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ORIGINAL ARTICLE

Visfatin Regulates Inflammatory Mediators in Mouse Intestinal Mucosa Through Toll-Like Receptors Signaling Under Lipopolysaccharide Stress

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Abstract

Visfatin is a multifunctional protein involved in inflammatory immune stress. The aim of current study was to explore the role of visfatin in lipopolysaccharide (LPS)-induced intestinal mucosal inflammation and to confirm its cellular effect in inflammatory immune response through silencing of Toll-like receptors (TLRs). We divided Kunming mice into three groups: Saline group, LPS group, and LPS + visfatin group and performed hematoxylin and eosin staining, immunohistochemistry, quantitative polymerase chain reaction, Western blot, enzyme linked immunosorbent assay and RNA-seq analysis. Pretreatment of visfatin improves LPS-stimulated reduction of tight junction protein 1 (ZO-1) and secretory immunoglobulin A, inhibits overexpression of Claudin-1 and vascular endothelial growth factor, and reduces intestinal mucosal damage and inflammation. RNA-seq analysis of cellular transcriptomes indicated that visfatin is involved in down-regulation of mRNA level of TLR4 as well as attenuation of protein levels of TLR8 and nucleotide-binding oligomerization domain-containing protein 2, revealing that visfatin could reduce intestinal mucosal inflammation through TLR signaling pathway in mice ileum. In RAW264.7 cells, the genes silencing of Toll/IL-1R family, such as TLR4, TLR2, and IL-1R1, was accompanied by decreased expressions of inflammatory factors (TNF-α, IL-1β, IL-6 and MCP-1) along with lower cellular visfatin levels. Hence, visfatin maintains the intestinal mucosal barrier structure and attenuates the intestinal mucosal inflammation through the TLR signaling pathway. Likewise, the Toll/IL-1R family regulates the release of visfatin, which can participate in the inflammatory reaction through the regulation of inflammatory factors.

Keywords Visfatin · Intestinal mucosa · Lipopolysaccharide · TLR · RAW264.7 cells

Introduction

Visfatin, a multifunctional protein, has the same enzymatic activity as that of nicotinamide phosphoribosyl transferase or pre-B cell clone enhancer (Chen et al. 2017). Visfatin showed protein expressions in leucocytes, especially in lysates of granulocytes and monocytes, and participates in the inflammatory response through immunomodulatory effects on immune cells (Friebe et al. 2011). Visfatin exerts dual effects on the inflammatory response, i.e., it may induce the inflammation in healthy mice, while it rescues the injury in the lipopolysaccharide (LPS) stressed mice (Xiao et al. 2015; Yang et al. 2015). The visfatin levels increased not only in mesenteric pathogenesis, but also significantly correlated with Crohn's disease severity (Starr et al. 2017), but recovered upon treatment (Waluga et al. 2014). Visfatin has great importance for mucosal immunity (Gerner et al. 2018) and plays dual role

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in the inflammation that may rescue the inflammatory conditions and apoptosis in the LPS-induced injury (Wu et al. 2018). However, the inhibition of nuclear factor kappa-light-chain enhancer of activated B (NF-κB) signaling dominantly reduced the bacterial induced upregulation of visfatin (Penke et al. 2015). On the other hand, visfatin activates NF-κB in macrophages (Brentano et al. 2007).

The intestinal mucosal barrier is composed of four barriers: mechanical, immune, chemical and biological. When the intestinal mucosal barrier has a complete structure and fully functional, intestinal epithelial cells can recognize exotic pathogens or harmful microorganisms and transmit signal to downstream, causing intracellular signal cascades and maintaining the integrity of the intestinal mucosal barrier and the gut homeostasis (Kau et al. 2011). Intestinal pathogenic infections and resulting inflammation may lead to epithelial damage and barrier disruption (Awad et al. 2017; Riddle and Porter 2012). LPS, a component of the outer membrane of Gram-negative bacteria, provokes immune stress through complement activation (Li et al. 2018). Exogenous LPS may alter the expression of Toll-like receptor (TLR)4 and levels of many pro-inflammatory cytokines including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6 (Kalaiyarasu et al. 2016). LPS triggers several distinct signaling pathways through expressions of TLRs in intestinal epithelial cell lines (Cario et al. 2000). TLRs regulate the synthesis and release of cytokines and coordinate the distribution of immune cells (Eissa et al. 2019). Increased expression of TLR4 in necrotizing enterocolitis (NEC) coordinates the balance between early intestinal injury and repair during the onset of NEC (Leaphart et al. 2007). The nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) also play key role in the intestinal mucosal barrier pathogenesis (Igarashi et al. 2015). Nevertheless, the relationship between visfatin and TLR signaling pathway in the intestinal mucosal inflammation is not fully clarified yet.

Therefore, this study was designed on pre-constructed LPS-induced intestinal mucosal inflammatory response in mice. Herein, we explored the relationship between visfatin and intestinal mucosal barrier during intestinal mucosal inflammatory response. The relationship between visfatin and Toll/IL-1R family was clarified through the RNA-sequencing results. The small interfer RNA (siRNA) technique was used to investigate the effect of visfatin in vitro inflammatory response. Hence, the aim of current investigation was to provide a theoretical basis for the prevention and treatment of inflammatory bowel diseases.

Materials and Methods

Animals

The appropriate local animal ethics committee of Huazhong Agricultural University approved all the protocols and the experimental procedures described in the current study. Male and female KunMing mice (6–8 weeks, SPF) were randomly divided into three groups. Each group with no less than three mice received an intraperitoneal injection as following: the saline group received single-dose injection of isotonic saline (0.2 ml) intraperitoneally (i.p.) for seven consecutive days. The LPS group received a single-dose injection of LPS (8 mg/kg bw i.p.) all at once. The visfatin + LPS group received single doses of visfatin (100 μg/kg bw. i.p.) for seven consecutive days following a single-dose injection of LPS via the tail vein (8 mg/kg bw). We sacrificed the mice and harvested ileum tissue specimens; part of the tissue was fixed with 4% paraformaldehyde and was subsequently embedded in paraffin. Stained sections were analyzed under a light microscope (BX51; Olympus, Tokyo, Japan). Other parts of ileum tissue specimens were saved at – 80 °C. These specimens subsequently tested by Western blot, quantitative polymerase chain reaction (q-PCR) and enzyme linked immunosorbent assay (ELISA). Data were analyzed using the Quantity One (Bio-Rad) software.

