Cellular Immunology 336 (2019) 40–47

Contents lists available at ScienceDirect

Cellular Immunology

journal homepage: www.elsevier.com/locate/ycimm

ER-stress regulates macrophage polarization through pancreatic EIF-2alpha kinase

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ARTICLE INFO

Keywords:
Macrophage
ER-stress
PERK
Phenotypes polarization

ABSTRACT

During the process of NAFLD progression, ER-stress is activated in macrophages and induces the pro-inflammatory polarization of macrophage. As one of the three ER membrane resident proteins, pancreatic eIF-2alpha kinase (PERK) plays an important role in ER stress, but its participation in macrophage polarization is largely unknown. In this study, we found that the PA mediated ER-stress activation could induce M1-type polarization in macrophages, and this phenotype polarization could be inhibited by ER-stress inhibitor 4-PBA as well as GSK2656157, an inhibitor of PERK. Moreover, the knockdown of PERK altered the STAT1 and STAT6 pathways in macrophages, which then led to the M1-to-M2 phenotypic shift. In summary, we found that PERK could regulate the phenotypic polarization of macrophages. This finding may provide new insight into the suppression of pathological progression of fatty liver or liver ischemia reperfusion injury induced by M1-type macrophages.

1. Introduction

Macrophage is a major component of natural immune system in human body. It plays critical roles in inflammation, host defense, and the maintenance of tissue homeostasis [1]. Activation of macrophages can be either pro-inflammatory (M1) that leads to tissue destruction, or anti-inflammatory (M2) that contributes to tissue regeneration and wound healing [2]. Different types of macrophages (M1 or M2) display highly distinct expression profiles of cytokines, enzymes and cell-surface markers [3]. Among the various factors that regulate macrophage polarization, cytokines play a critical role. Interferon-γ (IFN-γ), lipopolysaccharide (LPS), and Toll-like receptor (TLR) agonists can drive M1 phenotypic polarization, and IL-4 and IL-13 can induce M2 phenotype shift [4]. The M1-type macrophages are marked by TNFα, Il1β and NOS2, and the M2-type macrophage are marked by CD206, IL10, Arg1, etc [5,6].

Endoplasmic reticulum (ER) is a cellular organelle that plays crucial roles in lipid biosynthesis, intracellular Ca^{2+} homeostasis and the folding of secreted and transmembrane proteins. Several environmental and physiological stress conditions can induce endoplasmic reticulum stress (ER-stress), such as lipid accumulation, reactive oxidative stress (ROS), protein overload, Ca^{2+} leaking, iron imbalance, hypoxia and viral infections [7]. ER-stress is regulated by three ER membrane resident proteins, inositol-requiring kinase 1α (IRE1α), pancreatic eIF-2alpha kinase (PERK), and activating transcription factor 6 (ATF6) [8]. Among them, IRE1α and PERK are the central type-I transmembrane proteins for ER-stress [9]. PERK is a Ser/Thr kinase and its stress-sensing domain lies inside of ER lumen [10]. Similar to IRE1α, once ER stress is activated, Bip dissociates from the luminal domain of PERK and activates the oligomerization and autophosphorylation of the cytosolic kinase domain of PERK [11]. Then, PERK phosphorylates the α-subunit of eukaryotic translation initiation factor 2 (eIF2α), which leads to the general inhibition of protein translation and the induction of ATF4 (activating transcription factor 4), the transcription factor that controls UPR related genes [12]. Our previous study demonstrated that in fatty liver disease, ER-stress was activated in macrophages, resulting in the activation of IRE1α and M1-type polarization of macrophages, which then led to the increased ischemia reperfusion injury found in fatty liver

Abbreviations: ER-stress, endoplasmic reticulum stress; PERK, pancreatic eIF-2alpha kinase; IRE1α, inositol-requiring kinase 1α; ATF6, activating transcription factor 6; ATF4, activating transcription factor 4; XBP1, X-box binding protein 1; CHOP, C/EBP-homologous protein; PA, palmitic acid; BMDMs, bone marrow-derived macrophages; TNFα, tumor necrosis factor α; IL6, interleukin-6; IL1β, interleukin-1 β; STAT1, signal transducers and activators of transcription 1; STAT6, signal transducers and activators of transcription 6; JAK2, Janus Kinase 2; TLR4, toll-like receptor 4

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https://doi.org/10.1016/j.cellimm.2018.12.008
Received 22 August 2018; Received in revised form 4 December 2018; Accepted 20 December 2018
Available online 21 December 2018
0008-8749/

[13]. Moreover, recent study showed that the tumor necrosis factor-receptor associated factor 2 (TRAF2), an important mediator of IRE1α signaling system upon ER stress, was also activated in the macrophages stimulated with palmitic acid (PA) [14]. So far, preliminary studies have shown that both ATF6 and IRE1α, two of the three ER membrane resident proteins, are involved in phenotypic polarization of macrophages [15]. However, whether PERK also participates in this process is still unclear. Since PERK-eIF2α-ATF4 pathway regulates lipogenesis and hepatic steatosis, and PERK pathway is activated in the macrophages of fatty livers, it is possible that PERK pathway also participates in and regulates the macrophage polarization.

