Original Paper

Klf4 Alleviates Lipopolysaccharide-Induced Inflammation by Inducing Expression of MCP-1 Induced Protein 1 to Deubiquitinate TRAF6

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Key Words

Klf4 • MCPIP1 • Lipopolysaccharide • NF-κB • Deubiquitination

Abstract

Background/Aims: Inflammation is an essential component of innate immunity against pathogens, but is tightly regulated, such as by Krüppel-like factor 4 (Klf4), to prevent injury. Klf4 also regulates macrophage polarization, although the mechanisms underlying both functions are poorly understood. The aim of this study was to investigate whether and how Klf4 prevents unregulated inflammation. **Methods:** The bone marrow-derived macrophages and RAW264.7 cell line were used. Quantitative real-time PCR was used to determine inflammatory cytokines (IL-1β, TNF-α, IL-6 and MCP-1), Klf4 and MCPIP1 transcript levels. Extraction of nuclear and cytoplasmic proteins and Immunoblotting were used to determine Klf4, MCPIP1, relative kinases from NF-κB pathway and K63-linked polyubiquitins expression in nucleus and cytoplasm separately. Dual luciferase reporter assay was used to analyze whether Klf4 mediate MCPIP1 transcription. Immunoprecipitation was used to determine the protein interaction among Klf4, MCPIP1, TRAF6 and K63-linked polyubiquitins. Secretion of IL-1β and TNF-α into sera in mice was measured by Enzyme-linked immunosorbent assay. **Results:** We found that exposure to lipopolysaccharides suppresses Klf4 expression, even as it induces release of pro-inflammatory cytokines. Strikingly, Klf4 overexpression significantly lowered cytokine secretion and NF-κB signaling in the cytoplasm following exposure to lipopolysaccharide, even though Klf4 was exclusively nuclear. The cytoplasmic effects are likely mediated by MCP-1 induced protein 1 (MCPIP1), a deubiquitinase and a key modulator of inflammation that accumulates both in the nucleus and cytoplasm in response to Klf4. Indeed, binding between MCPIP1 and K63 polyubiquitins is attenuated in macrophages overexpressing Klf4, suggesting that MCPIP1 is an intermediary induced by Klf4 in the nucleus to remove K63 polyubiquitins from TRAF6 in the cytoplasm, and thereby impede NF-κB and inflammatory signaling. Importantly, Klf4

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overexpression in mice alleviated sepsis symptoms following exposure to lipopolysaccharides.

Conclusion: The data highlight Klf4 as an essential MCPIP1-dependent modulator of innate immunity that protects against excessive and self-destructive inflammation.

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Introduction

Inflammation plays an important role in the innate immune response to tissue injury or pathogens [1, 2], and is induced upon exposure to pathogen- or damage-associated molecular patterns [3, 4]. Indeed, innate immune cells such as macrophages induce inflammation and produce inflammatory cytokines, including IL-1 β , TNF, MCP-1, and IL-6, as a first line of defense against viruses or bacteria [5, 6]. However, unregulated inflammation may also lead to tissue injury, septic shock, autoimmune disease, and malignancy [7]. Therefore, the inflammatory response is precisely controlled at multiple levels to be effective against microbes but also to avoid self-injury.

Lipopolysaccharide, one of the most studied pathogen-associated molecular patterns, is found in the outer membrane of Gram-negative bacteria, and elicits a strong immune response in animals. Generally, lipopolysaccharides engage Toll-like receptor 4 (TLR4), and trigger multiple signaling cascades, ultimately causing a gamut of cell or tissue damage [8-10]. In one such cascade, binding of lipopolysaccharides to TLR4 triggers auto-ubiquitination of TNF receptor-associated factor 6 (TRAF6) with K63-linked ubiquitin chains [2, 11, 12]. Downstream of TRAF6, TAK1 and the adaptor proteins TAB2 and TAB3 activate the IKK complex, which in turn phosphorylates and hence targets I κ B for proteasomal degradation. This event frees NF- κ B (p50/p65), a transcription factor involved in both innate and adaptive immunity, to translocate to the nucleus, initiate transcription, and trigger release of inflammatory cytokines [13]. Therefore, regulation of ubiquitination of effectors in NF- κ B signaling plays a critical role in inflammation.

