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Eukaryotic Expression of Functionally Active Recombinant Porcine Toll-Like Receptor 5 from Flp-In-293 Cells

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Abstract: Toll-Like Receptor 5 (TLR5), a member of the TLR family, specifically recognizes flagellin which contributes to the motility of bacterial pathogens. Therefore, TLR5 plays a crucial role in host defense against flagellated bacteria. In the current study, researchers cloned the *TLR5* gene from Jiangquhai pigs (pTLR5). The pTLR5 open reading frame was fused with a FLAG tag by cloning amplified pTLR5 into the pcDNA3.1-FLAG vector. Flp-In-293 cells expressing pTLR5-FLAG (designated pTLR5-Flp-In-293) were obtained using the Flp recombinase system. β-galactosidase activity and zeocin resistance were lost in the pTLR5-Flp-In-293 cells but hygromycin B resistance was acquired. Reverse Transcription-Polymerase Chain Reaction (RT-PCR) and western blot analysis confirmed that pTLR5-FLAG was expressed in pTLR5-Flp-In-293 cells at both the transcriptional and protein levels. Indirect immunofluorescence assay showed that the pTLR5-FLAG protein was located in the cell membrane of pTLR5-Flp-In-293 cells. Stability analysis by fluorescence-activated cell sorting showed that compared with Flp-In-293 cells, the 1st, 10th and 20th generations of the recombinant cell line expressed consistent levels of pTLR5-FLAG protein. Recombinant pTLR5-Flp-In-293 cells stimulated with flagellin from *S. typhimurium* expressed high levels of IL-8 which indicated that pTLR5 protein expressed in the pTLR5-Flp-In-293 cell line was a functional TLR5 homolog.

Key words: Porcine TLR5, Flp-In-293 cell line, expression, identification, bacterial pathogens

INTRODUCTION

The Toll-Like Receptor (TLR) family is one of the best characterized groups of Pattern Recognition Receptors (PRRs). TLRs recognize Pathogen-Associated Molecular Patterns (PAMPs) on invading pathogens (Aderem and Ulevitch, 2000; Takeda *et al*., 2003). Upon activation by the recognition of PAMPs, TLRs initiate signaling cascades, resulting in the release of downstream proinflammatory cytokines and chemokines. This triggers inflammation in the host cell and induces migration of immune cells to the site of infection (Datta *et al*., 2003; Akira and Takeda, 2004). TLRs are the link between the innate and adaptive immune systems and play a critical role in host defense responses (Akira *et al*., 2001).

To date, 13 members of the TLR family have been identified in mammals (Lim and Staudt, 2013). The different TLRs recognize distinct pathogen components such as bacterial lipopolysaccharide, peptidoglycan, lipoproteins, viral double-stranded RNA, flagellin and unmethylated CpG DNA motifs in bacteria (Takeda and Akira, 2005). TLR5 is encoded by a single exon and specifically recognizes flagellin. Upon activation, TLR5 triggers the MyD88-dependent signaling pathway and activates nuclear factor κB (NF-κB) to stimulate transcription of several proinflammatory genes (Hayashi *et al*., 2001; Smith *et al*., 2003; Fang *et al*., 2012). TLR5 can be expressed in multiple cells of the immune system and promotes inflammatory responses. Previous studies have shown that TLR5-deficient mice have no inflammatory response to flagellin (Feuillet *et al*., 2006; Vijay-Kumar *et al*., 2007). TLR5 is important in the host defense against bacterial pathogens in humans and livestock and is involved in the recognition of *S. choleraesuis* in the course of infection in pigs (Shinkai *et al*., 2011).

In this study, a stable cell line expressing porcine TLR5 was established. Researchers expressed pTLR5 using the Flp-In-293 cell line which contains an integrated Flippase Recognition Target (FRT) site. The resultant pTLR5-Flp-In-293 cell line responded significantly to flagellin from *S. typhimurium*, indicating that the pTLR5

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protein expressed by the pTLR5-Flp-In-293 cells was a functional TLR5 homolog. The establishment of a stable Flp-In-293 cell line expressing functional porcine TLR5 will be beneficial for studying the relationship between TLR5 and innate immunity in pigs.