In this study, we focused on PERK signaling pathway in macrophages. We found that ER-stress and PERK pathway were induced in macrophages upon PA stimulation. The activation of ER-stress promoted macrophages to shift to pro-inflammatory type, and this polarization could be inhibited by 4-PBA or PERK inhibitor. In addition, inhibition of PERK by siRNA or GSK2656157 in macrophages decreased the M1-type and increased the M2-type polarization under LPS/IFNγ or IL4 stimulation. Altogether, our study revealed the important role of PERK in macrophage phenotypes polarization.

2. Materials and methods

2.1. Animals

Experiments were conducted on male C57BL/6J mice, which were purchased from the Animal Center of the Affiliated Drum Tower Hospital of Nanjing University Medical School and housed under specific pathogen-free conditions. The animal experiments were approved by the Institutional Animal Care and Use Committee of Nanjing University, China, based on the NIH Guide for the Care and Use of Laboratory Animals. All efforts were made to minimize suffering.

2.2. Cell culture

Bone marrow-derived macrophages (BMDMs) were obtained from the femur and tibia of 6–8-week-old C57BL/6 mouse, and cultured in RPMI 1640 medium containing 10% FBS and 20 ng/ml MCSF for a week.

2.3. Western blot analysis

Proteins were electrophoresed by SDS/PAGE (12% gel and 10% gel) and the blots were incubated overnight with primary antibody. The following primary antibodies were used: anti-PERK (Cell signal Technology, #3192), anti-ATF4 (Abcam, ab23760), anti-eIF2α (phospho S51) (Abcam, ab32157), anti-IRE1α (phospho Ser724) (Novus, NB100-2323), anti-XBP1s (Biolegend, 619501), anti-GRP78 (Abcam, ab21685), anti-GRP94 (Abcam, ab3674), anti-CHOP (Cell signal Technology, #2895), anti-ATF6α (Santa-Cruz, sc-432646), anti-STAT6 (Abcam, ab32520), anti-STAT6 (phospho Y641) (Abcam, ab28829), anti-phospho-STAT1 (Ser727 and Tyr701) (Cell signal Technology, #7649 and #8826), anti-TLR4 (Santa-Cruz, sc-293072), anti-JAK2 (Abcam, ab32101), and anti-GAPDH (Abcam, ab8245).

2.4. Oil Red O Stain Kit

To detect lipid accumulation in macrophages, Oil Red O Stain Kit (Jiancheng Bioengineering Institute, China) was used according to the manufacturer's instructions and visualized at light microscope.

2.5. PERK knockdown

The siRNA sequence of mouse IRE1α is as follows: 5′-GGACGAUC CUGCUUUGCAUTTAUGCAAAGCAGGAUGGUCCTT-3′. Cells were transiently transfected with siRNA using Lipofectamine2000 (Invitrogen, USA) according to the manufacturer's instructions.

2.6. Enzyme-linked immunosorbent assay (ELISA)

The levels of IL6 (eBioscience, USA), IL1β (R&D Systems, USA) and TNFα (eBioscience, USA) in cell culture supernatants were measured using commercially available ELISA kits according to the manufacturer's instructions.

2.7. Quantitative real-time polymerase chain reaction (qRT-PCR)

RNA of macrophages was extracted with TRIzol™ reagent (Life Technologies, USA) according to the manufacturer's instructions.

Reverse transcription was performed with PrimeScript™ RT Master Mix (Takara, Japan) according to the manufacturer's instructions. qRT-PCR was performed using TB Green™ *Premix Ex Taq*™ (Takara, Japan) and ABI PRISM 7500 real-time PCR System (Applied Biosystems, USA). Primers used for qPCR are are as follows: *β-Actin* forward: 5′-AGTGTG ACGTTGACATCCGTA-3′, reverse: 5′-GCCAGAGCAGTAATCTCCT TCT-3′; *PERK* forward: 5′-AGGAACATCGTAGGGGCTTT-3′, reverse: 5′-GAGTTGCAGACCCGAGCTAC-3′; *Arg1* forward: 5′-CTCCAAGCCAAA GTCCTTAGAG-3′, reverse: 5′-GGAGCTGTCATTAGGGACATCA-3′; *Il10* forward: 5′-GCTATGCTGCCTGCTCTTACT-3′, reverse: 5′-CCTGCTGAT CCTCATGCCA-3′; *Mgl1* forward: 5′-TGCAACAGCTGAGGAAGGACT TGA-3′, reverse: 5′-AACCAATAGCAGCTGCCTTCATGC-3′; *Mgl2* forward: 5′-GCATGAAGGCAGCTGCTATTGGTT-3′, reverse: 5′-TAGGCCC ATCCAGCTAAGCACATT-3′; *Chil3* forward: 5′-CAGGTCTGGCAATTCTT CTGAA-3′, reverse: 5′-GTCTTGCTCATGTGTGTAAGTGA-3′; *Tnfα* forward: 5′-GACGTGGAACTGGCAGAAGAG-3′, reverse: 5′-ACCGCCTGGA GTTCTGGAA-3′; *Il1β* forward: 5′-GCAACTGTTCCTGAACTCAACT-3′, reverse: 5′-ATCTTTTGGGGTCCGTCAACT-3′; *Ccl2* forward: 5′-TTAAAA ACCTGGATCGGAACCAA-3′, reverse: 5′-GCATTAGCTTCAGATTTACG GGT-3′; *Il6* forward: 5′-CCACGGCCTTCCCTACTTC-3′, reverse: 5′-TTG GGAGTGGTATCCTCTGTGA-3′; *iNos* forward: 5′-ACATCGACCCGTCCA CAGTAT-3′, reverse: 5′-CAGAGGGGTAGGCTTGTCTC-3′.

