although no systemic response in goats could be observed, PBMCs of *C. burnetii* infected goats respond differentially to *C. burnetii* stimulation compared to PBMC of uninfected goats. Stimulation with *E. coli* LPS did not lead to increased up-regulation of IFN-γ mRNA expression in the *C. burnetii* infected goats, which demonstrates that the observed IFN-γ response is specific for *C. burnetii*. Even though we hypothesized that *C. burnetii* infected PBMCs are translocated to the placenta, we assume that remaining memory T-cells in the peripheral blood can be activated and play a role in this immune response and increased IFN-γ mRNA expression.

The importance of TNF-α and IFN-γ during *C. burnetii* infection has been shown by Andoh *et al.*[24]. These authors demonstrated that IFN-$\gamma^{-/-}$ and TNF-$\alpha^{-/-}$ mice have respectively high and modest susceptibility to *C. burnetii* NM infection and that the disease progressed rapidly in these mice[24]. In humans, much research has been performed on the cytokine expression in chronic Q fever patients, a condition that in our opinion mostly resembles the *C. burnetii* re-stimulation of PBMCs from infected

goats. TNF-α mRNA and TNF-α protein levels are increased in *C. burnetii* re-stimulated monocytes of chronic Q fever patients compared to healthy controls[9]. Similarly, blood cells of chronic Q fever patients respond to *C. burnetii* stimulation with higher IFN-γ production[10, 22].

The observed up-regulation of IFN-γ mRNA expression after *C. burnetii* NM stimulation was also found for the inoculum strain *C. burnetii* 3262 and the other *C. burnetii* strains which indicates that the different *C. burnetii* strains contain corresponding antigens on their surfaces which are recognized by goat PBMCs. Similar cross reactivity was demonstrated by Arricau-Bouvery *et al.* in a vaccination study in goats[25]. Based on these results we decided to perform our stimulation experiments with *C. burnetii* Nine Mile. The concentrations of the different *C. burnetii* strains used in this experiment were not identical, due to time restrictions and culture difficulties. Therefore, it is not possible to compare the strains and the cytokine mRNA expression they induce. Further studies have to be performed to investigate the possible divers immune response against *C. burnetii* strains by goats.

PBMCs derived from goats infected with *C. burnetii* expressed significantly less or equal amounts of IL-10 mRNA compared to the PBMCs of non-infected goats. We did not expect this, as in humans high IL-10 protein levels are related to chronic Q fever infection, and in acute Q fever patients a slight increase of IL-10 production was observed compared to healthy controls[7, 11]. The lower expression of IL-10 mRNA in the infected goats can be explained by the higher levels of IFN-γ mRNA and IFN-γ protein, as these two cytokines have an antagonistic effect on each other. Our results indicate that PBMCs from infected goats actually did interact with *C. burnetii*, leading to an augmented cytokine mRNA expression after a second *C. burnetii* encounter. The amount of IL-10 mRNA exceeded the mRNA levels of the housekeeping gene much more (up to 400 fold) than for IL-β and IFN-γ. However, the fold induction of IL-10 (*C. burnetii* stimulated PBMCs divided by unstimulated PBMCs) did not differ from the IL-1β fold induction or was even lower in the infected goats at day 35 and 56 when compared with IFN-γ. For example, at day 56, the fold induction of IL-1β, IL-10 and IFN-γ in the infected goats was respectively 5.89, 5.04 and 24.21.

We can speculate that regarding the pro-inflammatory cytokines, TNF-α and IFN-γ, PBMCs of infected goats re-stimulated with *C. burnetii* show the same cytokine profile as chronic Q fever patients, while the IL-10 mRNA expression in infected goats is not up-regulated as found in the chronic Q fever patients. These differences in IL-10 expression could be an explanation for the divergent clinical outcomes in humans and goats during *C. burnetii* infection. Yet, is should be taken in to account that, in contrast with the human study subjects, the infected goats are pregnant which can influence the cytokine responses in general and upon encounter of micro-organisms. Furthermore, possible differences in experimental approaches should be considered, i.e. our conclusions are based on mRNA expression levels, while other studies rely on protein levels in particular.

An interesting finding is the decreased IFN-γ protein level in *C. burnetii* stimulated PBMCs and whole blood from infected goats at day 7 of the study. This reduction was not observed in the PBMCs and whole blood of naive goats and is inconsistent with the increased IFN-γ protein levels observed in the PBMCs obtained from infected goats later in the infection. It can be hypothesized that the initial *C. burnetii* infection in the goats is capable to immune modulate PBMCs to produce less IFN-γ. Other microorganism infections, like *Legionella pneumophila, Leishmania* and *Mycobacterium tuberculosis* are also capable of modulating the Th1 response during early phases of infection[26-28]. Modulation of the IFN-γ response early during the infection, could create a favorable situation for *C. burnetii* to escape the early immune response of the goats, and might therefore be a strategy to pass over to the placenta without being eliminated. PBMCs obtained from infected goats later in the infection did not show the IFN-γ decrease after re-stimulation *in vitro*. This could mean that the immune modulation of PBMCs *in vivo* only occurred early in infection and once the *Coxiella* bacteria have reached the placenta, no systemic immune modulation is needed, or all systemic *Coxiella* bacteria are eliminated. Antigen specific lymphocyte sequestration can also be an explanation for the lower IFN-γ levels in infected goats up to 7 days after the infection. However, our results suggests that the effector T-cell population (Th1) remained in the PBMC fraction during *Coxiella* infection, as the mRNA expression of IFN-γ increased. However, based on the results of IL-10 mRNA expression, we can hypothesize that the regulatory T-cells (Th2) could be transferred to the lymph nodes during *Coxiella* infection.

During first contact with *C. burnetii*, PBMCs of healthy pregnant goats are capable of recognizing *C. burnetii* and activate their early immune response *in vitro*. Further-

more, PBMCs from infected goats showed an augmented pro-inflammatory cytokine mRNA expression compared to PBMCs of naive goats after *C. burnetii* stimulation, whereas the anti-inflammatory cytokine IL-10 was down-regulated. The finding that PBMCs from goats that have previously had contact with *C. burnetii* react differently than naive PBMCs can be useful in future vaccine strategies. Finally, although the PBMC response as found in infected goats is strongly geared towards a pro-inflammatory state, the infection is not cleared and the goats will still suffer abortions and stillbirths.

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07

Coxiella burnetii isolates originating from infected cattle induce a more pronounced pro-inflammatory cytokine response compared to isolates from infected goats and sheep

SUBMITTED

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Abstract

The zoonotic bacterium *Coxiella burnetii* is the causative agent of Q fever. Goats and sheep are the main source for human Q fever outbreaks. Interestingly, although the prevalence of *C. burnetii* in cattle is much higher than in goats and sheep, infected cattle are only rarely associated with human outbreaks. In the present study we investigated whether the immune response of humans differs after contact with *C. burnetii* isolates originating from different host species. In addition, cytokine responses against *C. burnetii* isolates from acute and chronic Q fever patients were compared with *C. burnetii* isolates from animal origin.

Human peripheral blood mononuclear cells (PBMCs) were stimulated with 16 *C. burnetii* isolates with known MLVA genotype from goats, sheep, cattle, acute and chronic Q fever patients. Subsequently, IL-1β, TNF-α, IFN-γ, IL-1Ra, IL10, IL-17 and IL-22 were measured.

C. burnetii isolates originating from cattle induce significantly more IL-1β, TNF-α and IL-22 than the isolates from goats, sheep or chronic Q fever patients. A trend was observed of a higher cytokine induction by acute isolates than those from chronic Q fever patients. Comparing the cytokine induction of the isolates based on their MVLA genotype, instead of their origin, did not reveal any differences in response between the different MLVA genotypes.

The high pro-inflammatory cytokine response induced in human PBMCs by *C. burnetii* isolates from cattle may explain the low incidence of human Q fever outbreaks caused by cattle. The cytokine profile of PBMCs from healthy individuals stimulated with *C. burnetii* isolates derived from chronic Q fever patients resembles isolates from goats, while the acute human isolates induce a similar cytokine pattern as cattle isolates. Furthermore, cytokine responses seem to be more depending on host origin than on MLVA genotype.

Introduction

The zoonotic bacterium *Coxiella burnetii* caused a large outbreak in the Netherlands between 2007 and 2010. More than 4.000 acute Q fever cases were reported, but seroprevalence studies estimated at least >40.000 individuals infected with *C. burnetii*[1, 2]. Besides acute Q fever, *C. burnetii* infection can lead to chronic Q fever in 1-5% of the individuals after an initial infection[3]. The main source for Q fever in humans are sheep and goats. In contrast to the observed clinical symptoms in humans during *C. burnetii* infection, infection in ruminants is mostly asymptomatic. However, in pregnant sheep and goats infection may cause abortion, premature birth, dead or weak offspring and it may cause reproductive disorders in cattle[4, 5]. In 2012 a large review study was conducted in which, among others, the seroprevalence of *C. burnetii*-antibodies in goats, sheep and cattle was investigated[6]. Within-herd prevalence estimates for cattle, goat and sheep were respectively 20.8%, 40.0%, 56.9% (Bulgaria), 15.0%, 88.1%, 20.0% (France), 19.3%, 2.5%, 8.7% (Germany), and 21.0%, 7.8%, 3.5% (the Netherlands)[6]. The herd prevalence (a herd is considered positive when at least one animal in the herd was serologically confirmed) in the Netherlands was up to 37.0% in cattle, 17.8% in goats and 14.5% in sheep. In France, the herd prevalence for cattle, goats and sheep was respectively 73.0%, 40% and 89%[6]. More specifically for cattle in the Netherlands, Muskens *et al.*[7] identified that 268 of 341 cattle herds (78.6%) tested positive for *C. burnetii* antibodies and at 56.6% of the herds *C. burnetii* DNA was detected.

Despite the high percentage of (herd) seroprevalence of *C. burnetii* in cattle, only a minority of the human Q fever outbreaks are attributed to cattle[6]. In addition, while almost all commercial milk products from cattle contain *C. burnetii* DNA, to our knowledge no reports are available indicating that these farmers, their family or milk consumers developed Q fever[8]. Several studies indicate that specific genotypes of *C. burnetii* are associated with cattle[8-10]. However, cattle can also be infected with other genotypes, when more animal species are present on the same farm[11]. It can be hypothesized that humans can become infected by *C. burnetii* isolates originating from both cattle and goat, but infection with cattle isolates results in fewer, milder or no clinical symptoms compared with infections by goat

isolates. We hypothesize that the cattle *C. burnetii* isolates induce a more profound immune response in humans, which can eventually result in more effective elimination of the bacteria leading to absence of clinical disease.

To our knowledge, the current literature does not comprise studies focusing on the human immune response against different *C. burnetii* isolates. In order to check our hypothesis, we conducted the present study in which we used 16 *C. burnetii* isolates with either a different origin according to the host species and/or different MLVA genotype to investigate their cytokine induction upon stimulation of human peripheral blood mononuclear cells (PBMCs). As humans can get infected via either goat, sheep or cattle, *C. burnetii* isolates retrieved from these animals were included in the study. In addition, isolates from both acute and chronic Q fever patients were included to examine whether they induced a comparable cytokine pattern as the animal isolates. Our findings reveal that the origin of the *C. burnetii* isolates has a greater influence on the cytokine production by human PBMCs, compared to the MLVA genotype. Here we provide a possible immunological explanation why *C. burnetii* transmission from infected cattle are less likely to induce infection in humans, which can relate to the minimal numbers of human Q fever outbreaks caused by cattle as stated in the current literature.

Material and methods

C. burnetii isolates

The *C. burnetii* isolates X09003262-001 (3262), CbCVIC1, 8014160-001 (14160-001), 8014160-002 (14160-002) and 18430 were isolated from the placenta of goats or sheep that aborted at different farms during the Q fever outbreak in the Netherlands. The human *C. burnetii* isolates from the Netherlands, 42785537 and 334937 were isolated from the heart valve from two individual chronic Q fever patients from the Radboud University Medical Centre and Canisius Wilhelmina Hospital respectively. The other human isolates, Schperling, Henzerling, Herzberg and Scurry were a kind gift from Dimitrios Frangoulidis of the Bundeswehr Institute for Microbiology (Munich, Germany). These isolates were retrieved from acute Q fever patients, however they were not obtained in the Netherlands during the outbreak, and background information about the assumed origin (infection via goat, sheep or cattle) is missing. Four isolates were obtained in France from goats (1683CbC2 and CbG15D6), sheep (736Cb01) and cattle (701CbB1) and were kindly provided by A. Rodolakis (INRA, France). The second cattle isolate (CbBEB1) came from Belgium and was a kind gift from D. Fretin (Coda Cerva, Bruxelles). All isolates represent LPS phase I. LPS phase determination was performed by SDS-PAGE and silver staining, using purified phase I (RSA493) and phase II (RSA439) *C. burnetii* NM LPS (kindly provided by R. Toman) as controls[12, 13]. More details of the isolates, like origin of the host (referred throughout the article as 'origin'), country and MLVA genotype is described in Table 1. All isolates were cultured on Buffalo Green Monkey (BGM) cells as described previously and heat inactivated at 99 °C for 30 min[14]. All isolated were quantified by qPCR as described in detail by Roest *et al.*[15]. In short, the qPCR targets a single copy gene encoding a *C. burnetii*-specific hypothetical protein (gene bank number AY502846) using the forward primer 5'-ATAGCGCCAATCGAAATGGT-3', the reverse primer 5'- CTTGAATACCCATC-CCGAAGTC-3', and the NED-labelled probe 5'-CCCAGTAGGGCAGAAGACGTTC-CCC-3'. qPCR was performed on a 7500 Fast Real Time PCR system (Applied Biosystems, USA),

MLVA genotyping

MLVA genotyping was performed by using a selection of 12 loci: ms01, ms03, ms20, ms21, ms22, ms24, ms27, ms28, ms30, ms31, ms24 and ms36. The method of MLVA genotyping is described in detail by Roest *et al.* and Arricau-Bouvery *et al.*[10, 16]. A dendrogram based on the MLVA genotype of the isolates was made using BioNumerics 7.5.

