International Immunopharmacology 71 (2019) 198–204

Contents lists available at ScienceDirect

International Immunopharmacology

journal homepage: www.elsevier.com/locate/intimp

Nrf2 exerts mixed inflammation and glucose metabolism regulatory effects on murine RAW264.7 macrophages

Ling Ding[a], Xiaoyang Yuan[b], Jinhua Yan[a], Yi Huang[a], Mulin Xu[a], Zhen Yang[a], Ni Yang[a], Manting Wang[a], Cuntai Zhang[a], Le Zhang[a,*]

[a] Institute of Gerontology, Department of Geriatrics, Tongji Hospital, Tongji Medical College, Huazhong University of Science and Technology, 1095 JieFang Avenue, Wuhan, Hubei 430030, China
[b] Department of Neurology, Brain Aging and Cognitive, Neuroscience Laboratory of Hebei Province, The First Hospital of Hebei Medical University, Shijiazhuang 050031, China

ARTICLE INFO

Keywords:
Nuclear factor (erythroid-derived 2)-like 2
Murine RAW264.7 macrophages
Inflammation regulation
AKT signaling
Glucose metabolism regulation

ABSTRACT

Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) is a transcription factor that mediates a broad range of cellular antioxidative, detoxification and anti-inflammatory effects. However, the precise mechanism by which Nrf2 regulates inflammation and metabolism in macrophages remains controversial and unclear. To further clarify the roles of Nrf2 in inflammation and glucose metabolism regulation, retrovirus-mediated knockdown of Nrf2 was performed in murine RAW264.7 macrophages, and the cells were stimulated with 100 ng/mL lipopolysaccharide for 24 h for M1 activation. qPCR and western blotting results indicated that Nrf2 knockdown significantly enhanced expression of the inflammatory genes Il1a and Il1b in unstimulated macrophages and increased expression of the inflammatory genes Il1a, Il1b, Il6, Il10, Ccl2, Ccl22, and CD38 but decreased that of Tnfa and Tgfb1 in M1 macrophages. Nrf2 knockdown also significantly elevated IL6 and IL10 secretion by M1 macrophages. Western blotting showed that Nrf2 knockdown reduced iNOS protein levels in resting macrophages and enhanced CD38 protein levels in both resting and M1 macrophages. The differential regulation of these macrophage inflammation and polarization markers by Nrf2 reveals multiple roles for Nrf2 in regulating inflammation in macrophages. Moreover, Nrf2 knockdown increased the Glu4 protein level and decreased AKT and GSK3β protein phosphorylation in M1 macrophages, suggesting multiple roles for Nrf2 in regulating glucose metabolism in macrophages. Overall, our results are the first to demonstrate mixed inflammation and glucose metabolism regulatory effects of Nrf2 in macrophages that may occur independent of its classic function in redox regulation. These findings support the potential of Nrf2 as a therapeutic target for the prevention and treatment of inflammation- and obesity-associated syndromes, including diabetes and atherosclerosis.

1. Introduction

Nuclear factor (erythroid-derived 2)-like 2, also known as NFE2L2 or Nrf2, is a basic leucine zipper (bZIP) transcription factor that is traditionally considered a master regulator of the antioxidant defense system in cellular organisms [1–4]. Under static conditions, Nrf2 is retained in the cytoplasm by Kelch-like ECH-associated protein 1 (Keap1) and undergoes constant ubiquitination and proteasomal degradation. Upon oxidative or electrophilic stress, the Nrf2 protein is released from Keap1, rapidly accumulates in the cytoplasm and then translocates to the nucleus, where it forms heterodimers with other bZIP proteins, binds to antioxidant response elements in the promoter region of target genes, and induces expression of many cytoprotective antioxidant and phase II detoxifying genes, including *heme oxygenase 1* (*Hmox1* or *ho-1*) and *NAD(P)H:quinone oxidoreductase 1* (*nqo1*).

Extensive evidence to date indicates that the functions of Nrf2 are not limited to antioxidant and detoxification effects. Indeed, Nrf2 has also been implicated in many other molecular processes and diseases, including inflammatory responses, metabolic diseases, cancer and cell proliferation, senescence and survival [5–9]. However, the exact roles of Nrf2 in the inflammatory response, atherosclerosis and metabolic programming remain highly controversial.

Nrf2 deficiency can exacerbate inflammation and promote atherosclerosis and liver injury in a variety of murine models [10–13]. Paradoxically, Nrf2 deficiency was reported to prevent the early onset and development of atherosclerosis and to promote lipid metabolism in

* Corresponding author.
E-mail address: le_zhang@foxmail.com (L. Zhang).

https://doi.org/10.1016/j.intimp.2019.03.023
Received 18 December 2018; Received in revised form 14 February 2019; Accepted 9 March 2019
Available online 23 March 2019

ApoE knockout mice [14,15] and also to prevent diet-induced obesity and obesity-associated chronic inflammation in mouse adipose tissue [16–21].

Furthermore, controversial functions of Nrf2 in the regulation of inflammation and metabolism have also been observed in numerous Nrf2-activated cell and murine models. For example, genetic activation of Nrf2 via hypomorphic knockdown of Keap1 [22–24] or oral administration of Nrf2 inducers [24–27] represses inflammation and prevents the onset and development of diabetes mellitus and diabetes-associated disorders in diabetic mice. Conversely, enhanced activity and activation of Nrf2 have been reported to aggravate inflammation, worsen insulin resistance and promote hepatic steatosis and lipid accumulation in mice [27–29].

To address the controversial functions of Nrf2 in the regulation of inflammation and metabolism, Kobayashi et al. recently explored the Nrf2-mediated inflammatory response in Nrf2-activated macrophages [10]. In this elegant study, the authors revealed that Nrf2-mediated inhibition of pro-inflammatory genes is independent of its redox control and is not a secondary consequence of the elimination of reactive oxygen species (ROS) through production of antioxidant enzymes and proteins. Nrf2 can regulate the macrophage inflammatory response through direct binding to its binding motif (TGAG/CnnnGC motif or antioxidant responsive element, ARE) in inflammatory gene promoters in both unstimulated and M1-activated cells as well as through recruitment to form a regulatory protein complex with other transcription factors or cofactors without direct motif binding. These two regulatory mechanisms might coexist in an independent manner for transcriptional regulation of individual genes, and the interplay between these two mechanisms requires further elucidation.

