

## Both adiponectin and interleukin-10 inhibit LPS-induced activation of the NF- $\kappa$ B pathway in 3T3-L1 adipocytes

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### ABSTRACT

Adiponectin and interleukin 10 (IL-10) are adipokines that are predominantly secreted by differentiated adipocytes and are involved in energy homeostasis, insulin sensitivity, and the anti-inflammatory response. These two adipokines are reduced in obese subjects, which favors increased activation of nuclear factor kappa B (NF- $\kappa$ B) and leads to elevation of pro-inflammatory adipokines. However, the effects of adiponectin and IL-10 on NF- $\kappa$ B DNA binding activity (NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65) and proteins involved with the toll-like receptor (TLR-2 and TLR-4) pathway, such as MYD88 and TRAF6 expression, in lipopolysaccharide-treated 3T3-L1 adipocytes for 24 h elevated IL-6 levels; activated the NF- $\kappa$ B pathway cascade; increased protein expression of IL-6R, TLR-4, MYD88, and TRAF6; and increased the nuclear activity of NF- $\kappa$ B (p50 and p65) DNA binding. Adiponectin and IL-10 inhibited the elevation of IL-6 levels and activated NF- $\kappa$ B (p50 and p65) DNA binding. Taken together, the present results provide evidence that adiponectin and IL-10 have an important role in the anti-inflammatory response in adipocytes. In addition, inhibition of NF- $\kappa$ B signaling pathways may be an excellent strategy for the treatment of inflammation in obese individuals.

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### 1. Introduction

White adipose tissue plays a role in energy storage and insulation from environmental temperature and trauma. Recent advances in adipose biology have provided convincing evidence that adipocytes also secrete multiple proteins (i.e., adipokines) that influence metabolism in peripheral tissues [1,2]. Obesity has been shown to cause an increase in plasma concentrations of a number of pro-inflammatory (e.g., IL-6, TNF- $\alpha$ ) markers that are expressed and released by adipocytes [3]. In addition, the pro-inflammatory status in obesity promotes a decrease in anti-inflammatory adipokines, such as adiponectin and IL-10 plasma concentrations [4,5].

Previous studies [6,7] have shown that nuclear factor  $\kappa$ B (NF- $\kappa$ B) transcription factor is a key mediator of inflammation in adipose tissue. New data have shown a close relationship between toll-like receptor 4 (TLR-4) and activation of the NF- $\kappa$ B pathway, which leads to an elevation of pro-inflammatory adipokine gene and protein expression in adipose tissue [8,9].

Cani et al. [3] showed that increased endotoxin levels in obesity may be a key factor for the initiation of inflammation in adipose tissue, and the prototypical endotoxin, lipopolysaccharide (LPS), acts on TLR-4. Toll-like receptors are transmembrane proteins that play an important role in recognizing microbial pathogens and mediating whole body inflammation [10]. They are highly expressed in cells of the innate immune system [11]. In addition, TLR-2 and TLR-4 are also found in various other cell types, including adipocytes, hepatocytes, and myocytes [12,13].

Studies have shown that adiponectin and interleukin 10 (IL-10), two adipocyte-derived cytokines, act as potent inhibitors of inflammatory responses [6,7,14,15].

Zoico et al. [7] demonstrated that globular adiponectin and full-length adiponectin decreased NF- $\kappa$ B activity in 3T3-L1 adipocytes by 50% and 40%, respectively, compared with the NF- $\kappa$ B activation induced by LPS alone. This result demonstrated the important anti-inflammatory role of adiponectin to combat obesity-mediated inflammation.

Interestingly, Turner et al. [16] explored the anti-inflammatory effects of IL-10 in primary human cultures of differentiated adipocytes and found that IL-10 was ineffective against TLR-4-induced cytokine secretion. Human adipocytes, however, do not express the IL-10 receptor, which has been shown to respond to IL-10 in the murine 3T3-L1 adipocyte model.

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The effects of adiponectin and IL-10 on NF- $\kappa$ B DNA binding activity (NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65) and the expression of proteins involved in the signaling of the toll-like receptor (TLR-2 and TLR-4) pathway, such as MYD88 and TRAF6, in lipopolysaccharide-treated 3T3-L1 adipocytes are not clear.

In the present study, we analyzed the effects of adiponectin, IL-10, and the combination of adiponectin and IL-10 on TLR-2, TLR-4 and the NF- $\kappa$ B pathway in 3T3-L1 adipocytes in the presence of LPS.

## 2. Materials and methods

### 2.1. Cell culture

3T3-L1 cells were obtained from American Type Culture Collection and cultured at 37 °C in 5% CO<sub>2</sub>/95% humidified air. The cells were maintained in Dulbeccó's Eagle modified medium (DMEM) with 25 mM glucose, 1.0 mM pyruvate, 4.02 mM L-alanine-glutamine, and 10% fetal bovine serum (Gibco, New York, USA). Cell differentiation began 24 h after confluence and took 4 days in a medium containing 0.25  $\mu$ M dexamethasone, 0.5 mM 3-isobutyl-1-methylxanthine, and 5  $\mu$ g/mL insulin (Sigma). After differentiation, the cells were cultured for 8 days in growth medium containing 5  $\mu$ g/mL insulin.

### 2.2. Treatment

On the tenth day after differentiation, the cells were pretreated for a 24-h period with a medium containing 0.5% serum fetal bovine. The cells were harvested 24 h later. Cells were treated with adiponectin (50 ng/mL), IL-10 (5 ng/mL) or a combination of adiponectin (50 ng/mL) and IL-10 (5 ng/mL) for 24 h in the presence of LPS (100 ng/mL), and harvested 24 h later. In the control plates (C), the medium was changed, but no treatment was added. The cultured medium and adipocytes were collected in tubes and stored at –80 °C.

Preliminary experiments were performed to determine the concentrations of adiponectin and IL-10 (data not shown). In addition, we previously ran a time-course experiment (3, 6, 12, and 24 h of treatment).

