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THESE DE DOCTORAT

A TRANSCRIPTIONAL APPROACH TO DEFINE NEW BIOMARKERS: APPLICATION TO Q FEVER

Présentée par

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To the best person I have ever known

Kirshan Mehraj

May 18, 1949-April 21, 1999

He is my late father who was a wonderful human being: loving, kind, caring, clever, strong and supportive. He will always be in my heart.

I miss you everyday and every moment Baba.

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INTRODUCTION

Chapter I

Introduction

Cells of the myeloid lineage; such as monocytes, macrophages, dendritic cells and neutrophils, play an important role as first line defense against pathogens, inflammation-related diseases and also provide a critical contribution to tissue homeostasis and repair [1, 2]. In peculiar, the macrophages are highly heterogeneous, versatile and plastic cells. It has been proposed in 2000's years that murine macrophages acquire distinct functional phenotypes in response to environmental signals; such as bacterial lipopolysaccharide (LPS), microbial stimuli and cytokines [1, 3, 4]. They may undergo classical (known as M1) activation under stimulation by Toll-like receptor (TLR) ligands and/or interferon (IFN)-γ) or alternative (known as M2 activation) under stimulation by interleukin (IL)-4/IL-13 or glucocorticoids [1, 4]. M1 and M2 macrophages differ in terms of receptors, cytokine and chemokine expression, and effector functions. The M1 macrophages express receptors such as TLR2, TLR4, Fc receptors [1, 5], release inflammatory cytokines including TNF [1], produce reactive nitrogen and oxygen intermediates, and are highly microbicidal and tumoricidal [1, 4]. In contrast, the M2 macrophages express receptors such as mannose receptors, release immunoregulator cytokines such as IL-10, and are considered to be involved in parasite containment, promotion of tissue remodeling, tumor progression through their immunoregulatory functions [6, 7]. Unfortunately, the description of macrophage polarization with few canonical molecules does not account for the complexity of macrophage activation. In addition, whether the concept of M1/M2 polarization may be used to define the functional properties of human macrophages has only in part studied [4]. Hence, the validity of a concept defined in mice (M1/M2) is not demonstrated in humans.

An important question raised by the concept of the M1/M2 polarization of macrophages is the following: is this concept applicable to clinical situations? We reviewed the recent literature to reconsider macrophage polarization in human bacterial infections in light of the new high-throughput methods **(Chapter II).** We found that in vivo and in vitro data are often contradictory. For example, it has been demonstrated that *Mycobacterium tuberculosis* induces an M2 response in isolated macrophages but that the macrophages from patients with active tuberculosis infection are of M1 type. In addition, only monocytes, not tissue macrophages, are easily accessible in clinical investigations, and it is not known if the concept of macrophage polarization is applicable to monocytes. These reasons led us to examine whether the concept of macrophage polarization is applicable to circulating monocytes. For that purpose, we screened the transcripts by a global approach through whole genome microarrays and confirmed the expression of selected markers through quantitative real-time RT-PCR (qRT-PCR). When monocytes were stimulated for 6 hours, they exhibited early responses to IFN-γ and IL-4 that were not reducible to M1/M2 polarization. After 18 hours, the monocyte response was no longer polarized, and a late response signature was identified. We also tempted to use this new kinetic model to evaluate patients with acute Q fever or Q fever endocarditis. By selecting early- and late-related genes from each monocyte signature, we showed that specific biomarkers could be used to distinguish acute Q fever and Q fever endocarditis. These results suggest that a kinetic model of monocyte activation is relevant in the study of patients with infectious diseases **(Chapter III).**

Q fever is a disease caused by *Coxiella burnetii*, an obligate intracellular bacterium. Acute Q fever is spontaneously resolutive and is characterized by an efficient immune response. In contrast, chronic Q fever is characterized by dysregulated immune response, as demonstrated by the failure of *C. burnetii* to induce lymphoproliferation and the lack of granulomas [8-11]. The clinical expression of Q fever is affected by various host factors,

including gender and age. The male-to-female ratio of patients admitted to the hospital is about 2.5 in adults. In parallel, the rate of Q fever-related complications is higher in males than in females. Men represent 75% of the patients diagnosed with Q fever endocarditis, the major manifestation of chronic Q fever [8, 10]. On the other hand, it has been shown in mice that sex hormones play a role in the severity of *C. burnetii* infection [12] and that genes involved in the circadian rhythm are modulated in female mice, not in males [13]. Circadian fluctuations have also been reported in Haematopoietic stem cells (HSCs) in mice which were associated with the expression of the chemokine CXCL12 in the bone marrow microenvironment [14].

We hypothesized that circadian genes are differently modulated in men and women with Q fever. We showed that the transcriptional expression *Per2* gene was up-regulated in men with acute Q fever, not in women. In contrast to the expression of the *Per2* gene, the expression of the *Clock* and *Arntl* genes was not affected in men or women with Q fever compared with healthy volunteers. We conclude that circadian rhythm and immune response are associated in a gender-dependent manner in patients with Q fever **(Chapter IV).**

It has been recently demonstrated that the ligands of Numb proteins X1 and X2 (LNX1 and LNX2), when they are co-expressed in heterologous mammalian cell lines, regulate the level of the T-cell co-receptor CD8, which plays an essential role in T-cell-mediated immune response [15]. The importance of T $CD8^+$ cell response in *C. burnetii* infection has been recently reported [16]. Nude and SCID mice are highly sensitive to *C. burnetii* infection, whereas wild-type mice are resistant. We investigated the expression of LNX genes in blood from human patients with Q fever. We found a high level of LNX1 and LNX2 mRNAs in Q fever endocarditis, but not in acute Q fever. Our data suggest that LNXs may be used as complementary biomarkers for the prognosis of chronic Q fever **(Chapter V).**

Hepatitis E infection is generally an acute disease but it has been recently described as chronic in patients with kidney graft [17]. We found that chronic hepatitis E infection is specifically associated with an interferon-related transcriptional program **(Chapter VI).**

Chapter II

Macrophage polarization and bacterial infections

(Review)

Mège JL, Mehraj V, Capo C.

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Chapter II

Macrophage polarization and bacterial infections

Macrophages are antigen-presenting cells that also play important roles as sentinels for first line alerts or as mediators that guide the adaptive immune response [1, 18]. They are heterogeneous cells owing to different mechanisms governing their differentiation, tissue distribution, and responsiveness to stimuli [1, 3, 19]. Mirroring Th1/Th2 activation, murine macrophages are functionally polarized into two broad groups: Classical or M1 and alternate or M2 macrophages. Early studies described M1 as responsive to type 1 inflammatory cytokines and microbial products such as LPS [20]. Their inflammatory repertoire is characterized by the secretion of proinflammatory mediators and the release of reactive oxygen and nitrogen intermediates (ROI and RNI, respectively). In contrast, alternative activation of macrophages (M2) covers a continuum of functional states classified as M2a, induced by IL-4/IL-13, M2b, induced by immune complexes and some TLR agonists, and M2c, induced by IL-10 and glucocorticoid hormones [1, 21] (Figure 1). Recently, M2 macrophages have been characterized by the expression of alternative activation markers [3, 4, 6, 7]. M1 and M2 macrophages are distinguished by their receptors, cytokine and chemokine expression, and effector functions (Figure 1). The M1 macrophages are highly microbicidal and inflammatory, while M2 macrophages are immunomodulators (M2a and M2c) and are considered poorly microbicidal. Thus, macrophage activation can be either pro-inflammatory or anti-inflammatory. However, these extreme and simplified polarization states (M1 vs. M2) actually describe a complex process delineating a continuum of functional states. It has been reported recently that macrophage activation is plastic, rapid, and fully reversible, suggesting that macrophage populations are dynamic and may first take part in inflammation

and then participate in its resolution [22]. Consequently, macrophages display progressive functional changes resulting from changes in the microenvironment [23]. The model of M1/M2 polarization or continuum has been essentially established using murine macrophages and it is believed that it is applicable to human macrophages. In this review, we aimed to understand the role of macrophage polarization in human infectious diseases through reappraisal of macrophage polarization by high-throughput methods.

We described that the M1 polarization program is associated with gastrointestinal infections (e.g. typhoid fever and *Helicobacter pylori* gastritis) and active tuberculosis. On the other hand, an M2 signature is observed in lepromatous leprosy, Whipple's disease, and localized infections (keratitis, chronic rhinosinusitis). However, these findings could not be predicted from the analysis of the M1/M2 programs of macrophages stimulated in vitro. The reappraisal of macrophage polarization by high-throughput methods is critical to understanding the role of macrophage polarization in infectious diseases.

Figure 1. General concepts and properties of polarized macrophages, see [1]

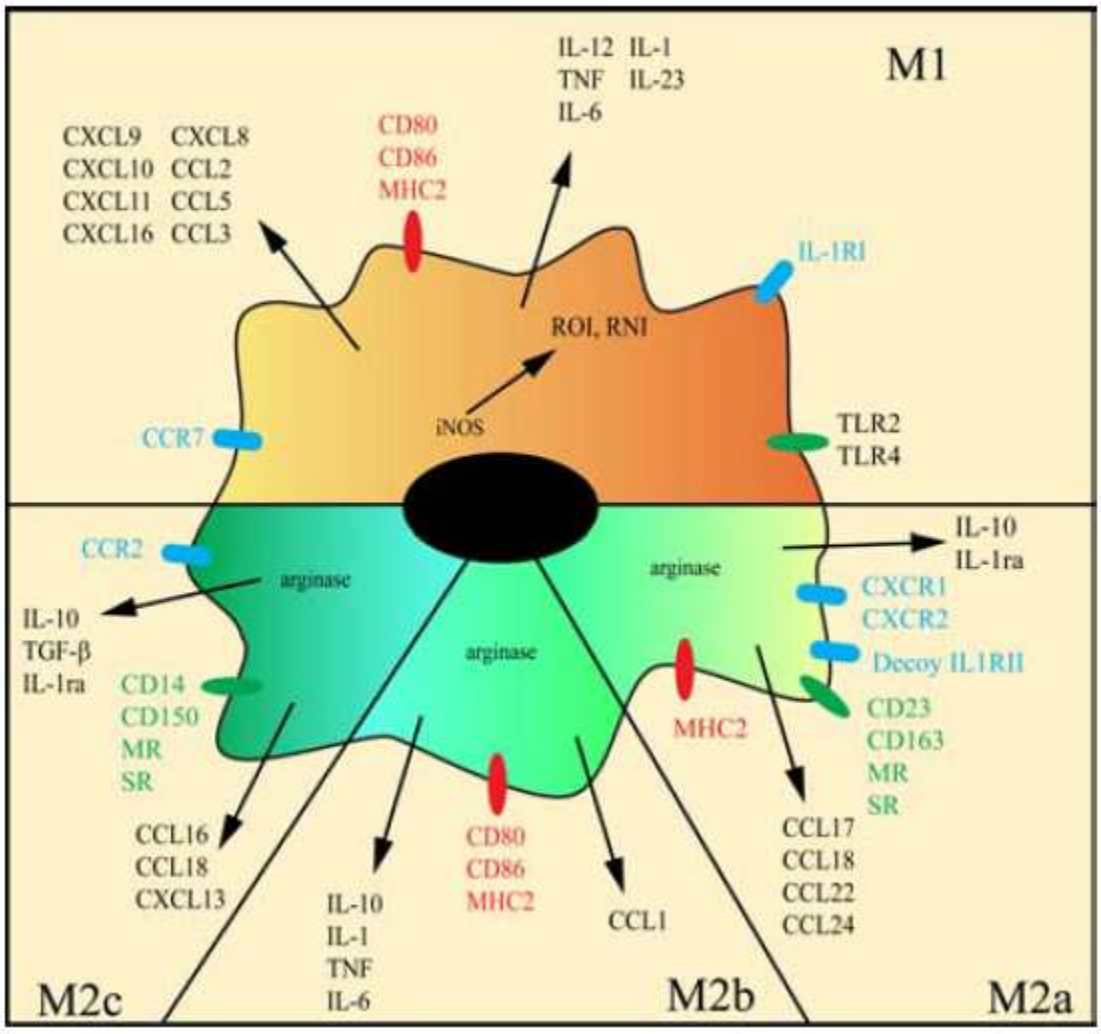

Figure taken from reference No. 1: Benoit M, Desnues B, Mege JL. Macrophage polarization in bacterial infections. J. Immunol. 2008, 181:3733-3739.

Macrophage polarization and bacterial infections

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Purpose of review

Macrophages are the first line of defense against pathogens, and the mode of their activation will determine the success or failure of the host response to pathogen aggression. Based on limited numbers of markers, activated macrophages can be classified as classically activated (M1) macrophages that support microbicidal activity or alternatively activated (M2) macrophages that are not competent to eliminate pathogens. The development of high-throughput gene expression methods affords a reappraisal of the concept of macrophage activation in human infectious diseases.

Recent findings

By combining microarray data and conventional approaches, it is becoming clear that the M1 polarization program is associated with gastrointestinal infections (e.g. typhoid fever and *Helicobacter pylori* gastritis) and active tuberculosis. An M2 signature is observed in lepromatous leprosy, Whipple's disease, and localized infections (keratitis, chronic rhinosinusitis). However, these findings could not be predicted from the analysis of the M1/M2 programs of macrophages stimulated *in vitro*.

Summary

The reappraisal of macrophage polarization by high-throughput methods is critical to understanding the role of macrophage polarization in infectious diseases. Only the identification of individual profiles will support promising therapeutic approaches based on target determination.

Keywords

bacterial pathogens, macrophage polarization, microarray

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Introduction

Macrophages are dynamic cells that are on the first line of defense against invading pathogens, where they act as effectors of the immune response. Monocytes, the precursors of macrophages and dendritic cells, and tissue macrophages are heterogeneous cell populations [1]. This heterogeneity likely supports specialization for different types of invading pathogens. Hence, it has been recently demonstrated that human monocyte subsets are specialized in their responses to bacteria or viruses [2]. For instance, alveolar, intestinal, and adipose tissue macrophages essentially exhibit immunoregulatory functions, whereas Kupffer cells, peritoneal macrophages, and macrophages from lymphoid organs are involved in the killing of microorganisms [3]. The functional heterogeneity of macrophages is also emphasized by the concept of macrophage polarization. Mirroring Th1–Th2 polarization, polarized macrophages can be classified as classically activated (M1) or alternatively activated (M2) macrophages. The M1/M2 polarization reflects the bidirectional macrophage–lymphocyte interaction: Th1 cells drive M1 polarization via interferon gamma (IFN-γ) and Th2 cells direct M2 polarization via interleukin (IL)-4 and IL-13 [4]. The identification of M1 and M2 macrophages relies on a combination of membrane receptors, cytokines, chemokines, and effector mediators [e.g. inducible nitric oxide synthase (iNOS)/arginase balance] [5].

The development of high-throughput gene expression microarray platforms has produced tremendous amounts of data, including those related to M1/M2 polarization [6]. As a consequence, it is now clear that the description of macrophage polarization with few canonical molecules does not account for the complexity of macrophage activation [4,7,8]. In addition, the use of blood samples as a pipeline for the immune system has boosted the research for peripheral signatures in patients [9]. In infected patients, blood transcriptional profiles may reflect the immune response against pathogens, and they appear instrumental for studying systemic or localized infections [10••]. The aim of this review is to reconsider macrophage polarization in human bacterial infections in light of the new high-throughput methods.

Septic syndromes: the model of tolerance to lipopolysaccharide

Sepsis involves the systemic inflammatory response to invading pathogens, which corresponds to an excessive

M1 program, followed by immune dysfunction reminiscent of lipopolysaccharide (LPS) tolerance and alternative activation of macrophages [11,12]. LPS-tolerant macrophages express molecules that are produced by M2 macrophages, such as IL-10, CCL2, CCL17, CCL22, and arginase, and lose the ability to mount an M1 response. This M2 phenotype is mediated by the p50 subunit of nuclear factor kappa B because its absence exacerbates M1-driven inflammation [13]. LPS tolerance may be counterbalanced via different mechanisms. For instance, IFN-γ overcomes LPS tolerance through its ability to recruit chromatin remodeling machinery and induce the expression of M1 genes [14]. Natural killer (NK) cells also impede M2 macrophages via IFN-γ production and/or a higher sensitivity of M2 macrophages to NK cell-induced lysis [15]. The role of M2 polarization in sepsis has recently been illustrated in a model of bacterial translocation in which mice are severely burned and orally infected with *Enterococcus faecalis*. In this context, overproduced CCL2 stimulates the conversion of resting macrophages toward M2 macrophages; both events are associated with a poor prognosis for infected mice [16•].

Gastrointestinal infectious diseases

Salmonella enterica serovar Typhi, the agent of typhoid fever, induces M1 polarization of human and murine macrophages, which is associated with control of the infection [12]. In patients with acute typhoid fever, the analysis of transcriptomic responses of circulating cells reveals that the transcripts related to the IFN-γ-mediated immune response are enriched. Typical M1 genes and M1-related genes are also enriched, whereas the genes associated with an M2 profile are not. This signature is replaced by an M2 signature in convalescent patients. Convalescent patients who retain this M2 signature may be more susceptible to re-infection, relapse or the establishment of a carrier state [17••].

Helicobacter pylori infection is often a source of chronic inflammation of the gastric mucosa, leading to gastric carcinoma in 1–2% of infected patients. Gastric disorder is associated with M1 polarization of macrophages. Indeed, iNOS contributes to carcinoma development, and a high level of CCL18 in gastric tumors is associated with prolonged patient survival [18]. A recent study using a mouse model of infection with a mouse-adapted *H. pylori* strain showed that gastric macrophages are polarized toward an M1 profile during the infection [19•]. In mice vaccinated with *H. pylori* lysate and the adjuvant cholera toxin, this M1 profile is amplified and is associated with protection. *H. pylori*-infected humans with uncomplicated gastritis have increased levels of M1 and M2 markers in the antrum. In patients with atrophic gastritis, M1 polarization is reinforced, as indicated by a strong increase in iNOS expression. This finding is reminiscent of the association of polymorphisms in the iNOS promoter leading to increased transcriptional activity and a higher incidence of gastric cancer in Japanese women [20]. Shifting macrophage polarization from an M1 to an M2 profile could represent a therapeutic approach in the treatment of chronic *H. pylori* infection.

Key points

- A better understanding of macrophage polarization needs high-throughput gene expression methods.
- Bacterial pathogens induce M1 or M2 polarization of isolated macrophages.
- M1 or M2 polarization programs are observed in bacterial infectious diseases, but discrepancies between *in vitro* and *in vivo* may be observed.
- The identification of individual profiles is needed to support therapeutic approaches of patients.

Tuberculosis

It is generally accepted that *Mycobacterium tuberculosis* interferes with M1 polarization through the secretion of virulence factors [12]. It has been recently reported that *M. tuberculosis* induces the expression of peroxisome proliferator-activated receptor (PPAR)-γ in monocyte-derived macrophages via a mannose receptor-dependent pathway [21•]. PPAR-γ is a ligand-activated nuclear transcription factor that is characteristic of M2 activation in macrophages [22] and may be critical for mycobacterial survival [21•]. It is unclear whether the induction of PPAR-γ by *M. tuberculosis* is sufficient to induce a permissive M2 profile or whether additional signals are required to orient macrophages toward an M2 profile in which mannose receptor is already upregulated. However, another experimental approach suggests that *M. tuberculosis* induces the M2 profile: *M. tuberculosis* infection in mice that express human IL-10 in the macrophage compartment reveals the presence of several features of M2 macrophages [23]. This model mimics tuberculosis reactivation and emphasizes the role of IL-10 and M2 polarization in the chronic evolution of tuberculosis, which likely overrides the M1 polarization observed during the acute phase of the disease.

The study of clinical samples may lead to divergent conclusions. Genome-wide transcriptional profiles of peripheral blood from patients with different clinical forms of tuberculosis have recently been reported [24••]. A hierarchical clustering analysis has shown that the transcriptional profiles of patients with active tuberculosis cluster independently compared with patients with latent tuberculosis or with healthy individuals. The transcriptional signature of patients with active tuberculosis is transient, because it is present at the beginning of the

disease and vanishes 1 year later. This specific signature consists of myeloid transcripts in which genes downstream of IFN-γ and type I IFN receptors are overexpressed. The genes that correspond to typical M1 and M1-related genes are present, but M2 genes are not. Interestingly, a similar signature is observed in a minority of patients with latent tuberculosis, suggesting that peripheral biomarkers may be predictors of the active expression of the disease.

The use of bacille Calmette–Guérin (BCG) as a human vaccine provides protection against severe infant tuberculosis, but it does not protect against lung tuberculosis in adults. Host responses in infants have been recently evaluated by measuring the transcriptomic response of circulating mononuclear cells to antigen re-stimulation [25•]. The specific signature includes the upregulation of M1-related genes (inflammatory genes) and the downregulation of genes known to be associated with an M2 profile (PPAR signaling pathway). Hence, the BCG vaccine skews macrophage polarization toward an M1 protective response.

Leprosy

Leprosy results from infection by *Mycobacterium leprae*, which is distinguished from *M. tuberculosis* by its decreased genome size [26]. The disease is characterized by both a clinical polarization (tuberculoid vs. lepromatous) and a polarization of the immune response (Th1 vs. Th2). IL-10 is critical for the pathophysiology of lepromatous leprosy. Higher levels of IL-10 and decreased TNF-to-IL-10 ratios associated with T-cell anergy correlate with bacillary growth. In addition, single-nucleotide polymorphisms in the IL-10 promoter are associated with high IL-10 production [27]. We have previously suggested that the overexpression of IL-10 by macrophages in lepromatous lesions could correspond to an M2 profile [12]. It has recently been demonstrated that the gene expression profile of lepromatous lesions is enriched for M2 genes compared with tuberculoid lesions, including CD36, CD163, scavenger receptor-A and macrophage receptor with collagenous structure (MARCO). By contrast, the antimicrobial program dominates in tuberculoid lesions [28••]. A phenotypic analysis also indicates that the majority of macrophages in lepromatous lesions express CD163. These macrophages, also called foamy macrophages, contain both mycobacteria and oxidized low-density lipoprotein, suggesting that they are polarized to an M2 phenotype. In contrast, the macrophages present in tuberculoid granulomas do not express CD163, which is suggestive of an M1 phenotype. Finally, the M2 program and the CD163 phenotype of macrophages present in lesions shift to an M1 program in patients who experience spontaneous conversion from the lepromatous to the tuberculoid form of the disease.

Whipple's disease: a model of systemic M2 infectious disease

Whipple's disease, a chronic multisystem infection caused by *Tropheryma whipplei*, is characterized by massive infiltration of the lamina propria by macrophages with periodic acid-Schiff inclusions. It has long been suspected that a specific immune deficiency, such as a Th2-biased polarization of immune responses, accounts for the bacterial persistence [29]. Our initial observation of an M2 macrophage signature in an intestinal biopsy of one patient with Whipple's disease [30] was recently confirmed using a large cohort of patients [31•]. Phenotypic and functional studies support the polarization of duodenal macrophages and circulating monocytes toward M2 cells. Indeed, the number of duodenal macrophages and circulating monocytes that express CD163 is increased in patients with Whipple's disease. CCL2 and IL-10 levels are also increased in duodenal lesions and peripheral blood, whereas iNOS expression and nitrite production are decreased. In addition to M2 polarization, *T. whipplei* induces a type I IFN response [32] that is associated with bacterial replication and cellular apoptosis, both of which are detrimental for the host. This finding is unique because type I IFN is typically associated with M1 polarization and bacterial clearance, as was recently shown for *Legionella pneumophila* [33]. Furthermore, it has recently been shown that the cross-talk between type I and type II IFNs affects host susceptibility to bacterial infections and that α/β IFNs downmodulate the expression of IFN-γ receptors [34]. Such cross-talk may contribute to macrophage re-polarization toward an M2 response, and similar mechanisms have been suggested for *M. tuberculosis* and *Listeria monocytogenes* infections [34].

Localized infectious diseases

We have selected two recent publications in which bacterial persistence has been associated with localized infectious diseases, namely keratitis and chronic rhinosinusitis with nasal polyps.

Keratitis caused by *Pseudomonas aeruginosa* is characterized by epithelial edema, stromal infiltrate, and corneal ulceration leading to vision loss. The genetic background of the host is critical, because BALB/c mice are resistant to murine keratitis, whereas C57BL/6 mice are susceptible to it [35•]. It has been shown recently that IL-33, a member of the IL-1 cytokine family that promotes or amplifies M2 chemokine expression [36], plays a major role in keratitis. Indeed, IL-33 is increased in the cornea of resistant mice, and its administration improves the course of disease and reduces infection in susceptible mice. IL-33 treatment decreases the production of type 1 cytokines, increases the production of type 2 cytokines,

Table 1 Confrontation of in-vitro and in-vivo studies

	M1	M2	Reference
In-vitro studies: interaction of bacteria with macrophages[a]			
Bacteria			
Escherichia coli (LPS)	Yes		[12]
Escherichia coli (LPS tolerance)		Yes	[13]
Helicobacter pylori	Yes		[19•]
Mycobacterium tuberculosis		Yes	[21•]
Legionella pneumophila	Yes		[33]
Tropheryma whipplei		Yes	[29]
Francisella novicida		Yes	[41]
In-vivo studies: clinical investigation[b]			
Infectious diseases			
Sepsis	Yes	Yes	[12]
Typhoid fever	Yes		[17••]
Typhoid fever relapses			[17••]
H. pylori, uncomplicated gastritis	Yes	Yes	[19•]
H. pylori, atrophic gastritis	Yes		[19•]
Active tuberculosis	Yes		[24••]
Active tuberculosis (minority subset)		Yes	[24••]
BCG-vaccinated infants	Yes		[25•]
Lepromatous leprosy		Yes	[28••]
Tuberculoid leprosy	Yes		[28••]
Whipple's disease		Yes	[31•]
Chronic rhinosinusitis with nasal polyps (*Staphylococcus aureus*)		Yes	[38•]

The comparison of these two types of studies reveals a major discrepancy between *Mycobacterium tuberculosis* infection and tuberculosis. BCG, bacille Calmette–Guèrin; LPS, lipopolysaccharide.
[a] Pathogens are incubated with macrophages, and we assess macrophage polarization. Note that the polarization profile of macrophages is stimulus-dependent.
[b] Patients are investigated, and we analyze markers of polarization or transcriptomic patterns.

and shifts macrophage polarization from an M1 to an M2 phenotype in the cornea [35•].

Chronic rhinosinusitis with nasal polyps is characterized by high rates of *Staphylococcus aureus* colonization. The presence of polyps is associated with Th2 inflammation, whereas a Th1 immune response is found in the absence of polyps [37]. Recent evidence suggests that the nasal tissue of patients with chronic rhinosinusitis and nasal polyps is enriched in macrophages that express M2 markers, such as CD163 [38•]. This enrichment may be secondary to IL-33, which is known to amplify the M2 polarization of alveolar macrophages [39] and is produced in excess in chronic rhinosinusitis with polyps [40]. In addition, M2 macrophages from nasal tissue poorly ingest *S. aureus* [38•], which likely favors bacterial colonization.

Conclusion

High-throughput methods for gene-expression studies combined with conventional approaches have permitted a reappraisal of the concept of macrophage polarization in infectious diseases. These studies reveal that macrophage activation in patients could not be predicted from the results of typical pathogen–macrophage interactions (Table 1). Investigation of macrophage polarization in individual patients could improve prognosis by permitting an adaptive therapeutic approach. Finally, recent data suggest that antibiotics, such as azithromycin, polarize macrophages toward an M2 phenotype in a mouse model of *P. aeruginosa* pulmonary infection [42•]. Additional developments aimed at limiting the pathogenic effect of an uncontrolled microbicidal M1 response may be promising.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:
- • of special interest
- •• of outstanding interest

Additional references related to this topic can also be found in the Current World Literature section in this issue (p. 292).

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Chapter III

Monocyte responses in the context of Q fever: from a static polarized model to a kinetic model of activation

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Chapter III

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The concept of M1/M2 polarization describes the responsiveness of macrophages to specific stimuli such as IFN-γ with or without LPS, IL-4 and immune complexes [1]. This concept is frequently used to describe the responsiveness of monocytes to diverse stimuli but we ignore if this concept is applicable to the stimulation of monocytes. In addition, M1/M2 polarization has been essentially based on in vitro experiments [4]. The in vivo relevance of this concept remains hypothetical. We created a transcriptional database of M1/M2 monocyte-derived macrophages through whole genome microarrays and qRT-PCR. Since macrophages are not easily accessible owing to being fixed in tissues and a biopsy is required in clinical situation, we extended our study to monocytes which are circulating cells and are easily accessible. We isolated CD14+ monocytes from blood donor buffy coats and stimulated these cells with the cytokines IFN- γ and IL-4 for 6 and 18 hours. Then, we extracted total RNA from these cells and we ran whole genome microarrays to monitor the transcriptional profile. Selected genes were analyzed by using qRT-PCRs to validate the microarray data. On the other hand, we obtained whole blood of acute and chronic Q fever patients in PAXgene tubes, and then we extracted total RNA from these samples and measured the expression of selected genes by using qRT-PCRs. Monocytes exhibited early (6-hour stimulation) patterns of activation specific to IFN-γ or IL-4 and a late (18-hour stimulation) pattern of activation common to both agonists. Because these responses were not reducible to M1/M2 polarization, we selected biomarkers associated with early and late responses of monocytes and tested the

relevance of these biomarkers in Q fever patients. We also carried out a follow-up study on most of the patients from the initial cohort. The early genes *NLRC5*, *RTP4*, and *RHOH*, which were modulated in response to IFN-γ, were up-regulated in patients with acute Q fever, and the expression levels of the *ALOX15, CLECSF1, CCL13,* and *CCL23* genes, which were associated with the late signature, were specifically increased in patients with Q fever endocarditis. In addition, we found that the *RHOH* and *ALOX15* genes were associated with the activity of acute Q fever and Q fever endocarditis, respectively. Our results show that the kinetic model of monocyte activation enables a dynamic approach for the evaluation of Q fever patients.