RAW264.7 Cells for RNA-seq

RAW264.7 cells were cultured in Dulbecco's modification of Eagle's medium Dulbecco (H-DMEM) medium containing 10% fetal bovine serum within a humidified incubator containing 5% CO_2 at 37 °C and passaged every 2–3 days to maintain growth. RAW264.7 cells were divided into four groups: Control group, LPS group, visfatin group and LPS + visfatin group. Complete medium was added to the control group, complete medium containing 200 ng/mL visfatin was added to the visfatin group, complete medium containing 10 μg/mL LPS was added to the LPS group, and complete medium containing 200 ng/mL visfatin and 10 μg/mL LPS was added to the LPS + visfatin group. Subsequently, the total RNA of RAW264.7 cells was isolated and RNA-seq analysis was performed in DGE format on the Illumia HiSeq platform after RNA extraction.

RAW264.7 Cells for Detection of Immune Response of Visfatin

RAW264.7 cells were divided into eight groups: control siRNA, TLR4 siRNA, TLR2 siRNA, IL-1R1 siRNA, control siRNA + LPS, TLR4 siRNA + LPS, TLR2 siRNA + LPS and

IL-1R1 siRNA + LPS groups. After siRNA was transfected into cells for 24 h, cells were stimulated with LPS (500 ng/mL) for 3, 6 and 12 h. The sequences of the siRNA are listed in Table 1. The expression of visfatin was detected by Western blot analysis and the inflammatory mediators such as IL-1β, IL-6, MCP-1 and TNF-α were examined by ELISA technique.

Cells Transfection

Experiment was performed according to the transfection reagent's instruction. For optimal siRNA-mediated silencing, we used 10–50 nM siRNA. The following conditions were given per well in a 6-well plate. We diluted 22–110 mol siRNA (for a final concentration of 10–50 nM per well) into 200 µl of jetPRIME® buffer mixing by pipetting up and down. We vortexed jetPRIME® reagent for 5 s and spin down before use and added 4 µl jetPRIME® reagent, vortexed for 10 s, spin down briefly and incubated for 10–15 min at room temperature. We added the transfection mixture to the cells in serum containing medium drop wise and gently rocked the plate back and forth and returned the plate to the incubator.

Hematoxylin and Eosin Staining

The histopathological examination of ileum tissue was carried out using the hematoxylin and eosin (H&E) staining. Briefly, sections of 4-µm thickness were affixed to glass slides, deparaffinized, and stained with hematoxylin and eosin staining. The slides were analyzed under a light microscope (OLYMPU, CX41, Olympus Corporation of Japan).

Immunohistochemical Staining

The ileum tissue was dewaxed, rehydrated, microwave irradiated, treated with 3% H_2O_2 at room temperature for 10 min, blocked with normal goat serum, incubated with the primary antibody overnight at 4 °C (dilution ratio 1:100), incubated with the secondary antibody at 37 °C for 30 min (dilution ratio 1:2), and stained by 3,3-diaminobenzidine (DAB). Finally, the slides were observed with light microscope.

Western Blot

The ileum tissue extracts were prepared with sample buffer. Equal amounts of total protein were mixed in SDS loading buffer, boiled for 10 min, and then subjected to SDS-PAGE. Total protein was electro-phoretically transferred onto polyvinylidene difluoride membranes (IPVH00010; Millipore, USA). Then the membranes were blocked with 5% defatted milk for 2 h at room temperature and incubated with rabbit anti-mouse NOD2 (1:600) and visfatin (1:800) at 4 °C overnight. Finally, the membranes were incubated with HRP conjugated secondary antibodies (1:5000). Quantification was performed using the software Quantity One (Bio-Rad, USA).

Real-Time PCR Analysis

The GenBank primer sequences for β-actin, IL-1β, TLR2, IL-1R1, TNF-α, TLR4, visfatin, vascular endothelial growth factor (VEGF), ZO-1, pIgR, IκBα, Claudin-1, H2-Q10, Ticam-1 are listed in Table 2. The total RNA extraction was conducted using RNAiso Plus Reagent (catalog# 9109, Takara, Japan) mRNA was reverse-transcripted to cDNA using PrimeScript RT reagent Kit with gDNA Eraser (Takara, Japan). The mRNA level of β-actin was measured as an internal control. The parameters used for PCR reactions were as follows: PCR activation at one cycle of 50 °C for 2 min, 95 °C for 5 min, followed by 40 cycles of 95 °C for 30 s and 60 °C for 30 s. All samples and controls were run in triplicate on an ABI 7000 Fast real-time PCR system. The quantitative RT-PCR data were analyzed by a comparative threshold method.

Table 1 Synthesis of TLR4-siRNA, TLR2-siRNA, IL-1R1-siRNA and control sequence (NC-siRNA)

Name	Sense (5′-3′)	Antisense (5′-3′)
NC siRNA	UUCUCCGAACGUGUCACGUTT	ACGUGACACGUUCGGAGAATT
Tlr2-mus-519	GCCUUGACCUGUCUUUCAATT	UUGAAAGACAGGUCAAGGCTT
Tlr2-mus-924	CCCAAAGUCUAAAGUCGAUTT	AUCGACUUUAGACUUUGGGTT
Tlr2-mus-2610	GCAAGAUAAUGAACACCAATT	UUGGUGUUCAUUAUCUUGCTT
Tlr4-mus-1567	CCUCCAUAGACUUCAAUUATT	UAAUUGAAGUCUAUGGAGGTT
Tlr4-mus-455	GGACAGCUUAUAACCUUAATT	UUAAGGUUAUAAGCUGUCCTT
Tlr4-mus-230	GCUAUAGCUUCUCCAAUUUTT	AAAUUGGAGAAGCUAUAGCTT
Il1r1-mus-645	GGAAGUCUUGUGUGCCCUUTT	AAGGGCACACAAGACUUCCTT
Il1r1-mus-1200	GCGCAUGUGCAGUUAAUAUTT	AUAUUAACUGCACAUGCGCTT
Il1r1-mus-1904	GCGGGACACUAAGGAGAAATT	UUUCUCCUUAGUGUCCCGCTT

Table 2 Primers used for real-time PCR Analysis

Genes	Forward primer(5′→3′)	Reverse primer(5′→3′)
β-actin	CACTGCCGCATCCTCTTCCTCCC	CAATAGTGATGACCTGGCCGT
visfatin	AATGTCTCCTTCGGTTCTGG	CCGCTGGTGTCCTATGTAAA
VEGF	TCATGCGGATCAAACCTCACCA	TGGTCTGCATTCACATCTGCTG
TLR4	TTCAGAGCCGTTGGTGTATC	CTCCCATTCCAGGTAGGTGT
ZO-1	TCTTCCATCATTTCGCTGTGT	TCTGAAACCATCAAGTCCACA
pIgR	TTGCCGAAGCTACAAGGGAG	AGGTCTGGCCTTGCTTTACC
IκBα	CGAGCAAATGGTGAAGGAGC	CCAAGTGGAGTGGAGTCTGC
Claudin-1	GGTGCCTGGAAGATGATGAGGTG	GCCACTAATGTCGCCAGACCTG
H2-Q10	CATCTGTGGTGGTGCCTCTTGG	ACATGATAGAGTCAGTGGAAGGAGGAG
Ticam-1	GACCTCAGCCTCTCATTATTCACCATG	CTCCGAACACTCAGTCTTGTCATCAG
TLR4	TTCAGAGCCGTTGGTGTATC	CTCCCATTCCAGGTAGGTGT
TLR2	GGGGCTTCACTTCTCTGCTT	ACAGCGTTTGCTGAAGAGGA
IL-1R1	AGCCCTCGGAATGAGACGATC	GAGAACGGCCCGTGACGTTG
IL-1β	GCCACCTTTTGACAGTGATG	GCTCTTGTTGATGTGCTGCT
TNF-α	TCTTCTCATTCCTGCTTGTGG	CACTTGGTGGTTTGCTACGAC

