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Original Research Article

Molecular characteristics of the protective effect of Bacillus Calmette-Guerin against neonatal sepsis

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Abstract

Purpose: *To investigate the molecular characteristics and potential mechanisms underlying the immune-protective effect of Bacillus Calmette-Guerin (BCG) against neonatal sepsis (NS).*
Methods: *Data on transcriptome sequencing of BCG infection, NS, post-BCG infection sepsis, and the respective controls were retrieved from public databases. Systems biology screening was used to identify differentially expressed genes (DEGs) amongst the groups, and common DEGs with high connectivity were selected using protein-protein interaction network analysis.*
Results: *A total of 15 common DEGs were related to the expressions of macrophages and neutrophils after BCG infection and NS infection. Some of the genes were involved in IL-4/IL-13 signal pathway and neutrophil degranulation pathway, and they were highly correlated with the expressions of TNF-alpha, IL-6 and type I interferon-response signal pathway. This may reduce the poor prognosis of NS after BCG infection.*
Conclusion: *These findings provide insights into how BCG-induced trained immunity may provide protection against NS.*

Keywords: *Bacillus Calmette-Guerin (BCG), Neonatal sepsis, Trained Immunity, Differentially expressed genes (DEGs), Transcriptome sequencing data*

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INTRODUCTION

Neonatal sepsis (NS) is a globally recognized health challenge [1]. It is a serious cause of death in neonates [2]. Indeed, NS has been recognized by the World Health Organization as a threat that needs priority intervention for future global health care [3]. Early effective infection treatment is the key to good prognosis of NS [4]. Therefore, poor prognosis of NS may be prevented through independent treatment of non-pathogenic infection. Bacillus Calmette-Guerin vaccine (BCG), an attenuated strain of *Mycobacterium tuberculosis*, is usually administered to neonates in the early postnatal period [5]. Long-term studies have shown that in addition to the expected protective effect against *Mycobacterium tuberculosis*, BCG also has a

non-specific protective effect on the host [6]. This process involves the innate immune protection in sexual immune cells and T and B lymphocytes [7]. With SARS pandemic, the debate on whether BCG may influence immune response to other unrelated pathogens has re-emerged [8]. In a meta-analysis, postnatal vaccination with BCG reduced the mortality rate of NS patients during hospitalization by 54 % (95 % confidence interval, 2 – 78 %) [9]. This non-specific protection may be caused by DEGs that are shared after BCG infection and pathogenesis of NS, i.e., one gene may be involved in multiple biological pathways. However, the shared differential gene characteristics and molecular processes between BCG infection and NS involved in heterologous protection have not been elucidated. Therefore, given the significant impact of NS on public health, systems biology was applied in this study to investigate the differentially expressed genes shared by the body and other pathogens after BCG infection, as well as the immune-protective effect against NS after BCG infection. The aim was to provide a potential target that might be used for reducing the poor prognosis of NS.

METHODS

General information

The datasets GSE69686 [10], GSE14408 [11] and GSE98550 [12] were downloaded from the high-throughput expression database, i.e., Gene Expression Omnibus (GEO; http://www.ncbi.nlm.nih.gov/geo/). Then, gene expression data and relevant clinical information were analyzed. The GSE69686 dataset contained neonatal peripheral blood samples from 64 cases of NS caused by bacterial or viral infection (experimental group) and 85 cases of normal (control group). The GSE14408 dataset contained neonatal peripheral blood samples from 5 cases of BCG infection (experimental group) and 5 cases of non-BCG infection (control group). The GSE98550 data set contained peripheral blood from 6 infants who developed sepsis after BCG infection (experimental group), and 6 infants who developed sepsis without BCG infection (control group). All data were high-throughput sequencing results.

Screening for shared differentially expressed genes

Following the standardization of the downloaded dataset using the LIMMA package in R, the ClusterProfiler package was used to match the probes with the corresponding genes [13]. Differentially expressed genes (DEGs) in NS group and control group, as well as BCG infection and control group, were screened using the LIMMA package. The DEGs were screened with adj.p < 0.05 and |FoldChange| >1 as the threshold, and then the intersection of DEGs in the two datasets was selected.

