The Low Expression of DUSP1 and HSPA5 Inhibits Chicken Immune Response and Causes Decreased Immunity under Heat Stress

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Abstract

Background As a major stressor, high temperatures negatively affect the poultry industry, through impairments to chicken immunity and production performance. The purpose of this study is to clarify how chicken immune systems responded to heat stress with and without immunization. In the present study, spleen and bursa of Fabricius of experimental chickens were subjected to RNA-seq. Key genes influencing immune response in heat-stressed chickens were identified and their functions validated. Results Immunized and heat-stressed chickens experienced a significant reduction in immune function. The expression of immune-related genes and heat stress-related genes in the spleen increased after immunization and decreased after heat stress, but in the bursa of Fabricius, few of these genes were differentially expressed after immunization and heat stress, indicating insensitivity to high temperature and the lack of vaccine processing. In the non-heat-stressed groups, spleen expression of DUSP1 and HSPA5 decreased significantly, suggesting their relationship to immunity. Upon DUSP1 or HSPA5 overexpression, the mRNA expression of MHC-I, MHC-II, CD80, CD86, CD1C, IL1B, IL6, and TLR4 was earlier than that under LPS stimulation only, indicating that DUSP1 or HSPA5 overexpression enhances HD11 recognition LPS. Inhibiting DUSP1 or HSPA5 expression, the mRNA expression levels of MHC-I, MHC-II, CD80, CD86, IL6 and TLR4 did not change significantly from LPS-stimulation-only levels but CD1C significantly decreased, suggesting that HD11 recognition of LPS is affected by DUSP1 or HSPA5 expression levels. Conclusions The inhibition of immune response due to lowly expressed DUSP1 and HSPA5 may be the cause of decreased immunity in chickens.

Background

Under high temperatures in summer, poultry would experience a host of health problems, such as reduced immunity and disease resistance, lower food intake, slow growth and development, abnormal endocrine functions, and increased mortality [1–3]. These issues seriously hamper intensive poultry production, leading to economic losses. High temperature usually causes heat stress that could influence chicken productive performances through reduced immunity. As a highly conserved protein family, the heat stress proteins (HSPs) are expressed under high temperatures or other environmental stresses, e.g., hypoxia, ultraviolet radiation, cold, viral infection, and starvation, and bind to non-protein molecules with exposed hydrophobic residues. What about HSP that affects immunity?

Mammalian HSP60, HSP70, HSP90, and gp96 can form bonds with lipopolysaccharides (LPS) [4]. In mammals, other functions of HSPs include inducing antigen-presenting cells (APCs) to secrete cytokines (e.g., IL–1 and IL–6), promoting dendritic cell maturation, and increasing MHC-I and MHC-II expression [5]. Extracellular HSPs can also promote cross-presentation of HSP-bound peptide antigens to MHC-I molecules in dendritic cells, activating antigen-specific cytotoxic T lymphocytes [6]. There are differences in chickens and mammals on immune genes. Transparent cells can recognize antigens but cannot present antigens in chickens, which are considered to be monocyte/macrophage systems, and that the function is not yet fully understood in the immune response [7]. The immune response of the HD11 cell line to LPS in the heat stress environment (up-regulation of CCL4, CCL5, IL1B, IL8 and iNOS) was higher

than in thermoneutral conditions [8]. Expression for the immune-related genes CCL4, CCL5, CD40, GM-CSF, IFN-g, IL−10, IL−12b, IL−1b, IL−6, IL−8, and iNOS was highly induced in response to LPS in the chicken bone marrow derived dendritic cells [9]. Microarray analysis on chicken DNA has revealed the transcriptome of *E. coli*-infected chicken HD11 (a macrophage cell line), and researchers speculate that chicken HSP70 is involved in immune-related pathways [10]. The participation of avian HSPs in immune response was further implied through the finding inhibition of intracellular HSP90 expression significantly decreased IFN-γ expression after CpG oligodeoxynucleotides (CpG-OND) immunostimulation of chicken HD11 cells, whereas HSP90-bound CpG-OND increased IFN-γ, IL −6, and MIP−3α expression, as well as nitrogen oxide (NO) levels [11]. Although HSPs can affect immune response, there is no report on how HSPs affect immunity in heat stress.

Therefore, in this study, specific pathogen-free (SPF) chickens were used as experimental materials, and their spleen and bursa of fabricius were collected for RNA-sequencing (RNA-seq) analysis. Through RNA-seq, we find the key genes that influence the immunity in chickens under heat stress and their effects were verified at the cellular level.

Methods

Experimental animal management

Six 28-d-old SPF chickens were inoculated with Newcastle disease vaccine (NDV, only as antigen) and served as experimental group, and three 28-d-old SPF chickens were not inoculated with Newcastle disease vaccine and served as control group. Antibody levels were detected after 7 d of rearing. The experimental chickens were divided into three groups: control group (24 ± 1℃ for 3 h), vaccination group (24 ± 1℃ for 3 h) and heat treatment vaccination group (36 ± 1℃ for 3 h), three 38-d-old SPF chickens in each group. Then, spleen and Bursa of Fabricius tissue of these chicks were harvested for RNA-seq. Blood samples were collected at −20 ℃ for preservation. Tissue samples were stored in liquid nitrogen.

Bloodletting method

The chicken was stunned with a stun gun and promptly exsanguinated through the jugular vein.

Antibody level determination and measurements of heat-resistance traits

Hemagglutination inhibition (HI) antibody levels were measured according to previously reported [12]. The corticosterone was detected using an enzyme-linked immunosorbent assay (ELISA) kit (Guangzhou Darui Antibody CO., LTD, Guangzhou, China) as per the manufacturer's instruction. The H/L value was counted by light microscope, and the expression of CD3+, CD4+, and CD8+ T cells was analysed using flow cytometry.

RNA-seq of spleen and bursa of Fabricius tissues

We performed RNA-seq (Illumina HiSeq 2500, SE50, 5×) on spleen and bursa of Fabricius samples from heat-stressed and immunized chickens (along with non-heat-stressed and non-immunized counterparts). Each treatment had three biological replicates, totalling 18 samples. The base mass fractions of sequencing reads for all samples were above Q30 (99.9% recognition rate of bases) (Supplemental Fig. 1A, B). In the base composition of each sequencing sample, C and G curves mostly overlapped, as did T and A curves. The sequencing process was stable and could be visualized as a horizontal line, indicating balanced base composition in each sequencing sample (Supplemental Fig. 1C, D). Sequenced reads were trimmed for adaptor sequence, and masked for low-complexity or low-quality sequence, then mapped to Gallus gallus (galgal4) whole genome using bowtie 2. RefSeq identifiers of candidate genes were matched to corresponding Ensembl identifiers (Ensembl release 75, www.ensembl.org). Differential expression analysis of two conditions/groups was performed using the DEseq. Genes with an adjusted P-value < 0.05 found by DEseq were assigned as differentially expressed. Gene Ontology (GO) enrichment analysis of the differentially expressed genes (DEGs) was implemented by the GOseq R packages based Wallenius non-central hyper-geometric distribution [13], which can adjust for gene length bias in DEGs. We used KOBAS software to test the statistical enrichment of differential expression genes in KEGG pathways [14].

Cell culture

The chicken HD11 macrophage cell line (gifted from Prof. Susan Lamont, Department of Animal Science, Iowa State University, Ames, IA, USA) was cultured at 37°C and 5% CO_2 in Roswell Park Memorial Institute 1640 medium (Gibco Life Technologies, Carlsbad, CA, USA) supplemented with 10% heat-inactivated fetal calf serum (PAA, Pashing, Austria), 10 mm HEPES, 0.1 mm non-essential amino acids, 2 mm glutamine, 1 mm sodium pyruvate, 100 U/mL penicillin, 100 µg/mL streptomycin, and 5×10^{-5} M 2-mercaptoethanol (pH 7.3).

Synthesis of siRNA and LPS stimulation

A set of four siRNA interfere fragments was synthesized by GenePharma (GenePharma, Suzhou, China) that targeted distinct sites within *HSPA5* and *DUSP1,* along with a positive control targeting glyceraldehyde–3-phosphate dehydrogenase (*GAPDH)* (sequences are shown in Supplemental Data 2).

Cells ($1 - 2 \times 10^5$) seeded in Costar 12-well plates (Corning, Palo Alto, CA, USA) were stimulated with 1 µg/mL LPS (*E. coli* endotoxin 0111:B4; Sigma, St. Louis, MO, USA). Cells were collected for RNA extraction 0, 2, 4, and 6 h post stimulation. To verify the role of *HSPA5* and *DUSP1* in LPS-induced activation, HD11 cells were transfected with an *HSPA5* and *DUSP1* overexpression plasmid or treated with

the siRNA interfere fragments targeting *HSPA5* and *DUSP1* for 48 h, then stimulated with LPS 1 μg/mL, and collected 0, 2, 4, and 6 h post stimulation. Triplicate samples were used in each group.

Overexpression plasmid construction and transfection

Chicken cDNA was synthesized from RNA using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). The chicken *DUSP1* open reading frame (the *Hind III* digestion site was added to the 5' end, and the *Xba I* restriction site was added to the 3' end) was synthesized by the company (Gene Create, Wuhan, China). The product was cloned into the pcDNA3.1 vector between the *Hind III* and *Xba I* restriction sites; The primer pair 5'- gggg*GGTCTC*tagtgGTTCGTGTGTGACGAGCGG –3' (forward) and 5'- ccg*GGTCTC*gtgggACACAACATTCAGAGATGCCAGT –3' (reverse) was used to amplify the chicken *HSPA5* open reading frame (uppercase in the primer sequences, annealing temperature is 56°C). The PCR product was cloned into the pSDS vector follow the Ruyilian™ instructions (Innovative Cellular Therapeutics, Shanghai, China); 0.8 μg plasmid and 3 μL Attractene (Qiagen, Düsseldorf, Germany) were mixed with 60 μL Opti-MEM (Gibco Life Technologies) and incubated for 12 min at room temperature, then transfected into cells.

