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FOXO3a acetylation regulates PINK1, mitophagy, inflammasome activation in murine palmitate-conditioned and diabetic macrophages

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Abstract

Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 inflammasome and mitophagy play an important role in cytokine release and diabetes progression; however, the role of saturated fatty acid that is induced under such conditions remains little explored. Therefore, the present study evaluates mechanisms regulating mitophagy and inflammasome activation in primary murine diabetic and palmitate-conditioned wild-type (WT) peritoneal macrophages. Peritoneal macrophage, from the diabetic mice and WT mice, challenged with LPS/ATP and palmitate/LPS/ATP, respectively, showed dysfunctional mitochondria as assessed by their membrane potential, mitochondrial reactive oxygen species (mtROS) production, and mitochondrial DNA (mtDNA) release. A defective mitophagy was observed in the diabetic and palmitate-conditioned macrophages stimulated with LPS/ATP as assessed by translocation of PTEN-induced kinase 1 (PINK1)/Parkin or p62 in the mitochondrial fraction. Consequently, increased apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization, caspase-1 activation, and IL1β secretion were observed in LPS/ATP stimulated diabetic and palmitate-conditioned macrophages. LPS/ATP induced Forkhead box O3a (FOXO3a) binding to PINK1 promoter and increased PINK1 mRNA expression in WT macrophages. However, PINK1 mRNA and protein expression were significantly decreased in diabetic and palmitate-conditioned macrophages in response to LPS/ATP. Palmitate-induced acetyl CoA promoted FOXO3a acetylation, which prevented LPS/ATP-induced FOXO3a binding to the PINK1 promoter. C646 (P300 inhibitor) and SRT1720 (SIRT1 activator) prevented FOXO3a acetylation and restored FOXO3a binding to the PINK1 promoter, PINK1 mRNA expression, and mitophagy in palmitate-conditioned macrophages treated with LPS/ATP. Also, a significant decrease in the LPS/ATP-induced mtROS production, mtDNA release, ASC oligomerization, caspase-1 activation, and IL-1β release was observed in the palmitate-conditioned macrophages. Similarly, modulation of FOXO3a acetylation also prevented LPS/ATP-induced mtDNA release and inflammasome

Abbreviations: ASC, Apoptosis-associated speck-like protein containing a caspase recruitment domain; DRP1, Dynamin related protein 1; FOXO3a, Forkhead box O3a; LC3B, Microtubule-associated proteins 1A/1B light chain 3B; NLRP3, Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3; PINK1, PTEN-induced kinase 1; ROS, Reactive oxygen species; TIM44, Translocase of inner mitochondrial membrane 44; TOM20, Translocase of outer mitochondrial membrane 20; WT, Wild-type

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activation in diabetic macrophages. Therefore, FOXO3a acetylation regulates PINK1-dependent mitophagy and inflammasome activation in the palmitate-conditioned and diabetic macrophages.

KEYWORDS
ASC oligomerization, diabetic mice, NLRP3 inflammasome, palmitate, PINK1-Parkin mitophagy

1 | INTRODUCTION

Nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome is a molecular machinery to promote caspase-1-dependent proteolytic processing and secretion of IL-1β and IL-18 from immune cells.[1] PAMPs mediated TLR4 signaling primes macrophages for NLRP3 inflammasome activation by promoting the proIL-1β, NLRP3 expression. Damage-associated molecular pattern (DAMPs) such as ATP, monosodium urate (MSU) crystal generate mitochondrial damage-associated signals that initiate NLRP3 interaction with apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), to form the NLRP3 inflammasome complex.[2] Mitochondrial damage is associated with a drop in mitochondrial membrane potential and enhanced mitochondrial reactive oxygen species (mtROS) production.[2,3] For NLRP3 inflammasome assembly, mtROS, mitochondrial DNA (mtDNA), and cytosolic presentation of cardiolipin serve as a direct signal to assemble NLRP3:ASC:pro-caspase-1 complex.[2] NLRP3 activators mediated mtROS production promote oxidation as well as the release of mtDNA into the cytosol.[3,4]

To avoid excess cellular damage, ROS-producing mitochondria are continuously removed by PTEN-induced kinase 1 (PINK1)/Parkin mediated mitophagy, a specialized process of autophagy. Hence, mitophagy negatively regulates NLRP3 inflammasome activation by the elimination of damaged mitochondria.[5,6] The previous studies suggest that the accumulation of damaged mitochondria resulted from autophagic dysfunction is associated with aging and metabolic diseases.[7,8] Furthermore, posttranslational modifications such as acetylation also play a cardinal role in NLRP3 inflammasome activation as well as autophagy.[9] Acetylation of α-tubulin regulates the transport of mitochondria to promote the interaction between ASC and NLRP3.[9] However, acetylation of autophagic proteins ATG5, ATG7, ATG12, and LC3 decrease autophagy and starvation-dependent SIRT1-induced deacetylation of these autophagy proteins promote autophagy.[10,11]

Saturated fatty acid, particularly palmitate is found up-regulated in plasma of type 2 diabetes (T2D) patients as well as diabetic mice.[12] Chronic elevation of free fatty acids in plasma promotes peripheral (muscle) insulin resistance by PKC mediated insulin receptor substrate (IRS)1 serine phosphorylation as well as hepatic insulin resistance by suppressing glycogenolysis.[12] Saturated fatty acids such as palmitate with stearic acid induce intracellular crystal formation in macrophages, which in turn activates the NLRP3 inflammasome via lysosomal destabilization.[13] The previous report also suggests that palmitate mediates NLRP3 inflammasome activation in LPS primed macrophages by inhibiting AMP kinase-induced autophagy.[14]

The role of the Forkhead box O3a (FOXO) transcription factor in mitophagy, as well as NLRP3 inflammasome, is well established.[15,16] Recently, the role of FOXO3a in the regulation of NLRP3 inflammasome in Kuffer cells was addressed.[16] FOXO3a mediated Bim expression decrease NLRP3 inflammasome activation by restoring the autophagy flux.[16] Little is known about the regulation of FOXO3a in diabetic macrophages and specifically in the regulation of mitophagy. Hence, this study aims to understand the mechanism of inflammasome activation in diabetes with special emphasis on mitophagy. We demonstrated that palmitate-induced acetylation of FOXO3a reduced PINK1 expression as well as PINK1-Parkin mediated mitophagy. The resultant increased mtROS and mtDNA release into cytosol due to the accumulation of damaged mitochondria enhances NLRP3 inflammasome activation in diabetic and palmitate-conditioned macrophages.

2 | MATERIAL AND METHODS

2.1 | Reagents

Brewer's thioglycolate, sodium palmitate, fat-free BSA, LPS from *Escherichia coli* 0111:B4, ATP, anti-PINK1, anti-histone-3, anti-β-actin, and anti-tubulin were purchased from Sigma-Aldrich (St Louis, MO, USA). DMEM, FBS, MitoSOX, MitoTracker deep red, and Mito Tracker green FM were obtained from Invitrogen (Carlsbad, CA, USA). Caspase-1/ICE Fluorometric assay kit and PicoProbe Acetyl CoA Fluorometric assay kit were from Biovision (San Francisco, CA, USA). BD OptEIA ELISA Kit for mouse IL-1β and TNFα were from BD Biosciences (San Diego, CA, USA). The anti-caspase-1 antibody was from Adipogen (Liestal, Switzerland). Anti-ASC, anti-p62, anti-phosphor-AKT (Ser473) and total AKT, anti-acetyl lysine, anti-FOXO3a were from Cell Signalling Technology (ZA Leiden, Netherland). Anti-Parkin, anti-VDAC1, anti-FOXO3a, and anti-MTCO2 were purchased from Abcam (Cambridge, United Kingdom). Protein A sepharose beads were obtained from GE Healthcare (Glattburgg, Switzerland). RNAiso plus reagent was from Takara (Shiga, Japan). Revert Aid first-strand cDNA

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synthesis kit and SYBER Green/ ROX qPCR Master Mix were from Thermo Scientific (Waltham, MA, USA).

2.2 | Animals

Male C57BL/6 (wild-type; WT), male db/db (diabetic mice), and db/+ (control mice) were obtained from the National Laboratory Animal Centre at the CSIR—Central Drug Research Institute (Lucknow, India). All animal procedures, used in this study, were approved by the Institutional Animal Ethics Committee (IAEC). The reference number of the IAEC protocol for db/+, db/db animal is IAEC/2019/101/Renew-0/Dated-14/06/2019. All the animals were provided with food and drinking water ad libitum and maintained at 23°C ± 2°C with humidity 50–60% and 12 h dark/light cycles.

Control and diabetic mice were checked for insulin resistance by the intraperitoneal glucose tolerance test (ipGTT) test. The ipGTT was performed after 12 h of fasting. A total of 2 g/kg of glucose was administered to mice i.p. and blood glucose was observed from the time of glucose injection to 120 min with an Accu-Chek Active blood glucometer.[17]

2.3 | Thioglycolate elicited macrophages isolation and treatment

To isolate the peritoneal macrophages from diabetic mice, respective control mice, and WT C57BL/6 mice, mice were i.p. injected with 2 ml of 4% Brewer's thioglycolate. After 96 h, peritoneal exudates were collected with an injection of cold PBS into the peritoneal cavity. The cells suspended in DMEM were supplemented with 10% FBS and 100 U/ml penicillin/streptomycin, plated in 6- or 12-well plates, incubated for 1 h, and washed twice with PBS. The remaining adherent cells were used for experiments.[18]

For conditioning with palmitate-BSA, cells were pre-incubated with the 250 μm palmitate-BSA solution for 16 h. For NLRP3 inflammasome activation in palmitate-conditioned macrophages, diabetic, and control macrophages, cells were primed with 500 ng/ml LPS for 4 h before stimulation with 5 mM ATP for 30 min.[19] For pharmacologic assessments of NLRP3 inflammasome activation, glibenclamide (50 μm), and VX765 (20 μm) are added after LPS treatment and followed by ATP treatment. C646 (20 μm), SRT1720 (10 μm), and etomoxir (40 μm) were added to culture 1 h before palmitate treatment.