MATERIALS AND METHODS

Cloning of porcine TLR5: Genomic DNA was extracted from the whole blood of a Jiangquhai breed pig which donated by Jiangsu Agri-Animal Husbandry Vocational College using a Universal Genomic DNA Extraction Kit Ver.3.0 (Takara Biotechnology Co., Ltd. China) according to the manufacturer's instructions. The coding region of the gene coding for porcine TLR5 (pTLR5), minus the stop codon was amplified using the genomic DNA as a template. The following primers were designed based on the porcine (*Sus scrofa*) TLR5 sequence (Genbank Accession No.: AB208697) and were used for thePCR:sense5'-TAGCTAGCATGGGAGACTGCCTGGT-3'and antisense 5'-TCCTCTAGAGGAGATGGTCACGCT TTG-3'. PCR was carried out using Prime STAR HS DNA Polymerase (Takara Biotechnology Co., Ltd. China) in a 25 μL reaction volume containing 2 μL (~100 ng) of genomic DNA template, 0.25 μL of PrimeSTAR HS DNA Polymerase, 5 μL of 5×PrimerSTAR Buffer (Mg^{2+} plus), 2 μL of dNTP Mixture (2.5 mM), 0.5 μL each of sense and antisense primers (10 μM). PCR was performed as follows: 1 cycle of 98°C for 5 min, 30 cycles of 98°C for 10 sec, 62°C for 15 sec and 72°C for 3 min followed by 1 cycle of 72°C for 10 min. The porcine TLR5 PCR fragment was purified and cloned into the pCR 2.1 vector using a TA cloning kit (Invitrogen, USA). The resultant vector was designated pCR2.1-TLR5 and the inserted porcine TLR5 fragment was confirmed by sequencing which performed by Genscript (Nanjing, China).

Construction of the pcDNA5/FRT-TLR5-FLAG expression vector: An expression construct, pcDNA5/FRT-TLR5-FLAG was made by cloning the TLR5-FLAG fragment into the NheI and ApaI sites of the pcDNA5/FRT expression vector (Invitrogen, USA). The coding region of the porcine *TLR5* gene minus the stop codon was excised from recombinant plasmid pCR2.1-TLR5 by digestion with NheI and XbaI (Takara Biotechnology Co., Ltd. China) and subcloned into the same restriction enzymes sites of the pcDNA3.1-FLAG construct (designated pcDNA3.1-TLR5-FLAG). The TLR5-FLAG fragment was digested out of recombinant plasmid pcDNA3.1-TLR5-FLAG using NheI and ApaI (Takara Biotechnology Co., Ltd. China) and subcloned into the same restriction enzymes sites of the pcDNA5/FRT expression vector (designated pcDNA5/ FRT-TLR5-FLAG).

Cell culture and generation of stable transfectants: The Flp-In transfection system (Invitrogen, USA) was used to produce stably transfected cells expressing pTLR5-FLAG. Flp-in-293 cells which are human embryonic kidney cells containing an integrated FRT site were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gibco, USA), 100 units mL^{-1} penicillin, 100 μg mL^{-1} streptomycin (Gibco, USA) and 100 μg mL^{-1} zeocin (Invitrogen, USA). Cells were cultured at 37°C in a humidified incubator with 5% CO_2. The 1 day prior to transfection, Flp-in-293 cells were seeded at a density of 5×10^5 cells mL^{-1} on six-well plates and grown in DMEM with 10% FBS. Transfections were performed with Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's instructions. Opti-MEM I (Gibco, USA) reduced-serum medium was used for the transfection with 1 μg pcDNA5/FRT-TLR5-FLAG, 9 μg pOG44 and 6 μL Lipofectamine 2000. At 48 h post-transfection, the cells were cultured with complete DMEM containing 120 μg mL^{-1} hygromycin B (Roche, Switzerland). Medium was subsequently changed every 2-3 days. Visible circular colonies were present about 3-4 weeks post-transfection. Hygromycin B-resistant foci were picked and pure cultures were established for further investigation (designated pTLR5-Flp-In-293).

