Expression profiles of genes involved in TLRs and NLRs signaling pathways of water buffaloes infected with *Fasciola gigantica*

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Abbreviations: ESP, excretory-secretory products; FABP, fatty acid binding protein; NLRs, NOD-like receptors; PBMC, peripheral blood mononuclear cell; PRRs, pattern-recognition receptors; TLRs, Toll-like receptors

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ABSTRACT

Infection of ruminants and humans with *Fasciola gigantica* is attracting increasing attention due to its economic impact and public health significance. However, little is known of innate immune responses during *F. gigantica* infection. Here, we investigated the expression profiles of genes involved in Toll-like receptors (TLRs) and NOD-like receptors (NLRs) signaling pathways in buffaloes infected with 500 *F. gigantica* metacercariae. Serum, liver and peripheral blood mononuclear cell (PBMC) samples were collected from infected and control buffaloes at 3, 10, 28, and 70 days post infection (dpi). Then, the levels of 12 cytokines in serum samples were evaluated by ELISA. Also, the levels of expression of 42 genes, related to TLRs and NLRs signaling, in liver and PBMCs were determined using custom RT^2 Profiler PCR Arrays. At 3 dpi, modest activation of TLR4 and TLR8 and the adaptor protein (TICAM1) was detected. At 10 dpi, NF-κB1 and Interferon Regulatory Factor signaling pathways were upregulated along with activation of TLR1, TLR2, TLR6, TLR10, TRAF6, IRF3, TBK1, CASP1, CD80, and IFNA1 in the liver, and inflammatory response with activated TLR4, TLR9, TICAM1, NF-κB1, NLRP3, CD86, IL-1B, IL-6, and IL-8 in PBMCs. At 28 dpi, there was increase in the levels of cytokines along with induction of NLRP1 and NLRP3 inflammasomes-dependent immune responses in the liver and PBMCs. At 70 dpi, *F. gigantica* activated TLRs and NLRs, and their downstream interacting molecules. The activation of TLR7/9 signaling (perhaps due to increased B-cell maturation and activation) and upregulation of NLRP3 gene were also detected. These findings indicate that *F. gigantica* alters the expression of TLRs and NLRs genes to evade host immune defenses. Elucidation of the roles of the downstream effectors interacting with these genes may aid in the development of new interventions to control disease caused by *F. gigantica* infection.

Keywords: *Fasciola gigantica*; Gene expression; Host-pathogen interaction; Pattern recognition receptors; Toll-like receptors

1. Introduction

Fasciolosis is a parasitic disease caused by *Fasciola gigantica* and *F. hepatica* in tropical and temperate countries, respectively. These liver flukes can cause significant economic losses when they affect economically important livestock species (Spithill et al., 1999). Infection with *Fasciola* can adversely affect the liver and cause significant reduction in the productivity, growth rate and even death of the affected animal (Keiser and Utzinger, 2009; Molina et al., 2006). Control of liver fluke infection has been challenging due to the lack of vaccines and increasing rate of drug resistance (Fairweather and Boray, 1999). These parasites are also common zoonotic agents (Keiser and Utzinger, 2007). Adult liver flukes have been recovered from the bile duct of humans (Inoue et al., 2007; Kanoksil et al., 2006; Sharifiyazdi et al., 2011). Also, infection has been linked to liver cirrhosis, fibrosis and cancer (Machicado et al., 2016), particularly in China, Korea, Africa, South America (Chen et al., 2013; Hotez et al., 2008). Despite the economic and public health importance of *F. gigantica* infection, its immunopathogenesis remains unclear.

The outcome of *Fasciola*-host interaction depends on the ability of the host immune system to limit parasite replication and the ability of the parasite to evade or suppress the host immune defenses in order to promote its own survival in the host (Girones et al., 2007; Rodriguez et al., 2015; Zhang et al., 2017b). *Fasciola* spp. are very efficient in modulating the host immune response (Girones et al., 2007). While *F. hepatica* infection elicits a dominant Th2/T-regulatory immune response in sheep (Fu et al., 2016), *F. gigantica* infection induces a mix of Th1/Th2 response with a Th2-biased pattern in mice, cattle and buffaloes (Chantree et al., 2013; Molina, 2005). Recent studies have revealed transcriptomic changes associated with host's immune response and metabolism in peripheral blood mononuclear cells (PBMCs) (Alvarez Rojas et al., 2015; 2016; Hacariz et al., 2015) and liver (Zhang et al., 2017b) in

response to *Fasciola* infection. Despite ongoing research on *F. gigantica* pathogenesis, little is known regarding the patterns of the host innate immune response to infection with *F. gigantica*. A better understanding of the immunoregulatory strategies employed by this parasite may guide the development of novel preventative and therapeutic interventions.

Innate pattern-recognition receptors (PRRs), such as the membrane-associated Toll-like receptors (TLRs), cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), RIG-I-like receptors (RLRs), C-type lectin receptors (CLRs), AIM2-like receptors (ALRs), and sequestosome 1/p62-like receptors (SLRs), are the first line of defense against microbial infections. These innate immune sensors can recognize pathogens, recruit innate immune cells and evoke adaptive immune responses (Cooney et al., 2010; Fukata et al., 2009). The interaction between *F. hepatica* and host TLRs, and interference with subsequent downstream host signaling has been suggested (Flynn and Mulcahy, 2008). This interaction is greatly influenced by the fluke's excretory-secretory products (ESP), which could affect the regulation of various TLR signaling pathways. For example, fatty acid binding protein (FABP) of *F. hepatica* can suppress the inflammatory immune response via targeting multiple TLR pathways (Ruiz-Jiménez and Espino, 2014). FABP of *F. hepatica* (Fh12) was shown to inhibit TLR4 activation and suppress the inflammatory cytokines (Martin et al., 2015). *F. hepatica* ESP was also shown to affect MyD88-dependent signaling pathway (Falcon et al., 2010). Ravidà et al., (2016) reported a role for CLRs in *F. hepatica* tegumental coat (FhTegs) interaction with bone marrow derived dendritic cells (DCs). Recently, mucin-derived peptide from *F. hepatica* (Fhmuc) was shown to enhance the stimulatory capacity of DCs and boost their ability to polarize T cell responses toward Th1 profiles, as well as increase the expression of TLR4 (Noya et al., 2017).

While several studies have investigated levels of host cytokines induced by *F. gigantica* infection (Chantree et al., 2013; Molina, 2005; Zhang et al., 2017a), PRRs involved in the

signaling pathways governing host immune responses remain unknown. Given the ability of *F. gigantica* flukes to subvert host immune defenses, persist within the host and cause chronic liver disease, deciphering the roles of innate immune receptors that mediate recognition of *F. gigantica* is key to understanding the immunological basis that may lead to the clearance of this parasite from host tissues. Therefore, in this study we used commercial quantitative RT^2 Profiler PCR Arrays to study the differential expression of 42 genes related to TLR- and NLR-mediated signal transduction and innate immunity in the liver and peripheral blood mononuclear cell (PBMCs) of buffaloes infected with *F. gigantica*. The levels of gene expression were determined in the liver and PBMC samples in order to capture a more comprehensive picture of the local and systemic immune responses against *F. gigantica* infection. In parallel to gene expression analysis, the levels of 12 serum cytokines were assessed using ELISA.

2. Materials and methods

2.1. Ethics Statement

The study was approved by The Animal Administration and Ethics Committee of Lanzhou Veterinary Research Institute, Chinese Academy of Agricultural Sciences. All animals were handled in strict accordance with good animal practice according to the Animal Ethics Procedures and Guidelines of the People's Republic of China.

2.2. Metacercariae

Eggs of *F. gigantica* were collected from the gall bladders of naturally infected buffaloes killed at local abattoirs in Guangxi Zhuang Autonomous Region, China, as previously described

(Zhang et al., 2017b). The collected eggs were incubated at 29 ºC for 11 days. The newly hatched miracidia were used to infect *Galba pervia* snails (3-5 miracidia/snail) maintained in a plastic culture plate for 2 hours. Then, infected snails were incubated in order to allow miracidia to develop to sporocysts, rediae and cercariae. After ~6 weeks fully-developed cercariae were shed from the snails and were harvested on 5×5 cm cellophane sheets and encysted into metacercariae.

2.3. Animals and experimental infection

Twenty-four (8-10-month-old) buffaloes were purchased from a water buffalo's farm in Guangxi Zhuang Autonomous Region, PR China. Animals were randomly divided into two groups: (i) 12 uninfected, control group and (ii) 12 infected group. Each group was further subdivided into 4 subgroups. Each subgroup of three buffaloes (infected or control) was examined at 3, 10, 28, and 70 days post infection (dpi) in order to assess the temporal alterations in the levels of serum cytokines and gene expression in liver and PBMC following *F. gigantica* infection. To rule out the possibility of any prior infection, fecal samples were examined microscopically for *Fasciola* eggs and sera were tested for anti-*F. gigantica* IgG and IgM antibodies using ELISA, as described previously (Chauvin et al., 1995; Zhang et al., 2017b). After acclimatization for 2 weeks, each buffalo was orally treated with a single dose of triclabendazole (Change Zhou Jialing Medicine Industry Co., Ltd., China) at a dose rate of 50 mg/kilogram of body weight in order to ensure liver fluke-free status of the buffaloes used in this study. Following a withdrawal time of four weeks, metacercariae encysted on cellophane sheets were washed several times with sterile phosphate buffered saline (PBS) and used immediately to infect 12 buffaloes (500 metacercariae/animal), as previously described (Molina

and Skerratt, 2005; Phalee et al., 2015). Control buffaloes were mock-inoculated with 0.85% NaCl solution without metacercariae.