2.4 | Palmitate-BSA solution stock preparation

A total of 10% fat-free BSA was prepared in Milli-Q water (MQ) and kept in a water bath at 37°C for 2 h and pH maintained to 7.4 by 1 N NaOH. A stock solution of 200 mM palmitate was prepared in 50% ethanol solution and heated at 70°C for 20 min until it dissolved completely. Now, a dissolved palmitate solution is added to 10% BSA immediately to prepare 5 mM palmitate-BSA stock. The solution was kept on the 37°C water-bath for 2 h and mixed at every 30 min interval.[14]

2.5 | ASC oligomerization assay

Macrophages were seeded in 6-well plates (1×10^6 cells/ well) and treated with different stimuli. After treatment, cells were centrifuged and the pellet was resuspended in 0.5 ml of ice-cold buffer containing 20 mM HEPES-KOH, pH 7.5, 150 mMKCl, 1% Nonidet P-40, 0.1 mM PMSF, and a protease inhibitor mixture. Cells were lysed by passing 10 times through a 21-gauge needle. Cell lysates were then centrifuged at 5000 $\times g$ for 10 min at 4°C. The supernatant was collected in a fresh tube and processed for analysis of ASC monomer and β-actin. The pellet obtained, was washed twice with PBS and resuspended in 50 μl of PBS (pH 8) and crosslinked with fresh disuccinimidylsuberate (4 mM) for 30 min. The crosslinking reaction was stopped with the addition of SDS sample buffer, and separated using 12% SDS-PAGE and immunoblotted using anti-mouse ASC antibodies.[18]

2.6 | Mitochondrial assay

In the last 15 min of ATP stimulation, peritoneal macrophages were incubated with MitoSOX or Mito-Tracker Green FM and Mito-Tracker deep Red. After treatment, cells were collected in PBS and acquired on a FACS Calibur flow cytometer (BD Biosciences) and analyzed with FlowJo (TreeStar, Ashland, OR, USA).[5]

2.7 | Caspase-1 detection assay

Briefly, after treatment with different agonists and inhibitors, peritoneal macrophages were pelleted down by centrifugation. Caspase-1 activity is measured in lysates by the caspase-1/ICE Fluorometric assay kit as per user instructions.[19]

2.8 | Immunoprecipitation assay

After treatments, cells were lysed in lysis buffer comprising 150 mM NaCl, 50 mM Tris (pH 7.4), 0.1 mM EDTA, 0.1 mM EGTA, 1% NP-40, and 0.2 mM PMSF with protease and phosphatase inhibitors. One microgram of the FOXO3a antibody is incubated with 350 μg of protein lysate on the rotator at 4°C, overnight. The next day, 25 μl of washed protein A-sepharose beads were added to capture immune complexes at 4°C, overnight. Immunoprecipitates on beads were washed thrice with the same lysis buffer at 4200 rpm/3 min. After the final wash, 30 μl of 2× SDS buffer was added to beads and boiled for 10 min at 94°C. The immunoprecipitated proteins were resolved by SDS-PAGE and blotted with an anti-acetyl lysine antibody.[20]

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2.9 | Mitochondria isolation

After treatment, cells were resuspended in 300–500 μl of Sucrose Tris base Magnesium chloride (STM) buffer consist of 50 mM Tris (pH 7.4), 350 mM sucrose, 5 mM $MgCl_2$, protease, and phosphatase inhibitor cocktails and homogenized for 1 min on ice with a tight-fitting Teflon pestle attached to a Potter (Sartorius, Gottingen, Germany) S homogenizer set on 600–1000 rpm. The homogenate was incubated on ice for 30 min and vortexed at maximum speed for 15 s and then centrifuged at 800 $\times g$ for 15 min. The supernatant was now centrifuged at 11,000 $\times g$ for 10 min/4°C. Pellets containing mitochondria were subsequently lysed with Tris buffer.[21]

2.10 | ELISA

The level of mouse IL-1β and TNFα cytokines in the supernatant was measured by using BD OptEIA ELISA Kit as per manual instructions of kit.[20]

2.11 | Western blotting

After the desired treatments, cells were lysed in Radioimmunoprecipitation assay (RIPA) lysis buffer containing 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl„ 0.001 M EDTA (pH 7.4), PMSF (100 mg/ml), sodium orthovanadate (2 mM), sodium fluoride (2 mM), 1% Triton X-100, and propidium iodide cocktail inhibitor. Bicinchoninic acid reagent was used to measure protein concentrations. SDS-PAGE was run and protein was transferred to the PVDF membrane. The blot was developed as previously described.[22]

2.12 | mtDNA isolation

After desired treatment, macrophages were homogenized with a Dounce homogenizer (Thomas scientific, Swedesboro, New Jersey, United States) in buffer containing 100 mM tricine-NaOH solution (pH 7.4), 0.25 M sucrose, 1 mM EDTA, and protease inhibitor. Then lysates were centrifuged at 13,000 rpm for 15 min, at 4°C. The protein concentration of the supernatant was normalized. The resultant supernatant (cytosolic fraction) is transferred to a new tube and an equal volume of absolute alcohol added. Now DNA is isolated with the help of DNA extraction kit protocol.[5]

2.13 | Immunofluorescent staining

Peritoneal macrophages were seeded at 0.2×10^6 cells per well in 8-well chamber slides. After the treatments, the cells were washed twice with sterile PBS and fixed with chilled acetone for 5 min and followed by incubation with chilled methanol for 5 min. Cells were washed with PBS and blocked in 2% BSA for 2 h. The cells were then incubated overnight with primary antibodies, anti-MtCO2, and anti-Parkin. The next day, slides were washed thrice with PBS and incubated with secondary fluorescent antibodies (anti-mouse Alexa Fluor-488, anti-rabbit Alexa Fluor-594) for 2 h at room temperature. After three washings with PBS, the slide was mounted with Fluoroshield with DAPI mounting medium. Images of samples were acquired through Olympus (Shinjuku City, Tokyo, Japan) BV61WI confocal microscope 24 h after mounting. The threshold Pearson's correlation coefficients (−1.0 to 1.0) for 30 cells/wells were analyzed and presented as mean ± SEM.[6]

2.14 | Acetyl CoA detection

After treatment, cells were lysed in sample buffer and deproteinized with perchloric acid. Acetyl CoA is detected in samples with the Pico-Probe Acetyl CoA Fluorometric assay kit (Biovision) as per manufacturer's instructions.[19]

2.15 | CHIP assay

CHIP assays were performed according to the previously described method.[23] Relative binding of FOXO3a to PINK1 promoter was assessed using qPCR primers specific to PINK1 promoter, using forward (5′-GGGGAGCTCGTTGTTGT-3′) and reverse primers (5′-GGGGCTAGCACAACAAC-3′).

2.16 | Real time PCR

After treatment, samples were prepared in RNAiso plus. Total RNA was extracted and reverse transcribed with the Revert Aid first-strand cDNA synthesis kit.[24] Real-time qPCR was performed in a LightCycler 480II system (Roche Applied Science, Branford, Connecticut, USA) using gene-specific primers and SYBR Green reagent. Gene expression was normalized to 18S rRNA. Primer sequences are as follows: mouse PINK1 forward 5′GAGCAGACTCCCAGTTCTCG3′, reverse 5′GTCCCACTCCAC AAGGATGT 3′; mouse 18S forward 5′GCAATTATTCCCCATGAACG3′, reverse 5′GGCCTCACTAAACCATC CAA3′; mouse mitochondria copy D loop forward 5′AATCTACCATCCTCCGTGAAACC3′, reverse 5′TCAGTTTAGCTACCCCCAAGTTTAA3′; and mouse nuclear copy B2m forward 5′ATGGGAAGCCGAACATACTG3′, reverse 5′CAGTCTCAGTGGGGGTGAAT3′.

2.17 | Statistical analysis

Data are expressed as mean ± SEM. The significance of the difference between groups or treatments was analyzed by performing an ANOVA, followed by the Bonferroni post hoc test. *P*-values equal to or less than 0.05 were considered statistically significant. All statistical

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analysis was performed with the Graph Pad Prism 5.0 program (GraphPad Inc., La Jolla, CA, USA).

3 | RESULTS

3.1 | Damaged mitochondria facilitate mtDNA release and NLRP3 inflammasome activation in diabetic macrophages

To investigate the role of damaged mitochondria in NLRP3 inflammasome activation under diabetic condition, we assessed the abundance of damaged mitochondria in response to NLRP3 stimuli (LPS and ATP) in macrophages, isolated from control mice ($AUC_{Glucose}$: 24,813 ± 1674 (mg/dl) × min)) and diabetic mice ($AUC_{Glucose}$: 72,634 ± 7575 (mg/dl) × min); Supporting Information Fig. S1A, B). Diabetic macrophages treated with NLRP3 stimuli showed a significant ($P < 0.05$) increase in damaged mitochondria (positive for mitotracker green) in comparison to control macrophages treated with NLRP3 stimuli (Fig. 1A, B). Mitophagy restrains NLRP3 inflammasome activation by digesting the dysfunctional pool of mitochondria.[6] NLRP3 stimuli induced Parkin-containing aggregates that were colocalized with mitochondria in control macrophages (Fig. 1C), whereas diabetic macrophages displayed diffuse Parkin localization and mitochondria in response to NLRP3 stimuli (Fig. 1C). The degree of colocalization of Parkin with mitochondria was measured by Pearson's correlation coefficient, representing the linear relationship of the signal intensity from the green and red channels. Diabetic macrophages treated with NLRP3 agonists showed a significant ($P < 0.01$) decrease in the degree of colocalization (Pearson's correlation coefficient) compared to control macrophages treated with NLRP3 agonist (Supporting Information Fig. S1C). Consistently, NLRP3 stimuli induced translocation of PINK1 and Parkin to mitochondria in the control macrophages whereas diabetic macrophages showed reduced LPS/ATP-induced PINK1 and Parkin translocation to the mitochondria (Supporting Information Fig. S1D). Degradation of mitochondrial inner and outer membrane proteins (translocase of inner mitochondrial membrane 44 [TIM44], translocase of outer mitochondrial membrane 20 [TOM20]) together with autophagy markers such as microtubule-associated proteins 1A/1B light chain 3B (LC3B)II is widely used to follow the mitophagy process.[25,26] To further substantiate reduced mitophagy in diabetic macrophages, the earlier mentioned parameters were assessed. Diabetic macrophages displayed accumulation of TIM44, TOM20, along with decreased LC3BII in response to NLRP3 stimuli compared to control macrophages treated with NLRP3 stimuli (Supporting Information Fig. S1E–G). The phosphorylation of dynamin related protein 1 (DRP1) at ser616 residue promotes its translocation to mitochondria to regulate mitochondrial fission, which is a prerequisite for mitophagy.[27,28] Diabetic macrophages showed decreased DRP1 phosphorylation in response to NLRP3 stimuli compared to control macrophages treated with NLRP3 stimuli (Supporting Information Fig. S1H). Damaged mitochondria are a prime source for mtROS and released mtDNA that promotes NLRP3 inflammasome complex formation.[5] NLRP3 agonist significantly ($P < 0.001$) increased mtROS and mtDNA release into the cytosol in diabetic macrophages compared to control macrophages stimulated with NLRP3 agonist (Fig. 1D,E). Accumulation of damaged mitochondria in diabetic macrophages correlated well with elevated mtROS production and enhanced mtDNA release. Further, we checked the NLRP3 inflammasome activation in control and diabetic macrophages by assessing ASC oligomerization, caspase-1 activation, and IL1β secretion. Diabetic macrophages treated with NLRP3 stimuli showed increased ASC oligomer formation (Fig. 1F) and significantly ($P < 0.05$) enhanced caspase-1 activation in both supernatant and lysate (Fig. 1G, Supporting Information Fig. S2A) compared to control macrophages stimulated with NLRP3 agonist. Consistently, NLRP3 agonist treatment in diabetic macrophages also significantly ($P < 0.001$) increased IL1β secretion in the supernatant compared with control macrophages stimulated with NLRP3 agonist, whereas TNFα level was not changed (Fig. 1H, Supporting Information Fig. S2B). NLRP3 inflammasome activation inhibitor (Glibenclamide) and caspase 1 inhibitor (VX-765) treatment significantly ($P < 0.01$, $P < 0.001$) reduced IL1β secretion from the diabetic macrophages in response to NLRP3 agonist (Supporting Information Fig. S2C). Because TLR4 mediated signaling primes the macrophages for inflammasome activation by promoting NLRP3 and IL1β mRNA expression6, their levels were assessed. NLRP3 and IL1β mRNA expression were significantly ($P < 0.001$, $P < 0.01$) up-regulated in the NLRP3 agonist stimulated diabetic macrophages when compared to the control macrophages stimulated in same manner (Supporting Information Fig. S2D, E).