Reverse Transcription-qPCR (RT-qPCR) analysis

Total RNA was extracted from cells using TRIzol reagent (Takara Bio Inc., Dalian, China), and first-strand cDNA was synthesized using iScript cDNA Synthesis kit (Bio-Rad). The cDNA samples were quantified by RT-qPCR using the CFX96 system with SsoFast EvaGreen Supermix (Bio-Rad), and reaction conditions were set according to the manufacturer's recommendations with an annealing temperature of 58–65°C(40 cycles). The primer sequences are shown in S Table 1.

Western blot analysis

Whole cell protein extract was prepared using cell lysis solution (DBI, Shanghai, China), and lysates were centrifuged at 12,000 × *g* for 10 min at 4°C. The supernatant was collected, and protein levels were measured using the bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Proteins were fractionated by sodium dodecyl sulphate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes, which were blocked with 5% non-fat dried milk (Amresco, Solon, OH, USA) in Tris-buffered saline with Triton X–100 (TBST) for 1 h at room temperature and incubated with primary antibodies against HSPA5 (ABIN363671), DUSP1 (ABIN2704732) and GAPDH (ABIN1573886, Antibodies-online, Aachen, Germany). After being washed in TBST buffer, membranes were incubated in horseradish peroxidase-conjugated secondary antibody (EarthOx, San Francisco, CA, USA), visualized by enhanced chemiluminescence, and scanned using FluorChem M (Proteinsimple, Santa Clara, CA, USA).

Statistical analysis

The RT-qPCR data were analysed by the 2-ΔΔCt method from three samples in each of three replicates[15]. Amplification efficiencies of the target (*HSPA5* and *DUSP1)* and reference (β-actin) mRNA sequences were approximately equal. *HSPA5* and *DUSP1* mRNA levels in all samples were normalized using the following formula: relative quantity of *HSPA5* or *DUSP1* mRNA = 2-ΔΔCt, where ΔΔCt corresponding to the difference between the ΔCt was measured for the mRNA level of each tissue. Here, ΔCt = CtHSPA5 or DUSP1 - Ctβ-actin. Western blotting results were analysed with the Gel-Pro Analyzer (Media Cybernetics, Rockville, MD, USA). Statistical significance was assessed using SAS v.8.2 (Cary, NC, USA) with least square method and $P < 0.05$ was considered as significant.

Results

Antibody changes pre- and post-immunization changes to antibodies, along with pre- and post-heat-stress changes to physiological indices

After the injection of Newcastle disease virus, antibody levels in heat-stressed SPF chicken rose at 7 d and 10 d post-immunization, antibody levels were significantly higher than pre-immunization levels, indicating immune response was ongoing. Moreover, acute heat stress did not appear to cause antibody changes immediately (Fig. 1A). Heat-stressed (36 ± 1ºC) chickens exhibited shortness of breath as a physical sign of experiencing high temperatures (Fig. 1B, C). They also had significantly higher concentration of H/L ($P < 0.001$) and corticosterone ($P < 0.001$) (Fig. 1D, E) and significantly lower concentration of CD4+/CD8+ ($P < 0.001$) than non-heat-stressed chickens (Fig. 1F), indicating a physiological stress response.

Gene expression differences in spleen and bursa of Fabricius tissues from different treatments

Across the spleen comparison groups (A and B), respectively, 22 (20 up-regulated, 2 down-regulated) and 35 (7 up-regulated, 28 down-regulated) differentially expressed genes were identified ($q < 0.05$). Of the 22 genes in A, 11 were functionally annotated (e.g., *FGB, IGSF11,* and *ALDOB),*, but the others were not. Expression differences were likely related to immune function in chickens. Of the 35 genes in B, 26 were functionally annotated (e.g., *GZMA, STAR,* and *DUSP1)* and 9 were not (Fig. 2A, B). Nonetheless, differential gene expression in Group B were likely associated with the immunoreaction in heat-stressed chickens.

In the bursa of Fabricius groups (C and D), 43 (12 up-regulated, 31 down-regulated) and 13 (5 up-regulated, eight down-regulated) genes, respectively, were differentially expressed ($q < 0.05$). Of the 43

genes in C, 16 were functionally annotated (e.g., *MAP2, CD69,* and *MYL4)* and 27 were not. And their differential expression may also be related to chicken immunity. Of the 13 genes in D, 9 were functionally annotated (e.g., *ABCB1LB, COL21A1,* and *CD69)* and 4 were not (Figure 2C, D).

GO enrichment analysis of differentially expressed genes in the spleen and bursa of Fabricius

Differentially expressed genes in spleen samples of Group A were enriched in the following biological processes: cellular protein complex assembly (4 genes, $q < 0.05$), platelet activation (3 genes, $q < 0.05$), and assembly of cellular macromolecule complexes (4 genes, $q < 0.05$) (Fig. 3A). In Group B, enriched biological processes included the assembly of cellular protein complexes (4 genes, $q < 0.05$), platelet activation (3 genes, $q < 0.05$), and protein aggregation (3 genes, $q < 0.05$) (Fig. 3B).

Differentially expressed genes in the bursa of Fabricius samples of Group C were enriched in the following biological processes: cellular processes (2 genes, $q < 0.05$), multicellular processes (1 genes, $q < 0.05$), and responses to stimuli (2 genes, $q < 0.05$) (Fig. 3C). In Group D, enrichment occurred in biological adhesion (1 genes, $q < 0.05$) and cellular processes (1 genes, $q < 0.05$) (Fig. 3D).

Differentially expressed genes associated with immune responses in the spleen and bursa of Fabricius

Six differentially expressed genes (including *IGSF11, ALDOB,* and *APOB)* were associated with spleen immune response after immunization. Their upregulation is associated with enhanced immunity, suggesting that immune raises disease resistance in chickens. Additionally, 15 differentially expressed genes (e.g., *GZMA, DUSP1,* and *HSPA5)* associated with immune response and heat stress were identified after 3 h of heat stimulation (Table 1). Their down-regulation is associated with reduced immunity, indicating the negative effects of heat stress on disease resistance in chickens.

In the bursa of Fabricius, *CD69* was the only immune-response-related gene identified after immunization. After 3 h of heat stimulation, 3 more differentially expressed genes (*COL21A1, CD69,* and *HSP90AB1)* associated with immune response were detected (Table 1). Contrasting with results from spleen samples, these data indicated that the bursa of Fabricius is insensitive to heat stress and the vaccine used.

KEGG pathway analysis of differentially expressed genes in the spleen and bursa of Fabricius

The results of KEGG pathway analysis showed that the 22 differentially expressed genes in Group A were significantly enriched in 8 pathways ($P < 0.05$), including complement and coagulation cascades, pentose phosphate pathway, and fat digestion and absorption ($P < 0.05$). In Group B, 35 genes were enriched in 7

pathways, such as glutathione metabolism, complement and coagulation cascades, and chemokine signalling pathways (Table 2). We noted that the pathway of complement and coagulation cascades was found in both groups. These pathways involve proteins mediating immune and inflammatory responses. Thus, KEGG results suggested that the genes in Groups A and B are associated with immune responses, supporting known spleen function.

The 43 differentially expressed genes in Group C were not enriched in any pathway, whereas the 13 differentially expressed genes in Group D were significantly enriched in the following 2 pathways, synaptic vesicle cycle and protein digestion and absorption (Table 2). These results further emphasize that the bursa of Fabricius does not respond to heat stress or the selected vaccine.

Ingenuity Pathway Analysis (IPA) network formed by differentially expressed genes in the spleen and bursa of Fabricius

The results of an IPA network analysis on Group A's 22 differentially expressed genes showed that they formed a significantly interactive gene network (Table 3), associated with cellular functions and maintenance, hematopoietic system development and function, as well as cell development. The 35 differentially expressed genes in Group B formed 2 significantly interactive gene networks (Table 3). The first was associated with neurological system development and function, cancer, as well as cardiovascular system development and function. The second was associated with cell motility, along with multiple immunity-related genes, including hematopoietic system development and functions, along with immune cell trafficking (Fig. 4). These networks in the spleen have strong association with immune response.

Group C's 43 differentially expressed genes also formed two significantly interactive gene networks (Table 3). The first was associated with cell migration, along with cellular functions, maintenance, and behaviour, whereas the second was associated with cell-to-cell signal transduction and interactions, digestive system development and function, as well as liver development and function. The 13 differentially expressed genes of Group D formed a single significantly interactive gene network (Table 3), associated with cell-to-cell signal transduction and interactions, as well as cell growth, proliferation, and migration. In line with data from our other analyses, these networks in the bursa of Fabricius have no strong association with immune response.

RT-qPCR analysis of the differentially expressed genes

To verify sequence accuracy, 20 differentially expressed genes were randomly selected from Groups A–D for RT-qPCR verification. The results indicated different fold changes (FC) from the sequencing data, but

consistent trends in differential expression (Table 4). Thus, the sequencing results appeared to be reliable.

The sequencing and RT-qPCR results all indicated that heat stress significantly decreased *DUSP1* (sequencing: FC = 0.29, P < 0.01; RT-qPCR: FC = 0.45, P < 0.01) and *HSPA5* (sequencing: FC = 0.52, P < 0.01; RT-qPCR: FC = 0.42, P < 0.01) mRNA expression from levels in the non-stressed group. Both KEGG and IPA analyses indicated that *DUSP1* is involved in immune-related pathways, and many reports have shown that *HSPA5* is associated with immune response. Thus, our subsequent experiments focused on verifying that *DUSP1* and *HSPA5* differential expression was associated with post-heat-stress immunity.