2.8. Immunocytofluorescence (ICF)

Immunocytofluorescence were performed as described previously [13]. Briefly, cells were seeded in sterile 12-well plates with slides placed in wells ahead. Then cells were fixed with 4% paraformaldehyde and rinsed with 0.01 M PBS. Fixed cells were permeablized with 0.3% Triton then blocked with 10% fetal sheep serum. Blocked cells were incubated with primary antibodies overnight at 4 °C. After rinsed with 0.05% Tween-20 in PBS, cells were incubated with corresponding secondary antibodies, followed by incubation with DAPI. After mounting, the slides were observed under immunofluorescence microscope (Leica, German). The following primary antibodies were used: anti-F4/80 (Abcam, ab60343), anti-CD206 (Abcam, ab8918), anti-iNOS (Abcam, ab15323).

2.9. Flow cytometry

For flow cytometry analysis, BMDMs were stained with fluorescence-conjugated antibodies according to the manufacturer's instructions. The following fluorescence-conjugated antibodies were used: APC anti-mouse F4/80 (Biolegend, 123115) and Isotype Ctrl Antibody (Biolegend, 400512). Analysis was performed using FlowJo software. (Tree Star)

2.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism software version 6.0. All data are expressed as mean ± standard error of the mean (SEM). Normally distributed data were tested by Student's *t* test. Differences between multiple groups were evaluated for significance

