European Journal of Cell Biology 98 (2019) 36–50

Contents lists available at ScienceDirect

European Journal of Cell Biology

journal homepage: www.elsevier.com/locate/ejcb

Research paper

Overexpression of Toll-like receptor 4 enhances LPS-induced inflammatory response and inhibits *Salmonella Typhimurium* growth in ovine macrophages

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ARTICLE INFO

Keywords:
Ovine macrophage
Overexpression TLR4
IRAK4
TBK1
Salmonella

ABSTRACT

The Toll-like receptor 4 (TLR4) plays a crucial role in innate inflammatory responses, as it recognizes gram-negative bacteria (or their products) and contributes greatly to host defense against invading pathogens. Though TLR4 overexpressing transgenic sheep, resistant to certain diseases related with gram-negative bacteria, had been bred in our previous research, the effects of overexpression of TLR4 on innate immune response remained unclear. In this study, TLR4 overexpressing ovine macrophages were obtained from peripheral blood, and it was found that the overexpression of *TLR4* initially promoted the production of proinflammatory cytokines *TNFα* and *IL-6* by activating TLR4-mediated IRAK4-dependent NF-κB and MAPK (JNK and ERK1/2) signaling following LPS stimulation. However, this effect was later impaired due to increased internalization of TLR4 into endosomal compartment of the macrophages. Then the overexpression of *TLR4* triggered TBK1-dependent interferon-regulatory factor-3 (IRF-3) expression, which in turn led to the induction of *IFN-β* and IFN-inducible genes (i.e.*IP10*, *IRG1* and *GARG16*). Understandably, an increased IFN-β level facilitated phosphorylation of STAT1 to induce expression of innate antiviral genes *Mx1* and *ISG15*, suggesting that *TLR4* overexpressing macrophages were equipped better against viral infection. Correspondingly, the bacterial burden in these macrophages, after infection with live *S. Typhimurium*, was decreased significantly. In summary, the results indicated that overexpression of TLR4 could enhance innate inflammatory responses, initiate the innate antiviral immunity, and control effectively *S. Typhimurium* growth in ovine macrophages.

1. Introduction

The innate immune response, involving immunocytes such as macrophages, neutrophils, dendritic cells, and monocytes(Akira and Takeda, 2004; Newton and Dixit, 2012), is the first line of host defense against invading pathogens. It plays a critical role in the initiation, maintenance, and resolution of inflammation(Newton and Dixit, 2012). In the process of inflammation, macrophages have three major functions, i.e. antigen presentation, phagocytosis and immunomodulation, and the activation and deactivation of macrophages are closely associated with this inflammatory process(Fujiwara and Kobayashi, 2005).

The Toll-like receptor 4 (TLR4), a member of Toll-like receptors family expressed mainly in innate immune cells, is critical for early host defense against invading pathogens and for inflammatory response by recognizing pathogen associated molecular patterns (PAMPS) on gram-negative bacterial surface or danger associated molecular patterns (DAMPS) released from tissue damage cells (Akira and Takeda, 2004; Newton and Dixit, 2012; Wittebole et al., 2010). As TLR4 recognizes specifically endotoxin lipopolysaccharide (LPS), a major component of gram-negative bacteria, it can induce macrophages and neutrophils to express a number of proinflammatory cytokines including TNFα, IL-8, IL-6, IL-1β and IL-12 (Ramachandran, 2014). Many studies had revealed that TLR4-mediated signaling was closely related with sepsis and organ injury outcome (Child et al., 2003; Eric *J.* Seeley et al., 2012; Pene et al., 2009; Roger et al., 2009; Wittebole et al., 2010; Zacharowski et al., 2006; Zhang et al. (2008)). That bacterial sepsis

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https://doi.org/10.1016/j.ejcb.2018.11.004
Received 28 June 2018; Received in revised form 6 November 2018; Accepted 28 November 2018

being impaired significantly in TLR4-deficient or treated neutralizing anti-TLR4 antibodies mice (Roger et al., 2009; Zacharowski et al., 2006) further confirmed that TLR4 can trigger proinflammatory response to LPS resulting in sepsis outcomes. Thus, when the body encounters pathogens or their components, the activation versus suppression of TLR4 in its innate immune system is critical importance for maintaining a balance between pro-inflammatory and anti-inflammatory response.

Upon LPS stimulation, TLR4 signaling is first activated through the myeloid differentiation primary-response protein 88 (MyD88)-dependent and MyD88-independent pathways. The binding of LPS to TLR4 on cell surface, involving the coreceptor MD2 and CD14, triggers the interaction between the TIR domain of TLR4 and TIR-containing adaptor protein MyD88 in the cytoplasm. As a result, MyD88 then activates the IL-1 receptor-associated kinase (IRAK) via death domain (Cao, 2016; Jenkins and Mansell, 2010), and the activated IRAK will engage TNF receptor-associated factor 6 (TRAF6), an E3 ubiquitin ligase, to touch off the polyubiquitin chain elongation and thereby activates the IKK complex (inhibitor of nuclear factor-κB (IκB)- kinase complex). The IκBs, inhibitor of NF-κB family that inactivates NF-κB in the cytoplasm, are phosphorylated by activated IKK and subsequently degradated by polyubiquitylation and proteasome system. Upon releasing from its inhibitor, NF-κB translocates into the nucleus to induce the expressions of pro-inflammatory cytokines, such as TNFα, IL-6 and IL-1β(Akira and Takeda, 2004; Beutler, 2004; Guijarro-Munoz et al., 2014; Newton and Dixit, 2012). Meanwhile, the interaction between IRAK and TRAF6 will promote the activation of transforming growth factor-activating kinase (TAK1). The TRAF6/TAK1 complexes recruit the TAK1 binding protein 2 (TAB2), and initiate mitogen-activated protein kinase (MAPK) signaling cascades, thus triggering the phosphorylation of p38, ERK1/2 and JNK (Moynagh, 2003) and therefore inducing the production of inflammatory cytokines including TNFα and IL-1β (Arthur and Ley, 2013; Zou and Shankar, 2015). In contrast, TLR4-mediated MyD88-independent/TRIF-dependent pathway is activated within the cytoplasm. The interaction between TIR domain of TLR4 and the adaptors protein TRIF induces the activation of TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1), and thereby promotes the phosphorylation of interferon (IFN)-regulatory factor 3 (IRF3). As a result, IRF3 then translocates into the nucleus to induce the expression of interferon-inducible genes, such as response gene 16 (GARG16), immunoresponsive gene 1 (IRG1), and the gene encoding CXC-chemokine ligand 10 (CXCL10, also known as IP10)(Akira and Takeda, 2004; Kawai et al., 2001; Newton and Dixit, 2012).

The endocytosis of TLR4 is an essential step for the signal transduction in innate immune response. After its binding with LPS, TLR4 migrates from cell membrane into endosomes of the cytoplasm, thereby activating the TRIF-dependent signal cascades which then induce the expression of interferon-inducible genes involved with inflammatory response (Balachandran, 2016; Zanoni et al., 2011). Findings from other researchers revealed that LPS induced the expression of IFN-β that functioned in cellular resistance to viral infection through TLR4 internalization pathway (Balachandran, 2016; Doyle et al., 2002; Hertzog et al., 2003). IFN-β binds to cell surface receptors known as the IFN-α receptors such as IFNAR1 and IFNAR2, which results in the activation of Janus tyrosine kinases, and then they phosphorylate signal transducers and activators of transcription (STAT), DNA-binding transcription factors, to engage with action of antiviral infections(Greenhill et al., 2011; Hertzog et al., 2003). Taken as a whole, the signal pathways mediated by TLR4 internalization not only regulate inflammatory response, but also promote cellular resistance to viral infection.

On the one hand, overstimulation of TLR4-mediated signaling will bring about devastating consequences, such as septic shock, organ failure, etc. Therefore, vigilant surveillance of TLR4 signaling is essential for effective immune response. On the other hand, the internalization of TLR4 into cytoplasm is one of the negative regulatory mechanism for preventing excessive inflammatory response in LPS-induced TLR4 signal pathway (Aerbajinai et al., 2013; Balachandran, 2016). In our previous research, TLR4 overexpressing transgenic sheep were bred for their resistance against diseases caused by gram-negative bacteria (Bai et al., 2015). However, the effect of TLR4 overexpression on innate immune responses remained incompletely elucidated. In this study, we examined the expression of LPS-induced proinflammatory cytokines in TLR4 overexpressing transgenic ovine macrophages in both the early and late stages. It was found that overexpression of TLR4 in early phase increased the production of LPS-stimulated proinflammatory cytokines by Myd88-dependent NF-κB and MAPK (ERK1/2 and JNK) signaling pathways. However, during the late phase upon LPS stimulation, the expression of proinflammatory cytokines was partially impaired and TLR4-mediated TRIF-dependent pathway was then activated to enhance cellular antiviral function through the internalization of TLR4. Meanwhile, the overexpression of TLR4 effectively inhibited *S. typhimurium* growth in blood macrophages (BMs).

2. Materials and methods

2.1. Isolation and culture of macrophages

In this study, 2 ~ 3 years old healthy *TLR4* overexpressing and wild type male sheep were provided by the Institute of Animal Science and Veterinary Medicine Tianjin Academy of Agricultural Sciences. Blood samples (10 ml) were collected aseptically from the jugular vein, and ovine peripheral blood lymphocyte separation solution (Tianjin Haoyang biological products, China) was added immediately thereafter. Following their isolation by density gradient centrifugation (2000 rpm for 20 min at 4 °C), the peripheral blood mononuclear cells (PBMC) were washed three times with sterile PBS. With the mononuclear cell pellet resuspended in RPMI 1640 medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, and 55 mM 2-mercaptoethanol at concentration of 1–2 × 10^6 cells/ml, cells were then cultured in plastic disposable flasks at 37 °C with 5% CO_2. The medium was changed every 3–4 days, and single adherent mononuclear cell was spontaneously differentiated into spindle-like macrophages to generate proliferating clone after 10 or 12 days (Fig. S1).

2.2. Immunofluorescent assay

The immunofluorescent assay was performed on monolayer cells in flat-bottomed 96-well tissue culture plates. Cells were washed three times with PBS and fixed with 4% paraformaldehyde for 20 min at 4 °C, and non-specific antibodies binding was blocked with 1% bovine serum albumin. The first antibodies CD14, F4/80 and CD11b (Bioss) were added and incubated at 4 °C for 12 h, then cells were washed three times with PBS before adding the fluorochrome-labelled second antibody (Bioss). Macrophages with fluorescent staining were observed with high content device (MDC) and the fluorescence intensity were evaluated and analyzed with high content analysis software (Molecular Device, USA).

