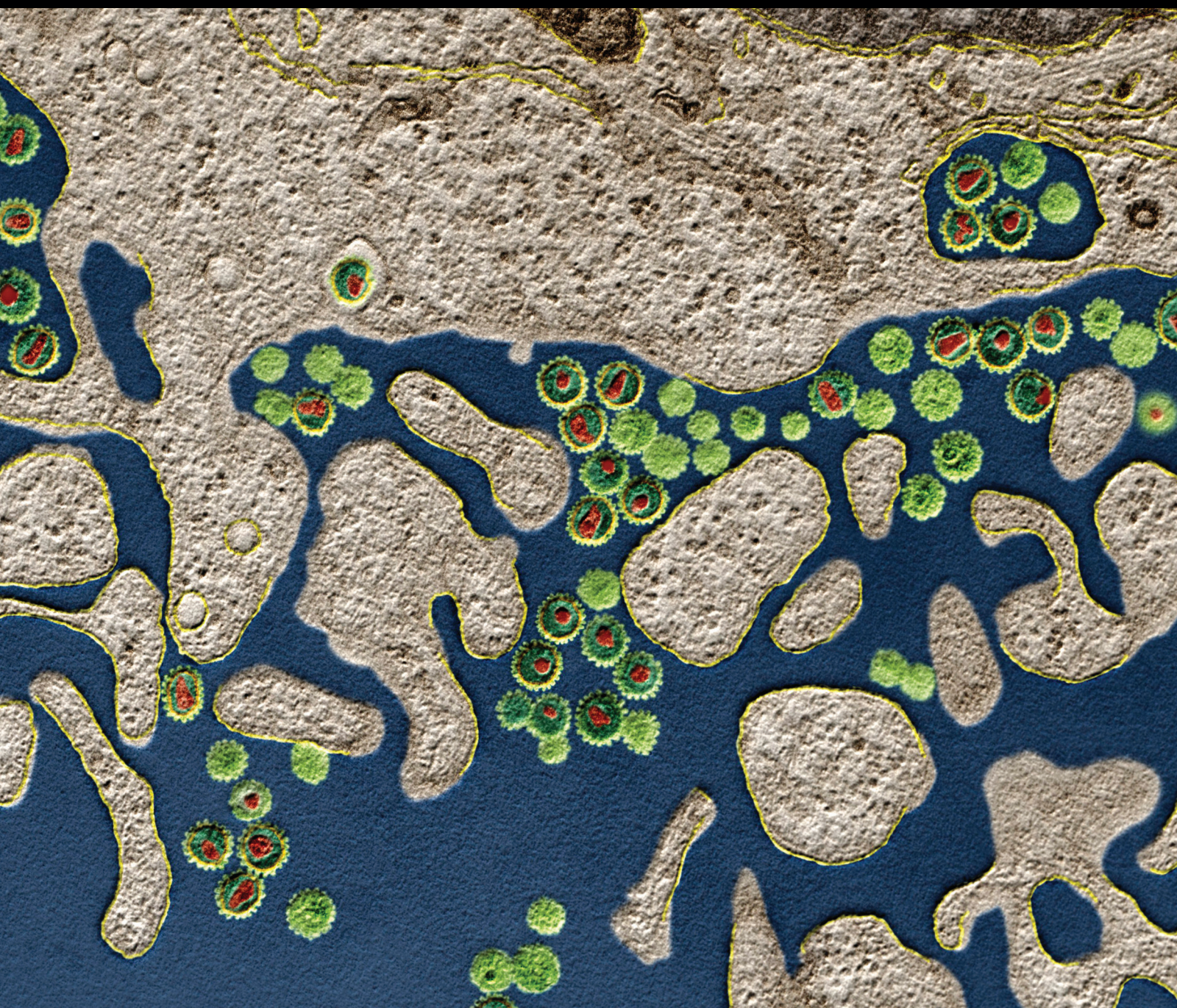
Journal of Immunology Research

Impact of Metabolism on Immune Responses

Lead Guest Editor: Abdallah Elkhal
Guest Editors: Hector Cetina-Biefer rodriguez and Miguel A. de la Fuente



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Editorial Board

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Editorial

Impact of Metabolism on Immune Responses

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Metabolic processes have been long seen as means to generate energy in a live organism. However, recent discoveries on the field of immunology and metabolism have shown an increasing interrelation between these processes. The field of immunometabolism is gaining momentum and presents a huge impact for the clinical practice. Of particular attention is the chronic inflammation state in obesity, as well as asthma to mention a few.

This special issue offers a selected and articulated overview of the examined topic. For this issue, we have included an interesting mix of reviews and original research articles, which underscore the relevance of metabolism in immune responses as observed in the listing below:

Y. Yuan et al. describe the immune regulators of lipid metabolism in obesity as well as the interplay between obesity and asthma. They further propose targeted therapies for direct and indirect immune regulators of lipid metabolism, which might help in the treatment of obesity-related asthma.

In the field of cardiovascular diseases, W. Zhou and M. Zhao underscored the importance of the Hippo-signaling pathway, known to regulate multiple organ development and diseases, and explored novel therapeutic approaches in this field.

In the research article by M. H. Lee et al., they explore the effects of sex and estrogens on the IFN signature of marrow-derived DCs in mice. Their findings show that estrogen enhances marrow-derived DC activation through IFN-dependent and -independent pathways, among other interesting findings, suggesting that immunometabolism plays a significant role in sex-biased diseases.

In his review, J. Kim explores molecular mechanisms responsible for molecular reprogramming in macrophages and T cells, further providing recent updates on functional modulation of immune cells by metabolic changes in the microenvironment.

H. Li et al. were able to show how intermittently high glucose potentiates activation and inflammatory responses through Toll-like receptor 4 and Th1 cells, suggesting a more detrimental role of glucose in a diabetic patient than generally assumed, in particular in diabetes-related vascular diseases.

H. Zhong et al. describe the role of obesity-induced metabolic dysfunction and its correlation to chronic inflammation. Furthermore, they analyze the role of microRNAs in obesity and suggest possible new targets in this field.

J. L. Grün et al. were able to show the plasticity of monocytes in response to metabolic syndrome risk factors such as high-density lipoprotein in human cells. These findings support the notion that metabolic dysfunction has a pivotal component in the systemic inflammatory response observed in cardiovascular diseases.

A. Mishra focuses on the metabolic processes of dendritic cells involved in the activation and differentiation of dendritic cells with special interest in the implications to control airway inflammation and adaptive immunity in asthma.

Acknowledgments

The guest editors hope the information collected in this special issue is of great interest for the readers and stimulates

further research in this interesting developing area of immunology. On a final note, we would like to extend our gratitude to the authors for the excellent and stimulating contribution of their work, as well as to all reviewers for all their inputs and suggestions regarding the published articles.

Abdallah Elkhal
Hector Rodriguez Cetina Biefer
Miguel A. de la Fuente

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Review Article

Obesity-Related Asthma: Immune Regulation and Potential Targeted Therapies

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Obesity, one of the most severe public health problems of the 21st century, is a common metabolic syndrome due to excess body fat. The incidence and severity of obesity-related asthma have undergone a dramatic increase. Because obesity-related asthma is poorly controlled using conventional therapies, alternative and complementary therapies are urgently needed. Lipid metabolism may be abnormal in obesity-related asthma, and immune modulation therapies need to be investigated. Herein, we describe the immune regulators of lipid metabolism in obesity as well as the interplay of obesity and asthma. These lay the foundations for targeted therapies in terms of direct and indirect immune regulators of lipid metabolism, which ultimately help provide effective control of obesity-related asthma with a feasible treatment strategy.

1. Introduction

Obesity is defined as an excess of body fat, and it is one of the main public health challenges worldwide. It increases the risk for certain diseases and disorders, including type 2 diabetes, hypertension, chronic kidney disease, cardiovascular diseases, certain types of cancer, and depression [1]. According to a previous report, approximately 13% of adults worldwide are obese [2]. In 2011–2014, 17% of people aged 2 to 19 years in the US were obese, and in 2011-2012, 38% were either overweight or obese; these are substantial increases in the past three decades [3]. Obesity in the US accounts for up to one-third of total mortality. A study from the Global Burden of Disease (GBD) revealed that the global obesity epidemic is worsening and is placing heavy public health and economic burdens in most regions of this planet. Thus, effective treatments for controlling obesity is necessary [1, 4].

Asthma is a chronic inflammatory disease characterized by variable symptoms of wheezing, shortness of breath, chest tightness, and/or cough and by variable expiratory airflow limitation. It is triggered by multiple factors such as exercise, allergen or irritant exposure, change in weather, or viral respiratory infections [5]. Previous studies have shown that obesity increases the severity of asthma, which demonstrates the close association between these two conditions [6, 7]. Global epidemiological studies on asthma and obesity have also shown that obesity-related asthma has reached an alarming level [8]. Asthma in obese patients is poorly controlled using standard asthma medications including oral corticosteroids, which is partial because the underlying metabolic mechanism, immune cells, and proteins involved in related signaling pathways may be unresponsive to corticosteroids [9]. Hence, additional treatments are urgently needed for the treatment of obesity-related asthma.

We have not yet reached a consensus concerning the accurate and comprehensive pathogenesis of obesity. However, lipid metabolism, which is a major part of energy homeostasis, is undoubtedly involved in the onset and development of obesity [4].

In this review, we summarize the association among lipid metabolism, obesity, and asthma. We also detail the roles of immune responses in lipid metabolism and the pathogenesis of obesity-related asthma. Ultimately, we propose various potential targeted therapies according to distinct cellular types and proteins involved in the regulation of lipid metabolism in obesity-related asthma.

2. Immune Modulators of Lipid Metabolism in Obesity

2.1. Sterol Regulatory Element-Binding Proteins and Lipid Metabolism. Sterol regulatory element-binding proteins (SREBPs) are significant transcription factors that regulate lipid biosynthesis [10]. This keeps the balance of cholesterol and fatty acids through the activation of gene-encoding enzymes [11]. Additionally, SREBPs play an important role as an interchange node within global signaling networks in a variety of physiological and pathophysiological processes [12]. SREBPs also improve the gene expression of low-density lipoprotein (LDL) receptors (LDLR), which are involved in sterol regulation [13–15]. SREBPs are divided into different isoforms, including SREBP1a, SREBP1c, and SREBP2. The physiological roles of SREBPs vary. SREBP1a is involved in global lipid synthesis and growth, SREBP1c is involved in fatty acid synthesis and energy storage, and SREBP2 is involved in the regulation of cholesterol synthesis [16]. Moreover, SREBPs are involved in a myriad of cellular processes and pathologies such as reactive oxygen species (ROS) production, endoplasmic reticulum stress, apoptosis, and autophagy [17]. Nevertheless, the underlying molecular mechanisms remain unclear and need further studies. SREBP1a might modulate the innate immune responses of macrophages, whereas SREBP2 is associated with cell phagocytosis and autophagy, indicating the significant role of SREBPs in the onset and development of chronic inflammatory diseases such as obesity [18–20]. These findings also suggest that targeting SREBPs may be clinically feasible and promising in the treatment of obesity.

2.2. Dipeptidyl Peptidase-4 and Lipid Metabolism. Dipeptidyl peptidase-4 (DPP-4), also known as T-cell surface marker CD26, is widely expressed in multiple cells, particularly in immune cells [20]. It cleaves various chemokines and peptide hormones involved in the regulation of immune response, and it plays an important role in the pathogenesis of inflammation [21, 22]. Previous studies reported on the function of DPP-4, which serves as a surface protease in T-cell activation [23]. Rufinatscha et al. found that in DPP-4 knockdown cells, the levels of triglyceride and peroxisome proliferator-activated receptor alpha (PPARα) were increased, while SREBP-1c expression was obviously decreased [24]. Similarly, Mulvihill found that inhibition of DPP4 significantly reduced postprandial lipoprotein secretion [25]. For example, one selective DPP-4 inhibitor, vildagliptin, led to a significant reduction in total triglyceride and apolipoprotein B-48 (apoB48) concentrations after a high-fat meal [26]. Recent studies showed that another DPP-4 inhibitor, anagliptin, could significantly decrease the expression level of SREBP2 messenger ribonucleic acid, which significantly decreased the plasma total cholesterol and triglyceride levels in anagliptin-treated mice. Both low-density lipoprotein cholesterol and very low-density lipoprotein cholesterol levels were also decreased significantly [27].

2.3. Nuclear Factor- (Erythroid-Derived 2-) Like2 and Lipid Metabolism. In addition to SREBP and DPP-4, nuclear factor-(erythroid-derived 2-) like2 (Nrf2), a basic leucine zipper transcription factor, is widely expressed in human and mouse tissues as a defense against exogenous and endogenous stimulation [28]. Obesity is a low-grade inflammatory disorder, and in a previous investigation, Nrf2 was reported to be involved in antiobesity activity. Nrf2 inducers mitigated the weight gain, insulin resistance, oxidative stress, and chronic inflammation induced by a high-fat diet (HFD) in mice [29]. In the same study, the weight-reducing and insulin-sensitizing effects of Nrf2 inducers were abrogated in $Nrf2^{-/-}$ mice, which indicates the importance of Nrf2 in host antiobesity activity. Consistent with the findings of this study, the Nrf2 inducer glucoraphanin increased energy expenditure, decreased lipid peroxidation, and activated M1-like macrophage accumulation and inflammation signaling in HFD-fed mice. Further experiments uncovered that glucoraphanin mitigates obesity by promoting fat browning, limiting metabolic endotoxemia-related chronic inflammation, and regulating redox stress, which suggests that Nrf2-targeted therapy may be clinically promising in the treatment of obesity [29].

2.4. Intestinal Microbiota and Lipid Metabolism. Bacterial bile salt hydrolase (BSH) enzymes in the gut play a significant role in the metabolism of bile acids [30]. Joyce et al. found that weight gain of mice with normal microbiota and the level of serum cholesterol and liver triglycerides are reduced by the expression of BSH, which indicated that using BSH may regulate host lipid metabolism [31]. A study showed that the levels of plasma triglycerides and muscle lipid (triglycerides and phospholipids) were significantly decreased in mice fed a diet with prebiotic compared to those that were fed a control diet. Additionally, they also found that the expression of muscle lipoprotein lipase mRNA increased in mice with prebiotic treatment, which may have resulted in the decrease in levels of plasma and muscle lipid [32]. Many studies have demonstrated that HFD feeding profoundly affects the gut microbial community [33–35]. Marc Schneeberger's research indicated that the number of *Akkermansia muciniphila* was reduced in mice with HFD treatment, which was significantly and positively correlated with fatty acid oxidation and browning. However, it was negatively associated with lipid synthesis, adiposity, and inflammatory markers [36]. The main route of cholesterol excretion is the conversion of cholesterol into bile acid in the liver, followed by excretion of bile acids through the feces. Bile is also considered to be bacteriostatic and to prevent overgrowth of small intestinal bacteria [37].

Control bacterial fermentation of dietary fiber in the colon produced short-chain fatty acids (SCFAs). SCFAs regulated the proliferation and apoptosis of cells, which affected intestinal permeability. Additionally, SCFAs could regulate anti-inflammatory effects on the intestinal epithelium by serving as ligands for a series of G-protein-coupled receptors (GPRs) [38]. Experiments have shown that SCFA levels are higher in feces of obese (ob/ob) mice and obese human subjects, which may be due to the reduction of colonic absorption of SCFA leading to obesity [39].

3. Obesity and Asthma

In recent years, the incidences of obesity and asthma have been rising with a parallel relationship. The presence of obesity increases the risk for several diseases including asthma [40–42]. Asthma is more common in obese than in nonobese people [43]. Studies have demonstrated that people with asthma have higher BMIs than those without asthma. [44] Moreover, some obese patients with asthma have significant respiratory symptoms and little eosinophilic airway inflammation. Similarly, concordant and growing evidence also supports the relationship between being overweight (defined by body mass index (BMI)) and having asthma [40–42]. Obesity has emerged as a serious risk factor for bronchial asthma, which indicates that obesity could cause or even worsen asthma. Additionally, asthma is more difficult to control in obese patients [45]. Therefore, this study focused on obesity-related asthma. Potential factors that affect the pathogenesis of obesity-related asthma are also summarized in the review (Figure 1).

3.1. Low-Grade Inflammation and Asthma. Obesity is regarded as an inflammatory disease [46]. Unlike typical inflammation, it is chronic low-grade systemic sterile inflammation that is characterized by only moderate upregulation of circulating proinflammatory factors and the absence of clinical symptoms of inflammation [47]. The inflammatory response may affect pulmonary function and thus worsen the asthma. In particular, macrophages play an important role in the occurrence and development of obesity-associated asthma. The HFD mice in obesity modelling increase the number of macrophages in the lungs and alveoli. Besides total cell count in bronchoalveolar lavage fluid (BALF), neutrophils and a few eosinophils also had increased counts [48]. In addition, the concentration of Th1 cytokines and IFN-γ also increased significantly in BALF [49]. In healthy adipose tissue, immune cells normally consist of CD4+ T-cell, regulatory T-cells (Treg), and type 2 macrophages (M2), which can regulate heat production, inflammation, and lipid metabolism. Nevertheless, in obese individuals, adipocyte hypertrophy and cytokine secretion result in a shift from M2 to M1 [50] and from Th2 to three different types: Th1, Th17, and CD8+ CTL. Obesity leads to increasing expression of the following: proinflammatory cells such as macrophages; integrins such as CD11b and CD11c; cytokines including TNF-α, IL-6, and nitric oxide synthase 2 (NOS2); and triggers such as Toll-like receptors (TLRs), metabolic endotoxemia, lipid spillover, and adipokines. These result in a shift from anti-inflammatory M2 type to proinflammatory M1 type [51]. The activation of NF-κB pathways caused by these cytokines then ensues, thereby inducing the overexpression of proinflammatory cytokines such as TNF-α and IL-6. The activation of adipose tissue macrophages (ATMs) is an amplification of the inflammatory process [52]. In obese humans, long-term nutritional excess can lead to adipose tissue hypertrophy, adipose tissue vascularization, hypoxia, and adipose tissue necrosis. It can also lead to the infiltration of macrophages into adipose tissue and the surrounding necrotic tissue, which also produces a wide variety of proinflammatory cytokines [53]. In addition, the secretion of inflammatory cytokines and activation of the NF-κB pathway trigger transcriptional expression of Nod-like receptor family pyrin domain containing 3 (NLRP3) and pro-IL-1β as well as the subsequent activation of NLRP3, causing macrophages to produce IL-1β. Overproduced saturated fatty acids in obese individuals can also stimulate NLRP3 inflammasome activation [54]. Inflammatory factors spread from adipose tissue into the blood circulation and then reach the lungs, which trigger airway inflammation and hyperresponsiveness. It has been reported that TNF-α can directly induce airway hyperresponsiveness (AHR) [55]. In addition, obesity can lead to abnormal fatty acid metabolism and increased fatty acid in blood by promoting increased expression of ACC1 and subsequently activation of RORγt to induce the differentiation of Th17 cells. IL-17 is produced by Th17 cells that bind with its receptors. IL-17 is able to improve the secretion of inflammatory cytokines such as IL-6, TNF-α, IL-8, CAM-1, and CM-CSF through the MAPK or NF-κB pathway [56]. The association between obesity and neutrophil count in sputum is significant; in addition, a recent cluster analysis has shown that the presence of obesity-related asthma is characterized by increased airway neutrophils [57–59]. In addition to this, IL-17, IL-6, and TNF-α secreted by Th17 cells recruit and activate neutrophils in the lungs [60]. Neutrophils play an important role in ATM recruitment and inflammation by degradation of insulin receptor substrate 1 or activation of the TLR-4 pathway [61]. In addition to Th17 cells, Th1 cells in abnormal adipose tissue of obese individuals can secrete cytokines including IL-2, IL-3, IFN-γ, and TNF-α, which may activate M1 and thereby activate neutrophils through the chemokines (C-X-C motif) ligand 8 (CXCL8) pathway. Obesity-associated AHR was independent of adaptive immunity. A few eosinophils but many neutrophils were found in sputum, which suggests that obesity-associated asthma may be allergen independent [9]. Apart from the immune responses mentioned above, innate lymphoid cells also participate in the pathogenesis of obesity-related asthma. Although ILC2s have been reported to promote AHR and airway inflammation in a previous study [62], other studies showed that ILC2s played a crucial role in the repair of the airway epithelium, metabolic regulation, and translation from white adipose tissue into beige adipose tissue in the lungs [63]. However, a lack of ILC2 protection in obesity-related asthma may be partially due to the replacement of the ILC2 response with ILC3 response. In a study involving diet-induced obese mice, ILC3s and Th17 as well as the corresponding release of IL-17 were observed in the BALF, which may promote the development of AHR [64]. Bronchial epithelial cells stimulated by

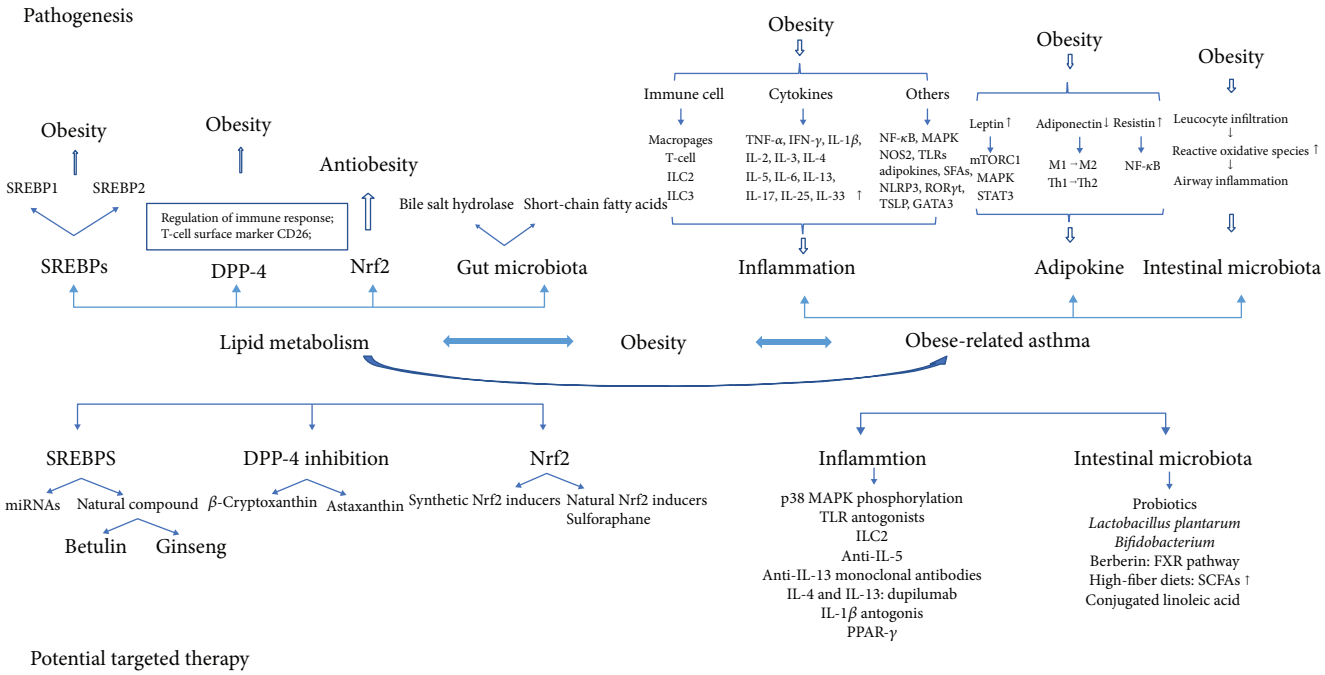

Figure 1: Possible connections among lipid metabolism, obesity, and obesity-related asthma. This figure is divided into two parts: the upper part about pathogenesis and the lower part about potential targeted therapy. The part on pathogenesis demonstrates a few major effects of immune regulators. The other introduces the corresponding possible targeted therapy including SREBPs, miRNAs, and the DPP-4 inhibitor.

exogenous substances such as ozone [65] or cytokines can secrete IL-25, IL-33, and thymic stromal lymphopoietin (TSLP), thereby activating ILC2 to produce type 2 cytokines such as IL-4, IL-5, and IL-13. The pathway depends on the activation of the transcription factor GATA3 [66]. Macrophages, neutrophils, ILC2, Th1, and Th17 secrete a wide variety of cytokines and chemokines, which act on bronchial smooth muscle and may subsequently cause a series of asthmatic symptoms including airway narrowing, airway remodeling, mucus hypersecretion, AHR, airway smooth muscle constriction and hypertrophy, and a rapid decline in lung function.

3.2. Adipokine Secretion and Asthma. Obesity-related low-grade inflammation originates from the adipose tissue, which enables the secretion of a variety of interleukins and adipokines such as leptin, adiponectin, and resistin. In turn, these factors can affect obesity-related inflammation and airway inflammation [67]. Therefore, adipose tissue can be considered as a typical endocrine organ [68]. The amount of leptin produced by adipose tissue is higher in obese subjects compared to their nonobese counterparts [69]. However, a previous study found that leptin function in obese patients did not change. Leptin tolerance may be a causative factor in obesity-related asthma [70]. Moreover, overproduced leptin can stimulate the production of proinflammatory mediators, such as TNF-α, IL-6 from the adipose tissue, and IFN-γ from Th1 immune responsive CD4+ T-cells. TNF-α and IFN-γ are the mediators associated with AHR in asthma. On the other hand, leptin may inhibit the function and proliferation of regulatory T-cells, which may impair the balance of Th1 and Th2 and promote the polarization of Th1-mediated autoimmune diseases and Th2-mediated immune diseases such as asthma [71, 72]. Leptin not only affects the innate immunity but also has a significant impact on the allergic inflammatory response in obese individuals. Leptin promotes the proliferation and survival of proallergic Th2 cells and ILC2 by activating mTORC1, MAPK, and STAT3 pathways, which leads to the production of type 2 cytokines such as IL-4, IL-5, and IL-13, which together contribute to allergic responses [73]. In short, IL-4 promotes the production of IgE and subsequently activates mast cells; IL-5 activates and recruits eosinophils. IL-13 can directly act on goblet cells, which results in the secretion of airway mucus, airway remolding, and AHR [74]. A study found that the release of airway mucus induced by IL-13 can be regulated through the JAK2-STAT3-MUNC18b pathway [75]. There is no evidence that leptin is a direct cause of asthma. However, several studies have demonstrated that leptin is correlated with obesity and asthma in both adults and children [69, 76, 77]. Adiponectin is an anti-inflammatory adipokine produced by adipose tissue [78]. Adiponectin participates in inflammation by downregulating proinflammatory cytokines including TNF-α, IL-6, and NF-κB and upregulating anti-inflammatory cytokines such as IL-1 receptor antagonists and IL-10. In addition, the decreased level of adiponectin reduces the inhibitory effect of bacterial lipopolysaccharide (LPS), thereby inducing macrophages to produce IFN-α, which induces the body's low-grade inflammatory reaction [79]. Adiponectin also induces a shift from M1 to M2 and from Th1 to Th2 and enhances the immunity and anti-inflammatory ability of body [80]. However, despite the enhanced inflammation in obese

individuals, their levels of adiponectin are low [46]. This may result in the decreased anti-inflammatory activity of adiponectin. In addition, the overexpression of proinflammatory cytokines may inhibit the secretion of adiponectin, which results in further reduction in the anti-inflammatory effect of adiponectin, thereby providing the appropriate immunological environment for the onset and development of asthma. Furthermore, the effect of distinct isoforms of adiponectin varies in obesity-related asthma cases. A recent study found that the higher concentration of low-molecular-weight (LMW) adiponectin and the lower ratio of middle molecular weight (MMW) adiponectin/total adiponectin were evidently associated with asthma [81]. However, the level of high-molecular-weight (HMW) adiponectin in serum is not related to the onset and development of obesity-associated asthma [67]. Resistin, like leptin, is another proinflammatory adipokine produced by adipose tissue. Obesity is a chronic low-grade inflammatory condition accompanied with the increased production of various inflammatory factors including resistin. Moreover, inflammatory factors such as IL-1, IL-6, TNF-α, and LPS can promote resistin expression via NF-κB-induced pathways. In turn, the proinflammatory cytokines including IL-6 and TNF-α can be promoted by resistin, thus decreasing obesity-associated inflammation [82]. These proinflammatory factors act on the lungs, which may lead to increased airway inflammation and asthma [83]. Through the comparison of serum leptin levels, Hassan et al. found that obese subjects with asthma showed higher resistin levels. With the increase in resistin levels, asthma severity is also accordingly increased [83]. Similarly, other studies have also demonstrated that the level of resistin and resistin/adiponectin ratio are proportionally increased in asthma and are even higher in obese subjects with asthma. In addition, the level of resistin and resistin/adiponectin ratio can also negatively predict lung function [67]. The above studies suggest that resistin can aggravate inflammation and promote the onset and deterioration of asthma. Thus, obesity may promote the occurrence of asthma and further aggravate asthma by promoting the increase of resistin. These studies probably uncovered a novel therapeutic target for obesity-related asthma.

3.3. Intestinal Microbiota Dysbiosis and Asthma. Intestinal microbiota mainly act as a biological barrier and are involved in immune regulation. Bacterial diversity increases mucosal immune defense [84]. However, obesity may result in intestinal microbiota dysbiosis and the reduction of bacterial diversity with an increase in Firmicutes and a reduction in Bacteroidetes [85], which are responsible for intestinal barrier and immune function damage. Thus, this results in weight gain, systemic inflammation, insulin resistance, and asthma [86]. Studies have reported that the concentration of LPS in plasma increased significantly in obese individuals, which may be due to the increase in intestinal permeability and excessive HFD [87]. Obesity-related low-grade inflammation and intestinal microbiota disorders can cause increased intestinal mucosal permeability [88]. LPS moves from the intestinal mucosa into the blood circulation, leading to endotoxemia. The related underlying mechanism is as follows: binding of LPS with TLR4 may activate the NF-κB pathway, which produces a wide variety of cytokines, including TNF-α and IL-6 [89]. These cytokines act on the lungs, which may result in AHR and asthma exacerbation. Diet also affects the intestinal microbiota, thereby influencing asthma. A high-fiber/low-fat diet can increase the circulating levels of SCFAs [87] that play a critical role in inhibiting inflammation by regulating the differentiation and activation of Treg [90, 91], inhibiting LPS-induced NF-κB activation, increasing TNF-α levels in neutrophils and macrophages, and inhibiting neutrophil production of proinflammatory reactive oxygen species (ROS) and TNF-α [92]. SCFAs stimulate intestinal epithelial proliferation and differentiation and could also contribute to the repair of the epithelial cell damage that is typical in asthma [92]. Moreover, SCFAs produced by the metabolism of dietary fiber from gut bacteria also promote the secretion of leptin to inhibit weight gain. However, a high-fat/low-fiber diet results in decreased levels of SCFAs and the imbalance of gut bacteria [93]. A study on an animal model reported that a low-fat/high-fiber diet promoted the production of beneficial bacteria such as Lactobacilli, Bifidobacteria, and Faecalibacterium and the production of SCFAs, especially butyrate. In contrast, the high-fat/low-fiber diet increased the Enterobacteriaceae, which are harmful to human health conditions [94]. High-fat/low-fiber diet-induced dysbacteriosis inhibits the function of regulatory T-cells through epigenetic modifications of the Forkhead box P3 (FoxP3) promoter and increases Th2-induced allergic airway inflammation. HFD-induced obesity may increase the risk for asthma via the changing gut bacteria. In addition, the obesity-induced dysbiosis of gut bacteria causes cholesterol metabolic disorders and the levels of bile acids are reduced in the gut, which impair the inhibition of NLRP3. NLRP3 activation induces IL-1β secretion primarily by M1 macrophage, which induces AHR that is considered as a major feature of asthma [95]. Many epidemiological studies have shown that with the increased number of caesarean section deliveries, there has also been an increase in obesity, type 1 diabetes, allergies, celiac disease, and some neurological disorders [96]. Caesarean-born children did not pass through the mother's birth canal, which causes intestinal microbial abnormalities, thus resulting in offspring obesity [97]. Studies found that compared with natural-born mice, caesarean-born mice gained more weight [98]. In addition, women who undergo caesarean section delivery usually use antibiotics preventively, which may be harmful to the baby's intestinal microbiota and may exacerbate obesity and other diseases. Another study found that ovalbumin-induced asthmatic mice had increased diversity of intestinal microbiota when exposed to microbes in early life. Moreover, IFN-γ levels and the ratio of IFN-γ and IL-4 were also increased significantly, which suggests that increased diversity of intestinal microbiota may induce Th1 response and inhibit airway inflammation in allergic asthma [99]. The results of these studies, taken together, indicate that a lack of microbial exposure in early life may cause obesity and asthma. Hence, we think that obesity caused by the lack of microbial exposure in early life may result in the risk and deterioration of asthma.

4. Potential Targeted Therapy of Obesity-Associated Asthma

There is a close association between obesity-associated asthma and several well-established risk factors for morbidity; thus, reversing the obesity-associated asthma is an urgent priority. We summarize herein potential targeted therapies for immune regulation, intestinal microbiota, and inflammation to control obesity-associated asthma (Figure 1). A better understanding of these different therapies will lead to future advances in the clinic.

4.1. Immune Regulator-Targeted Therapies. MicroRNAs (miRNAs), a class of noncoding RNAs, emerge as a novel treatment strategy for dyslipidemia and obesity via the modulation of sterols by SREBPs [100]. Among them, the most popular miRNA in the modulation of lipid metabolism is miR-33a, which is located in the intron of SREBF2. miR-33 regulates high-density lipoprotein (HDL) biogenesis and cholesterol efflux by downregulating the expression of ATP-binding cassette transporters [12]. miR-33 also targets SREBP1c, thereby affecting obesity [101]. A study using humanized knock-in mice observed the contribution of miR-33b to a relatively low HDL cholesterol level in human beings [101]. After induction by host SREBF genes, the miR-33 system facilitates lipid homeostasis by modulating the opposing regulator systems (plasma LDL versus HDL cholesterol and cholesterol versus fatty acid metabolism), indicating the therapeutic intervention of miR-33 in dyslipidemia [102]. Another example that has found clinical success in the regulation of lipid metabolism via SREBP is PCSK9 inhibitors, which indicate SREBP2 target PCSK9 in plasma and contribute to LDLR degradation [103, 104].

Intriguingly, extracts and natural compounds derived from some plants are also reported to possess antiobesity activity by targeting SREBP genes and other adipogenesis-related genes (such as PPARγ and ACC1). Betulin, a natural triterpene isolated from the bark of birch trees, was reported to inhibit the maturation of SREBP by inducing the interaction of SREBP cleavage-activating protein (SCAP) and insulin-induced gene protein (INSIG), which lowered the biosynthesis of cholesterol and fatty acid. In vivo, betulin ameliorated diet-induced obesity by decreasing the lipid contents in serum and tissues and concomitantly enhancing insulin sensitivity. Thus, this is a potential compound for the treatment of hyperlipidemia and obesity [105]. Ginseng, which has been used in traditional Chinese medicine for centuries, is a novel depressor against SREBP and other transcriptional factors such as PPARγ and ACC1. A South Korean research group found that compared with HFD mice, mice receiving HFD with Korean red ginseng extract (GE) (10 μg/ml) for eight weeks had decreased body weight, adipose tissue mass, and adipocyte size, which indicated significant antiobesity effect [106]. Additionally, data in vitro confirmed that GE and two major ginsenosides, Rb1 and Rg1, inhibited adipogenesis by lowering PPARγ, C/EBPα, and SCD1 expression at the gene level. Other studies have also reported significant antiobesity activity of Korean red ginseng by modulating transcription factors such as PPARγ and aP2, which decrease the amount of lipid accumulation and inhibit adipogenesis [107–109]. These observations suggested that natural herbs are abundant medical sources for targeting SREBPs and other transcriptional factors in the treatment of obesity-related asthma.

In addition to inhibitors targeting the transcriptional factor SREBP, two independent research groups highlighted the potential clinical application of DPP-4 inhibitors in the attenuation of macrophage cell-mediated or immune cell-mediated inflammation [110, 111]. DDP-4 inhibitor or carotenoids such as β-cryptoxanthin and astaxanthin facilitate the immune regulation of lipid homeostasis in vitro at least partly by the decline in M1 macrophage numbers and the increase in M2 macrophage numbers, which indicate potential application in the treatment of obesity-related asthma in clinical and scientific studies [112, 113]. There was evidence demonstrating the role of Nrf2 in the treatment of obesity. A wide variety of synthetic Nrf2 inducers including triterpnoid 2-cyano-3, 12-dioxoolean-1, 9-dien-28-oic acid- (CDDO-) imidazolide, dithiolethione analog, and oltipraz demonstrated a significant ameliorative effect on HFD-induced obesity [114, 115]. However, it seemed that synthetic Nrf2 inducers were not clinically available due to the significantly increased risks of heart failure and the composite cardiovascular outcomes (nonfatal myocardial infarction, nonfatal stroke, hospitalization for heart failure, or death from cardiovascular causes) [116]. Sulforaphane, an isothiocyanate derived from cruciferous vegetables, is one of the most potent naturally occurring Nrf2 inducer. The compound was reported to ameliorate obesity by the enhancement of energy expenditure and the reduction of metabolic endotoxemia, which were caused by the decline in inflammation and insulin resistance. Sulforaphane may be a promising treatment for obesity-related asthma [29].

4.2. Intestinal Microbiota-Targeted Therapies. Gut microbiota have been associated with obesity-activating innate immunity through the LPS Toll-like receptor 4 axis [117]. Gut microbiota dysbiosis may induce obesity-related asthma [118]. We hypothesized that it may be beneficial to improve or treat obesity-related asthma via the modulation of gut microbiota. Moreover, the metabolic products of intestinal microbiota have a significant effect on body metabolism and immune defense mechanisms. Probiotics have a significant effect on improving intestinal microbiota modulation and systemic immunity. Previous studies indicated the weight of mice fed with HFD was the highest in three groups of mice with HFD, mice with HFD plus probiotics, and mice with HFD plus *Lactobacillus plantarum* (LP). The mice in high-fat diet plus LP exhibited significantly lower IL-6 and endotoxin (ET) content. Moreover, it will preferably regulate intestinal microbiota and systemic immune function to feed the combination of *Lactobacillus plantarum* (LP) and *Lactobacillus fermentum* (LF) [119]. In view of systemic obesity-related inflammation, the use of probiotics in the treatment of obesity probably has a potential effect on improving asthma. Clinical evidence demonstrated that the microbiome of obese people lacked a cluster of abundant Bacteroides species. Animal experiments showed that gavage with *B. thetaiotaomicron* can reduce serum glutamate concentration, which

increased lipolysis and fatty acid oxidation process, thereby reducing fat accumulation and eventually achieving weight loss. Thus, it may be feasible to prevent obesity by regulating gut microbiota [120].

Obesity-related microbiota dysbiosis influences the production of bile acid, which is associated with lipid digestion and absorption [121]. Intestinal bile acids can be absorbed by bile acid binding resin (BAR) and likely improve obesity and metabolic disorders [122]. The binding of Berberine (BBR), extracted from the roots of *Rhizoma coptidis*, with gut and intestinal Farnesoid X receptor (FXR) lead to decreasing serum lipids in humans, hamsters, mice, and rats [123]. BBR inhibited bile salt hydrolase (BSH) activity in gut microbiota and activated FXR signaling pathway to alter bile acid metabolism, which may regulate lipid metabolism and then achieve weight loss. Therefore, BBR administration may be an effective strategy to improve obesity-related asthma [124].

Diet can also affect the intestinal microbiota balance and improve obesity and asthma. High-fiber diets can increase intestinal and circulating SCFA concentrations to suppress allergic inflammation and weight gain, which relies on the metabolism of intestinal microbiota [125]. Trompette et al. reported that high-fiber diet and SCFAs can shape immunological environment in the lungs by regulating the differentiation and activation of Treg. They can also affect the severity of allergic inflammation by inhibiting neutrophil production of proinflammatory reactive oxygen species (ROS) and TNF-α [90]. Thus, a diet with abundant dietary fermentable fiber may be beneficial for patients with obesity-related asthma. In addition, we summarized that increased activation of microbes producing SCFAs or the direct application of microbes producing SCFAs via fiber metabolism may also improve the symptoms of obesity-related asthma. Moreover, a prospective cohort study indicated that yogurt significantly prohibited weight gain, in particular among participants with higher fruit consumption, which may be due to a mechanism mediated by probiotics such as *Lactobacillus* and *Bifidobacterium* [126]. Additionally, gastrointestinal bacteria could metabolize foods such as beef, yogurt, and vegetable oils to produce conjugated linoleic acid (CLA) such as trans-10 and cis-12 CLA, which significantly prevented weight gain via mechanisms that increase adipocyte turnover and lead to the appearance of metabolically active beige adipocytes. Free linoleic acid and α-linolenic acid are converted to different CLA by *Bifidobacterium*, *Bifidobacterium pseudolongum* strain, and *Bifidobacterium breve* strains [127]. Therefore, CLA and CLA-associated compounds are a novel strategy to control weight that may be beneficial to improve obesity-related asthma.

4.3. Inflammation-Targeted Therapies. Obesity-related asthma is a chronic inflammatory disease accompanied with the disorder of proinflammatory and anti-inflammatory molecules driven by cytokines. Therefore, the regulation of cytokine secretion may be a new strategy to inhibit inflammation-induced obese-related asthma. MKP-1, a MAPK deactivator, can produce p38 MAPK phosphorylation in an irreversible manner, thereby inhibiting the occurrence of inflammation. Prabhala found that binding of dexamethasone or compounds interrupting the proteasome with MKP-1 can induce p38 MAPK phosphorylation and inhibit inflammatory cytokines in airway smooth muscle cells [128]. The interaction of p38 MAPK and MKP-1 may be a novel therapeutic target for obesity-associated asthma. Obesity-associated inflammation is mostly related to TLR4-NF-κB pathway [129]. TLR4 knockout or antagonists may be beneficial for obesity-related asthma. TLR4 knockout relieves HFD-induced phosphorylation of IKKβ, JNK, mTOR, and proinflammatory signaling molecules, which can alleviate obesity-associated inflammation. It might implicate the possible therapeutic potential of TLR4 in the management of asthma in HFD-induced obesity [130]. Furthermore, TLR antagonists have been used to treat metabolic diseases due to the beneficial effects of immune suppression in modern medicinal studies and applications. However, HFD-induced low-grade chronic inflammation may be an evolutional protective mechanism against pathogens. TLR antagonists or TLR knockout may inhibit the activity of host TLR, which probably increases the vulnerability to infection [131].

Chronic low-grade inflammation of the body induced by obesity also followed ILC2 activation. Cytokines derived from lung epithelial cells, such as IL-25, IL-33, and TSLP, can activate ILC2 to cause pulmonary injury. The activated ILC2s subsequently produce type 2 cytokines (IL-4, IL-5, and IL-13) and induce severe inflammation in the lungs [73]. Thus, inhibition of IL-25, IL-33, and TSLP possibly relieves type 2 inflammation. In a previous publication, a combination of an anti-TSLP antibody, AMG 157, and TSLP effectively obstructed the interplay between TSLP and its receptor, which may inhibit ILC2 activation [132]. Many clinical trials have revealed that antibodies against IL-5 or IL-5 receptor, IL-13, and IL-4Rα modestly reduced asthma exacerbations and improved lung function [133]. Anti-interleukin-5 (anti-IL-5) is a neutralizing antibody targeting IL-5, which is essential for eosinophil maturation and survival. Two anti-IL-5 drugs have been approved by the Food and Drug Administration (FDA) in the US: mepolizumab [134], by subcutaneous injection monthly, and reslizumab [135], by intravenous infusion monthly. Ramirez-Carrozzi et al. concluded that commissural inhibition of IL-13 and IL-33 pathway or IL-5 and IL-13 pathway was extremely effective to reduce type 2 inflammation in patients with severe asthma [136]. Of note, the blockade of interleukin-13 using the two anti-interleukin-13 monoclonal antibodies, lebrikizumab and tralokinumab, potentially improved airway inflammation and smooth-muscle reactivity, which may reduce FENO but increase circulating eosinophil counts. The growth in peripheral blood eosinophil counts have been reported previously to reflect blocking of IL-13 activity [137]. In 2015, in a phase II clinical study of using lebrikizumab to patients with moderate-to-severe uncontrolled asthma despite ICS therapy and an additional controller, subcutaneous administration of lebrikizumab taken every four weeks reduced asthma exacerbation rate by 60% compared with placebo in periostin-high patients and by 5% in periostin-low patients. In this study, despite improving lung function,

lebrikizumab treatments have not yet led to clinically meaningful placebo-corrected improvements in asthma symptoms or quality of life, potentially due to the limited power of the studies for these safety endpoints [138]. In 2017, the researchers had phase III clinical studies to provide further evidence of the safety and efficacy of lebrikizumab. Lebrikizumab, targeting IL-13 alone with biologics, has not shown a consistent reduction in asthma exacerbation. However, they confirmed that dupilumab, a medication simultaneously targeting at both IL-4 and IL-13 via blocking IL-4 receptor, has yielded more consistent results in reducing asthma exacerbations and improving lung function, especially in patients with increased blood eosinophils. Therefore, biologics targeting IL-4/IL-13 may be useful in patients with proof of T2-high asthma based on the presence of type 2 inflammation regardless of their baseline blood eosinophil levels [139]. Besides, late phase clinical trials of drugs targeting the IL-4/IL-13 pathways show promising results to achieve FDA-approved therapies. Doherty has said that TSLP can induce partial corticosteroid resistance, but under this condition, corticosteroid can still inhibit IL-33 to activate ILC2 [140]. Therefore, it is possible that corticosteroid can improve modestly obesity-related asthma. In addition, the function of ILC2 can be inhibited by Treg cells by the secreting cytokines of IL-10 and TGF-β in adipose tissue or via the direct contact of ILC2 with Treg, which can improve type 2 inflammation and deterioration of lung function. Therefore, taking effective measures to increase the number of Treg cells in asthmatic patients may reduce the inflammatory response induced by type 2 inflammatory cells [141]. In addition to ILC2, a team conducting experiments in mouse models of HFD concluded that NLRP3, IL-1β, and ILC3 cells facilitated obesity-related asthma by mediating inflammation [142]. In an experiment, after a short treatment of the IL-1β antagonist, anakinra, the symptom of AHR of obese mice induced by high-fat diets was improved [143]. Another team conducted a phase I clinical study, the result of which was that anakinra effectively reduced airway neutrophilic inflammation and caused no serious adverse events in a model of inhaled endotoxin LPS challenge. Thus, anakinra can be regarded as a potential therapeutic candidate for treatment of asthma with neutrophil advantage [144]. To date, it has not been investigated whether this type of treatment could be clinically applied in humans. In addition to inhibiting inflammatory cytokines, overall anti-inflammatory therapy may also be effective in obesity-related asthma. Similarly, obesity-related asthma frequently accompanies insulin resistance due to the lack of adiponectin. Calixto et al. conducted a study using obese mouse model fed with a HFD and found that metformin, a first-line treatment for diabetes, attenuated the exacerbation of the allergic eosinophilic inflammation [145]. In a retrospective cohort study, metformin users had a lower risk for asthma-related hospitalization and asthma exacerbation [146]. Hence, healthcare providers should consider metformin as a potential medication for patients with concurrent asthma and diabetes. It is well known that macrophages play an important role in the development and deterioration of obesity-related asthma and that peroxisome proliferator-activated receptors (PPARs) are expressed in monocytes/macrophages and adipose tissue. The activation of PPARs inhibits the shift from M2 to M1 and from Th2 to Th1 and also inhibits the secretion of proinflammatory cytokines such as IL-1β, IL-6, IL-10, IL-12, and TNF-α [147]. A study conducted by Yoon et al. demonstrated that the activation of PPAR-γ induced by apoptotic cell instillation over the course of bleomycin-induced lung injury can reverse the enhanced efferocytosis, the decreased expression of proinflammatory cytokines, and neutrophil recruitment, which likely inhibits inflammatory responses. Moreover, PPAR-γ activation may cause specific death of macrophages [148]. Therefore, PPAR-γ activation with PPAR-γ agonist or other PPAR-γ-stimulating compounds may reduce obesity-associated inflammation, thereby improving the severity of obesity-related asthma. To date, PPAR-γ agonists have not been studied clinically. Moreover, the regulation of adipokine level in obese individuals can also improve obesity-related asthma. Adiponectin secreted by adipose tissue is an anti-inflammatory adipokine and can promote the utilization of intracellular fatty acid and triglyceride-content reduction, whereas the level is decreased in obese individuals [149]. It has been demonstrated that adiponectin resistance observed in obese patients is due to the increased level of adiponectin in serum and the defective expression of adiponectin receptors in the lungs [150]. Despite some studies having shown no effect of recombinant adiponectin in animals, recombinant adiponectin may be a challenging therapeutic strategy for obesity-related asthma in the future.

