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—Original Article—

Palmitic acid activates NLRP3 inflammasome and induces placental inflammation during pregnancy in mice

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Abstract. Maternal obesity is one of the major risk factors for pregnancy complications and is associated with low-grade chronic systemic inflammation due to higher levels of pro-inflammatory cytokines such as interleukin (IL)-1β. Pregnant women with obesity have abnormal lipid profiles, characterized by higher levels of free fatty acids, especially palmitic acid (PA). Previously, we reported that PA stimulated IL-1β secretion via activation of NLRP3 inflammasome in human placental cells. These observations led us to hypothesize that higher levels of PA induce NLRP3 inflammasome activation and placental inflammation, resulting in pregnancy complications. However, the effects of PA on NLRP3 inflammasome during pregnancy *in vivo* remain unclear. Therefore, PA solutions were administered intravenously into pregnant mice on day 12 of gestation. Maternal body weight was significantly decreased and absorption rates were significantly higher in PA-injected mice. The administration of PA significantly increased IL-1β protein and the mRNA expression of NLRP3 inflammasome components (*NLRP3, ASC*, and caspase-1) within the placenta. In murine placental cell culture, PA significantly stimulated IL-1β secretion, and this secretion was suppressed by a specific NLRP3 inhibitor (MCC950). Simultaneously, the number of macrophages/monocytes and neutrophils, together with the mRNA expression of these chemokines increased significantly in the placentas of PA-treated mice. Treatment with PA induced ASC assembling and IL-1β secretion in macrophages, and this PA-induced IL-1β secretion was significantly suppressed in NLRP3-knockdown macrophages. These results indicate that transient higher levels of PA exposure in pregnant mice activates NLRP3 inflammasome and induces placental inflammation, resulting in the incidence of absorption.

Key words: Inflammation, NLRP3 inflammasome, Placenta, Pregnancy

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The incidence of maternal obesity has recently been on the rise around the world due to the modern lifestyle and obesity is a major risk factor in pregnancy complications such as gestational diabetes, spontaneous miscarriage, intrauterine growth restriction, and preeclampsia [1, 2]. Obesity is a low-grade chronic systemic inflammation and associated with insulin resistance, cardiovascular diseases, and diabetes [3]. Pregnant women with obesity are associated with elevated serum levels of pro-inflammatory cytokines such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α, compared to pregnant women with normal weight [4–7]. Pregnant women with obesity have abnormal lipid profiles, characterized by higher levels of free fatty acids (FFAs) [8, 9]. It has been reported that palmitic acid (PA), the most abundant saturated FFAs in the blood, promotes inflammatory responses by directly engaging toll-like receptors (TLR), and inducing nuclear factor-κB (NF-κB)-dependent production of inflammatory cytokines [10, 11]. Previously, we reported that PA caused inflammatory cytokine secretion, inducing IL-1β, IL-6, and IL-8 in human placental cells [12], suggesting that obesity-related PA accumulation induces placental inflammation.

Recently, there have been numerous reports of inflammasome mechanisms that control sterile inflammation involved in pregnancy pathologies [13–16]. Inflammasomes are large multi-protein complexes found in the cytosol, which play key roles in the production of the pivotal inflammatory cytokines, IL-1β and IL-18, and pyroptosis (inflammatory cell death) [17–19]. In particular, nucleotide-binding oligomerization domain, leucine-rich repeat-, and pyrin domain-containing 3 (NLRP3) inflammasome is a key mediator of sterile inflammation. Excessive activation of the NLRP3 inflammasome contributes to the pathogenesis of a wide variety of diseases, such as diabetes, atherosclerosis, and obesity-induced insulin resistance [11, 20–24]. The NLRP3 inflammasome is activated in obese liver and in non-alcoholic steatohepatitis, and these phenomena are inhibited

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in NLRP3 deleted mice [25, 26]. In addition, PA induces activation of the NLRP3 inflammasome, resulting in the secretion of mature IL-1β from macrophages, Kupffer cells, and human trophoblast cells [11, 12, 26]. In particular, PA causes intracellular crystallization, which in turn activates the NLRP3 inflammasome via lysosomal dysfunction in macrophages [27].

These observations led us to hypothesize that higher levels of PA in maternal tissue induce NLRP3 inflammasome activation and placental inflammation, resulting in association with pregnancy complications. However, the effects of PA on NLRP3 inflammasome and placental inflammation during pregnancy *in vivo* remain unclear. In the present study, we tested a working model in which PA induces NLRP3 inflammasome activation and placental inflammation *in vivo*.

