Crucial Role of Perilipin-3 (TIP47) in Formation of Lipid Droplets and PGE₂ Production in HL-60-Derived Neutrophils

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Abstract

Cytosolic lipid droplets (LDs), which are now recognized as multifunctional organelles, accumulate in leukocytes under various inflammatory conditions. However, little is known about the characteristic features of LDs in neutrophils. In this study, we show that perilipin-3 (PLIN3; formerly called TIP47) is involved in LD formation and the inflammatory response in HL-60-derived neutrophils. HL-60, a promyelocytic cell line, was differentiated into neutrophils via treatment with all-trans retinoic acid. After differentiation, cells were stimulated with Porphyromonas gingivalis lipopolysaccharide (P.g-LPS), a major pathogen in adult periodontitis. When HL-60-derived neutrophils were stimulated with P.g-LPS, LDs increased in both number and size. In the differentiated cells, PLIN3 was induced while PLIN1, PLIN2 and PLIN5 were not detected. PGE₂ production and the PLIN3 protein level were increased by the P.g-LPS treatment of the cells in a dose-dependent manner. When PLIN3 was down-regulated with siRNA treatment, LDs essentially disappeared and the level of PGE₂ secreted in the culture medium decreased by 65%. In addition, the suppression of PLIN3 repressed the PGE₂ producing enzymes; i.e., microsomal PGE synthase-1, -2 and cyclooxygenase-2. These findings indicate that PLIN3 has a pivotal role in LD-biogenesis in HL-60-derived neutrophils, and that PLIN3 is associated with the synthesis and secretion of PGE₂.

Introduction

Cytosolic lipid droplets (LDs), also called lipid bodies or adiposomes, are functional organelles that are ubiquitously expressed in a variety of cells. LDs are composed of triacylglycerol (TG) and/or cholesterol esters, phospholipids and associated proteins present at the LD surface [1–4]. LDs are involved in multiple intracellular processes, including membrane biosynthesis, lipid metabolism and vesicle trafficking, and ultimately play an important role in the energy balance of the entire body [5,6]. Although LDs are ubiquitously present in cells, the composition of the LD-associated proteins varies among cell types and changes under different physiological conditions. The LD-associated proteins contribute to the specific cellular functions of LDs. The perilipin family, conventionally called the PAT family, is a representative group of LD-associated proteins composed of five members. The recently proposed unified nomenclature designates them PLIN1 (the classic perilipin), PLIN2 (ADRP, ADIP, or adipophilin), PLIN3 (TIP47, PP17, or M6PRBP), PLIN4 (S3–12) and PLIN5 (MLDP, OXPAT, LSDP5, or PAT1) [7]. These proteins have two highly conserved regions; the PAT-1 domain locates at the N terminus and an 11-mer repeat locates at the C terminus and is named the PAT-2 domain [8,9]. PLIN1 is the most abundant LD protein in adipocytes, and it acts as a hormone-dependent switch in TG hydrolysis [10]. PLIN2 is involved in the accumulation of LDs in various cell types [10,11], PLIN2 and PLIN3 are both expressed ubiquitously in the body, while PLIN2 is relatively highly expressed in hepatic cells. Little is presently known about the functions of PLIN3. PLIN4 is expressed in adipocytes, yet it does not locate on the large, PLIN1-positive LDs in untreated adipocytes. Instead, it is abundantly found on punctate structures in the cytoplasm [11]. PLIN5 is particularly abundant in the heart and is essential for maintaining LDs in the cardiac and skeletal muscle cells by antagonizing lipases [12–14].

PLIN3 was initially described as a tail-interacting protein of 47 kDa (TIP47) as well as a mannose-6-phosphate receptor-binding protein (M6PRBP). The trafficking of lysosomal hydrolases to prelysosomes is controlled by a modification with oligosaccharide chains and then the subsequent binding with the mannose-6-phosphate receptor (M6PR). TIP47 binds to the cytosolic domain of the transmembrane protein M6PR that is required for M6PR transport from the endosome to the trans-Golgi network, and thus TIP47 has been thought to have a role in the
Materials and Methods
Antibodies and Reagents
Rabbit polyclonal antibodies (pAb) against prostaglandin E synthases (PGES), mPGES-1, mPGES-2 and cPGES, were purchased from the Cayman Chemical Co. (Ann Arbor, MI, USA). Goat anti-PLIN3 pAb, goat anti-cyclooxygenase (COX)-1 pAb and rabbit anti-COX-2 pAb were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Guinea pig pAb against PLIN1, PLIN2 and PLIN5 were from Progen Biotechnik (Heidelberg, Germany). Alexa Fluor 488 donkey anti-goat IgG, Alexa Fluor 488 donkey anti-rabbit IgG and Alexa Fluor 568 donkey anti-goat IgG were from Invitrogen (San Diego, CA, USA). LPS from P. gingivalis and E. coli were purchased from Invivogen (San Diego, CA, USA). All-trans retinoic acid (AtRA) was purchased from Sigma (St. Louis, MO, USA).

