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Letter to the Editor

RNF11 negatively regulates antiviral signaling and incites inflammatory response in chicken embryo fibroblast

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Abstract

RNF11 (RING finger protein 11) has been indicated to be related to numerous immunological diseases. As a neo-regulator in innate immunity, *RNF11* was proved to inhibit antiviral signaling and interferon-β (IFN-β) production in *human* cell line. Chicken antiviral signaling pathway is different with other species. However, there is little study about the role of *RNF11* in chicken antiviral signaling pathway and inflammatory response. This study was designed to identify the function of *RNF11* in chicken antiviral signaling pathway and inflammatory response. We overexpressed and interfered *RNF11* in *chicken* embryo fibroblasts (CEFs) and then used quantitative reverse transcription polymerase chain reaction (qRT-PCR) to investigate the poly (I:C)-induced production of *IFN-β* and inflammatory response factors *IL2* and *IL10*. The results indicated that *RNF11* extremely significantly decreased the production of *IFN-β* and highly significantly increased the messenger RNA (mRNA) levels of *IL2* and *IL10*, but depletion of *RNF11* obtained inverse results. Thus, we concluded that *RNF11* not only can inhibit antiviral signaling but also plays an important role in inflammatory response in *chicken*.

Keywords

antiviral signaling, *chicken* embryo fibroblast, inflammatory response, inhibition, *RNF11*

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Introduction

RNF11 (RING finger protein 11) is a highly conserved gene throughout evolution,[1] which belongs to the E3 ubiquitin ligases.[2] One of the most basic roles of *RNF11* is ubiquitin modification, which can interact with E2 conjugating enzymes or E3 ubiquitination ligase.[3] Previous studies revealed that *RNF11* acts as a regulator of transforming growth factor beta (TGF-β) signaling by modulating with Smurf2 and Smad4.[4] And recent study demonstrated that *RNF11* can inhibit melanocortin 3 and 4 receptor signaling[5] and promote the metastasis of murine melanoma cells.[6] It is also proposed to be an essential component of anti-inflammatory A20 ubiquitin-editing complex and a negative regulator of the nuclear factor-κB (NF-κB) in antiviral signaling pathways.[7]

RNF11 is a significant gene that relates to numerous immunological systems, and high-level expression of *RNF11* was found in breast cancer and Parkinson's disease, but the exact function of *RNF11* is poorly known in *chicken*, especially in *chicken* antiviral signaling pathways. In addition, a normal

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immune process is involved in NF-κB activity, but persistent activation of NF-κB signaling would promote inflammation, which could lead to many inflammatory diseases.[8] A recent study suggested that miR-19b-3p enhances the inflammatory response via targeting RNF11.[9] Thus, it is speculated that *RNF11* could regulate the inflammatory pathway. However, there is little report about the direct relationship between *RNF11* and inflammatory response. This study is designed to investigate the role of *RNF11* in *chicken* antiviral signaling and inflammatory response. *RNF11* was separately overexpressed and interfered with plasmids RNF11-GFP and short hairpin RNA (shRNA), and then, poly (I:C)-induced production of *IFN-β* and inflammatory response factors *IL2* (lymphokineinterleukin-2) and *IL10* (lymphokine interleukin-10) were investigated.

Materials and methods

Plasmids

The *RNF11* complementary DNA (cDNA) was synthesized in a gross volume of 20 μL according to the manufacturer's directions (TaKaRa, China). Then, *RNF11* cDNA was spliced into the pEGFPN1 vector (Clontech, CA, USA) with Xho1 and Hind III to generate RNF11-GFP (green fluorescent protein). The *RNF11* shRNA was purchased from BGI (China). The plasmids were extracted using endofree plasmid DNA extraction kit (OMEGA, USA).

Cell culture and transfections

The *chicken* embryo fibroblasts (CEFs) were obtained from 10-day hatching-specific-pathogen-free (SPF) eggs, which were purchased from a commercial company (Beijing Merial Vital Laboratory Animal Technology Co., Ltd., China). The CEFs collection processes were according to Beug and Graf.[10] The CEFs were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone, USA) and supplemented with 8% Fetal Bovine Serum and 1% streptomycin/penicillin. The cells were cultured at 37°C and 5% CO_2 concentration in a Thermostatic Incubator (Thermo Scientific Series 8000, Thermo Fisher Scientific, USA.). The *RNF11*-GFP (1.2 ng) or shRNA (1.2 ng) was transfected using Lipofectamine 2000 (Invitrogen, USA). After transfection for 4 h, liquid reagent was removed and cells were rinsed with 2 mL phosphate-buffered solution (PBS; 0.01M, pH = 7.4), and then, 2 mL of fresh culture solution was added to each individual well. Sequentially, the cells were cultured in an incubator. After transfection for 24 h, the CEFs were exposed to 0.1 μg/L poly (I:C) (Sigma-Aldrich, MO, USA) for 4 h.

