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RESEARCH ARTICLE

TLR4/MyD88 -mediated CCL2 production by lipopolysaccharide (endotoxin): Implications for metabolic inflammation

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Abstract

Background Obese human and mice were reported to have higher circularity endotoxin (LPS) levels as compared to their lean counter parts. The current study was aimed to reveal the molecular mechanisms underlying the LPS mediated induction of CCL2 in human monocytes/macrophages.

Methods Human monocytic cell line THP-1, THP-1 cells derived macrophages and primary macrophages were treated with LPS and TNF-α (positive control). CCL2 expression was determined with real-time RT-PCR and ELISA. THP-1-XBlue™ cells, THP-1-XBlue™-defMyD cells, TLR4 neutralization antibody, TLR4 siRNA and inhibitors for NF-kB and MAPK were used to study the signaling pathways. Phosphorylation of NF-kB and c-Jun was analyzed by ELISA.

Results LPS upregulates CCL2 expression at both mRNA (THP-1: 23.40 ± .071 Fold, $P < 0.0001$; THP-1-derived macrophages: 103 ± 0.56 Fold, < 0.0001; Primary macrophages: 48 ± 1.41 Fold, $P < 0.0005$) and protein (THP1 monocytes:1048 ± 5.67 pg/ml, $P < 0.0001$; THP-1-derived macrophages; 2014 ± 2.12, $P = 0.0001$; Primary macrophages: 859.5 ± 3.54, $P < 0.0001$) levels in human monocytic cells/macrophages. Neutralization of TLR4 blocked LPS-induced CCL-2 secretion ($P < 0.0001$). Silencing of TLR4 by siRNA also significantly reduced LPS-induced CCL-2 production. Furthermore, MyD88-Knockout cells treated with LPS did not produce CCL-2. NF-kB and c-Jun phosphorylation was noted in LPS treated cells.

Conclusion Overall, our data reveal that LPS induces CCL-2 in monocytes/macrophages via TLR4/MyD88 signaling which leads to the activation of NF-kB/AP-1 transcription factors.

Introduction

Gut microbial composition is changed by high fat diet (HFD) feeding along with alteration in the permeability of intestinal mucosa for bacterial derived endotoxin or Lipopolysaccharide (LPS) [1, 2]. Accordingly obese human and mice were reported to have higher circularity endotoxin levels as compared to their lean counter parts [3]. LPS is a heat stable glycolipid gram negative bacteria which contains a pathogen-associated molecular pattern (PAMP) called Lipid A. Binding of lipid A which upon binding to its cognate pattern recognition Toll-like receptor (TLR)-4 activates the proinflammatory signaling cascades and also induces oxidative stress [4]. TLR4 is a surface innate immune receptor found on immune cells including monocytes/macrophages as well as other cell types such as adipose tissue, skeletal muscle, and hepatocytes in mice and human [5–9]. Stimulation of TLRs triggers the activation of signaling pathways, resulting in the recruitment of several adaptor proteins to the toll/IL-1R homologous region (TIR) domain. The most well-known of these adaptors is MyD88 (myeloid differentiation factor 88). It is a main adaptor common to almost all TLRs except TLR3. Once recruited to the TLR TIR domain, MyD88 activates in turn IRAK (IL-1 receptor–associated kinases) family members and TRAF6 (tumor necrosis factor-alpha receptor–associated factor 6). These adaptor proteins are required for signaling the NF-κB/mitogen-activated protein kinase pathways [10–14].

Various proinflammatory cytokines and chemokines are involved directly or indirectly in the regulation of macrophage infiltration into the obese adipose tissue. Among inflammatory chemokines, CCL-2, produced by both monocytes/macrophages [15] and adipocytes [16], is a well-known chemokine that regulates macrophage accumulation in the adipose tissue [17]. Thus, CCL-2 plays a

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key role in the development of adipose tissue inflammation, insulin resistance and overall pathogenesis of metabolic diseases like obesity and type 2 diabetes (T2D). Studies show that CCL2 gene is regulated by different stimuli including LPS [18]. Elevated levels of both endotoxin and CCL-2 have been reported in obesity [3]. However, the molecular mechanism by which LPS induces CCL-2 in human monocytes/macrophages is not fully understood. Therefore, we demonstrate that LPS-stimulated release of CCL-2 from human monocytes/macrophages is mediated through the activation of NF-kB/AP-1 in TLR4/MyD88 dependent manner.