Cell Culture and Differentiation
The human promyelocytic leukemia cell line HL-60 was purchased from ATCC. Mouse Leydig tumor cell line MLTC1 and human hepatocellular carcinoma cell line Huh7 were purchased from RIKEN Health Research Resource Bank. HL-60 and MLTC1 were cultured in RPMI-1640 medium and Huh7 were cultured in DMEM-F-12 with 10% fetal bovine serum (FBS) (Gibco®, Life Technologies Co., Carlsbad, CA, USA) supplemented with 50 U/mL penicillin, 50 μg/mL streptomycin and 2 mM L-glutamine at 37°C in a humidified 5% CO2 atmosphere. HL-60 cells were induced to differentiate into mature neutrophils by incubation with 2 μM AtRA for four days and 82.9% of cells were differentiated into neutrophils judged by nitroblue tetrazolium reduction assay [31]. The amount of PGE2 in the culture medium was measured with a PGE2 EIA Kit (Cayman Chemical Co.) according to the manufacturer’s directions.

Lipid Droplet Staining
The HL-60 cells were attached on a slide glass by cytosin centrifugation at 800 rpm for 3 min. The cells were fixed with 4% paraformaldehyde. LPSs were then stained with BODIPY493/503 (Molecular probe®, Life Technologies Co., Carlsbad, CA, USA). The number and area of the BODIPY-labeled LDs were calculated using ImageJ software (NIH, Bethesda, MD, USA).

Lipid Analysis
Total lipids were extracted from HL-60 cells with chloroform/methanol/phosphate buffered saline (PBS) (1:1:0.9) and then dissolved in chloroform. The lipids in the cells were separated on thin-layer chromatography (TLC) developed with petroleum ether/diethyl ether/acetic acid (90:9:1, v/v/v) and then visualized with iodine vapor. The band intensity was calculated using ImageJ software (NIH, Bethesda, MA, USA).

Immunofluorescence Analysis
After being treated with or without P.g-LPS (10 μg/mL) for 12 h, HL-60 cells were adhered to the microscope slide by cytosin and were fixed with 4% paraformaldehyde for 10 min. The cells were then permeabilized with 0.01% digitonin in PBS for 20 min and blocked with 2% FBS in PBS for 1 h. Cells were incubated with a primary pAb against PLIN3, mPGES-1, mPGES-2, cPGES or COX-2 for 1 h at 37°C. After washing with PBS, cells were then incubated with Alexa Fluor 488- or 568-conjugated secondary antibodies for 1 h at 37°C. After a PBS wash, the cells were stained with Hoechst33258 for 10 min to visualize the nuclei, then the cells were observed under confocal microscopy (A1-si; Nikon).
Subcellular Fractionation

MLTC1 and Huh7 cells harvested from five 100 mm dishes were washed with PBS, resuspended in a hypotonic medium (20 mM Tris-Cl [pH 7.4], 1 mM EDTA, 10 mM sodium fluoride, with protease inhibitor cocktail) and incubated for 10 min on ice. Cells were homogenized by nitrogen cavitation. The homogenate was centrifuged at 1,000 ×g for 5 min at 4°C to obtain post-nuclear supernatant (PNS). For density gradient ultracentrifugation, PNS was adjusted to 20% sucrose and on top of that were layered buffers containing 5% sucrose and without sucrose. Centrifugation was carried out at 14,500 ×g for 1 h at 4°C. LD fractions were collected from the top and delipidated with cold acetone overnight at −20°C.

