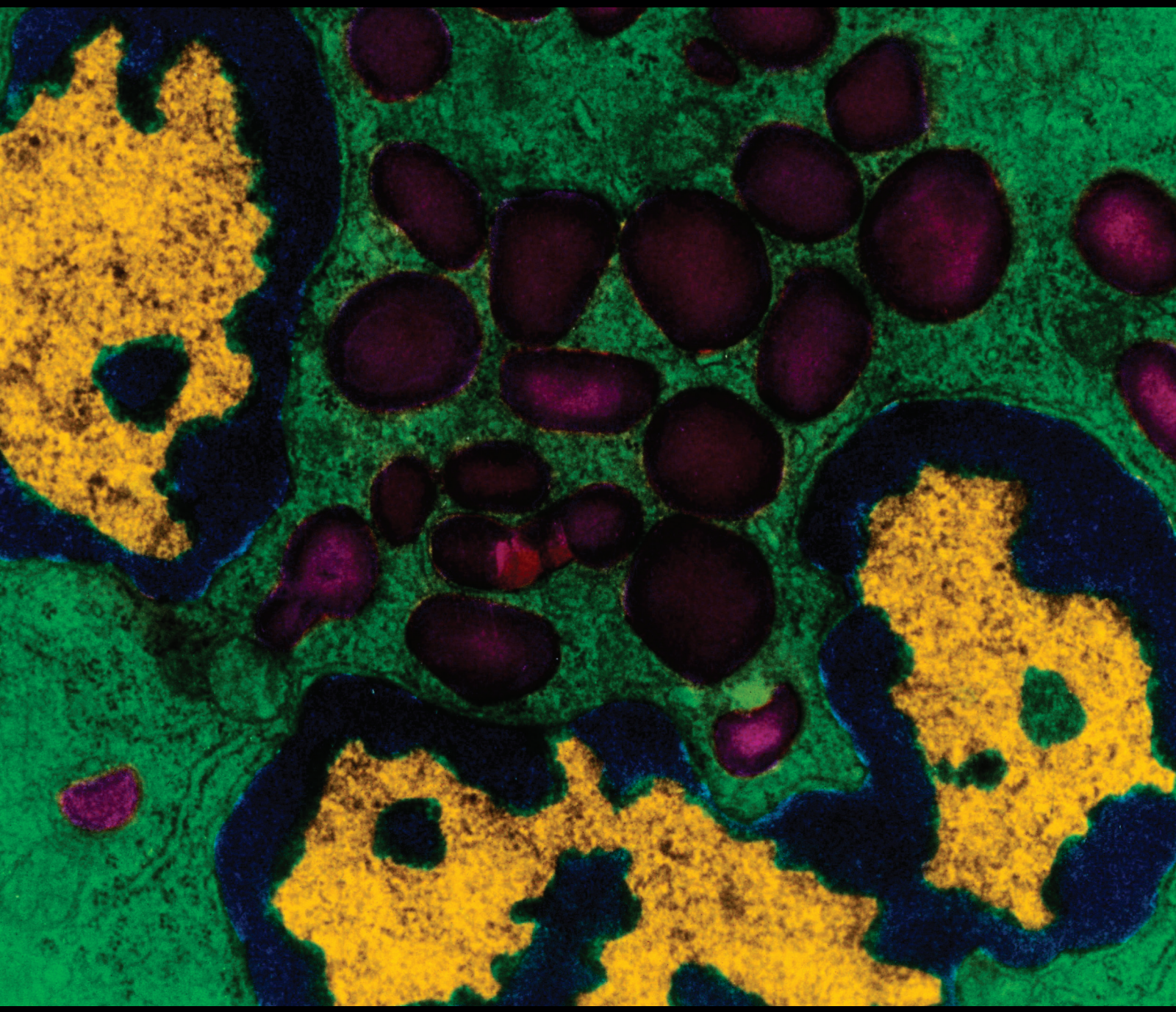
Mediators of Inflammation

Immunometabolism: Molecular Mechanisms, Diseases, and Therapies

Guest Editors: José Cesar Rosa Neto, Fabio Santos Lira, and William Tadeu Festuccia



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Editorial Board

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Editorial

Immunometabolism: Molecular Mechanisms, Diseases, and Therapies

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Several studies published over the last two decades have provided a good body of evidences supporting a central role of chronic low-grade inflammation as a major factor driving many of the metabolic complications commonly found in highly prevalent chronic diseases such as obesity, insulin resistance, and cancer. It was well established in these studies, for example, that activation of canonical inflammatory pathways is one of the major factors promoting the impairment in insulin signaling seen in obesity and type 2 diabetes, being responsible for the reduced glucose uptake and exacerbated lipolysis found in this condition. Such important role of inflammation in chronic diseases has motivated several studies aiming at understanding the mechanisms underlying its development and searching for efficient therapeutic strategies to minimize its metabolic consequences. In the present special issue, we gathered several original and review articles addressing potential nutritional, pharmacological, and behavioral strategies that could be used to counteract inflammatory process associated with a variety of diseases including obesity, periodontal disease, myocardial infarction, and pneumonia, among others. In a very interesting study, for example, E. G. Novoselova et al. experimentally tested individually or in combination several inhibitors of NFkB pathway and naturally occurring antioxidants as anti-inflammatory agents in vivo bringing new information about their therapeutic efficacy. In the same direction, M. S. Elburki et al. tested the appropriateness of using a novel chemically modified curcumin, a matrix metalloproteinase inhibitor with no antibiotic properties, as a therapeutic molecule for the treatment of periodontal disease with promising results towards the attenuation of alveolar bone loss.

In addition to the above-mentioned pharmacological agents, there are in this special issue two interesting studies reporting promising effects of nutrients and vitamins as anti-inflammatory molecules in vivo. In a very elegant study, Y. Chen et al. report the beneficial actions of vitamin C supplementation in the attenuation of the inflammation and oxidative stress induced by LPS in macrophages from humans with community-acquired pneumonia. Also important were the findings of C. O. Souza et al. showing that a supplementation with the monounsaturated palmitoleic acid markedly attenuates obesity-associated fat accumulation, inflammation, and insulin resistance in the liver, such effects that do not depend on the nuclear receptor peroxisome-proliferator activated receptor (PPAR) alpha.

As detailed in the three different manuscripts discussed below, behavioral strategies such as exercise training were also addressed in this special issue, as strategies to attenuate inflammatory processes. A. Correia et al., for example, report in an original study interesting effects of exercise training in different intensities as an attenuator of the changes induced by aging in skeletal muscle inflammation and metabolism. This study is followed by two comprehensive review articles

addressing the effects of exercise training on inflammation and autonomic dysfunction induced by myocardial infarction (B. Rodrigues et al.) and on lymphocyte metabolism and function in several conditions (F. Wasinski et al.). Finally, completing this special issue are the exciting findings of C. R. Balistreri et al. that report a strong association between toll-like receptor rs4986790 TLR4 polymorphism with development of type 2 diabetes in humans adding further support to the previous recognized role of this inflammatory pathway in the development of insulin resistance and S. Y. Gun et al. that extensively reviewed the role of interferons and regulatory factors as major components of the innate and adaptive responses fighting against malaria infection.

Overall, we believe that this special issue provides new insights into the complex interactions between inflammatory processes and underlying metabolic disarrangements commonly found in chronic diseases such as obesity, type 2 diabetes, and cancer, with an especial emphasis on the different pharmacological, nutritional, and behavioral strategies that could be used to prevent and/or attenuate such deleterious interrelationship.

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Research Article

Vitamin C Mitigates Oxidative Stress and Tumor Necrosis Factor-Alpha in Severe Community-Acquired Pneumonia and LPS-Induced Macrophages

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Oxidative stress is an important part of host innate immune response to foreign pathogens. However, the impact of vitamin C on oxidative stress and inflammation remains unclear in community-acquired pneumonia (CAP). We aimed to determine the effect of vitamin C on oxidative stress and inflammation. CAP patients were enrolled. Reactive oxygen species (ROS), DNA damage, superoxide dismutases (SOD) activity, tumor necrosis factor-alpha (TNF-α), and IL-6 were analyzed in CAP patients and LPS-stimulated macrophages cells. MH-S cells were transfected with RFP-LC3 plasmids. Autophagy was measured in LPS-stimulated macrophages cells. Severe CAP patients showed significantly increased ROS, DNA damage, TNF-α, and IL-6. SOD was significantly decreased in severe CAP. Vitamin C significantly decreased ROS, DNA damage, TNF-α, and IL-6. Vitamin C inhibited LPS-induced ROS, DNA damage, TNF-α, IL-6, and p38 in macrophages cells. Vitamin C inhibited autophagy in LPS-induced macrophages cells. These findings indicated that severe CAP exhibited significantly increased oxidative stress, DNA damage, and proinflammatory mediator. Vitamin C mitigated oxidative stress and proinflammatory mediator suggesting a possible mechanism for vitamin C in severe CAP.

1. Introduction

Oxidative stress is a key part of the chain of events leading to inflammation caused by bacterial infection. Granulocyte peroxidases play an important role in triggering oxidative stress [1]. Cystic fibrosis (CF) has been associated with oxidative stress, in particular during the chronic pulmonary infection with *Pseudomonas aeruginosa*, which is the main cause of morbidity and mortality in CF [2]. Respiratory syncytial virus (RSV) infection caused oxidative cell damage and cellular signaling in modulating virus-induced lung disease [3]. Reactive oxygen species (ROS) and oxidative stress are thought to play a central role in the etiology of cell dysfunction and tissue damage. ROS also modulate a number of cell signaling pathways resulting in transcription factor activation and inflammatory mediators' release [4]. ROS induce vascular cell adhesion molecule-1 (VCAM-1) signal transduction and VCAM-1-dependent inflammation is blocked by antioxidants [5]. A study reported the occurrence of higher oxidative stress in bacterial severe community-acquired pneumonia patients [6]. Antioxidants may affect pulmonary morbidity. Vitamin C significantly improved the "total respiratory score" in the most severely ill patients [7].

Lipopolysaccharide (LPS; endotoxin) is an important event that contributes to the elevation in reactive oxygen species [8]. LPS-induced acute lung injury (ALI) and aberrant proliferation of lung fibroblasts initiated in early disease stages are associated with PI3K-Akt pathway activation [9].

ROS induced DNA damage. Spontaneously, endogenous DNA damage can activate NFκB. Inhibiting the canonical NF-κB pathway exacerbated H_2O_2-induced A549 cell apoptosis [10]. Autophagic cell death plays a crucial role in infection. H5N1-infected lungs from a human cadaver, mice, and infected A549 human epithelial lung cells show the accumulation of autophagosomes. Blocked autophagic signaling increased the survival rate of mice and meliorated the acute lung injury and mortality caused by H5N1 infection [11]. Oxidative stress-induced DNA damage and autophagy remain unclear in pneumonia. In our present study, our findings indicated that severe CAP exhibited significantly increased oxidative stress and proinflammatory mediator. Vitamin C mitigated oxidative stress and proinflammatory mediator.

2. Material and Methods

2.1. Study Design and Subjects. The study was conducted at affiliated hospital of Luzhou medical college (a 3000-bed hospital in Luzhou City, Sichuan, China). All patients admitted to the hospital with pneumonia between July 2011 and June 2013 were recruited. Pneumonia was defined as a new infiltrate in chest radiography together with symptoms and signs of a lower respiratory tract infection: fever (>38°C), cough, and purulent sputum [12, 13]. Multiple severity scoring systems have been devised and evaluated in community-acquired pneumonia. Patients with pneumonia were classified into mild to moderate pneumonia (no severe pneumonia) and severe pneumonia according to the primary care summary of the British Thoracic Society Guidelines for the management of community-acquired pneumonia (CAP) in adults [14]. Patients who have a CRB-65 score of 3 or 4 were defined as severe pneumonia. This study was approved by the Luzhou Medical College Ethic Committee.

2.2. Mononuclear Cell Separation and Human Lung Tissue. Peripheral blood obtained from pneumonia and normal donor was processed for separation of mononuclear cells. Peripheral blood was diluted 1 : 1 with sterile phosphate-buffered saline (PBS), layered over Ficoll-Hypaque (GE Healthcare Bio-Sciences AB, Stockholm, Sweden), and centrifuged at 1500 rpm for 15 min at room temperature. Peripheral blood mononuclear cells (PBMC) were collected from the interphase layer and washed with PBS. PBMC were resuspended with RPMI-1640 medium. Cells were grown in RPMI-1640 medium containing 10% FCS (fetal calf serum). The lung tissues were collected by lung tissue biopsy with fiber bronchoscope. Tissues were quickly immersed in liquid nitrogen and transferred to −70°C refrigerator frozen storage.

2.3. MH-C Cells Culture. When murine alveolar macrophage *cell* lines (MH-C cells) were grown to 85% confluence in RPMI-1640 medium containing 10% FCS, the medium was replaced with serum-free RPMI-1640 culture medium. The cells were then treated with LPS or/and 100 nM vitamin C in serum-free culture medium for 24 hours.

2.4. Measurement of ROS, SOD, IL-6, and TNF-α. The cells were loaded with 10 μM H2DCF-DA (Invitrogen, Molecular Probes, USA) or 10 μM dihydroethidium (DHE, Invitrogen, Molecular Probes, USA) at 37°C for 30 minutes according to the manufacturer's instructions. After removing excess probes, the cells were kept at 37°C containing 5% CO_2. Fluorescence intensity was detected by Leica TCS SP5 confocal microscope (Leica, Germany). For each sample 10,000 events were collected. For lung tissues, add 0.1% ROS (DHE) probe about 10 edged up on the lung tissue of frozen section, after incubation for 30 minutes at 37°C, washing 2-3 times with phosphate-buffered saline (PBS) and observation by Leica TCS SP5 confocal microscope. Superoxide dismutases (SOD) activity was measured using the SOD assay kit WST (Nanjing jiancheng bioengineering institute, China). The ELISA kits of TNF-α and IL-6 were purchased from R&D Systems (Minneapolis, MN).

2.5. Measurement of DNA Damage and Cell Viability. The comet assay was used to measure DNA damage. The procedure of comet assay was performed [15]. 10 μL cells suspension containing 20,000 cells was mixed with 90 μL low-melting-point agarose (LMA) (Sigma) in PBS at 37°C and was layered onto slides which had been coated with normal melting point agarose. The slides were submersed in freshly prepared cold (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA-2Na, 10 mM Tris-HCl, pH 10–10.5, 1% Triton X-100, and 10% DMSO) for 2 hours. Slides were immersed in fresh electrophoresis buffer at 4°C for 30 min and then electrophoresed (25 V/300 Ma) for 25 min. After electrophoresis, the slides were stained with ethidium bromide. Slides were covered with a coverslip and analyzed using Leica TCS SP5 confocal microscope (Leica, Germany). Comet assay IV software was used to assess the DNA damage score. Cell viability was measured by 5-ethynyl-2′-deoxyuridine (EdU) assay using an EdU assay kit (Ribobio, Guangzhou, China) [16]. The cell nucleus was stained with 4′-6-diamidino-2-phenylindole (*DAPI*) reagents.

2.6. Cell Transfection. MH-S cells were transfected with RFP-LC3 or GFP-LC3 plasmids using Lipofectamine 2000 reagent (Invitrogen) in serum-free RPMI 1640 medium (Thermo Fisher Scientific) following the manufacturer's instructions [17]. RFP-LC3 and GFP-LC3 plasmids were kindly provided by Min Wu (the University of North Dakota, US).

2.7. Western Blot. Cells were homogenized in RIPA lysis buffer for western blot analysis. Lysates (20 μg) were run on 10% SDS polyacrylamide gel at 100 V for 2 hours and transferred to microporous polyvinylidene difluoride (PVDF) membrane at 100 mA for 2 hours. The membrane was blotted with phosphorylated (p)-P38, P38, TNF-α, LC3, BECN1, and β-actin antibodies (1 : 1000) (Santa Cruz Biotechnology, Inc.) and processed via enhanced chemiluminescence (Pierce).

2.8. Statistical Analysis. Data are expressed as mean ± standard error. Statistical analysis was performed using ANOVA

Table 1: Descriptive statistical analysis of the study groups.

	Control	Nonsevere pneumonia	Severe pneumonia
Number	15	15	15
Sex, m/f	8/7	9/6	11/4
Age, years	64.28 (4.12)	61.26 (3.19)	65.21 (4.56)
PaO_2 mmHg	—	99.8 (12.3)	41.2 (8.2)
$PaCO_2$ mmHg	—	35.2 (4.8)	43.2 (3.9)
PaO_2/FiO_2 mmHg	—	321 (35)	196 (42)
Klebsiella pneumoniae	—	2	2
Escherichia coli	—	3	2
Cinetobacter	—	3	4
Endotoxin (pg/mL)	—	28.13 (4.14)	39.34 (5.12)

Data are shown as means (SD).
M: male; F: female; FiO_2: fraction of inspired oxygen; ST: patient temperature during sampling.

(Tukey's post hoc) or Student's t-test and the level of significance was defined as $P < 0.05$ between any two groups. Mann-Whitney U test and Spearman's correlation with a two-tailed test were used for statistical analyses. The data were analyzed using SPSS 13.0 software [18].

3. Results

3.1. Description of Severe CAP. A total of 30 patients with community-acquired pneumonia were enrolled in the study. 15 patients had severe community-acquired pneumonia and 15 patients with community-acquired pneumonia showed nonsevere pneumonia. 15 cases were admitted to control cases. The baseline characteristics of these patients are described in Table 1. The mean age of severe community-acquired pneumonia or nonsevere community-acquired pneumonia was not significantly different with that in normal control. Severe community-acquired pneumonia shown decreased PaO_2 and PaO_2/FiO_2 compared to nonsevere community-acquired pneumonia ($P = 0.001$). The sputum culture showed the growth of *Klebsiella pneumonia*, *Escherichia coli*, and Acinetobacter. Endotoxins in severe CAP were significantly increased compared to nonsevere CAP.

3.2. Severe CAP Enhances Oxidative Stress in Lung. Community-acquired pneumonia had higher oxidative stress compared with patients without infection [19]. However, it remains unclear that oxidative stress involves severe degree of pneumonia. To evaluate the oxidative stress of lung in CAP, we measured the ROS in airway tissue from control groups or from CAP patients collected by bronchoscopy. CAP patients exhibited increased ROS in airway tissue. ROS level of severe CAP significantly increased compared with that of nonsevere CAP ($P = 0.0012$, Figure 1(b)).

3.3. Severe CAP Enhances ROS, TNF-α, and IL-6. It has been shown that protection against postinfluenza bacterial pneumonia is by increasing phagocyte recruitment and ROS production [20]. However, whether excessive ROS aggravated inflammation remains unclear. Using ROS probe, we found that CAP showed increased ROS compared to control in PBMC. Furthermore, severe CAP showed increased ROS density compared to nonsevere CAP ($P = 0.002$, Figure 2(a)). DNA damage in PBMC was detected by comet assay. DNA damage of PBMC exhibited different increases in CAP. A total damage score for each slide in severe CAP was significantly increased compared to nonsevere CAP ($P = 0.001$, Figure 2(b)). The SOD in severe CAP was significantly decreased compared to nonsevere CAP ($P = 0.001$, Figure 2(c)). The significant association was found between ROS and DNA damage in severe CAP ($r = 0.632$, $P = 0.007$, Figure 2(d)). There is significant negative correlation between SOD and ROS ($r = 0.632$, $P = 0.007$, Figure 2(e)). The TNF-α and IL-6 in severe CAP were significantly increased compared to nonsevere CAP ($P = 0.0003$ and 0.005, Figure 2(f)). The results indicated that severe CAP had higher oxidative stress, DNA damage, and proinflammatory mediator production.

3.4. Vitamin C Decreases ROS, TNF-α, and IL-6 in Severe CAP. Vitamin C effectively inhibited amfepramone-induced DNA damage [21], but whether vitamin C inhibited bacterial infection and induced ROS and DNA damage in severe CAP remains unclear. Our studies found that vitamin C decreased ROS and DNA damage scores compared with PBS-treated monocytes from severe CAP in vitro ($P = 0.0001$ and 0.00053, Figure 3(a)). Vitamin C decreased TNF-α and IL-6 compared with PBS-treated whole blood cells from severe CAP in vitro ($P = 0.006$ and 0.03, Figure 3(b)).

3.5. Vitamin C Inhibited LPS-Induced ROS, TNF-α, and P38 in MH-S Cell Lines. Reactive oxygen species produced during the innate immune response to LPS are important agents of antipathogen defense, but whether vitamin C regulates LPS-induced oxidative stress and proinflammatory mediators remains unclear [22]. In present studies, MH-S cells were stimulated with LPS for 24 hours and vitamin C was added. We found that LPS increased ROS-positive cells and DNA damage score compared to control cells. Vitamin C inhibited ROS-positive cells and DNA damage score compared to

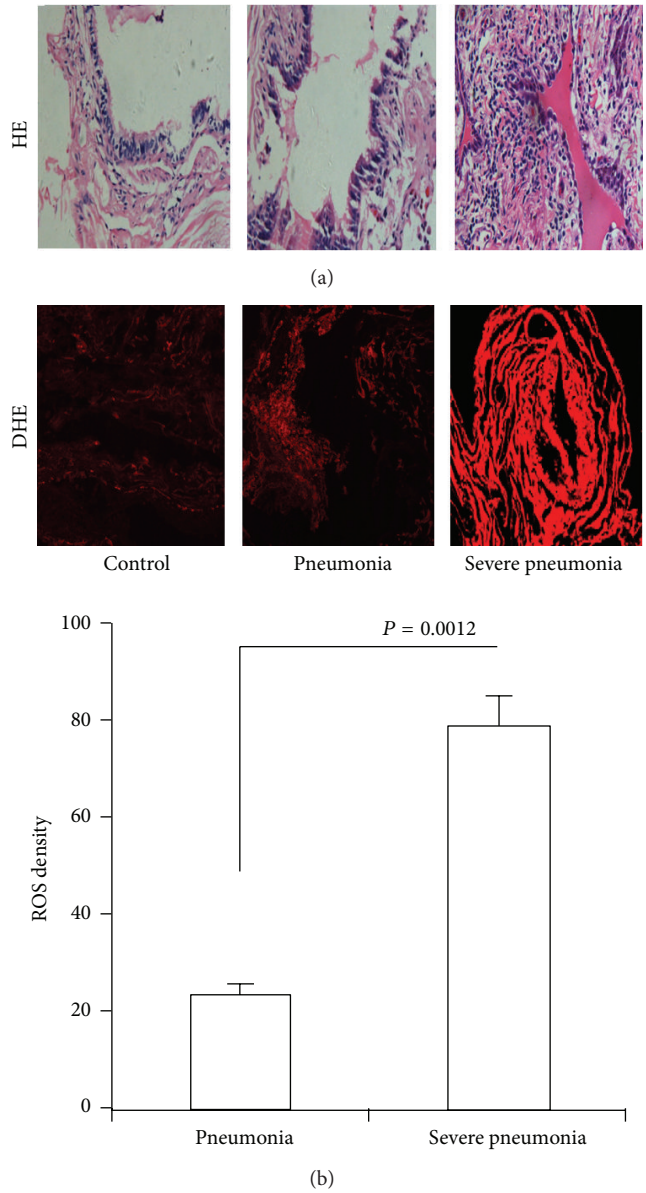

Figure 1: *Oxidative stress in severe CAP*. (a) HE staining of lung tissue from no severe CAP and severe CAP patients. (b) ROS were detected by confocal microscope using DHE probe. ROS level was represented by fluorescence intensity (×200).

LPS-stimulated cells (Figures 4(a) and 4(b), $P = 0.0002$ and 0.0001). Vitamin C increased MH-S cell viability in LPS-stimulated cells (Figure 4(c)). It has been shown that concentrations of TNF-α released from LPS-stimulated cells increased significantly [23]. Hydrogen peroxide induced TNF-α production in macrophages via activating p38 as oxidative stress-related signal pathways [24]. After vitamin C treatment, a significant decrease in TNF-α, P38, and p-P38 in LPS-stimulated cells was observed in present study (Figure 4(e), $P = 0.001$ and 0.0006).

3.6. Vitamin C Decreased LPS-Induced Autophagy in MH-S Cell Lines. It has been found that autophagy is required for an effective immune response against infection in vivo and enhances bacterial clearance during *Pseudomonas aeruginosa* lung infection [25]. LPS-induced autophagy is involved in the restriction of *Escherichia coli* in peritoneal mesothelial cells [26]. To determine whether LPS induces autophagy, MH-S cells were transfected with RFP-LC3 plasmids or GFP-LC3 plasmid. MH-S cells were stimulated with 10 μg/mL LPS for 12 h. According to previous report, the confocal microscopy images were used to semiquantitatively measure [17]. We observed that LPS induced LC3 punctation in the MH-S cells. H_2O_2 significantly increased LC3 punctation. However, vitamin C inhibited the increased LC3 punctation in LPS-induced cells (Figure 5(a)). We found that H_2O_2 significantly increased beclin-1 in LPS-stimulated cells (Figure 5(b)). The expression of LC3-II was increased in LPS-stimulated cells. Vitamin C inhibited increased LC3II in LPS-stimulated cells (Figure 5(c)).

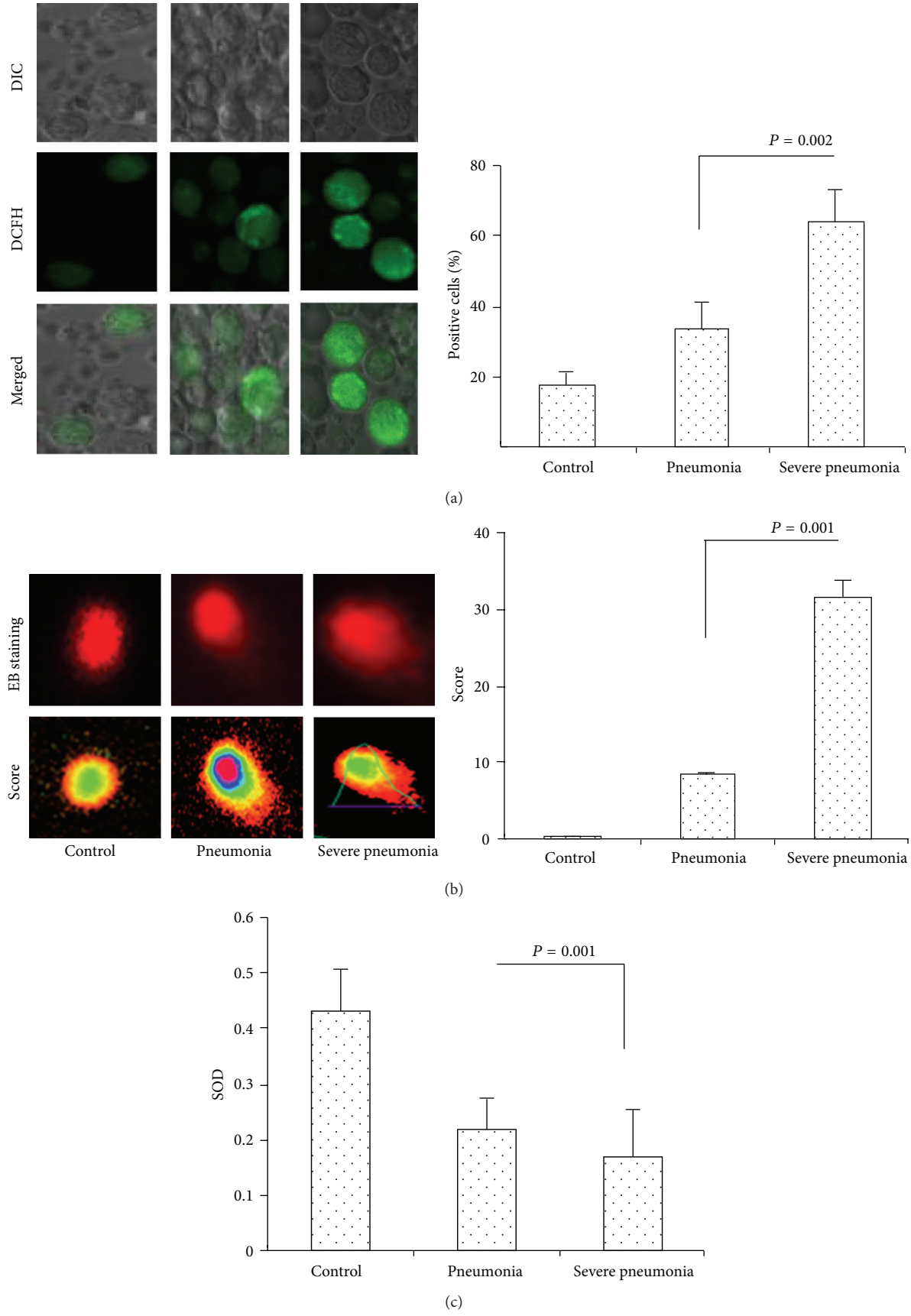

Figure 2: Continued.

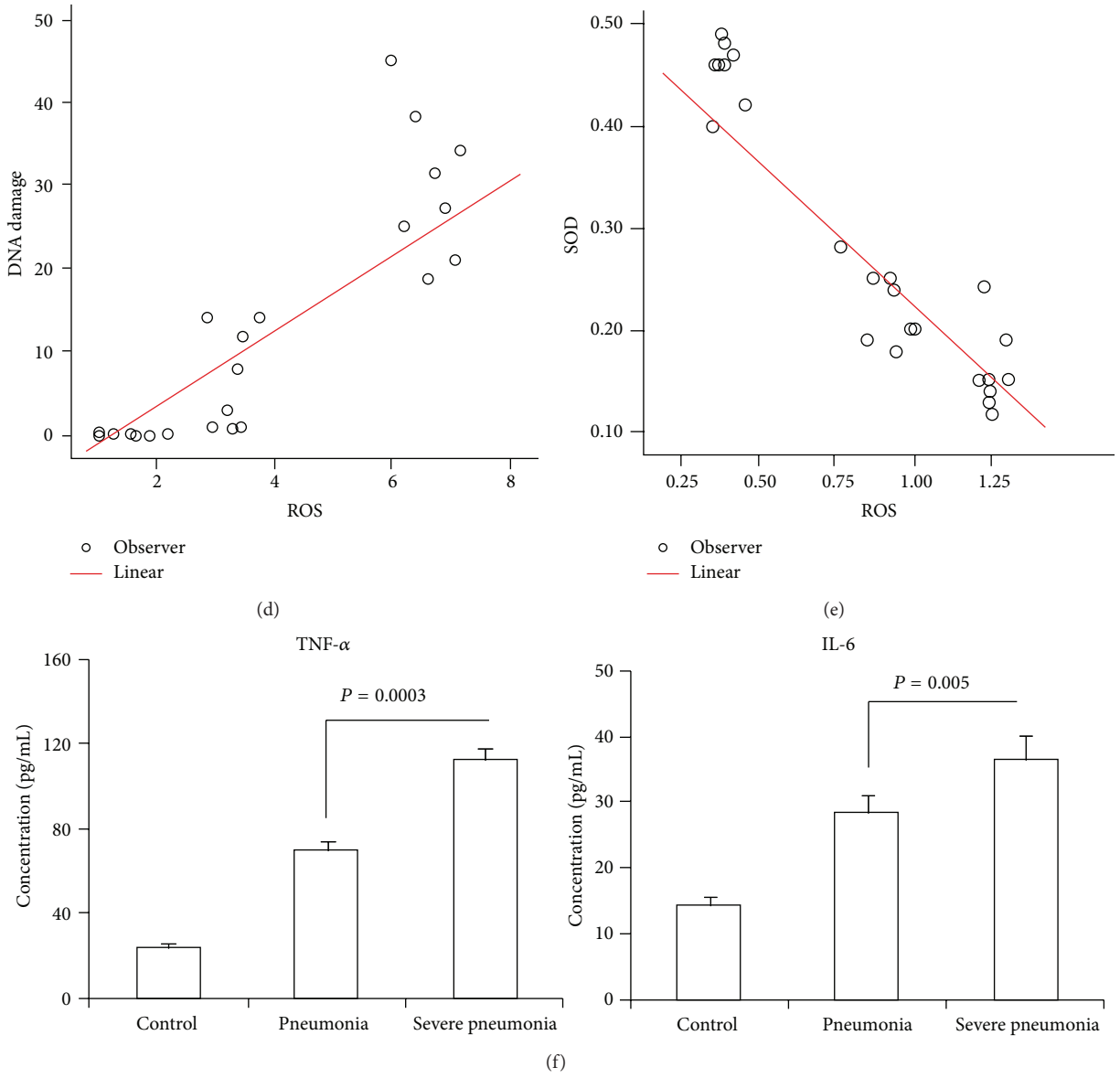

Figure 2: *Oxidative stress, DNA damage, TNF-α, and IL-6 in severe CAP.* (a) Intracellular ROS were detected by confocal microscope using DHE probe. ROS level was represented by fluorescence intensity (×400). (b) DNA damage was detected by comet assay using confocal microscope (×400). DNA damage score was analyzed with comet assay IV software. (c) SOD activity in serum was measured using the SOD assay kit WST. (d) Correlations between ROS and DNA damage were also evaluated. (e) Correlations between ROS and SOD were also evaluated. Spearman's correlation with a two-tailed test was used for statistical analyses. (f) Standard ELISA was performed to determine the levels of TNF-α and IL-6.

4. Discussion

Pneumonia is an infection of the lungs usually caused by bacteria and viruses [27]. Oxidative stress is an important part of host innate immune response to foreign pathogens [28]. Oxidative stress in the respiratory system increases the production of mediators of pulmonary inflammation. Such effects include increased expression of intercellular adhesion molecule 1 and interleukin-6 and hypersecretion of mucus [29]. Our study found that CAP exhibited increased ROS in lung tissue. In addition, severe CAP showed significantly increased ROS in lung. Therefore, these data indicated that oxidative stress involves severe degree of pneumonia.

It has been shown that respiratory syncytial virus infection induces significant downregulation of the airway antioxidant system in vivo, likely resulting in lung oxidative damage [30]. Antioxidants have been shown to be effective in preventing lung injury and protect against damage of other organs, such as heart, kidney, and liver in animal models of oxidative stress [31]. We found that ROS also increased in CAP PBMC. ROS significantly increased in severe CAP compared to CAP. Furthermore, CAP and severe

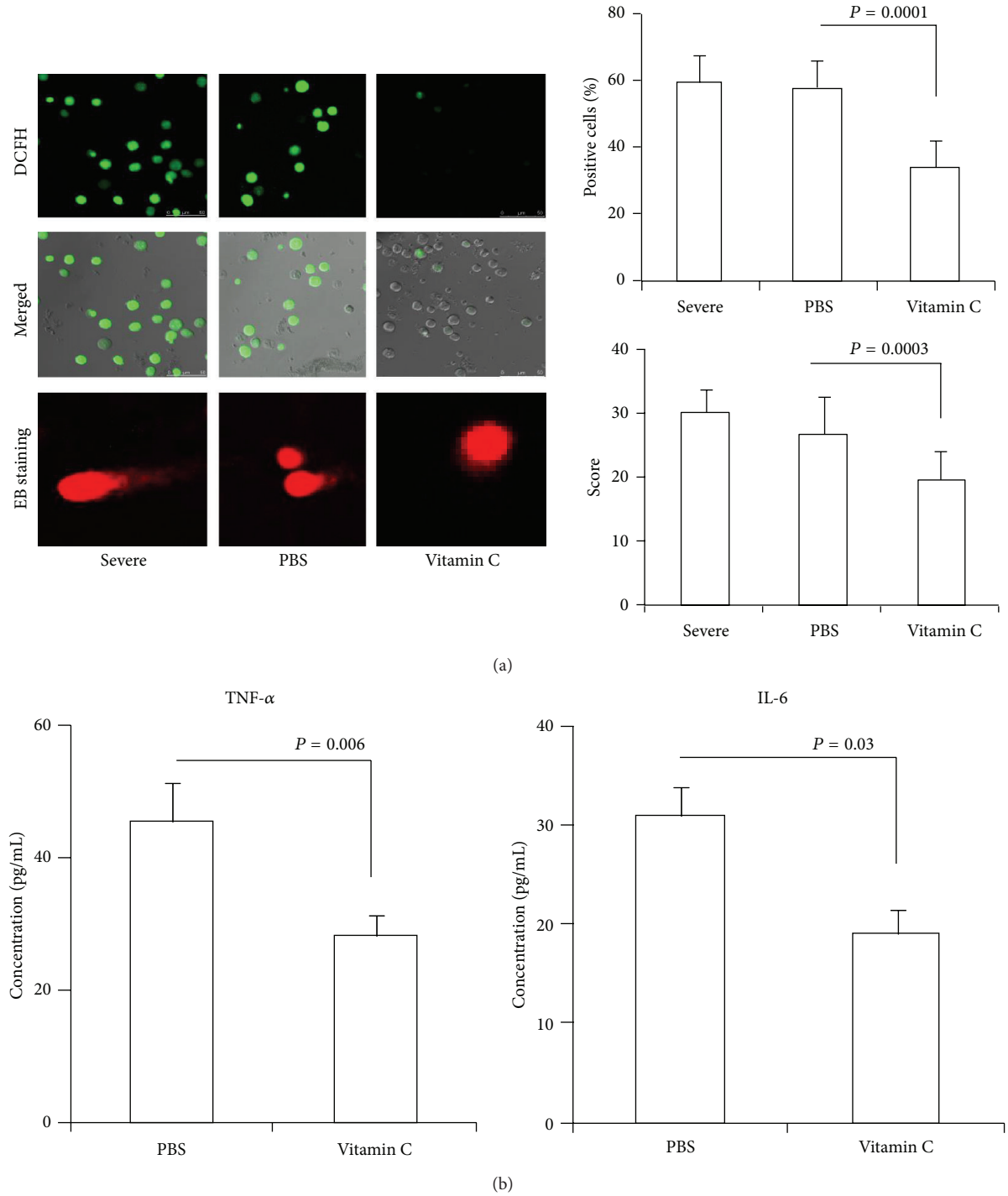

Figure 3: *The effect of vitamin C on ROS, DNA damage, TNF-α, and IL-6 in vitro*. Monocytes from severe CAP were treated with vitamin C for 12 h in vitro. (a) Intracellular ROS were detected by confocal microscope using DHE probe (×400). DNA damage was detected by comet assay using confocal microscope (×400). (b) Whole blood cells from severe CAP were treated with vitamin C for 12 h in vitro. Standard ELISA was performed to determine the levels of TNF-α and IL-6.

CAP exhibited DNA damage in PBMC. Severe CAP exhibited more DNA damage compared to CAP. Oxidative damage is correlated with superoxide dismutase (SOD) in the lung. Antioxidant treatment reverses organ failure in rat model of sepsis [31]. In our present study, we found that SOD was negatively correlated with ROS in severe CAP PBMC. Severe CAP exhibited more DNA damage in PBMC. The TNF-α and IL-6 in severe CAP were significantly increased. The results indicated that oxidative stress and DNA damage likely represent an important pathogenetic mechanism of severe CAP.

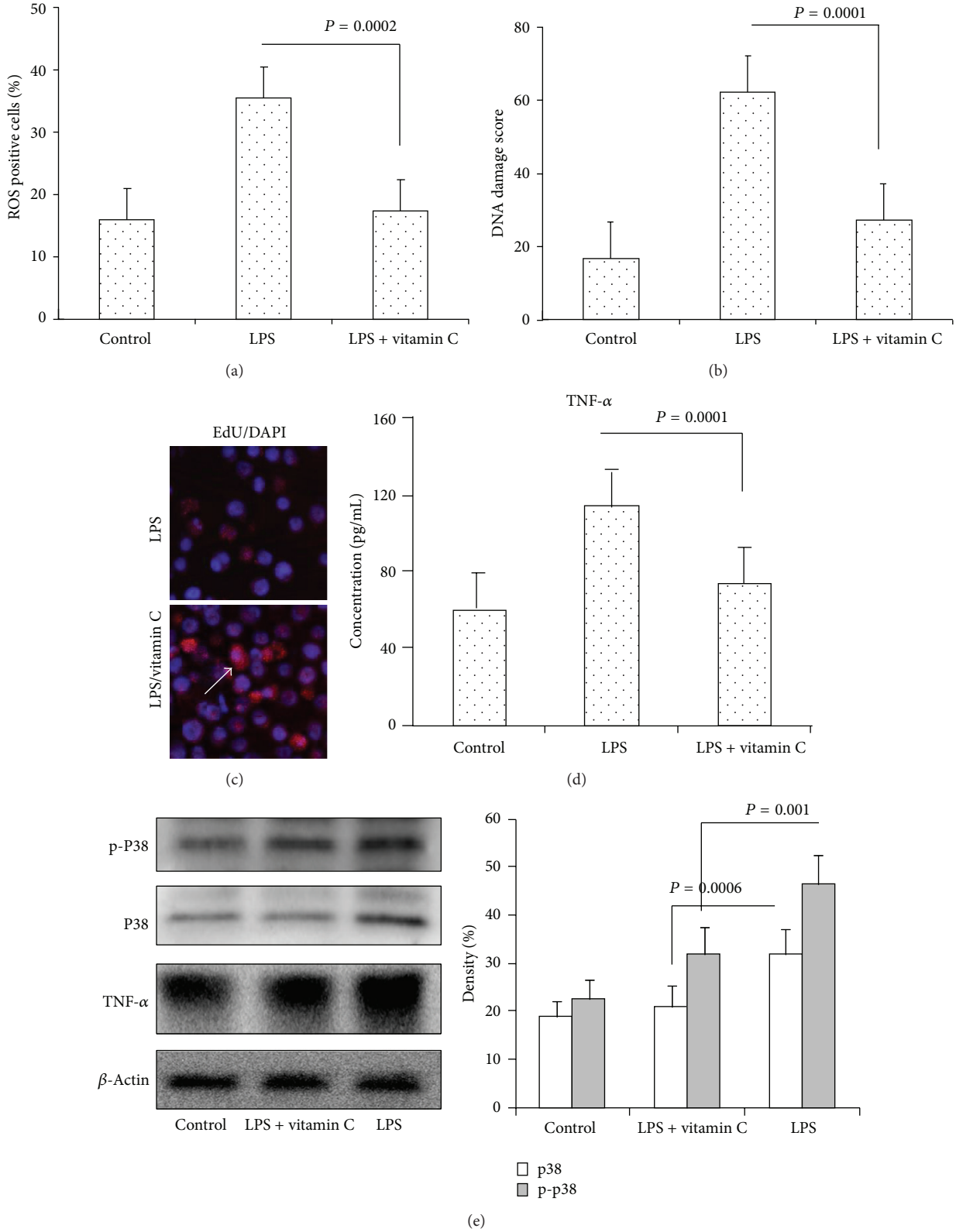

Figure 4: *The effect of vitamin C on oxidative stress and DNA damage in LPS-induced MH-S cells*. ROS and DNA damage in MH-S cells were measured in the presence or absence of vitamin C and treated with LPS for 12 hours. (a) ROS were detected by confocal microscope using DCFH-DA probe. ROS level was represented by fluorescence intensity. (b) DNA damage was measured by comet assay. DNA damage score was analyzed with comet assay IV software. (c) MH-S cells viability was detected by confocal microscope using Edu staining (×200). Arrow showed viability of cells. (d) TNF-α was measured by ELISA. (e) TNF-α, P38, and p-P38 were determined by western blotting. β-actin was used as the loading control.

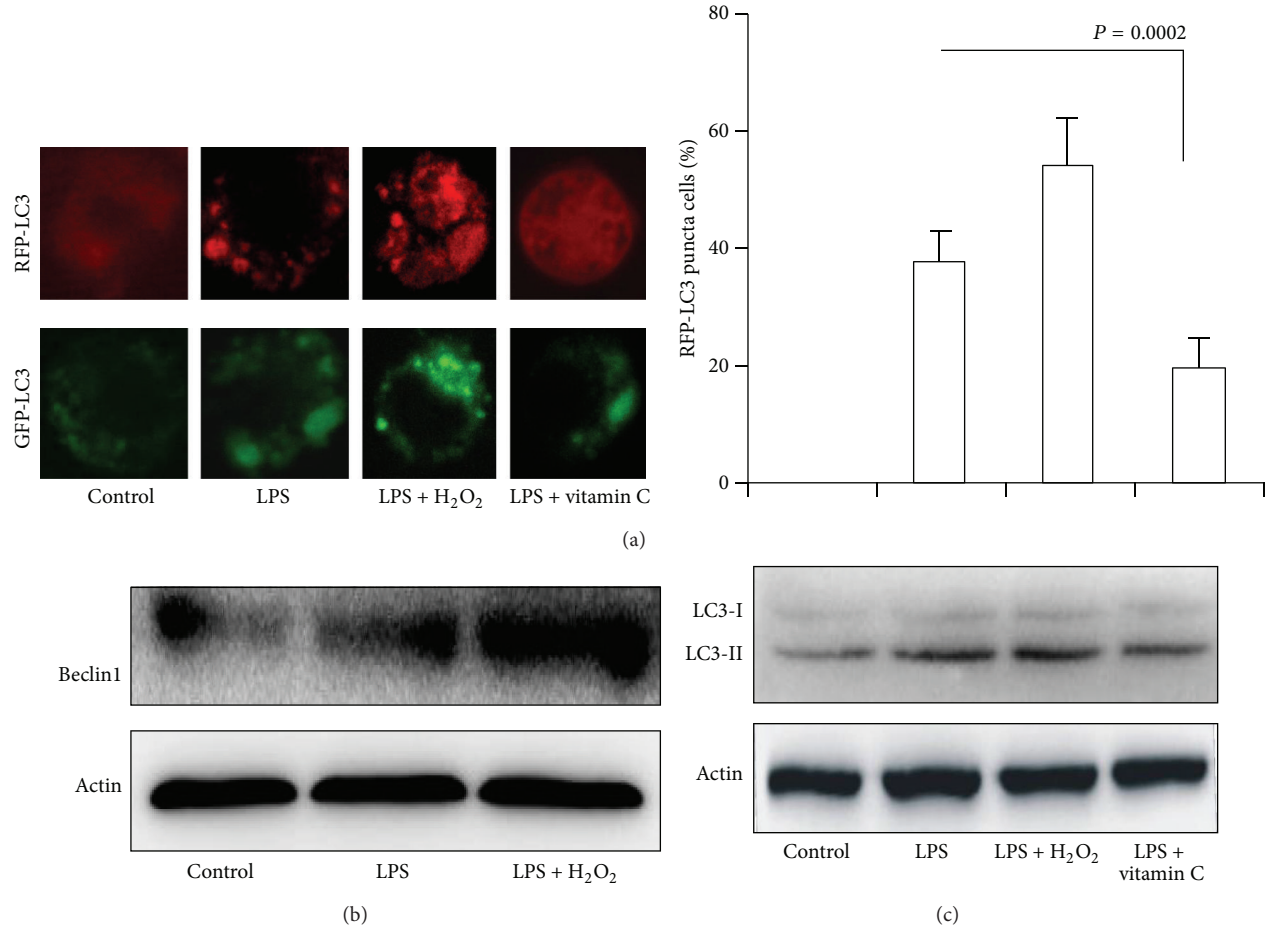

Figure 5: *The effect of vitamin C on LPS-induced autophagy in macrophages*. (a) MH-S cells were transfected with RFP-LC3 or GFP-LC3 plasmids for 24 hours. MH-S cells were cultured in the presence or absence of vitamin C and treated with LPS for 12 hours. (b) Western blotting of Beclin1; (c) western blotting of LC3. β-actin was probed as a loading control in (b) and (c).

The prophylactic use of vitamin C to prevent pneumonia should be further investigated in populations who have a high incidence of pneumonia [27]. Although it has been shown that vitamin C significantly improved the "total respiratory score" in the most severely ill patients, antioxidants may affect pulmonary morbidity. More research on vitamin C and other antioxidants seems to be warranted [7]. Antioxidants preserve macrophage phagocytosis of *Pseudomonas aeruginosa* during hyperoxia [32].

Our study is the first to report that vitamin C decreased ROS and DNA damage of severe CAP PBMC in vitro, and vitamin C decreased TNF-α and IL-6 in whole blood cells from severe CAP.

Reactive oxygen species (ROS) regulated inflammatory responses through the NF-κB pathway [33]. Oxidative stress may alter the balance between gene expression of proinflammatory mediators and antioxidant enzymes in favor of inflammatory mediators in the lung [34]. LPS (ligand of TLR4) induced tumor necrosis factor-α (TNF-α) production in macrophage lines. LPS-induced TNF-α may be a useful therapeutic candidate for the treatment of sepsis and other inflammatory diseases [35]. p38 activation were associated with LPS-induced TNF-α in macrophage [36]. N-acetylcysteine significantly improved zymosan-induced lung tissue damage and impaired lung function [37]. However, antioxidants increased the severity of peritonitis by decreasing the phagocytic efficiency, oxidative burst, and TNF-α production and increasing neutrophil infiltration. Antioxidants reduced the phagocytic efficacy of peritoneal macrophages and also decreased *E. coli*-induced oxidative burst in macrophages cells. Antioxidant supplementation during the course of bacterial infection is not recommended as it could be detrimental for the host [38]. Vitamin C is a novel regulator of neutrophil extracellular trap formation in sepsis. Vitamin C is protective in sepsis settings [39]. Our data indicated that LPS induced increase of ROS and DNA damage in macrophage cell lines. The expressions of TNF-α, p38, and of phosphorylation p38 were also increased in LPS-stimulated macrophages cells. Vitamin C reduced the ROS level and DNA damage degree and also decreased expressions of TNF-α, p38, and phosphorylation p38 in LPS-stimulated macrophages cells in vitro.

Autophagy pathway is activated under environmental stress conditions [40]. The previously reported autophagy in

vivo effectively regulates bacterial clearance of *P. aeruginosa* from the lung. Therapeutic intervention aimed at inducing autophagy with rapamycin correlates with decreased bacterial loads following *P. aeruginosa* lung infection in vivo [25]. LPS upregulates autophagy in hepatocytes; LC3II expression increased in both liver and hepatocytes after LPS and was dependent on TLR4 [41]. Indeed, our data convincingly showed that LC3 punctation increased in LPS-stimulated MH-S cells. H_2O_2 significantly increased LC3 punctation in LPS-stimulated MH-S cells. LC3 are widespread in the cells of various tissues, mainly expressed in autophagy body. LC3II expression increased in MH-S cells exposed to LPS and H_2O_2. Beclin1 expression increased in MH-S cells exposed to LPS and H_2O_2. The data indicated that oxidative stress unregulated autophagy, which might be useful for bacterial clearance. However, we found that vitamin C inhibited autophagy in MH-S cells exposed to LPS and H_2O_2. The effect of vitamin C on autophagy needs to be investigated.

In summary, we demonstrate that severe CAP exhibited significant increase of oxidative stress and proinflammatory mediators (TNF-α and IL-6) in lung and peripheral blood. Vitamin C inhibited ROS, DNA damage, TNF-α, and IL-6 from severe CAP in vitro. Vitamin C also reduced the ROS, DNA damage, and TNF-α production in LPS-stimulated macrophages cells. Oxidative stress unregulated LPS-induced autophagy in macrophages cells. Vitamin C inhibited autophagy in MH-S cells exposed to LPS and H_2O_2. Thus, our studies represent a novel mechanism of vitamin C by which it inhibited oxidative stress and proinflammatory mediators in severe pneumonia.

Abbreviations

ROS: Reactive oxygen species
LPS: Lipopolysaccharide
CAP: Community-acquired pneumonia
PBMC: Peripheral blood mononuclear cells
DHE: Dihydroethidium.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Guoping Li, Zhiguang Liu, and Nanshan Zhong conceived and designed the study. Yuanyuan Chen, Jiao Yuan, and Yuanyuan Wang performed the experiment. Guangyan Luo, Xiaoqiong Yang, and Xiaoyun Wang analyzed the data. Guoping Li wrote the paper. All authors read and approved the final paper. Yuanyuan Chen and Jiao Yuan contributed equally to this work.

Acknowledgment

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References

[1] P. V. Romero, B. Rodríguez, S. Martínez, R. Cañizares, D. Sepúlveda, and F. Manresa, "Analysis of oxidative stress in exhaled breath condensate from patients with severe pulmonary infections," *Archivos de Bronconeumologia*, vol. 42, no. 3, pp. 113–119, 2006.

[2] P. Ø. Jensen, J. Lykkesfeldt, T. Bjarnsholt, H. P. Hougen, N. Høiby, and O. Ciofu, "Poor antioxidant status exacerbates oxidative stress and inflammatory response to *Pseudomonas aeruginosa* lung infection in guinea pigs," *Basic & Clinical Pharmacology & Toxicology*, vol. 110, no. 4, pp. 353–358, 2012.

[3] Y. M. Hosakote, N. Komaravelli, N. Mautemps, T. Liu, R. P. Garofalo, and A. Casola, "Antioxidant mimetics modulate oxidative stress and cellular signaling in airway epithelial cells infected with respiratory syncytial virus," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 303, no. 11, pp. L991–L1000, 2012.

[4] W. Zhang, H. Wei, and B. Frei, "Genetic deficiency of NADPH oxidase does not diminish, but rather enhances, LPS-induced acute inflammatory responses in vivo," *Free Radical Biology and Medicine*, vol. 46, no. 6, pp. 791–798, 2009.

[5] J. M. Cook-Mills, M. E. Marchese, and H. Abdala-Valencia, "Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants," *Antioxidants & Redox Signaling*, vol. 15, no. 6, pp. 1607–1638, 2011.

[6] S. Trefler, A. Rodríguez, I. Martín-Loeches et al., "Oxidative stress in immunocompetent patients with severe community-acquired pneumonia. A pilot study," *Medicina Intensiva*, 2013.

[7] H. Hemilä, "Vitamin C and community-acquired pneumonia," *The American Journal of Respiratory and Critical Care Medicine*, vol. 184, no. 5, pp. 621–622, 2011.

[8] J. S. Tilstra, A. R. Robinson, J. Wang et al., "NF-κB inhibition delays DNA damage—induced senescence and aging in mice," *The Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2601–2612, 2012.

[9] L. A. Mandell, R. G. Wunderink, A. Anzueto et al., "Infectious diseases society of America/American thoracic society consensus guidelines on the management of community-acquired pneumonia in adults," *Clinical Infectious Diseases*, vol. 44, supplement 2, pp. S27–S72, 2007.

[10] E. H. Choi, M. Demerjian, D. Crumrine et al., "Glucocorticoid blockade reverses psychological stress-induced abnormalities in epidermal structure and function," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 291, no. 6, pp. R1657–R1662, 2006.

[11] R. P. Schleimer, A. P. Lane, and J. Kim, "Innate and acquired immunity and epithelial cell function in chronic rhinosinusitis," *Clinical Allergy and Immunology*, vol. 20, pp. 51–78, 2007.

[12] P. G. Holt and D. H. Strickland, "Interactions between innate and adaptive immunity in asthma pathogenesis: new perspectives from studies on acute exacerbations," *Journal of Allergy and Clinical Immunology*, vol. 125, no. 5, pp. 963–972, 2010.

[13] T. Lisboa, S. Blot, G. W. Waterer et al., "Radiologic progression of pulmonary infiltrates predicts a worse prognosis in severe community-acquired pneumonia than bacteremia," *Chest*, vol. 135, no. 1, pp. 165–172, 2009.

[14] M. L. Levy, I. L. Jeune, M. A. Woodhead, J. T. Macfarlane, and W. S. Lim, "Primary care summary of the British Thoracic Society guidelines for the management of community acquired

pneumonia in adults: 2009 update," *Primary Care Respiratory Journal*, vol. 19, no. 1, pp. 21–27, 2010.

[15] K. Shermatov, D. Zeyrek, F. Yildirim, M. Kilic, N. Cebi, and A. Kocyigit, "DNA damage in children exposed to secondhand cigarette smoke and its association with oxidative stress," *Indian Pediatrics*, vol. 49, no. 12, pp. 958–962, 2012.

[16] X. Fu, D. Tan, Z. Hou et al., "The effect of miR-338-3p on HBx deletion-mutant (HBx-d382) mediated liver-cell proliferation through CyclinD1 regulation," *PLoS ONE*, vol. 7, no. 8, Article ID e43204, 2012.

[17] K. Yuan, C. Huang, J. Fox et al., "Autophagy plays an essential role in the clearance of Pseudomonas aeruginosa by alveolar macrophages," *Journal of Cell Science*, vol. 125, part 2, pp. 507–515, 2012.

[18] G. Li, K. Yuan, C. Yan et al., "8-Oxoguanine-DNA glycosylase 1 deficiency modifies allergic airway inflammation by regulating STAT6 and IL-4 in cells and in mice," *Free Radical Biology and Medicine*, vol. 52, no. 2, pp. 392–401, 2012.

[19] S. Trefler, A. Rodríguez, I. Martín-Loeches et al., "Oxidative stress in immunocompetent patients with severe community-acquired pneumonia. A pilot study," *Medicina Intensiva*, vol. 38, no. 2, pp. 73–82, 2014.

[20] R. Subramaniam, P. F. Barnes, K. Fletcher et al., "Protecting against post-influenza bacterial pneumonia by increasing phagocyte recruitment and ROS production," *The Journal of Infectious Diseases*, 2014.

[21] M. F. Silva Nunes, R. da Silva Nunes, V. F. Silva Kahl, J. Moyses Reyes, and J. da Silva, "Use of buccal micronucleus assay to determine mutagenicity induced by amfepramone in humans and the protective effects of vitamin C," *Journal of Toxicology and Environmental Health A*, vol. 76, no. 19, pp. 1121–1128, 2013.

[22] S. Bozinovski, H. J. Seow, P. J. Crack, G. P. Anderson, and R. Vlahos, "Glutathione peroxidase-1 primes pro-inflammatory cytokine production after LPS challenge in vivo," *PLoS ONE*, vol. 7, no. 3, Article ID e33172, 2012.

[23] X. He, J. Shu, L. Xu, C. Lu, and A. Lu, "Inhibitory effect of Astragalus polysaccharides on lipopolysaccharide- induced TNF-α and IL-1β production in THP-1 cells," *Molecules*, vol. 17, no. 3, pp. 3155–3164, 2012.

[24] N. Nakao, T. Kurokawa, T. Nonami, G. Tumurkhuu, N. Koide, and T. Yokochi, "Hydrogen peroxide induces the production of tumor necrosis factor-α in RAW 264.7 macrophage cells via activation of p38 and stress-activated protein kinase," *Innate Immunity*, vol. 14, no. 3, pp. 190–196, 2008.

[25] R. D. Junkins, A. Shen, K. Rosen, C. McCormick, and T. Lin, "Autophagy Enhances Bacterial Clearance during *P. aeruginosa* Lung Infection," *PLoS ONE*, vol. 8, no. 8, Article ID e72263, 2013.

[26] J. Wang, X. Feng, Y. Zeng et al., "Lipopolysaccharide (LPS)-induced autophagy is involved in the restriction of *Escherichia coli* in peritoneal mesothelial cells," *BMC Microbiology*, vol. 13, article 255, 2013.

[27] H. Hemilä and P. Louhiala, "Vitamin C for preventing and treating pneumonia," *Cochrane Database of Systematic Reviews*, 2007.

[28] E. Kratzer, Y. Tian, N. Sarich et al., "Oxidative stress contributes to lung injury and barrier dysfunction via microtubule destabilization," *American Journal of Respiratory Cell and Molecular Biology*, vol. 47, no. 5, pp. 688–697, 2012.

[29] A. Valavanidis, T. Vlachogianni, K. Fiotakis, and S. Loridas, "Pulmonary oxidative stress, inflammation and cancer: Respirable particulate matter, fibrous dusts and ozone as major causes of lung carcinogenesis through reactive oxygen species mechanisms," *International Journal of Environmental Research and Public Health*, vol. 10, no. 9, pp. 3886–3907, 2013.

[30] Y. M. Hosakote, P. D. Jantzi, D. L. Esham et al., "Viral-mediated inhibition of antioxidant enzymes contributes to the pathogenesis of severe respiratory syncytial virus bronchiolitis," *American Journal of Respiratory and Critical Care Medicine*, vol. 183, no. 11, pp. 1550–1560, 2011.

[31] M. Andrades, C. Ritter, M. R. De Oliveira, E. L. Streck, J. C. Fonseca Moreira, and F. Dal-Pizzol, "Antioxidant treatment reverses organ failure in rat model of sepsis: Role of antioxidant enzymes imbalance, neutrophil infiltration, and oxidative stress," *Journal of Surgical Research*, vol. 167, no. 2, pp. e307–e313, 2011.

[32] D. M. P. Morrow, T. Entezari-Zaher, J. Romashko III et al., "Antioxidants preserve macrophage phagocytosis of Pseudomonas aeruginosa during hyperoxia," *Free Radical Biology and Medicine*, vol. 42, no. 9, pp. 1338–1349, 2007.

[33] W. Han, H. Li, J. Cai et al., "NADPH oxidase limits lipopolysaccharide-induced lung inflammation and injury in mice through reduction-oxidation regulation of NF-κB activity," *The Journal of Immunology*, vol. 190, no. 9, pp. 4786–4794, 2013.

[34] I. Rahman, "Oxidative stress and gene transcription in asthma and chronic obstructive pulmonary disease: antioxidant therapeutic targets," *Current Drug Targets: Inflammation & Allergy*, vol. 1, no. 3, pp. 291–315, 2002.

[35] Y. Song, H. Dou, W. Gong et al., "Bis-N-norgliovictin, a small-molecule compound from marine fungus, inhibits LPS-induced inflammation in macrophages and improves survival in sepsis," *European Journal of Pharmacology*, vol. 705, no. 1–3, pp. 49–60, 2013.

[36] H. Li, D. Hu, H. Fan et al., "β-arrestin 2 negatively regulates TLR4-triggered inflammatory signaling via targeting p38 MAPK and IL-10," *The Journal of Biological Chemistry*, 2014.

[37] H. W. Wang, W. Yang, J. Lu et al., "N-acetylcysteine administration is associated with reduced activation of NF-κB and preserves lung dendritic cells function in a zymosan-induced generalized inflammation model," *Journal of Clinical Immunology*, vol. 33, no. 3, pp. 649–660, 2013.

[38] M. Goswami, D. Sharma, N. M. Khan, R. Checker, S. K. Sandur, and N. Jawali, "Antioxidant supplementation enhances bacterial peritonitis in mice by inhibiting phagocytosis," *Journal of Medical Microbiology*, vol. 63, part 3, pp. 355–366, 2014.

[39] B. M. Mohammed, B. J. Fisher, D. Kraskauskas et al., "Vitamin C: a novel regulator of neutrophil extracellular trap formation," *Nutrients*, vol. 5, no. 8, pp. 3131–3151, 2013.

[40] R. Scherz-Shouval, E. Shvets, E. Fass, H. Shorer, L. Gil, and Z. Elazar, "Reactive oxygen species are essential for autophagy and specifically regulate the activity of Atg4," *The EMBO Journal*, vol. 26, no. 7, pp. 1749–1760, 2007.

[41] C. Chen, M. Deng, Q. Sun, P. Loughran, T. R. Billiar, and M. J. Scott, "Lipopolysaccharide stimulates p62-dependent autophagy-like aggregate clearance in hepatocytes," *BioMed Research International*, vol. 2014, Article ID 267350, 13 pages, 2014.

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Research Article

Palmitoleic Acid (N-7) Attenuates the Immunometabolic Disturbances Caused by a High-Fat Diet Independently of PPARα

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Palmitoleic acid (PMA) has anti-inflammatory and antidiabetic activities. Here we tested whether these effects of PMA on glucose homeostasis and liver inflammation, in mice fed with high-fat diet (HFD), are PPAR-α dependent. C57BL6 wild-type (WT) and PPAR-α-knockout (KO) mice fed with a standard diet (SD) or HFD for 12 weeks were treated after the 10th week with oleic acid (OLA, 300 mg/kg of b.w.) or PMA 300 mg/kg of b.w. Steatosis induced by HFD was associated with liver inflammation only in the KO mice, as shown by the increased hepatic levels of IL1-beta, IL-12, and TNF-α; however, the HFD increased the expression of TLR4 and decreased the expression of IL1-Ra in both genotypes. Treatment with palmitoleate markedly attenuated the insulin resistance induced by the HFD, increased glucose uptake and incorporation into muscle in vitro, reduced the serum levels of AST in WT mice, decreased the hepatic levels of IL1-beta and IL-12 in KO mice, reduced the expression of TLR-4 and increased the expression of IL-1Ra in WT mice, and reduced the phosphorylation of NF κB (p65) in the livers of KO mice. We conclude that palmitoleate attenuates diet-induced insulin resistance, liver inflammation, and damage through mechanisms that do not depend on PPAR-α.

1. Introduction

Chronic positive energy balance through inadequate dietary habits and a sedentary life style leads to an excessive accumulation of body fat, known as obesity, the prevalence of which is increasing alarmingly worldwide [1]. Obese individuals have a greater risk for the development of many chronic and highly incident diseases such as type 2 diabetes, dislipidemia, hepatic steatosis, and some types of cancer [2–6].

Among these diseases, nonalcoholic fatty liver disease (NAFLD), which is defined as excessive hepatic lipid accumulation, is one of the most common comorbidities associated with obesity and insulin resistance. Along with the obesity epidemic, the incidence of NAFLD is growing worldwide; NAFLD now affects an estimated 20–30% of the population of Western countries [1]. If not treated, hepatocellular steatosis may progress to more severe diseases such as nonalcoholic steatohepatitis (NASH), liver cirrhosis, and hepatocellular carcinoma [7, 8].

Consistent with the key role of the liver in the regulation of glucose metabolism, excessive fat accumulation in the liver promotes a local inflammatory process that is frequently associated with the development of tissue insulin resistance and major changes in glucose homeostasis. More specifically, hepatic steatosis and inflammation markedly impair the ability of insulin to inhibit liver glucose production, leading to hyperglycemia and hyperinsulinemia [9, 10]. Therefore, strategies to counteract hepatic steatosis, inflammation,

and increased hepatic glucose production are crucial to the prevention and treatment of chronic metabolic diseases.

Palmitoleic acid (16:1n7), a monounsaturated fatty acid (n-7) of 16 carbons that is produced in adipose tissue, has been shown to have important metabolic activities that improve whole body glucose homeostasis and insulin sensitivity [11]. Indeed, palmitoleic acids were shown to increase insulin-stimulated glucose uptake by the skeletal muscles [11] and to reduce liver steatosis, inflammation, and insulin resistance, thus attenuating high-fat diet-induced hepatic glucose production [12].

Mechanistically, palmitoleic acid reduces hepatic steatosis by inhibiting the expression of sterol regulatory element binding protein-1 (SREBP1), a transcription factor that is involved in the regulation of many enzymes involved in lipid synthesis, including fatty acid synthase (FAS) and stearoyl-CoA desaturase 1 (SCD1) [13]. Very recently, it was demonstrated that palmitoleic acid is a positive modulator of white adipose lipolysis through a mechanism that involves an increase in the content of the lipase adipose triglyceride lipase (ATGL) and requires the activation of nuclear receptor PPARα [14]. Peroxisome proliferator activated receptor α (PPARα), a transcription factor that is primarily expressed in the liver and modulates the transcription of enzymes related to β-oxidation, promoting the oxidation of fatty acids and an overall reduction in the deposition of ectopic triacylglycerol [15]. Furthermore, PPARα was also shown to suppress the expression of proinflammatory genes, primarily by inactivating the master proinflammatory transcription factor NFκB and thus reducing the production of cytokines and tissue inflammation [16].

Based on these findings, we tested the hypothesis that palmitoleic acid attenuates obesity-associated hepatic steatosis and inflammation by activating the nuclear receptor PPARα. For this, wild-type and PPARα-knockout mice were fed a high-fat diet, either untreated or treated with palmitoleic acid, and evaluated for hepatic steatosis and inflammation and whole body glucose homeostasis.

2. Materials and Methods

2.1. Animal Procedure. Male C57BL/6J wild-type (WT) and PPARα-knockout (KO) mice were obtained from the Jackson Laboratory and maintained on a 12:12 h light-dark cycle (lights on at 06:00). Beginning at 10 to 12 weeks of age, the mice were fed a high-fat diet (HFD, 59% of calories from fat, 15% from proteins, and 26% from carbohydrates) [17] or a low-fat diet (LFD, 9% of calories from fat, 15% from protein, and 76% from carbohydrate) [17] for 12 weeks. In the last 2 weeks, the HFD-fed mice were treated with oleic acid (300 mg/kg of body weight) or palmitoleic acid (300 mg/kg of body weight) daily by oral gavage. The doses and treatment regimen were based on previous studies [13, 14]. During the feeding/treatment period, the body weight and food intake of the mice were evaluated weekly. After 12 weeks, the mice were fasted for 4 hr and then sacrificed for the collection of blood and tissue samples. The epididymal, mesenteric, and retroperitoneal adipose tissues were dissected and weighed, and the total weight of these tissues was represented as adipose tissue index. The liver was weighed and stored for the further analysis of RNA and protein.

2.2. Analytical Procedures. Plasma total cholesterol, HDL cholesterol, triacylglycerol levels, and alanine aminotransferase activity were determined by enzymatic methods (Labtest, Lagoa Santa, MG, Brazil). The LDL levels were estimated using the Friedewald equation [18].

2.3. Histological Analyses. Small pieces of liver tissue were fixed with paraformaldehyde (10%), embedded in paraffin, and serially cross-sectioned. The slides were stained with hematoxylin and eosin to analyze steatosis [19].

2.4. Assessment of Triacylglycerol Levels in the Liver. Lipids were extracted from the livers with chloroform-methanol, as described by Folch et al. [20]. Tissue triacylglycerol levels in the lipid extract were determined by enzymatic assays (Labtest, Lagoa Santa, MG, Brazil).

2.5. Insulin and Glucose Tolerance Tests. Mice fasted for 4 hr received an intraperitoneal injection of insulin (1 U/kg body weight) or D-glucose (2 g/kg body weight). For the insulin tolerance tests, blood samples (5 μL) were collected from the tail vein before and at 10, 20, 30, and 40 min after the bolus insulin injection. The constant for plasma glucose clearance (KITT) was calculated by linear regression of the glycemic levels measured between 5 and 30 minutes after insulin injection; this is the interval in which the glucose linear decay phase occurs [21]. Similarly, for the glucose tolerance tests, blood samples were collected from the tail vein before and at 15, 30, 60, 90, and 120 min after the glucose bolus injection [22]. The differences in glycemia before and during glucose administration were used to calculate the areas under the curve (AUC). The levels of plasma glucose were measured using an Accu-Chek Performa glucometer (ROCHE, São Paulo, SP, Brazil).