Fluorescence microscopy and sampling

CEFs were observed using a fluorescent microscope (Nikon ECLIPSE 90i, Japan) after transfection. The GFP was observed under fluorescence microscope to denote the expression of *RNF11*. At 24 or 28 h post transfection, cells were correspondingly stimulated for 0 or 4 h by poly (I:C). Finally, cell samples were collected using TRIzol reagent (TaKaRa) for RNA extraction at two time points.

cDNA synthesis and real-time quantitative reverse transcription polymerase chain reaction

Total RNA was isolated from different time-point cells using TRIzol. cDNA was synthesized using a cDNA synthesis kit (TaKaRa) as per manufacturer's directions. *GAPDH* was selected as reference gene. The primer pairs are listed in Table 1. Each reaction was performed in triplicate, and qRT-PCR was performed in a 15 μL reaction mixtures with 1.5 μL cDNA, 6.5 μL SYBR Premix Ex Taq II (TaKaRa), 0.4 μL forward and reverse primer, and 6.2 μL of nuclease-free water. The quantitative real time polymerase chain reaction (qRT-PCR) were performed using CFX96™ (Bio-Rad, USA) with the following cycling conditions: 1 cycle at 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, 30 s at annealing temperature by reading of the plate, 72°C for 15 s, followed by 5 min at 72 °C for final extension. The annealing temperature for *RNF11, IFN-β, IL10, IL2*, and *GAPDH* was 54.8°C, 61°C, 61°C, 58.7°C and 56.8°C, respectively A melting curve analysis was performed at temperatures of 65 °C to 95 °C increasing at a rate of 0.5 °C/s.

Statistical analyses

All data in this study were presented as "Mean ± Standard Error of Mean (SEM)." The data represent the relative expression of target gene messenger RNA (mRNA). The mRNA levels of genes were calculated using the $2^{-\Delta\Delta Ct}$ method.[11] Statistical significance was assigned for *P*-value of less than 0.05 ($P < 0.05$). The significant difference analysis was performed using SAS8.0 software.

Table 1. The primer pairs for the genes in real-time qPCR.

Gene	Accession no.	Primer sequence 5′–3′	Product length (bp)
RNF11	NM_001006540.1	F:CCCTATCCGATTTCTGCC R:TGGTCTCCTTGGAGGTTCA	170
IFN-β	NM_001024836.1	F:CCTCAACCAGATCCAGCATTAC R:CCCAGGTACAAGCACTGTAGTT	167
IL10	NM_001004414.2	F: ATCCAGGGACGATGAACTT R: CTGATGACTGGTGCTGGT	154
IL2	NM_204153.1	F: GATCTTTGGCTGTATTTCGG R: CCTGGGTCTCAGTTGGTGT	167
GAPDH	NM_204305.1	F: AGGACCAGGTTGTCTCCTGT R: CCATCAAGTCCACAACACGG	153

Results

The photofluorography survey of the expression of RNF11

The *RNF11*-GFP and shRNA plasmids were transfected in cells, and the GFP was observed using fluorescence microscopy. The photographs were taken at 24 (Figure 1(A)) and 28 h post transfection (Figure 1(B)). The observable GFP in photographs show that plasmids were successfully transfected and expressed in CEFs.

Overexpression and interference of RNF11

To determine whether *RNF11* was overexpressed and depleted in CEFs, the mRNA level of *RNF11* was examined first. The result showed that *RNF11* was extremely significantly higher than empty vector (control) either at 24 or 28 h after *RNF11* overexpressed (Figure 2(a)). These data indicated that 158% and 116% of *RNF11* mRNA were upregulated at 24 and 28 h, respectively. Conversely, shRNA was used to deplete *RNF11*; the mRNA level of *RNF11* was extremely significantly lower than control at two time points (Figure 3(a)), and the knockdown efficiency was 58% and 62%, respectively. The qRT-PCR results indicated that *RNF11* is overexpressed or depleted in cells.

