

Comparative Gene Expression Analysis within Mouse Macrophage for Identifying Critical Pathways in Macrophage and *Brucella suis* Interaction

Jing Hu,¹ Tonglian Wang,² Hongbo Zhao,³ Yuzhu Song,² Qinqin Han,² Jinyang Zhang,² Tao Shou,¹ Fan Zhang,⁴ Xueshan Xia,² and Qiang Chen^{2,*}

¹Medical Oncology, The First People's Hospital of Yunnan Province, Kunming, P.R. China

²Research Center of Molecular Medicine of Yunnan Province, Faculty of Life Science and Technology, Kunming University of Science and Technology, Kunming, P.R. China

³Institute of Molecular and Clinical Medicine, Kunming Medical University, Kunming, P.R. China

⁴Department of Gastroenterology, The Third People's Hospital of Yunnan Province, Kunming, P.R. China

*Corresponding author: Qiang Chen, Research Center of Molecular Medicine of Yunnan Province, Faculty of Life Science and Technology, Kunming University of Science and Technology, No. 727 of South Jingsing Road, Chenggong New Town, Kunming, 650500, P.R. China. Tel: +86-87165939528, Fax: +86-87165939528, E-mail: chq@sjtu.edu.cn

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Abstract

Background: *Brucella* spp. are Gram-negative bacteria that cause a zoonotic disease called brucellosis in humans as well as many animals. *Brucella suis* (*B.suis*) is one of the greatest threats to the human health and food safety. Studying macrophage and *B. suis* interaction is critical for understanding the chronic infection mechanism. However, the interaction mechanisms, especially for molecular events triggered by *B. suis* infected macrophage, such as biological pathways, are still obscure.

Objectives: We will use gene set enrichment analysis (GSEA) to microarray in an attempt to find critical pathways in the interaction of macrophage and *B. suis*.

Methods: We applied a standardized microarray preprocessing and GSEA to 2 independent macrophage and *B. suis* interaction studies including smooth virulent *B. suis* strain S1330 (S1330) data sets and rough attenuated *B. suis* strain VTRS1 (VTRS1) data sets. Integrative analysis was used to find critical pathways for 2 independent macrophage and *B. suis* interaction data sets.

Results: The results demonstrated that for S1330 data sets, 8 and 13 common up- and down- regulated pathways were found in 4 interaction stages including 4h, 8h, 24h, and 48h post macrophage infected S1330 *B. suis*, and for VTRS1 data sets, we found 30 and 19 common up- and down- regulated pathways. Comparing the results of S1330 and VTRS1 data sets, 6 and 8 common up- and down-regulated pathways were identified.

Conclusions: The study of macrophage and *B. suis* interaction through pathway analysis highlighted genes weakly connected to the phenotype, and discovered common critical pathways in the process of macrophages and different phenotypes of *B. suis* interaction. The identified pathways will shed light on the understanding of the functional events within macrophage post infected *B. suis*.

Keywords: GSEA, Macrophage and *B. suis* Interaction, Microarray, Pathway Analysis

1. Background

Brucella spp., a Gram-negative and facultative intracellular bacteria, causes a zoonotic disease called brucellosis in humans and many animals including domestic pigs, cattle, wildlife camels, and so on by chronic macrophage infection (1). In 10 species reported (2), *Brucella suis* is one of 3 pathogenic bacteria (*B. melitensis*, *B. suis* and *B. abortus*) that are the biggest threat to human health and food safety. In many types of cells attacked, macrophages are the primary target cells attacked (3). Further studying the macrophage and *B. suis* interaction will contribute to understanding the pathogenesis.

To date, a large number of interaction studies showed that the fate of macrophages infected were modulated

by *Brucella*. Rough *Brucella* organisms induce caspase2-mediated macrophage death (1, 4), whereas smooth *Brucella* organisms inhibit macrophage apoptosis by inhibiting mitochondrial cell death pathway and caspase activation (4, 5). These results suggest that inhibition of macrophage death provide a hospitable intracellular niche for *Brucella* survival, while death induction promote *Brucella* release and dissemination (6-8). In addition, some studies observed that smooth *Brucella* dissociated into rough mutants that were cytotoxic to macrophage in macrophage and *Brucella* interaction (6), which suggested that smooth and rough *Brucella* should be synergistically responsible for macrophage egress and bacterial dissemination.

Despite extensive researches, the molecular events

such as critical biological pathways within macrophage in macrophage and *B. suis* interaction are not yet uncovered. Performing pathway analysis to gene expression profiles data from microarray will contribute to interpreting the results to insight into biological mechanisms including bacterial invasion, survival, and replication within macrophages.

2. Objectives

Gene set enrichment analysis (GSEA), a gene set analysis method including pathway analysis (9, 10), has been widely used to identify the significant differences between pre-defined gene sets in expression from controls and cases. Here, we will use GSEA to microarray in an attempt to find critical pathways, especially common critical pathways within macrophages in the interaction of macrophages and smooth or rough *B. suis*.

