Integrative Transcriptomic Analysis Reveals the Immune Mechanism for A CyHV-3-Resistant Common Carp Strain

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Word count: 5652

Number of figures: 7

Keywords: transcriptome, lncRNA, CyHV-3, resistant immune mechanism, common carp

ABSTRACT

Anti-disease breeding is becoming the most promising solution to cyprinid herpesvirus-3 (CyHV-3) infection, the major threat to common carp aquaculture. Mortality studies suggested that a breeding strain of common carp is resistant to this disease. This study illustrates the immune mechanisms involved in anti-CyHV-3 ability. An integrative analysis of the protein-coding genes and long non-coding RNAs (lncRNAs) using transcriptomic data was also performed. Tissues from the head kidney of common carp were extracted at day 0 (the healthy control) and day 7 after CyHV-3 infection (the survivors), and used to analyze the transcriptome through both Illumina and Pac-bio sequencing. Following analysis of the Kyoto Encyclopedia of Genes and Genomes pathways and Gene Ontology terms involved, the immune-related genes were merged. These genes were filtered using the current common carp immune gene library, and information on the immune process was detailed. Immune gene categories and their corresponding genes in different comparison groups were revealed. Also, the immunological Gene Ontology terms for lncRNA modulation were retained. The weighted gene co-expression network analysis was used for the regulation of immune genes lncRNA. The results demonstrated that the breeding carp strain develops marked resistance to CyHV-3 through a specific innate immune mechanism. The featured biological processes were autophagy, phagocytosis, cytotoxicity, and virus blockage by lectins and mucin 3 (MUC3). Moreover, the immune suppressive signals, such as suppression of interleukin 21 receptor (IL21R) on STAT3, PI3K mediated the inhibition of inflammation by dopamine upon infection, as well as the inhibition of NLR family CARD domain containing 3 (NLRC3) on STING during a steady state. Possible susceptible factors for CyHV-3, such as integrin beta 1 (ITGB1), toll-like receptor 18 (TLR18), and C-C motif chemokine ligand 4 (CCL4), were also revealed from the common strain. The results of this study suggested that the regulation of galectin 3 (LGALS3) and T cell leukemia homeobox 3 (TLX3) by lncRNA may play a role in the resistance mechanism. Therefore, immune factors that are immunogenetically insensitive or susceptible to CyHV-3 infection have been revealed.

INTRODUCTION

Cyprinid herpesvirus-3 (CyHV-3) infection is a major threat to common carp aquaculture (1), leading to widespread mortality and substantial economic loss. CyHV-3 is thought to cause death by weakening the host's immune system, resulting in susceptibility to pathogenic microbes (1). In common carp, clinical signs of the disease develop rapidly and may induce morbidity and mortality within a period of 6 to 10 days following infection (2).

Carp that survive a primary infection with CyHV-3 can be resistant to future infection with this virus. This immunity may be genetically passed onto offspring. Since

latency and life-long infections with CyHV-3 exist in carp (3, 4), genetic backgrounds are crucial in developing an understanding resistance against the virus. Experimental infections of carp from pure lines or crosses have indicated the existence of genetic background of resistance by divergent survival rates (2). For example, a markedly higher expression of immune-related genes involved in pathogen recognition, complement activation, major histocompatibility complex class I (MHC I)-restricted antigen presentation, and the development of adaptive mucosal immunity was noted in the more resistant R3 line. Higher activation of $CD8^+$ T cells was also observed (5). Additionally, carp strains of Asian origin, particularly Amur wild carp, currently being treated as *Cyprinus rubrofuscus*, were shown to be more resistant to CyHV-3 than strains originating from Europe and belonging to *Cyprinus carpio*, such as the Prerov scale carp or koi carp from a breed in the Czech Republic (6).

The immune response of carp to CyHV-3 involves both innate and adaptive aspects with the outcome of the disease largely depending on whether the balance is tipped in favor of the host's immune response or virus's evasion strategy (2). In general, transcriptomic analysis has revealed that three immune-related pathways, namely the mitogen-activated protein kinase (MAPK) signaling pathway, the innate immune response, and the cytokine-mediated signaling pathway, were highly involved in the infection with CyHV-3 (7). In red common carp × koi, the expression of interleukin 12 (IL12) p35, interferon (IFN) αβ, and toll-like receptor 9 (TLR9) may provide potential genes related to resistance against KHV (another term for CyHV-3) (8). However, the magnitude of type I IFN response did not correlate with a higher resistance in CyHV-3-infected carp, during the challenge test among different strains, although CyHV-3 infection can induce type I IFNs (6, 9). Regarding the innate resistance in carp, the mapped CyHV-3 survival quantitative trait loci have been reported mainly in *IL10* and *MHC II* (10). Recently, by quantitative trait locus mapping and genome-wide association study, *tumor necrosis factor-alpha* (*TNFA*), *hypoxia inducible factor 1 subunit alpha* (*HIF1A*), *galectin-8* (*LGALS8*), *rootletin*, and *paladin*, have also been related to resistance against CyHV-3 (11). Adaptive immunity through both cytotoxicity and immunoglobulin (Ig) secretion may be involved in resistance. Matthew et al. revealed that, in the anterior kidney, Ig secretion plays an important role in the resistance during the persistent infection or reactivation phases of CyHV-3 (1). In addition, CyHV-3 profoundly influences the expression of host miRNA, although the regulation of immune processes by miRNA in the clinical and latent phases differs (4). This suggests an important role of non-coding RNAs in anti-CyHV-3 immunity.

Based on our previous study, a strain of common carp has already shown a higher survival rate after breeding for four generations. In addition, it has displayed a better immunological index as well as a reduced virus load in immune organs, such as the kidneys and spleen (12, 13). However, there is a lack of systemic studies focusing on

a detailed network of the immune system for the anti-CyHV-3 immune mechanism in this resistant strain. The third-generation sequencing method, such as Pac-bio, can decode the genetic sequences that are markedly longer compared with the second-generation method, such as Illumina (14). Therefore, a higher quality assembly of transcripts, including both mRNA and non-coding RNAs, may provide a more detailed understanding of the mechanism of genetic resistance.

In this study, integrative transcriptomic analysis was performed for both mRNA and long non-coding RNA (lncRNA) during the CyHV-3 challenge. Immune-related transcripts from survivors and healthy controls, from either resistant or normal strains, were analyzed using the strategy shown in Figure 1. By comparing data from different groups for different strains obtained through both Illumina (New England Biolabs, Ipswich, MA, USA) and Pacific Biosciences (PacBio) sequencing, both immune-related differentially expressed genes (DEG) and lncRNA have been revealed for the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways and/or Gene Ontology (GO) terms involved. The immune gene-related lncRNA was also explored. This study may shed some light on fish breeding for virus-resistant strains, and facilitate disease control after CyHV-3 infection.

MATERIALS AND METHODS

Ethics Statement

All procedures involving animals in this study were conducted according to the guidelines for the care and use of laboratory animals of Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences (Harbin, China). The studies involving animals were reviewed and approved by the Committee for the Welfare and Ethics of Laboratory Animals of Heilongjiang River Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Animals and Virus Challenge

The fish used in this study were obtained from the Kuandian Research Base of Heilongjiang River Research Institute (Liaoning, China). The breeding resistant strain was the fourth generation after the selection of resistance to CyHV-3 (13). For the common carp involved in this study, both the CyHV-3-resistant strain and common strain were used for the virus challenge. Since CyHV-3 has a mucosal route of infection mainly via the skin rather than the gut (2), this study induced infection with CyHV-3 by adding the homogenate solution of internal organs from sick fish into the water of the tanks. To separate the control and experimental groups from either the resistant or common strains, four tanks (1.6 m × 1.2 m × 0.6 m) containing healthy juvenile common carp (~70 g; N=300 fish per tank), were used. The water temperature during the experiment was maintained at 25±1°C.

Sampling

A selection of common carp, from either the resistant or normal strains, were sacrificed. Head kidney samples were obtained at days 0 and 7 after challenge, representing the control and survivor carp. The samples (N=3) were collected immediately and soaked in 10 volumes of RNAlater (Qiagen, Hilden, Germany), for sequencing using Illumina (New England Biolabs). To better sequence and generate the lncRNAs, PacBio sequencing was applied to analyze the mix of liver, spleen, and kidney. The number of dead fish during the experiment was counted daily to calculate the mortality rate.

RNA Extraction

RNA was isolated using the AllPrep DNA/RNA FFPE Kit (Qiagen, Hilden, Germany), according to the instructions provided by the manufacturer. RNA degradation and contamination were monitored on 1% agarose gels. RNA purity was checked using the NanoPhotometer spectrophotometer (Implen Inc., Westlake Village, CA, USA). RNA concentration was measured using the Qubit RNA Assay Kit in a Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA). RNA integrity was assessed using the RNA Nano 6000 Assay Kit of the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, CA, USA).

Library Preparation and Sequencing for Transcriptomic Analysis

Optimized RNA-Seq strategies, including both PacBio and Illumina (New England Biolabs) sequencing (15), were used to more precisely resolve the sequence of transcripts. Firstly, the total RNA isolated from the head kidney was used to construct the cDNA library. Subsequently, the library was sequenced on a PacBio RS II platform (Biomarker Technologies, Beijing, China). For the PacBio Long Read Processing, raw reads were processed into error-corrected reads of insert using Iso-seq pipeline with minFullPass = 0 and minPredictedAccuracy = 0.90. Next, full-length, non-chemiric transcripts were determined by searching for the polyA tail signal and the 5’ and 3’ cDNA primers in reads of insert. Iterative Clustering for Error Correction was used to obtain consensus isoforms, and full-length consensus sequences from Iterative Clustering for Error Correction were polished using Quiver. High-quality transcripts with post-correction accuracy of >99% were retained for further analysis.

The illustration of transcripts obtained from the results of PacBio could provide reference transcriptional sequences for the assembly of Illumine sequencing data, to improve sequencing quality. Therefore, Illumina (New England Biolabs) sequencing was performed on all head kidney samples. The procedure used for the preparation of the gene library and sequencing of the transcriptome followed previously published methods (16). Briefly, sequencing libraries were generated using the NEBNext UltraTM RNA Library Prep Kit for Illumina (New England Biolabs), and the library quality was assessed on the Agilent Bioanalyzer 2100 system. The library preparations were sequenced on an Illumina platform, and 150 bp paired-end reads

were generated.

Annotation and Functional Analysis of Transcripts by Public Databases

All reads in the transcriptome data were mapped on the common carp genome (https://asia.ensembl.org/Cyprinus_carpio_german_mirror/Info/Index) (17) for annotation. The data from the Illumina platform were also used to check and replace errors in the data from the PacBio platform. Both the annotation of genes and lncRNAs were subsequently generated. The revealed common carp DEGs were annotated by GO terms and KEGG pathways, following a previously published protocol (16, 18). In brief, functional annotation and the classification of genes were determined by both employing local genes blasts against GO Consortium (http://geneontology.org/), Blast2GO (Bioinformatics Department, Prince Felipe Research Institute, Valencia, Spain), and KEGG (https://www.kegg.jp/kegg/pathway.html). Enrichment of the KEGG pathways was carried out for differently expressed immune genes, including both upregulated and downregulated genes for all comparison groups. For this purpose, the gene list of the current construct common carp immune gene library was used as follows. For the comparison groups, DEGs from intra-stain comparison groups A (survivors vs. controls in the resistant strain) and B (survivors vs. controls in the normal strain), as well as inter-strain comparison groups C (the comparison between survivors at day 7 for the two strains) and E (the comparison between control fish at day 0 for the two strains) were used. Vital genes for survival were investigated through group D, in which the genes without significant differences ($P > 0.05$) between the survivors from the resistant strain and the normal strain were compared with the transcripts from dying fish. LncRNAs were also annotated by GO terms.