Isolation of human peripheral blood mononuclear cells and stimulation with *C. burnetii*

PBMCs from healthy individuals were isolated according to standard protocols (Invitrogen, USA) as described previously[17]. $5x10^5$ cells were added to a round-bottom 96-well plate (Corning, The Netherlands) and incubated with RPMI medium and the 16 *C. burnetii* isolates ($1x10^7$/ml) in the presence of 10% human serum. After 24 hours, 48 hours and 7 days supernatants were harvested, depending on the cytokine to be measured, and stored at -20°C.

Cytokine measurements

Cytokine production was measured in the cell supernatants using ELISA according to the manufacturer's protocols. The following kits were used: IL-10 and IFN-γ (Sanquin, the Netherlands), TNF-α, IL-1β, IL-1Ra, IL-17 and IL-22 (R&D Systems, USA). Absorption was measured at 450 nm using a microplate reader (Bio-Rad Laboratories, the Netherlands). TNF-α and IL-1β were measured after 24h, IL-10 and IL-1Ra after 48h and IFN-γ, IL-17 and IL-22 were measured after 7d incubation.

Statistics

PBMCs of 11 healthy individuals were stimulated with the different *C. burnetii* isolates. The average cytokine production for each isolate was used in a one-way Anova to identify the differences between the groups. In case of IL-1β, TNF-α, IL-10, IL-1Ra, IL-17 and IL-22 the homogeneity of variances was ≥ 0.05, therefore the Bonferonni Post-Hoc test was used to compare the different groups. For IFN-γ, difference between the variances in the groups (<0.05) was calculated and the Dunnett T3 Post-Hoc test was used. The data are expressed as mean (± standard error of the mean). IBM SPSS 18 software was used to perform the statistical analyses and GraphPad Prism 5 software was used generate the graphs. Differences with a P value of < 0.05 were considered statistically significant.

Table 1: Characteristic of the *C. burnetii* isolates used in this study

Isolate	Country	Origin	Passage (in house)	MS 01	MS 03	MS 20	MS 21	MS 22	MS 24	MS 27	MS 28	MS 30	MS 31	MS 34	MS 36	MLVA genotype
Schperling	Kyrgyzstan	Human Acute Q fever	2	4	6	18	7	6	15	3	8	6	3	2	11	
Henzerling	Italy	Human Acute Q fever	3	4	7	19	6	6	7	3	3	5	2	3	13	
Herzberg	Greece	Human Acute Q fever	2	4	7	19				3	3	5	2	3		
Scurry	America	Human Chronic Q fever	4	4	6	15	6	6	9	3	4	6	4	2	4	
42785537	The Netherlands	Human Chronic Q fever	4	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
3345937	The Netherlands	Human Chronic Q fever	11	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
3262	The Netherlands	Goat	4	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
CbCVIC1	The Netherlands	Goat	13	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
14160-001	The Netherlands	Goat	14	3	6	15	6	6	13	2	7	6	3	9	4	CbNL12
1683CbC2	France	Goat	3	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
14160-002	The Netherlands	Goat	12	4	7	19	6	6	11	3	3	5	3	7	13	CbNL01
CbG15D6	France	Goat	3	3	6	15	6	6	13	2	7	6	3	9	4	CbNL12
18430	The Netherlands	Sheep	11		6		6	6	13	2	7	6	3	9	4	CbNL12
736Cb01	France	Sheep	5		6		7	7	11	4	5	6	3	2	2	
701CbB1	France	Cattle	5	3	6	15	6		13	2	7	6	3	9	4	CbNL12
CbBEB1	Belgium	Cattle	4		6	15	6	6	13	2	7	6	3	9	4	CbNL12

Results

***C. burnetii* isolates from cattle induce a pro-inflammatory cytokine response**

PBMCs stimulated with cattle isolates produced significantly more of the pro-inflammatory cytokines IL-1β and TNF-α, compared to the sheep, goat or isolates from patients with chronic Q fever (Figure 1A). In contrast, no significant differences were found between the cattle and the isolates from acute human cases. In case of IFN-γ, human PBMCs produced similar IFN-γ levels after stimulation with all different *C. burnetii* isolates. The general induction of IFN-γ was much lower than for IL-1β and TNF-α. The fold induction of IL-1β ranged from 10x in sheep and more than 40x in cattle isolates compared with no stimulation, whereas, the fold induction for IFN-γ was only 1.44x and 2.6x in sheep and cattle isolates respectively. Besides the high pro-inflammatory cytokine induction of cattle isolates, the isolates from acute human cases also produced high amounts of IL-1β and TNF-α. In case of IL-1β there was a significant difference between isolates from patients with acute Q fever compared to isolates derived from chronic Q fever patients ($p=0.038$) and sheep isolates ($p=0.035$) (Figure 1A).

In addition to the pro-inflammatory cytokines, the anti-inflammatory cytokines IL-10 and IL-Ra were measured. Isolates from acute Q fever patients induced the highest IL-10 and IL-1Ra in human PBMCs, followed by the cattle isolates (no significant differences). Comparing the isolates from chronic and acute Q fever patients shows a trend for a lower IL-10 ($p=0.072$) and IL-1Ra ($p=0.051$) in the isolates from chronic Q fever patients (Figure 1B). Finally the Th17 cytokines, IL-17 and IL-22 were measured. For IL-17, no differences were observed between the groups. In contrast, the IL-22 response was significantly higher in human PBMCs stimulated with cattle isolates compared to the other groups, the differences are significant for sheep ($p=0.002$) goat ($p=0.004$) and isolates from acute and chronic Q fever patients(both $p=0.001$) (Figure 1C). Comparison of the isolates derived from infected cattle and the isolates from individuals with acute Q fever shows that except for IL-22, there are no significant differences in cytokine responses in human PBMCs.

Three of the six goat isolates have a low passage number (3-4 times), while the other three isolates have been passed on BGM cells 12 to 14 times. Despite these differences in passage number, examination by SDS-PAGE and silver staining revealed that all isolates contained phase I LPS. Furthermore, statistical analyses between the low and high passage number goat isolates showed no significant difference in IL-1β and TNF-α production by human PBMCs upon stimulation (data not shown).

The MLVA genotype of *C. burnetii* isolates does not influence the cytokine production of human PBMCs

Besides analyzing the isolates based on their origin, the isolates were discriminated based on their MLVA genotype. Two main genotypes can be distinguished among our isolates. First the genotype, named CbNL01 by Roest *et al.*[10], was the dominant genotype found during the Dutch Q fever outbreak. In total six isolates from our study belong to this genotype, four goat and two isolates from chronic Q fever patients. The second main genotype in our study, referred to as CbNL12, contained two goat, one sheep and the two cattle isolates. The third group consist of the remaining individual genotypes, including the three acute human strains, one chronic human strain and a second sheep strain. The differences in MLVA genotype are visualized in Figure 2, which shows the minimal spanning tree of all *C. burnetii* isolates included in this study. Genotype CbNL12 differs 9 micro satellite (ms) loci from CbNL01 and is situated in a separate genotype. From three isolates, 701CbB1, CbBEB1 and 18430, one or two ms loci were missing. Based on the 10 known ms loci, these isolates belong to the CbNL12 MLVA genotype.

Comparison of the cytokine induction between the diverse genotypes showed no significant differences in the pro-inflammatory cytokines (IL-1β, TNF-α, IFN-γ) (Figure 3A) and other cytokines (IL-10, IL-1Ra, IL-17 and IL-22) (Figure 3B and 3C). Among the isolates with the CbNL12 MLVA genotype, the isolates originated from infected cattle produced more TNF-α, IL-1β, IL-10 and IL-1Ra (data not shown).

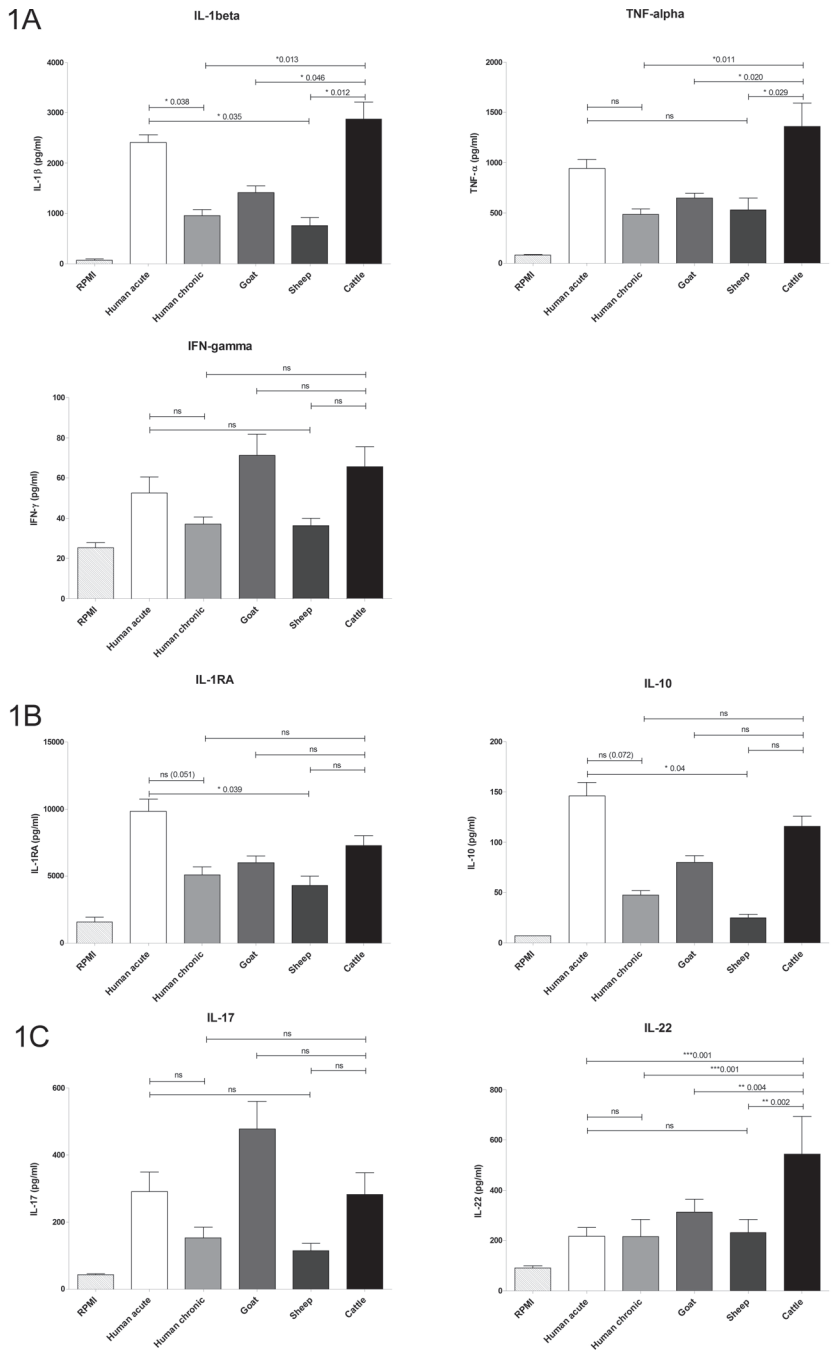


Figure 1: Cytokine profiles of the *C. burnetii* isolates grouped on their origin

PBMCs of 11 healthy individuals were stimulated with RPMI medium (striped bar) and 16 different *C. burnetii* isolates, see Table 1 for the details of the isolates. The following cytokines were measured using ELISA: IL-1β, TNF-α, IFN-γ **(1A)**, IL-Ra, IL-10 **(1B)**, IL-17 and IL-22 **(1C)**. The color of the bars match the following isolates; white (3x chronic human), light grey (3x acute human), medium grey (6x goat), dark grey (2x sheep), black (2x cattle). Statistical differences were tested using one-way ANOVA. In case of all cytokines, except IFN-γ, the Bonferonni Post-Hoc test was used. For IFN-γ, differences were calculates using Fishers Least Significant Difference (LSD). NS: Not Significant, *p<0.05, **p<0.01, ***p<0.001. Data is presented as mean ±SEM.

