Modular bioinformatics analysis demonstrates that a Toll-like receptor signaling pathway is involved in the regulation of macrophage polarization

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Abstract. In recent years, an increasing number of studies on the roles of macrophages in tumors, immune responses and metabolism have been published, in which macrophage polarization has been an extensively discussed topic. In the present study, differentially expressed genes in various types of macrophages were analyzed using the Gene Expression Omnibus database. Cluster analysis of differentially expressed genes was conducted, and a protein-protein interaction (PPI) network was constructed. Finally, modular analysis and functional enrichment analysis revealed that a Toll-like receptor (TLR) signaling pathway is involved in the regulation of macrophage polarization. Furthermore, the high-degree proteins in the PPI network that are involved in the molecular regulation of macrophage polarization are closely associated with proteins of the TLR signaling pathway. These results suggested that the TLR signaling pathways may be a principal direction of future research on the regulation of macrophage polarization.

Introduction

Macrophages are the primary effector cells of the immune system and are involved in inflammatory and anti-infective responses (1). They additionally serve an important role in tissue homeostasis, promote the growth of cells in tissues and are involved in the repair of tissue damage (2). Macrophages undergo different forms of polarization under the action of different inducers and are polarized to M1 or M2 macrophages (3). M1 macrophages are additionally known as classical pathway-activated macrophages. They are primarily induced by bacterial products and T helper (Th)1-type cytokines, including interferon (IFN)-γ. Their characteristic effects include eliminating intracellular microorganisms and producing large quantities of pro-inflammatory mediators (4). M2 macrophages are additionally termed alternative pathway-activated macrophages (5). The primary inducers of M2 macrophages are Th2-type cytokines [interleukin (IL)-4, IL-13 and IL-10], glucocorticoids and immunoglobulin complexes and Toll-like receptor (TLR) ligands (6). The principal effect of M2 macrophages is the suppression of inflammatory responses (7). Macrophage polarization commonly occurs during the pathogenesis and progression of inflammatory diseases, including cancer, obesity and cardiovascular diseases, and has a guiding significance for the prognosis of specific tumors (8-11).

In the present study, a protein-protein interaction (PPI) network was constructed based on differentially expressed genes in macrophages of different polarization types from the Gene Expression Omnibus (GEO) database. The aim of the present study was to identify important signaling pathways and gene groups that are involved in the regulation of macrophage polarization through modular analysis and functional enrichment analysis, which may provide specific novel insight for the treatment of human immune-associated diseases.

Materials and methods

Gene expression profiles of human M1 and M2 macrophages. Gene expression profiles of human M1 and M2 macrophages were downloaded from the GEO database [https://www.ncbi.nlm.nih.gov/geo/; accession nos. GSE18686 (12) and GSE35449 (13)]. Expression profile data of GSE18686 and GSE35449 were obtained from M1 and M2 macrophages cultured with inducer or without inducer, respectively (12,13). The GSE18686 and GSE35449 data sets were tested using the Illumina HumanHT-12 v3.0 Gene Expression BeadChip platform (Illumina, Inc., San Diego, CA, USA).

The data of M1 macrophages treated with lipopolysaccharide (LPS) and IFN-γ, M2 macrophages cultured with IL-4 and the corresponding control group (M0 macrophages)

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were selected from GSE18686, with six biological replicates included in each group. The data of the M1 and M2 macrophage groups in addition to the control group were selected from GSE35449, and seven biological replicates were included in each group.

Pretreatment of expression profile data. GSE18686 and GSE35449 expression matrix data sets were downloaded and pretreated using the quantile method in the lumi software package v2.32.0 (14,15). The mean value of the different probes that mapped to the same gene (GeneSymbol) was calculated. The GSE18686 data set consists of 48,802 probes, and a final total of 19,489 genes remained following the treatment. The GSE35449 data set consists of 48,797 probes, and 19,487 genes remained following the treatment. The gene expression data sets were extracted for M1 and M2 macrophages, according to the aforementioned methods.