Construction of the Common Carp Immune Gene Library

The common carp immune library was constructed by following our previously published method, which was applied to grass carp (16) and tilapia (18), with some adjustment for viral infection-related immune genes. The modifications were based on gene information obtained by blasting each sequence to databases, including NCBI NR database, as well as Swiss-Prot and Pfam databases. The common carp immune gene library contained information for immune genes at two levels. For the first level, nine categories of immune processes, namely “acute phase reactions”, “pattern recognition”, “antigen processing and regulators”, “complement system”, “inflammatory cytokines and receptors”, “adapters, effectors and signal transducers”, “innate immune cells related”, “T/B cell antigen activation”, and “other genes related to immune response”, were proposed. Subsequently, many categories of immune genes for each immune process (detailed in Table S1) were applied for the second level. The library was used to filter transcriptome data and obtain details of the immune processes and particular immune genes for each comparison group, during the GO term and KEGG pathway enrichment.

231
232 **Statistics Analysis**
233 The DEG from intra-strain comparison groups A and B, as well as inter-strain
234 comparison groups C and E were generated by comparing the RPKM (Reads Per
235 Kilobase of transcript, per Million mapped reads) for each gene during a steady or
236 inflammatory state, using the DESeq2 R package (1.16.1). To reveal the basic genes
237 for the fish to survive from CyHV-3 infection, the mRNAs of non-significant
238 difference ($P > 0.05$) between the survivors of both the resistant and normal strains,
239 were compared using transcripts from the dying fish, consequently obtaining the DEG
240 in group D. The resulting *P*-values were adjusted using the Benjamini and Hochberg
241 approach for controlling the false discovery rate. Genes with an adjusted $P < 0.05$
242 found by DESeq2 were assigned as being differentially expressed. The following
243 bioinformatics analysis was performed to select immune-related transcripts from the
244 common carp immune gene library, and construct the barplots for the major immune
245 processes and immune categories. The t-test was used to assess differences, with a
246 false discovery rate adjusted $P < 0.05$. Qualitative comparisons were performed
247 between samples by counting the number of DEG. The data were rearranged in
248 Microsoft EXCEL, and applied to plot charts by ggplot2 (2.2.1) using R language.
249
250 **Correlation Analysis between LncRNA and Genes Involved in Immune-related**
251 **GO**
252 The weighted gene co-expression network analysis (WGCNA) was performed using
253 the R package "WGCNA" (19). Specifically, all genes with an expression variance
254 ranked in the top 75 percentile of the data set were retained (20). The R package
255 WGCNA was used to construct the weighted gene co-expression network (21). A
256 matrix of signed Pearson correlations between all gene pairs was computed, and the
257 transformed matrix was used as input for linkage hierarchical clustering. Genes with
258 similar expression patterns were clustered together as one module. Subsequently,
259 using R package clusterProfiler (22), enriched GO terms for lncRNA-related protein-
260 coding genes were generated for the gene list of every module. The immune-related
261 GO terms were selected, and the immune genes, as well as related lncRNAs, were
262 classified into different comparison groups, to elucidate the detail lncRNA-mRNA
263 network for the differential anti-disease mechanism involved in the two analyzed
264 strains.
265
266 **Validation through Reverse Transcription-Polymerase Chain Reaction (RT-PCR)**
267 The mRNA samples used for transcriptome sequencing were also subjected to real-
268 time quantitative RT-PCR validation (n = 3), following a previously published
269 protocol [14, 16]. After RNA isolation, reverse transcription and quantitative PCR
270 (qPCR) experiments were carried out for 11 genes. Gene expression levels were
271 determined using TaqMan-based qPCR (Thermo Fisher Scientific, Waltham, MA,
272 USA), which was performed on the RotorGene system (Qiagen, Hilden, Germany).

The total RNA was reverse transcribed, and 60 ng were used for the One Taq RT PCR Kit (New England Biolabs) according to the instructions provided by the manufacturer for mRNA quantification and reverse transcription (Thermo Fisher Scientific, Waltham, MA, USA). For mRNA quantification, the resulting cDNA was pre-amplified using a TaqMan PreAmp Master Mix Kit (Thermo Fisher Scientific, Waltham, MA, USA). The relative levels of mRNA were determined from diluted cDNA and pre-amplified cDNA using qPCR and appropriate TaqMan probes. The expression levels were calculated after an efficiency correction (based on the results of a serial dilution) relative to those of endogenous controls.

RESULTS

Mortality Rate and Pathological Appearance

The number of dead fish was recorded from day 1 to day 15 after challenge (Table S1). The mortality rate was calculated for all groups (Table S2). For the challenged normal strain (Figure 2A), the mortality rate increased daily, except for a decrease observed on day 5. Mortality peaked at day 7 (69%) and decreased to a markedly low level thereafter. For the challenged resistant strain (Figure 2A), the mortality rate remained at a markedly low level, with the highest value (11%) recorded at day 7. The mortality rates of the two challenged groups were significantly different ($P = 0.000$). In the two unchallenged controls, there were no dead fish. Additionally, compared with the organs in survivors of the resistant strain, pathological swelling and hyperemia of the internal organs (particularly the spleen) was obvious in survivors from the normal strain on day 7 (Figure 2B).

Quality of Transcriptomic Data

Sequencing was performed with both the PacBio RS II and Illumina platforms to analyze the gene information of the common carp. Through PacBio sequencing, 19.49 G data were obtained. Of the 258,346 ccs, 79.79% were full-length sequences, and 17,769 polished high-quality isoforms were also revealed (Table S3, sheet 1). Meanwhile, the Illumina data were also characterized by high quality, and the information for each sample is provided in Table S3 (sheet 2).

Validation of Transcripts

A total of 24 reactions were performed to validate the transcripts of 11 genes, most of which were immune genes. The correlation analysis showed that fold changes between transcriptome and qPCR results correlated well (R^2=0.929718564). The gene ID, annotation, primers, and fold-change information are listed in Table S4.

GO Analysis of Differentially Expressed Transcripts among Tested Fish

The heatmap of all the tested head kidney samples (Figure S1) revealed significant differences across samples, including between the normal and resistant strains. Cluster analysis of the expression levels of immune genes demonstrated that survivors of the

normal strain had significantly differences versus those of the resistant strain. According to the enriched GO terms (Figures 3 and S2), the immune-related biological processes were “immune system process” in comparison of A–E and “cell killing” in comparison between A and C; the immune-related cellular component was “virion”. In comparisons between B and C, the immune-related molecular function was “antioxidant activity”. In comparisons between A, B and C, and “chemoattractant activity” in comparisons between A and B.

KEGG Analysis of Differentially Expressed Transcripts among Tested Fish

After filtering was conducted using the gene list of the common carp immune gene library (Table S1), the immune-related pathways appeared more clearly (Figure 4). Compared with group A, there were markedly more genes involved in immune-related pathways in groups B and C. The immune-related pathways found in different comparison groups were “phagosome”, “regulation of autophagy”, “ubiquitin mediated proteolysis”, and “plant-pathogen interaction” in group A (Figure 4A); “endocytosis”, “phagosome”, “FoxO signaling pathway”, “ubiquitin mediated proteolysis”, “DNA replication”, “fructose and mannose metabolism”, “oxidative phosphorylation”, and “plant-pathogen interaction” in group B (Figure 4B); and “endocytosis”, “phagosome”, “ubiquitin mediated proteolysis”, “DNA replication”, “fructose and mannose metabolism”, “oxidative phosphorylation”, and “plant-pathogen interaction” in group C (Figure 4C). Since the comparison of C highlighted the differences between both the survivors involved in comparisons A and B, a bubble chart was used to further clarify both the enrichment factors and gene numbers for the related pathways. The results (Figure 4D) showed that the most enriched immune-related pathways were “p53 signaling pathway”, “NOD-like receptor signaling pathway”, “lysosome”, “oxidative phosphorylation”, and “proteasome”. Of note, the pathways with the greatest number were “phagosome”, “proteasome”, and “oxidative phosphorylation”. Besides, some immune-related metabolic pathways, such as “glycolysis/gluconeogenesis” and “fructose and mannose metabolism”, were also revealed through filtering with the gene list of the common carp immune gene library in the KEGG pathway analysis for the comparison C (Figure 4D). Additionally, the pathway “dopaminergic synapse” (Figure S3) may indicate alteration of the dopaminergic signal during viral infection.

Classification of Immune-related DEG from Different Comparison Groups into Immune Process and Immune Gene Category

The current construction of the common carp immune gene library (Table S4) was used to classify the revealed immune transcripts and refine the involved immune processes and immune gene categories. To illustrate the immune process, the information for immune gene categories and the immune-related DEG in all comparisons were listed in Table 1, with the details for comparison groups A–E provided in Tables S5–S9, respectively. In group A (Figure 5A and Table S5), most

immune mRNAs were upregulated in the immune processes of “pattern recognition”, “inflammatory cytokines and receptors”, and “T/B cell antigen activation”. They were downregulated in the immune process of “inflammatory cytokines and receptors”, “complement system”, “adapters, effectors and signal transducers”, and “pattern recognition”. Interestingly, there were no genes in “antigen processing and regulators” for group A. In group A, there were only downregulated genes in “acute phase reactions”, as well as only upregulated genes in “innate immune cells related”. In group B (Figure 5A and Table S6), immune genes were upregulated in the immune processes, such as “other genes related to immune response”, “inflammatory cytokines and receptors”, and “adapters, effectors and signal transducers”. Meanwhile, the downregulated genes were involved in the immune processes, such as “pattern recognition”, “inflammatory cytokines and receptors” and “other genes related immune response”. The largest number of immune mRNAs was observed in group C (Figure 5A and Table S7). The upregulated genes were mainly involved in “inflammatory cytokines and receptors”, “other genes related to immune response”, “T/B cell antigen activation”, “pattern recognition”, and “adapters, effectors and signal transducers”, whereas the downregulated genes were mainly involved in “other genes related to immune response”, “adapters, effectors and signal transducers”, “inflammatory cytokines and receptors”, “T/B cell antigen activation”, “pattern recognition”, and “antigen processing and regulators”. In group D (Figure 5B and Table S8), without “acute phase reaction”, “innate immune cells related”, and “complement system”, there were only six immune processes. Among those, “T/B cell antigen activation” and “other genes related to immune response” exhibited the greatest number of DEG. In group E (Figure 5A and Table S9), there were no genes involved in “acute phase reaction” and “innate immune cells related”. Only upregulated genes were reported in “antigen processing and regulators” and “T/B cell antigen activation”, while only downregulated genes were noted in “complement system”. The upregulated genes in group E were mostly involved in “adapters, effectors and signal transducers”, and few in “inflammatory cytokines and receptors”, whereas the downregulated genes were mostly involved in “inflammatory cytokines and receptors”.

Venn-regional Analysis of Immune-related DEG from Different Comparison Groups

A Venn diagram was created to demonstrate the relationship between the immune-related DEG among different comparison groups (Figure 5C). The region-specific genes accounted for a large proportion of each comparison group. The region-specific DEG are listed in Table 2. The results in overlapping regions showed that the greatest number was observed between the overlap of B and C. There were a few overlapping genes between groups A and B, and some overlapping genes between A and C. Some regions had multiple overlaps that contained certain important genes. The overlapping regional DEGs are listed in Table 3 and details are provided in Table S10.

