Short communication

THE EFFECT OF BUFFALO CD14 shRNA ON THE GENE EXPRESSION OF TLR4 SIGNAL PATHWAY IN BUFFALO MONOCYTE/MACROPHAGES

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Abstract: CD14 plays a crucial role in the inflammatory response to lipopolysaccharide (LPS), which interacts with TLR4 and MD-2 to enable cell activation, resulting in inflammation. Upstream inhibition of the inflammation pathway mediated by bacterial LPS, toll-like receptor 4 (TLR4) and cluster of differentiation antigen 14 (CD14) was proven to be an effective therapeutic approach for attenuating harmful immune activation. To explore the effect of CD14 downregulation on the expression of TLR4 signaling pathway-related genes after LPS stimulation in buffalo (*Bubalus bubalis*) monocyte/macrophages, effective CD14 shRNA sequences were screened using qRT-PCR and FACS analysis with buffalo CD14 shRNA lentiviral recombinant plasmids (pSicoR-GFP-shRNA) and buffalo CD14 fusion expression plasmids (pDsRed-N1-buffalo CD14) co-transfected into HEK293T cells via liposomes. Of the tested shRNAs, shRNA-1041 revealed the highest knockdown efficiency (p < 0.01). When buffalo peripheral blood monocyte/macrophages were infected with shRNA-1041 lentivirus and stimulated with LPS, the expression of endogenous CD14 was significantly decreased by CD14 shRNA (p < 0.01), and the mRNA expression levels of TLR4, IL-6 and TNF-α were also significantly downregulated compared to the control groups (p < 0.01). These results

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demonstrated that the knockdown of endogenous CD14 had clear regulatory effects on the signal transduction of TLR4 after stimulation with LPS. These results may provide a better understanding of the molecular mechanisms of CD14 regulation in the development of several buffalo diseases.

Keywords: Buffalo CD14 gene, shRNA, TLR4 signal pathway, Gene expression, Monocyte/macrophages

INTRODUCTION

Cluster of differentiation antigen 14 (CD14) is a member of the glycoprotein family of cell surface receptor molecules. It is attached to the cell membrane via a glycosyl phosphatidylinositol (GPI) moiety, forming a 55-kDa glycoprotein [1, 2]. CD14 is a component of the innate immune system, and it is widely distributed on the monocyte/macrophage cell surface. There is also a low level of expression in neutrophils. In addition to the membrane-bound form (mCD14), CD14 also exists as a soluble form (sCD14) in the serum [3, 4].

Lipopolysaccharide (LPS) is present in the cell wall of Gram-negative bacteria and is a well-established pathogen-associated molecular pattern (PAMP) in the innate immune system [5, 6]. Binding between CD14 and LPS is a key step in bacterial infection. In the CD14-dependent signaling pathway, CD14 binds to LPS and facilitates the activation of the TLR4/NF-KB inflammatory pathway, resulting in the production of pro-inflammatory cytokines, including tumor necrosis factor alpha (TNF-a), interleukin-6 (IL-6), and interleukin-1 (IL-1) [7–10]. Any interference in the expression of these genes is likely to block the LPS-activated signaling pathway, in which the CD14 gene may play an important role [2, 3, 10]. Several studies have shown that upstream inhibition of the inflammation pathway mediated by bacterial LPS, toll-like receptor 4 (TLR4) and CD14 is an effective therapeutic approach for attenuating dysfunctional immune activation [4, 11].

The gene function of CD14 and several inflammatory cytokines has been widely explored in certain diseases that are caused by Gram-negative bacteria in humans, mice and livestock. Examples include endometritis, *Brucella* and mastitis [12–14]. Most of our understanding of the molecular regulatory mechanism of CD14 has been based on the results of mouse RAW264.7 macrophage studies [15–18]. However, there are tissue- and species-specific differences in TLR signaling and function between domestic animals, humans and rodents [19].

Buffalo (*Bubalus bubalis*) is an important species of dairy animal, especially in Southeast Asian countries with agriculture-based economies. As with other livestock, there are multiple reproductive and veterinary issues with this species. So far, there are very few reports on buffalo disease research, especially on inflammation caused by bacteria.

