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Expression patterns of innate immunity-related genes in response to polyinosinic:polycytidylic acid (poly[I:C]) stimulation in DF-1 chicken fibroblast cells

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Abstract

Polyinosinic:polycytidylic acid (poly[I:C]) can stimulate Toll-like receptor 3 (TLR3) signaling pathways. In this study, DF-1 cells were treated with poly(I:C) at various concentrations and time points to examine the comparative expression patterns of innate immune response genes. The viability of DF-1 cells decreased from 77.41% to 38.68% when cells were treated different dose of poly(I:C) from 0.1 μg/mL to 100 μg/mL for 24 h respectively. The expressions of *TLR3, TLR4, TLR7, TLR15, TLR21, IL1B*, and *IL10* were increased in dose- and time-dependent manners by poly(I:C) treatment. On the contrary, the expression patterns of *interferon regulatory factors 7* (*IRF7*), *Jun proto-oncogene*, *AP-1 transcription factor subunit* (*JUN*), *Nuclear Factor Kappa B Subunit 1* (*NF-κB1*), and *IL8L2* were varied; *IRF7* and *IL8L2* were increasingly expressed whereas the expressions of *JUN* and *NF-κB1* were decreased in a dose-dependent manner after they were early induced. In time-dependent analysis, *IRF7* expression was significantly upregulated from 3 h to 24 h, whereas *JUN* and *NF-κB1* expressions settled down from 6 h to 24 h after poly(I:C) treatment although they were induced at early time from 1 h to 3 h. Poly(I:C) treatment rapidly increased the expression of *IL8L2* from 3 h to 6 h with a plateau at 6 h and then the expression of *IL8L2* was dramatically decreased until 24 h after poly(I:C) treatment although the expression level was still higher than the non-treated control. These results may provide the basis for understanding host response to viral infection and its mimicry system in chickens.

Keywords: Poly(I:C), TLR, Immune response, Chicken, Gene expression

INTRODUCTION

Innate immunity is the first defense line against various pathogens through sensing pathogens, eliminating them, and activating adaptive immune response [1]. In sensing pathogens, nucleic acids (NAs) that are originated from pathogenic bacteria and viruses are recognized by innate immune receptor signaling, which are mediated by pattern recognition receptors (PRRs) including toll-like receptors (TLRs),

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Methodology: Jang HJ.
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Writing - original draft: Jang HJ.
Writing - review & editing: Song KD.

Ethics approval and consent to participate
This manuscript does not require IRB/IACUC approval because there are no human and animal participants.

retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) [2,3]. Among them, TLR3, TLR7/8, TLR9, and TLR13 of TLRs are known as nucleic acid NA-sensing TLRs. They primarily exist in endosome and respond to double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), single-stranded DNA, and bacterial ribosomal RNA respectively [4,5]. RIG-I, MDA5, and LGP2 are cytosolic NA receptors which detect dsRNA. RIG-I primarily responds to 5'-triphosphorylated blunt-ended RNA or dsRNA produced during RNA virus infections and MDA5 responds to long dsRNA [2]. LGP2 also seems to enhance initial MDA5-RNA interaction [6]. Complex with cognate PRRs and their ligands leads to the engagements of myeloid differentiation primary response 88 (MYD88), Toll/IL-1R homologous region (TIR) domain-containing adapter-inducing interferon-β (TRIF), or mitochondrial antiviral-signaling protein (MAVS). It activates transcription factors (TFs) such as interferon regulatory factor3 (IRF3), IRF7, nuclear factor kappa B (NF-κB), and activating protein 1 (AP-1) (ATF2/JUN) by orchestrating a combination of multi-protein complexes. The TFs induce to express inflammatory cytokines, chemokines and type I interferons [7–12].

Among the NA-sensing TLRs, chickens have obvious orthologues of *TLR3* and *TLR7* while *TLR8* has been disrupted by the insertion of a large CR1 repeat [13]. *TLR9* and *TLR13* were also absent [8,14]. In addition, *TLR15* and *TLR21* uniquely existed in chickens compared to human and mouse [15,16]. Chicken *TLR21* has recently been shown to recognize CpG motifs, suggesting a functional homologue to mammalian *TLR9* [17] whereas an virus-related agonist for *TLR15* remains unknown [18,19]. *MDA5* and *LGP2* are also present in chicken genome and their function seems to be similar to mammals whereas *RIG-I* is obviously absent [20,21]. It has been suggested that the lack of *RIG-I* caused a susceptibility for zoonotic RNA virus such as avian influenza in chickens [22]. Even if immune responses to NA have been comparatively well characterized in chickens, the precise mechanism remains to be elucidated.

