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Original Article

Angiopoietin-like 4 deficiency upregulates macrophage function through the dysregulation of cell-intrinsic fatty acid metabolism

Shizhen Ding[1,3*], Dandan Wu[1,3*], Quotao Lu[2], Li Qian[1,3], Yanbing Ding[2], George Liu[4], Xiaoqin Jia[3,5], Yu Zhang[3,5], Weiming Xiao[2,3,5], Weijuan Gong[1,2,3,5,6]

[1]Department of Immunology, School of Medicine, Yangzhou University, Yangzhou 225001, P. R. China; [2]Department of Gastroenterology, Affiliated Hospital, Yangzhou University, Yangzhou 225001, P. R. China; [3]Jiangsu Key Laboratory of Integrated Traditional Chinese and Western Medicine for Prevention and Treatment of Senile Diseases, Yangzhou 225001, P. R. China; [4]Key Laboratory of Molecular Cardiovascular Science of Ministry of Education, Institute of Cardiovascular Science, Peking University, Beijing 100191, P. R. China; [5]Jiangsu Key Laboratory of Zoonosis, Yangzhou 225001, P. R. China; [6]Jiangsu Co-innovation Center for Prevention and Control of Important Animal Infectious Diseases and Zoonoses, Yangzhou 225001, P. R. China. [*]Co-first authors.

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Abstract: Angiopoietin-like 4 (ANGPLT4) regulates lipid metabolism by inhibiting lipoprotein lipase. Abnormal ANGTPL4 levels are associated with metabolic syndrome, atherosclerosis, inflammation, and cancer. We show here that ANGPTL4-deficient mice have abnormally large numbers of macrophages in the spleen, and that these macrophages produce large amounts of TNF-α, CD86, and inducible nitric oxide synthase. However, recombinant ANGPTL4 protein did not inhibit macrophage function *ex vivo*. Glycolysis and fatty-acid synthesis were upregulated in ANGPTL4$^{-/-}$ macrophages, whereas fatty-acid oxidation was decreased. Elevated levels of free fatty acids in the cytoplasm of ANGPTL4$^{-/-}$ macrophages were confirmed. ANGPTL4$^{-/-}$ macrophages also displayed endoplasmic reticulum (ER) stress after stimulation with LPS. Protein kinase B (Akt)/mammalian target of rapamycin (mTOR) signaling was activated, but no major change in liver kinase B1 (LKB1)/adenosine 5'-monophosphate (AMP)- activated protein kinase (AMPK) phosphorylation was observed in ANGPTL4$^{-/-}$ macrophages. The modulation of fatty-acid metabolism prevented ER stress and the expression of inflammatory molecules, but the activation of ANGPTL4$^{-/-}$ macrophages was not restored by the inhibition of glycolysis. Thus, ANGPTL4 deficiency in macrophages results in ER stress due to the cell-intrinsic reprogramming of fatty-acid metabolism. Intracellular ANGPLT4 expression could thus be manipulated to modulate macrophage function.

Keywords: ANGPTL4, deficiency, macrophage, fatty acid, metabolism

Introduction

Angiopoietin-like 4 (ANGPTL4) is a member of the angiopoietin family with both metabolic and non-metabolic functions. It strongly inhibits lipoprotein lipase (LPL), an enzyme catalyzing triglyceride hydrolysis and regulating the uptake of circulating lipids into tissues [1, 2]. ANGPTL4-transgenic mice display hypertriglyceridemia [3], whereas circulating lipid levels are low in ANGPTL4 knockout mice [4]. ANGPTL4 has been detected in human adipose tissues, macrophages, placenta, liver, kidney and heart, and its expression is regulated by fasting, fatty acids, peroxisome proliferator-activated receptors (PPARs), glucocorticoids, and hypoxia [5, 6].

ANGPTL4 has been implicated in inflammation and cancer, including atherosclerosis [7], rheumatoid arthritis (RA) [8], influenza virus-induced lung injury [9], chronic obstructive pulmonary disease [10], diabetes [11], diabetic retinopathy [12], Alzheimer's disease [13], colorectal cancer, and hepatic cancer [2]. In *apoe*$^{-/-}$ mice, ANGPTL4 deficiency is associated with better lipid metabolism [14], the inhibition of foam cell formation and an alleviation of atherosclerosis.

Paradoxically, ANGPTL4$^{-/-}$ mice fed a high-fat diet develop fibrinopurulent peritonitis and ascites [15]. Furthermore, in *ldlr*$^{-/-}$ mice, global ANGPTL4 deficiency attenuates atherosclerosis progression, but hematopoietic ANGPTL4 deficiency promotes atherosclerosis [16]. ANGPTL4 overexpression prevents atherosclerosis progression [17]. In addition, collective data show that ANGPL4 promotes vascularisation, invasion and metastasis under hypoxic conditions in tumours. More detailed studies are therefore required to determine the role actually played by ANGTPL4 in metabolic syndrome, inflammation, and cancer.

ANGPTL4 expression levels are lower in activated macrophages [18]. ANGPTL4 deficiency in *ldlr*$^{-/-}$ mice not only promotes the expansion and survival of common myeloid progenitors (CMPs), but also enhances macrophage foam cell formation and polarizes macrophages towards an M1 inflammatory phenotype in response to cholesterol loading [16]. M1 polarization of macrophages is dependent on glycolysis, whereas M2 differentiation requires fatty-acid oxidation (FAO) [19]. Given the metabolic role of ANGTPL4, we investigated whether an absence of ANGPTL4 affected the intracellular glycolysis and fatty acid oxidation of macrophages and the effects of metabolic changes on macrophage function.

Materials and methods

Mice and reagents

ANGPTL4$^{-/-}$ mice (C57BL6 background) were purchased from the Mutant Mouse Resource and Research Center (MMRRC) of the National Institutes of Health (NIH). All mice were reared in the Comparative Medical Center of Yangzhou University and killed at the age of eight to 12 weeks. All animal experiments were approved by the Institutional Animal Care and Use Committee of Yangzhou University (Yangzhou, China). The following reagents were used: LPS (Sigma-Aldrich, St. Louis, US), 2-deoxy-D-glucose (Sigma-Aldrich), metformin, rapamycin, C75 (MedChemExpress, Monmouth Junction, USA), CP-641086 (AbMole, Boston, MA, USA), and recombinant ANGPTL4 (USACLOUD-CLONE, Hubei, China).

Anti-mouse antibodies for flow cytometry were obtained from BioLegend (San Diego, CA): F4/80 (BM8), CD16/32 (93), CD86 (GL-1), TNF-α (Mp6-XT22), CD4 (Rm4-5) and IFN-γ (XMG1.2). Antibodies for western blot analysis were obtained from Cell Signaling Technology (Boston, MA, USA): iNOS (D6B6S), p-ACC1 (Ser79) (D7D11), mTOR (7C10), p-mTOR (Ser2448) (D9C2), Akt (C67E7), p-Akt (S473) (D9E), LKB1 (D60C5), p-LKB1 (S428) (C67A3), AMPK (T172) (D63G4), p-AMPK (D4D6D), NF-κB p65 (D14E12), NF-κB p-p65 (C536) (93H1), p44/42 MAPK (Erk1/2) (137F5), p-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E), p-SAPK/JNK (Thr183/Tyr185) (81E11) , p-elf2a (S51) (D9G8), and β-actin (3H6G5). The following antibodies were obtained from Abcam (Cambridge, UK): Glut1 (D3J3A), CPT1 (EPR218-43-71-2F), LPL (LPL.A4).