Ubiquitin is a 76-amino acid polypeptide present in all eukaryotic cells [14-16]. The best characterized forms are K63 polyubiquitins, which mainly control protein-protein interactions [16-19]. Accumulating evidence show that the ubiquitin system, especially deubiquitinases, is essential in immunity [20-22]. For example, TRAF proteins are regulated by MCP-1 induced protein 1 (MCPIP1) [23, 24], a deubiquitinase and zinc-finger protein [2, 25, 26] that accumulates rapidly upon stimulation with pro-inflammatory agents such as lipopolysaccharides, IL-1, TNF, and MCP-1 [8, 26, 27]. Importantly, MCPIP1 was implicated in both physiological and pathological inflammation [2, 12, 28-31]. Nevertheless, the specific mechanisms linking MCPIP1 to lipopolysaccharide-induced inflammation remain to be identified.

Interestingly, the MCPIP1 promoter contains multiple response elements to Krüppel-like factor 4 (Klf4). Accordingly, IL-4 induces MCPIP1 expression via Klf4 [32, 33]. However, the specific regulation between Klf4 and MCPIP1 in the inflammatory response in macrophage is still unclear. Klf4 is well-known not only as a transcription factor essential for pluripotency [34], but also as a regulator of physiological and pathological processes [35, 36]. In particular, it plays important roles in macrophage polarization and innate immunology regulation. Notably, Klf4 was demonstrated to promote M2 polarization and inhibit coactivators required to activate NF- κ B. Moreover, Klf4-deficient macrophages show increased pro-inflammatory gene expression, enhanced bactericidal activity, and altered metabolism [33]. However, the mechanisms underlying this strict regulation are unclear, further research is needed to address whether and how Klf4 regulates NF- κ B signaling to control the inflammatory response in macrophages.

We now report that Klf4 alleviates the inflammatory reaction to lipopolysaccharide *in vitro* and *in vivo* by reducing the release of pro-inflammatory cytokines. We also found that Klf4 accumulates in the nucleus and indirectly inhibits NF- κ B signaling by inducing MCPIP1 and thereby elicit deubiquitination of TRAF6 in the cytoplasm. Collectively, our data highlight Klf4 as a promising regulator of inflammation and target of future research.

Materials and Methods

Animal experiments

Wild-type male C57BL/6 mice 6-8 weeks old were purchased from the Experimental Animal Center at Fourth Military Medical University, and housed in a temperature-controlled environment with a 12 h light/dark cycle at Department of Burns and Cutaneous Surgery, Xijing Hospital. All animal experiments were approved by and consistent with the principles of the Animal Research Ethics Board of Fourth Military Medical University, Xi'an, Shaanxi, China, as well as with local and national guidelines. Mice were transfected by injecting to caudal veins 60 µg/mouse of plasmids pGMLV-ZsGreen1 or pGMLV-Klf4 in 60 µL Micropoly-transfector Tissue Reagent (Micropoly, #MT215). Mice were challenged five days thereafter by peritoneal injection of 15 mg/kg lipopolysaccharide (Sigma, #L2880), and cytokine levels in peritoneal sera or tissue were quantified 6 h after injection. Survival rate was monitored every 12 h for 2 days.

Cells

Mouse RAW264.7 macrophages were obtained from American Type Culture Collection (#TIB-71), and cultured in RPMI 1640 (Corning Cellgro; #10-040-CVR) containing 10 % fetal bovine serum (Corning Cellgro; #35-076-CV). Bone marrow-derived macrophages were isolated from male C57BL/6 mice 6-8 weeks old, by culturing bone marrow for five days in RPMI 1640 supplemented with 10 % fetal bovine serum, 20 ng/mL macrophage colony-stimulating factor (PeproTech; #315-02), and 100 U/mL penicillin and streptomycin (Hyclone; #SV30010).

Plasmids and siRNAs

pGMLV-ZsGreen1, pGMLV-Klf4, pGMR-TK, and pGL3-MCPIP1-Luc were purchased from Hugu Science & Technologies. Mouse MCP1P1 siRNAs were purchased from Biomics Biotechnologies, and used at 20 µM by dilution in DEPC-treated water.