Polyinosinic:polycytidylic acid (poly[I:C]), viral like dsRNA, has generally been used to mimic NA-sensing responses of the innate immune system. Poly(I:C) is recognized by TLR3 and MDA-5, activate various TFs such as IRFs and NF-κB, and stimulates various cytokines and chemokine, IFNs and costimulatory factors in various species [10,11,23–26]. Poly(I:C) exhibited a toxicity in various tissues and cells [27,28]. Especially, the viability of chicken embryonic fibroblasts (CEFs) reduced to about 80% and below 50% with 1,000 μg/mL of poly(I:C) for 24 h and 72 h respectively and it suggested that poly(I:C) induced apoptosis of CEFs through the activation of caspase-3 and -8 by TNFRSF8 [29]. In addition, DF-1 cells, chicken fibroblast cell line modulated IRF7-related immune signaling pathways responding to poly(I:C) [30]. In this regard, chicken fibroblasts including DF-1 are a useful model to study *in vitro* immune responses which are stimulated by poly(I:C).

In this study, we examined the expression patterns of innate immune signaling-related genes such as canonical and non-canonical TLRs, the related TFs, cytokines, and immune-related effector molecules in chickens after poly(I:C) treatment. Our results could contribute to understanding the gene expression which is involved in NA-sensing and the related responses in chicken cells.

MATERIALS AND METHODS

Cell culture and poly(I:C) treatment

DF-1 chicken fibroblast cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA) and maintained in the Dulbecco's modified Eagle's medium with 10% fetal bovine serum (Biowest, Nuaillé, France). DF-1 cells were cultured at 37℃ in 5% CO_2 incubator. Poly(I:C) was purchased from Invivogen (San Diego, CA, USA) and was stocked according to the manufacturer's instruction and all poly(I:C) treatment was maintained under the culture condition

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of DF-1 cells.

Cell viability assay

Cell viability assays were performed using tetrazolium compound based CellTiter 96® AQueous One Solution Cell Proliferation (MTS) assay (Promega, Madison, WI, USA). MTS assay was then performed according to the manufacturer's instruction at 24 h after treatment at indicated concentrations of poly(I:C).

RNA extraction and quantitative RT-PCR

RNAs were isolated from DF-1 cells using RNA extraction kit (Invitrogen, CA, USA). For quantitative reverse transcription-polymerase chain reaction (qRT-PCR), 1 μg of total RNA was used for cDNA synthesis with Rever Tra Ace- α - first strand cDNA Synthesis Kit (Toyobo, Osaka, Japan). Sequence-specific primers (Table 1) were designed using the Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome). qRT- PCR was performed using the iCycler real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and SYBR Green (Bio-Rad, Hercules, CA, USA). Non-template wells without cDNA were included as negative controls. Each sample was tested in triplicate. The PCR conditions were 95℃ for 3 min, followed by 40 cycles at 95℃ for 10 s and 60℃ for 30 s, using a melting curve program (increasing temperature from 65℃ to 95℃ at a rate of 0.5℃ per 5 s) and continuous fluorescence measure-