RNA interference, a sequence-specific mRNA degradation induced by double-stranded RNA (dsRNA), is a common approach employed to specifically silence genes. RNA interference technology is widely used to study gene function and signaling pathways [20–22]. Lentiviral vectors, which possess the advantages of high transfection efficiency and resistance to degradation, have always been used to mediate RNA interference to improve the efficiency of gene knockdown. To investigate the effect of endogenous CD14 knockdown on related genes in the TLR4 signal transduction pathway at the immuno-related cellular level, we assessed the inhibitory effects of shRNA targeting of the buffalo CD14 gene. Buffalo peripheral blood monocyte/macrophages were infected using a buffalo CD14 shRNA lentivirus. The inhibitory effects on the expression of endogenous CD14 and other related genes after LPS stimulation were determined. The study provided valuable clues to explore the possibility of relieving the bacterial infection-triggered inflammatory response by regulating CD14 expression.

MATERIALS AND METHODS

Reagents and antibodies
All of the chemicals used in this study were purchased from Sigma-Aldrich, with the exception of TCM-199 powder, which was purchased from Gibco BRL. Dulbecco's modified Eagle's medium (DMEM) was purchased from Hyclone. LPS (purified lyophilized powder) from *Escherichia coli* O111:B4 was purchased from Sigma-Aldrich and was reconstituted in DMEM to the desired working concentrations prior to use. A specific anti-buffalo CD14 primary antibody was provided by the Animal Genetic Engineering Key Laboratory of Hainan University.

Cloning of the buffalo CD14 gene and construction of its expression vector
The buffalo (*Bubalus bubalis*) CD14 CDS fragment was generated using RT-PCR with the forward primer 5'GGAAAGAATCCACAGTCCAGCCG3' and the reverse primer 5'GCTGGTTGGTTGAGATGTCCTGG3'. The PCR fragment was subcloned into the pMD-18T vector (Takara). The buffalo CD14 CDS sequences were obtained after enzyme digestion and sequencing identification. These were inserted into pDsRed1-N1 vector (BD Biosciences Clontech) to construct the pDsRed1-N1-buffalo CD14 fusion expression vector, which was confirmed using restriction enzyme digestion. The expression vector was transfected into 293T cells using Lipofectamine LTX reagent, and cell lines that stably expressed the buffalo CD14 gene were obtained after G418 selection.

shRNA design and synthesis
Five different sites based on the cattle CD14 mRNA sequence (GenBank Accession No.NM_174008.1) were used to design the CD14 siRNA sequence using ABI siRNA online software (http://www.ambion.com). One random universal shRNA sequence was used as the scramble control (NC), which was provided by Open Biosystem (Cat. No. 1864). The frame of the 71-bp

oligonucleotide sequence of each shRNA fragment was 5'-XhoI CCGG shRNA (sense strand), TTGAAGAGA (loop structure), shRNA (antisense strand), and TTTTTT NotI-3' (Table 1). The synthesized shRNA fragments were inserted into the pSicoR-GFP vector (Invitrogen) by digestion with XhoI and NotI to construct the shRNA lentiviral expression vectors. They are referred to as pSicoR-GFP-CD14 shRNA (319/421/755/970/1041) and NC shRNA-1864. The shRNA fragments were synthesized by Nanjing GenScript Co. The constructed shRNA lentiviral vectors were confirmed by restriction enzyme digestion and sequencing.

Table 1. The designed shRNA sequences of the buffalo CD14 gene.