Materials and Methods

Animal model

All experiments were complied with the Ethics Committee on Animal Rights Protection and conducted following the Tokyo University of Agriculture Guide for Laboratory Animals (No.300099). We purchased Slc:ICR wild-type mice from Japan SLC (Shizuoka, Japan). All mice used in this study were female and aged 8–12 weeks. Mice were mated, and the presence of a vaginal plug was confirmed the next day, which was designated the first gestational day (GD). Body weight was measured on days 12–17 in pregnant mice.

Ethyl palmitic acid solution for the experiment was prepared following previous methods [27, 28]. Summarily, ethyl palmitate (Tokyo Chemical Industry, Tokyo, Japan) was dissolved with 1.6% lecithin (FUJIFILM Wako Pure Chemical, Osaka, Japan) and 3.3% glycerol in water to produce a mixture of 450 mM ethyl palmitate, 1.2% lecithin, and 2.5% glycerol. The mixture was then emulsified using a sonicator. The lecithin-glycerol-water solution was used as the vehicle. On GD12, ethyl palmitate solution (200 µl) was administered from the tail vein (administration of 90 µmol). Six hours later, on GD13, or GD17, the mice were sacrificed, and the fetuses, placentas, and spleen were excised and weighed. The fetal resorption rate was calculated with the equation: fetal resorption rate = (the number of resorbed fetuses)/(the number of viable fetuses + resorbed fetuses).

Murine placental cell isolation

Placental cells were isolated from pregnant mice on GD 16–17 as previously described with some modifications [14]. Summarily, the placental tissue was minced, vigorously agitated in phosphate buffered saline (PBS) supplemented with 1 µg/ml heparin for 30 sec, and centrifuged at 1,000 × *g* for 5 min. The pellet was collected and incubated in a 0.1% collagenase solution (FUJIFILM Wako Pure Chemical) in Dulbecco's Modified Eagle Medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) for 30 min at 37°C with gentle stirring. The pellet was then resuspended and filtered (through a 70 µm filter). The cells were washed and treated with erythrocyte-lysing buffer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and resuspended in PBS supplemented with 3% fetal bovine serum (FBS, Dainippon Pharmaceutical, Osaka, Japan) for flow cytometry analysis, and in DMEM supplemented with 5% FBS for culture experiments.

Murine placental cell experiment

Murine placental cells were plated at a concentration of 1×10^5 cells/well in a 24-well culture plate (Thermo Fisher Scientifc, Waltham, MA, USA) following previous our methods [14]. The day after seeding, non-adherent cells were removed. To check the existence of inflammatory cells, mRNA expression of inflammatory cytokines including *IL-1β* and white blood cell marker *CD45* in adherent cells were significantly lower compared with murine macrophages, indicating the removal of inflammatory cells (data not shown). The cells were treated with PA solution (400 µM) or with vehicle solution. After 6 h, the supernatant and cell lysate were collected for enzyme-linked immunosorbent assay (ELISA), real-time PCR, and western blot analysis. In the experiments for studying the role of NLRP3 inflammasome, murine placental cells were stimulated with PA solution in the absence or presence of the NLRP3 inhibitor MCC950 (50 µM, Sigma-Aldrich).

Stable ASC-GFP THP-1 cell experiment

THP-1 cells that stably expressed ASC-GFP were prepared based on methods in previous study [29]. ASC-GFP THP-1 cells were plated at a concentration of 1×10^5 cells/well in an 8-well chamber plate (Eppendorf, Hamburg, Germany). ASC-GFP THP-1 cells were differentiated with phorbol 12-myristate 13-acetate (PMA, 100 µM, Sigma-Aldrich) into macrophages for 24 h, and the cells were treated with PA solution at 400 µM or with the vehicle solution. After 6 h, ASC-GFP THP-1 cells were covered with VECTERSHIELD with 4′,6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA) and were observed using a fluorescence microscope (Leica Microsystems, Tokyo, Japan). The positive fluorescence staining cells for ASC speck-like formation were counted. Quantification was performed on five randomly chosen areas. The results represent the findings of at least 3 independent experiments.