Detection of differentially expressed genes. The limma package in Bioconductor (v3.36.2) (16) was employed to analyze genes that were differentially expressed between M1 and M0 or between M2 and M0 in GSE18686 and GSE35449. This method is comprised of the following steps: i) Constructing a design matrix for the preprocessed data; ii) estimating the number of folds in differential gene expression using a linear model; iii) followed by using a Bayesian approach for smoothing the standard deviation (17); and iv) finally utilizing different parameters for output of the differentially expressed genes. The log2 of fold change (log2FC) and P-values were used as parameters for the selection of differentially expressed genes; P<0.05 was considered to indicate a statistically significant difference, determined using a Student's t-test, and an absolute value of log2FC≥1 (the differential expression coefficient was 2). Subsequently, the differentially expressed genes in the intersection of GSE18686 and GSE35449 were selected.

Cluster analysis of differentially expressed genes in intersection. The gplots software package (v2.17.0) (18) was employed to calculate and construct thermal graphs from the cluster analysis of the differentially expressed genes in M1 (Fig. 1A and B) and M2 (Fig. 1C and D) in the intersection of GSE18686 and GSE35449. The expression values of these differentially expressed genes were analyzed for hierarchical clustering.

Modular analysis of differentially expressed genes in the intersection. To further analyze the differentially expressed genes at the molecular level, Search Tool for the Retrieval of Interacting Genes/Proteins v9.1 (https://string-db.org/) was used to obtain information regarding PPI pairs based on the differentially expressed genes in the GSE18686 and GSE35449 intersection and Cytoscape (v3.4.0) (19) to construct PPI networks. To obtain functional modules in the PPI networks, communities in the PPI networks were extracted using CFinder (v2.0.6) (20).

Functional enrichment analysis of each community. The candidate genes of each community were submitted to the Database for Annotation, Visualization and Integrated Discovery database (http://david.abcc.ncifcrf.gov/), and the complete genome of *Homo sapiens* was used for assessment and comparison as background genes. The 'Functional Annotation Tool' was used to obtain the Kyoto Encyclopedia of Genes and Genomes (KEGG; https://www.genome.jp/kegg/) pathway enrichment analysis results (the P-value cutoff was 0.05).

Results

Identification of differentially expressed genes in gene expression profiles. A total of 338 genes that were differentially expressed between the M1 test group and the M0 control group were obtained from GSE18686 when P<0.05 and the absolute log2FC value was ≥0.58; whereas, 636 genes that were differentially expressed between the M1 test group and the M0 control group were obtained from GSE35449 (Table I). A total of 151 differentially expressed genes were obtained from the intersection of differentially expressed genes from GSE18686 and GSE35449 between the M1 test group and the M0 control group (data not shown). Within the same threshold range as above, 273 genes that were differentially expressed between the M2 test group and the M0 control group were obtained in GSE18686; whereas, 1,171 genes that were differentially expressed between the M2 test group and the M0 control group were identified in GSE35449 (Table I). A total of 144 differentially expressed genes were obtained from the intersection of differentially expressed genes from GSE18686 and GSE35449 between the M2 test group and the M0 control group.

Cluster analysis of differentially expressed genes in intersection. The thermal graphs from the cluster analysis of the 151 differentially expressed genes between the M1 and M0 groups in the intersection of GSE18686 and GSE35449 are demonstrated in Fig. 1A and B. The majority of the differentially expressed genes in the intersection were upregulated in the M1 group when compared with their expression levels in the M0 samples. The thermal graphs from the cluster analysis of the 144 differentially expressed genes between the M2 and M0 groups in the intersection of GSE18686 and GSE35449 are demonstrated in Fig. 1C and D. The majority of the differentially expressed genes in the intersection were upregulated in the M2 group when compared with their expression levels in the M0 samples.