Name	Duplexes of DNA coding specific shRNA (5'-3')
CD14 shRNA-319	S 5'-CTCGAGCCGGGCAGTATGCTGACACAATCTTCAAG AGAGATTGTGTCAGCATACTGCTTTTTTGCGGCCGC-3' AS 5'-GCGGCCGCAAAAAAGCAGTATGCTGACACAATCTC TCTTGAAGATTGTGTCAGCATACTGCCCGGCTCGAG-3'
CD14 shRNA-421	S 5'-CTCGAGCCGGGTACTCTCGTCTCAAGGAATTCAAG AGATTCCTTGAGACGAGAGTACTTTTTTGCGGCCGC-3' AS 5'-GCGGCCGCAAAAAAGTACTCTCGTCTCAAGGAATC TCTTGAATTCCTTGAGACGAGAGTACCCGGCTCGAG-3
CD14 shRNA-755	S 5'-CTCGAGCCGGTATCTAGCGCTACGCAACGTTCAAG AGACGTTGCGTAGCGCTAGATATTTTTTGCGGCCGC-3' AS 5'-GCGGCCGCAAAAAATATCTAGCGCTACGCAACGTC TCTTGAACGTTGCGTAGCGCTAGATACCGGCTCGAG-3'
CD14 shRNA-970	S 5'-CTCGAGCCGGGCTCAGCGTGCTTGATCTCTTCAAG AGAGAGATCAAGCACGCTGAGCTTTTTTGCGGCCGC-3' AS 5'-GCGGCCGCAAAAAAGCTCAGCGTGCTTGATCTCTC TCTTGAAGAGATCAAGCACGCTGAGCCCGGCTCGAG-3'
CD14 shRNA-1041	S 5'-CTCGAGCCGGATGACCTGACTCTGGACGGTTCAAG AGACCGTCCAGAGTCAGGTCATTTTTTTGCGGCCGC-3' AS 5'-GCGGCCGCAAAAAAATGACCTGACTCTGGACGGTC TCTTGAACCGTCCAGAGTCAGGTCATCCGGCTCGAG-3
NC shRNA- 1864	S 5'-CTCGAGCCGGCCTAAGGTTAAGTCGCCCTCGCTCG AGCGAGGGCGACTTAACCTTAGGTTTTTTGGCGGCCGC-3' AS 5'-GCGGCCGCCAAAAAACCTAAGGTTAAGTCGCCCTC GCTCGAGCGAGGGCGACTTAACCTTAGGCCGGCTCGAG-3'

Isolation and identification of buffalo peripheral blood mononuclear macrophages

Fresh buffalo jugular vein blood was obtained from the local farm. We added 3.8% sodium citrate and then diluted it in an equal volume of PBS. The diluted whole blood was added to a Lympholyte-H cell separation solution (CEDARLANE) at a ratio of 2:1 and then centrifuged at 2000 rpm at 18–20ºC for 20 min. The cell pellet was washed twice with PBS and cultured in 1640 medium with 0.1% autologous serum prepared in a 5% CO_2 incubator at 37ºC for 2 h. Then, the cultured cells were washed twice with 1640 medium and cultured in 1640 medium with 10% FBS and 5% autologous serum prepared in a 5% CO_2 incubator at 37ºC for 3 to 4 days. The isolated buffalo monocyte/macrophages

were confirmed using morphological examination, the ink phagocytosis test and CD14 immunohistochemical analysis.

Screening of shRNA sequences targeting the buffalo CD14 gene
Lentiviral recombinant plasmids (pSicoR-GFP-shRNA) and the fusion expression vector (pDsRed-N1-buffalo CD14) were co-transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen) to screen for shRNA sequences that targeted the buffalo CD14 gene. The interference effects of each shRNA sequence on CD14 expression were examined under a fluorescence microscope (Nikon ECLIPSE, TE300) 72 h after transfection. CD14 levels were then determined using qRT-PCR and flow cytometry (FACS).

For qRT-PCR, the co-transfected cells were washed with 1 ml PBS, and the total RNA was extracted using Trizol solution (Invitrogen) and quantified using a Nanodrop ND1000 spectrophotometer (Labtech). For cDNA synthesis, the genomic DNA in the total RNA was first digested with DNaseI and then reverse transcribed into cDNA (Takara) according to the manufacturer's instructions. The cDNA was diluted to 100 ng/µl for subsequent Taqman quantitative PCR analysis (ABI 7500) using the probes and primers listed in Table 2. The PCR conditions were 94ºC for 30 sec followed by 40 cycles of 94ºC for 15 sec and 60ºC for 30 sec. Duplicate PCR experiments were performed for each transcript. The comparative Ct method was used for relative quantification of target gene expression levels (ABI Prism Sequence Detection System). The quantification was normalized to the Histone H2a gene. Within the log-linear phase region of the amplification curve, fold-changes in the relative mRNA expression of the target gene were determined using the formula $2^{-\Delta\Delta CT}$.

Table 2. The primers used for qRT-PCR analyses.

