AIX-MARSEILLE UNIVERSITE

FACULTE DE MEDECINE DE MARSEILLE

ECOLE DOCTORALE DES SCIENCES DE LA VIE ET DE LA SANTE

T H E S E

Présentée et publiquement soutenue devant

LA FACULTE DE MEDECINE DE MARSEILLE

Le 03 juillet 2012

Par M. Wiwit TANTIBHEDHYANGKUL

Date et lieu de naissance 09 mai 1981 à Bangkok

TITRE DE LA THESE :

Etude de la réponse immunitaire innée au cours de l'infection à *Orientia tsutsugamushi*

Pour obtenir le grade de DOCTORAT d'AIX-MARSEILLE UNIVERSITE

SPÉCIALITÉ : PATHOLOGIE HUMAINE / MALADIES INFECTIEUSES

Membres du Jury de la Thèse :

M. le Professeur Jean-Louis MEGE	Directeur de thèse
M. le Professeur Michel SIMONET	Rapporteur
Mme le Docteur Patricia RENESTO	Rapporteur
M. le Docteur Jean-Pierre GORVEL	Examinateur

Unité de Recherche sur les Maladies Infectieuses et Tropicales Emergentes CNRS-IRD UMR 6236

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Résumé

Orientia tsutsugamushi, l'agent pathogène responsable du typhus des broussailles, est une bactérie cytosolique qui envahit l'endothélium et les monocytes/macrophages. La réponse immune à l'infection par *O. tsutsugamushi* reste à ce jour mal connue. L'objectif de cette thèse est de mieux comprendre la réponse des cellules de la réponse immune innée humaine à *O. tsutsugamushi*. Nous avons montré que *O. tsutsugamushi* se réplique dans les monocytes humains. En utilisant un microarray portant sur la totalité du génome, nous avons également montré que les bactéries vivantes induisent de profondes modifications du profil transcriptionnel des monocytes. C'est ainsi que l'expression des gènes codant l'interféron de type I et des gènes stimulés par l'interféron est fortement augmentée. Les monocytes infectés expriment plusieurs gènes codant des cytokines et des chimiokines inflammatoires, ce qui montre qu'ils sont polarisés vers un phénotype M1 (*classically-activated phenotype*). Les bactéries vivantes induisent également la sécrétion de l'interleukine-1β et probablement l'activation des inflammasomes et de la caspase-1. *O. tsutsugamushi* affecte enfin l'expression des gènes associés à l'apoptose et induit la mort d'une partie des monocytes infectés. Nous avons en outre étudié le profil transcriptionnel de patients atteints d'un typhus des broussailles et avons trouvé une signature spécifique incluant la modulation de gènes de type M1 et de gènes stimulés par l'interféron. Nous avons finalement étudié la réponse des macrophages humains dérivés des monocytes à *O. tsutsugamushi*. Les réponses transcriptionnelles et fonctionnelles des macrophages sont globalement similaires à celles observées dans les monocytes circulants soumis à *O. tsutsugamushi* : elles incluent une réponse IFN de type I, l'expression de gènes codant des cytokines inflammatoires et la sécrétion d'interleukine-1β. Ces résultats accroissent notre compréhension de la pathogénèse du typhus des broussailles et révèlent des processus hautement inflammatoires.

Mots-clés : *Orientia tsutsugamushi*, scrub typhus, profil transcriptionnel, monocytes

Summary

Orientia tsutsugamushi, the causative pathogen of scrub typhus, is a cytosolic bacterium that invades endothelium and monocytes/macrophages. So far, the knowledge of immune response to *O. tsutsugamushi* is still limited. The objective of this thesis is to better understand the response of human innate immune cells against this pathogen. We demonstrated that *O. tsutsugamushi* was able to replicate in human monocytes. Using whole genome microarrays, we showed that live *O. tsutsugamushi* induced robust changes in the transcriptional profiles of monocytes. First, type I interferons and interferon-stimulated genes were remarkably up-regulated. Second, infected monocytes expressed several inflammatory cytokine and chemokine genes, and were polarized toward the classically-activated M1 phenotype. Third, live bacteria induced interleukin-1β secretion and likely inflammasome and caspase-1 activation. We also showed that *O. tsutsugamushi* altered the expression of apoptosis-related genes and induced cell death in monocytes. We extended our work to the study of the transcriptional profiles of patients with scrub typhus and found a specific signature in patients that included the modulation of M1-associated genes and interferon-stimulated genes. We finally studied the response of human monocyte-derived macrophages to *O. tsutsugamushi*. The transcriptional and functional responses of macrophages to *O. tsutsugamushi* were roughly similar to those observed in circulating monocytes including type I IFN response, pro-inflammatory cytokine gene expression and IL-1β secretion. Taken together, these data improve our understanding in the pathogenesis of scrub typhus in which highly inflammatory processes are involved.

Keywords : *Orientia tsutsugamushi*, scrub typhus, transcriptional profile, monocytes

Chapter 1

Introduction

Objectives of the thesis

Evidence suggests that *Orientia tsutsugamushi* induces highly inflammatory processes that likely play a critical role in the pathogenesis of scrub typhus; however, the underlying mechanisms remain unclear. In addition, monocytes and macrophages are target cells of *O. tsutsugamushi* but the interaction between this bacterium and human myeloid cells is not clearly understood. Therefore, the objectives of the thesis are 1) to determine the intracellular fate of *O. tsutsugamushi* in monocytes and macrophages; 2) to study the transcriptional response and cytokine secretion of monocytes and macrophages; 3) to characterize the transcriptional profiles in patients and compare them with those of *in vitro*-infected monocytes.

Epidemiology of scrub typhus and clinical features

Scrub typhus is caused by the obligate intracellular bacterium *Orientia tsutsugamushi*. This disease is a tropical rickettsiosis that is exclusively found in the Asia-Pacific region. It is transmitted to humans by the bite of a trombiculid mite larva (chigger). The chigger is an ectoparasite that feeds only once on vertebrate hosts. After the blood meal, the larva develops into the nymph and the adult, both of which are free-living arthropods in the soil. The adult mite then passes *O. tsutsugamushi* organisms to offsprings by transovarian transmission, thereby continuing the cycles of disease transmission. Rodents are reservoir hosts whereas humans are considered accidental hosts. The disease is endemic in areas such as scrub, small forests or agricultural fields where the surrounding environment is suitable for rodents and mite vectors [1, 2]. Scrub typhus is still a public health concern for people in endemic regions and for travelers returning from these areas [3].

Scrub typhus is a common cause of acute febrile illness in endemic areas. After an incubation period of 1-3 weeks, the patient develops signs and symptoms including fever, headache, myalgia, maculopapular rash, regional lymphadenopathy and hepatosplenomegaly. However, these symptoms are non-specific and cannot be distinguished from other systemic infections. The eschar at the site of chigger bite is quite specific to the disease but is not present in all patients. The clinical manifestations of scrub typhus vary in severity from mild and self-limiting to severe or even fatal. In severe cases, the patient may develop serious complications such as interstitial pneumonitis, meningoencephalitis, myocarditis, acute tubular necrosis, disseminated intravascular coagulation (DIC), multi-organ failure and death [1, 2].

The diagnosis of scrub typhus is usually made by indirect immunofluorescence assay. The diagnostic criteria are a 4-fold rise in the titer of specific IgG and/or the presence of

specific IgM. However, this serologic method is usually not sensitive enough at the early phase of the disease. Molecular techniques such as nested or real-time PCR from whole blood or buffy coats are quite sensitive, but are not routinely available in rural areas. Therefore, the diagnosis of scrub typhus is still problematic and may lead to delayed treatment and serious complications.

Doxycycline remains the drug of choice for treatment of scrub typhus. Chloramphenicol and azithromycin are alternative antibiotics in young children or pregnant patients in which doxycycline is contraindicated. Fever rapidly resolves within 24-48 hours after the initiation of antibiotic treatment [2, 4]. Rapid defervescence of symptoms is the hallmark of scrub typhus that is also helpful to diagnosis. Vaccines against scrub typhus are not currently available. Thus, the disease prevention is usually based on avoidance of arthropod bites.

The bacterium : *Orientia tsutsugamushi*

O. tsutsugamushi is an obligate intracellular bacterium that replicates in the cytosol of host cells. The genus belongs to the class alpha-Proteobacteria, the order Rickettsiales and the family Rickettsiaceae. *O. tsutsugamushi* has long been the single species in this genus. However, *Orientia chuto* has recently been proposed as a new species [5]. Based on serotypic or genotypic methods, *O. tsutsugamushi* is further classified into different strains such as Karp, Kato, Gilliam, Kuroki, Kawasaki and Boryong.

Due to remarkable genetic and phenotypic differences, *O. tsutsugamushi* has been classified as a new genus separate from the genus *Rickettsia*. Unlike *Rickettsia* species, *O. tsutsugamushi* has neither peptidoglycan nor lipopolysaccharide (LPS) in its cell wall and is thus fragile and very unstable in the extracellular environment. Its cell wall is composed of various outer membrane proteins that are distinct from those of *Rickettsia* spp. [6]. Among these proteins, the 56 kDa antigen is abundantly expressed and its variability can be used to classify the organism into different strains [1]. The full genome sequencing of *O. tsutsugamushi* strains Boryong and Ikeda has recently been completed. Their genomes are about 2 Mbp long and thus larger than those of *Rickettsia* spp. The genome of *O. tsutsugamushi* is recognized as the most highly repeated bacterial genome so far sequenced and also contains several amplified repetitive sequences, particularly the genes encoding conjugative type IV secretion systems (*tra* genes) [7, 8].

O. tsutsugamushi invades various cell types, particularly endothelial cells and macrophages. In experimental animals, *O. tsutsugamushi* can invade peritoneal mesothelial cells [9], macrophages [10] and polymorphonuclear leukocytes [11]. In humans, *O. tsutsugamushi* is detected in peripheral blood cells of patients with scrub typhus [12]. Autopsy findings have revealed the presence of *O. tsutsugamushi* in endothelial cells, tissue macrophages, cardiac myocytes and renal tubular cells [13, 14]. Cellular invasion of *O.*

tsutsugamushi results in perivascular mononuclear cell infiltration, which is the common histopathological feature of scrub typhus, subsequently leading to serious complications and organ failure. The mechanisms of cellular invasion and the pathogenesis of *O. tsutsugamushi* infection are discussed in the review article.

Monocytes and macrophages

Monocytes and macrophages are heterogenous cells of myeloid origin that are critical to host defense against pathogens. Monocytes are circulating cells that can differentiate into resident macrophages or dendritic cells. They can be classified into 3 subsets based on CD14 and CD16 expression. $CD14^{+}CD16^{-}$ and $CD14^{+}CD16^{+}$ monocytes are 'inflammatory' cells that secrete inflammatory cytokines in response to LPS or microbial stimuli. They represent about 90% of circulating monocytes. On the other hand, $CD14^{dim}CD16^{+}$ monocytes are 'patrolling' cells that play roles in immune surveillance, antiviral defense and autoimmunity [15]. Circulating monocytes may be involved in the dissemination of intracellular pathogens via blood circulation [16].

Monocytes are able to migrate toward tissues where they differentiate into macrophages. Macrophages are important effector cells in both innate and adaptive response. Interferon (IFN)-γ released by T cells and natural killer (NK) cells is known to activate macrophages that become able to eliminate microbes more effectively by producing reactive oxygen and nitrogen intermediates (ROI and RNI, respectively). Macrophages can be polarized towards classically activated M1 or alternatively activated M2 phenotypes in response to mediators or microbial stimuli. M1 macrophages are induced by IFN-γ or LPS and produce cytokines and chemokines (e.g. IL-12, IL-23, CXCL9 and CXCL10), which promote Th1 response and microbial killing. On the other hand, M2 macrophages comprising various forms of macrophage activation can be induced by IL-4, IL-10, IL-13 or glucocorticoid hormones. They express scavenger and mannose receptors, produce anti-inflammatory molecules (e.g. IL-10 and IL-1 receptor antagonist) and are generally involved in immunoregulation or Th2 response. Macrophage polarization can influence the outcome of infection [17].

Monocytes and macrophages can be targeted by intracellular pathogens. Some intracellular bacteria including *Mycobacterium* spp., *Salmonella enterica* and *Legionella pneumophila* can survive and replicate in phagosomes of macrophages. These bacteria develop some evasive strategies to resist phagosomal killing. In contrast, other bacteria, namely *Listeria monocytogenes*, *Francisella tularensis*, some *Rickettsia* and *O. tsutsugamushi*, rapidly escape from phagosomes into the cytosol, enabling them to avoid the lysosomal killing of monocytes and macrophages.

REVIEW ARTICLE

Pathogenesis of *Orientia tsutsugamushi* infection

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Pathogenesis of *Orientia tsutsugamushi* infection

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ABSTRACT

Scrub typhus is confined to countries in the Asia-Pacific region but is also a public health problem for individuals living in endemic regions and for travelers returning from these areas. The disease is caused by *Orientia tsutsugamushi*, an obligate intracellular bacterium that lives essentially in endothelial cells and monocytes/macrophages. Herein, we describe the mechanisms used by *O. tsutsugamushi* to invade endothelial cells as well as the molecular and functional consequences of this endothelial cell and monocyte/macrophage invasion by *O. tsutsugamushi* in terms of cytokine/chemokine production, the induction of cell death and the trigger of the adaptive immune response. Finally, we describe new, large-scale methodological approaches that aim to refine our understanding of *O. tsutsugamushi* infection in humans and animals, and may improve treatments for patients with scrub typhus.

INTRODUCTION

Scrub typhus is caused by *Orientia tsutsugamushi*, an obligate intracellular bacterium that is transmitted to human beings by the bites of trombiculid mites. The disease is confined to countries in the Asia-Pacific region and is a public health problem for individuals living in endemic regions and for travelers returning from these areas [1,2]. The disease is characterized by acute febrile illness, which can be complicated by disseminated intravascular coagulation, interstitial pneumonitis, neurological lesions and myocarditis [3,4]. *O. tsutsugamushi* exhibits characteristic features; it lacks both peptidoglycan and lipopolysaccharide (LPS) in its cell wall, which distinguishes it from *Rickettsia* species [5]. Consequently, *O. tsutsugamushi* is classified in a genus separate from that of *Rickettsiae* [6]. The complete genomes of the Boryong and Ikeda *O. tsutsugamushi* strains were recently sequenced, and these genomes were much larger than those of members of the *Rickettsiale*s order [7].

The aim of this chapter is to review recent data concerning the interaction of *O. tsutsugamushi* with its major cell targets, namely endothelial cells and monocytes/ macrophages as well as the consequences of host cell death and the nature of the protective adaptive immune response.

I - Interaction between *O. tsutsugamushi* and host cells

O. tsutsugamushi has been reported to infect many types of cells, including endothelial cells [8], monocytes/macrophages [9], neutrophils [10], lymphocytes and fibroblasts [8]. The consensus holds that endothelial cells and monocytes/macrophages are the most frequently observed cell targets in patients with scrub typhus and in animals infected with *O. tsutsugamushi* [8]. Indeed, *O. tsutsugamushi* is found in tissues rich in endothelial cells and macrophages [11]. In patients with scrub typhus, *O. tsutsugamushi* can be also detected in circulating monocytes [12].

I - 1. Invasion of endothelial cells by O. tsutsugamushi

Although the tropism of *O. tsutsugamushi* (and the *Rickettsia* species) for endothelial cells is shared with other bacteria such as *Listeria monocytogenes* [13], *Bartonella* species [14], *Chlamydia pneumoniae* [15], *Neisseria meningitidis* [16] and invasive strains of *Streptococcus pyogenes* [17], host cell invasion remains a critical feature of the bacterial pathogenicity of *O. tsutsugamushi*. We will focus on data using endothelial cell lines, but as the literature on this subject is relatively scarce, we will also include some findings from other nonphagocytic cell lines. The lack of LPS in the cell wall of *O. tsutsugamushi* begs the question of how the bacterium is recognized by endothelial cells. Two bacterial ligands have been described: the 56 kDa outer membrane protein of *O. tsutsugamushi* has been shown to interact with extracellular matrix components, such as fibronectin, which enable the necessary engagement of integrins for entry into endothelial cells [18], and the ScaC autotransporter membrane protein has been shown to promote the adherence of *O. tsutsugamushi* to nonphagocytic cell lines, including the human endothelial cell line ECV304, such that when ScaC is expressed in *Escherichia coli*, bacterial adherence is increased but bacterial invasion is not promoted [19]. The eucaryotic receptors for *O. tsutsugamushi* include the heparin sulfate moiety of the heparan sulfate proteoglycan (HSPG) [8]. Integrins are also likely candidates to bind *O. tsutsugamushi*, and the colocalization of *O. tsutsugamushi* with the α5β1 integrin has been reported in HeLa cells [20].

For nonphagocytic cells, the interaction of *O. tsutsugamushi* with various receptors leads to cytoskeletal reorganization and the formation of membrane protrusions [20], which are likely related to the ability of *O. tsutsugamushi* to activate

tyrosine kinases, such as fak and src family members. The interaction between *O. tsutsugamushi* and endothelial cells results in the production of cytokines and chemokines. In human dermal microvascular endothelial cells (HMEC-1), CCL2 (monocyte chemotactic protein-1, MCP-1), CCL5 (regulated upon activation, normal T-cell expressed and secreted, RANTES) and CXCL8 [interleukin (IL)-8] are produced in response to *O. tsutsugamushi* infection [21]. We have also shown that *O. tsutsugamushi* induces interferon (IFN)-β secretion by HMEC-1 cells (unpublished data). In ECV304 cells, a large panel of cytokines [IL-1, IL-6, IL-10, IL-15, tumor necrosis factor (TNF) and lymphotoxin alpha] and chemokines [CXCL1-3 (growth related oncogene, Gro), CCL5, CCL17 (thymus and activation-regulated chemokine, TARC) and CXCL8] are also induced [21]. In addition, the secretion of IL-32 by ECV304 cells infected with *O. tsutsugamushi* is dependent on the nucleotide oligomerization domain (NOD)-1 pathway, as NOD1 knockdown by siRNA results in decreased cytokine secretion [22]. It has been proposed that *O. tsutsugamushi* infection of endothelial cells results in the activation of the NOD1 pathway followed by IL-32 secretion, as the latter favours the production of IL-1β, IL-6 and CXCL8 as well as the expression of CD54 (inter-cellular adhesion molecule 1, ICAM-1) [8]. Because proteoglycans are lacking in *O. tsutsugamushi*, the bacterial ligand for NOD-1 remains unknown. IL-1β and IL-32 function synergistically [23] and may be critically involved in endothelial injury and inflammation, which are involved in the clinical complications of patients with scrub typhus.

O. tsutsugamushi invades ECV304 and L929 cells by clathrin-mediated endocytosis. Once inside the cells, the bacterium rapidly escapes from the phagosome into the cytosol [24]. Genome sequencing of *O. tsutsugamushi* revealed the presence of a hemolysin gene, tlyC, and a gene that potentially encodes phospholipase D; both are expressed by cultured *O. tsutsugamushi* and may account for phagosome release [25,26]. Within the cytosol, *O. tsutsugamushi* moves to the microtubule-organizing center in the perinuclear region [27]. The intracellular mobility of *O. tsutsugamushi* is distinct from that of other bacteria with an intracytosolic location, as *O. tsutsugamushi* does not mobilize filamentous actin [28].

I - 2. Infection of monocytes and macrophages by O. tsutsugamushi

O. tsutsugamushi is detected in the peripheral blood mononuclear cells [12] and buffy coats [29] of patients with scrub typhus, suggesting that circulating monocytes could be targeted by *O. tsutsugamushi*. We have shown that *O. tsutsugamushi* can infect and replicate in human monocytes [30]. Because *O. tsutsugamushi* is very unstable and quickly loses viability in an extracellular environment [31], monocyte invasion would be beneficial for bacterial survival and may contribute to bacterial dissemination from an infected site to the target cells within visceral organs.

As described for endothelial cells, *O. tsutsugamushi* is a potent activator of monocytes. Transcriptional profiling studies revealed that *O. tsutsugamushi*-infected monocytes are polarized toward a sustained M1 profile and exhibit a type I IFN response. Indeed, infected monocytes express a large panel of inflammatory cytokines (TNF, IL-1, IL-6, IL-12p40, IL-23p19 and IL-15) and chemokines (CCL3, CCL4, CC7, CCL8, CCL20, CXCL10 and CXCL11) [30], which are associated with the M1 status. The M1 status is often the result of macrophage stimulation with inflammatory cytokines (IFN-γ, TNF) and bacterial ligands, such as LPS, and this state confers microbicidal competence to macrophages [32]. However, the sustained polarization of monocytes that we observed is relatively unusual because the monocyte polarization status is thought to be transient (unpublished data). Thus, this state may contribute to the pathogenicity of *O. tsutsugamushi*.

The type I IFN response that is induced by *O. tsutsugamushi* involves type I IFN genes (IFN-β and some subtypes of IFN-α) and IFN-stimulated genes, such as 2'-5'-oligoadenylate synthetase (OAS) and myxovirus resistance genes (MX1 and MX2) [30]. The type I IFN response requires live bacteria, as heat-killed bacteria are unable to induce a type I IFN response. Recent evidence has demonstrated that recognition of cytosolic DNA by cytosolic DNA sensors triggers the type I IFN response in an interferon regulatory factor (IRF)-3-dependent manner [33], but it remains unclear how bacterial DNA is released into the cytosol of host cells. It has been shown that a small proportion of *L. monocytogenes* organisms found in the cytosol lose cell wall integrity and release DNA into the host cell cytosol [34]. As *O. tsutsugamushi* is very fragile due to the absence of peptidoglycan and LPS, cell wall rupture may occur after bacterial escape from the phagosome.

We also found that monocytes release high levels of IL-1β in response to live *O. tsutsugamushi* [30]. IL-1β mRNA expression can be upregulated by both live and heat-killed bacteria, but the secretion of the active cytokine requires live bacteria. These findings suggest that the infection of monocytes by live *O. tsutsugamushi* may trigger inflammasome activation followed by caspase-1 activation and IL-1β release; both responses have also been observed in *O. tsutsugamushi*-infected ECV304 cells [26]. The inflammasome is a macromolecular complex that can be activated by cytosolic pattern recognition receptors (PRRs), namely NOD-like receptors (NLRs) and absent in Melanoma (AIM)-2. AIM2 recognizes cytosolic DNA and forms a large inflammasome complex. The AIM2 inflammasome is critical for the innate immune response to cytosolic bacteria, including *Francisella tularensis* [35] and *L. monocytogenes* [36]. AIM2 is also an IFN-inducible protein [37] and is highly up-regulated following live *O. tsutsugamushi* infection [30]. As IL-1β is known to induce local and systemic inflammation [38], the IL-1β secreted by monocytes may be a key cytokine involved in the pathogenesis of scrub typhus.

Because inflammatory monocytes are the precursors of M1/inflammatory macrophages, we questioned whether the response of macrophages to *O. tsutsugamushi* could be specific. Reports from human autopsy specimens have demonstrated the presence of *O. tsutsugamushi* in Kupffer cells and macrophages located in the spleen, lymph nodes and liver capsule [39]. However, it is unclear whether these infected macrophages were derived from infected monocytes or were directly infected by *O. tsutsugamushi*. To answer this question, we have used murine macrophage cell lines, peritoneal macrophages and human macrophages. *O. tsutsugamushi* induces the expression of chemokine genes, including those encoding CCL2, CCL3 [macrophage inflammatory protein (MIP)-1α], CCL4 (MIP-1β), CCL5 and CXCL2 (MIP-2), in J774A.1 macrophages in an NF-κB-dependent manner [40,41]. *O. tsutsugamushi* also activates mitogen-activated protein kinase (MAPK) pathways, leading to the expression of TNF [42] and IFN-β [41]. Although the expression of TNF does not require live bacteria, the expression of IFN-β does depend on the presence of viable microorganisms [41]. Unlike several models of infection with Gram-negative bacteria, the secretion of TNF by peritoneal macrophages stimulated with *O. tsutsugamushi* does not depend on toll-like receptors (TLRs), a major family of PRRs, such as TLR-4 [42]. This is similar

to findings from *Rickettsia akari*-infected monocytes, which demonstrated that TLR-2 and TLR-4 were not required for TNF secretion [43]. *O. tsutsugamushi* also induces the production of anti-inflammatory mediators. Indeed, J774A.1 macrophages infected with live *O. tsutsugamushi* released lower levels of TNF and higher levels of IL-10 than LPS-treated cells, suggesting that *O. tsutsugamushi* stimulates the production of IL-10-inducing factors [44]. *O. tsutsugamushi* may use this suppressive mechanism to promote its own survival within murine macrophages. Indeed, the depletion of macrophages has been shown to enhance lymphocyte proliferation, which suggests that macrophages may mediate the inhibition of the lymphocyte response [45]. Our results obtained using human monocyte-derived macrophages (manuscript in preparation) demonstrated that the macrophage response is similar to what we had previously observed in circulating monocytes [30]. *O. tsutsugamushi* induces M1 polarization, the type I IFN response and IL-1β secretion in human monocyte-derived macrophages. The microorganism also had effects on the chemokines. The genes encoding the chemokines CCL3, CCL4, CCL5 and CCL8 (CCR5 ligands) as well as those encoding CXCL9, CXCL10 and CXCL11 (CXCR3 ligands) were upregulated following *O. tsutsugamushi* infection. Note that CCR5 and CXCR3 ligands are known to be chemoattractants for Th1 and cytotoxic lymphocytes), [46,47]. In addition, the genes encoding CXCL9, CXCL10 and CXCL11, which are considered IFN-stimulated genes [48], were overexpressed in patients with rickettsiosis and in *Rickettsia*-infected mice [49]. Thus, the type I IFN response is likely critical for mononuclear cell infiltration, a common pathologic finding in scrub typhus patients.

I - 3. O. tsutsugamushi *and modulation of cell death*

O. tsutsugamushi modulates cell death in its host cells by interfering with pro- and anti-apoptotic activities. *O. tsutsugamushi* has been shown to induce the apoptosis of heavily infected ECV304 endothelial cells [50,51], L929 cells [52], monocytes and macrophages [30]. Infected ECV304 cells undergoing cell death showed disruption of focal adhesion molecules (focal adhesion kinase and paxillin) and actin cytoskeletal damage [50]. Our results have shown that a small proportion of live *O. tsutsugamushi*-infected monocytes [30] and macrophages (manuscript in preparation) exhibit features of cell death as determined using the terminal deoxynucleotidyl transferase dUTP nick

end labeling (TUNEL) assay. Another study showed that *O. tsutsugamushi* prevents beauvericin-induced apoptosis of THP-1 macrophages by delaying the release of intracellular calcium from the endoplasmic reticulum [53]. This anti-apoptotic effect was independent of NF-κB, which is in contrast to that described for *Rickettsia*-infected endothelial cells [54]. It is likely that *O. tsutsugamushi* can have a dual effect on cell death depending on the time course of the infection; bacteria may be anti-apoptotic at the early stages of an infection but pro-apoptotic at later stages or in heavily infected cells.

The mechanisms by which *O. tsutsugamushi* can modulate cell death are only partially understood. Apoptosis may be induced by type I IFNs that are released by infected host cells. Indeed, the cell death/apoptosis-related IFN-stimulated genes (ISGs), including those for TRAIL/Apo2L, XAF-1, FAS and PML, are upregulated following *O. tsutsugamushi* infection and may promote apoptosis [55]. Fas/FasL interaction and perforin/granzymes secretion by cytotoxic lymphocytes may contribute to apoptosis in vivo. Pyroptosis, or inflammatory cell death, may also be a consequence of monocyte and macrophage infection by *O. tsutsugamushi*. The microbial components that are released into the cytosol of the host cell can trigger inflammasome and caspase-1 activation, leading to pyroptotic cell death accompanied by IL-1β secretion and inflammation. Pyroptosis is a component of the innate response that helps to clear intracellular bacteria, such as *Salmonella, Legionella, Francisella* and *Listeria* [56]. Understanding the cell death mechanisms induced by bacterial pathogens may be necessary to understand the immune suppression observed during severe or chronic infections [57]. Interestingly, lymphocyte apoptosis was observed in the lymph nodes and spleen of mice infected with the virulent Karp strain of *O. tsutsugamushi* but not with the less virulent Gilliam strain [58]. Thus, the role of T cell apoptosis in human scrub typhus requires further investigation.