Immune-related GO Terms and Correlation between Immune-related LncRNAs and Genes

The results of "WGCNA" were selected to determine the biological processes involved in CyHV-3-induced modulation of genes by lncRNAs, from an immune perspective. Among the currently divided 35 gene modules (Figure S4), only 14 modules could be classified according to the immune-related GO terms (Figure S5). Specifically, "double-stranded DNA binding", "receptor-mediated endocytosis", "response to bacterium", "lysosome", "defense response to fungus", "killing of cells of other organism", "oxygen carrier activity", "oxygen binding", "defense response to bacterium", "killing of cells of other organism", "NuA4 histone acetyltransferase complex", "defense response to gram-negative bacterium", "defense response to gram-positive bacterium", "immune response", "regulation of cytokine secretion", "cytolysis", "execution phase of apoptosis", "cellular response to gamma radiation", "nucleic acid metabolic process", "TOR signaling", "DNA replication", "ubiquitin-dependent protein catabolic process", "granulocyte differentiation", and "macrophage differentiation" were revealed after the "WGCNA" analysis. Furthermore, for the differentially expressed lncRNAs and their potential modulated genes in comparison groups (A–C), the immune-related GO terms are also demonstrated in Figure 6. Notably, "receptor-mediated endocytosis" and "lysosome" were found in both groups A and B. In addition, "ubiquitin-dependent protein catabolic process", "DNA replication", and "response to bacterium" were also found in group B. For group C, besides the GO terms found in groups A and B, "killing of cells of other organism", "defense response to fungus", and "double-stranded DNA binding" were also revealed. Specifically, for detailed correlations of lncRNAs and genes in groups A to C (Table S11), T cell leukemia homeobox 3 (TLX3) and LGALS3 were revealed, respectively, in the survivors of the resistant strain.

DISCUSSION

This study demonstrated the immune mechanisms of a CyHV-3-resistant strain of common carp. By comparing the survivors and healthy fish in either resistant or normal strains, we identified key genes and related lncRNAs involved in different immune processes and related downstream pathways. The network of these processes and pathways has been merged to improve clarity. Accordingly, the immunogenetically insensitive or susceptible factors to CyHV-3 infection were determined.

The current results indicated the components involved in the entry of the virus. Firstly, integrin acts as a herpesvirus receptor (23), and the integrin-dependent signalosome in herpesvirus-infected cells mediated or coactivated numerous inflammatory responses and signaling transductions (23). Therefore, the current finding that integrin beta 1 (ITGB1) was significantly downregulated in the resistant

strain compared with the normal strain, even during the steady status, may indicate that ITGB1 facilitates the binding of herpes virus glycoprotein for entry (24). This coincides with the finding that integrin signaling promotes the release of intracellular calcium stores and contributes to viral entry and cell-to-cell spreading via glycoprotein H, during herpes simplex virus infection in humans (25). Recent reports have also demonstrated that some integrins on lymphocytes, such as B cells, could facilitate the mucosa-specific homing (26). Secondly, higher TLR expression in survivors of the normal strain compared with that of the resistant strain also indicated its role in virus entry and downstream proinflammatory signaling. Of note, TLR4 signaling leads to the production of proinflammatory cytokines in human lymphatic endothelial cells (23). In addition, fish-specific and virus-responding TLR18 (27, 28), (the most overlapping gene among groups) was downregulated in survivors of the resistant strain, whereas it was upregulated in those of the normal stain. This suggested its potential ability to facilitate CyHV-3 infection. MAPK signaling, as the downstream process of pattern recognition receptors, could facilitate the tumor necrosis factor-alpha (TNFA)-induced suppressor of cytokine signaling 3 (SOCS3) expression. This can lead to both pro-inflammatory immune response and failure in growth (29), according to the upregulated genes upon infection observed in the normal strain in group B.

Non-specific binding of the virus by lectins played a protective role in preventing virus entry. Both upregulated ladderlectins and higher galectin expression were detected in survivors of resistant strains compared with normal strains. This is suggestive of their blocking ability for viral proteins (30, 31), such as glycoprotein (32). Moreover, as the head kidney is one of the major reticuloendothelial systems in fish (33), mucin was also found to be upregulated as the anti-virus barrier. This was shown in group B as the gel-forming mucin 5B (MUC5B) (34) was upregulated in the survivors of the normal strain. However, higher expression levels of membrane-bound MUC3 (35) were found in survivors of the resistant strain versus the normal strain. These findings suggested that more mucus was secreted, thereby causing tissue swelling upon CyHV-3 infection in the normal strain. For the survivors of the resistant strain, the membrane-bound mucin could effectively bind the virus with no gel.

Lectins are involved in the clearance of apoptotic cells via the complement pathway. Hence, in survivors, the complement components reported in the acute phase in common carp during CyHV-3 infection (36), could facilitate the phagocytic process via binding of MRC (Mannose-Receptor C) in fish (37). In the comparison of the survivors from groups A and B, the observed difference in the phagocytic process suggested the clearance of apoptosis cells, which was indicated by ubiquitin ligase in group A. Meanwhile, IL1, IL6, and IL8 were upregulated in group B. With regard to cytokine signaling, there were less proinflammatory cytokines and immune regulation-related cytokine receptors upregulated in the survivors of the resistant

strain compared with the normal strain. The interleukin 21 receptor (IL21R), which was upregulated in group A and downregulated in group B, indicated the control of inflammation by suppressing STAT3 signaling (38). The upregulated IL23R in group A can promote cytotoxic ability (39), which may immediately kill infected cells. The lack of antigen presentation in group A suggested that the infection was overcome before the amplification of CyHV-3. In contrast, in group B, numerous upregulated genes were involved in antigen presentation. Therefore, the survivors from breeding strains overcame the infection mainly through phagocytosis and cytotoxicity at the cellular level, without activating the typical process of proinflammation as in survivors from the normal strain.

Thus, there are different signatures between the two strains as survival strategies. For the survivors in the resistant strain, self-repairing related autophagy was detected as both the KEGG pathways “regulation of autophagy” and autophagy-related fish antiviral tripartite motif (TRIM) protein (40) were found in group A. These findings were in line with TRIMs, which were upregulated in group A and downregulated in group B. In the survivors from the resistant strain, the higher PI3K also suggests higher autophagy, since the PI3K/AKT/mTOR pathway enhances this process [28]. Fish TRIM may inhibit the activation of IFN and attenuate IFN regulatory factor (IRF) (41, 42). In addition, in carp, nuclear factor, interleukin 3 regulated (NFIL3) stimulated both proinflammatory (e.g., NF-kappa B [NF-κB]) and anti-inflammatory factors (e.g., IL10) (43). This may upregulate NFIL3 in group A, as suggested by the extensive activation of immune cells, enhancing immune regulation. The directed lymphocytes response could be present as there was upregulation of IL23R, which significantly enhances the expression of cytotoxic mediators (39), as well as cathepsin L (a component of lysosomes) (44). This indicated an enhanced activation of the cytotoxic ability of T cells in survivors from the resistant strain. For B cells, the secretion of Ig-related genes (e.g., polymeric immunoglobulin receptor [PIGR], Ig heavy chain and light chain) was also increased in group A. To fight the virus, the survivors from the normal strain developed typical inflammatory cascades, including pro-inflammatory cytokines (e.g., IL1, IL6, and IL8), as well as MAPK signals, which are profoundly involved in cell survival functions during viral infection (45). For the control of inflammation, the survivors from the common strain exhibited suppression of the IFN response, which was also reflected by the upregulated IRF8, the inhibitor of the MYD88-mediated NF-κB signaling pathway (46). Downstream hypoxia was also found, as indicated by the “p53 signaling pathway” and “oxidative phosphorylation”. The hypoxic status could also protect the fish from death, since p53 suppresses cell apoptosis (47), and HIF1A regulates virus-induced glycolytic genes (48).

Furthermore, the advantages of disease-resistant genes were reflected by comparing the survivors from the two strains, which can provide clues for breeding resistance to

CyHV-3. In group C there was more semaphorin, which is related to immunoregulation (49, 50), in survivors from resistant strains than normal strains. This indicated a possible tolerance of viral replication, without negative effects on the proliferation of host cells (51). There was less cyclin-dependent kinase inhibitor 1D (CDKN1D) in survivors from resistant strains compared with normal strains. This indicated that there was an interrupted circadian cell-cycle timing in the survivors from normal strains (52). The higher levels of STAT3 and several integrins in survivors from the normal strain may facilitate the effect of enhanced IL6 signaling. This was upregulated in group B through upregulation of integrins by STAT3 (53). Apart from the typical immune signals in group C, there was relatively higher PI3K activity, which was involved in the KEGG pathway “dopaminergic synapse”. This indicated that dopamine inhibited inflammation (54) in survivors from the resistant strain. This is because PI3K is dependent on the accumulation of DOPA decarboxylase, the enzyme involved in the production of dopamine, which is reduced by a viral infection [27]. Additionally, genes responsive to the secretion of immunoglobulin (e.g., PIGR and Ig light chain), which is important for anti-virus immunity, were common (in group D) between the two strains.

The comparison of two strains with healthy status suggested a basal anti-virus immunity. Among the upregulated genes in group E, the expression of NACHT, LRR, and PYD domains-containing protein 3 (NALP3) indicated a stronger ability to form the inflammasome in the resistant strain. The higher levels of NLR family CARD domain containing 3 (NLRC3), which is a negative regulator of the DNA sensor STING, suggest less sensitivity to the DNA virus in the resistant strain. The higher levels of MHC I indicate greater potential to activate response by natural killer cells upon herpes virus infection (55) in the resistant strain. For the potential T helper cell differentiation-related JAK/STAT pathway, the upregulation of both STAT3 and histone deacetylase (HDAC) indicated an easier differentiation of both T helper 17 and regulatory T directions (56), respectively, in the resistant strain even in the steady state (57, 58). Upregulation of HDAC in group E, that could inhibit the function of macrophages in fish (59), also suggests a more delicate immune regulation during the steady state in the resistant strain. However, in group E, among the downregulated genes, which indicated higher expression in the normal stain at a steady state, TLR18 and ITGB1 suggested susceptibility to virus binding. Moreover, C-C motif chemokine ligand 4 (CCL4), which can protect infected cells with viral burden (60), may be a risk factor for the normal strain.

The revealed regulation of resistance to CyHV-3 by lncRNA involved numerous biological processes. Among the revealed GO terms in group B, “DNA replication” could possibly indicate virus proliferation in fish. In survivors from the normal strain, the lncRNA-regulated genes were mainly involved in innate immune cell function (e.g., macrophage protein and cathepsin, as components of lysosomes) and cell

apoptosis. In survivors from the resistant strain, the only lncRNA-modulated gene, TLX3, has been shown to be strongly methylated. This indicates that TLX3 expression was suppressed during hepatitis B virus-related cancer (61). This suggests that TLX3 in survivors from a resistant strain may play a protective role, and participate in lymphocyte proliferation in the head kidney. Meanwhile, echoing recent findings regarding the participation of zebrafish galectin proteins in immunity against viral infection (32, 62), the expression of LGALS3 (the only lncRNA regulation-related immune gene found in group C), was markedly higher than that recorded in the normal strain upon infection. This suggested an ability of LGALS3 to block viral attachment.