3. Methods

3.1. Datasets

The interaction of macrophage and *B. suis* microarray datasets were searched and downloaded from NCBI GEO database (<http://www.ncbi.nlm.nih.gov/geo/>). Finally, data set GSE21117 (1), including 2 subsets of interaction of murine macrophage line and smooth or rough *B. suis* strains, was used in this study. Two phenotypes of *B. suis* were smooth virulent *B. suis* strain S1330, for short S1330 and rough attenuated *B. suis* strain VTRS1, for short VTRS1. For each subset in this study, data sets included 4 infection stages that respectively were 4h, 8h, 24h, and 48h post infection, and 0h post infection (uninfected group) was for control. Each phenotype or stage was treated as an independent data set. 8 case-control data sets including 4 smooth phenotype of case-control data sets and 4 rough phenotype of case-control data sets were included, and each data set was individually performed GSEA. The related information regarding the datasets such as cell line, sample size, and infection time, were listed in Table 1.

3.2. Data Preprocessing

Microarray data preprocessing was performed using software packages developed by Bioconductor in version 3.4 (<http://www.bioconductor.org>) (11) and R language in version 3.3.2 (<http://www.r-project.org>). All datasets were background adjusted, normalized, and log₂ probe-set intensities calculated using the robust multichip averaging (RMA) algorithm in version 1.52.0 affy package (12, 13). Some identifying genes failed to map any KEGG pathways that

were excluded from the further analysis. The data variability was measured using the interquartile range (IQR), and a cut-off was set from the resulting distribution of IQR values for all genes. Genes with IQR values under 0.5 were excluded. When multiple probe sets targeted the same gene, the probe set with the largest variability was kept for the next analysis. Pathway analysis was separately performed for each data set.

3.3. Gene Set Enrichment Analysis of Pathways

Gene set enrichment analysis was performed using the version 2.40.0 Category package in the Bioconductor project and the performing GSEA's purpose was to determine whether the members of a gene set *S* distributes randomly throughout the whole reference gene list *L* or was just primarily found at the top or bottom. A bigger merit of GSEA lies in the relative robustness to noise and outliers in the data. However, the fact is that a gene set including a large enough number of genes, typically 10 or more than 10, is true. Therefore, the gene set with less than 10 genes was removed.

3.4. Statistical Analysis

The t-statistic mean of the genes was computed in each remaining pathway. A permutation test was implemented 1000 times, and the pathways with P value ≤ 0.05 were identified to significantly change (14).

4. Results

4.1. Significant Pathways Identified Within Macrophages and 2 Phenotypes of *B. suis* Interaction

We compared 4 data sets to identify the significant pathways within macrophage infected S1330 *B. suis*. The reanalysis results for these data sets were showed in Table 2. Base on the permutation 0.05 P values, we found 21, 15, 56, and 91 up-regulated pathways and 36, 81, 48, and 64 down-regulated pathways in 4 interaction stages including 4h, 8h, 24h, and 48h post macrophage infected S1330 *B. suis*, separately. The overlap analysis showed that 8 common up- and 13 common down-regulated pathways were found. The overlaps of these pathways identified were showed in Figure 1A and 1B. We performed the same analysis for VTRS1 data sets as S1330 data sets, and the reanalysis results for these data sets also were showed in Table 2. We found 47, 44, 68, and 85 up-regulated pathways and 28, 104, 88, and 74 down-regulated pathways, separately. The overlaps among these pathways identified showed that 30 common up- and 19 common down-regulated pathways were found. The overlaps of these pathways were showed in Figure 1C and 1D.

Table 1. Experimental Design and Related Information of GSE21117 Microarray

Cell Line	Brucella Strain	Chip Platform	Probes	Time Post Infection (h)	No. of Arrays
J774.A1 cells ^a	<i>B. suis</i> strain S1330 (S1330) ^b	GPL1261	45k	0 (Control) ^c	3
				4	3
				8	3
				24	3
				48	3
J774.A1 cells ^a	<i>B. suis</i> strain VTRs1 (VTRs1) ^b	GPL1261	45k	0 (Control) ^c	3
				4	3
				8	3
				24	3
				48	3

^aJ774.A1 cells are murine macrophage-like cells.

^bS1330 and VTRs1 are represented the smooth virulent *B. suis* strain and rough attenuated *B. suis* strain, respectively.

^c0h post infection is control that shows that macrophages are uninfected.

Table 2. Analysis Results of Differentially Expressed Pathway Number

Classes	Process (h)	No. of Genes After Preprocessing	No. of Pathways Have Genes ≥ 10	Up-Regulated Pathway Number	Down-Regulated Pathway Number
S1330 strain ^a	4 ^b	1816	68	21	36
	8	2861	108	15	81
	24	5552	160	56	48
	48	6268	165	91	64
VTRs1 strain ^a	4	3578	107	47	28
	8	5839	159	44	104
	24	5433	159	68	88
	48	5996	164	85	73

^aS1330 and VTRs1 are represented the smooth virulent *B. suis* strain and rough attenuated *B. suis* strain, respectively.

^b4h, 8h, 24h and 48h are represented time post murine macrophage-like J774.A1 cells infected *B. suis*.