Materials and methods

Cell culture

THP-1 human monocytic cells were cultured and maintained in complete medium (RPMI-1640 culture medium containing 10% fetal bovine serum, 2 mM glutamine, 1 mM sodium pyruvate, 10 mM HEPES, 100μg/ml Normocin 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco, Invitrogen, Grand Island, NY, USA)) at 37 °C in 5% CO_2. NF-κB and AP-1 reporter THP-1-XBlue cells (expressing a secreted embryonic alkaline phosphatase (SEAP) and THP-1-XBlue™-defMyD cells (MyD88−/− THP-1 cells) were purchased from InvivoGen (InvivoGen, San Diego, CA, USA) and cultured as described earlier.

Macrophage preparation

Human PBMCs were isolated from whole blood donated by healthy volunteers using Ficoll-Paque (GE healthcare). To differentiate monocytes into macrophages, 3×10^6 PBMCs/mL in complete medium were plated in 12-well plates (Costar, Corning Incorporated, Corning, NY, USA). After 3 h of incubation, non-adherent cells were removed, and adherent cells were cultured in complete medium. THP-1 cells (2×10^5/ml) were differentiated using 10 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 2 days. After removing the PMA containing media, cells were incubated in fresh complete medium for one day, and used for subsequent treatments.

Cell stimulation

Cells were plated in 12-well plates at 1×10^6 cells/well concentration unless otherwise indicated. Cells were treated with LPS (10 ng/ml; Invivogen; InvivoGen, San Diego, CA, USA) or TNF-α (10 ng/ml; R&D Systems, USA) for 24 h at 37 °C. Cells were collected for RNA isolation and culture media were harvested for determining CCL2 levels and SEAP activity. Culture media were collected and stored at −80 °C.

TLR4 neutralization

THP-1 cells were incubated with neutralizing TLR4 mAb (2 μg/mL) or isotype mAb(IgA) for 40 min. Antibody-treated cells were incubated with LPS for 24 h. Cells and culture media were collected for further use.

Cell transfection

THP-1 cells were transfected separately with TLR4-siRNA (30 nM; OriGene Technologies, Inc. MD, USA), scramble (control) siRNA (30 nM; OriGene Technologies, Inc. MD, USA, USA) and pmaxGFP (0.5μg; Amaxa Noclecfector Kit V for THP-1, Lonza). Transfection experiments were carried out using Amaxa Electroporation System (Amaxa Inc., Germany), according to the manufacturer's protocol [13]. Effective suppression of constitutive TLR4 expression in THP-1 cells transfected with specific gene-targeted siRNA was confirmed by real time RT-PCR.

Measurement of NF-κB/AP-1 activity

THP-1 XBlue cells having reporter construct expressing SEAP for assessing the activation of transcription factors NF-κB and AP-1. Upon stimulation, NF-κB and AP-1 are activated and subsequently SEAP secretion is promoted. THP-1 XBlue cells were stimulated with LPS (10 ng/ml) or TNF-α (10 ng/ml; positive control) for 24 h at 37 °C. Levels of SEAP were detected in the culture media by incubation of supernatants with Quanti-Blue medium (InvivoGen, San Diego, CA, USA) for 4 h and quantification by ELISA (650 nm wave length).

Real time quantitative RT-PCR

Total RNA was extracted using RNeasy Mini Kit (Qiagen, Valencia. CA, USA) and the cDNA was synthesized using 1 μg of total RNA using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA, USA). 50 ng cDNA was amplified using Inventoried TaqMan Gene Expression Assay products (CCL-2: Hs00234140_m1; GAPDH: Hs03929097_g1) containing two gene-specific primers and one TaqMan MGB probe (6-FAM dye-labeled) using a TaqMan® Gene Expression Master Mix (Applied Biosystems, Foster city, CA, USA) in a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, USA). Target mRNA levels were normalized against control (GAPDH) mRNA and the amounts of CCL-2 mRNA relative to control were

calculated with ΔΔCt-method. Relative mRNA expression was expressed as fold expression over average of control gene expression. The expression level in control treatment was assumed to be 1. Values are presented as mean ± SEM. Results were analyzed statistically; $P < 0.05$ was considered significant.