2.3. Non-specific phagocytosis

Latex particle ingestion was carried out to evaluated the non-specific phagocytic capability of macrophages. At a ratio of 1:10, the latex particles (Sigma, USA) were added into the 96-well culture plates, and then incubated at 37 °C for 24 h. After washing three times with PBS, cells were stained with Hoechest 33,342 and ARG2 antibody, and fluorescent staining observed with high-content screening device. The phagocytosis and phagocytic rate were evaluated on the basis of at least 500 cells.

phagocytic rate = number of phagocytes /total number of cells.

2.4. Karyotype analysis

After 12–24 h of subculture, colchicine (2 μg/ml) was applied for at 37 °C for 6–8 h. The cells were then trypsinized in 3 ml and the cell suspensions poured into 15 ml centrifuge tubes with 8 ml 0.075 mol/L KCl solution for hypotonic treatment, and kept at room temperature for 30 min and centrifuged at 1000 rpm for 10 min. The cells were fixed with freshly orepared methanol-acetic acid (3:1) fixative, and the cool, moist microscope slides prepared and stained with Gimesa stain. The chromosomes were counted in 10 cells from each culture and 3 cells were photographed and karyotyped (MetaSystems, Ziess).

2.5. Southern blotting

The genetically modified (GM) sheep were identified by Southern blot (Roche Diagnostics, Mannheim, Germany) (Bai et al., 2015). The genomic DNA of ovine macrophages was extracted and digested by *HindIII*, and the integration of exogenous genes analyzed by Southern blotting. The probe was designed according to the DNA sequence of the 3' region of the third exon of TLR4 and 5' region of IRES, and then prepared by PCR using the following primer pairs: 5'-ACTGGTAAAGA ACTTGGAGGAGGG and 5'-GTTTCAGGTTCAGGGGGAGGTG-3'. The endogenous *TLR4* fragment measures 4700 bp, while the exogenous *TLR4* fragment 2771 bp.

2.6. Quantitative real-time polymerase chain reaction (qPCR)

1 μg/mL LPS (Sigma, USA) was used to stimulate the macrophages for 0, 0.5,1,2,4,8 and 12 h, and 15 μM Dynasore, 2 μM Amlexanox, 200 nM IRAK-4 Inhibitor (MCE, USA) were used to preprocess macrophages respectively. In addition, 50 μg/mL polyI:C (invivogen, USA) was used to stimulate the macrophages for 0, 2,4 and 8 h and the mRNA expression of related genes were detected and quantified using RT-PCR. Total RNA from stimulated macrophages was extracted with Trizol RNA reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA yield was evaluated by Nanodrop and integrity was assessed by gel electrophoresis demonstrating clear bands for 28 and 18 s rRNA. 1 μg of extracted RNA was reverse transcribed into cDNA with the Omniscript RT kit (Thermo, USA) by following the manufacturer's instructions. The cDNA was analyzed by qPCR, gene-specific primers, presented in the Table 1, were synthesized from Sangon (Shanghai, China). Each reaction was prepared with SYBR Green I nucleic acid stain (Promega, USA). RT-PCR amplification was carried out in MX3000 Q-PCR instrument (Agilent Technologies). Levels of mRNA expression were normalized to housekeeping gene *GAPDH* mRNA levels.

2.7. Flow cytometry

Single cell suspensions ($3–4 \times 10^6$ cells) were washed once with staining buffer (PBS containing 2.5% FBS) at 500 g at 4 °C for 5 min. After that, cells were blocked with 5% FBS for 30 min at 4 °C and stained for 20 min with anti-TLR4/PE and isotype control (Bioss, China) at 4 °C respectively. Cells were then washed additional three times with staining buffer, and data were acquired using BD FACS Calibur and analyzed with FlowJo software.

2.8. Western blotting

15 μM Dynasore, 2 μM Amlexanox, 200 nM IRAK-4 Inhibitor (MCE, USA) were used to preprocess macrophages respectively, 1 μg/ml LPS (Sigma, USA) was used to stimulate the macrophages for 0 h, 0.5 h, 1 h, 2 h and 4 h. Cultured macrophages ($3–4 \times 10^6$ cells/ml) washed twice with PBS and collected to a 1.5 ml tube. The nuclear protein and total protein were extracted (Invent Biotechnologies, USA and Beyotime, China) according to the manufacturer's protocols. Protein concentrations in the homogenates were determined with the micro BCA protein assay kit (Thermo Fisher Scientific, USA) by using an enzyme-labeling instrument. After being resuspended in SDS buffer and heated at 95 °C for 10 min, the protein samples, each approximately 10–20 μg, were loaded into separate lanes of a 6–10% gel for SDS-PAGE (Bio-Rad, USA). Upon electrophoretic separation, proteins were electro-transferred in transfer buffer to 0.22 μm polyvinylidene fluoride membranes (Millipore, USA), the membranes were then blocked with 2.5% bovine serum albumin (BSA) (Sigma, USA) in TBST (0.1% Tween in TBS) at 37 °C for 2 h and probed at 4 °C overnight with the antibodies: NF-κB (Abcam), p-Stat1, p42/44, p-JNK (Cell Signaling, USA), p-p38, IRAK4, IRF3 (Bioss), LMNB2, a-Tubulin (Beyotime). After washing three times with TBST, HRP-conjugated secondary antibodies (ZSGB-BOI, China) were applied at room temperature for 1 h. After washing again three times with TBST, the blot was developed with enhanced chemiluminescence system (Bio-Rad, USA) according to the manufacturer's instructions. The quantitative analysis was performed by ImageJ software and GraphPad Prism version 5.0 software.

2.9. Bacterial strain and culture

In this research, *Salmonella* Typhimurium CVCV541 was obtained from China Institute of Veterinary Drug Control Microbiological Culture Collection Center. The bacterial strain was cultured at 37 °C in Luria-Bertani (LB) broth to mid-log phase for later using.

2.10. Macrophages infection and CFU counts

In order to evaluate the phagocytic capability of the genetically modified (GM) and wild type (WT) macrophages, they were subjected to live *Salmonella*. Suspended and diluted to a concentration of 1×10^9/mL in RPMI 1640 medium (Thermo Fisher Scientific, USA) containing 10% fetal bovine serum (FBS) (Thermo Fisher Scientific, USA), *Salmonella* were then applied to infect the macrophages at two different multiplicity of infection (MOI) levels (1 and 5) respectively. After incubation for 30 min, the cells were washed three times. To eliminate extracellular bacteria, the RPMI-1640 supplemented with

Table 1
Gene-specific primers for quantitative polymerase chain reaction.

Gene	Forward primer	Reverse primer	Length (bp)
IRG1	AGGATGGTTCTGGATGCTCTG	TTGGTGGGAGCCTGAGTTCT	142
GARG16	GTGAGAAGTCTGGTTACCTGG	ATCCTTCCTCACAGTCCATC	166
IP10	CCTGGCACTGTGACTGTAAGC	TGGAGGGATGGCAGTGAAG	243
ISG15	GCAGTTCATCGCCCAGAAGA	ACCCTTGTCGTTCCTCACCA	187
MX1	AGTGAAGCCCAGATTGCCAT	CAAGTTGATGGTCTCCTGCCT	213
TNFα	TCTACTCGCAGGTCCTCTTC	TCGGCATAGTCCAGGTATTC	265
IL6	CTGCTGGTCTTCTGGAGTATC	TGTGGCTGGAGTGGTTATTAG	195
TLR4	ATCATCAGCGTGTCGGTTGTCA	GCAGCCAGCAAGAAGCATCAG	96
IFN-β	AATCGTCTGGAGCCAATCTG	GATGTTCAGTCACGGAGGT	220
GAPDH	CTGACCTGCCGCCTGGAGAAA	GTAGAAGAGTGAGTGTCGCTGTT	165

gentamicin (100 μg/mL) was added and kept for 1 h, and then replaced with less gentamincin (20 μg/mL). After 30 min, as a start (0 h) of salmonella invasion cells, the invasion index of GM and WT macrophages were observed. Meanwhile, after 12, 24, and 72 h, the cells were dissolved by 0.3% tritonX-100 (prepared in PBS) and the number of intracellular survival bacteria was verified by counting CFUs resulting from the plating of the dilutions.

Salmonella proliferation rate (%) = (the number of *Salmonella* within cells (24 h) – the number of *Salmonella* within cells (0 h))/ the number of *Salmonella* within cells (0 h) × 100%.

2.11. Statistical analysis

The data shown as means ± SD were obtained at least from three independent experiments. The differences observed among samples were determined by *t*-test, and differences would be considered statistically significant ($p < 0.05$) or extremely significant ($p < 0.01$).

3. Results

3.1. Isolation and identification of the macrophages from ovine peripheral blood

Involved in the inflammatory response against pathogenic infection, macrophages are key components in the innate immune system. To obtain monocyte-differentiated macrophages in vitro, Olivier's method for isolation of ovine blood monocyte-derived cells (Olivier et al., 2001) was modified. The isolated cells, spindle-like in form with rounded nuclei and several pseudopodia and vacuoles, bear strong morphologic resemblance to macrophages and thus should be so (Fig. 1A). For further confirmation, their phagocytosis was closely examined and the results showed that these cells possessed strong engulfing capacity, with phagocytic rate ranging around 78.55 ± 5.3 (Fig. 1B). Moreover, specific markers of macrophage, CD14, F4/80 and CD11b, were also detected on the cell membranes by antibodies staining (Fig. 1C, Fig. S2). Besides, the production of proinflammatory cytokines and chemokines in these cells also increased upon LPS stimulation (data not shown). Taking these observations together, the results showed clearly the cells derived from peripheral blood cultured in vitro were undoubtedly macrophages.

The karyotyping of BMs was performed at passage 20. As shown in Fig. 1D, each cell had 54 chromosomes, 6 large metacentrics, and 48 centrics. Since the number and morphology of chromosomes were quite normal, thus these BMs maintaining steady genome in vitro culture can be used as stable experimental materials for this research.

