Verification of key genes and pathways explores the anti-inflammation mechanism of NR4a1on macrophage through bioinformatics analysis

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Abstract

NR4a1 has been demonstrated to exert a protective role in various chronic inflammatory disease. The underlying mechanism has yet to be clarified. This research aimed to explore the anti-inflammatory mechanism of NR4a1on macrophage through bioinformatics analysis and further verified by a series of experiments. In this study, The GSMs (GEO samples) that LPS(lipopolysaccharide) treated macrophage derived from wild type and NR4a1 knockout mice were extracted from GSE68167 gene expression profiles in Gene Expression Omnibus (GEO) database. DEGs (Differentially expressed genes) screened out. The gene ontology (GO) and KEGG (Kyoto Encyclopedia of Genes and Genomes pathway) enrichment were conducted. PPI (Protein-protein interaction) network that comprised 49 nodes was mapped. The enrichment of biological process (BP) was principally and significantly revealed in signal transduction, inflammatory response and positive regulation of chemokine production. In terms of KEGG pathway, DEGs were primarily enriched in Cytokine-cytokine receptor interaction, JAK-STAT signaling pathway and cAMP signaling pathway. In the PPI network, IL10, IFNG, Fos, IL19, PDE4B NPY, Cnr1, MMP13, Rtn1, UCHL1 were selected as Hub gene. PDE4B and cAMP signaling pathway may associate with anti-inflammatory function of NR4a1. Our data showed that NR4a1 could promotes the protein level of cAMP, p-PKA and decreased p-p65, p-IκBα through down-regulating PDE4B. NR4a1-downregulated PDE4B expression notably decreased the LPS induced mRNA level of IL-6 and IL1β. This study also pointed the potential physiological processes regulated by NR4a1.Our findings supports that NR4a1 may be a considerable therapeutic target in inflammatory disease.

Introduction:

Inflammatory diseases are being considered a serious issue, for which inhibiting the prolonged and excessive macrophage-regulated inflammatory processes can serve as a considerable therapy [1,2]. The orphan nuclear receptor NR4a1, also known as Nurr7, NGFI, NAK-1, has been recognized as a key regulator in inflammation [2,3]. NR4a1 in all kinds of inflammatory diseases has been investigated, such as atherosclerosis, inflammatory bowel diseases, and rheumatoid arthritis, exerting a protective role against inflammation [4]. Inflammatory stimulation could promote the NR4a1 expression via NF-κB in macrophage [5]. However, NR4a1 exerts the anti-inflammatory effects depending on the negative regulator for NFκB [6-8]. NR4a1-KO macrophage displayed the increased productive in pro-inflammatory cytokines and this up-regulation was decreased by NFκB inhibitor [9,10]. Over-expressed NR4a1 exhibits increased IKK expression,which is a inhibitory role in NFκB signaling[9,10]. NR4a1 can directly have effect on p65 to suppress neuroinflammation [9,10]. NR4a1 is also considered a regulator in metabolism during inflammatory responses of immune cells [11,12]. Despite the several previous study, the understanding of downstream genes of NR4a1 remains lacked [13].

To investigate the functioning mechanism of NR4a1 in macrophage, the GSE681767 were employed for analysis. The GO and KEGG enrichment were conducted in DAVID online database. Protein-protein interaction (PPI) network was mapped by Cytoscape. The results showed that PDE4B and cAMP signaling pathway may play key roles in anti-inflammatory function of NR4a1. PDE4B serves as a selectively hydrolytic enzyme of second signal messenger cyclic adenosine monophosphate(cAMP) [14], of which the deficiency represses activation of nuclear factor kappa-B (NF-κB) in various cell stimulated with lipopolysaccharide(LPS) [15]. cAMP has been proved to play an anti-inflammatory function, employed as a solution of inflammation [14,16]. cAMP-activated PKA can inhibit the NFκB signaling through prohibiting the degradation of IκBα[14,17]. Πρειους στυδιες ηαε δεμονστρατεδ τηε ςλοσε ρελατιον οφ NP4α1′ς αντι-ινφλαμματορψ φυνςτιον ωιτη τηε ινηιβιτεδ ΝΦκΒ signaling [7,18,19]. Thus, we assumed that NR4a1 may regulates PDE4B expression to exert anti-inflammatory function.

