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Heme induces inflammatory injury by directly binding to the complex of myeloid differentiation protein 2 and toll-like receptor 4

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ABSTRACT

Heme, as an essential component of hemoproteins, is a prosthetic co-factor found in many cells, which is essential for physiologically vital oxygen transport. However, extracellular or circulatory heme is cytotoxic and triggers inflammation. Although the proinflammatory role of heme has been reported to be associated with Toll-like receptor 4 (TLR4) signaling, the exact mechanism remains unknown. Here, we show that heme promotes TLR4 signaling and inflammation *via* directly physically interacting with TLR4 and its adaptor protein myeloid differentiation protein 2 (MD2). Genetic loss of MD2 ameliorates heme-induced inflammation and inflammatory cytokine production in the spleen of MD2 knockout ($MD2^{-/-}$) mice. Using mouse macrophage RAW 264.7 cell line, we show that heme induces TLR4 dimerization and MD2/TLR4/MyD88 activation by physically interacting with TLR4 and MD2 *in vitro*. Genetic loss of MD2 inhibits heme-induced inflammation and MAPK/NF-κB pathway in mouse primary macrophages extracted from $MD2^{-/-}$ mice. Furthermore, pharmacological inhibition of MD2 by L6H9 ameliorates heme-induced inflammation in macrophages. These findings demonstrate that heme causes inflammation by directly binding to MD2/TLR4 complex, leading to activation of TLR4/MAPK/NF-κB signaling pathway and production of downstream effectors of inflammation.

1. Introduction

Red blood cells (RBCs) deliver oxygen to the tissues during their lifespan of about 120 days and are then phagocytosed by splenic macrophages and broken down into heme and iron (Ganz, 2012). Under physiological conditions, the released heme is normally cleared by the heme scavengers. However, an impaired or oversaturated scavenger system leads to the accumulation of heme in the circulation (Schaer et al., 2014). Heme is an essential molecule for all cells and functions as a prosthetic group for several hemoproteins such as hemoglobin, myoglobin and cytochromes (Dawson, 1988). These hemoproteins play important roles in gas exchange, electron transport and signal transduction (Dawson, 1988).

Besides its physiological significance, there is strong evidence indicating that heme is a potent pro-oxidant and pro-inflammatory molecule (Dutra and Bozza, 2014; Graça-Souza et al., 2002; Sawicki et al., 2015). Heme injection to experimental animals triggers local and systemic inflammation *in vivo* (Dutra and Bozza, 2014). In addition, heme activates innate immune cells *in vitro* acting as a chemoattractant, inducing cytokine production, reactive oxygen species (ROS) generation, causing significant inflammation (Dutra and Bozza, 2014; Graça-Souza et al., 2002) and toxicity (Sawicki et al., 2015). Hence, heme can be a toxic

Abbreviations: IB, immunoblotting; IP, immunoprecipitation; MD2, myeloid differentiation protein 2; MPM, mouse primary macrophage; MyD88, myeloid differentiation factor 88; NF-κB, nuclear factor-κB; Perls, prussian blue iron; RBCs, red blood cells; ROS, reactive oxygen species; SCD, sickle-cell disease; SPR, Surface plasmon resonance analysis; TLR4, toll like receptor 4.

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molecule once released from hemoproteins, which is observed in hemolytic diseases such as β-thalassemia, sickle-cell disease (SCD) and malaria (Schaer et al., 2014). However, the exact mechanism by which heme induces the innate immune system and causes inflammation is not fully understood.

It is reported that heme induces systemic inflammation *via* activation of nuclear factor-κB (NF-κB) pathway through toll-like receptor-4 (TLR4) signaling (Belcher et al., 2014). Myeloid differentiation protein 2 (MD2), which is the accessory protein of TLR4, is essential for the activation of TLR4 signaling in mediating inflammation (Kumagai et al., 2008; Wang et al., 2017). Further study shows that heme can bind to soluble MD2 to mediate inflammation in endothelial cells (Zhang et al., 2021). Besides, heme is reported to activate TLR4 signaling *via* bingding to amino acids of W23 and Y34 on MD2 *in vitro* (Belcher et al., 2020). However, the role of MD2 in heme-induced systemic inflammation *in vivo* and whether MD2 is the only binding target of heme needs further investigation.

In this study, we investigated the role of MD2 in overwhelming circulatory heme induced inflammation *in vivo*. We show that heme significantly induced inflammation in spleen tissue. The potential mechanism is associated with the direct interaction of heme with the complex of MD2/TLR4 rather than interaction only with MD2. Pharmacologic blockade of either TLR4 or MD2 could offer similar inhibitory effect against heme-induced activation of MD2/TLR4/MyD88 signaling. Our data suggest that MD2/TLR4 inhibitors could be potential therapeutic candidates for clinical diseases such as β-thalassemia, SCD and malaria.

2. Materials and methods

2.1. Reagents and chemicals

Recombinant human MD2 (rhMD2) and recombinant TLR4 (rhTLR4) proteins were purchased from R&D (Minneapolis, MN, USA). Plasmids of Flag-tagged TLR4, HA-tagged TLR4 and His-tagged MD2 were obtained from SinoBiological. Co. Ltd. (Beijing, China). Novel MD2 inhibitor, L6H9, was synthesized in our laboratory as described previously (Zhong et al., 2015). TAK-242 (#S7455), a TLR4 inhibitor, was obtained from Selleck (Shanghai, China). Heme (hemin chloride, #16009-13-5) was obtained from Aladdin (Shanghai, China). Heme was dissolved in 0.1 N NaOH and diluted in PBS at a final concentration of 10 mM, and pH was adjusted to 7.4 using 0.1 N HCl. Antibodies against Phospho-p44/42 (p-ERK; #4370), ERK (#4695), Phospho-p38 (#4511), p38 (#8690), Phospho-JNK (#9255), JNK (#9252), IκBα (#4812S), nuclear factor-κB p65 subunit (NF-κB p65; #8242S), and GADPH (#5174) were obtained from Cell Signaling Technology (Boston, MA, USA). Antibodies against Lamin B (#ab133741), MD2 (#ab24182), TLR4 (#ab22048), MyD88 (#ab2064) and TNF-α (#ab6671) were obtained from Abcam (Cambridge, UK). Antibodies against Flag (#H9658) and HA (#SAB4200071) were obtained from Sigma-Aldrich (St Louis, MO, USA). Secondary antibodies were obtained from Yeasen Biotech (Shanghai, China).

