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FoxO3 might be involved in the inflammatory response of human monocytes to lipopolysaccharide through regulating expression of toll like receptor 4

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Abstract

Objective—Previous studies have found that forkhead box o3 S574 phosphorylation status can regulate inflammation by inducing monocytes/macrophages apoptosis, and whether it directly affects the inflammatory response of monocytes has not been demonstrated. The aim of this study was to investigate the role of forkhead box o3 in inflammatory response of monocytes against lipopolysaccharide.

Methods—THP-1 cells were used to knock down or overexpress forkhead box o3 and its mutants, and then detect the activation of inflammatory cytokines expression and activation of nuclear factor kappa B after lipopolysaccharide treatment.

Results—The present study demonstrated that lipopolysaccharide can up-regulate forkhead box o3 protein expression, especially the non-phosphorylated form at S574, in a post-transcriptional way. Knockdown of forkhead box o3 attenuated lipopolysaccharide mediated nuclear factor kappa B activation and downstream inflammatory cytokines expression. When overexpressing forkhead box o3, only non-phosphorylated S574A forkhead box o3 mutant enhanced lipopolysaccharide induced nuclear factor kappa B activation and inflammatory cytokines expression. Further studies have found that S574A forkhead box o3 may promote toll like receptor 4 expression through binding and accelerating its transcriptional activity from promoter.

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Competing interests The authors declare that they have no competing interests.

Conclusion—There might be a positive feedback loop between lipopolysaccharide and forkhead box o3 in monocytes to promote the lipopolysaccharide mediated inflammatory response.

Keywords

Lipopolysaccharide; Fork head box O3; Monocyte; Nuclear factor kappa B; Toll like receptor 4

Introduction

As the essential molecule of the cell wall of gram-negative bacteria, lipopolysaccharides (LPS) is the most widely studied pathogen-associated molecular pattern (PAMP) involved in processes of multiple diseases such as sepsis [1], liver diseases [2], metabolic diseases [3], and autoimmune diseases [4]. Furthermore, LPS is the most important stimuli that induces recruitment, differentiation and inflammatory response of monocytes and macrophages through binding toll-like receptor 4 (TLR4) [5] to activate several pathways including nuclear factor kappa B (NF-κB) [6], mitogen-activated protein kinases (MAPK) [7-10], etc.

Forkhead box O3 (FoxO3) is a transcription factor involved in multiple biological processes including aging, cell cycle, cell death, autophagy, DNA repair and metabolism [11]. In addition, more and more evidences show the effects of FoxO3 on inflammatory response in dendritic cells [12, 13], T cells [14] and NK cells [15]. Some studies have found that FoxO3 could inhibit inflammatory responses through various mechanisms depending on different cell types, such as binding to RelA to prevent its translocation into nucleus in tumor associated dendritic cells [13] and repress transcription of interferon regulatory factor 7 (IRF7) in bone marrow macrophages [16], while others found that FoxO3 overexpression activates nuclear factor kappa B (NF-κB) to increased LPS mediated the transcription of IL-8 and TNF-α [17] in epithelial cells.

In our previous study, we found that LPS induces c-Jun N-terminal kinase (JNK) dependent phosphorylation of FoxO3 at serine 574, which mediated apoptosis of macrophages and monocytes through downregulation of Bcl-2 and upregulation of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) [18]. Although LPS induced p-S574-FoxO3 mediated apoptosis only occurred in about 10%-15% of macrophages and monocytes, it is critical for promoting anti-inflammatory macrophages differentiation [19]. In addition to affecting macrophages immune function by regulating cell death, FoxO3 can also play a regulatory role through directly affecting macrophage differentiation and immune responses. Bouzeyen etc. found that in *Mycobacterium tuberculosis*-infected macrophages, activating FoxO3 would promote the differentiation of macrophages into M1 subtype by inhibiting the expression of interleukin-10 (IL-10), while knocking down FoxO3 would downregulate the Th1 immune response induced by *mycobacterium tuberculosis* [20]. Using the mouse model of *Salmonella typhimurium* infection, Joseph etc. found that knockout of FoxO3 had no effect on macrophage proliferation and cell death. However, ERK activation mediated by *Salmonella typhimurium* infection could be prolonged by FoxO3 knockout, resulting in up-regulated IL-10, while down-regulating IL-12 and tumor necrosis factor alpha (TNF-α) [21]. These results suggested FoxO3 might play an important role in maintaining the Th1 immune response triggered by intracellular infection.

However, whether FoxO3 directly regulate inflammatory response of monocytes and macrophages against LPS needs further investigation, since the mechanisms by which LPS and intracellular bacteria induce macrophage immune responses are different. Therefore, in the present study, the role of FoxO3 in inflammatory response of monocytes against LPS and possible mechanisms has been partially studied.