NLRP3 knockdown THP-1 cells by CRISPR/Cas9-mediated genome editing

Human codon-optimized *Cas9* and single-guide RNA (sgRNA) expressing vectors (LentiCRIRPRv2) were obtained from Addgene (Cambridge, MA, USA). The sgRNA targeting NLRP3 was designed with CRISPR direct (http://crispr.dbcls.jp). The target sequences of sgRNA are 5'-GGGCGAGGAGCTGTTCACCG-3' (GFP) and 5'- GATCGCAGCGAAGATCCACA-3' (NLRP3). To prepare the lentiviral vectors, LentiX293T (Takara Bio, Shiga, Japan) cells were co-transfected together with LentiCRISPRv2, pLP1, pLP2, and pVSVG using PEI MAX (Polysciences, Warrington, PA, USA). Culture media containing the lentiviral vectors were collected 3 days after transfection. The collected media were filtered with a 0.45-µm filter and ultracentrifuged at 21,000 rpm using a SW55 Ti rotor (Beckman Coulter, Brea, CA, USA), and the pellets were resuspended in PBS containing 5% FBS. The lentivirus titer was measured using a Lentivirus qPCR Titer kit (Applied Biological Materials, Richmond, BC, Canada). To generate NLRP3-mutated cell, THP-1 cells (human monocyte/macrophage cell line) were incubated with lentiviral vectors for 16 h in the presence of 8 µg/ml polybrene (Sigma-Aldrich). The transduced cells were selected by incubating them with 2 µg/ml puromycin (Sigma-Aldrich) for 3 days. Protein expression of NLRP3 and β-actin (ACTB) was

determined by western blotting.

The mutated THP-1 cells targeting GFP or NLRP3 were plated at a concentration of 1×10^5 cells/well in a 48-well culture plate and differentiated with PMA into macrophages for 24 h, and the cells were treated with PA solution at 400 μM or with the vehicle solution. After 6 h, the supernatant was collected for ELISA.

Real-time RT-PCR

Total RNA was prepared using ISOGEN II (Nippon Gene Company, Toyama, Japan) according to the manufacturer's instructions and cDNA production were performed a commercial kit (ReverTra Ace; Toyobo, Tokyo, Japan). Real-time RT-PCR was performed using the CFX Connect™ Real Time PCR (Bio-Rad, Hercules, CA) to detect mRNA expressions of *Il1β*, *Il6*, *Nlrp3*, *Asc*, *Casp1*, *Tnfa*, *Ccl5*, *Cxcl2*, *CD45*, *Emr1*, *Ly6g*, *CD11c*, and *Gapdh*. Primers are listed in Supplementary Table 1 (online only). RT-qPCR was performed in duplicate with a final reaction volume of 20 μl containing 10 μl SYBR Green (Thunderbird SYBR qPCR Mix, Toyobo), 7.8 μl distilled water, 0.1 μl of each primers (forward and reverse at 100 μM), and 2 μl of cDNA template. The amplification program consisted of a 5 min denaturation at 95˚C followed by 40 cycles of amplification (95˚C for 10 sec, 56–60˚C for 10 sec, and 72˚C for 20 sec). Expression levels of each target gene were normalized to corresponding *Gapdh* threshold cycle (CT) values using the ΔΔ CT comparative method [30].

Determination of cytokines

Levels of IL-1β and IL-6 were determined using a human ELISA kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Flow cytometry analysis

To analyze the inflammatory cells within placental tissue of pregnant mice, five placentas from each pregnant mouse were used, as previously described [14]. Briefly, mice were sacrificed under general anesthesia. Placental cells were isolated from mice as described previously [14]. The placental cells were washed, treated with erythrocyte lysis buffer, and resuspended in PBS supplemented with 3% FBS for flow cytometry analysis. Cells were labeled with the following antibodies purchased from eBioscience (San Diego, CA, USA): phycoerythrin (PE)-conjugated anti-Ly6G, PE-conjugated anti-CD11b, and allophycocyanin-conjugated anti-CD45. The cells were examined by flow cytometry (NovoCyte flowcytmeter; ACEA Biosciences, San Diego, CA, USA). Isotype control antibodies were used as negative controls to exclude nonspecific background staining.

Western blot analysis

Lysates from the cell culture were prepared using RIPA buffer (FUJIFILM Wako Pure Chemical). The expression of NLRP3, and ACTB were analyzed using SDS-PAGE. After transfer onto polyvinylidene fluoride membranes, nonspecific antibody binding was blocked for 1 h at room temperature using Immunoblock (DS Pharma Biomedical, Osaka, Japan). Then, membranes were incubated for 24 h at 4°C with anti-NLRP3 antibody (1:1000, R&D) and anti-ACTB antibody (1:10000, Sigma-Aldrich), followed by an incubation for 1 h with secondary antibody conjugated horseradish peroxidase (HRP; 1:1000, GE Healthcare, UK, Buckinghamshire, UK). Immunoreactive bands were visualized by Western BLoT Quant HRP Substrate (GE Healthcare) using ImageQuant LAS 4000 (GE Healthcare). The results represent at least 3 independent experiments. Quantitative analysis of bands was performed using Image J (National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are expressed as mean ± standard error of the mean (SEM). Differences between treatment groups were identified using unpaired *t*-tests. A P-value of < 0.05 was considered to be statistically significant.