Macrophages are cells of the innate immune system that are present in all tissues, including the adipose tissue, the liver and the vasculature [30,31]. Macrophages secrete a broad spectrum of cytokines, chemokines, growth factors and extracellular vesicle-encapsulated material that can influence the function of neighboring cells through paracrine effects and the function of other tissues and organs through endocrine activity [30,31]. Because of their phenotypic flexibility, these cells have beneficial effects in tissue homeostasis [30,31], but they are also considered essential effector cells in the occurrence and development of obesity-associated metabolic disorders and atherosclerosis, which are associated with a state of chronic, low-grade inflammation [30–33]. As the principle source of inflammatory mediators, infiltration, expression and secretion of inflammatory cytokines in adipose tissue by macrophages precede the development of insulin resistance, underlying the etiology of obesity-related metabolic disorders [34–37]. Anti-inflammatory therapies through either inhibition of pro-inflammatory cytokines [38–41] or stimulation of anti-inflammatory cytokines [42,43] have been shown to have beneficial metabolic effects in patients with type 2 diabetes. Liver macrophages are crucial for the pathogenesis of acute and chronic liver diseases, in which they orchestrate inflammation, fibrosis, angiogenesis and tumor progression [44,45]. Macrophages are also fundamental contributors to the initiation and progression of atherosclerosis, which depend on local inflammation and lipid accumulation in the vascular wall [46–49]. Thus, understanding the finely tuned mechanisms underlying inflammation regulation in macrophages is essential for clarifying the controversial roles of Nrf2 in metabolic and inflammatory regulation in Nrf2-deficient [10–21] and Nrf2-activated [22–29] mice.

In the study reported by Kobayashi et al., the observed Nrf2-mediated inhibition of pro-inflammatory cytokines in M1 macrophages [10] provided deeper insight into the beneficial effects of Nrf2 activation [22–27] and the deleterious effects of Nrf2 deficiency [10–13] in mice. However, the findings were insufficient to explain the opposite outcomes of Nrf2 deficiency [14–21] or Nrf2 activation [27–29] in the regulation of inflammation and metabolism in mice.

In the present study, we knocked down Nrf2 expression in murine RAW 264.7 macrophages and evaluated effects on the inflammatory response and glucose regulation in these cells with the aim of gaining a comprehensive understanding of Nrf2-mediated inflammatory and metabolic regulation in macrophages.

2. Materials and methods

2.1. RAW 264.7 cell culture, transduction with retrovirus shRNA, and M1 stimulation

The RAW 264.7 murine macrophage cell line was obtained from American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Biological Industries, Israel) and 1% penicillin-streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. The sequences of the shRNA utilized in this study to target mouse Nrf2 gene were as follows: sense, 5′-GAAUUACAGUGUCUUAAUATT-3′, and antisense, 5′-UAUUAAGACACUGUAAUUCTT-3′. RAW 264.7 cells were transduced with according to the manufacturer's instructions. Briefly, RAW 264.7 cells were seeded at a density of $2.5 * 10^5$ in six-well plates for 1 day. Before transduction, 25 μL Nrf2 retrovirus carrying an anti-puromycin construct ($1 * 10^8$ U/mL) and control retrovirus with GFP RNA ($1 * 10^8$ U/mL) were separately added to 1 mL of culture medium. The retrovirus/medium complexes were added to cells, and 4 h later, the cells were supplemented with 1 mL normal medium. The vector-containing medium was replaced with normal culture medium at 24 h, and the cells were incubated for another 48 h. The percentage of GFP-positive cells was determined using a fluorescence microscope (Invitrogen EVOS FL Auto, Life Technologies, USA).

Cells carrying the Nrf2 retrovirus were selected using medium containing 2 μg/mL puromycin. The levels of Nrf2 mRNA in cells transduced with the Nrf2 retrovirus or the control retrovirus at each passage were measured and compared by qPCR analysis until efficiency of Nrf2 knockdown over 95% was attained. The retrovirus-transduced cells were continually cultured in a macrophage medium containing 1 μg/mL puromycin to maintain a mild selective pressure, and this cell pool was used for all experiments in this study.

To address the role of Nrf2 in inflammation regulation, we explored Nrf2 target genes in the inflammatory state using pro-inflammatory (M1)-activated macrophages stimulated with lipopolysaccharide (LPS, 100 ng/mL). Before each experiment, retrovirus-transduced cells were switched to puromycin-free starvation medium containing 2% FBS, cultured overnight (approximately ~12 h), and left untreated (Con) or incubated with 100 ng/mL LPS (M1) in normal medium for 24 h. The cells and culture medium were collected for experiments.

2.2. ELISA for inflammatory cytokine detection

The ELISA kit for IL6 was purchased from NeoBioscience (cat.#: EMC004(H).96, Shenzhen, China); the kit for IL10 was purchased from ExCell Bio (cat.#: EM005, Shanghai, China). Secretion of inflammatory cytokines by macrophages was assessed by measuring the concentration of cytokines in the culture medium by ELISA. The culture medium of untreated or LPS-treated cells was collected and centrifuged at 1000 rpm for 7 min to obtain cell-free supernatants. Aliquots of the cell-free supernatants were used to measure the concentration of the inflammatory cytokines IL6 and IL10 in the culture medium with cytokine sandwich ELISA kits according to the manufacturer's instructions.

2.3. Quantitative real-time polymerase chain reaction (qPCR) assay

Total RNA was extracted using a Hipure Total RNA Mini Kit (cat.#: R4111-03, Magen, China) and converted to cDNA with a ReverTra Ace qPCR RT kit (cat.#: FSQ-101, Toyobo, Japan). RNA quality was determined by the A260:A280 ratio, whereby RNA samples with A260:A280 ratios between 1.8 and 2.2 were considered to be pure and were used for reverse transcription. Quantitative real-time polymerase

chain reaction (qPCR) was performed using an ABI Step One Plus system (Applied Biosystems, USA) with SYBR green PCR mix (cat.#: QPK-201, Toyobo, Japan) with Gapdh and Actb as internal reference genes. Primer specificity was determined by melting curves and agarose gel electrophoresis of qPCR products. No-template controls were included in each batch of samples to evaluate contamination and unintended amplification. The sequences of primers used in this study and the range of the threshold cycle (Ct) for each pair of primers under our experimental conditions are listed in Supplementary Table 1. The fold change in relative mRNA expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Western blot analysis

Equal amounts of proteins were separated by gel electrophoresis and transferred onto a polyvinylidene difluoride membrane (Millipore, USA). The membrane was blocked in 5% nonfat milk for 1 h and subsequently probed with specific primary antibodies. The antibodies used in this study are listed in Supplementary Table 2. Proteins were detected via enhanced chemiluminescence (Boster, China) using a UVP imaging system (UVP, USA).

2.5. Statistical analysis

Statistical analyses were carried out with IBM SPSS 16.0 software, and the data are expressed as the means ± error of mean (SEM) of the number of replicates indicated. A paired *t*-test was used for direct analysis of two paired groups of ELISA data, and two-way analysis of variance (ANOVA) was employed for all other group analyses; $p < 0.05$ was considered statistically significant.