4.2. Integrative Analysis of 2 Phenotypes of *B. suis* and Macrophages Interaction

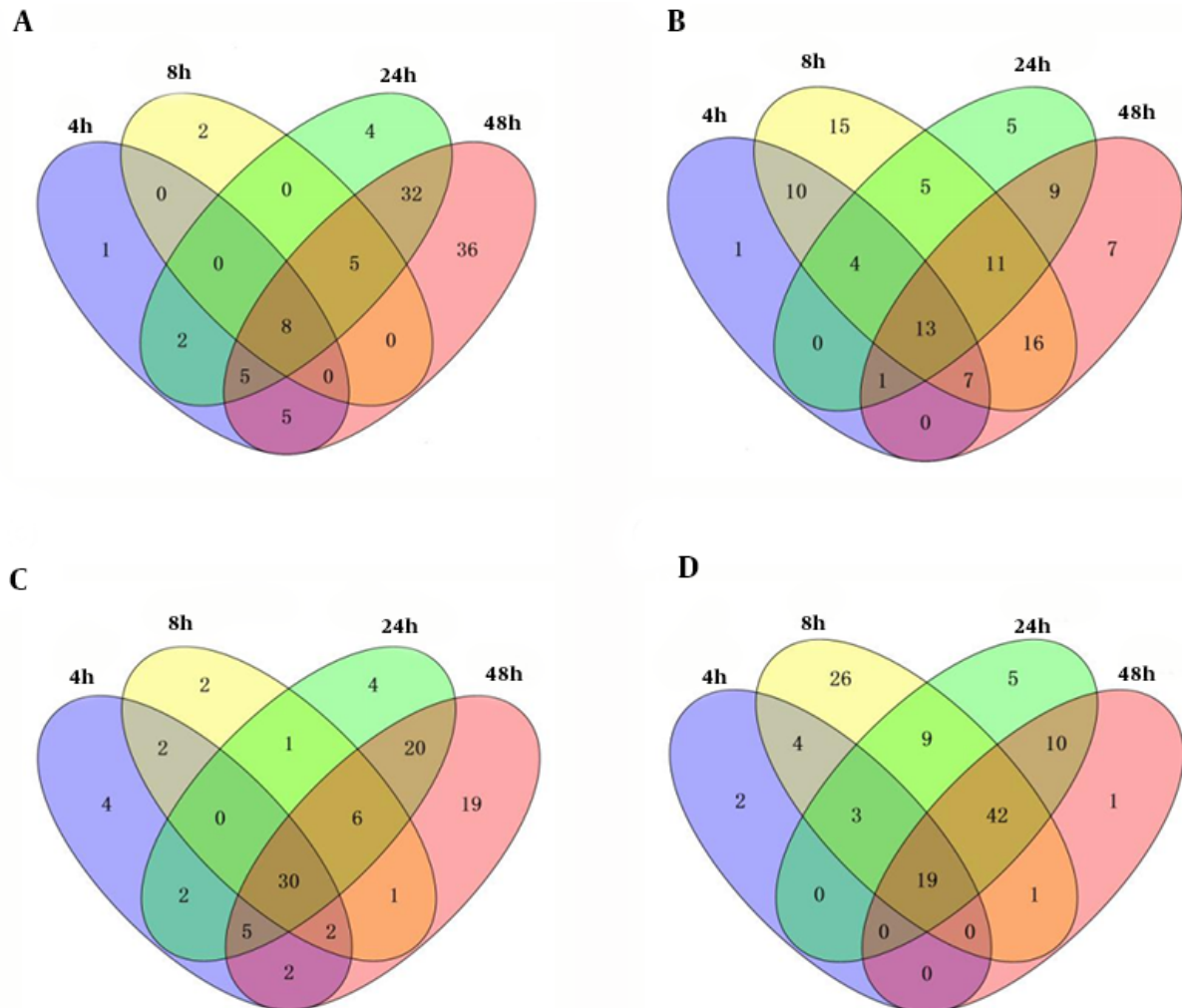
We compared the significant pathways of 2 phenotypes of *B. suis* and macrophages interaction. The significant pathways identified and overlaps were showed in [Figure 2](#) at the same stage post infection. We found 18, 13, 14, and 59 common up-regulated and 15, 62, 40, and 45 down-regulated pathways in 4 interaction stages including 4h, 8h, 24h, and 48h post infection, respectively. For 2 datasets in the 4 stages of interaction, we detected that 6 common significant pathways were up-regulated and 8 common significant pathways were down-regulated ([Figure 3](#)). The integrative results were listed in [Table 3](#). In the common significant pathways, functional protein association networks of sum set of genes within the up-regulated and

down-regulated pathways were showed in [Figure 4](#).

4.3. Dynamical Variation of Critical Significant Pathways

From [Table 3](#), we found some critical pathways related to immune system, immune disease and cell growth and death in common significant pathway list. These pathways included up-regulated toll-like receptor signaling pathways, RIG-I-like receptor signaling pathway, cytosolic DNA-sensing pathway as well as rheumatoid arthritis pathway, and down-regulated cell cycle pathway. We analyzed the dynamical change of these pathways in total interaction stages from 4h to 48h post infection. The results were presented in [Figure 5](#). From [Figure 5](#), for S1330 and VTRs1 strains, as a whole, in early interaction stages these critical pathways within macrophage had statistical significance ($P < 0.05$), most up-regulated pathways presented

Figure 1. Significant Pathways Identified and Overlap



4h, 8h, 24h and 48h are respectively represented 4h, 8h, 24h and 48h post macrophage infected *B. suis*. For each infection stage, we performed GSEA to generate p-values for each pathway and used a permutation test with 1000 times, and obtained the significant pathways with P values cut-off of ≤ 0.05 . A, GSEA detected 21, 15, 56 and 91 up-regulated pathways and 8 common were found in macrophage and S1330 *B. suis* interaction; b, GSEA detected 36, 81, 48 and 64 down-regulated pathways and 13 common were found in macrophage and S1330 *B. suis* interaction; C, GSEA detected 47, 44, 68 and 85 up-regulated pathways and 30 common were found in macrophage and VTRSI *B. suis* interaction; D, GSEA detected 28, 104, 88 and 73 down-regulated pathways and 19 common were found in macrophage and VTRSI *B. suis* interaction.