2.6. Insulin Response in Isolated Soleus Muscles. Soleus muscles from euthanized WT and KO mice were carefully isolated, weighed (8–10 mg), and attached to stainless steel clips to maintain resting tension. The muscles were preincubated in Krebs-Ringer bicarbonate buffer (KRBB) containing 5.69 mM glucose and 1% bovine serum albumin (BSA), pH 7.4, and pregassed (95% O_2, 5% CO_2) with agitation (100 oscillations/min). After these procedures, the muscles were transferred to fresh vials containing the same buffer containing 0.3 μCi/mL D-[U-^{14}C]-glucose and 0.2o μCi/mL 2-deoxy-D-[2,6-^{3}H]-glucose in the presence or absence of 7 nM insulin. After the incubation period, the samples were processed to measure the uptake of 2-deoxy-D-[2,6-^{3}H]-glucose, the incorporation of D-[^{14}C]-glucose, the synthesis of [^{14}C]-glycogen, and the decarboxylation of D-[^{14}C]-glucose, according to the methods described by Challiss et al. [23], Espinal et al. [24], and Leighton et al. [25].

2.7. Enzymatic Assays. Livers were homogenized (1 : 10, w/v) in SETH buffer, pH 7.4 (250 mM sucrose, 2 mM EDTA, 10 mM Trizma base, and 50 IU/mL heparin). The homogenates were centrifuged, and the supernatants were stored at −70°C for use in enzyme activity determination. The protein content of the homogenates was determined by the method described by Lowry and colleagues [26]. The activity of citrate synthase was assayed according to the method described by Srere, and the reaction was initiated by adding oxaloacetate (0.2 mM) to a mixture containing Tris (100 mM, pH 8.0), acetyl CoA (0.1 mM), dithiobis-2-nitrobenzoic acid (0.1 mM), Triton X-100 (0.1%), and 2 to 4 μg of supernatant protein and monitored at 412 nm for 3 min at 25°C [27]. The activity of succinate dehydrogenase was measured according to the method of Fischer and colleagues [28] as a decrease in the absorbance of the reactions at 600 nm caused by the reduction of 2,6-di-chloro-indophenol in the presence of phenazine methosulfate. The activity of malate dehydrogenase was measured as described by Kitto et al. [29]. The activity of NADH dehydrogenase (complex I) was evaluated according to Cassina and Radi [30], and the activity of the succinate: cytochrome c oxidoreductase (complex III) was determined using the method described by Fischer and colleagues [28].

2.8. Enzyme-Linked Immunosorbent Assay (ELISA). Liver tissue samples (80–100 mg) were carefully homogenized in RIPA buffer (0.625% Nonidet P-40, 0.625% sodium deoxycholate, 6.25 mM sodium phosphate, and 1 mM ethylenediaminetetraacetic acid at pH 7.4) containing 10 μg/mL protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The homogenates were centrifuged, the supernatant was utilized to determinate the protein concentration via Bradford assays (Bio-Rad, Hercules, CA, USA), and the protein levels of IL-1β, IL-8, IL-12, and TNF-α were measured by ELISA (DuoSet ELISA, R&D Systems, Minneapolis, MN, USA). For IL-1β, IL-8, IL-12, and TNF-α, the assay sensitivity was 5.0 pg/mL in a range of 31.2 to 2,000 pg/mL.

2.9. RNA Isolation, Reverse Transcription, and Real-Time PCR. The expression of hepatic genes related to fatty acid synthesis (ACC) and some factors involved in inflammation (IL-1Ra, TLR4 and TNF-α) was assessed by qRT-PCR with SYBR Green marker. For this, total RNA was extracted as described by Chomczynski and Sacchi [31] and quantified in a spectrophotometer (260 nm), and cDNA was synthesized from the total RNA using reverse transcriptase. The sequences of the primers are shown in Table 1; gene expression was quantified by the comparative method using the expression of GAPDH as standard [32].

2.10. Western Blotting. The livers were carefully homogenized in extraction buffer containing protease and phosphatase inhibitors. After proper centrifugation, the protein concentrations were determined by Bradford assay (Bio-Rad, Hercules, CA, USA). Aliquots of each sample with the same concentration of total protein (25 g) were then diluted in Laemmli buffer, subjected to electrophoresis on SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred from the gel to a nitrocellulose membrane. These membranes were incubated with antibodies against Toll-like receptor 4 (1 : 500), Phospho-NFκB p65 (Ser536) (1 : 500) (Cell Signaling Technologies, USA), or β-tubulin (1 : 1.000) (Santa Cruz Biotechnology, USA) and then incubated with an anti-IgG antibody conjugated with peroxidase. After the incubations, these membranes were incubated with the peroxidase substrate (ECL kit, Biorad, USA) and exposed to X-ray film.

2.11. Statistical Methods. The data are presented as mean ± SEM (standard error of the mean) and analyzed by one-way analysis of variance (one-way ANOVA) followed by Bonferroni posttests. Analyses were performed using GraphPad Prism 5.0 software. Differences were considered significant when $P < 0.05$.

3. Results

We did not observe any differences in the variables analyzed between WT and KO mice fed with the control SD diet (see Supplementary Table of the Supplementary Material available online at http://dx.doi.org/10.1155/2014/582197); therefore, for the sake of objectivity, we are showing only the data for WT mice fed with the standard control diet.

As expected, the HFD markedly increased the body weight gain of WT and KO mice, and this effect was of lower magnitude in the KO mice (40% versus 10%, resp.) (Table 1). This increased body weight gain induced by the HFD was associated with a marked increase in mouse adiposity, as evidenced by the greater sum of the masses of the major adipose deposits (epididymal, retroperitoneal, and inguinal, see adipose tissue index, Table 2). In contrast to the white adipose tissue, however, HFD feeding did not affect the mass of the brown adipose tissue in any of the groups tested (Table 2). The HFD significantly increased total cholesterol, and in KO mice, the estimated levels of LDL were higher (Table 2). The fasting glucose levels were higher in the animals that were subjected to the HFD, but the KO mice had lower glycemia than the WT mice (Table 2). Treatment with palmitoleic acid (PMA) did not alter the body weight, the weights of the white and brown adipose tissues, or the plasma lipid profile of either group, but treatment with palmitoleic acid reduced the fasting glucose levels in both WT and KO mice that were subjected to the HFD (Table 2).

Our results showed that independent of the mice genotype, HFD feeding reduced the peripheral responsiveness to insulin. WT and KO mice subjected to the HFD had a reduced glucose uptake, but WT mice had a reduced incorporation of glucose by insulin stimulation (Figures 1(a) and 1(b)). When compared to SD-fed WT mice, both WT and KO mice subjected to the HFD had a greater increase in glycemia in a glucose tolerance test (GTT) (Figure 1(c)), and this metric could be confirmed by the increase in the AUC (Figure 1(d)); however, compared to HFD-fed WT mice, the HFD-fed KO mice presented a higher glucose tolerance. In addition, the HFD impaired the response to insulin in WT

Table 1: Sequences of forward and reverse primers used for qRT-PCR.

Gene	Forward primer	Reverse primer
GAPDH	CAAGCTCATTTCCTGGTATGACA	GCCTCTCTTGCTCAGTGTCC
ACC	CCAGCAGATTGCCAACATC	ACTTCGGTACCTCTGCACCA
TLR4	TCCAGCCACTGAAGTTCT	CAGCAAAGTCCCTGATGA
TNF-α	TCTACTGAACTTCGGGGTGA	GATCTGAGTGTGAGGGTCTGG
IL-1Ra	GCAAGATGCAAGCCTTCAGA	CCTTGTAAGTACCCAGCAATGA

Table 2: Body weight (BW); tissue masses; and plasma levels of triacylglycerol, total cholesterol, HDL, estimated LDL, and fasting glycemia in wild-type (WT) and PPARα-knockout (KO) mice fed with a standard diet (SD) or a high fat diet (HFD) and either untreated or treated with oleic acid (HFD) or palmitoleic acid (HFD PMA).

	WT mice			PPARα-KO mice	
	SD	HFD	HFD PMA	HFD	HFD PMA
Initial BW (g)	26.33 ± 0.45 ($n = 6$)	26.87 ± 0.73 ($n = 6$)	27.2 ± 0.62 ($n = 6$)	26.71 ± 0.61 ($n = 4$)	25.73 ± 0.62 ($n = 5$)
10 wk BW (g)	29.80 ± 1.04 ($n = 6$)	39.83 ± 1.90* ($n = 6$)	39.39 ± 1.68* ($n = 6$)	34.80 ± 1.26* ($n = 4$)	33.98 ± 1.72* ($n = 5$)
Final BW (g)	28.66 ± 0.73 ($n = 6$)	38.82 ± 1.90* ($n = 6$)	38.4 ± 1.71* ($n = 6$)	33.39 ± 1.09* ($n = 4$)	32.47 ± 1.69* ($n = 5$)
Adiposity index (g)	1.33 ± 0.20 ($n = 6$)	3.51 ± 0.42* ($n = 5$)	3.82 ± 0.42* ($n = 6$)	2.41 ± 0.28$^{\$}$ ($n = 4$)	2.19 ± 0.40$^{\$}$ ($n = 5$)
Brown adipose tissue weight (g)	0.42 ± 0.05 ($n = 6$)	0.45 ± 0.04 ($n = 5$)	0.45 ± 0.07 ($n = 6$)	0.32 ± 0.03 ($n = 4$)	0.33 ± 0.04 ($n = 5$)
Liver weight (g)	1.05 ± 0.02 ($n = 6$)	1.19 ± 0.05* ($n = 5$)	1.19 ± 0.06* ($n = 6$)	1.26 ± 0.05* ($n = 4$)	1.23 ± 0.08* ($n = 5$)
Triacylglycerol (mg/dL)	75.39 ± 5.37 ($n = 6$)	87.86 ± 10.44 ($n = 4$)	88.25 ± 7.96 ($n = 6$)	94.28 ± 5.23 ($n = 4$)	91.05 ± 2.75 ($n = 5$)
Total cholesterol (mg/dL)	133.9 ± 7.18 ($n = 6$)	177.5 ± 8.68* ($n = 5$)	173.7 ± 4.45* ($n = 6$)	193.4 ± 19.99* ($n = 4$)	187.3 ± 14.64* ($n = 5$)
HDL cholesterol (mg/dL)	109.7 ± 5.32 ($n = 6$)	152.8 ± 12.14* ($n = 5$)	145.7 ± 12.54 ($n = 6$)	123.2 ± 7.65$^{\$}$ ($n = 4$)	111.4 ± 8.79$^{\$}$ ($n = 5$)
Estimated LDL (mg/dL)	21.28 ± 4.25 ($n = 4$)	20.72 ± 5.32 ($n = 4$)	31.34 ± 2.99 ($n = 4$)	51.37 ± 17.30$^{\$}$ ($n = 4$)	47.23 ± 10.88$^{\$}$ ($n = 4$)
Fasting glucose (mg/dL)	130.1 ± 13.66 ($n = 6$)	171.3 ± 9.58* ($n = 6$)	150.0 ± 10.25$^{\#}$ ($n = 6$)	124.7 ± 8.38$^{*\$\$\$}$ ($n = 4$)	98.0 ± 4.41$^{\#\$\$\$}$ ($n = 5$)

The data are presented as mean ± SEM. $^{*}P < 0.05$ versus WT SD; $^{\#}P < 0.05$ HFD versus HFD PMA; and $^{\$}P < 0.05$ and $^{\$\$\$}P < 0.001$ KO versus the respective WT control (one-way ANOVA followed by Bonferroni correction).

mice but not in KO mice (Figure 1(e)), who had a better responsiveness to insulin as shown by the diminished KITT (Figure 1(f)). Additionally, we observed that independent of the mouse genotype, palmitoleate markedly attenuated the insulin resistance induced by HFD, as evidenced by the increased glucose uptake (Figure 1(a)) and by the better response to insulin (Figure 1(h)) in both groups. The palmitoleate also increased the incorporation of glucose into muscle in vitro (Figure 1(b)) and improved the tolerance to glucose (Figure 1(d)) in WT mice but not in KO mice.

One of the tissues that was most affected by the HFD was the liver; indeed, the weight of the liver was markedly increased by the HFD in both WT and KO mice (Table 2); however, the HFD only promoted a significant increase in triacylglycerols in the livers of KO mice (Figure 2(a)), and we only observed severe steatosis in liver histological slices from KO mice fed with the HFD (Figure 3). Both WT and KO mice had an increase in the serum levels of the liver damage marker aspartate transaminase when subjected to the HFD (Figure 2(b)). PMA did not alter the weight of the liver or the ectopic accumulation of fat in the liver and did not seem to modulate hepatic steatosis, but this monounsaturated fatty acid almost completely restored the levels of aspartate transaminase in the serum of WT mice, indicating that there may be some protective effect of PMA on liver injury (Figures 2 and 3).

The activity of several Krebs cycle enzymes (citrate synthase (Figure 4(a)), succinate dehydrogenase (Figure 4(b)), and malate dehydrogenase (Figure 4(c))) and the activity of the electron transport chain complexes I and III (Figures 4(d) and 4(e)) were assessed. Surprisingly, of all these enzymes and complexes, the HFD only promoted a difference in the activity of malate dehydrogenase, and the effects of the HFD on the malate dehydrogenase activity of KO mice were even

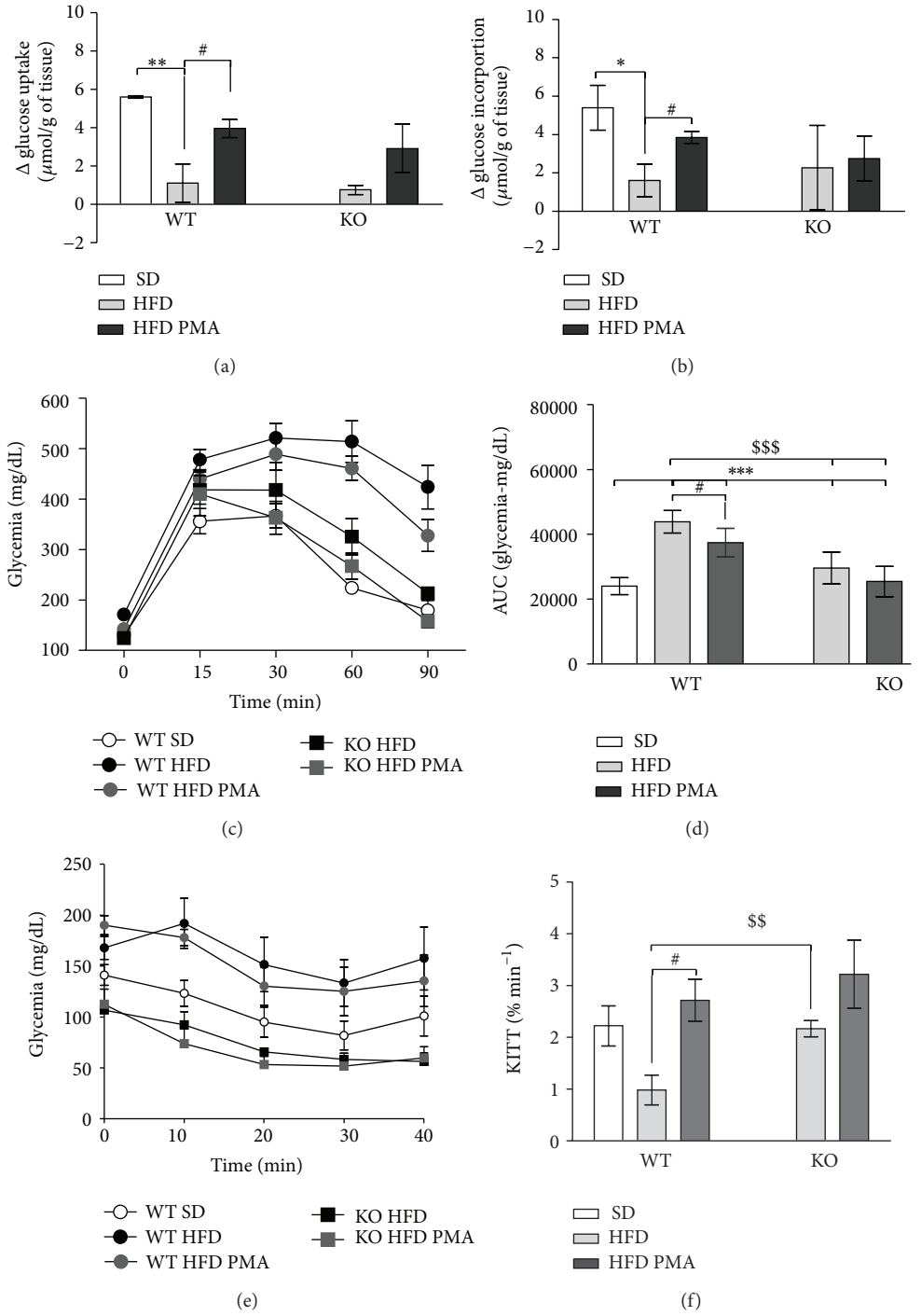

Figure 1: Delta (Δ) of glucose uptake (a) and incorporation (b), variation in glycemia in the glucose tolerance test (c), and respective area under curve (AUC) (d). Variation in glycemia in the insulin tolerance test (e). Respective constants for glucose clearance (f) of WT mice fed with a standard diet (SD) or WT and PPARα-knockout (KO) mice subjected to a high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA). The data are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ versus WT SD; $^{\#}P < 0.05$ HFD versus HFD PMA; and $^{\$\$}P < 0.01$ and $^{\$\$\$}P < 0.001$ KO versus the respective WT control (one-way ANOVA followed by Bonferroni correction).

greater. In spite of this, supplementation with palmitoleic acid only decreased the activity of malate dehydrogenase in WT mice (Figure 4(c)).

Surprisingly, the hepatic steatosis induced by the HFD was associated with liver inflammation in the KO mice but not in WT mice, as indicated by increased hepatic levels of IL-1β (Figure 5(a)), IL-12 (Figure 5(b)) and a trend toward higher levels of IL-8 (Figure 5(c)) and TNF-α (Figure 5(d)) in the livers of KO mice when compared to WT mice subjected to the SD. Furthermore, in KO mice, palmitoleate reduced the

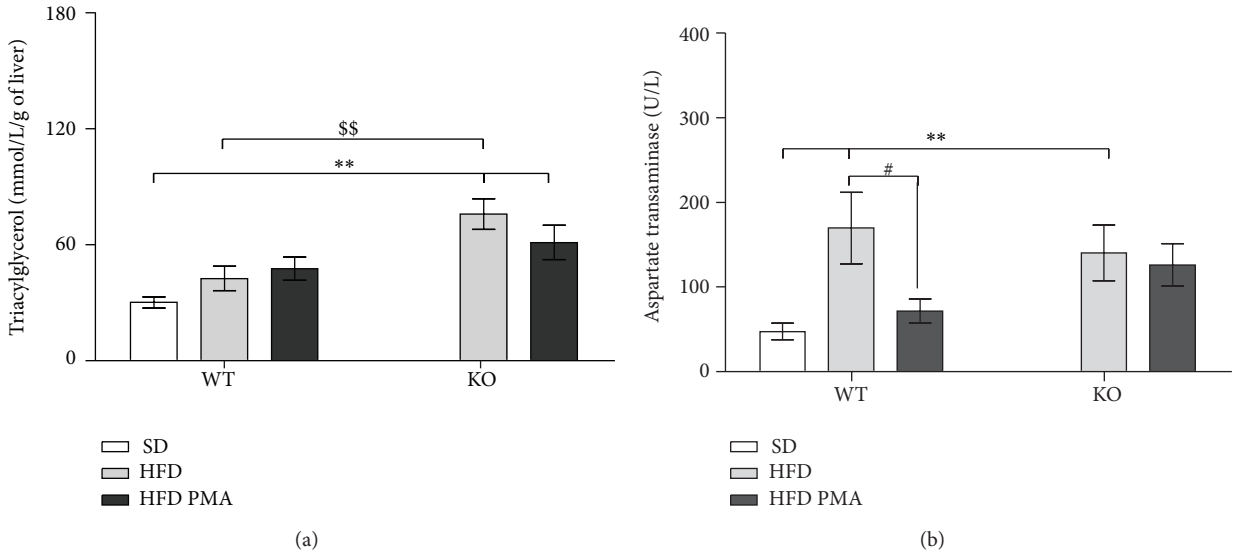

Figure 2: Triacylglycerol levels in the livers (a) and aspartate aminotransferase levels in the plasma (b) of WT mice fed with a standard diet (SD) or WT and PPARα-knockout (KO) subjected to a high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA). The data are presented as mean ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus WT SD; $^{\#}P < 0.05$ HFD versus HFD PMA; and $^{\$\$}P < 0.01$ KO versus the respective WT control (one-way ANOVA followed by Bonferroni correction).

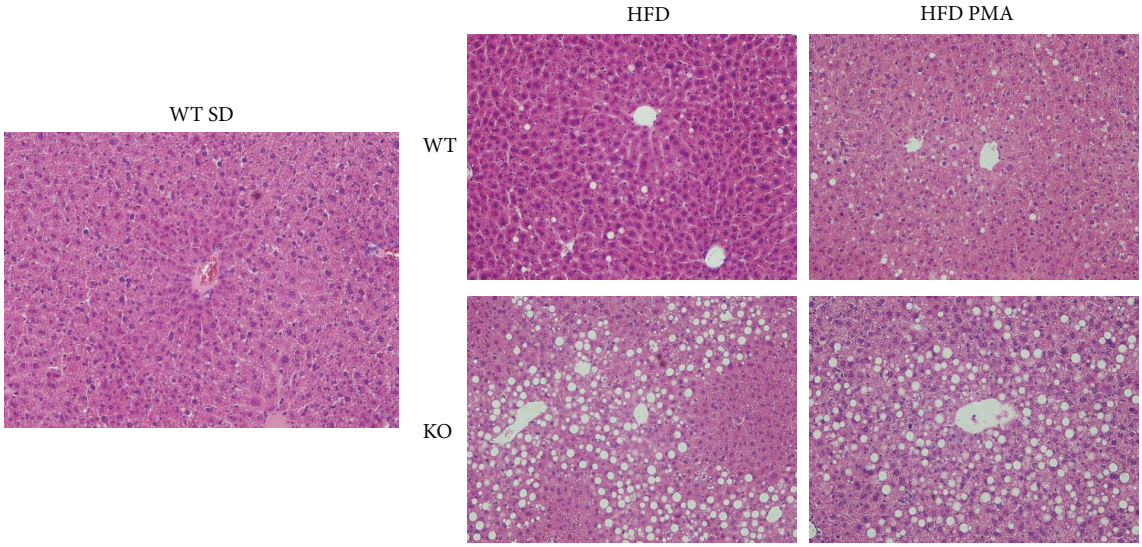

Figure 3: Histological slices of livers colored by hematoxylin and eosin (HE) at 40x magnification. Livers of WT mice fed with a standard diet (SD) or WT and PPARα-knockout (KO) mice submitted to a high fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA).

hepatic levels of IL1-β and IL-12 and caused a trend toward reduced levels of IL-8 and TNF-α in liver (Figure 5).

Although the HFD decreased the mRNA expression of ACC and IL-1Ra in WT mice (Figures 6(a) and 6(c)), the HFD upregulated the expression of TLR4 both in WT and KO mice (Figure 6(b)). In addition, the expression of TNFα was not modulated by the HFD but was increased only by the knockout of PPARα (Figure 6(d)). Palmitoleate did not affect the expression of ACC mRNA (Figure 6(a)), but it tended to reduce the hepatic expression of TLR-4 and TNFα (Figures 6(b) and 6(d)), reversed the decrease in IL-1Ra mRNA expression in WT, and dramatically increased IL-1Ra mRNA expression in KO mice (Figure 6(c)).

Along with increasing TLR4 mRNA levels, TLR4 protein levels and the phosphorylation of NFκB (p65) in serine were increased in KO mice that were fed with the HFD (Figure 7); treatment with palmitoleic acid decreased both of these inflammatory mediators, reducing the levels of TLR4 and the phosphorylation of NFKB (p65) (Figure 7).

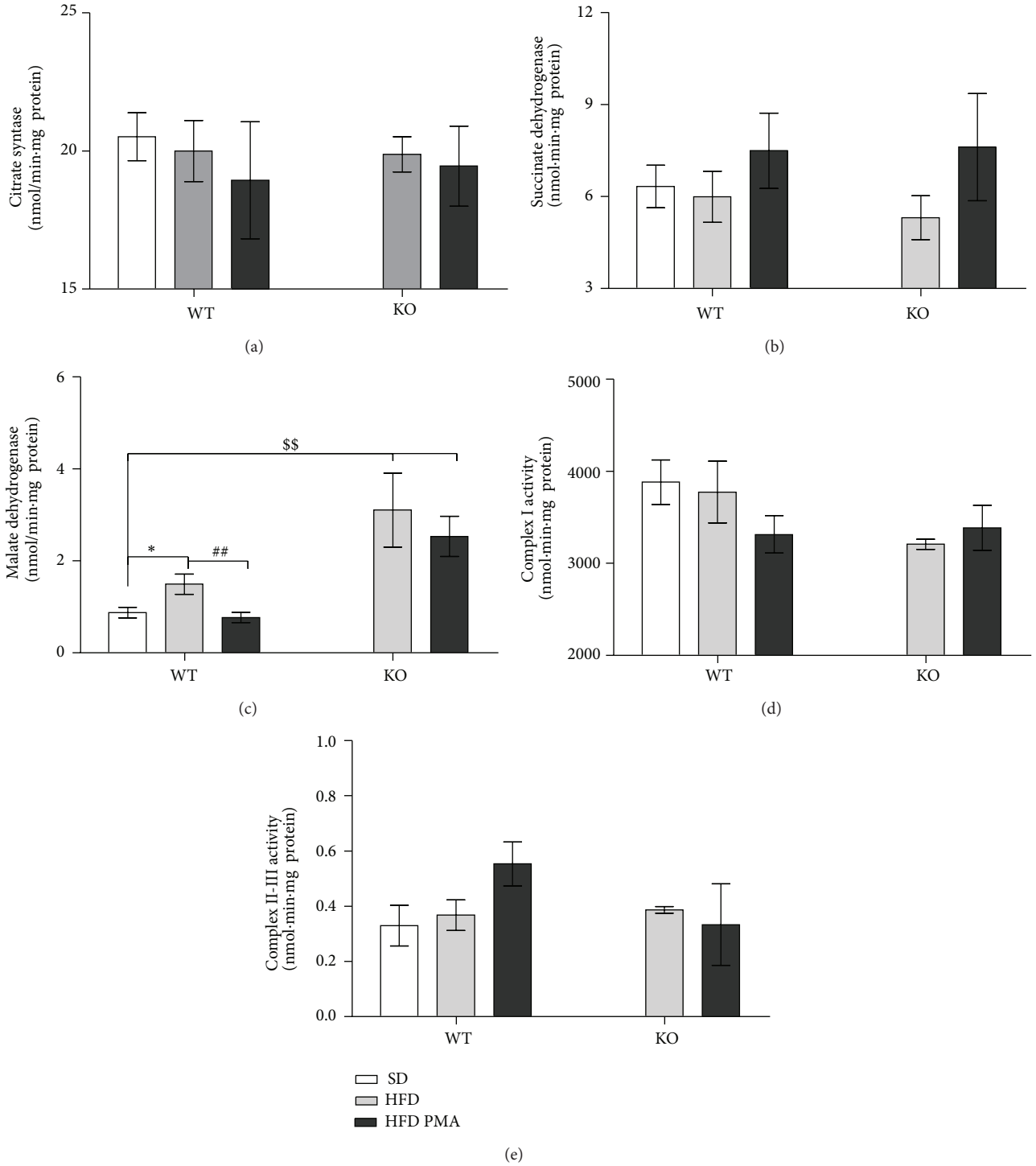

Figure 4: Activity of citrate synthase (a), succinate dehydrogenase (b), malate dehydrogenase (c), complex I (d), and complex II-III (e) in WT mice fed with standard diet (SD) or C57 and PPARα-knockout (KO) mice fed with a high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA). The data are presented as mean ± SEM. $^{*}P < 0.05$ versus WT SD; $^{\#\#}P < 0.01$ HFD versus HFD PMA; and $^{\$\$}P < 0.05$ KO versus the respective C57 control (one-way ANOVA followed by Bonferroni correction).

4. Discussion

In this study, we investigated the hypothesis that the nuclear receptor PPARα may be involved in the beneficial activities of palmitoleic acid in attenuating the development of HFD-induced hepatic steatosis and inflammation and disruptions of glucose homeostasis. In contrast to this hypothesis, our main findings indicated that palmitoleic acid exerts its

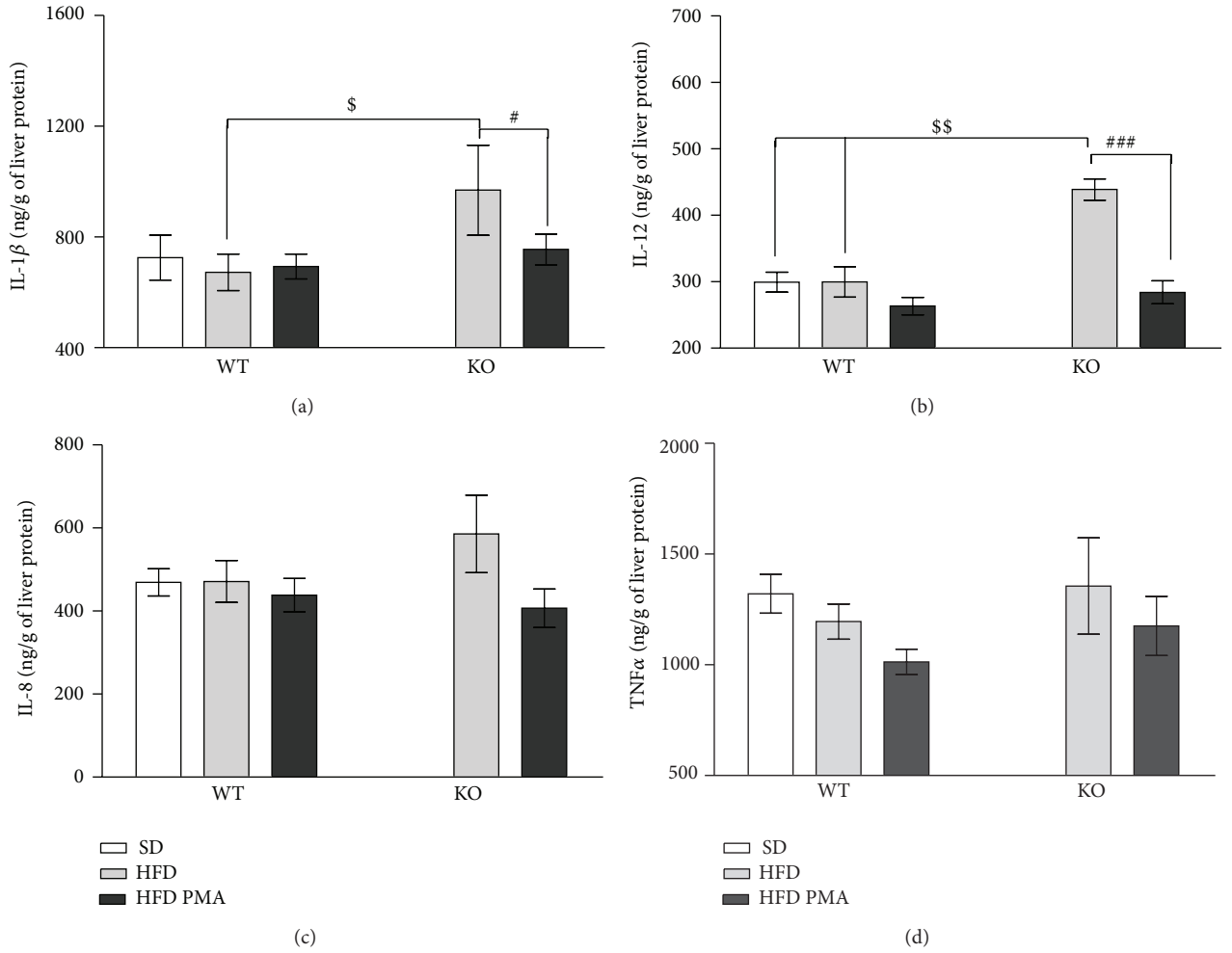

Figure 5: Hepatic levels of interleukin-1β (IL-1β) (a), interleukin-12 (IL-12) (b), interleukin-8 (IL-8) (c), and tumor necrosis factor-α (TNFα) (d) in WT mice fed with a standard diet (SD) or WT and PPARα-knockout (KO) mice fed with a high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA). The data are presented as mean ± SEM. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ HFD versus HFD PMA; $^{\$}P < 0.05$ and $^{\$\$}P < 0.01$ KO versus WT (one-way ANOVA followed by Bonferroni correction).

beneficial effects on hepatic inflammation and damage and improves insulin sensitivity through mechanisms that do not require PPARα.

The activation of PPARα seems to be a promising target in the treatment of NAFLD because PPARα is also a key regulator of the genes involved in fatty acid oxidation [33–36] and anti-inflammatory effects [37, 38]. Indeed, it has been previously shown that the lack of PPARα may cause an important hepatic steatosis and liver inflammation [34, 39]. In our study, we observed that the PPARα KO mice had exacerbated hepatic steatosis and liver inflammation after 12 weeks of exposure to a HFD. Consistent with this, Su et al. (2014) showed that defects in PPARα signaling induced mitochondrial and stress oxidative in mice fed with a high-fructose diet [40].

The effects of palmitoleic acid on NAFLD are controversial; while Guo et al. [12] showed that palmitoleic acid increased the deposition of fatty acids in the liver, Yang et al. [13] observed that it improved steatosis, reducing the ectopic deposition of triacylglycerols. In this work, we observed no effect of palmitoleic acid on steatosis or the ectopic deposition of triacylglycerols in liver caused by the HFD in both groups of mice, especially the KO mice, which had more pronounced NAFLD. The different effects of palmitoleic acid on NAFLD in these studies may be explained by the treatment times, dosages of PMA, and models used for the induction of steatosis.

We also observed other effects of palmitoleic acid, which is an important signaling molecule that is mainly produced by white adipose tissue and has been described as an insulin-sensitizing hormone that is capable of modulating several metabolic processes, such as glucose disposal and insulin sensitivity in skeletal muscle and lipids deposition in liver [11, 41]. Similarly, our findings indicate that palmitoleic acid increases the uptake of glucose in isolated muscles under stimulated conditions and improves the peripheral response to insulin in mice that are fed a HFD by mechanisms that are not regulated by PPARα.

Lipogenesis in hepatocytes is under the control of a series of critical genes, such as sterol regulatory element

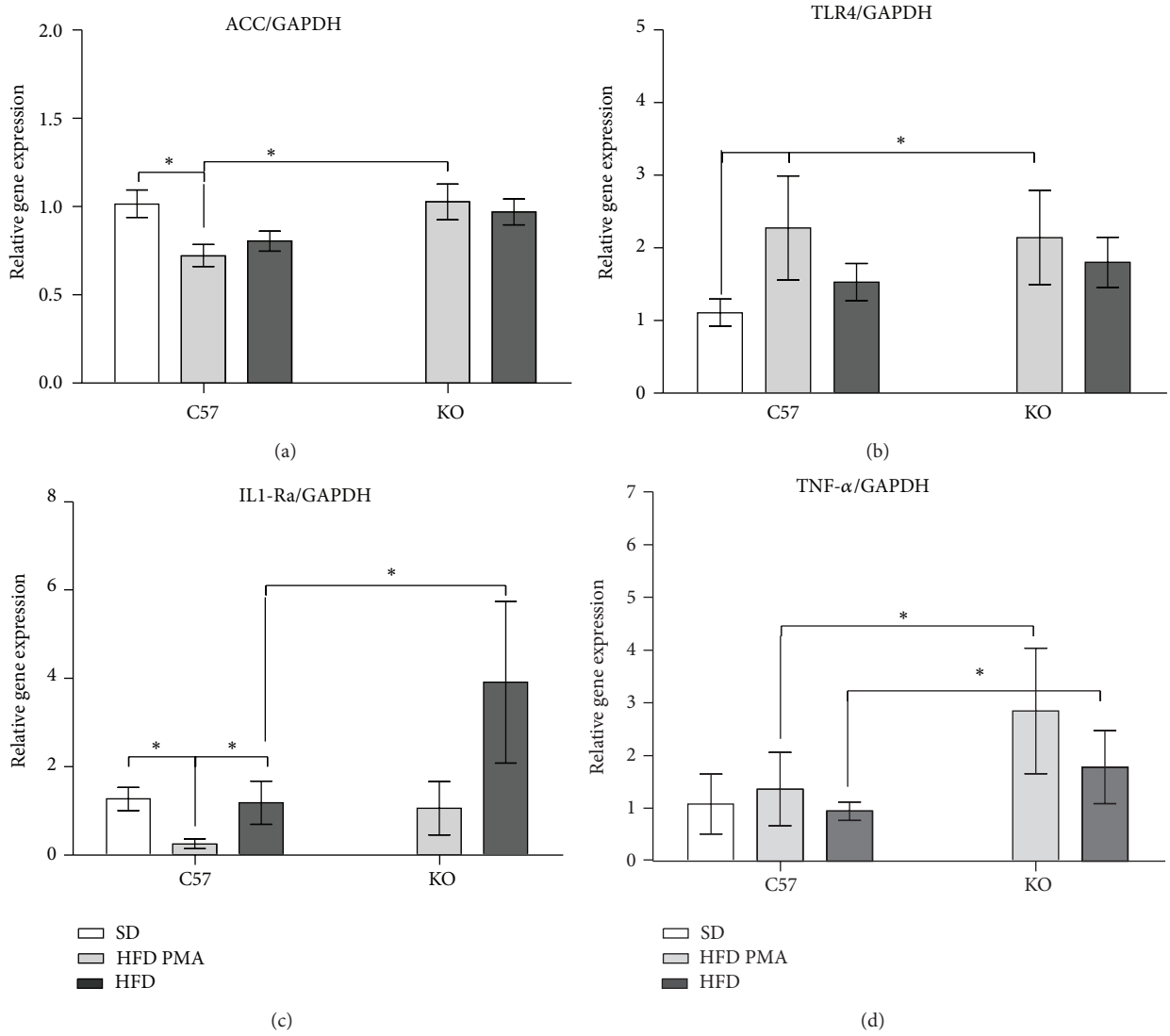

Figure 6: mRNA expression of acetyl-CoA carboxylase (ACC) (a), Toll-like receptor-4 (TLR-4) (b), antagonist receptor of interleukin-1 (IL-1Ra) (c), and tumor necrosis factor-α (TNF-α) (d) in WT mice fed with standard diet (SD) or WT and PPARα-knockout (KO) mice fed with a high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA). The data are presented as mean ± SEM. $^{*}P < 0.05$ versus SD; $^{\#}P < 0.05$ HFD versus HFD PMA; and $^{\$}P < 0.05$ KO versus the respective WT control (one-way ANOVA followed by Bonferroni correction).

binding protein-1 (SREBP1), fatty acid synthase (FAS), acetyl-CoA carboxylase enzymes (ACC), and stearoyl coenzyme A desaturase 1 (SCD-1). Of these, FAS and ACC seem to be particularly rate-limiting enzymes that are responsible for de novo lipogenesis, which may be increased in NAFLD [42, 43]. Concordantly, we observed that PPARα-KO mice had increases in the expression of ACC mRNA with consequently exacerbated ectopic deposition of fat in the liver, whereas WT mice had lower expression of ACC, which indicated a balancing regulatory mechanism in these animals, most likely via the activation of PPARα; this may explain why WT mice showed lower NAFLD and inflammation in response to the HFD. Combined with this increase in ACC, the hepatic levels of proinflammatory cytokines were increased in KO mice that were fed with the HFD.

The HFD increased the activity of malate dehydrogenase (MDH) in WT mice and promoted an even greater increase in the KO mice. A higher activity of this enzyme, which catalyzes the conversion of malate to oxaloacetate and the reverse reaction, could lead to an increase of malate levels in the mitochondria. Malate can be transported to the cytosol and converted by MDH to oxaloacetate, a precursor in the gluconeogenesis pathway [44, 45]. However, in addition to this increase in MDH activity, our group observed that PPARα KO mice subjected to a HFD had lower gluconeogenesis in pyruvate tolerance test (data not shown), indicating that this excess of Krebs cycle intermediates may be converted to other pathways, such as de novo lipogenesis, as indicated by the higher expression levels of ACC and by the more severe steatosis observed in the KO mice. Palmitoleic acid did

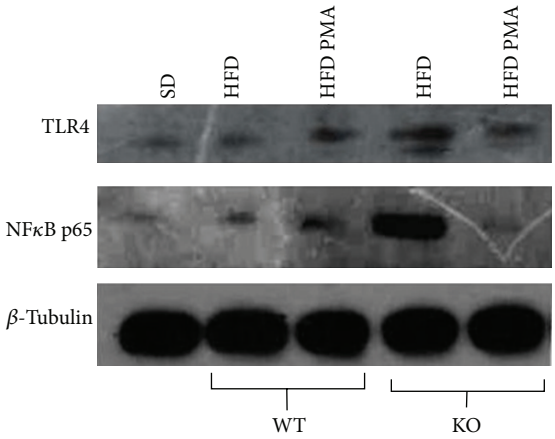

Figure 7: Protein levels of Toll-like receptor-4 (TLR-4) and serine phosphorylation of NFKB p65 in the liver of WT mice fed with a standard diet (SD) or WT and PPARα-knockout (KO) mice fed with high-fat diet and treated with oleic acid (HFD) or palmitoleic acid (HFD PMA).

not modulate the activity of the Krebs cycle enzymes or the electron chain transporter.

Although palmitoleic acid did not improve hepatic steatosis, this ω-7 fatty acid reduced the serum aspartate transaminase levels in both WT and PPARα-knockout mice, which indicates that the observed reduction in liver damage is independent of PPARα. Previous studies have reported that higher levels of AST indicate a greater degree of inflammation in the liver [46]; we observed an increase in the hepatic levels of proinflammatory cytokines in PPARα-knockout mice fed with the HFD, corroborating several studies [11, 14, 35, 39]. Surprisingly, palmitoleic acid diminishes the hepatic inflammation of these knockout mice, decreasing the levels of IL1-β and IL-12 in the liver, indicating once again that the beneficial effect of palmitoleic acid on the liver occurs by PPARα-independent mechanisms.

It has been reported that saturated fatty acids such palmitic acid (C16:0) could increase inflammation through TLR4 activation [47]. Indeed, some authors have shown that HFD supplementation also increased the expression of TLR4, suggesting that this receptor played a main role in the development of NAFLD [48, 49]. In the present study, we observed that HFD increased the expression of TLR4 mRNA in both WT and KO mice and increased TLR4 protein levels in the livers of KO mice, but palmitoleic acid reduced the hepatic mRNA and protein levels of TLR4, suggesting that this monounsaturated fatty acid has a strong anti-inflammatory effect.

The activation of PPARα has been described as an inhibitory mechanism for NFκB activation, consequently reducing the expression of proinflammatory genes and the production of proinflammatory cytokines, such TNF-α and IL-12 [50]. Consistent with this, we observed that the expression of TNF-α was increased in KO mice even when subjected to an SD. However because palmitoleic acid was capable of decreasing the serine phosphorylation of NFκB p65, which was increased by the HFD only in PPARα KO mice, we propose that the anti-inflammatory effect of palmitoleic acid involves other mechanisms for the inhibition of NFκB than the activation of PPARα. Indeed, the HFD decreased the expression of IL-1Ra in both genotypes, while palmitoleic acid increased the expression of IL-1Ra in both WT and KO mice. However, the expression of IL-1Ra was increased to a greater extent in KO mice that were subjected to the HFD and treated with palmitoleic acid. Other studies have shown that AMPK activation can elevate IL-1Ra levels independently of PPARα activation [51].

Therefore, we conclude that supplementation with palmitoleic acid but not with oleic acid can attenuate insulin resistance, liver damage, and inflammation induced by a HFD via mechanisms that do not depend on PPARα.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] S. Zelber-Sagi, D. Nitzan-Kaluski, Z. Halpern, and R. Oren, "Prevalence of primary non-alcoholic fatty liver disease in a population-based study and its association with biochemical and anthropometric measures," *Liver International*, vol. 26, no. 7, pp. 856–863, 2006.

[2] G. S. Hotamisligil and E. Erbay, "Nutrient sensing and inflammation in metabolic diseases," *Nature Reviews Immunology*, vol. 8, no. 12, pp. 923–934, 2008.

[3] Y. C. Wang, K. McPherson, T. Marsh, S. L. Gortmaker, and M. Brown, "Health and economic burden of the projected obesity trends in the USA and the UK," *The Lancet*, vol. 378, no. 9793, pp. 815–825, 2011.

[4] K. Makki, P. Froguel, and I. Wolowczuk, "Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines," *ISRN Inflammation*, vol. 2013, Article ID 139239, 12 pages, 2013.

[5] K. Singer, D. S. Eng, C. N. Lumeng, A. Gebremariam, and J. M. Lee, "The relationship between body fat mass percentiles and inflammation in children," *Obesity*, vol. 22, no. 5, pp. 1332–1336, 2014.

[6] L. Chen, D. J. Magliano, and P. Z. Zimmet, "The worldwide epidemiology of type 2 diabetes mellitus—present and future perspectives," *Nature Reviews Endocrinology*, vol. 8, no. 4, pp. 228–236, 2012.

[7] B. Q. Starley, C. J. Calcagno, and S. A. Harrison, "Nonalcoholic fatty liver disease and hepatocellular carcinoma: a weighty connection," *Hepatology*, vol. 51, no. 5, pp. 1820–1832, 2010.

[8] L. M. Pawella, M. Hashani, E. Eiteneuer et al., "Perilipin discerns chronic from acute hepatocellular steatosis," *Journal of Hepatology*, vol. 60, pp. 633–642, 2014.

[9] X. Leverve, "Hyperglycemia and oxidative stress: complex relationships with attractive prospects," *Intensive Care Medicine*, vol. 29, no. 4, pp. 511–514, 2003.

[10] M. Sanz, C. Sánchez-Martín, D. Detaille et al., "Acute mitochondrial actions of glitazones on the liver: a crucial parameter for their antidiabetic properties," *Cellular Physiology and Biochemistry*, vol. 28, no. 5, pp. 899–910, 2011.

[11] H. Cao, K. Gerhold, J. R. Mayers, M. M. Wiest, S. M. Watkins, and G. S. Hotamisligil, "Identification of a lipokine , a lipid hormone linking adipose tissue to systemic metabolism," *Cell*, vol. 134, no. 6, pp. 933–944, 2008.

[12] X. Guo, H. Li, H. Xu et al., "Palmitoleate induces hepatic steatosis but suppresses liver inflammatory response in mice," *PLoS ONE*, vol. 7, no. 6, Article ID e39286, 2012.

[13] Z. H. Yang, H. Miyahara, and A. Hatanaka, "Chronic administration of palmitoleic acid reduces insulin resistance and hepatic lipid accumulation in KK-Ay mice with genetic type 2 diabetes," *Lipids in Health and Disease*, vol. 10, article 120, 2011.

[14] A. Bolsoni-Lopes, W. T. Festuccia, T. S. Farias et al., "Palmitoleic acid (n-7) increases white adipocyte lipolysis and lipase content in a PPARα-dependent manner," *The American Journal of Physiology—Endocrinology and Metabolism*, vol. 305, no. 9, pp. E1093–E1102, 2013.

[15] M. Cherkaoui-Malki, S. Surapureddi, H. I. El Hajj, J. Vamecq, and P. Andreoletti, "Hepatic steatosis and peroxisomal fatty acid beta-oxidation," *Current Drug Metabolism*, vol. 13, no. 10, pp. 1412–1421, 2012.

[16] R. A. Daynes and D. C. Jones, "Emerging roles of PPARs in inflammation and immunity," *Nature Reviews Immunology*, vol. 2, no. 10, pp. 748–759, 2002.

[17] P. G. Reeves, F. H. Nielsen, and G. C. Fahey Jr., "AIN-93 purified diets for laboratory rodents: final report of the American Institute of Nutrition ad hoc writing committee on the reformulation of the AIN-76A rodent diet," *Journal of Nutrition*, vol. 123, no. 11, pp. 1939–1951, 1993.

[18] P. Srisawasdi, S. Chaloeysup, Y. Teerajetgul et al., "Estimation of plasma small dense LDL cholesterol from classic lipid measures," *American Journal of Clinical Pathology*, vol. 136, no. 1, pp. 20–29, 2011.

[19] Y. Yin, Z. Yu, M. Xia, X. Luo, X. Lu, and W. Ling, "Vitamin D attenuates high fat diet-induced hepatic steatosis in rats by modulating lipid metabolism," *European Journal of Clinical Investigation*, vol. 42, no. 11, pp. 1189–1196, 2012.

[20] J. Folch, M. Lees, and G. H. Sloane Stanley, "A simple method for the isolation and purification of total lipides from animal tissues," *The Journal of Biological Chemistry*, vol. 226, no. 1, pp. 497–509, 1957.

[21] E. Bonora, P. Moghetti, C. Zancanaro et al., "Estimates of in vivo insulin action in man: comparison of insulin tolerance tests with euglycemic and hyperglycemic glucose clamp studies," *Journal of Clinical Endocrinology and Metabolism*, vol. 68, no. 2, pp. 374–378, 1989.

[22] H. Bergmeyer and E. Bernet, "Determination of glucose with glucoseoxidase and peroxidase," in *Methods of Enzymatic Analysis*, H. Bergmeyer, Ed., pp. 1205–1215, Academic Press, New York, NY, USA, 1974.

[23] R. A. J. Challiss, F. J. Lozeman, B. Leighton, and E. A. Newsholme, "Effects of the β-adrenoceptor agonist isoprenaline on insulin-sensitivity in soleus muscle of the rat," *Biochemical Journal*, vol. 233, no. 2, pp. 377–381, 1986.

[24] J. Espinal, G. L. Dohm, and E. A. Newsholme, "Sensitivity to insulin of glycolysis and glycogen synthesis of isolated soleus-muscle strips from sedentary, exercised and exercise-trained rats," *Biochemical Journal*, vol. 212, no. 2, pp. 453–458, 1983.

[25] B. Leighton, L. Budohoski, F. J. Lozeman, R. A. Challiss, and E. A. Newsholme, "The effect of prostaglandins E1, E2 and F2 alpha and indomethacin on the sensitivity of glycolysis and glycogen synthesis to insulin in stripped soleus muscles of the rat," *The Biochemical journal*, vol. 227, no. 1, pp. 337–340, 1985.

[26] O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, "Protein measurement with the folin phenol reagent," *The Journal of biological chemistry*, vol. 193, no. 1, pp. 265–275, 1951.

[27] P. A. Srere, "Controls of citrate synthase activity," *Life Sciences*, vol. 15, no. 10, pp. 1695–1710, 1974.

[28] J. C. Fischer, W. Ruitenbeek, J. A. Berden et al., "Differential investigation of the capacity of succinate oxidation in human skeletal muscle," *Clinica Chimica Acta*, vol. 153, no. 1, pp. 23–36, 1985.

[29] G. B. Kitto, P. M. Wassarman, and N. O. Kaplan, "Enzymatically active conformers of mitochondrial malate dehydrogenase," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 56, no. 2, pp. 578–585, 1966.

[30] A. Cassina and R. Radi, "Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport," *Archives of Biochemistry and Biophysics*, vol. 328, no. 2, pp. 309–316, 1996.

[31] P. Chomczynski and N. Sacchi, "Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction," *Analytical Biochemistry*, vol. 162, no. 1, pp. 156–159, 1987.

[32] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}$T method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[33] S. Mandard, M. Müller, and S. Kersten, "Peroxisome proliferator-activated receptor α target genes," *Cellular and Molecular Life Sciences*, vol. 61, no. 4, pp. 393–416, 2004.

[34] R. Stienstra, S. Mandard, D. Patsouris, C. Maass, S. Kersten, and M. Müller, "Peroxisome proliferator-activated receptor α protects against obesity-induced hepatic inflammation," *Endocrinology*, vol. 148, no. 6, pp. 2753–2763, 2007.

[35] F. A. Monsalve, R. D. Pyarasani, F. Delgado-Lopez, and R. Moore-Carrasco, "Peroxisome proliferator-activated receptor targets for the treatment of metabolic diseases," *Mediators of Inflammation*, vol. 2013, Article ID 549627, 18 pages, 2013.

[36] E. Ip, G. Farrell, P. Hall, G. Robertson, and I. Leclercq, "Administration of the potent pparα agonist, Wy-14,643, reverses nutritional fibrosis and steatohepatitis in mice," *Hepatology*, vol. 39, no. 5, pp. 1286–1296, 2004.

[37] G. Tapia, R. Valenzuela, A. Espinosa et al., "N-3 long-chain PUFA supplementation prevents high fat diet induced mouse liver steatosis and inflammation in relation to PPAR-α upregulation and NF-κB DNA binding abrogation," *Molecular Nutrition & Food Research*, vol. 58, no. 6, pp. 1333–1341, 2014.

[38] P. Quintero and M. Arrese, "Nuclear control of inflammation and fibrosis in nonalcoholic steatohepatitis : therapeutic potential of dual peroxisome proliferator-activated receptor alpha/delta agonism," *Hepatology*, vol. 58, pp. 1881–1884, 2013.

[39] T. Hashimoto, T. Fujita, N. Usuda et al., "Peroxisomal and mitochondrial fatty acid β-oxidation in mice nullizygous for both peroxisome proliferator-activated receptor and peroxisomal fatty acyl-CoA oxidase: Genotype correlation with fatty liver phenotype," *Journal of Biological Chemistry*, vol. 274, no. 27, pp. 19228–19236, 1999.

[40] Q. Su, C. Baker, P. Christian et al., "Hepatic mitochondrial and ER stress induced by defective PPARα signaling in the pathogenesis of hepatic steatosis," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 306, no. 11, pp. E1264–E1273, 2014.

[41] N. Dimopoulos, M. Watson, K. Sakamoto, and H. S. Hundal, "Differential effects of palmitate and palmitoleate on insulin

action and glucose utilization in rat L6 skeletal muscle cells," *Biochemical Journal*, vol. 399, no. 3, pp. 473–481, 2006.

[42] S. H. Koo, "Nonalcoholic fatty liver disease: molecular mechanisms for the hepatic steatosis," *Clinical and Molecular Hepatology*, vol. 19, pp. 210–215, 2013.

[43] M. Nakamuta, M. Kohjima, S. Morizono et al., "Evaluation of fatty acid metabolism-related gene expression in nonalcoholic fatty liver disease," *International Journal of Molecular Medicine*, vol. 16, no. 4, pp. 631–635, 2005.

[44] S. Jitrapakdee, A. Wutthisathapornchai, J. C. Wallace, and M. J. MacDonald, "Regulation of insulin secretion: role of mitochondrial signalling," *Diabetologia*, vol. 53, no. 6, pp. 1019–1032, 2010.

[45] A. P. Rolo, J. S. Teodoro, and C. M. Palmeira, "Role of oxidative stress in the pathogenesis of nonalcoholic steatohepatitis," *Free Radical Biology and Medicine*, vol. 52, no. 1, pp. 59–69, 2012.

[46] O. F. Sharifov, X. Xu, A. Gaggar et al., "Anti-inflammatory mechanisms of apolipoprotein a-i mimetic peptide in acute respiratory distress syndrome secondary to sepsis," *PLoS ONE*, vol. 8, Article ID e64486, 2013.

[47] S. Huang, J. M. Rutkowsky, R. G. Snodgrass et al., "Saturated fatty acids activate TLR-mediated proinflammatory signaling pathways," *Journal of Lipid Research*, vol. 53, no. 9, pp. 2002–2013, 2012.

[48] Y. S. Roh and E. Seki, "Toll-like receptors in alcoholic liver disease, non-alcoholic steatohepatitis and carcinogenesis," *Journal of Gastroenterology and Hepatology*, vol. 28, supplement 1, pp. 38–42, 2013.

[49] T. le Roy, M. Llopis, P. Lepage et al., "Intestinal microbiota determines development of non-alcoholic fatty liver disease in mice," *Gut*, vol. 62, no. 12, pp. 1787–1794, 2013.

[50] R. Genolet, W. Wahli, and L. Michalik, "PPARs as drug targets to modulate inflammatory responses?" *Current Drug Targets: Inflammation and Allergy*, vol. 3, no. 4, pp. 361–375, 2004.

[51] A. Tomizawa, Y. Hattori, T. Inoue, S. Hattori, and K. Kasai, "Fenofibrate suppresses microvascular inflammation and apoptosis through adenosine monophosphate-activated protein kinase activation," *Metabolism: Clinical and Experimental*, vol. 60, no. 4, pp. 513–522, 2011.

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Review Article

Interferons and Interferon Regulatory Factors in Malaria

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Malaria is one of the most serious infectious diseases in humans and responsible for approximately 500 million clinical cases and 500 thousand deaths annually. Acquired adaptive immune responses control parasite replication and infection-induced pathologies. Most infections are clinically silent which reflects on the ability of adaptive immune mechanisms to prevent the disease. However, a minority of these can become severe and life-threatening, manifesting a range of overlapping syndromes of complex origins which could be induced by uncontrolled immune responses. Major players of the innate and adaptive responses are interferons. Here, we review their roles and the signaling pathways involved in their production and protection against infection and induced immunopathologies.

1. Introduction

Malaria, a mosquito-borne infectious disease transmitted by *Anopheles* mosquito, remains as one of the leading causes of morbidity and mortality worldwide, particularly in Africa, South-East Asia, and parts of South America [1]. When infected mosquito feeds on a human, the infective form of the *Plasmodium* parasite, sporozoites, is inoculated into the dermis of the host. Most of the motile sporozoites then leave the skin, travel through the blood circulation, and settle in the hepatocytes. During this liver phase, sporozoites undergo several asexual multiplications to form merozoites. Vesicles containing mature merozoites, merosomes, are released into the peripheral blood circulation and ruptured in the lungs to release thousands of merozoites into the blood circulation. These parasites infect red blood cells and initiate the erythrocytic phase [2]. Due to the exponential growth of the parasite, followed by massive destruction of erythrocytes, this stage is responsible for the common clinical manifestations of malaria such as fever, headaches, chills, and diaphoresis [3]. Usually the host immune response can control and eliminate the parasite, yet in some circumstance, patient's conditions deteriorate and develop severe systemic or organ-related pathological conditions such as anemia [4], hypoglycemia, febrile illness, respiratory distress [5], or cerebral malaria (CM) [6].

2. Innate Immunity to Pathogens

For the past decades, it was shown that the host immune response plays an important role in controlling the progression of malaria infection. The adaptive immunity, developed through repetitive infections during childhood, is pivotal in the elimination of *Plasmodium* parasite [7–10]. Yet, studies suggest that the host's ability to control the growth of parasites also relies on the innate immunity [11, 12]. Recent analysis of clinical records from neurosyphilis patients who underwent malaria therapy showed a controlled parasite density, irrespective of parasite strain, during the first and the second parasite inoculation which suggested the presence of a stable innate response [13]. In addition, peripheral blood mononuclear cells (PBMCs) from patients who had no prior exposure to malaria were able to produce proinflammatory cytokines, such as TNF-α, IL-12, and IFN-γ, within 10 hours of exposure to infected red blood cells (iRBCs) [14] demonstrating the activation of innate immune response against malaria parasite. However, proinflammatory cytokines are a double-edged sword. Under normal circumstances, they are essential for the control of parasite growth and sustained protection against the disease pathology, yet excessive and dysregulated production can lead to several immunopathologies [15, 16].

Human genetic diversity, parasite variability, and immune status of host generate various disease profiles of malaria

infections. Fortunately, only a fraction of malaria infection in human leads to pathologies [17]. This diversity in phenotypes is always associated with differences in measured biological and immune parameters. In addition, due to obvious ethnical reasons, analyses of these parameters are largely confined to peripheral blood (serum, plasma, and circulating cells). In most studies, only association but not causal mechanisms can be determined. Thus, malaria research mainly relies on mouse models to investigate the host immune response during malaria infection. Although these models cannot reflect all aspects of human infections, they allow the study of controlled experimental infections. There are 4 rodent malaria species, *P. berghei*, *P. chabaudi*, *P. vinckei*, and *P. yoelii*, 13 subspecies, and various strains and cloned lines [18]. These parasites were isolated from African thicket rats in Central and West Africa more than 50 years ago [19]. Depending on the host and parasite combinations, different disease profiles can be induced and host immune response will determine the outcome of infection (Table 1). These models, when used together with genetically deficient mice, allow in-depth study on protection against infection or immunopathogenesis. For example, the study of CM is hampered by the limited access to tissue samples and difficulty to perform *in vivo* experiments. Susceptible mice infected with *P. berghei* ANKA (PbA) manifest neurological abnormalities similar to human CM. In this model, termed experimental cerebral malaria (ECM), high production of proinflammatory cytokines, sequestration of parasite [20–23] and leukocytes, in particular $CD8^+$ T cells [24–26], and presentation of parasite antigen by brain microvessels [27] lead to the damage of the blood-brain barrier (BBB) and death. However, the role of innate immune responses in this pathology still remains to be determined.

When pathogens breach the skin or mucosal barriers, innate immune cells such as macrophages, mast cells, dendritic cells, and fibroblast, as well as circulating leukocytes, including monocytes and neutrophils, sense foreign agent using pattern recognition receptors (PRRs) that identify conserved pathogen-associated molecular patterns (PAMPs) on pathogens [28–30]. PRRs are either membrane-bound, such as toll-like receptors (TLRs) [28, 31–33] and C-type lectin receptors (CLRs) [28, 34–36], or free in the cytosol, such as NOD-like receptors (NLRs) [37–39] and RIG-I-like receptors (RLRs) [32, 40]. These PRRs are distinctly expressed on different cell populations which in turn influence the immunological repertoire elicited by a particular antigen. Professional antigen presenting cells, such as macrophage, B cells [41, 42], and dendritic cells [41, 43, 44], are well equipped with a wide spectrum of PRRs which enables this surveillance team to recognize a great variety of PAMPs and induce specific responses against each class of pathogens. For instance, in human, myeloid dendritic cells (mDCs) express all TLR1-10, but not TLR7, whereas plasmacytoid dendritic cells (pDCs) exclusively express TLR7 and TLR9 [41, 43, 44]. When activated, mDCs preferentially induces IL-12 while pDCs mainly produces IFN-α [44]. Other PRRs, such as dendritic cell-specific intracellular molecule-3-grabbing nonintegrin (DC-SIGN) [45] and DNGR-1 (Clec9A) [46], members of CLRs, expressed on immature DC were implicated in tolerogenic responses in some studies [47–49]. Besides professional antigen presenting cells, some epithelial cells are also furnished with PRRs. TLR2, TLR4, and TLR5 are widely found on pulmonary [50–53] and intestinal [54–56] epithelial cells. Since these surfaces are in continuous exposure to microbial challenges, strategic expression of these TLRs on these surfaces enables prompt recognition and response against bacterial infection. Vascular endothelial cells that line the entire circulatory system express also TLR4 [57, 58], RIG-I [59], and NOD-1 [60].

Upon positive PAMPs recognition, PRRs trigger a cascade of downstream signaling pathways that leads to nuclear translocation of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), activating protein-1 (AP-1), and interferon regulatory factors (IRFs) into the nucleus. These transcription factors modulate the production of inflammatory cytokines, chemokines, type I interferon (IFN-I), and some interferon-stimulated genes (ISGs) [127, 128], which in turn mobilize immune cells to target pathogens and eliminate infections. Most of these mechanisms have been identified for viral or bacterial infections [129, 130]. However, the precise mechanism by which the innate immune receptors and their signaling trigger the systemic inflammation and immune cells trafficking during malaria infection has yet to be fully uncovered. Here, we review the knowledge of the role of TLR-dependent and TLR-independent pathways and the modulation of IRFs in the activation of interferons (IFN) during malaria infection.

3. Recognition of Malarial Ligand by Host Receptors

Malaria parasite travels undetected in the circulation as it is encapsulated in the red blood cells. However, rupture of the matured forms of infected red blood cells exposes the parasite and releases malarial products which trigger host immune response [131–133]. This is evident by the paroxysms of fever and chills which coincide with the time of schizonts rupture [134]. The asexual erythrocytic stage of *Plasmodium* life cycle begins when merozoites are released from infected hepatocytes into the circulation. These merozoites infect red blood cells for source of nutrients and possibly also as a form of sanctuary from peripheral immune cells. Invasion is initiated by the initial contact of the parasite with red blood cells. Weak interactions of some glycosylphosphatidylinositol membrane anchors (GPIs) on the surface of merozoites [135] with receptors on red blood cells [136] trigger mechanisms that further commit the parasite to invasion [137, 138]. During invasion, most GPIs are shed from coat to facilitate entry into the target cells [139, 140]. As the parasite multiples and feeds on erythrocyte hemoglobin, it detoxifies hemoglobin heme by-product into Hemozoin (Hz) which is kept in the digestive vacuole (DV) [141–143]. Eventually, this DV, together with leftover host hemoglobin, is discharged into the circulation during egress of infective merozoites at the late schizonts stage in an explosive manner [138, 144]. Throughout this process of invasion and egress, the *Plasmodium* parasite continually scatters malarial products which could trigger the immune system.

Table 1: Combinations of different mouse backgrounds and parasite strains combinations allow the study of many disease profiles.

Mouse strain	Infection	Infection/pathology/protection	Ref.
C57BL/6 C57BL/6J C57BL/6N C57BL/6AnNCr 129/Ola x C57BL/6 129P2Sv/Ev 129 Sv/Ev x C57BL/6	*P. berghei* ANKA	ECM	[61–76]
	P. berghei ANKA-luc (231c11)		[21, 77]
	P. berghei ANKA-GFP		[78, 79]
	P. berghei ANKA clone 15cy1		[80, 81]
	P. berghei ANKA-GFP clone 15cy1		[82]
	P. berghei ANKA clone BdS		[26, 83]
	P. berghei ANKA sporozoite		[73]
	P. berghei ANKA clone 15cy1 sporozoite		[80, 84]
C57BL/6	*P. berghei* K173	Protection from ECM	[67]
	P. yoelii nigeriensis N67C	Lethal hyperparasitemia and severe anemia	[85]
	P. yoelii 17XNL	Protection from lethal hyperparasitemia and severe anemia	[86]
	P. yoelii yoelii 265 BY uncloned line		[87]
	P. yoelii nigeriensis N67		[85]
	P. berghei NK65	Liver injury	[88]
		Malaria-associated acute respiratory distress syndrome	[76]
	P. berghei NK65-GFP	Placental malaria	[89]
	P. chabaudi chabaudi AS	Protection in uncomplicated malaria	[77, 90]
	P. chabaudi AS		[76, 91]
	P. berghei ANKA-luc sporozoite	Liver-stage malaria	[92]
	P. berghei NK65 sporozoite		
	P. yoelii 17XNL sporozoite		
C57BL/6.C-*H2* ^{d}/bBy	*P. berghei* ANKA-GFP sporozoite	Liver-stage malaria	[93]
CBA/J	*P. berghei* ANKA	ECM	[75]
	P. yoelii 17X	Protection from lethal hyperparasitemia and severe anemia	[94]
	P. yoelii 17XNL		
CBA/T6	*P. berghei* ANKA	ECM	[95]
	P. berghei K173	Protection from ECM	
CBA/CaH	*P. chabaudi adami* 556 KA	Protection against blood-stage malaria	[96]
DBA/2	*P. berghei* ANKA	Protection from ECM Acute lung injury	[84]
129 Sv/Ev	*P. berghei* ANKA clone 1.4	ECM	[97]
	P. chabaudi chabaudi AS	Protection against blood-stage malaria	[98]
BALB/c	*P. berghei* ANKA	Protection from ECM	[65, 70, 74, 75, 84]
	P. berghei ANKA clone BdS		[26]
	P. berghei ANKA-GFP		[79]
BALB/cByJ SW	*P. yoelii* 17XL	Lethal hyperparasitemia and severe anemia	[94]
	P. yoelii 17XNL	Protection from lethal hyperparasitemia and severe anemia	

Extensive research has identified a few host receptors agonists from *Plasmodium* parasite which promote proinflammatory responses [61, 85, 99, 100, 102–106, 111, 112]. For the liver stage of the infection, *Plasmodium* RNA is the only malarial ligand discovered so far [92]. In the blood stage, several ligands have been identified, such as GPI [62, 99–103], Hz [63, 104, 145], CpG DNA bound on Hz [105], host fibrinogen [106], heme [107, 108], microparticles [109], AT-rich motifs in malarial genome [61], *Plasmodium* DNA/RNA [85], *P. falciparum* tyrosyl-tRNA synthetase (*Pf* TyrRS) [111], and *P. falciparum* high mobility group box protein (P*f* HMGB) [112]. All the different malarial ligands

Table 2: List of malarial ligands that stimulate different signaling molecules to trigger diverse immune responses and affect disease outcome in various experimental models.

Ligand	Signalling molecules involved	Cell types/mice	Immune responses/functions	Ref.
GPI	TLR1-TLR2 heterodimer	BMDM, PBMC	Stimulates production of TNF-α, IL-12, IL-6, and NO	[99]
	TLR2/TLR1, TLR4, MyD88, ERK1/2, p38, JNK1/2, NF-κB, AP-1 (c-Jun, ATF-2)	BMDM, PBMC, HEK, MPM		[62, 100, 101]
	MAPK2	BMDM	Stimulates production of TNF-α Controls production of IL-12	[102]
	IκB-ζ	BMDM	Involved in IL-12 expression	[103]
Hz (*Pf* 3D7)/ synthetic Hz	TLR9, MyD88	Murine splenocytes, BMDDC	Stimulates production of TNF-α, IL-12p40, MCP-1, and IL-6	[104]
		Knockout C57BL/6 or 129/Ola x C57BL/6	Increases serum level of MCP-1 and IL-6	
	TLR2, TLR9, MyD88	Knockout C57BL/6	Involved in ECM development Promotes parasite and leukocyte sequestration in brain sections Stimulates production of IFN-γ, TNF-α, and IL-12p40	[63]
Malarial CpG DNA (*Pf* 3D7)	TLR9, MyD88	BMDDC	Stimulates production of IL-12p40 and Rantes	[105]
Host fibrinogen	TLR4, CD11b/CD18-integrin	PBMC	Stimulates release of ROS, TNF, and MCP-1	[106]
Heme	TLR4, CD14, MyD88, IκB-α, ERK1/2, NF-κB	MPM, BMDDC, human monocyte-derived macrophages, PBMC	Stimulates production of TNF-α and KC Controls release of PGE_2	[107, 108]
MPs from infected mouse	TLR4, MyD88	BMDM	Upregulate expression of CD40 Stimulate production of TNF	[109]
Malarial AT-rich motif	STING, TBK1, IRF-3, IRF-7	BMDM, HEK293, knockout C57BL/6	Involved in ECM development Stimulates production of IFN-I, TNF-α, IL-6, and IL-15	[61]
Plasmodium DNA/RNA	MDA5, MAVS, RIG-1, CD14/IL-1R, p38	Knockout C57BL/6, RAW264.7	Stimulates production of IFN-I Controls parasitemia Prevents parasite sequestration in the brain capillaries and apoptosis in the spleen Promotes phagocytosis activity of macrophages	[85]
Unknown in PbA infection	TLR2/4, MyD88	Knockout C57BL/6	Involved in ECM development initiated with sporozoites Partially involved in ECM development initiated with iRBCs Regulates production of IFN-γ, MCP-1, TNF-α, and IL-10	[80]
Unknown ligand in *Py* 17XNL infection	TLR9, MyD88	Knockout C57BL/6	Controls parasitemia and promotes survival Essential Th1 development and cell-mediated immunity Stimulates production of TNF-α and IL-12 by DC Controls production of IL-10 and IL-4 by DC Induces cytotoxic activity in NK and $CD8^+$ T cells	[86]

Table 2: Continued.

