



## Basic nutritional investigation

# Oral free and dipeptide forms of glutamine supplementation attenuate oxidative stress and inflammation induced by endotoxemia

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## ABSTRACT

**Objective:** The aim of the present study was to determine the effects of oral supplementation with L-glutamine plus L-alanine (GLN+ALA), both in the free form and L-alanyl-L-glutamine dipeptide (DIP) in endotoxemic mice.

**Methods:** B6.<sub>129</sub> F<sub>2</sub>/J mice were subjected to endotoxemia (*Escherichia coli* lipopolysaccharide [LPS], 5 mg/kg, LPS group) and orally supplemented for 48 h with either L-glutamine (1 g/kg) plus L-alanine (0.61 g/kg) (GLN+ALA-LPS group) or 1.49 g/kg DIP (DIP-LPS group). Plasma glutamine, cytokines, and lymphocyte proliferation were measured. Liver and skeletal muscle glutamine, glutathione (GSH), oxidized GSH (GSSG), tissue lipoperoxidation (TBARS), and nuclear factor (NF)- $\kappa$ B–interleukin-1 receptor-associated kinase 1 (IRAK1)–Myeloid differentiation primary response gene 88 pathway also were determined.

**Results:** Endotoxemia depleted plasma (by 71%), muscle (by 44%), and liver (by 49%) glutamine concentrations (relative to the control group), which were restored in both GLN+ALA-LPS and DIP-LPS groups ( $P < 0.05$ ). Supplemented groups reestablished GSH content, intracellular redox status (GSSG/GSH ratio), and TBARS concentration in muscle and liver ( $P < 0.05$ ). T- and B-lymphocyte proliferation increased in supplemented groups compared with controls and LPS group ( $P < 0.05$ ). Tumor necrosis factor- $\alpha$ , interleukin (IL)-6, IL-1  $\beta$ , and IL-10 increased in LPS group but were attenuated by the supplements ( $P < 0.05$ ). Endotoxemic mice exhibited higher muscle gene expression of components of the NF- $\kappa$ B pathway, with the phosphorylation of  $\kappa$ B kinase- $\alpha/\beta$ . These returned to basal levels (relative to the control group) in both GLN+ALA-LPS and DIP-LPS groups ( $P < 0.05$ ). Higher mRNA of IRAK1 and MyD88 were observed in muscle of LPS group compared with the control and supplemented groups ( $P < 0.05$ ).

**Conclusion:** Oral supplementations with GLN+ALA or DIP are effective in attenuating oxidative stress and the proinflammatory responses induced by endotoxemia in mice.

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## Introduction

Sepsis is characterized by a severe inflammatory response to infection and it remains the leading cause of death in intensive care units worldwide [1]. The correct diagnosis and treatment of sepsis is complicated, but it must be fast because a delayed

approach increases the risk for death [2]. Bacterial endotoxin or lipopolysaccharide (LPS) is a major component of the cell walls of gram-negative bacteria, and experimentally mimics a number of physiological responses in animal models of sepsis. Oxidative stress and inflammatory reactions by the cytokine cascade are always a hallmark of human sepsis and LPS animal models, involving the nuclear factor (NF)- $\kappa$ B pathway [3,4]. The activation of the NF- $\kappa$ B pathway occurs through the enzymatic complex of  $\kappa$ B kinase (IKK), in which among others consists of two

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catalytic subunits (IKK $\alpha$  and IKK $\beta$ ) [5]. Stimuli like LPS, oxidative stress, cytokines, and chemokines induce the phosphorylation and degradation of the inhibitor  $\kappa$ B (I $\kappa$ B) protein, which results in the translocation of NF- $\kappa$ B to the nuclei, and transcription of several genes related to proinflammatory responses including, regulation of the NF- $\kappa$ B pathway itself, the myeloid differentiation primary response gene 88 (*MyD88*) and the interleukin-1 receptor-associated kinase 1 (IRAK1) [6,7]. On the other hand, *MyD88* and IRAK1 play an important role in the NF- $\kappa$ B activation cycle, contributing to the loss of homeostasis.

Due to increased requirements triggered by catabolic processes, high inflammatory and oxidative stress profiles in humans and animal models of sepsis (e.g., endotoxemia) lead to an imbalance of the body's most abundant amino acid, glutamine. Under stressful situations, such as sepsis, glutamine is termed a conditionally essential amino acid [8]. The concentration of glutamine in plasma and tissues fall sharply, especially in the liver and skeletal muscle, two major stores of the amino acid for the whole body [4,8]. Low plasma glutamine concentration is associated with poor clinical outcome and increased risk for mortality [4]. Suitable therapies and nutritional support in critical situations are needed to prevent initial sepsis leading to organ failure and the subsequent cascade of multiorgan failure. If the condition is diagnosed early and managed appropriately, providing optimal treatments, which include nutritional support, lives can be saved.

Because glutamine is a key substrate for cells, such as hepatocytes, enterocytes, muscle, and immune cells, previous studies suggested that the administration of L-glutamine might attenuate inflammation [9] and protect against a variety of cell/tissue injuries [10,11] or insults [12]. The mechanisms involved in the protective effects of L-glutamine supplementation include the antioxidant properties of the amino acid, mediated by glutathione (GSH) [4,13] and also specific molecular targets as transcription factors, mainly NF- $\kappa$ B [14]. The tripeptide GSH (L- $\gamma$ -glutamyl-L-cysteinylglycine) is the most important non-enzymatic soluble intracellular antioxidant and is dependent on the supply of glutamate from glutamine [15].

Given enterally, L-glutamine administration may provide physiological generation of other amino acid derivatives, such as citrulline [16] and arginine [17], which are decreased in sepsis [18]. Although results with oral or enteral administration of L-glutamine in its free form are still controversial and inconclusive, and because the amino acid is a preferred respiratory fuel and a precursor for protein synthesis in the gut epithelium, supplementations with L-glutamine in the dipeptide form, such L-alanyl-L-glutamine (DIP), seems to be more effective, providing an alternative non-invasive way to increase the concentration of glutamine in the body in some catabolic situations [19,20]. However, in many studies, despite administering glutamine amounts equivalent to those in the present study, solutions containing the same amino acids in the same quantities among groups were not usually tested. Previous work in animal models submitted to catabolic situations, such as intense, prolonged, and exhaustive physical exercise, oral supplementation with L-glutamine in the DIP form or in its free form along with free L-alanine (GLN+ALA) may restore total glutamine in the body, improving the intracellular redox status [10] and may result in decreased muscle damage and inflammation [11]. Here, we hypothesized that oral L-glutamine supplementations either in the DIP or GLN+ALA form would attenuate inflammation and oxidative stress induced by endotoxemia in mice. In this study, glutamine–glutathione axis, lymphocyte proliferation, and mRNA and/or protein expressions of *MyD88*, IRAK1, and NF- $\kappa$ B

pathway were measured in the two major stores of glutamine, the liver and the skeletal muscles.

