The transcriptional profiling identifies hub genes in immune subsets of patients with Behçet's syndrome

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Abstract

Objective

Behçet's syndrome (BS) is a variable vessel vasculitis characterised by heterogeneity of organ manifestations. Antigen-presenting cells, such as macrophages and T cells, play critical roles in their immunopathology. This study aimed to identify hub genes and biological processes in patients with BS.

Methods

We downloaded expression profiles, GSE61399, containing CD14$^+$ monocytes and CD4+T cells between BS and healthy controls from the Gene Expression Omnibus (GEO). We screened the differential expression genes (DEGs) by the GEO2R. The Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses were performed. Protein-protein interaction (PPI) network and core genes were analysed by the Search Tool for the Retrieval of Interacting Genes (STRING) and Cytoscape with Molecular Complex Detection (MCODE) plug-in tools.

Results

We identified 102 DEGs in CD14$^+$ monocytes and 48 in CD4$^+$ T cells. In monocytes, the gene enrichment was mainly involved in type I interferon signaling pathway, defense response to virus, cell chemotaxis, granulocyte chemotaxis, granulocyte migration, leukocyte chemotaxis, and neutrophil chemotaxis. The changed genes in CD4$^+$ cells were enriched in MyD88-dependent toll-like receptor signaling pathway, positive regulation of innate immune response, and IL1B production. In combination with PPI and Markov Cluster Algorithm (MCL), we defined three driving protein-protein modules, IL1B, CCL2, CCL4, CXCL2, CCL20, CXCL3, TLR6, CD83, IFIT3, and THBD as a set of hub genes in CD14$^+$ monocytes, associated with inflammation and thrombosis; CD300LF, CLEC5A, DMXL2, MS4A14, TMEM176A in CD4$^+$ T cells.

Conclusion

Our findings provide novel insights into the immune subsets related to the biological process in BS, which could contribute to identifying potential biomarkers and novel treatment strategies for BS.

Key words

Behçet's syndrome, bioinformatics analysis, differentially expressed genes, hub gene, CD14$^+$ monocytes, CD4$^+$ T-cells

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Introduction

Behçet's syndrome (BS) is a complex, variable vessel vasculitis. Patients usually present recurrent oral aphthae, genital ulcers, skin lesions, and ocular involvement (1). Demographic features and major organ involvement reflect heterogeneous characteristics (2, 3).

The specific aetiopathogenesis of BS remains elusive. The hypothesis is that environmental factors, such as infectious agents, trigger individuals with genetic backgrounds, such as human leukocyte antigen (HLA)-B51, eventually leading to systemic inflammation. Genome-wide association studies (GWAS) have revealed other gene loci, such as *IL10, IL23R-IL12RB2, CCR2*, and *STAT4* (4-7). T cells, innate immune cells, such as neutrophils, macrophages, and predominant cytokines, are the critical regulators of immune dysregulation (4, 5, 8-13). Inflammation promotes haemostasis and *vice versa* in BS (14).

Bioinformatics analysis identifying the molecular modules driving disease progression can significantly deepen the understanding of the mechanisms and provide helpful information for targeted therapies (15). The protein-protein association network is one of the most useful and broadly scoped network types. It encompasses all protein-coding genes in a given genome and highlights their functional associations (16). The expansion of protein-protein interaction databases and recent advances in RNA detection technologies have enabled routine analysis of intercellular signalling from gene expression measurements (17). Thus, bioinformatics analysis allowing for the construction and analysis of these networks at a tissue- or cell-type-specific resolution, has become a standard tool performed across diseases to identify and quantify (18).

Due to the lack of deep exploration of molecular mechanisms (19), interaction networks, and critical pathways at immune subset levels of BS, we aimed to uncover hub genes' biological functions and networks from the transcriptome data.

We selected the GSE61399 profile from the Gene Expression Omnibus (GEO) database and identified the differentially expressed genes (DEGs) by GEO2R. Subsequently, gene ontology (GO) and Kyoto Encyclopedia of Genes and Genome (KEGG) pathway analyses were performed, and a protein-protein interaction (PPI) network was constructed. Our findings on the hub genes and key pathways associated with inflammation and thrombosis may provide insights into diagnosis and treatment.

Methods and materials

Study design and data processing

The three microarray datasets (GSE61399 (20), GSE17114 (21)) and GSE48498 of patients with BS or Kawasaki disease and healthy control samples were collected from the GEO database (https://www.ncbi.nlm.nih.gov/geo/). GSE61399 contains a gene expression profile from two cell types sorted from PBMCs: CD4+ and $CD14^+$; The $CD4^+$ has three controls and nine patient samples; The $CD14^+$ data set included nine healthy controls (HC) and eight patient samples. Based on GeneChip Human Genome U133 Plus 2.0 microarrays (Affymetrix, Santa Clara, CA, USA) were used in differentially expressed genes (DEGs) analysis.