Monocyte responses in the context of Q fever:

from a static polarized model to a kinetic model of activation

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Abstract

Background: Q fever is caused by *Coxiella burnetii*, a bacterium that persists in M2-polarized macrophages. We wondered whether the concept of M1/M2 polarization is applicable to Q fever patients.

Methods: Monocytes from healthy controls were cultured with IFN-γ and IL-4, agonists of M1 and M2 macrophages, respectively, and their gene expression was assessed using whole-genome microarrays. Selected biomarkers were assessed in blood from Q fever patients by real-time RT-PCR.

Results: Monocytes exhibited early (6-hour) patterns of activation specific to IFN-γ or IL-4 and a late (18-hour) pattern of common activation. Because these responses were not reducible to M1/M2 polarization, we selected biomarkers and tested their relevance in Q fever patients. The early genes *NLRC5*, *RTP4*, and *RHOH*, which were modulated in response to IFN-γ, were up-regulated in patients with acute Q fever, and the expression levels of the late genes *ALOX15*, *CLECSF1*, *CCL13*, and *CCL23* were specifically increased in patients with Q fever endocarditis. The *RHOH* and *ALOX15* genes were associated with the activity of acute Q fever and Q fever endocarditis, respectively.

Conclusions: Our results show that the kinetic model of monocyte activation enables a dynamic approach for the evaluation of Q fever patients.

Key words: macrophage, microarray, monocyte, Q fever, polarization.

Introduction

Q fever is caused by *Coxiella burnetii*, an intracellular bacterium known for its myeloid tropism. This disease is characterized by a primary infection that may become chronic in patients with valvulopathy, pregnant women, and immunocompromised patients [1]. Although the primary infection is characterized by a Th1-type protective immune response, the chronic form of the disease is due to impaired cell-mediated immunity [2]. The analysis of *C. burnetii*-macrophage interaction has shown that macrophage polarization is critical for bacterial elimination or persistence [3].

The concept of macrophage polarization is instrumental in analyzing the activation of murine macrophages and, to a lesser degree, human macrophages [4]. M1 macrophages that are stimulated by interferon (IFN)-γ in the presence or absence of lipopolysaccharide (LPS) are considered inflammatory, microbicidal, and tumoricidal. M2 macrophages, which are induced by interleukin (IL)-4/IL-13, IL-10/Transforming Growth Factor (TGF)-β1, immune complexes, apoptotic cells, and corticosteroids, are poorly inflammatory and exhibit immunoregulatory functions [5]. Polarized macrophages are involved in innate immune response to pathogens [6], autoimmune diseases [7], and local immune suppression associated with tumors [8]. We previously showed that *C. burnetii* induces an atypical M2 program in macrophages that combined M2 markers, including TGF-β1, IL-10, CCL18, mannose receptor, and arginase, and some M1 markers, including IL-6 and CCL8 [9]. The uptake of apoptotic lymphocytes by macrophages stimulates an M2 program and the replication of *C. burnetii*. Both events are prevented in the presence of IFN-γ [10]. In vivo, the over-expression of IL-10 in transgenic mice enables bacterial persistence and mimics features of chronic Q fever, including the M2 polarization of macrophages [11]. These reports suggest that the chronic evolution of Q fever is related to the M2 polarization of macrophages, but direct evidence of such a relationship in infected patients is lacking. The concept of the M1/M2

polarization of macrophages seems pertinent only in few clinical situations, and in vivo and in vitro data are often contradictory [12]. In addition, only monocytes, not tissue macrophages, are easily accessible in clinical investigations, and it is not known if the concept of macrophage polarization is applicable to monocytes.

In this study, using a high-throughput microarray approach, we analyzed the transcriptional responses of circulating monocytes to IFN-γ and IL-4, canonical agonists of M1 and M2 macrophages, respectively. When monocytes were stimulated for 6 hours, they exhibited early responses to IFN-γ and IL-4 that were not reducible to M1/M2 polarization. After 18 hours, the monocyte response was no longer polarized, and a late response signature was identified. By selecting early- and late-related genes from each monocyte signature, we showed that specific biomarkers could be used to distinguish acute Q fever and Q fever endocarditis.

Methods

Patients

This study was approved by the Ethics Committee of the Aix-Marseille University and written informed consent was obtained from each subject. Q fever diagnosis was based upon epidemiological, clinical, and serological criteria [13]. We included 14 patients with acute Q fever with a mean age of 47 years (range, 28-69 years), 13 patients with chronic Q fever and endocarditis with a mean age of 52 years (range, 39-64 years). Six patients with acute Q fever and 9 patients with Q fever endocarditis were investigated twice: at the inclusion, after 5 (3-6) months for acute Q fever and after 16 (8-25) months for Q fever endocarditis. The control group consisted of 10 healthy controls with a mean age of 48 years (range, 32-59 years), 8 patients with classical Whipple's disease (mean age of 64 years, range: 53-69 years), 7 trauma patients with a mean age of 34 years (range, 24-37 years) and 5 trauma patients with

pneumonia (mean age of 36 years, range: 29-43 years) [14]. Samples were handled anonymously without knowledge of disease state until statistical analysis. Peripheral blood was drawn in PAXgene tubes (Qiagen), and RNA was extracted after DNase treatment (see below).

Monocytes and macrophages

Blood donor buffy coats were provided by the Etablissement Français du Sang (Marseille). Peripheral blood mononuclear cells were subjected to $CD14^+$ magnetic cell sorting (Miltenyi Biotec), which yielded monocytes with a purity higher than 95%, as assessed by flow cytometry. Monocytes (10^6 cells/assay) were differentiated into macrophages as previously described [9] and after differentiation, more than 95% of cells were macrophages as assessed by CD68 expression and CD14 down-regulation. Monocytes and monocyte-derived macrophages were stimulated with 20 ng/ml recombinant human IFN-γ (PeproTech) or IL-4 (AbCys) for 6 or 18 hours.

Microarrays

RNA was extracted with an RNeasy Mini kit (Qiagen) and analyzed with microarray chips (4x44K Whole Human Genome, Agilent Technologies, Massy, France) as recently described [15]. MIAME-compliant data were submitted to the Gene Expression Omnibus (GEO) of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/geo/) and can be assessed with the GEO series accession number GSE36537.

The data were analyzed with R and the Bioconductor software suites with Significance Analysis of Microarray and a multiclass model integrating the experimental design. Functional enrichment analysis was performed with DAVID as previously described [16]. To assess the M1/M2 polarization status of monocytes, 28 M1 genes and 17 M2 genes (**Table**

S1) were manually selected from the literature [4-6] and were found to be modulated in IFN-γ- or IL-4-stimulated macrophages. These genes were plotted above a virtual cell according to their cellular localization. The modulation of gene expression was explored by color coding. The ratio of gene expression in stimulated cells to gene expression in unstimulated cells is coded from red to blue, whereas the ratio of gene expression in IFN-γ-stimulated cells to gene expression in IL-4-stimulated cells is coded from pink to green. Cytoscape [17] and Inkscape were used to design the figures. These 45 genes were also analyzed by quantitative real-time RT-PCR (qRT-PCR) to confirm microarray results. The overall correlation coefficient for the two techniques was high ($R^2 = 0{\cdot}64$; $p = 0{\cdot}01$).

qRT-PCR

qRT-PCR experiments were performed with the MMLV RT Kit (Life Technologies) and the 7900HT Fast Real Time PCR System (Applied Biosystems) as recently described [15]. Gene-specific primers were designed with Primer3 [18]. The results were normalized to the result for the housekeeping gene β-actin and are expressed as ΔCt values ($\Delta Ct = Ct_{Target} - Ct_{Actin}$), where Ct is the number of PCR cycles. Correlation analysis for the microarray and qRT-PCR data was performed with the Spearman correlation coefficient. The significance of the differential expression in patients observed by qRT-PCR was assessed by the Wilcoxon rank sum test with a *p*-value cut-off of 0.05.

Results

Kinetics of transcriptional responses of monocytes to IFN-γand IL-4

The transcriptional responses of monocytes and macrophages to IFN-γ or IL-4 were investigated using whole-genome microarrays. The overall transcriptional response showed a completely different activation pattern in monocytes and macrophages. Principal component

analysis confirmed that the cell type (monocytes or macrophages), time (6 or 18 hours), and type of stimulation (IFN-γ or IL-4) influenced transcriptional modulation (**Figure 1, A-B**). Based on a multi-class model that integrates the cell type (macrophages or monocytes), length of stimulation (6 or 18 hours) and type of stimulation (IFN-γ or IL-4), 15,619 probes were modulated with an absolute FC of at least 2.0 and an false discovery rate (FDR) < 1% (assessed with Significance Analysis of Microarray). When filtering the dataset for an absolute FC≥4.0 and a FDR<1% 4,807 modulated probes (3,406 genes) were identified. These 4,807 modulated probes are represented as a heatmap (**Figure 1C**). Modulated genes were then filtered to retain those genes specifically modulated in monocytes (**Figure 1D**). We identified 587 probes whose targets were modulated in IFN-γ- or IL-4-stimulated monocytes compared with unstimulated monocytes. The early genes (6-hour stimulation) that were specifically up-regulated in response to IFN-γ or IL-4 included the targets of 59 probes (46 annotated genes) and the targets of 61 probes (39 annotated genes), respectively. In contrast to the early responses of monocytes to IFN-γ and IL-4, the patterns of the late response (18-hour stimulation) of monocytes to IFN-γ and IL-4 were indistinguishable and were characterized by a specific cluster of up-regulated genes that corresponded to 74 probes and 57 annotated genes (**Figure 1D**). Taken together, these results revealed a biphasic response of monocytes to IFN-γ and IL-4.

Polarization of early and late monocyte responses

We wondered if the polarization observed in macrophages is applicable to the early and late responses of monocytes. We studied the expression in monocytes of 28 and 17 genes considered to be markers of M1 and M2 polarization in macrophages [4-6]. In the early response of monocytes to IFN-γ, 16 M1 genes were up-regulated, but only one M2 gene was up-regulated (**Figure 2A**). In the early response of monocytes to IL-4, 10 M2 genes, but only

3 M1 genes, were up-regulated (**Figure 2C**). Hence, the early responses of monocytes to IFN-γ and IL-4 showed a clear dichotomy in the expression of M1 and M2 genes (**Figure 2E**). We then investigated whether the specific signatures of monocytes induced by IFN-γ and IL-4 included M1/M2 genes. In the early signature induced by IFN-γ (which included 46 genes), only 11 genes were M1 genes. Similarly, the early signature induced by IL-4, composed of 39 genes, included only 2 M2 genes, demonstrating that the early responses of monocytes are not reducible to M1/M2 polarization.

In the late response of monocytes to IFN-γ, most M1 genes that were up-regulated in the early response to IFN-γ returned to their basal levels or were down-regulated. Only one M1 gene remained up-regulated. In addition, 8 M2 genes were up-regulated in the late response to IFN-γ (compare **Figure 2, A** and **B**). The late response of monocytes to IL-4 was characterized by the up-regulation of one M1 gene, the down-regulation of 14 M1 genes, and the up-regulation of 10 M2 genes (compare **Figure 2, C** and **D**). The modulation of M1 and M2 genes was remarkably similar in the late responses of monocytes to IFN-γ and IL-4 (**Figure 2F**), demonstrating that the late responses of monocytes were not polarized as the responses of macrophages.

As a consequence, we hypothesized that the M1/M2 transcriptional program of macrophages may be insufficient to account for the clinical manifestation of Q fever. To test this hypothesis, we selected 6 M1-related genes (*IL15*, *CXCL9*, *IL2RA*, *IDO1* (*INDO*), *TNF*, *TNFSF10* (*TRAIL*)) and 6 M2-related genes (*CHN2*, *CTSC*, *CD209* (*DC-SIGN*), *FN1*, *HRH1*, *SLC4A7*), and we assessed their expression levels in Q fever patients. The expression levels of M1 and M2 genes (with the exception of *CD209*) were similar in healthy controls and patients with acute Q fever. In patients with Q fever endocarditis, the expression levels of 4 M1 genes were up-regulated, and the expression level of one M2 gene was down-regulated relative to the expression levels in healthy controls (**Figure S1**). These results show that the expression

of the M1/M2 genes does not characterize the evolution of Q fever.

Functional classification of early and late monocyte responses

Because the majority of the genes up-regulated in the early and late signatures of monocytes were not M1/M2 genes, we wondered if these genes belonged to distinct functional groups. Among the early genes specifically up-regulated in response to IFN-γ, we identified six different clusters including "apoptosis", "complement", "defense response", "IFN-related genes", "Jak/Stat pathway", and lipid metabolism". Six other functional clusters were identified among the early genes that were specifically up-regulated in response to IL-4. They included "cell adhesion", "C-type lectin", "cytoskeleton", "endocytosis and vesicular transport", "response to wounding", and "RNA binding". The late response of monocytes was organized in five different clusters including "Ig domain", "immune response", "Golgi apparatus", "signal transduction", and "Wnt pathway" (**Table 1**). These results demonstrated that the early and the late responses of monocytes to IFN-γ and IL-4 were characterized by specific functional signatures that may be useful to define new biomarkers of Q fever.

Specific biomarkers of Q fever

Among the early annotated genes, we selected four genes representative of the early IFN-γ response (*NLRC5*, *RTP4*, *C1S*, *GCH1*) and four genes representative of the early IL-4-response (*ITGB3*, *TREM1*, *NEDD4L*, *RHOH*), which are not described as M1 or M2 markers. The expression levels of these genes were compared between healthy controls and Q fever patients using qRT-PCR. In acute Q fever, two early genes associated with the IFN-γ response, *NLRC5* and *RTP4*, were significantly up-regulated. This up-regulation was specific of acute Q fever. Indeed, the expression of *NLRC5* and *RTP4* genes was not modulated in patients with Q fever endocarditis relative to healthy controls (**Figure 3**). In patients with

Whipple's disease, an infectious disease due to an intracellular bacterium with macrophage tropism, *NLRC5*, *RTP4*, and *RHOH* genes were not modulated (**Figure S2**). In trauma patients with or without pneumonia, who exhibited an intense inflammatory response, *NLRC5* and *RTP4* genes were down-regulated, suggesting that the modulation of early genes in acute Q fever was not a consequence of inflammatory response. Interestingly, the *ITGB3* and *TREM1* genes, which were associated with the early response to IL-4, were significantly down-regulated in patients with acute Q fever compared with controls. Only one early gene associated with the IL-4-response, *RHOH*, was significantly up-regulated in patients with acute Q fever (**Figure 3B**). Therefore, the up-regulation of *NLRC5*, *RTP4*, and *RHOH* genes may be considered biomarkers of acute Q fever.

A similar procedure was used to define biomarkers from the late signature of monocytes using eight genes belonging to different clusters. In acute Q fever patients, five genes (*ALOX15*, *CLEC4F*, *CCL23*, *BACE1*, *RAMP1*) were not differentially regulated relative to the expression levels in healthy controls (**Figure 4**), demonstrating that late biomarkers were unable to distinguish acute Q fever. By contrast, the *ALOX15*, *CLEC4F*, *CCL13*, *CCL23*, and *RAMP1* genes were significantly up-regulated in patients with Q fever endocarditis relative to healthy controls. Interestingly, the expression levels of *ALOX15*, *CLEC4F*, *CCL13*, and *CCL23* were significantly higher in patients with Q fever endocarditis than in patients with acute Q fever (**Figure 4**), suggesting that these genes may serve as specific biomarkers of Q fever endocarditis. We also found that two late genes, *GALNTL18* and *WNT5B*, which were not modulated in Q fever endocarditis patients, were down-regulated in acute Q fever patients, providing indirect evidence that late genes may be biomarkers of Q fever endocarditis. Again, late genes were not modulated in patients with patients with Whipple's disease. In trauma patients with or without pneumonia, these genes were down-modulated compared with healthy controls (**Figure S2**). These results demonstrate that *ALOX15*, *CCL13*,

and *CCL23* genes were specifically up-regulated in Q fever endocarditis.

Finally, we assessed the modulation of putative biomarkers during the follow-up of patients with Q fever. In patients with acute Q fever, the expression of *NLRC5* and *RTP4* genes remained up-regulated 5 months after the initial inclusion whereas the expression of *RHOH* gene was similar to controls after 5 months (**Figure 5A**). The modulation of CCL23, *CLECF4*, *ALOX15*, and *CCL13* genes was assessed in patients with Q fever endocarditis 16 months after the inclusion. The expression of *CCL13* and *CCL23* genes remained unchanged. By contrast, the expression of *ALOX15* and *CLECF4* genes decreased with time but only the expression of *ALOX15* gene returned to control levels (**Figure 5B**). Taken together, our results support the hypothesis that the kinetics model of monocyte activation is useful to assess the clinical manifestation of Q fever.

Discussion

The M2 polarization of macrophages has been considered critical for the persistence of *C. burnetii* [6,9,11]. Nevertheless, this concept needs to be confirmed in circulating monocytes. Different constraints impair the evaluation of patients with Q fever based on the concept of macrophage polarization. The isolation of macrophages is adapted for pathophysiological studies but is not convenient for routine use in clinical practice. As a consequence, the use of whole blood may be a practical alternative but requires the validation of monocyte biomarkers. In this study, we investigated the responses of monocytes to IFN-γ and IL-4, and we used these data to guide our analysis of whole blood from patients with Q fever.

The responses of monocytes to IFN-γ and IL-4 consisted of early and late phases of activation, a pattern that did not fit with the M1/M2 polarization model at the transcriptional level. The hallmarks of M1/M2 polarization were found in the early phases of monocyte activation, but the second phase of monocyte activation was common to the different modes

of stimulation. Our results were consistent with the modulation of early and late genes in macrophages stimulated with LPS [19] and the transient polarization observed in murine monocytes stimulated with *Listeria monocytogenes* [20].

In the early phase of the monocyte response to IFN-γ, we identified one set of up-regulated genes that included M1 genes, interferon-response genes, and genes related to inflammatory signalling, complement, and apoptosis. A second set of genes that were specifically up-regulated in IL-4-stimulated monocytes included M2-related genes, and a series of genes partitioned into different clusters associated with lectins, cell adhesion, endocytosis and the cytoskeleton. By contrast, the late response of monocytes was characterized by the up-regulation of numerous genes independent of the agonist, IFN-γ or IL-4. This late response was also distinct from the LPS tolerance that appears late in the inflammatory response and is associated with an M2 program [21]. The late response of monocytes may be considered a termination program with only some features of the M2 program. Our results were consistent with data obtained in a murine model of resolving and non-resolving peritonitis. In this model, peritoneal macrophages from mice with sustained inflammation may be referred to as M1 macrophages, and macrophages from mice with resolving inflammation produce high levels of immunoregulatory cytokines but also present hallmarks of M1 macrophages [22]. A recent transcriptional analysis confirmed that the peritoneal macrophages collected from mice with resolving inflammation exhibit a unique phenotype that is inconsistent with the conventional M1/M2 macrophage classification [23]. These results suggest a high level of plasticity in macrophages. The existence of a unique termination program in monocytes stimulated with IFN-γ and IL-4 may represent a parsimonious way to control the monocyte activation induced by a great variety of cytokines and infectious agents.

We showed that the model of M1/M2 polarization was not applicable to monocyte

responses in the context of Q fever. We selected M1-related and M2-related genes and tested these genes in whole blood. The expression levels of these genes were similar in patients with acute Q fever and healthy controls. Only a minority of these genes were up-regulated in patients with Q fever endocarditis. Such findings demonstrate that Q fever patients exhibited an activation program that cannot be classified as an M1 or M2 pattern, in contrast to the result of in vitro and in vivo studies using macrophages [9,10,24]. The differences in the transcriptional programs of monocytes and macrophages may explain why attempts to use markers defined as M1/M2 for macrophages are unsuccessful in clinical studies [25,26]. Only rare reports suggest that monocytes are polarized in infectious diseases. In addition, even though monocytes from patients with Whipple's disease overexpress CD163, other markers of M2 cells are lacking [27]. In patients with active tuberculosis [28] or in children vaccinated with BCG [29], a transient M1 profile is identified, whereas in vitro studies with macrophages suggest M2 polarization [12]. By contrast, monocytes stimulated with *Orientia tsutsugamushi* and patients with scrub typhus both exhibit an M1-type program [30].

Interestingly, the identification of early and late signatures of in vitro-stimulated monocytes allowed the identification of Q fever biomarkers. Some early genes specifically associated with acute Q fever, such as *NLRC5* and *RTP4*, were up-regulated by IFN-γ, suggesting that acute Q fever is controlled at least in part by an IFN-γ-mediated immune response. That these genes were not modulated in patients with Q fever endocarditis confirmed the defective Th1-response reported for Q fever endocarditis [31]. We also found that genes associated with IL-4 response, such as *ITGB3* and *TREM1*, were down-regulated in patients with acute Q fever, reinforcing our hypothesis that acute Q fever is associated with an efficient immune response. Moreover, we found that some late genes, including *ALOX15*, *CLEC4F*, *CCL13*, and *CCL23*, were specifically associated with Q fever endocarditis. Note that a few late genes were also related to the M2 program, a result that might lead to incorrect

conclusions about monocyte activation in the context of Q fever endocarditis. The modulation of early genes in acute Q fever and late genes in Q fever endocarditis seemed to be specific of Q fever because these genes were not modulated in patients with another infectious disease, the Whipple's disease, and patients with acute pathologies such as trauma or pneumonia. The follow-up of patients with Q fever revealed that the expression of *RHOH* and *ALOX15* genes was normalized after several months of disease's evolution, suggesting that these genes may be considered as biomarkers of the activity of acute Q fever and Q fever endocarditis, respectively.

In conclusion, we demonstrated that the concept of macrophage polarization is not usefull to describe monocyte ativation and is irrelevant to explore patients at the bedside. The kinetic approach of monocyte activation we developed allowed the identification of new biomarkers of acute Q fever and Q fever endocarditis, respectively. Additionally, two biomarkers were associated with the activity of the disease.

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Legends of figures

Figure 1. Principal component analysis and heatmap representation of modulated probes

Monocytes and macrophages were stimulated with IFN-γ or IL-4 (20 ng/ml), and microarray analysis was performed after RNA extraction. **A-B,** The impacts of the cell type and the length of stimulation (A), and those of cell stimulation by IFN-γ or IL-4 (B) were analyzed by principal component analysis. The distance along each axis represents the amount of variance in gene expression explained by the corresponding factor. **C,** The 4,807 probes modulated in monocytes and macrophages stimulated for different periods by IFN-γ or IL-4 (|FC|>4 and FDR<1%) are represented as a heatmap with probes in rows and samples in columns. Dendrograms show the results of the hierarchical clustering of the samples (top) and genes (left) (average linkage based on the Pearson correlation distance). **D,** Only genes modulated in stimulated monocytes compared with unstimulated monocytes were retained. Up-regulated genes specific to early stimulation with IFN-γ or IL-4 and those genes specifically up-regulated after 18 hours were selected. Specific signatures are identified as black lines on the right hand side. Gene expression levels are color-coded from blue to red.

Figure 2. Gene modulation of M1/M2 markers in stimulated monocytes

Monocytes were stimulated with IFN-γ or IL-4 for 6 hours. Genes involved in macrophage polarization (n = 45) were manually selected from the literature and plotted against a virtual cell according to their GO cellular component annotation. **A-D,** The ratios of gene expression in monocytes stimulated with IFN-γ (A, B) or IL-4 (C, D) for 6 (A, C) and 18 (B, D) hours to the gene expression in unstimulated monocytes are color-coded from blue (FC=-5) to red (FC=+5) at each node. **E-F,** M1/M2 markers were plotted against a virtual cell, and the ratios of the expression of these markers in monocytes stimulated with IFN-γ or IL-4 are color-

coded from green ($FC_{IFN\text{-}\gamma/IL\text{-}4}$ = -5) to pink ($FC_{IFN\text{-}\gamma/IL\text{-}4}$ = +5). Genes in white were up-regulated similarly by IFN-γ and IL-4.

Figure 3. Expression of early genes in patients with Q fever

The expression levels of genes included in the early signatures of monocytes induced by IFN-γ (**A**) or IL-4 (**B**) were tested in blood from healthy donors, patients with acute Q fever, and patients with Q fever endocarditis using qRT-PCR. Individual results are shown. Horizontal bars represent the median of each group, and significant differences between groups are shown. QF: Q fever. *: p-value < 0.05 ; **: p-value < 0.01

Figure 4. Expression of late genes in patients with Q fever

The expression levels of genes included in the late signature of monocytes induced by both IFN-γ and IL-4 were tested in blood from healthy donors, patients with acute Q fever, and patients with Q fever endocarditis using qRT-PCR. Individual results are shown. Horizontal bars represent the median of each group, and significant differences between groups are shown. QF: Q fever. *: *p*-value < 0.05 ; **: *p*-value < 0.01.

Figure 5. Expression of biomarkers according the evolution of Q fever

A, The expression of potential biomarkers of acute Q fever was analyzed in blood from healthy donors (**1**), patients with acute Q fever tested at the inclusion (**2**) and 5 months after inclusion (**3**). **B,** The expression of potential markers of Q fever endocarditis was analyzed in blood from healthy donors (**1**), patients with Q fever endocarditis tested at the inclusion (**2**) and 16 months after inclusion (**3**). Individual results of qRT-PCR data are shown. Horizontal bars represent the median of each group, and significant differences between groups are shown. *: *p*-value < 0.05 ; **: *p*-value < 0.01.