5. Conclusion

As obesity becomes more prevalent worldwide, obesity-related asthma is frequently observed in the whole population. In this review, we described the disturbed lipid metabolism and immune modulators of lipid metabolism in obesity such as SREBPs, DPP-4, and Nrf2. In addition, we also discussed several immune factors potentially contributing to the pathogenesis of obesity-related asthma including intestinal microbiota, immune regulator, and inflammation. According to these possible immune causes in the onset and development of obesity-related asthma, we summarized several promising targeted therapies in the treatment of obesity-related asthma, such as miRNAs and TLR antagonists, which may provide effective medical intervention strategy in controlling obesity-related asthma.

Conflicts of Interest

There is no conflict of interests regarding the publication of this review.

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Research Article

Conventional DCs from Male and Female Lupus-Prone B6.NZM Sle1/Sle2/Sle3 Mice Express an IFN Signature and Have a Higher Immunometabolism That Are Enhanced by Estrogen

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Type I interferons (IFN) are pathogenic in systemic lupus erythematosus (SLE) and were proposed to control the immunometabolism of dendritic cells (DCs). We previously reported that DCs from female lupus-prone mice constitutively overexpress IFN-responsive genes resembling the IFN signature found in SLE patients. As SLE has higher incidence in women than men, more so in women of reproductive age, estrogens are suggested to affect lupus pathogenesis. We investigated the effects of sex and estrogens on the IFN signature in conventional GM-CSF-bone marrow-derived DCs (cDCs), from male and female Triple Congenic B6.NZM.*Sle1*/*Sle2*/*Sle3* (TCSle) lupus-prone mice or from wild-type C57BL/6 mice, generated with titrations of 17-beta-estradiol (E2). We found that cDCs from prediseased TCSle male mice express the IFN signature as female TCSle cDCs do. Estrogens are necessary but not sufficient to express this IFN signature, but high doses of E2 can compensate for other steroidal components. E2 stimulation, regardless of sex, modulates type I IFN-dependent and type I IFN-independent activation of cDCs in response to TLR stimulation. Finally, we found that TCSle cDCs from both sexes have elevated markers of immunometabolism and estrogens enhance the metabolic pathways in cDCs, suggesting a mechanistic link between estrogens, immunometabolism, and the IFN signature in lupus.

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of complex pathogenesis characterized by autoantibodies against nucleic acids, chromatin, and ribonucleoproteins [1–3] as well as elevated type I IFN [1, 4]. Microarray analysis of PBMCs from SLE patients discovered the increased expression of IFN-responsive genes that coined the IFN signature [5–7]. Genome-wide association studies and genetic studies of families with SLE patients support a genetic dysregulation of IFN-α [8–12]. We have previously found that immune cells from NZM2410-derived Triple Congenic B6.NZM.*Sle1*/*Sle2*/*Sle3* (TCSle) [13] lupus-prone mice express a similar IFN signature *in vivo*, suggesting that these mice are a good tool to study the role of type I IFNs in lupus [14].

DNA and RNA are major autoAgs in lupus that can stimulate immune cells through TLR7 and TLR9 [15]. As major producers of type I IFNs in response to TLR7 and TLR9 signaling, plasmacytoid dendritic cells (pDCs) have been a major focus of SLE research [16–18], although recently, cDCs have been also appreciated. cDCs from SLE patients showed a dysregulated expression of immunomodulatory factors such as BLyS, CD86 [19–22], and PD-L1 [23] and induced greater allogeneic T cell proliferation than their healthy counterparts [20]. In TCSle mice, the IFN signature and *Ifn*β expression *in vivo* are high in cDCs but not in pDCs [14], and

TCSle-cDCs induce greater proliferation and IgM secretion from B cells *in vitro* [24]. Furthermore, murine studies with B6.*Sle1* mice demonstrated that TLR7 overexpression on cDCs, but not on pDCs, is responsible for driving splenomegaly and end organ damage [25]. Finally, modular transcriptional repertoire analysis of cells from SLE patients revealed an equal importance of IFN-β and IFN-α in the IFN signature [26], further underlying the importance of cDCs in lupus.

These cDC abnormalities could be due to an abnormal environment, intrinsic defects, or both in SLE. Among the possible environmental factors suspected to affect DC activation, sex hormones are important candidates. Indeed, they have long been thought to play a role in the pathogenesis of SLE, as the female to male prevalence ratio ranges from 4.3 : 1 to 13.6 : 1 [27]. As the onset of SLE is more frequent in women of childbearing age [27], estrogens could be responsible for this bias. In addition, Fulvestrant, a selective estrogen receptor modulator, decreased SLE disease activity index in a small clinical trial [28]. Murine models confirm a role for estrogens in lupus: overactivation of estrogen receptor-α (ERα) exacerbates lupus disease [29]. Further studies have determined that upon TLR7 or TLR9 stimulation, secretion of IFN-α from pDCs [30] and IL-6 from cDCs [31] is facilitated by estrogen/ERα. Different murine models of lupus with ERα deficiency have prolonged survival and reduced disease in females but not in males [32]. Early ovariectomy of female NZM2410 mice also reduced disease severity and splenic cDC numbers [33]. These results agree with the observations that 17-β-estradiol (E2) promotes dendritic cell differentiation through IFN regulatory factor (IRF) 4 in GM-CSF-derived DCs [34]. Interestingly, an opposite effect of estrogen is seen on Flt3 ligand-derived cDCs and pDCs: E2 inhibited the survival of DCs in Flt3 ligand in a dose-dependent manner [35]. As GM-CSF is involved in inflammatory pathways while Flt3 ligand is involved in homeostasis [36, 37], E2 is hypothesized to promote DC survival in inflammatory conditions. These studies were mostly performed in female mice; it remains unclear however how estrogens affect cDC activation in male lupus-prone mice and whether male cDCs express the IFN signature. Moreover, the mechanisms mediating the effects of estrogens on cDCs are not completely understood.

Recent studies have suggested a role for an increased immunometabolism in SLE [38]. Indeed, the pathways involved in the production of cellular energy were more activated in T cells from SLE patients and lupus-prone mice, and the metabolic inhibitors metformin and 2-deoxyglucose reduced disease severity in murine lupus [39–41]. To our knowledge, no study has determined the metabolic activity of DCs in SLE. Studies of wild-type murine cDCs indicate that, upon TLR stimulation, they undergo a metabolic shift towards aerobic glycolysis with a decline in oxidative phosphorylation regulated by nitric oxide [42, 43]. This change in the way in which cDCs produce their energy is necessary for cDCs to fully activate. In addition, type I IFNs were involved in the metabolic reprogramming that occurs during activation of wild-type cDCs [44] and pDCs [45] as important autocrine mediators. As E2 differentially regulates cDC survival in homeostasis and inflammation and the metabolic shift occurs during inflammation [35–37], it is important to investigate the role of estrogens in cDC metabolic changes in lupus.

We therefore investigated the effects of hormones, specifically E2, on the IFN-dependent and IFN-independent activation of cDCs from female and male lupus-prone mice. Different hormone receptor polymorphisms have been reported to modify SLE disease severity [46] and may affect different murine strains as well. Therefore, we investigated the effects of E2 and sex differences on cDC activation using TCSle mice [13] compared to its wild-type strain C67BL/6 (B6) to minimize possible differences in estrogen receptor signaling.

Our results demonstrate that cDCs from male TCSle mice express the IFN signature similar to cDCs from female lupus mice [14]. In addition, male and female cDCs are equally dependent on the presence of estrogen to activate and express the IFN signature. We found that estrogen is necessary but not sufficient for cDC expression of this IFN signature, but high doses of supplemented E2 can compensate for other steroidal components normally present in the culture media. Estrogen enhances cDC activation through the IFN-dependent and IFN-independent pathways in both wild-type and lupus cDCs. Finally, we found that lupus cDCs have higher metabolism than wild-type cDCs in both sexes and estrogen enhances the metabolic pathways in cDCs, suggesting a mechanistic link between estrogen, immunometabolism, and the IFN signature in lupus.

2. Material and Methods

2.1. Mice. C57BL/6 (B6) and NZM2410-derived Triple Congenic B6.*Sle1.Sle2.Sle3* (TCSle) [13] mice (purchased from The Jackson Laboratory, Bar Harbor, Maine) were bred and maintained in our animal facility. Studies were performed in accordance with the guidelines of the Institutional Animal Care and Use Committees of Temple University, a member of the American Association for the Accreditation of Laboratory Animal Care-accredited facilities. Age-matched female and male B6 and TCSle mice were used between 8 and 12 weeks of age, an age at which TCSle mice do not yet develop antinuclear autoantibodies in our animal facility.

2.2. In Vitro Bone Marrow-Derived Conventional Dendritic Cell (cDC) Cultures. Bone marrow precursors were flushed from the tibias and femurs of mice with 25-gauge needle and syringe, into a single-cell suspension, and then seeded at 10^6 cells/mL/well in 24-well plates (Corning Costar, Tewksbury, Massachusetts) in complete IMDM. IMDM containing phenol red (Corning) was supplemented with 10% FBS (Gemini Bio-Products, West Sacramento, California), 100 units/mL penicillin, 100 μg/mL streptomycin, 50 μg/mL gentamicin, 55 μM 2-ME (Life Technologies, Grand Island, NY), and 2 millimolar L-glutamine (Corning) for standard condition complete IMDM. Phenol red-free IMDM (Life Technologies) was supplemented with 10% charcoal-treated FBS (HyClone, Logan Utah or Omega Scientific, Tarzana, CA), penicillin/streptomycin, gentamicin, 2-ME

(Life Technologies), and L-glutamine (Corning) for hormone-depleted complete IMDM. All media were supplemented with supernatant of a hybridoma cell line secreting murine GM-CSF. 17-β-estradiol (E2 from Sigma-Aldrich) was reconstituted in ethanol and added to hormone-depleted complete IMDM at concentrations of 0.03 nanomolar (nM), 0.1 nM, or 50 nM E2 with a final concentration of 0.1% ethanol. E2-devoid conditions were also supplemented with 0.1% ethanol. Alternatively, Fulvestrant (1 μM or 100 nM in DMSO) and Tamoxifen (10 nM or 100 nM in ethanol) (Sigma-Aldrich) were added to standard condition media. One mL of media per well was added on day 2, and 1 mL of media was replaced on day 5 and each subsequent day until stimulation of cells. Resting cDCs were stimulated on day 7 of culture with 10 μg/mL CpG-B 1826 (IDT Biotechnologies, Coralville, IA) or 1 μg/mL R848 (Risiquimod, InvivoGen, San Diego, CA). Supernatants and cDCs were harvested at 6 or 24 hours post stimulation. One cDC culture from female and male B6 and TCSle mice was compared in the same experiment.

2.3. Quantitative RT-PCR. Gene expression in cDCs was analyzed by quantitative real-time RT-PCR in technical triplicates using TaqMan probes, as previously described [14, 47]. Briefly, total RNA was extracted using Qiagen RNeasy Plus kit (Qiagen, Valencia, CA) or Zymo quick kit (Zymo Research), following the manufacturer's protocols. cDNA was synthesized using the cDNA archive reverse transcription kit (Life Technologies). TaqMan primers and probes for *Cxcl10*, *Irf7*, *Isg15*, *Mx1*, and *Pdk1* were purchased from Applied Biosystems. *Cyclophilin* was used as the reference gene for normalization. The cycle threshold (Ct) method of relative quantification of gene expression was used for these TaqMan PCRs ($\Delta\Delta$Ct), and the normalized Ct values (against cyclophilin) were calibrated against the control sample (untreated WT female cDCs) in each experiment.

2.4. Flow Cytometry. cDCs were harvested 24 h post stimulation, washed in cold PBS, incubated with rat anti-mouse CD16/CD32 (clone 2.4G2, BioLegend) mAb for 10 min to block FcγRs, and then stained for 30 min on ice with allophycocyanin-conjugated hamster anti-mouse CD11c (N418, eBioscience), PE-Cyanin7-conjugated rat anti-mouse CD11b (M1/70, BD Bioscience), either PE-conjugated rat anti-mouse CD86 (GL1, BD Bioscience) or PE-conjugated rat anti-mouse CD115 (AFS98, BioLegend), FITC-conjugated hamster anti-mouse CD80 (16-10A1, eBioscience) or FITC-conjugated hamster anti-mouse CD40 (HM40–3, BD Biosciences), and FITC-conjugated rat anti-mouse MHCII (M5/114.15.2 eBioscience) or Biotin-conjugated rat anti-mouse MHCII (M5/114.15.2 eBioscience) followed by streptavidin PerCPCy5.5 (eBioscience) or Biotin-conjugated goat anti-mouse MERTK (polyclonal R&D Systems) followed by streptavidin FITC. In selected experiments, cDCs were stained with fixable viability dye (FVD) ef780 (Invitrogen) for 15 minutes in cold PBS and then washed before being incubated with the above antibodies. Cells were fixed with 1% formaldehyde before acquisition on a FACSCanto cytometer (BD Biosciences). FlowJo (FlowJo LLC, Ashland, Oregon) software was used for data analysis.

2.5. ELISA. ELISA analysis was performed following the manufacturer's protocols in technical duplicates. ELISA kits were used to measure the protein levels of murine TNF-α, IL-12p70, IL-6 (BD Biosciences), and CXCL10 (R&D Systems, Minneapolis, MN) in the supernatants of cDC cultures in medium alone or stimulated with TLR ligands. Absorbance was measured at 450 nm with a VERSA max microplate reader (Molecular Devices) with SoftMaxPro 6.5 software. Absorbance at 570 nanometers was measured and used as wavelength correction.

2.6. Griess Reaction. Nitrites, a proxy for nitric oxide, were measured in cDC culture supernatants by Griess reaction. Technical duplicates of supernatants and standards (sodium nitrite, Sigma-Aldrich) were titrated in a 96-well plate (Corning Costar). Griess reagent (Acros Organics, Geel, Belgium) was then added to each well at a 1 : 1 ratio and absorbance was measured at 550 nanometers with a VERSA max microplate reader (Molecular Devices) with SoftMax-Pro 6.5 software.

2.7. Statistical Analysis. Mean and SE were calculated by averaging the results of three to six independent experiments performed with independent bone marrow cultures obtained from individual mouse for each experiment. Prism software (GraphPad, San Diego, CA) was used for statistical analysis with the two-way unpaired *t*-test for comparison between two groups and the two-way ANOVA with Tukey multiple comparison for multiple groups. *P* values $p < 0.05$ were considered significant. *, Δ, and O represent $p < 0.05$. **, $\Delta\Delta$, and OO represent $p < 0.01$. ***, $\Delta\Delta\Delta$, and OOO represent $p < 0.001$.

3. Results

3.1. Conventional DCs from Young Lupus-Prone Male Mice Express the IFN Signature. To test whether male lupus-prone cDCs express the IFN signature, we generated conventional dendritic cells (cDCs) from bone marrow precursors of female and male C57BL/6 (B6) and TCSle mice [13], in the presence of the DC growth factor GM-CSF, as previously described [14]. At day 7 under these standard conditions of culture (standard FBS & IMDM containing phenol red [14, 47]), we detected a higher baseline expression of interferon-stimulated gene (ISG) RNAs, namely, *Cxcl10*, *Isg15*, and *Irf7*, in female TCSle cDC compared to female B6 cDCs, confirming our previous findings [14] (Figure 1(a)). Although *Mx1* did not reach significance, there was a trend towards higher level of expression in female TCSle compared to female B6 cDCs. No significant difference was found between females and males of the same strain. Interestingly, male TCSle cDCs expressed significantly higher levels of *Cxcl10*, *Isg15*, *Irf7*, and *Mx1* transcripts compared to male B6 cDCs and similar levels to female TCSle cDCs. This indicates that male cDCs express a similar IFN signature to female cDCs from lupus-prone mice (Figure 1(a)).

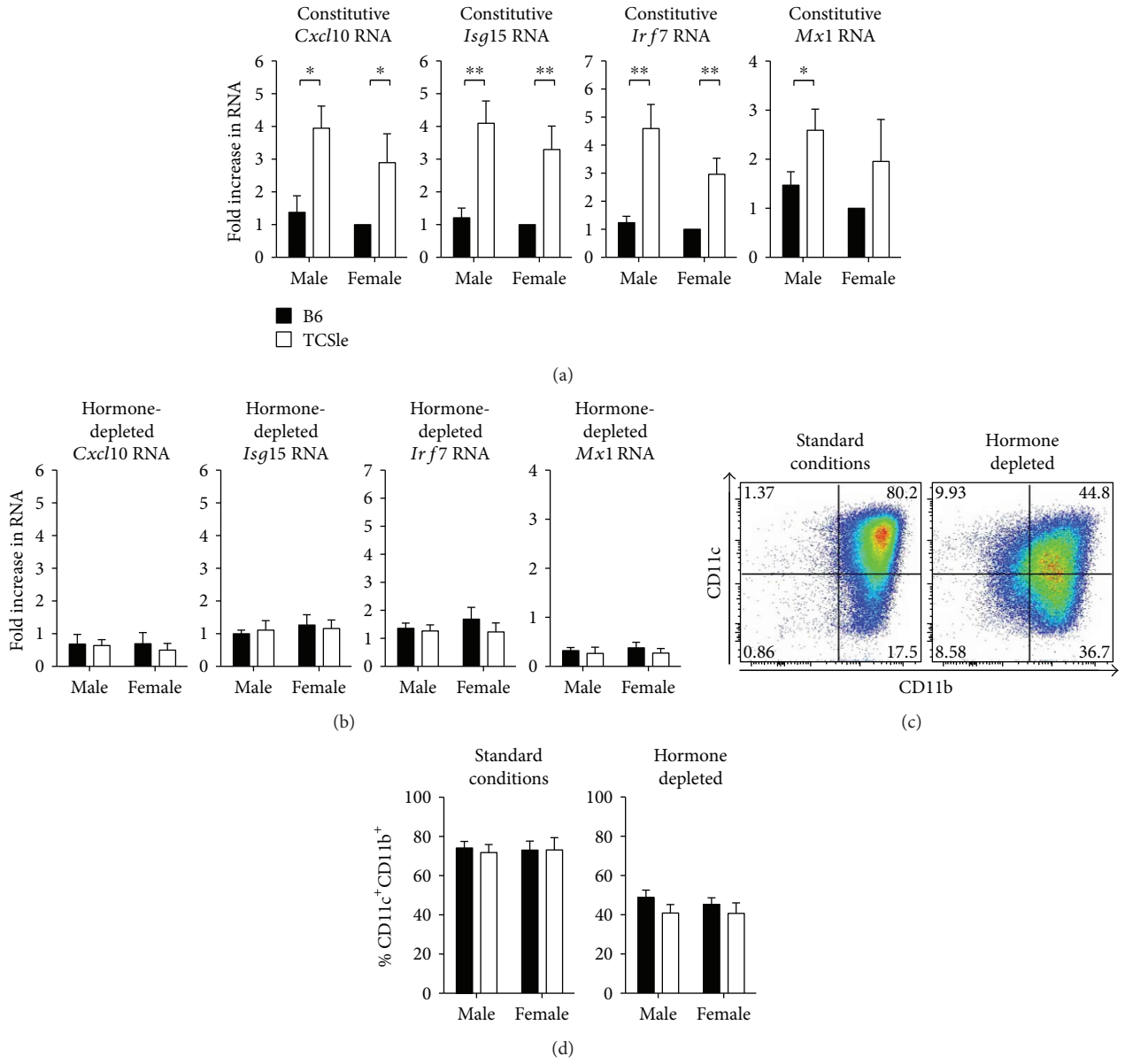

Figure 1: Conventional DCs from young lupus-prone male mice express the IFN signature. The IFN signature of TCSle cDCs is dependent on steroidal hormones. Bone marrow precursors from C57BL/6 (B6: black bars) or B6.NZM.Sle1.Sle2.Sle3 (TCSle: white bars) female and male mice were cultured with GM-CSF in standard phenol red/media conditions (a, c, d) or media lacking phenol red and void of steroids (charcoal-treated FBS: hormone depleted) (b, c, d). On day 7, cDCs were harvested and total RNA was isolated for qRT-PCR analysis (a, b). Genes were normalized to the housekeeping gene *cyclophilin*. Standard female B6 condition was set to 1. On day 7-8, cDCs were harvested and stained with antibodies against CD11c and CD11b and analyzed by flow cytometry (c, d). One representative plot of a B6 female in standard conditions (c, left) or hormone-depleted conditions (c, right). Unpaired *t*-test comparing male B6 to male TCSle or female B6 to female TCSle was used. Mean + SE values are from 6 independent experiments, using one mouse per strain per experiment. $^{*}p < 0.05$ and $^{**}p < 0.01$.

3.2. The IFN Signature of TCSle cDCs Is Dependent on Steroidal Hormones. To determine the importance of sex hormones on the IFN signature, we cultured cDCs in hormone-depleted conditions. A well-accepted protocol to study the role of estrogen on cell functions uses a medium enriched with FBS that had been previously treated with charcoal to deplete all the steroidal hormones, including estrogens [35, 48]. We analyzed the differentiation of cDCs from bone marrow precursors grown in GM-CSF-enriched complete IMDM containing charcoal-treated FBS, and we used phenol red-free IMDM because phenol red has been suggested to signal through the estrogen receptor [49]. At day 7 of culture, we measured the IFN signature and found that it was abrogated in both female and male TCSle cDCs grown in hormone-depleted conditions (Figure 1(b)), in contrast to the results obtained in standard culture conditions of the same experiments (Figure 1(a)). To ensure that the lack of IFN signature was not due to B6 and TCSle cDCs developing

at different rates in hormone-depleted conditions, we analyzed the expression of CD11c and CD11b as DC markers by flow cytometry (Figure 1(c); representative plot of B6 female). We found that the hormone-depleted conditions allowed the development of cDCs (~50% cDC after 7 days in culture) while standard culture conditions enhanced the frequency of cDCs (~75% cDC after 7 days). While there was a sizable reduction in the differentiation of DCs in hormone-depleted conditions compared to standard conditions, there was no significant difference between sexes and strains in cDC development within standard culture or hormone-depleted conditions (Figure 1(d)), indicating that the IFN signature in standard conditions was not due to a different ratio of B6 and TCSle cDC in culture. In summary, we used a protocol that allows the differentiation of bone marrow precursors into cDCs from both female and male B6 and TCSle mice in the absence of estrogens and other steroidal hormones. In these conditions, the lupus-prone cDCs lost the ability to express the IFN signature, while the baseline expression of ISGs by B6 cDCs of both sexes was not altered.

3.3. Estrogen Enhances the Development of cDCs of both Sexes of B6 and TCSle Mice and the IFN Signature in TCSle cDCs. Estrogen signaling through IRF4 has been shown to enhance B6 DC differentiation *in vitro* [50]. We therefore determined whether estrogen enhances cDC differentiation and the constitutive expression of the IFN signature in cDCs from female and male TCSle mice. We generated cDCs in hormone-depleted medium supplemented with 17-β-estradiol (E2) at doses comparable to those present in the serum of male mice (0.03 nanomolar (nM)), in the serum of female mice in diestrus, or in a regular fetal bovine serum (0.1 nM) [51, 52]. We also tested a superphysiological dose that is comparable to what is present in the serum of pregnant women (50 nM) [53]. The lack of clear data in mice does not allow us to state that the latter concentration is equivalent to pregnant mice [54]. We found that E2 supplementation increased cDC differentiation compared to the lack of supplement, even at the lowest dose tested, which is equivalent to what is present in the serum of male mice (Figure 2(a)). We found an increase in the percentages of $CD11c^+CD11b^+$ cDCs, indicating a clear ability of E2 to enhance cDC differentiation, as previously reported [50]. Furthermore, in both sexes and strains, the dose present in the serum of female mice in diestrus (0.1 nM E2) was sufficient to yield the percentage of cDCs that we obtain under standard conditions (Figure 2(a)). To determine whether changes in differentiation were associated with differences in cell viability, we stained cDCs with a fixable viability dye and analyzed them by flow cytometry (Figure 2(b)). Although standard conditions resulted in significantly higher cell viability than the other conditions, E2 was not found to alter the percent of live cells compared to 0 E2. We observed no significant difference in cDC differentiation between female and male mice of both wild-type and lupus-prone strains, when the cDCs were generated in the same conditions and with the same concentrations of E2. This suggests that cDC differentiation is not affected by sex or the genetic make-up of the lupus-prone mice, but rather by the hormonal milieu in which cDCs grow.

As hormone-depleted conditions prevent the expression of the IFN signature in TCSle cDCs (Figure 1(b)), we determined whether E2 supplementation could rescue this phenotype. Surprisingly, neither 0.03 nM E2 nor 0.1 nM E2 supported an IFN signature in both female and male TCSle cDCs (Figure 2(c)). While the addition of 0.1 nM E2 and 50 nM E2 yielded a normal cDC differentiation (Figure 2(a)), only the 50 nM dose was capable of inducing higher levels of ISG expression in TCSle compared to B6 cDCs in both sexes (Figure 2(c)). Therefore, while high levels of E2 strongly induce the IFN signature in TCSle cDCs that we detected in standard conditions, levels of E2 similar to those present in male and in female mice outside of pregnancy cannot compensate for the absence of other steroids or lipids found in standard culture conditions.

In addition to removing steroids, charcoal treatment also removes other lipids from FBS and may affect the ability of cells to synthetize molecules and adjust their metabolism. As metabolites have been shown to alter DC growth and differentiation [55], we additionally tested cDC differentiation in standard conditions in the presence of selective estrogen receptor modulators/degraders (SERM/SERD) Tamoxifen and Fulvestrant. These compounds ablate E2 signaling while all other metabolites, which are removed by charcoal treatment, remained in the culture. cDC differentiation from female B6 and TCSle mice was significantly reduced by both Fulvestrant and Tamoxifen (Figure 3(a)). This reduction brought the percent of cDCs down to levels seen in hormone-depleted conditions (comparing Figure 3(a) to Figures 1(c) and 2(a)). In addition, Fulvestrant and Tamoxifen reduced the expression of the IFN signature, and we show the gene *Mx1* as representative (Figure 3(b)), supporting the role of estrogens in these functions.

In prior reports, hormone-depleted conditions yielded poorer B6 DC differentiation [56] than what we have seen in our experiments. Since those studies utilized the medium RPMI while our lab uses IMDM for GM-CSF cDC cultures, we generated cDCs in hormone-depleted conditions with phenol red-free RPMI or IMDM to determine if these two media, which contain different levels of amino acids and glucose, would yield a different percentage of cDCs. As we expected, IMDM promoted greater differentiation of cDCs than RPMI (Figure 3(c)). As with IMDM (Figure 2(a)), 0.1 nM E2 supplementation in hormone-depleted RPMI conditions was able to increase cDC differentiation (Figure 3(c)), as previously reported [56], although not at the same levels with IMDM supplemented with the same E2 concentration.

In summary, E2 supplementation equally enhances cDC differentiation in female and male B6 and TCSle cDCs, with the IMDM medium further promoting cDC differentiation. However, despite the ability of 0.03 nM E2 and 0.1 nM E2 to enhance cDC differentiation, only 50 nM E2 can rescue the IFN signature to the levels observed in standard conditions. As inhibition of the estrogen receptor by SERM/SERD blocks the IFN signature in TCSle cDCs generated in standard conditions, we conclude that estrogen is necessary but not sufficient for the expression of the IFN signature in lupus

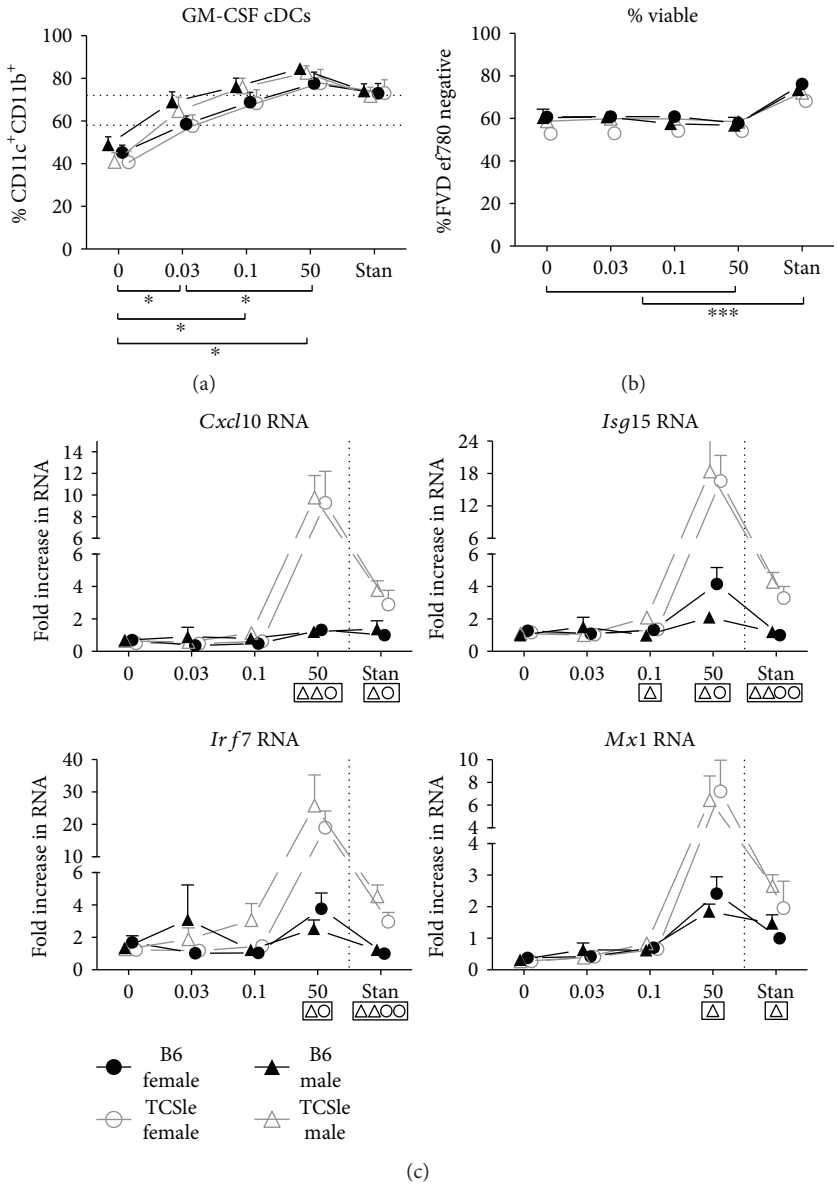

Figure 2: Estrogen enhances the development of cDCs of B6 and TCSle mice of both sexes and the constitutive IFN signature in TCSle cDCs. Bone marrow precursors from B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice were cultured with GM-CSF in standard phenol red/media conditions or media depleted of phenol red and devoid of steroids (charcoal-treated FBS: 0 E2) and supplemented with 0.03 nM, 0.1 nM, or 50 nM 17-β-estradiol (E2). On day 7-8, cDCs were harvested and stained with (a) antibodies against CD11c, CD11b, and (b) fixable viability dye and analyzed by flow cytometry or (c) total RNA was analyzed by qRT-PCR analysis. ISGs were normalized to the housekeeping gene *cyclophilin*. Standard condition female B6 was set to 1 in each experiment. Mean + SE values are from 6 independent experiments, using one mouse of each strain and sex per experiment. Two-way ANOVA analysis with Tukey multiple comparisons was used to calculate the significance of the effects of E2 treatment within each group of mice represented by brackets below the graph. A black star indicates statistical significance in all the fours curves representing both sexes and strains of cDCs. Two-way ANOVA analysis with Tukey multiple comparisons was used to compare differences between B6 and TCSle, and results are shown in a box surrounding the symbol Δ for significance between B6 and TCSle males or the symbol O for significance between B6 and TCSle females. *, Δ, and O represent $p < 0.05$. ΔΔ, and OO represent $p < 0.01$. *** represent $p < 0.001$.

and only higher doses of E2 can compensate for other steroidal components present in FBS.

3.4. The IFN Signature of TCSle cDCs Is Not Due to a Differential Heterogeneity of the GM-CSF cDCs. It has been previously shown that GM-CSF cDC cultures are composed of a heterogeneous population: $CD11c^{+}$ $MHCII^{Int}$, $CD11b^{High}$ monocyte-derived inflammatory DCs, and $CD11c^{+}$ $MHCII^{High}$ $CD11b^{Int}$ DCs. Since E2 titrations (Figure 2(a)) or SERM/SERDs (Figure 3(a)) could, respectively, increase or decrease the percent of $CD11c^{+}$ $CD11b^{+}$ cells, we determined if E2 affects the ratio between the

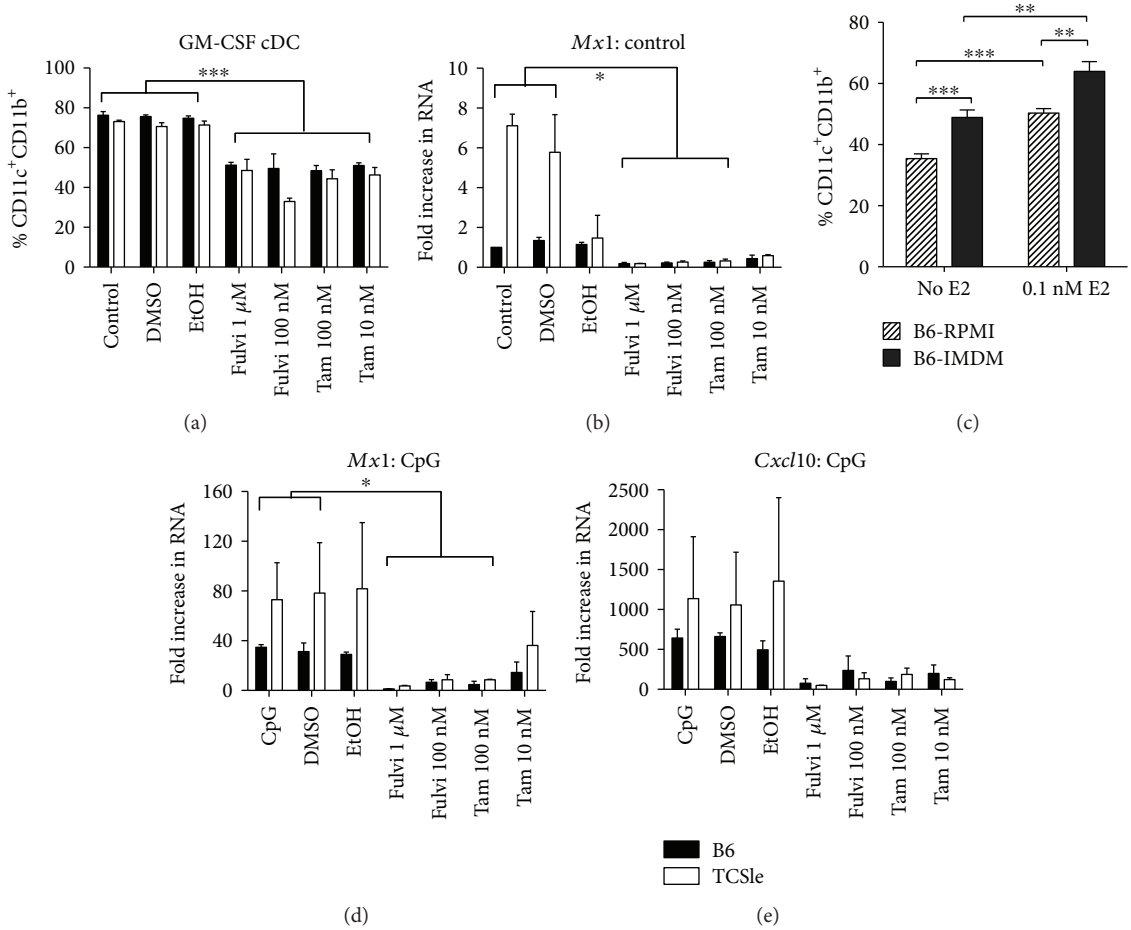

Figure 3: E2 inhibition reduces the development and IFN signature of TCSle cDCs. Bone marrow-derived cDCs from (a–e) B6 and (a, b, d, e) TCSle female mice were cultured with GM-CSF in standard phenol red IMDM (a–e) and RPMI (c) supplemented with Fulvestrant (Fulvi 1 μM or 100 nM in DMSO) and Tamoxifen (Tam 10 nM or 100 nM in ethanol: EtOH). (a, b, d, e) or with 0 or 0.1 nM E2 (c). On day 7-8, cDCs were harvested and stained with antibodies against CD11c and CD11b and analyzed by flow cytometry. On day 7, cDCs were stimulated with CpG B 1826 (d, e). Six hours post stimulation, cDCs were harvested and total RNA was isolated for qRT-PCR analysis (b, d, e). *Mx1* and *Cxcl10* RNAs were normalized to the RNA of the housekeeping gene *cyclophilin*. Standard female B6 control condition was set to 1. Unpaired *t*-test comparing baseline cDC to SERM-treated groups was used. Mean ± SE values are from 2 independent experiments, using one mouse per strain per experiment (a–e). Brackets indicate significance between controls (including DMSO and EtOH) and SERM-treated samples. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

reported CD11b^{int} and CD11b^{high} population of DCs. Representative gating of a B6 female is shown in Figure 4(a). Cells were first gated on CD11c^{+} MHCII^{+} (Figure 4(a), left) and then further divided between MHCII^{High} and MHCII^{Int} against CD11b (Figure 4(a), center). The MHCII^{High} population was high for CD86 (Figure 4(a), right (gray)) compared to the MHCII^{Int} (green) population. A similar trend was seen with CD40 (data not shown). Collectively, this suggested that the CD11c^{+} MHCII^{High} CD11b^{Int} cDCs are a spontaneously activated population as previously reported [57]. Interestingly, the MHCII^{High} population was not CD11b^{Int} as reported but higher in mean fluorescence intensity compared to the MHCII^{Int} population (Figure 4(a), center). We initially hypothesized that the TCSle cDCs may have an IFN signature because they were skewed towards a spontaneously activated MHCII^{High} DC population with E2 supplementation further enhancing this effect. However, in the conditions in which we saw an IFN signature (Figure 2(c): 50 nM E2 and standard conditions), there were less spontaneously activated MHCII^{High} TCSle DCs (Figure 4(b)) compared to B6 DCs and no difference between MHCII^{Int} TCSle DCs and B6 DCs (Figure 4(c)). Furthermore, when looking at an activation marker of these MHCII^{High} DCs, there were no differences between B6 and TCSle CD86 expression (Figure 4(d)). This spontaneous activation was modestly decreased with Fulvestrant. In contrast, the MHCII^{Int} DC population showed an E2-dependent, TCSle-specific, significant increase in CD86 (Figure 4(e)).

As these MHCII^{Int} CD11b^{+} CD11c^{+} have been referred to as more macrophage-like, we measured two surface markers: CD115, also known as M-CSF receptor, and MERTK. MHCII^{Int} DCs had an E2 dose-dependent increase in CD115 expression (Supplemental Figure 1A) which was neither strain nor sex specific (Supplemental Figure 1B).

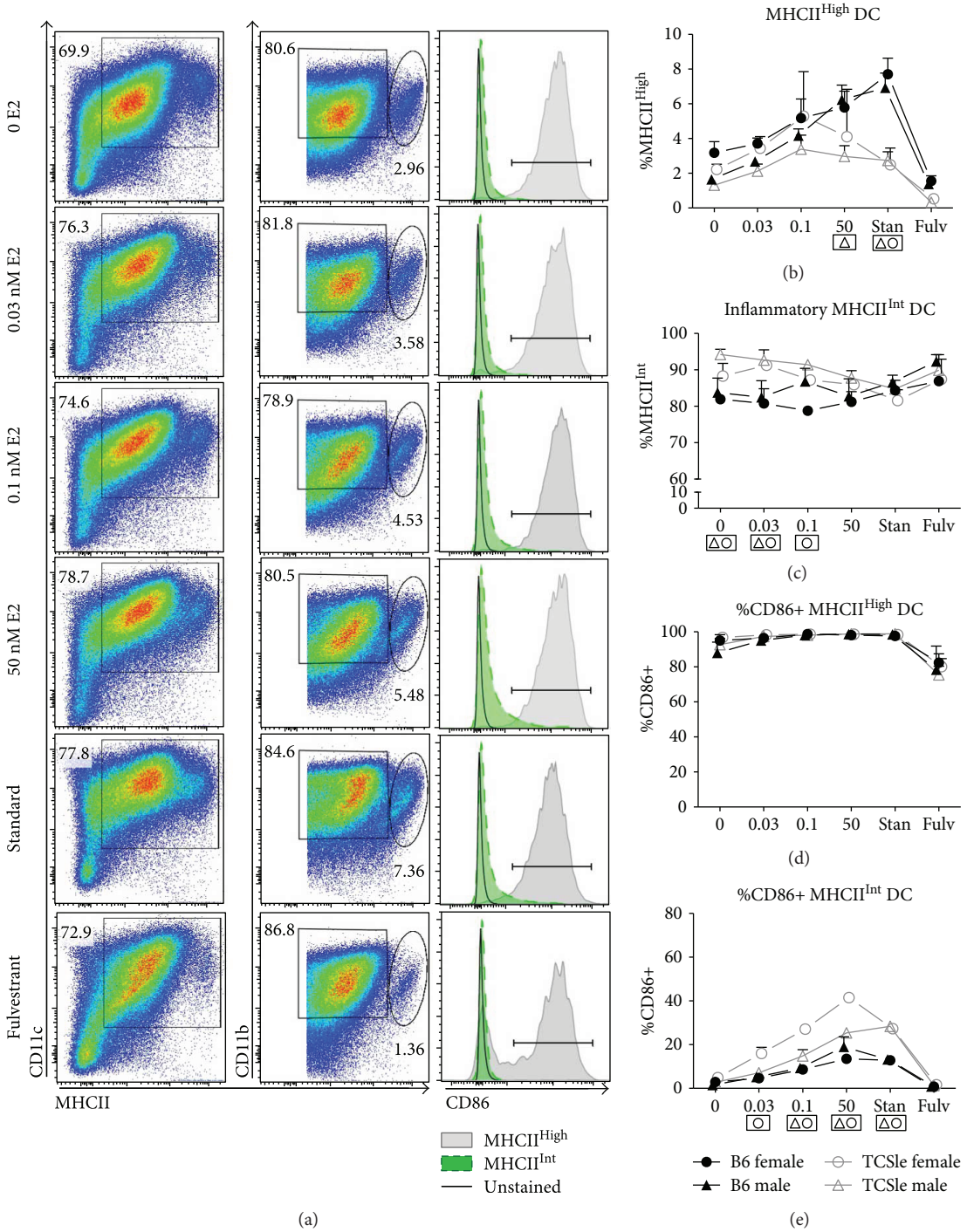

Figure 4: The IFN signature of TCSle cDCs is not due to a differential heterogeneity of the GM-CSF cDCs. Bone marrow precursors from B6 or TCSle female and male mice were cultured with GM-CSF in standard phenol red/media conditions or media depleted of phenol red and devoid of steroids (charcoal-treated FBS: 0 E2) and supplemented with 0.03 nM, 0.1 nM, or 50 nM 17-β-estradiol (E2). Standard conditions were also supplemented with 1 μM Fulvestrant. On day 8, cDCs were harvested and stained with fixable viability dye and antibodies against CD11c, CD11b, MHCII, and CD86. Samples were gated on singlets, live cells, $CD11c^+$ $MHCII^+$ (a, left), CD11b + versus $MHCII^{High}$ or $MHCII^{Int}$ (a, center), and CD86 (a, right). Gray histograms represent the $MHCII^{High}$ population, while green histograms represent the $MHCII^{Int}$ population and the black line represents an unstained population. (a) One representative plot of a B6 female culture is shown. Graphs of $\%MHCII^{High}$ (b) or $\%MHCII^{Int}$ (c) from the $CD11c^+$ gate from B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice. Graphs of %CD86 positive on $MHCII^{High}$ (d) or $MHCII^{Int}$ (e) populations. Mean + SE values are from 3 biological replicates, using one mouse of each strain and sex per experiment. Two-way ANOVA analysis with Tukey multiple comparisons was used to compare differences between B6 and TCSle, and results are shown in a box surrounding the symbol Δ for significance between B6 and TCSle males or the symbol O for significance between B6 and TCSle females. Δ and O represent $p < 0.05$. $\Delta\Delta$ and OO represent $p < 0.01$.