II - Adaptive Immune Response to *O. tsutsugamushi*

To understand how *O. tsutsugamushi* infection of endothelial cells and monocytes/macrophages affects the generation of the host immune response, it is necessary to review the features of infection in both animal models and patients with scrub typhus.

In mouse models of *O. tsutsugamushi* infection, the susceptibility or the resistance to *O. tsutsugamushi* is genetically controlled. After challenge with the Gilliam *O. tsutsugamushi* strain, susceptible C3H/HeN mice possess a greater number of macrophages and neutrophils but a lower number of lymphocytes in the peritoneal cavity than resistant BALB/c mice [59]. In addition, susceptible mice produce higher levels of cytokines [lymphotoxin β, TNF, IL-6, IFN-γ, transforming growth factor (TGF)-β1, macrophage migration inhibition factor (MIF), IFN-γ and IL-10] and chemokines (lymphotactin, CCL2, CCL3, CCL4, CCL5 and CXCL2) than do resistant mice after challenge with the Gilliam and Karp *O. tsutsugamushi* strains [59,60]. It is largely unknown whether the type I immune response induced by *O. tsutsugamushi* plays a role in the control of the infection. Initial publications found that the number of bacteria per cell as well as the percentage of infected cells were reduced in cytokine-treated macrophages during the initial phase of the infection [61]. The percentage of infected cells is lower for murine macrophages that have been pretreated with recombinant TNF, IFN-γ or the combination of TNF and IFN-γ than in untreated macrophages [62]. IFN-γ has been shown to partially inhibit the growth of the Gilliam *O. tsutsugamushi* strain in mouse fibroblasts [63], whereas TNF, but not IFN-γ, was shown to inhibit the growth of the Karp strain in mouse embryonic cell lines [62]. IFN-γ is produced by several cell types, including antigen-responsive T cells, found in the draining lymph nodes and spleens of infected mice [64,65]. The role of IFN-γ-producing T cells during *O. tsutsugamushi* infection was illustrated by the effectiveness of the adoptive transfer of specific IFN-γ-secreting T cells, which conferred protection against infection [66]. It is generally believed that cytosolic bacteria engage $CD8^+$ T cells known to produce IFN-γ and to be cytotoxic (CTLs) [67]. It has been reported that splenic T lymphocytes from mice infected with *O. tsutsugamushi* were able to lyse target cells in a major histocompatibility complex (MHC)-restricted manner [68]. These results are similar to those from mouse models of rickettsial infections where resistance

depended on the expression of markers of $CD8^+$-mediated anti-infectious immunity, including MHC class I molecules, perforin and IFN-γ [69].

Human studies have also revealed the importance of the inflammatory response during scrub typhus. In patients with scrub typhus, the serum levels of TNF, granulocyte colony-stimulating factor (G-CSF), macrophage CSF (M-CSF), IL-12p40, IL-15, IL-18, IFN-γ and IL-10 were elevated [70-73], and the mRNA expression of IL-1β, TNF, IL-6, IFN-γ and IL-10 was increased [72]. Another study also reported elevated levels of CXCL9 (also known as monokine induced by gamma interferon, Mig), CXCL10 (interferon gamma-induced protein 10, IP-10) and granzymes A and B [74]. CXCL9 and CXCL10 preferentially attract CTLs and natural killer (NK) cells, which express CXCR3, whereas granzymes A and B are the degranulation products of CTLs. The levels of TNF, IL-1β, IL-6, IFN-γ and IL-10 rapidly decrease following patient treatment with doxycycline, which correspond to early defervescence and the rapid improvement of symptoms [72]. An investigation of the transcriptomic profile of mononuclear cells from patients with scrub typhus revealed the modulation of more than 1,000 genes [30]. Most of the highly expressed genes were found to correspond to biological processes including DNA metabolism, the cell cycle and immune function. For those genes related to immune function, IFN-γ, AIM2, guanylate-binding protein 1 (GBP1), IFN-γ-inducible factor 16 (IFI16) and indoleamine-pyrrole 2 (INDO) were identified as upregulated genes. The transcriptomic profile of mononuclear cells from patients with scrub typhus was compared to that of monocytes infected in vitro with *O. tsutsugamushi*. This comparison revealed that the differential expression of genes in scrub typhus, such as those for type I and II IFNs and M1-associated genes, was directly related to *O. tsutsugamushi* infection. Finally, a comparison of the transcriptomic profiles between scrub typhus patients with patients with murine typhus, malaria or dengue highlighted the specificity of the scrub typhus signature relating to upregulation of CD8-responsive genes [30]. This specific transcriptomic signature of scrub typhus supports the role for $CD8^+$ T cells in the protection against *O. tsutsugamushi* in mice [68] and the activation of CTLs in patients with scrub typhus [74].

CONCLUSIONS

Scrub typhus is an infectious disease that is associated with overactivated endothelial cells and myeloid cells, which leads to a cytokine storm. This storm is related to the infection of host cells by *O. tsutsugamushi* and is likely critically related to the severe clinical complications of scrub typhus. The type I immune response is protective but may be also pathogenic. The combination of transcriptomic and proteomic approaches has revealed several novel features of *O. tsutsugamushi* infection, and these approaches are likely to provide new tools for the diagnosis of patients with scrub typhus.

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Chapter 2

Results

ARTICLE 1

***Orientia tsutsugamushi* Stimulates an Original Gene Expression Program in Monocytes : Relationship with Gene Expression in Patients with Scrub Typhus**

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In this article, we showed that *O. tsutsugamushi* invades and replicates in human monocytes. *O. tsutsugamushi* induces a substantial change in gene expression program of monocytes with about 4000 modulated genes. Notably, a type I IFN response, including IFN-β, some subtypes of IFN-α and several IFN-stimulated genes (ISGs), is induced by *O. tsutsugamushi*. Infected monocytes are polarized towards a classical M1 phenotype with the production of several cytokines and chemokines specific to M1 cells (e.g. TNF, IL-6, IL-12p40 and CXCL10). *O. tsutsugamushi* also triggers IL-1β release, the suggestive evidence of inflammasome activation. *O. tsutsugamushi* also modulates the expression of apoptosis-related genes and induces cell death in a minority of monocytes. Infection by live *O. tsutsugamushi* organisms is indispensable for type I IFN response, IL-1β release and cell death induction.

In the second part of the paper, we studied the transcriptional profiles of patients with scrub typhus and compared them with those of patients with other tropical diseases including murine typhus, malaria and dengue fever. We found that the expression profiles of 65 genes in scrub typhus are distinct from those in other diseases. The specific signature may be helpful to

diagnosis of scrub typhus. In addition, some of the modulated genes in *in vitro*-infected monocytes such as some ISGs are also modulated in patients with scrub typhus.

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PLoS NEGLECTED TROPICAL DISEASES

Orientia tsutsugamushi Stimulates an Original Gene Expression Program in Monocytes: Relationship with Gene Expression in Patients with Scrub Typhus

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Abstract

Orientia tsutsugamushi is the causal agent of scrub typhus, a public health problem in the Asia-Pacific region and a life-threatening disease. *O. tsutsugamushi* is an obligate intracellular bacterium that mainly infects endothelial cells. We demonstrated here that *O. tsutsugamushi* also replicated in monocytes isolated from healthy donors. In addition, *O. tsutsugamushi* altered the expression of more than 4,500 genes, as demonstrated by microarray analysis. The expression of type I interferon, interferon-stimulated genes and genes associated with the M1 polarization of macrophages was significantly upregulated. *O. tsutsugamushi* also induced the expression of apoptosis-related genes and promoted cell death in a small percentage of monocytes. Live organisms were indispensable to the type I interferon response and apoptosis and enhanced the expression of M1-associated cytokines. These data were related to the transcriptional changes detected in mononuclear cells isolated from patients with scrub typhus. Here, the microarray analyses revealed the upregulation of 613 genes, which included interferon-related genes, and some features of M1 polarization were observed in these patients, similar to what was observed in *O. tsutsugamushi*-stimulated monocytes in vitro. This is the first report demonstrating that monocytes are clearly polarized in vitro and ex vivo following exposure to *O. tsutsugamushi*. These results would improve our understanding of the pathogenesis of scrub typhus, during which interferon-mediated activation of monocytes and their subsequent polarization into an M1 phenotype appear critical. This study may give us a clue of new tools for the diagnosis of patients with scrub typhus.

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Introduction

Orientia tsutsugamushi is the causative agent of scrub typhus, a life-threatening disease characterized by fever, lymphadenopathy, rash and eschar that can be complicated by interstitial pneumonitis, meningitis and myocarditis [1]. The proper diagnosis of scrub typhus can be difficult due to the non-specific initial symptoms that are frequently found in other acute febrile illnesses. While scrub typhus is confined geographically to the Asia-Pacific region, a billion of people are at risk and one million new cases arise each year. As *O. tsutsugamushi* is transmitted to humans by the bite of larval trombiculid mites, people who inhabit regions infested with these vectors are at high risk for acquiring scrub typhus [2]. To date, no effective strategy has succeeded in generating long lasting, protective immunity to this particular infection despite aggressive attempts to develop a prophylactic vaccine [3].

Due to the significant genetic and phenotypic differences in its cell wall, including the absence of peptidoglycan and lipopolysaccharide (LPS), *O. tsutsugamushi* has been classified as a new genus that is distinct from the *Rickettsia* genus [4]. The complete genome sequence of two *O. tsutsugamushi* strains (Boryong and Ikeda) has recently been described. The *O. tsutsugamushi* genome contains several repetitive sequences, including genes for conjugative type IV secretion systems (*tra* genes) [5,6]. *O. tsutsugamushi* is an obligate intracellular bacterium that can invade a variety of cell types both in vitro and in vivo. It has been recently shown that *O. tsutsugamushi* can exploit $\alpha5\beta1$ integrin-mediated signaling and the actin cytoskeleton to invade HeLa cells [7]. Another study reported that following phagocytosis by L929 cells, *O. tsutsugamushi* rapidly escapes the phagosome and enters the cytosol [1]. *O. tsutsugamushi* also infects endothelial and fibroblast cell lines through clathrin-mediated endocytosis [8]. Once inside the cell, *O. tsutsugamushi* moves along microtubules to the microtubule-organizing center in

Author Summary

Scrub typhus, a life-threatening disease that occurs in the Asia-Pacific region, is a public health problem since a billion of people are at risk and one million new cases arise each year. *Orientia tsutsugamushi*, the causal agent of scrub typhus, is an obligate intracellular bacterium that mainly infects endothelial cells. We demonstrated here that *O. tsutsugamushi* grew in monocytes isolated from healthy donors and altered the expression of a large number of genes including interferon-related genes, genes associated with the M1 polarization of macrophages and apoptosis-related genes. Importantly, these data were related to the transcriptional changes detected in mononuclear cells isolated from patients with scrub typhus. Indeed, the microarray analyses revealed the upregulation of numerous genes, which included interferon-related genes, and some features of M1 polarization. This is the first report demonstrating that monocytes are clearly polarized in vitro and ex vivo following exposure to *O. tsutsugamushi*. These results improve our understanding of the pathogenesis of scrub typhus and may give us a clue of new tools for the diagnosis of patients with scrub typhus.

a dynein-dependent manner [9]. In experimental animals, *O. tsutsugamushi* infects peritoneal mesothelial cells [10], macrophages [11] and polymorphonuclear leukocytes [12]. In humans, *O. tsutsugamushi* has been detected in peripheral blood mononuclear cells (PBMCs) from patients with acute scrub typhus [13].

The mechanisms governing the interaction between *O. tsutsugamushi* and host cells are only partially understood. It has been recently demonstrated that the expression of approximately 30% of bacterial genes is modulated when *O. tsutsugamushi* is cultured in eukaryotic cells. When compared to the bacterial gene expression seen in the L929 fibroblast cell line, the expression of a number of bacterial genes involved in translation, protein folding and secretion is downregulated in J774 macrophages, and this decreased expression correlated with the reduced growth of *O. tsutsugamushi* in macrophages [14]. Infection with *O. tsutsugamushi* most likely has many effects on the human immune response. In vitro studies have shown that *O. tsutsugamushi* induces the expression of genes encoding chemokines, including MCP-1 (CCL2), RANTES (CCL5) and IL-8 (CXCL8), in human endothelial cells [15,16]. In patients with scrub typhus, the serum level of pro-inflammatory cytokines (e.g. TNF, IL-12p40, IL-15, IL-18 and IFN-γ) is also increased [17], demonstrating that *O. tsutsugamushi* infection is accompanied by an inflammatory response [18]. The circulating levels of chemokines such as CXCL9 (MIG) and CXCL10 (IP-10), which are known to attract Th1, cytotoxic T cells and NK cells, and molecules such as granzymes A or B, which are released following the degranulation of cytotoxic lymphocytes, are also increased [18].

In this paper, we report that *O. tsutsugamushi* induces large changes in gene transcription in naïve human monocytes. In addition to genes encoding inflammatory cytokines and chemokines, *O. tsutsugamushi* upregulates the expression of genes involved in type I IFN pathway and genes involved in apoptosis. Interestingly, these in vitro results were related to the expression of genes involved in the immune response, including the IFN response, in patients with scrub typhus. Our study highlights the role of IFN-mediated monocyte activation in the pathogenesis of scrub typhus.

Materials and Methods

Ethics Statement

Blood samples from patients and controls were collected after informed and written consent obtained from each participant, and the study was conducted with the approval of the Ethics Committee of Siriraj Hospital, Bangkok, Thailand.

Patients

Ten milliliters of blood was collected from patients with acute undifferentiated fever who were seen at Siriraj Hospital or Ban Mai Chaiyapot Hospital. The clinical status of each patient was recorded. Within two hours of blood drawing, PBMCs were separated by Ficoll density gradient centrifugation. The PBMCs were immediately lysed in Trizol reagent (Invitrogen, Carlsbad, CA), as recommended by the manufacturer, and the lysates were stored at -80°C until further analysis. The study participants were retrospectively divided into the following three groups: healthy controls (individuals without any of the four infections), patients with scrub typhus (n = 4) and an infected control group consisting of patients with murine typhus (n = 7), malaria (n = 4) or dengue (n = 7). Patients with evidence suggesting co-infections or those with malignancies were excluded. The diagnostic criteria for scrub typhus were the presence of circulating *O. tsutsugamushi*-specific IgM with a titer greater than 1:400 in serum from patients with acute disease and/or *O. tsutsugamushi*-specific IgG with at least a four-fold increase of titer. The criteria for murine typhus were a serum *Rickettsia typhi*-specific IgM titer greater than 1:400 in patients with acute disease and/or at least a four-fold increase of IgG titer. The criteria for dengue virus infection were a dengue virus-specific IgM titer greater than 1:1,280, as determined using enzyme linked immunosorbent assay (ELISA), and/or positive for dengue RNA using RT-PCR. Malaria infection was determined by the detection of *Plasmodium* species in blood films observed using a light microscope. Patients with murine typhus, malaria or dengue presented with a lower absolute number of leukocytes than patients with scrub typhus, but the lymphocyte/monocyte ratio was similar (Table S1). Ten healthy individuals were included in the study as controls, and the extracted RNA was pooled to reduce the effect of interindividual variability. The reproducibility of this procedure was tested using two different pools consisting of 5 individuals each.

O. tsutsugamushi culture and isolation

O. tsutsugamushi, strain Kato (CSUR R163), was propagated in L929 cells cultured in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine (Invitrogen, Cergy Pontoise, France), as recently described [19]. When almost 100% of the cells were infected, as determined using May Grünwald Giemsa (Merck, Darmstadt, Germany) staining, the cells were harvested, lysed using glass beads and centrifuged at $500\times g$ for 5 min to remove cell debris. The supernatants were centrifuged at $2{,}000\times g$ for 10 minutes to collect bacterial pellets. The isolated bacteria were frozen in MEM containing 20% FBS and 5% DMSO until use. The titer of the supernatants was determined as described previously [8,20] with slight modifications. Briefly, the bacteria were serially diluted five-fold and incubated with L929 cells grown in 24-well plates. After 2 hours, free bacteria were removed and the infected L929 cells were cultured in MEM containing 5% FBS and 0.4 µg/ml daunorubicin (Biomol, Hamburg, Germany), which partially inhibits cell growth, for 2 days [21]. The infection of the L929 cells was quantified using indirect immunofluorescence with pooled serum from Thai patients with scrub typhus at a dilution

of 1:400 and fluorescein isothiocyanate-conjugated goat anti-human IgG (BioMérieux, Marcy l'Etoile, France) diluted at 1:200 as a secondary antibody. The infected-cell counting units (ICUs) of *O. tsutsugamushi* were defined as (the total number of cells used in the infection)×(the percentage of infected cells)×(the dilution rate of the bacterial suspension)/100. In some experiments, *O. tsutsugamushi* organisms were killed by heating at 100°C for 5 minutes.

Infection of human monocytes

PBMCs were isolated from leukopacks (Etablissement Français du Sang, Marseille, France) over a Ficoll gradient (MSL, Eurobio, Les Ulis, France) and incubated in 24-well plates for 1 hour. Adherent cells were designed as monocytes since more than 90% of them expressed CD14, as previously described [22]. Monocytes (1.5×10^5 per assay) were incubated with 3×10^5 *O. tsutsugamushi* organisms in RPMI 1640 containing 10% FBS, 20 mM HEPES and 2 mM L-glutamine (Invitrogen) for 2 hours. The monocytes were then extensively washed to remove free organisms and cultured for the indicated times. The uptake and the intracellular fate of the *O. tsutsugamushi* organisms were determined using immunofluorescence and quantitative real-time PCR (qPCR). Immunofluorescence was performed using pooled serum from Thai patients with scrub typhus and a standard protocole. Cells were then examined by fluorescence and laser scanning microscopy using a confocal microscope (Leica TCS SP5, Heidelberg, Germany) as recently described [23].

To assess bacterial DNA, the monocytes were incubated in 0.1% Triton X-100, and DNA was extracted in a 150 µl volume using a QIAamp Tissue Kit (Qiagen, Courtaboeuf, France), as recommended by the manufacturer. The number of bacterial DNA copies was calculated using the Taqman system (Applied Biosystems, Warrington, UK) with a 5 µl DNA sample. The selected primers and probes were designed based on the available DNA sequence of *O. tsutsugamushi* strain Boryong (complete genome, GenBank ref. NC_009488.1) and were the following: forward (3235–3257), 5′-AAGCATAGGTTACAGCCTGG-WGA-3′; reverse (3346–3373), 5′-ACCCCAACGGATTTAA-TACTATATCWAC-3′; probe R (3307–3338), 5′-FAM-CCATCTTCAAGAAATGGCATATCTTCCTCAGG- TAMR-A-3′. The resulting PCR product was 139 bp in size. Negative controls consisted of DNA extracted from uninfected monocytes. Each PCR run included a standard curve generated from tenfold serial dilutions of a known concentration of *O. tsutsugamushi* DNA. The results are expressed as the total number of bacterial DNA copies.

Transcriptional profile of monocytes

Eight hours post-infection, total RNA was extracted from infected and uninfected monocytes using an RNeasy Mini kit (Qiagen) with DNase digestion, as recommended by the manufacturer. The quality of the isolated RNA was assessed by Agilent 2100 Bioanalyzer and an RNA 6000 Nano Kit (Agilent Technologies, Massy, France). The concentration of RNA was determined using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of eight RNA samples (four samples per condition) were then processed for microarray analysis. The RNA was amplified and Cy3-labeled cDNA was generated using the Agilent Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies), as recommended by the manufacturer. The amplified cDNA was processed and hybridized to 4×44K microarray slides (Agilent Technologies). The scanned images were analyzed with Feature Extraction Software 10.5.1 (Agilent) using default parameters. The data processing and analyses were performed using the Resolver software 7.1 (Rosetta Inpharmatics, Cambridge, MA) as previously described [24] and R and BioConductor softwares. The Rosetta intensity error model for single color microarrays was used to perform inter-array normalization. Statistical analyses were performed using the significance analysis of microarrays (SAM) [25]. The median false discovery rate was approximately 5%. Genes with an absolute fold change (FC) greater than 2 and a p value of the error model less than 0.01 were considered differentially modulated. The differentially expressed genes were classified based on their Gene Ontology (GO) category. The transcriptional profile of the infected monocytes was compared with the profile of monocytes stimulated with IFN-γ or IL-4 to assess their polarization status (manuscript in preparation) using R software and hierarchical clustering.

Minimum Information About a Microarray Experiment (MIAME)-compliant data sets are provided in the Gene Expression Omnibus (GEO) [26] at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) and can be assessed through GEO series accession number GSE24247 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE24247).

Transcriptional profile of patient PBMCs

The RNA from PBMCs lysed in TRIZOL was extracted using the RNeasy Mini Kit (Qiagen), and the RNA of ten healthy individuals was divided into two distinct pools. The RNA amplification for microarray analysis was performed using the Illumina TotalPrep RNA Amplification Kit (Ambion, Austin, TX), as recommended by the manufacturer. Five hundred nanograms of amplified cRNA was hybridized onto Human-6 v2 BeadChips (Illumina, San Diego, CA), which contained more than 46,000 probes targeting all known human transcripts. The hybridized chips were scanned on an Illumina BeadStation 500 and assessed for fluorescent signal intensity using Illumina Beadstudio software. Normalization and all analyses of microarray data were performed by GeneSpring GX 9 demo version (Agilent Technologies). Briefly, quantile normalization was applied to the raw signal intensities. Next, the probes in which the normalized expression level was below the twentieth percentile for every sample in any group of patients were excluded, leaving 38,630 probes for further analysis. We focused on two main sets of genes. The first one comprised scrub typhus-associated genes, which were identified by performing a Welch ANOVA with Benjamini-Hochberg correction [27] across the four disease groups. A post hoc Tukey's HSD test was further applied to the Welch ANOVA results. Genes that were differentially expressed in scrub typhus patients compared to the other groups were then selected using the intersection rule. Unsupervised hierarchical cluster was performed for all patient groups on the basis of the Euclidean distance and average linkage. The second gene set was composed of scrub typhus-responsive genes, which were genes whose mean expression level in patients with scrub typhus group was at least twofold greater than the expression in healthy controls. The significance of the GO enrichment was evaluated using the hypergeometric formula with Benjamini-Yekutieli correction [27,28]. As the transcriptomic profiles of patient PBMCs and those of naïve monocytes stimulated with *O. tsutsugamushi* were obtained using Illumina bead chips and Agilent microarrays, respectively, the two profiles were compared by building a virtual Agilent microarray. These genes were then analyzed using R software. The data are generated in compliance with the MIAME guidelines and have been deposited in the NCBI's Gene Expression Omnibus and are accessible using GEO Series accession number GSE16463 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=bxmzrwwgygmwahu&acc=GSE16463).

Quantitative real time RT-PCR and ELISA

Real time quantitative RT-PCR (qRT-PCR) of the genes of interest was carried out as previously described [24]. Briefly, total RNA was isolated from monocytes using a Qiagen kit, and cDNA synthesis was performed using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen), as recommended by the manufacturer. Real time PCR from cDNA templates was performed using Light Cycler-FastStart DNA Master^{PLUS} SYBR Green I (Roche Applied Science, Meylan, France). The sequences of the primers are provided in Table S2. The fold change of the target genes relative to β-actin was calculated using the $2^{-\Delta\Delta Ct}$ method, as described previously [29]. The level of IFN-β and TNF in the supernatants was analyzed by IFN-β and TNF ELISA kits (R&D Systems, Lille, France). The IL-1β level was determined using an IL-1β ELISA kit purchased from Diaclone (Besançon, France).

In situ cell death

Infected monocytes were fixed with 3% paraformaldehyde before being analyzed using the TdT-mediated dUTP nick-end labeling (TUNEL) assay. DNA strand breaks were labeled using an In Situ Cell Death Detection Kit, TMR red (Roche Applied Science), as recommended by the manufacturer. Nuclei were counter-stained with DAPI. The number of TUNEL-positive cells and DAPI-stained nuclei was determined using fluorescence microscopy. Cell death is expressed as the ratio of TUNEL-positive cells to DAPI-stained nuclei ×100.

Statistical analyses

Statistical analyses were performed using GraphPad Prism Sofware v. 5.01. The results are expressed as the mean ± SEM and were compared using the non-parametric Mann-Whitney *U* test: *p* values less than 0.05 were considered significant.

Results

O. tsutsugamushi replicates in human monocytes

As *O. tsutsugamushi* organisms have been detected in monocytes from patients with acute scrub typhus [13], we investigated whether they were able to invade and replicate within naïve monocytes. Monocytes were incubated with *O. tsutsugamushi* (two bacteria per cell) for 2 hours, extensively washed to remove free organisms and incubated for the indicated times. After 1 day, the number of bacterial DNA copies was approximately 1×10^5 (Figure 1A). Using immunofluorescence, the uptake of *O. tsutsugamushi* by monocytes could be readily observed (Figure 1B). The number of bacterial DNA copies steadily increased over the course of the 5-day experiment (Figure 1A). The replication of *O. tsutsugamushi* in monocytes was slower than in L929 cells (Figure 1A), a permissive control cell line. Indeed, the doubling time of *O. tsutsugamushi* within monocytes was approximately 18 hours compared with 10 hours in L929 cells. Time-dependent replication of *O. tsutsugamushi* within monocytes was also detected using indirect immunofluorescence (Figure 1B) and confocal microscopy (Figure 1C). These data demonstrate that *O. tsutsugamushi* was capable of replication in naïve monocytes.

Global transcriptome analysis of *O. tsutsugamushi*-infected monocytes

To understand how *O. tsutsugamushi* inhibits the microbicidal machinery of monocytes, we compared the transcriptional profile of unstimulated monocytes to that of monocytes stimulated with *O. tsutsugamushi* for 8 hours using whole genome microarrays.