3. Results

3.1. Nrf2 knockdown in macrophages

To investigate the effects of Nrf2 knockdown (NK) on the inflammatory response of macrophages, we first knocked down expression of the Nrf2 gene in murine RAW 264.7 cells via retrovirus-mediated transduction with Nrf2 shRNA (NK). Knockdown efficiency was confirmed by a > 95% decrease in the Nrf2 mRNA content and an over 70% decrease in the Nrf2 protein content in NK and NK-M1 cells compared with counterpart macrophages transduced with a retrovirus carrying the control (Con and M1 cells) (Fig. 1). The mRNA and protein levels of Nrf2 were relatively constant between resting (Con) and M1 macrophages, suggesting that LPS treatment of cells under our experimental conditions did not significantly affect Nrf2 gene or protein expression. The decrease in Nrf2 protein level in cells was accompanied by decreased levels of gene and protein of HO-1 and NQO1, two representative downstream targets in Nrf2-directed redox signaling (Fig. 1). As both the mRNA and protein levels of NQO1 increased in M1 cells compared to resting cells, it appears that mechanisms other than Nrf2 are able to stimulate NQO1 gene expression in M1 macrophages, consistent with our previous observation in AML12 hepatocytes that regulation of NQO1 transcription is not completely Nrf2-dependent [9]. The mRNA level of HO-1 was strongly increased in M1 cells compared to resting cells, though no significant difference was observed for the HO-1 protein level. The elevated level of HO-1 gene transcription did not increase HO-1 protein levels, suggesting the existence of a limiting step during post-transcriptional HO-1 protein synthesis and modification.

3.2. Nrf2 knockdown altered gene expression and secretion of inflammatory cytokines in M1 macrophages

The effects of Nrf2 knockdown on the gene expression and secretion of inflammatory cytokines involved in classical M1 and M2 macrophage activation were then assessed in both resting and M1 macrophages. These genes include the classical M1 macrophage markers Il1a, Il1b, Il6, Tnfa, Ccl2, iNOS and CD38 and the M2 macrophage markers Il10, Ccl22 and Tgfb1.

In the absence of LPS treatment, the expression levels of most of the inflammatory genes evaluated, except for Tnfa, Tgfb1 and CD38, remained relatively equivalent to the basal levels in both Con and NK cells (Fig. 2A, Table S3). Nonetheless, subtle but significant increases in Il1a and Il1b gene expression were observed in unstimulated cells after Nrf2 knockdown, suggesting that Nrf2 can repress Il1a and Il1b gene expression in resting macrophages.

LPS treatment stimulated the transcription of all inflammatory genes tested in M1 macrophages compared with resting cells. In M1 macrophages, Nrf2 knockdown resulted in significantly elevated Il1a, Il1b, Il6, Il10, Ccl2, Ccl22 and CD38 gene expression and significantly decreased Tnfa and Tgfb1 gene expression (Fig. 2A). The mixed outcomes of inflammatory genes in M1 macrophages following Nrf2 knockdown suggest that Nrf2 can mediate the inflammatory response through multiple pathways in macrophages.

Secretion of pro-inflammatory IL6 and anti-inflammatory IL10 by macrophages was also assessed by measuring their concentrations in the culture medium after 24 h of incubation in fresh medium (Fig. 2B, C). Due to technical limitations, IL6 and IL10 were not detectible in the culture medium of unstimulated resting cells, indicating that secretion of these cytokines by resting macrophages remained at very low levels, which were undetectable under our experimental conditions, and that Nrf2 knockdown did not significantly alter their secretion by resting cells. However, LPS stimulation dramatically enhanced secretion of both IL6 and IL10, and their levels were further enhanced by Nrf2 knockdown in M1 macrophages. This result is consistent with the transcriptional activation of these genes by Nrf2 knockdown in M1 macrophages, revealing that Nrf2 is able to mediate secretion of both

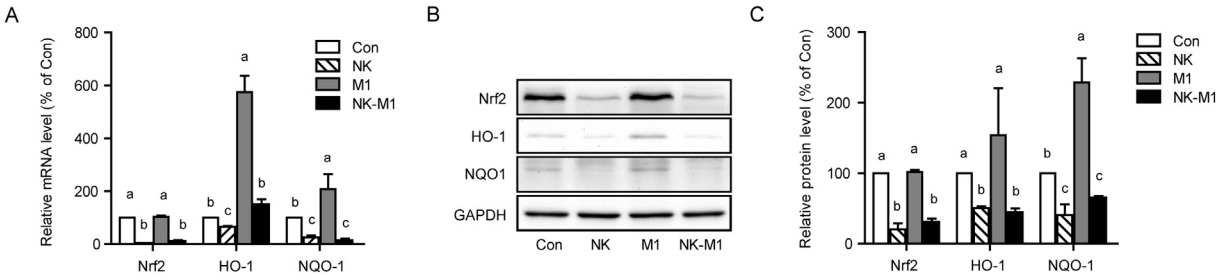

Fig. 1. Nrf2 knockdown in macrophages decreased levels of its downstream redox signaling targets HO-1 and NQO1. Nrf2 knockdown (NK) decreased the relative mRNA levels (A) and protein levels Nrf2, HO-1 and NQO1 in both resting (Con) and M1 macrophages, as shown by a representative western blot (B) and relative protein levels (C). The data are expressed as the mean ± SEM, n = 4/group. Different letters indicate differences among treatment groups ($p < 0.05$). Nrf2, nuclear factor E2-related factor 2; HO-1, heme oxygenase; NQO1, quinone oxidoreductase 1; Con, resting macrophages without LPS treatment; NK, Nrf2 knockdown in macrophages; M1, LPS-treated macrophages; NK-M1: LPS-treated macrophages with Nrf2 knockdown.