Results

Long-term effects of PA on dams and fetuses during pregnancy

To investigate the effects of PA during pregnancy, pregnant mice were administered intravenously with ethyl palmitate (450 mM) on GD12. Compared with vehicle-injected mice, the maternal body weight gain was significantly decreased after GD14 in PA-injected mice (Fig. 1A). On GD17, the absorption rate was significantly higher in PA-injected mice (Fig. 1B, 1 fetus/75 fetuses of 5 dams in vehicle-injected mice, 43 fetuses/62 fetuses of 5 dams in PA-injected mice), and most fetuses died *in utero*. Therefore, we did not investigate the effect of PA within the placenta because it was completely white and withered. The weight of spleen was significantly higher in PA-injected mice (Fig. 1C). The mRNA expression of *Il1β* and *Il6* in spleen tissue were stimulated by the administration of PA (Fig. 1D and E), indicating the occurrence of inflammatory responses by PA injection in pregnant mice.

Short-term effects of PA on dams and fetuses during pregnancy

To investigate the effects of PA on placentas, pregnant mice were administrated intravenously with ethyl palmitate on GD12, and the placenta was collected at 6 and 24 h after PA administration. The maternal body weight was gradually decreased and the absorption rate was gradually increased in a time-dependent manner in PA-injected mice compared to vehicle-injected mice (Fig. 2A and B, 0 fetuses/71 fetuses of 5 dams in vehicle-injected mice, 13 fetuses/51 fetuses of 5 dams at 6 h in PA-injected mice, 25 fetuses/61 fetuses of 5 dams at 24 h in PA-injected mice).

In the placenta, the administration of PA significantly increased IL-1β and IL-6 protein production as well as mRNA expression of *Il1β* and *Il6* (Fig. 2C–E and I). Also, the mRNA expression of NLRP3 inflammasome components such as *Nlrp3*, *Asc*, and *Casp1*, were significantly increased by PA administration within the placenta (Fig. 2F–H), indicating the possible role of PA in inducing NLRP3 inflammasome activation in the placenta. In addition, mRNA expression of *Tnfa* (one of the major inflammatory cytokines in the placenta), *Ccl5* (major chemoattractant for monocytes and T cells), and *Cxcl2* (major chemoattractant for neutrophils and monocytes) were also increased in the placenta by PA administration (Fig. 2J–L).

Short-term effects of PA on immune cell accumulation in placenta

Because it is widely accepted that placental inflammatory responses

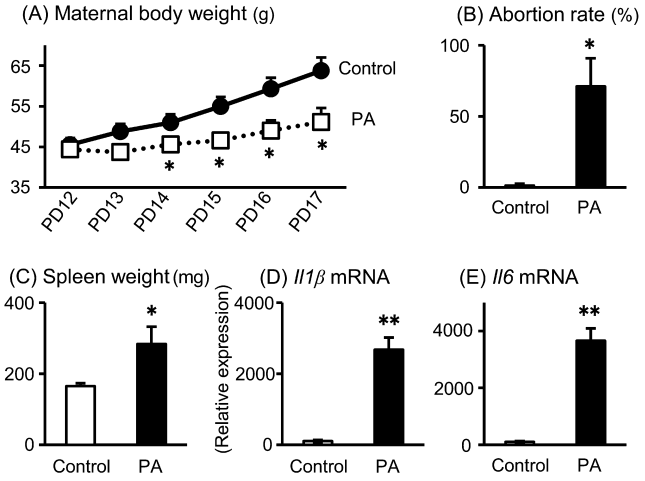

Fig. 1. Long-term effects of palmitic acid (PA) on dams and fetuses during pregnancy. Pregnant mice were injected intravenously with vehicle (control) or PA (450 mM) on gestational day (GD) 12. (A) Changes in maternal body weight were assessed (n = 5–6 per group). (B) Abortion rate was measured on GD 17 (n = 5–6 per group). (C) Spleen weights were assessed at GD 17 (n = 5–6 per group). (D and E) The *Il1β* and *Il6* mRNA levels in the spleen were assessed on GD 17 (n = 5–6 per group). Data are expressed as mean ± SEM. * P < 0.05 and ** P < 0.01 *vs*. vehicle.

