Original Paper

Insulin Influences LPS-Induced TNF- α and IL-6 Release Through Distinct Pathways in Mouse Macrophages from Different Compartments

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Key Words

Alloxan • Diabetes • Macrophage • PI3K • ERK • Akt

Abstract

Background/Aims: Diabetic subjects are more susceptible to infections, which is partially due to insulin deficiency and hyperglycemia. We hypothesized that insulin influences cytokine release by macrophages from diabetic C57BL/6 mice stimulated with lipopolysaccharides (LPS).

Methods: Bone marrow-derived macrophages (BMDM) and tissue-specific macrophages from diabetic (alloxan 60 mg/kg, i.v.) male C57BL/6 mice were stimulated by LPS (100 ng/mL) and/or treated by insulin (1 mU/mL). **Results:** Using BMDM from diabetic mice, we showed that LPS induced an increase in TNF- α and IL-6 release and p38, SAPK/JNK, ERK 1/2, and Akt (308-Thr and 473-Ser) phosphorylation but not in PKC α / β II and delta. Insulin increased TNF- α and IL-6 secretion in LPS-stimulated macrophages as well as p-p38, p-SAPK/JNK, p-ERK 1/2, p-PI3K (p55) and p-Akt (473-Ser) expression. Furthermore, PI3-kinase inhibition by wortmannin decreased TNF- α release, and inhibition by LY294002 decreased both TNF- α and IL-6 levels after LPS-insulin treatment. PD98059, which inhibits the ERK upstream activators MAPK kinase (MKK) 1 and MKK2, reduced the effect promoted by insulin in BMDM stimulated by LPS. In tissue-specific macrophages, insulin reduced LPS-induced TNF- α , IL-6 and IL-1 β secretion in alveolar and peritoneal macrophages. **Conclusion:** These data suggest that insulin through the modulation of PI3-kinase and ERK 1/2 pathways drive different responses in macrophages, thereby enhancing our understanding of the plasticity of these cells.

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Introduction

Insulin is a peptide hormone secreted by β cells located in pancreatic islets [1]. β cell destruction compromises insulin secretion, which deregulates glucose homeostasis and

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leads to a condition known as *diabetes mellitus* (DM) [1, 2]. According to the American Diabetes Association (ADA), infections are a particular issue for diabetic patients due to immune system failure [2], and after insulin has been introduced to treat diabetic patients, the number of infections and complications related to these populations has decreased [1-3].

Macrophages are multifunctional cells that are found virtually in every tissue of the body and play a pivotal role in inflammatory response secreting inflammatory mediators [4]. Macrophages have compromised functions in diabetic individuals, which is partially responsible for their enhanced susceptibility to infections [5]. Moreover, by using different experimental models of diabetes, such as type I diabetes induced by alloxan or streptozotocin, and insulin resistance mice model, helps to explain the high susceptibility to infections in these subjects through a decrease in the capacity of insulin to interact with insulin-sensitive tissues (such as liver, fat and muscle). Consequently, it may lead to impairment of glucose utilization with consequently hepatic glucose output [6-11]. In addition, Gram-negative bacteria are responsible for approximately 25% of reported infections in the diabetic population [12]. These microorganisms feature in the cell walls a unique component known as lipopolysaccharide (LPS) [13]. LPS activates macrophages by interacting with Toll-like receptor (TLR)4 signaling via myeloid differentiation primary response gene 88 (MyD88)- and TIR-domain-containing adapter-inducing interferon- β (TRIF)-dependent pathways that culminate in pro-inflammatory events due to interactions among multiple signaling pathways [13, 14].