To understand the interaction between common DEGs and mine the core regulatory genes, the STRING database was used to display the protein-protein interaction (PPI) [14], and the cut-off value of the interaction relationship between proteins was set at 0.35. The Cytohubba plug-in in Cytoscape software was used to select the common DEGs and sort them with topological network algorithm, and the key common DEGs were selected [15].

Impact of shared differential genes on immune response

To determine the impact of shared differential genes on the body's immune response, Gene Ontology (GO) enrichment analysis of key shared DEGs was first applied to ClusterProfiler package, Org.Hs.eg.db package and GOPlot package in R [16]. At the same time, enrichment analysis of key shared DEGs was carried out in the KEGG (http://www.kegg.jp) and Reactome (https://reactome.org) databases.

To understand the impact of key shared differential genes on immune cell response in the body, the GSEA package in R was used to analyze the expression of immune cells in NS and BCG infection groups [17]. Then, the relationship between key shared differential genes and immune cells was analyzed. The expression of key shared differential genes in immune cells were retrieved using correlation with ARCHS4 (https://amp.pharm.mssm.edu/archs4) [18].

Possible immuno-protection in sepsis after BCG infection

Studies have shown that increased amounts of cytokines such as TNF-α and IL-6 are produced when BCG is infected and then re-infected [19]. Therefore, the effect of TNF-α and IL-6 in the experimental group was analyzed in the GSE98550 data set. Then, the expressions of key DEGs shared between the experimental group and control group in the GSE98550 dataset were analyzed. Based on the results obtained, analysis was done on the relationship between the key shared DEGs and TNF-α, IL-6 and type I. The galluvial package in R [20] was used to establish the network relationship amongst immune cells, key shared DEGs and

signal pathways, to identify the possible immuno-protective effect of the key shared DEGs.

Statistical analysis

The SPSS 24.0 and R studio 4.1 software were used for statistical analysis, while the ggplot2 package in R was used for image generation and processing. Statistically significant differences were assumed at $p < 0.05$.

RESULTS

Screening of key shared differential genes

A total of 16,722 genes were identified in the GSE14408 dataset, amongst which were 394 DEGs between BCG infection and control group. As shown in Figure 1 A, the 394 DEGs comprised 122 up-regulated genes and 272 down-regulated genes. The heatmap of the GSE14408 dataset is shown in Figure 1 B.

A total of 18,860 genes were identified in the GSE69686 dataset, amongst which were 313 DEGs between NS and control group. The 313 DEGs consisted of 266 up-regulated genes and 47 down-regulated genes. These results and the heatmap for the GSE69686 dataset are shown in Figure 1 C and D, respectively. Figure 1 E shows that the GSE14408 dataset shared 25 DEGs with the GSE69686 dataset. The 25 DEGs comprised 4 co-upregulated genes and 21 genes that were downregulated in BCG infection but upregulated in NS. The results of PPI analysis on the 25 genes showed that there were 23 interacting proteins (Figure 1 F). The PPI results were imported into the Cytohubba plug-in of Cytoscape software, and the shared differential genes were sorted according to the principle of maximum centrality (Maximal Clique Centrality, MCC). A total of 15 highly connected hub genes were identified as the most important shared DEGs (Figure 1 G).