This time was determined as follows. The time course of the expression of genes encoding cytokines (TNF, IL-1β, IL-6, IL-12p40), chemokines (CXCL10, CXCL11), IFN-β and TNF-related apoptosis-inducing ligand (TRAIL/Apo2L/TNFSF10) was studied using qRT-PCR. Their expression was variable after 2 hours, was maximum after 8 hours and progressively decreased thereafter (Figure S1). Consequently, the time of 8 hours was used to stimulate monocytes with *O. tsutsugamushi*. We found that 4,762 genes were altered in response to *O. tsutsugamushi*: 2,380 genes were upregulated and 2,382 genes were downregulated with at least a twofold change and a *p* value less than 0.01. The differentially expressed genes were classified into different categories based on

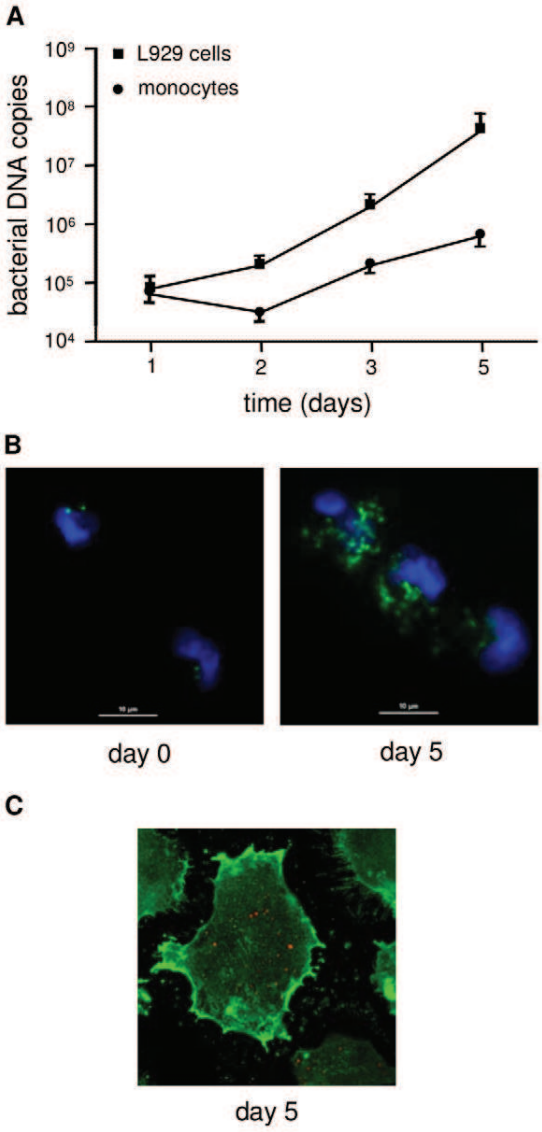

Figure 1. *O. tsutsugamushi* replication within monocytes. Monocytes and L929 cells were infected with *O. tsutsugamushi* (two viable bacteria per cell) for different periods of time. A. The number of bacterial DNA copies was determined using qRT-PCR. The data are expressed as the mean ± SD of two independent experiments performed in triplicate. B. Monocytes were infected with *O. tsutsugamushi,* and the bacteria were detected using indirect immunofluorescence. C. Monocytes infected with *O. tsutsugamushi* for 5 days were labeled with bodipy phallacidin to detect filamentous actin and bacteria were detected using indirect immunofluorescence (in red). One representative micrograph performed in confocal microscopy is shown.
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their GO according to their *p* value. Among the upregulated genes (2–100-fold increased) with an enrichment higher than 20% were genes involved in the immune response, the inflammatory response, chemotaxis, the anti-viral response and cell-cell signaling (Figure 2A). Other GO categories related to cellular processes, including apoptosis, cell proliferation, cell-cell signaling, receptor activity, signal transduction and transcription factor activity, exhibited lower enrichments, ranging from 10 to 20% (Figure 2A). Among the downregulated genes were genes involved in cell motility, chemotaxis, the cytoskeleton, the immune response, intracellular signaling, receptor activity and signal transduction (Figure 2B). Taken together, these data demonstrate that *O. tsutsugamushi* activated an important transcriptional program in human monocytes.

Analysis of the *O. tsutsugamushi*-induced type I IFN signature

As the GO category "response to virus" was specifically enriched following exposure to *O. tsutsugamushi*, we investigated the type I IFN pathway in monocytes. The genes encoding IFN-β, four subtypes of IFN-α and interferon-stimulated genes (ISGs) were upregulated in monocytes infected with *O. tsutsugamushi* (Table 1). These upregulated ISGs included the genes encoding CCL8, CXCL10 (IP10), CXCL11 (I-TAC), 2′5′ oligoadenylate synthetase (OAS 1–3, OASL), myxovirus resistance (MX1 and MX2), ISG15, ISG20, interferon regulatory factor 7 (IRF7), many interferon-induced proteins, the promyelocytic leukemia gene product (PML) and some metallothioneins. The upregulation of a type I IFN signature (the genes encoding IFN-β, IFN-α8, OAS1 and MX1) was confirmed using qRT-PCR in monocytes from three distinct donors incubated with *O. tsutsugamushi* for 8 (Figure 3A) and 24 hours (Figure 3B). Monocytes were also incubated with heat-killed *O. tsutsugamushi* for 8 hours, and the mRNA expression level of IFN-β and ISGs (MX1, CXCL10 and CXCL11) was determined using qRT-PCR. In contrast to live *O. tsutsugamushi*, heat-killed cells failed to induce the expression of IFN-β and ISGs (MX1, CXCL10 and CXCL11) in monocytes. We then investigated whether the *O. tsutsugamushi*-induced type I IFN signature was functional. Monocytes were incubated with *O. tsutsugamushi* for 24 hours, and the secretion of IFN-β was determined using ELISA. Monocytes infected with live *O. tsutsugamushi* produced 65±16 pg/ml IFN-β whereas those incubated with heat-killed bacteria were unable to produce IFN-β. These data clearly demonstrate that only live *O. tsutsugamushi* induced a sustained type I IFN response in human monocytes.

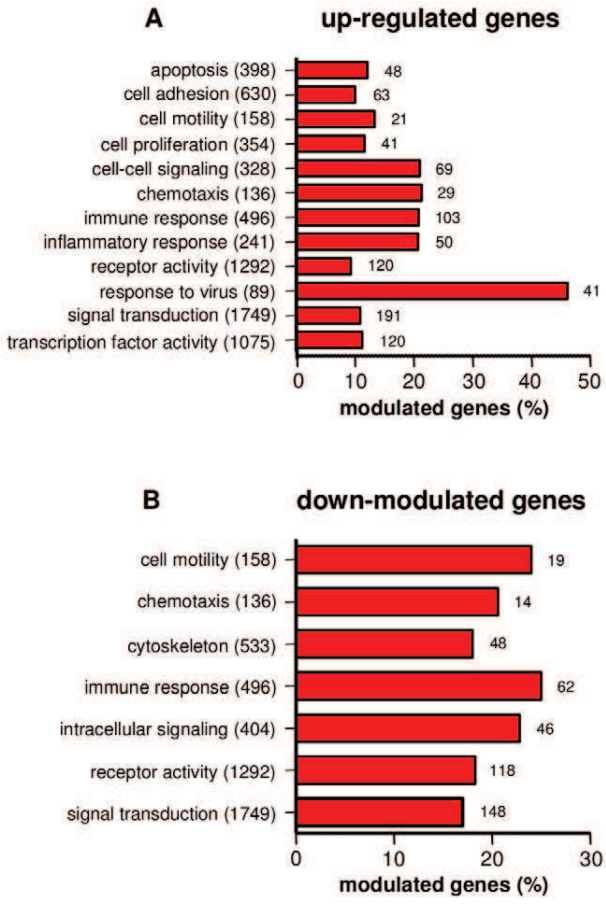

Figure 2. GO analysis of differentially expressed genes. Monocytes were stimulated with *O. tsutsugamushi* or mock stimulated for 8 hours, and the modulation of genes was analyzed using microarrays and GO term tools. The upregulated (A) and downregulated (B) genes were classified based on the major biological processes in which they are involved. The total number of genes present in each biological process and the number of differentially expressed genes are indicated. The results are expressed as the percentage of the upregulated or downregulated genes.
doi:10.1371/journal.pntd.0001028.g002

Analysis of the inflammatory response induced by *O. tsutsugamushi*

We next focused on the genes involved in the immune response (Table S3). Approximately 15% of the genes that were upregulated in response to *O. tsutsugamushi* were cytokines (14 genes) or chemokines (14 genes). The expression of the genes encoding pro-inflammatory cytokines, such as TNF, IL-1β, IL-6, IL-12p35, IL-12p40, IL-23p19 and IL-15, and chemokines, such as CXCL10, CXCL11 and CCL20, was determined using qRT-PCR. After 8 hours of stimulation with *O. tsutsugamushi*, the expression of these genes was markedly increased (Figure 3A). This upregulation was sustained, as the expression was similar at 8 and 24 hours (Figure 3B). Next, we examined if the inflammatory response was dependent on bacterial viability. Monocytes were stimulated with live or heat-killed *O. tsutsugamushi* for 8 hours. In contrast to the genes involved in the type I IFN response, the expression of genes encoding pro-inflammatory cytokines, such as TNF, IL-1β, IL-6, IL-12p40 and IL-23p19, was upregulated in response to both live and heat-killed *O. tsutsugamushi*, although the expression level of these cytokines was partially reduced in monocytes incubated with heat-killed bacteria (Figure 4A). The transcriptional response induced in monocytes by *O. tsutsugamushi* was accompanied by cytokine production: *O. tsutsugamushi* stimulated high levels of TNF (Figure 4B) and IL-1β (Figure 4C) production by monocytes in a manner that correlated with gene expression. Heat-killed organisms also induced the production of TNF, although it was significantly ($p<0.05$) less than the production induced by live organisms (Figure 4B). In contrast, heat-killed organisms were unable to induce IL-1β production (Figure 4C), despite the increased IL-1β mRNA expression (see Figure 4A).

Other genes, including CD40, CD70 and CD80, which play a major role in macrophage-T cell interactions, and indoleamine-pyrrole 2,3 dioxygenase (INDO), a multi-functional protein that plays a role in the intracellular killing of bacteria, were upregulated in response to *O. tsutsugamushi* (Table S3). The upregulation of the gene encoding INDO was confirmed in monocytes from three donors using RT-PCR (Figure 3). Notably, the expression of several molecules, including CD14, CD22, CCR2, IL16,

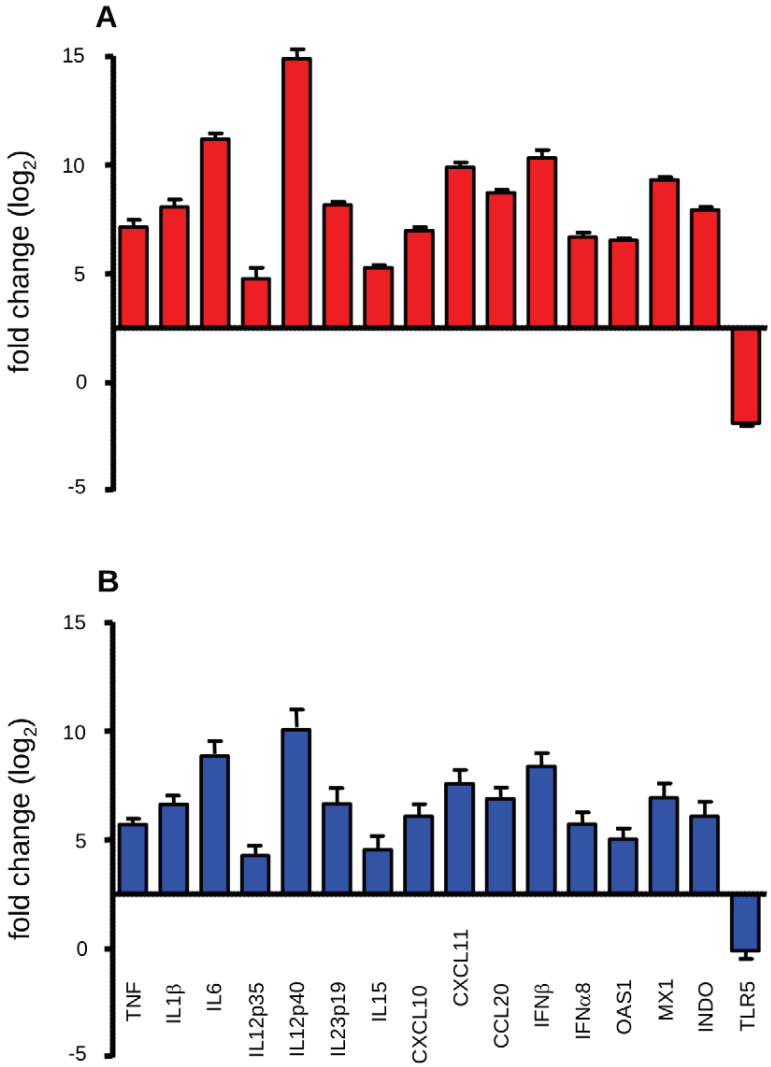

Figure 3. Quantitative RT-PCR of selected genes in stimulated monocytes. Monocytes were stimulated with or without *O. tsutsugamushi* for 8 (A) and 24 (B) hours. RNA was extracted, and qRT-PCR was performed on 16 genes involved in the immune response that were differentially expressing in the microarray experiments. The results, expressed as the $\log_2$ ratio of fold changes, are presented as the mean ± SEM of three experiments performed in triplicate.
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CLEC4A, CLEC10A and hepcidin antimicrobial peptides, was downregulated.

As the expression of a large number of chemokine and inflammatory cytokine genes associated with the M1 or M2 phenotype of macrophages was affected, we compared the transcriptomic profile of *O. tsutsugamushi*-infected monocytes with the list of M1 and M2 genes previously published [30]. We selected 32 and 28 genes representative of the M1 and M2 profiles, respectively (Table S4), in the genes associated with membrane receptors, cytokines, chemokines and apoptosis. Almost all of the genes characteristic of the M1 phenotype (29 of 32) were upregulated in response to *O. tsutsugamushi*. In contrast, M2 genes (except for CCL1, CCL23 and IL1RN) were either downregulated or remained unchanged. Taken together, these results demonstrate that *O. tsutsugamushi* induced a pro-inflammatory, M1 program in monocytes that did not require the presence of live organisms, which was in contrast to the type I IFN transcriptional signature.

Specificity of the M1 program induced by *O. tsutsugamushi*

As *O. tsutsugamushi* seems to induce an M1 program in monocytes, we compared this profile with the profile induced by IFN-γ, a canonical inducer of the M1 phenotype. The principal component analysis (Figure S2) and hierarchical clustering (Figure 5) revealed that the transcriptional pattern stimulated by *O. tsutsugamushi* was not identical to the program induced by IFN-γ. Approximately 76% of the genes altered in response to IFN-γ were also altered in response to *O. tsutsugamushi*, and 83% of the genes altered in response to *O. tsutsugamushi* were also altered in response to IFN-γ. Nevertheless, the expression of 572 and 413 genes was up- and downregulated in *O. tsutsugamushi*-stimulated cells compared with IFN-γ-stimulated cells, respectively. Among the genes upregulated in *O. tsutsugamushi*-stimulated monocytes, the GO analysis showed that genes involved in chromatin assembly, locomotion and lipid metabolism were enriched. Among the downregulated genes, we found a specific enrichment for genes in

Table 1. Modulated genes in the "response to virus" GO term.

Gene function and full gene name	Gene symbol	GenBank ID	Fold change
apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	APOBEC3G	NM_021822	3.1
chemokine (C-C motif) ligand 4	CCL4	NM_002984	16.9
chemokine (C-C motif) ligand 8	CCL8	NM_005623	74.8
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	DDX58	NM_014314	36.8
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	13.0
interferon, gamma-inducible protein 16	IFI16	NM_005531	3.5
interferon-induced protein 35	IFI35	NM_005533	7.6
interferon-induced protein 44	IFI44	NM_006417	29.4
interferon induced with helicase C domain 1	IFIH1	NM_022168	18.0
interferon, alpha 4	IFNA4	NM_021068	58.8
interferon, alpha 5	IFNA5	NM_002169	60.5
interferon, alpha 6	IFNA6	NM_021002	7.6
interferon, alpha 8	IFNA8	NM_002170	71.7
interferon, beta 1, fibroblast	IFNB1	NM_002176	71.7
interferon, gamma	IFNG	NM_000619	5.1
interleukin 23, alpha subunit p19	IL23A	NM_016584	5.1
interleukin 28 receptor, alpha (interferon, lambda receptor)	IL28RA	NM_170743	2.3
interleukin 29 (interferon, lambda 1)	IL29	NM_172140	5.7
interferon regulatory factor 7	IRF7	NM_004031	14.9
ISG15 ubiquitin-like modifier	ISG15	NM_005101	54.1
interferon stimulated exonuclease gene 20 kDa	ISG20	NM_002201	32.4
myxovirus (influenza virus) resistance 1	MX1	NM_002462	26.7
myxovirus (influenza virus) resistance 2	MX2	NM_002463	50.2
2′,5′-oligoadenylate synthetase 1, 40/46 kDa	OAS1	NM_002534	13.6
phospholipid scramblase 1	PLSCR1	NM_021105	5.0
v-rel reticuloendotheliosis viral oncogene homolog A	RELA	BC014095	3.1
signal transducer and activator of transcription 2, 113 kDa	STAT2	NM_005419	5.9
TANK-binding kinase 1	TBK1	NM_013254	2.0
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	13.9
tripartite motif-containing 22	TRIM22	NM_006074	8.4
tripartite motif-containing 5	TRIM5	NM_033034	10.7
tripartite motif-containing 5	TRIM5	NM_033092	13.6

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GO categories associated with cell activation, immune system processes and cell death. As determined using KEGG analysis, there was an over-representation of pathways associated with the immune response (14 of 18). Taken together, these findings suggest that while the response of monocytes to *O. tsutsugamushi* was polarized to an M1-like profile, it exhibited some specific features.

O. tsutsugamushi-induced monocyte apoptosis

The analysis of genes upregulated in response to *O. tsutsugamushi* revealed the enrichment of genes in four GO categories related to apoptosis, including apoptosis, anti-apoptosis, the induction of apoptosis and the regulation of apoptosis (Table S5). In each GO category, several genes encoding TNF family members and regulators of apoptosis, such as caspases and Bcl-2, were upregulated. The ability of *O. tsutsugamushi* to induce the apoptosis of monocytes was studied using TUNEL staining. Although no apoptosis was detected in monocytes incubated for 6 hours with *O. tsutsugamushi*, after 24 h, 4±1% of monocytes were apoptotic, and this percentage increased to 8±1% after 48 h. In contrast, less than 1% of monocytes were apoptotic in the absence of *O. tsutsugamushi*. Interestingly, heat-killed *O. tsutsugamushi* did not induce monocyte apoptosis during the same incubation periods (Table 2). Taken together, our data show that *O. tsutsugamushi* induced an apoptosis-related gene program and the apoptosis of a minority of monocytes.

Host response genes in scrub typhus infection

The transcriptional pattern of PBMCs from patients with scrub typhus (n = 4) was compared to the pattern in pooled PBMCs isolated from healthy controls using microarrays. Using an absolute value of a FC greater than 2.0, we identified 613 and 517 transcripts that were up- and downregulated in scrub typhus patients, respectively. Most of the highly expressed genes corresponded to biological process categories including DNA metabolism, the cell cycle and cellular component organization and biogenesis (Table 3). Of particular interest, the upregulated

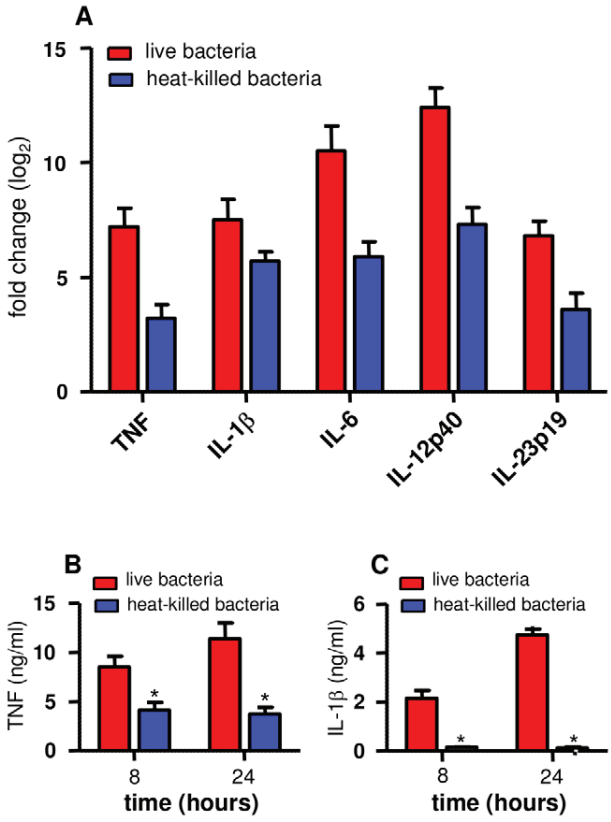

Figure 4. Bacterial viability and monocyte responses. Monocytes were stimulated with live or heat-killed *O. tsutsugamushi* for 8 (A) or 24 (B, C) hours. A. RNA was extracted, and qRT-PCR was performed to detect several genes that were differentially expressed in the microarray experiments. The results, expressed as the $\log_2$ ratio of fold changes, are presented as the mean ± SEM of two experiments performed in triplicate. B and C. Culture supernatants were analyzed for the presence of TNF (B) and IL-1β (C) using ELISAs. The results are expressed in ng/ml and are presented as the mean ± SD of two experiments performed in duplicate. *$p<0.05$.
doi:10.1371/journal.pntd.0001028.g004

genes involved in immune system process included IFN-γ (IFNG) and its related genes that encode absent in melanoma 2 (AIM2), guanylate binding protein 1(GBP1), IFN-γ-inducible protein 16 (IFI16) and indoleamine-pyrrole 2 (INDO). Significant enrichment in the downregulated genes was mainly observed in genes associated with immune-related processes, the inflammatory response and chemotaxis. To confirm the microarray-derived results, the level of 12 transcripts that were highly altered in patients with scrub typhus was re-assessed using qRT-PCR. The expression profiles detected using either technique were comparable, except for one gene, SSBP1. The transcriptomic profile of patients with scrub typhus was then compared to the profile of patients with murine typhus, malaria or dengue. Sixty-five probes corresponding to 63 genes were specifically expressed in patients with scrub typhus ($p<0.05$) (Table S6). The analysis of the microarray data by hierarchical clustering clearly showed that the four patients with scrub typhus were grouped together, separate from the other patients, while the transcriptional response of patients with murine typhus, malaria or dengue was more dispersed (Figure 6). These results clearly demonstrate that scrub typhus was characterized by a specific transcriptional signature. To reduce this transcriptional signature, the CBLB, LOC642161, CD8A and CD8B1 genes were selected because their expression was at least twofold greater in scrub typhus compared to the expression observed in the other infectious diseases; the FOSB gene was also selected because it was the only downregulated gene with the same fold difference. When hierarchical clustering was performed based on the expression of these five genes, the patients with scrub typhus were still grouped together, even though a patient with murine typhus also grouped in this cluster (Figure 7A). The expression of these five genes was then quantified using qRT-PCR and, as expected, the expression of CBLB, LOC64216, CD8A and CD8B1 was highly upregulated in patients with scrub typhus, while the expression of FOSB5 was downregulated (Figure 7B). This transcriptional profile was specific for scrub typhus, because the expression of these genes was completely different in murine typhus, malaria and dengue (Figure 7B).

Relationship between the transcriptomic profiles detected in patients with scrub typhus and the profiles in *in vitro*-infected monocytes

The transcriptional programs of PBMCs from patients with scrub typhus and those induced by *O. tutsugamushi* in monocytes were compared using Gene Symbol to allow the comparison between Illumina and Agilent data. The 2,015 probes differentially expressed in *O. tsutsugamushi*-stimulated monocytes detected using Agilent microarrays corresponded to 1,606 probes when Illumina assays were used. The differences were due to the fact that several Illumina probes are not annotated, leading to the impossibility to find these probes in Agilent probeset. In addition, Agilent probes were longer than those of Illumina. Interestingly, among the 1,606 probes that were modulated in *O. tsutsugamushi*-stimulated monocytes, 492 ($p<0.05$) and 184 probes ($p<0.01$) were also up- and downregulated, respectively, in patients with scrub typhus. This signature was clearly distinct from that of healthy controls (Figure 8). Among the 184 genes that were altered in scrub typhus, we found genes that were associated with important features of the monocyte response to *O. tsutsugamushi*, such as type I and II IFN and M1-associated genes (Table 3). These results suggest that the differential expression of genes in scrub typhus was related to *O. tsutsugamushi* infection.

Discussion

Our study is the first report demonstrating the replication of *O. tsutsugamushi* in primary human monocytes; however, the efficiency of bacterial replication was lower than the replication observed in permissive cell lines, such as L929 cells. The sustained presence of bacteria within monocytes may be beneficial for the dissemination to target tissues, as *O. tsutsugamushi* is known for its high level of genetic and antigenic variability [2].

The interaction of *O. tsutsugamushi* with monocytes resulted in a transcriptomic pattern in which the expression of a large number of genes was altered. The first feature of the response was the polarization of monocytes towards an M1 phenotype. The M1 phenotype has been largely described in macrophages stimulated by IFN-γ, TNF and/or microbial products and has been associated with microbicidal competence and the skewing of the adaptive immune response towards Th1/Th17 responses [31,32]. M1 phenotypes have been also described for macrophages stimulated with different bacterial pathogens including *Mycobacterium bovis* [33], *Legionella pneumophila* [34] and *Helicobacter pylori* [35] whereas *Mycobacterium tuberculosis* [36], *Coxiella burnetii* [37] and *Tropheryma whipplei* [29,38] induce M2 profiles. We demonstrate that the transcriptomic profile of monocytes infected with *O. tsutsugamushi* was not identical to the profile observed following

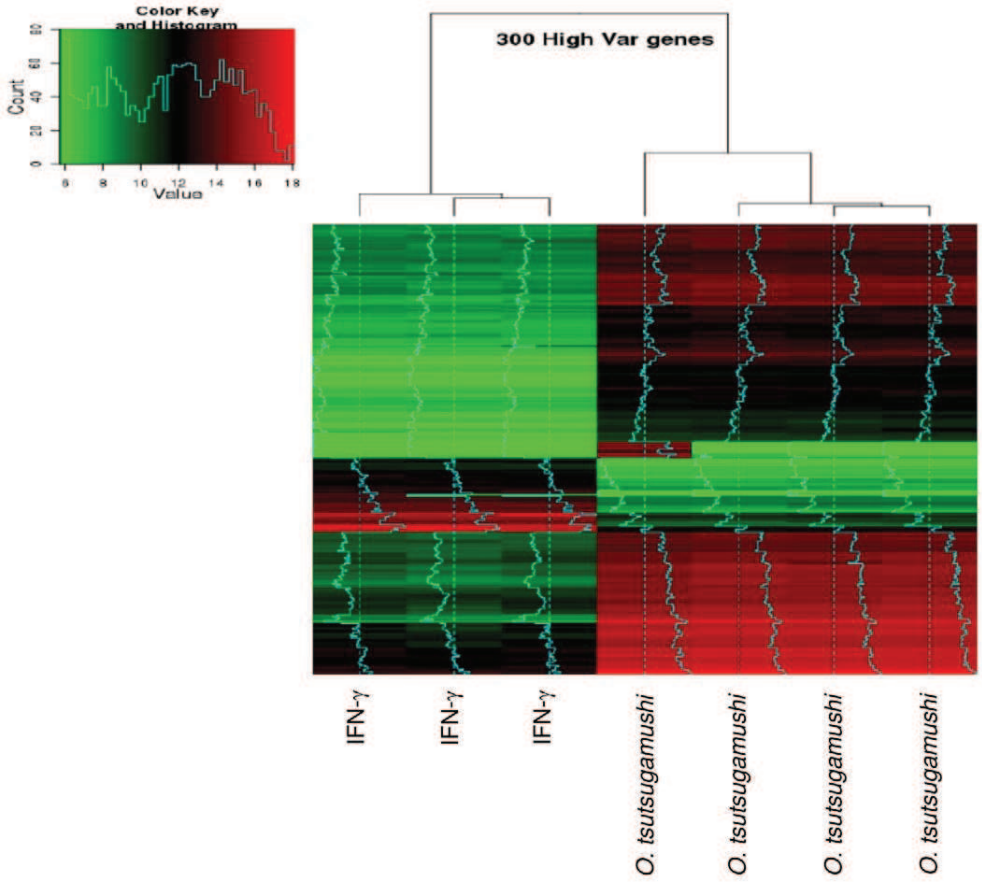

Figure 5. Hierarchical clustering of differentially expressed genes in stimulated monocytes. Monocytes were stimulated with *O. tsutsugamushi* or IFN-γ (500 UI/ml) for 8 hours, and genome-wide expression studies were performed using microarrays from Agilent Technologies. A hierarchical clustering consisting of 300 highly altered genes is shown.
doi:10.1371/journal.pntd.0001028.g005

induction with IFN-γ because many genes were differentially expressed. The M1 polarization of monocytes infected with *O. tsutsugamushi* was persistent as demonstrated by the sustained upregulation of inflammatory genes. It may be related to the increased level of pro-inflammatory cytokines that has been detected in patients with scrub typhus [17,18,39,40]. As activated or inflammatory monocytes play a major role in the dissemination of some pathogens, such as in the murine model of listeriosis [41] or human cytomegalovirus infection [42,43], we hypothesize that a similar phenomenon may occur in scrub typhus. In addition to the induction of an M1 phenotype, *O. tsutsugamushi* upregulated the expression of genes involved in polarized immune responses. They included the genes encoding IL-6, IL-12p40, IL-23p19, GM-CSF and CCL20. As IL-6, IL-12p40, IL-23p19 and GM-CSF are important for Th17 proliferation and/or differentiation and that CCL20 binds to CCR6 selectively expressed on Th17 cells [44], we can hypothesize that *O. tsutsugamushi* orients the immune response to a Th17 phenotype. Note that IL-17 levels are higher in patients with scrub typhus than in healthy controls [45]. It is well known that Th1 responses are also critical to protection against intracellular pathogens [46]. It is likely that the uncontrolled Th1 and Th17 responses to *O. tsutsugamushi* contribute to the pathophysiology of scrub typhus [47,48].

