Transcriptional Responses of *Leptospira interrogans* to Host Innate Immunity: Significant Changes in Metabolism, Oxygen Tolerance, and Outer Membrane

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**Abstract**

**Background:** *Leptospira interrogans* is the major causative agent of leptospirosis. Phagocytosis plays important roles in the innate immune responses to *L. interrogans* infection, and *L. interrogans* can evade the killing of phagocytes. However, little is known about the adaptation of *L. interrogans* during this process.

**Methodology/Principal Findings:** To better understand the interaction of pathogenic *Leptospira* and innate immunity, we employed microarray and comparative genomics analyzing the responses of *L. interrogans* to macrophage-derived cells. During this process, *L. interrogans* altered expressions of many genes involved in carbohydrate and lipid metabolism, energy production, signal transduction, transcription and translation, oxygen tolerance, and outer membrane proteins. Among them, the catalase gene expression was significantly up-regulated, suggesting it may contribute to resisting the oxidative pressure of the macrophages. The expressions of several major outer membrane protein (OMP) genes (e.g., ompL1, lipL32, lipL41, lipL48 and ompL47) were dramatically down-regulated (10–50 folds), consistent with previous observations that the major OMPs are differentially regulated in *vivo*. The persistent down-regulations of these major OMPs were validated by immunoblotting. Furthermore, to gain initial insight into the gene regulation mechanisms in *L. interrogans*, we re-defined the transcription factors (TFs) in the genome and identified the major OmpR TF gene (LB333) that is concurrently regulated with the major OMP genes, suggesting a potential role of LB333 in OMPs regulation.

**Conclusions/Significance:** This is the first report on global responses of pathogenic *Leptospira* to innate immunity, which revealed that the down-regulation of the major OMPs may be an immune evasion strategy of *L. interrogans*, and a putative TF may be involved in governing these down-regulations. Alterations of the leptospiral OMPs up interaction with host antigen-presenting cells (APCs) provide critical information for selection of vaccine candidates. In addition, genome-wide annotation and comparative analysis of TFs set a foundation for further studying regulatory networks in *Leptospira* spp.

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**Introduction**

Leptospirosis, which is characterized by hemorrhage, diarrhea, jaundice, severe renal impairment, and aseptic meningitis, etc., has emerged as a global zoonotic infectious disease in the past decade [1]. Several pathogenic *Leptospira* species cause infection, which include more than 15 genospecies and 230 serovars distributed geographically. Other free-living saprophytic *Leptospira* species, such as *Leptospira biflexa*, do not infect humans and animals. The pathogenic, saprophytic *Leptospira* and several other intermediate species all belong to the *Spirochaetes*, a unique phylum in eubacteria including other pathogens, such as *Borrelia burgdorferi* and *Treponema pallidum*. *Leptospira interrogans* is the most prevalent pathogenic *Leptospira* species which survives in natural environments and animal reservoir hosts, and infects humans through abrasions in the skin or mucous membrane. The main reservoir hosts of *L. interrogans* are wild rodents and domestic animals, which can persistently excrete *L. interrogans* through urine. The shed leptospiral cells can survive in moist soil and water for a long time before infecting a new host [2]. Therefore, *L. interrogans* adapts...
to diverse natural environments and evades host immune defense during infection to maintain transmission. This makes L. interrogans an important pathogen in understanding leptospirosis.

The genome sequences of L. interrogans (strain Lai 56601 and Fiocruz L1.1-130), pathogenic Leptospira borgpetersenii (strain L550 and JB197), and saprophytic L. biflexa (strain Patoc I Paris/Ames) have been released in the past few years [3,4,5,6]. The genome size of L. interrogans (~4.6M) is larger than those of L. borgpetersenii (~3.9M) and L. biflexa (~3.9M), which is consistent with the evidence that L. interrogans retained more genes from the common ancestor while acquiring exogenous genes during evolution. Comparative genomics have been performed to identify potential virulence genes in L. interrogans [7]. However, few virulence factors have been experimentally confirmed due to the lack of efficient methods for genetic manipulation of pathogenic Leptospira [8]. In addition, many of the putative functional genes are in multicyclics or families with high degree of redundancy, which further hampers virulence determinants using genetic approaches and molecular Koch’s postulate. For example, two major outer membrane protein genes, ligB [9] and lipL32 [10], which are highly conserved in pathogenic Leptospira and absent in non-pathogenic L. biflexa, have been inactivated in L. interrogans and verified to be dispensable for infection.

In comparison to the other pathogenic spirochetes, L. interrogans encodes more putative signal transduction and transcriptional regulation genes [11]. Several global gene expression studies have elucidated the transcriptional responses of L. interrogans to temperature, osmolality, and host serum [12,13,14,15]. Among these factors, osmotic stress was identified as a key signal affecting the leptospiral transcriptome. However, these microarray analyses identified few genes whose expression has been shown to be differentially regulated during mammalian infection by proteomics and other approaches [16,17,18]. In particular, several major OMPs genes (e.g., lipL32, qlp42 and boa22) are differentially regulated in vivo. This is likely due to the environmental factors in vivo are not the major signals Leptospira senses during mammalian infection. Therefore, global analysis of leptospiral gene expression in animal or infection models are vital to identify differentially regulated genes relevant to pathogenesis.

Co-cultivation of pathogenic Leptospira with host immune cells is widely used as an infection model to study leptospirosis [19,20]. Although pathogenic Leptospira is not considered a typical intracellular pathogen, recent studies showed that pathogenic Leptospira can attach, invade, and induce apoptosis of mammalian macrophages, and escape host innate immunity during the early stage of infection [21,22]. In addition, our study demonstrated differential survivability of L. interrogans within murine or human macrophages, which may contribute to the different severity between the mild chronic infection in reservoir animals and the acute lethal infection in humans [23]. Rapid uptake of L. interrogans by phagocytes were also verified by the naive zebrafish embryos model, suggesting that phagocytosis may be a key defense mechanism during the early stage of infection [24]. In this study, we performed microarray analysis on leptospiral gene expression in response to innate immune cells of murine and human origin. We found a dramatic influence of L. interrogans gene expression by host macrophage interaction, including genes of the major OMPs. A bioinformatic approach was used to determine regulators responsible for differential gene expression. This approach identified a putative OmpR transcription factor, which may be involved in the regulation of major OMP genes.

### Materials and Methods

#### Bacterial strain

L. interrogans Serovar Lai Strain Lai 56601 was obtained from the National Institute for the Control of Pharmaceutical and Biological Products, Beijing, China. For microarray hybridization purpose, a single colony was picked from the EMJH [25,26] plate (1% agar) and verified by 16S rDNA-specific and gyrB [DNA gysrase subunit B1 gene]-specific primer PCR and gene sequencing. The virulence of the L. interrogans isolate was restored by passage through Dunkin-Hartley ICO: DH (Poc) guinea pigs (10–12days old, weighing 120–150g each) before infection. As an in vitro control design, the isolate was cultured in liquid EMJH medium for 5 passages and named E0 sample after the EMJH medium. The culture condition of each passage was growth in 200ml liquid EMJH media at 28°C under aerobic conditions for 120 h to reach exponential growth phase. Three biological replicates (E0-1/2/3) were used for microarray purpose. Before sample collection, one volume of bacterial culture was mixed with a one-tenth volume of ice-cold phenol/EtOH stop solution [10% water-saturated phenol (pH<7.0) in ethanol] and chilled rapidly [27]. Leptospiral cells were harvested by centrifugation at 8,000 g, 4°C for 15 min. All animals were handled in strict accordance with good animal practice as defined by the relevant national and/or local animal welfare bodies, and all animal work was approved by the Animal Ethics Review Committee of Zhejiang University.

#### Host cell lines

Murine monoocyte-macrophage-like cell line J774A.1 and human acute monocytic leukemia cell line THP-1 were obtained from American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% (V/V) heat-inactivated fetal calf serum (FCS, Gibco/Invitrogen, Carlsbad, CA) with antibiotic, in a humidified 5% CO₂ atmosphere at 37°C. The suspended THP-1 cells were treated with 5 mM phosphor myristate acetate (PMA; Sigma-Aldrich St. Louis, MO) for 24h. After differentiation, the cells were washed three times with sterilized PBS buffer, and rested for 24h in new cell medium to ensure that they reverted to a resting phenotype before infection. All cells were cultured in
Infection models

The cultured mammalian cells were washed three times with sterilized PBS buffer to remove antibiotic, fresh media without antibiotics were added, and cultured for an additional 12 h before infection. Leptospiral cells were harvested by centrifugation at 8,000 g, 20°C for 15 min, and washed three times with sterilized PBS buffer. The leptospiral pellets were re-suspended in 37°C RPMI 1640 medium with 10% (V/V) heat-inactivated FCS and the bacterial numbers were counted with a Petroff-Hauser counting chamber (Fisher Scientifics, Houston, Texas). Then 10 ml of leptospiral suspension (10^6) were added into 10^7 macrophage cells (bacteria:cell = 100:1) and incubated in 5% CO_2 at 37°C for 12 h beforehand. Three biological replicates were designed for each sample for microarray purpose. The surviving macrophage cells were washed and dried according to the Roche NimbleGen standard procedure.

To guarantee the integrity of the total RNA, the survival of the total RNA was verified by darkfield microscope analysis (400x). Then the co-cultured L. interrogans samples were defined as J (J774A.1) and T (THP-1) samples respectively after the names of the mammalian cell lines. In order to evaluate the impact of mammalian cell culture medium on L. interrogans, RPMI 1640 medium controls (RPMI 1640 medium with 10% (V/V) heat-inactivated FCS) were introduced into experiments as M (RPMI medium controls). Then 10 ml of leptospiral suspension (10^6) were added into 10^7 macrophage cells (bacteria:cell = 100:1) and incubated in 5% CO_2 at 37°C. These co-cultured L. interrogans samples were defined as J (J774A.1) and T (THP-1) samples respectively after the names of the mammalian cell lines. In order to evaluate the impact of mammalian cell culture medium on L. interrogans, RPMI 1640 medium controls (RPMI 1640 medium with 10% (V/V) heat-inactivated FCS) were introduced into experiments as M (RPMI 1640) samples.