Antibodies

Antibodies to MCP1P1 (1:1, 000, #25009-1-AP), Klf4 (1:500, #11880-1-AP), H2AFY2 (1:1, 000, #17030-1-AP), and α-tubulin (1:1, 000, #11224-1-AP) were purchased from Proteintech, along with mouse anti-rabbit IgG light chain (1:5, 000, #SA00001-7). Anti-IKKγ (phospho ser85) (1:1, 000, #GTx32283) was purchased from GeneTex, while antibodies to IKKα/β (phospho Ser176/180) (1:1, 000, #2697S), TAK1 (1:1, 000, #5206), and TAK1 (phospho Thr184/187) (1:1, 000, #4511) were procured from Cell Signaling Technology. Antibodies to K63-linked ubiquitin (1:1, 000, #ab179434), TRAF6 (1:1, 000, #ab94720), NF-κB p65 (1:1, 000, #ab16502), IKKα/β (1:1, 000, #ab178870), and IKKγ (1:5, 000, #ab178872) were obtained from Abcam. Rabbit polyclonal antibodies to GAPDH (1:1, 000, #R1208-3), and goat anti-rabbit IgG (1:5, 000, #EK020) were obtained from Zhuangzhi Biotech.

Transient transfection

RAW264.7 macrophages grown to 60-80 % confluence were transfected for 24 h or 48 h with pGMLV-ZsGreen1, pGMLV-Klf4, pGMR-TK, or pGL3-MCPIP1-Luc in Opti-MEM (Thermo Gibco; #31985062) and Lipofectamine 3000 Reagent (Thermo Invitrogen; #L3000008), following the manufacturer's instructions. To silence MCP1P1, RAW264.7 macrophages grown to 60-80 % confluence were transfected with siRNAs or scrambled siRNAs at a 1:1 v:v mixture with Micropoly-transfector cell reagent (Micropoly; #MT110), following the manufacturer's instructions. After 48 h, cells were stimulated with 1 µg/mL lipopolysaccharide, and analyzed for mRNA or protein levels of relevant molecules.

Dual luciferase reporter assay

RAW264.7 cells at 60-80 % confluence were seeded in a 24-well plate, and co-transfected with pGL3-MCPIP1-Luc, pGMLV-ZsGreen1 (NC) or pGMLV-Klf4 (Klf4), and pGMR-TK at a ratio of 10:9:1. After 24 h, cells were washed with DPBS (Corning Cellgro; #21-031-CVR) and lysed in passive lysis buffer. Luciferase activity was measured in the lysate by the Dual Luciferase Reporter Assay System (Promega; #E1910) according to the manufacturer's instructions, and normalized to Renilla luciferase. Data were collected in triplicate from at least two experiments.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from cells or tissues using TRIzol Reagent (Thermo Invitrogen; #15596-026) in accordance with the manufacturer's protocol. RNA extracts with OD_{260/280} ratio 2.0 (0.5 mg) were reverse-transcribed by PrimeScript RT Master Mix (TaKaRa, #RR036A) at 37 °C for 15 minutes and at 85 °C for 5 seconds. Targets were then amplified in triplicate by quantitative RT-PCR using SYBR Green PCR Master Mix (TaKaRa; #RR820A) on a Bio-Rad iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad), normalized to GAPDH, and quantified by the comparative cycle threshold method ($2^{-\Delta\Delta CT}$). Primer sequences are listed in Table 1.

Protein isolation and immunoblotting

Cells were washed with ice-cold DPBS, lysed on ice for 30 minutes with RIPA buffer (HEART Biological Technology; #WB009), and centrifuged at 12,000 rpm for 10 minutes. Total protein was measured by BCA Protein Assay Kit (Thermo Scientific; #23225), mixed with 5× SDS-PAGE loading buffer (HEART Biological Technology; #R0993), and heated at 100 °C for 10 minutes. Samples (40 µg) were resolved by SDS-PAGE (HEART Biological Technology; #WB005), and immunoblotted with corresponding antibodies.

Extraction of nuclear and cytoplasmic proteins

Cells were lysed using Nuclear and Cytoplasmic Protein Extraction Kit (Yeasen; #20126ES50) in accordance with the manufacturer's protocol, and fractions were immunoblotted with appropriate antibodies.

Immunoprecipitation

Cells were lysed on ice for 30 minutes in Immunoprecipitation RIPA buffer (HEART Biological Technology; #WB065) emented with protease inhibitor, centrifuged at 12,000 rpm for 10 minutes, and immunoprecipitated with Protein A/G PLUS-Agarose Immunoprecipitation Reagent (Santa Cruz; #SC-2003) according to the manufacturer's instructions. Briefly, lysates were precleared by incubating on ice for 30 minutes with 1 µg control IgG and 20 µL Protein A/G PLUS-Agarose. Beads were pelleted by centrifugation at 2,500 rpm for 5 minutes at 4 °C. The resulting supernatant (approximately 100-500 µg total protein) was incubated with 1 µg primary antibody for 1 h, and then with 20 µL Protein A/G PLUS-Agarose overnight at 4 °C. Immunoprecipitates were collected by centrifugation at 2,500 rpm for 5 minutes at 4 °C, washed 4 times with Immunoprecipitation RIPA buffer, resuspended in 40 µL 1× loading buffer, and boiled for 10 minutes. Samples (20 µL) were then resolved by SDS-PAGE and immunoblotted with appropriate antibodies.

