Analysis of Immune Response of Chicken Primary Cells by Infection With Korean IBV Strain

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Research Article

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Abstract

Infectious bronchitis virus (IBV), an avian coronavirus, is highly contagious, which develops acute pathogenesis in multiple organs. Frequent recombination of spike (S) glycoprotein leads to vaccine strategies insignificant. To understand IBV pathogenesis, we analyzed genetic distance of the Korean IBV isolate to different coronaviruses, including SARS-Cov2. For comprehensive information of the immune responses during IBV infection, we infected primary chicken embryonic kidney cells and performed transcriptome analysis. We observed functional pathways for innate immunity are associated and confirmed mRNA expressions of element genes that coordinate early immune responses. Immune profile of the host cell may assist vaccine development.

1. Introduction

Avian infectious bronchitis virus (IBV) is a momentous coronavirus that develops infectious bronchitis (IB) in the upper respiratory tract and spread to remote organs [1, 2]. When it bears renal tropism, the infection induces interstitial nephritis and tubular pathology [3]. IBV is classified as gammacoronavirus while SARS-CoV-2 is classified as betacoronavirus. Swine coronavirus such as porcine epidemic diarrhea virus (PEDV) and porcine deltacoronavirus (PdCV) are classified as alpha- and deltacoronaviruses, respectively [4]. The spike (S) glycoprotein is a key structure that mediates the coronavirus invasion into the host cell. Specifically, it is a major inducer of host immune responses and determines host range and cell tropism [4-6]. The S glycoprotein is cleaved into S1 and S2 subunits and S1 protein facilitates viral attachment to the host cell receptor and the S2 protein mediates the fusion between the virus and cell membranes [5, 7]. Once the virus arrives inside host cells, genetic modification and recombination of the S1 protein induces various viral subtypes. Consequently, the vaccine strategy is excluded by those genetic variations and results in poor cross protection [1, 2, 8].

We previously reported a genetic feature of a Korean IBV isolate (K047-12) that has kidney tropism [9], hence this study is designed to evaluate the genetic feature of the K047-12 strain and analyze the host immune responses during viral infection. First, we performed a phylogenetic sequence analysis of the S1 protein compared with other coronaviruses. We then investigated the genetic change in the chicken embryonic kidney (CEK) cells after acute infection with K047-12. Transcriptome analysis is a useful tool to anticipate unknown feature of immune systems [10] that we examined various pathways and genes for immune regulation. Our investigation in host immune responses by genetically modified coronaviruses is to broaden the understanding of pathogenesis during chicken coronavirus infection. Moreover, comparing immune responses by coronavirus in animals to humans could contribute to prepare potential cross-species infection against modified viral strains.

2. Materials And Methods

The IBV K047-12 strain was isolated and propagated as reported previously [9]. Briefly, the virus was isolated from the cecal tonsil of a 6-week-old layer in 2012 from Pocheon city, Gyeonggi Province, Korea.

Then the virus was propagated in the SPF chicken embryos at embryo days (EDs) 9–11. The virus had been passaged three times in SPF embryonated eggs (Sungmin Farm, Korea).

Six representative coronaviruses and three IBV strains were chosen for this study. The nucleotide sequences of the S glycoprotein were downloaded from the NCBI GenBank database as follows: HCoV-NL63 (NC_005831.2), TGEV (NC_038861.1), PEDV (NC_003436.1), SARS-CoV-2 (NC_045512.2), Bat-SL-CoVZXC21 (MG772934.1), IBV Beau-R (AJ311317.1), IBV M41-CK (MK728875.1), IBV K047-12 (MK618759.1), and PDCV (MN058072.1). Sequences of S glycoprotein nucleotide were aligned with the Clustal W method using MEGA X software (version 10.2.2). The phylogenetic tree was constructed using the neighbor-joining method and 1,000 bootstrap replicates using MEGA X software.