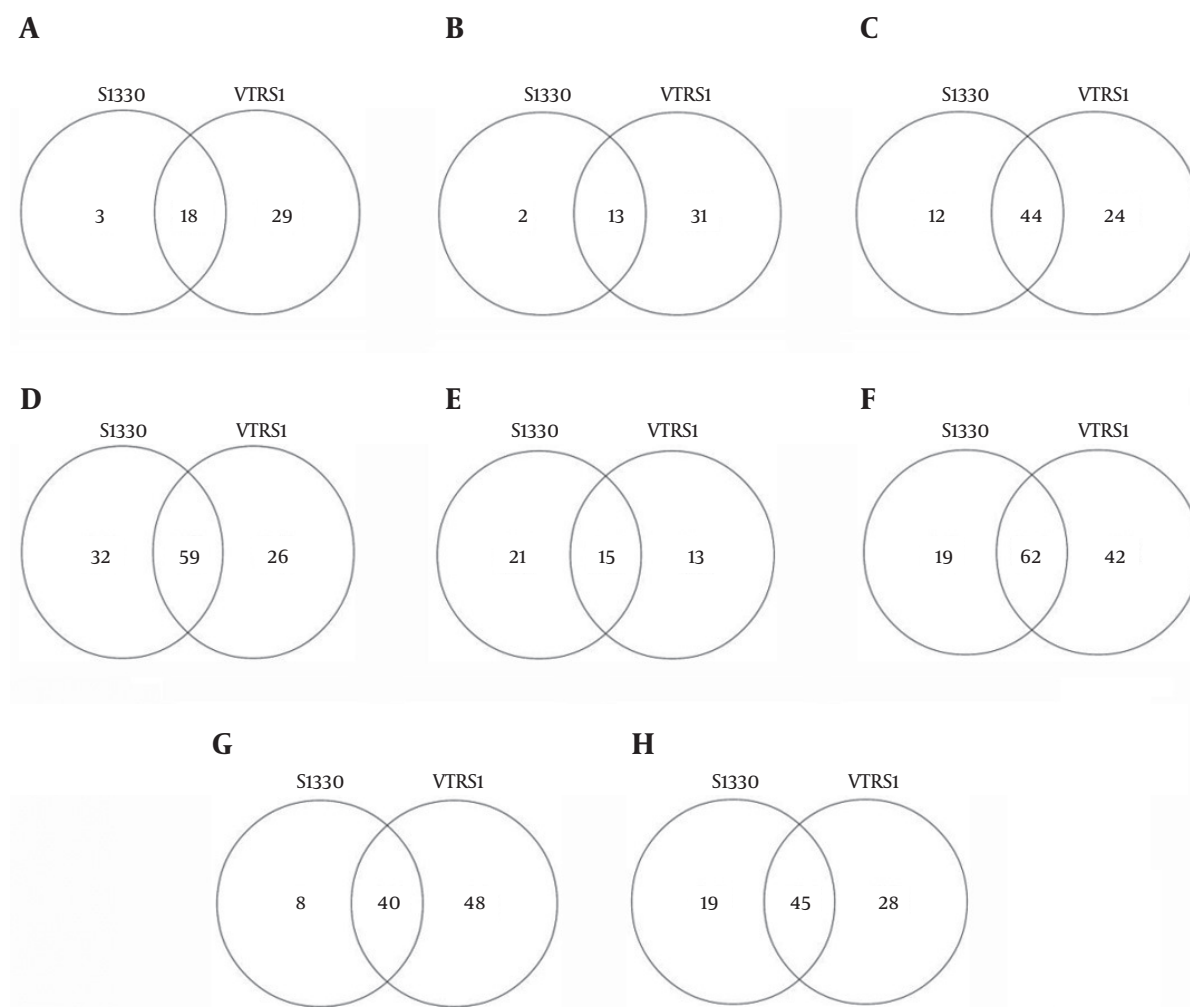
continuously to be up-regulated, than be down-regulated. Down-regulated cell cycle pathway was sequentially down-regulated.

5. Discussion

The host macrophages are the primary targets attacked (3). Studying macrophage and *B. suis* interaction is critical for the establishment of *B. suis* infection models (1). In the process of macrophage and *B. suis* interaction, many mechanisms are responsible for the successful infection

(15). However, most mechanisms are still obscure. The microarrays are a very powerful tool to illustrate the infection mechanisms (1, 4, 5, 16, 17). For the traditional single gene analysis method, the statistical analysis can only identify larger differently expressed genes, however, not find genes that made subtle contributions (10). In addition, the roles of identified genes need to be further discussed. Pathway analysis is able to put stress on genes weakly related to the phenotype and identify subtle genes by using univariate statistics (14).