Table 1. Lists of primers used to perform qRT-PCR

Target gene (accession number of NCBI)	Primer type 5' to 3'	Sequence
TLR3 (422720)	Forward	CCATTTTGAAGGGTGGAGAA
	Reverse	CCTGCTTCGAAGTCTCGTTC
TLR4 (417241)	Forward	TTCCAAGCACCAGATAGCAACATC
	Reverse	ACGGGTCACAGAAGAACTTAGGG
TLR7 (418638)	Forward	TTCTGGCCACAGATGTGACC
	Reverse	CCTTCAACTTGGCAGTGCAG
TLR15 (421219)	Forward	GTTCTCTCTCCCAGTTTTGTAAATAGC
	Reverse	GTGGTTCATTGGTTGTTTTTAGGAC
TLR21 (415623)	Forward	CAACAGACTGCTGGAGGTGA
	Reverse	TGCAGCTTCAGGTCGTACAG
IRF7 (396330)	Forward	GAGGATCCGGCCAAATGGAA
	Reverse	CCAAATCGTGGTGGTTGAGC
JUN (424673)	Forward	CCCGGTGTATGCCAATCTCA
	Reverse	CTCCTGCGACTCCATGTCAA
NF-κB1 (395587)	Forward	AGAAAAGCTGGGTCTTGGCA
	Reverse	CCATCTGTGTCAAAGCAGCG
IL1B (395196)	Forward	GGATTCTGAGCACACCACAGT
	Reverse	TCTGGTTGATGTCGAAGATGTC
IL8L2 (396495)	Forward	CCAAGCACACCTCTCTTCCA
	Reverse	GCAAGGTAGGACGCTGGTAA
IL10 (428264)	Forward	AGCAGATCAAGGAGACGTTC
	Reverse	ATCAGCAGGTACTCCTCGAT
GAPDH (374193)	Forward	TGCTGCCCAGAACATCATCC
	Reverse	ACGGCAGGTCAGGTCAACAA

ment. The qRT-PCR data were normalized relative to the expression of *GAPDH* and calculated using the 2 ΔΔCt method, where ΔΔCt = (Ct of the target gene – Ct of *GAPDH*) treatment – (Ct of the target gene – Ct of *GAPDH*) control [31].

Statistical analysis

Statistical significance ($p < 0.05$, $p < 0.01$, $p < 0.001$) of apparent differences in gene expression after poly(I:C) treatment was assessed by ANOVA and Tukey's multiple comparison test (GraphPad Prism 5.01, San Diego, CA, USA).

RESULTS AND DISCUSSION

Viability test of DF-1 cells in various concentrations of poly(I:C)

In this study, poly(I:C) treatment with different doses from 0.1 μg/mL to 100 μg/mL for 24 h decreased the viability of DF-1 cells (chicken fibroblasts cell line) by 77.41%, 57.63%, 56.28%, 46.69%, 43.06%, 43.19%, 44.22%, 43.32%, 38.9%, 39.19%, 38.25%, 38.1%, 36.85%, 37.73%, 38.42%, 37.17%, and 38.68% respectively, compared to the non-treated control. The statistical analysis showed significant difference at all the treated concentrations except the concentration of 0.1 μg/mL, compared to the non-treated control and no difference among the cell viabilities from 0.5 μg/mL to 100 μg/mL poly(I:C) ($p < 0.05$) (Fig. 1). These results suggested that poly(I:C) rapidly affected on the cell viability from 0.5 μg/mL and this effect was saturated from 0.5 μg/mL to 100 μg/mL. Thus, we supposed that DF-1 cells could be much more sensitive to poly(I:C) than primary cultured CEFs.