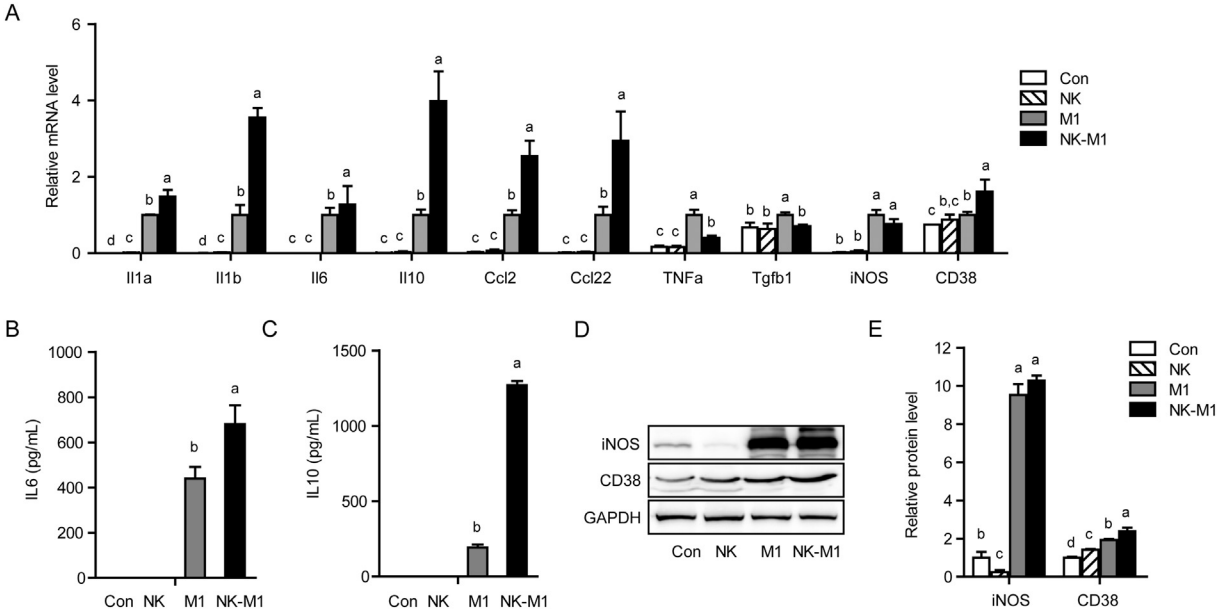

Fig. 2. Nrf2 knockdown altered gene expression and secretion of inflammatory cytokines in resting and M1 macrophages. (A) Nrf2 knockdown (NK) altered the mRNA levels of Il1a, Il1b, Il6, Il10, Ccl2, Ccl22, Tnfa, and Tgfb1 in both resting (Con) and M1 macrophages. The concentrations of IL6 (B) and IL10 (C) proteins in the supernatant of resting and M1 macrophages were measured by ELISA. The protein levels of iNOS and CD38 in resting (Con) and M1 macrophages are shown by a representative western blot (D) and relative protein levels (E). The data are expressed as the mean ± SEM, n = 4/group. Different letters indicate differences among treatment groups ($p < 0.05$).

pro-inflammatory (e.g., IL6) and anti-inflammatory (e.g., IL10) cytokines in M1 macrophages, which suggests a complex role for Nrf2 in the regulation of inflammatory homeostasis in the local environment.

Protein levels of M1 markers iNOS and CD38 were also evaluated by western blot analysis (Fig. 2D, E). In resting cells, Nrf2 knockdown significantly decreased the level of iNOS and increased that of CD38. Moreover, LPS treatment significantly enhanced the protein levels of both iNOS and CD38, and Nrf2 knockdown further increased the level of CD38 in M1 cells, without significantly affecting the level of iNOS.

3.3. Metabolism reprogramming induced by Nrf2 knockdown in M1 macrophages

A change in Nrf2 expression in mice, including both Nrf2 deficiency [10–21] and Nrf2 activation [22–29], may result in altered inflammation and metabolism regulation in mice. As the protein kinase B (AKT) pathway converges inflammatory and metabolic signals to regulate macrophage responses and modulate their activation phenotype [50], we also examined the protein levels and activation of certain key players possibly involved in Nrf2-mediated metabolic reprogramming, including glucose transporter type 4 (Glut4), AKT, glycogen synthase kinase 3β (GSK3β), mammalian target of rapamycin (mTOR) and microtubule-associated protein 1A/1B-light chain 3 (LC3), in both resting and M1 macrophages.

The level of Glut4 protein significantly increased upon LPS treatment and was further increased in both resting and M1 cells after Nrf2 knockdown. In contrast, the total protein levels of GSK3β and AKT remained relatively constant among all groups under our experimental conditions. Knockdown of Nrf2 significantly decreased p-GSK3β and p-AKT levels in both resting and M1 cells, but p-GSK3β and p-AKT levels remained relatively constant in M1 macrophages compared with resting cells (Fig. 3). No significant differences in the protein levels of p-mTOR, mTOR and LC3 were observed among the groups under our experimental conditions (Fig. 3).

4. Discussion

4.1. Nrf2 regulates inflammatory cytokines in resting macrophages

Previous ChIP-sequencing analysis has demonstrated direct binding of Nrf2 to the upstream region of Il1b and Il6 genes in both resting and M1 macrophages, and Nrf2 overexpression represses expression of Il1b and Il6 genes in M1 cells [10]. In this study, Nrf2 knockdown in resting macrophages resulted in subtle but significantly increased expression of the pro-inflammatory gene Il1b, confirming direct binding of Nrf2 to the promoter of this gene and further suggesting a repressive role of Nrf2 in regulating its expression, even in resting macrophages. In this study, the level of Il6 mRNA remained unchanged from the basal level in resting cells after Nrf2 knockdown, indicating that binding of Nrf2 to the upstream region of Il6 in resting cells did not have a repressive effect on basal Il6 expression in cells in this state.

Nrf2 knockdown also increased the level of Il1a mRNA in resting cells, suggesting that Nrf2 can also inhibit Il1a expression in resting cells. Because ChIP-sequencing analysis showed a lack of a significant Nrf2 binding pattern for Il1a in resting macrophages [10], Nrf2 likely inhibits Il1a and Il1b through different pathways in these cells.

In an attempt to eradicate bacterial, fungal, and viral infections by promoting nitric oxide (NO) production, both resting and M1 macrophages express iNOS [46,51]. In this study, we observed a decrease in iNOS protein levels after Nrf2 knockdown in resting macrophages, though iNOS mRNA levels remained relatively constant; this indicates that Nrf2 is able to increase the level of iNOS protein in resting macrophages through mechanisms independent of transcriptional regulation. This result also uncovers a protective role for Nrf2 in macrophages against infections under physiological conditions via stimulation of a host defense iNOS/NO-mediated process.

4.2. Nrf2-mediated macrophage polarization and inflammation regulation upon LPS treatment

IFNγ, inflammatory cytokines such as TNF and microbial products such as LPS drive, alone or in combination, classical M1 activation of