3.2 | Palmitate conditioning in macrophages inhibits mitophagy and promotes mtDNA release

The level of palmitate is highly enhanced in the serum of diabetic patients and mice.[12] To mimic a similar condition, WT macrophages were pretreated with palmitate and stimulated with NLRP3 agonist. NLRP3 stimuli significantly ($P < 0.001$) induced the appearance of damaged mitochondria (positive for mitotracker green) in WT macrophages compared to vehicle treated cells, which was further significantly ($P < 0.001$) enhanced in the palmitate-conditioned macrophages stimulated with NLRP3 stimuli (Fig. 2A, C). NLRP3 stimuli induced translocation of PINK1 and Parkin to mitochondria in WT macrophages. Consistent with decreased mitophagy in the diabetic macrophages, palmitate conditioning reduced LPS/ATP-induced PINK1 and Parkin translocation to the mitochondria in the WT macrophages (Fig. 2B). Parkin has E3 ubiquitin ligase activity to decorate damaged mitochondria with poly-Ub chains, which are recognized by the p62 UBA domain for mitophagic clearance.[29] Consistently, p62 is also decreased in the mitochondrial fraction of the palmitate-conditioned macrophages treated with NLRP3 agonist (Fig. 2B). Furthermore, NLRP3 stimuli also significantly ($P < 0.001$) reduced TIM44 and TOM20 levels, and increased ($P < 0.05$) LC3BII in WT macrophages (Supporting Information Fig. S3A–C). Whereas palmitate prevents LPS/ATP-induced decrease in TIM44 and TOM20 (Supporting Information Fig. S3A, B), and the increase in LC3BII protein expression (Supporting

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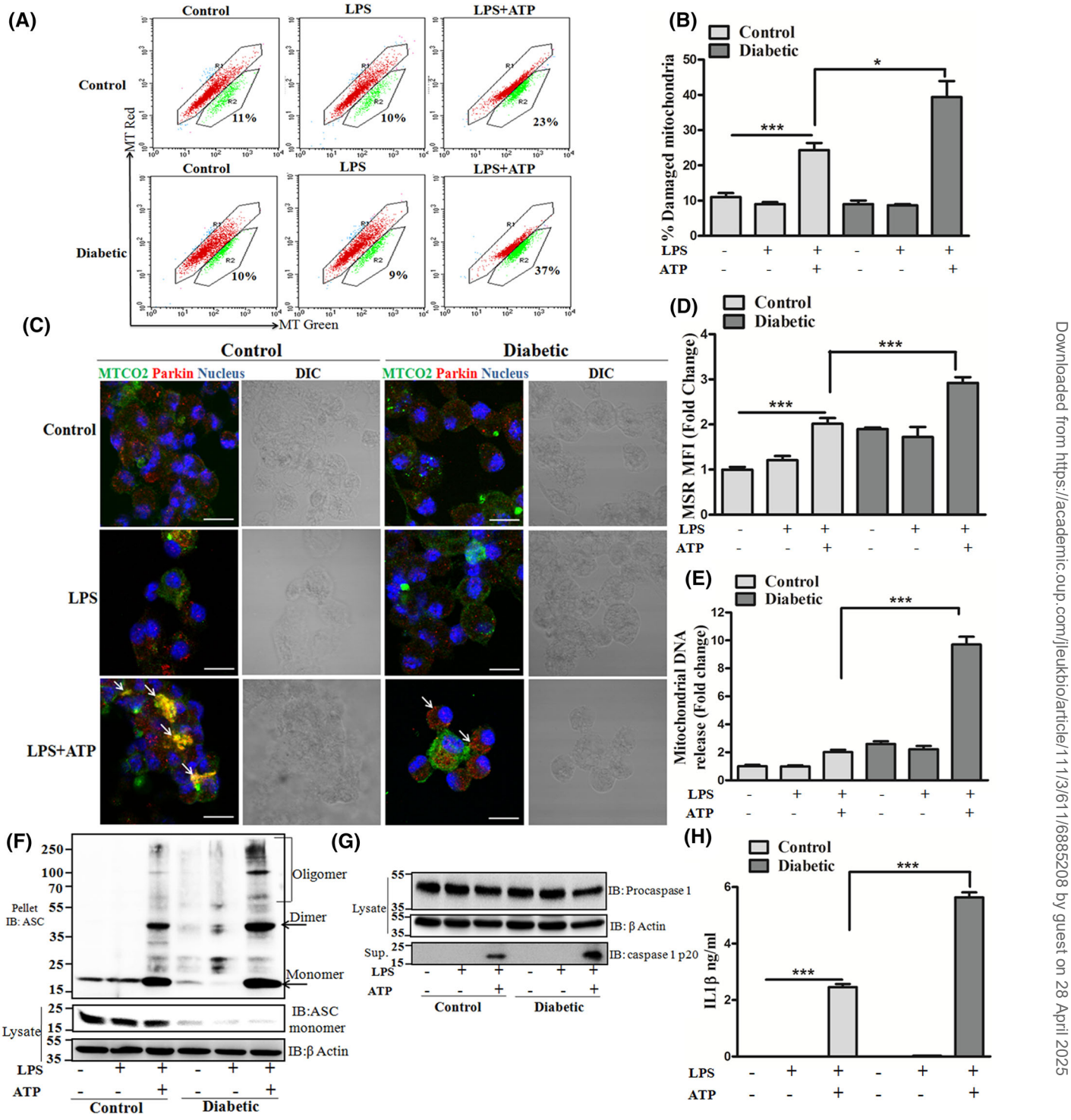

FIGURE 1 **Diabetic macrophages increased nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome activation**. Peritoneal macrophages from control and diabetic mice were treated with LPS (500 ng/ml) for 4 h and followed by ATP (5 mM) stimulation for 30 min. **(A)** Damaged mitochondria were quantified by flow cytometry labelling with mitotracker deep red and mitotracker green, which are taken up in a mitochondrial potential-dependent and -independent manner, respectively. Lower gate represent cells with damaged mitochondria, **(B)** Percentage of damaged mitochondria was analyzed from three independent experiments ($n = 3$). **(C)** Intracellular colocalization (orange to yellow) of Parkin (stained red) with mitochondria (stained green) merged with the nuclear stain DAPI (stained blue) in macrophages stimulated with LPS and ATP, examined by confocal microscopy (scale bar, 5 μm) ($n = 3$).**(D)** Mitochondrial ROS was measured by flow cytometry ($n = 3$). **(E)** mitochondrial DNA (mtDNA) release in the cytosol is quantified by RT-PCR ($n = 3$). Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization and redistribution assay in control and diabetic macrophages; Immunoblot analysis of **(F)** ASC in crosslinked pellets (upper panels) and ASC, β-actin in total cell lysates (lower panels) ($n = 3$), **(G)** active caspase-1 in the supernatant and procaspase-1, β-actin in cell lysates ($n = 3$). **(H)** IL1β measured in the supernatant by ELISA ($n = 3$). "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{*}P < 0.05$, $^{***}P < 0.001$

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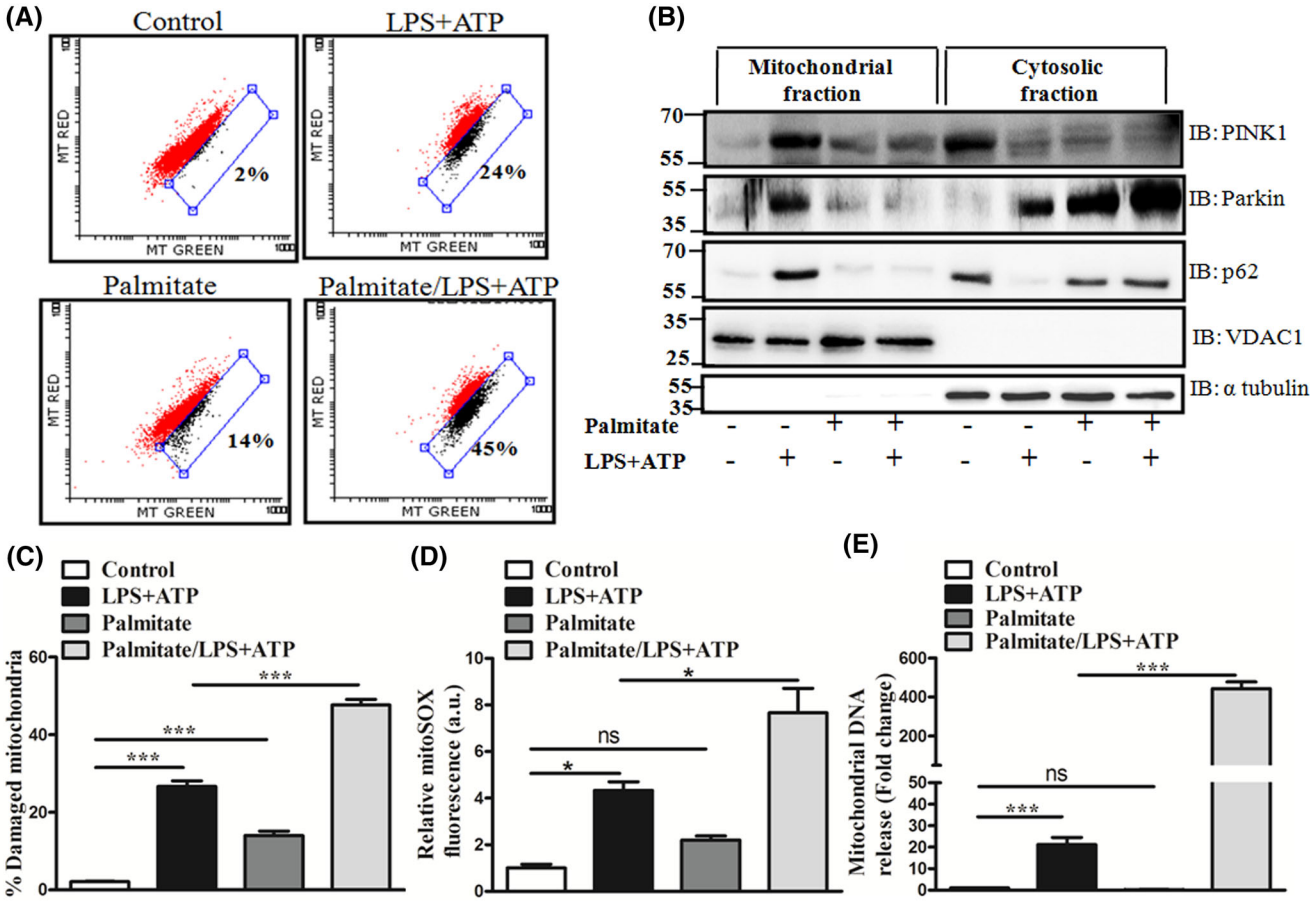

