EXPRESSION PROFILING AND IDENTIFICATION OF HOST RESPONSE GENES IN PATIENTS WITH SCRUB TYPHUS

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ABSTRACT

Scrub typhus, an infectious disease caused by *Orientia tsutsugamushi* (OT), is a potentially life-threatening illness that is a major cause of acute undifferentiated fever in the Asia-Pacific region. Early diagnosis of the disease is still a challenging clinical problem, and the molecular bases of the infection remain largely uncharacterized. Thus, genome-wide expression analyses were performed in patients with scrub typhus to assess the diagnostic potential of mRNA profiling for the disease and to investigate transcriptional responses of the host during the infection. As compared to other common infections with overlapping clinical features, 65 gene transcripts possessed a distinctive expression pattern for scrub typhus, which perfectly discriminated all the patients with scrub typhus from the rest. Using the profile of only five selected scrub-specific genes, the specified cases could still be grouped together. When a healthy control group was compared, 613 transcripts with ≥ 2-fold up-regulation in scrub typhus patients appeared to be enriched in cell cycle, cell division and immune response. The presence of IFN-γ and its related genes, such as *GBP1*, *IDO1*, and *FASLG*, in the list suggest that type 1 immune response was induced. Surprisingly, particular enrichment of 517 down-regulated transcripts was observed in immune system process, defense response and inflammation, and chemotaxis.

Among the scrub typhus-responsive genes being identified, *IDO1*, a tryptophan-catabolizing enzyme, which was previously reported for its role in host defense against various infectious pathogens, was selected for further study. An induction of IDO1 activity was first confirmed in patients with scrub typhus at both transcriptional and functional levels. Subsequent experiments in a human macrophage cell line THP-1 revealed that OT growth was limited by IFN-γ-mediated IDO1 activation and could be partly restored by 1-MT, an inhibitor of IDO1 enzyme. Excessive amount of tryptophan supplement did not only prevent the depression of OT growth but also dramatically increased the number of OT per host cell in and IDO1-active culture. Altogether, these data suggest that the induction of IDO1 upon scrub typhus infection might help restrict intracellular growth of OT via tryptophan deprivation.

KEY WORDS: SCRUB TYPHUS/ EXPRESSION PROFILING/ HOST RESPONSE/ INDOLEAMINE 2,3-DIOXYGENASE (IDO1)/ ORIENTIA TSUTSUGAMUSHI

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การศึกษารูปแบบการแสดงออกของยีนและการค้นหายีนที่ตอบสนองต่อการติดเชื้อในผู้ป่วยโรคไข้รากสาดใหญ่

EXPRESSION PROFILING AND IDENTIFICATION OF HOST RESPONSE GENES IN PATIENTS WITH SCRUB TYPHUS

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บทคัดย่อ

โรคไข้รากสาดใหญ่เป็นโรคที่อาจมีความรุนแรงถึงขั้นเสียชีวิตและเป็นสาเหตุหลักอย่างหนึ่งของอาการไข้ไม่ทราบสาเหตุในภูมิภาคเอเชียแปซิฟิก การให้การวินิจฉัยโรคตั้งแต่เนิ่นๆยังคงเป็นปัญหาทางคลินิกที่ท้าทาย พื้นฐานความรู้ในระดับโมเลกุลที่เกี่ยวกับการติดเชื้อชนิดนี้ส่วนใหญ่ก็ยังไม่เป็นที่กระจ่างนัก คณะผู้วิจัยจึงได้ทำการศึกษาวิเคราะห์รูปแบบการแสดงออกของยีนทั้งจีโนมในผู้ป่วยโรคไข้รากสาดใหญ่เพื่อประเมินความเป็นไปได้ของการใช้รูปแบบของ mRNA ในการวินิจฉัยโรค และเพื่อสำรวจการตอบสนองในระดับ transcription ของผู้ป่วยในระหว่างการติดเชื้อนี้ เมื่อเปรียบเทียบกับการติดเชื้อชนิดอื่นๆที่มีลักษณะทางคลินิกที่คล้ายคลึงกัน พบว่า transcript จำนวน 65 transcripts มีรูปแบบการแสดงออกของยีนที่แตกต่างออกไปเมื่อเปรียบเทียบกับโรคไข้รากสาดใหญ่ ซึ่งลักษณะการแสดงออกดังกล่าวสามารถแยกแยะผู้ป่วยโรคนี้จากผู้ป่วยติดเชื้อกลุ่มอื่นๆได้ เมื่อใช้รูปแบบการแสดงออกของยีนที่จำเพาะกับโรคนี้พียงแค่ 5 ยีน ผู้ป่วยโรคไข้รากสาดใหญ่ก็ยังสามารถถูกจัดให้อยู่รวมกลุ่มกันได้ จากการศึกษาเปรียบเทียบกับกลุ่มควบคุมที่ไม่เจ็บป่วย พบว่าในกลุ่มผู้ป่วยโรคไข้รากสาดใหญ่ มี transcript จำนวน 613 transcripts ที่มีระดับการแสดงออกสูงกว่ากลุ่มควบคุมอย่างน้อยสองเท่า ซึ่งส่วนใหญ่เป็นยีนที่มีบทบาทในระบวนการวัฏจักรของเซลล์ การแบ่งเซลล์ และการตอบสนองของภูมิคุ้มกัน โดยมียีน IFNG และยีนที่เกี่ยวข้องกันกับยีนนี้ เช่น *GBP1*, *IDO1* และ *FASLG* รวมอยู่ในรายการดังกล่าวด้วย สิ่งที่น่าประหลาดใจ คือ การสังเกตพบว่า transcript ที่มีการแสดงออกลดลงในผู้ป่วยโรคไข้รากสาดใหญ่จำนวน 517 transcripts เป็นยีนเกี่ยวกับกระบวนการในระบบภูมิคุ้มกัน กระบวนการตอบสนองเพื่อป้องกันตนเองและการอักเสบ และกระบวนการ chemotaxis เป็นหลัก

ในบรรดายีนที่ตอบสนองต่อการติดเชื้อไข้รากสาดใหญ่ที่ถูกค้นพบในการศึกษานี้ ยีน IDO1 ซึ่งผลิตเอ็นไซม์ที่สลายกรดอะมิโน tryptophan และมีบทบาทในการป้องกันเจ้าบ้านจากการติดเชื้อหลายชนิด เป็นยีนได้รับการคัดเลือกในการนำไปศึกษาในขั้นต่อไป คณะผู้วิจัยจึงได้ทำการศึกษาและยืนยันว่าระดับการทำงานของยีน IDO1 เพิ่มสูงขึ้นจริงในผู้ป่วยโรคไข้รากสาดใหญ่ทั้งในขั้น trancription และการทำหน้าที่ของเอ็นไซม์ การทดลองต่อมาในเซลล์ THP-1 ซึ่งเป็น macrophage cell line ของมนุษย์ พบว่าการเพิ่มจำนวนของเชื้อ OT ถูกจำกัดเมื่อกระตุ้นการทำงานของยีน IDO1 ด้วย IFN-γ และสามารถได้รับการฟื้นคืนบางส่วนเมื่อยับยั้งการทำงานของเอ็นไซม์นี้ด้วยสาร 1-MT การให้กรดอะมิโน tryptophan เสริมในปริมาณที่มากเกินพอไม่เพียงป้องกันการลดลงของจำนวนเชื้อ OT ในเซลล์ที่ได้รับการกระตุ้นการทำงานของยีน IDO1 แต่ยังเพิ่มสัดส่วนจำนวนเชื้อต่อเซลล์เจ้าบ้านอย่างมีนัยสำคัญ โดยรวมแล้วผลงานวิจัยชิ้นสนับสนุนว่าการทำงานของยีน IDO1 ที่เพิ่มขึ้นในระหว่างการติดเชื้อไข้รากสาดใหญ่น่าจะช่วยจำกัดการเพิ่มจำนวนของเชื้อ OT ภายในเซลล์ผ่านการพรากกรดอะมิโน tryptophan

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LIST OF ABBREVIATIONS

ºC	degree Celsius
:	per
/	per, to
x g	times gravitational force
>	more than
≥	more than or equal to
<	less than
≤	less than or equal to
=	equal
±	plus or minus
1-MT	1-methyl-L-tryptophan
ANOVA	analysis of variance
bp	base pair
cDNA	complementary deoxyribonucleic acid
CO_2	carbondioxide
cRNA	complementary ribonucleic acid
Ct	cycle threshold
d	day
DC	dendritic cell
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dT	deoxythymidine
EDTA	ethylenediaminetetraacetic acid
FBS	fetal bovine serum
FDR	false discovery rate
GO	gene ontology
hr	hour

LIST OF ABBREVIATIONS (cont.)

IFN-γ	Interferon-gamma
Ig	immunoglobulin
kDa	kilodalton
KEGG	Kyoto Encyclopedia of Genes and Genomes
LC-MS	liquid chromatography-mass spectrometry
L-Trp	L-tryptophan
M	molar
mM	millimolar
mg	milligram
min	minute
ml	milliliter
mm	millimeter
mRNA	messenger ribonucleic acid
NaCl	sodium chloride
ng	nanogram
nM	nanomolar
OT	*Orientia tsutsugamushi*
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffer saline
PCR	polymerase chain reaction
p.i.	post-infection
PMA	pharbol 12-myristate 13-acetate
qPCR	quantitative polymerase chain reaction
RT-PCR	reverse transcriptase polymerase chain reaction

LIST OF ABBREVIATIONS (cont.)

RNA	ribonucleic acid
RNase	ribonuclease
rpm	round per minute
rRNA	ribosomal ribonucleic acid
S	Svedberg unit
SD	standard deviation
SEM	standard error of the mean
Th	T-helper
TLR	Toll-like receptor
µg	microgram
µl	microliter
µm	micrometer

CHAPTER I
INTRODUCTION

Scrub typhus is a rural zoonosis infectious disease caused by *Orientia tsutsugamushi* (OT), an obligate intracellular gram-negative bacterium. After the organism is transmitted to human through the bite of a larval trombiculid mite, known as chigger, it mainly infects endothelial cells (1) and macrophages (2). The disease is one of major public health problems in Asia-Pacific region where approximately one billion people are at risk and one million cases are affected each year (3). Exposure to the field where its host vectors inhabit is an important risk factor for the disease but is difficult to avoid especially for people who live in rural areas or work in a farm. So far, no effective strategy has ever succeeded in providing long lasting immunity to this particular infection despite the long aggressive attempts to develop a vaccine for prophylaxis of scrub typhus (4).

Clinical diagnosis of scrub typhus is a critical problem for clinicians due to its non-specific initial symptoms. Acute flu-like fever, headache, rash and lymphadenopathy are common manifestations that could also be found in other acute febrile illnesses. Functional abnormalities of several organs including lung, heart and central nervous system are severe complications that might lead to a life-threatening disease. Because such outcome can be prevented by appropriate clinical management together with a proper antibiotic therapy, early recognition of the affected cases is a prerequisite. Unfortunately, the sensitivity of currently available tools, like serological assay and pathogen detection by real-time PCR, does not quite satisfy the need for early diagnosis.

Pathogenesis and host responses to scrub typhus are not clearly understood. Vascular and perivascular inflammation is the main pathological finding in the affected organs of patients who died of scrub typhus and probably explains a broad range of organ system involvement during the course of the disease (5-6). Therefore, cytokine profiles, as a major factor to shape the immune response and

regulate inflammatory reaction, have been particularly focused in earlier studies. Induction of IFN-γ has been consistently reported in patients with scrub typhus (7-9). It is a key mediator of type 1 immune response previously proved to play an important role in control of OT infection in experimental models (10-15). Up-regulation of number of inflammatory cytokines and chemokines, such as TNF-α, IL-1b, MCP-1, and MIP1a, was also observed upon OT infection and was proposed to be responsible for vascular inflammation during the illness (2, 16). However, it was shown that TNF-α might involve in restriction of OT growth (17), and suppression of its secretion upon the infection by a heat-sensitive component of OT was assumed an immunosuppressive strategy of the bacteria to evade host defense (18-19).

cDNA microarray is a powerful tool that was developed to allow a parallel investigation of transcriptional pattern of multiple related genes at a time. Seeing that host responses to a particular infection usually involve a diverse set of molecules functioning as an intricate network, such high-throughput technology is a promising tool to provide insight into molecular bases of an infection without bias on genes with known functions. In addition, a distinctive mRNA profile, which results from specific pattern of host-pathogen interactions, might also be discovered with the utilization of this powerful technology (20-21).

In the present study, we therefore performed genome-wide expression profiling of peripheral blood leukocytes from patients with scrub typhus. The aims of the study are to discover a scrub typhus-specific molecular signature that might be used as a diagnostic tool for the infection and to identify scrub typhus responsive host genes that might involve in the disease pathogenesis or host defensive mechanisms. To achieve both objectives, pattern of gene expression in patients with scrub typhus was compared to that in patients with other endemic infections with overlapping presentations and to that in healthy subjects, respectively. After being validated, the alteration of gene activity was confirmed at functional level. Since the investigated cell types in the present study are lymphocytes and monocytes, which are active immune cells against OT, a candidate gene involving in an immune system process or a host defensive mechanism is of particular interest. A hypothesis was generated based on its functions and was proved in a less complex system.

Objectives

1. To identify host response genes modulated upon scrub typhus infection
2. To study expression profile in peripheral blood leukocytes of scrub typhus infected patients when compared to other infections with overlapping clinical presentation
3. To generate a hypothesis on a role of a scrub typhus responsive gene during scrub typhus infection
4. To test the hypothesis instigated from microarray study in a cell-based model

CHAPTER II
LITERATURE REVIEW

2.1 An overview of scrub typhus

2.1.1 Etiology and epidemiology

Scrub typhus is a rural zoonosis infectious disease caused by *Orientia tsutsugamushi* (OT). The organism is an obligate intracellular gram-negative bacterium that is transmitted to human through the bites of larval trombiculid mites, known as chiggers. Thus, endemic zone of scrub typhus is geographically distributed in wide areas within Asia-Pacific region according to habitats of its host vector (22). The worldwide incidence has been estimated to be up to one million cases each year and one billion people may be at risk for the disease (3). In Thailand, scrub typhus also appeared to be a major cause of acute, undifferentiated fever in agricultural workers (23). However, the actual number of patients affected by the infection is expected to be larger than what have been reported since many cases of scrub typhus might not be recognized. Indeed, the prevalence of OT seropositivity among persons visiting a malaria clinic in western Thailand was reported to be as high as 59.5% (24).

2.1.2 Pathology and pathogenesis

Pathogenesis of scrub typhus has not been clearly elucidated. Studies in autopsy cases of scrub typhus have shown that vasculitis with perivasculitis is a common pathological finding in the infected patients (25-26). Correspondingly, immunohistochemical staining have revealed that endothelial cells were major targets in most organs evaluated; namely the skin, lungs, heart, kidneys, brain, pancreas, and appendix. OT-bearing macrophages were also found in the liver, spleen and lymph nodes, consistent with an earlier study that identified infected mononuclear leukocytes in peripheral blood of patients with acute scrub typhus(5, 27). An unexpected detection of OT harboured within cardiac muscle cells and renal tubular cells

suggested that the heart and kidneys could be directly injured by invasion of the intracellular organism as well (5).

Apart from vascular and perivascular inflammation, a concept of cytokine storm was also brought up to explain some dreadful complications presumed to be a consequence of overactivity of the immune response to the infection. The development of acute respiratory distress syndrome is one such case in that a minute degree of infection was observed in the pulmonary vasculature, which seems insufficient to cause extensive tissue injury to the lung (5). Hemophagocytosis syndrome is another example that may follow hyperactivation of macrophages as a result of excessive cytokine production (5, 9).

2.1.3 Clinical features

Scrub typhus is notorious for its non-specific manifestations. The clinical course can vary from a self-limited illness to a life-threatening disease depending on susceptibility of the host, virulence of the bacterial strain, or a combination of both factors (28). Acute undifferentiated fever is the most common presenting symptom. Headache and myalgia are complained in about 80% of the patients but do not rule out other acute infections (29). Eschar, a pathognomonic cigarette-burn-like lesion at the chigger bite site, was observed in as less as 3-7% (30-31) and probably up to more than 60% (32) of the infected patients, depending on the studied populations. Other presentations include rash, GI symptoms, chest pain, lymphadenopathy, and hepatosplenomegaly, which appear in less than half of the patients (Table 2.1) (29). Serious complications like myocarditis, pneumonitis, meningoencephalitis, and multiple organ failure may be developed particularly in untreated cases and usually lead to fatal outcome (33).

Table 2.1 Common signs and symptoms of scrub typhus (29)

Signs and symptoms	% of patients
Median (range) days of fever	7 (1-20)
Headache	84.4
Myalgia	76.3
Calf pain	41.1
Sore throat	41.9
Cough	45.6
Chest pain	17.4
Nausea	51.1
Vomiting	48.1
Abdominal pain	26.7
Diarrhea	23
Constipation	11.1
Alteration of consciousness	9.3
Rash	8.9
Injected pharynx	8.5
Calf muscle tenderness	10
Lymphadenopathy	
- Generalized	7.8
- Cervical lymph node enlargement	15.6
Lung’s crepitation	17
Abdominal tenderness	17.5
Hepatomegaly	14.4
Spelectomegaly	0.4

2.1.4 Host responses

In a scenario of an infection, a molecular sensing system is a prerequisite to detect an invading organism before a variety of signalling cascades is triggered and appropriate host defense mechanisms are initiated. Recently, it was suggested that NOD-1 might be responsible for intracellular detection of OT in human endothelial cell lines (34). IL-32 appears to be subsequently secreted and contributes to the production of a number of proinflammatory cytokines and chemokines, accompanied by surface expression of ICAM-1 in the infected cells (34). Since these changes in endothelial cells are crucial for instigation of inflammation, it was proposed that NOD1-IL-32 pathway might be a regulator of inflammatory reaction in the endothelium (34).

Seeing that disseminated inflammation is the main pathology observed in OT-infected mice as well as in patients with scrub typhus, cytokine and chemokine responses in endothelial cells and macrophages, as main targets of OT infection and major regulators of an inflammatory process, have been particularly focused. In murine macrophages, a neutrophil-attracting chemokine MIP-2 as well as several mononuclear leukocyte attractants like RANTES, MIP-1a, MIP-1b, and MCP-1 are induced by the infection and appears to involve NF-kB activation (2). By contrast, induction of MCP-1 in infected human endothelial cells is rather dependent on AP-1 transcription factor via activation of ERK1/2 and p38 MAPK pathway (16, 35). Recently, it was shown that production of IFN-β and TNF-α in OT-infected murine macrophages is also mediated by MAPK activation and is intriguingly influenced by viability of the ingested bacteria (36-37).

In animal models, the set of chemokines induced upon OT infection is consistent with previous studies in cell cultures, and their profile correlate well with kinetics of inflammatory cell infiltration in the affected tissue (38). Besides, the levels of these chemokines; T helper (Th)-1 cytokines like IFN-γ, IL-12, and TNF-α; and a Th-2 cytokine IL10, appear higher in OT susceptible mice as compared to their resistant counterparts, suggesting a role of hyperproduction of cytokines in susceptibility to the infection (39). Analysis of cytokine level in clinical samples concordantly revealed that a similar set of cytokines as well as IFN-γ inducing cytokines, and proinflammatory cytokines that also serve as endogenous pyrogens,

namely TNF-α, IL-1b and IL6, is up-regulated during scrub typhus (7-9, 40-41). The temporal decline of TNF-α, IFN-γ, IL-10 after doxycycline treatment also appeared to be in a good correlation with a decrement of body temperature (41).

IFN-γ and TNF-α are generally known to play a role in defense against a variety of intracellular infections. As a key regulator of macrophage activation, Th-1 cytokine response and development of cell-mediated immunity; IFN-γ has been proved to control OT infection in cell-based and animal models (10-15). An up-regulation of IFN-γ was also consistently reported by a number of studies in patients with scrub typhus (7-9). However, the role of TNF-α in OT infection is quite controversy, and its expression in different individuals with the illness appeared to be varied (7, 40). On one hand, recombinant TNF-α treatment was shown to inhibit intracellular growth of OT in mouse embryo cell lines, murine macrophage and bone marrow-derived macrophage (17). On the other hand, the cytokine was suspected to be responsible for immunopathogenesis and progression of the disease (9, 39). The latter conception was also supported by a recent report that showed a positive correlation between the concentration of TNF-α in acute sera and the severity of the infectious disease (40).

2.1.5 Diagnosis

Clinical diagnosis of scrub typhus is a challenging task since the disease is notorious for its non-specific manifestations, and delayed antibiotic treatment could increase the risk to develop serious complications. The presence of an eschar, a typical cigarette burn like lesion at chigger-bite site, can be very helpful in identifying patients with the infection. Unfortunately, its prevalence was reported to vary from 3-7% (30-31) to >60% (32) depending on the studied populations.

Immunofluorescence assay, which is a serological test for antigen-specific antibody, is currently considered a gold-standard diagnostic method for scrub typhus; however, early diagnosis could be made only if OT-specific IgM antibody titer is prominently high in acute serum. Unfortunately, it was reported that only 42% of scrub typhus patients developed an IgM antibody titer of ≥ 1:16 within the first week of infection, and the prevalence even dropped to 17% at a cutoff value of ≥ 1:160 (42). Convalescent serum is then required to confirm a diagnosis based on four-fold rising

criteria but is not particular useful to reinforce prompt management. Furthermore, high background of baseline antibody is prevalent in people who live in endemic area, causing difficulty in interpretation of an isolated acute antibody titer (43). Pathogen detection by molecular techniques is another promising diagnostic strategy, but the amount of bacterial DNA appeared not to reach a detectable level until five days after the onset of symptoms (7) and the sensitivity appeared to be only 40-60% (7, 44-45).

2.1.6 Treatment and prevention

OT is an obligate penicillin-resistant bacterium on account of the lack of peptidoglycan in its cell wall. Tetracyclin, doxycycline and chloramphenicol are effective drugs of choice that lead to dramatic defervescense within 24 hours in most patients (28, 41, 46). Other supportive treatment is also required to deal with developing complications before the disease processes is under control; even so, some fatal cases exist. Antibiotic resistance of the bacteria is one possibility to explain failure of the treatment as previously reported in northern Thailand (47). Other host and pathogen factors that might influence the outcome of the illness also deserve further investigation.

High antigenic variability of OT is notorious and also allows reinfection with a different strain of the organism in previously affected persons. Avoiding exposure to the forestry where its host vector inhabits is the most effective prophylactic measure but hardly practical for people who live or work in endemic area. Immunization is an interesting alternative strategy, yet no vaccine has ever succeeded in providing a long-lasting immunity to this particular infection so far (4).

2.2 Orientia tsutsugamushi

2.2.1 Characteristics of cellular structure and genome feature

OT is an obligate intracellular gram negative α-proteobacteria (48) with approximated size of 0.5-0.8 μm in width and 1.2-3.0 μm in length (49). It was formerly classified as a member in genus rickettsia on the basis of its morphology and life cycle. Later on, it was separated into a unique genus in family rickettsiaceae due to

three distinctive characters: 1) divergence of its 16s rRNA sequences from others rickettsia (48); 2) lack of peptidoglycan and lipopolysaccharide in its cell wall (50); and 3) a conversely thicker outer leaflet relative to the inner leaflet of its cell wall (49). So far OT is still the only member of genus orientia.

Another distinctive characteristic of OT lies in its genetic structure as revealed by recent whole-genome sequencing studies. First, a single circular chromosome of OT comprises approximately 2 Mb DNA sequences, which is much larger than those of other members of rickettsia family (51-52). Second, OT possesses a large number of repetitive sequences occupying more than 40% of its genome. The repeat density is about 10-200 folds higher than those of other rickettsia and also impressively greater than those of any other bacterial genome ever reported (52). Further characterization of these repetitive sequences revealed the presence of a large number of mobile genetic elements including integrases, transposases, reverse transcriptase, and components of conjugative type IV secretion system (51-52). The phonomenon does not only result in duplication of several transposable sequences-franking genes, like those encoding signaling proteins, transporters and putative host interaction proteins; but it is also accompanied by a subsequent disruption of native genes, particularly those with housekeeping functions, at the integration sites (51-52). Interestingly, the most extensive gene loss is observed in matabolic pathways; such as glycolysis, pentose phosphate pathway, acetyl-co A synthesis and de novo pyrimidine biosynthesis, suggesting a more host-dependent life cycle compared with other rickettsia (51, 53).

2.2.2 Life cycle in human

After inoculated into a human, OT initially infects the endothelium and proliferates at the bite site of a chigger. Subsequent release of inflammatory mediators by the infected cells leads to attraction of circulating leukocytes, which then become secondary targets of the infection and carry the organism to infect other cells in regional lymph nodes as well as remote organs (54).