ELISA

Concentrations of IL-1β, IL-6, MCP-1, TNF-α and TLR8 in samples were determined using commercially available antibodies' kits. The ELISA kits of IL-1β, IL-6 were purchased form Wuhan Yide Biotechnology Co., Ltd., China, while that of MCP-1, TNF-α form Wuhan Hualianke Biotechnology Co., Ltd., China. Protein standards and paired antibodies were according to manufacturer's instructions. Absorption was determined with a Microplate Reader at 450 nm.

Statistical Analyses

SPSS 17.0 and GraphPad Prism v5.0 softwares were used for statistical analysis. The data were expressed as mean ± SD values. Comparisons between continuous outcomes for two experimental groups were performed using two-tailed paired *t*-test tests. ANOVA was used for comparisons of outcome between three or more groups. The statistical significance test level was $P = 0.05$.

Results

LPS Incurred the Inflammation and the Increased Expression of Visfatin in Ileum

Control group showed the intact and clear structure and the longer intestinal villus in H&E staining. Moreover, intestinal villus to crypt ratios (V/C) was larger. Compared with saline group, the ileum with evident damage in LPS group was infiltrated by the inflammatory cell, the lamina propria was edematous, the serosal layer structure and the submucosa were loose, and the V/C value decreased ($P < 0.01$). However, the LPS + visfatin group improved the length and integrity in the ileum while compared to the LPS group ($P < 0.05$) (Fig. 1a, b).

Visfatin of saline group was mainly distributed in intestinal crypts, and slightly in intestinal villus lamina layer. However, compared with saline group, visfatin of LPS group was increased in the intestinal crypt and the lamina propria of the intestinal villus, and the transcriptional level of visfatin was significantly up-regulated ($P < 0.01$). Visfatin of the LPS + visfatin group decreased compared to the LPS group (Fig. 1c–e).

The Effect of Visfatin on Intestinal Mucosal Barrier

Zonula occludens-1 (ZO-1) was mainly distributed in intestinal villus epithelial cells and a small part of ZO-1 were observed in intestinal crypts. After LPS treatment, ZO-1 was significantly decreased in intestinal villus epithelial cells, while it was mainly concentrated in the intestinal crypt. However, ZO-1 was increased in epithelial cells and intestinal crypts of LPS + visfatin group compared to LPS group (Fig. 2a). The results of Western blot and q-PCR presented that the number of ZO-1 reduced in LPS group relative to Saline group ($P < 0.01$); Compared with LPS group, the mRNA and protein level of ZO-1 were increased in LPS + visfatin group (Fig. 2b–d). The detection of q-PCR showed that Claudin-1 in LPS group was higher than that saline group ($P < 0.001$). Compared with LPS group, the level of Claudin-1 in LPS + visfatin group showed a significant downtrend ($P < 0.001$) (Fig. 2g).

The level of secretory immunoglobulin A (sIgA) in saline group was detected around the intestinal gland and the lamina propria of the intestinal mucosa, whereas the level of sIgA in LPS group markedly declined, and the decrease

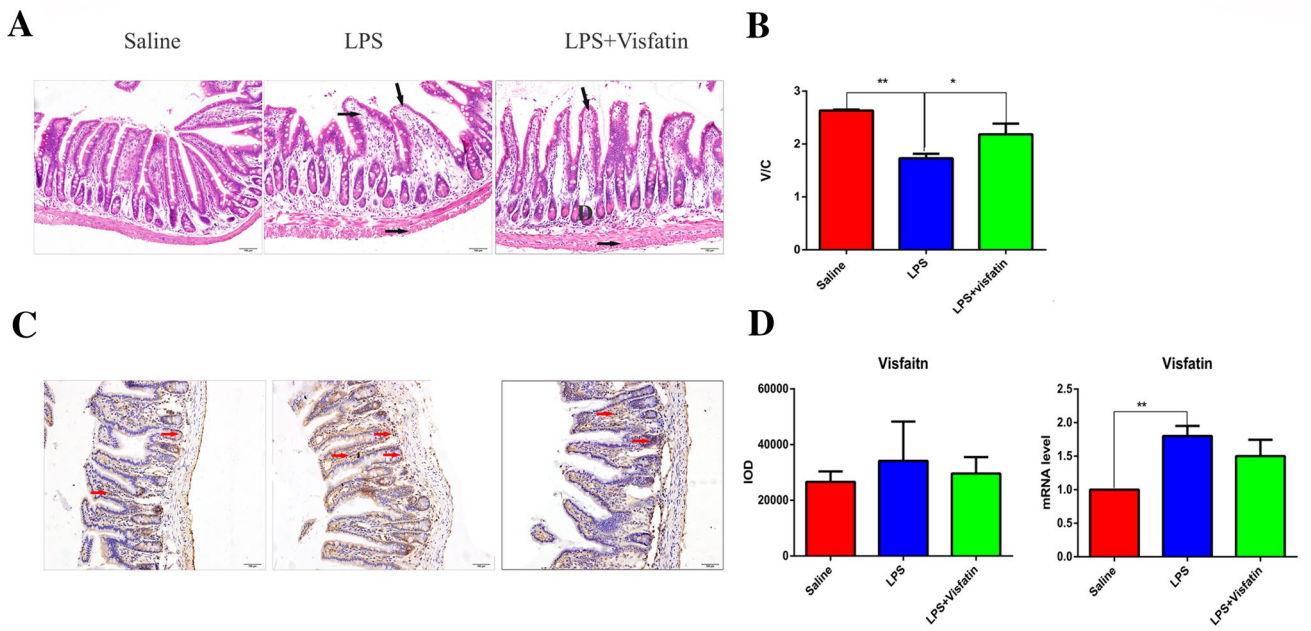

Fig. 1 Histo-morphological changes in ileum structure during intestinal mucosal injury. After LPS induced intestinal mucosal injury, the histological structure, intestinal villi length and crypt depth changed, the visfatin was increased. The visfatin injection rescued these LPS-induced changes in the intestinal mucosa. **a** The changes in histological structure of mouse ileum in different groups was detected by H&E staining and the bar of figure was 100 μm (×20). **b** Intestinal villus to crypt ratio (V/C), a significant index of intestinal digestion and absorption function and body health status was larger. The value of V/C in different groups of mouse ileum was detected using the image pro-plus software. To validate the effect of LPS-induced intestinal mucosal inflammation on visfatin expression, we examined the level of visfatin by immunohistochemistry (IHC) and q-PCR. **C**: The expression of visfatin in different groups of mouse ileum tissues (×20, IHC). **D**: The mRNA level and IOD values of visfatin in different groups. Note: compared with saline group, the difference was very highly significant, ***$P < 0.001$. Where the difference was *significant; **highly significant and ***very highly significant