LPS stimulates immune response in chicken HD11 cells

Compared with the control group, mRNA levels of *MHC-I* (P < 0.01) and *CD80* (P < 0.05) were very significantly or significantly increased at 4 h post-LPS stimulation. At 6 h post-stimulation, *MHC-II* (P < 0.01), *IL1B* (P < 0.05), *IL6* (P < 0.05), *TLR4* (P < 0.01), *CD1C* (P < 0.05), *CD80* (P < 0.05), and *CD86* (P < 0.01) mRNA levels were very significantly or significantly increased (P < 0.01) (Fig. 5A-H). LPS can induce immune response in chicken HD11 cells.

The effect of *DUSP1* on LPS recognition in HD11

The full-length coding sequence (CDS) of *DUSP1* is 1110 bp, and the pcDNA3.1 (+)-DUSP1 overexpression vector was confirmed via restriction enzyme digestion (Supplemental Fig. 2A). A 35-fold increase in mRNA (Fig. 6A) and an increased protein expression area (Fig. 6B) both clearly indicated *DUSP1* overexpression and confirmed that the overexpression vector can express genes in HD11. Next, RT-qPCR results showed that the mRNA expression of *MHC-I, MHC-II, CD80,* and *CD86* did not significantly differ before and after *DUSP1* overexpression (Fig. 6C). Thus, intracellular *DUSP1* overexpression does not influence macrophage activation without actual LPS addition.

Although *DUSP1* overexpression did not affect macrophage activation, it could affect HD11 recognition of LPS. Upon *DUSP1* overexpression, *TLR4* (P < 0.01) expression was significantly up-regulated at 2 h after LPS stimulation (Fig. 6E). Moreover, a two-fold decrease in duration before *TLR4* up-regulation occurred compared with LPS stimulation alone, indicating that HD11 recognition of LPS was enhanced with *DUSP1* assistance. We also found that *MHC-II* (P < 0.01) mRNA levels increased significantly at 4 h after LPS stimulation (Fig. 6D), while mRNA levels of *IL1B* (P < 0.05)Fig. 6F), *CD1C* (P < 0.01) (glycolipid antigen presenting molecule) (Fig. 6G), *CD80* (P < 0.05) (Fig. 6H), and *CD86* (P < 0.05) (Fig. 6I) increased significantly at 2 h after LPS stimulation. This timing was earlier than under LPS stimulation only. Together, these data indicated that *DUSP1* overexpression accelerates HD11 recognition of LPS and thus allows earlier LPS recognition, as compared with LPS stimulation alone.

The *DUSP1* interfering fragment 724 (DUSP1–724) was able to inhibit *DUSP1* expression at both mRNA and protein levels (Fig. 7A, B). After treatment with DUSP1–724, HD11 cells were stimulated with LPS, and the results within 6 h showed that *MHC-I* (Fig. 7C) and *MHC-II* (Fig. 7D) mRNA levels did not change significantly from LPS-stimulation-only levels. Thus, interfering with *DUSP1* can inhibit *MHC-I* and *MHC-II* expression in LPS-stimulated HD11 cells. We also found that *CD80 (*$P < 0.05$) (Fig. 7E) and *CD86* (Fig. 7F) mRNA expression was inhibited within 6 h post-LPS stimulation, followed by a significant decrease (also observed in and the mRNA expression of *CD1C* ($P < 0.01$) mRNA expression). In addition, we found that *IL6* expression (Fig. 7H) failed to change significantly within 6 h post-LPS stimulation, suggesting a decreased ability of HD11 cells to secrete cytokines upon interference of *DUSP1* expression. In summary, our data suggested that HD11 recognition of LPS is affected by *DUSP1* expression levels. Thus, interfering with *DUSP1* inhibits the ability of HD11 recognition LPS, causing downstream effects in the expression of other immune-related genes.

Effect of *HSPA5* on the ability of HD11 to recognize LPS

The full CDS length of chicken *HSPA5* is 1959 bp, and the pSDS-HSPA5 overexpression plasmid vector was confirmed with restriction enzyme digestion. We confirmed the successful construction of pSDS-HSPA5 through verifying a significant increase in *HSPA5* mRNA and protein levels (Fig. 8A, B). We tested whether changes to intracellular *HSPA5* expression can alter macrophage activation through fluorescent quantitative PCR. The mRNA expression level of intracellular *MHC-I, MHC-II, CD80,* and *CD86* did not change significantly before and after *HSPA5* overexpression (Fig. 8C), indicating that without LPS addition, *HSPA5* overexpression does not affect macrophage activation.

However, *HSPA5* overexpression affected LPS recognition by HD11. The mRNA levels (FC > 2) of *MHC-I (*$P < 0.01$), *MHC*-IIP < 0.01), *CD80 (*$P < 0.05$), *CD86 (*$P < 0.01$), *CD1C (*$P < 0.05$), *IL1B (*$P < 0.05$), *IL6 (*$P < 0.01$), and *TLR4 (*$P < 0.01$) (Fig. 8D - K) increased significantly at 2 h after LPS stimulation when *HSPA5* was overexpressed, compared with LPS stimulation alone. This increase occurred far earlier than the timing of MHC-II expression after LPS stimulation alone. Together, these data combine to demonstrate clearly that *HSPA5* can facilitate and accelerate HD11's LPS recognition.

After using *HSPA5* interfering fragment 183 (HSPA5–183) to interfere with *HSPA5* expression (Fig. 9A, B), HD11 cells were LPS-stimulated. The results within 6 h showed that *MHC-I (*Fig. 9C) and *MHC-II* (Fig. 9D) mRNA levels did not change significantly, compared with LPS stimulation alone. Moreover, *CD80* mRNA levels (Fig. 9G) were also inhibited within 6 h after LPS stimulation, whereas *CD1C (*$P < 0.05$) mRNA levels (Fig. 9E) decreased significantly at 2 h after LPS stimulation. We then found that *IL6* mRNA (Fig. 9F) did not change significantly within 6 h post-LPS stimulation, nor did *TLR4* mRNA (Fig. 9 H). In summary, these data allow us to propose that HD11 recognizes the rate of LPS is affected by *HSPA5* expression levels, and interference with *HSPA5* inhibits LPS recognition through inhibition of immune-related molecules (e.g., MHC, cytokines).

Discussion

Under high temperatures in summer, poultry would experience a host of health problems, such as reduced immunity and disease resistance [2]. We found that their differential expression probably reflected the immune response of chickens under heat stress. Most of these biological processes are related to immunity, suggesting that heat stress in spleen can cause changes in immunity. These biological processes have nothing to do with immunity, suggesting that heat stress in the bursa of Fabricius can't cause changes in immunity. Notably, only a few genes were differentially expressed after heat treatment in the bursa of Fabricius, indicating that the organ is less sensitive than the spleen to high temperatures.

Studies on the role of *HSPA5* (also referred to as *GRP78)* in the immune system showed that *GRP78* regulates inflammation and immune responses through multiple mechanisms [16], although such reports are predominantly in mammals. As a major endoplasmic reticulum chaperone molecule, *GRP78* facilitates chemokine and cytokine processing and secretion in mammalian cells [17], as well as being a necessary binding partner for cell-surface MHC-I [18]. But these studies are mostly in mammals, and there have been no studies in chickens. By RNA-seq analysis for chickens with antigens by heat treatment, we found that HSPA5 play an important role in immune cell activation.

However, until this report, it was unclear whether *HSPA5* facilitates immune cell activation in chickens, where *HSPA5* studies mainly used SNP loci and focused on the protein's role in apoptosis. For example, *GRP78* is required for cell proliferation and apoptosis inhibition of embryonic fibroblasts [19], and BiP / GRP78 plays a key role in avian reovirus-mediated apoptosis [20]. However, a rare study examining *GRP78* response to heat stress (35 °C) showed that *GRP78* mRNA levels in chicken heart, liver, brain, and leg muscles first increased (peaking at 3 h) and then decreased after, suggesting that the protein may function in avian immune response as well [21]. This study found that intracellular *HSPA5* overexpression did not directly affect the differential expression of antigen-presenting genes, but did accelerate and enhance LPS recognition by HD11. Interference with *HSPA5* could inhibit LPS recognition by HD11 (inhibit *MHC-II* expression). Our work, combined with previous research on avian *HSPA5* heat-stress response, indicates that chicken *HSPA5* may be involved in immune cell activation.

In mammals, some studies on *DUSP1* roles in immune response have shown that it negatively regulates inflammatory cytokine production [22]. *DUSP1* expression, for example, inhibited p38 activation and GATA–3 nuclear translocation, thereby impairing cytokine Th2 [23]. Furthermore, *DUSP1* attenuates STAT1 activation through the inhibition of miR155 expression and the induction of SOCS–1 [24], as well as possibly being involved in MAP kinase-independent tumorigenesis [25]. Finally, in DUSP1-deficient mice, glucocorticoids induce *DUSP1* expression to inhibit JNK and p38 activation. Therefore, *DUSP1* apparently contributes to the anti-inflammatory effects of glucocorticoids [26]. However, none is available on its potential role in immune cell activation of chicken. Similar to our results on *HSPA5,* we found that *DUSP1* overexpression did not directly affect the differential expression of antigen-presenting genes, but did accelerate LPS recognition by HD11, thus serving a key immune function. Interference with *DUSP1* could inhibit LPS recognition by HD11. Although the exact mechanism of DUSP1's role in antigen

presentation remains to be investigated, our data strongly suggests that *DUSP1* is important to immune response in chicken.

Conclusions

In summary, decreasing expression of *HSPA5* and *DUSP1* inhibited immune cell activation in chicken immune organs under heat stress. Thus, they may be key factors to understand in future investigations on decreased immunity in heat-stressed chicken.