CCL2 quantification

CCL-2 secreted protein levels in condition media of cells stimulated with LPS or TNF-α was quantified using sandwich ELISA following manufacturer's instructions (R&D systems, Minneapolis, USA).

Statistical analysis

The data were statistically analyzed using GraphPad Prism software (La Jolla, CA, USA). All data are shown as mean ± SEM values, unless otherwise indicated. Unpaired Student t-test was used to compare means between groups. For all analyses, P values <0.05 were considered significant.

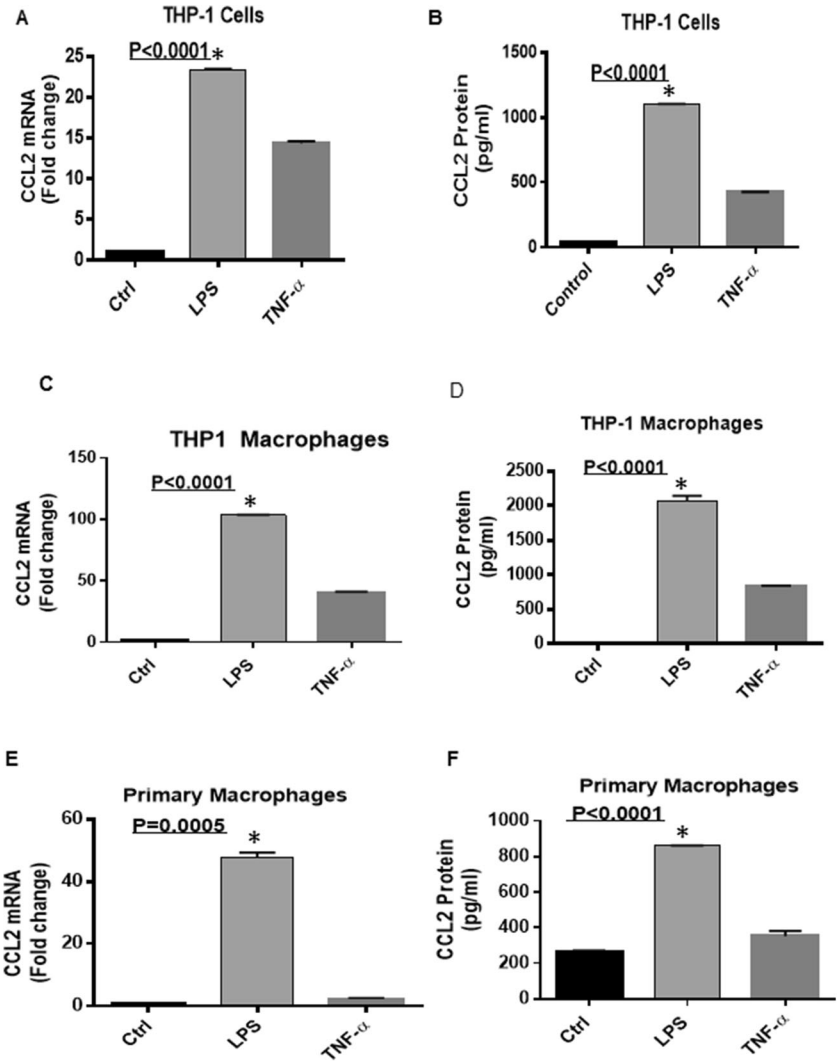

Fig. 1 Effect of LPS on CCL2 expression in human monocytic cells and macrophages. THP-1 cells were treated with LPS (10 ng/ml), TNF-α (10 ng/ml; positive control) and PBS Control = Ctrl) for 24 h. Cells and culture media were collected. Total RNA was isolated and CCL2 mRNA was quantified by real time PCR. Relative mRNA expression was expressed as fold expression over average of gene expression in BSA treated cells. LPS significantly increased the expression of CCL2 in THP-1 (A). Secreted CCL2 protein was increased in culture media was determined by ELISA. LPS induced high production of CCL2 compared to control (B). THP-1 cells were converted into macrophages and were treated with LPS or TNF- α for 24 h. Cells and culture media were collected. Real time PCR data showed increased CCL2 mRNA expression in LPS treated macrophages compared to control (C). LPS induced CCL2 protein in culture media (D). Primary macrophages were treated with LPS orTNF- α for 24 h. Cells and culture media were collected. Real time PCR data show that LPS increased expression of CCL2 (E). Secreted CCL2 in culture media was determined by ELISA and which was significantly upregulated by LPS (F). Data are shown as mean ± SEM of three independent experiments