## Materials and methods

### Mice and treatments

Eight-wk-old male isogenic B6.129 F<sub>2</sub>/J mice, obtained from the Animal House of the Faculty of Pharmaceutical Sciences at the University of São Paulo, were used and maintained under a 12-h light/dark cycle (lights on at 0700 h) at room temperature of 22°C  $\pm$  2°C and relative humidity of 60%. Mouse food intake and weight were monitored daily. Throughout the experiment, animals had free access to water and were fed with standard laboratory mouse chow (NUVILAB CR1, Nuvital Nutrients Ltd., Curitiba, Brazil) ad libitum. After 1 wk of acclimation (four mice/cage), the animals were randomly assigned to one of three groups: Endotoxemia (LPS group, 5 mg/kg body weight, intraperitoneal, of LPS from *Escherichia coli* strain 0127:B8, Sigma-Aldrich, USA; n = 12); endotoxemia subjected to 2 d of oral supplementation with a solution containing L-glutamine (1 g/kg body weight [BW] daily) and L-alanine (0.61 g/kg BW daily) both in their free forms (ALA+GLN-LPS group; n = 12); and endotoxemia subjected to 2 d of oral supplementation with the dipeptide L-alanyl-L-glutamine (DIP-LPS group; 1.49 g/kg BW daily; n = 12). Baseline parameters were obtained from control animals that were injected with phosphate-buffered saline (PBS) and received equivalent amounts of saline solution by gastric gavage (n = 12). Free L-glutamine and L-alanine were supplied by Ajinomoto Interamerican Industry and Commerce Ltd. (São Paulo, Brazil), whereas L-alanyl-L-glutamine DIP (Diamin solution, consisting of 20 mg of L-alanyl-L-glutamine [which equals 8.2 g L-alanine and 13.46 g L-glutamine] dissolved in 100 mL H<sub>2</sub>O) was manufactured and supplied by Fórmula Medicinal Ltd. (São Paulo, Brazil).

The amount of DIP was calculated so that the total amount of L-glutamine administered to the animals was the same as that of L-glutamine administered in its free form (1 g/kg BW daily, as previously reported [10,19]). In this sense, consequently, DIP-LPS and GLN+ALA-LPS groups received isocaloric and isonitrogenous treatments, respectively. Daily supplementations, including the control group, were administered through gastric gavage for a 48 h after induction of endotoxemia. The first gavage was initiated 2 h after induction of endotoxemia, then once daily in the morning, with the last given 3 h before completion of the 48-h period. This time schedule was followed to eliminate results that reflected an acute single-dose effect [21]. The mice were sacrificed by cervical dislocation under anesthesia (intraperitoneal) with 80 mg/kg ketamine hydrochloride and 15 mg/kg xylazine hydrochloride. All procedures were approved by the Ethics Committee on Animal Experimentation of the Faculty of Pharmaceutical Sciences, University of São Paulo, according to the guidelines of the Brazilian College on Animal Experimentation (protocol number CEUA/FCF/249).

### Biochemical analyses

Immediately after sacrifice, blood was heparin-collected and centrifuged, and the plasma samples were stored at –80°C for subsequent determinations, with the exception of lymphocyte proliferation. On the day of measurements, plasma was deproteinized (TCA) and immediately processed for glutamine and glutamate concentrations, which was determined spectrophotometrically by using a commercial kit (Sigma-Aldrich Chemical) adapted for microplate reader (Bio-Rad) [22]. Plasma tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-6, IL-1  $\beta$ , and IL-10 were evaluated using commercially available immunoassay kit Milliplex beads mapping SA-PE with 96 wells for Luminex 200 reader (Millipore, Hayward, CA, USA).

### Lymphocyte preparation and proliferation

The blood of mice was collected in heparin tubes and the peripheral blood mononuclear cells (PBMC; a mixture of monocytes and lymphocytes) and neutrophils were isolated by centrifugation. After collection, blood was diluted in PBS (1:1), and this suspension was layered on to Ficoll-Hypaque 1.084 and centrifuged for 20 min at 400 g and 4°C. PBMC were collected from the interphase and washed once with PBS and centrifuged for 10 min at 1000 g and warm temperature. The remaining erythrocytes were lysed with 150 mmol/L NH<sub>4</sub> Cl, 10 mmol/L NaHCO<sub>3</sub>, 0.1 mmol/L EDTA, pH 7.4. The PBMC were maintained in culture medium RPMI 1640 without phenol red (Sigma-Aldrich), pH 7.4, containing 2 mM L-glutamine, 25 mM of HEPES, supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma-Aldrich) and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL amphotericin B, Sigma-Aldrich) at humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C for 1 h to allow the adherence of monocytes to the plates. Afterward, the supernatant medium containing lymphocytes was collected to obtain a pure lymphocyte preparation (about 98%). The

number of cells was evaluated by counting in a Neubauer's chamber, and cell viability was determined by the Trypan blue dye exclusion technique.

For lymphocytes proliferation, cells ( $3 \times 10^5$  cells/mL) were plated in 96-well plates and cultured for 24 and 48 h in RPMI-1640 medium without phenol red (Sigma-Aldrich), pH 7.4, containing 2 mM L-glutamine, 25 mM HEPES, supplemented with 10% FBS and antibiotics (100 U/mL penicillin, 100 mg/mL streptomycin, 0.25 mg/mL anfotericin B, Sigma-Aldrich). The plates were incubated in a humidified atmosphere of 5% CO<sub>2</sub> and 95% air at 37°C and kept in the presence of sterile concanavalin A (ConA, 10 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) to a T-lymphocyte mitogen or LPS (strain 0111:B4, Sigma-Aldrich, St. Louis, MO, USA) to a B lymphocyte mitogen. At 0, 24, and 48 h of culture lymphocyte proliferation were evaluated using colorimetric microassay method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma-Aldrich) dissolved (5 mg/mL) in PBS. MTT solution was then added to each culture well, and the plates were incubated for 4 h at 37°C. The plate was then centrifuged and the medium was removed. Isopropyl alcohol (100 µL/each well) was added to solubilize the formazan dye and the absorbance was measured in microplate reader at 570 nm (Bio-Rad temperature-controlled Benchmark reader 340–750 nm UV/VIS, CA, EUA, Hercules, CA, USA).

#### Tissue preparations

The liver and the gastrocnemius muscle were removed and subsequently freeze-clamped in liquid nitrogen immediately after death for subsequent determination of protein, glutamine, glutamate, GSH, and oxidized GSH (GSSG) concentrations, thiobarbituric acid reactive substances (TBARS), gene/protein expression by Western Blot and mRNA expressions by real-time reverse transcription (RT) quantitative polymerase chain reaction (qPCR). For GSH and GSSG determinations, liver and gastrocnemius muscle were homogenized in 5% (w/v)-metaphosphoric acid (MPA) at 4 °C in the ratio of 1 mL/g fresh tissue. After vortex homogenization, samples were diluted (1:8 by volume) with cold MPA, centrifuged (15 000 g for 5 min at 4 °C) and the supernatant fractions were then assessed for GSH and GSSG content. Tissue glutamine and glutamate were TCA extracted and spectrophotometrically assayed (Sigma-Aldrich Diagnostics Inc. kit, St. Louis, MO, USA) [22]. Mean values are reported as micromoles of glutamine per gram of fresh tissue and as nanomoles of glutamine per milligram of protein.