2.4. Collection of liver and blood samples

For each time point, 3, 10, 28, and 70 dpi, three animals from each of the infected and control groups were sacrificed and their livers were quickly collected. Liver samples were washed in PBS, cut into small pieces, immediately frozen in liquid nitrogen, and kept in small tubes at –80 °C until analysis. Frozen liver samples homogenization was obtained using a TissueLyser LT (Qiagen, Germantown, MD). Venous blood samples were collected and were allowed to clot at ambient temperature for 30 min, followed by centrifugation at 1,700 ×*g* for 10 min at 20 °C. The serum layer was collected, divided into aliquots and frozen at –20 °C until use. Also, parallel blood samples were collected in 2-mL ethylenediaminetetraacetic acid (EDTA)-treated sterile tubes, and PBMCs were separated within 2 h after collection using the method described below.

2.5. Cytokine determination

The levels of 12 cytokines, namely tumor necrosis factor-α (TNF-α), transforming growth factor-β (TGF-β), interferon gamma (IFN-γ), interleukin-1β (IL-1β), IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, and IL-18, in the buffalo's serum were determined using ELISA (Bovine cytokine ELISA kit, Blue Gene Biotech Inc., Shanghai, China) following the manufacturer's instructions. Frozen serum samples were thawed immediately before the analysis. The assay procedure was similar for all cytokines. Briefly, 100 µl of standard or serum sample were added into each well in the antibody pre-coated microtiter plate. Also, 100 µl PBS were added to three

blank wells to control for any variation or contribution, of the plate itself to the measured optical density (OD). Then, 50 µl of enzyme conjugate were added into each well, mixed thoroughly and incubated for 1 hr at 37 °C. Then, the mixture was removed and all wells of the microtiter plate were washed 5X with PBS. For color development, 50 µl of each of Substrate A and Substrate B were added to each well including blank wells, followed by 10 min incubation at 37 °C in the dark. Finally, 50 µl of stop solution were added to each well and mixed with gentle tapping to terminate the reaction. OD of 450 nm (OD450) minus the background of plate absorbance was read on an ELISA microplate reader (BIO-RAD, Model 680). Even though the bovine cytokine ELISA kit has not been validated for use in buffaloes, the measured cytokines were within the detectable range of the kit.

2.6. Isolation of PBMCs

We used lymphocyte isolation kit (TBD Biological Technology Co., Tianjin, China) to obtain a suspension of PBMCs. Briefly, five ml diluent were added to 5 ml whole blood in a clean 10 ml glass centrifuge tube, followed by inversion of the tube several times to achieve good mixing. Then, 5 ml of the diluted blood was carefully overlaid over 5 ml lymphocyte separation medium without mixing of the two layers. Following centrifugation at 400 ×*g* for 30 min at ambient temperature, the layer containing the mononuclear cells was harvested carefully into a fresh tube and was mixed with 10 ml PBS. Following centrifugation at 250 ×*g* for 10 min at ambient temperature, supernatant was removed and cell pellet containing the isolated PBMCs (approximately 5×10^6 cells) was resuspended in 1 ml RNAlater and stored at –80 °C until analysis.

2.7. RNA isolation and qRT-PCR

Total RNA was isolated from homogenized liver and purified PBMC samples by RNeasy Mini Kit (Qiagen Gmbh, Hilden, Germany). The RNA samples were treated with 10 µl RNase-Free DNase to remove residual genomic DNA. The RNA quantity and purity was determined using NanoPhotometer® spectrophotometer at 260 nm/280 nm (Implen, CA, USA). Only RNA without DNA contamination and degradation of 26S rRNA was used for subsequent cDNA synthesis. One microgram of purified total RNA was used as a template to synthesize cDNA using First Strand cDNA Synthesis Kit according to the instructions of the manufacturer (Qiagen, Maryland, USA). The cDNA samples from liver and PBMCs were used as a template to quantify the expression level of 42 genes involved in TLR and NLR signaling pathways. Specifically, we examined genes related to TLRs (e.g. TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, TLR10), pathogen-specific responses (e.g. TICAM1, TBK1), TLR signaling (e.g. TRAF3, TRAF6, MYD88), signaling downstream of TLRs (e.g. [NFκB signaling: NFKB1], [Interferon Regulatory Factor (IRF) signaling: IFNG, IFNA1], [cytokine signaling: IL4, IL6, IL8, IL17A, IL18, IL13]), regulation of adaptive immunity (e.g. IL1B, IL12A, IL10), TLR interacting proteins and adaptors (e.g. RIPK2), and downstream effectors of TLR signaling (e.g. IRF3, IRF7). A list of all 42 gene targets investigated in the expression profiling analysis is provided (Table S1).

Commercial quantitative real-time PCR (qRT-PCR) primers used in the present study were provided by Qiagen Custom RT^2 Profiler PCR Array service. Quantitative mRNA measurements were performed with the RT^2 SYBR® Green ROX qPCR Mastermix in ABI

7500 real-time PCR cycler. The amplification program was performed using the following cycling conditions: initial denaturation for 10 min at 95 ºC, followed by 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. The data were normalized to β-actin housekeeping gene and the relative gene expression ratio of each gene was calculated by the cycle threshold ($\Delta\Delta C_T$) method according to the manufacturer's recommendations. We used an arbitrary cut-off of >1.5-fold change and ⩽0.5-fold change for upregulated and downregulated genes, respectively following the recommendation of QIAGEN "RT2 Profiler PCR Array Data Analysis v3.5 Handbook". Because fold change and statistical cut-offs can modulate the outcome of gene expression analysis (Dalman et al., 2012), we used less stringent cut-off values to avoid exclusion of genes that might have a biological relevance. Differential gene expression between infected and control samples was determined using an unpaired t-test with unequal variance and P-values of <0.05 were considered significant.

2.8. Statistical analysis

Statistical analysis of ELISA measurements was performed in GraphPad Prism version 6. One-way analysis of variance (ANOVA) followed by Tukey's multiple-comparison test was used to evaluate differences between groups. Two-way ANOVA followed by Bonferroni posttests was used to evaluate differences between the groups during the time course of infection. *P* values of < 0.05 (*), < 0.01 (**) and < 0.001 (***) were considered statistically significant.

3. Results

3.1. Confirmation of F. gigantica infection

At 70 dpi, livers collected from infected animals showed clear gross pathological lesions along with the presence of adult *F. gigantica* flukes, confirming the establishment of a successful infection in experimentally infected buffaloes. By contrast, livers of the control, uninfected, buffaloes appeared normal, without any pathological changes and were free of any *F. gigantica* flukes. Serological testing using ELISA confirmed the infection in the infected animals at 28 and 70 dpi.

3.2. Levels of serum cytokines

At 3 dpi, the levels of all cytokines, TNF-α, TGF-β, IFN-γ, IL-1B, IL-2, IL-4, IL-5, IL-6, IL-12, IL-13, IL-17, and IL-18, were significantly reduced in infected buffaloes, probably due to abnormally high levels of cytokines in control animals (Fig. 1). At 10 dpi, levels of all cytokines in infected buffaloes were comparable to the levels in control animals, except for IL-5 and IL-17, which was significantly reduced in infected buffaloes compared to controls. Interestingly, the levels of TGF-β, IL-4 and IL-17 were significantly increased in infected buffaloes compared to controls at 28 dpi. During the late stage of infection, 70 dpi, differences in the cytokines between infected and control buffaloes were not statistically significant ($P > 0.05$).

3.3. Expression of TLRs and NLRs genes

Expression analysis was successfully achieved for all 42 genes in PBMC samples and for 35 genes in liver samples. Our analysis identified many biologically relevant differentially expressed genes between infected and control animals (Tables S2 and S3). At 3 dpi, gene expression analysis showed that TIR-domain-containing adapter-inducing interferon-β (TRIF, also named as TICAM1) was upregulated in the liver and PBMCs of infected buffaloes compared to controls, whereas TLR4 and TLR8 showed high expression in PBMCs only (Fig. 2). TLR1, TLR5, TLR6, TLR7, TLR9, TLR10, Interferon regulatory factor (IRF) 3, IRF7, NF-κB1, TANK-binding kinase (TBK) 1, NLRP1, CASP1, CD40, CD80, CD86, IFNB1, IL-17A, IL-2, IL-8, TNF, nucleotide-binding and oligomerization domain containing 1 (NOD1), IFNA1, and PYCARD were downregulated in the liver, whereas TLR5 and IFNB1 were downregulated in PBMCs (Fig. 2). The genes whose expression was between 0.5 and 1.5 were deemed non-differentially expressed.

