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IFN-γ establishes interferon-stimulated gene-mediated antiviral state against Newcastle disease virus in chicken fibroblasts

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Abstract

Newcastle disease virus (NDV) causes severe economic losses through severe morbidity and mortality and poses a significant threat to the global poultry industry. Significant efforts have been made to develop novel vaccines and therapeutics; however, the interaction of NDV with the host is not yet fully understood. Interferons (IFNs), an integral component of innate immune signaling, act as the first line of defense against invading viruses. Compared with the mammalian repertoire of IFNs, limited information is available on the antiviral potential of IFNs in chickens. Here, we expressed chicken IFN-γ (chIFN-γ) using a baculovirus expression vector system, characterized its antiviral potential against NDV, and determined its antiviral potential. Priming of chicken embryo fibroblasts with chIFN-γ elicited an antiviral environment in primary cells, which was mainly due to interferon-stimulated genes (ISGs). A genome-wide transcriptomics approach was used to elucidate the possible signaling pathways associated with IFN-γ-induced immune responses. RNA-sequencing (RNA-seq) data revealed significant induction of ISG-associated pathways, activated temporal expression of ISGs, antiviral mediators, and transcriptional regulators in a cascade of antiviral responses. Collectively, we found that IFN-γ significantly elicited an antiviral response against NDV infection. These data provide a foundation for chIFN-γ-mediated antiviral responses and underpin functional annotation of these important chIFN-γ-induced antiviral influencers.

Key words: interferon, NDV, RNA-seq, interferon-stimulated gene, chicken embryo fibroblast

Introduction

Newcastle disease (ND) is a devastating global infectious disease that predominates in the poultry industry and results in severe economic losses by causing acute morbidity, reducing egg production, and significant mortality [1]. ND can infect 250 bird species via natural or experimental routes [2]. Wild migratory waterfowl, especially aquatic birds, can act as a reservoir host for most of the viruses that possess the pandemic potential and are known to be actively transmitted to other avian species. With the passage of time, it has gained serious attention with the viral evolution and re-emergence in the Newcastle disease virus (NDV)-infected susceptible birds [3–6]. To date, very few studies have been conducted to elucidate the NDV-mediated pathogenesis in birds, especially in the chickens. So, it is of great concern to regularly undermine and update the latest knowledge about NDV-induced immune responses in chicken. Specifically, the patterns of disease progression and the outcome significantly vary on viral strain and subtype. Therefore, the virulent NDV strain may cause severe lesions or even death in an infected chicken. Compared with chickens, ducks often show intermittent, prolonged NDV shedding [7]. Additionally, it has also been revealed that a strong humoral response is generated against NDV in chickens. Though previous studies have demonstrated that NDV replication

occurs in respiratory system and lymphoid organs of chickens, there are still loop holes in viral-mediated immune escape [7]. In the present study, we aimed to explore the NDV-mediated immune response in vital organs of chickens.

The innate immune response is the primary line of defense against invading pathogens. Upon viral invasion of host cells, the innate immune response is activated in an antigen-independent manner [2]. Interferons (IFNs) are the pleiotropic group of signaling proteins that are cytokines in nature and also function as natural immune boosters, antitumor factors, and antiviral molecules that play a fundamental role in stimulating an active antiviral environment within the host cells [8,9]. IFNs are broadly classified into three distinct groups based on their molecular architecture, pathway induction, and cell-receptor specificity [10].

IFNs are the integral component of the innate immune system, and their pattern of gene expression and antiviral properties are associated with specific receptor–ligand interactions in a specific biological environment [11]. In chickens, type I IFNs are secreted by developing cells (i.e. fibroblasts), type II IFNs are synthesized by immune cells, and type III IFNs are secreted in a variety of cells in a tissue-dependent manner [9]. In response to viral infection, numerous interferon-stimulated genes (ISGs) are activated, which elicits a broad antiviral environment in the host cells [12,13]. Regardless of the functional diversity, IFNs are involved in a cohesive, integrated, and precisely synchronized immune response cascade.

Similar to its mammalian orthologue, chIFN-γ is a 16.8-kDa cytokine composed of 169 amino acids (aa), a 19-aa signaling segment, and a 145-aa backbone [14]. Sensitive to heat (65°C) and low pH, chIFN-γ is secreted by immune cells (i.e. NK cells and T lymphocytes) [15] and is considered to be the major macrophage activator in *Aves* [9,16,17]. Previous studies have revealed that chIFN-γ can halt viral replication, enhance activation of both MHC-I and MHC-II molecules, and assist in antigen presentation and processing as well as removal of intracellular pathogens [18–22]. Moreover, chIFN-γ activates T-helper I-type immune responses [23]. IFN-γ and its adjuvant characteristics have potential antiviral activity against avian viral pathogens such as avian influenza virus (AIV), NDV, and Marek's disease virus (MDV), respectively [24–26]. Surprisingly, IFN-γ also facilitates the production of nitric oxide that is a significant marker of viral inhibition [27]. Identification of IFN-γ in other avian species including pigeon, duck, quail, guinea fowl, goose, pheasant, and turkey illustrates its role as a natural constituent of the innate immune system in *Aves* [19,28–30]. Like other chicken cytokines, the chIFN-γ signaling cascade is not clearly understood; however, it is thought to be similar to mammalian cytokines in that it also utilizes the classical Janus kinases-signal transducer and activator of transcription proteins (JAK-STAT) signaling pathway [31,32].

Numerous studies have been conducted to evaluate the mammalian immune responses generated by IFN-γ. Moreover, the immunomodulatory effect of IFN-γ as a vaccine adjuvant has been demonstrated [33]. However, enhanced protection and a boost in avian immune response were also observed in chickens. Administration of chIFN-γ along with fowlpox and DNA vaccines resulted in elevated antibody titer, improved cellular responses, and protection against NDV challenge in chickens and turkeys [25,34,35]. Similarly, chicken embryo fibroblasts (CEFs) do not have the key receptors required for type III IFNs (IFN-λ) [36]. Therefore, we explored the potential of IFN-γ to act against NDV in CEFs, *in vitro*. There is limited data on the nature and dynamics of immune responses induced by chIFN-γ that establishes an antiviral state that can then be used to guide the development of more reliable and advanced therapeutics in poultry, with the aim of preventing respiratory viral infection, replication, and shedding. To date, most of the commercially available IFN-γ preparations are produced recombinantly in *Escherichia coli*. Standardization of expression conditions and a lack of glycosylation render these IFN-γ preparations more protease-resistant compared with the glycosylated forms that have a shorter half-life in the blood. Endotoxin contamination, inclusion body formation, and complex protein purification from *E. coli* increase production costs of *E. coli*-derived IFN-γ [37,38].

In the present study, we employed the baculovirus expression vector system (BEVS) in *Bombyx mori*, also known as a silkworm, to express recombinant chicken IFN-γ (chIFN-γ) and characterized its antiviral potential against NDV (chicken-origin NDV, F48E9, genotype IX) in CEFs. By using the transcriptomic approach, we tried to analyze the variations in gene expression in CEFs during the antiviral experiments. Transcriptomic profiling revealed distinct upregulation of differentially expressed genes (DEGs) and temporal expression of ISGs and presented significant pathways involved in the cascade of avian immunity.