FIGURE 2 **Palmitate-conditioned macrophages to build up damaged mitochondria in response to nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) agonist**. Peritoneal macrophages from wild-type (WT) mice were conditioned with palmitate for 16 h and followed by treatment with LPS for 4 h and ATP for 30 min. **(A)** The number of damaged mitochondria was analyzed by flow cytometry labeling with mitotracker deep red and mitotracker green, **(C)** Percent damaged mitochondria were analyzed from three independent experiments ($n = 3$). **(B)** Immunoblot analysis of PTEN-induced kinase 1 (PINK1), Parkin, p62 in the mitochondrial and cytosolic fraction ($n = 3$). **(D)** Mitochondrial reactive oxygen species (mtROS) is analyzed by fluorescence microscopy ($n = 3$). **(E)** Mitochondrial DNA (mtDNA) release in the cytosol is quantified by RT-PCR ($n = 3$). "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{*}P < 0.05$, $^{***}P < 0.001$

Information Fig. S3C). Moreover, palmitate conditioning significantly ($P < 0.05$) decreased LPS/ATP-induced DRP1 phosphorylation (Supporting Information Fig. S3D). Similar to ex vivo results of diabetic macrophages, palmitate-conditioned macrophages treated with NLRP3 stimuli showed significant ($P < 0.05$, $P < 0.001$) increase in the mtROS and mtDNA release in comparison to macrophages treated with NLRP3 stimuli (Fig. 2D, E). These data suggest that palmitate conditioning augments mtROS and mtDNA release by decreasing mitophagy and increasing the accumulation of damaged mitochondria.

3.3 | Palmitate conditioning enhances ASC oligomerization and caspase-1 activation

The mtROS and mtDNA release were increased in the palmitate-conditioned macrophages, which prompted us to evaluate NLRP3 inflammasome activation in palmitate-conditioned cells. NLRP3 agonist treatment in the palmitate-conditioned macrophages increased ASC oligomer formation and significantly ($P < 0.05$) enhanced caspase-1 activation as compared to the macrophages stimulated with NLRP3 agonist (Fig. 3A–C). Consistently, NLRP3 agonist treatment in the palmitate-conditioned macrophages significantly ($P < 0.01$) increased IL1β secretion in the supernatant as compared to the macrophages stimulated with NLRP3 agonist (Fig. 3D). Palmitate-conditioned macrophages treated with NLRP3 agonist showed a significant ($P < 0.05$) increase in IL1β mRNA expression, whereas NLRP3 expression was not changed (Fig. 3E, F).

3.4 | Palmitate conditioning attenuates PINK1 expression

Our data clearly showed decreased mitophagy in the diabetic macrophages as well as in the palmitate-conditioned macrophages

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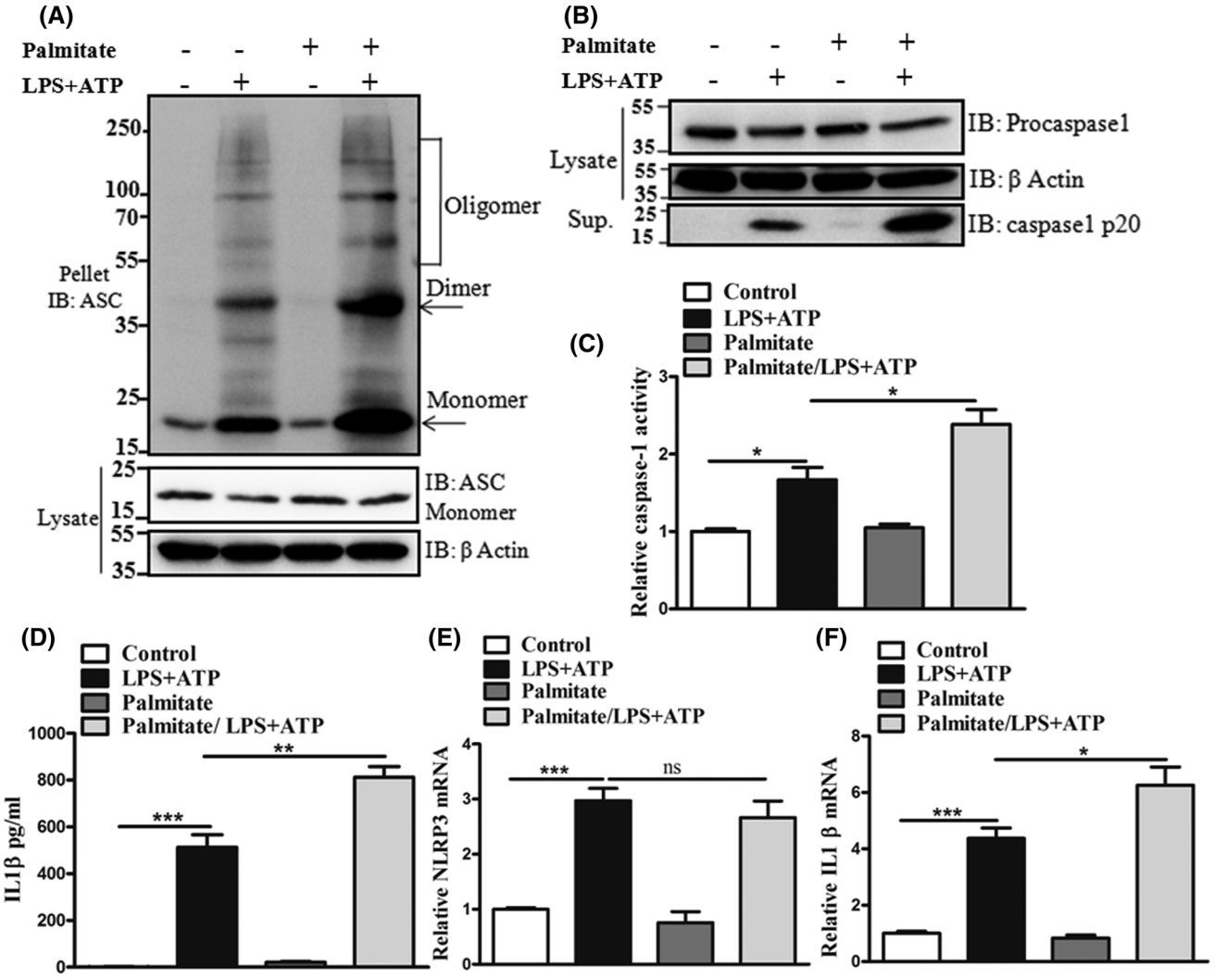

FIGURE 3 **Palmitate-conditioned macrophages showed enhanced nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome activation**. Peritoneal macrophages from wild-type (WT) mice were conditioned with palmitate for 16 h and followed by treatment with LPS for 4 h and ATP for 30 min. Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization and redistribution assay in peritoneal macrophages; immunoblot analysis of **(A)** ASC in crosslinked pellets (upper panels) and ASC, β-actin in total cell lysates (lower panels) ($n = 3$), **(B)** active caspase-1 in the supernatant and procaspase-1, β-actin in total cell lysates ($n = 3$). **(C)** Caspase-1 activity in lysate samples was measured by fluorometric assay ($n = 3$). **(D)** IL1β measured in the supernatant by ELISA ($n = 3$). **(E)** NLRP3 and **(F)** IL1β mRNA expression was measured by RT-PCR ($n = 5$). The value "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

in response to LPS/ATP (Figs. 1C, 2B). We decided to focus on the regulation of the mitophagy-related proteins (PINK1, Parkin) in the same scenario. Diabetic macrophages stimulated with NLRP3 agonist showed significantly ($P < 0.01$, $P < 0.001$) decreased PINK1 mRNA as well as protein expression compared to control macrophages treated with NLRP3 agonist (Fig. 4A, B). Similarly, NLRP3 stimuli significantly ($P < 0.01$) decreased PINK1 mRNA expression, as well as protein expression in the palmitate-conditioned macrophages compared to the macrophages treated with NLRP3 stimuli (Fig. 4C, D), whereas Parkin expression remains unchanged (Fig. 4E). These data suggest that altered PINK1 expression may be responsible for reduced mitophagy in both the diabetic and palmitate-conditioned macrophages.