At 10 dpi, TLR1, TLR2, TLR6, TLR10, TRAF6, IRF3, NF-κB1, TBK1, CASP1, CD80, and IFNA1 were upregulated in infected liver compared to control. In PBMCs, TLR4, TLR9, TICAM1, NF-κB1, NLRP3, CD86, IL-1B, IL-6, and IL-8 were more expressed in infected group compared to control (Fig. 3), whereas TLR3 and TLR8 were down-regulated in infected compared to control. The rest of genes with expression between 0.5 and 1.5 were deemed non-differentially expressed.

At 28 dpi, the levels of TLR3, TLR5, TLR8, TLR9, TLR10, TICAM1, TRAF3, IRF3, IRF7, NF-κB1, TBK1, NLRP1, NLRP3, CD40, IFNB1, IL-17A, IL-2, IL-8, TNF, NOD1, IFNA1, and PYCARD were upregulated in the liver of infected animals compared to controls. In PBMCs, expression of IRF7, IL-18 and IL-12A did not show any differences between infected and control samples, however the rest of the genes were slightly over expressed in infected relative to control samples (Fig. 4).

296 At 70 dpi, TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, MyD88,
297 TICAM1, TRAF3, IRF7, NOD2, NLRP1, NLRP3, CASP1, CD40, CD86, IFNB1, IL-17A, IL-
298 18, IL-2, IL-8, TNF, NOD1, IFNA1, and PYCARD were upregulated, and TBK1 and CD80
299 were downregulated in the liver of infected buffaloes compared to controls (Fig. 5). In PBMCs,
300 the levels of expression of all genes except IL-18 were downregulated.
301
302 **4. Discussion**
303
304 At present, the mechanisms by which *F. gigantica* engages with the innate immune system
305 and establish an infection have not yet been precisely elucidated. In the present study, we
306 examined the levels of cytokines in the serum of experimentally infected buffaloes at 3, 10, 28
307 and 70 dpi. Diminished host immune cytokines observed at 3 dpi is probably a mechanism
308 utilized by *F. gigantica* to facilitate host colonization. While it could be argued that this
309 apparent reduction in cytokines could be attributed to abnormally high level of cytokines in
310 control animals, this state of cytokine reduction at early stage of *F. gigantica* infection has been
311 observed in previous reports (Girones et al., 2007; Rodriguez et al., 2015; Zhang et al., 2017a).
312 Also, abnormally high level of cytokines was limited to control animals at 3 dpi, which indicates
313 that this might have been caused by another underpinning condition/infection specific to this
314 group. In an effort to counter the infection, the host seems to restore the immunological balance,
315 which was evident at 10 dpi. By 28 dpi, there was a significant increase in the level of three
316 cytokines (TGF-β, IL-4 and IL-17) in infected buffaloes, suggesting activation of
317 Th1/Th2/Th17 immune response. The inflammatory cascades triggered by *F. gigantica* must
318 be tightly coordinated in order to avoid severe pathology. Therefore, this coordinated response
319 is perhaps mediated by the parasite and/or the host to promote the parasite survival, while

minimizing host tissue damage. By 70 dpi a state of immune homeostasis was observed, which is probably needed for parasite persistence.

Next, we analyzed alterations in the expression of genes involved in the recognition of and host defense against *F. gigantica* infection using custom RT^2 Profiler PCR Array. TLRs and NLRs function as sensors for innate immune response. They detect various pathogen-associated molecular patterns (PAMPs), followed by activation of signal transduction cascades via recruiting and interacting with adaptors and other molecules in order to thwart damage induced by the infecting pathogen (Medzhitov, 2001). There are several adaptor molecules for TLR signaling, such as MyD88, TRIF, TRIF-related adaptor molecule (TRAM), TIRAP, and TRAF6 whose expression is controlled at the transcriptional level. MyD88 and TICAM1 are the major adaptors that bind to the intracellular domain of TLR, leading to the induction of the proinflammatory response via the activation of NF-κB. TLR signaling can also lead to IRF-dependent induction of type I interferons.

At 3 dpi, we detected an increase in the expression of TICAM1 gene in the liver and upregulation of TICAM1, TLR4 and TLR8 genes in PBMCs. Alteration in the expression of TLR4 has been detected in *F. hepatica* infection (Martin et al., 2015; Noya et al., 2017). TICAM1 gene encodes an adaptor protein possessing a Toll/interleukin-1 receptor (TIR) homology domain, which interacts with TLR3 and mediates the induction of IFN-β through the activation of NF-κB (Hardy et al., 2004). TLR4 (CD284) participates in signal transduction events induced by lipopolysaccharide (LPS) of gram-negative bacteria (Neyen and Lemaitre, 2016). Bacterial lipid A, a component of LPS molecule is specifically recognized by TLR4 and TLR2 of various host cells, leading to the production of TNF-α, various interleukins, oxygen radicals, and bioactive lipids (Alexander et al., 2010; Janeway and Medzhitov, 2002).

A structural similarity has been detected between glycolipid AL-II of *F. hepatica* and the bacterial lipid A, which has been suggested to be the reason for the similar biological activity

345 of both molecules in terms of recognition by TLR4 and TLR2 (Wuhrer et al., 2003). Therefore,
346 it is reasonable to hypothesize that *F. gigantica*-specific PAMP is recognized by TLR4, as
347 indicated by the activation of TLR4 and the adaptor TICAM1. In turn, this activates
348 transcription factors, such as NF-κB and IRF3, leading to expression of cytokines, such as TNF-
349 α, IL-1β, IL-6, IL-10, and IFN-γ (Palsson-McDermott and O'Neill, 2004). IL-6 cytokine plays
350 a role in the inflammatory response and maturation of B cells (Hunter and Jones, 2015). It is
351 unclear why there was no correlation between TLR4 and TICAM1 expression levels and level
352 of cytokine release. It is possible that, *F. gigantica* induces upregulation of TLR4, but in the
353 interim, diminishes buffalo's responsiveness to TLR4 activation by downregulating
354 lipopolysaccharide binding protein (LBP), as previously reported (Zhang et al., 2017b). At this
355 point, we cannot conclude whether TICAM via activation of NF-κB or some other mechanisms
356 is involved in the cytokine production. A future goal of this work will be to examine this
357 question by determining if NF-κB-dependent regulation of cytokine production is altered when
358 the TICAM expression is induced. The increased expression of TLR8 (CD288) in PBMCs was
359 anticipated because TLR8 gene is dominantly expressed in peripheral blood leukocytes.
360 At 10 dpi, expression analysis revealed upregulation of four TLRs, (TLR1, TLR2, TLR6,
361 and TLR10) in the liver of infected buffaloes. TLR1 (CD281) gene is ubiquitously expressed
362 and at higher levels than other TLR genes. TLR2 (CD282) gene promotes apoptosis in response
363 to bacterial lipoproteins (Quillard et al., 2015). TLR6 (CD286) interacts with TLR2 to mediate
364 cellular response to bacterial lipoproteins (Noreen and Arshad, 2015). TLR10 (CD209) is
365 highly expressed in lymphoid tissues (Chuang and Ulevitch, 2001). The activation of these four
366 TLRs, by as yet unknown *F. gigantica*-specific PAMPs, must have caused up-regulation of
367 signaling pathways to modulate the host's inflammatory response.
368 The subsequent downstream signaling events that follow the activation of these TLRs
369 included the induction of Interferon Regulatory Factor (IRF) signaling as indicated by increased

expression of IRF3, interferon alpha 1 (IFNA1), and CD80. IRF3 gene encodes a protein of the IRF family and forms a complex with CREBBP (Zhao et al., 2016). This complex translocates to the nucleus and triggers the transcription of interferons alpha and beta, as well as other interferon-induced genes (Honda et al., 2006). The activated CD80 gene, encodes protein that induces T-cell proliferation and cytokine production indicating the engagement of adaptive immunity (Peach et al., 1995). As a member of the TNF receptor associated factor (TRAF) protein family, TRAF6 mediates signaling from members of the Toll/IL-1 family (e.g. CD40) and the TNF receptor superfamily (Walsh et al., 2015). Also, it plays significant roles as a signal transducer in NF-κB pathway which activates IkappaB kinase (IKK) in response to proinflammatory cytokines and interacts with transforming growth factor (TGF) beta receptor complex (Walsh et al., 2015). Also, the transcription regulator, NF-κB1, was upregulated, probably in response to cytokines, TRAF6 activation, oxidant-free radicals or parasite products. Once triggered NF-κB1 translocates into the nucleus and induces the expression of genes participating in a wide range of biological functions (Balan and Locke, 2011). TBK1 (TANK binding kinase) has IKB kinase-like activity (i.e. phosphorylates serine residues on the IKB proteins, marking them for destruction via the ubiquitination pathway). IKB proteins are known to inactivate NF-κB by trapping it in the cytoplasm. IRF3 and TBK1 play a key role in triggering IFN response via activation of IFN-β (Liu et al., 2015). IFN-α exerts a pro-inflammatory function, which contributes to immune-mediated liver damage (Leifeld et al., 2001). CASP1 (Caspase1) is a member of the NF-κB signaling pathway and is known to be involved in the programmed cell death (Winkler and Rosen-Wolff, 2015).

