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Transcriptome of porcine alveolar macrophages activated by interferon-gamma and lipopolysaccharide

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ABSTRACT

The molecular repertoire of porcine alveolar macrophages (PAMs) is greatly affected by the microenvironment they are exposed to, and specifically by inflammatory cytokines, such as interferon gamma (IFN-γ) released by activated lymphocytes, and microbial products, such as lipopolysaccharide (LPS). In our previous study, we found that IFN-γ- and LPS-activated PAMs (M1) could inhibit porcine reproductive and respiratory syndrome virus (PRRSV) replication. In this study, comprehensive analysis of the expression profiles of the genes associated with the polarization of M0-type PAMs (resting) toward M1 phenotypes (activated by IFN-γ and LPS) led to the following main results: 1) 1551 and 1823 genes were upregulated or downregulated in M1-type PAMs, respectively, compared with M0-type PAMs; 2) Among these, genes encoding ASS1 and CRTAM were the most upregulated and downregulated, respectively; 3) Genes involved in cytokine-cytokine receptor interaction and the JAK/STAT signaling pathway were significantly upregulated, suggesting their critical role in cellular activation; and 4) Genes involved in antigen proteolysis and presentation (immunoproteasome subunits), and inhibition of virus replication (host restriction factors) were significantly upregulated, emphasizing the critical role of these cytokines in immunity. Thus, our results provide important information for future studies on the role of PAM polarization in modulation of infection.

1. Introduction

Functional diversity and plasticity are hallmarks of macrophages [1]. Polarized macrophages have both beneficial and detrimental roles in several diseases. Of particular interest is the classically activated polarized M1 macrophages induced by molecules, especially interferon γ (IFN-γ), and microbial stimuli such as lipopolysaccharide (LPS); the induction leads to *de novo* expression of genes that selectively alter macrophage functions in modulation of inflammation, infection, and tumor formation [2–4]. The protective role of M1 macrophages, which are associated with the control of infection, is beneficial during acute infections, such as those caused by *Listeria monocytogenes* and *Salmonella typhi* [5,6]; however, an excessive or prolonged M1 activation is deleterious for the host during many diseases, such as those caused by *Escherichia coli* and *Streptococcus sp.* [7].

Previous studies have analyzed expression profiles of genes during macrophage activation in humans and mice, that led to the identification of similarities and differences in the genetic program activated during macrophages polarization in the two species [8–10]. These studies demonstrated that on one hand, M1 macrophages, regardless of human or murine origin, are characterized by an IL-12^{high}, IL-23^{high}, IL-10^{low} phenotype, and are proficient producers of effector molecules, such as nitric oxide (NO) [11], and other pro-inflammatory cytokines, such as interleukin (IL)-1, IL-6, C-X-C motif chemokine (CXCL) 1–3, CXCL8-10, and type I IFN [12]. On the other hand, comparative studies have identified substantial differences in immune cell gene expression between human and mice. For example, differently from mouse macrophages, human macrophages do not strongly induce the expression of genes involved in arginine metabolism to produce NO in response to LPS [13]. To date, data on porcine mononuclear phagocytes are scarce. A study confirmed that upon LPS stimulation, the expression of lymphoattractant chemokines, such as CXCL9, CXCL11, and CXCL13, and proteins involved in tryptophan metabolism, such as

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indoleamine 2,3-dioxygenase (IDO) and kynurenic acid (KYN) is activated in pig bone marrow-derived macrophages (BMDM), as in human macrophages [13,14]. However, studying the molecular repertoire of other macrophages in response to different stimuli will provide important information for future studies on macrophage activation in pig, a species that is emerging as a comparable model of human innate immunity and disease.

Alveolar macrophages (AMs) play a critical role in the immediate defense against foreign agents and during adaptive immunity in the lung [15]. The activation and function of these cells is greatly affected by the microenvironment they are exposed to, and specifically by inflammatory cytokines released by lymphocytes, and microbial products. In our previous study, we have successfully established the model for *in vitro* porcine alveolar macrophage (PAM) polarization, and demonstrated that M1-type PAMs could inhibit porcine reproductive and respiratory syndrome virus (PRRSV) replication [16]. This observation suggests that cellular activation modulates host restriction and susceptibility to virus infection. Several studies reported that restriction factors were potent antiviral host proteins that confer protection against viruses, particularly retroviruses [17]. Additionally, the immunoproteasome is a highly efficient proteolytic machinery derived from the constitutive proteasome, and is crucial for the optimization of $CD8^+$ T lymphocyte-mediated immune responses during viral or bacterial infections [18].