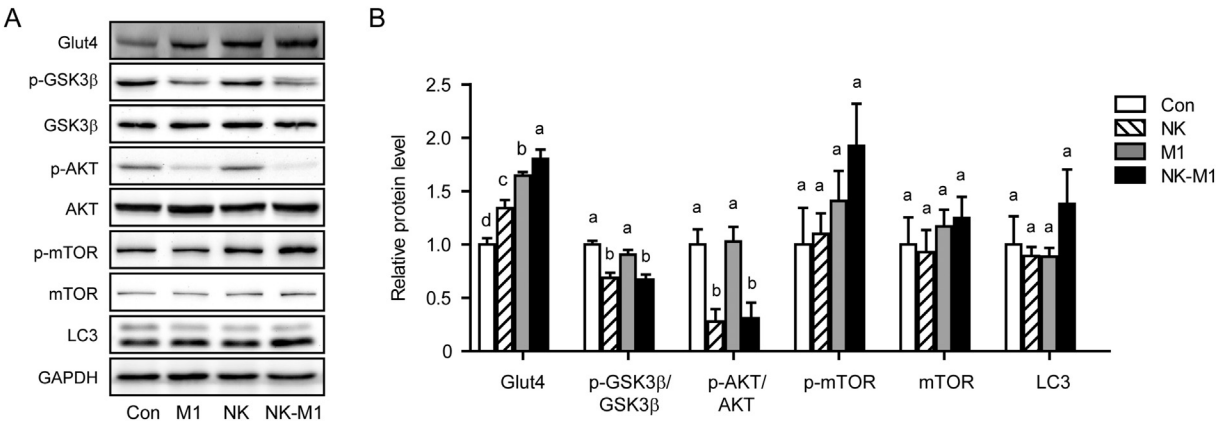

Fig. 3. Alteration of AKT and mTOR signaling induced by Nrf2 knockdown (NK) in macrophages. Representative western blots showing Glut4, p-GSK3β, GSK3β, p-AKT, AKT, p-mTOR, mTOR and LC3 protein expression (A) and relative protein levels (B) in resting (Con) and M1 macrophages. The data are expressed as the mean ± SEM, n = 4/group. Different letters indicate differences among treatment groups ($p < 0.05$). p-AKT, phosphorylated protein kinase B; GSK3β, glycogen synthase kinase 3β; mTOR, mammalian target of rapamycin (mTOR), LC3, microtubule-associated protein 1A/1B-light chain 3.

macrophages [46,52,53]. In this study, treatment of murine RAW264.7 macrophages with 100 ng/mL LPS was chosen for experiments to mimic clinical acute infection, such as that found in clinical chorioamnionitis [54]. Treatment of cells with 100 ng/mL LPS was sufficient to stimulate classical M1 activation of cells, as confirmed by global upregulation of M1 macrophage markers such as Il1a, Il1b, Il6, Ccl2, CD38 and iNOS under our experimental conditions (Fig. 2). Treatment of cells with 100 ng/mL LPS also allowed us to assess the secretion of inflammatory cytokines by cells after 24 h of incubation with LPS, without affecting cell viability (data not shown).

In a previous study [10], Nrf2 overexpression in M1 macrophages dramatically decreased Il1a, Il1b and Il6 gene expression; however, in the present study, Nrf2 knockdown in M1 macrophages increased the expression levels of these three genes in NK-M1 cells, confirming the repressive role of Nrf2 toward these pro-inflammatory genes [10]. Furthermore, the strong fluctuations in expression levels of these pro-inflammatory genes after Nrf2 knockdown or overexpression in M1 cells indicated that Nrf2-mediated inhibition is a major contributor to their regulation in M1 macrophages and that Nrf2-mediated inhibition is independent of its redox control, as the expression levels of the downstream Nrf2 redox targets Hmox1 and Nqo1 were both decreased in NK-M1 cells compared with M1 cells.

CD38 is a multifunctional ectoenzyme that contributes to the inflammatory response in a variety of inflammatory diseases in mice and humans [53,55–57], and its expression is upregulated in macrophages upon LPS treatment, promoting LPS-induced macrophage M1 polarization [53]. Upregulation of CD38 in LPS-treated macrophages was confirmed in this study (Fig. 2A, D, E). Moreover, Nrf2 knockdown further enhanced CD38 gene and protein expression in both resting and M1 macrophages, suggesting that Nrf2 inhibits M1 polarization in macrophages and relieves the CD38-mediated inflammatory response in inflammatory diseases through inhibition of CD38 expression.

Under our experimental conditions, Nrf2 knockdown increased expression of pro-inflammatory genes, including Il1a, Il1b, Il6, Ccl2, iNOS and CD38, increased that of the anti-inflammatory genes Il10 and Ccl22 and decreased that of the pro-inflammatory gene Tnfa and the anti-inflammatory gene Tgfb1. Cytokines and chemokines, including Il1a, Il1b, Il6, Tnfa, Ccl2, iNOS and CD38, are pro-inflammatory markers of M1 macrophages; Il10, Ccl22 and Tgfb1 are anti-inflammatory markers of M2 macrophages [46,58]. Hence, our study revealed a heterogeneity of Nrf2 function in the transcriptional regulation of both pro- and anti-inflammatory genes in LPS-stimulated macrophages and showed that Nrf2 knockdown drove macrophages to alternative activation/polarization upon LPS treatment, exhibiting mixed features of both classical M1-like and M2-like activation (Table 1) [32,33,46].

Table 1
Overview of NK-M1 induced macrophage polarization that exhibits mixed features of M1-like and M2-like activation.

Gene	Marker of the macrophage phenotype	Gene expression change in NK-M1 macrophages	NK-M1 induced macrophage polarization
Il1a	M1 [59]	↑	M1-like
Il1b	M1 [46]	↑	M1-like
Il6	M1 [46]	↑	M1-like
Il10	M2 [46]	↑	M2-like
Ccl2	M1 [46]	↑	M1-like
Ccl22	M2 [46]	↑	M2-like
Tnfa	M1 [46]	↓	M2-like
Tgfb1	M2 [46]	↓	M1-like
iNOS	M1 [46]	–	–
CD38	M1 [53]	↑	M1-like

IL6 is not only an essential pro-inflammatory cytokine implicated in the pathogenesis of a variety of diseases often associated with inflammation, immune dysfunction and tumors but is also a key component of the senescence-associated secretory phenotype (SASP) that promotes and develops aging and aging-related diseases, including atherosclerosis [46,49]. In contrast, IL10 is a pleiotropic anti-inflammatory cytokine that plays a central role in inflammatory bowel disease; by opposing the switch to the metabolic program induced by inflammatory stimuli, macrophages are as the main target cells of its inhibitory effects [60]. In this study, Nrf2 knockdown elevated secretion of both IL6 and IL10 protein by LPS-treated macrophages (Fig. 2C, D), which indicates that Nrf2 mediates the inflammatory response of macrophages by regulating secretion of both pro- and anti-inflammatory cytokines.

Although it is difficult to conclude which of the pro- and anti-inflammatory effects of Nrf2 are more dominant on a global level under our experimental conditions, our present findings support the notion that NK-M1 macrophages can be either pro-inflammatory or anti-inflammatory because Nrf2 can mediate expression of genes and secretion of inflammatory cytokines of both types.