Materials and Methods

Cells and viruses

This study was designed and performed in agreement with the animal ethics guidelines and endorsement of the Beijing Administration Office of Laboratory Animals (Beijing, China). CEFs were produced from 9- to 11-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) at the Biotechnology Research Institute Chinese Academy of Agricultural Sciences (Beijing, China) as previously described [39]. Nine-day-old SPF ECEs were acquired from Beijing Vital River Laboratory Animal Technology Co., Ltd (Beijing, China) and maintained at 37°C. Briefly, CEFs were cultured in Media 199 (M199) supplemented with 5% fetal bovine serum (FBS; Gibco, Carlsbad, USA), an antibiotic cocktail of 1% penicillin and streptomycin (P/S; Gibco) at 37°C in a 5% CO_2 incubator for 24 h. Similarly, the *B. mori*-derived cell line (Bm5) was cultured in TC100 culture media (Applichem, Darmstadt, Germany) supplemented with 10% FBS at 27°C as previously described [40]. For co-transfection, Bm5 cells were grown overnight with FBS-supplemented TC100 media to achieve 90% confluency (i.e. 1×10^6 cells per well) in a 6-well plate. TC100 media was used for washes to completely remove the FBS. A cocktail of transfection and co-transfection mixture was poured in it. Fresh FBS was added to the cell culture media 4–6 h post-transfection. A multiplicity of infection (MOI) of 0.1 was used to infect Bm5 cells over 1–2 h in order to perform viral amplification and expression. Velogenic NDV strain F48E9 (chicken-origin NDV, genotype IX) was prepared in 10-day-old SPF ECEs and maintained in our laboratory. Briefly, the virus was proliferated in 9- to 11-day-old SPF ECEs, titrated with Hemagglutination assay (HA) and stored at −80°C.

Expression of chIFN-γ

We have previously demonstrated the successful construction and development of a recombinant *B. mori* Bacmid (reBmBac) expression system [41]. The silkworm reBmBac gene expression system was used to construct and express recombinant chIFN-γ (GenBank: NM_205149.1; *Gallus gallus*). Briefly, the chIFN-γ gene was inserted into a plasmid containing a Pph promoter and ORF1629+ and then co-transfected with reBmBac into Bm5 cells. Similarly, the

Table 1. List of qPCR primer sequences used in this study

Gene	Forward primer (5′ to 3′)	Reverse primer (5′ to 3′)
IL-1β	GTGAGGCTCAACATTGCGCTGTA	TGTCCAGGCGGTAGAAGATGAAG
β-actin	CAACACAGTGCTGTCTGGTGGTA	ATCGTACTCCTGCTTGCTGATCC
Viperin	GGAGGCGGGAATGGAGAAAA	CAGCTGGCCTACAAATTCGC
Mx	AAGCCTGAGCATGAGCAGAA	TCTCAGGCTGTCAACAAGATCAA
JAK2	CAGATTTCAGGCCGTCATTT	ATCCAAGAGCTCCAGTTCGTAT
STAT1	TTGTAACTTCGCTATTGGTATTCC	TTCCGTGATGTGTCTTCCTTC
MyD88	AGGATGGTGGTCGTCATTTC	TTGGTGCAAGGATTGGTGTA
PKR	CCTCTGCTGGCCTTACTGTCA	AAGAGAGGCAGAAGGAATAATTTGCC
NDV copies test	AGTGATGTGCTCGGACCTTC	CCTGAGGAGAGGCATTTGCTA

recombinant virus containing the chIFN-γ gene was acquired 5 days after co-transfection. Likewise, chIFN-λ was harvested 100–120 h post pupae infection. Silkworm blood was diluted in phosphate-buffered saline (PBS) at a ratio of 1:10, lysed, sonicated, and centrifuged at 10,000 *g* for 10 min at 4°C. The supernatant containing fat bodies was discarded. The protein was filtered with a 0.22-μm syringe filter to remove any contamination, concentrated using a 10 K Microsep^{TM} Advance Centrifugal Device (Pall Corporation, New York, USA), and stored at −80°C. A plaque assay was performed to evaluate the recombination efficiency [40]. A luciferase assay kit (Promega, Madison, USA) was used to investigate the presence of luciferase in the purified protein that was quantified by Bradford assay [42]. The antiviral activity of chIFN-γ was evaluated using a recombinant vesicular stomatitis virus (VSV-GFP) in a GFP-reduction assay as previously described [43].

Western blot analysis

For sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE), a 20-μl sample containing 100 ug of recombinant protein was loaded on the gel. Blank silkworm blood treated in the same way was loaded as the negative control. After electrophoresis and transfer onto PVDF membrane, recombinant chIFN-γ expression was analyzed by western blot analysis using anti-IFN-γ antibody (Abcam, Cambridge, USA), according to the Protein Blotting Guide (Bio-Rad, Hercules, USA), as previously described [41].

Antiviral assay

To evaluate the anti-NDV activity of BEVS-derived chIFN-γ, CEFs were seeded in a 12-well plate and either left untreated or treated with the appropriate concentration of chIFN-γ for 24 h. Both the treatment, control cells were treated with the hemolymph preparations from the untreated silkworms. NDV at an MOI of 0.1 was incubated with CEFs for 2 h and then cultured with media in the absence of chIFN-γ for 36 h. The supernatant was harvested to analyze the inhibition kinetics of chIFN-γ against NDV infection. To study the pattern of ISG expression, CEFs were further stimulated with a serial dilution of IFNs for 12 h. CEFs were then treated with chIFN-γ at a concentration of 4.19×10^3 IU/ml. Cells were lysed for the extraction of total RNA.

Real-time quantitative PCR

Briefly, Trizol reagent (Invitrogen, Carlsbad, USA) was used to extract total RNA from supernatants or cell lysates as previously described [44]. Reverse transcription was performed using the M-MLV Reverse Transcriptase kit (Lot No. 0000293427; Promega) and quantitative PCR (qPCR) was performed on a Bio-Rad iQ5 thermal cycler (Version 2.0.148.60623) using power SYBR Green Real-time PCR Master Mix (Lot No. 441900; TOYOBO, Tokyo, Japan). All primers are presented in **Table 1**, with all reactions performed in triplicate. *β-Actin* was used as an endogenous control. All data were obtained using the Bio-Rad iQ5 thermal cycler, and gene expression was calculated relative to *β-actin* expression using the $2^{-\Delta\Delta CT}$ method. All the fold changes are from each treatment vs the untreated group [45].

RNA extraction and sequencing

For transcriptomic analysis, samples from virus-infected or mock-treated CEFs were treated with the control hemolymph preparations, and RNA extraction was performed using the Trizol method [44]. All experiments were carried out in triplicate. The quality of extracted RNA was determined by running the samples on a 1% (w/v) agarose gel. RNA integrity was measured using the RNA Nano 6000 Assay kit from the Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, USA). All the extracted RNA samples were submitted to Novogene (Beijing, China) for deep sequencing using the HiSeq X Ten (Illumina, San Diego, USA) and PE150 platforms. The generated RNA-seq raw data were submitted to the Sequence Read Archive (SRA) database at the National Centre for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/sra), and all the accession numbers are shown in **Supplementary Table S1**.

Sequencing quality

RNA-seq data from chIFN-γ-treated, NDV-treated, IFN + NDV-treated, and mock-treated CEFs were presented in **Table 2**. Once clusters were generated, the library preparation was run on an Illumina HiSeq X Ten Platform and 150 bp paired-end reads were generated. All the reads were aligned and mapped to the reference genome database (ftp://ftp.ensembl.org/pub/release-89/fasta/gallus_gallus/dna/). By using a reference-based approach, all of the individually mapped reads from each sample were assembled by StringTie (v1.3.3b) [46]. FeatureCountsv1.5.0-p3 was used to calculate the number of mapped reads per gene. Gene length and read count fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) of every gene was studied. Differential expression analysis was performed by utilizing the DESeq2 R package (version 1.16.1). All the *P*-values were further adjusted in order to control the false discovery rate, by employing Benjamini and Hochberg's approach. All the genes with adjusted *P*-values <0.05 and $|\log_2 \text{fold change}| > 1$ detected by DESeq2 were designated as the DEGs.