#### Measurement of GSH and GSSG

After preparation, tissue samples were spectrophotometrically (415 nm) assayed on a microplate reader (Bio-Rad temperature-controlled Benchmark reader 340–750 nm UV/VIS, CA, EUA, Hercules, CA, USA) by modification of the 5,5'-dithiobis (2-nitrobenzoic acid, Sigma-Aldrich Chemical) [DTNB]/GSSG reductase (Sigma-Aldrich Chemical) recycling method, using the *N*-ethylmaleimide (Sigma-Aldrich Chemical) conjugating technique for GSSG sample preparation [23]. Samples (10 µL), for both GSH and GSSG determinations, were assayed in 105-µL final volume in 96-well polystyrene plates (Corning) at 37°C in the presence of 10 mM DTNB, 0.17 mM β-NADPH (Sigma-Aldrich Chemical, St. Louis, MO, USA, dissolved in 0.5% [w/v] NaHCO<sub>3</sub> as a stabilizing agent) and 0.5 U/mL GSSG reductase (EC 1.6.4.2, Sigma-Aldrich Chemical) [24].

#### TBARS determination

The evaluation of TBARS was used as an index of lipoperoxidation in gastrocnemius tissue. The production of TBARS consists of acid-heating the lipid peroxidation end product, malondialdehyde (MDA), and reaction with thiobarbituric acid (TBA) in gastrocnemius tissue homogenates, as previously described [25]. TBARS were determined in 96-well polystyrene plates (Bio-Rad temperature-controlled Benchmark reader 340–750 nm UV/VIS, CA, EUA, Hercules, CA, USA) at 535 nm.

#### Western blotting analysis

Frozen gastrocnemius muscle was homogenized in ice-cold lysis buffer: 50 mM phosphate buffer (pH 7.0), 0.3 M sucrose, 0.5 mM dithiotreitol, 1 mM EDTA, 0.3 mM PMSF, 10 mM NaF, 1:100 Phosphatase Inhibitor Cocktail 1 to 2 (Sigma-Aldrich), and 1:100 Protease Inhibitor Cocktail (Sigma-Aldrich). Samples were then centrifuged (12 000 g at 4 °C for 20 min) for removal of insoluble materials. Lysates were combined with a sample buffer: 240 mM Tris (pH 6.8), 40% glycerol, 0.8% sodium dodecyl sulfate (SDS), 200 mM β-mercaptoethanol, and 0.02% bromophenol blue. Proteins (25 µg) were subjected to SDS- polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. SNAP i.d. (Millipore) quick immunoblot vacuum system was used and membranes were washed with water and then blocked in 0.5% (w/v) non-fat dry milk (Nestlé) in wash buffer PBS with Tween 20 (PBST). Membranes were washed three times with wash buffer and incubated for 10 min with the primary antibodies NF-κB p65 (1:1000 Cell Signaling Technology) and phosphorylated-IKKα/β Ser176/180

(1:1000 Cell Signaling Technology). Membranes were incubated for 1 h with peroxidase-labeled anti-rabbit immunoglobulin G antibodies (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:5000 in PBST buffer and 2% non-fat dry milk. Blots were visualized for 5 min in an ImageQuant 400 (GE Healthcare, Amersham, UK) image system, with a solution prepared with ECL-Advance Western blotting reagent (GE Healthcare, Amersham, UK). A horseradish peroxidase-labeled anti-βactin (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:5000 was used to verify correct protein loading.

#### mRNA extraction and real-time RT-PCR

Total RNA was isolated using RNeasy Mini column (Qiagen, Hilden, Germany) and purified by DNase digestion (Qiagen, USA) from 100 mg of gastrocnemius muscle after homogenization in 1 mL of reagent Qiazol (Qiagen, Hilden, Germany), according to manufacturer's recommendations. The spectrophotometric quantification of RNA was performed using NanoDrop system model 2000 c (Thermo Scientific, Waltham, MA, USA). To investigate the purity of the nucleic acid solution, additional readings were made at 230 and 280 nm for the calculation of ratios 260/230 and 260/280. Total RNA was also separated by agarose gel electrophoresis, and ribosomal bands were examined to ensure sample quality.

Total RNA was subjected to reverse transcription (RT) reaction with "primers" using RT mix RT2 First Strand Kit (Qiagen, Hilden, Germany). The reactions were incubated for 15 min at 42°C followed by a 5-min denaturation at 95°C. Synthesized cDNA was then frozen for subsequent qPCR in a 7500 qPCR real-time PCR device (Applied Biosystems) using Custom RT2 Profiler Array PCR (Qiagen, Hilden, Germany, Custom Catalog Number CAPM10824) and RT2 SYBR Green Mastermix (Qiagen, USA). In this customized plate, gene-specific primers were selected: NF-κB1 (NM\_008689, Qiagen Ref. PPM02930), NF-κB inhibitor alpha (NF-κBIA; NM\_010907, Qiagen Ref. PPM02943), MyD88 (NM\_010851, Qiagen Ref. PPM03399), IRAK1 (NM\_008363, Qiagen Ref. PPM03201), and the control gene glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; NM\_008084, Qiagen Ref. PPM02946). Analysis of results was performed by using the delta delta Ct method and *GAPDH* as the housekeeping gene.

#### Protein determination

Protein concentrations of liver and muscle preparation were measured by a previously described method [26] using bovine serum albumin as a standard.

#### Statistical analyses

Results were subjected to multivariate analysis of variance (ANOVA) to track type 1 errors through an array of univariate tests. Levene's test was used to detect deviations from homoscedasticity between the study groups. When one-way ANOVA detected significance, comparisons between nutritional treatments were made and, whenever *P*-values were <0.05, statistically significant differences were identified by the multiple comparison procedure of Tukey's Honestly Significant Difference (Tukey HSD). All statistical calculations were performed using PASW software version 18.0 (SPSS, Chicago, IL, USA).

## Results

### Body weight and food intake

The initial body weight did not differ among groups (23.8 ± 0.7 g). The final body weight, determined 48 h after intraperitoneal LPS injection was significantly lower (control, 23.6 ± 1.1 g versus LPS, 18.9 ± 0.7 g; *P* < 0.05), whereas glutamine supplementations did not significantly reverse this scenario (GLN+ALA-LPS, 20.6 ± 0.4 g; DIP-LPS, 20.8 ± 0.2 g). No differences were observed in gastrocnemius muscle weight between groups (data not show). LPS administration also significantly (*P* < 0.05) reduced daily food intake, which progressively fell by up to 87% after 48 h (control, 3.9 ± 0.1 g/d; LPS, 0.5 ± 0.1 g/d) and supplementations did not affect this profile (GLN+ALA-LPS, 0.5 ± 0.1 g/d; DIP-LPS, 0.6 ± 0.1 g/d).

### Plasma parameters

As depicted in Table 1, plasma glutamine concentrations were 71% lower in LPS animals than in controls (*P* < 0.05), whereas both glutamine supplementations reestablished glutaminemia.