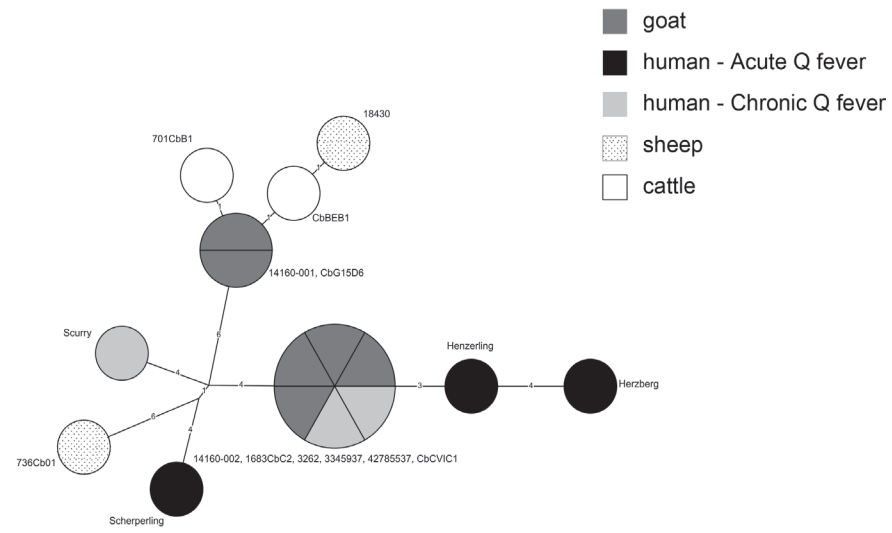
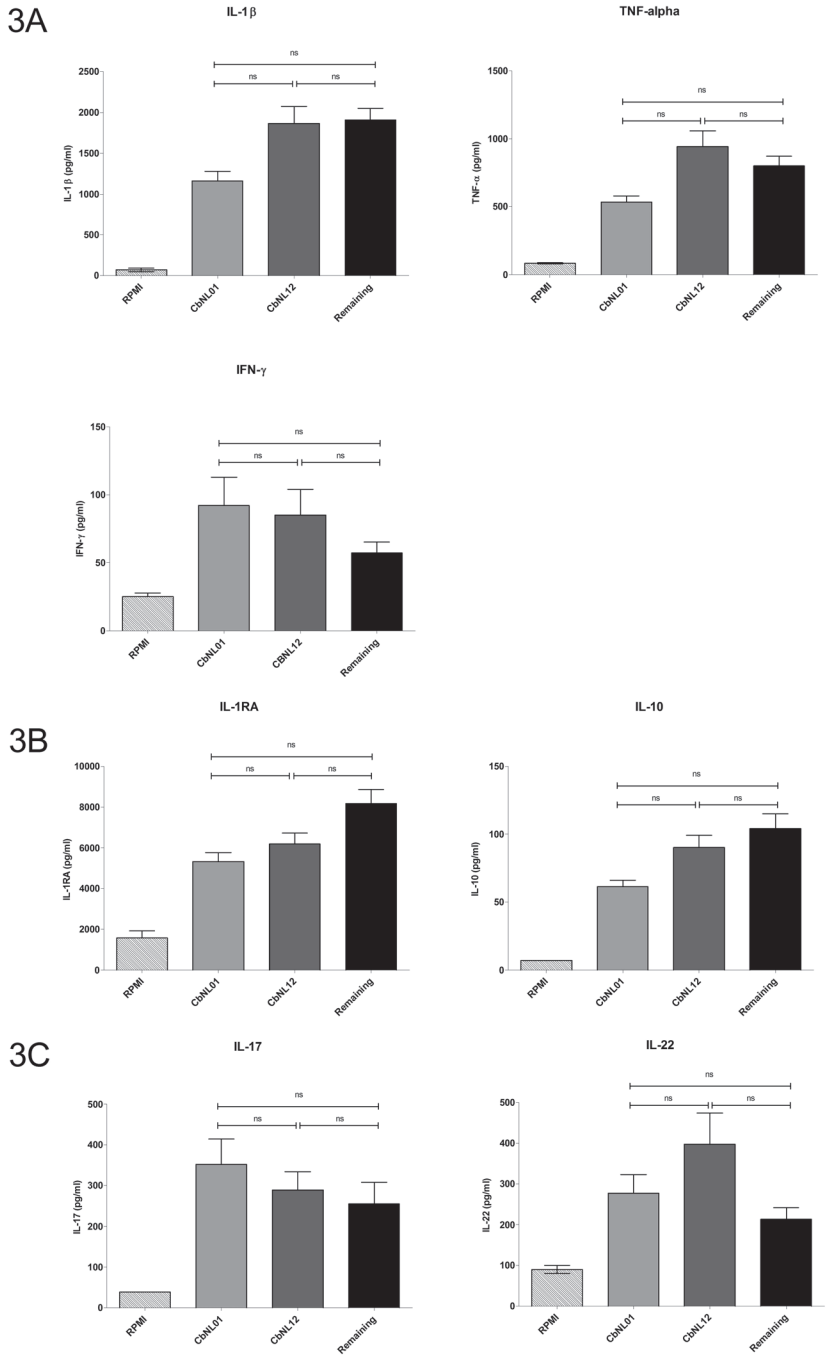


Figure 2: Minimum spanning tree analysis of MLVA genotypes from *C. burnetii* isolates used in the study

A total of 12 micro satellite loci (Table 1) were used to construct a dendrogram of 16 *C. burnetii* isolates. Numbers on the connecting lines refer to the distance between the nodes, e.g. 4 means four different loci. The size of the circles is proportional to the number of strains bearing the same genetic profile. Colors indicate the origin from which the isolates originate; goat (dark grey), human acute Q fever (black), human chronic Q fever (light grey), sheep (dashed) and cattle (white). Branch labels and connecting lines correspond to the number of different markers between the genotypes. Clustering by minimum spanning tree (MST, hypothetical, non-rooted) was performed with Bionumerics 7.5.



Discussion

C. burnetii causes Q fever in humans and a broad range of animals throughout the world. In the majority of the examined cases, goats and sheep are the most likely source for human Q fever outbreaks[6]. Interestingly, in the Netherlands and Germany, the herd prevalence of *C. burnetii* in cattle is much higher than in goat and sheep. This could suggest that infected cattle are a large threat for the human population[6]. However, in the Netherlands and other countries with known human Q fever outbreaks, cattle are scarcely the source of the outbreak[6]. We hypothesized that these cattle *C. burnetii* isolates induce a more profound immune response in humans, which can eventually result in more effective elimination of the bacteria, resulting in less clinical disease.

Our study revealed that the cytokine patterns induced in human PBMCs depends on the *C. burnetii* isolate host origin. Namely, the two cattle isolates induced higher amounts of IL-1β, TNF-α, and IL-22 in human PBMCs than the goat and sheep isolates. In addition, we observed that the MLVA genotype of the *C. burnetii* isolates is not related to the cytokine induction by PBMCs. As the cytokine patterns induced in human PBMCs by *C. burnetii* isolates (grouped on base of their MLVA genotype), revealed no significant differences between the groups.

There are several explanations for the observed differences in cytokine response of human PBMCs against *C. burnetii* isolates originating from different animal hosts, and for the observation that the MLVA genotypes used in this study do not effect these cytokine responses.

Figure 3: Cytokine profiles of the *C. burnetii* isolates grouped on their MLVA genotype

PBMCs of 11 healthy individuals were stimulated with RPMI medium (striped bar) and 16 different *C. burnetii* isolates, see Table 1 for the details of the isolates. The following cytokines were measured using ELISA: IL-1β, TNF-α, IFN-γ **(3A)**, IL-Ra, IL-10 **(3B)**, IL-17 and IL-22 **(3C)**. The color of the bars match the following isolates; white (6x CbNL01), grey (5x CbNL12), black (5x remaining MLVA genotypes). Statistical differences were tested using one-way ANOVA. In case of all cytokines, except IFN-γ, the Bonferonni Post-Hoc test was used. For IFN-γ, differences were calculates using Fishers Least Significant Difference (LSD). NS: Not Significant. Data is presented as mean ±SEM.

First is the common principle of the MLVA genotyping of the *C. burnetii* isolates. We observed that the two cattle isolates with MLVA genotype CbNL12 induced more pro- and anti-inflammatory cytokines in human PBMCs than the two goat and sheep isolates with the same MLVA genotype (data not shown). However, it is not possible to predict the cytokine induction by human PBMCs against a *C. burnetii* isolates based on its MLVA genotype. MLVA genotyping depends on a selection of loci containing tandem repeats are targeted. These loci do probably not code for antigens that are involved in the innate immune response. So, even though the MLVA genotype of the *C. burnetii* isolates is the same, other parts of the genome can differ which might result in diverse responses from host[18].

A second explanation for the observed differences in cytokine response upon stimulation by *C. burnetii* isolates coming from different host, is the expression of diverse virulence factors. Recently, D'Amato *et al.* indicated that *C. burnetii* Z3055, a clone linked to the Dutch Q fever outbreak isolate 3262, contains significantly more non synonymous gene mutations for proteins belonging to the categories of membrane proteins, ankyrin repeat domain-containing proteins, transcription factors and translational proteins[19]. They hypothesized that the epidemic potential of this isolate is based on modifications in the in antigenic surface proteins and growth rate, which results in a new serotype (classification of micro-organisms based on the cell surface antigens)[19]. Differences in membrane proteins or other virulence factors of *C. burnetii* can make individuals less or more susceptible for infection, in addition it could result in fewer or more clinical symptoms depending on the responsiveness of the immune system. It can be hypothesized that the *C. burnetii* isolates from cattle possess specific virulence factors, to which the human immune system is more profound to react.

The responsiveness of the human immune system is the third factor which can explain the induced cytokine differences between PBMCs stimulation with cattle isolates or goat and sheep isolates. This difference in cytokine induction may be explained by the ability of our immune system to mount an adequate pro-inflammatory response to these different strains. This was recently shown for *Cryptococcus* by Schoffelen *et al.*[20], as they concluded that PBMCs from healthy individuals induced a more pronounced inflammatory response against clinical heat-killed *C. gattii* isolates then to other *Cryptococcus* species and non-clinical *C. gattii*[20]. From our study we conclude that human PBMCs are able to induce a cytokine response against all *C. burnetii* isolates tested, independent the origin or MLVA genotype. There is evidence that insufficient induction of pro-inflammatory cytokines result in defective activation of the host immune response on invasion of the organism by pathogenic microbes, resulting in increased susceptibility to infections[21]. Of course, the virulence factors of the *C. burnetii* isolates, as mentioned above as a second explanation, and the responsiveness of the human immune system are linked to each other. The Q fever outbreak in the Netherlands can be explained by the fact that, besides possible differences in virulence of the isolate, the human immune system is not able to induce an optimal response, leading to a higher susceptibility to infection.

A stronger immune response by human PBMCs could lead to a more effective elimination of the bacteria and protection of the host, hence the infection does lead to minimal of no clinical symptoms. Which can explain the low incidence of Q fever outbreaks among humans caused by infected cattle. However, the more profound cytokine response against *C. burnetii* isolates from cattle is most likely not the only reason for the lower incidence of human infections. One can hypothesize that there is no or only limited exchange of genetic material between the niche of the goats and the niche of the cattle. Meaning that the niche of *C. burnetii* from cattle is separated from the niche of *C. burnetii* from goats, and potential virulence factors do not interchange. This hypothesis is strengthened by a study on the prevalence of *C. burnetii* DNA in cow milk and milk products from 18 countries throughout Europe and 10 non-European countries, which revealed the presence of only one predominant genotype[8]. It can be suggested that within this cattle population studied, which covers a widespread geographic area, there is exchange of the same *C. burnetii* isolate. It is possible that the infectious material of cattle does not come in contact with humans or goats, or the infective route of the infected material is not effective. In case the two niches are not in contact with each other, it allows genetic drift among the diverse populations. The route of infection is considered to play a role in the infectiveness of the strains. Infected goats and sheep suffer from abortion and large amounts of *C. burnetii* are shed during the delivery. Due to this large shedding of these infected particles, inhalation is the main infection route in humans. Infected cattle on the other hand, only incidentally show signs of abortion, but their milk products do contain *C. burnetii* which is consumed by humans[8]. Although most of the consumed cattle milk nowadays is pasteurized, Cerf *et al.* concluded in their study that ingestion of *C. burnetii*-contaminated milk may result

in serological conversion potentially indicating infection but not necessarily clinical disease[22]. Some studies do mention that infection via the oral route is possible, but it is infrequent and the doses of *C. burnetii* has to be very high[22].

IFN-γ induced by the cattle isolates was not significantly higher than in the isolates from either goats or sheep. This is an unexpected result as the other pro-inflammatory cytokines IL-1β and TNF-α were significant higher in the cattle isolates than in the isolates from either goats or sheep. One explanation for the observed difference could be the larger variation of IFN-γ production among the PBMCs of healthy individuals, resulting in less mutual differences between isolates of different origin (data not shown). PBMCs of 11 individuals were stimulated with the different isolates and we deliberately choose not to exclude the outliers in order to visualize the variance in cytokine responses. For example, our data shows, that there is a reasonable variation in IFN-γ, Il-17 and IL-22 induction per person. Secondly, as mentioned in the results, the general induction of IFN-γ was much lower than for IL-1β and TNF-α. Besides the significant differences in IL-1β and TNF-α induction of the cattle isolates, IL-22 was significant higher in these isolates than in the goat and sheep isolates. IL-22, in part synergistically with other cytokines such as TNF-α, activates the expression of antimicrobial proteins like defensins. Furthermore, IL-22 promotes the production of inflammatory mediators, such as granulocyte colony-stimulating factor, IL-1β and lipopolysaccharide-binding protein (LBP)[23]. The initiation of these immune responses by IL-1β, TNF-α and IL-22 can explain the better clearance of *C. burnetii* originating from cattle by humans.

The isolates from acute Q fever cases showed the same cytokine profile as the cattle isolates except for Il-22. Whoever these isolates were not retrieved from Dutch patients during the Q fever outbreak but from Kyrgyzstan, Italy and Greece. Unfortunately, there is only limited information on the background of these isolates, and we do not know whether these individuals were infected via cattle, sheep or goat. Although *C. burnetii* infection of human via cattle is less common, based on our study, we speculate that these acute human isolates originated from infected cattle. As they showed similar cytokine responses upon stimulation in human PBMCs and the symptoms of acute Q fever are mild and self-limiting. An inconvenience is that individuals with a possible *C. burnetii* infection via cattle may not see their general practitioner, as they don't experience clinical symptoms. Therefore, identification of *C. burnetii* infection in these individuals, based on serology, cannot be made and leads to less documented human Q fever cases.

