

Amino acid and dipeptide combination strongly attenuates endotoxemic cytokine production in human whole-blood monocytes

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Objective: The study reported here aimed to analyze the anti-inflammatory and immunomodulatory impact of arginine, glycine, glutamine, and the combination of these amino acids on the intracellular expression of proinflammatory cytokines during sepsis. These amino acids were tested on lipopolysaccharide (LPS)-stimulated human monocytes in a whole-blood system and examined using flow cytometry.

Materials and methods: The whole blood of twelve healthy volunteers processed immediately after withdrawal was incubated with arginine (2 and 5 mM), glycine (2 and 5 mM), glycyl-glutamine (2 and 5 mM), and the amino acid and dipeptide solution Glamin® (Fresenius Kabi, Bad Homburg, Germany) at three concentrations (5%, 10%, 20%), with or without LPS (0.2 ng/mL) stimulation. Cytokine-producing monocytes were phenotyped in whole blood and the intracellular expression of interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)- α was assessed by flow cytometry.

Results: In whole-blood samples from volunteers, used to best imitate physiological cellular interactions, the amino acid and dipeptide solution Glamin, containing arginine, glycine, glycyl-glutamine, was able to significantly and dose-dependently diminish LPS-induced production of proinflammatory cytokines such as IL-6, IL-8, and TNF- α in human monocytes. However, single incubation with the amino acids arginine, glycine, and glycyl-glutamine individually did not affect alterations in the expression of IL-6, IL-8, or TNF- α .

Conclusion: The amino acid and dipeptide solution Glamin, composed of glycine, arginine, and glycyl-glutamine, had strong anti-inflammatory and immunomodulatory effects during the induced experimental sepsis, as it significantly downregulated intracellular expression of proinflammatory cytokines in human whole-blood monocytes. However, only incubation with a combination of amino acids (ie, Glamin), rather than individual amino acids, demonstrated an inhibitory impact on cytokine production in LPS-stimulated monocytes. The results indicate that a combination of amino acids may potentiate the anti-inflammatory response, leading to a marked suppression of TNF- α , IL-6, and IL-8 during sepsis.

Keywords: sepsis, anti-inflammatory response, glycine, arginine, glycyl-glutamine, Glamin®

Introduction

Bacterial lipopolysaccharide (LPS) (or endotoxin) is a major component of the outer cell membrane of Gram-negative bacteria. It has remarkable potency as a signaling molecule that initiates the innate immune system's systemic liberation of proinflammatory cytokines.¹ About 80% of invading organisms are Gram-negative bacteria.² The possibility of removing this initiating agent early to attenuate the excessive activation of the innate immune response accompanied by the release of proinflammatory mediators has been viewed as a reasonable and optimal approach to sepsis, but anti-endotoxin

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strategies to significantly reduce the mortality rate in human septic shock so far remain unproven.^{1,3}

The amino acid arginine can modulate proinflammatory cytokine production and, subsequently, the immune response,^{4,5} most notably through immunomodulation via decreased proinflammatory cytokine production.^{6,7} Moreover, arginine can also serve as a substrate for polyamine synthesis, which is strongly involved in protein synthesis enhancement⁸ and could facilitate mucosal healing.^{9,10} Finally, arginine is the most important substrate in the formation of nitric oxide.¹¹ However, the European Society for Parenteral and Enteral Nutrition (ESPEN) guidelines do not currently recommend the application of arginine as a supplement in parenteral nutrition in adults, as there is no firm clinical evidence to support this additional supplementation with its putative advantages in situations of stress in the critically ill.¹² Further, there are insufficient data on safety as a result of the endogenous nitric oxide production by arginine that occurs when it produces citrulline during septic conditions.¹³

Glycine has similarly prevented inflammatory complications in several experimental models, such as ischemia/reperfusion, transplantation, shock, endotoxemia, and diabetes.^{14,15-21} Glycine is non-noxious and nontoxic with a median lethal dose of 2.6 g/kg when administered intravenously. Glycine has been shown to significantly blunt the inflammatory reaction of macrophages and neutrophils through binding to specific glycine-gated chloride channels.²²⁻²⁵ Glycine administration has been reported to reduce the induction of interleukin (IL)-6, tumor necrosis factor (TNF)- α , and mRNA, which are associated with attenuation in postoperative leukocyte recruitment. Glycine pretreatment has also been found to diminish molecular and cellular inflammatory events after major surgery or during sepsis.