Abbreviations

HSPs: Heat shock proteins; LPS: lipopolysaccharides; APCs: antigen-presenting cells; RNA-seq: RNAsequencing; SPF: specific pathogen-free; NDV: Newcastle disease vaccine; HI: Hemagglutination inhibition; RT-qPCR: Quantitative real-time PCR; IPA: Ingenuity Pathway Analysis; CDS: coding sequence; MHC: Major histocompatibility complex; GO: Gene ontology; KEGG: Kyoto encyclopedia of genes and genomes; FC: Fold Change

Declarations

Ethics approval and consent to participate

All experimental and animal management procedures were undertaken in accordance to the requirements of the animal care and ethics committee of the College of Animal Science, South China Agricultural University, P. R.China.

Consent to publish

This manuscript does not contain data involving human participants and therefore no consent for participation was needed.

Availability of data and materials

The datasets supporting the conclusions of this article are included within the article and its additional files. The supplementary data files can be accessed on National Center for Biotechnology Information (NCBI) via accession number GSE93224.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

WWZ: carried out experimental design and coordination, data analysis, drafted the manuscript. JGX and HML: helped with animal handling and sample collection and revised the manuscript in a critical way. QHN and LHZ: carried out the RNA-Seq study, collected raw data and performed statistical analysis, completed and revised the manuscript. XQZ and QBL: conceived and designed experimental study, made critical revisions on the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 The DEGs associated with immune responses in the spleen and bursa of Fabricius

Gene Symble	Gene Description	log2FC	pval	padj	↑or↓ Up or Down
Group A					
FGB	fibrinogen alpha chain	2.52	7.77E-06	0.0066	↑
FGA	fibrinogen beta chain	Inf	2.31E-11	5.89E-08	↑
FGG	fibrinogen gamma chain	5.02	7.60E-10	1.46E-06	↑
IGSF11	immunoglobulin superfamily member 11	2.59	7.14E-08	9.96E-05	↑
ALDOB	aldolase B, fructose-bisphosphate	6.11	1.29E-06	0.0013	↑
APOB	apolipoprotein B (including Ag(x) antigen)	Inf	2.08E-07	0.00026	↑
Group B					
GZMA	granzyme A (granzyme 1, cytotoxic T-lymphocyte-associated serine esterase 3)	2.12	6.52E-13	2.5E-09	↑
DUSP1	dual specificity phosphatase 1	-1.77	3.63E-19	5.59E-15	↓
FGB	fibrinogen beta chain	-2.29	8.22E-05	0.024	↓
FGA	fibrinogen alpha chain	#NAME?	1.55E-10	3.98E-07	↓
FGG	fibrinogen gamma chain	-4.40	7.31E-07	0.00055	↓
GC	group-specific component (vitamin D binding protein)	#NAME?	1.46E-05	0.0068	↓
APOLD1	apolipoprotein L domain containing 1	-2.80	2.39E-06	0.0015	↓
IGSF11	immunoglobulin superfamily member 11	-2.49	4.44E-07	0.00038	↓
ALDOB	aldolase B, fructose-bisphosphate	-6.09	4.13E-06	0.0023	↓
ADAMTS1	ADAM metallopeptidase with thrombospondin type 1 motif, 1	-1.40	5.84E-10	9.97E-07	↓
LOC396380	glutathione transferase	-1.19	0.000123	0.032	↓
APOB	apolipoprotein B (including Ag(x) antigen)	#NAME?	7.37E-07	0.00055	↓
GVIN1	GTPase, very large interferon inducible 1	1.38	1.81E-06	0.0012	↑

CCL17	chemokine (C-C motif) ligand 17	-1.39	0.000165	0.039	↓
HSPA5	heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)	-0.95	2.76E-05	0.011	↓
Group C					
CD69	CD69 molecule	3.51	1.9E-08	1.06E-05	↑
Group D					
COL21A1	collagen, type XXI, alpha 1	-1.51	3.25E-07	0.00054	↓
CD69	CD69 molecule	-2.91	3.88E-05	0.034	↓
HSP90AB1	heat shock protein 90kDa alpha (cytosolic), class B member 1	-0.71	3.89E-08	8.23E-05	↓

Note: #NAME? represent no expression in the experimental group; Inf represent no expression in the control group.

Table 2 The enriched KEGG pathway of DEGs

NO.	Pathway	P value
Group A		
1	Complement and coagulation cascades	0.001
2	Carbon fixation in photosynthetic organisms	0.018
3	Vitamin digestion and absorption	0.02
4	Fructose and mannose metabolism	0.022
5	Pentose phosphate pathway	0.022
6	Methane metabolism	0.025
7	Fat digestion and absorption	0.031
8	Glycolysis / Gluconeogenesis	0.043
Group B		
1	Ovarian steroidogenesis	0.0004
2	Vitamin digestion and absorption	0.002
3	Glutathione metabolism	0.009
4	Cyanoamino acid metabolism	0.013
5	Complement and coagulation cascades	0.019
6	Taurine and hypotaurine metabolism	0.019
7	Chemokine signaling pathway	0.039
Group D		
1	Synaptic vesicle cycle	0.025
2	Protein digestion and absorption	0.035

Table 3 DEGs interaction network of IPA in four alignment groups

NO.	network	Genes (count)
Group A		
1	cellular function and maintenance, hematological system development and function, cellular development	*BCL3, BCL, CD40, ↑CD69, CD3E, IL4, EL1B, INPP5D, MS4A1, POU2AF1, RIPK2, STAT6, TGFB1, TLR3* (14)
Group B		
1	Nervous system development and function□cancer□ cardiovascular system development and function	*FOXO1, ↑GZMA* (2)
2	cellular movement, hematological system development and function, immune cell trafficking	*CCL4, CXCL3, ↓DUSP1, ELF4, IL6, IL10, TGFB2, TNF* (8)
Group C		
1	cellular movement, cellular function and maintenance, behavior	*↓CA3, CCL15, CD59, ↑CD69, CTSG, ↓DCLK1, DCX, DRD2, GDNF, GRIN1□ HDC, LAMC2□ ↑MAP2□ MAPK1□ MBP, MME, ↑MMP7□MMP8□ NTF4□ PP1-C, SRC, ↑TAC1□ TACR1□ TFF2□ TGFA, TRPV1□ ↓TUBA1C* (27)
2	Cell-to-cell signaling and interaction, digestive system development and function, hepatic system development and function	*↓FER1L6□ HLX* (2)
Group D		
1	Cell-to-cell signaling and interaction□cellular growth and proliferation, cellular movement	*↑ABCB4, ACE, CALCA, ↓CD69, CNTF, CREM, CRH, CTSG, ↑ENPP2, FCER1A, GCG, GDNF, HDC, IL6, IL6R, MMP8, NFAT, NR4A1, SELE, ↓TAC1, TACR1, TFF2, TGFA, TH, TNF, TNFRSF11B, TNFSF13B, TRPV1* (28)

Table 4 RT-qPCR validation of DEGs

Gene	Log2(FC)_RNA-seq	Log2(FC)_qPCR
Group A		
MYL1	6.852	3.321
SYT8	2.386	1.988
FGB	2.519	1.769
ALDOB	3.027	2.256
IGSF11	2.592	2.941
Group B		
DUSP1	-1.768	-1.145
GZMA	2.118	1.586
CCL17	-1.395	-1.16
FGB	-2.286	-1.539
ALDOB	-6.087	-2.195
GGT1	-1.127	-1.041
HSPA5	-0.952	-1.233
IGSF11	-2.489	-1.615
Group C		
CD69	3.513	3.026
TAC1	7.934	4.075
MMP7	5.248	2.579
DCLK1	-2.429	-1.318
AADAT	-1.158	-1.502
MYBPC1	-3.555	-1.852
Group D		
CD69	-2.91	-1.699
TAC1	-7.333	-2.133
ABCB1LB	1.151	2.018
COL21A1	-1.511	-1.092
ENPP2	1.291	1.396

HSP90AB1	-0.712	-0.845

Log2(FC) > 0 indicates that the gene expression is up-regulated; Log2(FC) < 0 indicates that the gene expression is down-regulated.