Method

Microarray date and DEGs screen

In this research, GSE68167, a gene expression profile based on GPL6887 Illumina MouseWG-6 v2.0 expression beadchip composed of 18 samples was extracted from GEO database(www.ncbi.nlm.nih.gov), which contains gene expression profiles [20]. From these samples, the macrophage extracted from NR4a1 deficit or wild type mice treated with LPS 8 hours were chosen for analysis by GEO2R. LogFc>1 and P<0.05 were set as threshold to screen out the significant DEGs.

Functional enrichment analysis

The DEGs were analyzed by DAVID database(https://david.ncfcrf.gov), an online tool providing a functional annotation of genes and discover enriched functional-related gene group [21]. The Gene Ontology (GO) constructs the annotation of genes, gene products and sequence [22]. KEGG pathway analysis was carried out to explore the gene linking functional and genomic information [23]. P<0.05 were considered significant.

Integration of protein-protein interaction network and Hubgenes

The relationships between DEGs were assessed using STRING online database(https://cn.string-db.org) PPI network mapped by Cytoscape(https://cystoscape) [24]. All nodes were ranked by Degree in cytoHubba.

Cell culture and Transfection

RAW264.7 cells obtained from Zhong Qiao Xin Zhou Biotechnology were cultured in DMEM supplemented with 10% fetal bovine serum, 100 units/mL penicillin and 100 g/mL streptomycin at 37 C in 5% CO2. siNR4a1 and plasmid were Designed and produced by IGE biotechnology. The sequence of siNR4a1: Forward:GCCAUUAGAUGAGACCUAUdTdT Reverse:AUAGGGUCUCA

UCUAAUGGGCdTdT. siRNA and plasmid Transfection was conducted by lipofectamine 3000 (Thermo Fisher Scientific). After 40 hours transfection, cells were stimulated by LPS for another 8 hours for further testing.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR) assay

According to the manufacturer, total RNA was isolated from RAW264.7 cell by TRIzol (Cwbio). RNA was translated into cDNA using PrimeScript RT Master Mix(Takara Bio). The qRT-PCR was run on the LightCycler® 480 Software using TB Green® Premix Ex Taq II (TaKaRa, Bio) with gene specific primers. Gene expression level was calculated employing the 2^{-Ct} method. The primer sequences were listed in *Table S1* .

Western Blot

RAW264.7 cell were transfected by siRNA or plasmid, hydrolyzed in RIPA lysis buffer for 30 minutes after stimulated with LPS for 8 hours. The BCA protein were employed to detect the protein concentration, then separated by 10% SDS-PAGE. Membranes were blocked in 5% milk for 1 hour and then treated

with antibodies overnight. The primary antibodies were: anti-PED4B antibody, anti-cAMP antibody, anti-NR4a1 antibody were bought from Abcam Technology, USA. Anti-PKA antibody, anti-phosphorylated-PKA antibody, anti-p65 antibody, anti-phosphorylated-65 antibody, anti-IκBα antibody, anti-phosphorylated antibody, anti-GAPDH antibody were purchased from Cell Signaling Technology, USA. After washed and incubated with mouse or rabbits secondary antibodies for 1 hour, the protein bands were detected using the enhanced chemiluminescence reagents.

Statistical analysis

All data were expressed as mean ±SD. One-way analysis of variance (ANOVA) was employed to compare the multiple groups. Statistic package for social science (SPSS) version. 13.0 was employed for statistical analysis. When P value < 0.05, the differences were considered significant.