Poly (I:C) stimulation induced the production of IFN-β and IL2 and IL10

Cells were stimulated by poly (I:C) for 0 (unexposed) or 4 h. The mRNA level of genes was detected, which was induced by poly (I:C). The expressions of *IFN-β, IL2*, and *IL10* were significantly induced by poly (I:C), and *RNF11* was either overexpressed (Figure 4(a)) or depleted (Figure 4(b)). The results demonstrated that poly (I:C) stimulation significantly increased the production of *IFN-β, IL2*, and *IL10*.

RNF11 inhibits the production of IFN-β

This study first examined the effect of *RNF11* on *IFN-β* production. The results showed that overexpressing *RNF11* significantly decreased the production of *IFN-β* (Figure 2(b)) either at 24 or 28 h of post transfection. However, depletion of *RNF11* using shRNA increased the production of *IFN-β* (Figure 3(b)). The results indicated that *RNF11* can block the *IFN-β* production.

The effect of RNF11 on IL2 and IL10

This study examined the effect of *RNF11* on *IL2* and *IL10*. Overexpression of *RNF11* significantly increased *IL2* (Figure 2(c)) and *IL10* (Figure 2(d)). However, depletion of *RNF11* significantly decreased *IL2* (Figure 3(c)) and *IL10* (Figure 3(d)). Taken together, *RNF11* may play a key role in the inflammatory response.

Discussion

Innate immunity can impede viral infection and protect the organism from harm. Upon virus access host, the host pattern recognition receptors (PRRs) can detect it and the antiviral signaling pathway is promptly activated. *RNF11* appears to be a novel negative regulator of the antiviral pathway and blocks the production of IFN-β.[12] A recent study found out that Rnf11-like is an essential component of NF-κB signaling pathway for specification of the posterior somites in zebrafish embryos.[13] However,