In this study, we performed GSEA for 2 sets of

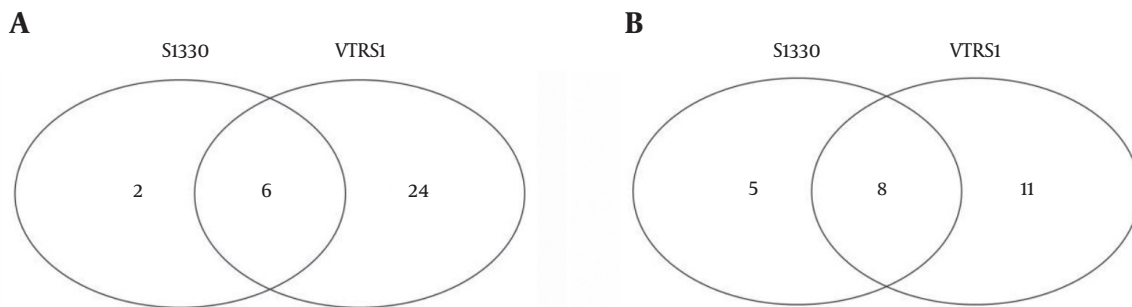
Figure 2. Pathway Analysis of Macrophages Infected between SI330 *B. suis* and VTRS1 *B. suis* at the Same Infection Stage.

SI330 and VTRS1 are respectively represented significant pathways identified of macrophages infected between SI330 and VTRS1 *B. suis*. A, B, C and D, showed the significant up-regulated pathways identified and overlap at 4h, 8h, 24h and 48h post infection, respectively; E, F, G and H, showed the significant down-regulated pathways identified and overlap at 4h, 8h, 24h and 48h post infection, respectively.

datasets to deeply understand the biological process of macrophages and smooth or rough *B. suis* interaction. In the original study, the results have showed that macrophages presented different responses to smooth and rough *B. suis* and discovered some genes with larger change in gene expression level (1, 4). This partly explained the molecular mechanism of macrophages and *B. suis* interaction. However, the critical genes made subtle expression change would be lost. Therefore, in this study, we studied the critical pathways and their dynamical change in macrophage and *B. suis* interaction.

Toll-like receptor signaling pathway was found to be up-regulated during the 4 interaction stages. Toll-like re-

ceptors (TLRs), the genes of specific families of pattern recognition receptors, are responsible for detecting microbial pathogens, generating innate immune responses and developing adaptive immunity, and have been extensively studied in macrophage as well as *Brucella* interaction (18-20). These studies demonstrated that TLRs play an important role in host resistance to *Brucella* infection or cooperate with each other (15, 21). Such as TLR6, one member of TLRs is important for triggering an innate immune response against *B. abortus* and further cooperates with TLR2 to activate NF- κ B signaling pathway (20). TLR4 and TLR9 also play a key role in macrophage and *Brucella* interaction (15). Such, we considered that the pathway related to im-

Figure 3. Common Significant Pathways and Overlap in Total Interaction Stages of Macrophages and S1330 and VTRSI *B. suis*.

S1330 and VTRSI are respectively represented common pathways identified post macrophages infected S1330 and VTRSI *B. suis*. A, We separately detected 8 and 30 common pathways within macrophages infected S1330 and VTRSI *B. suis*, and 6 common pathways were found; B, We separately detected 13 and 19 common pathways within macrophages infected S1330 and VTRSI *B. suis*, and 8 common pathways were found.

immune system played a critical role in macrophage and *B. suis* interaction, and might serve as a potential target for novel antibiotic drug.

Beyond toll-like receptor signaling pathway up-regulated, the other 2 up-regulated pathways related to immune system were RIG-I-like receptor signaling pathway and cytosolic DNA-sensing pathway. As similar to TLRs within toll-like receptor pathway, the receptors within the 2 pathways also belonged to specific families of pattern recognition receptors, which are respectively responsible for detecting viral pathogens as well as foreign DNA, and triggering innate immune responses. Currently, few published results directly showed RIG-I-like receptor signaling pathway, which was related to the interaction of macrophage and *Brucella*. Most published results proved that RIG-I-like receptor signaling pathway plays a vital role in RNA virus recognition (22-24). However, the functions of RIG-I-like receptor signaling pathway in defending against bacterial infection remain elusive. Lately, few studies showed that the role of RIG-I-like receptor signaling pathway in fighting against bacterial infection is a universal mechanism. RIG-I-like receptor signaling pathway is involved in the host immune response to several different types of bacterial pathogens, such as *Yersinia pestis* and so on (25). These studies provided theoretical supports for up-regulating RIG-I-like receptor signaling pathways in macrophage and *B. suis* interaction.

Cytosolic DNA-sensing pathway, a type of pathway generating innate immune response to DNA from DNA virus, bacteria or host cells, has been appreciated for many years where cytosolic DNA can evoke a Type I interferon response (26, 27). Some studies proved that DNA would be released into the cytoplasm from intracellular DNA-containing microbe such as *Mycobacterium tuberculosis* and *Francisella tularensis* during infections, and induced DNA immunity

(28, 29). Our results showed that cytosolic DNA-sensing pathway was up-regulated in the process of macrophage and *B. suis* interaction, which partly demonstrated that *B. suis* involved the similar interaction mechanisms as *Mycobacterium tuberculosis* and *Francisella tularensis* during infections. That is to say, in macrophage and *B. suis* interaction, part *B. suis* killed may be disintegrated, and release DNA into macrophage to induce DNA immunity producing (30).