3.2. Identification of overexpression of TLR4 in ovine blood macrophages

To confirm the integration of *TLR4* expression vector (Fig. 2A) into the genome of the transgenic sheep bred in our previous study [26], the transgene *TLR4* was detected by Southern blotting. Restriction enzyme Hind III was adopted to dissect the genomes of the *TLR4* GM and WT sheep, and four sheep were detected to be carriers of the exogenous *TLR4* gene (Fig. 2B). To further investigate the expression of *TLR4* in ovine GM macrophages, their mRNA and total protein were evaluated by RT-PCR and Western blot respectively. Compared with WT macrophages, the expression of *TLR4* in GM macrophages was increased distinctly on mRNA and protein levels (Fig. 2C, D, E). Besides, the content of TLR4 on macrophages membrane was tested by flow cytometry, and the results showed that the levels of TLR4 on GM macrophages membrane, compare with the WT macrophages, were remarkably higher (Fig. 2F, G). Taken together, the acquired results revealed that ovine macrophages stably overexpressing *TLR4* had been obtained undoubtedly.

3.3. Overexpression of TLR4 promoted activation of IRAK4-dependent signaling by LPS stimulation

To elucidate how overexpression of *TLR4* influenced the production of proinflammatory cytokines in BMs at early stage of LPS treatment, the expression of *TNFα* and *IL-6* initiated by LPS was examined by RT-PCR. The results showed that overexpression of *TLR4* in GM macrophages significantly promoted the expression of *TNFα* at 0.5 h after LPS stimulation, whereas it was decreased, though not statistically significant, following LPS stimulation (Fig. 3A). The production of LPS-induced *IL-6* in *TLR4* overexpressing BMs remained higher at time points of 0 h and 0.5 h, it decreased dramatically after 8 h of LPS treatment in comparison with WT macrophages (Fig. 3B). These results indicated that the overexpression of *TLR4* only promoted the production of proinflammatory cytokines during the early stage of LPS stimulation.

Since LPS-initiated production of TNFα and IL-6 was related to MyD88-dependent NF-κB or MAPK activation (Jenkins and Mansell, 2010) (Fig. 3C), the activation of NF-κB and MAPK signaling in *TLR4* overexpressing and WT BMs stimulated with LPS was estimated to investigate how overexpression of *TLR4* elevated LPS-induced proinflammatory cytokines levels. It was found that in *TLR4* overexpressing BMs the level of IRAK4, an upstream NF-κB and MAPK, was increased greatly after 0.5 h and 1 h of LPS treatment but decreased gradually after 2 h–4 h compared with the control group without treatment (Fig. 3D, E), and similar changing patterns of IRAK4 expression were also observed in WT BMs (Fig. 3D, E). However, the overexpression of *TLR4*, compared with WT BMs, greatly enhanced the expression of LPS-induced IRAK4 after 0.5 and 1 h of LPS treatment (Fig. 3D, E). Meanwhile, IRAK4 inhibitor I strongly suppressed the production of LPS-induced IRAK4 in *TLR4* overexpressing and WT BMs at all time points (Fig. 3D, F, G). These results indicated that the expression of LPS-induced *IL-6* and *TNFα* was mediated by TLR4 signaling pathway in an IRAK4-dependent manner.

As MAPK plays an essential role in transducing the effects of LPS initiation of inflammatory responses via the phosphorylation of a range of downstream substrates (Arthur and Ley, 2013), the LPS-induced MAPK activation status in *TLR4* overexpressing and WT BMs was then investigated. The phosphorylation of JNK was induced with LPS treatment for 1 h and 2 h in both *TLR4* overexpressing and WT BMs against the control group without LPS treatment (Fig. 3D, E). *TLR4* overexpressing BMs showed significantly higher phosphorylation of JNK than WT BMs at 1 h and 2 h after LPS stimulation (Fig. 3D, E), whereas the levels of LPS-stimulated phosphorylated JNK in *TLR4* overexpressing and WT BMs were significantly impaired by IRAK4 inhibitor I (Fig. 3D, F, G). These data suggested that the TLR4 promoted LPS-induced phosphorylation of JNK was IRAK4 dependent. Besides, the phosphorylation of ERK1/2 in *TLR4* overexpressing BMs remained obviously higher at 0.5 and 1 h, but lower at 2 and 4 h after LPS stimulation compared with the control group without LPS stimulation (Fig. 3D, E). Whereas, the levels of LPS-induced phosphorylated ERK1/2 in WT BMs increased from 0.5 h to 4 h compared with cells in the absence of LPS stimulation and was markedly lower than that in *TLR4* overexpressing BMs at 1 h, but higher at 2–4 h (Fig. 3D, E). These data indicated that the activation of LPS-induced phosphorylated ERK1/2 in *TLR4* overexpressing BMs was accelerated. In addition, IRAK4 inhibitor I could inhibit the phosphorylation of ERK1/2 in *TLR4* overexpressing and WT BMs at all time points post LPS stimulation (Fig. 3D, F, G). These evidences revealed that overexpression of *TLR4* could activate LPS-induced ERK1/2 signaling dependently on IRAK4. However, phosphorylation of p38 in TLR4 overexpressing BMs and WT BMs remained relatively constant at all time-points regardless of LPS treatment or not (Fig. 3D).

As TLR4 can induce the expression of proinflammatory cytokines via activation of NF-κB, a downstream IRAK4 (Guijarro-Munoz et al., 2014), so the effect of TLR4 on activation of LPS-triggered NF-κB was

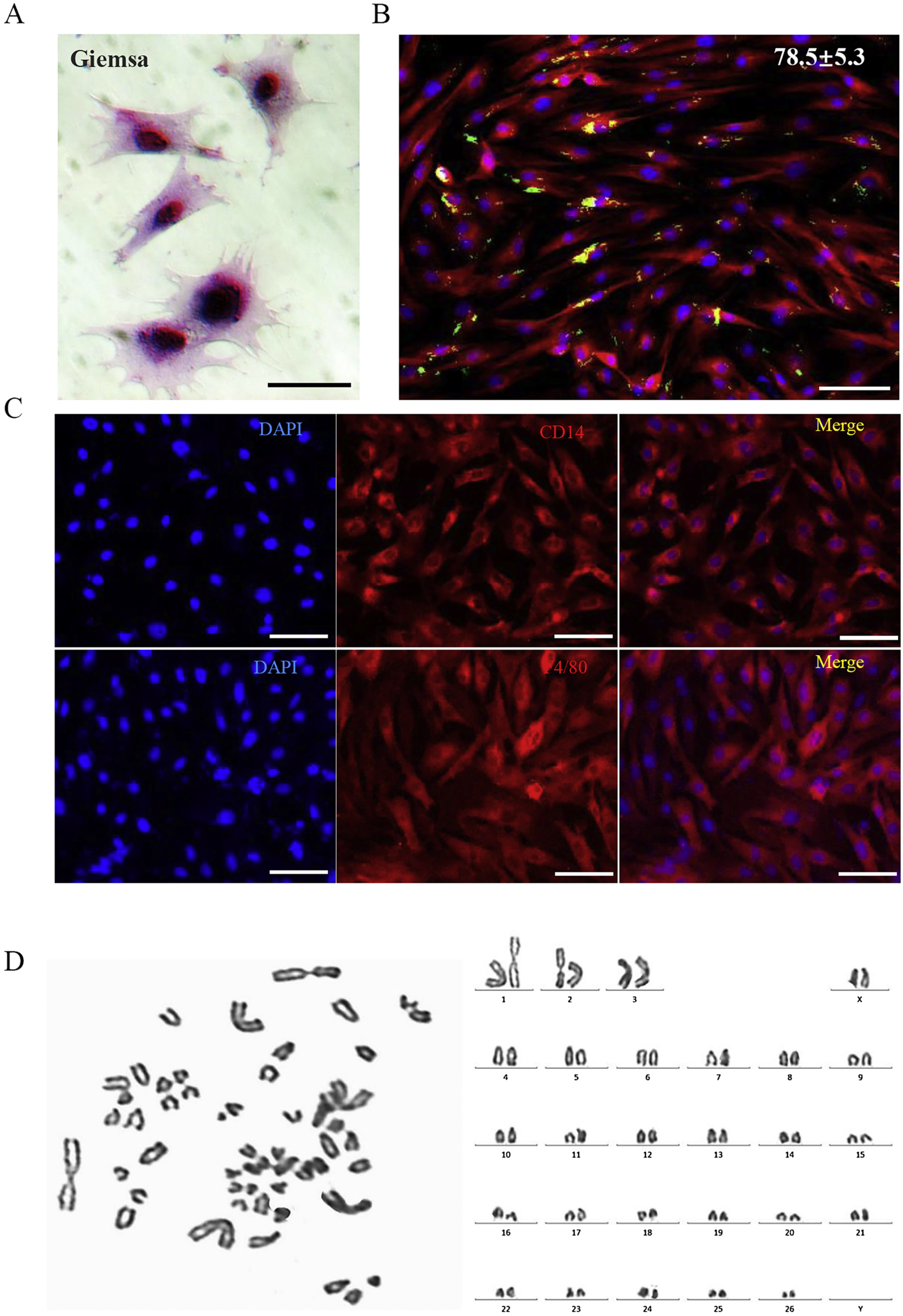


Fig. 1. Identification of ovine macrophages isolated from peripheral blood. (A) After being isolated from peripheral blood, the ovine BMs were cultured *in vitro* for 10–12 days, and then their morphology was highlighted by Giemsa staining and illustrated by microscopy. The isolated cells, long shuttle in form with rounded nucleus, have several pseudopodia and vacuoles. (B) The phagocytic capability of the cells was assessed by ingestion of latex particles, with the phagocytic rate calculated at least from 500 cells. (C) The cellular nuclei were stained by DAPI (Blue), and the expression of macrophage-specific proteins (CD14 and F4/80) were detected by immunofluorescence (Red). (D) The karyotype of BMs at passage 20 was analyzed. The chromosomes were photographed and counted by MetaSystems (scale bar: 100 μm). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