Therefore, based on the present findings, a hypothesis of immune mechanisms involved in both healthy controls and survivors from infection in both strains, has been proposed in Figure 7. In conclusion, the resistant strain of common carp showed a better ability to maintain immune homeostasis in both steady and inflammatory states, and displayed enhanced blockage of CyHV-3 infection compared with the normal strain. Since the modulation by lncRNA currently remains a hypothesis, further molecular evidence is needed to elucidate the mechanism of resistance. In addition, the finding that both the inhibition of inflammation by dopamine in the resistant strain and the disrupted bio-clock in the normal strain upon CyHV-3 infection suggested better growth performance for the resistant strain. Thus, the possible advantage in the resistant strain for growth performance warrants further study.

AUTHOR CONTRIBUTIONS

Lianyu Shi and Zhiying Jia conceived the project and designed the experiments. Nan Wu and Zhying Jia wrote the manuscript. Zhying Jia performed the experiments. Zhying Jia and Jiaxin Sun conducted the CyHV-3 infection experiment and collected samples. Chitao Li, Xuesong Hu and Yanlong Ge performed fish propagation and culture. Xiaona Jiang conducted the RT-PCR experiment. Nan Wu and Xiaoqin Xia coordinated the data analysis tasks. Nan Wu, Heng Li, Mijuan Shi, Xiaona Jiang, Weidong Ye, Ying Tang, and Yingyin Cheng analyzed the data. The manuscript was revised and approved by Xiao-Qin Xia and Lianyu Shi.

ACKNOWLEDGMENTS

This work was funded by grants from the National Key R&D Program of China (2018YFD0900302-6), The Natural Science Foundation of Heilongjiang Province (TD2019C004), China Aquaculture Research System (CARS-45-06), and Central Public-interest Scientific Institutional Basal Research Fund (2020TD31).

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

CONTRIBUTION TO THE FIELD STATEMENT

Cyprinid herpesvirus-3 (CyHV-3) is the major threat to common carp aquaculture. Experimental infections of carp from pure lines or crosses have indicated the existence of genetic background of resistance by divergent survival rates. The immune response of carp to CyHV-3 involves both innate and adaptive aspects. Even non-coding RNAs play an important role in anti-CyHV-3 immunity. However, there is a lack of systemic studies focusing on a detailed network of the immune system for the anti-CyHV-3 immune mechanism in common carp. Benefiting from a strain of common carp which has already shown a higher survival rate in CyHV-3 infection, this study systematically illustrated the immune mechanisms involved in anti-CyHV-3 ability on both mRNA and non-coding RNA levels, demonstrated that the carp may obtain significant resistance to CyHV-3 through a specific innate immune mechanism, and revealed the involved biological processes including autophagy, phagocytosis, cytotoxicity, blocking of the virus by lectins and MUC 3, and the immune suppressive signal transduction. The immune factors involved in resistant or susceptible to the disease shed light on prevention of CyHV-3 infection genetically.

DATA AVAILABILITY STATEMENT

The datasets, containing all clean data of this transcriptomic analysis, can be found in the Genome Sequence Archive (GSA) database (http://gsa.big.ac.cn/index.jsp) with the BioProject identifier <CRA003105>.

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FIGURE LEGENDS

Figure 1 Strategy for the identification of regulated immune genes in fish gut and liver by integrative analysis of both DEG and lncRNA using transcriptomics data. (**A**) Virus challenge was performed by infecting common carp through addition of sick fish tissue lysate into the tank. Subsequently, sampling of immune organs was conducted at day 0 (immediately before challenge) and day 7 post challenge, for transcriptomics analysis. (**B**) Immune-related annotation was carried out via bioinformatics analysis of the transcriptomics results using GO annotation and KEGG pathway enrichment, through filtering using the currently constructed common carp immune gene library. (**C**) Venn diagrams were generated using immune-related DEG data from comparisons of groups A–E. Next, Venn-regional analysis was applied to identify the immune-related anti-CyHV-3 or CyHV-3-resistant protein-coding genes or lncRNAs.
CyHV-3, cyprinid herpesvirus-3; DEG, differentially expressed gene; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long non-coding RNA

Figure 2 Differential appearance of general mortality and pathology between fish from the resistant and normal strains. (**A**) Comparison of daily mortality between the resistant and normal strains. The mortality was monitored within 15 days post challenge. N=300 per group. (**B**) The degree of swelling in the spleen was limited in the survivors from the resistant strain (a) compared with the markedly enlarged spleen observed in fish from the normal strain (b). The arrow indicates the spleen region.

Figure 3 GO terms comparing groups A, B, and C. The immune-related terms are labeled with stars.
GO, Gene Ontology

Figure 4 KEGG pathways comparing groups A, B, C, and D. The immune-related terms are labeled with arrows.
KEGG, Kyoto Encyclopedia of Genes and Genomes

Figure 5 The analysis of immune processes of DEG among different comparison groups.
DEG, differentially expressed gene

Figure 6 The GO terms involved in immune-related modular, which contain the matched lncRNA and genes. The arrows indicate the matched lncRNA and genes containing GO terms in black (**A**), blue (**B**), brown (**C**), grey60 (**D**), and turquoise (**E**). The star, triangle, and arrow represent the revealed GO term for Group A, Group B, and Group C, respectively.
GO, Gene Ontology; lncRNA, long non-coding RNA

Figure 7 Hypothesis of the carp immune mechanism in both breeding and normal strains either at the steady state or upon CyHV-3 infection. Group A: healthy control in the steady state in the breeding strain. Group B: survivors in an inflammatory state in the breeding strain. Group C: healthy control in the steady state in normal strain. Group D: survivors in an inflammatory state in the normal strain. The words in italics, bold, and green represent the biological process, cell type, and lncRNA-modulated target immune genes, respectively. The arrow denotes facilitation or promotion, and ---| denotes inhibition. The dashed line indicates a possible correlation.
CyHV-3, lncRNA, long non-coding RNA

Table 1 The immune processes and immune gene categories involved in differentially expressed mRNA for each comparison group

Compared group	Immune process	Immune gene category
Upregulated in group A (26)	Pattern recognition (6), complement system, inflammatory cytokines and receptors (5), adapters, effectors and signal transducers (4), innate immune cells related (2), T/B cell antigen activation (5), other genes related to immune response (3).	ladderlectin, LRR-containing proteins, MRC, C3, GVIN, IL21R, IL23R, integrin alpha, NFIL3, CSF, perforin, UBE, MMR, NCAM, Ig heavy chain, Ig light chain, PIGR, SEMA, cathepsin, TRIM, ubiquitin ligase.
Downregulated in group A (23)	Acute phase reactions (2), pattern recognition (4) complement system (4), inflammatory cytokines and receptors (5), adapters, effectors and signal transducers (4), T/B cell antigen activation (2), other genes related to immune response.	Fibrinogen, C-type lectin, galectin, TLR18, C3, CXCL, GVIN, IFI, IL1R, integrin alpha, NALP12, NLRC3, Ig light chain, TIP, myeloid cell related.
Upregulated in group B (155)	Pattern recognition (13), antigen processing and regulators (13), complement system (5), inflammatory cytokines and receptors (34), adapters, effectors and signal transducers (23), innate immune cells related (6), T/B cell antigen activation (15), other genes related to immune response (46).	FBXL, fucolectin, MPR, nattectin, NITR, TLR1, TLR18, TLR22, TLR25, TLR5, CDK, CDK related, CDKIs, HA, MHC I, MHC II, TGFB, TNF, TNFAIP3-interacting protein, C3, C3aR, C7, CCL, CCR, CFLAR, CXCL, CXCR, cytokine receptor, IFI, IFIT, IL1, IL1R, IL1R related, IL6, IL6R, IL8, IRF8, MX, NFIL3, MAPK, MAPKKK, NALP12, NLRC3, Programmed cell death protein, SOCS1, SOCS3, SOCS7, TRAF, TTRAP, UBE, MIF, NCF, NCK and related, NRAMP, BCL, BLNK, CD22, FCR, HDAC, HMG, Ig light chain, PBX, TAGAP, TCIRG1, AP-3, CARD, cathepsin, CEBP, hepcidin, HSP, HSP5, HSP70, lymphocyte antigen, mucin, myeloid cell related, PIN1, platelet related, TMED, ubiquitin ligase, ubiquitin related, UBL.
Downregulated in group B (102)	Acute phase reactions, pattern recognition (21), antigen processing and regulators (6), complement system (2), inflammatory cytokines and receptors (21), adapters, effectors and signal transducers (14), innate immune cells related (3), T/B cell antigen activation (14), other genes related to immune response (20).	Plasminogen, collectin, C-type lectin, galectin, Hepatic lectin, LRR-containing proteins, RBL, SITR, TLR13, CDK, VEGF, VEGFR, C3aR, CD59, ACRs, CCL, CCR, EGFR, FGFR, GVIN, IL17R, IL21R, IL2R, IL6R, IL8, integrin alpha, integrin beta, IRF related, TITIN, granzyme, MAPKKK, NALP12, NALP3, perforin, UBE, TRIM.
Upregulated in group C (357)	Acute phase reactions (3), pattern recognition (57), antigen processing and regulators (11), complement system (4), inflammatory cytokines and receptors (91), adapters, effectors	Fibrinogen, MBL, collectin, C-type lectin, FBXL, fucolectin, galectin, Hepatic lectin, intelectin, ladderlectin, LRR-containing proteins, MRC, NITR, RBL, scavenger receptor, SIGLEC, TLR8, TLR13, TLR3, TLR9, CDK, CDKIs, CIITA, LRMP, MHC I, MHC II, TGBR, VEGF, C3, C3aR, C5, C8, ACRs, CCL, CCR, CXCR, EGF, FGFR, GVIN, IFI, IFIT, IL10R, IL13R, IL17R, IL1R