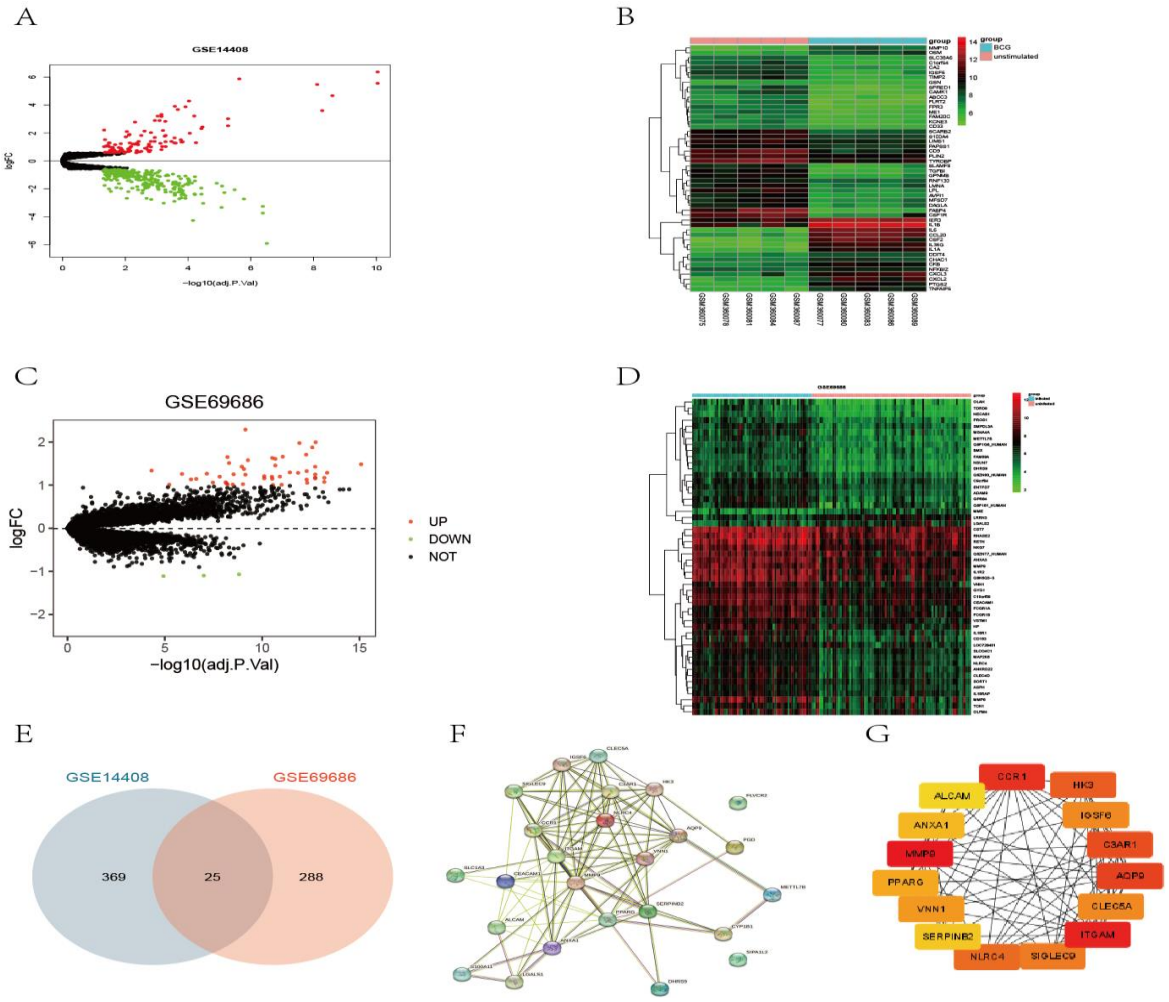

Figure 1: Screening of key shared DEGs. A: DEGs volcano map of GSE14408 dataset; B: DEGs heat map of GSE14408 dataset; C: DEGs volcano map of GSE68698 dataset; D: DEGs heat map of GSE68698 dataset. E: Common genes of DEGs in two datasets; F: PPI results. G: MCC showing the top 15 shared DEGs

Functions of key shared DEGs and their impact on immune response

The key shared DEGs were subjected to GO enrichment analysis. The molecular function (MF), cellular component (CC), and biological process (BP) are shown in Table 1. Pathway enrichment analysis was performed on the key shared DEGs. The enrichment results in KEGG and Reactome databases are shown in Table 2. The enrichment results suggest that the key shared DEGs are involved in signal pathways such as inflammation and post-stimulation immunity.

To further determine the effect of key shared differential genes on immune response, GSE69686 dataset and GSE14408 dataset were used to compare the immune cells in the groups. Secondly, analysis of the correlation between significantly changed immune cells and key shared DEGs was performed in order to understand the interaction between key shared DEGs and immune cells. The results showed that in GSE69686 dataset, NS caused significant and differential changes in immune cells except dendritic cells and mast cells (Figure 2 A and B). In the GSE14408 dataset, BCG infection caused significant and differential changes in macrophages, neutrophils, and Th1 cells (Figure 2 C and D). The major shared DEGs affected the expression levels of macrophages, neutrophils, granulocytes and Th1 cells (Figure 2 D), suggesting that NS induced a stronger immune response than BCG infection.