Flow cytometry

For the detection of intracellular cytokines, macrophages were cultured with the cell stimulation protein cocktail and the protein transport inhibitor cocktail from eBioscience (San Diego, CA, USA) for five hours. Cells were harvested, fixed and permeabilized with intracellular fixation buffer and permeabilization buffer, and were then stained with the appropriate antibodies and analyzed by flow cytometry. For the measurement of mitochondrial mass, macrophages were incubated at 37°C for 30 min with 100 nM MitoTracker green dye (Beyotime Biotechnology, China). Macrophages were loaded with 10 μM dichlorofluorescein diacetate (DCFH-DA) (Beyotime Biotechnology, China) for 20 min at 37°C in the dark, for the determination of intracellular ROS levels.

ELISA

Mouse ELISA (JYM0949MO 96T) kits for IL-10 and TNF-α were acquired from Bioswamp Biological Technology (Wuhan, China). The supernatants of ANGPTL4$^{-/-}$ and WT macrophages were collected after culture for 24 hours *ex vivo*. OD_{450} was determined, and the levels of IL-10 or TNF-α in the macrophage supernatants were then determined from a standard curve.

Western blot

Total cell protein was isolated after cell lysis in lysis buffer. The proteins were then separated by electrophoresis and transferred to polyvinyli-

dene fluoride membranes. The membranes were successively incubated with the corresponding primary antibodies at 4°C overnight and with horseradish peroxidase-conjugated secondary antibodies at room temperature for 1 h. The immunoreactive bands were routinely detected by enhanced chemiluminescence. Image-Pro Plus 6.0 software was used for gray-scale analysis.

Analysis of metabolism

Macrophages were treated with LPS and used to seed XF96-well plates (Agilent) at a density of 1.5×10^5 cells/well. The cells were centrifuged at 200×*g* for 1 minute. Plates were transferred to a CO_2-free incubator at 37°C for 25-30 minutes to ensure complete adhesion of the cells. The plates were then washed and incubated in Seahorse medium (Agilent) supplemented with 10 mM glucose, 2 mM glutamine, 1 mM pyruvate (Sigma). We used the Mitostress kit (Agilent) according to the manufacturer's instructions for the assessment of mitochondrial respiratory activity. We loaded the injection ports with 1 μM oligomycin, 2 μM FCCP, and 0.5 μM rotenone/antimycin A. For the assessment of glycolytic activities, cells were treated with 10 mM glucose, 1 μM oligomycin and 100 mM 2-DG. Measurements were made in basal conditions with an XF96 Extracellular Flux Analyzer (Agilent) and the results were processed with Wave v2.2.0 software.

Real-time PCR

Total RNA of macrophages was isolated with TRIzol reagent (Life Technologies; Carlsbad, CA, USA) and used for cDNA synthesis with the QuantiTect® reverse transcription kit (QIAGEN GmbH; Hilden, Germany). Quantitative PCR was performed on the cDNA with the QuaniNova™ SYBR® Green PCR kit (QIAGEN) on an ABI 7500 machine (PE Applied Biosystems, Carlsbad, CA, USA). The primers for ATF3, CHOP and GAPDH were designed with Premier 5.0 software. The sequences of the primer pairs were as follows: 5'-AAATTGCTGCTGCCAAGTGTCG-3' and 5'-GGTGTCCGTCCATTCTGAGCC-3' for *ATF3*; 5'-TTGCCCTCTTATTGGTCCAGC-3' and 5'-TAGCGACTGTTCTGTTCCCAC-3' for *CHOP*; 5'-CAAAATGGTGAAGGTCGGTGTG-3' and 5'-TGATGTTAGTGGGGTCTCGCTC-3' for *GAPDH*. Relative RNA levels were calculated by the $2^{-\Delta\Delta Ct}$ method after the normalization of *ATF3 and CHOP* mRNA levels against *GAPDH* mRNA.

Statistical analysis

Continuous variables are presented as means ± SD. Differences between groups were analyzed in unpaired, two-tailed Student's *t* tests. Data were analyzed by one-way ANOVA, followed by Dunnett's test, to compare the control and multiple treatment groups. Statistical significance is indicated as follows: *, $P<0.05$; **, $P<0.01$; and ***, $P<0.001$.

Results

Macrophage function is upregulated in ANGPTL4$^{-/-}$ mice

We first compared the frequencies of splenic macrophages between ANGPTL4-deficient mice and normal controls in physiological conditions. F4/80$^+$ and F4/80$^+$ CD16/32$^+$ (M1 macrophage) cell frequencies were very high in ANGPTL4$^{-/-}$ mice (**Figure 1A** and **1B**). Spleen macrophages were sorted and cultured *ex vivo*. TNF-α levels were higher in the supernatants of ANGPTL4$^{-/-}$ macrophages than in wild-type macrophage supernatants (WT), whereas IL-10 was present at similar levels in both ANGPTL4$^{-/-}$ and WT macrophage supernatants (**Figure 1C**). ANGPTL4$^{-/-}$ macrophages secreted more TNF-α than wild-type macrophages following LPS stimulation, as confirmed by flow cytometry (**Figure 1D**). CD86 expression was also higher on ANGPTL4$^{-/-}$ mouse macrophages than on WT macrophages, both before and after LPS stimulation (**Figure 1E**). Inducible nitric oxide synthase (iNOS), the typical effector molecule of M1 macrophages, was produced in larger amounts by ANGPTL4$^{-/-}$ macrophages than by wild-type macrophages (**Figure 1F**). Finally, we analyzed the stimulation of CD4$^+$ T-cell activation by macrophages from KO and WT mice. In both the presence and absence of LPS stimulation, the amount of IFN-γ secreted by CD4$^+$ T cells was greater after coculture with ANGPTL4$^{-/-}$ macrophages than after coculture with WT macrophages (**Figure 1G** and **1H**). These observations suggest that ANGPLT4-deficient mice had M1 macrophages and that their function was upregulated.

Exogenous ANGPTL4 has no effect on macrophage function ex vivo

Based on the upregulation of macrophage function in mice with ANGPTL4 deficiency, we investigated whether exogenous ANGPTL4