Results

DEG identification, KEGG and GO Enrichment

As displayed in heat map *Fig S1* , there are 108 DEGs in the heat map. KEGG and GO enrichment of DEGs were fabricated using DAVID online database. As *Fig1 (A,B,C)*shows, GO consisted of biological process (BP), cellular components (CC), molecular functions (MF). BP analysis of DEGs mainly shows signal transduction, positive regulation of inflammatory response and chemokine production. For CC terms, DEGs were enriched mainly in cytoplasm, membrane, and cytosol; while in protein binding, protein kinase binding, Calcium ion binding and collagen binding for MF. KEGG pathway shows the enrichment of DEGs mainly in cytokine-cytokine receptor, JAK-STAT signaling pathway and cAMP signaling pathway (*Fig1 D*).

PPI network and Hubgenes

All DEGs were analyzed by STRING online database. Using the Cytoscape software, 49 DEGs were mapped to the PPI network (*Fig 2*). The top 10 Hub genes referred to IL10, IFNG, Fos, IL19, NPY, Cnr1, PDE4B, MMP13, Rtn1, UCHL1, which were assessed and ranked by the plugin cytoHubba in Cytoscape (Table2)**.** Phosphodiesterase 4B (PDE4B), a member of phosphodiesterase (PDEs) family, serves as a selectively hydrolytic enzyme of second signal messenger cAMP(cyclic adenosine monophosphate)[15,25], playing a vital role in signal transduction of inflammation[15]. The inhibitor of PDE4 has demonstrated a prominent validity in treating inflammatory disease such as asthma, chronic obstructive pulmonary disease, inflammatory bowel disease, atopic dermatitis, rheumatoid arthritis, and other disease[15,26]. cAMP-activated PKA exerts an effect to inhibit NFκB signaling pathway [10,27,28]. PDE4B deficit macrophage exhibit the decreased NFκB activation and reactive oxygen species level induced by LPS, and the suppressed inflammatory response [14,29,30]. The anti-inflammatory ability of NR4a1 has association with NFκB signaling pathway. Therefore, we speculate that NR4a1 may regulate the PED4B expression with anti-inflammatory function and a close relation of NR4a1-regulated PDE4B to cAMP-PKA signaling and NFκB signaling pathway.

NR4a1 promotes the activation of cAMP-PKA signaling through down-regulating PED4B expression.

Our previous results implied a key role of PED4B in NR4a1-regulated inflammatory macrophage. First, we established the NR4a1 silenced or over-expressed RAW264.7 macrophage by transfecting pcDNA3.1-NR4a1 or siNR4a1 (*Fig S1*). The expression of PDE4B was appraised by western blot and RT-PCR. As shown in *Fig3 (A,B),* It can be seen that LPS prominently elevated the protein and mRNA level of PED4B. Compared to the LPS group, pcDNA3.1-NR4a1 group exhibited the decreased PED4B expression and the NR4a1 silenced group showed the increased. We further evaluated the components of cAMP signaling pathway by western blot(*Fig2 C,D*). LPS stimulation could significantly decrease the cAMP, p-PKA protein level. pcDNA3.1-NR4a1group exhibited higher cAMP, p-PKA protein level and the siNR4a1 group showed lower. However, cotransfection of pcDNA3.1-PDE4B and pcDNA3.1-NR4a1 group exhibited the decreased cAMP, p-PKA protein level compared to pcDNA3.1- NR4a1 group(*Fig2 E,F*) .

NR4a1 συπρεσσες τηε ΛΠΣ ινδυςεδ αςτιατιον οφ ΝΦκΒ σιγναλινγ πατηωαψ ανδ τηε ΙΛ-6 ανδ ΙΛ-1β μΡΝΑ λεελ βψ δοων-ρεγυλατινγ ΠΕΔ4Β εξπρεσσιον

Our previous discussion has been illustrated that the activation of NFκB signaling pathway regulated by NR4a1 and cAMP activated PKA. NF-κB, acting as a transcription factor, could manipulate a number of proinflammatory cytokines expression such as IL-6 IL1β [31,32]. We further detected the effects of NR4a1 and PDE4B on LPS induced activation of NFκB and the expression of IL-6 and IL1β. As depicted in*Fig4(A,B,E),* pcDNA3.1NR4a1 group displayed the decreased protein level of p-p65 and p-IκBα and mRNA level of IL-6 and IL-1β and the siNR4a1 group showed higher compared to LPS treated group. However, the protein level of p-p65 and p-IκBα and mRNA level of IL-6 and IL-1β of co-transfected group were higher than the pcDNA3.1-NR4a1 group(*Fig4 C,D,F*).