2.2. Cell culture

Human 293T cells were purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China), and were cultured in DMEM supplemented with 10 % FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Mouse macrophage cell line, RAW264.7, was purchased from Shanghai Institute of Biochemistry and Cell Biology (Shanghai, China). RAW264.7 cells were cultured in RPMI-1640 supplemented with 10 % FBS, 100 U/ml penicillin and 100 mg/ml streptomycin. Mouse primary macrophages (MPMs) were prepared from wild-type C57BL/6 (WT) or MD2-knockout ($MD2^{-/-}$) mice as described previously (Pan et al., 2012). MPMs were cultured in RPMI-1640 supplemented with 10 % FBS and 100 U/ml penicillin and 100 mg/ml streptomycin.

2.3. Cell transfection

Plasmid transfection was performed using Lipofectamine 2000 (Lipo 2000). Briefly, plasmids of Flag-tagged TLR4 (1 μg), HA-tagged TLR4 (1 μg) and His-tagged MD2 (1 μg) were mixed with Lipo 2000 in the ratio of 1:1. After mixing for 20 min, the mixture was added in the 293T cells. 6–8 h after transfection, medium was removed and fresh complete culture medium was added. Cells were then cultured for another 24 h before further experiments.

2.4. Animal studies

All animal study protocols were approved by Wenzhou Medical University Animal Policy and Welfare Committee and adhered to the NIH guidelines (Guide for the care and use of laboratory animals). Male C57BL/6 mice weighing 18–22 g were obtained from the Wenzhou Medical University Animal Centre. Male $MD2^{-/-}$ mice (B6.129P2-Ly96 KO) with C57BL/6 background were provided by Riken BioResource Center of Japan (Tsukuba, Ibaraki, Japan). Mice were randomly divided into 4 groups: **1)** WT (wild-type C57BL/6 mice injected with normal saline, n = 5), **2)** WT+Heme (wild type C57BL/6 mice injected with Heme, n = 5), **3)** $MD2^{-/-}$ ($MD2^{-/-}$ mice injected with normal saline, n = 5), **4)** $MD2^{-/-}$+Heme ($MD2^{-/-}$ mice injected with Heme, n = 5). Freshly prepared Heme was intravenously injected into the mice at a dose of 50 μM/kg for a total of 5-times in 15 days. After the injection, mice were sacrificed, and tissue samples were collected and immediately frozen for further studies.

2.5. Tissue staining and histology assay

Paraffin sections of spleen tissues were deparaffinized and rehydrated. Sections were then stained with Prussian blue iron (Perls, #G1422, Solarbio, Beijing, China), hematoxylin and eosin (H&E, #G1120, Solarbio) according to the manufacturer's instructions. For immunochemistry staining, spleen sections were deparaffinized, rehydrated and were then blocked with 1 % bovine serum albumin (BSA) for 30 min. Tissues were then incubated with anti-TNF-α (1:1000) antibodies at 4 °C overnight. After washing with PBS for 3 times, sections were incubated with horseradish peroxidase (HRP)-labeled secondary antibody (1:200) for 1 h at 37 °C, followed by DAB detection and hematoxylin counterstaining.

Immunofluorescence staining of p65 - RAW 264.7 cells or MPMs were pretreated with L6H9 (5 or 10 μM), and were then stimulated with heme (30 μM). Cells were fixed with 4 % paraformaldehyde and permeabilized with 0.1 % Triton for 10 min. Next, cells were blocked with 1 % bovine serum albumin and were then incubated with anti-p65 (1:500) antibody at 4 °C overnight. After washing with PBS for 3 times, cells were then incubated with FITC-conjugated secondary antibody and counterstained with DAPI. The stained cells were viewed using the Nikon fluorescence microscope (200× amplification; Nikon, Japan).

2.6. Surface plasmon resonance analysis

The binding of heme with rhMD2 or rhTLR4 was measured using the ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, Hercules, CA, USA) with an HTE sensor chip (ProteOn™, #176-5033). The target proteins were loaded to the biosensors. Different concentrations of heme (0, 0.390625, 0.78125, 1.5625, 3.125, 6.25, 12.5, 25, 50, or 100 μM) was added in the running buffer (PBS containing 0.1 % SDS and 5 % DMSO). After reaching baseline, sensors were moved to association step for 60 s and then dissociated for 60 s. The data were analyzed using ProteOn manager software. The binding kinetic parameters, including the K_D values, were calculated by global fitting of the kinetic data from various concentrations of heme using a 1:1 Langmuir

binding model.

2.7. LPS displacement ELISA assay

Firstly, a 96-well ELISA plates were coated with anti-MD2 antibody and incubated overnight at 4 °C. After washing with PBST, 3 % BSA was used for blocking. Following blocking, rhMD2 (1 μg/ml) diluted in 10 mM Tris-HCl buffer was added to the plate and incubated for 1.5 h at 25 °C. After washing with PBST, biotin-LPS (InvivoGen, San Diego, CA, USA) was added for 1 h with or without heme (3, 30, or 300 μM). HRP activity was then determined by SpectraMax M5 (Molecular Devices, Sunnyvale, CA, USA) at 450 nm after the addition of TMB substrate.

2.8. Bis-ANS displacement assay

In brief, bis-ANS (1 μM, Carlsbad, CA, USA) was incubated with rhMD2 (5 nM) in PBS to reach stable fluorescence units (RFU) at room temperature, and then different concentrations of heme (5, 10, 15, 20, 25, 30, 40 or 50 μM) were added. Fluorescence measurement were performed using SpectraMax M5 at 25 °C in 1 cm path-length quartz cuvettes.