Figure 1. The size and number of LDs in HL-60-derived neutrophils were increased by P.g-LPS. A: HL-60 cells were differentiated into neutrophils by treatment with 2 μM AtRA for 4 days and then cultured with or without 10 μg/mL P.g-LPS for 12 h. Cytosolic LDs were labeled with BODIPY493/503 and observed using confocal laser microscopy. B: BODIPY-stained LDs were quantified by the area of fluorescence per cell using NIH ImageJ software. The mean ± S.E. from at least three images were calculated. C: HL-60 neutrophils were incubated in the absence (C) or presence (L) of 10 μg/mL P.g-LPS for 12 h. The expression levels of PLIN1, PLIN2, PLIN3 and PLIN5 were detected by Western blotting. LDs recovered from the MLTC1 cells were used as a positive control for PLIN1 and PLIN5, and Huh7 LDs were for PLIN2 and PLIN3. D: HL-60 neutrophils were incubated in the absence or presence of 10 μg/mL P.g-LPS for 12 h. Total lipids extracted from the cells were separated on thin-layer chromatography. The lipids were visualized with iodine vapor, and the band intensity of TG was calculated using ImageJ. E: Differentiated HL-60 cells were treated with or without 10 μg/mL P.g-LPS for 12 h. Cells were fixed and labeled with BODIPY493/503 (green) and anti-PLIN3 pAb (red). Nuclei were stained with Hoechst 33258. The cells were observed under confocal laser microscopy. doi:10.1371/journal.pone.0071542.g001
Western Blot Analysis

Whole cell lysates derived from HL-60 neutrophils and the LDs of MLTC1 and Huh7 were subjected to 10% or 15% SDS-PAGE, and then transferred to polyvinylidene difluoride membranes. After blocking with 5% skim milk in PBS containing 0.1% Tween-20 (PBST), the membranes were incubated with a primary pAb in 1% skim milk overnight at 4°C. After washing with PBST, membranes were incubated with horseradish peroxidase-conjugated secondary pAb for 2 h at room temperature. The bands were detected with ECL-plus Western blotting detection reagent (GE Healthcare UK Ltd., Buckinghamshire, UK) and then visualized using X-ray film (RX-U; Fuji Film Co., Tokyo, Japan).

Transfection with Small Interfering RNA (siRNA)

Synthetic siRNA probes were purchased from Thermo Scientific (Chicago, USA). After treatment with 2 μM AtRA for four days, HL-60 cells (1×10^7 cells) were transfected with siRNA for human M6PRBP1 and non-targeting siRNA (ON-TARGETplus SMARTpool L-015979-00-0005 and #1 D-001810-01-05, respectively) at a final concentration of 250 nM using a Nucleofector™ electroporator (Amaxa biosystems, Germany) with Nucleofector Kit V (Lonza, Germany) according to the manufacturer’s directions. After transfection with siRNA, cells were cultured for 72 h and then cells were cultured in a new medium containing with P.g-LPS (10 μg/mL) for 12 h.

Statistical Analysis

Data are expressed as the mean ± standard deviation. Results were analyzed using Student’s t-test and Dunnett’s test. Statistical significance for all comparisons was assigned at P<0.05. Asterisks indicate values significantly different from the control: *P<0.05.
Results
LD Formation and TG Accumulation in HL-60-derived Neutrophils are Induced by P.g-LPS
HL-60 cells were treated with AtRA to inhibit the clonal growth and induce differentiation into neutrophils. After four days of incubation with 2 μM AtRA, HL-60-derived neutrophils were incubated with P.g-LPS for 12 h, then LD was visualized by staining the cells with BODIPY493/503. The number of LDs and LD area were analyzed using ImageJ software. There were almost no LDs observed in HL-60-derived neutrophils without any stimulation, but a number of LDs appeared after P.g-LPS treatment (Fig. 1A). The total area of LD after the stimulation with 10 μg/mL P.g-LPS resulted in a 38-fold increase compared
with the LD in the non-stimulated cells (Fig. 1B). The effect of P.g-LPS on LD formation was stronger than LPS from E. coli. Since 10 μg/mL E. coli-LPS caused cytotoxicity, we used P.g-LPS throughout this study. TLC analysis of the total lipid extract in the HL-60-derived neutrophils revealed that the major lipid which accumulated in the stimulated cells was TG and that TG increased 2.8-fold by stimulation with P.g-LPS (Fig. 1D).

The PLIN family proteins coat the surface of intracellular LDs in various cell types. Western blot analyses demonstrated that the P.g-LPS treatment of HL-60-derived neutrophils revealed that the major lipid which accumulated in the stimulated cells was TG and that TG increased 2.8-fold by stimulation with P.g-LPS (Fig. 1D).