Gene	Primer sequences	Fragment length (bp)
Histone H2a	Forward: 5'-AACAAGCTGCTGGGCAAAGT-3'	80
	Reverse: 5'-TTATGGTGGCTCTCCGTCTTCT-3'	
	Probe: 5'-CCCAACATCCAGGCCGTGCTG-3'	
CD14	Forward: 5'-CCGTTCAGTGGTAATGGT TGC-3'	100
	Reverse: 5'-TGG TGT CGG CTC CCT TGA G-3'	
	Probe: 5'-CCGCCCGCCACTGATCTTCCCACCTCTT-3'	
TLR4	Forward: 5'-CTGCCTGAGAACCGAGAGTTG-3'	300
	Reverse: 5'-GCTCCATGCACTGGTAACTAATGT-3'	
IL-6	Forward: 5'- ATCAGAACACTGATCCAGATCC-3'	300
	Reverse: 5'-CAAGGTTTCTCAGGATGAGG-3'	
TNF-α	Forward: 5'-GCTCCAGAAGTTGCTTGTGC-3'	300
	Reverse: 5'-AACCAGAGGGCTGTTGATGG-3'	
β-actin	Forward: 5'-GCCCTGGCACCCAGCACAAT-3'	150
	Reverse: 5'-GGAGGGGCCGGACTCATCGT-3'	

The CD14 protein expression level in co-transfected 293T cells was examined under a fluorescence microscope (Nikon ECLIPSE, TE300). The eGFP and RFP fluorescence signals were determined separately using FACS (BD Biosciences,

FACSCalibur), and water was used as a blank control. Cells that were co-transfected with pDsRed-N1-Buffalo CD14 plasmid plus pSicoR-GFP plasmid and pDsRed-N1-Buffalo CD14 plasmid plus pSicoR-GFP-1864 plasmid were respectively used as the blank and positive controls.

Lentivirus packaging and titer determination
Lentiviral particles were produced as previously described [23]. The pSicoR-GFP CD14 shRNA vector was co-transfected into 293T cells with VSVG and NRF plasmids using the calcium phosphate method. The co-transfection supernatant was harvested after transfection for 48–72 h at 37ºC and 5% CO_2. The supernatant was centrifuged (2000 rpm for 10 min at 4ºC) to remove cellular debris and subsequently filtered through a 0.45 μm membrane. The titers of the viral preparations were determined using a series dilution method in 293T cells.

The effects of CD14 shRNA on endogenous CD14 and related gene expression in buffalo peripheral blood mononuclear macrophages following LPS stimulation
First, the infection efficiency of vSicoR-GFP CD14 shRNA in buffalo peripheral blood macrophages was evaluated using one of two MOIs (100 or 300) with polybrene (final concentration: 6 μg/ml). The infection results were assayed under a fluorescence microscope (Nikon ECLIPSE, TE300) on the basis of the number of cells with GFP protein expression.
We also investigated the effect of LPS stimulation on buffalo macrophage growth and gene expression. After 7 days of viral infection, LPS at a final concentration of 1 μg/ml was added to the cells for 3 h. The cell growth status was observed under a fluorescence microscope, and the cells were then collected for RT-PCR analysis. The abundance of endogenous CD14 mRNA and that of related genes in all of the experimental samples was determined using $2^{-\Delta\Delta CT}$ relative quantification methods, similar to those previously described, with the exception of the addition of the SYBR green protocol (Takara). The mRNA expression levels of CD14, TLR4, IL-6 and TNF-α were normalized to ACTB. The PCR analyses were performed in triplicate. The blank control cells were not infected with virus and were stimulated with LPS. This group was compared to infected cells with or without LPS stimulation. The scramble control virus 1864 was used as a negative control.

Statistical analysis
Data on mRNA expression were analyzed using SPSS16.0 software. Single-factor analysis of variance was employed for pairwise comparisons. A p-value of less than 0.05 was considered to represent a statistically significant difference.

RESULTS

Cloning of the buffalo CD14 gene and the construction of its expression vector

The buffalo CD14 CDS fragment (1340 bp) was cloned into the pMD-18T vector. The sequence homology between buffalo and bovine CD14 CDS was 98%, with the different bases located in the non-coding sequence (data not shown). After digestion with SalI and SacII restriction enzymes, the buffalo CD14 CDS fragment was subcloned into the pDsRed1-N1 vector, which was digested with the same enzymes. The constructed CD14 fusion vector (pDsRed1-N1-buffalo CD14) was confirmed via restriction enzyme digestion and then transfected into 293T cells to verify RFP gene expression (Fig. 1). The confirmed expression plasmid was later used for shRNA screening.