RNA purification and ds cDNA synthesis

Leptospiral total RNA was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA), then purified with RNeasy Mini Kit (QIAGEN, Hilden, Germany) with on-column DNase digestion (QIAGEN, Hilden, Germany) according to the RNeasy Mini handbook. RNA quantity and integrity was determined using the RNA 6000 Nano Laboratory-on-a-Chip kit and the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). For each sample, about 10 μg of total RNA was mixed with 600 ng of random hexamer primers (TaKaRa, Otsu, Japan) and denatured at 65°C for 5 min. Then the first strand cDNA was synthesized using 2 μl (400 U) SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the protocol recommended by the manufacturer. The double strand cDNA (ds cDNA) sample was synthesized using the 2nd Strand Synthesis section of the M-MLV RTase cDNA Synthesis Kit (TaKaRa, Otsu, Japan) according to the manufacturer’s instructions. Following RNase H (Invitrogen, Carlsbad, CA) and RNase A (Ambion, Austin, TX) digestion for 1 h, ds cDNA sample was purified with the QIAquick PCR Purification Kit (QIAGEN, Hilden, Germany) according to the QIAquick Spin handbook.

Microarrays and hybridization

The L. interrogans Serovar Lai Strain Lai 56601-specific high-density, photolithography-based, mono-plex DNA microarray chip was designed and produced by Roche NimbleGen, Inc. Each slide consisted of a total of 385,000 oligonucleotide probes (60-mer each probe) which covered all predicted 4,727 ORFs of the whole genome (NC_004342 and NC_004343). In average, sixteen probes were designed for each ORF, which is one of the strength of this technology. Each probe pair consisted of a sequences matched to the ORF, and another adjacent sequence harbored mismatched bases for the determination of background and cross-hybridization. Note that the original annotation for L. interrogans Serovar Lai Strain Lai 56601 included more than 900 putative small ORFs (less than 150 bp). In contrast, the homologs of these small ORFs were not included in the later genome annotation for other 5 Leptospira strains. However, we found that some of these putative ORFs had very high level of expression (Data not shown). Thus, these small ORFs were included in our microarray analysis. For each hybridization, 1 μg of ds cDNA was labeled with Cy3-9mer Primers (TriLink Biotechnologies, San Diego, CA) using the Klenow fragment (New England Biolabs, Beverly, MA) exo-extending reaction. 1.5 μg of Labeled cDNA sample was individually hybridized to the microarray using the MAUI hybridization system from Roche NimbleGen, then washed and dried according to the Roche NimbleGen standard procedure.

Figure 1. Schematic representation of the macrophage infection models (A) and search tactics of specific transcription factors from the leptospiral genomes (B).

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Data extraction and statistical analysis

The microarrays were scanned using the Axon GenePix 4000B microarray scanner at 5-μm resolution. The data were extracted using Roche NimbleGen NimbleScan™ software and an algorithm (courtesy of Y. Qiu, University of Wisconsin School of Medicine) was applied to obtain a single measurement of signal intensity for each ORF. Data were normalized using the quantile normalization method and the total signal intensity of a given ORF was converted to estimates of transcript abundance by using the robust multiarray average (RMA) procedure. For all microarrays, a P value for each ORF was calculated by a two-tailed Welch's unpaired t test comparison of the five microarray replicates for each sampling condition. The changes of an ORF between two relevant sampling conditions were calculated as the division value of average signal intensity of 3 biological replicates: average J, T or M sample signal intensity at a certain time-point/average E sample signal intensity. Only fold changes of at least ±2 and P≤0.05 were considered significant and included in this report.

Validation of microarray data by quantitative real-time RT-PCR

Primers for randomly selected L. interrogans Serovar Lai Strain Lai 56601 genes (Table S1) were designed with Primer Premier software version 5 (Premier Biosoft International, Palo Alto, CA). New batches (in triplicate) of bacterial RNA were used in real-time RT-PCR. RT reaction mixtures contained 1 μg of total RNA, 300 ng of random hexamer primers (TaKaRa, Otsu, Japan), 0.5 μl (100 U) of Superscript III (Invitrogen, Carlsbad, CA) reverse transcriptase, and 500 μM concentrations each of dATP, dCTP, dGTP, and dTTP. After denaturation on 65°C for 5 min, the samples were incubated at 50°C for 1 h, followed by 10 min at 70°C to synthesize the first strand cDNA. 50 ng of cDNA were mixed with 12.5 μl of 2×SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). Assays were performed in triplicate with the ABI PRISM model 7500 sequence detection instrument. Amplicon quantification in real-time RT-PCR was performed by comparison with gene-specific standard curves constructed from known concentrations of each purified amplicon. The melting curve analysis was also used to evaluate that the construction of SYBR Green-bound DNA was gene-specific.

Extraction of total proteins and outer membrane proteins

The L. interrogans pellets were harvested from infection models at 1h, 2h, and 4h according to the method described previously in this report. Total lepotspiral protein was extracted with Triton X-100. For each sample, the leptospiral pellet was washed twice in 0.1 M PBS-5 mM MgCl2 and resuspended in 0.5 ml bacterial lysis buffer which was composed of 50 mM Tris-HCl (pH8.5), 2 mM EDTA, 100 mM NaCl, 0.5% Triton X-100 (Calbiochem, La Jolla, CA), 100 μg/ml lysozyme (Sigma-Aldrich St. Louis, MO) and 1 μl/ml protease inhibitor PMSF (Sigma-Aldrich St. Louis, MO). The suspension sample was subjected to three cycles of freezing, thawing and tip sonication, following by centrifugation at 10,000g for 10 min to exclude the indissoluble fragments. The soluble supernatant was dialyzed by 1% SDS and tested by SDS-PAGE electrophoresis. The leptospiral OMP samples were extracted by solubilization with 1% Triton X-114 (Calbiochem, La Jolla, CA) according to the method reported previously [29].

Recombinant proteins expression and immunization procedure

The full-length PCR products of the genes of LipL32 (LA2637), LipL41 (LA0616), OmpA (LB328), OmpL1 (LA3138), Mce (LA2055), FliH (LA2589), FliI (LA2592), FliY (LA2613), FliN (LA2069) were amplified from L. interrogans Serovar Lai Strain 56601 chromosomal DNA using gene-specific primers, inserted into pGEM-T easy vectors (Promega, Madison, WI) and verified by gene-sequencing. Then the target genes were double-digested and inserted into pET-12a (+) vectors (Merck Novagen, Nottingham, UK), verified by sequencing and expressed in E. coli Rosetta™ strain (Merck Novagen, Nottingham, UK) as N-terminal 6×His-tagged recombinant proteins. Recombinant proteins were purified by Ni-NTA agarose column (QiAGEN, Hilden, Germany), mixed with complete Freund's adjuvant (Sigma-Aldrich St. Louis, MO), and used to immunize SPF New Zealand rabbits at days 1, 15, 30, 45. On days 55, the blood samples were taken from the rabbits' hearts and the effect of antiserum was calculated by ELISA tests. The antiserum were diluted into appropriate solutions (1:800 for LipL32/41, OmpA, OmpL1, FliY and FliN, 1:400 for Mce, FliH, and FliI), and used in the following Western blotting analysis.

Verification of protein changes by Western blotting

Protein concentration was estimated by BCA protein assay (Pierce, Rockford, IL). For leptospiral lipoproteins (LipL32, LipL41) and outer membrane protein A (OmpA), the OMP samples were used in Western blotting analysis. For transmembrane protein (OmpL1), flagellar components (FliH, FliI, FliY and FliN) and intracellular protein (Mce), the total protein samples were used. Equivalent amounts of protein (1 μg) were separated by 1-D electrophoresis with 10% SDS-PAGE, and electrotransferred onto polyvinylidene difluoride membranes (PVDF membranes, Millipore, Billerica, MA) using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The membranes were blocked in TBST [20 mM Tris (pH 7.6), 157 mM NaCl, 0.1% Tween 20] containing 5% non-fat milk and probed with corresponding antibodies (1:2000) respectively overnight at 4°C. Then, the membranes were incubated with peroxidase-conjugated anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories Inc., PA) for 2 h at room temperature and visualized on X-ray film using enhanced chemiluminescence reagents (Millipore, Billerica, MA). The band intensities were estimated by densitometric scanning using the Gel Doc 2000 system (Bio-Rad, Redmond, WA) and Quantity One software. Data shown are from three independent experiments.

Genome annotation, cluster and pathway analysis of microarray data

To supply new functional annotation of the L. interrogans Serovar Lai Strain Lai 56601 genome, assignment of putative functions was performed by means of a combination of BLAST-based and HMM-based searches of the KEGG gene database and InterPro protein domain database. Hierarchical cluster analysis of microarray data was performed by the Cluster 3.0 software and visualized by the TreeView software [30]. Several similarity matrices, including correlation matrices [Correlation (uncentered), Absolute Correlation (uncentered), Spearman Rank Correlation, Kendall’s tau] and distance matrices (Euclidean distance and City-block distance) were used in cluster analysis to define the significantly changeable subclades. The KEGG pathway was introduced to analyze the changing magnitude in each biological pathway by gathering statistics from the significant gene expression changes. The proportion of regulated genes in the 14 leptospiral KEGG pathway was calculated and displayed by Microsoft Excel diagram.
Discovery and evolutionary analysis of transcription factors

Candidate TFs were collected by InterProScan program (http://www.ebi.ac.uk/Tools/InterProScan/) and the Gene Ontology terms were obtained from the InterProScan results using InterPro2GO (http://www.geneontology.org/external2go/interpro2go) [31]. Then, the DNA binding domains (DBDs) of the candidate TFs were used in further BLAST search against all *Leptospira* proteins to identify new TFs. All putative TFs were classified into different families according to categories of their DBDs. This strategy helped us to find new TFs that had not been defined before (Figure 1B). Finally, phylogenetic analyses of the whole TF sequences were performed using Neighbor-Joining method of MEGA 4.0 software to reveal the evolutionary relationship of the putative TFs [32].

Results and Discussion

Annotion of previous defined hypothetical ORFs

In order to conduct genome-wide transcriptional analyses of *L. interrogans*, we first performed an updated annotation using BLAST and InterProScan tools with up-to-date databases. Specifically, we focused on annotating the hypothetical ORFs, which comprises about 40% of total ORFs of the genome of *L. interrogans* Serovar Lai Strain Lai 56601 [3]. This annotation allowed us to assign putative functions to 375 hypothetical proteins (Table S2), and identified several functionally important homologs missing in the previously annotated genome. These included a ferrous iron transporter, outer membrane lipoprotein carrier protein LolA, phospholipase C, the SecE subunit for protein translocation complex, a cell wall hydrolase, and the flagellar hook-length control protein chromogranin. In addition, several ORFs involved in host-pathogen interaction were identified, including a haem oxygenase-like protein, type-III fibronectin, hemopexin, a prevent-lust-death protein, caspase catalytic proteins, a nitriase/cyanide hydratase, ricin B lectin, and cadherin-like protein.