Discussion

Previous studies has shown the noticeable anti-inflammatory effects of NR4a1. In this study, we explored the mechanism of NR4a1 through bioinformatics analysis. We employed the GSE68167 dataset and selected 108 DEGs to be analyzed using DAVID and KEGG pathway and GO term analysis. BP analysis mainly shows signal transduction, positive regulation of chemokine production and inflammatory response. CC terms displayed enrichment of DEGs mainly in cytoplasm, membrane, and cytosol; while MF indicated the protein binding, calcium ion binding and callagen binding. KEGG showed the enrichment of DEGs mainly in cytokine-cytokine receptor, JAK-STAT signaling pathway and cAMP signaling pathway. We used STRING and Cytoscape to construct the gene network, where 49 DEGs were mapped and top Hub gene

IFNG, IL10, Fos, IL19, PDE4B, Cnr1, MMP13, NPY, Rtn1, UCHL1 were ranked by CytoHubba. Among these Hub genes, IFNG encodes IFNγ, IL10 and IFNγ are considered the important inflammatory cytokine and NR4a1 defects macrophage showed the decreased IL10 while the increased IFNγ production [10,33,34]. NR4a1 regulates the expression of early gene, including Fos in cancer [35]. Fos together with c-jun forms AP-1 plays a key role in inflammatory type macrophage [36]. UCHL1 has been reported to exert the anti-inflammatory effects via MAPK and NFκB signaling pathway in LPS stimulated macrophage [37]. Phosphodiesterase 4B(PDE4B), a member of the phosphodiesterase (PDEs) family, serving as a selectively hydrolytic enzyme of the second signal messenger cyclic adenosine monophosphate (cAMP) to play a crucial role in cAMP signaling pathway [15,25]. PDE4B, NPY and FOS were found to be enriched in cAMP signaling pathway, where the former is critical in signal transduction of inflammation [15,25,30]. The inhibitor of PDE4B has been shown exert an effective validity in treating various inflammatory diseases [15]. PED4B defected macrophage shows the decreased LPS induced NFκB activation and reactive oxygen species level [30,38].

Our data demonstrated that NR4a1 showed the effects of increasing cAMP and p-PKA levels through down-regulating PED4B expression. We also found that NR4a1 can inhibit LPS induced NF-κB signaling activation and the mRNA level of IL-6 and IL-1β by down-regulating PED4B expression. The anti-inflammatory function of NR4a1 has been proved to be related to restrict NFκB signaling pathway [33,39] Interestingly, cAMP activated PKA could effectively inhibit the NFκB regulated gene transcription [10,27].Thus, we hypothesized that NR4a1 may regulates the NFκB signaling pathway through PDE4B-cAMP-PKA signaling, which is also worthy to further detecting. In addition, bioinformatics analysis showed that the effect of NR4a1 in macrophage may be related to Fos, UCHL1, MMP13. JAK-STAT signaling pathway and cytokine-cytokine interaction may be regulated by NR4a1.

Conclusion

Among the NR4a1 mediated several pathways, NFκB signaling lies one of the most crucial pathway. NR4a1 also manipulates the metabolism of inflammatory response of immune cells. In our study, we found that NR4a1 can promotes the cAMP signaling pathway and inhibits the LPS induced the activation of NFκB signaling and the mRNA level of IL-6 and IL-1β by down-regulating the PDE4B expression. Our study implied that NR4a1 plays a protective role with the potential to serve as an excellent therapy for inflammatory diseases.

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Author contributions

Juntao Tan : Validation, visualization and completed manuscripts. Teng Teng : Visualization and revised manuscripts. Chen Su, Tiegen Huang and Zhenni Xiao: Data curation. Xiaotian Luo: Supervision. Yixin Tang: Conceptualized projects and supervision.

Declaration of Competing Interest

None.

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