Ligand	Signalling molecules involved	Cell types/mice	Immune responses/functions	Ref.
Unknown	TLR7, TLR9	NK cells, $\gamma\delta$ T cells, $CD4^+$ T cells	TLR7 mediates IFN-γ production by NK cells 24 h after infection TLR7 stimulates production of IFN-I, IFN-γ, IL-10, and IL-12 TLR9 mediates IFN-γ production by $CD4^+$ cells and stimulates production of TNF 6 days after infection	[110]
Plasmodium RNA	MDA5, MAVS, IRF-3, IRF-7	Knockout C57BL/6J, BMDDC, MPH	Stimulates production of IFN-I Controls leukocyte recruitment which limits parasite growth in the liver and induction of erythrocytic stage infection	[92]
Pf TyrRs	Unknown	Mouse splenocytes, PBMC, RAW 264.7, THP-1	Stimulates production of TNF-α, IL-6, IL-1α, and IL-1β which upregulate expression of ICAM-1 and VCAM-1 receptors	[111]
Pf HMGB	Unknown	Mouse splenocytes, RAW 264.7	Stimulates production of TNF-α, IL-6, IL-8, and IL-1β and upregulates mRNA expression of iNOS	[112]

BMDM: mouse bone marrow-derived macrophages (C57BL/6 unless otherwise stated); BMDDC: mouse bone marrow-derived dendritic cells; PBMC: human peripheral blood mononuclear cells; HEK: human embryonic kidney epithelial cells; Hz: Hemozoin; iNOS: inducible nitric oxide synthetase; KC: keratinocyte chemokine; MCP-1: monocyte chemoattractant protein-1; MP: microparticles; MPH: mouse primary hepatocytes; MPM: murine peritoneal macrophages; PGE_2: prostaglandin E2; *Pf* TyrRS: *P. falciparum* tyrosyl-tRNA synthetase; *Pf* HMGB: *P. falciparum* high mobility group box protein; RAW264.7: murine macrophage-like cell line; ROS: reactive oxide species; THP-1: human monocytic leukemia cell line.

and their respective signaling molecules involved to induce an immune response are listed in Table 2. However, the exact roles of each of these factors remain to be established.

3.1. TLR-Dependent Signaling. TLRs are central in the sensing and responding to pathogens during innate immunity. Members of TLRs were originally identified in embryo of *Drosophila melanogaster* more than 20 years ago [146]. Later, Medzhitov et al. reported the first human homolog of the *Drosophila* toll protein that is involved in the activation of adaptive immunity [147]. To date, ten TLRs have been identified in human and twelve in mice [148]. In both human and mouse, TLRs 1, 2, 4, 5, and 6 are expressed on cell surface whereas TLRs 3, 7, 8, and 9 are found within the endosomal compartments. TLR10 is uniquely expressed in human [149] and localized on the surface of plasma membrane. TLRs 11, 12, and 13 are only functionally expressed in mice and expressed on the membrane of endosomes [150]. These TLRs recognize PAMPs ranging from DNA and RNA to bacterial products [151]. Subcellular localization of TLR ensures that different pathogenic antigens are promptly recognized by the correct receptor in order to induce proper immune responses and, at the same time, minimize accidental trigger of an autoimmune response. Upon ligand-receptor interactions, TLR signal transduction is initiated leading to production of interferons and induction of proinflammatory cytokines [31, 148, 151, 152].

3.1.1. TLR Polymorphism and Malaria. Studies on genetic epidemiology revealed that TLR polymorphism is associated with outcome of malaria infection (Table 3). A population study in the Amazonian region of Brazil demonstrated that single nucleotide polymorphisms in TLR1 and TLR6 are associated with incidence of mild malaria [114]. Genetic variations in TLR1 are also capable of influencing susceptibility to placental malaria in Ghanaian mothers [113]. Case control studies demonstrated that common polymorphism in TLR2 and TLR4 can affect CM development [115, 119]. Variants in TLR2 amongst uncomplicated malaria children in Uganda were associated with altered proinflammatory responses [115] and a particular single nucleotide polymorphism in TLR4 amongst African children is correlated with an altered responsiveness to the malarial ligand, GPI, which in turn determine risk to severe malaria [119]. On the other hand, another TLR4 variant assessed in Iran (Baluchi) [116], Burundi [117], Brazil [114], and Ghana [118] was not found to be involved in malaria infection or disease severity.

Effects of TLR9 polymorphism in malaria infection have been most extensively studied amongst all the TLRs. Human genetic studies in endemic regions found a strong correlation in most of TLR9 variants with parasite load in the peripheral circulation [114, 120]. However, association of TLR9 alleles with susceptibility to malaria infection and disease severity varies according to the single nucleotide polymorphism and the regions studied [114, 116–121]. For example, TLR9 T1237C rs5743836 was associated with susceptibility to malaria infection amongst people in Burundi but not in Ghana or Iran. And amongst Ghanaians, susceptibility to mild malaria was correlated with TLR9 T1486C rs187084, but not with TLR9 G2848A rs352140.

Table 3: Association of TLRs and adaptor molecules gene polymorphisms with susceptibility to malaria or pathology in human.

TLRs/ adaptors	SNPs	Association	Region	Ref.
TLR1	S248N rs4833095	Placental malaria and anemia	Ghana	[113]
	I602S	Susceptibility to malaria infection	Amazon	[114]
TLR2	Δ22	No association with serum cytokines (TNF, IFN-γ, IL-1β, IL-6, IL-10) levels	Uganda	[115]
		Susceptibility to cerebral malaria		
	GT_n	No association with serum cytokines (TNF, IFN-γ, IL-1β, IL-6, IL-10) levels		
		No association with cerebral malaria		
TLR4	D299G rs4986790	No association with susceptibility to malaria infection	Burundi, Amazon, Ghana, Iran	[114, 116–118]
		No association with risk of placental malaria	Ghana	[118]
		Maternal anemia		[119]
		Severe malaria		[116]
	T399I	No association with mild malaria	Iran	[119]
		Severe malaria	Ghana	
TLR6	S249P	Susceptibility to mild malaria	Amazon	[114]
TLR9	G1174A rs352139	No association with susceptibility to malaria infection	Burundi	[117]
		Susceptibility to mild malaria	Ghana	[120]
		Level of parasitemia		
		No association with serum TNFα level	Uganda	[121]
		No association with serum IFN-γ level in mild malaria children		
		Level of serum IFN-γ level in CM children		
	T1237C rs5743836	No association with susceptibility to malaria infection	Ghana, Iran	[116, 118, 120]
		No association with disease severity	Ghana	[119]
		No association with placental malaria		[118]
		Susceptibility to malaria infection	Burundi	[117]
		Level of parasitemia	Amazon, Ghana	[114, 120]
		No association with serum TNFα level	Uganda	[121]
		No association with serum IFN-γ level in mild malaria children		
		Level of serum IFN-γ level in CM children		
	T1486C rs187084	No association with susceptibility to malaria infection	Burundi, Ghana, Iran	[116–118, 120]
		No association with disease severity	Ghana	[119]
		No association with placental malaria		[118]
		No association with level of parasitemia		[120]
	G2848A rs352140	Level of parasitemia	Amazon	[114]
		No association with level of parasitemia	Ghana	[120]
		Susceptibility to mild malaria infection		
TIRAP	S180L rs8177374	No association with susceptibility to malaria or severity of infection	Burundi, Amazon	[114, 117]
		Mild malaria and severe malaria	Gambia, Vietnam, Kenya	[122]
		Mild malaria	Iran	[116]

CM: cerebral malaria; Δ22: 22 base pair deletion in the first untranslated exon; GT_n: GT dinucleotide repeat in the second intron; mild malaria: patients suffer fever with temperature greater than or equal to 38°C, malaise, muscular pain, headache, and parasite load greater than or equal to 5000 parasite/ul of blood; severe malaria: patients who suffer anaemia, prostration, respiratory distress, convulsions, and/or impaired consciousness; cerebral malaria (CM): patients who experience coma with *P. falciparum* on blood smear and have no other cause for coma.

Besides TLR, effects of single nucleotide polymorphism of coadaptor molecule, TIR domain-containing adaptor protein, TIRAP, on malaria infection were also investigated. A particular TIRAP variant was correlated with mild malaria amongst people living in Iran [116], Gambia, Vietnam, and Kenya [122]. However, when the same TIRAP alleles were sampled in Burundi and Amazon, no association with susceptibility to malaria or disease severity was observed [114, 117]. These findings suggest that variants in TLR are capable of altering disease outcome during malaria infection but polymorphism in the strains of *Plasmodium* in different regions could also account for the different association.

3.1.2. TLR in Malaria Infection. Purified GPI from *P. falciparum* iRBCs [153] was preferentially recognized by TLR2/TLR1 or TLR2/TLR6 heterodimer and, to a lesser extent, TLR4 *in vitro* [99, 101]. TLRs-GPI interactions trigger the recruitment of MyD88, which phosphorylates a series of mitogen-activated protein kinases (MAPKs) including extracellular-signal-regulated kinases 1/2 (ERK1/2), p38 MAPK, and c-Jun N-terminal kinases 1/2 (JNK1/2) [62, 100, 101]. Following that, nuclear translocations of transcription factor such as NF-κB and AP-1, comprising the activation of transcription factor-2/c-Jun (ATF-2/c-Jun) [100, 102], stimulate production of proinflammatory cytokines such as TNF-α, IL-6, IL-12, and nitric oxide (NO) [100, 102, 154]. Interaction of nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, zeta (IκB-ζ) with NF-kB, promotes IL-12 production [103]. However, the concentration of GPI on the surface of merozoites is too low to account for this potent stimulatory effect observed [155].

A study by Pichyangkul et al. described an unknown, heat labile, malarial product in the schizont-soluble fraction that is able to upregulate expression of CD86 and stimulate IFN-α production by human plasmacytoid dendritic cells. When mouse bone marrow-derived dendritic cells (BMDDC) were stimulated with the same schizont fraction, upregulated expressions of CD40, CD86, and IL-12 production were observed [156]. Later, this ligand was proposed to be a metabolic by-product, Hemozoin (Hz), that is present in *P. falciparum* schizont lysate [157]. It is recognized by TLR9 to induce production of proinflammatory cytokines such as TNF-α, IL-12p40, MCP-1, and IL-6 [104]. However, this discovery was refuted by Parroche et al. who suggested that Hz only serves as a vehicle to deliver the malarial DNA, a TLR9 ligand, to the endosome for TLR9 sensing [105]. Similarly, Barrera et al. also supported the claim that Hz is only a vehicle for other malarial ligands, such as host fibrinogen. In this case, instead of TLR9, TLR4 and CD11b/CD18-integrin on monocytes were shown to recognize these malarial ligands [106]. In response to these, Coban et al. have recently demonstrated that both DNase-treated natural Hz and synthetic Hz are recognized by TLR9 and able to elicit an immune response via MyD88 [145]. These highly discordant results are likely due to different methodologies adopted by each group to purify the malarial Hz. Parroche et al. and Sharma et al. purified free Hz biocrystals using a magnetic separator [61, 105], whereas both Barrera et al. and Coban et al. utilized different protocols that largely consist of various chemical and mechanical procedures to obtain the natural Hz [104]. Despite disagreement on the ligand that stimulates the immune response, *in vitro* studies by both Parroche et al. and Coban et al. agreed upon the importance of TLR9 in *P. falciparum* infection. During *P. falciparum* infection, activation of TLR9 mediates production of IL-12 and IFN-γ. These proinflammatory cytokines in turn enhance expression of TLR and prime the signaling pathway to be more sensitive to TLR agonist [158].

Heme is released into the circulation when cell-free hemoglobin, from ruptured schizonts, is oxidized by reactive oxygen species (ROS) or other free radicals present in the plasma. The prosthetic group is recognized by TLR4, along with coreceptor CD14 [107, 108]. This interaction triggers MyD88 recruitment, IκBα degradation, ERK1/2 phosphorylation, and eventually NF-κB activation. Endotoxin contamination was abolished through the use of polymyxin B, anti-TLR4/MD2, and lipid A antagonist which inhibit effect of lipopolysaccharide (LPS) [108]. A study on a population of *P. vivax*-infected Brazilians discovered a correlation between high concentrations of heme in the plasma with disease severity. This is mediated through the activation of antioxidant enzyme Cu/Zn superoxide dismutase (SOD-1) which impairs production of anti-inflammatory mediators, such as Prostaglandin E2 (PGE_2) and TGF-β, by PBMCs [107]. In the same study, plasma level of proinflammatory cytokine, TNF-α, was found to be positively correlated with total heme and SOD-1 [107]. Heme can be detrimental in other ways such as its toxic effects on endothelial cells [159–161] and hepatocytes [162]. At the same time, it promotes survival [163], activation [164], and migration [165] of polymorph nuclear cells. Taken together, it seems to suggest that free heme in the plasma could engage in multiple signaling pathways to promote a proinflammatory immune response, which possibly exacerbates the malaria infection.

Microparticles (MPs) are submicron vesicles produced through membrane budding during immune-activation or cell death. Extensive studies revealed that MPs are capable of influencing biological functions such as expression of adhesion molecules by endothelial cells [166] and leukocyte recruitment [167]. In addition, these minute vesicles also play a part in pathology, for instance, by inducing nitric oxide synthesis [168, 169] and delivering mRNA into other cells [170, 171]. During malaria infection, MP derived from iRBCs affects disease progression by strongly inducing bone marrow-derived macrophages (BMDM) to produce TNF [109, 172]. *In vitro* study revealed that MP, and not LPS contamination, engages in a TLR4-MyD88 signaling pathway to induce this immune response. Since MP could contain other parasite proteins, like GPI and Hz, in the vesicles, it was not surprising that both TLR2 and TLR9 were also found to be involved in the induction of this MP-mediated signaling pathway. In fact, synergic engagement of all these TLRs with MP and other parasite ligands stimulated a proinflammatory response stronger than that induced by iRBCs [109].

The TLR signaling pathway is also involved in the development of placental malaria [89]. Study of *P. berghei* NK65-induced placental malaria in mice showed that MyD88 is

essential in the production of proinflammatory cytokines such as IL-6, IFN-γ, and TNF-α. In addition, the MyD88 pathway also affects the survival rates of pups from malaria-infected mothers [89]. Unfortunately, no specific TLR or malarial ligands were identified to account for the activation of this MyD88 signaling pathway.

3.1.3. TLR in ECM. Optimal production of proinflammatory cytokines can control parasite growth but overwhelming secretion of these soluble mediators can lead to immunopathologies such as cerebral malaria. Human population studies have demonstrated that single nucleotide polymorphism in TLRs can affect susceptibility to cerebral malaria [113–121]. However, no exact mechanism can be derived from such studies. Using the murine model of ECM, specific immune responses upon TLR-ligand interactions can be studied.

TLR2/TLR4, which recognizes malarial GPI, despite playing no role in the early stage of *P. chabaudi* infection (IFN-I secretion in this model is mediated by TLR7) [110], is important in ECM. The absence of these receptors leads to an attenuated proinflammatory response and protection from ECM lethality [63, 80]. However, different model seems to display varying degrees of reliance on this signaling pathway to induce ECM. Using wild-type (WT) and TLR2-knockout (KO) or MyD88-KO in C57BL/6 background mice infected with 10^6 fresh PbAiRBCs intraperitoneally, Coban et al. [63] showed that ECM pathogenesis totally relies on TLR2-MyD88 signaling pathway. Activation of this pathway led to sequestration of parasites and infiltration of pathogenic T cells into the brain, two important factors responsible for damaging the brain endothelial cells. On the contrary, in this model, TLR4 was shown not to be involved in ECM development. Conversely, Kordes et al. showed that WT and TLR2/4 double knockout (DKO) in C57BL/6 background mice, infected with 10^4 fresh PbA(clone 15cy1) iRBCs intravenously, trigger a proinflammatory response that is partially dependent on TLR2/4-MyD88 signaling pathway to cause ECM [80]. Unlike blood-stage infection, intravenous inoculation of PbA (clone 15cy1) sporozoites has absolute dependence on MyD88-dependent TLR2/4 pathway to develop ECM [80]. Inconsistency in the involvement of TLR2/4 in these models could be attributed to different infection regimens or the parasite strain/clones used. In a separate study, it was revealed that heme engages with TLR4-MyD88 signaling pathway to secrete TNF-α in mouse peritoneal macrophages and BMDDC. In fact, heme can also engage in a TLR4-independent pathway to induce production of ROS, expression of heme oxygenase-1 (HO-1), and recruitment of neutrophils [108]. Besides TLR2/4, TLR9 is also shown to play a role in ECM [63]. Coban et al. demonstrated that ECM pathogenesis relied on TLR9-MyD88 signaling to induce systemic proinflammatory responses and sequestration of parasite, Hz, and leukocytes in the brain [63].

In addition, TLR9 was discovered to work in synergy with TLR7 to induce IFN-I and IFN-γ production in mice infected with many other strains of *Plasmodium*, like *P. chabaudi*, *P. berghei NK65*, *P. berghei K173*, *P. yoelii YM* (*Py*YM), *P. yoelii 17X* (*Py*17X), and *P. vinckei petteri* infection [90, 110]. C57BL/6 mice infected with *Py17XNL* were shown to rely on TLR9-MyD88 signaling pathway to induce production of proinflammatory cytokine and increase commitment to Th1 and cytolytic activity by NK and T cells [86].

Despite all these findings that supported the involvement of TLR2/TLR4/TLR9 in ECM development, Lepenies et al. held a different opinion. Using triple TLR2/4/9 KO mice on C57BL/6 mice, intraperitoneally inoculated with PbA iRBCs that was maintained through alternate cyclic passage in *Anopheles stephensi* mosquito and BALB/c mice, they demonstrated that ECM induction is independent of TLR2/4/9 [64]. Such disparity in research findings could be due to the unique maintenance of parasite strain/clones used. Similarly, in spite of all these studies that demonstrated the importance of TLR, the study by Togbe et al. [78] casts some doubt on the importance of TLR cascade in the development of ECM. In his study, C57BL/6 mice were infected with a cloned line of PbA tagged with GFP. Results showed that deficiency in TLR did not prevent development in ECM, lungs, or liver pathology. Once again, these conflicting results emphasized the diversity of the immune response to malaria infection in different animal models [65].

3.2. TLR-Independent Signaling. In the erythrocytic stage, AT-rich motifs in the *P. falciparum* genome can also induce secretion of IFN-I, TNF-α, IL-6, and IL-15 via a TLR-independent pathway. This ligand engages in a distinct signaling pathway that involves cytoplasmic nucleic sensor STING, downstream kinase TBK1, and interferon regulatory factor (IRF) 3/7 [61]. Besides, *Pf*TyrRS [111] and P*f*HMGB [112] are two other malarial ligands that were shown to induce proinflammatory activity. However, more studies are needed to identify the specific receptors that recognize these two ligands. Other cytoplasmic signaling molecules, such as the member of RLR family and its adaptor molecule, melanoma differentiation-associated protein 5/mitochondrial antiviral signaling protein (MDA5/MAVS), were also associated with IFN-I signaling during acute phase of nonlethal *P. yoelii nigeriensis* N67 (*PyN* N67) infection in C57BL/6 mice [85]. On the contrary, STING and MAVS were found to be redundant in early *P. chabaudi* infection [110] and *P. yoelii* liver-stage infection [126].

Host innate immune response does not only exist in the erythrocytic stage of the parasite life cycle. In fact, control of parasitic growth starts as early as in the asymptomatic liver stage [173, 174]. Liver resident cells of C57BL/6 and BALB/c mice were shown to induce IFN-I production upon infection with PbA or *Py*17XNL sporozoite, independent of TLR-MyD88 pathway [92]. The *Plasmodium* RNA was found to be identified by MDA5, which typically recognizes double stranded DNA. This triggers the assembly of MAVS, which mediates downstream production of IFN-I. Liver-stage specific IFN-I mobilizes leukocytes into the vicinity of infected hepatocytes to limit parasite load in the liver and consequently influences the induction of erythrocytic stage infection. Besides MDA5, there are other unknown malarial

ligands that signal through Mavs as evident by the differential IFN-I response in MDA5$^{-/-}$ and MAVS$^{-/-}$ mice [92].

4. Interferons

Recognition of PAMPs by PRRs triggers a cascade of downstream signaling pathways which stimulates production of IFN-I, IFN-γ, and many other proinflammatory mediators. The IFN compartment comprises 3 classes, namely, IFN-I, IFN-II, and IFN-III. IFN are renowned for their antiviral properties and share common secondary structure. Yet each class of IFN binds to distinct multichain receptor complexes. They engage in different JAK-STAT molecules, drive the expression of different interferon stimulated response elements (ISRE) and/or interferon-gamma activated sequences (GAS) elements [175], and induce various interferon stimulated genes (ISGs), which in turn regulate development, host defense, and signaling [176]. In humans and mice, the IFN-I family comprises 13 types of IFN-α and 1 IFN-β [177]. IFN-II consists of solely interferon gamma (IFN-γ), while there are 3 types of IFN-III, namely, IFNλ1, IFNλ2, and IFNλ3 [175].

4.1. Type II Interferon (IFN-γ). IFN-γ is the only form of type II IFN. It regulates several components of the immune system such as antigen presentation [178–181], antimicrobial mechanism [182–184], leukocyte development [185], and immune cells trafficking [186, 187]. It is the most widely studied interferon in malaria infection since it is primarily involved in host defense against intracellular pathogens. Its protective role as an immune mediator emerged as early as in the liver stage [126, 188–193]. *In vitro* study of human recombinant IFN-γ treatment on *P. berghei* sporozoites-infected murine hepatocytes [190] or human hepatoma cells [189] identified an inhibitory effect of IFN-γ on parasite multiplication. Further *in vivo* study validated the importance of IFN-γ in protective immunity as it inhibits intracellular development of parasite within hepatocytes following challenge with *P. berghei* [194], *P. yoelii* [191], or *P. vivax* sporozoites [188] in mice and chimpanzee, respectively. Recently, Miller et al. demonstrated that IFN-γ secreted in primary *P. yoelii* sporozoite infection is the key innate mediator that controls liver-stage parasite growth in a secondary infection [126]. Above all, this inhibitory effect of IFN-γ on parasite development in liver stage extends and influences the initiation of blood stage parasite growth [126].

IFN-γ also plays a crucial protective role during blood-stage infection of various parasite strains. Administration of exogenous recombinant IFN-γ leads to control of parasite growth in *P. chabaudi adami* 556KA-infected CBA/CaH mice. After infection has been resolved, continuous IFN-γ treatment fully protected these mice from subsequent infection [96]. Lower level of parasitemia was also observed in IFN-γ treated SW mice that were infected with lethal strain of *P. yoelii*. In addition, these treated mice also exhibited better survival outcome [94]. *P. chabaudi* AS-infected mice treated with monoclonal antibody against IFN-γ had less control of parasite multiplication [195], once again suggesting that IFN-γ is essential for limiting parasite growth. Similar findings were observed in *P. chabaudi* AS-infected mice that were deficient in IFN-γ receptor. However, these mice had lower survival rates as compared to the WT controls [91]. This suggests that IFN-γ production at different period during infection could alter survival outcome. In *P. berghei* infection, IFN-γ also plays a protective role by mediating parasite clearance [196]. Population study of children in Papua New Guinea showed that high and early IFN-γ responses seem to protect from symptomatic malaria [197].

However, production of high level of IFN-γ during parasite blood stage development is associated with predisposition to severe malaria, such as CM. Studies in animal model of ECM corroborated findings from human study that IFN-γ is essential for the development of CM [66]. IFN-γ signaling in the brain regulates expression of adhesion molecules which influence parasites and leukocytes sequestration in the brain microvessels [97]. At the same time, there is also evidence that IFN-γ promotes trafficking of leukocytes, including pathogenic CD8$^+$ T cells, to the brain [81, 83]. IFN-γ is essential in both protective immunity and pathogenesis of severe diseases [198]. Whether it protects or harms the host depends on when and where it is produced [67].

4.2. Type I Interferon (IFN-α/β). Unlike IFN-γ, IFN-I is only starting to gain more attention with increasing evidence that supports its role in protection [87, 199, 200]. This cytokine regulates various immune mechanisms such as MHC expression [201], antigen presentation [201], and T cell expansion [202, 203]. Furthermore, IFN-I can also modulate production of IFN-γ [204] and prime IFN-γ-mediated immune responses [205]. The earliest report which revealed its significance in malaria demonstrated that exogenous administration of unpurified mouse serum IFN was able to protect CF-1 mice from PbA sporozoite infection [206]. Although the experiment suggested a role for IFN-I, this unpurified mouse serum contains mediators other than IFN-I [207], such as IL-6, which have activity against *Plasmodium* liver stages [208]. Following that, a study of treatment with recombinant human IFN-α, which cross-reacts with mouse cells, did not show any effect on near matured (42 h) liver stage after a challenge with *P. yoelii* sporozoite [87]. However, recent analysis of liver transcriptome obtained from PbA [92] or Py [92, 126] sporozoite-infected C57BL/6 or BALB/c mice revealed an upregulation of genes expressions that are linked to IFN signaling. IFN-I was found to act during the very late phase of the liver stage and this liver specific IFN-I production partially limits parasite growth in the liver and influences initiation of erythrocytic stage infection [92]. In mice, *P. yoelii* and *P. berghei* liver stages last a minimum of 48 h and 51 h, respectively [209], and the effect of IFN-I was only apparent after 48 h but not 42 h after sporozoite infection. Interestingly, the IFN-I effect was indirect and mainly mediates the recruitment of leukocytes around liver-stage parasites. IFN-γ-secreting immune cells, in particular CD1d-restricted NKT cells, are the main players responsible for the innate elimination of liver stage [126]. Leukocyte-mediated inhibition of liver-stage parasite further leads to a reduced development of parasitemia [92]. More importantly,

this innate immune response can facilitate parasite elimination in subsequent liver-stage infection [126, 191]. In fact, early production of IFN-I prior to infection can impair parasite establishment [92].

Compared to liver-stage infection, IFN-I has a more striking role against blood stage parasites. Previous work shows that treatment of C57BL/6 mice with pure recombinant IFN-α inhibits *P. yoelii* or *P. berghei* blood stage development [87]. This effect was indirect and mediated by IFN-γ [210]. During early stage of *P. chabaudi* infection, IFN-I induced by the infection plays a pathogenic role by suppressing IFN-γ producing $CD4^+$ T cells that control parasite load in C57BL/6 [77] but not in 129 Sv/Ev mice [98]. These results are not contradictory but suggest different levels of IFN-I and the duration of action is essential for proper immune response to control parasite growth.

In human, polymorphism in the receptor of IFN-I, IFNAR, has been shown to be robustly associated with progression of CM [79, 200]. It was further revealed that peripheral blood mononuclear cells from Malawian children recovering from severe malaria had higher expression of genes involved in interferon pathway [211]. In murine model, recombinant IFN-α [210] or IFN-β [68] treatment protected mice from ECM death. When PbA-infected C57BL/6J mice were administered with recombinant human IFNα, increased level of IFN-γ in treated mice was observed, which was linked to improved control of parasitemia and survival [210]. On the other hand, IFN-β treatment prevented ECM death by suppressing the expression of chemokine receptor CXCR3, the production of IFN-γ, and chemokine ligand CXCL9. Consequently, decreased T cells migrate and sequester in the brain thereby preserving a better vascular integrity of blood brain barrier as compared to nontreated WT controls [68]. However, IFN-I induced endogenously during *Plasmodium* infection plays a pathogenic role in ECM development. Absence of IFN-I signaling, in mice deficient in IFNAR, either delayed [82] or fully protected [77, 79] the mice from ECM. Haque et al. [77] attributed this protection to a restrained parasite growth in the absence of IFN-I pathway whereas Ball et al. [79] and Palomo et al. [82] concluded that deficiency in IFN-I signaling reduced sequestration of pathogenic T cells in the brain. All these conflicting data suggest that effects of IFN-I might rely on precise level and timing of expression of systemic IFN-I.

5. Interferon Regulatory Factors

Both IFN-I and IFN-γ are essential in the immune response against malaria infection. Production of IFNs is triggered upon recognition of malaria antigen by receptors as discussed above. Although an array of receptors and downstream signaling molecules have been implicated, all signaling pathways ultimately converge to a few downstream transcription factors, such as IRFs, which regulate gene expression of IFNs. The family of IRFs comprised 9 members, namely, IRF1-9. Each IRF binds to a unique set of ISRE to stimulate transcription of diverse genes that are translated into functional proteins [212, 213]. The diverse roles of a few IRFs in malaria infection have been uncovered recently (Table 4).

5.1. Interferon Regulatory Factor 1. The first member of the IRF family identified that binds to the promoter region of IFN-β gene is IRF-1. This transcription factor is expressed in many cells types. It mediates signaling of both IFN-I and particularly IFN-γ, a strong inducer of IRF-1 expression. IRF-1 regulates antigen presentation, monocyte/macrophage differentiation, T cell development, and B cell growth [214] and promotes Th1 response [215]. In humans, the IRF-1 gene is located in chromosome 5q31-33 region and variation in 5q31-33 region was associated with variations in parasite density during *P. falciparum* erythrocytic infection [216]. A subsequent study in West African ethnic groups identified that polymorphisms in IRF-1 gene could lead to differential abilities to control *P. falciparum* infection [123]. Despite the fact that Mangano et al. discovered a correlation between IRF-1 and control of *P. falciparum* infection [123], they found no association of this transcription factor in the development of severe malaria pathology amongst African children [124]. Using mice deficient in IRF-1, Tan et al. showed that IRF-1 is essential in limiting parasite growth and survival outcome of PbA infection [125]. Further animal studies also demonstrated that IRF-1 regulates antigen presentation [214] and is indispensable in the pathogenesis of ECM. When infected with PbA, mice deficient in IRF1 were partially protected from ECM with a lesser control in parasite growth in the circulation [69]. Microarray analysis of brains from ECM-susceptible C57BL/6 mice as compared to ECM-resistant BALB/c mice revealed an increase of IRF-1 gene expression [70]. Similarly, IRF-1 gene expression was higher in brain from CBA/T6 mice infected with ECM-causing PbA parasite than with non-ECM causing *P. berghei* K173 parasite [95]. With this evidence, there is a need to further investigate the exact implication of IRF-1 in these different immune mechanisms which are essential for ECM pathogenesis [27].

Recently, Wu et al. also demonstrated that higher expression of IRF-1 gene and production of IFN-I enabled better control of parasitemia in nonlethal *P. yoelii* N67-infected mice than in lethal *P. yoelii* N67C-infected mice [85]. However, the role of IRF-1 in IFN-β signaling remains controversial as stimulation of splenocytes from IRF-1 deficient mice with malarial genome-alike AT-rich oligonucleotides did not abrogate IFN-β production [61]. This discrepancy could be due to the use of different malaria ligand to induce IFN-I production comforting previous speculation of multiple signaling pathway to produce IFN-I [217].

5.2. Interferon Regulatory Factor 3/7. Among the 9 IRFs, IRF-3 and IRF-7 are the master regulators of IFN-I. They are responsible for driving the initial transcription of IFN-I during early stage of infection. Induction of IFN-I is generated through a biphasic mechanism which warrants transcriptional efficiency and diversity of targeted genes. IRF-3 is constitutively expressed in the cytoplasm of all cells and resides as an inactive form. Upon phosphorylation, activated IRF-3 translocates into the nucleus and forms enhanceosome

Table 4: Diverse roles of different IRFs in malaria infection.

	Host /model	Infection	Functions	Ref.
IRF-1	Human	*P. falciparum*	Controls parasitemia	[123]
			Not involved in development of severe malaria	[124]
	Mice	*Py nigeriensis* N67 iRBCs	Plays a role in IFN-I signaling	[85]
		PbA iRBCs	Involved in ECM development Controls parasitemia	[69]
			Involved in ECM development Promotes parasitemia	[125]
			Regulates production of IFN-γ and IL12p4 Controls $CD8^+$ T cells numbers	[72]
			Plays a role in ECM development	[70, 95]
	Ex vivo	AT-rich oligonucleotides	No effect on IFN-β production by splenocytes	[61]
IRF-3	Mice	*P. yoelii* sporozoite	Mediates IFN-I-induced innate response during liver-stage infection	[126]
		P. chabaudi iRBCs	Mediates splenic IFN-α, but not IFN-β, transcription in red pulp macrophages	[90]
			Not involved in IFN-I production	[110]
IRF-5	*Ex vivo*	AT-rich oligonucleotides	Not involved in IFN-β production by splenocytes	[61]
IRF-7	Mice	*Py nigeriensis* N67 iRBCs	Plays a role in IFN-I signaling	[85]
		PbA iRBCs	Plays a role in ECM development	[70, 71]
		P.chabaudi iRBCs	Mediates splenic IFN-I transcription in red pulp macrophages	[90]
			Involved in IFN-I production	[110]
IRF-3 and IRF-7	Mice	PbA sporozoite	Mediate IFN-I response in liver-stage infection Control parasite load in the liver	[92]
		PbA iRBCs	Involved in ECM development	
	Ex vivo	AT-rich oligonucleotides	Mediate IFN-β production by splenocytes	[61]
		Pf iRBCs	Mediate IFN-β production by macrophages	
IRF-8	Mice	PbA iRBCs	Plays a role in ECM development	[95]
			Regulates production of proinflammatory cytokines Mediates IFN-I production Controls antigen processing and presentation and chemotaxis	[72]
IRF-9	Mice	*Py nigeriensis* N67 iRBCs	Plays a role in IFN-I signaling	[85]
		PbA iRBCs	Plays a role in ECM development	[72]

with other transcription factors, namely, NF-kB and AP-1 [218], which will lead to IFN-β transcription [219–221]. On the other hand, IRF-7 is expressed at very low levels in the cytoplasm of most cells. Positive feedback of IFN-β increases IRF-7 expression. Like IRF-3, it undergoes nuclear translocation and forms heterodimer with IRF-3 to bind with ISRE. Unlike IRF3, IRF-7 induces maximal transcription of both IFN-α and IFN-β [222]. The role of IRF-3 and IRF-7 in malaria infection remains poorly defined.

When mice deficient in either IRF-3 or IRF-7 were infected with PbA sporozoite, significant impairment in IFN-I response was observed. Consequently, these deficient mice had higher parasite load in the liver and peripheral circulation as compared to their WT counterparts. Initiation of blood-stage infection was also found to be 1-day earlier in the KO as compared to WT mice [92]. On the other hand, only mice deficient in IRF-3 displayed marked impairment in the control of parasite burden in the liver upon secondary *P. yoelii* sporozoite infection [126]. Such disparity could be attributed to the strain of parasite or the time point measured in each study. Since IRF-3 stimulates IFN-β production [219–221] and IRF-7 induces both IFN-α and IFN-β production [222], the significance of IRFs in each infection model could possibly hint on the importance of IFN-α and/or IFN-β at different window of the liver-stage infection. When stimulated with infected red blood cells or AT-rich motif derived from genome of *P. falciparum*, splenocytes obtained from mice deficient in both IRF-3 and IRF-7 had attenuated IFN-β production, demonstrating a role for one or both of these factors in IFN-β production [61].

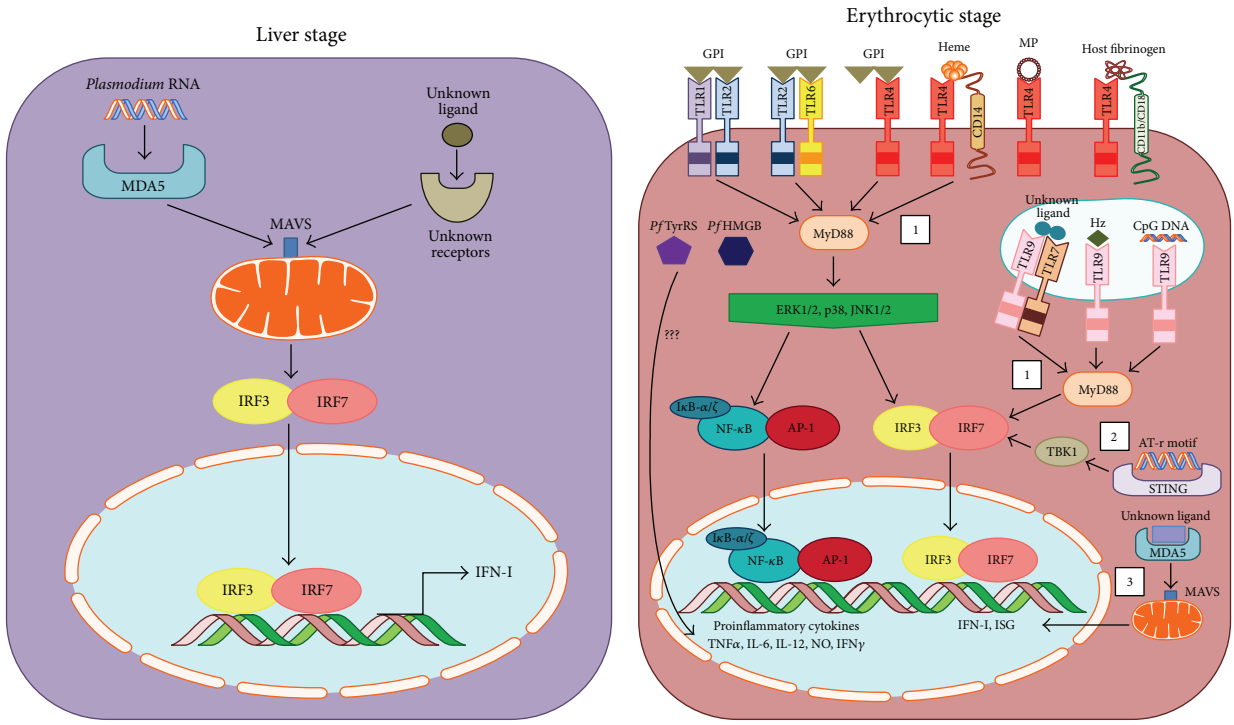

Figure 1: (a) Signaling pathway induced by malarial ligand during liver-stage infection. *Plasmodium* RNA is recognized by MDA5 (melanoma differentiation-association protein 5) present in the cytoplasm. Ligand-receptor interaction triggers assembly of MAVS (mitochondrial antiviral signaling protein) that aggregate on the surface of mitochondria. This eventually leads to the activation of both IRF-3 and IRF-7 which regulate transcription of IFN-I. Besides MDA5, activation of other receptors can also trigger aggregation of MAVS. However, this specific receptor and its corresponding malarial ligand have yet to be identified. (b) Signaling pathway induced by malarial ligand during erythrocytic-stage infection. Surface TLR4 recognizes a number of malarial ligands such as GPI (glycosylphosphatidylinositol membrane anchor) and MP (microparticles). Together with CD14 or CD11b/CD18 integrin, it can recognize heme and host fibrinogen, respectively. Both TLR heterodimer TLR1/TLR2 and TLR2/TLR6 recognize GPI. Within the endosomal compartment, Hz (Hemozoin) and CpG DNA are recognized by TLR9. In addition, TLR7/TLR9 heterodimer has been proposed to recognize an unknown malarial ligand. These ligand-receptor interactions trigger 3 proposed pathways. (1) TLR-dependent pathway involves the recruitment of MyD88 (myeloid differentiation primary gene 88) to TLR, which phosphorylates downstream MAPKs (mitogen-activating protein kinases), such as ERK1/2 (extracellular-signal-regulated kinases 1/2), p38 MAPK, and JNK1/2 (c-Jun N-terminal kinases 1/2). Subsequently, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) and AP-1 (activating protein-1) translocate into the nucleus and stimulate production of proinflammatory cytokines. At the same time, phosphorylated MAPKs or MyD88 can induce activation of IRF-3 and IRF-7 to transcribe IFN-I and ISGs (interferon stimulated genes). (2) Activation of TLR-independent pathway triggered by AT-rich motif present in the *plasmodial* genome engages STING, TBK, IRF-3, and IRF-7. (3) Another TLR-independent pathway involves MDA5 and MAVS. *Pf*TyrRS (*P. falciparum* tyrosyl-tRNA synthetase) and *Pf*HMGB (*P. falciparum* high mobility group box protein) were shown to induce proinflammatory responses but the exact signaling pathways have yet to be identified.

In mice infected with *P. chabaudi*, early IFN-α production by red pulp macrophages is dependent on both IRF-3 and IRF-7. Intriguingly, contrary to what is observed with viruses [223–226], IFN-β production was independent of IRF-3 [90, 110], suggesting an alternate pathway of activation for malaria parasite. Microarray analysis of brain from ECM-susceptible mice showed a higher transcriptional activity of IRF-7 than ECM-resistant [70] and uninfected control mice [71]. Double IRF-3/IRF-7 deficient mice infected with PbA were resistant to ECM upon infection [61] confirming a role for IFN-I in ECM. However, the precise functions of IRF-3 and IRF-7 in CM remained to be determined.

5.3. Interferon Regulatory Factor 8. IRF-8 is one of the unique IRFs that is only expressed in immune cells [227]. Unlike IRF-3 and IRF-7, expression of IRF-8 is induced by IFN-γ instead of IFN-I. This transcription factor coordinates growth and differentiation of myeloid cells, such as macrophages and dendritic cells, and production of proinflammatory cytokines, such as IFN-I and IL-12p40 [227]. Together with IRF-1 [228], IRF-8 directs transcription programs in immune cells towards a Th1-dominated response [229]. Since ECM is a Th1-mediated pathology [230–232], it is not surprising that amplification in IRF-8 gene expression was observed in the brains of ECM-susceptible PbA-infected CBA/T6 mice [95]. Mice with dysfunctional IRF-8 are protected from ECM due to downregulated transcriptional activity of many IRF-8-dependent genes which are essential in various aspects of the immune response during PbA infection. These modulated genes are involved in antigen processing and presentation,

chemotaxis, maturation of phagosomes, and production of proinflammatory cytokines [72]. Though both reports concurred that IRF-8 is involved in ECM development, further research is mandatory to ascertain its role in human CM.

5.4. Other Interferon Regulatory Factors. Apart from IRF-1, IRF-3, IRF-7, and IRF-8, some studies have also briefly explored the role of IRF-5 and IRF-9 in malaria infection. IRF-5 is expressed in B cells and dendritic cells. Like IRF-7, it is mainly regulated by IFN-I. This transcription factor interacts with IRF-1, IRF-3, and IRF-7 to induce expression of proinflammatory cytokines [233, 234]. The only report on IRF-5 in malaria infection revealed that it is dispensable in the production of IFN-β by splenocytes in response to stimulation by AT-rich oligonucleotides that resemble those in the malarial genome [61]. Another member, IRF-9, is expressed constitutively in many cell types and unlike the rest of the IRFs, it functions only when it dimerizes with STAT1 and STAT2 to form an active trimeric complex, known as ISGF3. This complex binds to ISRE and activates ISGs [235, 236]. During nonlethal *Py*N67 infection, IRF-9 participates in the production of IFN-I to control parasite growth [85]. A robust IRF-8-dependent amplification of IRF-9 was detected in brain of mice infected with PbA [72]. These separate studies seem to hint on the possibility of more IRFs involvement in the immune response during malaria infection.

6. Future Perspectives

In Figure 1, we illustrate the different malarial ligands and the various signaling pathways triggered to produce IFN-I and proinflammatory cytokines in the liver and erythrocytic stages. Though controversial, these studies demonstrated that IFN-I [77] and IFN-γ [94] produced during infection may modulate the course of disease progression. However, the same immune response that initially protects the host could inevitably contribute to the pathogenesis of severe malaria [66, 82, 94].

Thus far, the most effective malaria treatment is administration of antimalarial drugs, Chloroquine (CQ) or Artemisinin (ART) and its derivatives, which solely targets the parasite. But the emergence of CQ/ART-resistance parasite species rendered these treatments increasingly ineffective [237, 238]. In the recent years, increased knowledge of the host immune response uncovers a potential to employ host-directed therapy in malaria infection. In fact, immunotherapy has emerged as a hot topic for both research and treatment against a diverse array of disease over the last few centuries [239–241]. Specifically, interferon therapy has been widely reported to treat cancer [242–246] and viral infections [247–249]. Recently, a synthetic innate defense regulator-1018 (IDR-1018) adjunctive treatment, in combination with antimalarial drug, demonstrated efficacy against ECM [250]. Taken together, these data offer the possibility of interferon treatment as an immunotherapy for malaria infection. Thus, dissecting the innate signaling pathways and their corresponding cytokine responses would provide further insights into the induction of adaptive immune response and offer some directions on vaccine or drug developments.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Sin Yee Gun, Carla Claser, Kevin Shyong Wei Tan, and Laurent Rénia contributed equally to the paper.

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References

[1] WHO, "World malaria report 2013 shows major progress in fight against malaria, calls for sustained financing," Tech. Rep., 2013.

[2] A. F. Cowman and S. H. I. Kappe, "Malaria's stealth shuttle," *Science*, vol. 313, no. 5791, pp. 1245–1246, 2006.

[3] M. Giboda, J. Gutvirth, M. Maloveská, F. Kosina, M. Hocmanová, and V. Struncová, "Imported malaria diagnostic and clinical features," *Bratislavske Lekarske Listy*, vol. 88, no. 1, pp. 104–111, 1987.

[4] D. J. Perkins, T. Were, G. C. Davenport, P. Kempaiah, J. B. Hittner, and J. M. Ong'echa, "Severe malarial anemia: innate immunity and pathogenesis," *International Journal of Biological Sciences*, vol. 7, no. 9, pp. 1427–1442, 2011.

[5] W. R. J. Taylor, J. Hanson, G. D. H. Turner, N. J. White, and A. M. Dondorp, "Respiratory manifestations of malaria," *Chest*, vol. 142, no. 2, pp. 492–505, 2012.

[6] R. Idro, N. E. Jenkins, and C. R. J. Newton, "Pathogenesis, clinical features, and neurological outcome of cerebral malaria," *The Lancet Neurology*, vol. 4, no. 12, pp. 827–840, 2005.

[7] L. Rénia, M. S. Marussig, D. Grillot et al., "*In vitro* activity of $CD4^+$ and $CD8^+$ T lymphocytes from mice immunized with a synthetic malaria peptide," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 88, no. 18, pp. 7963–7967, 1991.

[8] L. Schofield and G. E. Grau, "Immunological processes in malaria pathogenesis," *Nature Reviews Immunology*, vol. 5, no. 9, pp. 722–735, 2005.

[9] J. M. Horne-Debets, R. Faleiro, D. S. Karunarathne et al., "PD-1 dependent exhaustion of CD8+ T cells drives chronic malaria," *Cell Reports*, vol. 5, no. 5, pp. 1204–1213, 2013.

[10] K. J. Ewer, G. A. O'Hara, C. J. A. Duncan et al., "Protective $CD8^+$ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation," *Nature Communications*, vol. 4, article 2836, 2013.

[11] K. Artavanis-Tsakonas and E. M. Riley, "Innate immune response to malaria: rapid induction of IFN-γ from human NK

cells by live Plasmodium falciparum-infected erythrocytes," *The Journal of Immunology*, vol. 169, no. 6, pp. 2956–2963, 2002.

[12] J. Santhanam, L. Råberg, and N. Jon Savill, "Immune-mediated competition in rodent Malaria is most likely caused by induced changes in innate immune clearance of merozoites," *PLoS Computional Biology*, vol. 10, no. 1, Article ID e1003416, 2014.

[13] L. Molineaux, M. Träuble, W. E. Collins, G. M. Jeffery, and K. Dietz, "Malaria therapy reinculation data suggest individual variation of an innate immune response and independent acquisition of antiparasitic and antitoxic immunities," *Transactions of the Royal Society of Tropical Medicine and Hygiene*, vol. 96, no. 2, pp. 205–209, 2002.

[14] I. G. Scragg, "Early cytokine induction by *Plasmodium falciparum* is not a classical endotoxin-like process," *European Journal of Immunology*, vol. 29, no. 8, pp. 2636–2644, 1999.

[15] H. Brown, G. Turner, S. Rogerson et al., "Cytokine expression in the brain in human cerebral malaria," *Journal of Infectious Diseases*, vol. 180, no. 5, pp. 1742–1746, 1999.

[16] I. Angulo and M. Fresno, "Cytokines in the pathogenesis of and protection against malaria," *Clinical and Diagnostic Laboratory Immunology*, vol. 9, no. 6, pp. 1145–1152, 2002.

[17] E. Tjitra, N. M. Anstey, P. Sugiarto et al., "Multidrug-resistant Plasmodium vivax associated with severe and fatal malaria: a prospective study in Papua, Indonesia," *PLoS Medicine*, vol. 5, no. 6, pp. 0890–0899, 2008.

[18] S. Ahamada, M. Wery, and R. Hamers, "Rodent malaria parasites: molecular karyotypes characterize species, subspecies and lines," *Parasite*, vol. 1, no. 1, pp. 31–38, 1994.

[19] S. L. Perkins, I. N. Sarkar, and R. Carter, "The phylogeny of rodent malaria parasites: simultaneous analysis across three genomes," *Infection, Genetics and Evolution*, vol. 7, no. 1, pp. 74–83, 2007.

[20] F. H. Amante, A. Haque, A. C. Stanley et al., "Immune-mediated mechanisms of parasite tissue sequestration during experimental cerebral malaria," *Journal of Immunology*, vol. 185, no. 6, pp. 3632–3642, 2010.

[21] C. Claser, B. Malleret, S. Y. Gun et al., "$Cd8^+$ T cells and IFN-γ mediate the time-dependent accumulation of infected red blood cells in deep organs during experimental cerebral malaria," *PLoS ONE*, vol. 6, no. 4, Article ID e18720, 2011.

[22] J. A. McQuillan, A. J. Mitchell, Y. F. Ho et al., "Coincident parasite and CD8 T cell sequestration is required for development of experimental cerebral malaria," *International Journal for Parasitology*, vol. 41, no. 2, pp. 155–163, 2011.

[23] F. El-Assaad, J. Whewaya, A. J. Mitchell, and et al, "Cytoadherence of *Plasmodium* berghei-infected red blood cells to murine brain and lung microvascular endothelial cells *in vitro*," *Infection and Immunity*, vol. 81, no. 11, pp. 3984–91, 2013.

[24] C. R. J. C. Newton, T. T. Hien, and N. White, "Cerebral malaria," *Journal of Neurology Neurosurgery & Psychiatry*, vol. 69, no. 4, pp. 433–441, 2000.

[25] S. C. Wassmer, V. Combes, and G. E. Grau, "Pathophysiology of cerebral malaria: Role of host cells in the modulation of cytoadhesion," *Annals of the New York Academy of Sciences*, vol. 992, pp. 30–38, 2003.

[26] E. Belnoue, M. Kayibanda, A. M. Vigario et al., "On the pathogenic role of brain-sequestered alphabeta CD8+ T cells in experimental cerebral malaria," *Journal of Immunology*, vol. 169, no. 11, pp. 6369–6375, 2002.

[27] S. W. Howland, C. M. Poh, S. Y. Gun et al., "Brain microvessel cross-presentation is a hallmark of experimental cerebral malaria," *EMBO Molecular Medicine*, vol. 5, no. 7, pp. 984–999, 2013.

[28] R. Medzhitov and J. Janeway C., "Advances in immunology: innate immunity," *The New England Journal of Medicine*, vol. 343, no. 5, pp. 338–344, 2000.

[29] R. Medzhitov and C. Janeway Jr., "Innate immune recognition: Mechanisms and pathways," *Immunological Reviews*, vol. 173, pp. 89–97, 2000.

[30] S. Akira, S. Uematsu, and O. Takeuchi, "Pathogen recognition and innate immunity," *Cell*, vol. 124, no. 4, pp. 783–801, 2006.

[31] T. Vasselon and P. A. Detmers, "Toll receptors: a central element in innate immune responses," *Infection and Immunity*, vol. 70, no. 3, pp. 1033–1041, 2002.

[32] T. Kawai and S. Akira, "Toll-like receptor and RIG-1-like receptor signaling," *Annals of the New York Academy of Sciences*, vol. 1143, pp. 1–20, 2008.

[33] S. Chtarbanova and J. Imler, "Microbial sensing by toll receptors: a historical perspective," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 31, no. 8, pp. 1734–1738, 2011.

[34] A. Cambi and C. G. Figdor, "Dual function of C-type lectin-like receptors in the immune system," *Current Opinion in Cell Biology*, vol. 15, no. 5, pp. 539–546, 2003.

[35] E. Pyz, A. S. J. Marshall, S. Gordon, and G. D. Brown, "C-type lectin-like receptors on myeloid cells," *Annals of Medicine*, vol. 38, no. 4, pp. 242–251, 2006.

[36] T. B. H. Geijtenbeek and S. I. Gringhuis, "Signalling through C-type lectin receptors: shaping immune responses," *Nature Reviews Immunology*, vol. 9, no. 7, pp. 465–479, 2009.

[37] T.-D. Kanneganti, M. Lamkanfi, and G. Núñez, "Intracellular NOD-like receptors in host defense and disease," *Immunity*, vol. 27, no. 4, pp. 549–559, 2007.

[38] M. Proell, S. J. Riedl, J. H. Fritz, A. M. Rojas, and R. Schwarzenbacher, "The Nod-Like Receptor (NLR) family: a tale of similarities and differences," *PLoS ONE*, vol. 3, no. 4, Article ID e2119, 2008.

[39] K. Geddes, J. G. Magalhães, and S. E. Girardin, "Unleashing the therapeutic potential of NOD-like receptors," *Nature Reviews Drug Discovery*, vol. 8, no. 6, pp. 465–479, 2009.

[40] Y. M. Loo and M. Gale Jr., "Immune Signaling by RIG-I-like Receptors," *Immunity*, vol. 34, no. 5, pp. 680–692, 2011.

[41] M. Muzio, D. Bosisio, N. Polentarutti et al., "Differential expression and regulation of toll-like receptors (TLR) in human leukocytes: selective expression of TLR3 in dendritic cells," *Journal of Immunology*, vol. 164, no. 11, pp. 5998–6004, 2000.

[42] V. Hornung, S. Rothenfusser, S. Britsch et al., "Quantitative expression of toll-like receptor 1-10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides," *Journal of Immunology*, vol. 168, no. 9, pp. 4531–4537, 2002.

[43] D. Jarrossay, G. Napolitani, M. Colonna, F. Sallusto, and A. Lanzavecchia, "Specialization and complementarity in microbial molecule recognition by human myeloid and plasmacytoid dendritic cells," *European Journal of Immunology*, vol. 31, no. 11, pp. 3388–3393, 2001.

[44] T. Ito, R. Amakawa, T. Kaisho et al., "Interferon-α and interleukin-12 are induced differentially by toll-like receptor 7 ligands in human blood dendritic cell subsets," *Journal of Experimental Medicine*, vol. 195, no. 11, pp. 1507–1512, 2002.

[45] T. B. H. Geijtenbeek, R. Torensma, S. J. Van Vliet et al., "Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses," *Cell*, vol. 100, no. 5, pp. 575–585, 2000.

[46] L. F. Poulin, Y. Reyal, H. Uronen-Hansson et al., "DNGR-1 is a specific and universal marker of mouse and human Batf3-dependent dendritic cells in lymphoid and nonlymphoid tissues," *Blood*, vol. 119, no. 25, pp. 6052–6062, 2012.

[47] H. H. Smits, A. Engering, D. Van Der Kleij et al., "Selective probiotic bacteria induce IL-10-producing regulatory T cells in vitro by modulating dendritic cell function through dendritic cell-specific intercellular adhesion molecule 3-grabbing nonintegrin," *Journal of Allergy and Clinical Immunology*, vol. 115, no. 6, pp. 1260–1267, 2005.

[48] T. B. Geijtenbeek, S. J. van Vliet, E. A. Koppel et al., "Mycobacteria target DC-SIGN to suppress dendritic cell function," *Journal of Experimental Medicine*, vol. 197, no. 1, pp. 7–17, 2003.

[49] M. P. Bergman, A. Engering, H. H. Smits et al., "Helicobacter pylori modulates the T helper cell 1/T helper cell 2 balance through phase-variable interaction between lipopolysaccharide and DC-SIGN," *Journal of Experimental Medicine*, vol. 200, no. 8, pp. 979–990, 2004.

[50] J. Li, Z. Ma, Z. Tang, T. Stevens, B. Pitt, and S. Li, "CpG DNA-mediated immune response in pulmonary endothelial cells," *The American Journal of Physiology—Lung Cellular and Molecular Physiology*, vol. 287, no. 3, pp. L552–L558, 2004.

[51] L. Guillott, S. Medjane, K. Le-Barillec et al., "Response of human pulmonary epithelial cells to lipopolysaccharide involves toll-like receptor 4 (TLR4)-dependent signaling pathways: Evidence for an intracellular compartmentalization of TLR4," *The Journal of Biological Chemistry*, vol. 279, no. 4, pp. 2712–2718, 2004.

[52] D. Droemann, T. Goldmann, D. Branscheid et al., "Toll-like receptor 2 is expressed by alveolar epithelial cells type II and macrophages in the human lung," *Histochemistry and Cell Biology*, vol. 119, no. 2, pp. 103–108, 2003.

[53] S. Claeys, T. de Belder, G. Holtappels et al., "Human β-defensins and toll-like receptors in the upper airway," *Allergy*, vol. 58, no. 8, pp. 748–753, 2003.

[54] C. Maaser, J. Heidemann, C. Von Eiff et al., "Human intestinal microvascular endothelial cells express Toll-like receptor 5: a binding partner for bacterial flagellin," *Journal of Immunology*, vol. 172, no. 8, pp. 5056–5062, 2004.

[55] E. Cario and D. K. Podolsky, "Differential alteration in intestinal epithelial cell expression of Toll-like receptor 3 (TLR3) and TLR4 in inflammatory bowel disease," *Infection and Immunity*, vol. 68, no. 12, pp. 7010–7017, 2000.

[56] A. T. Gewirtz, T. A. Navas, S. Lyons, P. J. Godowski, and J. L. Madara, "Cutting edge: bacterial flagellin activates basolaterally expressed TLR5 to induce epithelial proinflammatory gene expression," *Journal of Immunology*, vol. 167, no. 4, pp. 1882–1885, 2001.

[57] S. Frantz, L. Kobzik, Y. Kim et al., "Toll4 (TLR4) expression in cardiac myocytes in normal and failing myocardium," *Journal of Clinical Investigation*, vol. 104, no. 3, pp. 271–280, 1999.

[58] Y. Bulut, E. Faure, L. Thomas et al., "Chlamydial heat shock protein 60 activates macrophages and endothelial cells through toll-like receptor 4 and MD2 in a MyD88-dependent pathway," *The Journal of Immunology*, vol. 168, no. 3, pp. 1435–1440, 2002.

[59] T. Imaizumi, S. Aratani, T. Nakajima et al., "Retinoic acid-inducible gene-I is induced in endothelial cells by LPS and regulates expression of COX-2," *Biochemical and Biophysical Research Communications*, vol. 292, no. 1, pp. 274–279, 2002.

[60] B. Opitz, S. Förster, A. C. Hocke et al., "Nod1-mediated endothelial cell activation by Chlamydophila pneumoniae," *Circulation Research*, vol. 96, no. 3, pp. 319–326, 2005.

[61] S. Sharma, R. B. DeOliveira, P. Kalantari et al., "Innate immune recognition of an AT-rich stem-loop DNA motif in the Plasmodium falciparum genome," *Immunity*, vol. 35, no. 2, pp. 194–207, 2011.

[62] Z. Lu, L. Serghides, S. N. Patel et al., "Disruption of JNK2 decreases the cytokine response to *Plasmodium falciparum* glycosylphosphatidylinositol in vitro and confers protection in a cerebral malaria model," *Journal of Immunology*, vol. 177, no. 9, pp. 6344–6352, 2006.

[63] C. Coban, S. Uematsu, N. Arisue et al., "Pathological role of Toll-like receptor signaling in cerebral malaria," *International Immunology*, vol. 19, no. 1, pp. 67–79, 2007.

[64] B. Lepenies, J. P. Cramer, G. D. Burchard, H. Wagner, C. J. Kirschning, and T. Jacobs, "Induction of experimental cerebral malaria is independent of TLR2/4/9," *Medical Microbiology and Immunology*, vol. 197, no. 1, pp. 39–44, 2008.

[65] J. W. Griffith, C. O'Connor, K. Bernard, T. Town, D. R. Goldstein, and R. Bucala, "Toll-like receptor modulation of murine cerebral malaria is dependent on the genetic background of the host," *Journal of Infectious Diseases*, vol. 196, no. 10, pp. 1553–1564, 2007.

[66] W. Rudin, N. Favre, G. Bordmann, and B. Ryffel, "Interferon-γ is essential for the development of cerebral malaria," *European Journal of Immunology*, vol. 27, no. 4, pp. 810–815, 1997.

[67] A. J. Mitchell, A. M. Hansen, L. Hee et al., "Early cytokine production is associated with protection from murine cerebral malaria," *Infection and Immunity*, vol. 73, no. 9, pp. 5645–5653, 2005.

[68] C. N. Morrell, K. Srivastava, A. Swaim et al., "Beta interferon suppresses the development of experimental cerebral malaria," *Infection and Immunity*, vol. 79, no. 4, pp. 1750–1758, 2011.

[69] G. Senaldi, C. L. Shaklee, J. Guo et al., "Protection against the mortality associated with disease models mediated by TNF and IFN-γ in mice lacking IFN regulatory factor-1," *Journal of Immunology*, vol. 163, no. 12, pp. 6820–6826, 1999.

[70] F. E. Lovegrove, S. A. Gharib, S. N. Patel, C. A. Hawkes, K. C. Kain, and W. C. Liles, "Expression microarray analysis implicates apoptosis and interferon-responsive mechanisms in susceptibility to experimental cerebral malaria," *The American Journal of Pathology*, vol. 171, no. 6, pp. 1894–1903, 2007.

[71] A. C. Sexton, R. T. Good, D. S. Hansen et al., "Transcriptional profiling reveals suppressed erythropoiesis, up-regulated glycolysis, and interferon-associated responses in murine malaria," *Journal of Infectious Diseases*, vol. 189, no. 7, pp. 1245–1256, 2004.

[72] J. Berghout, D. Langlais, and P. Gros, "Irf8-regulated genomic responses drive pathological inflammation during cerebral Malaria," *PLoS Pathogens*, vol. 9, no. 7, p. 11, 2013.

[73] J. C. R. Hafalla, J. Burgold, A. Dorhoi et al., "Experimental cerebral malaria develops independently of caspase recruitment domain-containing protein 9 signaling," *Infection and Immunity*, vol. 80, no. 3, pp. 1274–1279, 2012.

[74] F. E. Lovegrove, L. Peña-Castillo, N. Mohammad, W. C. Liles, T. R. Hughes, and K. C. Kain, "Simultaneous host and parasite expression profiling identifies tissue-specific transcriptional programs associated with susceptibility or resistance to experimental cerebral malaria," *BMC Genomics*, vol. 7, article 295, 2006.

[75] N. F. Delahaye, N. Coltel, D. Puthier et al., "Gene expression analysis reveals early changes in several molecular pathways in cerebral malaria-susceptible mice versus cerebral malaria-resistant mice," *BMC Genomics*, vol. 8, article 452, 2007.

[76] P. E. van den Steen, N. Geurts, K. Deroost et al., "Immunopathology and dexamethasone therapy in a new model for malaria-associated acute respiratory distress syndrome," *American Journal of Respiratory and Critical Care Medicine*, vol. 181, no. 9, pp. 957–968, 2010.

[77] A. Haque, S. E. Best, A. Ammerdorffer et al., "Type I interferons suppress CD4 + T-cell-dependent parasite control during blood-stage Plasmodium infection," *European Journal of Immunology*, vol. 41, no. 9, pp. 2688–2698, 2011.

[78] D. Togbe, L. Schofield, G. E. Grau et al., "Murine cerebral malaria development is independent of toll-like receptor signaling," *The American Journal of Pathology*, vol. 170, no. 5, pp. 1640–1648, 2007.

[79] E. A. Ball, M. R. Sambo, M. Martins et al., "IFNAR1 controls progression to cerebral malaria in children and CD8 + T cell brain pathology in plasmodium berghei-infected mice," *Journal of Immunology*, vol. 190, no. 10, pp. 5118–5127, 2013.

[80] M. Kordes, K. Matuschewski, and J. C. R. Hafalla, "Caspase-1 activation of interleukin-1ß (IL-1ß) and IL-18 Is dispensable for induction of experimental cerebral malaria," *Infection and Immunity*, vol. 79, no. 9, pp. 3633–3641, 2011.

[81] A. Villegas-Mendez, R. Greig, T. N. Shaw et al., "IFN-γ-producing $CD4^+$ T cells promote experimental cerebral malaria by modulating $CD8^+$ T cell accumulation within the brain," *The Journal of Immunology*, vol. 189, no. 2, pp. 968–979, 2012.

[82] J. Palomo, M. Fauconnier, L. Coquard et al., "Type I interferons contribute to experimental cerebral malaria development in response to sporozoite or blood-stage *Plasmodium berghei* ANKA," *European Journal of Immunology*, vol. 43, no. 10, pp. 2683–2695, 2013.

[83] E. Belnoue, S. M. Potter, D. S. Rosa et al., "Control of pathogenic $CD8^+$ T cell migration to the brain by IFN-γ during experimental cerebral malaria," *Parasite Immunology*, vol. 30, no. 10, pp. 544–553, 2008.

[84] S. Epiphanio, M. G. Campos, A. Pamplona et al., "VEGF promotes malaria-associated acute lung injury in mice," *PLoS Pathogens*, vol. 6, no. 5, Article ID e1000916, 2010.

[85] J. Wu, L. Tian, X. Yu et al., "Strain-specific innate immune signaling pathways determine malaria parasitemia dynamics and host mortality," *Proceedings of the National Academy of Sciences*, vol. 111, no. 4, pp. E511–E520, 2014.

[86] N. M. Gowda, X. Wu, and D. C. Gowda, "TLR9 and MyD88 are crucial for the development of protective immunity to malaria," *The Journal of Immunology*, vol. 188, no. 10, pp. 5073–5085, 2012.

[87] A. M. Vigário, E. Belnoue, A. Cumano et al., "Inhibition of Plasmodium yoelii blood-stage malaria by interferon α through the inhibition of the production of its target cell, the reticulocyte," *Blood*, vol. 97, no. 12, pp. 3966–3971, 2001.

[88] K. Adachi, H. Tsutsui, S. I. Kashiwamura et al., "Plasmodium berghei infection in mice induces liver injury by an IL-12- and toll-like receptor/myeloid differentiation factor 88-dependent mechanism," *Journal of Immunology*, vol. 167, no. 10, pp. 5928–5934, 2001.

[89] R. Barboza, A. S. Reis, L. G. da Silva et al., "MyD88 signaling is directly involved in the development of murine placental malaria," *Infection and Immunity*, 2013.

[90] C. C. Kim, C. S. Nelson, E. B. Wilson, B. Hou, A. L. DeFranco, and J. L. DeRisi, "Splenic red pulp macrophages produce type I interferons as early sentinels of malaria infection but are dispensable for control," *PLoS ONE*, vol. 7, no. 10, Article ID e48126, 2012.

[91] Z. Su and M. M. Stevenson, "Central role of endogenous gamma interferon in protective immunity against blood-stage Plasmodium chabaudi AS infection," *Infection and Immunity*, vol. 68, no. 8, pp. 4399–4406, 2000.

[92] P. Liehl, V. Zuzarte-Luís, J. Chan et al., "Host-cell sensors for Plasmodium activate innate immunity against liver-stage infection," *Nature Medicine*, vol. 20, no. 1, pp. 47–53, 2014.

[93] L. A. Gonçalves, L. Rodrigues-Duarte, J. Rodo, L. Vieira de Moraes, I. Marques, and C. Penha-Gonçalves, "TREM2 governs Kupffer cell activation and explains belr1 genetic resistance to malaria liver stage infection," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, pp. 19531–19536, 2013.

[94] H. L. Shear, R. Srinivasan, T. Nolan, and C. Ng, "Role of IFN-γ in lethal and nonlethal malaria in susceptible and resistant murine hosts," *Journal of Immunology*, vol. 143, no. 6, pp. 2038–2044, 1989.

[95] J. Miu, N. H. Hunt, and H. J. Ball, "Predominance of interferon-related responses in the brain during murine malaria, as identified by microarray analysis," *Infection and Immunity*, vol. 76, no. 5, pp. 1812–1824, 2008.

[96] I. A. Clark, N. H. Hunt, G. A. Butcher, and W. B. Cowden, "Inhibition of murine malaria (Plasmodium chabaudi) in vivo by recombinant interferon-γ or tumor necrosis factor, and its enhancement by butylated hydroxyanisole," *The Journal of Immunology*, vol. 139, no. 10, pp. 3493–3496, 1987.

[97] V. Amani, A. M. Vigário, E. Belnoue et al., "Involvement of IFN-γ receptor-mediated signaling in pathology and anti-malarial immunity induced by Plasmodium berghei infection," *European Journal of Immunology*, vol. 30, no. 6, pp. 1646–1655, 2000.

[98] C. Voisine, B. Mastelic, A. Sponaas, and J. Langhorne, "Classical $CD11c^+$ dendritic cells, not plasmacytoid dendritic cells, induce T cell responses to *Plasmodium chabaudi* malaria," *International Journal for Parasitology*, vol. 40, no. 6, pp. 711–719, 2010.

[99] J. Zhu, G. Krishnegowda, G. Li, and D. Channe Gowda, "Proinflammatory responses by glycosylphosphatidylinositols (GPIs) of Plasmodium falciparum are mainly mediated through the recognition of TLR2/TLR1," *Experimental Parasitology*, vol. 128, no. 3, pp. 205–211, 2011.

[100] J. Zhu, G. Krishnegowda, and D. C. Gowda, "Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: The requirement of extracellular signal-regulated kinase, p38, c-Jun N-terminal kinase and NF-κB pathways for the expression of proinflammatory cytokines and nitric oxide," *Journal of Biological Chemistry*, vol. 280, no. 9, pp. 8617–8627, 2005.

[101] G. Krishnegowda, A. M. Hajjar, J. Zhu et al., "Induction of proinflammatory responses in macrophages by the glycosylphosphatidylinositols of Plasmodium falciparum: cell signaling receptors, glycosylphosphatidylinositol (GPI) structural requirement, and regulation of GPI activity," *Journal of Biological Chemistry*, vol. 280, no. 9, pp. 8606–8616, 2005.

[102] J. Zhu, X. Wu, S. Goel et al., "MAPK-activated protein kinase 2 differentially regulates plasmodium falciparum glycosylphosphatidylinositol-inducedproduction of tumor necrosis Factor-α and interleukin-12 in macrophages," *The Journal of Biological Chemistry*, vol. 284, no. 23, pp. 15756–15761, 2009.