Enzyme-linked immunosorbent assay (ELISA)

Mice were anesthetized by intraperitoneal injection of 1 % pentobarbital sodium (5 mL/kg). Blood was collected from the eyes, and centrifuged at 3,000 rpm for 10 min. TNF-α (#EMC102a.48) and IL-1β (#EMC001b.48) in sera were quantified in triplicate by mouse ELISA kits according to the manufacturer's instructions (Neobioscience).

Statistical analysis

Data were analyzed in GraphPad Prism version 4.0 (GraphPad Software, La Jolla, CA, USA). Each experiment was repeated at least three times, and data are reported as mean ± standard error of the

Table 1. Names and sequences of primers used for quantitative real-time-polymerase chain reaction

Name	Species	Sense	Antisense
TNF-α	Mouse	TATGGCCCAGACCCTCACA	GGAGTAGACAAGGTACAACCCATC
IL-1β	Mouse	CAACCAACAAGTGATATTCTCCATG	GATCCACACTCTCCAGCTGCA
IL-6	Mouse	CAACGATGATGCACTTGCAGA	CTCCAGGTAGCTATGGTACTCCAGA
MCP-1	Mouse	AGCAGCAGGTGTCCCAAAGA	GTGCTGAAGACCTTAGGGCAGA
Klf4	Mouse	GCATGTGCCCCAAGATTAAG	GTGACAGTCCCTGCTGTTCA
MCP1P1	Mouse	CCACCTACCCATCCAGAGAG	GGGAAGACACCACACAGCTT
GAPDH	Mouse	TGTGTCCGTCGTGGATCTGA	TTGCTGTTGAAGTCGCAGGAG

mean. Statistical significance was determined by Student's *t* test or one-way analysis of variance. Kaplan–Meier survival was assessed by log-rank test. A *P* value less than 0.05 was considered statistically significant.

Results

Klf4 is an essential inhibitor of inflammation

As innate immune cells, macrophages represent the first line of defense. These cells are highly plastic, and acquire various phenotypes in response to environmental cues [5]. In mouse RAW264.7 and bone marrow-derived macrophages exposed to 1 µg/mL lipopolysaccharide and characterized at 0 h, 0.5 h, 1 h, 2 h, 6 h, 12 h, and 24 h (Fig. 1 A and B), the pro-inflammatory cytokines *TNF-α*, *IL-1β*, *IL-6*, and *MCP-1* were upregulated to various extents, peaking at about 2 h after stimulation. These data confirm that macrophages are activated and acquire a pro-inflammatory phenotype in response to lipopolysaccharides.

On the other hand, *Klf4* plays important roles in macrophage polarization and innate immunology regulation. However, the mechanisms by which *Klf4* regulates inflammation are not fully understood. Strikingly, *Klf4* mRNA and protein levels were significantly attenuated in RAW264.7 and bone marrow-derived macrophages exposed to lipopolysaccharides. Indeed, *Klf4* expression reached the lowest levels about 2 to 6 hours after stimulation, and coincided with upregulation of pro-inflammatory genes (Fig. 1 A–D).

We then constructed a plasmid (pGMLV-*Klf4*) to overexpress *Klf4* in RAW264.7 macrophages, with pGMLV-ZsGreen1 as negative control (Fig. 1 E–G). As shown in Fig. 1 H, activation of pro-inflammatory marker genes following exposure to lipopolysaccharide was significantly ameliorated in macrophages overexpressing *Klf4*, indicating strong anti-inflammatory activity. These results collectively show that *Klf4* expression diminishes in parallel to lipopolysaccharide-mediated activation of pro-inflammatory genes, while *Klf4* overexpression blocks the effects of lipopolysaccharide.