**Table 1**  
Glutamine and glutamate content in plasma, liver, and skeletal muscle

	Experimental Groups			
	CTRL	LPS	GLN+ALA-LPS	DIP-LPS
<b>Plasma variables</b>				
Glutamine (mmol/L)	0.66 ± 0.07	0.19 ± 0.04 <sup>†</sup>	0.65 ± 0.12*	0.58 ± 0.08*
Glutamate (mmol/L)	0.11 ± 0.01	0.10 ± 0.02	0.10 ± 0.01	0.10 ± 0.01
Glutamine/Glutamate	6.54 ± 0.27	2.69 ± 0.71 <sup>†</sup>	6.53 ± 0.98*	5.89 ± 0.59*
<b>Tissue variables in liver</b>				
Glutamine (μmol/g fresh tissue)	6.18 ± 0.32	3.13 ± 0.10 <sup>†</sup>	6.72 ± 0.41*	6.37 ± 0.49*
Glutamate (μmol/g fresh tissue)	3.73 ± 0.41	2.90 ± 0.37	3.65 ± 0.36	3.35 ± 0.34
Glutamine/Glutamate	1.84 ± 0.12	1.18 ± 0.15 <sup>†</sup>	2.01 ± 0.10*	2.13 ± 0.15*
Glutamine (nmol/mg protein)	26.29 ± 1.07	13.80 ± 0.51 <sup>†</sup>	27.01 ± 1.44*	27.30 ± 1.30*
Glutamate (nmol/mg protein)	13.74 ± 0.49	12.07 ± 1.28	14.84 ± 1.21	14.50 ± 0.76
Glutamine/Glutamate	1.91 ± 0.05	1.18 ± 0.10 <sup>†</sup>	1.82 ± 0.07*	1.89 ± 0.04*
<b>Tissue variables in skeletal muscle</b>				
Glutamine (μmol/g fresh tissue)	4.56 ± 0.17	2.56 ± 0.19 <sup>†</sup>	5.50 ± 0.46*	5.19 ± 0.32*
Glutamate (μmol/g fresh tissue)	0.39 ± 0.06	0.41 ± 0.08	0.55 ± 0.07	0.45 ± 0.04
Glutamine/Glutamate	11.99 ± 0.83	6.33 ± 0.61 <sup>†</sup>	13.11 ± 1.58*	11.09 ± 0.50*
Glutamine (nmol/mg protein)	62.69 ± 2.12	26.64 ± 1.81 <sup>†</sup>	67.73 ± 6.12*	62.88 ± 4.48*
Glutamate (nmol/mg protein)	5.63 ± 0.30	4.44 ± 0.57	5.74 ± 0.23	5.24 ± 0.30
Glutamine/Glutamate	11.19 ± 0.25	6.24 ± 0.42 <sup>†</sup>	11.73 ± 0.62*	11.97 ± 0.31*

ALA, L-alanine; CTRL, control; DIP, dipeptide; GLN, L-glutamine; LPS, lipopolysaccharide; PBS, phosphate buffered saline.

B6<sub>129</sub> F<sub>2</sub>/J mice (12/group) were made endotoxemic (LPS) or PBS-injected (CTRL). Supplemented animals were orally administered (for 48 h after LPS injection), on a daily basis, with either a solution containing L-glutamine plus L-alanine (GLN+ALA-LPS), both in their free forms or L-alanyl-L-glutamine dipeptide (DIP-LPS). Data are reported as mean ± SEM.

Data are reported as mean ± SEM.

\* P < 0.05 for comparison with CTRL group.

† P < 0.05 for comparison with LPS animals.

On the contrary, plasma glutamate concentrations were not affected by LPS treatment or by glutamine supplementation. Therefore, glutamine to glutamate plasma ratio, which is an indicator of the potential ( $\Delta G < 0$ ) for glutamine flux through glutaminase and glutamate dehydrogenase pathway, was also recovered by both glutamine supplementations to LPS-treated mice (Table 1). The inflammatory profile was measured by the concentration of plasma TNF- $\alpha$  (Fig. 1A), IL-6 (Fig. 1A), IL-1  $\beta$  (Fig. 1B), and IL-10 (Fig. 1B). LPS administration raised the concentration of all plasma cytokines compared with the control group ( $P < 0.05$ ). Plasma TNF- $\alpha$ , IL-6, and IL-10 were significantly increased in the GLN+ALA-LPS and DIP-LPS groups compared with the controls ( $P < 0.05$ ). However, both nutritional interventions significantly attenuated the inflammatory response, when compared with LPS group (Fig. 1A, B, respectively;  $P < 0.05$ ).

#### Glutamine and glutamate in liver and skeletal muscle

LPS treatment evoked a decrease in glutamine content in both liver and gastrocnemius muscle (by 49% and 44%, respectively) as calculated in terms of  $\mu\text{mol/g}$  of fresh tissue and when expressed as nmol/mg of tissue protein (Table 1;  $P < 0.05$ ). Furthermore, as observed for plasma, both glutamine supplementations restored the amount of L-glutamine found in liver and muscle. Interestingly, the supplementations elicited an even higher amount (by 10%–20%) of glutamine in both tissues of LPS-treated animals ( $P < 0.05$ ). Because glutamate contents remained unaltered, the ratio of glutamine to glutamate in liver and skeletal muscle was lower in the LPS group and was restored in LPS animals treated with the supplements (Table 1;  $P < 0.05$ ).

#### Lymphocyte proliferation

ConA- and LPS-stimulated lymphocyte proliferations were significantly higher in 24 and 48 h of incubation in both

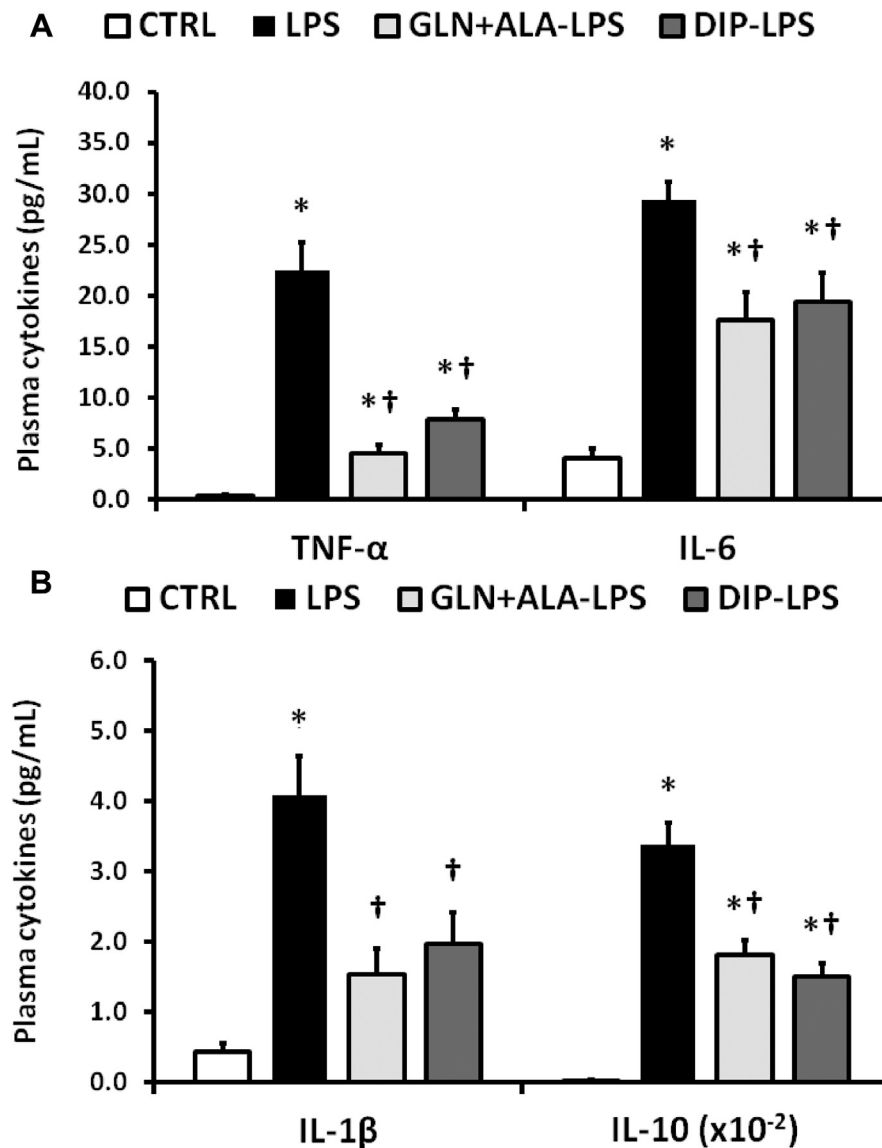
supplemented groups compared with controls and the LPS group (Fig. 2A, B, respectively).