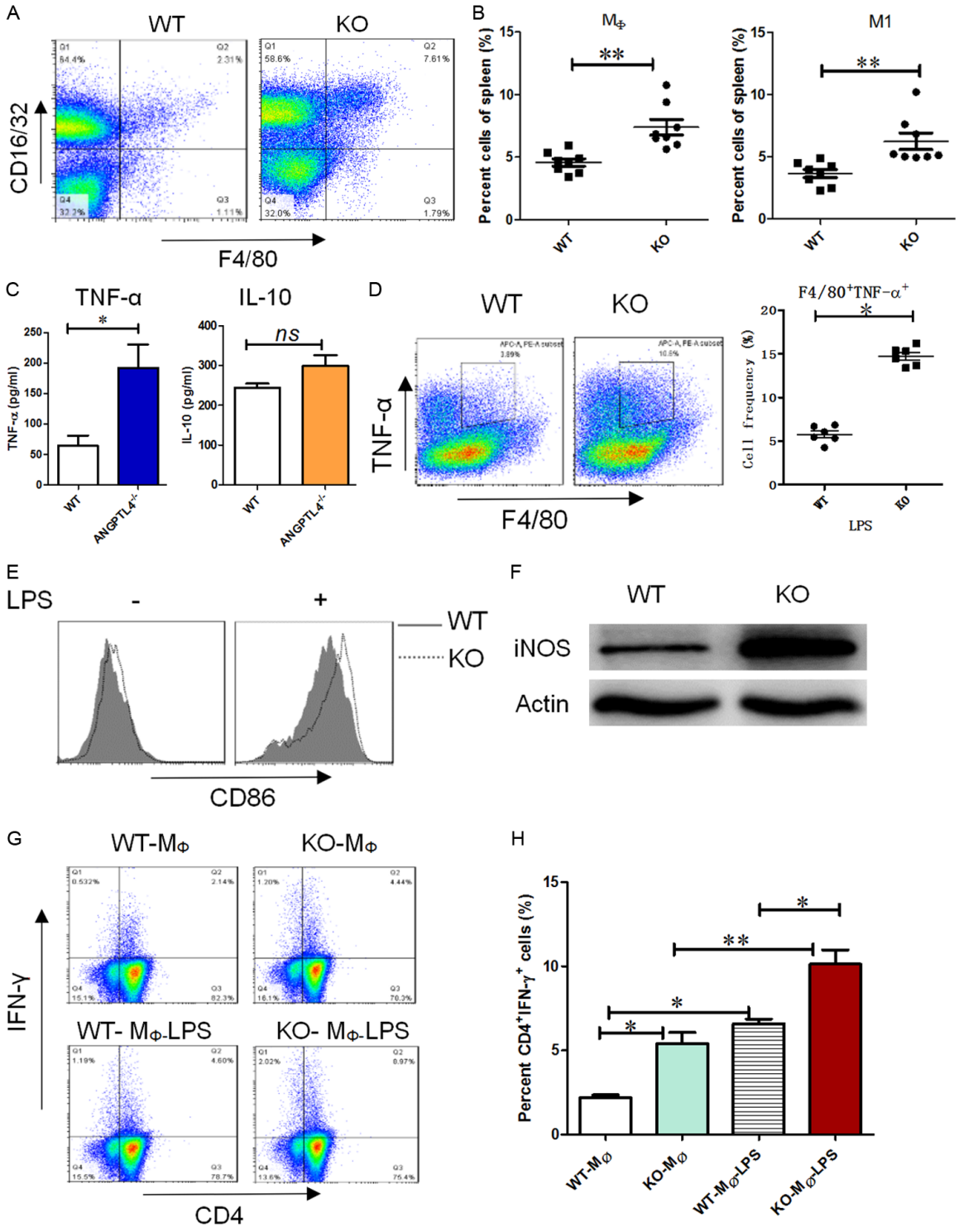


Figure 1. Upregulation of the frequency and function of macrophages in ANGPTL4$^{-/-}$ mice. (A) F4/80^{+} CD16/32^{+} cells detected by flow cytometry. (B) Splenic M_{ϕ} and M1 cell frequencies. (C) Secretion of TNF-α and IL-10 by macrophages, detected by ELISA. (D) TNF-α production determined by intracellular staining and flow cytometry. (E) CD86 expression before and after LPS stimulation. (F) Production of iNOS by macrophages, as assessed by western blotting. (G), IFN-γ secretion by CD4^{+} T cells in response to macrophage stimulation, and (H), statistical analysis. All experiments were carried out at least three times. *, $P<0.05$; **, $P<0.01$; *ns*, not significant.

modulated macrophage activity. The splenic $F4/80^+$ $CD16/32^+$ cell frequency of WT mice was not markedly changed by treatment with recombinant ANGPTL4 (rANGPTL4, 1 μg/ml) (**Figure 2A**). We also observed no major change in the TNF-α production and CD86 expression of WT macrophages following stimulation with rANGPTL4 (**Figure 2B** and **2C**). The macrophages of ANGPTL4$^{-/-}$ mice displayed no marked change in TNF-α production (**Figure 2D**) and CD86 expression (**Figure 2E**) in response to treatment with various doses of recombinant ANGPTL4. The IFN-γ secretion of $CD4^+$ T cells was also largely unmodified by co-culture with rANGPTL4-treated macrophages (**Figure 2F** and **2G**). Thus, exogenous ANGTPL4 had no effect on the expression of TNF-α and CD86 by normal macrophages, and the addition of rANGPTL4 did not downregulate ANGTPL4$^{-/-}$ macrophage function. Thus, the upregulation of ANGTPL4$^{-/-}$ macrophages was due to intracellular alterations.

Upregulated glycolysis in ANGPTL4$^{-/-}$ macrophages

ANGPTL4 modulates cell metabolism. We therefore analyzed glucose uptake and glycolysis in macrophages. In the presence and absence of LPS stimulation, ANGTPL4$^{-/-}$ macrophages displayed markedly higher levels of activity than wild-type macrophages, for both glucose transporter 1 (Glut1) and the rate-limiting enzyme in glycolysis, hexokinase II (HK II) (**Figure 3A** and **3B**). Similarly, the extracellular acidification rate (EACR), which reflects glycolysis activity, was higher in the macrophages of ANGTPL4$^{-/-}$ mice than in wild-type macrophages (**Figure 3C**). However, the treatment of macrophages with various doses of 2-deoxy-D-glucose (2-DG), had no marked effect on either TNF-α, IL-1β, or CD86 levels, although their levels were higher in ANGTPL4$^{-/-}$ macrophages than in wild-type macrophages (**Figure 3D-G**). It could be inferred that the enhancement of glycolysis in ANGTPL4$^{-/-}$ macrophages was not the cause of macrophage activities demonstrated by TNF-α, IL-1β, or CD86 expression, but as a result from activation.

Changes to the fatty-acid metabolism of ANGPTL4$^{-/-}$ macrophages

We then analyzed fatty-acid oxidation (FAO) and synthesis (FAS) in ANGTPL4$^{-/-}$ macrophages. The rate of FAO is highly dependent on the entry of acyl groups into the mitochondria, and thus on the activity of carnitine palmitoyltransferase-1 (CPT1). CPT1 staining was much weaker in ANGTPL4$^{-/-}$ macrophages than in WT macrophages in physiological conditions (**Figure 4A** and **4B**). Acetyl-coA carboxylase-1 (ACC1) is present in cell cytosol and is the rate-limiting enzyme in fatty-acid synthesis. Phosphorylated ACC1 levels were markedly higher in ANGTPL4$^{-/-}$ macrophages than in wild-type macrophages, particularly after LPS stimulation (**Figure 4A** and **4B**). Sterol regulatory element binding protein-1 (SREBP1) regulates several target genes related to the synthesis of fatty acids from acetyl-CoA ANGTPL4$^{-/-}$ macrophages had higher levels of SREBP1 than wild-type macrophages, especially before LPS stimulation (**Figure 4C** and **4D**). Thus, FAS was upregulated, whereas FAO was downregulated, in ANGTPL4$^{-/-}$ macrophages. Similarly, LPL, the substrate of ANGPTL4, was present at lower levels in ANGPTL4$^{-/-}$ macrophages, consistent with a lower level of free fatty acids in the cytoplasm in physiological conditions (**Figure 4C** and **4D**).