The kidney tissue of SPF embryonated eggs at ED 18 were collected. Single cells from kidney (1×10^6 cells/mL) were obtained and cultured in DMEM supplemented with 10% FBS. Monolayers of primary kidney cells were prepared and split into K047-12 infected and uninfected control groups. After five days of incubation, half of the medium was replaced with medium containing an IBV virus suspension for the infection group while the equal amount of fresh media was replaced in the control group. Cells were harvested after 48 hours post infection and stored in Tri reagent (Ambion, USA) for RNA extraction. The RNA from each group was sent to Macrogen Inc (Seoul, Korea) for RNA sequencing. The RNA was purified, fragmented, and reverse-transcripted before amplification by PCR for sequencing. The expression profiles for transcript were calculated as the fragments per kb of transcript per million mapped reads (FPKM). The filtered data was normalized by TMM normalization using the calcNormFactors method in the edgeR R library. The criteria for significant differentially expressed (DE) genes between two groups was as follows; l-fold change 1 ≥ 1.5, raw *p* value <0.05. The statistical significance of DE genes was determined using exactTest using edgeR. Gene ontology (GO) and functional annotation analysis for the differentially expressed genes was performed using gProfiler. The significant data were analyzed based on KEGG pathway enrichment. The transcriptome data for IBV strains (M41-CK, Beau-R, K047-12) were analyzed by hierarchical clustering (Euclidean distance and average linkage). The analysis was performed using Cluster 3.0 software and Java Treeview 1.2.0. The DE genes data for the M41-CK and Beau-R strains were acquired from a recent study [1].

Ten genes associated with immune responses were selected for validation at the mRNA level. The RNA was reverse-transcribed into cDNA using Accupower RT mix (Bioneer, Korea) according to the manufacturer's protocol. Quantitative PCR was conducted using a Maxima SYBR Green/Rox qPCR master mix kit (Thermo Fisher, USA). All reactions were set up in duplicate. The reaction conditions were 50°C for 2 min, 95°C for 10 min, and 40 amplification cycles consisting of 95°C for 15 s, 55°C *(b-actin, Il1b, Il10, Il12a, Tlr4, Tlr7, Ccr2, Ccr7*), or 50°C (*Il6r, Il2rh, Il1rap*) for 1 min. The melt curve stage was performed at 95°C for 15 s, 60°C for 1 min, and 95°C for 15 s. The threshold cycle (CT) values were collected. The gene expression was calculated using the formula $2^{-\Delta\Delta CT}$ and mRNA levels were normalized against β-actin. Primers were made based on the published chicken genome sequences in NCBI. The primer sequences were sourced from published papers or designed by a primer designing tool (Primer 3 and BLAST, NCBI) and are listed in Table 1.

3. Results

Using the GenBank database, we analyzed the genetic differences of the Korean IBV isolate (K047-12) to other coronaviruses. As expected, the sequence of the Korean IBV isolate was most similar to standard IBV strains. Of interest, IBV strains showed highest similarity to SARS-CoV-2, and bat coronavirus. On the other hand, alpha- and deltacoronavirus were less correlated in terms of their S1 protein sequences (Fig. 1A). This result identifies the adjacent genetic distance between IBV and SARS-Cov-2 and potential cross antigenicity between human and chicken. Because the K047-12 strain is adapted to renal damage, we infected the virus on a primary chicken kidney cell and performed RNA sequencing to comprehensively investigate genetic regulation. Among 17,026 total genes, we identified 1,084 DE genes that showed greater than 1.5-fold change. Within those, we recognized 787 upregulated genes and 297 downregulated genes by volcano plot (Fig. 1B).

We further classified the DE genes using the GO enrichment analysis and significant changes were detected in biological process and molecular function. In the biological process, K047-12 infection significantly changed about 150 genes that regulate leukocyte activation, inflammatory response, and cytokine-mediated signaling pathway. Arrangement by molecular function revealed that pathways for cytokines and receptors were highly expressed by IBV infection (Fig. 2A). In addition, we examined functional classification by KEGG pathway analysis. In accordance with the GO enrichment analysis, the top-ranked functions were closely correlated with major immune responses, including metabolic pathway, cytokine-cytokine receptor interaction, and the toll-like receptor (TLR) signaling pathway (Fig. 2B). The functional classification demonstrates that the K047-12 infection in primary renal cells essentially activates the immune system.