Materials and methods

Cell culture and viral infection

THP-1 human monocytes were purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in RPMI1640 medium supplemented with 10% fetal bovine serum (Gibco) and 50 μM 2-mercaptoethanol (Gibco) at 37 °C in an atmosphere containing 5% CO2.

Lentiviral vectors ovexpressing wild type FoxO3, point mutated FoxO3 S574A and S574D were constructed and kept in our lab as described previously [18]. Non-target control vector, lentiviral shRNA vectors against FoxO3(MISSION TRC shRNA TRCN0000040100, TRCN0000040098), psPAX2 (packaging vector), pMD2.G (envelop vector) and the control vector pLKO.1 were from Sigma Aldrich and co-transfected into 293FT cells by X-tremeGENE HP (Roche) according to the manufacture's instruction. At 24 h, 48 h and 72 h post transfection, medium were collected and stored at −80°C before use. For preparation of FoxO3 knockdown cells, THP-1 cells were transduced with lentiviral FoxO3 shRNA supernatants supplemented with polybrene (4 μg/ ml). Pools of shRNA-transduced cells were selected by adding 5 μg/ml puromycin (Gibco, NY, USA) to the culture medium. FoxO3 expression level was determined by western blot 72 h after infection.

Antibodies

A custom p-FoxO3-S-574 rabbit polyclonal antibody was generated by Epitomics using the peptide SAKHQQQS(PO3H2)PVSQSM for immunization as described previously [18]. Phospho-specific antibodies were subsequently purified through antigen affinity columns. Primary antibodies against FoxO1, FoxO3, P21, phosphorylated-P65, total P65, phosphorylated-IκB, total IκB and phosphorylated-Iκκα/β were purchased from Cell Signaling Technology (Danvers, MA, USA). GAPDH antibody and normal Ig control were from Santa Cruz. HA antibody was from Abcam. Secondary antibodies were from Invitrogen. LPS (*E.coli*, serotype O55:B5) was from Enzo.

Protein isolation and western blot

Whole-cell lysates were prepared from cells that had been washed with PBS and harvested by centrifugation. Cell pellets were resuspended in RIPA lysis buffer containing 50 mM Tris, pH 7.5, 150 mM sodium chloride, 1% NP-40, 0.2% sodium dodecyl sulfate, 0.5% sodium deoxycholate, 0.1 mM ethylenediaminetetraacetic acid, and 1% protease and phosphatase inhibitors (Sigma-Aldrich) on ice for 30 min with occasionally vortex. The lysates were then centrifuged at 14,000×*g* for 15 min; supernatants were collected, and protein concentrations were measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). Cell lysates (40 μg) were separated by 10% SDS–polyacrylamide gel electrophoresis and

transferred to polyvinylidene difluoride membranes (Immobilon-P membranes; Millipore, Billerica, MA, USA). Membranes were blocked with blocking buffer (5% non-fat milk in TBST) for 1 h at room temperature. After incubation with appropriate primary antibodies overnight at 4 °C, membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies, and protein bands were detected using the SuperSignal West Femto substrate (Thermofisher) with the ODYSSEY Fc, Dual-Mode Imaging system (Li-COR, Lincoln, NE, USA).

Real time PCR

RNA was extracted and purified from cultured cells using RNeasy Mini kit (Qiagen, Valencia, CA). cDNA was generated with RNA reverse transcription kit (Applied Biosystems, Warrington, UK). Quantitative RT-PCR was performed in a CFX96 real-time system (Bio-Rad, Hercules, CA) using specific sense and antisense primers in 20 μl reaction volumes containing 10 μl SYBR Green PCR master mix (Bio-Rad), 10 μl of 1 μM primer stock and 40 ng of cDNA. PCR was performed at following condition: 95 °C for 10 min, followed by 40 cycles at 95 °C for 10 s, 58 °C for 20 s and 72 °C for 30 s. The relative amounts of mRNA were calculated by the $2^{-\Delta\Delta Ct}$ method. The following primers were listed in Supplementary Table 1.