[103] J. Zhu, R. Weinberg, X. Wu, N. M. Gowda, T. Muta, and D. C. Gowda, "Ikappab-zeta plays an important role in the ERK-dependent dysregulation of malaria parasite GPI-induced IL-12 expression," *IUBMB Life*, vol. 64, no. 2, pp. 187–193, 2012.

[104] C. Coban, K. J. Ishii, T. Kawai et al., "Toll-like receptor 9 mediates innate immune activation by the malaria pigment hemozoin," *The Journal of Experimental Medicine*, vol. 201, no. 1, pp. 19–25, 2005.

[105] P. Parroche, F. N. Lauw, N. Goutagny et al., "Malaria hemozoin is immunologically inert but radically enhances innate responses by presenting malaria DNA to Toll-like receptor 9," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 6, pp. 1919–1924, 2007.

[106] V. Barrera, O. A. Skorokhod, D. Baci, G. Gremo, P. Arese, and E. Schwarzer, "Host fibrinogen stably bound to hemozoin rapidly activates monocytes via TLR-4 and CD11b/CD18-integrin: A new paradigm of hemozoin action," *Blood*, vol. 117, no. 21, pp. 5674–5682, 2011.

[107] B. B. Andrade, T. Araújo-Santos, N. F. Luz et al., "Heme impairs prostaglandin E2 and TGF-β production by human mononuclear cells via Cu/Zn superoxide dismutase: insight into the pathogenesis of severe malaria," *The Journal of Immunology*, vol. 185, no. 2, pp. 1196–1204, 2010.

[108] R. T. Figueiredo, P. L. Fernandez, D. S. Mourao-Sa et al., "Characterization of heme as activator of toll-like receptor 4," *The Journal of Biological Chemistry*, vol. 282, no. 28, pp. 20221–20229, 2007.

[109] K. N. Couper, T. Barnes, J. C. R. Hafalla et al., "Parasite-derived plasma microparticles contribute significantly to malaria infection-induced inflammation through potent macrophage stimulation," *PLoS Pathogens*, vol. 6, no. 1, Article ID e1000744, 2010.

[110] A. Baccarella, M. F. Fontana, E. Chen, and C. C. Kim, "Toll-like receptor 7 mediates early innate immune responses to malaria," *Infection and Immunity*, vol. 81, no. 12, pp. 4431–4442, 2013.

[111] T. K. Bhatt, A. Sharma, S. Khan et al., "Malaria parasite tyrosyl-tRNA synthetase secretion triggers pro-inflammatory responses," *Nature Communications*, vol. 2, no. 1, article 530, 2011.

[112] K. Kumar, A. Singal, M. M. A. Rizvi, and V. S. Chauhan, "High mobility group box (HMGB) proteins of Plasmodium falciparum: DNA binding proteins with pro-inflammatory activity," *Parasitology International*, vol. 57, no. 2, pp. 150–157, 2008.

[113] L. Hamann, G. Bedu-Addo, T. A. Eggelte, R. R. Schumann, and F. P. Mockenhaupt, "The toll-like receptor 1 variant S248N influences placental malaria," *Infection, Genetics and Evolution*, vol. 10, no. 6, pp. 785–789, 2010.

[114] F. M. S. Leoratti, L. Farias, F. P. Alves et al., "Variants in the toll-like receptor signaling pathway and clinical outcomes of malaria," *The Journal of Infectious Diseases*, vol. 198, no. 5, pp. 772–780, 2008.

[115] J. A. Greene, N. Sam-Agudu, C. C. John, R. O. Opoka, P. A. Zimmerman, and J. W. Kazura, "Toll-like receptor polymorphisms and cerebral malaria: TLR2 Δ22 polymorphism is associated with protection from cerebral malaria in a case control study," *Malaria Journal*, vol. 11, no. 47, pp. 1475–2875, 2012.

[116] S. Zakeri, S. Pirahmadi, A. A. Mehrizi, and N. D. Djadid, "Genetic variation of *TLR-4*, *TLR-9* and *TIRAP* genes in Iranian malaria patients," *Malaria Journal*, vol. 10, article 77, 2011.

[117] S. Esposito, C. G. Molteni, A. Zampiero et al., "Role of polymorphisms of toll-like receptor (TLR) 4, TLR9, toll-interleukin 1 receptor domain containing adaptor protein (TIRAP) and FCGR2A genes in malaria susceptibility and severity in Burundian children," *Malaria Journal*, vol. 11, article 196, 2012.

[118] F. P. Mockenhaupt, L. Hamann, C. Von Gaertner et al., "Common polymorphisms of toll-like receptors 4 and 9 are associated with the clinical manifestation of malaria during pregnancy," *Journal of Infectious Diseases*, vol. 194, no. 2, pp. 184–188, 2006.

[119] F. P. Mockenhaupt, J. P. Cramer, L. Hamann et al., "Toll-like receptor (TLR) polymorphisms in African children: common TLR-4 variants predispose to severe malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 1, pp. 177–182, 2006.

[120] A. H. Omar, M. Yasunami, A. Yamazaki et al., "Toll-like receptor 9 (TLR9) polymorphism associated with symptomatic malaria: a cohort study," *Malaria Journal*, vol. 11, article 168, pp. 1475–2875, 2012.

[121] N. A. Sam-Agudu, J. A. Greene, R. O. Opoka et al., "TLR9 polymorphisms are associated with altered IFN-γlevels in children with cerebral malaria," *The American Journal of Tropical Medicine and Hygiene*, vol. 82, no. 4, pp. 548–555, 2010.

[122] C. C. Khor, S. J. Chapman, F. O. Vannberg et al., "A Mal functional variant is associated with protection against invasive pneumococcal disease, bacteremia, malaria and tuberculosis," *Nature Genetics*, vol. 39, no. 4, pp. 523–528, 2007.

[123] V. D. Mangano, G. Luoni, K. A. Rockett et al., "Interferon regulatory factor-1 polymorphisms are associated with the control of *Plasmodium falciparum* infection," *Genes & Immunity*, vol. 9, no. 2, pp. 122–129, 2008.

[124] V. D. Mangano, T. G. Clark, S. Auburn et al., "Lack of association of *interferon regulatory factor 1* with severe malaria in affected child-parental trio studies across three African populations," *PLoS ONE*, vol. 4, no. 1, Article ID e4206, 2009.

[125] R. S. Tan, C. Feng, Y. Asano, and A. U. Kara, "Altered immune response of interferon regulatory factor 1-deficient mice against Plasmodium berghei blood-stage malaria infection," *Infection and Immunity*, vol. 67, no. 5, pp. 2277–2283, 1999.

[126] J. L. Miller, B. K. Sack, M. Baldwin, A. M. Vaughan, and S. H. Kappe, "Interferon-mediated innate immune responses against malaria parasite liver stages," *Cell Reports*, vol. 7, no. 2, pp. 436–447, 2014.

[127] N. J. Gay and M. Gangloff, "Structure and function of toll receptors and their ligands," *Annual Review of Biochemistry*, vol. 76, pp. 141–145, 2007.

[128] O. Takeuchi and S. Akira, "Pattern recognition receptors and inflammation," *Cell*, vol. 140, no. 6, pp. 805–820, 2010.

[129] E. M. Pietras, S. K. Saha, and G. Cheng, "The interferon response to bacterial and viral infections," *Journal of Endotoxin Research*, vol. 12, no. 4, pp. 246–250, 2006.

[130] A. K. Perry, G. Chen, D. Zheng, H. Tang, and G. Cheng, "The host type I interferon response to viral and bacterial infections," *Cell Research*, vol. 15, no. 6, pp. 407–422, 2005.

[131] J. Currier, H. Beck, B. Currie, and M. F. Good, "Antigens released at schizont burst stimulate Plasmodium falciparum-specific CD4+ T cells from non-exposed donors: Potential for cross-reactive memory T cells to cause disease," *International Immunology*, vol. 7, no. 5, pp. 821–833, 1995.

[132] K. P. O'Dea and G. Pasvol, "Optimal tumor necrosis factor induction by Plasmodium falciparum requires the highly localized release of parasite products," *Infection and Immunity*, vol. 71, no. 6, pp. 3155–3164, 2003.

[133] D. Kwiatkowski, J. G. Cannon, K. R. Manogue, A. Cerami, C. A. Dinarello, and B. M. Greenwood, "Tumour necrosis factor production in Falciparum malaria and its association with schizont rupture," *Clinical and Experimental Immunology*, vol. 77, no. 3, pp. 361–366, 1989.

[134] S. F. Kitchen, "Symptomology: general considerations," in *Malariology: A Comprehensive Survey of all Aspects of this Group of Diseases from a Global Standpoint*, Saunders, Philadelphia, Pa, USA, 1949.

[135] P. Gerold, L. Schofield, M. J. Blackman, A. A. Holder, and R. T. Schwarz, "Structural analysis of the glycosylphosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*," *Molecular and Biochemical Parasitology*, vol. 75, no. 2, pp. 131–143, 1996.

[136] V. K. Goel, X. Li, H. Chen, S. C. Liu, A. H. Chishti, and S. S. Oh, "Band 3 is a host receptor binding merozoite surface protein 1 during the *Plasmodium falciparum* invasion of erythrocytes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 9, pp. 5164–5169, 2003.

[137] D. T. Riglar, D. Richard, D. W. Wilson et al., "Super-resolution dissection of coordinated events during malaria parasite invasion of the human erythrocyte," *Cell Host and Microbe*, vol. 9, no. 1, pp. 9–20, 2011.

[138] J. A. Dvorak, L. H. Miller, W. C. Whitehouse, and T. Shiroishi, "Invasion of erythrocytes by malaria merozoites," *Science*, vol. 187, no. 4178, pp. 748–750, 1975.

[139] M. J. Boyle, C. Langer, J. A. Chan et al., "Sequential processing of merozoite surface proteins during and after erythrocyte invasion by *Plasmodium falciparum*," *Infection and Immunity*, vol. 82, no. 3, pp. 924–936, 2014.

[140] M. Aikawa, L. H. Miller, J. Johnson, and J. Rabbege, "Erythrocyte entry by malarial parasites. A moving junction between erythrocyte and parasite," *Journal of Cell Biology*, vol. 77, no. 1, pp. 72–82, 1978.

[141] M. Chugh, V. Sundararaman, S. Kumar et al., "Protein complex directs hemoglobin-to-hemozoin formation in *Plasmodium falciparum*," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 110, no. 14, pp. 5392–5397, 2013.

[142] S. Kapishnikov, A. Weiner, E. Shimoni et al., "Oriented nucleation of hemozoin at the digestive vacuole membrane in Plasmodium falciparum," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 28, pp. 11188–11193, 2012.

[143] S. Kapishnikov, A. Weiner, E. Shimoni, G. Schneider, M. Elbaum, and L. Leiserowitz, "Digestive vacuole membrane in *Plasmodium falciparum* infected erythrocytes: relevance to templated nucleation of hemozoin," *Langmuir*, vol. 29, no. 47, pp. 14595–14602, 2013.

[144] M. Abkarian, G. Massiera, L. Berry, M. Roques, and C. Braun-Breton, "A novel mechanism for egress of malarial parasites from red blood cells," *Blood*, vol. 117, no. 15, pp. 4118–4124, 2011.

[145] C. Coban, Y. Igari, M. Yagi et al., "Immunogenicity of whole-parasite vaccines against *Plasmodium falciparum* involves malarial hemozoin and host TLR9," *Cell Host and Microbe*, vol. 7, no. 1, pp. 50–61, 2010.

[146] C. Hashimoto, K. L. Hudson, and K. V. Anderson, "The Toll gene of drosophila, required for dorsal-ventral embryonic polarity, appears to encode a transmembrane protein," *Cell*, vol. 52, no. 2, pp. 269–279, 1988.

[147] R. Medzhitov, P. Preston-Hurlburt, and C. A. Janeway Jr., "A human homologue of the Drosophila toll protein signals activation of adaptive immunity," *Nature*, vol. 388, no. 6640, pp. 394–397, 1997.

[148] S. Pandey and D. K. Agrawal, "Immunobiology of Toll-like receptors: emerging trends," *Immunology and Cell Biology*, vol. 84, no. 4, pp. 333–341, 2006.

[149] T. Chuang and R. J. Ulevitch, "Identification of hTLR10: a novel human Toll-like receptor preferentially expressed in immune cells," *Biochimica et Biophysica Acta: Gene Structure and Expression*, vol. 1518, no. 1-2, pp. 157–161, 2001.

[150] F. Yarovinsky, "Innate immunity to Toxoplasma gondii infection," *Nature Reviews Immunology*, vol. 14, no. 2, pp. 109–121, 2014.

[151] T. Kawai and S. Akira, "Pathogen recognition with Toll-like receptors," *Current Opinion in Immunology*, vol. 17, no. 4, pp. 338–344, 2005.

[152] T. Kawai and S. Akira, "TLR signaling," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 816–825, 2006.

[153] L. Schofield and F. Hackett, "Signal transduction in host cells by a glycosylphosphatidylinositol toxin of malaria parasites," *Journal of Experimental Medicine*, vol. 177, no. 1, pp. 145–153, 1993.

[154] S. D. Tachado, P. Gerold, M. J. McConville et al., "Glycosylphosphatidylinositol toxin of Plasmodium induces nitric oxide synthase expression in macrophages and vascular endothelial cells by a protein tyrosine kinase-dependent and protein kinase C-dependent signaling pathway," *Journal of Immunology*, vol. 156, no. 5, pp. 1897–1907, 1996.

[155] X. Wu, N. M. Gowda, S. Kumar, and D. C. Gowda, "Protein-DNA complex is the exclusive malaria parasite component that activates dendritic cells and triggers innate immune responses," *The Journal of Immunology*, vol. 184, no. 8, pp. 4338–4348, 2010.

[156] S. Pichyangkul, K. Yongvanitchit, U. Kum-Arb et al., "Malaria blood stage parasites activate human plasmacytoid dendritic cells and murine dendritic cells through a toll-like receptor 9-dependent pathway," *Journal of Immunology*, vol. 172, no. 8, pp. 4926–4933, 2004.

[157] E. Hempelmann and T. J. Egan, "Pigment biocrystallization in Plasmodium falciparum," *Trends in Parasitology*, vol. 18, no. 1, article 11, 2002.

[158] B. S. Franklin, P. Parroche, M. A. Ataíde et al., "Malaria primes the innate immune response due to interferon-γ induced enhancement of toll-like receptor expression and function," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 14, pp. 5789–5794, 2009.

[159] V. Jeney, J. Balla, A. Yachie et al., "Pro-oxidant and cytotoxic effects of circulating heme," *Blood*, vol. 100, no. 3, pp. 879–887, 2002.

[160] J. Balla, G. Balla, V. Jeney, G. Kakuk, H. S. Jacob, and G. M. Vercellotti, "Ferriporphyrins and endothelium: a 2-edged sword—promotion of oxidation and induction of cytoprotectants," *Blood*, vol. 95, no. 11, pp. 3442–3450, 2000.

[161] J. Balla, H. S. Jacob, G. Balla, K. Nath, J. W. Eaton, and G. M. Vercellotti, "Endothelial-cell heme uptake from heme proteins: induction of sensitization and desensitization to oxidant damage," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 90, no. 20, pp. 9285–9289, 1993.

[162] E. Seixas, R. Gozzelino, Â. Chora et al., "Heme oxygenase-1 affords protection against noncerebral forms of severe malaria," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 37, pp. 15837–15842, 2009.

[163] M. A. Arruda, A. G. Rossi, M. S. De Freitas, C. Barja-Fidalgo, and A. V. Graça-Souza, "Heme inhibits human neutrophil apoptosis: involvement of phosphoinositide 3-kinase, MAPK, and NF-κB," *Journal of Immunology*, vol. 173, no. 3, pp. 2023–2030, 2004.

[164] A. V. Graça-Souza, M. A. B. Arruda, M. S. de Freitas, C. Barja-Fidalgo, and P. L. Oliveira, "Neutrophil activation by heme: implications for inflammatory processes," *Blood*, vol. 99, no. 11, pp. 4160–4165, 2002.

[165] B. N. Porto, L. S. Alves, P. L. Fernández et al., "Heme induces neutrophil migration and reactive oxygen species generation through signaling pathways characteristic of chemotactic receptors," *Journal of Biological Chemistry*, vol. 282, no. 33, pp. 24430–24436, 2007.

[166] S. Nomura, N. N. Tandon, T. Nakamura, J. Cone, S. Fukuhara, and J. Kambayashi, "High-shear-stress-induced activation of platelets and microparticles enhances expression of cell adhesion molecules in THP-1 and endothelial cells," *Atherosclerosis*, vol. 158, no. 2, pp. 277–287, 2001.

[167] S. F. Mause, P. von Hundelshausen, A. Zernecke, R. R. Koenen, and C. Weber, "Platelet microparticles: a transcellular delivery system for RANTES promoting monocyte recruitment on endothelium," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 25, no. 7, pp. 1512–1518, 2005.

[168] N. Amabile, A. P. Guérin, A. Leroyer et al., "Circulating endothelial microparticles are associated with vascular dysfunction in patients with end-stage renal failure," *Journal of the American Society of Nephrology*, vol. 16, no. 11, pp. 3381–3388, 2005.

[169] C. M. Boulanger, A. Scoazec, T. Ebrahimian et al., "Circulating microparticles from patients with myocardial infarction cause endothelial dysfunction," *Circulation*, vol. 104, no. 22, pp. 2649–2652, 2001.

[170] B. Fritzsching, B. Schwer, J. Kartenbeck, A. Pedal, V. Horejsi, and M. Ott, "Release and intercellular transfer of cell surface CD81 via microparticles," *Journal of Immunology*, vol. 169, no. 10, pp. 5531–5537, 2002.

[171] M. Baj-Krzyworzeka, R. Szatanek, K. Węglarczyk et al., "Tumour-derived microvesicles carry several surface determinants and mRNA of tumour cells and transfer some of these determinants to monocytes," *Cancer Immunology, Immunotherapy*, vol. 55, no. 7, pp. 808–818, 2006.

[172] V. Combes, N. Coltel, M. Alibert et al., "ABCA1 gene deletion protects against cerebral malaria: potential pathogenic role of microparticles in neuropathology," *The American Journal of Pathology*, vol. 166, no. 1, pp. 295–302, 2005.

[173] Z. M. Khan, C. Ng, and J. P. Vanderberg, "Early hepatic stages of *Plasmodium berghei*: release of circumsporozoite protein and host cellular inflammatory response," *Infection and Immunity*, vol. 60, no. 1, pp. 264–270, 1992.

[174] S. Epiphanio, S. A. Mikolajczak, L. A. Gonçalves et al., "Heme oxygenase-1 is an anti-inflammatory host factor that promotes murine plasmodium liver Infection," *Cell Host and Microbe*, vol. 3, no. 5, pp. 331–338, 2008.

[175] N. A. De Weerd and T. Nguyen, "The interferons and their receptors—distribution and regulation," *Immunology and Cell Biology*, vol. 90, no. 5, pp. 483–491, 2012.

[176] M. J. de Veer, M. Holko, M. Frevel et al., "Functional classification of interferon-stimulated genes identified using microarrays," *Journal of Leukocyte Biology*, vol. 69, no. 6, pp. 912–920, 2001.

[177] G. Trinchieri, "Type I interferon: friend or foe?" *Journal of Experimental Medicine*, vol. 207, no. 10, pp. 2053–2063, 2010.

[178] H. Hisamatsu, N. Shimbara, Y. Saito et al., "Newly identified pair of proteasomal subunits regulated reciprocally by interferon γ," *Journal of Experimental Medicine*, vol. 183, no. 4, pp. 1807–1816, 1996.

[179] B. Boes, H. Hengel, T. Ruppert, G. Multhaup, U. H. Koszinowski, and P. Kloetzel, "Interferon γ stimulation modulates the proteolytic activity and cleavage site preference of 20S mouse proteasomes," *The Journal of Experimental Medicine*, vol. 179, no. 3, pp. 901–909, 1994.

[180] L. A. Cramer, S. L. Nelson, and M. J. Klemsz, "Synergistic induction of the Tap-1 gene by IFN-γ and lipopolysaccharide in macrophages is regulated by STAT1," *The Journal of Immunology*, vol. 165, no. 6, pp. 3190–3197, 2000.

[181] D. Wallach, M. Fellous, and M. Revel, "Preferential effect of γ interferon on the synthesis of HLA antigens and their mRNAs in human cells," *Nature*, vol. 299, no. 5886, pp. 833–836, 1982.

[182] D. V. Erbe, J. E. Collins, L. Shen, R. F. Graziano, and M. W. Fanger, "The effect of cytokines on the expression and function of Fc receptors for IgG on human myeloid cells," *Molecular Immunology*, vol. 27, no. 1, pp. 57–67, 1990.

[183] R. C. Strunk, F. S. Cole, D. H. Perlmutter, and H. R. Colten, "γ-Interferon increases expression of class III complement genes C2 and factor B in human monocytes and in murine fibroblasts transfected with human C2 and factor B genes," *Journal of Biological Chemistry*, vol. 260, no. 28, pp. 15280–15285, 1985.

[184] D. A. Drevets, P. J. M. Leenen, and P. A. Campbell, "Complement receptor type 3 mediates phagocytosis and killing of Listeria monocytogenes by a TNF-α- and IFN-γ-stimulated macrophage precursor hybrid," *Cellular Immunology*, vol. 169, no. 1, pp. 1–6, 1996.

[185] A. Yoshida, Y. Koide, M. Uchijima, and T. O. Yoshida, "IFN-γ induces IL-12 mRNA expression by a murine macrophage cell line, J774," *Biochemical and Biophysical Research Communications*, vol. 198, no. 3, pp. 857–861, 1994.

[186] M. P. Gil, E. Bohn, A. K. O'Guin et al., "Biologic consequences of Stat1-independent IFN signaling," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 12, pp. 6680–6685, 2001.

[187] D. D. Taub, A. R. Lloyd, K. Conlon et al., "Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells," *The Journal of Experimental Medicine*, vol. 177, no. 6, pp. 1809–1814, 1993.

[188] A. Ferreira, L. Schofield, V. Enea et al., "Inhibition of development of exoerythrocytic froms of malaria parasites by γ-interferon," *Science*, vol. 232, no. 4752, pp. 881–884, 1986.

[189] L. Schofield, A. Ferreira, R. Altszuler, V. Nussenzweig, and R. S. Nussenzweig, "Interferon-γ inhibits the intrahepatocytic development of malaria parasites in vitro," *The Journal of Immunology*, vol. 139, no. 6, pp. 2020–2025, 1987.

[190] S. Mellouk, S. J. Green, C. A. Nacy, and S. L. Hoffman, "IFN-γ inhibits development of Plasmodium berghei exoerythrocytic stages in hepatocytes by an L-arginine-dependent effector mechanism," *Journal of Immunology*, vol. 146, no. 11, pp. 3971–3976, 1991.

[191] A. K. Nussler, L. Renia, V. Pasquetto, F. Miltgen, H. Matile, and D. Mazier, "In vivo induction of the nitric oxide pathway in hepatocytes after injection with irradiated malaria sporozoites, malaria blood parasites or adjuvants," *European Journal of Immunology*, vol. 23, no. 4, pp. 882–887, 1993.

[192] A. J. F. Luty, B. Lell, R. Schmidt-Ott et al., "Interferon-γ responses are associated with resistance to reinfection with Plasmodium falciparum in young African children," *Journal of Infectious Diseases*, vol. 179, no. 4, pp. 980–988, 1999.

[193] B. Perlaza, J. Sauzet, K. Brahimi, L. Benmohamed, and P. Druilhe, "Interferon-γ, a valuable surrogate marker of Plasmodium falciparum pre-erythrocytic stages protective immunity," *Malaria Journal*, vol. 10, article 27, 2011.

[194] L. Schofield, J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig, "Gamma Interferon, CD8+ T cells and antibodies required for immunity to malaria sporozoites," *Nature*, vol. 330, no. 6149, pp. 664–666, 1987.

[195] M. M. Stevenson, M. F. Tam, M. Belosevic, P. H. van der Meide, and J. E. Podoba, "Role of endogenous gamma interferon in host response to infection with blood-stage Plasmodium chabaudi AS," *Infection and Immunity*, vol. 58, no. 10, pp. 3225–3232, 1990.

[196] T. Yoneto, T. Yoshimoto, C. Wang et al., "Gamma interferon production is critical for protective immunity to infection with blood-stage Plasmodium berghei XAT but neither NO production nor NK cell activation is critical," *Infection and Immunity*, vol. 67, no. 5, pp. 2349–2356, 1999.

[197] M. C. D'Ombrain, L. J. Robinson, D. I. Stanisic et al., "Association of early interferon-γ production with immunity to clinical malaria: a longitudinal study among Papua New Guinean children," *Clinical Infectious Diseases*, vol. 47, no. 11, pp. 1380–1387, 2008.

[198] S. Inoue, M. Niikura, S. Mineo, and F. Kobayashi, "Roles of IFN-gamma and gammadelta T Cells in protective immunity against blood-stage malaria," *Frontiers in Immunology*, vol. 4, article 258, 2013.

[199] A. J. F. Luty, D. J. Perkins, B. Lell et al., "Low interleukin-12 activity in severe Plasmodium falciparum malaria," *Infection and Immunity*, vol. 68, no. 7, pp. 3909–3915, 2000.

[200] C. Aucan, A. J. Walley, B. J. W. Hennig et al., "Interferon-alpha receptor-1 (IFNAR1) variants are associated with protection against cerebral malaria in The Gambia," *Genes & Immunity*, vol. 4, no. 4, pp. 275–282, 2003.

[201] D. P. Simmons, P. A. Wearsch, D. H. Canaday et al., "Type I IFN drives a distinctive dendritic cell maturation phenotype that allows continued class II MHC synthesis and antigen processing," *Journal of Immunology*, vol. 188, no. 7, pp. 3116–3126, 2012.

[202] J. M. Curtsinger, J. O. Valenzuela, P. Agarwal, D. Lins, and M. F. Mescher, "Cutting edge: type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation," *Journal of Immunology*, vol. 174, no. 8, pp. 4465–4469, 2005.

[203] P. Agarwal, A. Raghavan, S. L. Nandiwada et al., "Gene regulation and chromatin remodeling by IL-12 and type I IFN in programming for CD8 T cell effector function and memory," *Journal of Immunology*, vol. 183, no. 3, pp. 1695–1704, 2009.

[204] C. A. Hunter, K. E. Gabriel, T. Radzanowski, L. E. Neyer, and J. S. Remington, "Type I interferons enhance production of IFN-γ by NK cells," *Immunology Letters*, vol. 59, no. 1, pp. 1–5, 1997.

[205] D. J. Gough, N. L. Messina, L. Hii et al., "Functional crosstalk between type I and II interferon through the regulated expression of STAT1," *PLoS Biology*, vol. 8, no. 4, Article ID e1000361, 2010.

[206] R. I. Jahiel, J. Vilcek, and R. S. Nussenzweig, "Exogenous interferon protects mice against Plasmodium berghei malaria," *Nature*, vol. 227, no. 5265, pp. 1350–1351, 1970.

[207] J. Van Damme, M. R. Schaafsma, W. E. Fibbe, J. H. F. Falkenburg, G. Opdenakker, and A. Billiau, "Simultaneous production of interleukin 6, interferon-β and colony-stimulating activity by fibroblasts after viral and bacterial infection," *European Journal of Immunology*, vol. 19, no. 1, pp. 163–168, 1989.

[208] A. Nussler, J.-ac. Drapier, L. Renia et al., "L-Arginine-dependent destruction of intrahepatic malaria parasites in response to tumor necrosis factor and/or interleukin 6 stimulation," *European Journal of Immunology*, vol. 21, no. 1, pp. 227–230, 1991.

[209] I. Landau and P. Gautret, "Animal models: rodents," in *Malaria: Parasite Biology, Pathogenesis, and Protection*, I. W. Sherman, Ed., pp. 401–417, ASM Press, Washington, DC, USA, 1998.

[210] A. M. Vigário, E. Belnoue, A. C. Grüner et al., "Recombinant human IFN-α inhibits cerebral malaria and reduces parasite burden in mice," *Journal of Immunology*, vol. 178, no. 10, pp. 6416–6425, 2007.

[211] M. Krupka, K. Seydel, C. M. Feintuch et al., "Mild Plasmodium falciparum malaria following an episode of severe malaria is associated with induction of the interferon pathway in Malawian children," *Infection and Immunity*, vol. 80, no. 3, pp. 1150–1155, 2012.

[212] T. Taniguchi, K. Ogasawara, A. Takaoka, and N. Tanaka, "IRF family of transcription factors as regulators of host defense," *Annual Review of Immunology*, vol. 19, pp. 623–655, 2001.

[213] K. Ozato, P. Tailor, and T. Kubota, "The interferon regulatory factor family in host defense: mechanism of action," *Journal of Biological Chemistry*, vol. 282, no. 28, pp. 20065–20069, 2007.

[214] A. Kröger, M. Köster, K. Schroeder, H. Hauser, and P. P. Mueller, "Activities of IRF-1," *Journal of Interferon and Cytokine Research*, vol. 22, no. 1, pp. 5–14, 2002.

[215] K. Honda, A. Takaoka, and T. Taniguchi, "Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors," *Immunity*, vol. 25, no. 3, pp. 349–360, 2006.

[216] P. Rihet, Y. Traoré, L. Abel, C. Aucan, T. Traoré-Leroux, and F. Fumoux, "Malaria in humans: plasmodium falciparum blood infection levels are linked to chromosome 5q31-q33," *American Journal of Human Genetics*, vol. 63, no. 2, pp. 498–505, 1998.

[217] K. Honda, H. Yanai, A. Takaoka, and T. Taniguchi, "Regulation of the type I IFN induction: a current view," *International Immunology*, vol. 17, no. 11, pp. 1367–1378, 2005.

[218] Y. Liu, L. Zeng, A. T. Tian et al., "Endoplasmic reticulum stress regulates the innate immunity critical transcription factor IRF3," *Journal of Immunology*, vol. 189, no. 9, pp. 4630–4639, 2012.

[219] M. Yoneyama, W. Suhara, Y. Fukuhara, M. Fukuda, E. Nishida, and T. Fujita, "Direct triggering of the type I interferon system by virus infection: activation of a transcription factor complex containing IRF-3 and CBP/p300," *EMBO Journal*, vol. 17, no. 4, pp. 1087–1095, 1998.

[220] M. G. Wathelet, C. H. Lin, B. S. Parekh, L. V. Ronco, P. M. Howley, and T. Maniatis, "Virus infection induces the assembly of coordinately activated transcription factors on the IFN-β enhancer in vivo," *Molecular Cell*, vol. 1, no. 4, pp. 507–518, 1998.

[221] J. Yie, K. Senger, and D. Thanos, "Mechanism by which the IFN-β enhanceosome activates transcription," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 96, no. 23, pp. 13108–13113, 1999.

[222] M. Sato, N. Hata, M. Asagiri, T. Nakaya, T. Taniguchi, and N. Tanaka, "Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7," *FEBS Letters*, vol. 441, no. 1, pp. 106–110, 1998.

[223] D. L. Noah, M. A. Blum, and B. Sherry, "Interferon regulatory factor 3 is required for viral induction of beta interferon in primary cardiac myocyte cultures," *Journal of Virology*, vol. 73, no. 12, pp. 10208–10213, 1999.

[224] C. F. Basler, A. Mikulasova, L. Martinez-Sobrido et al., "The Ebola virus VP35 protein inhibits activation of interferon regulatory factor 3," *Journal of Virology*, vol. 77, no. 14, pp. 7945–7956, 2003.

[225] J. Ye and T. Maniatis, "Negative regulation of interferon-β gene expression during acute and persistent virus infections," *PLoS ONE*, vol. 6, no. 6, Article ID e20681, 2011.

[226] M. Sagong and C. Lee, "Porcine reproductive and respiratory syndrome virus nucleocapsid protein modulates interferon-β production by inhibiting IRF3 activation in immortalized porcine alveolar macrophages," *Archives of Virology*, vol. 156, no. 12, pp. 2187–2195, 2011.

[227] T. Tamura and K. Ozato, "ICSBP/IRF-8: its regulatory roles in the development of myeloid cells," *Journal of Interferon and Cytokine Research*, vol. 22, no. 1, pp. 145–152, 2002.

[228] M. Horiuchi, A. Itoh, D. Pleasure, K. Ozato, and T. Itoh, "Cooperative contributions of interferon regulatory factor 1 (IRF1) and IRF8 to interferon-γ-mediated cytotoxic effects on oligodendroglial progenitor cells," *Journal of Neuroinflammation*, vol. 8, article 8, pp. 1742–2094, 2011.

[229] P. H. Driggers, D. L. Ennist, S. L. Gleason et al., "An interferon γ-regulated protein that binds the interferon- inducible enhancer element of major histocompatibility complex class I genes," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 10, pp. 3743–3747, 1990.

[230] J. Nitcheu, O. Bonduelle, C. Combadiere et al., "Perforin-dependent brain-infiltrating cytotoxic $CD8^+$ T lymphocytes mediate experimental cerebral malaria pathogenesis," *Journal of Immunology*, vol. 170, no. 4, pp. 2221–2228, 2003.

[231] G. L. Suidan, J. R. Mcdole, Y. Chen, I. Pirko, and A. J. Johnson, "Induction of blood brain barrier tight junction protein alterations by CD8 T cells," *PLoS ONE*, vol. 3, no. 8, Article ID e3037, 2008.

[232] M. Fauconnier, J. Palomo, M. L. Bourigault et al., "IL-12Rβ2 is essential for the development of experimental cerebral malaria," *The Journal of Immunology*, vol. 188, no. 4, pp. 1905–1914, 2012.

[233] B. J. Barnes, P. A. Moore, and P. M. Pitha, "Virus-specific activation of a novel Interferon regulatory factor, IRF-5, results in the induction of distinct interferon alpha genes," *The Journal of Biological Chemistry*, vol. 276, no. 26, pp. 23382–23390, 2001.

[234] B. J. Barnes, M. J. Kellum, A. E. Field, and P. M. Pitha, "Multiple regulatory domains of IRF-5 control activation, cellular localization, and induction of chemokines that mediate recruitment of T lymphocytes," *Molecular and Cellular Biology*, vol. 22, no. 16, pp. 5721–5740, 2002.

[235] X. Y. Fu, D. S. Kessler, S. A. Veals, D. E. Levy, and J. E. Darnell Jr., "ISGF3, the transcriptional activator induced by interferon α, consists of multiple interacting polypeptide chains," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 87, no. 21, pp. 8555–8559, 1990.

[236] S. A. Qureshi, M. Salditt-Georgieff, and J. E. Darnell Jr., "Tyrosine-phosphorylated Stat1 and Stat2 plus a 48-kDa protein all contact DNA in forming interferon-stimulated-gene factor 3," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 92, no. 9, pp. 3829–3833, 1995.

[237] C. J. Sutherland, A. Alloueche, J. Curtis et al., "Gambian children successfully treated with chloroquine can harbor and transmit *Plasmodium falciparum* gametocytes carrying resistance genes," *The American Journal of Tropical Medicine and Hygiene*, vol. 67, no. 6, pp. 578–585, 2002.

[238] K. Na-Bangchang, P. Muhamad, R. Ruaengweerayut, W. Chaijaroenkul, and J. Karbwang, "Identification of resistance of *Plasmodium falciparum* to artesunate-mefloquine combination in an area along the Thai-Myanmar border: integration of clinico-parasitological response, systemic drug exposure, and *in vitro* parasite sensitivity," *Malaria Journal*, vol. 12, no. 1, article 263, 2013.

[239] J. Hadden, "Recent thoughts on the immunotherapy of infectious diseases including HIV infection," in *Microbial Infections*, H. Friedman, T. Klein, and H. Yamaguchi, Eds., pp. 13–22, Springer, 1992.

[240] M. Jeffrey and M. Jacobson, "Immunotherapy for infectious diseases," in *Infectious Disease*, V. S. Georgiev, Ed., p. 331, Humana Press, New York, NY, USA, 2002.

[241] V. Kak, V. Sundareshan, J. Modi, and N. M. Khardori, "Immunotherapies in infectious diseases," *Medical Clinics of North America*, vol. 96, no. 3, pp. 455–474, 2012.

[242] M. Talpaz, H. M. Kantarjian, K. McCredie, J. M. Trujillo, M. J. Keating, and J. U. Gutterman, "Hematologic remission and cytogenetic improvement induced by recombinant human interferon alpha(A) in chronic myelogenous leukemia," *The New England Journal of Medicine*, vol. 314, no. 17, pp. 1065–1069, 1986.

[243] D. Goldstein and J. Laszlo, "The role of interferon in cancer therapy: a current perspective," *Ca: A Cancer Journal for Clinicians*, vol. 38, no. 5, pp. 258–277, 1988.

[244] J. M. Kirkwood, M. S. Ernstoff, C. A. Davis, M. Reiss, R. Ferraresi, and S. A. Rudnick, "Comparison of intramuscular and intravenous recombinant alpha-2 interferon in melanoma and other cancers," *Annals of Internal Medicine*, vol. 103, no. 1, pp. 32–36, 1985.

[245] L. Repetto, P. G. Giannessi, E. Campora et al., "Tamoxifen and interferon-beta for the treatment of metastatic breast cancer," *Breast Cancer Research and Treatment*, vol. 39, no. 2, pp. 235–238, 1996.

[246] G. H. Windbichler, H. Hausmaninger, W. Stummvoll et al., "Interferon-gamma in the first-line therapy of ovarian cancer: a randomized phase III trial," *British Journal of Cancer*, vol. 82, no. 6, pp. 1138–1144, 2000.

[247] M. L. Shiffman, "Pegylated interferons: what role will they play in the treatment of chronic hepatitis C?" *Current Gastroenterology Reports*, vol. 3, no. 1, pp. 30–37, 2001.

[248] G. Chander, M. S. Sulkowski, M. W. Jenckes et al., "Treatment of chronic hepatitis C: a systematic review," *Hepatology*, vol. 36, no. 5, supplement 1, pp. S135–S144, 2002.

[249] J. Yang, Y. G. Pu, Z. M. Zeng et al., "Interferon for the treatment of genital warts: a systematic review," *BMC Infectious Diseases*, vol. 9, no. 156, pp. 1471–2334, 2009.

[250] A. H. Achtman, S. Pilat, C. W. Law et al., "Effective adjunctive therapy by an innate defense regulatory peptide in a preclinical model of severe malaria," *Science Translational Medicine*, vol. 4, no. 135, Article ID 135ra64, 2012.

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Research Article

A Novel Chemically Modified Curcumin Reduces Severity of Experimental Periodontal Disease in Rats: Initial Observations

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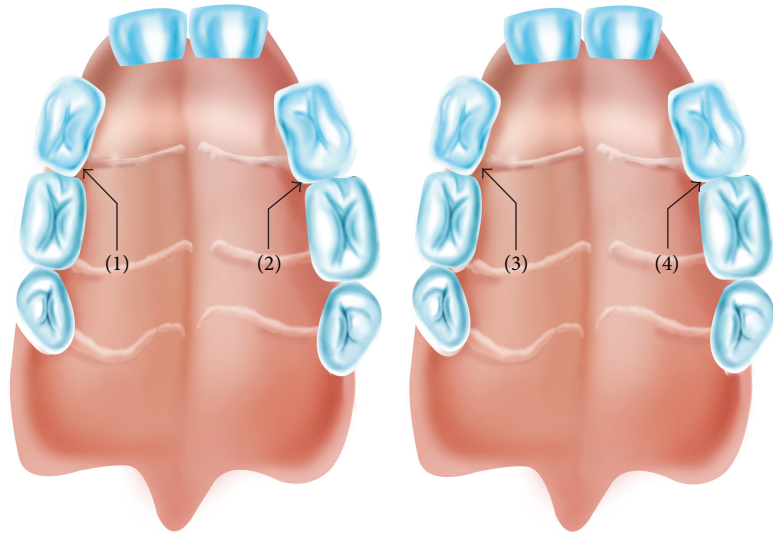
Tetracycline-based matrix metalloproteinase- (MMP-) inhibitors are currently approved for two inflammatory diseases, periodontitis and rosacea. The current study addresses the therapeutic potential of a novel pleiotropic MMP-inhibitor not based on an antibiotic. To induce experimental periodontitis, endotoxin (LPS) was repeatedly injected into the gingiva of rats on one side of the maxilla; the contralateral (control) side received saline injections. Two groups of rats were treated by daily oral intubation with a chemically modified curcumin, CMC 2.24, for two weeks; the control groups received vehicle alone. After sacrifice, gingiva, blood, and maxilla were collected, the jaws were defleshed, and periodontal (alveolar) bone loss was quantified morphometrically and by μ-CT scan. The gingivae were pooled per experimental group, extracted, and analyzed for MMPs (gelatin zymography; western blot) and for cytokines (e.g., IL-1β; ELISA); serum and plasma samples were analyzed for cytokines and MMP-8. The LPS-induced pathologically excessive bone loss was reduced to normal levels based on either morphometric ($P = 0.003$) or μ-CT ($P = 0.008$) analysis. A similar response was seen for MMPs and cytokines in the gingiva and blood. This initial study, on a novel triketonic zinc-binding CMC, indicates potential efficacy on inflammatory mediators and alveolar bone loss in experimental periodontitis and warrants future therapeutic and pharmacokinetic investigations.

1. Introduction

Over the past several decades, numerous studies have described pharmacologic strategies to utilize matrix metalloproteinase-inhibitors (MMP-Is) to prevent connective tissue breakdown associated with various inflammatory and other diseases, for example, periodontitis, arthritis, osteoporosis, cardiovascular disease, and cancer [1–4]. Recently, these have also included less obvious strategies such as (but not limited to) blocking MMP-mediated cleavage of insulin receptors in type-2 diabetics to improve insulin sensitivity [5] and to reduce HbA1c levels [6]. However, to date, the only orally (systemically) administered MMP-Is approved by the US-FDA and other national regulatory agencies (Europe and Canada) are those based on the surprising nonantimicrobial properties of the tetracycline antibiotics [4, 7–9]. In this regard, studies on experimental animals and on human subjects have demonstrated the efficacy of nonantimicrobial tetracycline formulations, as pleiotropic MMP-Is, in periodontal and other diseases [4, 7, 9, 10]. In addition to demonstrating that these medications, which include two formulations of subantimicrobial-dose doxycycline (both FDA-approved), can inhibit collagenolysis, connective tissue destruction, and bone resorption in the diseased periodontal tissues, other therapeutic mechanisms have also been identified. These include suppressed expression of inflammatory mediators such as the cytokines (e.g., IL-1β, TNF-α, and IL-6), prostaglandins, reactive oxygen species (e.g., HOCl), and nitric oxide, the latter reflecting the inhibition of inducible nitric oxide synthase [11, 12].

Given this background, a search has been underway for new drug molecules which exhibit a similar active site for MMP-inhibition as the tetracyclines "but with a different phenolic superstructure" [11]. With this strategy in mind, the



Group 1: PBS + vehicle
Group 2: LPS + vehicle
Group 3: PBS + CMC 2.24
Group 4: LPS + CMC 2.24

Figure 1: Diagrammatic representation of the four experimental groups using "split-mouth" protocol.

therapeutic potential of the tetracycline diketonic, metal-ion binding site [8, 9] has been expanded by the recent development of a new series of compounds with a similar zinc-binding moiety, which are bicyclic rather than tetracyclic, that is, the chemically modified curcumins or CMCs. The structures of these compounds, their potency and mechanisms of action as MMP-Is, and their zinc-binding (and other) characteristics have been described recently, and a "lead" compound has been identified [11, 13, 14]. This compound, CMC 2.24, is a phenylamino carbonyl curcumin, is triketonic (which enhances its zinc-binding characteristics) in contrast to the diketonic active site on both the tetracyclines and on traditional/natural curcumin compounds, and has shown evidence of efficacy in vitro, in cell and organ culture, and in animal models of chronic inflammatory and other diseases [13–15]. As additional background, recent studies have shown that natural/unmodified curcumin administered to rats with experimentally induced periodontal disease was effective in reducing inflammatory mediators and MMPs in the gingiva and periodontal ligament but was ineffective in reducing the excessive resorption and loss of alveolar bone [16]. Accordingly, the current report describes the first of a series of studies which examined the efficacy of CMC 2.24 as a pleiotropic MMP-I in several rat models of periodontitis with a particular focus on its ability to inhibit pathologic alveolar bone loss. Moreover, because of the long-standing interest in the link between the oral disease, periodontitis, and systemic inflammation (the latter associated with increased risk for various diseases, notably cardiovascular disease and more severe diabetes [4, 17]), the effects of treatment with this novel compound on biomarkers in the circulation were also examined.

2. Materials and Methods

2.1. Experimental Periodontal Disease Model. Eleven male Holtzman rats (*Rattus norvegicus albinus*) weighing 150–250 g were maintained under pathogen-free conditions with controlled temperature (21 ± 1°C) and humidity (65–70%) and a 12 h light-dark cycle. Food and water were provided *ad libitum* throughout the experiment. General anesthesia was induced by inhalation of an isoflurane/oxygen mixture. 30 μg of lipopolysaccharide (LPS) from *Escherichia coli* (strain 055:B5; Sigma Chem Co., St. Louis, MO, USA) diluted in phosphate buffered saline (PBS) was injected into the palatal gingiva (3 μL volume per injection) using a Hamilton microsyringe (Agilent, Santa Clara, CA, USA) as described by us previously [18]. These LPS injections were made into the palatal tissue between the upper 1st and 2nd molars, on the left side of the animal, three times a week for 14 days (a total of 6 injections and 180 μg of LPS in each site). The opposite side received injections of the same volume of PBS vehicle and served as the control site ("split-mouth" protocol; see Figure 1). At the end of the experimental period, the animals were sacrificed by CO_2 inhalation and samples were collected as described below. Also at the time of sacrifice, blood samples were collected and the serum and plasma were separated by standard procedure and analyzed for MMPs and cytokines as described below. The study protocol was previously approved by the Institution's Committees (Araraquara-UNESP, SP, Brazil, and Stony Brook University, NY, USA) for Experimental Animal Use.

2.2. Experimental Groups. The effects of CMC 2.24 (a phenylamino carbonyl curcumin) were assessed in a "prophylactic" model (the efficacy of this compound in a "therapeutic"

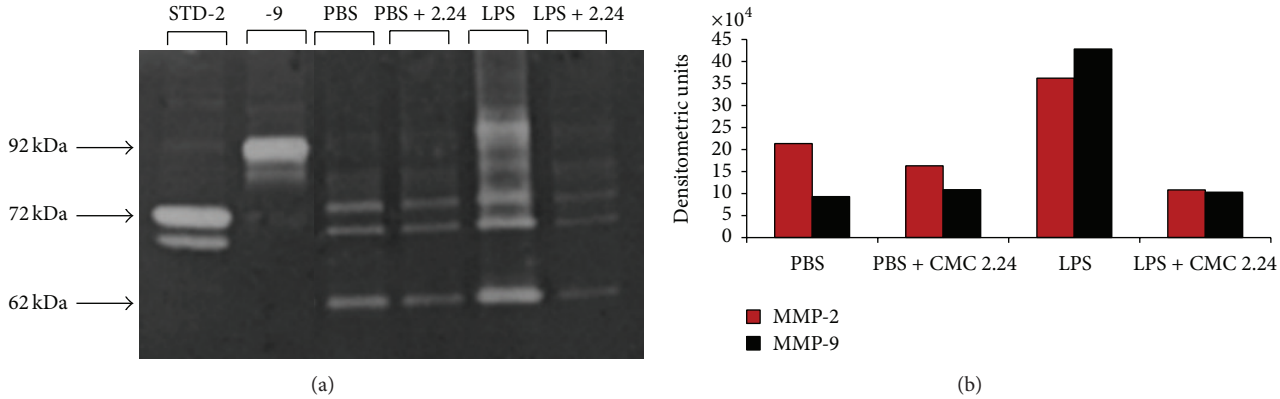


Figure 2: (a) Gelatin zymography of partially purified extract of gingiva from each experimental group showing the effect of orally administered CMC 2.24 on gingival MMPs (-2, -9). In groups 1 and 3, all rats received PBS injections into the gingiva plus oral administration of either vehicle alone (group 1) or CMC 2.24 (group 3). In groups 2 and 4, all rats received LPS injections into the gingiva plus oral administration of either vehicle alone (group 2) or CMC 2.24 (group 4). (b) Densitometric analysis of gingival MMPs (-2, -9).

model will be assessed in future studies) in which the induction of periodontal disease by LPS injections was carried out during the same period of time (14 days) as the daily oral administration of CMC 2.24 (30 mg/kg) or the vehicle-control. The test compound and the vehicle-control (a 1 mL suspension of 2% carboxymethyl cellulose) were both administered once per day over the 14-day protocol by oral intubation. The rats and their periodontal tissues were randomly distributed into the following experimental groups as illustrated in Figure 1.

Group 1—gingiva injected with PBS in rats systemically administered vehicle alone ($n = 5$); group 2—gingiva injected with *E. coli* LPS in the vehicle-treated rats ($n = 5$) (note: with this "split-mouth" design, group 1 and group 2 tissues involve the same 5 rats); group 3—gingiva injected with PBS in rats systemically administered the test medication (CMC 2.24; $n = 6$); and group 4—gingiva injected with *E. coli* LPS in rats systemically administered CMC 2.24 ($n = 6$) (as above, groups 3 and 4 involve the same 6 rats). However, for the μ-CT analysis, additional rats were added to each experimental group resulting in $n = 10$ rats per group.

2.3. Gingival Tissue Extract and Its Partial Purification. The gingival tissues from the hemimaxilla of each rat were excised and pooled per experimental group (5-6 rats per group) as described by us previously [19, 20]. The pooling of gingival tissues for each group was necessary because individual rats do not yield sufficient gingiva for enzyme analyses. The gingival tissues were extracted and the MMPs were partially purified as described by us previously [19, 20]. In brief, the samples were homogenized (all procedures at 4°C) with a glass grinder (Kontes, Glass Co., Vineland, NJ) attached to a T-Line Lab stirrer (Model 106 Taboys Engineering Corp., NJ) in 50 mM Tris-HCl buffer (pH 7.6) containing 5 M urea, 0.2 M NaCl, and 5 mM $CaCl_2$ and then extracted overnight and centrifuged at 15,000 rpm for 1 h. The supernatants were collected and dialyzed exhaustively against 50 mM Tris buffer (pH 7.8) containing 0.2 M NaCl and 5 mM $CaCl_2$. Ammonium sulfate was added to the dialysate to produce 60% saturation, allowed to stand overnight, and the precipitate containing the MMPs was collected by centrifugation at 15,000 rpm for 90 min. The pellets were then dissolved in the Tris buffer (pH 7.8) containing NaCl, $CaCl_2$, and 0.05% Brij and exhaustively dialyzed against the same buffer. Protein content of the extracts was determined by Bio-Rad Protein Assay.

2.4. Zymographic Assay of MMP-2 (Gelatinase A) and MMP-9 (Gelatinase B). The relative levels of the higher molecular weight proforms and the lower molecular weight activated forms of MMP-2 and MMP-9, in the pooled gingival extracts from each of the four experimental groups (Figure 2), were determined by zymography (the gelatin zymography system was purchased from Invitrogen Corp., Carlsbad, CA). In brief, all samples were run under nonreducing denaturing conditions on the gelatin zymography system containing polyacrylamide copolymerized with gelatin at a final concentration of 1 mg/mL. After electrophoresis, the gels were washed in 2.5% Triton X-100 and incubated at 37°C overnight in the assay buffer (40 mM Tris, 200 mM NaCl, and 10 mM $CaCl_2$; pH 7.5). After incubation, the gels were stained with SimplyBlue SafeStain (Invitrogen Corp., Carlsbad, CA). Clear zones of lysis against a blue background indicate gelatinolytic activity, as described by us previously [11, 21, 22]. Densitometric analysis of the gelatinolytic bands was carried out using the Scientific Imaging system (KODAK ID 3.5, Rochester, NY).

2.5. Alveolar Bone Loss Measurements. Since this is a major outcome in the experimental periodontal disease model and since reducing alveolar bone loss is a key therapeutic goal in treating human inflammatory periodontal disease, two methods were used to assess the effects of CMC 2.24 on this inflammatory-driven bone loss model.

2.5.1. Morphometric Analysis of Alveolar Bone Loss. As described previously [23], the soft tissues were carefully dissected to maintain the integrity of the maxillary bone

specimens. These were then completely defleshed by immersion in 8% sodium hypochlorite for 4 h followed by gentle mechanical scavenging of the remaining soft tissue. After washing in running water, the specimens were immediately dried with compressed air. To distinguish the cementum-enamel junction (CEJ), 1% aqueous methylene blue solution (Sigma-Aldrich, Saint Louis, MO, USA) was applied to the specimens for 1 min and then washed in running water. The specimens were fixed on 3 mm thick red dental wax with their palatal surface facing up. Standardized orientation was achieved by positioning the specimens with the palatal cusp tip of the first and second molars superimposed on the corresponding buccal cusp tips (i.e., occlusal plane perpendicular to the ground). To validate measurement conversions, a millimeter ruler was positioned on the wax and photographed with all specimens. The specimens were positioned under a stereomicroscope (Leica MZ6, Buffalo Grove, IL, USA) and digital images were obtained at 25x magnification using a 6.1-megapixel color digital camera coupled to the microscope.

A single examiner, who was not aware of the experimental group allocation of the specimens, carried out all morphometric measurements of alveolar bone loss by delineating the area of exposed root surface of the first and second molars using an image analysis software (Leica Application Suite, v3.8.0, Leica Microsystems, Buffalo Grove, IL, USA) and the results were converted to mm^2 using measurement of the reference millimeter grid. The area of exposed root surface in each specimen was averaged according to the experimental groups. Intraexaminer calibration was performed by evaluating repeated measurements of 10 nonstudy images presenting alveolar bone loss similar to the present study. The intraclass correlation showed a 96.8% reproducibility.

2.5.2. Microcomputerized Tomography (μ-CT). Upon sacrifice, the hemimaxillae of the rats were dissected including teeth and surrounding soft tissues, fixed for 18–24 h in 10% neutral buffered formalin at 4°C, washed in distilled water, and transferred to 70% ethanol. This procedure allowed us to use these same specimens for the histological assessments used in subsequent studies (Guimaraes et al., in preparation). These samples were scanned on a microcomputer tomograph (Skyscan 1176, SkyScan, Aartselaar, Belgium) using 18 μm slices. The digital radiographic images of each sample were reconstructed into a three-dimensional model (NRecon Software, SkyScan, Aartselaar, Belgium) consisting of a matrix of $18 \times 18 \times 18\,\mu$m and a standardized gray scale value to visualize only mineralized tissues. Using the software package Dataviewer\CTan\CTvol (Skyscan, Aartselaar, Belgium), the reconstructed tridimensional matrix of each sample was initially reoriented in a standardized manner on three planes: sagittal, coronal, and transversal. Subsequently, a cubic region of interest (ROI) of 9.72 mm^3 was defined using standardized dimensions and anatomical landmarks: cementum-enamel junction of the first molar as the coronal limit extending vertically 1.5 mm apically, an anteroposterior dimension of 3 mm from the distal aspect of the mesial root of the first molar, and the transversal (buccolingual thickness) dimension of 2.16 mm (120 slices of 18 μm each). This ROI included the first molar, half of the second molar, and also approximately 1 mm from the most palatal aspect of the first molar crown (including the palatal bone adjacent to the first and second molar teeth which was the site of LPS injections). We determined the relative volume of this ROI occupied by mineralized tissue in each sample. The data was averaged for each experimental group and compared by nonpaired t-tests using Welch's correction for unequal variances. Significance level was set to 95%.

2.6. Immunoblotting for Measurement of MMP-8 in Plasma and Gingival Extracts. MMP-8 levels in plasma and gingival extracts, the latter prepared as described above, were determined by western blot analysis. In brief, samples were reduced, boiled, subjected to SDS/PAGE, and transferred to polyvinylidene difluoride (PVDF) membrane (Amersham Pharmacia Biotech Inc., Piscataway, NJ). Blots were blocked with 5% nonfat dry milk for 2 h at room temperature. The membranes where then incubated with polyclonal antibodies specific for MMP-8 (Abcam PLC, Cambridge, MA) overnight at 4°C. Blots were washed and incubated with secondary antibodies purchased from Thermo Scientific for 2 h at room temperature. Detection of the bands was carried out on radiographic film by using SuperSignal West Dura Extended Duration Chemiluminescent substrate (Thermo Fisher Scientific Inc., Waltham, MA). The band densities were quantified by scanning on a laser densitometer [24]. To assess the levels of inactive (proform) and smaller molecular weight active forms of the MMP-8 (collagenase-2), bands corresponding to both molecular weight forms were quantitated, and the data is expressed as densitometric units and as the ratios of inactive/active forms. Recombinant rat MMP-8 (source: mouse myeloma cell line, NSO derived) from R&D Systems (Minneapolis, MN) was used as a standard for western blot analysis of the rat plasma samples. This MMP-8 standard was incubated for 4 hours at room temperature, in the presence or absence of 1 mM amino phenyl mercuric acetate (APMA), a known activator of higher molecular weight pro-MMPs into the lower molecular weight activated forms [20].

2.7. ELISA for Measurement of MMP-13 in Plasma and Gingival Extracts. The level of MMP-13 was measured in the gingival tissue extracts and plasma of each rat by Enzyme-Linked Immunosorbent Assay (ELISA). This assay was performed according to the manufacturer's instructions (TSZ Scientific LLC, Framingham, MA). Blood samples from animals in each experimental group were assayed in duplicate.

2.8. Measurement of Gingival Tissue and Serum Levels of Bone Resorptive Cytokines. The levels of 3 bone resorptive cytokines (IL-1β, IL-6, and TNF-α) were measured in serum and gingival tissue extracts by Enzyme-Linked Immunosorbent Assays (ELISAs). These assays were performed according to the manufacturer's instructions (R&D systems, Minneapolis, MN), and the results were normalized to the total concentration of protein in the samples. Blood samples from animals in each experimental group were assayed in duplicate.

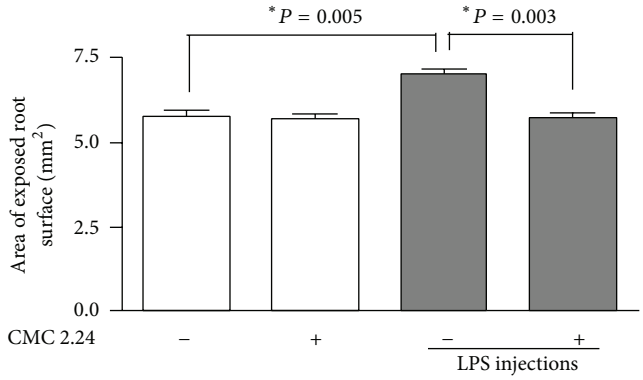


Figure 3: Direct measurements on defleshed hemimaxillae demonstrate that CMC 2.24 significantly inhibits alveolar bone resorption in the in vivo model of LPS-induced periodontal disease. The bar graph presents the results of the percent of exposed root surface, which is directly proportional to the extent of bone loss, according to the experimental group. LPS caused bone loss as indicated by the significant increase of the area of exposed root surface, whereas simultaneous systemic administration of CMC 2.24 significantly reduced this area, indicating an attenuation of inflammatory-driven bone resorption. Differences between experimental conditions are indicated by the brackets and ∗ (unpaired t-test for independent samples with Welch's correction for unequal variances).

3. Results

3.1. Local/Oral Measurements: Gingiva and Alveolar Bone. The levels of both MMP-2 (72 kDa progelatinase) and MMP-9 (92 kDa progelatinase) were assessed by gelatin zymography in pooled gingival tissue from half-jaws of rats from each experimental group (Figure 2). LPS-induced periodontal disease dramatically increased MMP-2 and MMP-9 levels in the pooled gingival tissue, while lower levels of the pro- (higher molecular weight) and activated (lower molecular weight) forms of these gelatinases were seen in the gingival tissue from all of the other experimental groups. Treatment of the rats with systemically administered CMC 2.24 appeared to "normalize" the pathologically excessive levels of the various molecular weight forms of these gelatinolytic MMPs in the LPS-injected gingiva assessed either visually (Figure 2(a)) or by densitometric analysis of the zymograms (Figure 2(b)). Some reduction of these MMP proteinases by CMC 2.24 administration was also seen in the gingiva from the rats without LPS injections (Figures 2(a) and 2(b)).

In a pattern reminiscent of the zymograms described above and based on morphometric analysis of alveolar bone height loss which measured the area of exposed root relative to the cementoenamel junction as a fixed anatomical landmark, LPS injections into the gingiva significantly (P = 0.005) increased alveolar bone loss (Figure 3). Moreover, when the LPS-injected rats were treated by oral administration of CMC 2.24, alveolar bone loss was significantly reduced (P = 0.003) back to the normal level seen in the rats not exposed to gingival LPS injections. Note that CMC 2.24 treatment did not affect alveolar bone loss in the control rats receiving injections of PBS vehicle rather than LPS (Figure 3).

To confirm and expand these data on alveolar bone loss in the four experimental groups (Figure 1), additional measurements using μ-CT were carried out. As shown in Figure 4, these data again demonstrate that LPS increased the loss of bone in the AOI and that CMC 2.24 administration reduced this bone loss to the level seen in the control rats in which the gingivae were injected with PBS instead of LPS.

Analysis of IL-1β in extracts of the pooled gingival tissues indicated that LPS injections markedly increased the level of this proinflammatory cytokine since it was not detectable in the extracts of the PBS-injected gingival tissue (Figure 5(a)). Moreover, CMC 2.24 administration reduced the pathologically excessive levels of IL-1β in the gingiva by 93% (Figure 5(a)). Similar concentrations of IL-6 were detected in the gingival tissues from the different groups of rats; however, the LPS injections did not appear to affect these levels and CMC 2.24 treatment only slightly reduced the levels of this cytokine by about 15% (data not shown). TNF-α was undetectable in both gingival extracts and serum (see below).

3.2. Systemic Measurements: Plasma and Serum. In the experimental protocol used in the current study (a "split-mouth" design), MMP-8 (neutrophil-type collagenase, collagenase-2) and MMP-13 (collagenase-3) were both detected in the plasma samples from the different groups of rats but neither was detected in the gingiva (see Section 4). Based on western blot analysis, the plasma samples from the LPS-injected rats (half-jaw only) which were treated by oral administration of CMC 2.24 appeared to exhibit reduced levels of activated, lower molecular weight forms of MMP-8 compared to the plasma from the LPS-treated rats administered with the vehicle alone (controls) (Figure 6(a)). Based on the densitometric analysis of these western blots (Figure 6(a)), the plasma of the CMC 2.24-treated rats with LPS-induced periodontitis exhibited a ratio of pro/active MMP-8 of 2.52 ± 0.20 (SEM) which was 89.5% higher than the ratio, 1.33 ± 0.05, seen in the plasma from the vehicle-treated LPS-periodontitis rats (Figure 6(b)), and this inhibition of activation of the precursor (latent) form of MMP-8 by the CMC2.24 treatment was statistically significant (P = 0.024). Note that a 4-hour incubation of the standard recombinant rat MMP-8 with 1 mM APMA, a known activator of pro-MMPs in vitro [20], converted the higher molecular weight pro-MMP-8 into the smaller molecular weight activated form of this leukocyte-type collagenase (see Figure 6(a)).

The plasma levels of MMP-13 assessed by ELISA were found to be about 1.1 μg/mL. Administration of CMC 2.24 to the LPS-periodontitis rats appeared to slightly reduce the levels of this collagenase in the plasma; however, this effect was not statistically significant (data not shown).

Regarding the proinflammatory cytokines in the serum (Figure 5(b)), because of the "split-mouth" design (see Figure 1), there were no serum samples from rats without gingival LPS injection. However, the levels of IL-1β in the serum of these LPS-exposed rats (about 30 pg/mL) were significantly (P = 0.03) reduced to undetectable levels by CMC 2.24 administration, a pattern similar to that seen in the gingival tissues (Figure 5(a)).

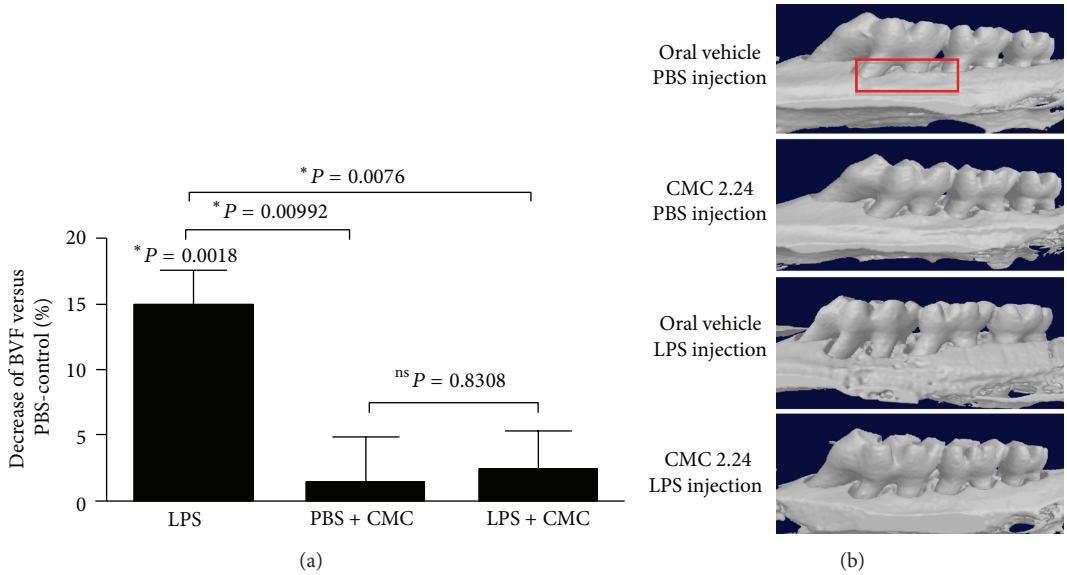


FIGURE 4: μ-CT data confirming that CMC 2.24 significantly inhibits alveolar bone resorption in the in vivo model of LPS-induced periodontal disease. Rats received either 2% carboxymethylcellulose vehicle or 30 mg/Kg of CMC 2.24 by oral intubation daily for 2 weeks. Contralateral LPS (3 μL, 30 μg) or PBS (3 μL) vehicle injections were performed 3 times/week for 14 days at the palatal aspect of first molars (see Figure 1). The bar graph presents the results of the μ-CT analysis as the change in the bone volume fraction (BVF) in the standardized ROI (bidimensionally shown as a red box in the representative image of the control) in comparison to vehicle-treated/PBS-injected samples (BVF in these samples was set to 100% since these were assumed to present no inflammatory bone resorption). Bars indicate average and standard deviations. *Significant difference in comparison to PBS-injected/vehicle-treated control. Differences between experimental conditions are indicated by the brackets and $*$ (unpaired t-test for independent samples with Welch's correction for unequal variances). Images in (b) show three-dimensional rendering of the mineralized tissues in representative samples.

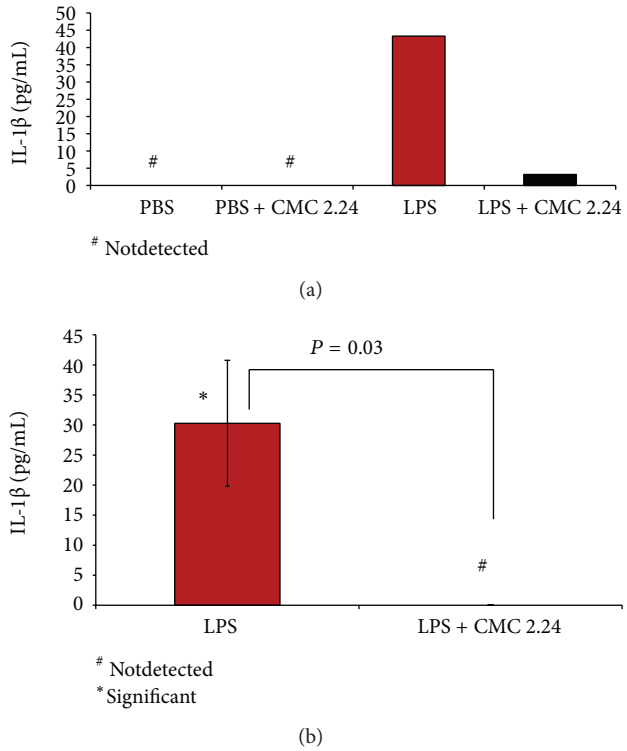


FIGURE 5: (a) The effect of CMC therapy on IL-1β in rat gingiva (top) and (b) serum (bottom) measured by ELISA.

IL-6 showed higher concentrations in the serum (about 95 pg/mL) than IL-1β in the LPS-periodontitis rats, and, again, CMC 2.24 appeared to reduce the level of this cytokine. However, this lesser effect (about 18% reduction) was not statistically significant (data not shown).