Results

LPS upregulates CCL2 in monocytes/macrophages

Here, our data show that LPS significantly stimulates CCL2 gene expression at both mRNA (23.40 ± 0.071 Fold; $P < 0.0001$; (Fig. 1a) and protein (1048 ± 5.67 pg/ml; $P < 0.0001$; (Fig. 1b)) levels in human monocytic cells. Since macrophages are major players in adipose tissue inflammation, we incubated monocyte-derived macrophages and primary macrophages with LPS. Our results indicate that LPS upregulates CCL2 in macrophages at both mRNA (THP-1-derived macrophages: 103 ± 0.56 Fold; $P < 0.0001$ (Fig. 1c); Primary macrophages: 48 ± 1.41 Fold; $P = 0.0005$, (Fig. 1e)) and protein (THP-1-derived macrophages: 2014 ± 2.12 pg/ml; P, 0.0001, (Fig. 1d); Primary macrophages: 859.5 ± 3.54 pg/ml $P < 0.0003$ (Fig. 1f)) levels.

LPS mediated production of CCL2 requires TLR4

Our data show that neutralization of TLR4 significantly inhibited LPS-induced CCL2 expression THP-1 cells at both mRNA ($P = 0.0001$; Fig. 2a) and protein ($P < 0.0001$; Fig. 2b) levels, while no CCL2 suppression was observed in cells treated with isotype control antibody. Additionally, when TLR4 expression was inhibited in THP-1 cells by silencing TLR4 with TLR4-specific siRNA (*P: 0.0073;* Fig. 2c) LPS-induced CCL2 mRNA/protein expression was significantly suppressed when compared with scramble siRNA-transfected control cells ($P < 0.0001$; Fig. 2d and e). Therefore, our data