At the cellular level, invasive mechanism of OT seems varied depending on host cell types. For example, it was found that OT exploits phagocytic pathway to invade PMN (55), whereas its entry into non-phagocytes requires clathrin-mediated

endocytosis (56). However, it was recently revealed that OT could also induce phagocytosis in non-phagocytes for its internalization through activation of integrin signaling (57). Fibronectin is utilized as a linker for an indirect interaction between TSA 56 kDa surface protein of OT and integrin receptor on host cell, which ultimately results in actin reorganization and subsequent uptake of adhering bacteria (57-58). Other surface cell antigen (Sca) proteins of the bacterium were also suspected to facilitate attachment and internalization of the bacteria during its invasion (59).

Subsequent to its entry into a host cell, OT seems to escape from phagosome or endosome by unidentified mechanisms (60). Microtubules and dynein are then utilized for its movement to perinuclear area, where it replicates by binary fission (18) with a doubling time of 9-18 hours (61). The transmission to other cells might occur immediately by direct entry into neighboring cells or might be a following event after the outer host membrane has been shed out (22). However, penetration into a new host cell is needed soon after budding out since OT is more fragile in extracellular environment due to the lack of peptidoglycan in its cell wall (50, 62).

2.2.3 Survival strategies

As an obligate intracellular parasite, OT has evolved multiple adaptations to survive in such hostile environment. First, it needs effective strategies to gain nutrients and essential material for its activities, particularly when a variety of functional metabolic genes has been lost from its genome as mentioned earlier (51, 53). Coincidently it was found that a number of putative genes for transporter and host-cell interacting protein have been amplified, which was surmised to advantage its nutritional attainment (51). However, such hypothesis still needs to be proved, and the predicted role of these gene products still remains illusive.

Evasion from host defensive mechanism is another crucial capability of an invading parasite for its survival in body of the host. As previously described, OT intriguingly leaves phagosome into intracellular compartment early after its entry in order to avoid getting killed and being presented to the host immune system (60). However, the tactic does not guarantee its safety since some non-viable organisms trapped in phagosome were also observed (55, 63); thus, other immune evasion strategies are required. For example, it was found that a heat-sensitive component of

OT can suppress production of TNF-α by indirectly inducing the release of IL-10, an anti-inflammatory cytokine with negative effect on type I immune responses and clearance of intracellular pathogen (18-19). Such findings were also supported by a recent in vivo study that observed a positive relationship between the concentration of IL-10 and the bacterial load in patients with scrub typhus (7).

Moreover, it was found that OT inhibits host cell apoptosis to preserve its intracellular hideouts by retarding calcium release that causes endonuclease activation with subsequent apoptosis of the infected host cell (64). Paradoxically, an earlier histochemical study in OT-infected mice revealed prominent apoptotic changes of lymphocytes inside the spleen and regional lymph nodes of the affected animals which was suspected to be induced by the infection and probably responsible for immunosuppression during the illness (65). The latter finding is also concordant with the down-regulation of a major antiapoptotic factor, bcl2, simultaneous with disassembly of focal adhesion and apoptosis observed in infected endothelial cells (66).

2.3 Microarray technology

2.3.1 Basic principles

Microarray is a high-throughput technology that works on a basis of a specific interaction of complementary bases on two particular nucleotide strands and aims to simultenously quantify multiple gene transcripts at a time. A chip comprises a vast number of gene-specific oligonucleotide probes immobilized on a solid support to capture each particular species of labelled nucleotide targets in an inquired sample. The amount of each transcript can be then implied from the signal intensity of its specific probes hybridized in order to reflect the transcriptional activity of that particular gene in a specimen.

2.3.2 Potential applications

After the completion of the human genome project and the advent of this powerful technology, an analysis of genome-wide expression has come to a reality.

Investigation of transcriptional pattern of multiple related genes in parallel has allowed unravelling the complexity of biological networks as demonstrated in a variety of research area like cancer (67-69), autoimmune diseases (70-72), and infection (73-75). Without a requirement of prior knowledge, an unbiased analysis of global gene expression could contribute to the generation of a novel hypothesis for further study and the discovery of biomarkers and new drug targets as well (76-77). Additionally, gene expression profiling appeared to hold a great potential to be utilized in verification and subclassification of patients, as proved in several types of solid and hematologic malignancies (69, 78-79), and also in prediction of their clinical courses and potential responses to treatment (80-82).

2.3.3 Genome-wide expression profiling in infectious diseases

As an output of an activation of host defensive programs counteracted by an active transcriptional modulation by the pathogen, an investigation on host gene expression may shed the light on a complex interplay between the host and the invading organism. An example for this notion includes an in vitro study that elucidated molecular mechanisms of macrophage activation programs in response to a number of pathogenic bacteria and their components and also evidenced a transcriptional modification of IL-12 by Mycobacterium tuberculosis (75).

In vivo, a trace of host responses does not only lie in the cells at the affected site but also in circulating leukocytes, which are another group of highly active players in the battle of infection. Moreover, peripheral blood is an easily accessible specimen and holds clinically relevant information that might help clarify the underlying pathogenesis and pathophysiology of the illness. A genome-wide expression study in peripheral blood leukocytes of malaria infected patients, for example, has revealed common and divergent immune signalling pathways in presymptomatic and symptomatic stages of the infection (83). In dengue viral infection, correlation of expression profiles to clinical outcomes also appeared to provide more understanding about pathogenic mechanisms of such a disease with variable severity (84-86).

The diagnostic application of microarray analysis was also adopted for infectious diseases as it was presumed that a disease-specific profile could be extracted

from a mixing set of specific and non-specific host responses upon an infection. To prove this concept, global gene expression was explored and compared amomg patients with acute infection caused by influenza A, gram-negative bacteria, and gram-positive bacteria (20). It was demonstrated that differential mRNA profiles could be identified and also successfully differentiated patients with a particular infection form the others, suggesting the potential of transcriptional signatures to assist in diagnosis of infectious diseases (20).

CHAPTER III
MATERIALS AND METHODS

3.1 Materials

3.1.1 List of chemicals and reagents

1) Lymphoprep *(*Axis-Shield PoC AS, Oslo, Norway)
2) PBS
3) Trizol reagent (Invitrogen, Calsbad, CA)
4) LightCycler Fast start DNA Master SYBR Green I (Roche Diagnastic GmB, Mannheim, Germany)
5) Phenol
6) Chloroform
7) Superscript III First strand dDNA synthesis (Invitrogen, Calsbad, CA)
8) Totalprep RNA amplification (Ambion, Carlsbad, CA)
9) RNeasy mini kit (Qiagen, Valencia, CA)
10) Human-6 v2 BeadChips (Illumina)
11) RPMI1640 (Gibco, Auckland, NZ)
12) Fetal bovine serum (Biochorm AG, Berlin)
13) Phorbol myristate acetate (Sigma-Aldrich, USA)
14) L-tryptophan (Sigma-Aldrich, USA)
15) L-kynurenine (Sigma-Aldrich, USA)
16) 0.25% Trypsin-EDTA (Gibco, Auckland, NZ)
17) 1-methyl-L-tryptophan (Sigma-Aldrich, USA)
18) Recombinant human Interferon-γ (R&D Systems, Minneapolis, MN, USA)

3.1.2 Clinical samples

Clinical samples were derived from the following groups of subjects with an approval by the Ethical Committee of Siriraj Hospital in 2007.

1) Patients who came to Siriraj Hospital or Bann Mai Chaiyapoj Hospital and presented with acute undifferentiated fever as defined by three main characteristics: i) body temperature > 37.5 ºC; ii) no sign and symptom of upper respiratory tract infection; iii) no evidence of localized infection

2) Healthy blood donors who volunteered to participate in this study

3) Patients with a definite diagnosis for scrub typhus by serological as determined by immunofluorescent assay for OT-specific Ab detection (see the diagnostic criteria in Section 3.2.1.2)

3.1.3 Cell cultures

1) THP-1 cell line (ATCC TIB-202) was kindly provided by Dr.Angkana Chaiprasert.

2) L-929 cell line was kindly provided by Dr.Sontana Siritantikorn.

3) OT strain Kato was kindly provided by Prof. Jean-Louis Mege and Dr. Wiwit Tantipattayangkul.

3.2 Methods

Part I

3.2.1 Genome-wide expression study in peripheral blood mononuclear cells (PBMCs)

3.2.1.1 Collection and processing of blood samples

The project was approved by ethical committee of Siriraj Hospital in 2007. Ten ml of EDTA blood was drawn from each participating subject at the first encounter with a written informed consent. Clinical characteristics of the patients at the day of blood collection including day of fever, body temperature and differential white blood cell count were also recorded. Within two hours after blood collection, PBMCs were isolated by standard Ficol gradient centrifugation method. Briefly, blood samples were diluted with 10 ml of PBS before being gently layered over Lymphoprep. After centrifugation at 2000 rpm for 30 minutes without brake, white ring of PBMCs at the interphase was collected into a fresh tube. The isolated cells were washed twice with PBS to remove the remaining Lymphoprep before being lysed in 1 ml of Trizol reagent. The samples were stored at -80 °C until being used in the next step.

3.2.1.2 Selection of samples for microarray study

Samples from participants with confirmed diagnosis for scrub typhus, or other common endemic infectious diseases; namely murine typhus, dengue fever or malaria, were retrospectively selected to be further processed for microarray study. Diagnostic criteria for each infectious disease are given as follow:

- Scrub typhus: *O.tsutsugamushi*-specific IgM titer ≥1:400 in acute serum or ≥ four-fold rising of antibody titer in paired sera.
- Murine typhus: *Rickettsia typhi*-specific IgM titer ≥1:400 in acute serum or ≥ four-fold rising of antibody titer in paired sera.
- Dengue viral infection: dengue virus-specific IgM positive in acute serum (by ELISA) /or ≥ four-fold rising of dengue virus-specific IgG in paired sera.

▪ Malaria: *Plasmodium spp*. detected in blood films under light microscope.

However, patients with evidence of co-infection or cancer as co-morbidity were excluded from the study.

3.2.1.3 Extraction and purification of total RNA

Total RNA were extracted from frozen PBMCs in TRIZOL® reagent derived from healthy blood donors and the selected patients, as indicated in Section 3.2.1.2, in accordance to the manufacturer's instruction. Briefly, 0.2 mL of chloroform was added per 1 mL of TRIZOL® reagent. The mixture was shook vigorously for 15 seconds and incubated at room temperature for 2-3 minutes. After 15 minutes of 12,000 x g centrifugation at 4 °C, colorless upper aqueous phase containing RNA was aspirated and transferred into a fresh tube. Total RNA was precipitated with 0.5 mL of isopropanol before being washed with 75% RNase-free ethanol and finally dissolved in RNase-free water. Then, the extracted RNA was purified using RNeasy mini kit (Qiagen, Valencia, CA) in accordance to the manufacturer's protocol. For healthy control group, two pools of unpurified RNA were generated by mixing an equal amount of RNA sample from five healthy males and five healthy females before similar purification process was performed. The quality of purified RNA was determined by gel electrophoresis before being used in microarray study.

3.2.1.4 Amplification of cRNA

Biotin-labelled cRNA was prepared from 500 ng of each purified RNA sample according to Illumina Total Prep RNA Amplification's protocol. Briefly, double-stranded cDNA was synthesized from purified RNA using T7 promoter-linked oligo (dT) primers. After being purified by column purification, T7 promoter-containing cDNA was used as a template for in vitro transcription which continued for fourteen hours to synthesize biotin-labelled cRNA. The yields of cRNA were quantified by NanoDrop spectrophotometer.

3.2.1.5 Hybridization and scanning of BeadChips

Five-hundred nanograms of each biotinylated cRNA sample was loaded onto individual Human-6 v2 BeadChips (Illumina), which inquires into >46,000 transcripts across humuan genome. Hybridization of the samples was allowed for sixteen hours at 58 ºC in a hybridization oven. Then, the chips were washed and

stained with steptavidin-Cy5 before being scanned by Illumina BeadStation 500 instrument. Fluorescent signals derived from the scanner were finally converted into raw signal intensities with background subtraction by Illumina BeadStudio software.

3.2.1.6 Pre-analytical data processing

Raw signal intensity data were imported into GeneSpring GX 9.0 software (Agilent). Quantile normalization was then applied to the data. All 24 samples were classified into five groups according to their causative infection: scrub typhus, murine typhus, malaria, dengue fever and healthy control. To filter out data with low signal to noise ratio, probes with the expression level lower than 20^{th} percentile rank in all samples of any groups were excluded from further analysis.

3.2.1.7 Analysis of microarray data

To identify scrub typhus-specific genes, Welch ANOVA with Benjamini-Hochberg correction (87) was used to test across four groups of the diseases. P-value < 0.05 at false discovery rate (FDR) $< 5\%$ was considered statistically significant. Tukey post hoc test was then applied to subsequent Welch ANOVA result, and genes differentially expressed in scrub typhus as compared to the other three disease groups were selected by intersection rule. Analyses of pathway and gene ontology of the gene set were performed, using DAVID online software (88-89). In an attempt to reduce the number of scrub typhus discriminating genes, a criterion based on fold difference was further applied to scrub typhus-specific gene set previously identified by statistical criteria. To evaluate discriminative potential of these gene sets, their expression profiles were used to perform hierarchical clustering of all samples, based on Euclidean distance and average linkage rule.

To identify scrub typhus responsive genes, levels of gene expression in scrub typhus group were compared to those of healthy control group. Gene expression exhibiting at least two-fold difference between the two conditions was considered significant. Finally, functional annotation of selected gene sets were performed based on Gene Ontology (GO) (90) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases (91-92), using DAVID online software (88-89).

3.2.2 Validation of expression levels by quantitative PCR (qPCR)

3.2.2.1 Synthesis of cDNA

cDNA samples that would be used in the same subsequent qPCR experiment were simultaneously synthesized from each RNA sample using SuperScript™ III First-Strand Synthesis System (Invitrogen) in accordance to the manufacturer's protocol. Briefly, dNTPs, oligo dT primers, and an equal amount of total RNA sample ranging from 200 ng to 5 µg was assembled in a 0.2-µl microcentrifuge tube and incubated at 65 ºC for five minutes to denature all the components. Afterwards, the mixture was immediately placed on ice for more than one minute. Then, a cDNA synthesis reaction master mix was prepared before being aliquoted into the denatured mixture on ice. All the mixture was incubated at 50 ºC for 50 minutes followed by five minutes of incubation at 85 ºC to terminate the reaction. After the mixture was cooled on ice, RNase H was added to each tube and incubated at 37 ºC for 20 minutes to remove the RNA template from the cDNA-RNA hybrid. The synthesized cDNA samples were stored at -20 ºC until being used in subsequent qPCR.

3.2.2.2 Gene specific amplification by qPCR

Gene-specific primers used in analysis of gene expression were designed to bind at exon-exon junctions or across two different exons of the target cDNA sequence using Oligio 6.7 software (Molecular Biology Insights). The specificity of each primer pair was checked by NCBI/Primer-BLAST tool. Ten-µl quantitative real-time PCR reaction mixture was prepared using LightCycler® FastStart DNA Master SYBR Green I reagents (Roche Applied Science), a pair of gene specific primers and two µl of an appropriate dilution of cDNA sample. Then, real-time PCR was performed in duplicate using LightCycler 480 Instrument.

Table 3.1 Primer pairs for quantitative PCR

Genes	Forward primers (5' → 3')	Reverse primers (5' → 3')	Product size (bp)
CD8A	CCTTTACTGCAACCACAGGA	AGGAAGGATCTCAGTTTGAAG	145
CD8B	ACTTCTGCATGATCGTCGG	AGGGTGGACTTCTTGGTG	109
CBLB	CCCTTTGTTGATCTAGCAAGTG	GTGCCTGTGAACCATCTGAA	133
LOC642161	CTCAAAATGCCCCTCCTTTC	GGTTCTGTGAGTCCTGCTT	139
FOSB	CAGCAGCTAAATGCAGGA	TTTGGAGCTCGGCGATCT	114
IFI27	TCCTTCTTTGGGTCTGGCT	CCACACTGGTCACTGCTGA	112
TIMD4	TTGTTTGTGGCGTTTCTC	GCCGTCTTCGTCTTCCCT	135
ANKRD22	GCAGACCCCACAATAAAGAATAAGC	TTAACTTTTTCTGTATCTCCATCGG	141
CCL3	AGGACACGGGCAGCAGAC	CGTGTCAGCAGCAAGTGA	169
CCL3L3	TCTGCAACCAGGTCCTC	TGGGCTTGGAGCACTG	135
FCER1A	CATTACAAATGCCACAGTTG	AGCAAACAGAATCACCACCA	166
HLA-DRB5	TCACCCAACAGGACTCG	GAGTTGCTGAACCGGTAG	157
IL1B	ATCTGTACCTGTCCTGCGTGTT	GGAACTGGGCAGACTCAAATTC	156
SSBP1	AGACAACATGGCACAGAATATCAG	GTTGCTTGTCGCCTCACATTA	148
TNF	ACGCTCTTCTGCCTGCT	CGAGAAGATGATCTGACTGCC	110
CCL20	GAAGGCTGTGACATCAATGCT	TTCCATTCCAGAAAAGCCAC	151
CCL3L1	GAAGGACGCAGGCAGCAA	TGTCAGCAGCAAGTGGTG	172
TBP	CAGGAGCCAAGAGTGAAGAA	CCTTATAGGAAACTTCACATCACAG	143
IDO1	CAAATCCACGATCATGTGAACC	AGAACCCTTCATACACCAGAC	112
IFNG	TGGGTTCTCTTGGCTGTTACTG	TTTTGTCACTCTCCTCTTTCC	151
[†]*MTHFR*	AAGGAGAAGGTGTCTGCGGGCGC	AAGATCCCGGGGACGATGGGG	128
[†,§]*OT47kDa*	ATCTTACTCAGGCATAAGTT	TAACATACCACGACGAATTT	97

Note: [†] The primer pairs were used for an analysis of OT growth (see Section 3.2.4.6).

[§] The primer pair was specifically designed for 47 kDa-protein gene based on DNA sequences of Kato strain (accession: L11697).

3.2.2.3 Analysis of qPCR data

In the analysis of qPCR data, relative quantification was performed by LightCycler 480 software based on comparative ΔΔCt method. Expression of a housekeeping gene, *TATA box binding protein* (*TBP*), was used as a reference level. Expression ratio between each target gene and *TBP* was normalized to that of healthy control group.

Part II

3.2.3 Study of IDO1 activity in patients' sera and culture media

3.2.3.1 Collection of serum samples

Acute serum samples were derived from patients with acute febrile illness at the first visit to Siriraj Hospital with a written informed consent. Definite diagnosis of scrub typhus was determined using the following serological criteria: OT-specific IgM ≥ 1:400 in acute sera or ≥ four-fold rising of OT-specific antibody in paired sera collected two weeks apart. The sera were stored at -80ºC until being processed for liquid chromatography-mass spectrometry (LC-MS) analysis. As a control group, serum samples from ten healthy blood donors were also derived and handled in a similar manner.

3.2.3.2 LC-MS analysis of L-tryptophan and L-kynurenine

Analysis of L-tryptophan and L-kynurenine was performed using a validated high performance liquid chromatography with tandem mass spectrometry (HPLC-MS/MS) method in accordance with the USFDA guidelines [U.S. Department of Health and Human Services Food and Drug Administration Center for Drug Evaluation and Research 2001]. Sample preparation was performed by protein precipitation technique. Briefly, 10,000 ng/ml 3-nitro-L-tyrosine, as an internal standard (IS), was added into 100 µl of every serum sample and culture media to be tested, as well as corresponding types of specimens spiked with tryptophan and kynurenine standards. After well mixed for 10 seconds, all samples underwent protein precipitation process by mixing with 150 µl of 10% TCA followed by centrifugation at 10,000 rpm for 10 minutes. The organic layer was transferred into a new vial before

five µl of each specimen was injected into the LC-MS/MS system. Chromatographic separation was carried out on LC-MS/MS with C18, 2.5 µm (50 x 3.00 mm i.d.). A mobile phase consisting of acetonitrile and 0.1% formic acid (Gradient condition) was delivered at a flow rate of 0.2 mL/min. Mass spectra were obtained using a Quattro Premier XE mass spectrometer (Micromass. UK) equipped with electrospray ionisation (ESI) source. The mass spectrometer was operated in multiple reaction monitoring (MRM) mode. Sample introduction and ionization was performed by electrospray ionization in the positive ion mode for L-tryptophan and L-kynurenine and IS. The mass transition ion-pair for L-tryptophan [*M*+H]+ ions was selected as *m*/*z* 205.08 > 188.00 and 205.08 > 146.08. The mass transition ion-pair for 3-nitro-L-tyrosine [*M*+H]+ ions was selected as *m*/*z* 227.02 > 181.03. The data acquisition was ascertained by Masslynx 4.1 software. The detectable ranges of L-tryptophan and L-kynurenine in both human serum and culture media were 0.05 – 50 ng/ml and 0.15 – 10 ng/ml respectively. The best linear fit was achieved with a 1/x weighting factor, showing a mean correlation coefficient (r^2) ≥ 0.998.

3.2.3.3 Statistical analysis

Serum levels of L-tryptophan and L-kynurenine in scrub typhus and control groups were compared using Mann Whitney test. To refelct IDO1 activity in every individual, the proportion of L-kynurenine level to L-tryptophan level was also calculated. The ratios derived from both groups of subjects were then compared using the same statistics.

For culture media derived from the in vitro experiments, described in the following section; statistical significance of the difference in L-tryptophan level, L-kynurenine level, as well as L-kynurenine/L-tryptophan ratio between any two groups of treatment was determined using unpaired t-test with or without Welch's correction as appropriate.

3.2.4 Investigation of IDO1 functions in an in vitro model of OT infection

3.2.4.1 Maintenance of cell lines

L929, a mouse fibroblast cell line, was cultured in RPMI-1640 media supplemented with 10% FBS in a humidified 5% CO_2 environment at 37 ºC.

Every three to four days, the cell culture was trypsinized and split to appropriate confluence using 0.25% trypsin/EDTA.

THP-1, a human monocytic cell line, was cultured in RPMI-1640 media supplemented with 10% FBS in a humidified 5% CO_2 environment at 37 ºC. Every three to four days, the culture was split and fresh media was added to maintain the density of cell suspension below 10^6 cells/ml.

3.2.4.2 Preparation of OT stock

OT Standard Kato strain was propagated using L929 as a host cell. Briefly, frozen OT-infected host cells were thawed in a water bath at 37 ºC; then, one-millimeter glass beads and vortex were used to break the host cells. OT-containing suspension was transferred to a conical tube and was centrifuged at 500 x g for five minutes to pellet cell lysate while leave extracellular OT in supernatant. Lysate-free OT inoculum was then added onto L929 monolayers which were incubated further in a humidified 5% CO_2 environment at 37 ºC. Stale media was replaced with fresh one every seven day. When more than 90% of the culture were infected as determined by Giemsa staining, mostly at ten to fourteen days post inoculation, the cells were disrupted using 1.0-mm-diameter glass beads and a vortex. To propagate the organisms further, the disrupted cell suspension was used to continue another cycle of inoculation onto L929 monolayers. To prepare OT inoculum for subsequent experiments, stale media was replaced with three ml of fresh media. The infected cells were then dislodged from the flask and were broken down with 1.0-mm-diameter glass beads and a vortex. The disrupted cell suspension ensuing from multiple parallel cultures was pooled together and stored as small aliquots in liquid nitrogen. Before being used in experiments, a few aliquots of frozen OT inoculum were thawed in water bath at 37 ºC. Afterwards, the cell suspension was thoroughly mixed and broken down once again with glass beads and vortex before being centrifuged at 500 x g for five minutes to sediment host cell lysate. Finally, subsequent OT-containing supernatant was immediately used to infect target cells.

3.2.4.3 Cell culture and in vitro infection

Twenty-four hours prior to an in vitro infection experiment, one ml of THP-1 cell suspension (2 x 10^5 cells/ml) was transferred into each well of 24-well plate and treated with 100 nM pharbol 12-myristate 13-acetate (PMA) to

induce cell adherence. When IDO1 expression was required, the cells were be treated with 20 ng/ml IFN-γ, along with 1 mM 1-methyl-L-tryptophan (1-MT), 400 μg or 1 mg of L-tryptophan supplement, or neither. On the day of the experiment, the culture media was aspirated. Two hundreds and fifty μl of OT-containing inoculum, which was prepared as previously described in Section 3.2.4.2, was added to the cell culture. Infection process was facilitated by centrifugation at 1,450 x g for five minutes and further incubation for one hour in a humidified 5% CO_2 atmosphere at 37°C. The inoculum was then replaced with one ml of fresh PMA-containing media, together with additional treatments similar to each pre-infectious condition for each culture; and this time point was designated as 0 hour p.i.. At specified time points, culture media was discarded or collected for determination of IDO activity. Floating cell particles and extracellular organisms were sedimented by centrifugation at 10,000 rpm for 10 minutes. Subsequent supernatant was transferred to a new microcentrifugation tube and stored at -80 ºC until being processed for LC-MS analysis, as describe in Section 3.2.3.2. The cell layer was then rinsed three times with PBS to wash out the extracellular organisms. Finally, the infected cells were collected and further processed for RNA, as described in Section 3.2.1.3, or for DNA, as described in Section 3.2.4.5.