Figure 1

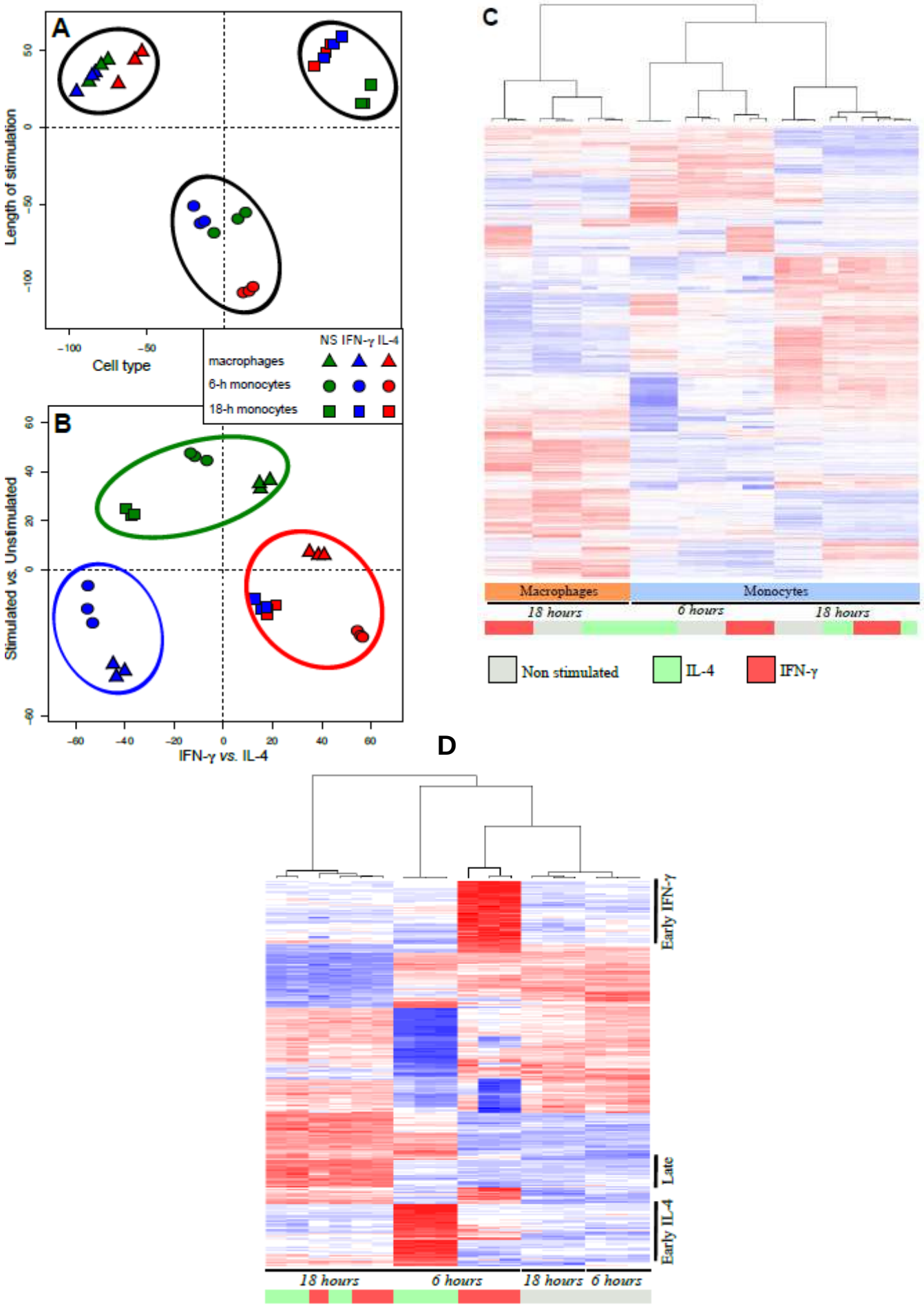

A
Length of stimulation
Cell type
NS IFN-γ IL-4
macrophages
6-h monocytes
18-h monocytes
B
Stimulated vs. Unstimulated
IFN-γ vs. IL-4
C
Macrophages
Monocytes
18 hours
6 hours
18 hours
Non stimulated
IL-4
IFN-γ
D
Early IFN-γ
Late
Early IL-4
18 hours
6 hours
18 hours
6 hours

Figure 2

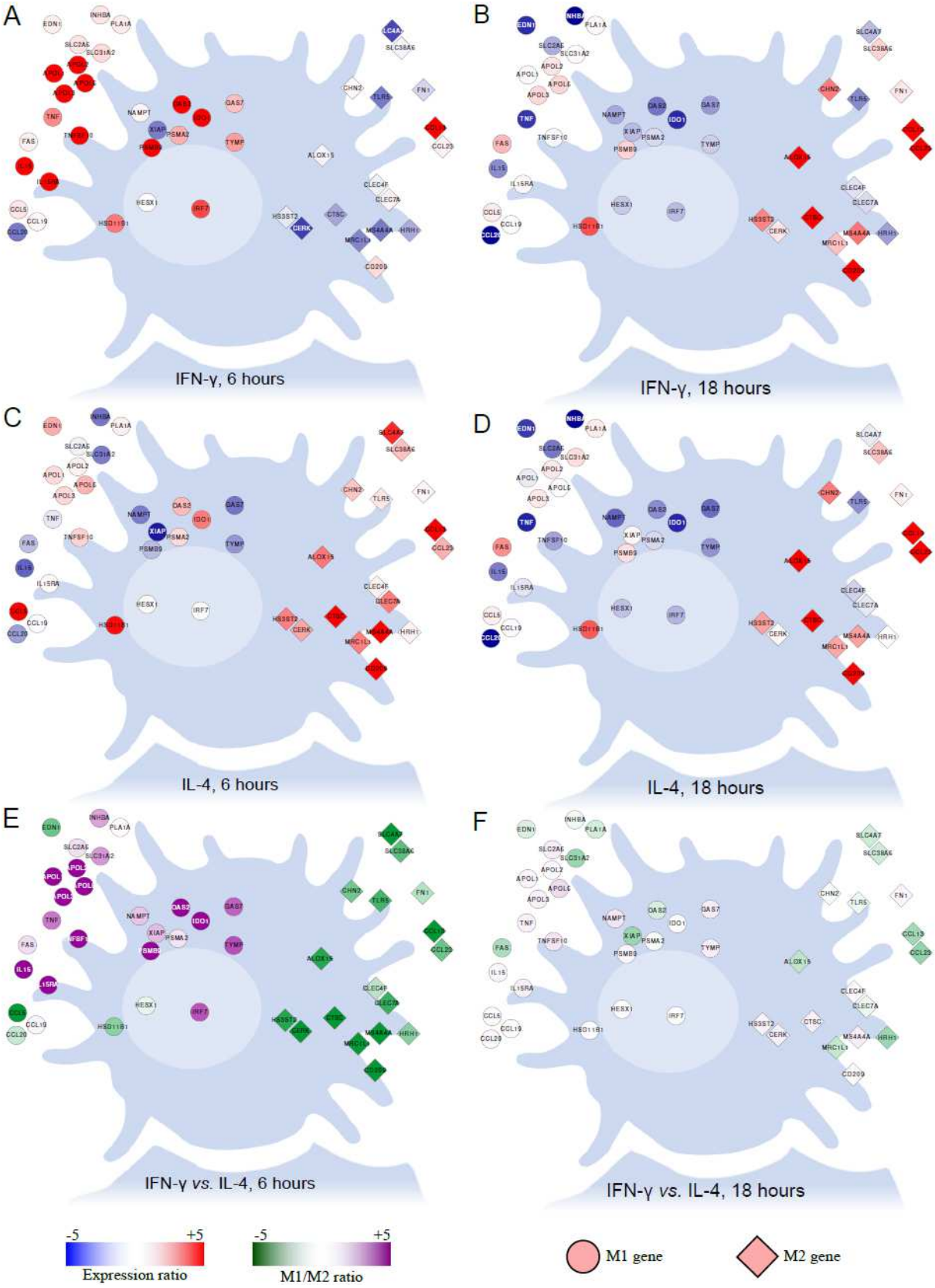

A
IFN-γ, 6 hours
B
IFN-γ, 18 hours
C
IL-4, 6 hours
D
IL-4, 18 hours
E
IFN-γ vs. IL-4, 6 hours
F
IFN-γ vs. IL-4, 18 hours
-5
+5
Expression ratio
-5
+5
M1/M2 ratio
M1 gene
M2 gene

Figure 3

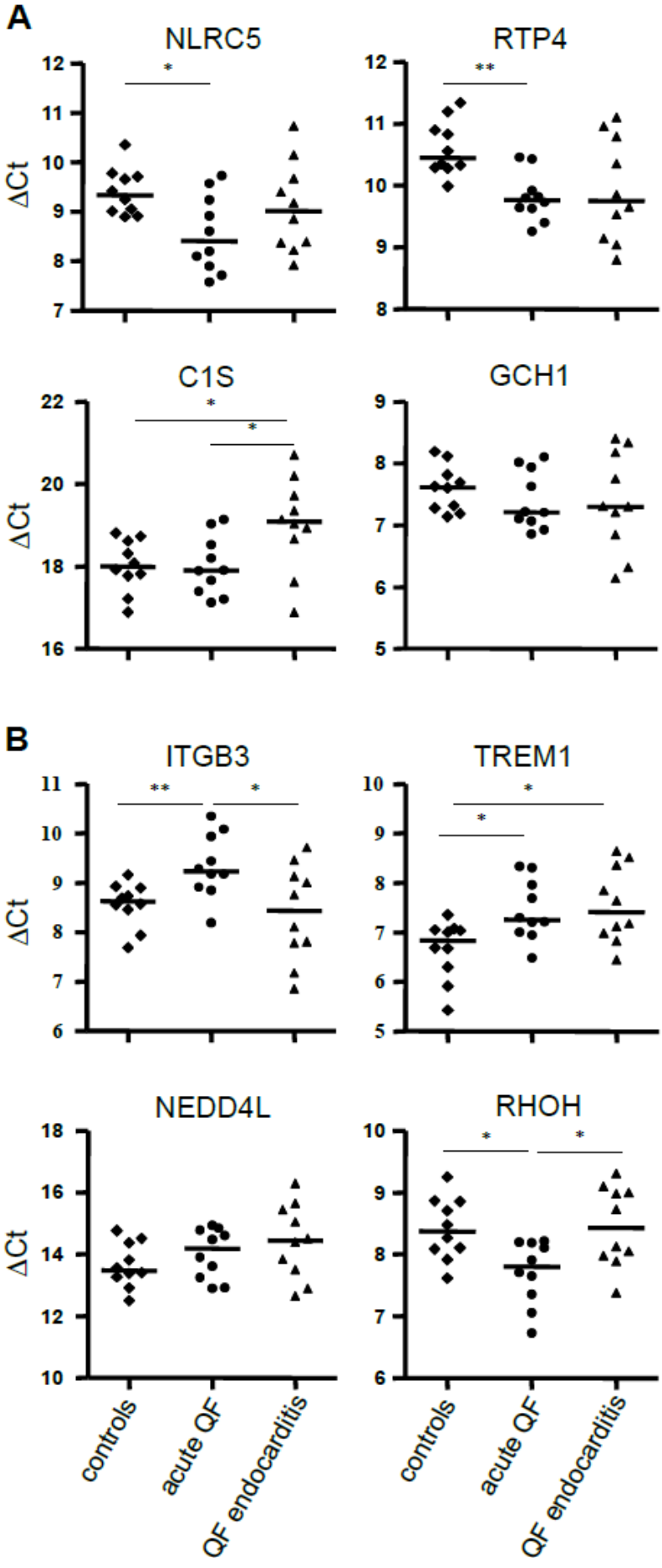

A
NLRC5
RTP4
C1S
GCH1
ΔCt
B
ITGB3
TREM1
NEDD4L
RHOH
ΔCt
controls
acute QF
QF endocarditis

Figure 4

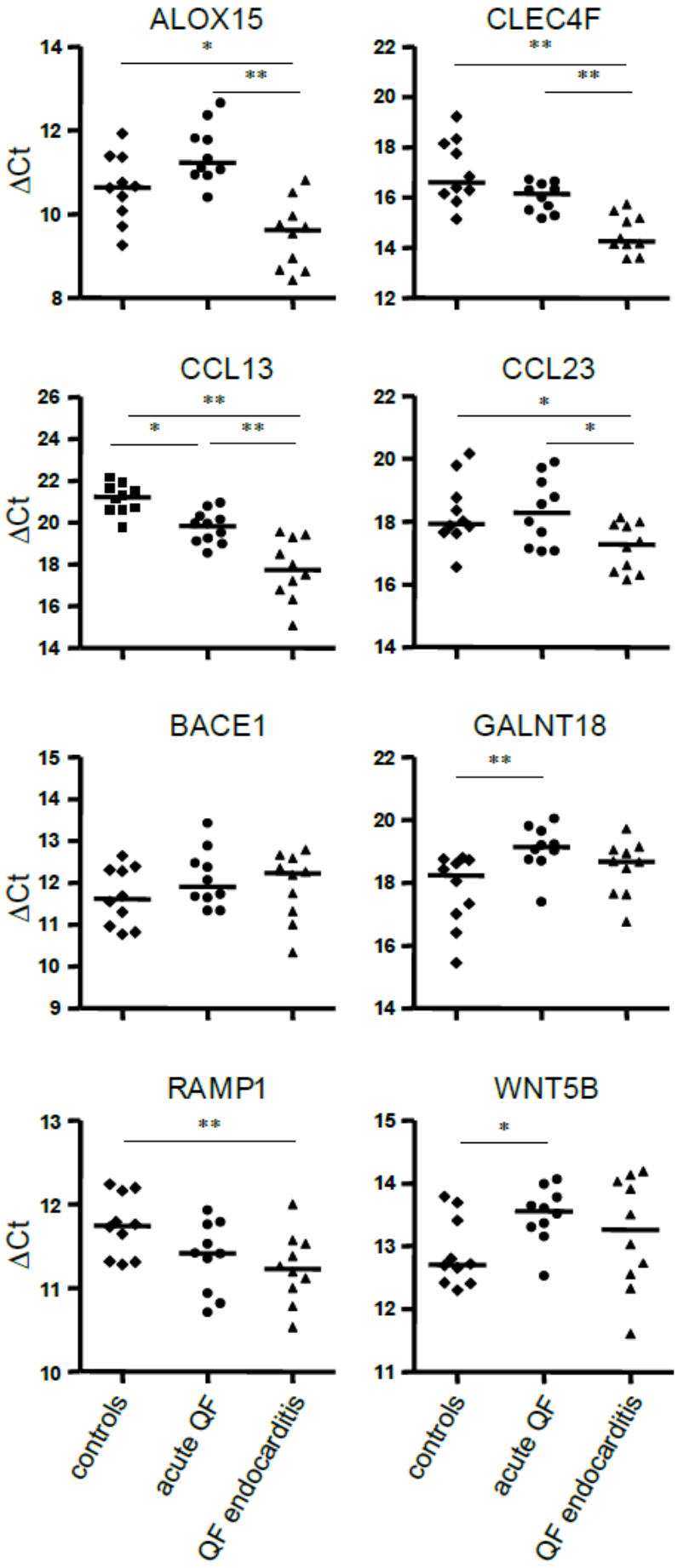

ALOX15
CLEC4F
CCL13
CCL23
BACE1
GALNT18
RAMP1
WNT5B
ΔCt
controls
acute QF
QF endocarditis

Figure 5

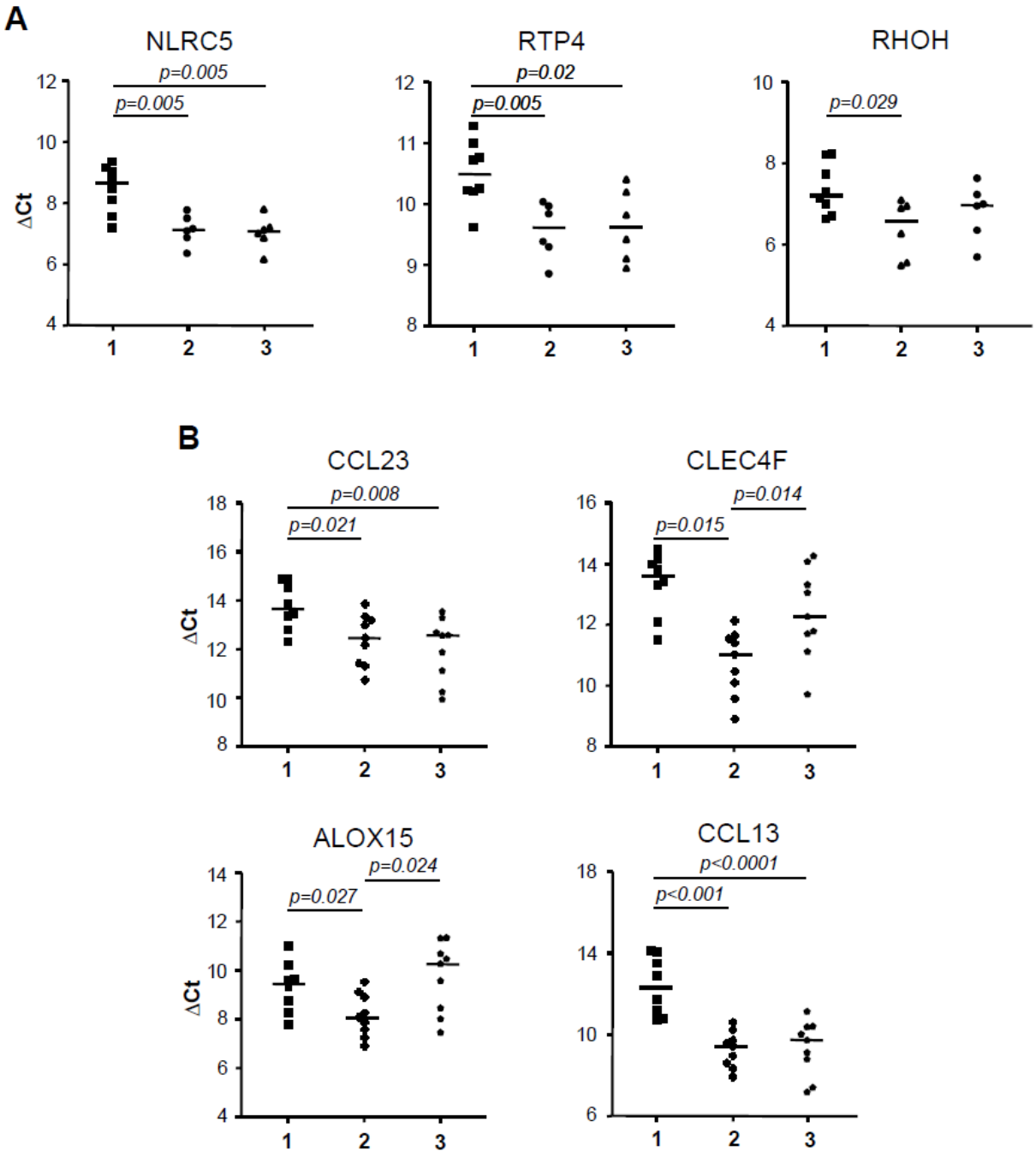
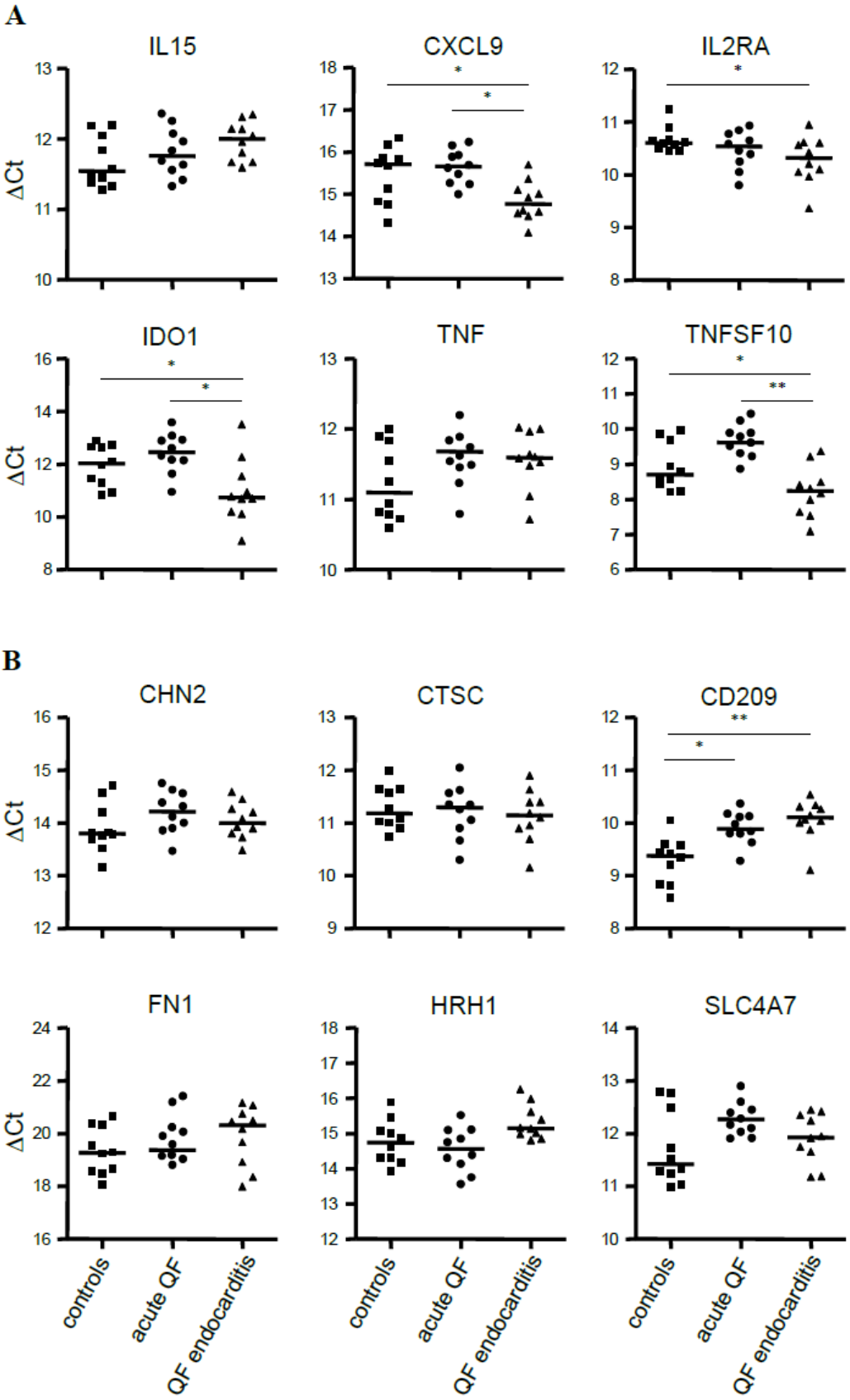
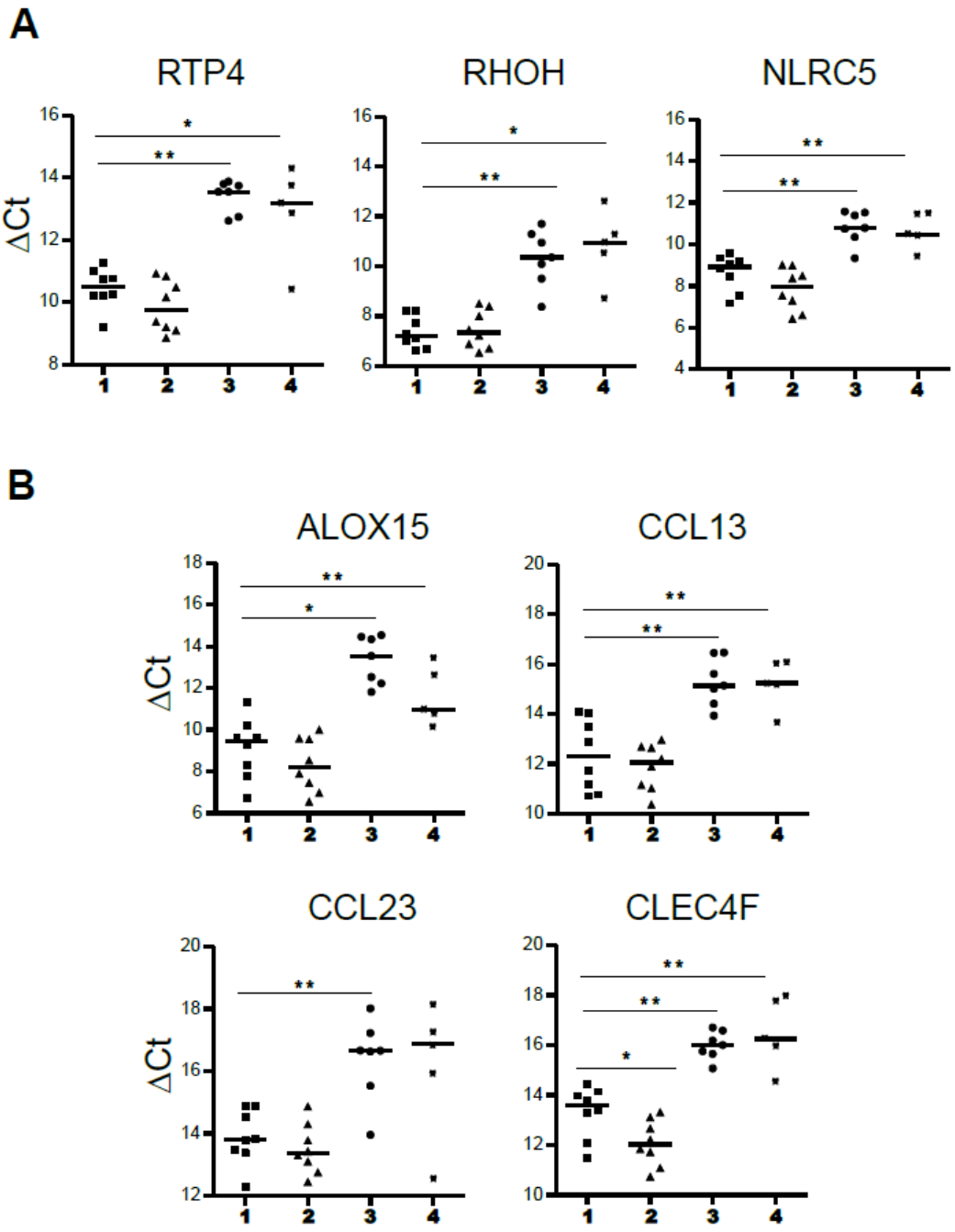

A
NLRC5
p=0.005
p=0.005
RTP4
p=0.02
p=0.005
RHOH
p=0.029
ΔCt
B
CCL23
p=0.008
p=0.021
CLEC4F
p=0.014
p=0.015
ALOX15
p=0.024
p=0.027
CCL13
p<0.0001
p<0.001

Figure S1. Expression of M1 and M2 genes in patients with Q fever



The expression levels of M1 genes (**A**) and M2 genes (**B**) were tested in blood from healthy donors, patients with acute Q fever, and patients with Q fever endocarditis using qRT-PCR. Individual results are shown, and horizontal bars represent the median of each group. QF: Q fever. *: p-value < 0.05 ; **: p-value < 0.01

Figure S2. Expression of biomarkers in different pathologies



The expression of biomarkers was studied in blood from healthy donors (1), patients with Whipple disease (2), trauma (3), and pneumonia (4) using qRT-PCR. Individual results are shown. Horizontal bars represent the median of each group. *: p-value < 0.05, **: p-value < 0.01

Table 1: Functional classification of genes belonging to specific signatures

Type and duration of stimulus **Functional categories**	**Modulated genes**
Early IFN-γ signature	
Jak/Stat pathway	JAK2, IL7, STAT1, IL15, PML, IL15RA
Defense response	IL7, IL15, IL15RA, HLA-DRB6, JAK2, STAT1, PML, C1S, GCH1, APOL1, APOL2, APOL3, RSAD2
Apoptosis	CASP1, CASP5, CARD16, CARD17, AIM2, PML
Complement	C1S, SERPING1
Lipid metabolism	APOL1, APOL2, APOL3
IFN-related	IRF1, IRF5, NLRC5, IFI35, ETV7, HIVEP2, RTP4
Early IL-4 signature	
RNA binding	DDX3Y, DDX43, EIF1AY, RAVER2, RPS4Y1, RPS4Y2, TEP1, RNASE6
Endocytosis & vesicular transport	CD93, EHD2, CCL5, STAB1, RAB15, NEDD4L
Cytoskeleton	FRMD4A, PDLIM2, ARHGAP26, ITGB3, MSN
Response to wounding	CCL5, CD93, HLA-DQA1, TREM1, THBD, FCGR2A, SLAMF1, RHOH
C-type lectin	CD93, STAB1, THBD
Cell adhesion	CD93, ITGB3, STAB1, THBD, MSN
Late signature	
Immune response	F13A1, MAL, MAF, CD1D, FCERA1, ALOX15, CACNB4, CCL23, CD36, TREM2, CXCL14, CCL3
Signal transduction	CCL23, CXCL14, FCER1A, CACNB4, DACT1, FZD7, RAMP1, GFRA2, WNT5B, CLEC4F
Golgi apparatus	CD36, GALNTL4, BACE1, CXCL14, GGTA1, PCSK5, TMEM130
Wnt pathway	DACT1, FDZ7, WNT5B
Ig domain	CD1D, FCER1A, AMICA1, PDGFRL, TREM2, UNC5B

The genes included in the early and late signatures of monocytes were analyzed with the functional analysis tools of DAVID. Major functional clusters are listed with corresponding genes.

Chapter IV

Overexpression of the *Per2* gene in male patients with acute Q fever

Mehraj V., Textoris J., Capo C., Raoult D., Leone M., Mege J.L.

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Chapter IV

Overexpression of the *Per2* gene in male patients with acute Q fever

Gender, age and various other host factors are well known to affect the clinical expression of Q fever, a worldwide zoonosis due to *Coxiella burnetii* [24]. Among human adult patients hospitalized primarily with Q fever, the number of males is about 2.5 times the number of females (7-9, 23). Similarly, the males are more likely to suffer from Q fever-related complications than females [8-10, 24]. Men represent 75% of the patients diagnosed with *C. burnetii* endocarditis, the major manifestation of chronic Q fever. It has been previously shown that sex hormones play a role in the pathophysiology of *C. burnetii* infection in mice. In females, the bacterial load is increased in ovariectomized mice, reaching levels found in males, and 17-β estradiol administration restores the bacterial load to levels found in intact females [12]. It has been also reported that 80% of modulated genes were differently modulated in males and females [13]: among these genes, major components of the circadian rhythm pathway are affected *by C. burnetii* infection in females but not in males. Indeed, the transcriptional expression of *Clock* and *Arntl* genes was down-regulated, whereas that of *Per2* gene was up-regulated in females (12).

Circadian rhythms maintain homeostasis by coordinating the action of physiological systems. They are associated with physiological changes over a course of time [25]. Circadian rhythms are therefore important to control the behavior of organisms related to environmental changes [26, 27]. Alterations of circadian rhythms have been observed in many diseases such as cardiovascular disorders (diabetes, hypertension, …), cancers, neurological disorders (learning and memory dysfunctions), and psychiatric disorders (depression, bipolar disease) [28, 29]. A number of genes are known to regulate the circadian rhythm [30, 31]. This

includes *CLOCK*, *ARNTL* (also known as *BMAL1*) and *PER2*. These genes are an important part of the peripheral molecular clock complex that regulates the circadian expression of many genes (Figure 2).

To our knowledge, the circadian rhythm is involved in a great number of biological phenomena (figure 3, [32]) but had never been described in human infectious diseases. In mice, haematopoietic stem cells (HSCs) and their progenitors are reported to follow a circadian pattern, peaking 5 h after the initiation of light and reaching a nadir 5 h after darkness [14]. While in the bone marrow microenvironment, these cells fluctuate in antiphase with the expression of the chemokine CXCL12. The cyclical release of HSCs and expression of Cxcl12 are regulated by core genes of the molecular clock through circadian noradrenaline secretion by the sympathetic nervous system [14]. Although the circadian rhythm is inversed in mice and humans, we hypothesized that circadian genes are differently modulated in men and women with Q fever. We showed that the expression of the *Per2* gene was specifically increased in men with acute Q fever. This result may indicate for the first time that a gene involved in the circadian clock is differentially modulated in men and women infected with a bacterial pathogen such as *C. burnetii*.

Figure 2. Schematic representation of the negative feedback loop that controls the rhythmic circadian expression of the peripheral molecular clock components. CRG: Clock-Regulated Genes.