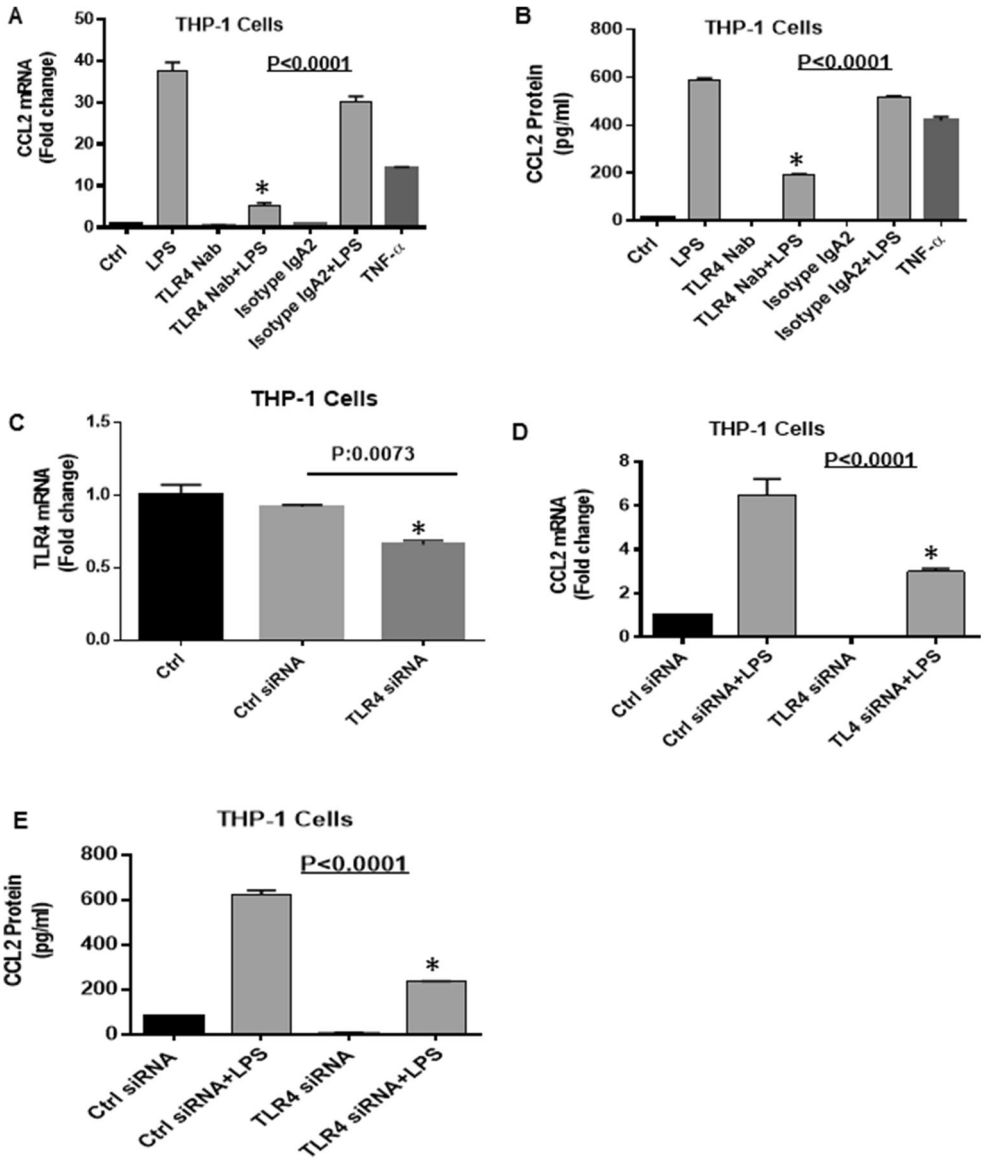

Fig. 2 Inhibition of TLR4 down-regulates the LPS induced CCL2. Antibody-treated cells were stimulated with LPS and incubated for 24 h. Cells and culture media were collected. Real time PCR data showed that neutralization of TLR4 significantly suppress LPS induction of CCL2 (A) and reduced CCL2 protein was determined in the culture media by ELISA (B). TLR4 siRNA transfected THP-1 cells showed reduced expression of TLR4 mRNA compared to the cells transfected with control siRNA (C). LPS induced CCL2 expression was significantly inhibited in TLR4 deficient cells at both mRNA (D) and protein (E) levels

show that LPS activates CCL2 gene/protein expression by signaling through the TLR4 receptor.

LPS regulates CCL2 expression via MyD88

Our results show that LPS-induced CCL2 expression was not observed in MyD88$^{-/-}$ THP-1 monocytic cells at both mRNA (Fig. 3a) and protein (Fig. 3b) levels), whereas the TNF-α-induce CCL2 expression was not affected in MyD88$^{-/-}$ cells as it was independent of MyD88 pathway (Fig. 3a and b). Likewise, MyD88 deficient macrophages did not show response to LPS (Fig. 3c and d). These observations further confirmed the role of TLR signaling in LPS induced CCL2 up-regulation.

Involvement of NF-kB and AP-1 in LPS-mediated production of CCL2

Our data show that THP-1 cells stimulation with LPS increased NF-kB and c-Jun (a major component of AP-1 transcription) phosphorylation Fig. 4a; and Fig. 4b**,** respectively). As expected, NF-κB/AP-1 activity was strongly induced in LPS-treated cells ($P < 0.014$; Fig. 4c) and this induction was associated with CCL2 secretion **($P = 0.0003$; Fig. 4d).** In addition, the lack of MyD88 also led to reduced NF-κB/AP-1 activity following LPS treatment (Fig. 4e). These data suggest that LPS-induced up-regulation of CCL2 gene expression in monocytic cells involves activation of the NF-κB/AP-1 transcription factors.