LPS stimulation leads to earlier mitogen-activated protein kinase (MAPK) activation by MyD88 [15]. MAPK are a group of molecules that regulate diverse cellular programs by relaying extracellular signals to intracellular responses [15, 16]. The most studied MAPK are extracellular-signal-regulated kinase (ERK 1/2), stress-activated protein kinase/Jun amino-terminal kinase (SAPK/JNK) and p38 [16]. ERK1/2 activation occurs through mitogen-activated protein kinase kinase (MEK)1 and MEK2 and involves the release of tumor progression locus 2 (Tpl2) from subunit 105, which is crucial for cytokine release [17]. SAPK/JNK and p38 activation by LPS promotes the synthesis and release of pro-inflammatory mediators by macrophages [18]. In addition, p38 activates mitogen-activated protein kinase-activated protein kinase 2 (MK2), which indirectly regulates Akt activity by controlling phosphatidylinositol 3, 4,5-trisphosphate (PIP₃) availability; therefore, the down-regulation of MK2 diminishes PIP₃ production and decreases Akt activity [19]. PI3K/Akt signaling plays an important role in cell survival [20, 21]. PI3K consists of a catalytic subunit (p110) and two regulatory subunits (p85 and p55) [20] and phosphorylates phosphatidylinositol 4, 5-bisphosphate (PIP₂) to generate PIP₃. PIP₃ is responsible for recruiting both phosphoinositide-dependent protein kinase 1 (PDK1) and Akt to the cell membrane, where PDK1 phosphorylates Akt [22]. Akt comprises a family of serine-threonine protein kinases that regulate cellular responses [23]. In an inflammatory process, PI3K/Akt signaling-controls macrophage response during the course of immune response [21, 23].

Insulin is primarily involved in metabolism regulation, and acts modulating immune cell responses via insulin receptors [24]. We hypothesize that insulin might play a role in regulating cytokine secretion by macrophages from diabetic mice. In this study, we performed an *in vitro* study to investigate the insulin effects in LPS-induced cytokine release by macrophages from different compartments. We found that insulin did not affect LPS-stimulated tumoral necrosis factor (TNF)- α and interleukin (IL)-6 released by bone marrow-derived macrophages (BMDM) from non-diabetic mice, although this hormone increased the cytokine release by LPS-stimulated BMDM from diabetic mice. In addition, insulin increased LPS-induced p-p38, p-SAPK/JNK, p-ERK 1/2, p-PI3K (p55) and p-Akt (473-Ser) levels. In tissue-specific macrophages, insulin decreased LPS-induced TNF- α , IL-6 and IL-1 β secretion by both alveolar and peritoneal macrophages. In our study, we demonstrated that insulin plays an important role in macrophages from diabetic mice by distinctly influencing the macrophages response from different tissues.

Materials and Methods

Animals and ethics statement

Forty-two specific pathogen-free male C57BL/6 mice (8-12 weeks old, wild-type phenotype, and weighing 25 ± 2 g at baseline) were used. The animals were maintained at $23 \pm 2^\circ\text{C}$ under a 12-h light:dark cycle. Food and water were provided *ad libitum* before and during the experimental period. This study was conducted in strict accordance with the principles and guidelines of the National Council for the Control of Animal Experimentation (CONCEA) and approved by the Ethics Committee on Animal Use (CEUA) at the School of Pharmaceutical Sciences (FCF), University of São Paulo, Brazil (protocol number: CEUA/FCF/339). Cellular content was collected under ketamine/xylazine anesthesia, and all efforts were made to minimize animal suffering.

Induction of diabetes mellitus

Diabetes mellitus was induced via intravenous injection of 60 mg/kg of alloxan monohydrate (Sigma Chemical Co, St. Louis, Mo) dissolved in physiological saline (0.9% NaCl), as previously described [25]. After 10 days, the glycemia of the animals was measured using Accu-Chek Advantage II (Roche Diagnostics, São Paulo, SP, Brazil). Only animals with glycaemia levels above 300 mg/dL were considered diabetic for this study.

Cell isolation and cell culture

Bone marrow-derived macrophages. BMDM were obtained as previously described [26]. Briefly, bone marrow was collected from femurs and cultured in RPMI-1640 (Gibco, Gaithersburg, MD, USA) medium supplemented with 20% fetal bovine serum (FBS) (Sigma Chemical Co, St. Louis, Mo, USA), 30% L929-cell conditioned medium and gentamicin (Gibco, Gaithersburg, MD, USA) (40 mg/L), at 37°C in a 5% CO_2 atmosphere. After four days, the cells received a fresh supplemented medium and were incubated for three additional days. Macrophages were collected from the dishes using cold phosphate buffered saline (PBS) and incubated in RPMI-1640 medium supplemented with 10% FBS, 5% L929-cell conditioned medium and gentamicin (Gibco, Gaithersburg, MD, USA) (40 mg/L), at 37°C in a 5% CO_2 atmosphere. The next day, the cells were washed with warm PBS to remove non-adherent cells and FBS.