3.2.4.4 Analysis of *IDO1* expression by qPCR

Following the protocols in Section 3.2.2.1, cDNA was synthesized from RNA samples derived from the in vitro experiments in Section 3.2.4.3. It was then used as a template for specific amplification of TBP and IDO1 gene transcripts by qPCR technique as described in Section 3.2.2.2. Relative quantification analysis of qPCR data was performed by LightCycler 480 software based on comparative ΔΔCt method using IDO1 as a target gene and TBP as a reference gene. The mean expression ratio of IDO1 and TBP of mock-infected culture harvested at 0 hour p.i. was used to normalize those of the other cultures.

3.2.4.5 DNA extraction

DNA was isolated from THP-1 cell culture using phenol/chloroform method. Firstly, 200 μl of Tris-EDTA buffer (20:5) was filled to a cell layer in culture plates, followed by addition of 10 μl of 10%SDS and 10 μl of 2mg/ml proteinase K. After pipetting up and down vigorously, the mixture was

incubated further at 50 ºC for a few hours to allow complete cell lysis. Then, it was cooled to room temperature and transferred to a 1.5-ml microcentrifugation tube. To isolate nucleic acids from other components, 200 µl of equilibrated phenol (pH 8.0) and 200 µl of chloroform-isoamyl alcohol (24:1) was added to each tube, and the sample was mixed thoroughly but gently by inversion. After being centrifuged at 2,500 rpm for 10 minutes at 4 ºC, the mixture was separated into two layers: an upper aqueous phase, which contains nucleic acids; and a lower organic phase, where proteins and lipids reside. Then, the lower portion was suctioned out, and another round of nucleic acids isolation was repeated. To remove remaining contaminants from the aqueous phase, 400 µl of chloroform-isoamyl alcohol (24:1) was added; the mixture was thoroughly mixed and underwent 10-minute centrifugation using the same setting. Afterwards, subsequent organic phase was suctioned out, and the process of decontamination was repeated another one more time. To precipitate DNA, the resulting nucleic-acid-containing solution was gently mixed with 40 µl of 4 M NaCl and 800 µl of chilled absolute ethanol before being centrifuged at 10,000 rpm for 10 minutes at 4 ºC. The supernatant was then removed, and DNA pellet was washed with 1 ml of 70% ethanol, followed by 10,000-rpm centrifugation at 4 ºC for two minutes. Afterwards, the supernatant was carefully aspirated, and the DNA pellet was allowed to air dry. Finally, DNA was dissolved in deionized sterile distilled water before the concentration was determined by NanoDrop spectrophotometer.

3.2.4.6 Analysis of OT growth by qPCR

To assess the degree of OT growth in each culture indirectly, the number of OT was quantified in relation to the number of host cells. Comparative ΔΔCt method was adopted, using *OT47kDa* of OT as a target gene and *Methylenetetrahydrofolate reductase* (*MTHFR*) of the host as a reference gene. A DNA sample derived from section 3.2.4.5, which comprise a mixture of host's and OT's genomic DNA, was used as a template. Primer pairs for *OT 47 kDa* and *MTHFR* were provided in Table 3.1, and details of gene-specific amplification protocol were described in Section 3.2.2.2. Finally, the ratio of OT 47 kDa/ MTHFR in each culture was normalized to that of the infected culture without IFN-γ treatment at 6 hours p.i.. The normalized value was designated as "infection index", which reflects an extent of OT harboured in each culture.

3.2.4.7 Statistical analysis

Statistical significance of the difference in normalized expression level of *IDO1* and infection indexes between any two groups of treatment was determined using unpaired t-test with or without Welch's correction as appropriate.

CHAPTER IV
RESULTS

Part I

4.1 Genome-wide expression profiling

4.1.1 Enrollment and characteristics of the patients

Totally 75 patients with acute undifferentiated fever were enrolled into the study. Only 23 met the inclusion criteria, but one was excluded because he also has cancer. Finally, 22 patients were selected to undergo microarray study and were then classified into four groups according to the confirmed diagnosis. Two pools of RNA samples derived from five healthy males and five healthy females were additionally included as a group of healthy control.

Characteristics of patients in each group were given in Table 4.1. The number of leukocytes in scrub typhus was pronouncedly greater than that in the other groups of infection ($P < 0.01$). Patients with dengue fever came to see the doctor earlier than those with scrub typhus or with murine typhus did ($P < 0.05$). However, lymphocyte/monocyte ratios, which represent cellular constituents of the samples being studied, were not significantly different among the four groups of the patients.

Table 4.1 Characteristics of patients in each group of diseases

	Scrub typhus	Malaria	Murine typhus	Dengue fever
N	4	4	7	7
Age	46 ± 18.4	26.5 ± 6.0	41.7 ± 14.5	31.9 ± 18.1
Male : Female	2:2	2:2	1:6	4:3
Mean fever day (range)	10.5 (3-20)	5.25 (3-7)	9.71 (3-15)	2.29 (1-4)*,†
Body temperature (°C)	38.0 ± 0.8	38.8 ± 0.6	37.8 ± 1.0	38.9 ± 0.9
WBC (x10^3/µl)	13.5 ± 2.4	4.1 ± 1.3***,†	8.1 ± 2.4**	5.1 ± 2.6***
Platelet (x10^3/µl)	182.5 ± 69.3	37 ± 19.1††	221.7 ± 120.1	131.3 ± 65.3
Neutrophil (%)	76.8 ± 9.7	67.6 ± 9.0	72.1 ± 12.4	65.2 ± 20.6
Lymphocyte (%)	17.4 ± 12.1	17.7 ± 11.4	20.8 ± 12.1	16.8 ± 15.7
Monocyte (%)	4.3 ± 2.5	7.1 ± 2.5	5.1 ± 2.1	6.9 ± 4.5
Lymphocyte/Monocyte	2.0 ± 4.9	2.5 ± 4.6	4.1 ± 5.8	2.4 ± 3.8

NOTE: Data represents mean values of each group of the patients ± standard deviation.

* $P < .05$ as compared to the mean value of scrub typhus group

** $P < .01$ as compared to the mean value of scrub typhus group

*** $P < .001$ as compared to the mean value of scrub typhus group

† $P < .05$ as compared to the mean value of murine typhus group

†† $P < .01$ as compared to the mean value of murine typhus group

4.1.2 Processing of intensity signals

All gene-specific probes in a chip were ranked by their signal intensities for each sample. To filter out data with low signal/noise ratio, the probes that were ordered in the rank below 20th percentile for all samples of any groups were excluded from subsequent analysis. Ultimately, 38,630 out of 48,701 probes passed the filtering criteria to be further normalized. Post-normalization data of every sample were comparably distributed as depicted in Figure 4.1.

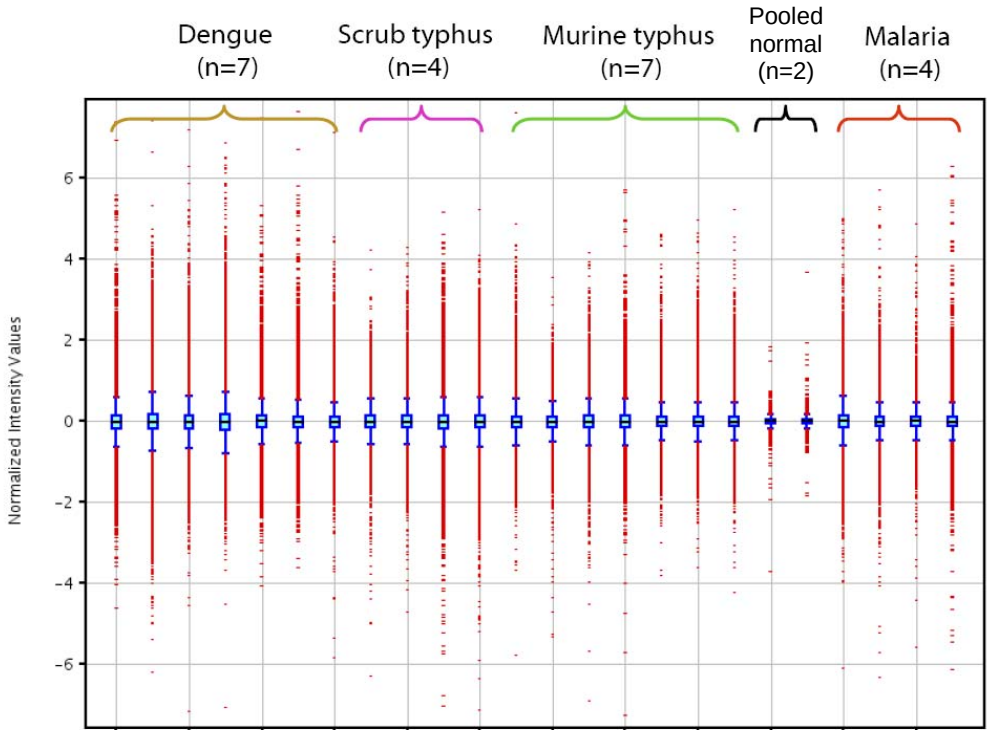

Figure 4.1 Box plots of post-normalization data of all 24 samples. Distribution of normalized expression values of all probes passing the filtering criteria was shown.

4.1.3 Analysis of gene expression

4.1.3.1 Identification of scrub typhus specific genes

To find a gene set that was distinctively expressed during OT infection, averaged levels of gene expression in patients with scrub typhus was compared to those with malaria, murine typhus and dengue fever. Sixty-five transcripts representing 63 genes were differentially expressed in patients with scrub typhus infection ($P < 0.05$) as listed in Table 4.2. Compared with the other groups of patients, nine of them were expressed at relatively lower levels in scrub typhus, while the rest were conversely expressed at higher levels. To characterize the biological significance of these genes, GO and pathway analysis was performed. As shown in Table 4.3, scrub typhus-specific genes were significantly enriched in T cell activation (fold enrichment = 13.03, P = 0.001), T cell receptor complex (fold enrichment = 82.85, P = 0.001) and T cell signaling pathway (fold enrichment = 13.85, P = 0.0003).

To test whether the profiles of these transcripts could be used as a molecular signature for scrub typhus, hierarchical clustering of the samples was performed. As illustrated in Figure 4.2a, all four patients with scrub typhus were correctly grouped together but separated from the others. In search of a smaller gene set with high discriminative power, a criterion on relative fold difference was further applied to scrub typhus-specific gene set. *CD8A*, *CD8B1*, *LOC642161* and *CBLB* were genes whose expression in scrub typhus was at least two folds greater than that in all the other three diseases (Table 4.2). *FOSB* was the only down-regulated gene with the similar magnitude of fold difference (Table 4.2). When hierarchical clustering was performed based on the expression of these five genes; scrub typhus infected patients were still gathered together despite inclusion of a patient with murine typhus in the cluster (Figure 4.2b).

Table 4.2 List of 63 scrub typhus-specific genes

Gene symbol	Definition	Accession No.	[a] Fold difference				[b] p-value
			ST vs DF	ST vs ML	SC vs MT	SC vs HC	
BCL11A	B-cell CLL/lymphoma 11A (zinc finger protein)	NM_138559.1	-1.55	-1.77	-1.46	-2.07	0.041
CORO1B	coronin, actin binding protein, 1B	NM_020441.2	-1.43	-1.88	-1.59	-1.32	0.013
FOSB*	FBJ murine osteosarcoma viral oncogene homolog B	NM_006732.1	-5.40	-4.24	-3.89	-3.43	0.047
GAGE6	G antigen 6	NM_001476.1	-1.11	-1.21	-1.08	-1.04	0.032
LDLRAP1	low density lipoprotein receptor adaptor protein 1	NM_015627.1	-1.43	-2.34	-2.02	-3.44	0.019
LOC653374		XM_930086.1	-2.68	-2.00	-1.49	-1.33	0.001
RAPGEF3	Rap guanine nucleotide exchange factor (GEF) 3	NM_006105.3	-1.21	-1.28	-1.14	-1.34	0.036
SLC22A18	solute carrier family 22, member 18	NM_183233.1	-1.94	-1.99	-1.65	-1.19	0.030
Hs.552434		AA399558	-1.40	-1.52	-1.29	-1.26	0.014
ADD3	adducin 3 (gamma)	NM_016824.3	2.30	2.11	1.46	1.44	0.019
ATP13A3	ATPase type 13A3	XM_942079.1	1.34	1.39	1.31	1.62	0.039
AYTL2	acyltransferase like 2	NM_024830.3	2.08	1.74	1.45	1.71	0.047
CBLB*	Cas-Br-M (murine) ecotropic retroviral transforming sequence b	NM_170662.3	4.33	2.62	2.02	1.94	0.044
CD3G	CD3g molecule, gamma (CD3-TCR complex)	NM_000073.1	5.59	2.32	1.94	2.88	0.023
CD8A*	CD8 antigen, alpha chain	NM_001768.4	8.58	5.45	2.59	3.87	0.035
CD8B1*	CD8 antigen, beta chain	NM_172213.1	7.81	3.13	2.31	2.89	0.032
DLD	dihydrolipoamide dehydrogenase	NM_000108.2	1.32	1.30	1.28	1.51	0.016

Table 4.2 List of 63 scrub typhus-specific genes (cont.)

Gene symbol	Definition	Accession No.	[a] Fold difference				[b] p-value
			ST vs DF	ST vs ML	SC vs MT	SC vs HC	
DLG1	discs, large homolog 1 (Drosophila)	NM_004087.1	1.38	1.48	1.23	1.36	0.025
DNTTIP2	deoxynucleotidyltransferase, terminal, interacting protein 2	NM_014597.3	1.40	1.71	1.41	1.73	0.031
FAM107B	family with sequence similarity 107, member B	NM_031453.2	2.10	1.73	1.38	1.31	0.003
FAM108B1	family with sequence similarity 108, member B1frame 77	NM_001025780.1	1.70	2.58	1.69	1.85	0.027
FBXO4	F-box protein 4	NM_033484.1	1.29	1.30	1.22	1.20	0.022
FLJ20160		NM_017694.2	2.08	1.71	1.55	2.17	0.047
LOC285513		NM_198281.1	2.65	2.25	1.79	2.60	0.002
LOC642161*		XM_936316.1	6.91	3.35	2.26	2.51	0.044
LOC649801		XM_938871.1	2.01	2.65	1.68	2.30	0.003
LOC651633		XM_940830.1	1.85	2.15	1.69	1.84	0.031
LOC652282		XM_941697.1	1.28	1.33	1.23	1.34	0.044
LOC653115		XM_926415.1	1.72	2.45	1.69	1.74	0.013
LOC653663		XM_930490.1	1.12	1.14	1.15	1.13	0.044
LOC653675		XM_933197.1	1.42	1.40	1.27	1.61	0.032
MACF1	microtubule-actin crosslinking factor 1	NM_012090.3	2.18	3.09	1.87	2.10	0.023
NUP54	nucleoporin 54kDa	NM_017426.2	1.52	1.91	1.44	1.71	0.003
NXF5	nuclear RNA export factor 5	NM_033155.1	1.14	1.22	1.15	1.14	0.012

Table 4.2 List of 63 scrub typhus-specific genes (cont.)

Gene symbol	Definition	Accession No.	[a] Fold difference				[b] p-value
			ST vs DF	ST vs ML	SC vs MT	SC vs HC	
NXT2	nuclear transport factor 2-like export factor 2	NM_018698.3	1.54	1.56	1.42	1.53	0.013
PLEKHF1	pleckstrin homology domain containing, family F (with FYVE domain), member 1	NM_024310.2	3.13	1.95	1.69	1.89	0.032
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	NM_178588.1	1.49	1.61	1.36	1.54	0.040
PPP2R5C	protein phosphatase 2, regulatory subunit B', gamma isoform	NM_178587.1	1.60	2.01	1.59	2.00	0.001
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	NM_182948.2	1.41	1.51	1.26	1.48	0.003
PRKACB	protein kinase, cAMP-dependent, catalytic, beta	NM_207578.1	2.30	2.16	1.50	1.83	0.041
PRPS1L1	phosphoribosyl pyrophosphate synthetase 1-like 1	NM_175886.2	1.34	1.47	1.34	1.46	0.039
RASA2	RAS p21 protein activator 2	NM_006506.2	1.43	1.89	1.44	1.27	0.010
RKHD2	ring finger and KH domain containing 2	NM_016626.2	1.73	1.86	1.45	1.21	0.004
RORA	RAR-related orphan receptor A	NM_134262.1	2.56	3.14	1.83	2.88	0.019
SLC25A32	solute carrier family 25	NM_030780.2	1.38	1.32	1.33	1.31	0.037
SRPK2	SFRS protein kinase 2	NM_182691.1	1.84	1.87	1.57	1.70	0.025
SUMO2	SMT3 suppressor of mif two 3 homolog 2 (S. cerevisiae)	NM_001005849.1	1.60	1.76	1.40	1.54	0.002
SYNE2	spectrin repeat containing, nuclear envelop 2	NM_182910.1	1.20	1.28	1.23	1.41	0.034
SYTL2	synaptotagmin-like 2	NM_032379.3	1.27	1.32	1.24	1.13	0.028
TCEA1	transcription elongation factor A (SII)	NM_006756.2	1.85	2.21	1.57	2.17	0.024

Table 4.2 List of 63 scrub typhus-specific genes (cont.)

Gene symbol	Definition	Accession No.	[a] Fold difference				[b] p-value
			ST vs DF	ST vs ML	SC vs MT	SC vs HC	
TCERG1	transcription elongation regulator 1	NM_006706.2	1.42	1.52	1.30	1.10	0.032
TOPBP1	topoisomerase (DNA) II binding protein 1	NM_007027.2	1.71	2.41	1.51	1.67	0.049
UBE1L2	ubiquitin-like modifier activating enzyme 6	NM_018227.3	1.46	1.70	1.44	1.51	0.038
UGCGL1	UDP-glucose ceramide glucosyltransferase-like 1	NM_020120.2	1.57	2.31	1.53	1.78	0.015
UHMK1	U2AF homology motif (UHM) kinase 1	NM_175866.2	1.55	2.06	1.67	1.37	0.030
ZBTB2	zinc finger and BTB domain containing 2	NM_020861.1	1.65	2.05	1.45	1.68	0.012
ZMYND11	zinc finger, MYND domain containing 11	NM_212479.1	1.33	1.42	1.22	1.31	0.049
ZNF326	zinc finger protein 326	NM_182976.1	1.48	2.33	1.53	2.10	0.008
ZNF644	zinc finger protein 644	NM_201269.1	2.22	3.26	1.95	2.60	0.001
Hs.561954		BU624523	1.62	2.36	1.57	1.77	0.041
Hs.373705		BX117372	1.48	1.46	1.40	-1.01	0.038
Hs.562182		BX404805	1.61	1.64	1.44	1.24	0.031
Hs.573541		BX951899	2.43	1.71	1.55	1.93	0.010
Hs.567392		BX537622	1.33	1.47	1.21	1.47	0.030
Hs.250648		CR603272	1.52	1.84	1.48	1.61	0.019

[a] Negative values indicate that mean expression levels of transcripts were lower in scrub typhus group (SC) compared with those of the others (DF = Dengue fever, ML = Malaria, MT = Murine typhus, HC = healthy control), where as positive values represent a reverse relationship

[b] p-values were derived by Welch ANOVA with 5% FDR

Table 4.3 GO and pathway analysis of 63 scrub typhus-specific genes

Accession	Term	Count	Fold Enrichment	P-value	[a] Corrected P-value	FDR	Examples of Genes
Biological process							
GO:0042110	T cell activation	5	13.03	0.001	0.27	0.77	*BCL11A, CD8A,CBLB, CD3G, CD8B1*
GO:0046649	lymphocyte activation	5	8.25	0.003	0.58	4.09	*BCL11A, CD8A,CBLB, CD3G, CD8B1*
Cellular component							
GO:0005622	intracellular	42	1.27	0.003	0.25	4.13	*UGCGL1, PLEKHF1, TOPBP1, TCEA1, RAPGEF3, NXF5, DLG1, ADD3, PPP2R5C, RORA, SYNE2, CORO1B, BCL11A,, SUMO2, UBE1L2, DLD, RASA2, CBLB, AYTL2, LDLRAP1, MACF1, FOSB, TCERG1, ZBTB2, CD8B1, PRKACB, RKHD2, PRKACB*
GO:0042101	T cell receptor complex	3	82.85	0.001	0.09	0.67	*CD8A, CD3G, CD8B1*
Molecular function							
GO:0005488	binding	45	1.18	0.002	0.34	2.55	*SLC25A32, GAGE6, PPP2R5C, CD3G, SYTL2, NXF5, DLG1, ADD3, PPP2R5C, SLC22A18, NUP54, PRPS1L1, ATP13A3,CD8A, BCL11A, FBXO4, SRPK2, ZNF326, SUMO2, UBE1L2, DLD, RASA2, NXT2, CBLB, FOSB, TCERG1, ZBTB2, CD8B1*
Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway							
hsa04660	T cell receptor signaling pathway	5	13.85	0.0003	0.01	0.27	*CD8A,CBLB, CD3G, DLG1, CD8B1*

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction.

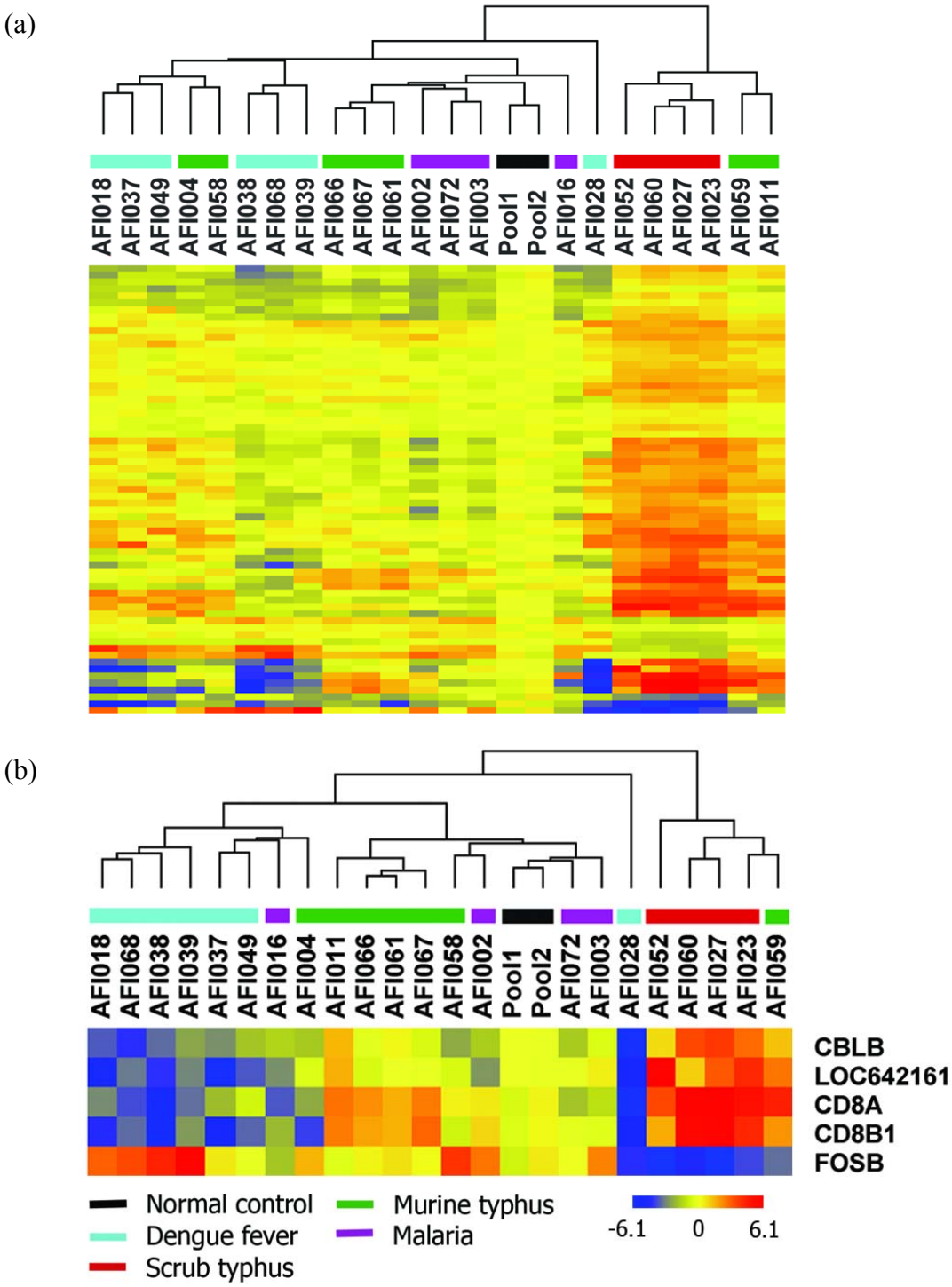

Figure 4.2 Unsupervised hierarchical clustering of all samples based on scrub typhus specific genes. The profile of sixty-five (a) and five (b) scrub typhus-specific transcripts was used to perform the clustering. Normalized expression levels in each sample were baseline-adjusted to the mean expression of healthy control and color-scaled ranging from red, representing high expression, to blue, indicating low expression.