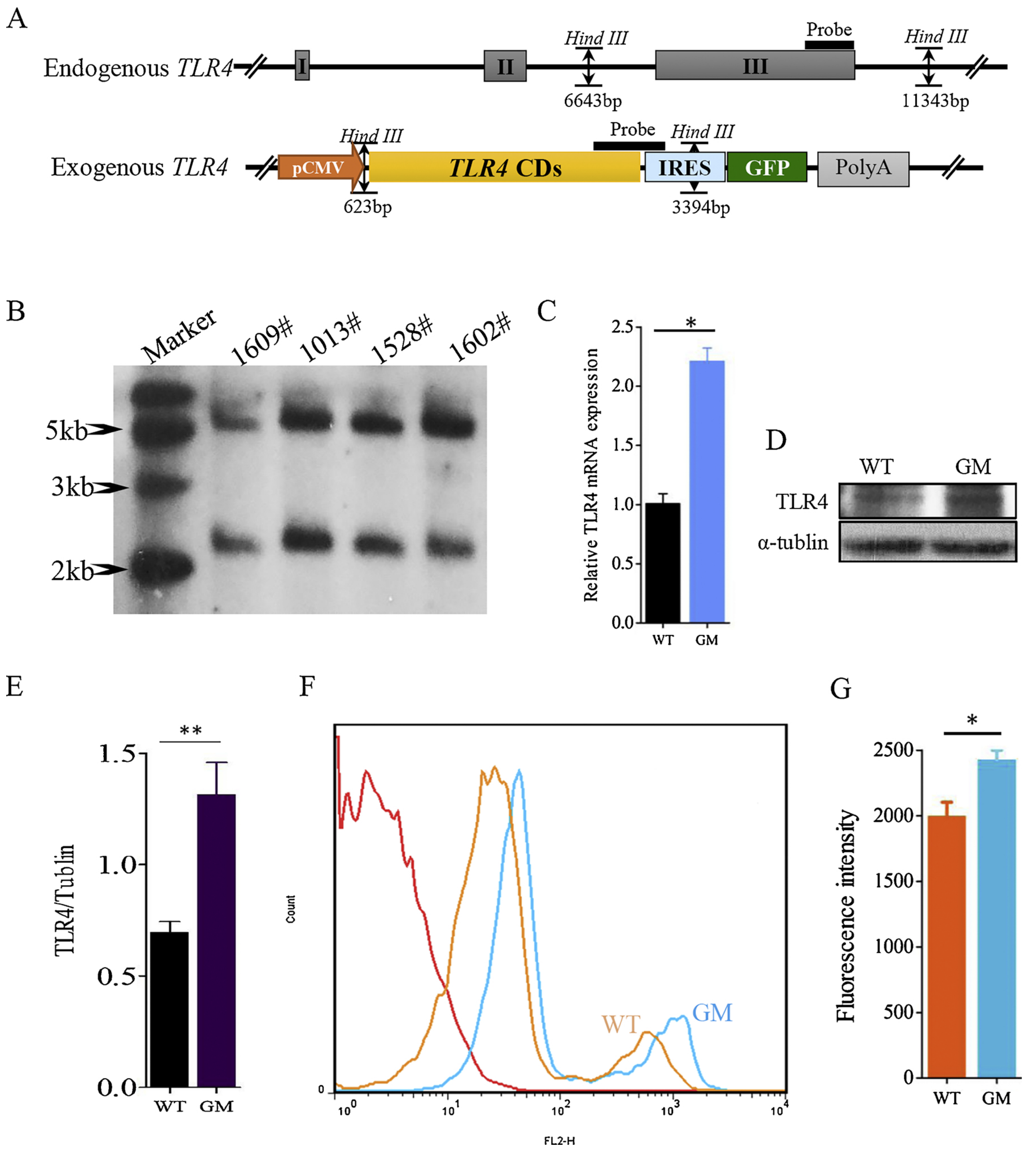
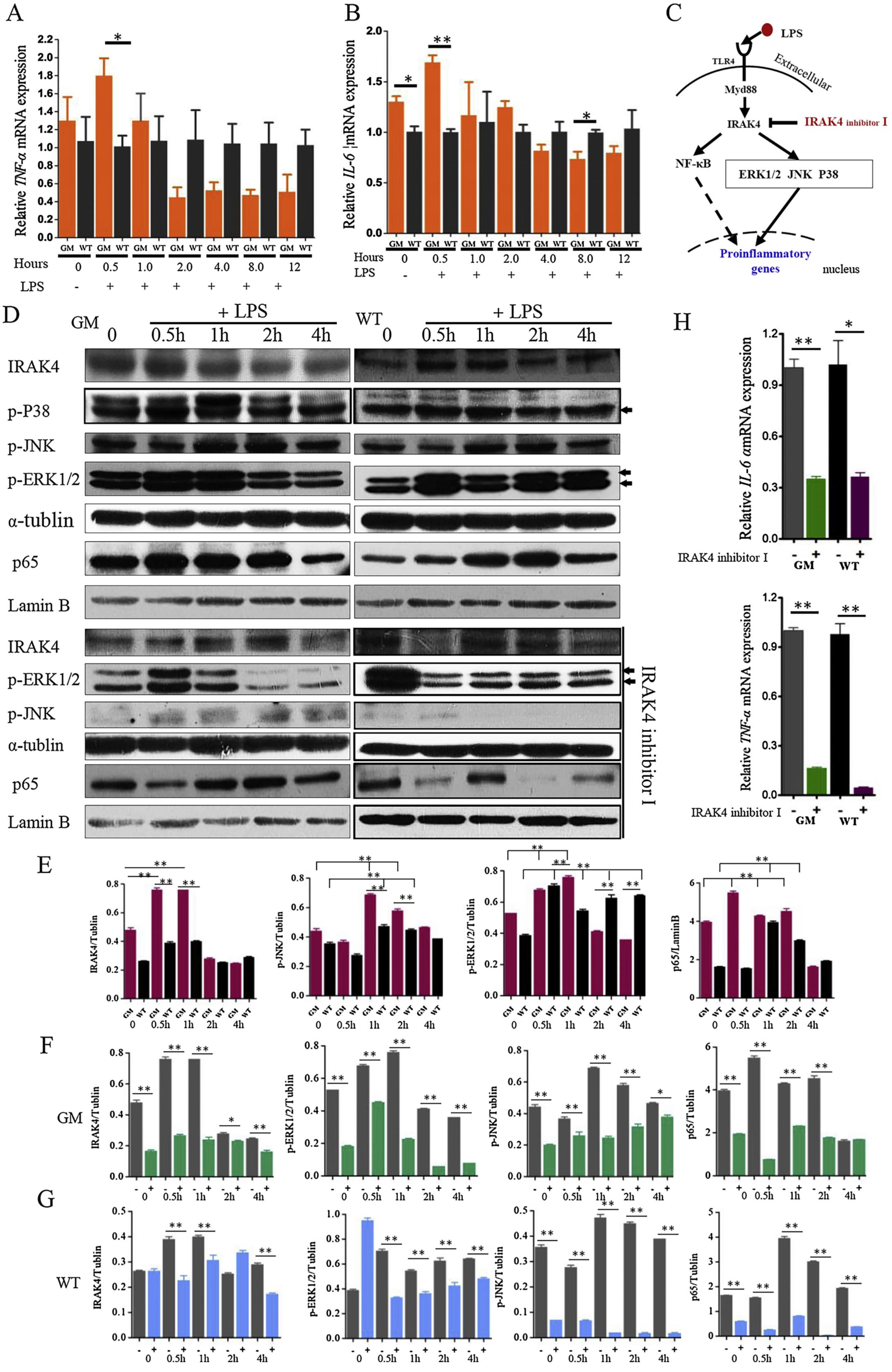


Fig. 2. Detection of overexpression of *TLR4* in ovine blood macrophages. The genomic integration and expression of exogenous *TLR4* were examined by different approaches. (A) Schematic diagram of the endogenous and exogenous *TLR4* gene structures. The endogenous *TLR4* consists of three exons and produced one 4700-bp fragment upon Hind III digestion, while the constructed exogenous *TLR4* vector, composed of the pCMV element controlling *TLR4* expression and IRES segment positioning between *TLR4* CDs and *GFP*, produced a 2771-bp fragment after Hind III digestion. The Southern blotting probe was designed according to the DNA sequence of the 3' region of the third exon of *TLR4* and the 5' region of IRES. (B) The integration of *TLR4* transgene into the cell genome was detected by Southern blotting. Four *TLR4* transgenic sheep were identified to carry both the endogenous 4700-bp *TLR4* band and the exogenous 2771-bp *TLR4* band. (C) The expression of *TLR4* mRNA in GM BMs was evaluated by qRT-PCR and proved to be 2.3 times of the expression in GM BMs (*$p < 0.05$). (D) The TLR4 protein level in both GM and WT BMs was examined by Western blotting. Compared with WT BMs, the TLR4 protein level in GM BMs was saliently higher. (E) The data of western blot were analyzed statistically (**$p < 0.01$). (F) The TLR4 content on the WT and GM BMs membrane was quantified by flow cytometry. (G) The fluorescence intensity showed that the TLR4 content on GM BMs membrane was remarkably higher than that on WT BMs membrane (*$p < 0.05$).

evaluated. After 0.5 h with LPS treatment, pronouncedly enhanced nuclear translocation of NF-κB subunit p65 was then detected in *TLR4* overexpressing BMs (Fig. 3D, E), and this effect sustained for 2 h and then attenuated distinctly by IRAK4 inhibitor I (Fig. 3D, F). In contrast, the content of NF-κB p65 within WT BMs was only increased after 1 and 2 h of LPS treatment and was impaired by IRAK4 inhibitor I at all time (Fig. 3D, G), which suggested that the NF-κB activation started slower than the process within *TLR4* overexpressing BMs. Moreover, it was found that production of *TNFα* and *IL-6* in *TLR4* overexpressing and WT BMs was distinctly impaired with IRAK4 inhibitor I (Fig. 3H). Collectively, these results indicated that TLR4 could promote the early expression of LPS-induced proinflammatory cytokines via activation of IRAK4-dependent JNK, ERK1/2 and NF-κB signaling.