At 10 dpi, inflammatory response was evident in PBMCs as indicated by increased expression of CD86, NLRP3, NF-κB1, TICAM1, TLR4, TLR9, IL-1β, IL-6, IL-8. CD86 molecule is expressed by antigen-presenting cells and acts as a ligand for CD28 antigen for activation of T-cell and binds with cytotoxic T-lymphocyte-associated protein 4 to suppress T-

cell activation (Gu et al., 2012). NOD-like receptor (NLR)P3 inflammasome plays a role in the regulation of inflammation, immune response, and apoptosis, and is linked to NF-κB signaling (Boaru et al., 2015). TICAM1 induces IFN-β through activation of NF-κB. Increased expression of TLR4 (CD284) has been implicated in signal transduction events induced by LPS. Also, TLR9 (CD289) was upregulated and was predominantly expressed in PBMCs. IL-1β is a major mediator of the inflammatory response, and participates in a range of cellular activities, including cell proliferation, differentiation, and apoptosis (Netea et al., 2015). IL-6 cytokine plays a role in the inflammatory response and maturation of B cells (Hunter and Jones, 2015). IL-8, secreted by several cell types, mediates the inflammatory response, and functions as a chemoattractant and a potent angiogenic factor (Singh et al., 2013).

At 28 dpi, the majority of the examined genes were upregulated especially TLR3, TLR10, TRAF3, TICAM1, IFNA1, NLRP1, and NLRP3 in the liver and IL-10 in PBMCs. TRAF3, TNF receptor associated factor 3, functions in lymphotoxin-beta receptor signaling complex, which induces NF-κB activation and cell death (Hu et al., 2016). Interestingly, NF-κB activation induced the expression of the inflammasomes NLRP1 and NLRP3 (and their adaptor protein PYCARD), leading to activation of caspase-1 and subsequent processing of bioactive IL-1β (Srinivasula et al., 2002).

At 70 dpi, there was an increase in the expression of NLRP1 and NLRP3 inflammasomes, which induce caspase-1-dependent maturation of IL-1β (Fernandes-Alnemri et al., 2009; Halle et al., 2008). The activation of nucleotide-binding oligomerization domain 2 (NOD2) protein, which senses pathogens, may contribute to host defense via the production of pro-inflammatory cytokines (Keestra-Gounder et al., 2016). The activation of the inflammasome in parasitic diseases, such as malaria, leishmaniosis, trypanosomiasis, amoebiasis, naegleriasis, and toxoplasmosis, has been investigated (Zamboni and Lima-Junior, 2015). To our knowledge, this is the first documentation of the involvement of inflammasomes in *F. gigantica* infection

in buffaloes. We also detected activation of TLR7-and TLR9 genes, which along with increased gene expression of the surface marker CD40 can provide co-stimulatory signals for increased proliferation and activation of B cells, which is critical for the promotion of Th2 immune response. This pattern of Th1/Th2 immune response associated with late infection reflects the concomitant upregulation of the pro- and anti-inflammatory cytokines, B cells activation and the levels of pro- and anti-inflammatory gene expression.

In conclusion, the study resulted in several important findings. First, early infection induced modest activation of TLR4 and TLR8 and TICAM1. Second, at 10 dpi, NF-κB (TRAF6, NFKB1, TBK1, CASP1) and Interferon Regulatory Factor (IRF; IRF3, IFNA1, CD80) signaling pathways were upregulated in response to activation of TLR1, TLR2, TLR6, and TLR10 in the liver, and inflammatory response (IL-8, CD86, NLRP3, NFKB1, TICAM-1) and activated TLR4 and 9 in PBMCs. However, it is not yet possible to be sure that higher gene expression equates to increased cytokine production. Third, at 28 dpi, marked enhancement in the immune response was detected in both liver and PBMC samples, including inflammasome-dependent innate immune responses mediated by NLRP1 and NLRP3. Enhancement of the immune response correlated with increased levels of cytokines. Fourth, at 70 dpi, *F. gigantica* induced a local immune response in hepatic tissue mediated by TLRs and NLRs and their downstream interacting and adaptor molecules, which correlated with a systemic Th1/Th2 immune response. These data uncovered new immune signaling cascades and expression patterns of TLRs and NLRs during *F. gigantica* infection and should be important for understanding the interaction of *F. gigantica* with the innate immune system of buffaloes. Further investigation of how *F. gigantica* infection and these innate PRRs' signaling pathways intersect may provide new insights into the roles of the pathways involved, their constituent proteins, which ultimately may reveal new functions of these innate immunity pathways that could be manipulated therapeutically.

Conflict of interest

The authors declare that they have no potential conflicts of interest.

Acknowledgements

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632 **Figure Legends**
633

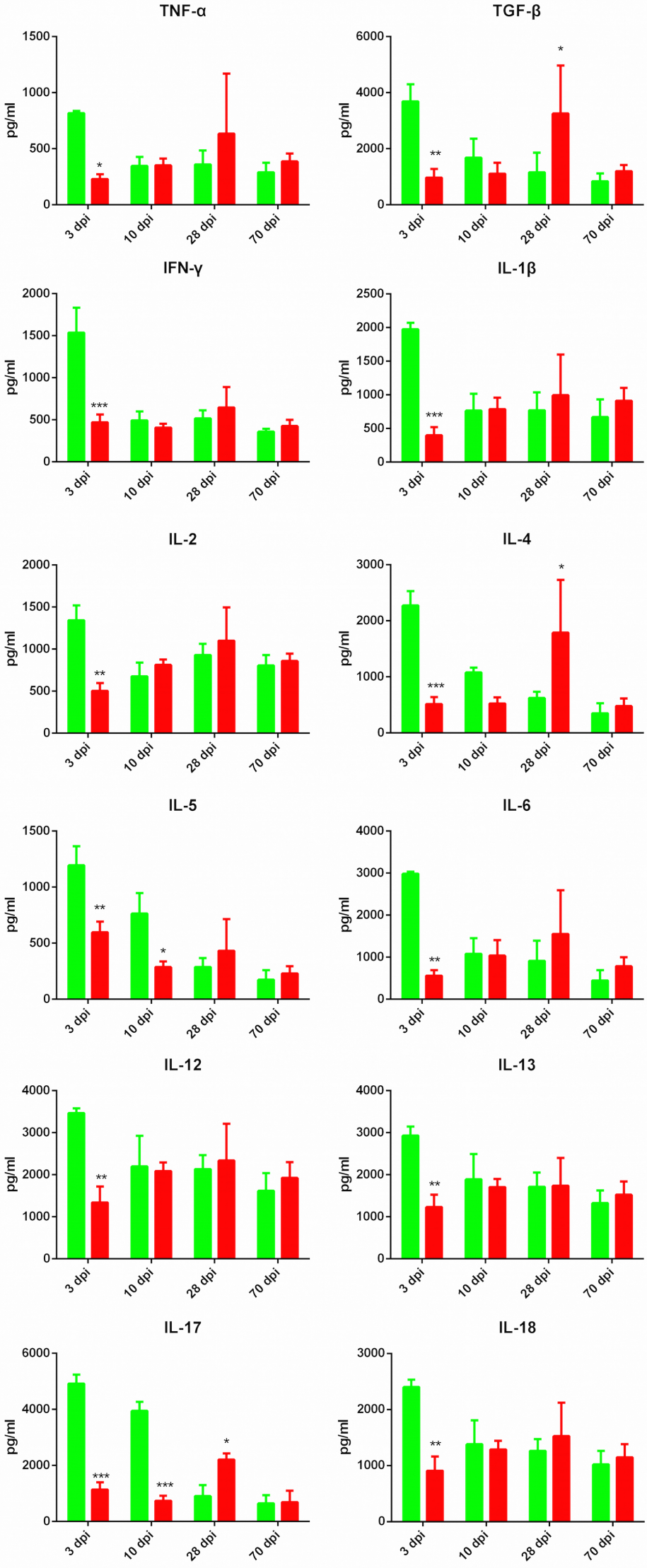
634 **Fig. 1.** The effect of *Fasciola gigantica* on the levels of the 12 indicated cytokines in the serum
635 of experimentally infected buffaloes. Red and green bars represent infected and control groups,
636 respectively. Data are expressed as means ± SD from three different animals (*, $P < 0.05$; **,
637 $P < 0.01$; ***, $P < 0.001$, compared with control, uninfected animals).
638

639 **Fig. 2.** Results of the qRT-PCR analysis showing the levels of expression of immunity-related
640 genes in the liver (A) and peripheral blood mononuclear cells (B) of buffaloes infected with
641 *Fasciola gigantica* at 3 dpi. Red and green colors represent upregulated (fold change > 1.5) and
642 downregulated (fold change ≤ 0.5) genes between infected and uninfected buffaloes,
643 respectively. Black bars represent genes whose expression was between 0.5 and 1.5 and were
644 considered non-differentially expressed. Expression levels were normalized against the
645 reference housekeeping gene *β-actin*.
646

647 **Fig. 3.** qRT-PCR results showing the levels of expression of immunity-related genes in the liver
648 (A) and peripheral blood mononuclear cells (B) of buffaloes infected with *Fasciola gigantica*
649 compared to uninfected buffaloes at 10 dpi. Red, up-regulation; green, down-regulation; black,
650 no change. Fold changes were computed for Ct values of amplified mRNA in comparison to
651 those of housekeeping gene *β-actin*.
652

653 **Fig. 4.** qRT-PCR results showing the levels of expression of immunity-related genes in the liver
654 (A) and peripheral blood mononuclear cells (B) of buffaloes infected with *Fasciola gigantica*
655 compared to uninfected buffaloes at 28 dpi. Red, up-regulation; green, down-regulation; black,
656 no change. Fold changes were computed for Ct values of amplified mRNA in comparison to
657 those of housekeeping gene *β-actin*.