4.3. Effects of Nrf2 knockdown on glucose metabolism in M1 macrophages

Macrophages dramatically alter their metabolic pathway from oxidative to glycolytic when activated by LPS, and this change is essential for the production of pro-inflammatory mediators when energy is required [59]. Consistent with previous studies [60,61], LPS treatment increased the Glut4 protein level in M1 cells compared to resting cells

(Fig. 3), suggesting increased Glut4-mediated glucose uptake and energy requirement during macrophage M1 activation. Nrf2 knockdown further increased the level of Glut4 protein in both resting and M1 cells (Fig. 3), consistent with the increased expression and secretion of most inflammatory cytokines in NK-M1 cells (Fig. 2). These results suggest that Nrf2 suppresses the elevated metabolic program in macrophages upon LPS treatment [60,61].

AKT may promote glycogenesis by inactivating GSK3α/β via phosphorylation [9,62]. We found that Nrf2 knockdown significantly decreased AKT and GSK3β phosphorylation, suggesting that Nrf2 promotes AKT activation and phosphorylation of downstream GSK3β, thus inhibiting GSK3β-mediated glycogenesis, in macrophages.

The mTOR pathway, another downstream effector of AKT activation, plays critical roles in coordinating metabolism, inflammation and autophagy signals in macrophages [50,63], and mTOR is a vital negative controller of cell autophagy associated with LC3 [64]. In the present study, Nrf2 knockdown did not significantly affect the levels of mTOR or LC3 protein or mTOR protein phosphorylation (Fig. 3), suggesting that mTOR signaling was not strongly affected by Nrf2 knockdown under our experimental conditions.

IL10 may oppose the switch to the metabolic program induced by inflammatory stimuli in macrophages by inhibiting glucose uptake, suppressing mTOR signaling and promoting autophagy in macrophages [60]. In this study, Nrf2 knockdown resulted in dramatic elevation of Il10 gene expression and protein secretion in M1 macrophages (Fig. 3), which indicates that Nrf2 may enhance glucose uptake and mTOR signaling through inhibition of IL10 expression and secretion.

Thus, our results reveal a mixed function for Nrf2 in the regulation of glucose metabolism in M1 macrophages, including downregulation of Glut4-mediated glucose uptake and AKT/GSK3β-mediated glycogenesis on the one hand and upregulation of glucose metabolism through inhibition of IL10 on the other hand.

4.4. Time-dependent inflammatory responses of macrophages upon LPS treatment

Wang et al. reported roles for Nrf2 in the differential regulation of pro-inflammatory gene expression in LPS-treated peritoneal macrophages extracted from $Nrf2^{-/-}$ and $Nrf2^{+/+}$ mice [65]. Our study confirms most of their observations, including upregulation of Il1b and Il6 genes and downregulation of the TNFa gene; an exception is iNOS gene expression, which remained relatively unchanged in NK-M1 cells compared to that in M1 cells (Fig. 2A). However, Wang et al. extracted mRNA from cells for qPCR analysis 8 h after the beginning of LPS treatment [65], whereas we extracted RNA for qPCR analysis at 24 h after LPS treatment. Therefore, the difference between the 2 studies may be explained by the existence of a time dependency of iNOS gene expression that peaked at 6 h after the beginning of LPS treatment and then attenuated over time [65]. The expression profile of pro-inflammatory TNFa in macrophages during LPS treatment also exhibited a time dependency similar to that of iNOS [65]. Moreover, macrophages exhibit differential desensitization of a variety of LPS-induced inflammatory responses, including both pro- and anti-inflammatory genes [54,66]. As macrophages are capable of tightly coordinating their metabolic programs to adjust their immunological and bioenergetic functional requirements, including polarization and activation [67,68], we speculate that there is a time dependency of metabolism reprogramming in macrophages during LPS treatment.

Although Nrf2-mediated inhibition of pro-inflammatory genes [10] appears insufficient to explain the beneficial effects of Nrf2 deficiency [14–21] or the deleterious effects of Nrf2 activation [27–29] on the regulation of inflammation and metabolism in mice, our revelation of the mixed functions of Nrf2 in regulating inflammation and glucose metabolism in NK-M1 cells may provide a plausible explanation for the controversial role of Nrf2 in mice. In other words, under different experimental conditions, particular environmental stressors, including mouse dietary and hygiene conditions and the time of exposure to stressors, might activate macrophages to attain different inflammatory and metabolic states through differential activation of pro-/anti-inflammatory and pro-/anti-glucose metabolic genes. Such a mechanism may consequently contribute to the beneficial, deleterious or even mixed effects on inflammation and metabolism regulation observed in Nrf2-deficient [10–21] and Nrf2-activated [22–29] mice in previous studies. Overall, the present study revealed for the first time mixed functions for Nrf2 in inflammation and glucose metabolism in macrophages, which may occur independently of its classic function in redox regulation. The findings provide a plausible explanation for the controversial role of Nrf2 in inflammation and metabolism regulation in mice and support the potential of Nrf2 as a therapeutic target for the prevention and treatment of inflammation- and obesity-associated syndromes, including diabetes and atherosclerosis.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was supported by the National Natural Science Foundation of China to L. Z. (grant numbers 81570411, 81873533) and C. Z. (grant numbers 81571377, 81873811).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2019.03.023.

References

[1] H. Motohashi, M. Yamamoto, Nrf2-Keap1 defines a physiologically important stress response mechanism, Trends Mol. Med. 10 (2004) 549–557.
[2] T.W. Kensler, N. Wakabayashi, S. Biswal, Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway, Annu. Rev. Pharmacol. Toxicol. 47 (2007) 89–116.
[3] K. Itoh, N. Wakabayashi, Y. Katoh, et al., Keap1 represses nuclear activation of antioxidant responsive elements by Nrf2 through binding to the amino-terminal Neh2 domain, Genes Dev. 13 (1999) 76–86.
[4] M. Kobayashi, M. Yamamoto, Nrf2-Keap1 regulation of cellular defense mechanisms against electrophiles and reactive oxygen species, Adv. Enzym. Regul. 46 (2006) 113–140.
[5] G.P. Sykiotis, D. Bohmann, Stress-activated cap'n'collar transcription factors in aging and human disease, Sci. Signal. 3 (2010) re3.
[6] N. Wakabayashi, S.L. Slocum, J.J. Skoko, S. Shin, T.W. Kensler, When NRF2 talks, who's listening? Antioxid. Redox Signal. 13 (2010) 1649–1663.
[7] D.V. Chartoumpekis, T.W. Kensler, New player on an old field; the keap1/Nrf2 pathway as a target for treatment of type 2 diabetes and metabolic syndrome, Curr. Diabetes Rev. 9 (2013) 137–145.
[8] Y. Huang, W. Li, Z.Y. Su, A.N. Kong, The complexity of the Nrf2 pathway: beyond the antioxidant response, J. Nutr. Biochem. 26 (2015) 1401–1413.
[9] X. Yuan, H. Huang, Y. Huang, et al., Nuclear factor E2-related factor 2 knockdown enhances glucose uptake and alters glucose metabolism in AML12 hepatocytes, Exp. Biol. Med. 242 (2017) 930–938.
[10] E.H. Kobayashi, T. Suzuki, R. Funayama, et al., Nrf2 suppresses macrophage inflammatory response by blocking proinflammatory cytokine transcription, Nat. Commun. 7 (2016) 11624.
[11] A.R. Collins, A.A. Gupte, R. Ji, et al., Myeloid deletion of nuclear factor erythroid 2-related factor 2 increases atherosclerosis and liver injury, Arterioscler. Thromb. Vasc. Biol. 32 (2012) 2839–2846.
[12] A.K. Ruotsalainen, M. Inkala, M.E. Partanen, et al., The absence of macrophage Nrf2 promotes early atherogenesis, Cardiovasc. Res. 98 (2013) 107–115.
[13] S. Freigang, F. Ampenberger, G. Spohn, et al., Nrf2 is essential for cholesterol crystal-induced inflammasome activation and exacerbation of atherosclerosis, Eur. J. Immunol. 41 (2011) 2040–2051.
[14] T.E. Sussan, J. Jun, R. Thimmulappa, et al., Disruption of Nrf2, a key inducer of antioxidant defenses, attenuates ApoE-mediated atherosclerosis in mice, PLoS One 3 (2008) e3791.
[15] B. Barajas, N. Che, F. Yin, et al., NF-E2-related factor 2 promotes atherosclerosis by effects on plasma lipoproteins and cholesterol transport that overshadow antioxidant protection, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 58–66.
[16] D.V. Chartoumpekis, P.G. Ziros, A.I. Psyrogiannis, et al., Nrf2 represses FGF21 during long-term high-fat diet-induced obesity in mice, Diabetes 60 (2011)