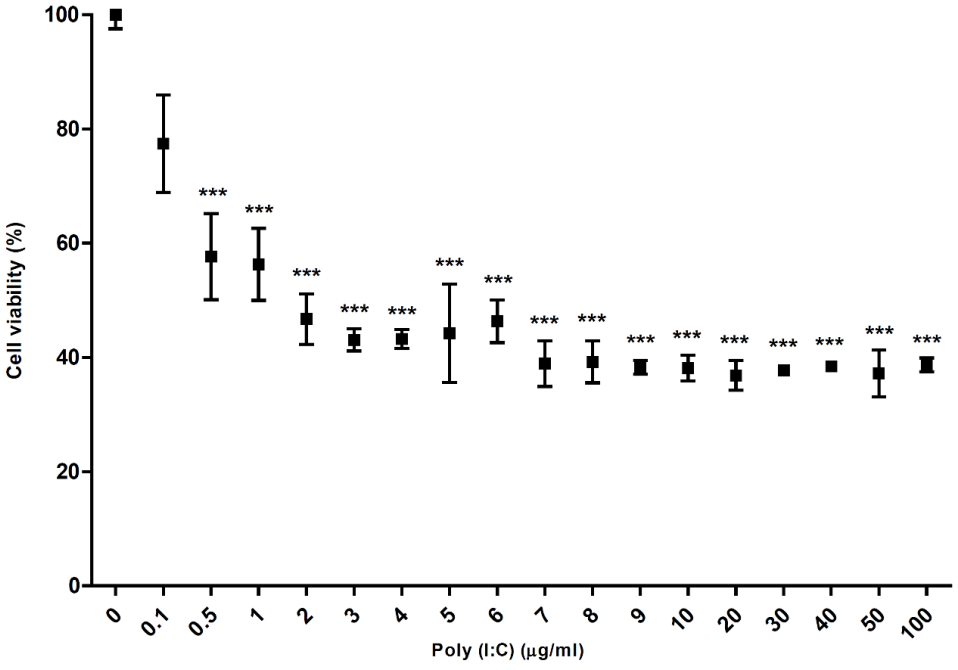

Fig. 1. The viability and morphology of DF-1 cells in the poly(I:C)-treated conditions with various concentrations of poly(I:C) for 24 h. The statistical analysis was performed to assess statistical significance between each concentration and the non-treated control. Error bars were expressed as SEM $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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Dose- and time-dependent expression patterns of TLRs by poly(I:C) treatment

TLR3 and TLR7 are known as NA-sensing TLRs while the function of TLR4 is associated with the recognition of endotoxins molecules, in particular lipopolysaccharide from gram-negative bacteria [13,32]. Recently, the several studies have shown that TLR3, 4, and 7 mediated the responses to the viral-associated PAMPs such as poly(I:C), F protein of Respiratory Syncytial Virus (RSV), and imidazoquinolines, antiviral therapeutic compounds, respectively [33–38]. In addition, it has been reported that selective activation of TLR3/4-IRF3 pathway was associated with potential inhibition of viral replication [39]. *TLR15*, an avian-specific TLR, has been reported to be induced by salmonella, mycoplasma, and even Marek's Disease Virus (MDV) [16,18,19,40]; however, the specifically virus-associated agonist was still unknown [41]. Instead of mammalian *TLR9* which was missing from the chicken genome, chicken TLR21 acted as a functional homologue to the mammalian TLR9 to recognize CpG [17]. Poly(I:C) and CpG ODN (CpG-motif containing oligodeoxydinucleotide) synergized the expression of pro-inflammatory cytokines and chemokines and the production of nitric oxide in chicken monocytes [42,43].

To investigate chicken TLRs expressions in response to poly(I:C) treatment, the expressions of chicken TLRs were analyzed dose and time-dependently. From the analysis, the expressions of *TLR3, 4, 7, 15*, and *21* were significantly induced at the poly(I:C) concentrations of 5 μg/mL and 10 μg/mL for 24 h (Fig. 2A). In addition, the expression levels of *TLR3, 4, 7, 15*, and *21* were significantly increased with 10 μg/mL poly(I:C) at 12 h and 24 h after poly(I:C) treatment (Fig. 2B). Therefore, we suggested that poly(I:C) was directly targeted at these TLRs in DF-1 cells to stimulate immune responses.

Dose- and time-dependent expression patterns of TLR signaling-associated transcription factors (TFs) by poly(I:C) treatment

TLRs which recognize their ligands activated conserved TFs including AP-1, NF-κB, and IRFs through the interplay of complex TLR signaling pathways [44–47]. Among the AP-1 family, JUN that was a target protein of c-Jun N-terminal kinase (JNK) was regarded as a key factor in TLR signaling [47]. Among NF-κB protein complex, NF-κB1 (also known as p50) was known to have DNA binding activity for the promoter region of its target genes [48]. Among IRFs, IRF3 and IRF7 were activated by various ligands, such as poly(I:C), LPS, and virus infection and mainly controlled type-I IFN expression [49]. In mammalian, type I IFNs-mediated signaling pathways were dependent on the stimulus and the responding cell types. TLR signaling pathways associated with type I IFN, TLR3 and TLR4 induced type I IFN production in various cell types in a manner dependent on TIR-domain-containing adaptor protein inducing IFN β (TRIF) whereas TLR7, TLR8 and TLR9 induced type I IFN production in dendritic cells via a pathway dependent on MYD88. Eventually they can activate some common signaling molecules including TNF receptor-associated factor 3 (TRAF3) and IRF3 and IRF7 [49,50]. Additionally, poly(I:C) treatment increased *IRF7* and *type-I IFN* (*IFNA*) in DF-1 cells [25].