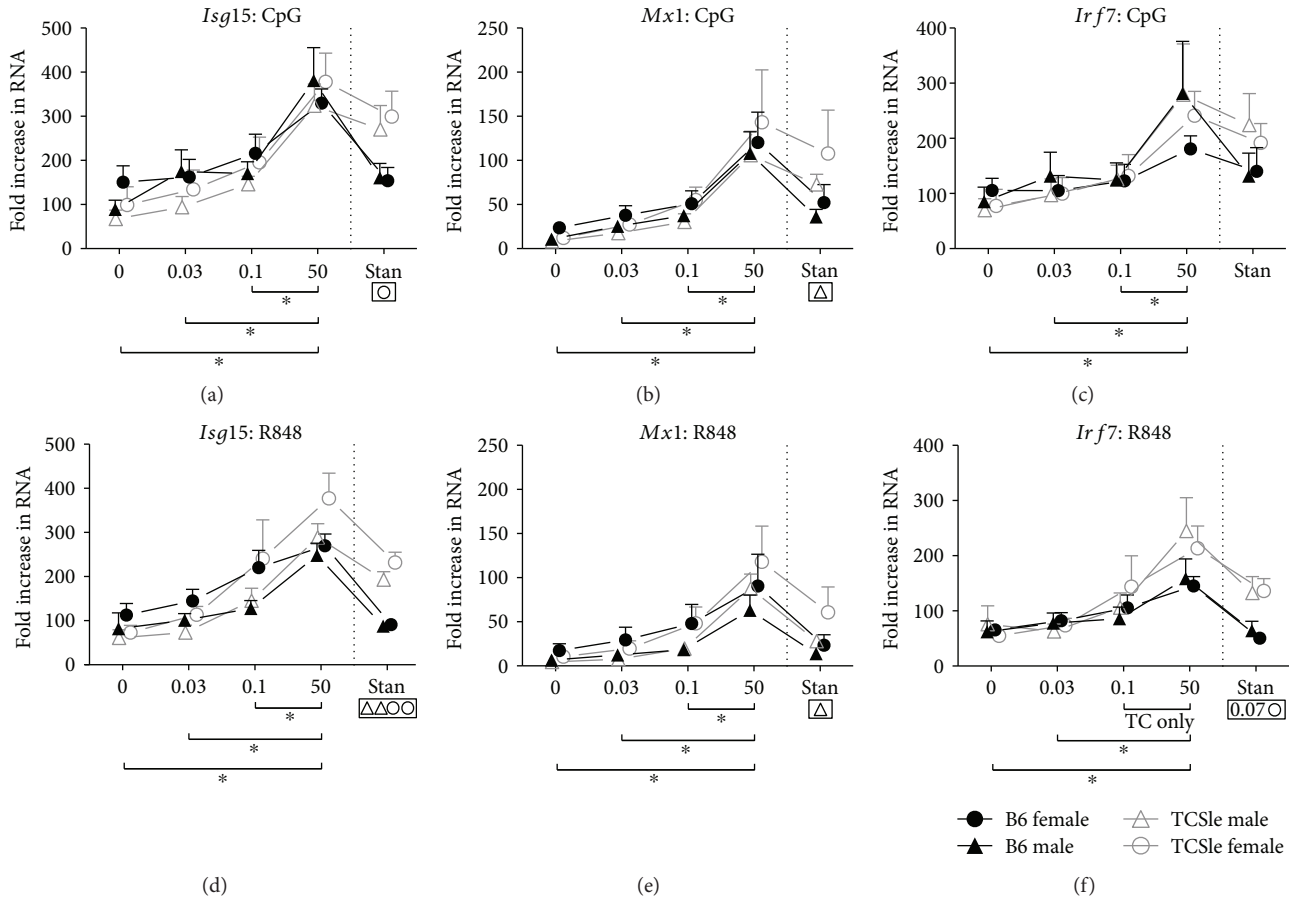

Figure 5: Estrogen enhances the upregulation of ISGs in response to TLR stimulation. B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice were cultured with GM-CSF in standard phenol red/media conditions or media depleted of phenol red and void of steroids (charcoal-treated FBS: 0 E2) supplemented with 0.03 nM, 0.1 nM, or 50 nM E2. On day 7, cDCs were stimulated with CpG B 1826 (10 μg/mL) (a–c) or R848 (1 μg/mL) (d–f). Six hours post stimulation, cDCs were harvested for qRT-PCR analysis. ISGs were normalized to the housekeeping gene *cyclophilin*. Standard female B6 control condition without stimulation (not shown) was set to 1 in each experiment. Mean + SE values are from 6 independent experiments, using one mouse of each strain and sex per experiment. Two-way ANOVA analysis with Tukey multiple comparisons was used to calculate the significance of the effects of E2 treatment within each group of mice and the results are represented by brackets below the graph. A black star indicates statistical significance in all the fours curves representing both sexes and strains of cDCs. Two-way ANOVA analysis with Tukey multiple comparisons was used to compare differences between B6 and TCSle, and results are shown in a box surrounding the symbol Δ for significance between B6 and TCSle males or the symbol O for significance between B6 and TCSle females. *, Δ, and O represent $p < 0.05$. ΔΔ, and OO represent $p < 0.01$.

In contrast, we did not observe any MERTK staining in these experiments (data not shown), confirming our previous experience with GM-CSF cDC cultures done in the lab (unpublished data), although bone marrow-derived macrophage cultures generated in M-CSF-enriched medium regularly stained MERTK positive (unpublished data). In addition, the number of MHCII^{High} DCs in our culture ranged from 2–7% whereas Helft et al. reported around 30% [57]. We hypothesize that these differences in CD11b MFI, MER expression, and ratio of subsets may be attributed to culture media: while RPMI is a common media for GM-CSF DC cultures, we utilize IMDM, a richer medium, which yields a higher ratio of cDCs (Figure 3(c)) and may shift the culture towards a stronger dendritic cell phenotype.

In conclusion, while our GM-CSF DC cultures result in a heterogeneous population of inflammatory cDCs, the increase in IFN-responsive genes in the TCSle cDCs compared to B6 cDCs is not due to a change in cDC differentiation nor to an increase in the spontaneously activated MHCII^{High} cDCs, but rather to an augmented ability to express ISGs.

3.5. Estrogen Enhances the Upregulation of ISGs in Response to TLR Stimulation. Nucleic acids are potent stimulators of the innate immunity and contribute to the IFN signature by stimulating DCs through TLR7/8 and TLR9 [14]. We therefore tested whether E2 enhanced the response to R848 and CpG, respective agonists of TLR7/8 and TLR9. We found that cDCs grown in hormone-depleted 0 nM E2 were able to respond to both CpG and R848 by upregulating the expression of the ISGs *Isg15*, *Mx1*, and *Irf7* (Figures 5(a)–5(f)). The highest concentration of 50 nM

E2 further increased the expression of the ISGs *Isg15*, *Mx1*, and *Irf7* as compared to lower doses of E2 upon CpG stimulation (Figures 5(a)–5(c)) in both sexes and strains of mice. The highest E2 dose was similarly potent in enhancing *Isg15* and *Mx1* expression upon R848 stimulation (Figures 5(d) and 5(e)). Interestingly, while 50 nM E2 induced higher *Irf7* expression upon R848 stimulation, there was no statistical difference between 0.1 nM E2 and 50 nM E2 female and male B6 cDCs (Figure 5(f)). In contrast, TCSle cDCs were capable of further increasing *Irf7* expression with 50 nM E2. This may indicate an upper limit of *Irf7* expression in B6 cDCs that TCSle cDCs can surpass with higher doses of E2. Although we did not see consistent statistical significance because of variation between experiments, we observed a trend of higher expression of ISGs in TCSle cDC after CpG and R848 stimulation compared to B6 cDCs in standard conditions (Figures 5(a)–5(f): significance is shown in boxes below the graph). This suggests that both female and male TCSle cDCs increase their IFN signature upon TLR stimulation, as we have reported before in female TCSle cDCs [14]. In hormone-depleted E2-supplemented cDCs, the differences between TCSle and B6 cDCs of both sexes disappeared, suggesting that estrogen is necessary but not sufficient for the amplification of the IFN signature expression by TLR ligands in lupus. These conclusions are also supported by the findings that Fulvestrant and Tamoxifen, which inhibit E2 signaling, reduced the TLR-dependent upregulation of the ISGs *Mx1* and *Cxcl10* in cDCs generated in standard conditions (Figures 3(d) and 3(e)).

3.6. Estrogen Enhances the Upregulation of cDC Activation Markers in Response to TLR Stimulation. As costimulatory molecules are dysregulated in SLE cDCs [19–21, 58] and can lead to abnormal T and B cell proliferation [20, 24], we next analyzed the effects of E2 on the expression of the surface activation markers CD40, CD86, and CD80 on cDCs, measured by flow cytometry (Figure 6). Constitutive expression of CD40 and CD86 was significantly elevated in TCSle female cDCs compared to B6 female cDCs in standard conditions (Figures 6(a) and 6(d): significance is shown in boxes below the graph), confirming the results from Figure 4(e). We did not find this difference in our previous work [58], although this may be due to the culture conditions in the past, when we used to grow cDCs in medium enriched with IL-4. Upon CpG and R848 stimulation, the percentage of $CD40^+$ (Figures 6(b) and 6(c)), $CD86^+$ (Figures 6(e) and 6(f)), and $CD80^+$ (Figures 6(h) and 6(i)) cDCs significantly increased in standard culture conditions for all mice compared to unstimulated conditions (representative plot for CpG effects on TCSle is shown in Figure 6(j)). CpG and R848 stimulation induced higher CD40 expression in TCSle female but not in TCSle male cDCs as compared to B6 cDCs (Figures 6(b) and 6(c)). Upon CpG stimulation, CD86 and CD80 expression was significantly upregulated in male and female TCSle cDCs as compared to B6 cDCs in standard conditions, confirming that TCSle cDCs have a higher response to TLR7–9 compared to B6 cDCs as we have shown in gene expression (Figure 5).

CpG and R848 stimulation was able to increase CD40 and CD86 expression of cDCs cultured with 0.03 nM, 0.1 nM, and 50 nM E2. A horizontal dashed line provides visual aid to compare baseline to stimulated levels of CD40 (Figures 6(a)–6(c)) and CD86 (Figures 6(d)–6(f)). In contrast, only 50 nM E2 promoted CD80 activation in response to CpG and R848 (Figures 6(g)–6(i)). Increasing E2 doses further enhanced CD40 and CD86 expression (Figures 6(b), 6(c), 6(e), and 6(f): significance is represented by brackets below the graph).

Collectively, E2 supports the upregulation of costimulatory molecules CD40 and CD86 in response to TLR7 and 9 stimulation. The absence of any difference between females and males when cultured with the same concentration of E2 indicates that the E2-specific hormonal environment, not the sex of the cDCs, dictates activation levels. However, female TCSle cDCs show higher expression of costimulatory molecules than female B6 cDCs in standard conditions while male DCs did not show consistent strain differences. Therefore, the upregulation of costimulatory molecules in cDCs is controlled by E2 in a sex-independent manner and by additional factors in a sex-dependent manner.

3.7. Estrogen Enhances CXCL10 Chemokine Production Not Predicted by Cxcl10 RNA. We have previously reported that cDCs from TCSle female mice, grown in standard conditions, secrete significantly more CXCL10 chemokine than B6 cDCs upon CpG and R848 stimulation, as part of the IFN signature [14]. Therefore, we hypothesized that male TCSle cDCs can also secrete elevated levels of CXCL10 protein upon the same stimulations. Indeed, we found that upon CpG (Figure 7(a)) and R848 (Figure 7(b)) stimulation, CXCL10 production was significantly elevated in female and male TCSle cDCs as compared to their B6 cDC counterparts under standard conditions (significance is shown in boxes below each graph), further supporting the idea that male cDCs have an IFN signature that is amplified by TLR7–9 stimulation as female cDCs do.

Furthermore, CXCL10 production upon CpG stimulation was elevated in both female and male TCSle cDCs as compared to B6 DCs at 0.1 nM E2 and 50 nM E2, although it did not reach significance (Figure 7(a)). Moreover, CXCL10 production by TCSle cDCs was significantly higher than that by B6 cDCs in 50 nM E2 conditions upon R848 stimulation with a similar trend at 0.1 nM E2 (Figure 7(b)), suggesting that estrogen can enhance the amplification of the IFN signature induced by TLR7 and TLR9 stimulation.

While only higher concentrations of E2 promoted a difference between TCSle and B6 in CXCL10 production, even 0.03 nM E2 was sufficient to enhance CpG-induced production of CXCL10 compared to hormone-depleted conditions in both sexes and strains (Figure 7(a): significance is shown in brackets below each graph). Interestingly, putting aside the enhancing effects of E2, there was still significant production of CXCL10 in hormone-depleted conditions upon CpG stimulation (Figure 7(a)). In contrast, upon R848 stimulation, hormone-depleted conditions and 0.03 nM E2 were

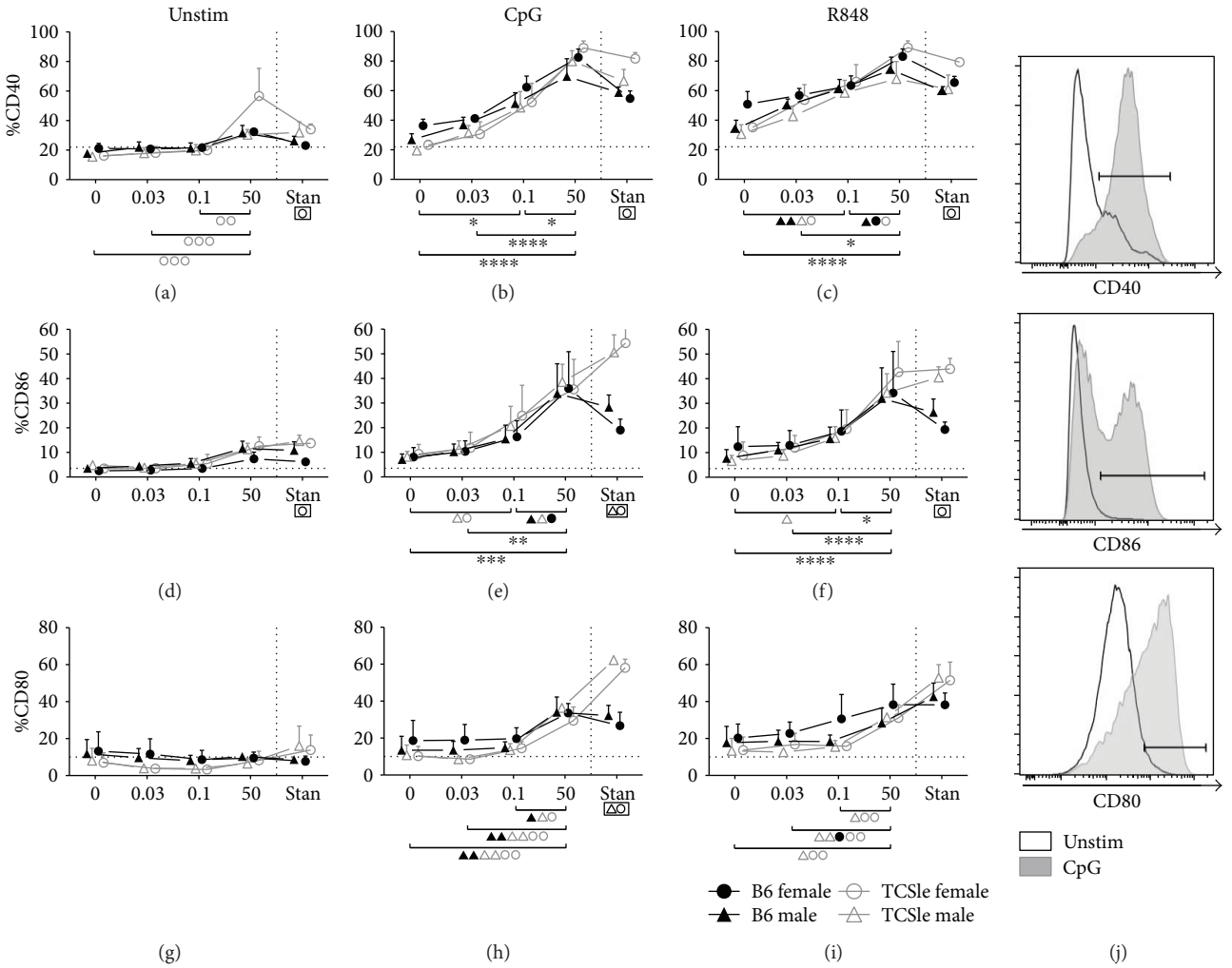

Figure 6: Estrogen enhances the upregulation of cDC activation markers in response to TLR stimulation. Bone marrow precursors from B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice were cultured with GM-CSF in standard phenol red/media conditions or media depleted in phenol red and void of steroids (charcoal-treated FBS: 0 E2) supplemented with 0.03 nM, 0.1 nM, or 50 nM E2. On day 7, cDCs were stimulated with CpG (10 μg/mL) (b, e, h) or R848 (1 μg/mL) (c, f, i). cDCs were harvested 24 hours post stimulation, stained, and gated on singlets, live cells, and $CD11c^+$ $CD11b^+$ (gating shown in Figure 1(c)) before analyzing costimulatory molecules (a–i). Representative histogram plots of CD40, CD86, and CD80 on unstimulated (black line) or CpG-stimulated (gray histogram) female TCSle cDCs from standard conditions (j). Mean + SE values are from 3 (female cDCs) or 4 (male cDCs) independent experiments, using one mouse of each strain and sex per experiment. Two-way ANOVA analysis with Tukey multiple comparisons was used to calculate the significance of the effects of E2 treatment within each group of mice, represented by brackets below the graph. Black * indicates significance within all 4 groups while symbols represent significance within a single group. Two-way ANOVA analysis with Tukey multiple comparisons was used to compare differences between B6 and TCSle, and results are shown in a box surrounding the symbol Δ for significance between B6 and TCSle males or the symbol O for significance between B6 and TCSle females. *, Δ, and O represent $p < 0.05$. **, ΔΔ, and OO represent $p < 0.01$. *** represents $p < 0.001$ and ****$p < 0.0001$.

not capable of inducing as robust of a CXCL10 response (Figure 7(b)), indicating that E2 may affect CXCL10 production in response to CpG and R848 differently. To confirm this hypothesis, we cultured female TCSle cDCs in standard culture conditions with Tamoxifen and Fulvestrant to inhibit estrogen receptor signaling. In concordance with the response to CpG seen in hormone-depleted conditions and E2 supplemented, both high and low doses of Tamoxifen can reduce CXCL10 production but are unable to ablate the CXCL10 response to CpG stimulation (Figure 7(c)). Similarly, a low dose of Fulvestrant reduced CXCL10 production to similar levels seen in hormone-depleted conditions, while the higher dose of Fulvestrant prevented CpG-induced CXCL10 production (Figure 7(c)). We found that both low and high doses of Tamoxifen and Fulvestrant ablated the CXCL10 response to R848 (Figure 7(d)), suggesting that TLR7-induced CXCL10 is more estrogen dependent than TLR9-induced CXCL10.

We have shown in Figure 5 that only the highest dose of 50 nM of E2 could enhance the RNA expression of ISGs upon CpG and R848 and it was not sufficient for the expression of a significant difference between TCSle and B6 cDCs. In

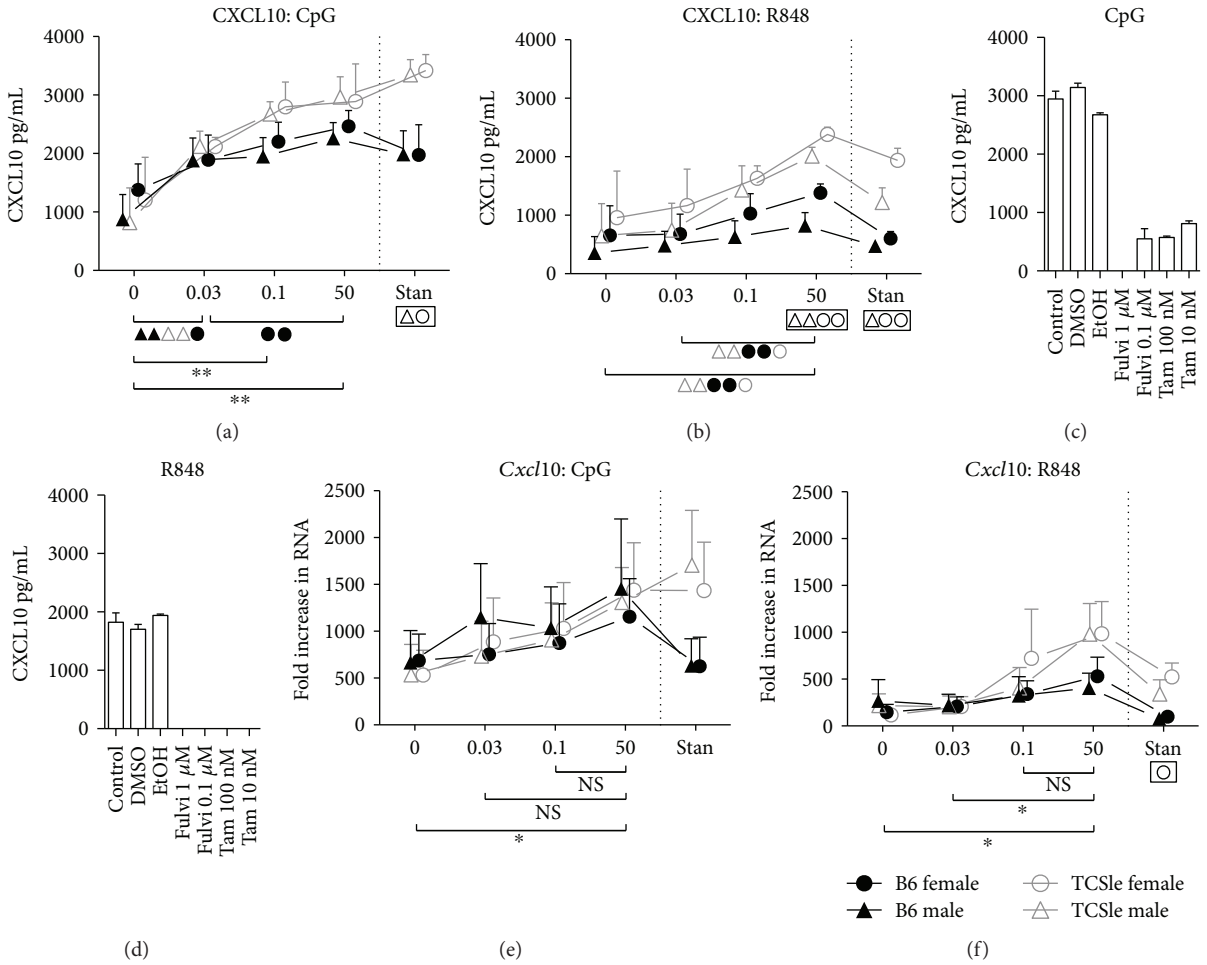

Figure 7: Estrogen enhances CXCL10 chemokine production not predicted by *Cxcl10* RNA. Bone marrow precursors from B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice were cultured in standard conditions or hormone-depleted conditions supplemented with 0.03 nM, 0.1 nM, or 50 nM E2 (a, b, e, f). TCSle cDCs were cultured in standard conditions supplemented with Fulvestrant (Fulvi 1 μM or 100 nM in DMSO) and Tamoxifen (Tam 10 nM or 100 nM in ethanol) (c, d). On day 7, cDCs were stimulated with CpG (10 μg/mL) (a, c, e) or R848 (1 μg/mL) (b, d, f). Supernatants were harvested 24 hours post stimulation and analyzed by ELISA for CXCL10 protein levels (a–d). Total RNA was isolated 6 hours post stimulation and analyzed by qRT-PCR (e, f). Mean + SE values are from 3 (female cDCs) or 4 (male cDCs) independent experiments or (c and d) 2 independent experiments using one mouse per strain per experiment (a, b, e, f). Two-way ANOVA analysis with Tukey multiple comparisons was used to calculate the significance of the effects of E2 treatment within each group of mice, represented by brackets below the graph. Black * indicates significance within all 4 groups while symbols represent significance within a single group. Two-way ANOVA analysis with Tukey multiple comparisons was used to compare differences between B6 and TCSle, and results are shown in a box surrounding the symbol Δ for significance between B6 and TCSle males or the symbol O for significance between B6 and TCSle females. *, Δ, and O represent $p < 0.05$. **, ΔΔ, and OO represent $p < 0.01$.

Figures 7(a) and 7(b) instead, we show that all 0.03 nM, 0.1 nM, and 50 nM could enhance the TLR9-induced production of ISG CXCL10 as protein and 50 nM allowed the TCSle cDCs to secrete significantly higher amounts of CXCL10 upon TLR-7 stimulation than B6 cDCs. To solve the contrast between the results presented in Figure 5 and Figure 7 [59], we determined if the regulation of CXCL10 production by E2 was detectable at the RNA level. Surprisingly, the ability of E2 to enhance CXCL10 production upon CpG stimulation was not mirrored in *Cxcl10* transcript levels (Figure 7(e)), as only 50 nM E2 supported elevated *Cxcl10* levels of expression as compared to hormone-depleted conditions. However, upon R848 stimulation, *Cxcl10* RNA levels more closely mirrored the production of CXCL10 protein (Figure 7(f)).

In summary, estrogen enhances the production of the chemokine CXCL10 in a dose-dependent manner, as we have observed with the upregulation of costimulatory molecules, in both strains and sexes of cDCs (Figure 6). E2 also strengthens the amplification of the IFN signature induced by TLR7 and TLR9 stimulation in lupus cDCs. Since we found different susceptibilities to estrogen in the production of CXCL10 protein levels versus *Cxcl10* transcripts upon CpG versus R848 stimulation and since the results of the RNA expression of CXCL10 were in agreement with the results of the gene expression of the other ISGs shown in

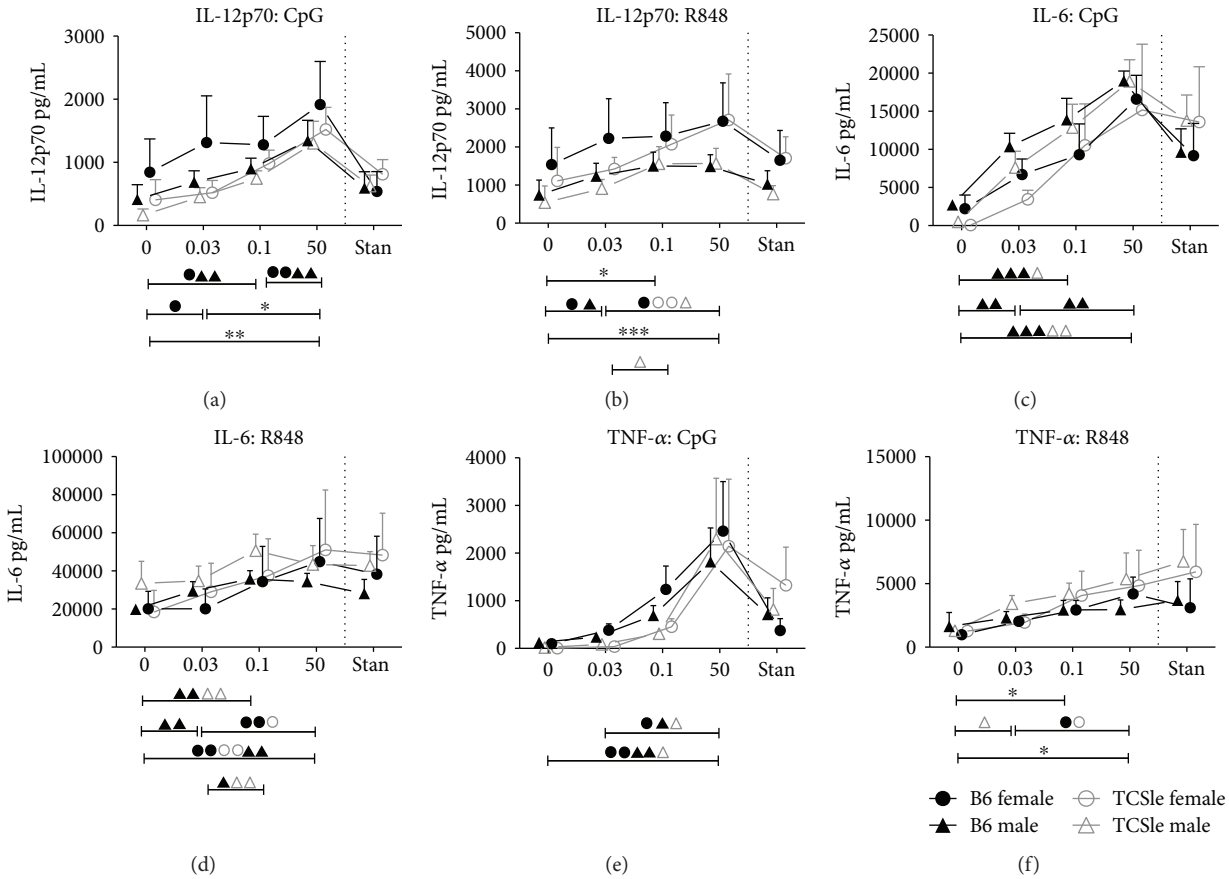

Figure 8: E2 enhances both IFN-dependent and IFN-independent cytokine production. Bone marrow precursors from B6 (black closed symbols) or TCSle (gray open symbols) female (circle) and male (triangle) mice were cultured with GM-CSF in standard phenol red/media conditions or media depleted in phenol red and void of steroids (charcoal-treated FBS: 0 E2) supplemented with 0.03 nM, 0.1 nM, or 50 nM E2. On day 7, cDCs were stimulated with CpG (10 μg/mL) (a, c, e) or R848 (1 μg/mL) (b, d, f). Supernatants were harvested and analyzed by ELISA 24 hours (IL-12p70 (a, b)) or 6 hours (IL-6 (c, d) and TNF-α (e, f)) post stimulation. Mean + SE values are from 3 (female cDCs) or 4 (male cDCs) independent experiments, using one mouse of each strain and sex per experiment. Two-way ANOVA analysis with Tukey multiple comparisons, used to compare differences between B6 and TCSle, did not reveal significance between the strains. Two-way ANOVA analysis with Tukey multiple comparisons was used to calculate the significance of the effects of E2 treatment within each group of mice, represented by brackets below the graph. Black * indicates significance within all 4 groups while symbols represent significance within a single group. *, Δ, and O represent $p < 0.05$. **, ΔΔ, and OO represent $p < 0.01$. *** represents $p < 0.001$.

Figure 5, we propose that the regulation of CXCL10, and possibly other ISGs, by E2 occurs at both the transcriptional and posttranscriptional levels.

3.8. E2 Enhances both IFN-Dependent and IFN-Independent Cytokine Production. We have shown so far that E2 regulates the expression of ISGs (Figures 2–7), costimulatory molecules (Figure 6), and the chemokine CXCL10 (Figure 7), equally in female and male cDCs, and contributes to the differences between TCSle and B6 cDCs. We have previously reported that ISGs, some costimulatory molecules, and CXCL10 are regulated in a type I IFN/STAT-2-dependent manner [47]. To assess whether E2 can also affect IFN-independent functions in TCSle cDCs, we measured the levels of IL-12p70, IL-6, and TNF-α, cytokines that we have previously shown to be IFN independent [47]. We analyzed the same supernatants in which we had measured CXCL10 (Figure 7). As with CXCL10, the secretion of IL-12p70 upon CpG (Figure 8(a)) or R848 (Figure 8(b)) stimulation was enhanced by 50 nM E2 (significance is shown in brackets below each graph) in both female and male B6 and TCSle cDC. In addition, 0.1 nM E2 significantly enhanced the IL-12p70 response to R848 but not CpG in all mice (Figure 8(b)). Although it did not reach significance for all mice, there was a trend towards 0.03 nM E2 supporting a stronger IL-12p70 response upon CpG and R848 stimulation compared to hormone-depleted conditions. In similar fashion to IL-12p70 and CXCL10, increasing E2 titration increased the secretion of IL-6 (Figures 8(c) and 8(d)) and TNF-α (Figures 8(e) and 8(f)) upon CpG and R848 stimulation, although it did not reach significance for all mice. In contrast to CXCL10, the secretion of IL-12p70, IL-6, and TNF-α, IFN-independent cytokines, was produced in equal amounts by TCSle and B6 cDCs (Figure 8), confirming our previous report [14], and did not show sex differences. Moreover, the enhancement of these cytokines by estrogen was similar in both strains and sexes.

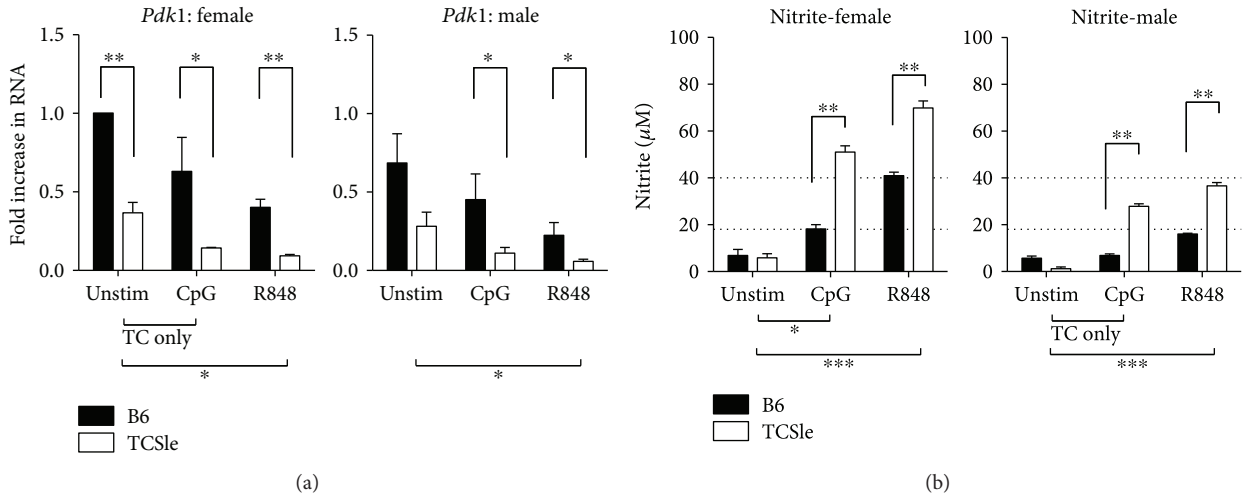

Figure 9: Female and male TCSle cDCs have a higher immunometabolism. Bone marrow-derived cDCs from B6 and TCSle female and male mice were cultured in standard conditions. On day 7, cDCs were stimulated with CpG (10 μg/mL) or R848 (1 μg/mL). cDCs were harvested 6 hours post stimulation for qRT-PCR analysis. *Pdk1* was normalized to the housekeeping gene *cyclophilin* (a). Standard female unstimulated B6 condition was set to 1 in each experiment. Supernatants were harvested at the 24-hour time point and analyzed using the Griess reaction to measure nitrites (b). Dotted lines at 18 and 40 represent female B6 levels after CpG and R848 stimulation. Mean + SE values are from 3 independent experiments, using one mouse of each strain and sex per experiment (a, b). Two-way ANOVA analysis with Tukey multiple comparisons was used to determine significant activation by CpG or R848 within each group of mice represented by brackets and * below the graph. Two-way ANOVA analysis with Tukey multiple comparisons, used to compare differences between B6 and TCSle, is represented by brackets and * above the bars. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$.

In summary, E2 enhances the production of IFN-independent cytokines in both sexes in wild-type and lupus-prone strains. These results indicate that the enhancement by estrogen of the response of cDCs to TLR7 and TLR9 ligands involves both the IFN-dependent and IFN-independent pathways, suggesting that estrogen affects more than one mechanism, possibly upstream of the production of type I IFNs.

3.9. E2 TCSle cDCs Show a Higher Energy Metabolism. It was recently shown that T cells from TCSle female mice have an elevated metabolic state, with measures of increased glycolysis and oxidative phosphorylation, as compared to B6 T cells [41]. No data is available on the metabolic state of TCSle cDCs. To determine the effects of E2 on cDC metabolism, we chose two biomarkers of metabolic activation of the cDCs, Pdk1, and nitric oxide (NO).

Pdk1 (pyruvate dehydrogenase kinase 1) is an inhibitory regulator of the Krebs cycle and controls the amplitude of both the oxidative phosphorylation and fatty acid synthesis. It is downregulated upon activation to increase energy metabolism [60]. We analyzed the expression of *Pdk1* first in standard conditions and found that TCSle cDCs have a decreased expression of *Pdk1* in both sexes, with the baseline levels as low as the levels in B6 cDCs upon activation. TCSle *Pdk1* levels were further decreased upon activation by CpG and R848, suggesting that TCSle cDCs have a higher baseline energy metabolism that can further increase upon activation (Figure 9(a)). This is the first evidence that lupus-prone cDCs have a higher metabolism than wild-type cDCs, and such difference was equally present in female and male cDCs.

Nitric oxide (NO) is synthesized from arginine by inducible nitric oxide synthase (iNOS) [61] and can act as a microbicidal agent [62]. Six to 24 hours after TLR stimulation, cDCs have been shown to produce NO, which participates in sustaining the metabolic shift toward aerobic glycolysis in activated iNOS-expressing cDCs [43]. We measured nitrite as a proxy for NO as reported [63]. Six hours after CpG or R848 stimulation, cDCs did not produce significant levels of NO (data not shown). After 24 hours of stimulation with CpG or R848 in standard conditions, we found high levels of nitrite in the supernatants of all the cDCs, confirming previous reports that TLR stimulation induces NO production in cDCs [43]. TCSle cDCs secreted significantly higher levels of nitrite than B6 cDCs, both from females and males (Figure 9(b)), suggesting a stronger metabolism and commitment to glycolysis in TCSle cDCs. Furthermore, after CpG or R848 stimulation, female cDCs of both strains produced higher levels of nitrite than male cDCs (Figure 9(b): dotted lines at 18 and 40 represent female B6 levels after CpG and R848 stimulation), suggesting a novel sex bias in cDC metabolism.

3.10. E2 Enhances the Higher Energy Metabolism of TCSle cDCs. Since we have found that E2 can modulate the development and the activation of female and male B6 and TCSle cDCs, we hypothesized that E2 could also modulate the metabolic state of cDCs. The significant differences in nitrite levels linked to activation, sex, and strain (Figures 9(b) and 10(a)) were mostly lost in hormone-depleted conditions, in which cDCs of both strains and sexes secreted very modest amounts of NO upon R848 stimulation and none upon CpGs (Figures 10(a) and 10(b)). With the

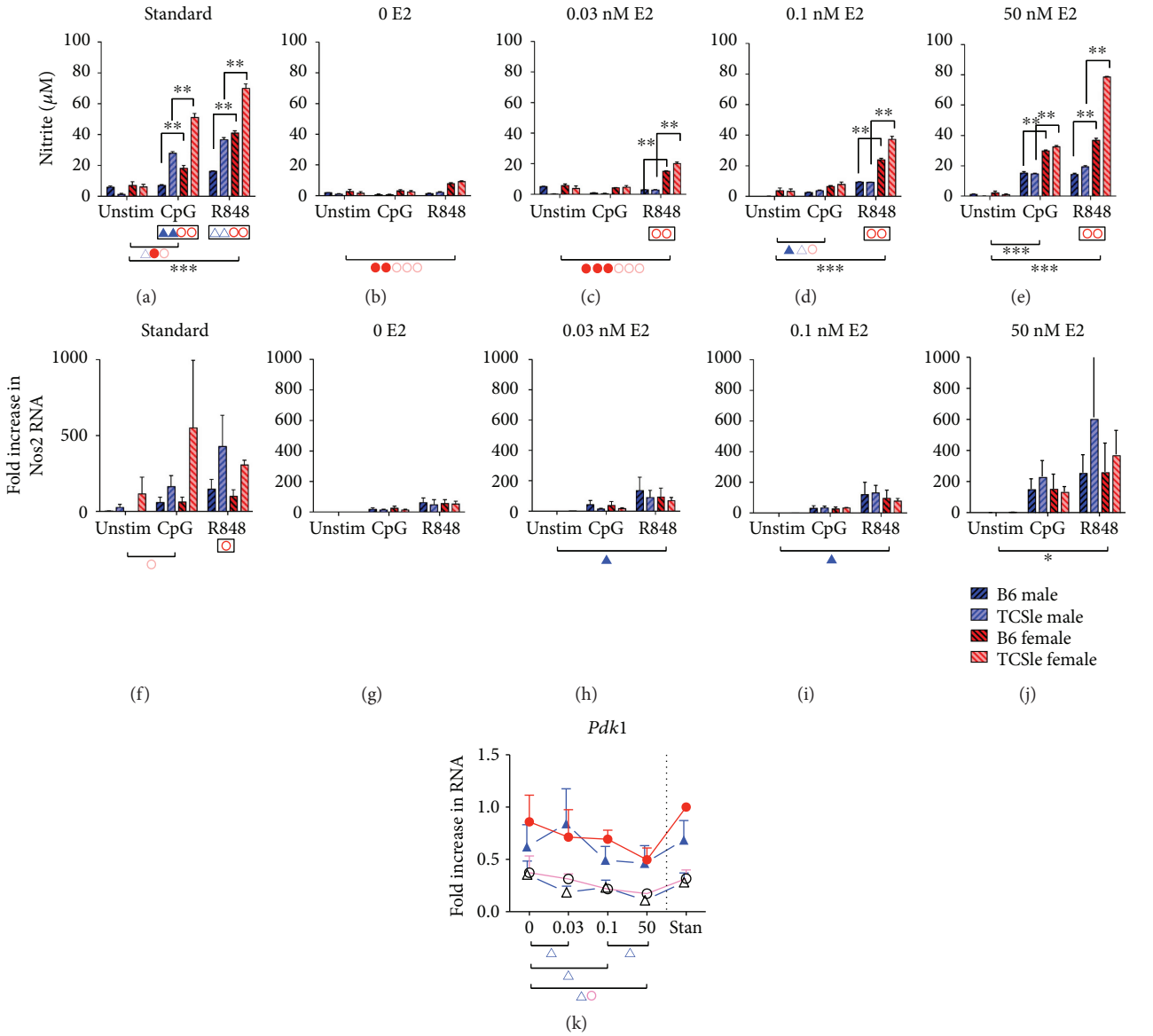

Figure 10: E2 enhances the higher energy metabolism of TCSle cDCs. cDCs from B6 (darker color) or TCSle (lighter color) female (red/orange) and male (blue/azur) mice were cultured in standard conditions or in hormone-depleted conditions supplemented with 0.03 nM, 0.1 nM, or 50 nM E2 (a–k). On day 7, cDC were stimulated with CpG B 1826 (10 μg/mL) or R848 (1 μg/mL). Supernatants were harvested and analyzed using the Griess reaction 24 hours after stimulation (a–e). Six hours post stimulation, cDCs were harvested for qRT-PCR analysis (f–j). *Nos2* (f–j) and *Pdk1* (k) genes were normalized to the housekeeping gene *cyclophilin*. Standard female B6 condition was set to 1. Mean + SE values are from 3 independent experiments, using one mouse per strain per experiment. Two-way ANOVA analysis with Tukey multiple comparisons was used to determine the significant activation by CpG or R848 within each group of mice, represented by brackets below the graph. Black * indicates significance within all 4 groups while individual colors/symbols represent significance within a single group (a–k). Two-way ANOVA analysis with Tukey multiple comparisons, used to compare differences between females and males, is represented by brackets and * above the graph (a–e). Tukey multiple comparisons, used to compare differences between B6 and TCSle, is represented by a box surrounding red Δ symbol for B6 and TCSle females or a blue O symbol for B6 and TCSle males (a–j). Two-way ANOVA analysis with Tukey multiple comparisons is used to determine the effects of E2 treatment on cDC differentiation within each group of mice represented by brackets and symbols below the graph (j). *, Δ, and O represent $p < 0.05$. **, ΔΔ, and OO represent $p < 0.01$. *** represents $p < 0.001$

addition of 0.03 nM E2, the strain bias seen upon R848 stimulation in standard conditions returned, as female TCSle cDCs produced significantly more nitrite than female B6 cDCs (Figure 10(c): significance is shown in the box below the axis). The 0.1 nM E2 dose was sufficient for nitrite production upon both CpG and R848 stimulation, although sex and strain differences were significant only upon R848 stimulation (Figures 10(c) and 10(d)). Finally, 50 nM E2 can recreate the majority of trends seen in standard conditions: a significant increase in the production of nitrite upon CpG and R848 both in female and in male B6 and TCSle cDCs (Figure 10(e)). We observed a significant higher

production of nitrite by female than male cDCs in both strains and a significant higher production of nitrite by TCSle cDCs than B6 cDCs. However, the difference between B6 and TCSle cDCs seen in standard conditions (Figures 9(b) and 10(a)) was not rescued upon CpG stimulation (Figures 10(b)–10(e)).