We carried out GO, KEGG pathway enrichment analysis, PPI network analysis, and graph clustering to find the possible functions for molecules of similarity. We evaluate their roles as biomarkers for the diagnosis and validate them in GSE17114 (14 controls and 15 BS patients) (Fig. 1). The Ethics Committee of the Huadong Hospital, Fudan University, approved this study.

DEGs selection

GEO2R (http://www.ncbi.nlm.nih.gov/geo/geo2r) was applied to perform DEGs analysis between BS and control groups. DEGs were deemed as genes with a p-value <0.05 and $|\log FC| \geq 1.0$.

GO and KEGG enrichment analysis

Functional enrichment of DEGs was analysed in the GO three domains: biological process (BP), cellular component (CC), molecular function (MF), and the KEGG pathway database. We analysed significantly upregulated and downregulated DEGs using online software (Enrichr, http://amp.pharm.mssm.

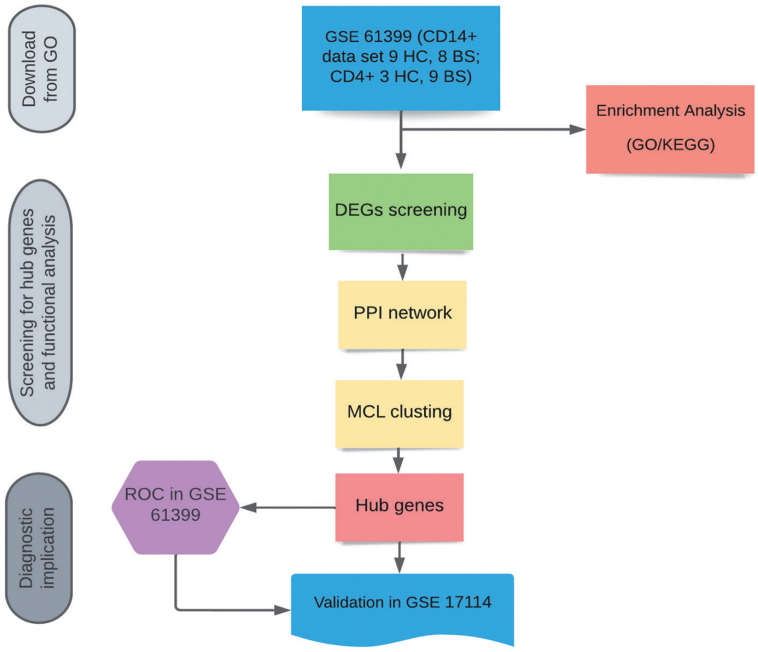

Fig. 1. Flow diagram of exploring hub genes and diagnostic markers in BD.