In up-regulated pathways, we also detected that rheumatoid arthritis pathway was up-regulated. Several studies have demonstrated that the brucellosis caused *Brucella* spp involved a diversity of clinical signs and symptoms including arthritis (31-35). Furthermore, some studies showed the relationship of brucellosis and rheumatic symptoms (32). However, these studies only described that some clinical symptoms of brucellosis patients were related to arthritis or rheumatoid arthritis. The mechanisms of arthritis caused *Brucella* are still unclear. In this study, we observed that many genes were significantly differently expressed within the rheumatoid arthritis pathway, in which many inflammatory and proinflammatory factors including interleukin 15, 6, 1beta, and tumor necrosis factor receptor and ligand superfamily members were up-regulated. Due to the fact that rheumatoid arthritis is caused by means of the body's immune system attacking the joints (36), rheumatoid arthritis pathway up-regulated may be one of the main reasons leading to joint pain. We speculated that the macrophages and *B. suis* interaction disturbed the pathway related to immune system, which makes the normal macrophage function disorder, and generates inflammatory and proinflammatory factors to promote the arthritis (37).

In down-regulated pathways, we found that the cell cycle pathway was down-regulated. A large number of stud-

Table 3. Common Pathways Categories Identified by GSEA

Entry	Pathway Name	Class	Gene Number ^a	Genes ^b
Up regulated				
04620 ^c	Toll-like receptor signaling pathway	Immune system	14	<i>Ikbke, Ccl5, Ccl3, Cd86, Ccl4, Tlr7, Pik3cg, Fos, Pik3r1, Cd40, Tlr4, Ilib, Nfkbia, Tlr8</i>
04622	RIG-I-like receptor signaling pathway	Immune system	3	<i>Ikbke, Nfkbib, Nfkbia</i>
04623	Cytosolic DNA-sensing pathway	Immune system	6	<i>Ikbke, Ccl5, Nfkbib, Ccl4, Ilib, Nfkbia</i>
05140	Leishmaniasis	Infectious diseases	6	<i>Fos, Ilib, Nfkbia, Nfkbib, Ptgs2, Tlr4</i>
05323	Rheumatoid arthritis	Immune diseases	6	<i>Cd86, Fos, Ilib, Ccl3, Ccl5, Tlr4</i>
04920	Adipocytokine signaling pathway	Endocrine system	5	<i>Socs3, Nfkbia, Nfkbib, Acsl3, Pck2</i>
Genes included in all common up-regulated pathways			19	<i>Ikbke, Ccl5, Ccl3, Cd86, Ccl4, Tlr7, Pik3cg, Fos, Pik3r1, Cd40, Tlr4, Ilib, Nfkbia, Tlr8, Nfkbib, Ptgs2, Socs3, Acsl3, Pck2</i>
Down regulated				
04110	Cell cycle	Cell growth and death	16	<i>Atm, Ccnd1, Ccne2, Cdkn1a, Chek1, Gadd45a, Anapc1, Gadd45b, Rbl1, Wee1, Cdc6, Skp2, Gsk3b, Ccnh, E2f2, Atr</i>
04114	Oocyte meiosis	Cell growth and death	4	<i>Ccne2, Itpr1, Anapc1, Prkacb</i>
04914	Progesterone-mediated oocyte maturation	Endocrine system	4	<i>Anapc1, Pik3r1, Prkacb, Pik3cg</i>
05210	Colorectal cancer	Cancers	7	<i>Ccnd1, Fos, Msh6, Pik3r1, Tgfb2, Pik3cg, Gsk3b</i>
05212	Pancreatic cancer	Cancers	5	<i>Ccnd1, Pik3r1, Tgfb2, Pik3cg, E2f2</i>
05223	Non-small cell lung cancer	Cancers	6	<i>Ccnd1, Pik3r1, Sost, Pik3cg, Stk4, E2f2</i>
03040	Spliceosome	Transcription	5	<i>Srsf10, Srsf3, Prpf19, Prpf40a, U2surp, Tra2a</i>
04070	Phosphatidylinositol signaling system	Signal transduction	6	<i>Itpr1, Pik3r1, Pikfyve, Pip4k2a, Pten, Pik3cg</i>
Genes included in all common down-regulated pathways			34	<i>Atm, Ccnd1, Ccne2, Cdkn1a, Chek1, Gadd45a, Anapc1, Gadd45b, Rbl1, Wee1, Cdc6, Skp2, Gsk3b, Ccnh, E2f2, Atr, Itpr1, Prkacb, Pik3r1, Pik3cg, Fos, Msh6, Tgfb2, Sost, Stk4, Srsf10, Srsf3, Prpf19, Prpf40a, U2surp, Tra2a, Pikfyve, Pip4k2a, Pten</i>

^a Number of common genes included within the pathways.

^b Overlapping genes within the pathway.