Fig. 1. Klf4 negatively regulated pro-inflammatory response in BMDMs and RAW264.7 macrophages upon stimulation with LPS. A and B. Inflammatory cytokines and *Klf4* mRNA levels in (A) RAW264.7 macrophages and (B) BMDMs after different time points (0 h, 0.5 h, 1 h, 2 h, 6 h, 12 h and 24 h) of stimulation with LPS (1 µg/ml). Gene expression levels were analyzed by quantitative real time PCR (qRT-PCR) and normalized to those in untreated cells. C and D. *Klf4* protein levels after LPS treatment both in RAW264.7 macrophages and BMDMs, and normalized against GAPDH. E–G. *Klf4* (E) mRNA levels and (F and G) protein levels in RAW264.7 macrophages after transient transfection of plasmid pGMLV-Green1 as negative control (NC) or pGMLV-*Klf4* (*Klf4*) 48 h after transfection. H. Analysis of inflammatory cytokines transcripts by qRT-PCR following 2 h LPS stimulation in RAW264.7 macrophages transfected with NC or *Klf4*, and normalized against GAPDH. Data are shown as mean ± SEM. * *p* < 0.05, ** *p* < 0.01 vs. negative control. Data are representative of at least three independent experiments.

Nuclear Klf4 suppresses cytoplasmic NF- κ B signaling

Furthermore, we found that phosphorylation of molecules in the NF- κ B pathway, including TAK1, IKK- α/β , and IKK- γ , was significantly enhanced in macrophages treated with 1 μ g/mL lipopolysaccharide for various durations. In line with this result, expression of K63-linked polyubiquitins was also enhanced (Fig. 2 A and B). Strikingly, activation of these kinases in response to lipopolysaccharide was less pronounced in RAW264.7 macrophages transiently transfected for 48 h with pGMLV-Klf4 to overexpress Klf4 (Fig. 2 B). We note that bone marrow-derived macrophages were inefficiently transfected, and were thus unsuitable for similar analysis.

Klf4 accumulated exclusively in the nucleus under basal conditions or under lipopolysaccharide stimulation, while IKK complexes and TAK1 were cytoplasmic. Further, Klf4 expression reduced the translocation of p65 to the nucleus following exposure to lipopolysaccharide (Fig. 2 C). Collectively, these results indicate that Klf4 ameliorates NF- κ B signaling only indirectly.

Klf4 mediates MCP1 transcription to regulate NF- κ B

The deubiquitinase MCP1 inhibits NF- κ B activation, and is driven by a promoter that contains multiple Klf4 sites [32]. As shown in Fig. 3 A to C, transcript and protein levels of MCP1 were both elevated in RAW264.7 macrophages transiently overexpressing Klf4, implying that Klf4 boosts MCP1 expression. Similarly, RAW264.7 macrophages transfected with a dual luciferase reporter vector under the control of the *MCP1* promoter, expressed luciferase only upon Klf4 overexpression (Fig. 3 D). Moreover, immunoblotting of subcellular fractions revealed that MCP1 accumulates both in the nucleus and cytoplasm, and is upregulated upon Klf4 overexpression under both basal conditions and under lipopolysaccharide stimulation. Klf4 overexpression also reduced p65 translocation to the nucleus following exposure to lipopolysaccharide (Fig. 3 E and F). Together, these results indicate that Klf4 regulates the expression of MCP1, which then translocates to the cytoplasm to regulate NF- κ B.

Fig. 2. Klf4 accumulates in nucleus and negatively regulates NF- κ B pathway upon LPS stimuli. A. Immunoblotting analysis of p-TAK1, TAK1, p-IKK α/β , IKK α/β , p-IKK γ , IKK γ , Klf4 and K63 polyubiquitin chains expression in BMDMs either uninfected or infected with LPS for indicated time points, and GAPDH as loading control. B. NC and Klf4 overexpressed Raw264.7 macrophages either unstimulated or stimulated with LPS for indicated time points were analyzed by immunoblotting for p-TAK1, TAK1, p-IKK α/β , IKK α/β , p-IKK γ , IKK γ , Klf4, K63 polyubiquitin chains, and GAPDH as loading control. C. Cytosolic and nuclear extracts of NC and Klf4 overexpressed Raw264.7 macrophages that either unstimulated or stimulated with LPS for indicated time points were analyzed by immunoblotting for p-TAK1, TAK1, p-IKK α/β , IKK α/β , p-IKK γ , IKK γ , Klf4, P65, H2A and α -tubulin. Purity of cytoplasmic and nuclear fraction is depicted by α -tubulin and H2A as loading control. Data are representative of at least three independent experiments.