4.1.3.2 Identification of scrub typhus responsive genes

To investigate transcriptional changes in response to scrub typhus infection, the expression pattern of scrub typhus infected patients was compared to that of healthy donors. Genes with at least two-fold difference between the two groups were focused.

a) Up-regulated genes in scrub typhus

613 transcripts were identified to be up-regulated in patients with scrub typhus. GO analysis revealed particular enrichment of these genes, with the highest degree of more than 20 folds, in a number of cellular processes related to cell cycle, DNA replication and cell division (Table 4.4). Correspondingly, the gene set appears to include cellular components with a role in chromosomal segregation; such as kinetochore (fold enrichment = 10.61), spindle microtubule (fold enrichment = 11.02), spindle pole body (fold enrichment = 26.63), and condensin complex (fold enrichment = 23.67) (Table 4.5). Similar findings were also derived from pathway analysis as shown in Table 4.6. Examples of the up-regulated genes involved in cell cycle (Figure 4.3) and DNA replication pathways (Figure 4.4) include several kinesin family members (*KIF11*, *KIF15, KIF23*, *KIF2C*), cell cycle division genes (*CDC2, CDC20, CDC7*), and cyclin family (*CCNA2, CCNB1, CCNB2, CCNF*).

In addition, an abundance of immune-related genes was also observed in the up-regulated gene list; even though the degree of enrichment of these processes was not so striking as that of cell cycle and cell division (Table 4.4). When molecular function was focused, enrichment of genes participating in MHC-class I protein binding was observed with a fold of enrichment up to 11.06 (Table 4.7). Some members of this group include *CD8A*, *CD8B*, *PSMB8*, *TUBB2C*, and *TUBB*. Of noted, *IFNG* and several interferon-inducible genes; such as a transcription factor *STAT1*, *IDO1*, *FASLG, GBP1,* and *CTLA4,* are among immune-related genes that were induced upon the infection (Table 4.8). Interestingly, expression levels of most of these genes appeared lowest in AFI052, who was the earliest to present at the hospital since the third day of his illness (Table 4.8).

Table 4.4 GO analysis of up-regulated genes in scrub typhus according to GO biological processes

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0034508	centromere complex assembly	4	0.74	21.73	1.48×10^{-2}
GO:0006268	DNA unwinding during replication	7	1.30	16.30	1.02×10^{-4}
GO:0040001	establishment of mitotic spindle localization	4	0.74	16.30	3.56×10^{-2}
GO:0007052	mitotic spindle organization	7	1.30	15.21	1.55×10^{-4}
GO:0007076	mitotic chromosome condensation	6	1.11	15.05	1.08×10^{-3}
GO:0007094	mitotic cell cycle spindle assembly checkpoint	5	0.93	14.82	7.65×10^{-3}
GO:0045841	negative regulation of mitotic metaphase/anaphase transition	5	0.93	14.82	7.65×10^{-3}
GO:0045839	negative regulation of mitosis	5	0.93	13.58	1.06×10^{-2}
GO:0031577	spindle checkpoint	5	0.93	13.58	1.06×10^{-2}
GO:0051784	negative regulation of nuclear division	5	0.93	13.58	1.06×10^{-2}
GO:0007051	spindle organization	18	3.33	13.04	9.44×10^{-13}
GO:0000070	mitotic sister chromatid segregation	14	2.59	12.68	2.51×10^{-9}
GO:0032508	DNA duplex unwinding	7	1.30	12.68	4.88×10^{-4}
GO:0032392	DNA geometric change	7	1.30	12.68	4.88×10^{-4}
GO:0000819	sister chromatid segregation	14	2.59	12.34	3.25×10^{-9}
GO:0006270	DNA replication initiation	6	1.11	12.23	2.97×10^{-3}
GO:0007059	chromosome segregation	26	4.81	10.46	1.47×10^{-16}
GO:0030071	regulation of mitotic metaphase/anaphase transition	7	1.30	10.37	1.45×10^{-3}
GO:0000724	double-strand break repair via homologous recombination	6	1.11	10.29	7.00×10^{-3}
GO:0000725	recombinational repair	6	1.11	10.29	7.00×10^{-3}
GO:0030261	chromosome condensation	7	1.30	9.13	2.98×10^{-3}
GO:0010948	negative regulation of cell cycle process	7	1.30	9.13	2.98×10^{-3}
GO:0007067	Mitosis	61	11.30	9.04	6.33×10^{-38}
GO:0000280	nuclear division	61	11.30	9.04	6.33×10^{-38}
GO:0000087	M phase of mitotic cell cycle	61	11.30	8.88	1.71×10^{-37}

Table 4.4 GO analysis of up-regulated genes in scrub typhus according to biological processes (cont.)

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0048285	organelle fission	61	11.30	8.68	6.07×10^{-37}
GO:0006261	DNA-dependent DNA replication	15	2.78	8.43	1.33×10^{-7}
GO:0007093	mitotic cell cycle checkpoint	11	2.04	8.34	3.09×10^{-5}
GO:0000279	M phase	78	14.44	7.73	2.23×10^{-43}
GO:0000075	cell cycle checkpoint	20	3.70	7.16	3.10×10^{-9}
GO:0000278	mitotic cell cycle	77	14.26	6.78	4.06×10^{-39}
GO:0051258	protein polymerization	10	1.85	6.65	6.30×10^{-4}
GO:0051301	cell division	60	11.11	6.63	2.54×10^{-29}
GO:0022403	cell cycle phase	84	15.56	6.61	5.71×10^{-42}
GO:0010564	regulation of cell cycle process	22	4.07	6.29	3.31×10^{-9}
GO:0031570	DNA integrity checkpoint	10	1.85	6.27	9.85×10^{-4}
GO:0006260	DNA replication	35	6.48	6.01	8.31×10^{-15}
GO:0006323	DNA packaging	21	3.89	5.85	3.64×10^{-8}
GO:0007088	regulation of mitosis	10	1.85	5.82	1.65×10^{-3}
GO:0051783	regulation of nuclear division	10	1.85	5.82	1.65×10^{-3}
GO:0000226	microtubule cytoskeleton organization	26	4.81	5.77	3.19×10^{-10}
GO:0051321	meiotic cell cycle	17	3.15	5.54	4.47×10^{-6}
GO:0051439	regulation of ubiquitin-protein ligase activity during mitotic cell cycle	12	2.22	5.51	4.40×10^{-4}
GO:0000077	DNA damage checkpoint	8	1.48	5.43	1.61×10^{-2}
GO:0070507	regulation of microtubule cytoskeleton organization	7	1.30	5.43	4.04×10^{-2}
GO:0022402	cell cycle process	94	17.41	5.42	5.08×10^{-40}
GO:0065004	protein-DNA complex assembly	15	2.78	5.37	3.70×10^{-5}
GO:0007126	meiosis	16	2.96	5.32	1.81×10^{-5}
GO:0051327	M phase of meiotic cell cycle	16	2.96	5.32	1.81×10^{-5}
GO:0048015	phosphoinositide-mediated signaling	14	2.59	5.19	1.29×10^{-4}
GO:0007017	microtubule-based process	39	7.22	5.03	3.87×10^{-14}
GO:0051438	regulation of ubiquitin-protein ligase activity	12	2.22	5.02	9.64×10^{-4}

Table 4.4 GO analysis of up-regulated genes in scrub typhus according to biological processes (cont.)

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0031145	anaphase-promoting complex-dependent proteasomal ubiquitin-dependent protein catabolic process	10	1.85	5.02	5.04×10^{-3}
GO:0007018	microtubule-based movement	17	3.15	4.90	2.06×10^{-5}
GO:0031497	chromatin assembly	13	2.41	4.87	5.50×10^{-4}
GO:0051340	regulation of ligase activity	12	2.22	4.83	1.29×10^{-3}
GO:0051437	positive regulation of ubiquitin-protein ligase activity during mitotic cell cycle	10	1.85	4.79	6.95×10^{-3}
GO:0007049	cell cycle	113	20.93	4.75	4.61×10^{-43}
GO:0006302	double-strand break repair	9	1.67	4.73	1.57×10^{-2}
GO:0006310	DNA recombination	15	2.78	4.66	1.79×10^{-4}
GO:0006334	nucleosome assembly	12	2.22	4.66	1.71×10^{-3}
GO:0031398	positive regulation of protein ubiquitination	12	2.22	4.66	1.71×10^{-3}
GO:0002449	lymphocyte mediated immunity	10	1.85	4.66	8.45×10^{-3}
GO:0051443	positive regulation of ubiquitin-protein ligase activity	10	1.85	4.66	8.45×10^{-3}
GO:0031396	regulation of protein ubiquitination	14	2.59	4.56	4.85×10^{-4}
GO:0051436	negative regulation of ubiquitin-protein ligase activity during mitotic cell cycle	9	1.67	4.51	2.11×10^{-2}
GO:0007346	regulation of mitotic cell cycle	21	3.89	4.50	3.17×10^{-6}
GO:0051351	positive regulation of ligase activity	10	1.85	4.47	1.10×10^{-2}
GO:0051352	negative regulation of ligase activity	9	1.67	4.38	2.52×10^{-2}
GO:0051444	negative regulation of ubiquitin-protein ligase activity	9	1.67	4.38	2.52×10^{-2}
GO:0034728	nucleosome organization	12	2.22	4.21	4.18×10^{-3}
GO:0051726	regulation of cell cycle	42	7.78	4.14	2.46×10^{-12}
GO:0031397	negative regulation of protein ubiquitination	9	1.67	3.96	4.43×10^{-2}
GO:0010498	proteasomal protein catabolic process	12	2.22	3.84	8.75×10^{-3}
GO:0043161	proteasomal ubiquitin-dependent protein catabolic process	12	2.22	3.84	8.75×10^{-3}

Table 4.4 GO analysis of up-regulated genes in scrub typhus according to biological processes (cont.)

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0043623	cellular protein complex assembly	19	3.52	3.82	1.21×10^{-4}
GO:0051329	interphase of mitotic cell cycle	12	2.22	3.80	9.40×10^{-3}
GO:0002443	leukocyte mediated immunity	10	1.85	3.79	3.14×10^{-2}
GO:0051325	interphase	12	2.22	3.69	1.13×10^{-2}
GO:0006259	DNA metabolic process	55	10.19	3.54	1.08×10^{-13}
GO:0006333	chromatin assembly or disassembly	13	2.41	3.34	1.46×10^{-2}
GO:0034621	cellular macromolecular complex subunit organization	36	6.67	3.29	1.07×10^{-7}
GO:0034622	cellular macromolecular complex assembly	32	5.93	3.28	1.04×10^{-6}
GO:0006974	response to DNA damage stimulus	37	6.85	3.23	9.84×10^{-8}
GO:0006281	DNA repair	28	5.19	3.21	1.32×10^{-5}
GO:0006457	protein folding	15	2.78	2.76	2.83×10^{-2}
GO:0033043	regulation of organelle organization	18	3.33	2.70	1.10×10^{-2}
GO:0051276	chromosome organization	39	7.22	2.62	7.74×10^{-6}
GO:0006996	organelle organization	105	19.44	2.57	1.20×10^{-17}
GO:0008283	cell proliferation	32	5.93	2.39	5.22×10^{-4}
GO:0070271	protein complex biogenesis	36	6.67	2.32	2.66×10^{-4}
GO:0006461	protein complex assembly	36	6.67	2.32	2.66×10^{-4}
GO:0007010	cytoskeleton organization	31	5.74	2.32	1.19×10^{-3}
GO:0043933	macromolecular complex subunit organization	50	9.26	2.30	5.89×10^{-6}
GO:0065003	macromolecular complex assembly	47	8.70	2.30	1.29×10^{-5}
GO:0033554	cellular response to stress	40	7.41	2.30	1.01×10^{-4}
GO:0006955	immune response	47	8.70	2.22	3.23×10^{-5}
GO:0009607	response to biotic stimulus	24	4.44	2.04	4.20×10^{-2}
GO:0002682	regulation of immune system process	24	4.44	2.03	4.28×10^{-2}
GO:0002376	immune system process	61	11.30	1.99	2.11×10^{-5}
GO:0016043	cellular component organization	144	26.67	1.88	4.70×10^{-13}
GO:0022607	cellular component assembly	50	9.26	1.84	1.51×10^{-3}
GO:0044085	cellular component biogenesis	56	10.37	1.82	6.43×10^{-4}
GO:0051716	cellular response to stimulus	44	8.15	1.75	1.11×10^{-2}

Table 4.4 GO analysis of up-regulated genes in scrub typhus according to biological processes (cont.)

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0051704	multi-organism process	36	6.67	1.72	4.42×10^{-2}
GO:0006950	response to stress	86	15.93	1.66	1.02×10^{-4}
GO:0050896	response to stimulus	137	25.37	1.28	2.28×10^{-2}
GO:0009987	cellular process	364	67.41	1.13	7.69×10^{-5}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.5 GO analysis of up-regulated genes in scrub typhus according to cellular component

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0005816	spindle pole body	3	0.56	26.63	3.61×10^{-2}
GO:0031262	Ndc80 complex	3	0.56	26.63	3.61×10^{-2}
GO:0000796	condensin complex	4	0.74	23.67	3.91×10^{-3}
GO:0000940	outer kinetochore of condensed chromosome	5	0.93	19.73	6.73×10^{-4}
GO:0000777	condensed chromosome kinetochore	22	4.07	13.47	7.70×10^{-17}
GO:0000779	condensed chromosome, centromeric region	25	4.63	13.45	4.85×10^{-19}
GO:0005876	spindle microtubule	9	1.67	11.02	1.04×10^{-5}
GO:0000776	kinetochore	23	4.26	10.61	3.55×10^{-15}
GO:0000793	condensed chromosome	38	7.04	10.46	4.43×10^{-25}
GO:0000922	spindle pole	10	1.85	10.44	3.46×10^{-6}
GO:0005881	cytoplasmic microtubule	5	0.93	9.86	1.18×10^{-2}
GO:0030496	midbody	5	0.93	9.86	1.18×10^{-2}
GO:0000775	chromosome, centromeric region	34	6.30	9.74	2.15×10^{-21}
GO:0005819	spindle	30	5.56	7.25	3.77×10^{-15}
GO:0044427	chromosomal part	58	10.74	5.34	2.76×10^{-23}
GO:0005694	chromosome	69	12.78	5.33	1.13×10^{-27}
GO:0000794	condensed nuclear chromosome	7	1.30	5.07	2.00×10^{-2}
GO:0000228	nuclear chromosome	21	3.89	4.60	3.83×10^{-7}
GO:0044450	microtubule organizing center part	7	1.30	4.44	3.66×10^{-2}
GO:0015630	microtubule cytoskeleton	60	11.11	3.88	1.36×10^{-17}
GO:0044454	nuclear chromosome part	12	2.22	3.49	6.00×10^{-3}
GO:0005874	microtubule	26	4.81	3.37	3.09×10^{-6}
GO:0005815	microtubule organizing center	24	4.44	3.37	9.39×10^{-6}
GO:0000785	chromatin	19	3.52	3.37	1.47×10^{-4}
GO:0005813	centrosome	21	3.89	3.33	5.92×10^{-5}
GO:0005654	nucleoplasm	59	10.93	2.38	1.94×10^{-8}
GO:0044430	cytoskeletal part	63	11.67	2.35	8.04×10^{-9}
GO:0044428	nuclear part	112	20.74	2.18	2.59×10^{-14}

Table 4.5 GO analysis of up-regulated genes in scrub typhus according to cellular component (cont.)

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0031981	nuclear lumen	89	16.48	2.18	5.23×10^{-11}
GO:0043228	non-membrane-bounded organelle	150	27.78	2.05	8.86×10^{-18}
GO:0043232	intracellular non-membrane-bounded organelle	150	27.78	2.05	8.86×10^{-18}
GO:0070013	intracellular organelle lumen	97	17.96	1.94	3.10×10^{-9}
GO:0031974	membrane-enclosed lumen	100	18.52	1.91	2.96×10^{-9}
GO:0043233	organelle lumen	98	18.15	1.91	4.60×10^{-9}
GO:0005856	Cytoskeleton	71	13.15	1.83	9.53×10^{-6}
GO:0044446	intracellular organelle part	207	38.33	1.74	7.58×10^{-18}
GO:0005829	Cytosol	65	12.04	1.74	1.49×10^{-4}
GO:0044422	organelle part	207	38.33	1.73	1.21×10^{-17}
GO:0005730	Nucleolus	34	6.30	1.73	2.05×10^{-2}
GO:0043234	protein complex	122	22.59	1.67	4.72×10^{-8}
GO:0032991	macromolecular complex	141	26.11	1.59	5.07×10^{-8}
GO:0005634	Nucleus	218	40.37	1.52	2.45×10^{-12}
GO:0043231	intracellular membrane-bounded organelle	285	52.78	1.27	7.67×10^{-8}
GO:0043227	membrane-bounded organelle	285	52.78	1.27	8.26×10^{-8}
GO:0043229	intracellular organelle	315	58.33	1.25	1.36×10^{-8}
GO:0043226	Organelle	315	58.33	1.24	1.59×10^{-8}
GO:0044424	intracellular part	366	67.78	1.22	2.18×10^{-11}
GO:0005622	Intracellular	374	69.26	1.21	3.79×10^{-11}
GO:0005737	Cytoplasm	248	45.93	1.20	4.34×10^{-4}
GO:0044464	cell part	433	80.19	1.04	9.28×10^{-3}
GO:0005623	Cell	433	80.19	1.04	9.21×10^{-3}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.6 Pathway analysis of up-regulated genes in scrub typhus

KEGG Accession	Pathway	Count	%	Fold Enrichment	[a] Corrected P-value
hsa03030	DNA replication	9	1.67	6.98	1.16×10^{-3}
hsa04110	Cell cycle	25	4.63	5.59	6.70×10^{-10}
hsa04114	Oocyte meiosis	16	2.96	4.06	3.90×10^{-4}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5 %.

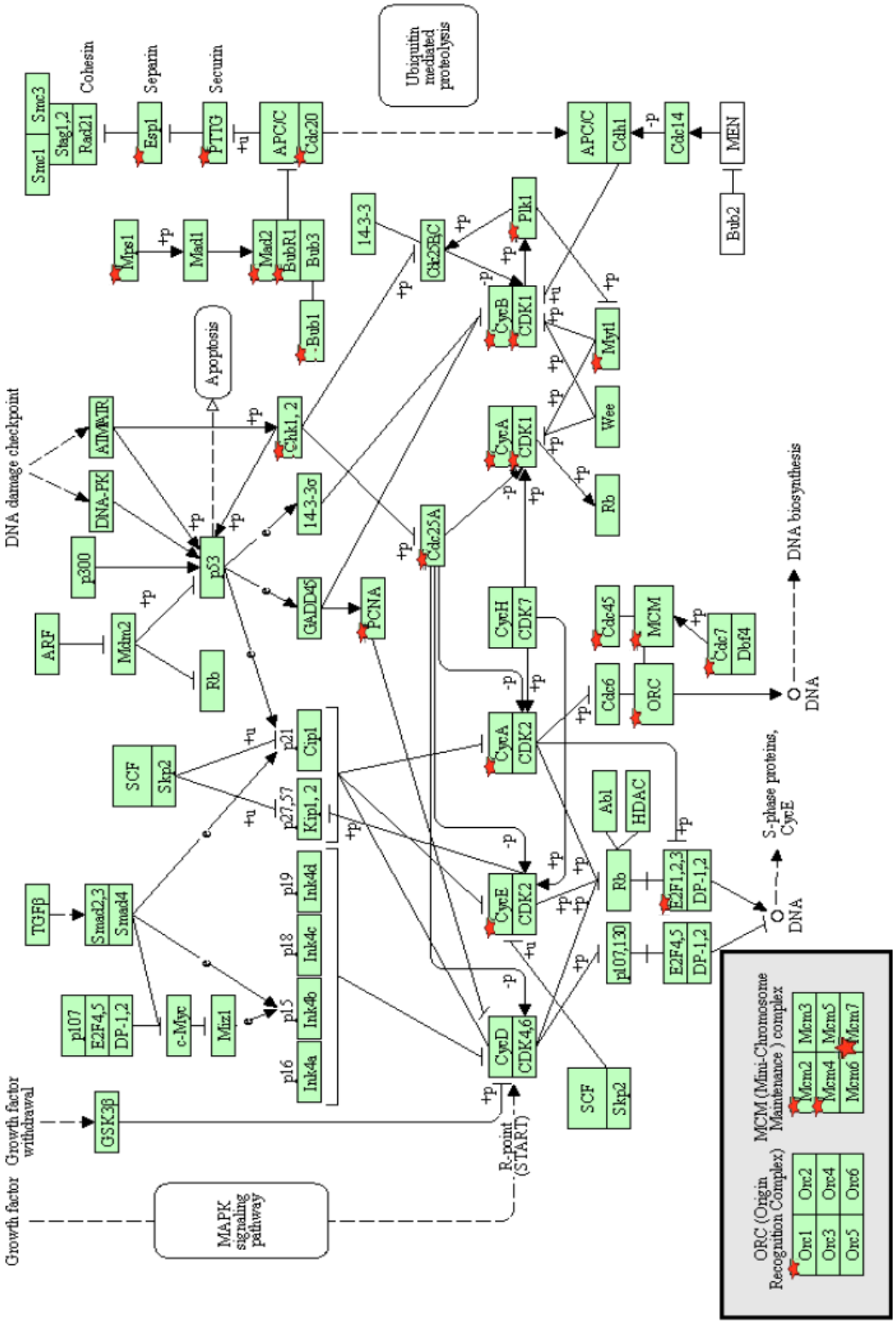

Figure 4.3 Cell cycle pathway. Up-regulated genes in scrub typhus were marked with stars. (Adapted from KEGG pathway) (91-92)

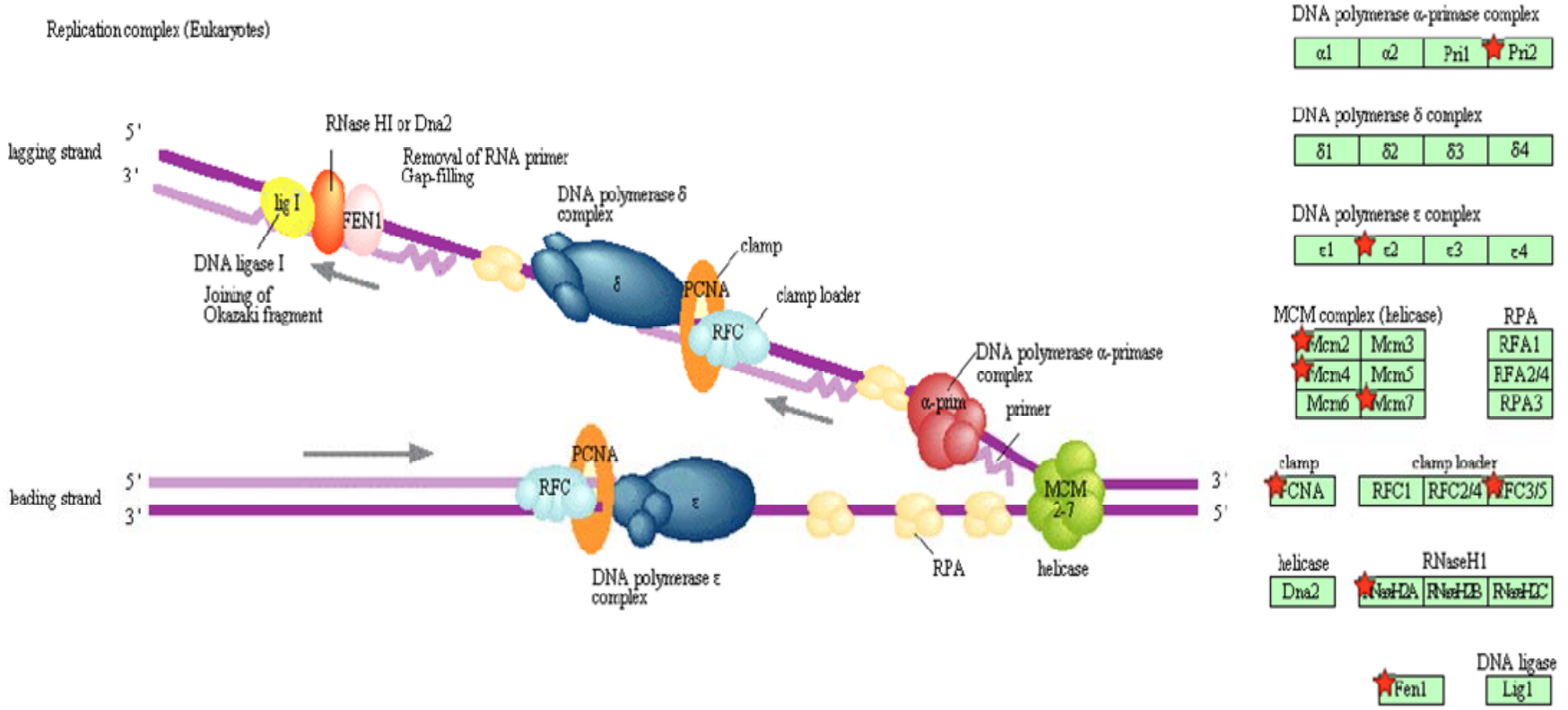

Figure 4.4 DNA replication pathway. Up-regulated genes in scrub typhus were marked with stars. (Adapted from KEGG pathway) (91-92)