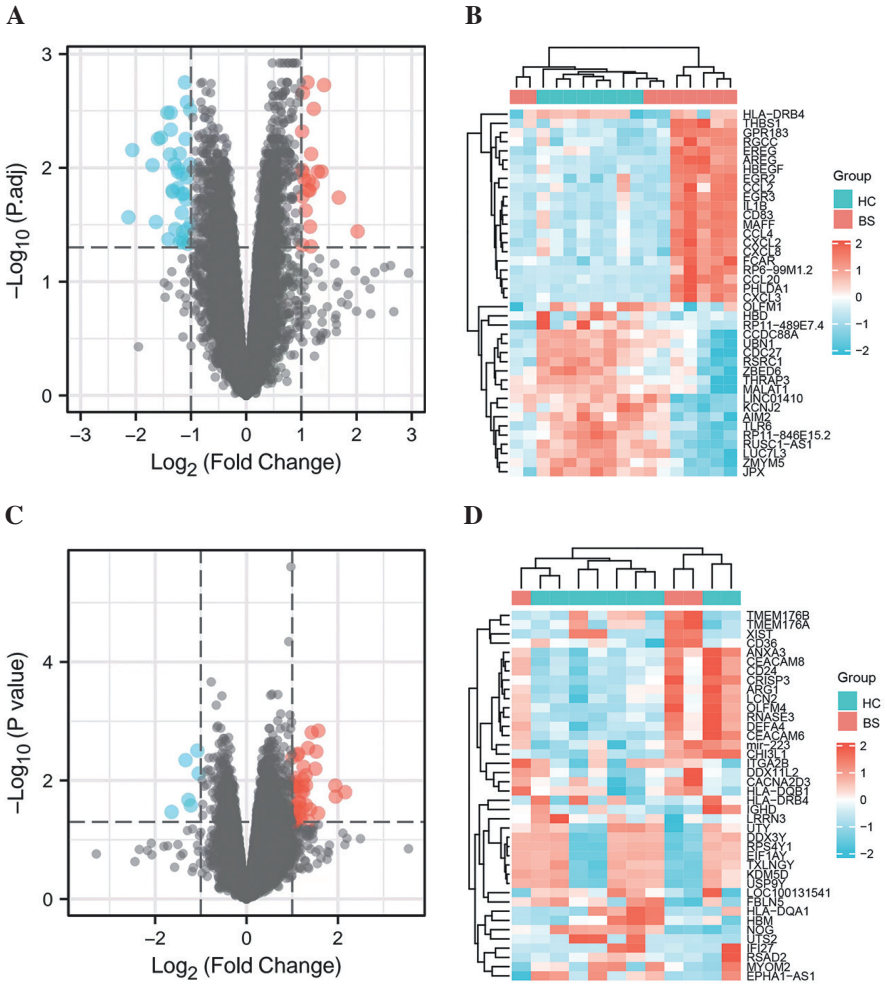

Fig. 2. Differentially expressed genes (DEGs) in CD14+ monocytes are displayed by volcano plot (**A**) and heatmap (**B**). DEGs in CD4+ T cells are shown by volcano plot (**C**) and heatmap (**D**).

edu/Enrichr/). An enriched term was considered, with a *p*-value <0.05.

PPI establishment and identification of hub genes

We evaluated the PPI network by Search Tools for the Retrieval of Interacting Genes, STRING v. 11.5, https://string-db.org/, using a confidence score >0.40 to screen the PPI pairs. (22)

We used Markov Cluster algorithm (MCL) to identify clusters in the PPI network to generate the similarity matrix of functional modules. After executing the clustering, closely functional related genes were grouped into the same cliques as candidate modules.

We applied CytoHubba and then extracted the key Hubba nodes ranked by maximum clique centrality (MCC) in Cytoscape software. (23) Furthermore, we screened the top ten genes ranked by the combined score in the PPI network and node genes filtered in CytoHubba analysis, together with genes exported using the MCODE programme. (24)

Diagnostic prediction and validation of hub genes

ROC curves were rendered using the R package "pROC", and DEGs between BS and HC were visualised using "ggpubr." To validate the expression pattern and diagnostic value of the selected hub genes, we verified the performance of the combination of critical genes in differentiating patients with BS from HC in GSE17114 by ROC analysis.

Results

Identification of DEGs in immune subsets

The volcanic map and the circular network analysis map obtained with Metascape showed that the distribution of their DEGs is different. From CD14+ cells, 102 DEGs were identified, including 54 up and 48 down-regulated genes (Fig. 2A-B). In CD4+ T cells, 48 DEGs were identified, including 42 up and six down-regulated genes shown in the volcano and heat maps (Fig. 2C-D).