Zeocin resistance and β-galactosidase activity assays: The pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells (control cells) were cultured on 24-well plates with complete DMEM containing 100 μg mL^{-1} zeocin for 4 days to test for zeocin sensitivity. The 1 day prior to β-galactosidase activity assays, cells were seeded at a density of 5×10^5 cells mL^{-1} on six-well plates and grown in complete DMEM containing 120 μg mL^{-1} hygromycin B. β-galactosidase activity assays were performed using a β-Gal Staining Kit (Invitrogen, USA) according to the manufacturer's instructions. Cells were examined under a microscope to detect the development of blue color.

Detection of porcine TLR5 mRNA in pTLR5-Flp-In-293 cells by Reverse Transcription-Polymerase Chain Reaction (RT-PCR): Total RNA was extracted from pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells (control cells) using TRIzoL Reagent (Invitrogen, USA) according to the manufacturer's instructions. To avoid contamination with genomic DNA, total RNA samples were treated with RNase-Free DNase I (Takara Biotechnology Co., Ltd. China). First-strand cDNA was synthesized using M-MLV reverse transcriptase (Invitrogen, USA) according to the manufacturer's instructions. Porcine TLR5 mRNA was amplified by PCR using the following primers: sense 5'-TAGCTAG CATGGGAGACTGCCTGGT-3'and antisense 5'-TCCTCT

AGAGGAGATGGTCACGCTTTG-3'. PCR was carried out using Recombinant Taq DNA Polymerase (Takara Biotechnology Co., Ltd. China) in a 25 μL reaction volume containing 2 μL (~100 ng) of cDNA template, 0.125 μL of Recombinant Taq DNA Polymerase, 2.5 μL of 10×PCR Buffer, 2 μL of dNTP Mixture (2.5 mM), 0.5 μL each of sense and antisense primers (10 μM). PCR was performed as follows: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, 63°C for 30 sec and 72°C for 3 min followed by 1 cycle of 72°C for 10 min. The PCR products were run on a 1.0% (w/v) agarose gel and detected with ethidium bromide under UV light.

Western blot: The pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells were collected and treated with a lysis buffer (BI Yuntian, China). The protein concentration was measured using a BCA protein assay kit (BI Yuntian, China) according to the manufacturer's protocol. The 15 μg of each protein extract was separated by SDS-PAGE using a 12% polyacrylamide gel. Following SDS-PAGE, the contents of the gel were electrotransferred to a nitrocellulose membrane. The membranes were then blocked with 2% BSA in PBST at 4°C overnight. After blocking, the membranes were probed with specific primary antibodies washed and then incubated with a secondary anti-body. To detect the TLR5-FLAG fusion protein, nitrocellulose membranes were treated with mouse polyclonal anti-porcine TLR5 anti-body (1:500 dilution) or monoclonal anti-FLAG anti-body (1:1000 dilution, Sigma, USA) as the primary anti-body and subsequently with HRP-conjugated goat anti-mouse IgG (1:3000 dilution, Sigma, USA) as the secondary anti-body. The membranes were then washed and signals were detected using Super Signal West Pico Chemiluminescent Substrate (Pierce, USA) according to the manufacturer's instructions.

Indirect immunofluorescence analysis for expression and distribution of TLR5-FLAG: pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells (control cells) were grown in six-well plates until 80% confluence was reached. Cells were washed twice with PBS and then fixed in PBS containing 2% formaldehyde for 1 h at room temperature, followed by two rinses in PBS. Nonspecific anti-body interactions were blocked with PBS containing 2% BSA for 30 min. To detect the TLR5-FLAG fusion protein, cells were treated with mouse polyclonal anti-porcine TLR5 anti-body (1:100 dilution) or monoclonal anti-FLAG anti-body (1:1000 dilution, Sigma, USA) as the primary anti-body and subsequently with Alexa Fluor 488-conjugated anti-mouse IgG anti-body (1:1000 dilution, Invitrogen, USA). Fluorescence was visualized using a Leica DMI3000B fluorescence microscope (Leica Microsystems, Germany).