(caption on next page)

Fig. 3. Overexpression of *TLR4* promoted activation of LPS-induced MyD88-dependent signaling. (A) and (B) The expression of TNFα and IL-6 mRNA in BMs was examined by qRT-PCR at different time points (0.5, 1, 2, 4, 8, and 12 h) after LPS treatment (*$p < 0.05$). (C) The schematic diagram illustrated the TLR4 mediated MyD88-dependent NF-κB and MAPK signaling pathways. (D) The activation of proteins involved in NF-κB and MAPK signaling in GM and WT BMs was detected by immunoblot at different time points (0, 0.5, 1, 2, 4 h) after LPS stimulation. LPS-induced IRAK4 levels and phosphorylated p38, JNK and ERK1/2 were analyzed with IRAK4 inhibitor I pretreatment or not, respectively. The LPS-induced NF-κB subunit p65 content within cellular nuclear was examined with IRAK4 inhibitor I pretreatment or not respectively, with immunoblots for tubulin serving as loading controls. The arrows indicated target bands. (E) The data of western blot in GM and WT BMs were analyzed statistically (*$p < 0.05$, **$p < 0.01$). (F) The data of western blot, in GM BMs with IRAK4 inhibitor I or not, were analyzed statistically (*$p < 0.05$, **$p < 0.01$). (G) The data of western blot, in WT BMs with IRAK4 inhibitor I or not, were analyzed statistically (*$p < 0.05$, **$p < 0.01$). (H) The expression of LPS-induced *TNFα* and *IL-6* mRNA in GM and WT BMs was examined by qRT-PCR with IRAK4 inhibitor I pretreatment or not respectively. (**$p < 0.01$).

3.4. Overexpression of TLR4 accelerated activation of LPS-induced TBK1-dependent pathway via its internalization

Compare with the WT BMs, the expressions of *TNFα* and *IL-6* in *TLR4* overexpressing BMs were much lower after 1 h of LPS stimulation (Fig. 3A, B), and such decreased expression might result from the intensified internalization of TLR4 on membranes, as its internalization into cytoplasm had been reported as one of the negative regulatory mechanisms for impairing inflammatory response to LPS (Balachandran, 2016). To test our hypothesis, the LPS-induced endocytosis of extracellular TLR4 was first detected by flow cytometry, in reference to the cellular loss of surface expression of TLR4 as an index (Zanoni et al., 2011). And the index of LPS-induced endocytosis of TLR4 in GM BMs only got prominently higher since 2 h with LPS treatment compared with WT BMs (Fig. 4A). Moreover, it was also observed that the diminution of membranous TLR4 was obviously impaired by dynasore (Fig. 4B), an inhibitor of dynamin GTPases that mainly controls the endocytic processes in mammalian cells (Kagan et al., 2008). These data showed that overexpression of *TLR4* had facilitated its own LPS-induced internalization in late stage.

The LPS-TLR4 complexes, once forming endosome, then trigger the TRIF-dependent signaling pathway, and thereby induce the activation of TRAF-family-member-associated NF-κB activator (TANK)-binding kinase 1 (TBK1) and lead to the nuclear translocation of IRF3 and then induction of GARG16, IRG1and IP10 (Kagan et al., 2008; Tan et al., 2015) (Fig. 4C). The intranuclear IRF3 content in both *TLR4* overexpressing BMs and WT BMs increased markedly from 1 to 8 h after LPS treatment compared with control group (0 h), but its content level in *TLR4* overexpressing BMs was higher than that in WT BMs (Fig. 4D, E). At the same time, overexpression of *TLR4* also promoted the expression of IRF3-dependent GARG16, IRG1and IP10 from 1 h to last detection time point after LPS treatment compared with WT BMs (Fig. 4J), which demonstrated that TLR4 overexpression had activated LPS-induced TBK1-dependent signaling at late phase.

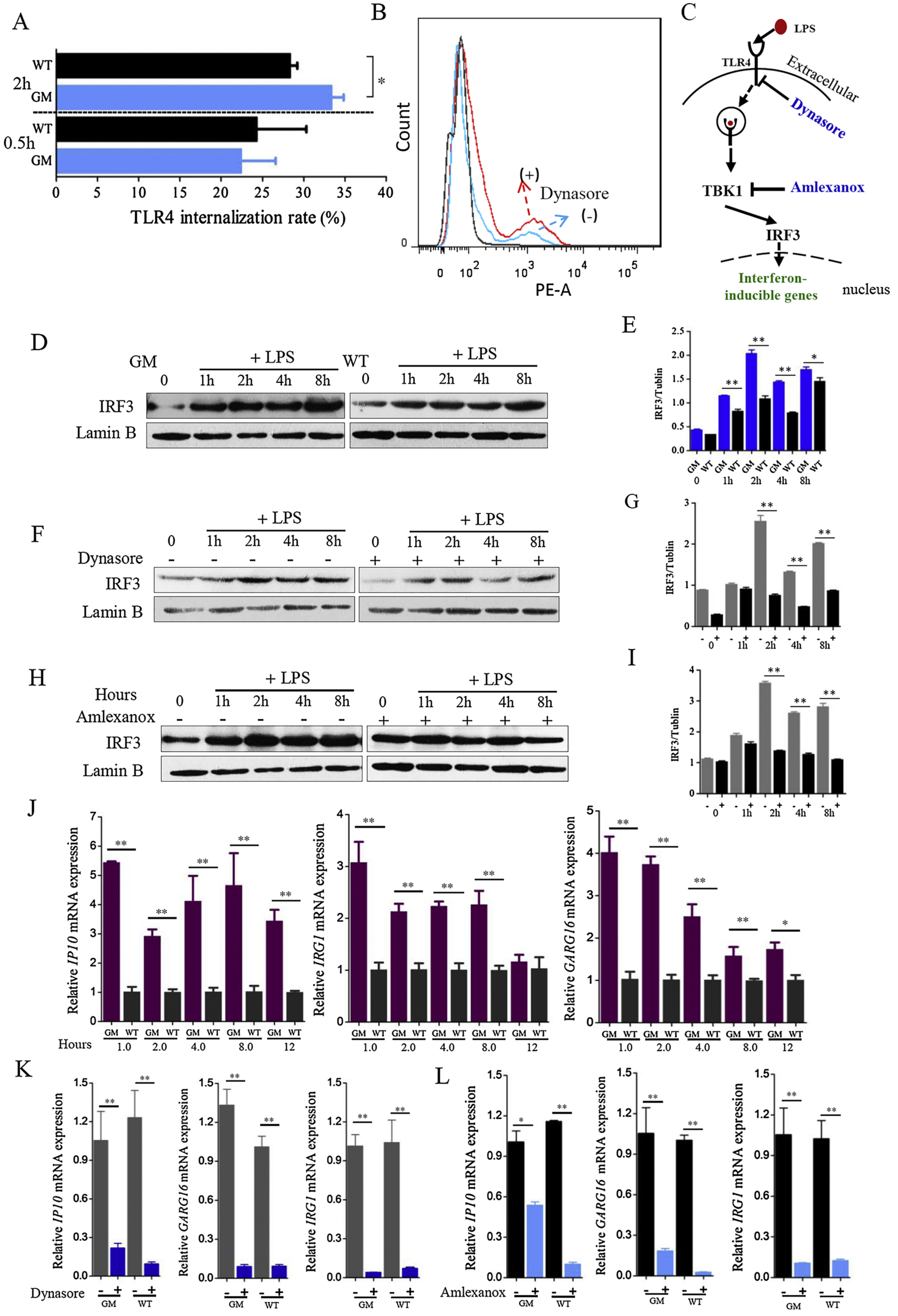
Meanwhile, nuclear import of IRF3 in *TLR4* overexpressing BMs treated with LPS, in comparison with the control group without treatment, was decreased pronouncedly as a result of the endocytosis inhibitor Dynasore (Fig. 4F, G). Moreover, Dynasore was also found accountable for the significant drop in expression of LPS-induced GARG16, IRG1and IP10 in *TLR4* overexpressing BMs and WT BMs (Fig. 4K). In addition, the activation of LPS-induced IRF3 in *TLR4* overexpressing BMs was obviously weakened by TBK1 inhibitor Amlexanox (Fig. 4H, I), and the expression of GARG16, IRG1 and IP10 in both *TLR4* overexpressing BMs and WT BMs was also inhibited by Amlexanox could (Fig. 4L). These data suggested that endocytosis of TLR4 would first activate LPS-induced TRIF-dependent IRF3 signaling pathway, then promoted the expression of GARG16, IRG1and IP10. To sum up, these results proved that overexpression of *TLR4* could enhance endocytosis of TLR4 and activate TBK1-dependent IRF3 signaling in BMs upon LPS stimulation.

3.5. Overexpression of TLR4 triggered LPS-initiated antiviral signaling

As the production of IFN-β could be induced by TRIF-dependent IRF3 activation (Akira and Takeda, 2004) (Fig. 5A), the expression of LPS-induced *IFN-β* in *TLR4* overexpressing and WT BMs was then examined. As expected, the mRNA level of IRF3's target gene *IFN-β* in *TLR4* overexpressing BMs increased significantly at 2, 4, and 8 h after LPS treatment than that in WT BMs (Fig. 5B), and Dynasore treatment could dramatically attenuate the mRNA levels of LPS-induced *IFN-β* in *TLR4* overexpressing BMs (Fig. 5C), which further confirmed that the induction of IFN-β operated independently from the endocytosis of TLR4. Similarly, the effect of TLR4 overexpression on promoting the expression of IFN-β was notably impaired by TBK1 inhibitor Amlexanox in BMs (Fig. 5D), demonstrating that TLR4-TBK1-IRF3-mediated signaling modulated the expression of IFN-β. Taken together, overexpression of *TLR4* could promote the expression of LPS-induced IFN-β through TLR4 endocytosis and activate TBK1-IRF3-dependent pathway. To further explore the effect of TLR4 on the antiviral performances of BMs, downstream functions of increased IFN-β should also be examined. After binding to type I IFN receptor (IFNAR), IFN-β first would activate the JAK pathway, and then phosphorylate the signal transducers and activators of transcription (STAT) to initiate expression of antiviral genes, for example, Mx1 and ISG15 (Greenhill et al., 2011; Kagan et al., 2008) (Fig. 5A). Indeed, in contrast to the group without LPS treatment, phosphorylation of LPS-induced STAT1 in *TLR4* overexpressing BMs increased significantly from 2 to 8 h, with the phosphorylation reaching peak at 2 h (Fig. 5E, F). Whereas the phosphorylation of LPS-induced STAT1 in WT BMs only intensified significantly at 2 h compared with the group without LPS treatment, its intensity being markedly lower than that in *TLR4* overexpressing BMs (Fig. 5E, F), thus supporting a scenario that IFN-β increase resulting from overexpression of *TLR4* prompted autocrine activation of IFNAR and then enhanced phosphorylation of STAT1 in response to LPS stimulation. Finally, the activated status of pSTAT1-dependent antivirus related genes were examined, and it was found that significant differences existed in the mRNA levels of LPS-induced *Mx1* and *ISG15* between *TLR4* overexpressing BMs and WT BMs from 2 to 8 h (Fig. 5G), which revealed that TLR4 could enhance the expression of LPS-induced antiviral genes *Mx1* and *ISG15*. Besides, the expression of *Mx1* and *ISG15* increased in *TLR4* overexpressing BMs and WT BMs at different time after poly I:C stimulation compared to 0 h control. However, the fold change of *TLR4* overexpressing BMs was higher than that of WT BMs (Fig.S3). Thus, it can be confirmed that TLR4 could prompt the activation of antiviral IRF3-IFN-β-pSTAT signal cascade by LPS-induced TLR4 internalization.