GO function and KEGG pathway enrichment analyses of DEGs

We performed functional enrichment analysis to analyse the biological

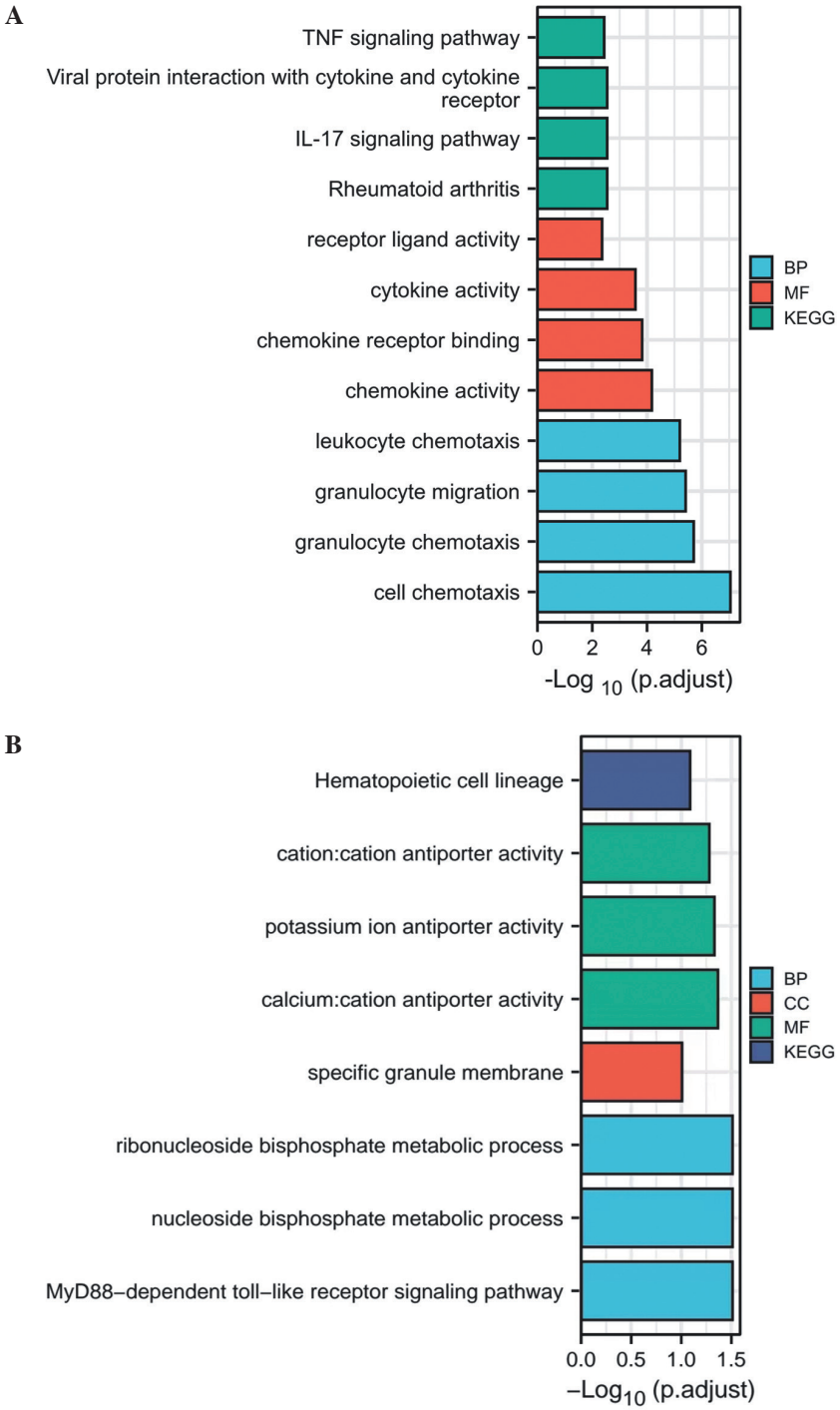

Fig. 3. (**A**). Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) annotation of DEGs.
(**B**). Histogram of GO and KEGG analysis of DEGs in CD14⁺ monocytes. (B) Histogram of GO and KEGG analysis in CD4⁺ T cells. Biological process (BP), cellular component (CC), molecular function (MF).

classification of DEGs. GO analysis showed that the downregulated genes from CD14⁺ cells were mainly enriched in type I interferon signalling pathway, cellular response to type I interferon, and defense response to virus. In contrast, the upregulated genes were significantly involved in cell chemotaxis, granulocyte chemotaxis, granulocyte migration, leukocyte chemotaxis, and neutrophil chemotaxis. KEGG pathway analysis indicates that the downregulated genes were mainly enriched in tyrosine metabolism. In contrast, the upregulated genes were primarily enriched in the IL-17 signalling pathway, rheumatoid arthritis, viral protein interaction with cytokine and cytokine receptor, TNF signalling pathway, and cytokine-cytokine receptor interaction. Myd88-dependent toll-like receptor signalling pathway, regulation of toll-like receptor signalling pathway, and regulation of macrophage cytokine production were enriched in CD4⁺ T cells (Fig. 3A-B).

PPI network construction, module analysis and identification of hub genes

In CD14+ monocytes, the PPI network contains 91 nodes and 94 edges, with an average node degree of 2.07 and avg. local clustering coefficient 0.468, PPI enrichment *p*-value <1.0e-16.

We identified three driver modules based on the PPI network's Markov Cluster Algorithm (MCL) clustering (Fig. 4A): the first module contains *AIM2, CCL20, CD83, IL1B*, and *TLR6*, enriched in natural killer cell chemotaxis regulation, IL1B production, positive regulation of endothelial cell apoptotic process, T cell migration, and Neutrophil chemotaxis (Fig. 4B). The second module includes *EPSTI1, IFI27, IFI6, IFIT3, RTP4*, and *ZBP1*. The gene enrichment study indicted type 1 interferon signalling pathway, defense response to virus, and innate immune response. The third module consists of *EGR2, EGR3, NR4A1, NR4A3,* and *TRIB1*. This module's members are enriched in the cellular response to corticotropin-releasing hormone stimulus, cell migration involved in sprouting angiogenesis, and cellular response to vascular endothelial growth factor stimulus.

In CD4+ T cells, the PPI network contains 40 nodes and ten edges, with an average node degree of 3 and avg. local clustering coefficient 0.354, PPI enrichment *p*-value = 0.000654.

Based on MCLclustering of the PPI network, the driver module involving *CD300LF, CLEC5A, DMXL2, MS4A14,* and *TMEM176A*, is enriched in the negative regulation of the myd88-dependent toll-like receptor signalling pathway (Fig. 4C).