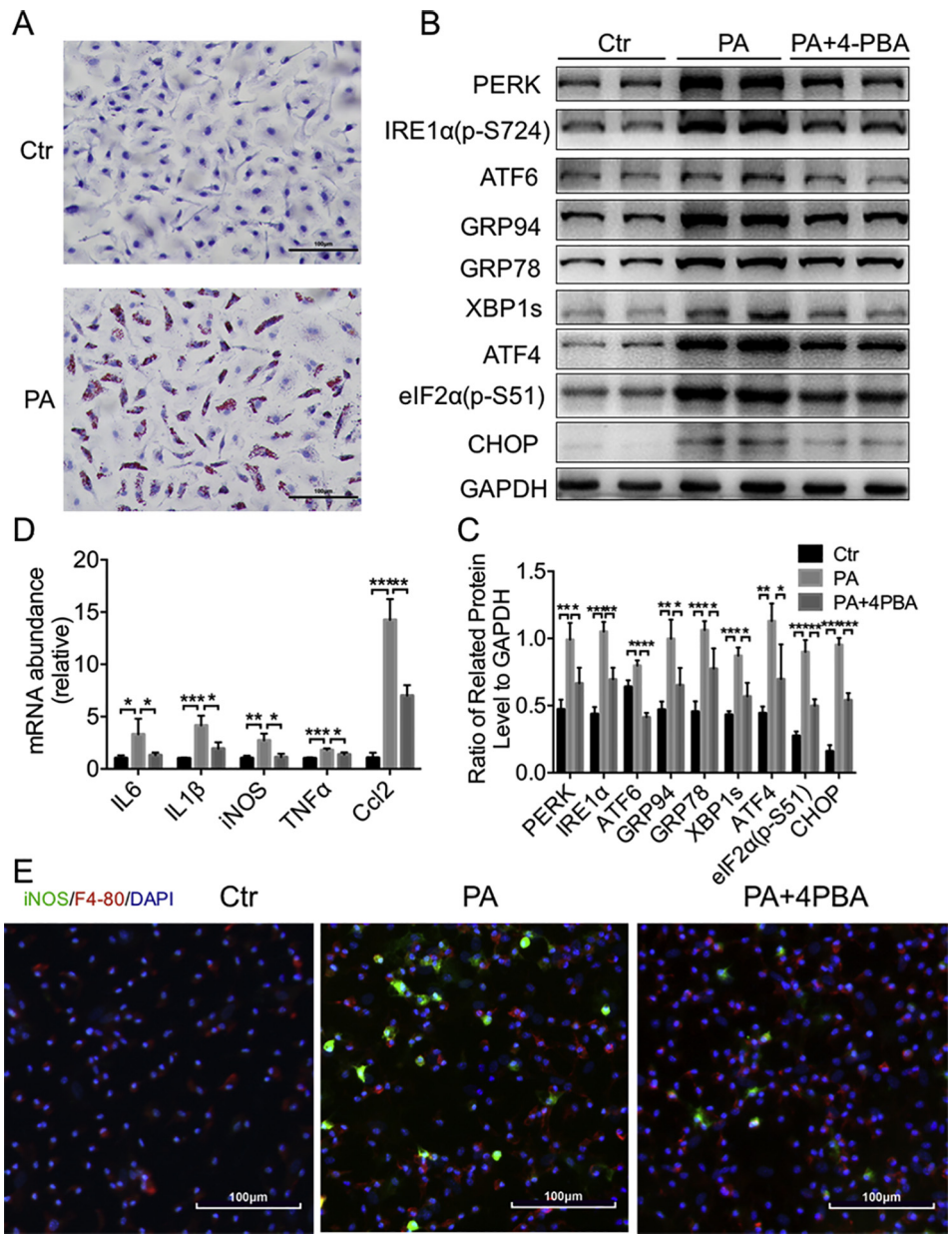

Fig. 1. Palmitic acid induced ER-stress of macrophages and M1 phenotypes polarization. A Representative Oil red staining of BMDMs treated with PA or PBS for 24 h. Scale bars, 100 μm. B,C Immunoblot analysis of ER stress markers IRE1α, PERK, ATF6, GRP78, GRP94, spliced XBP1, ATF4, eIF2α and CHOP treated with PA for 24 h with or without 4-PBA. Protein levels were normalized to GAPDH and analyzed. D qPCR analysis of macrophages inflammation genes treated with PA for 24 h with or without 4-PBA. (n = 4 per group) E Representative immunofluorescence staining of iNOS in macrophages. Scale bars, 100 μm. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

using a one-way ANOVA combined with Bonferroni's post hoc test. P-value < 0.05 was considered statistically significant.

3. Results

3.1. Palmitic acid induced ER-stress and M1 phenotypes polarization in macrophages.

Our previous study found that in fatty liver disease, ER-stress was activated in macrophages. Therefore, to further investigate the effect of fatty acid on ER-stress in macrophages, we extracted BMDMs and FACS was used to verify that the cells obtained from mice are predominantly BMDMs. (Supplement Fig. S1). Then BMDMs were stimulated with PA. After 24 h of stimulation, lipids accumulation was found in cytoplasm (Fig. 1A). As expected, ER-stress was activated in these macrophages, demonstrated by upregulation of IRE1α, ATF6, PERK, GRP78, GRP94 and other downstream proteins (Fig. 1B and C). In addition, C/EBP-homologous protein (CHOP) was also induced, suggesting that ER-stress related apoptosis was activated (Fig. 1B and C). We then detected the macrophage polarization through qPCR and found that the levels of M1-type markers, such as *IL6, IL1β, Ccl2, TNFα* and *iNOS,* were all increased after PA stimulation (Fig. 1D). Moreover, immunofluorescence staining of iNOS further horted the idea that ER-stress activation via PA could promote M1 phenotypic transformation of macrophages (Fig. 1E). To further test this idea, we then asked whether inhibiting ER-stress could reverse this process. 4-PBA, an ER-stress inhibitor, was applied after PA stimulation, and the ER-stress was indeed suppressed, demonstrated by the downregulation of all three membrane resident proteins (Fig. 1B and C). Consistently, the levels of M1-type markers were all inhibited (Fig. 1D), as well as the immunofluorescence staining of iNOS (Fig. 1E), suggesting that M1 phenotypic transformation was suppressed. Except for PA, we also treated BMDMs with oleic acid, but ER-stress was not activated under this treatment (Data not shown). In conclusion, PA activated ER-stress in macrophages and promoted its M1 phenotypic transformation.