Table 4.7 GO analysis of up-regulated genes in scrub typhus according to molecular function

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0042288	MHC class I protein binding	5	0.93	11.06	2.88×10^{-2}
GO:0042287	MHC protein binding	6	1.11	8.49	2.15×10^{-2}
GO:0003697	single-stranded DNA binding	8	1.48	5.15	2.98×10^{-2}
GO:0003924	GTPase activity	17	3.15	2.85	1.29×10^{-2}
GO:0017111	nucleoside-triphosphatase activity	46	8.52	2.24	4.25×10^{-5}
GO:0005525	GTP binding	23	4.26	2.19	2.85×10^{-2}
GO:0016462	pyrophosphatase activity	46	8.52	2.15	1.11×10^{-4}
GO:0016818	hydrolase activity, acting on acid anhydrides, in phosphorus-containing anhydrides	46	8.52	2.14	1.15×10^{-4}
GO:0016817	hydrolase activity, acting on acid anhydrides	46	8.52	2.13	1.21×10^{-4}
GO:0019001	guanyl nucleotide binding	23	4.26	2.13	3.86×10^{-2}
GO:0032561	guanyl ribonucleotide binding	23	4.26	2.13	3.86×10^{-2}
GO:0005524	ATP binding	86	15.93	2.06	1.69×10^{-8}
GO:0032559	adenyl ribonucleotide binding	86	15.93	2.03	2.65×10^{-8}
GO:0032555	purine ribonucleotide binding	104	19.26	2.00	1.75×10^{-9}
GO:0032553	ribonucleotide binding	104	19.26	2.00	1.75×10^{-9}
GO:0030554	adenyl nucleotide binding	87	16.11	1.95	1.08×10^{-7}
GO:0017076	purine nucleotide binding	105	19.44	1.94	5.34×10^{-9}
GO:0001883	purine nucleoside binding	88	16.30	1.94	1.18×10^{-7}
GO:0001882	nucleoside binding	88	16.30	1.93	1.25×10^{-7}
GO:0000166	nucleotide binding	116	21.48	1.83	8.05×10^{-9}
GO:0016787	hydrolase activity	94	17.41	1.46	4.82×10^{-3}
GO:0003824	catalytic activity	189	35.00	1.29	7.10×10^{-4}
GO:0005515	protein binding	289	53.52	1.25	3.50×10^{-7}
GO:0005488	binding	386	71.48	1.09	3.96×10^{-4}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
ADA	adenosine deaminase	NM_000022.2	2.10	0.10	1.70	1.32	1.16
[†] *AIM2*	absent in melanoma 2	NM_004833.1	4.17	1.34	2.52	2.65	1.73
BAK1	BCL2-antagonist/killer 1	NM_001188.2	2.41	0.58	1.95	1.50	1.05
C1QA	complement component 1 q subcomponent alpha polypeptide	NM_015991.1	5.45	1.98	2.51	2.59	2.71
C1QB	complement component 1 q subcomponent beta polypeptide	NM_000491.2	9.56	3.08	3.04	3.35	3.57
C1QC	complement component 1 q subcomponent C chain	NM_172369.2	7.31	2.39	3.15	2.84	3.10
C3AR1	complement component 3a receptor 1	NM_004054.2	2.09	2.80	0.20	0.77	0.48
C6orf190	chromosome 6 open reading frame 190	NM_001010923.1	2.35	0.40	1.79	1.21	1.54
CAMP	cathelicidin antimicrobial peptide	NM_004345.3	2.06	1.20	-1.74	2.72	1.99
CCNB2	cyclin B2	NM_004701.2	8.15	1.20	3.97	3.72	3.22
CD164	CD164 antigen sialomucin	NM_006016.3	2.02	1.27	0.83	1.15	0.82
CD38	CD38 antigen	NM_001775.2	4.07	0.53	2.64	2.87	2.07
CD3G	CD3G antigen gamma polypeptide	NM_000073.1	2.88	1.09	1.86	1.45	1.71
*CD8A**	CD8 antigen alpha polypeptide (p32)	NM_001768.4	3.04	0.90	2.01	1.42	1.87
CD8B1	CD8 antigen beta polypeptide 1 (p37)	NM_172213.1	2.89	0.59	2.13	1.48	1.92

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
CEACAM8	carcinoembryonic antigen-related cell adhesion molecule 8	NM_001816.2	3.08	0.99	-0.75	4.27	1.98
CLEC5A	C-type lectin domain family 5 member A	NM_013252.2	2.49	1.75	0.98	1.44	1.10
CLEC6A	C-type lectin domain family 6 member A	NM_001007033.1	2.18	2.30	0.90	0.82	0.47
CST7	cystatin F	NM_003650.2	2.24	1.13	1.41	1.13	0.99
† *CTLA4*	cytotoxic T-lymphocyte-associated protein 4	NM_005214.2	2.69	0.62	1.95	1.44	1.70
CTSC	cathepsin C	NM_001814.2	2.25	0.96	1.42	1.12	1.17
DPP4	dipeptidyl-peptidase 4	NM_001935.3	2.17	-0.33	1.51	1.74	1.55
EBI3	Epstein-Barr virus induced gene 3	NM_005755.2	2.05	1.80	0.50	1.02	0.83
EBP	emopamil binding protein	NM_006579.1	2.01	0.49	1.18	0.90	1.46
EXO1	exonuclease 1	NM_003686.3	4.38	0.66	2.74	2.47	2.66
† *FASLG*	Fas ligand	NM_000639.1	2.42	0.59	1.70	1.43	1.37
FCGR1A	Fc fragment of IgG high affinity Ia receptor	NM_000566.2	3.58	1.98	1.24	2.34	1.81
† *GBP1*	guanylate binding protein 1 interferon-inducible 67kDa	NM_002053.1	4.48	1.33	2.35	2.64	2.34
† *GBP3*	guanylate binding protein 3	NM_018284.1	2.63	1.14	1.15	2.28	1.01
† *GBP4*	guanylate binding protein 4	NM_052941.2	2.86	0.76	1.74	2.02	1.54
† *GBP5*	guanylate binding protein 5	NM_052942.2	3.50	0.83	2.03	2.40	1.97

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	NM_000161.2	2.55	1.25	1.32	1.79	1.04
GCH1	GTP cyclohydrolase 1 (dopa-responsive dystonia)	NM_001024024.1	2.39	1.22	1.21	1.44	1.15
GPR65	G protein-coupled receptor 65	NM_003608.2	2.15	1.85	0.53	1.22	0.82
HELLS	helicase lymphoid-specific	NM_018063.3	2.09	0.00	1.50	1.37	1.37
HIST1H4C	histone 1 H4c	NM_003542.3	2.12	0.08	1.47	1.50	1.29
HIST1H4H	histone 1 H4h	NM_003543.3	2.02	0.69	1.50	0.46	1.40
HIST1H4K	histone 1 H4k	NM_003541.2	2.30	0.76	1.64	0.99	1.42
HLA-G	HLA-G histocompatibility antigen class I G	NM_002127.3	2.44	1.56	1.23	1.36	1.00
HOXB4	homeo box B4	NM_024015.3	2.22	0.24	1.77	1.55	1.03
HSPA4	heat shock 70kDa protein 4	NM_002154.3	3.27	1.31	1.75	2.29	1.48
HSPB1	heat shock 27kDa protein 1	NM_001540.2	2.09	2.03	1.24	1.52	-0.54
HSPCAL3	PREDICTED: heat shock 90kDa protein 1 alpha-like 3 transcript variant 3	XM_934529.1	2.20	0.68	1.38	2.05	0.44
*HSPD1**	heat shock 60kDa protein 1 (chaperonin)	NM_002156.4	2.06	0.22	1.58	1.76	0.60
HSPE1	heat shock 10kDa protein 1	NM_002157.1	2.17	0.38	1.78	1.68	0.63
† *IFI16*	interferon gamma-inducible protein 16	NM_005531.1	2.10	0.83	1.10	1.28	1.07

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
† *IFNG*	interferon gamma	NM_000619.2	4.07	0.79	2.80	3.12	1.40
IL12RB2	interleukin 12 receptor beta 2	NM_001559.2	2.90	0.13	2.04	2.14	1.84
IL1R2	interleukin 1 receptor type II	NM_173343.1	2.90	0.69	1.17	3.26	1.03
IL2RG	interleukin 2 receptor gamma	NM_000206.1	2.07	0.76	1.27	1.30	0.88
IL32	interleukin 32	NM_001012632.1	2.45	0.59	1.98	1.43	1.17
† *IDO1*	indoleamine-pyrrole 23 dioxygenase	NM_002164.3	2.97	0.41	2.15	3.66	0.08
LAG3	lymphocyte-activation gene 3	NM_002286.4	6.72	1.11	3.70	3.20	2.99
LCN2	lipocalin 2	NM_005564.2	2.39	0.53	-0.25	3.69	1.06
LOC642443	PREDICTED: similar to Alcohol dehydrogenase class III chi chain	XM_931030.1	2.06	0.80	1.33	0.97	1.07
LOC643300	PREDICTED: similar to 60 kDa heat shock protein mitochondrial precursor	XM_931981.1	3.92	0.85	2.93	2.76	1.34
LOC647000	PREDICTED: similar to tubulin beta 5	XM_929980.1	2.42	0.44	1.81	1.61	1.24
LOC652025	PREDICTED: similar to tubulin beta 5	XM_941325.1	2.12	0.35	1.48	1.30	1.21
LOC652694	PREDICTED: similar to Ig kappa chain V-I region HK102 precursor	XM_942302.1	3.72	0.65	2.76	2.68	1.49
LTF	lactotransferrin	NM_002343.2	5.70	2.09	0.22	5.16	2.57
† *MMP9*	matrix metallopeptidase 9	NM_004994.2	4.30	1.63	-0.32	4.64	2.48

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
NME1	non-metastatic cells 1 protein	NM_198175.1	2.47	0.09	1.89	1.77	1.48
† *OAS1*	2'5'-oligoadenylate synthetase 1 40/46kDa	NM_016816.2	2.10	1.01	1.19	0.57	1.51
† *OAS2*	2'-5'-oligoadenylate synthetase 2 69/71kDa	NM_016817.2	2.19	1.39	1.34	0.89	0.90
*OASL**	2'-5'-oligoadenylate synthetase-like	NM_003733.2	2.56	1.64	1.40	1.12	1.27
ORM1	orosomucoid 1	NM_000607.1	2.63	0.86	0.17	1.94	2.62
PDCD1	programmed cell death 1	NM_005018.1	2.31	0.73	1.66	1.63	0.83
PGLYRP1	peptidoglycan recognition protein 1	NM_005091.1	3.95	1.39	-0.34	4.44	2.44
PLSCR1	phospholipid scramblase 1	NM_021105.1	2.57	1.67	1.15	1.40	1.24
† *PSMB8*	proteasome (prosome macropain) subunit beta type 8 (large multifunctional peptidase 7)	NM_004159.4	2.56	0.65	1.74	1.54	1.49
† *PSMB8*	proteasome (prosome macropain) subunit beta type 8 (large multifunctional peptidase 7)	NM_148919.3	2.51	0.74	1.70	1.54	1.33
PTPN22	protein tyrosine phosphatase non-receptor type 22 (lymphoid)	NM_012411.2	2.01	0.48	1.36	1.11	1.08
PTPRC	protein tyrosine phosphatase receptor type C	NM_080922.1	4.97	2.49	2.35	2.02	2.40

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
PTPRC	protein tyrosine phosphatase receptor type C	NM_002838.2	2.44	1.92	1.06	1.09	1.08
RGS1	regulator of G-protein signalling 1	NM_002922.3	3.17	1.37	1.68	0.89	2.71
RNASE3	ribonuclease RNase A family 3	NM_002935.2	2.33	1.69	-0.14	2.66	0.68
RORA	RAR-related orphan receptor A	NM_134262.1	2.88	1.78	1.46	1.12	1.74
S100A12	S100 calcium binding protein A12	NM_005621.1	2.10	1.39	-0.63	2.32	1.20
† *SELP*	selectin P	NM_003005.2	2.01	1.27	0.27	1.75	0.75
SERPING1	serpin peptidase inhibitor clade G (C1 inhibitor) member 1 (angioedema hereditary)	NM_000062.2	3.29	2.35	1.26	2.43	0.83
SFXN1	sideroflexin 1	NM_022754.4	2.56	0.49	1.93	1.37	1.64
SLAMF7	SLAM family member 7	NM_021181.3	4.45	1.99	2.31	2.25	2.06
SPON2	spondin 2 extracellular matrix protein	NM_012445.1	2.03	0.14	1.48	1.57	0.89
† *STAT1**	signal transducer and activator of transcription 1 91kDa	NM_139266.1	2.25	0.80	1.14	1.34	1.38
TCEA1	transcription elongation factor A (SII) 1	NM_006756.2	2.17	0.83	1.28	1.25	1.12

Table 4.8 List of immune-related genes with ≥2-fold up-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
TNFSF7	tumor necrosis factor	NM_001252.2	2.46	0.49	1.92	1.70	1.09
TUBB	tubulin beta	NM_178014.2	2.70	0.66	1.88	1.65	1.53
TUBB2C	tubulin beta 2C	NM_006088.5	2.30	0.52	1.73	1.66	0.88
	Predicted: similar to immune-responsive gene 1 (LOC341720)	XM_292184	2.32	0.28	0.53	1.03	3.03

NOTE: The list is generated by a combination of gene members in the terms: immune system process, immune response, lymphocyte mediated immunity, leukocyte mediated immunity, response to biotic stimulus, and regulation of immune system process.

[a] Grey boxes mark the patient with lowest expression level for each gene.

[b] Sample number of scrub typhus infected patients with day of fever at the time of blood collection shown in parentheses.

[†] IFN-γ-inducible genes as identified by the presence of GAS or ISRE in their promoter regions (93)

* Average expression levels were shown for genes with two different probes quantifying the same transcript.

b) Down-regulated genes in scrub typhus

As compared to healthy controls, expression levels of 517 transcripts appeared to be depressed in patients with scrub typhus. Analysis of GO biological process of the down-regulated gene set revealed significant enrichment in various subcategories of immune system processes; such as regulation of NF-kB import into the nucleus, positive regulation of cytokine production, B-cell activation, humoral immune response, and inflammatory process, and chemotaxis (Table 4.9). Examples of genes in these groups include several proinflammatory cytokines, like *TNF*, *CCL3*, *IL1B*, and *IL8*; components of transcription factor AP-1, *FOS* and *JUN*; as well as a transcriptional co-activator of NF-kB, *BCL3* (Table 4.10). Consistently, prominent enrichment of genes with chemokine and cytokine activity was also demonstrated by GO analysis of molecular function (Table 4.11). The levels of inflammatory cytokines were least depressed in AFI060, who was the latest among scrub typhus group to present to the hospital according to his fever day (Table 4.10).

When cellular component was concerned, several genes in MHC class II protein complex; namely *HLA-DMB, HLA-DOA, HLA-DOB, HLA-DRB1, HLA-DRB4,* and *HLA-DRB5*, appeared to be down-regulated (Table 4.12). Interestingly, several pairs of genes with opposite roles in humoral immune response appeared to be down-regulated; such examples include, *HLA-DM* and *HLA-DO*, which act in reverse in peptide loading onto MHC class II; as well as *CD300LB* and *CD300LF*, which are an activating and an inhibitory receptor of immunoglobulin, respectively, expressed by myeloid cells.

In an analysis of pathway, the down-regulated genes appeared to be enriched in NOD-like signaling pathway, asthma, allograft rejection & GVH disease (Table 4.13). Most enrichment score of these pathways were contributed by a similar set of genes including *TNF*, *IL1B*, MCH class II (*HLA-DRB1* and *HLA-DRB5*), and *CD40L*, as represented by NOD-like signaling pathway (Figure 4.5).

Table 4.9 GO analysis of down-regulated genes in scrub typhus according to biological process

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0042345	regulation of NF-kappaB import into nucleus	6	1.35	13.56	8.93×10^{-3}
GO:0042113	B cell activation	13	2.93	6.96	9.34×10^{-5}
GO:0006959	humoral immune response	11	2.48	5.66	5.42×10^{-3}
GO:0001819	positive regulation of cytokine production	11	2.48	4.97	1.02×10^{-2}
GO:0030098	lymphocyte differentiation	11	2.48	4.34	2.05×10^{-2}
GO:0002521	leukocyte differentiation	13	2.93	4.04	1.17×10^{-2}
GO:0006954	inflammatory response	32	7.21	4.01	5.29×10^{-8}
GO:0006935	Chemotaxis	15	3.38	3.81	6.73×10^{-3}
GO:0042330	Taxis	15	3.38	3.81	6.73×10^{-3}
GO:0046649	lymphocyte activation	17	3.83	3.48	5.93×10^{-3}
GO:0006955	immune response	58	13.06	3.42	9.85×10^{-13}
GO:0045321	leukocyte activation	19	4.28	3.19	5.97×10^{-3}
GO:0006952	defense response	47	10.59	3.11	8.97×10^{-9}
GO:0050776	regulation of immune response	17	3.83	3.06	1.61×10^{-2}
GO:0051240	positive regulation of multicellular organismal process	18	4.05	3.00	1.28×10^{-2}
GO:0001775	cell activation	21	4.73	2.98	6.13×10^{-3}
GO:0002684	positive regulation of immune system process	17	3.83	2.91	2.41×10^{-2}
GO:0002376	immune system process	71	15.99	2.89	4.92×10^{-13}
GO:0009611	response to wounding	37	8.33	2.84	1.37×10^{-5}
GO:0007626	locomotory behavior	19	4.28	2.82	1.55×10^{-2}
GO:0008285	negative regulation of cell proliferation	23	5.18	2.59	1.16×10^{-2}
GO:0002682	regulation of immune system process	23	5.18	2.43	2.13×10^{-2}
GO:0007610	behavior	27	6.08	2.34	1.16×10^{-2}
GO:0009605	response to external stimulus	51	11.49	2.27	2.52×10^{-5}
GO:0048583	regulation of response to stimulus	25	5.63	2.19	4.13×10^{-2}
GO:0006950	response to stress	69	15.54	1.67	5.51×10^{-3}
GO:0050789	regulation of biological process	206	46.40	1.18	4.05×10^{-2}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Positive regulation of cytokine production & Regulation of NF-kB import into nucleus							
[§, †] *BCL3*	B-cell CLL/lymphoma 3	NM_005178.2	2.47	1.04	-3.32	-3.32	0.38
CARD9	caspase recruitment domain family member 9	NM_052813.2	2.53	-0.47	-1.99	-1.78	-1.12
[†] *CD40LG*	CD40 ligand	NM_000074.2	2.69	-1.09	-1.14	-1.44	-2.05
[§] *CD83*	CD83 antigen	NM_004233.2	9.02	-2.24	-4.55	-4.83	-1.07
CIAS1	cold autoinflammatory syndrome 1	NM_004895.3	4.12	-0.99	-3.36	-2.86	-0.97
EDAR	ectodysplasin A receptor	NM_022336.1	2.54	-1.06	-1.62	-1.43	-1.26
Hs.516646	cDNA FLJ44370 fis clone TRACH3008902	AK126342	2.44	-1.06	-0.98	-2.18	-0.93
IL1A	interleukin 1 alpha	NM_000575.3	4.30	-2.85	-2.47	-2.69	-0.41
IL1B	interleukin 1 beta	NM_000576.2	11.66	-3.20	-4.82	-5.65	-0.51
NALP12	NACHT leucine rich repeat and PYD containing 12	NM_033297.1	2.06	-0.41	-1.75	-1.38	-0.64
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	NM_020529.1	2.43	-1.12	-2.12	-1.86	-0.02
RARA	retinoic acid receptor alpha	NM_001024809.2	2.27	-0.03	-1.97	-1.47	-1.26
[§] *TNF*	tumor necrosis factor (TNF superfamily member 2)	NM_000594.2	13.40	-4.19	-5.19	-5.21	-0.40

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Inflammatory process & Chemotaxis							
CCL20	chemokine (C-C motif) ligand 20	NM_004591.1	15.66	-4.37	-4.97	-4.98	-1.56
CCL3	chemokine (C-C motif) ligand 3	NM_002983.1	15.81	-4.98	-4.55	-5.22	-1.18
CCL3L1	chemokine (C-C motif) ligand 3-like 1	NM_021006.4	44.04	-6.29	-6.33	-6.78	-2.44
CCL3L3	chemokine (C-C motif) ligand 3-like 3	NM_001001437.2	17.94	-4.95	-4.84	-5.54	-1.32
CCL4	chemokine (C-C motif) ligand 4	NM_002984.1	3.10	-2.07	-1.79	-1.49	-1.18
CCL4L1	chemokine (C-C motif) ligand 4-like 1	NM_001001435.2	3.30	-2.12	-1.88	-1.62	-1.26
CCL8	chemokine (C-C motif) ligand 8	NM_005623.2	2.07	-0.90	-1.30	-0.92	-1.10
CCR3	chemokine (C-C motif) receptor 3	NM_178329.1	2.98	-0.94	-1.69	-1.93	-1.74
[§] *CCR6*	chemokine (C-C motif) receptor 6	NM_031409.2	3.02	-0.46	-2.14	-2.53	-1.26
CCR7	chemokine (C-C motif) receptor 7	NM_001838.2	4.40	-0.73	-2.88	-2.62	-2.31
CD163	CD163 antigen	NM_203416.1	2.31	0.07	-1.65	-1.81	-1.43
CD302	CD302 antigen	NM_014880.3	2.73	-0.26	-2.51	-2.27	-0.76
[†] *CD40LG*	CD40 ligand	NM_000074.2	2.69	-1.09	-1.14	-1.44	-2.05
[§] *CFD*	complement factor D (adipsin)	NM_001928.2	2.87	-0.26	-2.32	-2.28	-1.22
CIAS1	cold autoinflammatory syndrome 1	NM_004895.3	4.12	-0.99	-3.36	-2.86	-0.97
[§] *CR2*	complement component (3d/Epstein Barr virus) receptor 2	NM_001877.3	2.20	-1.17	-1.13	-1.22	-1.04

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Inflammatory process & Chemotaxis (cont.)							
CXCL1	chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)	NM_001511.1	3.11	-1.78	-2.05	-1.99	-0.73
CXCL16	chemokine (C-X-C motif) ligand 16	NM_022059.1	2.38	0.22	-2.07	-2.17	-0.99
CXCL2	chemokine (C-X-C motif) ligand 2	NM_002089.1	7.33	-4.38	-3.34	-4.11	0.33
EPHX2	epoxide hydrolase 2 cytoplasmic	NM_001979.4	2.47	-1.02	-1.18	-1.34	-1.67
FOS	v-fos FBJ murine osteosarcoma viral oncogene homolog	NM_005252.2	3.69	-1.35	-2.23	-1.15	-2.80
IL1A	interleukin 1 alpha	NM_000575.3	4.30	-2.85	-2.47	-2.69	-0.41
IL1B	interleukin 1 beta	NM_000576.2	11.66	-3.20	-4.82	-5.65	-0.51
*IL1RN**	interleukin 1 receptor antagonist	NM_173842.1	4.10	-1.19	-3.51	-2.70	-0.64
IL23A	interleukin 23 alpha subunit p19	NM_016584.2	3.09	-1.33	-2.02	-1.95	-1.21
IL8	interleukin 8	NM_000584.2	7.11	-2.78	-3.60	-4.52	-0.41
LOC643939	PREDICTED: similar to Small inducible cytokine A4 precursor (CCL4)	XM_934361.1	2.38	-1.45	-1.27	-1.32	-0.96
[§] *LY86*	lymphocyte antigen 86	NM_004271.3	2.61	-0.40	-2.27	-1.95	-0.92