A search through ARCHS4 tissue revealed that 15 keys shared DEGs were expressed in macrophages and neutrophils. This is consistent with the changes after BCG infection, suggesting that macrophages and neutrophils may be the main immune cells that produce non-heterologous protective mechanisms after BCG infection.

Table 1: GO enrichment analysis of key shared differential gene

Ontology	ID	Description	*P*-value	Adjusted *P*-value	Gene
BP	GO:0043312	Neutrophil degranulation	0.000189	0.023638	ITGAM, MMP9, VNN1
BP	GO:0002283	Neutrophil activation involved in immune response	0.000193	0.023638	ITGAM, MMP9, VNN1
BP	GO:1902107	Positive regulation of leukocyte differentiation	9.39E-05	0.023638	ANXA1, CCR1, VNN1
CC	GO:0009897	External side of plasma membrane	6.78E-05	0.003932	ALCAM, ANXA1, CCR1, ITGAM
MF	GO:0098641	Cadherin binding is involved in cell-cell adhesion	7.16E-05	0.007233	ANXA1
MF	GO:0098632	Cell-cell adhesion mediator activity	0.000507	0.019048	ANXA1
MF	GO:0048306	Calcium-dependent protein binding	0.000754	0.019048	ANXA1

Table 2: Enrichment analysis of key shared differential gene signaling pathways

Pathway	ID	Description	*P*-value	Adjusted *p*-value	Gene
Reactome	R-HSA-6798695	Neutrophil Degranulation	6.455e-8	0.000007230	ITGAM, MMP9, VNN1, CLEC5A, C3AR1, SIGLEC9
Reactome	R-HSA-449147	Signaling by interleukins	0.0002130	0.005964	CCR1, ITGAM, MMP9, SERPINB2, ANXA1
Reactome	R-HSA-6785807	Interleukin-4 and interleukin-13 signaling	0.0003143	0.007040	ITGAM, MMP9, ANXA1
KEGG	map04610	Complement and coagulation cascades	0.0001593	0.01131	ITGAM, C3AR1, SERPINB2
KEGG	map04613	Neutrophil extracellular trap formation	0.001639	0.04058	ITGAM, AQP9, SIGLEC9
KEGG	map05134	Legionellosis	0.002295	0.04074	ITGAM, NLRC4