To identify the precise action of the K047-12 strain in a host immune system, we clustered and compared the gene expression among IBV strains. From a previous publication, we retrieved the raw data for gene expression from primary renal cells that were infected with M41-CK or Beau-R [1]. We then clustered genes by functions for immune response, cytokine responses, and cell activation. As shown in Fig.1A, the S1 genetic distance of M41-CK was closer to Beau-R than K047-12. However, K047-12-activating genes were more analogous to M41-CK than the Beau-R strain (Fig. 3A). This conflicting coherence suggests that the immune system is not completely coded by S1 sequence during infection. During acute infection, TLR activation turns on intracellular signals to stimulate effector cytokines and their receptors followed by cytotoxic T cell generation which is critical for protection, but an exacerbated cytokine secretion is detrimental to the host [11, 12]. Based on functional classification, we determined highly regulated genes that are associated with innate immunity (Table 2). To verify the transcriptome analysis, we designed primers for ten genes and confirmed their expression at the mRNA level. By K047-12 infection, genetic levels of *il1b, il10, il1rap,* and *il2rg* for inflammatory signaling, *tlr4* and *tlr7* for RNA virus-specific PRRs, and *ccr2* and *ccr7* for lymphocyte migration were significantly increased (Figure 3B). Nevertheless a few gene expressions are not significantly changed but showed increasing trend overall. This result validates kidney cells is a source of critical immune regulators during early stage of IBV infection.

4. Discussion

Complete prevention of IB by vaccination has not been very successful due to the rise of variant virus strains. The respiratory tract is the primary organ affected by IBV invasion, but the genetic modification of the S1 protein expands the range of infection to remote tissues [13-15]. Earlier IBV studies focused on viral activity rather than the host-pathogen interaction. Recently we isolated a novel IBV strain with kidney tropism and reported its genetic characteristics by comparing IBV isolates from different regions [9]. Current study, we examined the genetic correlation of the K047-12 strain with coronaviruses from different host species. Interestingly, IBV showed a relative closeness to betacoronavirus such as SARS-CoV2 and bat coronavirus. Considering the repeated emergence of new variants amid the coronavirus pandemic (COVID-19), we speculate on the appearance of a zoonotic coronavirus through IBV similar to avian influenza virus (AIV). Hence, we asked how host cells react with IBV and regulate innate immune response. Infection by kidney-adapted IBV is detrimental to chickens and develops a systemic pathology. As the first line of defense, innate immunity is activated within two days after antigenic stimulation. At this initial stage of IBV infection, cytokines and chemokines are produced and co-stimulatory pathways are activated [3, 16, 17]. Then we speculated the transcriptome analysis of infected kidney tissue by an IBV strain with renal tropism might be helpful to understand the pathogenesis of IB. For precise genetic information for innate immunity during kidney infection, we infected a Korean IBV isolate (K047-12) on primary kidney cells for 48 hours and analyzed genes in immune functions. We first detected expression of 1,084 genes that were significantly changed, while 787 genes were enhanced but 297 genes were suppressed. Assuming upregulated genes are linked to initial immune responses, functional significance by GO enrichment and the KEGG pathway were examined. Functions for innate immunity regulation, such as leukocyte activation, inflammatory response, and cytokine-mediated signaling pathway, are notably activated by K047-12. The study that infected the M41-CK strain also observed comparable gene regulation. For example, *rsad2, snx10, stat1, tnip2, il6, il8l1 and dll1* genes that are associated with cytokine pathway were enhanced by kidney-adapted IBV [1]. Despite of the genetic variance of the S1 gene, IBVs might utilize common pathways for host protection against IBV. However, it is debatable whether certain genetic modifications significantly contribute to the pathogenicity and host immune reaction. Although the modification of a few amino acids is enough to affect the infectivity [18, 19], additional studies are necessary to understand precise immune regulation. Moreover, our examination suggests that the relationship between the S1 protein variation and host response is not always correlated. In a phylogenetic tree analysis for the S1 protein sequence, the genetic distances between K047-12 to M41-CK strain and K047-12 to the Beau-R strain were not different (Fig.1A). According to the immunological classification, however, K047-12 was closer to M41-CK than the Beau-R strain (Fig.3A). This observation emphasizes the advantage of transcriptome analysis in host cells over the viral examination. Identification of major cytokine regulation during acute IBV infection opens paths to figure out following adaptive immunity that manipulate systemic inflammation and we pinpointed substantial genes for innate immunity. In the M41-CK strain infection, *il6* and *il1b* were activated in lung tissue when an IBV stain for kidney tropism accelerated immune modulators, such as *il6, il10ra, il17ra, ccl4,* and *ccl17* [3, 20, 21]. Among the top twenty list of highly regulated genes, we also found overlapping genes