Table 2. *O. tsutsugamushi*-induced apoptosis.

Time (hours)	control	*O. tsutsugamushi*	heat-killed *O. tsutsugamushi*
6	not detectable	not detectable	not detectable
24	<1%	4±1%	<1%
48	<1%	8±1%	<1%

Monocytes were incubated with *O. tsutsugamushi* for different periods. Apoptosis was revealed by TUNEL assay and fluorescence microscopy. The results expressed as the ratio of TUNEL-positive cells and DAPI-stained nuclei ×100 are the mean ± SD of three different experiments.
doi:10.1371/journal.pntd.0001028.t002

The second feature of the transcriptional program induced by *O. tsutsugamushi* in human monocytes was the upregulation of genes belonging to the "response to virus" GO category; these genes essentially corresponded to the type I IFN genes and ISGs. The release of IFN-β and the expression of ISGs have been reported in response to LPS and intracellular bacteria, such as *Chlamydia* sp., *Salmonella enteritica* serovar typhimurium [49], *T. whipplei* [50] and *Francisella tularensis* [51]. We recently reported that *Rickettsia prowazekii*, an intracellular bacterium related to *O. tsutsugamushi*, stimulates a type I IFN response in endothelial cell lines [52]. *Listeria monocytogenes* stimulates an IRF-3-dependent cytosolic response consisting of IFN-β and several ISGs [53]. It is tempting to speculate that the production of IFN-β and the expression of ISGs, at least in part, are related to the cytosolic location of the

Table 3. Enriched biological processes in scrub typhus.

Biological process[a]	Examples of genes
Up-regulated genes	
Immune system process	AIM2^{+}, C1QA, C1QB, C1QC, CD164, CD8B1, CEACAM8, CLEC6A, CST7, CTLA4, CTSC, DPP4, FASLG, FCGR1A, GBP1, BP3, GBP4, GBP5, GPR65, IFI16^{+}, IFI27^{+}, IFNG, IL1R2, IL2RG, IL32, INDO^{+}, OAS1^{+}, OAS2^{+}, OASL^{+}, PSMB8^{+}, PTPRC, RGS1, SERPING1, SPON2, TNFSF7, TUBB, TUBB2C
Response to stress	ATRX, BRCA1, CCNA2, CHAF1B, CHEK1, DCLRE1A, EXO1, FEN1, GTSE1, H2AFX, HMGB1, HMGB2, HSPA4, HSPB1, HSPD1, HSPE1, NEIL3, PCNA, POLE2, POLQ, PTTG1, RAD51, RAD51AP1, RAD54L, RECQL, SFPQ, TOP2A, TYMS, UHRF1
DNA metabolic process	CDC45L, CDC7, CDT1, DTYMK, KPNA2, MCM2, MCM4, MCM7, ORC1L, Pfs2,PRIM2A, RFC3, RNASEH2A, RRM2, TK1, TOP1
Cell cycle process	ASPM, BCAT1, BUB1, BUB1B, CCNB1, CCNB2, CCNF, CDC2, CDC20, CDC25A, CDC1, CDCA5, CDKN3, CENPE, CENPF, CET, CNAP1, E2F1, ESPL1, HCAP-G, KIF11, KIF15, KIF23, KIF2C, KNTC2, MACF1, MAD2L1, NEK2, PBK, PLK1, PTTG1, SMC2L1, SPAG5, STK6, STMN1, TPX2, TTK, TUBG1, UBE2C
Cellular component organization and biogenesis	BUB1B, CENPA, CKS2, GTSE1, GZMB, HIST1H1C, HIST1H2BD, HIST1H3C, KIF14, KIF20A, KIF4A, PACSIN1, PPP2R1B, RNF19, SMARCA5, TAF9, TUBA3, TUBB3, TUBB4Q, TUBB8, ZWINT
Down-regulated genes	
Immune system process	BCL2, BRDG1, CCL3L3, CD163, CD1C, CD79B, CLC, CLEC4A, CLEC4C, CSF1R, CXCL16, EBI2, FCER1A, FTH1, HLA-DMB, HLA-DOA, HLA-DOB, HLA-DRB1, HLA-DRB5, IL23A, IL4R, KLRB1, KLRF1, LILRA3, LTB, LY86, MS4A1, MS4A2, NR4A2, OSM, POU2F2, TCF7, TNF*, TNFRSF25, VIPR1
Defense response and inflammatory process	CD40LG, CD79A, CD83, CIAS1, EPHX2, FOS, IL1B, IL1RN, LY86, NALP1, NCR3, PLA2G7, PTX3, RNASE6, TCEA3, TLR10
Chemotaxis	CCL20, CCL3, CCL3L1, CCL4, CCL8, CCR3, CCR6, CCR7, CXCL1, CXCL16, CXCL2, IL1A, IL8, ROBO3

Enriched biological processes in scrub typhus with the list of modulated genes (FC>2.0) are shown.
[a]Determined by hypergeometric formula with Benjamini-Yekutieli correction [25,26].
[+]Genes induced by interferons.
doi:10.1371/journal.pntd.0001028.t003

bacteria. Indeed, *L. monocytogenes* [54] and *O. tsutsugamushi* [55] are known to reside in the cytosol. Among the genes upregulated in response to *O. tsutsugamushi*, we detected TBK-1 and IRF-7. TBK-1 is involved in IFN-β production after cytoplasmic recognition of *L. monocytogenes* [56], and IRF-7 is increased after infection and promotes an amplification loop of IFN-β production [49]. It is likely that a substantial number of genes included in the ''response to virus'' GO category are controlled by IFN-β and not directly dependent on *O. tsutsugamushi* infection. In LPS-stimulated cells, a considerable number of the differentially expressed genes are due to type I IFN synthesis and signaling through IFN receptors [49]. The effects of type I IFNs can be beneficial or detrimental to host defense against bacterial infections [49]. Type I IFNs activate NK cells and cytotoxic T cells (CTLs), which are critical to clear cytosolic pathogens, including *Rickettsia* spp. [57,58]. In addition, type I IFNs sensitize the host cells to apoptosis [59] through the induction of ISGs, such as TRAIL, FAS, XIAP-associated factor-1 (XAF-1), caspase-8, protein kinase R (PKR), 2′5′oligoadenylate synthase (OAS), phospholipid scramblase and the promyelocytic leukemia gene product [60]. All of these genes were upregulated in monocytes infected with *O. tsutsugamushi*. In contrast, type I IFNs are detrimental to the host during *L. monocytogenes* infection. They contribute to macrophage cell death [61] and sensitize T lymphocytes to apoptosis induced by listeriolysin O [62]. As a result, IRF3$^{-/-}$ and IFNAR$^{-/-}$ mice show increased resistance to *L. monocytogenes* compared to wild type mice [56]. IFN-β has been shown to inhibit the in vitro replication of *Francisella tularensis* in murine macrophages [63]. Recently, our group has shown that the type I IFN response is detrimental to murine macrophages infected with *Tropheryma whipplei*. Indeed, macrophage apoptosis and bacterial replication are inhibited in IFNAR$^{-/-}$ macrophages compared with wild type macrophages [50]. A previous study has shown that type I IFN inhibited *O. tsutsugamushi* replication depending on the bacterial strain and the genetic background of host cells [64]. However, further studies are required to determine the exact role of type I IFNs in *O. tsutsugamushi* infection.

The third prominent feature of the infection of human monocytes with *O. tsutsugamushi* was the enrichment in apoptosis-related genes but only a minority of *O. tsutsugamushi*-infected monocytes were apoptotic. Cell death has already been described in lymphocytes, lymph nodes and endothelial cell lines in response to *O. tsutsugamushi* [65]. In vivo, cell death is prominent in mice susceptible to *O. tsutsugamushi*, but it is not detected in resistant mice [65]. Our results were apparently contradictory with those of Kim et al. obtained with the THP-1 macrophage cell line in which *O. tsutsugamushi* inhibits transiently the cell death induced by apoptosis promoters. In addition, the ability to prevent apoptosis is not related to bacterial virulence [66]. We hypothesized that the apparent discrepancy between gene expression and low level of apoptosis is associated to the modulation of the genes belonging to apoptosis and anti-apoptosis GO terms in inflammatory conditions [67]. The precise mechanisms used by *O. tsutsugamushi* to affect the cell death of monocytes remain to be determined. Apoptosis can result from inflammasome activation that involves caspase-1 activation and IL-1β secretion [68]. In this study, we demonstrate that live *O. tsutsugamushi* induced IL-1β secretion by monocytes, whereas heat-killed bacteria stimulated the expression of the gene encoding IL-1β but did not induce secretion of active IL-1β. Similar to what has been reported for *F. tularensis* [51] and *T. whipplei* [50], type I IFNs may promote apoptosis in monocytes infected with *O. tsutsugamushi*. We hypothesize that *O. tsutsugamushi* stimulates inflammasome activation via IFN-β release when the bacteria reach the cytosol.

Lastly, we extended our analysis of the host response to *O. tsutsugamushi* to patients with scrub typhus. For that purpose, we analyzed the transcriptional response of whole PBMCs from patients with scrub typhus and then compared this response to that observed in monocytes stimulated with *O. tsutsugamushi*. In patients with scrub typhus, we observed a significant upregulation of IFN-γ, a type II IFN, and its related genes, suggesting an important role for the type II IFN pathway in the response to *O. tsutsugamushi*

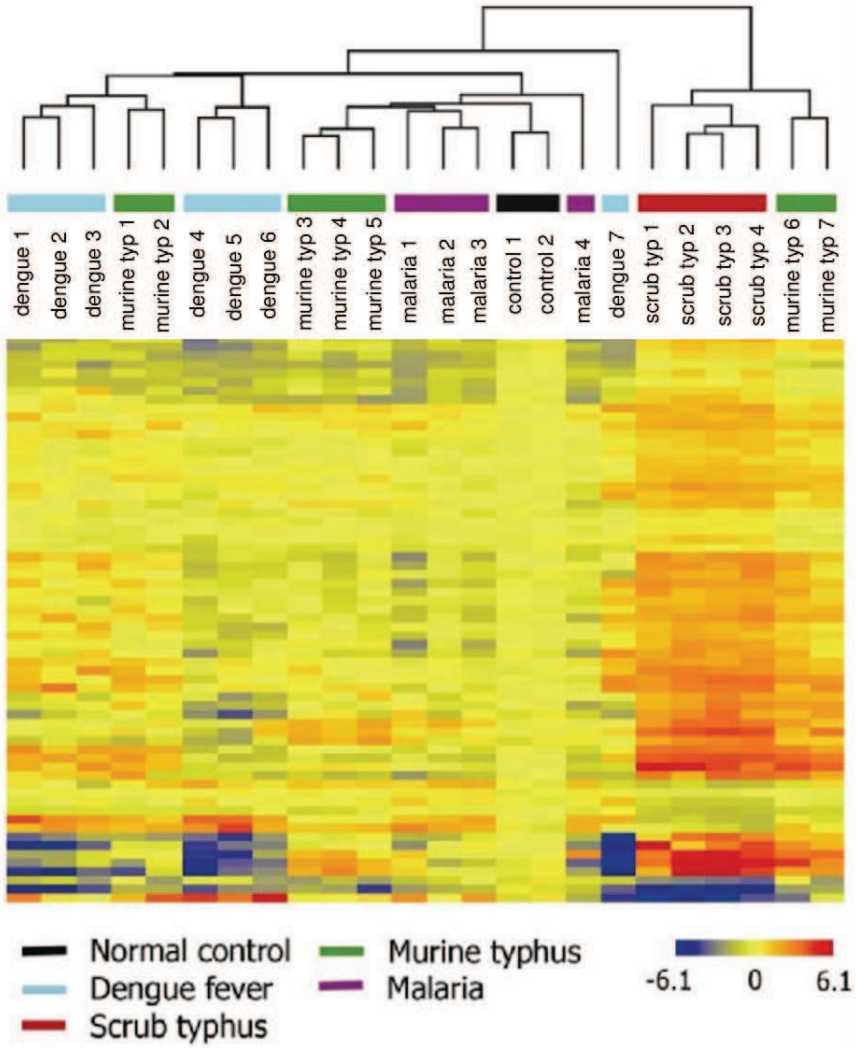

Figure 6. Hierarchical clustering in patients with scrub typhus. RNA was isolated from PBMCs from healthy controls and patients with different infectious diseases, and microarray studies were performed using Illumina Human-6 v2 BeadChips. The unsupervised hierarchical clustering of 22 patients and 2 RNA pools from healthy controls was performed based on the expression of 65 genes specific to scrub typhus (typ). The normalized expression level in each sample was baseline-adjusted to the mean expression level of the healthy control group and color-scaled, with red indicating increased expression and blue indicating decreased expression.
doi:10.1371/journal.pntd.0001028.g006

infection. IFN-γ is a key cytokine in macrophage activation and Th1 responses, and these cells are necessary to clear *O. tsutsugamushi* infection [20]. IFN-γ can also directly exert an inhibitory effect on the intracellular replication of *O. tsutsugamushi* in non-immune cells [64]. Indeed, increased IFN-γ production correlates with the acquisition of resistance to *O. tsutsugamushi* infection in immune mice [69]. We hypothesize that the increased production of IFN-γ is, at least in part, a consequence of the elevated number of $CD8^+$ T cells and NK cells observed in patients with scrub typhus [70]. This hypothesis is also in agreement with the increased expression of genes encoding the CD8 subunits and the abundance of gene transcripts involved in cell cycle and cell division. All together, these data emphasize the role of type II IFN and cell-mediated immunity in the protection against *O. tsutsugamushi* infection.

The transcriptional signature of patients with scrub typhus included the differential expression of the CBLB, LOC642161, CD8A, CD8B1 and FOSB genes, when compared to healthy controls and patients with other infectious diseases. Among these genes, CBLB is interesting, as there is increasing evidence that it has important functions in the immune system. The CBLB gene encodes the E3 ubiquitin ligase, Cbl-b, which controls peripheral T cell activation and tolerance by regulating CD28 co-stimulatory signaling [71,72]. In infectious diseases, the increased expression of Cbl-b in many chronic infections is believed to be due to a defective immune response [15–17]. Cbl-b also modulates the stability of bacterial effector proteins essential for virulence, as recently reported in *Pseudomonas aeruginosa* infection [24]. The enhanced expression of Cbl-b in patients with scrub typhus may suggest a role for it in the degradation of bacterial products or in the immune evasion of *O. tsutsugamushi*, but it may also represent a regulatory mechanism of the immune response to prevent over-activation of T cells. Independent of the putative role of these five genes in the pathophysiology of scrub typhus, their specific alteration in scrub typhus may be useful to improve the diagnosis of this infection.

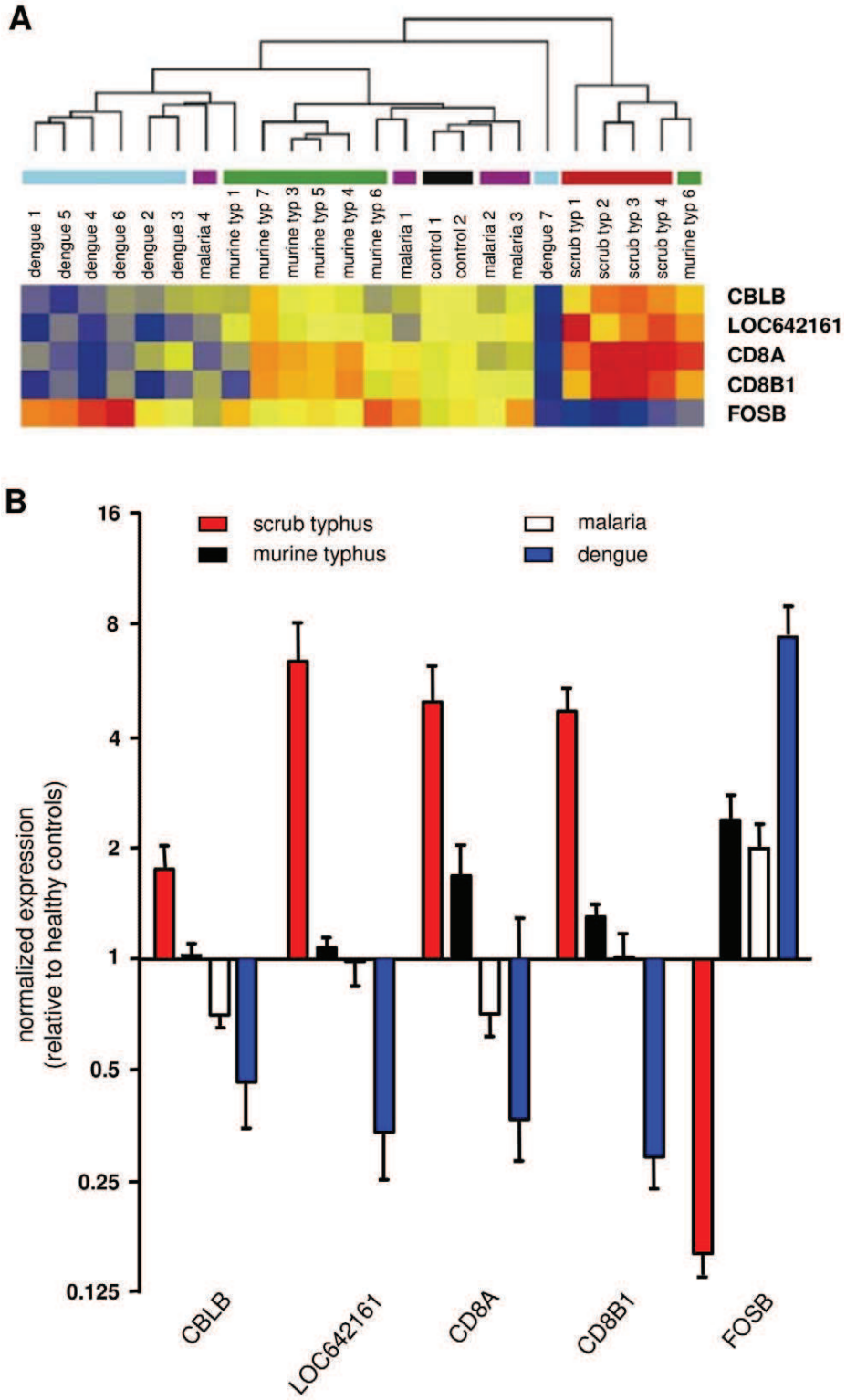

Figure 7. Reduced transcriptional signature of scrub typhus. The microarrays performed on RNA isolated from healthy controls or patients with infectious diseases suggested that five genes can be used as a specific signature of scrub typhus. A. The unsupervised hierarchical clustering of 22 patients and 2 pools of RNA from healthy controls was performed based on the expression of these five genes. The normalized expression level in each sample was baseline-adjusted to the mean expression level of the healthy control group and color-scaled, with red indicating increased expression and blue indicating decreased expression. B. The expression of the five genes was assessed using qRT-PCR. The results, expressed as the $\log_2$ ratio of fold changes, are presented as the mean ± SEM of all the patients in each group performed in duplicate.
doi:10.1371/journal.pntd.0001028.g007

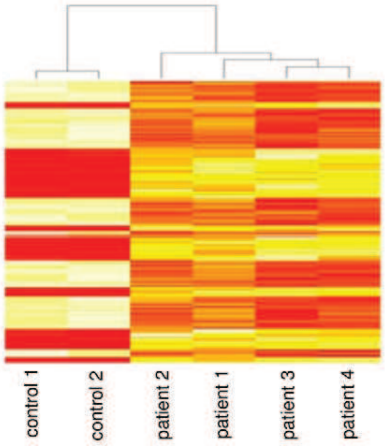

Figure 8. Comparison between patient blood samples and in vitro-infected monocytes. Monocytes from healthy donors were stimulated with *O. tsutsugamushi* for 8 hours, and Agilent microarrays were used to detect the differential expression of 2,015 genes that corresponded to 1,606 genes in the Illumina microarrays. Among these genes, 184 (250 probes) were altered in patients with scrub typhus with a p value less than 0.01. The hierarchical clustering of these genes demonstrates that the resulting transcriptional signature was specific to scrub typhus.
doi:10.1371/journal.pntd.0001028.g008

In conclusion, we report the global transcriptional response of monocytes in response to *O. tsutsugamushi*. *O. tsutsugamushi* induced a specific M1 phenotype and stimulated a type I IFN response. Type I and II IFNs and the M1 signature were also found in the PBMCs from patients with scrub typhus, suggesting that these molecules may be associated with the inflammatory complications of scrub typhus.

Supporting Information

Figure S1 Time course of gene modulation.
(PDF)

Figure S2 PCA and Venn diagram.
(PDF)

Table S1 Characteristics of patients in each group of infectious diseases.
(PDF)

Table S2 Nucleotide sequences of oligonucleotide primers.
(PDF)

Table S3 Modulated genes in the "immune response" GO term.
(PDF)

Table S4 M1 and M2 genes in *O. tsutsugamushi*-stimulated monocytes.
(PDF)

Table S5 Apoptosis-related genes in *O. tsutsugamushi*-stimulated monocytes.
(PDF)

Table S6 List of the 65 transcripts specific for scrub typhus.
(PDF)

Author Contributions

Conceived and designed the experiments: D Raoult Y Suputtamongkol C Capo C Limwongse JL Mege. Performed the experiments: W Tantibhedhyangkul T Prachason D Waywa E Ghigo. Analyzed the data: W Tantibhedhyangkul T Prachason D Waywa A El Filali W Thongnoppakhun D Raoult Y Suputtamongkol C Limwongse JL Mege. Contributed reagents/materials/analysis tools: D Waywa A El Filali E Ghigo W Thongnoppakhun. Wrote the paper: W Tantibhedhyangkul T Prachason Y Suputtamongkol C Capo C Limwongse JL Mege.

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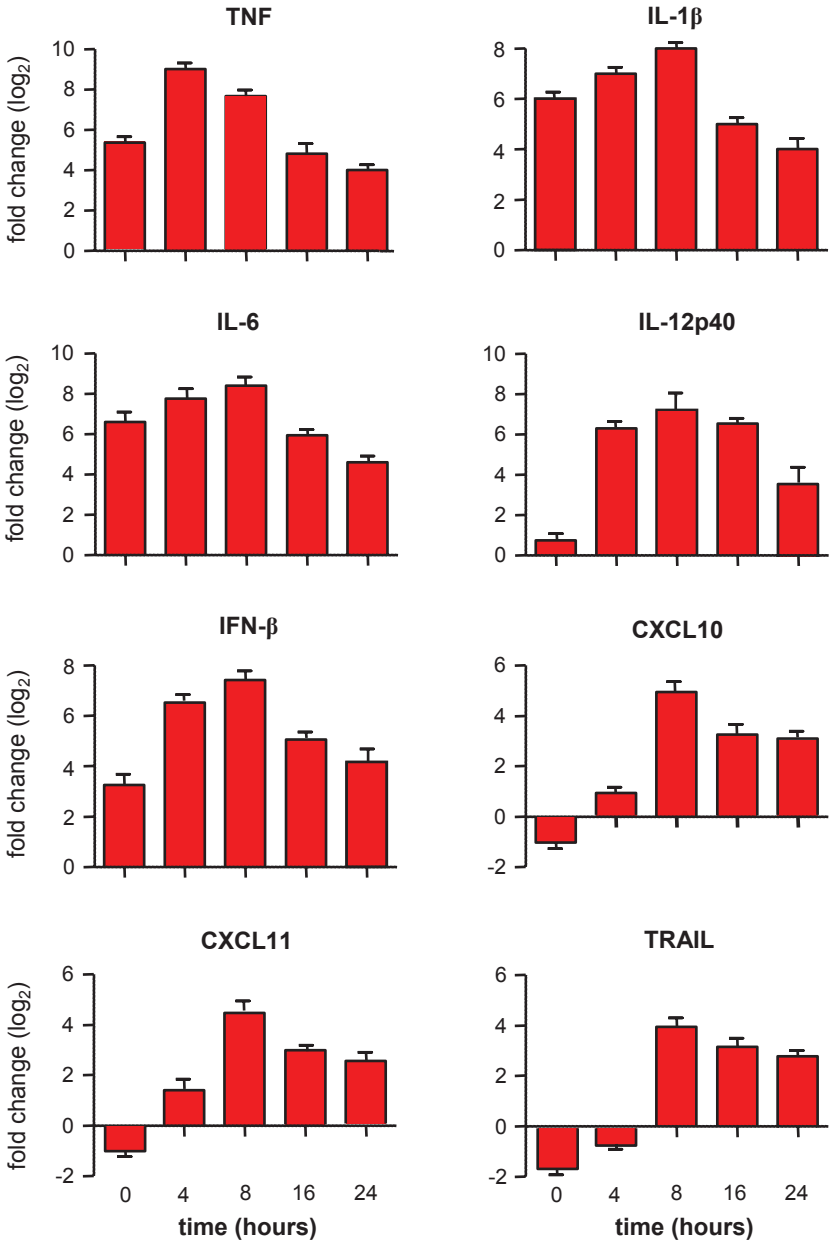

new Figure S1

Figure S2. PCA and Venn diagram

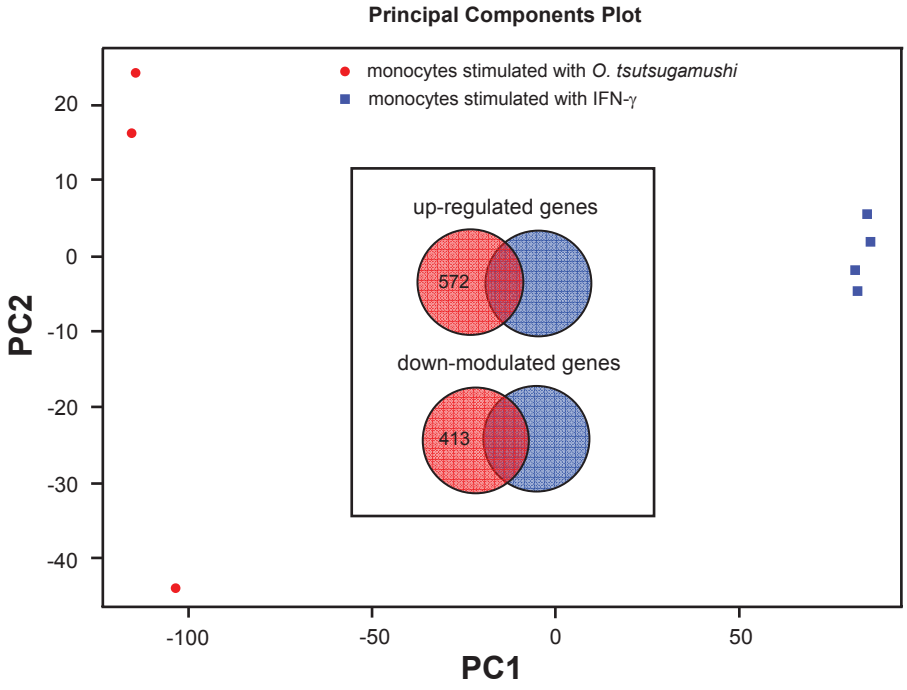

Table S1. Characteristics of patients in each group of infectious diseases

	scrub typhus	**murine typhus**	**malaria**	**dengue**
Number	4	7	4	7
Age	46 ± 18.4	41.7 ± 14.5	26.5 ± 6.0	31.9 ± 18.1
Male : Female	2:2	1:6	2:2	4:3
Mean fever day (range)	10.5 (3-20)	9.7 3-15	5.3 (3-7)	2.3 (1-4)*
Body temperature (°C)	38.0 ± 0.8	37.8 ± 1.0	38.8 ± 0.6	38.9 ± 0.9
WBC (x 10^3/μl)	13.5 ± 2.4	8.1 ± 2.4**	4.1 ± 1.3***	5.1 ± 2.6***
Platelets (x 10^3/μl)	182.5 ± 69.3	221.7 ± 120.1	37 ± 19.1	131.33 ± 65.34
Neutrophils (%)	76.8 ± 9.7	72.1 ± 12.4	67.6 ± 9.0	65.2 ± 20.6
Lymphocytes (%)	17.4 ± 12.1	20.8 ± 12.1	17.9 ± 11.4	16.8 ± 15.7
Monocytes (%)	4.3 ± 2.5	5.1 ± 2.1	7.1 ± 2.5	6.9 ± 4.5
Lymphocytes/Monocytes	2.0 ± 4.9	4.1 ± 5.8	2.5 ± 4.6	2.4 ± 3.8

Data are mean values of each group of patients with standard deviation.