are accompanied by pregnancy complications [31], we examined the degree of inflammatory cell infiltration in the placentas of PA-treated mice by flow cytometry analysis. The numbers of macrophages/monocytes ($CD45^+CD11b^+$) and neutrophils ($CD45^+Ly6G^+$) were significantly increased in the placentas of PA-treated mice compared to control mice (Fig. 3A and B). Similar with the flow cytometric data, mRNA expressions of *CD45* (a marker of leukocytes), *Emr1* (a marker of macrophages/monocytes), and *Ly6g* (a marker of neutrophils) were significantly higher in the placentas of mice in the PA-injected group than those in the vehicle-treated group (Fig. 3C–E). However, there were no differences in *CD11c* mRNA expression (a marker of dendritic cells) between treated groups (Fig. 3F). These data indicate that immune cells such as macrophages/monocytes and neutrophils are accumulated in the placenta by PA administration.

Effects of PA on IL-1β secretion in murine placental cells

Previously, we reported that PA could increase IL-1β production and secretion via NLRP3 inflammasome activation in human placental cells [12]. To determine the possible role of NLRP3 inflammasome in murine placental cells, we utilized a specific inhibitor of NLRP3 (MCC950). Palmitic acid (400 μM) significantly stimulated IL-1β secretion and its mRNA expression (Fig. 4A and B). Additionally, treatment with the NLRP3 inhibitor significantly suppressed IL-1β

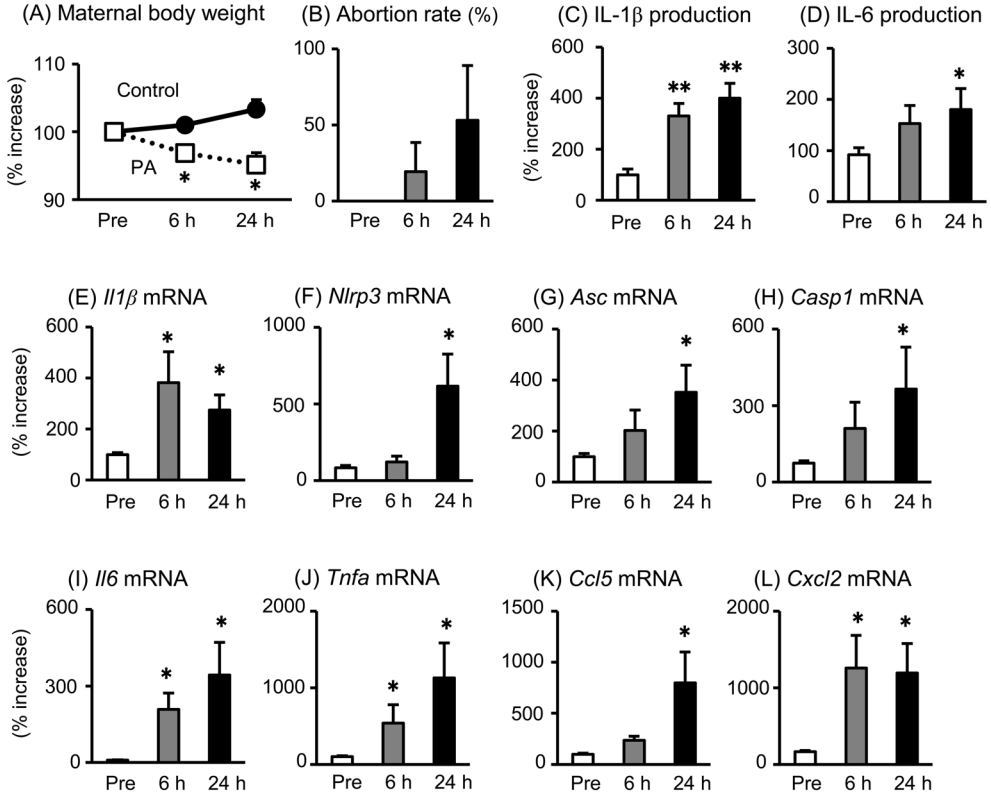

Fig. 2. Short-term effects of palmitic acid (PA) on dams and fetuses during pregnancy. Pregnant mice were injected intravenously with vehicle (control) or PA (450 mM) on gestational day (GD) 12. (A) Changes in maternal body weight were assessed (n = 5 per group). (B) Abortion rate was measured. (C and D) The IL-1β and IL-6 protein levels in the placenta were assessed (n = 5 per group). (E–L) The *Il1β*, *Nlrp3*, *Asc*, *Casp1*, *Il6*, *Tnfa*, *Ccl5* and *Cxcl2* mRNA levels in the placenta were assessed (n = 5 per group). Data are expressed as mean ± SEM. * P < 0.05 *vs*. vehicle.