in the lamina propria was the most obvious. As compared to LPS group, the distribution of sIgA was significantly expanded in LPS + visfatin group ($P < 0.01$) (Fig. 2e, f). VEGF was mainly expressed in intestinal crypts and only a little in the central chyle of lamina propria. After LPS stimulation, elevated VEGF was expressed in intestinal crypts, intestinal villus epithelial cells and intestinal villus lamina propria ($P < 0.0001$). Compared with LPS group, VEGF of LPS + visfatin group was remarkably decreased in intestinal villus epithelial cells and intestinal villus lamina propria ($P < 0.05$) (Fig. 2h–j).

The Effect of Visfatin on the Key Factors of Innate Immune Signaling Pathway in Intestinal Mucosal Inflammation

According to the RNA-seq results, the genes such as IκBα, TLR8, TICAM-1, and NOD2 were mainly enriched in the TLR and NLR signaling pathways related to intestinal mucosal immunity. Nevertheless, H2-Q10 was the only differentially expressed gene in antigen presenting signaling pathway. The up-regulated genes are represented with red color, while the down-regulated genes with green color in supplementary data file S1-TLR and NLR pathways and file S2-cluster analysis diagram.

Six key genes in the TLR, NLR and antigen presentation signaling pathways were selected to validate the results of RNA-Seq data analysis. Q-PCR results presented that the level of TLR4 in LPS group was significantly up-regulated compared to saline group ($P < 0.0001$), and remarkably decreased in LPS + visfatin group compared with LPS group ($P < 0.05$) (Fig. 3a). The change of TICAM-1 and IκBα were consistent with TLR4 (Fig. 3b, c). H2-Q10 had an upward trend in saline group, LPS group and LPS + visfatin group. The expression of H2-Q10 in LPS group was significantly higher than that in Saline group ($P < 0.01$) (Fig. 3d). The results of Western blot and ELISA showed that NOD2 and TLR8 expressed in LPS group were higher than that in saline group. As compared to LPS group, the expression of NOD2 and TLR8 was decreased in LPS + visfatin group (Fig. 3e–g).

Effects of the Silence of TLR4, TLR2 and IL-1R1 on Visfatin in Inflammatory Response

After transfection of different concentrations of FAM-siRNA, RAW264.7 cells showed green fluorescence under

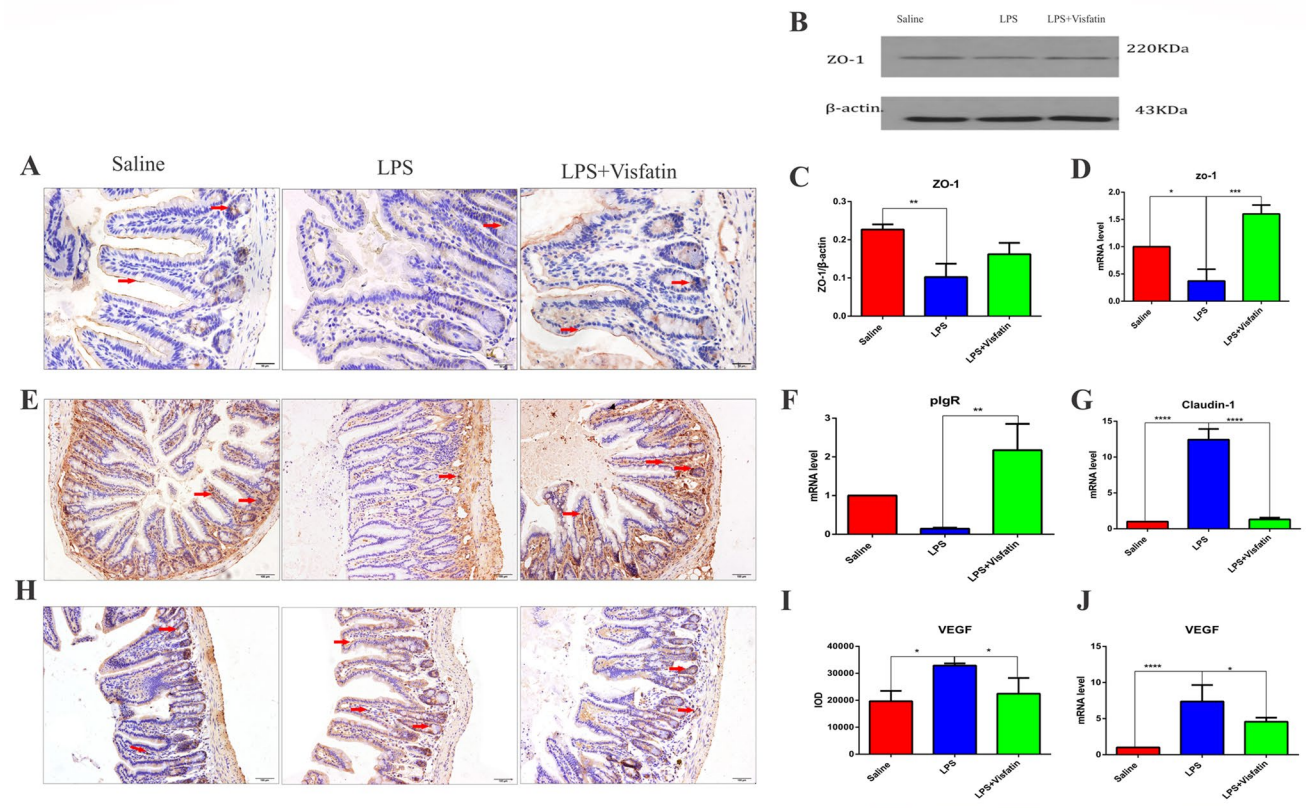

Fig. 2 Altered levels of ZO-1, sIgA and VEGF in intestinal mucosal injury. To examine the effect of visfatin on intestinal mucosal barrier we selected four factors ZO-1, sIgA, Claudin-1, and VEGF to detect. ZO-1, sIgA, Claudin-1, and VEGF act as important components of the intestinal mucosal barrier that play critical role in the structural and functional integrity of the intestinal mucosal barrier. **a** The level of ZO-1 in different groups of mouse ileum (×40, IHC). **b–d** The protein and mRNA level of ZO-1 in different groups were detected by the Western blot and q-PCR. **e** Secretory immunoglobulin A (sIgA) maintain intestinal mucosal immunity barrier stability. The expression of sIgA in different groups of mouse ileum (×20, IHC). **f** The mRNA level of pIgR in different groups of mouse ileum. **g** The mRNA level of Claudin-1 in different groups of ileum tissue. **h** The positioning expression of VEGF in different groups of ileum (×20, IHC). **i–j** The IOD values and mRNA level of VEGF in different groups. Note: The difference was significant, $^{*}P < 0.05$, the difference was highly significant $^{**}P < 0.01$, the difference was very highly significant, $^{***}P < 0.001$ and $^{****}P < 0.0001$

