A novel increase in resistin levels induced by TLR4 deficiency inhibits lipopolysaccharide-induced IL-1β and TNF-α expression in C57BL/6J mice

Bin Han1*, Rong Li1,2,3*, Meizhen Wang1,4*, Hongping Chen5, Lixia Jiang6, Renping Liu1, Yujuan Zhang1, Yulin Liu1, Fucai Wang1, Qiaofa Shi1,2,3

1Department of Medical Microbiology and Immunology and Laboratory of Infection and Immunity, School of Basic Medical Science, Nanchang University, Nanchang, Jiangxi 330006, PR China; 2Jiangxi Province Key Laboratory of Infection and Immunity, Nanchang, Jiangxi 330006, PR China; 3Jiangxi Province Key Laboratory of Tumor Pathogenesis and Molecular Pathology, Nanchang, Jiangxi 330006, PR China; 4The Hospital of Nanchang University, Nanchang, Jiangxi 330006, PR China; 5Department of Histology and Embryology, Medical College of Nanchang University, 603 Bayi Road, Nanchang, Jiangxi 330006, PR China; 6Department of Clinical Examination, the First Affiliated Hospital of Gannan Medical University, Ganzhou, Jiangxi 341000, PR China. *Equal contributors.

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Abstract: Background: The activation of Toll-like receptor 4 (TLR4) by lipopolysaccharide (LPS) induces inflammatory cytokine production. Resistin is associated with chronic inflammation of the vascular system. However, the effects of TLR4 on resistin expression are not yet clear. Methods: Initially, resistin, IL-1β and TNF-α levels in wild type and TLR4-/- mice treated with or without LPS were examined using qPCR and ELISA, and the T lymphocyte profile was investigated using qPCR. Then, wild-type C57BL/6J mice were intraperitoneally injected with hRetn alone or in combination with LPS, and IL-1β and TNF-α levels were examined using qPCR and ELISA. Results: Resistin was consistently highly expressed in the spleen and serum of TLR4-/- mice more than wild-type mice (P < 0.05). This expression pattern was significantly attenuated 24 h post intraperitoneal injection of 5 mg/kg LPS (P < 0.05). However, TNF-α and IL-1β levels did not increase and were not obviously altered by LPS treatment in TLR4-/- mice. Compared with the wild-type C57BL/6J mice, significantly increased levels of RORγt, FoxP3, Tbet and GATA3 mRNA that were observed in splenic lymphocytes from TLR4-/- mice using qPCR, and their levels significantly decreased following stimulation with 5 mg/kg LPS for 24 h (P < 0.05). Treatment with 25 mg/kg recombinant human resistin for 12 h deceased LPS-induced IL-1β and TNF-α expression in wild-type C57BL/6J mice to some extent (P < 0.05). Conclusion: TLR4 deficiency increases resistin expression, while resistin inhibits LPS-induced IL-1β and TNF-α expression in C57BL/6J mice under some conditions.

Keywords: Toll-like receptor 4, lipopolysaccharide, inflammation, human resistin

Introduction

Toll-like receptor 4 (TLR4) is a pattern recognition receptor (PRR) that recognizes multiple types of pathogen-associated molecular patterns (PAMPs), such as LPS, and initiates intracellular signaling that induces the expression of related proinflammatory factors [1]. Lipopolysaccharide (LPS), an important component of the outer membrane of gram-negative bacteria, is an essential factor that induces the overexpression of proinflammatory factors by binding to TLR4. Resistin (also called FIZZ3) is a cysteine-rich adipokine that belongs to the family of resistin-like molecules (RELMs), which also includes three other members named RELMα (FIZZ1), RELMβ (FIZZ2) and RELMγ. The resistin protein forms a dimer through disulfide bonds, and then three dimers form a hexamer [2]. Rodent resistin is mainly secreted by white adipose tissue, but human resistin is mainly secreted by mononuclear/macrophages, lymphocytes, bone marrow, etc. [3]. It was named “resistin” because it produces insulin resistance [4], which is closely related to the occurrence of type 2 diabetes. In some non-bacterial inflammatory diseases, such as rheumatoid arthritis [5], degenerative disc disease [6], and
Atherosclerosis [7], resistin levels are significantly increased and positively correlate with disease severity.

Resistin has long been considered a pro-inflammatory factor, but recent studies have reported that resistin-like proteins also competitively bind TLR4 [6] and protect against endotoxic shock by blocking the LPS-TLR4 interaction [8]. Based on these findings, resistin may possess somewhat contradictory antibacterial and anti-inflammatory activities. In the present study, we first reported that TLR4 knockout mice had increased resistin expression, and resistin inhibited LPS-induced IL-1β and TNF-α expression and reduced the level of the inflammatory response in C57BL/6J mice.