3.2. PERK inhibitor suppressed M1 phenotypic transformation of macrophages.

The above results showed that ER-stress activation via PA promoted

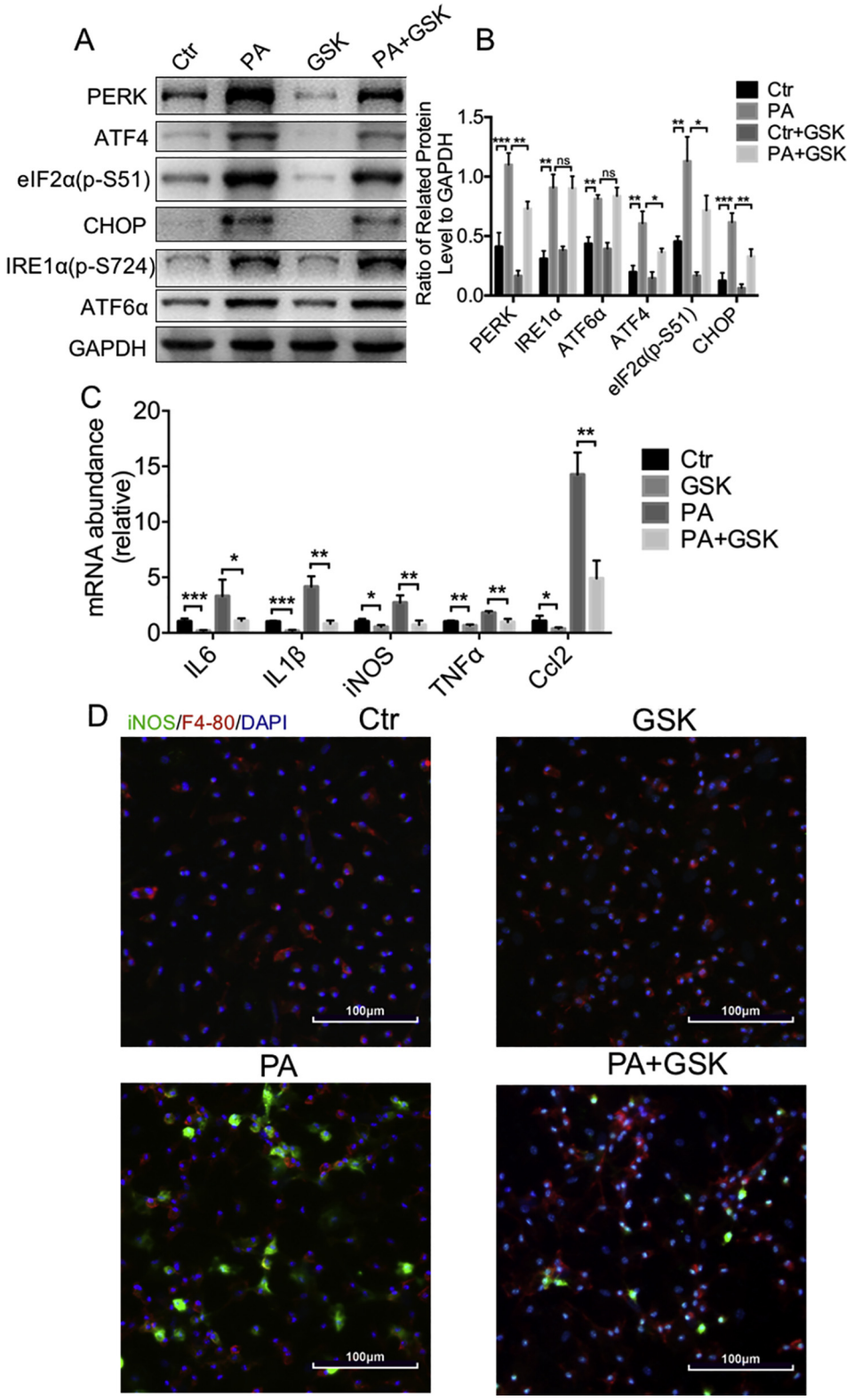

Fig. 2. PERK inhibitor suppressed M1 phenotypic transformation of macrophages induced by PA. A, B Immunoblot analysis of PERK, ATF4, eIF2α and CHOP treated with PA for 24 h with or without GSK2656157 (GSK). Protein levels were normalized to GAPDH and analyzed. C qPCR analysis of macrophages inflammation genes treated with PA for 24 h with or without GSK. (n = 4 per group) D Representative immunofluorescence staining of iNOS in macrophages. Scale bars, 100 μm. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

M1 phenotypic transformation of macrophages, and this process could be inhibited by 4-PBA. Next, we explored the underlying mechanisms of how ER-stress induces macrophage M1-transformation. Our previous study has demonstrated that IRE1α played an important role in regulating M1-M2 macrophage phenotypic transformation. However, whether PERK also participates in the phenotype transformation is still unclear. Therefore, to investigate the role of PERK in macrophage phenotypic transformation, GSK2656157, a PERK inhibitor, was