Figures

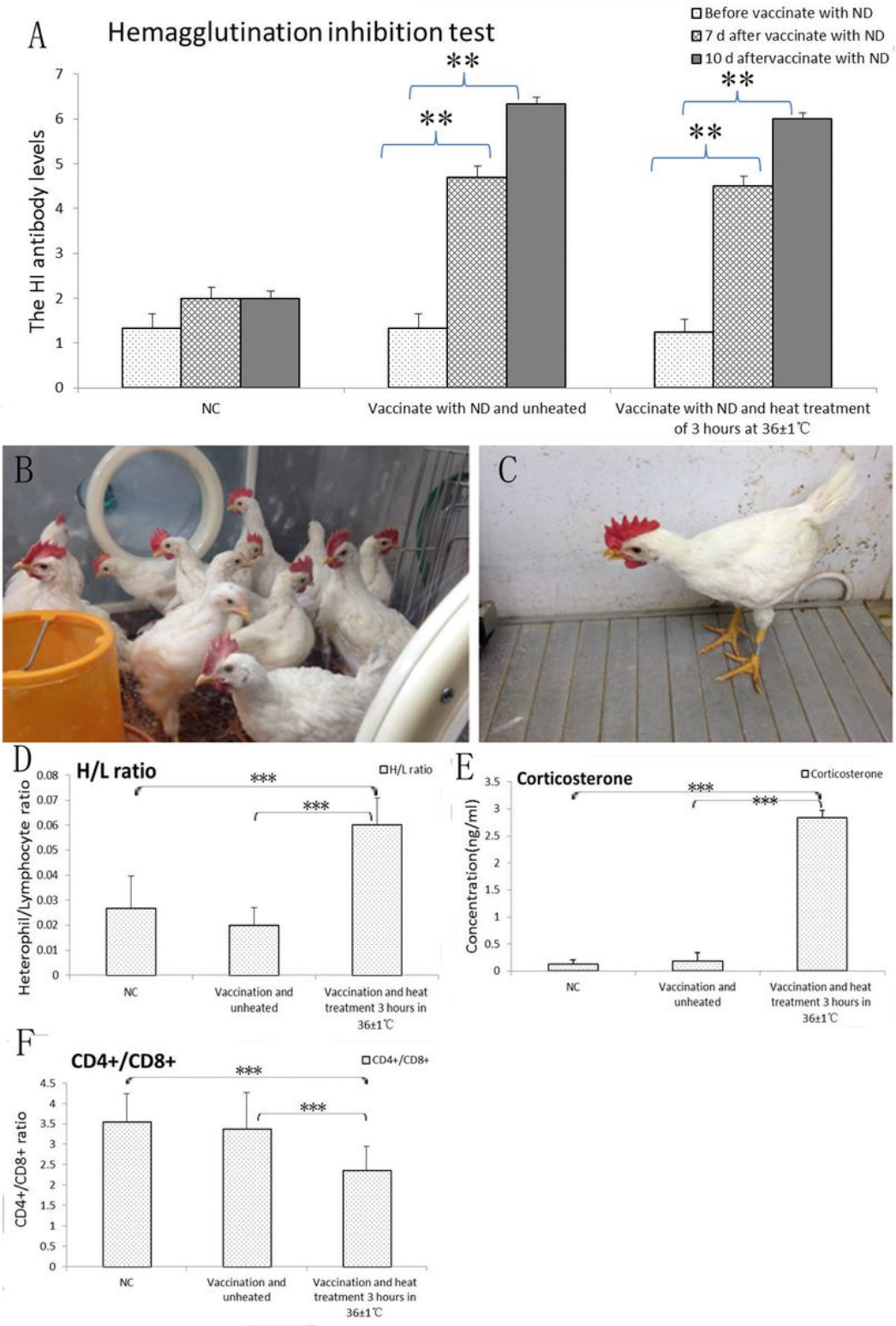

Figure 1

Antibody levels and heat stress detection. After the injection of Newcastle disease virus, antibody levels were significantly higher than pre-immunization levels, indicating that immune response (A). Heat-stressed (36 ± 1°C) chickens exhibited shortness of breath as a physical sign of experiencing high temperatures ($P < 0.001$) (B, C). They also had significantly higher concentration of H/L and corticosterone (D, E) and significantly lower concentration of CD4+/CD8+ ($P < 0.001$) than non-heat-stressed chickens (F). NC represent control.

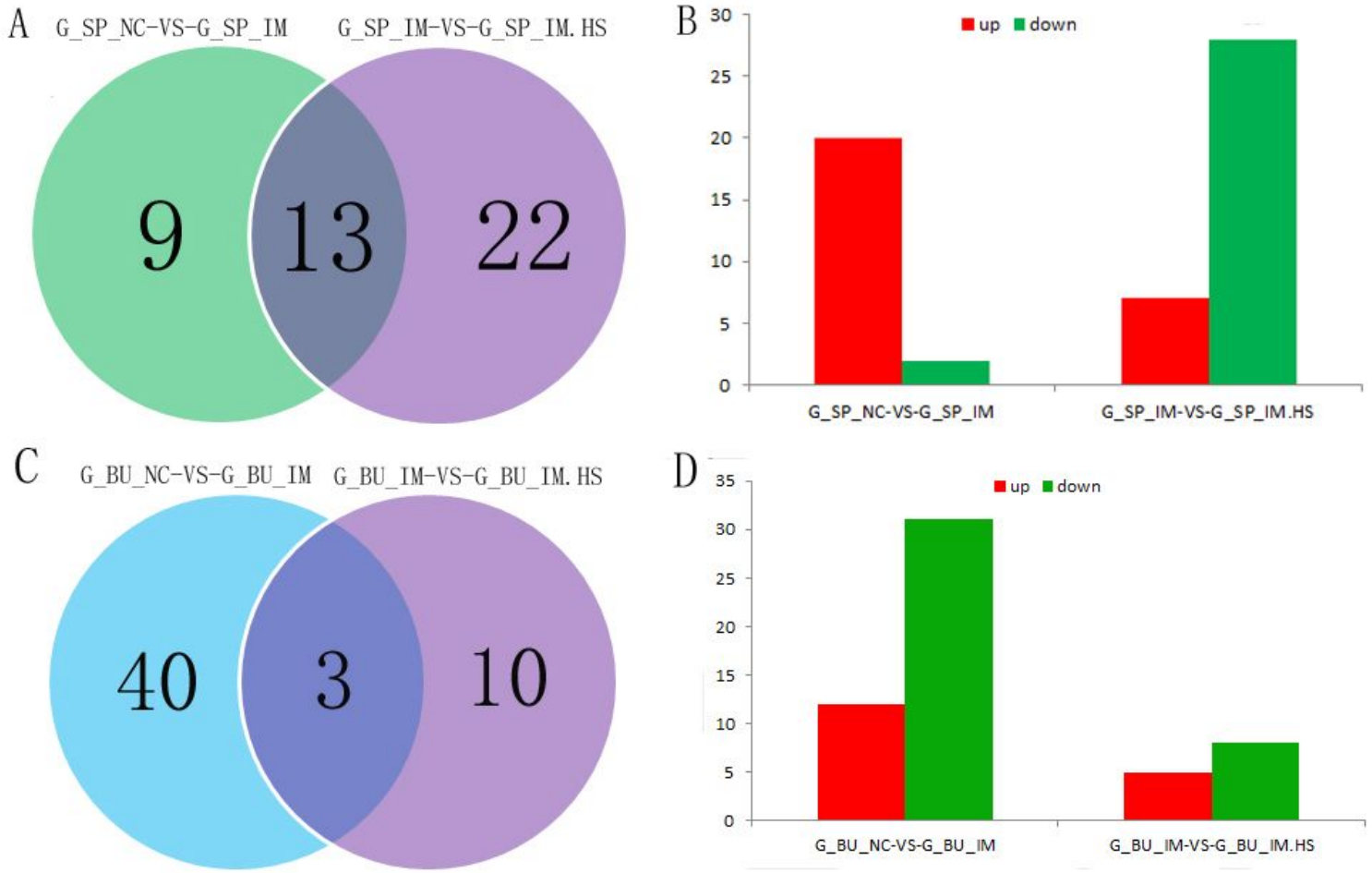

Figure 2

Differentially expressed genes among four contrasts. Across the spleen comparison groups (A, B), respectively, 22 (20 up-regulated, two down-regulated) and 35 (seven up-regulated, 28 down-regulated) differentially expressed genes were identified. In the bursa of Fabricius groups (C, D), 43 (12 up-regulated, 31 down-regulated) and 13 (five up-regulated, eight down-regulated) genes, respectively, were differentially expressed. A, C: DEGs are unique or shared among two contrasts; B, D: DEGs are up or down in the latter of each contrast.