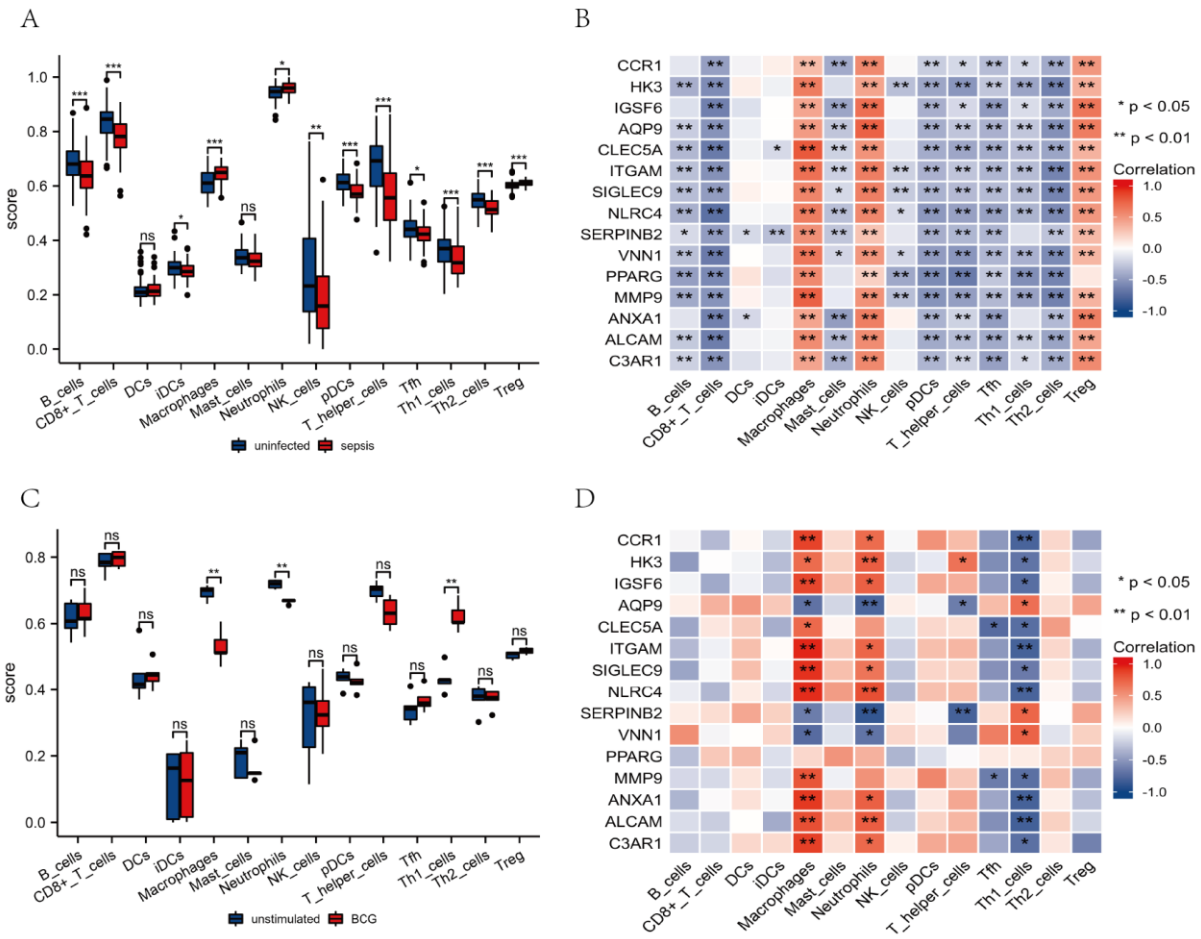

Figure 2: Effect of key shared DEGs on the immune response. A: Group expression of immune cells in GSE69686. B: Effect of key shared DEGs on immune cells in NS. C: Group expression of immune cells in GSE14408. D: Effect of key shared DEGs on immune cells after BCG infection. *P < 0.05; **p < 0.01; ***p < 0.001

Key shared DEGs may be involved in immune protection in sepsis after BCG infection

In order to determine changes in the expression levels of cytokines when sepsis occurred after BCG infection, the expression levels of TNF-α and IL-6 in the GSE98550 dataset samples were first compared. The results showed that sepsis occurred after BCG infection. The expression levels of the two cytokines, i.e., TNF-α and IL-6 were significantly higher than those in children with sepsis without BCG infection (Figure 3 A). Once again, this proved that the body raises the production levels of cytokines when sepsis occurs after BCG infection.

Secondly, a comparison of the group expressions of 15 key shared DEGs in the GSE98550 dataset samples showed no significant differences in ANXA1, PPARG, ALCAM and AQP9 between the experimental group and control group (p > 0.05). The remaining 11 key shared DEGs, in addition to SERPINB2, were significantly increased in sepsis after BCG infection, while the expression levels of other key shared DEGs were significantly decreased in sepsis after BCG infection (p < 0.05; Figure 3 B).

The enrichment analysis of key shared differential gene pathways showed that 6 genes (ITGAM, MMP9, VNN1, CLEC5A, C3AR1 and SIGLEC9) were involved in neutrophil degranulation and IL-4/IL-13 signaling. These genes were significantly correlated with the expression levels of TNF-α, IL-6 and type I interferon response (IFN-1 RESPONSE) (p < 0. 05; Figure 3 C), suggesting that the 6 key shared DEGs may be involved in immune protection when sepsis occurs after BCG infection.

The pathway enrichment of the key shared DEGs was combined with their expressions in immune cells in order to draw a network diagram showing the possible molecular characteristics and mechanism underlying the immune protection against NS after BCG infection (Figure 3 D).