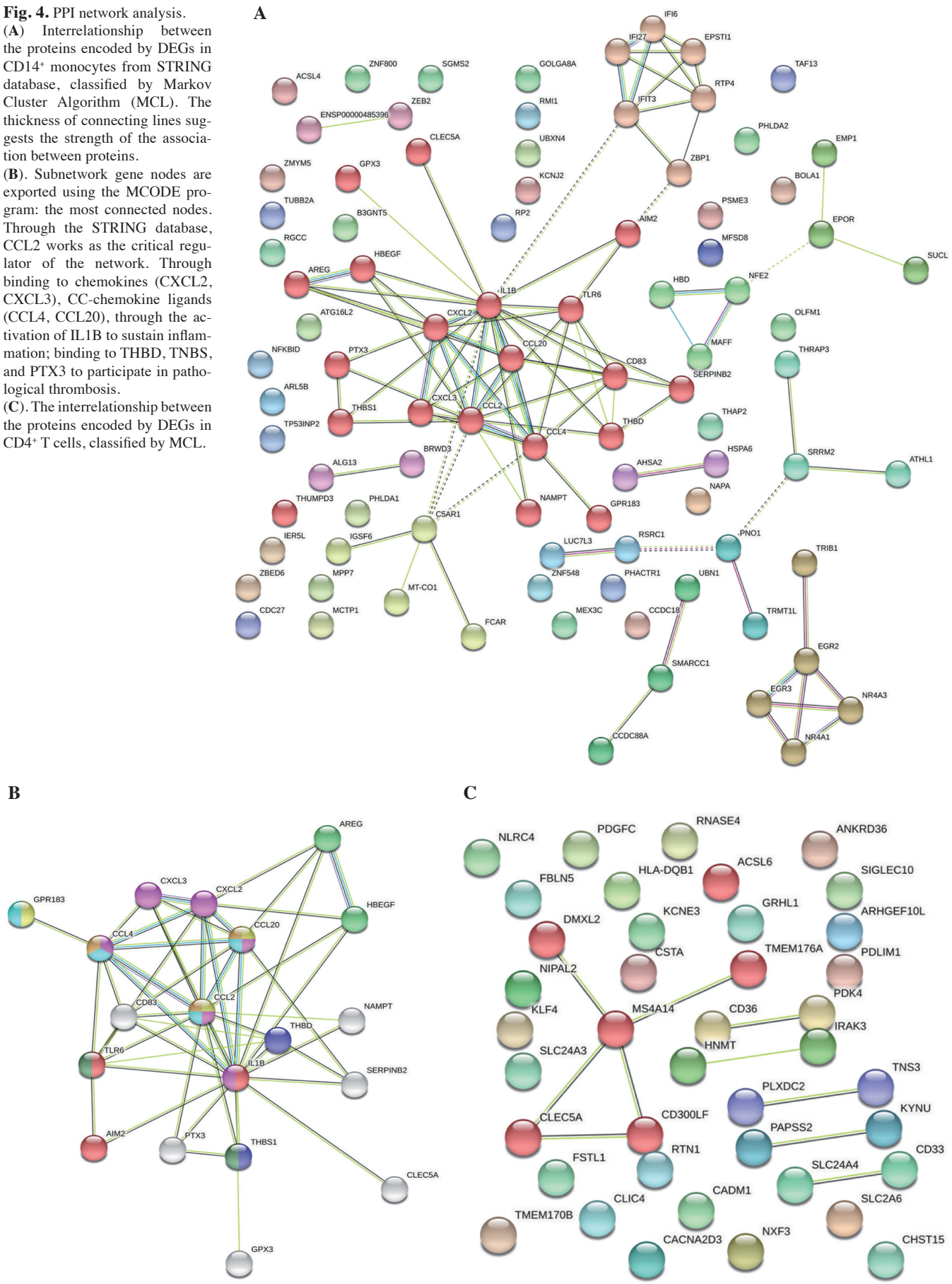

Fig. 4. PPI network analysis.
(**A**) Interrelationship between the proteins encoded by DEGs in CD14$^+$ monocytes from STRING database, classified by Markov Cluster Algorithm (MCL). The thickness of connecting lines suggests the strength of the association between proteins.
(**B**). Subnetwork gene nodes are exported using the MCODE program: the most connected nodes. Through the STRING database, CCL2 works as the critical regulator of the network. Through binding to chemokines (CXCL2, CXCL3), CC-chemokine ligands (CCL4, CCL20), through the activation of IL1B to sustain inflammation; binding to THBD, TNBS, and PTX3 to participate in pathological thrombosis.
(**C**). The interrelationship between the proteins encoded by DEGs in CD4$^+$ T cells, classified by MCL.

Table I. Hubba nodes are ranked by maximum clique centrality (MCC) in CytoHubba.

Rank	Name	Score
1	*IL1B*	257
2	*CCL2*	236
3	*CCL4*	199
4	*CXCL2*	162
5	*CCL20*	152
6	*CXCL3*	120
7	*TLR6*	74
8	*CD83*	72
9	*IFIT3*	27
10	*THBD*	26

By a combined score by CytoHubba and the Molecular Complex Detection (MCODE) in Cytoscape, we filtered the top 10 hub genes *IL1B, CCL2, CCL4, CXCL2, CCL20, CXCL3, TLR6, CD83, IFIT3*, and *THBD* (Table I).