4. Discussion

This paper advances a novel therapeutic strategy which uses systemically administered medications as adjunctive therapy to modulate the host response in periodontal disease (periodontal therapy has traditionally only focused on locally suppressing the pathogenic microorganisms in the oral biofilm), with applications for other chronic inflammatory diseases as well (see below). The clinical application of this strategy began with the discovery that tetracyclines (TCs), unexpectedly, can inhibit host-derived MMPs, inflammatory mediators (e.g., the cytokine IL-1β), and collagen degradation including bone resorption; and by mechanisms not dependent on the antibacterial properties of these drugs [4, 7–10]. Soon thereafter, doxycycline was found to be a more potent MMP-inhibitor than other tetracycline antibiotics, including minocycline and tetracycline itself, and was subsequently developed and approved as a nonantibiotic low-dose formulation for long-term administration to patients with chronic periodontitis and the dermatologic inflammatory disease, rosacea [4, 9]. Based on these earlier and the current studies, the nontetracycline chemically modified curcumin

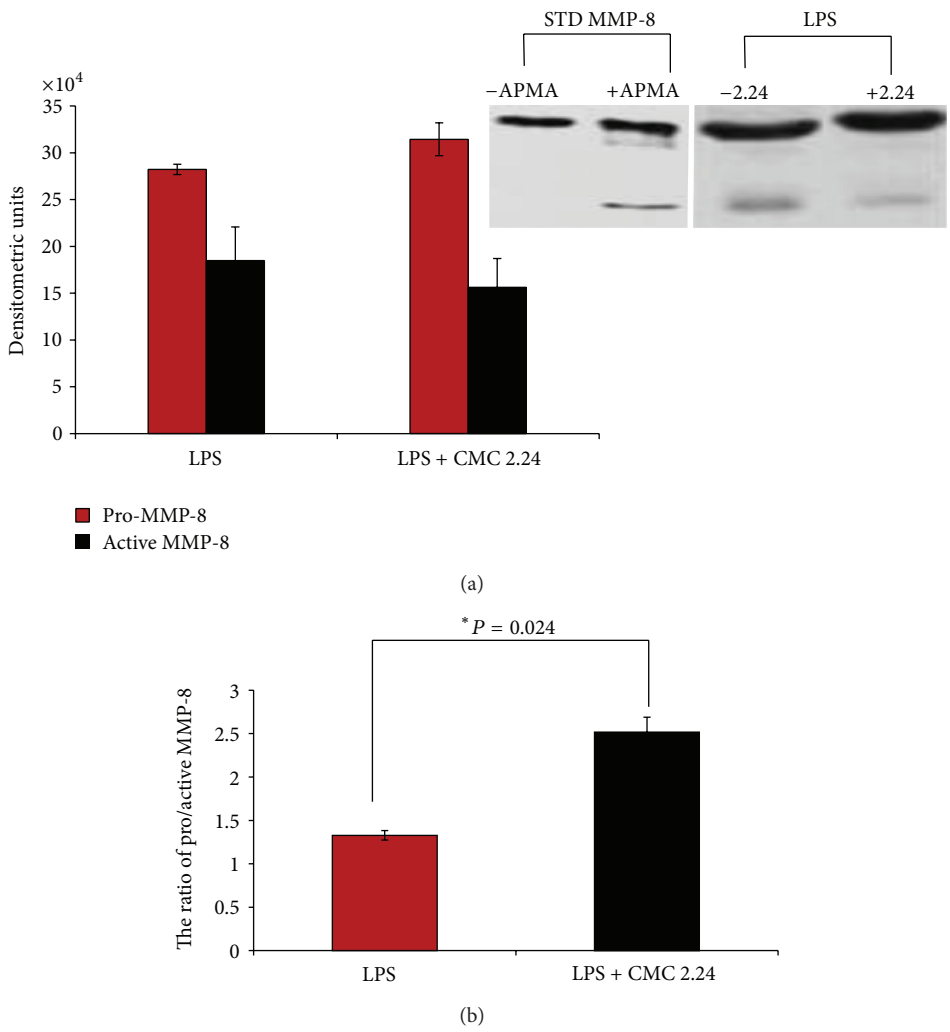


Figure 6: (a) Densitometric analysis of western blots of MMP-8 in plasma from untreated LPS- injected rats (LPS) and LPS-injected rats treated with CMC 2.24 (LPS + CMC 2.24). Each value represents the mean of MMP-8 ± the standard error of the mean (SEM); representative western blots of MMP-8 in plasma from untreated and CMC 2.24-treated rats are shown in the insert. (b) The ratio of pro/active MMP-8 calculated from densitometric analysis shown in (a) above.

(discussed below) appears to be as, or more, potent an MMP-inhibitor compound compared to doxycycline [4, 8, 9, 13]. As one example, the IC_{50} (the concentration of the compound required to inhibit 50% of MMP activity in vitro) of doxycycline has been reported to be approximately 15 μM [8, 9]. In contrast, recent studies by our group have demonstrated IC_{50} levels of CMC 2.24 at even lower μM levels (2–5 μM) when tested in vitro against MMPs such as MMP-8 (leukocyte-type collagenase), MMP-9 (leukocyte-type gelatinase), MMP-12 (macrophage metalloelastase), and MMP-14 (membrane-type MMP) [13]. However, a significant disadvantage of the approved subantimicrobial-dose formulations of doxycycline is that NO increase in the dose of this tetracycline can be prescribed to the patient (which might be desirable in order to, possibly, enhance the efficacy of this treatment in collagen-destructive diseases, e.g., periodontitis) because the low nonantibiotic blood levels of the drug (<1 μg/mL) produced by this formulation cannot be exceeded in order to prevent an important side-effect, namely, the emergence of tetracycline-resistant or pan-antibiotic-resistant bacteria [4]. In contrast, the potential strategy of long-term administration of CMC 2.24, for inflammatory diseases, would not be undermined by this strict, low-dose, limitation because this compound is not an antibiotic like the tetracyclines.

As described earlier (see Section 1), natural curcumin has a similar active site (i.e., the diketone zinc-binding moiety) as the tetracyclines and can also modulate the host response including MMP-inhibition and suppression of inflammatory mediators [25–31], although it is ineffective against alveolar bone loss (see below). However, the chemically modified curcumin, CMC 2.24, tested in the current in vivo study, has a modified active site which is triketonic as detailed by us in previous studies by Zhang et al. [13, 14] and does effectively inhibit bone loss.

Recently, newer host-modulating medications have also been investigated as adjunctive treatment for periodontal

disease and related medical disorders. These, in particular, have included (1) the resolvins such as the polyunsaturated fatty acids [32] which do not suppress the acute inflammatory response required by the host to combat infection, but which do prevent the tissue-destructive prolongation of this process, and (2) the subject of the current study, the chemically modified curcumins (CMCs). Of importance, the latter have shown improved solubility, zinc-binding, and biological effects in comparison with natural curcumin [13, 14]. Development of these CMCs is based on maintaining a similar active site for MMP-inhibition as that of the tetracyclines but with a different phenolic superstructure [11], which most recently resulted in the development of a new series of compounds with a triketonic zinc-binding moiety, which are still bicyclic rather than tetracyclic, that is, the chemically modified curcumins or CMCs. A series of these triketonic CMCs have been developed including CMC 2.5 (a methoxy carbonyl curcumin [11]) which, in turn, has been superseded by a more potent MMP-I compound, CMC 2.24, a phenylamino carbonyl curcumin; the latter has shown evidence of efficacy (and safety) in vitro, in cell and tissue culture, and in vivo models of several diseases including arthritis, diabetes, and cancer [13–15, 33].

The current study is the first to demonstrate efficacy of this compound, CMC 2.24, in an animal model of experimental periodontitis. Evidence of the onset and progression of this disease, induced by several injections of LPS into the gingiva of the rat, included dramatic increases in several forms (both pro- and activated) of connective tissue-destructive MMP-2 (72 kDa) and MMP-9 (92 kDa) gelatinases, elevated levels of the inflammatory cytokine often associated with periodontitis, IL-1β, and, most importantly in this model, a significant increase in alveolar bone loss, assessed morphometrically and by μ-CT, in the same jaws as the increase in gingival inflammatory mediators and MMPs (the impact of this local inflammatory disease and this experimental treatment on systemic levels of mediators is discussed below).

The potent efficacy of CMC 2.24 was demonstrated by (i) the statistically significant reduction of the LPS-induced, pathologically elevated alveolar bone loss down to the levels seen in the healthy controls and (ii) the essentially complete reduction of the pro- and activated, pathologically excessive levels of MMP-2, MMP-9, and IL-1β, in the inflamed gingival tissues back down to the un- (or barely) detectable levels seen in the control gingiva. As a result of the profound efficacy of this novel compound in this initial study, we now have a rationale to initiate studies using a modified animal model of experimental periodontal disease, which does not use "split-mouth" design, and in a periodontitis model in which the CMC 2.24 is administered therapeutically (after the disease has been established) rather than prophylactically as in the current study. In a more recent study in which alveolar bone loss was assessed at the cellular level histomorphometrically and histochemically, a similar pattern of change was seen, namely, that LPS injection increased osteoclast-mediated bone resorption and that CMC 2.24 inhibited this mechanism of alveolar bone loss (Guimaraes et al., in preparation). The potency of the biological effects of CMC 2.24 at an oral dose of 30 mg/kg is further demonstrated by the fact that, in previous experiments, we have not observed a significant decrease of inflammatory-driven bone resorption with 100 mg/kg dose of natural curcumin [16]. Interestingly, in recent experiments, we found that daily administration of 400 mg/kg of natural curcumin significantly reduced inflammatory-driven bone resorption in this model, but this dose of natural curcumin is more than 10-fold higher than the dose of CMC 2.24 administered in the current study (Guimaraes et al., in preparation).

Regarding insights into the mechanisms, plus the impact of this local disease and its treatment on the systemic condition of the host, we also observed the following: (i) the apparent reduction of IL-1β by CMC 2.24 treatment in the pooled gingival tissues was paralleled by a dramatic and significant reduction in this inflammatory mediator in the systemic circulation of the same animals, and (ii) for CMC 2.24 treatment, although it did not appear to alter the total levels of MMP-8 (neutrophil-type collagenase) in the blood samples of the LPS-injected rats, it did significantly reduce the ratio of the lower molecular weight, activated, collagen-destructive forms of this collagenase relative to the higher molecular weight, inactive, proforms of this MMP (note that, in the current experiment, MMP-8 could not be detected in the pooled gingival tissue). Mechanisms could include the ability of CMC 2.24 to inhibit other neutral proteinases such as plasmin, elastase, and MMP-1 which are known to cleave the amino-terminal propeptide domain of pro-MMP-8, converting it into the smaller molecular weight activated forms [9, 20]. Of relevance to the mechanisms involving CMCs ability to inhibit pro-MMP activation, recent studies (S. Simon et al., unpublished data) indicate that 2.24 can inhibit serine neutral proteinases (i.e., neutrophil elastase) which could explain the reduced conversion of pro- into smaller molecular weight activated MMPs which was observed in the current study in the systemic circulation. Still another possible mechanism involves the potential of this compound to inhibit the production of reactive oxygen metabolites (e.g., hypochlorous acid, HOCl). These are known to mediate proteinase activation by dissociating the thiol group in the propeptide domain [20]. This mechanism is significant because MMP-8 is largely derived from the degranulation of polymorphonuclear leukocytes, and, in the human periodontal pocket, MMP-8 constitutes about 80–90% of the total collagenase in this lesion; MMP-13 is the second most dominant collagenase in the periodontal pocket in humans, contributing about 10–20% of the total, and is thought to be derived from the junctional epithelium and bone cells [7, 34]. However, in the rat, MMP-13 is analogous to the constitutive collagenase, MMP-1, in humans and likely plays a role in physiologic turnover of collagen rather than the pathological degradation of collagen during periodontitis. In this regard, MMP-13 also could not be detected in the inflamed gingival tissues in the rats in the current study and, although it was detected in the plasma, was not reduced by CMC 2.24 treatment suggesting a preferential effect of the test compound on pathologically elevated rather than on constitutive levels of these MMPs. Additional mechanisms include the ability of natural curcumins to inhibit various signaling pathways and transcription factors involved in the

expression of inflammatory mediators (AP-1, MAPK, NF-kB, and STAT3) resulting in a decrease in the expression of the inactive proforms of the MMPs and of inflammatory cytokines and, ultimately, a marked change in the microenvironment [25, 35, 36].

5. Conclusions

The results of this initial study indicate that the oral administration of a novel, triketonic phenylamino carbonyl curcumin (CMC 2.24), to rats with endotoxin- (LPS-) induced periodontitis, is a significant and potent inhibitor of both pathologic alveolar bone loss and its inflammatory and collagen-destructive mediators. Moreover, this chemically modified curcumin appears to have additional benefits by reducing the impact of this local inflammatory disease on systemic biomarkers of the host without (apparently) negatively affecting the mediators of constitutive connective tissue turnover. Studies are now underway to expand these observations in additional rat models of experimental inflammatory periodontal disease with a particular focus on CMC 2.24 effects (i) on the cellular mechanisms of alveolar bone loss; (ii) in a model in which the test medication is administered therapeutically (i.e., after the disease has been established) rather than prophylactically; and (iii) on the pharmacokinetics (such as peak blood levels; serum half-life) of this novel compound.

Conflict of Interests

Lorne M. Golub is listed as an inventor on several related patents and these have been fully assigned to his institution, Stony Brook University. Francis Johnson is also listed as an inventor on several related patents which have been fully assigned to Stony Brook University and to Chem-Master Int. Inc. on a shared basis. He declares that he has no conflict of interests, financial or otherwise, with regard to the publication of this paper. All other authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] J. Hu, P. E. Van den Steen, Q. A. Sang, and G. Opdenakker, "Matrix metalloproteinase inhibitors as therapy for inflammatory and vascular diseases," *Nature Reviews Drug Discovery*, vol. 6, no. 6, pp. 480–498, 2007.

[2] T. Sorsa, L. Tjäderhane, Y. T. Konttinen et al., "Matrix metalloproteinases: contribution to pathogenesis, diagnosis and treatment of periodontal inflammation," *Annals of Medicine*, vol. 38, no. 5, pp. 306–321, 2006.

[3] C. M. Overall and C. López-Otín, "Strategies for MMP inhibition in cancer: innovations for the post-trial era," *Nature Reviews Cancer*, vol. 2, pp. 657–672, 2002.

[4] Y. Gu, C. Walker, M. E. Ryan, J. B. Payne, and L. M. Golub, "Non-antibacterial tetracycline formulations: clinical applications in dentistry and medicine," *Journal of Oral Microbiology*, vol. 4, Article ID 19227, 2012.

[5] K. Frankwich, C. Tibble, M. Torres-Gonzalez et al., "Proof of concept: matrix metalloproteinase inhibitor decreases inflammation and improves muscle insulin sensitivity in people with type 2 diabetes," *Journal of Inflammation*, vol. 9, article 35, 2012.

[6] S. P. Engebretson and J. Hey-Hadavi, "Sub-antimicrobial doxycycline for periodontitis reduces hemoglobin A1c in subjects with type 2 diabetes: a pilot study," *Pharmacological Research*, vol. 64, no. 6, pp. 624–629, 2011.

[7] L. M. Golub, H. M. Lee, R. A. Greenwald et al., "A matrix metalloproteinase inhibitor reduces bone-type collagen degradation fragments and specific collagenases in gingival crevicular fluid during adult periodontitis," *Inflammation Research*, vol. 46, no. 8, pp. 310–319, 1997.

[8] L. M. Golub, N. S. Ramamurthy, T. F. McNamara, R. A. Greenwald, and B. R. Rifkin, "Tetracyclines inhibit connective tissue breakdown: new therapeutic implications for an old family of drugs," *Critical Reviews in Oral Biology and Medicine*, vol. 2, no. 3, pp. 297–321, 1991.

[9] L. M. Golub, H. M. Lee, M. E. Ryan, W. V. Giannobile, J. Payne, and T. Sorsa, "Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms," *Advances in dental research*, vol. 12, no. 2, pp. 12–26, 1998.

[10] J. B. Payne and L. M. Golub, "Using tetracyclines to treat osteoporotic/osteopenic bone loss: from the basic science laboratory to the clinic," *Pharmacological Research*, vol. 63, no. 2, pp. 121–129, 2011.

[11] Y. Gu, H. M. Lee, N. Napolitano et al., "4-methoxycarbonyl curcumin: a unique inhibitor of both inflammatory mediators and periodontal inflammation," *Mediators of Inflammation*, vol. 2013, Article ID 329740, 10 pages, 2013.

[12] A. R. Amin, M. Attur, and S. B. Abramson, "Nitric oxide synthase and cyclooxygenases: distribution, regulation, and intervention in arthritis," *Current Opinion in Rheumatology*, vol. 11, no. 3, pp. 202–209, 1999.

[13] Y. Zhang, Y. Gu, H. M. Lee et al., "Design, synthesis and biological activity of new polyenolic inhibitors of matrix metalloproteinases: a focus on chemically-modified curcumins," *Current Medicinal Chemistry*, vol. 19, no. 25, pp. 4348–4358, 2012.

[14] Y. Zhang, L. M. Golub, F. Johnson, and A. Wishnia, "PKa, Zinc- and serum albumin-binding of curcumin and two novel biologically-active chemically-modified curcumins," *Current Medicinal Chemistry*, vol. 19, no. 25, pp. 4367–4375, 2012.

[15] G. I. Botchkina, E. S. Zuniga, R. H. Rowehl et al., "Prostate cancer stem cell-targeted efficacy of a new-generation taxoid , SBT-1214 and novel polyenolic zinc-binding curcuminoid, CMC2.24," *PLoS ONE*, vol. 8, no. 9, Article ID e69884, 2013.

[16] M. R. Guimarães, L. S. Coimbra, S. G. De Aquino, L. C. Spolidorio, K. L. Kirkwood, and C. Rossa, "Potent anti-inflammatory

effects of systemically administered curcumin modulate periodontal disease *in vivo*: curcumin inhibits periodontal disease *in vivo*," *Journal of Periodontal Research*, vol. 46, no. 2, pp. 269–279, 2011.

[17] J. B. Payne, L. M. Golub, J. A. Stoner et al., "The effect of subantimicrobial-dosedoxycycline periodontal therapy on serum biomarkers of systemic inflammation: A randomized, double-masked, placebo-controlled clinical trial," *Journal of the American Dental Association*, vol. 142, no. 3, pp. 262–273, 2011.

[18] S. Garcia de Aquino, F. R. Manzolli Leite, D. R. Stach-Machado, J. A. Francisco da Silva, L. C. Spolidorio, and C. Rossa Jr., "Signaling pathways associated with the expression of inflammatory mediators activated during the course of two models of experimental periodontitis," *Life Sciences*, vol. 84, no. 21-22, pp. 745–754, 2009.

[19] N. S. Ramamurthy and L. M. Golub, "Diabetes increases collagenase activity in extracts of rat gingiva and skin," *Journal of Periodontal Research*, vol. 18, no. 1, pp. 23–30, 1983.

[20] L. M. Golub, R. T. Evans, T. F. McNamara, H. M. Lee, and N. S. Ramamurthy, "A non-antimicrobial tetracycline inhibits gingival matrix metalloproteinases and bone loss in Porphyromonas gingivalis-induced periodontitis in rats," *Annals of the New York Academy of Sciences*, vol. 732, pp. 96–111, 1994.

[21] H. Lee, S. G. Ciancio, G. Tüter, M. E. Ryan, E. Komaroff, and L. M. Golub, "Subantimicrobial dose doxycycline efficacy as a matrix metalloproteinase inhibitor in chronic periodontitis patient is enhanced when combined with a non-steriodal anti-inflammatory drug," *Journal of Periodontology*, vol. 75, no. 3, pp. 453–463, 2004.

[22] D. L. Brown, K. K. Desai, B. A. Vakili, C. Nouneh, H. Lee, and L. M. Golub, "Clinical and biochemical results of the metalloproteinase inhibition with subantimicrobial doses of doxycycline to prevent acute coronary syndromes (MIDAS) pilot trial," *Arteriosclerosis, Thrombosis, and Vascular Biology*, vol. 24, no. 4, pp. 733–738, 2004.

[23] D. M. de Souza, F. A. de Prado, M. A. de Prado, R. F. da Rocha, and Y. R. de Carvalho, "Evaluation of two morphometric methods of bone loss percentages caused by periodontitis in rats in different locations," *Journal of Applied Oral Science*, vol. 18, no. 5, pp. 493–497, 2010.

[24] L. M. Golub, T. Sorsa, H. M. Lee et al., "Doxycycline inhibits neutrophil (PMN)-type matrix metalloproteinases in human adult periodontitis gingiva," *Journal of Clinical Periodontology*, vol. 22, no. 2, pp. 100–109, 1995.

[25] A. C. Bharti, Y. Takada, and B. B. Aggarwal, "Curcumin (diferuloylmethane) inhibits receptor activator of NF- κB ligand-induced NF- κB activation in osteoclast precursors and suppresses osteoclastogenesis," *The Journal of Immunology*, vol. 172, no. 10, pp. 5940–5947, 2004.

[26] G. Grynkiewicz and P. Ślifirski, "Curcumin and curcuminoids in quest for medicinal status," *Acta Biochimica Polonica*, vol. 59, no. 2, pp. 201–212, 2012.

[27] M. Woo, S. Jung, S. Kim et al., "Curcumin suppresses phorbol ester-induced matrix metalloproteinase-9 expression by inhibiting the PKC to MAPK signaling pathways in human astroglioma cells," *Biochemical and Biophysical Research Communications*, vol. 335, no. 4, pp. 1017–1025, 2005.

[28] A. Banerji, J. Chakrabarti, A. Mitra, and A. Chatterjee, "Effect of curcumin on gelatinase a (MMP-2) activity in B16F10 melanoma cells," *Cancer Letters*, vol. 211, no. 2, pp. 235–242, 2004.

[29] M. Shakibaei, T. John, G. Schulze-Tanzil, I. Lehmann, and A. Mobasheri, "Suppression of NF-κB activation by curcumin leads to inhibition of expression of cyclo-oxygenase-2 and matrix metalloproteinase-9 in human articular chondrocytes: implications for the treatment of osteoarthritis," *Biochemical Pharmacology*, vol. 73, no. 9, pp. 1434–1445, 2007.

[30] G. Kaur, N. Tirkey, S. Bharrhan, V. Chanana, P. Rishi, and K. Chopra, "Inhibition of oxidative stress and cytokine activity by curcumin in amelioration of endotoxin-induced experimental hepatoxicity in rodents," *Clinical and Experimental Immunology*, vol. 145, no. 2, pp. 313–321, 2006.

[31] A. N. Begum, M. R. Jones, G. P. Lim et al., "Curcumin structure-function, bioavailability, and efficacy in models of neuroinflammation and Alzheimer's disease," *Journal of Pharmacology and Experimental Therapeutics*, vol. 326, no. 1, pp. 196–208, 2008.

[32] C. N. Serhan, N. Chiang, and T. E. Van Dyke, "Resolving inflammation: dual anti-inflammatory and pro-resolution lipid mediators," *Nature Reviews Immunology*, vol. 8, no. 5, pp. 349–361, 2008.

[33] E. Katzap, M. J. Goldstein, N. V. Shah et al., "The chondroprotective properties of curcumin (*Curcuma longa*) and curcumin derived polyenolic zinc binding inhibitors against IL-1β and OsM -induced chrondrolysis," *Transactions of the Orthopedic Research Society*, vol. 36, 2011.

[34] L. M. Golub, H. M. Lee, J. A. Stoner et al., "Doxycycline effects on serum bone biomarkers in post-menopausal women," *Journal of Dental Research*, vol. 89, no. 6, pp. 644–649, 2010.

[35] M. R. Guimarães, S. G. de Aquino, L. S. Coimbra, L. C. Spolidorio, K. L. Kirkwood, and C. Rossa Jr., "Curcumin modulates the immune response associated with LPS-induced periodontal disease in rats," *Innate Immunity*, vol. 18, no. 1, pp. 155–163, 2012.

[36] J. A. C. de Souza, C. Rossa Junior, G. P. Garlet, A. V. B. Nogueira, and J. A. Cirelli, "Modulation of host cell signaling pathways as a therapeutic approach in periodontal disease," *Journal of Applied Oral Science*, vol. 20, no. 2, pp. 128–138, 2012.

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Research Article

Anti-Inflammatory Effects of IKK Inhibitor XII, Thymulin, and Fat-Soluble Antioxidants in LPS-Treated Mice

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The present study was designed to compare the anti-inflammatory effects of several agents applied *in vivo*, namely, a synthetic inhibitor of the NF-κB cascade, fat-soluble antioxidants, and the thymic peptide thymulin. Cytokine response in LPS-treated mice was analysed in tandem with the following parameters: the synthesis of inducible forms of the heat shock proteins HSP72 and HSP90α; activity of the NF-κB and SAPK/JNK signalling pathways; and TLR4 expression. Inflammation-bearing Balb/c male mice were pretreated with an inhibitor of IKK-α/β kinases (IKK Inhibitor XII); with thymulin; with dietary coenzyme Q_9, α-tocopherol, and β-carotene; or with combinations of the inhibitor and peptide or antioxidants. Comparable anti-inflammatory effects were observed in inflammation-bearing mice treated separately with thymulin or with dietary antioxidants administered daily for two weeks before LPS treatment. When LPS-injected mice were treated with the inhibitor and antioxidants together, neither plasma cytokines, signal proteins, nor heat shock proteins recovered more efficiently than when mice were treated with these agents separately. In contrast to antioxidant diet, the thymulin was shown to increase the effect of IKK Inhibitor XII in preventing IKK activation in LPS-treated mice.

1. Introduction

The nuclear factor kappa B (NF-κB) family consists of transcription factors activated in response to a variety of cellular stressors [1]. The transcription factor NF-κB is a key component of the cellular response to damage, stress, and inflammation [2, 3]. The NF-κB proteins consist of 5 subunits, which bind to DNA as a dimer; the most common of these subunits is the p65/p50 heterodimer. NF-κB activation via the canonical pathway is mediated by inhibitory kappa B (IκB) kinase (IKK), which is activated in response to a variety of pathogenic factors [4]. Activated IKK phosphorylates an inhibitory protein, IκB, which results in IκB degradation and thereby allows NF-κB to translocate to the nucleus and to induce the synthesis of proinflammatory molecules. Studies that have utilised a direct blockade of the enzymatic activities of IKK, JNK, or p38 MAPK have demonstrated the great potential for such treatments to elicit anti-inflammatory effects. Selective inhibitors for different signal pathways, including the NF-κB cascade, are therefore potentially useful in the treatment of inflammation. We have recently reported that synthetic inhibitors of Toll-like receptor 4 (TLR-4), stress-activated protein kinase JNK (SAPK/JNK), and NF-κB signalling (CLI-095, SP600125, and IKK Inhibitor XII, resp.) reduced the *in vitro* effect of LPS on macrophage-like RAW 264.7 cells. Among these three studied inhibitors, the suppressor of the NF-κB cascade, IKK Inhibitor XII, has been shown to be the most effective antitoxic agent *in vitro* [5].

The first aim of present study was to determine whether IKK Inhibitor XII is effective *in vivo* and how the effectiveness of this inhibitor is affected when used in combination with other anti-inflammatory agents. To address these knowledge gaps, we used a mouse model of acute septic inflammation induced by LPS from the Gram-negative bacteria *Escherichia coli*. Numerous studies have reported an increase in the production of reactive oxygen species during sepsis. It is well known that mutual cross-talk exists between reactive oxygen species and NF-κB signalling [6]. Since antioxidants

act not individually, but synergistically, a mixture of vitamin E, ubiquinone Q_9 , and β-carotene was added to the animal diet. In addition, in an attempt to gain more insight on the effects of each fat-soluble antioxidant, we studied coenzyme Q_9, α-tocopherol, and β-carotene in cellular model. Earlier we have shown that oxidative stress linked with acute inflammation can be prevented by the mixed dietary fat-soluble antioxidants coenzyme Q_9, α-tocopherol, and β-carotene [7]. The second aim of the present study was to test if these antioxidants can reinforce IKK Inhibitor XII activity.

Finally, the third aim of study was screening the anti-inflammatory activity of IKK Inhibitor XII in combination with the thymic peptide thymulin. The activity of this peptide has been reported not only in the thymus but also in the bloodstream, affecting extrathymic immune cells [8]. At present, the role of thymic peptides in the control of septic inflammation is unclear. It has been shown that thymic peptides can affect signalling pathways during both normal conditions and systemic inflammation [9]. Other authors believe that the mechanisms of the anti-inflammatory effects of thymic peptides, including thymulin, may involve NF-κB and p38 signalling cascades, which play crucial roles in inflammation [10, 11]. Recently we have demonstrated that thymulin *in vitro* and *in vivo* affected the NF-κB cascade in tandem with the production of proinflammatory cytokines, nitric oxide, and heat shock proteins in immune cells [12, 13]. We hypothesised that the antioxidants and/or thymulin could act as adjuvants, strengthening the anti-inflammatory effect of IKK Inhibitor XII.

2. Materials and Methods

2.1. Protocol of Experiments. Two independent series of *in vivo* experiments were performed. The first series included seven groups, with each group consisting of 4 mice, and the results of the study are shown on Figures 1 and 2. The second series performed at another point of time, included five groups, with each group consisting of 4 mice, and the results of the study are shown on Figures 3 and 4.

2.2. Animals, Animal Inflammation Model, Diet, Thymulin, and Inhibition of the NF-κB Cascade. Male Balb/c 8- to 10-week-old (25–27 g) mice were maintained under standard laboratory conditions (20–21°C, 10–14 h light-dark cycle, and 65% humidity) with food and water available *ad libitum*. The standard food pellets contained a balanced diet with proteins, vitamins, and minerals. The procedures followed were approved by the ethics committee of the institution and were in accordance with the Guidelines for Ethical Conduct in the Care and Use of Animals. A diet enriched with β-carotene (2 mg/kg body weight), α-tocopherol (2 mg/kg body weight), and ubiquinone Q_9 (8 mg/kg body weight) was administered daily for 15 days prior to LPS treatment. All antioxidants were purchased from Sigma, USA. The antioxidant formula used took into account the approximate levels of these antioxidants in the animals' tissues [14–16], such that each compound did not exceed its physiological level in mice. The antioxidant mixture was stirred into 2 g of pellets and was given daily before feeding at 8 am; antioxidants were fed to each mouse individually. Before morning feeding, animals in the other groups were fed pellets without antioxidants.

Inflammation was induced by a single intraperitoneal injection of lipopolysaccharide (LPS) from *Escherichia coli* (Serotype 026.B6, "Sigma," USA) (2.5 mg per kg body weight). Thymulin solution (1.5 mg/kg) was injected intraperitoneally 1.5 hr before LPS treatment and was prepared from serum thymic factor (American Peptides, USA), to which an equimolar concentration of $ZnCl_2$ was added [17]. IKK Inhibitor XII, at concentrations ranging from 5 to 20 mg/kg was injected intraperitoneally 1 h prior to LPS treatment. Mice were decapitated 6 h after LPS injection in parallel with the corresponding control groups. All measurements were carried out individually for each mouse, with nine replicates.

2.3. Blood Plasma and Cells. Plasma was isolated from blood collected during the decapitation of animals. The blood samples were kept for 3–5 h at 4°C and centrifuged at 200 g; supernatants were then collected for cytokine assays. Lymphocytes from the spleen were isolated in 199 medium (Sigma, USA) containing 1% 1 M HEPES solution, 100 μg/mL streptomycin, and 10% fetal bovine serum. Erythrocytes were lysed in Tris-buffered ammonium chloride (0.01 M Tris-HCl with 0.15 M NaCl—0.83% NH_4Cl, 9 : 1). After washing, the samples were stored at a concentration of 1×10^8 cells/mL in RPMI 1640 medium at −20°C until Western blotting was performed.

2.4. ELISA. ELISA was used to determine the concentration of cytokines in blood plasma. ELISA Development Kits for mouse TNF-α, IL-1α, IL-6, IL-17, IL-10, and IFN-γ (Peprotech, USA) were used. To visualise binding, 100 μL of ABTS green dye (Sigma, USA) dissolved in 0.05 M citrate buffer (pH 4.0) with 0.01% hydrogen peroxide was added. The optical density was measured at 405 nm with a plate spectrophotometer (Multiscan EX, Thermo Electron Corporation).

2.5. Western Blot Analysis. To prepare specimens, 1×10^8 splenic cells were lysed using an ultrasonic disintegrator with constant stirring for 2 min. The total protein concentration of each sample was then determined by the Bradford method. The proteins in each sample were then precipitated in acetone, solubilised, boiled for 5 min, and stored at −70°C. Proteins were resolved by electrophoresis over a 10% PAGE gel and then transferred from the gel onto a nitrocellulose membrane (GE Healthcare, Amersham, UK) in a transblot chamber. After blocking, the membrane was exposed for 2 hr to antibodies against the following mouse proteins: HSP70 antibody (rabbit anti-mouse HSP 72, clone SPA-812, inducible form, StressGen), HSP90 antibody (rabbit anti-Hsp90α [Hsp86], StressGen), phospho-NF-κB antibody (phospho-NF-κB p65 [Ser 536], #3031, Cell Signaling Technology, Danvers, MA), rabbit phospho- IKKα/β antibody II (Ser 176/180 (Cell Signaling Technology, USA), rabbit phospho-SAPK/JNK antibody to synthetic phospho-peptide SAPK/JNK, or rabbit TLR4 antibody (#2246, Cell Signaling Technology, USA). After washing, the nitrocellulose membranes were incubated

for 1 hr with the anti-rabbit biotinylated antibody (Jackson ImmunoResearch, West Grove, PA), and peroxidase-conjugated streptavidin was added for 1 hr. The loading control was a rabbit monoclonal antibody against a synthetic peptide near the carboxy terminus of human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling). An ECL-plus chemiluminescent cocktail (Amersham/GE) was then used to develop the blots according to the manufacturer's instructions, and the blot was then exposed to film. Quantitative evaluation of protein bands was then performed using the Qapa computer program (Pushchino, Russia).

2.6. In Vitro Addition of Antioxidants. Fifty μM ubiquinone, 10 μM α-tocopherol, or 10 μM β-carotene were added to the cultivating medium as water-alcohol emulsions (for Q_9 and α-tocopherol) or a water-Tween 85 emulsion (for β-carotene). Samples with medium supplemented only with the equivalent amount of alcohol or Tween 85 served as controls. The cells were cultivated for 24 h at 37°C in a humidified atmosphere containing 5% CO_2. In all cases, the final alcohol and Tween 85 concentrations did not exceed 1% in control or experimental samples.

2.7. Blood Glucose Measurements. Blood glucose levels were assayed in all groups of mice prior to manipulations and at 1, 2, and 6 h after LPS treatment. During glucose measurement, the tail tip (2-3 mm) was excised and massaged to harvest a small volume of blood (1.0–10 μL) which was placed into the hole of a blood glucose test strip (Accu-Chek Performa, Germany). Glucose measurements were obtained using a rapid blood glucose meter (Accu-Chek Performa, Germany).

2.8. Statistical Analysis. Statistical analysis was performed using Statistica/Win 6.0 software (Tulsa, OK). One-way analysis of variance (ANOVA) followed by a post hoc Tukey test was used to determine the significance of differences among groups, with P values ≤ 0.05 considered significant. All values were expressed as means (±SE).

3. Results

Initial experiments were performed to reveal the abilities of each of used antioxidants (β-carotene, α-tocopherol, and ubiquinone Q_9) to eliminate reactive oxygen species in murine peritoneal macrophages. It is well known that oxidative stress linked with LPS treatment results in proinflammatory cytokine response, including extremely TNF-α production [18]. So, we tested the protective effect of each of antioxidants by measuring the production of TNF-α level in LPS-treated macrophages as shown in Table 1.

The series of observations strongly indicated that separately the addition of the ubiquinone, α-tocopherol, and β-carotene to the culture medium *in vitro* significantly decreased the TNF production in LPS-treated macrophages. In addition, effect of three mixed compounds was rather more appreciable, indicating synergistic interaction between fat-soluble antioxidants.

In each experimental group, blood glucose levels decreased to 70%–80% compared to controls one hour after LPS treatment, but rapidly recovered to the control level, being unaltered at 2 and 6 h. In addition, no substantial differences in blood glucose levels were found between experimental groups (data not shown). We can assume a glucose-independent mechanism of anti-inflammatory effects of agents studied.

3.1. The Effects of Dietary Antioxidants and IKK Inhibitor XII on LPS-Treated Mice Immunity

3.1.1. Plasma Cytokines in LPS-Treated Mice Presubjected to Dietary Antioxidants and IKK Inhibitor XII. To examine the amounts of proinflammatory molecules associated with acute inflammation and the protective effect of the inhibitor and diet, the plasma cytokine concentrations were measured concurrently in seven mouse groups treated with IKK Inhibitor XII, dietary antioxidants, or a combination thereof. Treatment with LPS alone resulted in an expected increase in plasma proinflammatory cytokines (e.g., IL-1α, IFN-γ, and TNF-α). There was more than 2-fold increase in plasma TNF-α levels and a more than 3-fold increase in IFN-γ levels (Figure 1).

Additionally, levels of the anti-inflammatory cytokine IL-10 increased by approximately 1.5-fold. This finding agrees with previously reported data that demonstrated that LPS *in vivo* increased IL-10 production in peritoneal mouse macrophages [7].

In mice pretreated with different concentrations of IKK Inhibitor XII, plasma cytokine values were significantly decreased, and the maximal effect was observed at a concentration of 20 mg/kg. The pretreatment of mice with dietary antioxidants affected cellular responses to LPS; in cells from mice treated with antioxidant-rich diet, plasma cytokine values were reduced (Figure 1). It should be noted that the effects of antioxidants on the inflammatory response measured by cytokine production were less suppressive than the effects induced by the IKK inhibitor; the difference, however, was statistically significant for IL-1α, IL-10, and IFN-γ values. Additionally, when mice were simultaneously exposed to the inhibitor and antioxidants, nonadditive effects from the IKK inhibitor and dietary antioxidants were observed.

3.1.2. Signal and Stress Proteins in LPS-Treated Mice Presubjected to Dietary Antioxidants and IKK Inhibitor XII. To examine the extent of NF-κB activation associated with inflammation, the levels of the phosphorylated dimer p65/RelA and phosphorylated IKK-α/β were measured by the immunoblotting of splenic lymphocytes. There was a more than 2-fold increase in phosphorylated p65, which correlated with a significant (more than 7-fold) increase in the level of phosphorylated IKK in these cells (Figure 2). The pretreatment of mice separately either with the inhibitor or with dietary antioxidants significantly decreased the phosphorylation of both p65 and IκB, and the combination of the inhibitor and antioxidants did not result in an additive effect.

Table 1: Effect of *in vitro* added antioxidants on TNF-α production in LPS-treated peritoneal macrophages from mice.

Control 1	Control 2	LPS	LPS + Q_9	LPS + β-carotene	LPS + α-tocopherol	LPS + antioxidant's mixture
20.2 ± 2.5	23 ± 2.6	80.1 ± 9.8[a]	32.4 ± 4.0[b]	35.2 ± 4.1[ab]	29.5 ± 3.1[b]	18.1 ± 2.5[bc]

Macrophages were cultivated for 24 h; 50 μM of ubiquinone, 10 μM α-tocopherol, and 10 μM β-carotene were added in cultivating medium as water-alcohol emulsions (for Q_9 and α-tocopherol) or water-Tween 85 emulsion (for β-carotene). The samples with only medium supplemented with the equal amounts of alcohol or Tween 85 served as controls (control 1 and control 2, correspondingly); then 2.5 μg/mL lipopolysaccharide (LPS) from *Escherichia coli* was added to cells. TNF-α concentration shown in pg/mL was measured in the cell-free supernatants by ELISA kit. The final alcohol and Tween 85 concentrations did not exceed 1% in control or experimental samples. Each of values is the average mean ± S.D. from 12 duplicates.

[a]Significantly different from control, $P < 0.05$; [b]significantly different from LPS-treated cells, $P < 0.05$; [c]significantly different from LPS + Q_9, LPS + β-carotene, and LPS + α-tocopherol-treated cells, $P < 0.05$.

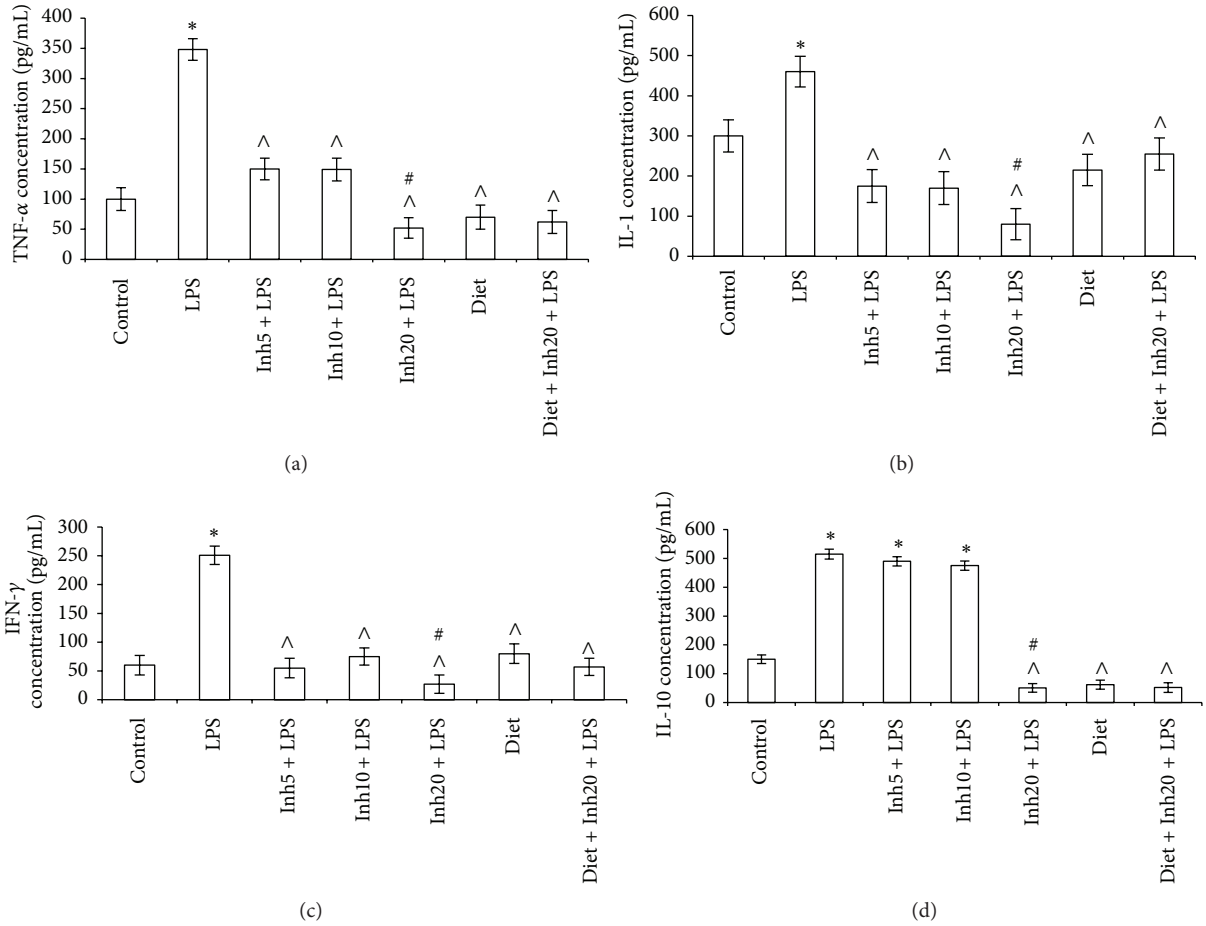

Figure 1: Plasma cytokine values in inflammation-bearing mice treated with antioxidant-rich diet and IKK Inhibitor XII. Seven mouse groups were used: inflammation-bearing (IB) mice; three groups of IB mice pretreated with inhibitor at concentration 5 mg/kg (Inh5), 10 mg/kg (Inh10), or 20 (Inh20) mg/kg; IB mice pretreated with antioxidant-rich diet; IB mice pretreated with antioxidant-rich diet plus inhibitor; and untreated controls. Each group consisted of 4 mice, which was examined individually. Each value is average mean ± SD from four independent experiments; the measurements were made for each individual mouse in six duplicates. Data are expressed in pg/mL of plasma. * Significantly different from control, $P < 0.05$. ^Significantly different from LPS-group, $P < 0.05$. # Significantly different from Inh(5) or Inh (10) plus LPS-group, $P < 0.05$.

To elucidate the role of an essential receptor for LPS and TLR4, we analysed cellular TLR4 levels using Western blot analysis. As shown in Figure 2, the addition of LPS induced an expected significant increase in TLR4 levels in splenic cells. However, in the cells of mice pretreated with IKK Inhibitor XII or with antioxidants, these increases in TLR4 expression were completely ablated, indicating that the TLR4 pathway can be interregulated via NF-κB signalling as well as by the redox balance.

Additionally, to assess the level of stress response in LPS-treated mice, the expression of two inducible heat shock proteins, Hsp72 and Hsp90-б, was studied in splenic lymphocytes. LPS induced a 4-fold rise in Hsp90 expression and a more than 8-fold increase in Hsp72 production (Figure 2).

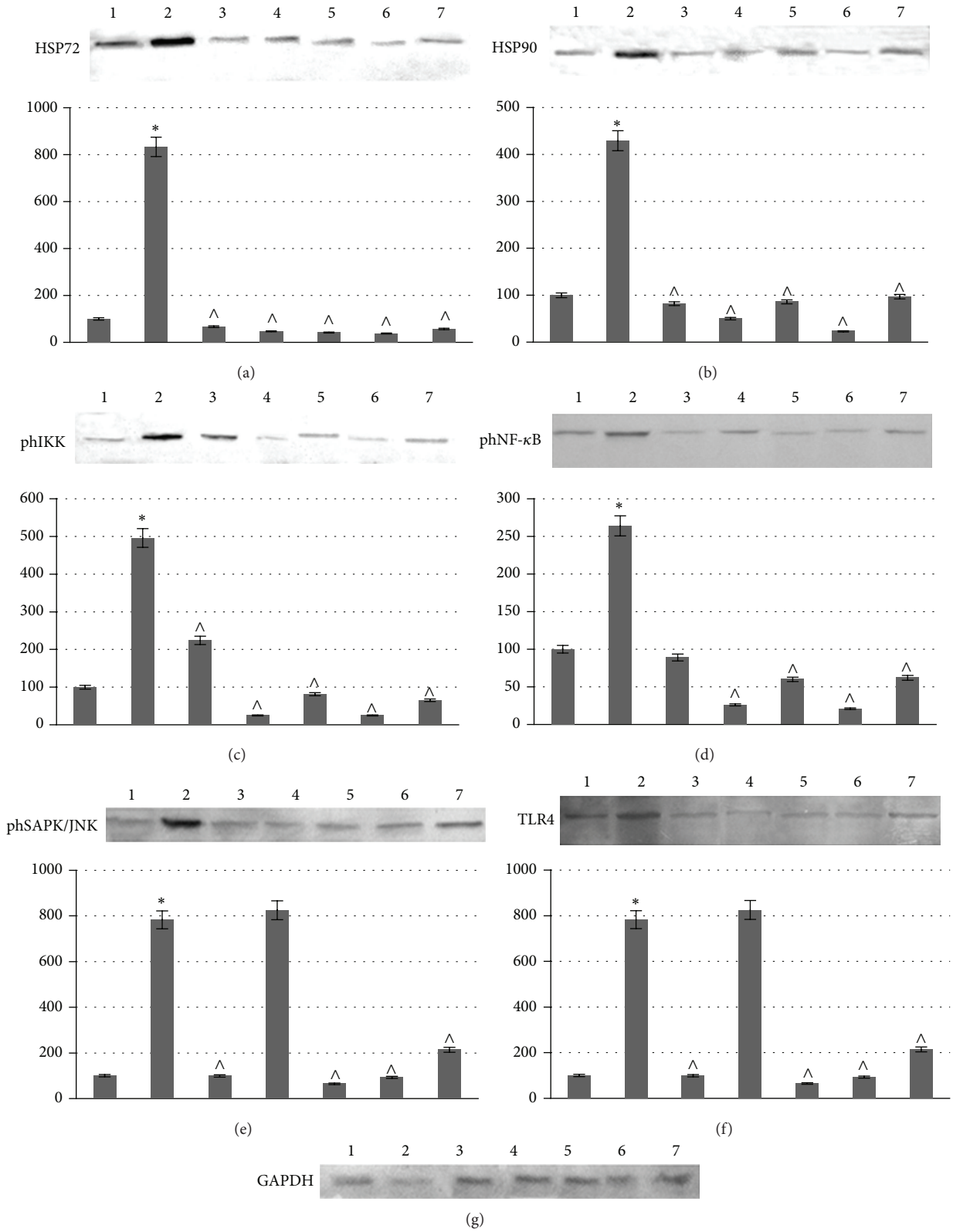

Figure 2: The effects of IKK Inhibitor XII and antioxidant-rich diet on phosphorylation of RelA, IKK, and SAPK/JNK and on the expression of TLR4 and heat shock proteins in the splenic lymphocytes from inflammation-bearing mice. The animal's groups that was indicated in Figure 1 were used (1, control; 2, IB mice; 3, IB + Inh5; 4, IB + Inh10; 5, IB + Inh20; 6, IB + diet; 7, IB + diet + Inh20). Western blot analysis of extracts from isolated mice lymphocytes was provided using corresponding antibodies or anti-GAPDH antibody (bottom). Blot pictures show a single representative experiment from four independent experiments. Histograms below protein bands show protein levels calculated as mean relative units correspondingly to internal control and are the results of blots densitometry by program QAPA from four independent experiments. *Significantly different from control, $P < 0.05$. ^Significantly different from LPS-group, $P < 0.05$.

Again, the pretreatment of mice with IKK Inhibitor XII or with antioxidants resulted in the complete ablation of Hsp72 and Hsp90-α spikes in cells after injection with LPS. Additionally, these two simultaneously applied treatments did not have a reciprocal interaction, as demonstrated by the measurement of both heat shock proteins, of cytokines, and of signalling proteins.

3.2. The Effects of Thymulin and IKK Inhibitor XII on LPS-Treated Mice Immunity

3.2.1. Plasma Cytokines in LPS-Treated Mice Subjected to Thymulin and IKK Inhibitor XII. To determine whether thymulin with or without IKK Inhibitor XII can downregulate the *in vivo* response to LPS, the values of plasma IL-6, IL-17, IFN-γ, and TNF-α were measured in LPS-injected mice using five mouse groups indicated in the legend to Figure 3. Note that other panel of cytokines was tested in these experiments based on our previous data on thymulin activity. Indeed, these cytokines are predisposed to thymulin control [19].

Based on the above results, the optimal concentration of 20 mg/kg inhibitor was used in this experimental series examining the combined effect of thymulin with IKK Inhibitor XII. These experiments confirmed that an increase in plasma TNF-α levels occurs after LPS treatment, supporting the results of the experiments displayed in Figure 1. The pretreatment with thymulin or with IKK Inhibitor XII decreased the accumulation of the TNF-α in plasma. The concentration of plasma TNF-α in mice pretreated with the inhibitor and thymulin together did not significantly differ from that measured in LPS-treated mice. There was a more than 3-fold increase in plasma IFN-γ in mice treated only with LPS; both IKK Inhibitor XII and thymulin prevented this increase. The combined effect of the inhibitor and thymulin in preventing LPS-mediated increases of plasma IFN-γ was no greater than the individual activity of each agent.

In these experiments, we detected a significant increase in the concentration of IL-6 in LPS-treated mice, which was partly but not significantly decreased by pretreating the mice with IKK Inhibitor XII or with thymulin, demonstrating that these treatments could not completely ablate plasma IL-6 levels. It is important to note that when these two agents were administered together, IL-6 levels were still not completely normalised. In addition, there was a more than 3-fold increase in plasma IL-17 from LPS-treated mice, but the inhibitor and thymulin, alone or in combination, could not prevent the increase in IL-17 (Figure 3).

3.2.2. Signal and Stress Proteins in LPS-Treated Mice Subjected to Thymulin and IKK Inhibitor XII. Similar to results from the first experimental series, the treatment of mice with LPS resulted in the increased phosphorylation of RelA and IKK (Figure 4).

Thymulin and particularly IKK Inhibitor XII reduced IKK phosphorylation. Interestingly, compared to separate treatments with peptide or with inhibitor, the combined treatment with thymulin and the inhibitor resulted in an apparent increase in the protective effect directed towards the prevention of excessive IKK phosphorylation. It has been previously shown that IKK Inhibitor XII *in vitro* affects the SAPK/JNK pathway in RAW 264.7 cells [5]. To examine the *in vivo* effects of the inhibitor and thymulin on the activity of SAPK/JNK signalling, the levels of phosphorylated SAPK/JNK were measured in lymphocytes from 5 animal groups. In contrast to linear RAW 264.7 cells, which generate two phosphorylated isoforms, JNK1 and JNK2, *ex vivo* cells expressed only a single form of phosphorylated SAPK/JNK. LPS induced an approximately 7-fold increase in SAPK/JNK phosphorylation, and in mice separately treated with thymulin or with IKK Inhibitor XII, the spike in phosphorylated SAPK/JNK significantly decreased. It should be noted that the combined treatment with thymulin and inhibitor resulted in a summation of their effects and the normalisation of SAPK/JNK phosphorylation.

There was also a reduction in cellular Hsp72 expression in cells from inflammation-bearing mice that were pretreated with thymulin or the inhibitor (Figure 4). Collectively, these data demonstrate that the pharmacological inhibition of IKK/NF-κB and SAPK/JNK activation leads to the attenuation of LPS-related inflammation.

4. Discussion

Over the last decade, an inhibitor of the NF-κB signalling pathway, IKK Inhibitor XII, has been discovered and investigated for its clinical potential in inflammatory diseases [18]. This inhibitor is a synthetic small hydrophobic molecule that can freely penetrate into the cell. IKK Inhibitor XII, a cell-permeable amino-diarylbenzamide compound, acts as a potential ATP site targeting inhibitor against IKK-1 and IKK-2. Interestingly, IKK Inhibitor XII, a selective inhibitor of NF-κB signalling that functions by decreasing NF-κB phosphorylation and thus reducing the probability of translocation of the NF-κB into the nucleus, also prevented SAPK/JNK activation in LPS-treated cells (Figure 2). These results coincide with findings demonstrating that SAPK/JNK signalling is regulated by the NF-κB pathway [20, 21]. Indeed, it is generally suggested that NF-κB activation plays a critical role in LPS-induced responses and that the suppression of NF-κB pathway activity prevents its ability to activate the transcription of a wide variety of genes encoding immunologically relevant proteins. It is also known that targeting IKK also may affect other pathways in addition to NF-κB. For example, IKK affects p53, FOXO3A, and HIF-1 in addition to NF-κB [22, 23].

We demonstrated that the pharmacological inhibition of the IKK/NF-κB pathway by IKK Inhibitor XII delays the proinflammatory response in LPS-treated mice. Thus, many processes are shown to be altered in mice undergoing inflammation, including increases in cytokine production, the stimulation of heat shock proteins and TLR4 expression, and the activation of NF-κB and SAPK/JNK signalling, were at least partially corrected *in vivo* by NF-κB inhibition. This finding provides additional experimental evidence that an increase of NF-κB activity plays a causal role in LPS-related inflammation.

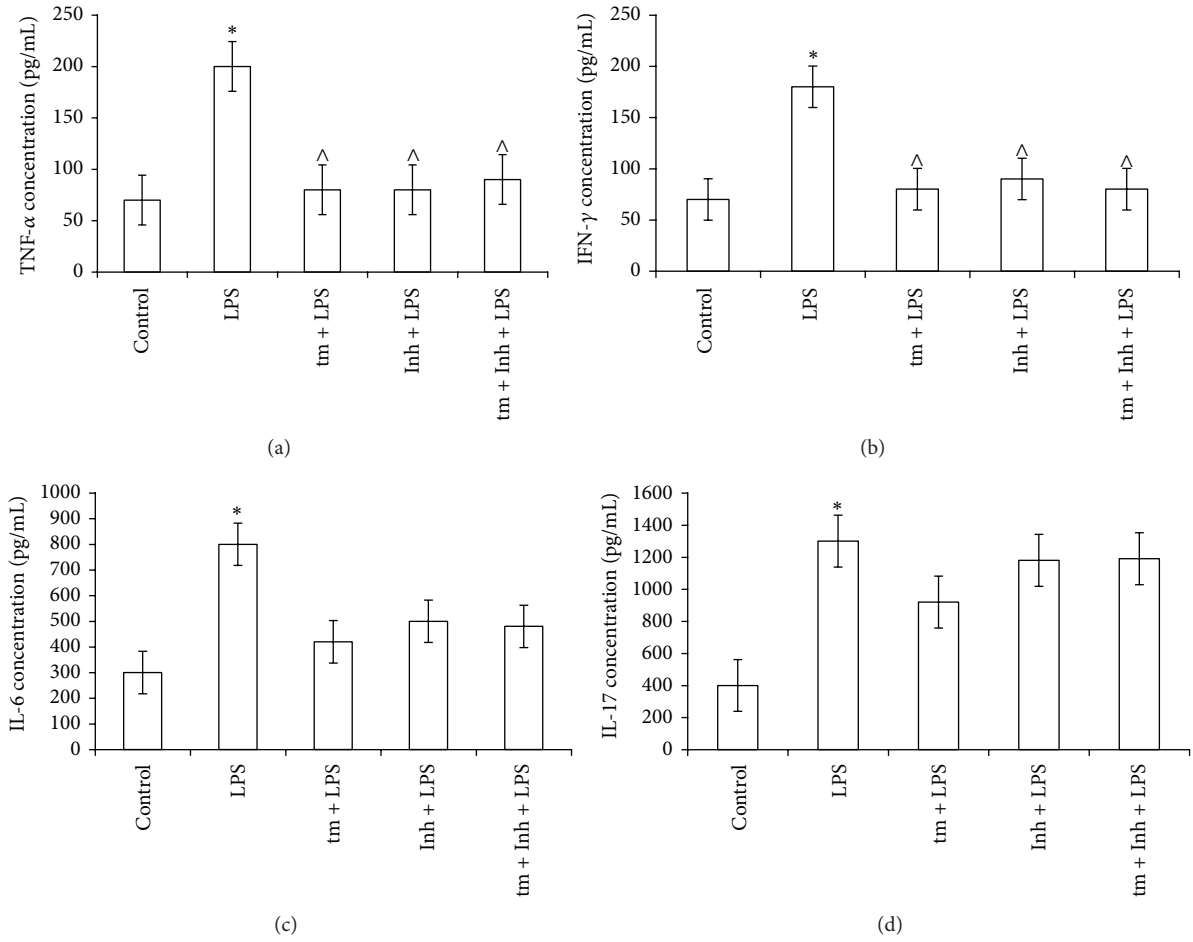

Figure 3: Plasma cytokine values in inflammation-bearing mice treated with thymulin and IKK Inhibitor XII. Five mouse groups were used: IB mice; IB mice pretreated with 20 mg/kg inhibitor; IB mice pretreated with thymulin; IB mice pretreated with thymulin plus inhibitor; and untreated controls. Each group consisted of 4 animals, which were examined individually. Each value is average mean ± SD from four mice; the measurements were made for each individual mouse in six duplicates. Data are expressed in pg/mL of plasma. *Significantly different from control, $P < 0.05$. ^Significantly different from LPS-group, $P < 0.05$.

Our data show that a diet supplemented with antioxidants prevents NF-κB activation in mice treated with LPS. It should be noted that several previous studies using similar animal inflammation models demonstrated that pretreatment with antioxidant N-acetylcysteine or N-acetylcysteine combined with α-tocopherol before endotoxin administration resulted in a decrease in NF-κB activation [24, 25], lowered TNF-α release, and increased survival [26]. In addition, the administration of N-acetylcysteine with vitamin E and β-carotene reduced lipid peroxidation and restored GSH levels in endotoxic rats [27]. Moreover, it was demonstrated that the genetic reduction of NF-κB reduced the amount of mitochondrial-derived ROS [28].

In the present study, we hypothesised that an excess of coenzyme Q in the diet, along with other fat-soluble vitamins, could be very useful due to the unique properties of ubiquinones as suppressors of cholesterol synthesis, thereby enhancing membrane fluidity. A significant decrease in cholesterol synthesis and a subsequent drop in the cholesterol concentration of the cellular membranes were observed in rats fed a diet supplemented with ubiquinone Q_9. Additionally, the inhibition of cholesterol synthesis in lymphocytes cultivated *in vitro* with coenzyme Q_9 was demonstrated [29, 30]. Interestingly, our data indicated that the preintervention with dietary antioxidants decreased the magnitude of the inflammatory response, but diet did not amplify the effect of IKK Inhibitor XII. These results demonstrating nonadditive anti-inflammatory effects of IKK Inhibitor XII and dietary antioxidants suggest antioxidant activity of the studied inhibitor. Thus, it can theoretically be assumed that there was a coincidental interaction between the protective mechanisms of the inhibitor and antioxidants, which may both be mediated via the reduction of reactive oxygen species. This assumption is consistent with recent data demonstrating that the inhibition of NF-κB activity reduces oxidative stress and damage *in vitro* and *in vivo* [28].

Thymic peptide thymulin, which is a metallopeptide consisting of a nonapeptide (Glu-Ala-Lys-Ser-Gln-Gly-Gly-Ser-Asp) that couples with zinc ions, mostly has inhibitory effects on immune responses [12, 31] and also stimulates endocrine

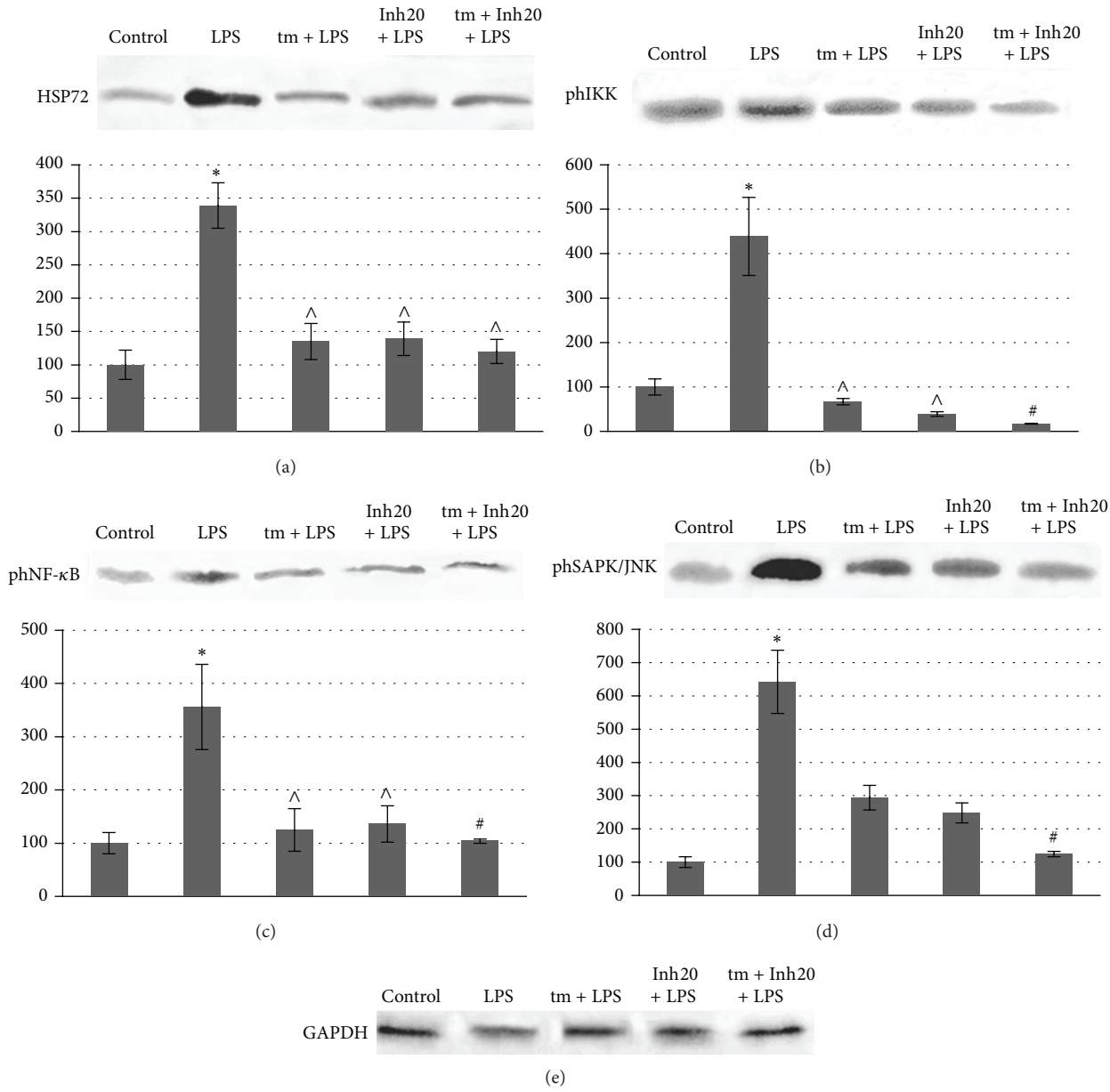

Figure 4: The effects of IKK Inhibitor XII and thymulin on phosphorylation of RelA, IKK, SAPK/JNK, and expression of Hsp72 in the splenocytes from inflammation-bearing mice. The animal's groups that indicated in Figure 3 were used. Western blot analysis of extracts from isolated mice lymphocytes was provided using corresponding antibodies or anti-GAPDH antibody as loading control (bottom). Blot pictures show a single representative experiment for four independent experiments. Histograms below protein bands show protein levels calculated as mean relative units correspondingly to internal control and are the results of blots densitometry by program QAPA from four independent experiments. *Significantly different from control, $P < 0.05$. ^Significantly different from LPS-group, $P < 0.05$. #Significantly different from (Inh+LPS)-group, $P < 0.05$.

systems, indicating its role in the recovery from inflammatory conditions [32]. Using thymulin as an immune-modulator in the second experimental line, we tested a repertoire of cytokines, including IL-6 and IL-17. Although IL-17 was identified more than 15 years ago as a product of $CD4^+$ T cells, it was only recently proven that IL-17 is preferentially produced by a subset of T cells, specifically Th17, which have been shown to be very important in the pathogenesis of autoimmune disease. Additionally, it has been shown recently that thymulin significantly reduces the disease severity in mice with acute experimental autoimmune encephalomyelitis [33]. IL-17 and IL-6 are important in many disorders characterised by immune self-recognition, and IL-6 is known to induce the differentiation of Th17 cells [19, 34], but the role of Th17 cells in septic inflammation is still unclear. We demonstrated that the separate administration of the inhibitor and thymulin or their combination did not significantly change the plasma IL-17 concentration. Other cytokines, namely,

TNF-alpha and IFN-gamma, were significantly decreased in mice that were pretreated with thymulin, IKK Inhibitor XII, or the combination thereof. These data support the assumption that Th17 cells do not play an active role in inflammatory pathogenesis induced by LPS. The plasma cytokine observations certainly do not provide a complete picture regarding the response to damage. It is possible that the release of cytokines is temporally differentiated, and the extent of release and utilisation seems to be nonsynchronous for different cytokines. Indeed, it was reported that after the intravenous administration of endotoxin in humans, plasma IL-6 increased slowly compared with plasma TNF-alpha [35, 36].

Alternately, monitoring short-lived signal and stress proteins in the cells enables a more complete evaluation of cellular responses. Indeed, inhibition of the IKK/NF-κB pathway using IKK Inhibitor XII delayed phosphorylation of both independent steps of NF-κB signalling, IKK, and p65. In addition, activation of the SAPK/JNK pathway also decreased in cells from LPS-plus-inhibitor-treated mice. The results of the present study demonstrate that thymulin enhances the activity of the IKK Inhibitor XII. Taken together, these results demonstrate that reducing NF-κB activity using both thymulin and IKK Inhibitor XII decreases the inflammatory response *in vivo*. The inhibition of NF-κB using thymulin plus NF-κB inhibitor offers what we believe to be a novel strategy for delaying or attenuating inflammatory diseases in patients with SIRS (systemic inflammatory response syndrome) or sepsis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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References

[1] S. Miyamoto, "Nuclear initiated NF-κB signaling: NEMO and ATM take center stage," *Cell Research*, vol. 21, no. 1, pp. 116–130, 2011.

[2] M. S. Hayden and S. Ghosh, "Shared principles in NF-κB signaling," *Cell*, vol. 132, no. 3, pp. 344–362, 2008.

[3] H. Oh and S. Ghosh, "NF-κB: roles and regulation in different $CD4^+$ T-cell subsets," *Immunological Reviews*, vol. 252, no. 1, pp. 41–51, 2013.

[4] M. S. Hayden, A. P. West, and S. Ghosh, "NF-κB and the immune response," *Oncogene*, vol. 25, no. 51, pp. 6758–6780, 2006.

[5] O. V. Glushkova, S. B. Parfenyuk, M. O. Khrenov et al., "Inhibitors of TLR-4, NF-κB, and SAPK/JNK signaling reduce the toxic effect of lipopolysaccharide on RAW 264.7 cells," *Journal of Immunotoxicology*, vol. 10, no. 2, pp. 133–140, 2013.

[6] C. Bubici, S. Papa, K. Dean, and G. Franzoso, "Mutual cross-talk between reactive oxygen species and nuclear factor-kappa B: molecular basis and biological significance," *Oncogene*, vol. 25, no. 51, pp. 6731–6748, 2006.

[7] E. G. Novoselova, S. M. Lunin, T. V. Novoselova et al., "Naturally occurring antioxidant nutrients reduce inflammatory response in mice," *European Journal of Pharmacology*, vol. 615, no. 1-3, pp. 234–240, 2009.

[8] B. Safieh-Garabedian, M. Dardenne, J. M. Pléau, and N. E. Saadé, "Potent analgesic and anti-inflammatory actions of a novel thymulin-related peptide in the rat," *British Journal of Pharmacology*, vol. 136, no. 6, pp. 947–955, 2002.

[9] E. G. Novoselova, S. M. Lunin, M. O. Khrenov, T. V. Novoselova, and E. E. Fesenko, "Involvement of NF-κB transcription factor in the antiinflammatory activity of thymic peptides," *Doklady Biological Sciences*, vol. 428, no. 1, pp. 484–486, 2009.

[10] J. J. Haddad, "Thymulin and zinc (Zn^{2+})-mediated inhibition of endotoxin-induced production of proinflammatory cytokines and NF-κB nuclear translocation and activation in the alveolar epithelium: unraveling the molecular immunomodulatory, anti-inflammatory effect of thymulin/Zn^{2+} in vitro," *Molecular Immunology*, vol. 47, no. 2-3, pp. 205–214, 2009.

[11] T. Henriques-Coelho, S. M. Oliveira, R. S. Moura et al., "Thymulin inhibits monocrotaline-induced pulmonary hypertension modulating interleukin-6 expression and suppressing p38 pathway," *Endocrinology*, vol. 149, no. 9, pp. 4367–4373, 2008.

[12] S. M. Lunin, M. O. Khrenov, T. V. Novoselova, S. B. Parfenyuk, and E. G. Novoselova, "Thymulin, a thymic peptide, prevents the overproduction of pro-inflammatory cytokines and heat shock protein Hsp70 in inflammation-bearing mice," *Immunological Investigations*, vol. 37, no. 8, pp. 858–870, 2008.

[13] S. M. Lunin, O. V. Glushkova, M. O. Khrenov et al., "Thymus peptides regulate activity of RAW 264.7 macrophage cells: inhibitory analysis and a role of signal cascades," *Expert Opinion on Therapeutic Targets*, vol. 15, no. 12, pp. 1337–1346, 2011.

[14] F. L. Grane, "Distribution of ubiquinone," in *Biochemistry of Ubiquinones*, R. Morton, Ed., pp. 184–206, New York, NY, USA, 1965.

[15] M. Alexander, H. Newmark, and R. G. Miller, "Oral beta-carotene can increase the number of $OKT4^+$ cells in human blood," *Immunology Letters*, vol. 9, no. 4, pp. 221–224, 1985.

[16] K. Folkers, "Basic chemical research on coenzyme Q and integrated clinical research on therapy of diseases," in *Coenzyme Q*, G. Lenaz, Ed., pp. 457–479, John Wiley & Sons, Chichester, UK, 1985.

[17] M. Dardenne, J. M. Pleau, B. Nabarra et al., "Contribution of zinc and other metals to the biological activity of the serum thymic factor," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 79, no. 17, pp. 5370–5373, 1982.

[18] M. L. Schmitz, S. Bacher, and M. Kracht, "IκB-independent control of NF-κB activity by modulatory phosphorylations," *Trends in Biochemical Sciences*, vol. 26, no. 3, pp. 186–190, 2001.

[19] S. M. Lunin, O. V. Glushkova, M. O. Khrenov et al., "Thymic peptides restrain the inflammatory response in mice with

experimental autoimmune encephalomyelitis," *Immunobiology*, vol. 218, no. 3, pp. 402–407, 2013.