The cytokine pattern of PBMCs stimulated with isolates from chronic Q fever patients were similar to that of goat isolates. Based on these findings it is possible to speculate that some *C. burnetii* isolates are inducing acute Q fever, while other *C. burnetii* isolates are responsible for chronic Q fever infections. This hypothesis is strengthened by several studies demonstrating that *C. burnetii* isolates originating from humans with acute Q fever differ in plasmid type[24], lipopolysaccharide profiles[25], and chromosomal DNA restriction endonuclease fragment patterns[26] from many isolates originating from chronic Q fever. In addition, a 28-kDa membrane-associated protein was identified and demonstrated that the acute disease antigen A (adaA) gene is expressed in acute isolates but not carried by chronic isolates, suggesting that adaA may be a virulence factor related to acute Q fever[27]. In contrast, other studies indicated that no specific gene(s) on plasmids are responsible for a specific virulence phenotype[28, 29]. Moreover, during the Dutch Q fever outbreak we assume that the majority of the infected individuals, both acute as chronic, are infected with the same *C. burnetii* MLVA genotype originating from goats. Meaning that one *C. burnetii* MLVA genotype can induce either acute or chronic Q fever infections in humans. In our opinion, the disease outcome of *C. burnetii* infections in humans is multi-factorial. Besides the findings presents in this study, we recently also described that single nucleotide polymorphisms in specific pattern recognition receptors increase the chance to develop chronic Q fever[30]. Taking all this into account, the severity of the infection is influenced among others by the origin and virulence of the *C. burnetii* isolate, as well as the immune status and genetic background of the infected individual. This is strengthened by the fact that many diverse *C. burnetii* isolates are found in goats, which are not detected in human samples. It implies that only the origin of the isolate does not predict the possibility for human infection and disease outcome.

In conclusion, in the present study we investigated the potential of *C. burnetii* isolates from different species of origin and MLVA genotypes to induce cytokine production in human PBMCs. We demonstrated that isolates derived from cattle and from acute Q fever patients induced higher amounts of the pro-inflammatory cytokines TNF-α, IL-1β and IL-22 compared to isolates from other sources and patients with chronic Q fever. Interestingly, this could not be related to differences

in MLVA genotype. The observation that cattle isolates elicit higher amounts of pro-inflammatory cytokines may explain the epidemiological observation that Q fever in humans is seldom related to cattle as a source for human Q fever. Although it must be noted that the strains from acute patients were all not-related to the present outbreak. The observation that isolates from patient with acute Q fever induce a more pronounced pro-inflammatory cytokine response compared to patients with chronic Q fever may signify the importance of the inflammatory response to completely eradicates *C. burnetii* from the host after the initial infection in order to prevent chronic infections.

Acknowledgments

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08

Summary and general discussion

First there was the Q fever outbreak in the Netherlands, secondly an outbreak of researchers occurred.

Maarten van Calmhout (KennisCafé – Ziek van een dier)

Summary

The aim of this thesis was to answer the following questions: how is *C. burnetii* recognized and initiates immune responses in goats versus humans? And how does *C. burnetii* lead to persistence and abortion in goats and chronic disease in humans? Understanding the immunological host response to *C. burnetii* could eventually result in better diagnostic tests for humans and animals, and treatment for patients with Q fever.

Chapter 1 provides background knowledge of Q fever and *Coxiella burnetii*. The zoonotic bacterium *Coxiella burnetii* is the causative agent of Q fever, causing acute or chronic infections in humans and abortions in infected pregnant goats. The Q in Q fever stands for query, as in 1935 the discoverers of the disease did not know what kind of organism caused the illness among humans. Eighty years later much more is known about the *C. burnetii* and the disease it causes. Research increased exponentially when the Netherlands experienced the world largest Q fever outbreak worldwide from 2007 till 2010. The Dutch Q fever outbreak led to many projects investigating all different fields of *C. burnetii*, such as epidemiology, Q fever fatigue syndrome, Q fever in domestic animals, optimizing diagnostics test and so on. Throughout this thesis, the reference strain *C. burnetii* Nine Mile and the Dutch outbreak isolate *C. burnetii* 3262 were mostly used. These two *C. burnetii* strains, and all other *C. burnetii* isolates implemented in the studies, were heat inactivated prior usage.

In **Chapter 2** we examined the involvement of the complement system in the immune response towards *C. burnetii*. The complement system is part of the humoral innate immune response and plays an important role in the elimination of bacteria. The complement system can be activated via three different pathways; the classical pathway, the lectin pathway and the alternative pathway. The classical pathway is initiated by antibodies, the lectin pathway is activated via mannose-binding lectin (MBL), and many bacteria, viruses and fungi can initiate the alternative pathway. Activation of all three pathways eventually leads to the formation the opsonin C3b, the anaphylatoxins C3a and C5a and the terminal complement complex (TCC)[1, 2].

We demonstrated that in healthy, seronegative individuals the alternative pathway was activated by *C. burnetii*, while both the classical and lectin pathway were not involved. In the presence of *C. burnetii* antibodies both classical and alternative pathways were activated. Production of monocyte-derived cytokines such as IL-1β, TNF-α and IL-6 was in part dependent on the activation of the complement system. This was mediated by the anaphylatoxin C5a, which was formed after activation of the alternative pathway by *C. burnetii*. Although *C. burnetii* expresses sugars on its LPS that can be recognized by MBL (D-mannose and N-acetyl-D-glucosamine), no role was observed for MBL in complement activation and induction of monocyte-derived cytokines[3, 4]. Of interest, we did find that MBL augmented induction of T-cell-derived cytokines. This suggest that in case of *C. burnetii* stimulation, MBL interacts predominantly with T-cells to induce T-cell-derived cytokines.

Next to the complement system, recognition of micro-organisms by pattern recognition receptors (PRRs) is very important to initiate a proper innate immune response. PRRs recognize unique pathogen-associated molecular patterns (PAMPs) on the surfaces of microorganisms and activate host-defense mechanisms, such as phagocytosis and cytokine signaling[5]. The main PRRs involved in recognition of bacteria are Toll-like receptors (TLRs; TLR1, TLR2, TLR4, and TLR6) and nucleotide-binding oligomerization domain receptors 1 (NOD1) and NOD2.

In **Chapter 3** we showed that both the *C. burnetii* Nine Mile (NM) strain and the Dutch outbreak isolate *C. burnetii* 3262 were able to induce cytokine production via TLR2 signaling, which suggests that both strains have PAMPs that can activate TLR2. This also accounts for NOD2, which was also identified to be important for the recognition of both strains. Besides these similarities, we also detected some differences between the two strains. Analyses of human PBMCs suggested that *C. burnetii* 3262 was recognized by TLR6, while this was not the case for *C. burnetii* NM. This may be explained by a different binding site of *C. burnetii* 3262 to TLR6, compared with *C. burnetii* NM. Our study showed that in humans both *C. burnetii* NM and *C. burnetii* 3262 were recognized by TLR1, TLR2, and NOD2 and induced a robust pro-inflammatory cytokine response.

In **Chapter 4** we discussed the role of TLR10, which recently has been found to suppress cytokine induction by TLR2 ligands[6]. We showed that HEK293 cells,

transfected with TLR2, TLR10 or TLR2/TLR10, and human PBMCs in the presence of anti-TLR10, showed higher cytokine responses in the absence of TLR10 upon *C. burnetii* stimulation. The function of *C. burnetii* as strong TLR2 ligand was confirmed in this study, as well as the suppressive effect of TLR10.

The findings described in the above chapters were based on *in vitro* studies, using cell lines, human PBMCs or knockout mice. The next step was to investigate whether these findings could be linked to the development of chronic Q fever in humans. In **Chapter 5** we focused on the genetic variation within the TLRs, NLRs and downstream signaling pathways. In total, 25 SNPs in TLR1, TLR2, TLR4, TLR6, TLR8, TIRAP, MYD88, NOD2, ITGAV, ITGB3, and ITGAM were genotyped. It showed that among 139 chronic Q fever patients and 220 controls specific SNPs in NOD2 (1007finsC), TLR1 (R80T) and MyD88 (−938C>A) were associated with the development of chronic Q fever. Besides that TLR1 (R80T) was significantly less frequently present in the patients with proven chronic Q fever than in the control cohort, the presence of this SNP also resulted in lower IL-10 production. This suggests that having lower IL-10 production upon contact with *C. burnetii* may protect against chronic Q fever[7]. The same chronic Q fever and control cohort was used to investigate the effect of SNPs in *MBL2* and *TLR10*. In **Chapter 4** the distribution of two common SNPs in *TLR10* was examined, but both were not associated with the development of chronic Q fever. In **Chapter 2** we showed that the distribution of five SNPs in *MBL2* did not differ between the chronic Q fever patients and the control group. Also, the different MBL haplotypes, which are depending on the genetic background and have a direct effect on MBL serum levels, did not differ between the two groups. To summarize, we identified that individuals with specific SNPS in *NOD2*, *TLR1* and *MyD88* have a higher chance to develop chronic Q fever. In contrast, even though *in vitro* studies on these gene show effect concerning *C. burnetii* recognition, no role for was found for SNPs in *TLR2, TLR6, TLR10* and *MBL2*.

Much of the Q fever research focused on the immune response against *C. burnetii* in humans, while less is known about the responses in goats. More knowledge about this can be useful, as goats are one of the main sources of human Q fever infections. Furthermore, they do not show any symptoms during the *C. burnetii* infection itself, which could indicate that their immune system reacts differently towards *C. burnetii* infection than the human immune system. In **Chapter 6** we showed that naive goats have an increased mRNA expression of IL-1β, TNF-α, IL10 and IFN-γ upon *C. burnetii* stimulation. In addition, TLR2 expression was also strongly up-regulated which corresponds with our findings in humans. During experimental *C. burnetii* infection of the goats, we observed an increase in TNF-α and IFN-γ in *C. burnetii*-infected goats compared to naive goats. Goat PBMCs initiated a robust pro-inflammatory immune response against *C. burnetii in vitro*. Furthermore, PBMCs from *C. burnetii*-infected goats showed augmented pro-inflammatory cytokine responses compared to PBMCs from non-infected goats. However, despite this pro-inflammatory response, goats were not capable of clearing the *C. burnetii* infection.

In the previous chapters we used the Dutch outbreak isolate *C. burnetii* 3262 and the reference strain *C. burnetii* NM. We observed some differences in recognition, as *C. burnetii* 3262 was recognized by TLR6 while *C. burnetii* NM was not, and the inhibitory effect of TLR10 on cytokine induction was more prominent after *C. burnetii* 3262 than *C. burnetii* NM stimulation. During the Q fever outbreak many *C. burnetii* isolates derived from goats, sheep and in a lesser extent from cattle were collected by the Central Veterinary Institute, part of Wageningen University and Research Centre. These isolates and the isolates obtained from chronic Q fever patients collected by the Radboud University Medical Centre and the Canisius-Wilhelmina Hospital were cultured in-house. In **Chapter 7** we used these strains to answer the question whether strains with a diverse origin induced a different cytokine profile upon stimulation of human PBMCs. *C. burnetii* isolates from cattle induced significant more of the pro-inflammatory cytokines, IL-1β, TNF-α and IL-22 than the isolates from goat, sheep or acute Q fever patients. Furthermore, a trend was observed of a higher cytokine induction by acute isolates than those from chronic Q fever patients. When the cytokine profile of the different isolates was distinguished based on their MLVA genotype, no differences in cytokine induction was observed. Literature showed that most human Q fever outbreaks came from infected sheep or goats, and not or in lesser extent from infected cattle[8]. Based on our findings, we can suggest that the reduced pathogenic potential of cattle isolates may be caused by a stronger initial immune response from the human host.

General discussion

The goal of this thesis was to elucidate the recognition and responses of *C. burnetii* by the immune system of healthy individuals and pregnant goats. After this fundamental understanding of the role of important innate immune processes, we implemented our findings to answer the question why only a small proportion of the individuals with an initial infection developed chronic Q fever and whether *C. burnetii* isolates origin from different hosts, such as goat, sheep and cattle, induce a diverse immune response in human PBMCs.

Only very few publications focused on the recognition of *C. burnetii* by PRRs. A few studies showed the important role of TLR2 and NOD2 in the recognition of *C. burnetii* NM and the induction of immune responses in humans and mice[9-11]. A weakness of these studies, for example in the case of NOD2, was that the results were based on mice studies alone. In addition, no literature was available regarding other PRRs like TLR1, TLR6 and TLR10. In goats, only one study examined the cytokine expression upon *C. burnetii* infection and no literature is available on the role of TLRs[12]. The advantage of our research was the comparison between human, mice and goat using *in vitro* experiments as well as examining the effects of SNPs in the concerned PRRs in humans. This enabled us to identify whether *C. burnetii* induces a generic immune response in different hosts. Furthermore, we tackled existing knowledge gaps in literature by investigating the role of TLR1, TLR6 and TLR10 in humans (Chapter 3 and 4). We examined the cytokine response and the upregulation of TLR1, TLR2, TLR4 and TLR6 during *C. burnetii* infection in pregnant goats (Chapter 6). In addition, we were the first to examine the cytokine induction in human PBMCs upon stimulation with *C. burnetii* isolates coming from different animal species (Chapter 7).

Combining our findings with the existing literature, we conclude that the TLR1/TLR2 heterodimer and NOD2 play an important role in the induction of cytokine responses in healthy individuals by both *C. burnetii* NM and *C. burnetii* 3262. In addition, TLR6 is involved in the recognition of the Dutch outbreak strain *C. burnetii* 3262 as well (Chapter 3). Despite the proper recognition of *C. burnetii* 3262 by these TLRs, it was able to cause a large outbreak of Q fever among humans in the Netherlands. Therefore, we can conclude that an efficient response of *C. burnetii* 3262 via PRRs does not prevent initial infection in humans. We can hypothesize that the additional recognition of *C. burnetii* 3262 by TLR6 and the induced cytokine response can lead to a higher phagocytosis of the bacteria by macrophages. The engulfment of *C. burnetii* by these immune cells can be in favor of the bacteria, as it replicates in macrophages. The advanced uptake by macrophages which facilitates the replication of *C. burnetii* can lead to more intense infection in the individual.