Several studies have outlined that glutamine is essentially involved in intracellular nitrogen transport;²⁶⁻²⁸ intermediary metabolism; cellular redox mechanisms; and the production of nucleotides, glucose, and glutathione.²⁹⁻³³ A meta-analysis of 14 randomized trials including 737 patients has shown that the administration of glutamine to surgical and critically ill adult patients reduces infectious complications and shortens length of hospital stay without any adverse effect on mortality.³⁴ In addition, externally delivered glutamine results in a reduction in pneumonia, bacterial superinfections, and severe sepsis after polytrauma,³⁵ as well as a decreased incidence of bacterial abdominal translocation that in turn leads to a reduction in sepsis.³⁶⁻³⁸ Glutamine-containing dipeptides (glycyl-glutamine), as these are hydrolyzed immediately after

infusion into their components glutamine and glycine, are more stable and soluble, therefore providing the opportunity to improve the nitrogen balance more quickly and, thus, the outcome in critically ill patients.^{12,39-41} An infusion solution for parenteral nutrition containing essential and nonessential amino acids such as Glamin® (Fresenius Kabi, Bad Homburg, Germany) (composition: arginine 11.3 g, glycine 10.3 g, glycyl-glutamine 30.3 g, among other ingredients) positively affects and ensures the optimal utilization of the externally applied amino acids and dipeptides leading to immunomodulatory effects in clinical trials.⁴² Therefore, the ESPEN guidelines highly recommend adding a glutamine solution to the standard enteral formula for burn and trauma patients and to administer glutamine when parenteral nutrition is indicated in intensive care unit patients.¹²

The available data concerning the immunomodulating effects of arginine, glycine, and glutamine are only based on suppression of TNF- α , IL-6, and IL-8 in human and rat peripheral blood mononuclear cells and human coronary artery endothelial cells.⁴³⁻⁴⁹ These results were obtained in supernatants using enzyme-linked immunosorbent assay after peripheral blood mononuclear cells and human coronary artery endothelial cells were isolated from whole blood by density gradient separation.

Monocytes, as an essential part of the immune and inflammation systems, are a major source of proinflammatory cytokines such as TNF- α , IL-6, and IL-8⁴⁸⁻⁵⁰ after stimulation with LPS. Cytokines have been shown to be critical mediators of septic shock and are associated with increased mortality. As far as the authors are aware, there are no available data on the effect of arginine, glycine, glycyl-glutamine, and Glamin on intracellular cytokine production in single cells, as assessed by flow cytometry. Further, no investigation has examined the effects of glycine, arginine, and glutamine on whole blood, which retains all blood components including serum. We therefore developed a system employing the stimulation of whole peripheral blood followed by identification of monocytes using monoclonal antibodies to their cell-surface markers and by detection of cytokines using intracellular staining, as a physiological *ex vivo* approach.⁵¹

The investigation reported here was performed to explore the effects of arginine, glycine, glycyl-glutamine, and Glamin on the expression of IL-6, IL-8 and TNF- α in monocytes under non-septic conditions in an *ex vivo* model with whole blood, imitating best physiological conditions. Further, the investigation of the effects of arginine, glycine, glycyl-glutamine, and Glamin on the expression of proinflammatory cytokines IL-6, IL-8, and TNF- α in monocytes during

endotoxemia was also one of the study's objectives. Lastly, we also wanted to examine the alterations of human monocytes at the level of the single cell using fluorescence-activated cell sorter analysis.

Methods

The Ethics Committee of the Martin Luther University of Halle approved this investigation. After informed consent was obtained from twelve healthy volunteers (average age, 31.2 ± 1.8 years), intravenous blood samples (200 μ L) were collected in sterile tubes prefilled with lepirudin (Refludan®; CSL Behring, Marburg Germany; final concentration, 1 μ g/mL) directly before stimulation and processed immediately under sterile conditions (Heraclean, Heraeus, Germany).

Two hundred microliters of whole blood was diluted 1:5 with 800 μ L of Roswell Park Memorial Institute medium 1640 Cell Culture Medium (Life Technologies, Carlsbad, CA, USA) in 5 mL Falcon® polystyrene tubes (BD, Franklin Lakes, NJ, USA). Thereafter, 1 μ L of GolgiPlug™ (PharMingen, San Diego, CA, USA), a protein transport inhibitor containing brefeldin A, was added to each tube.⁵² Blood was stimulated with LPS (055:B5) from *Escherichia coli* (Sigma-Aldrich, St Louis, MO, USA) (final concentration 0.2 ng/mL) and incubated with arginine, glycine, glycyl-glutamine, and Glamin (final concentrations in each experiment were 2 mM and 5 mM for glycine, glycyl-glutamine, and arginine; for Glamin, the final concentration in each experiment was 5%, 10%, and 20%). Samples were incubated at 37°C in a CO₂ (5%) humidified atmosphere for 3 hours.