Material and methods

Animals and animal care

6 to 8 weeks old male TLR4-deficient C57BL/6J mice and corresponding control mice were purchased from the Model Animal Research Center of Nanjing University. Subsequently, lack of the TLR-4 mRNA in TLR4-deficient C57BL/6J mice was confirmed using real-time PCR. All mice were fed a normal diet. After adaptive feeding for 3 days, we divided all mice into 2 experiments. In the first experiment designed to investigate the effect of TLR4 deficiency on the expression of proinflammatory factors, including resistin, IL-1β and TNF-α; 10 mice of each genotype were randomly sorted into a control subgroup and LPS treatment subgroup (n = 5 animals per subgroup). The LPS group was intraperitoneally injected with 5 mg/kg LPS (from Escherichia coli O55:B5, Sigma, CA, USA). The control group was intraperitoneally injected with the same amount of sterile PBS (approximately 0.1 ml). After 24 h, the mice were sacrificed, the spleen was removed, and blood was collected via retro-orbital puncture. After the blood had naturally coagulated, the serum was collected by centrifugation at 3000 rpm for 10 min, and frozen at -80°C until further examination.

All experimental protocols for animal care and use were approved by the Institutional Animal Care and Use Committee at Nanchang University, Jiangxi, China.

Quantitative real-time PCR

The mice in each group were treated with the drugs described above for 24 h, and the mouse spleens were aseptically removed and placed in a glass homogenizer. Total RNA was extracted from the spleens using a commercially available acid-phenol reagent (TRizol, Thermo Fisher, USA) after thorough grinding on ice. Potential DNase contamination was removed with DNase-free (Ambion, Austin, TX). The control group was intraperitoneally injected with the same amount of sterile PBS (approximately 0.1 ml). After 24 h, blood was collected via retro-orbital puncture and placed on ice. Serum was separated by centrifugation at 3000 rpm for 10 min, collected and frozen at -80°C until analysis. At the same time, the mice were sacrificed, and their spleens were isolated for real-time PCR and western blot analyses.

In the second experiment designed to determine the effects of a hRetn intervention on LPS-induced IL-1β and TNF-α expression, another 20 male wild-type C57BL/6J mice (described above) aged 6-8 weeks were randomly divided into a blank control group (C group), LPS treatment group (LPS group), resistin control group (hRetn group), and resistin + LPS intervention group (LPS + hRetn group), with 5 mice in each group. In this experiment, the complete treatment was performed in 2 steps. First, the mice in the hRetn and resistin + LPS groups were intraperitoneally injected with 25 mg/kg hRetn (PeproTech, USA). The purity was greater than 98% by HPLC, and the LPS concentration was less than 1 EU/mg. The other two groups (C group and LPS group) were injected with the same amount of sterile PBS (approximately 0.1 ml). After 12 h, the LPS and LPS + hRetn groups were intraperitoneally injected with 5 mg/kg LPS. The other groups (C group and hRetn group) were injected with 0.1 ml of sterile PBS. After 24 h, the mice were sacrificed, the spleen was removed, and blood was collected via retro-orbital puncture. After the blood had naturally coagulated, the serum was collected by centrifugation at 3000 rpm for 10 min, and frozen at -80°C until further examination.

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**Western blot**

Initially, 10 ml of protease inhibitor (PMSF; Solarbio, Beijing, China) were added to each 1 ml of protein lysis buffer according to the instructions of the total protein extraction kit (Solarbio). Then, 0.1 g of spleen tissue isolated from mice was placed in a glass homogenizer and 1 ml of protein lysis buffer was added immediately. After thorough grinding on ice, total proteins were collected into a 1.5 ml EP tube and centrifuged at 1200 × g per min for 30 min at 4°C. The supernatant was harvested into another 1.5 ml tube and the protein concentration was measured using the BCA method (Solarbio, Beijing, China). Twenty micrograms of each protein sample were loaded into the wells of the gel to ensure uniform loading. The samples were electrophoresed on a 15% SDS PAGE gel and transferred to a PVDF membrane (Millipore, Billerica, USA). The membrane strips were blocked with 5% skim milk at room temperature for 2 h, and then incubated with a rabbit anti-mouse resistin antibody (1:1000 dilution) (Omnimabs, USA) overnight at 4°C. A sheep anti-rabbit IgG antibody (1:10,000 dilution) (Omnimabs, USA) was incubated with the membrane at room temperature for 1.5 h, followed by exposure to a hypersensitive chemiluminescence substrate and visualization using a Bio-Rad instrument (Bio-Rad, USA). ImageJ software was used to test the gray value of the immunoblot bands, and the relative expression level of the target protein was measured by the ratio of the target protein bands to the corresponding internal reference protein bands.