To reveal TFs which are associated with TLR signaling responded to poly(I:C), the expressions of *IRF7*, *JUN*, and *NF-κB1* were analyzed in DF-1 cells at different doses of poly(I:C) and time points. From the dose-dependent treatment, *IRF7* and *NF-κB1* expressions were significantly increased at 5 μg/mL and 10 μg/mL and 5 μg/mL of the poly(I:C) treatment for 24 h, respectively. Whereas the expression of *JUN* was significantly decreased at 1 μg/mL, 5 μg/mL, and 10 μg/mL of poly(I:C) for 24 h (Fig. 3A). When the expressions of *IRF7*, *JUN*, and *NF-κB1* were analyzed with 10 μg/mL poly(I:C) according to time course, the expression of *IRF7* steadily increased from 3 h to 24 h after poly(I:C) treatment. *JUN* and *NF-κB1* expressions were commonly increased from 1 h to 3 h after poly(I:C) treatment, but were decreased from 6 h to 24 h after poly(I:C) treatment

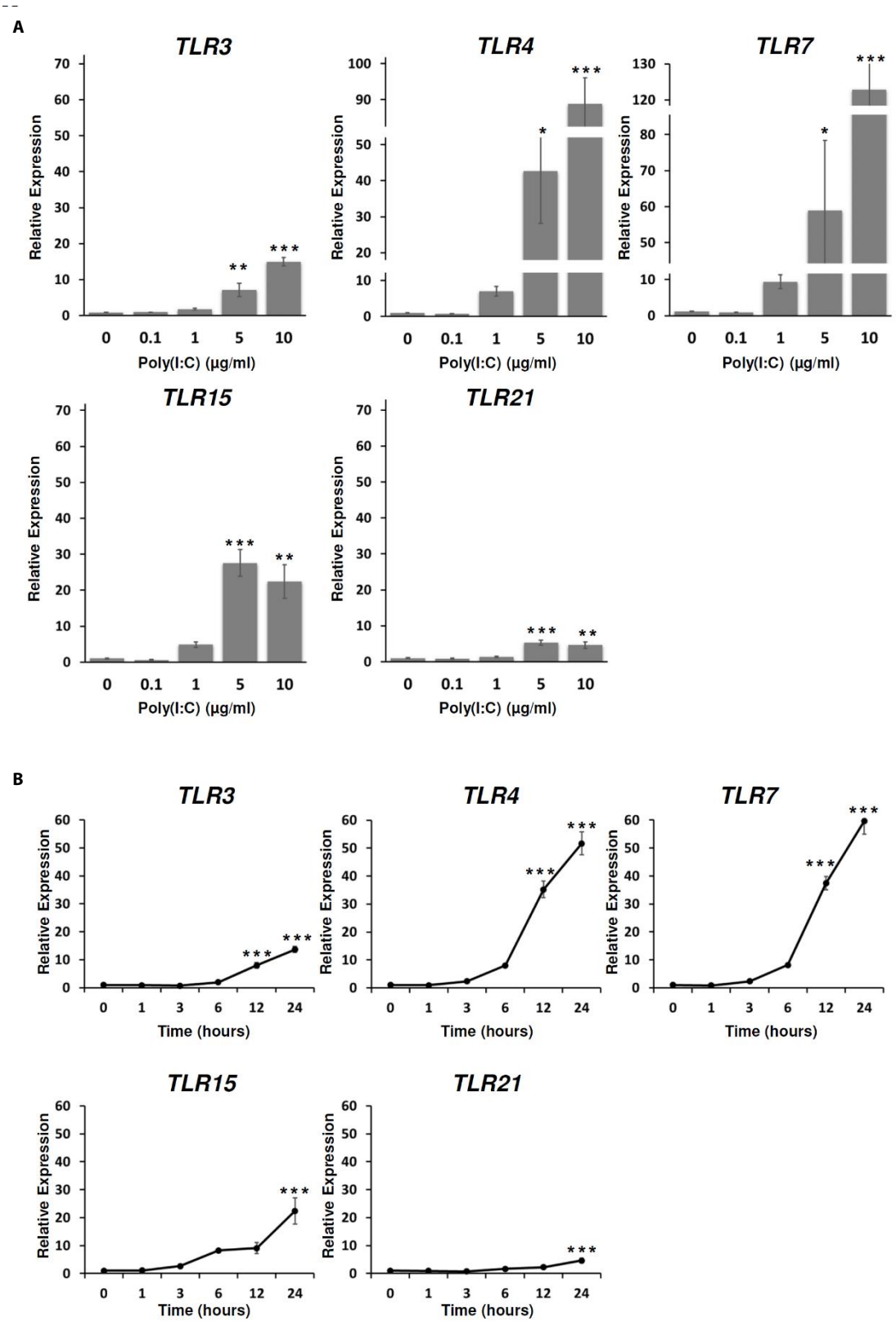

Fig. 2. Dose- and time-dependent expression patterns of TLRs by poly(I:C) treatment. The expressions of *TLR3, 4, 7, 15*, and *21* in DF-1 cells were analyzed in poly(I:C)-treated conditions with concentrations of 0, 0.1, 1, 5, and 10 μg/mL for 24 h (A) and with concentration of 10 μg/mL for 1, 3, 6, 12, and 24 h (B). The statistical analysis was performed to assess statistical significance between each treated condition and the non-treated control. Error bars were expressed as SEM $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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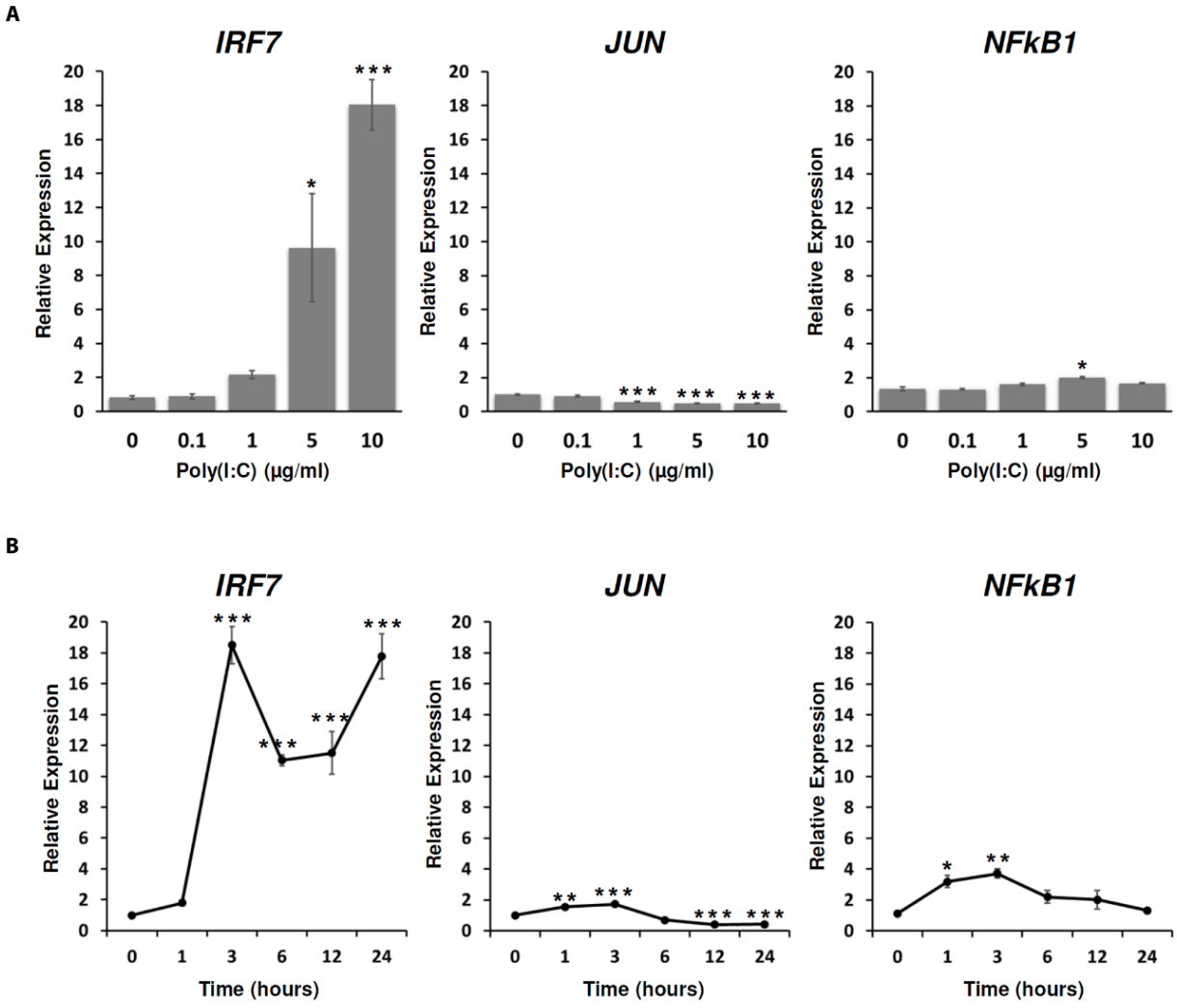