2.9. Molecular docking

A molecular interaction between heme and MD2/TLR4 complex was performed using AutoDock version 4.2.6. The crystal structure of human MD2-lipid IVa complex (PDB code 3vq2) was obtained from Protein Data Bank. The AutoDock Tools version 1.5.6 package was applied to generate the docking input files. A 60 × 60 × 60 point's grid box with a spacing of 0.375 Å between the grid points was implemented. The affinity maps of MD2 were calculated by AutoGrid. One hundred Lamarckian Genetic Algorithm runs with default parameter settings were processed. The hydrogen bonds and bond lengths within the complex of protein-ligand conformations were analyzed.

2.10. Real-time quantitative PCR

Cells or spleen tissues were homogenized in TRIZOL (Invitrogen, Carlsbad, CA) for total RNA extraction. Reverse transcription was performed using a two-step PrimeScript RT Reagent Kit with gDNA Eraser (TAKARA, Japan). Quantitative PCR was performed using platinum SYBR Green qPCR SuperMix-UDG Invitrogen (Shanghai, China) on Eppendorf Mastercycler (Eppendorf, Hamburg, Germany). Primers for the target genes (Supplementary Table S1) were obtained from Invitrogen. The mRNA levels of target genes were normalized to β-actin.

2.11. Western blot

After treatments, cells were either lysed to collect total proteins or nuclear proteins. The concentrations of proteins were determined by the Bradford assay (Bio-Rad, CA). Cell lysates were separated by 10 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to a polyvinylidene fluoride membrane (Bio-Rad Laboratory, Hercules, CA). After blocking with non-fat milk for 1.5 h, membranes were then incubated with specific primary antibodies. Immunoreactive bands were detected by incubating with horseradish peroxidase-labeled secondary antibody for 2 h at room temperature and visualizing using enhanced chemiluminescence reagents (Bio-Rad).

2.12. Immunoprecipitation

The MD2/TLR4/MyD88 complex was detected *via* co-IP assay. After treatment, cells were lysed and incubated with anti-TLR4 antibody at 4 °C overnight, followed by immunoprecipitation with protein G-sepharose beads at 4 °C for 4 h. The samples were prepared and used for Western blot analysis. Immunoblots of MD2 and MyD88 (IB) were detected with plots of TLR4 (IP) as the loading control. For TLR4 dimerization assay, Flag-tagged TLR4, HA-tagged TLR4 and His-tagged MD2 plasmids were used. Plasmid transfection was performed as described (Chen et al., 2020). After treatment, cell lysates were collected and incubated with anti-Flag antibody at 4 °C overnight and were then immunoprecipitated with protein G-sepharose beads at 4 °C for 4 h. The samples were prepared and used for immunoblotting analysis. Immunoblots of HA (IB) were detected with plots of Flag (IP) as the loading control.

2.13. Determination of cytokines of IL-6 and TNF-α by ELISA assay

Cytokine IL-6 and TNF-α in culture media and spleen tissues were determined using mouse IL-6 and TNF-α ELISA kit (Bioscience, San Diego, CA, USA) according to the manufacturer's instructions. Data were normalized to the amounts of total proteins from lysates.

2.14. Statistical analysis

All experiments are randomized and blinded. All data are reported as Mean ± SEM. Statistical analysis was performed using GraphPad Prism 8.0 software (San Diego, CA, USA). One-way ANOVA followed by Dunnett's *post hoc* test was used when comparing more than 2 groups of data. $P < 0.05$ was considered significant.

3. Results

3.1. Loss of MD2 ameliorates heme-induced inflammation in spleen tissues

To identify the role of MD2 in heme-induced systemic inflammation *in vivo*, a mouse model induced by intravenous injection of heme was utilized. An increased heme content in spleen tissues was observed in both the WT and $MD2^{-/-}$ mice after heme injection, as revealed by Perl staining (Fig. 1A, Supplementary Fig. S1A). H&E staining shows morphological abnormality mostly located in the lymphoid nodule of heme-treated WT mice (Fig. 1B). TNFα expression was increased after heme treatment in the WT mice (Fig. 1C, Supplementary Fig. S1B). All these changes were significantly reversed in $MD2^{-/-}$ mice (Fig. 1A–C, Supplementary Fig. S1). In addition, mRNA and protein levels of TNFα and IL-6 were found in the spleen of heme-treated WT mice, which were significantly reduced in the spleen of heme-treated $MD2^{-/-}$ mice (Fig. 1D-E). Overall, these data indicate that MD2 knockout inhibits heme-induced injury and inflammation in spleen.

3.2. MD2 mediates heme-induced TLR4 dimerization and MD2/TLR4/MyD88 activation in vitro

In order to evaluate the effect of heme on MD2/TLR4/MyD88 pathway, we next detected the interaction among MD2, TLR4 and MyD88, as well as the TLR4 dimerization. As shown in Fig. 2A, heme treatment induced significant MD2/TLR4 interaction for all the three tested time-points (Fig. 2A). Next, RAW264.7 cells were treated with L6H9 (chemical structure was shown in Fig. 6 and Supplementary Fig. S2), which directly binds to MD2 and prevents MD2-mediated TLR4 activation (Chen et al., 2020) and TAK242, which binds TLR4 and disrupts the interaction of TLR4 with adaptor molecules, thereby inhibiting MD2 and TLR4 activity, respectively (Belcher et al., 2014). Pretreatment with either L6H9 or TAK242 showed significant reduction in the formation of TLR4/MD2/MyD88 complex, indicating reduced physical interaction (Fig. 2B). Further, to understand the effect of heme on TLR4 dimerization, 293T cells were transfected with HA-tagged and Flag-tagged TLR4 and then further transfected with or without His-tagged MD2. As shown in Fig. 2C, heme failed to induce TLR4 dimerization without the presence of MD2 in 293T cells (Fig. 2C). Similarly, L6H9 or TAK242-treatment significantly inhibited