**ELISA**

According to the instructions of the ELISA kit (Multisciences, Hangzhou, China), the standards and mouse serum samples were separately diluted, and three replicate wells were prepared for each sample. One hundred microliters of each sample was added to the wells and incubated at 37°C for 1.5 h. After carefully washing the wells with PBST, 100 μl of the working biotin-labeled antibody solution was added to each well and incubated at 37°C for 1 h. The wells were washed, and 100 μl of the avidin-peroxidase complex working solution was added to each well and incubated at 37°C for 0.5 h. Next, 90 ml of the TMB color development solution was added to each well and incubated at 37°C for 20 min in the dark. The color development of the wells was terminated by adding 100 ml of TMB stop solution to each well, and the absorbance was measured at 450 nm using a microplate reader. The concentrations of resistin and the proinflammatory factors IL-1β and TNF-α were calculated from the standard curve.

**Statistical analysis**

Measured data are presented as the mean ± standard deviation (x ± s) from at least three independent experiments. Data were analyzed using one-way analysis of variance analysis (ANOVA) and paired-t tests were used to compare the difference between groups with SPSS 20.0 statistical software (version 20.0; SPSS, Inc., Chicago, IL, USA). P-values < 0.05 were considered statistically significant.

**Results**

**TLR4 gene knockout increased the expression of resistin**

Resistin is an adipokine that has been extensively studied for its roles in glucose and lipid metabolism. Resistin levels are increased in subjects with some chronic nonbacterial inflam-

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**Table 1. Mouse primer sequences used for quantitative real-time PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Primer direction</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Resistin</td>
<td>Forward</td>
<td>CGATGAAGCCATCGACAAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGGAGGCAACCTCCCTCC</td>
</tr>
<tr>
<td>Tbet</td>
<td>Forward</td>
<td>AGCATGAGGCGCCACACTC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CACTCGGCGGGGTATGAG</td>
</tr>
<tr>
<td>GATA3</td>
<td>Forward</td>
<td>ATGCCCTGCGGACTCTCACC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGTGGTGCTGTGACAGTGCG</td>
</tr>
<tr>
<td>RORγt</td>
<td>Forward</td>
<td>ATGTCGCGAGTGGCTCAA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGAGAAGCTTGGCGACTTG</td>
</tr>
<tr>
<td>FoxP3</td>
<td>Forward</td>
<td>CGCTCAGATGGGTCACA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATTTGCAGCAGGGTGAGG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward</td>
<td>CCAGATGAGGACATGAGCA</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CCGAAGCTCTGAGTGCAGTG</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>ACTGGAGAAGAGAAGGCAC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CTGGCAGAACAGAGATGAG</td>
</tr>
<tr>
<td>β-actin</td>
<td>Forward</td>
<td>TAGAGCGAAGTACTGCTGT</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCCGACTGTCGATACTCCT</td>
</tr>
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</table>
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Figure 1. TLR4 knockout in C57BL/6J mice increases endogenous resistin levels but not IL-1β and TNF-α levels. WT (WT-LPS) and TLR4-/- (KO-LPS) mice were intraperitoneally injected with 5 mg/kg LPS. After 24 h, the total RNA and proteins were extracted from the spleen on ice using TRIzol (Thermo Fisher, USA) and protein lysis buffer, respectively. Levels of the resistin (A), IL-1β (C) and TNF-α (E) mRNA in the spleen were measured using real-time fluorescence quantitative PCR detection and normalized to β-actin. Serum concentrations of resistin (B), IL-1β (D) and TNF-α (F) were measured using ELISAs. (n = 5; *P < 0.05; **P < 0.01; ***P < 0.001; and ns, not significant).