Fig. 5. qRT-PCR results showing the levels of expression of immunity-related genes in the liver (A) and peripheral blood mononuclear cells (B) of buffaloes infected with *Fasciola gigantica* compared to uninfected buffaloes at 70 dpi. Red, up-regulation; green, down-regulation; black, no change. Fold changes were computed for Ct values of amplified mRNA in comparison to those of housekeeping gene *β-actin*.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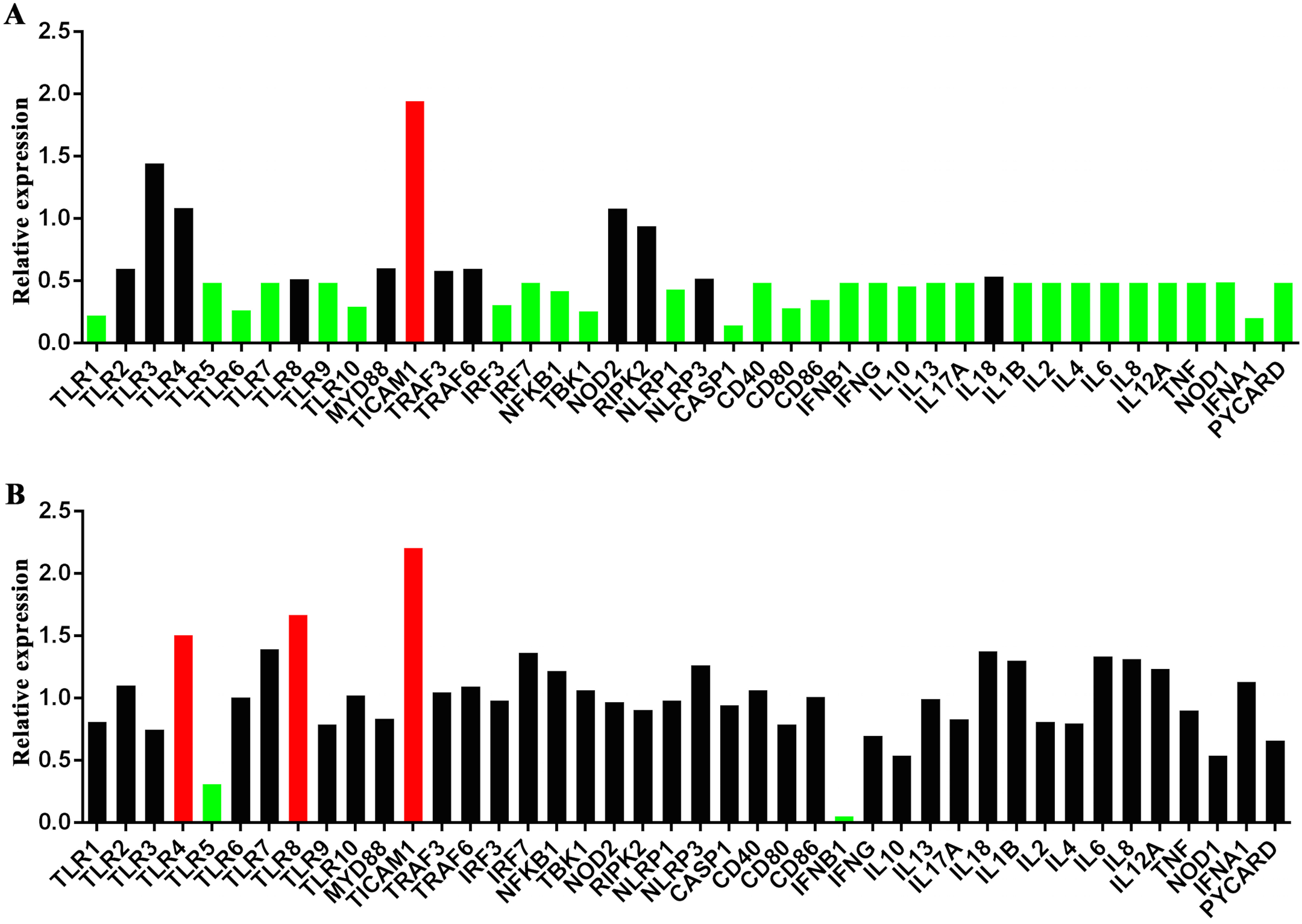
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IRF3
IRF7
NFKB1
TBK1
NOD2
RIPK2
NLRP1
NLRP3
CASP1
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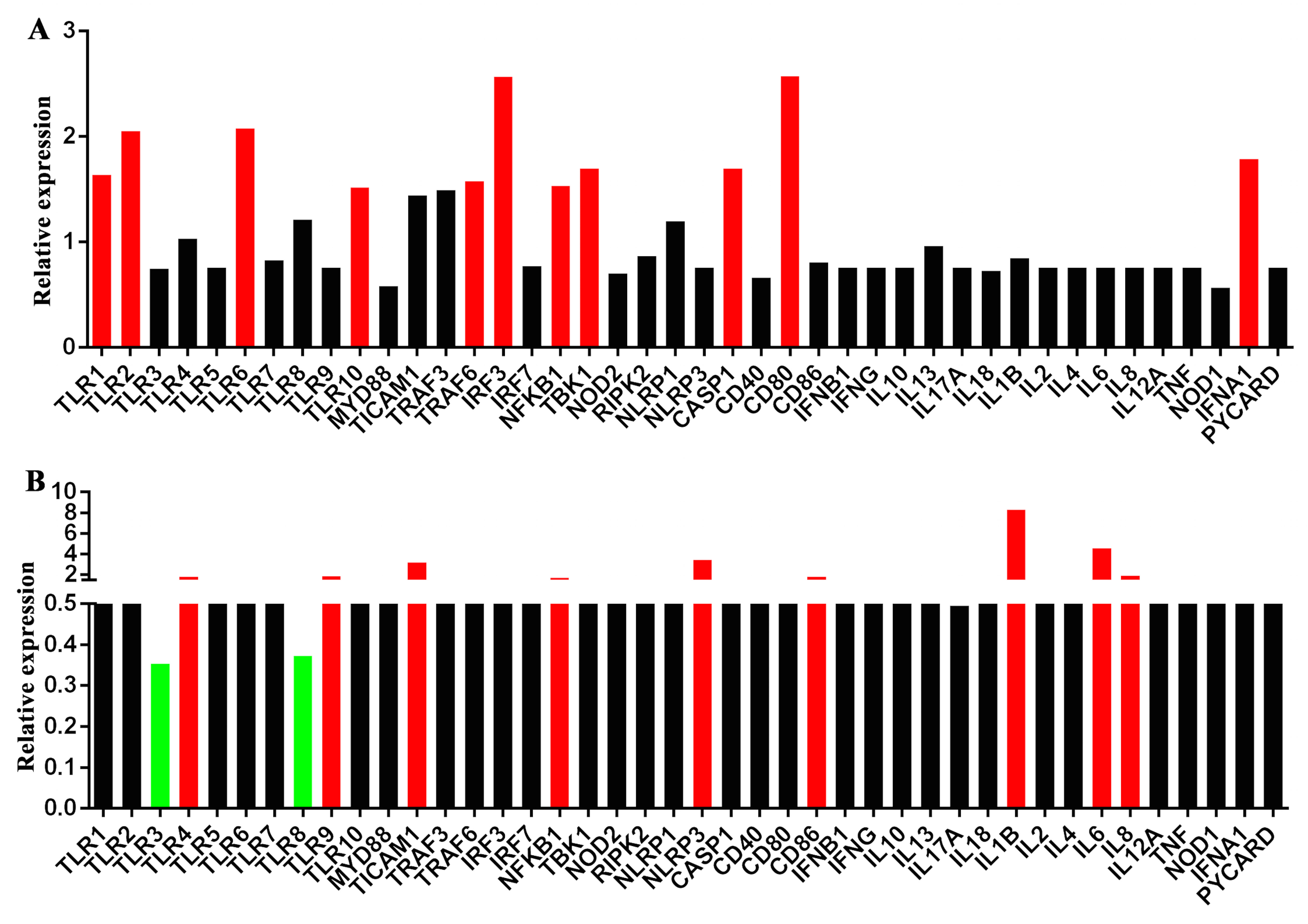
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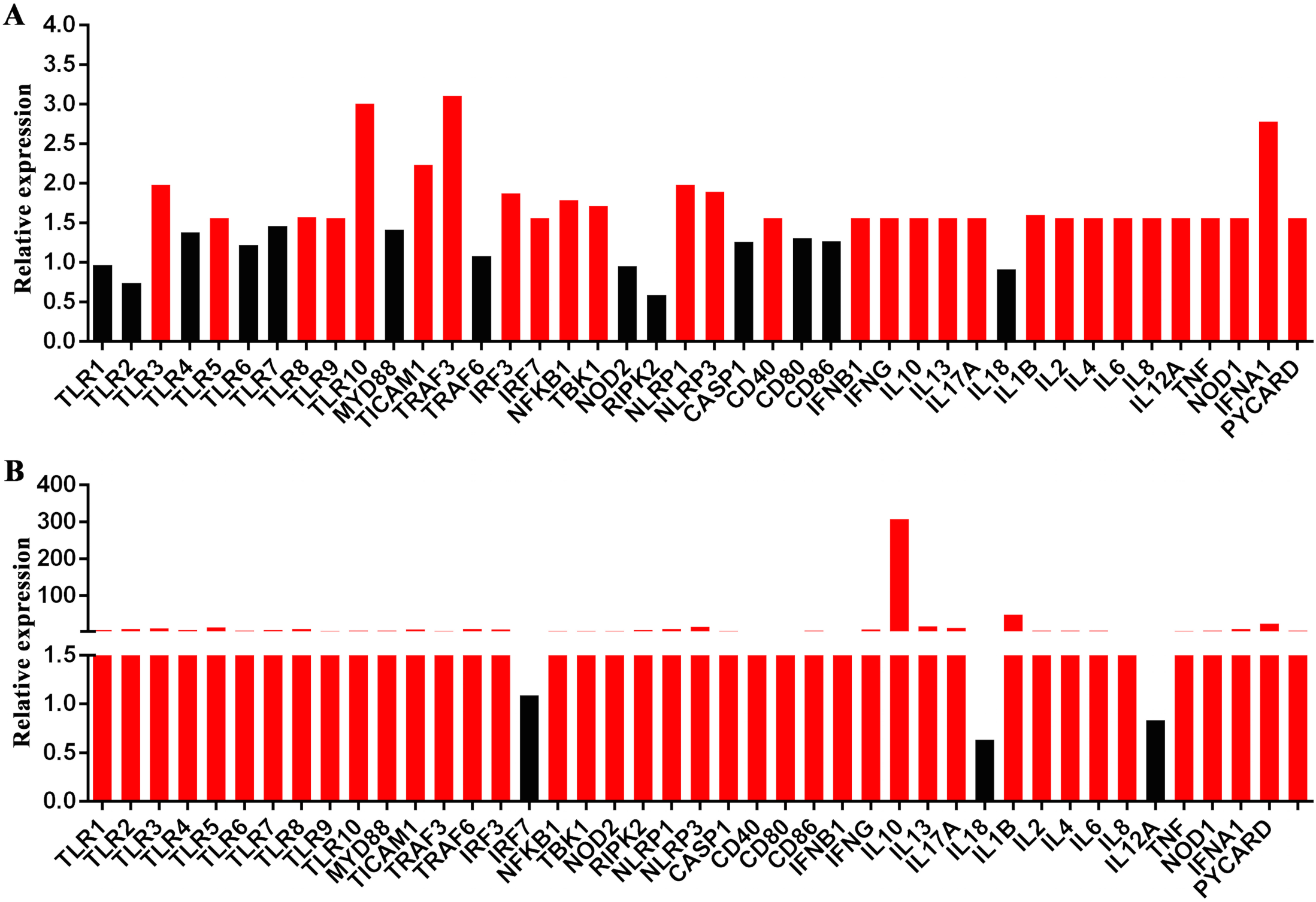
IFNA1
PYCARD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Relative expression
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3.0
2.5
2.0
1.5
1.0
0.5
0.0