Chromatin immunoprecipitation (ChIP) assay

THP-1 cells were infected with lentivirus to knockdown and (or) overexpress FoxO3 (WT, S574A and S574D). After 48 h post infection, cells were treated with formaldehyde at a final concentration of 1% for 10 min to crosslink DNA and proteins. The crosslinking reaction was stopped by adding glycine at 0.125 mol/l final concentration for 5 min at room temperature. Cells were rinsed twice with ice-cold 1 × PBS and resuspended in cell lysis buffer (10 mM Tris–HCL (PH 8.0), 10 mM NaCl, 3 mM $MaCl_2$, 0.5% NP-40 and protease inhibitors) and incubated on ice for 15 min. The cell suspension was vortexed briefly every 5 min to aid in release of nuclei. Nuclei were collected by centrifuge, resuspended in nuclei lysis buffer (1% SDS, 5 mmol/l EDTA, 50 mmol/l Tris–HCl (PH 8.0) and protease inhibitors) and sonicated to generate chromatin to an average length of ~ 200 to 500 bp (10 × 15 s at 55% maximum potency). After centrifugation at 12,000×*g* for 10 min at 4 °C, samples (400 μg of protein extracts) were immunoprecipitated overnight at 4 °C with 2 μg anti-HA ChIP-grade antibody (Abcam) or 3 μl anti-FoxO3 (clone 75D8 from Cell Signaling Technology, Danvers, MA, USA). Normal mouse or rabbit IgG was used as a negative control of immunoprecipitation. One percent of supernatant from the immunoprecipitation was saved as total input of chromatin and was processed with the eluted immunoprecipitates beginning at the crosslink reversal step. Next, 20μL magnetic beads (Dynabeads M-280 Sheep anti Mouse IgG, Invitrogen) were added into each sample and incubated at 4 °C for 4 h with rotation. Immunoprecipitates were washed with ChIP Low Salt Buffer (0.1% SDS, 1% Triton-100, 2 mM EDTA, 50 mM HEPES, 150 mM NaCl, PH 7.5), ChIP High Salt Buffer (0.1% SDS, 1% Triton-100, 2 mM EDTA, 50 mM Hepes, 500 mM NaCl, PH7.5), ChIP LiCl buffer (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholated, 1 mM EDTA, 10 mM Tris–HCl, PH 8.0) and TE buffer (10 mM Tris–HCl, 1 mM EDTA). Immunocomplexes were eluted with 90μL elution buffer (1% SDS, 50 mmol/l $NaHCO_3$) and 10μg of RNase A was added to the pooled eluates; crosslinks were reverted by incubation at 65 °C for at least

6 h. Samples were added with 1 μl of 20 μg/μl proteinase K and incubated for 2 h at 45 °C. After incubation at 95 °C for 10 min, samples were purified with Qiagen PCR purify kit. DNA samples were amplified with target promoter-specific primers using PCR analysis. The primers used in ChIP were listed in Supplementary Table 2.

Luciferase reporter assays

The hTLR4 promoter containing two binding sites (BS) confirmed by ChIP and it was synthesized by Synbio Technologies Company (Suzhou, China) and then cloned into pGL4.10 luc2 reporter vector (Promega). Hela cells growing in 12-well plates were co-transfected with pGL4-hTLR4 full length promoter (or truncated pGL4-hTLR4 lacking BS2 (pGL4-hTLR4-ΔBS2)) plasmids and FoxO3 plasmids. After 48 h post transfection, the relative luciferase activity was detected by the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer instructions.

Statistics

Each experiment was repeated at least three times with two technical replicates each unless indicated otherwise, as this was generally sufficient to achieve statistical significance for differences. Statistical significance between groups was calculated by using one-way analysis of variance, followed by Turkey's test and statistical significance between the two groups was calculated by two-tailed unpaired Student's t-test using commercially available statistical software (SigmaPlot 11.0 for Windows; Systat Software, Inc., San Jose, CA, USA). Data are presented as means ± standard deviations, and differences with P values of lower than 0.05 were considered statistically significant.

Results

LPS up-regulated FoxO3 protein level in human monocytes

Our previous study showed LPS could phosphorylate FoxO3 at S574 through JNK pathway [18]. LPS also up-regulated total FoxO3 protein level in THP-1 monocytes (Fig. 1A) in post transcriptional way since mRNA level of FoxO3 had no changes post LPS stimulation (Fig. 1B). Then, cycloheximide (CHX) was used to inhibit protein synthesis to evaluate whether LPS regulated total FoxO3 protein through affecting FoxO3 degradation. As shown in Fig. 1C, no differences in CHX plus LPS group were observed compared with CHX group, suggesting LPS mediated upregulation of FoxO3 level was also not caused by reducing its degradation. Therefore, the mechanisms of LPS mediated upregulation of FoxO3 protein expression, such as affecting the stability of mRNA, or regulation at translational level, needs to be further elucidated. Further, fold changes of phosphorylated FoxO3 at S574 were significantly lower than total FoxO3 (Fig. 1D, E), indicating newly FoxO3 protein induced by LPS was dominated by non-phosphorylated FoxO3 at S574.

FoxO3 knockdown attenuated the inflammatory cytokines expression and NF-κB activation induced by LPS in THP-1 cells

Since LPS was capable of upregulating FoxO3 expression in monocytes, it's reasonable to speculate that FoxO3 plays a role in LPS mediated monocytes inflammatory response. TNF-α (Fig. 2A), IL-1β (Fig. 2B), IL-6 (Fig. 2C) and IL-8 (Fig. 2D) mRNA expression after

6 h post LPS (10 ng/ml) stimulation was compared between control and FoxO3 KD groups, and inflammation cytokines expression was significantly lower in FoxO3 deficient THP-1 monocytes. Comparison of NF-κB activity was also performed and similar results with cytokines expression were observed as shown in Fig. 2E. Phosphorylation of P65, IκBα and Iκκα/β induced by LPS was significantly attenuated by in FoxO3 KD THP-1 monocytes.