Levels of CD36, a fatty-acid receptor, were not affected by LPS stimulation, suggesting that exogenous fatty-acid uptake is unaffected in ANGTPL4$^{-/-}$ macrophages (**Figure 4E**). Oxygen consumption rates (OCR) were also determined for the analysis of oxidative phosphorylation (OXPHOS) in macrophages. No marked differences in basal respiration (OCR before oligomycin) and ATP production (OCR after oligomycin) were observed between the two kinds of macrophages, but ANGTPL4$^{-/-}$ macrophages had a lower maximal respiration rate (OCR after FCCP) and spare respiratory capacity (OCR after antimycin A and rotenone treatment) (**Figure 4F**), indicating that OXPHOS levels were lower in ANGTPL4$^{-/-}$ macrophages.

Mitochondrial mass and reactive oxygen species (ROS) levels were higher in ANGTPL4$^{-/-}$ macrophages than in the macrophages of WT mice, both before and after LPS stimulation (**Figure 4G** and **4H**). Higher levels of ROS production were therefore associated with the inflammatory function of ANGTPL4$^{-/-}$ macrophages. Cytoplasmic free fatty acid levels were increased in ANGTPL4$^{-/-}$ macrophages before and after the LPS stimulation (**Figure 4I**). We then investigated whether the alterations to fatty-acid metabolism in ANGTPL4$^{-/-}$ macro-

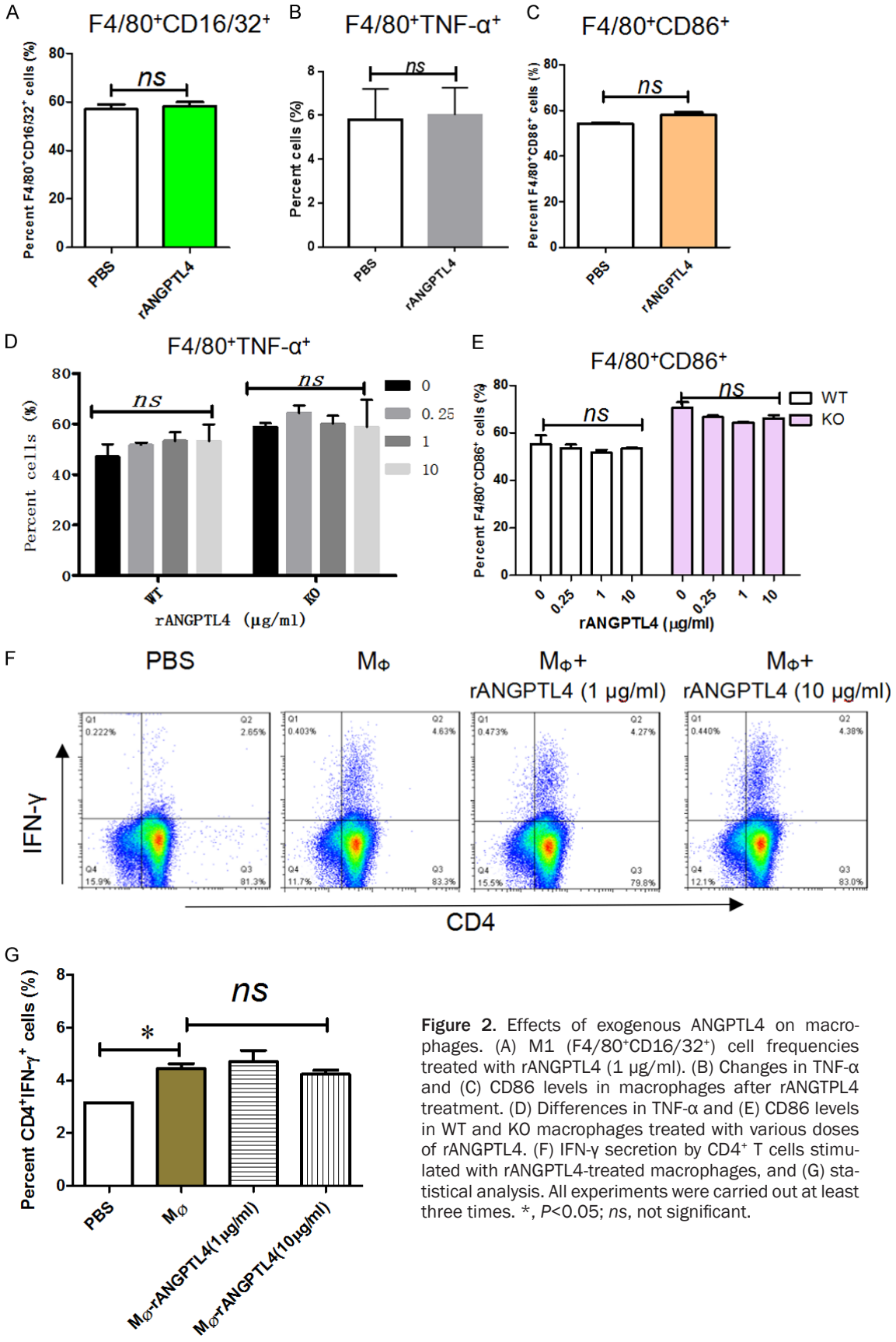
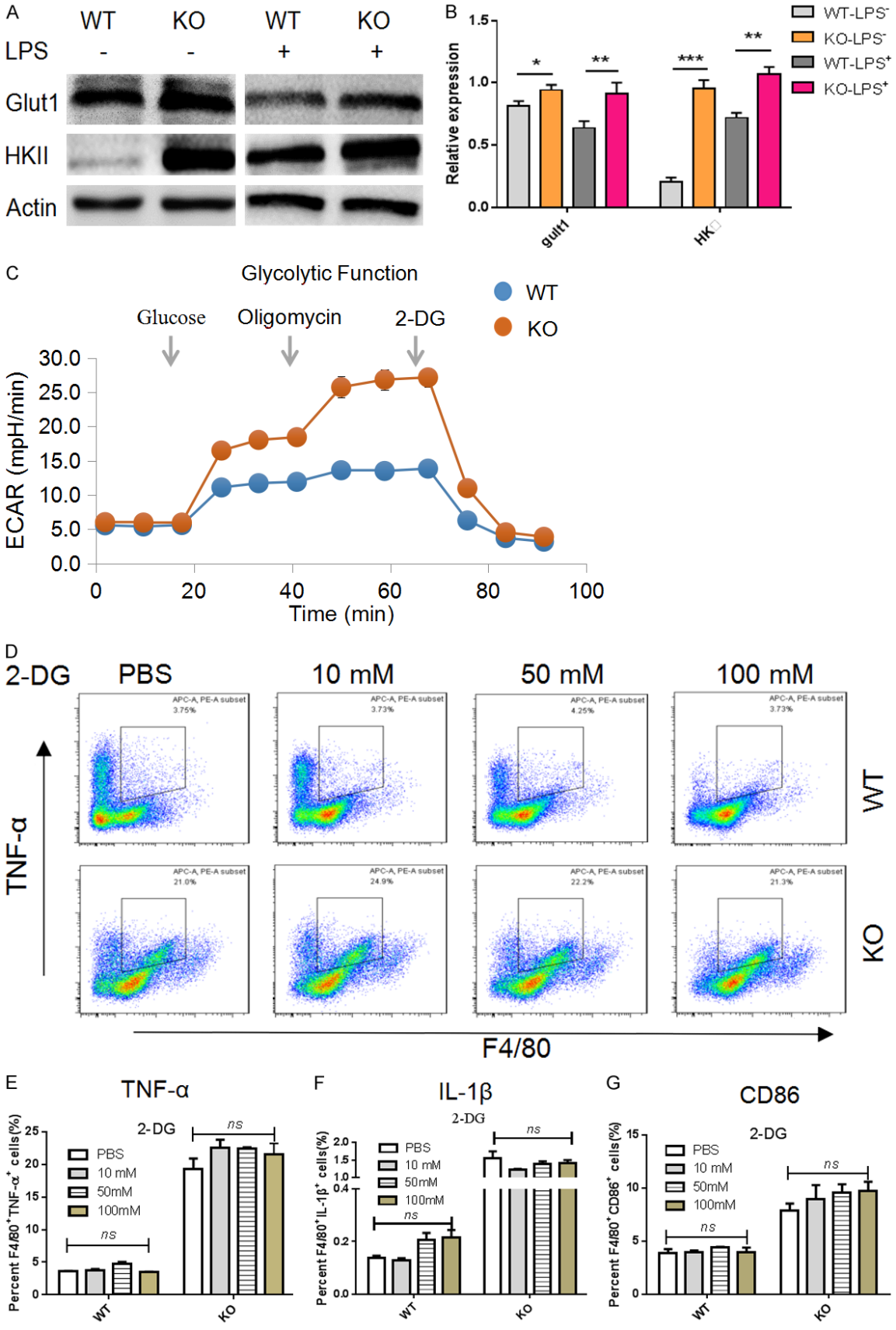