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Inflammatory process & Chemotaxis (cont.)							
[§] *MS4A2*	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	NM_000139.2	2.94	-0.58	-2.04	-1.77	-1.84
NCR3	natural cytotoxicity triggering receptor 3	NM_147130.1	2.29	-1.70	-1.56	-1.28	-0.24
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta	NM_001005474.1	3.68	-1.72	-2.84	-2.22	-0.74
NFKBIZ	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor zeta	NM_031419.2	2.14	-1.01	-1.79	-1.48	-0.11
PLA2G7	phospholipase A2 group VII (platelet-activating factor acetylhydrolase plasma)	NM_005084.2	3.03	0.72	-2.45	-2.76	-1.91
PTX3	pentraxin-related gene rapidly induced by IL-1 beta	NM_002852.2	2.02	-0.09	-2.44	-1.73	0.21
ROBO3	roundabout axon guidance receptor homolog 3 (Drosophila)	NM_022370.2	2.03	-0.80	-1.04	-1.12	-1.14
RXRA	retinoid X receptor alpha	NM_002957.3	2.38	-0.15	-2.13	-1.69	-1.03
TLR10	toll-like receptor 10	NM_030956.2	3.12	-1.51	-1.96	-1.90	-1.20
[§] *TNF*	tumor necrosis factor (TNF superfamily member 2)	NM_000594.2	13.40	-4.19	-5.19	-5.21	-0.40

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Leukocyte activation & Leukocyte differentiation							
† *BANK1*	B-cell scaffold protein with ankyrin repeats 1	NM_017935.2	2.91	-0.79	-2.31	-1.90	-1.17
† *BCL11A*	B-cell CLL/lymphoma 11A (zinc finger protein)	NM_138559.1	2.07	-0.86	-1.08	-1.23	-1.02
† *BCL11A*	B-cell CLL/lymphoma 11A (zinc finger protein)	NM_022893.2	2.60	-1.28	-1.53	-1.47	-1.24
§, † *BCL2*	B-cell CLL/lymphoma 2	NM_000633.2	3.25	-0.25	-3.04	-2.35	-1.16
§, † *BCL3*	B-cell CLL/lymphoma 3	NM_005178.2	2.47	1.04	-3.32	-3.32	0.38
† *BLR1*	Burkitt lymphoma receptor 1 GTP binding protein (chemokine (C-X-C motif) receptor 5)	NM_032966.1	3.01	-0.56	-2.16	-2.58	-1.06
C1QR1	complement component 1 q subcomponent receptor 1	NM_012072.2	2.74	0.16	-2.46	-2.39	-1.12
† *CD40LG*	CD40 ligand	NM_000074.2	2.69	-1.09	-1.14	-1.44	-2.05
† *CD79A**	CD79A antigen	NM_001783.2	2.81	-1.14	-1.71	-1.83	-1.28
CEBPA	CCAAT/enhancer binding protein (C/EBP) alpha	NM_004364.2	2.12	-0.44	-1.54	-1.14	-1.21
§, † *EBI2*	Epstein-Barr virus induced gene 2	NM_004951.3	3.67	-1.12	-2.27	-1.90	-2.22
EGR1	early growth response 1	NM_001964.2	2.68	-2.10	-1.46	-0.35	-1.79
† *FLJ33641*	hypothetical protein FLJ33641	NM_152687.1	3.62	-0.51	-2.54	-2.47	-1.92
IL23A	interleukin 23 alpha subunit p19	NM_016584.2	3.09	-1.33	-2.02	-1.95	-1.21
† *IL7R*	PREDICTED: interleukin 7 receptor	XM_937367.1	2.25	-1.08	-0.94	-1.15	-1.51

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Leukocyte activation & Leukocyte differentiation (cont.)							
IL8	interleukin 8	NM_000584.2	7.11	-2.78	-3.60	-4.52	-0.41
† *KLF6**	Kruppel-like factor 6	NM_001008490.1	2.56	-1.23	-1.74	-1.15	-1.26
† *KLF6*	Kruppel-like factor 6	NM_001300.4	2.52	-0.91	-1.97	-1.19	-1.26
† *MS4A1*	membrane-spanning 4-domains subfamily A member 1	NM_152866.2	2.54	-1.00	-1.76	-1.88	-0.73
RPL22	ribosomal protein L22	NM_000983.3	2.31	-1.15	-1.30	-1.31	-1.06
SOX4	SRY (sex determining region Y)-box 4	NM_003107.2	2.21	-0.87	-1.20	-1.36	-1.13
† *SWAP70*	SWAP-70 protein	NM_015055.1	2.05	0.28	-2.19	-1.63	-0.60
§ *TNF*	tumor necrosis factor (TNF superfamily member 2)	NM_000594.2	13.40	-4.19	-5.19	-5.21	-0.40
Regulation of immune response & Regulation of immune system process							
CA2	carbonic anhydrase II	NM_000067.1	2.10	0.20	-1.56	-1.03	-1.89
CARD9	caspase recruitment domain family member 9	NM_052813.2	2.53	-0.47	-1.99	-1.78	-1.12
CD19	CD19 antigen	NM_001770.3	2.21	-1.27	-1.25	-0.96	-1.09
† *CD40LG*	CD40 ligand	NM_000074.2	2.69	-1.09	-1.14	-1.44	-2.05
† *CD79A**	CD79A antigen	NM_001783.2	2.81	-1.14	-1.71	-1.83	-1.28
§ *CD83*	CD83 antigen	NM_004233.2	9.02	-2.24	-4.55	-4.83	-1.07
CDKN1A	cyclin-dependent kinase inhibitor 1A (p21 Cip1)	NM_078467.1	2.08	-1.21	-1.16	-1.10	-0.74

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Regulation of immune response & Regulation of immune system process (cont.)							
[§] *CFD*	complement factor D (adipsin)	NM_001928.2	2.87	-0.26	-2.32	-2.28	-1.22
[§] *CR2*	complement component (3d/Epstein Barr virus) receptor 2	NM_001877.3	2.20	-1.17	-1.13	-1.22	-1.04
FCER1A	Fc fragment of IgE high affinity I receptor for; alpha polypeptide	NM_002001.2	15.71	-1.39	-4.90	-4.92	-4.69
FCER2	Fc fragment of IgE low affinity II receptor for (CD23)	NM_002002.3	2.46	-1.08	-1.40	-1.67	-1.04
HLA-DOA	major histocompatibility complex class II DO alpha	NM_002119.3	2.64	-1.69	-1.32	-1.54	-1.06
IL13RA1	interleukin 13 receptor alpha 1	NM_001560.2	2.81	-0.68	-2.23	-1.87	-1.17
IL1B	interleukin 1 beta	NM_000576.2	11.66	-3.20	-4.82	-5.65	-0.51
IL4R	interleukin 4 receptor	NM_000418.2	2.09	-0.28	-1.73	-1.49	-0.75
[†] *IL7R*	PREDICTED: interleukin 7 receptor	XM_937367.1	2.25	-1.08	-0.94	-1.15	-1.51
JUN	v-jun sarcoma virus 17 oncogene homolog	NM_002228.3	2.27	-1.48	-1.06	-1.14	-1.06
[§] *MS4A2*	membrane-spanning 4-domains, subfamily A, member 2 (Fc fragment of IgE, high affinity I, receptor for; beta polypeptide)	NM_000139.2	2.94	-0.58	-2.04	-1.77	-1.84

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Regulation of immune response & Regulation of immune system process (cont.)							
NCR3	natural cytotoxicity triggering receptor 3	NM_147130.1	2.29	-1.70	-1.56	-1.28	-0.24
NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha	NM_020529.1	2.43	-1.12	-2.12	-1.86	-0.02
RARA	retinoic acid receptor alpha	NM_001024809.2	2.27	-0.03	-1.97	-1.47	-1.26
[§] *TNF*	tumor necrosis factor (TNF superfamily member 2)	NM_000594.2	13.40	-4.19	-5.19	-5.21	-0.40
ZFP36	zinc finger protein 36 C3H type homolog (mouse)	NM_003407.1	2.41	-1.33	-1.76	-1.69	-0.30
Other immune-related processes							
CD160	CD160 antigen	NM_007053.2	2.62	-0.41	-1.99	-2.05	-1.12
CD300LB	CD300 antigen like family member B	NM_174892.1	2.59	-0.86	-1.75	-1.49	-1.39
CD1C	CD1C antigen c polypeptide	NM_001765.1	3.79	-1.62	-2.10	-2.12	-1.85
CD300LF	CD300 antigen like family member F	NM_139018.2	2.20	-0.11	-2.00	-1.28	-1.17
CD79B	CD79B antigen	NM_021602.1	2.06	-0.78	-1.29	-1.17	-0.94
CLEC4A	C-type lectin domain family 4 member A	NM_194448.1	3.29	-0.04	-2.80	-2.60	-1.42
CLEC4C	C-type lectin domain family 4 member C	NM_203503.1	2.04	-0.95	-1.11	-1.12	-0.94
CRIP2	cysteine-rich protein 2	NM_001312.2	2.85	-0.77	-1.84	-1.70	-1.73
CST3	Cystatin C	NM_000099.2	2.20	-0.07	-1.84	-1.54	-1.11
FAIM3	Fas apoptotic inhibitory molecule 3	NM_005449.3	2.04	-0.87	-0.82	-1.22	-1.21

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Other immune-related processes (cont.)							
FCGRT	Fc fragment of IgG receptor transporter alpha	NM_004107.3	2.85	-0.28	-2.57	-2.08	-1.12
FTH1	ferritin heavy polypeptide 1	NM_002032.2	2.70	-0.41	-2.20	-1.99	-1.14
GNG7	guanine nucleotide binding protein (G protein) gamma 7	NM_052847.1	3.18	-1.16	-2.13	-1.85	-1.54
HLA-DMB	major histocompatibility complex class II DM beta	NM_002118.3	2.08	-1.18	-1.29	-1.03	-0.72
HLA-DOB	major histocompatibility complex class II DO beta	NM_002120.2	2.39	-1.39	-0.49	-1.89	-1.26
HLA-DRB1	major histocompatibility complex class II DR beta 1	NM_002124.1	4.96	0.37	-4.79	-4.76	-0.06
HLA-DRB5	major histocompatibility complex class II DR beta 5	NM_002125.3	14.72	-4.02	-5.90	-5.74	0.14
Hs.567460	T cell receptor alpha locus (cDNA clone MGC:23964 IMAGE:4687209) complete cds	BC020840	2.36	-0.04	0.31	-2.01	-3.21
IBRDC3	IBR domain containing 3	NM_153341.1	2.40	-0.79	-1.96	-1.31	-1.00
LILRA3	leukocyte immunoglobulin-like receptor subfamily A	NM_006865.2	2.20	1.86	-1.04	-1.21	-4.16
LTB	lymphotoxin beta (TNF superfamily, member 3)	NM_002341.1	3.82	-0.89	-2.41	-3.28	-1.15

Table 4.10 List of immune-related genes with >2-fold down-regulation in patients with scrub typhus (cont.)

Symbol	Definition	Accession	Average fold change	Normalized expression value [a]			
				AFI052 [b] (day 3)	AFI027 [b] (day 5)	AFI023 [b] (day 14)	AFI060 [b] (day 20)
Other immune-related processes (cont.)							
NALP1	NACHT leucine rich repeat and PYD (pyrin domain) containing 1	NM_001033053.1	2.16	-0.85	-1.23	-1.07	-1.29
OSM	oncostatin M	NM_020530.3	2.35	-0.59	-2.24	-1.49	-0.60
[§] *POU2F2*	POU domain class 2 transcription factor 2	NM_002698.1	2.08	-0.16	-1.62	-1.61	-0.84
RNASE6	ribonuclease RNase A family k6	NM_005615.2	2.75	-0.32	-2.01	-2.33	-1.17
TCF7	transcription factor 7 (T-cell specific, HMG-box)	NM_201633.1	2.60	-0.58	-1.75	-1.76	-1.43
VIPR1	vasoactive intestinal peptide receptor 1	NM_004624.2	4.29	-1.50	-2.34	-2.52	-2.05

[a] Grey boxes mark the patient with highest expression level for each gene.

[b] Sample number of scrub typhus infected patients with day of fever at the time of blood collection in parentheses.

[†] Genes that are also present in "GO:0042113_B cell activation" list

[§] Genes that are also present in "GO:0006959_Humoral immune response" list

* Average expression levels were shown for genes with two probes quantifying the same transcript.

Table 4.11 GO analysis of down-regulated genes in scrub typhus according to molecular function

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0008009	chemokine activity	10	2.25	9.20	6.00×10^{-4}
GO:0042379	chemokine receptor binding	10	2.25	8.63	5.25×10^{-4}
GO:0019955	cytokine binding	12	2.70	4.66	5.02×10^{-3}
GO:0001664	G-protein-coupled receptor binding	11	2.48	4.19	2.38×10^{-2}
GO:0005125	cytokine activity	18	4.05	3.90	5.48×10^{-4}
GO:0004872	receptor activity	73	16.44	1.68	9.81×10^{-4}
GO:0060089	molecular transducer activity	87	19.59	1.62	6.29×10^{-4}
GO:0004871	signal transducer activity	87	19.59	1.62	6.29×10^{-4}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.12 GO analysis of down-regulated genes in scrub typhus according to cellular component

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
GO:0042613	MHC class II protein complex	5	1.13	7.51	1.41×10^{-1}
GO:0042611	MHC protein complex	7	1.58	5.35	7.66×10^{-2}
GO:0009897	external side of plasma membrane	11	2.48	2.82	1.76×10^{-1}
GO:0005615	extracellular space	36	8.11	2.29	2.32×10^{-3}
GO:0009986	cell surface	16	3.60	2.00	3.52×10^{-1}
GO:0044421	extracellular region part	39	8.78	1.77	6.68×10^{-2}
GO:0031226	intrinsic to plasma membrane	45	10.14	1.61	9.22×10^{-2}
GO:0005887	integral to plasma membrane	44	9.91	1.61	8.79×10^{-2}
GO:0044459	plasma membrane part	73	16.44	1.44	7.05×10^{-2}
GO:0005886	plasma membrane	118	26.58	1.36	2.06×10^{-2}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

Table 4.13 Pathway analysis of down-regulated genes in scrub typhus

GO Accession	GO Term	Count	%	Fold Enrichment	[a] Corrected P-value
hsa05310	Asthma	9	2.03	11.52	2.24×10^{-5}
hsa05332	Graft-versus-host disease	8	1.80	7.61	1.49×10^{-3}
hsa05330	Allograft rejection	7	1.58	7.22	4.85×10^{-3}
hsa04940	Type I diabetes mellitus	8	1.80	7.07	2.03×10^{-3}
hsa04621	NOD-like receptor signaling pathway	11	2.48	6.59	1.30×10^{-4}
hsa04640	Hematopoietic cell lineage	15	3.38	6.47	2.54×10^{-6}
hsa04060	Cytokine-cytokine receptor interaction	29	6.53	4.11	1.55×10^{-8}
hsa04062	Chemokine signaling pathway	16	3.60	3.18	2.02×10^{-3}

Note: [a] P-values were corrected for multiple hypothesis testing by Benjamini-Hochberg correction at FDR < 5%.

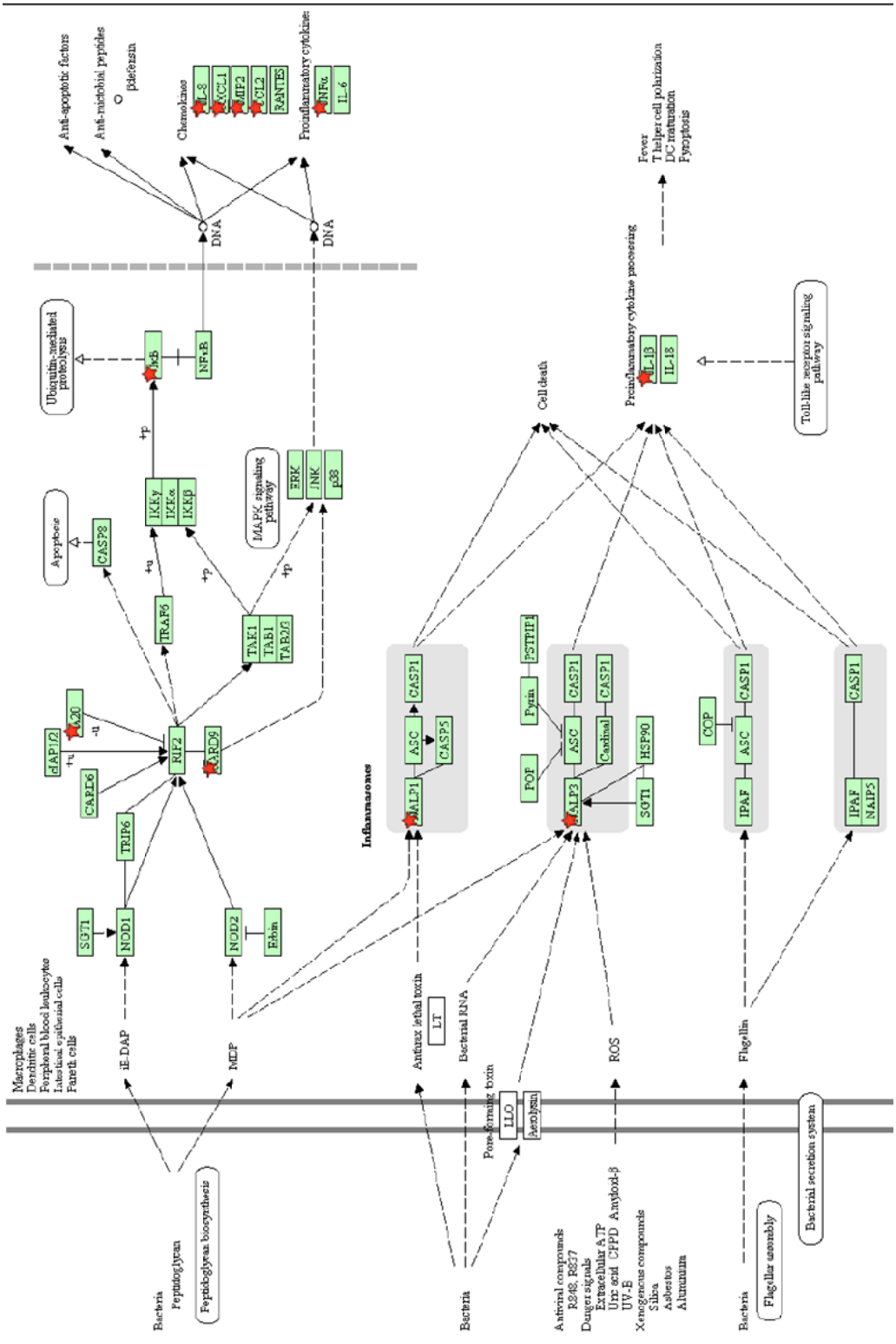

Figure 4.5 NOD-like receptor pathway. Down-regulated genes in scrub typhus were marked with stars. (Adapted from KEGG pathway) (91-92)

4.2 Validation of gene expression by qPCR

To confirm the findings from the microarray study, five scrub typhus-specific genes and twelve scrub typhus responsive genes were requantified using quantitative real-time PCR technique. As shown in Figure 4.6, expression of *CD8A*, *CD8B1*, *LOC642161* and *CBLB,* which were identified as specific up-regulated genes for scrub typhus by microarray analysis, appeared to be significantly higher in scrub typhus group compared with the other infections. Concordantly, *FOSB* was also specifically down-regulated in scrub typhus. When scrub typhus responsive genes were focused, the profile was similar to that derived from microarrays for all genes except *SSBP1* (Table 4.14).

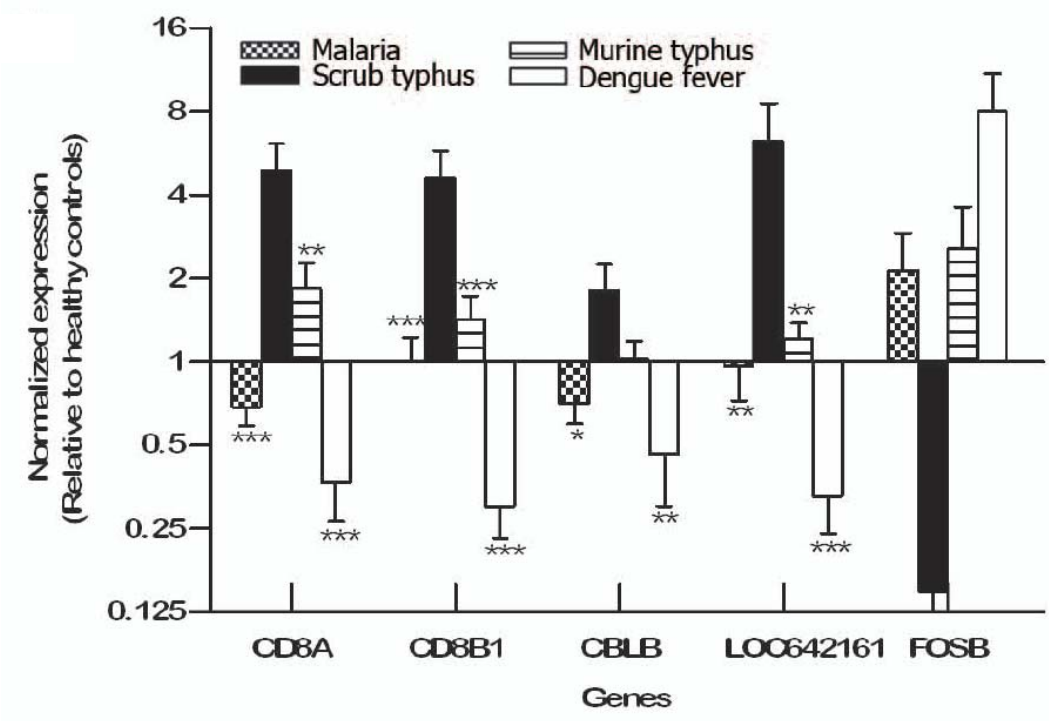

Figure 4.6 Expression profiles of five scrub typhus-specific genes validated by qPCR. Expression values of each sample were calibrated by those of healthy controls. The asterisks indicate significant difference of expression level of each group as compared to that of scrub typhus group by one-way ANOVA with Tukey post hoc test.
(*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$)

Table 4.14 Comparison of gene expression levels of scrub typhus responsive genes as quantified by microarray and qPCR

Gene symbol	Gene name	Accession No.	Fold change[a]	
			Microarray	qPCR
ANKRD22	ankyrin repeat domain 22	NM_144590.1	14.63	21.57
IFI27	Interferon alpha-inducible protein 27	NM_005532.3	10.12	47.01
TIMD4	T-cell immunoglobulin and mucin domain containing 4	NM_138379.1	13.83	33.86
CCL20	chemokine (C-C motif) ligand 20	NM_004591.1	-15.66	-6.84
CCL3	chemokine (C-C motif) ligand 3	NM_002983.1	-15.81	-9.90
CCL3L1	chemokine (C-C motif) ligand 3-like 1	NM_021006.4	-44.04	-18.96
CCL3L3	chemokine (C-C motif) ligand 3-like 3	NM_001001437.2	-17.94	-13.14
FCER1A	Fc fragment of IgE, high affinity I, receptor for; alpha polypeptide	NM_002001.2	-15.71	-9.28
HLA-DRB5	major histocompatibility complex, class II, DR beta5	NM_002125.3	-14.72	-2.40
IL1B	interleukin 1, beta	NM_000576.2	-11.66	-4.66
SSBP1	single-stranded DNA binding protein 1		-12.33	1.42
TNF	tumor necrosis factor (TNF superfamily, member 2)	NM_000594.2	-13.40	-2.46

Note: [a] Positive value indicates up-regulation, whereas negative value indicates down-regulation of a transcript in scrub typhus as compared with healthy control group.