Stability analysis of pTLR5-Flp-In-293 cells by flow cytometry: pTLR5-Flp-In-293 cells were cultured with complete DMEM containing 120 μg mL^{-1} hygromycin B and continually passaged. To examine the stability of the expressed protein in the pTLR5-Flp-In-293 cells, cells were frozen at the 1st, 10th and 20th generations. Flow cytometry was then used to detect the TLR5-FLAG fusion protein from each generation. Cells were treated with monoclonal anti-FLAG anti-body (1:200 dilution, Sigma, USA) as the primary anti-body and subsequently with the FITC-conjugated anti-mouse IgG anti-body (1:100 dilution, Sigma, USA). Fluorescence Activated Cell Sorting (FACS) analyses were performed using a FACSAria cytofluorometer (BD Biosciences, USA).

TLR5 activation assay: pTLR5-Flp-In-293 cells and untransfected Flp-in-293 cells (control cells) were seeded at a density of 5×10^4 cells/well on a flat-bottom 96-well plate and incubated overnight. The cells were washed twice and then stimulated for 5 h with purified flagellin from *S. typhimurium* (Enzo Life Sciences, USA) at a final concentration of 10 ng mL^{-1}. The amount of human IL-8 secreted into the supernatant was quantified by Human IL-8 ELISA kit (BD, USA) according to the manufacturer's protocol.

Statistical analysis: Statistical analyses were performed using a two-tailed Student's t test. A $p<0.05$ was considered significant. All the statistical values were calculated using Prism (GraphPad Software Inc.).

RESULTS AND DISCUSSION

The pcDNA5/FRT-TLR5-FLAG construct successfully integrated into the FRT site in the genome of Flp-In-293 cells: To identify integration of the pcDNA5/FRT-TLR5-FLAG construct into the genome of Flp-In-293 cells, zeocin resistance and β-galactosidase activity assays were performed. pTLR5-Flp-In-293 cells did not survive in complete DMEM containing 100 μg mL^{-1} zeocin (Fig. 1), indicating that pTLR5-Flp-In-293 cells lost zeocin resistance. In addition, pTLR5-Flp-In-293 cells appeared white in color following β-galactosidase staining (Fig. 1), suggesting that the cells had also lost β-galactosidase activity. Hygromycin

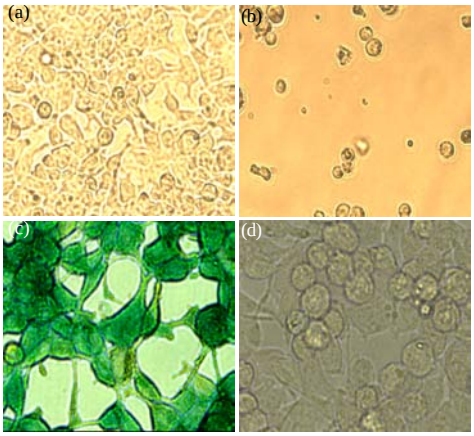

Fig. 1: Phenotype of the stable pTLR5-Flp-In-293 cell line. For analysis of zeocin resistance, untransfected Flp-In-293 cells and a) pTLR5-Flp-In-293 cells; b) were cultured with complete DMEM containing 100 μg mL^{-1} zeocin for 4 days. The cell state was then visualized by light microscope. For analysis of β-galactosidase activity, untransfected Flp-In-293 cells and c) pTLR5-Flp-In-293 cells; d) were stained with a β-Gal Staining Kit according to the manufacturer's instructions. Cell color was checked by light microscope

resistance, zeocin sensitivity and lack of β-galactosidase activity confirmed that the pcDNA5/FRT-TLR5-FLAG construct had successfully integrated into the FRT site in the genome of Flp-In-293 cells.