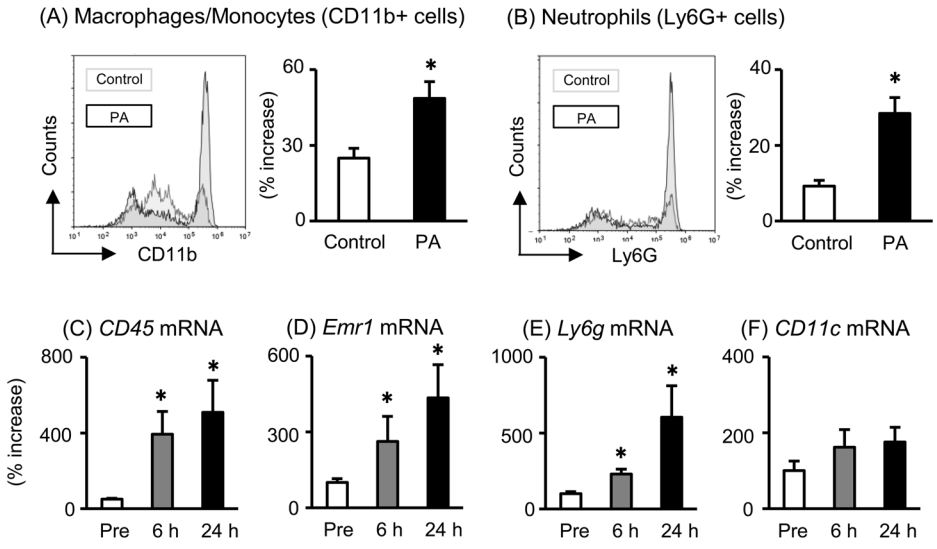

Fig. 3. Short-term effects of palmitic acid (PA) on immune cell accumulation in placenta. Pregnant mice were injected intravenously with vehicle (control) or PA (450 mM) on gestational day (GD) 12. (A and B) Percentages of monocytes or macrophages ($CD45^+/CD11b^+$ cells) and neutrophils ($CD45^+/Ly6G^+$ cells) in the placenta were analyzed by flow cytometry (n = 5 per group). (C–F) The *CD45*, *Emr1*, *Ly6g* and *CD11c* mRNA levels in the placenta were assessed (n = 5 per group). Data are expressed as mean ± SEM. * $P < 0.05$ *vs*. vehicle.

secretion and its PA-treatment induced mRNA expression (Fig. 4A and B).

Effects of PA on activation of NLRP3 inflammasome in THP1 cells

In vivo experiment, the administration of PA led to the accumulation of immune cells including macrophages/monocytes and neutrophils within the placenta. Consequently, we next investigated the effect of PA on activation of NLRP3 inflammasome using THP-1 cells (macrophage/monocyte cell line) *in vitro*.

After inflammasome activation, ASC assembles into a large protein complex, which is termed "speck-like formation". Hence, ASC speck formation can be used as a simple upstream readout for inflammasome activation [32]. A core component of the inflammasome pathway, ASC, was found to form ASC speck upon PA treatment of THP-1 cells (Fig. 5A).

Finally, to examine the role of NLRP3, we performed NLRP3 genome editing in THP-1 cells using a CRISPR/Cas9 system. We confirmed a reduction of NLRP3 protein in cells transfected with NLRP3 sgRNA (Fig. 5B). We subsequently examined whether NLRP3 affected PA-induced IL-1β secretion and found that PA-induced IL-1β secretion was significantly suppressed in NLRP3-knockdown THP-1 cells (Fig. 5C).