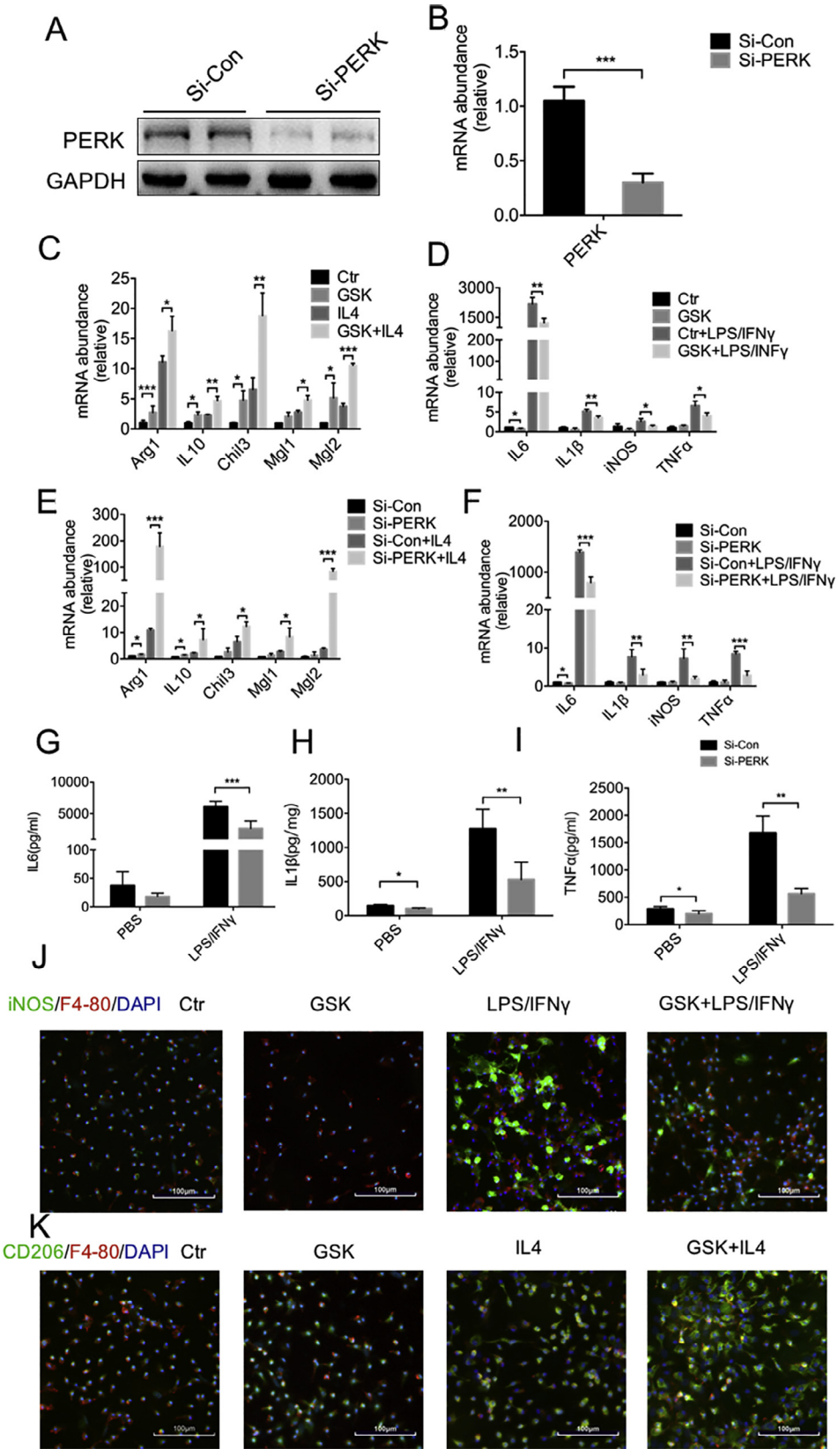

Fig. 3. PERK played an important role in governing M1-M2 macrophage phenotypic transformation. A, B Immunoblot and qPCR analysis of PERK with Si-Con or Si-PERK. (n = 3–4 per group) C, D BMDMs were cultured with LPS (100 ng/ml, Sigma-Aldrich, USA) and IFNγ (100 IU/m, PeproTech, USA) for 24 h or IL4 (20 ng/ml, PeproTech, USA) for 24 h to induce BMDMs phenotypic transformation. qPCR analysis of proinflammation and antiinflammation genes in BMDMs that treated with or without GSK after LPS/IFNγ or IL-4 stimulation. (n = 4–5 per group) E, F qPCR analysis of proinflammation and antiinflammation genes in BMDMs that si-PERK or si-Con with LPS/IFNγ or IL-4 stimulation. G-I IL6, IL1β and TNFα in cell supernatant were measured after stimulation with LPS/IFNγ for 6 h (n = 6 per group). J, K Representative immunofluorescence staining of iNOS and CD206 . Scale bars, 100 μm. Data are mean ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

applied on the stimulated BMDMs. As shown in Fig. 2A and B, GSK2656157 effectively inhibited PERK, as well as ATF4 and eIF2α, the downstream genes of PERK. The apoptosis related protein CHOP was also decreased. The levels of another two ER membrane resident proteins, IRE1α and ATF6, were not changed when treated with GSK2656157. We then tested the phenotype markers, and found that M1-type markers were significantly reduced (Fig. 2C). Similar result was found in immunofluorescence staining of iNOS (Fig. 2D). Altogether, these results showed that GSK2656157 suppressed the PA induced M1 phenotypic transformation of macrophages, indicating that