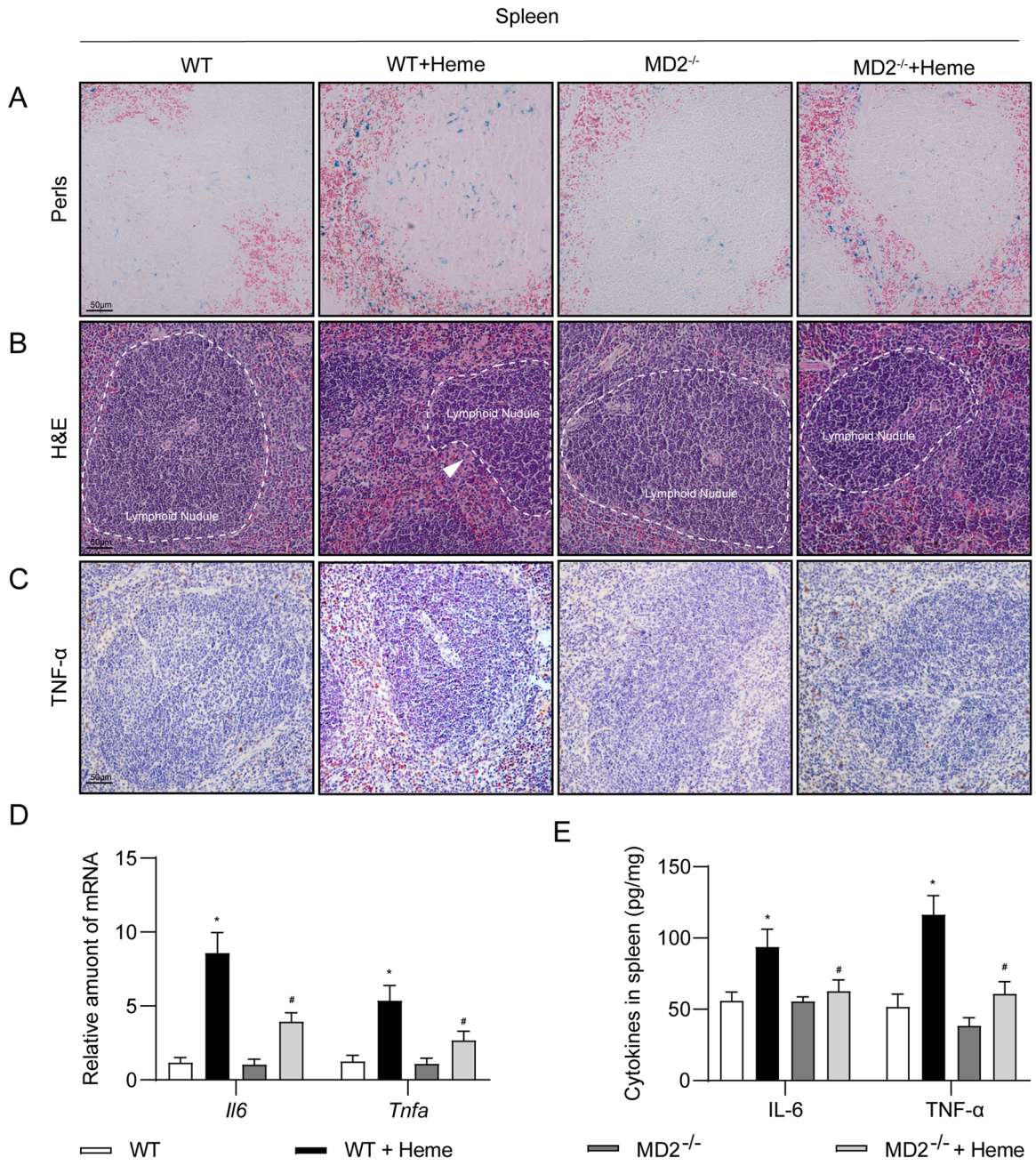

Fig. 1. MD2 knockout ameliorates heme-induced inflammation in spleen tissues. WT or MD2$^{-/-}$ mice were injected with 50 μM/kg heme or saline for 5 times in 15 days. Mice were sacrificed and spleen tissues were collected for further study. Prussian blue iron staining (A), H&E staining (B) and Immunochemistry staining of TNF-α (C) in spleen tissues from different groups of mice. Dotted area shows lymphoid nodule and arrow shows the morphological abnormality of the lymphoid nodule. Relative mRNA (D) and protein (E) levels of IL-6 and TNFα. Data are shown as mean ± SEM, n = 5; *, $P < 0.05$, compared to the WT group; #, $P < 0.05$, compared with the WT + Heme group.

heme-induced TLR4 dimerization in transfected 293T cells (Fig. 2D). Collectively, these results demonstrate that MD2 participated in heme-induced TLR4 signaling.

3.3. Heme directly binds to MD2 and TLR4

In order to unravel how MD2 participated in heme-induced TLR4 signaling, we tested the direct binding between heme and MD2/TLR4 complex. SPR assay demonstrated that heme physically interacted with MD2 with a K_D value of 4.76×10^{-6} M (Fig. 3A). We confirmed this interaction using Bis-ANS displacement assay. Our data show that heme competitively bound to the hydrophobic pocket of rhMD2 in a dose-dependent manner (Supplementary Fig. S3). We further confirmed heme binding to MD2 using ELISA assay. Consistently, heme displaced Biotin-LPS in a dose-dependent manner, which further confirms the binding between heme and MD2 (Fig. 3B). We next determined whether heme directly binds to rhTLR4. As revealed by SPR assay, we also found a direct binding between heme and rhTLR4 with a K_D value of 8.81×10^{-6} M (Fig. 3C), which was comparable with the interaction between heme and rhMD2. Moreover, the result of molecular docking confirms that heme binds to the junction area of MD2 and TLR4 (Fig. 3D).