Figure 2. Effects of exogenous ANGPTL4 on macrophages. (A) M1 ($F4/80^+CD16/32^+$) cell frequencies treated with rANGPTL4 (1 μg/ml). (B) Changes in TNF-α and (C) CD86 levels in macrophages after rANGTPL4 treatment. (D) Differences in TNF-α and (E) CD86 levels in WT and KO macrophages treated with various doses of rANGPTL4. (F) IFN-γ secretion by $CD4^+$ T cells stimulated with rANGPTL4-treated macrophages, and (G) statistical analysis. All experiments were carried out at least three times. *, $P<0.05$; *ns*, not significant.


A
WT
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Glut1
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WT-LPS-
KO-LPS-
WT-LPS+
KO-LPS+
glut1
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Glycolytic Function
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2-DG
WT
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ECAR (mpH/min)
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Time (min)
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WT
KO
F
IL-1β
2-DG
Percent F4/80+IL-1β+ cells(%)
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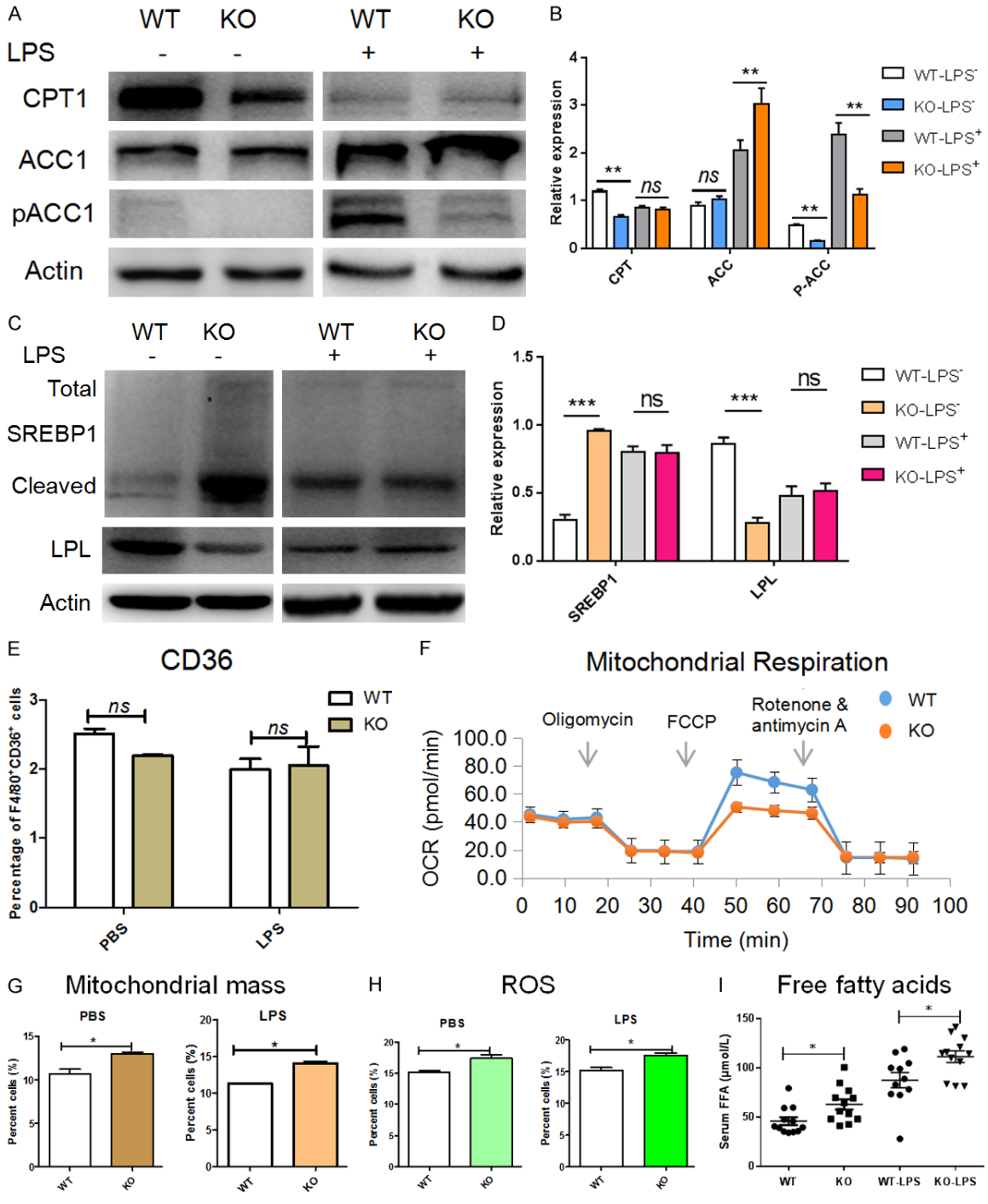
CD86
2-DG
Percent F4/80+CD86+ cells(%)

Figure 3. Glycolysis in ANGPTL4$^{-/-}$ macrophages. (A) GLUT1 and HK II levels in macrophages, as assessed by western blotting, and (B) statistical analysis. (C) Extracellular acidification rate (ECAR) of macrophages. (D) TNF-α production by macrophages treated with 2-DG, and (E) statistical analysis. (F) IL-1β and (G) CD86 expression of macrophages treated with 2-DG. All experiments were performed three times. *, P<0.05; **, P<0.01; ***, P<0.001; *ns*, not significant.