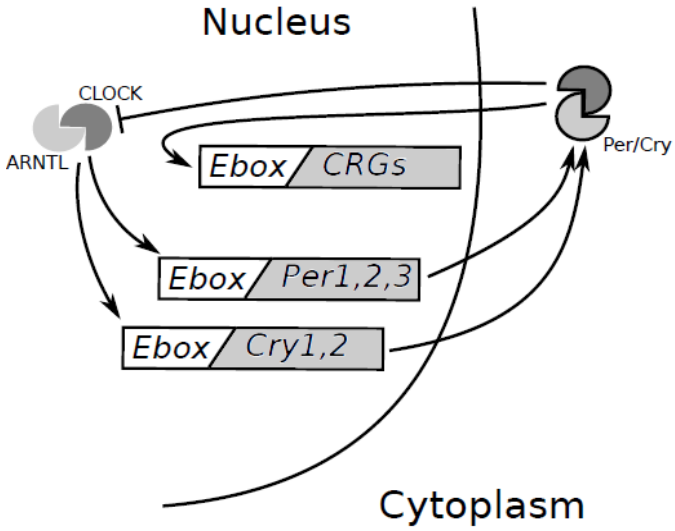
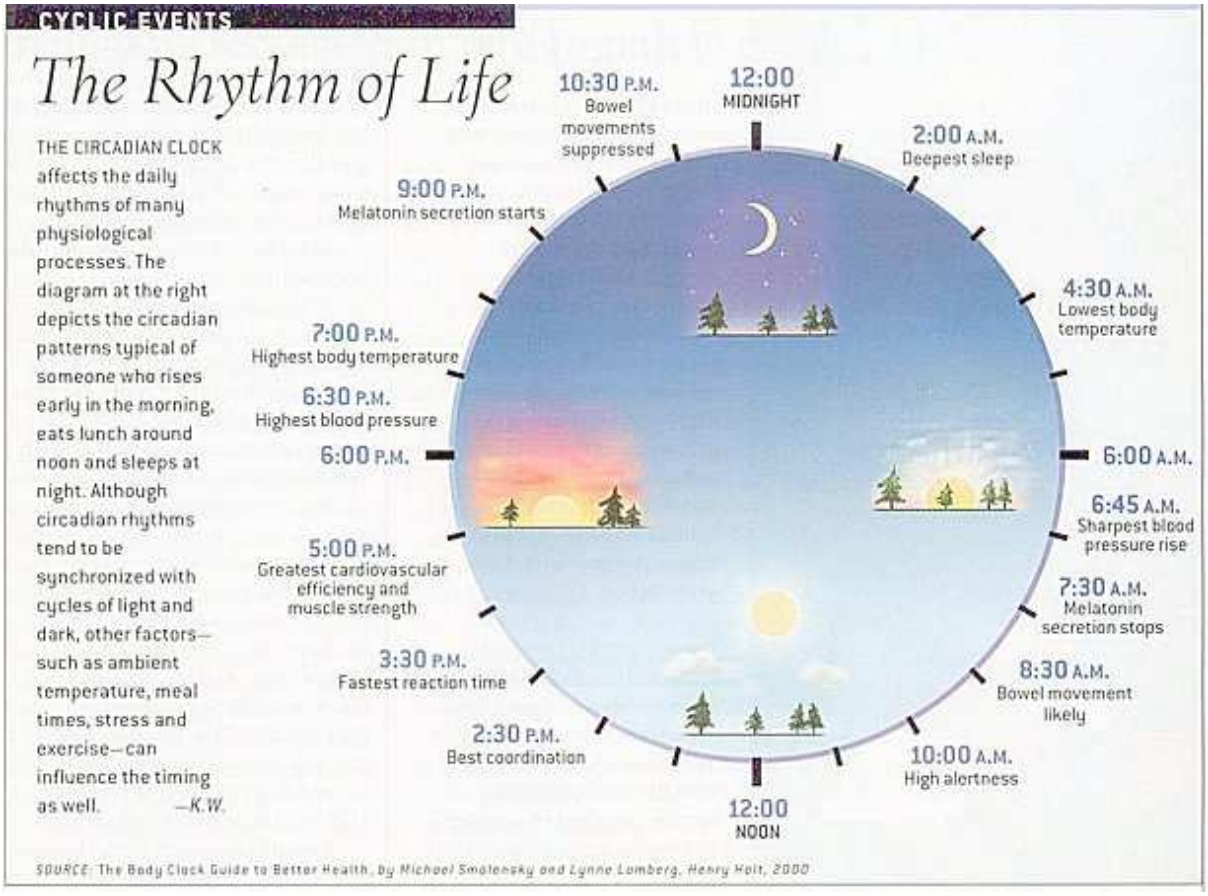


Figure 3. Representation of circadian rhythm over 24-hour human daily activities.



Reference: [32] Smolensky M, Lamberg L. The Body Clock Guide to Better Health: How to Use Your Body's Natural Clock to Fight Illness and Achieve Maximum Health. Henry Holt and Company, May 1, 2001.

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BRIEF REPORT

Overexpression of the *Per2* Gene in Male Patients with Acute Q Fever

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The prevalence of Q fever is higher in men than in women. Because the expression of circadian clock genes differs in male and female mice infected with *Coxiella burnetii*, we hypothesized that circadian genes are differently modulated in men and women with Q fever. The expression of the *Per2* gene was significantly ($P = .01$) increased in males with acute Q fever compared with healthy volunteers. No significant difference was observed in females. We showed for the first time that gender altered the expression of a circadian gene, *Per2*, in an infectious disease.

The clinical expression of Q fever, a worldwide zoonosis due to *Coxiella burnetii*, is affected by various host factors, including gender and age [1]. The male-to-female ratio of patients admitted to the hospital is 2.45 in adults [1]. In parallel, the rate of Q fever-related complications is higher in males than in females [1]. Men represent 75% of the patients diagnosed with *C. burnetii* endocarditis, the major manifestation of chronic Q fever [1].

In mice, it has been shown that sex hormones play a role in the pathophysiology of *C. burnetii* infection. The bacterial load is increased in ovariectomized mice, reaching levels found in males, and estradiol administration restores the bacterial load to levels found in intact females [2]. We have also shown that major components of the circadian rhythm pathway are affected by *C. burnetii* infection in females. The transcriptional expression of *Clock* and *Arntl* genes is downregulated, whereas that of *Per2* gene is upregulated in females but not in males [3]. It is likely that the circadian rhythm and the immune response are related [4]. Rodent models show that the circadian oscillation of clock genes affects interferon-γ production through *Per2* regulation or the activity of natural killer cells [5, 6].

To our knowledge, little is known regarding the relation between clock gene expression and human infectious diseases. This relation has never been investigated in Q fever patients. Here, we hypothesized that the expression of the *Clock*, *Arntl*, and *Per2* genes may be affected by *C. burnetii* infection in patients with acute and chronic Q fever. We showed that the expression of the *Per2* gene was specifically increased in men with acute Q fever. This result may indicate for the first time that a gene involved in the circadian clock is differentially modulated in men and women infected with a bacterial pathogen such as *C. burnetii*.

METHODS

The study was conducted with the approval of the Ethics Committee of the Aix-Marseille University, Marseille, France. Informed written consent was obtained from each participant. Blood samples from patients undergoing infectious disease consultations and healthy volunteers were collected in PAXgene Blood RNA tubes (Qiagen, Courtaboeuf, France). The samples were collected from 9:00 am to 11:00 am. The diagnosis of Q fever was performed as reported previously [7]. Q fever patients were split into "Acute" and "Endocarditis" according to their underlying disease [8]. The gene expression in blood samples was determined with real-time quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) as recently described [9]. In brief, total RNA was extracted after DNase digestion, according to the manufacturer's recommendations (Invitrogen, Life Technologies, Saint-Aubin, France). Real-time PCR with cDNA templates was performed using Light Cycler-FastStart DNA Master^{PLUS} SYBR Green I (Roche Diagnostics, Basel, Switzerland). The primers were designed with the free Web software Primer3 (http://frodo.wi.mit.edu/). The primers consisted of the forward sequences 5′-CGGAGT TAGAGATGG-TGGAAGA-3′, 5′-TACTGAGGAAAGGGAG GAGAGG-3′ and 5′-CCCTCTACCTGCTCAAA-GAAAA-3′ and the reverse sequences 5′-GGGACTGGAAA-ATGCT GAGTT-3′, 5′-AAGGATA-ACAGCAAAGCAGAGG-3′ and 5′-GCCCTCTGGTCT-ACAAAAACAA-3′ for the *Per2*, *Clock*, and *Arntl* genes, respectively. Quantitative RT-PCR experiments were performed using the gene encoding β-actin as the

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reference housekeeping gene. For each patient, the cycle threshold (CT) values of the genes of interest were normalized with the patient's CT values of β-actin to calculate the ΔCT. The results are expressed as the median and were compared using the nonparametric Mann–Whitney *U* test. A *P* value less than .05 was considered significant.

RESULTS

Our cohort consisted of 14 men (mean age of 51 ± 4 years), including 9 patients with acute Q fever and 5 with Q fever–related endocarditis; 6 women (mean age of 35 ± 3 years), including 5 patients with acute Q fever and 1 with Q fever–related endocarditis; and 12 healthy volunteers, including 6 men (mean age of 41 ± 4 years) and 6 women (mean age of 39 ± 5 years). With respect to gender, age did not differ significantly between the patients and the healthy volunteers. In contrast, among the patients, the men were older than the women (51 ± 4 vs 35 ± 3 years; $P = .02$). No difference was noted between men and women in the healthy volunteers.

In men, the expression of the *Per2* gene was significantly higher in the patients with acute Q fever than in the healthy volunteers (FC = 3.1; $P = .01$). No significant difference of expression was observed between Q fever–related endocarditis and healthy controls ($P = .18$) (Figure 1*A*). In women, the *Per2* expression ratio between acute Q fever and healthy controls did not reach a significant level (FC = 1.8; $P = .18$) (Figure 1*B*). In contrast to the expression of the *Per2* gene, the expression of the *Clock* and *Arntl* genes was not affected in men or women with Q fever compared with healthy volunteers (Figure 1*C*–*F*).

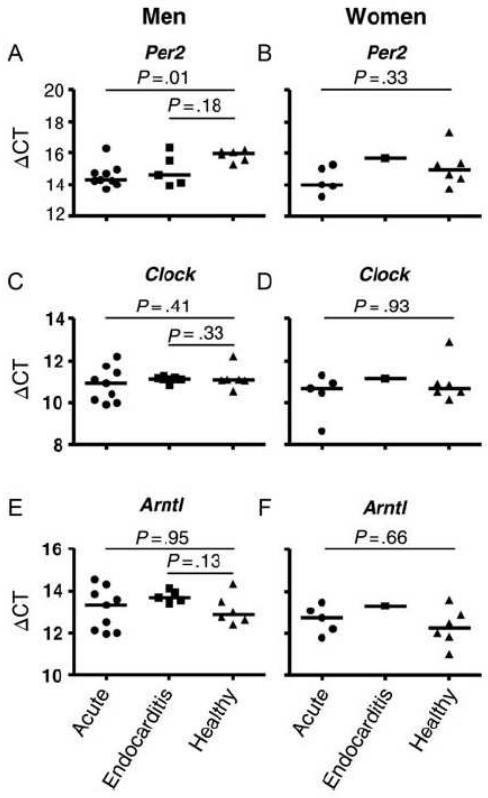

Figure 1. Gene expression in Q fever patients and healthy volunteers according to gender. Blood samples were collected and analyzed for the expression of the *Per2*, *Clock*, and *Arntl* genes with qRT-PCR in men (*A*, *C*, and *E*, respectively) and women (*B*, *D*, and *F*, respectively). The results were normalized with the β-actin gene. Acute: patients with acute Q fever. Endocarditis: patients with *C. burnetii* endocarditis. Healthy: healthy volunteers. Abbreviations: CT, cycle threshold; qRT-PCR, quantitative reverse transcriptase polymerase chain reaction.

DISCUSSION

To our knowledge, we showed significant changes in circadian clock–related gene expression in a human infectious disease for the first time. Indeed, the expression of the *Per2* gene was increased in men but not in women with acute Q fever. This result confirmed that *C. burnetii* infection partly affects the circadian clock, as demonstrated in mice [3]. This effect was independent of the time of blood collection because blood was sampled within a 2-hour period.

The human infection shows striking differences compared with the murine model of *C. burnetii* infection. First, the expression of the *Per2* gene is upregulated in female mice [3], but it is obvious that the circadian clock is inverted in rodents and humans [10]. Second, the expression of the *Arntl* and *Clock* genes is modulated in female mice infected with *C. burnetii*. However, this finding was not confirmed in the present study. These differences may be induced by specificities at the tissue level. Indeed, the expression of the *Per2*, *Arntl*, and *Clock* genes was studied in the liver of mice, whereas blood samples were collected in humans. Note that significant differences have been reported in the expression of the *Clock* gene between peripheral tissues and blood [11].

In humans, the exposure to *C. burnetii* is similar for both genders, although the prevalence of Q fever is higher in men than in women [1]. This explains the small number of women included in our study. Murine models have shown that the bacterial load is higher in the spleen of males than in females [2]. In *Drosophila*, phagocytosis is circadian-regulated by the protein Timeless. This protein appears to modulate an upstream event in phagocytosis [12]. Because the survival of *C. burnetii* in macrophages is mediated by a subversion of receptor-mediated phagocytosis [13], this result suggests that the impaired bacterial clearance in males may be related to these circadian clock alterations [2, 3]. Future studies are required to test this hypothesis.

Interestingly, the expression of the *Per2* gene was increased in men with acute Q fever but not in those with *C. burnetii* endocarditis, suggesting that the expression of the *Per2* gene is related to the clinical status of the patient. It has been demonstrated that the immune response of patients with acute Q fever is largely different from that of patients with *C. burnetii* endocarditis [8].

Among the factors affecting the chronic evolution of Q fever, aging may be essential. Indeed, in mice, aging is associated with a reduced amplitude of circadian clock related–gene expression [14]. Age affects both the expression of circadian clock–related genes and the response of mice to *C. burnetii* infection [15]. In our cohort, the female patients were younger than the male patients. Future studies are required to identify the correlation between age and gender in the expression of the *Per2* gene.

In conclusion, we reported for the first time that the expression of the *Per2* gene was increased in men with acute Q fever but not in women. This gender dimorphism may be related to the higher incidence and severity of Q fever in men than in women. This finding provides new perspectives to investigate the pathophysiology of Q fever.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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Chapter V

The ligands of Numb proteins X1 and X2 are specific markers for chronic Q fever

Mehraj V, Boucherit N, Ben Amara A, Capo C, Bonatti S, Mege JL, Mottola G, Ghigo E. FEMS Immunol. Med. Microbiol.

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Chapter V

The ligands of Numb proteins X1 and X2 are specific markers for chronic Q fever

The enzymes E3 ubiquitin ligases give specificity to the ubiquitination process, which results in protein degradation [33, 34]. From the recently published data in the last few years, it has been reported that these enzymes play an important function in regulating immune response [35-37]. Two of E3 ubiquitin ligases are the ligands of Numb proteins X1 and X2 (LNX1 and LNX2), which have been described as interactors and regulators of the T-cell co-receptor CD8 by promoting its ubiquitination and endocytosis [15]. The ligands of Numb proteins are reported to regulate the ubiquitination and endocytosis of T-cell co-receptor CD8, which plays an essential role in T-cell-mediated immune response. CD8 has been reported to be expressed on the surface of many cells including intestinal T cells, γδ T cells, NK cells etc... [38, 39]. CD8 is also required for the activation of cytotoxic T lymphocytes by stabilizing the interaction between T cell receptors (TCR) and class II major histocompatibility complex present on antigen-presenting cells [40]. The importance of $CD8^+$ T cell response in *C. burnetii* infection has been recently reported [16]. Nude and SCID mice are highly sensitive to *C. burnetii* infection, whereas wild type mice are resistant. The reconstitution of SCID mice with $CD4^+$ T cells or $CD8^+$ T cells is sufficient to control infection but, surprisingly, $CD8^+$ T cells play a more significant role in controlling splenomegaly.

In this study, we investigated the expression of *LNX* genes in patients with Q fever to tempt to identify specific biomarkers. We show a high level of *LNX1* and *LNX2* transcripts in Q fever endocarditis, but not in acute Q fever, suggesting that they may be used as specific markers of Q fever endocarditis.

FEMS IMMUNOLOGY & MEDICAL MICROBIOLOGY

SHORT COMMUNICATION

The ligands of Numb proteins X1 and X2 are specific markers for chronic Q fever

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LNX; Q Fever; CD8.

Abstract

Q fever is a disease caused by *Coxiella burnetii*, an obligate intracellular bacterium. Acute Q fever is spontaneously resolutive and is characterized by an efficient immune response. In contrast, chronic Q fever is characterized by dysregulated immune response, as demonstrated by the failure of *C. burnetii* to induce lymphoproliferation and the lack of granulomas. Recently, it has been demonstrated that when co-expressed in heterologous mammalian cell lines, the ligands of Numb proteins X1 and X2 (LNX1 and LNX2) regulate the level of the T-cell co-receptor CD8, which plays an essential role in T-cell-mediated immune response. We decided to investigate the expression of LNX1 and LNX2 genes in patients with acute or chronic Q fever. Interestingly, we found a high level of LNX1 and LNX2 mRNAs in endocarditis, the principal manifestation of chronic Q fever, but not in acute Q fever. Our data suggest that LNXs may be used as complementary biomarkers to follow the prognosis of chronic Q fever.

Introduction

Q fever is a zoonosis caused by *Coxiella burnetii*, an obligate intracellular gram-negative bacterium. While acute Q fever is spontaneously resolutive, the chronic disease is characterized by impaired immune response. The control of infection in patients with primary Q fever involves a systemic cell-mediated immune response and granuloma formation with an essential role for interferon-γ in the protection against *C. burnetii*. In contrast, chronic Q fever that essentially manifests as an endocarditis is characterized by defective cell-mediated immune response with defective formation of granulomas, failure to induce lympho-proliferation and interferon-γ production, and over-production of interleukin-10. Although T cells are essential for *C. burnetii* clearance (Andoh *et al.*, 2007), the mechanisms leading to T-cell responses to *C. burnetii* remain poorly understood.

E3 ubiquitin ligases give specificity to the ubiquitination process, which results in protein degradation (Mukhopadhyay & Riezman, 2007; Piper & Luzio, 2007). In the last years, it has been emerging that they play an important function in regulating immune response (Gomez-Martin *et al.*, 2008; Bhoj & Chen, 2009; Deshaies & Joazeiro, 2009). In particular, the ligands of Numb proteins X1 and X2 (LNX1 and LNX2) have been described as interactors and regulators of the T-cell co-receptor CD8 by promoting its ubiquitination and endocytosis (D'Agostino *et al.*, in press). CD8 is expressed on the surface of intestinal T cells, γδ T cells, thymic T-cell precursors, NK cells, thymocytes and peripheral T cells (Irie *et al.*, 1995; Gangadharan & Cheroutre, 2004). The activation of cytotoxic T lymphocytes requires CD8 that stabilizes the interaction between lymphocyte TCR and class I major histocompatibility complex present on antigen-presenting cells. Indeed, the development of cytotoxic T lymphocytes is greatly reduced in $CD8\alpha^{-/-}$ mice (Fung-Leung *et al.*, 1991) and defects in CD8α and β expression are observed in immunodeficiencies and Wiskott–Aldrich syndrome (Kawabata *et al.*, 1996; Schmitz *et al.*, 1998; de la Calle-Martin *et al.*, 2001).

The importance of T $CD8^+$ cell response in *C. burnetii* infection has been recently reported (Read *et al.*, 2010). Nude and SCID mice are highly sensitive to *C. burnetii* infection, whereas wild type mice are resistant. The reconstitution of SCID mice with $CD4^+$ T cells or $CD8^+$ T cells is sufficient to control infection but, surprisingly, $CD8^+$

T cells play a more significant role in controlling splenomegaly. Thus, in this study, in order to identify biomarkers for the Q fever, we decided to investigate the expression of LNX genes in patients with Q fever. Written, informed consent was obtained from each subject, and the present study was approved by the Ethics Committee of the Université de la Méditerranée. Total mRNA was extracted from blood collected in PAXgene blood RNA tubes. The cDNAs were prepared by reverse transcription and the expression of LNX1 and LNX2 genes was assessed by quantitative real time PCR on Smart Cycler system Cepheid, using syber green method as previously described (Benoit *et al.*, 2008). Specific primers were: for LNX1, forward 5′-GGAATTACCACGGTGCTTGTAT-3′, reverse 5′-TGTATGC-TGGTGTTCCTTCAAC-3′; for LNX2, forward 5′-TCTTCATAAACG-GGACTCTGGT-3′, reverse 5′GCT-GCTATGATTTCCTGCTTCT-3′; β-actin, forward 5′-GG-AAATCGTGCGT-GACATTA-3′, reverse 5′AG GAAGGA-AGGCTGGAAGAG-3′ (D'Agostino *et al.*, in press). LNX1 and LNX2 mRNAs were detected in the blood from healthy donors (Fig 1a). In healthy controls ($n = 3$) the average cycle threshold (CT) values for LNX1 and LNX2 genes were 25.3 and 23.9, respectively (Fig 1a). Patients with acute Q fever ($n = 10$) were diagnosed by detection of IgM and IgG against *C. burnetii* and patients with chronic Q fever ($n = 10$) using modified Duke's criteria (Dupont *et al.*, 1994). The expression of LNX1 and LNX2 genes in these patients was compared to the one in the healthy controls by using fold change (FC) values calculated as follows: $FC = 2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = (Ct\ Target - Ct\ Actin)_{patient} - (Ct\ Target - Ct\ Actin)_{control}$. In patients with acute Q fever, the value of FC was 1.4 ± 1.1 and 2.4 ± 1.4 for LNX1 (Fig 1b) and LNX2 (1c), respectively, demonstrating that the expression of these genes was not affected in acute Q fever. In contrast, the expression of LNX1 and LNX2 genes was increased in patients with chronic Q fever. Indeed, we found a significantly increased FC value (11.6 ± 2.6, $P < 0.0001$) for the expression of LNX1 in patients with chronic Q fever compared with acute Q fever patients, as determined using Mann–Whitney *U*-test (Fig 1b). Similar increase (FC value: 10.7 ± 3.4, $P < 0.0001$) was observed for LNX2 gene as well (Fig 1c). The increased expression of LNX1 and LNX2 genes in patients with chronic Q fever was not due to different number of $CD8^+$ T cell in patients with acute or chronic Q fever (Fig 1d).

It has been recently reported that T $CD8^+$ cell response is important in the control of *C. burnetii* infection (Read *et al.*, 2010). It is likely that the role of $CD8^+$ T cells in *C. burnetii* infection is related to their ability to produce IFN-γ, which may also be produced by Th1 $CD4^+$ T cells. The essential role of IFN-γ in protection against

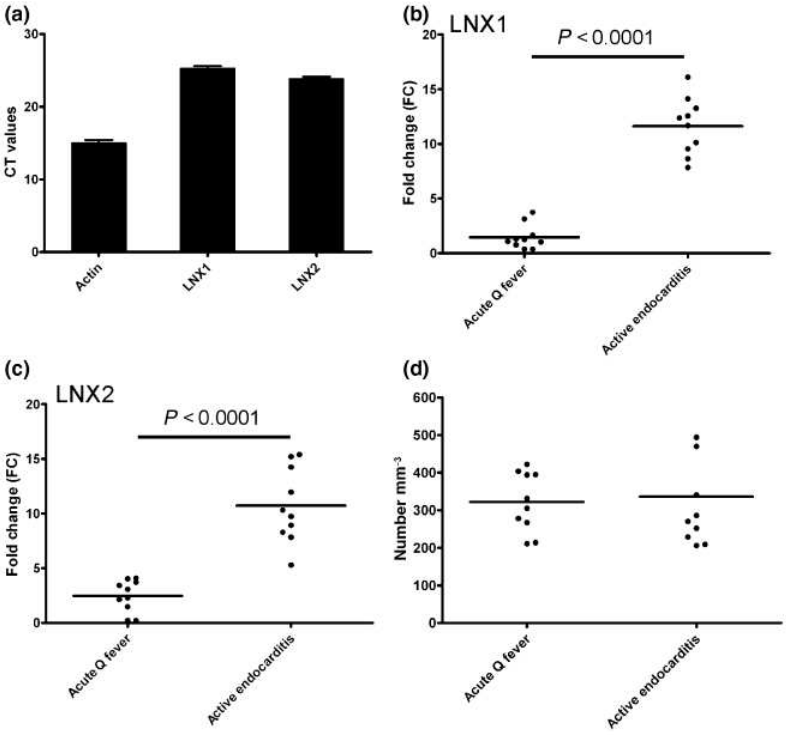

Fig. 1. Expression of LNXs in Q fever patients. The expression of LNX1 and LNX2 genes in controls (a) and in patients with Q fever (b, c) was monitored by quantitative RT-PCR. Results representative of three different experiments performed in each patient are expressed as Fold Change. (d) Numeration of $CD8^+$ T cells in Q fever patients. In (b) and (c) the *P* values are < 0.0001.

C. burnetii is supported by the high mortality rate observed in IFN-$\gamma^{-/-}$ mice infected with *C. burnetii*.

To our knowledge, it is the first time that the expression of LNXs was studied in an infectious disease. Our results show that LNX1 and LNX2 genes were over-expressed in chronic Q fever but not in acute Q fever. Although the direct evidence that LNX1 and LNX2 do regulate CD8 protein expression in lymphocytes is still lacking (D'Agostino *et al.*, in press), our results strongly support this hypothesis and suggest that LNX1 and LNX2 overexpression might reflect an alteration in CD8 level and might explain the imbalance of immune response in chronic Q fever through their action on T $CD8^+$ cells. Moreover, our data envisage the possibility in future studies to use also the monitoring of LNX genes expression together with the previously described biomarkers of chronic Q fever.

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Authors' contribution

G.M. and E.G. contributed equally to this work.

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Chapter VI

Chronic hepatitis E infection is specifically associated with an interferon-related transcriptional program

Moal V, Textoris J, Ben Amara A, Mehraj V, Berland Y, Colson P, Mege JL. **J. Infect. Dis. 2013; 207(1):125-32.**

Chapter VI

Chronic hepatitis E infection is specifically associated with an interferon-related transcriptional program

Hepatitis E virus (HEV) is a small non-enveloped RNA virus, which is the leading cause of waterborne acute hepatitis in adults in developing countries [41, 42]. It is an emerging causative agent of autochthonous acute hepatitis in humans in European countries [41, 42]. In most of the cases HEV hepatitis is an acute, self-limiting infection. In some cases the disease exhibits a severity that may lead to fulminant hepatitis. Recent studies suggest HEV as a new causative agent of chronic hepatitis in solid organ transplant recipients throughout the world [43-47]. The incidence of HEV infection after kidney transplantation has been estimated to be 2.7 cases/100 person-years [48], and half of the kidney transplant recipients infected with HEV develop chronic HEV infection (CHE) [17]. Rapid progression to cirrhosis, liver transplantation, and death has also been reported [43, 45, 49]. Clinical studies [50-52] suggest that the occurrence and persistence of chronic HEV infection are related to the immunological status of patients.

In this prospective study, the transcriptional profiles of whole blood from kidney transplant recipients with CHE and matched kidney transplant recipients without viral infection were compared. Microarray studies demonstrated that patients with CHE exhibited a specific peripheral signature including 30 up-regulated genes, 25 of which are annotated interferon-stimulated genes (ISGs). The up-regulation of 5 type I ISGs selected from the CHE signature was confirmed by qRT-PCR. Interestingly, the expression level of these genes was associated with the persistence of HEV infection. These results suggest that the persistence of HEV infection is related to increased expression of ISGs and that studying the peripheral

transcriptional signature may be useful in understanding the chronic evolution of HEV infection in kidney transplant recipients.

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MAJOR ARTICLE

Chronic Hepatitis E Virus Infection Is Specifically Associated With an Interferon-Related Transcriptional Program

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Background. Hepatitis E virus (HEV) is a new causative agent of chronic hepatitis in solid organ transplant recipients. Clinical studies suggest that the occurrence and persistence of chronic HEV infection are related to the immunological status of patients.

Methods. We used whole-genome microarray and quantitative reverse transcription polymerase chain reaction (qRT-PCR) to compare the transcriptional profiles of whole blood from 8 kidney transplant recipients with chronic HEV infection and 8 matched kidney transplant recipients without HEV infection.

Results. We found that 30 genes in HEV-infected patients were upregulated, compared with those in control patients, as determined by microarray analysis. In contrast, no genes were downregulated. The 30 upregulated genes included 25 interferon-stimulated genes. Increased expression of the genes that encode IFIT1, IFI44L, RSAD2, EPSTI1, and ISG15 was confirmed by qRT-PCR. Interestingly, the expression levels of these genes were associated with the persistence of HEV infection.

Conclusions. Increased expression of interferon-stimulated genes may favor the persistence of an HEV infection. Whether the expression of interferon-stimulated genes is a marker of ongoing viremia or independent prognostic marker of HEV clearance needs further investigations.

Clinical Trials Registration. NCT01090232.

Hepatitis E virus (HEV) is a small nonenveloped virus, and its genome is a single-stranded, linear, positive-sense RNA [1]. In developing countries, HEV is a leading cause of waterborne acute hepatitis in adults [2, 3]. In Europe, HEV causes a porcine zoonosis and is an emerging causative agent of autochthonous acute hepatitis, which is caused by HEV genotype 3. HEV genotypes 1 and 2 are involved in sporadic cases and major outbreaks of HEV infection in Asia, Africa, and Central America [2, 3]. Although HEV hepatitis is an acute, self-limiting infection, the disease exhibits a severity varying from unapparent infection to fulminant hepatitis. Chronic HEV infections were first described in solid organ transplant recipients in 2008 [4, 5] and have been subsequently reported by several European teams [6–8]. This emerging disease is defined as a persistent viral infection, with hepatitis lasting for >6 months. The incidence of HEV infection after kidney transplantation has been estimated to be 2.7 cases/100 person-years [9], and half of the kidney transplant recipients infected with HEV develop chronic HEV infection (CHE) [10]. Rapid progression to cirrhosis, liver transplantation, and death has also been reported [5, 6, 11]. No curative treatment for HEV hepatitis is currently recommended. Both pegylated interferon and ribavirin were effective in treating CHE in few patients, but interferon is contraindicated in kidney transplant recipients [12, 13].