including *il1b, il10, il12a, il1rap, il2rg, il6r, tlr4m tl47, ccr2,* and *ccr7*. We then further validated the mRNA expression of those genes using real-time PCR and identified *il1b* and *il10* as signature genes. Produced by mesangial cell, IL-10 is a critical cytokine for pathophysiology in renal disorders. Nonetheless of its anti-inflammatory action, IL-10 is upregulated during nephritis where the bloom of inflammatory lymphocytes to be controlled and regulatory T cells are required [22, 23]. Along with IL-6, IL-1β is also secreted from renal mesangial cells and mediates inflammation. These are key activators for Th17 cell generation that develops numerous renal disorders [24, 25]

Detecting PAMPs, TLR activation signals for proinflammatory cytokine production [26]. For instance, TLR3 and TLR7 are critical mediators for IBV recognition and initiation of innate immune response [20]. TLR3 and TLR4 are also activated in SARS coronavirus infection [27, 28]. Considering TLR4 and TLR7 promotion by K047-12, we speculate that IBV might share in common with betacoronavirus in the regulation of PRR recognition. The knowledge from rodent and human immunology in regulation of the chemokine receptors is frequently applied in chicken study. CCR2 receives the CCL2 signal and activates monocyte migration for viral clearance thus its deficiency is fatal during infection [29]. CCR7 is activated by CCL19 and CCL21, and a study examined its critical role for T migration in chickens [30]. In addition, human patients with SARS coronavirus infections were activated by CCR2 and CCR7 expression [31, 32]. We also observed *ccr2* and *ccr7* activation by K047-12 which suggests that the avian immune system activates comparable chemokine receptors to mouse and human and directs immune cell migration and host protection.

A genetic modification endows IBV for various tissue tropisms, which makes it difficult to save animals from the endemic outbreak. IBV strains with renal tropism are lethal because they also attack both urogenital organs and systemic organs [33]. Therefore, to understand the immune responses of target organs against viral infection, we applied the transcriptome analysis of primary renal cells. In specific, we found the genetic profile in immunologic function for pathogen recognition, cytokine regulation, and signals is closely associated with innate immune responses during viral infection. In this regard, this study can be utilized to identify unknown immune pathways that avian viruses prefer to utilize. Therefore, our results could expand the knowledge in avian immunology that is applied for vaccine strategies and prevention of new variant viruses.

Declarations

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Conflict of interest/Competing interests

The authors declare that they have no conflicts of interest.

Authors' Contributions

Conceptualization: Kwon HM, Park J; Data curation: Lee R, Jung JS, Park J; Formal analysis: Lee R, Jung JS, Yeo J, Park J; Investigation: Lee R, Jung JS, Park J; Methodology: Lee R, Jung JS, J Yeo, Park J; Project administration: Kwon HM; Park J; Supervision: Park J; Validation: Lee R, Jung JS, Park J; Writing - original draft: Lee R, Jung JS, Park J

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Tables

Table 1.