Fig. 1. Map of DsRed1-N1-buffalo CD14 plasmid and its transfection effects in HEK293T cells. Left: white light; right: fluorescence light. Magnification 10×.

Screening of buffalo CD14 shRNA

Five buffalo CD14 shRNA lentiviral pSicoR-GFP-CD14 shRNA plasmids and the pDsRed-N1-buffalo CD14 fusion expression vector were co-transfected into HEK293T cells using Lipofectamine 2000 at a ratio of 8:1. After 72 h of transfection, the fluorescent expression of green and red in the transfected cells was examined under a fluorescent microscope (Fig. 2). With the exception of the blank control, all of the transfected cells had nearly the same degree of green fluorescence, indicating that the shRNA vectors had the same transfection efficiency. The pSicoR-GFP-NC shRNA-1864-negative transfected group exhibited the same red fluorescence signal as the no-shRNA vector transfected group. The number of red cells in all of the pSicoR-GFP-CD14 shRNA and pDsRed-N1-buffalo CD14 co-transfected groups decreased by different degrees. A clear reduction in the number of red cells was observed in the pSicoR-GFP-CD14 shRNA-319, -421 and -1041 groups, indicating that these groups had a higher interference efficiency.
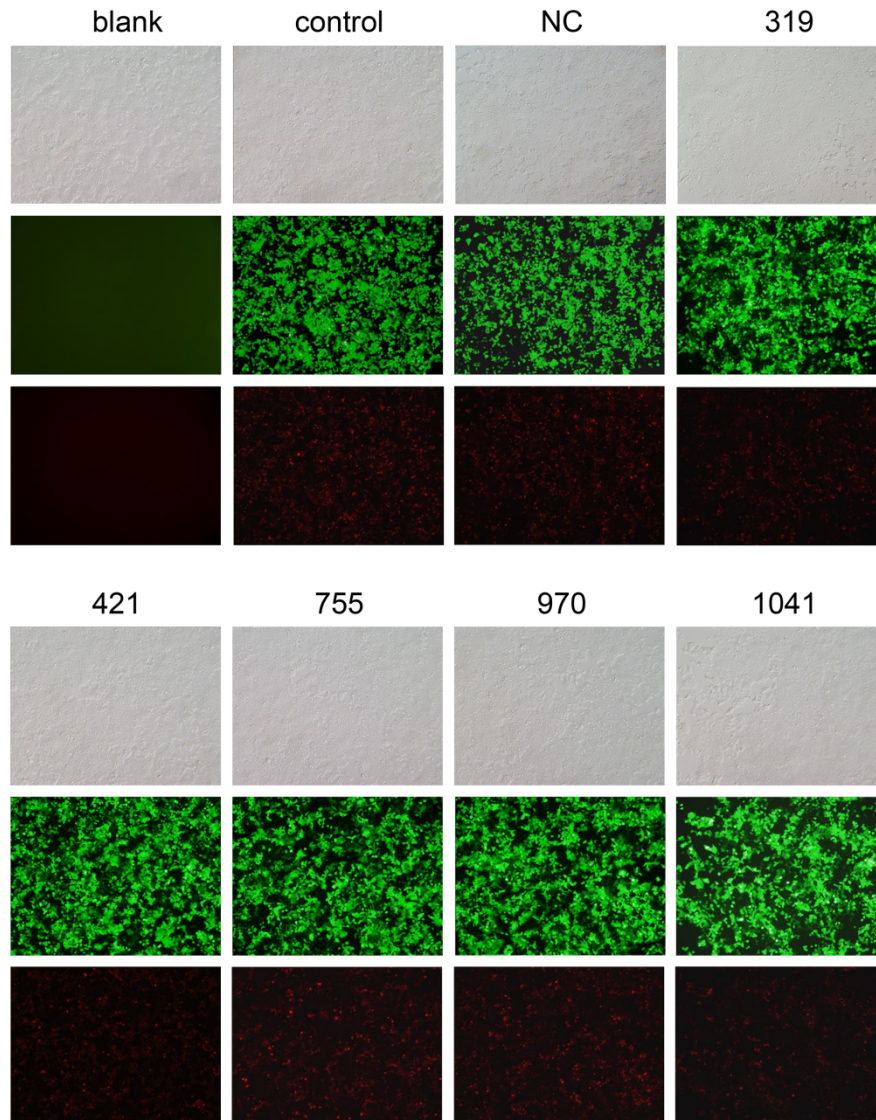
Fig. 2. The green and red fluorescent expression of each co-transfected 293T cell under a fluorescent microscope (magnification 10×). Samples – Blank: no plasmid added; Control: pDsRed1-N1 plus pSicoR-GFP; NC: pDsRed-N1-buffalo CD14 plus pSicoR-GFP-shRNA-1864; 319/421/755/970/1041: pSicoR-GFP-CD14 shRNA plasmid (-319/421/755/970/1041) with pDsRed-N1-buffalo CD14 at a ratio of 8:1.