Recombinant fusion protein TLR5-FLAG is expressed in pTLR5-Flp-In-293 cells: The expression of porcine TLR5 mRNA in pTLR5-Flp-In-293 cells was examined by RT-PCR. PCR products of predicted sizes for porcine TLR5 (2571 bp) were obtained from the pTLR5-Flp-In-293 cells and not from the untransfected Flp-In-293 cells (Fig. 2a). Western blot analysis confirmed the expression of pTLR5-FLAG fusion protein using both the monoclonal anti-FLAG anti-body and the polyclonal anti-porcine TLR5 anti-body (Fig. 2b). Indirect immunofluorescence analysis was used to show the expression and localization of pTLR5-FLAG fusion protein using the monoclonal anti-FLAG anti-body (Fig. 2c) and the polyclonal anti-porcine TLR5 anti-body (Fig. 2d). Results showed that TLR5-FLAG fusion protein was expressed on the plasma membrane of pTLR5-Flp-In-293 cells.

pTLR5-Flp-In-293 cells can stably express porcine TLR5: To evaluate expressed protein stability in pTLR5-Flp-In-293 cells, researchers harvested 1st, 10th and 20th generation cells and examined the expression of fusion protein pTLR5-FLAG by flow cytometry. The percentages of pTLR5-Flp-In-293 cells expressing pTLR5-FLAG protein were 82.6, 83.6 and 82.3% in the 1st, 10th and 20th generation samples (Fig. 3).

Functional analysis of porcine TLR5 in the pTLR5-Flp-In-293 cells: To evaluate the response of porcine TLR5 to flagellin, the effect of flagellin stimulation on human IL-8 secretion was determined. The level of IL-8 secretion in pTLR5-Flp-In-293 cells 5 h post-flagellin stimulation was significantly higher than the level in untransfected Flp-In-293 cells ($p<0.05$) (Fig. 4).

Toll-like receptors are key PRRs in microbial infection and play a important role in the activation of innate immunity and the development of adaptive immunity through their interaction with ligands (Takeda *et al*., 2003; Zhang *et al*., 2004). Along with the elucidation of the various functions of TLR molecules in humans and mice there has been considerable interest in the identification of the *TLR* genes and the genes encoding molecules involved in ligand recognition by TLRs in livestock including pigs and cattle (Vijay-Kumar *et al*., 2007; Uenishi and Shinkai, 2009). Genes corresponding to all 10 of the human TLRs have also been fully cloned in pigs (Uddin *et al*., 2013). As in other animals, chicken (Gupta *et al*., 2013), bovine (Silva *et al*., 2012) and porcine TLR5 molecules recognize and respond to flagellin which induces the production of chemokines and play an important role in host defense against flagellated bacteria. Porcine TLR5 is involved in the recognition of *S. choleraesuis* in the course of infection in pigs and the genetic variation of porcine TLR5 can affect recognition of the ligand and signal transduction of the receptor and alter the susceptibility/resistance of the host to infectious diseases (Shinkai *et al*., 2011; Yang *et al*., 2013). Understanding the role of TLR5 in porcine health and response to disease is very important. The first step in elucidating the role of TLR5 is to study the relationship between TLR5 and pathogens.

To achieve this researchers need to express functionally active porcine TLR5. The Flp-In-293 cell line allows rapid generation of stable cell lines and ensures high level expression of the target protein. Many previous studies have successfully expressed functionally active proteins using this system (Harris *et al*., 2007; Wakabayashi *et al*., 2007; Filipski *et al*., 2008).

In this study, researchers selected Flp-In-293 human embryonic kidney cells to prepare a stable cell line expressing porcine TLR5. The stable cell line were