	and signal transducers (50), innate immune cells related (6), T/B cell antigen activation (64), other genes related to immune response (70).	related, IL21R, IL23R, IL2R, IL6R, IL8, integrin alpha, integrin beta, IRF4, IRF5, IRF7, MX, NFIL3, TITIN, XCR, CBX4, CSF, CSFR, granzyme, MAPK, MAPKKK, NALP12, NALP3, NDRG1, NLRC3, perforin, Programmed cell death protein, TRAF, TRAIL, UBE, MCP, MMR, NCAM, B-cell CLL/lymphoma, CD2, CD22, CD276, CD4, CD6, FCR, GATA and related, HDAC, Ig heavy chain, Ig light chain, immunoglobulin superfamily, JAG, LAG, MAL, NFAT, PIGR, PKC, SEMA, T-bet, VSIG, VTCN, ZAP, ACTININ, cathepsin, CD302, HSPb1, lymphocyte antigen, lymphocyte related, mucin, myeloid cell related, nectin, palladin, PI3K, rootletin, RTK, TIAM, ubiquitin ligase, ubiquitin related, TRIM, UBL.
Downregulated in group C (303)	Acute phase reactions (2), pattern recognition (27), antigen processing and regulators (25), complement system (11), inflammatory cytokines and receptors (55), adapters, effectors and signal transducers (58), innate immune cells related (7), T/B cell antigen activation (37), other genes related to immune response (81).	Macroglobulin, CD209, c-type lectin, FBXL, galectin, intelectin, LPS-anchor protein, LRR-containing proteins, MPR, TLR1, TLR18, TLR22, TLR25, TLR4, TLR5, CDK, CDK related, CDKIs, HA, MHC II, TGFB, TNF, TNFAIP3-interacting protein, C3, C3aR, C4, C7, CD59, ACRs, CCL, CCR, CD11, CFLAR, CXCL, CXCR, cytokine receptor, FAM, FGF, GVIN, HB-EGF, IL1, IL12, IL12R,IL1R, IL1R related, IL2R, IL6, IL6R, IL8, integrin alpha, integrin beta, IRF1, IRF8, NFIL3, CSF, CSFR, JAK1, MAPK, MAPKKK, NALP12, NALP3, NLRC3, Programmed cell death protein, SOCS1, SOCS3, SOCS7, STAT3, UBE, MIF, NCF, NCK and related, NRAMP, BAFF, B-cell CLL/lymphoma, BCL, BLNK, CD22, CD276, FCR, HDAC, HMG, Ig light chain, PBX, PIGR, SEMA, TAGAP, TAL, TCIRG1, TCIRG2, TCIRG3, VSIG, WWP, ACTININ, AP-3, CARD, caspase, cathepsin, CEBP, hepcidin, HSP, HSP4, HSP5, HSP70, HSP90, Kruppel-like factor, lymphocyte antigen, MALT1, mucin, myeloid cell related, PAXILLIN, PIN1, platelet related, TMED, ubiquitin ligase, ubiquitin related, TRIM, UBL.
All in group D (48)	Pattern recognition (4), antigen processing and regulators (7), inflammatory cytokines and receptors (7), adapters, effectors and signal transducers (4) T/B cell antigen activation (14), other genes related to immune response (12).	LRR-containing proteins, VEGFR, VEGF, CDKIs, CDK, NFIL3, LIFR, integrin beta, FAM, CCR, CD166, MAPK, CD97, SEMA, PIGR, NFAT, Ig light chain, HMG, HDAC, BTG, BCL, ubiquitin ligase, platelet related, HIF1a, CEBP.
Upregulated in group E (12)	Pattern recognition, antigen processing and regulators, inflammatory cytokines and receptors (3), adapters, effectors and signal transducers (4), T/B cell antigen activation, other genes related to immune response (2).	MRC, MHC I, GVIN, IL21R, integrin alpha, NALP3, NLRC3, perforin, STAT3, HDAC, platelet related, ubiquitin related.
Downregulated in group E (7)	Pattern recognition, complement system, inflammatory cytokines and receptors (3), adapters, effectors and	TLR18, C3, CCL, IFI, integrin beta, perforin, myeloid cell related.

	signal transducers, other genes related to immune response.

Notes: the number (>1) of DEG is shown in the brackets. The full names of all gene abbreviations could be found in the Tables S5–S9.

911 **TABLE 2** Comparison of group-specific DEG in the Venn regional analysis

Comparison group (DEG No.)	Immune process (DEG NO.)	Immune gene category
Up-regulated in group A specific (7)	inflammatory cytokines and receptors，innate immune cells related，pattern recognition，T/B cell antigen activation（3），other genes related to immune response	NFIL3，MMR，cathepsin，MRC，Ig heavy chain，SEMA
Downregulated in group A specific (10)	acute phase reactions （2），inflammatory cytokines and receptors （2），adapters, effectors and signal transducers （3），T/B cell antigen activation （2）, other genes related to immune response	Fibrinogen，NLRC3，GVIN，integrin alpha，myeloid cell related，Ig light chain，TIP
Upregulated in group B specific (29)	pattern recognition (5), antigen processing and regulators, complement system, inflammatory cytokines and receptors (5), adapters, effectors and signal transducers (8), T/B cell antigen activation (2), other genes related to immune response (7)	MAPK, NLRC3, programmed cell death protein, SOCS3, TRAF, TTRAP, MHC I, C3, IFI, IFIT, MX, NFIL3, CEBP, mucin, myeloid cell related, ubiquitin ligase, TRIM, ubiquitin related, Fucolectin, nattectin, NITR, TLR1, BCL, HMG
Downregulated in group B specific (36)	acute phase reactions，pattern recognition (9), antigen processing and regulators, complement system, inflammatory cytokines and receptors (7), adapters, effectors and signal transducers (3), innate immune cells related, T/B cell antigen activation (3), other genes related to immune response (10)	Plasminogen, NALP12, perforin, UBE, VEGFR, CD59, CCL, EGFR, integrin alpha, integrin beta, IRF related, NCAM, ACTININ, CD33, HSP90, Kruppel-like factor, MALT1, ubiquitin ligase, UBL, C-type lectin, galectin, Hepatic lectin, LRR-containing proteins, RBL, SITR, B-cell CLL/lymphoma, Ig heavy chain, TCR
Upregulated in group C specific (278)	acute phase reactions (3), pattern recognition (41), antigen processing and regulators (9), complement system (2) inflammatory cytokines and receptors (74), adapters, effectors and signal transducers (35), innate immune cells related (3), T/B cell antigen activation (51), other genes related to immune response (60)	Fibrinogen, MBL, CBX4, CSFR, granzyme, MAPK, MAPKKK, NALP12, NALP3, NDRG1, NLRC3, perforin, Programmed cell death protein, TRAF, TRAIL, UBE, CDK, CDKIs, CIITA, LRMP, MHC I, MHC II, TGBR, C5, C8, CCL, CCR, CXCR, EGF, FGFR, GVIN, IFI, IFIT, IL10R, IL13R, IL17R, IL1R related, IL21R, IL23R, IL2R, IL6R, IL8, integrin alpha, integrin beta, IRF4, IRF5, IRF7, MX, NFIL3, XCR, MCP, MMR, NCAM, cathepsin, CD302, HSPb1, lymphocyte antigen, lymphocyte related, mucin, myeloid cell related, nectin, paladin, PI3K, rootletin, TIAM, TRIM, ubiquitin ligase, ubiquitin related, collectin, C-type lectin, FBXL, Fucolectin, galectin, Hepatic lectin, intelectin, LRR-containing proteins, MRC, NITR, scavenger receptor, SIGLEC, TLR 8, TLR3, TLR9, B-cell CLL/lymphoma, CD2, CD22, CD276, CD4, CD6, FCR, GATA and related, HDAC, Ig heavy chain, immunoglobulin superfamily, JAG, LAG, MAL,

		NFAT, PIGR, PKC, SEMA, T-bet, VSIG, VTCN, ZAP
Downregulated in group C specific (172)	acute phase reactions (2), pattern recognition (16), antigen processing and regulators (13), complement system (5), inflammatory cytokines and receptors (26), adapters, effectors and signal transducers (42), innate immune cells related, T/B cell antigen activation (24), other genes related to immune response (43)	Macroglobulin, CSF, CSFR, JAK1, MAPK, MAPKKK, NALP3, NLRC3, Programmed cell death protein, SOCS1, SOCS3, STAT3, UBE, CDK, CDK related, CDKIs, MHC II, TNF, TNFAIP3-interacting protein, C3aR, C4, C7, CD59, ACRs, CD11, CXCL, FAM, FGF, GVIN, HB-EGF, IL1, IL12, IL12R, IL1R related, IL2R, IL6, IL6R, integrin alpha, integrin beta, IRF1, NFIL3, NCK and related, ACTININ, AP-3, CARD, caspase, cathepsin, CEBP, HSP4, HSP70, HSP90, Kruppel-like factor, MALT1, mucin, myeloid cell related, PAXILLIN, platelet related, TMED, ubiquitin ligase, ubiquitin related, TRIM, UBL, CD209, C-type lectin, FBXL, intelectin, LPS-anchor protein, LRR-containing proteins, MPR, TLR4, BAFF, B-cell CLL/lymphoma, BCL, BLNK, CD22, CD276, FCR, HDAC, Ig light chain, PBX, PIGR, SEMA, TAL, TCIRG2, TCIRG3, VSIG, WWP
All in group D specific (44)	pattern recognition (4), antigen processing and regulators (4), inflammatory cytokines and receptors (7), adapters, effectors and signal transducers (4), T/B cell antigen activation (14), other genes related to immune response (11)	CD166, CD97, MAPK, CDK, CDKIs, VEGF, VEGFR, CCR, FAM, integrin beta, LIFR, NFIL3, CEBP, HIF1a, platelet related, ubiquitin ligase, LRR-containing proteins, BCL, BTG, HDAC, HMG, Ig light chain, NFAT, PIGR, SEMA
Upregulated in group E specific (9)	pattern recognition, antigen processing and regulators, inflammatory cytokines and receptors, adapters, effectors and signal transducers (3), T/B cell antigen activation, other genes related to immune response (2),	NLRC3, platelet related, GVIN, HDAC, MHC I, MRC, ubiquitin related, NALP3, STAT3
Downregulated in group E specific (2)	inflammatory cytokines and receptors, other genes related to immune response	integrin beta, myeloid cell related

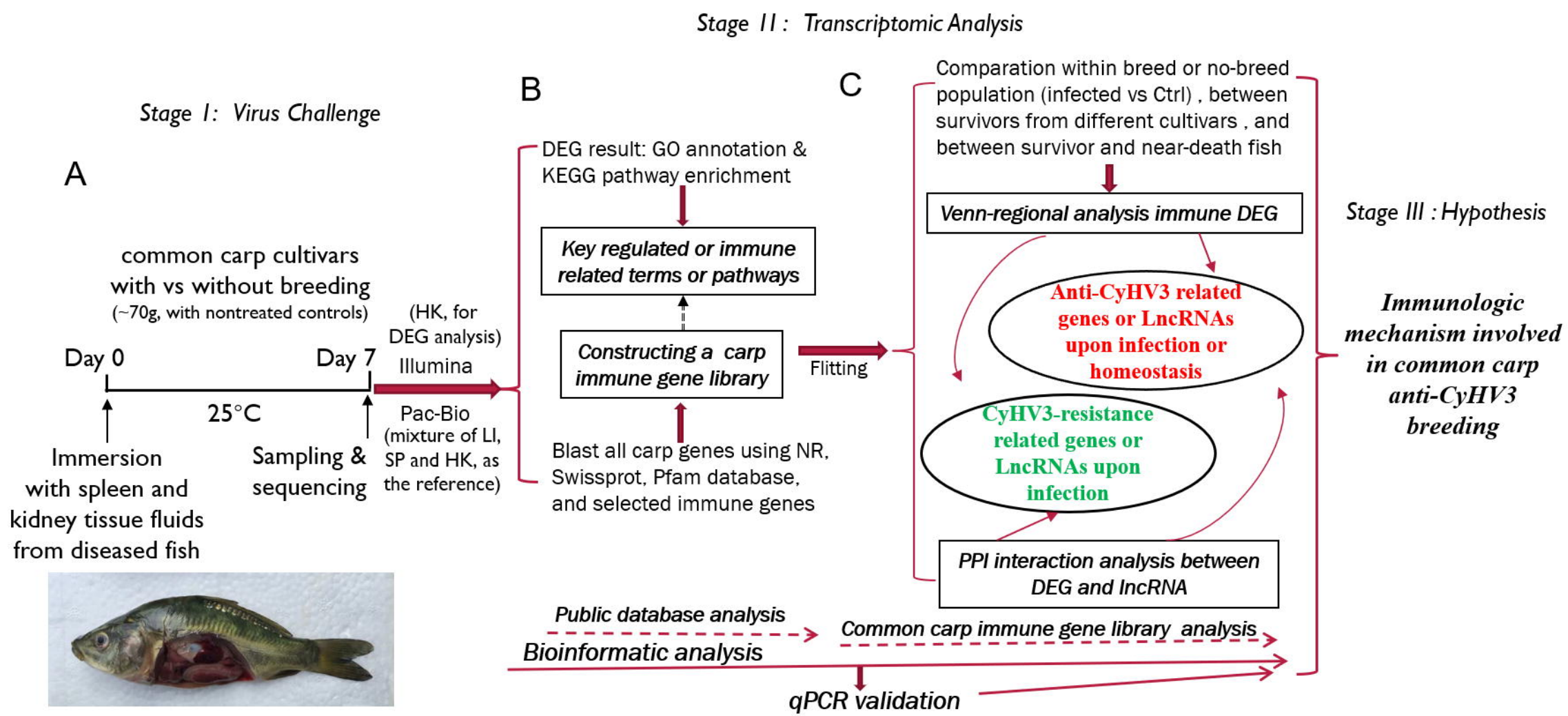
Notes: the number (>1) of DEG is shown in the brackets. The full names of all gene abbreviations could be found in the Tables S10.