3.5 | Palmitate conditioning promotes FOXO3a acetylation and inhibits its recruitment to PINK1 promoter

Inactivation of Akt signaling increases PINK1 transcription by FOXO3a binding to FOXO consensus DNA-binding site "GTTGTTGT" on the PINK1 promoter.[30] Palmitate-conditioned macrophages treated with NLRP3 agonist showed a similar level of AKT phosphorylation as in the cells treated with NLRP3 agonist, without any change of FOXO3a nuclear translocation (Supporting Information Fig. S4A, B). P300 mediates acetylation of FOXO3a by using acetyl CoA and abrogates the FOXO3a transcriptional activity.[31,32] Palmitate-conditioned

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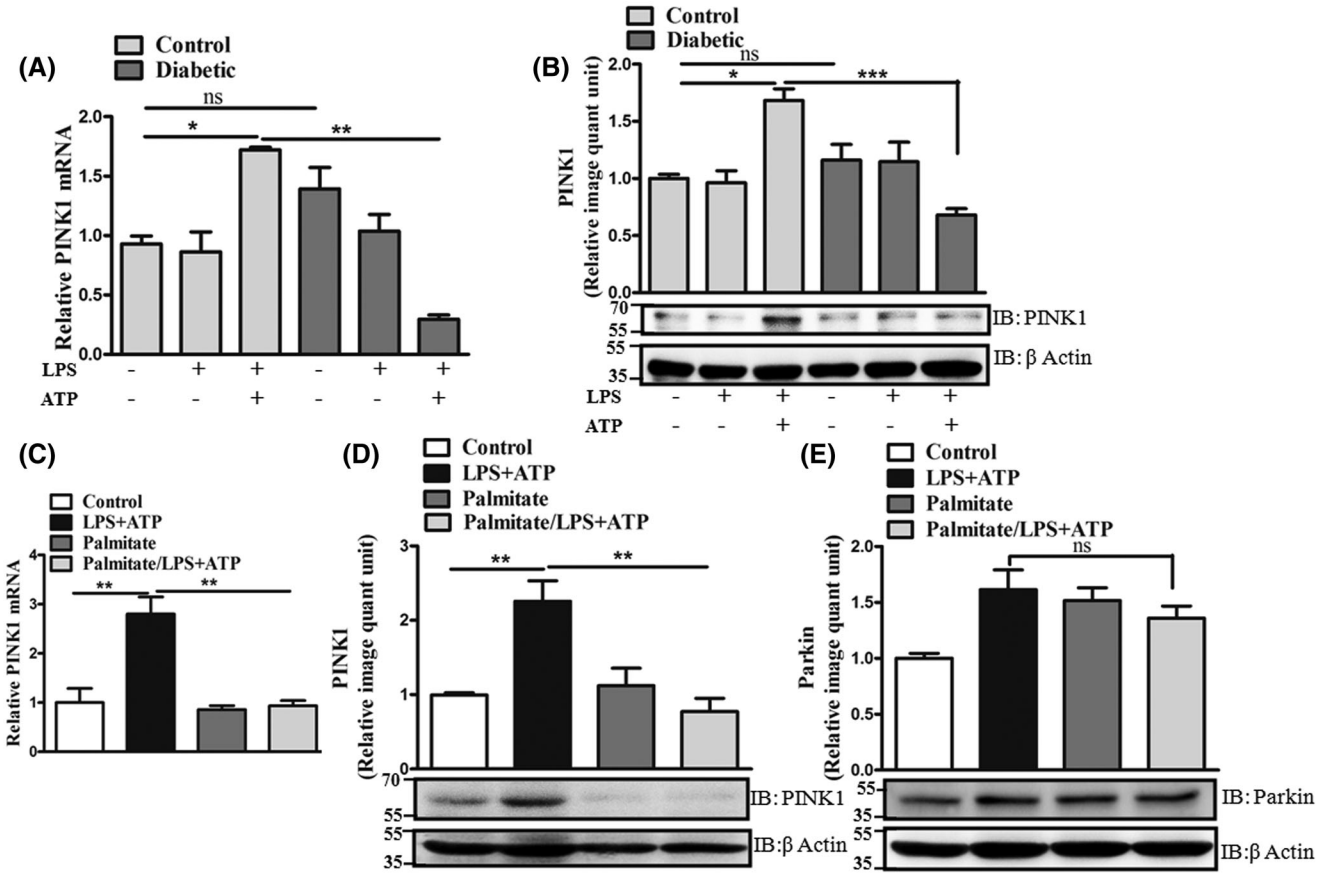

FIGURE 4 **Palmitate conditioning attenuates PTEN-induced kinase 1 (PINK1) expression in response to nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) agonist**. Diabetic macrophages were treated with LPS (4 h) and ATP (30 min); **(A)**RT-PCR analysis of PINK1 mRNA ($n = 3$), and **(B)** immunoblot analysis of PINK1 in total cell lysates ($n = 3$). Palmitate-conditioned macrophages were treated with LPS for 4 h and ATP for 30 min; **(C)** RT-PCR analysis of PINK1 mRNA ($n = 3$), Immunoblot analysis of **(D)** PINK1 ($n = 4$), **(E)** Parkin ($n = 3$) in the total cell lysates. The value "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

macrophages stimulated with NLRP3 agonist showed a significant ($P < 0.001$) increase in the acetyl CoA level compared to the macrophages treated with NLRP3 stimuli (Fig. 5A). Inhibition of fatty acid oxidation with etomoxir significantly ($P < 0.001$) decreased acetyl CoA level in palmitate-conditioned macrophages treated with NLRP3 stimuli (Fig. 5A). Further, we checked the acetylation of FOXO3a and found that NLRP3 agonist treatment in the palmitate-conditioned macrophages significantly ($P < 0.001$) increased acetylation of FOXO3a compared to the macrophages treated with NLRP3 stimuli (Fig. 5B). P300 inhibitor- C646 and SIRT1 activator (deacetylate FOXO)[33]- SRT1720 were used to attenuate acetylation of FOXO3a. Pretreatment with C646, SRT1720, and etomoxir significantly decreased acetylation of FOXO3a in the palmitate-conditioned macrophages stimulated with NLRP3 agonist (Fig. 5C). Next, we checked FOXO3a binding to PINK1 promoter and our data showed that palmitate-conditioned macrophages treated with NLRP3 agonist showed a significant ($P < 0.01$) decrease in FOXO3a binding to the PINK1 promoter (Fig. 5D). Interestingly, pretreatment with C646 and SRT1720 recovered the FOXO3a binding to PINK1 promoter (Fig. 5D) and PINK1 mRNA, protein expression (Fig. 5E, F) in NLRP3 agonist stimulated palmitate-conditioned macrophages, to the levels similar to NLRP3 agonist alone treated cells. These data suggest that FOXO3a acetylation in the palmitate-conditioned macrophages reduced PINK1 expression by decreasing PINK1 promoter binding.

3.6 | Modulation of FOXO3a acetylation by pharmacologic intervention recovers mitophagy and attenuates mtDNA release

Palmitate-conditioned macrophages treated with NLRP3 stimuli displayed the diffuse cytosolic distribution of Parkin and mitochondria (Fig. 6A). Acetylation modulators (C646 and SRT1720) and etomoxir pretreatment increased Parkin colocalization with mitochondria as well as Pearson's correlation coefficient in palmitate-conditioned macrophages treated with NLRP3 agonist (Fig. 6A, B). Furthermore, acetylation modulators (C646 and SRT1720) and etomoxir pretreatment reduced ($P < 0.001$, $P < 0.01$, $P < 0.05$) TIM44 and TOM20 levels (Supporting Information Fig. S5A, B) as well as increased ($P < 0.001$, $P < 0.01$, $P < 0.05$) LC3BII and DRP1 phosphorylation

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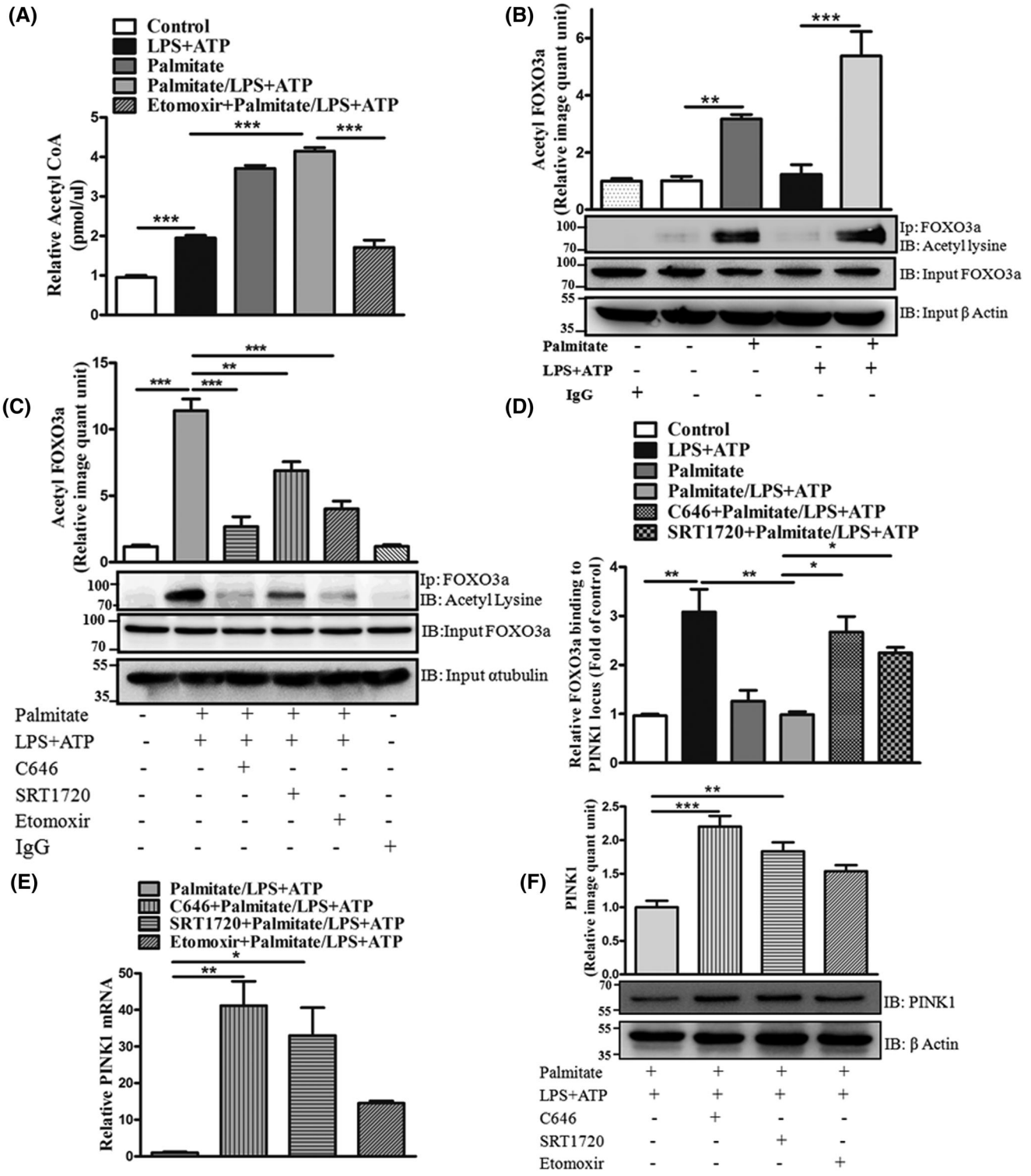