### 2.3. Determination of the IL-6 level in the adipocyte culture medium

Quantitative assessment of IL-6 levels in the culture medium was performed using an enzyme linked immunosorbent assay (ELISA) (DuoSet ELISA, R&D Systems, Minneapolis, MN). The IL-6 (DY506) assay sensitivity was found to be 5.0 pg/mL, and the range was 31.2–2000 pg/mL. The intra- and inter-assay variability of the IL-6 kit was 2.7–5.2%. All samples were run in triplicate, and the mean value was used for analysis. The protein concentration of 3T3-L1 adipocytes was determined by the Bradford assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. The results are expressed in pg/ $\mu$ g protein.

### 2.4. Protein analysis by Western blotting

3T3-L1 adipocyte cells were homogenized in 1.0 mL of solubilization buffer at 4 °C [1% Triton X-100, 100 mM Tris-HCl (pH 7.4), 100 mM sodium pyrophosphate, 100 mM sodium fluoride, 10 mM EDTA, 10 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mg aprotinin/mL]. Insoluble material was removed by centrifugation for 30 min at 9000g in a 70 Ti rotor (Beckman, Fullerton, CA, USA) at 4 °C. The protein concentration of the supernatants was determined with a BCA assay (Bio-Rad, Hercules, CA, USA). Proteins were denatured by boiling (5 min) in

Laemmli sample buffer [17] containing 100 mM DTT and subjected to 10% SDS-PAGE in a Bio-Rad miniature slab gel apparatus.

Electrotransfer of proteins from the gel to nitrocellulose membranes was performed for ~1.30 h/4 gels at 15 V (constant) in a Bio-Rad semi-dry transfer apparatus. Nonspecific protein binding to the nitrocellulose was reduced by preincubation for 2 h at 22 °C in blocking buffer (5% nonfat dry milk, 10 mM Tris, 150 mM NaCl, and 0.02% Tween 20). The nitrocellulose membranes were incubated overnight at 4 °C with antibodies against IL-6R, TLR-2, TLR-4, MYD88, TRAF6, and alpha-tubulin (Santa Cruz Biotechnology, CA, USA), which were all diluted 1:1000 in blocking buffer with 1% BSA. After incubation, the membranes were washed for 30 min in blocking buffer without BSA. The blots were subsequently incubated with peroxidase-conjugated secondary antibody for 1 h at 22 °C. For the evaluation of protein loading, membranes were stripped and reblotted with anti-alpha-tubulin antibody as appropriate. Specific bands were detected by chemiluminescence, and visualization/capture was performed by exposure of the membranes to RX films. Band intensities were quantified by optical densitometry of developed autoradiographs (Scion Image software, Scion Corporation, Frederick, MD, USA).

### 2.5. Nuclear extraction

3T3-L1 adipocytes were rapidly removed and homogenized in accordance with the manufacturer's instructions for the Panomics Nuclear Extraction Kit (AY2002). The nuclear fraction was stored at –80 °C.

### 2.6. NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65 DNA binding assay

Nuclear-localized NF- $\kappa$ B was quantified using a Transcription Factor ELISA Kit to detect the DNA binding of the p50 and p65 subunits of NF- $\kappa$ B (Panomics, Fremont, CA; EK 1110 and 1120, respectively). All reagents required for preparing nuclear extracts and performing ELISA assays were included, and all reagents were used as described by the manufacturer.

### 2.7. Statistical analysis

The results are expressed as the mean  $\pm$  S.E.M. We used Student's *t* test to compare the treatment effects (control vs. LPS; LPS vs. LPS + adiponectin; LPS vs. LPS + IL-10; and LPS vs. LPS + adiponectin and IL-10). Values of *p* < 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. Time course

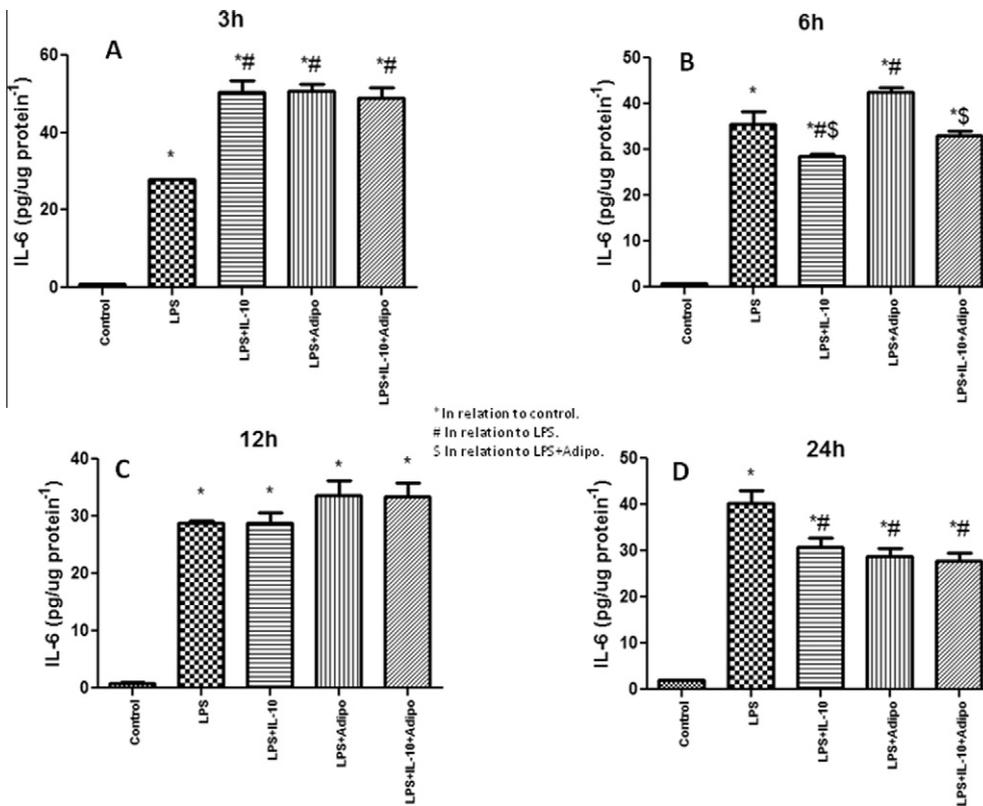
Fig. 1A–D shows that the IL-6 levels in the culture medium after 3, 6, 12, and 24 h were higher in the LPS, LPS + adiponectin, LPS + IL-10, and LPS + adiponectin and IL-10 groups compared with the control group (*p* < 0.05).