915 **TABLE 3** Overlapping genes in the Venn regional analysis

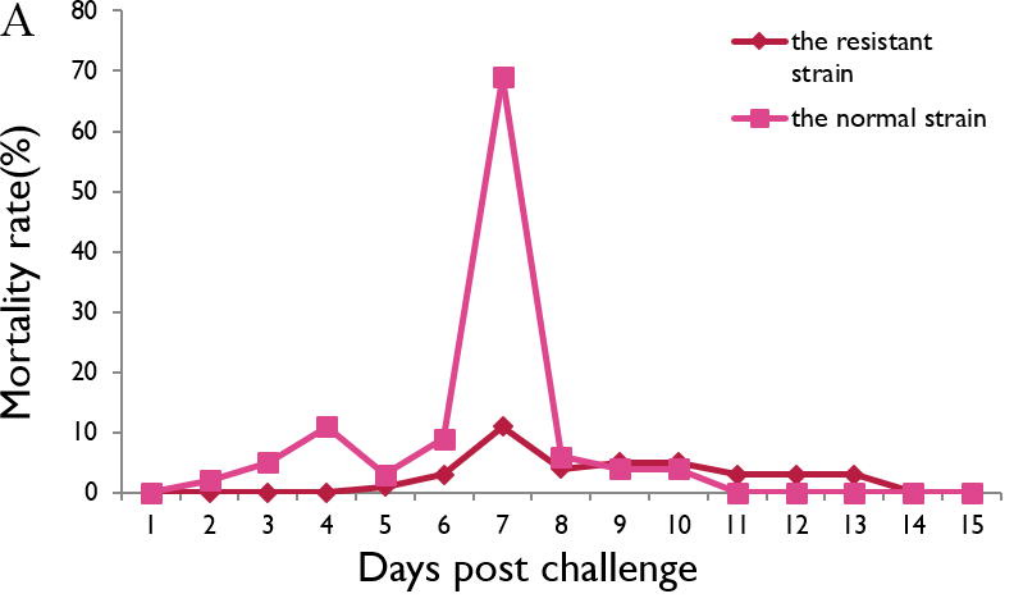
Overlapping regions (DEG NO.)	Immune process (DEG NO.)	Immune gene category
Group A & C specific (23)	pattern recognition (7), complement system (4), inflammatory cytokines and receptors (3), adapters, effectors and signal transducers (4), innate immune cells related, T/B cell antigen activation (2), other genes related to immune response (2)	CSF, NALP12, UBE, C3, GVIN, IL23R, integrin alpha, NCAM, ubiquitin ligase, C-type lectin, galectin, ladderlectin, LRR-containing proteins, MRC, PIGR
Group A & E specific (1)	inflammatory cytokines and receptors	IFI
Group B & C specific (176)	pattern recognition (18), antigen processing and regulators (14), complement system (4), inflammatory cytokines and receptors (38), adapters, effectors and signal transducers (23), innate immune cells related (8), T/B cell antigen activation (24), other genes related to immune response (47)	Granzyme, MAPK, MAPKKK, NALP12, NALP3, NLRC3, perforin, Programmed cell death protein, SOCS1, SOCS3, SOCS7, UBE, CDK, CDK related, CDKIs, HA, MHC II, TGFB, TNF, TNFAIP3-interacting protein, VEGF, C3aR, C7, ACRs, CCL, CCR, CFLAR, CXCL, CXCR, cytokine receptor, FGFR, GVIN, IL1, IL17R, IL1R related, IL2R, IL6, IL6R, IL8, integrin alpha, IRF8, NFIL3, TITIN, MIF, NCAM, NCF, NCK and related, NRAMP, ACTININ, AP-3, CARD, cathepsin, CEBP, hepcidin, HSP, HSP5, HSP70,lymphocyte antigen, paladin, PIN1, platelet related, RTK, TMED, TRIM, ubiquitin ligase, ubiquitin related, UBL, collectin, C-type lectin, FBXL, galectin, LRR-containing proteins, MPR, RBL, TLR1, TLR13, TLR22, TLR25, TLR5, BCL, BLNK, CD22, FCR, HDAC, HMG, Ig heavy chain, Ig light chain, JAG, NFAT, PBX, SEMA, TAGAP, TCIRG1
Group B & D (4)	antigen processing and regulators (3), other genes related to immune response	CEBP, VEGFR
Group B & E specific (1)	inflammatory cytokines and receptors	integrin alpha
Group C & E specific (1)	inflammatory cytokines and receptors	CCL
Group A & B & C specific (5)	pattern recognition, inflammatory cytokines and receptors (3), adapters, effectors and signal transducers	LRR-containing proteins, CXCL, IL1R, perforin
Group A & B & E (1)	complement system	C3
Group B & C & E (2)	adapters, effectors and signal transducers (2)	perforin
Group A & B & C	pattern recognition, inflammatory cytokines and	TLR18, IL21R

& E (2)	receptors,

Notes: the number (>1) of lncRNAs or mRNAs is shown in the brackets. The full names of all gene abbreviations could be found in the Tables S10.


Stage II : Transcriptomic Analysis
Stage I: Virus Challenge
A
common carp cultivars
with vs without breeding
(~70g, with nontreated controls)
Day 0
Day 7
25°C
Immersion
with spleen and
kidney tissue fluids
from diseased fish
Sampling &
sequencing
(HK, for
DEG analysis)
Illumina
Pac-Bio
(mixture of LI,
SP and HK, as
the reference)
B
DEG result: GO annotation &
KEGG pathway enrichment
Key regulated or immune
related terms or pathways
Constructing a carp
immune gene library
Blast all carp genes using NR,
Swissprot, Pfam database,
and selected immune genes
Flitting
C
Comparation within breed or no-breed
population (infected vs Ctrl) , between
survivors from different cultivars , and
between survivor and near-death fish
Venn-regional analysis immune DEG
Anti-CyHV3 related
genes or LncRNAs
upon infection or
homeostasis
CyHV3-resistance
related genes or
LncRNAs upon
infection
PPI interaction analysis between
DEG and lncRNA
Public database analysis
Common carp immune gene library analysis
Bioinformatic analysis
qPCR validation
Stage III : Hypothesis
Immunologic
mechanism involved
in common carp
anti-CyHV3
breeding

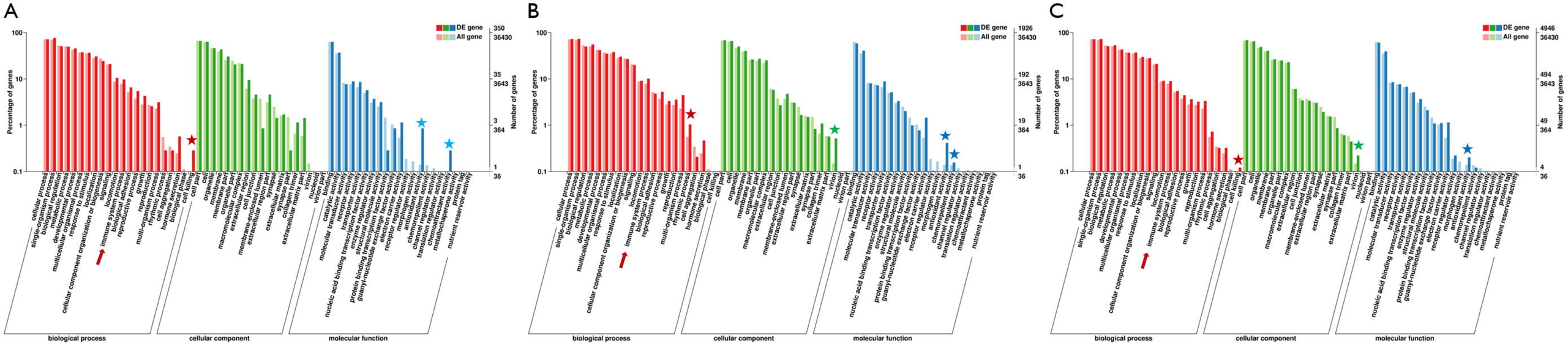
A

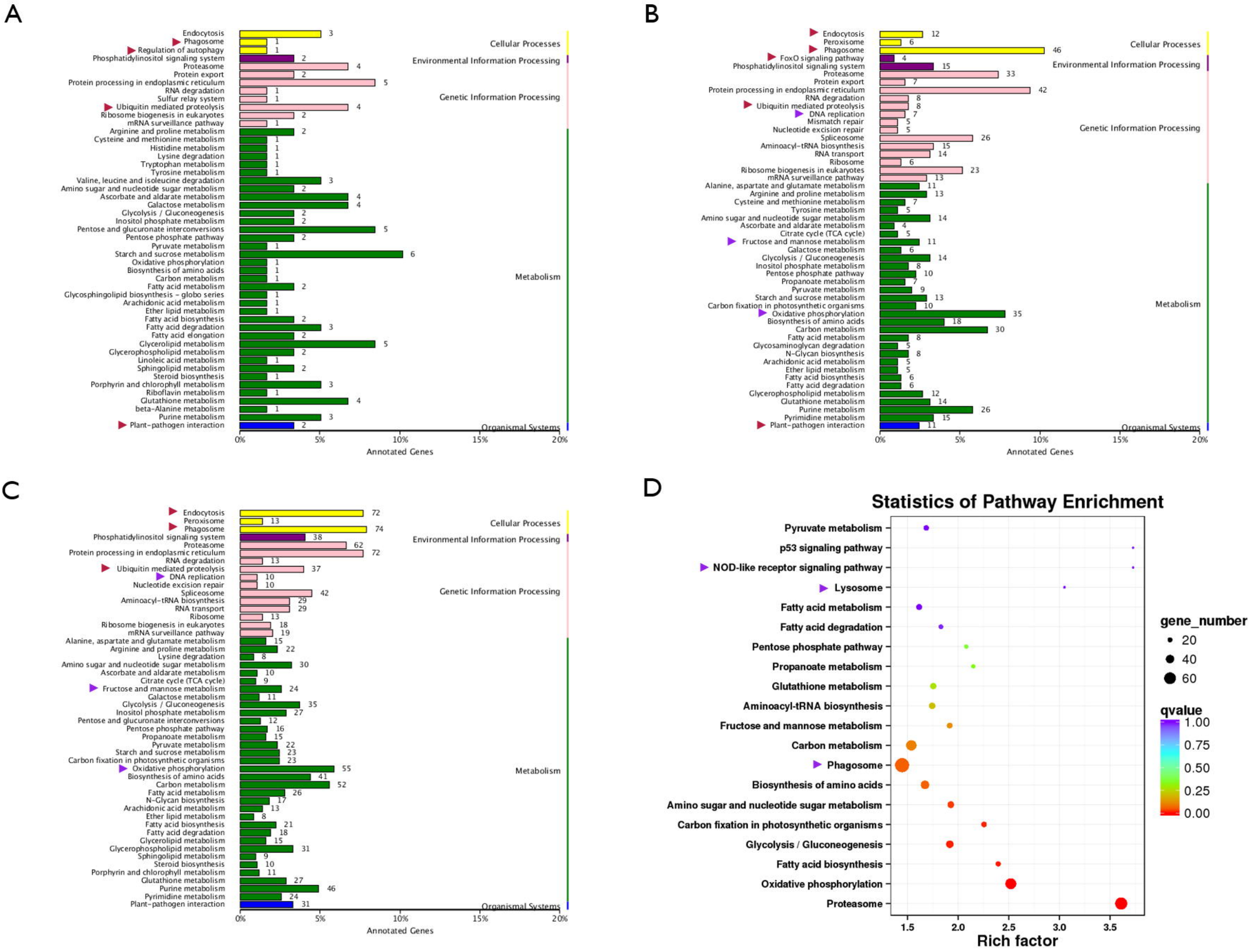

Mortality rate(%)
80
70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
Days post challenge
the resistant strain
the normal strain