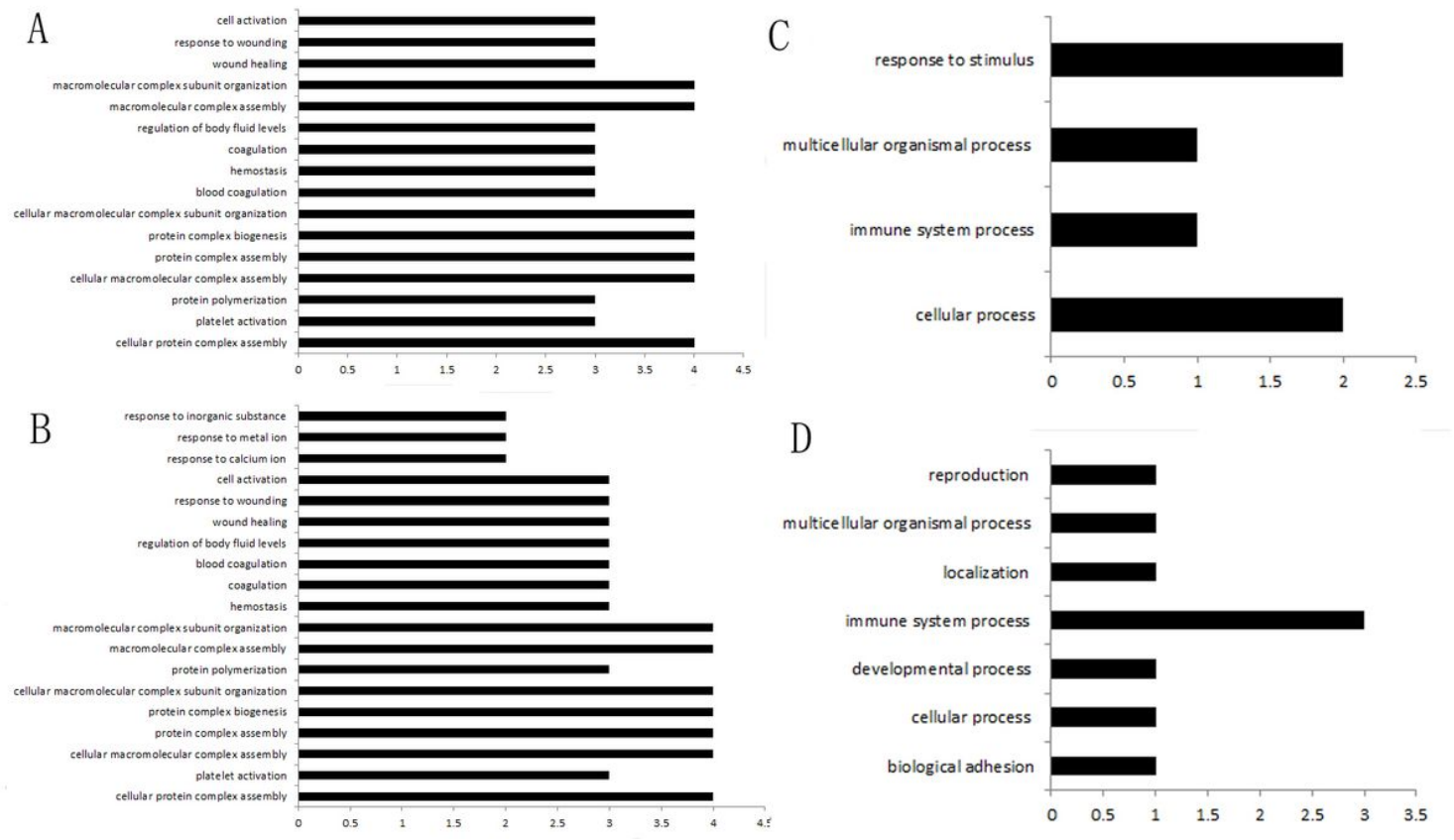

Figure 3

The biological processes of DEGs enrichment in different treatments. DEGs in the spleen samples were enriched in the following biological processes, cellular protein complex assembly, platelet activation, and assembly of cellular macromolecule complexes. (A), and enriched biological processes included the assembly of cellular protein complexes, platelet activation, etc. (B). DEGs in the bursa of Fabricius samples were enriched in the following biological processes, cellular processes, multicellular processes, etc. (C), and enrichment occurred in bioadhesion and cellular processes (D).

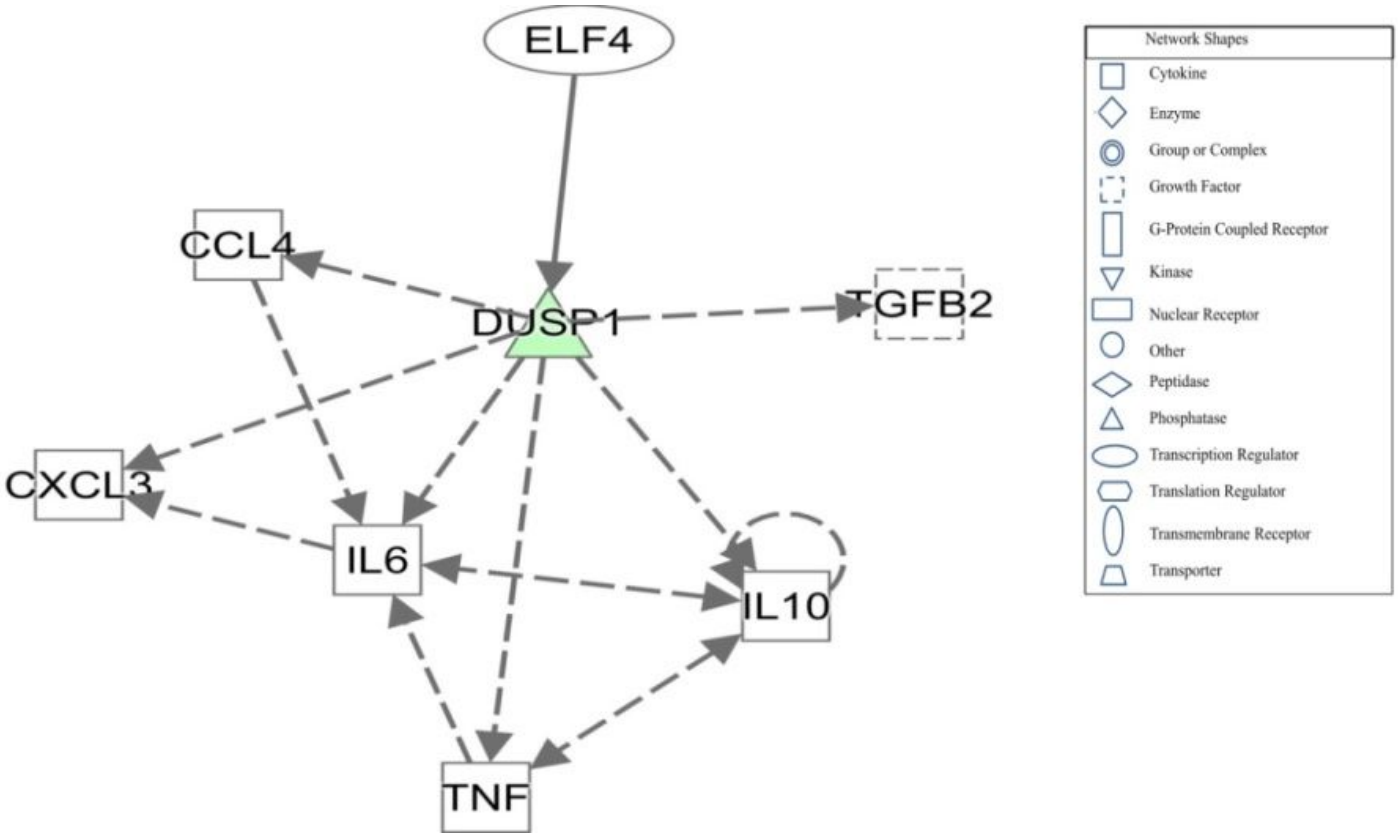

Figure 4

IPA network of differential gene enrichment. The 35 differentially expressed genes in Group B formed significantly interactive gene networks. Genes and IPA network were associated with cellular movement, hematological system development and function, and immune cell trafficking. Colored in green are down-regulated in White Leghorn. Color intensity correlates with the size of the FC, in which the color is the darker, and the variance is the greater.

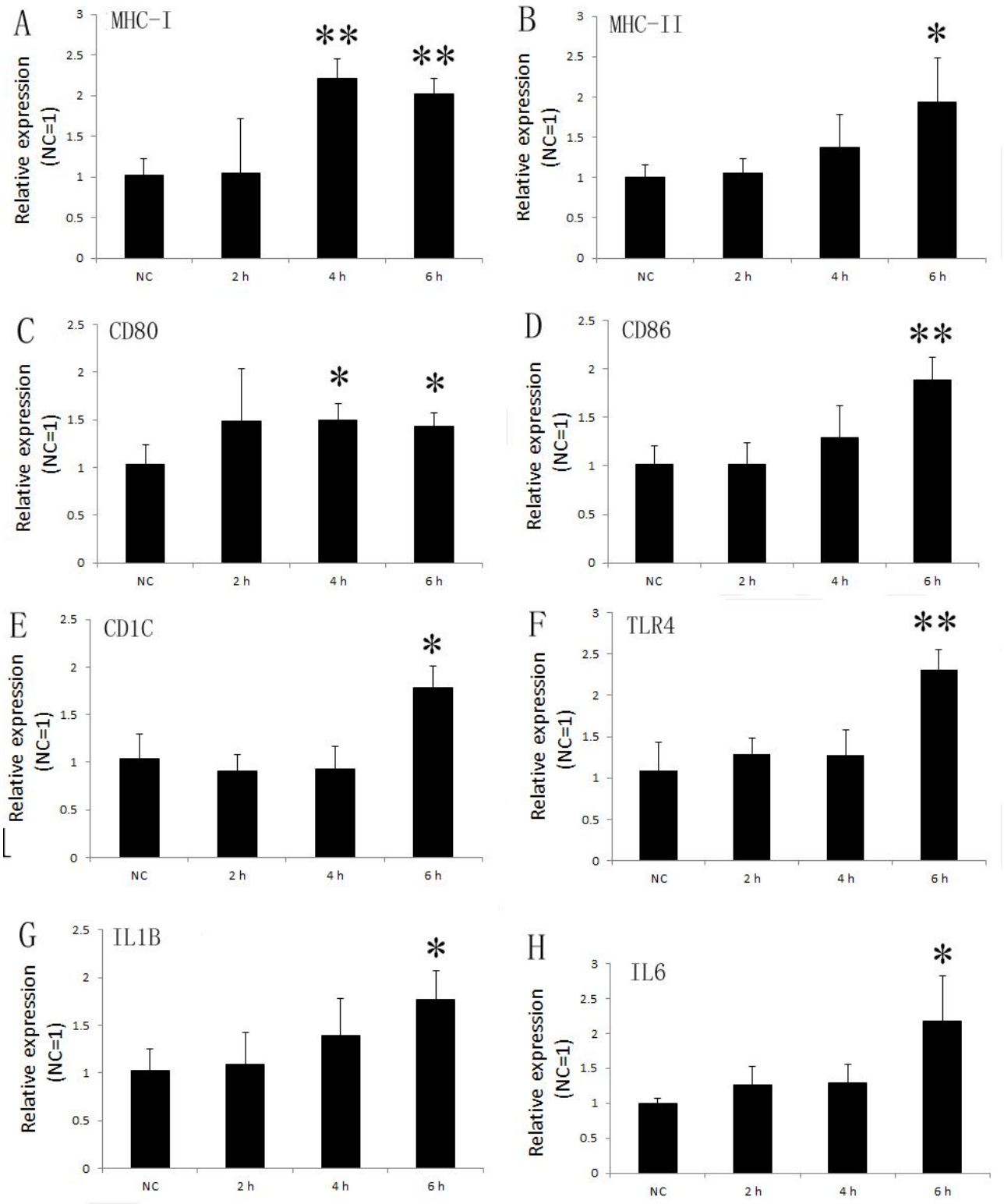

Figure 5

The expression detection of related genes after LPS-stimulated HD11. Compared with the control group, mRNA levels of MHC-I and CD80 were very significantly or significantly increased at 4 h post-LPS stimulation. At 6 h post-stimulation, MHC-II, IL1B, IL6 , TLR4, CD1C, and CD86 mRNA levels were very significantly or significantly increased (Fig. 5A-H). Furthermore, MHC-II expression on HD11 cell surfaces significantly increased at 4 h after LPS stimulation (Fig. 5I). A-H: Cells were collected for RNA extraction

after stimulated 2 h, 4 h and 8 h by 1 μg/mL LPS, RT-qPCR was used to detect the expression level of related genes. I: Cells were collected for labeled MHC-II antibody after stimulated 2 h, 4 h and 6 h by 1μg/mL LPS, the expression of MHC-II was detected by flow cytometry (FCM). NC is control group, 2 h, 4 h and 6 h is the stimulation time by LPS.