Construction of PPI networks based on differentially expressed intersection genes. The PPI network based on the intersection of the differentially expressed genes between the M1 and M0 groups was constructed. This network consists of 94 protein nodes and 523 PPIs. As demonstrated in the network diagram, the proteins encoded by these intersecting differentially expressed genes exhibit a complex association. In this PPI network, the 10 highest degree proteins were signal transducer and activator of transcription (STAT)1 (degree=41), guanylate-binding protein (GBP)1 (degree=35), GBP5 (degree=35), C-X-C motif chemokine 10 (CXCL10; degree=35), interferon-induced protein with tetratricopeptide (IFIT)2 (degree=32), interferon regulator factor (IRF)7 (degree=32), IFIT3 (degree=32), ubiquitin-like protein ISG15 (ISG15; degree=31), IRF1 (degree=31) and interferon-induced helicase C domain-containing protein 1
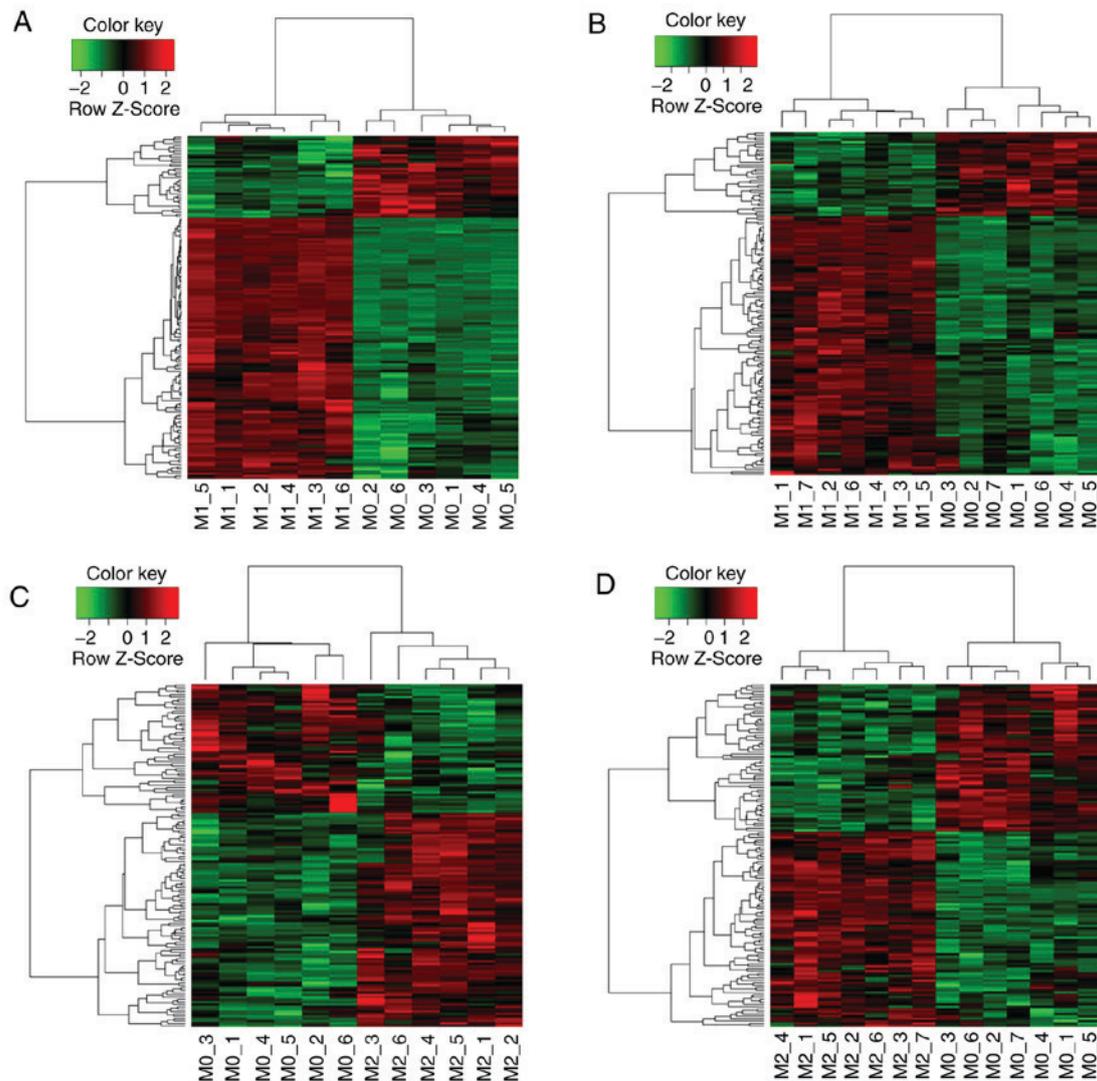
Figure 1. Cluster analysis of intersecting differentially expressed genes. (A) Thermal graph from cluster analysis of the 151 intersecting differentially expressed genes of GSE18686 in the M1 vs. M0 group. (B) Thermal graph from cluster analysis of the 151 intersecting differentially expressed genes of GSE35449 in the M1 vs. M0 group. (C) Thermal graph from cluster analysis of the 144 intersecting differentially expressed genes of GSE18686 in the M2 vs. M0 group. (D) Thermal graph from cluster analysis of the 144 intersecting differentially expressed genes of GSE35449 in the M2 vs. M0 group. Red indicates upregulated gene expression and green indicates downregulated gene expression. Each column in the thermal graph represents a sample and each row represents a gene.