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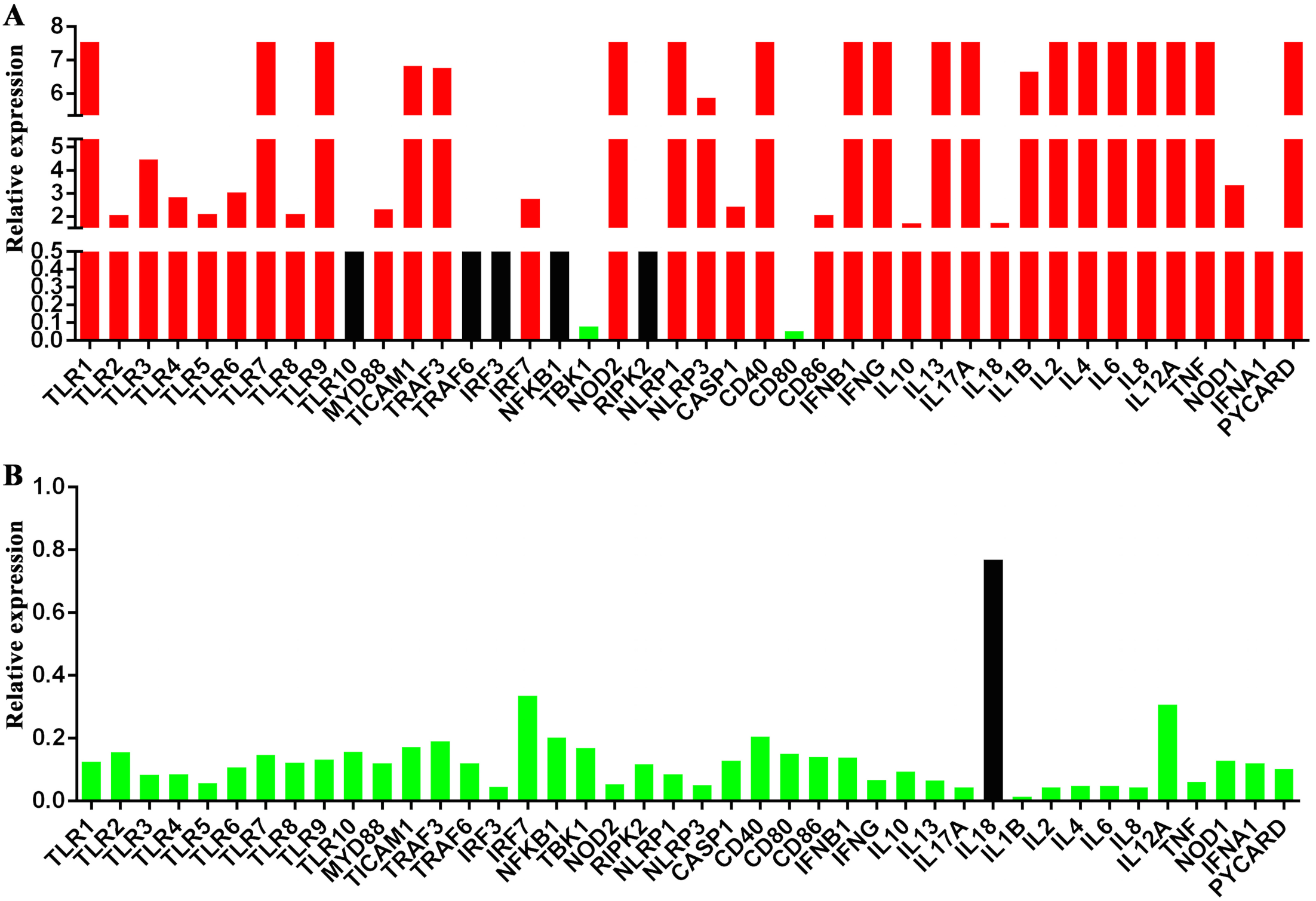
B
Relative expression
400
300
200
100
1.5
1.0
0.5
0.0
TLR1 TLR2 TLR3 TLR4 TLR5 TLR6 TLR7 TLR8 TLR9 TLR10 MYD88 TICAM1 TRAF3 TRAF6 IRF3 IRF7 NFKB1 TBK1 NOD2 RIPK2 NLRP1 NLRP3 CASP1 CD40 CD80 CD86 IFNB1 IFNG IL10 IL13 IL17A IL18 IL1B IL2 IL4 IL6 IL8 IL12A TNF NOD1 IFNA1 PYCARD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TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10
MYD88
TICAM1
TRAF3
TRAF6
IRF3
IRF7
NFKB1
TBK1
NOD2
RIPK2
NLRP1
NLRP3
CASP1
CD40
CD80
CD86
IFNB1
IFNG
IL10
IL13
IL17A
IL18
IL1B
IL2
IL4
IL6
IL8
IL12A
TNF
NOD1
IFNA1
PYCARD
B
Relative expression
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0.8
0.6
0.4
0.2
0.0
TLR1
TLR2
TLR3
TLR4
TLR5
TLR6
TLR7
TLR8
TLR9
TLR10
MYD88
TICAM1
TRAF3
TRAF6
IRF3
IRF7
NFKB1
TBK1
NOD2
RIPK2
NLRP1
NLRP3
CASP1
CD40
CD80
CD86
IFNB1
IFNG
IL10
IL13
IL17A
IL18
IL1B
IL2
IL4
IL6
IL8
IL12A
TNF
NOD1
IFNA1
PYCARD