Table 2. Sequence quality analysis

Sample name	Raw reads*	Clean reads#	Clean bases†	Error rate (%)	Q20 (%)	Q30 (%)	GC content (%)
blank_1	45,603,864	44,408,320	6.66G	0.03	96.02	90.63	55.89
blank_2	50,563,436	49,243,494	7.39G	0.04	94.29	87.25	56.75
blank_3	42,471,178	41,431,374	6.21G	0.03	96.3	91.18	55.56
ChIFN-γ_1	42,859,388	41,724,680	6.26G	0.03	95.82	90.23	56.53
ChIFN-γ_2	42,607,444	41,582,060	6.24G	0.03	95.49	89.65	56.94
ChIFN-γ_3	43,388,104	42,283,724	6.34G	0.03	95.56	89.71	55.26
NDV_1	42,350,418	41,436,090	6.22G	0.03	96.02	90.55	55.15
NDV_2	44,446,912	43,372,152	6.51G	0.03	96.18	90.88	54.3
NDV_3	44,418,518	43,424,590	6.51G	0.03	95.86	90.22	54.42
ChIFN-γ_NDV_1	43,291,544	42,319,700	6.35G	0.03	96.14	90.76	54.76
ChIFN-γ_NDV_2	46,201,160	45,031,870	6.75G	0.03	96.16	90.84	55.04
ChIFN-ã_NDV_3	45,123,376	43,911,684	6.59G	0.03	95.96	90.41	54.84

*Raw reads: all data were generated by one sequencing; #clean reads, the remaining reads after deletion of low-quality reads and reads with adapters or poly-N > 10%; †clean bases, the sequence number multiplied by the stretch of the sequencing and transformed to present G units.

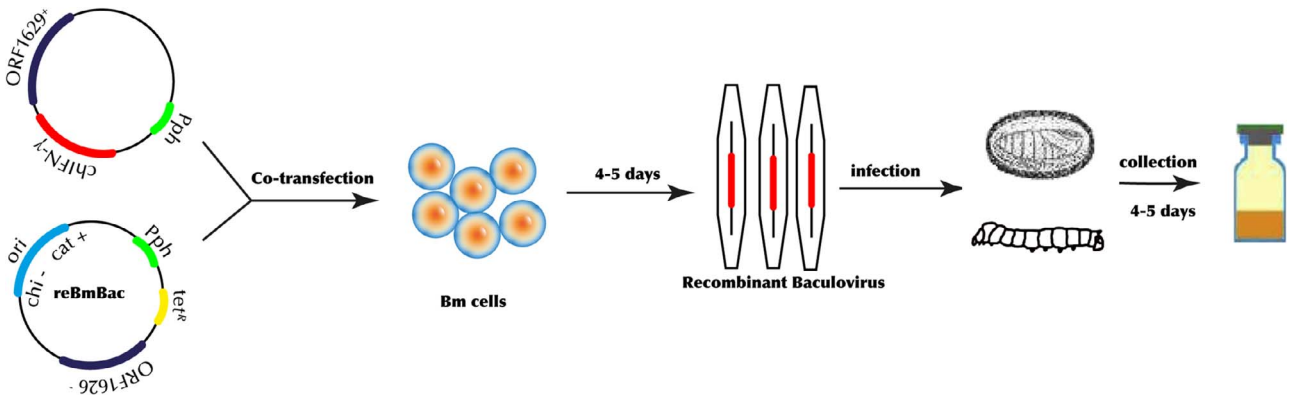

Figure 1. Schematic representation of the silkworm BEVS It demonstrated the construction of recombinant baculovirus and expression of recombinant protein.

DEGs, Gene Ontology and Kyoto Encyclopedia of Genes and Genomes enrichment analysis

In order to predict gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG), we used the pathway enrichment ClusterProfiler R package (http://www.genome.jp/kegg/). Entire GO terms and KEGG pathways were designated as significantly enriched if they displayed an adjusted *P*-value <0.05.

Statistical analysis

GraphPad Prism 5.0 was used to perform statistical analysis. Differences between two treatments were analyzed using an unpaired *t*-test. If the adjusted *P*-value <0.05, difference was termed as significant.

Results

Expression of chIFN-γ in BEVS

BEVS was used for the construction and expression of chIFN-γ. A Luciferase reporter gene was used to assess the expression efficiency as previously described [41]. Co-transfection of the pVL1393-luc plasmid and reBmBac DNA in Bm5 cells are presented in Fig. 1. To investigate the optimal viral strain for luciferase expression, a viral plaque assay was performed. The supernatant from Bm5 cells containing recombinant BmNPV (reBm-luc) was acquired 5 days post-transfection. Additionally, after 100–120 h, all proteins were harvested from silkworm pupae and 50 μg protein from larval hemolymph was used for luciferase assays. Luminescence observed from hemolymph was $(4.33 \pm 0.61) \times 10^8$ relative light units (RLUs) in comparison to 150–300 RLUs from the mock controls. Results from western blot analysis further confirmed the successful expression of the *chIFN-γ* gene in BEVS (Supplementary Fig. S1).

The antiviral effect of recombinant chIFN-γ was measured by the VSV-GFP reporter assay. It was found that the BEVS expressed chIFN-γ was biologically active with remarkable anti-VSV activity in CEFs. It was also found that 3.2×10^6 IU/ml chIFN-γ was present in the silkworm hemolymph (Supplementary Table S2). Previous studies have shown that antiviral units (AUs) of recombinant IFNs are closely associated with the cell type and viruses used in the reporter assay. First, we evaluated the antiviral potential of chIFN-γ against the F48E9 NDV strain. Briefly, CEFs were pre-treated with increasing concentrations of chIFN-γ for 24 h before NDV infection. The chIFN-γ treatment significantly inhibited NDV replication in CEFs in a dose-dependent manner (Supplementary Table S2). Next, to analyze the stages of NDV infection and the extent of viral inhibition by chIFN-γ treatment, we collected supernatant at different hours post-infection (hpi). Intriguingly, we observed that pre-treatment with chIFN-γ at a concentration of 4.19×10^3 IU/ml significantly reduced the viral replication in CEFs at a regular interval (12, 24, and 36 hpi) (Fig. 2). These results suggested that chIFN-γ has significant antiviral activity against NDV strain F48E9 in CEFs.

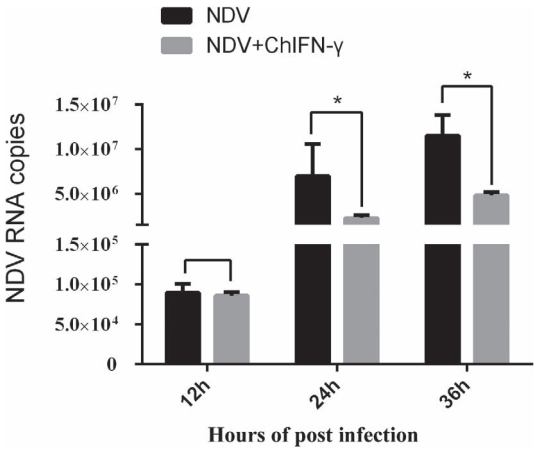

Figure 2. chIFN-γ inhibited NDV (strain F48E9) infection in CEFs CEFs were either left untreated or treated with chIFN-γ (4.19×10^3 IU/ml) for 24 h before infection with NDV (strain F48E9). The supernatant containing NDV RNA was measured by RT-qPCR at 12, 24, and 36 h post-infection. Surprisingly, we observed that pre-treatment with chIFN-γ at a concentration of 4.19×10^3 IU/ml significantly reduced the viral replication in CEFs at a regular interval (12, 24, and 36 hpi). The results are presented as the mean ± SEM ($n=3$). *P-value <0.05 by unpaired t-test.