(IFIH1; degree=2; Fig. 2). These 10 proteins are significant nodes in the network. A previous study demonstrated that IFN signaling activates the IRF/STAT signaling pathways through STAT1, leading to the transformation of macrophages to the M1 type (21).

The PPI network based on the intersection of the differentially expressed genes between the M2 and M0 groups is demonstrated in Fig. 3. This network consists of 56 protein nodes and 72 PPIs. The 10 highest degree proteins in this PPI network were monocyte differentiation antigen CD14 (CD14; degree=8), arachidonate 5-lipoxygenase-activating protein (ALOX5AP; degree=8), myeloid differentiation primary response gene 88 (MYD88; degree=7), cyclin-dependent kinase inhibitor 1A (CDKN1A; degree=6), protein S100-A9 (S100A9; degree=32), cytochrome b-245 heavy chain (CYBB; degree=6), cyclin-A2 (CCNA2; degree=5), protein S100-A8 (S100A8; degree=5), thymidine kinase, cytosolic (TK1; degree=5) and TLR7 (degree=5).

Modular analysis and KEGG functional analysis of each module. In the M1 vs. M0 group, communities in the PPI network were identified using CFinder software. When K=5, three communities were identified, and there were 432, 15 and 14 pairs of interacting genes in the first, second and third communities, respectively.

In the M2 vs. M0 group, communities in the PPI network were identified using CFinder software. When K=3, five communities were identified, and there were 23, 3, 5, 3 and 12 pairs of interacting genes in the first, second, third, fourth and fifth communities, respectively (data not shown).

The results from KEGG functional analysis of each module are demonstrated in Fig. 4. Enrichment was obtained in community 1 of the M1 group and in communities 1, 3 and 5 of the M2 group. Community 1 of the M1 group and communities 1 and 3 of the M2 group demonstrated functions primarily enriched in the 'toll-like receptor signaling pathway'. Detailed information regarding gene-interaction pairs and the degree of gene nodes in these three communities is presented in Fig. 5.

Table I. Differentially expressed genes between M1 experimental group and M0 control group.

A, GSE18686

Contrast group	Number of differentially expressed genes	Number of upregulated genes	Number of downregulated genes
M1 vs. M0	338	249	89
M2 vs. M0	273	181	92