The increase in NO production requires the upregulation of inducible NOS2 expression [61]. Since we have previously found that E2 regulates CXCL10 production at both the transcriptional and translational levels (Figure 7), we determined whether *Nos2* transcript levels followed the trend observed with nitrite levels. cDCs generated in standard culture conditions showed higher expression of *Nos2* by female TCSle cDCs and to a lesser extent by male TCSle cDCs (Figure 10(f)), compared to the levels of *Nos2* expressed by both female and male B6 cDCs. Stimulation with CpG and R848 increased *Nos2* levels, with a higher upregulation in TCSle cDCs than B6 cDCs. cDCs generated in hormone-depleted conditions were unable to support significant increase in *Nos2* levels (Figure 10(g)), while increasing titrations of E2 modestly increased *Nos2* levels in response to CpG and R848 stimulation (Figures 10(h)–10(j)). The 50 nM concentration of E2 could rescue the expression of *Nos2* transcripts to the same level detected in standard conditions, although the differences between B6 and TCSle cDCs were lost. The sex bias towards higher levels of nitrite in female cDCs was not observed with *Nos2* gene expression. As we have seen for the gene expression of ISGs (Figures 5 and 7), the regulation by estrogen is present at the transcriptional and posttranscriptional levels, with strain difference being more affected by the latter.

When we looked at the effects of estrogen on the expression of *Pdk1* transcripts, we found that the hormone depletion did not change the gene expression of *Pdk1*. E2 supplementation decreased the expression in TCSle cDCs in both sexes. Although it did not reach significance, a similar trend was seen with B6 cDCs in both sexes (Figure 10(k)). These results suggest that estrogen can increase the energy metabolism of TCSle cDCs in the absence of other hormones, by decreasing the expression of the inhibitor Pdk1.

In summary, we present the first evidence that lupus cDCs have higher immunometabolism than wild-type cDCs. Specifically, we found decreased levels of the metabolic negative regulator *Pdk1* and increased secretion of NO. While *Pdk1* levels were decreased equally in female and male TCSle cDCs, a sign that both TCSle cDCs equally have increased metabolism of pyruvate [64, 65], NO levels were higher in female than in male cDCs of both strains, suggesting a hierarchy of strength in aerobic glycolysis. TLR stimulation further increased the energy metabolism of both TCSle and B6 cDCs, indicating both a higher baseline and an elevated potential for metabolic activation in TCSle cDCs. Interestingly, nitric oxide production and *Nos2* transcription by cDCs require E2 signaling for cell activation. However, while little differences were seen between sex and strains with *Nos2* transcripts, there is both a sex and strain bias in nitric oxide production, suggesting the ability of E2 to further regulate inducible NOS and the metabolic state at the posttranscriptional level.

4. Discussion

Both in SLE patients and in most murine models of lupus, males are less likely to develop disease, but they have higher incidence of renal disorders [66], skin complications [67], and disease severity [68]. Despite this, lupus research has focused predominantly on females due to higher disease prevalence. This gender bias in lupus research does not serve the population of men with SLE well and does not allow us to fully understand the pathogenesis of this complex disease. We have previously reported that cDCs from TCSle female mice show an intrinsic IFN signature that precedes the development of autoimmunity [14]. Here, we show that the IFN signature is equally present in female and male cDCs grown in standard culture conditions and that it is steroidal hormone dependent. We found that E2 enhances the expression not only of the ISGs (Figures 2, 3 and 5) but also of costimulatory molecules (Figure 6), the chemokine CXCL10 (Figure 7), and the proinflammatory cytokines IL-12p70, TNF-α, and IL-6 (Figure 8), equally in B6 and TCSle cDCs. When we cultured cDCs with doses of E2 equivalent to what is present in the serum of murine males (0.03 nM) and diestrus female mice (0.1 nM), we found minimal differences between B6 and TCSle cDCs, indicating that TCSle have a normal sensitivity to the effects of estrogen. The finding that the levels of E2 present during human pregnancy (50 nM) were capable of matching or surpassing the ISG, cytokine, and costimulatory molecule expression found in standard conditions suggests that high doses of estrogen can compensate for the absence of other hormones in the medium. Nevertheless, 50 nM E2 was inconsistently capable of yielding differences between B6 and TCSle cDCs, indicating that other components found in standard conditions further mold the TCSle IFN signature. These conclusions were confirmed by the results obtained by adding the SERM/SERD Tamoxifen and Fulvestrant to the standard conditions. Interestingly, these inhibitors reduced *Cxcl10* transcript levels and CXCL10 protein levels to below what was observed in hormone-depleted conditions, hinting at other inhibitory factors in standard conditions that may regulate these responses (Figures 3 and 7). Therefore, we propose that E2 is necessary but not sufficient to the expression of the IFN signature by cDCs and that other factors, especially other steroidal hormones, which are eliminated by the treatment of FBS with charcoal, may contribute to the differences between B6 and TCSle cDCs in standard conditions. Two likely candidates as inhibitors of immune responses are androgens and glucocorticoids, as studies have reported that androgens suppress the activation of key cells of the innate and adaptive immunity [69] and polymorphisms conferring signaling resistance in the androgen receptor inversely correlate with severity of chronic damage [46]. Glucocorticoids have been shown to inhibit DC activation [70] and promote a tolerogenic phenotype [71] although the stimulation through TLR-7 and TLR-9 can confer resistance to glucocorticoids in dendritic cells by promoting NFκB activation in both human cells and murine models of SLE [18]. Moreover, progesterone has been suggested to counteract the effects of estrogens on DC functions in vivo [72]. On

the other hand, possible steroidal candidates as immunostimulators are prostaglandins like PGE_2 that can act as a proinflammatory agent in several models of inflammatory/autoimmune disease, promoting cDC activation and Th17 development [73, 74].

Recently, there was a renewed interest on the heterogeneity of the GM-CSF bone marrow-derived cDCs [57, 75]. Our results add new details to that discussion with the variation of the medium used, IMDM versus RPMI, and the effects of estrogens and steroidal hormones on cDCs so generated. We propose that a rich medium like IMDM promotes the generation of a population of inflammatory cDCs, with a majority of immature or nonactivated $CD11^+$ $CD11b^+$ and MHC class II intermediate cells and a minority of $CD11^+$ $CD11b^+$ and MHC class II high cells that spontaneously activated in culture and express higher levels of costimulatory molecules like CD86 (Figure 4) but do not produce yet any cytokines (data not shown). We consider these two populations a gradient of activation of the same cDCs, since MHC class II and costimulatory molecules increase upon TLR stimulation without important changes in the lineage markers, like CD11c, CD11b, and CD115. We do not consider these cell macrophages because they do not express MERTK (data not show). Our results are not really in conflict with those of Helft et al. since they measured *Mertk* RNA and did not show surface protein staining [57], leaving the possibility that even in their protocols, the GM-CSF BMDCs do not express MERTK receptors. No important differences in cDC heterogeneity were measurable between TCSle and B6 cDCs, leading us to conclude that the IFN signature in TCSle cDCs is not due a different cDC composition, but rather to an augmented ability to express ISGs.

Studies in SLE patients indicate dysregulation of costimulatory factors on cDCs such as CD86 [19–21]. Furthermore, inflammatory cytokines such as IL-12 [76], IL-6, and TNF-α [77] are dysregulated in SLE. TLR9 and TLR7, respective sensors for CpG and R848, are implicated in SLE [15]. We have previously shown that the response to CpG and R848 stimulation in terms of upregulation of ISGs, CXCL10, and CD86 is type I IFN/STAT-2-dependent, while IL-12, IL-6, and TNF-α were regulated in a type I IFN/STAT-2-independent manner [47]. A previous study of pediatric SLE patients reported an IFN signature in both female and males [6] while a recent study of treatment-naïve girls and boys with childhood onset SLE further described a "TNF signature" present in boys but absent in girls [78], suggesting that sex may affect disease differently by promoting type I IFN-dependent and type I IFNs-independent pathways. Recent studies have also shown that prior to diagnosis of SLE, there is elevated type II IFN in the serum of patients [79]. Furthermore, advanced analysis of the IFN signature in adult SLE patients has revealed that there is a modular signature composed of both type I and type II IFN signatures [26]. Collectively, this may explain the clinical efficacy of targeting type I IFN in SLE: while multiple type I IFN-blocking agents in clinical trial are capable of reducing the IFN signature in SLE patients, their effects on the disease are not consistent [80]. We show here that E2 affects both type I IFN-dependent and type I IFN-independent cytokines and costimulatory molecules in both sexes (Figures 6 and 8), indicating that E2 modulates both type I IFN-dependent and type I IFN-independent pathways in both females and males. This suggests that E2 modulation may be beneficial in treating SLE in both sexes.

We found that E2 modulation of cDC activation is different at the transcriptional and posttranscriptional levels, suggesting that E2 affects DC activation through more than one pathway. Indeed, while the highest dose of 50 nM of E2 was required to enhance RNA expression of ISGs, physiologic doses of E2 were sufficient to enhance the production of proteins, either ISGs like CXCL10 or costimulatory molecules and inflammatory cytokines. This pattern of posttranscriptional regulation has been shown to be modulated through effects on the immunometabolism [42].

Recent studies on the intracellular metabolism of DCs have revealed the critical role of the metabolic reprogramming in the response of DCs to environmental changes, from hypoxia to danger signals and cytokines [81]. It was shown that murine wild-type cDCs rapidly increase their glycolytic rate soon after TLR engagement, with a short-term increase in mitochondrial respiration. The upregulation of glycolysis during the early phase of DC activation is essential for NADPH regeneration, fatty acid synthesis, and the enlargement of the endoplasmic reticulum and Golgi. These processes are necessary to produce proinflammatory protein mediators, while the upregulation of RNA transcription seems to require lesser metabolic changes. In iNOS-expressing DCs, such as the murine GM-CSF-derived cDCs, a progressive decrease of the oxidative phosphorylation is induced by the nitric oxide that is produced after the first 8 hours of TLR stimulation. Thus, 24 hours after stimulation, cDCs depend almost exclusively on glycolysis to sustain their function and survival.

Immunogenicity and tolerogenicity of DCs have been proposed to be promoted by anabolic and catabolic processes, respectively. The higher state of activation of TCSle cDCs, which we show here, and of T cells that Wu et al. have reported [45], could be sustained by a higher energy metabolism. Indeed, the result that *Pdk1*, an inhibitory regulator of the Krebs cycle that controls both the oxidative phosphorylation and fatty acid synthesis, is decreased in TCSle cDCs, which is the first evidence that TCSle cDCs have a constitutive higher immunometabolism even in the absence of any exogenous stimulation. The increase in nitric oxide (NO) in TCSle cDCs, which is also used as biomarker of metabolic activation of cDCs [43], indicates that TCSle cDCs respond to activation with a greater shift towards glycolysis. Altogether, these results suggest that, compared to B6 cDCs, TCSle cDCs have a higher baseline mitochondrial respiration and they can generate a stronger aerobic glycolysis upon stimulation with nucleic acids. Since it has been recently suggested that type I IFNs are required for the metabolic shift to glycolysis that occurs during cDC activation [44], we speculate that the IFN signature may contribute to the higher metabolism of TCSle cDCs.

Estrogens have been shown to affect oxidative phosphorylation, glycolytic enzymes, and the glucose uptake upstream of glycolysis in several tissues in the body, especially in the

brain [82], while less was known in immune cells. We show here that E2 can further decrease the expression of *Pdk1* in TCSle cDCs and increase the production of nitric oxide, suggesting a novel role for estrogen in increasing the immunometabolism (Figure 10). Moreover, we found that E2 promotes elevated NO production by cDCs in a sex-dependent manner, with female cDCs producing more NO than male cDCs (Figures 9 and 10). This sex bias was even present in cDCs generated with hormone-depleted medium supplemented with physiologic doses of E2, suggesting that the immunometabolism is more affected by E2 than by other hormones present in FBS in a sex-dependent manner.

In conclusion, we show that male TCSle cDCs express the same IFN signature as female TCSle cDCs and respond to the enhancing effects of estrogen, and yet, estrogen alone is not sufficient to recapitulate the difference between TCSle and B6 cDCs. These results suggest that the effect of estrogens on just the IFN signature cannot per se justify the higher incidence of lupus in females, while the effects of estrogens on IFN-dependent and IFN-independent pathways, in conjugation with additional hormonal and environmental factors, are likely necessary for the full development of lupus in genetically susceptible individuals. The results indicating that SERM/SERD inhibit the expression of the IFN signature in cDCs of both sexes *in vitro* warrant that testing the effects of SERM/SERD should be performed in both male and female mice in preclinical studies. Together, the novel data showing a higher immunometabolism in TCSle cDCs, along with the sex bias in NO production and its estrogen dependence, suggest that the immunometabolism may be an important mechanism for the sex bias in lupus pathogenesis and a possible novel therapeutic target in lupus.

5. Summary

β-Estradiol modulates baseline and TLR-induced activation of lupus conventional dendritic cells; it increases the IFN signature and DC immunometabolism.

Abbreviations

B6:	C57BL/6
cDC:	Conventional dendritic cell
DC:	Dendritic cells
ERα:	Estrogen receptor-α
E2:	17-beta-estradiol
ISG:	Interferon-stimulated gene
IRF:	IFN regulatory factor
nM:	Nanomolar
NO:	Nitric oxide
NOS:	Nitric oxide synthase
pDC:	Plasmacytoid dendritic cells
Pdk1:	Pyruvate dehydrogenase kinase 1
R848:	Risiquimod
SERM/SERD:	Selective estrogen receptor modulators/ degraders
SLE:	Systemic lupus erythematosus
TCSle:	NZM2410-derived Triple Congenic B6. NZM.*Sle1*/*Sle2*/*Sle3*.

Disclosure

An early version of this work was presented as a poster at the Annual Meeting of the American Association of Immunologists (AAI), in Seattle, WA, on May 13–17, 2016.

Conflicts of Interest

The authors declare no conflicts of interest.

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Supplementary Materials

Supplemental Figure 1: shows the surface expression of MHC class II and CD115 as markers of differentiation of inflammatory dendritic cells in dendritic cells generated in the presence of different concentrations of estrogens. *(Supplementary Materials)*

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Research Article

Intermittent High Glucose Exacerbates A-FABP Activation and Inflammatory Response through TLR4-JNK Signaling in THP-1 Cells

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Background. Glucose fluctuation confers additional risks on diabetes-related vascular diseases, but the underlying mechanisms are unknown. Macrophage activation mediated by TLR4-JNK signaling plays an important role during the progress of diabetes. In the present study, we hypothesize that glucose fluctuation results in macrophage inflammation through TLR4-JNK signaling pathways. *Methods*. THP-1 cells were treated with normal glucose (5 mM), constant high glucose (25 mM), and intermittent high glucose (rotation per 6 h in 5 mM or 25 mM) for 24 h. The mRNA and protein expression levels of TLR4, p-JNK, and adipocyte fatty acid-binding protein (A-FABP) were determined, and the proinflammatory cytokines TNF-α and IL-1β were quantified. *Results*. In constant high glucose, TLR4 expression and JNK phosphorylation levels increased, and this effect was more pronounced in intermittent high glucose. Accordingly, the expression of A-FABP and the release of the proinflammatory cytokines TNF-α and IL-1β also increased in response to constant high glucose, an effect that also was more evident in intermittent high glucose. The inhibition of p-JNK by SP600125 did not attenuate TLR4 expression, but totally inhibited both A-FABP expression and the production of the proinflammatory cytokines TNF-α and IL-1β in both constant and intermittent high glucose. *Conclusions*. Intermittent high glucose potentiates A-FABP activation and inflammatory responses via TLR4/p-JNK signaling in THP-1 cells. These findings suggest a more detrimental impact of glucose fluctuation on macrophage inflammation in diabetes-related vascular diseases than thus far generally assumed.

1. Introduction

Accumulating evidence from epidemiological and interventional research has pointed toward the deleterious impact of hyperglycemia on the progress of diabetic complications [1–3]. However, more recent clinical studies have shown that glucose fluctuation plays a more important role in conferring additional risks on micro- and macrovascular diabetic complications [4–6] than merely high glucose levels do. In a study following 566 elderly patients with type 2 diabetes mellitus, glucose instability measured as the coefficient of variation of fasting plasma glucose concentrations for 3 years was found to independently correlate with cardiovascular-related mortality [7]. Glucose fluctuation not only exists in and accelerates the disease progress of diabetes, but also impairs the recovery of coexistent diseases [8, 9]. In a study of sepsis, the glycemic lability index was reported to be the best indicator to predict mortality, even after adjustment for confounders including the number of organ failures and hypoglycemia [8]. In addition, daily glucose fluctuation was also shown to affect vessel healing in coronary artery disease patients who had received everolimus-eluting stent implantation [10]. In spite of the fact that abundant clinical literature has depicted a critical role of glucose fluctuation in disease

progress, irrespective of diabetes, the underlying mechanism by which glucose fluctuation exacerbates the disease progress remains unclear so far.

The macrophage, an inflammatory cell essential for the initiation and development of inflammation, is a key element in hyperglycemia-induced damage [11]. Current studies on macrophages focus predominantly on the impairment by consistent hyperglycemia. In primary human monocyte-derived macrophages, hyperglycemia induces the production of the prototype M_1 cytokines tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and IL-6, but inhibits the M_2 cytokines IL-1Ra and C-C motif chemokine ligand 18 (CCL18) during macrophage differentiation [12]. High glucose levels have been shown to markedly repress the expression of silent mating type information regulation 2 homolog 1 (SIRT1) and promote the release of proinflammatory cytokines in RAW264.7 macrophages [13]. While extensive research has been carried out on the effects of fluctuating hyperglycemia in patients with diabetes, coronary artery disease, and sepsis, few studies have examined the effect of fluctuating hyperglycemia on macrophage functioning. In this regard, previous studies in rodents reported that repetitive glucose fluctuation evoked more monocyte adhesion to the endothelium than stable glucose [14, 15].

Adipocyte fatty acid-binding protein (A-FABP, also referred to as FABP4, aP2) is highly expressed in adipocytes and macrophages and is extensively involved in inflammation and glucose and lipid metabolism [16]. A-FABP mainly functions as an intracellular transport protein for fatty acids, thereby regulating cellular lipid metabolism and lipid signals [17]. In macrophages, several proinflammatory stimuli have been reported to upregulate A-FABP expression, including oxidized low-density lipoproteins, toll-like receptor (TLR) agonists, and PPARγ agonists [18–20]. In this regard, lipopolysaccharide (LPS), a TLR4 ligand, stimulates A-FABP expression and the activated A-FABP, reciprocally, enhances the LPS-TLR4 signaling-evoked c-Jun N-terminal kinase (JNK) inflammatory pathways [21]. In addition, prolonged hyperglycemia has been shown to induce A-FABP expression in mesangial cells and trigger the release of proinflammatory cytokines [22]. The concentration of A-FABP in circulation was found to strongly associate with diabetes, atherosclerosis, and coronary heart disease; it has been identified as a good biomarker in the prediction of the development of both diabetes and metabolic syndrome [23]. However, the impact of intermittent hyperglycemia on A-FABP expression and the involved inflammatory pathways in macrophages remain unknown.

Considering the notion that fluctuating hyperglycemia may exert more detrimental effects in patients with diabetes or other diseases, the aim of the present study is to compare the effects of persistent and of intermittent hyperglycemia on TLR4 and A-FABP expression and on the inflammatory response in THP-1 macrophages. The present study shows that intermittent hyperglycemia results in higher A-FABP expression and in an increased release of proinflammatory cytokines through TLR4-JNK pathways. These findings strongly indicate that the prevention of fluctuating hyperglycemia is essential to reduce macrophage activation and JNK inhibition may be a potential therapeutic method to inhibit macrophage activation and the resulting inflammatory response.

2. Results

2.1. Intermittent High Glucose Greatly Induces Upregulation of TLR4 and p-JNK in THP-1 Cells. As shown in Figure 1, very low expression levels of TLR4 mRNA (Figure 1(a)) and protein (Figure 1(b)) were detected in the constant or intermittent mannitol-treated or normal glucose-treated THP-1 cells. The TLR4 mRNA and protein levels were comparable in these three groups. In contrast, in the persistent presence of 25 mM of glucose, the expression of the TLR4 gene and protein increased as compared with the constant or intermittent mannitol-treated, or normal-glucose group ($p < 0.05$, high-Glu versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group). However, intermittent high glucose (5 mM glucose for 6 hours and 25 mM glucose for 6 hours per cycle) resulted in more potent upregulation of TLR4 mRNA and protein levels ($p < 0.01$, normal-/high-Glu versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group). The increased TLR4 expression by fluctuating high glucose was lower than the effect of the TLR ligand LPS, which resulted in a 5–7-fold upregulation of TLR4 mRNA and protein levels in the concentration of 100 ng/ml ($p < 0.05$, normal-/high-Glu group versus LPS group).

As JNK is a downstream signal of TLR4, in the present study we next investigated the effect of high glucose on JNK phosphorylation. As shown in Figure 2, LPS resulted in a more than 6-fold increase in JNK phosphorylation as compared with the constant or intermittent mannitol-treated, or normal-glucose group ($p < 0.001$, LPS group versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group). Similarly, persistent high glucose led to a milder increase in JNK phosphorylation ($p < 0.05$ high-Glu group versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group). However, intermittent high glucose activated p-JNK expression to a higher extent as compared with persistent high-glucose treatment ($p < 0.05$, normal-/high-Glu group versus high-Glu group).

2.2. Intermittent High Glucose Greatly Upregulates A-FABP Expression and Induces Proinflammatory Cytokine Release. Our previous studies have shown that LPS induces A-FABP activation and the following release of cytokines through the TLR4-JNK pathway [21]. Consistent with the previous study, LPS (100 ng/ml) dramatically increased the expression of A-FABP mRNA and protein ($p < 0.001$, LPS group versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group) (Figure 3). Persistent high glucose also induced the upregulation of A-FABP to a much lower extent than LPS treatment ($p < 0.01$, high-Glu group versus LPS group). In contrast, intermittent high glucose caused a more robust upregulation of A-FABP as compared with persistent high glucose ($p < 0.05$ normal-/high-Glu group versus high-Glu group), but less than that of LPS treatment ($p < 0.05$, normal-/high-Glu group versus LPS group).

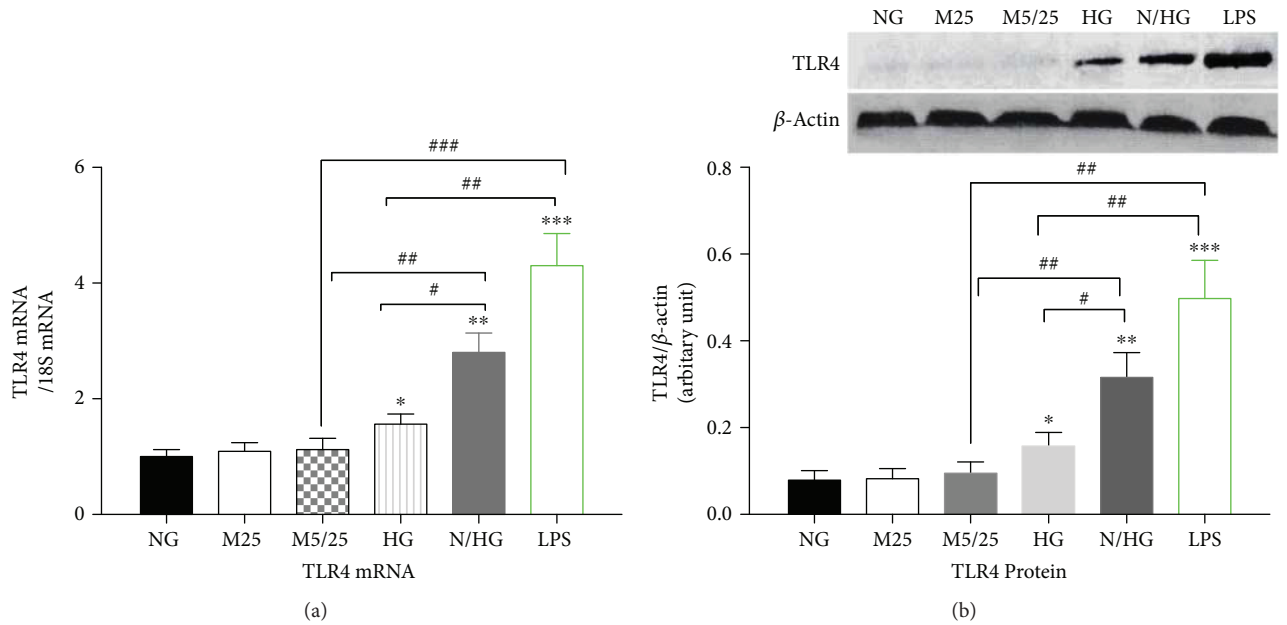

Figure 1: Constant high glucose, intermittent high glucose, and LPS individually induced a robust increase in the expression of TLR4 in THP-1 cells. (a) The mRNA levels of TLR4 were examined 24 h after the treatment of THP-1 cells. (b) The protein levels of TLR4 were examined 24 h after the treatment of THP-1 cells. NG, normal glucose (5 mM); M25, mannitol (25 mM); M5/25, intermittent mannitol (25–5 mM); HG, constant high glucose (25 mM); N/HG, intermittent glucose (25–5 mM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus ctrl, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ ($n = 4$ for each group).

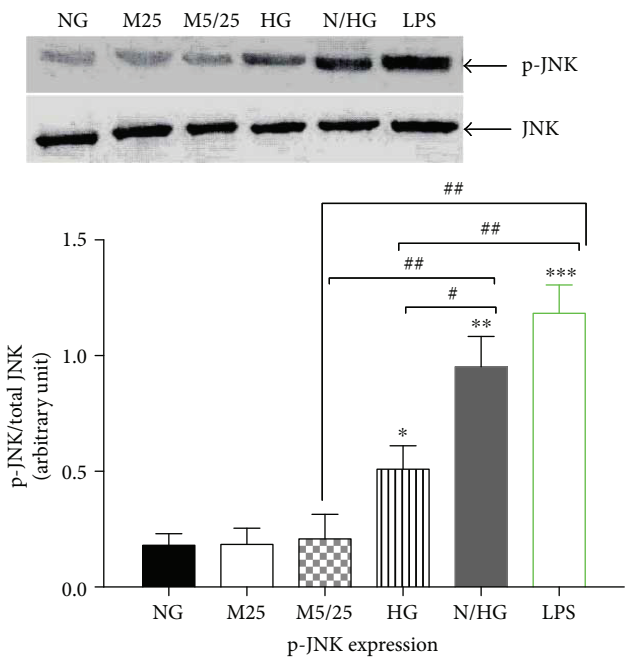

Figure 2: Constant high glucose, intermittent high glucose, and LPS individually increased phosphorylation of JNK in THP-1 cells. NG, normal glucose (5 mM); M25, mannitol (25 mM); M5/25, intermittent mannitol (5–25 mM); HG, constant high glucose (25 mM); N/HG, intermittent glucose (5–25 mM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus ctrl, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ ($n = 4$ for each group).

Like the activation of A-FABP, LPS treatment remarkably increased the supernatant TNF-α and IL-1β levels (Figures 4(a) and 4(b)) ($p < 0.001$, LPS group versus 25 mM Man group, versus 5/25 mM Man group, or versus normal-Glu group). Be it to a lower extent, persistent or intermittent high glucose also increased the supernatant levels of TNF-α (Figure 4(a)) and IL-1β (Figure 4(b)). However, intermittent high glucose increased the levels of these two cytokines more than persistent high-glucose treatment did ($p < 0.05$, normal-/high-Glu group versus high-Glu group).

2.3. JNK Inhibitor SP600125 Did Not Attenuate the Activation of TLR4 by Persistent and Intermittent High Glucose. To further explore the role of TLR4-JNK pathways on the activation of A-FABP and the following inflammatory response, the JNK inhibitor SP600125 was used to inhibit JNK phosphorylation. As shown in Figure 5, all three concentrations of SP600125 tested in the present study (10 μM, 20 μM, and 50 μM) almost completely prevented the increased JNK phosphorylation induced by LPS treatment ($p < 0.01$, 0 μM group versus 10 μM group, versus 20 μM group, or versus 50 μM group), suggesting that SP600125 is a potent inhibitor of JNK activity.

In sharp contrast, SP600125 treatment (20 μM) did not inhibit the upregulation of TLR4 mRNA induced by intermittent high glucose or LPS treatment (Figure 6(a)) ($p < 0.05$, normal-/high-Glu group versus high-Glu group; $p < 0.01$, LPS group versus high-Glu group). Similarly, the upregulation of TLR4 induced by intermittent high glucose or LPS treatment was not changed after SP600125 treatment (Figure 6(b)).

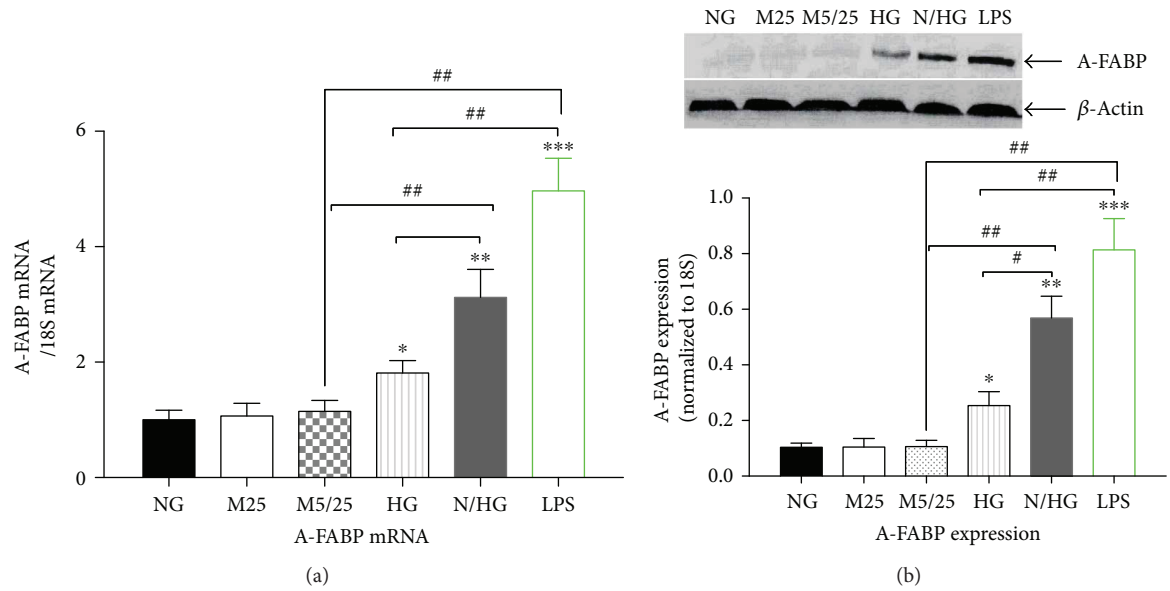

Figure 3: Constant high glucose, intermittent high glucose, and LPS individually induced a robust increase in the expression of A-FABP in THP-1 cells. (a) The mRNA levels of A-FABP were examined 24 h after the treatment of THP-1 cells. (b) The protein levels of A-FABP were examined 24 h after the treatment of THP-1 cells. NG, normal glucose (5 mM); M25, mannitol (25 mM); M5/25, intermittent mannitol (5–25 mM); HG, constant high glucose (25 mM); N/HG, intermittent glucose (5–25 mM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus ctrl, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ ($n = 4$ for each group).

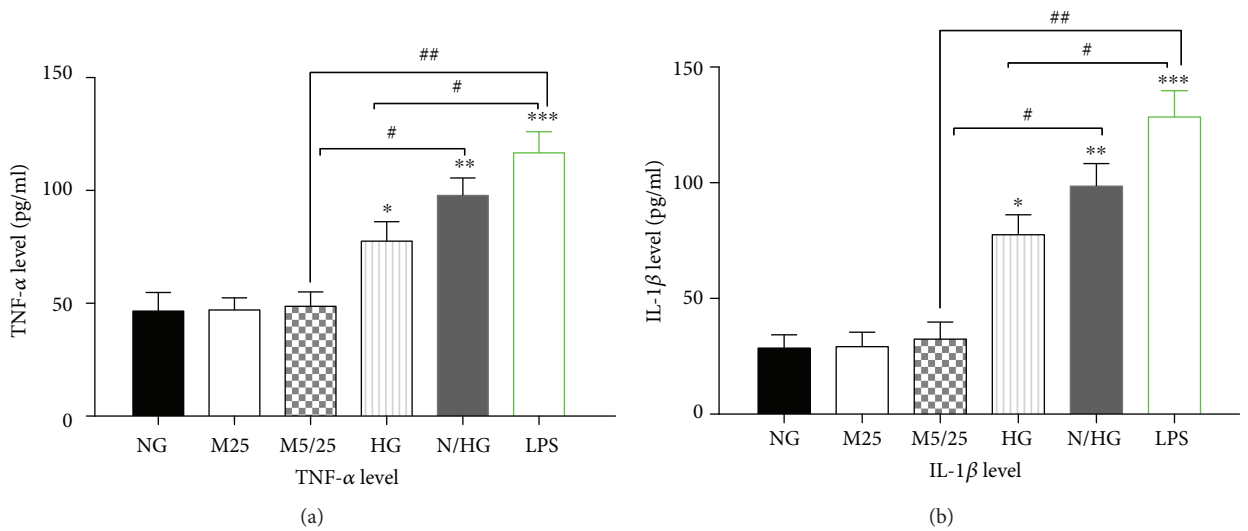

Figure 4: Constant high glucose, intermittent high glucose, and LPS individually increased the production of inflammatory cytokines TNF-α and IL-1β in THP-1 cells. (a) The TNF-α levels were determined 24 h after the treatment of THP-1 cells. (b) The IL-1β levels were determined 24 h after the treatment of THP-1 cells. NG, normal glucose (5 mM); M25, mannitol (25 mM); M5/25, intermittent mannitol (5–25 mM); HG, constant high glucose (25 mM); N/HG, intermittent glucose (5–25 mM). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus ctrl, $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ ($n = 4$ for each group).

2.4. SP600125 Inhibited the Activation of A-FABP and Reduced the Release of TNF-α and IL-1β Induced by Persistent and Intermittent High Glucose. In the present study, we next investigated the effect of SP600125 on the high-glucose-evoked upregulation of A-FABP and the increased TNF-α and IL-1β supernatant levels. As shown in Figure 7(a), in the presence of 20 μM SP600125, A-FABP mRNA levels in high-Glu, normal-/high-Glu, and LPS groups were much lower than their match groups without SP600125 treatment, respectively ($p < 0.05$, high Glu + SP600125 versus high Glu; $p < 0.01$, normal/high Glu + SP600126 versus normal/high Glu and LPS + SP600125 versus LPS). Figure 7(b) shows the dramatic inhibition of A-FABP protein expression after SP600125 treatment ($p < 0.01$, high Glu versus high Glu + SP600125, or versus normal/high Glu + SP600125, or versus LPS). These results suggest that SP600125 remarkably inhibits A-FABP

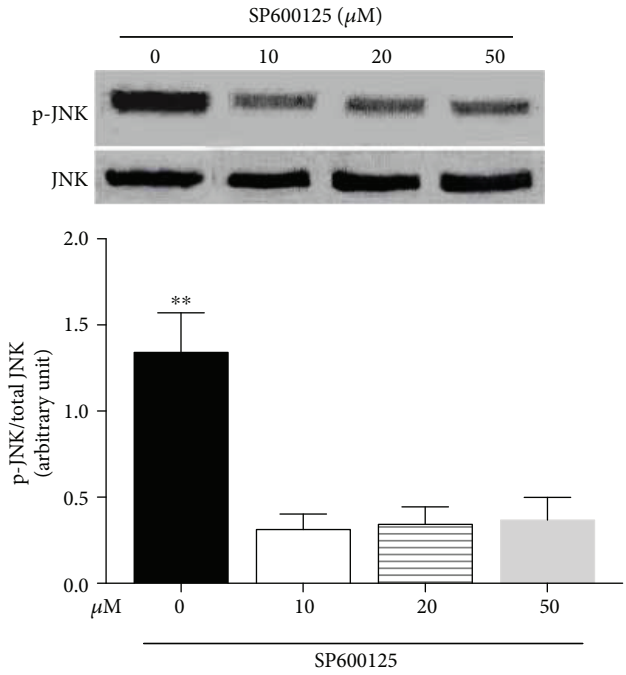

Figure 5: SP600125 treatment at different concentrations downregulated the increased JNK phosphorylation induced by LPS treatment. The levels of JNK phosphorylation in THP-1 cells pretreated with 10 μM, 20 μM, or 50 μM SP600125 for 2 h followed by stimulation with LPS. $^{**}p < 0.01$, 0 μM group versus 10 μM group, versus 20 μM group, or versus 50 μM group ($n = 4$ for each group).

activation upon the treatment of persistent or intermittent high glucose.

Figures 7(c) and 7(d) show that SP600125 greatly inhibits the release of TNF-α (Figure 7(c)) and IL-1β (Figure 7(d)) induced by the persistent or intermittent high glucose ($p < 0.05$, high Glu + SP600125 versus high Glu and normal/high Glu + SP600125 versus normal/high Glu). Notably, the inhibitory effect on TNF-α and IL-1β release of SP600125 was milder than its inhibitory effect on A-FABP expression (Figures 7(a) and 7(b)).

3. Discussion

Glucose level variability is emerging as an important index of diabetes control, even though its underlying mechanisms on diabetic complications and the immune system are only beginning to be elucidated. The current study shows that both consistent and intermittent hyperglycemia increase A-FABP mRNA and protein expression, in parallel with the production of proinflammatory cytokines TNF-α and IL-1β, the upregulation of which is mediated by upregulated phosphorylation of JNK in response to high or fluctuating glucose. However, intermittent hyperglycemia appeared to worsen the aberrant production of A-FABP and the proinflammatory effects of high glucose on THP-1 cells.

A growing body of literature has revealed the close involvement of chronic low-grade inflammation and of the innate immune system in the development of diabetes and its vascular complications. In epidemiologic studies, a variety of circulating inflammatory markers, such as IL-1β, have been identified as strong predictors of type 2 diabetes [24–26]. Moreover, monocytes isolated from patients with type 1 diabetes also present a proinflammatory phenotype and secrete higher levels of inflammatory cytokines, such as IL-6 and IL-1β, compared with nondiabetic individuals [27]. Hyperglycemia can cause an M_1/M_2 imbalance, switching macrophage polarization towards a proinflammatory M_1 phenotype [12, 28–31]. In the present study, we have also detected that high glucose increases the level of TNF-α and IL-1β, markers of M_1 macrophages, confirming that high glucose induces the inflammatory response in macrophages. More importantly, intermittent high glucose caused a more robust upregulation of TNF-α and IL-1β. These findings strongly indicate that, as compared with persistent high glucose, violent fluctuation of glucose exerts more detrimental inflammatory responses in patients.

Most studies on diabetes-related diseases were performed with treatment of consistent high glucose levels. These studies did not mimic an actual clinical context, where most diabetic patients experience violent fluctuations of blood glucose levels every day. Indeed, glucose oscillation is equally important as HbA_1c in the control of diabetes and it has been reported to be a good predictor for the development of diabetic complications in a growing body of literature. However, compared with numerous studies on the impact of consistent hyperglycemia on macrophages, there is little knowledge available on the questions whether intermittent hyperglycemia causes aberrant activation of macrophages and whether there is a difference in the extent of inflammation between cases of consistent and intermittent hyperglycemia. The more severe inflammatory responses likely to be caused by intermittent hyperglycemia have been reported in monocytes and umbilical endothelial cells [32, 33]. Notably, in the previous study of monocytes, the author also used THP-1 cells, but merely stimulated differentiation into the monocyte form of THP-1 cells under high glucose conditions, without the addition of PMA for differentiation into macrophages [33]. Indeed, after reaching the tissue, monocytes differentiate into macrophages, which play a number of critical roles in diabetes, atherosclerosis, and other health issues. Because macrophages and monocytes exhibit distinct phenotypes, we further looked into the responses of macrophages to intermittent and consistent high glucose. Compared with a previous study in monocytes [33], both consistent and intermittent hyperglycemia increased a more dramatic production of the inflammatory cytokines TNF-α and IL-1β in macrophages, and this proinflammatory effect is more pronounced in response to intermittent hyperglycemia. This finding further supports the deleterious effects of glucose fluctuation on macrophage inflammation in micro- and macrodiabetic complications.

The present study has mimicked glucose toxicity under consistent and intermittent high glucose conditions to clarify the relationship between A-FABP and glucose toxicity in the control of macrophage inflammation. Hyperglycemia is shown to induce inflammation through different signaling cascades, including (i) Rho-associated coiled-coil forming

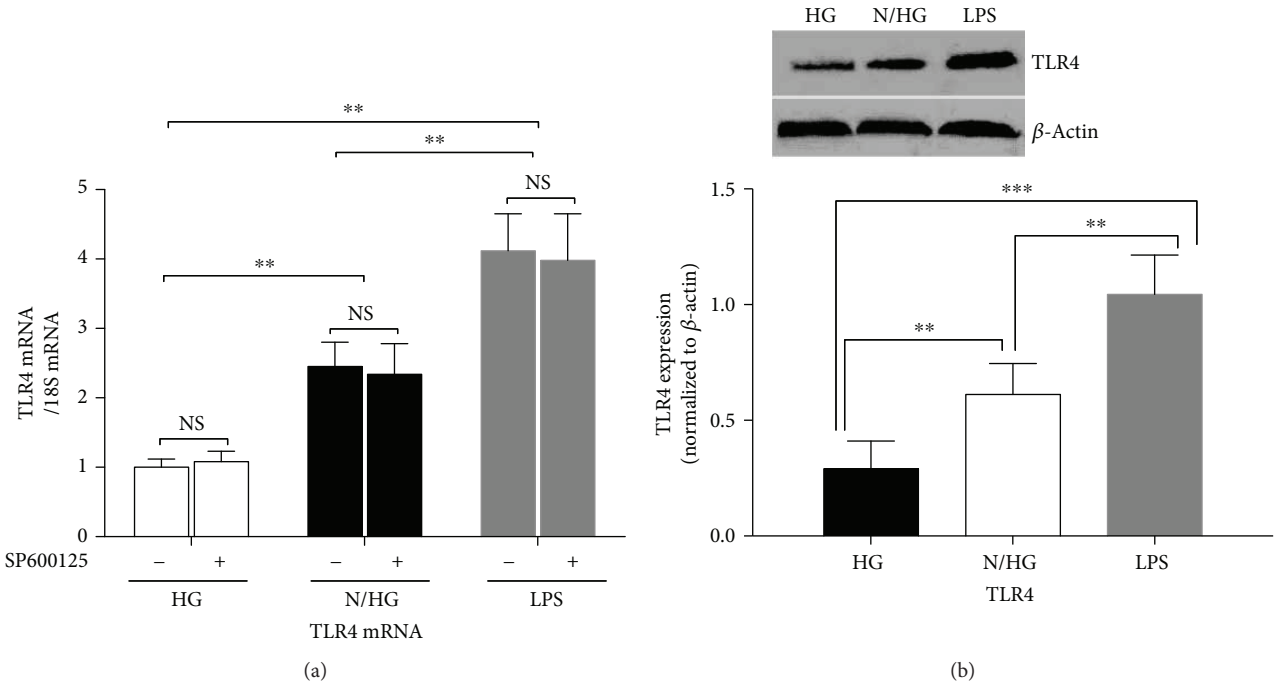

Figure 6: JNK inhibitor SP600125 did not inhibit the activation of TLR4 by persistent or intermittent high glucose. THP-1 cells were pretreated with SP600125 (20 μM) for 2 h followed by stimulation with intermittent high glucose, persistent high glucose, or LPS. Then the expression of TLR4 was examined. $^{**}p < 0.01$, $^{***}p < 0.001$ ($n = 4$ for each group).

kinase (ROCK)-dependent JNK and extracellular regulated protein kinase (ERK) phosphorylation in RAW264.7 macrophages [28], (ii) signal transducer and activator of transcription 3 (STAT3) in retinal endothelial cells [34], and (iii) TLR2 and TLR4 in human microvascular retinal endothelial cells [35]. LPS activates the JNK activator protein-1 (AP-1) signaling pathway and leads to the upregulation of A-FABP and the release of proinflammatory cytokines in macrophages. Consistently, we have shown that p-JNK is activated by consistent or intermittent high glucose; pharmaceutical inhibition of p-JNK completely abolished glucose-induced A-FABP expression and the production of the inflammatory cytokines TNF-α and IL-1β. This indicates that p-JNK is involved in hyperglycemia-induced macrophage inflammation.