*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, as compared to the scrub typhus group.

Table S2. Nucleotide sequences of oligonucleotide primers

Symbol	Forward primers	Reverse primers
TNF	5'- CCCGACTATCTCGACTTTGC-3'	5'-AGGTTGAGGGTGTCTGAAGGA-3'
IL1β	5'-GCTGAGGAAGATGCTGGTTC-3'	5'-TCCATATCCTGTCCCTGGAG-3'
IL6	5'-TACCCCCAGGAGAAGATTCC-3'	5'-TTTTCTGCCAGTGCCTCTTT-3'
IL12p35	5'-TCAGCAACATGCTCCAGAAGGC-3'	5'-TGCATTCATGGTCTTGAACTCCACC-3'
IL12p40	5'-AGGGGACAACAAGGAGTATGAGT-3'	5'-AGGGAGAAGTAGGAATGTGGAGT-3'
IL23p19	5'-AGGAGAAGAGGGAGATGAAGAGAC-3'	5'-GCTATCAGGGAGCAGAGAAGG-3'
IL15	5'-GTTAGCAGATAGCCAGCCCATAC-3'	5'-TACTCAAAGCCACGGTAAATCC-3'
CXCL10	5'-AAGCAGTTAGCAAGGAAAGGTC-3'	5'-TTGAAGCAGGGTCAGAACATC-3'
CXCL11	5'-TATAGCCTTGGCTGTGATATTGTG-3'	5'-CTGCCACTTTCACTGCTTTTACC-3'
CCL20	5'-GACATCAATGCTATCATCTTTCAC-3'	5'-GCTATGTCCAATTCCATTCCA-3'
IFNβ	5'-GACGCCGCATTGACCATCTA-3'	5'-CCTTAGGATTTCCACTCTGACT-3'
IFNα8	5'-ATGACCTGGAGTCCTGTGTGAT-3'	5'-GATCTCATGATTTCTGCTCTGACAAC-3'
OAS1	5'-TCCGCCTAGTCAAGCACTGGTA-3'	5'-CCTGGGCTGTGTTGAAATGTGT-3'
MX1	5'-GCCACCACAGAGGCTCTCAG-3'	5'-CTCAGCTGGTCCTGGATCTCCT-3'
INDO	5'-TGCTGGTGGAGGACATGCTG-3'	5'-TGAAAGGACAAACTCACGGACTGA-3'
TLR5	5'-CTTGTCCCAGTACCAGTTGATGA-3'	5'-AGGAGATGGTTGCTACAGTTTG-3'
CBLB	5'-CCCTTTGTTGATCTAGCAAGTG-3'	5'-GTGCCTGTGAACCATCTGAA-3'
LOC642161	5'-CTCAAAATGCCCCTCCTT TC-3'	5'-GGTTCTGTGAGTCCTGCTT-3'
CD8A	5'-CCTTTACTGCAACCACAGGA-3'	5'-AGGAAGGATCTCAGTTTGAAG-3'
CD8B1	5'-ACTTCTGCATGATCGTCG G-3'	5'-AGGGTGGACTTCTTGGTG-3'
FOSB	5'-CAGCAGCTAAATGCAGGA-3'	5'-TTTGGAGCTCGGCGATCT-3'

Table S3. Modulated genes in the "immune response" GO term

Gene function and full gene name	Gene symbol	GenBank ID	FC
adenosine deaminase	ADA	NM_000022	3.8
absent in melanoma 2	AIM2	NM_004833	8.1
aquaporin 9	AQP9	AQP9	3.6
chemokine (C-C motif) ligand 1	**CCL1**	**NM_002981**	**20.3**
chemokine (C-C motif) ligand 3	**CCL3**	**NM_002983**	**9.0**
chemokine (C-C motif) ligand 4	**CCL4**	**NM_002984**	**16.9**
chemokine (C-C motif) ligand 7	**CCL7**	**NM_006273**	**23.2**
chemokine (C-C motif) ligand 8	**CCL8**	**NM_005623**	**74.9**
chemokine (C-C motif) ligand 20	**CCL20**	**NM_004591**	**13.6**
CD274 molecule	CD274	NM_014143	16.6
CD40 molecule, TNF receptor superfamily member 5	CD40	NM_001250	6.0
CD70 molecule	CD70	NM_001252	3.8
CD80 molecule	CD80	NM_005191	21.3
C-type lectin domain family 4, member D	CLEC4D	NM_080387	5.6
C-type lectin domain family 4, member E	CLEC4E	NM_014358	7.5
colony stimulating factor 2 (granulocyte-macrophage)	CSF2	NM_000758	7.8
colony stimulating factor 3 (granulocyte)	CSF3	NM_000759	97.5
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	**CXCL1**	**NM_001511**	**4.8**
chemokine (C-X-C motif) ligand 2	**CXCL2**	**NM_002089**	**2.8**
chemokine (C-X-C motif) ligand 3	**CXCL3**	**NM_002090**	**5.6**
chemokine (C-X-C motif) ligand 5	**CXCL5**	**NM_002994**	**2.7**
chemokine (C-X-C motif) ligand 10	**CXCL10**	**NM_001565**	**16.3**
chemokine (C-X-C motif) ligand 11	**CXCL11**	**NM_005409**	**98.2**
chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	**CXCL13**	**NM_006419**	**2.3**
chemokine (C-X-C motif) ligand 14	**CXCL14**	**NM_004887**	**4.1**
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	13.0
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.3
guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	NM_002053	5.9
guanylate binding protein 3	GBP3	NM_018284	5.2
guanylate binding protein 5	GBP5	NM_052942	4.5
guanylate binding protein 7	GBP7	NM_207398	15.4
GTP binding protein overexpressed in skeletal muscle	GEM	NM_005261	3.0
GTP binding protein 1	GTPBP1	NM_004286	3.8
interferon, alpha-inducible protein 27	IFI27	NM_005532	24.1
interferon-induced protein 35	IFI35	NM_005533	7.6
interferon, alpha-inducible protein 6	IFI6	NM_022873	10.2
interferon-induced protein with tetratricopeptide repeats 1	IFIT1	NM_001548	100.0
interferon-induced protein with tetratricopeptide repeats 2	IFIT2	NM_001547	49.2
interferon-induced protein with tetratricopeptide repeats 3	IFIT3	NM_001549	31.3
interferon-induced protein with tetratricopeptide repeats 5	IFIT5	NM_012420	9.7
interferon induced transmembrane protein 1 (9-27)	IFITM1	NM_003641	14.7
interferon induced transmembrane protein 2 (1-8D)	IFITM2	NM_006435	8.8
interferon induced transmembrane protein 3 (1-8U)	IFITM3	NM_021034	10.6
interleukin 12B (p40)	**IL 12B**	**NM_002187**	**> 100**
interleukin 15	**IL15**	**NM_172174**	**3.6**
interleukin 18 (interferon-gamma-inducing factor)	**IL18**	**NM_001562**	**3.7**
interleukin 19	**IL19**	**NM_153758**	**35.9**

interleukin 1, alpha	**IL1A**	**NM_000575**	**9.0**
interleukin 1, beta	**IL1B**	**NM_000576**	**20.7**
interleukin 1 family, member 5 (delta)	**IL1F5**	**NM_012275**	**28.3**
interleukin 1 family, member 9	**IL1F9**	**NM_019618**	**100.0**
interleukin 1 receptor antagonist	**IL1RN**	**BC068441**	**17.8**
interleukin 1 receptor antagonist	**IL1RN**	**NM_173842**	**37.9**
interleukin 7 receptor	**IL7R**	**NM_002185**	**4.5**
interleukin 6	**IL6**	**NM_000600**	**> 100**
interleukin 8	**IL8**	**NM_000584**	**2.7**
interleukin 23, alpha subunit p19	**IL23A**	**NM_016584**	**5.1**
indoleamine-pyrrole 2,3 dioxygenase	INDO	NM_002164	15.6
interferon regulatory factor 2	IRF2	NM_002199	2.6
interferon regulatory factor 7	IRF7	NM_004031	14.9
ISG15 ubiquitin-like modifier	ISG15	NM_005101	54.1
MHC class I polypeptide-related sequence B	MICB	NM_005931	2.9
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	NM_002462	26.7
myxovirus (influenza virus) resistance 2 (mouse)	MX2	NM_002463	50.2
2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	NM_002534	13.6
2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	NM_016817	26.2
2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	NM_006187	21.7
2'-5'-oligoadenylate synthetase-like	OASL	NM_003733	36.3
oncostatin M	OSM	NM_020530	4.2
regulator of G-protein signaling 1	RGS1	NM_002922	4.7
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	NM_006379	2.7
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	TAP1	NM_000593	2.9
tumor necrosis factor receptor superfamily, member 4	TNFRSF4	NM_003327	4.8
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_001561	5.8
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	78.7
tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	NM_005118	10.4
tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811	8.1
tripartite motif-containing 22	TRIM22	NM_006074	8.4
chemokine (C-C motif) receptor 2	CCR2	NM_000647	-2.5
chemokine (C-C motif) receptor 2	CCR2	NM_000648	-2.8
CD14 molecule	CD14	NM_000591	-2.9
CD22 molecule	CD22	NM_001771	-3.7
class II, major histocompatibility complex, transactivator	CIITA	U18259	-3.6
C-type lectin domain family 10, member A	CLEC10A	NM_182906	-4.4
C-type lectin domain family 4, member A	CLEC4A	NM_016184	-3.6
cytotoxic and regulatory T cell molecule	CRTAM	NM_019604	-2.7
hepcidin antimicrobial peptide	HAMP	NM_021175	-4.7
major histocompatibility complex, class II, DM beta	HLA-DMB	BC035650	-10.2
interleukin 16 (lymphocyte chemoattractant factor)	IL16	NM_172217	-5.4
interleukin 16 (lymphocyte chemoattractant factor)	IL16	NM_004513	-3.3
Notch homolog 1, translocation-associated (Drosophila)	NOTCH1	NM_017617	-4.0
ring finger protein 125	RNF125	NM_017831	-5.4
signaling threshold regulating transmembrane adaptor 1	SIT1	NM_014450	-4.1
transcription factor 7 (T-cell specific, HMG-box)	TCF7	NM_003202	-2.5
tumor necrosis factor (ligand) superfamily, member 11	TNFSF11	NM_003701	-2.1

The genes encoding chemokines and cytokines were presented in bold.

Table S4. M1 and M2 genes in *O. tsutsugamushi*-stimulated monocytes

Gene category and name	**Gene symbol**	Fold change
M1 genes		
Chemokine (C-C motif) receptor 7	CCR7	2.6
Interleukin 2 receptor α chain	IL2RA	1.9
Interleukin 7 receptor	IL7R	4.5
Interleukin 15 receptor α chain	IL15RA	15.6
Chemokine (C-C motif) ligand 2	CCL2	nc
Chemokine (C-C motif) ligand 3	CCL3	9.0
Chemokine (C-C motif) ligand 4	CCL4	16.9
Chemokine (C-C motif) ligand 5	CCL5	1.5
Chemokine (C-C motif) ligand 15	CCL15	nc
Chemokine (C-C motif) ligand 19	CCL19	nc
Chemokine (C-C motif) ligand 20	CCL20	13.6
Chemokine (C-X-C motif) ligand 8	CXCL8, IL8	2.7
Chemokine (C-X-C motif) ligand 9	CXCL9	nc
Chemokine (C-X-C motif) ligand 10	CXCL10	16.3
Chemokine (C-X-C motif) ligand 11	CXCL11	98.1
Chemokine (C-X-C motif) ligand 16	CXCL16	nc
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	1.6
Interleukin 1α	IL1A	9.0
Interleukin 1β	**IL1B**	**20.6**
Interleukin 6	**IL6**	**> 100**
Interleukin 12A	**IL12A**	**3.0**
Interleukin 12B	**IL12B**	**> 100**
Interleukin 15	**IL15**	**3.6**
Interleukin 23 α subunit p19	**IL23A**	**5.1**
Tumor necrosis factor ligand superfamily, member 2	**TNF**	**13.9**
Tumor necrosis factor ligand superfamily, member 10	TRAIL	78.7
Visfatin	PBEF1	7.0
Apoptosis-related genes		
BCL2-related protein A1	BCL2A1	nc
Fas (TNF receptor superfamily, member 6)	FAS	3.3
Baculoviral IAP repeat-containing 3	BIRC3	1.5
growth arrest and DNA-damage-inducible, gamma	GADD45G	2.0
XIAP associated factor-1	XAF1	13.2
M2 genes		
CD36	CD36	-2
CD163	CD163	-1.8
CD209	DCSIGN	nc
Chemokine (C-X-C motif) receptor 4	CXCR4	-3.8
C-type lectin receptor DCL-1	DCL-1	-1.5
C-type lectin superfamily member 12	DECTIN1	-1.9
Fcε-RII	CD23	-2.5
G protein-coupled receptor 105	P2RY14	-1.7
G protein-coupled receptor 86	GPR86	-5.1
Histamine receptor H1	HRH1	1.7
macrophage scavenger receptor 1	MSR1	1.7
Macrophage scavenger receptor 1	MSR1	nc
Mannose receptor C type 1	MRC1	-2.2
Membrane-spanning 4-domains, subfamily A, member 4	MS4A4A	-1.7
Membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	-3
Purinergic receptor P2Y5	P2RY5	-1.6
Transforming growth factor β receptor II	TGFBR2	-3.6

Transforming growth factor β receptor II	TGFBR2	-3.9
Chemokine (C-C motif) ligand 1	CCL1	20.3
Chemokine (C-C motif) ligand 13	CCL13	2.0
Chemokine (C-C motif) ligand 17	CCL17	nc
Chemokine (C-C motif) ligand 18	CCL18	nc
Chemokine (C-C motif) ligand 22	CCL22	nc
Chemokine (C-C motif) ligand 23	CCL23	4.7
Chemokine (C-C motif) ligand 24	CCL24	nc
Interleukin 1 receptor antagonist	IL1RN	37.9
Interleukin 10	IL10	1.6

M1 and M2 genes are referred to specific transcriptional profiles provided in references 28 and 29. The expression of the genes in bold letters was confirmed by qRT-PCR. nc : not changed. FC in microarray.

Table S5. Apoptosis-related genes in *O. tsutsugamushi*-stimulated monocytes

Gene function and full gene name	Gene symbol	GenBank ID	FC
Apoptosis			
adenosine A2a receptor	ADORA2A	NM_000675	13.4
apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	NM_032797	2.4
AXIN1 up-regulated 1	AXUD1	NM_033027	12,2
B-cell receptor-associated protein 29	BCAP29	NM_001008407	2.5
baculoviral IAP repeat-containing 4	BIRC4	NM_001167	2.3
CD40 molecule, TNF receptor superfamily member 5	CD40	NM_001250	6.0
catenin, beta like 1	CTNNBL1	NM_030877	2.4
damage-regulated autophagy modulator	DRAM	BC018435	5.2
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	13.0
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.3
immediate early response 3	IER3	NM_003897	5.3
interleukin 19	IL19	NM_153758	35.9
interleukin 1, alpha	IL1A	NM_000575	9.0
interleukin 1, beta	IL1B	NM_000576	20.7
Janus kinase 2 (a protein tyrosine kinase)	JAK2	NM_004972	4.9
lipopolysaccharide-induced TNF factor	LITAF	NM_004862	2.2
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	NM_003998	3.1
oncostatin M	OSM	NM_020530	4.2
pleckstrin homology-like domain, family A, member 2	PHLDA2	NM_003311	4.9
protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	NM_014330	3.1
receptor (TNFRSF)-interacting serine-threonine kinase 1	RIPK1	NM_003804	3.2
serine/threonine kinase 3 (STE20 homolog, yeast)	STK3	NM_006281	2.5
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022173	2.5
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022037	2.6
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	13.9
tumor necrosis factor, alpha-induced protein 3	TNFAIP3	NM_006290	4.6
tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	NM_003844	4.3
tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	NM_003840	2.9
tumor necrosis factor receptor superfamily, member 18	TNFRSF18	NM_148901	3.6
tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811	8.1
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	78.7
tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	NM_005118	10.4
ubiquitin-conjugating enzyme E2Z	UBE2Z	NM_023079	2.4
Anti-apoptosis			
annexin A1	ANXA1	NM_000700	2.6
baculoviral IAP repeat-containing 4	BIRC4	NM_001167	2.3
CASP8 and FADD-like apoptosis regulator	CFLAR	NM_003879	3.2
CASP8 and FADD-like apoptosis regulator	CFLAR	AF009616	5.8
immediate early response 3	IER3	NM_003897	5.3
interferon, alpha-inducible protein 6	IFI6	NM_022873	10.2
interleukin 1, alpha	IL1A	NM_000575	9.0
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	NM_003998	3.1
v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	RELA	BC014095	3.1
serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	NM_002575	13.9
suppressor of cytokine signaling 3	SOCS3	NM_003955	11.7
sphingosine kinase 1	SPHK1	NM_021972	2.6
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	13.9

tumor necrosis factor, alpha-induced protein 3	TNFAIP3	NM_006290	4.6
tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	NM_003840	2.9
tumor necrosis factor receptor superfamily, member 18	TNFRSF18	NM_148901	3.6
Induction of apoptosis			
apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	NM_032797	2.4
BCL2-antagonist/killer 1	BAK1	NM_001188	2.3
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032977	5.7
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032974	3.3
CD70 molecule	CD70	NM_001252	3.8
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.3
interferon, beta 1, fibroblast	IFNB1	NM_002176	71.7
interleukin 19	IL19	NM_153758	35.9
inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	AK001903	51.5
inhibin, beta A	INHBA	NM_002192	39.0
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	NM_002462	26.7
pleiomorphic adenoma gene-like 1	PLAGL1	NM_006718	1.9
phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	NM_021127	4.5
promyelocytic leukemia	PML	NM_002675	7.3
promyelocytic leukemia	PML	NM_033238	10.3
promyelocytic leukemia	PML	NM_033244	9.5
promyelocytic leukemia	PML	NM_033247	8.1
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022173	2.5
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022037	2.6
toll-like receptor 2	TLR2	NM_003264	3,1
tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	NM_003844	4.3
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_003811	5.8
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	78.7
Regulation of apoptosis			
BCL2-antagonist/killer 1	BAK1	NM_001188	2.3
BCL2-like 14 (apoptosis facilitator)	BCL2L14	NM_030766	20.6
B-cell translocation gene 1, anti-proliferative	BTG1	NM_001731	3.7
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032977	5.7
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032974	3.3
CASP8 and FADD-like apoptosis regulator	CFLAR	NM_003879	3.2
CASP8 and FADD-like apoptosis regulator	CFLAR	AF009616	5.8
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.3
interferon induced with helicase C domain 1	IFIH1	NM_022168	18.0
nucleotide-binding oligomerization domain containing 2	NOD2	NM_022162	2.0
TNF receptor-associated factor 1	TRAF1	NM_005658	6.7

Table S6. List of the 65 transcripts specific for scrub typhus

down-modulated genes in scrub typhus				
BCL11A	CORO1B	FOSB	GAGE6	LDLRAP1
RAPGEF3	SLC22A18	Hs.552434	LOC653374	

up-regulated genes in scrub typhus				
ADD3	FAM107B	RASA2	UBE1L2	Hs.562182
ATP13A3	FBXO4	RKHD2	UGCGL1	Hs.250648
AYTL2	FLJ20160	RORA	UHMK1	LOC285513
C9orf77	MACF1	SLC25A32	ZBTB2	LOC642161
CBLB	NUP54	SRPK2	ZMYND11	LOC649801
CD3G	NXF5	SUMO2	ZNF326	LOC651633
CD8A	NXT2	SYNE2	ZNF644	LOC652282
CD8B1	PLEKHF1	SYTL2	Hs.561954	LOC653115
DLD	PPP2R5C[a]	TCEA1	Hs.573541	LOC653663
DLG1	PRKACB[a]	TCERG1	Hs.373705	LOC653675
DNTTIP2	PRPS1L1	TOPBP1	Hs.567392	

Down-modulated and up-regulated genes in scrub typhus were compared to murine typhus, malaria and dengue, and were identified by Welch ANOVA ($P < 0.05$, FDR < 5%) followed by Tukey HSD post hoc test. [a]Two isoforms of gene transcripts are included in the list.

ARTICLE 2

Analysis of the transcriptional program induced in macrophages by *Orientia tsutsugamushi*, the agent of scrub typhus

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(Manuscript in preparation)

In this article, we studied the response of monocyte-derived macrophages (MDMs) to *O. tsutsugamushi* because it is well known that the response of MDMs to bacterial infection may be largely different from that of monocytes. *O. tsutsugamushi* replicates in MDMs as in monocytes. Type I IFN response, up-regulation of M1-associated genes and IL-1β secretion were found in MDMs, although the number of modulated-genes and the magnitude of the response are lower than those in monocytes. Finally, we showed that live organisms induce the cell death of a small proportion of MDMs.

Analysis of the transcriptional program induced in macrophages by *Orientia tsutsugamushi*, the agent of scrub typhus

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Abstract

Scrub typhus is a life-threatening disease caused by *Orientia tsutsugamushi*, a bacterial pathogen that mainly infects endothelial cells both *in vitro and in vivo*. Evidence suggests that the interaction of *O. tsutsugamushi* with myeloid cells may play a pivotal role in *O. tsutsugamushi* infection. We showed here that *O. tsutsugamushi* can infect human monocyte-derived macrophages. Microarray data also showed that *O. tsutsugamushi* affected the expression of 2,568 genes including type I interferon, interferon-stimulated genes, inflammatory genes and apoptosis-related genes in macrophages. The analysis of M1 and M2 genes revealed that *O. tsutsugamushi* induced an M1-type response. This transcriptional signature was accompanied by functional consequences such as release of inflammatory cytokines and macrophage apoptosis. Live *O.tsutsugamushi* organisms were necessary for type I interferon response and macrophage apoptosis and increased the levels of inflammatory response. The signature induced by *O. tsutsugamushi* in human macrophages was reminiscent of that of circulating monocytes. Our results suggest that the inflammatory response induced by *O. tsutsugamushi* may account for the local and systemic inflammation observed in scrub typhus.

Keywords: *Orientia tsutsugamushi*, macrophages, type I interferon, inflammation, apoptosis.

Introduction

Orientia tsutsugamushi is the agent of scrub typhus, a tropical infectious disease responsible of one million new cases each year and exclusively found in the Asia-Pacific region. Scrub typhus is characterized by fever, rash, eschar, pneumonitis, meningitis and disseminated intravascular coagulation, which may lead to multiorgan failure and death of patients (1). As *O. tsutsugamushi* is transmitted to humans by the bite of larval trombiculid mites, a billion of people are at risk to contract infection (2).

O. tsutsugamushi belongs to a genus that has been separated from the *Rickettsia* genus on the basis of genetic and phenotypic differences including the absence of peptidoglycan and lipopolysaccharide (LPS) in its cell wall (3). The genome of the bacterial strains Boryong and Ikeda has been recently sequenced (4, 5). *O. tsutsugamushi* is considered as an intracellular pathogen that mainly infects endothelial cells both in vitro (6) and in vivo (7). It also appears that the interaction of *O. tsutsugamushi* with myeloid cells plays a pivotal role in *O. tsutsugamushi* infection (8). First, *O. tsutsugamushi* infects and multiplies in mouse peritoneal macrophages (9, 10). *O. tsutsugamushi* leads to the death of infected mice unless peritoneal macrophages had been activated in vivo (11). Second, in autopsy tissues of patients with clinical suspicion of scrub typhus, *O. tsutsugamushi* organisms are located in endothelial cells from different organs in macrophages from liver and spleen (7). We recently showed that *O. tsutsugamushi* replicates in human monocytes isolated from healthy donors and alters the expression of more than 4,500 genes as demonstrated by microarray analysis (12).

The mechanisms that govern local and systemic inflammation in scrub typhus are only in part elucidated. In patients with scrub typhus, the circulating levels of inflammatory cytokines and chemokines are increased (13-15). Similarly, the susceptibility of mice to *O. tsutsugamushi* infection is associated with the over-production of cytokines and chemokines (8, 16). The expression of genes associated with the inflammatory response and interferon-

stimulated genes (ISGs) is upregulated in human monocytes stimulated with *O. tsutsugamushi*. In addition, several features of M1 polarization are observed in circulating leukocytes from patients with scrub typhus (12). However, the functional properties of circulating monocytes and those of tissue macrophages are largely different even if they belong to the same cell lineage. Indeed, monocytes express high levels of CD14 but not CD68 whereas monocyte-derived macrophages lose CD14 but acquire CD68. The macrophage response to infection may be distinct from that of monocytes. *Tropheryma whipplei*, the agent of Whipple's disease, is eliminated by monocytes and replicates within macrophages (17). *Coxiella burnetii*, the agent of Q fever, survives but is unable to replicate within monocytes whereas organisms replicate within macrophages. In addition, *C. burnetii* induces an M1 polarization in monocytes and an atypical M2 polarization in macrophages (18).

In this paper, we showed that *O. tsutsugamushi* replicated within human monocyte-derived macrophages (MDMs). Bacteria induced a robust transcriptional response consisting of genes encoding interferon-stimulated genes (ISGs), apoptosis-related genes and inflammatory mediators. This M1-type response was unable to eradicate infection, suggesting that the exacerbated inflammatory response induced by *O. tsutsugamushi* may be related to the inflammatory complications of scrub typhus.