3.6. Overexpression of TLR4 contributed to the clearance of S. Typhimurium within BMs

Being the major components of the mononuclear phagocytic system (MPS), macrophage is not only attacked as target cells by salmonella, but also essential for host immune defense against invading salmonella. TLR4-mediated signaling pathway is of critical importance in controlling S. Typhimurium growth in macrophages (Arpaia et al., 2011; Talbot et al., 2009), we determined the effect of TLR4 on control of *S. Typhimurium* growth within BMs. *TLR4* overexpressing BMs and WT BMs were infected with live *S. Typhimurium* at two different MOI levels (1 and 5). The bacterial burden (the number of *Salmonella* per well at 30 min after salmonella infection) upon *TLR4* overexpressing BMs, in



(caption on next page)

Fig. 4. Overexpression of *TLR4* accelerated activation of LPS-induced TRIF-dependent pathway. (A) The LPS-induced endocytosis of extracellular TLR4 was detected by a highly sensitive flow cytometry, using the loss of the cell surface expression as an index for TLR4 endocytosis. The TLR4 internalization rate of GM BMs and WT BMs was calculated 0.5 and 2 h after LPS treatment. (B) The amount of surface TLR4 on GM BMs was tested with dynasore treatment or not after LPS stimulation. (C) The schematic diagram illustrated the TLR4 mediated TRIF-dependent IRF3 signaling pathway and the inhibitor for dynamin and TBK1. (D) The levels of nuclear import of IRF3 in GM and WT BMs were detected by immunoblot at different time points (0, 1, 2, 4, 8 h) respectively after LPS stimulation, with Lamin B serving as a loading control. (E) The data of western blot in GM and WT BMs were analyzed statistically (*$p < 0.05$, **$p < 0.01$). (F) The levels of nuclear import of IRF3 in GM or WT BMs were detected by immunoblot at different time points (0, 1, 2, 4, 8 h) after LPS stimulation with dynamin inhibitor dynasore treatment or not respectively. (G) The data of western blot, in GM BMs with dynasore treatment or not respectively, were analyzed statistically (**$p < 0.01$). (H) The levels of nuclear import of IRF3 in GM or WT BMs were detected by immunoblot at different durations (0, 1, 2, 4, 8 h) of LPS stimulation with TBK1 inhibitor Amlexanox treatment or not. (I) The data of western blot, in GM BMs with Amlexanox treatment or not respectively, were analyzed statistically (**$p < 0.01$). (J) The expression of *GARG16*, *IRG1*and *IP10* mRNA in GM or WT BMs was examined by qRT-PCR at different durations (1, 2, 4, 8, and 12 h) of LPS treatment (*$p < 0.05$, **$p < 0.01$). (K) The expression of *GARG16*, *IRG1*and *IP10* mRNA in GM or WT BMs was examined by qRT-PCR after 2 h of LPS stimulation with dynasore treatment or not (**$p < 0.01$). (L) The expression of *GARG16*, *IRG1*and *IP10* mRNA in GM or WT BMs was examined by qRT-PCR after 2 h of LPS stimulation with Amlexanox treatment or not (*$p < 0.05$, **$p < 0.01$).

comparison with WT BMs, was significantly higher at a MOI of 5 but not at 1 (Fig. 6A), thus showing that overexpression of *TLR4* can enhance the infection rate of *S. Typhimurium* in BMs at a higher MOI. To investigate the role TLR4 plays in controlling bacterial growth, the proliferation rate of *S. Typhimurium* in *TLR4* overexpressing BMs and WT BMs after 12, 24, and 72 h post infection were examined. The proliferation rate of *S. Typhimurium* within WT BMs exhibited a dynamic pattern, as it reached its peak after 24 h post infection but declined rapidly after 72 h regardless of MOI level, while the rate within *TLR4* overexpressing BMs showed sustained declining from 12 to 72 h post infection. The proliferation rate of *S. Typhimurium* within *TLR4* overexpressing BMs at 12 h post infection appeared significantly higher than that of WT BMs, but it declined much lower at 24 and 72 h post infection (Fig. 6B, C). Taken together, these data demonstrated that TLR4 can govern *S. Typhimurium* growth effectively within BMs, though a surge of *S. Typhimurium* would occur in the initial stage post infection.

Considering that TLR4 signaling can control early *S. Typhimurium* growth in mouse typhoid by TRIF-dependent pathway(Talbot et al., 2009), we therefore assessed the effect of LPS-induced, TRIF-dependent signaling on TLR4 overexpression controlling *S. Typhimurium* growth. The results mentioned above reveal that overexpression of *TLR4* could activate TRIF-dependent signaling greatly at 2 h post LPS treatment. Pretreated with LPS for 2 h, both *TLR4* overexpressing BMs and WT BMs were then subjected to *S. Typhimurium* infection at a MOI of 5, and the bacterial growth rate within *TLR4* overexpressing BMs was obviously lower than that in WT BMs after 24 h post infection (Fig. 6D). Meanwhile, LPS pretreatment could distinctly control *S. Typhimurium* growth within *TLR4* overexpressing BMs after 12 h post challenge compared the control group without LPS pretreatment (Fig. 6D). Collectively, these results demonstrated that *TLR4* overexpression might activate TRIF-dependent signaling to control effectively *S. Typhimurium* growth within BMs.

4. Discussion

Being critical for host defense against invading pathogens, TLR4 plays a key in the innate immune system, and study has shown that its deficiency or dysfunction in mice increased their susceptibility to intracellular bacteria (Arpaia et al., 2011; Li and Cherayil, 2003). In our previous research, transgenic sheep overexpressing *TLR4* were bred to improve their resistance against such certain bacterial diseases, such as brucellosis and salmonellosis (Bai et al., 2015). By using the macrophages isolated from peripheral blood of these transgenic sheep, this study investigated the expression of *TLR4* at transcription and translation levels was observed to be significantly higher than that in WT BMs (Fig. 1).

To elucidate the mechanism involved with the TLR4-triggered inflammatory response, the effect of overexpression of *TLR4* on the proinflammatory mediator TNF-α was explored. Consistent with other research that TNF-α was activated in macrophages and monocytes in response to LPS within 1 h (Ira Adams-Chapman and Stoll (2001)), our results showed that overexpression of *TLR4* promoted the high-level production of TNF-α mRNA at 0.5 h but not at other time points after LPS treatment in comparison with WT BMs (Fig. 2), contributing to initiating a brisk inflammatory response for pathogen containment and elimination (Rothe et al., 1993; Serbina et al., 2003; White et al., 2000). Besides, it was also found that increased production of LPS-induced TNF-α mRNA at early stage in *TLR4* overexpressing BMs was highly dependent upon the activation of both NF-κB and MAPK (ERK1/2 and JNK) signaling, as NF-κB and ERK1/2 signaling were activated in *TLR4* overexpressing BMs at 0.5 h after LPS treatment, while JNK signaling was delayed and activated at 1 h after LPS treatment (Fig. 2). Thus, overexpression of *TLR4* activated NF-κB and ERK1/2 signaling was responsible for LPS-induced early expression of TNF-α.

Based on the fact that the activation of NF-κB and ERK1/2 in *TLR4* overexpressing BMs gradually attenuated as the duration of LPS treatment lengthened and LPS-induced TNF-α mRNA levels also showed a modest decrease from 1 to 12 h after LPS treatment (Fig. 2), it can then be concluded that a negative regulated mechanism for activation of TLR4 signaling might occur in *TLR4* overexpressing BMs, and the internalization of the receptor into cytoplasm was quite accountable for this negative regulation as it had been reported to impair inflammatory response in LPS-induced TLR4 signal pathway (Aerbajinai et al., 2013; Balachandran, 2016). TLRs could be internalized into subcellular organelles for degradation and termination of inflammatory signaling (Cao, 2016). This study found that the internalization of surface TLR4 increased greatly at 2 h after LPS treatment in *TLR4* overexpressing BMs compared to that in WT BMs in dynamin-dependent fashion (Fig. 3). Consistent with other studies (Ghosh et al., 2015; Zanoni et al., 2011), out results suggested that the activation of TLR4-mediated Myd88-dependent signaling was impaired via internalization pathway, as TLR4-mediated TRIF-dependent pathway could be activated upon TLR4 endocytosis, thus leading to IRF3 translocation to the nucleus and subsequently inducing expression of GARG16, IRG1and IP10 (Husebye et al., 2006; Kagan et al., 2008; Kawai et al., 2001). Indeed, it was found that *TLR4* overexpression facilitated activation of LPS-induced IRF3, but the activation could also be impaired by dynamin inhibitor Dynasore and TBK1 inhibitor Amlexanox (Fig. 4), which demonstrated that the activation of LPS-induced IRF3 required dynamin-dependent TLR4 internalization and subsequently TRIF-dependent signaling.

As a potent antimicrobial agent against *E. coli* and *L. monocytogenes* infection (Cole et al., 2001), IP10 plays an important role in recruiting activated T cells into site of tissue inflammation, as IP10-deficient mice were reported to have impaired T cell responses (Dufour et al., 2002; Sauty et al., 1999). Compared with WT BMs, *TLR4* overexpressing BMs produced a high-level IP10 after LPS stimulation (Fig. 4), indicating that *TLR4* overexpressing sheep could enhance T cell response effectively and thus contributed greatly to host defense against pathogens. Here, our study showed that the expression of IRG1 was strictly regulated in *TLR4* overexpressing BMs with LPS treatment (Fig. 4), which was similar to previous report that LPS-tolerized mouse macrophages exhibited an increased the production of IRG1(Li et al., 2013).