The pattern of gene expression post-chIFN-γ treatment

Transcriptomics was used to explore the possible cellular and molecular signaling associated with IFN-induced immune regulation. Transcriptomes from NDV- and chIFN-γ-treated CEFs were compared with mock conditions to identify DEGs. The variation in DEGs reflects immune signaling, cellular structure, molecular transportation, and biochemical pathways.

Previous studies have demonstrated that chIFN-γ signaling occurs via specific IFN-γ receptors and functions as a channel between innate and adaptive immunity [47,48]. Studies have also revealed that chIFN-γ provides protection against chicken anemia virus and MDV, further highlighting its diverse antiviral potential and suggesting that it plays a significant role in downstream signaling in the chicken immune system [9]. Therefore, we stimulated CEFs with silkworm-derived chIFN-γ and investigated its antiviral potential in chicken fibroblasts.

Additionally, we profiled the patterns of gene expression and compared them with mock conditions by RNA-seq. There was an overall moderate to high gene expression in the distinct treatment groups; in Group 1 (NDV-treated CEF vs mock), 1845 genes out of 4121 DEGs were upregulated and 2276 genes were downregulated (Fig. 3A); in Group 2 (chIFN-γ-treated CEF vs mock), 365 genes out of 433 DEGs were upregulated and 68 genes were downregulated (Fig. 3B); and in Group 3 (chIFN-γ + NDV treated CEF vs mock) 3140 genes out of 6455 DEGs were upregulated and 3315 genes were downregulated (Fig. 3C). We compared chIFN-γ + NDV-treated CEFs with NDV-treated CEFs (i.e. Group 3 vs Group 4) and found that 1384 out of 1946 genes were upregulated, while 562 genes downregulated (Fig. 3D). The significant differences in gene abundance were documented and illustrated as hierarchical clustering (Fig. 4A). Briefly, the transcripts demonstrated significant differences in the treatment groups compared with the control group. Heat map analysis presented a visual comparison of the transcripts. In the NDV-treated group, significant gene upregulation occurred. A similar pattern was observed in chIFN-γ-treated CEFs, but at a lower level. However, a remarkable change was seen in chIFN-γ + NDV-treated CEFs, where a significant cohort of genes was distinctly upregulated. Fascinatingly, a significant number of genes overlapped in the three treatment groups (Fig. 4B). Furthermore, we used qPCR quantification to validate the pattern of expression for DEGs. The qRT-PCR results shown in Fig. 5 demonstrated that the most crucial ISGs, including viperin, MyD88, STAT1, Mx, and PKR tested in this study, are significantly upregulated. Moreover, the expressions of IL1B and JAK2 were greater in the treated group than those in the control group (adjusted P-values >0.05), which is also consistent with the findings of the RNA-seq data. Collectively, these findings strongly supported the antiviral role of ISGs in the IFN-mediated immune response. DEGs were further characterized on the basis of their abundance and fold change (Supplementary Table S1).

Taken together, we observed fundamental upregulation of integral cytokine, chemokine, and antiviral genes (*IL6*, *IL7*, *IL12A*, *IL15*, *STAT1*, *JAK2*, *IL1B*, *IL12B*, *JUN*, *SOCS3*, *TLR3*, *MX1*, *RSAD2*, *IFIH1*, *IRF7*, *MYD88*, *NFKB1*, *NFKBIA*, *BF1*, *BF2*, and *DMB1*) in the NDV + IFN-γ group (Supplementary Table S1). These genes are linked and closely related in terms of functionality (Fig. 6). These genes are also associated with apoptosis, cytokine–cytokine receptor interactions, cellular proliferation. and differentiation, as well as antiviral responses. The expressions of a diverse subset of genes identified in this study are in agreement to the previously reported literature where a moderate antiviral functionality is observed against NDV [12,49,50]. Moreover, chIFN-γ is unique in its functionality in the cascade of pathogenic attack against host cells, because it is significantly active in creating an overall antiviral environment against invading pathogens.

In order to deeply undermine the possible functionality of the observed novel genes found in the cohort of DEGs, we further analyzed and predicted the specific functions associated with each novel gene. Briefly, both the novel gene 00068 and novel gene 00025 were hypothetical proteins Y956_03374. Similarly, the novel gene 00372 was designated as peroxisome proliferator-activated receptor gamma coactivator-related protein 1-like isoform and the novel gene 00577 as paramyosin-like, while partial and novel gene 00372 is similar to PPRC1. The protein encoded by gene 00372 is identical to PPAR-gamma coactivator 1 (PPARGC1/PGC-1), a protein that is broadly involved in the activation of mitochondrial biogenesis via interaction with respiratory factor 1 (NRF1) in response to proliferative signals. It is believed that alternative splicing results in multiple transcript variants [51]. In the mammals, this PRC-dependent stress cascade is initiated during apoptosis and senescence; however, its exact functional role in chicken needs to be further explored.

Comparative analysis of DEGs

In order to classify the functions associated with the identified DEGs, GO enrichment analysis was performed. Based on sequence homology, the transcripts for DEGs were grouped into three broad categories, i.e. cellular components, molecular functions, and biological processes (Fig. 7), by using DESeq2 [52].

The functional distribution of DEGs in each treatment group was evaluated by comparing the transcript profiles. All analysis here was subject to an adjusted P-value of <0.05. In NDV-treated CEFs, 48 out of 3465 GO terms were significant (Fig. 7A). In the chIFN-γ-treated CEFs, 18 out of 1730 GO terms were significant (Fig. 7B). In NDV + chIFN-γ-treated CEFs, 90 out of 3886 GO terms were significant (Fig. 7C). In the NDV + chIFN-γ-treated vs NDV-treated group, 26 out of 2766 GO terms were significant (Fig. 7D).