#### Oxidative stress parameters (GSH, GSSG, GSSG to GSH ratio, and TBARS)

In both hepatic and muscular tissues, LPS promoted a reduction in GSH (by 54% and 64%, respectively), leading to a threatening extreme oxidative milieu (voltage rising 2.7-fold from  $-0.024$  V to  $-0.009$  V at  $37^\circ\text{C}$ ). However, in such tissues, both nutritional interventions reduced the oxidative imbalance making GSSG to GSH ratios lower compared with the LPS group (Table 2;  $P < 0.05$ ). GSSG based in nmol/mg protein and the GSSG to GSH ratio in the GLN+ALA-LPS group was increased ( $P < 0.05$ ) compared with controls and the DIP-LPS group (Table 2). The effects of LPS administration in GSH metabolism were paralleled by a rise in TBARS of 188% and 294% in the liver and skeletal muscle, respectively, which was abolished by glutamine supplementations (Table 2;  $P < 0.05$ ).

#### Western blot and mRNA analysis of inflammatory response

LPS administration increased the muscle gene expression of total NF- $\kappa$ B p65 and the phosphorylation of IKK- $\alpha/\beta$  (by 50% and 180%, respectively) compared with controls (Fig. 3A, B;  $P < 0.05$ ). This proinflammatory response elicited by LPS was reinforced by an increase in mRNA expression of NF- $\kappa$ B1 and decreased expression of NF- $\kappa$ BIA in muscle (Fig. 4A;  $P < 0.05$ ). However, both nutritional interventions were able to attenuate the gene expression of total NF- $\kappa$ B p65 (Fig. 2A) and phosphorylation of IKK- $\alpha/\beta$  (Fig. 3B), confirmed by normal mRNA expression (relative to the control group) of NF- $\kappa$ B1 and NF- $\kappa$ BIA (Fig. 4A;  $P < 0.05$ ). In the same tissue of all endotoxemic groups, genes related to proinflammatory responses were significantly increased, such as *MyD88* and *IRAK1*, compared with the control group (Fig. 4A;  $P < 0.05$ ). However, compared with the LPS group, these responses were attenuated by the supplements (Fig. 4A;  $P < 0.05$ ).



**Fig. 1.** Plasma concentrations of TNF- $\alpha$  (A), IL-6 (A), IL-1  $\beta$  (B), and IL-10  $\times 10^{-2}$  (B). B6.129 F<sub>2</sub>/J mice (12/group) were made endotoxemic (LPS) or PBS-injected (CTRL). Supplemented animals were orally administered (for 48 h after LPS injection), on a daily basis, with either a solution containing L-glutamine plus L-alanine (GLN+ALA-LPS), both in their free forms or L-alanyl-L-glutamine dipeptide (DIP-LPS). Data are reported as mean  $\pm$  SEM. \* $P < 0.05$  for comparison with CTRL group. † $P < 0.05$  for comparison with LPS animals.

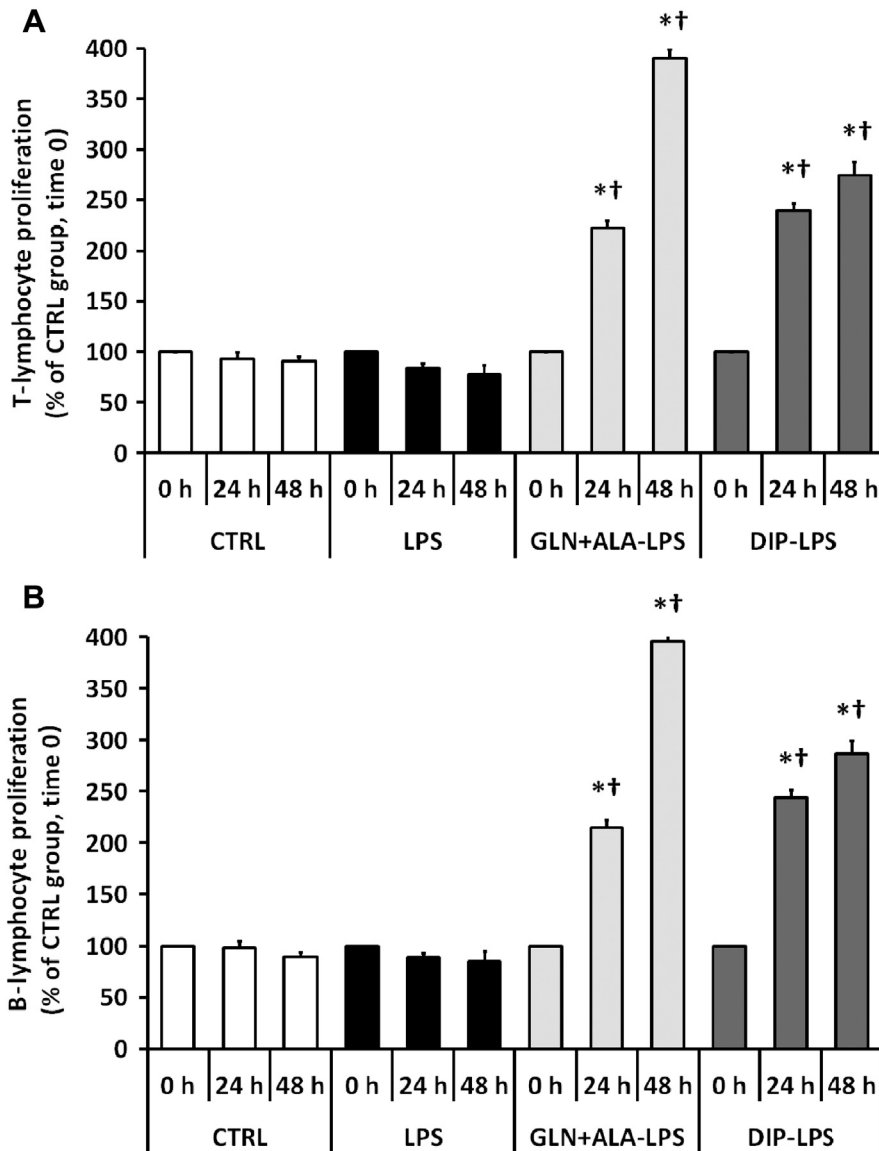
In the liver, mRNA of NF- $\kappa$ B1 and NF- $\kappa$ BIA were elicited by LPS administration, however, it was attenuated by supplements (Fig. 4B;  $P < 0.05$ ). Although *MyD88* in the liver was not responsive to LPS and supplements, *IRAK1* was significantly increased ( $P < 0.05$ ) in the LPS group and returned to normal levels in the GLN+ALA-LPS and DIP-LPS groups (Fig. 4B;  $P < 0.05$ ).