Part II

4.3 Literature review of a candidate gene

Seeing that a number of IFN-γ inducible genes were up-regulated during scrub typhus infection, concordant to an increased serum level of IFN-γ previously reported in patients with scrub typhus; studies involving IFN-γ inducible genes and infections were searched in the literature. Among these, IDO1 is an interesting gene with a growing number of publications for its roles in host defense against various infectious organisms. For this reason, it was selected to be reviewed in more details. A hypothesis was then proposed and tested in a cell-based model as described in the next section.

4.3.1 Indoleamine-2,3-dioxygenase (IDO1) and kynurenine pathway

IDO1 is a well-conserved gene in mammals (94) encoding an intracellular heme-containing dioxygenase (95) that is an initial and rate-limiting enzyme of kynurenine pathway, a major catabolic pathway of tryptophan (Figure 4.7). The enzyme catalyzes oxygenation of tryptophan to form n-formyl kynurenine, which is then rapidly broken down into a more stable product kynurenine (96). After being generated, kynurenine can be either transported to the extracellular compartment or further metabolized intracellularly into a number of products. The ultimate pattern of matabolites being produced is varied depending on the collection of downstream enzymes in each cell type. However, the ratio of kynurenine to tryptophan in sera and in culture media is generally used as an indicator of IDO1 activity in vivo and in vitro, respectively.

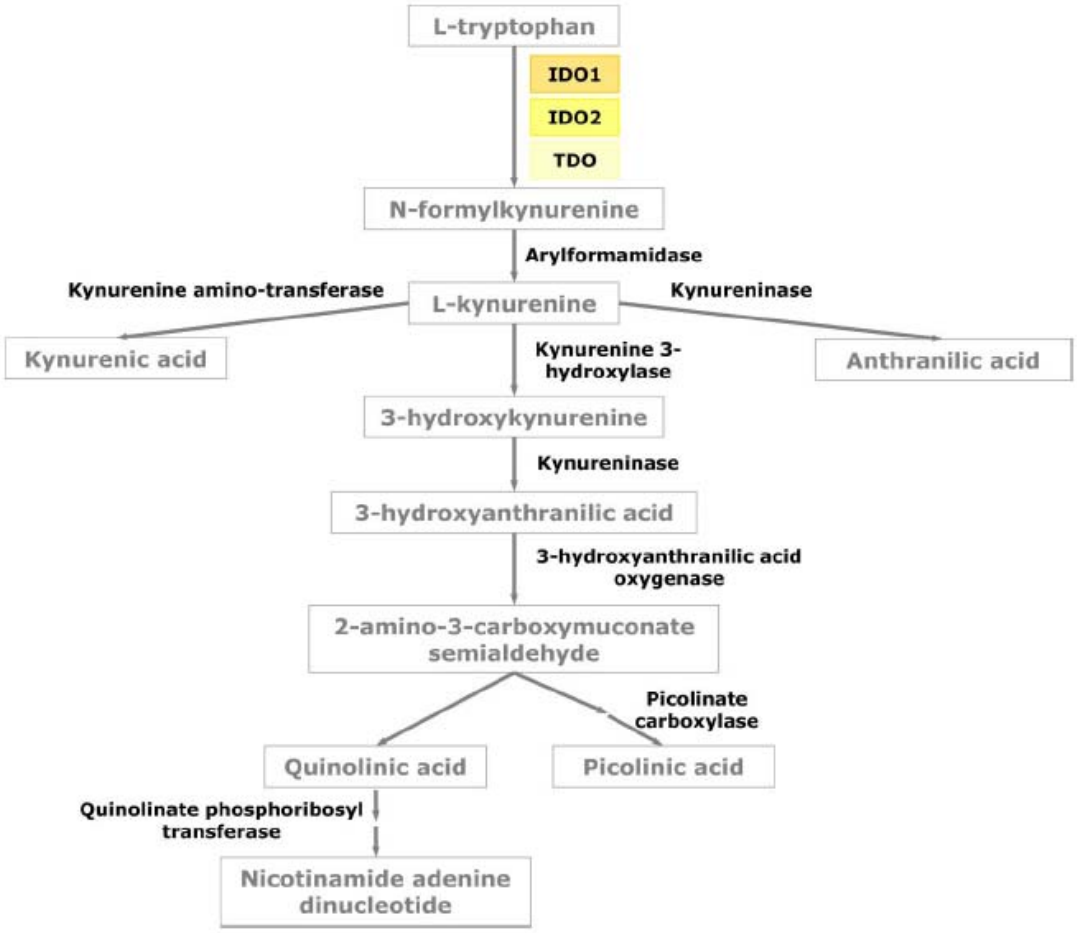

Figure 4.7 The kynurenine pathway of tryptophan catabolism (97)

4.3.2 Regulation of IDO1 expression and IDO1 activity

In human, IDO1 expression can be detected in various tissues but appears to be relatively high in the lung, small intestine and placenta (98). Unlike another tryptophan-degrading enzyme TDO, which is mainly expressed in the liver, IDO1 expression can be induced by various immunological signals in a variety of cell types including fibroblast, endothelial cell, monocytes, macrophage, dentritic cells. Given that the gene promotor contains two IFN-stimulated response elements (ISRE) and an IFN-γ-activated site (GAS), which respond to the IFN-γ-activated trascription factors STAT-1 and IRF-1 respectively; IFN-γ appears to be one of the most potent inducers that has been widely used in a number of functional studies of IDO1. (99-102). Via activation of ISRE but not GAS, IFN-α/β can also enhance the transcription of IDO1 relatively to a lesser extent (100-101, 103-104). Other cytokines reported to regulate IDO1 expression include the proinflammatory cytokines TNF-α and IL-1, which appear to potentiate IFN-γ mediated IDO1 expression despites their inablility to induce the gene transcription by themselves; and IL-4, which inversely exhibits an inhibitory effect on IDO1 activity in human monocytes. The effect of TGF-β on IDO1 expression appears to be cell type specific since it was shown to exert a negative regulatory effect on human fibroblast but conversely enhance the enzyme activity in DC.

Apart from secreted cytokines, signaling downstream to TLR9 as well as various membrane-anchored molecules was also reported to affect transcription of IDO1 in dendritic cells (DC). For example, the binding of B7 molecules to the inhibitory receptor CTLA-4, either in a soluble form or expressed on regulatory T cells, enhances IDO1 expression in DC in an IFN-γ dependent manner (105-106). In contrast, engagement of the same DC-borne ligand with another receptor CD28 on T cells inhibits IFN-γ-mediated IDO1 activation through IL-6 production, which effect can be restored by transcritional silencing of suppressor of cytokine signaling-3 (SOC3) (107-108). Interestingly, the interaction of glucocorticoid-induced tumore necrosis factor receptor ligand (GITRL) with its partner appears to induce IDO1 expression via non-canonical NF-kB pathway and lead to the development of tolerogenic phenotype of the ligand-bearing DC (109).

4.3.3 The roles of IDO1 in infection

Since tryptophan is the rarest essential amino acid in a cell, deprivation of tryptophan becomes a potential stratagy of the host to control the population of an invading microorganism unable to synthesize the amino acid itself. According to this conception, IDO1 was first suggested to be a gene with an anti-microbial function by Pfefferkorn and collegues as they observed that growth retardation of the organism is associated with tryptophan degradation in IFN-γ-treated human fibroblast and can be reversed by supplementation of L-tryptophan (110). Such depletion of the amino acid was then confirmed to be a consequence of induction of IDO1 gene transcription following IFN-γ stimulation. Similar anti-toxoplasma activity of IDO1 induced by IFN-γ was also observed in a broad-range of cell types such as human glioblastoma cell line (111), primary endothelial cells (112), uroepithelial cells (113), as well as monocyte-derived macrophage (114). Later on, IDO1-mediated microbial-stasis was also reported in a number of intracellular and extracellular infections including *Chlamydia spp.* (115), *Mycobacterium avium* (116), HSV (117-118), measles virus (119), CMV (120), Dengue (121), group B Streptococcus (122), Enterococci (123), and *Staphylococcus aureus* (124).

Apart from tryptophan depletion, it was revealed that IDO1 also indirectly exerts antimicrobial activity via certain downstream catabolites of kynurenine pathway (125-127). For example, IDO1 activation with subsequent production of 3-hydroxy-kynurenine was shown to limit replication of *Trypanosoma cruzi* in vivo, and treatment of the infected mice with the metabolite can improve resistance to the infection as well as survival of the host (125). In another study, it was shown that not only 3-hydroxy-DL-kynurenine but also picolinic acid, 3-hydroxyanthranilic acid, and quinolinic acid can inhibit the growth of methicillin-resistant *S. aureus*, *S. epidermidis*, *Escherichia coli*, and multidrug-resistant *Pseudomonas aeruginosa* in vascular allograft, even though the latter two appears to be less potent (126).

Despite a protective role against infection, IDO1 activation was paradoxically shown to play a role in immunosuppression. This was originally proposed by a key study showing that IDO1-mediated tryptophan degradation prevents allogenic fetal rejection in mice (128). It was later demonstrated in vitro that the enzyme exerts anti-proliferative effects on T cells, NK cells, as well as tumor cells via

degradation of tryptophan and production of downstream metabolites, resembling its impact on microorganisms (129-132). Indeed, IDO1 expression in certain DC subsets was shown to induce tolerogenic response to antigenic stimuli through a variety of mechanisms, including induction of T cell anergy (133), apoptosis (134) and regulatory T cell differentiation (135). When such concept is applied to a scenario of infection, it seems that IDO1 activation in certain setting may contribute to ineffective development of adaptive immunity and allow the organisms to persist. For example, it was found that HIV-induced IDO1 activation observed in peripheral blood of HIV-infected patients is mainly contributed by plasmacytoid DC subpopulation and also responsible for unresponsiveness of CD4-positive T cell to TCR stimulation in vitro (136). The levels of IDO1 expression in PBMCs from these patients also appears to correlate with their viral loads, which further supports a link between IDO1, T cell dysfunction, and ineffective viral control (136).

4.3.4 Studies of IDO1 in rickettsial infection

It was reported that IFN-γ-mediated IDO1 activation inhibits the growth of *R. conorii* in vitro and such restriction can be relieved by tryptophan supplementation (137). In contrast, proliferation of *R. prowazekii* appears insensitive to IDO1-mediated tryptophan depletion (138). An up-regulation of IDO1 was also observed at the skin lesion of patients with Mediterranean spotted fever, an illness caused by *R.* conorii infection, and appears to positively correlate with IFN-γ and TNF-α expression (139). However, the role of IDO1 in OT infection has never been directly investigated.

4.3.5 Hypothesis: IFN-γ-mediated activation of IDO1 restricts OT growth

Seeing that OT lacks of enzyme to generate tryptophan which is an essential material for its expansion (53), we supposed that up-regulation of IDO1 upon OT infection could result in tryptophan depletion concurrent with kynurenine formation, either or both of which leads to restrict OT growth in host cells. To test the hypothesis, we first validated an induction of IDO1 in peripheral blood of patients with scrub typhus at both transcriptional and functional levels. Since monocytes are a major target of OT infection and also appear to be the main source of active IDO1 in

human peripheral blood in normal condition (140) as well as upon stimulation with IFN-γ (141), human monocytic cell line THP-1 was used as a model in subsequent in vitro infection experiments. After IDO1 expression and its activity was checked, an effect of IDO1 induction on OT growth were investigated in the cell-based model.

4.4 Validation of *IFNG* and *IDO1* gene expression in clinical samples

To confirm the induction of *IFNG* and *IDO1* gene expression as observed in the microarray study, mRNA levels of these genes were requantified using qPCR. As shown in Table 4.15, average expression levels of *IFNG* and *IDO1* in patients with scrub typhus were about 10 and 15 folds greater, respectively, than those in healthy control. Even though statistical significance of the difference was not reached, both genes were harmoniously expressed at higher level in all four scrub typhus patients compared with healthy subjects (Table 4.15).

Table 4.15 Expression levels of *IFNG* and *IDO1* as quantified by qPCR

Sample No.	*IFNG*			*IDO1*		
	Normalized expression	mean ± SD	*P*	Normalized expression	mean ± SD	*P*
AFI023	18.10	10.22 ± 7.00	0.078	45.95	15.41 ± 20.85	0.26
AFI027	13.81			11.55		
AFI052	2.74			2.70		
AFI060	6.23			1.45		
Pool1	1.02	1.00 ± 0.02		0.99	1.00 ± 0.01	
Pool2	0.98			1.01		

4.5 Functional activity of IDO1 in patients with scrub typhus

To check whether the up-regulation of IDO1 actually led to an increased activity of IDO1 enzyme, the levels of L-tryptophan and L-kynurenine were determined in sera from another set of scrub typhus patients (n = 20) using LC-MS. As shown in Figure 4.8a and 4.8b, serum level of L-Tryptophan was significantly lower in scrub typhus infected patients as compared to healthy individuals (P = 0.0146), whereas a reverse trend was observed for serum L-kynurenine level (P = 0.0002). To see how IDO1 activity differs between the two groups of subjects, the ratio of L-kynurenine to L-tryptophan was evaluated. As shown in Figure 4.8c, the enzyme activity in patients with scrub typhus was about nine times higher than that of healthy controls ($P < 0.0001$)

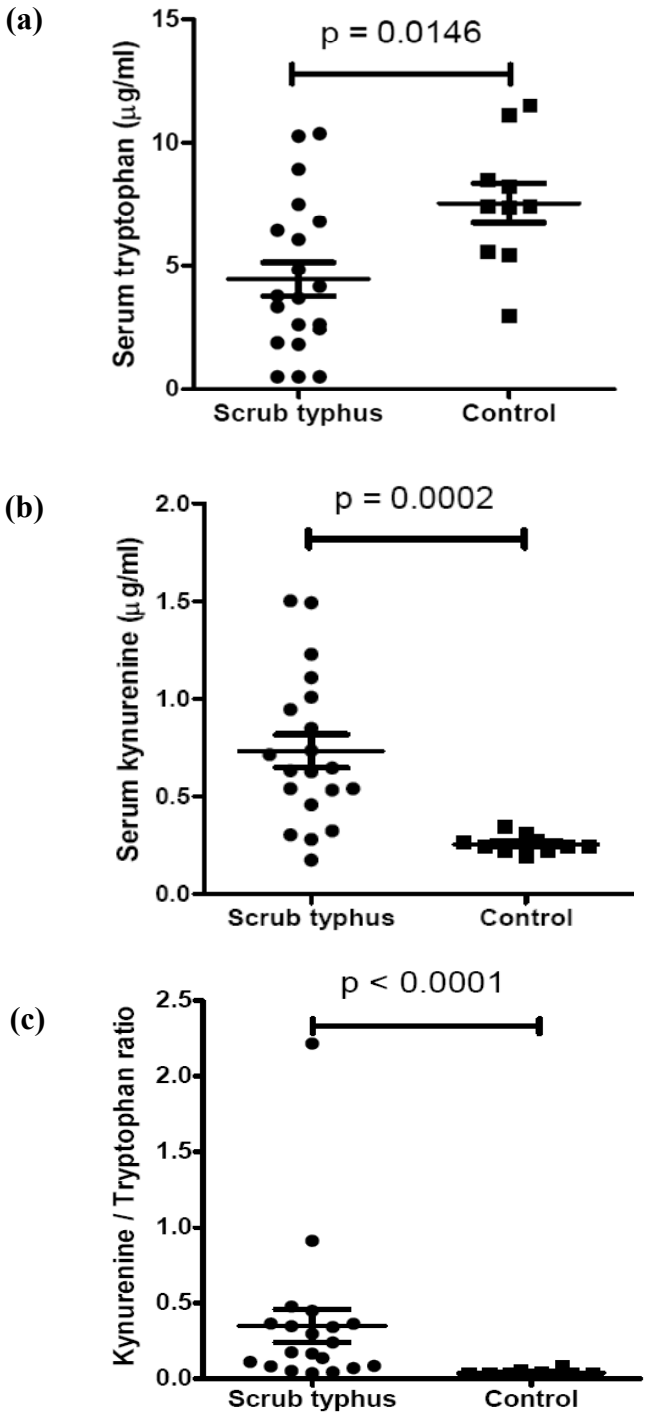

Figure 4.8 Assessment of IDO1 activity in patients' sera. Serum concentrations of L-tryptophan (a) and L-kynurenine (b) in patients with scrub typhus (n = 20) were compared with those in healthy donors (n =10). L-kynurenine/L-tryptophan ratios (c) were calculated to reflect IDO1 activity. Data were derived from triplicate measurement.

4.6 Expression of *IDO1* in THP1 cell

To prove our hypothesis on the role of IDO1 in OT infection, we first evaluated the profile of the gene expression in the experimental models. As shown in Figure 4.9a, expression of *IDO1* was evidently induced in OT-infected THP-1 at 24 hours p.i. ($P < 0.0001$), and rose up to 18 times greater than mock-infected cultures at 120 hour p.i. ($P = 0.0001$). IFN-γ treatment dramatically enhanced transcription of *IDO1* to even more than 2000 folds since 6 hours p.i. ($P < 0.0007$), but the expression dropped to a level comparable to that of unstimulated infected cultures at 72 and 120 hour p.i. (Figure 4.9b). The degree of *IDO1* induction in cultures co-treated with IFN-γ and 1-MT was lower when compared to IFN-γ treatment alone at 6 and 24 hours p.i. ($P = 0.077$ and 0.001 respectively), yet overall kinetics of IDO1 expression in both conditions were quite similar (Figure 4.9b).

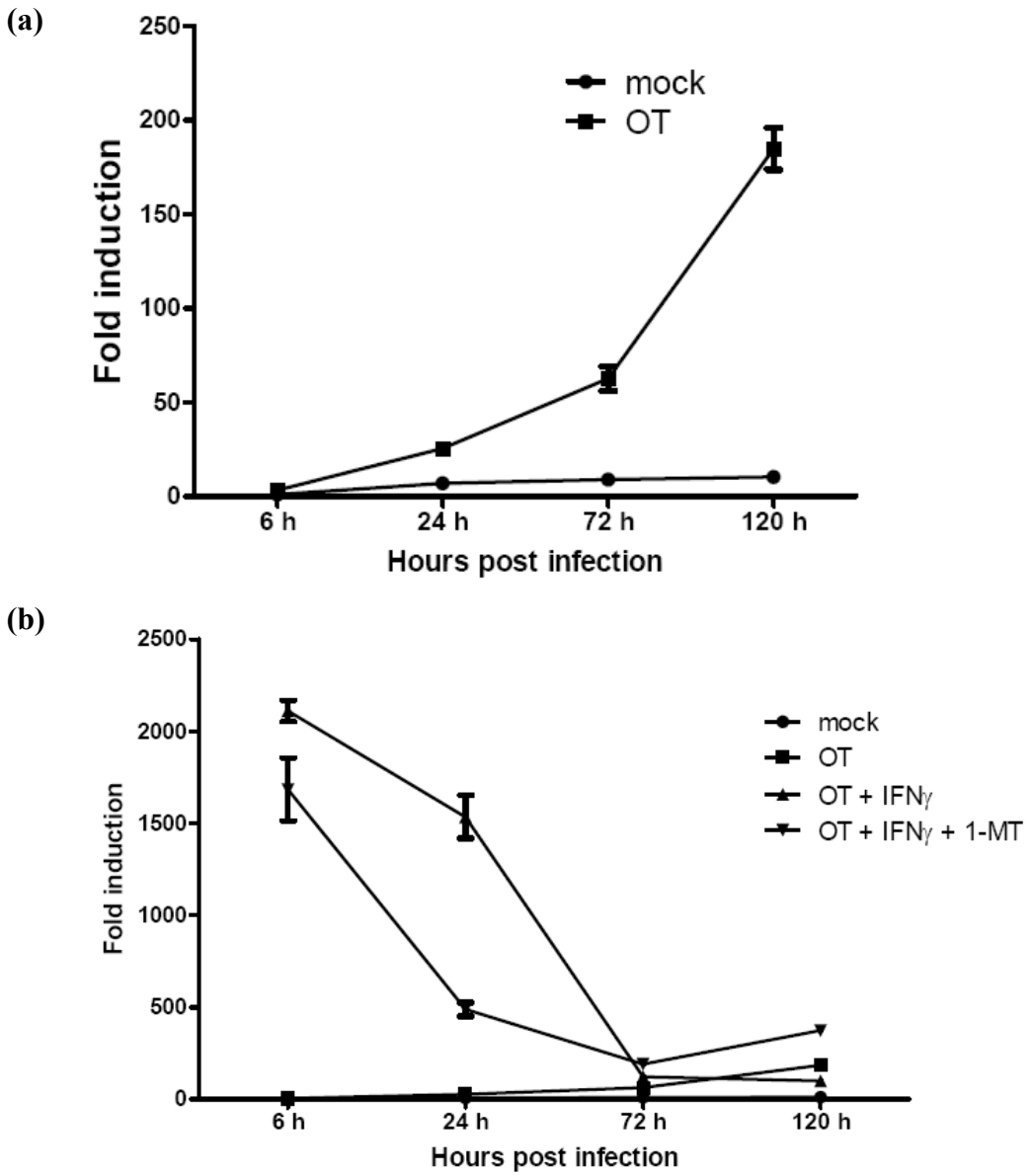

Figure 4.9 Kinetics of *IDO1* expression in THP-1 cells. Before being infected with OT, THP-1 cells were treated with IFNγ alone, IFNγ combined with 1-MT, or neither. Mock-infected THP-1 cells were used as a control. Fold induction represents levels of *IDO1* expression in each culture condition at indicated time points relative to that in mock-infected cells at 6 hours p.i.. For clearer illustration, data of OT-infected and mock-infected cultures are exclusively presented in (a), and those of all four culture conditions are shown in (b).

4.7 Functional activity of IDO1 in THP1 cells

To assess functional activity of IDO1 in a cell model, the concentration of L-tryptophan and L-kynrenine in culture supernatant of THP1 cells was determined at corresponding time points. As shown in Figure 4.10a, the level of L-tryptophan in OT-infected cultures was relatively lower than that of mock infection at 6 hours p.i. (P = 0.001) yet pronouncedly greater than that of IFN-γ treated ones (P = 0.0011). Co-treatment of IFN-γ and 1-MT retarded the rate of tryptophan consumption as compared with IFN-γ treatment alone at the same time point (P = 0.0334). However, levels of the amino acid in supernatant fell below the limit of detection in all culture conditions by 24 hours p.i. (Figure 4.10a).

Despite the different rate of tryptophan consumption, the dynamic of kynurenine level in OT-infected cultures was comparable to that in mock-infected counterparts, as shown in Figure 4.10b. IFN-γ treatment increased the level of kynurenine at the first 6 and 24 hours p.i. (P = 0.0027 and 0.0009 respectively), but the level became comparable to that of mock infection at later stages (Figure 4.10b). Unexpectedly, a continuous rise of kynurenine level was observed in 1-MT treated cultures (Figure 4.10b). Since the difference of L-tryptophan levels among the four culture conditions was unremarkable in comparison with that of L-kynurenine levels, profiles of L-kynurenine/L-tryptophan ratio resembled that of L-kynurenine as shown in Figure 4.10c.