Overexpression of FoxO3 mutant non-phosphorylated at S574 enhanced LPS mediated cytokines expression and NF-κB activation

To further confirm the role of FoxO3 in monocytes response to LPS, we over-expressed wild type (WT) FoxO3, its isoforms S574A and S574D in THP-1 monocytes using lentivirus. Interestingly, only the S574A FoxO3 mutant that could not to be phosphorylated enhanced the LPS induced inflammatory cytokines expression, while WT and S574D FoxO3 had no effect (Fig. 3A-D). Activation of NF-κB confirmed by western blot showed the similar results, phosphorylation of P65, IκBα and Iκκα/β could be significantly enhanced in S574A overexpressed cells to LPS (Fig. 3E). To determine whether FoxO3 regulates the activity of NF-κB by regulating nuclear translocation of P65, we used immunofluorescence to detect the translocation of total P65 after LPS stimulation. There were no significant differences in P65 translocation post LPS among FoxO3 WT and mutant groups (Supplementary Fig. 1).

TLR4 was up-regulated in S574A FoxO3 overexpressed THP-1 monocytes

To further elucidate the mechanisms of S574A FoxO3 enhanced LPS mediated NF-κB activation and its downstream genes expression, key molecules of NF-κB pathway and its regulators were screened. As shown in Fig. 4A, relative expression of TLR4, MyD88 and TRIF was significantly higher in S574A FoxO3 overexpressed THP-1 cells compared with control, WT FoxO3 and S574D FoxO3 groups. However, FoxO3 KD only down-regulated the relative expression of TLR4 mRNA, which was corresponding to the changes of LPS mediated inflammatory cytokines expression and NF-κB activation regulated by FoxO3 mutants. Therefore, TLR4 was chosen to be further investigated and protein expression of TLR4 was consistent with mRNA level (Fig. 4B).

S574A FoxO3 binds and accelerate the transcription from TLR4 promoter

To confirm the transcriptional regulation of FoxO3 on TLR4, ChIP assay was recruited to see whether S574A FoxO3 can specifically bind TLR4 promoter. Based on the predicted binding sites on TLR4 promoter, 11 pairs of corresponding primers targeting binding sites were designed. As shown in Fig. 5, although WT and S574D FoxO3 could also bind to TLR4 promoter at − 519 to − 510 bp, − 659 to − 650, − 808 to − 800, − 1498 to − 1490, − 1962 to − 1954, − 2156 to − 2148 and − 2989 to − 2980 bp from TSS, combined with above results showing WT and S574D FoxO3 overexpression had no effects on TLR4 mRNA expression, these bindings may have no regulatory effects on TLR4 transcription. Therefore, two predicted BSs [(BS1: − 1385 to − 1377, GAAATAAG) and (BS2: − 1567 to − 1559, TGAAAACA)] might be involved in S574A FoxO3 mediated TLR4 mRNA upregulation. Further, the luciferase reporter assay was used to confirm the impact of S574A FoxO3 on these two BSs. As shown in Fig. 5E, it is suggested that S574A FoxO3 only promotes the transcription of TLR4 by binding to BS2, while binding to BS1 has no effect on the

transcription of TLR4, since S574A FoxO3 didn't affect the transcriptional activity in BS2 deletion mutation.

Discussion

FoxO3 is mainly regulated by post-translational modifications with multiple signaling pathways including PI3K/Akt [22], SGK1 [23], casein kinase 1α [24], DYRK1 [25], AMPK [26] and IκκβIκκβ [27]. In the absence of environment signals or growth factors, FoxO members localize to the nucleus. After PI3K mediated activation of the serine/threonine kinase Akt, FoxO3 are rapidly phosphorylated at T32, S253 and S315, and then bind to 14-3-3 chaperone protein, which sequester FoxO3 within the cytoplasm, leading to its functional inactivation [22, 28, 29]. However, in some cell types such as acute myeloid leukemia cells, FoxO3 is in constant inactive state due to cytoplasmic localization and ubiquitin-dependent degradation mediated by IκκβIκκβ induced phosphorylation at S644. In HE-29 intestinal epithelial cells, LPS was found to induce FoxO3 degradation by activating PI3K [29]. However, in this study, LPS up-regulated FoxO3 instantly in human monocytes with IκκαIκκα/βIκκβ phosphorylation at post-transcription level, without increased FoxO3 degradation, indicating LPS might affect FoxO3 translational efficiency or FoxO3 mRNA stability in human monocytes. As the most important one of LPS induced inflammatory cytokine secreted from monocytes, the expression of TNF-α is regulated at both transcriptional and post-transcriptional levels [30, 31]. LPS could activate NF-κB to increase TNF-α mRNA expression, and also enhance the translational efficiency from mRNA to protein through activation of P38 [32]. Therefore, whether LPS mediated P38 activation enhances translational efficiency of FoxO3 needs further confirmation.