2465–2473.
[17] P. Xue, Y. Hou, Y. Chen, et al., Adipose deficiency of Nrf2 in ob/ob mice results in severe metabolic syndrome, Diabetes 62 (2013) 845–854.
[18] J. Xu, A.C. Donepudi, V.R. More, et al., Deficiency in Nrf2 transcription factor decreases adipose tissue mass and hepatic lipid accumulation in leptin-deficient mice, Obesity 23 (2015) 335–344.
[19] J. Pi, L. Leung, P. Xue, et al., Deficiency in the nuclear factor E2-related factor-2 transcription factor results in impaired adipogenesis and protects against diet-induced obesity, J. Biol. Chem. 285 (2010) 9292–9300.
[20] L. Zhang, K. Dasuri, S.O. Fernandez-Kim, A.J. Bruce-Keller, J.N. Keller, Adipose-specific ablation of Nrf2 transiently delayed high-fat diet-induced obesity by altering glucose, lipid and energy metabolism of male mice, Am. J. Transl. Res. 8 (2016) 5309–5319.
[21] K. Schneider, J. Valdez, J. Nguyen, et al., Increased energy expenditure, Ucp1 expression, and resistance to diet-induced obesity in mice lacking nuclear factor-erythroid-2-related transcription factor-2 (Nrf2), J. Biol. Chem. 291 (2016) 7754–7766.
[22] X. Kong, R. Thimmulappa, F. Craciun, et al., Enhancing Nrf2 pathway by disruption of Keap1 in myeloid leukocytes protects against sepsis, Am. J. Respir. Crit. Care Med. 184 (2011) 928–938.
[23] S.L. Slocum, J.J. Skoko, N. Wakabayashi, et al., Keap1/Nrf2 pathway activation leads to a repressed hepatic gluconeogenic and lipogenic program in mice on a high-fat diet, Arch. Biochem. Biophys. 591 (2016) 57–65.
[24] A. Uruno, Y. Furusawa, Y. Yagishita, et al., The Keap1-Nrf2 system prevents onset of diabetes mellitus, Mol. Cell. Biol. 33 (2013) 2996–3010.
[25] S. Shin, J. Wakabayashi, M.S. Yates, et al., Role of Nrf2 in prevention of high-fat diet-induced obesity by synthetic triterpenoid CDDO-imidazolide, Eur. J. Pharmacol. 620 (2009) 138–144.
[26] E. Bhakkiyalakshmi, D. Sireesh, M. Sakthivadivel, S. Sivasubramanian, P. Gunasekaran, K.M. Ramkumar, Anti-hyperlipidemic and anti-peroxidative role of pterostilbene via Nrf2 signaling in experimental diabetes, Eur. J. Pharmacol. 777 (2016) 9–16.
[27] C. Guo, Y. Xia, P. Niu, et al., Silica nanoparticles induce oxidative stress, inflammation, and endothelial dysfunction in vitro via activation of the MAPK/Nrf2 pathway and nuclear factor-kappaB signaling, Int. J. Nanomedicine 10 (2015) 1463–1477.
[28] J. Xu, S.R. Kulkarni, A.C. Donepudi, V.R. More, A.L. Slitt, Enhanced Nrf2 activity worsens insulin resistance, impairs lipid accumulation in adipose tissue, and increases hepatic steatosis in leptin-deficient mice, Diabetes 61 (2012) 3208–3218.
[29] A. Gerstgrasser, H. Melhem, I. Leonardi, et al., Cell-specific activation of the Nrf2 antioxidant pathway increases mucosal inflammation in acute but not in chronic colitis, J. Crohns Colitis 11 (2017) 485–499.
[30] K. Man, V.I. Kutyavin, A. Chawla, Tissue immunometabolism: development, physiology, and pathobiology, Cell Metab. 25 (2017) 11–26.
[31] S. Gordon, A. Pluddemann, F. Martinez Estrada, Macrophage heterogeneity in tissues: phenotypic diversity and functions, Immunol. Rev. 262 (2014) 36–55.
[32] K.R. Peterson, M.A. Cottam, A.J. Kennedy, A.H. Hasty, Macrophage-targeted therapeutics for metabolic disease, Trends Pharmacol. Sci. 39 (2018) 536–546.
[33] J.C. McNelis, J.M. Olefsky, Macrophages, immunity, and metabolic disease, Immunity 41 (2014) 36–48.
[34] S.U. Amano, J.L. Cohen, P. Vangala, et al., Local proliferation of macrophages contributes to obesity-associated adipose tissue inflammation, Cell Metab. 19 (2014) 162–171.
[35] T. Suganami, Y. Ogawa, Adipose tissue macrophages: their role in adipose tissue remodeling, J. Leukoc. Biol. 88 (2010) 33–39.
[36] L. Russo, C.N. Lumeng, Properties and functions of adipose tissue macrophages in obesity, Immunology 155 (2018) 407–417.
[37] M. Aouadi, M. Tencerova, P. Vangala, et al., Gene silencing in adipose tissue macrophages regulates whole-body metabolism in obese mice, Proc. Natl. Acad. Sci. U. S. A. 110 (2013) 8278–8283.
[38] M.S. Akash, Q. Shen, K. Rehman, S. Chen, Interleukin-1 receptor antagonist: a new therapy for type 2 diabetes mellitus, J. Pharm. Sci. 101 (2012) 1647–1658.
[39] C.M. Larsen, M. Faulenbach, A. Vaag, et al., Interleukin-1-receptor antagonist in type 2 diabetes mellitus, N. Engl. J. Med. 356 (2007) 1517–1526.
[40] C. Cavelti-Weder, A. Babians-Brunner, C. Keller, et al., Effects of gevokizumab on glycemia and inflammatory markers in type 2 diabetes, Diabetes Care 35 (2012) 1654–1662.
[41] A.B. Goldfine, V. Fonseca, K.A. Jablonski, et al., The effects of salsalate on glycemic control in patients with type 2 diabetes: a randomized trial, Ann. Intern. Med. 152 (2010) 346–357.