Fig. 3. Dose- and time-dependent expression patterns of TLR signaling-associated transcription factors (TFs) by poly(I:C) treatment. The expressions of IRF7, JUN, and NF-κB1 in DF-1 cells were analyzed in poly(I:C)-treated conditions with concentrations of 0, 0.1, 1, 5, and 10 μg/mL for 24 h (A) and with concentration of 10 μg/mL for 1, 3, 6, 12, and 24 h (B). The statistical analysis was performed to assess statistical significance between each treated condition and the non-treated control. Error bars were expressed as SEM $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Fig. 3B). These results suggested that *TLR3* stimulation by poly(I:C) induced *IRF7* transcription, whereas the expressions of *JUN* and *NF-κB1* were gradually decreased and maintained to the ground state although they were rapidly induced within 1 h after the poly(I:C) treatment. Thus, we speculated that poly(I:C) may mainly induce immune-effector genes by *IRF7*-mediated signaling pathway after the recognition by TLRs such as *TLR3, 4, 7, 15*, and *21* in 24 h after the treatment while direct or indirect pathways may exist to acutely induce *JUN* and *NF-κB1*. The further study is necessary to prove the activation of TLR pathway-mediated TFs.

Dose- and time-dependent expression patterns of immune-related effector molecules by poly(I:C) treatment

From TLRs recognizing their ligands, the activated TFs can induce a variety of interferons, cytokines and chemokines [9,39]. During the immune responses, cytokine and chemokine families acted as extracellular molecular regulators which mediated immune cell recruitment and participated in complex intracellular signaling processes [9]. Among them, IL1B belonging to IL1 family

and IL10 have been known as a pro-inflammatory and an anti-inflammatory cytokine respectively. These cytokines were induced by viral infections [9,51–53]. IL8, a critical inflammatory chemokine was also upregulated by various viral infection in human epithelial cells [54].

To examine whether the expressions of immune-related effector genes are affected by poly(I:C) treatment, *IL1B, IL8L2* (*chicken IL8-like 2*), and *IL10* expressions were analyzed after the poly(I:C) treatment at different dose and time points . From the analysis, the expressions of *IL1B, IL8L2*, and *IL10* were significantly increased by poly(I:C) treatments from 5 μg/mL to 10 μg/mL for 24 h (Fig. 4A). In time-dependent analysis, the expressions of *IL1B*, and *IL10* were significantly increased from 12 h to 24 h after the poly(I:C) treatment (Fig. 4B). Unlike *IL1B* and *IL10*, the expression of *IL8L2* showed the rapid increase at 3 h after the poly(I:C) treatment and reached to the plateau at 6 h after the poly(I:C) treatment. In addition, it was continuously decreased from 12 h to 24 h after the poly(I:C) treatment compared to the expression of *IL8L2* at 6 h after the poly(I:C) treatment although the expressions of *IL8L2* at 12 h and 24 h after the poly(I:C) treatment were still higher