The next question we asked ourselves was whether either TLR1, TLR2 or TLR6 are involved in the development of chronic Q fever. Our findings indicate that individuals with a SNP in TLR1, but not in TLR2 or TLR6 have a higher chance to develop chronic Q fever infection (Chapter 4). In healthy goats, we observed an increased mRNA expression of TLR1, TLR4, TLR6 and especially TLR2 in *C. burnetii* stimulated PBMCs (Chapter 6)[13]. Next to the up-regulated mRNA expression of TLRs, we examined the cytokine response induced by *C. burnetii* in humans and goats. Human as well as goat PBMCs are able to induce a cytokine response, specifically TNF-α and IFN-γ, upon encounter with *C. burnetii*. Regardless of the present recognition by PRRs and the initiation of a cytokine response, a small proportion of the infected humans develop chronic Q fever and infected goats suffer from adverse pregnancies. An explanation is that other parts of the host immune response are less activated during *C. burnetii* infection, for example the adaptive immune response which includes T-cells and B-cells. In addition, as we described in Chapter 5, individuals can bear polymorphisms in genes involved in *C. burnetii* recognition, which can lead to a higher chance to develop chronic Q fever infection. Furthermore, *C. burnetii* itself can possess strategies to avoid the immune responses of the host, as this has been characterized in many other micro-organisms[14].

The whole blood IFN-γ assay in goats showed a higher IFN-γ response in pregnant goats experimentally infected with *C. burnetii* than naive goats (Chapter 6). Similar findings were observed in humans by Schoffelen *et al.*, as the IFN-γ response was significantly higher in individuals with a chronic infection compared to individuals with a previous Q fever infection[15, 16]. However, we do not know how the *C. burnetii*-induced IFN-γ production relates to the IFN-γ induction by viruses or other bacteria in these chronic Q fever patients. It is possible that the IFN-γ produc-

tion in chronic Q fever patients is not sufficient to effectively clear the bacterium, and not all chronic Q fever patients induced these large amounts of IFN-γ upon *C. burnetii* stimulation. Treatment of chronic Q fever patients with additional IFN-γ may therefore result in regression of the disease. To test this hypothesis, a chronic Q fever patient (with a low IFN-γ response) recently underwent IFN-γ treatment in the Radboudumc. We hypothesize that an IFN-γ treatment could also potentially resolve *C. burnetii* infection in goat. However, in contrast to humans, IFN-γ treatment of *C. burnetii* infected goats would not be cost-effective.

In literature, it is often mentioned that 1-5% of the individuals with an initial infection will develop chronic fever. We know that individuals with preexisting cardiac valvulo-pathy, vascular grafts and aneurysms and immunosuppression have a higher chance to develop chronic Q fever. In addition, our study showed that individuals with a specific SNP in either *MyD88*, *NOD2* or *TLR1* have a significantly higher chance to develop chronic Q fever (Chapter 5)[17]. Although TLR2 is an important receptor in the recognition of *C. burnetii*, polymorphisms in the TLR2 gene were not associated with a higher chance to develop chronic Q fever. In addition, SNPs in TLR2 and TLR4 were also not involved in the development of acute Q fever, as demonstrated by Everett *et al.*[18]. An explanation that the SNPs in TLR2 and TLR4 we and others examined did not play a role in the development of acute and chronic Q fever, can be that they did not interfere with the binding site of *C. burnetii*, resulting in no functional effect of the SNP on *C. burnetii* recognition.

A question rising from literature and our own findings, is whether we could compare the immune recognition and responses between goats and human? Based on the induction of the pro-inflammatory cytokines, TNF-α and IFN-γ, we can hypothesize that PBMCs of infected goats show a similar cytokine profile to chronic Q fever patients, while the IL-10 mRNA expression in infected goats is not up-regulated as found in the chronic Q fever patients[7, 19]. These differences in IL-10 expression could be an explanation for the divergent clinical outcomes in humans and goats during *C. burnetii* infection. However, it should be taken in to account that the experimental set-up differed between the two hosts. The main difference was that in our study goats were experimentally infected with *C. burnetii*, while the chronic Q fever patients in the studies of our colleagues were infected naturally. In addition, in goats we measured mainly mRNA expression of cytokines, while human studies are predominantly based on protein expression. mRNA expression does not always reflects the actual amount of proteins produced. Furthermore, the goats in the study were pregnant. Pregnancy is a necessity for goats to develop clinical Q fever, as non pregnant goats do not show any symptoms of the infection. Until recently, pregnancy in humans was considered a risk factor to develop chronic Q fever. However, studies performed during the Dutch Q fever outbreak did not show clear evidence for an increased probability to develop chronic Q fever during pregnancy[20, 21]. Based on the above mentioned differences and similarities between the goats and human immune response against *C. burnetii*, it is difficult to give an answer on the question whether the immune response is comparable. They are two independent models, which should be used to answer questions related to their own environmental background and situation. Though, with our approach to investigate the response in both models, we can conclude that *C. burnetii* itself is able to induce an innate immune response, and can be recognized in both humans and goat. We recommend that future studies on the *C. burnetii*-induced immune response in humans focus on *in vitro* studies using human immune cells, and we expect that much more information can be obtained from genetic studies among Q fever patients. Studies that focus on the *C. burnetii* infections and the immune responses in goats and other animal hosts are important and should not be neglected. Because of the current intensive herding, and the fact that humans and ruminants live close together, possible future outbreaks can be expected, when the protective vaccination of goats is abandoned. More knowledge on the epidemiology, transmission and immune responses in the animals hosts is always appreciated and useful to prevent new outbreaks, find better diagnostics or treatments.

As *C. burnetii* is been classified as a group B bioterrorism agent, experiments with the live bacteria need to performed in a level 3 laboratory. To our knowledge, no studies are published comparing both live and heat killed *C. burnetii* and the effect on cytokine induction and recognition by PRRs. We decided to use heat inactivated *C. burnetii* in our studies for two reasons. First, we investigated the initial recognition of *C. burnetii*: does stimulation of human and goat PBMCs with *C. burnetii* induce a cytokine response, and which PRRs of these PBMCs are involved. We did not examine the replication or trafficking inside the immune cells. Secondly, it was a practical consideration: we were able to use one single batch of heat inactivated *C. burnetii* for all experiments in this thesis. There might be a possibility that our findings do not apply to live *C. burnetii* as heat inactivation may influence

the antigen exposure on bacteria and the interaction with the immune system may be altered. For example, in previous studies investigating the role of TLR2 in *C. burnetii* recognition, live *C. burnetii* was used to infect TLR2 specific knockout mice. However, the important role of TLR2 was identified both in these previous studies and in our study, showing the indifference of the usage of live or heat inactivated *C. burnetii*[9, 10, 22]. In addition, both live and heat inactivated *C. burnetii* have been used in previous human cytokine studies, to which we could compare our findings[19, 23].

In the studies conducted for this thesis we made use of both *C. burnetii* NM RSA 493 and the Dutch outbreak strain *C. burnetii* 3262. We chose these particular strains, because the former is the laboratory reference strain and used by other research groups. And the latter caused the Dutch outbreak which led to the culling of many goats, and acute and chronic infections in humans. As mentioned in the above summary, we did identify some differences between the Nine Mile and the Dutch outbreak isolate. One frequently asked question is what determines these differences and whether we can speculate about the different gene expression of these two strains. At the moment, the only comparison can be made based on MLVA genotype, which indeed differs at several loci. Additional information about the genetic background will come available upon the publication of the de novo sequence of the Dutch outbreak isolate *C. burnetii* 3262. Genome comparison between *C. burnetii* NM and *C. burnetii* 3262 could identify differences in the genetic parts corresponding for virulence factors like LPS or proteins. In addition, it could be used to compare possible changes like polymorphisms in genes coding for PRRs.

C. burnetii 3262 represents the CbNL01 MLVA genotype, which was the predominant genotype found during the Q fever outbreak in the Netherlands and caused the largest outbreak of Q fever ever reported worldwide. Besides increased exposure of humans to this pathogen due to intensive goat herding[24], it may be hypothesized that this strain is less effectively recognized by PRRs. Inadequate recognition of *C. burnetii* 3262 can subsequently result in fewer responses such as the induction of pro-inflammatory cytokines, phagocytosis and elimination of the bacteria. In the end this could explain the more infectious and disease causing features of *C. burnetii* 3262. Our results however indicate that *C. burnetii* 3262 is able to induce

an adequate immune response in PBMCs and is recognized by TLR1, TLR2 and, in contrast to *C. burnetii* NM, also TLR6. We therefore conclude that the hypothesis that *C. burnetii* 3262 was able to cause disease in many individuals due to altered recognition by PRRs and diminished cytokine responses, is less likely.

An interesting finding is the cytokine profile of *C. burnetii* isolates with a cattle origin, which shows the highest production of pro-inflammatory cytokine in human PBMCs once compared with isolates coming from either human, goats or sheep. It is not known what makes these cattle isolates less virulent. It can be questioned whether they lack several escape strategies or contain a lesser stimulatory LPS or other surface antigens, which enables the hosts immune system to respond more effectively. Structural comparison studies or *in vivo* studies with different isolates can provide more answers. The findings of this study can also be used in the prediction of potential Q fever outbreaks. We know from literature that cattle are less often the source of Q fever infection in humans, and our study relates to this as the human immune systems reacts with a high pro-inflammatory cytokine production upon stimulation with cattle related *C. burnetii* isolates. During the Dutch Q fever outbreak, many pregnant goats were culled, as they were a potential source to infect humans with *C. burnetii*. In contrast, from our study we can assume that during a potential Q fever outbreak among cattle, there is less need to cull these animals as infected individuals will have a more effective immune response (expecting to result in no or limited clinic) against *C. burnetii* isolates derived from cattle. From the point of infected human individuals, specific serodiagnostics could be developed to identify the strains origin and relate it to its virulence and chance to initiate a chronic infection. If we are able to characterize these host dependent virulence factors, diagnostics test could be developed to define the origin of the *C. burnetii* strains and the expected clinical cause in humans.

One of the research questions of this thesis was to investigate the immune recognition and response in pregnant goats and why *C. burnetii* infection leads to abortion, without additional clinical symptoms. We found that goats induce a pro-inflammatory cytokine response upon stimulation with *C. burnetii* and the mRNA of TLRs is also upregulated. It seems that the goat immune system is able to induce a proper initial recognition and immune response, both humoral and cellular[12, 13]. Our studies did show that in the first week of infection in pregnant goats, IFN-γ production was reduced[13](Chapter 6). It can be hypothesized that *C. burnetii* suppresses

IFN-γ responses early during infection, making it possible for the bacterium to be transported to the trophoblasts in the placenta without being noticed by the immune system. However, more research is needed to answer the question how the *C. burnetii* infection eventually leads to abortion and still birth. For example, to investigate the role of IFN-γ on *C. burnetii* replication and survival. It is known from literature that IFN-γ facilitates *C. burnetii* killing, however these experiments were performed in THP-1 (monocyte-like) cell-line, and not in cells more specifically related to the placenta[25, 26]. In addition, a successful pregnancy depends largely on mechanisms by which the immune system of the mother is made tolerant to the semi-allogeneic fetus. The effect of *C. burnetii* on immunomodulators such as arginase and tryptophan during pregnancy have not been investigated yet and could provide new insights.

In the end, the final question remains why infection with *C. burnetii* results in chronic infection in some of the infected individuals. In my opinion, the answer can only partly be found in the hosts immune response to the *C. burnetii* isolate itself. It is a combination of factors, including the initial dose of *C. burnetii* infection, the virulence and origin of the *C. burnetii* isolate, and the immune status, presence of predisposing vascular factors and genetic background of the infected individual. There remain many possibilities to investigate in the future, in which these aspects should all be considered and combined.

Recommendations and future scientific perspectives

The results presented in this thesis contribute to answer a broad range of questions and knowledge gaps in Q fever infections. We gained more information about the immune response against *C. burnetii* in humans and goats and our findings are useful for other researchers in the Q fever field and future studies in both the veterinary as the human field. By understanding more about the immunological response to *C. burnetii*, we eventually hope to find better diagnostic tests for humans and animals and treatments for patients with Q fever.

One of the problems is the diagnoses of *C. burnetii* infection in goats, as it is difficult to determine whether goats suffer from a previous or active infection. We have identified that goats are able to induce a pro-inflammatory cytokine response after encounter with *C. burnetii*. Diagnostics for early detection of *C. burnetii* infection in goats seems to be possible by measuring *C. burnetii* specific induced IFN-γ in whole blood, as we show that infected goats induce significant lower IFN-γ (Chapter 6). However, these possibilities to measure IFN-γ and the application in these purposes need to be further investigated.

In this thesis we demonstrated that individuals with a specific SNP in either MyD88, NOD2 or TLR1 have a higher chance to develop chronic Q fever (Chapter 5). These findings can be implemented in the diagnosis of chronic Q fever, and making it possible to detect individuals with a larger preposition to develop chronic Q fever in an early stage. If it is known whether a patient with acute Q fever and risk factors to develop chronic infection carries such a mutation, prophylactic treatment may be considered. Another advantages of the genetic study we performed was the ability to obtain and use a large collection of DNA samples from chronic Q fever patients and matched controls. With approximately 140 samples of chronic Q fever patients, it is the world largest cohort and the samples are very precious for further studies. In the present studies, we only investigated the effect of genetic variation in specific recognition receptors on the development of chronic Q fever. In future studies it would be interesting to compare healthy *C. burnetii* seronegative individuals with individuals with an acute Q fever infection. Even a comparison can be made between acute Q fever patients with and without hospitalization. It can be hypothesized that during Q fever infection, individuals with SNPs in genes involved in the initial recognition, such as cytokines and PRRs, are more often hospitalized than individuals without these SNPs. A previous study did not find an association with SNPs in TLR2 and TLR4 with disease manifestations in acute Q fever[18]. The role of TLR1, NOD2 and MyD88, which are linked to the development of chronic Q fever, are not investigated so far. The findings will provide more information about which receptors and pathways are involved in the initial recognition of *C. burnetii*.