Our data also show that A-FABP levels increase during the inflammatory response induced by consistent or intermittent hyperglycemia in macrophages. A-FABP belongs to the fatty acid-binding protein family and has been identified as a critical mediator linking diabetes and cardiovascular diseases, partly through its proinflammatory actions in macrophages [16]. Mice deficient in FABP4 and FABP5 reveal a striking phenotype with strong protection against diet-induced obesity, insulin resistance, type 2 diabetes and fatty liver disease [36]. However, the influence of high glucose, consistent or intermittent, on the expression of A-FABP in macrophages remains unclear. In our experiments, the exposure of THP-1 cells to high glucose concentrations induced an increase in mRNA and protein expression of A-FABP, which was higher in intermittent high glucose than in consistent high glucose. In line with our results, Yao et al. treated human mesangial cells with stable high glucose (30 mM) for 24 hours, which dramatically increased A-FABP mRNA and protein expression. Consistent with our findings, a close connection between A-FABP and p-JNK has been reported in the LPS-evoked inflammatory response. After the exposure of RAW264.7 cells to LPS, activated JNK increases A-FABP mRNA and protein expression through the binding of a phosphorylated site of c-Jun to a highly conserved AP-1 *cis*-element within the A-FABP gene promoter [21]. It should be noted that many diabetes patients are subjected to infection or sepsis, in which the TLR4 pathway may be activated to a certain extent. Our study suggests that, under this condition, the avoidance of fluctuating high glucose levels is more important to control the strong inflammatory response than lowering blood glucose levels.

4. Conclusion

In summary, this study shows that the exposure of THP-1 cells to intermittent high glucose results in a more robust overproduction of A-FABP and proinflammatory cytokines than stable high glucose, which is mediated by the TLR4/p-JNK signaling cascade. These findings suggest a more ominous impact of glucose fluctuation on macrophage functioning in diabetes-related vascular diseases and strongly imply that prevention of violent glucose fluctuations is critical for the delay of diabetes-related diseases.

5. Materials and Methods

5.1. Cell Culture and Treatment. THP-1 human monocytic cells (Shanghai Institute of Cell Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) were cultured in RPMI-1640 medium (Invitrogen, Carlsbad,

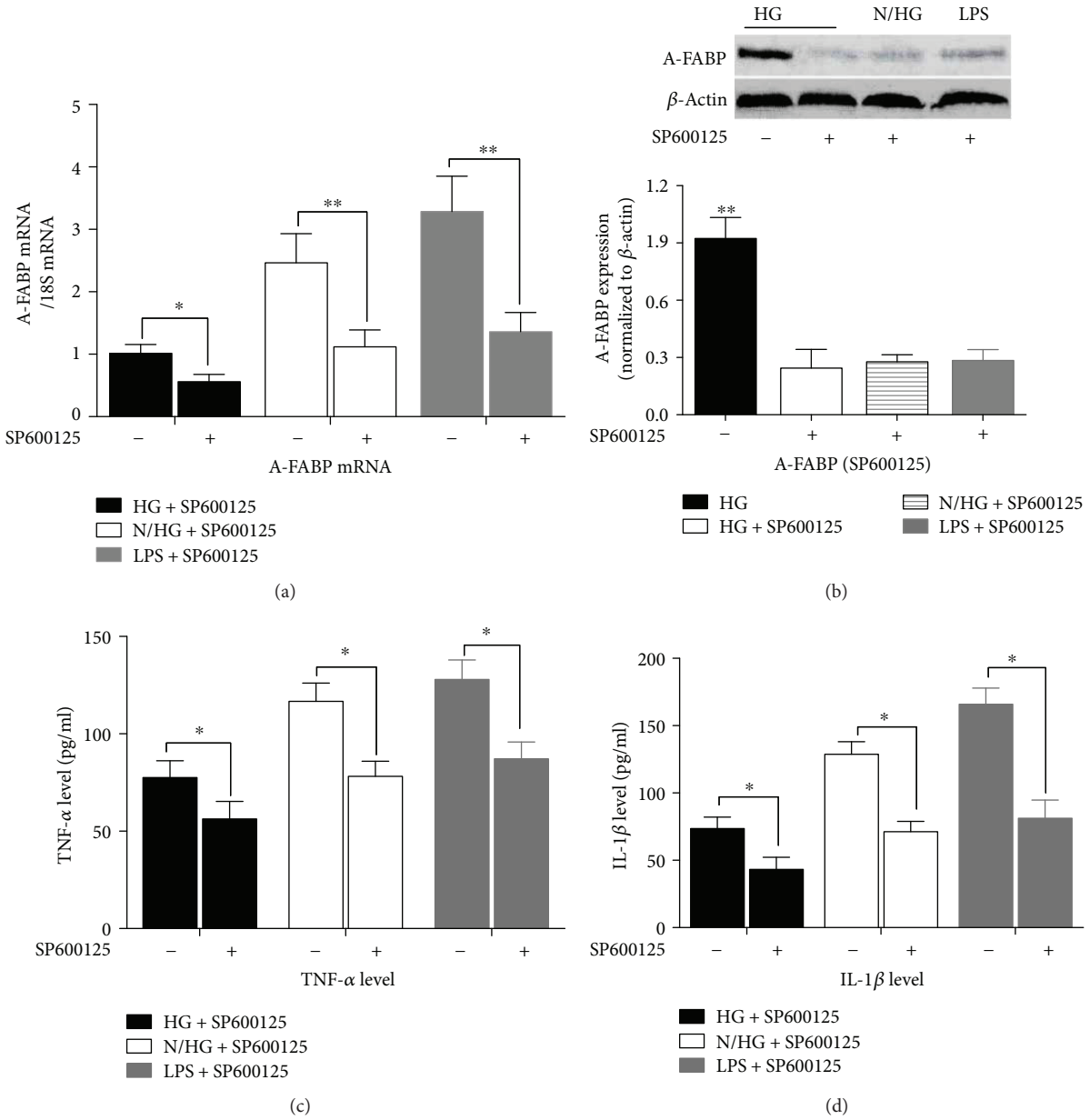

Figure 7: SP600125 (20 μM) treatment greatly inhibited the production of A-FABP and inflammatory cytokines induced by intermittent high glucose or LPS treatment. (a) The levels of A-FABP mRNA and (b) A-FABP protein were analyzed in THP-1 cells pretreated with 20 μM SP600125 for 2 h followed by stimulation with glucose or LPS. (c) The release of TNF-α in THP-1 cells pretreated with SP600125 followed by stimulation with glucose or LPS. (d) Effects of SP600125 on the production of IL-1β in THP-1 cells after glucose or LPS treatment for 24 h. $^*p < 0.05$, $^{**}p < 0.01$ ($n = 4$ for each group).

USA) supplemented with 10% FBS and 1% P/S, at 37°C under 5% CO_2. The cells were differentiated to macrophages by treatment with 100 nM phorbol-12-myristate-13-acetate (PMA) (Sigma-Aldrich, St. Louis, MO, USA) in RPMI-1640 supplemented with 10% FBS and 1% P/S for 72 h. After 72 h of differentiation, cells were divided into four groups and incubated for 24 h as follows: (1) constant 5 mM glucose, (2) constant 25 mM glucose, (3) alternating normal (5 mM) and high (25 mM) glucose medium every 6 h, and (4) 100 ng/ml LPS. Osmotic control was assured by treating THP-1 cells with equimolar concentrations of mannitol (Sigma-Aldrich, St. Louis, MO), both continuously and in an alternating manner.

5.2. Real-Time Polymerase Chain Reaction. Real-time polymerase chain reaction (PCR) was performed to detect the gene expression of A-FABP, TLR4, and β-actin. The PCR sequence pairs were: AACCTTAGATGGGGGTGTCC and ATGCGAACTTCAGTCCAGGT (A-FABP); GCCGAC AGGATGCAGAAGGAG and AAGCATTTGCGGTGGA CGATG (β-actin); AAGCCGAAAGGTGATTGTTG and CTGAGCAGGGTCTTCTCCAC (TLR4). PCR reaction was

carried out using 15 ng of cDNA, 200 nM of each primer and SYBR green PCR master mix (Roche, Germany). Cycling conditions included 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 60 s at 60°C.

5.3. Western Blotting Analysis. After treatment, protein extracts for total cellular fractions were isolated in a cell lysis buffer (Cell Signaling Technology, Beverly, MA, USA) supplemented with 0.1 mg PMSF and a 1/100 dilution of protease and phosphatase inhibitor cocktails (Sigma-Aldrich, St. Louis, MO). Scraped samples were then centrifuged at 10000 rpm for 15 min at 4°C. The supernatants were used for Western blotting. Protein concentrations were measured using the Bio-Rad protein dye microassay (Bio-Rad, Hercules, USA). Proteins were separated on a 12% SDS-PAGE gel and then transferred onto PVDF membranes. The membrane was blocked with a solution of TBS and 5% fat-free milk for 1 h, then incubated overnight with rabbit anti-A-FABP, rabbit anti-phospho-JNK, rabbit anti-JNK, rabbit anti-TLR4 (all from Cell Signaling Technology, Beverly, MA, USA), or monoclonal mouse anti-β-actin (Santa Cruz Biotechnology, Heidelberg, Germany). The blot was then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG in TBS for 2 h at room temperature. The membrane was then exposed to film before development.

5.4. TNF-α and IL-1β Quantification. Conditioned media from cultured cells were collected at the indicated time points. The quantitative detection of soluble TNF-α and IL-1β was determined using commercially available ELISA kits (BioSource International, USA), in accordance with the manufacturer's instructions.

5.5. Statistical Analysis. Data are expressed as mean ± standard error of the mean (SEM). Statistical analyses were performed using the paired Student's t test or one-way analysis of variance (ANOVA) followed by Bonferroni analysis where appropriate. Statistical significance was arbitrarily declared at p values below 0.05. All analyses were performed using SPSS version 20 (SPSS Inc., Chicago, IL).

Disclosure

Hui Li and Han-Ying Luo should be considered co-first authors.

Conflicts of Interest

The authors declare no conflict of interest.

Authors' Contributions

Hui Li and Han-Ying Luo contributed equally to this work.

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Research Article

High-Density Lipoprotein Reduction Differentially Modulates to Classical and Nonclassical Monocyte Subpopulations in Metabolic Syndrome Patients and in LPS-Stimulated Primary Human Monocytes *In Vitro*

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The effect of metabolic syndrome on human monocyte subpopulations has not yet been studied. Our main goal was to examine monocyte subpopulations in metabolic syndrome patients, while also identifying the risk factors that could directly influence these cells. Eighty-six subjects were divided into metabolic syndrome patients and controls. Monocyte subpopulations were quantified by flow cytometry, and interleukin- (IL-) 1β secretion levels were measured by ELISA. Primary human monocytes were cultured in low or elevated concentrations of high-density lipoprotein (HDL) and stimulated with lipopolysaccharide (LPS). The nonclassical monocyte (NCM) percentage was significantly increased in metabolic syndrome patients as compared to controls, whereas classical monocytes (CM) were reduced. Among all metabolic syndrome risk factors, HDL reduction exhibited the most important correlation with monocyte subpopulations and then was studied *in vitro*. Low HDL concentration reduced the CM percentage, whereas it increased the NCM percentage and IL-1β secretion in LPS-treated monocytes. The LPS effect was abolished when monocytes were cultured in elevated HDL concentrations. Concurring with *in vitro* results, IL-1β serum values significantly increased in metabolic syndrome patients with low HDL levels as compared to metabolic syndrome patients without HDL reduction. Our data demonstrate that HDL directly modulates monocyte subpopulations in metabolic syndrome.

1. Introduction

In humans, circulating monocytes have been sorted into three different subpopulations according to the cell surface expression of CD14 and CD16 [1, 2]. The vast majority of circulating monocytes show high expression of CD14 and no expression of CD16 ($CD14^{high}CD16^{-}$) and constitute the fraction of classical monocytes (CM). Monocytes producing CD16 are divided into two subgroups: intermediate monocytes (IM) that show high expression of CD14 and also show CD16 expression ($CD14^{high}CD16^{+}$) and nonclassical monocytes (NCM) that exhibit low CD14 levels accompanied by CD16 expression ($CD14^{low}CD16^{+}$) [1]. Monocyte subpopulations also show different immunological functions. The NCM subset has been shown to produce high amounts of interleukin- (IL-) 1β under basal conditions or in response to lipopolysaccharide (LPS) stimulation, which has led to a postulation that NCM exerts the most important inflammatory functions in circulation [3–5]. On the contrary, CM and IM subpopulations have been demonstrated to participate in endothelial adhesion and cell migration by preferably expressing chemokines and chemokine receptors without showing prominent inflammatory roles [1, 5, 6]. Thus, IL-1β production is considered to be a marker of inflammatory activity in human nonclassical monocytes.

Monocyte subsets are typically modulated by immune factors including tumor necrosis factor alpha (TNF-α) and Toll-like receptor (TLR) 2, TLR4, and TLR8 ligands [4, 7]. However, recent data show that dynamic changes in monocyte subpopulations can be influenced not only by immune agents but also by different pathophysiological conditions such as obesity [3, 4]. Indeed, a previous work of Devevre et al. has shown that an increase in body mass index (BMI) is capable of decreasing the CM percentage while also increasing the number of NCM, when comparing normal-weight controls with morbidly obese patients [3]. Interestingly, this study did not only show a direct relationship between body weight gain and increased NCM percentage but also revealed that this immune cell subpopulation seems to be the main monocytic source of IL-1β in obese individuals. Additional studies have consistently reported an increased percentage of NCM in subjects with BMI $> 30\,kg/m^2$ as compared to normal-weight individuals, which has brought to light the association between monocyte subpopulations and obesity [8]. However, it is a well-known fact that obesity is strongly linked to the development of metabolic abnormalities that are encompassed in metabolic syndrome [9]. Metabolic syndrome is a clustering of factors including abdominal obesity, hyperglycemia, high levels of blood pressure and triglycerides, and low concentration of high-density lipoproteins (HDL) that significantly increase the risk of having a cardiovascular event [10–12]. Therefore, it is reasonable to assume that metabolic syndrome may also have major effects upon monocyte subpopulations by mechanisms with the ability to alter the fragile balance among classical, intermediate, and nonclassical monocytes. Thereby, our main goal was to examine the percentages of classical, intermediate, and nonclassical monocytes in subjects with metabolic syndrome while also identifying the risk factors that could directly contribute to alter the monocyte subpopulation balance by performing *in vitro* cultures using primary human monocytes.

2. Materials and Methods

2.1. Subjects. Eighty-six women and men between 20 and 60 years old attending to the Blood Bank of the General Hospital of Mexico "Dr. Eduardo Liceaga" were included in the study. All participants provided written informed consent, previously approved by the institutional ethical committee of the General Hospital of Mexico, which guaranteed that the study was conducted in accordance with the principles described in the Helsinki Declaration. Subjects were excluded if they had previous diagnosis of diabetes mellitus, cardiovascular diseases, acute or chronic hepatic disease, acute or chronic renal disease, inflammatory or autoimmune disorders, acute or chronic infectious diseases, cancer, and endocrine disorders. We also excluded HIV-, HCV-, and HBV-seropositive patients, pregnant or lactating women, and subjects under any kind of anti-inflammatory, antiaggregant, and antihypertensive medication. All of the individuals enrolled in the study received full medical evaluation, including clinical history and physical examination by a physician.

2.2. Anthropometric and Metabolic Measurements. In all participants, BMI, waist circumference, and body fat percentage were recorded. The BMI is a result of dividing weight by height squared (kg/m^2). Waist circumference was obtained from each study subject by measuring at the midpoint between the lower rib margin and the iliac crest using a conventional tape in centimeters (cm). Body fat percentage was individually recorded by means of using a body composition analyzer (TANITA® Body Composition Analyzer, Model TBF-300A, Tokyo, Japan). Systolic blood pressure was individually measured using a digital automatic blood pressure monitor (OMRON Healthcare, Germany). Blood samples were individually obtained from each subject after an 8 h overnight fasting and collected into pyrogen-free tubes (Vacutainer, BD Diagnostics, NJ, USA) at room temperature. Collection tubes were then centrifuged at $1800\,g$ for 10 min, and serum samples were obtained and stored at −80°C in numerous aliquots until use. Serum glucose levels were measured in triplicate by the glucose oxidase assay, following the manufacturer's instructions (Megazyme International, Ireland). Serum insulin levels were measured in triplicate by enzyme-linked immunosorbent assay (ELISA), following the manufacturer's instructions (Abnova Corporation, Taiwan). The estimate of insulin resistance was individually calculated by means of the homeostasis model assessment of insulin resistance (HOMA-IR). Total cholesterol, triglyceride, low-density lipoproteins (LDL), and high-density lipoproteins (HDL) levels were individually measured in triplicate by enzymatic assays according to the manufacturer's instructions (Roche Diagnostics, Mannheim, Germany). IL-1β was measured in serum samples and culture supernatants in triplicate by Sandwich ELISA (PeproTech, Mexico) using serum samples diluted 1 : 250 in PBS 1x (Sigma-Aldrich, Mexico) and culture supernatants

without any dilution. All of the biochemical measurements were performed at the same time in order to avoid procedural variations.

2.3. Diagnosis of Metabolic Syndrome. Diagnosis of metabolic syndrome was performed according to the criteria of the National Cholesterol Education Program's Adult Treatment Panel III report (ATP III) [13], when three of five of the following factors were present: central obesity denoted by a waist circumference greater than 80 cm in women and 90 cm in men (cut-off points recommended for the Hispano-American population [14]), hypertriglyceridemia (circulating triglyceride levels > 150 mg/dL), decreased serum values of HDL-cholesterol (serum HDL < 40 mg/dL in men and 50 mg/dL in women), blood pressure higher than 120/80 mmHg, and hyperglycemia (fasting blood glucose > 100 mg/dL). According to the presence or absence of metabolic syndrome, participants were divided into metabolic syndrome and control groups.

2.4. White Blood Cell Isolation and Characterization of Monocyte Subpopulations by Flow Cytometry. Six milliliters of venous blood was obtained from each participant and collected into tubes containing EDTA (Vacutainer™, BD Diagnostics, NJ, USA). Collection tubes were then centrifuged at 1800g for 10 minutes and white blood cells (WBCs) separated using a micropipette. Total WBCs were separately placed into 1.6 mL pyrogen-free Eppendorf tubes containing 1 mL of ACK Lysing Buffer (Life Technologies, USA) and incubated at 4°C for 8 minutes. Immediately after, each sample was centrifuged at 1800g/4°C for 4 minutes and cell pellets washed twice with PBS 1x (Sigma-Aldrich, Mexico). After an additional centrifugation step and removal of the supernatant, each cell pellet was resuspended in 50 L of PBS 1x (Sigma-Aldrich, Mexico). On each test, 3 μL of Human TruStrain Reagent (BioLegend Inc., USA) was added to 2×10^5 WBCs and then incubated for 10 minutes on ice. Immediately after, WBCs were incubated with anti-CD14 PE/Cy7 and anti-CD16 FITC (BioLegend Inc., USA) for 30 minutes on ice for posterior analysis on a FACSCanto II flow cytometer (BD Biosciences, Mexico) by means of BD FACSDiva™ software 6.0, acquiring 1×10^5 monocyte events per test in duplicate. PE/Cy7 mouse IgG2 and FITC mouse IgG1 were used as isotype control antibodies for cell surface staining of CD14 and CD16, respectively. For gating strategy, white blood cells were firstly gated for singlets on a forward scatter height (FSC-H)/forward scatter area (FSC-A) density plot. Then, lymphocyte, granulocyte, and monocyte populations were gated on a FSC-A/side scatter area (SSC-A) plot. On the monocyte gate, living cells were further gated using the Live/Dead Aqua stain (Thermo Fisher Scientific Inc., USA). Living monocytes were then gated to determine CD14- and CD16-positive expression. Assessment of monocyte subpopulations was performed according to the cell surface expression of CD14 and CD16, as follows: $CD14^{high}CD16^{-}$, classical monocytes; $CD14^{high}CD16^{+}$, intermediate monocytes; and $CD14^{low}CD16^{+}$, nonclassical monocytes.

2.5. HDL Removal for In Vitro Cultures. Blood samples were collected into pyrogen-free tubes (Vacutainer, BD Diagnostics, NJ, USA) from nine volunteers with diagnosis of metabolic syndrome that specifically included central obesity, fasting hyperglycemia, increased triglyceride levels, and low HDL concentration. Collection tubes were then centrifuged at 1800g for 10 min and serum samples obtained. Serum fractions were treated with HDL precipitation buffer (Abcam, Cambridge, MA) for HDL removal, as previously reported [15]. HDL removal was verified by Western blot using an anti-HDL polyclonal antibody that specifically recognizes a 31 kDa protein, according to the manufacturer's instructions (Abcam, Cambridge, MA). Resulting serum samples were supplemented using the same amount of RPMI 1640 medium containing 2 mM L-glutamine and 50 μg/mL gentamicin (Sigma-Aldrich, Mexico), and then 0.77 mmol/L or 1.55 mmol/L purified HDL (Sigma-Aldrich, Mexico) was added. HDL-enriched culture media were differentially used to simulate the low (0.77 mmol/L = 30 mg /dL) and high (1.55 mmol/L = 60 mg/dL) HDL levels found in metabolic syndrome patients and healthy subjects, respectively. A negative control with total absence of HDL was included in all different *in vitro* culture conditions.

2.6. In Vitro Cultures of Primary Human Monocytes and LPS Stimulation. For *in vitro* studies, blood samples were collected into tubes containing EDTA (Vacutainer, BD Diagnostics, NJ, USA) from the same nine donors with diagnosis of metabolic syndrome mentioned above. Blood samples were separately diluted 1 : 2 with phosphate saline buffer 1x (PBS 1x, Sigma-Aldrich, Mexico) for posterior isolation of WBCs by density gradient centrifugation (Sigma-Aldrich, Mexico). Monocytes were then isolated from WBCs by CD14-positive selection using magnetic columns (Miltenyi Biotec, Germany). Purified monocytes were placed in 0.77 mmol/L or 1.55 mmol/L HDL-enriched culture media in 12-well cell-culture plates (CoStar, USA), at a density of 1×10^6 monocytes per well. Each culture well was incubated with or without gram-negative bacteria-derived LPS (Sigma-Aldrich, Mexico) at 1 μg/mL for six hours at 37°C in humidified 5% CO_2 atmosphere. After 6 h incubation, LPS-stimulated and untreated monocytes were obtained and resuspended in 50 μL of PBS 1x for being incubated with anti-CD14 PE/Cy7 and anti-CD16 FITC (BioLegend Inc., USA) antibodies as described above. For gating strategy, untreated and LPS-treated cells were firstly gated for singlets on a FSC-H/FSC-A density plot. On the monocyte gate, living untreated and LPS-treated cells were further gated using the Live/Dead Aqua stain. Living monocytes were then gated to determine CD14- and CD16-positive expression and identify monocyte subpopulations as follows: $CD14^{high}CD16^{-}$, classical monocytes; $CD14^{high}CD16^{+}$, intermediate monocytes; and $CD14^{low}CD16^{+}$, nonclassical monocytes. The cell viability rate was determined based on the Live/Dead Aqua stain and ranged from 91 to 96% for all acquired cell samples, without showing significant differences between untreated and LPS-treated monocytes. FACS analysis was performed in a FACSCanto II flow cytometer (BD Biosciences, Mexico) by means of BD FACSDiva software 6.0, acquiring 1×10^5

Table 1: Demographical and metabolic parameters of the study population.

Parameters	Control	Metabolic syndrome	P value
Gender (W/M)	17/25	16/28	0.313
Age (years)	49.25 ± 5.88	48.38 ± 5.47	0.296
BMI (kg/m^2)	26.12 ± 4.09	29.92 ± 5.26	0.006
Waist circumference (cm)	90.18 ± 9.22	100.41 ± 10.71	0.004
Body fat (%)	27.38 ± 7.63	33.35 ± 10.13	0.012
SBP (mmHg)	124.0 ± 2.47	126.0 ± 5.61	0.306
FBG (mg/dL)	82.37 ± 18.74	106.50 ± 23.48	0.001
Insulin (mU/L)	13.67 ± 5.30	13.90 ± 3.82	0.428
HOMA-IR	2.77 ± 1.21	3.63 ± 1.26	0.005
Total cholesterol (mg/dL)	209.04 ± 41.49	200.26 ± 32.87	0.204
Triglycerides (mg/dL)	165.04 ± 95.12	235.53 ± 95.26	0.006
HDL (mg/dL)	53.20 ± 13.34	38.53 ± 8.62	0.001
LDL (mg/dL)	116.95 ± 33.45	109.50 ± 29.46	0.203

Data are expressed as mean ± standard deviation. The Shapiro-Wilk test was used to estimate normality in data distribution. Significant differences were estimated by means of performing Student's t-test with the exception of women/men proportion in each group, which was estimated by means of the chi-squared test. Differences were considered significant when $P < 0.05$. W: women; M: men; BMI: body mass index; SBP: systolic blood pressure; FBG: fasting blood glucose; HOMA-IR: homeostatic model assessment of insulin resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein. Diagnosis of metabolic syndrome was performed according to the ATP III criteria, when three of five of the following factors were present: central obesity denoted by a waist circumference greater than 80 cm in women and 90 cm in men, circulating triglyceride levels > 150 mg/dL, serum HDL < 40 mg/dL in men and 50 mg/dL in women, blood pressure higher than 120/80 mmHg, fasting blood glucose > 100 mg/dL.

monocyte events per test in duplicate. Assessment of monocyte subpopulations was performed as described above and supernatants collected for posterior IL-1β measuring by ELISA as mentioned before.

2.7. Statistical Analysis. The Shapiro-Wilk test was performed to estimate normality in data distribution and then proceed to perform Student's t-test to compare metabolic syndrome patients and healthy subjects in terms of BMI, waist circumference, body fat percentage, fasting glucose, fasting insulin, HOMA-IR, systolic blood pressure, total cholesterol, triglycerides, LDL, HDL, IL-1β, CM, IM, and NCM. Significant differences in women/men proportion were estimated by means of the chi-squared test. One-way ANOVA, followed by a post hoc Tukey test, was used to compare the serum levels of IL-1β in subjects with different numbers of metabolic syndrome risk factors. Data were expressed as median ± standard deviation. Pearson's correlation coefficients were calculated for examining the association of CM, IM, and NCM with anthropometric, biochemical, and immunological parameters in the study population. Pearson's correlation results were expressed as coefficients (r) and P values. Differences were considered significant when $P < 0.05$. All statistical analyses were performed using the GraphPad Prism 6.01 software.

3. Results

Eighty-six participants were included in the study, and no significant differences were seen between controls and subjects with metabolic syndrome in age, women/men proportion, systolic blood pressure, plasma insulin, total cholesterol, and LDL levels (Table 1). On the contrary, metabolic syndrome subjects had higher BMI than control individuals (29.92 ± 5.26 versus 26.12 ± 4.09, resp.), while waist circumference was also significantly elevated in this study group (100.41 ± 10.71 versus 90.18 ± 9.22, resp.). Furthermore, body fat percentage exhibited a significant 21% increase in metabolic syndrome subjects with respect to control individuals (33.35 ± 10.13 versus 27.38 ± 7.63 percent, resp.) (Table 1). Similarly, fasting blood glucose concentration was 30% increased in patients with metabolic syndrome as compared to controls (106.50 ± 23.48 versus 82.37 ± 18.74, resp.), while HOMA-IR value was raised by 31% (3.63 ± 1.26 versus 2.77 ± 1.21, resp.). In the same sense, triglycerides were 42% augmented in the metabolic syndrome group as compared to controls (235.53 ± 95.26 versus 165.04 ± 95.12, resp.), whereas HDL levels showed a clear 27% reduction in the scenario of metabolic syndrome with respect to normal conditions (38.53 ± 8.62 versus 53.20 ± 13.34, resp.) (Table 1). According to the ATP III criteria, the prevalence of metabolic syndrome in our study population was 58%, which suggests that half of the apparently normal subjects enrolled into the study had higher cardiovascular risk. In the metabolic syndrome group, the most frequently seen risk factor was central obesity (89%, which means that 9 in 10 metabolic syndrome patients were abdominally obese), followed by HDL reduction (78%), hypertriglyceridemia (52%), hyperglycemia (18%), and high blood pressure (13%).

Monocyte subpopulations were analyzed according to CD14 and CD16 cell surface expression by flow cytometry (Figure 1). Representative dot plots showing CD14 and CD16 expression in classical (CM), intermediate (IM), and nonclassical (NCM) monocyte subsets from control individuals and subjects with metabolic syndrome can be seen in Figures 1(a) and 1(b), respectively. The percentage of CM ($CD14^{high}CD16^{-}$) showed a significant 15% decrease in subjects with metabolic syndrome as compared to controls

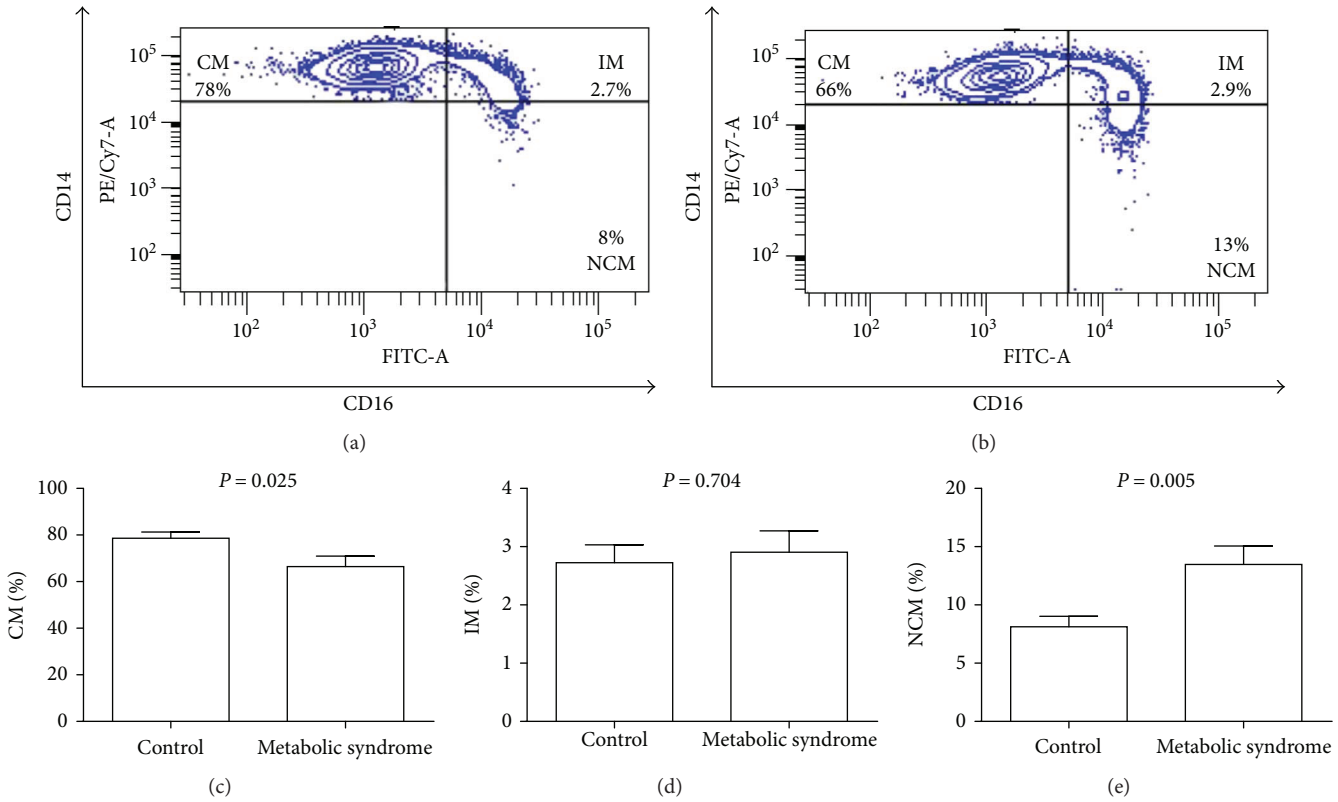

Figure 1: Percentage of classical, intermediate, and nonclassical monocytes in metabolic syndrome patients and control subjects. Representative flow cytometry dot plots showing the percentage of classical (CM), intermediate (IM), and nonclassical monocytes (NCM) in control subjects (a) and patients with metabolic syndrome (b). The CM percentage is significantly decreased in metabolic syndrome patients as compared to controls (c). The IM percentage showed no significant differences between metabolic syndrome patients and controls (d). The NCM percentage is significantly increased in metabolic syndrome patients as compared to controls (e). For gating strategy, white blood cells were firstly gated for singlets on a FSC-H/FSC-A density plot. Then, lymphocyte, granulocyte, and monocyte populations were gated on a FSC-A/SSC-A plot. On the monocyte gate, living cells were further gated using the Live/Dead Aqua stain. Living monocytes were then gated to determine CD14- and CD16-positive expression and identify monocyte subpopulations as follows: $CD14^{high}CD16^{-}$, classical monocytes; $CD14^{high}CD16^{+}$, intermediate monocytes; and $CD14^{low}CD16^{+}$, nonclassical monocytes. In panels (c)–(e), data are expressed as mean ± standard deviation. Significant differences were estimated by means of performing Student's *t*-test. Differences were considered significant when $P < 0.05$. Diagnosis of metabolic syndrome was performed according to the ATP III criteria, when three of five of the following factors were present: central obesity denoted by a waist circumference greater than 80 cm in women and 90 cm in men, hypertriglyceridemia (circulating triglyceride levels > 150 mg/dL), decreased serum values of HDL-cholesterol (serum HDL < 40 mg/dL in men and 50 mg/dL in women), blood pressure higher than 120/80 mmHg, and hyperglycemia (fasting blood glucose > 100 mg/dL).

(66.46 ± 4.5 versus 78.62 ± 2.68, resp.) (Figure 1(c)). The IM subpopulation ($CD14^{high}CD16^{+}$) did not show any significant difference between controls and metabolic syndrome subjects (2.72 ± 0.30 versus 2.90 ± 0.36, resp.) (Figure 1(d)). In contrast, the percentage of NCM expressing CD16 and low CD14 levels exhibited a significant 65% increase in the metabolic syndrome group as compared to controls (13.47 ± 1.59 versus 8.12 ± 0.89, resp.) (Figure 1(e)).

After evaluating the percentage of classical, intermediate, and nonclassical monocyte subsets in subjects with metabolic syndrome and controls, we attempted to identify what anthropometric and metabolic variables had a significant correlation level with these innate immune cells (Table 2). In control individuals without metabolic syndrome, we saw that anthropometric parameters such as BMI, waist circumference, and body fat percentage had the strongest correlation with monocyte subpopulations (Table 2). In contrast, anthropometric parameters were shown to lose statistical correlations with monocyte subpopulations in the scenario of metabolic syndrome. However, we found a remarkable emerging association between HDL and classical and nonclassical monocytes in subjects with metabolic syndrome. In fact, a moderate relationship between increasing percentage of CM and elevated HDL levels was seen in metabolic syndrome patients ($r = 0.531$, $P = 0.013$) (Table 2). On the opposite, the NCM subpopulation showed a strong inverse association with increased concentrations of HDL ($r = -0.621$, $P = 0.009$) (Table 2). Interestingly, the inverse relationship between CM and NCM was significantly stronger when studied in patients with metabolic syndrome than in control individuals ($r = -0.727$, $P < 0.001$, and $r = -0.585$, $P = 0.001$, resp.) (Table 2).

By means of performing statistical analyses, HDL was identified as the molecule exerting the most important

Table 2: Correlation coefficients of monocyte subpopulations with anthropometric, metabolic, and cellular parameters in patients with metabolic syndrome and controls.

Parameters	Control						Metabolic syndrome					
	CM		IM		NCM		CM		IM		NCM	
	r	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Age	0.242	0.126	0.046	0.414	−0.217	0.153	0.078	0.351	0.203	0.159	−0.028	0.446
BMI	*−0.523*	*0.004*	0.140	0.256	*0.592*	*0.001*	0.037	0.427	−0.077	0.354	0.034	0.433
WC	−0.323	0.065	0.220	0.155	*0.353*	*0.049*	0.103	0.307	0.011	0.477	−0.011	0.477
Body fat	*−0.363*	*0.043*	0.236	0.139	*0.438*	*0.018*	0.240	0.118	−0.005	0.488	−0.051	0.402
SBP	0.024	0.548	0.017	0.471	0.057	0.210	0.051	0.412	0.072	0.347	0.054	0.207
FBG	−0.237	0.132	−0.342	0.059	0.326	0.081	0.139	0.249	0.150	0.231	−0.082	0.344
Insulin	−0.012	0.476	−0.006	0.487	−0.052	0.403	−0.066	0.373	0.103	0.306	0.025	0.451
HOMA-IR	−0.042	0.421	−0.161	0.224	0.098	0.323	0.017	0.465	0.172	0.199	−0.005	0.489
Cholesterol	−0.204	0.169	0.171	0.212	−0.093	0.332	0.010	0.478	0.008	0.484	−0.175	0.196
Triglycerides	−0.111	0.306	−0.018	0.466	−0.187	0.196	−0.009	0.481	0.185	0.181	−0.154	0.226
HDL	−0.032	0.440	−0.180	0.198	0.086	0.343	*0.531*	*0.013*	0.209	0.152	*−0.621*	*0.009*
LDL	−0.214	0.156	0.249	0.120	−0.046	0.414	−0.144	0.240	−0.158	0.220	0.026	0.449
CM	—	—	0.325	0.060	*−0.585*	*0.001*	—	—	0.237	0.121	*−0.727*	*<0.001*
IM	0.319	0.064	—	—	0.084	0.695	0.237	0.121	—	—	−0.166	0.207
NCM	*−0.585*	*0.001*	0.084	0.347	—	—	*−0.727*	*<0.001*	−0.166	0.207	—	—

Coefficients (r) and P values were calculated by Pearson's correlation model. The correlation level was considered significant when $P < 0.05$. Significant associations are marked in italics. CM: classical monocytes; IM: intermediate monocytes; NCM: nonclassical monocytes; BMI: body mass index; WC: waist circumference; SBP: systolic blood pressure; FBG: fasting blood glucose; HOMA-IR: homeostatic model assessment of insulin resistance; HDL: high-density lipoprotein; LDL: low-density lipoprotein. Diagnosis of metabolic syndrome was performed according to the ATP III criteria, when three of five of the following factors were present: central obesity denoted by a waist circumference greater than 80 cm in women and 90 cm in men, hypertriglyceridemia (circulating triglyceride levels > 150 mg/dL), decreased serum values of HDL-cholesterol (serum HDL < 40 mg/dL in men and 50 mg/dL in women), blood pressure higher than 120/80 mmHg, and hyperglycemia (fasting blood glucose > 100 mg/dL).

association with classical and nonclassical monocytes in metabolic syndrome. For this reason, our next step was to study the effect of HDL on the monocyte subpopulation dynamic by conducting *in vitro* cell culture experiments (Figure 2). Figure 2(a) illustrates a representative polyacrylamide gel showing a metabolic syndrome patient's serum sample in which HDL was totally removed (left) and then reconstituted with 0.77 mmol/L (middle) or 1.55 mmol/L HDL (right) (Figure 2(a)). In culture conditions using low HDL levels (30 mg/dL), LPS stimulation induced a 17% reduction in the CM percentage as compared to untreated cells (Figure 2(b), middle panel). In contrast, the effect of LPS on this monocyte subset was abolished when using HDL concentrations that resembled those found in healthy subjects without metabolic syndrome (60 mg/dL) (Figure 2(b), right panel). The IM percentage tended to decrease when treated with LPS either in low HDL level or in normal HDL concentration without showing significant differences (Figure 2(c)). On the contrary, in culture conditions mimicking the HDL reduction that is observed in metabolic syndrome patients, LPS stimulation promoted a 40% increase in the NCM percentage with respect to untreated cells (Figure 2(d), middle panel). Interestingly, the LPS-stimulated NCM increase was revoked when monocytes were cultured in the presence of high HDL levels mimicking those found in healthy individuals without metabolic syndrome (Figure 2(d), right panel). Interestingly, classical and nonclassical monocytes cultured in the absence of HDL showed a similar response pattern than that found in 30 mg/dL HDL (left panels at Figures 2(b) and 2(d), resp.). As previously mentioned, IL-1β is a key cytokine that is mainly produced by NCM in response to LPS. In parallel to the NCM increase, LPS stimulation also enhanced IL-1β secretion by 216% in monocytes cultured in zero and 30 mg/dL HDL (Figure 2(e), left and middle panels, resp.). However, the effect of LPS on IL-1β production was 1.5-fold decreased when tested in 60 mg/dL HDL (Figure 2(e), right panel).

Since not only monocyte subpopulations were modified by HDL but also their ability to produce IL-1β, we decided to measure IL-1β serum levels in the metabolic syndrome population (Figure 3). Abdominally obese patients with metabolic syndrome showed similar IL-1β serum levels than those found in metabolic syndrome patients without central obesity (Figure 3(a)). No significant differences in the IL-1β serum levels were also seen in metabolic syndrome patients with and without high blood pressure or hyperglycemia (Figures 3(b) and 3(c), resp.). Metabolic syndrome patients with hypertriglyceridemia exhibited a nonsignificant increase in serum IL-1β as compared to metabolic syndrome patients without abnormally high triglyceride values (Figure 3(d)). On the opposite, the serum levels of IL-1β showed a significant 1.5-fold increase when studied in metabolic syndrome patients with low HDL levels as compared to metabolic syndrome patients without showing HDL reduction (Figure 3(e)).

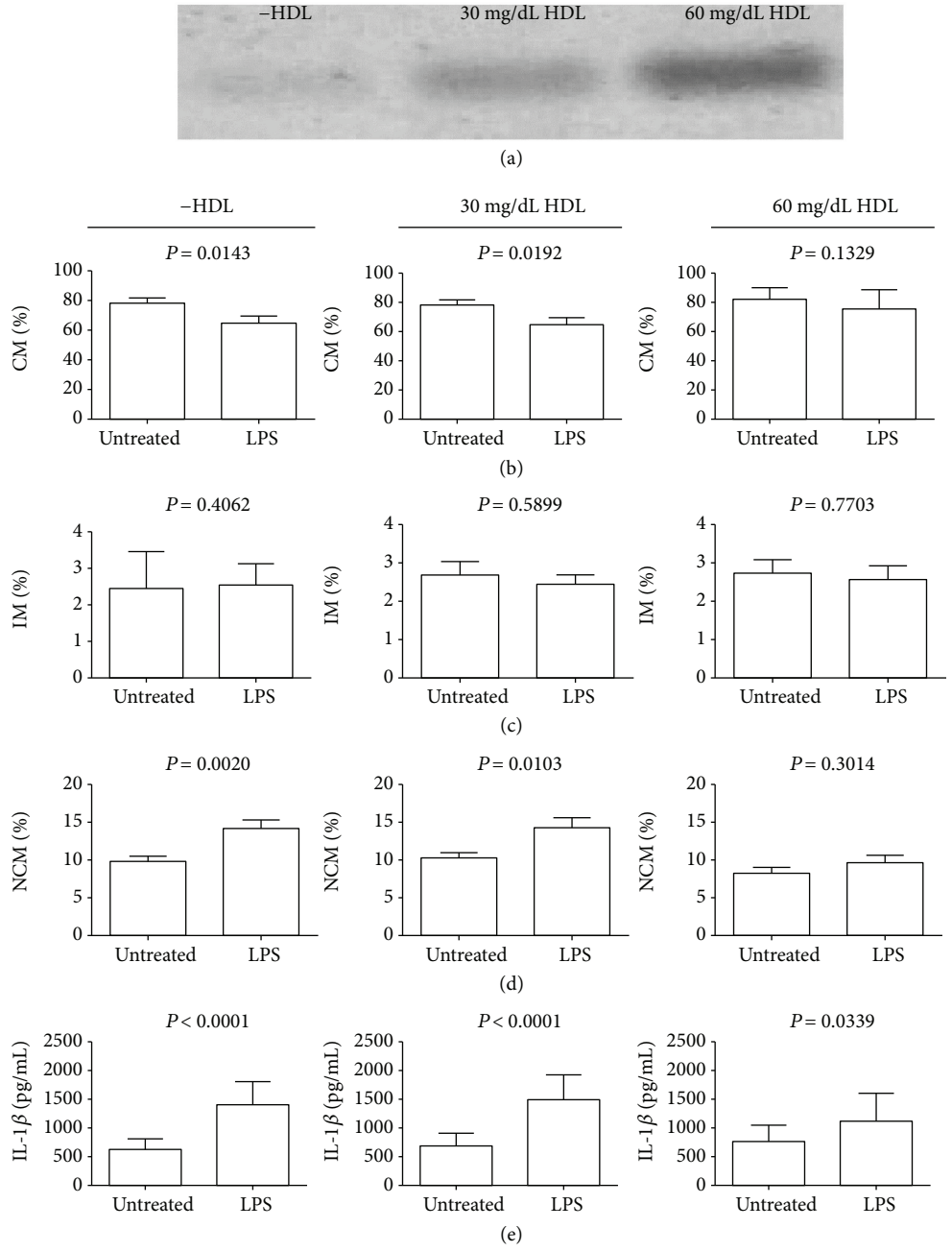

Figure 2: Effect of HDL on LPS-stimulated primary human monocytes *in vitro*. Representative polyacrylamide gel showing a metabolic syndrome patient's serum sample in which HDL was totally removed (−HDL) and then reconstituted with 0.77 mmol/L (30 mg/dL) or 1.55 mmol/L (60 mg/dL) HDL (a). As compared to untreated cells, LPS stimulation induced reduction in the CM percentage in low HDL levels (zero and 30 mg/dL) (b, left and middle panels, resp.). In contrast, the effect of LPS on the CM percentage was abolished in 60 mg/dL HDL that resembled a high HDL concentration (b, right panel). LPS stimulation did not significantly modify the IM percentage neither in low nor in high HDL concentrations (c, left, middle and right panels, resp.). As compared to untreated cells, LPS stimulation increased the NCM percentage in zero and 30 mg/dL HDL (d, left and middle panels, resp.). On the contrary, the effect of LPS on the NCM percentage was abolished in high HDL concentrations (d, right panel). As compared to untreated cells, LPS stimulation increased IL-1β production in primary human monocytes cultured in low HDL concentrations (e, left and middle panels, resp.). In contrast, the effect of LPS on IL-1β production was 1.5-fold reduced in 60 mg/dL HDL (e, right panel). Monocytes were isolated from white blood cells by CD14-positive selection using magnetic columns and placed in 0.77 mmol/L (30 mg/dL) or 1.55 mmol/L (60 mg/dL) HDL-enriched culture media (1×10^6 monocytes per well), in the presence or absence of gram-negative bacteria-derived LPS at 1 μg/mL for six hours at 37°C. After this time, monocytes were incubated with anti-CD14 PE/Cy7 and anti-CD16 FITC as described. For the gating strategy, untreated and LPS-treated cells were firstly gated for singlets on a FSC-H/FSC-A density plot. On the monocyte gate, living untreated and LPS-treated cells were further gated using the Live/Dead Aqua stain. Living monocytes were then gated to determine CD14- and CD16-positive expression and identify monocyte subpopulations as follows: $CD14^{high}CD16^{-}$, classical monocytes; $CD14^{high}CD16^{+}$, intermediate monocytes; and $CD14^{low}CD16^{+}$, nonclassical monocytes. In (b–e), data are expressed as mean ± standard deviation. Significant differences were considered when $P < 0.05$.