Tissue-specific macrophages: alveolar and peritoneal macrophages. Alveolar and peritoneal macrophages were obtained by lung lavage and peritoneal lavage, respectively, using sterilized cold PBS pH 7.4. The cells were allowed to adhere for 2 h (37°C , 5% CO_2), followed by three washes with warm PBS. The cells were cultured overnight in RPMI-1640 containing 10% FBS. The next day, the cells were washed with warm PBS to remove non-adherent cells and FBS.

Cell treatments

BMDM and tissue-specific macrophages were seeded and incubated in RPMI 1640 (Sigma Chemical Co, St. Louis, Mo, USA). The cells were treated at a final concentration of 1 mU/mL of insulin from bovine pancreas [27] (Sigma Chemical Co, St. Louis, Mo, USA) concomitantly stimulated with or without LPS (100 ng/mL) from *Escherichia coli* (serotype 055:B5) (Sigma Chemical Co, St. Louis, Mo, USA) for different time point in different assays. For the inhibition assay, BMDM from diabetic animals were pre-treated with inhibitors for PI3K (10 μM LY294002 and/or 10 nM wortmannin); ERK1/2 (2 μM PD98059) and/or p38 (10 μM SB20190) for 1 h before LPS and/or insulin addition [28]. PI3K inhibitors were added together with LPS and/or insulin. All pharmacological inhibitors were purchased from Cell Signaling Technologies (Danvers, MA, USA).

Cell viability assay

The viability of BMDM from diabetic mice was evaluated after 24 h using propidium iodide (PI) staining. Macrophages were detached from the plate with 10 mM PBS and EDTA solution. Cells were labeled with anti-mouse PI (Thermo Fisher Scientific Inc., Rockford, IL, USA) at a 1/100 dilution, acquired on a BD FACSCanto II flow cytometer (São Paulo, SP, Brazil) and analyzed using FlowJo Software 9.9.5 (Treestar, Ashland, OR, USA).

Electrophoresis and immunoblotting

Protein concentrations were determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., Rockford, IL). Protein samples containing 20 µg were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes using the Amersham Te 70 Pwr Semi-Dry Transfer system (Amersham Biosciences Corp., Piscataway, NJ, USA). For immunoblotting, nitrocellulose membranes were incubated in Tris-buffered saline-Tween (TBS-T) buffer (150 mM NaCl, 20 mM Tris, 1% Tween 20, pH 7.4) containing 5% non-fat milk dried milk for 1 h. Afterwards, the membranes were washed with TBS-T buffer three times for 5 min each. Then, the membranes were incubated for 12-14 h with primary antibodies (1:1000 dilution) against phospho-ERK 1/2 MAP Kinase (Thr183/Tyr185), phospho-p38 MAP Kinase (Thr180/Tyr182), phospho-Akt (308-Thr), phospho-Akt (473-Ser), phospho-PI3 Kinase p85 (Tyr458)/p55 (Tyr199), Phospho-PKCα/β II (Thr638/641) and phospho-PKCdelta (Tyr311) diluted in 5% bovine serum albumin in TBS-T at 4°C. The antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Membranes were incubated with anti-rabbit secondary antibody (1:10000) for 1 h (Abcam), and developed using enhanced chemiluminescence detection over the nitrocellulose membrane. Band densities were determined by densitometric analysis using Image Studio Lite Version 5.2. The density values of the bands were normalized in each lane to that of β-actin or glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Sigma Chemical Co, St. Louis, Mo, USA).

Enzyme-linked immunosorbent assay

Cells were cultured at different time points, and supernatants were collected, centrifuged, and analyzed by enzyme-linked immunosorbent assay (ELISA) using cytokine-specific kits (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

The data are presented as the mean ± SEM from at least three independent experiments. Statistical analyses were performed using GraphPad 6 software (San Diego, CA, USA), and Student's *t*-test and analysis of variance (ANOVA) followed by the Tukey-Kramer or Holm-Sidak test to perform comparisons. A *p* value lower than 0.05 was considered statistically significant.