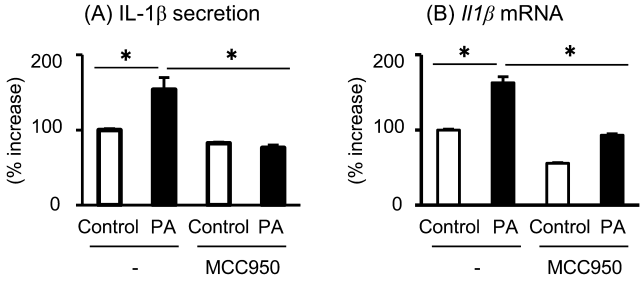

Fig. 4. Effects of palmitic acid (PA) on IL-1β secretion in murine placental cells. Isolated murine placental cells were incubated for 24 h with vehicle or PA solution (400 μM). MCC950 were treated to inhibit NLRP3 inflammasome activation. (A) IL-1β levels in the supernatant were determined using ELISA. (B) The *Il1β* mRNA levels were measured using qRT-PCR. Data are expressed as mean ± SEM (n = 4 per group). * $P < 0.05$ *vs*. vehicle.

Discussion

Our novel results demonstrated that transient high level exposure to PA in pregnant mice activated NLPR3 inflammasome and induced placental inflammation (inflammatory cytokine production and immune cell accumulation), resulting in the increased absorption. Previously, when emulsified ethyl palmitate ester solution (600 mM) was infused into mice, the serum levels of PA was significantly increased to about 400 μM [28]. In addition, circulating levels of PA was 250–500 μM in high-fat feeding obesity mice [33, 34]. These serum PA levels are similar to its levels in women with obesity [35], therefore, in the present study, we administrated similar dose of PA with references to these reports. Indeed, FFA levels are elevated in the plasma of pregnant women with obesity [9]. Plasma PA levels in pregnant women with obesity-related diseases such as preeclampsia and fetal growth restriction (FGR) are higher than that in non-obese healthy pregnant women [36]. Moreover, mature IL-1β secretion is higher in the placenta and adipose tissues of obesity pregnant women compared to non-obese healthy pregnant women [6, 37]. The maternal body mass index positively correlates with active IL-1β expression in the placenta [4, 38]. PA induces NF-κB activation and

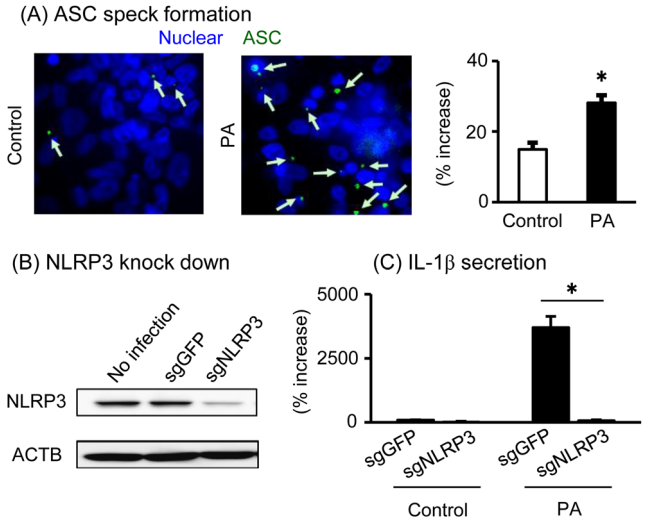

Fig. 5. Effects of palmitic acid (PA) on activation of NLRP3 inflammasome in THP-1 cells. (A) Stable ASC-GFP THP-1 cells were differentiated into macrophages by PMA, and the cells were treated with PA solution at 400 μM or with the vehicle. After 6 h, the positive fluorescence staining cells for ASC speck-like formation were counted. (B) THP-1 cells were transfected with sgRNA targeting GFP or NLRP3. NLRP3 and ACTB protein levels were assessed in the cell lysates using western blotting. Untransfected THP-1 cells were used as a negative controls. These THP-1 cells were differentiated into macrophages by PMA and treated with PA solution at 400 μM or with the vehicle. IL-1β levels in the supernatant were determined using ELISA. Data are expressed as mean ± SEM (n = 4 per group). * $P < 0.05$ *vs.* vehicle.

directly stimulates the secretion of mature IL-1β by placental cells in humans and mice [12, 39]. In addition, we previously found that PA activated caspase-3 and induced annexin V-expression in human placental cells, indicating that PA induces apoptosis in the placenta [12]. These findings suggest that maternal obesity-related increased levels of PA induce placental inflammation.