After stimulation, cell samples were washed once with 1 mL of CellWash (PharMingen) per tube followed by centrifugation at 500 g for 5 minutes. The supernatants were removed. The pellets were vortexed, washed with 1 mL of staining buffer (PharMingen) per tube, and vortexed again, followed by centrifugation at 500 g for 5 minutes. After removing the supernatants, a pellet of approximately 100 μ L was vortexed again and 10 μ L of fluorescein isothiocyanate-conjugated CD14 (PharMingen) was added. The samples were stored at room temperature in the dark for 10 minutes. Thereafter, the samples were twice washed with staining buffer.⁵²

For fixation, the cell pellets were resuspended in 250 μ L of Cytofix/Cytoperm™ (PharMingen) and stored at 4°C in the dark for 10 minutes. The thoroughly resuspended, fixed, and permeabilized cells (100 μ L) were then mixed with 10 μ L per tube of phycoerythrin-conjugated anti-human IL-6, IL-8,

or TNF- α or an appropriate isotype control (PharMingen) and incubated at 4°C in the dark for 30 minutes. After incubation, the cells were washed twice with Perm/Wash-Buffer (PharMingen) and resuspended in 300 μ L of staining buffer.⁵²

Flow cytometric analyses were performed with a BD Biosciences FACSCalibur™ flow cytometer using Cellquest™ software (v 3.3) (San Jose, CA, USA). The CD14⁺ monocytes were identified by immunofluorescence. At least 20,000 CD14⁺ monocytes were analyzed per sample. The CD14⁺ monocytes were gated for subsequent measuring of intracellular cytokines with phycoerythrin-labeled antibodies. Unstimulated samples and isotype controls were used as negative controls.

Ten measurements were undertaken per experiment. For analysis, the number of cytokine-positive monocytes and mean fluorescence intensities were assessed. Statistical analyses were performed using autonomous office for statistics Mogk and Reitze (MoReData) with SPSS (v 11.0.1; IBM Corporation, Armonk, NY, USA) software. Normal distribution of data was examined using the Shapiro–Wilk test. Data values for cytokine-positive monocytes and total fluorescence values were taken as mean $\pm$ standard error of the mean (SEM). The significance for each titration curve was calculated for repeated measurements to the significant levels of $P < 0.05$, $P < 0.01$, and $P < 0.001$. Pairwise comparisons were Bonferroni adjusted. The figures in this paper were created using Origin software from OriginLab (Northampton, UK).

Results

To analyze if arginine, glycine, glycyl-glutamine, and Glamin under non-septic conditions alter the expression of proinflammatory cytokines (IL-6, IL-8, TNF- α) in monocytes, whole blood was incubated with these substances without LPS. IL-6, IL-8, and TNF- α production were not affected by glycine, glycyl-glutamine, arginine (Figure 1) or Glamin (Figure 2). The percentage of gated monocytes, which was comparable to their fraction in a white blood cell count, did not decrease during incubation with any amino acid solution (glycine, glycyl-glutamine, arginine, Glamin) at any concentration (2 or 5 mM for glutamine, glycyl-glutamine, arginine; 5%, 10%, 20% for Glamin; data only shown for 5 mM glutamine, glycyl-glutamine, arginine and Glamin 20%). These results show that a decrease in cytokine-positive cells resulting from a possible toxic effect of amino acids in higher concentrations can be excluded.

In contrast, LPS-stimulated human monocytes showed a strong liberation of IL-6, IL-8, and TNF- α expression. The portion of CD14⁺ cytokine-producing cells (IL-6: 0.30 ± 0.03