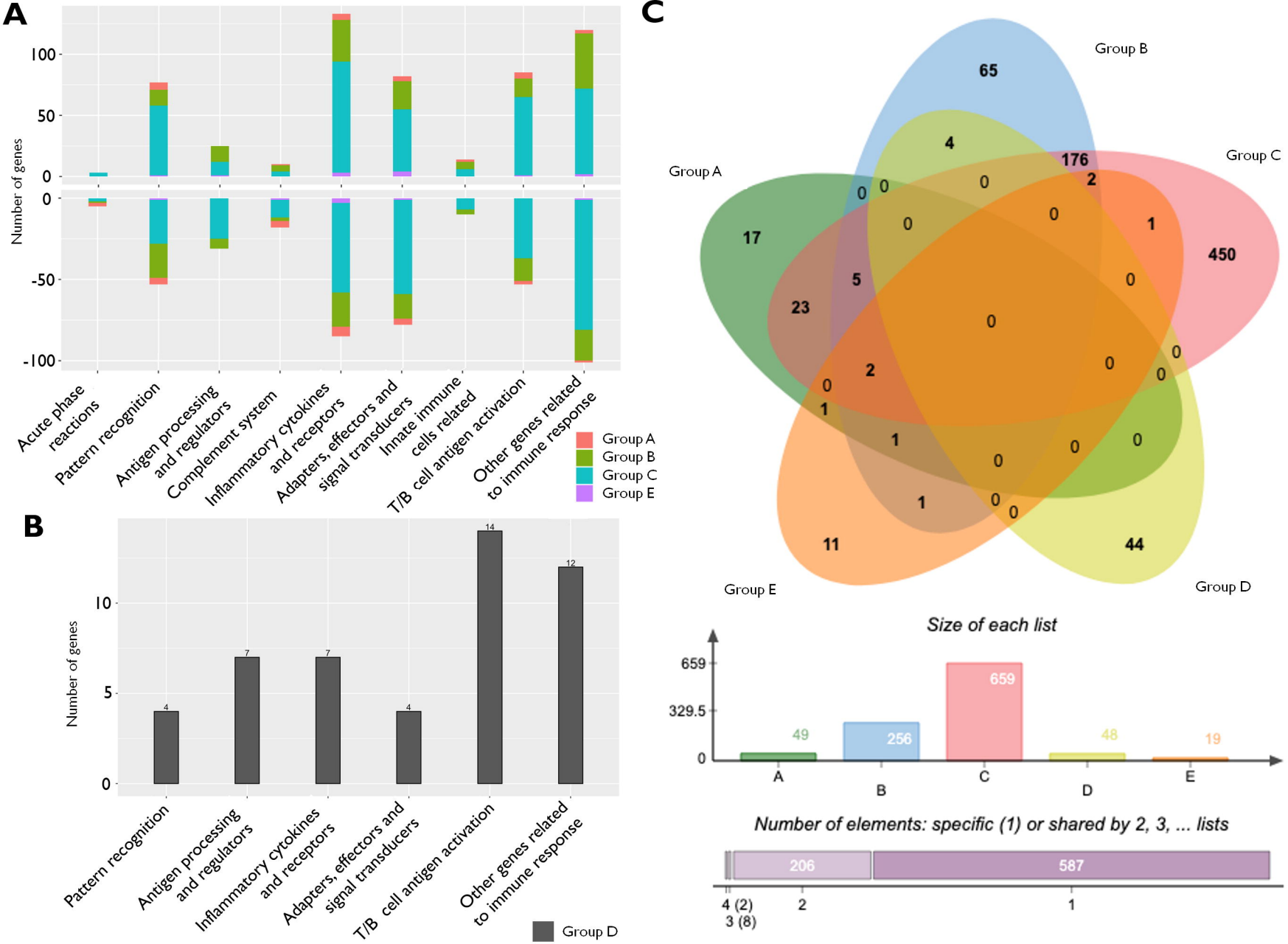
B

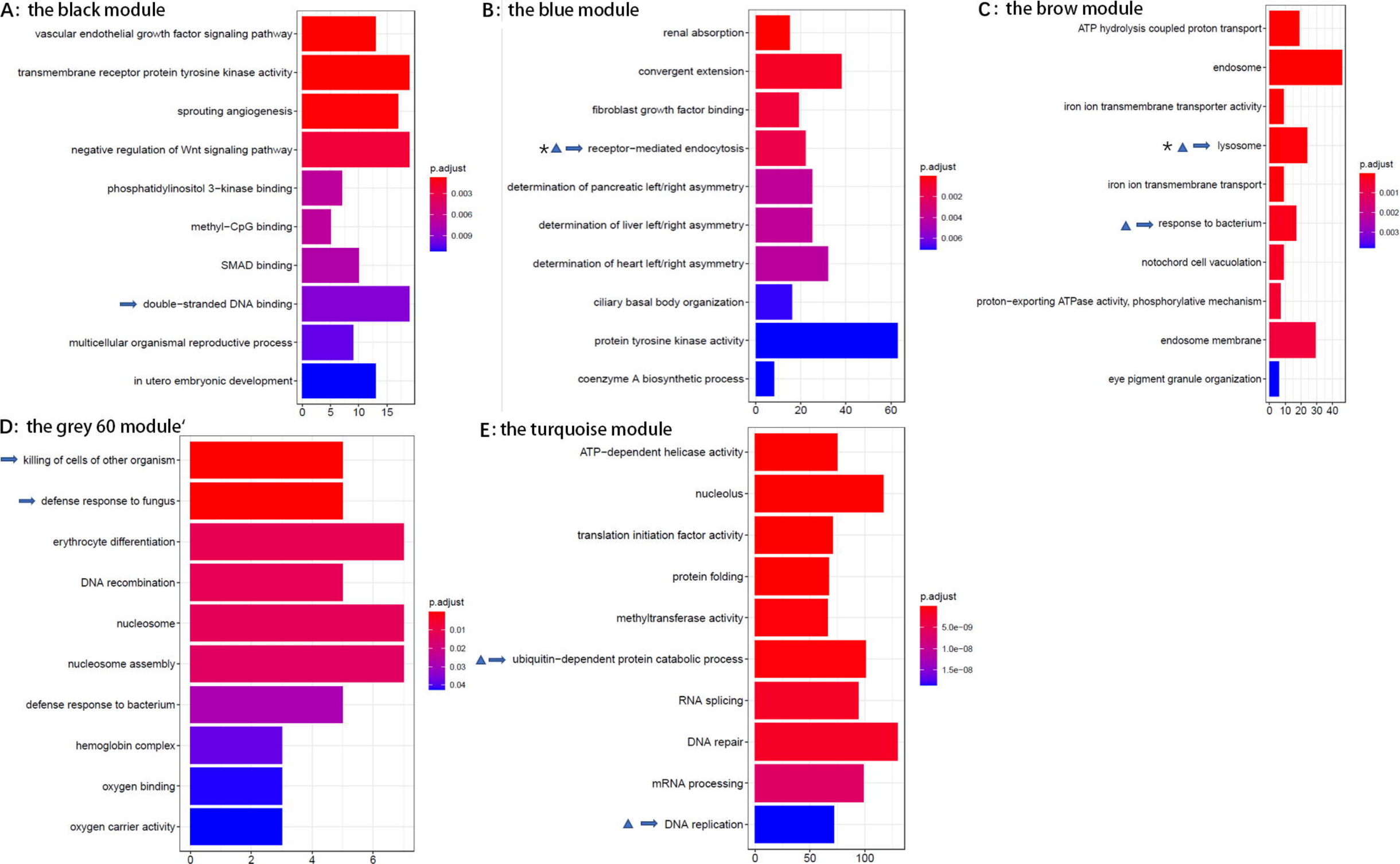
a

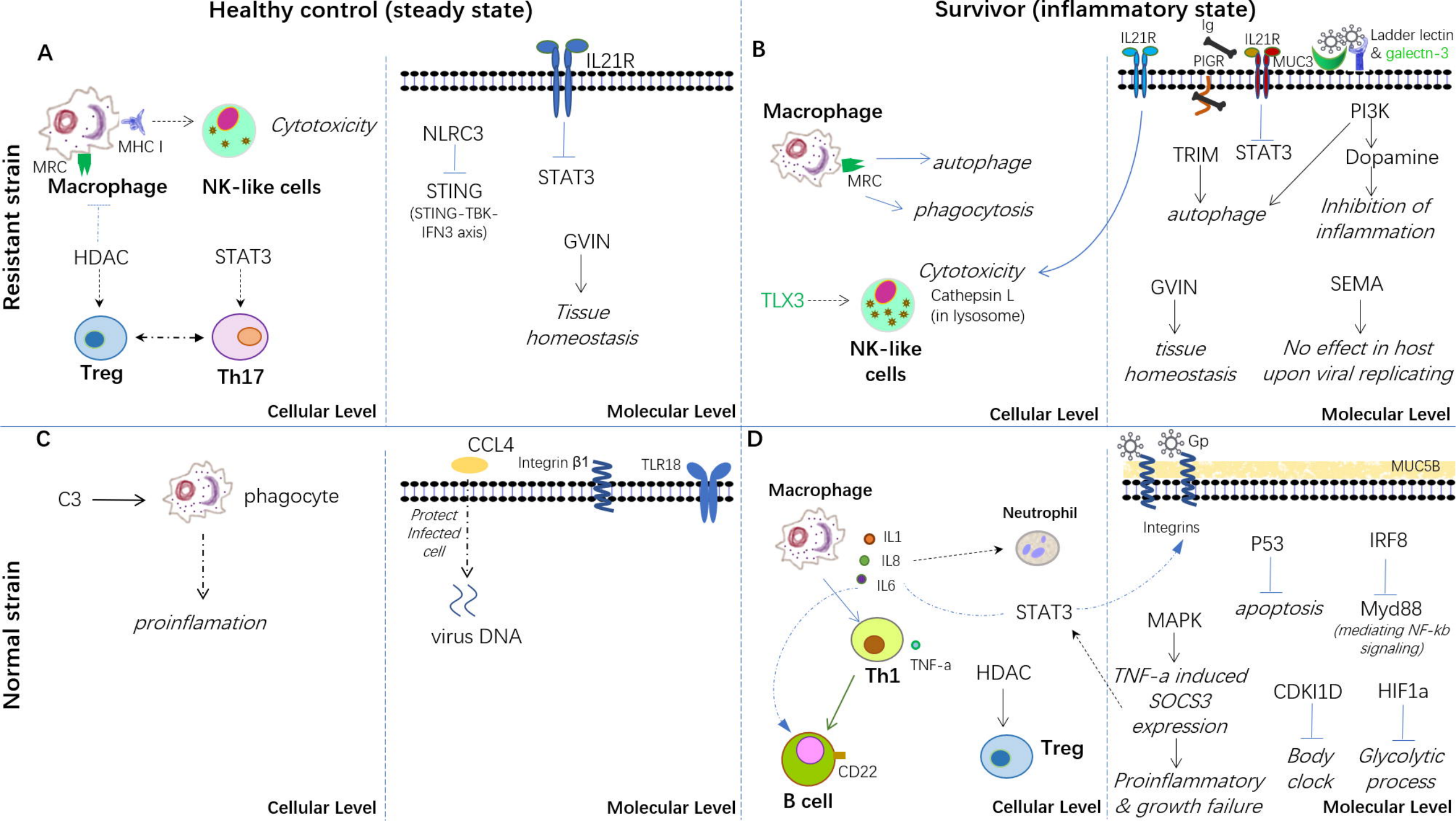
b


A
B
C
DE gene
All gene
Percentage of genes
Number of genes
biological process
cellular component
molecular function