[42] E.G. Hong, H.J. Ko, Y.R. Cho, et al., Interleukin-10 prevents diet-induced insulin resistance by attenuating macrophage and cytokine response in skeletal muscle, Diabetes 58 (2009) 2525–2535.
[43] C. Herder, M. Carstensen, D.M. Ouwens, Anti-inflammatory cytokines and risk of type 2 diabetes, Diabetes Obes. Metab. 15 (Suppl. 3) (2013) 39–50.
[44] C. Ju, F. Tacke, Hepatic macrophages in homeostasis and liver diseases: from pathogenesis to novel therapeutic strategies, Cell. Mol. Immunol. 13 (2016) 316–327.
[45] O. Krenkel, F. Tacke, Liver macrophages in tissue homeostasis and disease, Nat. Rev. Immunol. 17 (2017) 306–321.
[46] S. Colin, G. Chinetti-Gbaguidi, B. Staels, Macrophage phenotypes in atherosclerosis, Immunol. Rev. 262 (2014) 153–166.
[47] X.L. Tian, Y. Li, Endothelial cell senescence and age-related vascular diseases, J. Genet. Genomics 41 (2014) 485–495.
[48] I. Tabas, K.E. Bornfeldt, Macrophage phenotype and function in different stages of atherosclerosis, Circ. Res. 118 (2016) 653–667.
[49] G. Chinetti-Gbaguidi, S. Colin, B. Staels, Macrophage subsets in atherosclerosis, Nat. Rev. Cardiol. 12 (2015) 10–17.
[50] E. Vergadi, E. Ieronymaki, K. Lyroni, K. Vaporidi, C. Tsatsanis, Akt signaling pathway in macrophage activation and M1/M2 polarization, J. Immunol. 198 (2017) 1006–1014.
[51] P. Kubes, Inducible nitric oxide synthase: a little bit of good in all of us, Gut 47 (2000) 6–9.
[52] A. Mantovani, C. Garlanda, M. Locati, Macrophage diversity and polarization in atherosclerosis: a question of balance, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 1419–1423.
[53] B. Shu, Y. Feng, Y. Gui, et al., Blockade of CD38 diminishes lipopolysaccharide-induced macrophage classical activation and acute kidney injury involving NF-kappaB signaling suppression, Cell. Signal. 42 (2018) 249–258.
[54] M.L. Kim, C. Maloney, N. Klimova, et al., Repeated lipopolysaccharide exposure leads to placental endotoxin tolerance, Am. J. Reprod. Immunol. (2018) e13080 [Epub ahead of print].
[55] Y. Qian, C. Chen, L. Ma, et al., CD38 deficiency promotes inflammatory response through activating Sirt1/NF-kappaB-mediated inhibition of TLR2 expression in macrophages, Mediat. Inflamm. 2018 (2018) 8736949.
[56] D.A. Deshpande, A.G.P. Guedes, F.E. Lund, S. Subramanian, T.F. Walseth, M.S. Kannan, CD38 in the pathogenesis of allergic airway disease: potential therapeutic targets, Pharmacol. Ther. 172 (2017) 116–126.
[57] M.S. Viegas, A. do Carmo, T. Silva, et al., CD38 plays a role in effective containment of mycobacteria within granulomata and polarization of Th1 immune responses against Mycobacterium avium, Microbes Infect. 9 (2007) 847–854.
[58] B. Mahdavian Delavary, W.M. van der Veer, M. van Egmond, F.B. Niessen, R.H. Beelen, Macrophages in skin injury and repair, Immunobiology 216 (2011) 753–762.
[59] A. Shapouri-Moghaddam, S. Mohammadian, H. Vazini, et al., Macrophage plasticity, polarization, and function in health and disease, J. Cell. Physiol. 233 (2018) 6425–6440.
[60] W.K.E. Ip, N. Hoshi, D.S. Shouval, S. Snapper, R. Medzhitov, Anti-inflammatory effect of IL-10 mediated by metabolic reprogramming of macrophages, Science 356 (2017) 513–519.
[61] M. Fukuzumi, H. Shinomiya, Y. Shimizu, K. Ohishi, S. Utsumi, Endotoxin-induced enhancement of glucose influx into murine peritoneal macrophages via GLUT1, Infect. Immun. 64 (1996) 108–112.
[62] M. Lu, M. Wan, K.F. Leavens, et al., Insulin regulates liver metabolism in vivo in the absence of hepatic Akt and Foxo1, Nat. Med. 18 (2012) 388–395.
[63] B.D. Manning, A. Toker, AKT/PKB signaling: navigating the network, Cell 169 (2017) 381–405.
[64] F.B. Feng, H.Y. Qiu, Effects of Artesunate on chondrocyte proliferation, apoptosis and autophagy through the PI3K/AKT/mTOR signaling pathway in rat models with rheumatoid arthritis, Biomed. Pharmacother. 102 (2018) 1209–1220.
[65] H. Wang, T.O. Khor, C.L. Saw, et al., Role of Nrf2 in suppressing LPS-induced inflammation in mouse peritoneal macrophages by polyunsaturated fatty acids docosahexaenoic acid and eicosapentaenoic acid, Mol. Pharm. 7 (2010) 2185–2193.
[66] A. Kaufmann, D. Gemsa, H. Sprenger, Differential desensitization of lipopolysaccharide-inducible chemokine gene expression in human monocytes and macrophages, Eur. J. Immunol. 30 (2000) 1562–1567.
[67] M.I. Stunault, G. Bories, R.R. Guinamard, S. Ivanov, Metabolism plays a key role during macrophage activation, Mediat. Inflamm. 2018 (2018) 2426138.
[68] L. Liu, Y. Lu, J. Martinez, et al., Proinflammatory signal suppresses proliferation and shifts macrophage metabolism from Myc-dependent to HIF1alpha-dependent, Proc. Natl. Acad. Sci. U. S. A. 113 (2016) 1564–1569.