fluorescence microscope, indicating that siRNA was successfully transfected into RAW264.7 cells (Fig. 4a). The results of q-PCR showed that TLR4-230, TLR2-519 and IL1R1-1904 could, respectively, serve as the best RNA interference sequences for silencing TLR4, TLR2 and IL-1R1 gene expression (Fig. 4b). Moreover, siRNA with the concentration of 45 nM presented the best silence effect (Fig. 4c). Based on previous report (Gao et al. 2019), mice were treated with those concentrations of LPS (0–2000 ng/mL) to determine its appropriate concentration for the construction of vitro inflammation model. Expression of TNF-α and IL-1β were significantly promoted by different concentrations of LPS. At a concentration of 500 ng/mL, LPS up-regulated TNF-α and IL-1β to a peak (Fig. 4d).

After siRNA transfection, the expression of TLR4 in the TLR4 siRNA group was lower than that in the control siRNA group ($P < 0.01$). Compared with control siRNA group, the level of TLR2 was decreased in the TLR2 siRNA group, the IL-1R1 was also decreased in the IL-1R1 siRNA group, but the difference was extremely significant ($P < 0.01$) (Fig. 5a–c). When the concentration of siRNA was 45 nM and the concentration of LPS was 500 ng/mL, compared to control siRNA + LPS group, visfatin was down-regulated in TLR4siRNA + LPS group, TLR-2siRNA + LPS group and IL-1R1siRNA group ($P < 0.01$) (Fig. 5d–f). In addition, siRNA of TLR4, TLR2 and IL-1R1 were transfected into RAW264.7 cells for 24 h, and the dynamic changes of visfatin expression were observed, respectively, after LPS treatment for 3, 6 and 12 h. The result of Western blot showed that compared with the control siRNA + LPS group, visfatin was decreased in TLR4 siRNA + LPS group, TLR2 siRNA + LPS group and IL-1R1siRNA + LPS group. The expression of visfatin in TLR2 siRNA + LPS group and IL-1R1siRNA + LPS group was downregulated remarkably at 6 and 12 h ($P < 0.05$) (Fig. 5g–l).

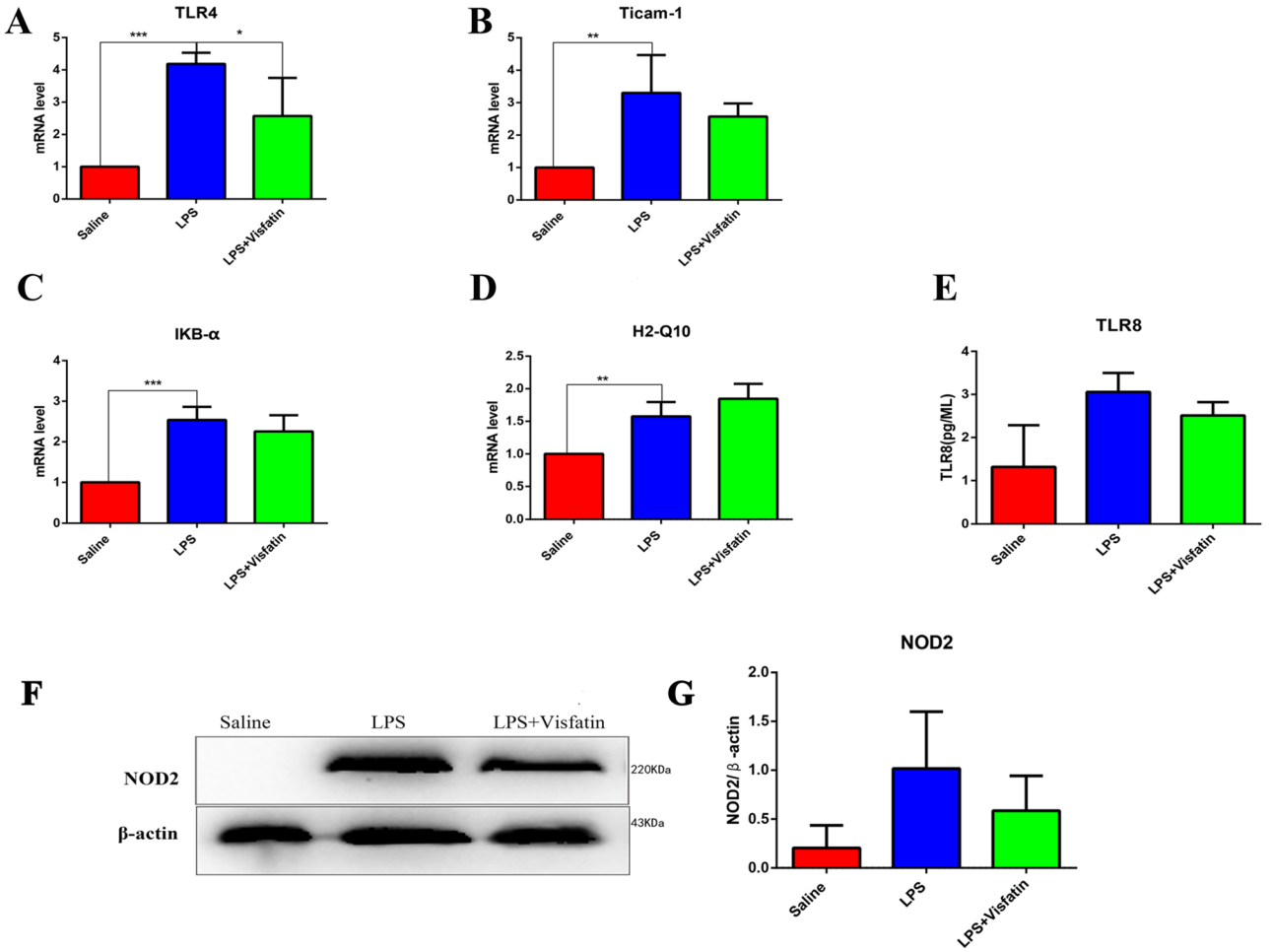

Fig. 3 Expression of key genes in innate immune signaling pathway of ileum. We further studied the mechanisms of the beneficial effects by visfatin on immune-related mRNAs and proteins upregulated after LPS stimulation. Western bot, ELISA and q-PCR techniques were used to detect the expression of key genes in innate immune signaling pathway of ileum. **a**–**d** The mRNA level of key genes in intestinal mucosal immune signaling pathway. **e** and **g** The protein level of NOD2 in ileum. **h** The expression of TLR8 in different experimental mouse ileum. Note: The difference was significant, $*P<0.05$, the difference was highly significant $**P<0.01$, the difference was very highly significant, $***P<0.001$