Foxo3 has been shown to regulate monocyte and macrophage differentiation and immune responses in intracellular bacterial infections such as *Mycobacterium tuberculosis* and *Salmonella typhimurium* by affecting the expression of IL-10 [20, 21]. However, the mechanisms by which intracellular and extracellular infections initiate monocyte and macrophage differentiation and immune responses are different. As an important pathogenic substance of extracellular Gram-negative bacterial infection, LPS mainly mediates immune response by activating NF-κB and MAPK path-ways, etc. During the intracellular infection of *Salmonella typhimurium*, however, the impact of FoxO3 on the immune response of monocytes depends on the expression of IL-10, while it has no effects on P65, P38 and JNK [21].

The impact of FoxO3 on NF-κB activation is different in various cell types. Our present results showed that LPS induced inflammatory response in human monocytes while up-regulating FoxO3 expression, and could be significantly attenuated by FoxO3 knockdown. As the most important signaling pathway in monocytes inflammatory response to LPS, NF-κB activation showed the similar results with inflammation cytokines expression. All these results indicated the positive role of FoxO3 in LPS mediated NF-κB activation. However, when FoxO3 was overexpressed in human monocytes, only S574A FoxO3 enhanced the LPS-induced NF-κB activation and downstream cytokines, while WT and S574D FoxO3 mutants had no effect. Hae-Young Lee et al. [33] found that FoxO3 could suppress NF-κB activation by promoting IκB degradation in endothelial cells. In other epithelial cell types

including 293 T, Hela and PC3 cells, FoxO3 could transcriptionally up-regulated Bcl-10 to enhance IκκB phosphorylation and NF-κB activation [17]. The present study showed that FoxO3 act upstream of IκB because of the changes of IκκB phosphorylation without regulating Bcl-10. In addition to regulating gene expression at the transcriptional level, FoxO3 was also found directly bind to P65 in cytoplasm to inhibit its nuclear translocation to suppress NF-κB activation in dendritic cells [13]. However, we did not find that phosphorylation status of S574 had an effect on P65 translocation in monocytes post LPS stimulation. Next, by screening related genes, we found that FoxO3 S574A may enhance LPS-mediated NF-κB activation and inflammatory response by up-regulating TLR4.

Combined with the results of up-regulation of monocytes FoxO3 expression by LPS was dominated by the non-phosphorylated form of S574, which may be a new positive feedback mechanism for LPS-induced inflammatory response in human monocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Data availability

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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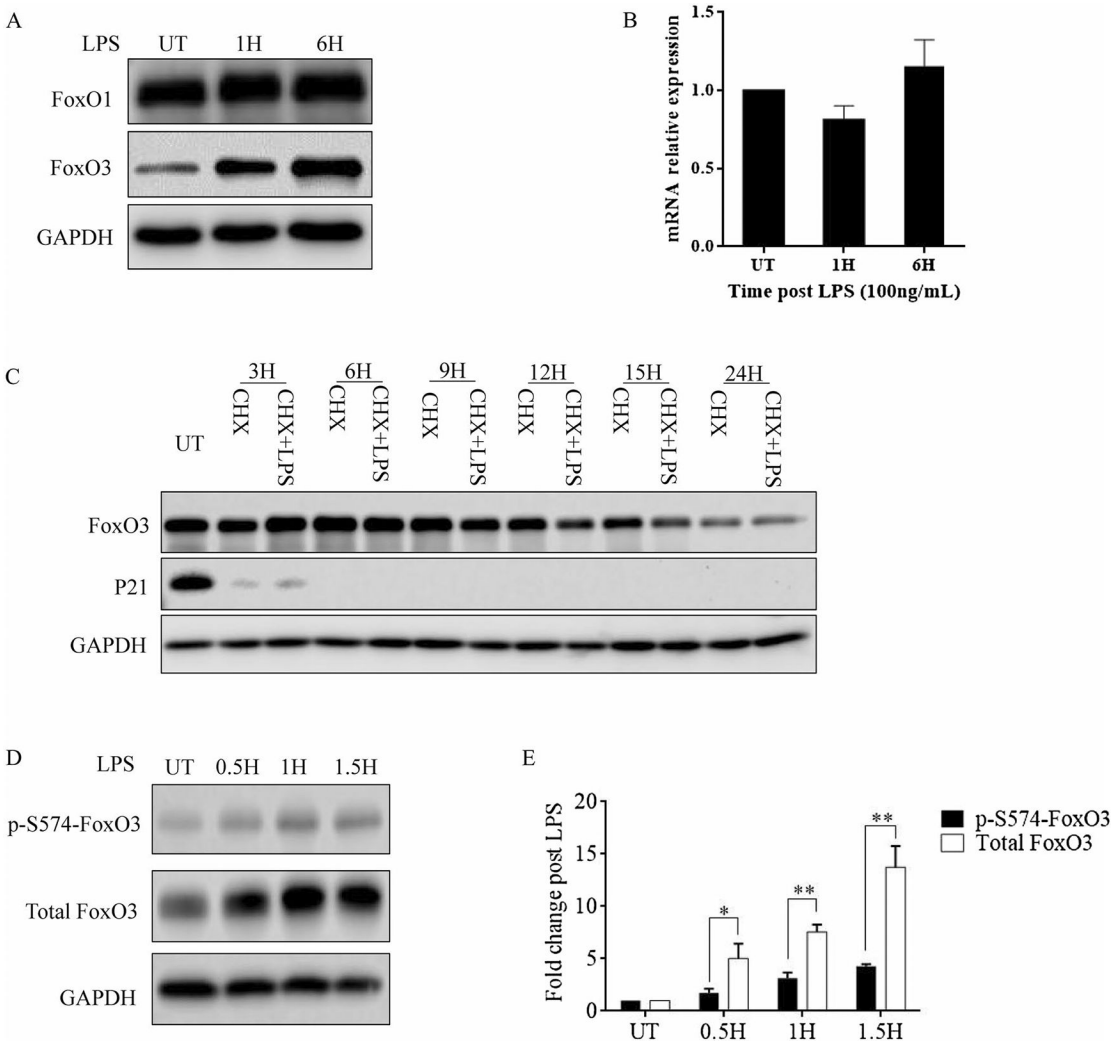