Microarray experimental design

To study global transcriptional responses of *L. interrogans* upon interaction with the host innate immune system, we chose two different mammalian cell lines: murine monocyte-macrophage-like cell line J774A.1 and human acute monocytic leukemia cell line THP-1. This represented interaction with macrophage of either natural mammalian reservoir or human host. Briefly, a clone of *L. interrogans* Serovar Lai Strain Lai 56601 was used to infect a guinea pig and subsequently recovered from kidney tissue. Isolated infectious spirochetes were split to two samples. One sample was kept under *in vitro* cultivation and passage in EMJH medium for 5 passages (approximately one month) then subjected to RNA extraction and defined as *in vitro* cultivation sample (sample E0). The other sample was divided into three groups and co-cultured with cell lines J774A.1, THP-1, or RPMI 1640 medium with 10% heat-inactivated FCS designated as samples J, T, and M, respectively. After 45 or 90 minutes, spirochetes were harvested, subjected to RNA extraction, and cDNA synthesis (Figure 1A). Each cDNA sample was labeled and used for a single hybridization. Average signal intensity of 3 biological replicates was calculated as a valid data for each sample (E0, J45, J90, T45, T90, M45 or M90). The transcriptional fold-changes of ORFs in J, T or M samples were calculated relative to sample E0. Analysis of differentially expressed genes were confined to those with changes ≥2-fold (P<0.05). The microarray raw data are available at http://cibex.nig.ac.jp/ under CIBEX accession no. CBX129.

Global transcriptomic analysis

Based on the correlation and distance similarity matrices of Cluster 3.0 software, *L. interrogans* genes differentially expressed upon interaction with macrophages were grouped into several clades (Figure 2A). Clades of up-regulation and down-regulation of gene expression occurred mainly in comparison between the *in vitro* cultivated spirochetes and bacteria that interacted with either mouse-derived macrophage or human macrophage cell lines, not in comparison between the *in vitro* cultivated spirochetes and bacteria in RPMI 1640 medium controls. In other words, incubation of spirochetes with the cell culture medium alone had little influence in gene expression (M45/E0 and M90/E0). In addition, the cluster analysis showed that T45/E0, T90/E0 and J45/E0 were closely related, whereas J90/E0 was similar to M45/E0 and M90/E0, both in correlation matrices and in distance matrices. This suggested a more transient change of gene expression profile in *L. interrogans* upon interaction with murine macrophages (J90/E0) than with human-derived macrophage cells (T90/E0). About 65% of down-regulated genes and 45% up-regulated genes were hypothetical protein genes, which was consistent with previous transcriptomic results that these genes are often regulated in different environments [12,14].

Cluster analysis revealed a distinct clade, Clade 1, containing a set of highly down-regulated genes (30 to 50-fold) (Figure 2B). Intriguingly, some of the well-studied major OMP and lipoprotein genes fell into Clade 1, including LA0505 (ompL47) [33], LA0616 (lipL41) [34], LA3240 (lipL48) [35], LA3138 (ompL1) [36] and LA2637 (lipL32) [17,18,29,38,39]. The down-regulation of Clade 1 was mainly due to the interaction with host macrophage, which was different from the moderately down-regulated Clade 2. The Clade 2 differed from Clade 1 because its moderate down-regulation was also observed in the RPMI 1640 medium controls. Another distinct clade, Clade 3, included a set of highly up-regulated genes (3 to 7-fold) which may contributed to oxygen tolerance, such as the fatty acid desaturase gene (LA0502), the 2-Cys thioredoxin peroxidase gene (LA2809) and the catalase gene (LA1859). These genes were regulated not only upon interaction with macrophages, but also in RPMI 1640 medium controls. Interestingly, the Clade 4 included several chaperone and heat shock protein genes, such as GrpE gene (LA3704), HSP20 genes (LA1563 and LA1564), 60 kDa chaperonin gene (LA2655), 10 kDa chaperonin gene (LA2654), and chaperone protein DnaK gene (LA3705), etc. These genes were persistently up-regulated in RPMI 1640 medium controls (M45/E0, M90/E0) and maybe due to the elevated temperature [12], but transiently down-regulated upon interaction with host macrophage cells at the 45 min time-point (T45/E0, J45/E0). At the 90 min time-point, *L. interrogans* moderately up-regulated this clade upon interaction with murine J774A.1 cell lines (J90/E0), but remained down-regulated upon interaction with the human THP-1 cell lines (T90/E0). The implication and mechanism of this differential regulation of Clade 4 upon interaction with murine vs. human cell lines are still unclear. In addition, two moderately down-regulated clades, Clade 5 and Clade 6, were defined for discussions in the corresponding section below.

Validation of the microarray data by qRT-PCR was shown in Figure 3. To evaluate the alterations in leptospiral biological pathways, proportions of up-regulated and down-regulated genes in KEGG pathways were calculated (Figure 4). Transcription and translation systems, carbohydrate, energy and lipid metabolism, and signal transduction systems exhibited significant down-regulation patterns, while biosynthesis of secondary metabolites, membrane transport and metabolism of cofactors and vitamins showed up-regulation patterns in our infection models.

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addition, several important KEGG sub-pathways were significantly altered. Most genes of the citric acid cycle (TCA cycle), flagellar assembly, oxidative phosphorylation, fatty acid metabolism and ribosome synthesis were down-regulated, while most genes of starch and sucrose metabolism, porphyrin metabolism, and two-component systems were up-regulated (Data not shown). Categories exhibiting significant regulation are discussed below in detail.

Energy, carbohydrate, and lipid metabolism

Most of the genes involved in this category were down-regulated upon interaction with macrophages. First, genes involved in oxidative phosphorylation were down-regulated. These changes included a significant down-regulation (3-6 folds) of a putative operon (LA0242-0244, included in down-regulated Clade 5) encoding cytochrome caa3 oxidase subunit II, cytochrome C oxidase polypeptide I and cytochrome C oxidase polypeptide III (Table S2). Previous reports showed that the leptospiral cytochrome C oxidase polypeptide III is replaced by an alternative subunit that has weak oxygen affinity, which contributes to the low rate of oxidative phosphorylation in *L. interrogans* [4]. The reduced expression of this operon observed suggested that *L. interrogans* reduced its O2 requirement upon interaction with host cells. Furthermore, most genes of the citric acid cycle (TCA cycle) as well as a putative operon encoding the leptospiral F0F1 ATP synthetase subunits (LA2780-LA2782) [4], were significantly down-regulated upon interaction with macrophages.

*Leptospira* can not utilize glucose and other sugars as carbon source. Instead, it degrades long chain fatty acid through beta-oxidation for carbon and energy production. In fact, tween-80, not glucose, are essential ingredients of the semi-synthetic EMJH medium for *in vitro* growth of *Leptospira*. Consistent with this, recent comparative genomics analyses on six leptospiral genomes revealed that *Leptospira* has limited sugar transport system [3,7]. As such, *L. interrogans* encodes more genes of the fatty acid metabolism genes than *Escherichia coli* K12. There are three homologs of long-chain-fatty-acid CoA ligase (LA0106, LA2177, and LA2309), which are the rate-limiting enzymes for fatty acid degradation [3]. Our microarray data revealed that expression of LA0106 and LA2309 were down-regulated (2-8 folds) during interaction with host cells. In addition, one of glycerol utilizing genes, LA0587, encoding lactonizing lipase, was dramatically down-regulated (4-9 folds). These results are consistent with down-regulation of its oxidative metabolism pathways described above.

In addition to the general trend of gene down-regulation, few genes in this category were up-regulated upon interaction with macrophages. Genes involved in nitrogen metabolism [2-nitropropane dioxygenase (LA2772), glutamate synthase (LB286)], were up-regulated. One of the genes in methane metabolism, the catalase gene (LA1859), was also significantly up-regulated 4-7 fold persistently. Since catalase is also involved in tryptophan metabolism and oxidative resistance, it remains to be determined the exact role the catalase plays during the initial stage of leptospiral infection. In addition, three genes in starch and sucrose metabolism [CDP-glucose 4,6-dehydratase (LA1632), alpha-glucoisidase II (LA294), glucose-6-phosphate isomerase (LA3888)] and three genes in glycolysis/gluconeogenesis [phosphoglyceromutase (LA0439), dihydrodipoidate dehydrogenase (LA2115), a

Figure 2. Genome-wide transcriptional changes of the *L. interrogans* Serovar Lai Strain Lai 56601 in the infection models. Cluster analysis (Euclidean distance) revealed several distinct subclades in the whole transcriptomics (A). The subgroup of most highly down-regulated genes was defined as Clade 1, which included several major outer membrane protein genes, such as ompL47 (LA0505), lipL41 (LA0616), lipL48 (LA3240), ompL1 (LA3138), and lipL32 (LA2637), etc. The most significantly up-regulated genes were included in Clade 3. The Clades 2, 4, 5 and 6 included the moderately down-regulated genes (B). doi:10.1371/journal.pntd.0000857.g002
probable alcohol dehydrogenase (LA2361), were up-regulated. *L. interrogans* Strain Lai 56601 possesses the genes involved in the biosynthesis of unsaturated fatty acids pathway (KEGG pathway: lil01040), which suggests that this strain may synthesize some unsaturated fatty acids de novo. In contrast, *B. burgdorferi* has no unsaturated fatty acids biosynthesis genes, and instead scavenges polyunsaturated fatty acids from BSK II growth medium or hosts [40]. The putative rate-limiting enzyme in this pathway, omega-6 fatty acid desaturase gene (delta-12 desaturase gene, desl, LA0502), was dramatically up-regulated in RPMI 1640 medium controls (>10-fold). The relatively modest and late up-regulation of this gene upon interaction with macrophages may reflect the culturing conditions and micro-environments, such as elevated temperature and osmolarity [12,15]. Considering that the RPMI 1640 medium controls and host cells were all eutrophic in unsaturated fatty acids, the implication and mechanism of the up-regulations of *desL* were still unclear.