Materials and Methods

Bacteria and cell culture

O. tsutsugamushi, strain Kato (CSUR R163), was propagated and titered in L929 cells as recently described (12). In some experiments, *O. tsutsugamushi* organisms were killed by heating at 100°C for 5 minutes (HK bacteria). Peripheral blood mononuclear cells (PBMCs) were isolated from leukopacks (Etablissement Français du Sang, Marseille, France) over a Ficoll gradient (MSL, Eurobio, Les Ulis, France). Monocytes were isolated by adherence in 24-well plates. They were then differentiated into macrophages using a procedure described elsewhere (19) with slight modifications. Briefly, monocytes were cultivated in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine (Invitrogen, Cergy Pontoise, France) and 10% heat-inactivated human AB sera (Lonza, Verviers, Belgium) for three days and human serum was replaced by 10% fetal bovine serum (FBS, Invitrogen) for additional periods ranging from 4 to 6 days. Cultivated cells were designated as MDMs since more than 90% of them did not express CD14 but expressed CD68.

Infection procedure

MDMs (1.5×10^5 cells per assay) were incubated in RPMI 1640 containing 25 mM HEPES, 2 mM L-glutamine and 10% heat-inactivated FBS in the presence of *O. tsutsugamushi* for 2 hours. MDMs were then washed to discard unbound organisms and cultivated for different periods. The intracellular fate of bacterial pathogens was determined using quantitative real-time PCR (qPCR), as recently described (12). To assess bacterial DNA, MDMs were lyzed in 0.1% Triton X-100, and DNA was extracted in a 100 µl volume using a QIAamp Tissue Kit (Qiagen, Courtaboeuf, France) and stored at -20°C. qPCR was performed using 5 µl of DNA extract, specific primers and probes and the Taqman system (Applied Biosystems,

Warrington, UK). The results are expressed as the total number of bacterial DNA copies determined from serial dilutions of a known concentration of bacterial DNA.

Microarrays

MDMs (4×10^5 cells per assay) were incubated with *O. tsutsugamushi* at a bacterium-to-cell ratio of 20:1 for 8 hours. RNAs were extracted from infected and uninfected MDMs using an RNeasy Mini kit (Qiagen) with a DNase digestion step. The quality and the quantity of isolated RNAs were assessed as recently described (12). Eight RNA samples (four samples per condition) were processed for microarray analysis. The amplification of RNAs and Cy3 labeling of cDNAs were generated using the Low RNA Input Fluorescent Linear Amplification Kit (Agilent Technologies, Massy, France). cDNAs were hybridized to 4×44K microarray slides (Agilent Technologies). The scanned images were analyzed with the Feature Extraction Software 10.5.1 (Agilent Technologies). The data processing and analyses were performed using the Resolver software 7.1 (Rosetta Inpharmatics, Cambridge, MA), R and BioConductor softwares, as recently described (12). Statistical analyses were performed using the significance analysis of microarrays (SAM) and the genes with an absolute fold change (FC) greater than 2.0 and a *p* value less than 0.01 were considered differentially modulated. Results were expressed as $\log_2$ FC. The differentially expressed genes were classified based on their Gene Ontology (GO) category.

Quantitative real time RT-PCR

Quantitative real time RT-PCR (qRT-PCR) was performed on selected genes as recently described (12). In brief, total RNAs were isolated from MDMs using a Qiagen kit, and cDNA synthesis was performed using an oligo(dT) primer and M-MLV reverse transcriptase (Invitrogen). qPCR was then carried out using specific primers (see the list in **Table S1**) and

Light Cycler-FastStart DNA MasterPLUS SYBR Green I (Roche Applied Science, Meylan, France). The FC of genes relative to β-actin gene was calculated using the $2^{-\Delta\Delta Ct}$ method.

TdT-mediated dUTP nick-end labeling (TUNEL) assay

Infected MDMs were fixed with 3% paraformaldehyde and cell death was determined using In Situ Cell Death Detection Kit, TMR red (Roche Applied Science) as recently described (12). The number of TUNEL-positive cells and DAPI-stained nuclei were determined using fluorescence microscopy. Cell death is expressed as the number of TUNEL-positive cells relative to the number of DAPI-stained nuclei.

Immunoassays

The amounts of TNF and IFN-β in MDM supernatants were determined by TNF and IFN-β ELISA kits purchased from R&D Systems, Lille, France. IL-1β amounts were determined using a specific ELISA kit provided by Diaclone (Besançon, France).

Statistical analysis

The results were compared using the non-parametric Mann-Whitney *U* test and are expressed as means ± standard deviation (SD). Differences were considered significant when *p* values were less than 0.05.

Results

Intracellular fate of O. tsutsugamushi *in MDMs*

The intracellular replication of *O. tsutsugamushi* was determined by incubating MDMs with organisms (2 per cell) for two hours. MDMs were then washed to remove free organisms (time considered as day 0) and cultured for 5 days. At day 0, the number of bacterial DNA

copies was $6.9 \pm 1.5 \times 10^4$ and approximately increased two fold each supplementary day (**Figure 1**), demonstrating that MDMs are a replicative niche for *O. tsutsugamushi*.

Global transcriptome analysis of O. tsutsugamushi-*infected MDMs*

The transcriptional profiles of uninfected MDMs and MDMs stimulated with *O. tsutsugamushi* for 8 hours were analyzed using whole genome microarrays. We found that 2,568 genes (1,513 upregulated and 1,055 downregulated) were modulated in response to *O. tsutsugamushi*. These modulated genes were then classified according GO terms. Among the upregulated genes, the enrichment of genes involved in anti-viral response, chemotaxis, inflammatory response and immune response comprised between 20 and 40% (**Figure 2A**), suggesting that these pathways play an important role in the response of MDMs to *O. tsutsugamushi*. GO categories related to cell-cell signaling, cell motility, cell proliferation, apoptosis, signal transduction, cell adhesion, receptor activity and transcription factor activity exhibited lower enrichments (**Figure 2A**). Among the downregulated genes, the enrichment of genes involved in chemotaxis, cytoskeleton, immune response, intracellular signaling, cell motility, receptor activity and signal transduction was comprised between 4 and 8% (**Figure 2B**). Taken together, these data showed that *O. tsutsugamushi* induced substantial changes in the transcriptional program of MDMs.

Analysis of the type I IFN response induced by O.tsutsugamushi

The genes belonging to the GO category "response to virus" mainly consisted of genes involved in type I IFN pathway (**Table S2**). Indeed, the genes encoding IFN-β, three subtypes of IFN-α and ISGs were upregulated in MDMs infected with *O. tsutsugamushi*. The upregulated ISGs included the genes encoding CCL4 (MIP-1β), CCL5 (RANTES), CCL8, 2'5' oligoadenylate synthetase (OAS 1-3, OASL), myxovirus resistance (MX1 and MX2),

ISG15, ISG20, interferon regulatory factor 7 (IRF7). The upregulation of IFN-β, OAS1, MX1, and CCL5 was confirmed by qRT-PCR performed on MDMs from three different donors (**Figure 3A**). Note that the upregulated expression of these genes was long-lasting since it was still observed after 18 hours (**Figure 3B**). In contrast to live *O. tsutsugamushi*, HK bacteria were unable to induce the expression of IFN-β and ISGs such as MX1 (**Figure S1**). Finally, the type I IFN signature of *O. tsutsugamushi*-stimulated MDMs was functional. Indeed, the release of IFN-β by MDMs stimulated with *O. tsutsugamushi* for 24 and 48 hours was 33 ± 9 and 40 ± 3 pg/ml, respectively. In contrast, HK bacteria were unable to induce IFN-β release by MDMs. Taken together, these results indicate that live *O. tsutsugamushi* induced a type I IFN response in MDMs.

Analysis of inflammatory response in O. tsutsugamushi-*infected MDMs*

The enrichment of 'immune response' GO term concerned the genes involved in chemotaxis (17 genes), inflammatory response (23 genes encoding cytokines and related molecules) and microbicidal responses (guanylate binding proteins, cathepsin G, indoleamine-pyrrole 2,3 dioxygenase (INDO) (**Table S3**). These results were confirmed by qRT-PCR using a series of genes that encode inflammatory cytokines (TNF, IL-1β, IL-16, IL-12p40, IL-23p19, IL-15, IFN-β) and chemokines (CCL5, CCL20, CXCL10, CXCL11). As reported above, these genes were upregulated after 8 hours (**Figure 3A**) and 18 hours (**Figure 3B**). The robust expression of inflammatory cytokines and chemokines in response to *O. tsutsugamushi* suggested that MDMs were polarized toward an M1 phenotype. Consequently, we compared the transcriptional profile of *O. tsutsugamushi*-infected MDMs with M1/M2 profiles. We selected 32 genes as representative of M1 macrophages and 28 genes as representative of M2 macrophages. Twenty-five M1 genes (**Table 1**) and only two M2 genes were up-regulated following *O. tsutsugamushi* infection (**Table 2**).

Next, we determined whether the expression of inflammatory genes was dependent on bacterial viability. HK *O. tsutsugamushi* organisms were unable to modulate the expression of the genes encoding CXCL10 and CXCL11 (**Figure S1**). The expression of the genes encoding TNF, IL-6 and IL-12p40 was reduced as compared to the modulation of these genes induced by live organisms whereas the expression of IL-1β and IL-23p19 genes was similar in macrophages stimulated with live and HK organisms, respectively (compare **Figure S1** and **Figure 3A**).

Finally, the inflammatory signature of macrophages stimulated with *O. tsutsugamushi* was functional. Indeed, macrophages released high amounts of TNF and IL-1β in response to live organisms whereas HK organisms induced the release of lower amounts of TNF and were unable to induce the release of IL-1β **(Table 3)**. Taken together, these results suggest that *O. tsutsugamushi* induced a transcriptional and functional M1 profile in macrophages.

O. tsutsugamushi *induces MDM apoptosis*

The analysis of upregulated genes showed the enrichment of genes in apoptosis GO terms including apoptosis, anti-apoptosis, induction of apoptosis and regulation of apoptosis (**Table S4**). They included FAS, XAF1, TNF superfamily members and apoptosis regulators such as BAK1 and CFLAR. The upregulated expression of the genes encoding XAF1 and TRAIL induced by *O. tsutsugamushi* in MDMs stimulated for 8 hours was confirmed by qRT-PCR (**Figure 3A**) and, again, this upregulation was persistent (**Figure 3B**). We then studied MDM apoptosis using TUNEL staining. In response to live *O. tsutsugamushi*, 11.8 ± 1.2 and 18.3 ± 1.4% of MDMs were apoptotic after 24 and 48 hours, respectively. In contrast, HK organisms were unable to induce the apoptosis of MDMs (about 1% of control MDMs and MDMs incubated with HK *O. tsutsugamushi* were found apoptotic after 24 and 48 hours). Taken

together, our data indicate that *O. tsutsugamushi* altered the apoptosis gene program in MDMs leading to their death.

Discussion

We showed in this paper that *O. tsutsugamushi* was able to replicate within human monocyte-derived macrophages. The bacterial uptake by MDMs was slightly lower than that observed in monocytes but bacterial replication was higher in MDMs than that found in monocytes (12). These findings suggest that the mechanisms that govern the entry and the intracellular fate of *O. tsutsugamushi* within MDMs and monocytes might be partly different. It has been found that *O. tsutsugamushi* is present in tissue macrophages from autopsied patients with scrub typhus (20, 21). The intracellular survival of *O. tsutsugamushi* in macrophages may contribute to infection in organs such as liver, spleen and lymph nodes from patients with scrub typhus. The mechanisms of *O. tsutsugamushi* internalization are partially understood. *O. tsutsugamushi* binds non-phagocytic mammalian cells through bacterial 56-kDa type-specific surface antigen. Heparin sulfate proteoglycans are candidate receptors for cell invasion (8). It has been recently proposed that nucleotide-binding oligomerization domain-containing protein (NOD) 1 may be involved in the recognition of *O. tsutsugamushi* by endothelial cells despite the absence of identified bacterial ligand of NOD1 because peptidoglycan, known to bind NOD1, is absent from *O. tsutsugamushi* (22).

The global transcriptomic analysis showed that *O. tsutsugamushi* organisms induced substantial changes in the transcriptional program of macrophages that remained lower than that in monocytes (23) Nevertheless, the comparison of the transcriptional responses of macrophages (our results) and monocytes revealed an enrichment with the same GO terms. However, the transcriptomic response of macrophages to *O. tsutsugamushi* associated the upregulation of type I IFNs, several interferon-stimulated genes (ISGs) and M1 polarization

as reported in monocytes (23). There are some differences between infected monocytes and macrophages. The gene encoding IFN4 was modulated in monocytes but not in macrophages. Note that the level of gene expression was markedly higher in monocytes than in MDMs. The expression of IFN-β and ISGs completely depended on *O. tsutsugamushi* viability since HK bacteria failed to induce type I IFN response in MDMs as found in monocytes (23). We can suppose that the expression of IFN-β and ISGs is related to the cytosolic localization of live organisms as found for other pathogens (24-26).

Numerous genes belonging to the immune response GO term were modulated in macrophages. They included genes encoding inflammatory cytokines and chemokines. It has been clearly demonstrated that *O. tsutsugamushi* susceptible mice over-produce inflammatory cytokines and chemokines compared with resistant mice (27). To more precisely define the inflammatory response of macrophages to *O. tsutsugamushi*, we compared the program of *O. tsutsugamushi*-stimulated macrophages with the list of M1 and M2 genes previously established (28). We found that *O. tsutsugamushi* induced an M1-type program because 23/32 M1 genes were modulated in macrophages. This M1-type program presented some differences with that elicited by *O. tsutsugamushi* in monocytes (23). Indeed, the upregulation of the genes encoding CCR7, IL-2 receptor α chain and IL-23p19 was more pronounced in macrophages than in monocytes whereas that of IL-6 was strikingly higher in monocytes than in macrophages. The transcriptional responses detected by qRT-PCR of *O. tsutsugamushi*-stimulated macrophages and monocytes confirmed microarray data. The genes encoding inflammatory cytokines and chemokines were upregulated but the expression of some of them was higher (CXCL10, CXCL11) or lower (IL-1β, IL-12p40) in MDMs. Finally, the amounts of released inflammatory cytokines by MDMs and monocytes were also different: they increased with time in monocytes but decreased in MDMs. In addition, a few number of genes belonging to the immune response GO term (e.g. CD14) were modulated only in monocytes

whereas other genes including CCL5, CCL19, CXCL9, cathepsin G, CD1d and CD6 were modulated only in MDMs. Finally, the level of modulation of numerous genes belonging to the immune response GO term was lower in MDMs than in monocytes.

The apoptosis GO term including FAS, XAF1, TNF superfamily members and apoptosis regulators such as BAK1 and CFLAR was enriched in MDMs. Some differences characterized the response of MDMs to *O. tsutsugamushi* and that of monocytes (12). Indeed, the up-regulation of some genes such as NFKBIA was observed in MDMs but not in monocytes. In addition, the level of upregulation of some genes was clearly different: SERPINB2 gene was highly upregulated in MDMs but moderately in monocytes; conversely, the upregulation of IL19 and TNFSF10 genes was higher in monocytes than in MDMs stimulated with *O. tsutsugamushi*. Note that XAF1 and TNFSF10 are apoptosis inducers that can be upregulated by type I IFNs (29). The enrichment in apoptosis-related genes program induced by *O. tsutsugamushi* was accompanied by the apoptosis of MDMs that was higher than that observed in monocytes. Interestingly, HK bacteria that were not cytosolic were unable to induce MDM apoptosis. As apoptosis can result from caspase-1 activation and IL-1β production (30), we hypothesize that *O. tsutsugamushi* stimulates inflammasome activation when the organisms reach the cytosol.

In conclusion, we demonstrate here that human macrophages are targeted in vitro by *O. tsutsugamushi*, reinforcing the idea that tissue macrophages may be infected during scrub typhus. *O. tsutsugamushi* stimulated a type I IFN response likely through bacterial location within cytoplasm and an M1-type response that were unable to eradicate infection. This exacerbated inflammatory response may be related to the inflammatory complications of scrub typhus.

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Legends of Figures

Figure 1. *O. tsutsugamushi* infection of MDMs

MDMs were incubated with viable *O. tsutsugamushi* for two hours. They were then washed to remove free organisms and cultured for 5 days. The number of bacterial DNA copies was determined by qPCR. The results are the mean ± SD of three different experiments.

Figure 2. GO annotation of regulated genes in MDMs

MDMs were stimulated with *O. tsutsugamushi* for 8 hours. The genes that were differentially expressed in stimulated MDMs were subjected to GO annotation. The major identified biological processes are shown with the total number of genes within each biological process. A, upregulated genes; B, downregulated genes.

Figure 3. Effect of *O. tsutsugamushi* on the transcriptional response of MDMs

MDMs were stimulated with viable *O. tsutsugamushi* for 8 (A) and 18 hours (B). RNA was extracted and qRT-PCR was performed on different genes found to be regulated in microarray experiments. The results expressed in relative expression (stimulated vs. unstimulated conditions) represent the mean ± SD of 3 independent experiments performed in triplicate.

	number of bacterial DNA
day 0	$6.9 \pm 1.5 \times 10^4$
day 1	$8.4 \pm 1.8 \times 10^4$
day 2	$16 \pm 2.8 \times 10^4$
day 3	$43 \pm 6.9 \times 10^4$
day 5	$35 \pm 2.2 \times 10^5$

Figure 1

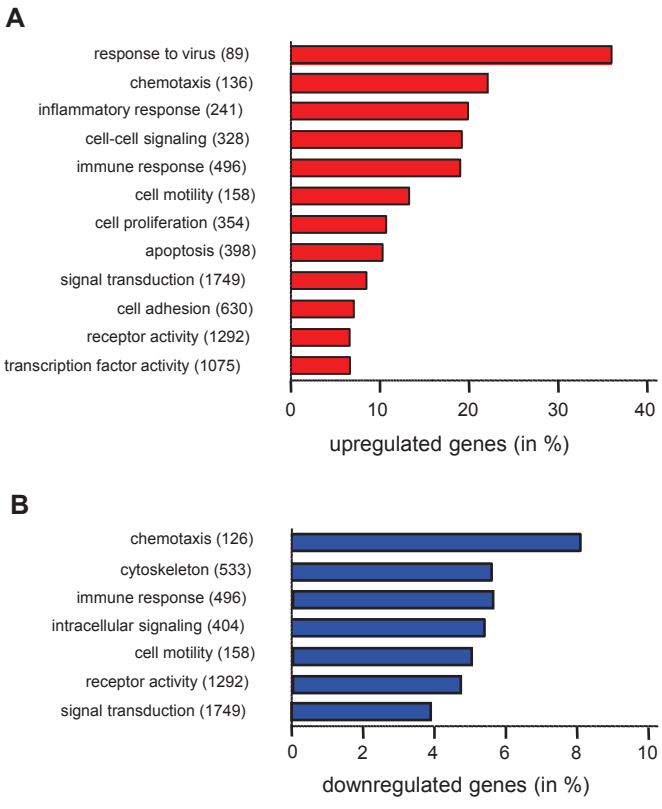

Figure 2

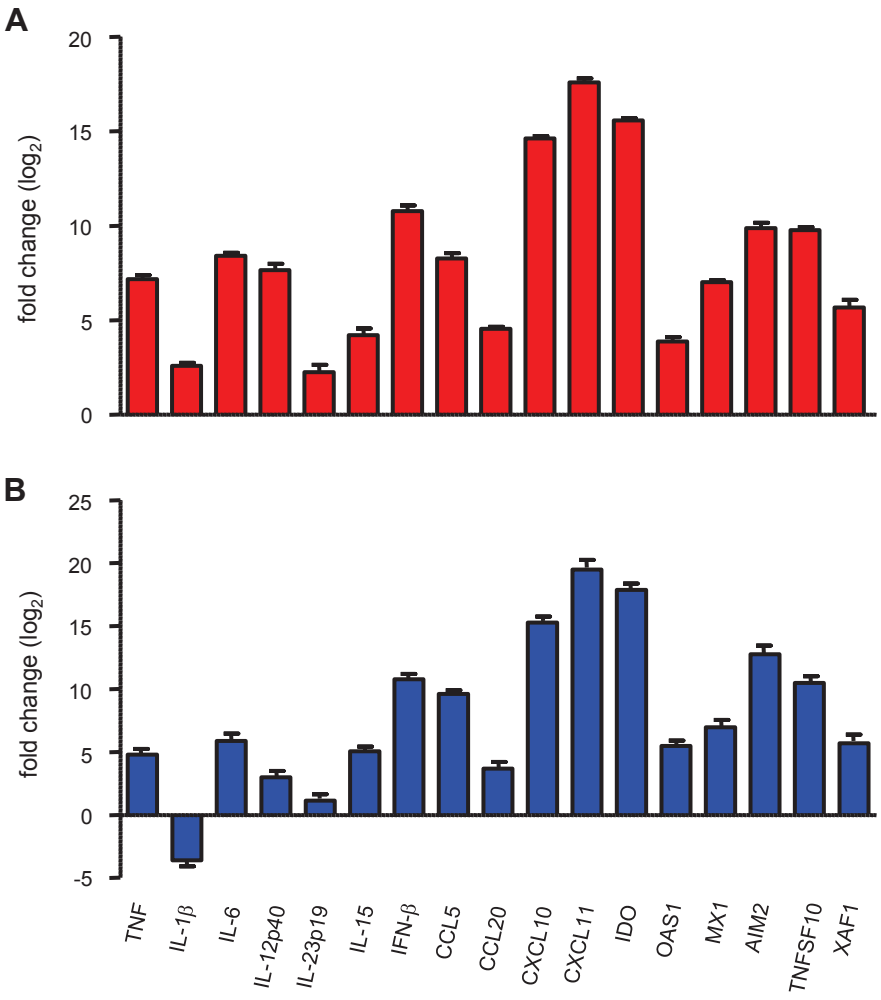
A
fold change (log2)
20
15
10
5
0
B
fold change (log2)
25
20
15
10
5
0
-5
TNF
IL-1β
IL-6
IL-12p40
IL-23p19
IL-15
IFN-β
CCL5
CCL20
CXCL10
CXCL11
IDO
OAS1
MX1
AIM2
TNFSF10
XAF1

Figure 3

Figure S1. Effect of HK *O. tsutsugamushi* on the transcriptional response of MDMs
MDMs were stimulated with HK organisms for 8 hours. RNA was extracted and qRT-PCR was performed. The results that are expressed in relative expression (stimulated vs. unstimulated conditions) represent the mean ± SD of 3 independent experiments performed in triplicate.

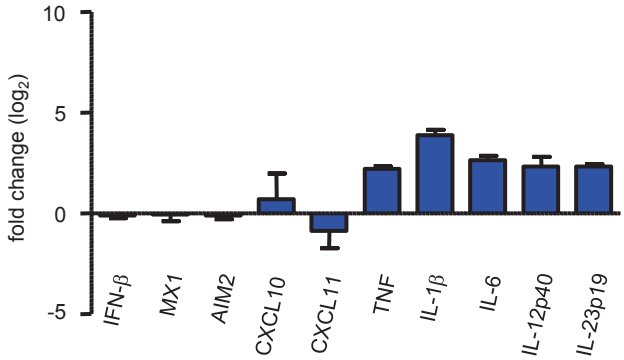

Table 1. Modulated genes according M1 genes

Gene category and name	Gene symbol	FC
Membrane receptors		
Chemokine (C-C motif) receptor 7	CCR7	23.4
Interleukin 2 receptor α chain	IL2RA	7.6
Interleukin 7 receptor	IL7R	3.5
Interleukin 15 receptor α chain	IL15RA	5.7
Cytokines and chemokines		
Chemokine (C-C motif) ligand 2	CCL2	nc
Chemokine (C-C motif) ligand 3	CCL3	4.7
Chemokine (C-C motif) ligand 4	CCL4	3.8
Chemokine (C-C motif) ligand 5	CCL5	2.9
Chemokine (C-C motif) ligand 15	CCL15	nc
Chemokine (C-C motif) ligand 19	CCL19	3.2
Chemokine (C-C motif) ligand 20	CCL20	3.7
Chemokine (C-X-C motif) ligand 8	CXCL8, IL8	8.0
Chemokine (C-X-C motif) ligand 9	CXCL9	2.5
Chemokine (C-X-C motif) ligand 10	CXCL10	8.3
Chemokine (C-X-C motif) ligand 11	CXCL11	48.0
Chemokine (C-X-C motif) ligand 16	CXCL16	nc
Endothelial cell growth factor 1 (platelet-derived)	ECGF1	nc
Interleukin 1α	IL1A	15.0
Interleukin 1β	IL1B	16.6
Interleukin 6	IL6	9.7
Interleukin 12A	IL12A	1.7
Interleukin 12B	IL12B	75.8
Interleukin 15	IL15	4.1
Interleukin 23 α subunit p19	IL23A	12.4
Tumor necrosis factor ligand superfamily, member 2	TNF	4.9
Tumor necrosis factor ligand superfamily, member 10	TRAIL	19.1
Visfatin	PBEF1	5.1
Apoptosis-related genes		
BCL2-related protein A1	BCL2A1	1.7
Fas (TNF receptor superfamily, member 6)	FAS	3.7
Baculoviral IAP repeat-containing 3	BIRC3	2.5
growth arrest and DNA-damage-inducible, gamma	GADD45G	1.8
XIAP associated factor-1	XAF1	5.0

M1 genes are referred to specific transcriptional profiles provided in references 19 and 30. The values of FC were obtained in microarray. nc : not changed.

Table 2. Modulated genes according M2 genes

Gene category and name	Gene symbol	FC
Membrane receptors		
CD36	CD36	-1.6
CD163	CD163	nc
CD209	DCSIGN	nc
Chemokine (C-X-C motif) receptor 4	CXCR4	1.8
C-type lectin receptor DCL-1	DCL-1	nc
C-type lectin superfamily member 12	DECTIN1	nc
Fcε-RII	CD23	nc
G protein-coupled receptor 105	P2RY14	-2.9
G protein-coupled receptor 86	GPR86	-4.9
Histamine receptor H1	HRH1	nc
macrophage scavenger receptor 1	MSR1	1.7
macrophage scavenger receptor 1	MSR1	nc
Mannose receptor C type 1	MRC1	nc
Membrane-spanning 4-domains, subfamily A, member 4	MS4A4A	-1.5
Membrane-spanning 4-domains, subfamily A, member 6A	MS4A6A	-1.5
Purinergic receptor P2Y5	P2RY5	-1.8
Toll-like receptor 5	TLR5	-6.9
Transforming growth factor β receptor II	TGFBR2	-2.4
Transforming growth factor β receptor II	TGFBR2	-3
Cytokines and chemokines		
Chemokine (C-C motif) ligand 1	CCL1	6.0
Chemokine (C-C motif) ligand 13	CCL13	-1.6
Chemokine (C-C motif) ligand 17	CCL17	nc
Chemokine (C-C motif) ligand 18	CCL18	nc
Chemokine (C-C motif) ligand 22	CCL22	nc
Chemokine (C-C motif) ligand 23	CCL23	1.9
Chemokine (C-C motif) ligand 24	CCL24	nc
Interleukin 1 receptor antagonist	IL1RN	8.5
Interleukin 10	IL10	nc

M2 genes are referred to specific transcriptional profiles provided in references 19 and 30. The values of FC were obtained in microarray. nc : not changed.

Table 3. Release of inflammatory cytokines

	TNF		IL-1β	
	live bacteria	HK bacteria	live bacteria	HK bacteria
8 hours	7286 ± 712	911 ± 102	533 ± 11	< 5
24 hours	3329 ± 464	28 ± 3	410 ± 35	< 5

MDMs were stimulated with live or HK *O. tsutsugamushi* for 8 and 24 hours. Culture supernatants were analyzed for the presence of TNF and IL-1β using specific immunoassays. The results are expressed in pg/ml and are presented as the mean ± SD of three experiments performed in duplicate.