A final recommendation based on our research concerns the diverse clinical outcome in humans infected with different *C. burnetii* isolates. We observed higher pro-inflammatory cytokine response in human PBMCs stimulated with cattle

derived *C. burnetii* isolates than with *C. burnetii* isolated from goats, which means that the human immune response has a different ability to react on these isolates. In addition, we observed that isolates with the same MLVA genotype, induced a different cytokine response in human PBMCs. The identification and classification of *C. burnetii* isolates is currently based on MLVA genotyping, however it does not provide any information about the possible virulence of the isolate. For our knowledge about the classification of *C. burnetii*, *C. burnetii* infection and future (serological) diagnostics (as mentioned in the general discussion section), investigating the virulence factors of *C. burnetii* isolates is very interesting. It will give us more information which virulent factors of *C. burnetii* are important for human infection, and potential new vaccines or treatment can be adjusted to these findings.

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09

Nederlandse samenvatting

Eerst was er de Q-koorts uitbraak in Nederland,
daarna volgde er een uitbraak van onderzoekers.

Maarten van Calmhout (KennisCafé – Ziek van een dier)

Samenvatting

Het doel van dit proefschrift was om de volgende vragen te beantwoorden: hoe wordt *C. burnetii* herkend en hoe activeert het een immuun reactie in geiten en mensen? En hoe kan *C. burnetii*-infectie leiden tot persistentie en abortus in geiten en chronische infectie in mensen? Meer kennis over de immunologische reactie van de gastheer kan leiden tot betere diagnostiek in mens en dier, en uiteindelijk tot doelgerichtere behandeling van patiënten met chronische Q koorts.

Hoofdstuk 1 geeft achtergrond informatie over Q-koorts en *Coxiella burnetii*. De zoönotische bacterie *Coxiella burnetii* is de veroorzaker van Q-koorts, wat kan leiden tot acute en chronische infecties in mensen en abortussen in geïnfecteerde drachtige geiten. De Q in Q koorts staat voor 'query' ('onbekend'), omdat de onderzoekers tijdens de ontdekking in 1935 geen idee hadden wat voor soort organisme de ziekte in mensen veroorzaakte. Tachtig jaar later is er veel meer bekend over *C. burnetii* en de ziekte die het veroorzaakt. Het onderzoek naar Q-koorts is exponentieel gestegen toen Nederland van 2007 tot en met 2010 te maken kreeg met de grootste Q-koorts uitbraak wereldwijd. De Nederlandse Q-koorts uitbraak leidde tot veel verschillende onderzoeken, waaronder epidemiologie, het Q-koorts vermoeidheid syndroom, Q-koorts in huisdieren, het optimaliseren van diagnostische testen, etc.

In **Hoofdstuk 2** hebben we de rol van het complement system binnen het immuun response tegen *C. burnetii* onderzocht. Het complement systeem is onderdeel van het humorale aangeboren immuun response en speelt een belangrijke rol bij de eliminatie van bacteriën. Het complement systeem kan geactiveerd worden via drie verschillende wegen; de klassieke weg, de lectine weg en de alternatieve weg. De klassieke weg wordt geactiveerd door antilichamen, de lectine weg door mannose-bindend lectine (MBL), en vele bacteriën, virussen en schimmels kunnen de alternatieve weg activeren. Uiteindelijk leidt de activatie van alle drie de wegen tot de formatie van opsonin C3b, de anaphylatoxines C3a en C5a en het terminale complement complex (TCC)[1, 2]. We laten zien dat in gezonde, seronegatieve individuen de alternatieve weg werd geactiveerd door *C. burnetii*, terwijl zowel de klassieke als de lectine weg niet betrokken waren. In de aanwezigheid van anti-

lichamen tegen *C. burnetii* werden zowel de klassieke als de alternatieve weg geactiveerd. De productie van IL-1β, TNF-α en IL-6 was gedeeltelijk afhankelijk van de activatie van het complement systeem. Dit werd gemedieerd door C5a, welke was gevormd na de activatie van de alternatieve weg door *C. burnetii*. Hoewel *C. burnetii* LPS bepaalde suikers bevat die kunnen worden herkend door MBL (D-mannose en N-acetyl-D-glucosamine), vonden we geen rol voor MBL in de activatie van het complement systeem of in de inductie van IL-1β, TNF-α en IL-6 [3, 4]. Een interessant observatie was dat MBL wel de inductie van T-cellen-geproduceerde cytokinen verhoogde. Dit suggereert dat in geval van *C. burnetii* stimulatie, de interactie van MBL vooral plaats vindt met T-cellen om vervolgens T-cellen-geproduceerde cytokinen (IL-17, IL-22 en IFN-γ) te induceren.

Naast het complement system, spelen de patroon herkennings receptoren (pattern recognition receptors (PRRs)) een zeer belangrijke rol bij het initiëren van een goed aangeboren immuun respons. Deze PRRs kunnen unieke componenten op het oppervlak van micro-organismen herkennen (pathogen-associated molecular patterns (PAMPs)), en vervolgens het immuun response van de gastheer activeren[5]. De belangrijkste PRRs in de herkenning van bacteriën zijn de Toll-like receptoren (TLRs; TLR1, TLR2, TLR4, en TLR6) en de nucleotide-binding oligomerization domain receptoren 1 (NOD1) en NOD2.
In **Hoofdstuk 3** laten we zien dat zowel de *C. burnetii* Nine Mile (NM) stam en de Nederlandse uitbraak isolaat *C. burnetii* 3262 in staat zijn cytokines te induceren via TLR2. Dit betekent dat beide stammen PAMPs bezitten die TLR2 kunnen activeren. Hetzelfde geldt voor NOD2, welke ook belangrijk is voor de herkenning van beide stammen. Naast deze overeenkomsten tussen de twee *C. burnetii*-stammen, hebben we ook enkele verschillen gevonden. Ons onderzoek, waarin gebruik wordt gemaakt van humane perifeer bloed mononucleaire cellen (PBMCs), suggereert dat *C. burnetii* 3262 wordt herkend door TLR6, terwijl dit niet geldt voor *C. burnetii* NM. Dit zou verklaard kunnen worden doordat TLR6 verschillende bindingsplekken heeft voor *C. burnetii* 3262 en *C. burnetii* NM. Onze studie laat zien dat in gezonde vrijwilligers, *C. burnetii* NM en *C. burnetii* 3262 worden herkend door TLR1, TLR2 en NOD2, en dat beide stammen een robuust pro-inflammatoir cytokine response induceren.

In **Hoofdstuk 4** bediscussieerden we de rol van TLR10, deze receptor onderdrukt de cytokine productie door TLR2 liganden[6]. We laten zien dat HEK293 cellen, getransfecteerd met TLR2, TLR10 of TLR2/TLR10 en humane PBMCs in de aanwezigheid van anti-TLR10, een verhoogd cytokine response laten zien na *C. burnetii* stimulatie in de afwezigheid van TLR10. Deze studie bevestigd zowel de functie van *C. burnetii* als een sterke TLR2 ligand, als het onderdrukkende effect van TLR10.

De resultaten die we in de bovenstaande hoofdstukken hebben beschreven zijn gebaseerd op *in vitro* studies, zoals cellijnen, humane PBMC of knock-out muizen. De volgende stap was om te onderzoeken of deze bevindingen konden worden gekoppeld aan de ontwikkeling van een chronische Q-koorts infectie in patiënten. In **Hoofdstuk 5** lag de aandacht op de genetische variatie binnen de TLRs, NLRs en adaptor moleculen en de ontwikkeling van chronische Q-koorts. In totaal zijn er 25 single nuceotide polymorphisms (SNPs) gegenotypeerd, in TLR1, TLR2, TLR4, TLR6, TLR8, TIRAP, MYD88, NOD2, ITGAV, ITGB3, en ITGAM. Ons onderzoek onder 139 chronische Q-koorts patiënten en 220 controles, toonde aan dat specifieke SNPs in NOD2 (1007finsC), TLR1 (R80T) en MyD88 (−938C>A) geassocieerd zijn met de ontwikkeling van chronische Q-koorts. Behalve dat TLR1 (R80T) significant minder aanwezig was in de patiënten met een bewezen chronische Q-koorts, leidt de aanwezigheid van deze SNP ook tot minder IL-10 productie. Dit suggereert dat een verminderde IL-10 productie na contact met *C. burnetii* mogelijk kan beschermen tegen de ontwikkeling van chronische Q-koorts[7].

Hetzelfde cohort van chronische Q-koorts patiënten hebben we gebruikt om het effect van de SNPs in *MLB2* en *TLR10* te onderzoeken. In **Hoofdstuk 4** onderzochten we de verdeling van twee bekende SNPs in *TLR10*, maar beide zijn niet geassocieerd met het ontstaan van chronische Q-koorts. In **Hoofdstuk 2** laten we zien dat de distributie van vijf SNPs in *MBL2* niet verschilt tussen chronische Q-koorts patiënten en de controle groep. Ook de verschillende MBL haplotypen, welke afhankelijk zijn van de genetische achtergrond en een direct effect op de MBL serum levels hebben, verschillen niet tussen beide groepen. Samenvattend laten de genetische studies zien dat individuen met specifieke SNPs in *NOD2, TLR1* en *MyD88* een grotere kans hebben om chronische Q-koorts te ontwikkelen. In tegenstelling tot onze *in vitro* studies, die een rol voor *TLR2, TLR6, TLR10* en *MBL2* in de herkenning van *C. burnetii* lieten zien, hebben bepaalde SNPs in deze genen geen invloed op de ontwikkeling van chronische Q-koorts.

Veel van het Q-koorts onderzoek is gericht op het immuun response tegen *C. burnetii* in mensen, terwijl er veel minder bekend is over het response in geiten. Het kan echter erg nuttig zijn om meer kennis hierover te verkrijgen, omdat geiten een van de belangrijkste bronnen zijn van humane Q-koorts besmettingen. Daarnaast vertonen geiten geen symptomen wanneer zij geïnfecteerd zijn, wat zou kunnen betekenen dat hun immuun systeem anders reageert tijdens *C. burnetii*-infectie dan het humane immuun systeem. In **Hoofdstuk 6** toonden we aan dat PBMCs van naïeve geiten een verhoogde mRNA expressie hebben van IL-1β, TNF-α, IL10 en IFN-γ na *C. burnetii* stimulatie. Daarnaast was de expressie van TLR2 ook sterk verhoogd, wat overeenkomt met onze bevindingen in mensen. Gedurende experimentele infectie van geiten met *C. burnetii* vonden wij een verhoogde TNF-α en IFN-γ in *C. burnetii* geïnfecteerde geiten in vergelijking met naïeve geiten. PBMCs van geiten initiëren een robuust pro-inflammatoir immuun response tegen *C. burnetii in vitro*. Maar, ondanks deze pro-inflammatoire reactie zijn geiten niet in staat om de *C. burnetii* te klaren.

In de bovengenoemde hoofdstukken hebben we gebruik gemaakt van de Nederlandse uitbraak isolaat *C. burnetii* 3262 en de referentie stam *C. burnetii* NM. We hebben enkele verschillen ontdekt tussen deze stammen, namelijk dat *C. burnetii* 3262 wordt herkend door TLR6 terwijl dit niet het geval was voor *C. burnetii* NM. En het inhiberende effect van TLR10 op de cytokine productie had een groter effect na stimulatie met *C. burnetii* 3262 dan *C. burnetii* NM. Tijdens de Q-koorts uitbraak in Nederland, zijn er veel *C. burnetii*-isolaten afkomstig van geiten, schapen en in mindere mate ook van runderen, verzameld door het Centraal Veterinair Instituut (CVI, Wageningen University and Research Centre). Deze dierlijke isolaten, en de isolaten verkregen uit chronische Q-koorts patiënten uit het Radboud University Medical Centre en het Canisius-Wilhelmina Ziekenhuis zijn in het CVI opgewerkt. In **Hoofdstuk 7** hebben we deze stammen gebruikt om de vraag te beantwoorden of isolaten met een verschillende herkomst (geit, schaap, rund, acute en chronische Q-koorts patiënten) een verschillend cytokine profiel laten zijn na stimulatie van humane PBMCs. We vonden dat *C. burnetii*-isolaten afkomstig van runderen significant meer pro-inflammatoire cytokines, IL-1β, TNF-α en IL-22, produceren dan de isolaten afkomstig van geiten, schapen of acute Q koorts patiënten. Daarnaast laten de isolaten van acute Q-koorts patiënten een trend zien van een hogere cytokine inductie dan de isolaten van chronische Q-koorts patiënten. Wanneer het

cytokine profiel van de isolaten wordt ingedeeld op basis van hun MLVA genotype (in plaats van herkomst), waren er geen verschillen tussen de isolaten en de cytokine inductie te zien. De huidige literatuur laat zien dat de meeste humane Q-koorts uitbraken werden veroorzaakt door geïnfecteerde geiten en schapen, en in mindere mate door geïnfecteerde runderen[8]. Gebaseerd op onze bevindingen, kunnen we suggereren dat de verminderde pathogene potentie van runder isolaten kan worden veroorzaakt door een sterker immuun reactie in de humane gastheer.

Algemene discussie

Het doel van dit proefschrift was om een beter en vernieuwend inzicht te krijgen hoe *C. burnetii* wordt herkend door het immuun systeem en welke immuun reacties het initieert in gezonde individuen en drachtige geiten. De resultaten van ons fundamentele onderzoek hebben we gebruikt om de vraag te beantwoorden waarom slechts een klein gedeelte van de individuen met een initiële infectie chronische Q-koorts ontwikkeld, en of *C. burnetii*-isolaten met een diverse herkomst, zoals geit, schaap of rund, een verschillend immuun response genereren in humane PBMCs.