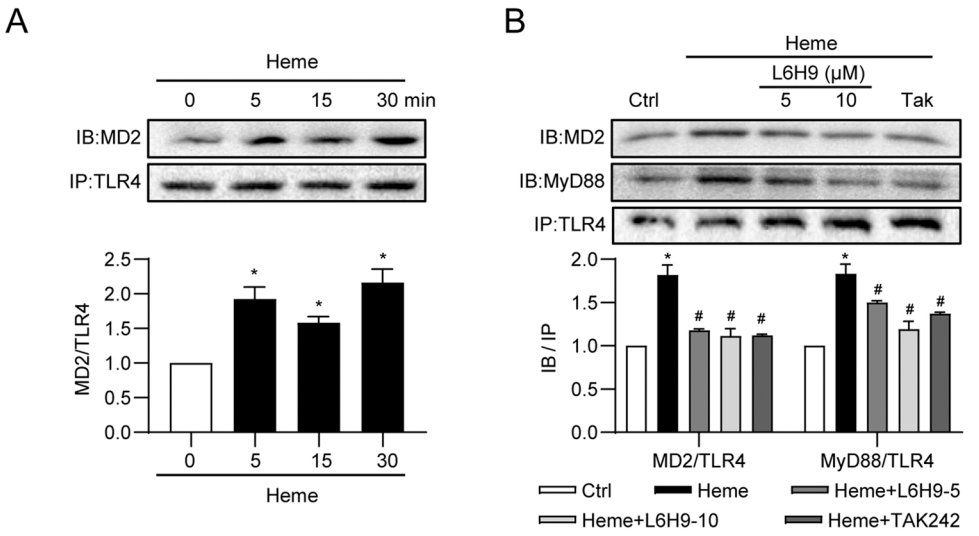

Fig. 2. Heme induces TLR4 dimerization and MD2/TLR4/MyD88 activation *in vitro*. (A) RAW264.7 cells were treated with heme (30 μM) for various time points. TLR4 was immunoprecipitated (IP) and levels of MD2 were detected by immunoblotting (IB). (B) RAW264.7 cells pretreated with L6H9 (5 or 10 μM) or TAK-242 (400 nM) for 1 h, followed by heme stimulation (30 μM) for 5 min. TLR4 was IP and levels of MD2 and MyD88 were detected by IB. (C) 293T cells were transfected with HA-tagged TLR4 and Flag-tagged TLR4 with or without the transfection of His-tagged MD2. Cells were treated with heme (30 μM) for 5 min. Flag-TLR4 was IP and levels of HA-TLR4 were detected by IB. (D) 293T cells were transfected with HA-tagged TLR4, Flag-tagged TLR4 and His-tagged MD2. Cells were then pretreated with (5 or 10 μM) or TAK-242 (400 nM) for 1 h, followed by heme stimulation (30 μM) for 5 min. Flag-TLR4 was IP and levels of HA-TLR4 were detected by IB. Representative blots and densitometric quantification are shown. Data are shown as mean ± SEM, n = 3; *, $P < 0.05$; ns, $P > 0.05$, compared to the control cells (0 or Ctrl); #, $P < 0.05$, compared to the heme-treated cells (Heme).

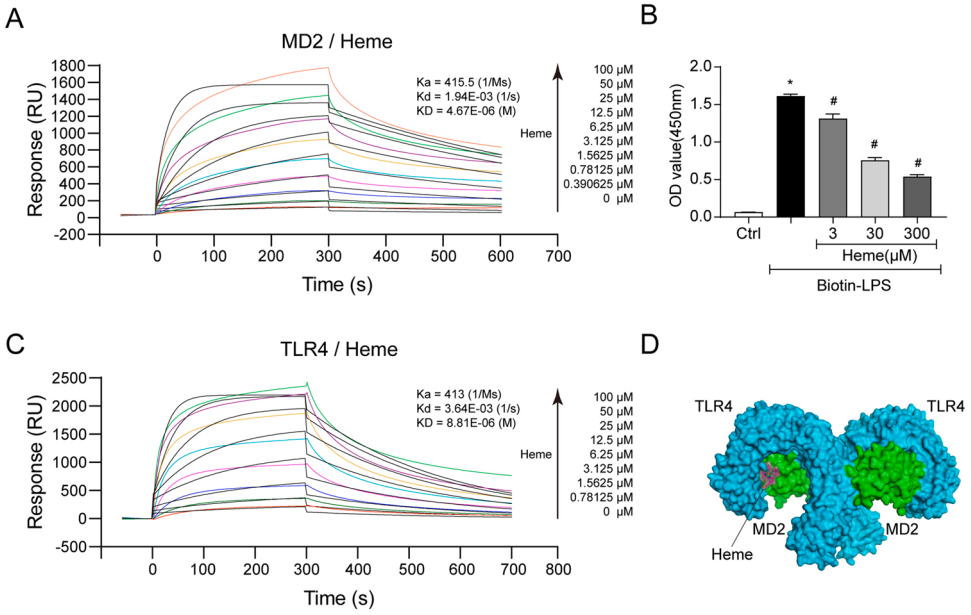

Fig. 3. Heme directly binds to MD2/TLR4 complex. (A) SPR analysis of the binding between Heme and rhMD2. (B) As determined by ELISA, heme dose-dependently inhibited the binding of biotin-LPS to rhMD2. Data are shown as mean ± SEM, n = 3; *, $P < 0.05$, compared to the control group; #, $P < 0.05$, compared to the Biotin-LPS group.