MCP1 acts as a negative regulator in the inflammatory response via modulation of NF- κ B signaling

MCP1 is widely reported to suppress the inflammatory response by deubiquitinating inflammatory proteins [12, 24]. In line with this model, we found that exposure to lipopolysaccharide dramatically enhanced MCP1 expression in macrophages, as shown in Figure 4 A to C, especially at 2 to 6 hours after exposure. We then synthesized three small interfering RNAs against MCP1 (siMCP1), of which siMCP1-2 and siMCP1-3 markedly reduced MCP1 expression after transient transfection for 48 h, in comparison to scrambled siRNA (siNC). In cells transfected with siMCP1-3 for 48 h (Fig. 4 D to F), the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and MCP-1 were notably upregulated at mRNA levels both in normal conditions and in response to lipopolysaccharide (Fig. 4 G), suggesting that MCP1 knockdown elicits hyper-inflammation. In addition, IKK complexes, TAK1, and K63-linked polyubiquitins were elevated to various extents in macrophages transfected with siMCP1-3 for 48 h and then challenged with 1 μ g/mL lipopolysaccharide for 0, 30, 60, and 180 min (Fig. 4 H). Taken together, these results indicate that MCP1 suppresses the inflammatory response to lipopolysaccharide by modulating NF- κ B signaling.

Klf4 indirectly inactivates TRAF6 via MCP1

Recent studies identified TRAF proteins as essential in the response to lipopolysaccharide. For example, binding of lipopolysaccharide to TLR4 triggers recruitment of MyD88 and IRAK1/4, which subsequently recruit TRAF6 to initiate downstream signaling [6]. In RAW264.7 macrophages transfected with negative control or Klf4 plasmid for 48 h and then

Fig. 3. Klf4 induced MCP1 transcription to regulate NF- κ B activation. A-C. MCP1 (A) mRNA levels and (B and C) protein levels in RAW264.7 macrophages after transient transfection of plasmid pGMLV-Green1 as negative control (NC) or pGMLV-Klf4 (Klf4) for 48 h, and normalized against GAPDH. D. RAW264.7 macrophages were cotransfected with MCP1 promoter luciferase reporter vector pGL3-MCP1-Luc, pGMLV-Green1 (NC)/ pGMLV-Klf4 (Klf4) and pGMR-TK at ratio 10:9:1 for 24 h, and luciferase activity was measured in the lysate by the Dual-luciferase reporter Assay System and normalized to Renilla luciferase assay. Overexpression of Klf4 enhanced MCP1 luciferase reporter activity. E and F. Cytosolic and nuclear extracts of NC and Klf4 overexpressed Raw264.7 macrophages that either uninfected or infected with LPS for 2 h were analyzed by immunoblotting for MCP1, Klf4, P65, H2A and α -tubulin. Purity of cytoplasmic and nuclear fraction is depicted by α -tubulin and H2A as loading control. Data are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. negative control. Data are representative of at least three independent experiments.

stimulated with lipopolysaccharide, we found by immunoprecipitation that Klf4 coprecipitates with MCP1, but not with TRAF6 or K63-linked polyubiquitins (Fig. 5 A). Moreover, we found that TRAF6 coprecipitates with K63 polyubiquitins and MCP1, but not with Klf4, and that accumulation of K63 polyubiquitins was lower in cells overexpressing Klf4 (Fig. 5 B). Moreover, pull-down experiments showed that cytoplasmic MCP1 was in complex with TRAF6 and K63 polyubiquitins, but not with Klf4. On the other hand, nuclear MCP1 was associated with Klf4, but not TRAF6 and K63 polyubiquitins (Fig. 5 C). Together, these data imply that increased Klf4 expression elevates MCP1 activity in the nucleus to attenuate TRAF6 deubiquitination in the cytoplasm, and thereby suppress NF- κ B signaling in response to lipopolysaccharide (Fig. 7).

Klf4 overexpression in vivo alleviates inflammation in septic mice

The role of Klf4 in inflammation was investigated *in vivo* using mice transfected for five days with pGMLV-ZsGreen1 or pGMLV-Klf4 by injection through the caudal vein. These mice were then treated with 15 mg/kg lipopolysaccharide by intraperitoneal injection. We found that survival 48 h after exposure was higher in mice overexpressing Klf4 (approximately 70 %) than in mice transfected with pGMLV-Green1 (approximately 40 %, Fig. 6 A). Moreover, secretion of inflammatory cytokines into sera, including TNF- α and IL-1 β , was well-controlled in mice overexpressing Klf4 6 h after exposure to lipopolysaccharide (Fig. 6 B). Analysis of the liver, lungs, and kidney, which are the organs most susceptible to septic injury, indicated that although Klf4 mRNA levels in the kidney were comparable among groups, levels in the liver and lungs were > 200- and 15-fold higher in mice overexpressing Klf4 than in control mice (Fig. 6C), regardless of exposure to lipopolysaccharide. However, lipopolysaccharide-induced expression of pro-inflammatory mRNAs in the liver, including TNF- α , IL-1 β ,