B, GSE35449

Contrast group	Number of differentially expressed genes	Number of upregulated genes	Number of downregulated genes
M1 vs. M0	636	298	338
M2 vs. M0	1,171	399	772
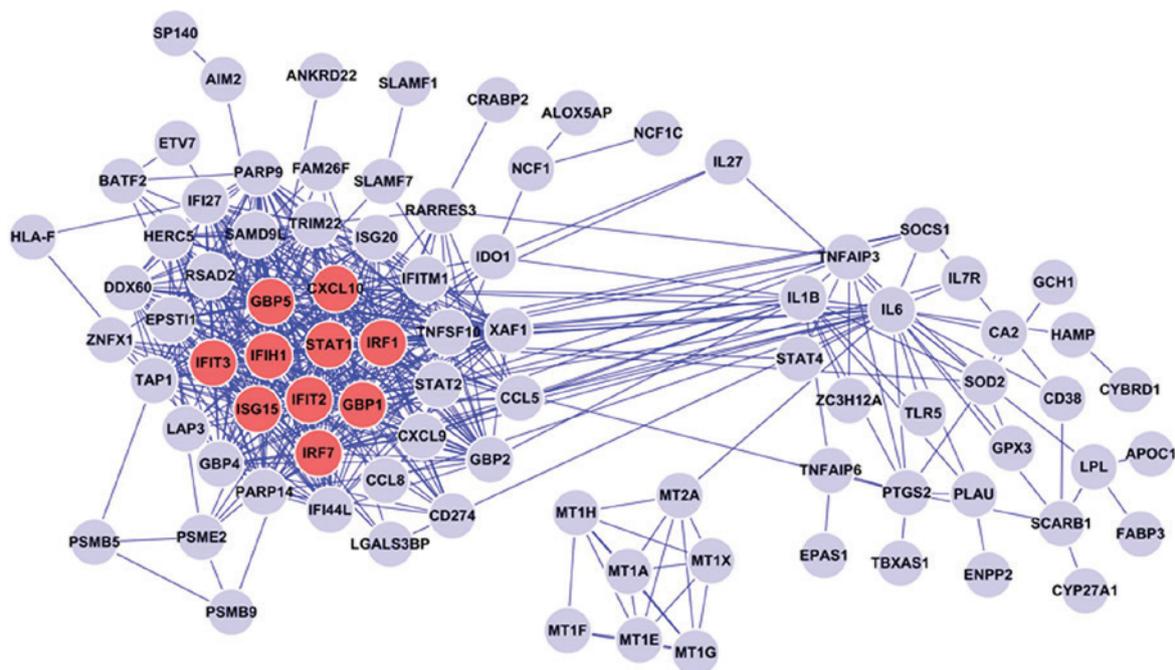
Figure 2. PPI network constructed based on the genes with differential expression between the M2 and M0 groups in the intersection of GSE18686 and GSE35449. The network is undirected, where a circular node represents a protein and a gray line represents an interaction between nodes. Red nodes indicate proteins with the highest degree in the PPI network. PPI, protein-protein interaction.

Discussion

Macrophages are multifunctional cells that perform different functions depending on the type and state of the tissues where they reside. Dysregulation of macrophage functions may lead to numerous diseases, including infectious diseases and immune disorders. In addition, macrophages serve a role in the destruction of endocrine pancreatic-cells in the autoimmune response of type 1 diabetes (21), metabolic diseases (22) and malignancies (23). The transition between macrophage polarization types serves a significant and pivotal role in the progression of these diseases (24). Therefore, the identification of the molecules and molecular groups associated with the

dynamic processes of macrophages is crucial for the elucidation of the molecular basis of disease progression and the design of novel macrophage-based therapeutic strategies.

The concept of macrophage polarization types has been described as a dynamic, stepwise and continuous process of alteration from M1 to M2 (25-27). Macrophage polarization is classified into M1 (classical) and M2 (alternative) types, which is currently an intense area of research in the study of macrophage function (3,28). The present study employed gene expression profiling data of M1 and M2 macrophages in the GEO database to evaluate protein-protein interrelationships and construct PPI networks through a more efficient method based on protein semantic similarity (29). However,
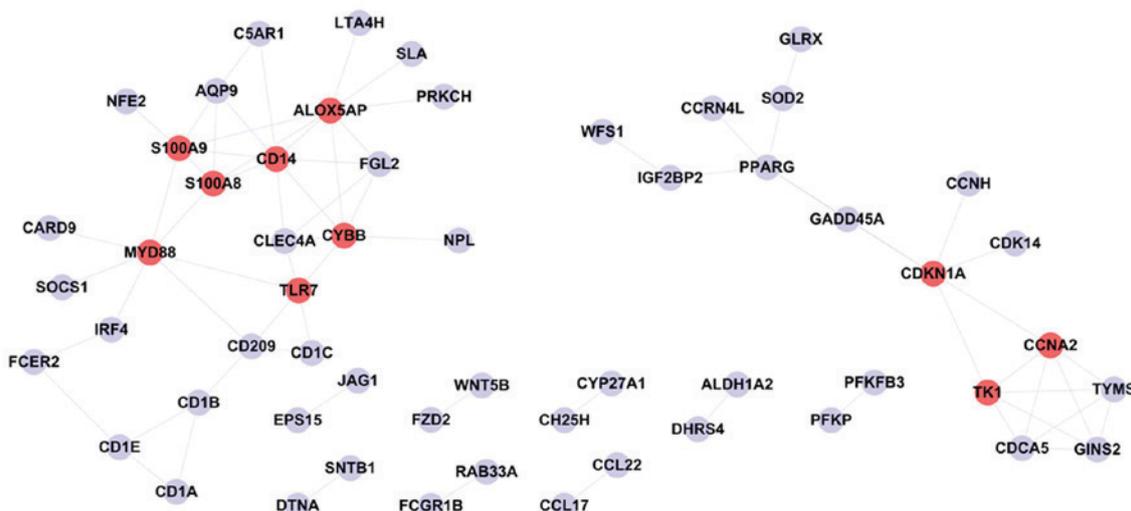
Figure 3. The PPI network based on the interaction of the differentially expressed genes between the M2 and M0 groups. This network consists of 56 protein nodes and 72 PPIs. The 10 highest degree proteins in this PPI network are presented as red nodes, where a circular node represents a protein and a gray line represents an interaction between nodes. PPI, protein-protein interaction.
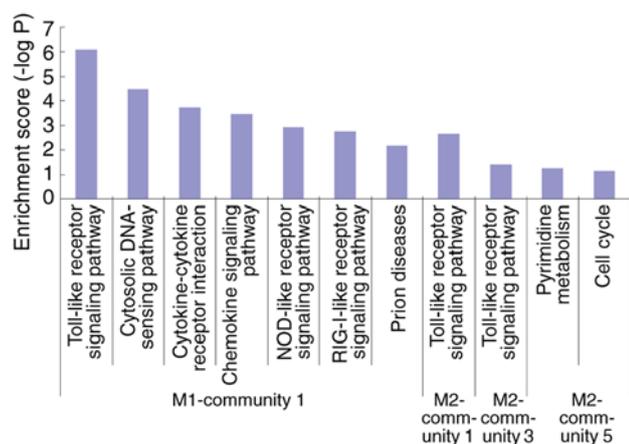
Figure 4. Kyoto Encyclopedia of Genes and Genomes enrichment analysis of the three communities of the M1 group and the five communities of the M2 group. The functions of community 1 of the M1 group and communities 1 and 3 of the M2 group were primarily enriched in Toll-like receptor signaling pathway proteins. NOD, nucleotide oligomerization domain.