3.4. Loss of MD2 inhibits heme-induced inflammation and MAPK/NF-κB pathway in mouse primary macrophages

Given the role of heme as well as MD2/TLR4/MyD88 pathway in inflammation, we next tested whether heme-MD2 interaction is required to induce inflammation *in vitro*. We observed an increase of phosphorylated ERK, JNK and p38 in the heme-treated MPMs extracted from the WT (WT MPMs) but not in the MPM extracted from the $MD2^{-/-}$ mice ($MD2^{-/-}$ MPMs, Fig. 4A, Supplementary Fig. S4A-C). Next, we evaluated the effect of heme-MD2 interaction on NF-κB pathways by measuring the p65 translocation to the nucleus, as well as the protein level of IκB-α. Our immunoblotting data confirm significantly higher protein levels of nuclear p65 and reduced level of IκB-α, indicating the activation of NF-κB pathway in the heme-treated WT MPMs in comparison to the $MD2^{-/-}$ MPMs (Fig. 4B, Supplementary Fig. S4D-E). Similarly, our immunofluorescence assay also confirms that MD2 knockout significantly prevented the heme-induced increase of p65 nuclear translocation (Fig. 4C, Supplementary Fig. S4F). Both the mRNA and protein levels of IL-6 and TNFα were significantly increased in heme-treated WT MPMs but were markedly reduced in the heme-treated $MD2^{-/-}$ MPMs (Fig. 4D-E). In summary, MD2 knockout inhibited heme-induced inflammation and MAPK/NF-κB activation in macrophages.

3.5. MD2 inhibitor ameliorates heme-induced inflammation in macrophages

Finally, we evaluated the inhibitory effects of L6H9, an MD2 inhibitor, against heme-induced cytokine production and MAPK/NF-κB pathway activation. As expected, L6H9 significantly inhibited heme-induced activation of MAPK pathways (Fig. 5A, Supplementary Fig. S5A-C). In addition, heme-induced activation of NF-κB signaling was also abolished after treatment of L6H9, characterized by inhibition of the p65 nuclear translocation and IκB-α degradation (Fig. 5B-C, Supplementary Fig. S5D-F). Similarly, heme-induced overexpression and secretion of TNF-α and IL-6 were also significantly inhibited by L6H9 in heme-treated macrophages. Collectively, these *in vitro* results reveal that MD2 inhibitor offered similar protective effects as MD2 knockout in blockade of heme-induced inflammation.

4. Discussion

Heme is an essential molecule in many cells. However, heme can cause life-threatening situation when it escapes into the circulation warranting in-depth mechanistic investigation. The main findings of this study include the discovery that heme directly binds to MD2/TLR4 complex and activates MD2/TLR4/MyD88 pathway, leading to exacerbated activation of MAPK and NF-κB signaling and production of downstream effectors of inflammation (Fig. 6). We also demonstrate that genetic or pharmacologic blockade of MD2 effectively inhibits heme-induced MD2/TLR4/MyD88 activation, leading to reduced heme-induced inflammatory molecules (Fig. 6).

Heme, as a prototypical damage-associated molecular pattern, targets different immune and non-immune cells (Bozza and Jeney, 2020). The role of the innate immune receptor, TLR4, in heme-induced inflammation has been confirmed. For example, it was reported that heme activates TLR4 and NF-κB signaling in endothelial cells triggering vaso-occlusion in sickle cell disease mice, but not in normal mice (Belcher et al., 2014). More importantly, the effect of heme was mimicked by TLR4 ligand LPS, demonstrating that hemolysis and associated vaso-occlusion in sickle cell disease (SCD) mice was mediated by TLR4 signaling, leading to NF-κB activation (Belcher et al., 2014). Heme promotes neutrophil and macrophages migration by acting as a chemotactic molecule and activates macrophages to secrete pleiotropic cytokines, such as TNF-α and IL-1β (Dutra and Bozza, 2014). These cytokines regulate cell death, recruitment of immune cells to the inflamed tissues and induce the production of acute-phase proteins (Dutra and Bozza, 2014). Heme is also reported to induce TNF-α secretion in monocyte/macrophages through TLR4 signaling (Figueiredo et al., 2007).

The interaction between TLR4 and its co-receptor MD2 activates TLR4 pathway (Rajamanickam et al., 2020). The importance of TLR4/MD2 complex can be understood by the observed higher level of MD2/TLR4 pathway activation in diabetes-associated inflamed heart, where loss of MD2 inhibited the activation of TLR4 pathway and downregulated MAPK/NF-κB pathways, leading to reduced inflammatory cytokine production and thereby reducing myocardial inflammation (Wang et al., 2020). Furthermore, it was reported that heme could bind to soluble MD2 and modulate endothelial inflammation in sickle cell disease (Zhang et al., 2021). *In vitro* assay confirms the binding of heme with MD2 at amino acids W23 and Y34 on MD2 (Belcher et al., 2020). In this study, we extended the role of MD2 in regulating heme-induced inflammation. Our data demonstrated for the first time that heme not only binds to MD2, but also binds to TLR4 with similar affinity to regulating inflammation.

It is important to note that hemolysis, which leads to heme generation, mainly occurs in the spleen (Klei et al., 2020). And systemic inflammation is often characterized by its level in spleen tissue (Gotoh et al., 2017). Hence, we investigated whether MD2 is involved in the systemic inflammation under excessive circulatory heme condition in spleen tissue. Our data show that heme induced significant hemosiderin and abnormal lymphoid nodule, which were further associated with increased inflammation and increased inflammatory cytokine production. Besides, deletion of MD2 offered significant protection and decreased inflammation in spleen tissue. This was similar to the previous findings that deletion of TLR4 protected the mice from heme-induced inflammatory injury in spleen *via* regulating M1 macrophage-dependent inflammation (Belcher et al., 2014). Since MD2 is the accessory protein of TLR4, they may share the same pathway in regulating heme-induced inflammation. Using MD2 and TLR4 specific inhibitors, L6H9 and TAK-242, we further evaluated inhibition of MD2 or TLR4 on TLR4 dimerization and the formation of MD2/TLR4/MyD88 complex. Interestingly, our *in vitro* data demonstrate that L6H9 and TAK-242 offered similar inhibitory effects on heme-induced TLR4 dimerization and the formation of MD2/TLR4/MyD88 complex. However, strict comparison of TLR4 or MD2 inhibition on heme-induced inflammation needs further *in vivo* and *in vitro* investigation.