Interestingly, the IL-6 level was lower in LPS group after 3 h compared with the other treatments (*p* < 0.05).

After 6 h, IL-10 and LPS reduced the IL-6 level compared with LPS alone. Conversely, LPS + adiponectin increased the IL-6 level compared with LPS alone, LPS + IL-10 and LPS + IL-10 and adiponectin (*p* < 0.05).

In addition, adipokines incubated with LPS for 24 h induced an approximately 25% decrease in the IL-6 level in the culture medium.

We observed similar results for NF- $\kappa$ Bp50 nuclear activity in adipocytes after 3 and 6 h of treatment among groups (Fig. 2A



**Fig. 1.** Time course of IL-6 release in the culture medium of 3T3-L1 adipocytes treated with LPS, LPS + IL-10, LPS + adiponectin.  $n = 6$  for all groups. \*Values are means  $\pm$  SE. \* $p < 0.05$  in relation to control, # $p < 0.05$  in relation to LPS.

and B); however, LPS treatment for 12 and 24 h caused an increase in NF- $\kappa$ Bp50 nuclear activity in adipocytes compared with the other groups ( $p < 0.01$ ) (Fig. 2C and D).

The NF- $\kappa$ Bp65 nuclear activity in adipocytes was increased by LPS treatment at all examined time points compared with control and adipokine-treated groups. The addition of adiponectin, IL-10 or adiponectin + IL-10 decreased the effect of LPS (Fig. 3A–D) on NF- $\kappa$ Bp65 nuclear activity in adipocytes. After 6, 12 and 24 h of adipokine treatment, this parameter was similar to the control group (Fig. 3B–D).

After determining the best time response for our treatments with adipokines, we chose to assess the 24-h time point, and we divided our study into three distinct experiments. We assessed the effects of adiponectin, IL-10, and adiponectin combined with IL-10 on TLR-2, TLR-4 and on the NF- $\kappa$ B pathway in adipocytes in the presence the LPS.

Previous studies have shown that LPS induces NF- $\kappa$ B activation and IL-6 production in adipocytes [1,6,7,18]. In addition, we observed that LPS increased TLR-4, MYD88, and TRAF6 expression. These data demonstrate a classic LPS-mediated pro-inflammatory response in adipocytes. These results are shown in Figs. 4A–D and 5A–D.

Figs. 6A–D and 7A–D show the effects of adiponectin on the LPS-induced inflammatory response in adipocytes. Compared with LPS alone, adiponectin reduced IL-6 levels and both NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65 nuclear activity in adipocytes ( $p < 0.05$ ). In addition, adiponectin increased TLR-2, MYD88, and TRAF6 protein expression compared with LPS alone ( $p < 0.05$ ).

Figs. 8A–D and 9A–D show the effects of IL-10 on the LPS-induced inflammatory response in adipocytes. Compared with LPS alone, IL-10 reduced the IL-6 level and NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65 nuclear activity in adipocytes ( $p < 0.05$ ). We did not observe any effects of IL-10 on TLR-2, MYD88, or TRAF6 protein expression.

Figs. 10A–D and 11A–D show the effects of adiponectin combined with IL-10 on the LPS-induced inflammatory response in adipocytes. Compared with LPS alone, the combination of adiponectin and IL-10 reduced the IL-6 level, MYD88 protein expression and NF- $\kappa$ Bp50 and NF- $\kappa$ Bp65 nuclear activity in adipocytes ( $p < 0.01$ ).

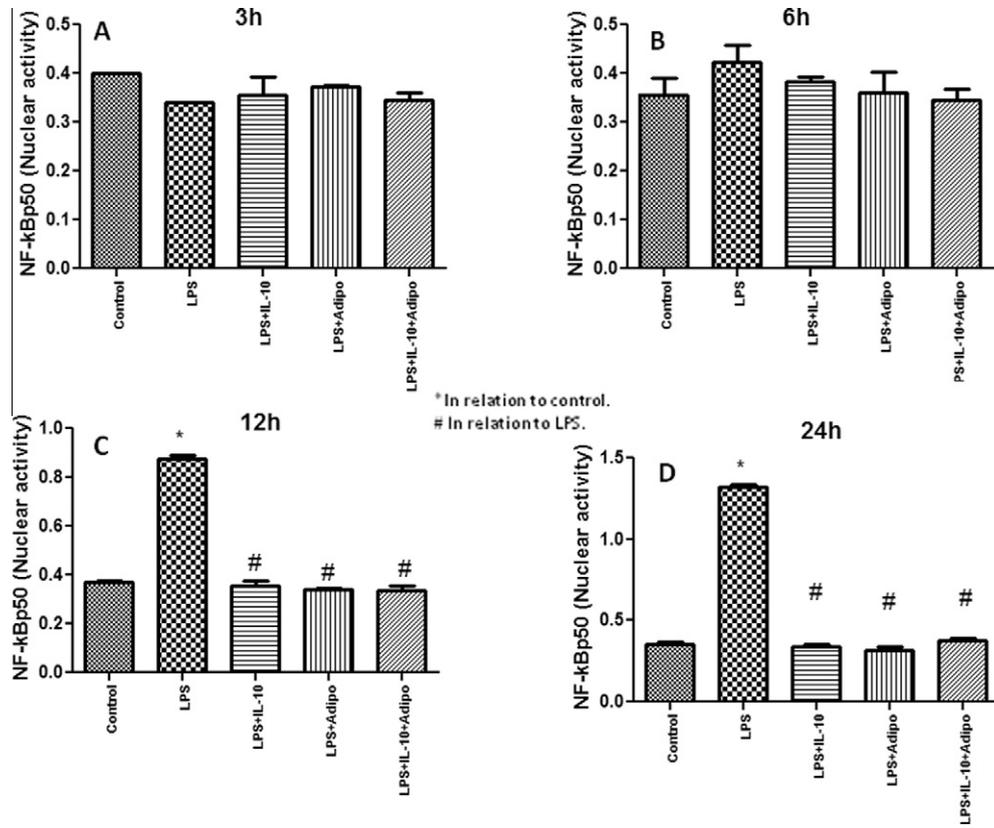
#### 4. Discussion

The present study shows that the treatment with anti-inflammatory adipokines was effective in reducing the activation of inflammatory pathways, especially the NF- $\kappa$ B pathway. In addition, anti-inflammatory adipokines decreased IL-6 levels.