The expression of PLIN3 and PGE2 production was induced by P.g-LPS

To investigate the inflammatory responses in the cells, the amount of PGE2 in the culture medium was measured by enzyme-linked immunoassay. HL-60-derived neutrophils were treated with various concentrations of P.g-LPS for 12 h. Western blot analysis revealed that the expression of PLIN3 was increased by P.g-LPS in a concentration-dependent manner (Fig. 2A, B). It was clearly shown that P.g-LPS treatment induced PGE2 production, and the amount of PGE2 increased in accord with the P.g-LPS concentration (Fig. 2C).

PGE2 is produced from arachidonic acid by cyclooxygenase (COX) and PGE2 synthase (PGES), with each of these enzymes having several isozymes. We examined the changes in the expression of mPGES-1, mPGES-2, COX-1 and COX-2 by Western blot analysis, and found that only COX-2 was increased concomitantly with PLIN3 induction by P.g-LPS in a concentration-dependent manner (Fig. 2A, D). The enzyme mPGES-1 was induced by P.g-LPS, but was suppressed at the highest concentration.
Differentiated HL-60 cells treated with or without *P. g*.LPS were immunostained and observed microscopically. *P. g*.LPS treatment increased the expression of PLIN3 under the same conditions that the expressions of mPGES-1 and COX-2 were induced, but mPGES-2 and cPGES were largely unaffected (Fig. 3). These observations correspond well with those in Fig. 2. The intracellular distribution of PLIN3 did not match that of the PGE2 synthesizing enzymes.

The Knockdown of PLIN3 Reduces LD Formation and the Production of PGE2

To determine whether PLIN3 is related to the production of PGE2, RNAi experiments were carried out to knock down PLIN3 expression. Cells transfected with either PLIN3 siRNA or control siRNA were stimulated with ATRA. LDs essentially disappeared in the cells when treated with PLIN3 siRNA (Fig. 4A). PGE2

Figure 5. PLIN3 knockdown reduced the PGE2 synthesizing enzymes. After PLIN3 or control siRNA treatment of HL-60-derived neutrophils for 2 days, cells were stimulated with *P. g*.LPS for 12 h, as in Fig. 4. Cells were fixed and then double stained with anti-PLIN3 and either COX-2, anti-mPGES-1, or anti-mPGES-2 pAbs. Nuclei were stained with Hoechst33258. The cells were observed under confocal laser microscopy. doi:10.1371/journal.pone.0071542.g005
production was significantly decreased by PLIN3 knockdown (Fig. 4B). Furthermore, the suppression of PLIN3 reduced the protein levels of mPGES-1, mPGES-2 and COX-2 by 75%, 59% and 90%, respectively compared with non-targeting siRNA (Fig. 4C).

We next investigated the behavior of these PGE2 producing enzymes, when PLIN3 expression was knocked down. Fig. 5 shows that the suppression of PLIN3 leads to the reduction of mPGES-1, mPGES-2 and COX-2, which is in good correspondence with the results of Western blot (Fig. 4).

Discussion

Recently LDs have been paid attention as a functional organelle related to cellular lipid metabolism. LD-associated proteins have been extensively studied and it is well known that the PAT family proteins are the major LD-associated proteins in a variety of cell types, however, little is presently known about the functions of PLIN3. To the best of our knowledge this is the first to report PLIN3 is a crucial protein component of LD involved in LD formation and PGE2 production in HL-60-derived neutrophils.

Some of the LD-associated proteins have been extensively studied. In particular, PLIN1 is a primary LD-associated protein in adipocytes, and it has a crucial role in the regulation of lipolysis by associating with CGI-58 and adipose TG lipase [23,35]. PLIN2, which is highly expressed in hepatocytes, macrophages and premature adipogenic fibroblasts, is considered to be expressed ubiquitously. The expression level of PLIN2 is closely related to lipid accumulation in cells, indicating it has a role in the formation of the LD structure [36,37]. PLIN3 is specifically expressed in cardiac and skeletal muscles as major LD-associated protein [12]. LDs in muscle cells are generally small and located in very proximity with mitochondria. PLIN3-KO mice exhibit intolerance to energy expenditure, and PLIN5 plays a crucial role as a distributor of the energy for β-oxidation [38]. Although PLIN3 and PLIN4 are members of the PAT family proteins, their functions have yet to be clarified.