Table S1

List of the 42 TLR and NLR-related genes whose expression profiles were investigated in the present study.

Gene Symbol *	Full Name of gene target
TLR1	Toll-like receptor 1
TLR2	Toll-like receptor 2
TLR3	Toll-like receptor 3
TLR4	Toll-like receptor 4
TLR5	Toll-like receptor 5
TLR6	Toll-like receptor 6
TLR7	Toll-like receptor 7
TLR8	Toll-like receptor 8
TLR9	Toll-like receptor 9
TLR10	Toll-like receptor 10
MYD88	Myeloid differentiation primary response gene (88)
TICAM1	Toll-like receptor adaptor molecule 1
TRAF3	TNF receptor-associated factor 3
TRAF6	TNF receptor-associated factor 6
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
TBK1	TANK-binding kinase 1
NOD2	Nucleotide-binding oligomerization domain containing 2
RIPK2	Receptor-interacting serine-threonine kinase 2
NLRP1	NLR family, pyrin domain containing 1
NLRP3	NLR family, pyrin domain containing 3
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CD40	CD40 molecule, TNF receptor superfamily member 5
CD80	CD80 molecule
CD86	CD86 molecule
IFNB1	Interferon, beta 1, fibroblast
IFNG	Interferon, gamma
IL10	Interleukin 10
IL13	Interleukin 13
IL17A	Interleukin 17A
IL18	Interleukin 18 (interferon-gamma-inducing factor)
IL1B	Interleukin 1, beta
IL2	Interleukin 2

IL4	Interleukin 4
IL6	Interleukin 6 (interferon, beta 2)
IL8	Interleukin 8
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)
TNF	Tumor necrosis factor
NOD1	Nucleotide-binding oligomerization domain containing 1
IFNA1	Interferon, alpha, leukocyte
PYCARD	PYD and CARD domain containing

* Expression levels of the above listed 42 genes were normalized against beta actin (ACTB) as the reference housekeeping gene.

Table S2. Quantitative real time PCR expression data for immunity-related genes in the liver of buffaloes infected with *Fasciola gigantica*.

Gene Symbol	Full Name of the Gene	3 DPI		10 DPI		28 DPI		70 DPI	
		Control	Infected	Control	Infected	Control	Infected	Control	Infected
TLR1	Toll-like receptor 1	5.81±3.54	7.42±3.97	8.22±0.96	7.87±0.31	8.17±1.59	7.94±1.85	8.67±0.96	5.79±0.61*
TLR2	Toll-like receptor 2	5.51±3.26	4.46±6.05	7.84±1.08	7.55±0.49	7.55±0.86	7.49±1.62	6.16±1.9	5.79±0.61
TLR3	Toll-like receptor 3	4.9±6.08	6.29±3.33	7.47±0.4	7.89±0.7	8.26±1.37	7.11±1.19	7.95±0.11	5.79±0.61*
TLR4	Toll-like receptor 4	5.85±2.6	6.05±3.29	7.15±1.6	7.1±0.4	8±1.22	7.2±1.59	7.3±1.04	5.79±0.61
TLR5	Toll-like receptor 5	7.25±3.96	6.33±6.61	8.59±1.22	8.99±1.02	7.76±1.23	7.71±1.71	6.22±1.67	5.79±0.61
TLR6	Toll-like receptor 6	6.28±3.14	7.26±3.83	7.85±1.09	6.92±0.23	7.97±1.13	7.52±1.59	7.4±0.89	5.79±0.61
TLR7	Toll-like receptor 7	4.46±8.57	3±12.34	8.56±1.19	8.56±1.56	7.77±1.23	7.32±1.62	8.71±1.03	5.79±0.61*
TLR8	Toll-like receptor 8	0.9±10.62	5.58±4.11	7.85±1	7.82±0.37	8.16±1.38	6.86±0.98	6.88±1.89	5.79±0.61
TLR9	Toll-like receptor 9	7.25±3.96	7.46±4.69	8.59±1.22	8.93±1.09	7.93±1.21	7.98±1.88	8.71±1.03	5.54±0.56*
TLR10	Toll-like receptor 10	5.41±3.53	6.52±3.29	7.77±1.14	7.47±0.87	8.55±1.7	6.71±0.79	5.29±4.14	5.76±0.58
MYD88	Myeloid differentiation primary response gene (88)	5.85±2.69	5.42±5.73	7.86±1.08	8.14±0.59	7.88±1.21	7.98±1.88	6.89±1.2	5.79±0.61
TICAM1	Toll-like receptor adaptor molecule 1	6.22±5.67	6.79±4.39	8.59±1.22	8.06±1.71	8.1±1.27	6.77±1.05	8.34±1.44	5.79±0.61*
TRAF3	TNF receptor-associated factor 3	7.18±3.97	6.48±5.18	8.22±0.96	8.01±0.95	8.23±1.35	7.27±1.34	8.55±1.08	5.79±0.61*
TRAF6	TNF receptor-associated factor 6	4.84±1.84	1.82±7.86	5.52±0.61	4.86±0.19	5.81±0.32	5.69±0.3	4.97±0.38	5.42±0.59
IRF3	Interferon regulatory factor 3	3.39±3.22	4.91±2.05	4.49±0.46	3.13±1.23	4.86±1.21	3.95±0.84	3.28±1.41	4.7±1.48
IRF7	Interferon regulatory factor 7	7.25±3.96	6.66±6.06	8.59±1.22	8.32±0.92	8.62±1.76	7.24±1.64	6.94±1.39	5.79±0.61
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	2.32±4.65	3.26±3.91	4.51±0.5	3.89±0.28	4.62±0.66	3.78±0.69	3.76±0.67	4.18±1.71
TBK1	TANK-binding kinase 1	0.8±4.5	3.86±0.95	3.13±0.78	2.36±0.55	4.17±0.74	3.39±1.06	2.19±0.32	5.32±0.7*
NOD2	Nucleotide-binding oligomerization domain containing 2	6.74±3.34	6.47±5.41	8.55±1.18	8.57±0.4	7.52±1.37	7.94±1.86	8.71±1.03	5.79±0.61*
RIPK2	Receptor-interacting serine-threonine kinase 2	4.51±1.4	4.59±1.53	5.08±1.65	5.28±0.27	5.44±0.8	5.57±0.49	5.11±1.2	5.79±0.61
NLRP1	NLR family, pyrin domain containing 1	5.26±7.06	7.5±4.06	8.45±1.33	8.19±0.73	8.23±1.35	7.18±1.2	8.64±1	5.79±0.61*
NLRP3	NLR family, pyrin domain containing 3	6.88±3.49	7.66±4.27	8.22±0.96	8.7±0.58	8.22±1.34	7.69±1.68	7.71±1.31	5.79±0.61
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	5.84±2.88	7.12±3.7	8±1.11	7.24±0.68	8.02±1.2	7.49±1.57	7.07±1.71	5.79±0.61
CD40	CD40 molecule, TNF receptor superfamily member 5	6.81±3.41	7.57±4.4	8.31±1.14	8.58±0.45	8.04±1.24	7.6±1.65	8.71±1.03	5.79±0.61*
CD80	CD80 molecule	5.46±4.97	7.26±4.01	7.94±0.94	6.84±0.75	7.97±1.18	7.23±1.24	1.63±1.06	5.79±0.61
CD86	CD86 molecule	5.05±3.53	6.08±3.86	7.72±1.13	8.02±0.41	7.93±1.17	6.95±1.07	6.84±1.07	5.79±0.61
IFNB1	Interferon, beta 1, fibroblast	7.25±3.96	7.5±4.63	8.59±1.22	8.99±1.02	8.62±1.76	7.98±1.88	8.71±1.03	5.79±0.61*
IL17A	Interleukin 17A	6.85±4.62	7.7±4.29	8.59±1.22	8.99±1.02	8.62±1.76	7.98±1.88	8.71±1.03	5.79±0.61*
IL18	Interleukin 18 (interferon-gamma-inducing factor)	3.69±1.64	3.56±3.27	4.62±0.65	5.08±0.53	6.06±1.3	6.19±0.66	6.14±0.23	5.35±0.55
IL2	Interleukin 2	7.25±3.96	6.98±5.51	8.59±1.22	8.99±1.02	8.62±1.76	7.94±1.86	8.71±1.03	5.79±0.61*
IL8	Interleukin 8	4.55±8.51	7.7±4.29	8.59±1.22	8.99±1.02	8.62±1.76	7.98±1.88	8.71±1.03	5.79±0.61*
TNF	Tumor necrosis factor	4.42±5.4	6.66±6.04	8.59±1.22	8.99±1.02	8.62±1.76	7.98±1.88	8.71±1.03	5.79±0.61*
NOD1	Nucleotide-binding oligomerization domain containing 1	5.74±2.56	6.31±3.84	7.94±0.73	8.62±0.47	8.26±1.38	7.9±1.82	7.47±0.57	5.79±0.61
IFNA1	Interferon, alpha, leukocyte	3.96±4.1	5.47±4.15	6.21±0.68	5.37±0.55	6.8±0.22	5.32±0.59	5.28±0.44	5.16±0.73
PYCARD	PYD and CARD domain containing	6.88±4.56	5.52±8	8.59±1.22	8.83±1.24	8.62±1.76	7.98±1.88	8.71±1.03	5.79±0.61*

* $P<0.05$ vs control. Data are the average ΔCt ± SD. $n = 3$ buffaloes per group. Expression levels were normalized against the reference housekeeping gene β-actin.