**Oxygen tolerance and DNA repair**

*L. interrogans* must evade oxidative killing mediated by host cells including macrophages. However, the *L. interrogans* genome has only few predicted genes involved in resistance to oxidative stress and reactive oxygen species (ROS). All four pathogenic leptospiral genomes lack homologues of *fog*, *nfo*, *nei* or superoxide dismutase (*sod*) [3,5]. *L. interrogans* Strain Lai 56601 has glutathione peroxidase genes (LA1007, LA4299) and thiol peroxidase gene (LA0862), but their level of expression were very low and did not have significant change upon interaction with macrophages. Pathogenic *Leptospira* also have cytochrome C oxidase genes, which may be involved in protection from O_2 stress. Our microarray results showed a significant down-regulation of these genes (LA0242-0244), suggesting they may not be important for resistance to oxidative killing in our models.

Catalase is one of the proteins that plays an important role in resisting oxidative killing by phagocytes [41]. Both pathogenic and non-pathogenic *L. biflexa* have catalase genes in their genomes, but they are not homologs and belong to different enzyme groups: *L. interrogans* has a heme-containing *katE* homolog, whereas *L. biflexa* has a heme-containing dual functioning peroxidase/catalase *katG* homolog [42]. Our microarray result showed that expression of *katE* was very high and further up-regulated during interaction with host cells. However, this up-regulation appeared not to be the result of direct interaction with macrophages, but rather due to other host factors such as elevated temperature and mammalian serum, since *katE* gene expression was also increased in the M samples. This was consistent with previous reports that these factors can influence catalase expression in *Leptospira* [12,15]. Interestingly, it was reported that non-pathogenic *Leptospira* is more susceptible to H_2O_2 killing in vitro [43], which suggests *katE* may play an important role in *Leptospira* infection.

In addition, the high expression and up-regulation of 2-Cys thioredoxin peroxidase gene (LA2809) indicated this gene may contribute to resisting oxidative stress. It was significantly up-
regulated in RPMI 1640 medium (4–8 folds) and moderately up-regulated upon interaction with macrophages (2–3 folds). Cluster analysis revealed that this gene, the fatty acid desaturase gene and the catalase gene were assembled into a same clade, Clade 3 (Figure 2B). However, considering that it was not regulated by elevated temperature and host serum in previous studies [12,15], the mechanism of its up-regulation may be different from those of the fatty acid desaturase and the catalase.

All six Leptospira genomes contain integrated DNA repair systems, such as the base-excision repair, the photo reactivation, and the SOS repair, etc [7]. Unexpectedly, no significant up-regulation of these genes in *L. interrogans* was found upon interaction with macrophages. The only major change was the down-regulation of the major recombinase A gene (rocA, LA2179) in the homologous recombination pathway. The implication of this change remains unclear and suggested that *L. interrogans* experienced limited DNA damage under our conditions.

**Signal transduction, chemotaxis and motility**

A distinct feature of *L. interrogans* relative to other spirochetal pathogens such as *B. burgdorferi* and *T. pallidum*, is that it has more two-component signal transduction systems (with more than 26 pairs of histidine kinases and response regulators). Regulation of two-component systems often occurs at the level of phosphorylation, not at the level of transcription. As expected, few obvious changes were observed in the microarray analysis for the histidine kinase and response regulator genes except that a two-component response regulator gene (LA2548) and a neighboring sensory kinase and response regulator genes (LA2549) were moderately up-regulated at the 45 min time-point. The only change in the GGDEF sensory system was that the GGDEF sensory box/GGDEF family protein (LA2931) was moderately up-regulated upon interaction with macrophages (2–3 folds). Cluster analysis revealed that this gene, the fatty acid desaturase gene and the phosphate-binding signal transduction related genes were differentially expressed. The regulation temporarily at the 45 min time-point. In addition, other transduction histidine kinase gene (LA2549) were moderately up-regulation of the major recombinase A gene (rocA, LA2179) in the homologous recombination pathway. The implication of this change remains unclear and suggested that *L. interrogans* experienced limited DNA damage under our conditions.

**LPS and O-antigen synthesis**

Unlike other none-lipopolysaccharide (LPS) spirochetes, *Leptospira* contains an intact and conserved biosynthesis system of LPS. Leptospiral LPS can activate host cells through TLR-2, which is distinct from other bacterial LPS [1,46]. The structure of leptospiral lipid A and oligosaccharide can be modified [47,48], which may attribute to the complicated diversity of more than 200 serovars. About 80% of ORFs involved in the leptospiral LPS synthesis pathway are similar to those of *E. coli*, which may indicate the highly conserved evolution of this genome locus [3]. However, unlike other gram-negative bacteria in which the lipid A biosynthesis genes are in an operon, these genes are scattered across the genome in *L. interrogans*. The O antigen synthesis genes remains clustered in the rfb operon with 103 kb in size [7].

Overall, genes in this category are evolutionarily conserved and showed limited changes. These changes included moderate up-regulation of UDP-N-acetylgalactosamine acyltransferase gene (lpxD2, LA4326), several O-antigen synthesis genes [glucose-1-phosphate thymidylyltransferase (rfbB, LA3802), dTDP-glucose 4,6-dehydratase (rfbB3, LA1606), CDP-glucose 4,6-dehydratase (rfB6, LA1632)], and two of the O-antigen assembly genes [UDP-glucose lipid carrier transferase (rhpP2, LA2509), polysaccharide biosynthesis protein (rfbX, LA1649)], and down-regulation of O-antigen polymerase gene (rfb, LA1648). Note that the rate-limiting enzyme gene, lpxA (LA3949) had no change in our infection models.

**Sphingomyelinases and hemolysins**

Leptospiral sphingomyelinases and hemolysins are hypothesized to degrade the host cell membrane and acquire nutrition [3]. Some were verified to be haemolytic enzymes or pore-forming cytotoxins [49,50]. Pathogenic *L. interrogans* and *L. borgpetersenii* has 5 and 3 sphingomyelinase genes respectively, while non-pathogenic *L. biflexa* has no such genes [6]. Recent data showed that the major secreted sphingomyelinase C precursor gene (sph2, LA1029) is host-inducible during infection [51,52]. Our microarray data showed that the sph2 was expressed at additional high level even in spirochetes cultivated in the EMJH medium. The sph2, along with another sphingomyelinase C precursor gene, sph1 (LA1027), were further up-regulated either in the RPMI 1640 medium controls or upon interaction with macrophage cells. Up-regulation of these genes were likely due to changes in osmolarity, since it was previously shown that that expression of Sph2 increases when *L. interrogans* was grown in a physiologically relevant osmotic concentration [32].

Regarding non-sphingomyelinase hemolysins, one of the putative hemolysis genes, which is present only in pathogenic *Leptospira*, tlyC (LA3937), was up-regulated upon interaction with macrophages. The hemolytic activity of TlyC remains controversial. One earlier study showed that TlyC had hemolytic activity [50]. A recent study from another group reported that TlyC had no hemolytic activity, but it was a surface protein that mediates interaction with host extracellular matrix (ECM) [53]. Neverthe-
Table 1. Category of leptospiral ORFs which were up-regulated at least 3-fold in infection models.