[20] J. A. Christopher, B. G. Avitabile, P. Bamborough et al., "The discovery of 2-amino-3,5-diarylbenzamide inhibitors of IKK-α and IKK-β kinases," *Bioorganic and Medicinal Chemistry Letters*, vol. 17, no. 14, pp. 3972–3977, 2007.

[21] S. Papa, C. Bubici, F. Zazzeroni et al., "The NF-κB-mediated control of the JNK cascade in the antagonism of programmed cell death in health and disease," *Cell Death and Differentiation*, vol. 13, no. 5, pp. 712–729, 2006.

[22] S. Papa, F. Zazzeroni, C. G. Pham, C. Bubici, and G. Franzoso, "Linking JNK signaling to NF-κB: a key to survival," *Journal of Cell Science*, vol. 117, no. 22, pp. 5197–5208, 2004.

[23] W. Lim, J. Cho, H.-Y. Kwon, Y. Park, M.-R. Rhyu, and Y. Lee, "Hypoxia-inducible factor 1α activates and is inhibited by unoccupied estrogen receptor β," *FEBS Letters*, vol. 583, no. 8, pp. 1314–1318, 2009.

[24] C. Scheidereit, "IκB kinase complexes: gateways to NF-κB activation and transcription," *Oncogene*, vol. 25, no. 51, pp. 6685–6705, 2006.

[25] T. S. Blackwell, T. R. Blackwell, E. P. Holden, B. W. Christman, and J. W. Christman, "In vivo antioxidant treatment suppresses nuclear factor-κB activation and neutrophilic lung inflammation," *Journal of Immunology*, vol. 157, no. 4, pp. 1630–1637, 1996.

[26] E. S. Fox, J. S. Brower, J. M. Bellezzo, and K. A. Leingang, "N-acetylcysteine and α-tocopherol reverse the inflammatory response in activated rat kupffer cells," *Journal of Immunology*, vol. 158, no. 11, pp. 5418–5423, 1997.

[27] H. Zhang, H. Spapen, M. Benlabed, W. A. Buurman, and J.-L. Vincent, "Protective effects of N-acetyl-L-cysteine in endotoxemia," *American Journal of Physiology*, vol. 266, no. 5, pp. H1746–H1754, 1994.

[28] A. A. Kheir-Eldin, T. K. Motawi, M. Z. Gad, and H. M. Abd-Elgawad, "Protective effect of vitamin E, β-carotene and N-acetylcysteine from the brain oxidative stress induced in rats by lipopolysaccharide," *International Journal of Biochemistry and Cell Biology*, vol. 33, no. 5, pp. 475–482, 2001.

[29] J. S. Tilstra, A. R. Robinson, J. Wang et al., "NF-κB inhibition delays DNA damage—induced senescence and aging in mice," *Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2601–2612, 2012.

[30] E. G. Novoselova, "The role of ubiquinones in the regulation of lipid metabolism in rat thymocytes," *FEBS Letters*, vol. 249, no. 2, pp. 371–374, 1989.

[31] E. G. Novoselova, V. R. Makar, and N. V. Semiletova, "Effect of dietary ubiquinone on immune functions and lipid metabolism in rat T lymphocytes," *Bulletin of Molecular Biology and Medicine*, vol. 21, pp. 326–334, 1996.

[32] M. Dardenne, N. Saade, and B. Safieh-Garabedian, "Role of thymulin or its analogue as a new analgesic molecule," *Annals of the New York Academy of Sciences*, vol. 1088, pp. 153–163, 2006.

[33] S. M. Lunin and E. G. Novoselova, "Thymus hormones as prospective anti-inflammatory agents," *Expert Opinion on Therapeutic Targets*, vol. 14, no. 8, pp. 775–786, 2010.

[34] Z. Chen, A. Laurence, and J. J. O'Shea, "Signal transduction pathways and transcriptional regulation in the control of Th17 differentiation," *Seminars in Immunology*, vol. 19, no. 6, pp. 400–408, 2007.

[35] D. B. Kuhns, W. G. Alvord, and J. I. Gallin, "Increased circulating cytokines, cytokine antagonists, and E-selectin after intravenous administration of endotoxin in humans," *Journal of Infectious Diseases*, vol. 171, no. 1, pp. 145–152, 1995.

[36] D. Pajkrt, A. Manten, T. van der Poll et al., "Modulation of cytokine release and neutrophil function by granulocyte colony-stimulating factor during endotoxemia in humans," *Blood*, vol. 90, no. 4, pp. 1415–1424, 1997.

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Review Article

Role of Exercise Training on Autonomic Changes and Inflammatory Profile Induced by Myocardial Infarction

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The cardiovascular autonomic imbalance in patients after myocardial infarction (MI) provides a significant increase in mortality rate, and seems to precede metabolic, hormonal, and immunological changes. Moreover, the reduction in the parasympathetic function has been associated with inflammatory response in different pathological conditions. Over the years, most of the studies have indicated the exercise training (ET) as an important nonpharmacological tool in the management of autonomic dysfunction and reduction in inflammatory profile after a myocardial infarction. In this work, we reviewed the effects of ET on autonomic imbalance after MI, and its consequences, particularly, in the post-MI inflammatory profile. Clinical and experimental evidence regarding relationship between alterations in autonomic regulation and local or systemic inflammation response after MI were also discussed.

1. Introduction

Since the 50s, when cardiovascular diseases (CVDs) exceeded 50% as a cause of mortality worldwide, a detailed search for better understanding of the risk factors was initiated with the Framingham study. Cigarette smoking, hypertension, hypercholesterolemia, diabetes mellitus, physical inactivity, and obesity were identified as the main threats [1] and prevention strategies were initiated.

Despite all technological and scientific advances in the management and prevention of CVD, it remains the leading cause of morbidity and mortality in developed countries and it is fast becoming a major health challenge in developing countries, contributing significantly to high costs in public health [2]. Coronary artery disease (CAD) has a broad spectrum of clinical manifestations, in particular acute coronary syndromes (ACS), that is, unstable angina and acute myocardial infarction (MI). MI is estimated to occur in the US every 44 seconds and about 49% of CVD deaths in the country are attributed to cardiac ischemic events [2].

Both during and after MI, neurohumoral changes occur in order to minimize the consequences of reduced ventricular function, which is caused by the obstruction of blood flow in the left ventricle (LV) of patients who had experienced an ischemic event. On the other hand, chronically, autonomic imbalance is a key element in the pathophysiology of heart failure (HF) after a MI [3–6]. Thus, strategies in order to detect, prevent, and attenuate the cardiovascular autonomic dysfunction, particularly associated with reduced vagal activity, have been seen as important interventions in the management of the changes triggered by MI.

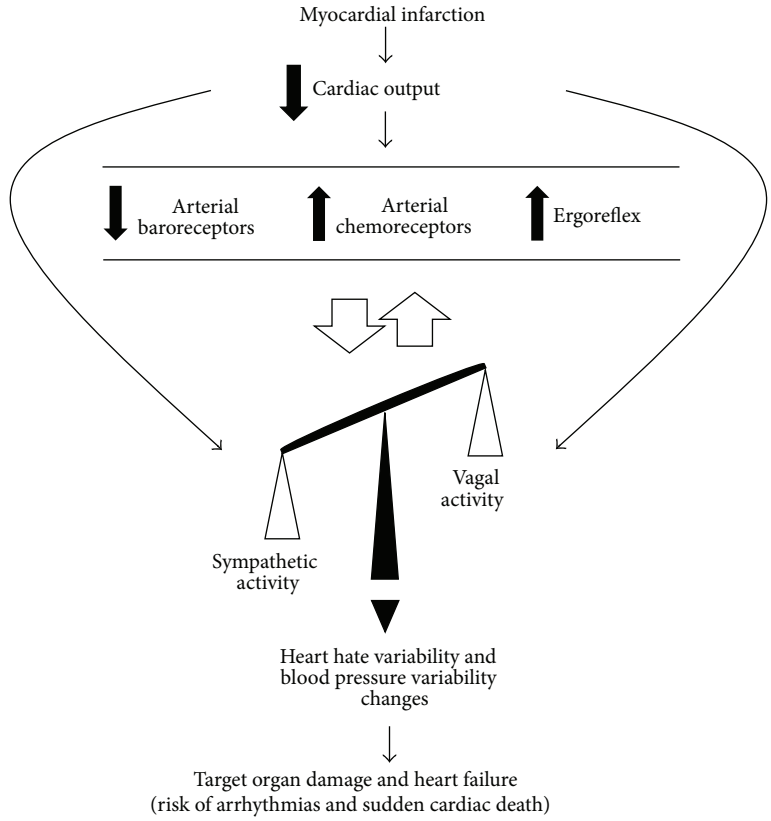

Figure 1: Cardiovascular reflexes impairment and cardiac autonomic nervous system imbalance after myocardial infarction.

The metabolic, cardiovascular, autonomic, and anti-inflammatory benefits of a physically active life style have led many researchers to suggest exercise training (ET) as an important nonpharmacological tool in the prevention and treatment of CVD [7–11]. The effectiveness of ET as a great tool in the treatment of patients with established CAD (either with or without MI) has been widely reported in the literature [7, 12–16]. In this sense, the purpose of this paper was to review the effects of ET on autonomic imbalance and inflammatory profile after MI. Clinical and experimental evidence, or lack of them, regarding relationship between alterations on cardiac autonomic regulation and local or systemic inflammation response after MI will be discussed.

2. Myocardial Infarction and Autonomic Dysfunction

As illustrated in Figure 1, after MI, the reduction in cardiac output is associated with an imbalance of the autonomic nervous system in favor of increased sympathetic activity and reduced vagal activity and is usually accompanied by abnormalities in the cardiorespiratory reflex control, that is, impairment of baroreflex sensitivity and function, and increased activation of ergoreflex and chemoreflex [3, 5, 17, 18]. The sympathoinhibition by the arterial baroreflex is significantly suppressed, whereas sympathoexcitatory reflexes, including the cardiac sympathetic afferent reflex, arterial chemoreceptor, and cardiopulmonary reflexes, are augmented [19]. Thus, the change in cardiovascular reflexes leads to a generalized activation of the sympathetic nervous system after MI in order to change the heart and peripheral hemodynamics. These changes are initially necessary; however, chronically, they are associated with reduced heart rate variability and increased blood pressure variability, which contributes with target organ damage, heart failure development, risk of arrhythmias, and sudden cardiac death [20].

In this sense, the reflex control of circulation commanded by arterial pressure receptors has been recognized as an important predictor of cardiovascular risk after cardiac event. The ATRAMI study (autonomic tone and reflexes after myocardial infarction) has provided clinical evidence of the prognostic value of baroreflex sensitivity and heart rate variability (HRV) in the mortality rate after MI, regardless of ejection fraction and ventricular arrhythmias [3]. Furthermore, in a study undertaken by Kleiger et al. [21], at the Multicenter Post Myocardial Infarction Program, it was found that the individuals who had lower HRV displayed a higher relative risk of mortality (five times) as compared with those who had higher HRV.

Additionally, experimental data have also pointed to a loss of autonomic function in animals after coronary artery occlusion. Indeed, our group has consistently demonstrated that after chronic MI the animals displayed baroreflex dysfunction and reduction in total HRV and in parasympathetic

modulation, as well as increased sympathetic modulation in relation to the noninfarcted animals [19, 22–28]. Furthermore, the changes of hormonal/signaling factors levels at specific sites (angiotensin II, nitric oxide, reactive oxygen species, arginine vasopressin, endothelin-1, atrial natriuretic peptide, prostaglandins, and aldosterone), as well as the increased concentration of proinflammatory cytokines in the central nervous system, may be potential candidate mechanisms underlying the increased sympathetic outflow [29–33].

The clinical prognosis of increased sympathetic activity after myocardial ischemia and HF is now well established [34], and, as such, beta adrenergic blockade has become a standard element in the therapy of these patients [35]. In contrast, the reduction in parasympathetic tonus, although already demonstrated more than 40 years [36], has received less attention. In this sense, more recently, experimental studies have demonstrated that vagal stimulation promoted an antifibrillatory effect and reduced mortality rate in animal models of HF [37, 38]. On the other hand, our group has recently tested the effectiveness of treatment with pyridostigmine bromide, an acetylcholinesterase inhibitor, for 7 days (0.14 mg/mL/day), on autonomic function in rats after MI. The MI treated group improved vagal tonus and decreased sympathetic tonus and MI area in relation to the MI placebo group [39].

Thus, interventions to detect, prevent, or attenuate cardiovascular autonomic dysfunction (particularly reduced vagal activity) have been welcomed as new and important strategies in the management of the MI-induced changes.

3. Autonomic Dysfunction and Inflammatory Response

Inflammatory processes combined with cytokine release are important steps in response to tissue injury and play an active role in cardiac remodeling and function after MI. Inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-α), interleukin-1β (IL-1β), interleukin-6 (IL-6), and interleukin-10 (IL-10), are released after acute ischemic injury and may regulate survival or apoptosis of myocytes, as well as triggering additional inflammatory cell response [40]. In this sense, Ammirati et al. [41] have observed 109 patients during an acute MI and found that those with additional elevations of IL-6 and IL-10 displayed a poor prognosis when compared to individuals with lower levels of these cytokines. Thus, the authors suggested that the degree of acute inflammation after ischemia may indicate a poor prognosis in MI patients.

Chronically, cytokines may mediate cardiac remodeling and repair, enabling the formation of collagen and matrix metalloproteinase, associated with integrin regulation, angiogenesis, and mobilization of progenitor cells [40]. After the initial increase of proinflammatory cytokines in the infarcted area, their levels usually decline toward baseline values one week after MI [42]. However, according to Ono et al. [42], if the size of MI is large, or if there are other stress factors in course, the gene expression of cytokines may remain significantly elevated in noninfarcted region following 20 weeks of MI. Furthermore, these researchers showed that the cytokines levels, such as IL-1β, are associated with ventricular diastolic diameter increase and collagen deposition in the infarcted area after 8 and 20 weeks of MI. Thus, acutely and/or chronically, the release of proinflammatory cytokines adversely affects the LV function, exerts a negative inotropic effect [43], induces abnormalities in cardiac metabolism, and promotes myocardial remodeling, leading to HF [44, 45]. Additionally, activation of the immune system promotes the development of endothelial dysfunction and skeletal muscle apoptosis in HF [46, 47].

The origin of immune activation after ischemia has been dealt with by various research studies but remains unclear. There are at least five hypotheses addressing the underlying mechanism of inflammatory response [48]: (1) the failure of the myocardium *per se* would be the main source of cytokine production [49]; (2) the circulatory decompensation would lead to increased intestinal translocation of bacterial endotoxin (lipopolysaccharide) to the systemic circulation, which in turn would activate circulating immune cells [50]; (3) the main source of proinflammatory mediators would be the body tissues exposed to hypoxia [51, 52]; (4) immune activation would be a consequence of increased [53] sympathetic stimulation; and (5) reduction in parasympathetic participation would work as the primary mediator of the inflammatory response activation [48].

Studies by Kevin J. Tracey group [54–62] have lent strength to the hypothesis of a direct relation between parasympathetic activation and immune system response. This group initially argued that the activation of the vagus nerve (electrically or by cholinergic agonists) would reduce inflammatory response in experimental models of sepsis, and subsequently they postulated the theory of "inflammatory reflex," linking aspects related to neuroimmunomodulation. The reflex arc, mediated by the nervous system, is composed by efferent via, integrative areas of central nervous system, and efferent via. Briefly, Kevin J. Tracey's group observed that inflammatory mediators (cytokines) produced in peripheral tissues may warn the central nervous system by a direct central action or by afferent stimulation of the vagus nerve. In this model, the integration of signals takes place in the central areas, triggering the activation of the parasympathetic efferent pathway, mediated mainly by the vagus nerve, whose neurotransmitter is acetylcholine. The cholinergic pathway innervates various components of the immune system (reticuloendothelial system), such as lymph nodes, liver, heart, spleen, and gastrointestinal tract. The activation of the vagus nerve leads to reduced production of cytokines, which in turn decreases the inflammatory response in models of septic and aseptic inflammation [55].

According to the "inflammatory reflex" theory, the vagus nerve seems to be the most important element in the efferent arm. In this sense, Blalock [63] has suggested that the immune system linked in such way to the central nervous system works as a "sixth sense," being able to detect microbial invasion and other inflammatory substances and to retransmit this information to the brain, triggering responses that would interfere in the initial process. In fact, studies have viewed the parasympathetic hyperactivity, brought about by either drugs

or direct vagal stimulation, as a mechanism that reduces the release of cytokines and reactive oxygen species during an inflammatory process [56, 64].

In this context, Borovikova et al. [65] have also demonstrated that injection of endotoxin (lipopolysaccharide) in animals that underwent vagus nerve stimulation resulted in reduced systemic release of inflammatory cytokines and macrophages, without affecting the release of interleukin-10 (IL-10). However, the vagus nerve transection abolished this protection. Accordingly, in humans, significantly reduced HRV is associated with elevated levels of inflammatory cytokines (IL-6) and C-reactive protein (CRP) [66]. Lanza et al. [67] have shown that serum CRP levels were significantly associated with reduced HRV in patients with unstable angina. This association has also been observed in healthy individuals and patients with stable coronary artery disease and HF [68]. Thus, in accordance with "inflammatory reflex" theory, inflammatory products produced in ischemic ventricle activate afferent signals that are relayed to the nucleus tractus solitaries, and subsequent activation of vagus efferent activity would inhibit cytokine synthesis through the cholinergic anti-inflammatory pathway (Figure 2). However, as previously mentioned there is an imbalance in favor of increased sympathetic activity and reduced vagal activity after MI. Thus, it is possible to suggest that cardiovascular autonomic imbalance after ischemic event may blunt "inflammatory reflex" by reducing cholinergic anti-inflammatory pathway. However, despite the fact that some suggestions are pointed out on this direction in the literature [66–68], there is no strong evidence to support a cause-effect relationship.

In this sense, preventing and/or attenuating autonomic dysfunction triggered by MI would result in a less intense inflammatory response and, as such, cardiac and peripheral structure and function would be preserved.

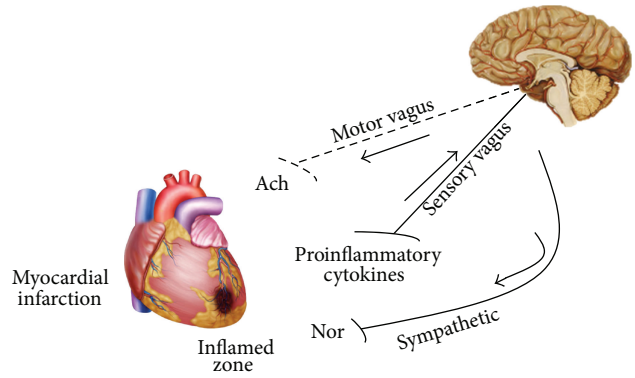

Figure 2: Blunted "inflammatory reflex" by vagal efferent function reduction after myocardial infarction (adapted from Tracey [55]). Ach: acetylcholine; Nor: noradrenaline.

4. Aerobic Exercise Training as Therapy

In recent years, our laboratory has investigated the changes induced by MI alone [25, 27] or associated with different risk factors, such as hypertension [69, 70], ovarian hormone deprivation [23], sinoaortic denervation [24], and diabetes [26–28, 71–73]. These studies have also tested different therapeutic approaches in the management of MI-induced changes. Among these therapeutic approaches, some deserve greater emphasis: therapy involving stem cells and mesenchymal bone marrow both *in situ* [70] and intravenously [69], gene therapy with vascular endothelium-derived growth factor (VEGF) [73], and ET [22, 23, 25, 27].

Moderate intensity aerobic ET is responsible for structural and hemodynamic adaptations in the cardiovascular system and promotes adjustments in autonomic nervous system. This can be exemplified by cardiac adaptations such as increase of stroke volume [74, 75], adjustments in diastolic and systolic functions [75], and positive changes in cavities diameter and ventricular mass [76, 77] as well as alterations in the rest heart rate (HR) [77].

Resting bradycardia is associated with a decrease in intrinsic HR as well as an altered autonomic balance, leading to parasympathetic dominance [78]. This is thought to be mediated, at least in part, by an increase in cardiac vagal tone [79]. The mechanisms by which exercise produces changes in autonomic control have yet to be fully understood; however, there is evidence of alterations in the central afferent and efferent pathways and in the effector organs (receptor function) [80, 81].

Aerobic ET is largely recommended for patients with cardiac disease, including those who have experienced an MI. A meta-analysis, based on 48 randomized controlled trials (8940 patients), showed that cardiac rehabilitation programs based on aerobic exercises reduced all-cause mortality by 20% and CVD mortality by 26% in patients after MI, angina pectoris, and/or CAD [14]. Additionally, in another meta-analysis based on 34 studies, the authors observed that aerobic ET (predominantly) after MI, even for a short period (1–3 months), was an effective therapeutic strategy, since the observed benefits extended far beyond the intervention period [16].

One of the most important adaptations to the ET, generally associated with reduced mortality rate, is the improvement in autonomic nervous system regulation. Classic research studies such as Billman et al. [12] and Hull Jr. et al. [13] have suggested that this intervention reduces mortality in individuals after an MI, particularly when associated with increased vagal component and decreased sympathetic activity. La Rovere et al. [82], using head-up tilt testing to evaluate heart rate variability (HRV) in MI patients, have observed that aerobically trained individuals presented a better autonomic response to orthostatic stress, with reduction in vagal modulation and an increase in sympathetic vasoconstrictor outflow due to initiation of the baroreceptors. Moreover, Iellamo et al. [83], in the first randomized controlled study in the area, have observed that aerobic ET results in marked enhancement of both baroreflex sensitivity and HRV in patients with coronary artery disease. These researchers have also suggested that the improvement in baroreflex sensitivity associated with aerobic ET is not limited to patients with prior MI but extends to coronary patients without MI, for whom risk-reducing strategies designed to avoid subsequent lethal events might be of paramount importance.

In la Rovere et al. [7] study, 95 consecutive male patients, survivors of a first uncomplicated MI, underwent four weeks of ET. The researchers found that aerobic ET was associated with increased survival together with an adequate modulation of the autonomic balance toward increase in vagal activity, as revealed by the increase in baroreflex sensitivity. In this context, several clinical studies have reported improvements in cardiovascular autonomic control, particularly in HRV, after ET among MI patients. Sandercock et al. [15] have observed that after eight-week cardiac rehabilitation program, MI participants had significant increases in HRV parameters as compared with those not participating in the training program. In an elegant study, Malfatto et al. [84] have reported improvement in HRV following 8 weeks of aerobic ET. After this period, participants were encouraged to continue exercising at home two to three times per week. After one year of training program participation, improvements in HRV index were still relevant. More recently, six months of aerobic (predominantly) ET significantly decreased muscle sympathetic nerve activity and the low-frequency component of systolic arterial pressure and increased the baroreflex sensitivity in MI patients. These changes were so marked that the differences between patients with MI and the normal control group were no longer observed after ET. These findings highlight the clinical importance of this nonpharmacological therapy based on ET in the long-term treatment of patients with MI [85].

In an experimental setting, our group has studied the role of aerobic ET in functional, biochemical, and molecular alterations, as well as in the mortality rate after MI in rats. Rondon et al. [22] have investigated the role of ET in MI-induced heart failure rats and demonstrated that aerobic ET increased peak oxygen uptake and the high frequency band of HRV, while reducing the low frequency band of HRV. In addition, these authors also observed that after ET protocol the heart failure animals presented improvement in baroreflex control of heart rate and renal sympathetic nerve activity, associated with increased aortic depressor nerve activity. However, we should bear in mind that, although these data suggest an association between increased aortic depressor nerve activity and improvement in baroreflex control, the cause-effect relationship remains unclear. Moreover, Jorge et al. [25] have demonstrated that early aerobic ET intervention (one week) after MI induced an improvement in LV systolic and diastolic functions. In the study, we have also observed a normalization of hemodynamic and regional blood flows and an improvement in cardiovascular autonomic function associated with increased baroreflex sensitivity. These benefits, in turn, resulted in an increase in functional capacity and a reduction in mortality rate in trained infarcted animals. Similar results were also demonstrated in streptozotocin-diabetic rats undergoing MI, for which aerobic ET promoted, in addition to the benefits observed in autonomic function, positive changes in the expression of proteins associated with intracellular calcium handling [86]. Studying female ovariectomized rats undergoing MI, Flores et al. [23] have demonstrated that eight weeks of aerobic ET was able to improve resting hemodynamic status and reflex control of the circulation (arterial and cardiopulmonary baroreflex), possibly associated with the increase in vagal component observed in the study.

In line with la Rovere et al. [7] study, in which aerobic ET was associated with improvement in baroreflex sensitivity and increased survival rate in a 10-year followup, our group tested the hypothesis that autonomic ET benefits might remain for an extended period, even during detraining. Accordingly, in a study by Barboza et al. [27], MI animals underwent three months of aerobic ET (starting one week after MI) and one month of detraining. The authors demonstrated that one month of detraining did not alter the beneficial effects of ET on the MI area, LV morphometry and function, and baroreflex sensitivity, as well as overall survival rate in MI-detrained animals. These findings indicate not only that aerobic ET is an effective tool in the management of cardiovascular and autonomic MI derangements, but also that these positive changes were extended even beyond one month of detraining in rats.

In addition to the benefits of ET on autonomic function, it is now well established that cytokine production by exercise is different from that observed in response to severe infections or tissue injury, since exercises usually do not provide expressive changes in the classic proinflammatory cytokines, TNF-α and IL-1β [87]. During exercise, IL-6 is the first cytokine present in the circulation, exponentially increasing when the exercise is in course, and fast declining after the exercise period [88–90]. In this sense, there is evidence in the literature that IL-6 may exert anti-inflammatory effects, since circulating IL-6 triggers an inhibitory effect on TNF-α and IL-1 production [91] and stimulates the production of IL-1 receptor antagonist and IL-10 [92]. IL-10 and IL-1 receptor antagonist production after exercise, in turn, contributes to the inhibition of the synthesis of a large spectrum of proinflammatory cytokines by different cells, particularly the ones of monocytic lineage [93].

Actually, the protective effects of ET on inflammatory markers have been widely discussed [94]. In this sense, Adamopoulos et al. [95] have demonstrated a reduction in inflammatory markers after 12 weeks of aerobic ET in patients with moderate to severe HF, suggesting a correlation between improvement in exercise tolerance and attenuation ofinflammatory process. In an experimental model of HF, Batista Jr. et al. [96] have shown that aerobic ET in infarcted rats increased the ratio IL-10/tumor necrosis factor-α (TNF-α) in the soleus muscle of animals, emphasizing the anti-inflammatory effect of exercise after an ischemic event.

Individuals with increased risk of CAD presented reduction in atherogenic cytokines, as interleukin-1 (IL-1), IL-6, TNF-α, and C reactive protein, as well as improving atheroprotective cytokines, as IL-10, and transforming growth factor beta-1 after an aerobic ET program [97]. The reduction in plasma TNF-α, IL-6, and their receptors has been demonstrated in aerobically trained HF patients [98], suggesting an attenuation in the chronic inflammatory condition mediated by a regulation in peripheral inflammatory response [95, 98]. Similarly, the association between aerobic and resistance ET decreased the concentration of TNF-α receptors I and II in patients with HF [99].

Thus, aerobic ET seems to be an important strategy in the management MI-induced changes and HF, especially with regard to improving cardiovascular autonomic control and reducing chronic inflammatory response, consequently reducing cardiac work, decreasing risk of fatal arrhythmias, and increasing survival in affected individuals.

In order to understand the mechanisms associated with autonomic adjustments and anti-inflammatory role of exercise fully, it is necessary to do a more detailed search of the nature, intensity, and duration of ET after MI. The beneficial effects of aerobic ET are well known; however, autonomic and anti-inflammatory effects of resistance ET or high intensity aerobic ET are poorly defined and remain areas for future investigations. In this direction, our group recently demonstrated that resistance ET (moderate intensity) induced additional benefits in the low frequency band (+50%) and high frequency band of HRV (+45%), as well as in the low frequency band of systolic blood pressure variability (−46%) of trained infarcted rats compared to sedentary [100]. These findings suggest that resistance ET was effective in reducing cardiac and peripheral sympathetic modulation, as well as increasing cardiac parasympathetic modulation of infarcted rats.

5. Exercise Training on "Inflammatory Reflex" Response after Myocardial Infarction

Despite all data generated on the anti-inflammatory role of the exercise, the underlying mechanisms associated with improved inflammatory profile in patients after an ischemic event have yet to be fully understood. During the last decade, studies on the anti-inflammatory effects of exercise have pointed to three possible mechanisms [87, 101–106]: reduction in visceral fat mass; increased production and release of anti-inflammatory cytokines from contracting skeletal muscle (myokines); and reduced expression of toll-like receptors (TLRs) on monocytes and macrophages. Regarding the first mechanism, ET is able to reduce low-grade and systemic inflammation via a decrease in proinflammatory adipokine secretion, such as TNF, leptin, retinol-binding protein 4, lipocalin 2, IL-6, and IL-18, which is a direct result of lowering the amount of fat stored in abdominal and visceral depots [107–110].

Related to the second proposed mechanism, during exercise it seems that IL-6, produced by contractile skeletal muscle via a TNF-independent pathway, stimulates systemic appearance of anti-inflammatory cytokines, such as IL-1 receptor antagonist and IL-10, and inhibits the liberation of proinflammatory cytokine TNF-α [101, 111]. Additionally, IL-6 may be associated with increase of lipid turnover, stimulating lipolysis as well as fat oxidation [101]. In line with the third proposed mechanism about the anti-inflammatory effects of ET, it has been evidenced that TLRs may be involved in the link between a sedentary lifestyle and inflammation and disease [112]. Studies have observed that blood monocytes from trained individuals present decreased TLR4 expression, which is associated with decreased inflammatory cytokine production, and reduced inflammatory response to endotoxin stimulation *in vitro* [103, 105, 113].

On the other hand, as previously mentioned, the "inflammatory reflex" proposed by Tracey [55] seems to have an important role in the development of the inflammatory process, since it is in line with the suggestion that the activation of the vagus nerve leads to a reduced production of cytokines by the reticuloendothelial system, such as lymph nodes, liver, heart, spleen, and gastrointestinal tract. ET, in turn, modulates cardiac autonomic control with reduction in sympathetic tonus and increases the role of the vagal tonus [7, 15, 85], thereby positively affecting the prognosis of MI patients. Thus, we suggest that in addition to the reduction in visceral fat mass, increased production anti-inflammatory myokines, and reduced expression of TLRs on monocytes and macrophages, the increase in vagal activation induced by ET may be an important mechanism that would explain, at least in part, the improvement in the inflammatory status of animals or patients after MI.

As evidenced in Figure 3, we proposed that chronic benefits of ET would be associated with significant improvements in baroreflex sensitivity, resulting in the increase in vagal activity and reduction in sympathetic activity (as observed to the heart and vessels [7, 12, 13, 15, 22, 23, 25, 27, 78, 82–86]) to important organs (reticulum endothelial system and other tissues), including brain, heart, liver, spleen, and gastrointestinal (GI) tract. This autonomic remodeling, in turn, would be directly associated with improved local and systemic inflammatory profile, reducing cardiovascular risk and enhancing the survival rate in patients who had suffered a MI. When studying infarcted rats undergoing 3 months of moderate-intensity aerobic ET, preliminary data from our group has noted that HRV parameters such as the high frequency band and root mean square of successive difference (RMSSD)—indexes of cardiac parasympathetic modulation—were negatively correlated with cardiac levels of TNF-α and IL-6 (unpublished data). Furthermore, the low frequency band of systolic blood pressure variability, an indicator of vascular sympathetic modulation, was positively associated with the TNF-α and IL-6 levels in the adipose tissue of these animals (unpublished data). Taken together, our preliminary findings suggest that ET can increase cardiac vagal modulation, as well as reducing sympathetic peripheral modulation, leading to a reduction in the inflammatory profile of infarcted rats.

Despite the vast literature raised in this review, many questions still remain about the benefits of ET after MI on cardiovascular autonomic function and inflammatory response, or if there is a direct relationship between them. At present, we do not know whether there is a cause-effect relationship between the improvement in vagal cholinergic activity promoted by ET and reduction in cardiac and systemic inflammatory status after a cardiac ischemic event. Or even, what is the relative importance of these mechanisms on cardiac morphology and function, as well as in the prognosis of infarcted patients.

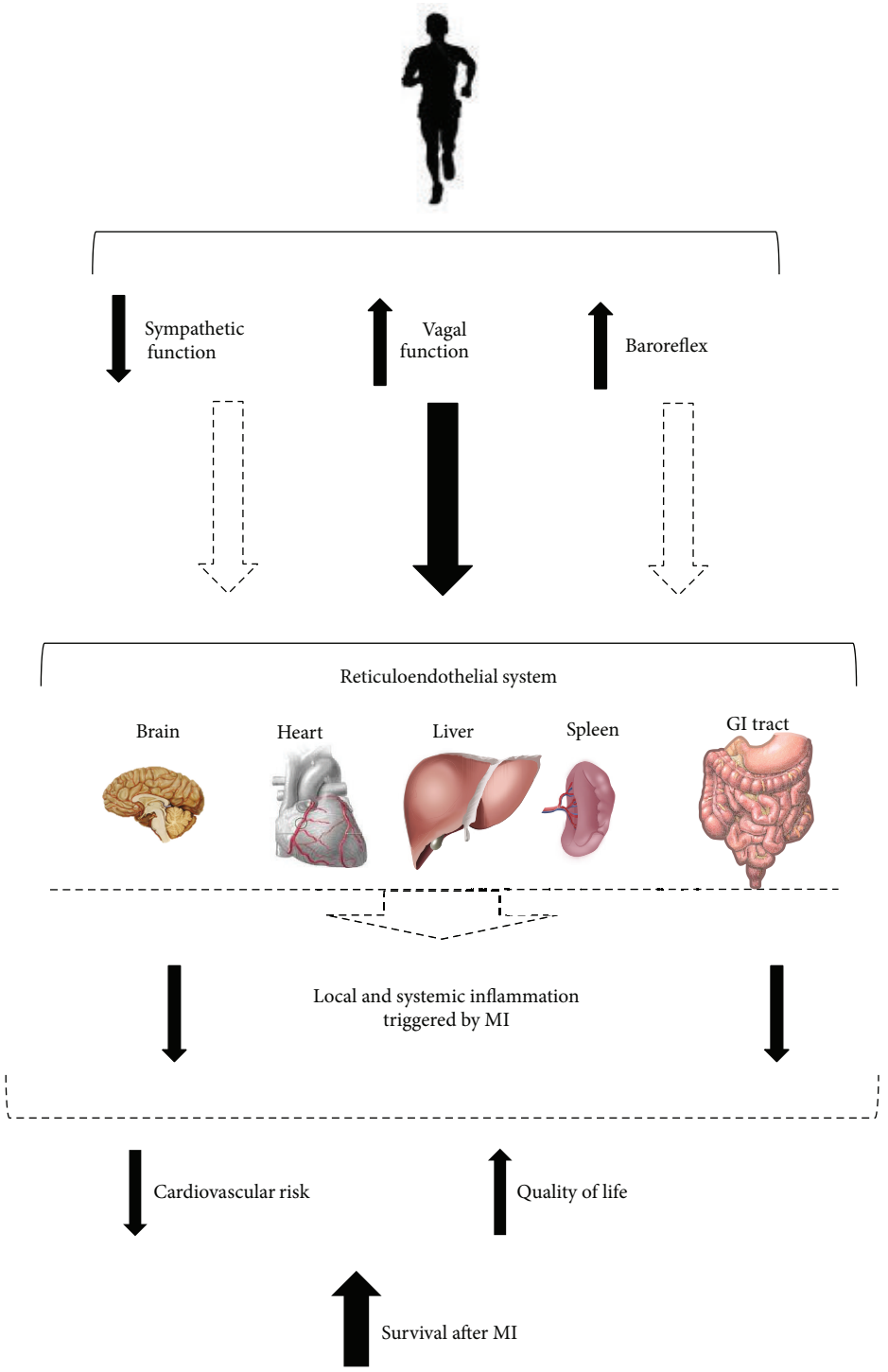

Figure 3: Possible mechanisms associated with inflammatory profile reduction in patients after myocardial infarction undergoing exercise training.

Intuitively, we can expect that many anti-inflammatory mechanisms of exercise, known or unknown, are acting in combination in order to better respond to different stimulations applied. Furthermore, it is possible that the involved mechanisms and the magnitude of the benefits found in autonomic function and inflammatory profile will depend on the mode, intensity and duration of exercise performed. However, the vast majority of the studies presented in this review used the moderate intensity aerobic ET. The absence of studies comparing different intensities, durations, and modalities of ET and its possible relationship with the anti-inflammatory cholinergic

pathway opens new perspectives for working in exercise cardiology.

6. Conclusions

There is consistent experimental and clinical evidence that the autonomic nervous system and inflammatory response play a key role in cardiac and peripheral dysfunctions after myocardial infarction. Exercise training has been associated with increased vagal tonus/modulation, as well as with reduction in sympathetic tonus/modulation and inflammatory profile in heart failure or after myocardial infarction. In recent years, a direct relationship between vagal activity and inflammatory status has been proposed. Here, we suggest that exercise training may improve autonomic function to different sites, leading to a reduction in the proinflammatory response observed after myocardial infarction. However, further experimental and clinical investigations are needed to test the role of exercise training in the modulation of this "inflammatory reflex" after myocardial infarction. The results of these future studies will improve the management of cardiovascular risk, quality of life, and survival of this population.

Conflict of Interests

The authors have no conflict of interests to declare.

Authors' Contribution

Kátia De Angelis and Maria-Cláudia Irigoyen contributed equally to this paper.

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References

[1] G. Thiene and C. Basso, "Myocardial infarction: a paradigm of success in modern medicine," *Cardiovascular Pathology*, vol. 19, no. 1, pp. 1–5, 2010.

[2] A. S. Go, D. Mozaffarian, V. L. Roger et al., "Heart disease and stroke statistics–2014 update: a report from the American Heart Association," *Circulation*, vol. 129, no. 3, pp. e28–e292, 2014.

[3] M. T. la Rovere, J. T. Bigger Jr., F. I. Marcus, A. Mortara, and P. J. Schwartz, "Baroreflex sensitivity and heart-rate variability in prediction of total cardiac mortality after myocardial infarction," *The Lancet*, vol. 351, no. 9101, pp. 478–484, 1998.

[4] E. Braunwald and M. R. Bristow, "Congestive heart failure: fifty years of progress," *Circulation*, vol. 102, no. 20, pp. 14–23, 2000.

[5] C. E. Negrao and H. R. Middlekauff, "Adaptations in autonomic function during exercise training in heart failure," *Heart Failure Reviews*, vol. 13, no. 1, pp. 51–60, 2008.

[6] K. Thygesen, J. S. Alpert, A. S. Jaffe et al., "Third universal definition of myocardial infarction," *Circulation*, vol. 126, no. 16, pp. 2020–2035, 2012.

[7] M. T. la Rovere, C. Bersano, M. Gnemmi, G. Specchia, and P. J. Schwartz, "Exercise-induced increase in baroreflex sensitivity predicts improved prognosis after myocardial infarction," *Circulation*, vol. 106, no. 8, pp. 945–949, 2002.

[8] B. K. Pedersen and B. Saltin, "Evidence for prescribing exercise as therapy in chronic disease," *Scandinavian Journal of Medicine and Science in Sports*, vol. 16, no. 1, pp. 3–63, 2006.

[9] T. A. Lakka and D. E. Laaksonen, "Physical activity in prevention and treatment of the metabolic syndrome," *Applied Physiology, Nutrition and Metabolism*, vol. 32, no. 1, pp. 76–88, 2007.

[10] S. Ertek and A. Cicero, "Impact of physical activity on inflammation: effects on cardiovascular disease risk and other inflammatory conditions," *Archives of Medical Science*, vol. 8, no. 5, pp. 794–804, 2012.

[11] N. Mann and A. Rosenzweig, "Can exercise teach Us how to treat heart disease?" *Circulation*, vol. 126, no. 22, pp. 2625–2635, 2012.

[12] G. E. Billman, P. J. Schwartz, and H. L. Stone, "The effects of daily exercise on susceptibility to sudden cardiac death," *Circulation*, vol. 69, no. 6, pp. 1182–1189, 1984.

[13] S. S. Hull Jr., E. Vanoli, P. B. Adamson, R. L. Verrier, R. D. Foreman, and P. J. Schwartz, "Exercise training confers anticipatory protection from sudden death during acute myocardial ischemia," *Circulation*, vol. 89, no. 2, pp. 548–552, 1994.

[14] R. S. Taylor, A. Brown, S. Ebrahim et al., "Exercise-based rehabilitation for patients with coronary heart disease: systematic review and meta-analysis of randomized controlled trials," *American Journal of Medicine*, vol. 116, no. 10, pp. 682–692, 2004.

[15] G. R. H. Sandercock, R. Grocott-Mason, and D. A. Brodie, "Changes in short-term measures of heart rate variability after eight weeks of cardiac rehabilitation," *Clinical Autonomic Research*, vol. 17, no. 1, pp. 39–45, 2007.

[16] P. R. Lawler, K. B. Filion, and M. J. Eisenberg, "Efficacy of exercise-based cardiac rehabilitation post-myocardial infarction: a systematic review and meta-analysis of randomized controlled trials," *American Heart Journal*, vol. 162, no. 4, pp. 571.e2–584.e2, 2011.

[17] P. P. Ponikowski, T. P. Chua, D. P. Francis, A. Capucci, A. J. S. Coats, and M. F. Piepoli, "Muscle ergoreceptor overactivity reflects deterioration in clinical status and cardiorespiratory reflex control in chronic heart failure," *Circulation*, vol. 104, no. 19, pp. 2324–2330, 2001.

[18] P. Ponikowski, T. P. Chua, S. D. Anker et al., "Peripheral chemoreceptor hypersensitivity an ominous sign in patients with chronic heart failure," *Circulation*, vol. 104, no. 5, pp. 544–549, 2001.

[19] J. E. D. C. Lacerda, F. M. Consolim-Colombo, E. D. Moreira et al., "Influence of cardiopulmonary reflex on the sympathetic activity during myocardial infarction," *Autonomic Neuroscience: Basic and Clinical*, vol. 133, no. 2, pp. 128–135, 2007.

[20] J. S. Floras, "Sympathetic nervous system activation in human heart failure. Clinical implications of an updated model," *Journal of the American College of Cardiology*, vol. 54, no. 5, pp. 375–385, 2009.

[21] R. E. Kleiger, J. P. Miller, J. T. Bigger Jr., and A. J. Moss, "Decreased heart rate variability and its association with increased mortality after acute myocardial infarction," *The American Journal of Cardiology*, vol. 59, no. 4, pp. 256–262, 1987.

[22] E. Rondon, M. S. Brasileiro-Santos, E. D. Moreira et al., "Exercise training improves aortic depressor nerve sensitivity in rats with ischemia-induced heart failure," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 291, no. 6, pp. H2801–H2806, 2006.

[23] L. J. Flores, D. Figueroa, I. C. Sanches et al., "Effects of exercise training on autonomic dysfunction management in an experimental model of menopause and myocardial infarction," *Menopause*, vol. 17, no. 4, pp. 712–717, 2010.

[24] C. Mostarda, B. Rodrigues, M. Vane et al., "Autonomic impairment after myocardial infarction: role in cardiac remodelling and mortality," *Clinical and Experimental Pharmacology and Physiology*, vol. 37, no. 4, pp. 447–452, 2010.

[25] L. Jorge, B. Rodrigues, K. T. Rosa et al., "Cardiac and peripheral adjustments induced by early exercise training intervention were associated with autonomic improvement in infarcted rats: role in functional capacity and mortality," *European Heart Journal*, vol. 32, no. 7, pp. 904–912, 2011.

[26] B. Rodrigues, K. T. Rosa, A. Medeiros et al., "Hyperglycemia can delay left ventricular dysfunction but not autonomic damage after myocardial infarction in rodents," *Cardiovascular Diabetology*, vol. 10, article 26, 2011.

[27] C. A. Barboza, L. Y. Rocha, C. T. Mostarda et al., "Ventricular and autonomic benefits of exercise training persist after detraining in infarcted rats," *European Journal of Applied Physiology*, vol. 113, no. 5, pp. 1137–1146, 2013.

[28] B. Rodrigues, C. T. Mostarda, L. Jorge et al., "Impact of myocardial infarction on cardiac autonomic function in diabetic rats," *Journal of Diabetes and Its Complications*, vol. 27, no. 1, pp. 16–22, 2013.

[29] J. Francis, Y. Chu, A. K. Johnson et al., "Acute myocardial infarction induces hypothalamic cytokine synthesis," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 286, no. 6, pp. H2264–H2271, 2004.

[30] T. Kishi, Y. Hirooka, Y. Kimura, K. Ito, H. Shimokawa, and A. Takeshita, "Increased reactive oxygen species in rostral ventrolateral medulla contribute to neural mechanisms of hypertension in stroke-prone spontaneously hypertensive rats," *Circulation*, vol. 109, no. 19, pp. 2357–2362, 2004.

[31] I. H. Zucker, H. D. Schultz, K. P. Patel, W. Wang, and L. Gao, "Regulation of central angiotensin type 1 receptors and sympathetic outflow in heart failure," *American Journal of Physiology. Heart and Circulatory Physiology*, vol. 297, no. 5, pp. H1557–H1566, 2009.

[32] T. Kishi, Y. Hirooka, S. Konno, K. Ogawa, and K. Sunagawa, "Angiotensin II type 1 receptor-activated caspase-3 through ras/mitogen-activated protein kinase/extracellular signal-regulated kinase in the rostral ventrolateral medulla is involved in sympathoexcitation in stroke-prone spontaneously hypertensive rats," *Hypertension*, vol. 55, no. 2, pp. 291–297, 2010.

[33] K. Ogawa, Y. Hirooka, T. Kishi, and K. Sunagawa, "Brain AT1 receptor activates the sympathetic nervous system through toll-like receptor 4 in mice with heart failure," *Journal of Cardiovascular Pharmacology*, vol. 58, no. 5, pp. 543–549, 2011.

[34] J. S. Floras, "Sympathetic activation in human heart failure: diverse mechanisms, therapeutic opportunities," *Acta Physiologica Scandinavica*, vol. 177, no. 3, pp. 391–398, 2003.

[35] M. Packer, M. R. Bristow, J. N. Cohn et al., "The effect of carvedilol on morbidity and mortality in patients with chronic heart failure," *New England Journal of Medicine*, vol. 334, no. 21, pp. 1349–1355, 1996.

[36] D. L. Eckberg, M. Drabinsky, and E. Braunwald, "Defective cardiac parasympathetic control in patients with heart disease," *New England Journal of Medicine*, vol. 285, no. 16, pp. 877–883, 1971.

[37] M. Li, C. Zheng, T. Sato, T. Kawada, M. Sugimachi, and K. Sunagawa, "Vagal nerve stimulation markedly improves long-term survival after chronic heart failure in rats," *Circulation*, vol. 109, no. 1, pp. 120–124, 2004.

[38] Y. Zhang, Z. B. Popović, S. Bibevski et al., "Chronic vagus nerve stimulation improves autonomic control and attenuates systemic inflammation and heart failure progression in a canine high-rate pacing model," *Circulation: Heart Failure*, vol. 2, no. 6, pp. 692–699, 2009.

[39] R. N. de la Fuente, B. Rodrigues, I. C. Moraes-Silva et al., "Cholinergic stimulation with pyridostigmine improves autonomic function in infarcted rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 40, no. 9, pp. 610–616, 2013.

[40] M. Nian, P. Lee, N. Khaper, and P. Liu, "Inflammatory cytokines and postmyocardial infarction remodeling," *Circulation Research*, vol. 94, no. 12, pp. 1543–1553, 2004.

[41] E. Ammirati, C. V. Cannistraci, N. A. Cristell et al., "Identification and predictive value of interleukin-6+ interleukin-10+ and interleukin-6-interleukin-10+ cytokine patterns in st-elevation acute myocardial infarction," *Circulation Research*, vol. 111, no. 10, pp. 1336–1348, 2012.

[42] K. Ono, A. Matsumori, T. Shioi, Y. Furukawa, and S. Sasayama, "Cytokine gene expression after myocardial lnfarction in rat hearts: possible implication in left ventricular remodeling," *Circulation*, vol. 98, no. 2, pp. 149–156, 1998.

[43] D. R. Meldrum, "Tumor necrosis factor in the heart," *American Journal of Physiology. Regulatory Integrative and Comparative Physiology*, vol. 274, no. 3, pp. R577–R595, 1998.

[44] D. L. Mann, "Inflammatory mediators and the failing heart: past, present, and the foreseeable future," *Circulation Research*, vol. 91, no. 11, pp. 988–998, 2002.

[45] A. Diwan, T. Tran, A. Misra, and D. L. Mann, "Inflammatory mediators and the failing heart: a translational approach," *Current Molecular Medicine*, vol. 3, no. 2, pp. 161–182, 2003.

[46] S. D. Anker, P. P. Ponikowski, A. L. Clark et al., "Cytokines and neurohormones relating to body composition alterations in the wasting syndrome of chronic heart failure," *European Heart Journal*, vol. 20, no. 9, pp. 683–693, 1999.

[47] R. Sharma, A. J. S. Coats, and S. D. Anker, "The role of inflammatory mediators in chronic heart failure: cytokines, nitric oxide, and endothelin-1," *International Journal of Cardiology*, vol. 72, no. 2, pp. 175–186, 2000.

[48] E. A. Jankowska, P. Ponikowski, M. F. Piepoli, W. Banasiak, S. D. Anker, and P. A. Poole-Wilson, "Autonomic imbalance and immune activation in chronic heart failure—pathophysiological links," *Cardiovascular Research*, vol. 70, no. 3, pp. 434–445, 2006.

[49] G. Torre-Amione, S. Kapadia, J. Lee et al., "Tumor necrosis factor-α and tumor necrosis factor receptors in the failing human heart," *Circulation*, vol. 93, no. 4, pp. 704–711, 1996.

[50] J. Niebauer, H.-D. Volk, M. Kemp et al., "Endotoxin and immune activation in chronic heart failure: a prospective cohort study," *The Lancet*, vol. 353, no. 9167, pp. 1838–1842, 1999.

[51] D. Hasper, M. Hummel, F. X. Kleber, I. Reindl, and H.-D. Volk, "Systemic inflammation in patients with heart failure," *European Heart Journal*, vol. 19, no. 5, pp. 761–765, 1998.

[52] T. Bachetti and R. Ferrari, "The dynamic balance between heart function and immune activation," *European Heart Journal*, vol. 19, no. 5, pp. 681–682, 1998.

[53] U. Müller-Werdan and K. Werdan, "Immune modulation by catecholamines—a potential mechanism of cytokine release in heart failure?" *Herz*, vol. 25, no. 3, pp. 271–273, 2000.

[54] F. Rangwala, R. C. Drisdel, S. Rakhilin et al., "Neuronal α-bungarotoxin receptors differ structurally from other nicotinic acetylcholine receptors," *Journal of Neuroscience*, vol. 17, no. 21, pp. 8201–8212, 1997.

[55] K. J. Tracey, "The inflammatory reflex," *Nature*, vol. 420, no. 6917, pp. 853–859, 2002.

[56] H. Wang, H. Liao, M. Ochani et al., "Cholinergic agonists inhibit HMGB1 release and improve survival in experimental sepsis," *Nature Medicine*, vol. 10, no. 11, pp. 1216–1221, 2004.

[57] V. A. Pavlov, M. Ochani, M. Gallowitsch-Puerta et al., "Central muscarinic cholinergic regulation of the systemic inflammatory response during endotoxemia," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 103, no. 13, pp. 5219–5223, 2006.

[58] K. J. Tracey, "Physiology and immunology of the cholinergic antiinflammatory pathway," *Journal of Clinical Investigation*, vol. 117, no. 2, pp. 289–296, 2007.

[59] R. P. Sloan, H. McCreath, K. J. Tracey, S. Sidney, K. Liu, and T. Seeman, "RR interval variability is inversely related to inflammatory markers: the CARDIA study," *Molecular Medicine*, vol. 13, no. 3-4, pp. 178–184, 2007.

[60] M. Rosas-Ballina and K. J. Tracey, "The neurology of the immune system: neural reflexes regulate immunity," *Neuron*, vol. 64, no. 1, pp. 28–32, 2009.

[61] K. J. Tracey, "Reflex control of immunity," *Nature Reviews Immunology*, vol. 9, no. 6, pp. 418–428, 2009.

[62] J. M. Huston and K. J. Tracey, "The pulse of inflammation: heart rate variability, the cholinergic anti-inflammatory pathway and implications for therapy," *Journal of Internal Medicine*, vol. 269, no. 1, pp. 45–53, 2011.

[63] J. E. Blalock, "The immune system as the sixth sense," *Journal of Internal Medicine*, vol. 257, no. 2, pp. 126–138, 2005.

[64] T. Tsutsumi, T. Ide, M. Yamato et al., "Modulation of the myocardial redox state by vagal nerve stimulation after experimental myocardial infarction," *Cardiovascular Research*, vol. 77, no. 4, pp. 713–721, 2008.

[65] L. V. Borovikova, S. Ivanova, M. Zhang et al., "Vagus nerve stimulation attenuates the systemic inflammatory response to endotoxin," *Nature*, vol. 405, no. 6785, pp. 458–462, 2000.

[66] D. Aronson, M. A. Mittleman, and A. J. Burger, "Interleukin-6 levels are inversely correlated with heart rate variability in patients with decompensated heart failure," *Journal of Cardiovascular Electrophysiology*, vol. 12, no. 3, pp. 294–300, 2001.

[67] G. A. Lanza, G. A. Sgueglia, D. Cianflone et al., "Relation of heart rate variability to serum levels of C-reactive protein in patients with unstable angina pectoris," *American Journal of Cardiology*, vol. 97, no. 12, pp. 1702–1706, 2006.

[68] M. H. Shishehbor, C. Alves, and V. Rajagopal, "Inflammation: implications for understanding the heart-brain connection," *Cleveland Clinic Journal of Medicine*, vol. 74, no. 1, pp. 37–41, 2007.

[69] L. M. G. de Macedo Braga, K. Rosa, B. Rodrigues et al., "Systemic delivery of adult stem cells improves cardiac function in spontaneously hypertensive rats," *Clinical and Experimental Pharmacology and Physiology*, vol. 35, no. 2, pp. 113–119, 2008.

[70] L. M. G. De Macedo Braga, S. Lacchini, B. D. Schaan et al., "In situ delivery of bone marrow cells and mesenchymal stem cells improves cardiovascular function in hypertensive rats submitted to myocardial infarction," *Journal of Biomedical Science*, vol. 15, no. 3, pp. 365–374, 2008.

[71] C. Malfitano, T. C. Alba Loureiro, B. Rodrigues et al., "Hyperglycaemia protects the heart after myocardial infarction: aspects of programmed cell survival and cell death," *European Journal of Heart Failure*, vol. 12, no. 7, pp. 659–667, 2010.

[72] B. Rodrigues, D. M. T. Figueroa, J. Fang et al., "Short-term diabetes attenuates left ventricular dysfunction and mortality rates after myocardial infarction in rodents," *Clinics*, vol. 66, no. 8, pp. 1437–1442, 2011.

[73] B. Rodrigues, C. T. Mostarda, K. T. Rosa et al., "VEGF165 gene therapy improves left ventricular function and exercise capacity in diabetic rats after myocardial infarction: impact on mortality rate," *Journal of Diabetes & Metabolism*, vol. 4, article 006, 2011.

[74] M. U. P. Brandao, M. Wajngarten, E. Rondon, M. C. P. Giorgi, F. Hironaka, and C. E. Negrao, "Left ventricular function during dynamic exercise in untrained and moderately trained subjects," *Journal of Applied Physiology*, vol. 75, no. 5, pp. 1989–1995, 1993.

[75] A. D'Andrea, P. Caso, S. Severino et al., "Effects of different training protocols on left ventricular myocardial function in competitive athletes: a Doppler tissue imaging study," *Italian Heart Journal*, vol. 3, no. 1, pp. 34–40, 2002.

[76] L. M. Shapiro and R. G. Smith, "Effect of training on left ventricular structure and function. An echocardiographic study," *British Heart Journal*, vol. 50, no. 6, pp. 534–539, 1983.

[77] A. M. Dart, I. T. Meredith, and G. L. Jennings, "Effects of 4 weeks endurance training on cardiac left ventricular structure and function," *Clinical and Experimental Pharmacology and Physiology*, vol. 19, no. 11, pp. 777–783, 1992.

[78] M. L. Smith, D. L. Hudson, H. M. Graitzer, and P. B. Raven, "Exercise training bradycardia: the role of autonomic balance," *Medicine and Science in Sports and Exercise*, vol. 21, no. 1, pp. 40–44, 1989.

[79] K. de Angelis, R. B. Wichi, W. R. A. Jesus et al., "Exercise training changes autonomic cardiovascular balance in mice," *Journal of Applied Physiology*, vol. 96, no. 6, pp. 2174–2178, 2004.

[80] P. C. Brum, G. J. Justo da Silva, E. Dias Moreira, F. Ida, C. E. Negrão, and E. M. Krieger, "Exercise training increases baroreceptor gain sensitivity in normal and hypertensive rats," *Hypertension*, vol. 36, no. 6, pp. 1018–1022, 2000.

[81] F. Favret, K. K. Henderson, R. L. Clancy, J.-P. Richalet, and N. C. Gonzalez, "Exercise training alters the effect of chronic hypoxia on myocardial adrenergic and muscarinic receptor number," *Journal of Applied Physiology*, vol. 91, no. 3, pp. 1283–1288, 2001.

[82] M. T. la Rovere, A. Mortara, G. Sandrone, and F. Lombardi, "Autonomic nervous system adaptations to short-term exercise training," *Chest*, vol. 101, no. 5, supplement, pp. 299S–303S, 1992.

[83] F. Iellamo, J. M. Legramante, M. Massaro, G. Raimondi, and A. Galante, "Effects of a residential exercise training on baroreflex sensitivity and heart rate variability in patients with coronary artery disease: a randomized, controlled study," *Circulation*, vol. 102, no. 21, pp. 2588–2592, 2000.

[84] G. Malfatto, M. Facchini, R. Bragato, G. Branzi, L. Sala, and G. Leonetti, "Short and long term effects of exercise training on the tonic autonomic modulation of heart rate variability after myocardial infarction," *European Heart Journal*, vol. 17, no. 4, pp. 532–538, 1996.

[85] D. G. Martinez, J. C. Nicolau, R. L. Lage et al., "Effects of long-term exercise training on autonomic control in myocardial

infarction patients," *Hypertension*, vol. 58, no. 6, pp. 1049–1056, 2011.

[86] B. Rodrigues, L. Jorge, C. T. Mostarda et al., "Aerobic exercise training delays cardiac dysfunction and improves autonomic control of circulation in diabetic rats undergoing myocardial infarction," *Journal of Cardiac Failure*, vol. 18, no. 9, pp. 734–744, 2012.

[87] N. P. Walsh, M. Gleeson, R. J. Shephard et al., "Position statement part one: immune function and exercise," *Exercise Immunology Review*, vol. 17, pp. 6–63, 2011.

[88] B. K. Pedersen and L. Hoffman-Goetz, "Exercise and the immune system: regulation, integration, and adaptation," *Physiological Reviews*, vol. 80, no. 3, pp. 1055–1081, 2000.

[89] K. Suzuki, S. Nakaji, M. Yamada, M. Totsuka, K. Sato, and K. Sugawara, "Systemic inflammatory response to exhaustive exercise. Cytokine kinetics," *Exercise Immunology Review*, vol. 8, pp. 6–48, 2002.

[90] M. A. Febbraio and B. K. Pedersen, "Muscle-derived interleukin-6: mechanisms for activation and possible biological roles," *FASEB Journal*, vol. 16, no. 11, pp. 1335–1347, 2002.

[91] R. Schindler, J. Mancilla, S. Endres, R. Ghorbani, S. C. Clark, and C. A. Dinarello, "Correlations and interactions in the production of interleukin-6 (IL-6), IL-1, and tumor necrosis factor (TNF) in human blood mononuclear cells: IL-6 suppresses IL-1 and TNF," *Blood*, vol. 75, no. 1, pp. 40–47, 1990.

[92] A. Steensberg, C. P. Fischer, C. Keller, K. Møller, and B. K. Pedersen, "IL-6 enhances plasma IL-1ra, IL-10, and cortisol in humans," *American Journal of Physiology. Endocrinology and Metabolism*, vol. 285, no. 2, pp. E433–E437, 2003.

[93] M. Pretolani, "Interleukin-10: an anti-inflammatory cytokine with therapeutic potential," *Clinical and Experimental Allergy*, vol. 29, no. 9, pp. 1164–1171, 1999.

[94] A. M. W. Petersen and B. K. Pedersen, "The anti-inflammatory effect of exercise," *Journal of Applied Physiology*, vol. 98, no. 4, pp. 1154–1162, 2005.

[95] S. Adamopoulos, J. Parissis, C. Kroupis et al., "Physical training reduces peripheral markers of inflammation in patients with chronic heart failure," *European Heart Journal*, vol. 22, no. 9, pp. 791–797, 2001.

[96] M. L. Batista Jr., J. C. Rosa, R. D. Lopes et al., "Exercise training changes IL-10/TNF-α ratio in the skeletal muscle of post-MI rats," *Cytokine*, vol. 49, no. 1, pp. 102–108, 2010.

[97] J. K. Smith, R. Dykes, J. E. Douglas, G. Krishnaswamy, and S. Berk, "Long-term exercise and atherogenic activity of blood mononuclear cells in persons at risk of developing ischemic heart disease," *Journal of the American Medical Association*, vol. 281, no. 18, pp. 1722–1727, 1999.

[98] S. Adamopoulos, J. Parissis, D. Karatzas et al., "Physical training modulates proinflammatory cytokines and the soluble Fas/soluble Fas ligand system in patients with chronic heart failure," *Journal of the American College of Cardiology*, vol. 39, no. 4, pp. 653–663, 2002.

[99] V. M. Conraads, P. Beckers, J. Bosmans et al., "Combined endurance/resistance training reduces plasma TNF-α receptor levels in patients with chronic heart failure and coronary artery disease," *European Heart Journal*, vol. 23, no. 23, pp. 1854–1860, 2002.

[100] C. F. Grans, D. J. Feriani, M. E. V. Abssamra et al., "Resistance training after myocardial infarction in rats: role on cardiac and autonomic function," *Arquivos Brasileiros de Cardiologia*. In press.

[101] C. Brandt and B. K. Pedersen, "The role of exercise-induced myokines in muscle homeostasis and the defense against chronic diseases," *Journal of Biomedicine and Biotechnology*, vol. 2010, Article ID 520258, 6 pages, 2010.

[102] N. Mathur and B. K. Pedersen, "Exercise as a mean to control low-grade systemic inflammation," *Mediators of Inflammation*, vol. 2008, Article ID 109502, 6 pages, 2008.

[103] M. G. Flynn and B. K. McFarlin, "Toll-like receptor 4: link to the anti-inflammatory effects of exercise?" *Exercise and Sport Sciences Reviews*, vol. 34, no. 4, pp. 176–181, 2006.

[104] B. K. Pedersen and M. A. Febbraio, "Muscle as an endocrine organ: focus on muscle-derived interleukin-6," *Physiological Reviews*, vol. 88, no. 4, pp. 1379–1406, 2008.

[105] M. Gleeson, B. McFarlin, and M. Flynn, "Exercise and toll-like receptors," *Exercise Immunology Review*, vol. 12, pp. 34–53, 2006.

[106] N. Kawanishi, H. Yano, Y. Yokogawa, and K. Suzuki, "Exercise training inhibits inflammation in adipose tissue via both suppression of macrophage infiltration and acceleration of phenotypic switching from M1 to M2 macrophages in high-fat-diet-induced obese mice," *Exercise Immunology Review*, vol. 16, pp. 105–118, 2010.

[107] J. S. Yudkin, "Inflammation, obesity, and the metabolic syndrome," *Hormone and Metabolic Research*, vol. 39, no. 10, pp. 707–709, 2007.

[108] P. P. Mujumdar, P. J. Duerksen-Hughes, A. F. Firek, and D. A. Hessinger, "Long-term, progressive, aerobic training increases adiponectin in middle-aged, overweight, untrained males and females," *Scandinavian Journal of Clinical and Laboratory Investigation*, vol. 71, no. 2, pp. 101–107, 2011.

[109] O. Ben Ounis, M. Elloumi, G. Lac et al., "Two-month effects of individualized exercise training with or without caloric restriction on plasma adipocytokine levels in obese female adolescents," *Annales d'Endocrinologie*, vol. 70, no. 4, pp. 235–241, 2009.

[110] S. Lim, H. C. Sung, I.-K. Jeong et al., "Insulin-sensitizing effects of exercise on adiponectin and retinol-binding protein-4 concentrations in young and middle-aged women," *Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 6, pp. 2263–2268, 2008.

[111] R. B. Nunes, J. P. Alves, L. P. Kessler, and P. dal Lago, "Aerobic exercise improves the inflammatory profile correlated with cardiac remodeling and function in chronic heart failure rats," *Clinics*, vol. 68, no. 6, pp. 876–882, 2013.

[112] M. Gleeson, N. C. Bishop, D. J. Stensel, M. R. Lindley, S. S. Mastana, and M. A. Nimmo, "The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease," *Nature Reviews Immunology*, vol. 11, no. 9, pp. 607–610, 2011.

[113] G. I. Lancaster, Q. Khan, P. Drysdale et al., "The physiological regulation of toll-like receptor expression and function in humans," *Journal of Physiology*, vol. 563, no. 3, pp. 945–955, 2005.

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Research Article

Treadmill Training Increases SIRT-1 and PGC-1α Protein Levels and AMPK Phosphorylation in Quadriceps of Middle-Aged Rats in an Intensity-Dependent Manner

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The present study investigated the effects of running at 0.8 or 1.2 km/h on inflammatory proteins (i.e., protein levels of TNF-α, IL-1β, and NF-κB) and metabolic proteins (i.e., protein levels of SIRT-1 and PGC-1α, and AMPK phosphorylation) in quadriceps of rats. Male Wistar rats at 3 (young) and 18 months (middle-aged rats) of age were divided into nonexercised (NE) and exercised at 0.8 or 1.2 km/h. The rats were trained on treadmill, 50 min per day, 5 days per week, during 8 weeks. Forty-eight hours after the last training session, muscles were removed, homogenized, and analyzed using biochemical and western blot techniques. Our results showed that: (a) running at 0.8 km/h decreased the inflammatory proteins and increased the metabolic proteins compared with NE rats; (b) these responses were lower for the inflammatory proteins and higher for the metabolic proteins in young rats compared with middle-aged rats; (c) running at 1.2 km/h decreased the inflammatory proteins and increased the metabolic proteins compared with 0.8 km/h; (d) these responses were similar between young and middle-aged rats when trained at 1.2 km. In summary, the age-related increases in inflammatory proteins, and the age-related declines in metabolic proteins can be reversed and largely improved by treadmill training.

1. Introduction

The mitochondrial function impairment in skeletal muscle is one of the physiological limitations of aging [1]. However, there is a considerable variability of aging impact on mitochondrial function [2–6]. Sarcopenia and muscle fatigability in response to aging are associated with the increase of both the reactive oxygen species (ROS) production and the mitochondrial apoptotic susceptibility, as well as the decrease of transcriptional drive for mitochondrial biogenesis [3]. However, the precise underlying mechanisms of these processes remain unclear. Some common regulatory mechanisms that include silent information regulators like sirtuins have been studied [7–9]. The silent information regulator 2 homolog

1 (SIRT-1) may influence the aging processes and many age-associated diseases, including metabolic disorders such as diabetes. SIRT-1 is downregulated in human senescent cells, suggesting that SIRT-1 may be required to extend the replicative life span [10].

Aging processes are orchestrated in part by powerful deacetylators Sirts [7]. For example, the deacetylation of lysine residues of the histone tails by Sirt induces closed chromatin configuration and transcriptional silencing [8]. Besides histone deacetylation, SIRT-1 targets a number of transcription factors such as peroxisome proliferator-activated receptor gamma coactivator-1alpha (PGC-1α), which is involved in mitochondrial biogenesis and skeletal muscle differentiation [9, 11]. Thus, SIRT-1-mediated PGC-1α deacetylation is a key factor of mitochondrial biogenesis activation [9, 11]. The PGC-1α family of regulated coactivators plays a central role in a regulatory network governing the transcriptional control of mitochondrial biogenesis and respiratory function. These coactivators target multiple transcription factors, including nuclear respiratory factors (NRF) 1 and 2 and the orphan nuclear hormone receptor (estrogen-related receptor alpha—ERRα), among others [12]. In addition, they themselves are the targets of coactivator and corepressor complexes that regulate gene expression through chromatin remodeling.

The proposed role for PGC-1α as a regulator of mitochondrial biogenesis is supported by experiments that verified functional improvements in both cultured cells [12] and transgenic mice [13]. The PGC-1α expression is modulated by extracellular signals that control metabolism, differentiation, or cell growth. In addition, in some cases, PGC-1α activity is regulated by posttranslational modification by the energy sensors, such as SIRT-1 and AMP-activated protein kinase (AMPK).

AMPK is emerging as a crucial regulator of whole-body energy balance [14]. In skeletal muscle, after being activated, AMPK regulates both the fatty-acid oxidation by the phosphorylation of acetyl-CoA carboxylase (ACC) and the mitochondrial biogenesis by the increase of the expression of vital proteins for proper mitochondrial function, such as citrate synthase and succinate dehydrogenase [15, 16]. In addition, AMPK promotes mitochondrial biogenesis by the increase of both PGC-1α levels and other associated mitochondrial proteins [17–20].

The activation of AMPK (e.g., by exercise) triggers an increase in the NAD^+/NADH ratio, which activates SIRT-1. AMPK also induces PGC-1α phosphorylation and primes it for subsequent deacetylation by SIRT-1 [21]. The impact of AMPK and SIRT-1 on the PGC-1α acetylation status and other transcriptional regulators will modulate mitochondrial function and activity [21]. In fact, multiple endogenous and exogenous factors regulate mitochondrial biogenesis by PGC-1α, SIRT-1, and AMPK, including physical exercise. Physical exercise is known to induce metabolic adaptations in skeletal muscle via activation of these molecules [3, 22]. However, in the aging process, the influence of different training intensities on molecular alterations remains unclear. Thus, this study was designed to test the hypothesis that high training intensity is more effective than low training intensity in restoring SIRT-1, AMPK, PGC-1α, and related metabolic enzymes that decrease with aging. Based on the fact that aging is related to increased circulating proinflammatory and lower anti-inflammatory cytokines [23] and knowing that besides the role as an activator of mitochondrial biogenesis, PGC-1α also acts as a suppressor of inflammatory cytokines [24], we also investigated the responses of the tumor necrosis factor alpha (TNF-α), interleukin 1β (IL-1β), and nuclear factor kappa-B (NF-κB) to different training intensities in young and middle-aged Wistar rats.