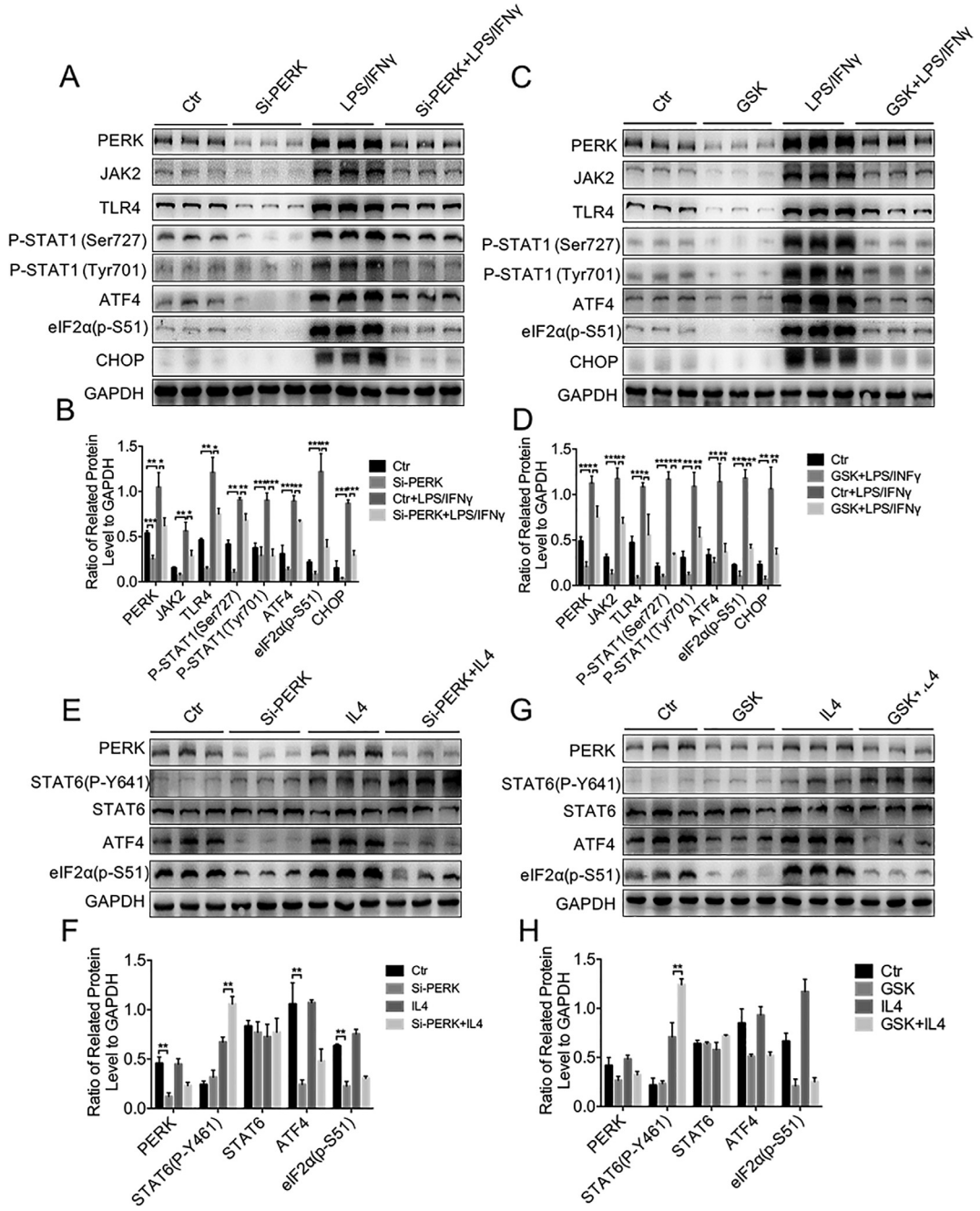

Fig. 4. PERK knockdown regulated STAT1 and STAT6 pathway to govern M1-M2 macrophage phenotypic transformation. A, B Immunoblot analysis of JAK2, STAT1 and TLR4 indicates that PERK knockdown can inhibit JAK2, STAT1 and TLR4 in the present of LPS/IFNγ. Protein levels were normalized to GAPDH and analyzed. C, D Immunoblot analysis of JAK2, STAT1, TLR4 with or without GSK stimulation in the present of LPS/IFNγ. Protein levels were normalized to GAPDH and analyzed. E, F Immunoblot analysis of STAT6 indicates PERK knockdown can upregulate the level of STAT6 (phospho Y641) in the present of IL4. Protein levels were normalized to GAPDH and analyzed. G, H Immunoblot analysis of STAT6 (phospho Y641) with or without GSK stimulation. Protein levels were normalized to GAPDH and analyzed. Data are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001.

PERK may play an important role in regulating macrophage phenotypic transformation.

3.3. PERK played an important role in regulating M1-M2 macrophage phenotypic transformation.

To further verify the role of PERK in regulating M1-M2 macrophage phenotypic transformation, we used siRNA to knock down PERK in BMDMs. The levels of PERK protein and mRNA were both significantly reduced by siRNA (Fig. 3A and B). Since lipopolysaccharide (LPS)/IFNγ and IL-4 were established methods to induce macrophage polarization [16], we used LPS/IFNγ and IL-4 to stimulate BMDMs in order to mimic M1 and M2 polarization. M1-markers (*IL6, IL1β, TNFα, NOS2*) were increased following LPS/IFNγ stimulation, and M2-markers (*IL10, Arg1, Chil3, Mgl1, Mgl2*) were increased with IL4 stimulation (Fig. 3C–F). When BMDMs were treated with PERK siRNA, the expression of M1-markers was reduced and the expression of M2-markers was elevated (Fig. 3E and F). Consistently, the BMDMs pretreated with GSK2656157 also exhibited reduced pro-inflammatory transformation and enhanced anti-inflammatory transformation under LPS/IFNγ or IL4 stimulations (Fig. 3C and D). From the BMDMs culture medium supernatant, we found the levels of TNFα, IL6 and IL1β were decreased after LPS/IFNγ

stimulation when PERK was knocked down (Fig. 3G–I), and the immunofluorescence staining of iNOS showed consistent results (Fig. 3J and K). In summary, PERK played an important role in regulating the macrophage phenotypic transformation induced by LPS/IFNγ and IL-4.