Fig. 1.

LPS up-regulated FoxO3 expression of THP-1 cells in a post-transcriptional manner. **A** FoxO1 and FoxO3 protein expression in THP-1 cells after 0.5H, 1H and 1.5H post LPS (10 ng/ml) stimulation. **B** FoxO3 mRNA level of THP-1 cells at 1H and 6H post LPS (10 ng/ml) treatment. **C** FoxO3 protein post CHX alone and CHX plus LPS and P21 was as positive control. **D** Phosphorylated FoxO3 at S574 and total FoxO3 protein level of THP-1 cells post LPS (10 ng/ml) treatment. **E** Fold changes of p-S574-FoxO3 and total FoxO3 post LPS (10 ng/ml) treatment. *UT* untreated. All data were presented as mean ± SEM, n = 3. *P < 0.05, **P < 0.01

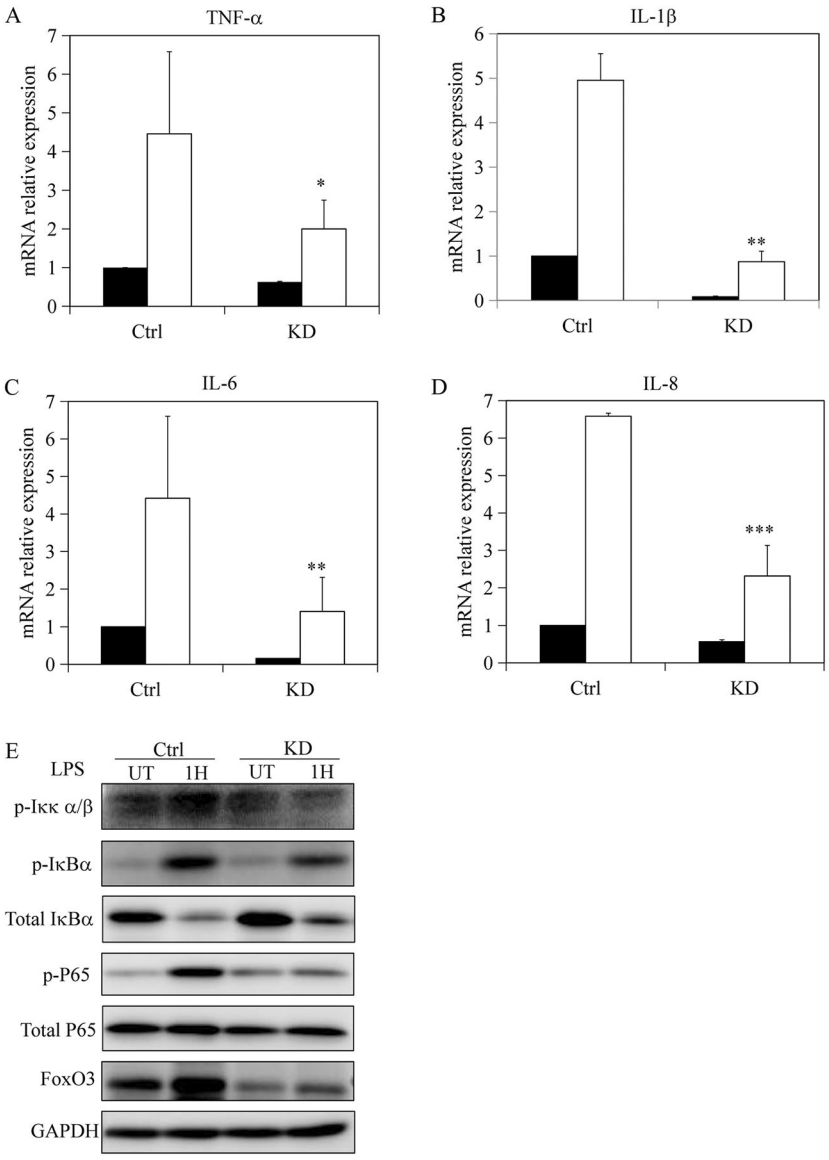

Fig. 2.

FoxO3 KD attenuated LPS mediated inflammatory response in THP-1cells. LPS (10 ng/ml) induced cytokines expression including TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and IL-8 (**D**) at 6H post treatment in FoxO3 KD and control (Ctrl) THP-1 cells. E. NF-κB activation in THP-1cells after 1 h post LPS (10 ng/ml) treatment. UT: untreated. All data were presented as mean ± SEM, n = 3. *P < 0.05, **P < 0.01, ***P < 0.001

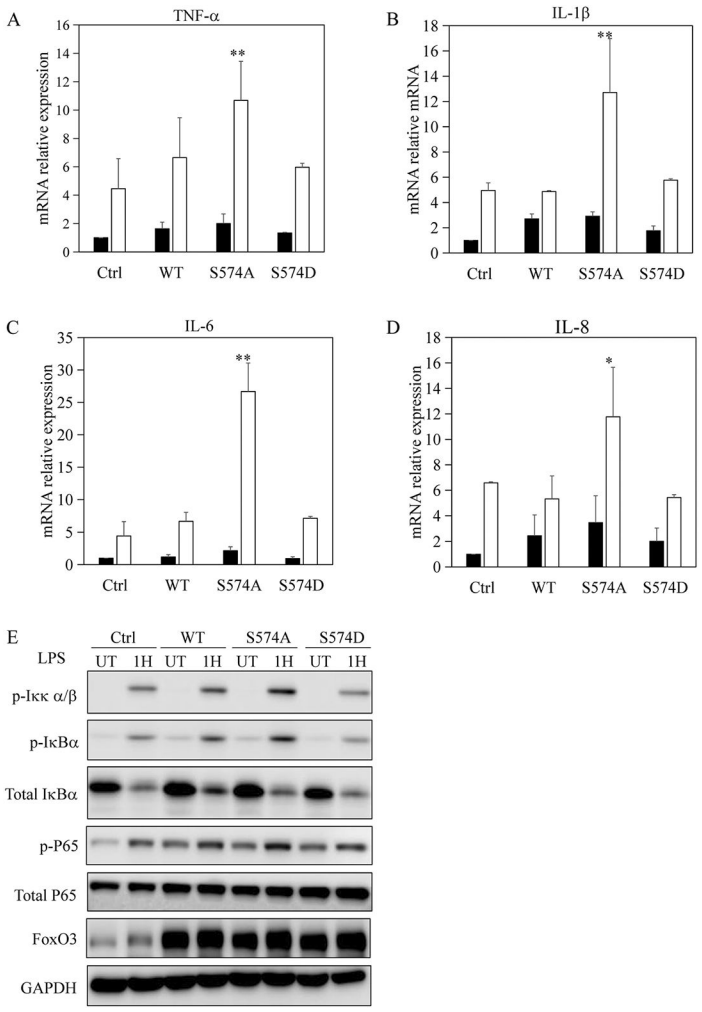

Fig. 3.

Overexpression of S574A FoxO3 mutant enhanced LPS mediated inflammatory response in THP-1 cells. LPS (10 ng/ml) induced cytokines expression including TNF-α (**A**), IL-1β (**B**), IL-6 (**C**) and IL-8 (**D**) at 6H post treatment in THP-1 cells overexpressed with control (Ctrl) WT, S574A and S574D FoxO3. E. NF-κB activation in THP-1cells after 1 h post LPS (10 ng/ml) treatment. UT: untreated. All data were presented as mean ± SEM, n = 3. *P < 0.05, **P < 0.01