| Clade ID | ORF ID | M45/E0 mean fold | J45/E0 mean fold | T45/E0 mean fold | M90/E0 mean fold | J90/E0 mean fold | T90/E0 mean fold | Function and description of gene product |
|----------|--------|------------------|------------------|------------------|------------------|------------------|------------------|------------------------------------------|
| 3        | LA0268 | 5.3              | 1.56             | 1.11             | 16.71            | 6.18             | 1.95             | hypothetical protein                     |
|          | LA0273 | 0.79             | 2.65             | 3.11             | 1.24             | 1.4              | 2.07             | lipoprotein releasing system transmembrane protein LolC |
|          | LA0330 | 0.38             | 3.21             | 2.51             | 0.58             | 0.98             | 1.46             | penicillin G acylase precursor (Penicillin G amidase, Penicillin G amidohydrolase) |
|          | LA0356 | 0.64             | 3.01             | 1.6              | 0.88             | 1.45             | 2.56             | hypothetical protein                     |
|          | LA0366 | 0.58             | 2.25             | 3                | 1.19             | 1.33             | 2.15             | phosphoserine aminotransferase (catalyzes the formation of 3-phosphonooxypyruvate and glutamate from O-phospho-L-serine and 2-oxoglutarate) |
| 3        | LA0502 | 3.36             | 0.96             | 0.95             | 14.96            | 5.84             | 1.98             | fatty acid desaturase (Delta 12 desaturase) |
|          | LA0625 | 0.8              | 2.03             | 3.21             | 1.27             | 0.74             | 1.61             | DNA helicase RecQ                         |
|          | LA0635 | 0.59             | 2.81             | 3.3              | 1.07             | 0.88             | 2.17             | S-layer-like array protein                |
|          | LA0650 | 0.43             | 2.9              | 3.13             | 0.67             | 1.01             | 2.34             | rhomboid family protein                   |
|          | LA0662 | 0.72             | 2.26             | 3.08             | 0.84             | 0.8              | 1.55             | chemotaxis motA protein                   |
|          | LA0701 | 0.67             | 3.09             | 2.67             | 0.87             | 0.92             | 1.96             | leucine-rich repeat containing protein    |
|          | LA0784 | 0.89             | 1.89             | 3.07             | 1.35             | 1.16             | 1.57             | hypothetical protein                      |
|          | LA0884 | 0.55             | 2.59             | 3.11             | 0.84             | 1.26             | 1.78             | NADH dehydrogenase L, N subunit           |
|          | LA1122 | 0.6              | 3.13             | 3.09             | 0.92             | 1.22             | 1.81             | putative outer membrane protein           |
|          | LA1334 | 0.63             | 2.39             | 3.81             | 0.83             | 1.15             | 1.72             | putative oxidoreductase                   |
|          | LA1854 | 0.59             | 3.03             | 2.5              | 0.79             | 1.04             | 2.86             | hypothetical protein                      |
| 3        | LA1859 | 1.71             | 1.85             | 1.76             | 7.51             | 4.69             | 5.28             | catalase                                  |
|          | LA1933 | 0.8              | 3                | 2.75             | 1.17             | 1.13             | 1.76             | tetracycline resistance protein           |
|          | LA1937 | 0.43             | 2.64             | 3.07             | 0.53             | 1.14             | 3.12             | predicted transcriptional regulator, copG family |
|          | LA1944 | 0.49             | 3.41             | 4.27             | 0.54             | 1.64             | 2.17             | putative lipoprotein                      |
|          | LA1979 | 0.94             | 1.61             | 1.35             | 0.98             | 3.08             | 1.55             | putative glycosyl transferase             |
|          | LA2032 | 0.41             | 2.56             | 2.6              | 0.48             | 1.08             | 3.02             | predicted transcriptional regulator, copG family |
|          | LA2156 | 0.6              | 2.63             | 3.04             | 0.97             | 1.56             | 1.97             | aminotransferase                          |
|          | LA2275 | 0.56             | 3.11             | 3.02             | 1.02             | 1.15             | 1.96             | dedA protein                              |
|          | LA2277 | 0.93             | 3.07             | 2.16             | 1.15             | 1.39             | 2.11             | hypothetical protein                      |
|          | LA2444 | 0.7              | 3.66             | 3.35             | 0.92             | 1.52             | 2.16             | putative outer membrane protein           |
|          | LA2654 | 4.01             | 0.15             | 0.22             | 3.22             | 3.47             | 1.05             | 10 kDa chaperonin                         |
|          | LA2659 | 0.98             | 2.77             | 3.59             | 1.43             | 1.51             | 2.32             | hypothetical protein (Isopentenyl-diphosphate delta-isomerase, FMN-dependent, ATP-grasp fold) |
| 3        | LA2824 | 3.74             | 1.94             | 2.41             | 10.09            | 3.73             | 2.22             | hypothetical protein (DoxX)               |
|          | LA2875 | 0.49             | 3.18             | 3.51             | 0.72             | 1.28             | 2.31             | hypothetical protein                      |
|          | LA2931 | 0.5              | 3.32             | 2.88             | 0.5              | 1.07             | 2.12             | sensory box/GGDEF family protein          |
|          | LA3075 | 0.77             | 3.42             | 2.66             | 1.13             | 1.42             | 2.5              | surface protein Lk90-like protein (LigC)   |
|          | LA3078 | 0.82             | 3.03             | 1.9              | 0.97             | 1.09             | 1.61             | sterol desaturase family protein          |
|          | LA3189 | 0.73             | 4.11             | 2.68             | 1.36             | 1.76             | 2.84             | hypothetical protein (CRISPR-associated protein, Cas6-related) |
|          | LA3197 | 0.61             | 3.21             | 2.71             | 0.47             | 0.89             | 2.35             | Type I restriction enzyme EcoR1248 M protein |
|          | LA3198 | 0.71             | 3.15             | 2.72             | 0.75             | 1.06             | 2.57             | Type I restriction enzyme Ecop1 specificity protein |
|          | LA3199 | 0.74             | 3.21             | 2.81             | 0.77             | 1.07             | 2.45             | anticondon nuclease                        |
|          | LA3216 | 0.65             | 2.51             | 3.06             | 1.08             | 1.59             | 2.15             | octoprenyl-diphosphate synthase            |
| 3        | LA3248 | 0.94             | 2.71             | 2.08             | 3.02             | 8.68             | 2.77             | hypothetical protein                      |
| 3        | LA3283 | 0.93             | 2.69             | 1.86             | 2.91             | 8.6              | 2.74             | hypothetical protein                      |
|          | LA3287 | 0.64             | 3.84             | 2.28             | 0.8              | 1.11             | 2                | hypothetical protein                      |
|          | LA3353 | 0.89             | 1.27             | 1.08             | 0.91             | 4.73             | 1.15             | hypothetical protein                      |
|          | LA3414 | 0.51             | 3.3              | 3.2              | 0.9              | 1.86             | 3.1              | hypothetical protein                      |
|          | LA3574 | 0.65             | 2.47             | 3.08             | 0.74             | 0.99             | 1.79             | flagellar protein FIL                    |
|          | LA3630 | 0.7              | 2.59             | 3.28             | 0.91             | 1.36             | 2.54             | probable transport ATP-binding protein msbA |
Recent study shows that MviN (Muj) of lipid II flippase [54]. In this study, most of these genes are largely unknown, with exception for during infection and facilitates the infection of this study supports the hypothesis that it may be expressed in infection models [J774A.1 cell model (J774A.1)] were estimated by Western blotting band intensities.

Figure 5. Verification of the leptospiral protein changes by Western blotting. The leptospiral samples at 1, 2 and 4 hour in the infection models [J774A.1 cell model (A) and THP-1 cell model (B)] were harvested for semi-quantitative protein assay. The protein expression levels of LipL41 (LA0616), LipL32 (LA2637), Mce (LA2055), OmpA (LB328), OmpP1 (LA3138), FliH (LA2589), FliI (LA2592), FliY (LA2613) and FlIN (LA2069) were estimated by Western blotting band intensities.

Table 1. Cont.

| Clade ID | ORF ID | 
|---------|--------|---------|
|         |        | M45/E0 fold | J45/E0 fold | T45/E0 fold | M90/E0 fold | J90/E0 fold | T90/E0 fold | Function and description of gene product |
|---------|--------|-------------|-------------|-------------|-------------|-------------|-------------|----------------------------------------|
| LA3726  | 0.82   | 3.19        | 2.49        | 0.96        | 1.39        | 2.66        |            | hypothetical protein (Cadherin-like) |
| LA3735  | 0.63   | 3.15        | 2.05        | 1.07        | 1.53        | 1.88        |            | putative lipoprotein                 |
| LA3736  | 0.77   | 3.33        | 2.43        | 0.86        | 1.23        | 1.3         |            | TPR-repeat-containing proteins        |
| LA3801  | 0.94   | 3.21        | 2.9         | 0.92        | 1.1         | 2.02        |            | glucosamine-fructose-6-phosphate aminotransferase (Hexosephosphate aminotransferase; D-fructose-6-phosphate amidotransferase) (GFA) (L-glutamine-D-fructose-6-phosphate amidotransferase; Glucosamine-6-phosphate synthase) |

The ORFs up-regulated at least 3-fold in J or T samples were included in this table. The supplementary annotations generated in this study were showed in brackets. doi:10.1371/journal.pntd.0000857.g005

Table 1.

| ORF ID | Clade ID | 
|--------|----------|---------|----------------|
|        |          | ORF     | mean | fold | mean | fold | mean | fold | Function and description of gene product |
|        |          | ID      |      |      |      |      |      |      |----------------------------------------|
| LA3982 | 3        |         | 7.03 | 1.53 | 0.93 | 14.92 | 6.08 | 1.64 | hypothetical protein |
| LA4034 |          |         | 0.66 | 3.04 | 0.98 | 3.15 | 4.01 | 0.77 | putative lipoprotein |
| LA4046 |          |         | 1.28 | 2.68 | 2.39 | 1.03 | 1.22 | 2.28 | hypothetical protein |
| LA4128 |          |         | 0.64 | 3.03 | 0.98 | 3.15 | 3.58 | 1.03 | hypothetical protein |
| LA4141 |          |         | 0.67 | 3.04 | 0.98 | 3.15 | 4.01 | 0.77 | putative lipoprotein |
| LA4142 |          |         | 0.64 | 3.37 | 4.01 | 0.77 | 1.25 | 2.4 | putative lipoprotein |
| LA4148 |          |         | 0.87 | 3.42 | 2.54 | 1.09 | 1.85 | 2.67 | hypothetical protein |
| LB350  |          | 0.68    | 2.91 | 3.19 | 1.15 | 0.84 | 2.2 | hypothetical protein |

Several adhesion or invasion-related genes were annotated in the original L. interrogans Serovar Lai Strain Lai 56601 genome, including mce, mva, atsE and mviN [3]. The precise functions for most of these genes are largely unknown, with exception for mviN. Recent study shows that MviN (Muj) of E. coli is a peptidoglycan lipid II flippase [54]. In this study, mviN, as well as another putative virulence factor, mce, were up-regulated in L. interrogans upon interaction with macrophages.

L. interrogans possesses several other virulence-associated genes such as a collagenase gene (LA0872), a PAF acetylhydrolase gene (pafAH, LA2144) [55], a von Willebrand factor type A gene (vwa, LA0697), and a paraoxonase gene (pon, LA0399). The homologs of pafAH, vwa and pon are also present in saprophyte Leptospira [7]. These genes were largely unchanged in this model, with the except that vwa and pon were transiently up-regulated upon interaction with murine macrophages [J samples].

Iron acquisition is one of the essential survival strategies many pathogens possess for establishing infection in mammalian hosts [56]. TonB-dependent receptors are associated with iron acquisition [57] and may transport heme and hemoprotein [58]. Some of these outer membrane receptor genes (LA0572, LA2641 and LA3258) were up-regulated, while another receptor gene (LA3242) was down-regulated (Table S2) upon interaction with macrophages. The iron resources of EMJH and RPMI 1640 medium controls (with 10% heat-inactivated FCS) are somewhat different. The available iron in the EMJH medium is free Fe3+, whereas in the mammalian cell culture medium, iron source is Fe3+ in transferrin (TRF, siderophilin) provided by serum. This difference may also affect leptospiral iron transportation and uptake. For example, hemO (LB186) in Clade 6, the virulence gene encoding heme oxygenase for iron acquisition from hemoglobin [59,60], was transiently up-regulated only in the RPMI 1640 medium controls. This regulation could be due to a difference in iron status among the EMJH medium, the RPMI 1640 medium controls, and the macrophage-containing cell culture medium.

Outer membrane proteins and lipoproteins

The major components on the surface of Leptospira are transmembrane OMPs and lipoproteins [39]. These OMPs are functionally and structurally important for nutrition uptake, signal transduction, cell stabilization, and immunogenicity [61]. A mutant lacking ompA-like outer membrane protein, Loa22
had been generated, and showed that this gene was essential for \textit{L. interrogans} pathogenesis [62]. Several major OMPs had been employed for developing subunit vaccines or serological tests for leptospirosis [37,63,64,65,66,67].