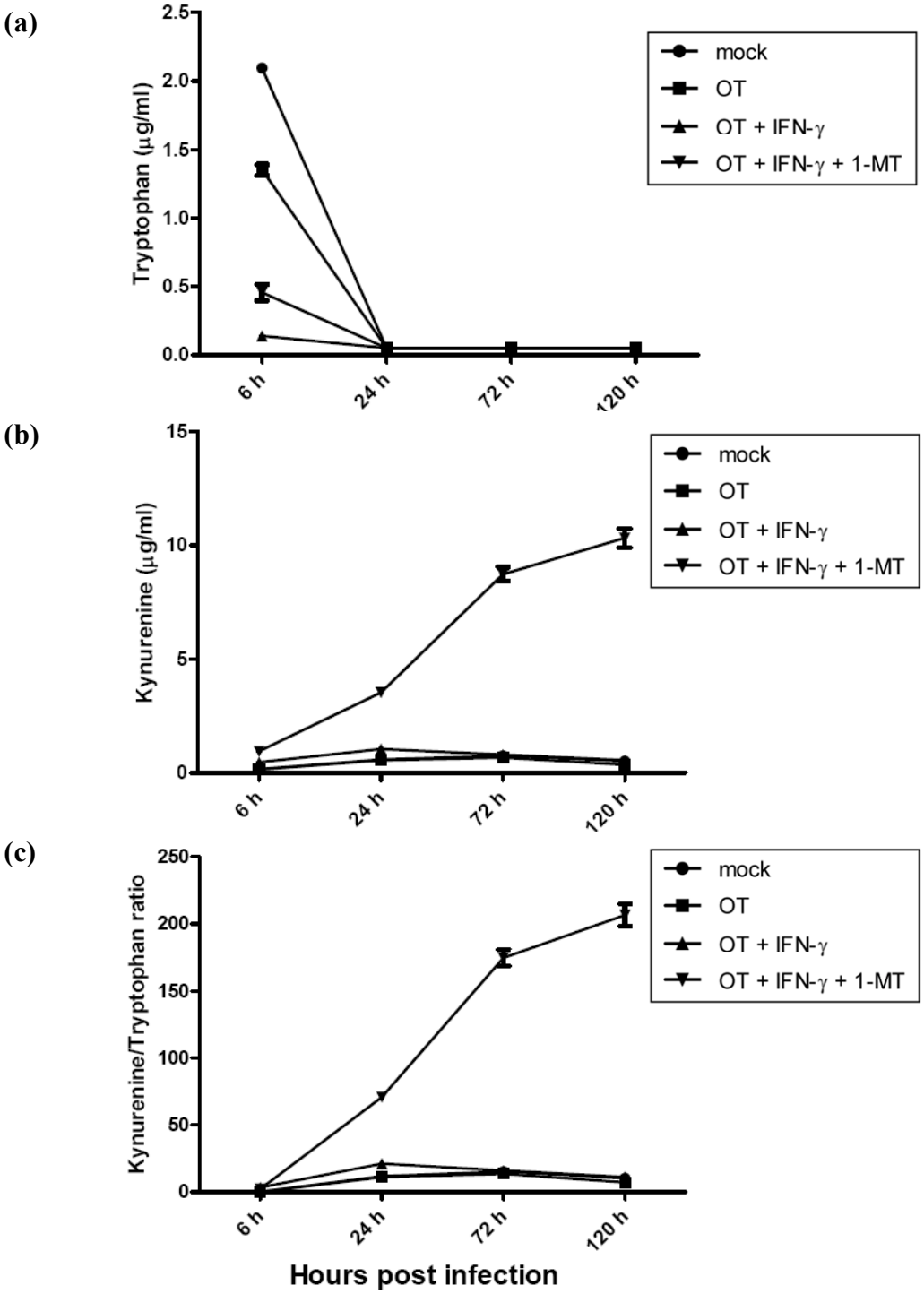

Figure 4.10 Assessment of IDO1 activity in OT-infected THP-1 cultures. Before being infected with OT, THP-1 cells were treated with IFNγ alone , IFNγ combined with 1-MT , or neither. Mock-infected THP-1 cells were used as a control. Levels of L-tryptophan (a) and L-kynurenine (b) were measured in culture supernatant at 6, 24, 72, and 120 hours p.i.. IDO1 activity was reflected by the ratio of L-Kynurenine to L-tryptophan (c) at each indicated time point. Means ± SEM from triplicate cultures are shown.

4.8 OT growth in cell cultures

To investigate the effect of IDO1 activation on OT growth; infection index, which is the relative copy number of OT47 kDa gene per a host gene, was determined in OT-infected cultures with or without IFN-γ-induced IDO1 activation. As shown in Figure 4.11a, the infection index was significantly lowered by IFN-γ treatment at five days p.i. (P = 0.0273). When IDO1 activity in IFN-γ treated cultures was inhibited by 1-MT, the infection index was partially restored. Addition of L-tryptophan in the culture media at the concentration of 400 μg/ml could not revert suppressive effect of IFN-γ-induced IDO1 induction (Figure 4.11a). However, supplementation of the amino acid at 1 mg/ml markedly increased the infection index to even higher level than that in IFN-γ-unstimulated cultures as early as 3 days p.i. (P = 0.0326) (Figure 4.11b).

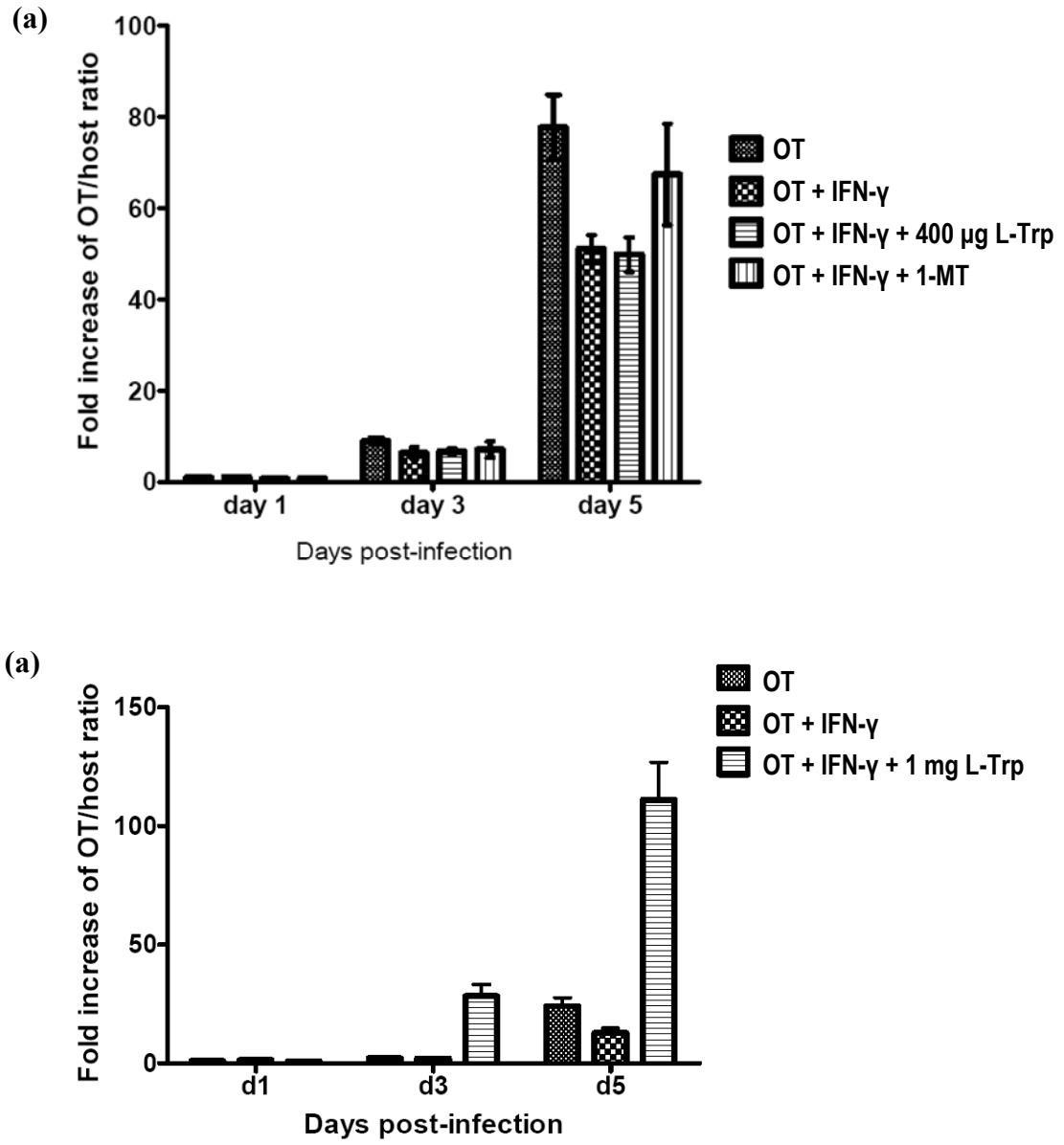

Figure 4.11 Assessment of OT growth in THP-1 cultures. Before being infected with OT, THP-1 cells were treated with IFNγ alone, IFNγ combined with 1-MT, or IFNγ supplemented with L-tryptophan (L-Trp) at 400 µg/ml (a) or 1 mg/ml (b). The infection index represents the degree of OT growth in each culture condition at indicated time points relative to that in mock-infected cells at 1 day p.i.. Means ± SEM from triplicate cultures are shown.

CHAPTER V
DISCUSSION

5.1 Comparative analysis of genome-wide expression

In a scenario of infection, transcriptional changes inside human leukocytes are informative traces of complex interplay between the host and the invading pathogen. While a defined set of host genes is activated in an attempt to fight against the infection, the microorganism might possess a capability to modulate some transcriptional programs to evade the immunity and survive in a hostile environment. The balance between host-pathogen specific interactions could contribute to a unique mRNA profile that might help specify the causative infectious agent. This notion has been proved by several studies (20-21, 84, 86), which prompted us to seek for such a signature for scrub typhus, a common cause of acute undifferentiated fever in rural Asia-Pacific region.

With the power of genome-wide expression analysis, we identified a set of 65 transcripts whose expression pattern was significantly distinct in scrub typhus as compared to that of the other endemic infections and could be used to discriminate all scrub typhus cases from the rest despite their similar clinical presentations. Further filtration with the degree of relative difference in expression resulted in five scrub typhus associated genes that could also clustered patients with the disease together, suggesting a diagnostic potential of this small subset of genes for scrub typhus infection. Even though the variation among individuals may account for the observed difference in gene expression, it has been demonstrated that the size of effect is much smaller than that contributed by the disease and might be of minimal concern (142). Moreover, the homogeneity of expression profile in our scrub typhus patients is of noted despite the varied days of illness at blood collection, ranging from day three to day twenty. This may imply that the distinctive expression pattern in peripheral blood leukocytes might be more sensitive for early diagnosis of scrub typhus than other approaches like real-time PCR, which has been reported to detect the presence of the

organism in patient's blood no sooner than five days after symptom onset (7). However, a study in more subjects with a variety of clinical settings is required to prove the discriminative power of this subset of genes. A proper diagnostic criterion must also be set up before sensitivity and specificity of the strategy can be evaluated. Furthermore, it is compelling to investigate whether protein expression of these genes correlates with the mRNA levels and could be applicable into clinical uses.

To characterize the scrub typhus-specific gene set, gene ontology and pathway analysis was performed. Interestingly, it was found that genes in the list were particularly enriched in T cell activation, T cell receptor complex, and T cell receptor signaling pathway. The presence of CD3G and both subunits of CD8 antigen in the list suggests that cytotoxic T cell is a prominently active population upon the infection, which is concordant to an earlier study that observed an elevation of the number of CD8+ cells in scrub typhus infected patients when compared to other non-rickettsial acute infections (143). From these data, we proposed that characterization of lymphocyte subpopulation in patient's peripheral blood might provide an informative clue in diagnosis of scrub typhus.

Among the set of uniquely over-expressed genes in scrub typhus, *CBLB* is an interesting one with a growing number of evidences for its important functions in immune system. The gene encodes an E3 ubiquitin ligase Cbl-b, which plays a critical role in the control of peripheral T cell activation and tolerance by negatively regulating CD28 costimulation signaling (144-145). In infectious diseases, up-regulation of Cbl-b is observed in many chronic infections and believed to implicate in unresponsiveness of immune response (146-148). Moreover, it also plays a part in host defense by modulating the stability of bacterial effector proteins essential for its virulence as recently reported in *Pseudomonas aeruginosa* infection (149). Since the role of this protein has never been addressed in scrub typhus, it should be investigated whether the up-regulation observed in scrub typhus infected patients could have a role in degradation of bacterial products or implicate in immune evasion of the bacteria. However, it may simply reflect a regulatory mechanism of the immune system in order to prevent over-activation of T cells.

5.2 Host responses to scrub typhus

Focusing on immune responses to scrub typhus, a significant up-regulation of IFN-γ and IFN-inducible genes was observed. Interestingly, we noticed that STAT-1, which is a key transcription factor for cellular responses to IFN-γ and Th-1 development; and GCH1 (GTP-cyclohydrolase I), which is responsible for formation of neopterin, a marker commonly used for IFN-γ and cell-mediated immune activation, also appeared to be induced. Even though it was known that the level of gene transcription might not always correlate with the level of its protein encoded, our findings are consistent with preexisting reports that showed an elevated level of IFN-γ in serum of the infected patients; which further support the role of IFN-γ in host responses to scrub typhus.

IFN-γ is known as a key cytokine in macrophage activation and T helper (Th)-1 response, both of which are essential host responses against OT (10, 13-14). Besides, it has been shown to directly inhibit intracellular growth of OT in non-immune host cells (150). A kinetic study on the development of protective immunity against OT has revealed that an increase in IFN-γ production was parallel to the acquisition of resistance to OT infection in immunized mice (12). This indicated that adaptive immunity should be involved in IFN-γ production. In the present study, we harmoniously support such notion considering that expression level of most IFN-related genes seemed lowest in the patient No. AFI052, whose blood was collected since OT-specific IgM titer was not yet marked. Furthermore, concomitant overexpression of CD8 subunits and those genes involving in cell cycle and cell division was also observed, implying that the up-regulation of IFN-γ was, at least in part, a consequence of an expansion of CD8+ T-cells and probably NK cells in response to scrub typhus. Indeed, the increased number of CT cells and NK cells has been previously reported in scrub typhus infected patients (143). Altogether these data emphasize on the role of type II IFN and cell-mediated immunity in protection against OT infection.

Disseminated vasculitis with perivasculitis is major pathology that leads to dysfunction of the affected organs in scrub typhus infected patients (25-26). For this reason, hyperproduction of cytokines, as the key mediators of inflammation, has been implicated in pathogenesis of the disease. In the present study, we surprisingly

observed a marked down-regulation of several chemoattractants and proinflammatory cytokines including TNF-α, IL1-B, IL8, and CCL3; accompanied with concordant low expression of FOS and JUN, both of which composes AP-1, a transcription factor with a role in cytokine production. Such findings contrasted with previous studies in mouse models and cell lines that reported an induction of these cytokine-encoding genes during OT infection (2, 16, 38-39). The discrepancy might be explained by the difference in timing and systems being studied. While earlier works inspected early responses of infected cells or inflammatory cells at the site of infection, where cytokines are mainly produced; we examined transcriptional patterns inside circulating leukocytes of the patients after several days of fever, which are equivalent to more than a week after first acquisition of the infection. At this late time point, depletion or feedback inhibition of gene expression might have already occurred subsequent to prior massive production of their protein products, similar to what have been addressed in patients with severe sepsis (151-153). From our data, the explanation seems unlikely since the patient who was the latest to present to the hospital according to the day of his illness exhibited the least severe degree of inflammatory cytokine depression as compared with the others. On the other hand, it is also possible that the down-regulation of inflammatory cytokines, particularly TNF-α, might have resulted from an immunosuppression actively induced by the organism. This hypothesis was supported by an observation that found an elevation of TNF-α in convalescent serum of scrub typhus patients when compared to the acute one (9). Subsequent studies in murine macrophage have demonstrated that OT inhibits TNF-α production by inducing IL-10 secretion (18-19). A parallel study of cytokine expression at both mRNA and protein levels in the same set of samples shall clarify this obscure finding in human.

5.3 Further study of IDO1

Activation of IDO1 is a defensive mechanism downstream to IFN-γ that has been proved to limit the growth of various infectious pathogens in both immune and non-immune cells in vitro (111-112, 116-119). It also appears to be induced in human subjects infected with some particular pathogens, such as *Leishmania guyanensis* (154), *R. conorii* (139), HIV-1 (136) and dengue virus (121). According to the fact that OT lacks tryptophan-synthesizing enzyme (53), IDO1-mediated deprivation of tryptophan is a potential protective mechanism to limit the activity of this particular organism.

In the present study, we first observed an up-regulation of *IFNG* and *IDO1* in peripheral blood leukocytes of patients with scrub typhus as compared to healthy subjects. Functional activity of IDO1 in the affected patients was concordantly higher than that in healthy subjects, confirming that IDO1 was functionally active upon the illness. Further experiment in a human macrophage-like cell line also showed that transcription of *IDO1* could directly be induced by OT infection early after infection. However, a decreased level of L-tryptophan without a concomitant elevation of L-kynurenine in OT-infected cultures as compared with mock infection may imply that higher rate of tryptophan consumption was contributed by increased utilization of the amino acid by the organism for its intracellular activity rather than enhancement of tryptophan catabolism by IDO1 enzyme. We postulated that the increased IDO1 activity in vivo was a secondary event following the release of IFN-γ rather than a direct induction by the infection itself. For this reason, THP-1 was treated with IFN-γ prior to the infection to imitate the infection in human body, in which other IFN-γ-producing immune cells, such as NK cells, γδT cells,Th-1 cells and cytotoxic T cells, are also present. This is sensible considering that the rise of IFN-γ has been consistently observed in patients with scrub typhus by a number of earlier studies (7-9)

After pre-treatment with exogenous IFN-γ, a marked increase in transcription of IDO1 along with its functional activity was detected as early as 6 hours p.i.. Concurrently, the intracellular number of OT was significantly depressed by IFN-γ treatment at 5 days p.i.. Even though IFN-γ is known to cause multiple changes in macrophage biology that influence the outcome of an intracellular infection, our data suggest that IFN-γ-mediated IDO1 activation is responsible for growth restriction

of OT to some extent since the number of OT per host cell was partially restored by a competitive inhibitor of the enzyme. However, it must be noted that induction of IDO1 only limited the rate of OT proliferation, but neither froze the growth nor reduced the number of the intracellular organism. For this reason, development of adaptive immune mechanisms seems to be required for eradication of the infection.

From earlier studies, anti-microbial activity of IDO1 has been explained by two non-mutually exclusive mechanisms: deprivation of tryptophan and formation of its metabolites, collectively known as kynurenines. The first mechanism is based on the fact that tryptophan is the rarest essential amino acid in a human cell; therefore IDO1-mediated tryptophan degradation, which limits the availability of the amino acid to be exploited by the organism, could lead to restraint of reproduction of tryptophan-sensitive microorganisms. In this study, we demonstrated that tryptophan supplementation at 400 μg/ml caused no change in the infection index of IFN-γ treated culture, whereas replenishment of the amino acid up to 1 mg/ml did not just restore but accelerated the growth of OT since the third day post infection. For this reason, it is not surprising why an earlier study that supplemented the culture media with 100 μg/ml of trytophan failed to rescue the growth of OT in IFN-γ-treated murine embryonic cell lines and refuted the role of tryptophan deprivation in control of OT infection (150). Indeed, the number of the organisms in the culture with no IFN-γ treatment in the same experiment was demonstrated to be significantly increased by such small dose of tryptophan supplementation (150), which supports the dependence of OT proliferation on the availability of L-tryptophan similar to ours. Altogether, these data implies that IDO1-mediated tryptophan deprivation can restrict OT growth; but restoration or enhancement of its reproduction requires adequate replenishment of tryptophan that allows an excess of the amino acid to be present in IDO1-active environment long enough for exploitation by this particularly slow-growing organism. However, it should be aware that comparing the findings derived from experiments in murine and human cell models might not be straightforward since defensive mechanisms to an infection may vary from species to species. For example, it was found that IDO1, but not inducible nitric oxide synthase, is essential in control of various infectious organisms in human mesenchymal stromal cells, while a reversed scenario was revealed for a similar cell type derived from mice (155). In much the

same way, it is also possible that IDO1 is critical in control of OT growth in human macrophages but not in murine embryonic cells.

For the second mechanism involving IDO1-mediated kynurenine formation, it has been proved that some tryptophan metabolites, particularly 3-hydroxy-DL-kynurenine and α-picolinic acid but not L-kynurenine, can exert anti-microbial activity against several extracellular bacteria in a dose-dependent manner (126). In our experiments, the role of these metabolites in OT control was not directly investigated. However, supplementation of tryptophan into the culture, which was assumed to result in greater amount of metabolites produced in the system, did not increase the extent of OT growth restriction. Thus, we suppose that that formation of kynurenines is unlikely to be involved in suppression of OT growth.

Despite the key findings that have already been discussed, we are also aware of some factors that could influence the interpretation of our experimental data. Firstly, the infection index, used as an indicator of OT growth in this study, was derived by a relative quantification method; it is therefore not only sensitive to a change in the number of OT but also to that of host cells at the time of the assessment. In the cultures supplemented with 1-mg tryptophan, we observed a reduction in the number of host cells by time, which might be partly responsible for a marked increase in the infection index of the condition compared with the others. Such cell loss could be a consequence of overwhelming expansion of OT in tryptophan-rich environment, excessive accumulation of toxic metabolites in the culture system, or both. Secondly, the use of kynurenine to tryptophan ratio as an indicator of IDO1 activity in THP-1 cells was unfortunately limited by the drop of L-tryptophan below the detectable level since the first day post infection. Moreover, some ambiguity in the data interpretation also resulted from the surprising rise of kynurenine level in the culture supernatant of cells treated with 1-MT. Unlike other cultures whose kynurenine level similarly reached its equilibrium at 72 hours p.i., the concentration of kynurenine in 1-MT treated cultures continuously rose beyond 120 hour p.i.. This might reflect a higher rate of kynurenine formation exceeding the capacity of downstream enzymes to catabolize the molecule into downstream metabolite even in the presence of the IDO1-specific inhibitor. However, it is unlikely that the other two tryptophan-degrading enzymes, IDO-2 and tryptophan 2,3-dioxygenase (TDO), were responsible for such

phenomenon because the former appears to be expressed as an inactive form in human dendritic cells (156) and tumor cells (157), while the latter is exclusively expressed by hepatocytes (158). Other possible explanations might include an incidental blockage of a downstream kynurenine-degrading enzyme by 1-MT treatment, which results in accumulation of kynurenine. However, further investigation is needed to determine exact underlying mechanisms.

Apart from anti-microbial activity, IDO1 also appears to be involved in suppression of immune response as well as development of immunological tolerance. This conception stems from a key finding that demonstrated a role of IDO1-mediated tryptophan degradation in prevention of allogeneic fetal rejection (128). It was later revealed that the enzyme exerts anti-proliferative effects on T cells, NK cells and tumor cells via degradation of tryptophan as well as production of its downstream metabolites, resembling its impact on microorganisms (129-132). Then, the issue has been actively concentrated in a variety of research areas, including infection, transplantation, autoimmunity, and cancer, with the hope to develop effective therapeutic strategies for these conditions. In certain DC subsets, IDO1 expression also appears to induce tolerogenic response to antigenic stimuli through other varieties of mechanisms, including induction of T cell anergy (133), apoptosis (134) and differentiation of regulatory T cell (135). Based on our data derived from genome-wide expression study, such anti-proliferation of immune cells seemed unlikely to happen in vivo since we still observed marked leukocytosis as well as up-regulation of genes in cell division process and leukocyte activation among patients with scrub typhus. Nevertheless, further investigation is warranted to see whether IDO1 activation upon the infection also leads to tolerogenic responses or other modulation of the immune system.

Recently, an association between an increased IDO1 function and development of severe complications, like septic shock and multiple organ failure was reported in patients with major trauma (159), sepsis (140), and bacteremia (160). It was also shown that the level of enzymatic activity in peripheral blood could predict severity and fatality of the patients (140, 159-160). According to the regulatory roles of IDO1 in the immune system mentioned earlier, hyperactivity of the enzyme is suspected to be responsible for immune dysregulation and gives rise to such serious

complications (159, 161). Development of similar life-threatening conditions is quite common in severe cases of scrub typhus yet remains unpredictable. However, it was recently reported that high loads of OT's DNA in peripheral blood of the infected patients are associated with increased severity of the disease (44). Taken these data together, it is compelling to investigate the relationships between IDO1 activity, OT load and clinical manifestations of the infected patients, as well as to evaluate the potential of IDO1 as a prognostic predictor of scrub typhus.

CHAPTER VI
CONCLUSIONS

We provided here the first genome-wide expression analysis in scrub typhus infected patients and identified sets of genes that could differentiate scrub typhus from other infections with overlapping clinical features. Despite the limited number of samples, this is a proof-of-principle study to demonstrate the diagnostic potential of mRNA expression profiling for scrub typhus. Further evaluation on practicality and discriminative power of the strategy is necessary and requires more subjects with diverse clinical settings. Additionally, we deciphered transcriptional changes during scrub typhus infection surprising that did not only emphasize on host responses previously demonstrated elsewhere but also introduced novel hypotheses for this incompletely characterized disease. Key findings include an up-regulation of genes in cell cycle and cell division, which reflects an active proliferation of mononuclear cells during the illness; an induction of IFN-γ and its related genes, which stresses on the role of type-1 immune response upon the infection; as well as a surprising down-regulation of proinflammatory cytokines and chemokines.

In the second part of our study, IDO1, one of IFN-γ inducible genes in the up-regulated gene list, was further focused given its interesting roles in host defense against various infections. For the first time, IDO1 activation was demonstrated in patients with scrub typhus at both transcriptional and functional levels. We also proved in subsequent experiments that IDO1-mediated tryptophan deprivation was involved in control of OT growth. However, further studies are deserved to investigate other emerging immune-related effects of IDO1 activation on the outcome of OT infection in a more complex experimental model as well as in patients with scrub typhus.

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1. Tantibhedhyangkul W, **Prachason T**, Waywa D, El Filali A, Ghigo E, et al. (2011) Orientia tsutsugamushi stimulates an original gene expression program in monocytes: relationship with gene expression in patients with scrub typhus. PLoS Negl Trop Dis 5: e1028.