## Discussion

Sepsis affects about 18 million individuals worldwide, with mortality rates of 25% to 30%, and the numbers of cases are increasing annually [1,2]. Gram-negative bacteria and their endotoxins or LPS, play a pivotal role in sepsis, especially in triggering inflammation and oxidative stress [27], which also affects glutamine metabolism [13,28]. The increase in catabolic processes stimulates immune cells to consume high

amounts of glutamine, which is related to immune cell survival and proliferation, leading to an imbalance of whole-body defenses [4,8]. Hence, the possible benefits of oral L-glutamine supplementation, independently of its dipeptide or free form along with free L-alanine were evaluated in the present study.

Endotoxemia produced a severe decrease in plasma, liver, and skeletal muscle glutamine concentrations, which were restored by both glutamine supplementations. Because plasma and tissue glutamate levels remained unaltered in L-glutamine supplemented LPS animals, the glutamine to glutamate ratio was reestablished. These effects in glutamine availability were related to higher plasmatic lymphocytes proliferation (T and B). Glutamine is used at high rates by leukocytes (particularly lymphocytes), because this amino acid provides a substrate for purine and pyrimidine synthesis, glutaminolysis pathways, and intermediate metabolism of amino acids [8]. However, lymphocytes do not possess the enzyme glutamine synthetase, which



**Fig. 2.** Effect of a solution containing L-glutamine plus L-alanine (GLN+ALA-LPS), both in their free forms or L-alanyl-L-glutamine dipeptide (DIP-LPS) on lymphocyte proliferation. Lymphocytes were plated and cultured for a period of 0, 24, and 48 h in the presence of sterile concanavalin A (ConA)—a T-lymphocyte mitogen (A) or lipopolysaccharide (LPS)—a B-lymphocyte mitogen (B). Data are reported as mean  $\pm$  SEM. \* $P < 0.05$  for comparison with CTRL group. † $P < 0.05$  for comparison with LPS animals.

catalyzes the synthesis of glutamine from ammonia and glutamate, and therefore these cells are unable to synthesize glutamine. Liver and skeletal muscles are two important sites of glutamine production and storage in the body [15]. Leukocytes are highly dependent of liver and skeletal muscles because they can release glutamine into the bloodstream to satisfy their metabolic requirements. Other studies are in agreement with our results with the effects of L-glutamine supplementation on lymphocyte proliferation and differentiation in different catabolic and immune suppressor situations [29,30].

Decreased glutamine concentrations, especially in liver and skeletal muscles may compromise *de novo* synthesis of GSH since glutamine is the immediate precursor of glutamate, even if cysteine and glycine were maintained at relatively constant levels [15]. The rise in GSH content evoked by the supplements (by 59%–82% relative to LPS animals) and the consequent decrease in GSSG to GSH ratio, an index of intracellular redox

status [31], make liver and skeletal muscle redox status much more balanced. The GSH system is quantitatively the most important reactive oxygen species/reactive nitrogen species scavenger and has many metabolic functions with the ability to protect cells against lipid peroxidation caused by hydrogen peroxide and free radicals. TBARS concentration, a parameter of lipid peroxidation, was higher in liver and skeletal muscle of LPS-treated animals compared with controls, and L-glutamine supplementations reversed this scenario, illustrating the benefits of GSH concentration and the decreased intracellular redox status.

As a cause or consequence, oxidative stress is always a hallmark of human sepsis [4] and in LPS-stimulated animal models [9] induced by mitochondrial dysfunction, LPS stimulation, and/or cytokine production [3]. Oxidative stress can cause DNA damage [27] and trigger the redox pathways for transcriptional activation, such the increased activation of NF- $\kappa$ B. The NF- $\kappa$ B

**Table 2**  
TBARS, GSH, and GSSG content in liver and skeletal muscle

	Experimental groups			
	CTRL	LPS	GLN+ALA-LPS	DIP-LPS
Tissue variables in liver				
GSH ( $\mu\text{mol/g}$ fresh tissue)	3.01 $\pm$ 0.12	1.09 $\pm$ 0.16*	2.25 $\pm$ 0.30 <sup>†</sup>	2.48 $\pm$ 0.25 <sup>†</sup>
GSSG ( $\mu\text{mol/g}$ fresh tissue)	1.05 $\pm$ 0.12	1.46 $\pm$ 0.12*	1.30 $\pm$ 0.02	0.99 $\pm$ 0.06 <sup>†</sup>
GSSG/GSH	0.34 $\pm$ 0.04	1.47 $\pm$ 0.31*	0.59 $\pm$ 0.05 <sup>†</sup>	0.41 $\pm$ 0.07 <sup>†</sup>
GSH (nmol/mg protein)	12.2 $\pm$ 0.50	5.03 $\pm$ 0.50*	10.93 $\pm$ 0.60 <sup>†</sup>	12.13 $\pm$ 0.50 <sup>†</sup>
GSSG (nmol/mg protein)	4.05 $\pm$ 0.18	7.03 $\pm$ 0.52*	5.63 $\pm$ 0.14* <sup>†</sup>	4.07 $\pm$ 0.12 <sup>†,‡</sup>
GSSG/GSH	0.32 $\pm$ 0.02	1.41 $\pm$ 0.05*	0.52 $\pm$ 0.04* <sup>†</sup>	0.34 $\pm$ 0.01 <sup>†,‡</sup>
TBARS (nmol/g fresh tissue)	1.70 $\pm$ 0.15	4.89 $\pm$ 0.21*	2.15 $\pm$ 0.19 <sup>†</sup>	2.37 $\pm$ 0.33 <sup>†</sup>
Tissue variables in skeletal muscle				
GSH ( $\mu\text{mol/g}$ fresh tissue)	3.05 $\pm$ 0.44	1.40 $\pm$ 0.14*	2.59 $\pm$ 0.10 <sup>†</sup>	3.01 $\pm$ 0.38 <sup>†</sup>
GSSG ( $\mu\text{mol/g}$ fresh tissue)	0.43 $\pm$ 0.03	0.67 $\pm$ 0.06*	0.46 $\pm$ 0.04 <sup>†</sup>	0.41 $\pm$ 0.05 <sup>†</sup>
GSSG/GSH	0.16 $\pm$ 0.04	0.52 $\pm$ 0.06*	0.18 $\pm$ 0.01 <sup>†</sup>	0.14 $\pm$ 0.01 <sup>†</sup>
GSH (nmol/mg protein)	38.75 $\pm$ 0.52	16.61 $\pm$ 1.22*	33.88 $\pm$ 2.10 <sup>†</sup>	37.23 $\pm$ 3.19 <sup>†</sup>
GSSG (nmol/mg protein)	5.17 $\pm$ 0.16	8.12 $\pm$ 0.31*	5.15 $\pm$ 0.16 <sup>†</sup>	5.06 $\pm$ 0.40 <sup>†</sup>
GSSG/GSH	0.13 $\pm$ 0.01	0.51 $\pm$ 0.02*	0.15 $\pm$ 0.01 <sup>†</sup>	0.14 $\pm$ 0.01 <sup>†</sup>
TBARS (nmol/g fresh tissue)	1.20 $\pm$ 0.08	4.73 $\pm$ 0.35*	1.54 $\pm$ 0.10 <sup>†</sup>	1.87 $\pm$ 0.10 <sup>†</sup>

ALA, L-alanine; CTRL, control; DIP, dipeptide; GLN, L-glutamine; GSH, glutathione; GSSG, oxidized glutathione; LPS, lipopolysaccharide; PBS, phosphate buffered saline; TBARS, thiobarbituric acid reactive substances

B6.<sub>129</sub> F<sub>2</sub>/J mice (12/group) were made endotoxemic (LPS) or PBS-injected (CTRL). Supplemented animals were orally administered (for 48 h after LPS injection), on a daily basis, with either a solution containing L-glutamine plus L-alanine (GLN+ALA-LPS), both in their free forms or L-alanyl-L-glutamine dipeptide (DIP-LPS)

Data are reported as mean  $\pm$  SEM

\*  $P < 0.05$  for comparison with CTRL group.