FIGURE 5 **Palmitate conditioning increases the acetylation of FOXO3a and inhibits its recruitment to the PTEN-induced kinase 1 (PINK1) promoter**. Peritoneal macrophages from wild-type (WT) mice were pretreated with etomoxir (Fatty acid oxidation inhibitor) 1 h before palmitate treatment and followed by LPS (4 h) and ATP (30 min) treatment, **(A)** Acetyl CoA level was detected in total cell lysates ($n = 3$). **(B)** FOXO3a was immunoprecipitated with anti-FOXO3a antibody from whole cell lysate and immune-blotted with anti-acetyl lysine antibody ($n = 3$). **(C)** Peritoneal macrophages from WT mice were pretreated with C646 (p300 inhibitor), SRT1720 (SIRT 1 activator), etomoxir 1 h before Palmitate treatment and followed by LPS and ATP treatment; and immunoblot analysis was done for acetyl FOXO3a ($n = 3$), **(D)** FOXO3a-DNA complexes were crosslinked by formaldehyde and immunoprecipitated with anti-FOXO3a antibody, PINK1 promoter enrichment was analyzed by RT-PCR and normalized with input DNA ($n = 3$), **(E)** RT-PCR analysis of PINK1 mRNA expression ($n = 3$), **(F)** Immunoblot analysis of PINK1 in total cell lysates ($n = 3$). The value "n" indicates number of biologic replicates. Data represented as mean ± SEM, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

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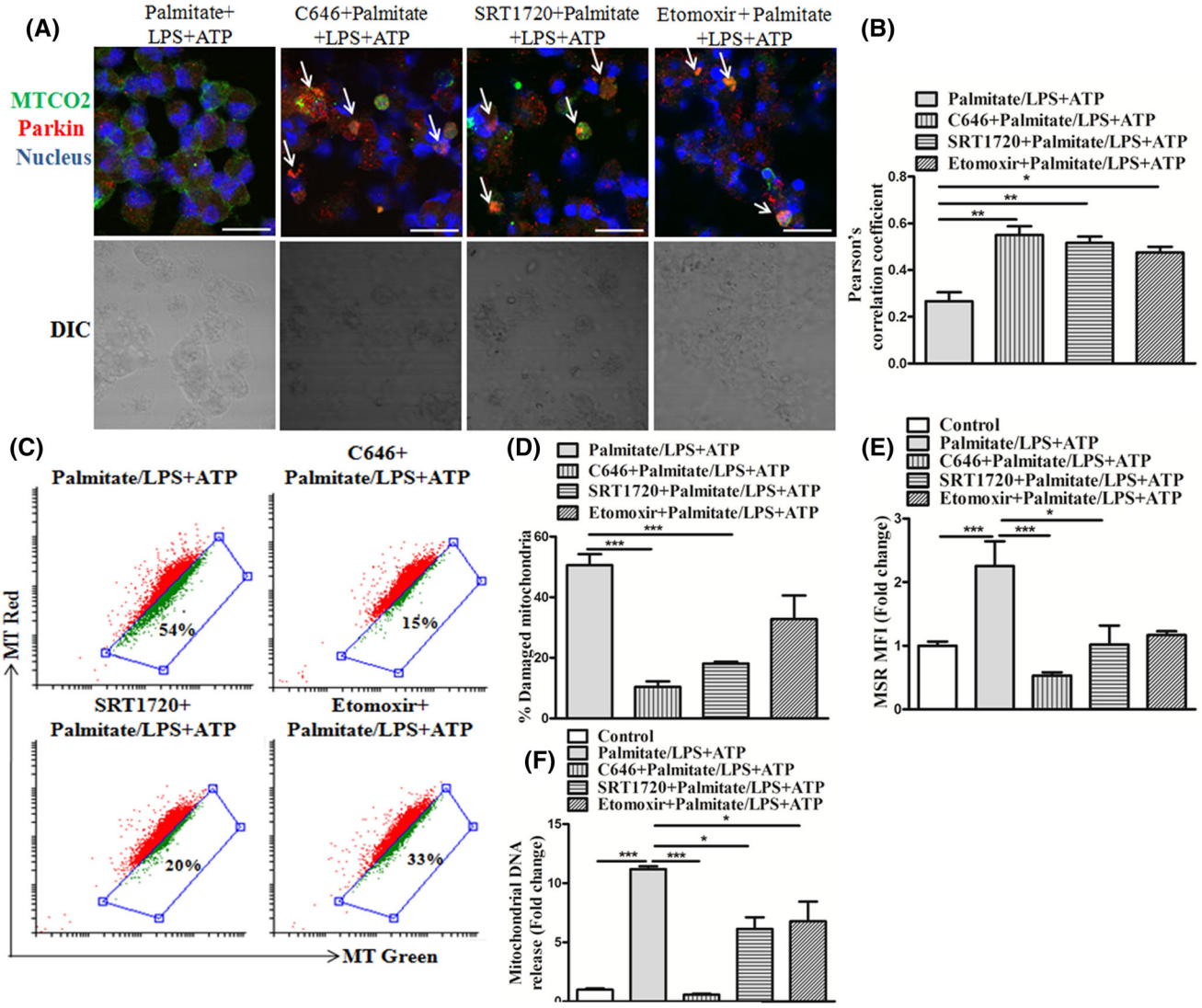

FIGURE 6 **Modulation of FOXO3a acetylation recovers mitophagy and attenuate mitochondrial reactive oxygen species (mtROS) and mitochondrial DNA (mtDNA) release.** Peritoneal macrophages from wild-type (WT) mice were pretreated with C646, SRT1720, and etomoxir 1 h before palmitate treatment and followed by LPS (4 h) and ATP (30 min) treatment. **(A)** Intracellular colocalization (oranges to yellow) of Parkin (stained with red) with mitochondria (stained with green) merged with nuclear stain DAPI (stained with blue), examined by confocal microscopy (scale bar, 5 μm). **(B)** The Pearson correlation coefficient indexes Parkin and MTCO2 colocalization intensity were determined in three independent experiments($n = 3$). **(C)** Damaged mitochondria were analyzed by flow cytometry labeling with mitotracker deep red and mitotracker green. Gates represent cells with damaged mitochondria. **(D)** Percent of damaged mitochondria was analyzed from 3 independent experiments ($n = 3$). **(E)** Mitochondrial ROS was measured by flow cytometry ($n = 3$). **(F)** mtDNA release was quantified by RT-PCR ($n = 3$). The value "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$

(Supporting Information Fig. S5C, D) in palmitate-conditioned macrophages treated with NLRP3 agonists when compared with respective controls. Consistent with the increased mitophagy, pretreatment with C646 and SRT1720, etomoxir decreased accumulation of damaged mitochondria ($P < 0.001$, Fig. 6C, D), mtROS ($P < 0.001$, $P < 0.05$; Fig. 6E) and mtDNA release ($P < 0.001$, $P < 0.05$; Fig. 6F) in the palmitate-conditioned macrophages stimulated with NLRP3 agonist when compared with respective control. Therefore current data suggest FOXO3a acetylation as a putative mechanism that leads to decreased mitophagy and enhanced mtROS and mtDNA release in palmitate-conditioned macrophages.

3.7 | Modulation of FOXO3a acetylation attenuates NLRP3 inflammasome activation

Because acetylation modulators decreased mtROS and mtDNA release in the palmitate-conditioned macrophages treated with NLRP3 agonist, we assessed the effect of FOXO3a acetylation modulation on NLRP3 inflammasome activation in palmitate-conditioned macrophages. Pretreatment with C646, SRT1720, and etomoxir decreased ASC oligomer formation (Fig. 7A) and caspase-1 activation ($P < 0.01$, $P < 0.001$) (Fig. 7B, C) in the palmitate-conditioned macrophages treated with NLRP3 agonist. Similarly, pretreatment

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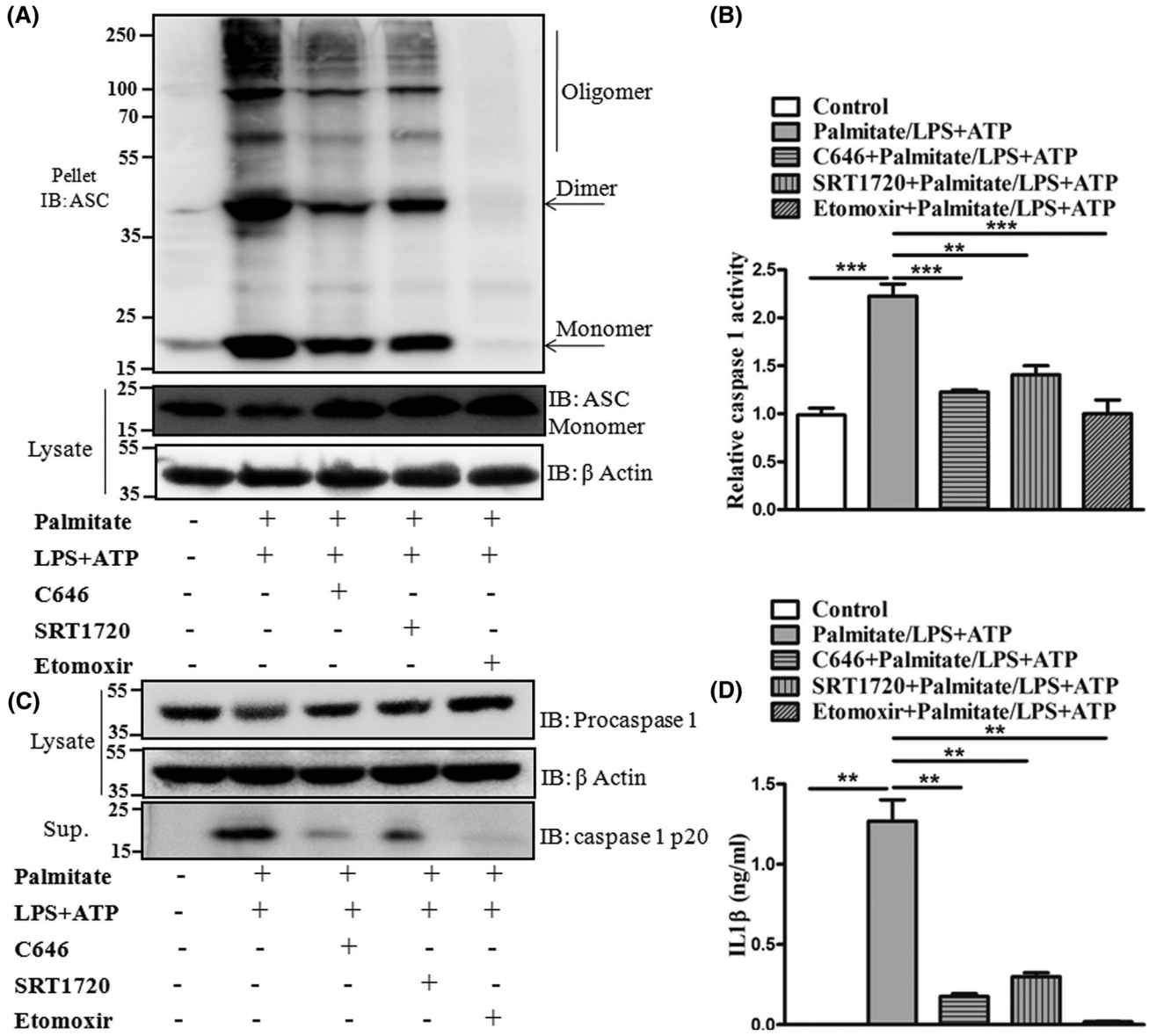