In the present study, comprehensive analysis of the expression profiles of genes associated with the polarization of M0-type PAMs (resting) toward M1 phenotypes (activated by IFN-γ and LPS) was performed using the BGISEQ-500 sequencer [19]. Transcriptome profiling will reveal important information about activation of polarized PAMs and may identify novel molecules and signatures not only for future studies on the role of PAM polarization in respiratory diseases affecting pig, but also for the role of PAM polarization in modulation of infection.

2. Materials and methods

2.1. Cell isolation, culture, and treatment

All animal studies were approved by the Animal Care and Use Committee of Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, China. PAMs were obtained and cultivated as described in previous study [20]. The cells were treated using recombinant porcine IFN-γ (R&D systems, Minneapolis, MN, USA) at a concentration of 50 ng/mL, and LPS (R&D systems) at a concentration of 100 ng/mL in culture medium for 24 h prior to analyses; these concentrations were based on the optimal stimulus conditions found in our previous study [16]. For transcriptomal analyses, the four replicates of each cell culture were prepared.

2.2. Next-generation sequencing using the BGISEQ-500 sequencer

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. DNase I (Invitrogen) was used to degrade double-stranded and single-stranded contaminant DNA. RNA-Sequencing (RNA-Seq, BGISEQ-500 sequencing platform, Beijing Genomics Institute, Beijing, China) was used to analyze the gene expression profile in M0-(no IFNγ/LPS treatment) and M1-type (with IFN-γ/LPS treatment) PAMs. The sequencing libraries were constructed as described in previous study [19].

2.3. Functional characterization of the differentially expressed genes

Differentially expressed genes identified in this study were analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) tools [21] (http://david.abcc.ncifcrf.gov) for enrichment analysis of the significant overrepresentation of gene ontology (GO), for biological processes (BP), and analysis of the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway category, respectively [22].

2.4. Quantitative PCR (qPCR) validation of gene expression data

qPCR analysis was carried out using the SYBR Green 2.0 × PCR reaction mix (Takara, Shiga, Japan). Sequences and annealing temperatures of primer pairs are indicated in Supplementary Table 1. All RNA samples were assessed in quadruplicate and averaged. Data were collected and analyzed with an MxPro 3005p qPCR system (Stratagene, La Jolla, CA, USA). Target genes were normalized to the reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results are expressed as relative fold of stimulated over control group (calibrator), based on the $2^{-\Delta\Delta Ct}$ method [23].

2.5. Statistical analysis

All statistical analyses were performed using the Mann-Whitney test. The results were considered statistically significant or very significant when P values were less than 0.05 or 0.01, respectively. All graphs were generated using the GraphPad Software (Version 7.02, GraphPad Prism, San Diego, CA, USA).

3. Results

3.1. Transcriptome profiling of PAMs upon IFN-γ and LPS stimulation

To investigate for genes that were differentially expressed in M1-and M0-type PAMs, we determined and compared the gene expression profiles of M1-and M0- PAMs using the BIGSEQ-500 sequencer. Significantly different expressed genes were identified through volcano plot and fold-change filtering. Hierarchical clustering was performed to show the distinguishable gene expression pattern among samples. Transcriptome profiling revealed that 1551 and 1823 genes were upregulated and downregulated in M1-type PAMs upon IFN-γ and LPS stimulation, respectively (Fig. 1A and B).

The pathway involvement and ontology of the differentially expressed genes were analyzed using the gene annotation enrichment analysis and GO annotation tools from DAVID, respectively. For genes that were upregulated in M1-type PAMs, gene annotation enrichment analysis revealed that the most frequently enriched KEGG pathways were related to cytokine-cytokine receptor interaction, followed by the Janus kinase/signal transducers and activators of transcription (JAK/STAT), TNF, RIG-I-like receptor and Toll-like receptor (TLR) signaling pathways (Fig. 1C). For genes that were downregulated by IFN-γ and LPS, fewer KEGG pathways were identified; among them, the transforming growth factor (TGF)-β signaling pathway, followed by cytokine-cytokine receptor interaction (Fig. 1D).

3.2. Identification of the most differentially expressed genes in M1-type PAMs

Subsequently, the genes whose expression was the most upregulated or downregulated were analyzed, respectively. Among the

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