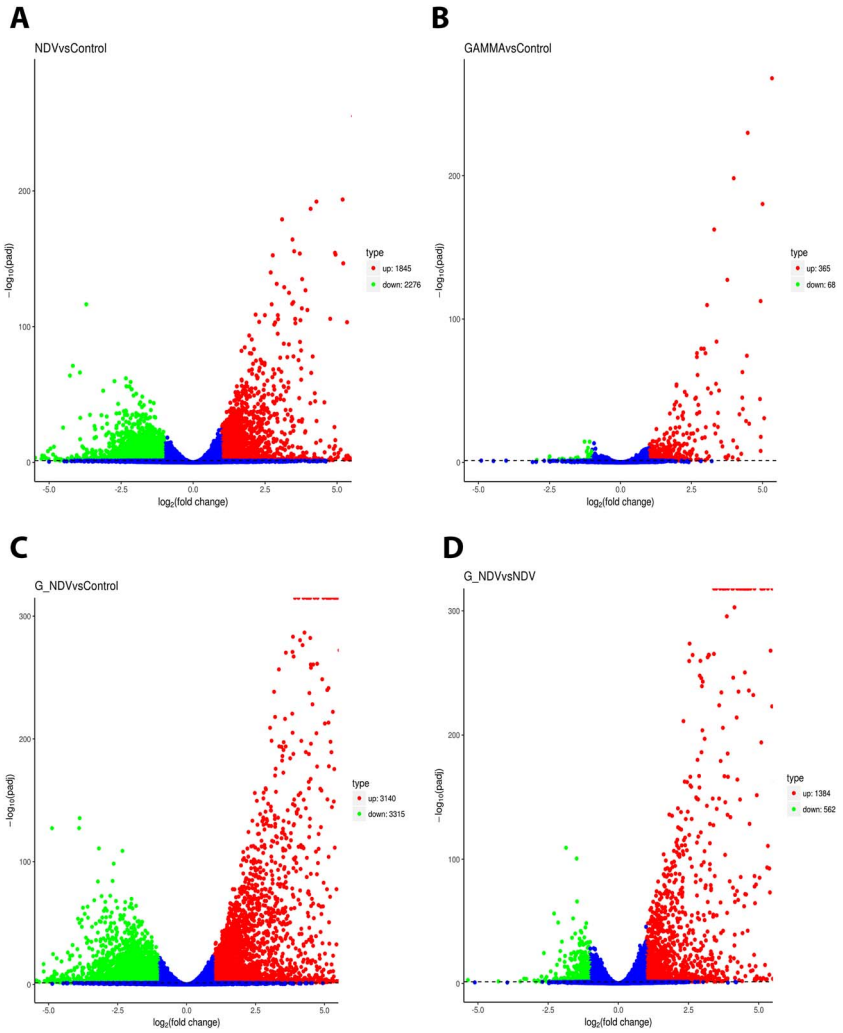

Figure 3. Gene expression illustration volcano plots and a Venn diagram (A) DEGs in NDV-treated CEFs. (B) DEGs in chIFN-γ-treated CEFs. (C) DEGs in chIFN-γ + NDV-treated CEFs. (D) DEGs in chIFN-γ + NDV-treated CEFS with NDV treated CEFS (chIFN-γ + NDV vs NDV). Red and green dots demonstrate upregulated and downregulated DEGs, respectively. Differential expression patterns represent the temporal expression of genes expressed in the four groups.

Terms associated with biological processes were significantly enriched by comparing the transcriptional profiles of chIFN-γ-treated group with those of the control group. These biological processes include immune response, immune system process, antigen processing and presentation, regulation of innate immune response, activation of immune response, and regulation of immune system process. The cellular components included MHC protein complex, MHC class II protein complex, and membrane-related dynamics. The enriched terms associated with molecular functions were chemokine and cytokine activity, cytokine receptor binding, regulatory region DNA binding, and regulatory region nucleic acid binding (Fig. 7B). In NDV-treated CEFs, the significantly enriched GO terms in biological processes were mainly linked to regulatory processes related to nucleic acids and proteins, as well as gene expression. Among the molecular functions, cytokine activity, cytokine receptor binding, transcription factor activity, and sequence-specific DNA binding were the most significantly enriched GO terms (Fig. 7A). GO analysis of NDV + chIFN-γ-treated CEFs showed that among biological processes, regulatory processes related to biosynthesis, metabolism, and nucleic acid synthesis were highly enriched. Similarly, chemokine activity, organic cyclic compound binding, heterocyclic compound binding, and catalytic activity were the most significantly enriched GO terms in molecular functions (Fig. 7C). Some of the highly significant biological processes observed in the NDV + chIFN-γ vs NDV treatment groups include immune responses, immune system processes, cytokine activity, chemokine activity, chemokine receptor binding, cytokine receptor binding, MHC protein complex, MHC class II protein complex, and antigen processing and presentation (Fig. 7D). In short, this analysis showed that distinct biological processes were enriched in all treatment groups, suggesting that diverse biological processes are initiated in response to NDV, IFN-γ, and NDV + IFN-γ treatment. However, further experiments are required to address these differences in a comprehensive manner.

KEGG pathway enrichment

To further characterize and classify the predicted functions of all the upregulated DEGs, data were further analyzed for KEGG pathway enrichment analysis. The analyzed data are presented in Supplementary Table S3, adjusted P-value <0.05. In all three experimental groups, significant upregulation in gene expression was observed in biological signaling including the Toll-like

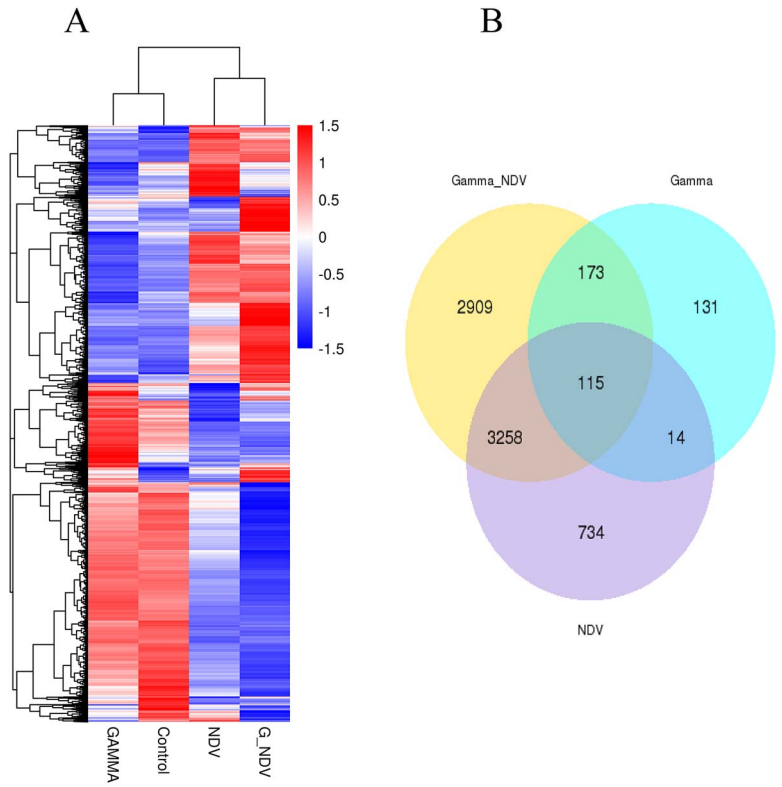

Figure 4. A hierarchical heat map representing the expressional values of DEGs in the different groups (A) The color bar represents the level of differential expression compared with the control group. GAMMA (chIFN-γ-treated CEF), Control (only CEF with blank silkworm blood), NDV (NDV-treated CEF), and G_NDV (chIFN-γ + NDV-treated CEF). Red and blue colors represent up and down-regulation, respectively. (B) Venn diagram representing the common gene distribution among the three distinct groups.