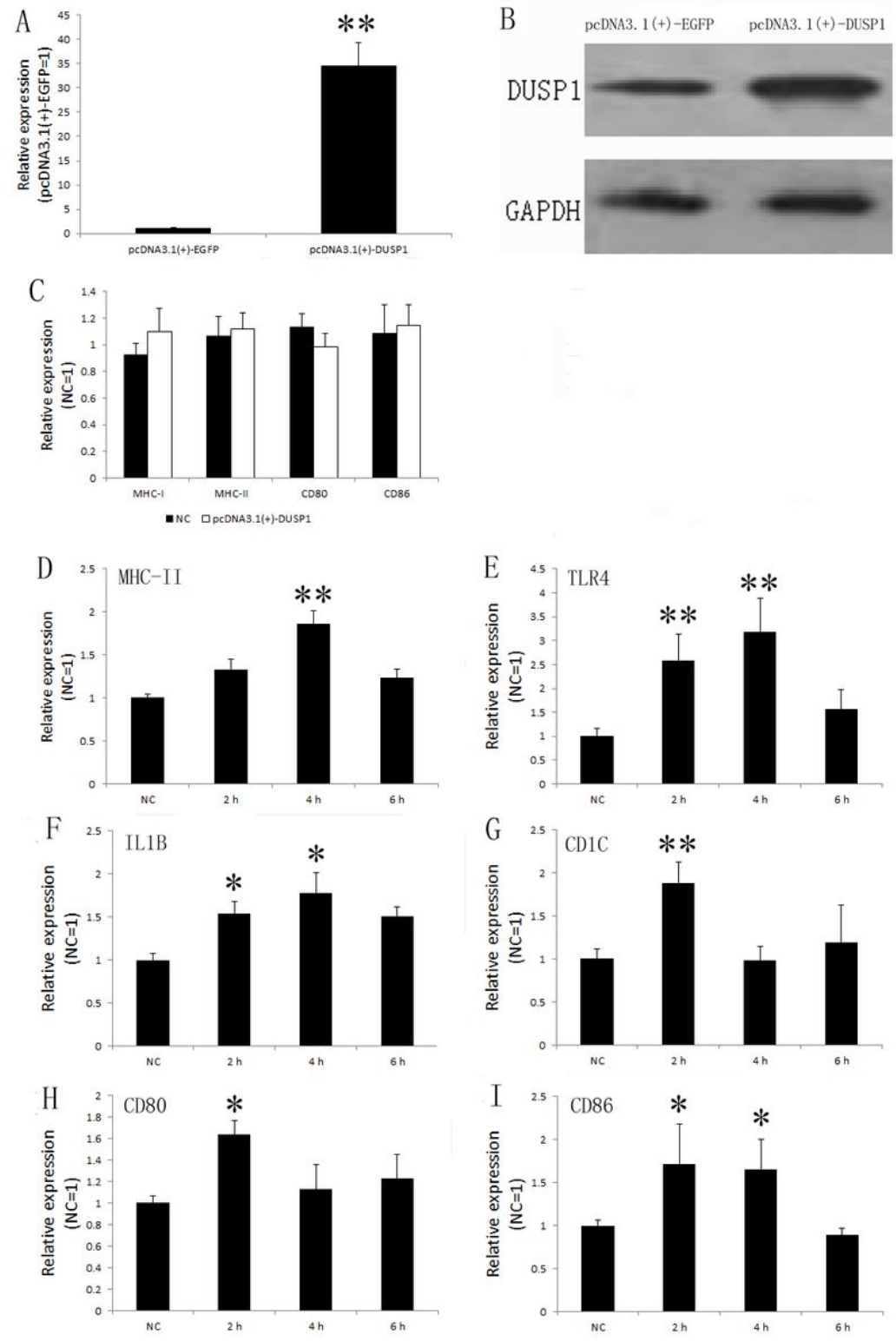

Figure 6

DUSP1 overexpression accelerates HD11 recognition of LPS and thus causes allows earlier LPS presentation. The overexpression vector can express genes in HD11 (A, B). Intracellular DUSP1 overexpression does not influence the expression of antigen-presentation-related genes without actual antigen addition(C). As exogenous antigens are mainly presented through the MHC-II pathway, we examined MHC-II presence on HD11 surfaces and found that after DUSP1 overexpression (D-J). A, B: pcDNA3.1(+)-DUSP1 represent overexpression, pcDNA3.1(+)-EGFP represent control; C: pcDNA3.1(+)-DUSP1 represent cell that transfected with overexpression DUSP1 plasmid, NC represent negative control cells that only pulsed with transfection reagent; D: NC was added MHC-II antibody but no LPS stimulation, and 2 h, 4 h and 6 h is the stimulation time by LPS; E-J: NC was cell that transfected with overexpression plasmid of DUSP1 but without stimulation with LPS, and 2, 4 and 6 h was stimulation time with LPS after transfected with overexpression DUSP1 plasmid.

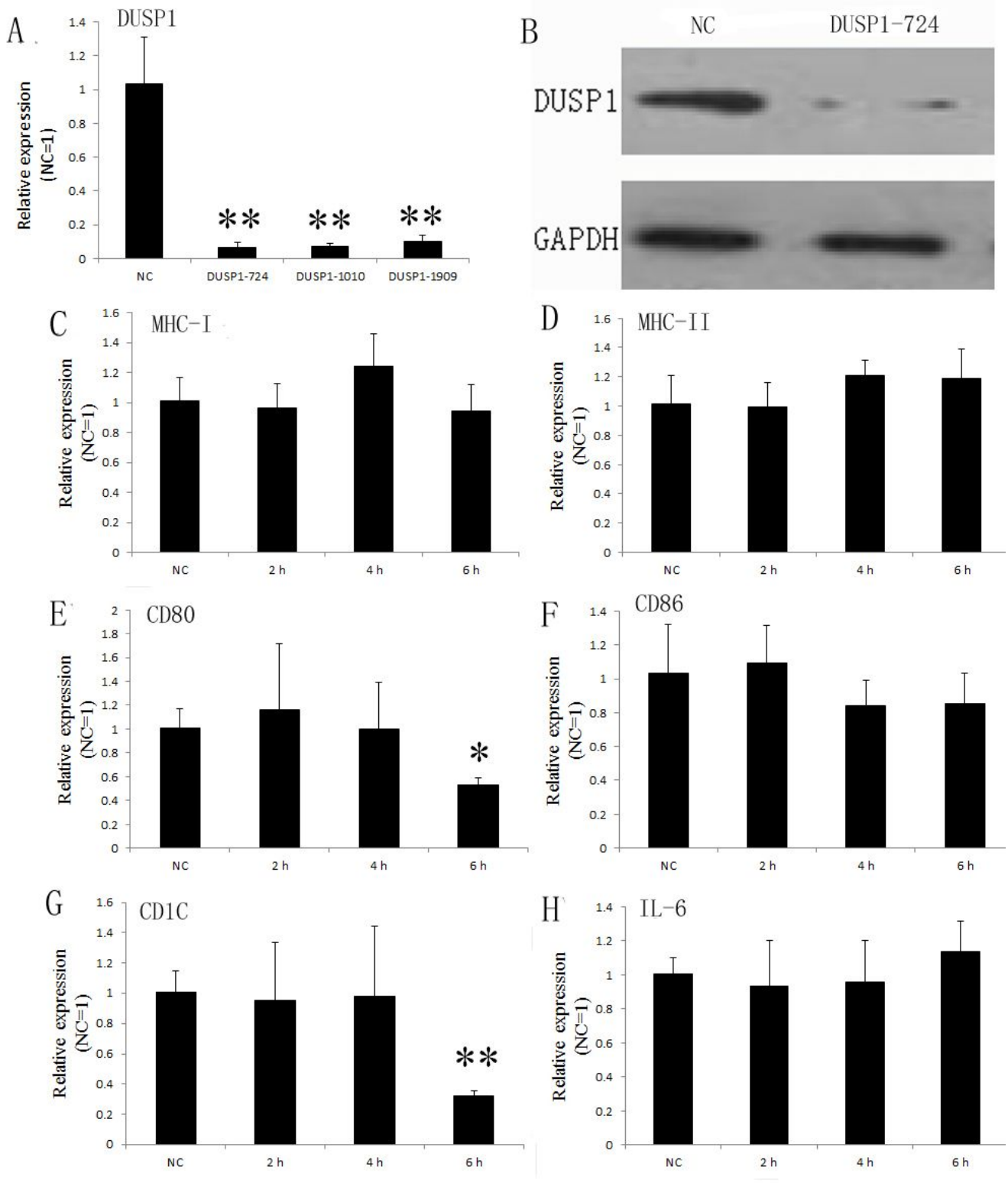

Figure 7

HD11 presentation of LPS is affected by DUSP1 expression levels. The DUSP1 interfering fragment is valid (A, B). Interfering with DUSP1 inhibits the ability of HD11 ability for LPS presentation, causing downstream effects in the expression of other immune-related genes(C-I). A, B: Interference effect of DUSP1 interference fragment; C-H: Detection of HD11 presented LPS after DUSP1 was inhibited, NC was cell that transfected with DUSP1-724 but without stimulation with LPS; 2, 4 and 6 h was stimulation time

with LPS after transfected with DUSP1-724; I: The expression of MHC-II on HD11 surface was detected by flow cytometry.