Slechts enkele publicaties waren gericht op de herkenning van *C. burnetii* door PRRs. Enkele studies lieten zien dat TLR2 en NOD2 een belangrijke rol spelen in de herkenning van *C. burnetii* NM en in de inductie van immuun reacties in mensen en muizen[9-11]. Een nadeel van deze studies, bijvoorbeeld in geval van de NOD2 studie, was dat de resultaten alleen waren gebaseerd op muis studies. Daarnaast was er geen literatuur aanwezig waarin de rol van andere PRRs, zoals TLR1, TLR6 en TLR10 zijn onderzocht. In geiten is er slechts één studie die de cytokine expressie laat zien in drachtige geiten na *C. burnetii* infectie, maar er bestaat geen literatuur over de rol van TLRs[12]. Het voordeel van ons onderzoek was dat we de vergelijking tussen humaan, muizen en geiten kunnen maken via *in vitro* experimenten, maar ook gebruik hebben gemaakt van de effecten van SNPs in betrokken PRRs in mensen. Hierdoor was het mogelijk om te bepalen of *C. burnetii* een algemeen immuun response genereert in verschillende gastheren. Daarnaast hebben we de huidige 'knowledge gaps' in de literatuur aangepakt door onderzoek te doen naar de rol van TLR1, TLR6 en TLR10 in mensen (Hoofdstuk 3 en 4). We onderzochten het cytokine response en de expressie van TLR1, TLR2, TLR4 en TLR6 tijdens *C. burnetii* infectie in drachtige geiten (Hoofdstuk 6). Daarnaast zijn we de eerste die onderzocht hebben hoe het cytokine response van humane PBMCs is wanneer zij worden gestimuleerd met *C. burnetii*-isolaten afkomstig van verschillende dieren (Hoofdstuk 7).

Als we onze bevindingen combineren met de bestaande literatuur, kunnen we concluderen dat de TLR1/TLR2 heterodimer en NOD2 een belangrijke rol spelen bij de initiatie van een cytokine response in gezonde personen in de herkenning

van zowel *C. burnetii* NM als *C. burnetii* 3262. Daarnaast is TLR6 betrokken bij de herkenning van de Nederlandse uitbraak stam *C. burnetii* 3262 (Hoofdstuk 3). Ondanks een goede herkenning van *C. burnetii* door deze TLRs, is het in staat geweest om een grote uitbraak te creëren onder individuen in Nederland. We kunnen hieruit concluderen dan een efficiënte reactie tegen *C. burnetii* 3262 via PRRs een infectie in mensen niet weerhoudt. Een hypothese is dat de extra herkenning van *C. burnetii* 3262 door TLR6 en het geïnduceerde cytokine response kan leiden tot een hogere opname van de bacterie door macrofagen. Deze opname van *C. burnetii* door immuun cellen kan in het voordeel van de bacterie zijn omdat het repliceert in macrofagen. Deze opname door macrofagen faciliteert de replicatie van *C. burnetii* en kan zo leiden tot een meer intensieve infectie in mensen.

De volgende vraag die we ons zelf stelden was of TRL1, TLR2 en TLR6 betrokken zijn bij het ontwikkelen van chronische Q-koorts. Onze bevindingen laten zien dat personen met een SNP in TLR1, maar niet in TLR2 of TLR6, een grotere kans hebben om chronische Q-koorts te ontwikkelen (Hoofdstuk 5). In gezonde geiten zagen we een hogere mRNA expressie van TLR1, TLR4, TLR6 en vooral TLR2 in *C. burnetii* gestimuleerde PBMCs (Hoofdstuk 6)[13]. Naast de verhoogde mRNA expressie van TLRs hebben we het door *C. burnetii* geïnduceerde cytokine response onderzocht in geiten en mensen. Zowel de PBMCs van mensen als geiten zijn in staat om een cytokine response te induceren, voornamelijk TNF-α en IFN-γ, na contact met *C. burnetii*. Ongeacht de functionele herkenning van PRRs en de initiatie van een immuun response, ontwikkelt een klein deel van de geïnfecteerde personen een chronische infectie en kan *C. burnetii* infectie in drachtige geiten tot abortus leiden. Een verklaring hiervoor kan zijn dat andere delen van het immuun systeem minder geactiveerd worden tijdens *C. burnetii* infectie, bijvoorbeeld het adaptieve immuun response welke onder andere T- en B cellen bevat. Daarnaast, zoals we beschrijven in Hoofdstuk 5, kunnen individuen polymorphismen in specifieke genen hebben die betrokken zijn bij de herkenning van *C. burnetii*, wat tot een grotere kans van de ontwikkeling van chronische Q-koorts leidt. Ook kan *C. burnetii* zelf strategieën bezitten die het mogelijk maken om het immuun systeem van de gastheer te omzeilen, zoals beschreven is bij vele andere micro-organismen[14].

De vol bloed IFN-γ assay in geiten liet een hoger IFN-γ response zien in drachtige geiten experimenteel geïnfecteerd met *C. burnetii* dan naïeve geiten (Hoofstuk 6).

Schoffelen *et al.* zagen de zelfde bevindingen in mensen, waar de IFN-γ response significant hoger was in individuen met een chronische Q-koorts dan individuen met een doorgemaakte Q-koorts infectie[15, 16]. We weten niet hoe deze *C. burnetii* geïnduceerde IFN-γ productie relateert tot andere bacteriën of virussen in chronische Q-koorts patiënten. Het is mogelijk dat de IFN-γ productie in chronische Q-koorts patiënten niet genoeg is om de bacterie effectief te verwijderen en daarnaast produceren niet alle chronische Q-koorts patiënten hogere hoeveelheden van IFN-γ. Het behandelen van chronische Q-koorts patiënten met extra IFN-γ kan wellicht leiden tot regressie van de ziekte. Om deze hypothese te testen is een chronische Q-koorts patiënt (met een lage IFN-γ response) recentelijk behandelt met IFN-γ in het Radboudumc. In principe verwachten we dat IFN-γ behandeling van geïnfecteerde geiten ook de infecte kan helpen te overkomen. Maar, in tegenstelling tot bij mensen, is IFN-γ behandeling van *C. burnetii* geïnfecteerde geiten niet kosten effectief.

In de literatuur wordt vaak gezegd dat 1-5% van de individuen met een initiële infectie chronische Q-koorts ontwikkelen. Het is bekend dat personen met een aneurysma, hartklepafwijking, vaatprothese of immunosuppressie een hogere kans hebben op het ontwikkelen van chronische Q-koorts. Daarnaast laat onze studie zien dat individuen met een specifieke SNP in *MyD88, NOD2* of *TLR1* ook een hogere kans hebben op chronische infectie (Hoofdstuk 5)[17]. Hoewel TLR2 een belangrijke receptor is in de herkenning van *C. burnetii* zijn polymorphismen in het TLR2 gen niet geassocieerd met een hogere kans op het ontwikkelen van chronische Q-koorts. Tevens is er ook geen associatie gevonden tussen polymorphismen in TLR2 en TLR4 en acute Q-koorts, zoals Everett *et al.* aantonen[18]. Een verklaring dat de SNPs in TLR2 en TLR4 die wij en andere hebben onderzocht geen rol spelen in de ontwikkeling van acute of chronische Q-koorts kan zijn dat ze niet interfereren met de bindingsplaats van *C. burnetii*, en daarom geen functioneel effect hebben.

Kijkend naar de huidige literatuur en onze eigen bevindingen is de vraag of we de immuun herkenning en responses kunnen vergelijken tussen mensen en geiten. Gebaseerd op de inductie van de pro-inflammatoire cytokines, TNF-α en IFN-γ, kunnen we hypothetiseren dat PBMCs van geïnfecteerde geiten een zelfde cytokine profiel laten zien als chronische Q-koorts patiënten. Daarentegen is de IL-10 mRNA expressie niet verhoogd in geïnfecteerde geiten, maar observeren we wel

een verhoogd IL-10 response in chronische Q-koorts patiënten[7, 19]. Deze verschillen in IL-10 kunnen de diverse klinische ziektebeelden tijdens *C. burnetii* infectie in geiten en mensen verklaren. Al moet er rekening mee worden gehouden dat de experimentele set-up verschillend is tussen de twee groepen. Als eerste zijn de geiten in onze studie experimenteel geïnfecteerd met *C. burnetii*, terwijl de chronische Q-koorts patiënten op natuurlijke wijze zijn geïnfecteerd. Daarnaast hebben we in geiten vooral de mRNA expressie gemeten van cytokines, terwijl de humane studies met name zijn gericht op eiwit expressie. mRNA expressie komt niet altijd overeen met de daadwerkelijke hoeveelheid van geproduceerde eiwitten. Een ander verschil binnen de studies is dat de geiten drachtig waren. Dracht is een vereiste voor geiten om klinische Q-koorts te ontwikkelen, aangezien niet drachtige geiten geen symptomen van infectie laten zien. Tot recentelijk werd zwangerschap in mensen gezien als een risico factor voor de ontwikkeling van chronische Q-koorts. Maar studies tijdens de Nederlandse Q-koorts uitbraak laten geen duidelijke associatie zien tussen een verhoogde kans op chronische Q-koorts en zwangerschap[20, 21]. Gebaseerd op bovenstaande genoemde verschillen en overeenkomsten tussen het immuun response van geiten en mensen tegen *C. burnetii* is het moeilijk te beantwoorden of het immuun response vergelijkbaar is. Het zijn twee onafhankelijk modellen, welke gebruikt kunnen worden om vragen te beantwoorden gerelateerd aan hun eigen milieu en achtergrond situatie. Alhoewel, uit onze aanpak om in beide modellen het immuun response tegen *C. burnetii* te onderzoeken, kunnen we concluderen dat *C. burnetii* op zichzelf in staat in een immuun response te initiëren en dat het wordt herkend in zowel geiten als mensen. We raden aan dat de toekomstige studies betreffende het *C. burnetii* geïnduceerd immuun response in mensen zich richten op *in vitro* studies met humane immuun cellen en we verwachten dat veel meer informatie uit genetische studies met Q-koorts patiënten kan worden gehaald. Onderzoeken die zich richten op *C. burnetii* infectie en het immuun response in geiten en andere dierlijke gastheren zijn belangrijk en moeten niet worden genegeerd. Vanwege de huidige intensieve veehouderij en het feit dat mensen en vee dichter op elkaar leven, kunnen mogelijke uitbraken worden verwacht wanneer de beschermende vaccinatie van geiten achterwegen wordt gelaten. Meer kennis over de epidemiologie, transmissie en immuun reacties in de dierlijke gastheren wordt gewaardeerd en is bruikbaar om nieuwe uitbraken te voorkomen en om de huidige diagnostiek en behandeling te verbeteren.

Omdat *C. burnetii* is geclassificeerd als een groep B bioterrorisme agent, moeten experimenten met levende bacteriën worden uitgevoerd in een level 3 laboratorium. Zo ver we weten, zijn er geen studies gepubliceerd die levende en hitte geïnactiveerde *C. burnetii* vergelijken in relatie tot het effect op cytokine response en herkenning door PRRs. We hebben besloten om hitte geïnactiveerde *C. burnetii* te gebruiken in onze studies vanwege twee redenen. Ten eerste onderzochten we de initiële herkenning van *C. burnetii*: zorgt stimulatie van humane en geiten PBMCs met *C. burnetii* voor een cytokine response en welke PRRs van deze PBMCs zijn betrokken bij de herkenning? We hebben niet gekeken naar de replicatie of beweging van *C. burnetii* in de immuun cellen. Daarnaast was het een praktische overweging, door het gebruik van hitte geïnactiveerde *C. burnetii* konden we gebruik maken van één batch voor alle experimenten in dit proefschrift. Het is mogelijk dat onze bevindingen niet overeenkomen met levende *C. burnetii*, omdat de hitte inactivatie de antigenen expositie op de bacteriën kunnen veranderen en de interactie met het immuun system kan beïnvloeden. In andere studies is de rol van TLR2 in *C. burnetii* herkenning bijvoorbeeld onderzocht door TLR2 specifieke knock out muizen te infecteren met levend *C. burnetii*. De belangrijke rol van TLR2 was zowel gedemonstreerd in deze studies als in onze eigen studie, wat aantoont dat er geen verschil was tussen het gebruik van levens of hitte geïnactiveerd *C. burnetii*[9, 10, 22]. Ook werden zowel levend als hitte geïnactiveerd *C. burnetii* gebruikt in humane cytokine studies, zodat we onze huidige bevindingen kunnen vergelijken[19, 23].

In de studies uitgevoerd voor dit proefschrift hebben we gebruik gemaakt van zowel *C. burnetii* NM RSA 493 en de Nederlandse uitbraak stam *C. burnetii* 3262. We hebben gekozen voor deze twee stammen, omdat de eerste de referentie stam is die ook wordt gebruikt in andere laboratoria. De tweede stam was verantwoordelijk voor de Q-koorts uitbraak in Nederland, wat leidde tot het ruimen van veel geiten en acute en chronische Q-koorts infecties in mensen. Zoals vermeld in de bovenstaande samenvatting, hebben we enkele verschillen gevonden tussen deze twee stammen. Een veel gestelde vraag is wat deze verschillen veroorzaakt en of we kunnen speculeren over een verschillende gen expressie van deze twee stammen. Op dit moment is de enige vergelijking die we kunnen maken gebaseerd op het MLVA genotype, welke inderdaad verschilt op verschillende loci. Meer informatie over de genetische achtergrond zal beschikbaar komen wanneer de de novo sequence van de Nederlandse uitbraakstam *C. burnetii* 3262 wordt gepubliceerd. Genoom verge-

lijking tussen *C. burnetii* NM en *C. burnetii* 3262 zou verschillen kunnen aantonen in genetische delen die corresponderen voor virulentie factoren zoals LPS of eiwitten. Daarnaast zou het gebruikt kunnen worden om mogelijke veranderingen, zoals polymorphisms in genen die coderen voor PRRs, te vergelijken.