<sup>†</sup>  $P < 0.05$  for comparison with LPS animals.

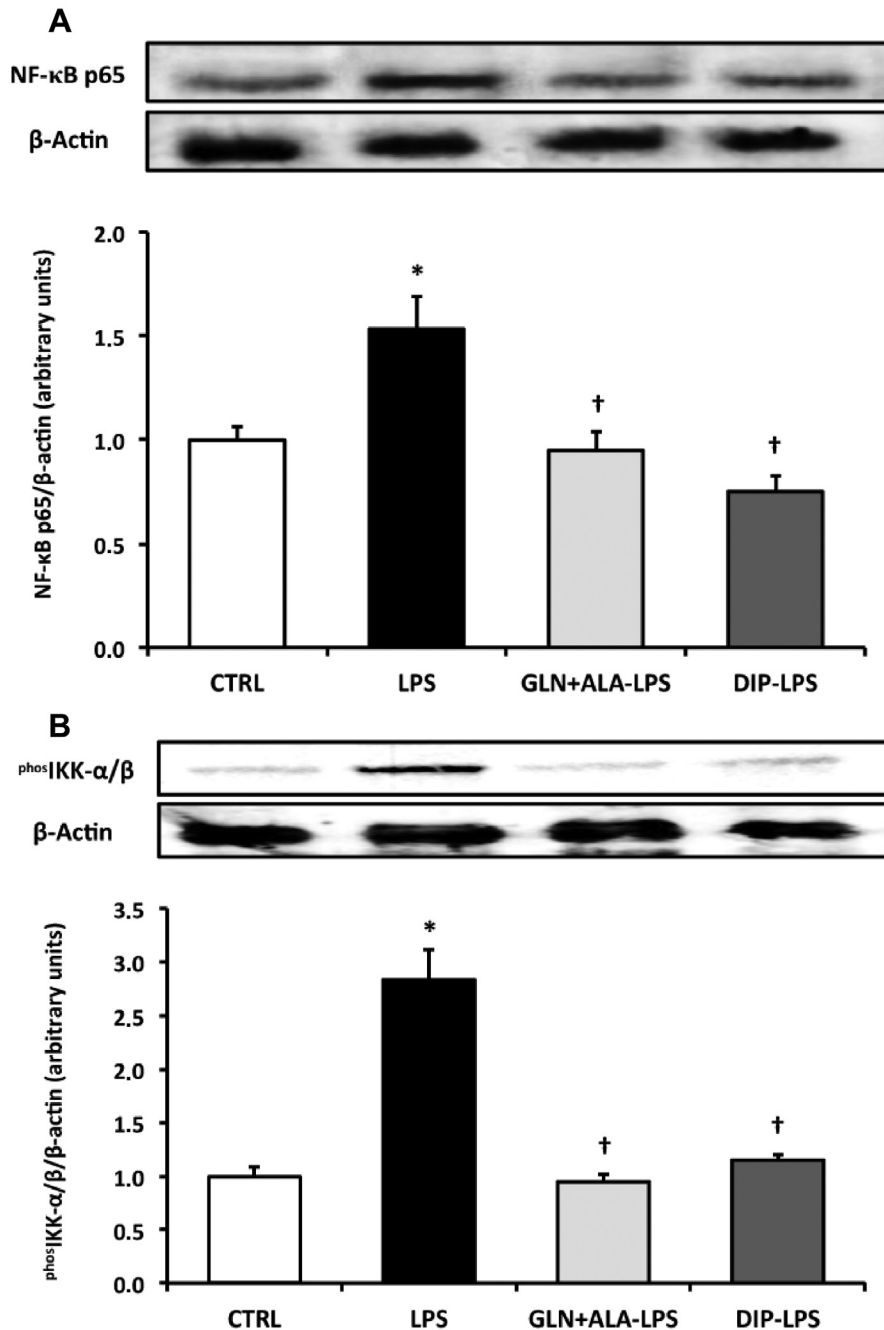
<sup>‡</sup>  $P < 0.05$  for comparison with GLN+ALA-LPS group.

family is essential to regulate immune system functions, activating important genes necessary to provide appropriate responses [5]. However, increased and prolonged activation of NF- $\kappa$ B, especially in immune cells (e.g., macrophages), results in the overexpression of mediator proteins, such TNF- $\alpha$  and IL-1  $\beta$  [31], thereby unbalancing cellular homeostasis and may contribute to the deleterious effects observed in sepsis [3]. In accordance with the literature, our LPS group demonstrated higher concentrations of plasma TNF- $\alpha$ , IL-6, IL-1  $\beta$ , and IL-10 compared with the controls. These inflammatory changes are associated with hormone responses, which stimulate muscle loss leading to decreased plasma glutamine [15], a fact that was observed in our study in the LPS group.

*In vitro* and *in vivo* studies reports that L-glutamine supplementation is capable of attenuating the excessive production of cytokines, such TNF- $\alpha$  and their effects [9,11,32]. In the present study, the groups supplemented with L-glutamine in DIP or GLN+ALA form exhibited an increase in the proinflammatory profile induced by LPS inoculation, however, these responses were much lower than that observed in the LPS group. These effects have been associated with decreased activation of NF- $\kappa$ B pathway. NF- $\kappa$ B exists in the cytoplasm in an inactive form, bound with an inhibitory protein from the I $\kappa$ B family. The phosphorylation of IKK- $\alpha/\beta$ , releases NF- $\kappa$ B from I $\kappa$ B, which permits NF- $\kappa$ B to translocate to the nucleus activating the transcription of target genes related to inflammatory response. In skeletal muscle, total NF- $\kappa$ B p65 and the phosphorylation of IKK- $\alpha/\beta$  was attenuated by the supplements, which resulted in less mRNA of NF- $\kappa$ B1 being transcribed. The subunit composition of NF- $\kappa$ B can vary, although NF- $\kappa$ B p65 (Rel A) and NF- $\kappa$ B p50 (NF- $\kappa$ B1) are the classical NF- $\kappa$ B pathway components studied in inflammation [5]. Furthermore, we observed that oral L-glutamine supplementation, independently of its form (dipeptide or free) diminished the effects of LPS in muscle and liver mRNA of NF- $\kappa$ B1A, a gene that encodes for I $\kappa$ B $\alpha$ . An imbalance between NF- $\kappa$ B and I $\kappa$ B is a critical step for the overexpression of inflammatory mediators.

Various mechanisms can activate NF- $\kappa$ B pathway, which includes MyD88 and IRAK1 proteins. These intracellular proteins are central adaptors for the majority Toll-like receptors cascade, acting as a link between the receptors and downstream kinases [7]. The replacement of glutamine by both oral supplementations attenuated the mRNA response of MyD88 and IRAK1 in muscle and IRAK1 in liver, when compared with LPS group. This is consistent with other studies that also found that treatments with L-glutamine down-regulated intestinal [33] and kidney [7] MyD88 expressions. On the other hand, the complex family of IRAK1 and its variants appear to be involved in the development of septic shock [6]. Given the results of the present study, supplementation of L-glutamine may have potential effects in decreasing the uncontrolled inflammation mediated by IRAK1 and NF- $\kappa$ B pathway.