TLR4 gene knockout promotes the differentiation of T lymphocytes in the mouse spleen

As the largest immune organ in the body, the spleen collects a large number of immune cells and is an important site of the immune response. TLR4 is the major receptor for LPS and is a bridge between innate and adaptive immunity. We examined the expression of the lymphocyte-specific transcription factors Tbet, GATA3, FoxP3, and RORγt in the spleens of wild-type and TLR4-/- mice to evaluate the effect of the TLR4 deficiency on the differentiation of Th1, Th2, Treg and Th17 cells, respectively. The expression of all of these T lymphocyte-specific transcription factors was significantly upregulated in TLR4-/- mice (P < 0.05) (Figure 3), indicating that TLR4 knockout significantly increased levels of the resistin mRNA and protein in the spleen of TLR4-/− mice, compared with wild-type mice (Figure 1A) (P < 0.05). Surprisingly, resistin expression in both TLR4-/− and wild-type C57BL/6J mice was partially attenuated by a 5 mg/kg LPS treatment (Figure 1A). This pattern of resistin expression in TLR4-/− C57BL/6J mice was also consistently confirmed by ELISA (Figure 1B) and western blotting (Figure 2). However, IL-1β (Figure 1C and 1D) and TNF-α (Figure 1E and 1F) levels in the spleen and serum did not increase and showed no fluctuation upon the LPS treatment in TLR4-deficiency mice (P > 0.05). Thus, the TLR4 gene knockdown increased resistin expression but not IL-1β and TNF-α expression in C57BL/6J mice.

TLR4 gene knockout promotes the differentiation of T lymphocytes in the mouse spleen

As the largest immune organ in the body, the spleen collects a large number of immune cells and is an important site of the immune response. TLR4 is the major receptor for LPS and is a bridge between innate and adaptive immunity. We examined the expression of the lymphocyte-specific transcription factors Tbet, GATA3, FoxP3, and RORγt in the spleens of wild-type and TLR4-/- mice to evaluate the effect of the TLR4 deficiency on the differentiation of Th1, Th2, Treg and Th17 cells, respectively. The expression of all of these T lymphocyte-specific transcription factors was significantly upregulated in TLR4-/- mice (P < 0.05) (Figure 3), indicating that TLR4 knockout
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May induce Th cell differentiation and development. The expression of FoxP3, Tbet, RORγt and GATA3 mRNA was significantly decreased in TLR4-/- mice that received an intraperitoneal injection of 5 mg/kg LPS. The altered expression patterns of T lymphocyte-specific transcription factors in TLR4-/- mice were amazingly the same as the changes of resistin expression. Based on these results, TLR4 knockout exerted certain effects on the differentiation and development of various T lymphocytes in C57BL/6J mice.

Resistin inhibits the LPS-induced inflammatory response in C57BL/6J mice

The endogenous expression of resistin, but not IL-1β and TNF-α, was significantly increased and unexpectedly decreased, respectively, following treatment with LPS in TLR4-deficient and wild type C57BL/6J mice. At the same time, the mRNA levels of the T lymphocyte-specific transcription factors FoxP3, RORγt and GATA3 were consistently decreased in a similar manner as resistin in TLR4-deficient and wild type C57BL/6J mice in response to the LPS treatment. All these results implied that resistin might decrease LPS-induced inflammation by regulating the immune reaction. Therefore, we treated wild-type C57BL/6J mice with recombinant human resistin to evaluate the effect of the increased resistin levels on LPS-induced IL-1β and TNF-α expression in wild type C57BL/6J mice. The results showed that the treatment of 25 mg/kg recombinant human resistin protein for 12 h significantly reduced LPS-induced expression of the IL-1β (Figure 4A) and TNF-α mRNA levels (Figure 4B) and serum levels of the secreted proteins in wild-type mice (Figure 4C and 4D). Thus, resistin inhibited LPS-induced IL-1β and TNF-α expression in wild-type C57BL/6J mice to some extent.

Discussion

LPS is a component of the outer wall of gram-negative bacteria that interacts with cell surface receptors such as TLR4 to stimulate a strong immune response in the host and the release of a large number of inflammatory factors, causing endotoxemia. Severe endotoxemia leads to systemic inflammatory response syndrome (SIRS), multiple organ dysfunction syndrome (MODS), and even multiple organ failure (MOF) [9]. Resistin is an adipokine that regulates glucose and lipid metabolism. Resistin reduces the sensitivity of tissues and organs to insulin and promotes the pathogenesis of type 2 diabetes [4]. Studies have also reported elevated levels of resistin in subjects with some non-bacterial inflammatory diseases, such as rheumatoid arthritis and atherosclerosis. Therefore, these studies identified resistin as a proinflammatory factor. TLR4 is the major receptor for LPS. After this gene is knocked out, the LPS-induced activation of the TLR4/MyD88/NFκB signaling pathway is inhibited [10], thereby reducing the inflammatory response.