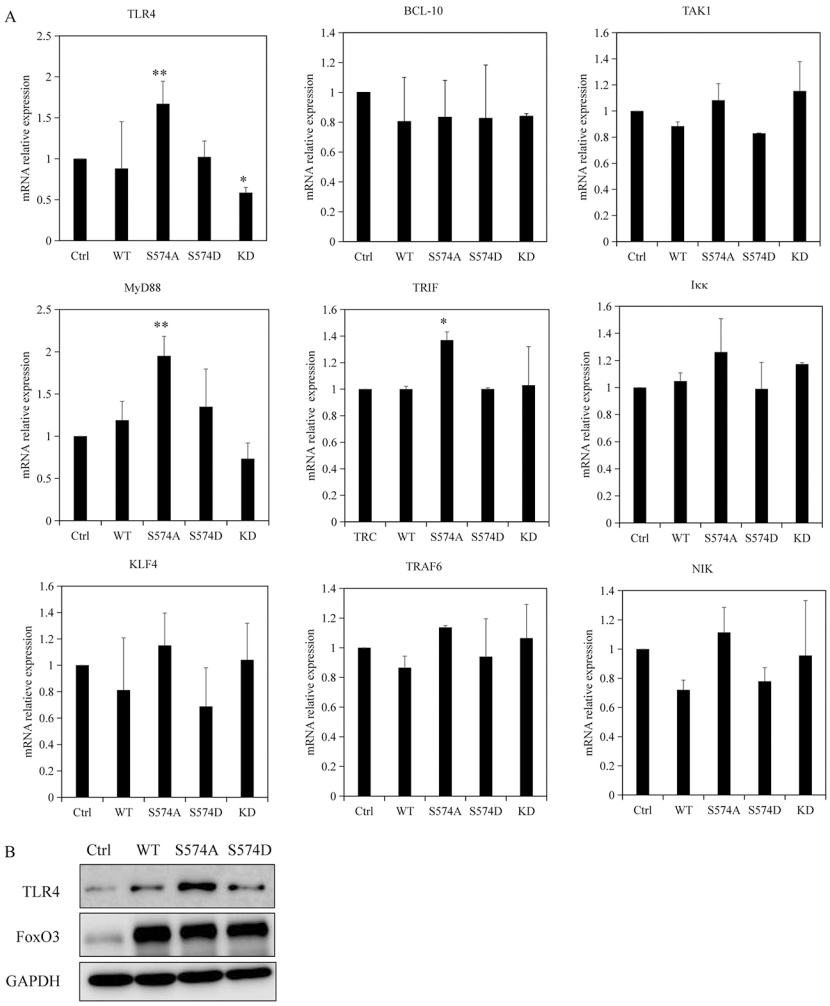

Fig. 4.

Effects of FoxO3 on mRNA expression of key molecules involved in NF-κB signal pathway. **A** mRNA level of key molecules of NF-kB pathway in THP-1 cells overexpressed with WT, S574A, S574D FoxO3 and FoxO3 KD groups. **B** TLR4 protein level in THP-1 cells overexpressed with WT, S574A and S574D FoxO3. All data were presented as mean ± SEM, n = 3. *P < 0.05, **P < 0.01

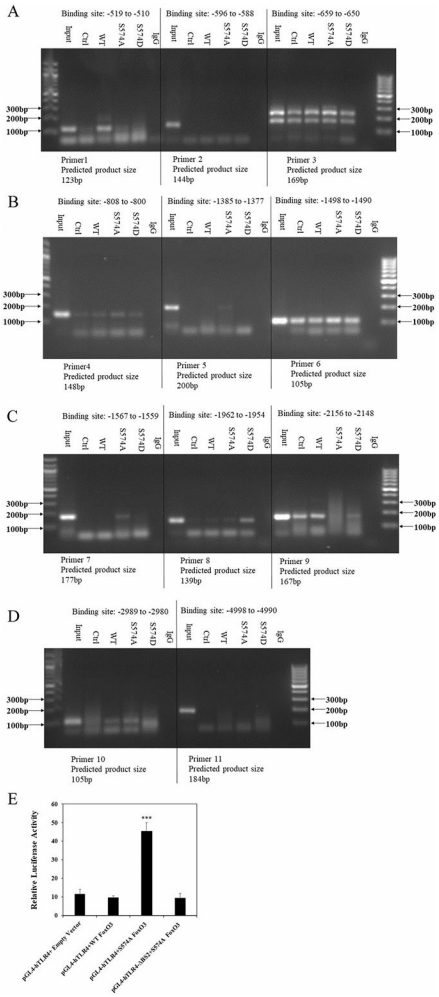

Fig. 5.

S574A FoxO3 binds and accelerate transcriptional activity from TLR4 promoter. **A**–**D** Gel electrophoresis of PCR product using primers targeting predicted binding sites located at TLR4 promoter for FoxO3. **E** Hela cells were co-transfected with empty vector, WT FoxO3, S574A FoxO3, mutant FoxO1 plasmid vectors together with pGL-hTLR4 or its deletion mutant lacking binding site 2. Relative luciferase activity was tested after 48 h post transfection. All data were presented as mean ± SEM, n = 3. ***P < 0.001