2. Materials and Methods

2.1. Animals Groups. Male Wistar rats at 2 (young) and 18 (middle-aged) months were used during the experiments. The current investigation followed the university guidelines for the use of animals in experimental studies (protocol number 92/2009). The procedures used in this study received approval from the Research Ethics Committee of Universidade do Extremo Sul Catarinense (Criciúma, Brazil). Young and middle-aged animals were divided into the following groups ($n = 6$): nonexercised (NE), exercised at 0.8 km/h (0.8 km/h), and exercised at 1.2 km/h (1.2 km/h). We selected middle-aged rats in order to study molecular and physiological changes related to mitochondrial function during a life period that still allows preventive actions that can lead to healthy aging. All animals were maintained at temperatures ranging from 20 to 25°C, with a 12-hour light/dark cycle and fed on a standard rodent chow *ad libitum*.

2.2. Descriptive Characteristics of Young and Middle-Aged Rats before Exercise Protocols. Before the beginning of the exercise protocols, one set of young and middle-aged rats ($n = 8$) was evaluated for the following parameters: body weight (g), blood glucose (mg/dL), epididymal fat (g/100 g), and serum insulin (ng/mL). The rats were anesthetized with an intraperitoneal (i.p) injection of ketamine chlorohydrate (50 mg/kg; Syntec, Cotia, SP, Brazil) and xylazine (20 mg/kg; Syntec, Cotia, SP, Brazil), and the blood was collected from the cava vein. Serum was separated by centrifugation (1,100 ×g) for 15 min at 4°C and stored at −80°C for further analysis. The epididymal fat was surgically removed, weighted, and expressed as g/100 g of rats' body weight. While rest blood glucose (mg/dL) was measured by a glucometer (Advantage, Boehringer Mannheim, Irvine, CA), the rest serum insulin was determined using a commercially available Enzyme Linked Immunosorbent Assay (ELISA) kit (Crystal Chem Inc., Chicago, IL).

2.3. Exercise Protocols. All animals were habituated on a nine-channel motor-drive treadmill (Insight EP 131, Ribeirão Preto, Brazil) at a velocity of 0.6 km/h for 10 min/day during 1 week in order to reduce stress during the training period. The rats did not receive any electric stimulus to run, but manual stimulation was applied. The exercise group performed a running program at constant speed of 0.8 km/h or 1.2 km/h without inclination for 50 min, 5 days per week, during 8 weeks. The nontrained rats were placed on the switched-off treadmill for the same 8 weeks. Forty-eight hours after

the last training sessions, the rats were anesthetized with an intraperitoneal (i.p) injection of ketamine chlorohydrate (50 mg/kg; Syntec, Cotia, SP, Brazil) and xylazine (20 mg/kg; Syntec, Cotia, SP, Brazil), and quadriceps was removed for biochemical and immunoblotting analyses.

2.4. Protein Analysis by Immunoblotting. The superficial (i.e., rectus femoris composed of type I: 1%, type IIa: 25%, and type IIb: 74%) and deep quadriceps (i.e., vastus intermedius composed of type I: 59%, type IIa: 40%, and type IIb: 1%) [25] were homogenized together in extraction buffer (1% Triton-X 100, 100 mMTris, pH 7.4, containing 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium vanadate, 2 mM PMSF, and 0.1 mg of aprotinin/mL) at 4°C (Polytron MR 2100, Kinematica, Switzerland). The extracts were centrifuged at 11,000 rpm at 4°C (5804R, Eppendorf AG, Hamburg, Germany) for 40 min to remove insoluble material, and the supernatants of this tissue were used for protein quantification, according to the Bradford method. Proteins were denatured by boiling in Laemmli sample buffer containing 100 mM DTT, run on SDS-PAGE, and transferred to nitrocellulose membranes. Membranes were blocked, probed, and blotted with primary antibodies. Antibodies used for immunoblotting were antiphospho (Thr172) AMPK, antiphospho (Ser79) ACC, anti-AMPK, and anti-ACC antibodies (Cell Signaling Technology, Beverly, MA, USA); anti-SIRT-1, anti-PGC-1α, anti-CPT1, anti-SDH, citrate synthase, anti-Cyt-C, and β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); and anti-TNF-α, anti-IL-1β, and anti-NF-κB (Abcam Biotechnology, Eugene, Oregon, USA). The original membrane was stripped and reblotted with β-actin as loading protein. Chemiluminescent detection was performed with horseradish peroxidase-conjugate secondary antibodies (Thermo Scientific, Rockford, IL, USA). Autoradiographs of membranes were taken for visualization of protein bands. The results of the blots are presented as direct comparisons of the area of the apparent bands in autoradiographs and quantified by densitometry using the Scion Image software (Scion Image software, ScionCorp, Frederick, MD).

2.5. Activity of Krebs Cycle Enzymes

2.5.1. Citrate Synthase Activity. Citrate synthase activity was assayed according to the method described by Shepherd and Garland [26]. The reaction mixture contained 100 mMTris, pH 8.0, 100 mM acetyl CoA, 100 mM 5,5′-di-thiobis-(2-nitrobenzoic acid), 0.1% triton X-100, and 2–4 μg supernatant protein, and it was initiated with 100 μM oxaloacetate and monitored at 412 nm for 3 min at 25°C.

2.5.2. Succinate Dehydrogenase Activity. Succinate dehydrogenase activity was determined according to the method of Fischer et al. [27], measured by the following decrease in absorbance due to the reduction of 2,6-dichloroindophenol (2,6-DCIP) at 600 nm with 700 nm as the reference wave length (ε = 19.1 mM^{-1} cm^{-1}) in the presence of phenazine methosulphate (PMS). The reaction mixture consisting of 40 mM potassium phosphate, pH 7.4, 16 mM succinate, and 8 μM 2,6-DCIP was preincubated with 40–80 μg homogenate protein at 30°C for 20 min. Subsequently, 4 mM sodium azide, 7 μM rotenone, and 40 μM 2,6-DCIP were added, and the reaction was initiated by adding 1 mM PMS and was monitored for 5 min.

2.6. Activity of Mitochondrial Respiratory Chain Enzymes

2.6.1. Complex I Activity. NADH dehydrogenase (complex I) was evaluated according to Cassina and Radi [28] by determining the rate of NADH-dependent ferricyanide reduction at λ = 420 nm.

2.6.2. Complex II Activity. The activity of succinate-2,6-dichloroindophenol- (DCIP-) oxidoreductase (complex II) was determined using the method described by Fischer et al. [27]. Complex II activity was measured by following the decrease in absorbance due to the reduction of 2,6-DCIP at λ = 600 nm.

2.6.3. Complex II-III Activity. The activity of succinate: cytochrome *c* oxidoreductase (complex III) was determined using the method described by Fischer et al. [27]. Complex II-III activity was measured by cytochrome *c* reduction using succinate as substrate at λ = 550 nm.

2.6.4. Complex IV Activity. The activity of cytochrome *c* oxidase (complex IV) was assayed according to the method described by Rustin et al. [29] and measured by following the decrease in absorbance due to the oxidation of previously reduced cytochrome *c* (prepared by reduction of cytochrome with $NaBH_4$ and HCl) at λ = 550 nm with 580 nm as the reference wavelength (ε = 19.1 mM^{-1} cm^{-1}). The activities of the mitochondrial respiratory chain complexes were expressed as nmol/min^{-1}/mg of $protein^{-1}$.

2.7. Statistical Analysis. In the present study, the hypothesis is that molecular biomarkers are affected by the following factors: (1) age: young versus middle-aged and (2) training intensity: nontrained versus 0.8 km/h speed versus 1.2 speed km/h. The results about the descriptive characteristics of young and middle-aged rats before exercise protocols were expressed as mean and standard error median (SEM) and were evaluated using the unpaired Student's *t*-test. While the results about the western blot analyses were expressed as the mean area of the apparent band ± SEM (arbitrary units were calculated as area versus density), the activity of Krebs cycle enzymes and the activity of mitochondrial respiratory chain enzymes were expressed as nmol/min/mg of protein ± SEM. Differences between the groups for the mentioned parameters were evaluated using two-way analysis of variance (ANOVA) followed by the Bonferroni *post hoc* test. A probability of less than 0.05 was considered to be significant. The software used for the analysis of the data was the Statistical Package for the Social Sciences (SPSS) version 16.0 for Windows.

Table 1: Descriptive characteristics of young and middle-aged rats ($n = 8$) before exercise protocols expressed as mean ± SEM.

Parameters	Young rats ($n = 8$)	Middle-aged rats ($n = 8$)
Body weight (g)	218.9 ± 10.02	526 ± 12.31*
Epididymal fat (g/100 g)	1.2 ± 0.2	3.9 ± 0.4*
Serum insulin (ng/mL)	1.3 ± 0.5	4.2 ± 0.7*
Blood glucose (mg/dL)	87.7 ± 7.54	92.9 ± 6.92

* $P < 0.05$ significant difference versus young rats.

3. Results

3.1. Descriptive Characteristics of Young and Middle-Aged Rats before Exercise Protocols. According to Table 1, the middle-aged rats presented higher values of body weight (526 ± 12.31 versus 218.9 ± 10.02 g), epididymal fat (3.9 ± 0.4 versus 1.2 ± 0.2 g/100 g), and serum insulin (4.2 ± 0.7 versus 1.3 ± 0.5 ng/mL) compared to young rats.

3.2. TNF-α, IL-1β, and NF-κB Protein Levels in Quadriceps of Young and Middle-Aged Rats after Exercise Training at 0.8 and 1.2 km/h. The protein levels of TNF-α, IL-1β, and NF-κB were higher in the quadriceps of middle-aged NE compared to young NE rats (Figures 1(a)–1(c)). Both groups (i.e., young and middle-aged rats) were trained at 0.8 km/h which decreased the protein levels of TNF-α, IL-1β, and NF-κB compared to their respective NE groups; however, the middle-aged rats presented higher protein levels of TNF-α, IL-1β, and NF-κB compared to young rats at the 0.8 km/h training intensity (Figures 1(a)–1(c)). In addition, both groups were trained at 1.2 km/h which decreased the protein levels of TNF-α, IL-1β, and NF-κB compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figures 1(a)–1(c)). β-actin protein levels were similar between the groups (Figures 1(a)–1(c)—lower panels).

3.3. SIRT-1 and PGC-1α Protein Levels and AMPK and ACC Phosphorylation in Quadriceps of Young and Middle-Aged Rats after Exercise Training at 0.8 and 1.2 km/h. We evaluated pivotal molecules involved in mitochondrial function and oxidative metabolism. While AMPK phosphorylation decreased in the quadriceps of middle-aged NE compared to young NE rats (Figure 2(a)), no significant differences were observed between these groups (i.e., young and middle aged rats) at NE situation for ACC phosphorylation (Figure 2(b)). Both groups (i.e., young and middle-aged rats) were trained at 0.8 km/h which increased AMPK and ACC phosphorylations compared to their respective NE groups; however, the middle-aged rats presented lower AMPK and ACC phosphorylations compared to the young rats at the 0.8 km/h training intensity. In addition, both groups were trained at 1.2 km/h which increased the AMPK and ACC phosphorylation compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figures 2(a) and 2(b)). Total AMPK and ACC protein levels were similar between the groups (Figures 2(a) and 2(b)—lower panels).

SIRT-1 and PGC-1α protein levels decreased in the quadriceps of middle-aged NE rats compared to young NE rats (Figures 2(c) and 2(d)). Both groups (i.e., young and middle-aged rats) were trained at 0.8 km/h which increased the protein levels of SIRT-1 and PGC-1α compared to their respective NE groups; however, the middle-aged rats presented lower protein levels of SIRT-1 and PGC-1α compared to young rats at the 0.8 km/h training intensity. In addition, both groups were trained at 1.2 km/h which increased the protein levels of SIRT-1 and PGC-1α compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figures 2(c) and 2(d)).

3.4. CPT1, Cyt-C, and SDH Protein Levels and SDH and Citrate Synthase Activities in Quadriceps of Young and Middle-Aged Rats after Exercise Training at 0.8 and 1.2 km/h. Subsequently, we determined the protein levels and activities of important metabolic enzymes. Lower protein levels of CPT1 and Cyt-c were observed in the quadriceps of middle-aged NE rats compared to young NE rats. Both groups (i.e., young and middle-aged rats) were trained at 0.8 km/h which increased the protein levels of CPT1 and Cyt-c compared to their respective NE groups; however, the middle-aged rats presented lower protein levels of CPT1 and Cyt-c compared to young rats at the 0.8 km/h. In addition, both groups were trained at 1.2 km/h which increased the protein levels of CPT1 and Cyt-c compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figures 3(a) and 3(b)). The SDH protein levels did not present significant differences between young and middle-aged rats for the studied experimental situations (i.e., NE, 0.8 and 12 km/h; Figure 3(c)). The SDH activity was not different between young and middle-aged rats for the NE situation. The young rats were trained at 0.8 km/h which increased the SDH activity compared to their respective NE group. On the other hand, SDH activity was lower in middle-aged rats compared to young rats at the 0.8 km/h training intensity. In addition, middle-aged rats were trained at 1.2 km/h which increased the SDH activity compared to their respective 0.8 km/h group, but no significant differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figure 3(d)).

The protein levels and activity of citrate synthase were lower in the quadriceps of middle-aged NE rats compared to young NE rats. Both groups (i.e., young and middle-aged rats) were trained at 0.8 km/h which increased the protein levels of citrate synthase compared to their respective NE groups; however, the middle-aged rats presented lower protein levels and activity of citrate synthase compared to young rats at the 0.8 km/h. In addition, both groups were trained at 1.2 km/h which increased the protein levels and activity of citrate synthase compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figures 3(e) and 3(f)).

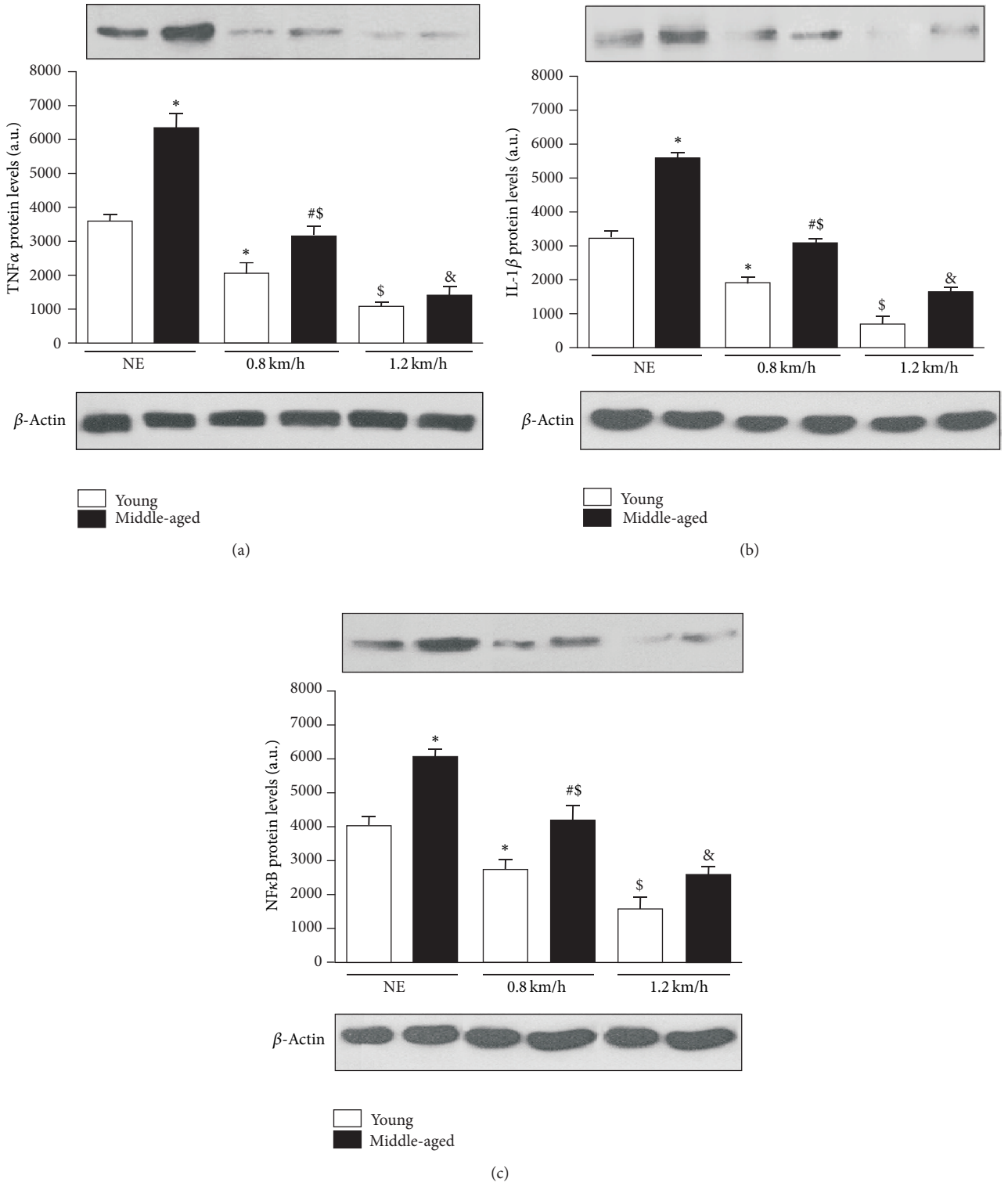

Figure 1: Effects of exercise protocols performed in different intensities on the TNF-α, IL-1β, and NF-κB protein levels in the quadriceps of young and middle-aged rats. Protein levels of TNF-α (a), IL-1β (b), and NF-κB (c). Upper panels show representative blots of these proteins. Lower panels show representative blots of β-actin (a)–(c) protein levels. The results of scanning densitometry are expressed as arbitrary units. Bars represent means ± SEM of six rats. $^{*}P < 0.05$ versus young NE rats, $^{\#}P < 0.05$ versus middle-aged NE rats, $^{\$}P < 0.05$ versus young rats at 0.8 km/h, and $^{\&}P < 0.05$ versus middle-aged rats at 0.8 km/h.

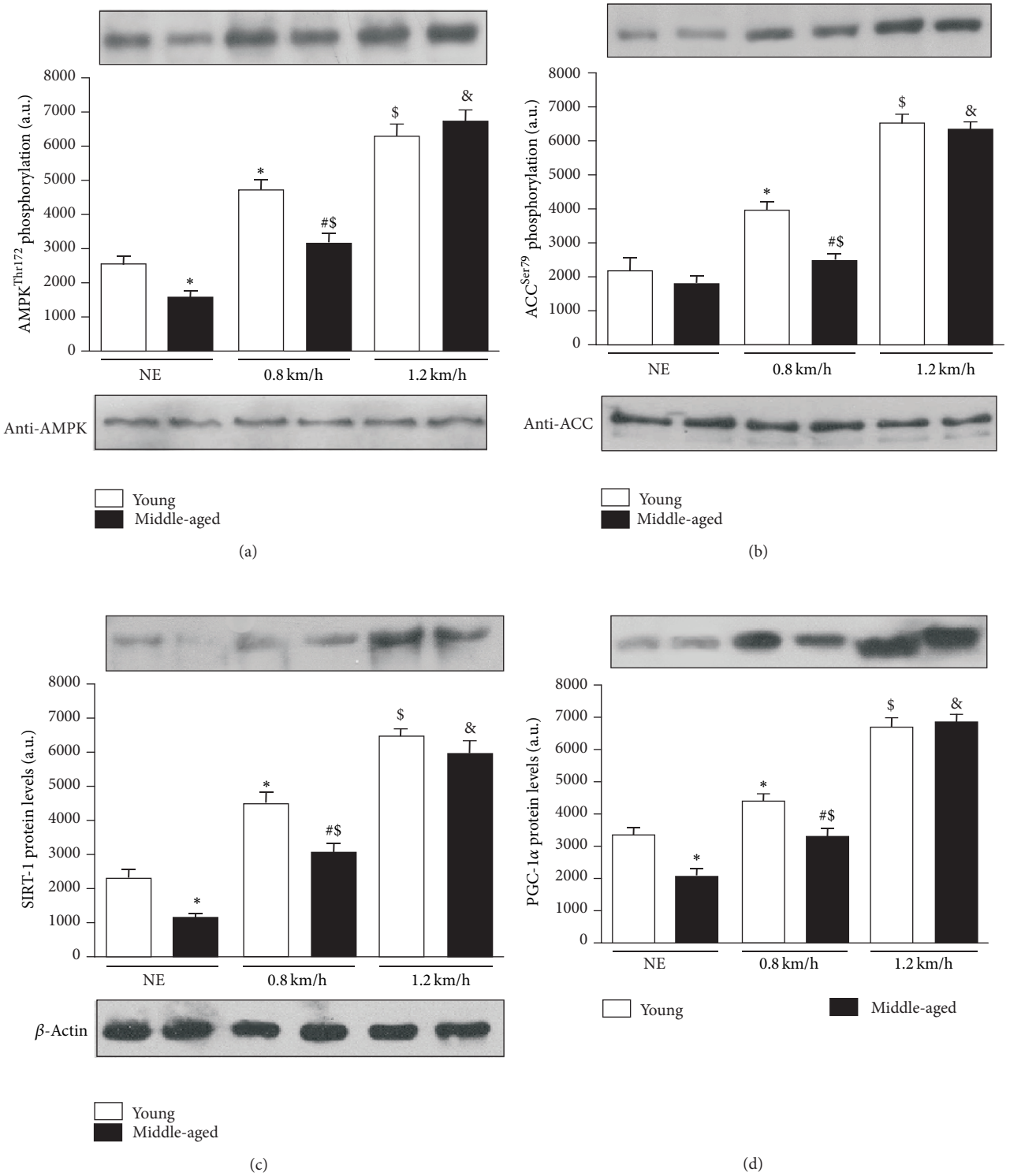

Figure 2: Effects of exercise protocols performed in different intensities on the AMPK and ACC phosphorylation and SIRT-1 and PGC-1α protein levels in the quadriceps of young and middle-aged rats. Phosphorylation of the AMPK (a) and ACC (b) and protein levels of SIRT-1 (c) and PGC-1α (d). Upper panels show representative blots of these proteins. Lower panels show representative blots of total AMPK (a) and total ACC (b) protein levels. The results of scanning densitometry are expressed as arbitrary units. Bars represent means ± SEM of six rats. $^{*}P < 0.05$ versus young NE rats, $^{\#}P < 0.05$ versus middle-aged NE rats, $^{\$}P < 0.05$ versus young rats at 0.8 km/h, and $^{\&}P < 0.05$ versus middle-aged rats at 0.8 km/h.

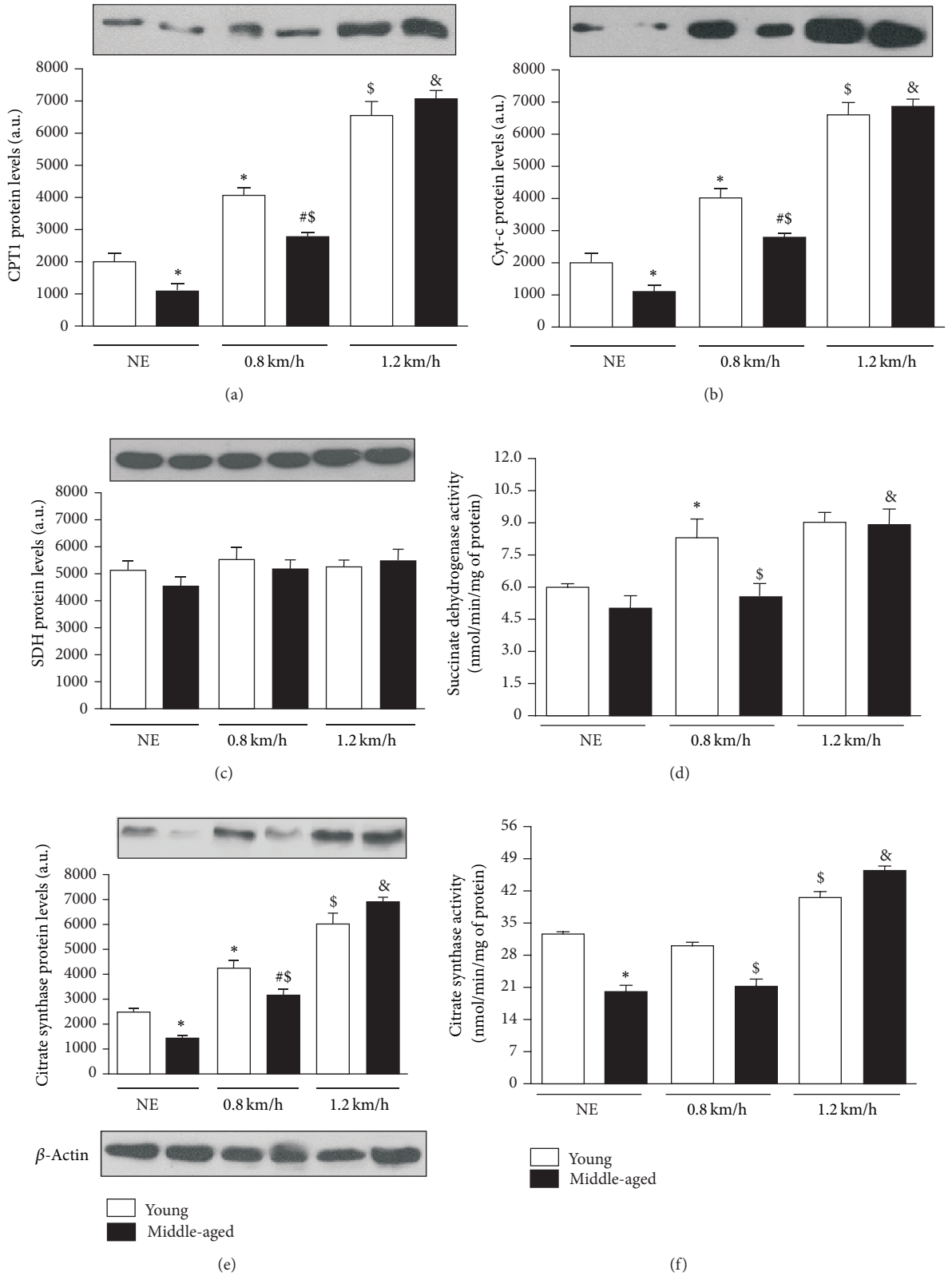

Figure 3: Effects of exercise protocols performed in different intensities on the CPT1, Cyt-c, citrate synthase, and SDH protein levels and SDH and citrate synthase activity in the quadriceps of young and middle-aged rats. Protein levels of CPT1 (a), Cyt-c (b), SDH (c), and citrate synthase (e). Upper panels show representative blots of these proteins. The results of scanning densitometry are expressed as arbitrary units. The activities of succinate dehydrogenase (d) and citrate synthase (f) are expressed as nmol/min/mg of protein. Bars represent means ± SEM of six rats. $^{*}P < 0.05$ versus young NE rats, $^{\#}P < 0.05$ versus middle-aged NE rats, $^{\$}P < 0.05$ versus young rats at 0.8 km/h, and $^{\&}P < 0.05$ versus middle-aged rats at 0.8 km/h.

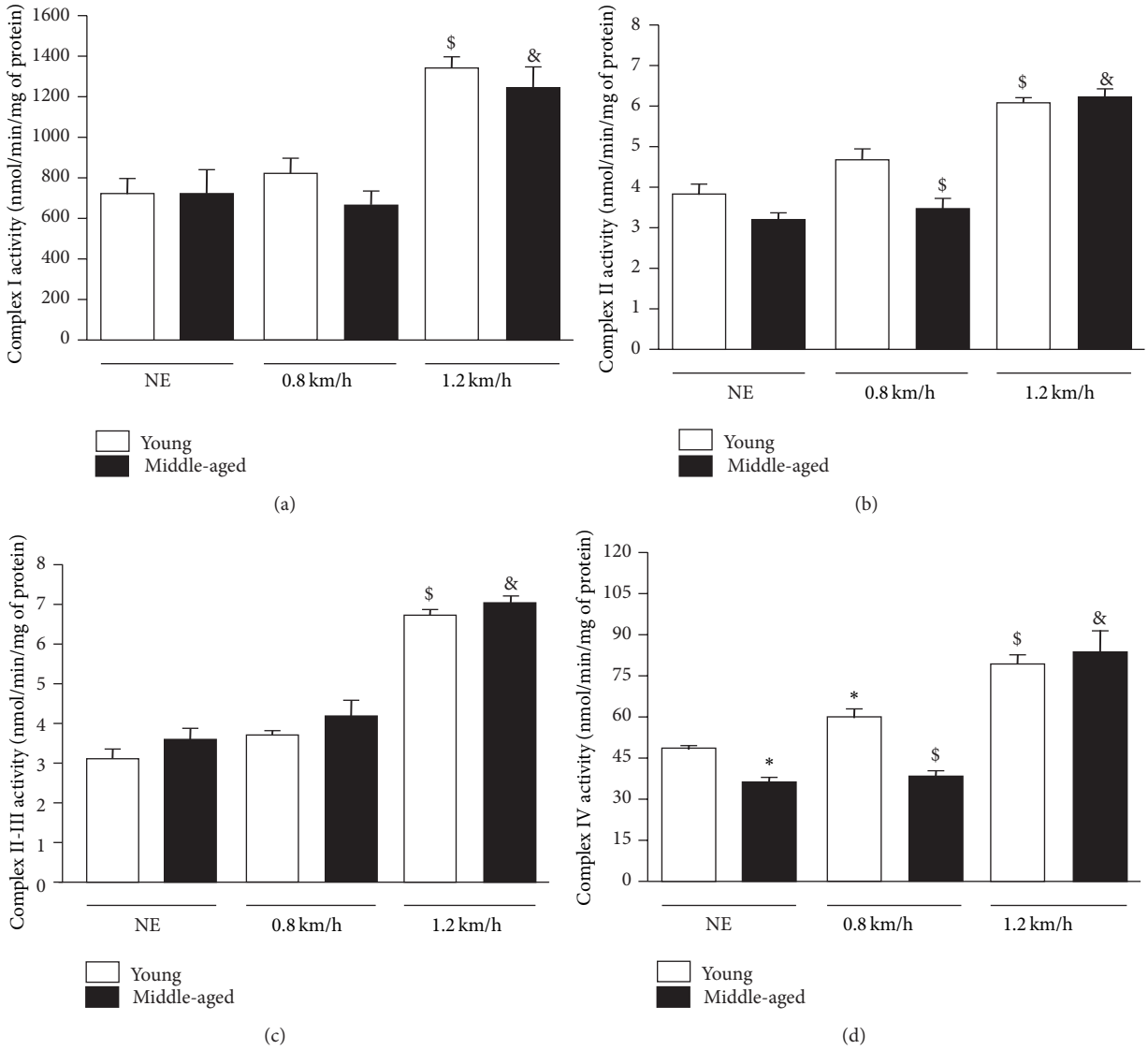

Figure 4: Effects of exercise protocols performed in different intensities on the complexes I, II, II-III, and IV activities in the quadriceps of young and middle-aged rats. Complexes I (a), II (b), II-III (c), and IV (d) activities are expressed as nmol/min/mg of protein. $^{*}P < 0.05$ versus young NE rats, $^{\#}P < 0.05$ versus middle-aged NE rats, $^{\$}P < 0.05$ versus young rats at 0.8 km/h, and $^{\&}P < 0.05$ versus middle-aged rats at 0.8 km/h.

3.5. Mitochondrial Complexes Activities in Quadriceps in Young and Middle-Aged Rats after Exercise Training at 0.8 and 1.2 km/h. The young and middle-aged rats did not present significant alterations for the complexes I, II, and II-III activities at the NE and 0.8 km/h situations. However, the young and middle-aged rats were trained at 1.2 km/h which increased the complexes I, II, and II-III activities compared to their respective 0.8 km/h groups. In addition, for these complexes, we did not observe significant differences between young and middle-aged rats at the 1.2 km/h training intensity (Figures 4(a)–4(c)). The complex II activity was higher in young rats compared to middle-aged rats at the 0.8 km/h training intensity (Figure 4(b)). The complex IV activity decreased in the quadriceps of middle-aged NE rats compared to young NE rats. The young rats were trained at 0.8 km/h which increased the complex IV activity compared to their respective NE group. The complex IV activity of the middle-aged rats was lower compared to young rats at the 0.8 km/h training intensity. In addition, both groups (i.e., young and middle-aged rats) were trained at 1.2 km/h which increased the complex IV activity compared to their respective 0.8 km/h groups, but no differences were observed between young and middle-aged rats at the 1.2 km/h training intensity (Figure 4(d)).

4. Discussion

The main findings of the present investigation are (a) the aging process is associated with the increase of TNF-α, IL-1β,

and NF-κB protein levels and with the decrease of the AMPK phosphorylation and SIRT-1 and PGC-1α protein levels; (b) the young and middle-aged rats were trained at 0.8 km/h which decreased the TNF-α, IL-1β, and NF-κB protein levels and increased the AMPK phosphorylation and SIRT-1 and PGC-1α protein levels compared to their respective NE groups, although the young rats presented lower values (for TNF-α, IL-1β, and NF-κB) and higher values (for SIRT-1, PGC-1α, and AMPK) compared to the middle-aged rats inside this specific training intensity; (c) the young and middle-aged rats were trained at 1.2 km/h which decreased the TNF-α, IL-1β, and NF-κB protein levels and increased the AMPK phosphorylation and SIRT-1 and PGC-1α protein levels compared to their respective 0.8 km/h groups; (d) interestingly, at this training intensity (i.e., 1.2 km/h), the behavior of the analyzed molecules was not different between young and middle-aged rats.

The inflamm-aging process is associated with high levels of TNF-α, IL-1β, and NF-κB in skeletal muscle of elderly individuals and rodents [23, 30]. The present results are in accordance with Della Gata et al. [23] showing that exercise training is able to revert partially the high levels of cytokines induced by the inflamm-aging process. On the other hand, up to today, this is the first investigation showing that high training intensities lead to better responses of TNF-α, IL-1β, and NF-κB in skeletal muscle of middle-aged rats. These responses may be linked to the increases observed in the protein levels of PGC-1α, once this molecule acts as a suppressor of inflammatory cytokines [24].

Aging process is associated with the reduction of the muscle functionality that, at least in part, is linked to mitochondrial dysfunction [1, 31–34]. Some studies have shown that key molecules are involved in this process, such as SIRT-1, AMPK, and PGC-1α [9–12, 22, 32]. The role of SIRT-1 in skeletal muscle is mainly attributed to its ability to deacetylate and activate PGC-1α [9, 12, 22]. PGC-1α orchestrates the genetic program that allows skeletal muscle cell adaptation to meet the energy demands. Ectopic expression of PGC-1α in myotubes increases the respiratory chain gene expression (i.e., cytochrome c) and promotes mitochondrial biogenesis [12]. Furthermore, it is well known that AMPK upregulates SIRT-1 activity in skeletal muscles [21, 35]. The AMPK activity inhibition prevents the deacetylation of PGC-1α by SIRT-1 in skeletal muscle cells in response to glucose deprivation, decreasing the mitochondrial complex activity and damaging the fatty acid oxidation metabolism [21, 35]. In addition, the study of AMPK deficient mice evidenced the impairment of PGC-1α deacetylation in correlation with a failure of muscle metabolism adaptation in response to exercise [21, 35].

Thus, in the present investigation, we tested the hypothesis that high training intensity is more effective than low training intensity in restoring SIRT-1, AMPK, PGC-1α, and related metabolic enzymes that decrease with aging. In summary, these molecules increased when the experimental groups were trained at 0.8 km/h (i.e., compared to their respective NE groups) and at 1.2 km/h (i.e., compared to their respective 0.8 km/h groups). In addition, these molecules were higher in young rats compared to middle-aged rats at the 0.8 km/h training intensity but were not different at the 1.2 km/h training intensity. These findings suggest that middle-aged rats will present the same molecular responses as young rats when the training intensity is high. In accordance with the elegant study of Koltai et al. [6], we demonstrated that treadmill training may reverse the negative effects of aging on pivotal molecules that are associated with the mitochondrial control of skeletal muscle of rats. In fact, Koltai et al. [6] also verified that treadmill training increased the SIRT-1 activity in both young and aged rats (26 months of age).

The elegant study of Cantó et al. [35] showed that AMPK activation precedes and determines the changes in SIRT-1 activity in situations of energy stress. The regulation of the acetylation levels of transcriptional regulators through the AMPK/SIRT-1 axis provides a mechanism by which mitochondrial and lipid oxidation genes can be rapidly and selectively controlled in response to energy levels [21, 34, 36]. In the present investigation, we observed that the phosphorylation of AMPK and the protein levels of SIRT-1 and PGC-1α decreased in the quadriceps of NE middle-aged rats compared to the NE young rats. On the other hand, these proteins increased when the experimental groups were trained at 0.8 km/h compared to their respective NE groups, although the results of the young rats were higher compared to the middle-aged rats at 0.8 km/h training intensity. However, these proteins remained unchanged between young and middle-aged rats at 1.2 km/h training intensity. Tobina et al. [24] cited that AMPK is activated by the decrease in the ATP/AMP ratio and phosphocreatine (CP) and glycogen levels. On the other hand, the CP and glycogen depletion are dependent on the exercise intensity [37, 38]. Although we did not measure the CP and glycogen concentrations in the present paper, it is possible to hypothesize that the higher training intensity led to higher depletion of these substrates and was responsible for the higher AMPK activation.

Similar results were observed for CPT1 and Cyt-c protein levels. Interestingly, Suwa et al. [39] observed that the protein expressions of SIRT-1 but not PGC-1α increased in response to training. The differences between our data and the results of Suwa et al. [39] may be related to the differences in the exercise protocols and age of the studied rats. Aging process may impair the oxidative capacity of skeletal muscles [33]. The mitochondrial respiratory chain is composed of four respiratory complexes (complexes I–IV), and each one is capable of catalyzing electron transfers in a partial reaction of the respiratory chain. It is known that citrate synthase and succinate dehydrogenase activities can be used to estimate mitochondrial content [40] and mitochondrial complexes can reflect the mitochondrial oxidative capacity. In accordance with the results of AMPK phosphorylation, SIRT-1 and PGC-1α protein levels, the activity and protein levels of citrate synthase, and the activities of the mitochondrial complexes I, II, II-III, and IV also increased in both groups (i.e., young and middle-aged rats) at the 1.2 km/h training intensity.

Studying AMPK knockout mice, Jørgensen et al. [41] reported significant reductions in mitochondrial markers (i.e., citrate synthase activity and protein contents of one or more complexes in the mitochondrial respiratory chain). On the other hand, SIRT-1 played a crucial role in the ability of AMPK increasing the mitochondrial respiration, once the

long-term AICAR effects on cellular oxygen consumption were blunted by knocking-down SIRT-1 [35]. In addition, the authors also observed a decrease in the lipid oxidation and an increase in alternative substrate oxidation [35]. In fact, the direct oleate oxidation measurement confirmed that the AICAR chronic effects on lipid oxidation were blunted in myotubes when the SIRT-1 expression was knocked down [35]. Therefore, the AMPK and SIRT-1 signaling pathways have similar effects on lifespan, aging, and metabolism. Like SIRT-1, AMPK has been considered as one of several molecules involved in the mammalian longevity regulation [42].

Other investigations have verified the training effects on the current studied parameters in aged rats [22, 43]. For example, Ljubicic and Hood [44] examined a very extreme form of muscle activation, chronic electrical stimulation, in rats (i.e., 36 months of age), and they verified that the mitochondrial biogenesis signaling response was compromised. These data indicate that high exercise intensity (or muscle activation) in aged rats is not sufficient to correct the age-related decline in the muscle aerobic plasticity. Recently, Bayod et al. [22] observed that treadmill training during 36 weeks increased the protein content and activity of SIRT-1 and the protein expression of PGC-1α in the heart, muscle, and liver of aged rats. However, no changes in AMPK activation or mitochondrial biogenesis were found after 36-week treadmill training.

The aging process is associated with the decline of both AMPK and SIRT-1 activities. For example, the AMPK activation by AICAR or exercise is blunted in skeletal muscles of old rats (i.e., 28 months of age). Furthermore, the mitochondrial biogenesis was also reduced after chronic activation of AMPK with α-guanidinopropionic acid (α-GPA) [19]. On the other hand, SIRT-1 protein levels are diminished in mouse embryonic fibroblasts that exhibit premature senescence [45]. However, as previously mentioned, there is a considerable variability of aging impact on mitochondrial function [2–6]. The different types of studied rodents, their respective aging phases, and the analyzed parameters may be considered by the discrepancies mentioned above, justifying the new investigations to elucidate the relationship between aging and its molecular consequences. In addition, the selection of middle-aged rats to study molecular and physiological changes related to mitochondrial function is justified because this life period still allows the use of preventive actions that can lead to healthy aging.

In summary, we conclude that age-related increases in TNF-α, IL-1β, and NF-κB and age-related declines in the phosphorylation of AMPK and in the protein levels of SIRT-1 and PGC-1α in skeletal muscle can be reversed and largely improved by treadmill exercise training. In addition, the present data demonstrated that to achieve changes in muscle of middle-aged rats that are similar to young rats, high training intensities are necessary.

Conflict of Interests

The authors have no conflict of interests.

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References

[1] I. R. Lanza and K. S. Nair, "Regulation of skeletal muscle mitochondrial function: genes to proteins," *Acta Physiologica*, vol. 199, no. 4, pp. 529–547, 2010.

[2] E. Hütter, M. Skovbro, B. Lener et al., "Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle," *Aging Cell*, vol. 6, no. 2, pp. 245–256, 2007.

[3] B. Chabi, V. Ljubicic, K. J. Menzies, J. H. Huang, A. Saleem, and D. A. Hood, "Mitochondrial function and apoptotic susceptibility in aging skeletal muscle," *Aging Cell*, vol. 7, no. 1, pp. 2–12, 2008.

[4] M. Picard, D. Ritchie, K. J. Wright et al., "Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers," *Aging Cell*, vol. 9, no. 6, pp. 1032–1046, 2010.

[5] M. Picard, D. Ritchie, M. M. Thomas, K. J. Wright, and R. T. Hepple, "Alterations in intrinsic mitochondrial function with aging are fiber type-specific and do not explain differential atrophy between muscles," *Aging Cell*, vol. 10, no. 6, pp. 1047–1055, 2011.

[6] E. Koltai, N. Hart, A. W. Taylor et al., "Age-associated declines in mitochondrial biogenesis and protein quality control factors are minimized by exercise training," *American Journal of Physiology: Regulatory, Integrative and Comparative Physiology*, vol. 303, no. 2, pp. 127–134, 2012.

[7] M. Porcu and A. Chiarugi, "The emerging therapeutic potential of sirtuin-interacting drugs: from cell death to lifespan extension," *Trends in Pharmacological Sciences*, vol. 26, no. 2, pp. 94–103, 2005.

[8] M. D. Shahbazian and M. Grunstein, "Functions of Site-Specific histone acetylation and deacetylation," *Annual Review of Biochemistry*, vol. 76, pp. 75–100, 2007.

[9] S. Lavu, O. Boss, P. J. Elliott, and P. D. Lambert, "Sirtuins—novel therapeutic targets to treat age-associated diseases," *Nature Reviews Drug Discovery*, vol. 7, no. 10, pp. 841–853, 2008.

[10] E. Michishita, J. Y. Park, J. M. Burneskis, J. C. Barrett, and I. Horikawa, "Evolutionarily conserved and nonconserved cellular localizations and functions of human SIRT proteins," *Molecular Biology of the Cell*, vol. 16, no. 10, pp. 4623–4635, 2005.

[11] M. Fulco, R. L. Schiltz, S. Iezzi et al., "Sir2 regulates skeletal muscle differentiation as a potential sensor of the redox state," *Molecular Cell*, vol. 12, no. 1, pp. 51–62, 2003.

[12] Z. Wu, P. Puigserver, U. Andersson et al., "Mechanisms controlling mitochondrial biogenesis and respiration through the thermogenic coactivator PGC-1," *Cell*, vol. 98, no. 1, pp. 115–124, 1999.

[13] J. J. Lehman, P. M. Barger, A. Kovacs, J. E. Saffitz, D. M. Medeiros, and D. P. Kelly, "Peroxisome proliferator-activated receptor γ coactivator-1 promotes cardiac mitochondrial biogenesis," *The Journal of Clinical Investigation*, vol. 106, no. 7, pp. 847–856, 2000.

[14] B. B. Kahn, T. Alquier, D. Carling, and D. G. Hardie, "AMP-activated protein kinase: ancient energy gauge provides clues to

modern understanding of metabolism," *Cell Metabolism*, vol. 1, no. 1, pp. 15–25, 2005.

[15] G. F. Merrill, E. J. Kurth, D. G. Hardie, and W. W. Winder, "AICA riboside increases AMP-activated protein kinase, fatty acid oxidation, and glucose uptake in rat muscle," *The American Journal of Physiology*, vol. 273, no. 6, pp. 1107–1112, 1997.

[16] W. W. Winder, H. A. Wilson, D. G. Hardie, B. B. Rasmussen, C. A. Hutber, and G. B. Call, "Phosphorylation of rat muscle acetyl-CoA carboxylase by AMP-activated protein kinase and protein kinase," *Journal of Applied Physiology*, vol. 82, no. 1, pp. 219–225, 1997.

[17] R. Bergeron, J. M. Ren, K. S. Cadman et al., "Chronic activation of AMP kinase results in NRF-1 activation and mitochondrial biogenesis," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 281, no. 6, pp. E1340–E1346, 2001.

[18] H. Zong, J. M. Ren, L. H. Young et al., "AMP kinase is required for mitochondrial biogenesis in skeletal muscle in response to chronic energy deprivation," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 99, no. 25, pp. 15983–15987, 2002.

[19] R. M. Reznick, H. Zong, J. Li et al., "Aging-associated reductions in AMP-activated protein kinase activity and mitochondrial biogenesis," *Cell Metabolism*, vol. 5, no. 2, pp. 151–156, 2007.

[20] F. R. Jornayvaz and G. I. Shulman, "Regulation of mitochondrial biogenesis," *Essays in Biochemistry*, vol. 47, pp. 69–84, 2010.

[21] C. Cantó, L. Q. Jiang, A. S. Deshmukh et al., "Interdependence of AMPK and SIRT1 for metabolic adaptation to fasting and exercise in skeletal muscle," *Cell Metabolism*, vol. 11, no. 3, pp. 213–219, 2010.

[22] S. Bayod, J. Del Valle, J. F. Lalanza et al., "Long-term physical exercise induces changes in sirtuin 1 pathway and oxidative parameters in adult rat tissues," *Experimental Gerontology*, vol. 47, no. 12, pp. 925–935, 2012.

[23] P. A. Della Gata, A. P. Garnham, J. M. Peake, and D. Cameron-Smith, "Effect of exercise training on skeletal muscle cytokine expression in the elderly," *Brain, Behavior, and Immunity*. In press.

[24] T. Tobina, K. Yoshioka, A. Hirata, S. Mori, A. Kiyonaga, and H. Tanaka, "Peroxisomal proliferator-activated receptor gamma co-activator-1 alpha gene expression increases above the lactate threshold in human skeletal muscle," *The Journal of Sports Medicine and Physical Fitness*, vol. 51, no. 4, pp. 683–688, 2011.

[25] R. B. Armstrong and R. O. Phelps, "Muscle fiber type composition of the rat hindlimb," *American Journal of Anatomy*, vol. 171, no. 3, pp. 259–272, 1984.

[26] D. Shepherd and P. B. Garland, "The kinetic properties of citrate synthase from rat liver mitochondria," *The Biochemical Journal*, vol. 114, no. 3, pp. 597–610, 1969.

[27] J. C. Fischer, W. Ruitenbeek, and J. A. Berden, "Differential investigation of the capacity of succinate oxidation in human skeletal muscle," *Clinica Chimica Acta*, vol. 153, no. 1, pp. 23–26, 1985.

[28] A. Cassina and R. Radi, "Differential inhibitory action of nitric oxide and peroxynitrite on mitochondrial electron transport," *Archives of Biochemistry and Biophysics*, vol. 328, no. 2, pp. 309–316, 1996.

[29] P. Rustin, D. Chretien, T. Bourgeron et al., "Biochemical and molecular investigations in respiratory chain deficiencies," *Clinica Chimica Acta*, vol. 228, no. 1, pp. 35–51, 1994.

[30] J. S. Tilstra, A. R. Robinson, J. Wang et al., "NF-κB inhibition delays DNA damage-induced senescence and aging in mice," *The Journal of Clinical Investigation*, vol. 122, no. 7, pp. 2601–2612, 2012.

[31] P. Gianni, K. J. Jan, M. J. Douglas, P. M. Stuart, and M. A. Tarnopolsky, "Oxidative stress and the mitochondrial theory of aging in human skeletal muscle," *Experimental Gerontology*, vol. 39, no. 9, pp. 1391–1400, 2004.

[32] K. R. Short, M. L. Bigelow, J. Kahl et al., "Decline in skeletal muscle mitochondrial function with aging in humans," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 102, no. 15, pp. 5618–5623, 2005.

[33] E. Bua, J. Johnson, A. Herbst et al., "Mitochondrial DNA-deletion mutations accumulate intracellularly to detrimental levels in aged human skeletal muscle fibers," *American Journal of Human Genetics*, vol. 79, no. 3, pp. 469–480, 2006.

[34] P. S. Pardo and A. M. Boriek, "The physiological roles of Sirt1 in skeletal muscle," *Aging*, vol. 3, no. 4, pp. 430–437, 2011.

[35] C. Cantó, Z. Gerhart-Hines, J. N. Feige et al., "AMPK regulates energy expenditure by modulating NAD+ metabolism and SIRT1 activity," *Nature*, vol. 458, no. 7241, pp. 1056–1060, 2009.

[36] J. N. Feige, M. Lagouge, C. Canto et al., "Specific SIRT1 activation mimics low energy levels and protects against diet-induced metabolic disorders by enhancing fat oxidation," *Cell Metabolism*, vol. 8, no. 5, pp. 347–358, 2008.

[37] T. E. Jensen and E. A. Richter, "Regulation of glucose and glycogen metabolism during and after exercise," *Journal of Physiology*, vol. 590, no. 5, pp. 1069–1076, 2012.

[38] M. J. McKenna, J. Bangsbo, and J.-M. Renaud, "Muscle K^+, Na^+, and Cl^- disturbances and Na^+-K^+ pump inactivation: implications for fatigue," *Journal of Applied Physiology*, vol. 104, no. 1, pp. 288–295, 2008.

[39] M. Suwa, H. Nakano, Z. Radak, and S. Kumagai, "Endurance exercise increases the SIRT1 and peroxisome proliferator-activated receptor γ coactivator-1α protein expressions in rat skeletal muscle," *Metabolism: Clinical and Experimental*, vol. 57, no. 7, pp. 986–998, 2008.

[40] U. F. Rasmussen, P. Krustrup, M. Kjær, and H. N. Rasmussen, "Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria," *Experimental Gerontology*, vol. 38, no. 8, pp. 877–886, 2003.

[41] S. B. Jørgensen, J. F. P. Wojtaszewski, B. Viollet et al., "Effects of α-AMPK knockout on exercise-induced gene activation in mouse skeletal muscle," *FASEB Journal*, vol. 19, no. 9, pp. 1146–1148, 2005.

[42] M. F. McCarty, "Chronic activation of AMP-activated kinase as a strategy for slowing aging," *Medical Hypotheses*, vol. 63, no. 2, pp. 334–339, 2004.

[43] A. C. Betik, M. M. Thomas, K. J. Wright, C. D. Riel, and R. T. Hepple, "Exercise training from late middle age until senescence does not attenuate the declines in skeletal muscle aerobic function," *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 297, no. 3, pp. 744–755, 2009.

[44] V. Ljubicic and D. A. Hood, "Diminished contraction-induced intracellular signaling towards mitochondrial biogenesis in aged skeletal muscle," *Aging Cell*, vol. 8, no. 4, pp. 394–404, 2009.

[45] T. Sasaki, B. Maier, A. Bartke, and H. Scrable, "Progressive loss of SIRT1 with cell cycle withdrawal," *Aging Cell*, vol. 5, no. 5, pp. 413–422, 2006.

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Review Article

Lymphocyte Glucose and Glutamine Metabolism as Targets of the Anti-Inflammatory and Immunomodulatory Effects of Exercise

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Glucose and glutamine are important energetic and biosynthetic nutrients for T and B lymphocytes. These cells consume both nutrients at high rates in a function-dependent manner. In other words, the pathways that control lymphocyte function and survival directly control the glucose and glutamine metabolic pathways. Therefore, lymphocytes in different functional states reprogram their glucose and glutamine metabolism to balance their requirement for ATP and macromolecule production. The tight association between metabolism and function in these cells was suggested to introduce the possibility of several pathologies resulting from the inability of lymphocytes to meet their nutrient demands under a given condition. In fact, disruptions in lymphocyte metabolism and function have been observed in different inflammatory, metabolic, and autoimmune pathologies. Regular physical exercise and physical activity offer protection against several chronic pathologies, and this benefit has been associated with the anti-inflammatory and immunomodulatory effects of exercise/physical activity. Chronic exercise induces changes in lymphocyte functionality and substrate metabolism. In the present review, we discuss whether the beneficial effects of exercise on lymphocyte function in health and disease are associated with modulation of the glucose and glutamine metabolic pathways.

1. Glucose and Glutamine Metabolism and Lymphocyte Function

Activated lymphocytes undergo a rapid burst in cellular proliferative, biosynthetic, and secretory activities and must obtain metabolic substrates to attempt this dramatic increase in metabolism [1]. Their insignificant intracellular store of nutrients obligates lymphocytes to markedly increase the uptake of metabolic substrates from their microenvironment [2]. Although lymphocytes are able to use glucose, glutamine, ketone bodies, and fatty acids (FA), it was determined that glucose and glutamine are quantitatively the most important fuel for activated lymphocytes [3].

Regarding the new metabolic demands of activated lymphocytes, glucose is initially retained in the cell by phosphorylation into glucose 6-phosphate by hexokinases (HKs) [2]. From there, glucose 6-phosphate can be used as a substrate by aerobic glycolysis or by the pentose-phosphate pathway (PPP). In the PPP, glucose 6-phosphate serves to generate ribose (for the synthesis of RNA and DNA) and NADPH (for FA synthesis) [1, 2].

For glucose 6-phosphate that enters aerobic glycolysis, the molecule is converted to pyruvate, after which it can be converted to lactate or acetyl-CoA or be fully oxidized [3]. The majority is converted to lactate (approximately 91%) [4–7], while most of the remaining pyruvate is converted to acetyl-CoA, which has a central role in membrane biogenesis [8], serving as a precursor to phospholipids, cholesterol, and triacylglycerol [1, 3]. Thus, only a small percentage of glucose 6-phosphate is fully oxidized in lymphocytes [3].

In this scenario, the removal of citrate (pyruvate converted to acetyl-CoA plus oxaloacetate) from the tricarboxylic acid (TCA) cycle for biosynthetic reactions imposes the need to continue replenishing intermediates to maintain this cycle's function [2, 9]. Thus, beyond glucose, activated lymphocytes also increase their update of glutamine and convert it to glutamate, which is in turn converted to α-ketoglutarate via glutamate dehydrogenase [3]. In addition to replenishing intermediates to maintain the TCA cycle using glutamate, lymphocytes also convert glutamine to aspartate and ammonia, providing biosynthetic precursors, purines, and pyrimidines, for the synthesis of DNA and RNA [3]. Finally, a limited percentage of glutamine can be converted to lactate or be fully oxidized [3]. In fact, although oxidative phosphorylation still occurs in effector T lymphocytes [8], it seems that, of the glutamine (and glucose) utilized by these cells, only approximately 1.5% is oxidized [3].

In accordance with the importance of glucose and glutamine in activated lymphocytes, early studies of these cells demonstrated that, to meet the new bioenergetic and biosynthetic demands imposed by activation, lymphocytes also increase the maximal activity levels of enzymes, such as HK, glucose-6-phosphate dehydrogenase (G6PDH) and phosphate-dependent glutaminase (GLUTase), which are key enzymes in the glycolysis, pentose-phosphate, and glutaminolysis pathways, respectively. The mitochondrial enzyme citrate synthase (CS), an important enzyme in the TCA cycle, is also affected [6, 7]. Comparatively, B cell metabolism has been less well investigated than T cell metabolism; however, the metabolic characteristics of both lymphocyte types might be similar [2, 10].

An outstanding feature of lymphocytes is that these cells utilize glucose and glutamine at high rates in a strictly function-dependent manner [7]. Furthermore, more recently, studies of direct modifications to T lymphocyte metabolic pathways demonstrated that metabolic reprogramming and lymphocyte activation are intricately linked, as cellular metabolism was found to be directly controlled by the signaling pathways that drive cell survival and activity [10]. Notably, Pearce and colleagues [8] stated that the reasons why T cells would adopt specific metabolic programs and the impacts of such programs on cell function and immunological responses were unclear.

In this sense, T lymphocytes adopt a metabolic program that reflects their energetic and biosynthetic needs in specific states, ranging from resting to memory cell conversion. For resting lymphocytes, it is worth mentioning that, despite the name, these cells continuously migrate through secondary lymphoid tissues to maintain immune surveillance prior to activation; to accomplish this activity, these cells rely on the oxidative metabolism of glucose, amino acids, and lipids [9]. However, as previously discussed, once activated, lymphocytes grow, proliferate, differentiate, and adapt to stress [8], and the mixed oxidative metabolism associated with a naïve resting state, which preferentially generates ATP, does not support these new functions [11]. Thus, the prioritization of the synthesis of macromolecules, rather than ATP production, explains the already-mentioned dependence of activated lymphocytes on aerobic glycolysis. In other words, the cell replaces its previous efficient ATP production (resting state) with efficient and rapid macromolecule biosynthesis (activated state) [11].

Additional support for the finding that cell metabolism is a key regulator of lymphocyte function and differentiation [11] has been provided by the patterns of "fuel usage" and transcriptional and posttranscriptional factors that control metabolism in the various activated T cell lineages. The helper T cell lineages Th1, Th2, and Th17 all exhibited increased aerobic glycolysis (as previously mentioned for activated lymphocytes), the posttranscriptional regulator mTORC1 was found to control glucose metabolism in Th1 and Th17 T cells, whereas mTORC2 controlled glucose in Th2 cells [9]. Th17 glucose metabolism is also controlled by the transcriptional regulator HIF-1α [9]. T regulatory (Treg) cells exhibit lipid oxidation as a primary metabolic phenotype, which is controlled by AMPK. Similarly, memory T cells also oxidize lipids, although in these cells, this metabolic phenotype is controlled by the posttranscriptional regulators TRAF6 and AMPK [11].

2. Lymphocyte Metabolic Dysregulation and Disease

The signals and stimuli that normally control the immune system (IS) can be affected by conditions such as obesity and type 2 diabetes (T2D) [12]. In this sense, it was proposed that the direct control of lymphocyte metabolism mediated by survival and activity-related signaling pathways could introduce the potential for metabolic changes to promote diseases [10]. More specifically, the inability of cell metabolism to meet the energetic and biosynthetic demands of lymphocytes could disrupt immune functionality, a process that has been observed in several immunological diseases [10].

For example, the inhibition of glycolytic metabolism can suppress cell proliferation and cytokine production and also compromise effector T cell differentiation [13]. In contrast, mitogen-induced T cell activation can reflect the glycemic statuses and insulin levels of type 1 diabetes and T2D patients [14]. Furthermore, hyperglycemia and ketoacidosis were found to increase the levels of proinflammatory cytokines and the numbers of activated T lymphocytes in diabetic patients [14].

Lymphocyte metabolic and/or functional dysregulation has been observed in a diet-induced obesity (DIO) model [15]. These phenomena were reported to promote reductions in the Treg and Th2 cell populations and increases in the resident inflammatory lymphocyte population [15]. Similarly, our laboratory recently reported that dendritic cells

cultivated under leptin-free conditions exhibited a different phenotype from that of wild type cells; this phenotype was characterized by a reduced ability to induce $CD4^+$ cell proliferation, while inducing increased Treg and Th17 cell differentiation [16]. These results proved useful for increasing our understanding of whether leptin can induce beneficial (increased Treg cell numbers) or detrimental (increased Th17 numbers) clinical outcomes [16].

In lymphocytes from Graves' disease patients, the maximal activity levels of HK, G6PDH, CS, and GLUTase were all reduced [17]. *In vitro*, thyroid hormones were found to increase glucose and glutamine metabolism in the lymphocytes from these patients [17]. Additionally, it was reported that concomitant acute and chronic infections in patients with several diseases, such as cancer or asthma, are associated with an imbalance of T1 (T helper type 1 and T cytotoxic type 1) and T2 (T helper type 2 and T cytotoxic type 2) immune functions [18, 19].

In graft-versus-host disease (GVHD), T lymphocytes are activated within a systemic inflammatory environment containing ubiquitous antigens [20]. One of the few studies that investigated T lymphocyte metabolism *in vivo* demonstrated that cells activated under GVHD conditions became highly dependent on lipid metabolism, rather than exhibiting the expected increase in glycolytic metabolism [20]. In accordance with the results of that study, the FA metabolism dependence of allogeneic T cells distinguishes these cells metabolically from other activated T cell subsets, thus providing targets for therapeutic intervention (e.g., blockade of FA transport, inhibition of FA oxidation, and limitation of fuel sources) [20].

Cancer is another condition associated with inflammation. In fact, the concept that an inflammatory tissue injury could induce neoplasia and the existence of a close relationship between carcinogenesis and inflammation were initially postulated by the Greek physician Galenus approximately 2000 years ago [21]. Current estimations suggest that approximately 25% of cancers require a chronic inflammatory microenvironment for development [21]. Additionally, obesity, which is associated with chronic low-grade inflammation, increases the risk of developing certain types of cancers [22].

Therefore, in accordance with the hypothesis suggested by Caro-Maldonato and coworkers [10], lymphocytes from animals and humans with cancer are expected to exhibit metabolic dysregulation or mismatches. In fact, cells from primary effusion lymphoma (PEL), a subtype of B cell non-Hodgkin's lymphoma with a median patient survival duration of 6 months, provide support for this hypothesis [23]. *In vitro*, these cells exhibit an increased dependence on aerobic glycolysis through the PI3 K/AKT/mTOR pathways that control glycolysis via GLUT1 [24]. Additionally, PEL cells actively convert glucose to FAs via increased FA synthase activity. In fact, these cells, as well as those from other B cell non-Hodgkin's lymphoma subtypes, are dependent on FA synthase to such an extent that Bhatt and coworkers [23] suggested the possibility of using this enzyme as a unique molecular treatment target in these cancers.

Three possible outcomes of the occurrence of a mismatch or insufficient fuel usage in T lymphocytes were described [10]. The first was altered or inhibited Th1, Th2, and Th17 differentiation. The second was the inhibition of proliferation or induction of cellular senescence. The third was the induction of cell death. In accordance with these results, the above-described outcomes for T and B lymphocytes have been observed in several immunological diseases and have also provided opportunities to selectively modulate specific immune functions by targeting glucose, lipid, and amino acid metabolism [10].

3. Mediators of Exercise-Induced Immunomodulation

3.1. Catecholamines and Cortisol. The studies mentioned in this section were designed in accordance with two main research approaches that have been proposed to establish a link between exercise and the IS: a metabolic approach, which considers plasma glutamine concentrations/metabolism, and a neuroendocrine approach, which considers changes in the levels of immunomodulatory hormones and neurotransmitters [25].

Interestingly, *in vitro* studies demonstrated that the "stress hormones" adrenaline and cortisol are able to modulate lymphocyte metabolism. The organism exerts tight control over the internal environment, and any subtle disruption of the regulated limits triggers physiological feedback mechanisms to reestablish the internal milieu [26]. Among these mechanisms is integration of the nervous and endocrine systems (or the neuroendocrine system, NES) with IS [27], which is systematically controlled by the NES [26]. Of note, the integrated communication between the NES and the IS possibly consists of the sharing of common signaling proteins and their corresponding receptors [28, 29].

In the face of any stimuli able to disrupt homeostasis, the response of the NES is invariably (regardless of the nature of the stress) the same: activation of the sympathoadrenal (SA) system and consequent release of catecholamines (i.e., adrenaline and noradrenaline) and activation of the hypothalamic-pituitary-adrenal (HPA) axis, which, in humans, results in cortisol production and release [30, 31].

The stereotypic response of the NES to stress [32] aids in understanding of the effects of exercise upon the IS because among the several molecules (e.g., hormones, cytokines) able to affect immune cell function, catecholamines, and cortisol appear to be particularly involved in exercise-induced immune responses [33]. Specifically, activation of the SA system occurs several seconds after exercise initiation, whereas the HPA response and the secretion of cortisol often need 20–30 minutes before beginning [34]. Additionally, catecholamines appear to be responsible for the initial effects of acute exercise on the IS (e.g., the migration of lymphocyte subpopulations) [35], whereas cortisol appears to exert its effects within a period of at least 2 hours [35].

An interesting feature of exercise is that in accordance with Fragala et al.'s [36] work exercise presents a unique stress

on the homeostatic conditions, and this stress is specific to the nature and configurations of the protocol and associated elements (i.e., the environmental conditions or nutritional status). That is, the magnitude of NES activation in response to exercise stress is determined by the intensity and duration of such exercise [37]. Consequently, the exercise protocols that most affect the IS are those in which the intensity and duration (acute variables) and frequency (chronic variable) are higher. Not surprisingly, aerobic exercise protocols with longer durations (>1.5 hours) and greater intensities (55/60–75% of VO_2max) induce greater release of catecholamines and cortisol in comparison with aerobic exercise at lower intensity [38].

Discussing the effects of different intensities, durations, and modes of exercise on the response of the NES is obviously beyond the scope of this review. However, briefly, noradrenaline presents a curvilinear increase in response to acute exercise as workload augments, while adrenaline increases at workloads over 60% VO_2max [39]. Regarding cortisol, mild-intensity, moderate-duration aerobic exercise does not appear to alter its levels. However, exercises with intensity above 85% VO_2max [40, 41] or with a duration greater than 60 minutes [42] typically lead to increases in cortisol secretion.

Rosa and coworkers [43] previously demonstrated the ability of adrenaline to increase the proliferative index of mesenteric lymphocytes and to concomitantly augment the maximal activities of HK, GLUTase, and CS, as well as glucose and glutamine consumption. However, the excess of systemic catecholamines induced by high-intensity, exhaustive exercise could have an immunosuppressive effect, such as a reduction in the plasma levels of interferon-α (IFNα) and an antiviral cytokine [44]. In addition to its antiviral property, IFNα has antiapoptotic and antiproliferative effects on activated lymphocytes [45]. Regarding the effects of this cytokine on lymphocyte metabolism, we demonstrated that the ability of IFNα to limit T and B cell proliferation could be explained by the suppression of glucose and glutamine metabolism and reduced maximal G6PDH, CS, and GLUTase activities [46].

The immunosuppressive effect of glucocorticoids on mesenteric lymphocytes is associated with a 40% reduction in pyruvate utilization due to inhibition of pyruvate dehydrogenase's maximal activity [47].

Additionally, as mentioned, cortisol increases are typically observed during high-intensity exercises. We recently demonstrated that basketball players participating in an official game (stressful exercise) presented increases in salivary cortisol and a reduction in levels of interleukin (IL)-21, a cytokine that stimulates immunoglobulin A-secreting cells [48]. Additionally, in accordance with the immunosuppressive ability of cortisol, it was observed that the numbers and proliferative ability of circulating lymphocytes are affected by a single bout of intense exercise [38]. For example, a 30–50% decrease in the lymphocyte count occurs at 30 minutes after exercise [38].

Regarding the proliferative index of lymphocytes, our results demonstrated that participation in very intense exercise protocols, an Olympic triathlon (swimming for 1.5 km, cycling for 40 km, and running for 10 km) [49, 50] or a simulated cycling competition (6 sets of 20 minutes at 90% of the individual's anaerobic threshold) [51] reduced the proliferative indices of T and B lymphocytes and plasma glutamine levels. These changes were associated with reduced cytokine production (e.g., IL-1, IL-2, IL-4, tumor necrosis factor (TNF)α, and interferon (IFN)γ) by mononuclear peripheral cells in response to mitogens. It was proposed that the IFNγ/IL-4 ratio in the culture supernatants of stimulated T cells could act as an objective indicator for monitoring the Th1/Th2 balance (cell-mediated/humoral immunity) [19]. As such, these studies suggested that an intense bout of acute exercise could affect this balance [19, 49–51].

To maintain homeostasis in the face of all organic changes induced by physical exercise (e.g., increased body temperature, dehydration, ion imbalances, hypoxia, and blood pressure changes), other hormones (e.g., insulin, growth hormone, aldosterone, glucagon, and thyroxin) are necessary in addition to cortisol and catecholamines during exercise and recovery [32]. Although the involvement of these hormones in exercise's ability to modulate the IS needs further investigation, certain evidence reinforces possible involvement. For example, insulin can control the metabolism and functionality of lymphocytes [1], and its infusion into critically ill patients is used to achieve tight hyperglycemic control and to fight systemic inflammation [52]. Thyroid hormones in turn stimulate aerobic glycolysis, glutamine consumption, and aerobic metabolism in human lymphocytes [17].

3.2. Cytokines. As previously discussed regarding the effect of an excess of catecholamines on plasma TNFα [44], the plasma levels of other cytokines are affected by exercise [27], and it has been suggested that the impact of exercise on cytokine production could partially explain how this stressor modulates the IS [32]. Regarding this concept, the cytokine that is most responsive to exercise is IL-6, whose levels increase up to 100 fold as the duration of exercise progresses [53]. Interestingly, the source of this cytokine is the skeletal muscle [54]. The increase in IL-6 levels is followed by an increase in the levels of an IL-1 receptor antagonist that is an inhibitor of the inflammatory cytokines IL-1 and IL-10, important anti-inflammatory cytokines [22].

3.3. Other Mediators. In addition to the neuroendocrine and immune responses to exercise mentioned above, exercise may modulate immunity in alternative ways. One of these ways is exercise-induced muscle damage, which results in the secretion of inflammatory cytokines by innate immune cells [33]. Another way is the effect of exercise upon plasma glutamine levels [55]. Thus, the reduction in the levels of this amino acid after strenuous exercise could be related to immunosuppression, whereas the increase in glutamine levels induced by chronic moderate-intensity exercise would induce a positive effect upon immune function [56].

Finally, it is worth mentioning that, in many respects, the responses and adaptations to chronic exercise are the result of the cumulative influence of repeated acute exercise bouts [57]. Thus, to understand how exercise training can modulate

lymphocyte function as well as metabolism, it is important to know the acute effects of exercise.

4. Anti-Inflammatory and Immunomodulatory Effects of Chronic Exercise

Regarding the chronic effects of physical exercise, the protection that it offers against all-cause mortality is known to occur primarily due to the ability of exercise to protect against atherosclerosis, T2D, colon cancer, and breast cancer [22]. The fact that the above-mentioned diseases seem to associate with low-grade chronic inflammation and the finding that acute exercise increases the systemic levels of several anti-inflammatory cytokines (especially exercise that is not high intensity or exhaustive) suggest the possibility that regular exercise can protect against the chronic conditions associated with low-grade inflammation via an anti-inflammatory effect [22].