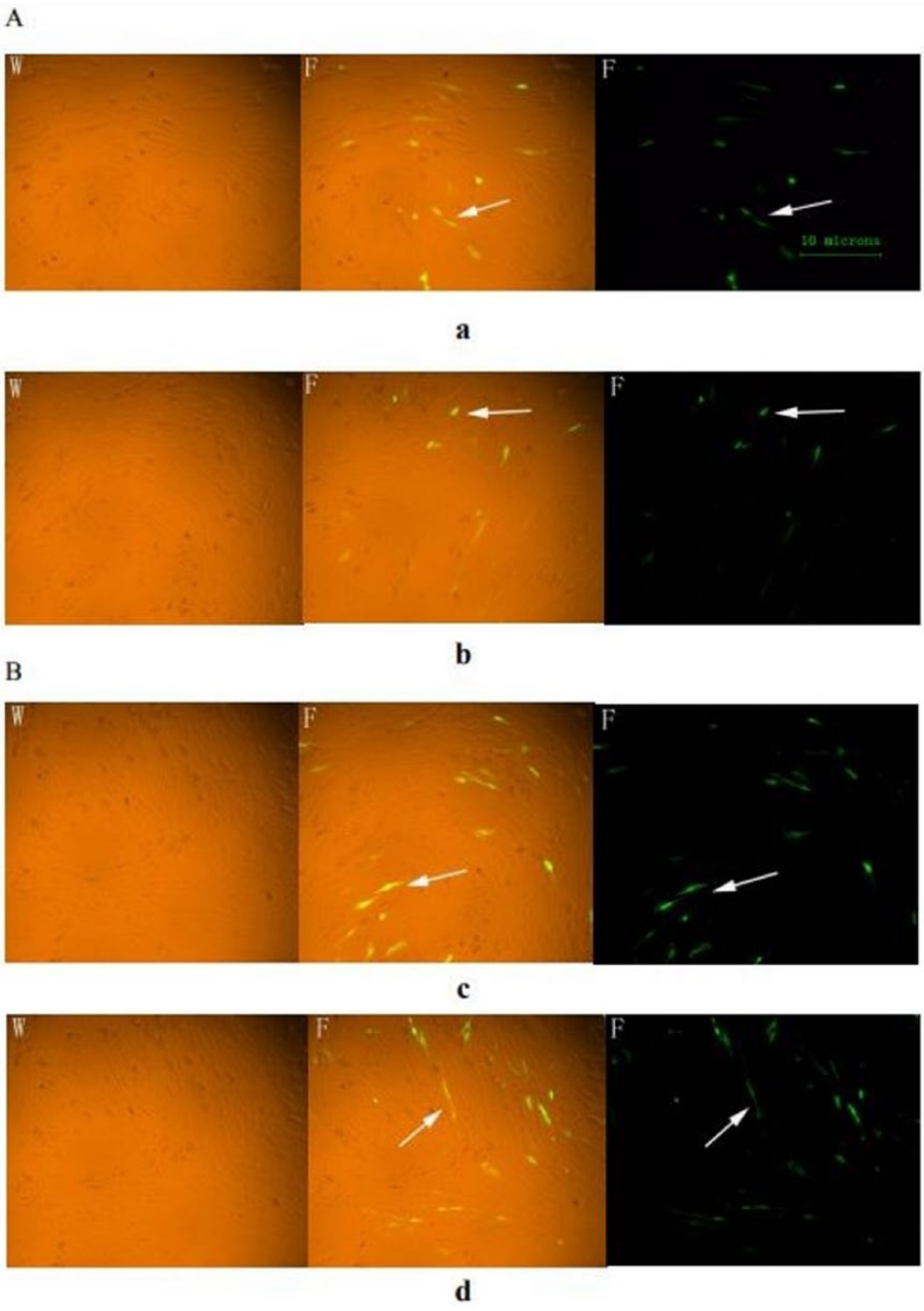

Figure 1. The photofluorography of chicken embryo fibroblasts. The photographs were taken under white (W) or fluorescence light (F) (scale represents 10 μm, 200×). (A) Photographs were taken at 24 h post transfection; (B) Photographs were taken at 28 h post transfection. Plasmids were transfected with empty vectors (a, c) and RNF11-GFP (b, d).

chicken antiviral signaling pathway is different from other species; for instance, RIG-I is absent in *chicken*. Thus, it is unknown whether *chicken RNF11* plays the same role. Previous studies have demonstrated that overexpression of *RNF11* downregulated IFN-β in *human* and *mouse* cells,[14] and knockdown of *RNF11*-like gene using RNF11-like-specific morpholino caused elevating transcripts of NF-κB target gene in zebrafish embryos.[13] This study obtained a similar result: *RNF11* may decrease the *IFN-β* production and acts as a negative regulator of innate antiviral signaling in *chicken*. Although this study showed a desired conclusion, all data were just testified the endogenous levels of mRNA expression but barring protein levels. Thus, further studies would be necessary in the future.