The interference effects of each shRNA sequence on the buffalo CD14 gene were verified using qRT-PCR and FACS analyses. Compared with the blank control, the expression of the CD14 gene in 1864 shRNA-negative control cells did not change. The CD14 expression in all of the co-transfected groups of

pSicoR-GFP-CD14 shRNA and pDsRed-N1-buffalo CD14 decreased to varying degrees. pSicoR-GFP-CD14 shRNA-319 and shRNA-1041 had significant inhibitory effects on CD14 expression, achieving a reduction of 58.8% and 69.1%, respectively (Fig. 3A). In the FACS analysis, the number of cells with red and green fluorescence was also quantified. These results revealed that the blank control group had no red cells, and compared with the blank control, the number of red cells in the 1864 shRNA-negative control group was similar. The number of red cells in all of the other co-transfected groups also clearly decreased, with the number of red cells in the co-transfected CD14 shRNA-319 and CD14 shRNA-1041 groups decreasing significantly ($p < 0.01$). Moreover, the interference efficiency reached 59.9% and 64.6%, respectively, when it was calculated using the average fluorescence value (Fig. 3B).
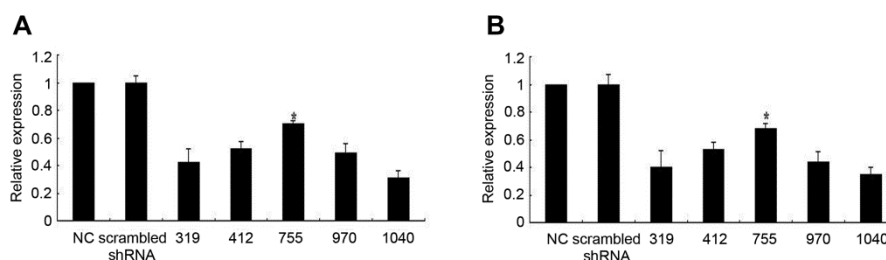

Fig. 3. Inhibition effects of each shRNA sequence on the buffalo CD14 gene in co-transfected 293T cells using qPCR and FACS analysis. A – Results for CD14 mRNA expression obtained using qRT-PCR. B – The numbers of red cells found using FACS analysis. Note: the values for the columns with asterisks are significantly different, $p < 0.01$.

Isolation and identification of buffalo peripheral blood mononuclear macrophages

To explore the effect of CD14 knockdown on the expression of related genes in buffalo immune cells, buffalo peripheral blood mononuclear macrophages were isolated from samples taken from the jugular vein and separated using Lympholyte-H solution. The morphology of the isolated and purified buffalo blood mononuclear macrophages was confirmed using Wright's stain after 5 days of culture. The purified cells were circular, oval or triangular but also exhibited other irregular shapes. The nuclei were round and located at one end of the cytoplasm, indicating that the isolated cells exhibited typical morphological characteristics of mononuclear macrophages (Fig. 4A and B). The purified cells were further identified in ink phagocytic experiments. Many black ink particles were observed in the cell cytoplasm (Fig. 4C and D), indicating that the obtained mononuclear macrophages had strong phagocytic ability.