Fig. 4. A-C. MCP1 (A) mRNA levels and (B and C) protein levels in RAW264.7 macrophages after different points of stimulation with LPS (1 μ g/ml), and normalized against GAPDH. D-F. Gene silencing effects of three different siRNA fragments targeting MCP1 (siMCP1) on RAW264.7 macrophages through qRT-PCR (D) and immunoblotting (E and F) analysis respectively. G. Analysis of inflammatory cytokines transcripts by qRT-PCR followed by 2 h LPS stimulation in RAW264.7 macrophages transfected with siNC or siMCP1. H. RAW264.7 macrophages silenced MCP1 either unstimulated or stimulated with LPS for indicated time points were analyzed by immunoblotting for p-TAK1, TAK1, p-IKK α /IKK β , IKK α /IKK β , p-IKK γ , IKK γ , MCP1, K63 polyubiquitin chains, and GAPDH as loading control. Data are shown as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ vs. negative control. Data are representative of at least three independent experiments.

Fig. 5. Klf4 indirectly deubiquitinates K63 poly ubiquitins on TRAF6 via regulation of MCP1. A and B. RAW264.7 macrophages transfected NC or Klf4 either unstimulated or stimulated with LPS for 2 h were immunoprecipitated with anti-Klf4, anti-TRAF6 or IgG control. Pulldowns and corresponding whole-cell lysates were then immunoblotted for MCP1, Klf4 TRAF6, K63 poly ubiquitins and GAPDH (loading control). C. Cytoplasmic and nuclear extracts of NC and Klf4 overexpressed RAW264.7 macrophages that either uninfected or infected with LPS for 2 h were immunoprecipitated with anti-MCP1 or IgG control. Pulldowns and corresponding Cytoplasmic and nuclear lysates were then immunoblotted for MCP1, Klf4 TRAF6, K63 poly ubiquitins, H2A and α -tubulin. Purity of cytoplasmic and nuclear fraction is depicted by α -tubulin and H2A loading control. Data are representative of at least three independent experiments.

Fig. 6. Klf4 overexpression ameliorates inflammation in LPS-induced septic mice. A. C57BL/6 mice were transfected pGMLV-Green1 (NC, n = 10) or pGMLV-Klf4 (Klf4, n = 10) through injection of caudal veins for 5 days then inoculated via intraperitoneal route with saline or LPS (15 mg/kg) to observe their morbidity. The mice survival is represented in a Kaplan–Meier plot. Statistical analysis on survival curves were performed using log-rank test. B. mice transfected with NC (n = 5) or Klf4 (n = 5) were inoculated via intraperitoneal route with saline or LPS (15 mg/kg). 6 h later, sera were analyzed by enzyme-linked immuno sorbent assay (ELISA) for TNF- α and IL-1 β levels. C. qRT-PCR analysis of transcript for Klf4 gene in livers, lungs and kidneys of mice transfected with NC (n = 6) or Klf4 (n = 6) 6 h after inoculation of LPS. D and E. qRT-PCR analysis of transcripts for inflammatory cytokines TNF- α , IL-1 β , IL-6 and MCP-1 genes in livers (D) and lungs (E) of mice transfected with NC (n = 6) and Klf4 (n = 6) 6 h after inoculation of LPS. Data are shown as mean $\pm$ SEM. * p<0.05, ** p<0.01 vs. negative control.

IL-6, and MCP-1, was remarkably lower in the former (Fig. 6D). Similar results, except IL-6, were observed in the lungs (Fig. 6E), but not in the kidney (data not shown). Together, these data show that Klf4 is an important inhibitor of the inflammatory response, and partially protects mice from lipopolysaccharide-induced sepsis.