Discussion

CCL2 is a major chemokine which plays a major role in metabolic inflammation. Dysregulation in CCL2 production leads to the pathogenesis of several inflammatory disorders [19]. In obesity, CCL2 may facilitate the migration of immune cells into the adipose tissue and could enhance obesity related inflammation [17]. Obese human and mice were reported to have higher circularity endotoxin (LPS) levels as compared to their lean counter parts. LPS induces gene expression of several cytokines and chemokines including CCL2. High level of LPS in plasma of ob/ob mice enhances the expression inflammatory mediators including PAI-1, IL-1, TNF-, STAMP2, NADP Hox, CCL-2, and F4/80 which leads metabolic inflammation [18]. Bacterial endotoxins may regulate the development of the metabolic and vascular abnormalities generally observed in obesity and diabetes-associated complications [20]. LPS derived from E coli and Pseudomonas induces the production of proinflammatory cytokines/chemokines such as IL-1β, IL-6, TNF-α and CCL-2 and IL-8 [21, 22]. However, the mechanistic role of LPS in regulating CCL2 production in monocytic cells is not well defined. Our data show that LPS induces CCL2 expression at both mRNA and protein levels in human monocytic THP-1 cells.

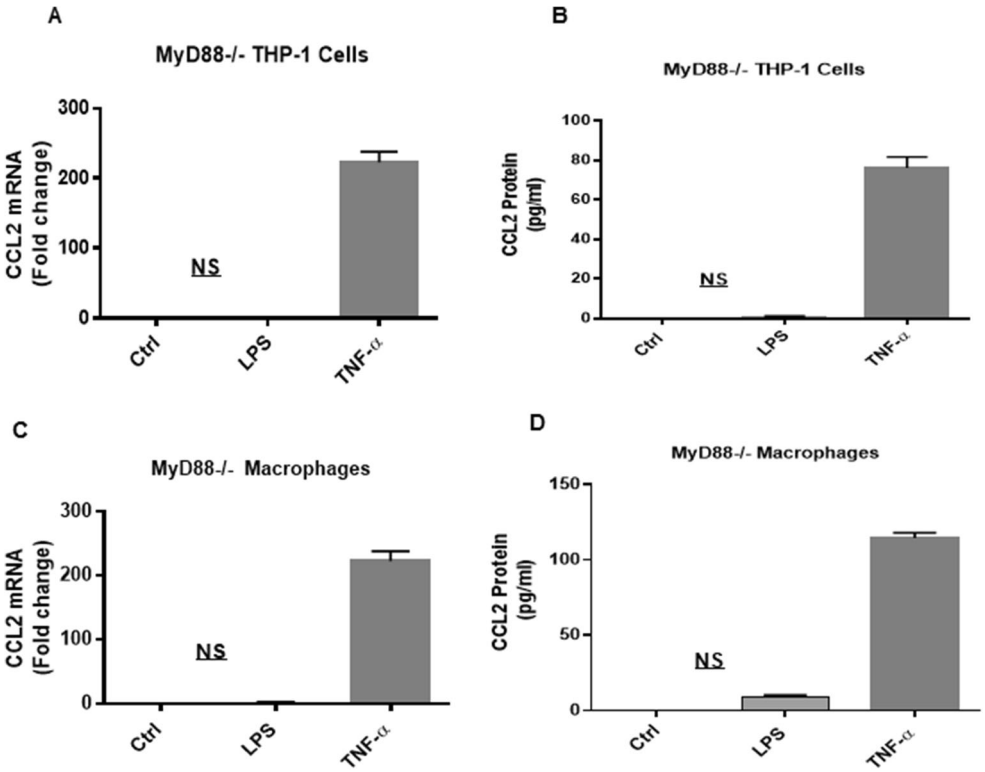

Fig. 3 MyD88 deficiency reduced the LPS induced production of CCL2. THP-1 XBlue defMyD cells (cells deficient in MyD88 activity) were treated with LPS (10 ng/ml) or PBS (Vehicle; control) or TNF-α (10 ng/ml; MyD88 independent stimulus). Cells and culture media were collected after 24 h. Real time PCR and ELISA data showed that LPS failed to induce CCL2 at both mRNA (A) and secreted protein (B) levels. XBlue defMyD cells derived macrophages and were treated with LPS (10 ng/ml) or TNF-α (10 ng/ml). CCL2 gene expression was not induced by LPS in MyD88 deficient cells (C and D)