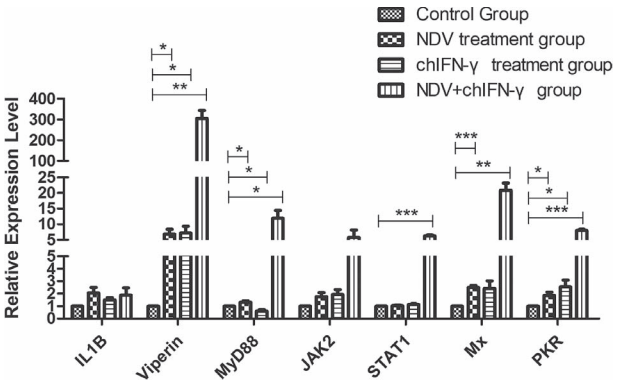

Figure 5. Verification of the relative expression levels by quantitative real-time PCR Expression patterns of selected DEGs associated with the immune response as determined by qPCR. The x-axis shows the annotations of the selected genes. The y-axis shows expression levels that are normalized to *β-actin* expression. Gene expression was quantified relative to *β-actin* expression using the $2^{-\Delta\Delta CT}$ method. The results are presented as the mean ± SEM (three biological replicates and three technical replicates). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ by unpaired *t*-test.

receptor (TLR) signaling pathway (*STAT1/TLR3/IRF7*), influenza A (*BLB1/STAT1/TLR3/IRF7/MX1/RSAD2/DMB2/IFIH1*), and herpes simplex infection (*BLB1*, *CD74*, *STAT1*, *TRAF5*, *IL15*, *TLR3*, *IRF7*, *TAP2*, *BF1*, *TAP1*, *DMB2*, *IFIH1*, and *BF2*) (Fig. 8). Similarly, cytokine–cytokine receptor interaction, herpes simplex infection, JAK-STAT, and *Salmonella* infection signaling pathways also demonstrated significant upregulation in terms of cytokines, interleukins, ISGs, and transcription modulators, respectively (Fig. 8D). Major genes including *IL1β*, *IL6*, *IL8*, *CCL4*, *CCL5*, *STAT1*, *TLR3*, *IRF7*, *IFITM-1*, *IFITM-3*, *IFITM-5*, *STAT1*, *NF-kB*, *MX1*, *RSAD2 (viperin)*, *MDA5*, and *ZAP* were identified in this study (Supplementary Table S1).

Interestingly, among all those crucial genes related to innate immunity identified in the present study, we found that TLR3, TLR7, IL1B, IRF7, IL8, STAT1, and MHC class I and II molecules are exactly the same as those observed in previously studies where similar gene expression was shown in CEFs treated with NDV (genotype VII and IX) [53,54]. In short, our results demonstrated a low level (non-significant) expression of IFN-α and IFN-β. However, compared with IFN-α and IFN-β, IFN-γ was significantly expressed in response to NDV, which is in agreement with the results of a previous study [26].

Discussion

In the current study, we presented the transcriptomic profile of DEGs in primary fibroblasts after treatment with chIFN-γ and the release of subsequent pro-inflammatory mediators in response to the treatment. This significant response is due to sustained and swift signaling through highly specific receptor–ligand interactions and expressions of chemokines and cytokines, which results in a potent antiviral environment. Surprisingly, we observed an overall increase in TLR signaling in all treatment groups and the temporal expression of numerous genes in TLR-mediated immune responses. We also reported a comprehensive gene expression analysis of

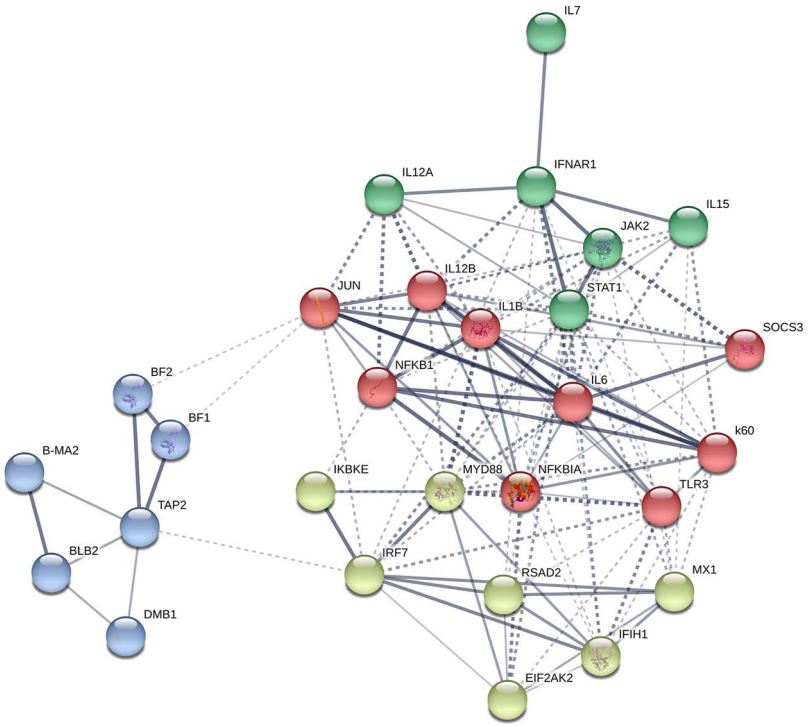

Figure 6. Gene network describing the association of genes observed in the present study (kmeans = 4) String protein network demonstrating the interlinked genes associated with immune response, immune system process, MHC protein complex, MHC class II protein complex, cytokine activity, and cytokine receptor binding (NDV + IFN-γ group) (https://string-db.org/).

eukaryote-derived chIFN-γ-induced gene expression in live silkworms. Due to the advantages of BEVS system over the *E. coli* expression system [55], we used BEVS to express chIFN-γ. We reported the successful cloning, expression, and purification of chIFN-γ using the BEVS system, and presented data that confirmed the antiviral potential of chIFN-γ.

Type I IFNs do not provide sufficient protection against members of avian *Paramyxoviridae* including velogenic NDV [49]. Therefore, the aim of this study was to explore IFN-γ activity against NDV *in vitro* in CEFs. IFN-γ plays a significant role in physiological functions, and there is evidence supporting the fundamental role of IFN-γ in the avian family of IFNs [32].

A large amount of ISGs are induced by chIFN-γ, which changes the traditional view that primary CEFs are optimally responsive to IFN-γ [56]. In fact, a previous study found that chIFN-γ had antiviral activity against MDV [30]. It has been suggested that chIFN-γ treatment elicits a broad set of antiviral ISGs, especially in the viperin gene (RSAD2), a potent antiviral gene that is expressed in response to viral pathogens [57]. Additionally, overexpression of RSAD2 in IFN-γ-treated CEFs further confirmed the induction of ISGs following chIFN-γ treatment *in vitro*. Furthermore, studies have also shown that in chicken IFNs, cell-type specificity is unusual and distinct from mammalian IFNs in receptor–ligand interactions. It has recently been shown that chIFN-γ induces ISGs and sustains a potent antiviral environment in host cells [58]. Interestingly, all three types of chicken IFNs (IFN-α, IFN-β, and IFN-γ) are upregulated in response to NDV infection [53]. However, upon infection with velogenic NDV, IFN-γ is significantly upgraded in the bursa of Fabricius, while expression of IFN-γ does not markedly reduce the proliferation and replication of NDV *in vitro* [26,59]. Here, GO and KEGG analyses revealed overlaying cellular and molecular mechanisms. Mx, one of the most significant ISGs in host–pathogen interactions, is mainly responsible for creating a barrier against different bacterial and viral pathogens [60]. The upregulation of Mx expression in response to chIFN-γ treatment is characteristic for all ISGs and confirms ISG expression *in vivo*.

Microarray and RNA-seq technologies have been used to investigate the effect of type I chicken interferon (IFN-α) treatment on ISGs in primary fibroblast cells, showing that treatment with IFN-α causes significant upregulation of ISGs [12]. Expression of IFN-inducible transmembrane protein-1 (IFITM-1) following IFN treatment further showed that it is possible to stop viral propagation by simply obstructing the entry into a cell [61]. It has also been reported that changes in cell membrane fluidity are due to IFITM whereby it either generates curvature in the membrane or interferes with the cholesterol homeostasis within the host cells [61]. We observed that the upregulation of IFITM-1 in chIFN-γ-treated CEFs further increased the sequential expression of ISGs post-IFN treatment. More research is required to further explore the potential role of chIFN-γ as a novel and potent therapy against viral pathogens in the poultry industry.