Primer information for qRT-PCR

Target	Forward primer (5'-3')	Reverse primer (3'-5')	Reference
β-actin	TTGTCCACCGCAAATGCTTC	AAGCCATGCCAATCTCGTCT	This study
Il-1β	ACAGAGATGGCGTTCGTTCCCGA	TCAGCTCGACGCTGTCGATGT	[34]
Il-10	CTGTCACCGCTTCTTCACCT	ATCAGCAGGTACTCCTCGAT	[35]
Il-12α	CAGAGCTGGGGAACCTCAAG	CATCTCTGCAGTGAGGGCAC	This study
Tlr4	TCTTTCAAGGTGCCACA	GGATATGCTTGTTTCCAC	This study
Tlr7	TCTGGACTTCTCTAACAACA	AATCTCATTCTCATTCATCATC	[36]
Ccr2	ATGCCAACAACAACGTTTGA	TGTTGCCTATGAAGCCAAA	[37]
Ccr7	CGGCTGAAGACCATGACAGA	CAGCCTGAACGATGGCAAAG	This study
Il6r	CGCCTGCTGGTGGAAGA	TTCACCCGGCAGACGAATTT	This study
Il2rg	CTACGTGCGCAGCAAGATCA	GACACTGTGAGGACACTGCGG	This study
Il1rap	TCGTGGATGCCAAAGTCACA	AGTGCTTGAGGCCGAATCTT	This study

Table 2.

Fold change of highly upregulated genes by IBV infection.

Gene	Description	Fold change (Infected / Control)
Il2rg	interleukin 2 receptor subunit gamma	1.6447
Tf	transferrin (ovotransferrin)	1.6469
Tlr4	toll like receptor 4	1.6504
Il6r	interleukin 6 receptor	1.7668
Tlr15	toll-like receptor 15	1.7790
Tlr7	toll like receptor 7	1.8052
Lcp1	lymphocyte cytosolic protein 1	1.8258
Lbp	lipopolysaccharide binding protein	1.8319
Il1rap	interleukin 1 receptor accessory protein	1.8490
Stab1	stabilin 1, transcript variant X2	1.8967
Il8l2	interleukin 8-like 2	1.9669
Tlr2b	toll-like receptor 2 family member B	1.9793
Cybb	cytochrome b-245 beta chain	1.9834
Mmr1l4	macrophage mannose receptor 1-like 4	2.2063
Cd48	CD48 molecule	2.2107
Ccl26	C-C motif chemokine ligand 26	2.2521
Ccll8	chemokine	2.3019
Il1r2	interleukin 1 receptor type 2	2.3726
Ccll7	chemokine	2.3810
Cd80	CD80 molecule	2.4126
Cd72ag	CD72 antigen	3.0760
Ccr2	C-C motif chemokine receptor 2	3.1495
Marco	macrophage receptor with collagenous structure	3.3576
Selp	selectin P, transcript variant X2	3.9415
Ccl4	chemokine (C-C motif) ligand 4	4.0789
Il1b	interleukin 1, beta	4.2180

Ccr7	C-C motif chemokine receptor 7	4.2953
Il8l1	interleukin 8-like 1	4.5621
Avd	avidin	5.3144
Il4i1	interleukin 4 induced 1	9.7158