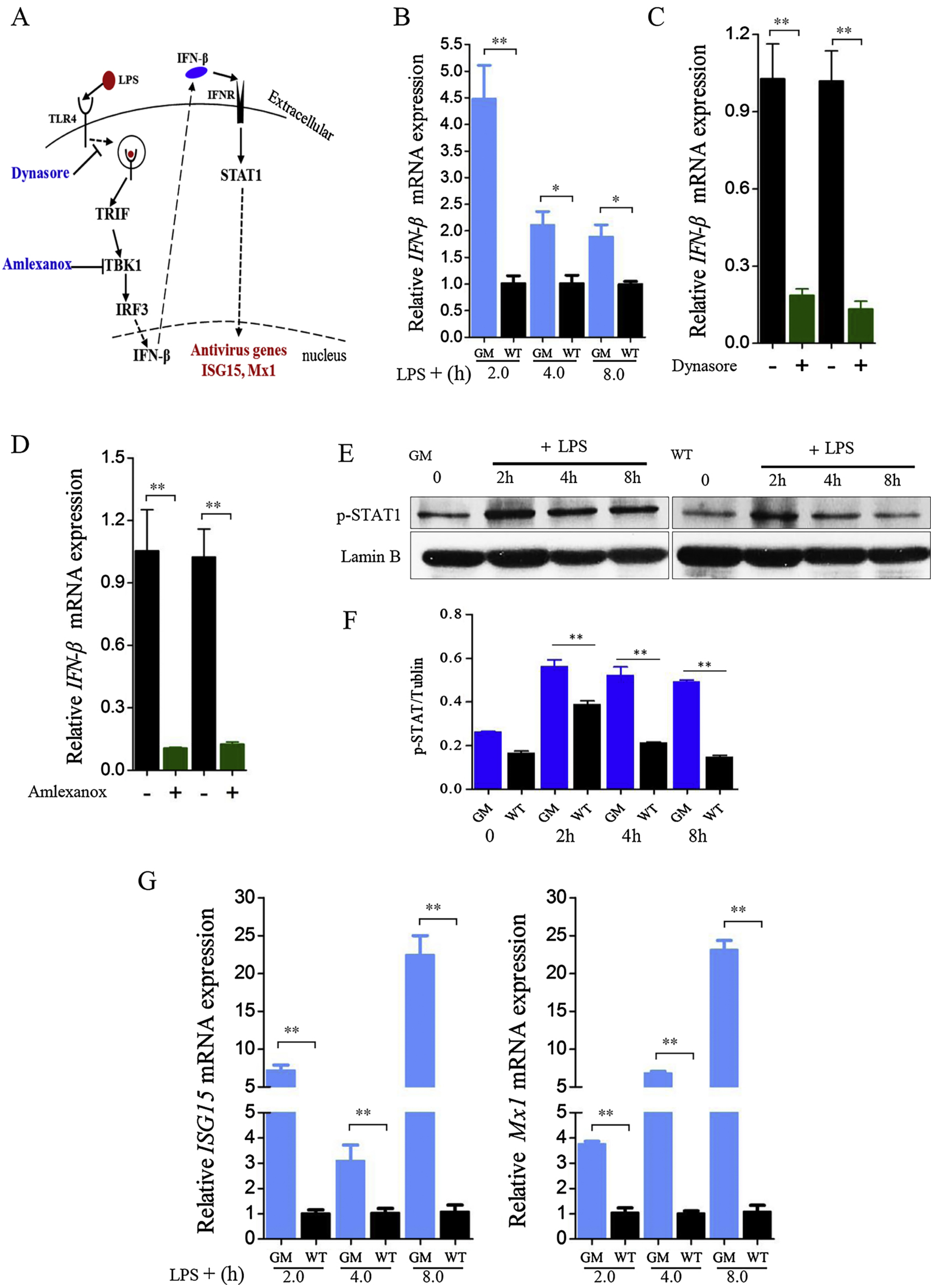


Fig. 5. Overexpression of *TLR4* triggered LPS-initiated antiviral signaling in BMs. (A) The schematic diagram for TLR4 mediated TRIF-dependent IRF3 signaling pathway and IFN-β activating STAT signaling pathway. (B) The expression of *IFN-β* mRNA in GM or WT BMs was examined by qRT-PCR respectively after 2, 4, and 8 h of LPS stimulation ($*p < 0.05$, $**p < 0.01$). (C) The expression of *IFN-β* mRNA in GM BMs was examined by qRT-PCR after 2 h of LPS stimulation with dynasore treatment or not respectively ($*p < 0.05$, $**p < 0.01$). (D) The expression of *IFN-β* mRNA in GM BMs was examined by qRT-PCR after 2 h of LPS stimulation with Amlexanox treatment or not respectively ($*p < 0.05$, $**p < 0.01$). (E) The phosphorylated STAT1 in GM or WT BMs was analyzed by immunoblot at different durations (0, 2, 4, 8 h) of LPS stimulation, with Lamin B serving as a loading control. (F) The data of western blot in GM and WT BMs were analyzed statistically ($*p < 0.05$, $**p < 0.01$). (G) The mRNA levels of LPS-induced Mx1 and ISG15 in GM BMs and WT BMs were examined by qRT-PCR after 2, 4, and 8 h of LPS stimulation ($**p < 0.01$).

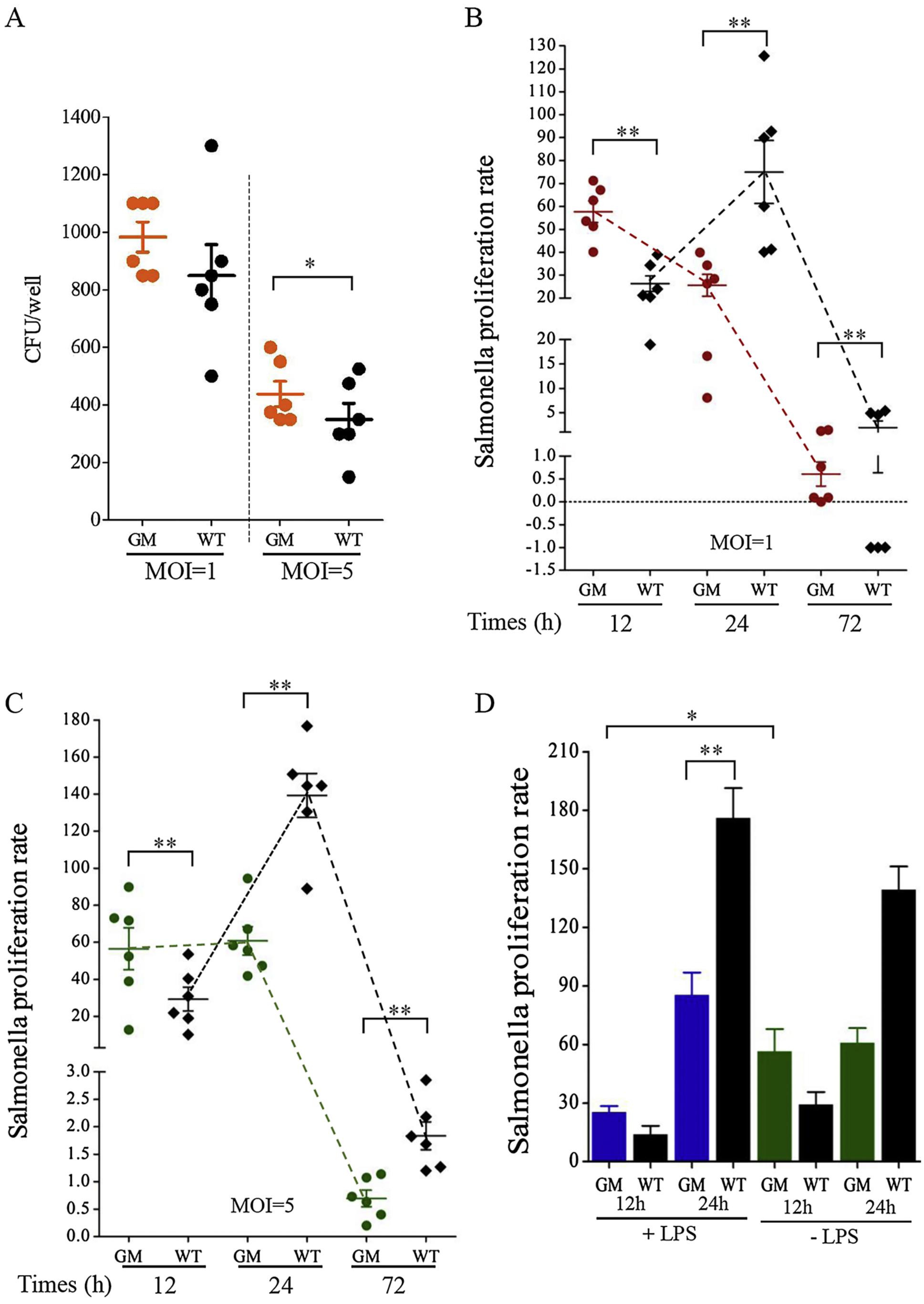


Fig. 6. Overexpression of *TLR4* helped to eradicate *S. Typhimurium* within BMs. (A) The bacterial burden (the number of per milliliter lysis) within GM or WT BMs was tested respectively by CFU count after 30 min post infection with *S. Typhimurium* at different MOI (1 and 5) (*p < 0.05). (B) The bacterial burden within GM and WT BMs was tested after 12, 24, and 72 h post infection at a MOI of 1, and the proliferation rate was calculated (**p < 0.01). (C) The bacterial burden within GM or WT BMs was tested after 12, 24, and 72 h post infection with *S. Typhimurium* at a MOI of 5, and the proliferation rate was calculated (**p < 0.01). (D) After pretreatment with LPS for 2 h, GM BMs or WT BMs were then infected with *S. Typhimurium* (MOI = 5). The bacterial burden was tested after 12 h and 24 h post infection, and the proliferation rate of *S. Typhimurium* was calculated (*p < 0.05, **p < 0.01).