A
Endocytosis 3
Phagosome 1
Regulation of autophagy 1
Phosphatidylinositol signaling system 2
Proteasome 4
Protein export 2
Protein processing in endoplasmic reticulum 5
RNA degradation 1
Sulfur relay system 1
Ubiquitin mediated proteolysis 4
Ribosome biogenesis in eukaryotes 2
mRNA surveillance pathway 1
Arginine and proline metabolism 2
Cysteine and methionine metabolism 1
Histidine metabolism 1
Lysine degradation 1
Tryptophan metabolism 1
Tyrosine metabolism 1
Valine, leucine and isoleucine degradation 3
Amino sugar and nucleotide sugar metabolism 2
Ascorbate and aldarate metabolism 4
Galactose metabolism 4
Glycolysis / Gluconeogenesis 2
Inositol phosphate metabolism 2
Pentose and glucuronate interconversions 5
Pentose phosphate pathway 2
Pyruvate metabolism 1
Starch and sucrose metabolism 6
Oxidative phosphorylation 1
Biosynthesis of amino acids 1
Carbon metabolism 1
Fatty acid metabolism 2
Glycosphingolipid biosynthesis - globo series 1
Arachidonic acid metabolism 1
Ether lipid metabolism 1
Fatty acid biosynthesis 2
Fatty acid degradation 3
Fatty acid elongation 2
Glycerolipid metabolism 5
Glycerophospholipid metabolism 2
Linoleic acid metabolism 1
Sphingolipid metabolism 2
Steroid biosynthesis 1
Porphyrin and chlorophyll metabolism 3
Riboflavin metabolism 1
Glutathione metabolism 4
beta-Alanine metabolism 1
Purine metabolism 3
Plant-pathogen interaction 2
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Metabolism
Organismal Systems
0% 5% 10% 15% 20%
Annotated Genes
B
Endocytosis 12
Peroxisome 6
Phagosome 46
FoxO signaling pathway 4
Phosphatidylinositol signaling system 15
Proteasome 33
Protein export 7
Protein processing in endoplasmic reticulum 42
RNA degradation 8
Ubiquitin mediated proteolysis 8
DNA replication 7
Mismatch repair 5
Nucleotide excision repair 5
Spliceosome 26
Aminoacyl-tRNA biosynthesis 15
RNA transport 14
Ribosome 6
Ribosome biogenesis in eukaryotes 23
mRNA surveillance pathway 13
Alanine, aspartate and glutamate metabolism 11
Arginine and proline metabolism 13
Cysteine and methionine metabolism 7
Tyrosine metabolism 5
Amino sugar and nucleotide sugar metabolism 14
Ascorbate and aldarate metabolism 4
Citrate cycle (TCA cycle) 5
Fructose and mannose metabolism 11
Galactose metabolism 6
Glycolysis / Gluconeogenesis 14
Inositol phosphate metabolism 8
Pentose phosphate pathway 10
Propanoate metabolism 7
Pyruvate metabolism 9
Starch and sucrose metabolism 13
Carbon fixation in photosynthetic organisms 10
Oxidative phosphorylation 35
Biosynthesis of amino acids 18
Carbon metabolism 30
Fatty acid metabolism 8
Glycosaminoglycan degradation 5
N-Glycan biosynthesis 8
Arachidonic acid metabolism 5
Ether lipid metabolism 5
Fatty acid biosynthesis 6
Fatty acid degradation 6
Glycerophospholipid metabolism 12
Glutathione metabolism 14
Purine metabolism 26
Pyrimidine metabolism 15
Plant-pathogen interaction 11
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Metabolism
Organismal Systems
0% 5% 10% 15% 20%
Annotated Genes
C
Endocytosis 72
Peroxisome 13
Phagosome 74
Phosphatidylinositol signaling system 38
Proteasome 62
Protein processing in endoplasmic reticulum 72
RNA degradation 13
Ubiquitin mediated proteolysis 37
DNA replication 10
Nucleotide excision repair 10
Spliceosome 42
Aminoacyl-tRNA biosynthesis 29
RNA transport 29
Ribosome 13
Ribosome biogenesis in eukaryotes 18
mRNA surveillance pathway 19
Alanine, aspartate and glutamate metabolism 15
Arginine and proline metabolism 22
Lysine degradation 8
Amino sugar and nucleotide sugar metabolism 30
Ascorbate and aldarate metabolism 10
Citrate cycle (TCA cycle) 9
Fructose and mannose metabolism 24
Galactose metabolism 11
Glycolysis / Gluconeogenesis 35
Inositol phosphate metabolism 27
Pentose and glucuronate interconversions 12
Pentose phosphate pathway 16
Propanoate metabolism 15
Pyruvate metabolism 22
Starch and sucrose metabolism 23
Carbon fixation in photosynthetic organisms 23
Oxidative phosphorylation 55
Biosynthesis of amino acids 41
Carbon metabolism 52
Fatty acid metabolism 26
N-Glycan biosynthesis 17
Arachidonic acid metabolism 13
Ether lipid metabolism 8
Fatty acid biosynthesis 21
Fatty acid degradation 18
Glycerolipid metabolism 15
Glycerophospholipid metabolism 31
Sphingolipid metabolism 9
Steroid biosynthesis 10
Porphyrin and chlorophyll metabolism 11
Glutathione metabolism 27
Purine metabolism 46
Pyrimidine metabolism 24
Plant-pathogen interaction 31
Cellular Processes
Environmental Information Processing
Genetic Information Processing
Metabolism
Organismal Systems
0% 5% 10% 15% 20%
Annotated Genes
D
Statistics of Pathway Enrichment
Pyruvate metabolism
p53 signaling pathway
NOD-like receptor signaling pathway
Lysosome
Fatty acid metabolism
Fatty acid degradation
Pentose phosphate pathway
Propanoate metabolism
Glutathione metabolism
Aminoacyl-tRNA biosynthesis
Fructose and mannose metabolism
Carbon metabolism
Phagosome
Biosynthesis of amino acids
Amino sugar and nucleotide sugar metabolism
Carbon fixation in photosynthetic organisms
Glycolysis / Gluconeogenesis
Fatty acid biosynthesis
Oxidative phosphorylation
Proteasome
1.5 2.0 2.5 3.0 3.5
Rich factor
gene_number
20
40
60
qvalue
1.00
0.75
0.50
0.25
0.00


A
Number of genes
100
50
0
0
-50
-100
Acute phase reactions
Pattern recognition
Antigen processing and regulators
Complement system
Inflammatory cytokines and receptors
Adapters, effectors and signal transducers
Innate immune cells related
T/B cell antigen activation
Other genes related to immune response
Group A
Group B
Group C
Group E
B
Number of genes
10
5
0
4
7
7
4
14
12
Pattern recognition
Antigen processing and regulators
Inflammatory cytokines and receptors
Adapters, effectors and signal transducers
T/B cell antigen activation
Other genes related to immune response
Group D
C
Group A
Group B
Group C
Group D
Group E
65
4
176
2
17
450
5
23
2
11
44
Size of each list
659
329.5
0
49
256
659
48
19
A
B
C
D
E
Number of elements: specific (1) or shared by 2, 3, ... lists
206
587
4 (2)
3 (8)
2
1


A: the black module
vascular endothelial growth factor signaling pathway
transmembrane receptor protein tyrosine kinase activity
sprouting angiogenesis
negative regulation of Wnt signaling pathway
phosphatidylinositol 3-kinase binding
methyl-CpG binding
SMAD binding
double-stranded DNA binding
multicellular organismal reproductive process
in utero embryonic development
0 5 10 15
p.adjust
0.003
0.006
0.009
B: the blue module
renal absorption
convergent extension
fibroblast growth factor binding
receptor-mediated endocytosis
determination of pancreatic left/right asymmetry
determination of liver left/right asymmetry
determination of heart left/right asymmetry
ciliary basal body organization
protein tyrosine kinase activity
coenzyme A biosynthetic process
0 20 40 60
p.adjust
0.002
0.004
0.006
C: the brow module
ATP hydrolysis coupled proton transport
endosome
iron ion transmembrane transporter activity
lysosome
iron ion transmembrane transport
response to bacterium
notochord cell vacuolation
proton-exporting ATPase activity, phosphorylative mechanism
endosome membrane
eye pigment granule organization
0 10 20 30 40
p.adjust
0.001
0.002
0.003
D: the grey 60 module
killing of cells of other organism
defense response to fungus
erythrocyte differentiation
DNA recombination
nucleosome
nucleosome assembly
defense response to bacterium
hemoglobin complex
oxygen binding
oxygen carrier activity
0 2 4 6
p.adjust
0.01
0.02
0.03
0.04
E: the turquoise module
ATP-dependent helicase activity
nucleolus
translation initiation factor activity
protein folding
methyltransferase activity
ubiquitin-dependent protein catabolic process
RNA splicing
DNA repair
mRNA processing
DNA replication
0 50 100
p.adjust
5.0e-09
1.0e-08
1.5e-08


Healthy control (steady state)
Survivor (inflammatory state)
Resistant strain
Normal strain
A
MRC
MHC I
Macrophage
Cytotoxicity
NK-like cells
HDAC
STAT3
Treg
Th17
Cellular Level
IL21R
NLRC3
STING
(STING-TBK-IFN3 axis)
STAT3
GVIN
Tissue homeostasis
Molecular Level
B
Macrophage
MRC
autophage
phagocytosis
Cytotoxicity
Cathepsin L
(in lysosome)
TLX3
NK-like cells
Cellular Level
IL21R
Ig
PIGR
IL21R
MUC3
Ladder lectin
& galectn-3
PI3K
TRIM
STAT3
Dopamine
autophage
Inhibition of inflammation
GVIN
tissue homeostasis
SEMA
No effect in host upon viral replicating
Molecular Level
C
C3
phagocyte
proinflamation
Cellular Level
CCL4
Integrin β1
TLR18
Protect Infected cell
virus DNA
Molecular Level
D
Macrophage
IL1
IL8
IL6
Neutrophil
STAT3
Th1
TNF-a
HDAC
Treg
CD22
B cell
Cellular Level
Gp
MUC5B
Integrins
P53
apoptosis
IRF8
Myd88
(mediating NF-kb signaling)
MAPK
TNF-a induced SOCS3 expression
Proinflammatory & growth failure
CDKI1D
Body clock
HIF1a
Glycolytic process
Molecular Level