In addition to its documented role in inflammation, heme is also a well-known pro-oxidant damage associated molecule pattern (DAMP) (Erdei et al., 2018). In our current study, we mainly focused on the inflammatory role of heme based on its ability to bind MD2/TLR4 complex. Whether pro-oxidant role of heme also plays an additive role in heme-induced inflammation remains unclear. MD2 has already been reported to regulate oxidative stress, while inflammation is tightly associated with reactive oxygen species generation (Huang et al., 2021). All these findings suggest the potential role of MD2 in heme-induced oxidative injury, which may help the full understanding of mechanism of heme-induced inflammation in the future. Besides, we confirmed for the direct binding of heme with MD2/TLR4 complex, however, the accurate binding sites of heme with MD2/TLR4 complex were unclear. X-ray crystal structure analysis of this trimer complex would be helpful. All these would need our further efforts in the future.

5. Conclusion

In summary, our results identify a novel mechanism of heme action in inflammation, which is the direct physical interaction of heme with MD2/TLR4 complex, leading to the activation of TLR4 signaling and thereby increasing inflammation. This study indicates that MD2 and TLR4 can be promising targets to treat heme-associated diseases, such as β-thalassemia, SCD and malaria. And the MD2 inhibitor, L6H9, could also be an effective candidate for treatment of heme-related clinical diseases.

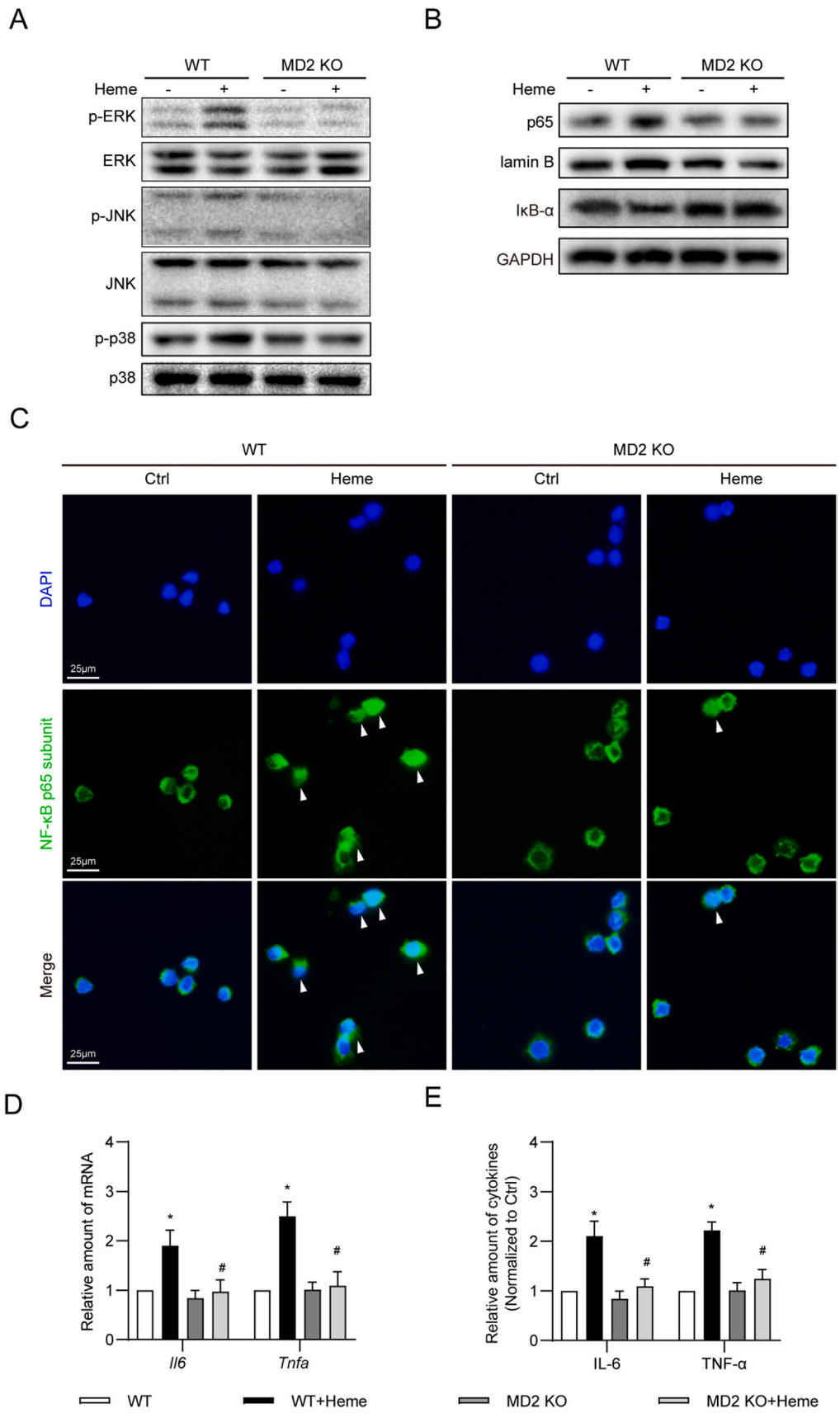

Fig. 4. MD2 knockout ameliorates heme-induced inflammation in mouse primary macrophages. MPMs were extracted from WT or MD2$^{-/-}$ mice, followed by heme stimulation for 1 h. MD2-knockout inhibited heme-induced activation of MAPK (A) and NF-κB (B) pathways. (C) MD2-knockout inhibited p65-subunit nuclear translocation. Cells were stained with p65 (green) and were further counterstained with DAPI (blue) [scale bar = 25 μm]. (D-E) MPMs were extracted from WT or MD2$^{-/-}$ mice, followed by heme stimulation for 8 or 24 h. MD2-knockout suppressed heme-induced inflammatory genes transcription and expression *in vitro*. Data are shown as mean ± SEM, n = 3; *, $P < 0.05$, compared to the WT group; #, $P < 0.05$, compared to the heme-treated WT group (WT + heme).