Another important aspect is related to the type of supplementation. In studies with L-glutamine supplementation, the amino acid was given parenterally by itself or as part of total parenteral nutrition (TPN), which results in normalization of the availability of total-body glutamine, with less inflammation and immune suppression [13,20,30]. However, because IV or TPN solutions may expose the patient to enhanced risk for infections, enteral alternatives should be chosen. Although results with oral or enteral administration of L-glutamine in its free form are still controversial and inconclusive, L-alanyl-L-glutamine supplementation seems to be more effective and hydrolytically stable, providing an alternative way to increase the concentration of glutamine in the body [19]. This effect has been attributed to the active transport through glycopeptides transport protein 1 (Pept-1), which is located exclusively in the luminal membrane, has broad substrate specificity, and actively transports dipeptides and tripeptides in the intestines of humans and animals [34]. Once transported, the dipeptide could be metabolized by the enterocytes or escape of its intracellular hydrolysis, allowing high glutamine and alanine concentrations to be rapidly achieved in the plasma. However, in the present work oral supplementations with L-glutamine plus L-alanine, both in its free form

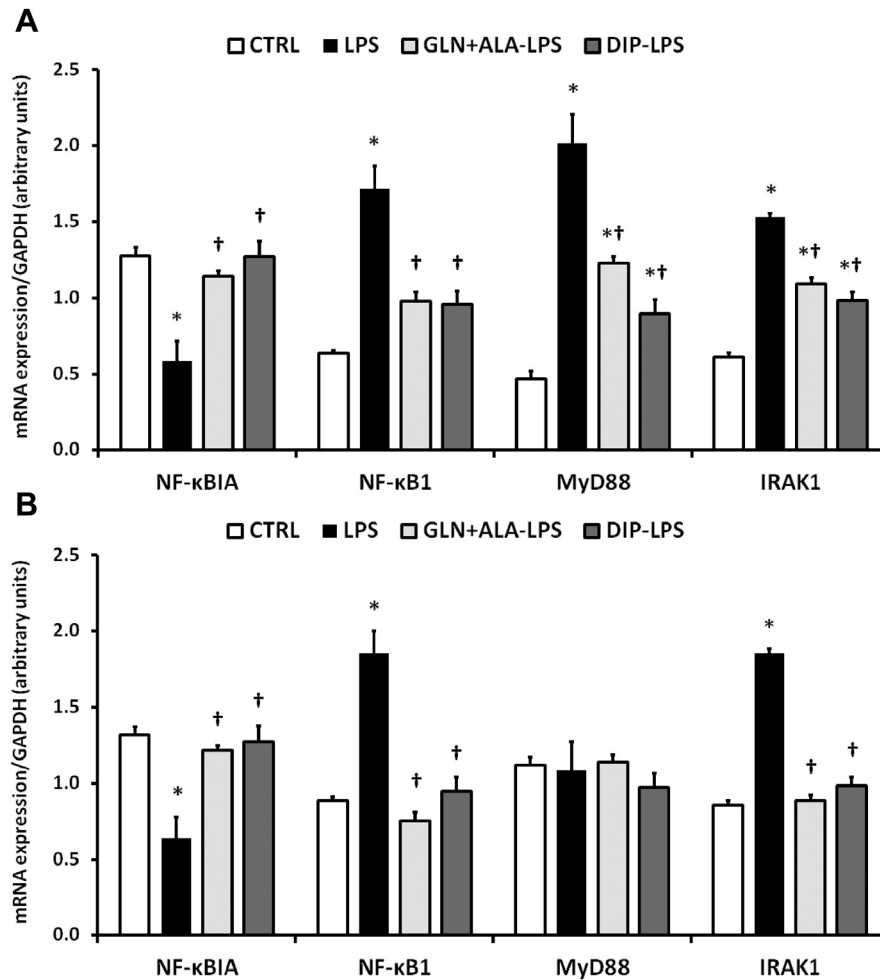


**Fig. 3.** Total NF-κB p65 (A) and phosphorylated IKK-α/β (B) expression in gastrocnemius skeletal muscle, measured using Western blot analysis. Animals and treatment groups were as shown in the table legends. After treatments and killing, tissues were surgically excised and immediately freeze-clamped under liquid nitrogen for Western blot analysis as described in the Methods section. Values are reported as mean ± SEM. \* $P < 0.05$  for comparison with CTRL group. † $P < 0.05$  for comparison with LPS animals.

and a dipeptide form had the same metabolic pattern of effects, which indicates that other mechanisms of transport are involved. These may include paracellular movement or cell-penetrating peptides and amino acids [35]. More recently, it has been demonstrated that citrulline and arginine are important end products of glutamine metabolism in the gut that involve mitochondrial enzymes [16,17]. As glutamine is a coregulator of citrulline and arginine, both can be reduced in catabolic states, especially in sepsis [18]. However, the effects of glutamine supplementation in the synthesis of these precursors and its contribution to the whole body are still debated.

According to other works [28], our results of plasma and tissues glutamine concentrations indicate that the beneficial effect of L-glutamine supplementation may be due to its delivery to the peripheral tissues and L-alanine can contribute to this effect [36]. Our experimental approach does not allow for the unraveling of the mechanism by which L-glutamine and L-alanine (separately or in conjunction) reestablished glutamine metabolism in endotoxemic animals, although it has been known that these two amino acids work in parallel. Once released by active muscles, alanine and glutamine may respond for up to 60% of the overall amino acid bulk into the blood.





**Fig. 4.** Gene expression in gastrocnemius skeletal muscle (A) and liver (B), measured using RT-qPCR. Animals and treatment groups were as shown in the table legends. After treatments and killing, tissues were surgically excised and immediately freeze-clamped under liquid nitrogen for reverse transcription and real-time PCR analysis as described in the Methods section. Values are reported as mean  $\pm$  SEM. \* $P < 0.05$  for comparison with CTRL group. † $P < 0.05$  for the comparison with LPS animals. NF- $\kappa$ BIA, nuclear factor- $\kappa$ B inhibitor alpha; NF- $\kappa$ B1, nuclear factor- $\kappa$ B1; MyD88, myeloid differentiation primary response gene 88; IRAK, interleukin-1 receptor-associated kinase 1.

Moreover, L-alanine is rapidly metabolized via alanine aminotransferase to pyruvate, with concomitant production of glutamate from 2-oxoglutarate, which contribute to antioxidant defense, such GSH production [37]. Based on the results presented here and in other studies [10,37,38], we suggest that the presence of L-alanine in the DIP form or in its free form can spare glutamine metabolism, allowing the latter to be used by high-demand tissues [36] under endotoxemia. In vivo studies support this mechanistic effect because both DIP and L-glutamine in its free form [10,11], in conjunction with other amino acids [39,40], can enhance whole-body glutamine status in health and catabolic situations.

## Conclusion

Our findings indicate that oral supplementation with L-glutamine, independently of its form as a dipeptide, L-alanyl-L-glutamine or as an equivalent mixture of free L-glutamine and free L-alanine can restore glutamine availability in the body, promoting antioxidant and anti-inflammatory effects via the NF- $\kappa$ B pathway in mice subjected to endotoxemia.

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