Diagnostic performance of hub genes and validation

We used receiver operator characteristic (ROC) analysis to independently examine the performance of six essential genes in diagnosing patients with BS from healthy controls (HC) in GSE61399. The area under the curve (AUC) ranged from 0.72 to 0.94 (Fig. 5A). We performed the combinations of the top three hub genes in GSE61399, with an AUC of 0.96, CI 0.88-1.00 (Fig. 5B). Additionally, we test three hub genes separately and in combination for the diagnostic value in the validation cohort (GSE17114, Fig. 5 C-D).

We found that CCL2, CXCL3, and CD83 from the hub genes could become the potential diagnostic markers for BS to differentiate from Kawasaki disease patients in the positive validation cohort (GSE48498), AUC ranged from 0.58 to 0.71 (Fig. 5E).

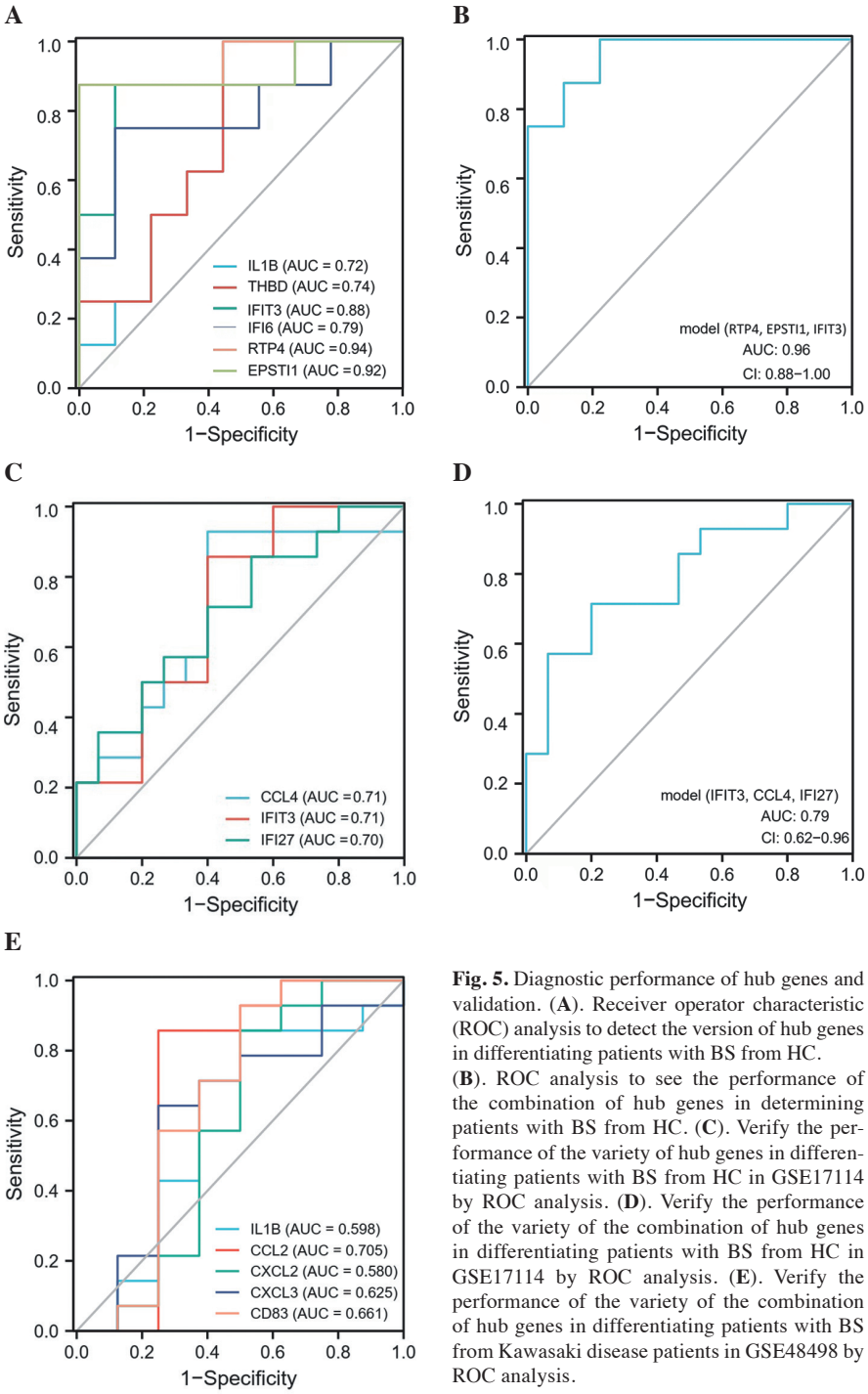

Fig. 5. Diagnostic performance of hub genes and validation. (**A**). Receiver operator characteristic (ROC) analysis to detect the version of hub genes in differentiating patients with BS from HC. (**B**). ROC analysis to see the performance of the combination of hub genes in determining patients with BS from HC. (**C**). Verify the performance of the variety of hub genes in differentiating patients with BS from HC in GSE17114 by ROC analysis. (**D**). Verify the performance of the variety of the combination of hub genes in differentiating patients with BS from HC in GSE17114 by ROC analysis. (**E**). Verify the performance of the variety of the combination of hub genes in differentiating patients with BS from Kawasaki disease patients in GSE48498 by ROC analysis.