FIGURE 7 **Attenuation of FOXO3a acetylation inhibits nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome activation**. Peritoneal macrophages from wild-type (WT) mice were pretreated with C646, SRT1720, and etomoxir 1 h before palmitate treatment and followed by LPS (4 h) and ATP (30 min) treatment. **(A)** Apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization and redistribution assay in peritoneal macrophages; Immunoblot analysis of ASC in crosslinked pellets (upper panels) and ASC, β-actin in cell lysates (lower panels) ($n = 3$). **(B)** Caspase-1 activity in lysate measured by fluorometric assay ($n = 3$). **(C)** Immunoblot analysis of active caspase-1 in supernatant and procaspase-1, β-actin in total cell lysates ($n = 3$). **(D)** IL1β measured in the supernatant by ELISA ($n = 3$). The value "n" indicates number of biologic replicates. Data represented as mean ± SEM,$^{**}P < 0.01$, $^{***}P < 0.001$

with C646, SRT1720, and etomoxir also significantly ($P < 0.01$) decreased IL1β secretion from the similar experiment (Fig. 7D). Now, to assess the effect of these acetylation modulators on inflammasome priming, the NLRP3, IL1β mRNA expression, and secretory TNFα production were assessed. Pretreatment with C646, SRT1720, and etomoxir did not affect NLRP3 agonist-induced NLRP3, IL1β expression, and TNFα level in palmitate-conditioned macrophages (Supporting Information Fig. S6A–C). These results suggest that inhibition of FOXO3a acetylation repressed NLRP3 inflammasome activation.

3.8 | Modulation of FOXO3a acetylation in diabetic macrophages attenuates NLRP3 inflammasome activation

Next, we checked the acetylation of FOXO3a in the diabetic macrophages and its effect on NLRP3 inflammasome activation. As expected, diabetic macrophages treated with NLRP3 agonists showed a significant ($P < 0.001$) increase in FOXO3a acetylation (Fig. 8A). Pretreatment with C646 and SRT1720 in diabetic macrophages showed a significant ($P < 0.01$) decrease in NLRP3 agonist induced

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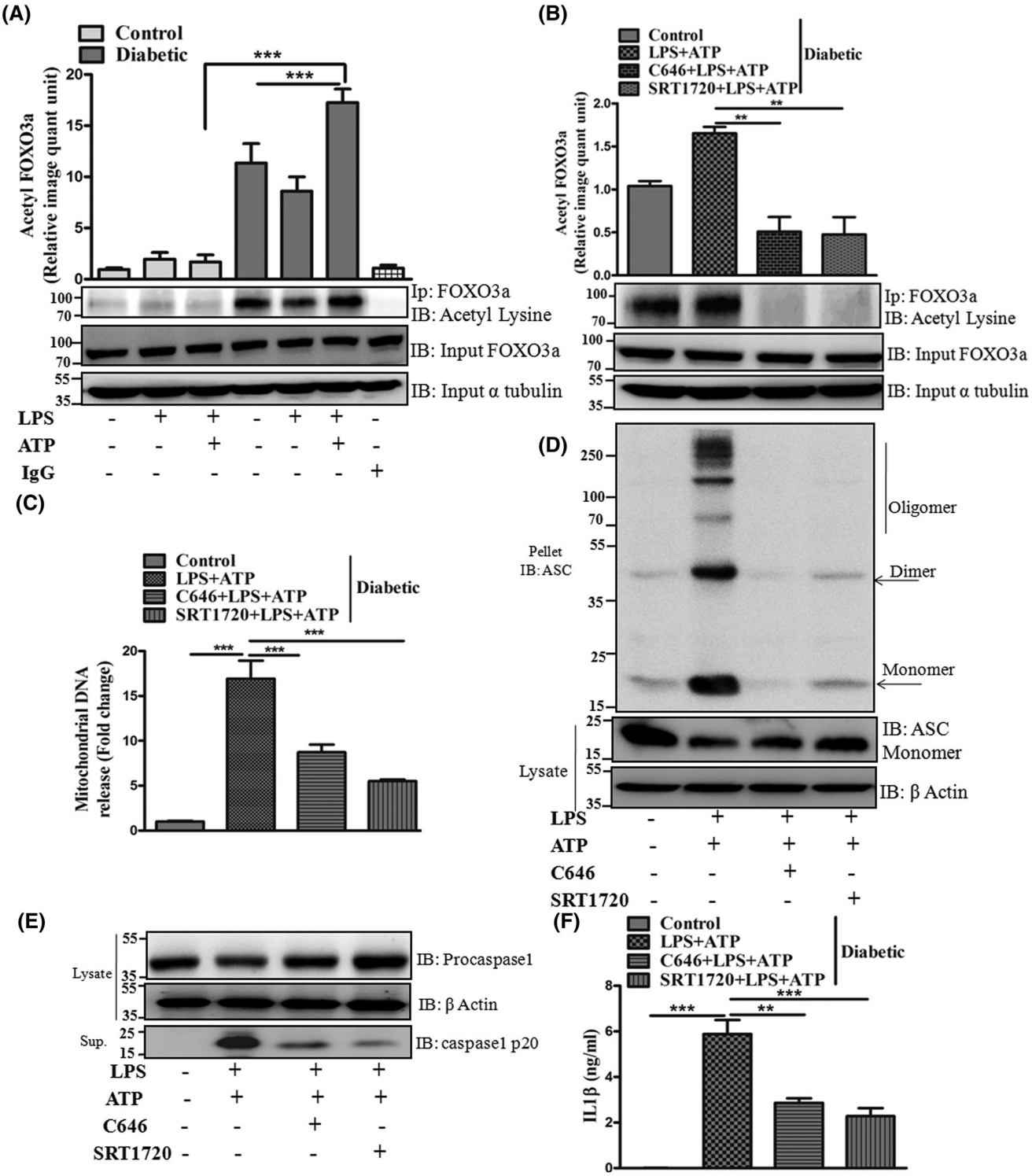

FIGURE 8 **Attenuation of FOXO3a acetylation in diabetic macrophages inhibits nucleotide-binding oligomerization domain, leucine-rich repeat, and pyrin domain containing 3 (NLRP3) inflammasome activation**. Control and diabetic macrophages were treated with LPS for 4 h and ATP for 30 min; **(A)** FOXO3a was immunoprecipitated with anti-FOXO3 antibody from whole cell lysate and immune-blotted with anti-acetyl lysine antibody ($n = 3$). Diabetic macrophages pretreated with C646, SRT1720 and followed by LPS and ATP treatment; **(B)** Immunoblot analysis of acetylated FOXO3a was done as in (A) ($n = 3$), **(C)** mitochondrial DNA (mtDNA) release was quantified by RT-PCR ($n = 3$), **(D)** apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) oligomerization and redistribution assay in diabetic macrophages; immunoblot analysis of ASC in crosslinked pellets (upper panels) and ASC, β-actin in cell lysates (lower panels) ($n = 3$), **(E)** active caspase-1 in the supernatant and procaspase-1, β-actin in total cell lysates ($n = 3$). **(F)** IL1β measured in supernatant from diabetic macrophages by ELISA ($n = 3$). The value "n" indicates number of biologic replicates. Data represented as mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$

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FOXO3a acetylation (Fig. 8B). Consequently, C646 and SRT1720 pretreatment in diabetic macrophages displayed reduced NLRP3 agonist induced mtDNA release ($P < 0.001$, Fig. 8C), ASC oligomerization (Fig. 8D), caspase-1 activation (Fig. 8E), and IL1β secretion ($P < 0.01$, $P < 0.001$, Fig. 8F). Furthermore, pretreatment of diabetic macrophages with etomoxir decreased ASC oligomerization, caspase-1 activation, and IL1β secretion ($P < 0.01$, Supporting Information Fig. S6D–F). These results establish the role of FOXO3a acetylation in enhanced NLRP3 inflammasome activation in the diabetic macrophages.

4 | DISCUSSION

Many inflammatory cytokines, including TNFα and IL-6, have been demonstrated as important inflammatory and prediabetic mediators; however, anti-inflammatory-based therapeutic approaches, targeting these two cytokines, in T2D and insulin resistance seem suboptimal. In contrast, a recent study indicates a concrete and promising therapeutic effect of anakinra (IL1 receptor blocker) on T2D patients in the clinical trial.[34] Thus, exaggerated IL1β production and the associated effect on muscle and liver cell metabolism are key phenomena of the pathogenesis of diabetes.[34,12] Apart from its key role in T2D pathogenesis, sustained and enhanced inflammasome activation in diabetic macrophages has been shown to impair wound healing in T2D patients as well as in db/db mice.[35,36] The current study explored the key signaling events associated with increased IL1β production in the diabetic macrophages.