Results

Type 1 diabetes experimental model

After 10 days of alloxan injection, the mice exhibited a significant reduction in body weight (before alloxan injection: 26.11 ± 0.38 g; 10 days thereafter: 23.82 ± 0.29 g, *n* = 17; *P* < 0.001) and elevated blood glucose levels (before alloxan injection: 156.8 ± 2.4 mg/dL; 10 days thereafter: 591.4 ± 3.90, *n* = 17; *P* < 0.001).

Insulin amplifies cytokine secretion by LPS-stimulated BMDM from diabetic mice

To understand the insulin effect under LPS stimulation in macrophages from non-diabetic and diabetic mice, we measured in LPS-induced TNF-α and IL-6 secretion by BMDM. Insulin did not alter cytokine release by LPS-stimulated BMDM from non-diabetic mice (data not shown). Then, we investigated TNF-α (Fig. 1A) and IL-6 (Fig. 1B) release in a kinetic study using BMDM from diabetic mice. Compared to LPS-stimulated cytokine production, insulin treatment boosted LPS-induced TNF-α release by BMDM after 3, 6 and 12 h; IL-6 concentrations increased after 6 and 12 h under these same conditions. However, insulin did not alter TNF-α and IL-6 secretion after 24 h of LPS stimulation.

Insulin does not affect the viability of BMDM from diabetic mice

We assessed whether cell viability was altered by insulin (1 mU/mL) and/or LPS (100 ng/mL). We stained BMDM from diabetic mice with PI and measured cell viability after 24 h via flow cytometry (Fig. 1C). Incubation for 24 h did not affect BMDM viability. Thus, we next investigated protein phosphorylation after insulin treatment and LPS stimulus in BMDM from diabetic animals.

Fig. 1. Insulin enhances LPS-induced TNF- α and IL-6 secretion in bone marrow-derived macrophages from diabetic mice without altering cell viability. Bone marrow-derived macrophages (BMDM) from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) in a kinetic study. (A) TNF- α and (B) IL-6 secretion by BMDM from diabetic mice was determined by ELISA. (C) BMDM viability from diabetic mice after 24 h was determined via flow cytometry after propidium iodide staining. The data represent the mean $\pm$ SEM from five independent experiments. * P <0.05 versus vehicle control; ** P <0.05 versus LPS.

Fig. 2. Insulin enhances LPS-induced MAPK phosphorylation in bone marrow-derived macrophages from diabetic mice. Bone marrow-derived macrophages (BMDM) from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) for 60 min. (A) p38, ERK 1/2 (p42/p44), SAPK/JNK (p46/p54) and GAPDH protein levels in BMDM from diabetic mice were determined by Western blotting. Relative (B) p38, (C) p42 MAPK, (D) p44 MAPK, (E) p46 SAPK/JNK and (F) p54 SAPK/JNK levels were determined by densitometry

(GAPDH was used as a control). For all conditions, data represent the mean $\pm$ SEM from five independent experiments. * P <0.05 versus vehicle control; ** P <0.05 versus LPS.

Insulin enhances LPS-induced MAPK phosphorylation in BMDM from diabetic mice

To understand the effects of insulin on LPS-induced signaling pathways, we checked MAPK phosphorylated proteins in BMDM from diabetic mice using Western blotting (Fig. 2A). We analyzed p38 (Fig. 2B); p42 (Fig. 2C) and p44 ERK (Fig. 2D), also known as ERK 1/2, p46 (Fig. 2E) and p54 SAPK/JNK (Fig. 2F). We found that LPS induced MAPK activation but that insulin alone did not. Simultaneously, insulin treatment increased LPS-induced MAPK phosphorylation in diabetic BMDM, suggesting that MAPK may be involved in the enhancement of cytokine release by insulin.

Effects of insulin on LPS-induced PI3K/Akt signaling

The effect of insulin on LPS-induced PI3K/Akt phosphorylation in BMDM from diabetic mice was evaluated using Western blotting (Fig. 3A). As a control, neither LPS nor insulin

Fig. 3. - Insulin enhances LPS-induced PI3K/Akt phosphorylation in bone marrow-derived macrophages from diabetic mice. Bone marrow-derived macrophages (BMDM) from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) for 60 min. (A) PI3K p85 and p55, Akt (308-Thr), Akt (473-Ser), and β -actin protein levels in BMDM from diabetic mice were determined by Western blotting. Relative (B) PI3K p85, (C) PI3K p55, (D) Akt (308-Thr) and (E) Akt (473-Ser) levels

were determined by densitometry (β -actin was used as a control). In all circumstances, the data represent the mean $\pm$ SEM from five independent experiments. * $P < 0.05$ versus vehicle control; ** $P < 0.05$ versus LPS.