Based on genome annotation and lipoprotein prediction, there are at least 150 predicted lipoprotein genes (including predicted lipoproteins on inner and outer membrane) and about 100 predicted transmembrane OMP genes defined in the genomes of \textit{L. interrogans} [3,4,11,68,69]. Cluster analysis of these 255 OMPs and lipoprotein genes revealed that half of these genes were up-regulated, while 40\% of them were down-regulated (Figure 6). It was interesting to note that most of these changes were due to the interaction with macrophage rather than in the RPMI 1640 medium controls. Thus, it was likely that the membrane profiles underwent a series of dramatic changes upon interaction with macrophages. Most of the up-regulated genes were moderately regulated and almost all of these genes were putative transmembrane OMPs and putative lipoproteins not previously verified by proteomics [68]. One of the well-studied up-regulated genes was \textit{lig} genes. The leptospiral \textit{lig} genes encode several surface Lk90-like proteins containing immunoglobulin-like repeats, including LigA, LigB and LigC [7,70,71]. The \textit{L. interrogans} Serovar Lai Strain Lai 56601 has LigB (LA3778) and LigC (LA9075) [3], whereas \textit{L. interrogans} serovar Copenhageni Strain Fiocruz L1-130 has LigA and LigB. Expression of \textit{lig}B is up-regulated during infection, and LigB has been suggested as a putative virulence factor [72]. However, recent inactivation of \textit{lig}B in \textit{L. interrogans} Serovar Copenhageni Strain Fiocruz L1-130 did not affect leptospiral pathogenicity [9]. It was proposed that loss of LigB was compensated by the presence of LigA [8]. In this study, both expression of \textit{lig}B and \textit{lig}C in \textit{L. interrogans} Serovar Lai Strain Lai 56601 were up-regulated either upon interaction with macrophages or in the RPMI 1640 medium controls alone. This result was consistent with the increased expression of \textit{lig}B by evaluated temperature, host serum and during infection [15,73]. Concurrent up-regulation of LigB and LigC also supported the functional compensatory hypothesis among LigA, LigB, and LigC.

Based on our microarray data, the most abundantly expressed genes in E0 samples were well-studied major transmembrane OMPs and surface lipoproteins (Table, S2), which was consistent with results of previous proteomics study [29]. The most interesting result was the dramatic down-regulation (10–50 fold) of these major OMPs upon interaction with macrophages (Table 2). These genes clustered into two unique subclades based on Euclidean distance (Figure 6). The most highly down-regulated subclade included the genes of LipL41 (LA0616), LipL48 (LA3240), a putative OMPs (LA1538), OmpL1 (LA3138) and LipL32 (LA2637). Another highly down-regulated subclade included the genes of LipL21 (LA0011), LipL46 (LA2024), two putative outer membrane proteins (LA0100 and LA2066), LipL45 (LA2295), putative lipoprotein qLp42 (LA0136), Loo22 (LA0222), LruB (LA3469) and LruA/LipL71 (LA3097). In addition, most of these genes were members of the above-mentioned Clade 1 in Cluster analysis of whole transcriptomics data (Figure 2B). The virulence gene \textit{loa22} gene was down-regulated by 2–4 fold upon interaction with macrophages, and was included in the moderately down-regulated Clade 6. This down-regulation was contrary to an earlier report that expression of \textit{loa22} was up-regulated by host serum [15]. Notably, this was not due to incubation in the RPMI 1640 medium controls, as down-

Figure 6. Sequential changes of the predicted leptospiral OMP genes. The balance between up-regulation (red) and down-regulation (green) indicated that \textit{L. interrogans} altered its membrane in the infection models. The highly down-regulated transmembrane OMP and lipoprotein genes were clustered into two distinct subclades. The most highly down-regulated subclade included the genes of LipL41 (LA0616), LipL48 (LA3240), a putative OMPs (LA1538), OmpL1 (LA3138) and LipL32 (LA2637). Another highly down-regulated subclade included the genes of LipL21 (LA0011), LipL46 (LA2024), two putative outer membrane proteins (LA0100 and LA2066), LipL45 (LA2295), putative lipoprotein qLp42 (LA0136), Loo22 (LA0222), LruB (LA3469) and LruA/LipL71 (LA3097). doi:10.1371/journal.pntd.0000857.g006
## Table 2. Category of leptospiral ORFs which were down-regulated at least 5-fold in infection models.