Figures

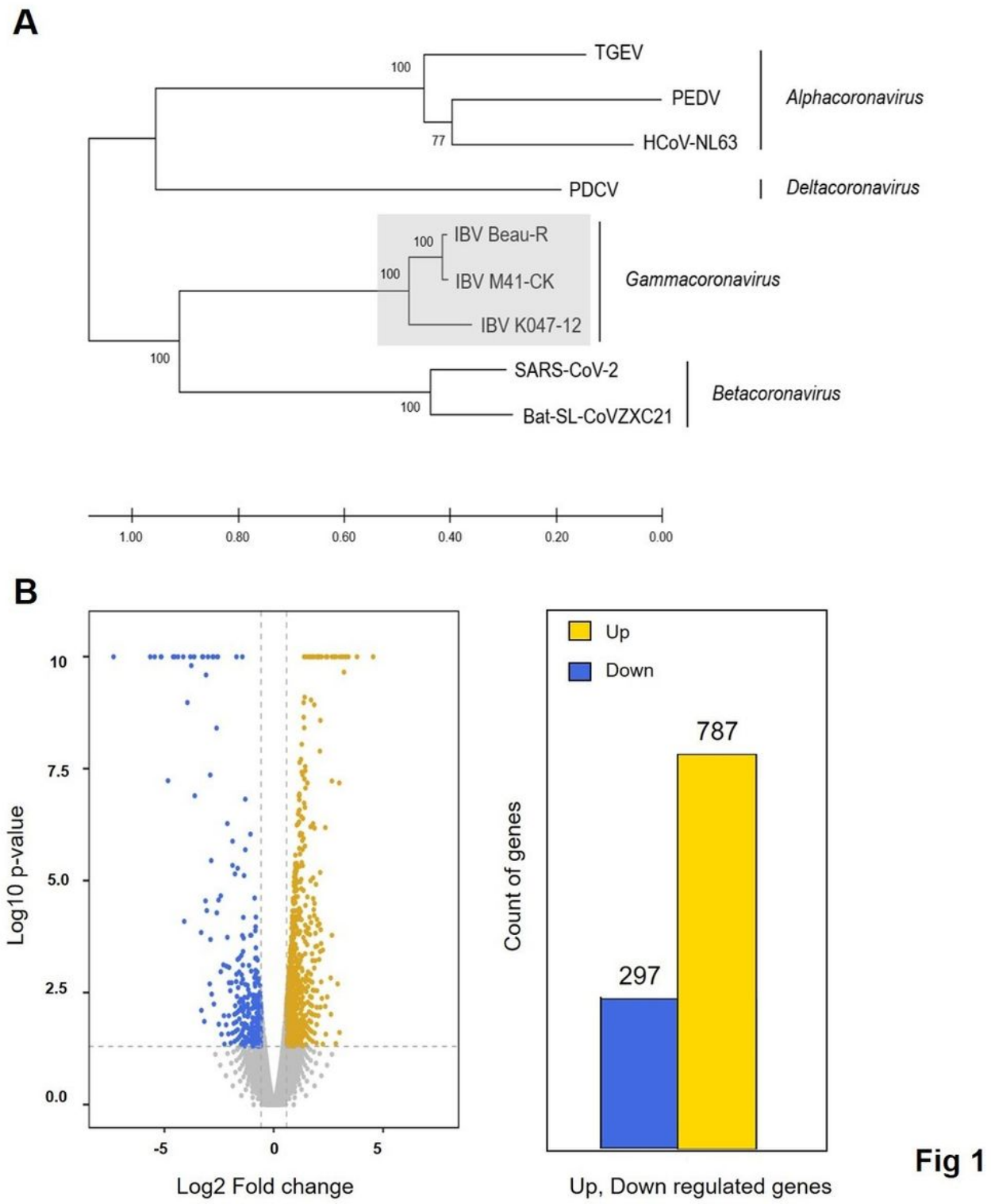

Figure 1

Phylogenetic analysis of IBV 047-12 and identification of differentially expressed (DE) genes during infection. (A) The phylogenetic tree was constructed using MEGA X software (version 10.2.2) with the neighbor-joining method (bootstrapping for 1,000 replicates). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the genetic distances used to infer the

phylogenetic tree. The IBV strains are highlighted. (B) Volcano plot showed p values derived by comparison on the log 2-fold change of expression level between control and infected group. The blue color represents downregulated genes and the yellow bar represents upregulated genes (left). The number of DE genes was shown in the histogram (right). The criteria for significant DE genes was as follows; l-fold change 1 ≥ 1.5, raw p value < 0.05.