The evolution of HEV infection toward CHE seems to be dependent on the immunological status of the

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patient. Indeed, CHE has been described only in severely immunocompromised patients, namely solid organ transplant recipients, human immunodeficiency virus–infected patients [14, 15], and individuals with hematological diseases [12]. In solid organ transplant recipients, CHE development has been found to be related to the dose or the type of immunosuppressive drugs taken [10, 16], and one-third of CHE patients clear HEV after reduction of the dose of immunosuppressive drugs [10]. The mechanisms leading to CHE are poorly understood. CHE has been recently associated with impaired HEV-specific T-cell responses in organ transplant recipients [17]. On the other hand, a microarray analysis performed on the livers of chimpanzees that were experimentally infected with HEV showed enhanced expression of interferon-stimulated genes (ISGs), which suggests activation of the interferon response by HEV [18].

In the prospective study presented here, we compared the transcriptional profiles of whole blood from kidney transplant recipients with CHE and matched kidney transplant recipients without viral infection. Microarray studies demonstrated that patients with CHE exhibited a specific peripheral signature including 30 upregulated genes, 25 of which are annotated ISGs. The upregulation of 5 type I ISGs selected from the CHE signature was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Interestingly, the expression level of these genes was associated with the persistence of HEV infection. These results suggest that the persistence of HEV infection is related to increased expression of ISGs and that studying the peripheral transcriptional signature may be useful in understanding the chronic evolution of HEV infection in kidney transplant recipients.

MATERIALS AND METHODS

Patients

The registry number of the clinical trial is NCT01090232. The study protocol was approved by the Comité de Protection des Personnes Sud Méditerranée I, the ethical committee of the Assistance Publique-Hôpitaux de Marseille. Patients signed an informed consent form to participate in the study.

Sixteen kidney transplant recipients monitored at the Marseille University Hospital were enrolled in the study from May to November 2010. Eight control patients and 8 patients with CHE (CHE patients) were matched for sex, age, time since kidney transplantation, and immunosuppressive treatment (presence of a calcineurin inhibitor). CHE was defined by the persistence of HEV RNA in the blood for >6 months. The date of HEV infection was considered to be the first date on which elevated liver enzyme levels were observed. Consumption of uncooked pig liver sausage, which has been identified as a risk factor for HEV transmission to humans [19], was reported in 5 patients. The mode of HEV transmission for the 3 remaining patients was not known. At inclusion, no CHE patient had received antiviral treatment. Control patients were free of acute or chronic viral infection at the time of inclusion. Serological determination of hepatitis B virus surface antigen, human immunodeficiency virus, and hepatitis C virus (HCV) was negative for all patients. Their clinical characteristics are presented in Table 1. Briefly, the median age of patients was 55 years (range, 44–77 years). The median time since kidney transplantation at inclusion was 87 months (range, 43–182 months). The median time since HEV infection was 44 months (range, 12–75 months) in CHE patients. Results of blood liver tests were normal for all of the control patients. The numbers of peripheral leukocytes were similar in CHE and control patients.

The virological status of CHE patients was determined as follows. Serial dilutions of a plasmid containing a specific HEV region were used for HEV RNA quantification. Serum HEV RNA was detected using an in-house qRT-PCR assay and sequencing of the partial open reading frame 2 of the viral genome, as described previously [19]. Phylogenetic analysis revealed that all of the viral strains belonged to genotype 3. Four patients were infected with subtype 3f, 3 were infected with subtype 3c and 1 was infected with subtype 3e.

Microarray Analysis

Whole blood (2.5 mL) was collected from patients by use of PAXgene blood RNA tubes (Qiagen), and samples were stored at −80°C before RNA extraction. Microarrays were performed as described [20]. In brief, the RNA was extracted using the RNeasy Mini Kit (Qiagen) after a DNase I step to eliminate DNA contaminants. The quantity and the quality of RNA were assessed using a Nanodrop (Thermo Science) and a 2100 Bioanalyzer (Agilent Technologies), respectively. The microarray study was performed using Agilent 4X44 K Whole Human Genome microarrays, which include 45 000 probes. The RNA was labeled with cyanine-3 CTP by using an Agilent Low RNA Input Fluorescent Amplification kit. The samples were deposited on microarray slides, and the hybridization was performed using the QIamp labeling kit. The slides were scanned with a G2505B DNA microarray scanner (Agilent Technologies) at a resolution of 5 µm. The images were analyzed with Feature Extraction Software 10.5.1.

The microarray data analysis was performed, and the figures were created using R (v.2.13) and the Bioconductor software suite. The raw data were filtered and normalized using the *Agi4 × 44PreProcess* library. Unsupervised and supervised analyses were carried out using hierarchical clustering, principal component analysis (*made4* library) [21] and the significance analysis of microarray (SAM) algorithm (*siggenes* library) [22]. Genes were considered to be differentially expressed when the false-discovery rate [23] was <10%, and the absolute fold-change (FC) was >2.0. Functional

Table 1. Characteristics of Patients With Chronic Hepatitis E Virus Infection (CHE) and Control Patients at Inclusion

Patient, by Group	Age (y)	Sex	Time Since Event (mo)		HEV Load (log_{10} copies/mL)[a]	ALT Level (IU/L)[b]	AST Level (IU/L)[c]	GGT Level (IU/L)[d]	TB Level (μmol/L)[e]	Immunosuppressive Treatment[f]
			Kidney Transplantation	HEV Infection						
CHE										
A	60	M	152	75	6.7[g]	80	65	119	17	Tac, Pr
B	52	F	43	30	7.6	39	25	18	NA	Tac, MPA, Pr
C	46	M	72	45	7.9[h]	9	21	50	13	Tac, MPA, Pr
D	56	M	62	47	3.2	16	16	28	11	Tac, MPA, Pr
E	68	M	182	48	6.0	72	56	35	13	CsA, Pr
F	47	M	75	13	NA	43	36	60	NA	Tac, MPA, Pr
G	47	M	85	21	3.8	24	28	19	24	Tac, MPA, Pr
H	76	M	160	43	5.9	169	114	NA	NA	Tac, Pr
Control										
A	60	M	167	…	…	11	16	NA	NA	Tac, MPA, Pr
B	50	F	44	…	…	22	27	39	NA	Tac, MPA, Pr
C	47	M	71	…	…	28	20	37	NA	CsA, MPA, Pr
D	58	M	62	…	…	19	26	26	20	CsA, Aza, Pr
E	44	M	94	…	…	44	25	NA	NA	Tac, Pr
F	46	M	88	…	…	14	15	10	15	CsA, Aza, Pr
G	68	M	176	…	…	21	23	53	NA	CsA
H	74	M	133	…	…	18	13	33	NA	CsA, Pr

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; Aza: azathioprine, CsA, cyclosporin; GGT, gamma-glutamyltranspeptidase; MPA, mycophenolic acid; NA, not available; Tac, tacrolimus; TB, total bilirubin; Pr, prednisone.

[a] At inclusion, except for CHE patients A and C.

[b] Reference, 3–41 IU/L.

[c] Reference, 0–38 IU/L.

[d] Reference, 8–60 IU/L.

[e] Reference, <26 μmol/L.

[f] Received at the time of HEV diagnosis.

[g] Measured 32 days before inclusion.

[h] Measured 10 days before inclusion.

enrichment analysis was performed on selected genes with the DAVID bioinformatics tool [24], using the Gene Ontology (GO) [25], INTERPRO [26], and KEGG pathways [27, 28]. Key words were selected when the corrected *P* value (Benjamini-Hochberg) for enrichment was <.01. The data were generated in compliance with the Minimum Information About a Microarray Experiment (MIAME) guidelines and were deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (accession number GSE36539).

qRT-PCR

qRT-PCR was carried out as described elsewhere [20]. Reverse transcription of 100 ng of RNA was performed with the MMLV-RT kit (Invitrogen). Complementary DNA was obtained using oligo(dT) primers and MMLV reverse transcriptase (Invitrogen), and qPCR experiments were performed using SYBR Green Fast Master Mix (Roche Diagnostics) and a SmartCycler (Cepheid). The primers were designed using Primer3 [29], and their sequences are provided in Supplementary Table 1. The results were normalized using the housekeeping gene β-actin and are expressed as $FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = [(Ct^{Target} - Ct^{Actin})_{CHE} - (Ct^{Target} - Ct^{Actin})_{control}]$, as described elsewhere [30].

Statistical Analyses

Qualitative parameters were expressed as the median and min-max range. Quantitative parameters were expressed as absolute counts and percentages. Differences between groups were assessed for statistical significance with the nonparametric Mann-Whitney *U* test, with a *P* value cutoff of .05. The experimental data were presented as medians and interquartile ranges.

RESULTS

Transcriptional Signature of CHE Patients

A whole-genome microarray approach was used to define the peripheral transcriptional signature of CHE. The principal

component analysis showed that the overall gene expression of CHE patients and control patients was organized in 2 different groups (Figure 1*A*). Supervised analysis using the SAM algorithm identified 41 probes, corresponding to 30 genes that were upregulated in CHE patients, compared with control patients (Figure 1*B*). In contrast, no genes were downregulated. The ratio of gene expression ranged from 2.5- to 15.2-fold between CHE patients and matched control patients. Of note, 1 CHE patient (D_P) showed an atypical pattern of gene expression, regardless of the gene studied.

When we performed functional enrichment analysis of the 30 genes that were upregulated in CHE patients, we found that 20 genes were related to the innate immune response. When we compared the CHE transcriptional signature with the recently established Interferome database, which contains 1996 ISGs [31], we found that 25 of the 30 genes that were upregulated in CHE patients (83% of the total number of modulated genes) were annotated ISGs. The DAVID tool isolated 2 particular groups of genes associated with the interferon pathway. The first group was composed of 4 genes that are known to encode interferon-induced proteins with tetratricopeptide repeats (IFIT1, IFIT2, IFIT3, and IFIT5). The second group was composed of 3 genes that share 2′-5′-oligoadenylate synthetase–related domains (*OAS2*, *OAS3*, and *OASL*). The other upregulated genes of the interferon pathway included the genes that encode CXCL10, a ubiquitin-like modifier (ISG15), HERC5, interferon-induced protein 44-like (IFI44L), MX1, radical S-adenosyl methionine domain containing 2 (RSAD2), epithelial stromal interaction 1 (EPSTI1), and XAF1. The expression levels of 5 genes involved in the type I interferon response (*ISG15*, *IFIT1*, *IFI44L*, *RSAD2*, and *EPSTI1*) that were upregulated in the microarray were also determined by qRT-PCR. The microarray and qRT-PCR results were strongly correlated ($R^2 = 0.91$, $P = .0008$). We found that expression of the *ISG15*, *IFIT1*, *IFI44L*, *RSAD2*, and *EPSTI1* was upregulated in 7 CHE patients as compared to 7 matched control patients (Figure 2). Of note, both techniques indicated a higher interindividual variability of expression in CHE patients, compared with controls. Taken together, these results showed that the CHE patients exhibited a specific transcriptional program in which interferon-stimulated effectors were prominent.

CHE Signature and HEV Persistence

One can hypothesize that the interindividual variability between CHE patients that was found in the microarray and qRT-PCR may be related to the course of HEV infection. To address this issue, we sorted the CHE patients into 3 groups: the first one included patients who experienced early HEV clearance within 6 months after inclusion in the study

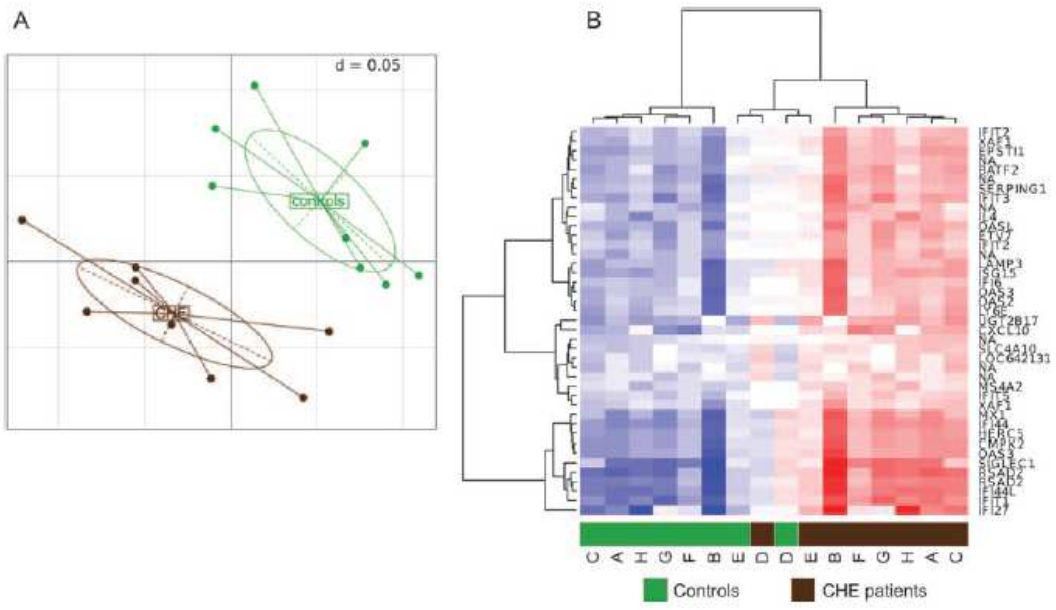

Figure 1. Chronic hepatitis E virus infection (CHE)–associated signature of gene expression. *A*, The graphical representation of principal component analysis shows a clear separation between CHE patients and matched controls. *B*, The gene expression signature associated with CHE is represented on a heat map. The CHE patients and control patients are represented in columns. The expression levels of the genes are color coded from blue to red. Dendrograms on the top (samples) and the left (genes) show the results of the hierarchical clustering that was computed with Pearson correlation distance and average linkage.

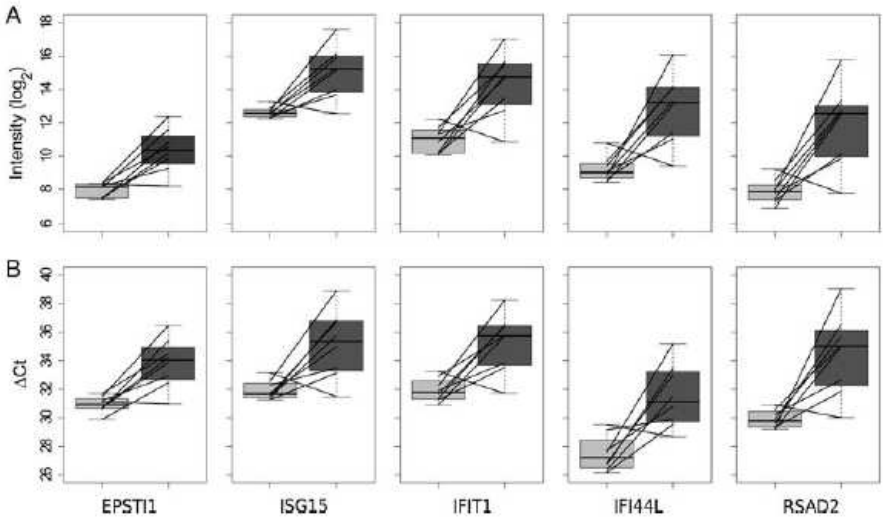

Figure 2. Validation of the microarray results by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Gene expression levels from the microarray (*A*) and qRT-PCR (*B*) are represented as box plots. Patients with chronic hepatitis E virus infection (CHE) are shown in light gray, and control patients are shown in dark grey. Pairings between the CHE and control patients are illustrated by straight lines. Ct, cycle threshold.

(median time, 4 months; range, 1.3–4.6 months), the second group included patients who experienced HEV clearance with a delay of >6 months after inclusion (median time, 11.5 months; range, 8.9–17.4 months), and the last group included patients whose HEV infection had not resolved at the time of data analysis (>17.4 months after inclusion). The patterns of gene expression observed by microarray differed considerably between the 3 groups of CHE patients. The expression of *ISG15*, *IFIT1*, *IFI44L*, *RSAD2*, and *EPSTI1* was significantly ($P \leq .01$) higher in patients who had not cleared the HEV infection than in patients who cleared the HEV infection early on (Figure 3). The ratios of gene expression ranged from 6.6-fold, for *EPSTI1*, to 25.6-fold, for *RSAD2*. The expression of *ISG15*, *IFIT1*, *IFI44L*, *RSAD2*, and *EPSTI1* was intermediate in patients who cleared HEV infection in its late stages. Similarly, the determination of gene expression levels by qRT-PCR showed that these genes were more highly expressed in patients who did not clear the HEV infection than in patients who had cleared the HEV infection in its early phases.

Finally, we attempted to analyze the interindividual variability between CHE patients and the viral load. The HEV RNA level was measured in 7 CHE patients. The HEV load was the lowest in patients who experienced early clearance of HEV infection, intermediate in patients who cleared HEV infection in its late stages, and highest in patients who had not cleared HEV infection (Figure 3). Taken together, these results suggest that upregulated expression of *EPSTI1*, *ISG15*, *IFIT1*, *IFI44L*, and *RSAD2* is associated with persistent HEV infection.

DISCUSSION

CHE has been increasingly described in kidney transplant recipients since 2008 [4, 5], but the underlying mechanisms of this new disease remain obscure. In this study, we compared the transcriptional profiles of whole blood from kidney transplant recipients with CHE and matched kidney transplant recipients without HEV infection. We found that CHE patients were characterized by a specific transcriptional signature consisting of 30 upregulated genes. Of note, 1 CHE patient showed an atypical gene expression pattern, compared with the other patients, but we were unable to explain this apparent discrepancy on the basis of clinical and biological data. Our results were close to those obtained in a microarray study conducted on liver biopsy specimens from chimpanzees. In that study, Yu et al found that 58 genes were upregulated and no genes were downmodulated in chimpanzees with acute HEV infection [18]. Interestingly, 20 genes that were upregulated in our study were also upregulated in chimpanzees, suggesting that the peripheral signature that we found in humans characterizes HEV infection.

The specific signature of CHE included 25 genes annotated as ISGs, which are known to be involved in antiviral activity. In our study, we found the genes encoding CXCL10 (also known as IP-10) and ISG15 to be upregulated. CXCL10 is a chemokine that attracts T lymphocytes, natural killer cells, and monocytes [32]. CXCL10 has been reported to be essential in the development of a protective response against viral infection [33]. ISG15, a ubiquitin homolog, has numerous

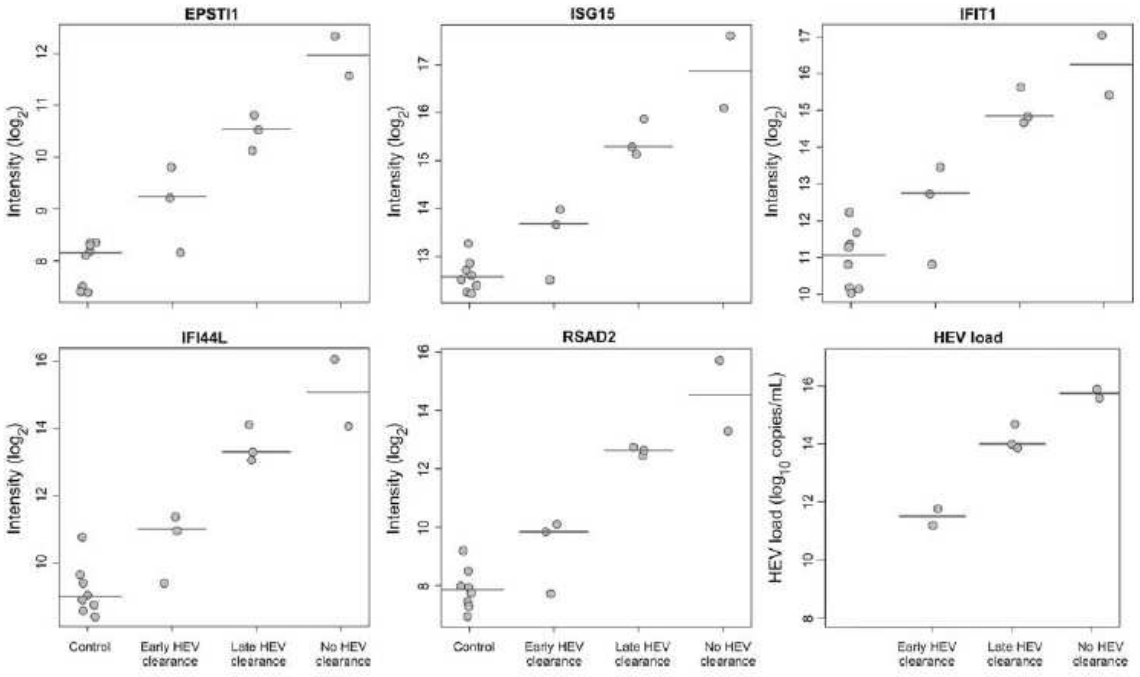

Figure 3. Gene expression signatures and persistence of hepatitis E virus (HEV) infection. Strip charts illustrate the relationship between expression levels of genes, as determined by microarray and the persistence of HEV infection. The strip chart (bottom, right) illustrates the relationship between blood HEV load and the persistence of HEV infection. The patients with chronic HEV infection were separated into 3 groups according to the time of HEV clearance (early, late, or no HEV clearance at the time of the analysis). The horizontal grey bars illustrate the median of each group.

target proteins that are important in the interferon response. These targets include transcription factors, such as Janus kinase 1 and signal transducer and activator of transcription 1 (STAT1); pattern-recognition receptors, such as retinoic acid–inducible gene I; and antiviral effector proteins, such as MX1, PKR, and RNaseL [34]. It has been demonstrated that increased levels of ISG15 lead to an increase in the antiviral activity of interferon [35], suggesting that the upregulated expression of *ISG15* in CHE patients is related to high antiviral activity. Other ISGs, such as *OAS2*, *OAS3*, *OASL*, and *MX1*, which were found to be upregulated in CHE patients, encode proteins known to be antiviral effectors [34]. In addition to these genes, other ISGs were upregulated. These included the genes that encode HERC5, a ligase that conjugates ISG15 to protein substrates; RSAD2 (viperin), one of the most highly induced interferon effector proteins; the IFIT complex (*IFIT1*, *IFIT2*, *IFIT3*, and *IFIT5* genes), which antagonizes viruses by sequestering specific viral nucleic acids [36]; and IFI44L, a paralog of IFI44, a 44-kD protein originally identified as the HCV-associated microtubular aggregate in the hepatocytes of chimpanzees infected with HCV [37]. Of note, it has been recently demonstrated that IFI44L has high anti-HCV antiviral activity [38]. Other genes that were upregulated in CHE patients encode proteins such as XAF1 and BATF2 (SARI), which have been reported to mediate apoptosis [39, 40] and may constitute a line of defense of immune cells against viruses [41]. To our knowledge, no antiviral function has been described for EPSTI1, another ISG expressed in tissues characterized by extensive epithelial-stromal interaction [42, 43]. Our results were consistent with the activation of the interferon system reported after acute HEV infection in chimpanzees [18]. In contrast, during persistent HEV infection of an epithelial cell line, it has been found that HEV inhibits type I interferon signaling through STAT1 phosphorylation. Interestingly, the expression of *MX1* and *OAS* genes is similar in infected and noninfected cells [44], whereas these genes were upregulated in CHE patients. It is likely that this in vitro model of HEV infection is not convenient for studying patients with CHE. We can hypothesize that the upregulated expression of ISGs in CHE patients is related to activation of the interferon pathway, as previously described for human viral infections, such as HCV infection [45–47].

We found that the ISG response of patients who did not clear their HEV infection was largely higher than the ISG response of patients who cleared HEV infection quickly. These results showed that kidney transplant recipients did not

spontaneously resolve CHE despite activation of the interferon system. It is likely that the increased expression of ISGs in CHE patients favors viral persistence through induction of a refractory state of the interferon signaling pathway. This hypothesis is supported by earlier observations demonstrating that strong induction of ISGs in the liver does not systematically lead to the resolution of chronic HCV infection; in fact, this induction impedes the response to interferon therapy [45–47]. It has been suggested that patients with chronic HCV infection exhibit defects in the downstream steps of ISG transcription, rendering them resistant to both endogenous interferon and interferon therapy [45]. In CHE, we presume that the absence of the few critical ISGs directly involved in antiviral activity prevents the elimination of HEV or that posttranscriptional modifications of critical ISG proteins alter their antiviral activity. Ribavirin, which was found effective to treat CHE in few kidney transplant recipients, has been reported to enhance the antiviral effects of interferon in chronic HCV infection through ISGs [48]. Nonetheless, it is difficult to speculate on the antiviral mechanism of ribavirin in HEV infection. Indeed, HEV clearance is rapidly achieved after ribavirin monotherapy in kidney transplant recipients [12] whereas ribavirin monotherapy is not efficient against HCV [48].

Finally, we found that the HEV loads were higher in patients who did not clear their HEV infection than in patients who cleared HEV infection, suggesting that timing of HEV clearance was associated with HEV loads. As the level of expression of selected ISGs was associated to the timing of HEV clearance, ISG expression may be a marker of ongoing viremia or an independent prognostic marker of HEV clearance. The absence of sequential assessment of ISGs did not allow the discrimination between these hypotheses. Further studies are needed to elucidate the role of ISGs in CHE.

In conclusion, the blood of kidney transplant recipients with CHE exhibited a specific transcriptional signature associated with a dysregulated interferon response that is correlated with a persistent HEV infection. Our results opened new ways to explore the mechanisms of chronic HEV infection in kidney transplant recipients and to improve the monitoring of these patients.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. All authors: No reported conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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CONCLUSIONS AND PERSPECTIVES

Conclusions and Perspectives

Transcriptional studies using high-throughput methods, such as whole genome microarrays, coupled with conventional approaches, such as quantitative real-time RT-PCR (qRT-PCR), are highly useful to re-evaluate the concept of macrophage polarization in infectious diseases [4]. The available literature on patients reveals that macrophage activation could not be predicted from the results of typical pathogen-macrophage interactions. The reappraisal of macrophage polarization by high-throughput methods is critical to understanding the role of macrophage polarization in infectious diseases. Only the identification of individual profiles will support promising therapeutic approaches based on target determination.

Our studies used the responses of monocytes to IFN-γ and IL-4 to guide the analysis of whole blood from patients with Q fever. First, we used microarray to compare monocytes and monocyte-derived macrophages. We found that the response of macrophages to IFN-γ and IL-4 was polarized while the responsiveness of monocytes was completely different from that of macrophages. The analysis of monocyte responses revealed two phases: an early phase (6 hour-stimulation) and a late phase (18 hour-stimulation). The response of the early phase was dependent on the agonist (IFN-γ and IL-4), suggesting that it may be related to the M1/M2 polarization observed in macrophages. We therefore tested this hypothesis using a panel of genes considered as specific of M1 and M2 macrophages. We found that 587 genes were modulated in IFN-γ- or IL-4-stimulated monocytes compared with unstimulated monocytes. We also studied the genes that were specifically modulated in the early response of monocytes to IFN-γ and IL-4: 46 and 39 genes were up-regulated, respectively. Among these up-regulated genes, only 16 M1 and 10 M2 genes were up-regulated, highlighting that the apparent polarization of monocytes was not similar to the M1/M2 polarization of

macrophages. The second phase of monocyte activation that was common to IFN-γ and IL-4 consisted of 57 up-regulated genes. It is noteworthy that macrophages are fully polarized into M1 or M2 macrophages when they are stimulated with IFN-γ and IL-4 for 18 hours (Martinez [4] and our results), demonstrating that the responsiveness of monocytes was completely different from that of macrophages. Second, we took advantage of these monocyte signatures to identify putative Q fever biomarkers. We selected 4 genes specific of the early IFN-γ signature (*NLRC5*, *RTP4*, *C1S*, *GCH1*), 4 genes specific of the early IL-4 signature (*ITGB3*, *TREM1*, *NEDD4L*, *RHOH*), and 4 genes specific of the late signature (*ALOX15*, *CLEC4F*, *CCL13*, *CCL23*) of monocytes, and we tested their modulation in the blood of patients with Q fever using qRT-PCR. The *NLRC5*, *RTP4* and *RHOH* genes present in the early signature were up-regulated in acute Q fever, but not in Q fever endocarditis. Conversely, the *ALOX15*, *CLEC4F*, *CCL13*, and *CCL23* genes present in the late signature of monocytes were specifically associated with Q fever endocarditis. Interestingly, the follow-up of patients showed that the expression of *NLRC5* and *RTP4* genes remained up-regulated 5 months after the initial inclusion in patients with acute Q fever whereas the expression of *RHOH* gene was similar to controls after 5 months. The modulation of *CCL23*, *CLECF4*, *ALOX15*, and *CCL13* genes was assessed in patients with Q fever endocarditis 16 months after the inclusion. The expression of *CCL13* and *CCL23* genes remained unchanged. By contrast, the expression of *ALOX15* and *CLECF4* genes decreased with time but only the expression of *ALOX15* gene returned to control levels. These results suggest that some biomarkers, namely *RHOH* and *ALOX15* genes, were associated with the activity of acute Q fever and Q fever endocarditis, respectively. Since our study was conducted on a small group of patients with limited clinical information and follow up data, well designed prospective studies on a larger cohort of patients will be set up to further explore the relevance of the present biomarker series for their potential in clinical use.