In mammals, IFN-γ acts as a crucial IFN that is mainly responsible for diverse biological functions including macrophage activation, demonstrates immunomodulatory activity, and is directly associated with antigen presentation and processing, antitumor, and antimicrobial pathways. Similarly, it has been observed that this IFN has a direct impact on the maturation and differentiation of lymphoid cells, the activity of natural killer (NK) cells, and the regulation of immunoglobulin production by B cells [62]. It has been established that IFN-γ treatment initiates a complex downstream signaling cascade in a cell that results in the production of signal transducers, especially STAT1, tumor suppressor gene (IRF-1), IL-12, CXCL10,

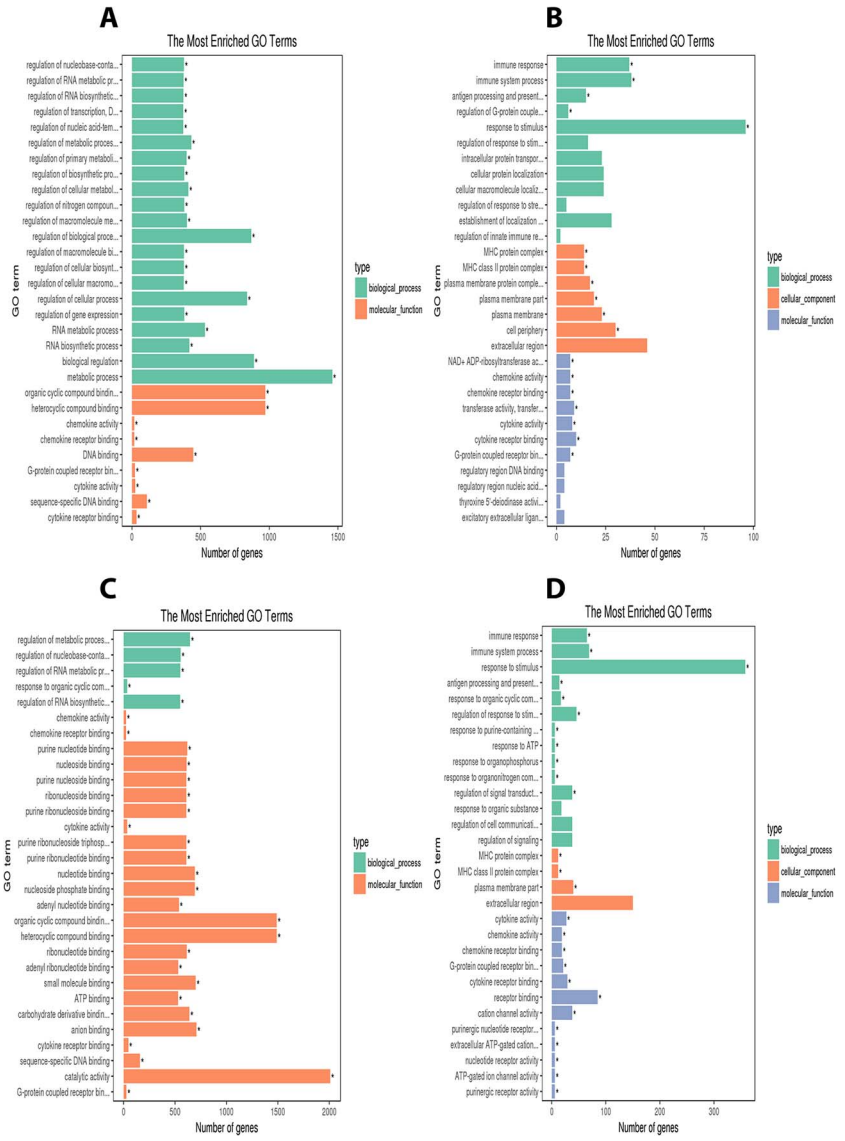

Figure 7. GO enrichment analysis for DEGs (A) GO analyses of DEGs in NDV-treated CEF compared with control. (B) GO analyses of DEGs in chIFN-γ-treated CEFs compared with control. (C) GO analyses of DEGs in NDV + chIFN-γ-treated CEF compared with control. (D) GO analyses of DEGs in NDV + chIFN-γ-treated CEFs compared with NDV-treated CEF. Among various enriched GO terms, cytokine-mediated immune processes were the most enriched processes based on the criteria ($P < 0.05$).

chemoattractants including (CCL2, CCL3, CCL4) and ISGs [62,63]. Fascinatingly, the same phenomenon was observed in IFN-γ-treated CEFs, where significant upregulation of STAT1, IRF-1, and other associated genes was reported. GO and KEGG pathway enrichment analyses revealed distinct biological features in all treatment groups. In order to replicate in the host cells, viruses need to breach the immune and metabolic processes of the host cells [38]. We found this to be the case when we observed significant variations in cellular metabolic processes (i.e. 1281 genes upregulated and 1033 genes downregulated) in NDV-treated CEFs. However, in response to IFN-γ treatment, the most highly significant GO terms were mainly associated with immunity. Interestingly, in NDV + IFN-γ-treated cells, a significant number of DEGs were found to enrich in both metabolic and immune processes. Moreover, co-treatment of chIFN-γ and NDV synergistically upregulated ISG expression compared with only NDV treatment. Upregulation of crucial biological processes, key immune responses, and metabolic processes in this context requires further investigation. Nonetheless, our findings revealed the fundamental cellular and molecular basis involved in chIFN-γ-mediated immune response.

Melanoma differentiation-associated gene 5 (MDA5), a crucial pattern recognition receptor (PRR) in birds, is activated by both long and short double-stranded RNA (dsRNA) viruses [64]. MDA5 mimics the function of RIG-1, which is absent in *Aves*, by recognizing both long and short dsRNA. Therefore, communication between the laboratory of genetics and physiology 2 (LGP2) and MDA5 induces a potent antiviral response in chickens [65]. Significant upregulation of MDA5 in response to chIFN-γ treatment suggested that chIFN-γ stimulates PRRs that in turn elicit an antiviral environment against RNA viruses (e.g. AIV, NDV, etc.). Additionally, as type-1 transmembrane proteins, TLRs are highly conserved [66]. Compared with a human genome that contains TLR1 to TLR10, the chicken genome