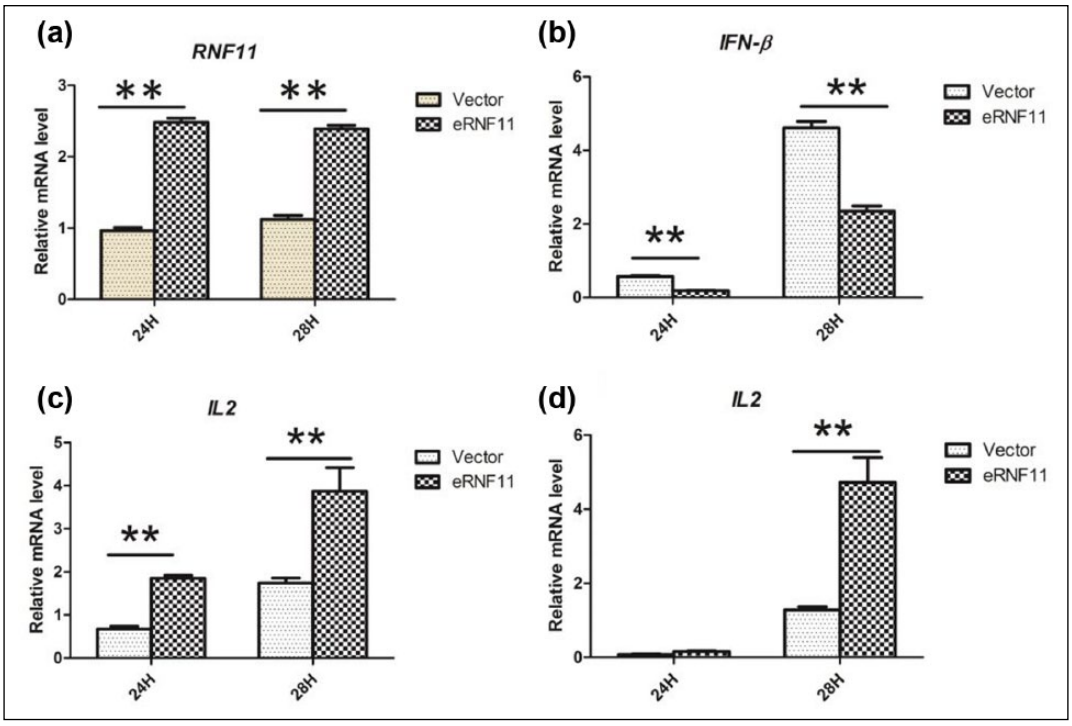

Figure 2. Overexpressing of RNF11 gene affects the production of IFN-β, IL2 and IL10. The CEFs were firstly transfected with RNF11-GFP (1.2 μg; eRNF11) or empty vector (1.2 μg) and then exposured under poly (I:C; 10 μg) at 24 h post transfection. The relative expression of RNF11 (a), IFN-β (b), IL2 (c) and IL10 (d) were detected at 24 or 28 h post transfection. The significant difference were represented by stars (**indicates $P < 0.01$ and *indicates $P < 0.05$). Error bars indicate SE.

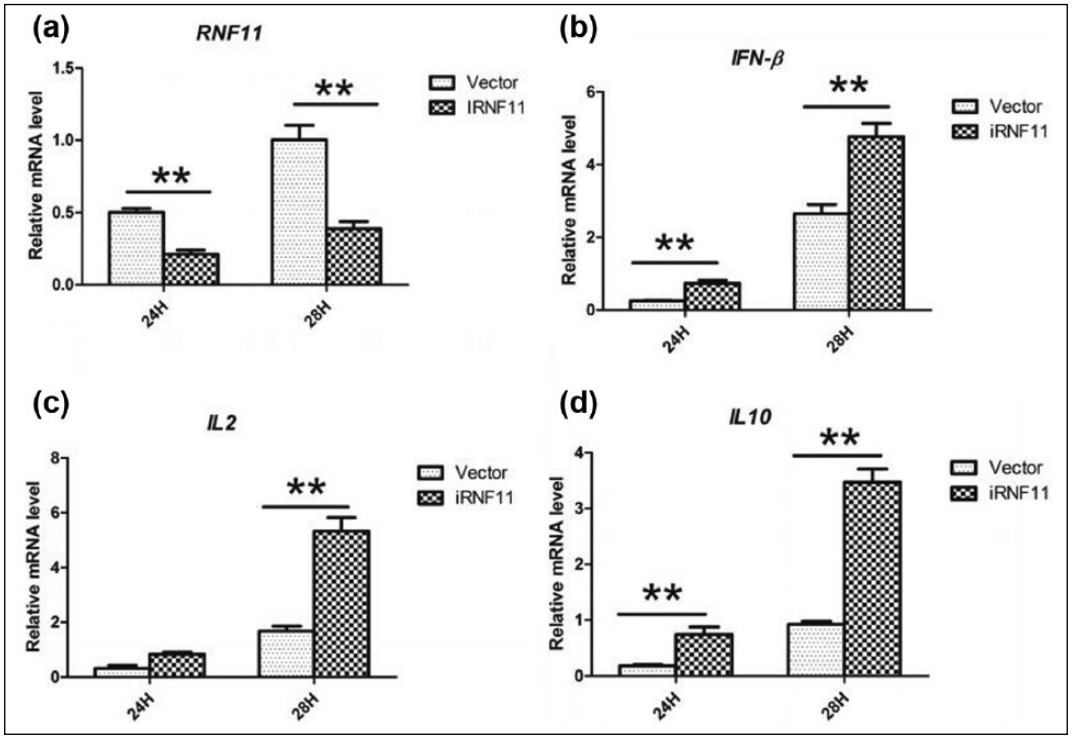

Figure 3. Depletion of RNF11 with short hairpin RNA (shRNA) conversely affects the production of IFN-β, IL2 and IL10. The CEFs were transfected with RNF11 shRNA (1.2 μg; iRNF11) or control (1.2 μg; vector) and then exposured under poly (I:C; 10 μg) at 24 h post transfection. We detected the relative expression of RNF11 (a), IFN-β (b), IL2 (c) and IL10 (d) at 24 or 28 h post transfection. The significant difference were represented by stars (** indicates $P < 0.01$ and * indicates $P < 0.05$). Error bars indicate SE.