| Clade ID | ORF ID | M45/E0 mean fold | J45/E0 mean fold | T45/E0 mean fold | T90/E0 mean fold | Function and description of gene product |
|----------|--------|-------------------|------------------|------------------|------------------|------------------------------------------|
| 5        | LA0011 | 1.5               | -7.69            | -3.13            | -5.56            | putative lipoprotein (LipL21)             |
| 5        | LA0100 | 3.17              | -7.69            | -5.26            | 0.75             | putative outer membrane protein          |
| 5        | LA0242 | 0.72              | -5.88            | -6.25            | 1.17             | cytochrome cca3 oxidase subunit II       |
| 5        | LA0243 | 0.78              | -5.26            | -6.67            | 1.21             | cytochrome C oxidase polypeptide I       |
| 5        | LA0244 | 0.83              | -3.45            | -3.13            | 1.21             | cytochrome C oxidase polypeptide III (Cytochrome AA3 subunit 3) |
| 2        | LA0296 | -2.56             | -4.17            | -3.85            | -5.88            | alcohol dehydrogenase                    |
| 5        | LA0411 | 0.93              | -5.88            | -5               | 1.2              | electron transfer flavoprotein alpha-subunit (Alpha-ETF; Electron transfer flavoprotein large subunit; ETF55) |
| 1        | LA0412 | 0.84              | -14.29           | -11.11           | 1.19             | electron transfer flavoprotein beta-subunit (Beta-ETF; Electron transfer flavoprotein small subunit; ETF55) |
| 1        | LA0505 | 1.92              | -9.09            | -8.33            | 1.17             | probable glycosyl hydrolase (OmpL47)     |
| 2        | LA0587 | 0.86              | -5               | -4.76            | -7.69            | lactonizing lipase (Triacylglycerol lipase) |
| 1        | LA0616 | 1.26              | -14.29           | -11.11           | 0.82             | outer membrane lipoprotein lipL41        |
| 5        | LA0737 | 1.23              | -11.11           | -6.25            | 1.21             | elongation factor Tu                      |
| 1        | LA0738 | 1.24              | -14.29           | -9.09            | 1.25             | ribosomal protein S10                    |
| 5        | LA0739 | 1.18              | -7.14            | -5.88            | 1.36             | ribosomal protein L3                     |
| 5        | LA0751 | 1.38              | -3.33            | -3.23            | 1.35             | ribosomal protein L5                     |
| 5        | LA0755 | 1.07              | -2.56            | -2.44            | 0.54             | ribosomal protein L18                    |
| 5        | LA0756 | 1.05              | -2.7             | -2.22            | 0.68             | ribosomal protein S5                     |
| 5        | LA0757 | 0.88              | -2.63            | -3.85            | 0.78             | ribosomal protein L30                    |
| 5        | LA1084 | 1.21              | -3.7             | -2.04            | 0.66             | hypothetical protein                     |
| 2        | LA1101 | -2.33             | -4.35            | -3.23            | -5               | succinyl-CoA synthetase alpha subunit     |
| 2        | LA1102 | 0.54              | -5               | -4.35            | -4.17            | succinyl-CoA synthetase beta chain        |
| 5        | LA1202 | 0.93              | -3.85            | -3.85            | 0.58             | hypothetical protein                     |
| 5        | LA1313 | 0.91              | -5.88            | -5.56            | 0.83             | glutamine synthetase (Glutamate-ammonia ligase) |
| 1        | LA1402 | 2.41              | -6.25            | -2.86            | 1.04             | -16.67                                    |
| 5        | LA1403 | -2.33             | -3.7             | -6.67            | 1.26             | hypothetical protein                     |
| 2        | LA1471 | -2.7              | -3.33            | -2.86            | -8.33            | hypothetical protein                     |
| 5        | LA1532 | 0.52              | -5               | -3.45            | 0.8              | fructose-bisphosphate aldolase (catalyzes the formation of glyceraldehyde 3-phosphate from fructose 1,6-bisphosphate) |
| 1        | LA1538 | 1.05              | -33.33           | -20              | 1.22             | putative outer membrane protein          |
| 1        | LA1539 | 0.95              | -50              | -50              | 1.19             | putative phosphosyltransferase            |
| 4        | LA1563 | 4.47              | -6.25            | -3.33            | 2.91             | class II heat shock protein (HSP20)       |
| 4        | LA1564 | 2.02              | -11.11           | -2.94            | 1.71             | class II heat shock protein (HSP20)       |
| 5        | LA1676 | 1.23              | -4.76            | -5.56            | 0.52             | single-stranded DNA-binding protein      |
| 5        | LA1677 | 1.15              | -5               | -5.26            | 1.05             | single-stranded DNA-binding protein      |
| 5        | LA1678 | 1.1               | -4.17            | -5               | 2.44             | ribosomal protein L9                     |
| 1        | LA1718 | 1.23              | -4               | 0.55             | 0.65             | hypothetical protein                     |
| 1        | LA1719 | 2.69              | -6.67            | -5               | 1.91             | cysteine synthase (O-acetylserine sulphydrylase; O-acetylserine (Thiol)-lyase) |
| 2        | LA1883 | 0.52              | -2.94            | -2.38            | -4.17            | hypothetical protein                     |
| 2        | LA1897 | 0.76              | -6.25            | -4.55            | -3.33            | succinate dehydrogenase (Converts succinate to fumarate as part of the TCA cycle. It is the only membrane bound enzyme in the TCA cycle) |
| 1        | LA1901 | 1.25              | -3.85            | 0.53             | 0.67             | hypothetical protein                     |
| 2        | LA1920 | 0.45              | -6.25            | -5.26            | -5               | RNA-binding protein                      |
| 4        | LA2017 | 1.47              | -16.67           | -4.17            | 0.95             | periplasmic flagellin (flaB1)             |
| 4        | LA2019 | 1.88              | -20              | -3.03            | 2.27             | periplasmic flagellin (flaB2)             |
| 5        | LA2024 | 1.5               | -4.55            | -3.85            | 1.36             | hypothetical protein                     |
| 5        | LA2138 | 2.22              | -6.25            | -3.03            | 2.67             | hypothetical protein                     |
| Clade ID | ORF ID | M45/E0 mean fold | J45/E0 mean fold | T45/E0 mean fold | M90/E0 mean fold | J90/E0 mean fold | T90/E0 mean fold | Function and description of gene product |
|---------|--------|------------------|------------------|-----------------|-----------------|-----------------|-----------------|------------------------------------------|
| 5       | LA2179 | 2.66             | -5.56            | -3.45           | -2.5            | -4.55           | -5.56           | recombinase A (catalyzes the hydrolysis of ATP in the presence of single-stranded DNA, the ATP-dependent uptake of single-stranded DNA by duplex DNA, and the ATP-dependent hybridization of homologous single-stranded DNAs) |
|         | LA2181 | 1.27             | -3.7             | 0.53            | 0.67            | 0.76            | -5.26           | hypothetical protein |
|         | LA2239 | 1.25             | -4               | 0.57            | 0.66            | 0.85            | -5              | hypothetical protein |
|         | LA2295 | 1.35             | -10              | -4              | 1.41            | 0.59            | -5.88           | Lpl45 protein |
| 2       | LA2309 | -2.17            | -10              | -7.14           | -9.09           | -8.33           | -6.25           | long-chain-fatty-acid CoA ligase |
|         | LA2360 | 0.89             | -4.35            | -4              | -7.69           | -9.09           | -4.76           | ribonucleotide-diphosphate reductase alpha subunit (Catalyzes the rate-limiting step in dNTP synthesis) |
| 1       | LA2418 | 0.98             | -16.67           | -9.09           | -2.27           | -4.76           | -6.67           | possible hook-associated protein, flagellin family |
| 5       | LA2458 | 0.55             | -6.67            | -5              | 1.08            | -4.76           | -5.26           | hypothetical protein |
| 1       | LA2637 | 1.86             | -33.33           | -12.5           | 1.09            | -20             | -33.33          | LipL32 protein |
| 4       | LA2654 | 4.01             | -6.67            | -4.55           | 3.22            | 3.47            | 1.05            | 10 kDa chaperonin (Protein Cpn10) (Protein GROES) (Heat shock 10 kDa protein) |
| 4       | LA2655 | 2.17             | -6.67            | -2.86           | 1.8             | 1.56            | 0.64            | 60 kDa chaperonin (Protein Cpn60) (groEL protein) (Heat shock 58 kDa protein) |
| 5       | LA2781 | 1.06             | -6.67            | -4.35           | 0.84            | -6.67           | -6.67           | ATP synthase F0, B subunit |
| 2       | LA2834 | -3.57            | -5.88            | -5.56           | -2.86           | -5.56           | -4.76           | adenylate cyclase |
| 2       | LA2835 | -3.45            | -9.09            | -6.67           | -3.13           | -6.25           | -6.25           | hypothetical protein (FMN-binding split barrel, related; Pyridoxamine 5'-phosphate oxidase-related, FMN-binding core) |
| 2       | LA2859 | -2.27            | -5               | -3.23           | -2.17           | -2.86           | -3.33           | hypothetical protein |
|         | LA2888 | 1.2              | -4               | 0.56            | 0.71            | 0.9             | -5              | hypothetical protein |
|         | LA3081 | 1.23             | -3.85            | 0.54            | 0.67            | 0.87            | -5              | hypothetical protein |
| 1       | LA3138 | 0.8              | -20              | -12.5           | 1.12            | -20             | -16.67          | transmembrane outer membrane protein L1 (OmpL1) |
| 5       | LA3143 | 0.97             | -6.67            | -3.85           | 0.87            | -2.78           | -4              | acyl-CoA dehydrogenase |
| 1       | LA3240 | 1.19             | -16.67           | -8.33           | 1.2             | -12.5           | -14.29          | hypothetical protein (LipL48) |
| 2       | LA3263 | 0.87             | -4.76            | -4.35           | 0.94            | -8.33           | -4.76           | hypothetical protein |
| 2       | LA3264 | 1.28             | -7.69            | -5.56           | 1.16            | -9.09           | -6.67           | hypothetical protein (Cytochrome c, monohaem; Cytochrome c, alcohol dehydrogenase-like subunit) |
| 1       | LA3265 | 0.72             | -9.09            | -10             | 0.86            | -10             | -9.09           | hypothetical protein |
| 2       | LA3266 | 0.95             | -8.33            | -6.25           | 1.13            | -12.5           | -8.33           | molybdopterin oxireductase |
| 2       | LA3267 | 1.35             | -8.33            | -6.67           | 1.19            | -12.5           | -8.33           | molybdopterin oxireductase, iron-sulfur binding subunit |
| 2       | LA3268 | 0.81             | -6.67            | -5.56           | 1.12            | -7.69           | -7.69           | cytochrome c3 (Cytochrome c7; Cytochrome c551.5) |
| 5       | LA3298 | 1.46             | -7.14            | -2.86           | 0.66            | -3.13           | -4.76           | 30S ribosomal protein S2 (Essential for binding of S1 to the small ribosomal subunit) |
| 5       | LA3379 | 2.26             | -6.67            | -3.45           | 0.7             | -3.57           | -4.35           | flagellar filament outer layer protein A |
|         | LA3380 | 2.45             | -6.67            | -2.7            | 2.57            | 0.8             | -3.03           | flagellar filament outer layer protein A |
| 5       | LA3417 | 0.75             | -4.55            | -4.17           | 0.54            | -3.03           | -5              | 30S ribosomal protein S12 (Important for translational accuracy. Interacts with and stabilizes bases of the 16S rRNA that are involved in rRNA selection in the A site and with the mRNA backbone. Located at the interface of the 3OS and 5OS subunits, it traverses the body of the 3OS s) |
| 5       | LA3419 | 0.97             | -4               | -3.33           | 1.31            | -5.26           | -4.35           | DNA-directed RNA polymerase beta' subunit (DNA-dependent RNA polymerase catalyzes the transcription of DNA into RNA using the four ribonucleoside triphosphates as substrates) |
|         | LA3426 | 1.66             | -5.26            | -2.33           | 1.98            | -2.22           | -2.94           | hypothetical protein (SecE subunit of protein translocation complex; Protein secE/sec61-gamma protein) |
| 4       | LA3705 | 2.88             | -6.25            | -3.23           | 1.39            | 1.2             | 0.86            | chaperone protein dnaK |
| 5       | LA3707 | 1.7              | -4.17            | -5              | 1.02            | 0.65            | -2.27           | hypothetical protein |
|         | LA3793 | 2.76             | -6.67            | -3.85           | 4.32            | -2.08           | -3.7            | hypothetical protein (Acyl-CoA N-acyltransferase) |
|         | LA3829 | 3.68             | -8.33            | -5.56           | 2.79            | -3.33           | -10             | hypothetical protein |
|         | LA3961 | 2.06             | -6.25            | -4.35           | 1.64            | -2.04           | -4.55           | hypothetical protein (OmpL36) |
| 5       | LA4067 | 1.08             | -5.26            | -4.55           | 1.31            | -4.55           | -4.55           | isocitrate dehydrogenase (Converts isocitrate to alpha ketoglutarate) |
The regulation did not occur in M samples. The persistent down-regulation of these major OMPs was further validated by immunoblotting in this study (Figure 5).

It is well established that leptospiral major OMPs are differentially expressed in semi-in vivo conditions or during infection [17,18,74]. We found that the expression levels of leptospiral OMPs were different in EMJH, Korthof and other leptospiral culture mediums (Date not shown). However, factors that contribute to differential regulation of these major surface proteins were largely unknown. Previous studies indicated that the common factors, including temperature, osmolarity, iron, or host serum, did not significantly influence expression of these major OMPs [12,13,14,15,59]. Our results indicated that the interaction with host cells was an important factor in triggering differential expression of major OMPs in L. interrogans, a phenomenon not achievable using leptospiral culture media. Since most of these surface proteins are major antigens and the macrophage is an important antigen presenting cell of the host, down-regulation of this group of proteins was likely an immune evasion mechanism of L. interrogans [75].

The ORFs down-regulated at least 5-fold in J or T samples were included in this table. The mean fold values were inverted into negative reciprocal values when the fold changes were 0.5 or less. The supplementary annotations generated in this study were showed in brackets. Clade ID: the clade ID for the significantly regulated ORF defined in genome-wide cluster analysis (Figure 2B).

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| Clade ID | ORF ID | M45/E0 mean fold | J45/E0 mean fold | T45/E0 mean fold | M90/E0 mean fold | J90/E0 mean fold | T90/E0 mean fold | Function and description of gene product |
|----------|--------|------------------|------------------|------------------|------------------|------------------|------------------|-----------------------------------------|
| 4        | LB0099 | 1.52             | −14.29           | −6.67            | 3.54             | 1.34             | −2.27            | hypothetical protein                     |
| 5        | LB106  | 2.55             | −5.26            | −4.17            | −2.17            | −2.27            | −3.57            | S-adenosyl-L-homocysteine hydrolase (catalyzes the formation of L-homocysteine from S-adenosyl-L-homocysteine) |
| 2        | LB327  | 0.73             | −4.76            | −3.85            | −4               | −7.14            | −4.55            | aconitate hydratase                      |

Identification of potential transcription factors involving in differential gene expression

The above-mentioned significant changes of gene expression, especially the dramatic down-regulations of the major OMPs, created a problem when determining which regulation systems were involved in differential gene expression. The first step to solve this problem is to identify the TFs which were directly involved in the major changes. More than forty sigma factors, anti-sigma factors, and anti-sigma factor antagonists were defined in the genome of L. interrogans Serovar Lai Strain Lai 56601. Their expression levels were unchangeable upon interaction with host cells in our microarray study (Table S2). Especially, Sigma S (RpoS), the sigma factor that plays a key role in differential gene expression in another well-studied spirochete, B. burgdorferi, is not present in the six released Leptospira genomes [76]. The anti-sigma factors can control sigma factors activity at post-translation level, and may be involved in the major OMPs regulation.