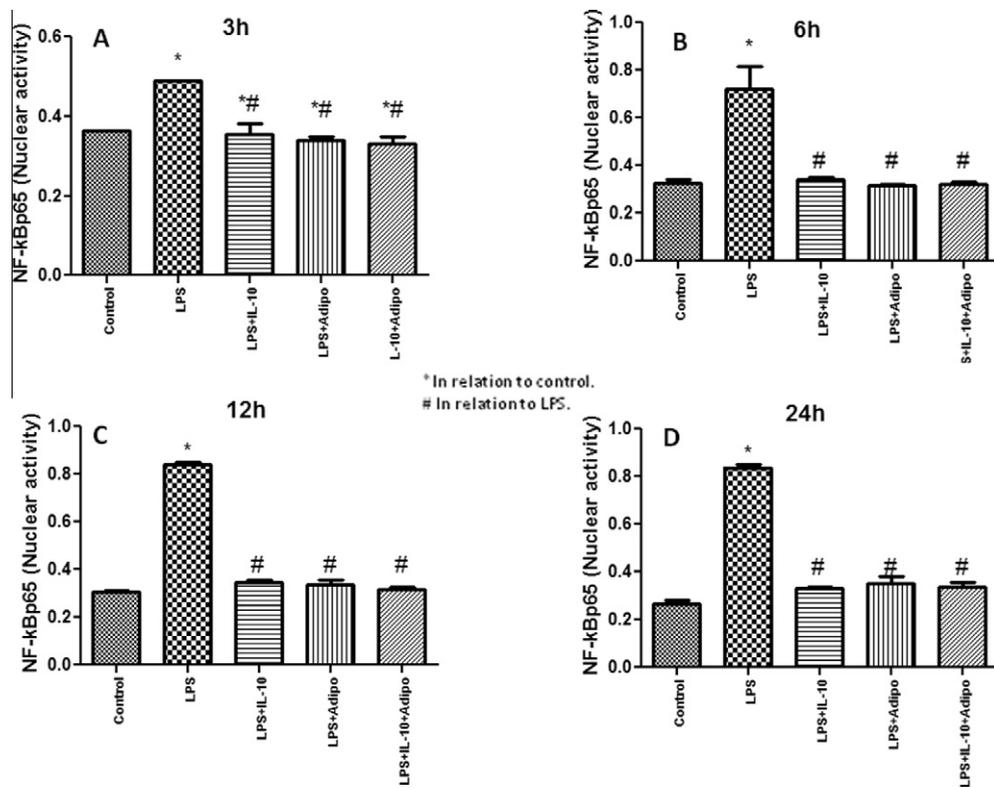
In agreement with previous studies, LPS caused an increase in IL-6, IL-6R, TLR-4, MYD88, and TRAF6 protein expression and in the nuclear activity of NF- $\kappa$ B (p50 and p65) DNA binding [6,7,19].

One of the questions addressed in the present study was how adiponectin would affect the inflammatory response in the presence of LPS. We observed that the addition of adiponectin reduced NF- $\kappa$ B (both p50 and p65) activation.

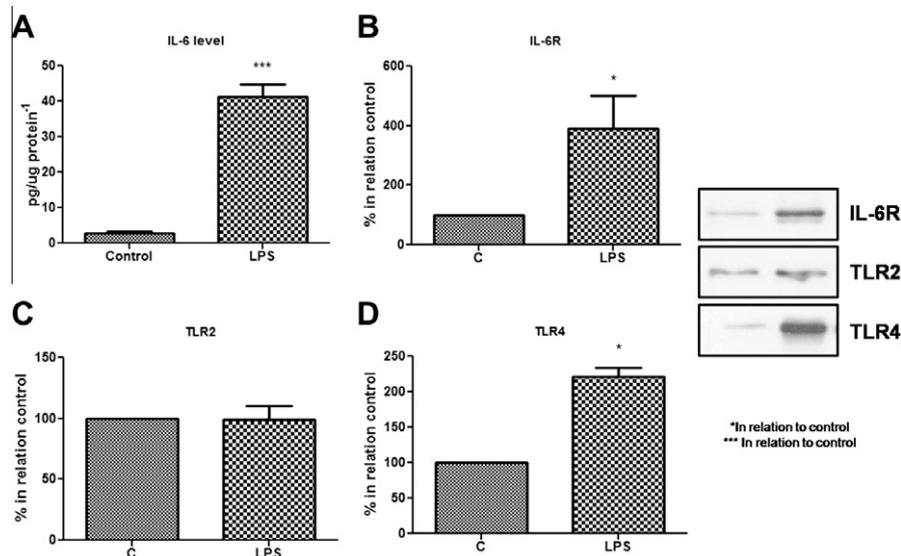
Ajuwon and Spurlock [6] showed that adiponectin may be a local regulator of inflammation in the adipocyte and adipose tissue via its regulation of the NF- $\kappa$ B and PPAR $\gamma$ 2 transcription factors. They used primary adipocytes from pig subcutaneous adipose tissue with or without treatment with LPS and adiponectin. Although LPS induced an increase in NF- $\kappa$ B activation, adiponectin suppressed both NF- $\kappa$ B activation and the induction of IL-6 expression by LPS. Similar results were obtained in 3T3-L1 adipocytes. In addition, adiponectin antagonized the LPS-induced increase in TNF- $\alpha$  mRNA expression and lead towards a reduction of its accumulation in the culture media in 3T3-L1 adipocytes. Adiponectin also induced an upregulation of PPAR $\gamma$ 2 mRNA.