it is critical to identify functional modules in the PPI network for analysis with the aim of identifying cell functions (30). The subsequent modular and KEGG enrichment analyses of each module demonstrated that the majority of the M1 and M2 modules were primarily involved in the TLR signaling pathway, suggesting that this pathway is involved in the regulation of macrophage M1 and M2 polarization.

Macrophages are the sensing cells of the immune system and are crucial mediators of the inflammatory response. TLRs, which serve an important role in the body's defense against specific pathogenic microorganisms, are the best-characterized inducers of acute inflammation (31). The initial characterization of enhancers involved in LPS-inducible gene expression in macrophages is based on the ability of stimulus-activated translational factors, including NF-κB and IRFs (32). The TLR/NF-κB and Janus kinase/STAT signaling pathways (33) are involved in the regulation of macrophage polarization.

In the majority of cases, the TLR/NF-κB signaling pathway promotes M1-type macrophage polarization when external microorganisms invade. However, this signaling pathway may additionally select the type of macrophage polarization based on the subunit composition of NF-κB. When NF-κB is activated in the form of p65/p50, macrophages increase pro-inflammatory cytokine production (34), and M1 macrophages are formed. When NF-κB is activated in the form of p50/p50, M2 macrophages are formed, which occurs in tumor-associated and LPS-tolerant macrophages (35). In addition, TLR serves an important role in the regulation of post-transcriptional polarization of macrophages.