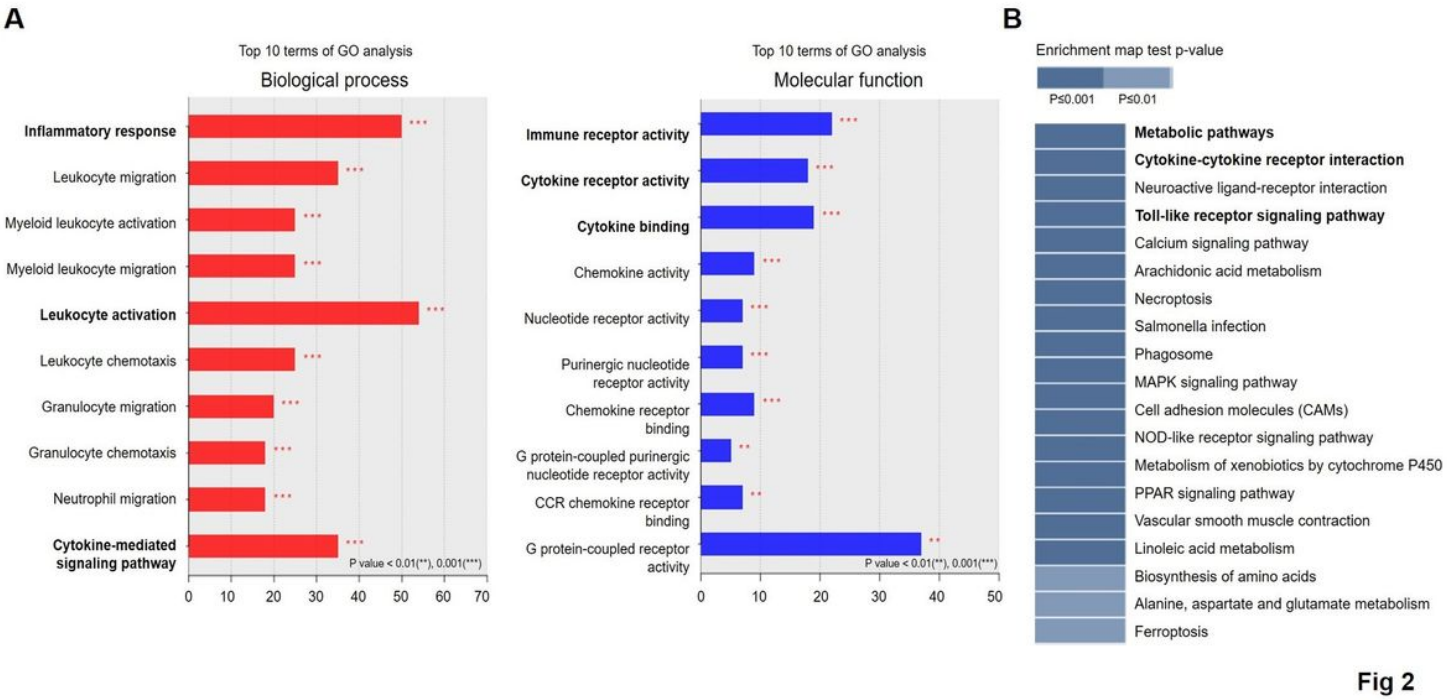

Figure 2

Functional classification of DE genes. (A) DE genes were assorted by GO enrichment analysis. The top 10 terms were shown within biological processes and molecular function. (B) The heatmap was a result of gene-set enrichment analysis using a modified Fisher's exact test on each pathway map. The legend shows p values where a p value < 0.05 indicates a significantly enriched pathway term.