Effects of the Silence of TLR4, TLR2 and IL1R1 on the Expression of Inflammatory Factors

Following siRNA transfection, we stimulated RAW264.7 for 3, 6 and 12 h with LPS and detected the expression of TNF-α, IL-1β, IL-6 and MCP-1 in the culture supernatant by ELISA. Compared with the controls iRNA + LPS group, the inflammatory factors such as TNF-α and IL-6, IL-1β and MCP-1 were significantly decreased in the TLR4 siRNA + LPS, TLR2 siRNA + LPS and IL-1R1 siRNA + LPS groups ($P<0.01$). TLR4 siRNA, TLR2 siRNA and IL-1R1 siRNA significantly down regulated TNF-α (LPS stimulation for 6 h) and IL-6 (LPS treatment for 12 h) ($P<0.01$). TLR4 siRNA, TLR2 siRNA and IL-1R1 siRNA inhibited IL-1β (LPS treatment for 6 and 12 h) ($P>0.05$) and MCP-1 (LPS stimulation for 3 h) ($P<0.01$) (Fig. 6a–d).

Discussion

Pathogenic intestinal infections could cause epithelial injury and barrier destruction (Chen et al. 2019). Herein, after LPS stimulation, intestinal villi were damaged and villus-length/crypt-depth was reduced. The structural alterations of intact intestinal villi is a symbol of the intestinal barrier injury such as LPS-induced injury (Zhuang et al. 2019). Visfatin as a multifunctional protein (Brentano et al. 2007), promotes immune cells survival and involve in the inflammatory response (Moschen et al. 2007). Interestingly, visfatin is also an important pro-inflammatory factor, which expresses its high levels in the inflammatory response and controls the expression of many cytokines (Apostolopoulos et al. 2016; Hong et al. 2016; Wu et al. 2018; Yun et al. 2014). Consistent with these reports, LPS induced the intestinal barrier

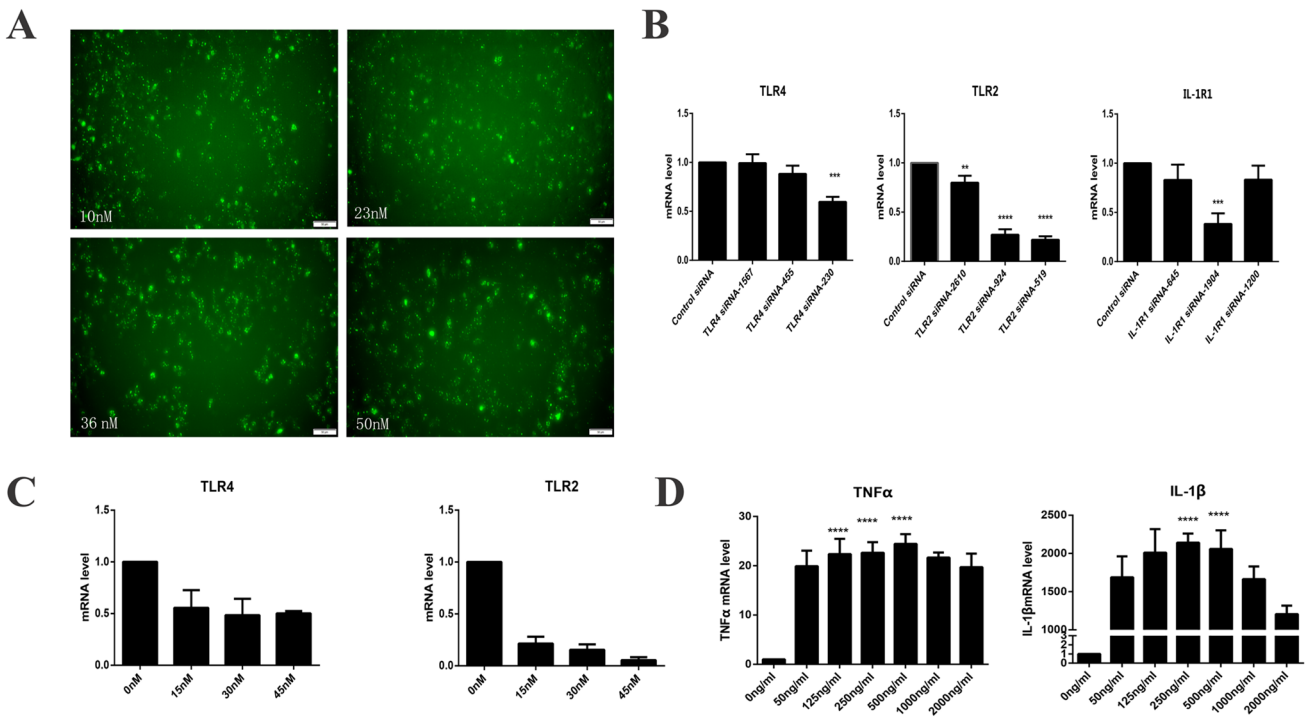

Fig. 4 RAW264.7 Cells and cell transfection. Specifying siRNAs targeting TLR4, TLR2 and IL-1R1 have the close relation to the expression of visfatin in inflammatory response. **a** The detection of FAM-NC siRNA transfection. **b** The expression of TLR4, TLR2 and IL-1R1 after transfection of RAW264.7 cells. **c** The expression of TLR4 and TLR2 after different siRNA transfection concentrations. **d** The expression of TNF-α and IL-1β induced by LPS. Note: compared with control siRNA group, $^{**}P<0.01$, $^{***}P<0.001$, $^{****}P<0.0001$. Compared with 0 ng/ml group, the difference was very highly significant, $^{****}P<0.0001$

injury and inflammation in our results. Moreover, visfatin regulates the synthesis of nicotinamide adenine dinucleotide (NAD) (Gerner et al. 2018). LPS induces inflammatory damage while promoting visfatin expression (Adyshev et al. 2014). Hence, the inflammation caused by bacteria is closely associated with visfatin.