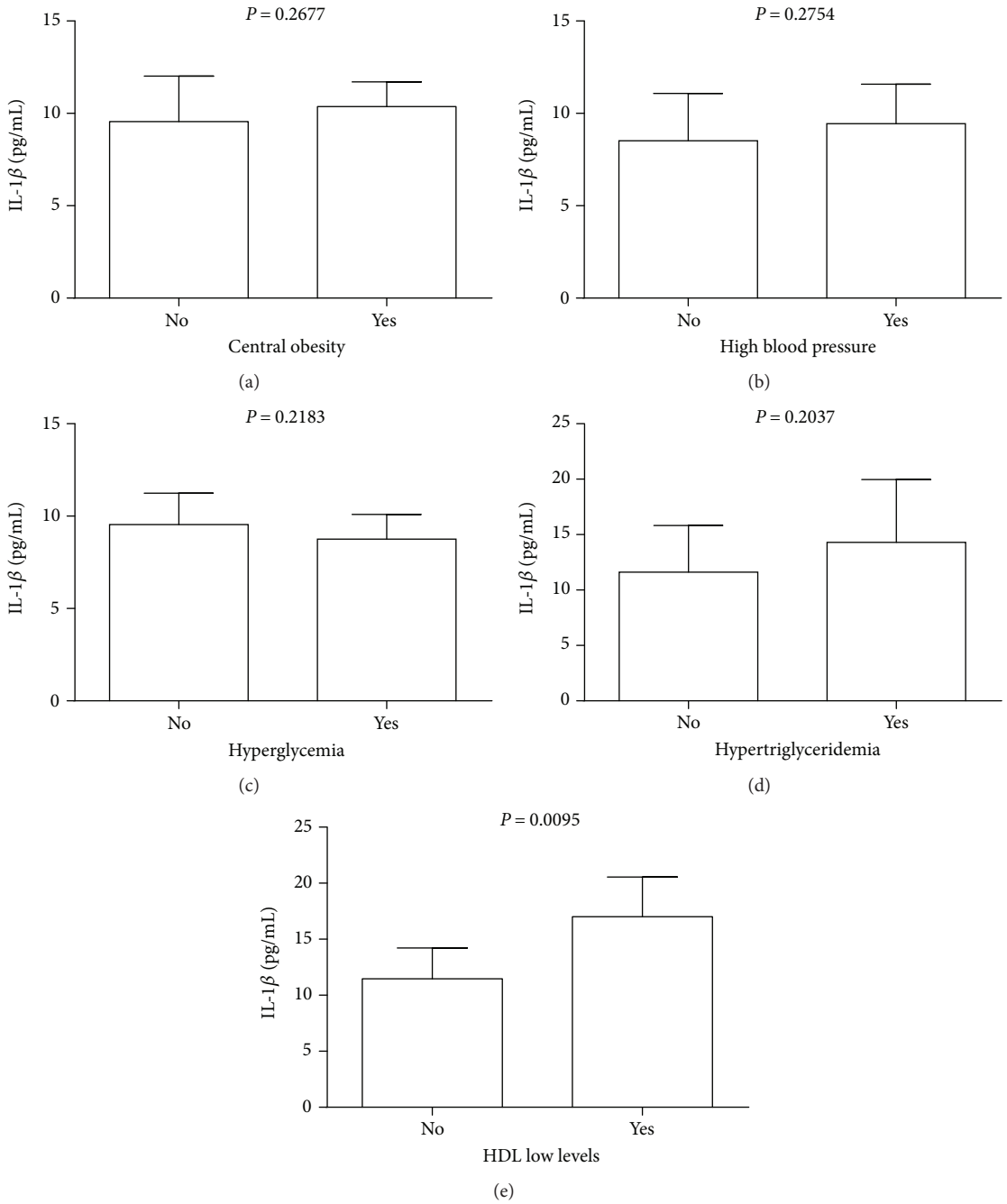

Figure 3: Serum levels of IL-1β in the study patients according to different metabolic syndrome risk factors. (a) Metabolic syndrome patients displaying central obesity ($n = 39$) showed similar IL-1β serum levels than did metabolic syndrome patients that had a normal waist circumference ($n = 5$). (b) The serum levels of IL-1β did not show significant differences in metabolic syndrome patients with elevated blood pressure ($n = 6$) as compared to metabolic syndrome patients with normal blood pressure ($n = 38$). (c) Metabolic syndrome patients showing fasting hyperglycemia ($n = 8$) exhibited similar IL-1β circulating levels than did metabolic syndrome patients with normal glycemic values ($n = 36$). (d) The serum levels of IL-1β tended to increase in metabolic syndrome patients with hypertriglyceridemia ($n = 23$) but did not show significant differences with respect to metabolic syndrome patients showing normal triglyceride values ($n = 21$). (e) In contrast, IL-1β was significantly increased in metabolic syndrome patients with HDL low levels ($n = 34$) as compared to metabolic syndrome patients exhibiting normal HDL values ($n = 10$). Data are expressed as mean ± standard deviation. Significant differences were estimated by means of performing the Mann–Whitney test. Differences were considered significant when $P < 0.05$. Central obesity was diagnosed when women and men had a waist circumference greater than 80 cm and 90 cm, respectively. High blood pressure was diagnosed in women and men with blood pressure values higher than 120/80 mmHg. Hyperglycemia was diagnosed in women and men with fasting blood glucose greater than 100 mg/dL. Hypertriglyceridemia was diagnosed in women and men with triglyceride values higher than 150 mg/dL. Decreased serum values of HDL were established in women and men with HDL serum values lower than 50 mg/dL and 40 mg/dL, respectively.

4. Discussion

Previous studies have demonstrated a monocyte subset imbalance in morbidly obese patients that is mainly characterized by increased percentages of nonclassical monocytes [3]. Nevertheless, obesity is frequently accompanied by cardiovascular risk factors that are encompassed in the metabolic syndrome [10–12]. For this reason, we attempted to examine the monocyte subpopulation percentages when considering the presence of metabolic syndrome.

As expected, our data show that BMI, waist circumference, and body fat percentage are directly related to increased NCM percentage and decreased CM amount, which concurs with previous works reporting that obesity is associated with imbalance in monocyte subsets [3]. However, this association was only seen in overweight and obese subjects that did not meet the rest of criteria for being diagnosed with metabolic syndrome. Beyond obesity, in the scenario of metabolic syndrome, we found decreasing percentages of classical monocytes and prominent elevations in the nonclassical monocyte subpopulation that were related to HDL levels and showed no association with obesity-related anthropometric parameters. In this context, it is reasonable to consider the fact that the monocyte subpopulation dynamic is not only modified by obesity but is also due to other factors such as those encompassed in the metabolic syndrome, especially the serum levels of HDL.

HDL is a protein of blood plasma that is capable of binding to lipid molecules such as triglycerides and cholesterol and participates in the cholesterol clearance—the main reason why HDL is also called the "good cholesterol" [16, 17]. High serum levels of HDL have been related to low cardiovascular risk, whereas a reduction in this lipoprotein accompanied by increased levels of LDL is considered to increase the risk of having metabolic syndrome, heart disease, or stroke [16, 18]. In our study, HDL showed a positive correlation with classical monocytes but a negative relationship with nonclassical monocytes. In other words, the present results suggest that elevated HDL levels may restrict the proportion of the inflammatory nonclassical monocytes but also favor expanding the classical monocyte subset that has no prominent inflammatory actions. However, the idea that HDL seems to be associated with imbalance between classical and nonclassical monocyte subsets was conceived after performing a merely statistical approach. In view of the fact that statistical correlation models do not allow us to find a causative role for HDL in modulating monocyte subsets, we decided to culture blood-isolated monocytes in the presence of high and low HDL levels *in vitro*. To simulate the microenvironment found in metabolic syndrome, we used serum samples from subjects having HDL reduction, central obesity, hyperglycemia, and hypertriglyceridemia, in which we specifically removed HDL. Then, we deliberately supplemented each serum sample with 0.77 mmol/L (30 mg/dL) or 1.55 mmol/L (60 mg/dL) purified HDL for having monocyte culture conditions with low and high HDL levels, respectively. In this form, we were able to corroborate our *in vivo* findings by studying the effect of HDL on LPS-stimulated monocytes *in vitro*. As expected, in culture conditions simulating the microenvironment found in metabolic syndrome with low HDL levels, LPS stimulation was able to induce decreased CM percentage and at the same time increased the NCM subset, which concurs with Mukherjee et al. who reported the same result [5]. In contrast, when the HDL concentration was deliberately increased until the levels found in healthy subjects without metabolic syndrome were reached, the effect of LPS on classical and nonclassical monocytes was abolished. As mentioned, numerous studies have suggested that HDL is positively associated with classical monocytes but at the same time negatively related to nonclassical monocytes. In this sense, previous studies have demonstrated that nonclassical monocyte percentages increase as HDL plasma levels decrease in adults and children with familial hypercholesterolemia [19, 20]. Concomitantly, a positive correlation between HDL plasma levels and classical monocytes was also reported by Christensen et al. [20]. Additional studies have also confirmed that HDL levels negatively correlate with increasing percentage of NCM [21–23], which concurs with present results. Interestingly, our findings also demonstrate that absence of HDL has similar *in vitro* effects on monocyte subpopulations than those found when using 30 mg/dL HDL, which suggest that HDL may prevent raising nonclassical monocytes in concentrations above 60 mg/dL. To the best of our knowledge, this is the first report to not only suggest an association between HDL levels and monocyte subpopulations but also demonstrate that low HDL levels directly contribute to a decrease in CM and an increase in NCM in response to prototypical stimuli such as LPS. This finding brings to light the importance of studying the probable role of HDL in the regulation of the CD14 and CD16 expression in human monocytes, which is the primary feature defining classical, intermediate, and nonclassical monocyte subpopulations.

CD14 is a surface protein that preferably expresses on human monocytes and macrophages [24]. CD14 forms the trimeric LPS receptor complex together with TLR4 and MD-2, aimed at recognizing bacterial endotoxins and consequently triggering the NFκB-dependent inflammatory cytokine expression such as TNF-α, IL-6, and IL-1β [24–26]. Notably, it has been reported that infusion with reconstituted HDL is able to reduce CD14 expression in peripheral monocytes of healthy volunteers while also decreasing the serum concentrations of TNF-α and IL-6 [27]. It is worth mentioning that monocytes isolated from reconstituted HDL-treated healthy volunteers still showed decreased expression of CD14 even upon *in vitro* stimulation with LPS [27]. Similarly, Galbois et al. demonstrated that CD14 expression is diminished in circulating monocytes of cirrhotic patients and healthy controls after having been cultured in the presence of HDL [28]. In the specific case of CD16 (an Fcγ receptor that binds to Fc fragments of immunoglobulin G in the cell surface of innate immune cells [1]), HDL levels have an apparent association with CD16 expression in monocytes of patients with stable coronary artery disease [21–23]. However, to the best of our knowledge, there are no experimental works studying the possible mechanisms by which HDL is linked to CD16 expression in human monocytes. Altogether, this information suggests that HDL may exert its effects on monocyte

subpopulations by modulating the expression of CD14, and probably CD16, via TLR4 and NFκB activation. Nevertheless, it is important to note there are not yet concluding studies aimed at characterizing the possible mechanism by which HDL is able to impact monocyte subpopulations, and the discussion of these results makes no attempt to conjecture beyond that. On the other hand, the idea that HDL exerts its effects by activating prominent inflammatory pathways could not only involve the dynamic changes found in monocyte subsets but also the ability of monocytes to produce key proinflammatory cytokines such as IL-1β.

IL-1β is a cytokine predominantly produced by nonclassical monocytes and activated macrophages with prominent roles in the regulation of the inflammatory response as well as cell differentiation and apoptosis [3, 5]. Our results show that LPS-stimulated IL-1β production in cultured primary human monocytes is favored when HDL is totally absent or in low levels. In other words, culture conditions mimicking the levels of HDL that are found in metabolic syndrome do not only increase the NCM percentage but also enhance production of IL-1β. Concurring with this notion, restoration of the HDL concentration until reaching the levels found in healthy subjects without metabolic syndrome was able to diminish the LPS-stimulated IL-1β production in cultured primary human monocytes *in vitro*. These findings are consistent with prior studies suggesting that high HDL values, as those seen in metabollicaly healthy individuals, seem to exert anti-inflammatory actions, whereas low HDL levels may predispose the organism to more robust proinflammatory responses [17–19, 27]. However, it is important to proceed with caution in trying to transfer our *in vitro* results that support an effect of HDL on monocyte subpopulations toward a much more complicated clinical scenario such as metabolic syndrome, in which monocytes subsets are not only in contact with HDL but also in the presence of many other risk factors. For this reason, it is still of enormous importance to clarify whether high HDL levels have prominent anti-inflammatory actions in transversal and prospective clinical trials aimed at understanding the role of HDL in metabolic syndrome and its comorbidities.

IL-1β has been shown to be primarily produced by nonclassical monocytes either under basal conditions or in response to LPS [3, 5]. On the contrary, classical monocytes are mainly involved in cell migration functions by predominantly expressing chemokine receptors without showing significant IL-1β production [5, 29–31]. Therefore, we want to speculate that nonclassical monocytes were the main cellular source of IL-1β in our *in vitro* experiments, a notion that concurs with the fact that while this monocyte subpopulation increased the IL-1β production also augmented and both of them were downregulated by HDL. These *in vitro* findings agree with our *in vivo* results showing that serum values of IL-1β significantly increased in metabolic syndrome patients with low HDL levels as compared to metabolic syndrome patients without HDL reduction. Thus, present data suggest a direct relationship among increased percentage of nonclassical monocytes, elevated concentrations of IL-1β, and low HDL levels in patients with metabolic syndrome. In this sense, a previous work reported an association between IL-β gene polymorphisms and metabolic syndrome in patients with coronary heart disease [32]. Furthermore, Al-Shorman et al. recently showed that IL-1β serum levels are significantly elevated in obese adolescents with metabolic syndrome as compared to normal-weight controls without metabolic alterations [33]. Altogether, this information concurs with our data that demonstrate elevation in the serum levels of IL-1β in patients with metabolic syndrome. However, to the best of our knowledge this is the first report suggesting that nonclassical monocytes could be a main cellular source of IL-1β in subjects with metabolic syndrome that show low HDL levels. The idea that both nonclassical monocytes and IL-1β may exert their prominent inflammatory actions in metabolic syndrome patients is congruent with numerous works showing that systemic inflammation is linked to the pathogenesis of metabolic dysfunction [7, 18, 34, 35]. However, IL-1β was only measured in serum samples and direct evidence regarding the role of nonclassical monocytes in IL-1β production in metabolic syndrome patients remains to be further elucidated; therefore, discussion of these results makes no attempt to conjecture beyond that.

A final phenomenon captured in our study is that both classical and nonclassical monocytes show a strong interdependent relationship, most of all in the setting of metabolic syndrome. It has been previously demonstrated that M1 macrophages can be shifted toward M2 macrophages, and vice versa, in response to the cell microenvironment [36–38]. However, there is scant information supporting a possible shift between monocytes with no prominent inflammatory actions and monocytes primed to inflammatory activities. In this sense, our results suggest that classical monocytes may be converted into nonclassical monocytes in response to metabolic syndrome risk factors such as HDL, thus supporting the notion that monocytes and macrophages may be primed toward proinflammatory activation profiles in the early stages of metabolic dysfunction. The possibility that monocyte subsets can be influenced by metabolic syndrome risk factors should be taken into consideration when designing molecular therapeutic interventions aimed at switching nonclassical monocytes into classical monocytes in patients at higher cardiovascular risk such as individuals with metabolic syndrome.

5. Conclusions

Our data demonstrate for the first time that HDL reduction directly contributes to an increase in the nonclassical monocyte subpopulation and concomitantly a decrease in the classical monocyte percentage in patients with metabolic syndrome and in LPS-stimulated primary human monocytes *in vitro*. In this work, HDL reduction was also shown to induce higher IL-1β production in LPS-stimulated primary human monocytes and associate with increased IL-1β serum levels in patients with metabolic syndrome. Altogether, these findings support the notion that metabolic dysfunction has a pivotal component in the systemic inflammatory response that is mediated by dynamic changes in monocyte subpopulations. The exact molecular mechanisms by which HDL is able to modulate monocyte subpopulations and IL-

1β production remain to be elucidated. The potential impact of understanding the role of metabolic signals in immune cell activation adds a compelling degree of urgency to further studies.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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Review Article

Regulation of Immune Cell Functions by Metabolic Reprogramming

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Recent findings show that the metabolic status of immune cells can determine immune responses. Metabolic reprogramming between aerobic glycolysis and oxidative phosphorylation, previously speculated as exclusively observable in cancer cells, exists in various types of immune and stromal cells in many different pathological conditions other than cancer. The microenvironments of cancer, obese adipose, and wound-repairing tissues share common features of inflammatory reactions. In addition, the metabolic changes in macrophages and T cells are now regarded as crucial for the functional plasticity of the immune cells and responsible for the progression and regression of many pathological processes, notably cancer. It is possible that metabolic changes in the microenvironment induced by other cellular components are responsible for the functional plasticity of immune cells. This review explores the molecular mechanisms responsible for metabolic reprogramming in macrophages and T cells and also provides a summary of recent updates with regard to the functional modulation of the immune cells by metabolic changes in the microenvironment, notably the tumor microenvironment.

1. Introduction

Pleiotropic interactions between various cells are responsible for the maintenance and disturbance of homeostasis in the tissue microenvironment of physiological and pathological conditions. For example, from early carcinogenesis to progression and metastasis, cancer cells interact with various types of stromal cells, for example, cancer-associated fibroblasts, endothelial cells, and immune cells in the tumor microenvironment (TME). The TME is flooded with cytokines and growth factors responsible for "smoldering persistent inflammation." This reactive stroma is a well-characterized component of the TME that shows similarities to the repair response in injured tissue [1]. Recent findings revealed that various immune cell subsets are dominant regulators of the delicate balance between homeostasis and disturbance in the tissue microenvironment [2–5]. For example, macrophages can form a major component of immune cell infiltrate in the TME, constituting as much as half of a tumor mass [6, 7]. Immune responses of M1 and M2 macrophages describe the opposing activities of killing or repairing. The typical M1 macrophages drive inflammation and show high antigen presentation, high production of inflammatory cytokines such as IL-12 and IL-23, and high production of nitric oxide (NO) and reactive oxygen intermediates. In contrast, M2-type responses are the "resting" phenotype and are observed in the resolution of inflammation without infections, tissue remodeling, and repair. It has been widely accepted that IFNγ alone or with microbial LPS or cytokines such as GM-CSF and TNF induces classically activated M1 macrophages, and IL-4, IL-6, IL-10, IL-13, IL-21, IL-33, immune complexes, and Notch can induce the M2 form of macrophage activation [8, 9]. Notably, truly polarized macrophages are rare [10–13] and tumor-associated macrophages (TAMs) can be also described as M(IL-4), M(Ig), M(IL-10), M(GC: glucocorticoid), M(IFNγ), M(LPS), and

so forth, according to a recently attempted nomenclature based on specific activation standard [12]. Evidence supports a tumor-promoting role of TAMs, and high frequencies of TAMs are generally associated with poor prognosis in most human cancers [2, 14, 15]. TAMs infiltrating established tumors generally show the properties of an M2-like activated anti-inflammatory, protumoral properties rather than M1-like activated proinflammatory, antitumoral phagocytic properties [16–18].

Macrophages can also form a major component of immune cell infiltrate in obese adipose tissue (AT), constituting as much as 40% of all AT cells [19]. In the progression of obesity, a switch from M2-like to M1-like activation of the macrophage population occurs and inflammatory cytokines such as tumor necrosis factor (TNF) contribute to insulin resistance in adipocytes characterized by an impaired insulin response such as hypertriglyceridemia and elevated fasting glucose [20, 21]. In addition, lymphoid as well as myeloid cells infiltrates and expands in the liver tissue and the obese AT and these immune cell subsets are responsible for the development of obesity-related metabolic dysregulation due to excessive nutrient intake and exacerbation of low-grade inflammatory changes in the microenvironment. $CD8^+$ T cells also promote inflammation and metabolic disturbance in the AT [22]. In addition to macrophages and T cells, neutrophils and mast cells can also disturb the homeostasis in the tissue microenvironment.

Many recent findings in the field of immunometabolism now show that metabolic status in immune cells can determine various types of immune responses. Immune cells have remarkably diverse functions and cellular activities that are associated with distinct metabolic demands. The traditional simple concept of production of cellular ATP is that glycolysis generates two molecules of ATPs from one molecule of glucose. Glycolysis metabolizes glucose to pyruvate first, and the pyruvate is further metabolized to carbon dioxide, NADH, and $FADH_2$ in the mitochondria. The reducing equivalents (NADH and $FADH_2$) drive oxidative phosphorylation (OXPHOS) for more ATP synthesis. In the 1920s, it was demonstrated that cancer tissues can metabolize, even in aerobic conditions, about tenfolds more glucose to produce lactate than normal tissues can and this is known as aerobic glycolysis or the Warburg effect [23]. Since pyruvate is metabolized to lactate and secrete, lactate appears to be wasted in aerobic glycolysis. However, lactate secretion out of cells allows increased continuous glucose influx from the generation of NAD^+ and resultant accumulation of glycolytic intermediates facilitates biomass synthesis for rapidly proliferating cells. Since the observation and dramatic revitalization of the Warburg effect, the dominant glycolysis and relatively reduced OXPHOS were thought to be confined to cancer cells. However, recent findings clearly show that the Warburg effect-like metabolic reprogramming also exists in rapidly proliferating cells including various types of immune cells, most notably in macrophages and T cells, and determines the function of the immune cell subsets in disease conditions such as those in inflamed tissue or cancer [24–27].

2. Metabolic Regulation of Macrophage Phenotypes

The function of macrophages is not limited to the maintenance of homeostasis in the tissue microenvironment but also includes many activities such as cytokine production and phagocytosis upon their activation. Importantly, macrophages are famous for their plasticity and adoption of various activation states in response to their functional requirements signaled from their microenvironment. For example, an innate arm of the immune system can have an important capacity to adapt after challenged with pathogens [28]. This is known as innate immune memory or trained immunity. Trained immunity from epigenetic reprogramming of macrophages shows high glucose consumption and a high ratio of NAD^+ to its reduced form NADH, reflecting a shift in metabolism with an increase in glycolysis and M1-like activation of macrophages, dependent on the activation of mTOR through the Akt-HIF-1α pathway [29]. M2-like activated macrophages exploit fatty acid oxidation (FAO) to fuel OXPHOS rather than aerobic glycolysis for ATP production [30–32].

Of note, HIF1α and NFκB drive the M1 phenotypes [33, 34] and PGC1β, and peroxisome proliferator-activated receptors and STAT6 drive the M2 phenotypes (Figure 1) [35–38]. Phosphorylation and activation of a nutritional sensor, AMPK, regulate mitochondrial biogenesis via deacetylation of regulating proteins, including SIRT1 with NAD^+, and suppress HIF1α and NFκB [38–40]. AMPK and NAD^+-SIRT1-PGC1β signaling are key factors for nutritional state-dependent M1/M2-like activation of macrophages in inflammatory conditions [39, 41]. HIF-1α also enhances the lactate dehydrogenase- (LDH-) mediated conversion of pyruvate-to-lactate [42] and increases expression of GLUT1, GLUT3, and MCT4 to increase glucose uptake and expression of pyruvate kinase M2 (PKM2), resulting in an increase in the secretion of lactate and uncoupled glycolysis and oxidative phosphorylation [43, 44] (Figure 2). Pyruvate dehydrogenase (PDH) inactivation from phosphorylation by pyruvate dehydrogenase kinases (PDKs) prevents pyruvate from entering the mitochondrial Krebs cycle [45]. HIF-1α transcriptionally activates the PDKs [46, 47].

LPS-activated dendritic cells and M1-like activated macrophages show enhanced aerobic glycolysis, flux through the pentose phosphate pathway, and fatty acid synthesis but have incomplete OXPHOS at the level of succinate dehydrogenase (SDH) and isocitrate dehydrogenase, blocking the synthesis of mitochondrial ATP. In these cells, glucose is used for the biosynthesis of large quantities of cytokines and effector molecules, and inactivation of OXPHOS directs metabolites from the Krebs cycle for inflammatory reaction [32, 48]. Accumulation of succinate and citrate from the truncated OXPHOS leads to stabilization of HIF1α by limiting prolyl hydroxylase activity to maintain a proinflammatory, antitumoral response [49–51].

Recently, itaconic acid-mediated inhibition of SDH has also been found as a driver for succinate accumulation in LPS-stimulated M1-like activated proinflammatory

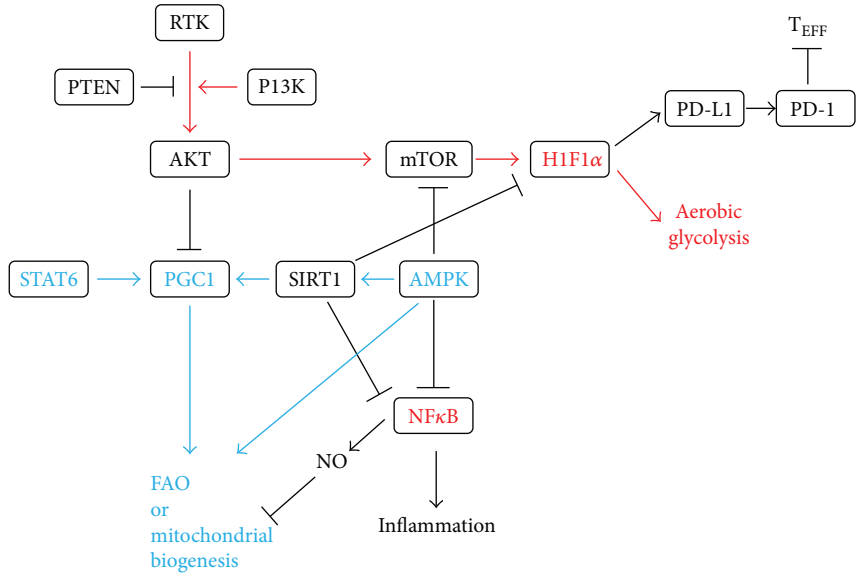

Figure 1: Regulation of metabolic rewiring in macrophages. PGC1 is important for FAO and mitochondrial biogenesis (shown in blue) and HIF1α for aerobic glycolysis (shown in red). Active SIRT1 can inhibit inflammation and glycolytic metabolism and promote mitochondrial biogenesis and FAO. Interaction of PD-L1 and PD-1 induces FAO and suppresses aerobic glycolysis and immune functions in Teff. PD-1: programmed death-1; PD-L1: programmed death ligand-1; RTK: receptor tyrosine kinase; T_{EFF}: effector T cell.

macrophages [52]. Immunoresponsive gene 1 (*Irg1*) is highly expressed in mammalian macrophages during inflammation and *Irg1* gene silencing in macrophages results in significantly decreased intracellular itaconic acid levels as well as significantly reduced antimicrobial activity during bacterial infections [53].

High intracellular iron levels in M1-like activated macrophages stabilize HIF1α through low levels of ferroportin and high levels of H-ferritin, involved in iron export and storage, respectively [54, 55]. Hemeoxygenase-1 (HO-1) catabolizes heme to ferrous ion, biliverdin, and carbon monooxide, and suppression of HO-1 results in M2-like activation of TAMs [56].

HIF1α can also be stabilized from nitrosylation with peroxynitrites from increased iNOS [57], favoring aerobic glycolysis in M1 phenotypes. NFκB transcriptionally activates proinflammatory genes including iNOS, which forms NO in the presence of arginine. Peroxynitrite, formed from NO and superoxide anions in the mitochondria, nitrosylates iron-sulfur proteins in the mitochondrial electron transport chain, and the resultant nitrosylation can inhibit OXPHOS [58], also favoring aerobic glycolysis in M1 phenotypes. Unlike iNOS-mediated catabolism of arginine to NO in M1-like activated macrophages, M2-like activated macrophages catalyze arginine to urea and ornithine by arginase 1 (ARG1); ARG1 is a representative marker for M2-like activation. As NO production is limited in M2-like activated macrophages, the nitrosylation-mediated inhibition of OXPHOS is dampened, now favoring M2 phenotypes [48]. Although HIF1α drives the M1 phenotypes in hypoxic conditions, lactate produced by cancer cells, as a by-product of aerobic glycolysis, has an unexpected critical function in HIF1α-dependent expression of ARG1 and resultant M2-like activation of TAMs in normoxic conditions [59] (Figure 3). These findings clearly indicate highly interconnected signaling for the conservation of HIF-1-centered metabolic phenotypes.

As stated, M2-like activated macrophages show lowered glycolysis and enhanced FAO to fuel OXPHOS. Th2 cytokine and IL-4-induced PGC1β increase mitochondrial biogenesis and FAO in a STAT6-dependent manner [38, 41, 60]. PGC1β plays a key role in increasing mitochondrial biogenesis and OXPHOS by upregulating the expression of FAO-involved genes [41]. IL-4-/IL-13-stimulated macrophages express PFKFB1, which produces a low level of a glycolytic activator, fructose 2,6 bisphosphate [61, 62]. In IL-4-stimulated macrophages, fatty acid sources such as LDL and VLDL are taken up via the scavenger receptor CD36 and metabolized in the lysosome. The CD36-mediated lysosomal lipolysis is essential for the M2-like activation [31].

An orphan nuclear receptor, estrogen-related receptor α (ESRRα), is required for the increased mitochondrial biogenesis [63]. Importantly, ESRRα-deficient macrophages show a decrease in phagosomal maturation and antimicrobial activity [64]. Another study reported an M1-like phenotype of increased glycolysis but impaired mitochondrial respiratory function and biosynthesis as a result of ESRRα deficiency [65]. Interestingly, VLDLR expression is a determinant factor in inflammation and in M1-like activation of macrophages in AT [66].

In spite of our knowledge gained from macrophages in inflammatory disease conditions, our understanding of the metabolic regulations in TAMs is surprisingly limited and the signals involved in communication between tumors and macrophages are still poorly defined [67]. However, emerging evidence strongly indicates that the metabolic reprogramming of macrophages is closely related to the protumoral or antitumoral function of macrophages [68, 69] and that unraveling the TAM phenotype might lead to the

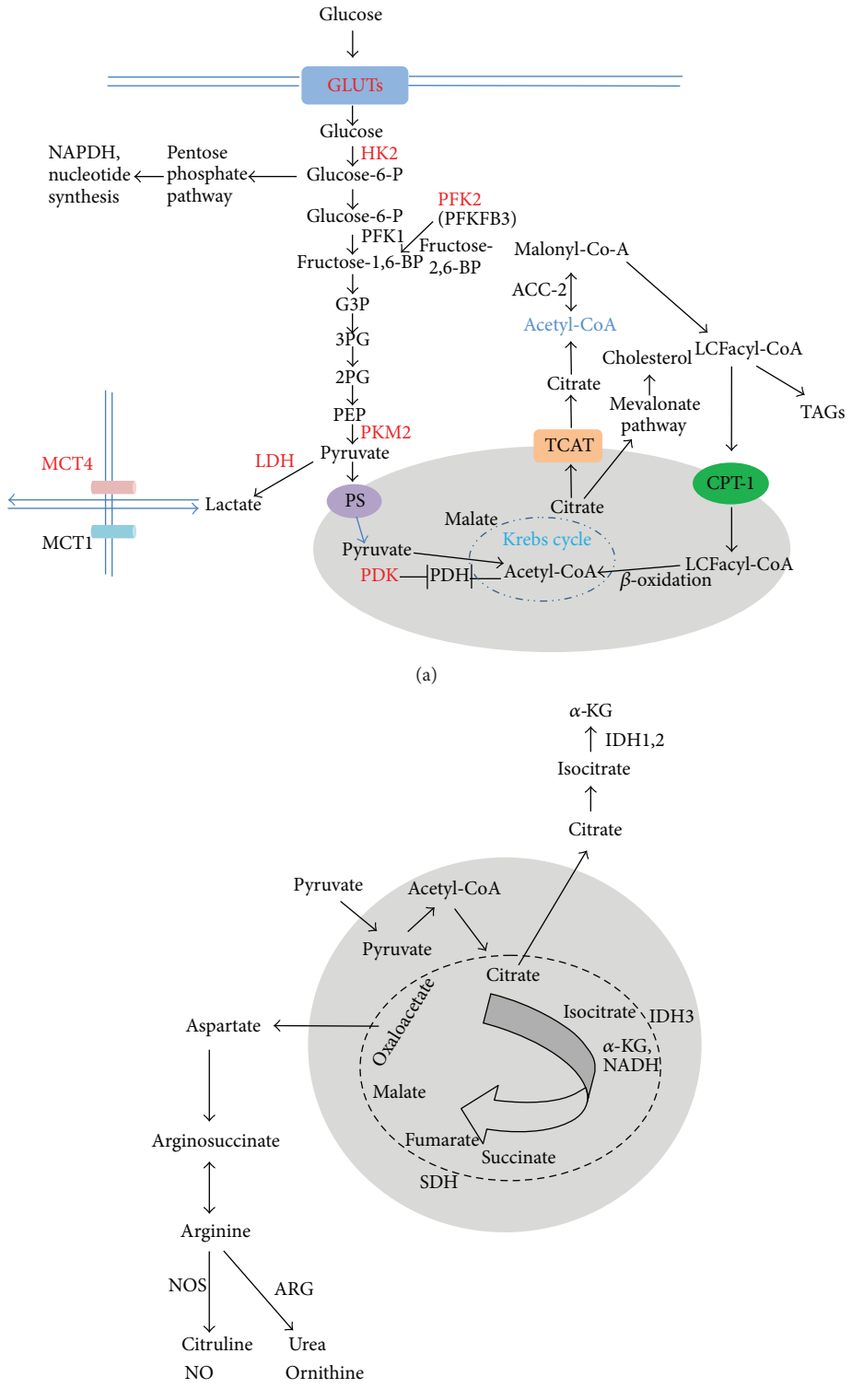

Figure 2: Metabolism of glucose and fatty acid at a glance. (a) Stabilization of HIF-1α upregulates GLUTs, HK2, PFK2, PKM2, LDH, PDK, and MCT4 shown in red. ACC: acetyl-CoA carboxylase; ARG: arginase; CPT-1: carnitine palmitoyltransferase 1; FAT: fatty acid translocase; G3P: glyceraldehyde 3-phosphate; GLUT: glucose transporter; HK2: hexokinase 2; IDH: isocitrate dehydrogenase; LCFacyl-CoAs: long-chain fatty acyl-CoAs; MCT: monocarboxylate transporter; 2PG: 2-phosphoglycerate; 3PG: 3-phosphoglycerate; PEP: phosphoenolpyruvate; PDH: pyruvate dehydrogenase; PDK: pyruvate dehydrogenase kinase; PFK: phosphofructokinase; PS: pyruvate symporter; SDH: succinate dehydrogenase; TAG: triacylglyceride; TCAT: tricarboxylic acid transporter. (b) A schematic of the Krebs cycle and metabolites exported out of the mitochondria. Arginine is metabolized to urea and ornithine in M2-like macrophages that do not express NOS. ARG: arginase; NOS: nitric oxide synthase.

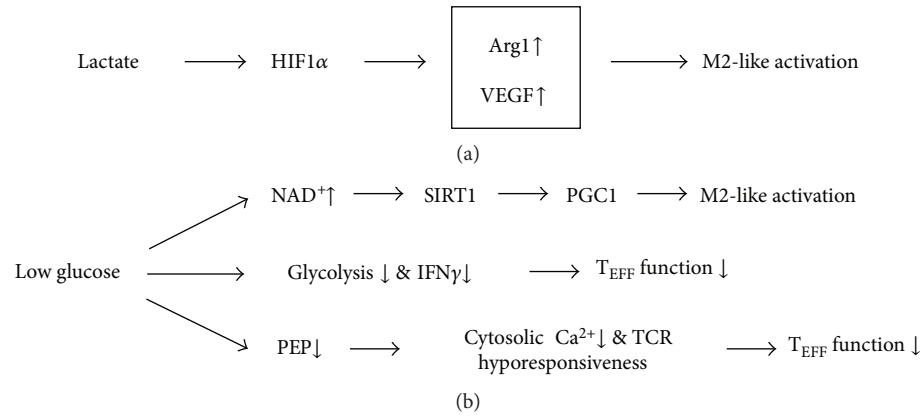

Figure 3: Metabolic changes in the TME-regulating immune cell function. (a) Lactate produced by cancer cells, as a by-product of aerobic glycolysis, has a critical function in inducing M2-like activation of TAMs. (b) A low-glucose microenvironment via multiple signaling pathways regulates activation state of macrophages and T cells. TCR: T cell receptor; VEGF: vascular endothelial growth factor.

identification of alternative, novel metabolic targets for TAM-directed intervention. Recently, it was shown that lactate produced by cancer cells has a critical function in inducing M2-like activation of TAMs [59] (Figure 3). Acidification of the TME by lactate increases level of ARG1, a representative M2 marker, in macrophages, which limits the proinflammatory, antitumoral response of TAMs and, importantly, the proliferation and activation of T cells [59, 70]. Also, de novo fatty acid synthesis in cancer cells increases fatty acid levels in the TME to promote the generation of immunosuppressive, regulatory T cells (Tregs) and M2-like TAMs, favoring survival of cancer cells [71]. Expression level of vitamin D receptor (VDR) negatively correlates with metastasis in breast cancer, and suppression of VDR by TNFα can mediate the prometastatic effects of TAMs through enhancement of the β-catenin pathway [72].

3. Metabolic Regulation of T Cells

Multiple studies have shown that distinct metabolic programs in $CD4^+$ T cell subsets can be manipulated *in vivo* to control Treg and effector T cells (T_{EFF}) development in inflammatory diseases [73–76]. A transcription factor, Myc, shows a dominant role in driving metabolic reprogramming in activated T cells by promoting glycolysis and glutaminolysis and suppressing FAO [75]. mTOR increases expression of HIF-1α, which facilitates the expression of critical glycolytic enzymes and promotes differentiation and activation of T cells [76].

A "shift" from OXPHOS to aerobic glycolysis is a hallmark of T cell activation [25]. T cells, if not activated, show low levels of metabolic requirements, use OXPHOS to maximize production of ATP as an energy source, and engage scarcely in biosynthesis, while activated T cells use aerobic glycolysis to produce effector molecules for rapid cellular proliferation [32].

In order to facilitate proper immunological response upon encounter of antigenic stimuli, it is vital that T cells should differentiate into T_{EFF} and clonally expand rapidly to ensure prompt reaction. Glycolysis promotes the differentiation of activated $CD4^+$ T cells into T_{EFF} [73]. Activated T cells also consume glutamine to fuel the Krebs cycle to support the production of biomass and ATP [77]. Clonal expansion is achieved from upregulation of glycolysis and OXPHOS together. In addition to high level of glycolysis, increased mitochondrial flux and production of ROS are also required for initiation of the clonal expansion [78]. After differentiation, T_{EFF} cells, Th1, Th2, and Th17 cells, remain highly glycolytic [73].

When the antigenic stimuli are eliminated, most T_{EFF} cells die, leaving behind a small antigen-specific T cell population that becomes memory T cells (Tm). Quiescent Tm with the CD8 coreceptor exploits FAO to fuel OXPHOS rather than aerobic glycolysis for ATP production [32, 73]. Instead of utilizing extracellular lipids for energy generation, Tm metabolizes de novo generated fatty acids, synthesized from extracellular glucose and intracellularly stored during the previous effector phase [79]. Enforcing FAO with activation of AMPK or inhibiting mTOR results in increased numbers of Tm [80–82]. Mitochondrial oxidative metabolism supports immunosuppression and lineage commitment of Tregs [83–85]. Tregs with increased glycolysis are more proliferative yet have reduced ability to maintain FOXP3 expression and suppress inflammation [84].

4. Nonmetabolic Function of Glycolytic Enzymes in Immune Cells

In addition to their canonical, metabolic functions in glycolysis, recent studies uncovered nonmetabolic functions of glycolytic enzymes such as hexokinase 2 (HK2), phosphoglucose isomerase, and GAPDH, connecting metabolic states to apoptosis, gene transcription, protein kinase activity, and the mTOR signaling pathway [86]. Briefly, the interaction between HK2 and voltage-dependent anion channel (VDAC1) reduces the release of proapoptotic proteins and prevents cancer cells from undergoing apoptosis [87]; phosphoglucose isomerase exerts its antiapoptosis effect by suppressing the expression of Apaf-1 and caspase-9 genes, thereby indirectly regulating the formation of the

apoptosome [88, 89]. GAPDH exerts controversial pro- and antiapoptotic effects through interaction with VDAC1 and induction of autophagy, respectively [86, 90].

Other studies have shown that GAPDH, HK, and enolase are also RNA-binding proteins [91–93] and the "REM (RNA–enzyme–metabolite) hypothesis" proposes a regulatory interaction between gene expression and cellular metabolism by RNA-binding metabolic enzymes [94, 95]. It is notable that many glycolytic enzymes, formerly known to exclusively function in glycolytic metabolic events in the cytoplasm or mitochondria, have now been shown to regulate transcription and translation [86, 91]. Indeed, numerous metabolic enzymes that also function in glycolysis, fatty acid synthesis, and the Krebs cycle are also RNA-binding proteins [96], although the significance for immune response is not clearly known except for that of GAPDH [95] and enolase [32, 83]. Recently, a REM connection with GAPDH was proven in T cell activation [25]. GAPDH is diverted to glycolysis and translation of IFNγ and IL-2 is not perturbed in highly glycolytic T cells. However, when aerobic glycolysis is blocked, GAPDH binds to IFNγ and IL-2 mRNA in $CD4^+$ T cells to suppress their translation [25]. In myeloid cells, GAPDH is a component of the IFNγ-activated inhibitor of translation (GAIT) complex that controls translation of inflammatory genes [97, 98]. High glycolytic flux suppresses the interaction between GAPDH and Rheb and thus allows Rheb to activate mTORC1 and stimulate cell growth [99]. By modulating expression of Foxp3-splicing variants with exon 2(Foxp3-E2), enolase-1-mediated glycolysis controls induction of human Tregs with a potent immunosuppressive function [83]. When glycolysis is inhibited, enolase-1 translocates to the nucleus and represses expression of the Foxp3-E2 splice variant in Tregs and suppresses Treg induction.

The level of the glycolytic intermediate phosphoenolpyruvate (PEP) is controlled by a balance between enolase-mediated formation of PEP and pyruvate kinase-mediated conversion to pyruvate. PKM2 exists either as an inactive dimer or as more active tetramer, and the transition between the two conformations is subject to posttranslational modifications [100]. Dimeric PKM2, previously regarded as crucial for metabolic reprogramming exclusively in cancer cell, is also important in promoting aerobic glycolysis in immune cells [101, 102]. Enhanced expression of dimeric PKM2 reduces the rate of PEP conversion to pyruvate and results in an accumulation of glycolytic products that can be otherwise metabolized in biosynthetic pathways [32]. Importantly, PEP enhances antitumor effector functions in activated T cells by regulating $Ca2^+$ import into the endoplasmic reticulum, thus sustaining translocation of nuclear factor of activated T cells (NFAT) into the nucleus and the expression of a set of genes that are required for T cell activation [103].

These findings imply that a direct and strong interaction exists between the nonmetabolic function of glycolytic enzymes and the generation of immune responses and also that enhanced glycolysis sustains antitumoral and proinflammatory functions via highly interconnected signaling in immune cells.

5. Metabolic Changes in the TME Influencing Immune Cell Functions

The microenvironment determines the metabolism of immune cells, which in turn adjust to a broad spectrum of configurations to meet the demands of various cellular activities. For example, changes in the metabolic profiles of immune cells by cancer cells can alter the function of the immune cells [103, 104]. A protracted aerobic glycolysis acidifies and destabilizes the TME and this is consistent with the view of the tumor as an unhealed wound [105]. Similarities of utilizing nutrients and engaging metabolic regulation to sustain cellular proliferation and survival are shared by cancer and immune cells. Notably, nutritional competition between cancer cells and antitumoral immune cells in the TME shifts the activation and differentiation status of T-cells to favoring the survival of cancer cells [103, 104, 106, 107].

Recently, it was shown that lactate produced by cancer cells, as a by-product of aerobic glycolysis, has a critical function in signaling that induces M2-like activation of TAMs [59] (Figure 3). Interestingly, lactate-induced M2-like activation was from HIF1α-dependent expression of ARG1. Depletion of glucose and a glucose-rich hypoxic ROS environment favor M2-like activation and M1-like activation of TAMs, respectively, and depletion of glucose can disarm T cells in the TME [27, 104]. Low levels of ATP from dietary restrictions or energy consumption induces nicotinamide phosphoribosyltransferase that generates NAD^+, which is a key factor for SIRT1 activation. SIRT1 acetylates and activates PGC1β to increase OXPHOS [35, 67, 108]. Pyruvate is metabolized by LDH-A, producing lactate and NAD^+. NAD^+ acts as an electron acceptor in the Krebs cycle and the electron transport system in mitochondria. It appears feasible from these findings that glucose-depleted, low ATP, and NAD^+-rich states (in cachexic patient with advanced cancer) may drive the M2-like activation of macrophages, while the macrophage population still retains its phagocytic activity in maintaining biosynthesis with molecules acquired from their microenvironment [32]. For the identification of alternative, novel targets for TAM-directed intervention, it would be necessary to show whether these events can predominantly happen in TAMs of the TME.