^c The number is represented pathway entry number, and the “mmu” is omitted in the front of the number.

ies showed that during the viral infection a number of viruses encode proteins to target cell cycle regulators and press cell cycle arrest (38-40). Furthermore, some studies showed that during bacterial infection the cell cycle of macrophage was arrested (41-43). For macrophage and *Brucella* interaction, some studies revealed that *Brucella* inhibited transcription of various host genes involved in cell cycling (16), which indicated that cell cycle pathway within macrophage might be down-regulated in macrophage and *Brucella* interaction. Our GSEA results showed that cell cycle pathway would be down-regulated in *B. suis* and macrophage interaction. Down-regulated cell cycle would subvert the cellular machinery that controlled replication

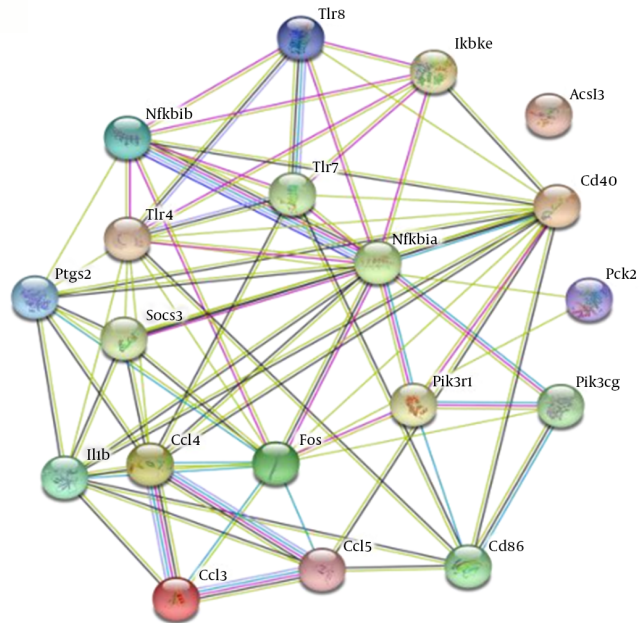
of macrophage (39), which would benefit bacterial survival and growth within the host (44).

Beyond common critical pathways discussed above, we observed that metabolic pathways were down-regulated in the majority of interaction stages. The metabolic pathways belong to a global pathway involved in a series of chemical reactions of material metabolism (45). When infection, some studies showed that pathways or members related to lipid, sugar, and protein metabolism were changed (46-48). It is easy to understand that pathogen infection will result in metabolic disturbance of host or cell (49, 50).

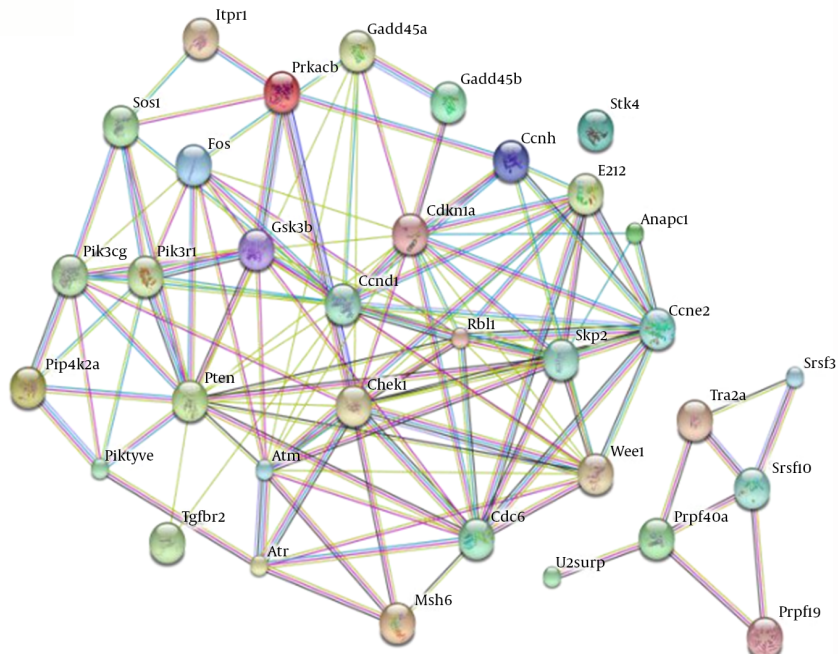
The interaction of macrophage and *B. suis* is extremely intricate. The infection process involves a series of im-