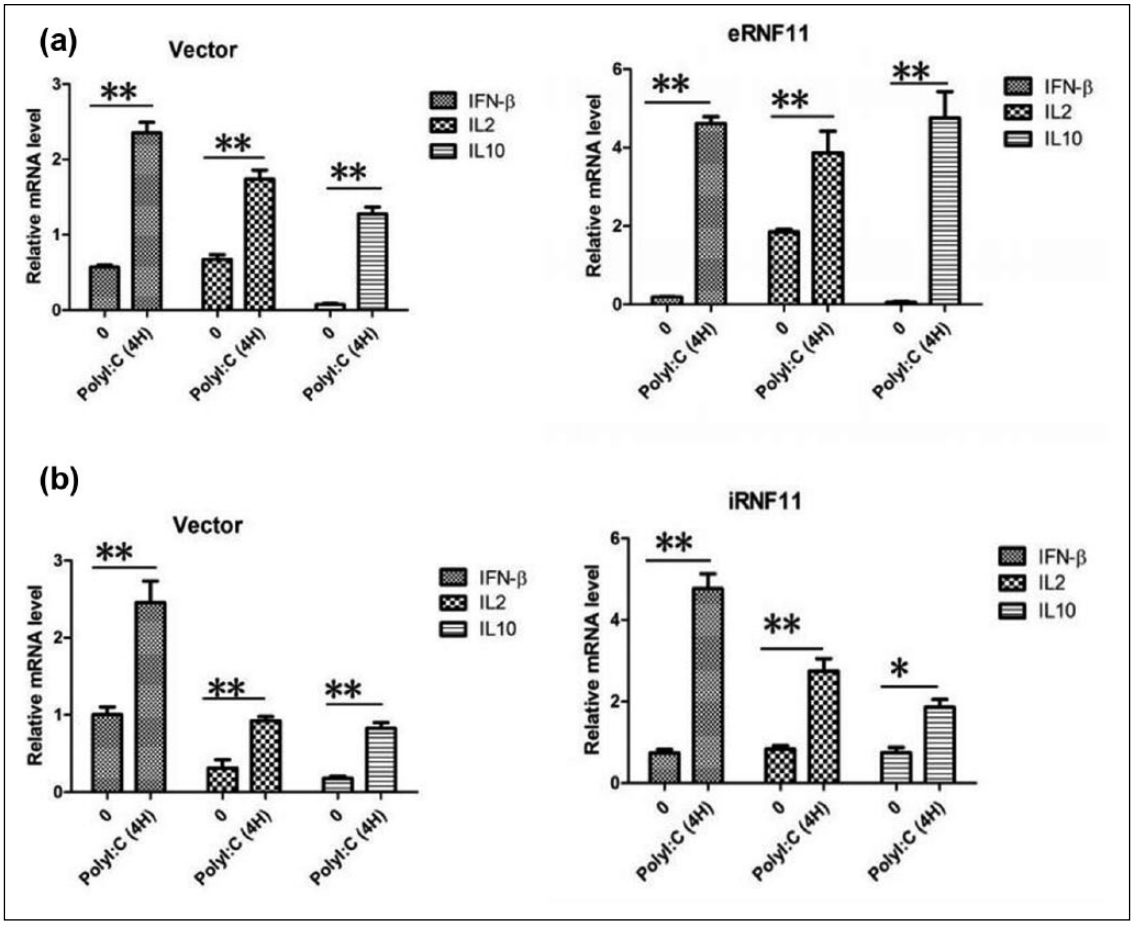

Figure 4. Poly (I:C) transfection induces the production of IFN-β, IL2 and IL10. Chicken embryo fibroblasts (CEFs) were transfected with empty vector or (a) RNF11-GFP and (b) shRNA. Poly (I:C) (4H) represents cells were stimulated by poly (I:C) for 4 h and "0" represents cells were unexposed under poly (I:C). The significant difference were represented by stars (** indicates $P < 0.01$ and * indicates $P < 0.05$). Error bars indicate SE.

Cytokines play a critical role in innate and adaptive immune responses. *IL2* and *IL10* are members of the interleukin family which can cause the proliferation of activated T-cell clones[15] and participate in inflammatory response. Charoenthongtrakul et al.[14] provided in vivo evidence on the important role of *RNF11* in the resolution of inflammation. However, there was no clear study on the effect of *RNF11* gene on inflammatory factors. Therefore, this study examined the effect of *RNF11* on inflammatory factors *IL2* and *IL10*. The results demonstrated that *RNF11* may not only inhibit antiviral signaling but also play an important role in inflammatory response.

In conclusion, this study demonstrated that *RNF11* not only can inhibit antiviral signaling but also can play an important role in inflammatory response in *chicken*.

Acknowledgements

K.T. and X.L. contributed equally to this work.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

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