In the present study, we showed that PA stimulated IL-1β secretion depending on NLRP3 inflammasome activation in placental cells and macrophages. PA induces cellular inflammation and activates NLRP3 inflammasome by several known mechanisms [40]. First, PA acts to promote inflammatory responses by directly engaging TLRs (TLR2, TLR4, and TLR6) [28, 41]. These then induce mRNA expression of inflammation-related factors, including NLRP3 and IL-1β via activation of transcription factors (NF-κB and AP-1) [10, 11]. Subsequently, PA directly activates the NLRP3 inflammasome via induction of co-localization of NLRP3-ASC-caspase-1, inducing IL-1β secretion in macrophages and Kupffer cells [26, 42]. Importantly, PA causes intracellular crystallization, which in turn activates the NLRP3 inflammasome via lysosomal dysfunction in macrophages [27]. PA also induces NLRP3 inflammasome activation by generating reactive oxygen species by mitochondrial dysfunction and inducing autophagy dysfunction, resulting in secretion of mature IL-1β [11, 12, 43]. In addition, Pan *et al*. [42] reported that PA induced mitochondrial DNA release from the mitochondria to the cytoplasm, and then NLRP3 inflammasome activation was mediated by sensing mitochondrial dysfunction through direct binding of NLRP3 to mitochondrial DNA in mouse Kupffer cells. It is thought that PA activates the NLRP3 inflammasome due to the complex mechanisms described above, leading to the development of various diseases.

Inflammation is critical for pregnancy because excessive inflammatory responses directly induce adverse outcomes, such as abortion, preterm birth, and fetal death. Intrauterine administration of IL-1β induced the expression of inflammatory cytokines in the placenta, fetal membranes, and amniotic fluid, and resulted in preterm birth and marked neonatal mortality [44]. Importantly, treatment with a small peptide noncompetitive IL-1 receptor antagonist abolished these IL-1β-induced adverse outcomes. On the other hand, there is emerging evidence that lipopolysaccharide (LPS) administration induces various types of pregnancy complications via excessive inflammatory responses including IL-1β secretion [5, 45, 46]. Diet-induced obesity alters the gut microbial composition, thereby causing gut microbiota-derived LPS to leak into the whole body, and inducing chronic inflammatory responses [46, 47]. Indeed, plasma endotoxin (e.g., LPS) and inflammatory cytokine levels are higher in pregnant women with obesity than in lean pregnant women [48]. These findings indicate that the tissues of pregnant women with obesity, including the placenta, are chronically exposed to endotoxins, such as LPS, thus, inducing low-grade inflammation. Like PA, LPS drastically stimulates IL-1β secretion from the human placenta [49]. Therefore, we hypothesized that obesity-related IL-1β via PA and LPS is an important driver of pregnancy complications, such as placental inflammation, preterm birth, and abortion.

A limitation of this study is that although we showed the administration of PA during pregnancy induced acute and transient inflammatory responses, we could not evaluate the chronic effects of PA during pregnancy. Although we administered similar dose of PA with references to its levels in women with obesity [35], the transient increase of PA may lead to a strong inflammatory response induction such as absorption in the present study. Moreover, because the administration of PA induced the increase in inflammatory cytokines not only in the placenta but also spleen, the systemic inflammatory responses by PA and secondary effects by cytokines *in vivo* may be overlapping manner and these effects cannot be distinctly evaluated. Maternal obesity is associated with pregnancy complications such as preeclampsia and FGR [1], but these complications may be induced by chronic inflammatory responses. Therefore, it is necessary to investigate the long-term and persistent effects of PA administration during pregnancy or the effect of high fat diet-induced obesity during pregnancy on placental inflammation and pregnancy outcomes. In addition, we could not evaluate the detail role of NLRP3 inflammasome in PA-induced pregnancy complications *in vivo*. Further investigations are needed to investigate the importance of NLRP3 inflammasome *in vivo*, using mice deficient in the NLRP3 inflammasome constituent genes or a specific NLRP3 inhibitor (e.g. MCC950).

In conclusion, we have demonstrated that the administration of PA activates the NLRP3 inflammasome and causes accumulation of inflammatory immune cells, thus, inducing excessive acute inflammation in mouse placenta. These adverse effects of PA may be associated with pregnancy complications, such as preterm birth and abortion. Because the FFAs and PA levels are increased in

pregnant women with later occurrence of preeclampsia [34], chronic stimulation by PA during pregnancy may induce preeclampsia in mice model. We will investigate the role of chronic stimulation of PA on NLRP3 inflammasome *in vivo*, using mice deficient in the NLRP3 inflammasome constituent genes or a specific NLRP3 inhibitor, in the future.

Conflict of interest: The authors declare no conflict of interest.

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