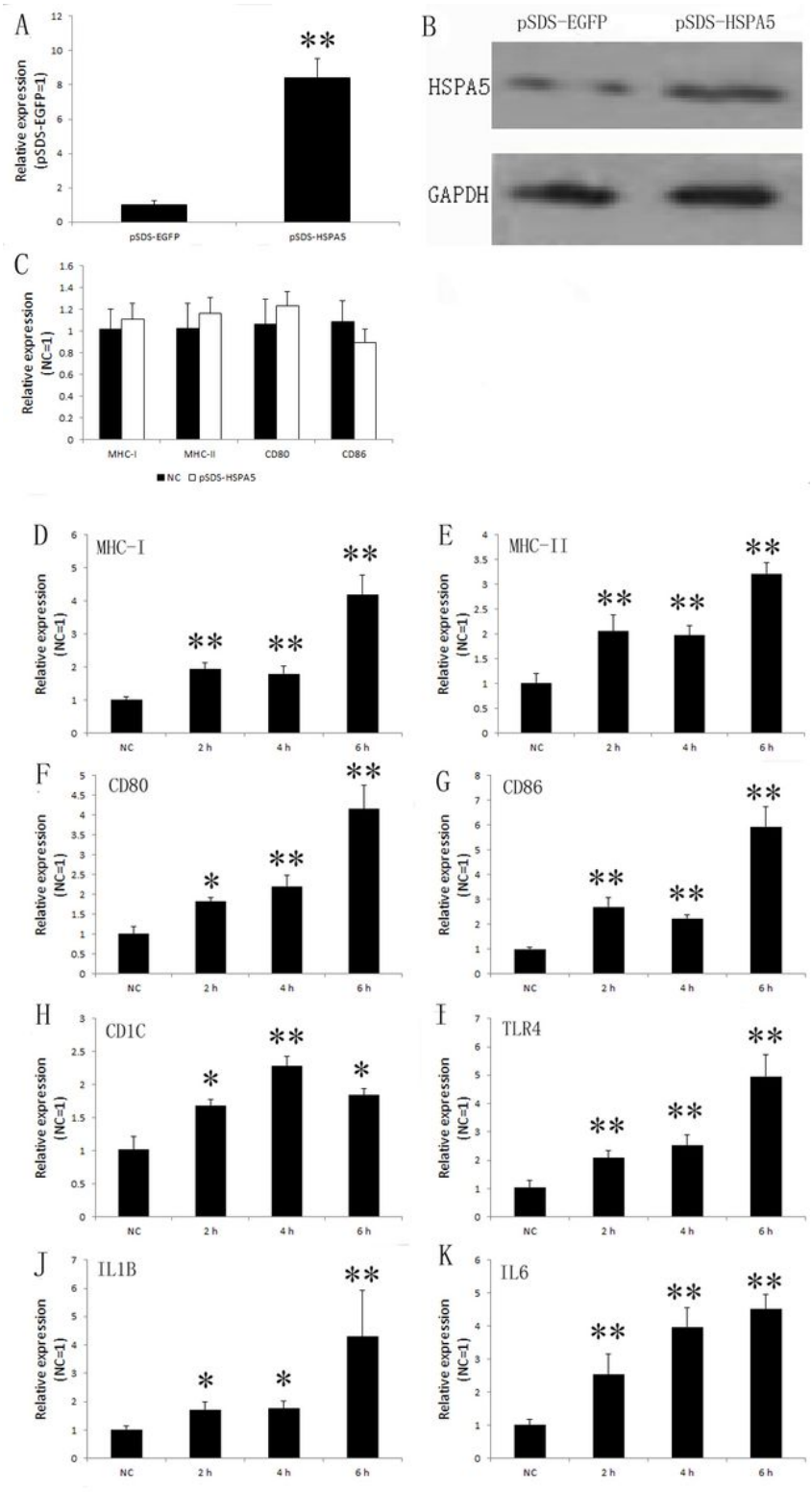

Figure 8

HSPA5 overexpression accelerates HD11 recognition of LPS and thus causes allows earlier LPS presentation. The overexpression vector can express genes in HD11 (A, B). Intracellular HSPA5 overexpression does not influence the expression of antigen-presentation-related genes without actual

antigen addition(C). As exogenous antigens are mainly presented through the MHC-II pathway, we examined MHC-II presence on HD11 surfaces and found that after HSPA5 overexpression (D-L). A,B: pSDS-HSPA5 represent overexpression, pSDS-EGFP represent control; C: pSDS-HSPA5 represent cell that transfected with overexpression plasmid pSDS-HSPA5, NC represent negative control cells that only pulsed with transfection reagent; D: NC was added MHC-II antibody but no LPS stimulation, and 2 h, 4 h and 6 h is the stimulation time by LPS ; E-L: NC was cell that transfected with overexpression plasmid of HSPA5 but without stimulation with LPS, and 2, 4 and 6 h was stimulation time with LPS after transfected with overexpression HSPA5 plasmid.

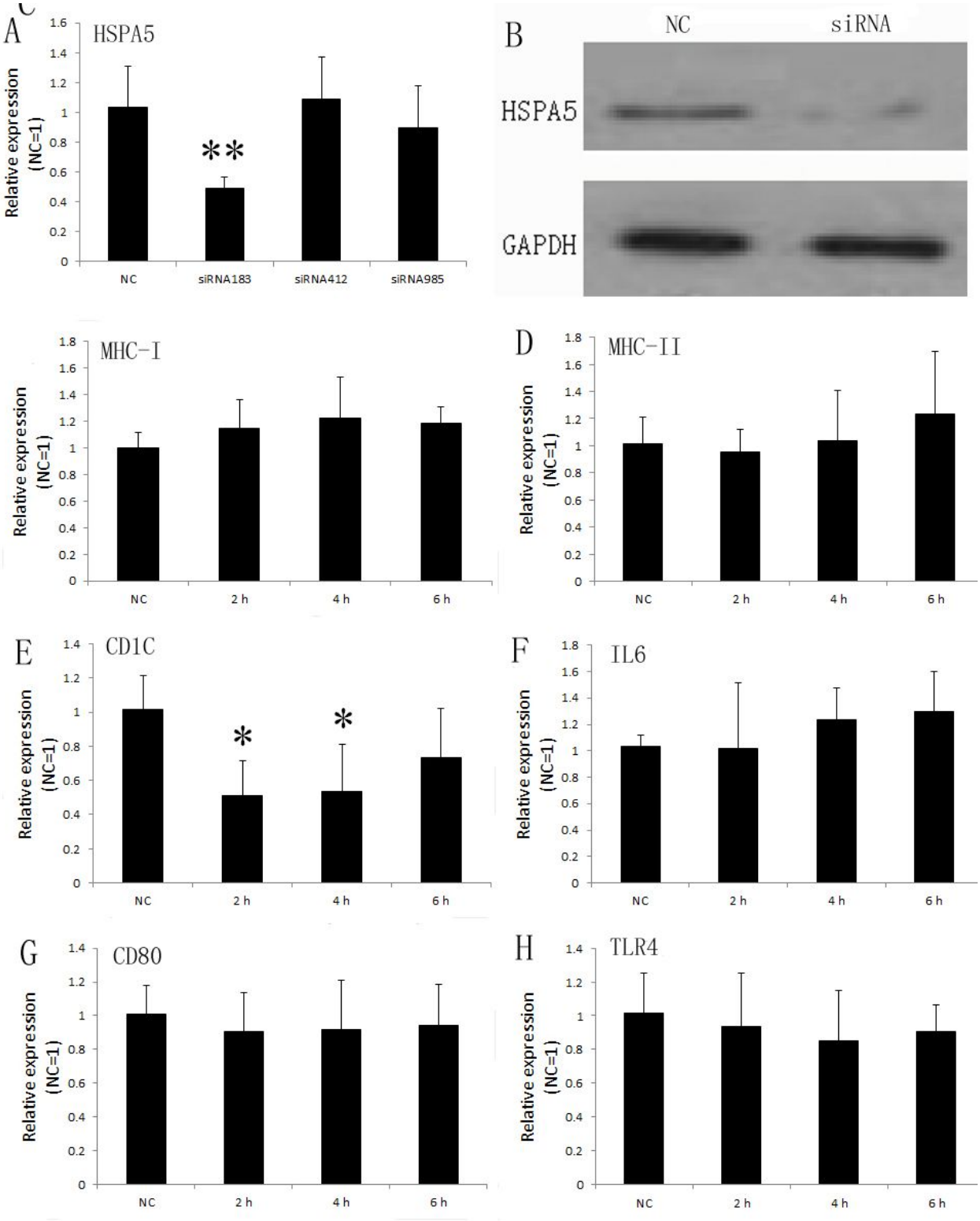

Figure 9

HD11 presentation of LPS is affected by HSPA5 expression levels. The HSPA5 interfering fragment is valid (A, B). Interfering with HSPA5 inhibits the ability of HD11 ability for LPS presentation, causing downstream effects in the expression of other immune-related genes(C-I). A: Interference effect detection of 3 HSPA5 interference fragment by RT-qPCR; B: Interference effect detection of HSPA5-183 by western blot; C-H: NC was cell that transfected with HSPA5-183 but without stimulation with LPS, 2, 4 and 6 h was

stimulation time with LPS after transfected with HSPA5-183; I: NC was added MHC-II antibody but no LPS stimulation, and 2 h, 4 h and 6 h is the stimulation time.

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