The CD14 phenotypes of purified buffalo mononuclear macrophages were detected using immunohistochemistry. Under a fluorescence microscope, the purified cells exhibited green staining on the cell surface. Moreover, the negative control cells exhibited no green signal (Fig. 4E and F). These results demonstrated that the purified cells were buffalo mononuclear macrophages.
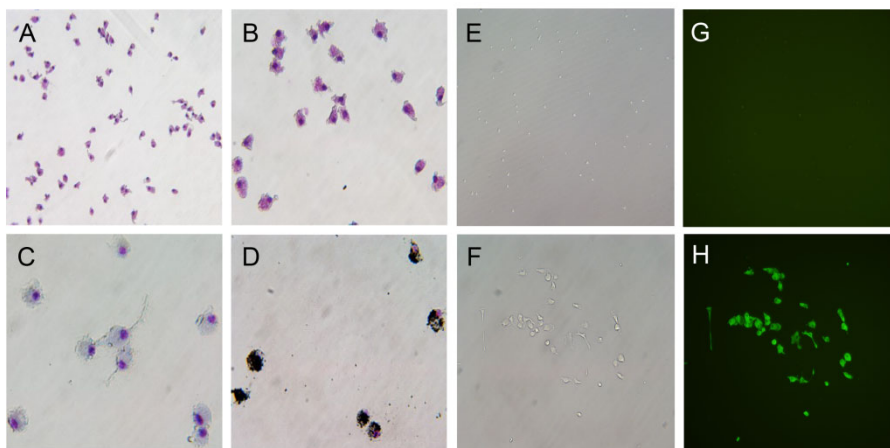
Fig. 4. Identification of purified buffalo monocyte/macrophages. A, B − Wright staining, magnification 400×. C, D − phagocytic detection, magnification 400×. C − Control. D − Phagocytizing. E, F, G, H − Immunohistochemistry analysis of CD14 antibody. E, G − buffalo fibroblast cells under bright and fluorescent microscope respectively, magnification 100×, F, H − purified buffalo monocyte/macrophages under bright and fluorescent microscope respectively, magnification 200×.

The effects of CD14 shRNA on the gene expression of the TLR4 pathway in buffalo peripheral blood mononuclear macrophages after LPS stimulation

We evaluated the infection efficiency of vSicoR-GFP CD14 shRNA-1041 in buffalo peripheral blood macrophages. Two MOIs (100 or 300) were used to infect the cells to optimize the experimental conditions. We found that 7 days after infection, the cells in the MOI 300 group exhibited a higher number of GFP-positive cells than the MOI 100 group under conditions that did not influence cell growth. With MOI 300, 7 days after infection, 60% of the cells were GFP-positive (data not shown). When 1 µg/ml LPS was added for 3 h, the morphology of the cells clearly changed: the cytoplasm of the cells contained a large number of lysosomal granules, with their pseudopod extended and displaying a typical inflammatory response (data not shown). Therefore, MOI 300, 7 days infection and 1 µg/ml LPS added for 3 h were the conditions used for subsequent experiments.

Under the above experimental conditions, the effects of buffalo CD14 shRNA-1041 on the endogenous CD14 gene expression and its related genes in buffalo peripheral blood mononuclear macrophages following LPS stimulation were determined using qRT-PCR. No shRNA was used as a blank control and the 1864 scrambled shRNA was used as a negative control. The results demonstrated that LPS stimulation could significantly increase the mRNA expression of endogenous CD14, TNF-α, IL-6 and TLR4 genes in all of the control groups ($p < 0.01$). Compared with the two control groups, the expression of CD14 and TLR4 in the cells of the CD14 shRNA-1041 infection group was significantly downregulated ($p < 0.01$) by LPS stimulation, but there was no

difference for CD14 and TLR4 expression between the LPS-treated and non-treated cells infected with the CD14 shRNA-1041 virus. IL-6 and TNF-α expression was also significantly decreased after stimulation with LPS in cells in the CD14 shRNA-1041 infection groups ($p < 0.01$), and a significant difference was observed between the LPS-treated and untreated cells infected by the CD14 shRNA-1041 virus ($p < 0.01$; Fig. 5).
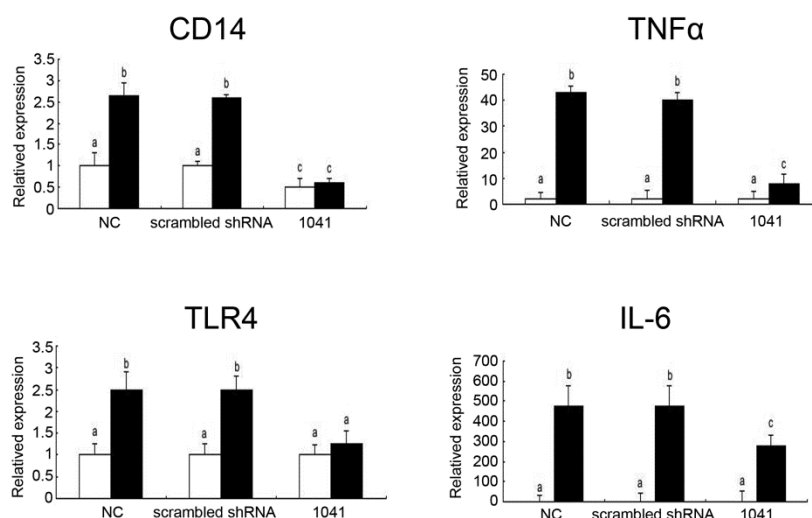