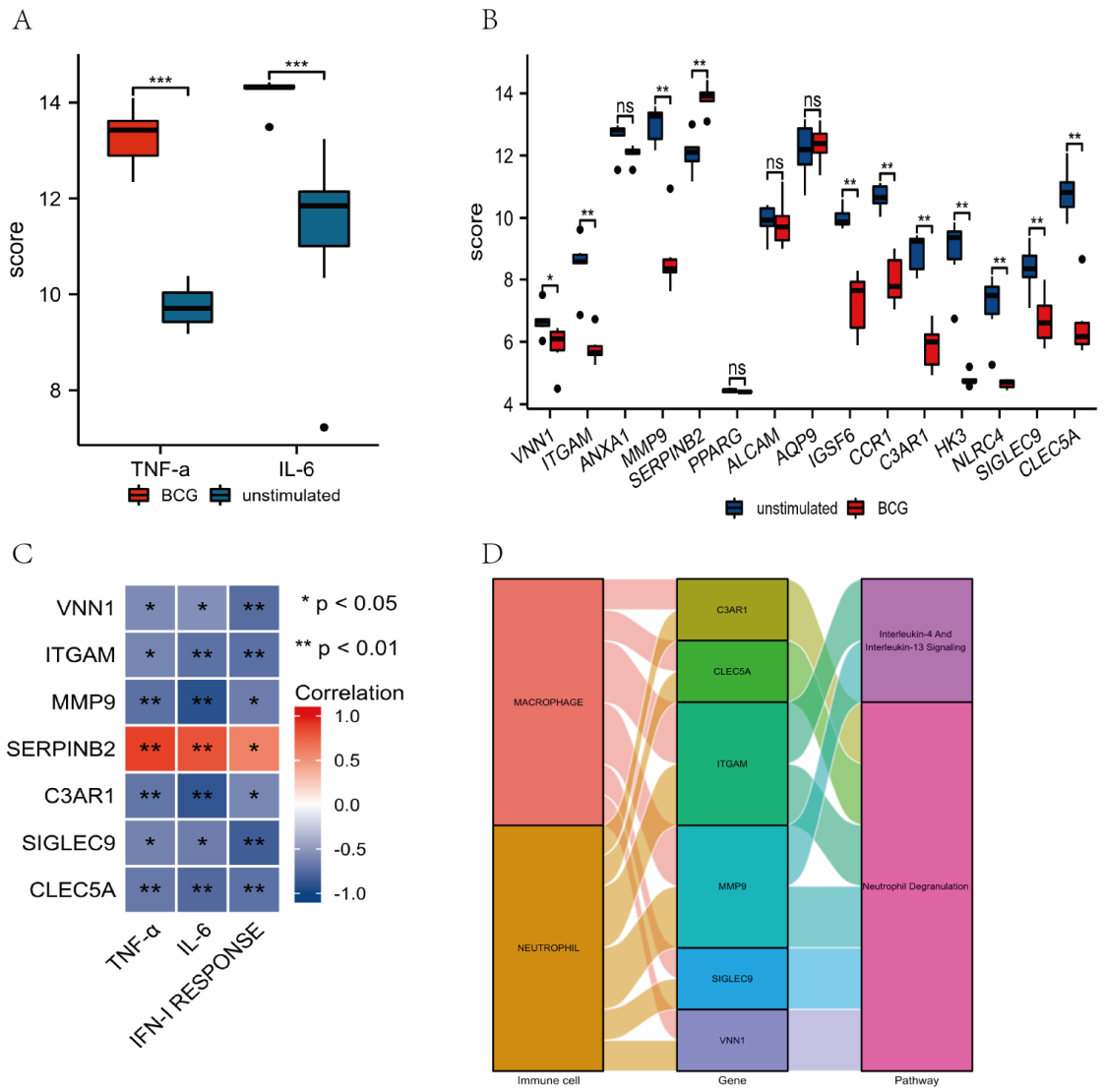

Figure 3: Validation of expressions of key shared DEGs. A: Comparison of expression levels of TNF-α and IL-6 after reinfection after BCG stimulation. B: Comparison of key shared DEGs between groups. C: Expressions of 6 key shared DEGs, expression levels of TNF-α and IL-6 and analysis of correlation with type I interferon signaling pathway. D: Pathway enrichment results of key shared DEGs and their expressions in immune cells. *P < 0.05; **p < 0.01; ***p < 0.001