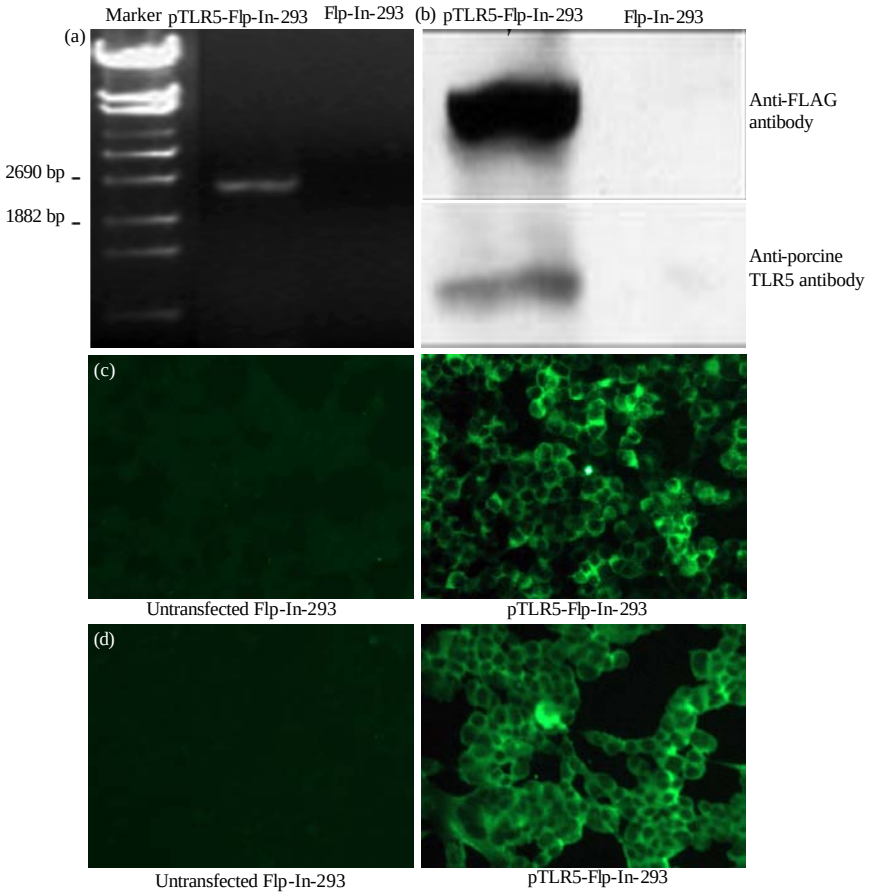

Fig. 2: Detection of pTLR5-FLAG expression in pTLR5-Flp-In-293 cells at the transcriptional and protein levels; the mRNA level was analyzed by RT-PCR using total RNA extracted from pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells; a) the pTLR5-FLAG fusion protein expressed in pTLR5-Flp-In-293 cells was detected by Western blot using the monoclonal anti-FLAG anti-body and the polyclonal anti-porcine TLR5 anti-body; b) the expression and localization of pTLR5-FLAG fusion protein in pTLR5-Flp-In-293 cells was detected by indirect immunofluorescence analysis using the monoclonal anti-FLAG ant-ibody; or c) polyclonal anti-porcine TLR5 anti-body; d) as the primary anti-body and subsequently with the Alexa Fluor 488-conjugated anti-mouse IgG anti-body. Fluorescence was visualized using a fluorescence microscope

generated using a Flp recombinase system which allows expression of Flp recombinase, resulting in integration of the pcDNA5/FRT plasmid containing target gene into the genome via the FRT sites. Researchers introduced a FLAG-tag into the C-terminal of pTLR5 which facilitated the detection of pTLR5 expression. RT-PCR, Western blot and indirect immunofluorescence assays showed that pTLR5 was successfully expressed in the generated pTLR5-Flp-In-293 cell line. Monitoring of the pTLR5-Flp-In-293 cell line by flow cytometry every ten passages showed that pTLR5 was stably overexpressed for at least 20 passages. Flagellin, a major component of bacterial flagella is recognized by TLR5, resulting in activation of NF-κB and mitogen-activated protein kinases and the production of inflammatory cytokines (Yoshino *et al*., 2013). Flagellin can stimulate epithelial cells to release key inflammatory mediators such as IL-8 (Lopez-Boado *et al*., 2005; Im *et al*., 2009). IL-8 plays an important role in innate immune responses by recruiting neutrophils, lymphocytes and eosinophils to the site of infection. In this study, recombinant pTLR5-Flp-In-293 cells secreted significantly higher levels of IL-8 than the untransfected Flp-In-293 cells stimulated by flagellin from *S. typhimurium*. This result implied that pTLR5 protein expressed by the pTLR5-Flp-In-293 cell line could recognize *S. typhimurium* flagellin and that the expressed protein was a functional TLR5 homolog. Therefore, the recombinant pTLR5-Flp-In-293 cells could mediate innate immune and inflammatory responses induced by flagellin. The establishment of a stable recombinant