C. burnetii 3262 representeert de CBNL01 MLVA genotype, welke domineerde tijdens de Q-koorts uitbraak in Nederland en verantwoordelijk was voor de grootste Q-koorts uitbraak wereldwijd ooit gerapporteerd. Naast dat individuen een hogere kans hebben om in aanraking te komen met dit pathogeen vanwege de intensieve veehouderij[24], kan er ook gehypothetiseerd worden dat deze stam minder goed herkend wordt door PRRs. Een mindere herkenning van *C. burnetii* 3262 kan vervolgens leiden tot minder reacties zoals de inductie van pro-inflammatoire cytokines, fagocytose en de eliminatie van bacteriën. Uiteindelijk kan dit de meer infectieuze en ziekte veroorzakende kenmerken van *C. burnetii* 3262 verklaren. Maar onze resultaten laten zien dat *C. burnetii* 3262 in staat is een adequate immuun response te induceren in PBMCs en herkend wordt door TLR1, TLR2, en in tegenstelling tot *C. burnetii* NM, ook TLR6. Daarom kunnen we concluderen dat een veranderde herkenning door PRRs van *C. burnetii* een minder aannemelijke hypothese is.

Een interessante bevinding is dat het cytokine profiel van *C. burnetii* isolaten afkomstig van runderen, welke een hoog pro-inflammatoire cytokine response produceren in humane PBMCs dan isolaten afkomstig van mensen, schapen of geiten. Het is niet bekend wat deze runder isolaten minder virulent maakt. Wellicht bevatten zij minder escape strategieën of hebben zij een minder stimulerend LPS of andere oppervlakte antigenen, wat een beter immuun response van de gastheer mogelijk maakt. Structurele vergelijking van de isolaten of *in vivo* studies kunnen meer antwoorden geven. De bevinden van deze studie kunnen ook gebruikt worden om potentiele Q-koorts uitbraken te voorspellen. We weten uit de literatuur dat geïnfecteerde runderen minder vaak de oorzaak zijn van Q-koorts infecties in mensen. Onze studie relateert hieraan, en laat zien dat het humane immuun systeem met een hoge pro-inflammatoire cytokine response produceert na contact met *C. burnetii* isolaten uit runderen. Tijdens de Nederlandse Q-koorts uitbraak zijn er veel geiten geruimd omdat zij een potentiele bron waren voor humane infectie. Uit onze studie kan er echter geconcludeerd worden dat tijdens een mogelijke Q-koorts uitbraak onder runderen er minder reden is om deze dieren te ruimen, aangezien geïnfecteerde individuen een effectiever immuun response (waarschijnlijk resulterend in geen of milde kliniek) hebben tegen *C. burnetii* isolaten afkomstig van runderen. Met het oog op geïnfecteerde individuen kan er wellicht specifieke serodiagnostiek worden ontwikkelt om de herkomst van het *C. burnetii* isolaat te bepalen en vervolgens te relateren aan de virulentie en de kans om een chronische infectie te veroorzaken. Als het namelijk mogelijk is om deze host afhankelijke virulentie factoren te karakteriseren, kunnen diagnostische testen worden ontwikkeld die de herkomst van de *C. burnetii* isolaten kunnen bepalen en het verwachtte klinische ziekte verloop in mensen vaststellen.

Een van de onderzoeksvragen van dit proefschrift was om de immuun herkenning en response in drachtige geiten te onderzoeken en te beantwoorden waarom *C. burnetii* infectie kan leiden tot abortus zonder andere klinische symptomen. We vonden dat geiten een pro-inflammatoire cytokine response induceren na stimulatie met *C. burnetii* en dat de mRNA van TLRs ook is verhoogd. Het lijkt er op dat het immuun systeem van geiten in staat is goed te reageren tegen *C. burnetii*, zowel het humorale als cellulaire response[12, 13]. Onze studie toonde aan dat tijdens de eerste week van infectie van drachtige geiten, de IFN- γ productie was verminderd[13](Hoofdstuk 6).

Er kan gesuggereerd worden dat *C. burnetii* de IFN-γ responses in het begin van de infectie onderdrukt, wat het voor de bacterie mogelijk maakt om getransporteerd te worden naar de throfoblasten in de placenta zonder herkend te worden door het immuun systeem. Maar, er is meer onderzoek nodig om de vraag te beantwoorden hoe *C. burnetii* infectie uiteindelijk kan leiden tot abortus en doodgeborene. Bijvoorbeeld de rol van IFN-γ op de replicatie en overleving van *C. burnetii* kan worden onderzocht. Vanuit de literatuur is het bekend dat IFN-γ de eliminatie van *C. burnetii* faciliteert, maar deze experimenten zijn uitgevoerd met THP-1 (monocyt-achtige) cellijnen en niet in cellen die meer specifiek gerelateerd zijn aan de placenta[25, 26]. Daarnaast hangt een succesvolle zwangerschap grotendeels af van mechanismen die het immuun systeem van de moeder tolerant maken tegen de semi-allogeneic foetus. Het effect van *C. burnetii* op immunomodulatoren zoals arginase en thryptophan tijdens de zwangerschap zijn nog niet onderzocht en kunnen nieuwe inzichten geven.

Een van de laatste vragen die overblijft is waarom infectie met *C. burnetii* in sommige geïnfecteerde personen tot een chronische infectie leidt. Mijn inziens kan het antwoord op deze vraag slechts deels gevonden worden in het immuun response van de gastheer. Het is een combinatie van verschillende factoren, waaronder de initiële dosis van *C. burnetii* tijdens infectie, de virulentie en de herkomst van het *C. burnetii* isolaat, en de immuun status, aanwezige of aanleg voor vasculaire factoren en de genetische achtergrond van de geïnfecteerde individu. Er zijn nog veel mogelijkheden die kunnen worden onderzocht in de toekomst, waarbij men rekening zal moeten houden met al deze aspecten.

Aanbevelingen en toekomstperspectieven

De resultaten die we in deze thesis beschrijven dragen bij aan het beantwoorden van een breed arsenaal van vragen en knowledge gaps in Q-koorts infecties. We hebben meer informatie verkregen over de immuun reactie tegen *C. burnetii* infecties in mensen en geiten, en onze bevindingen zijn nuttig voor andere onderzoekers in het Q-koorts veld en toekomstige onderzoeken, zowel in het veterinaire als het humane veld. Uiteindelijk zal een beter begrip van het immunologische response tegen *C. burnetii* kunnen leiden tot betere diagnostische testen voor mensen en dieren, en behandelingen voor patiënten met Q-koorts.

Een van de problemen binnen de diagnostiek van *C. burnetii* infectie in geiten is om te bepalen of een geit een actieve of een doorgemaakte infectie heeft. Onze studie laat zien dat geiten in staat zijn een pro-inflammatoire cytokine response te induceren na contact met *C. burnetii*. Voor vroege diagnostiek van *C. burnetii* in geiten is het wellicht mogelijk om specifiek geïnduceerd IFN-γ te meten in volbloed, aangezien geïnfecteerde geiten aanzienlijk minder IFN-γ induceren (Hoofdstuk 6). Al moeten deze eventuele mogelijkheden wel verder worden onderzocht voor dit doeleinde.

In dit proefschrift tonen we aan dat individuen met een specifieke SNPs in MyD88, NOD2 of TLR1 een grotere kans hebben om chronische Q-koorts te ontwikkelen (Hoofdstuk 5). Deze bevindingen kunnen worden geïmplementeerd in de diagnose van chronische Q-koorts en maakt het wellicht mogelijk om individuen met een grotere aanleg voor de ontwikkeling van chronische Q-koorts in een vroeg stadium te ontdekken. Wanneer we weten dat een patiënt met acute Q-koorts en risicofactoren voor het ontwikkelingen van chronische Q-koorts zulke mutaties bijdraagt, kan er vroegtijdig met preventieve behandeling worden gestart. Een ander voordeel van de genetische studie die we hebben uitgevoerd is de beschikbaarheid van een grote collectie DNA samples van chronische Q-koorts patienten en gematchte controles. De ruim 140 verzamelde samples van chronische Q-koorts patiënten is de grootste in zijn soort en erg waardevol voor toekomstige studies. In de huidige studies hebben we alleen het effect van genetische variatie onderzocht binnen specifieke receptoren naar de ontwikkeling van chronische Q-koorts. Het is interessant om in

toekomstige studies gezonde *C. burnetii* seronegatieve personen te vergelijken met individuen die een acute Q-koorts infectie hebben. Er kan zelfs onderscheid worden gemaakt tussen acute Q-koorts patiënten met of zonder ziekenhuis opname. Een hypothese is dat gedurende een Q-koorts infectie, individuen met SNPs in de genen die betrokken zijn bij de initiële herkenning, zoals cytokines en PRRs, eerder worden opgenomen in het ziekenhuis dan patiënten zonder deze SNPs. Een reeds gepubliceerde studie vond geen associatie tussen aanwezige SNPs in TLR2 en TLR4, en het ziekte manifestatie in acute Q-koorts[18]. De rol van TLR1, NOD2 en MyD88, welke gerelateerd zijn aan de ontwikkeling van chronische Q-koorts, zijn tot nu toe nog niet onderzocht in acute Q-koorts. De resultaten van deze onderzoeken zullen meer informatie opleveren over welke receptoren en wegen betrokken zijn bij de initiële herkenning van *C. burnetii*.

Een laatste aanbeveling gebaseerd op ons onderzoek betreft de diverse klinische beelden in personen geïnfecteerd met verschillende *C. burnetii*-isolaten. We observeerden een hoger cytokine response in humane PBMCs die gestimuleerd waren met *C. burnetii*-isolaten afkomstig van runderen dan van de isolaten afkomstig van geiten. Dit betekent dat het humane immuun systeem anders kan reageren op verschillende *C. burnetii*-isolaten. Daarnaast zagen we dat isolaten met hetzelfde MLVA genotype een verschillende cytokine response induceren in humane PBMCs. De identificatie en classificatie van *C. burnetii*-isolaten is momenteel gebaseerd op MLVA genotypering, maar deze techniek biedt geen informatie over de eventuele virulentie van het isolaat. Voor onze algemene kennis over de classificatie van *C. burnetii*, zijn zowel *C. burnetii* infectie en toekomstige (serologische) diagnostiek (zoals bediscussieerd in de algemene discussie) als onderzoek naar de virulentie factoren van *C. burnetii* erg interessant. Het geeft ons meer informatie welke virulentie factoren van *C. burnetii* een belangrijke rol spelen tijdens humane infectie en mogelijke nieuwe vaccinaties of behandelingen kunnen worden aangepast op deze bevindingen.

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10

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Curriculum Vitae

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Curriculum Vitae

Anne Ammerdorffer was born on the 25th of March 1986 in Rotterdam, the Netherlands, and spend her youth in the small village of Dinteloord. She attended the Gymnasium Juvenaat in Bergen op Zoom and obtained her diploma in 2004, with a major in Nature and Health.

After graduating pre-university school, she moved to Wageningen to study Biology at the Wageningen University and Research centre. During her Bachelor she choose the direction of Cell biology and completed a minor in 'human and animal infectious diseases'. She performed her Master thesis at the Department of Virology at the WageningenUR under supervision of dr. Gorben Pijlman. She investigated the functional characterization of the dengue virus inhibitor of apoptosis. For her Master internship she moved to Brisbane, Australia, to work at the Infection and Immunology Department of the Queensland Institute of Medical Research (QIMR). Here she investigated the Type I Interferon responses during experimental cerebral malaria under supervision of dr. Ashraful Haque.
During and after her studies she was an active member of the Wageningen Student Rowing Association Argo and participated in many committees. In 2007-2008, she was the secretary of the rowing club during a fulltime board year.

After completing her studies, she started her PhD on Q fever entitled: 'Host and bacterial factors of Q fever in humans and goats'. The project was a collaboration between the Internal Medicine Department of the Radboudumc (Nijmegen, the Netherlands) and the Central Veterinary Institute (Lelystad, the Netherlands, part of WageningenUR). The main aim of the project was to define which human or goat immunological events determine persistence of *Coxiella burnetii* and to investigate how the bacterium is recognized. The project focused on the role of the innate immune response, e.g. pattern recognition receptors, cytokine induction and the complement pathway. The project was supervised by prof. dr. Leo Joosten (Radboudumc), dr. Tom Sprong (Radboudumc, CWZ), dr. Annemarie Rebel (WageningenUR) and dr. Hendrik-Jan Roest (CVI).

Since September 2015 she works as a postdoctoral researcher in the laboratory of dr. David Baud in Lausanne, Switzerland. She investigates the immunopathology of *Waddlia chrondrophila,* a *Chlamydia* related intracellular bacterium associated with adverse pregnancy outcomes. It is a collaboration between the Department of Gynecology & Obstetrics and the Institute of Microbiology from the University Hospital (CHUV) in Lausanne. In the summer of 2016 she interrupted her postdoctoral studies for a role as Study Coordinator to test a new hematology analyzer from Roche (Cobas M511), a collaboration between Roche Diagnostics Hematology (Boston, USA) and the Department of Clinical Chemistry from the Erasmus Medical Centre (Rotterdam, the Netherlands).

Besides her professional experiences, she is enrolled in the training program for recognition as a SMBWO Immunologist. For this training she participated in the ENII Summer School on Advanced Immunology, performed an internship at the Department of Laboratory Medicine of the Radboudumc, and followed the course 'Pathofysiology' at the Radboud University.

Colophon

The research in this thesis was performed at the Department of Internal Medicine of the Radboud University Medical Centre, Nijmegen, the Netherlands and the Central Veterinary Institute (part of Wageningen University), Lelystad, the Netherlands.

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