Discussion

This study analysed immune subsets of BS patients of mRNA microarray data sets. A total of 150 DEGs were screened, with 48 DEGs in $CD4^+$ T cells and 102 DEGs in CD14 monocytes. Enrichment analysis of GO and KEGG indicated that in $CD4^+$ T cells, DEGs were mainly enriched in MyD88-dependent toll-like receptor signalling pathway, positive regulation of innate immune response, and IL1B production; While DEGs in $CD14^+$ cells mostly participated in type I interferon signalling pathway, cellular response to type I interferon, cell chemotaxis, granulocyte chemotaxis, granulocyte migration, leukocyte chemotaxis, and neutrophil chemotaxis. A single hub gene or a combination in $CD14^+$ monocytes could be biological marks for an accurate BS diagnosis.

Whether BS is a condition linking autoinflammation or autoimmunity is an ongoing debate (25). Hyperactivation of neutrophils and M1 macrophages are the features of BS (26, 27). Consistent with our findings, a recent mRNA sequencing study identified that most DEGs comprised an abundance of *CC*- and *CXC*-chemokines in leucocyte recruitment to peripheral tissues, especially neutrophils (28). Similarly, a previous microarray study in whole blood cells showed a strong enrichment of genes involved in

Table II. Functional enrichments in module 1 are classified by String Markov Cluster algorithm (MCL).

GO-term	description	count in network	strength	false discovery rate	bubble
GO:0032611	interleukin-1 beta production	3 of 15	2.31	0.00016	
GO:0051918	Negative regulation of fibrinolysis	2 of 10	2.31	0.0056	
GO:0045741	Positive regulation of epidermal growth factor-activated receptor activity	2 of 13	2.2	0.0084	
GO:0072678	T cell migration	3 of 32	1.98	0.0008	
GO:0030593	Neutrophil chemotaxis	6 of 74	1.92	1.60E-07	
GO:0048247	Lymphocyte chemotaxis	4 of 50	1.92	6.85E-05	
GO:0043032	Positive regulation of macrophage activation	2 of 25	1.92	0.0236	
GO:0002548	Monocyte chemotaxis	3 of 43	1.86	0.0015	

Table III. the enrichment process in Module 1 in CD14+ monocytes by the Search Tool for the Retrieval of Interacting Genes (STRING) analysis.

Term description	Observed gene count	Background gene count	Strength	False discovery rate	Matching proteins in your network (labels)
Regulation of natural killer cell chemotaxis	2	9	2.36	0.0047	CCL2,CCL4
interleukin-1 beta production	3	15	2.31	0.00016	IL1B,AIM2,TLR6
Negative regulation of fibrinolysis	2	10	2.31	0.0056	THBS1, THBD
Positive regulation of epidermal growth factor-activated receptor activity	2	13	2.2	0.0084	HBEGF, AREG
Positive regulation of nitric-oxide synthase biosynthetic process	2	16	2.11	0.0115	NAMPT, CCL2
Negative regulation of glycoprotein metabolic process	2	17	2.08	0.0124	HBEGF, PTX3
Positive regulation of endothelial cell apoptotic process	2	20	2.01	0.0161	CCL2, THBS1
T cell migration	3	32	1.98	0.0008	CCL2,CCL20,GPR183
Positive regulation of signalling receptor activity	3	36	1.93	0.0011	CCL2,HBEGF,AREG
Neutrophil chemotaxis	6	74	1.92	1.60E-07	CCL2,IL1B,CXCL3,CCL20, CXCL2,CCL4
Lymphocyte chemotaxis	4	50	1.92	6.85E-05	CCL2,CCL20,GPR183,CCL4
Positive regulation of macrophage activation	2	25	1.92	0.0236	THBS1, TLR6
Glial cell activation	3	41	1.88	0.0014	NAMPT,IL1B,TLR6
Monocyte chemotaxis	3	43	1.86	0.0015	CCL2,CCL20,CCL4
Positive regulation of nitric oxide biosynthetic process	3	44	1.85	0.0016	IL1B,PTX3,TLR6
Regulation of fatty acid transport	2	30	1.84	0.0309	THBS1, IL1B
Chemokine-mediated signaling pathway	5	80	1.81	8.46E-06	CCL2,CXCL3,CCL20,CXCL 2,CCL4
Microglial cell activation	2	32	1.81	0.0337	NAMPT, TLR6
Positive regulation of interleukin-2 production	2	32	1.81	0.0337	IL1B,CD83
Negative regulation of cell junction assembly	2	32	1.81	0.0337	THBS1, IL1B
Lipopolysaccharide-mediated signalling pathway	2	34	1.78	0.0363	CCL2, IL1B
Protein kinase b signaling	2	38	1.73	0.0426	CCL2, IL1B
Positive regulation of lymphocyte migration	2	38	1.73	0.0426	CCL20,CCL4
Positive regulation of wound healing	3	59	1.72	0.0031	HBEGF,THBS1,THBD
Cellular response to amyloid-beta	2	39	1.72	0.0444	NAMPT, TLR6
Leukocyte chemotaxis	7	142	1.71	1.28E-07	CCL2,IL1B,CXCL3,CCL20, GPR183,CXCL2,CCL4
Regulation of lymphocyte migration	3	65	1.68	0.0039	CCL2,CCL20,CCL4
Positive regulation of phagocytosis	3	66	1.67	0.004	CCL2,IL1B,PTX3
Cell chemotaxis	8	204	1.61	3.99E-08	CCL2,HBEGF,IL1B,CXCL3, CCL20,GPR183,CXCL2,CCL4
Positive regulation of reactive oxygen species metabolic process	4	103	1.6	0.00055	THBS1,IL1B,PTX3,TLR6