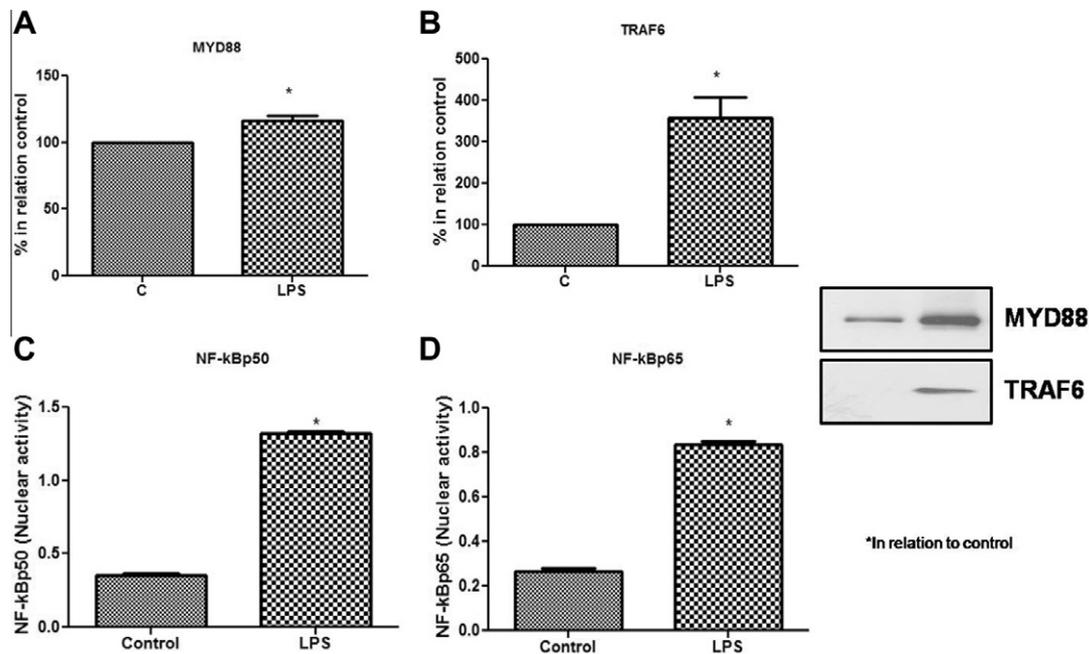
**Fig. 2.** Time course of DNA-binding activity of NF-kBp50 in 3T3-L1 adipocytes treated with LPS, LPS + IL-10, LPS + adiponectin.  $n = 6$  for all groups. \*Values are means  $\pm$  SE. \* $p < 0.05$  in relation to control, # $p < 0.05$  in relation to LPS.



**Fig. 3.** Time course of DNA-binding activity of NF-kBp65 in 3T3-L1 adipocytes treated with LPS, LPS + IL-10, LPS + adiponectin.  $n = 6$  for all groups. Values are means  $\pm$  SE. \* $p < 0.05$  in relation to control, # $p < 0.05$  in relation to LPS.



**Fig. 4.** Effect of LPS for 24 h on the IL-6 level in culture medium and on the 3T3-L1 protein expression of IL-6R, TLR-2, and TLR-4.  $n = 6$  for all groups. Values are means  $\pm$  SE. \* $p < 0.05$  and \*\*\* $p < 0.0001$  in relation to control.

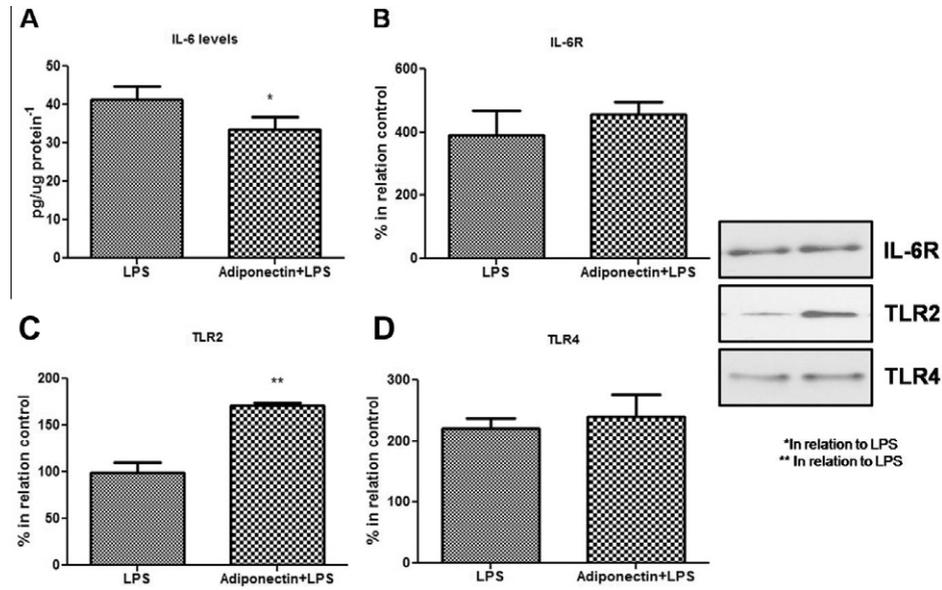


**Fig. 5.** Effect of LPS for 24 h on the 3T3-L1 protein expression of MYD88, TRAF6, NF-kBp50, NF-kBp65.  $n = 6$  for all groups. Values are means  $\pm$  SE. \* $p < 0.05$  and \*\*\* $p < 0.0001$  in relation to control.