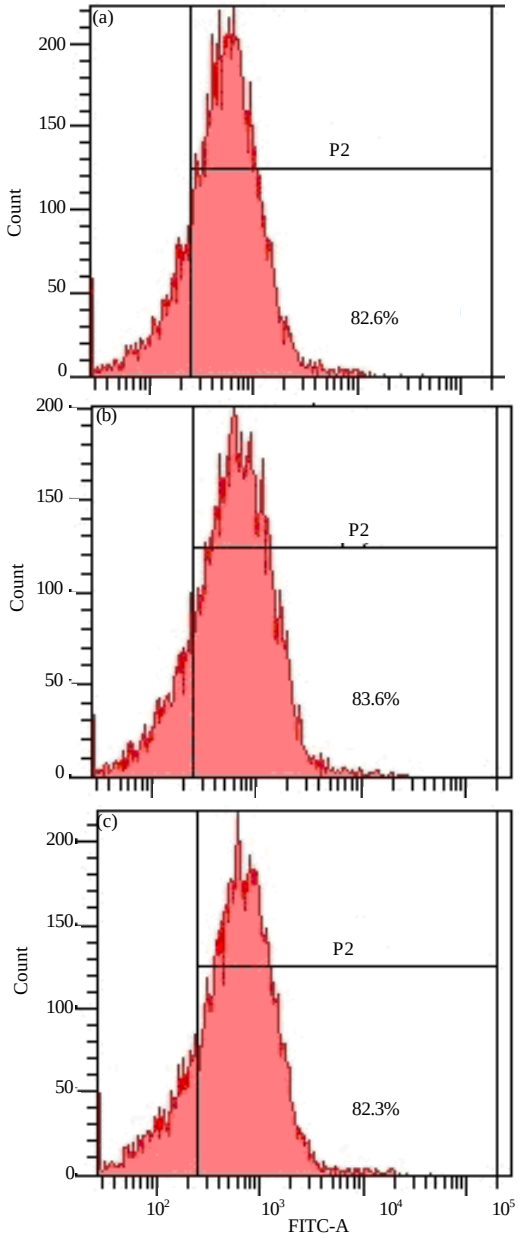

Fig. 3: Flow cytometry analysis of recombinant pTLR5-Flp-In-293 cell line stability. The 1st generation; a) 10th generation; b) and 20th generation; c) pTLR5-Flp-In-293 cells were treated with the monoclonal anti-FLAG anti-body as the primary anti-body and subsequently with the FITC-conjugated anti-mouse IgG anti-body. The stability of pTLR5-Flp-In-293 cells expressing pTLR5-FLAG fusion protein was examined by flow cytometry

pTLR5-Flp-In-293 cell line will be beneficial to study the relationship between porcine TLR5 and innate immunity.

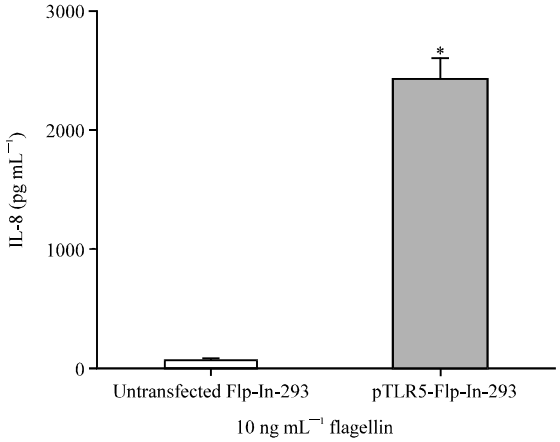

Fig. 4: IL-8 production by flagellin-stimulated pTLR5-Flp-In-293 cells. The pTLR5-Flp-In-293 cells and untransfected Flp-In-293 cells were stimulated for 5 h with 10 ng mL 1 purified flagellin from S. typhimurium. Levels of IL-8 were detected by ELISA. The results are the means±SD of three independent experiments performed in triplicate. *, $p<0.05$ determined by a two-tailed Student's t-test relative to the untransfected Flp-In-293 cells

CONCLUSION

In this study, researchers successfully established a stable transgenic cell line to express functionally active recombinant porcine TLR5. The pTLR5-Flp-In-293 cell line responded to flagellin from *S. typhimurium* via TLR5 and secreted high levels of IL-8.

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