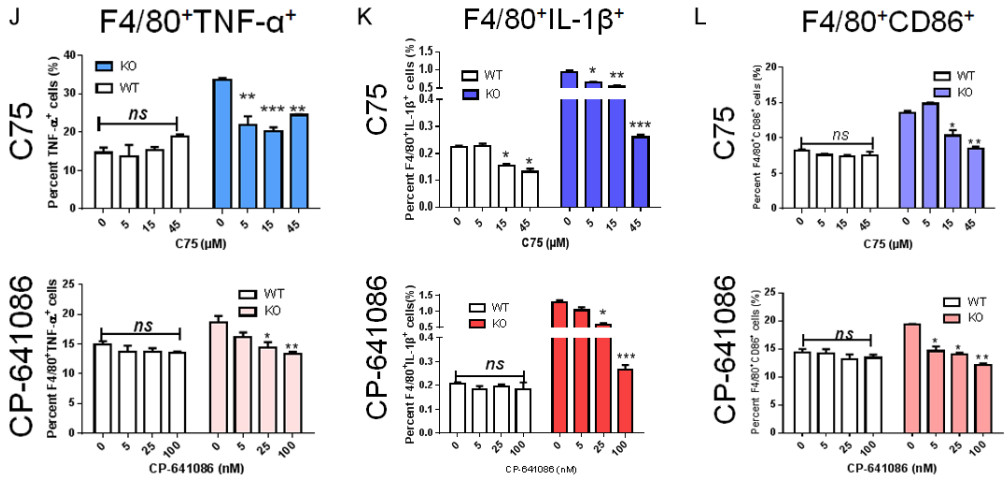
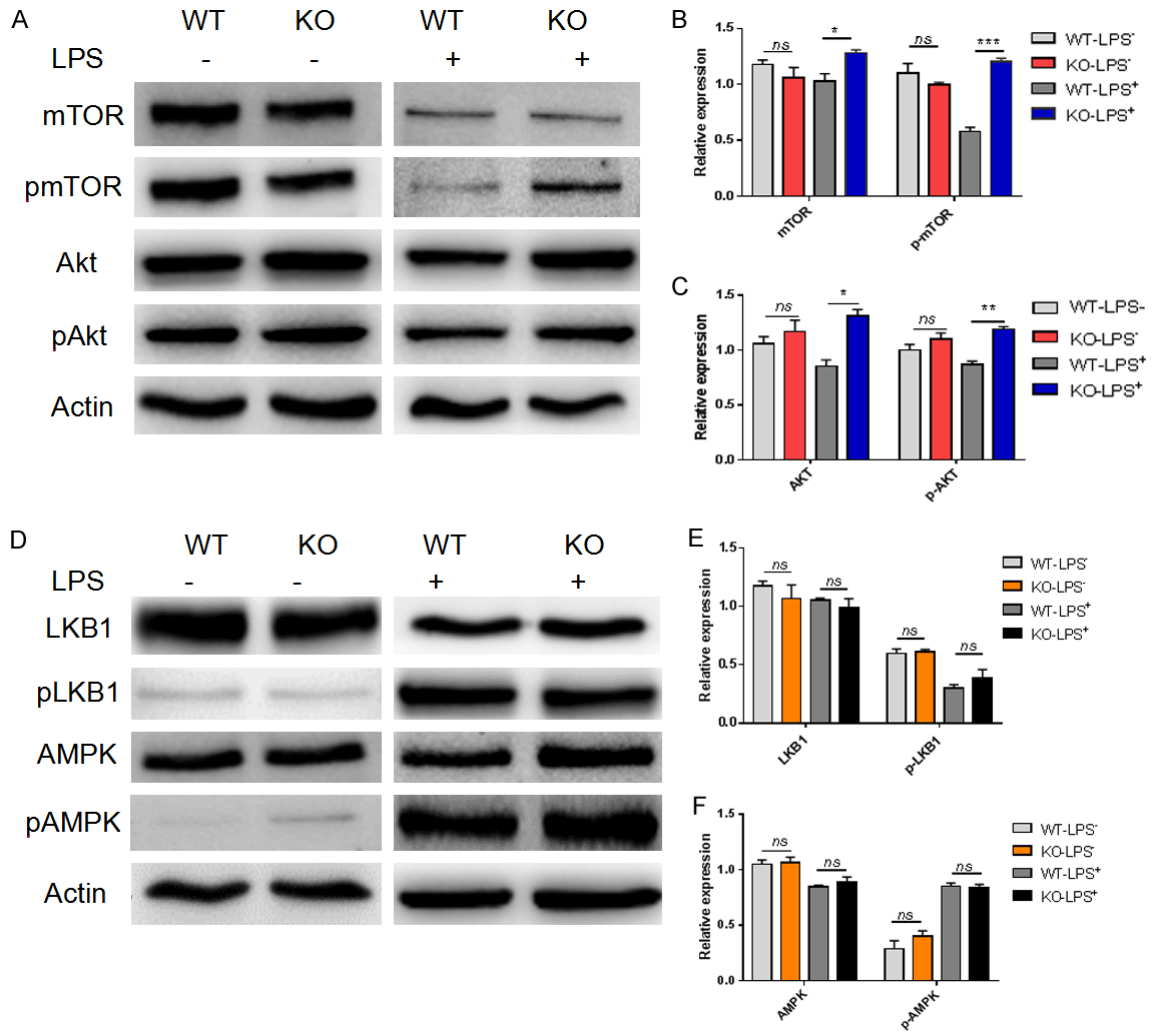


Figure 4. Fatty-acid metabolism of ANGPTL4$^{-/-}$ macrophages. (A) CPT1 and pACC1 levels of macrophages, as determined by western blotting and (B) statistical analysis. (C) Representative results for SREBP1 and LPL in macrophages, and (D) statistics. (E) CD36 expression on macrophages before and after LPS stimulation. (F) Oxygen consumption rate (OCR) of macrophages. (G) Mitochondrial mass, (H) intracellular ROS levels, and (I) intracellular free fatty acids of fresh and LPS-activated macrophages. (J) TNF-α, (K) IL-1β, and (L) CD86 levels of macrophages treated with a CPT1 activator (C75) or an ACC1 inhibitor (CP-641086). All experiments were performed three times. *, P<0.05; **, P<0.01; ***, P<0.001; *ns*, not significant.