Recent in vitro studies have confirmed that TLR4 is also a receptor for resistin [6, 11], and resistin competes with LPS for binding to TLR4 [12], which may be one of the mechanisms by which resistin exerts its anti-inflammatory effects. In studies of adipose tissue and adipocytes, LPS inhibited resistin expression in adi-
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**TLR4 Knockdown and Resistin Expression**

In our study, TLR4 knockout increased resistin expression and levels of the FoxP3, RORγt, and GATA3 mRNA in TLR4-/- C57BL/6J mice, changes that were consistently attenuated to some extent after LPS stimulation in TLR4 knockout mice. However, the levels of the typical proinflammatory factors IL-1β and TNF-α were not increased and showed no fluctuations following LPS treatment in TLR4-/- mice. Thus, resistin plays an important role in inhibiting the inflammatory response induced by LPS. This result is consistent with findings reported in the literature [12]. Then, we administered the recombinant human resistin protein to the wild-type mouse model of endotoxemia to evaluate the effect of the TLR4 deficiency-induced increase in resistin levels on the expression of proinflammatory factors. An in vivo injection of human resistin inhibited the LPS-induced expression of IL-1β and TNF-α, thereby exerting an inhibitory effect on inflammation.

**Resistin and Parasitic Infections**

Recently, human and mouse resistin and resistin-like molecule β (RELMβ) were shown to selectively recognize gram-negative bacteria in the gut and bind to negatively charged lipids on the surface of bacteria. The membrane surface forms transmembrane pores with a uniform size, leading to bacterial lysis. This biological function plays an important role in the ability of intestinal mucusal epithelial cells to resist intestinal bacterial invasion and maintain intestinal mucusal barrier function [14]. According to Jang et al [15], elevated resistin expression induced by parasitic infections increases the parasite load in animal models and the tolerance of the experimental model to parasitic infections. Furthermore, resistin competes with LPS/MD2 for TLR4, significantly increasing the survival rate of mice with endotoxic shock [8]. Although previous studies have confirmed elevated levels of resistin in patients with diabetes and have shown that resistin promotes diabetes, resistin reduces the production of reactive oxygen species (ROS) in patients with diabetes mellitus complicated with *M. tuberculosis* and inhibits mycobacteria-induced inflammatory reactions [16]. Moreover, human resistin enhances neutrophil-mediated inflam-

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**Figure 3.** Effects of TLR4 knockout and the LPS treatment on splenic T lymphocyte differentiation in mice. TLR4 knockout mice and wild-type control mice were intraperitoneally injected with 5 mg/kg LPS, and the blank control group was injected with 0.1 ml of sterile PBS. After 24 h, the total RNA was extracted from the spleen and detected using real-time fluorescence quantitative PCR. The expression of the following mRNAs was detected: (A) Treg-specific transcription factor FoxP3, (B) Th7-specific transcription factor RORγt, (C) Th1-specific transcription factor Tbet, and (D) Th2-specific transcription factor GATA3. The expression of the four T lymphocyte-specific transcription factors was upregulated in TLR4-/- C57BL/6J mice. After intraperitoneal injection of 5 mg/kg LPS, splenic mRNA levels of the T cell-specific transcription factors FoxP3, RORγt, Tbet, and GATA3 were significantly decreased in TLR4-/- mice, indicating that LPS altered the differentiation of various T lymphocytes in a TLR4-independent manner in mice. (n = 5; *P < 0.05; **P < 0.01; ***P < 0.001; and ns, not significant).
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Inflammatory responses and aggravates LPS-induced lung injury by inhibiting the MAPK signaling pathway in LPS-induced inflammatory models of human resistin transgenic mice [17]. However, in these studies, the doses and duration of LPS and resistin differed, which may be the main explanations for different experimental conclusions. Interestingly, clinical studies have observed higher resistin expression in patients with various types of cancer, although some studies have used cancer cell lines to prove that resistin promotes tumor growth by increasing the expression of certain proteins or enhancing the activity of some enzymes [18-20]. However, further studies are needed to determine whether the inhibition of immune functions mediated by increased resistin levels affects tumor immunity. Combined with some existing studies, the resistin family of molecules exerts bactericidal and anti-inflammatory effects and is an integral component of innate immunity. This study provides a useful explanation to improve primary understanding of the regulatory effects of resistin on immunity.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Qiaofa Shi, Department of Immunology, Medical College of Nanchang University, 461 Bayi Road, Nanchang, Jiangxi 330006, PR China. Tel: +86 079186360563; E-mail: sqf741@sina.com

References


[3] Patel L, Buckels AC, Kinghorn IJ, Murdoch PR, Holbrook JD, Plumpton C, Macphee CH and
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