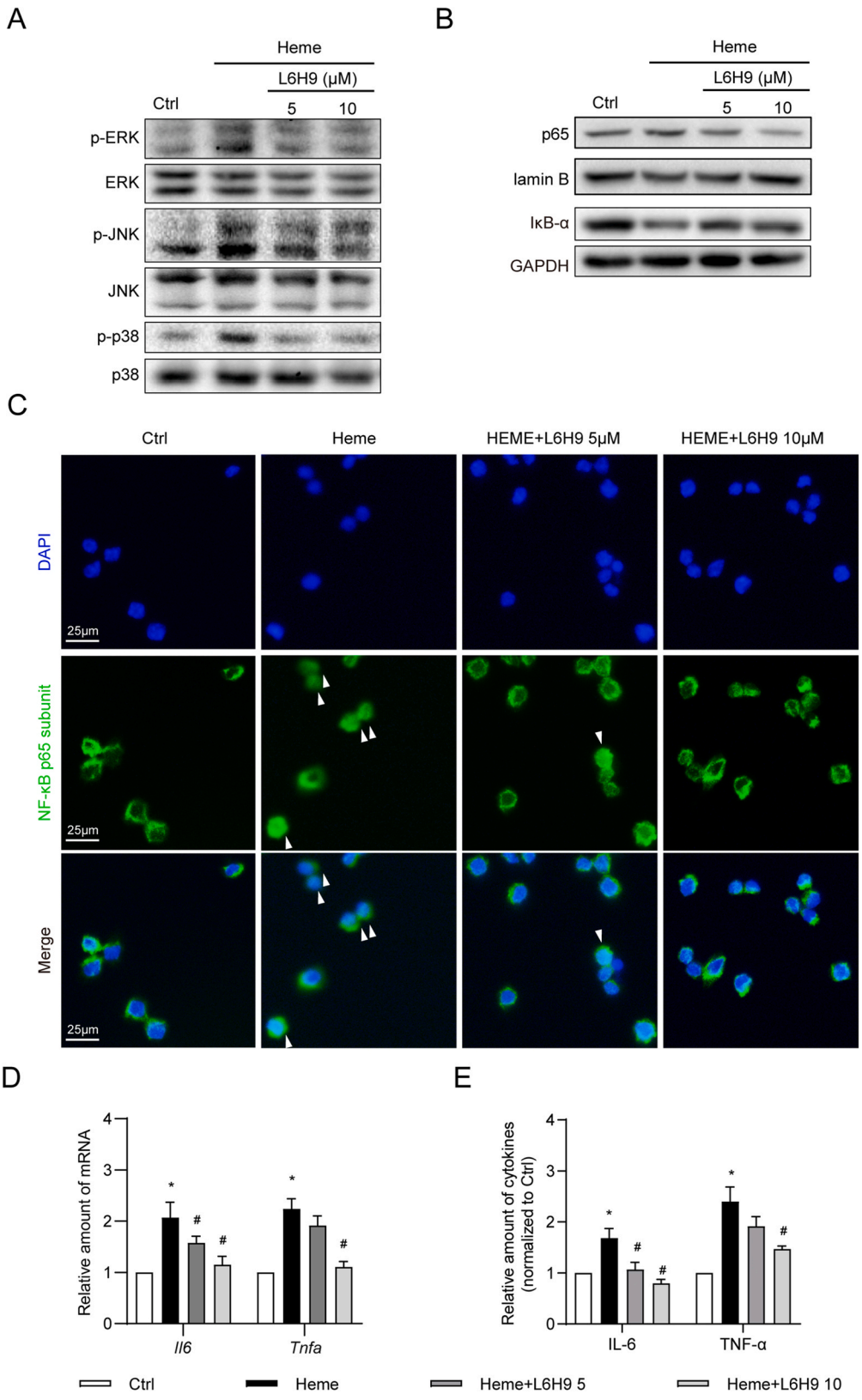

Fig. 5. MD2 inhibitor ameliorates heme-induced inflammation *via* reduced activation of MAPK and NF-κB pathways in macrophages. (A-B) RAW264.7 cells were pretreated with L6H9 (5 or 10 μM) for 1 h, followed by heme stimulation (30 μM) for 1 h. Cell lysates were collected to evaluate the activation of MAPK (A) and NF-κB (B) pathways. (C) L6H9 inhibited p65-subunit nuclear translocation. Cells were stained with p65 (green) and were further counterstained with DAPI (blue) [scale bar = 25 μm]. (D-E) RAW264.7 cells were pretreated with L6H9 (5 or 10 μM) for 1 h, followed by heme incubation (30 μM) for 8 or 24 h. The mRNA (D) and protein (E) levels of IL-6 and TNFα were determined. Data are shown as mean ± SEM, n = 3; *, $P < 0.05$, compared to the control group (Ctrl); #, $P < 0.05$, compared to the heme-treated heme group.

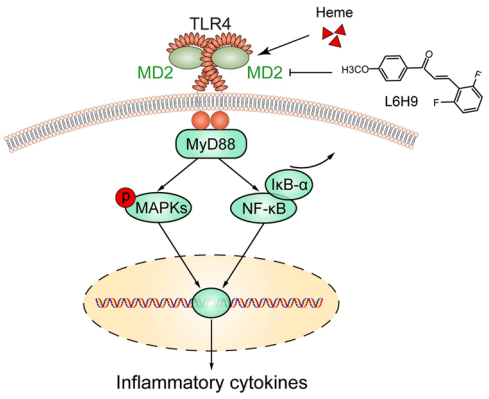

Fig. 6. Schematic illustration. Heme induces inflammation *via* direct binding to MD2/TLR4 complex and activating TLR4 signaling.

Authors contribution

All the authors reviewed and approved the manuscript. J.L., J.Q., G. L. and Y.W. conceived the study and designed the experiments. K.L., J.L., L.H., X.D., and Y.J., performed the experiments. K.L., A.S., V.P., G.L. and Y.W. analyzed the data. K.L., J.L., Q.C. and Y.W. wrote the manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxlet.2022.09.007.

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