The microarray results obtained on monocytes open new perspectives in infectious diseases. Hence, we explored only a small part of genes from the early IFN-γ signature and genes from the late signature of monocytes by qRT-PCR. The other up-regulated (or down-modulated) genes may also be useful to define new biomarkers. We can hypothesize that some of the markers from the early response may allow clinicians to assess the pathophysiological responses to acute inflammatory diseases whereas the biomarkers from the late monocyte signature may be useful for analyzing chronic inflammatory and infectious pathologies. An adaptive therapeutic approach based on studies on monocyte signature could improve the prognosis of patients in a number of diseases. Additional developments aimed at limiting the pathogenic effect of an uncontrolled microbicidal M1 response may be also promising. We proposed the early and late response genes to be used as biomarkers, but have to be confirmed in a larger prospective cohort of patients with Q fever or in patients suffering from other infectious diseases.

It is known that gender plays an important role in the clinical expression of Q fever. Males are at a higher risk and the rate of Q fever-related complications is also higher in males than in females. It has been previously shown that major components of the circadian rhythm pathway are affected by *C. burnetii* infection in mice and these changes were controlled by sex hormones. We analyzed three genes (*Per2*, *ARNTL*, and *CLOCK*) involved in the circadian pathway in patients with Q fever. We showed that the expression of the *Per2* gene was specifically increased in men with acute Q fever compared with healthy volunteers. In contrast, the expression of *Clock* and *ARNTL* genes was not affected in men or women with Q fever compared with healthy volunteers. The relationship between the specific over-expression of *Per2* gene in men with acute Q fever and the higher incidence and severity of Q fever in men than in women needs further investigations. Nevertheless, to our knowledge it is the first time that it is demonstrated that a gene involved in circadian rhythm (over-expression

of *PER2* gene) is modulated in an infectious disease. This opens new perspectives in the understanding of infectious diseases. Further studies are required to determine which infectious diseases are associated with the modulation of the circadian cycle and how the circadian rhythm is affected in these diseases.

The enzymes E3 ubiquitin ligases including LNX1 and LNX2 degrade proteins and are known to regulate immune response. Particulary, LNX1 and LNX2 interact with CD8, which is expressed on a variety of cells including intestinal T cells, thymic T-cell precursors, NK cells, thymocytes and peripheral T cells. We found that *LNX1* and *LNX2* genes were over-expressed in Q fever endocarditis but not in acute Q fever. Although the direct evidence that LNX1 and LNX2 regulate the expression of CD8 protein in lymphocytes is still lacking [15], our results strongly support this hypothesis and suggest that LNX1 and LNX2 over-expression might reflect an alteration in CD8 level and might explain the imbalance of immune response in chronic Q fever through their action on T $CD8^+$ cells. Future prospective clinical studies with larger sample size are proposed to monitor *LNX* genes expression over the course of the disease.

In developing countries one of the leading causes of waterborne acute hepatitis is infection with hepatitis E virus (HEV). In European countries, HEV is an emerging causative agent of autochthonous acute hepatitis. In most of the cases, HEV hepatitis is an acute, self-limiting infection but, in some cases, the disease exhibits a severity varying from unapparent infection to fulminant hepatitis. In 2008 chronic HEV infections (CHE) were described in solid organ transplant recipients. This emerging disease is defined as a persistent viral infection, with hepatitis lasting for at least 6 months. The incidence of HEV infection after kidney transplantation has been estimated to be 2.7 cases/100 person-years and half of the kidney transplant recipients infected with HEV develop CHE. In severe cases, rapid progression of HEV disease to cirrhosis, liver transplantation, and death has also been reported. No curative

treatment for HEV hepatitis is currently recommended. Both pegylated interferon and ribavirin were effective in treating CHE in few patients, but interferon is contraindicated in kidney transplant recipients. Current literature coming from clinical studies suggests that the occurrence and persistence of CHE are related to the immunological status of patients. We compared the transcriptional profiles of whole blood from eight kidney transplant recipients with CHE and eight kidney transplant recipients without viral infection by microarray. Patients with CHE exhibited a specific peripheral signature including 30 up-regulated genes, 25 of which are annotated interferon-stimulated genes (ISGs). We then studied the expression levels of 5 genes involved in the type I interferon response (*ISG15*, *IFIT1*, *IFI44L*, *RSAD2*, and *EPSTI1*) by qRT-PCR. We found that the blood of kidney transplant recipients with CHE exhibited a specific transcriptional signature associated with a dysregulated interferon response that is related to persistent HEV infection. These results open new ways to explore the mechanisms of chronic HEV infection in kidney transplant recipients and to improve the monitoring of these patients.

Annexure I

Blood leukocytes and macrophages of various phenotypes have distinct abilities to form podosomes and to migrate in 3D environments

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Annexure I

Blood leukocytes and macrophages of various phenotypes have distinct abilities to form podosomes and to migrate in 3D environments

Leukocytes migrate through most tissues in the body, a process which takes place in 3D environments. It has been previously shown [53, 54] that macrophages use the amoeboid migration mode in porous matrices such as fibrillar collagen I and the mesenchymal mode involving podosomes and matrix proteolysis in dense matrices such as Matrigel. Whether such plasticity may apply to other leukocytes and to all subsets of macrophages is unknown. This study is a comparative analysis of the in vitro 3D migration modes adopted by primary human leukocytes. Blood-derived monocytes, neutrophils, and T lymphocytes were found to use the amoeboid mode in a porous fibrillar collagen I matrix but were unable to infiltrate dense Matrigel and to form podosomes. M2-polarized macrophages and elicited peritoneal macrophages formed podosome rosettes, degraded the ECM and infiltrated both matrices. In contrast, M1 macrophages were motionless in 2D and 3D environments, whilst resident macrophages, devoid of podosomes, were only able to use the amoeboid mode. In conclusion, we demonstrate that leukocytes use the amoeboid mode to migrate through porous matrices and that the macrophages may adopt the mesenchymal mode that permits migration through dense matrices.

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Blood leukocytes and macrophages of various phenotypes have distinct abilities to form podosomes and to migrate in 3D environments

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ABSTRACT

Leukocytes migrate through most tissues in the body, a process which takes place in 3D environments. We have previously shown that macrophages use the amoeboid migration mode in porous matrices such as fibrillar collagen I and the mesenchymal mode involving podosomes and matrix proteolysis in dense matrices such as Matrigel. Whether such a plasticity may apply to other leukocytes and to all subsets of macrophages is unknown. Here, we therefore provide a comparative analysis of the *in vitro* 3D migration modes adopted by primary human leukocytes. Blood-derived monocytes, neutrophils and T lymphocytes were found to use the amoeboid mode in a porous fibrillar collagen I matrix but were unable to infiltrate dense Matrigel and to form podosomes. M2-polarized macrophages and elicited peritoneal macrophages formed podosome rosettes, degraded the ECM and infiltrated both matrices. In contrast, M1 macrophages were motionless in 2D and 3D environments, whilst resident macrophages, devoid of podosomes, were only able to use the amoeboid mode. Thus, we conclude that whereas all leukocytes use the amoeboid mode to migrate through porous matrices, it is only certain macrophages that can adopt the mesenchymal mode that permits migration through dense matrices. Interestingly, the acquisition of mesenchymal migration capacity by macrophages correlates with the presence of podosomes and with their capacity to organize those as rosettes, which appears to be modulated by their differentiation and polarization states. As a perspective, specific control of the mesenchymal migration would be a potential target for therapeutic approaches aiming at decreasing macrophage tissue infiltration.

Introduction

Trafficking of leukocytes is a key process for immune cell development and host defence (reviewed in Friedl and Weigelin, 2008). Leukocytes are able to migrate through most tissues in the body and, *in vivo*, they encounter both 2-dimensional (2D) and 3-dimensional (3D) environments. 2D migration takes place along surfaces such as inner vessel walls and inner epithelial surfaces, whereas 3D migration takes place inside tissues composed of cells and extracellular matrix (ECM) protein scaffolds with heterogeneous composition and architecture.

We have shown that macrophages derived from blood monocytes (MDMs) can use either the amoeboid migration mode, a movement of rounded or ellipsoid cells that squeeze in and glide along the pores and gaps of a porous ECM, *e.g.* fibrillar collagen I (Friedl and Weigelin, 2008; Van Goethem et al., 2010) or the mesenchymal migration mode characterized by elongated cell morphology and requirement for proteases to progress in a non-porous environment such as Matrigel or gelled collagen I (Van Goethem et al., 2010). In environments with heterogeneous architecture, they combine the two migration modes (Guiet et al., 2011). From those studies, we concluded that the architecture of the matrix dictates the choice of the migration mode. Macrophages form podosomes constitutively in 2D environments (Linder, 2007).

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Podosomes are characterized as cell structures with proteolytic activity directed at the ECM, with a core of F-actin surrounded by a ring of adhesion receptors and actin-associated proteins such as vinculin and paxillin. In dense matrices, macrophages dig holes and form specialized F-actin rich structures called 3D podosomes at the tip of cell protrusions where proteolytic degradation of the matrix takes place (Van Goethem et al., 2010, 2011). We have previously proposed that podosomes are an essential requirement of the mesenchymal mode (Cougoule et al., 2010; Guiet et al., 2012), for the following reasons. First, 3D-podosomes are not formed in macrophages that use the amoeboid migration mode (Van Goethem et al., 2010). Second, Hck and Filamin A are two proteins involved in podosome stability, podosome organization as rosettes and ECM degradation, and both proteins are necessary for mesenchymal migration of macrophages but dispensable for amoeboid migration.

Macrophages can adopt the mesenchymal mode to infiltrate dense matrices (Van Goethem et al., 2010). The other leukocytes have been described to use the amoeboid migration mode (Friedl and Weigelin, 2008; Sabeh et al., 2009) but whether they can adopt the mesenchymal mode has not been investigated. Neutrophils have been described to use the amoeboid mode to infiltrate fibrillar collagen I (Sabeh et al., 2009) and podosome-related structures have been observed in murine neutrophils but the organization of vinculin as a ring and matrix proteolytic activity have not been documented to unequivocally characterize these structures (Calle et al., 2008; Szczur et al., 2006). T lymphocytes have been described to use the amoeboid migration mode in fibrillar collagen I (Wolf et al., 2003) and to also use podosome-related structures to palpate the surface of the endothelium to do transcellular diapedesis (Carman et al., 2007) and antigen recognition (Sage et al., 2012) but these structures have not been shown to have a proteolytic activity, a requisite to call them podosomes. Podosome structures have been largely characterized in monocyte-derived cells such as macrophages, osteoclasts and dendritic cells (Linder et al., 2011), but whether monocytes form podosome structures was never investigated. Thus, we investigated the ability of human blood-derived T lymphocytes, neutrophils and monocytes to migrate in a dense matrix in relation to their capacity to form podosome structures.

Macrophages exhibit various phenotypes as they polarize in response to environmental cues and can be broadly classified in two main groups: classically activated macrophages (or M1) and alternatively activated macrophages (or M2) (Delavary et al., 2011; Martinez et al., 2008, 2009; Mosser and Edwards, 2008; Murray and Wynn, 2011). Macrophages differentiate as M1 macrophages in response to IFN-γ and microbial products such as LPS. They typically take part in the initial immune response to invading microorganisms and promote T helper (Th) 1 immunity. M2 macrophages are obtained in response to IL4 or IL13 and are involved in the resolution phase of inflammation and tissue healing by promoting Th2 immunity (Delavary et al., 2011; Mege et al., 2011). Moreover, M2 macrophages have impaired phagocytosis capacity (Krysko et al., 2011; Varin et al., 2010). Because *in vitro* polarization of macrophages triggers phenotypes at the two extremes of a continuum of phenotypes which occur *in vivo*, we also studied the migration of macrophages collected from the peritoneal cavity in resting and acute inflammatory conditions was also studied.

For this study we thus set out to explore the 3D migration capacity of a variety of blood-derived leukocyte populations such as neutrophils, T lymphocytes and monocytes in porous (fibrillar collagen I) and dense (Matrigel) matrices polymerized *in vitro*. Since macrophages constitute a heterogeneous population *in vivo*, we also examined different macrophage subpopulations.

Materials and methods

Cell preparations

Blood samples from healthy donors were obtained following standard ethical procedures and with the approval of the concerned Internal Review Boards.

Human monocytes: Monocytes were isolated from blood (Etablissement Français du Sang, Toulouse, France) as previously described (Van Goethem et al., 2010). Those were then maintained in RPMI 1640 medium supplemented with 1% FCS and seeded on matrices polymerized in transwells (5×10^4) or ECM-coated coverslips (10^5).

Human neutrophils: Neutrophils were isolated from the same source by the dextran-Ficoll method as previously described (Le Cabec and Maridonneau-Parini, 1994). Cells were then maintained in RPMI 1640 supplemented with HEPES (10 mM), pH 7.4 for 20 min at 37 °C in suspension for recovery and layered down on matrices (5×10^4) or on ECM-coated coverslips (10^5). FCS at 1% final concentration was added after 1 h.

Human macrophages: Human macrophages derived from CD14-sorted monocytes (MDMs) were differentiated in the presence of M-CSF (20 ng/mL, Peprotech) as previously described (Van Goethem et al., 2010). After 7 days of differentiation, macrophages were polarized using either IFN-γ 20 ng/mL (100 U/mL-Roche) or IL4 (20 ng/mL-Miltenyi Biotec) for 18 h to obtain M1 and M2 polarized macrophages, respectively. TNFα (10 ng/mL) was also used to induce M1 polarization. All culture media contained M-CSF at 10 ng/mL during the polarization processes. MDMs had been serum starved for 4 h, harvested as previously described (Van Goethem et al., 2010), suspended in RPMI 1640 medium supplemented with 1% FCS, M-CSF (10 ng/mL) and the indicated cytokine and seeded on matrices (3×10^4) or ECM-coated coverslips (10^5).

Human lymphocytes: $CD4^+$ T cells were isolated by negative depletion using Rosette Sep (StemCell Technologies), cultured and expanded in RPMI 1640, 5% human serum, 100 U/mL IL2 in the presence of anti-CD3/CD28 coated Dynabeads (Invitrogen), with a bead:cell ratio of 1:1. Fresh IL2 was added every 3–4 days. $CD4^+$ T cells were restimulated as above every 19–21 days. When $CD4^+$ T cells were used, 14–21 days after activation, they were regaining a resting phenotype. Cell purity was assessed by FACS analysis (FACScan, Becton Dickinson) using PE-CY5-labelled anti-CD4 mAb (BD Biosciences) and was routinely between 95 and 99%. Cells were suspended in RPMI 1640 supplemented with 0.5% human serum and seeded on matrices (5×10^4) or ECM-coated coverslips (10^5).

Murine macrophages: Bone marrow-derived macrophages (BMDMs) from C57Bl6/J mice were obtained as previously described (Cougoule et al., 2010) and polarized using murine recombinant cytokines (ImmunoTools) according to the protocol described above for human macrophages. BMDMs had been serum starved for 4 h, harvested as previously described (Cougoule et al., 2010), suspended in RMPI 1640 supplemented with 1% FCS, 10 ng/mL M-CSF and the indicated cytokine, and seeded on matrices (5×10^4) or ECM-coated coverslips (10^5).

Tissue macrophages: C57Bl6/J mice were purchased from Charles River Inc. All experiments were performed with 6–12-week old female mice according to animal protocols approved by the Animal Care and Use committee of the Institut de Pharmacologie et de Biologie Structurale. Resident cells were collected by peritoneal lavages performed in control mice as previously described (Cougoule et al., 2010). Peritoneal elicited cells were collected 4 days after an intra-peritoneal injection of 0.5 mL of 4% thioglycollate Brewer (Sigma) as previously described (Cougoule et al., 2010). Collected cells were spun down (300 g) for 5 min, the pellet was suspended in RPMI 1640 medium and cells were layered down

on matrices (5×10^4) or ECM-coated coverslips (10^5). Medium was supplemented with 1% FCS after 1 h.

Matrix preparations

Collagen I (Nutragen – Nutacon; 2 mg/mL final concentration) or Matrigel (BD Biosciences, batches from 8 to 12 mg/mL) were polymerized as a thick layer (1–1.5 mm) in the upper Transwell chambers, as described (Van Goethem et al., 2010).

3D migration

Cells were seeded on the top of the matrices at the indicated concentration (see above). For all the cell types studied, the lower chambers were filled with RPMI 1640 medium supplemented with 10% FCS. For T lymphocytes and monocytes/macrophages, CXCL12 (1 μg/mL) and M-CSF (50 ng/mL) were added respectively. The percentage of 3D migration in fibrillar collagen I and in Matrigel was manually quantified after 24 h and 72 h, respectively using the ImageJ software.

2D migration

2D migration was performed in uncoated-8 μm porous transwells (BD Biosciences). Cells had been serum starved for 3 h. M1 or M2 polarized (3×10^4) MDMs were seeded in the upper chamber. The lower chamber was filled with 1 mL of RPMI 1640 supplemented with 10% of FCS and 50 ng/mL M-CSF. The migration assay was carried out overnight. The cells on the upper face were then removed using a cotton swab and the cells on the lower side were fixed with paraformaldehyde (3.7%; Sigma), permeabilized with Triton X-100 (0.1%; Sigma) and stained with Texas Red-coupled phalloidin (1/1000, Molecular Probes, Invitrogen) and DAPI [4,6-diamidino-2-phenylindole] (5 ng/mL, Sigma) to visualize F-actin and nuclei. The number of cells was then counted with a Leica DM-RB fluorescence microscope.

Matrix degradation assay

Coverslips were coated with 0.2 mg/mL FITC coupled-gelatin (Molecular Probes) as previously described (Cougoule et al., 2010). Macrophages or other leukocytes were cultured on FITC-coupled gelatin. For T lymphocytes, the gelatin-FITC had been coated with either fibronectin or ICAM-1 (10 μg/mL) for 1 h at 37 °C. After 24 h in culture, cells were fixed, processed for F-actin staining and observed by fluorescence microscopy. Quantification of the matrix degradation was assessed as previously described (Cougoule et al., 2010).

Immunofluorescence microscopy

BMDMs, murine tissue macrophages and other leukocytes had been cultured for 24 h on glass coverslips coated either with fibronectin, fibrinogen (10 μg/mL; Sigma) or nothing, or with ICAM-1 Fc chimera (10 μg/mL, R&D system) for T lymphocytes. Based on our previous data showing that macrophages spread on glass, coated or not with ECM proteins, spontaneously form podosomes after 15–30 min, we investigated podosome formation in blood-derived leukocytes at different time points starting from 60 min till 24 h. Cells were fixed with paraformaldehyde (3.7%; Sigma), permeabilized with Triton X-100 (0.1%; Sigma), and stained with anti-vinculin antibody (clone HVin-1, dilution 1/300; Sigma) followed by Alexa488-conjugated goat anti-mouse immunoglobulin G (1/1000; Cell Signalling Technology), Texas Red-coupled phalloidin and DAPI. Slides were visualized with a Leica DM-RB fluorescence microscope or with an LSM710 confocal microscope equipped with an x63-1.4 oil immersion Plan-Apochromat objective (CarlZeiss AG, Jena, Germany).

Table 1
Sequence primers used to characterize the M1 and M2 marker expression.

Primer	Sequence
Human markers	
hCXCL9-R	TCT-TTT-GGC-TGA-CCT-GTT-TCT-C
hCXCL9-L	AGT-GGT-GTT-CTT-TTC-CTC-TTG-G
hINDO-R	CAA-AAT-AGG-AGG-CAG-TTC-CAG-T
hINDO-L	TCA-TCT-CAC-AGA-CCA-CAA-GTC-A
hTNF-R	AGG-AGG-GGG-TAA-TAA-AGG-GAT-T
hTNF-L	CAT-CTA-TCT-GGG-AGG-GGT-CTT-C
hIL-15R	GTT-AGC-AGA-TAG-CCA-GCC-CAT-AC
hIL-15 L	TAC-TCA-AAG-CCA-CGG-TAA-ATC-C
hALOX15-R	GGG-GGC-TGA-AAT-AAC-CAA-AG
hALOX15-L	AAC-TTC-CAC-CAG-GCT-TCT-CTC
hCCL13-R	ATG-TGA-AGC-AGC-AAG-TAG-ATG-G
hCCL13-L	GAG-CAG-AGA-GGC-AAA-GAA-ACA
hDCSIGN-R	CAG-CAG-AGG-AAA-GAG-AGA-GAG-G
hDCSIGN-L	AGA-AGG-GTA-GGA-CTG-GAT-GTT-G
hFN1-R	CCA-CAG-AGT-AGA-CCA-CAC-CAC-T
hFN1-L	ACA-CCT-GGA-GCA-AGA-AGG-ATA-A
Murine markers	
mTNF-R	TCTGGAAAGGTCTGAAGGTAGGA
mTNF-L	AGAAACACAAGATGCTCGGACA
mCXCL10-R	CGT-CAT-TTT-CTG-CCT-CAT-CCT
mCXCL10-L	TCT-GCT-CAT-CAT-TCT-TTT-TCA-TCG
mIL12p40-R	GACACGCCTGAAGAAGATGAC
mIL12p40-L	GCCATTCCACATGTCACTGC
mMR-R	CATGAGGCTTCTCCTGCTTCTG
mMR-L (Mrc1	TTGCCGTCTGAACTGAGATGG
mYIM1/2-R	CCACTGAAGTCATCCATGTC
mYIM1/2-L	GGGCATACCTTTATCCTGAG
mArg2-R	TGGATCAAACCTTGCCTCTC
mArg2-L	GCCGATCAAATGTCTGTTCC

For each species, the M1 and M2 markers are presented in regular and italic case, respectively.

M1/M2 transcriptional profiles of macrophages

RNAs were extracted from MDMs, BMDMs, resident and elicited mouse macrophages using RNeasy Mini Kit (Qiagen) with a DNase I step to eliminate DNA contaminants, as recently described (Ben Amara et al., 2010). The quantity and the quality of RNA were assessed using Nanodrop (Thermo Science, Maurens-Scopont, France) and 2100 Bioanalyser (Agilent Technologies, Massy, France), respectively. Real-time quantitative RT-PCR (qRT-PCR) was carried out as recently described (Ben Amara et al., 2010). In brief, reverse transcription of 150 ng of RNA was performed with the MMLV-RT kit (Invitrogen). cDNA was obtained using oligo(dT) primers and M-MLV reverse transcriptase (Invitrogen) and qPCR experiments were performed using SYBR Green Fast Master Mix (Roche Diagnostics, Meylan, France) and a SmartCycler (Cepheid, Maurens-Scopont, France). The M1 and M2 genes were selected according to a previously published list of M1 and M2 genes (Martinez et al., 2006; Tantibhedhyangkul et al., 2011). The primers were designed using Primer3 (http://frodo.wi.mit.edu/primer3) (Table 1). Results were normalized using the housekeeping gene β-actin and are expressed as fold change ($FC = 2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (CtTarget - CtActin)_{assay} - (CtTarget - CtActin)_{control}$.

Podosome life time measurement

CD14-sorted human monocytes were seeded in 8-wells coverglass Labtek chambers (10^5 cells/wells) and differentiated in the presence of M-CSF (20 ng/mL). At day 3, cells were washed twice with PBS and transduced with a mCherry-Lifeact lentiviral vector to visualize F-actin structures. Briefly, the transduction was initiated by adding 200 μL/well of a mix of RPMI 1640 without FCS and the

lentiviral vector (10^6 effective viral particles for 10^6 macrophages). After 1h30, 400 μL of complete medium was added. At day 6, the culture medium was removed and MDMs were polarized as described above. After 24 h, cells were imaged using an inverted microscope (Leica DMIRB, Leica Microsystems) equipped with a motorized stage and an incubator chamber to maintain the temperature and CO_2 concentration constant. Images were acquired with the Metamorph software, every 20 s in one z-plane for 40 min, and on 10 separate fields per cell type. Quantification of podosome life-span was measured manually from two independent experiments, using the ImageJ software for podosomes appearing and disappearing during the time-course of the experiment and results were expressed as the mean ± SEM of 100 podosomes from 10 cells.

Statistics

All data in the text and figures are expressed as mean (±SEM) and unpaired *T*-test statistical analysis was carried out with the Prism 4.0 software. A *p* value of less than .05 was considered significant ($^*p < .05$, $^{**}p < .01$, $^{***}p < .001$).

Results

T lymphocytes, neutrophils and monocytes use the amoeboid but not the mesenchymal migration mode

To study the 3D migration ability of leukocytes, we used two different matrices with distinct architectures which were polymerized as thick layers (>1 mm) to create a 3D environment (Van Goethem et al., 2010). Fibrillar collagen I is a porous matrix into which macrophages and other leukocytes have been shown to use the amoeboid migration mode (Sabeh et al., 2009; Van Goethem et al., 2010; Wolf et al., 2003). Matrigel is purified from a mouse sarcoma which, when used at 10 mg/mL, forms a dense and poorly porous extracellular matrix. In Matrigel, MDMs and BMDMs have been shown to migrate using the protease-dependent mesenchymal mode (Cougoule et al., 2010; Van Goethem et al., 2010) whilst neutrophils have been shown not to migrate into it (Steadman et al., 1997).

T cell motility thought 3D matrices was induced by the chemokine CXCL12, a ligand for CXCR4. We mainly studied cultured primary human $CD4^+$ T cells. Those express CXCR4 homogeneously whilst freshly isolated T lymphocytes express heterogeneous CXCR4 levels (Bleul et al., 1997). T lymphocytes infiltrated fibrillar collagen I and exhibited a pear shape characteristic of the amoeboid migration mode (Fig. 1A and B, left panel, arrow) but they did not infiltrate Matrigel (Fig. 1A and B, right panel). Similarly, freshly isolated total T cells were unable to infiltrate Matrigel but migrated readily in fibrillar collagen matrices (data not shown). Next, the presence of podosomes was examined. To optimize cell spreading, a prerequisite for podosome formation, T lymphocytes were seeded on ICAM-I coated coverslips and treated or not with PMA for 24 h. Even under these conditions, no podosome structure could be observed in T lymphocytes. Some displayed actin dots, but those were not surrounded by vinculin, a hallmark of podosomes (Fig. 1C, inset a) and failed to degrade the gelatin-FITC covered with ICAM-1 (Fig. 1D) or fibronectin (data not shown). Treatment with PMA, although it increased cell spreading, still did not trigger proteolytic activity towards ECM (data not shown). Thus, despite our efforts to provide favourable conditions, we could not get T lymphocytes neither to form podosomes, nor to exhibit proteolytic activity on ECM nor to migrate into Matrigel.

Neutrophils freshly isolated from blood infiltrated fibrillar collagen I (Fig. 1A and B) with a rounded cell shape but did not infiltrate Matrigel (Fig. 1A and B). Occasionally, a few F-actin dots with almost a regular ring of vinculin were noticed (Fig. 1C, inset b), but on glass, on fibronectin or vitronectin, neutrophils generally failed to form podosomes (Fig. 1C, inset a), and failed to degrade the matrix of FITC-gelatin (Fig. 1D). As the diapedesis step could be critical "to switch" cells to a competent migration phenotype in dense matrices, we then tested the migration ability of inflammatory neutrophils harvested from the mouse peritoneal cavity 12 h after injection of thioglycollate. At this time point, neutrophils accounted for 77.4 ± 4.7% ($n = 5$) of the collected cells (Fig. S1). Despite the inflammatory context from which they were obtained, elicited neutrophils still did not infiltrate Matrigel and did not form podosomes (data not shown).

Similarly, freshly isolated monocytes infiltrated the matrix of fibrillar collagen I with the typical amoeboid rounded cell shape (Fig. 1A and B) but not Matrigel (Fig. 1A and B). They formed F-actin dots which were not surrounded by vinculin and thus were not considered as podosomes (Fig. 1C). After 4–5 days on Matrigel, some monocytes did acquire the capacity to infiltrate the matrix with the characteristic elongated cell shape (Fig. 1B). At day 6, the percentage of cells into the matrix was quantified (Fig. 1A) and was similar to that previously described for macrophages after 7 days of differentiation in culture dishes (Van Goethem et al., 2010), suggesting that differentiation is required to infiltrate dense matrices.

Thus, none of the leukocytes studied here were found to have the capacity to infiltrate Matrigel or to form podosomes, suggesting that these cells are unable to use the protease-dependent mesenchymal mode.

In vitro polarized M1 or M2 human and mouse macrophages display distinct 3D migration abilities

Next, we investigated whether the 3D migration ability of monocyte-derived macrophages (MDMs) was regulated during macrophage polarization.

Human M1 and M2 MDMs were generated by IFN-γ- and IL4-treatment, respectively. Using a combination of 8 polarization markers, we checked that IFN-γ- and IL4-stimulated macrophages exhibited typical M1 and M2 profiles when compared to non-polarized MDMs that we called here M0 (Fig. 2A).

The migration ability of M1 and M2-polarized MDMs was examined in comparison with M0. M2 MDMs were able to migrate into fibrillar collagen I and Matrigel (Fig. 2B) with the typical cell shape of amoeboid and mesenchymal migration, respectively (Fig. 2C). They exhibited comparable migration ability than M0 macrophages (Fig. 2B and C). In marked contrast, IFN-γ-polarized M1 MDMs were unable to migrate into fibrillar collagen I or in Matrigel (Fig. 2B), and stayed at the top of the matrices where they displayed a rounded cell shape (Fig. 2C). To ensure that inhibition of the migration was the result of M1 polarization, MDMs were also polarized using TNFα (Mantovani et al., 2004). TNFα-treated MDMs did not migrate in fibrillar collagen I or in Matrigel (data not shown) confirming that M1 macrophages are defective in both migration modes.