DISCUSSION

Neonatal sepsis (NS) is still one of the major causes of severe neonatal complications in the NICU. The pathophysiology of the disease is relatively complex [21]. The vaccination of newborns with BCG not only effectively prevents disseminated tuberculosis, but also reduces neonatal mortality [22]. However, the mechanism of action of BCG is still unclear. Therefore, this study applied systems biology analysis and used the corresponding expression profile chip in the public database to identify 6 key shared DEGs that may be directly involved in the immune protection of NS after BCG infection.

The results of this study showed significant differences in the abundance of expressions of immune cells after BCG infection and NS in macrophages and neutrophils. After BCG, the macrophages and neutrophils showed low expression levels, while macrophages and neutrophils showed high expression levels in NS, suggesting that there are different immune response processes between BCG infection and NS. The decrease in the abundance of innate immune cells such as macrophages after BCG infection may be related to the induction of pyroptosis [23].

At present, it is believed that the molecular mechanism of heterologous protection induced by BCG is mainly realized by the joint participation of pathogen-associated molecular patterns (PAMP) and pattern recognition receptors (PRR) [24]. After BCG infection,

intracellular PRRs are involved in the protective activities of monocytes/macrophages against secondary infection in which large amounts of TNF-α and IL-6 are produced upon secondary stimulation by various PRR ligands [19]. This is also consistent with the results of the analysis of the GSE98550 dataset in this study. At the same time, in the GSE98550 dataset, the trend in the expression levels of some immune cells during sepsis after BCG infection was consistent with the corresponding trend after BCG infection, but it was opposite to the expression trend in NS, which also suggests that BCG infection has the same effect on the expression level of immune cells. The protective effect of NS may be related to the immune response produced by these immune cells. The group expression levels of 11 of the 15 key shared DEGs differed significantly in the GSE98550 dataset, which is different from the expression levels in NS. It can be speculated that the changes in expression levels of the key shared DEGs may be caused by BCG infection. Although these 11 genes belong to different families, they are all involved in immuno-regulation. Six of these genes were significantly enriched in the neutrophil degranulation and IL-4/IL-13 pathways. It has been shown that IL-4 and IL-13 are key cytokines for the induction and maintenance of type II inflammatory responses, and they induce Th2 T cell differentiation and M2 macrophage polarization [25]. In a study, an *in vitro* model of BCG-infected macrophages pre-stimulated with IL-4 and IL-13 was found to secrete pro-inflammatory cytokines when secondary stimulation with mycobacterial ligands was increased [26], indicating that it may be one of the mechanisms involved in BCG-induced immune protection.

In addition, the involvement of neutrophils in immuno-protection has been confirmed in another study [27]. Neutrophils play a role in immuno-protection by producing high levels of reactive oxygen species (ROS) through pre-stimulation, but this process requires the type I interferon response pathway as a regulator. However, in this study, the 6 key shared DEGs were significantly correlated with type I interferon signaling pathways. Again, this confirms that these genes may be involved in immune protection after BCG infection, thereby reducing the adverse effects of NS.

Limitations of this study

This study was based only on bioinformatics analysis. Therefore, the findings need to be confirmed through experimental studies on cells and experimental animals. Moreover, multi-omics studies should be considered for in-depth mining to reveal more comprehensively the mechanism underlying the immuno-protective effect of BCG against NS.

CONCLUSION

This study has revealed the possible molecular features and related mechanisms underlying the immuno-protective effect of BCG against NS. It has provided a new reference for heterologous protection by BCG and a potential target for the development of new neonatal protective vaccines.

DECLARATIONS

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Funding

None provided.

Ethical approval

None provided.

Availability of data and materials

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

Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

We declare that this work was done by the authors named in this article, and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Chen Kou designs of study, drafting of the initial manuscript, and revision of the manuscript. Chen Kou collected the GEO data and performed data analysis. Adong Shen supervised the data collection, and critically reviewed and revised the manuscript.

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