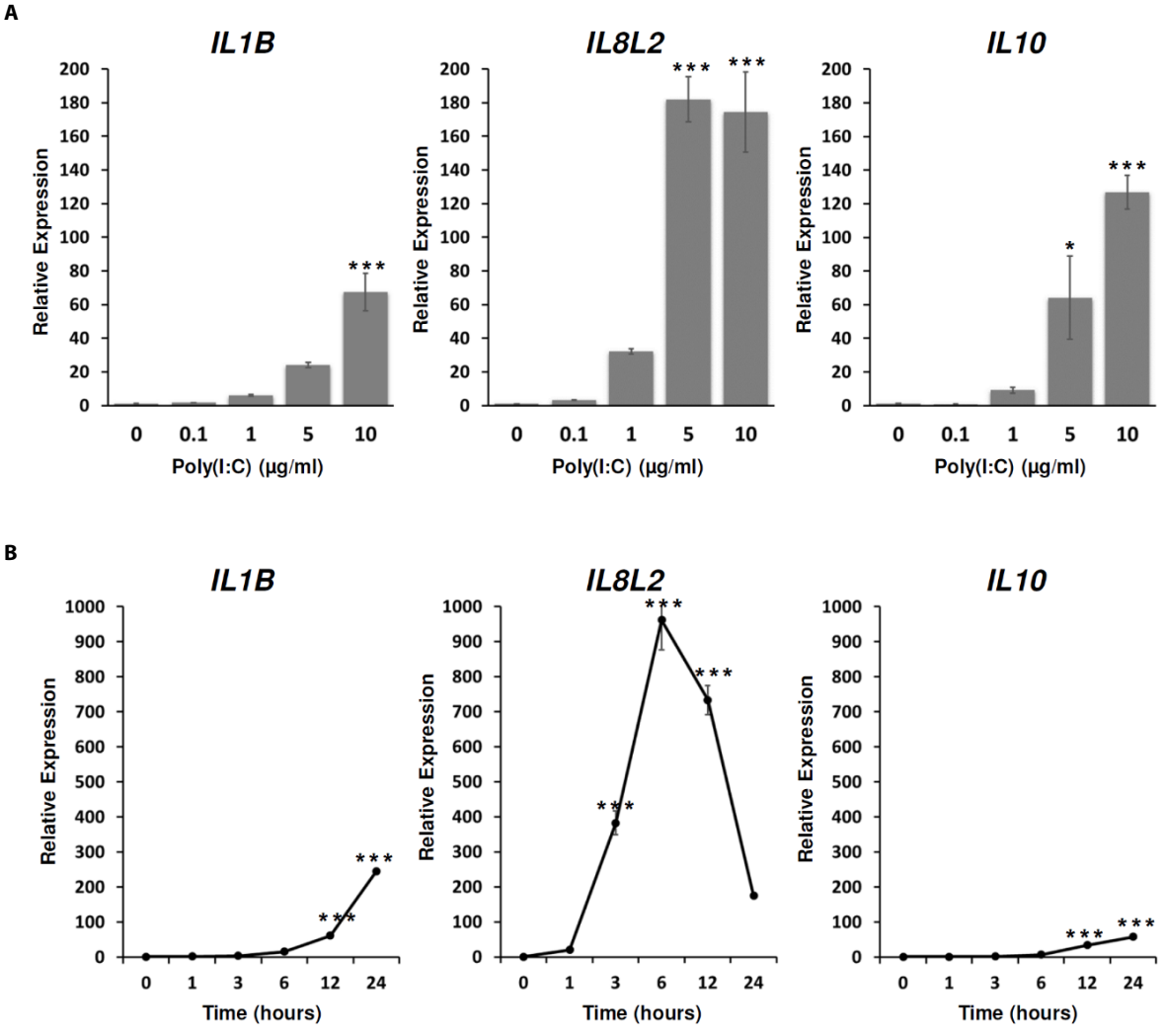

Fig. 4. Dose- and time-dependent expression patterns of immune-related effector molecules by poly(I:C) treatment. The expressions of IL1B, IL8L2, and IL10 in DF-1 cells were analyzed in poly(I:C)-treated conditions with concentrations of 0, 0.1, 1, 5, and 10 μg/mL for 24 h (A) and with concentration of 10 μg/mL for 1, 3, 6, 12, and 24 h (B). The statistical analysis was performed to assess statistical significance between each treated condition and the non-treated control. Error bars were expressed as SEM $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

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than the non-treated control (Fig. 4B). This result suggested that the inductions of *IL1B*, *IL8L2*, and *IL10* in DF-1 cells could be mediated by TLR-signaling pathways. In addition, *IL8L2* could more sensitively respond to poly(I:C) and be inhibited by other feedback systems compared to IL1B and IL10.

Conclusively, we suggested the distinct TLR signaling pathways which responded to poly(I:C) in chicken-originated cell line (DF-1) compared to mammalian TLRs for NA-sensing and their signaling pathways. Our results could contribute to understanding NA-sensing and subsequent immune signaling pathways in chicken cells.

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