In accordance with certain authors, the potential underlying mechanisms of the anti-inflammatory effects of regular moderate exercise primarily include a reduction in visceral fat mass, increased production, and release of myokines and reduced expression of Toll-like receptors on monocytes and macrophages; however, these mechanisms likely also include inhibition of monocyte and macrophage infiltration into adipose tissues, a reduction in the number of circulating proinflammatory monocytes and an increase in the number of circulating Treg cells [58]. The modulation of lymphocyte metabolism by exercise, however, is not included on this list.

In accordance with the proposal that regular moderate-intensity exercise has beneficial anti-inflammatory (and immunomodulatory) effects, it seems that when chronic, this exercise intensity is able to reverse the age-associated reduction in Th1 cell numbers or Th1-cell derived cytokine levels that are normally observed in older adults [19]. Moreover, other studies have reported that moderate exercise training increased the production of Th1 response-associated cytokines in both humans and rats [59–63]. Still, experimental evidence suggests that chronic moderate exercise could normalize IL-4 concentrations and increase IL-2 concentrations in a heart failure model in which the T2-type response had been initially elevated [64].

The evidence suggests that high-intensity exercise could be immunosuppressive [12]. It was demonstrated that when chronic, this level of exercise stimulates a type 2 T cell phenotype in trained individuals [19] and that this tendency might be associated with the high incidence of upper respiratory infection episodes among athletes [12]. In fact, two recent studies have suggested that lifelong participation in high volumes of intense exercise could compromise immune functionality [65, 66]. Together, these studies reported that young athletes had fewer $CD4^+$ T lymphocytes and that these cells exhibited reduced functionality and a higher degree of differentiation in comparison with those from young nonathletes. Regarding $CD8^+$ T lymphocytes, it was observed that despite, the higher number of these cells, there was a lower frequency of thymic emigration [65]. However, this altered lymphocyte functionality observed in young athletes was not found in elderly athletes; however, in the latter group, natural killer cells exhibited increased activation and degranulation. Therefore, it is possible that, in elderly individuals, the IS can adapt itself to the detrimental effects of lifelong exhaustive exercise [65].

Together, these studies suggest that chronic exercise affects lymphocyte functioning. Thus, considering the strict relationship between function and metabolism in these cells, it is important to understand how glucose and glutamine consumption in lymphocytes is modulated by regular moderate- and high-intensity exercise.

5. Chronic Exercise-Mediated Modulation of Lymphocyte Nutrient Metabolism

Evidence suggests that these effects of exercise are at least partly due to the ability of exercise to modulate cell nutrient metabolism and particularly glucose and glutamine metabolism.

For example, Navarro and colleagues [67] demonstrated the ability of chronic moderate-intensity exercise (eight weeks of treadmill running) to modulate the activation, proliferation, cytokine production, and glucose and glutamine metabolism of T and B lymphocytes.

In support of the statement that lymphocytes utilize glucose and glutamine at high rates according to their specific immune functions [68], the exercise-induced metabolic changes observed by Navarro and colleagues [45] were accompanied by concomitant alterations in functionality. For example, increased expression of IL-2 and its receptor (IL-2R) and decreased expression of IL-4 and its receptor (IL-4R) were observed in T cells relative to B lymphocytes. These data suggest that chronic moderate exercise in healthy animals primarily enhances the Th1 response phenotype [67]. As several immunological disorders have been associated with a dysregulated Th1/Th2 balance [69], this is an important finding.

Regarding humoral immunity, chronic moderate exercise was shown to increase IgG production in lymphocytes from trained rats compared with lymphocytes from sedentary animals, thus indicating an improvement in humoral immunity. In support of the finding that both cellular and humoral immune functions improved in response to exercise, increases in the expression and modulation of CD8, CD54, and CD30 were observed, potentially indicating improvements in both types of immunity [67].

It was observed that the changes in lymphocyte function were accompanied by a differential effect of moderate exercise on T and B lymphocyte metabolism [67]. Specifically, T lymphocytes increased glutamine utilization by shifting the metabolism of this amino acid to an aerobic pathway (as previously mentioned herein, only a minor percentage of glutamine is oxidized in lymphocytes). Concomitantly, these cells reduced their glucose consumption and lactate production levels (lymphocytes typically convert most of their glucose to lactate). In contrast, B lymphocytes exhibited increases in both glucose and glutamine consumption, although only glutamine aerobic metabolism was increased

[67]. All of these lymphocytic changes were possible because key glucose and glutamine metabolic enzymes were targets of the modulatory effect of chronic exercise. Therefore, in accordance with enhanced aerobic glutamine metabolism, the maximal activities of GLUTase and CS increased in T lymphocytes' response to exercise. In addition to these 2 enzymes, the maximal activities of HK and G6PDH were also augmented in B lymphocytes in response to chronic exercise [67].

The effects of chronic moderate-intensity exercise were also investigated in animal models of chronic diseases. Recently, activated T lymphocytes were proposed as a model to understand carcinogenesis [70]. Through changes in transporter expression and isozyme switching, both activated lymphocytes and cancer cells become highly glycolytic and glutamine dependent to promote growth, proliferation, and differentiation. Therefore, an understanding of the metabolism of activated T cells could facilitate the identification of new therapeutic strategies that would selectively target tumor metabolism or inflammatory immune responses [70].

Thus, the effects of exercise were investigated in lymphocytes obtained from Walker-256 tumor-bearing rats [71]. In response to the tumor, the metabolism and function of these cells were compromised; T and B cells from the tumor-bearing rats exhibited lower proliferative indices relative to those of cells from sedentary animals [71] and increased glucose consumption and lactate production in comparison with cells from control animals. Eight weeks of moderate-intensity treadmill running suppressed tumor growth and reversed the repressive effects of the Walker-256 tumors on the lymphocytes' proliferative indices. Additionally, exercise training reversed the effect of the tumor by reducing glucose consumption and lactate production while counterbalancing the effects of the disease on the maximal activities of G6PDH, HK, and CS. Therefore, the immunomodulatory effects of exercise were characterized by a reversion of the tumor-induced changes. Finally, the exercise-induced effects on lymphocyte function and metabolism were accompanied by altered plasma hormone levels (e.g., growth hormone, testosterone, and corticosterone) and beneficial changes in cytokine levels (e.g., IL-1, IL-2) [71].

Rheumatoid arthritis (RA) is an autoimmune disease that causes several disturbances in immunological functioning [72, 73]. Previously, it had been speculated that because certain immune functions are exacerbated in RA, improved IS functioning with exercise could theoretically be detrimental to RA patients. However, the opposite was found to be true, as studies performed in the 1990s regarding exercise and inflammatory disease demonstrated that nearly any type of exercise was superior to a sedentary lifestyle for RA patients [74, 75]. Additionally, it was observed that exercise appeared to be beneficial for individuals with RA because of its anti-inflammatory effects [22, 74].

Therefore, we decided to verify whether alterations in glucose and glutamine metabolism were present in a model of experimental arthritis (collagen-induced arthritis, CIA) and whether a chronic swim training regimen could counterbalance the deleterious effects of RA by modulating the metabolism of these nutrients [62]. Initially, we observed that lymphocytes from CIA animals consumed more glucose, despite exhibiting reduced lactate production relative to lymphocytes from healthy animals, thus indicating that CIA induced "defective" lymphocyte activation [62]. Additionally, CIA reduced glutamine consumption and glutamate/aspartate production, and these metabolic changes were associated with an elevated proliferative index in the cells from CIA animals. However, an eight-week moderate-intensity swim training regimen reduced the proliferative index and glucose consumption of lymphocytes from the CIA animals and increased their glutamine metabolism (Figure 1).

To obtain a more complete understanding of the immunomodulatory effects of exercise, we also analyzed the plasma levels of certain hormones. Trained CIA rats exhibited lower levels of the proinflammatory hormone prolactin and higher levels of the immunosuppressive hormones progesterone and corticosterone [62]. Interestingly, chronic exercise also increased the plasma levels of IL-2, a cytokine that can both initiate and terminate inflammation under different conditions, as well as increased plasma levels of glutamine [76]. Taken together, the data from the trained CIA animals suggested that the ability of swim training to counterbalance several effects of CIA resulted from the modulation of lymphocyte metabolism and the balance between proinflammatory and anti-inflammatory hormones and cytokines.

Despite the common belief that high-intensity exercise is immunosuppressive [12], it is important to note that, in this case, "high intensity" means chronic exercise of high intensity and volume, such as those physical training regimens used by athletes [12]. That is, it is possible to speculate that high-intensity exercise at a low/moderate volume would induce beneficial effects on the IS because this type of exercise would allow the organism to become adapted to it.

In support of this speculation, an eight-week anaerobic jumping training (a high-intensity exercise) regimen was reported to increase the expression of the proapoptotic protein Bax and reduce the expression of the antiapoptotic protein Bcl-2; these findings were associated with reduced Walker-256 tumor growth consequent to apoptosis [77]. The authors attributed their findings to unknown interference in the Walker-256 tumor cells. Subsequently, our group observed that Walker-256 tumor-bearing rats subjected to high-intensity running training for an 8-week period (85% VO_2max thirty minutes per day for five days) exhibited a 40% reduction in tumor growth and a 35.5% increase in lifespan relative to sedentary, tumor-bearing rats [63]. These changes were accompanied by reduced lactate production in the Walker-256 tumor cells, suggesting that the tumor cells had become less glycolytic. Moreover, in the trained animals, the tumor cells exhibited increased glutamine consumption and glutamate and aspartate production. Despite these findings, glutamine consumption due to aerobic metabolism was reduced [63].

Regarding lymphocyte function and metabolism, the same study demonstrated that the exercise training protocol counterbalanced the effects of the Walker-256 tumors on lymphocyte metabolism [63]. Lymphocytes from trained animals exhibited an increased proliferative index, reduced

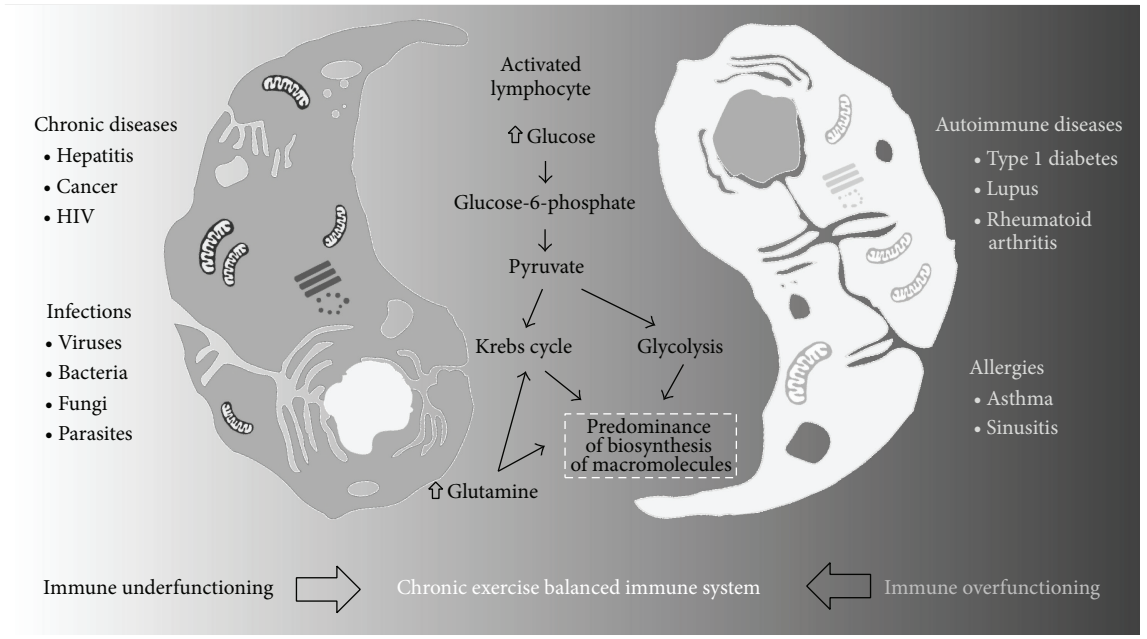

Figure 1: Exercise counterbalances lymphocyte metabolic dysregulation, modulating several components of glycolysis and glutaminolysis. HIV—human immunodeficiency virus.

glucose consumption (aerobic and anaerobic), and reduced lactate production in comparison with immune cells from sedentary, tumor-bearing rats. Similarly, the high-intensity exercise program reversed the tumor-induced effects on glutamine metabolism [63]. Notably, the modulatory effect of exercise was accompanied by increased cytokine levels (e.g., IL-1, IL-2, and TNFα) and changes in plasma hormone levels (e.g., increased corticosterone levels, reduced growth hormone levels) [63]. Therefore, the immunomodulatory effects of exercise occurred in response to a complex interaction of hormones, cytokines, and metabolic changes.

6. Conclusion

Acute exercise and chronic exercise affect lymphocyte function in a manner associated with the modulation of glucose and glutamine metabolism. Although further studies are necessary, the primary experimental evidence suggests that the well-known anti-inflammatory and immunomodulatory effects of exercise are at least partly characterized by the ability of chronic exercise to adjust the energetic and biosynthetic demands of lymphocytes in response to physiological and pathological conditions.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

References

[1] P. C. Calder, G. Dimitriadis, and P. Newsholme, "Glucose metabolism in lymphoid and inflammatory cells and tissues," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 10, no. 4, pp. 531–540, 2007.

[2] K. Ganeshan and A. Chawla, "Metabolic regulation of immune responses," *Annual Review of Immunology*, vol. 32, pp. 609–634, 2014.

[3] R. Curi, P. Newsholme, T. C. Pithon-Curi et al., "Metabolic fate of glutamine in lymphocytes, macrophages and neutrophils," *Brazilian Journal of Medical and Biological Research*, vol. 32, no. 1, pp. 15–21, 1999.

[4] D. A. Hume, J. L. Radik, E. Ferber, and M. J. Weidemann, "Aerobic glycolysis and lymphocyte transformation," *The Biochemical Journal*, vol. 174, no. 3, pp. 703–709, 1978.

[5] T. O. Tollefsbol and H. J. Cohen, "Culture kinetics of glycolytic enzyme induction, glucose utilization, and thymidine incorporation of extended-exposure phytohemagglutinin-stimulated human lymphocytes," *Journal of Cellular Physiology*, vol. 122, no. 1, pp. 98–104, 1985.

[6] M. S. M. Ardawi and E. A. Newsholme, "Maximum activities of some enzymes of glycolysis, the tricarboxylic acid cycle and ketone-body and glutamine utilization pathways in lymphocytes of the rat," *The Biochemical Journal*, vol. 208, no. 3, pp. 743–748, 1982.

[7] M. S. Ardawi and E. A. Newsholme, "Metabolism in lymphocytes and its importance in the immune response," *Essays in Biochemistry*, vol. 21, pp. 1–44, 1985.

[8] E. L. Pearce, M. C. Poffenberger, C. H. Chang, and R. G. Jones, "Fueling immunity: insights into metabolism and lymphocyte function," *Science*, vol. 342, no. 6155, Article ID 124245, 2013.

[9] V. A. Gerriets and J. C. Rathmell, "Metabolic pathways in T cell fate and function," *Trends in Immunology*, vol. 33, no. 4, pp. 168–172, 2012.

[10] A. Caro-Maldonado, V. A. Gerriets, and J. C. Rathmell, "Matched and mismatched metabolic fuels in lymphocyte

function," *Seminars in Immunology*, vol. 24, no. 6, pp. 405–413, 2012.

[11] N. J. Maciver, R. D. Michalek, and J. C. Rathmell, "Metabolic regulation of T lymphocytes," *Annual Review of Immunology*, vol. 31, pp. 259–283, 2013.

[12] M. Gleeson, N. Bishop, M. Oliveira, T. McCauley, P. Tauler, and A. S. Muhamad, "Respiratory infection risk in athletes: association with antigen-stimulated IL-10 production and salivary IgA secretion," *Scandinavian Journal of Medicine and Science in Sports*, vol. 22, no. 3, pp. 410–417, 2012.

[13] R. D. Michalek, V. A. Gerriets, S. R. Jacobs et al., "Cutting edge: distinct glycolytic and lipid oxidative metabolic programs are essential for effector and regulatory $CD4^+$ T cell subsets," *Journal of Immunology*, vol. 186, no. 6, pp. 3299–3303, 2011.

[14] F. Stentz and A. E. Kitabchi, "Activated T lymphocytes in type 2 diabetes: implications from in vitro studies," *Current Drug Targets*, vol. 4, no. 6, pp. 493–503, 2003.

[15] E. K. Anderson, D. A. Gutierrez, A. Kennedy, and A. H. Hasty, "Weight cycling increases T-cell accumulation in adipose tissue and impairs systemic glucose tolerance," *Diabetes*, vol. 62, no. 9, pp. 3180–3188, 2013.

[16] P. M. Moraes-Vieira, R. A. Larocca, E. J. Bassi et al., "Leptin deficiency impairs maturation of dendritic cells and enhances induction of regulatory T and Th17 cells," *European Journal of Immunology*, vol. 44, no. 3, pp. 794–806, 2014.

[17] M. C. Werner, L. F. Costa Rosa, J. H. Romaldini, and R. Curi, "Metabolism of glucose and glutamine in lymphocytes from Graves' hyperthyroid patients: influence of methimazole treatment," *Cell Biochemistry and Function*, vol. 14, no. 2, pp. 97–104, 1996.

[18] I. J. Elenkov, "Glucocorticoids and the Th1/Th2 balance," *Annals of the New York Academy of Sciences*, vol. 1024, pp. 138–146, 2004.

[19] G. Zhao, S. Zhou, A. Davie, and Q. Su, "Effects of moderate and high intensity exercise on T1/T2 balance," *Exercise Immunology Review*, vol. 18, pp. 98–114, 2012.

[20] C. A. Byersdorfer, V. Tkachev, A. W. Opipari et al., "Effector T cells require fatty acid metabolism during murine graft-versus-host disease," *Blood*, vol. 122, no. 18, pp. 3230–3237, 2013.

[21] L. Antonioli, C. Blandizzi, P. Pacher, and G. Hasko, "Immunity, inflammation and cancer: a leading role for adenosine," *Nature Reviews Cancer*, vol. 13, no. 12, pp. 842–857, 2013.

[22] A. M. W. Petersen and B. K. Pedersen, "The anti-inflammatory effect of exercise," *Journal of Applied Physiology*, vol. 98, no. 4, pp. 1154–1162, 2005.

[23] A. P. Bhatt, S. R. Jacobs, A. J. Freemerman et al., "Dysregulation of fatty acid synthesis and glycolysis in non-Hodgkin lymphoma," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 109, no. 29, pp. 11818–11823, 2012.

[24] H. L. Wieman, J. A. Wofford, and J. C. Rathmell, "Cytokine stimulation promotes glucose uptake via phosphatidylinositol-3 kinase/Akt regulation of Glut1 activity and trafficking," *Molecular Biology of the Cell*, vol. 18, no. 4, pp. 1437–1446, 2007.

[25] J. C. Rosa Neto, F. S. Lira, M. T. de Mello, and R. V. T. Santos, "Importance of exercise immunology in health promotion," *Amino Acids*, vol. 41, no. 5, pp. 1165–1172, 2011.

[26] K. Mašek, J. Slánský, P. Petrovický, and J. W. Hadden, "Neuroendocrine immune interactions in health and disease," *International Immunopharmacology*, vol. 3, no. 8, pp. 1235–1246, 2003.

[27] J. J. Haddad, N. E. Saadé, and B. Safieh-Garabedian, "Cytokines and neuro-immune-endocrine interactions: a role for the hypothalamic-pituitary-adrenal revolving axis," *Journal of Neuroimmunology*, vol. 133, no. 1-2, pp. 1–19, 2002.

[28] D. A. Weigent, D. J. J. Carr, and J. E. Blalock, "Bidirectional communication between the neuroendocrine and immune systems. Common hormones and hormone receptors," *Annals of the New York Academy of Sciences*, vol. 579, pp. 17–27, 1990.

[29] J. Edwin Blalock, "Shared ligands and receptors as a molecular mechanism for communication between the immune and neuroendocrine systems," *Annals of the New York Academy of Sciences*, vol. 741, pp. 292–298, 1994.

[30] G. P. Chrousos and P. W. Gold, "The concepts of stress and stress system disorders: overview of physical and behavioral homeostasis," *Journal of the American Medical Association*, vol. 267, no. 9, pp. 1244–1252, 1992.

[31] C. Tsigos and G. P. Chrousos, "Hypothalamic-pituitary-adrenal axis, neuroendocrine factors and stress," *Journal of Psychosomatic Research*, vol. 53, no. 4, pp. 865–871, 2002.

[32] K. E. Habib, P. W. Gold, and G. P. Chrousos, "Neuroendocrinology of stress," *Endocrinology and Metabolism Clinics of North America*, vol. 30, no. 3, pp. 695–728, 2001.

[33] B. K. Pedersen, H. Bruunsgaard, M. Klokker et al., "Exercise-induced immunomodulation—possible roles of neuroendocrine and metabolic factors," *International Journal of Sports Medicine*, vol. 18, supplement 1, pp. S2–S7, 1997.

[34] P. A. Deuster, G. P. Chrousos, A. Luger et al., "Hormonal and metabolic responses of untrained, moderately trained, and highly trained men to three exercise intensities," *Metabolism: Clinical and Experimental*, vol. 38, no. 2, pp. 141–148, 1989.

[35] B. K. Pedersen, T. Rohde, and K. Ostrowski, "Recovery of the immune system after exercise," *Acta Physiologica Scandinavica*, vol. 162, no. 3, pp. 325–332, 1998.

[36] M. S. Fragala, W. J. Kraemer, C. R. Denegar, C. M. Maresh, A. M. Mastro, and J. S. Volek, "Neuroendocrine-immune interactions and responses to exercise," *Sports Medicine*, vol. 41, no. 8, pp. 621–639, 2011.

[37] D. A. Weigent and J. E. Blalock, "Interactions between the neuroendocrine and immune systems: common hormones and receptors," *Immunological Reviews*, vol. 100, pp. 79–108, 1987.

[38] M. Gleeson, "Immune function in sport and exercise," *Journal of Applied Physiology*, vol. 103, no. 2, pp. 693–699, 2007.

[39] H. E. Webb, M. L. Weldy, E. C. Fabianke-Kadue, G. R. Orndorff, G. H. Kamimori, and E. O. Acevedo, "Psychological stress during exercise: cardiorespiratory and hormonal responses," *European Journal of Applied Physiology*, vol. 104, no. 6, pp. 973–981, 2008.

[40] G. A. Wittert, D. E. Stewart, M. P. Graves et al., "Plasma corticotrophin releasing factor and vasopressin responses to exercise in normal man," *Clinical Endocrinology*, vol. 35, no. 4, pp. 311–317, 1991.

[41] F. Petraglia, C. Barletta, F. Facchinetti et al., "Response of circulating adrenocorticotropin, beta-endorphin, beta-lipotropin and cortisol to athletic competition," *Acta Endocrinologica*, vol. 118, no. 3, pp. 332–336, 1988.

[42] W. J. Inder, J. Hellemans, M. P. Swanney, T. C. R. Prickett, and R. A. Donald, "Prolonged exercise increases peripheral plasma ACTH, CRH, and AVP in male athletes," *Journal of Applied Physiology*, vol. 85, no. 3, pp. 835–841, 1998.

[43] L. F. Rosa, "Effect of adrenaline on lymphocyte metabolism and function. A mechanism involving cAMP and hydrogen peroxide," *Cell Biochemistry and Function*, vol. 15, no. 2, pp. 103–112, 1997.

[44] H. Yano, M. Uchida, R. Nakai et al., "Exhaustive exercise reduces TNF-α and IFN-α production in response to R-848 via toll-like receptor 7 in mice," *European Journal of Applied Physiology*, vol. 110, no. 4, pp. 797–803, 2010.

[45] R. Gimeno, C.-K. Lee, C. Schindler, and D. E. Levy, "Stat1 and Stat2 but not Stat3 arbitrate contradictory growth signals elicited by alpha/beta interferon in T lymphocytes," *Molecular and Cellular Biology*, vol. 25, no. 13, pp. 5456–5465, 2005.

[46] R. F. P. Bacurau, F. Navarro, A. V. N. Bacurau et al., "Changes in glucose and glutamine lymphocyte metabolisms induced by type I interferon α," *Mediators of Inflammation*, vol. 2010, Article ID 364290, 6 pages, 2010.

[47] M. A. R. Serrano, R. Curi, M. Parry-Billings, J. F. Williams, and E. A. Newsholme, "Effects of glucocorticoids on lymphocyte metabolism," *American Journal of Physiology: Endocrinology and Metabolism*, vol. 264, no. 1, part 1, pp. E24–E28, 1993.

[48] A. Moreira, R. F. Bacurau, M. H. Napimoga et al., "Salivary IL-21 and IgA responses to a competitive match in elite basketball players," *Biology of Sport*, vol. 30, no. 4, pp. 243–247, 2013.

[49] R. A. Bassit, L. A. Sawada, R. F. P. Bacurau, F. Navarro, and L. F. Costa Rosa, "The effect of BCAA supplementation upon the immune response of triathletes," *Medicine and Science in Sports and Exercise*, vol. 32, no. 7, pp. 1214–1219, 2000.

[50] R. A. Bassit, L. A. Sawada, R. F. P. Bacurau et al., "Branched-chain amino acid supplementation and the immune response of long-distance athletes," *Nutrition*, vol. 18, no. 5, pp. 376–379, 2002.

[51] R. F. Bacurau, R. A. Bassit, L. Sawada, F. Navarro, E. Martins Jr., and L. F. Costa Rosa, "Carbohydrate supplementation during intense exercise and the immune response of cyclists," *Clinical Nutrition*, vol. 21, no. 5, pp. 423–429, 2002.

[52] C. Lazzeri, R. Tarquini, F. Giunta, and G. F. Gensini, "Glucose dysmetabolism and prognosis in critical illness," *Internal and Emergency Medicine*, vol. 4, no. 2, pp. 147–156, 2009.

[53] L. Rosendal, K. Søgaard, M. Kjær, G. Sjøgaard, H. Langberg, and J. Kristiansen, "Increase in interstitial interleukin-6 of human skeletal muscle with repetitive low-force exercise," *Journal of Applied Physiology*, vol. 98, no. 2, pp. 477–481, 2005.

[54] P. Muñoz-Cánoves, C. Scheele, B. K. Pedersen, and A. L. Serrano, "Interleukin-6 myokine signaling in skeletal muscle: a double-edged sword?" *FEBS Journal*, vol. 280, no. 17, pp. 4131–4148, 2013.

[55] E. A. Newsholme and M. Parry-Billings, "Properties of glutamine release from muscle and its importance for the immune system," *Journal of Parenteral and Enteral Nutrition*, vol. 14, supplement 4, pp. 63S–67S, 1990.

[56] F. Agostini and G. Biolo, "Effect of physical activity on glutamine metabolism," *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 13, no. 1, pp. 58–64, 2010.

[57] P. D. Thompson, S. F. Crouse, B. Goodpaster, D. Kelley, N. Moyna, and L. Pescatello, "The acute versus the chronic response to exercise," *Medicine and Science in Sports and Exercise*, vol. 33, supplement 6, pp. S438–S453, 2001.

[58] M. Gleeson, N. C. Bishop, D. J. Stensel, M. R. Lindley, S. S. Mastana, and M. A. Nimmo, "The anti-inflammatory effects of exercise: mechanisms and implications for the prevention and treatment of disease," *Nature Reviews Immunology*, vol. 11, no. 9, pp. 607–610, 2011.

[59] M. L. Kohut, G. W. Boehm, and J. A. Moynihan, "Moderate exercise is associated with enhanced antigen-specific cytokine, but not IgM antibody production in aged mice," *Mechanisms of Ageing and Development*, vol. 122, no. 11, pp. 1135–1150, 2001.

[60] N. P. Walsh, M. Gleeson, R. J. Shephard et al., "Position statement part one: immune function and exercise," *Exercise Immunology Review*, vol. 17, pp. 6–63, 2011.

[61] M. Bental and C. Deutsch, "Metabolic changes in activated T cells: an NMR study of human peripheral blood lymphocytes," *Magnetic Resonance in Medicine*, vol. 29, no. 3, pp. 317–326, 1993.

[62] F. Navarro, A. V. N. Bacurau, S. S. Almeida et al., "Exercise prevents the effects of experimental arthritis on the metabolism and function of immune cells," *Cell Biochemistry and Function*, vol. 28, no. 4, pp. 266–273, 2010.

[63] A. V. N. Bacurau, M. A. Belmonte, F. Navarro et al., "Effect of a high-intensity exercise training on the metabolism and function of macrophages and lymphocytes of walker 256 tumor-bearing rats," *Experimental Biology and Medicine*, vol. 232, no. 10, pp. 1289–1299, 2007.

[64] M. L. Batista Jr., R. V. T. Santos, L. M. Cunha et al., "Changes in the pro-inflammatory cytokine production and peritoneal macrophage function in rats with chronic heart failure," *Cytokine*, vol. 34, no. 5-6, pp. 284–290, 2006.

[65] A. Prieto-Hinojosa, A. Knight, C. Compton, M. Gleeson, and P. J. Travers, "Reduced thymic output in elite athletes," *Brain, Behavior, and Immunity*, 2014.

[66] M. A. Moro-Garcia, B. Fernandez-Garcia, A. Echeverria et al., "Frequent participation in high volume exercise throughout life is associated with a more differentiated adaptive immune response," *Brain, Behavior, and Immunity*, 2013.

[67] F. Navarro, A. V. N. Bacurau, G. B. Pereira et al., "Moderate exercise increases the metabolism and immune function of lymphocytes in rats," *European Journal of Applied Physiology*, vol. 113, no. 5, pp. 1343–1352, 2013.

[68] P. Newsholme, "Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection?" *Journal of Nutrition*, vol. 131, supplement 9, pp. 2515S–2524S, 2001.

[69] G. M. Shearer, "Th1/Th2 changes in aging," *Mechanisms of Ageing and Development*, vol. 94, no. 1–3, pp. 1–5, 1997.

[70] A. N. Macintyre and J. C. Rathmell, "Activated lymphocytes as a metabolic model for carcinogenesis," *Cancer & Metabolism*, vol. 1, article 5, 2013.

[71] R. F. Bacurau, M. A. Belmonte, M. C. Seelaender, and L. F. Costa Rosa, "Effect of a moderate intensity exercise training protocol on the metabolism of macrophages and lymphocytes of tumour-bearing rats," *Cell Biochemistry and Function*, vol. 18, no. 4, pp. 249–258, 2000.

[72] R. Roubenoff, J. Walsmith, N. Lundgren, L. Snydman, G. J. Dolnikowski, and S. Roberts, "Low physical activity reduces total energy expenditure in women with rheumatoid arthritis: implications for dietary intake recommendations," *American Journal of Clinical Nutrition*, vol. 76, no. 4, pp. 774–779, 2002.

[73] E. D. Harris Jr., "Rheumatoid arthritis. Pathophysiology and implications for therapy," *The New England Journal of Medicine*, vol. 322, no. 18, pp. 1277–1289, 1990.

[74] R. Roubenoff, "Exercise and inflammatory disease," *Arthritis and Rheumatism*, vol. 49, no. 2, pp. 263–266, 2003.

[75] A. Finckh, M. Iversen, and M. H. Liang, "The exercise prescription in rheumatoid arthritis: primum non nocere," *Arthritis and Rheumatism*, vol. 48, no. 9, pp. 2393–2395, 2003.

[76] K. K. Hoyer, H. Dooms, L. Barron, and A. K. Abbas, "Interleukin-2 in the development and control of inflammatory disease," *Immunological Reviews*, vol. 226, no. 1, pp. 19–28, 2008.

[77] C. de Lima, L. Alves, F. Iagher et al., "Tumor growth reduction in walker 256 tumorbearing rats performing anaerobic exercise: participation of Bcl-2, Bax, apoptosis, and peroxidation," *Applied Physiology, Nutrition and Metabolism*, vol. 36, no. 4, pp. 533–538, 2011.

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Research Article

Evidences of +896 A/G TLR4 Polymorphism as an Indicative of Prevalence of Complications in T2DM Patients

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T2DM is today considered as world-wide health problem, with complications responsible of an enhanced mortality and morbidity. Thus, new strategies for its prevention and therapy are necessary. For this reason, the research interest has focused its attention on TLR4 and its polymorphisms, particularly the rs4986790. However, no conclusive findings have been reported until now about the role of this polymorphism in development of T2DM and its complications, even if a recent meta-analysis showed its T2DM association in Caucasians. In this study, we sought to evaluate the weight of rs4986790 polymorphism in the risk of the major T2DM complications, including 367 T2DM patients complicated for the 55.6%. Patients with A/A and A/G TLR4 genotypes showed significant differences in complication's prevalence. In particular, AG carriers had higher risk prevalence for neuropathy ($P = 0.026$), lower limb arteriopathy ($P = 0.013$), and the major cardiovascular pathologies ($P = 0.017$). Their cumulative risk was significant ($P = 0.01$), with a threefold risk to develop neuropathy, lower limb arteriopathy, and major cardiovascular events in AG cases compared to AA cases. The adjusted OR for the confounding variables was 3.788 (95% CI: 1.642–8.741). Thus, the rs4986790 polymorphism may be an indicative of prevalence of complications in T2DM patients.

1. Introduction

Type 2 diabetes mellitus (T2DM) is becoming a common worldwide disease with epidemic proportions in many populations [1]. Environmental changes promoting unhealthy behaviours and development of obesity and overweight around the world have been suggested as the principal causes [2]. In addition, related diabetes complications (i.e. chronic arterial disease of the lower limbs, carotid arterial diseases, ischemic heart diseases, neuropathies, nephropathy, chronic kidney failure) are responsible of an increased morbidity and mortality [1]. Thus, the knowledge of the pathophysiological

mechanisms involved in the occurrence of T2DM and related complications is crucial for successful prevention and new therapeutic treatments.

It is well-recognised that defective insulin secretion of pancreatic-β cells and diminished insulin sensitivity in peripheral tissues characterise T2DM. In addition, recent evidence considers the occurrence of T2DM and its complications as the result of a state of chronic, systemic, and low grade of inflammation in accordance with metainflammation hypothesis [3, 4]. Elevated levels of several circulating inflammatory molecules constitute a common feature in the natural course of diabetes [5, 6]. Accordingly, pancreatic-β cells, under certain pathological condition, produce and release the proinflammatory cytokine interleukin-1β (IL-1β). IL-1β can in turn impair β-cell function and induce apoptosis [7, 8]. In the recent years, it has been also proposed that T2DM may be the consequence of the stimulation of Toll-like receptors (TLRs), a family of pattern-recognition receptors able to detect microbial conserved components and trigger protective host responses, and implicated in mediating chronic inflammatory diseases, including obesity and diabetes [2–4, 9]. Indeed, they also recognize endogenous ligands (i.e., endogenous damage-associated molecular patterns—DAMPs), such as saturated fatty acids and necrotic cell products [10, 11]. Interestingly, the activation of TLR4, one of the best known TLR member, expressed in several tissue cells, such as cells of the pancreatic islets (i.e., β-cells and resident macrophages), can induce both insulin resistance, pancreatic β-cell dysfunction, and alteration of glucose homeostasis [2, 12–14]. The TLR4 activation seems also to be exacerbated by the low-grade of circulating endotoxemia (circulating lipopolysaccharide-LPS) correlated with the altered gut microbiota, which characterizes subjects with metabolic diseases, such as T2DM [2]. In particular, it has been recently demonstrated that LPS inhibits β-cell gene expression of insulin in a TLR4-manner and via Nuclear Factor (NF)-κB signaling in pancreatic islets [15]. This crucial role of TLR4 has been confirmed by data demonstrating that deletions or mutations in TLR4 gene (MIM: 603030) protect against fatty acid-induced insulin resistance and diet-induced obesity [16–18]. A lot of single nucleotide polymorphisms (SNPs) were described in the TLR4 coding region. The +896 A>G SNP (rs4986790) induces the substitution of Asp299Gly amino acids, modifying the normal structure of the extracellular region of the TLR4. Thus, different +896 TLR4 genotypes may be associated with decreased ligand recognition or protein interaction and decreased responsiveness to LPS [9, 19]. Interestingly, a recent meta-analysis showed a significant association between +896 TLR4 SNP and T2DM and metabolic syndrome, in Caucasians [20]. A significant association was also reported between the Asp299Gly polymorphism of the TLR4 gene and early onset of diabetic retinopathy. T2DM patients carrying AG/GG genotypes showed an increased risk of developing retinopathy compared with patients carrying AA genotypes [21].

Overall, although Asp299Gly polymorphism of the TLR4 gene is a well-recognised genetic risk factor in some age-related diseases [9], only few data have been reported for T2DM complications, such as neuropathy, retinopathy, ischemic heart disease, and coronary artery disease [22–27], and no data were reported on chronic kidney and other T2DM-related cardiovascular diseases, such as carotid arterial and cerebrovascular diseases and lower limb arteriopathy in Caucasian populations.

In order to clarify the weight of +896 TLR4 A/G polymorphism as potential predisposing or protective genetic factor in the major T2DM complications (neuropathy, nephropathy, chronic kidney failure, chronic arterial disease of the lower limbs, carotid arterial diseases, and ischemic heart diseases) in the Caucasian population, we analysed 367 patients affected by T2DM and with complications for the 55.6%.

2. Subjects and Methods

2.1. Subject Populations. Three hundred and sixty-seven diabetic patients were enrolled. Informed consent was obtained from each subject. The study protocol was approved by the Ethics Committee of the INRCA Hospital. T2DM was diagnosed according to the American Diabetes Association Criteria [28]. Inclusion criteria were body max index (BMI) $<40\,kg/m^2$, age from 35 to 85 years, ability, and willingness to give written informed consent and to comply with the requirements of the study. Information collected included data on vital signs, anthropometric factors, medical history, and behaviours as well as physical activity. DNA was collected from participants providing consent to use genetic material (100 percent of the sample). The presence/absence of diabetic complications was evidenced as follows: diabetic retinopathy by fundoscopy through dilated pupils and/or fluorescence angiography; renal impairment, defined as an estimated glomerular filtration rate (eGFR) <60 mL/min per $1.73\,m^2$ evaluated using Cockcroft-Gault equation [29]; neuropathy established by electromyography; ischemic heart disease defined by clinical history and/or ischemic electrocardiographic alterations; peripheral vascular disease including atherosclerosis obliterations and cerebrovascular disease on the basis of history, physical examinations and Doppler velocimetry technique. Hypertension was defined as a systolic blood pressure >140 mmHg and/or a diastolic blood pressure >90 mmHg, measured while the subjects were sitting, which was confirmed in at least three different occasions. BMI was calculated as weight (kg)/height (m^2). All the selected subjects were Italian and consumed a Mediterranean diet. Overnight fasting venous blood samples of all subjects were collected from 8:00 to 9:00 a.m. in plain, EDTA, heparin, and citrate added tubes. The samples were either analyzed immediately or stored at −80°C for no more than 30 days.

2.2. Laboratory Assays. Blood concentration of fasting glucose, low and high density lipoprotein (LDL and HDL) cholesterol, and triglycerides was measured using commercially available kits on a Roche/Hitachi 912 (Roche Diagnostics, Switzerland). Insulin, C-reactive protein (CRP), apoliprotein-A1, and-B100 (Apo-A1 and Apo-B) levels were assessed using immunochemical methods and an Access Analyzer (Beckman Coulter, CA, USA). Creatinine was measured by Jaffè method, fibrinogen by Clauss method,

and urea by a colorimetric method. Glycosylated hemoglobin (HbA1c) levels were measured in all subjects using an HPLC auto-analyzer Adams HA 8160 (Menarini, Italy). All these determinations were performed according to the manufacturer's specifications, and quality control was within the recommended precision for each test.

2.3. Assessment of Insulin Resistance. Insulin resistance was estimated using the homeostasis model assessment (HOMA-IR) as described by Matthews et al. [30] and validated by several authors for epidemiological studies [18]. HOMA-IR was calculated as the product of fasting glucose (mmol/L) and fasting insulin (mU/L) divided by 22.5.

2.4. Genotyping. DNA samples of 367 diabetic subjects were extracted from peripheral blood samples collected in tripotassium EDTA and purified by using a QIAamp Blood DNA Maxi kit (Qiagen, Dusseldorf, Germany). Samples were genotyped for TLR4 Asp299Gly (+896 A/G TLR4; rs4986790). The procedure for detecting the +896 A/G TLR4 SNP was based on Restriction Fragment Length Polymorphism-PCR (RFLP-PCR), restriction cleavage with NcoI (New England Biolabs, USA), and separation of the DNA fragments by electrophoresis, as previously described [31].

2.5. Statistical Analysis. Data were reported as mean (Standard Deviation) for continuous variables and as percentages (n) for categorical variables. The skewed distributions (triglycerides, fasting insulin, high-sensitivity C-reactive protein, fibrinogen, and creatinine) were log-transformed before statistical analyses to achieve a normal distribution. Differences between patients without complication and those having at least one complication were compared using Student's t-test with Bonferroni correction for continuous variables and χ^2 test for categorical variables.

In order to create a dependent binary variable for the next logistic regression model, we considered neuropathy, lower limb arteriopathy, and the major cardiovascular events (MACE) (i.e., carotid arterial diseases, cerebrovascular, and ischemic heart diseases) complications together ("0" = no complications; "1" = at least one of the three complications).

Logistic regression models were performed to estimate the adjusted risk of having at least one of three complications when AG carrier. Results were expressed as odds ratios (OR) with 95% CI. Two covariates, urea and LDL cholesterol, were strongly correlated, respectively, with creatinine and Apo-B (Pearson's $r > 0.5$). They were removed as they had less explanatory power than the other two. Data were analyzed with SPSS/Win program (version 19.0; Spss Inc., Chicago, IL). Probability values lower than 0.05 were considered statistically significant. The reported P values were two tailed in all calculations.

3. Results

3.1. Patient Characteristics. The 367 T2DM diabetics were characterized to have a mean (Standard Deviation) age of 66 (7.9) years, be males for the 56.7% (precisely 208), and they were affected by T2DM complications for the 55.6%, including neuropathy, nephropathy, chronic kidney failure, chronic arterial disease of the lower limbs, and MACE (carotid arterial diseases, cerebrovascular and ischemic heart diseases). In particular, we reported in the Table 1 the comparisons of anthropometric and biochemical characteristics between patients without complication and those having at least one complication (204 versus 163; 55.6% versus 44.4%, resp.). Thus, we observed that complicated patients were males for the major number than those without complications. In addition, they had an older age and showed higher values of biochemical variables, including fasting glucose, creatinine, urea, HbA1c, total and LDL cholesterol, and Apo-A1.

3.2. Anthropometric and Biochemical Characteristics of T2DM Patients Stratified for the A/A and A/G TLR4 Genotypes. Genotyping the T2DM patients for +896 A/G TLR4 SNP, we observed that they predominantly had the A/A wild type genotype (91.5%; 336). The A/G genotype was observed only in 31 patients (8.5%), while nobody had the G/G genotype. Stratifying the T2DM patients according to these genotypes, no statistical significant differences were detected in their anthropometric and biochemical characteristics, with exception of total and LDL cholesterol values. Higher values of total and LDL cholesterol were assessed in patients with A/A genotype with a $P = 0.052$ and $P = 0.044$, respectively (Table 2), by evidencing a borderline association.

3.3. The Role of TLR4 Genotypes in T2DM Complications. With the aim to evaluate the role of +896 A/G TLR4 SNP on the predisposition of T2DM complications observed in the population studied, we compared their prevalence in positive A/A TLR4 individuals versus those positive for A/G TLR4 genotype. A higher significant prevalence was detected for neuropathy, lower limb arteriopathy, and MACE in positive A/G TLR4 patients, when compared with those positive for A/A TLR4 genotype (see Table 3).

In order to analyse the association between complications and TRL4 polymorphism we considered neuropathy, MACE, and lower limb arteriopathy complications together ("0" = no complications; "1" = at least one of these three complications). The association between complication in the previous three significant variables and TRL4 polymorphism was significant (χ^2 test = 10.697; $P = 0.01$). Thus, we calculated the crude risk to be complicated in patients with AG genotype obtaining the following results: OR = 3.403; 95% CI 1.577–7.344.

In addition, we compared the mean values of the main studied parameters among complicated (at least 1 of 3 complications) and noncomplicated groups, using Student's t-test with Bonferroni correction for continuous variables and χ^2 test for categorical variables. We found the following significant parameters: HbA1c, urea, creatinine, LDL cholesterol, Apo-A1, and Apo-B ($P < 0.001$).

Furthermore, we applied a binary logistic regression model to estimate the adjusted risk of at least one of three complications when AG carrier (Table 4). From the model Azotemia and LDL variables were removed, being redundant

Table 1: Anthropometric and biochemical characteristics of complicated and no-complicated T2DM patients.

	No-complicated cases (n = 163)	Complicated cases (n = 204)	P
Age (years)	64.03 (8.21)	67.58 (7.36)	<0.001
Male(*)	49.7 (81)	62.3 (127)	0.016
BMI (kg/m^2)	28.71 (4.67)	28.71 (4.27)	0.998
Total cholesterol (mg/dL)	202.72 (36.35)	210.86 (37.43)	0.037
HDL-cholesterol (mg/dL)	53.98 (14.78)	51.17 (14.55)	0.069
Triglycerides (mg/dL)	127.97 (91.02)	138.22 (97.91)	0.373
Fasting glucose (mg/dL)	151.74 (36.94)	169.46 (50.84)	<0.001
HbA1C (%)	7.08 (10.04)	7.65 (1.25)	<0.001
Fasting insulin (uiU/mL)	6.82 (5.06)	6.87 (5.99)	0.691
WBC (10^3/L)	6.61 (1.54)	6.78 (1.73)	0.307
High-sensitivity C-reactive protein (mg/dL)	4.16 (4.73)	4.20 (6.24)	0.849
Fibrinogen (mg/dL)	301.32 (84.07)	305.96 (74.81)	0.477
Creatinine (mg/dL)	0.85 (0.17)	0.98 (0.37)	<0.001
HOMA-IR (mg/dL ∗ uiU/mL)	2.61 (2.42)	2.91 (2.78)	0.267
Urea (mg/dL)	38.56 (9.28)	42.41 (14.20)	0.003
LDL cholesterol (mg/dL)	113.38 (27.06)	120.60 (32.53)	0.024
Apo-A1 (mg/dL)	161.41 (34.67)	170.06 (35.24)	0.020
Apo-B (mg/dL)	99.93 (24.38)	104.94 (27.00)	0.068

Variables are expressed as mean (Standard Deviation).
(*)Categorical variable expressed as percentage (n).

Table 2: Anthropometric and biochemical characteristics in T2DM patients stratified for the A/A and A/G TLR4 genotypes.

	A/A TLR4 positive individuals (n = 336)	A/G TLR4 positive individuals (n = 31)	P
Age (years)	66.01 (8.01)	65.93 (7.18)	0.958
Male(*)	55.7 (187)	67.7 (21)	0.195
BMI (kg/m^2)	28.59 (4.44)	29.96 (4.38)	0.102
Total cholesterol (mg/dL)	207.48 (37.27)	193.94 (33.70)	0.052
HDL-cholesterol (mg/dL)	52.35 (14.51)	53.13 (16.83)	0.778
Triglycerides (mg/dL)	133.78 (95.54)	132.45 (89.52)	0.822
Fasting glucose (mg/dL)	161.45 (45.95)	163.13 (47.28)	0.846
HbA1C (%)	7.40 (1.18)	7.38 (1.41)	0.940
Fasting insuline (uiU/mL)	6.91 (5.73)	6.17 (3.73)	0.550
WBC (10^3/L)	6.70 (1.65)	6.77 (1.67)	0.826
High-sensitivity C-reactive protein (mg/dL)	4.20 (5.74)	3.97 (4.04)	0.971
Fibrinogen (mg/dL)	302.02 (78.95)	324.06 (77.87)	0.117
Creatinine (mg/dL)	0.92 (0.31)	0.90 (0.25)	0.726
HOMA-IR (mg/dL ∗ uiU/mL)	2.81 (2.71)	2.47 (1.43)	0.499
Urea (mg/dL)	40.91 (12.67)	38.42 (8.69)	0.284
LDL cholesterol (mg/dL)	117.54 (30.43)	106.05 (28.74)	0.044
Apo-A1 (mg/dL)	165.90 (35.26)	157.81 (34.36)	0.221
Apo-B (mg/dL)	102.54 (25.87)	97.68 (27.05)	0.319

Variables are expressed as mean (Standard Deviation).
(*)Categorical variable expressed as percentage (n).

Table 3: Prevalence of complications in T2DM patients stratified for A/A TLR4 and A/G genotypes.

	A/A TLR4 positive cases	A/G TLR4 positive cases	*P*
Complicated cases	54.2 (182)	71 (22)	0.071
Neuropathy	18.8 (63)	35.5 (11)	0.026
Nephropathy	12.8 (43)	16.1 (5)	0.599
Kidney failure	3.6 (12)	3.2 (1)	0.921
Retinopathy	30.4 (102)	32.3 (10)	0.826
Lower limb arteriopathy	5.1 (17)	16.1 (5)	0.013
MACE	17.9 (60)	35.5 (11)	0.017

Variables are expressed as percentage (*n*).

Table 4: Binary logistic regression model with Odds Ratio (OR) and 95% confidence intervals of at least one of three complications (neuropathy, lower limb arteriopathy, and MACE).

		Study sample	
	OR	95% CI	*P*
TRL4 "A/G" genotype	3.788	1.642–8.741	0.002
Age	1.449	1.027–1.097	<0.001
HbA1c	0.989	1.180–1.780	<0.001
Apo-A1	3.202	0.982–0.996	0.002
Creatinine	0.992	1.248–8.216	0.016
Apo-B	0.983	0.983–1.001	0.067

(Azotemia/creatinine $r = 0.647$; LDL/Apo-B $r = 0.784$). Thus, we observed the adjusted risk at least one of three complications when AG carrier (OR = 3.788; 95% CI 1.642–8.741) (see Table 4).

4. Discussion

T2DM is today considered as world-wide health problem, as demonstrated by continuous increase of its incidence essentially linked to obesity and overweight in growing and constant augment in various populations, such as the Caucasian populations [1]. In addition, the T2DM individuals have an enhanced mortality and morbidity due to T2DM-related complications, including particularly chronic arterial diseases of the lower limbs, carotid arterial diseases, cerebrovascular, coronary and ischemic heart diseases, neuropathies, nephropathy, and chronic kidney failure [1]. This implies the necessity to develop new strategies for prevention and therapy of both T2DM and its complications, even if the diet and physical activity represent until now the main basis in their prevention and management. This condition is leading different researchers to identify appropriate genetic and molecular factors as potential biomarkers and therapeutic targets, which might permit the early identification of at-high risk individuals for both T2DM and its complications. The attention has been particularly focused on inflammatory/immune pathways, including the TLR4 pathway, since the occurrence of T2DM and its complications is now considered as the result of a state of chronic, systemic, and low grade of inflammation in accordance with metainflammation hypothesis [2–4]. The focus on TLR4 pathway derives by different literature data. It has been demonstrated that dietary macronutrients (i.e., fats and sugars) are able to activate this pathway [2]. In addition, long-term intake of diets rich in fats and carbohydrates has been evidenced to provoke an exacerbated expression and activity of TLR4 in human monocytes along with increases in superoxide generation, NF-κB activity, and proinflammatory factors and with a significant correlation with HbA1c levels [32–38]. Other studies performed in animal models showed that over-nutrition or pathogen infections induce an increased TLR4 expression in tissues and cell types modulating energy homeostasis and insulin action, including adipose tissue, pancreatic islets, muscle, gut, endothelial and smooth muscle cells of arteries, brain, kidney, and liver [2, 34–36]. As result, insulin resistance, pancreatic β-cell dysfunction and alteration of glucose homeostasis, increased production of reactive oxygen species of polymorphonuclear leukocytes, and modulation of natural killer cell functions have been found [2, 12–14, 37–41]. The immune dysfunctions observed seem to clarify the high susceptibility to infections of lower respiratory and urinary tracts, skin, and mucous membranes observed in T2DM cases [42]. In the complex, these conditions determine and feed as a vicious cycle a chronic systemic low-grade inflammation, which seems to be responsible for the onset of metabolic diseases, such as T2DM and its related complications [43]. In contrast, it has been demonstrated that insulin reduces LPS-induced TLR4 expression and activation and oxidative stress [44, 45]. In addition, recent investigation supports the idea of involvement of intestinal bacteria in the onset of T2DM and its complications. Specific intestinal bacteria seem to operate as LPS sources mediating LPS release and/or bacteria translocation into the circulation due to vulnerable microbial barrier and the increased intestinal permeability and to play a role in systemic inflammation and onset and progression of T2DM. Pancreatic β cells express significant levels of TLR4 which recognize LPS or intestinal bacteria [45, 46].

Based on these recent evidences, TLR4 seems to have the role of hub in the chronic inflammation observed in T2DM complications, as currently affirmed by Dasu group [47]. In addition, its activity is modulated by genetic variations, principally SNPs, such as +896 A>G. This SNP determines a blunted immune response against viral and bacterial infections or other exogenous (fats and sugars) endogenous molecules characterized by a reduced production of proinflammatory cytokines [9, 19]. A recent meta-analysis evidenced a significant association of AG/GG genotypes with decreased metabolic disorder risk [20]. In contrast, few and inconsistent literature data have been reported on its capacity to be a predisposing or protective genetic factor for T2DM-related complications, that is, neuropathy, retinopathy, ischemic heart disease, and coronary artery disease [22–27]. No literature data exist on TLR4 role in the T2DM-associated chronic kidney and other T2DM-related cardiovascular diseases, such as carotid arterial and cerebrovascular diseases, lower limb arteriopathy, in Caucasian populations, although it is well recognised in other age-related diseases [9]. Thus, the key aim of the present

study was to analyze the weight of +896 TLR4 A>G SNP as potential predisposing or protective genetic risk factor in the major T2DM complications evaluating a population of 367 patients affected by T2DM and with complications for the 55.6%, including neuropathy, nephropathy, chronic kidney failure, chronic arterial disease of the lower limbs, and MACE. Complicated T2DM patients were characterized to be prevalently males (62.3%), to have an older age (67.58 versus 64.03 in noncomplicated cases), and to show higher values of biochemical variables, such as fasting glucose, creatinine, urea, HbA1c, total and LDL cholesterol, and Apo-A1 (Table 1). In addition, 91.5% of cases had the of A/A TLR4 genotype and 8.5% had the A/G genotype, while nobody had the G/G genotype. No associations were observed between A/A and A/G TLR4 genotypes and their anthropometric and biochemical characteristics, with exception of total and LDL cholesterol values (Table 2). In particular, a borderline association was evidenced (Table 2).

Evaluating the role of +896 A/G TLR4 SNP on the predisposition of T2DM complications, interesting data were, however, detected. In particular, diabetic carriers of AG genotype had a major susceptibility for neuropathy, lower limb arteriopathy, and MACE, as reported in Table 3. Their cumulative risk was significant ($P = 0.01$), with a threefold risk to develop neuropathy, lower limb arteriopathy, and major cardiovascular events in AG cases compared to AA cases (crude OR = 3.403; 95% CI: 1.577–7.344). In addition, we applied a binary logistic regression model to estimate the risk, adjusted for confounding variables (HbA1c, urea, creatinine, LDL cholesterol, Apo-A1, and Apo-B), of having at least one complication of three when AG carrier. The adjusted OR was 3.788 (95% CI: 1.642–8.741) as shown in Table 4. This underlines the remarkable role of this SNP in inducing T2DM complications independently from other biological risk factors known to favour the onset of these complications.

5. Limitations

The major limitations of the present study are the relative small sample size and the necessity to confirm and validate our data in larger populations of different genetic at least one of three complications when AG carrier. Despite these limitations, our study represents the first to have analyzed the weight of the TLR4 SNPs in of +896 TLR4 A/G polymorphism as potential predisposing or protective genetic risk factor in the major T2DM complications (neuropathy, nephropathy, chronic kidney failure, chronic arterial disease of the lower limbs, carotid arterial diseases, and ischemic heart diseases) in the Caucasian populations. However, further studies are required to obtain more conclusive results and to consider the rs4986790 TLR4 SNP a biomarker and the TLR4 pathway as target for new therapeutic treatment aimed to prevent or delay the T2DM complications.

6. Conclusions

In the light of the results obtained, a possible explanation of significant predisposition in the development of diabetic complications in AG versus AA genotype carriers is likely due to a compromised immune control against the infectious diseases. Supporting this hypothesis, we recently demonstrated that the genetic control of infectious diseases has a significant role in determining different trajectories to reach longevity in centenarians [48]. Thus, genetic background and consequently genetic factors might have a key role in both onset and progression of T2DM-related complications. As consequence, our results might open new perspectives for the analysis of susceptibility factors and prevention for T2DM-related complications. Actually, these findings might prompt studies on pharmacological strategies to prevent or delay the development of T2DM complications in predisposed subjects. In addition, they lead to considering the rs4986790 TLR4 SNP an optimal biomarker to identify at-risk individuals for T2DM and T2DM-related complications. Thus, it may be an indicative of prevalence of complications in T2DM patients.

Abbreviations

Apo-A1:	Apolipoprotein-A1
Apo-B:	Apolipoprotein-B100
BMI:	Body max index
DAMPs:	Endogenous danger-associated molecular patterns
CRP:	C-reactive protein
eGFR:	Glomerular filtration rate
HbA1c:	Glycosylated hemoglobin
HDL:	High density lipoprotein
HOMA-IR:	Homeostasis model assessment
IL-1β:	Interleukin-1β
LDL:	Low density lipoprotein
LPS:	Lipopolysaccharide
LRR:	Leucine-Rich-Repeat
MACE:	Major cardiovascular events
NF-κB:	Nuclear factor-κB
NK:	Natural killer
OR:	Odds ratios
TLR-4:	Toll-like receptor-4
SNP:	Single nucleotide polymorphisms
T2DM:	Type 2 diabetes mellitus.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Balistreri and Testa made substantial contributions to conception and design. Boemi, Bonfigli, Caruso, and Testa provided study materials/patients and collected and assembled the data. Balistreri performed the genetic analyses. Spazzafumo and Fabietti performed statistical analysis. In the data interpretation, Balistreri, Ceriello, Genovese, Franceschi, Caruso, Lio, and Testa were involved. Balistreri and Testa were involved in drafting the paper. Balistreri, Bonfigli,

Candore, Olivieri, Ceriello, Genovese, and Testa contributed to the critical revision. Balistreri and Testa gave the final approval of the version to be published. All authors participated in the study, and they read and approved the final version of the paper. Carmela Rita Balistreri and Roberto Testa contributed equally to this study.

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References

[1] "International diabetes federation," in *Updated Diabetes Atlas*, N. Unwin, D. Whiting, L. Guariguata, G. Ghyoot, and D. Gan, Eds., Brussels, Belgium, 5th edition, 2011.

[2] C. R. Balistreri, C. Caruso, and G. Candore, "The role of adipose tissue and adipokines in obesity-related inflammatory diseases," *Mediators of Inflammation*, vol. 2010, Article ID 802078, 19 pages, 2010.

[3] G. S. Hotamisligil, "Inflammation and metabolic disorders," *Nature*, vol. 444, no. 7121, pp. 860–867, 2006.

[4] E. Lontchi-Yimagou, E. Sobngwi, T. E. Matsha, and A. P. Kengne, "Diabetes mellitus and inflammation," *Current Diabetes Report*, vol. 13, pp. 435–444, 2013.

[5] H. Kolb and T. Mandrup-Poulsen, "An immune origin of type 2 diabetes?" *Diabetologia*, vol. 48, no. 6, pp. 1038–1050, 2005.

[6] S. E. Shoelson, J. Lee, and A. B. Goldfine, "Inflammation and insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 7, pp. 1793–1801, 2006.

[7] T. Okada, W. L. Chong, J. Hu et al., "Insulin receptors in β-cells are critical for islet compensatory growth response to insulin resistance," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 104, no. 21, pp. 8977–8982, 2007.

[8] Q. Wang and T. Jin, "The role of insulin signaling in the development of β-cell dysfunction and diabetes," *Islets*, vol. 1, no. 2, pp. 95–101, 2009.

[9] C. R. Balistreri, G. Colonna-Romano, D. Lio, G. Candore, and C. Caruso, "TLR4 polymorphisms and ageing: implications for the pathophysiology of age-related diseases," *Journal of Clinical Immunology*, vol. 29, no. 4, pp. 406–415, 2009.

[10] S. Sirisinha, "Insight into the mechanisms regulating immune-homeostasis in health and disease," *Asian Pacific Journal of Allergy and Immunology*, vol. 29, no. 1, pp. 1–14, 2011.

[11] K. Newton and V. M. Dixit, "Signaling in innate immunity and inflammation," *Cold Spring Harbor Perspectives in Biology*, vol. 4, no. 3, Article ID 006049, 2012.

[12] M. J. Hutton, G. Soukhatcheva, J. D. Johnson, and C. B. Verchere, "Role of the TLR signalling molecule TRIF in beta-cell function and glucose homeostasis," *Islets*, vol. 2, no. 2, pp. 104–111, 2010.

[13] H. M. Garay-Malpartida, R. F. Mourão, M. Mantovani, I. A. Santos, M. C. Sogayar, and A. C. Goldberg, "Toll-like receptor 4 (TLR4) expression in human and murine pancreatic beta-cells affects cell viability and insulin homeostasis," *BMC Immunology*, vol. 12, article 18, 2011.

[14] H. Ellingsgaard, I. Hauselmann, B. Schuler et al., "Interleukin-6 enhances insulin secretion by increasing glucagon-like peptide-1 secretion from L cells and alpha cells," *Nature Medicine*, vol. 17, no. 11, pp. 1481–1489, 2011.

[15] J. Amyot, M. Semache, M. Ferdaoussi, G. Fontés, and V. Poitout, "Lipopolysaccharides impair insulin gene expression in isolated islets of langerhans via toll-like receptor-4 and nf-κb signalling," *PLoS ONE*, vol. 7, no. 4, Article ID e36200, 2012.

[16] H. Shi, M. V. Kokoeva, K. Inouye, I. Tzameli, H. Yin, and J. S. Flier, "TLR4 links innate immunity and fatty acid-induced insulin resistance," *Journal of Clinical Investigation*, vol. 116, no. 11, pp. 3015–3025, 2006.

[17] D. M. L. Tsukumo, M. A. Carvalho-Filho, J. B. C. Carvalheira et al., "Loss-of-function mutation in toll-like receptor 4 prevents diet-induced obesity and insulin resistance," *Diabetes*, vol. 56, no. 8, pp. 1986–1998, 2007.

[18] T. Suganami, T. Mieda, M. Itoh, Y. Shimoda, Y. Kamei, and Y. Ogawa, "Attenuation of obesity-induced adipose tissue inflammation in C3H/HeJ mice carrying a Toll-like receptor 4 mutation," *Biochemical and Biophysical Research Communications*, vol. 354, no. 1, pp. 45–49, 2007.

[19] C. R. Balistreri, C. Caruso, F. Listì, G. Colonna-Romano, D. Lio, and G. Candore, "LPS-mediated production of pro/anti-inflammatory cytokines and eicosanoids in whole blood samples: biological effects of +896A/G TLR4 polymorphism in a Sicilian population of healthy subjects," *Mechanisms of Ageing and Development*, vol. 132, no. 3, pp. 86–92, 2011.

[20] F. S. Belforte, F. Coluccio Leskow, E. Poskus, and A. Penas Steinhardt, "Toll-like receptor 4 D299G polymorphism in metabolic disorders: a meta-analysis," *Molecular Biology Reports*, vol. 40, pp. 3015–3020, 2013.

[21] M. Buraczynska, I. Baranowicz-Gaszczyk, J. Tarach, and A. Ksiazek, "Toll-like receptor 4 gene polymorphism and early onset of diabetic retinopathy in patients with type 2 diabetes," *Human Immunology*, vol. 70, no. 2, pp. 121–124, 2009.

[22] F. Lirussi, "The global challenge of type 2 diabetes and the strategies for response in ethnic minority groups," *Diabetes/Metabolism Research and Reviews*, vol. 26, no. 6, pp. 421–432, 2010.

[23] P. W. F. Wilson, J. B. Meigs, L. Sullivan, C. S. Fox, D. M. Nathan, and R. B. D'Agostino Sr., "Prediction of incident diabetes mellitus in middle-aged adults: the framingham offspring study," *Archives of Internal Medicine*, vol. 167, no. 10, pp. 1068–1074, 2007.

[24] P. J. Talmud, A. D. Hingorani, J. A. Cooper et al., "Utility of genetic and non-genetic risk factors in prediction of type 2 diabetes: whitehall II prospective cohort study," *British Medical Journal*, vol. 340, Article ID b4838, 2010.

[25] M. J. Kolek, J. F. Carlquist, J. B. Muhlestein et al., "Toll-like receptor 4 gene Asp299Gly polymorphism is associated with reductions in vascular inflammation, angiographic coronary artery disease, and clinical diabetes," *American Heart Journal*, vol. 148, no. 6, pp. 1034–1040, 2004.

[26] G. Rudofsky Jr., P. Reismann, S. Witte et al., "Asp299Gly and Thr399Ile genotypes of the TLR4 Gene are associated with a reduced prevalence of diabetic neuropathy in patients with Type 2 diabetes," *Diabetes Care*, vol. 27, no. 1, pp. 179–183, 2004.

[27] F. Liu, W. Lu, Q. Qian, W. Qi, J. Hu, and B. Feng, "Frequency of TLR 2, 4, and 9 gene polymorphisms in Chinese population and their susceptibility to type 2 diabetes and coronary artery disease," *Journal of Biomedice and Biotechnology*, vol. 2012, Article ID 373945, 7 pages, 2012.

[28] American Diabetes Association, "Diagnosis and classification of diabetes mellitus," *Diabetes Care*, vol. 30, pp. S42–S47, 2007.

[29] D. W. Cockcroft and M. H. Gault, "Prediction of creatinine clearance from serum creatinine," *Nephron*, vol. 16, no. 1, pp. 31–41, 1976.

[30] D. R. Matthews, J. P. Hosker, and A. S. Rudenski, "Homeostasis model assessment: insulin resistance and β-cell function from fasting plasma glucose and insulin concentrations in man," *Diabetologia*, vol. 28, no. 7, pp. 412–419, 1985.

[31] C. R. Balistreri, M. P. Grimaldi, M. Chiappelli et al., "Association between the polymorphisms of TLR4 and CD14 genes and Alzheimer's disease," *Current Pharmaceutical Design*, vol. 14, no. 26, pp. 2672–2677, 2008.

[32] M. R. Dasu, S. Devaraj, L. Zhao, D. H. Hwang, and I. Jialal, "High glucose induces toll-like receptor expression in human monocytes Mechanism of activation," *Diabetes*, vol. 57, no. 11, pp. 3090–3098, 2008.

[33] M. R. Dasu and I. Jialal, "Free fatty acids in the presence of high glucose amplify monocyte inflammation via Toll-like receptors," *American Journal of Physiology—Endocrinology and Metabolism*, vol. 300, no. 1, pp. E145–E154, 2011.

[34] M. R. Dasu, S. Ramirez, and R. R. Isseroff, "Toll-like receptors and diabetes: a therapeutic perspective," *Clinical Science*, vol. 122, no. 5, pp. 203–214, 2012.

[35] J. J. Kim and D. D. Sears, "TLR4 and insulin resistance," *Gastroenterology Research and Practice*, vol. 2010, Article ID 212563, 11 pages, 2010.

[36] M. Ladefoged, K. Buschard, and A. M. Hansen, "Increased expression of toll-like receptor 4 and inflammatory cytokines, interleukin-6 in particular, in islets from a mouse model of obesity and type 2 diabetes," *Acta Pathologica, Microbiologica, et Immunologica Scandinavica*, vol. 121, pp. 531–538, 2013.

[37] M. E. Stegenga, S. N. van der Crabben, R. M. E. Blümer et al., "Hyperglycemia enhances coagulation and reduces neutrophil degranulation, whereas hyperinsulinemia inhibits fibrinolysis during human endotoxemia," *Blood*, vol. 112, no. 1, pp. 82–89, 2008.

[38] M.-N. Peraldi, J. Berrou, N. Dulphy et al., "Oxidative stress mediates a reduced expression of the activating receptor NKG2D in NK cells from end-stage renal disease patients," *Journal of Immunology*, vol. 182, no. 3, pp. 1696–1705, 2009.

[39] K. Zhang and R. J. Kaufman, "From endoplasmic-reticulum stress to the inflammatory response," *Nature*, vol. 454, no. 7203, pp. 455–462, 2008.

[40] J. Berrou, S. Fougeray, and M. Venot, "Natural killer cell function, an important target for infection and tumor protection, is impaired in type 2 diabetes," *PLoS ONE*, vol. 8, Article ID e62418, 2013.

[41] L. M. A. J. Muller, K. J. Gorter, E. Hak et al., "Increased risk of common infections in patients with type 1 and type 2 diabetes mellitus," *Clinical Infectious Diseases*, vol. 41, no. 3, pp. 281–288, 2005.

[42] E. Lontchi-Yimagou, E. Sobngwi, T. E. Matsha, and A. P. Kengne, "Diabetes mellitus and inflammation," *Current Diabetes Reports*, vol. 13, pp. 435–444, 2013.

[43] P. Dandona, H. Ghanim, A. Bandyopadhyay et al., "Insulin suppresses endotoxin-induced oxidative, nitrosative, and inflammatory stress in humans," *Diabetes Care*, vol. 33, no. 11, pp. 2416–2423, 2010.

[44] P. Dandona, A. Chaudhuri, H. Ghanim, and P. Mohanty, "Insulin as an anti-inflammatory and antiatherogenic modulator," *Journal of the American College of Cardiology*, vol. 53, no. 5, pp. S14–S20, 2009.

[45] E. Esteve, W. Ricart, and J.-M. Fernández-Real, "Gut microbiota interactions with obesity, insulin resistance and type 2 diabetes: did gut microbiote co-evolve with insulin resistance?" *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 14, no. 5, pp. 483–490, 2011.

[46] C. Erridge, "Diet, commensals and the intestine as sources of pathogen-associated molecular patterns in atherosclerosis, type 2 diabetes and non-alcoholic fatty liver disease," *Atherosclerosis*, vol. 216, no. 1, pp. 1–6, 2011.

[47] S. Rosa Ramirez and M. Ravi Krishna Dasu ", "Toll-like receptors and diabetes complications: recent advances," *Current Diabetes Review*, vol. 8, pp. 480–488, 2012.

[48] C. R. Balistreri, G. Candore, G. Accardi et al., "Genetics of longevity. Data from the studies on Sicilian centenarians," *Immunity & Ageing*, vol. 9, article 8, 2012.