Fig. 4. Insulin and/or LPS do not alter PKC phosphorylation in bone marrow-derived macrophages from diabetic mice. Bone marrow-derived macrophages (BMDM) from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) for 60 min. (A) PKC α/β , PKC delta and β -actin protein levels in BMDM from diabetic mice were determined by Western blotting. Relative (B) PKC α/β and (C) PKC delta levels were determined by densitometry (β -actin was used as a control). For all conditions, the data represent the mean $\pm$ SEM from five independent experiments.

alone altered p85 (Fig. 3B) and p55 (Fig. 3C) phosphorylation. Insulin induced higher phosphorylation in the catalytic p55 domain, but not in p85 in LPS-stimulated macrophages.

Fig. 5. Insulin enhances LPS-induced cytokine secretion in bone marrow-derived macrophages from diabetic mice via PI3K and ERK1/2. Bone marrow-derived macrophages (BMDM) were pre-treated with selective inhibitors of (A) (C) PI3K (10 nM wortmannin and 10 μ M LY294002) and (B) (D) ERK1/2 (2 μ M PD98059) or p38 (SB20190 10 μ M) and then incubated with insulin and/or LPS. BMDM from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) for 6 h. (A) (B) TNF- α and (C) (D) IL-6 secretion by BMDM from diabetic mice was determined by ELISA. The data represent the mean $\pm$ SEM from four independent experiments. * P <0.05 versus vehicle control; ** P <0.05 versus LPS; *** P <0.05 versus Ins + LPS.

To further understand this phenomenon, we examined Akt phosphorylation at both 308-Thr (Fig. 3D) and 473-Ser (Fig. 3E). LPS induced Akt phosphorylation at both 308-Thr and 473-Ser, whereas insulin alone did not alter Akt phosphorylation. Simultaneously, insulin treatment of LPS-stimulated BMDM increased Akt phosphorylation at 473-Ser.

Effects of insulin on LPS-induced PKC activation

To understand the effects of insulin treatment on LPS-induced PKC phosphorylation in BMDM from diabetic mice (Fig. 4A), we analyzed by Western blot the phosphorylation of PKC isoforms α/β II (Fig. 4B) and delta (Fig. 4C) and found out that insulin treatment did not activate PKC isoforms from diabetic mice with or without LPS stimulation.

Insulin enhances TNF- α and IL-6 secretion by LPS-stimulated BMDM via PI3K and ERK

We use pharmacological inhibitors to investigate the importance of PI3K phosphorylation in insulin-enhanced cytokine secretion. BMDM were treated with two specific PI3K inhibitors, wortmannin and LY294002. The effect of insulin on LPS-induced TNF- α (Fig. 5A) and IL-6 (Fig. 5C) by BMDM from diabetic mice was abolished by LY294002, whereas wortmannin abolished only TNF- α release. In addition, we investigated insulin amplification of LPS-induced TNF- α and IL-6 release by using two well-established pharmacological inhibitors of MEK1/2 (PD98059) and p38 (SB20190), which are responsible for inactivating ERK1/2 and p38, respectively. Pharmacological ERK inhibition had no effect on LPS-induced cytokine release, whereas p38 inhibition reduced LPS-induced TNF- α (Fig. 5B) and IL-6 (Fig. 5D) release. The effects of insulin on LPS-dependent TNF- α and IL-6 release by BMDM from diabetic mice were abolished by PD98056 (ERK 1/2 inhibition), while SB20190 (p38 inhibition) reduced the effect of insulin on LPS-induced TNF- α and IL-6 release. Taken together, these data indicate that insulin enhances TNF- α and IL-6 concentrations in BMDM stimulated with LPS via the PI3K and ERK pathways.