A recent study observed that hypoxia-induced upregulation of the immunosuppressive programmed death ligand-1(PD-L1) is directly mediated by HIF1α [109]. In the TME, cancer cells, macrophages, and dendritic cells express PD-L1, a notable ligand for immune checkpoint, programmed cell death-1(PD-1) in T_{EFF}. The interaction of PD-1 and PD-L1 directly inhibits glycolysis and promotes lipolysis and FAO in T cells, resulting in failure of the antitumoral function of T cells [110] (Figure 1). The lessons that application of immune checkpoint blockade antibodies against cytotoxic T lymphocyte antigen-4 (CTLA-4), PD-1, and PD-L1, which are used clinically, restore glucose in the TME, permitting T cell glycolysis and IFNγ production clearly show that nutrient availability in the microenvironment can change the metabolic status of immune cells. Another study also

revealed that PD-1 expression by TAMs correlates with protumoral activity, and blockage of PD-1–PD-L1 *in vivo* increases phagocytosis, reduces growth of cancer cells, and increases the survival of mice in mouse models of cancer in a macrophage-dependent way [111]. In addition, blocking PD-L1 directly on cancer cells decreases glycolysis and restores glucose in the TME, resulting in allowance of antitumoral function of T cells from glycolysis and IFNγ production [104].

In conclusion, these findings indicate that signals such as cytokines, growth factors, hypoxia, and nutrient availability that emanate from the microenvironment can induce metabolic changes in immune cell subsets, resulting in changes in immune functions and pathological responses. An interesting perspective is whether immune cells can supply their microenvironment with lactate and antioxidative resources as stromal cells in the TME are able to (the reverse Warburg effect). Considering their preponderance in the TME, there is an ample possibility that metabolic changes in a large subgroup of macrophages or T cells may also affect the metabolic state of their microenvironment and functions of other cellular components.

6. Potential Metabolic Targets for the Manipulation of Immune Cell Function

Since the function of immune cells is dependent on a delicate metabolic balance, results of many clinical trials performed with inhibitors of metabolic enzymes and oncogenes will provide valuable insights for the prospect of immunomodulation by specific metabolic regulation [112]. Of note, results from targeting cancer metabolism *in vivo* have been disappointing and less prominent than results from targeting immune cell metabolism [85]. The PKM2 inhibitor, TLN-232, was tested in a clinical trial for refractory renal cell carcinoma (NCT00422786). Inactive dimeric PKM2 activates the mTORC1 signaling pathway by phosphorylating the mTOR inhibitor, AKT1S1, and leads to an accelerated oncogenic growth and autophagy inhibition of cancer cells [113]. In line with this, increase in the tetrameric, active form of PKM2, attenuated the LPS-induced proinflammatory M1-like macrophage phenotypes while promoting M2-like macrophage phenotypes [114]. Many AMPK activators are now tested in clinical and preclinical studies for diabetes, cancer, and cardiovascular disease [115]. Importantly, AMPK stimulation inhibiting mTORC1 was sufficient to decrease Glut1 and increase generation of Tregs in an animal model, implying AMPK activation as a potential manipulable checkpoint for immune response [73]. REDD1, an inhibitor of mTOR, is highly expressed in M2-like TAMs. Inhibition of REDD1 stimulates glycolysis in the TAMs and competition of glucose between TAMs and endothelial cells prevents vascular hyperactivation and promotes the formation of quiescent vascular junctions in the TME [69]. Suppression of REDD1 was attempted in phase 2 clinical trial (NCT00713518) for the treatment of neovascularization in AMD patients. Nitrosylation of HIF1α prevents its degradation. If denitrosylation of HIF1α is observed, its modulation may be potentially applicable for the inhibition of glycolytic enzymes and the alleviation of M1-like phenotypes.

Isoprenylation of ubiquinone is important for OXPHOS and isoprenylation of Ras, Rho, and Rab guanosine triphosphatases is involved in immunological synapse formation, migration, proliferation, and cytotoxic effector response of T cells. The intracellular availability of sterols is crucial for isoprenylation modification of proteins for plasma membrane attachment and represents a checkpoint for metabolic reprogramming that modulates T cell responses [116]. Statin and other chemical inhibitors of the mevalonate pathway can suppress isoprenylation of Rho proteins [117] and have been tested in many clinical trials.

Clinical trials involving agents that inhibit PD-L1 and PD-1 are now being performed. Atezolizumab is the sole member of this class currently approved for the treatment of bladder cancer, but approvals for avelumab, durvalumab, nivolumab, and pembrolizumab in the treatment of various cancer are anticipated in the near future [118]. Therefore, it appears possible that the combined use of metabolism-targeting reagents with immune checkpoint inhibitors can alter the activation and differentiation of T cells.

7. Conclusions

Immunity and metabolism advance together. Considering the significant contribution of immune cell functions in promoting and suppressing various types of disease progression, repolarization of immune cells from the potential targets stated above shows an ample possibility to become novel therapeutic approaches. Extension of our knowledge of the functional plasticity of macrophages and T cells spanning from inflammation biology to cancer immunology and the persistent reprogramming effect achievable from stable epigenetic changes in the metabolic pathways of macrophages [29] and potentially T cells by potential modulators may provide new information for immune therapeutic strategies applicable for different disease conditions. Importantly, cancer cells and host primary cell constituents such as immune cells and stromal cells can form microanatomical compartments within the cancer tissue to regulate metabolic needs, immune surveillance, survival, invasion, and metastasis. Indeed, different signals from particular locations in the TME seem to influence activation of TAMs and T cells and overall tumor prognosis [119]. TAMs can be diverse within the microanatomical compartments, including the accumulation of M1-like activated cells with protumoral properties in hypoxic areas [120] and differences in inflammatory components and pathways between tumors originating in distinct anatomical sites [120, 121]. The notion that metabolic competition between cancer cells, immune cells, and other stromal cells can determine function and fate of each cell subset proposing that identification of which of specific niches in the microenvironment can impede immune cells from proper metabolic engagement will encourage significant contributions to this research field. Generation of metabolically fit T cells prior to adoptive cell transfer will improve T cell-based immunotherapy against cancer by surviving the unfavorable, hostile TME. Furthermore, successful therapies

targeting the function of macrophages and T cells will require identification of targets that specifically allow metabolic reprogramming of immune cells while, at the same time, not causing an increase in proliferation and survival of cancer cells or systemic inflammatory changes or autoimmunity. Our understanding of the metabolic regulations in B cells is surprisingly limited, and the mechanisms about how cellular metabolism supports and regulates function of B cells are still poorly defined. B cell immunometabolism is anticipated to become an exciting research field.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

How Hippo Signaling Pathway Modulates Cardiovascular Development and Diseases

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Cardiovascular disease remains the leading cause of death around the globe. Cardiac deterioration is associated with irreversible cardiomyocyte loss. Understanding how the cardiovascular system develops and the pathological processes of cardiac disease will contribute to finding novel and preventive therapeutic methods. The canonical Hippo tumor suppressor pathway in mammalian cells is primarily composed of the MST1/2-SAV1-LATS1/2-MOB1-YAP/TAZ cascade. Continuing research on this pathway has identified other factors like RASSF1A, Nf2, MAP4Ks, and NDR1/2, further enriching our knowledge of the Hippo-YAP pathway. YAP, the core effecter of the Hippo pathway, may accumulate in the nucleus and initiate transcriptional activity if the pathway is inhibited. The role of Hippo signaling has been widely investigated in organ development and cancers. A heart of normal size and function which is critical for survival could not be generated without the proper regulation of the Hippo tumor suppressor pathway. Recent research has demonstrated a novel role of Hippo signaling in cardiovascular disease in the context of development, hypertrophy, angiogenesis, regeneration, apoptosis, and autophagy. In this review, we summarize the current knowledge of how Hippo signaling modulates pathological processes in cardiovascular disease and discuss potential molecular therapeutic targets.

1. Introduction

Heart disease continues to be the main risk of death in both developed and developing countries. Heart malformation could lead to embryonic or postnatal death, and strenuous stimulations like pressure overload and/or ischemia could cause irreversible damage. It has been shown that cardiomyocytes rapidly change from the proliferative state into hypertrophy at postnatal day 3 or 4 [1]. The regenerative ability of cardiomyocytes has been demonstrated in young human hearts [2], providing evidence that can be used toward heart regeneration therapy. However, due to the massive cell loss and the limited potential of cardiomyocyte proliferation in heart diseases, optimization of cardiac regeneration treatments remains challenging.

The Hippo signaling pathway primarily consists of the MST1/2-SAV1-LATS1/2-MOB1-YAP/TAZ cascade, known to regulate multiple organ development and diseases [3, 4]. In addition, NDR was recently included as a novel member in the cascade [5, 6]. YAP dephosphorylation leads to its inactivation, followed by cytoplasmic retention when the Hippo pathway is "switched on." However, when the pathway is "switched off," YAP is phosphorylated and accumulates in the nucleus, promoting cellular proliferation, metastasis, or regeneration [3, 4].

Interestingly, the Hippo pathway participates in diverse physiological and pathological processes in the heart spanning heart development, apoptosis, hypertrophy, autophagy, angiogenesis, and cardiomyocyte regeneration [7]. The purpose of this review is to summarize the current findings of

the Hippo signaling cascade in cardiac development, apoptosis, hypertrophy, autophagy, angiogenesis, and cardiomyocyte regeneration. Moreover, we will explore novel therapeutic approaches in the field.

2. Hippo-YAP Pathway

The classical Hippo pathway was first characterized in *Drosophila*, identifying the major effectors like Hippo, Warts, Yorkie, and Mats [7]. The counterparts of these kinases in mammalian cells are MST1/2, LATS1/2, YAP/TAZ, and MOB1, respectively [7]. Here, we will discuss the most widely studied core cascade, namely, the MST1/2-SAV1-LATS1/2-MOB1-YAP/TAZ signaling pathway components. When the Hippo cassette is switched on, the activated MST1/2 (also termed STK4/3) phosphorylates LATS1/2, which in turn could cause phosphorylation of the major effectors YAP/TAZ [8]. MST1/2 is regulated by SAV1 protein, whereas MOB1 may interact with LATS1/2 [9]. Once YAP is phosphorylated, it can either be held in retention in the cytoplasm by protein 14-3-3 [8] or undergo degradation [10]. On the contrary, when the Hippo pathway is at the "off" state, YAP can no longer be phosphorylated, causing it to accumulate in the nucleus where it forms a complex together with TEAD (Transcriptional Enhancer Associated Domain) and initiates further biochemical activities [9]. Apart from the regular Hippo-YAP axis, novel kinases like NDR1/2 (STK38/STK38L), MAP4Ks, and CK1 are also included in the network [5, 6, 10]. Activated MAP4Ks may also phosphorylate both LATS1/2 and NDR1/2 [5]. While LATS phosphorylates YAP on five serine residues: S61, S109, S127, S164, and S381, NDR directly phosphorylates YAP on S127, restraining it from shuttling into the nucleus [6, 10]. Interestingly, recent research has identified another player which executes opposite effects on YAP in comparison to NDR and LATS activity [11]. Nemo-like kinase (NLK), a member of the nonclassic MAP-kinase family, phosphorylates YAP at the S128 residue. On the one hand, it deters YAP from binding with 14-3-3, and on the other hand, it reduces the phosphorylation of YAP at S127, thus promoting YAP nuclear localization [11]. What is more, the activities of LATS1/2 and NDR1/2 both rely on MOB1 [5]. The important work from Zhao et al. has provided deeper insights into the modulation of YAP protein [10]. The authors found that, after being phosphorylated on the S381 site, YAP was subjected to further phosphorylation by CK1 on S384, facilitating the degradation of YAP [10].

There are two additional renowned molecules positively regulating MST1/2. One of them is RASSF1 (Ras association domain family 1), a tumor suppressor member protein [12]. RASSF1A may keep MST1/2 at its phosphorylated state and prevent dephosphorylation by binding to MST1/2 [12]. The other molecule is Nf2 (neurofibromin2), a tumor suppressor and a proapoptosis kinase, which positively regulates the Hippo signaling through activation of MST1 [13]. The summary of the Hippo-Yap signaling pathway is shown in Figure 1.

The Hippo-YAP pathway primarily functions in cell proliferation and apoptosis, controlling the organ size [4]. Cell polarity, cell contact, other mechanical forces, and soluble factors were identified as key Hippo signaling regulators [3, 4]. To our knowledge, whether YAP could exert biochemical functions depends on its location. Intriguingly, cell density could also affect the YAP localization and vice versa [8]. When YAP is upregulated, cell growth may be stimulated without the constraints of cell-contact inhibition [8]. Except for numerous upstream factors regulating the Hippo pathway, the cascade itself has a negative feedback loop. YAP coupled with TEAD in the nucleus may augment the expression of Nf2, LAST2, and MST1, which together may ensure negative control on YAP [4].

3. Role of the Hippo Tumor Suppressor Pathway in Cardiovascular Disease

The summary of how the Hippo-YAP pathway participates in cardiovascular development, hypertrophy, apoptosis, autophagy, angiogenesis, and regeneration is listed in Table 1.

3.1. The Hippo-YAP Signaling in Cardiovascular Development. Numerous studies have documented the essential role of the Hippo tumor suppressor pathway in organ development and tumorigenesis. In 2011, Xin et al. showed that deletion of YAP in mice containing the Nkx2.5-Cre resulted in reduced cardiomyocyte proliferative ability and eventually led to embryonic death at stage 10.5. On the other hand, cardiomyocyte numbers in the newborn mice were significantly increased when YAP expression was increased with adenovirus expressing YAPS112A [14]. In addition, the proproliferation function of YAP was achieved via activation of the insulin-like growth factor pathway [14]. Heallen et al. reported that cardiac-specific knockout of SAV1 inactivates the Hippo signaling, evident by decreased phosphorylated YAP level but not total YAP, leading to an enlarged heart without alterations in cell size. Similar results were found in MST1/2 and LATS2 knockout mice [15]. To deepen insights into the underlying mechanisms, their group also reported that the Hippo pathway exerts its antigrowth effects through suppression of Wnt signaling [15]. While ventricular septal defect (VSD) was observed in some of the SAV1 mutant hearts [15], VSD also occurred in cardiac/vascular smooth muscle cell-(SMC-) specific YAP ablation mice, which may harbor other severe vascular malformations like dysplastic arterial wall, hypogenetic brachiocephalic artery, and retroesophageal right subclavian artery [16]. These abnormalities may ultimately lead to perinatal fatality [16]. To further elucidate on the role of Hippo signaling in coronary development, Singh et al. induced epicardial-specific deletion of YAP and TAZ with Sema3dGFPCre+/−. The epicardium is one of the important sources of coronary vasculogenesis [17]. Results showed that YAP/TAZ-null mice embryos had attenuated differentiation of the epicardial cell into coronary endothelial cells which caused embryonic lethality between stages E11.5 and E12.5 [17]. These data emphasize the significant role of the Hippo pathway in normal cardiovascular development.

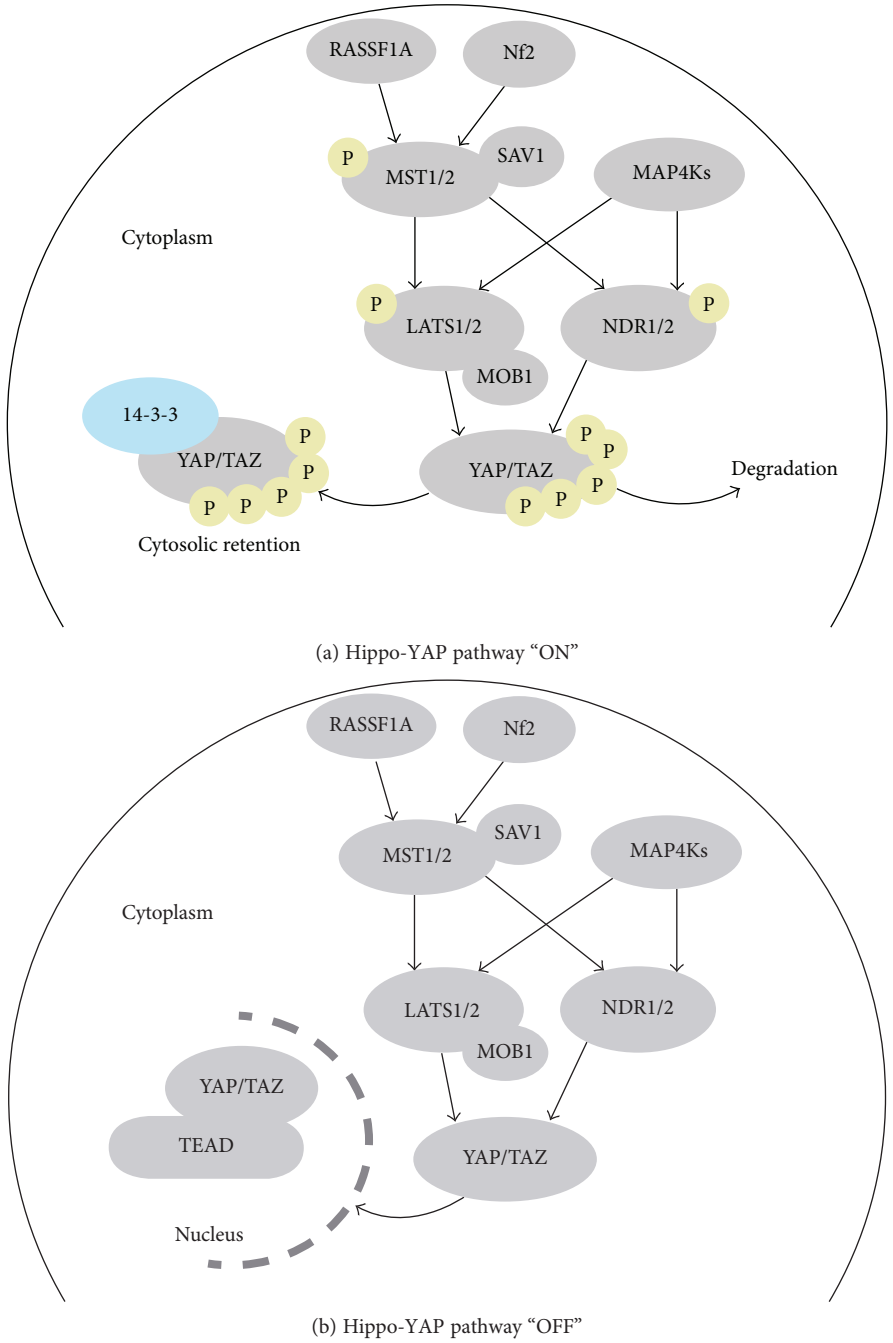

Figure 1: The overview of Hippo-Yap signaling pathway. (a) YAP and TAZ are phosphorylated and held in cytoplasm when the Hippo-YAP pathway is at the "ON" status. (b) Unphosphorylated YAP and TAZ accumulate in the nucleus with TEAD when the Hippo-Yap pathway is at the "OFF" status.

3.2. The Hippo-YAP Signaling in Cardiomyocyte Hypertrophy and Apoptosis. Cardiomyocytes and fibroblasts are two critical components of the heart. In response to hypertension or other pressure overload diseases, cardiac hypertrophy occurs, resulting in enlargement of cell size, enrichment of cell number, or both. However, given that adult cardiomyocytes have limited proliferation ability, cardiomyocyte hypertrophy rather than hyperplasia have more commonly been observed. To investigate the possible role of Hippo signaling in cardiomyocyte hypertrophy and apoptosis, we discuss its key components separately.

To start with MST1, Lin et al. have summarized that altered expression levels of MST1 could not affect the size of cardiomyocytes but an upregulated MST1 level increased cardiomyocyte apoptosis [18]. Other researcher groups have reported that the RASSF1A/MST1 pathway exerts rather different results in cardiomyocyte and fibroblast. That is, in a setting of pressure overload, activated RASSF1A/MST1

Table 1: Role of the Hippo tumor suppressor pathway in cardiovascular disease.

	Effecter	Methods	Outcomes	Ref.
Cardiovascular development	MST1/2	Cardiac-specific knockout of MST1/2	Enlarged hearts; without alteration of cell size	[15]
	LATS2	Cardiac-specific knockout of LATS2	Enlarged hearts; without alteration of cell size	[15]
	SAV1	Cardiac-specific knockout of SAV1	Enlarged ventricular chambers; thickened ventricular walls; without alteration of cell size	[15]
	YAP	Deplete YAP with Nkx2.5-Cre	Reduced cardiomyocyte proliferative ability; embryonic death at embryonic stage 10.5	[14]
		Overexpress YAP with adenovirus	Cardiomyocytes number increased significantly in newborn mice	[14]
		Cardiac/vascular smooth muscle cell-specific ablation of YAP	Vascular malformations like ventricular septal defect etc.; might result in perinatal fatality	[16]
		Epicardial-specific deletion of YAP/TAZ with Sema3dGFPCre+/−	Attenuated differentiation of the epicardial cell into coronary endothelial cells; embryonic death between E11.5 and E12.5	[17]
Cardiomyocyte hypertrophy and apoptosis	RASSF1A	Generated RASSF1A transgenic (TG)/(L308P) RASSF1A TG mice with adenoviral system; subjected them to pressure overload	Increased MST1 phosphorylation; promotes cardiomyocyte apoptosis; reduced the proliferation ability of fibroblast and cardiac hypertrophy	[19]
	MST1	Upregulation of MST1	Enhanced cardiomyocyte apoptosis	[18]
	MST2	MST2 knockout	Attenuated cardiac hypertrophy	[20]
		MST2 overexpression	Increased cardiac hypertrophy	[20]
	LATS1	Mutation of LATS1 using siRNA	Encouraged cardiac hypertrophy	[22]
	LATS2	Transduced Ad-LATS2 or Ad-LacZ into cultured myocytes; generated LATS2 and DN-LATS2 TG mice using the α-myosin heavy chain promoter	Dose dependently increased apoptosis and reduced cardiac myocyte size in vitro; negatively regulated ventricular chamber size in vivo	[21]
Cardiomyocyte hypertrophy and apoptosis	YAP	Cardiac-specific activation of YAP using adenoassociated virus subtype 9 (AAV9) after MI	Improved cardiac function without causing hypertrophy; enhanced survival	[24]
		Cardiac-specific inactivation of YAP1 using α-MHC Cre recombinase transgenic mice; transduced cardiomyocytes with YAP1 or LacZ adenovirus	Caused increased cardiomyocyte apoptosis in YAP(−/−) at baseline; YAP expression induced cardiomyocyte hypertrophy	[25]
Angiogenesis	LATS1/2	Coinjection of mRNAs encoding Angiomotin p130 and mRNAs encoding LATS2	Induced angiogenesis defects in zebrafish embryos	[30]
	YAP	Knock-down of YAP by siRNA	Significantly reduced the tube formation or sprouting ability of endothelial cells	[28]
		Upregulation of YAP	Induce robust angiogenesis	[28]
Heart regeneration	YAP	Cardiac-specific YAP knockout in MI mice	The infract area was broader and cardiomyocytes were less robust	[32]
		Cardiac-specific upregulation of YAP in MI mice with adenoassociated virus serotype 9	Rescued the cardiomyocyte number and cardiac function	[33]
		Compared Pitx2-deficient mice and Pitx2-overexpressing mice when subjected to apex dissection	Pitx2-deficient mice fail to repair while Pitx2-overexpressing mice showed functional recovery	[35]
Cardiomyocyte autophagy	MST1/2	Inhibition of MST1 phosphorylation with Melatonin, oncostatin M etc.	Promoted cardiac function, enhanced autophagy, and weakened apoptosis	[46–50]
		Phosphorylation of LC3 by MST1/2	Promoted the fusion step of autophagy	[51]
	NDR1	Interact with Beclin1	Function in the early stage of autophagy	[52]

may lead to cardiomyocyte apoptosis, while suppressing the proliferation ability of fibroblast leading to reduced cardiac hypertrophy [19]. This interesting finding may indicate that specific inhibition of the RASSF1A/MST1 pathway in cardiomyocytes rather than fibroblasts could be a novel therapeutic target [19]. Based on the evidence that MST2 knockout mice showed attenuated hypertrophy while MST2 overexpression lead to increased hypertrophy, researchers concluded that MST2 took part in cardiac hypertrophy [20]. However, such effect was exerted through the

Raf1/ERK1/2 pathway but not by alteration of YAP [20]. When it comes to LATS1/2, Matsui et al. reported that LATS2 contributed to MST1-mediated apoptosis and antihypertrophy in the heart but upregulation of LATS2 alone did not affect cardiac apoptosis at baseline [21]. Moreover, LATS2 reduced cardiomyocyte size in vitro in a dose-dependent manner, and it negatively modulated cardiac hypertrophy in response to pressure overload [21]. Consistently, silencing LATS1 with siRNA enhanced the paracrine secretion of cardiac fibroblasts when under mechanical stress stimulation, leading to cardiac hypertrophy [22]. As for YAP, the major effector of the Hippo pathway, recent data has shown that YAP activation plays an essential role in cell proliferation of both fetal and postnatal heart while not affecting cardiomyocyte size in both physiological and pathological settings [23]. Lin et al. also found that in the context of myocardial infarction (MI), YAP activation exerted its cardioprotective function via stimulation of cell proliferation without causing hypertrophy [24]. However, another team which also investigated the role of YAP in cardiomyocytes found that YAP also induced cardiac hypertrophy in addition to its antiapoptotic and proproliferative role [25]. While YAP negatively controls cardiomyocyte apoptosis through activation of Akt [25], it could also encourage compensatory cardiomyocyte hypertrophy via upregulation of miR-206 [26], both favoring cell survival.

In summary, the Hippo pathway which primarily functions through the YAP effector protein, mediates cardiac hypertrophy and apoptosis, although it seems that whether YAP could induce hypertrophy depends on different backgrounds. However, noteworthily, the core components of the Hippo cascade might cause hypertrophy or apoptosis via other pathways without translocating the YAP protein.

3.3. The Hippo-YAP Signaling in Angiogenesis. Angiogenesis, a process that produces neovessels, is important in the context of cardiac ischemia. Given that endothelial cells (ECs) play a major part in angiogenesis and the Hippo-YAP pathway participates in EC survival, proliferation, and migration [27], abundant evidence has supported the idea that Hippo signaling could tune the production of new blood vessels. Choi et al. demonstrated that the tube formation or sprouting ability of ECs was noticeably diminished after was YAP knocked down by short interfering RNA [28]. As previously shown, Zhao et al. found that YAP localization was mediated by cell-cell contact at least partially driven via the Hippo pathway [8]. Interestingly, Choi et al. discovered that phosphorylation of YAP in ECs was regulated by VE-cadherin through Akt but not mediated by LATS1/2 [28]. Moreover, upregulation of YAP could induce robust angiogenesis by transcriptional modulation of angiopoietin-2 [28]. Similarly, Marti et al. revealed another transcriptional target of YAP in cholangiocarcinoma which enhances neovascularization, that is, microfibrillar-associated protein 5 (MFAP5) [29]. Apart from YAP, Dai et al. helped define that Angiomotin is an alternative target of LATS1/2. Angiomotin phosphorylation on Serine175 mediated by LATS1/2 negatively regulates angiogenesis in zebrafish embryos [30]. Furthermore, Yuan et al. demonstrated that palmitic acid interfered with the Hippo pathway by blocking YAP from shuttling into the nucleus leading to attenuated angiogenesis. This process was mainly driven by palmitic acid damage on mitochondria [31]. In conclusion, these data support the proangiogenesis role of YAP. Surprisingly, the activated LATS1/2 could negatively modulate angiogenesis via regulation of YAP and other targets.

3.4. The Hippo-YAP Signaling in Heart Regeneration. Myocardial infarction (MI) is a devastating disease around the world because it causes irreversible cell loss in the heart. The limited proliferation capacity of the adult cardiomyocytes makes the management of MI rather challenging. In recent years, therapies aiming at promoting the regeneration ability of the heart have attracted much spotlight. Among these therapies, harnessing the Hippo pathway might be beneficial. Xin et al. investigated the role of YAP in the context of MI [32]. When neonatal hearts were subjected to left anterior descending coronary artery ligation, mice in the cardiac-specific YAP knockout group showed broader infarcted area with reduced functioning cardiomyocytes. Furthermore, the proproliferation and prosurvival function of YAP was observed in a postnatal MI model [32]. Lin et al. used an adeno-associated virus serotype 9 (AAV9) to specifically upregulate YAP in an in vivo heart model. The authors successfully identified a direct target of YAP, the Pik3cb, which regulates the Hippo signaling through the PI3K-Akt pathway [33]. By increasing the expression level of YAP, the cardiomyocyte number and heart function were restored after MI at least partially through Pik3cb [33]. Moreover, Tian et al. provided novel insights into microRNA-based heart regeneration therapy [34]. They identified a cluster of microRNA, miR302–367, which drives the cardiomyocyte to reenter the cell cycle, thus inducing cardiac proliferation after MI, fractionally due to inhibition of the Hippo pathway [34]. However, constitutive expression of the miRNA was not beneficial. To overcome this problem, Tian et al. found that utilization of miR302–367 could achieve the same desired outcome but with minimum side effects [34]. Intriguingly, Tao et al. described another transcription factor, the paired-like homeodomain transcription factor 2 (Pitx2), but with subsidiary function in the regulation of YAP [35]. Subsequent studies showed that Pitx2-deficient mice failed to regrow. Furthermore, Pitx2-overexpressing in mice after apex dissection showed successful functional recovery in the heart. This study indicates a heart-regenerating role of Pitx2, probably achieved through YAP interaction to promote cardiomyocyte entry into the S-phase [35]. Together, these data underline the indispensable role of the Hippo-YAP pathway in heart regeneration.

3.5. The Hippo-YAP Signaling in Cardiomyocyte Autophagy. The term autophagy was first described by Christian de Duve in 1963, referring to a self-protective process in which damaged organelles and defective proteins were decomposed and recycled [36]. There are grossly three kinds of autophagy,

macroautophagy, microautophagy, and chaperone-mediated autophagy [37]. However, here we will primarily discuss macroautophagy (referred to as autophagy in this article) since it is the most studied pathway. There are several phases in the autophagy process. Stimulation like starvation (especially depletion of amino acids) could induce the initial step of autophagy [37], through the inactivation of mTORC1 (i.e., the mammalian target of rapamycin complex 1), which could in turn positively regulate the ULK1 complex [38]. On the other hand, when stimulated by insulin or other growth factors, class I PI3K-AKT could inhibit autophagy via two separate pathways: activation of mTORC1 and suppression of Beclin1-VPS34 complex [38]. After initiation, products destined for degradation are recruited, and the isolated membrane elongates, closes, and eventually fuses with lysosomes [36]. The membrane of autophagosomes comes from endoplasmic reticulum, Golgi complex, mitochondria, and plasma membrane. The formation of autophagosomes is primarily governed by ATG proteins, among which the Atg12-Atg5-Atg16L1 complex and LC3I-PE complex (LC3II) are equally essential [36–38]. More details regarding ATG proteins are described in Choi et al.'s outstanding review [38]. In physiological conditions, autophagy maintains cellular homeostasis, controls the quality of mitochondria, and contributes to organ development by degrading nonfunctional organelles and proteins. This checkpoint control assures that degradation is not simply a waste. Autophagy can process cellular components into amino acids, lipids, and sugar constituents, which later could be utilized in protein synthesis or production of glycogen or act as a direct supply for ATP [38, 39]. The significance of autophagy in cardiac development was confirmed by evidence that specific ATG5 knockout in the heart could ultimately lead to malformation and dysfunction of the heart, resulting from loss of autophagy [40]. Furthermore, ATG5 upregulation increased lifespan probably due to enhanced autophagy [41].

MST1, a proapoptotic effector in cardiomyocytes [7], concomitantly modulated autophagy in response to stress [42]. Apart from its central role in the Hippo-YAP pathway, MST1 also phosphorylates Beclin1 on the Thr108 residue in the BH3 domain, disturbing the Beclin1-PI3K class III complex, thus reducing autophagy flux [42]. Of note, the Beclin1 phosphorylation strengthens the stability of the Beclin1-Bcl-2 complex, thus promoting apoptosis [42]. Current reports indicate that MST1 may be involved in the pathogenesis of atherosclerosis progression [43], diabetic coronary microvascular dysfunction [44], and diabetic cardiomyopathy [45] by increasing apoptosis and suppressing autophagy. The indispensable role of MST1 in cardiac autophagy was demonstrated by Sun et al.'s research [46–50]. In mice subjected to diabetic cardiomyopathy or myocardial infarction (MI) induced-surgery, Melatonin promoted heart function by enhancing autophagy and weakening apoptosis, as indicated by the elevated expression levels of Beclin1, Atg5, LC3II, and decreased p62. Moreover, this effect was observed by inhibition of MST1 phosphorylation, and it could be abolished in the MST1 double knockout mice [46, 49]. Similarly, the protective effect of oncostatin M (OSM) in MI mice model was subverted by knockout of MST1 [47]. Furthermore, Sirt3, a downstream regulator of MST1, was found to be a key modulator of autophagic flux in cardiomyocytes under the treatment of Polydatin in an MI model [50]. However, a recent study has identified LC3 as a novel target of MST1/2, and phosphorylation of LC3 on its Thr50 residue by MST1/2 can promote the fusion step of autophagy [51].

NDR1, another proapoptotic protein, plays an essential role in autophagy [5, 52] by resembling LATS1/2. NDR1 functions as the positive upstream phosphorylation regulator of YAP [5]. NDR1 is required in the early stage of autophagy through interactions with Beclin1 [52]. Overactivation of NDR1 may cause apoptosis even though it was in the case of autophagy induction [52]. To investigate mechanisms balancing between apoptosis and autophagy, Joffre et al. [52] successfully revealed that RalB restrained the activity of NDR1 by preventing hyperactivation.

Moreover, it has been shown that YAP also participates in the autophagy process, playing a protective role in breast cancer [53]. However, its role in cardiomyocytes is worthy of further elucidation.

4. Possible Therapeutic Insights

Homeostasis has vital importance in maintaining the normal physical function of the cardiovascular system, thus Hippo signaling, and autophagy can play an important role. Discovering that autophagy has a cardioprotective role in MI, Kanamori et al. suggested a therapeutic role in treating MI [54]. Recent studies have confirmed this theory and demonstrated that Melatonin, Luteolin, and OSM can enhance the autophagic flux and suppress apoptosis by subverting phosphorylation of MST1, ultimately attenuating cardiac dysfunction after MI [46–48]. Moreover, Melatonin can also exert a similar effect on diabetic cardiomyopathy [49], while Polydatin demonstrated to beneficially elevate autophagic flux and limit cellular apoptosis in the context of MI [50]. Fan et al. described an interesting phenomenon. AS-1 (hydrocinnamoyl-L-valyl pyrrolidine), a TIR/BB-loop mimetic, can protect cardiomyocytes from hypertrophy in the context of pressure overload by partially increasing phosphorylation of LATS1 levels [22]. Moreover, acetylation of VGLL4, a tumor suppressor, can stimulate the combination activity of YAP and TEAD, providing an interesting insight of their dual role in heart regeneration [55].

In conclusion, our current understanding of the Hippo pathway and its role in the cardiac field remains insufficient. Furthermore, the Hippo pathway might have a different role in different types of cells. Further knowledge about its underlying mechanisms may help identify novel therapeutic targets.

Conflicts of Interest

The authors declare no competing financial interests.

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Review Article

Role of MicroRNAs in Obesity-Induced Metabolic Disorder and Immune Response

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In all living organisms, metabolic homeostasis and the immune system are the most fundamental requirements for survival. Recently, obesity has become a global public health issue, which is the cardinal risk factor for metabolic disorder. Many diseases emanating from obesity-induced metabolic dysfunction are responsible for the activated immune system, including innate and adaptive responses. Of note, inflammation is the manifest accountant signal. Deeply studied microRNAs (miRNAs) have participated in many pathways involved in metabolism and immune responses to protect cells from multiple harmful stimulants, and they play an important role in determining the progress through targeting different inflammatory pathways. Thus, immune response and metabolic regulation are highly integrated with miRNAs. Collectively, miRNAs are the new targets for therapy in immune dysfunction.

1. Introduction

Obesity is the result of imbalanced energy intake and expenditure, which is defined as abnormal or excessive ectopic fat accumulation in peripheral tissues that may impair health. It is estimated that by 2030, the overweight adults (body mass index (BMI) > 25 kg/m^2) are projected to be 1.35 billion, and 573 million of these are considered clinically obese (BMI > 30 kg/m^2) in the world [1]. Obesity plays an important role in the dysfunction of the liver, cardiac, pulmonary, endocrine, and reproductive systems, resulting in serious metabolic disorders, such as diabetes, fatty liver disease, atherosclerosis, and some cancers. This imposes a spectacular burden on personal health, society, and economy. Treatments of the escalating obesity and metabolic disorder have been a long journey which requires efforts from each level of society. Further, medical therapy and surgery are also powerful measures to shape tackling and curbing programs.

Since the beginning of life, metabolic response and immune system are highly interwoven for tissue and organismal health. It was reported that immune cells, such as macrophages and mast cells, infiltrated adipose tissue in obese animal models [2], suggesting an immunological nature of metabolic disease. This observation can clarify another study which showed that some diabetic patients treated with aspirin exhibited rapid improvement in glucose homeostasis [3]. On the other hand, dysimmunity is paramount for metabolic disorder. Fox et al. reported that patients with meningitis exhibited a transient diabetic syndrome [4]. Another study also found that treatment with lipopolysaccharide in dogs caused resistance to insulin by abrogating the ability of insulin to induce glucose uptake in the muscle [5]. Besides, it was recognized that acute infection in human patients was associated with decreased binding of insulin to the insulin receptor in isolated blood cells [6]. Hence, delicate regulation of these pathways is vital for cell homeostasis.

miRNAs are small noncoding, endogenous, single-stranded RNAs usually consisting of 18–25 nucleotides that regulate gene expression through repression or degradation

of targeted mRNAs at the posttranscriptional level [7]. It is estimated that about 30–50% of protein-coding genes are regulated by miRNAs [8]. Disrupted expression of miRNAs participating in cell process is related to many diseases, such as obesity-induced hyperlipidemia, nonalcoholic fatty liver disease (NAFLD), type 2 diabetes mellitus (T2DM), and atherosclerosis, through regulation of multiple genes [9]. In the immune system, the feedback networks serve to regulate protein expression at a steady state and conditions of environmental stress that are necessary for fate commitment [10]. Therefore, miRNAs implicated in immune system development and function have a potential role in the therapeutics for immune-related diseases.

In this review, we focus on obesity-induced metabolic disorder with the goal to illustrate the links with immune response and the role of miRNAs and therefore to develop effective therapeutic strategies.

2. miRNAs and Obesity-Induced Metabolic Disorder

Obesity is the primary target for prevention and treatment as elevated serum concentration of lipid in obese subjects may lead to severe disturbances (lipotoxicity) and inevitable metabolic disorder. Adipose tissue is the main organ for lipid storage; however, excess calories will change endocrine functions of adipocytes and the ectopic fat accumulation in peripheral tissues, such as liver, skeletal muscle, pancreatic β-cells, and kidney [11], will lead to lipotoxic stress and low-grade inflammation, accompanied by metabolic disorder.

In the last few years, there has been a growing interest in the role of miRNAs in the development of obesity-induced metabolic disorder. These miRNAs play important roles in physiologic and pathophysiological conditions which participate in cell differentiation, proliferation, apoptosis, hematopoiesis, limb morphogenesis, and important metabolic pathways, such as insulin secretion, triglyceride and cholesterol biosynthesis, and oxidative stress [12, 13]. Among these, it is shown that miR-103, miR-107, and miR-143 accelerate fat cell development [14]. miR-935, miR-4772, miR-223, and miR-376b are reporters of diet-induced obesity [15]. And mice lacking miR-378 are resistant to obesity and exhibit enhanced mitochondrial fatty acid metabolism and elevated oxidative capacity in insulin target tissues [16]. miR-221, miR-28, and miR-486 are associated with BMI, percentage fat mass, waist, and regional fat distribution [17]. In addition, miR-126, miR-15a, miR-29b, miR-223, and miR-28-3p are related to T2DM, and miR-155, miR-302a, and miR-712 are related to atherosclerosis [18].

3. Inflammation and Immune Response in Obesity-Induced Metabolic Disorder

The living organisms activate the immune system composed of cell lineages residing in lymphoid organs or vary tissues and transit through the peripheral blood against infectious pathogens. Inflammation is a self-protective response with the goal to clear antigens and return the system back to a normal baseline, which recruits leukocytes to fat, but lacks many of the cardinal signs of classic inflammation, such as dolor, rubor, calor, and tumor. Immune activation is recurrent in superimposed metabolic disorder on obesity with tonic low-grade inflammation. Innate and adaptive immune responses are different kinds of immunity interacting with additional cells to form dynamic cellular communities in tissues. Innate immunity is an intrinsic, cell-autonomous response representing the first barrier of fast-acting defense against pathogens, while adaptive immune response stimulates antigen-specific receptor molecules expressed by T and B lymphocytes [19]. Excess lipid in obese individuals is the main cause of metabolic disorder. Likewise, it may also influence the ability of the immune system. Thus, it is vital to evaluate the role of immune response and inflammation in the obesity-induced metabolic disorder.

All metabolic tissues contain resident populations of immune cells, and all cells with normal metabolism perform cell-type-specific biological functions involved in immune responses against ambient environment [20], which gave birth to the concepts of "immunometabolism" [21] and "metainflammation" [22]. Immunometabolism is proposed to depict metabolism connected to immunity and the metabolic impact on immune cell function, while metainflammation is a discipline of chronic low-grade inflammatory response to obesity.

There are multiple signaling pathways participating in promoting obesity-derived diseases and involved in the progress of inflammation. Lipid can act directly on cells of the innate system to promote the development of Th2-type responses associated with allergy or through CD1 to capture and present lipid antigen restricted to T lymphocytes, which can promote allergic reactions [23, 24]. Macrophages are important for lipid sensing and induction of the inflammatory programming from an anomalous activation of the innate immune system. In the presence of a continuous nutritional surplus, foreign pathogen molecules such as lipid or saturated fatty acids are sensed by lipid transporter, pattern recognition receptors (PRRs, such as Toll-like receptors (TLRs), and Nod-like receptors (NLRs)) or other cytokine receptors to initiate a defense response. Intracellular lipids are recognized to ligate several immune receptors by TLRs and subsequently induce inflammatory activity and inflammatory gene transcription, resulting in the production and secretion of cytokines such as tumor necrosis factor (TNF) and interleukin 6 (IL-6) [25], which are overexpressed in the adipose tissue of obese mice providing the first clear link between obesity and induced metabolic disorder [26]. IκB kinase-β as the downstream target and activation of IKKβ/NF-κB is crucial in inflammation in the obese state. Besides, in insulin-responsive tissues, JNK is activated by fatty acids, insulin, hyperglycemia, and inflammatory cytokines [27]. Another downstream pathway is endoplasmic reticulum (ER) stress, which activates unfolded protein response and governs multiple metabolic responses [28]. In addition, lipid recognized by NLRs activated Caspase1 and ultimately resulted in ROS activation and the release of IL-1β and IL-

18 [29]. Thus, the lipid accumulation tissues which are populated by macrophages and other immune cells give rise to chronic activation of inflammatory pathways in the setting of obesity (Figure 1). Further work is needed to show the precise cell signal for deep understanding of the response against pathogen infections.

3.1. Hyperlipidemia and Atherosclerosis. Hyperlipidemia, a chronic disorder with high levels of triglyceride (TG, hypertriglyceridemia), total cholesterol (TC, hypercholesterolemia), and low-density lipoprotein cholesterol (LDLC) and a decreased level of high-density lipoprotein cholesterol (HDLC), is a manifest consequence of obesity. Lipid droplets are absorbed by intestine cells and transported to tissues for storage and expenditure. Thus, regulation of lipid absorption, generation, and expenditure is crucial in determining circulating lipid levels. To understand the prevalence of hyperlipidemia in China, Li et al. determined TG, TC, HDLC, and LDLC levels in fasting serum for 97,409 subjects who were selected by multistage stratified cluster random sampling from 162 surveillance points of 31 provinces in 2010. After the complex weighting, data showed that prevalence of hypertriglyceridemia, hypercholesterolemia, high blood LDLC, and low blood HDLC in Chinese adults was 11.3%, 3.3%, 2.1%, and 44.8%, respectively [30].

Atherosclerosis is a result of fatty streak lesions initiated by macrophages forming foam cells trapped beneath the endothelial cell lining in the artery [31]. It is enhanced after continued recruitment of immune cells and subsequent proliferation and migration of smooth muscle cells to larger fibrofatty plaques, followed with significant narrowing of the arterial lumen, leading to chronic syndromes, such as cardiovascular disease [32]. The major clinical manifestations of atherosclerosis include ischemic heart disease, ischemic stroke, and peripheral arterial disease. It is the leading cause of death worldwide which is declared by the World Health Organization to highlight its prevalence threat to public health.