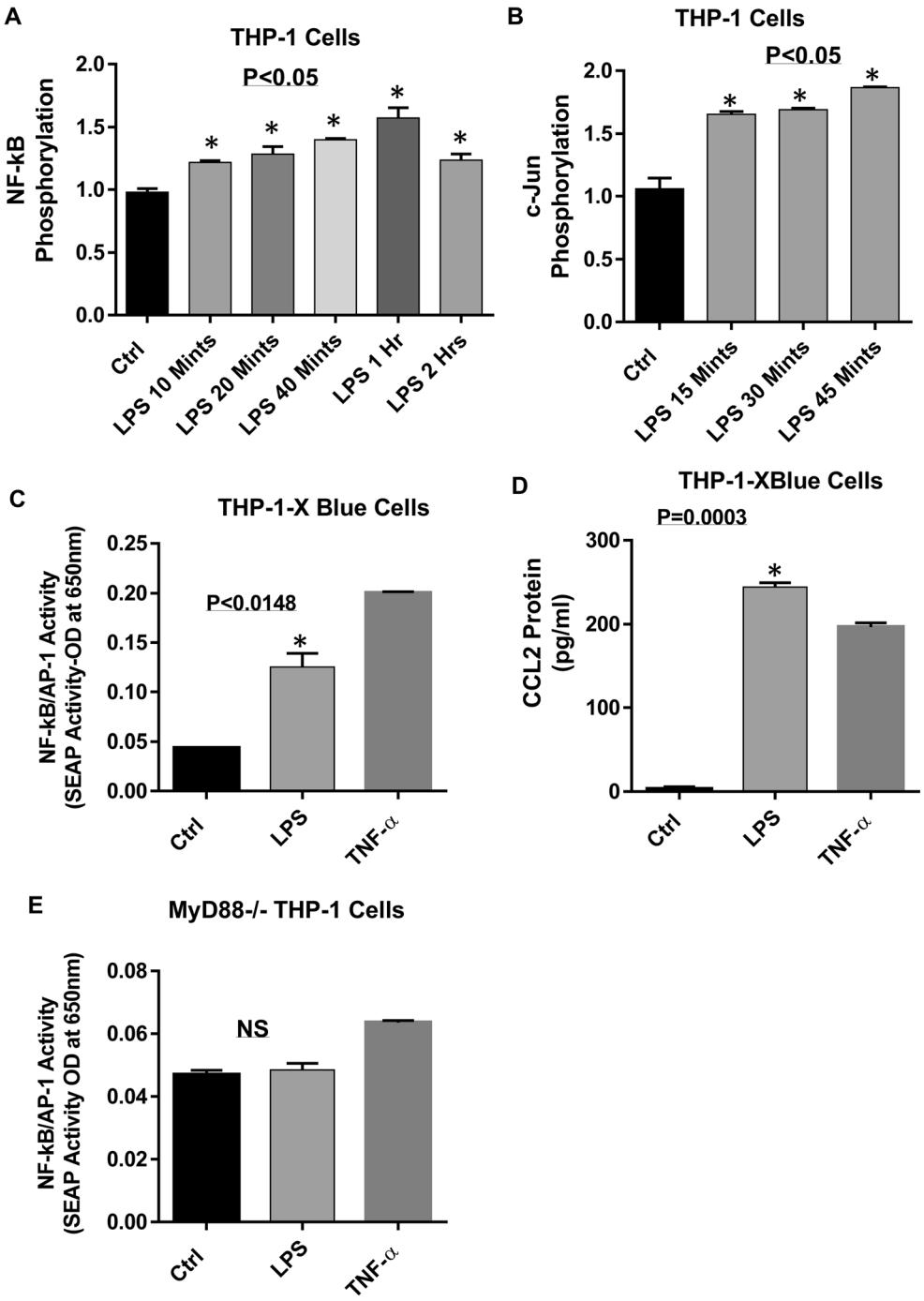

Fig. 4 LPS activates NF-kB and AP-1 transcription factors. THP- 1 cells were treated with LPS for different time points and cell lysates were prepared as described in methods. Samples were run for Quantikine assay on PathScan Sandwich Elisa kit. Our data showed that phosphorylated NF-kB and c-Jun was seen in LPS treated cells (4A and 4B). THP-1-XBlue cells (THP-1 cells stably expressing a secreted embryonic alkaline phosphatase (SEAP) reporter inducible by NF-κB and AP-1) were treated with LPS or PBSor TNF-α for 24 h. Culture media were collected. Cell culture media were assayed for SEAP reporter activity (degree of NF-κB / AP-1 activation) along with CCL2 production. LPS increased NF-kB/AP-1 activity as compared to control (C and D). Similarly, THP- 1-XBlue™-defMyD cells (Cells deficient in MyD88 activity) were treated with LPS (10 ng/ml) or TNF-α (10 ng/ml) for 24 h. SEAP reporter activity (degree of NF-κB /AP-1 activation) was determined in the cell culture media. MyD88 deficiency inhibits the LPS induced activation of NF-kB/AP-1 (E). The results obtained from three independent experiments are shown. The data are presented as mean ± SD

Furthermore, LPS-induced CCL2 production was dependent on the TLR4. The stimulation of TLR4 by LPS induces the release of critical proinflammatory cytokines that participate in the activation of numerous immune responses [4].

Our data further show that LPS-induced CCL2 gene expression was regulated by a mechanism that depended on MyD88. MyD88 is a key adaptor protein which is common to all TLRs except TLR3. The interaction between TLRs and MyD88 is required for signaling via the MAPK/NF-κB pathways. It is supported by our previous findings which have shown that FSL-1 induces TLR-2-dependent MMP-9 gene expression which requires the recruitment of MyD88 and leads to activation of MEK1/2 /ERK 1/2, MEK5/ERK5, JNK, p38 MAPK and NF-κB/AP-1. Similarly, palmitate produces TLR4-dependent activation of MMP-9 gene expression, which requires the recruitment of MyD88 leading to activation of NF-kB/AP-1 transcription factors. It has been reported that HKLM activates TLR2 and NF-κB/AP-1 signaling pathways, leading to up-regulation of MMP-9 production in THP-1 cells. [10–14]. Our data also show that MyD88 is a