To test the 2D migration ability of M1 and M2 macrophages, a standard transwell assay was used. As shown in Fig. 2D, M1 MDMs were also motionless in those conditions, while M2 were migrating even more efficiently than M0 macrophages. Taken together, these results show that M1 MDMs were defective for both 3D and 2D migration whereas M2 MDMs remained competent for all types of migration: 2D and 3D migration into fibrillar collagen I and Matrigel. The presence of podosomes was examined in M1 and M2 MDMs layered on fibrinogen. Individual podosomes were observed in 100% of the M1 and M2 macrophages. Live observations of mCherry-Lifeact-expressing M1 and M2 MDMs revealed that the lifetime of individual podosomes in M1 MDMs was 625 ± 59 s while it was 411 ± 42 s in M2 macrophages ($p = 0.0037$, $n = 100$ podosomes/condition) (to compare with podosomes in

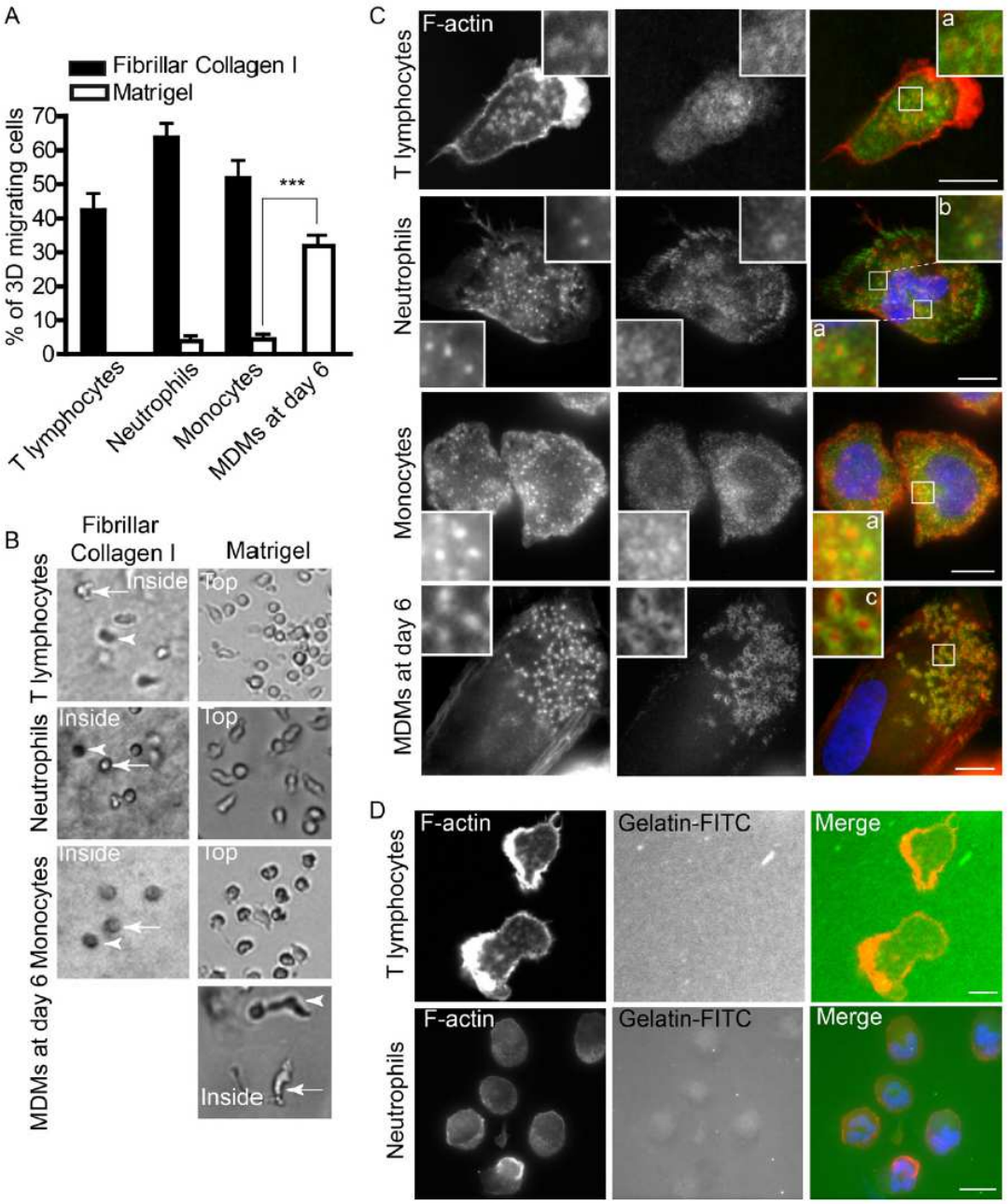

Fig. 1. T lymphocytes, neutrophils and monocytes only use the amoeboid migration mode. (A) Cells were seeded at the top of a thick layer of fibrillar collagen I or Matrigel and the 3D cell migration was quantified after 24 h and 72 h, respectively. T lymphocytes, neutrophils and monocytes infiltrated the matrix of fibrillar collagen I but did not infiltrate Matrigel. After 6 days on the matrix, monocyte-derived macrophages (MDMs) which had acquired the capacity to infiltrate Matrigel were quantified as described in the section "Materials and methods". Results are expressed as mean ± SEM of three independent experiments performed in triplicates, $^{***}p < 0.001$. (B) T lymphocytes, neutrophils and monocytes migrating either inside fibrillar collagen I (left panels, arrows) or on top of the Matrigel matrix (right panels, arrows) are shown. Arrowheads show cells at a distinct depth in the matrices. (C) T lymphocytes were seeded on ICAM-I coated coverslips (without PMA treatment) for 24 h, neutrophils were seeded on glass coverslips for 1 h, monocytes and macrophages were seeded on glass coverslips for 24 h. All cells were fixed and permeabilized. F-actin was stained with Texas Red-coupled phalloidin, vinculin was stained with primary and AlexaFluor 488-coupled secondary antibodies, and nuclei with DAPI. T lymphocytes, neutrophils and monocytes formed F-actin dots but vinculin did not form a ring around the actin core (insets (a)). Inset (b) illustrates the presence of few F-actin dots with a quite regular ring of vinculin in neutrophils. Inset (c) illustrates the organization of classical podosomes with a ring of vinculin in MDMs after 6 days of culture. Scale bars: 10 μm, insets – 3× magnification. (D) T lymphocytes and neutrophils seeded on FITC-coupled gelatin coated on glass coverslips and cultured either overnight or for 5 h, respectively, were fixed and stained for F-actin and nuclei and microscopically examined. Neither T lymphocytes nor neutrophils degraded FITC-coupled gelatin. Scale bars: 10 μm.

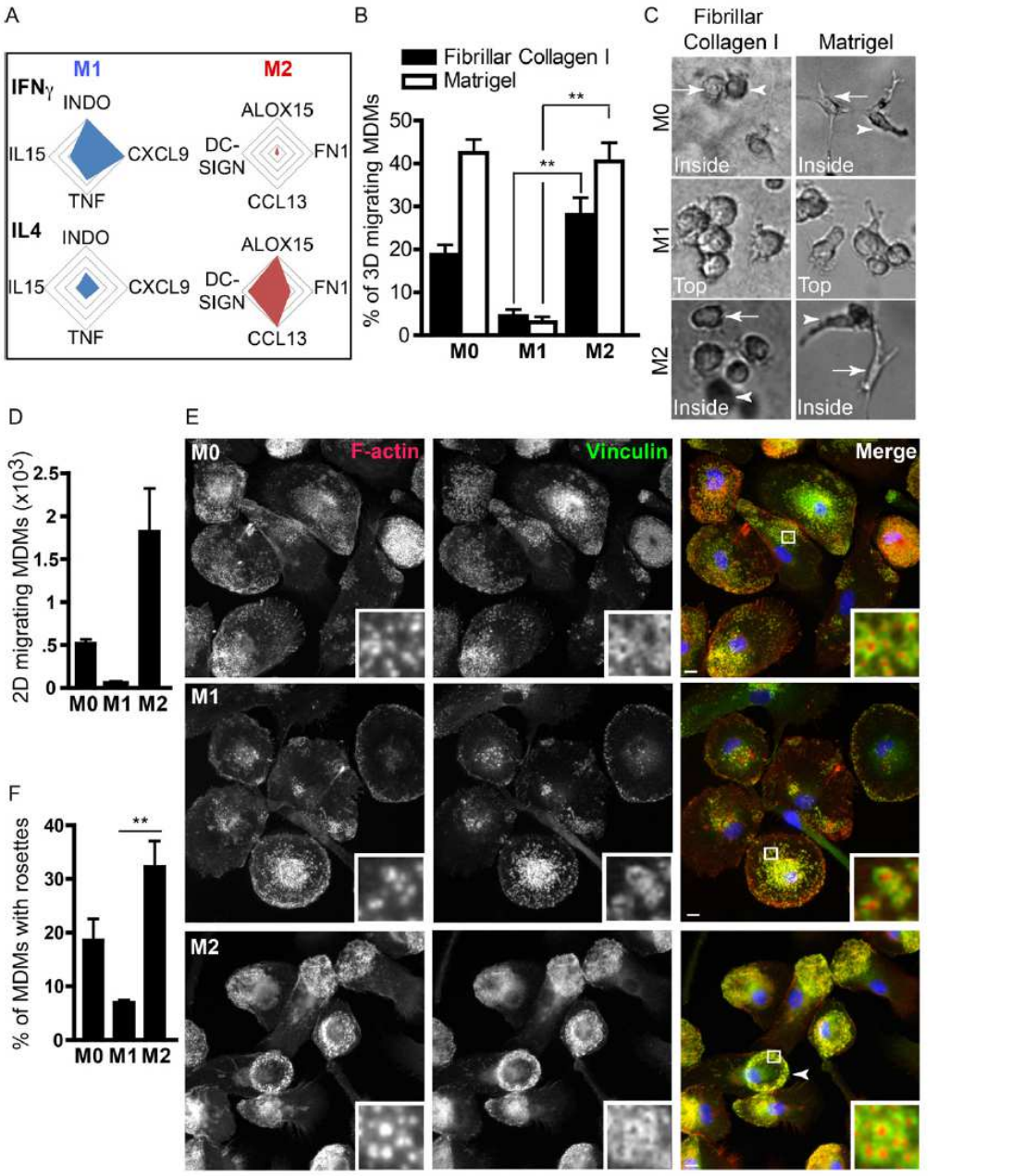

Fig. 2. M2 but not M1 human macrophages migrate in 3D matrices. MDMs were stimulated with nothing (M0), IFN-γ or IL4 to induce M1 and M2 polarization respectively. (A) Total mRNA was extracted and the expression of 4 M1-related genes (IL15, INDO, TNF, CXCL9) and M2-related genes (DC-SIGN, ALOX15, CCL13, FN1) was measured by real time RT-PCR. Results are expressed as fold change (FC) compared to M0 macrophages and represent the mean of three experiments. They are presented as radar plots with a maximum increase of 4 folds. (B) M0, M1 and M2 MDMs were harvested and seeded on top of a thick layer of fibrillar collagen I or Matrigel and cell migration was quantified from triplicate samples after 24 h and 72 h, respectively. Results are expressed as mean ± SEM of four independent experiments, $^{**}p < 0.01$. (C) M0 and M2 MDMs are shown inside fibrillar collagen I or in Matrigel while M1 macrophages which mostly do not infiltrate matrices are shown at the top. Arrowheads show cells deeper in the matrices. (D) 2D migration of M0, M1 and M2 MDMs through uncoated transwells. MDMs that reached the lower chamber were microscopically counted. Results are expressed as mean ± SEM of two independent experiments. (E) M0, M1 and M2 MDMs were seeded on fibrinogen-coated glass coverslips for 24 h, fixed, permeabilized and F-actin was stained with Texas Red-coupled phalloidin, vinculin with primary and AlexaFluor 488-coupled secondary antibodies, and nuclei with DAPI. M1 MDMs harbor individual podosomes (inset) while M2 MDMs harbor podosome rosettes (arrowhead and inset). Scale bar = 10 μm, insets = 6× magnification. (F) Quantification of podosome rosettes in M0, M1 and M2 MDMs. Podosome structures were counted in at least 100 cells in duplicate samples, in three separate experiments. $^{**}p < 0.01$.

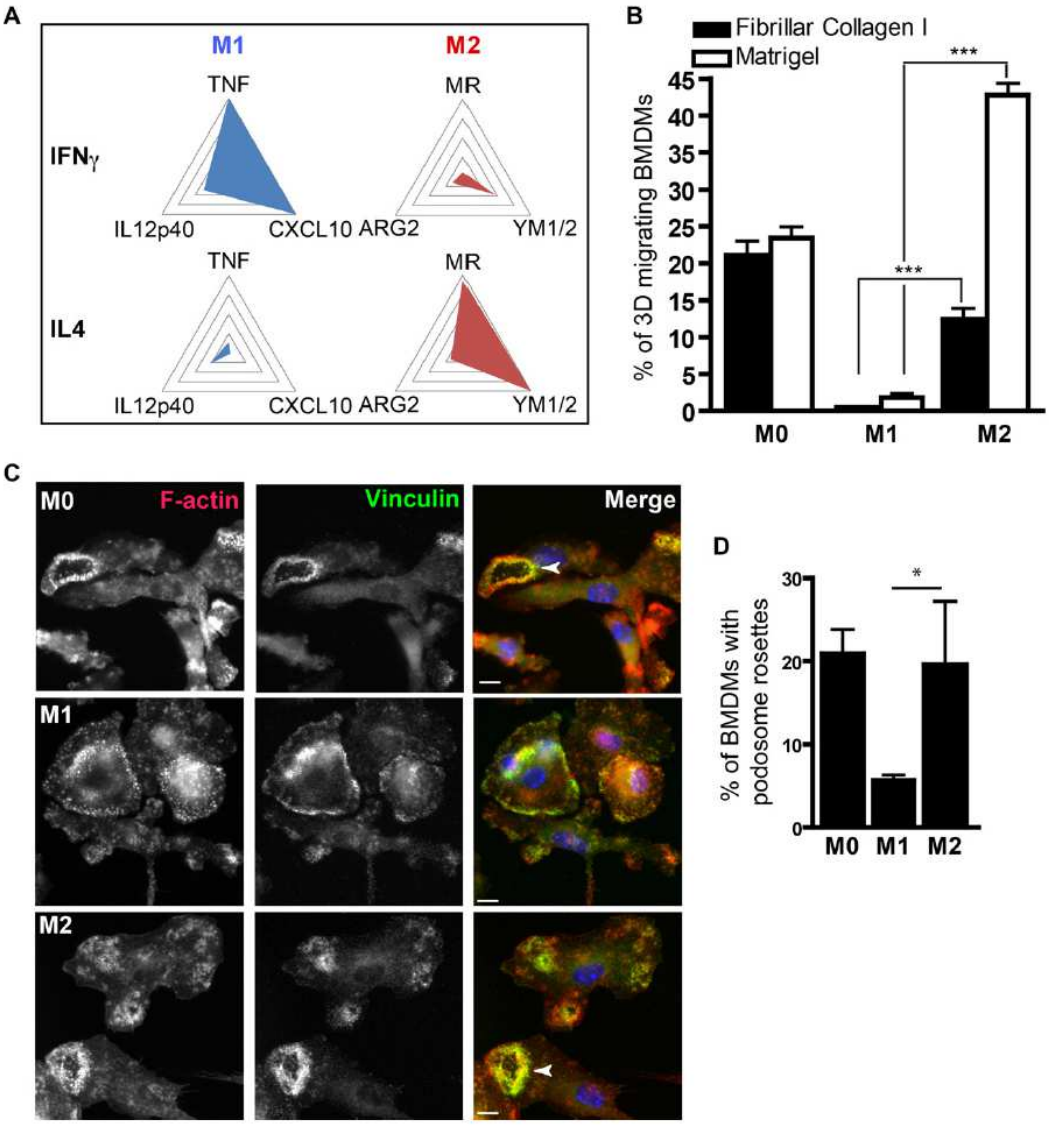

Fig. 3. M2 but not M1 mouse macrophages migrate in 3D matrices. Mouse bone marrow-derived macrophages (BMDMs) were stimulated with nothing, IFN-γ or IL4 to induce M0, M1 and M2 polarization, respectively. (A) Total mRNA was extracted and the expression of 3 M1-related genes (TNFα, CXCL10, IL12p40) and M2-related genes (Manose Receptor (MR), YM1/2, Arginase 2 (ARG2)) was measured by real time RT-PCR. The results are expressed as fold change compared to M0 BMDMs and represent the mean of three experiments. They are presented as radar plots with a maximum increase of 4 folds. (B) M0, M1 and M2 BMDMs were harvested and seeded on top of a thick layer of fibrillar collagen I or Matrigel and the 3D cell migration was quantified after 24 h and 72 h respectively from triplicate samples. Results are expressed as mean ± SEM of three independent experiments, $^{***}p < 0.001$. (C) M0, M1 and M2 BMDMs were seeded on fibronectin-coated glass coverslips for 24 h, fixed, permeabilized and F-actin was stained with Texas Red-coupled phalloidin, vinculin with primary and AlexaFluor 488-coupled secondary antibodies, and nuclei with DAPI. M1 BMDMs form few podosome rosettes while M0 and M2 BMDMs harbor podosome rosettes (arrowheads). Scale bar – 10 μm. (D) Quantification of podosome rosettes in M0, M1 and M2 BMDMs. Podosome structures were counted in at least 100 cells in duplicate samples, in three independent experiments. $^{*}p < 0.05$.

M0 macrophages which is approximately 400 s (Bhuwania et al., 2012)). These results indicate that M1 macrophages have a reduced podosome turnover compared to M2 . In murine bone marrow derived macrophages (BMDMs), we have previously reported that the organization of podosomes as rosettes is required for mesenchymal migration (Cougoule et al., 2010). Thus, we quantified podosome rosettes in M0, M1 and M2 MDMs layered on fibrinogen. As shown in Fig. 2E and F, similarly to M0, M2 MDMs efficiently organized their podosomes as clusters and rosettes while M1 MDMs hardly formed these structures. The presence of rosettes has been correlated with enhanced ECM proteolytic activity (Cougoule et al., 2010) but somewhat surprisingly, M1 MDMs were found to degrade gelatin-FITC with the same efficiency as M0 and M2 cells (Fig. 4C). One noticeable difference, however, was that M1 MDMs harbored a "sitting" cell shape on gelatin-FITC with a large degradation area under the cells, whereas M2 MDMs harbored a

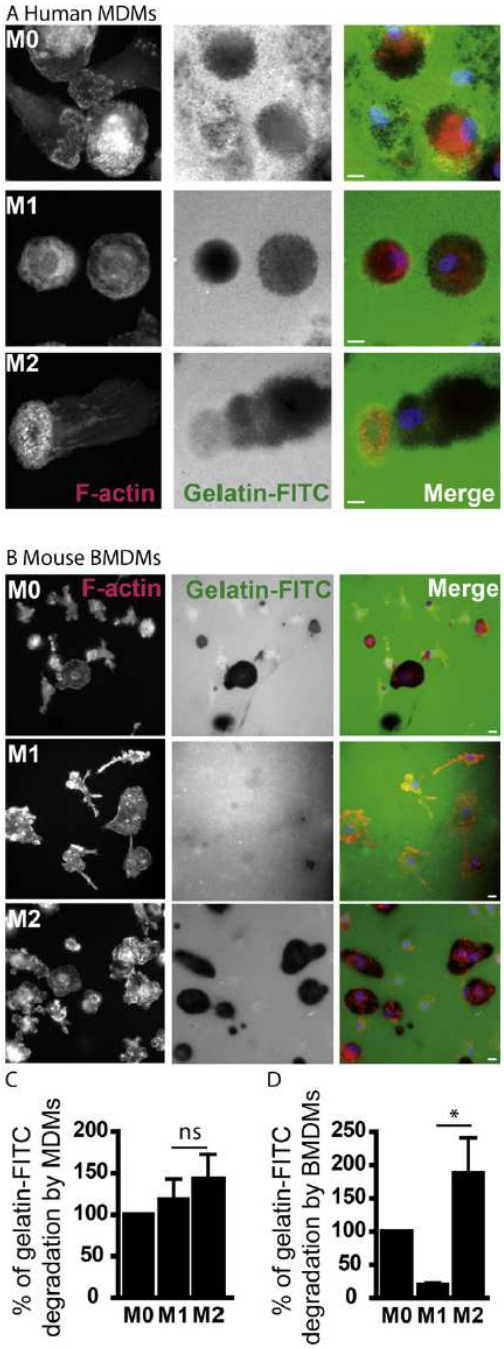

Fig. 4. (A) M0, M1 and M2 MDMs were seeded on FITC-coupled gelatin coated on glass coverslips, cultured overnight, fixed and stained for F-actin and nuclei and microscopically examined. Scale bar: 10 μm. (B) M1 BMDMs are defective in gelatin-FITC degradation. M0, M1 and M2 BMDMs were seeded on FITC-coupled gelatin coated on glass coverslips and incubated overnight, fixed and stained for F-actin and nuclei. Scale bar: 10 μm. (C) Quantification of gelatin-FITC degradation by M0, M1 and M2 MDMs. The percentage of degradation corresponds to the number of pixels of degradation for 100 pixels of cell surface. Results are expressed as mean ± SEM ($n = 3$). (D) Quantification of the percentage of gelatin-FITC degradation by M0, M1 and M2 BMDMs. The percentage of degradation corresponds to the number of pixels of degradation for 100 pixels of cell surface. Results are expressed as mean ± SEM ($n = 3$). *$p < 0.05$.

"migrating" phenotype characterized by a polarized shape with podosomes or podosome rosettes localized at the leading edge (Fig. 4A). Thus the proteolytic activity of M1 macrophages which form more stable podosomes appeared to be similar to M2 cells which form podosome rosettes and are motile.

Differences have been described between human and murine polarized macrophages (Martinez et al., 2006). We thus investigated the migration capacity of polarized mouse macrophages derived from bone marrow (BMDMs). Those were polarized in M1 and M2 BMDMs, as controlled by analysis of the expression of six polarization markers (Fig. 3A). Similarly to human macrophages, M1 BMDMs did not migrate into fibrillar collagen I or Matrigel while M2 BMDMs did and even better than M0 macrophages in Matrigel (Fig. 3B). In addition, 100% of mouse M1 and M2 macrophages formed individual podosomes when layered on fibronectin but only M2 BMDMs organized their podosomes as rosettes (Fig. 3C and D). The only difference between human and mouse cells we observed in our experiments was that mouse M1 BMDMs showed relatively low gelatin-FITC degradation capacity (Fig. 4B and D). Taken together, these results indicate that the M1 polarization process is characterized by a motionless phenotype, the inability to organize podosomes as rosettes and an heterogeneous capacity to degrade the matrix, while M2 polarized cells are able to migrate effectively in both matrices, form rosettes and degrade the matrix.

The migration ability of elicited macrophages differs from that of resident macrophages

We next examined the ability of resident and elicited macrophages harvested from the mouse peritoneal cavity to migrate in 3D matrices, to form podosome structures and degrade gelatin-FITC. Elicited macrophages were collected 4 days after intraperitoneal injection of thioglycollate.

We compared the expression of 6 biomarkers between elicited and resident macrophages to characterize their polarization profiles. As shown in Fig. 5A , elicited macrophages displayed a combined expression of the M1 and M2 markers.

While both cell types migrated in fibrillar collagen I, only elicited macrophages migrated in Matrigel (Fig. 5B and C), formed podosome rosettes (Fig. 5D and E) and degraded the gelatin-FITC matrix (Fig. 5F and G).

Thus resident and elicited macrophages which are known to exert distinct functionalities (Gordon and Mantovani, 2011) exhibit distinct migration behavior, providing further information about the phenotypic heterogeneity of tissue macrophages.

Discussion

The data presented herein extend our knowledge of the 3D migration ability of several leukocyte populations into thick extracellular matrices. We observe that (i) circulating leukocytes use the amoeboid mode, do not form podosomes and are unable to infiltrate dense matrices and (ii) the migration of macrophages, which have been previously shown to use the amoeboid and the mesenchymal mode in porous and dense matrices, respectively, is strongly influenced by polarization and the tissue environment.

3D migration takes place when leukocytes enter interstitial tissues beyond vessels and the basal membrane. The architecture of interstitial tissues is heterogeneous, ranging from loose fibrillar regions to dense compact connective tissue with submicron

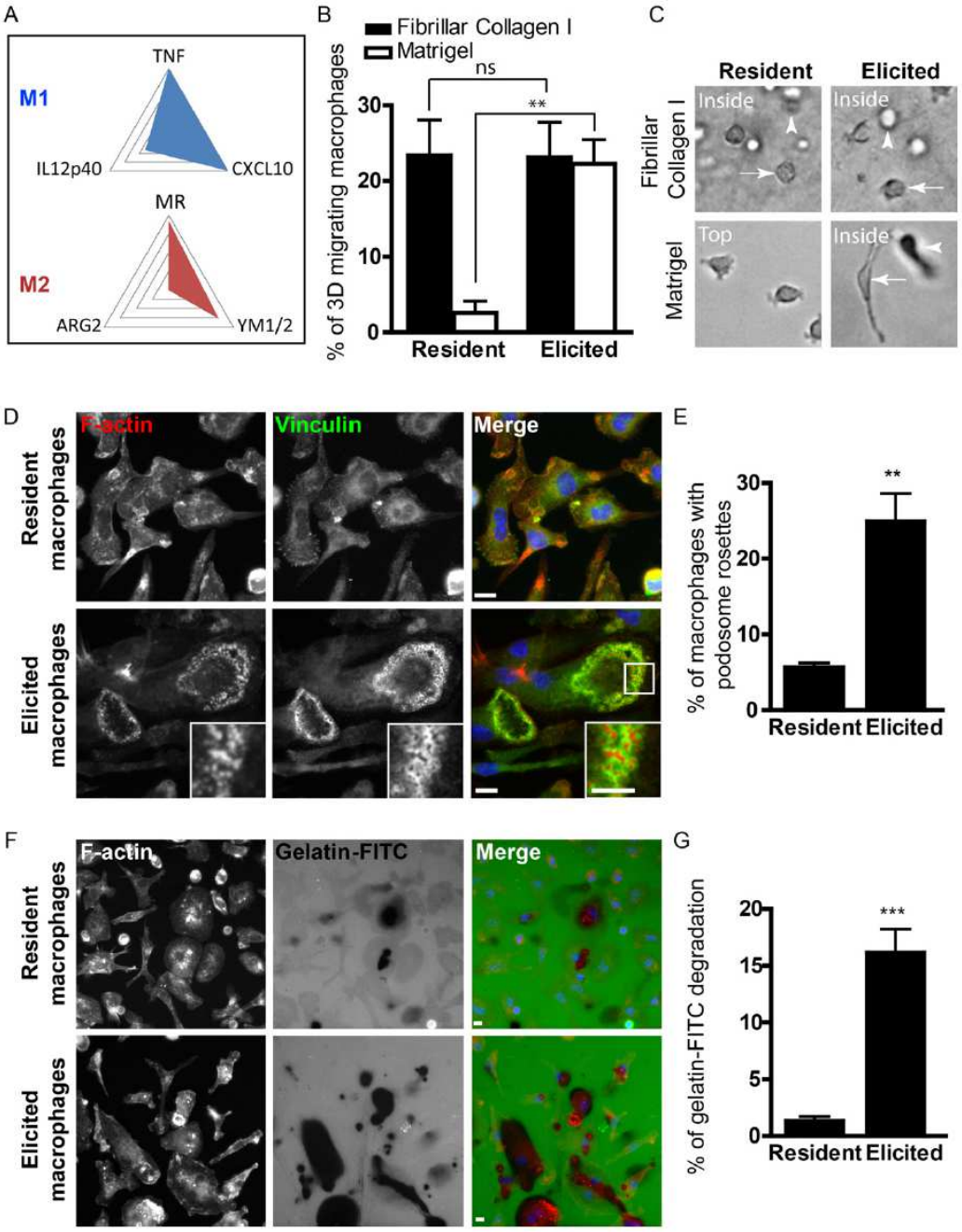

Fig. 5. Elicited but not resident macrophages use the mesenchymal migration mode. Resident and elicited macrophages were harvested from mouse peritoneal cavity. Elicited macrophages were collected 4 days after an intraperitoneal injection of thioglycollate. (A) Total mRNA was extracted from resident and elicited macrophages and the expression of 3 M1 related genes (TNF, CXCL10, IL12p40) and 3 M2 related genes (MR, YM1/2, ARG2) was measured by real time RT-PCR. The results are expressed as FC of elicited on resident macrophages and represent the mean of 3 experiments. They are presented as radar plots with a maximum increase of 4 folds. (B) Resident and elicited macrophages were harvested and seeded on top of a thick layer of fibrillar collagen I or Matrigel and 3D cell migration was quantified after 24 h and 72 h respectively from triplicate samples. Results are expressed as mean ± SEM of five independent experiments, $^{***}p < 0.001$. (C) Resident macrophages migrating inside fibrillar collagen I (arrow) or on top of Matrigel are shown. Elicited macrophages migrating inside fibrillar collagen I or Matrigel are shown (arrow). Arrowheads show cells at a different depth in matrices. (D) Tissue macrophages were seeded on fibronectin-coated glass coverslips for 24 h, fixed, permeabilized and F-actin was stained with Texas Red-coupled

spacing (Egeblad et al., 2010; Larsen et al., 2006). Thus two matrices which have been described in detail in a previous report (Van Goethem et al., 2010) were used to mimic the architectural heterogeneity of interstitial tissues. In fibrillar collagen I, neutrophils, T lymphocytes and human macrophages have been described to use the amoeboid migration mode (Nourshargh et al., 2010; Sabeh et al., 2009; Van Goethem et al., 2010; Wolf et al., 2003). Herein, we compared, in the same experiments, the migration capacity of leukocytes in porous fibrillar collagen I and in a dense Matrigel. We confirm the ability of monocytes, neutrophils and T lymphocytes to migrate using the amoeboid mode in fibrillar collagen I, and we show that they do not infiltrate Matrigel. Using the experimental conditions which trigger the formation of podosomes in macrophages, dendritic cells and osteoclasts (Linder et al., 2011), namely on glass coated or not with ECM proteins, we observed that T lymphocytes, neutrophils and monocytes do not form podosomes, according to the definition of these cell structures which implicates an actin dot surrounded by a ring of vinculin and a proteolytic activity towards the ECM (Linder et al., 2011). It has been reported that T lymphocytes form podosome-like structures during trans-cellular migration through the endothelium (Carman et al., 2007) and scanning for antigen on antigen presenting cells (Sage et al., 2012). In those experiments, however, the proteolytic activity of those structures was not established, a requisite for qualifying these structures as podosomes.