B. burgdorferi has only a few of specific TFs, while there are lots of specific TF homology defined in the leptospiral genomes, which increased the possibility that specific TFs also played important

![Figure 7. Domain structures of all predicted leptospiral specific transcription factors.](https://www.plosntds.org/10.1371/journal.pntd.0000857.g007)
roles in the regulation of the major OMPs. The leptospiral signal transduction proteins had recently been classified by domain-based rules in MiST2 database (http://mistdb.com/) [77]. In this database, the putative TFs were classified into several catalogs, such as one-component proteins, two-component proteins, and response regulators, etc., but not systematized into specific TF families named after their original function. In this study, the DBD definitions were obtained from InterPro integrative protein signature database by InterProScan program and well annotated by InterPro2Go. The InterPro database integrates PROSITE, PRINTS, Pfam, ProDom, SMART, TIGRFAMs, PIR superfamilies, SUPERFAMILY Gene3D and PANTHER databases, which guaranteed the accuracy of the definition of the functional DBD domains [31]. All putative TFs were classified into specific TF families based on the original definition of the DBD domains, which enabled us to predict the potential function of the putative TFs. In addition, the phylogenetic tree for each of the TF families was constructed based on the whole TF sequences, which helped us to compare the TF homologs within specific families, and identify the specific TFs that only existed in pathogenic Leptospira, which may be associated with leptospiral pathogenesis (Figure 1B).

Overall, the total number of specific TFs of non-pathogenic L. biflexa was almost twice than that of pathogenic Leptospira species (Table S3). That is, L. biflexa had about 100 specific TFs, while L. interrogans and L. borgpetersenii had less than 50 specific TFs. In addition, L. biflexa had higher proportions of TFs (TFs/ORFs) than pathogenic Leptospira, which is consistent with its strong survivability and high growth rate [6]. Based on domain analysis, 18 specific TF families were defined in six released leptospiral genomes (Figure 7). Several TF families were not found in all leptospiral genomes. The HTH_11 family existed only in L. interrogans, and the MerR, LytR, LyS, Cpr and GntR families existed only in L. biflexa. The CopG families existed in L. borgpetersenii and L. biflexa, but was absent in L. interrogans. (The previous definitions of CopG TFs in the genome of L. interrogans Lai 56601 were not precise, and there were no CopG TFs in the genome of L. interrogans Serovar Copenhageni Strain Fiocruz L1-130). Based on the microarray date in this study, several specific TF genes with high expression levels were identified, such as LB333 of the OmpR family, LA3094 of the Fur family, LA1447 of the LexA family, LA9090 of the MarR family and LA3531 of the ArsR family, etc., which may contribute to the major regulation in our microarray study.

Most specific TF families of non-pathogenic L. biflexa were larger than those of the pathogenic Leptospira. The only exception was that the OmpR TF family of pathogenic Leptospira species (L. interrogans and L. borgpetersenii), which was larger than that of L. biflexa. Considering that the OmpR TF was first defined as a regulator of outer membrane porin genes (ompC and ompF) in E. coli [78,79], it is possible that the leptospiral OmpR TFs were also involved in the regulation of the porins or other membrane proteins. Pathogenic Leptospira may regulate OMPs more efficiently than non-pathogenic L. biflexa. Furthermore, this OMP regulation may be related with leptospiral pathogenesis. If so, it would be consistent with the down-regulation of the major OMPs observed herein and previously [18].

The molecular phylogeny of OmpR family revealed four monophyletic origins in all six Leptospira spp. (Figure 8A). Two exceptions were that LA3108 homologs were only found in L. interrogans, and LA1919 homologs only existed in pathogenic Leptospira species (L. interrogans and L. borgpetersenii). Based on domain analysis (Figure 7), LA1919 was supposed to encode a TF with seven putative transmembrane regions, which was seldom present in prokaryotes but common in eukaryotes. Of note, LB333 was the unique OmpR TF gene which was highly-expressed in EMJH and RPMI 1640 medium (E0, M45 and M90), but significantly down-regulated to almost zero in infection models (J45, J90, T45 and T90). (B).

Figure 8. Molecular evolution and gene regulation of leptospiral OmpR transcription factors. The molecular evolutionary tree was constructed using the Neighbor-Joining method implemented in the MEGA 4.0 program with confidences of topology summarized from 1000 bootstrap replications based on the whole sequences of OmpRs. Only the bootstrap values larger than 50% were shown on the branches. Orthologous OmpRs sharing among all of the six Leptospira genomes syntenies were marked in a yellow green. lil: L. interrogans Serovar Lai Strain Lai 56601; lic: L. interrogans Serovar Copenhageni Strain Fiocruz L1-130; lbj: L. borgpetersenii Serovar Hardjo-bovis Strain JB197; lbi: L. biflexa. The molecular evolutionary tree was constructed using the Neighbor-Joining method implemented in the MEGA 4.0 program with confidences of topology summarized from 1000 bootstrap replications based on the whole sequences of OmpRs. Only the bootstrap values larger than 50% were shown on the branches. Orthologous OmpRs sharing among all of the six Leptospira genomes syntenies were marked in a yellow green. lil: L. interrogans Serovar Lai Strain Lai 56601; lic: L. interrogans Serovar Copenhageni Strain Fiocruz L1-130; lbj: L. borgpetersenii Serovar Hardjo-bovis Strain JB197; lbi: L. biflexa.
most abundantly expressed TF gene and the only OmpR TF gene highly expressed in EMJH medium, but was down-regulated significantly upon interaction with macrophages (Figure 8B).

Furthermore, it was concomitantly down-regulated with the group of major OMP genes including ompL1, lipL32, lipL41, lipL40 and ompL47, implying LB333 contributed to the differential regulation of this group of genes.

Concluding remarks

The global transcriptomic analyses of pathogens using a bacteria-host cell interaction model provide new understandings of immune evasion and pathogenesis for many bacterial pathogens [80,81,82]. Of note, a host-adapted model using a dialysis membrane chamber (DMC) for the Lyme disease spirochete, B. burgdorferi, has been used for genome-wide analysis of transcriptome in response to host-specific signals. However, this method can not be used for L. interrogans Serovar Lai Strain Lai 56601 as it can not survive and replicate within DMC (Date not shown). In this study, we adopted macrophage-derived cell models to analyze the leptospiral transcriptomic changes upon interaction with host cells or to host-specific signals. The adaptability to host microenvironments and immune evasion mechanism of L. interrogans revealed in this study were significantly different from those of the previous in vitro studies. Although the main virulence factors of Leptospira remain largely unknown due to the high difficulty of genetic manipulation, these significant changes of L. interrogans in different microenvironments led us to recommend that further research should be performed under conditions that imitate in vivo conditions as much as possible.

One of the major observations in this study was the dramatic down-regulation of major OMPs (e.g., LipL32, OmpL1, LipL41 and LipL48) upon interaction with host cells. This was in contrast to previous microarray analyses using various growth conditions, such as varied temperature, osmolality, or the presence of host serum, which did not observe changes in expression of these genes. The fact that these genes, including LipL32, LipL41, and LipL21, etc., have been shown differentially regulated during infection, suggests that interaction with host is a key signal for regulating expression of these genes [17,18]. Our observations in this study suggested that down-regulation of these major OMPs may be important for the immune evasion of pathogenic Leptospira, a strategy similar to B. burgdorferi [33,34]. These changes in the OMPs profile also suggested that host cells induce a substantial change in surface protein profile, which is important when considering vaccine candidates against leptospirosis [80].

Regulation of the major OMP genes of L. interrogans appeared to be different from that of B. burgdorferi. The expression levels of the major leptospiral OMPs, such as LipL32, LipL41, and OmpL1, etc., were relatively stable and not responsive to common environmental cues, whereas the major surface lipoprotein OspC of B. burgdorferi, is induced by elevated temperature, lowered pH, higher cell density, the presence of CO2 or host serum. Regulation of OspC is directly controlled by sigma S (RpoS), which is further controlled by transcription factors including Rrp2, RpoN (sigma N), and BosR [76,83]. L. interrogans does not have rpoN homologue in its genome. The specific TFs may play important roles in gene regulation of the major leptospiral OMPs. Co-regulation of LB333 with the major OMPs suggests that LB333 may be involved in such regulation. In addition, the comparative and evolutionary relationship of all the leptospiral specific TFs revealed in this study also facilitates further research on identifying the regulation networks in Leptospira spp.

This report focused on the common regulation of L. interrogans Strain Lai 56601 infecting the murine and human macrophage cell lines, especially the persistent down-regulation of the major OMPs. However, several differences in the transcriptomic changes of L. interrogans Strain 56601 infecting the murine vs. human macrophage cell lines were also observed. Cluster analysis on the global transcriptomics (Figure 2A) showed that the J90/E0 was somewhat similar to M45/E0 and M90/E0, which indicated that most of the gene regulations in L. interrogans upon interaction with murine macrophages (J90) were not persistent changes. For example, several genes of anti-anti-sigma factor (anti-sigma factor antagonists), ArsR TF, heat shock proteins, and chaperonins in Clade 4 were differently regulated in J and T samples, which may contribute to the differential regulations upon interaction with murine vs. human macrophages both at transcription level and at translation level. In addition, the major flagellin genes, flaB1/2 (LA2017 and LA2019), were persistently down-regulated in T samples, but only transiently down-regulated in J samples. Whether this difference contributed to the different behaviors of L. interrogans infecting murine vs. human macrophages remains unclear [23].

One limitation of our study was that macrophage cell lines instead of primary macrophages were used as infection models. However, our previous study had revealed that the behaviors of L. interrogans Strain Lai 56601 in the immortalized macrophage models were same as those in the primary macrophage models [23]. In addition, the high homogeneity and culturability of cell lines guaranteed the data reliability and repeatability in the highly sensitive microarray analysis. Therefore, only macrophage cell lines were employed as infection models in this leptospiral transcriptomics research. Another limitation was that the transcriptional responses revealed by microarray, qRT-PCR and Western blotting in this study only displayed the average and general regulations of all leptospiral cells infecting host cells. In fact, there were some individual differences among the invasive leptospiral cells morphologically [23]. Further cellular proteome analysis may complement the understanding of the individual regulation of pathogenic Leptospira during infection [86].

Supporting Information

Table S1 Primers for quantitative real-time RT-PCR validation. Found at: doi:10.1371/journal.pntd.0000857.s001 (0.03 MB DOC)

Table S2 The microarray data summary of the L. interrogans Serovar Lai Strain Lai 56601. Found at: doi:10.1371/journal.pntd.0000857.s002 (0.48 MB ZIP)

Table S3 Catalog and evolutionary analysis of all leptospiral specific transcription factors. Found at: doi:10.1371/journal.pntd.0000857.s003 (0.08 MB ZIP)

Author Contributions

Conceived and designed the experiments: FX JY. Performed the experiments: FX HD ZW WH AS. Analyzed the data: FX JW XFY. Contributed reagents/materials/analysis tools: FX JY. Wrote the paper: FX BT XFY JY.

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