Fig. 6. Insulin inhibits LPS-induced pro-inflammatory cytokine secretion in alveolar and peritoneal macrophages from diabetic mice. Alveolar and peritoneal macrophages from diabetic mice were incubated with insulin (1 mU/mL) and simultaneously stimulated with LPS (100 ng/mL) for 30 min and 3 h. (A) IL-6 and (B) TNF-α secretion by alveolar macrophages at 3 h min. (C) IL-6 secretion by peritoneal macrophages at 30 min. (D) TNF-α, (E) IL-6 and (F) IL-1β secretion by peritoneal macrophages at 3 h. Cytokine levels were determined by ELISA. The data represent the mean ± SEM from five independent experiments. *P<0.05 versus vehicle control; **P<0.05 versus LPS.

Insulin reduces LPS-induced pro-inflammatory cytokine secretion by alveolar and peritoneal macrophages from diabetic mice

We also investigated the effect of insulin on LPS-induced pro-inflammatory cytokine release in tissue-specific macrophage, alveolar and peritoneal macrophages. In alveolar macrophages (AM), LPS induced higher levels of TNF-α (Fig. 6A) and IL-6 (Fig. 6B) release compared to the control. Insulin reduced TNF-α and IL-6 secretion in AMs stimulated with LPS after 3 h. Peritoneal macrophages (PM) stimulated with LPS released higher levels of TNF-α (Fig. 6C), IL-1β (Fig. 6D) and IL-6 (Fig. 6E-6F) compared to controls. In addition, insulin decreased the LPS-induced release of IL-6 after 30 min and IL-1β after 3 h. Insulin alone did not affect TNF-α and IL-6 concentrations after 3 h, suggesting that insulin distinctly modulates the LPS response of resident macrophages from different tissues in diabetic mice.

Discussion

Accordingly with our findings, insulin positively regulated TNF-α and IL-6 secretion by up-regulating p-p38, p-SAPK/JNK, p-ERK 1/2, p-PI3K (p55) and p-Akt (473-Ser). Furthermore, PI3-kinase inhibition by wortmannin decreased TNF-α release, and inhibition by LY294002 decreased both TNF-α and IL-6 after LPS-insulin treatment. PD98059, which inhibits MKK1 and MKK2, reduced the insulin effect on LPS-induced cytokine release in BMDM. In tissue-specific macrophages, insulin reduced LPS-induced TNF-α, IL-6 by AM and IL-6 and IL-1β secretion by PM.

Macrophages play a key role to regulate and avoid a prolonged inflammatory response exerting strict control against pathogens [4]. Cytokine release is an important process in homeostasis [4], and dysregulation in this mechanism in diabetic patients can be associated with high rates of infection [5]. After an extended period, the effects of diabetes itself including hyperglycemia and insulin deficiency can disrupt macrophage function [2, 29]. Our results demonstrated that insulin increased LPS-induced cytokine secretion by BMDM, but decreased cytokine release in AM and PM, suggesting a modulatory effect promoted by insulin. In addition, insulin promoted distinctly effects in macrophages from different compartments and it can be due to macrophage actions according to the tissue microenvironment. It has recently been discussed extensively by Davies and Taylor [30], and by Perdiguero and Geissmann [31]. By acting on non-diabetic [27] and diabetic mononuclear cells [24], it has been demonstrated that insulin plays additional roles beyond glucose uptake [32] and seems to have important immunomodulatory effects in macrophages. Moreover, insulin binds to its own receptor and has different roles depending on the cell type. In 3T3-L1 adipocytes, insulin increases p38 phosphorylation [33], whereas the opposite occurs in neuronal cells [34]. In addition, different concentrations of insulin activate p38 and ERK 1/2 without activating SAPK/JNK in vascular smooth muscle cells [35]. However, insulin-treated AM display no MAPK and Akt phosphorylation, and therefore do not exhibit alterations in TNF- α secretion [27]. In the present study, we showed that insulin alone had no effect on macrophages from diabetic mice.