Table S1. Nucleotide sequences of oligonucleotide primers

Symbol	Forward primers	Reverse primers
GAPDH	5’-GGTGGTCTCCTCTGACTTCAACA-3’	5’-GTTGCTGTAGCCAAATTCGTTGT-3’
AIM2	5’-GGTTTGTTTGTAGTCCAGAAGGTA-3’	5’-GTGCTATGAACTCCAGATGTCAG-3’
CCL5	5’-CTGCTGCTTTGCCTACATTGC-3’	5’-GTTCAGGTTCAAGGACTCTCCATC-3’
CCL20	5'-GACATCAATGCTATCATCTTTCAC-3'	5'-GCTATGTCCAATTCCATTCCA-3'
CXCL10	5'-AAGCAGTTAGCAAGGAAAGGTC-3'	5'-TTGAAGCAGGGTCAGAACATC-3'
CXCL11	5'-TATAGCCTTGGCTGTGATATTGTG-3'	5'-CTGCCACTTTCACTGCTTTTACC-3'
IFNβ	5'-GACGCCGCATTGACCATCTA-3'	5'-CCTTAGGATTTCCACTCTGACT-3'
IL-1β	5'-GCTGAGGAAGATGCTGGTTC-3'	5'-TCCATATCCTGTCCCTGGAG-3'
IL-6	5'-TACCCCCAGGAGAAGATTCC-3'	5'-TTTTCTGCCAGTGCCTCTTT-3'
IL-12p40	5'-AGGGGACAACAAGGAGTATGAGT-3'	5'-AGGGAGAAGTAGGAATGTGGAGT-3'
IL-15	5'-GTTAGCAGATAGCCAGCCCATAC-3'	5'-TACTCAAAGCCACGGTAAATCC-3'
IL-23p19	5'-AGGAGAAGAGGGAGATGAAGAGAC-3'	5'-GCTATCAGGGAGCAGAGAAGG-3'
INDO	5'-TGCTGGTGGAGGACATGCTG-3'	5'-TGAAAGGACAAACTCACGGACTGA-3'
MX1	5'-GCCACCACAGAGGCTCTCAG-3'	5'-CTCAGCTGGTCCTGGATCTCCT-3'
OAS1	5'-TCCGCCTAGTCAAGCACTGGTA-3'	5'-CCTGGGCTGTGTTGAAATGTGT-3'
TNF	5'- CCCGACTATCTCGACTTTGC-3'	5'-AGGTTGAGGGTGTCTGAAGGA-3'
TRAIL	5’-GGCTAACTGACCTGGAAAGAAA-3’	5’-TTTGGTTGTGGCTGCTCTACT-3’
XAF1	5’-GAGCTCCACGAGTCCTACTGT-3’	3’-CTCTGAGTCTGGACAACATTTACC-3’

Table S2. Modulated genes in the "response to virus" GO term

Gene function and full gene name	Gene symbol	GenBank ID	FC
apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3G	APOBEC3G	NM_021822	2.6
chemokine (C-C motif) ligand 4	CCL4	NM_002984	3.8
chemokine (C-C motif) ligand 5	CCL5	NM_002985	2.9
chemokine (C-C motif) ligand 8	CCL8	NM_005623	7.2
chemokine (C-C motif) ligand 19	CCL19	NM_006274	3.2
DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	DDX58	NM_014314	20.7
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	6.3
interferon-induced protein 35	IFI35	NM_005533	3.7
interferon-induced protein 44	IFI44	NM_006417	10.2
interferon induced with helicase C domain 1	IFIH1	NM_022168	9.5
interferon, alpha 5	IFNA5	NM_002169	3.1
interferon, alpha 6	IFNA6	NM_021002	2.0
interferon, alpha 8	IFNA8	NM_002170	5.9
interferon, beta 1, fibroblast	IFNB1	NM_002176	5.8
interferon, gamma	IFNG	NM_000619	6.1
interleukin 23, alpha subunit p19	IL23A	NM_016584	12.4
interleukin 28 receptor, alpha (interferon, lambda receptor)	IL28RA	NM_170743	4.1
interleukin 29 (interferon, lambda 1)	IL29	NM_172140	2.3
interferon regulatory factor 7	IRF7	NM_004031	11.6
ISG15 ubiquitin-like modifier	ISG15	NM_005101	21.4
interferon stimulated exonuclease gene 20kDa	ISG20	NM_002201	8.2
myxovirus (influenza virus) resistance 1	MX1	NM_002462	12.3
myxovirus (influenza virus) resistance 2	MX2	NM_002463	14.8
2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	NM_002534	5.4
phospholipid scramblase 1	PLSCR1	NM_021105	3.7
v-rel reticuloendotheliosis viral oncogene homolog A	RELA	BC014095	2.4
signal transducer and activator of transcription 2, 113kDa	STAT2	NM_005419	3.6
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	4.9
tripartite motif-containing 22	TRIM22	NM_006074	3.2
tripartite motif-containing 5	TRIM5	NM_033034	2.4
tripartite motif-containing 5	TRIM5	NM_033092	4.0

Table S3. Modulated genes in the "immune response" GO term

Gene function and full gene name	Gene symbol	GenBank ID	FC
Immune response			
adenosine deaminase	ADA	NM_000022	4.2
absent in melanoma 2	AIM2	NM_004833	3.6
aquaporin 9	AQP9	AQP9	3.3
chemokine (C-C motif) ligand 1	CCL1	NM_002981	6.0
chemokine (C-C motif) ligand 3	CCL3	NM_002983	4.7
chemokine (C-C motif) ligand 4	CCL4	NM_002984	3.8
chemokine (C-C motif) ligand 5	CCL5	NM_002985	2.9
chemokine (C-C motif) ligand 7	CCL7	NM_006273	8.3
chemokine (C-C motif) ligand 8	CCL8	NM_005623	7.2
chemokine (C-C motif) ligand 19	CCL19	NM_006274	3.2
chemokine (C-C motif) ligand 20	CCL20	NM_004591	3.7
CD274 molecule	CD274	NM_014143	12.1
CD40 molecule, TNF receptor superfamily member 5	CD40	NM_001250	3.3
CD70 molecule	CD70	NM_001252	2.1
CD80 molecule	CD80	NM_005191	8.6
C-type lectin domain family 4, member D	CLEC4D	NM_080387	5.4
C-type lectin domain family 4, member E	CLEC4E	NM_014358	7.9
colony stimulating factor 2 (granulocyte-macrophage)	CSF2	NM_000758	4.8
colony stimulating factor 3 (granulocyte)	CSF3	NM_000759	54.7
cathepsin G	CTSG	NM_001911	2.6
chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	CXCL1	NM_001511	2.9
chemokine (C-X-C motif) ligand 2	CXCL2	NM_002089	6.0
chemokine (C-X-C motif) ligand 3	CXCL3	NM_002090	7.6
chemokine (C-X-C motif) ligand 5	CXCL5	NM_002994	5.3
chemokine (C-X-C motif) ligand 9	CXCL9	NM_002416	2.5
chemokine (C-X-C motif) ligand 10	CXCL10	NM_001565	8.3
chemokine (C-X-C motif) ligand 11	CXCL11	NM_005409	48.0
chemokine (C-X-C motif) ligand 13 (B-cell chemoattractant)	CXCL13	NM_006419	3.0
chemokine (C-X-C motif) ligand 14	CXCL14	NM_004887	2.7
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	6.3
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.7
guanylate binding protein 1, interferon-inducible, 67kDa	GBP1	NM_002053	5.1
guanylate binding protein 3	GBP3	NM_018284	3.4
guanylate binding protein 5	GBP5	NM_052942	4.5
guanylate binding protein 7	GBP7	NM_207398	58.3
GTP binding protein overexpressed in skeletal muscle	GEM	NM_005261	4.4
GTP binding protein 1	GTPBP1	NM_004286	2.9
interferon, alpha-inducible protein 27	IFI27	NM_005532	2.8
interferon-induced protein 35	IFI35	NM_005533	3.7
interferon, alpha-inducible protein 6	IFI6	NM_022873	2.6
interferon-induced protein with tetratricopeptide repeats 1	IFIT1	NM_001548	100.0
interferon-induced protein with tetratricopeptide repeats 2	IFIT2	NM_001547	19.9
interferon-induced protein with tetratricopeptide repeats 3	IFIT3	NM_001549	14.9
interferon-induced protein with tetratricopeptide repeats 5	IFIT5	NM_012420	7.6
interferon induced transmembrane protein 1 (9-27)	IFITM1	NM_003641	5.6
interferon induced transmembrane protein 2 (1-8D)	IFITM2	NM_006435	3.1
interferon induced transmembrane protein 3 (1-8U)	IFITM3	NM_021034	3.7
interleukin 1, alpha	IL1A	NM_000575	15.0
interleukin 1, beta	IL1B	NM_000576	16.7
interleukin 6	IL6	NM_000600	9.7
interleukin 8	IL8	NM_000584	8.0
interleukin 12A (p35)	IL12A	NM_000882	1.7

interleukin 12B (p40)	IL12B	NM_002187	75.8
interleukin 15	IL15	NM_172174	4.1
interleukin 18 (interferon-gamma-inducing factor)	IL18	NM_001562	2.7
interleukin 19	IL19	NM_153758	5.1
interleukin 23A (p19)	IL23A	NM_016584	12.4
interleukin 1 family, member 5 (delta)	IL1F5	NM_012275	9.5
interleukin 1 family, member 9	IL1F9	NM_019618	100.0
interleukin 1 receptor antagonist	IL1RN	BC068441	5.5
interleukin 1 receptor antagonist	IL1RN	NM_173842	8.5
interleukin 7 receptor	IL7R	NM_002185	3.5
indoleamine-pyrrole 2,3 dioxygenase	INDO	NM_002164	34.9
interferon regulatory factor 2	IRF2	NM_002199	2.1
interferon regulatory factor 7	IRF7	NM_004031	11.6
ISG15 ubiquitin-like modifier	ISG15	NM_005101	21.4
MHC class I polypeptide-related sequence B	MICB	NM_005931	2.4
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	NM_002462	12.3
myxovirus (influenza virus) resistance 2 (mouse)	MX2	NM_002463	14.8
2',5'-oligoadenylate synthetase 1, 40/46kDa	OAS1	NM_002534	5.4
2'-5'-oligoadenylate synthetase 2, 69/71kDa	OAS2	NM_016817	8.2
2'-5'-oligoadenylate synthetase 3, 100kDa	OAS3	NM_006187	7.4
2'-5'-oligoadenylate synthetase-like	OASL	NM_003733	44.7
oncostatin M	OSM	NM_020530	5.4
regulator of G-protein signaling 1	RGS1	NM_002922	3.2
sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3C	SEMA3C	NM_006379	3.3
transporter 1, ATP-binding cassette, sub-family B (MDR/TAP)	TAP1	NM_000593	2.2
tumor necrosis factor receptor superfamily, member 4	TNFRSF4	NM_003327	2.3
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_001561	10.3
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	19.1
tumor necrosis factor (ligand) superfamily, member 15	TNFSF15	NM_005118	20.5
tumor necrosis factor (ligand) superfamily, member 4 (tax-transcriptionally activated glycoprotein 1, 34kDa)	TNFSF4	NM_003326	3.9
tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811	2.8
tripartite motif-containing 22	TRIM22	NM_006074	3.2
chemokine (C-C motif) receptor 2	CCR2	NM_000647	-2.0
chemokine (C-C motif) receptor 2	CCR2	NM_000648	-2.1
CD1d molecule	CD1d	NM_001766	-4.2
CD6 molecule	CD6	NM_006725	-3.1
C-type lectin domain family 10, member A	CLEC10A	NM_182906	-2.1
C-type lectin domain family 4, member A	CLEC4A	NM_016184	-2.4
cytotoxic and regulatory T cell molecule	CRTAM	NM_019604	-2.3
hepcidin antimicrobial peptide	HAMP	NM_021175	-5.9
major histocompatibility complex, class II, DM beta	HLA-DMB	BC035650	-4.3
interleukin 16 (lymphocyte chemoattractant factor)	IL16	NM_172217	-11.0
interleukin 16 (lymphocyte chemoattractant factor)	IL16	NM_004513	-4.9
Notch homolog 1, translocation-associated (Drosophila)	NOTCH1	NM_017617	-2.7
ring finger protein 125	RNF125	NM_017831	-3.2
signaling threshold regulating transmembrane adaptor 1	SIT1	NM_014450	-3.6
transcription factor 7 (T-cell specific, HMG-box)	TCF7	NM_003202	-2.2
tumor necrosis factor (ligand) superfamily, member 11	TNFSF11	NM_003701	-2.2

Table S4. Modulated genes in the "apoptosis" GO term

Gene function and full gene name	Gene symbol	GenBank ID	FC
Apoptosis			
adenosine A2a receptor	ADORA2A	NM_000675	6.1
apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	NM_032797	2.7
AXIN1 up-regulated 1	AXUD1	NM_033027	5.6
B-cell receptor-associated protein 29	BCAP29	NM_001008407	2.1
baculoviral IAP repeat-containing 4	BIRC4	NM_001167	2.1
CD40 molecule, TNF receptor superfamily member 5	CD40	NM_001250	3.3
catenin, beta like 1	CTNNBL1	NM_030877	2.2
damage-regulated autophagy modulator	DRAM	BC018435	3.2
eukaryotic translation initiation factor 2-alpha kinase 2	EIF2AK2	NM_002759	6.3
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.7
immediate early response 3	IER3	NM_003897	4.8
interleukin 19	IL19	NM_153758	5.1
interleukin 1, alpha	IL1A	NM_000575	15.0
interleukin 1, beta	IL1B	NM_000576	16.7
Janus kinase 2 (a protein tyrosine kinase)	JAK2	NM_004972	3.1
lipopolysaccharide-induced TNF factor	LITAF	NM_004862	2.4
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	NM_003998	2.6
nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	NFKBIA	NM_020529	2.4
oncostatin M	OSM	NM_020530	5.4
pleckstrin homology-like domain, family A, member 2	PHLDA2	NM_003311	3.1
protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	NM_014330	3.1
receptor (TNFRSF)-interacting serine-threonine kinase 1	RIPK1	NM_003804	2.7
serine/threonine kinase 3 (STE20 homolog, yeast)	STK3	NM_006281	2.9
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022173	2.4
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022037	2.7
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	4.9
tumor necrosis factor, alpha-induced protein 3	TNFAIP3	NM_006290	5.9
tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	NM_003844	3.1
tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	NM_003840	2.8
tumor necrosis factor receptor superfamily, member 18	TNFRSF18	NM_148901	2.2
tumor necrosis factor (ligand) superfamily, member 9	TNFSF9	NM_003811	2.8
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	19.1
ubiquitin-conjugating enzyme E2Z	UBE2Z	NM_023079	2.5
Anti-apoptosis			
Annexin A1	ANXA1	NM_000700	1.9
baculoviral IAP repeat-containing 4	BIRC4	NM_001167	2.1
CASP8 and FADD-like apoptosis regulator	CFLAR	NM_003879	4.1
CASP8 and FADD-like apoptosis regulator	CFLAR	AF009616	6.0
immediate early response 3	IER3	NM_003897	4.8
interferon, alpha-inducible protein 6	IFI6	NM_022873	2.6
interleukin 1, alpha	IL1A	NM_000575	15.0
nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (p105)	NFKB1	NM_003998	2.6
prokineticin 2	PROK2	NM_021935	3.9
v-rel reticuloendotheliosis viral oncogene homolog A, nuclear factor of kappa light polypeptide gene enhancer in B-cells 3, p65 (avian)	RELA	BC014095	2.4
serpin peptidase inhibitor, clade B (ovalbumin), member 2	SERPINB2	NM_002575	88.3
serpin peptidase inhibitor, clade B (ovalbumin), member 9	SERPINB9	NM_004155	3.8
suppressor of cytokine signaling 3	SOCS3	NM_003955	3.9

sphingosine kinase 1	SPHK1	NM_021972	3.5
tumor necrosis factor (TNF superfamily, member 2)	TNF	NM_000594	4.9
tumor necrosis factor, alpha-induced protein 3	TNFAIP3	NM_006290	5.9
tumor necrosis factor receptor superfamily, member 10d, decoy with truncated death domain	TNFRSF10D	NM_003840	2.8
tumor necrosis factor receptor superfamily, member 18	TNFRSF18	NM_148901	2.2
Induction of apoptosis			
apoptosis-inducing factor, mitochondrion-associated, 2	AIFM2	NM_032797	2.7
BCL2-antagonist/killer 1	BAK1	NM_001188	2.1
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032977	3.6
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032974	2.2
CD70 molecule	CD70	NM_001252	2.1
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.7
interferon, beta 1, fibroblast	IFNB1	NM_002176	5.8
interleukin 19	IL19	NM_153758	5.1
Inhibin, beta A (activin A, activin AB alpha polypeptide)	INHBA	AK001903	29.2
inhibin, beta A	INHBA	NM_002192	22.7
myxovirus (influenza virus) resistance 1, interferon-inducible protein p78 (mouse)	MX1	NM_002462	12.3
Pleiomorphic adenoma gene-like 1	PLAGL1	NM_006718	2.3
phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1	NM_021127	9.2
promyelocytic leukemia	PML	NM_002675	3.5
promyelocytic leukemia	PML	NM_033238	5.0
promyelocytic leukemia	PML	NM_033244	6.2
promyelocytic leukemia	PML	NM_033247	3.9
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022173	2.4
TIA1 cytotoxic granule-associated RNA binding protein	TIA1	NM_022037	2.7
toll-like receptor 2	TLR2	NM_003264	3.1
tumor necrosis factor receptor superfamily, member 10a	TNFRSF10A	NM_003844	3.1
tumor necrosis factor receptor superfamily, member 9	TNFRSF9	NM_003811	10.3
tumor necrosis factor (ligand) superfamily, member 10	TNFSF10	NM_003810	19.1
XIAP associated factor - 1	XAF1	NM_017523	5.0
Regulation of apoptosis			
BCL2-antagonist/killer 1	BAK1	NM_001188	2.1
BCL2-like 14 (apoptosis facilitator)	BCL2L14	NM_030766	33.7
B-cell translocation gene 1, anti-proliferative	BTG1	NM_001731	3.5
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032977	3.6
caspase 10, apoptosis-related cysteine peptidase	CASP10	NM_032974	2.2
CASP8 and FADD-like apoptosis regulator	CFLAR	NM_003879	4.1
CASP8 and FADD-like apoptosis regulator	CFLAR	AF009616	6.0
Fas (TNF receptor superfamily, member 6)	FAS	NM_000043	3.7
interferon induced with helicase C domain 1	IFIH1	NM_022168	9.5
serine/threonine protein kinase MST4	MST4	NM_016542	4.7
nucleotide-binding oligomerization domain containing 2	NOD2	NM_022162	4.9
TNF receptor-associated factor 1	TRAF1	NM_005658	4.5

Chapter 3

Discussion and Perspectives

Discussion and Perspectives

Orientia tsutsugamushi is a cytosolic bacterium that is able to invade endothelial and myeloid cells. However, little is known about the interaction between this bacterium and monocytes/macrophages. We showed that *O. tsutsugamushi* replicates in monocytes/ macrophages and strongly induces innate immune response.

We have shown for the first time that *O. tsutsugamushi* invades monocytes and macrophages and replicates in the cytosol of these host cells. As *O. tsutsugamushi* is very unstable and quickly loses its viability in extracellular environment [18], the intracellular survival of *O. tsutsugamushi* in monocytes may be beneficial to bacterial dissemination from dermal infected sites to target cells in visceral organs. *O. tsutsugamushi* is also able to replicate in monocyte-derived macrophages (MDMs). This may explain the pathologic findings in patients that show the organisms in tissue macrophages present in liver, spleen and lymph nodes. It is likely that infected macrophages promote the inflammatory processes in these organs.

The prominent feature of the transcriptional response of *O. tsutsugamushi*-infected monocytes and MDMs is the up-regulation of genes from the GO category ‘response to virus’. These genes include IFN-β, some subtypes of IFN-α and several ISGs such as OAS, MX1-2, interferon-induced proteins, CXCL10 and CXCL11. The type I IFN response is specific to infection with live organisms that are able to escape into the cytosol. The type I IFN signature is also observed in host response to other cytosolic bacteria namely *L. monocytogenes* [19, 20], *F. tularensis* [21] and *R. conorii* [22]. Recent evidence indicates that double stranded DNA is recognized by cytosolic DNA sensors and triggers IFN-β expression in an IRF-3 dependent manner [23]. Then, IFN-β binds specific receptors (IFNAR) and further induces the expression of IFN-α and ISGs, thus amplifying the type I IFN response [24]. The positive feedback mechanism of type I IFNs can explain the sustained expression of type I IFNs and

ISGs observed in infected monocytes and MDMs. Type I IFNs can have protective or detrimental effects in host defense against bacterial infections [24]. Type I IFNs activate NK cells and cytotoxic T cells (CTLs) and promote their functional activities. In addition, type I IFNs sensitize host cells to cell death through the induction of ISGs, such as TRAIL, FAS, XIAP-associated factor-1 (XAF-1), caspase-8, protein kinase R (PKR), 2'- 5'oligoadenylate synthase (OAS), phospholipid scramblase and the promyelocytic leukemia gene product (PML) [25], all of which are up-regulated in monocytes and MDMs infected with *O. tsutsugamushi*. IFN-β was shown to inhibit the *in vitro* replication of *F. tularensis* in murine macrophages [26] and *R. conorii* in human endothelial cells [22]. Inhibition of IFN-β by neutralizing antibodies results in increased bacterial replication, whereas addition of exogenous IFN-β inhibits bacterial replication [22]. On the other hand, type I IFNs can be detrimental to the host defense. In *L. monocytogenes* infection, type I IFNs play a critical role in macrophage cell death [27] and sensitize T lymphocytes to apoptosis induced by listeriolysin O [28]. Consequently, $IRF3^{-/-}$ and $IFNAR1^{-/-}$ mice are more resistant to *L. monocytogenes* infection than wild type mice and exhibit decreased apoptosis in spleen [29]. Recently, our group has shown that the type I IFN response is detrimental to *Tropheryma whipplei* infection in murine macrophages. Macrophage apoptosis and bacterial replication are decreased in $IFNAR^{-/-}$ macrophages compared with wild type controls [30]. A previous study showed that type I IFNs inhibit *O. tsutsugamushi* replication in mouse cell lines, depending on the bacterial strain and the genetic background of mice ; however, this inhibitory effect is subtle and variable [31]. Therefore, further studies are required to determine the precise role of type I IFNs in *O. tsutsugamushi* infection.

Another feature of transcriptional profiles is that *O. tsutsugamushi*-infected monocytes and MDMs are polarized towards an M1 phenotype. The classically activated M1 phenotype is described in macrophages stimulated by IFN-γ, TNF and/or microbial products such as LPS

and is associated with microbicidal competence and Th1 response. Several M1 genes such as TNF, IL-1, IL-6, IL-12p40, IL-15, CXCL10, CXCL11 and INDO are up-regulated following *O. tsutsugamushi* infection. We showed that the transcriptional profile of *O. tsutsugamushi*-infected monocytes is clearly different from the profile of IFN-γ stimulated monocytes. The number of modulated genes is largely different and the expression of M1 genes by *O. tsutsugamushi*-infected monocytes is more persistent compared to IFN-γ-stimulated monocytes. The increased expression of cytokines and chemokines can be related to the increased levels of pro-inflammatory cytokines in patients with scrub typhus [32-35]. Although the up-regulation of cytokine and chemokine genes is not restricted to infection by live bacteria, the inflammatory response is induced at higher levels by live bacteria compared with heat-killed bacteria, suggesting that the inflammatory response to *O. tsutsugamushi* is largely dependent on the cytosolic location of bacteria. This finding is comparable to the previous study of *Rickettsia akari*-infected monocytes that suggests the importance of cytosolic pattern recognition receptors (PRRs) in rickettsial infections [36]. Regarding the chemokine expression, the up-regulated chemokines CCL3, CCL4, CCL5 and CCL8 (CCR5 ligands) and CXCL9, CXCL10 and CXCL11 (CXCR3 ligands) are chemoattractants for Th1 and cytotoxic lymphocytes [37, 38] and thus may contribute to the perivascular lymphocyte infiltration in visceral organs. In addition, CXCL9-CXCL11 are also ISGs [39] and their expression are dependent on live *O. tsutsugamushi* organisms. The strong up-regulation of inflammatory cytokines by innate immune cells can explain the inflammatory processes in patients that lead to the clinical manifestations and complications of scrub typhus.

We also showed that *O. tsutsugamushi* induces IL-1β secretion by both monocytes and MDMs. Similar to the type I IFN response, the IL-1β release is only induced by live bacteria. On the other hand, heat-killed bacteria can up-regulate IL-1β mRNA expression but fail to induce the release of active IL-1β. Infected monocytes secrete much higher levels of IL-1β

than do macrophages. The IL-1β secretion is the suggestive evidence of inflammasome and caspase-1 activation, which is another important feature of innate immune system activation. A previous study demonstrated that caspase-1 activation and IL-1β secretion follow *O. tsutsugamushi* infection in an endothelial cell line [40]. IL-1β may be one of the key cytokines involved in the pathogenesis of scrub typhus. Inflammasome is a macromolecular complex that can be activated by cytosolic PRRs including some types of NLRs (NOD-like receptors) and AIM2 (Absent in melanoma 2). Once activated, inflammasome triggers caspase-1 activation, which then mediates the cleavage of pro-IL-1β and IL-18, leading to the release of active cytokines. Caspase-1 also trigger the process of cell death [41]. Inflammasome activation is now recognized as a fundamental feature of innate immune system activation.

We found that live *O. tsutsugamushi* organisms induce cell death in a minority of monocytes and MDMs. *O. tsutsugamushi* also induces apoptosis in heavily infected human endothelial cell line ECV304 [42, 43] and L929 cells [44]. However, one previous study showed that *O. tsutsugamushi* prevents the beauvericin-induced apoptosis of THP-1 macrophages by delaying the release of intracellular calcium from the endoplasmic reticulum. Heat-stable molecules of *O. tsutsugamushi* are likely responsible for the inhibition of apoptosis because heat-killed bacteria have greater anti-apoptotic activities compared with live bacteria [45]. The mechanisms of cell death are complex and multiple. Cell death by apoptosis can be induced by type I IFNs. Several ISGs such as TRAIL/Apo2L, XAF-1, and caspase-8 genes, which are implicated in apoptosis process [25], are up-regulated following *O. tsutsugamushi* infection. *O. tsutsugamushi* probably triggers cell death by pyroptosis, the caspase-1-dependent cell death accompanied by IL-1β release and inflammatory response. Pyroptosis is observed in a variety of bacterial infections including *Salmonella*, *Shigella*, *Francisella* and *Listeria* [46]. Cell death helps to restrict the growth of intracellular pathogens. It is likely that the cell death following live *O. tsutsugamushi* infection is another

aspect of host defense mechanisms against cytosolic bacteria, although *O. tsutsugamushi* may use some mechanisms to partially inhibit cell death. We can hypothesize that the effect of *O. tsutsugamushi* infection on cell death depends on the time course of the infection: *O. tsutsugamushi* may exhibit an anti-apoptosis activity at the early stage of infection but may induce cell death at later stage or in heavily-infected cells.

We studied the transcriptional profiles of peripheral blood mononuclear cells of patients with scrub typhus. The expression profiles of some genes in scrub typhus patients are quite specific and distinct from those in patients with murine typhus, malaria and dengue fever. This specific signature may be helpful to the diagnosis of scrub typhus. IFN-γ (type II IFN), the key cytokine in Th1 response, is up-regulated in patients with scrub typhus. In addition, some M1-related genes and ISGs are up-regulated in patients with scrub typhus. These data can be related to data obtained with *O. tsutsugamushi*-infected monocytes and emphasize the important roles of type I and type II IFNs and cell-mediated immunity in response to *O. tsutsugamushi* infection.