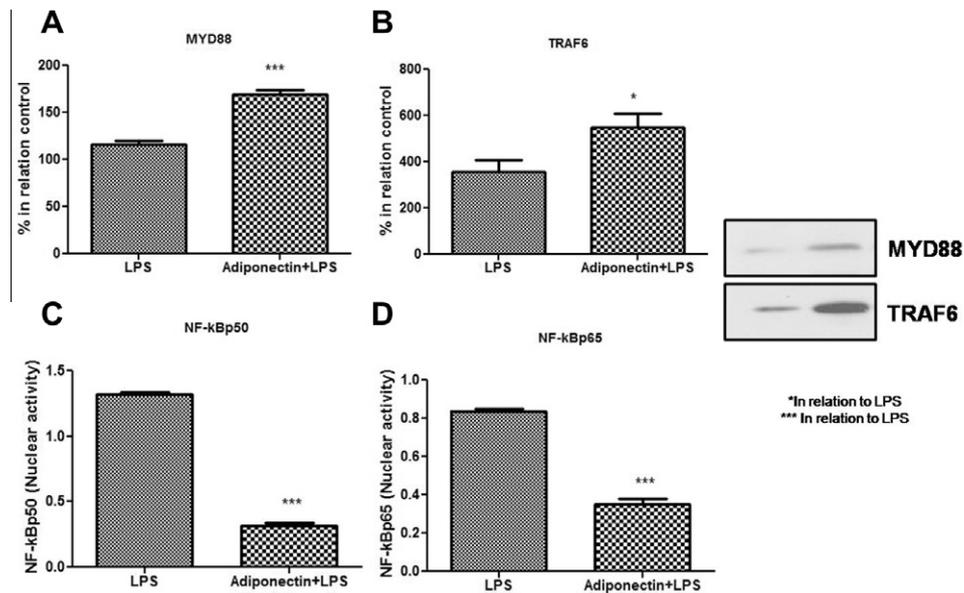
Similar results were found in a study by Zoico et al. [7], which showed that adiponectin (two isoforms of adiponectin: globular and full length) significantly suppressed LPS-induced expression of IL-6 mRNA in adipocytes and reduced the concentration of IL-6 in culture media. Adiponectin pretreatment significantly reduced the increase in monocyte chemoattractant protein 1 (MCP-1) mRNA in adipocytes exposed to LPS. In culture media, the increase in MCP-1 detected after LPS stimulation was significantly attenuated after pretreatment with adiponectin. In 3T3-L1, adiponectin reduced NF- $\kappa$ B activity by 50% compared with the NF- $\kappa$ B activation induced by LPS alone. Moreover, adiponectin significantly attenuated IkappaB-alpha and IKK gene expression.

Using macrophages, Park et al. [20] demonstrated the mechanism by which adiponectin suppresses the inflammatory pathway. They showed that adiponectin initially increases TNF- $\alpha$  production by macrophages via ERK1/2, Egr-1, and NF- $\kappa$ B-dependent mechanisms, which leads to increased expression of IL-10 and an eventual dampening of LPS-mediated cytokine production.

Traditionally, LPS is specific for TLR-4, and TLR-2 is a receptor for bacterial lipoproteins [21]. Lin et al. [12] reported that acute LPS induced TLR-2 expression, which was consistent with the notion that TLR-4, but not TLR-2, is constitutively present on the cell surface of 3T3-L1 adipocytes. In addition, TLR-4 activation results in induction of TLR-2, and this newly synthesized TLR-2



**Fig. 6.** Effect of adiponectin treatment for 24 h on IL-6 level in the culture medium and on protein expression of IL-6R, TLR-2, and TLR-4 in 3T3-L1 adipocyte treated with LPS. *n* = 6 for all groups. Values are means ± SE. \**p* < 0.05 in relation to LPS.



**Fig. 7.** Effect of adiponectin treatment for 24 h on protein expression of MYD88, TRAF6, NF-kBp50, NF-kBp65 in 3T3-L1 adipocyte treated with LPS. *n* = 6 for all groups. Values are means ± SE. \**p* < 0.05 in relation to LPS.

translocates to the cell surface where it can contribute to increased signaling.

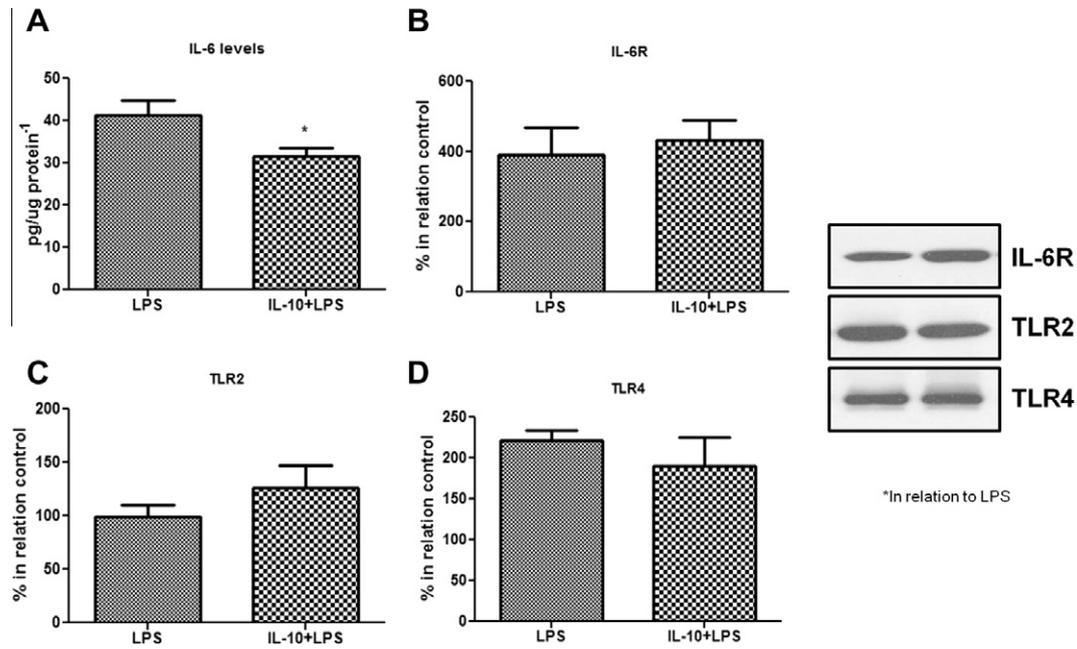
Unexpectedly, adiponectin addition to the culture medium of 3T3-L1 adipocytes increased protein expression of TLR-2, MYD88, and TRAF6 compared with the levels induced by LPS alone, which demonstrated that the reduced NF-κB (both p50 and p65) activation caused by adiponectin was not related to an effect on the TLR-4 pathway; however, this could be associated with a decrease in IL-6.