Discussion

Infection by bacteria, viruses, fungi, parasites, and other pathogens elicit local and systemic inflammatory responses [37, 38], of which sepsis is a leading cause of mortality in adults and children [39, 40]. Since inflammation and innate immunity against bacteria are hallmarks of sepsis [41, 42], enhancing the latter while mitigating the former is a worthwhile therapeutic strategy [43]. One possibility is to exploit Klf4, a zinc-finger transcription factor that was shown to either promote [44, 45] or inhibit inflammatory signaling [33, 46]. In light of these contradictory results, we now present strong evidence that Klf4 overexpression, at least under our experimental conditions, significantly suppresses the inflammatory response of macrophages to lipopolysaccharide, markedly decreasing the release of pro-inflammatory cytokines such as TNF- α , IL-1 β , IL-6, and MCP-1. Nevertheless, further *in vivo* experiments in animals and even in humans are still needed to conclusively demonstrate that Klf4 is anti-inflammatory.

Importantly, Klf4 appears to inhibit lipopolysaccharide-induced inflammation via NF- κ B, as evidenced by a dramatic decrease in phosphorylation of TAK1, IKK α / β , and IKK γ , as well as in the expression of K63 polyubiquitin chains. However, we also found that while the affected proteins are cytoplasmic, Klf4 is nuclear. Immunoprecipitation also indicated that Klf4 did not bind any of the affected proteins, implying that Klf4 does not directly regulate NF- κ B effector proteins. Subsequently, we found the Klf4 avidly binds MCP1, which, in turn interacts with a protein upstream to NF- κ B such as TRAF6. Indeed, MCP1 was previously shown to inhibit lipopolysaccharide-induced JNK and NF- κ B signaling by deubiquitinating TRAF proteins [2, 12, 22].

Strikingly, MCP1 is itself induced during inflammation [32], and by inflammatory signals such as NF- κ B, IL-1b, TNF- α , and IL-6 [8, 27], although it is not clear how MCP1 is regulated, or whether it provides negative feedback regulation. We believe that such a feedback mechanism is likely. Indeed, we observed that Klf4 overexpression also upregulates MCP1 both at the mRNA and protein level, and that Klf4 stimulates expression of a luciferase reporter gene controlled by the MCP1 promoter. In addition, our data also indicate that loss of MCP1 significantly enhances the release of the pro-inflammatory cytokines TNF- α , IL-1 β , IL-6, and MCP-1, reinforcing that notion that MCP1 is anti-inflammatory. Therefore, Klf4 appears to modulate lipopolysaccharide-induced inflammation via MCP1.

In line with *in vitro* data, mice overexpressing Klf4 were less sensitive to lipopolysaccharide, secreting lower levels of pro-inflammatory cytokines into sera, including TNF- α and IL-1 β , in response to exposure. Most importantly, Klf4 overexpression ameliorated the severity of

Fig. 7. Schematic of proposed mechanism.

clinical symptoms (data not shown) and reduced mortality. However, the protective effects of Klf4 were observed only in the lungs and liver, but not in the kidney, even though all three are severely damaged during sepsis. This result correlates with Klf4 levels, which were variable in these tissues perhaps as a result of variable dissemination of the plasmid. For example, we found that Klf4 overexpression was much stronger in the liver than in the lungs and kidney of the same mice, as was previously observed [47]. In addition, specific cellular conditions or greater reparative capacity in the liver may enhance the effects of Klf4. In any case, these observations suggest that the protective effects of Klf4 against inflammation may differ depending on tissue type.

Some major issues remain to be addressed. For example, why is Klf4 suppressed in response to lipopolysaccharide? One possibility is that miR-7 or other factors control Klf4 expression to modulate physiological process in macrophages, which are under study in our group. The mechanisms underlying MCP1 upregulation but Klf4 suppression response to lipopolysaccharide should also be clarified. On the other hand, MCP1 may also modify Klf4 ubiquitination, and thereby alter its regulatory effects on cellular processes such as cell growth, proliferation, and differentiation [48, 49]. Indeed, Klf4 sumoylation promotes IL-4-induced macrophage M2 polarization [50]. Finally, the long-term effects of Klf4 against lipopolysaccharide-induced inflammation remain unknown.

Taken together, the data demonstrate that Klf4 modulates lipopolysaccharide-induced inflammation and tissue damage both *in vivo* and *in vitro* by suppressing the release of pro-inflammatory cytokines, inducing MCP1, and inhibiting NF- κ B signaling. Our findings not only provide strong evidence that Klf4 is involved in innate immunity and sepsis, but also highlight new research opportunities into the role of Klf4 in various inflammatory processes and disorders, as well as into its potential as therapeutic target.

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Disclosure Statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential Disclosure Statement.

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