Figure 4. Association Network of Gene Sumset in Significant Regulated Pathways

A



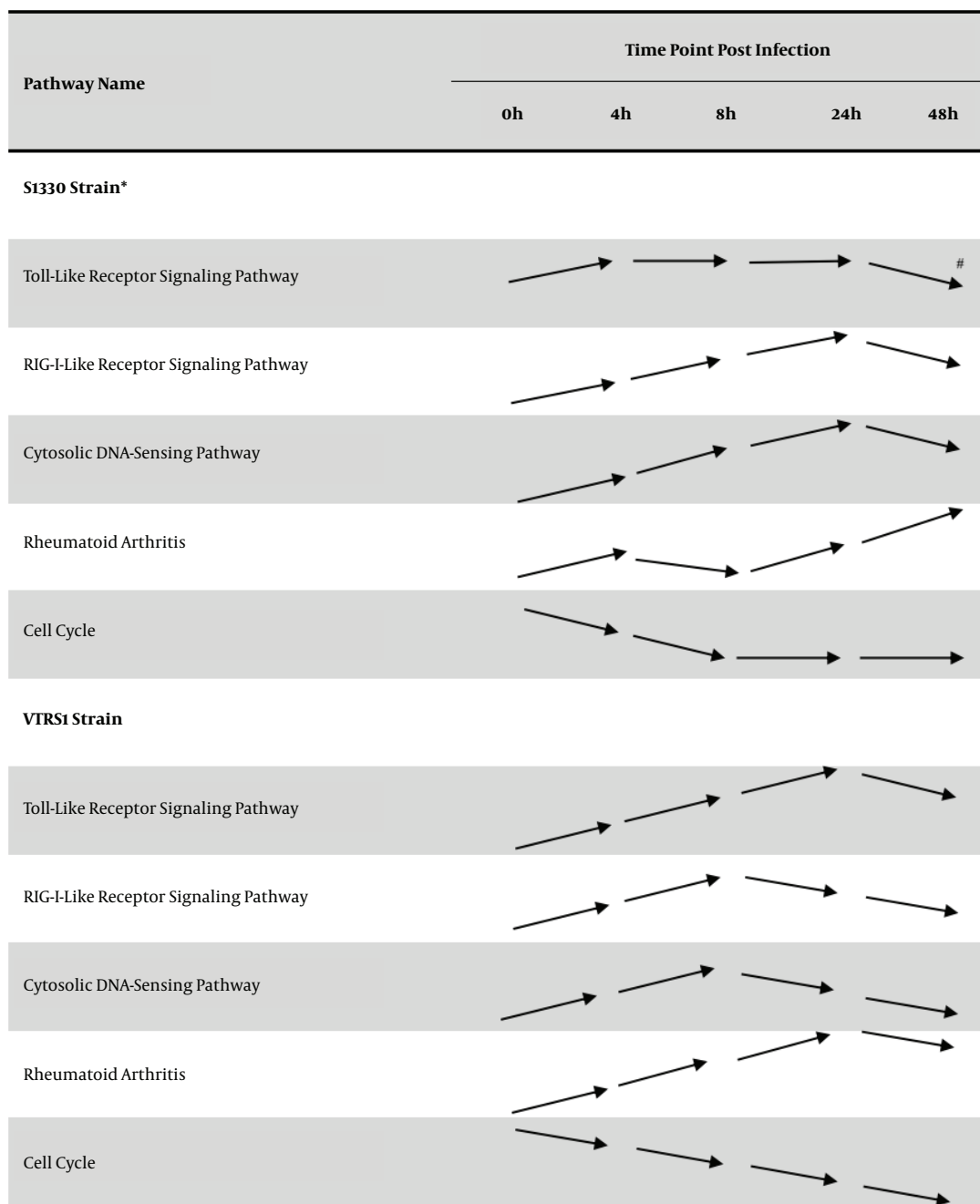
B



A, Association network of gene sumset in common up-regulated pathways; B, Association network of gene sumset in common down-regulated pathways.

munological and inflammatory reaction events within macrophage against *B. suis* infection. Inversely, *B. suis* have evolved a unique strategy to modify and create a novel intracellular environment for surviving and multiplying. A

deeper understanding of the interaction mechanisms can be reached by focusing on regulation of gene sets rather than on a large number of single genes. By means of standardized microarray preprocessing and GSEA, we found

Figure 5. Dynamical Change of Common Critical Pathways

* S1330 strain and VTRS1 strain are represented murine macrophage-like J774.A1 cells infected by smooth virulent *B. suis* strain and rough attenuated *B. suis* strain, respectively.
 # Arrow line is represented the regulated change of over-represented pathway. The parallel arrow shows that the pathway has no significant change in the latter infection stage than that in the former stage. The arrow line with raising end shows that the pathway is significantly up-regulated in the latter infection stage than that in the former stage. The arrow line with dropping end shows that the pathway is significantly down-regulated in the latter infection stage than that in the former stage.

the concordance to identify many biological mechanisms involved in interaction of macrophage and *B. suis* in some

infection stages. The identified corresponding pathways will provide some important insights into immunologi-

cal reactions within macrophage post infected *B. suis*. Further, these results will provide some clues for discovering novel wide-spectrum antibiotic drug targets for helping to more efficiently prevent and control brucellosis. Next, more studies will focus on the specific genes within related pathways and gene interaction to improve the understanding of macrophage and *B. suis* interaction.

6. Conclusions

Through pathway analysis based GSEA, we found some critical biological pathways involved in the process of macrophage and *B. suis* interaction. These results will contribute to deeply understand the interaction mechanisms, and help to efficiently prevent and control brucellosis. Nevertheless, the number of the array datasets used only contains 2 datasets and the array datasets have a limited set of probes of the mouse genome. Thus, further studies with arrays covering the entire repertoire of mouse genes will be needed to update the current results.

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Footnotes

Authors' Contribution: Jing Hu searched the microarray from NCBI GEO database. Qiang Chen and Hongbo Zhao performed the data processing and GSEA with the help of Tonglian Wang, Yuzhu Song, Qinqin Han, Jinyang Zhang. Qiang Chen wrote the manuscript. Tao Shou, Fan Zhang and Xueshan Xia revised the manuscript.

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