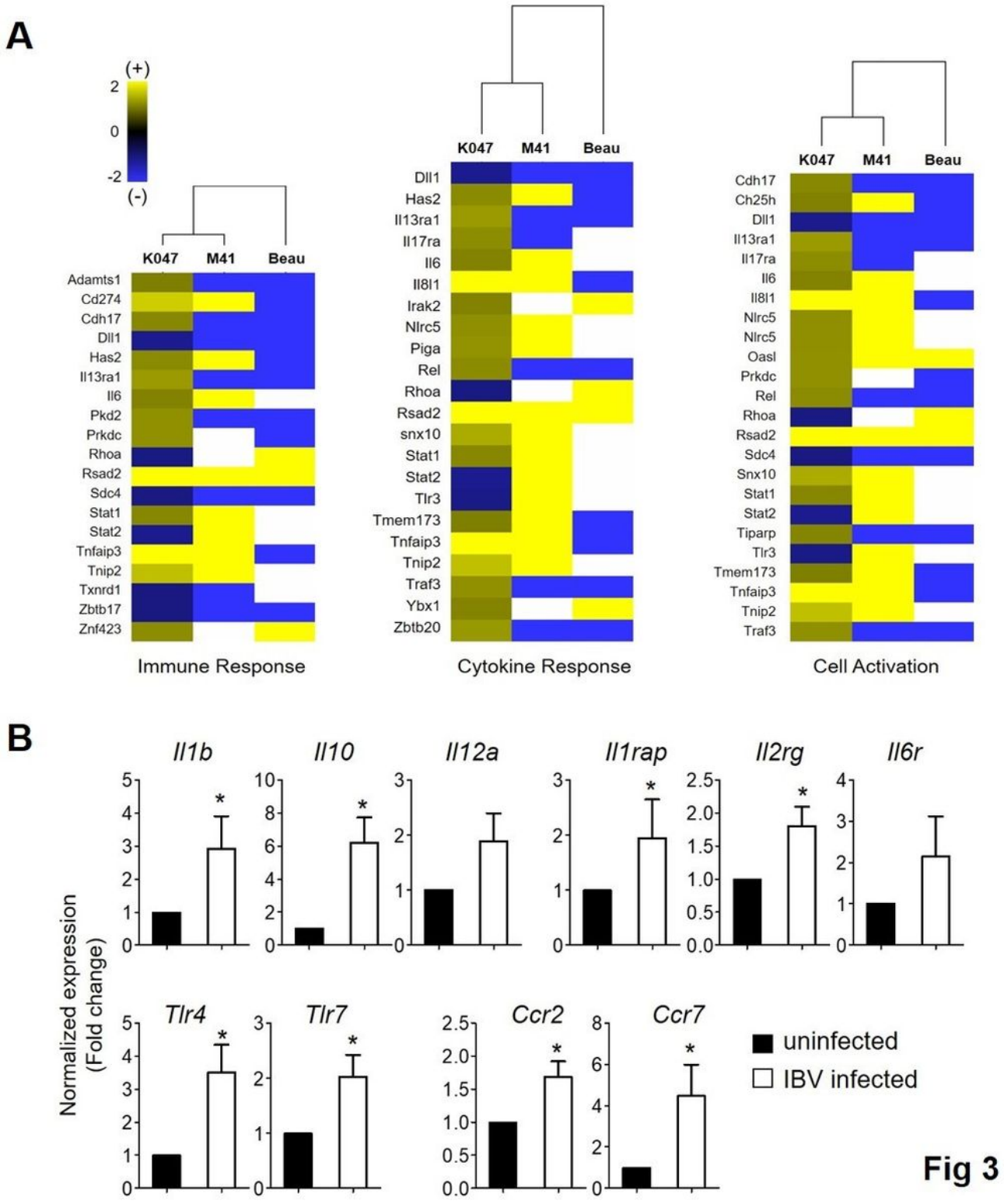

Figure 3

Assorted DE genes by immunologic function. (A) Hierarchical clustering of differential expression profiles of genes in K047-12, M41-CK, and Beau-R strains. Gene profiles were classified based on GO enrichment. The diagram indicates the correlation of the gene expression levels from all samples compared against each other. Color represents the relative expression levels of genes, where yellow and blue refer to higher and lower expression, respectively (white color for null data). (B) Genes for innate immunity were

validated by qRT-PCR. Cytokine, and receptor related molecules (upper) and TLRs and chemokine receptors (lower) expressed in primary chicken kidney cells are shown. Combined data from 6-7 experiments are shown and error bars are SEM. *$p \leq 0.05$ from uninfected groups by nonparametric and paired t test.