That neutrophils, monocytes and T lymphocytes are unable to infiltrate Matrigel is surprising as these cells are expected to migrate in most tissues. One hypothesis is that steps of differentiation might be missing. For our experiments, we have used cells isolated from blood which were then differentiated *in vitro*. Thus, compared to the *in vivo* context, diapedesis is by-passed and this step could be critical "to switch" cells to a competent migration phenotype in dense matrices. Using elicited neutrophils which had accomplished this step of diapedesis, however, we found that those cells still did not form podosomes, nor did T lymphocytes differentiated with anti-CD3/CD28 Abs in the presence of IL2 for 2–3 weeks acquire the capacity to migrate in Matrigel. Since neutrophils and lymphocytes are small cells which might glide into small pores (Van Goethem et al., 2011), another hypothesis is that leukocytes unable to use the mesenchymal mode would only infiltrate loose areas in tissues. In agreement with this proposal, a recent report on lung tumor biopsies shows that T lymphocytes are unable to cross dense fibers of ECM proteins surrounding tumor islets and remain in regions of loose tissue (Salmon et al., 2012). It should be noted that neutrophils have a segmented nucleus which might facilitate infiltration into poorly porous tissues. In fact, a recent report shows that in tumor microenvironments, the nuclei of infiltrated neutrophils are even more segmented than control neutrophils (Fridlender et al., 2009), suggesting that, by modifying their nuclear morphology, neutrophils could more easily squeeze in poorly porous tissues such as tumors (Egeblad et al., 2010) using the amoeboid migration. In the case of migration into the dense Matrigel matrix, used in our experiments at approximately 10 mg/mL, the size of the pores is apparently too narrow (see a scanning electron microscopy picture of the architecture of Matrigel in Van Goethem et al. (2010)) to allow neutrophil migration. A previous report has shown that neutrophils can infiltrate Matrigel in a protease-independent manner when it is diluted to 3 mg/mL (Steadman et al., 1997) indicating that a lower density allows neutrophils to infiltrate Matrigel using the amoeboid mode.

In conclusion, these three types of leukocytes are unable to migrate in matrices which require the formation of paths in a protease-dependent manner, while they migrate in porous 3D environments using the amoeboid mode. Thus, if the same rules apply *in vivo*, we would expect to find these cells only in porous tissue areas.

In marked contrast, when monocytes (from human blood) differentiate in macrophages, they become able to infiltrate Matrigel using the mesenchymal migration mode (Van Goethem et al., 2010, this report). Similar observations have been made with mouse macrophages differentiated from bone marrow (Cougoule et al., 2010). Because macrophages have a large plasticity, we examined the migration of polarized and of tissue macrophages to get further insight into their respective 3D migration abilities. Macrophages polarized as M1 or M2 exhibit distinct functions. M1 macrophages are involved in the early phase of the inflammatory response (Bystrom et al., 2008; Mege et al., 2011), they release inflammatory mediators and bactericidal products and they actively ingest microorganisms and cellular debris (Liddiard et al., 2011). We report that M1 macrophages do not migrate in 2D and 3D environments. Despite the fact that distinct molecular mechanisms govern 2D and 3D migration (Harunaga and Yamada, 2011), these results suggest that a common step may be inactivated. The observation that macrophages become motionless under inflammatory processes is important as it might contribute to maintain macrophages at the site of infection to clean out bacteria and release toxic bactericidal products only in limited tissue areas (Laskin et al., 2011). M1 BMDMs have been shown to adhere to extracellular matrices to a higher extent than M2 (Vereyken et al., 2011) and, in the same line, we report that podosomes in M1 cells are more stable than in M2 macrophages. M2 macrophages have been characterized as anti-inflammatory cells with healing properties to curtail inflammation after an infection or an injury and promote to return the body to homeostasis (Delavary et al., 2011; Serhan and Savill, 2005). It has been reported that mouse M2 BMDMs have a higher 2D motility towards conditioned medium than their M1 counterpart (Vereyken et al., 2011). Similar results were obtained here with human M2 MDMs, and we showed further that mouse and human M2 macrophages can migrate in both fibrillar collagen I and Matrigel, and form podosomes that they organize as rosettes. Interestingly, M2 polarization induces an increase in fibronectin transcript which is involved in cell adhesion and migration processes and in cathepsin C, a lysosomal protease which could be involved in mesenchymal migration (Martinez et al., 2006; Verollet et al., 2011). Moreover, Hck, which regulates the formation of podosome rosettes and the protease-dependent 3D migration of macrophages (Cougoule et al., 2010), is activated during the process of M2-polarization (Bhattacharjee et al., 2011). M2 macrophages are also associated with pathological contexts such as persistent infections (tuberculosis, leprosy, Whipple's disease (Mege et al., 2011). In the light of our results, it would be interesting to examine whether M2 macrophages facilitate host dissemination of pathogens. M2 macrophages are also described in chronic autoimmunity, obesity, fibrosis and cancer (Fairweather and Cihakova, 2009; Gordon and Martinez, 2010; Mosser and Edwards, 2008; Sica and Mantovani, 2012)). In several tumor models, macrophages have

phalloidin, vinculin with primary and AlexaFluor 488-coupled secondary antibodies, and nuclei with DAPI. Elicited macrophages organized their podosomes as rosettes (insets). Scale bars: 10 μm, inset – 3× magnification. (E) Quantification of podosome rosettes in resident and elicited macrophages. Podosome structures were counted in at least 100 cells in duplicate samples ($n = 5$), $^{**}p < 0.01$. (F) Resident and elicited macrophages were seeded on FITC-coupled gelatin coated on glass coverslips and incubated overnight, fixed and stained for F-actin and nuclei. Resident macrophages poorly degrade FITC-coupled gelatin. Elicited macrophages degraded large areas of gelatin-FITC. Scale bars: 10 μm. (G) Quantification of FITC-coupled gelatin degradation by resident and elicited macrophages. The percentage of degradation corresponds to the number of pixels of degradation for 100 pixels of cell surface. Results are expressed as mean ± SEM ($n = 5$), $^{***}p < 0.001$.

been shown to be polarized as M2 and to enhance malignancy and tumor cell invasiveness (Qian and Pollard, 2010; Ruffell et al., 2012) notably by remodeling the extracellular matrix to open paths to tumor cells (Gocheva et al., 2010; Guiet et al., 2011).

Despite the fact that *in vitro* polarization is clearly a simplified model, results on cell migration provide new striking differences between M1 and M2 macrophages, namely that 2D and 3D migration is an exclusive property of M2 macrophages.

We also report that resident and elicited peritoneal macrophages have distinct migration abilities. Elicited peritoneal macrophages, harvested 4 days after thioglycollate injection, were found to up-regulate both M1 and M2 markers as previously reported (Schif-Zuck et al., 2011; Stables et al., 2011) and to migrate in both fibrillar collagen I and Matrigel. At this time point, the inflammatory response initiated by thioglycollate is reaching the resolution phase (C. Lastrucci et al., unpublished data) and macrophages are thus not expected to die locally but to migrate to the draining lymph nodes (Bellingan et al., 1996; Randolph, 2008). For this, motile functions in 3D environments would be necessary.

Resident macrophages originate either from blood monocytes which migrate to tissues in non-inflammatory conditions or from resident cells which self-renew by transient proliferation after inflammatory episodes (Davies et al., 2011; Jenkins et al., 2011). One of the functions of resident macrophages is to sample the tissue in search of foreign particles. We show that peritoneal resident macrophages migrate in fibrillar collagen I but not in Matrigel. As previously described (Isaac et al., 2010), we observed that resident macrophages do not form podosome rosettes and they do not degrade gelatin-FITC. Consequently, in contrast to elicited macrophages, residents have a limited migration ability which might confine them to their tissue of residence. In future, it will be interesting to explore whether resident macrophages collected from tissues with distinct biophysical properties (porosity, stiffness) have distinct migration abilities compared to peritoneal macrophages.

We have previously shown that migration of human and mouse macrophages in dense matrices such as Matrigel or gelled collagen I is: (i) inhibited by protease inhibitors, (ii) independent of ROCK, a key effector of the amoeboid migration, (iii) dependent on the macrophage ability to organize its podosomes as rosettes and to degrade the ECM, (iv) characterized by an elongated cell shape with 3D podosomes at the tip of cell protrusions (Van Goethem et al., 2010, 2011; Verollet et al., 2011). This mode of migration has been called mesenchymal migration (Cougoule et al., 2010; Van Goethem et al., 2010, 2011; Verollet et al., 2011) by analogy to a migration mode used by tumor cells (Wolf and Friedl, 2011). Taken together with our previous studies, we show that cells which are able to infiltrate Matrigel with the characteristic mesenchymal phenotype (MDMs, BMDMs, human and mouse M2 macrophages, mouse elicited macrophages) form podosomes, organize them as rosettes and degrade the ECM while cells which do not infiltrate Matrigel (T lymphocytes, neutrophils, monocytes and resident macrophages) do not form podosomes. Although podosomes are required for the mesenchymal mode, the presence of podosomes does not certify the mesenchymal migration ability since M1 MDMs do form podosomes and degrade the matrix but are motionless. Interestingly, M1 macrophages do not organize their podosomes as rosettes. Hck and Filamin A, which regulate the mesenchymal but not the amoeboid migration mode, also regulate the organization of podosomes as rosettes (Cougoule et al., 2010; Guiet et al., 2012), further supporting the view that these podosome suprastructures are involved in the 3D migration of macrophages in dense matrices (Van Goethem et al., 2011).

In conclusion, this is the first study compiling a detailed analysis of the capacity of human primary blood-derived leukocytes and macrophages to migrate in 3D environments, form podosomes, organize them as rosettes and degrade the ECM. Our results highlight that none of the blood-derived leukocytes studied here do form podosomes or infiltrate a dense matrix of concentrated Matrigel. Thus we suspect that these leukocytes would more likely localize in loose areas of tissues. In addition, our results extend our knowledge on the diversity of macrophage phenotypes by showing that M2 and elicited macrophages use the mesenchymal mode while M1 polarized and resident macrophages do not. Tissues infiltrated by macrophages are often a negative sign of disease progression, including for cancer and chronic inflammation. These diseases are also often characterized by tissue densification (Egeblad et al., 2010) which would likely require the mesenchymal migration. A potential outcome of our work is to consider pathways regulating specifically the mesenchymal migration of macrophages as potential targets for therapeutic approaches.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ejcb.2012.07.002.

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Annexure II

Modulation of key circadian cycle genes among trauma patients in ICU

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Annexure II

Modulation of key circadian cycle genes among trauma patients in ICU

Circadian rhythm is known to play an important role in physiological functions including the immune system biology [25-27]. The variations in many biological parameters dependent on time have been reported to play a fundamental role in disease processes [28]. Our previous study on Q fever patients showed that genes of the peripheral molecular clock were modulated in a gender-dependent manner. Circadian rhythms affect many variables in healthy individuals: heart rate, blood pressure, body temperature and hormone levels (such as melatonin, cortisol). This is a key feature of the biological pathways which are under circadian control and regulate the immune response [55, 56]. Indeed, many components of the inflammatory response (leukocytes, cytokines, hormones) follow diurnal patterns [57, 58], and clock-related genes exhibit circadian patterns in neutrophils [31]. These observations support the hypothesis that the time of day can affect the patient outcomes in specific diseases [59-61].

In this study, we included severe trauma patients on the first day after admission in intensive care units because intensive care unit patients, and particularly those with brain injury, exhibit abolished or desynchronized patterns across daytime. The whole blood expression of the molecular clock components *ARNTL*, *CLOCK* and *PER2* genes was assessed in male and female healthy volunteers or patients with trauma using qRT-PCR. We observed a significant over-expression of *ARNTL* and *CLOCK* genes in male trauma patients compared with healthy volunteers. *ARNTL* was also significantly up regulated in male trauma patients as compared to female trauma patients. We report for the first time a gender-related modulation of molecular

clock genes in blood after severe trauma. These results emphasize the role of circadian rhythm in the immune response in trauma patients.

Early Gender-Specific Modulation of the Molecular Clock in Trauma

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Abstract

Immune system biology, as well as most of physiological functions, is tightly link to circadian rhythms. Time of day dependent variations of many biological variables also play a fundamental role in the disease process. In Q fever, we previously showed that genes of the peripheral molecular clock were modulated in a gender-dependent manner. Here, we included severe trauma patients on the first day after admission in intensive care unit. Using quantitative real time PCR, the whole blood expression of the molecular clock components *ARNTL*, *CLOCK* and *PER2* was assessed in male and female trauma patients. Healthy volunteers of both gender were used as controls. We observed a significant over-expression of both components (*ARNTL* and *CLOCK*) in male trauma patients. We report for the first time a gender-related modulation of the molecular clock genes in blood after severe trauma. These results emphasize the role of circadian rhythm in the immune response in trauma patients.

Keywords (3-5) : Trauma, Circadian Rhythm, Gender, Inflammation, Per2, Clock, Arntl

Introduction

Circadian rhythms maintain homeostasis by coordinating the action of physiological systems. They are associated with physiological changes over a course of time (1). Circadian rhythms are therefore important to control the behavior of organisms related to environmental changes (2,3). Alterations of circadian rhythms have been observed in many diseases such as cardiovascular disorders (diabetes, hypertension, …), cancers, neurological disorders (learning and memory dysfunctions), psychiatric disorders (depression, bipolar disease, …) and infectious diseases (4,5). A number of genes are known to regulate the circadian rhythms (6,7). This includes *CLOCK*, *ARNTL* (also known as BMAL1) and *PER2*. These genes are part of a peripheral molecular clock complex that regulates the circadian expression of many genes (**Figure 1A**). *CLOCK*, *ARNTL* and *PER2* have recently been reported as differentially modulated in infectious (8–10) and non-infectious diseases (11,12). Medical interventions, like sedation, can also alter circadian rhythms (13).

Gender is another variable that influence host immune response to pathogens. Whereas men often exhibit strong inflammatory responses to pathogens, women are shown to shape strong cell-mediated and humoral responses to antigenic challenges (11). A number of infectious diseases are more severe in men than in women (14–16). We recently found that *Coxiella burnetii* infection was associated with a gender-specific modulation of *CLOCK*, *ARNTL* and *PER2* genes (17,18). It is unclear whether these changes are related to the presence of bacteria or to the development of the inflammatory response. We hypothesized that the gender-related modulation of these genes is not restricted to Q fever but may be induced by the inflammatory response, independently of infection. We therefore explored transcriptional data obtained in trauma-patients (19). Our results suggest that the expression of the *CLOCK* and *ARNTL* genes was significantly increased in male trauma patients. We show for the first time a gender-related modulation of the molecular clock genes in blood after severe trauma.

Materials and Methods

Trauma patients admitted to the intensive care unit of Nord Hospital (Marseille, France) and healthy volunteers were included. The study was approved by ethic committee (Comité de Protection des Personnes Sud-Méditerranée II, no. 206–005). Informed written consent was obtained from each study participant by a next-of-kin. The patient himself/herself later approved the consent after recovering his conscience and free-will.

Blood samples from the study participants were collected in PAXgene Blood RNA Tubes (Qiagen™, Courtaboeuf, France). The samples were collected between 9:00 am and 11:00 am. The gene expression in blood samples was determined with real-time quantitative RT-PCR (qRT-PCR) as recently described (17). In brief, total RNA was extracted after DNase digestion, according to the manufacturer's recommendations (Invitrogen™, Life Technologies, Saint-Aubin, France). Real-time PCR with cDNA templates was performed using Light Cycler-FastStart DNA MasterPLUS SYBR Green I (Roche Diagnostics, Basel, Switzerland). The primers were designed with the free web software Primer3 (20). The primers consisted of the forward sequences 5'-CGGAGTTAGAGATGGTGGAAGA-3', 5'-TACTGAGGAAAGGGAGGAGAGG-3' and 5'-CCCTCTACCTGCTCAAAGAAAA-3' and the reverse sequences 5'-GGGACTGGAAAATGCTGAGTT-3', 5'-AAGGATAACAGCAAAGCAGAGG-3' and 5'-GCCCTCTGGTCTACAAAAACAA-3' for the Per2, Clock and Arntl genes, respectively. qRT-PCR experiments were performed using the gene encoding β-actin (forward sequence 5'-GGAAATCGTGCGTGACATTA-3' and reverse sequence 5'-AGGAAGGAAGGCTGGAAGAG-3') as the reference housekeeping gene.

The study participants were analyzed in four groups, accordingly to gender and trauma status. For each patient and control, the cycle threshold (Ct) values of the genes of interest were normalized with their own Ct values of β-actin to calculate the ΔCt. The results are

expressed as medians and were compared using the non-parametric Mann-Whitney *U* test. A *p* value less than 0.05 was considered significant.

Results

Twenty trauma patients (median age of 36 [29-44] years) and 19 healthy volunteers (median age of 39 [29-46] years) were included in the study. Among patients, 12 men and 8 women had similar severity scores (**Table 1**). Most of these trauma patients had severe brain injury with a median initial Glasgow coma scale score at 6 [4-8]. Clinical and biological variables were similar between male and female trauma patients, with the exception of serum creatinine levels (**Table 1**).

In healthy volunteers, the expression of *CLOCK* was significantly lower in male than in female (**Figure 1C**). The expression of *ARNTL* and *PER2* was similar in both gender (**Figure 1B and 1D**). In trauma patients, *ARNTL* was significantly up-regulated in male whereas it tend to be less expressed in female (**Figure 1B**). In addition, the expression of *CLOCK* was significantly higher in male with trauma. The expression level of *CLOCK* in female with trauma was similar to control female (**Figure 1C**). Taken together, both components (*ARNTL* and *CLOCK*) are over expressed in male with trauma.

Discussion

To our knowledge, this is the first report showing significant gender-related modulation in circadian clock gene expression in trauma patients. *CLOCK* and *ARNTL* genes, coding for a major transcription factor of the peripheral molecular clock (21,22), were over-expressed in male trauma patients. In addition, the down-regulation of *ARNTL* expression in female trauma patients may amplify the difference in clock-related gene expression between both gender in trauma patients.

Circadian rhythms affect many variables in healthy individuals: heart rate, blood pressure, body temperature and hormone levels (such as melatonin, cortisol). For these variables, intensive care unit patients, and particularly those with brain injury, exhibit abolished or desynchronized patterns across daytime (13,23). This is a key feature of the biological pathways which are under circadian control and regulate the immune response (24,25). Indeed, many components of the inflammatory response (leukocytes, cytokines, hormones) follow diurnal patterns (26,27), and clock-related genes exhibit circadian patterns in neutrophils (7). These observations support the hypothesis that the time of day can affect the patient outcomes in specific diseases (28–30). Our results suggest that the inflammatory response occurring after severe trauma is associated with a modulation of the molecular clock in leukocytes. This up-regulation of clock genes was also reported in suprachiasmatic nuclei in a male rat model of traumatic brain injury (5).

Our preliminary data underline that, in whole blood, gender affects the molecular clock. Indeed, the over-expression of *ARNTL* and *CLOCK* was only observed in male trauma patients. Gender-specific circadian patterns are reported for many variables (31). We previously showed in a murine model of Q fever that, after infection, the molecular clock was differentially modulated between male and female (18). We confirmed these findings in blood samples from Q fever patients (17). The clinical expression of Q fever is known to be

associated with a strong sexual dimorphism. Interestingly, our results suggest that this gender-related modulation of the circadian clock may be rather related to the host inflammatory response than to a specific infection.

As suggested elsewhere (32), these preliminary data support the need to include gender in the pathophysiological models of host inflammatory response. Future investigations are needed to confirm the altered expression patterns of these genes during the intensive care unit stay. Associations with the patient outcomes like immune response and cognitive dysfunction after intensive care unit stay should be examined.

In conclusion, we report for the first time a gender-related modulation of *ARNTL* and *CLOCK* genes in the blood of severe trauma patients. Our previous results were obtained in an infectious model, Q fever, where gender-dependency is well known. The present data support a role for a gender-specific modulation of circadian rhythm in inflammation and infections. Moreover, these data underline the need to take into account variables such as gender to customize the management of trauma patients.

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Figure 1: Expression of *ARNTL*, *CLOCK* and *PER2* in trauma patients and healthy volunteers according to gender. A: schematic representation of the negative feedback loop that controls the rhythmic circadian expression of the peripheral molecular clock components. CRG: Clock Regulated Genes. **B, C, D.** Gene expression ws assessed by qRT-PCR in healthy volunteers and trauma patients and represented as delta Ct values according to gender. As lower Δ-Ct values represent a higher expression, y-axis was reversed to allow an easy visual interpretation of the modulation between groups.

Figure 1

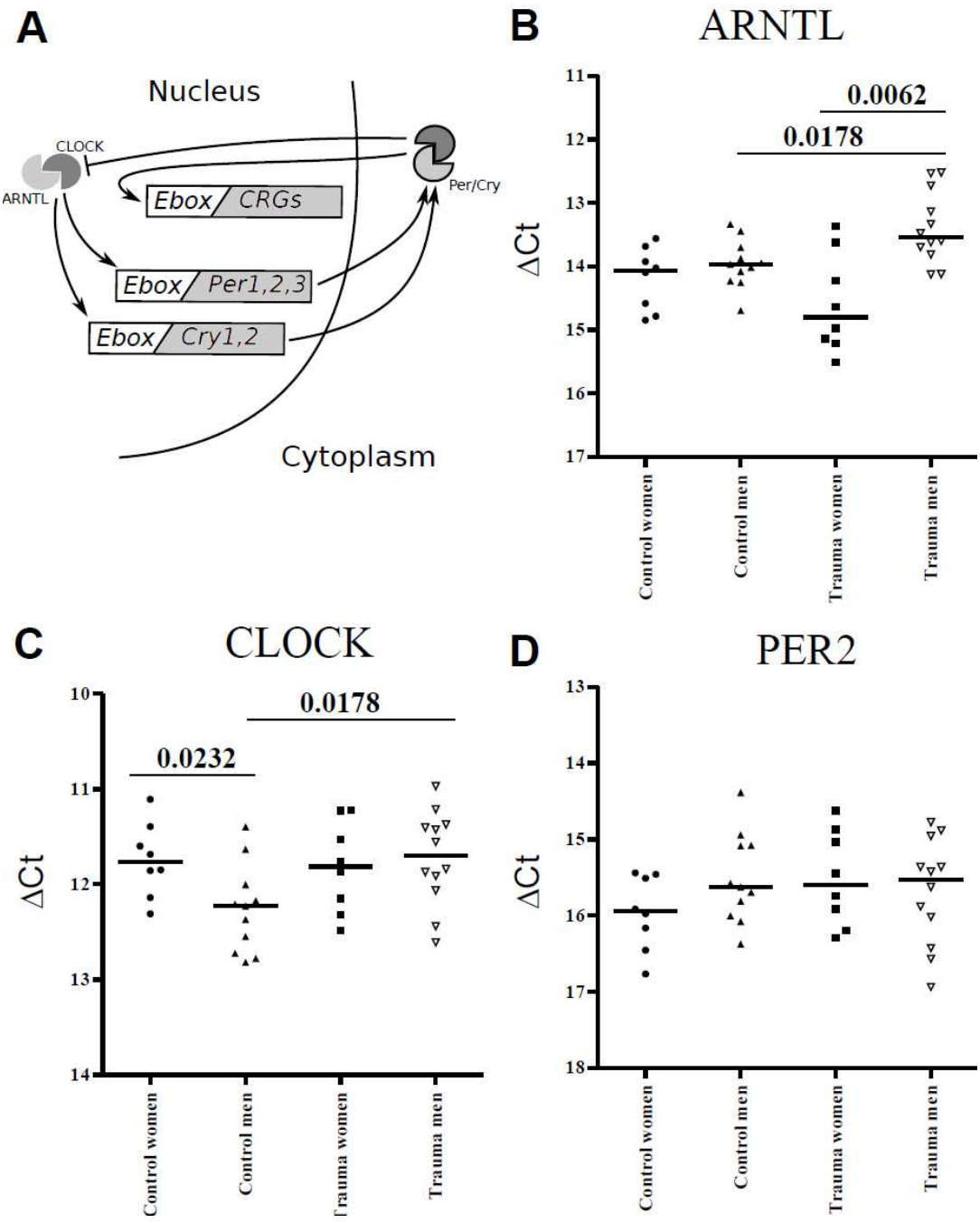

Table 1. Comparison of demographic, clinical and biological characteristics of trauma patients according to gender

Characteristics	**Male (n=12) Median [IQR]**	**Female (n=8) Median (IQR)**	**p**
Age in years	36 [29-45]	37 [23-44]	0.67
BMI	25 [23-26]	20 [18-24]	0.11
SAPS2	41 [35-53]	59 [35-60]	0.34
ISS	33 [25-41]	36 [26-43]	0.65
GCS	6 [4-11]	6 [5-9]	0.81
Hemoglobin (g/dL)	11 [10-13]	10 [7.2-11]	0.15
WBCs (10^9/L)	16 [10-19]	12 [8-19]	0.49
Neutrophils (10^9/L)	14 [8-17]	10 [6.8-16]	0.42
Lymphocytes (10^9/L)	1.2 [1-1.7]	1.4 [0.9-1.8]	0.73
Monocytes (10^9/L)	1.0 [0.8-1.2]	0.5 [0.4-0.8]	0.05
Platelets (10^9/L)	161 [133-176]	179 [122-279]	0.44
Fibrinogen (g/L)	1.4 [1-2.4]	1.8 [1.3-2.3]	0.36
Serum Protein (g/L)	52 [42-62]	52 [46-63]	0.82
Serum Albumin (g/L)	26 [20-38]	28 [22-33]	0.63
Serum Creatinine (µmol/L)	84 [67-99]	64 [51-78]	0.03*

BMI: Body Mass Index; SAPS2: Simplified Acute Physiological Score; ISS: Injury Severity Score; GCS: Glasgow Coma Scale; WBC: White Blood Cell. * **$p<0.05$**

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ABSTRACT

Macrophages are functionally polarized into inflammatory and microbicidal (M1) and immunoregulatory (M2) cells. If circulating monocytes may be polarized is not known. We determined the transcriptional signatures of human monocytes stimulated with IFN-γ and IL-4, known to induce the polarization of macrophages into M1 and M2 cells, respectively, using microarrays and real-time RT-PCR. We found that monocytes exhibited an early pattern of activation specific to IFN-γ or IL-4 and a late pattern of activation common to both agonists. The selected biomarkers of early and late responses were tested in patients with Q fever. We showed that the kinetic model of monocyte activation enables a dynamic approach for the evaluation of patients with acute Q fever or Q fever endocarditis. On the other hand, it is known that the prevalence of Q fever is related to sex and is higher in men than in women. Based on previous studies on an experimental model of infection by *Coxiella burnetii*, the agent of Q fever, we hypothesized that circadian genes are differently modulated in men and women with Q fever. We showed that the expression of the *Per2* gene was significantly increased in males with acute Q fever compared with healthy volunteers but did not differ in females with Q fever and healthy females. These results suggest that that the modulation of circadian genes is associated with a human infectious disease. We also found that the expression of *LNX1* and *LNX2* genes that encode two enzymes involved in protein degradation is increased in Q fever endocarditis but not in acute Q fever.

Key words: monocytes, polarization, Q fever, endocarditis, circadian genes.

RESUME

Les macrophages sont fonctionnellement polarisés en macrophages inflammatoires et microbicides (M1) ou immunorégulateurs (M2). Que les monocytes circulants soient polarisables n'est pas démontré. Nous avons étudié la signature transcriptionnelle par microarray et RT-PCR en temps réel de monocytes humains stimulés par l'IFN-γ, un inducteur des macrophages M1 et l'IL-4, un inducteur des macrophages M2. Leur profil de réponse précoce est dépendent de l'agoniste alors que la réponse tardive des monocytes est similaire qu'ils soient stimulés par l'IFN-γ ou l'IL-4. Cette approche dynamique de la réponse monocytaire permet probablement une étude bien plus pertinente des patients atteints d'une fièvre Q que le modèle de polarisation macrophagique. Par ailleurs, la prévalence de la fièvre Q est plus importante chez l'homme que chez la femme. Comme il a été montré que des gènes associés au cycle circadien sont modulés chez les souris infectées par *Coxiella burnetii*, la bactérie responsable de la fièvre Q, nous avons étudié ces gènes au cours de la fièvre Q. C'est ainsi que le gène *Per2* est fortement exprimé chez les hommes atteints d'une fièvre Q aiguë. Ces résultats suggèrent donc que la modulation de gènes circadiens est associée à une maladie infectieuse chez l'homme. L'expression des gènes *LNX1* and *LNX2*, qui codent deux enzymes impliquées dans le catabolisme des protéines, est accrue dans l'endocardite de la fièvre Q mais pas dans la fièvre Q aiguë.

Mots clés: monocytes, polarisation, fièvre Q, endocardite, gènes circadiens.