Table S3. Quantitative real time PCR expression data for immunity-related genes in the peripheral blood mononuclear cells of buffaloes infected with *Fasciola gigantica*.

Gene Symbol	Full Name of the Gene	3 DPI		10 DPI		28 DPI		70 DPI	
		Control	Infected	Control	Infected	Control	Infected	Control	Infected
TLR1	Toll-like receptor 1	3.78±3.68	4.08±2.6	5.88±3.45	6.32±1.63	6.76±0.23	4.19±5.37	3.76±3.06	6.74±1.82
TLR2	Toll-like receptor 2	3.55±3.03	3.41±1.45	5.54±3.1	5.01±1.51	7.38±0.65	4.5±4.95	3.57±2.75	6.25±0.32
TLR3	Toll-like receptor 3	4.5±3.98	4.81±3.68	6.47±4.19	7.34±2.71	7.94±0.14	4.48±4.89	3.96±3.47	7.84±2.48
TLR4	Toll-like receptor 4	3.06±2.47	2.46±0.47	4.81±2.82	3.97±1.2	6.07±0.46	3.58±3.64	3.01±1.74	6.25±0.74
TLR5	Toll-like receptor 5	4.94±3.9	6.56±2.44	6.77±4.76	7.6±2.44	7.94±0.14	4.13±1.94	4.75±2.78	9.54±3.69
TLR6	Toll-like receptor 6	4.64±3.68	4.63±2.99	6.13±3.61	6.57±2.42	6.62±0.43	4.49±4.95	3.92±3.41	7.13±2.22
TLR7	Toll-like receptor 7	5.49±3.13	5.32±2.96	7.22±3.82	7.79±2.2	7.89±0.2	5.41±4.9	4.97±2.63	8.37±2.73
TLR8	Toll-like receptor 8	5.04±3.51	4.6±3.03	6.56±4.25	7.42±2.59	7.66±0.25	4.63±5.15	4.66±3.03	8.33±1.76
TLR9	Toll-like receptor 9	5.45±3.17	5.79±3.02	7.24±2.9	7.01±1.66	7.94±0.14	5.5±5.09	5.19±2.33	7.8±1.85
TLR10	Toll-like receptor 10	4.63±3.89	4.9±3.44	6.28±3.93	6.76±2.59	6.7±0.09	4.49±4.95	4±3.43	6.99±2.33
MYD88	Myeloid differentiation primary response gene (88)	4.72±2.71	4.98±2.25	5.7±2.63	6.05±1.07	6.76±0.04	4.29±3.25	4.25±2.25	7.29±1.19
TICAM1	Toll-like receptor adaptor molecule 1	7.26±1.62	6.45±2.35	7.87±2.96	7.2±2.58	7.94±0.14	4.99±4.93	6.35±1.48	8.51±1.91
TRAF3	TNF receptor-associated factor 3	4.7±2.09	4.63±1.48	5.5±2.01	5.06±1.08	6.43±0.72	4.51±3.53	4.29±1.45	6.68±1.64
TRAF6	TNF receptor-associated factor 6	4.39±3.17	4.26±1.51	5.37±2.97	5.23±1.36	7.22±0.07	4±4.24	3.52±2.06	6.58±1.44
IRF3	Interferon regulatory factor 3	5.13±3.43	5.17±3.54	6.92±4.43	7.31±2.85	7.89±0.07	5.03±5.6	4.32±3.2	8.61±2.12
IRF7	Interferon regulatory factor 7	8.16±1.06	8.13±1.49	8.71±1.05	8.56±1.29	7.94±0.14	7.15±2.75	7.33±0.54	8.64±2.13
NFKB1	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1	3.12±1.64	2.83±0.97	4.03±1.78	3.24±1.42	4.86±0.11	3.34±3.75	2.62±1.07	4.92±1.19
TBK1	TANK-binding kinase 1	3.19±2.48	3.1±1.74	4.38±2.64	4.24±1.67	5.7±0.33	3.82±4.47	3.28±2.24	5.84±1.58
NOD2	Nucleotide-binding oligomerization domain containing 2	4.57±3.89	4.62±2.99	6.57±4.03	7.01±2.28	6.96±0.03	4.94±5.73	4.1±3.35	7.99±1.96
RIPK2	Receptor-interacting serine-threonine kinase 2	3.72±2.06	3.85±1.85	4.87±2.51	4.47±0.96	6.04±0.43	3.49±3.53	2.96±1.65	6.04±1.28
NLRP1	NLR family, pyrin domain containing 1	4.62±3.55	4.64±3.03	6.71±4.19	6.99±2.93	7.42±0.87	4.49±4.95	4.21±3.27	8.31±2.51
NLRP3	NLR family, pyrin domain containing 3	3.69±2.74	3.35±1.45	6.02±3.54	4.22±1.57	7.5±0.49	4.02±4.27	3.39±2.14	7.03±0.38
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)	4.46±3.16	4.54±1.75	6.4±3.33	5.82±1.17	7.18±0.61	5.24±4.59	4.62±2.82	7.57±1.45
CD40	CD40 molecule, TNF receptor superfamily member 5	3.96±2.66	3.87±1.81	5.21±2.89	5.02±1.09	5.09±0.67	3.99±4.25	3.47±2.55	5.75±1.84
CD80	CD80 molecule	4.44±3.14	4.78±1.93	4.97±2.71	5.22±1.33	5.84±0.15	4.68±4.62	3.91±1.69	6.64±2
CD86	CD86 molecule	4.06±2.6	4.04±1.82	6.08±3.55	5.23±1.37	6.85±0.2	4.5±4.95	3.6±2.08	6.42±1.34
IFNB1	Interferon, beta 1, fibroblast	7.19±3.52	9.58±1.01	7.7±3.23	8.34±1.68	7.56±0.66	5.6±4.94	6.69±0.88	9.54±3.69
IFNG	Interferon, gamma	4.06±3.92	4.57±3.01	6.67±4.67	7.05±3.07	7.76±0.12	4.91±5.55	3.98±3.45	8.32±2.58
IL10	Interleukin 10	4.99±3.58	6.51±3.69	5.99±3.08	5.64±1.31	7.94±0.14	-0.33±3.83	5.28±2.23	7.76±0.67
IL13	Interleukin 13	4.63±3.77	4.64±3	6.9±4.54	7.09±3.13	7.89±0.07	3.93±4.54	4±3.44	8.11±2.24
IL17A	Interleukin 17A	4.61±3.91	4.96±3.54	6.91±4.43	7.28±2.89	7.94±0.14	4.43±5.03	4.29±3.21	8.7±2.33
IL18	Interleukin 18 (interferon-gamma-inducing factor)	9.51±0.52	9.41±0.92	9.1±1.43	8.74±1.58	7.94±0.14	8.6±0.7	8.43±0.59	8.5±1.9
IL1B	Interleukin 1, beta	1.22±1.19	0.84±1.06	4.09±2.03	1.04±1.08	7.9±0.08	2.37±2.08	-0.24±2.34	5.14±3
IL2	Interleukin 2	4.81±3.77	5.2±3.57	6.91±4.45	7.22±2.97	7.94±0.14	5.18±5.53	4.11±3.34	8.75±2.4
IL4	Interleukin 4	5.01±3.56	5.34±3.42	6.95±4.46	7.45±2.65	7.94±0.14	5.16±5.57	4.43±3.07	9.12±3.11
IL6	Interleukin 6 (interferon, beta 2)	4.27±3.06	3.85±1.6	6.95±4.46	5.52±1.83	7.9±0.08	5.18±5.54	3.78±2.29	7.5±0.35
IL8	Interleukin 8	6.72±1.61	6.32±0.61	7.58±2.42	6.95±1.11	7.94±0.14	6.54±2.33	4.52±1.69	7.65±0.51
IL12A	Interleukin 12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1, p35)	8.79±0.8	8.48±1.25	8.72±1.73	8.82±1.6	7.94±0.14	7.66±2.02	7.36±1.11	9.49±3.6
TNF	Tumor necrosis factor	6.17±2.08	6.31±1.47	7.95±2.83	8.43±1.56	7.94±0.14	6.17±3.03	5.49±1.31	8.38±1.69
NOD1	Nucleotide-binding oligomerization domain containing 1	6.28±2.48	6.85±2.25	7.67±3.28	8.56±1.59	7.94±0.14	5.88±3.52	5.92±1.77	8.51±1.91
IFNA1	Interferon, alpha, leukocyte	4.5±3.93	4.32±2.42	6.71±4.19	6.85±2.84	7.51±0.32	4.43±4.86	4.1±3.39	7.45±1.95
PYCARD	PYD and CARD domain containing	4.48±4	5.07±3.57	6.95±4.47	7.3±2.85	7.9±0.08	3.42±3.43	4.38±3.13	8.35±2.89

* Data are the average ΔCt ± SD. n = 3 buffaloes per group. Expression levels were normalized against the reference housekeeping gene β-actin.