One possible interpretation is that TLR-4 recruits TLR-2 into a complex. Alternatively, TLR-4 activation could result in the activation of intracellular effectors that could associate with TLR-2. This interaction may also be involved in other intracellular signaling pathways because both IL-6 and NF-κB are reduced. In addition, TLR-2, MYD88, and TRAF6 were

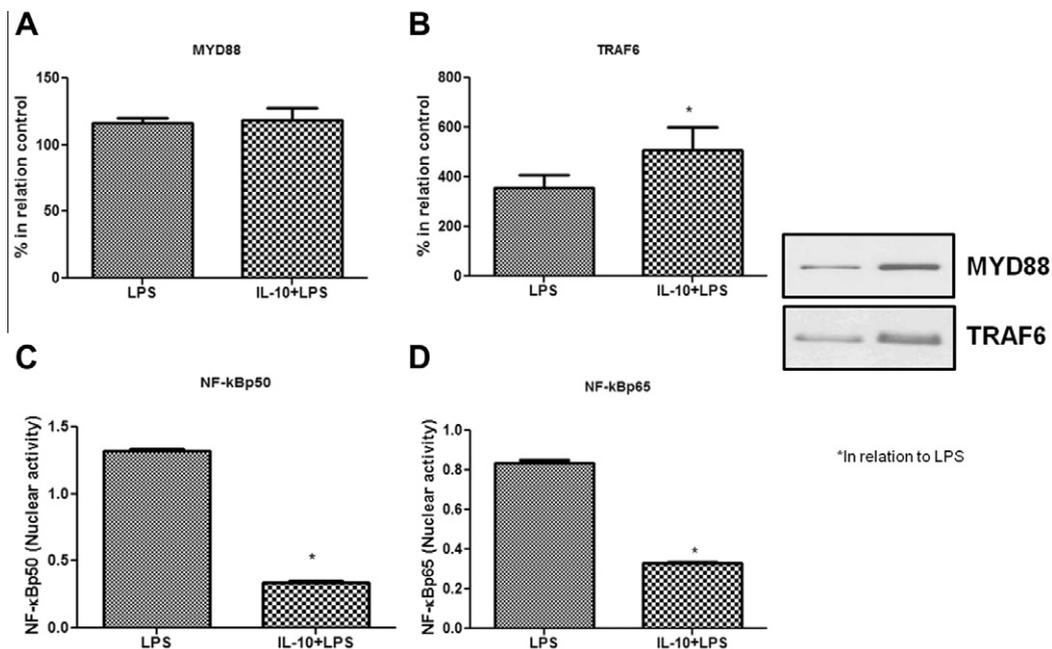
increased, and they could participate in other noninflammatory pathways.

The present study also investigated the effects of IL-10 on the LPS-induced inflammatory response. We observed that IL-10 reduced the IL-6 level and NF-κB (both p50 and p65) DNA binding. In contrast to adiponectin, fewer studies have been conducted to investigate the effects of IL-10 in adipocytes and the inflammatory response.

Interleukin 10 inhibits the production of several cytokines, such as TNF-α, IL-1β, and IL-6, in a variety of cell types. Several studies have shown that the production of IL-10 is increased in inflammatory processes and predominantly plays an immune modulating role in these conditions [15,19]. In obesity, adiponectin and IL-10 serum levels are decreased, which leads to a pro-inflammatory status [4].



**Fig. 8.** Effect of IL-10 treatment for 24 h on IL-6 level in the culture medium and on protein expression of IL-6R, TLR-2, and TLR-4 in 3T3-L1 adipocyte treated with LPS. *n* = 6 for all groups. Values are means ± SE. \**p* < 0.05 in relation to LPS.



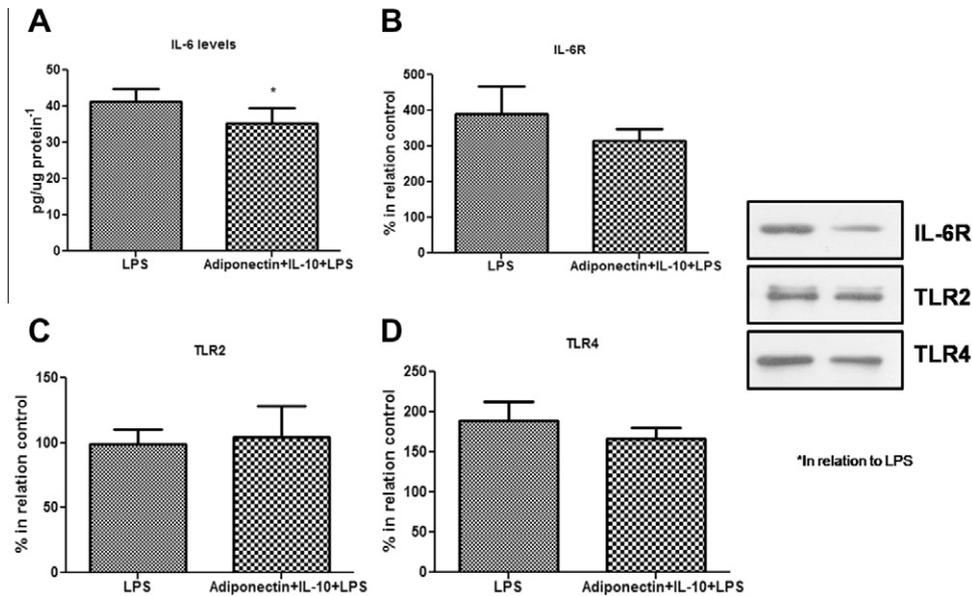
**Fig. 9.** Effect of IL-10 treatment for 24 h on protein expression of MYD88, TRAF6, NF-kBp50, NF-kBp65 in 3T3-L1 adipocyte treated with LPS. *n* = 6 for all groups. Values are means ± SE. \**p* < 0.05 in relation to LPS.

Compared with control cells, Bradley et al. [22] showed that 3T3-L1 adipocytes incubated with palmitic acid for 24 h exhibited a 70% increase in TNF- $\alpha$  production and up to a 75% decrease in IL-10 production. Furthermore, NF- $\kappa$ B DNA binding activity increased fourfold in response to palmitic acid.