In the present study, the macrophage polarization regulatory gene profile was efficiently extracted from the accessible online database and a PPI network map of M1 and M2 macrophages, based on genes demonstrating significantly upregulated expression identified by cluster analysis of differentially expressed genes was constructed. The network map demonstrated that the first 10 high-degree proteins in the PPI network of the M1-type polarized macrophages were STAT1, GBP1, GBP5, CXCL10, IFIT2, IRF7, IFIT3, ISG15, IRF1 and IFIH1. A PPI network map of M2-type polarized macrophages was constructed using the same method, and the first 10 high-degree proteins were CD14, ALOX5AP, MYD88, CDKN1A, S100A9, CYBB, CCNA2, S100A8, TK1 and TLR7. The majority of the high-degree proteins in the PPI network that are involved in the molecular regulation of macrophage polarization are closely associated with proteins involved in the TLR signaling pathway. It was identified that the TLR7 signaling pathway serves an important role in the regulation of macrophage polarization. These results are consistent with a previous study, which demonstrated that the TLR family regulates M1 and M2 differentiation, in addition to demonstrating the potential importance of TLR7 in virus-induced M2 polarization (36). Furthermore, other high-degree proteins identified during comparison not only help suggest the modulating role of the TLR pathway during macrophage polarization; however, may additionally function individually
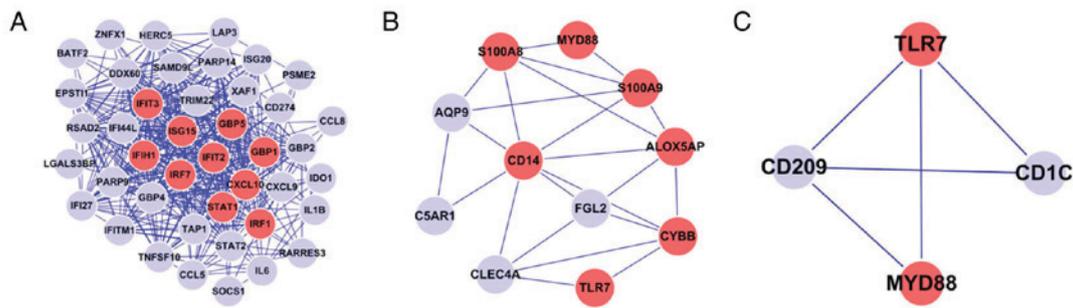
Figure 5. Sub-networks constructed based on the differentially expressed genes identified in the communities of interest in the protein-protein interaction network using CFinder software. The network is undirected, where a circular node represents a protein and a gray line represents an interaction between nodes. (A) A sub-PPI network was identified in the community 1 of the M1 group and is constructed of 42 protein nodes, 10 of which are colored red to indicate highest degree. (B) The PPI network consists of 11 proteins from community 1 of the M2 group, including seven of the previously identified highest degree proteins and 24 PPIs. (C) The PPI network derives from community 3 of the M2 group. PPI, protein-protein interaction.

in the program and link macrophage polarization to crucial biological programs, including immune response and malignancy progression.

In a previous study of mouse bone marrow-derived macrophages, macrophages were mock transfected and their transcriptional products analyzed (37). IFN responses were the primary reactions identified at 5 and 24 h after transfection, and the expression levels of IFN-mediated proteins (IFIH1, IFIT2 and IFIT3), the GBP protein family (GBP1 and GBP5), the transcription factor STAT1 and cytokine CXCL10 were detected. Increased expression of *IRF1* was additionally detected in the transcriptional products of macrophages stimulated by IFN-γ (37).

LPS, as well as IFN-γ and TNF Th1-type cytokines, mediate the classical macrophage activation pathway (33). It has been demonstrated that LPS mediates the expression of *IFIH1*, *GBP5*, *IRF1*, *IRF7*, *IFIT2*, *IFIT3* and *CXCL10* and that their expression is due to pro-inflammatory responses of M1 macrophages. The expression of a number of genes stimulated by LPS is mediated by IFN-γ and not IL-4 or IL-10. These genes include *GBP5*, *IFIH1*, *IFIT2*, *IFIT3* and *STAT1* (38). During the replication of *Orientia tsutsugamushi* in macrophages, the expression of genes involved in M1 macrophage polarization is upregulated. When macrophages are infected by *Orientia tsutsugamushi*, the expression of IFN-stimulated genes, including *CXCL10*, *IRF7* and *ISG15*, is increased (39). Exposure to LPS increases blood TNF levels via the canonical TLR4-associated NF-κB signaling pathway, resulting in inflammation. TLR4 recognizes LPS in the canonical NF-κB signaling pathway and initiates a signaling cascade. This leads to the activation of NF-κB and the expression of pro-inflammatory cytokines. Therefore, the complex transcriptional programme induced in macrophages following LPS stimulation is a product of the coordinated action of the transcription factors (40), and inhibition of the TLR4-NF-κB signaling pathway may shift M1 macrophage polarization toward the M2 phenotype.