PLIN3 and PLIN2 are expressed abundantly in macrophages and other types of cells, and their expression is thought to be ubiquitous [5,39]. It is a surprising observation in this study that PLIN3 was found to be the predominant PAT protein in HL-60-derived neutrophils while PLIN2 was under the detection limit. In addition, P.g-LPS treatment induced LD formation as well as PLIN3 expression, and the suppression of PLIN3 by siRNA dramatically reduced LD formation. These results strongly suggest that PLIN3 has an ability to both generate and maintain LDs. In our preliminary experiments, both PLIN2 and PIN3 were expressed in human neutrophils from peripheral blood, suggesting HL-60 may be lacking in PLIN2.

Buers, et al. reported that LD-associated PLIN3 increased when PLIN2 was suppressed by siRNA treatment in THP-1 macrophages [40]. This observation can be explained if PLIN3 has a capacity to compensate for the loss of PLIN2 in maintaining the LD structure. The absence of PLIN2, which could compensate the role of PLIN3, is likely to be one reason that PLIN3 is crucial for the formation of LD in HL-60-derived neutrophils. In THP-1 macrophages knockdown of PLIN3 reduced the cellular amount of TG by 10–20%, but had no effect on cholesterol [40], suggesting that PLIN3 plays only a limited role in lipid accumulation in macrophage LDs. Interestingly, the intracellular distribution of PLIN2 and PLIN3 overlapped only slightly, and knockdown of PLIN2 reduced cholesterol rather than TG [41]. A very recent report showed that PLIN3 was induced by treatment of RAW264.7 macrophages with insulin but not with free fatty acids [42]. The LD particles formed with PLIN3 appear to be regulated differently in PLIN2-dependent LD.

While E. coli-LPS is recognized by toll-like receptor 4 (TLR4), P.g-LPS is reported to activate TLR2 [43]. Recently, Gu, et al. reported that PLIN3 expression was induced by a TLR-9 stimulating compound, ODN1826, in RAW264.7 murine macrophages [41]. The stimulating pathways for PLIN3 expression may be different in different cell types.

Another finding of this study is that suppression of the PLIN3 protein dramatically reduced PGE2 production. In the literature, PGE2 production, which is carried out through three enzymatic steps, is thought to take place in intracellular membranes including the endoplasmic reticulum and perinuclear membranes [44]. Recent reports have suggested that PGE2 might be produced in LDs from the observation that some of the PGE2 producing enzymes co-localize with the PAT family proteins in macrophages or cosinophils [25,45]. In this study we examined the co-localization of PGE2 producing enzymes with PLIN3 carefully, however, we did not observe any evidence that PLIN3 co-localizes with PGE2 synthesizing enzymes.

COX-2 is known to be an inducible protein and is a rate-limiting enzyme in PGE2 production. Under our experimental conditions, COX-2 was up-regulated by the P.g-LPS stimulus, and strongly suppressed by knockdown of PLIN3 expression. The mechanism of COX-2 regulation by PLIN3 is not clear yet. One possibility is that LD may be needed for the organization of the membrane domains on ER and/or nuclear membranes that in turn stabilizes the PGE2 producing enzymes. This would explain the result that not only COX-2, but also the mPGES-1 and mPGES-2 proteins were all suppressed by knockdown of PLIN3. Alternatively, since PLIN3 is actually involved in the sorting of lysosomal proteins [15,16], it may control the trafficking of some proteins between intracellular membranes and LDs.

In conclusion, this study provides evidence for a functional role of PLIN3 in LD formation and PGE2 production. Further study is needed to answer a number of unresolved issues. We would like to obtain insight into the mechanisms by which PLIN3 regulates LD biogenesis and expression of PGE2-producing enzymes by identifying proteins which interact with PLIN3, for example, using proteomic analysis.

Author Contributions

Conceived and designed the experiments: FN TY HI. Performed the experiments: FN RK TA TO TY. Analyzed the data: FN TY HI. Contributed reagents/materials/analysis tools: SH MY HI. Wrote the paper: FN TY HI.