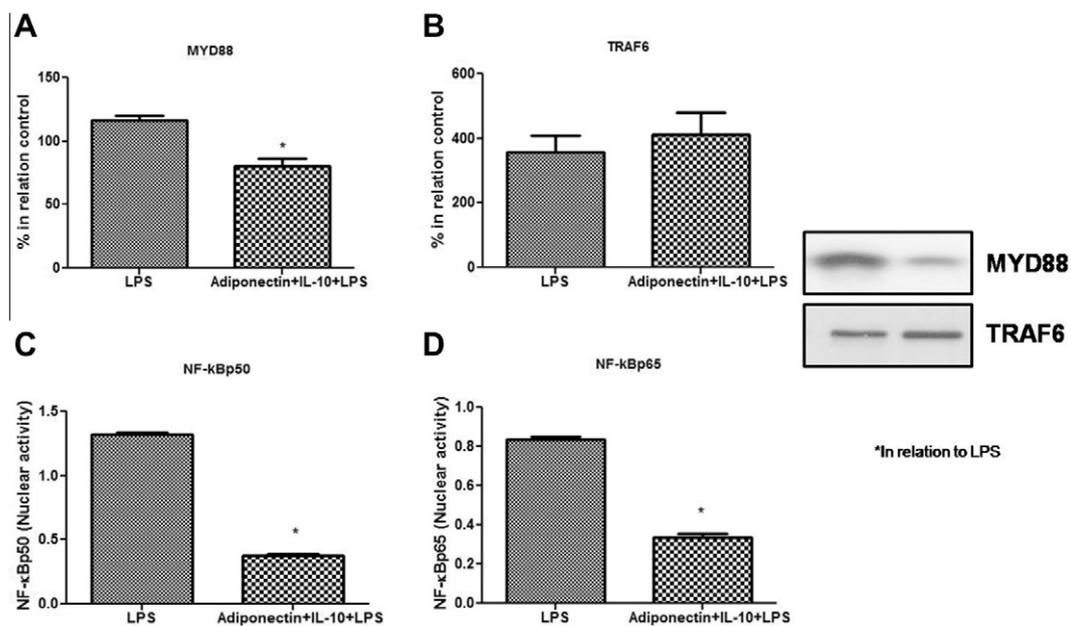
Turner et al. [16] examined the anti-inflammatory effects of IL-10 in primary human adipocytes and showed that IL-10 did not inhibit TLR-4-induced cytokine secretion. Interestingly, a different result was observed in the 3T3-L1 adipocyte model. The authors suggested that the receptor for IL-10 was absent in human

subcutaneous adipocytes. However, recently it has been shown in human primary adipocytes the gene expression of IL-10R $\alpha$  and IL-10R $\beta$  [23]. Taken together, the studies suggest that IL-10 may act on human adipocytes, depending on the conditions and the primary adipose pad from which these cells are derived.

Cintra et al. [24] administered an endogenous IL-10 inhibitor for 5 days to male Swiss mice and demonstrated an increase in hepatic expression of inflammatory markers, such as TNF- $\alpha$ , IL-6, IL-1 $\beta$ , and F4/80. This increase in inflammatory markers was accompanied by a significant negative modulation of insulin signal



**Fig. 10.** Effect of IL-10 + adiponectin treatment for 24 h on IL-6 level in the culture medium and on protein expression of IL-6R, TLR-2, and TLR-4 in 3T3-L1 adipocyte treated with LPS.  $n = 6$  for all groups. Values are means  $\pm$  SE. \* $p < 0.05$  in relation to LPS.



**Fig. 11.** Effect of IL-10 + adiponectin treatment for 24 h on protein expression of MYD88, TRAF6, NF-kBp50, NF-kBp65 in 3T3-L1 adipocyte treated with LPS.  $n = 6$  for all groups. Values are means  $\pm$  SE. \* $p < 0.05$  in relation to LPS.

transduction through the insulin receptor/IRS1-IRS2/PI3-kinase/Akt/FOXO1 pathway and through an increase in hepatic signaling proteins involved in gluconeogenesis and lipid synthesis.

Strategies such as energy restriction and exercise training have been adopted to promote increases in the IL-10 serum level and adipose tissue production, which would reduce the inflammatory status [4,15,19,25]. The pathway that mediates this IL-10 effect in 3T3-L1 adipocytes, however, is unknown. We demonstrated that the addition of IL-10 to the culture medium decreased NF- $\kappa$ B DNA binding activity in 3T3-L1 adipocytes independent of the TLR pathway.

We also examined the effects of adiponectin combined with IL-10 on the LPS-induced inflammatory response. Interestingly, we observed that the combination of adiponectin and IL-10 reduced IL-6 levels, the protein expression of MYD88, and NF- $\kappa$ B (both p50 and p65) DNA binding.

Almost all TLRs have a common signaling pathway in which MYD88 adaptor molecules form a molecular complex with TLR-initiated signaling events. MYD88 also interacts with downstream IL-1R-associated kinase (IRAKs) [26], and TRAF6 regulates distinct processes of innate and adaptive immunity mediated by I $\kappa$ B kinases (IKK) that regulate NF- $\kappa$ B [27,28]. The combination of

adiponectin and IL-10 altered the MYD88-dependent pathway, which led to a decrease in NF- $\kappa$ B (both p50 and p65) DNA binding. Tsan and Gao [21] reported that TLR1/2, TLR2/6, and TLR4 (but not other TLRs) induced NF- $\kappa$ B activation through a MYD88-dependent pathway.

More studies are needed to fully elucidate the comprehensive pathway involved with anti-inflammatory responses in adipocytes and confirm their importance *in vivo*.

In summary, we demonstrated that adiponectin, IL-10 and the combination of adiponectin and IL-10 all reduced NF- $\kappa$ B (both p50 and p65) DNA binding in 3T3-L1 adipocytes exposed to LPS, which may have resulted from a reduction in IL-6 production rather than from an inhibition of the TLR-4 pathway. The present results suggest that anti-inflammatory adipokines may be utilized as a strategy to reduce the pro-inflammatory state in obese people.

## Acknowledgment

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