main regulatory adaptor molecule in LPS-induced AP-1/NF-κB activation. AP-1/NF-kB are key transcription factors downstream of the TLR signaling cascade. We further confirmed the involvement of NF-κB/AP1 in LPS-induced CCL2 production by using culture media obtained from LPS-treated THP-1 cells, which expressed a reporter driven by NF-κB and AP-1 response elements, to analyze NF-κB/AP-1 activity. We also found phosphorylated c-Jun and NF-kB components following the stimulation of monocytic cells with LPS. LPS induces production and expression of IL-6, IL-8, and MMP-1 via the TLR4, MAPK and PI3K/Akt signaling pathways in nasal poly fibroblasts. Additionally, expression of IL-6, IL-8, and MMP-1 is stimulated by LPS via TLR4 in nasal polyp organism cultures [21]. This observation indicates that LPS exposure may be involved in the pathogenesis in the remodeling of nasal polyposis. The interaction of different ligands with the TLRs has been shown to be involved in CCL2 production.

Conclusion

We conclude that LPS acts as a ligand for TLR4 and it induces TLR4-dependent activation of CCL2 gene expression which requires MyD88 recruitment and activation of NF-κB/AP-1 transcription factors.

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Author contributions NA performed experiments, analyzed the data and participated in writing the paper. AW, SS, and SK participated in performing experiments. AH contributed in scientific discussions and critically reviewed/edited the manuscript. SA participated in arranging data and preparing graphs. JT critically reviewed and commented the manuscript. SS participated in designing experiments. RA conceived the idea, designed the experiments, analyzed the data, and wrote the manuscript.

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Compliance with ethical standards

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

Abbreviations *AP-1,* Activating protein-1; *ATCC,* American Type Culture Collection; *CCL-2,* Chemokine (C-C motif) ligand −2; *ELISA,* Enzyme-linked immunosorbent assay; *GAPDH,* Glyceraldehyde-3-phosphate dehydrogenase; *LPS,* Lipopolysaccharide; *MAPK,* Mitogen-activated protein kinase; *MyD88,* Myeloid differentiation factor 88; *NF-κB,* Nuclear factor-kappaB; *PBMC,* Peripheral blood mononuclear cells; *PCR,* Polymerase chain reaction; *SEAP,* Secreted embryonic alkaline phosphatase; *THP-1,* A human monocytic cell line; *TLRs,* Toll-like receptors; *TNF-α,* Tumor necrosis factor-alpha

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