Excessive inflammation may bring devastating consequences such as lethal sepsis and organ damage. IRG1 significantly suppressed TLR-triggered production of proinflammatory cytokines TNF-α, IL-6, and IFN-β in LPS-tolerized macrophages(Li et al., 2013), indicating IRG1 could impair the inflammation. Considering all sides, it can then be inferred that overexpression of *TLR4* could enhance the production of IRG1 to suppress excessive inflammatory responses to maintain the balance between pro-inflammatory and anti-inflammatory responses. Besides, IRG1 also functioned inside the mitochondria of activated macrophage lineage cells to enhance the utilization of energetically efficient fatty acid β-oxidation, resulting in increasing oxidative phosphorylation (OXPHOS) that amplifies mitochondria derived reactive oxygen species (mROS) production and thereby enhances bactericidal activity of macrophages (Hall et al., 2013). IRG1 expression levels correlate closely with the amounts of itaconic acid, a metabolite inhibiting the growth of such bacteria,as *Salmonella enterica* and *Mycobacterium tuberculosis* that produce isocitrate lyase. Moreover, Irg1 gene silencing in macrophages resulted in significant decrease in intracellular itaconic acid and antimicrobial activity during bacterial infection (Michelucci et al., 2013). These evidences demonstrated that overexpression of *TLR4* could help eliminate pathogenic bacteria within macrophages.

Other studies have demonstrated that TLR4 signaling mediated a specific gene program responsible for innate antiviral responses(Doyle et al., 2002; Hertzog et al., 2003), and LPS could directly inhibit the replication of murine γ herpesvirus 68 (MHV68) (Doyle et al., 2002). Accordingly, the effect of overexpression of *TLR4* on the activation of genes involved in host innate antiviral responses was examined. Activation of the transcription IRF3 was essential for innate immune response to viral infection (Andersen et al., 2008). The activation IRF3

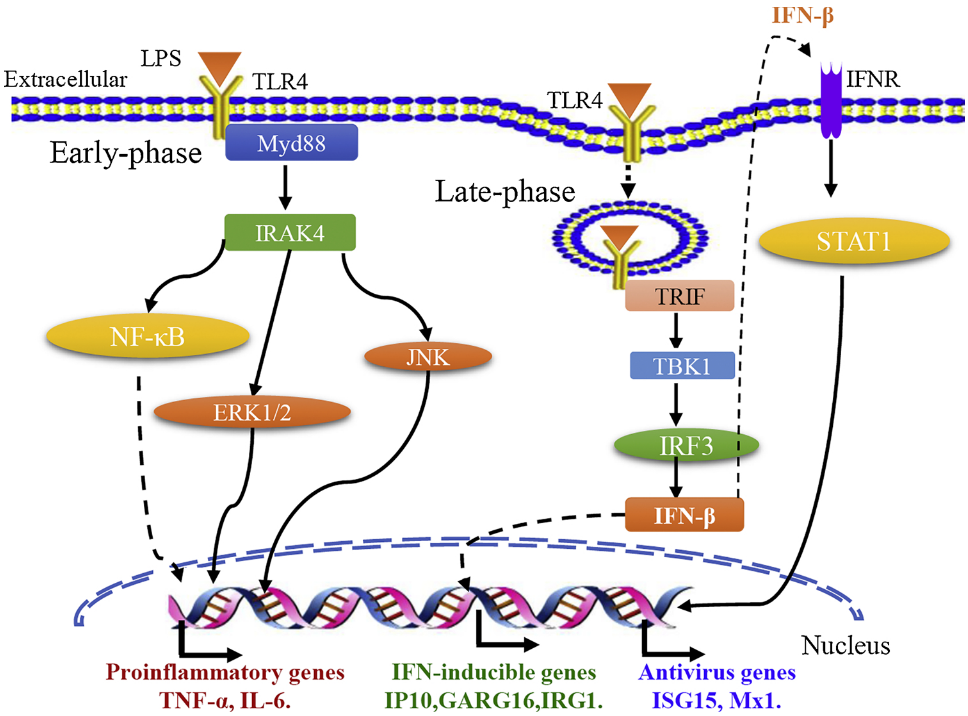


Fig. 7. Model of overexpression of *TLR4* activating Myd88-dependent/independent pathways. TLR4 overexpression at early stage activated the LPS-induced Myd88-dependent NF-κB and MAPK (ERK1/2 and JNK) signaling pathway, thereby induced the expression of proinflammatory genes. While at late stage, TLR4 overexpression activates LPS-induced TRIF-dependent IRF3 signaling pathway to induce IFN-inducible genes. Meanwhile, IFN-β, induced by IRF3, activated the phosphorylation of STAT1 and thus promotes the expressions of several antiviral genes.

levels upon LPS stimulation in *TLR4* overexpressing MBs were clearly higher than that in WT BMs (Fig. 4), suggesting that *TLR4* overexpression could promote innate antiviral effect by upregulating IRF3 expression. IFN-β was a key factor for host defense against various viral infections, and its expression was highly dependent on IRF3 (Hertzog et al., 2003; Sooryanarain et al., 2017). Our data showed that *TLR4* overexpression increased the expression of LPS-induced IFN-β by TBK1-IRF3 signaling (Fig. 5), which further confirmed the results observed in other related studies (Doyle et al., 2002). After binding to IFNAR receptor, IFN-β then activated STAT pathway to induce a bunch of secondary response genes by an autocrine/paracrine loop(Doyle et al., 2002; Hertzog et al., 2003). Our results and other data indicated that LPS could induce the rapid phosphorylation of STAT1 after 2 h of treatment (Doyle et al., 2002), and the phosphorylation rate in TLR4 overexpressing BMs was higher compared with WT BMs after LPS treatment (Fig. 5), which further revealed that *TLR4* overexpressing BMs might possess better antiviral capability against bacterial infection.

Recent research have shown that ISG15 plays an antiviral role against many pathogens in vitro and in vivo (Dai et al., 2011), as mice lacking ISG15 were highly susceptible to several viruses including vaccinia virus (VACV) (Baldanta et al., 2017), Dengue virus (DENV), West Nile virus (WNV) (Dai et al., 2011), influenza B virus (Morales et al., 2015) and Hepatitis E virus (HEV) (Sooryanarain et al., 2017). As the expression of ISG15, an IFN α/β-induced ubiquitin-like protein, was pSTAT1-dependent (Hertzog et al., 2003), our results showed that the expression of LPS-induced ISG15 in *TLR4* overexpressing BMs was distinctly increased at all time points compared that in WT BMs (Fig. 5), revealing that the overexpression of *TLR4* could help protect host against viral infection. Mx1, other IFN-β-induced antiviral protein, was also regulated by pSTAT1 (Doyle et al., 2002), and it controlled the viral replication in infected cells. Knock-in mice carrying a fully functional Mx1 gene presented a protective antiviral response by controlling the expression of key modulator molecules associated with influenza virus lethality (Cilloniz et al., 2012; Salomon et al., 2007). Moreover, Mx1 protein could disrupt the replication of foot-and-mouth disease virus (FMDV) (Cai et al., 2013). This effect was evidently attenuated when Mx1 gene in the bovine primary fetal tracheal epithelial cells was knocked out (Wang et al., 2016). These studies demonstrated that Mx1, a key factor in controlling viral replication, a certain part in host defense against influenza virus and FMDV infection. It has been reported that TLR4-specific upregulation of IFN-β could activate STAT1 and was responsible for the secondary upregulation of Mx1 (Doyle et al., 2002), which indicated that LPS could enhance the expression of Mx1 and promote antiviral immunity. Similarly, our data showed *TLR4* overexpressing BMs outstandingly enhanced the expression of LPS-induced Mx1 (Fig. 5). Taken together, overexpression of *TLR4* could promote immune responses by the IRF3-IFN-β-pSTAT-induced expression of antiviral genes.

TLR4-deficient mice have increased mortality following infection with live Gram-negative bacteria such as *S. Typhimurium* or *Escherichia coli*, suggesting that TLR4 signaling is important for protective immune responses (Arpaia et al., 2011; Vazquez-Torres et al., 2004). On evaluating the effect of overexpression of *TLR4* on controlling *S. Typhimurium* growth within BMs, the study revealed that the proliferation of bacteria within TLR4 overexpressing BMs was depressed dramatically on all time points post challenge when compared with WT BMs (Fig. 6), thus prompting *TLR4* overexpression could enhance the BMs' capability to eradicate *S. Typhimurium*.

Understandably, there exist several mechanisms in which TLR4 participates to control *S. Typhimurium* growth. First, as Myd88$^{-/-}$ mice failed to control effectively the growth of *S. Typhimurium* (Talbot et al., 2009), it then suggested that TLR4/Myd88-dependent signaling was essential for control of bacterial growth. As *TLR4* overexpression enhanced activation of TLR4/Myd88-dependent signaling, thus it might help control bacterial growth. Second, though the bacterial burden upon the TRIF$^{-/-}$ mice were significantly higher than that of the wildtype mice, they were still able to control sublethal *S. Typhimurium* infection (Talbot et al., 2009). Moreover, Type I IFN signaling, activated in a TLR4/TRIF-dependent fashion, was testified to be crucial for host protection against *S. typhimurium* infection (Mancuso et al., 2007; Nirmal Robinson et al., 2012). The data of this study showed that *TLR4* overexpression could activate higher-level expression of IFN-β in BMs, and TLR4/TRIF-dependent activation following LPS pretreatment in *TLR4* overexpressing BMs clearly inhibited bacterial growth (Fig. 6), revealing that such inhibition resulting from *TLR4* overexpression might be associated with increased IFN-β expression level. Third, IRG1 could boost the bactericidal activity of macrophage-lineage cells by regulating β-Oxidation-dependent mitochondrial ROS production (Hall et al., 2013). As the IRG1 level was strikingly higher in *TLR4* over-expressing BMs, thus overexpression of *TLR4* might inhibit bacterial growth by increased IRG1.

In conclusion, this study revealed that overexpression of *TLR4* could facilitate LPS-induced inflammatory responses at early stage via Myd88-dependent NF-κB and MAPK (ERK1/2 and JNK) signaling pathways, while TRIF-dependent IRF3 signaling was activated at late stage to regulate inflammation and antiviral effects (Fig. 7). Meanwhile, overexpression of *TLR4* could effectively inhibits *S. typhimurium* growth within BMs during the late stage.

Conflict of interest

The authors have declared that no competing interest exists.

Acknowledgements

These studies were supported by National Transgenic Creature Breeding Grand Project, China(2016ZX08008-003) and Tianjin Science and Technology Project, China (16ZXZYNC00050).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ejcb.2018.11.004.

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