3.4. PERK regulated STAT1 and STAT6 pathway to govern M1-M2 macrophage phenotypic transformation.

We next explored the mechanisms by which PERK regulated macrophages phenotypic transformation. It has been shown that STAT1 is associated with M1 phenotypic polarization and STAT6 is related to M2 phenotype transformation [17,18]. Moreover, IRE1α could regulate both STAT1 and STAT6 to govern M1-M2 macrophage phenotypic transformation. Thus, we decided to examine whether PERK also regulates macrophage phenotypic transformation through STAT1 and STAT6, just like IRE1α. As shown in Fig. 4A–D, STAT1 was activated after LPS/IFNγ stimulation, as well as JAK2 and TLR4, which were components in STAT1 signaling pathway. STAT6 was activated upon IL4 treatment (Fig. 4E–H). However, when PERK was knocked down, the levels of activated STAT1 was significantly reduced while STAT6 was upregulated (Fig. 4A, B, E, F). Consistently, JAK2 and TLR4 were also inhibited by PERK knockdown, and CHOP, a gene that participates in M1-phenotypic transformation [19], was suppressed by PERK knockdown as well. We also used GSK2656157 to inhibit PERK and found similar effects (Fig. 4C, D, G, H). In conclusion, PERK regulates M1-M2 phenotypic transformation of macrophages through the STAT1 and STAT6 signaling pathways.

4. Discussion

Macrophage is the major component of innate immune cells. It responds to acute infection as phagocytes and secrets inflammatory cytokines [20]. Interestingly, macrophages can display different phenotypes in different microenvironments, such as pro-inflammatory type (M1) and anti-inflammatory type (M2). The accumulation of M1-type macrophages has been shown to contribute to inflammation and ischemia reperfusion injury, while the M2-type macrophages can promote allergy, fibrosis and cancer [21,22]. The pro-inflammatory macrophages (M1-type) can secrete inflammatory cytokines and chemotactic factors such as IL6, IL1β, TNFα, CCl2, which participate in the development of various diseases. Recent studies indicated that the M1 phenotype polarization of macrophages play an important role in the pathological progression of NAFLD and the ischemia reperfusion injury of solid organs [23,24]. Therefore, targeting the pathways regulating macrophage phenotype polarization represents a novel therapeutic direction.

Our previous study found that ER-stress was activated in macrophages during the the pathological progression of NAFLD. In this study, we used PA to stimulate macrophages in order to mimic the context of fatty liver. We found that PA stimulation could activate ER-stress in macrophages, which is consistent with our previous results. The PERK-eIF2α-ATF4 pathway, one of the three ER-stress signaling transducers, has been shown to regulate lipogenesis and hepatic steatosis [25]. Recent report suggested that activation of PERK-eIF2α pathway via antipsychotic drugs (APDs) could increase the intracellular lipid accumulation through SREBP-1c and SREBP-2 [26]. ATF4 is also involved in regulating cell death by inducing the apoptosis related transcription factor CHOP [27]. Previous studies have found that ATF6 deletion could reduce the production of pro-inflammatory cytokines from ER-stressed macrophages, and thereby inhibit IRI [15]. Also, IRE1α was found to play an important role in macrophage phenotypic transformation. However, whether PERK is involved in macrophage phenotypic transformation is still unknown. Therefore, in this study, we investigated the role of PERK-eIF2α-ATF4 in macrophage phenotypic shift. We found that the PA induced ER-stress in macrophages promoted M1-phenotypic shift, and this shift could be reversed by PERK inhibitor GSK2656157. Moreover, knockdown of PERK in macrophages could inhibit the M1-type polarization induced by LPS/IFNγ and promote the M2-phenotypic shift induced by IL4. Therefore, PERK played an important role in regulating M1-M2 macrophage phenotypic transformation.

It has been reported that STAT1 is associated with M1 polarization in macrophages and STAT6 is related to M2 polarization. Also, LPS/IFNγ and IL4 are closely connected to these two phenotypic changes of macrophages. LPS binds to the Toll-like receptor 4 (TLR4) on macrophage surface and IFNγ binds to the IFNγ receptor associated with Janus kinases (Jaks), which then activate STAT1 and promote M1 macrophage polarization [28,29]. IL-4 binds to the IL-4 receptor and activates Jak-STAT6 signaling, which then promotes M2 macrophage polarization [30]. Consistent with these findings, in our analysis, we found that PERK knockdown could significantly inhibit the STAT1 activation induced by LPS and enhance the STAT6 activation stimulated by IL4. Thus, PERK might regulate macrophage phenotypic transformation via affecting STAT1 and STAT6 pathways.

In summary, our study revealed a new mechanism by which ER-stress activation led to phenotypic transformation of macrophages. What's more, it may provide us with a potential new way to treat disease induced by M1-type macrophages.

Acknowledgment

This project was supported by grants from the National Natural Science Foundation of China (NSFC) (Grant Nos. 81670561 and 81670566), Nanjing Medical Science and Technology Development Key Project (Grant No. ZKX14025), Fundamental Research Funds for the Central Universities (Grant No. 021414380360).

Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cellimm.2018.12.008.

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