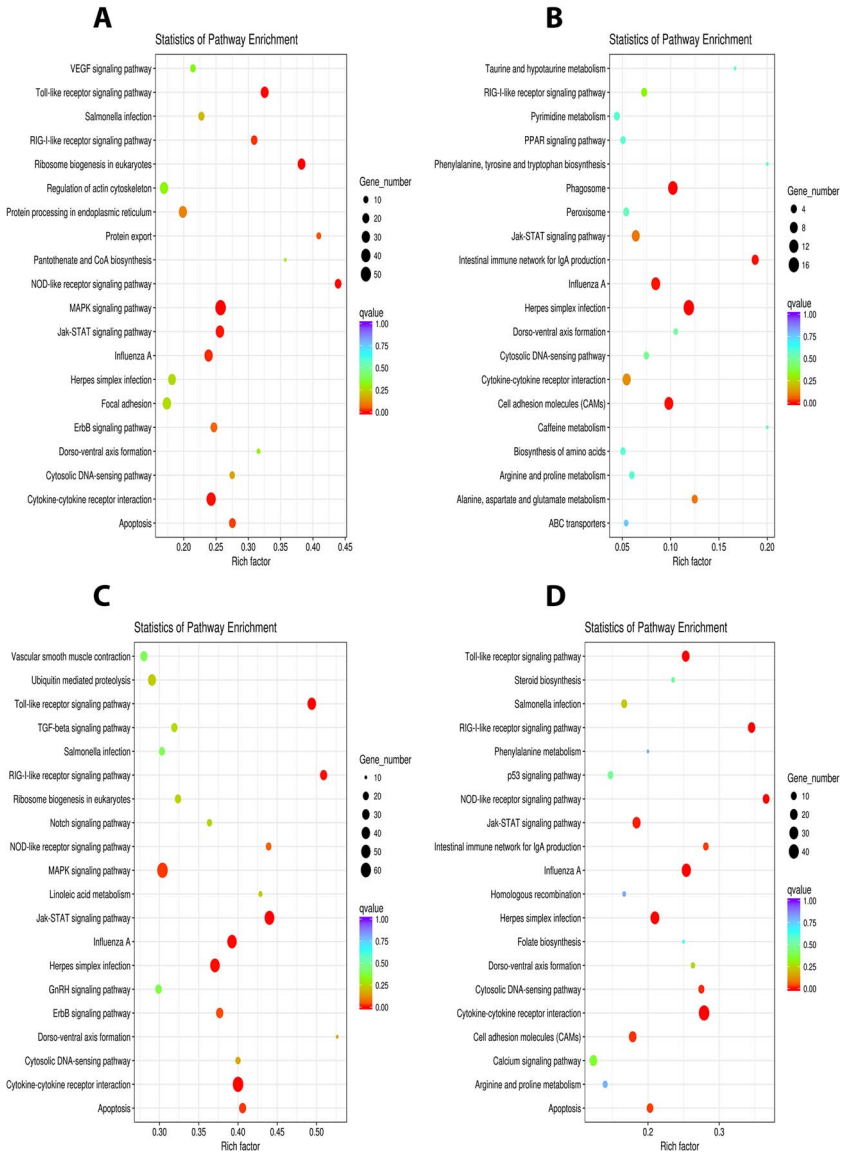

Figure 8. KEGG pathway enrichment analysis for all upregulated DEGs (A) KEGG pathway enrichment in NDV-treated CEFs compared with the control group. (B) KEGG pathway enrichment in chIFN-γ-treated CEFs compared with control group. (C) KEGG pathway enrichment in NDV + chIFN-γ-treated CEFs compared with control group. (D) KEGG pathway enrichment in NDV + chIFN-γ-treated CEFs compared with NDV-treated CEFs group. Circles represent the number of genes, while the colors illustrate the magnitude of richness factor.

contains single genes of TLR3, TLR4, TLR5, TLR7, two genes of TLR2 isoforms (chTLR2 type I and II), and two TLR1/TLR6/TLR10 orthologues [67]. Interestingly, as the chicken genome is devoid of TLR8 (non-functional) and TLR9, only TLR3 and TLR7 are crucial in RNA viral recognition and sensing [68]. Overexpression and upregulation of TLR7 in response to chIFN-γ further changes the role of chIFN-γ in the avian innate immune response in primary cells. Considerable upregulation of TLRs post-chIFN-γ treatment in CEFs further increases the expression of ISGs in a temporal fashion. Additional comprehensive research is necessary to evaluate chIFN-γ as an alternative, effective, and unique therapy for the poultry industry.

The expressions of crucial genes related to innate immunity identified in the present study are exactly the same as those observed in previously performed studies where similar patterns of gene expression were shown in CEFs treated with chIFN-α [12]. Surprisingly, the upregulation of IFIT5, STAT1, Mx1, RSAD2, IL15, ZC3HAV1, and IRF8 further suggested that a common pattern of ISGs expression occurred in both IFN-α- and IFN-γ-mediated immune response in CEFs. Additionally, the upregulation of potent ISGs, including RSAD2 (viperin), IFIT5, and Mx1, demonstrated that an antiviral environment has been generated in the IFN-treated cells, which could stop the propagation of viral pathogen. Similarly, upregulation of identical chemokines, cytokines, and transcription modulators strongly suggested that patterns of gene expression are in common between IFN-α- and IFN-γ-treated CEFs. Although this study primarily focused on IFN-γ, a more precise and comprehensive study including a broad set of chicken IFNs can now be possible to elucidate the IFN-mediated immune response in chicken cells. More work is required to understand the innate immune

signaling cascade associated with type II IFNs. Here, we identified some uncharacterized genes that are involved in avian immunity. Furthermore, *in vitro* CEF treatment with chIFN-γ resulted in a rapid surge of pro-inflammatory cytokines. Cytokine gene expression is widely used as a landmark of immune signaling due to its crucial role in immune signaling. We identified some previously described genes including chemokine ligand 1 (CCL1; ENSGALT00000003670) [69]. Chemokines are essential cytokines that are critical for avian immune responses where they govern the transportation of myeloid and lymphoid cells to the target organs [70]. CCL1 is secreted by monocytes and plays an essential role in the activation of macrophages and T lymphocytes [71]. Likewise, CCL20 is involved in eliciting early immune responses by recruiting lymphoid cells [72]. Similarly, upregulation of CCL1, CCL4, and CCL5 in CEFs suggested that they play fundamental roles in innate immunity in chickens.

The expressions of crucial genes, including TLR3, TLR7, IL1B, IRF7, IL8, STAT1, and MHC class I and II molecules, are characteristic of NDV infection in chicken cells. However, in previously performed studies, a significant expression of type I IFNs (INF-α and IFN-β) was observed against NDV infection [53,54]. Surprisingly, in our experimental settings, we were unable to observe this response. This may be due to multiple reasons, including the use of different viral genotypes, the origin and source of virus, and the type of cells used to determine the NDV-mediated pathogenesis in different bird species [53]. Collectively, based on our experimental findings, we can postulate that the temporal induction of IFNs in response to NDV infection in CEFs is dependent on certain factors, including viral genotype, viral source, cell type, mode of infection, and time points of NDV infection in cells. CEFs are an invaluable tool for investigating the interactions between avian viral pathogens and immune-related genes. Since viral infection can be controlled in a cell culture system, specific changes in molecular and cellular architecture can be observed and characterized. Previous studies have demonstrated that IFN-γ inhibits influenza virus replication [73,74]. Different research groups have tried to investigate the antiviral role of IFNs, but none have managed to elucidate the chIFN-γ-induced antiviral response against NDV infection in CEFs. In this work, in order to evaluate the immune state elicited by chIFN-γ in CEFs, the released NDV viral particles were counted in culture medium with and without chIFN-γ treatment. A reduction in viral load indicated that chIFN-γ actively reduced the NDV viral population. Significantly, published reports showed that ISGs have antiviral activities against NDV infection [9]. These results suggested that stimulation of the innate immune response prior to NDV infection inhibits viral propagation and the release of viral progeny. These findings provided a better insight into the innate immune responses triggered by deadly viral pathogens.

Innate immune signaling in *Aves* is an extremely diverse, multifaceted, integrated, and precise process that relies on many different aspects. All the genes observed in this study govern protein interactions and are directly associated with avian immunity. However, additional investigation and validation of a cohort of immune-associated genes are required to understand the avian immune response against invading viral pathogens. An extensive and inclusive study involving a broad range of genes related to immunity and several IFNs that compare and connect the unified function is required to provide a comprehensive understanding of the innate immune response cascade. Additional investigations using animal models to evaluate whether related arrays of immune response exist in chicken against chIFN-γ are also significant and will further provide a deeper insight into immune signaling in *Aves*.

Supplementary Data

Supplementary data is available at *Acta Biochimica et Biophysica Sinica* online.

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