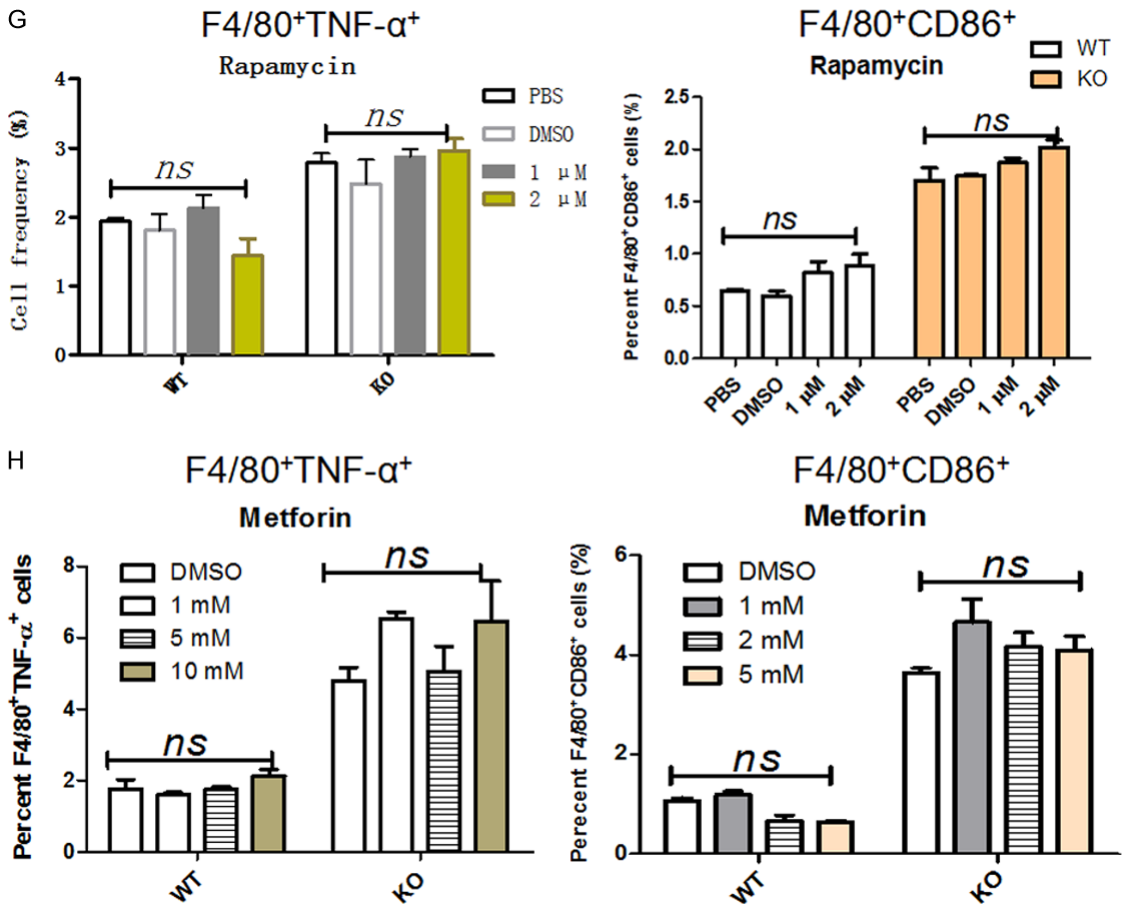


Figure 5. Signaling events in ANGPTL4$^{-/-}$ macrophages. (A-C) Differences in the levels of Akt/mTOR and (D-F) LKB1/AMPK signaling of macrophages before and after LPS stimulation. (G) Levels of TNF-α and CD86 in macrophages treated with the mTOR inhibitor (rapamycin) or (H) the AMPK stimulator (metformin). All experiments were carried out three times. *, P<0.05; **, P<0.01; ***, P<0.001; ns, not significant.

phages contributed to these functional changes. Following the treatment of cells with C75 to stimulate CPT-1, the TNF-α, IL-1β, and CD86 levels of ANGTPL4$^{-/-}$ macrophages were restored to WT levels (**Figure 4J-L**). Another reagent (CP-640186), an ACC1 inhibitor, also decreased the TNF-α and CD86 levels of ANGTPL4$^{-/-}$ macrophages to normal levels (**Figure 4J-L**). Thus, the upregulation of ANGTPL4$^{-/-}$ macrophage function was involved in the dysregulation of intracellular fatty-acid metabolism.

Molecular mechanisms of ANGPTL4$^{-/-}$ macrophage activation

Cell metabolism is strongly regulated by mTOR and the signaling molecules with which it is associated. The activation of mTOR signaling promotes glycolysis and fatty-acid synthesis [20]. In the absence of LPS stimulation, mTOR and its phosphorylated form were present at similar levels in ANGTPL4$^{-/-}$ and wild-type macrophages. Following LPS stimulation, mTOR was strongly activated in ANGTPL4$^{-/-}$ macrophages (**Figure 5A** and **5B**). Total and phosphorylated AKT levels simultaneously increased in ANGTPL4$^{-/-}$ macrophages (**Figure 5A** and **5C**). The LKB1 and AMPK signaling pathway also regulates mTOR activation. However, LKB1 and AMPK1 levels were similar in ANGTPL4$^{-/-}$ and wild-type macrophages, in the presence or absence of LPS stimulation (**Figure 5D-F**). Surprisingly, the treatment of macrophages with rapamycin (an inhibitor of mTOR) had no effect on the TNF-α and CD86 levels of ANGTPL4$^{-/-}$ macrophages (**Figure 5G**). An AMPK stimulator (metformin) also had no effect on the TNF-α and CD86 levels of ANGTPL4$^{-/-}$ macrophages (**Figure 5H**). Thus, the stronger