Mitophagy is a type of selective autophagy that specifically targets damaged mitochondria for degradation.[37,38] The most explored pathway of mitophagy is regulated by PINK1 and Parkin.[37] PINK1 accumulates on the surface of depolarized mitochondria where it simultaneously recruits Parkin, having E3 ubiquitin ligase activity. This provides the basis of multiple signaling pathways that culminate in the engulfment of dysfunctional mitochondria by autophagosomes and subsequent degradation by lysosomes.[37,38]

Several studies present evidence to support the IGF-I/PI3K/Akt pathway in regulating the FOXO3a protein.[39,40] However, increasing evidence also demonstrates that FOXO3a signaling can be controlled by protein-protein interactions and additional posttranslational modifications, which are distinct from Akt mediated phosphorylation.[41,31] FOXO3a acetylation and deacetylation also affect the transcriptional activity of FOXO3a. p300 interacts with FOXO3a and regulates FOXO3a activity by acetylation in skeletal muscle indicating that the acetyl-transferase activity of p300 is both essential and enough to repress FOXO3a activity.[31] Inactivation of the Akt pathway increases PINK1 expression by facilitating FOXO3a binding to the PINK1 promoter.[30] Consistent with the previous reports we also observed that NLRP3 inflammasome activation in macrophages occurred with decreased Akt phosphorylation and increased FOXO3a translocation to the nucleus and subsequently it augments PINK1 expression by its binding to the PINK1 promoter. Previous reports suggest that PINK1 expression is reduced during palmitate-induced insulin resistance in both HepG2 cells and the db/db mice or high fat diet (HFD) fed mice during the insulin-resistant condition.[42] Hepatic cells of *ob/ob* mice showed reduced PINK1/PARKIN mediated mitophagy under metabolic stress caused by ad libitum feeding of chow diets.[43] Similarly, in the present study, PINK1 expression and mitophagy were decreased in both the palmitate-conditioned macrophages and the diabetic macrophages in response to NLRP3 stimuli. This was accompanied by an increased number of damaged mitochondria and mtDNA release in both the palmitate-conditioned macrophages and the diabetic macrophages in response to NLRP3 stimuli. Free circulating mtDNA was also found in the serum of T2D patients.[44] Studies on clinical and experimental models indicate that damage and loss of mtDNA are emerging as a molecular signature of metabolic diseases such as T2D and obesity.[45]

Maintenance of a high level of acetyl CoA in the cytosol inhibits autophagy because of the acetylation of multiple components of autophagy protein by P300 protein.[32] The depletion of acetyl CoA reduced the activity of the acetyl-transferase P300, thereby increasing autophagy.[32] Palmitate is a free fatty acid that converts to eight molecules of acetyl CoA upon β oxidation. In the present study, palmitate conditioning in macrophages increased acetyl CoA level and acetylation of FOXO3a, which was inhibited by etomoxir treatment. FOXO3a acetylation reduced its binding to the PINK1 promoter and hence decreased PINK1 expression. We observed that attenuation of FOXO3a acetylation by P300 inhibitor and SIRT1 activator decreased caspase-1 activation and IL1β release by increasing the PINK1 mediated mitophagy. P300 inhibitor treatment in HFD fed mice and ob/ob mice improve glucose tolerance and insulin resistance by deacetylating IRS1/2.[46] It can be speculated that a P300 inhibitor might affect insulin resistance by inhibiting FOXO3a acetylation and subsequent NLRP3 inflammasome in macrophages of diabetic mice. Modulation of FOXO3a acetylation by etomoxir also decreased inflammasome activation and resulted in reduced ASC oligomerization, caspase-1 activation and IL1β release. However, a dramatic decrease in the pellet ASC monomer could be due to the profound effect of the inhibitor on inflammasome activation leading to complete inhibition of ASC translocation and oligomerization. Besides this, etomoxir may affect overall ASC pool by inhibiting its expression. In a previous study, etomoxir decreased LPS-induced ASC protein expression in human colon epithelial cancer cells.[47]

Mitophagy engages a sequence of events: mitochondrial fission, autophagosome formation, and subsequent fusion with the lysosome. pDRP1^{Ser616} primarily regulates mitochondrial fission to initiate mitophagy.[48] A recent study indicates that PINK1 phosphorylates DRP1^{Ser616} to regulate mitochondrial fission.[49] We observed decreased PINK1 and pDRP1^{Ser616} expression in both diabetic and palmitate-conditioned macrophages in response to NLRP3 agonist. It might be possible that decreased PINK1 expression may be responsible for reduced phosphorylation of DRP1^{Ser616}, and hence modulate mitochondrial dynamics in our study. Furthermore, treatment with acetylation modulators recovered both PINK1 and p-DRP1^{Ser616} expressions and hence increased mitophagy in palmitate-conditioned macrophages treated with NLRP3 agonist.

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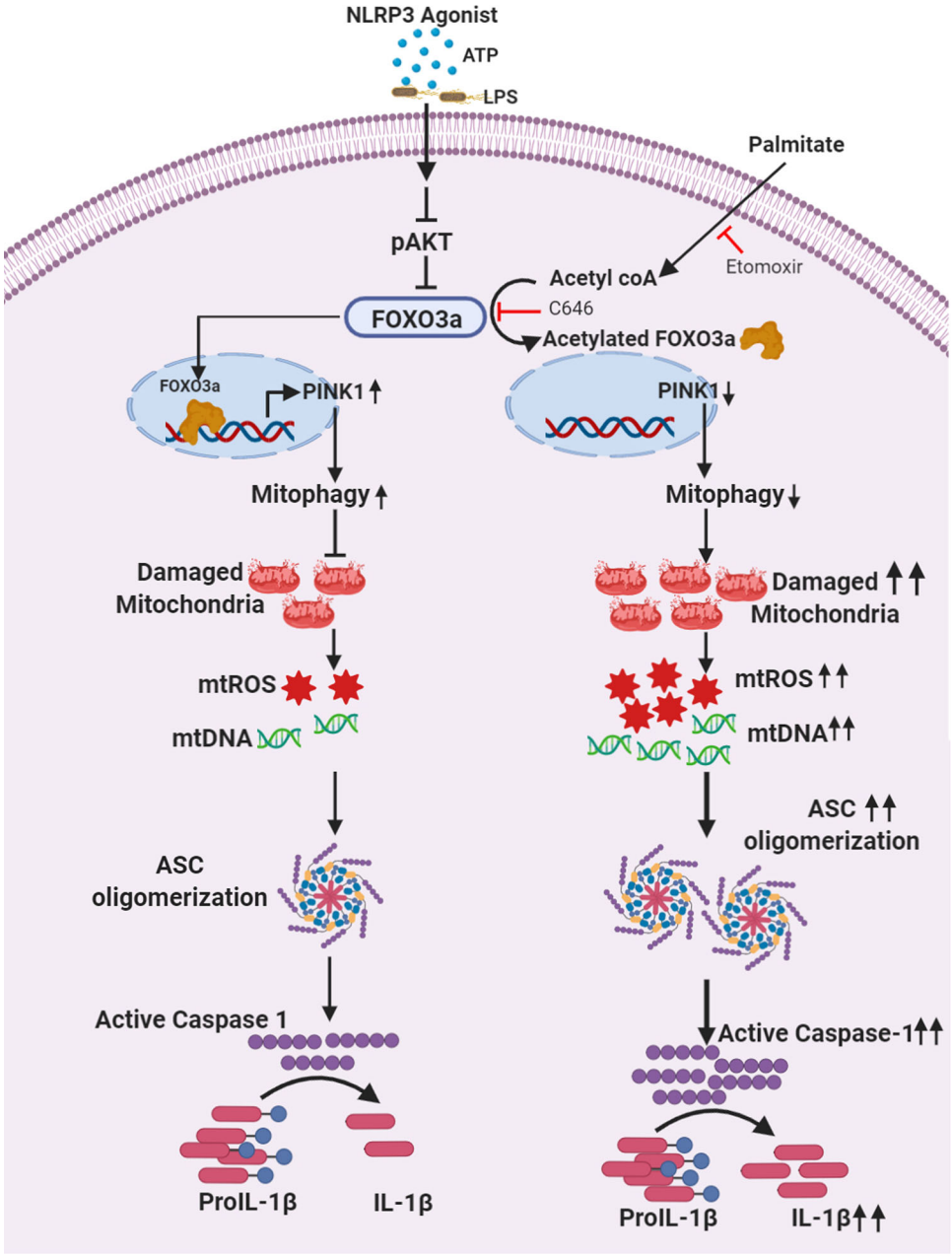

FIGURE 9 **Schematic illustration of the proposed sequence of events in palmitate conditioning macrophages**. Palmitate conditioning inhibits PTEN-induced kinase 1 (PINK1) mediated mitophagy by FOXO3a acetylation to exacerbate NLRP3 inflammasome activation

Mitochondrial ROS activates inflammasome and also contributes to its priming by promoting Hif1α-mediated Pro-IL1β transcription.[50,51] It can be speculated that increased Pro-IL1β expression in the palmitate-conditioned macrophages might be due to increasing mtROS at basal level and after treatment with LPS/ATP. In palmitate-conditioned macrophages, NLRP3 mRNA expression was not changed; it indicates that increased mtROS and mtDNA are only accountable for exacerbated NLRP3 inflammasome activation. Diet-induced obesity in mice increased intestinal permeability, which sustains plasma concentration of LPS.[46] The increased basal level of mtROS and sustained LPS in plasma might be increasing basal NLRP3 and IL1β mRNA expression in diabetic macrophages. It can be concluded that increased NLRP3 mRNA expression with enhanced mtROS and mtDNA in cytosol upholds the NLRP3 inflammasome activation in the diabetic macrophages.

A few, mainly in vitro studies indicate a possible beneficial role of IL-1β in insulin secretion and β-cell survival.[52,53] While the deleterious effect of IL-1β high-dose and long-term exposure on islet function and mass is also well documented, indicating the dynamic nature of IL-1β.[54,55] Pancreatic β-cells have the highest expression of the IL-1 receptor in comparison to any tissue,[56] and the IL-1 receptor is the most abundantly expressed surface receptor on β-cells.[57] Hence, exaggerated inflammasome activation and IL1β production from diabetic macrophages can contribute to islet cell damage or

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death and therefore lead to the development of insulin-dependent T2D.

Evidence from other studies also suggests the importance of FOXO3a and mitophagy in neurodegenerative diseases.[58] Amyloid precursor protein intracellular domain enhances PINK1 transactivation in a FOXO3a-dependent manner and influences mitophagy and mitochondrial dynamics in cells and in vivo Alzheimer's disease model.[58] A recent study showed that palmitate is increased in the cerebrospinal fluid of obese patients with mild cognitive impairment.[59] Therefore, it can be speculated that the earlier-observed phenotype in diabetic condition might be associated with neurodegenerative changes.

In summary, the present work illustrated that palmitate and diabetic condition exacerbated an inflammatory IL-1β response by modulating mitochondrial homeostasis in macrophages. Palmitate-induced FOXO3a acetylation prevents FOXO3a binding to the PINK1 promoter, and therefore decreases PINK1 mediated mitophagy and increases NLRP3 inflammasome activation (Fig. 9). Modulation of FOXO3a acetylation restored FOXO3a binding to PINK1 promoter and PINK1 mediated mitophagy and thus attenuated NLRP3 inflammasome activation under diabetic condition.

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DISCLOSURES

The authors declare no conflicts of interest.

AUTHORSHIP

P.G. planned and executed the experiments and drafted the manuscript; G.S. helped in the execution of the experiments; A.L. provided critical inputs on the manuscript; and M.K.B. conceptualized the work and was involved in data interpretation and manuscript formulation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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