References

1. Buza PT, Magdaheis KG, Weller PF (2009) Leukocyte lipid bodies: Biogenesis and functions in inflammation. Biochim Biophys Acta 1791: 540–551.
2. Tauchi-Sato K, Okuda S, Houou T, Taguchi R, Fujimoto T (2002) The surface of lipid droplets is a phospholipid monolayer with a unique fatty acid composition. J Biol Chem 277: 44507–44512.
3. Martin S, Parzon RG (2006) Lipid droplets: a unified view of a dynamic organelle. Nat Rev Mol Cell Biol 7: 373–378.
4. Goodman JM (2009) Demonstrated and inferred metabolism associated with cytosolic lipid droplets. J Lipid Res 50: 2140–2156.
5. Lu X, Gruss-Gray J, Copeland NG, Gilbert DJ, Jenkins NA, et al. (2001) The murine perilipin gene: the lipid droplet-associated perilipins derive from tissue-specific, mRNA splice variants and define a gene family of ancient origin. Mamm Genome 12: 741–749.
6. Thiele C, Spandid J (2000) Cell biology of lipid droplets. Curr Opin Cell Biol 20: 378–385.

7. Kimmel AR, Brasaeuml DL, McAndrews-Hill M, Szataly C, Londos C (2010) Adoption of PERILIPIN as a unifying nomenclature for the mammalian PAT-family of intracellular lipid storage droplet proteins. J Lipid Res 51: 468–471.

8. Szataly C, Xu G, Dorward H, Tansey JT, Contreras JA, et al. (2003) Perilipin A is essential for the translocation of hormone-sensitive lipase during lipolytic activation. J Cell Biol 161: 1093–1103.

9. Martinez-Rous J, Anderson JR, Tessler D, Lapiplonne A, Chang BH, et al. (2000) Absence of perilipin results in leanness and reversing obesity in Lepr/db/db mice. Nat Genet 26: 474–479.

10. Bozek PE, Tansey J, Welte MA (2009) PAT proteins, an ancient family of lipid droplet proteins that regulate cellular lipid stores. Biochim Biophys Acta 1791: 419–440.

11. Wolins NE, Skinner JR, Scherfush MJ, Ztekov A, Bloch KG, et al. (2003) Adipocyte protein S3–12 coats nascent lipid droplets. J Biol Chem 278: 37713–37723.

12. Yamaguchi T, Matsushita S, Motojima K, Hirose F, Osumi T (2006) MLDP, a novel PAT family protein localized to lipid droplets and enriched in the heart, is regulated by peroxisome proliferator-activated receptor alpha. J Biol Chem 281: 14272–14280.

13. Wolins NE, Quaynor BK, Skinner JR, Ztekov A, Croce MA, et al. (2006) OX-PAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. Diabetes 55: 3413–3422.

14. Dalen KT, Dahl T, Holter E, Arntsen B, Londos C, et al. (2007) LSDP5 is a cargo selection device for mannos-6-phosphate receptor trafficking. Cell 93: 433–443.

15. Hickenbottom SJ, Kimmel AR, Londos C, Hurley JH (2004) Structure of a lipid droplet is an important organelle for hepatitis C virus production. Nat Cell Biol 6: 1089–1097.

16. Fan B, Gu J-Q, Yan R, Zhang H, Fen S, et al. (2013) PPARgamma-deficient mutant shows a marked decrease in toll-like receptor 2-mediated signaling. Infect Immun 73: 2157–2163.

17. Li K, Cerami A, Sander C, Simon RM, Albrecht M, et al. (2005) Lipooylsaccharide preparation extracted from Porphyromonas gingivalis lipoprotein-deficient mutant shows a marked decrease in toll-like receptor 2-mediated signaling. Infect Immun 73: 2157–2163.

18. Santos TA, Prates DB, Andrade RB, Nascimento DO, Clanciiero J, et al. (2010) Lutzomyia longipalpis saliva triggers lipid body formation and preadaptation in murine macrophages. PLoS Negl Trop Dis 4: e873.

19. Daveu DP, Tanner A, Page RC (1997) The microbial challenge in periodontitis. Periodontol 2000 14: 12–32.

20. Socarsky SS, Hallage AD (1992) The bacterial etiology of destructive periodontal disease: current concepts. J Periodontol 63: 322–331.

21. Hayashi C, Vierek J, Hua N, Phnakiadou A, Madrigal AG, et al. (2011) Porphyromonas gingivalis accelerates inflammatory atherosclerosis in the innominate artery of Apoe deficient mice. Atherosclerosis 215: 52–59.

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