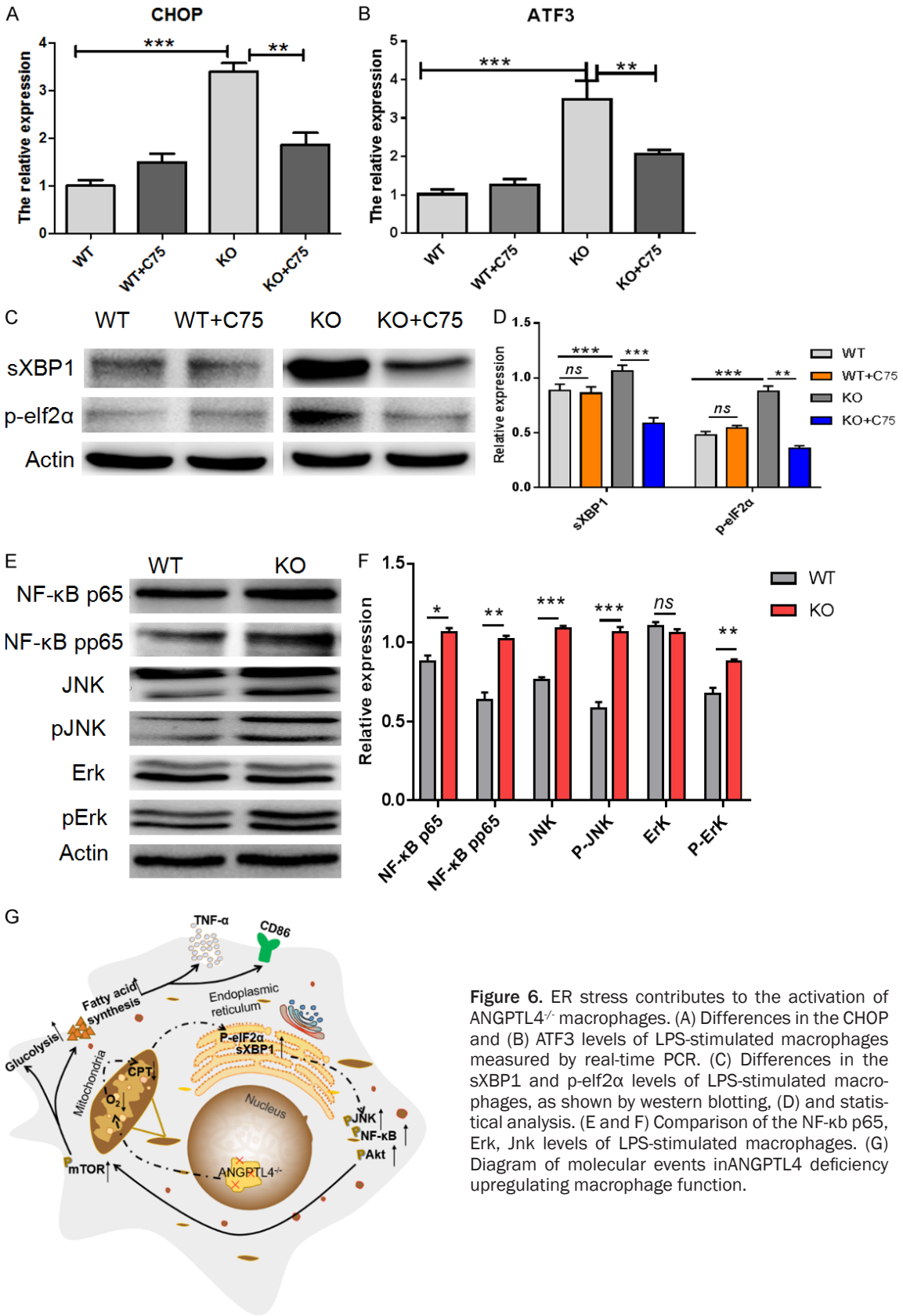


Figure 6. ER stress contributes to the activation of ANGPTL4$^{-/-}$ macrophages. (A) Differences in the CHOP and (B) ATF3 levels of LPS-stimulated macrophages measured by real-time PCR. (C) Differences in the sXBP1 and p-elf2α levels of LPS-stimulated macrophages, as shown by western blotting, (D) and statistical analysis. (E and F) Comparison of the NF-κb p65, Erk, Jnk levels of LPS-stimulated macrophages. (G) Diagram of molecular events inANGPTL4 deficiency upregulating macrophage function.

activation of Akt and mTOR may contribute little to the change in ANGTPL4$^{-/-}$ macrophage function.

Contributions of the endoplasmic reticulum stress induced by FA metabolism

CPT1 knockdown in macrophages increases endoplasmic reticulum (ER) stress [21]. We investigated the levels of molecular markers of ER stress in ANGTPL4$^{-/-}$ macrophages. Levels of activating transcription-3 (ATF3) and CHOP transcription were substantially higher in the macrophages of KO mice than in wild-type mice. C75 treatment decreased the transcription of both ATF3 and CHOP (**Figure 6A** and **6B**). Meanwhile, of the transcription of spliced X-box-binding protein 1 (sXBP1) and phosphorylated translational initiation factor 2 in eukaryotes (eIF2α) were higher in ANGTPL4$^{-/-}$ macrophages but were restored to normal levels following C75 treatment (**Figure 6C** and **6D**). ER stress usually leads to inflammatory responses. The strong activation of signaling molecules, such as NF-κB, Jnk, and Erk, was also confirmed in LPS-stimulated ANGTPL4$^{-/-}$ macrophages (**Figure 6E** and **6F**). Overall, these data indicate that ER stress in ANGTPL4$^{-/-}$ macrophages results from abnormal fatty-acid metabolism.

Discussion

ANGPTL4 deficiency was associated with an upregulation of macrophage function due to abnormal fatty-acid metabolism. However, exogenous ANGPTL4 had no effect on TNF-α and CD86 levels in macrophages from either ANGPTL4-knockout of wild-type mice. Glycolysis and mTOR activation were enhanced in ANGTPL4$^{-/-}$ macrophages, but inhibitors of these two processes did not block macrophage activation. Cytoplasmic levels of free fatty acids were remarkably elevated in ANGTPL4$^{-/-}$ macrophages. In addition, the ER stress, and the increases in TNF-α and CD86 levels in ANGTPL4$^{-/-}$ macrophages were blocked by treatment with a CPT1 stimulator (C75) or an ACC1 inhibitor (CP-640186). This study addressed the role of endogenous ANGPTL4 in macrophages for regulating the autologous metabolism of fatty acids, and deficiency of intracellular ANGPTL4 impacts activities of macrophages.

Associations of ANGPTL4 gene polymorphisms with serum lipid levels [22], metabolic syndrome [23], coronary artery disease [24], ischemic stroke [25], atorvastatin cholesterol-lowering responses [26], cancer invasiveness [27, 28], and type 2 diabetes [29, 30] have been reported. The tagged single-nucleotide polymorphisms (SNPs) implicated in disease risk include rs116843064, rs4076317, rs1044250, rs7255436, rs2967605, and rs2278236. Some ANGPTL4 genotypes with the A allele of rs116843064 or the G allele of rs1044250 are positively associated with higher serum lipid levels, whereas other genotypes, with the G allele of rs116843064 or the T allele of rs1044250, have protective effects, resulting in lower serum triglyceride (TG) concentrations. A large meta-analysis based on 1000 Genomes data also highlighted the existence of a damaging exonic variant of ANGPTL4 (rs116843064) determining fasting TG levels [22]. Transgenic ANGPTL4 mice have higher serum TG and total cholesterol (TC) levels, but ANGPTL4-knockout mice have lower serum TG and TC levels [4]. The variations of macrophage function and intracellular fatty acid metabolism in macrophages from patients with the protective allele of *ANGPTL4* remain unknown. Detailed clinical studies may reveal both positive and negative effects of ANGPTL4.

Hyperlipidemic mice with an ANGPTL4 deletion in hematopoietic cells had a larger CMP population, due to higher rates of proliferation and lower rates of apoptosis. ANGPTL4-deficient CMPs had a higher lipid raft content, and, therefore, a greater requirement for fatty acids. These CMPs had higher levels of CD36 expression, enabling them to take up more fatty acids, but no intracellular fatty-acid synthesis was detected. The CD36 expression ofANGPTL4$^{-/-}$ macrophages was unaffected by LPS stimulation, but these cells displayed high levels of fatty-acid synthesis and low levels of fatty-acid oxidation. These results confirm the greater lipid requirement of ANGPTL4$^{-/-}$ macrophages. Recombinant ANGPTL4 promoted the induction of M2 macrophages *ex vivo* [31]. However, exogenous ANGTPL4 did not inhibit the TNF-α and CD86 expression of macrophages. The involvement of different cytokines in macrophage polarization may account for this discrepancy.

When peritoneal ANGPTL4$^{-/-}$ macrophages were incubated with chyle, they displayed stronger inflammatory gene expression and ER stress than wild-type macrophages in similar conditions [15]. Our findings demonstrate that ANGPTL4 deficiency increases the sensitivity of macrophages to ER stress following activation by LPS. The inhibition of CPT1 in macrophages increases proinflammatory cytokine expression and ER stress responses to palmitate treatment [20]. CPT1 and ACC1 inhibit each other in cells. ANGPTL4$^{-/-}$ macrophages displayed an increase in FAS and a decrease in FAO after LPS treatment. The use of a CPT1 stimulator (C75) and an ACC1 inhibitor (CP-640186) prevented ER stress and inflammation, confirming the contribution of the dysregulation of fatty-acid metabolism to ER stress and inflammation in ANGPTL4$^{-/-}$ macrophages.

In conclusion, we show that ANGPTL4 deficiency in macrophages leads to the cell-intrinsic upregulation of FAS and downregulation of FAO. This dysregulation of fatty-acid metabolism induces ER stress and inflammation. The ER stress leads to an activation of Akt-mTOR signaling, which stimulates glycolysis and fatty-acid synthesis in a positive feedback loop. The entire process is illustrated diagrammatically in **Figure 6G**. This study deepens our understanding of ANGPTL4 activity in the modulation of macrophage function.

Disclosure of conflict of interest

None.

Address correspondence to: Weijuan Gong, Department of Immunology, School of Medicine, Jiangyang Road 136, Yangzhou, Jiangsu Province, China. Tel: +86-514-87978820; Fax: +86-514-87341733; E-mail: wjgong@yzu.edu.cn

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