Zo-1 regulates paracellular permeability by binding to actin and myosin (Kosińska and Andlauer 2013). Claudin-1 is involved in the regulation of epithelial ion and small molecules (Martínez et al. 2012). We found the main distribution of ZO-1 transferred from intestinal villus epithelial cells to intestinal crypts in LPS group. Degradation of ZO-1 caused by LPS leads to the active cytoskeleton damage in the intestinal mucosa (Van Itallie et al. 2009). TLR2 favors integrity of ZO-1-associated intestinal epithelial barrier (Cario et al. 2004). Herein, LPS also up-regulated Claudin-1 and inhibited ZO-1, which revealed that LPS induces the tight junction protein imbalance in the intestinal mechanical barrier (Shin and Kim 2018). Whereas, ZO-1 is abundantly expressed in the model of inflammation induced by trinitrobenzenesulfonic acid (Reinecke et al. 2012). sIgA plays critical role in intestinal mucosal immune barrier (Corthésy 2010). We found sIgA distribution around the intestinal gland and the lamina propria of the intestinal mucosa. LPS inhibits sIgA expression contributing to intestinal mucosal immune suppression (Liu et al. 2009a). In a prior report, VEGF promotes angiogenesis in the presence of acute inflammation (Karmpaliotis et al. 2002). Hence, it is evident that pretreatment of visfatin functions in mouse intestinal mucosal inflammatory injury and improves the secretion of sIgA in the ileum by antagonizing LPS.

H2-Q10 binds to the Ly49 receptor to regulate natural killer cells (Sullivan et al. 2016). TLR8 promotes the expression of TLR4 / TLR2 and MyD88 in adipose tissue (Ahmad et al. 2016). Visfatin functions to decrease TLR8 and NOD2 in intestinal mucosal injury (Kruis et al. 2014). Herein, visfatin significantly inhibited the expression of TLR4 by indirectly reducing TLR8 expression in ileum of mouse. While, visfatin can down-regulate NOD2 and exogenous visfatin could inhibit the inflammation of LPS-induced immune stress (Zhou et al. 2017). Thus, visfatin can reduce intestinal mucosal inflammatory response through the TLR4 signaling pathway and through TLR8 and NOD2 signaling indirectly.

TLRs activate host defense by initiating inflammatory response via TLR4 signaling (Lu et al. 2008) and play a critical role in inflammatory reactions and tissue homeostasis (Kayisoglu et al. 2020). TLRs trigger regulation of adapter molecules MyD88 and TRIF in the small intestine

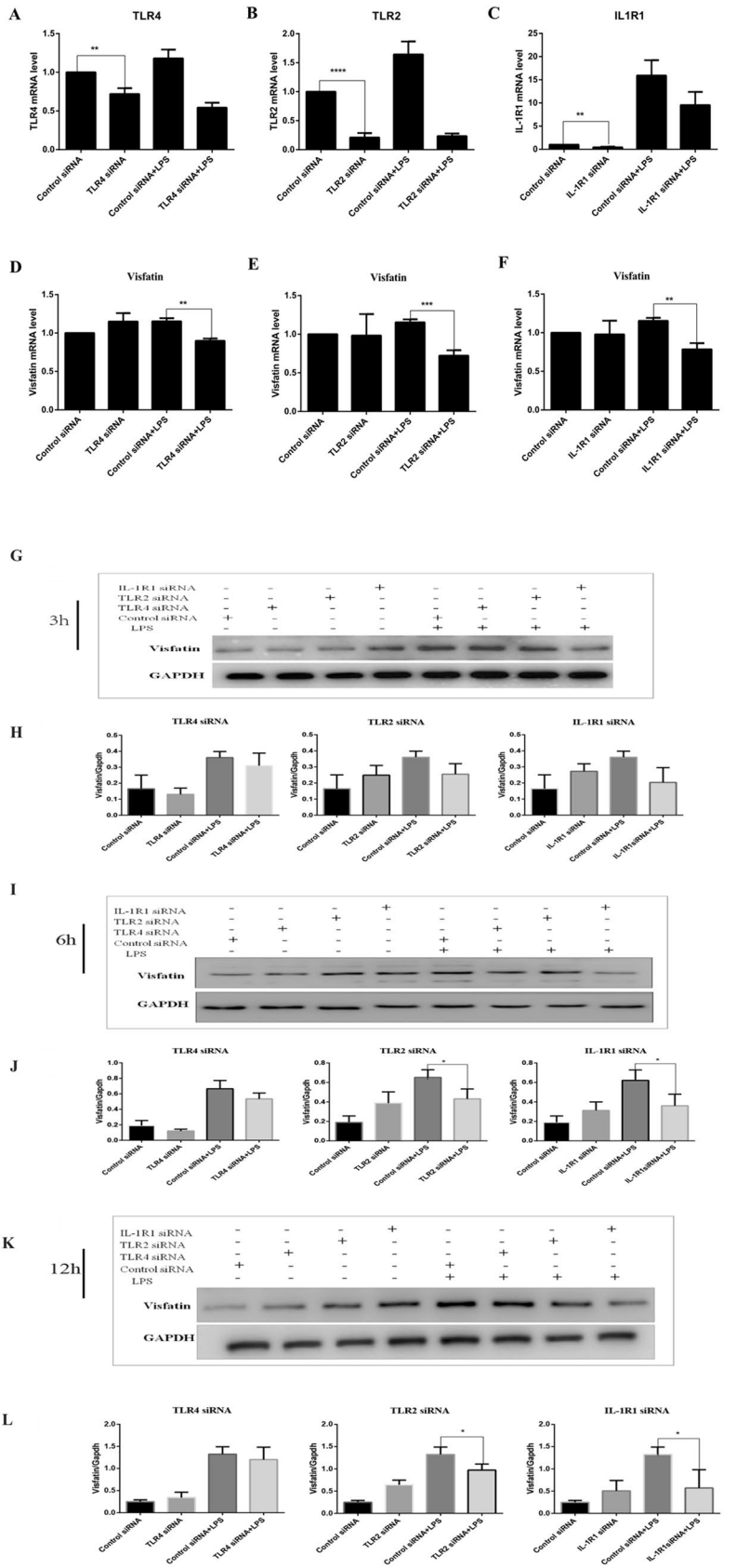

Fig. 5 Expression of visfatin in inflammatory response. **a–f** The mRNA level of visfatin in inflammation after the siRNA of TLR4, TLR2 and IL-1R1 transfection. **g–l** Western blot analysis of total visfatin expression at different time points after LPS stimulation. Note: compared with control siRNA + LPS group, the difference was significant, $*P < 0.05$, ($n = 3$ per group)