Atherosclerotic lesions recruit inflamed endothelial cells in postcapillary venues, such as intracellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1. Macrophage scavenger receptor type A expressed by immune cells recognizes and facilitates the phagocytosis of specific surface molecules of pathogens. Besides, CD36 and TLRs are also receptors regulated by macrophages and endothelial cells contributing to inflammation [33, 34], which can provide a link between systemic inflammation and local infection in driving plaque growth or engendering atherosclerotic plaque instability. The role of inflammatory cytokines and mediators influence the development of atherosclerotic lesions [35]. In addition, interferon-γ and IL-18 are two Th1 cytokines involved in proatherogenic reaction. Recent studies show that IL-18 receptor is expressed in multiple immune cells within human atherosclerotic plaques, while intraperitoneal injection of recombinant IL-18 increased atherosclerotic-lesion size twofold in $ApoE^{-/-}$ mice [36, 37].

3.2. NAFLD. NAFLD is a pathologic syndrome ranging from simple steatosis through steatohepatitis to fibrosis and cirrhosis which are characterized by excess fat accumulation in hepatocytes that is associated with an enlargement of the liver (hepatomegaly) accompanied by inflammation, leading to loss of metabolic competency as reduced mitochondrial β-oxidation capacity and induced endoplasmic reticulum stress, oxidative stress, and hepatocyte apoptosis. It is the major risk factor of chronic liver disease in the developed countries as the prevalence of steatosis in patients with obesity is about 75% [38]. In a US community, the incidence of NAFLD diagnosis increased 5-fold from 1997 to 2014 [39]; therefore, it was expected that within the next decade, NAFLD-associated hepatic disorder could be the most common. In general, nearly 10–20% of NAFLD patients will progress to nonalcoholic steatohepatitis (NASH) and 8–25% of NASH patients may develop liver cirrhosis. Up to 2.8% of NASH cases may further develop into end-stage liver disease or hepatocellular carcinoma [40].

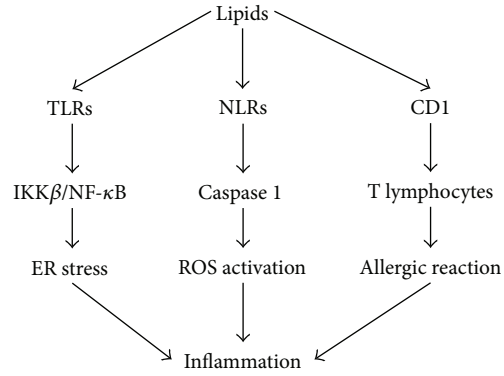

Figure 1: Inflammation pathways induced by lipid. Lipid can be recognized by multiple molecules resident in cellular membrane, such as TLRs, NLRs, and CD1, to activate different signal pathways and ultimately induce inflammation. TLRs are responsible for IKKβ/NF-κB and ER stress augment, while NLRs activate Caspase1 expression and induce ROS production. Besides, lipid captured by CD1 can be presented to T lymphocytes directly. All these signals participate in translating lipids to inflammatory response.

NAFLD is characterized of hepatic lipid accumulation accompanied with inflammation. Acute immune response and coordinated network of multiple cell types are essential for maintaining metabolic homeostasis. In lipid accumulation tissues, aggregation macrophages predominantly assume a classical proinflammatory activation state (M1) through Th1 responses while reducing an alternative macrophage activation state (M2) generated by Th2 cytokines which promotes fibrotic responses [41, 42], resulting in the suppressed recruitment of eosinophils and attenuation of classical NF-κB-dependent activation pathways [43, 44], leading to low-grade inflammation. Many of the signaling pathways such as TLR, JNK, and ER stress were elevated in steatotic liver inducing inflammation and metabolic dysfunction. In addition, M1/Th1 cytokines are increased mediated by immune cells recruited to the liver.

3.3. T2DM. Glucose homeostasis is controlled by multiple organ system, including brain, pancreas, and peripheral

tissues (such as liver, adipose tissue, and skeletal muscle). In the fasted state, release of glucose from liver is a key for euglycemia. Circulatory glucose originates from hydrolysis of glycogen (the polysaccharide storage form of glucose) in the liver as well as from gluconeogenesis (de novo production of glucose from non-glucose-derived carbon precursors). As a compensatory response to postprandial hyperglycemia, plasma insulin concentration rises to maintain normal glucose homeostasis by inducing glucose uptake in the skeletal muscle and liver while simultaneously inhibiting hepatic glucose production [45]. However, nutritional excess enhances the secretion of insulin but blunts the response of organs to insulin and ultimately results in the clinical manifestation of T2DM, the most studied multifactorial metabolic disorder associated with obesity. The global prevalence of T2DM is rapidly increasing, and epidemiologists predict that the number of patients will double in the second half of the twentieth century by 2030 in China [46]. The number of people with diabetes mellitus is projected to rise to 439 million globally, which represents 7.7% of the total adult population of the world's adults [47].

T2DM is relevant to synergistic action by multiple organs, such as pancreatic islets, liver, adipose tissue, and skeletal muscle, which coordinated to determine circulatory glucose level and insulin action. Pancreatic islets are the critical cells for insulin secretion. When the lipid is overwhelmed, macrophages are recruited and produce proinflammatory cytokines to induce inflammation, which result in blunted β-cell function, reduced insulin secretion, and cell apoptosis, leading to decreased islet mass. Excess lipid in liver, adipose tissue, and skeletal muscle has causal relationship with insulin resistance. The increased adipose tissue mass is related to an estimated excess of 20–30 million macrophages that accumulate with each kilogram of excess fat in humans [48]. The inflammatory cytokines are increased in obesity coupled with myocytes' capacity in response to inflammatory and metabolism [49, 50]. Infiltrating macrophages accumulated in muscle induce M1 activation [51].

Taken together, at the molecular and cellular levels, excess nutrients such as lipid can induce secretion of cytokines and trigger inflammatory responses in obesity-induced disorder.

4. Role of miRNAs in Metabolic Disorder and Immunity

There are many miRNAs enriched in immune response dysfunction to affect immunity [52]. miR-125 has a vital role in maintaining normal inflammatory cytokine output, which targets several mRNAs that are important in development and apoptosis, thereby altering immune cell biology in complex ways. Overexpression of miR-125a decreases cell apoptosis and increases total number of bone marrow cells [53]. Beyond this, many others have been linked to the modulation of immune cell development. A recent study shows that ectopic expression of miR-142 has been found to increase production of T lymphocytes *in vitro* [54]. Besides, miR-221 and miR-222 are downregulated during erythropoiesis, thus relieving repression of their target, which encodes the stem cell factor receptor c-Kit [55].

In addition to function on gene expression participating in immune response, miRNAs also influence metabolism. For example, miR-100, miR-130, and miR-155 which is positive in macrophage infiltration are inhibited with adipocyte differentiation [18]. miR-155 is ubiquitously expressed, not only in many haemopoietic cell types but also in human reproductive tissues, fibroblasts, epithelial tissues, and central nervous system [56]. The miR-155 is encoded by a gene originally isolated near a common retroviral integration site-induced lymphomas [57]. It is found that this miRNA is upregulated in atherosclerosis which is coordinated with lipid and inflammation [58]. Also, its expression is downregulated in mature immune cells and increased in adaptive macrophages after exposure to inflammatory cytokines [59, 60]. The importance of proper regulation of miR-155 expression is exemplified by its much higher expression in response to infection [61–63].

miR-33 is the typical miRNA abundant in lipoprotein particles which is crucial in lipid metabolism [64]. Targets of miR-33 include key enzymes of fatty acid uptake and metabolism such as CPT-1, AMPK, and β-hydroxyacyl-CoA dehydrogenase [65]. Moreover, overexpression of miR-33 significantly inhibits cellular fatty acid oxidation and enhances mitochondrial oxidative capacity and ATP production [66, 67]. Further, numerous studies have regarded miR-33 as the therapy target of obesity and induced metabolic disorder [68]. At the same time, a recent study shows that miR-33 regulates the innate immune response via ATP-binding cassette transporter [69]. Consistent with this, $Abca1^{-/-}$ and $Abcg1^{-/-}$ macrophages have increased TLR proinflammatory responses, which indicate that miR-33 augment TLR signaling in macrophages via a raft cholesterol-dependent mechanism. Another study shows that miR-33 controls adaptive fibrotic response in the remodeling heart by preserving lipid raft cholesterol [70].

5. Conclusions and Future Therapeutic Directions

miRNAs are now widely regarded as playing a critical role in regulating homeostasis of obesity-induced metabolic disorder and immune response by fine tuning the expression of a network of genes through posttranscriptional regulation. Specific miRNA expression profiles can be utilized as biomarkers for diagnosis, prognostic purposes, and clinical development in various diseases [71]. However, studies in demonstrating the therapy role of miRNAs in metabolic disorder and dysimmunity are lagging.

In this review, we analyze the important role of miRNAs in obesity-induced metabolic disorder and immune response. We listed many diseases induced by obesity, such as NAFLD, T2DM, hyperlipidemia, and atherosclerosis, which have affinity with miRNAs. These miRNAs participate in many pathways and regulate metabolism progression, including insulin secretion, triglyceride and cholesterol biosynthesis, and oxidative stress. Moreover, metabolic disorder accounts for dysimmunity as ectopic and excess lipid accumulation

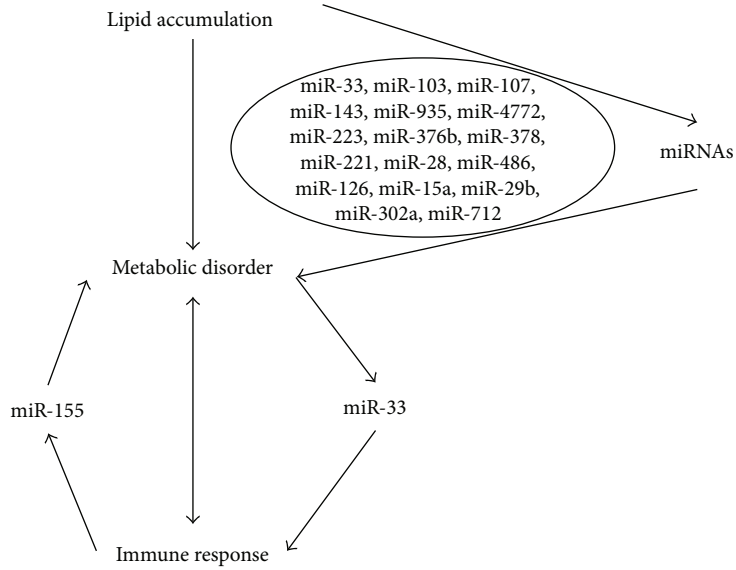

Figure 2: Role of miRNAs in obesity-induced metabolic disorder and immune response. Many miRNAs are regulated by lipid accumulation and play a mediatory effect in coupling obesity and metabolic disorder. Moreover, they also mediate the induction of immune response. Here, we illustrated two typical miRNAs to show the critical role of miRNAs in the interactions of metabolic disorder and immune response.

in cell can be detected by multiple signals, such as TLRs, NLRs, and CD1, to initiate inflammatory response. And dysimmunity is accompanied by metabolic disorder as patients with meningitis exhibited an instant diabetic syndrome. Further, miRNAs play a crucial role in coupling metabolism and immunity. As shown before, miRNAs regulated by immune response can regulate the development of obesity-induced metabolic disorder. On the contrary, immune response regulated by metabolism is mediated by miRNAs (Figure 2).

Thus, it is expected that a better understanding of miRNAs in obesity-induced disorder and immune response will lead to the discovery of the potential therapy role of miRNAs in metabolic and immune-related disorder. And further work needs to accelerate the clinical use of miRNAs.

Abbreviations

BMI: Body mass index
ER: Endoplasmic reticulum
HDLC: High-density lipoprotein cholesterol
IL: Interleukin
LDLC: Low-density lipoprotein cholesterol
miRNAs: MicroRNAs
NAFLD: Nonalcoholic fatty liver disease
NASH: Nonalcoholic steatohepatitis
NLRs: Nod-like receptors
PRRs: Pattern recognition receptors
TC: Total cholesterol
TG: Triglyceride
TLRs: Toll-like receptors
TNF: Tumor necrosis factor
T2DM: Type 2 diabetes mellitus.

Conflicts of Interest

The authors declare no conflict of interest.

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Review Article

Metabolic Plasticity in Dendritic Cell Responses: Implications in Allergic Asthma

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Dendritic cells (DCs) are highly specialized in antigen presentation and play a pivotal role in the initiation, progression, and perpetuation of adaptive immune responses. Emerging immune pathways are being recognized increasingly for DCs and their subsets that differentially regulate T lymphocyte function based on the type and interactions with the antigen. However, these interactions not only alter the signaling process and DC function but also render metabolic plasticity. The current review focuses on the metabolic cues of DCs that coordinate DC activation and differentiation and discuss whether targeting these fundamental cellular processes have implications to control airway inflammation and adaptive immunity. Therefore, strategies using metabolism-based therapeutic manipulation of DC functions could be developed into novel treatments for airway inflammation and asthma.

1. Introduction

Dendritic cells (DCs) are heterogeneous population of rare hematopoietic cells that coevolved ontogenically with the emergence of the adaptive immune system during evolution. Myeloid-derived effector cells, such as DCs have a rapid turnover in general. For example, under normal steady-state conditions, the average residence time of airway epithelial DCs is only 3 days, suggesting that these cells are continuously made by the hematopoietic system [1]. DCs differentiate in the bone marrow (BM) from hematopoietic stem cells (HSCs), which give rise to a common dendritic progenitor (CDP) that is restricted to the DC fate based on their potential of FMS-like tyrosine kinase 3 (Flt3) expression and ability to respond to Flt3 ligand (Flt3L) for maintenance. The CDP produces preplasmacytoid DCs (pDCs) and preconventional DCs (cDCs), the latter of which leaves the BM and circulates in the blood before entering the lung tissue and further develops into DC subsets (*for review of DC subsets and nomenclature see* Merad et al.) [2–4]. The identification of distinct dendritic cell subset has fostered the concepts of distinct mechanism of tolerance and immunogenicity in adaptive immune response, which are triggered by pathogens, allergens, infections, and inflammation. Importantly, lung DCs are located mainly at the basolateral side of the epithelium and able to sense the surrounding tissues as well as the alveolar airspace to capture antigens and migrate to the lung-draining mediastinal lymph nodes, to maintain lung immune homeostasis in steady state and inflammation [5]. Under noninflammatory conditions, DCs remain in a quiescent resting state and are poorly immunogenic, whereas environmental triggers such as allergen, germline-encoded pattern recognition receptor (PRR) ligands, for example, Toll-like receptor (TLR) ligands, viruses, growth factors (granulocyte macrophage colony-stimulating factors (GM-CSF)), or inflammatory cytokines trigger the process of activation and maturation of DCs and make them highly immunogenic. In particular, the major lung $CD11b^+$ cDC (conventional/classical dendritic cells (cDC2)) subset has recently been shown to initiate and effectively generate Th2-mediated adaptive immune responses to inhaled aeroallergens, such as house dust mite (HDM) [6–8]. Similar and overlapping functions of $CD103^+$ lung DCs (cDC1) have been suggested to be associated with Th1 or Th2 responses in airway inflammation, whereas plasmacytoid DCs have poised towards type I interferon production, viral clearance, and tolerance [9–12]. In

aggregate, monocyte-derived dendritic cells (moDCs; originally branched from common monocyte progenitors (cMoP) in the BM) maintained in the lung are capable to initiate Th2 immune responses in asthma [3, 7].

It is increasingly recognized that the activation and immune-priming function of DCs are coupled to profound alterations of the cellular metabolic state and are comprised of eliciting a DC-specific response. Moreover, evidence is accumulating for distinct metabolic requirements of these DC subsets for their immune-priming and immune-polarizing function that regulates airway inflammation. This metabolic plasticity of DC function in adaptive immunity could pave the way for our current understanding in asthma initiation, perpetuation, and progression. This review discusses how DC metabolism is interlinked with the adaptive immune pathways and whether these signaling cues are attractive targets for developing novel treatments for asthma and airway disease.

2. Metabolic Plasticity of the DC Progenitors

Dual metabolic regulation by long-term HSCs (LT-HSCs) during self-renewal and quiescence primarily relies on anaerobic glycolysis. Lineage-committed progenitors rapidly enter into the Krebs cycle to meet the unique set of bioenergetic demands required for cell proliferation and differentiation via the mitochondrial oxidative phosphorylation (OXPHOS) switch. Interestingly, several observations have identified that mitochondria in HSCs are relatively inactive and produce less mitochondrial reactive oxygen species (mit-ROS) as compared to the progenitor cells [13]. In the bone marrow, HSC entry into cell cycle is triggered by increased levels of ROS that corresponds to the metabolic switch from glycolysis to mitochondrial OXPHOS. This metabolic reprogramming provides the robust energy required for HSC differentiation into committed progenitors [13, 14]. Studies using the conditional inactivation of protein tyrosine phosphatase mitochondrial 1 (*Ptpmt1*) in HSCs show rapid inactivation of differentiation and cell divisions, which slows down the mitochondrial respiration and increases the anaerobic glycolysis. The substrates of *ptpmt1*, such as phosphatidylinositol phosphates (PIPs), activate the mitochondrial uncoupling protein 2 (UCP 2), thereby blocking the glucose-derived mitochondrial pyruvate oxidation in *Ptpmt1*-deficient HSCs and progenitors [15]. Mechanistically, the exit from quiescence of HSCs is sensed by low level of DNA damage or stress that is regulated by the master transducer of the DNA damage response signaling pathways, such as ATM (ataxia telangiectasia mutated). This, in turn, blocks the BH3-interacting domain death agonist BID (an effector of the ATM kinase in DNA damage response) to enter into differentiation [16]. Furthermore, mitochondrial carrier homolog 2 (MTCH2), an outer mitochondrial membrane receptor, acts as a negative regulator to increase mitochondrial metabolism and ROS productions, thereby determining the HSC fate [17, 18]. Collectively, these findings confirm the key drivers of HSC fate decision in which a low level of ATP through anaerobic glycolysis is required to prevent ROS generation and to maintain quiescence. In contrast, high ATP and ROS levels through fatty acid oxidation (FAO) pathway and tricarboxylic acid (TCA) cycle determine commitment and drive the differentiation through asymmetric cell divisions.

In the bone marrow, DC-committed progenitors (common dendritic cell progenitors or CDPs) originate from HSCs that give rise to preconventional DCs (pre-cDCs) and plasmacytoid DCs (pDCs) [19, 20]. The pre-cDCs further differentiated into cDC1 and cDC2 lineage and exit the BM and migrate to peripheral organs, such as the lung [21]. In contrast, the human monocyte-derived DCs (moDCs; equivalent to mouse Ly6C^{hi} moDCs) that originate from common monocyte progenitors (cMoP) are activated by LPS or CD40 ligand (CD40L) via peroxisome proliferator-activated receptor gamma (PPAR-γ) pathways for maturation and type 2 immune responses [22]. Increased expressions of the transcription factor PPAR-γ and PPAR-γ coactivator 1α (PGC1α) have also been implicated in the process of moDC differentiation through active lipid metabolism and mitochondrial biogenesis [23–25]. The ability of monocytes to differentiate into moDCs is primarily dependent on increased citrate synthase activity and the conversion of mitochondrial citrate to cytosolic acetyl-CoA, an important intermediate in the process of fatty acid synthesis [26]. This suggests that differentiations of DCs are integrated and regulated by mitochondrial functions and metabolic pathways of fatty acid biosynthesis. Analysis of mouse DC progenitors in the presence of the serine/threonine kinase, mammalian target of rapamycin complex 1 (mTORC1), impairs Flt3L-driven differentiation and mobilization of pDCs and cDCs from the bone marrow both *in vitro* and *in vivo* [27–29]. Consistent with mTOR function in DC development, a recent study by Wang et al. has identified tuberous sclerosis 1 (Tsc1) (a modulator of mTOR1 and mTOR2 activities), which acts as a negative regulator and blocks the differentiation of cDCs and pDCs from DC progenitors. This is further associated with increased expression of the transcription factor Myc, thereby is regulated in the biosynthetic and bioenergetic programs for DC development [30]. The expression of Myc is coupled by mTORC1 and is crucial for activation and expression of proteins involved in glycolytic pathway [31]. In particular, the Myc paralogue MYCL expression is increased in DC progenitors, indicating the broader importance of Myc in mitochondrial biogenesis and respiration [32, 33]. Altogether, these results address the fundamental bioenergetic properties of DC-committed progenitors and the regulatory pathways of DC differentiation. Further investigations in this area would highlight the metabolic framework of CDPs, pre-cDCs, and pre-pDCs in the context of their mobilization from the bone marrow into the bloodstream and tissue differentiation during steady state and inflammation.

3. Metabolic Sensors of DC Metabolism

The metabolic sensors on DCs translate the information of the cellular energy levels into the biological responses by different signaling modules. The important exogenous key metabolites that trigger the nutrient-sensitive anabolic and/or catabolic pathways to support DC differentiation and function are discussed here.

The recent identification of surface markers for both human and mouse DCs has enabled their purification with high efficiency. Currently, the state-of-the-art seahorse flux analyzer platform, developed by Agilent, is equipped to perform highly accurate real-time measurement of extracellular acidification (ECAR) and oxidative phosphorylation (OCR) as surrogate measures of cellular glycolysis and mitochondrial respiration, respectively. These fundamental bioenergetic measurements have enabled us to further study DC metabolism during steady-state and inflammatory condition in different tissues. The nutrient-sensitive PI3K-AKT-mTOR pathway promotes the production of ROS and regulates DC activation and proliferation by modulating glycolysis and anabolic metabolism. Compensatory to the glycolytic regulation, the Krebs/TCA cycle activity slows down and increases pentose phosphate pathway (PPP), thereby fuel nicotinamide adenine dinucleotide phosphate (NADPH) for the NADPH oxidase enzyme to generate ROS [34, 35]. Importantly, a regulatory role for the coenzyme β-nicotinamide adenine dinucleotide (NAD^+) and its reduced form NADH in primary T cell development, proliferation, and differentiation have been recently reported [36–38]. However, similar roles for NAD^+ and its reduced forms in DC immunometabolism are yet to be investigated. The activation of PI3K by growth factors and nutrients, such as glucose and amino acids, further increases the cellular levels of phosphatidylinositol-3, 4-biphosphate (PtdIns (4,5) P_2) and PtdIns-3, 4, 5-triphosphate (PtdIns (3,4,5) P_3). This induces a conformational change in AKT [39]. Studies using an inhibitor of mTOR, Tsc1-deficient cDCs (cDC2), indicate increased expression of the DC maturation markers CD80, CD40, and CD86 that identifies the essential role of the tumor suppressor Tsc 1 as a negative regulator of mTOR signaling for DC maturation [30]. PI3K-mTOR signaling pathway has essential roles in IFN-α production from pDCs and has been shown to selectively inhibit the transcriptional activation of IL6, TNF-α, and IL10 cytokines in the presence of the mTOR inhibitor rapamycin during TLR-mediated DC activation [40–43]. Another important key player in DC metabolism is hypoxia-inducible factor 1α (HIF1α), a transcription factor that acts as a sensor to low oxygen availability. HIF1α has been shown to be associated with TLR-dependent activation of DCs [44–46]. The prolyl hydroxylase 2 (PHD2) hydroxylates the two proresidues that are present in the O_2-dependent degradation domain of the HIF1α, which primes HIF1α for degradation under normoxic conditions. In contrast, when O_2 levels are low (hypoxic conditions), this hydroxylation is inhibited, which prevents the degradation of HIF1α and promotes the accumulation of HIF1α and HIF1β complexes on the promoters of glycolytic genes [47]. Notably, the LPS-induced IL-1β production and NLRP3 inflammasome activation in macrophages are mediated through metabolic reprogramming [48, 49]. Macrophage-derived mature IL-1β productions require two consecutive signals. Activation of HIF1α promotes the transcription of pro-IL-1β and is driven by accumulation of the TCA/Krebs cycle intermediate succinate [50], whereas the second signal is generated by multiple stimuli including ATP to activate NLRP3 inflammasomes, which is regulated via the glycolytic enzyme hexokinase [51]. This is important, since the macrophage-derived IL-1β production and NLRP3 activation have been shown to expand the IL-17-producing type 3 innate lymphoid cells (ILC3 cells) in obesity-associated airway hyperactivity [52]. Although HIF1α modulates the expression of several glycolytic enzymes and activates glycolysis, however, studies indicate that transient TLR-mediated DC activation does not rely on mTOR-HIF1α signaling. Unsurprisingly, in contrast to PI3K signaling, the early TLR-dependent DC activation is mediated by downstream AKT signaling nodes, which phosphorylates and activates the rate-limiting enzyme hexokinase 2 (HK2) by a TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor-κB kinase subunit-ε (IKK ε) [53–55]. Collectively, these findings confirm that the TLR-dependent activation of DCs induces an early event of metabolic alterations. These bioenergetic demands are solely driven by glycolysis to further synthesize more fatty acids. Importantly, the metabolic alterations of DCs upon activation are supportive of a role for expansion of ER and Golgi to adopt secretory state, via an AKT-dependent signaling pathway. The cellular levels of AMP/ATP ratios determine the activation of the metabolic sensor AMP kinase (AMPK) and modulate the phosphorylation and activation, thereby inhibiting mTORC1 and antagonize the fatty acid synthesis pathways [56]. This in turn drives the catabolic processes, such as activating PGC1α and mitochondrial OXPHOS which are crucial in regulating DC activation [57, 58]. Several interesting observations regarding DC activation using AMPK-deficient DCs were made during these studies. First, AMPK knockdown increases TLR-induced glucose consumption and myeloid DC activation via CD40 signaling. Second, the effect of TLR-induced changes could be reversed by pharmacological activation of AMPK. Third, in the presence of resveratrol, activation of sirtuin1 (SIRT1) and PGC 1α suppresses HIF1α, which reduces mitochondrial membrane potential and ATP levels rendering DCs more tolerogenic [59, 60]. This is important since SIRT1 regulates the production of IL 27 and IFN-β through deacetylation of the transcription factor interferon regulatory factor 1 (IRF 1) and thereby regulates Th17-mediated immune pathways [61]. In aggregate, SIRT 1 function has been shown to promote the Th2-immune responses in airway inflammation by suppressing PPAR-γ activity in DCs [62]. Fourth, knockdown of the downstream partners of PGC 1α, for example, the transcriptional factor nuclear factor erythroid 2-related factor 2 (NRF2) or PPAR-γ in DCs enhances maturation, dysregulates redox homeostasis, and contributes to development and priming of $CD4^+$ T cells [63–65]. These findings collectively support the concepts that AMPK-mediated signaling pathways are potentially linked to metabolic changes in DC activation and promote tolerogenicity via PGC 1α activation to facilitate catabolic pathways.

4. Metabolic Plasticity of DC Activation

Resting or immature DCs are characterized by their reduced cytokine secretory capacity, priming, and ability to activate T cells, which solely fuel from ATP productions by oxidative

phosphorylation (OXPHOS) in mitochondria. These immature DCs express the germ-line encoded pattern-recognition receptors (PRRs), such as Toll-like receptors (TLRs), C-type lectin receptors (CLRs), that patrol and rapidly recognize and respond to the inflammatory triggers like environmental antigens, TLR ligands, and microbial products in the peripheral tissues under noninflammatory conditions [11]. It is noteworthy, although a "glycolytic burst" has long been demonstrated as a metabolic signature of TLR4 stimulation, more complex metabolic changes exist and are driven by whole microorganism and multiple TLR activation [66]. Therefore, the metabolic reprogramming of these cells will be of fundamental importance in the context of the tissue environment, nutrient availability, and disease state *in vivo* [67].

As summarized in Table 1, following encounter with the danger signals, DCs become activated, which are characterized by their enhanced ability to capture and process antigens and present the antigen-derived peptides to T cells. This in turn induces the genes encoding for cytokines, chemokines, and costimulatory molecules to exert T cell-specific immune responses. It has been recognized that homologous process of metabolic programming does exist in immature DCs as developing $CD8^+$ T cells employ glucose to fuel the demand for fatty acid biosynthesis and glycolysis in their quiescent state to rapidly assemble and respond to restimulation by antigens [57, 68, 69]. However, several studies have identified that the metabolic reprogramming following TLR-induced activation of DCs and T cells differs strikingly. Intriguingly, the glycolytic surge in early activations of DCs does not employ ATP for additional necessary bioenergetic resources as compared to the T cells that primarily count on mitochondrial OXPHOS necessary for their activation [70, 71]. Rather crucial to this early rapid "glycolytic burst," TLR activations in DCs are necessary for the de novo fatty acid biosynthesis via glucose-dependent citrate metabolism, which renders DCs with an immunogenic phenotype [44, 54, 72]. The early glycolytic changes are fundamental feature of activated moDCs, $CD11b^+$ cDCs (cDC2), and $CD8\alpha^+$ DCs (cDC1), and are primarily mediated via AKT-dependent signaling pathways. Furthermore, this contributes to the kinase-dependent activation of the rate-limiting glycolytic enzyme HK-II and its association with mitochondria [54]. Importantly, during inflammation, activation of DCs in response to TLR triggers is associated with an increased glycolysis that concomitantly shuts off mitochondrial OXPHOS. This glycolytic shift is both contributed from extracellular and intracellular glucose resources to meet the metabolic demands of DC immune activation and to support the synthesis of cytokine secretions [26, 73, 74]. Furthermore, activation-induced metabolic shift promotes the expression of inducible nitric oxide synthase (iNOS) and nitric oxide (NO) productions. This is mediated via PI (3) K signaling through mTORC1 which renders the inflammatory DCs for long-term commitment towards aerobic glycolysis and anabolic metabolism [75, 76]. In contrast, the TLR-induced long-term glycolytic changes are potentially associated with the mTOR-HIF-α axis, via iNOS expression which shuts off electron transport chain (ETC) by nitric oxide (NO) [44, 46, 77]. Taken together, these studies imply the two important metabolic states that exist during DC activation. The early TLR-driven, NO-independent rapid changes in glycolysis are mediated via AKT-driven activation of key glycolytic enzyme HK-II. In aggregate, the long-term NO-dependent commitment of glycolysis is supported by mTORC1 and induction of HIF1α (Figure 1).

5. Lung DC Subsets and Airway Inflammation

Distinct surface marker expressions and functional properties of DCs have enabled researchers to identify and define different DC subsets that play a crucial role in promoting Th2 immune responses in allergic asthma [6, 7, 78]. Committed DC precursor develops from CDPs in the BM and expresses the hematopoietic cytokine receptor Flt3. CDPs generate pre-cDCs which differentiated into lineage-specific cDC1 and cDC2 and circulate in the bloodstream and lung tissues [4]. Based on their distinct biological functions and surface expressions of integrin molecules, two major lung DC subsets, $CD103^+$ DCs (cDC1) and $CD11b^+$ (cDC2) DCs, have recently been recognized to play pivotal functions in allergic immune responses [6, 7, 79–81]. Although, $CD103^+$ DCs (cDC1) are primarily involved with viral antigens to cross-present $CD8^+$ T cells, however, it has recently been showed that in response to an innocuous inhaled antigen house dust mite (HDM) this subset of DCs is particularly capable to prime and mount Th2 immune response [82, 83]. Interestingly, the $CD11b^+$ lung DCs (cDC2) are more efficient to trap soluble antigens and to present to $CD4^+$ T cells, thereby initiating and producing Th2 immune response in allergic airway inflammations. The $CD11b^+$ DC subset in the airway pool is also contributed by circulating monocytes in steady state and also during inflammation, which originates as $Ly6C^{hi}CCR2^{hi}$ monocytes from common monocyte progenitors (cMoP) in the BM [19, 79]. These monocyte-derived DCs (moDCs) are primary responders to dose-dependent HDM-induced TLR4 activation and hence migrate to draining the mediastinal lymph node (MLN) to mount Th2 immune response [7]. In contrast, the pDC subset in the lung is apparently involved to balance airway inflammation through interactions with the regulatory T cells (T_{regs}).

6. Targeting Metabolic Sensors: Implications in Allergic Airway Disease

Allergic asthma is a Th2 disorder of the lung and is manifested by elevated airway inflammation, mucous cell metaplasia with mucous overproductions, airway hyperresponsiveness, and airway remodeling. Airways in severe asthmatics are characterized by increased degree of eosinophilia and elevated numbers of effector $CD4^+$ cells in bronchoalveolar lavage that produce canonical set of Th2 cytokines, such as IL4, IL5, and IL13 [84, 85]. The hallmark features of allergic "type-2 high" asthma phenotype in humans are closely manifested in several experimental murine models of allergic asthma, such as administration of either the aeroallergen house dust mite (HDM) or model antigen ovalbumin (OVA), and have

Table 1: Metabolic changes associated with dendritic cell maturation and effector functions*.

References	Cell types	Activation signals	Molecular effect	Metabolic shifts	Immune response
Bajwa et al. [101]	Plasmacytoid DCs (pDCs)	Influenza (flu) and respiratory virus	HIF1α translocation (↑)	Glycolysis (↑)	Interferon alpha (IFN-α) production and antiviral activity
Wu et al. [102]	Plasmacytoid DCs (pDCs)	TLR-9 agonist (CpG)	PPAR-α-dependent FAO activity (↑)	OXPHOS (↑)	Interferon alpha (IFN-α) production and antiviral activity
Pantel et al. [103]	$CD11C^{+}MHC\ II^{+}DX5^{-}$ $B220^{-}$ splenic DCs	TLR-3 agonist (poly I:C)	HIF1α expression (↑)	Glycolysis (↑)	Interferon α/β receptor-(IFNAR-) mediated DC maturation
Malinarich et al. [104]	Monocyte-derived DCs (moDCs)	TLR-4 agonist (LPS)	FAO activity (↑) ATP (↓)	OXPHOS (↑) Glycolytic capacity (↑)	Induce tolerance, negatively regulates immunogenicity
Everts et al. [54]	GM-DCs	TLR-4 agonist (LPS)	HK-II activation	Glycolysis (↑) (early events: within minutes of stimulation)	Activation and function of DC
Everts et al. [75] Amiel et al. [105]	Monocyte-derived DCs (moDCs)	TLR-4 agonist (LPS)	iNOS (↑) mTOR-dependent NO (↓) production	OXPHOS (↓) Glycolysis (↑) (late events: after 24 hours of stimulation)	DC survival and inflammation
Boukhaled et al. [106]	DCs constitutively expressing transcriptional repressor: PCGF6	TLR agonist (LPS)	Active transcriptional silencing by histone demethylation	Glycolysis (↓)	Maintenance of DC quiescence
Wang et al. [30]	Tsc1-deficient bone marrow-derived DCs	Spontaneous	mTORC-1 activity (↓)	Glycolysis (↑) OXPHOS (↑)	Checkpoint of DC development and differentiation; DC-mediated T cell response

*Note that this list is not exhaustive. DC: dendritic cell; HIF1α: hypoxia-inducible factor 1α; PPAR-α: peroxisome proliferator-activated receptor-α; FAO: fatty acid oxidase; TLR: toll-like receptor; HK-II: hexokinase II; PCGF6: polycomb group factor 6; Tsc1: tuberous sclerosis 1; ROS: reactive oxygen species; iNOS: inducible nitric oxide synthase; ATP: adenosine triphosphate; mTORC 1: mammalian target of rapamycin complex I.

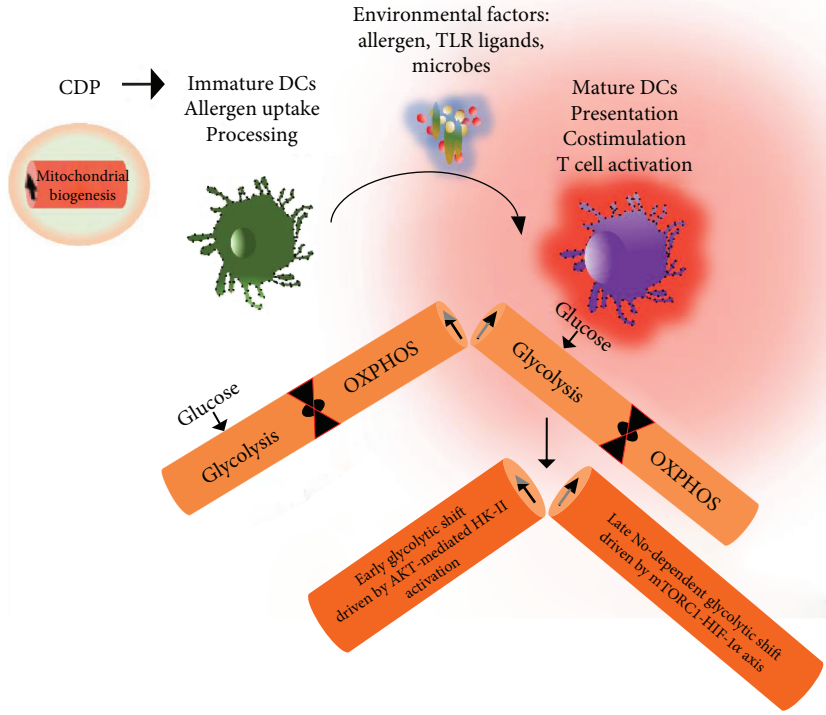

Figure 1: Basic preferences of DC metabolism. DCs originates from the common dendritic progenitors (CDPs) in the bone marrow (BM) that use mitochondrial oxidative phosphorylation (OXPHOS) as a key metabolic energy source and have increased mitochondrial biogenesis. These DC-committed progenitors egress the BM and circulate in the bloodstream and tissues as naïve immature DCs that promoted mitochondrial OXPHOS and shifted with the metabolic preferences upon allergen uptake and toll-like receptor (TLR) activation. The early glycolytic surge in activated DCs is primarily mediated via AKT pathways that phosphorylate and activate hexokinase II (the rate-limiting enzyme of the glycolytic pathway), whereas a late-occurring event of increased glycolysis is maintained by activated mTORC-HIF1α and is NO-dependent. NO: nitric oxide; mTORC1: mammalian target of rapamycin complex 1; HIF1α: hypoxia-inducible factor 1-alpha.

been extensively reviewed elsewhere [84, 86, 87]. Here, we describe the current understanding of targeting the important metabolic sensors and signaling pathways to manipulate the adaptive immune responses in allergic asthma (Table 2).

Mouse model-based studies of targeting serine/threonine kinase mammalian target of rapamycin (mTOR) have provided several key insights into the role of mTOR-dependent signaling pathway in the pathogenesis of allergic asthma. Studies using the macrolide product rapamycin (a potent inhibitor of mTOR) in HDM-induced asthma model showed different outcomes in attenuating airway inflammation [88, 89]. For example, surprisingly intranasal administration of HDM with concomitant rapamycin treatment almost significantly attenuated the infiltration of inflammatory cells in particular eosinophils in the BALF and reduction of the Th2 cytokines, such as IL4, IL5, and IL13 levels. The treatment of rapamycin in the induction model also blocked AHR, goblet cell metaplasia, and IgE and activated T cell numbers. However, when rapamycin was treated later following HDM, it failed to reverse and in contrast exacerbated airway inflammation and AHR. These studies confirmed the context-dependent effect of mTOR inhibition by rapamycin treatment and identified the crucial importance of mTOR-mediated signaling pathways in the setting of allergic asthma [88–90].

Moreover, studies using OVA-challenged high-fat diet-induced obese allergic mice have reported that oral treatment with the antihyperglycemic drug (an AMPK activator) metformin significantly abrogates the exacerbation of airway inflammation and lung eosinophilia by NF-κB-dependent iNOS expression [91]. Consistent with this, increased activation of AMPK and reduced oxidative stress were found in metformin-treated allergic mice, whereas, upon allergen challenge, the heterozygous AMPKα1-deficient mice showed increased airway inflammation and eosinophil infiltration in the lung [91, 92]. Intriguingly, several studies have identified the importance of the nuclear receptor peroxisome proliferator-activated receptor- (PPAR-) mediated pathways in allergic asthma [65, 93, 94]. Activation of PPARs in DCs by a selective agonist rosiglitazone significantly reduced the migration of DCs from the lung epithelia to the draining lymph nodes and thus inhibited the OVA-specific T cell proliferation [95, 96]. Dendritic cell-specific PPAR activation prevents the Th2-dependent eosinophilic airway inflammation by inducing the anti-inflammatory cytokine IL10 productions. However, the beneficial effect of PPAR activation in mouse asthma models has translated into several discrepancies in the study outcomes from randomized human asthmatic trials. Although in two short-term randomized controlled asthma studies, rosiglitazone has shown to improve

Table 2: Immunological effects of targeting important metabolic signaling pathways in asthma*.

Metabolic sensors/ target	Asthma model	Examples of agents/treatments	Asthma phenotype	References
mTOR	OVA-induced	mTORC inhibitor rapamycin derivative (SAR 943)	(↓) airway inflammation, Th2 cytokine production, mucous cell metaplasia, and AHR (↓) $CD4^+$ T cell numbers, however, failed to decrease AHR and eosinophilia	Fujitani et al. [90] Eynott et al. [107]
mTOR	Induction and treatment-model of HDM-mediated asthma	mTORC inhibitor Rapamycin	Airway inflammation, Th2 cytokine production, mucous cell metaplasia, and AHR (↓) in induction model, however, exacerbated AHR and airway inflammation when rapamycin administered later in a treatment model	Fredriksson et al. [88] Mushaben et al. [89]
Rheb1	OVA-induced	Myeloid-specific Rheb1 deletion	(↑) eosinophilic airway inflammation, mucous production, and AHR	Kai et al. [108]
AMPK	Obese asthma model: high fat-fed diet + OVA	AMPK activator metformin	(↓) BAL eosinophil counts and iNOS expression in lung	Calixto et al. [91]
AMPK/PPAR-γ	OVA-induced	SRT1720; synthetic SRT 1 activator	(↓) BAL eosinophil counts and type 2 cytokine productions	Ichikawa et al. [109]
PPAR-γ	FITC-OVA	PPAR-γ agonist rosiglitazone	Inhibits the migration of DCs from airway mucosa to draining lymph nodes and decrease priming of T cells	Angeli et al. [95]
PPAR-γ	OVA-pulsed DCs	PPAR-γ agonist rosiglitazone	(↓) BAL eosinophil counts and OVA-specific T cell proliferation (↑) interleukin 10 (IL10) production by T cells	Hammad et al. [96]
PPAR-γ	Obese asthmatics model of a two-center, 12-week randomized double-blinded trial	PPAR-γ agonist pioglitazone	No significant difference in asthma control and treatment group in lung function, (↑) body weight by pioglitazone treatment	Dixon et al. [94]

*Note that this list is not exhaustive. Rheb: Ras homolog enriched in the brain (small GTPase downstream target of tuberous sclerosis complex (TSC) 1/2 and upstream activator of mTORC1); OVA: ovalbumin; HDM: house dust mite; mTORC 1: mammalian target of rapamycin complex I; AMPK: AMP-activated protein kinase; PPAR-α: peroxisome proliferator-activated receptor-α; BAL: bronchoalveolar lavage; AHR: airway hyperresponsiveness; iNOS: inducible nitric oxide synthase.

the airway inflammation modestly in allergen challenge or smokers with asthma [97, 98]. However, activation of PPARs by pioglitazone failed to improve the mild airway inflammation in obese asthmatics [93, 94]. Collectively, these studies point out the important regulatory role of cooperative immune-metabolic signaling pathways and imply that targeting these metabolic sensors to alter allergic airway inflammation might hold promise as an effective therapeutic strategy for the treatment of asthma. However, future studies warrant more mechanistic research to address the metabolic effects of targeting these metabolic sensors in a cell-specific manner by lung immune cells and structural cells to potentially render adaptive immune response.

7. Discussion

Asthma treatment broadly relies on drugs that predominantly aims at relaxing airway smooth muscle (bronchodilators) or used to attenuate airway inflammations (anti-inflammatory drugs). Currently, newer medications, such as leukotriene modifiers along with drug combinations like inhaled corticosteroids with long-acting β-adrenergic agonists, are effective as controller therapy. Long-term asthma management with increasing dosage of corticosteroids has potentially become a concern for their adverse effects (refractory-asthma or steroid-unresponsive asthma). Treatments with long-acting β-adrenergic agonists, leukotriene modifiers, or anti-IgE therapy suppress airway inflammation and thereby facilitate and reduce the dose and use of inhaled corticosteroids. However, they do not completely cure asthmatic inflammation. Therefore, a comprehensive understanding of how metabolism dictates immune cell fate in DCs are an unmet need to develop novel controller therapeutic options, such as metabolism-based approaches to improve the overall efficacy of asthma management.

A successful airway immune response relies on effective antigen uptake and presentation by DCs to T cells. Targeting metabolic pathways to increase DC function and to modulate immune effector pathways has now come into focus. While inhibition of mTOR pathways in DCs have specific cellular effect on DC maturation and development in cDCs, pDCs, and moDCs [27–29, 99, 100], the administration of mTOR inhibitors in attenuating airway inflammation has varying effects, which abated response in induction model and exacerbation in treatment model [88, 89]. These discrepancies of outcomes are possibly caused by dose, timing, and/or off-target effects. Thus, it is clear that modulating DC-specific mTOR signaling pathways possesses extremely potent immune conditioning effects. In addition to therapies aimed at mTOR, in therapies aimed at other metabolic sensors, such as AMPK, PPARs might prove to be effective when attacking the altered metabolic energetics of DCs [28, 58, 62, 95].

8. Conclusions

Emerging immune pathways in asthma pathogenesis have added a new dimension to our understanding of the unique adaptations that contribute to initiate and propagate the adaptive immune responses in airway inflammation. There is a growing appreciation that in order to be effective for antigen uptake, migration to draining lymph node, and antigen presentations, DCs must undergo the correct metabolic reprogramming to initiate appropriate T cell-mediated immune response. Although little research has focused on manipulating DC metabolism to promote activation and priming capacity, evidence is accumulating which addresses how the metabolic perturbations are interlinked to the fundamental properties of DC function and thereby facilitate immune responses. Therefore, integrating DC metabolism in immune therapy design for asthma and airway inflammation promises a novel strategy and can be used to alter the allergen-induced adaptive immune responses.

Conflicts of Interest

The author has declared that no competing interests exist.

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