inflammation, IL- and TLR-signalling, with high levels of chemokines (*e.g. CXCL1, CCL2, CCL3, CXCL8*) at protein levels (29).

The crosstalk between innate and adaptive immune subsets drives chronic inflammation, which depends on a complex web of functional associations between biomolecules. Protein-protein interactions are critical due to their versatility, specificity, and adaptability. By STRING database, we observed a set of hub genes, including *CCL2*, and *CCL20*, enriched in neutrophils, T cells migration; *IL1B, CCL2, CCL20, CXCL2*, and *CXCL3*, are involved in neutrophils chemotaxis (Fig. 5E, Table II). We could assume that the immune subsets form a sustained inflammatory condition through chemokine signalling.

Despite continuous efforts to characterise the biological background of BS, our understanding of the pathogenesis is still limited. BS may lie at the crossroad between autoinflammatory and autoimmune syndromes (30). Autoinflammatory and autoimmune diseases share many characteristics, as they are systemic diseases with a pathological process directed against the self-anti-

gen, frequently involving multiple organs indicating immune system overactivity. Two main mechanisms could trigger a sustained immune response. Initially, infection or alarm signals of injured host cells activate the innate immune system and directly causes tissue inflammation. In contrast, dysregulation of innate and adaptive immunity is operative in the latter.

The central role of innate immunity has been suggested by DEGs enriched in MyD88-dependent toll-like receptor signalling pathway (activates *NF-κB* and *MAPKs* for the induction of inflammatory cytokine genes), positive regulation of innate immune response and IL1B production, type I interferon signalling pathway, cellular response to type I interferon, cell chemotaxis, granulocyte. In addition, IL-1 gene cluster polymorphisms are related to BS risk, contributing to enhanced inflammatory reactivity (31).

Inflammation-induced thrombosis is another feature of BS. Generally, neutrophil activation promotes fibrinogen oxidation and thrombus formation. We observed three critical upregulation genes, *thrombomodulin (THBD), thrombospondin 1 (THBS1),* and *pentraxin 3 (PTX3)*, involved in platelet aggregation, platelet-endothelial interactions, angiogenesis, tissue remodelling.

There are several advantages and limitations in the present study. As for the strength, we identified DEGs in the mRNA profiles from subsets of immune cells, which generated significant DEGs. The limitations include: first, due to the rarity of studies in mRNA expressions, we had just one data set of GSE61399. Second, DNA microarrays have limited probe availability, lower detection of splice junctions, and rare or novel transcripts. Third, we aimed to identify a diagnostic marker in discerning BS patients from healthy subjects and other systemic vasculitides. Considering comparing data from the same platform, we included GSE17114 and GSE48498. However, The samples of cohorts were PBMCs, not $CD4^+$ T cells or CD 14+ monocytes, which could lead to diminished particular DEGs. In the present study, we employed a comprehensive bioinformatics analysis to explore the transcriptional profiles of BS. Recently, single-cell RNA sequencing revealed distinct T cells and monocyte subpopulations in patients with BS (32). In the future, we will use our validation cohort to study the hub genes in the context of single-cell level.

Conclusion

The study identified hub genes of inflammation and thrombosis pathways by bioinformatics analysis from gene expression profiles of immune cell subsets in BS. However, these preliminary findings of the complex transcriptional networks of BS deserve future studies to elucidate the mechanisms underlying the dysregulated expression of target genes and their exact roles in the immune context.

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