Cytokines, such as TNF- α is enhanced in the presence of LPS and has influences on BMDM viability [36]. We found that LPS stimulation with or without insulin did not affect the viability of BMDM from diabetic mice, despite the change in the concentration of TNF- α . In addition, macrophages from different compartments have different surveillance profiles. PI3K/Akt signaling and ERK 1/2 phosphorylation play a protective, anti-apoptotic role in osteoclasts, which is diminished when PI3K and ERK 1/2 are inhibited, thus demonstrating the importance of both molecules in signal transduction [37]. Moreover, the role of p38 appears to be ambiguous, as p38 seems to activate Akt indirectly; their activation culminates in either death or survival depending on the cell type [37, 38]. ERK 1/2, p38, and SAPK/JNK activation by LPS promotes synthesis and release of pro-inflammatory mediators by M1 macrophages during the inflammatory response [16, 18]. In the present study, insulin treatment promoted higher cytokine release by LPS-stimulated BMDM from diabetic mice but not in LPS-stimulated BMDM from non-diabetic mice. Insulin appears to work together with LPS in PI3K p55 catalytic subunit phosphorylation to generate more PIP₃ from PIP₂. Insulin also selectively enhanced Akt phosphorylation at 473-Ser but not at 308-Thr. Somehow, the PI3K/Akt pathway appears connected with MAPK due to the higher levels of p38, ERK 1/2 and SAPK/JNK phosphorylation. As such, we suggest the existence of a bridge that connects LPS stimulation and insulin treatment that boosts the release of pro-inflammatory mediators by BMDM from diabetic mice. Furthermore, our macrophage studies using pharmacological inhibitors of PI3K, ERK 1/2 and p38 helped us identify the proteins involved in the effect of insulin on LPS stimulation. We found that PI3K and ERK 1/2 inhibition significantly reduced the ability of insulin to boost LPS-induced cytokine release by BMDM from diabetic mice. Consistent with our results, insulin treatment *in vitro* in LPS- and interferon (IFN)- γ -stimulated non-diabetic PM promoted higher levels of IL-6 and TNF- α secretion, which was blocked by ERK inhibition [29].

In non-diabetic AM, insulin inhibits LPS-induced p38 and ERK 1/2 MAPK, PKC and Akt phosphorylation, resulting in reduced TNF- α levels [27]. Nevertheless, high concentrations of insulin promote ERK 1/2 but not Akt phosphorylation in PM; these high concentrations do not alter cytokine release [29]. However, according to our results in tissue-specific macrophages from diabetic mice, insulin promoted a reduction in LPS-induced TNF- α and IL-6 released by AM, while IL 1- β and IL-6 secreted levels by PM. Under high glucose concentration, primary microglia showed increased sensitivity *in vitro* to LPS stimulus due to higher TLR4 expression and inflammatory cytokines secretion [34]. Moreover, in mononuclear cells from of type 1 diabetes patients, insulin infusion suppresses while glucose

infusion induces TLR4 expression [39], which make stronger the concept that both insulin and glucose might modulate the macrophage functions.

Conclusion

Taken together, these data indicate that insulin drives different responses through distinct pathways in macrophages, thereby enhancing our understanding of the plasticity of these cells.

Abbreviations

ADA (American Diabetes Association); AM (Alveolar Macrophage); BMDM (Bone marrow-derived macrophage); CEUA (Ethics Committee on Animal Use); CONCEA (National Council for the Control of Animal Experimentation); DM (Diabetes mellitus); ELISA (Enzyme-linked immunosorbent assay); ERK 1/2 (Extracellular-signal-regulated kinases); FBS (Fetal Bovine Serum); FCF (School of Pharmaceutical Sciences); GAPDH (Glyceraldehyde 3-phosphate dehydrogenase); IL (Interleukin); IFN (Interferon); LPS (Lipopolysaccharide); MAPK (Mitogen-activated protein kinase); MEK (Mitogen-activated protein kinase kinase); MK2 (Mitogen-activated protein kinase-activated protein kinase 2); MyD88 (Myeloid differentiation primary response gene 88); PBS (Phosphate Buffered Saline); PDK1 (Phosphoinositide-dependent protein kinase 1); PI (Propidium Iodide); PI3K (Phosphoinositide 3-kinase); PIP₂ (Phosphatidylinositol 4,5-bisphosphate); PIP₃ (Phosphatidylinositol 3,4,5-trisphosphate); PKC (Protein Kinase C); PM (Peritoneal Macrophage); SAPK/JNK (Stress-activated protein kinases/Jun amino-terminal kinases); TLR (Toll-like receptor); TNF (Tumoral necrosis factor); Tpl2 (Tumor Progression Locus 2); TRIF (TIR-domain-containing adapter-inducing interferon- β); TSB-T (Tris-buffered Saline-Tween).

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Disclosure Statement

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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