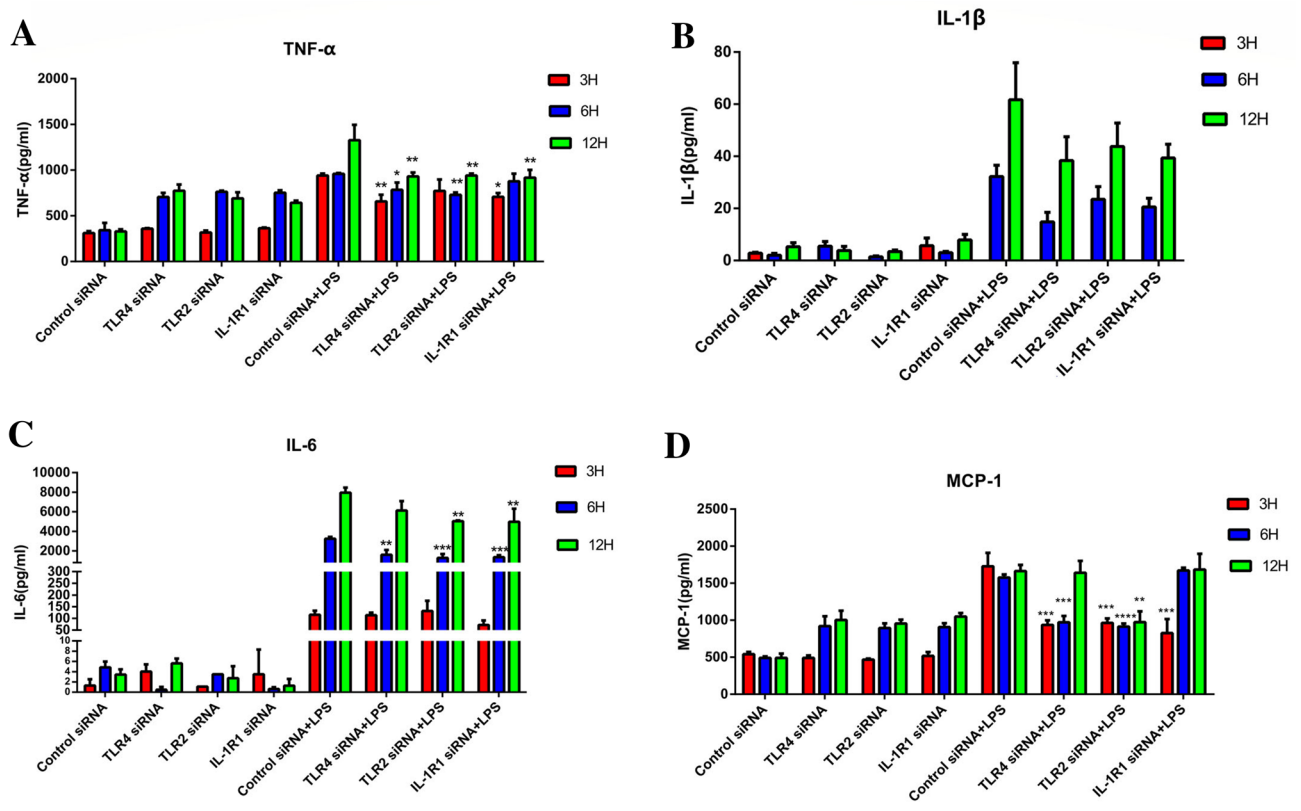

Fig. 6 Expression of inflammatory factors in RAW264.7after TLR4, TLR2 and IL1R1 silencing. Specifying siRNAs targeting TLR4, TLR2 and IL-1R1 were associated with the expression of inflammatory factors in RAW264.7 cells. **a**–**d** The effect of TLR4, TLR2 and IL-1R1 on the expression of TNF-α, IL-1β, IL-6 and MCP-1induced by LPS. Note: compared with control siRNA + LPS group, the difference was significant, $*P<0.05$, the difference was highly significant $**P<0.01$, the difference was very highly significant $***P<0.001$ and $***P<0.0001$

via gut microbiota (Hörmann et al. 2014). TLR signaling also activates the genes of host defense in intestinal epithelium (Price et al. 2018). Crohn's disease bacteria in the patient's stool effectively upregulates TLR4 expression (Gu et al. 2018). Herein, we showed that visfatin was highest in control siRNA + LPS group. By contrast, the lacking of TLR4, TLR2 and IL-1R1, especially TLR2 and IL-1R1, were down-regulated visfatin expression at 6 and 12 h in LPS stimulation. IL-1R1 initiates the IL-1 signaling cascade to participate in intracellular signal transduction (Sims 2002). Thus, Toll/IL-1R family has a strong regulatory effect on visfatin.

The silencing of TLR4, TLR2 and IL-1R1 gene expression could down-regulate inflammatory factors, while IL-1R1 blockage reduced inflammatory factors in serum (Gehrke et al. 2018), indicating that deletion of IL-1R1 may not affect activation of TLR4 signaling pathway. Increased visfatin in chronic obstructive pulmonary disease promotes TNF-α expression (Liu et al. 2009c). In visfatin knockout cells, IL-1β is significantly reduced (Liu et al. 2009b). Visfatin inhibitor in endotoxemia limit the inhibition of neutrophil enzymatic activity (Busso et al. 2008). The inhibition of MCP-1 secretion is not only regulated through NF-κB and AP-1 but also mediate signal pathway (Viedt et al. 2000). Thus, the decrease of visfatin mainly caused by the silence of TLR4, TLR2 and IL-1R1, played a key role in the immune inflammatory response. Moreover, the inflammatory factors decreased after silencing of TLR4, TLR2 and IL1-R1 and hence visfatin might participate in the TLR signaling pathway to regulate the release of inflammatory factors.

In conclusion, visfatin not only regulates the tight junction proteins Claudin-1 and ZO-1, but also VEGF and sIgA to participate in the intestinal mucosal barrier repair and protection. Visfatin activates TLRs and NLRs signaling and mainly monitor the expression of TLR4 to participate in the inflammatory response of intestinal mucosal injury. Secondly, TLR4, TLR2 and IL-1R1 regulate visfatin expression during LPS-induced inflammatory response in vitro. Furthermore, visfatin could regulate inflammatory factors such as TNF-α, IL-6 and IL-1β to participate in the LPS-induced inflammatory response.

Conclusion

Visfatin regulates not only the tight junction proteins Claudin-1, ZO-1, but also VEGF and sIgA to participate in the intestinal mucosal barrier repair and protection. On the cellular and molecular pathways, visfatin activates TLRs and NLRs signaling pathways, and mainly monitor the expression of TLR4 to participate the inflammatory response in intestinal mucosal injury. Secondly, TLR4, TLR2 and IL-1R1 regulate visfatin expression during LPS-induced inflammatory response in vitro, and TLR2 and IL-1R1 are dominating. Furthermore, visfatin could regulate inflammatory factors such as TNF-α, IL-6 and IL-1β to participate in the LPS-induced inflammatory response.

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Declaration

Conflict of Interest The authors declare that they have no conflicts of interest.

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