Fig. 5. The expression of CD14, TLR4, IL-6 and TNF-α genes in buffalo peripheral blood monocyte/macrophages infected with CD14 shRNA-1041 virus. White column: no LPS. Black column: with LPS. Note: the values for columns with different letters are significantly different, $p < 0.01$.

DISCUSSION

Macrophages are cells produced by the differentiation of monocytes in tissues. Monocytes and macrophages are phagocytes, and they play a pivotal role in the immune response, both in the non-specific defense (innate immunity) and specific defense mechanisms (adaptive immunity) of vertebrates. Peripheral blood monocytes/macrophages in humans and certain important animals have been successfully isolated [5, 10, 24, 25]. They were used to investigate the mechanisms and potential for prevention of several inflammatory diseases. The mouse RAW264.7 cell line has been consistently used in related studies [18, 26, 27]. There are many tissue- and species-specific differences between domestic animals and humans or rodents. Therefore, to investigate the effect of endogenous CD14 knockdown on related genes of the TLR4 signal transduction pathway in cells of the buffalo immune system, buffalo peripheral blood monocytes/macrophages were isolated and identified. The isolated cells exhibited a typical morphology of monocytes/macrophages, expressed CD14 protein and exhibited an inflammatory response to LPS stimulation. The

obtained monocytes/macrophages also provided specific cellular materials for the study of other immune-related diseases in buffalo.

CD14 plays a very important role in the animal immune response, which binds to LPS and facilitates the activation of the TLR4/NF-KB inflammatory pathway, resulting in the production of pro-atherogenic cytokines, including tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 (IL-1) [10]. The function of human, mouse and cattle CD14 has been previously explored in certain immune-related cells and tissues, such as the endometrium and mammary tissue [14, 18, 19]. However, less is known regarding the role of CD14 in buffalo. There was one recent research report about the role of CD14 in the endometrial tissue of buffaloes [12].

In this study, we determined that treatment of buffalo mononuclear macrophages with LPS had a significant effect on the expression of endogenous CD14 and TLR4 signaling pathway-related genes. Moreover, the downregulation of endogenous CD14 expression by LPS stimulation decreased the expression of several important cytokines in the TLR4 signaling pathway, a result that is consistent with many previous findings. Moreover, LPS stimulation significantly upregulated CD14 gene expression, and the downregulation of CD14 expression decreased the production of TNF-a and IL-6 in macrophages in response to LPS [10, 28–31]. Several studies have also reported that CD14 expression or the number of CD14-positive cells is correlated with certain livestock diseases. We observed that the number of IL6 (475-fold) and TNF-α (22-fold) transcripts varied in response to LPS stimulation and was higher compared to the change in the number of CD14 (2.7-fold) and TLR4 (2.5-fold) transcripts.

These results are consistent with those from previous studies of practical buffalo inflammatory disease. Loyi observed the differential expression of several pro-inflammatory cytokines in the endometrium of buffalo with clinical and sub-clinical endometritis [12, 32], and a significant upregulation of CD14 (1- to 2-fold), IL-6 (15- to 36-fold) and TNF-α (10- to 11-fold) mRNA in SCE. Our results indicate that the expression pattern of CD14 and other related cytokine genes in cultured buffalo mononuclear macrophages in response to LPS stimulation is similar to that reported for in vivo expression. In addition, buffalo CD14 shRNA-1041 significantly downregulated the TNF-α, TLR4 and IL-6 genes. These results further support the use of in vitro cultured buffalo mononuclear macrophages to model the function of CD14 and other genes in the inflammatory response caused by Gram-negative bacteria. CD14 expression and that of other related cytokines may be used to diagnose diseases that are caused by bacteria.

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