Finally, we are studying the response of dendritic cells to *O. tsutsugamushi* infection. *O. tsutsugamushi* replicates in human monocyte-derived dendritic cells. The expression of CD80, CD86, CD83, HLA-DR and HLA-ABC, which are surface markers of mature DCs, is largely increased in dendritic cells infected with *O. tsutsugamushi*, demonstrating that *O. tsutsugamushi* induces the maturation of dendritic cells, in sharp contrast with other bacterial pathogens such as *T. whipplei*, the agent of Whipple's disease. The maturation of *O. tsutsugamushi*-infected dendritic cells is also attested by reduced endocytosis activity, powerful allogenic T cell stimulation and inflammatory response, including NF-κB and MAPK-p38 activation and up-regulated expression of inflammatory cytokines. Microarray results showed that the genes in GO categories of response to virus and inflammatory response are modulated following *O. tsutsugamushi* infection and are thus comparable to

transcriptional profiles found in monocytes and MDMs. Taken together, these preliminary results suggest that dendritic cells play an important role in the inflammatory processes that characterize *O. tsutsugamushi* infection.

Many questions about the pathogenesis of scrub typhus are still open for further investigations. The determination of the nature of pattern recognition receptors (PRRs) that recognize *O. tsutsugamushi* needs new studies. Cytosolic DNA sensors such as DLM-1(ZBP1) [47] and RNA polymerase III /RIG-I pathway [48, 49] are likely candidate receptors responsible for type I IFN response. Another DNA sensor, AIM2 inflammasome, is likely responsible for IL-1β secretion and cell death, and is crucial for innate immunity to other cytosolic bacteria such as *F. tularensis* and *L. monocytogenes* [50, 51]. AIM2 is also an IFN-inducible protein [52] that is up-regulated in live *O. tsutsugamushi*-infected monocytes and MDMs and, importantly, in patients with scrub typhus. As a consequence, the study of the role of AIM2 in *O. tsutsugamushi* infection needs further investigations. Other PRRs including Toll-like receptors or NOD-like receptors may be involved in the recognition of *O. tsutsugamushi* by host cells. The bacterial ligands that activate the innate immune response also remain to be determined. *O. tsutsugamushi* DNA and cell wall proteins are likely ligands for immune cells. Bacteriostatic antibiotics that inhibit bacterial synthesis (e.g. doxycycline and chloramphenicol) rapidly improve the clinical symptoms of patients with scrub typhus [2, 4]. This observation suggests that bacterial proteins are critically involved in inflammatory processes induced by *O. tsutsugamushi*. Bacterial proteins involved in the induction and/or modulation of host response are yet to be determined. Type IV secretion system (T4SS) may play critical roles in bacterial survival in host cells. Indeed, T4SS is expressed by *O. tsutsugamushi* cultured in L929 cells [53]. The mechanisms of *O. tsutsugamushi* escape into the cytosol remain unclear. The mechanisms leading to the cell death induced by *O. tsutsugamushi* also merit further investigation because they may involve apoptosis, pyroptosis

and/or autophagy. The identification of *O. tsutsugamushi* epitopes recognized by $CD8^+$ T cells may be useful to understand how patients with scrub typhus can be re-infected by different strains of *O. tsutsugamushi*. Future research will possibly be applied for the development of vaccines and may ameliorate the treatment of serious complications of scrub typhus.

Chapter 4

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Chapter 5

Annexe

Intrinsic Fluoroquinolone resistance in *Orientia tsutsugamushi*

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Fluoroquinolones are broad spectrum antibiotics which inhibit bacterial gyrase (topoisomerase II) and are generally effective against intracellular bacteria. However, available full genome sequences of *O. tsutsugamushi* displayed the intrinsic Ser83Leu mutation in *gyr*A gene, which is associated with fluoroquinolone resistance in other gram negative bacteria. Therefore, we determined the *in vitro* susceptibility of *O. tsutsugamushi* strain Kato to ciprofloxacin and ofloxacin and sequenced the quinolone determining resistance domain in *gyr*A of *O. tsutsugamushi* isolated from patients. We found that *O. tsutsugamushi* was resistant to ciprofloxacin and ofloxacin with minimal inhibitory concentration (MIC) of 8 μg/ml. All sequences of the isolates exhibited the Ser83Leu mutation. These results re-emphasize the usefulness of *in silico* analysis for the prediction of antibiotic resistance and discourage the use of fluoroquinolones for the empirical treatment of undifferentiated febrile illness in the endemic areas of scrub typhus.

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ABSTRACT

Scrub typhus is a public health concern for a population of over a billion humans, with an estimated incidence of one million cases/year in endemic areas. Although doxycycline remains the standard therapy, fluoroquinolones have been used successfully in a few patients. However, there is also clinical evidence that fluoroquinolones are ineffective in the treatment of scrub typhus. To clarify this matter, we determined the in vitro susceptibility of *Orientia tsutsugamushi* strain Kato to ciprofloxacin and ofloxacin and sequenced the quinolone resistance-determining region (QRDR) of the *gyrA* gene, the target of fluoroquinolones, of 18 fresh isolates from the Lao PDR. *Orientia tsutsugamushi* strain Kato was resistant to ciprofloxacin and ofloxacin in vitro (minimum inhibitory concentration = 8 μg/mL). All sequences obtained, including those from the two available genomes of *O. tsutsugamushi* (strains Boryong and Ikeda), had a Ser83Leu mutation in their QRDR domain that is known to be associated with fluoroquinolone resistance. These findings re-emphasise the usefulness of in silico analysis for the prediction of antibiotic resistance and suggest that fluoroquinolones should not be used in the treatment of scrub typhus.

1. Introduction

Orientia tsutsugamushi, the agent of scrub typhus, is a small Gram-negative obligate intracellular bacterium of the alpha subgroup of Proteobacteria, transmitted to humans by the bite of trombiculid mites. The disease is a public health concern for a population of over a billion humans in a geographical triangle extending from northern Japan and far eastern Russia in the north, to northern Australia in the south and Pakistan in the west [1–3]. The estimated incidence of the disease in endemic areas is one million cases/year. Although doxycycline and chloramphenicol remain the gold standard therapy for scrub typhus, ciprofloxacin has been shown to be effective in a mouse model [4] and in some anecdotal clinical cases [5–7]. However, recent reports of clinical failure and deaths as well as abortions in pregnant women with fluoroquinolone therapy suggest drug resistance in these cases [8–10]. Fluoroquinolone activity is due to inhibition of bacterial DNA gyrase (topoisomerase II) and topoisomerase IV. It has been demonstrated that resistance to quinolones in intracellular bacteria was mainly due to point mutations in the quinolone resistance-determining region (QRDR) of DNA gyrase (*gyrA*) [11,12]. Surprisingly, there are only a few reports regarding in vitro and/or in vivo antibiotic susceptibility in cell culture or animal models of *O. tsutsugamushi*, especially for fluoroquinolones. In the in vitro work of Kelly et al. [13], although they determined precisely the doxycycline minimum inhibitory concentration (MIC) against *O. tsutsugamushi* Karp strain, they tested only one high concentration of ciprofloxacin (4 μg/mL) that was found to be effective in vitro. Similarly, McClain et al. [4] found that ciprofloxacin was effective in preventing death in a mouse model of scrub typhus, but the appropriateness of such a mouse model to human disease is uncertain and the dose (mg/kg body weight) of ciprofloxacin administered to mice was approximately six times the adult human dosage. With a lack of definitive data regarding the susceptibility of *O. tsutsugamushi* to fluoroquinolones, the objectives of this study were: (i) to determine the MIC of ciprofloxacin and ofloxacin against *O. tsutsugamushi* strain Kato using a modified real-time quantitative polymerase chain reaction (qPCR) assay previously used for *Rickettsia* spp. and *Coxiella burnetii* [14,15]; (ii) to amplify and sequence the *gyrA* gene of recent human *O. tsutsugamushi* isolates from Lao PDR (Laos); and (iii) to compare these sequences with those retrieved from available full sequenced genomes of strains Boryong [16] and Ikeda [17] in order to decipher the possible molecular mechanism of resistance to fluoroquinolones.

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2. Materials and methods

2.1. Patients and rickettsial culture

Patients were recruited to a study of the causes of fever [1]. Buffy coats, prepared from 5 mL of whole ethylene diamine tetra-acetic acid (EDTA)-anticoagulated venous blood from patients with suspected scrub typhus and serum immunoglobulin M (IgM) against *O. tsutsugamushi* detected by the scrub typhus immunochromatographic test (PanBio, Sinnamon Park, QLD, Australia), were cultured in Vero cells and L929 cells at 35 °C in 10% fetal calf serum/RPMI in a 5% CO_2 incubator [18]. DNA was extracted using High Pure PCR Template Preparation Kit (Roche Diagnostics, Meylan, France) and sent to URMITE (Marseille, France).

2.2. Preparation of Orientia tsutsugamushi inocula

Orientia tsutsugamushi strain Kato (CSUR R163) was propagated in monolayers of L929 cells grown in minimum essential media (MEM) supplemented with 5% fetal bovine serum (FBS) and 2 mM L-glutamine (Invitrogen, Cergy-Pontoise, France). When almost 100% of the floating cells were infected, as determined by Giemsa (Merck, Darmstadt, Germany) staining, cells were harvested, disrupted by glass beads (diameter 3 mm) and centrifuged at 500 × *g* for 5 min at 4 °C. The supernatant containing *O. tsutsugamushi* was centrifuged again at 2000 × *g* for 10 min at 4 °C to collect the bacterial pellet. *Orientia tsutsugamushi* was cryopreserved in MEM containing 20% FBS and 5% dimethyl sulphoxide (DMSO) and stored at −80 °C until use. The infectivity titre of inocula was determined as described previously [19] with slight modification. Briefly, the inoculum was five-fold serially diluted and inoculated onto L929 cells grown in a 24-well plate. After 2 h of inoculation, the inoculum was removed and replaced by new media containing 0.4 μg/mL daunorubicin (BIOMOL, Lausen, Switzerland), which partially inhibits the growth of host cells [20]. After 2 days of incubation, cells were collected and stained by indirect immunofluorescence assay using pooled human sera from Thai patients [2] with scrub typhus at a dilution of 1/400. Fluorescein isothiocyanate (FITC)-conjugated goat anti-human IgG (FluolineG; bioMérieux, Marcy l'Etoile, France) diluted at 1/200 was used as the secondary antibody. The infected cell counting units (ICU) of *O. tsutsugamushi* were calculated from the dilution in the well in which 5–25% of cells were infected, using the formula ICU = total number of cells × percentage of infected cells/100 × dilution factor.

2.3. Antibiotic susceptibility testing

Growth of intracellular *O. tsutsugamushi* in cell culture with different concentrations of antibiotics was determined using real-time PCR with a TaqMan® probe as previously described for *Rickettsia* [15]. Briefly, 3×10^5 ICU of *O. tsutsugamushi* was inoculated onto monolayers of 1.5×10^5 L929 cells grown in 24-well plates. After incubation for 2 h, the inoculum was removed and infected cells were washed with phosphate-buffered saline. Infected cells were grown in media without antibiotic or with two-fold serial dilutions of antibiotics and were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cell monolayers were harvested by trypsinisation and the cell suspension was centrifuged to collect cell pellets. Samples were collected every 24 h throughout the 5-day experiments. Experiments were repeated twice and each experiment was performed in duplicate. Cell pellets containing intracellular *O. tsutsugamushi* were processed for DNA extraction using QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The DNA copy number of *O. tsutsugamushi* was quantified using a real-time PCR targeting the *rpoB* gene. The sequence of the primers and the TaqMan probe were as follows: rpoB F (3235–3257), 5′-AAG CAT AGG TTA CAG CCT GGW GA-3′; rpoB R (3346–3373), 5′-ACC CCA ACG GAT TTA ATA CTA TAT CWA C-3′; and rpoB probe R (3307–3338), 5′-FAM-CCA TCT TCA AGA AAT GGC ATA TCT TCC TCA GG-TAMRA-3′. The real-time PCR mixture contained 1× QuantiTect Probe PCR Master Mix (Qiagen), 0.5 μM forward and reverse primers, 0.2 μM probe, sterile distilled water and 5 μL of DNA. Real-time PCR was performed in a Smart Cycler Instrument (Cepheid, Paris, France) with the following conditions: initial denaturation at 95 °C for 15 min, followed by 40 cycles of denaturation at 94 °C for 5 s and combined annealing and extension at 60 °C for 60 min with the acquisition of fluorescence. The number of DNA copies was calculated using the standard curve technique.

2.4. Determination of the quinolone resistance-determining region of gyrA and protein sequences alignment

Eighteen strains of *O. tsutsugamushi* cultured from the blood of Lao patients with scrub typhus [1] were used for determination of the QRDR sequence of *gyrA*. DNA was extracted from the blood sample (buffy coat layer) using a QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's instructions. Primers used for partial *gyrA* amplification and sequencing were as follows: TsgyrAF, 5′-TATGCTATGAGCGTAATAGT-3′; and TsgyrAR, 5′-TGCCATTCCTACTGCAATTC-3′. Finally, the partial *gyrA* sequences of the QRDR of *O. tsutsugamushi* were aligned using the CLUSTAL W program (http://www.ebi.ac.uk/clustalw/) to look at possible

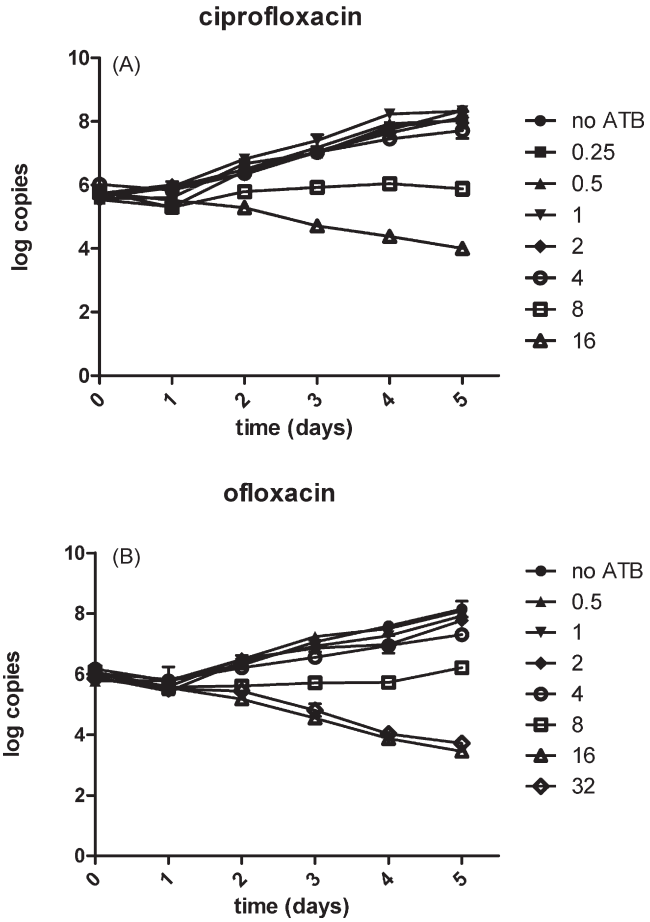

Fig. 1. Growth curves of *Orientia tsutsugamushi* in L929 cells with different concentrations of (A) ciprofloxacin and (B) ofloxacin. The number of bacteria was expressed as log DNA copies. Experiments were repeated twice and each experiment was performed in duplicate. Data in the figure represent mean ± standard deviation.

mutations known to be associated with fluoroquinolone resistance. The *gyrA* protein sequences of *O. tsutsugamushi* strain Boryong (OTBS_0252) and Ikeda (OTT_1379) and *Escherichia coli* K12 (b2231) retrieved at the KEGG website (http://www.genome.jp/) were used for comparison of positions within the gene.

3. Results and discussion

In the real-time qPCR assay, the MIC determined in duplicate against ciprofloxacin and ofloxacin for *O. tsutsugamushi* strain Kato was 8 μg/mL (Fig. 1). Experiments were carried out twice to confirm the results. Compared with *E. coli*, the QRDR region of *gyrA* of the 18 Lao *O. tsutsugamushi* isolates as well as those retrieved from the KEGG website (strains Boryong and Ikeda) displayed an intrinsic Ser83Leu mutation (Fig. 2). Moreover, 19 of the 21 sequences had a Thr88Ser mutation, 1 isolate had a Thr88Ala mutation and all 21 protein sequences had an Ile89Leu mutation. Thus, looking at in vitro and in silico results, it was found that *O. tsutsugamushi* strain Kato was intrinsically resistant to ciprofloxacin and that all available sequences of the *O. tsutsugamushi* QRDR domain of *gyrA* had an intrinsic mutation at position 83 known to be associated with fluoroquinolone resistance, as has been established in other intracellular bacteria including *Ehrlichia* spp., *Bartonella* spp. and *Tropheryma whipplei* [11,12]. Therefore, these data provide evidence that *O. tsutsugamushi* is naturally resistant to fluoroquinolones, explaining clinical failures reported using such antibiotics in the treatment of scrub typhus. Indeed, there are several reports demonstrating that ciprofloxacin is not effective in the treatment of scrub typhus (Table 1). In an outbreak in Southern India, among 28 patients serologically confirmed to have

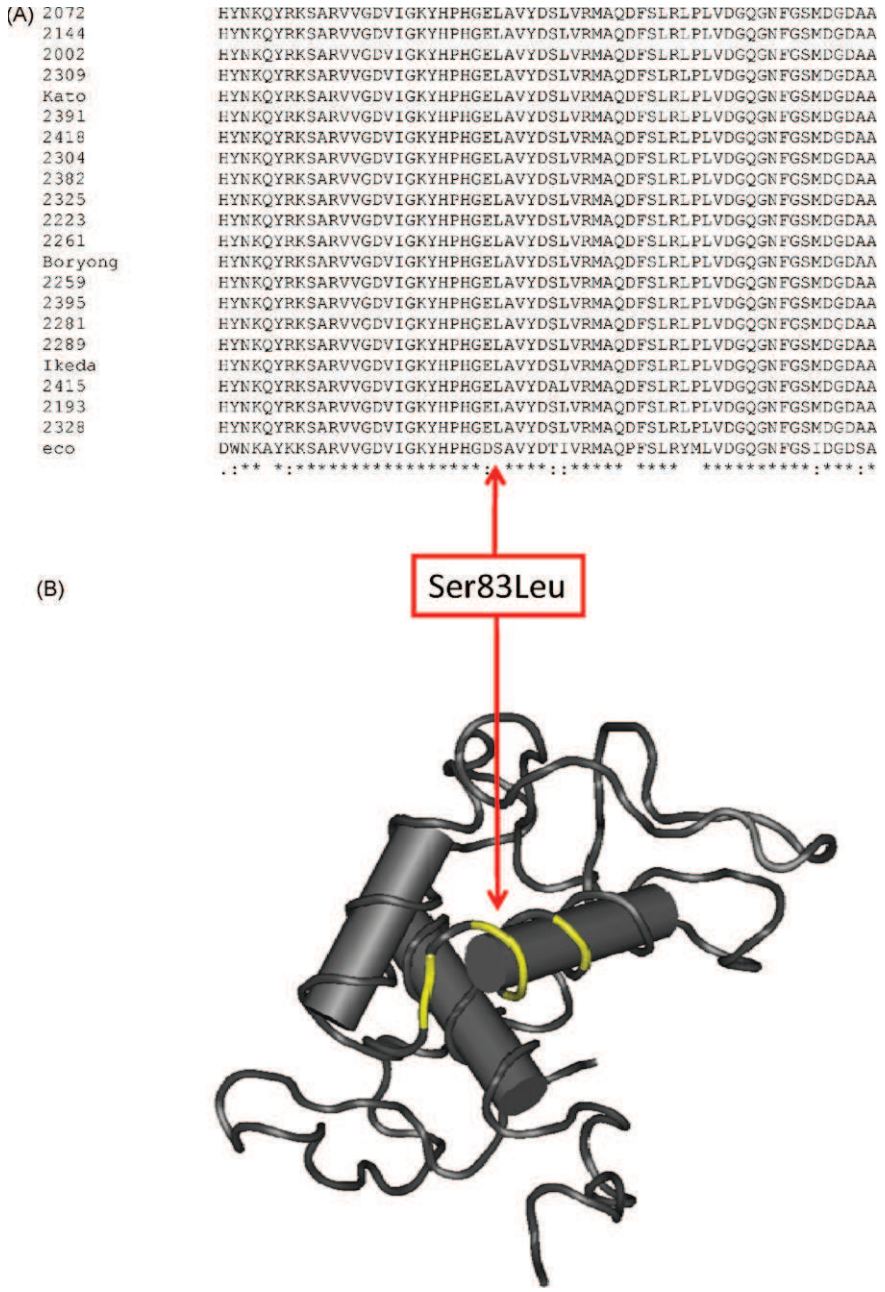

Fig. 2. (A) DNA sequence alignment of the quinolone resistance-determining region (QRDR) domain of the *gyrA* genes of *Orientia tsutsugamushi* strains from Laos and from genomes of strains Boryong and Ikeda showing the Ser83Leu mutation (*Escherichia coli* numbering); and (B) three-dimensional representation of DNA gyrase showing position 83 in the QRDR.

Table 1
Epidemiological and clinical data on fluoroquinolone therapy for scrub typhus.

Patient [age (years)]	Country	Antibiotic	Outcome	Reference
Female (32)	Thailand	Cefotaxime + ofloxacin	Cured	[5]
Male (34)	Sri Lanka	Doxycycline + ciprofloxacin	Cured	[7]
Male (20)	Korea	Pefloxacin + cefazolin	Clinical deterioration	[20]
Female (17)[a]	India	Ciprofloxacin	No improvement; stillbirth	[8]
Female (20)[a]	India	Ciprofloxacin	No improvement; stillbirth	[8]
Female (24)[a]	India	Ciprofloxacin + ampicillin	No improvement; low-birth-weight baby	[8]
Female (21)[a]	India	Ciprofloxacin	No improvement; abortion	[8]
Male and female patients ($n = 5$)	India	Ciprofloxacin	Longer time to defervescence	[9]
Male and female patients ($n = 71$)	Taiwan	Levofloxacin	Longer time to defervescence; 4 deaths	[10]

[a] Pregnant women.

scrub typhus, 17 patients treated with doxycycline and 2 patients treated with chloramphenicol recovered in 1–3 days [9]. However, in five patients who received ciprofloxacin, fever subsided only after 5 days. Three patients (10.7%) died, including one patient treated with doxycycline and two with fluoroquinolones, indicating a possibility of drug resistance [9]. Similarly, we have reported five pregnant Indian patients with scrub typhus, four of whom were treated initially with ciprofloxacin [8]. Three women had stillbirths, one an abortion and one a low-birth-weight baby [8]. Scrub typhus has also been transmitted by needle stick from a patient who received pefloxacin [21]. Finally, Tsai et al. [10] have shown recently in a retrospective case series of 132 patients with scrub typhus that although treatment with levofloxacin was effective in 71 patients, the patients had a significantly longer time to defervescence compared with 61 patients treated with tetracyclines. Moreover, for patients with severe scrub typhus, higher mortality was observed in the levofloxacin-treated group [10]. There are three reports of apparent successful scrub typhus therapy with fluoroquinolones [5,6,22], although the evidence for scrub typhus as the aetiology of the disease is uncertain for one patient [5]. Our in vitro and in silico findings re-emphasise the usefulness of such analysis for prediction of antibiotic resistance in intracellular bacteria. These results suggest that fluoroquinolones should not be used for the treatment of scrub typhus. Fluoroquinolones remain efficacious for the therapy of uncomplicated typhoid fever in much of Asia. Therefore, with the difficulty in clinically distinguishing typhus from typhoid, innate resistance in *O. tsutsugamushi* to fluoroquinolones has important practical implications for the empirical treatment of undifferentiated fever in Southeast Asia, suggesting that fluoroquinolones are unlikely to have sufficiently broad pathogen coverage.

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Summary

Orientia tsutsugamushi, the causative pathogen of scrub typhus, is a cytosolic bacterium that invades endothelium and monocytes/macrophages. So far, the knowledge of immune response to *O. tsutsugamushi* is still limited. The objective of this thesis is to better understand the response of human innate immune cells against this pathogen. We demonstrated that *O. tsutsugamushi* was able to replicate in human monocytes. Using whole genome microarrays, we showed that live *O. tsutsugamushi* induced robust changes in the transcriptional profiles of monocytes. First, type I interferons and interferon-stimulated genes were remarkably up-regulated. Second, infected monocytes expressed several inflammatory cytokine and chemokine genes, and were polarized toward the classically-activated M1 phenotype. Third, live bacteria induced interleukin-1β secretion and likely inflammasome and caspase-1 activation. We also showed that *O. tsutsugamushi* altered the expression of apoptosis-related genes and induced cell death in monocytes. We extended our work to the study of the transcriptional profiles of patients with scrub typhus and found a specific signature in patients that included the modulation of M1-associated genes and interferon-stimulated genes. We finally studied the response of human monocyte-derived macrophages to *O. tsutsugamushi*. The transcriptional and functional responses of macrophages to *O. tsutsugamushi* were roughly similar to those observed in circulating monocytes including type I IFN response, pro-inflammatory cytokine gene expression and IL-1β secretion. Taken together, these data improve our understanding in the pathogenesis of scrub typhus in which highly inflammatory processes are involved.

Keywords : *Orientia tsutsugamushi*, scrub typhus, transcriptional profile, monocytes

Résumé

Orientia tsutsugamushi, l'agent pathogène responsable du typhus des broussailles, est une bactérie cytosolique qui envahit l'endothélium et les monocytes/macrophages. La réponse immune à l'infection par *O. tsutsugamushi* reste à ce jour mal connue. L'objectif de cette thèse est de mieux comprendre la réponse des cellules de la réponse immune innée humaine à *O. tsutsugamushi*. Nous avons montré que *O. tsutsugamushi* se réplique dans les monocytes humains. En utilisant un microarray portant sur la totalité du génome, nous avons également montré que les bactéries vivantes induisent de profondes modifications du profil transcriptionnel des monocytes. C'est ainsi que l'expression des gènes codant l'interféron de type I et des gènes stimulés par l'interféron est fortement augmentée. Les monocytes infectés expriment plusieurs gènes codant des cytokines et des chimiokines inflammatoires, ce qui montre qu'ils sont polarisés vers un phénotype M1 (*classically-activated phenotype*). Les bactéries vivantes induisent également la sécrétion de l'interleukine-1β et probablement l'activation des inflammasomes et de la caspase-1. *O. tsutsugamushi* affecte enfin l'expression des gènes associés à l'apoptose et induit la mort d'une partie des monocytes infectés. Nous avons en outre étudié le profil transcriptionnel de patients atteints d'un typhus des broussailles et avons trouvé une signature spécifique incluant la modulation de gènes de type M1 et de gènes stimulés par l'interféron. Nous avons finalement étudié la réponse des macrophages humains dérivés des monocytes à *O. tsutsugamushi*. Les réponses transcriptionnelles et fonctionnelles des macrophages sont globalement similaires à celles observées dans les monocytes circulants soumis à *O. tsutsugamushi* : elles incluent une réponse IFN de type I, l'expression de gènes codant des cytokines inflammatoires et la sécrétion d'interleukine-1β. Ces résultats accroissent notre compréhension de la pathogénèse du typhus des broussailles et révèlent des processus hautement inflammatoires.

Mots-clés : *Orientia tsutsugamushi*, scrub typhus, profil transcriptionnel, monocytes