The TLR signaling pathway upregulates the expression of pro-inflammatory gene products by activating STAT1 and NF-κB (41), thereby regulating M1 macrophage polarization. STAT1 has been demonstrated to be an important regulator of the biological responses of different TLRs. The expression of inflammatory cytokines mediated by TLR2, TLR4,

TLR7, TLR8 or TLR9 is suppressed in STAT1-deficient macrophages (42). In addition, activation of IRF3 is selectively coupled to TLR3 and TLR4 (43). Previous studies have demonstrated that IFN is involved in the regulation of TLR-triggered gene expression, including the TLR4 upregulation of *IRF7* expression through TLR-, activator- and interferon-mediated signaling pathways and the upregulation of *IRF1* expression through TLR9 (44,45). The present results further suggested that TLR7 serves as a mediator of M2 macrophage polarization. In contrast to the well-known macrophage polarization-associated TLR transmembrane family members, including TLR4, TLR7 is localized in the endosomal compartment, along with TLR3/9, and is associated with the viral-induced immune response (46). A recent study demonstrated that Hepatitis C virus-induced TLR7 stimulation results in monocyte differentiation and M2 macrophage polarization (36), which is consistent with the present study and further suggests that TLR7 stimulated macrophage polarization associated with chronic liver disease pathogenesis.

A previous study demonstrated that CDKN1A, or p21, serves an important role in the production of IL-1β and the progression of inflammatory diseases (47). CDKN1A-deficient mice are more prone to endotoxic shock, and CDKN1A-deficient macrophages produce more IL-1β. CDKN1A was additionally demonstrated to suppress the stimulatory effect of macrophages on inflammatory stimulation factors, and CDKN1A-deficient macrophages produce more inflammatory response factors, which promote M1 macrophage polarization (47). In contrast, the presence of CDKN1A regulates M2 macrophage polarization. It was identified that CDKN1A inhibits the activation of macrophages by TLR (47). The carboxyl-terminal domain of CDKN1A inhibits macrophage function by enhancing protein kinase B phosphorylation, which suppresses p38 activation, thereby inhibiting the production of inflammatory cytokines in macrophages activated by TLR (48). Consequently, CDKN1A limits macrophage activation in inflammatory reactive diseases, including rheumatoid arthritis. Previous studies demonstrated that CDKN1A-deficient macrophages exhibit enhanced activation by TLR agonists compared with control macrophages, regardless of the study background (47,49), which confirms the inhibitory effect of CDKN1A on TLR.

CCNA2 is essential for the initiation of DNA replication, transcription and regulation of the cell cycle and has been

reported to be a key regulator of cell differentiation (50). It was reported that the expression of microRNA (miR)-125b is downregulated during macrophage TLR4 signaling (51). TLR4 activates macrophages to produce pro-inflammatory cytokines; whereas, mmu-miR-125b reduces the production of nitric oxide in activated macrophages, promotes the growth of tumor cells, and, at least partially, inhibits the expression of CCNA2 (52).

MYD88 is an adapter protein that transduces intracellular signals induced by TLRs and IL-1 receptors (IL-1Rs) and serves a critical role in TLR/IL-1R-mediated immune responses (53). It has been demonstrated that MYD88 is essential for most TLR signaling pathways (54) and serves an important role in the activation of the signaling pathways induced by all TLRs/IL-1Rs (except TLR3) (55). Therefore, MYD88 is a suitable target for abnormal regulation of the TLR/IL-1R signaling pathways under pathological conditions (56,57). The absence of MYD88 in macrophages leads to a reduction in pro-inflammatory cytokine production mediated by TLR2, TLR5, TLR7 and TLR9 (58-61).

In conclusion, the present study used bioinformatics analysis to demonstrate that the TLR signaling pathway serves an important role in the regulation of macrophage polarization and that the high-degree proteins in the PPI network involved in molecular regulation of macrophage polarization are closely associated with proteins of the TLR signaling pathway, suggesting that the TLR signaling pathway will be an important direction for future studies of macrophage function in a systematic view. The aforementioned proteins that were identified may serve as a primary focus and may provide a useful reference for the intervention and regulation of macrophage polarization.

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Availability of data and materials

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

BM, YY, ZL, DZ and WZ analyzed the Gene Expression Omnibus database, conducted the cluster analysis of differentially expressed genes and constructed the protein-protein interaction networks. BM and YY were primary contributors in writing the manuscript. YJ and DX conceived the study. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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