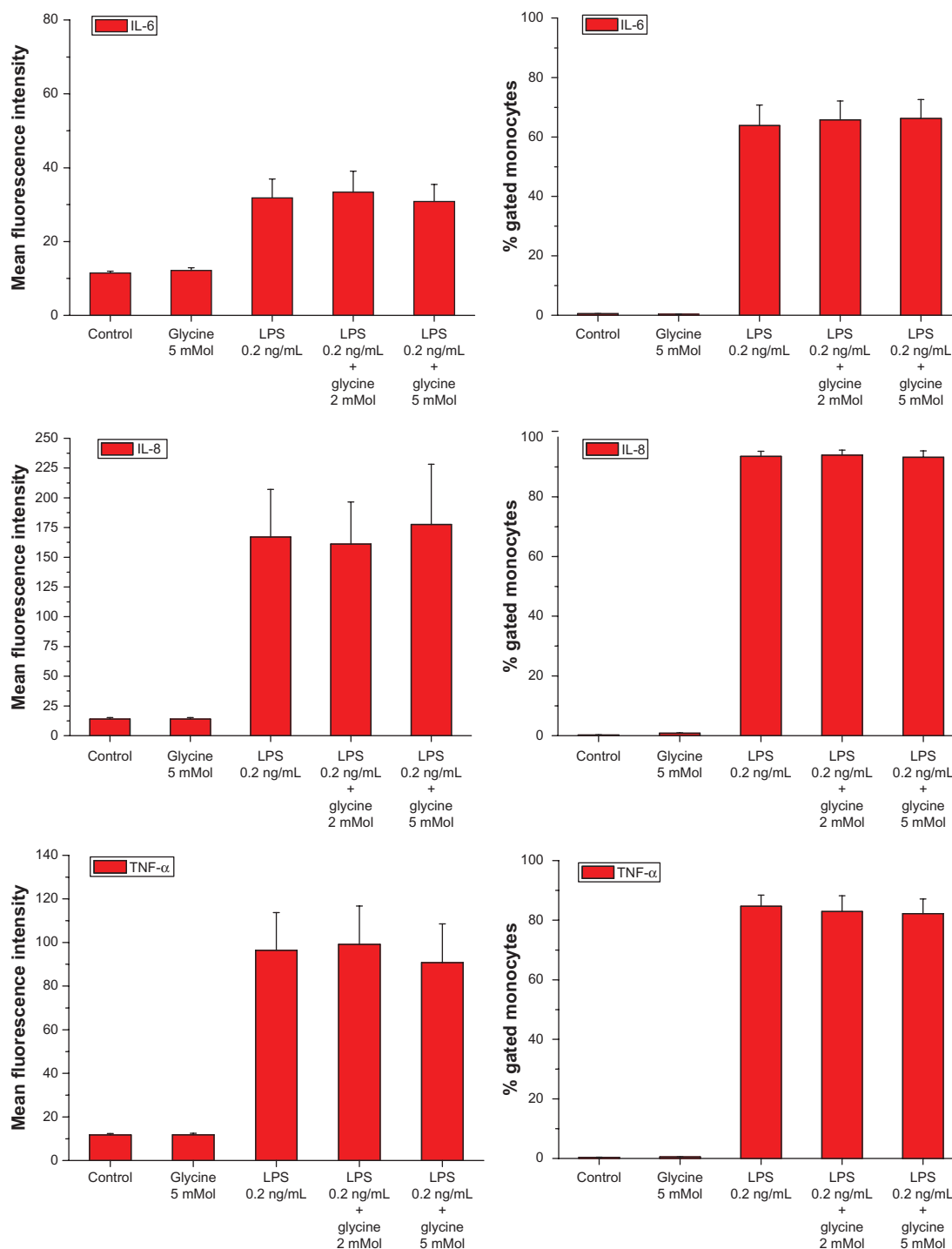


Figure 1 Effect of lipopolysaccharide (LPS) alone and LPS + glycine at two different concentrations (2 and 5 mM) on intracellular interleukin (IL)-6, IL-8, and tumor necrosis factor- α expression in monocytes taken from human whole blood measured by flow cytometry.

Notes: Figure shows the portion (%) of cytokine-producing CD14⁺ cells on total CD14⁺ cells and mean fluorescence intensity, representing the intracellular cytokine expression of each monocyte. N = 10 experiments per group. Data values are mean $\pm$ standard error of the mean.

to $64.10\% \pm 6.20\%$; IL-8: 0.20 ± 0.03 to $93.20\% \pm 2.20\%$; TNF- α : 0.20 ± 0.03 to $83.80\% \pm 5.80\%$, as well as the mean fluorescence intensity (MFI) (IL-6: 12.2 ± 0.3 to 31.1 ± 4.8 ; IL-8: 13.1 ± 0.3 to 162.2 ± 41.2 ; TNF- α : 11.9 ± 0.2 to 95.2 ± 18.8), which represent the intracellular

cytokine expression of each monocyte, increased significantly (Figures 1 and 2).

To investigate if arginine, glycine, and glycyl-glutamine are involved in the regulation of IL-6, IL-8, and TNF- α expression, both the portion of CD14⁺ cytokine-producing

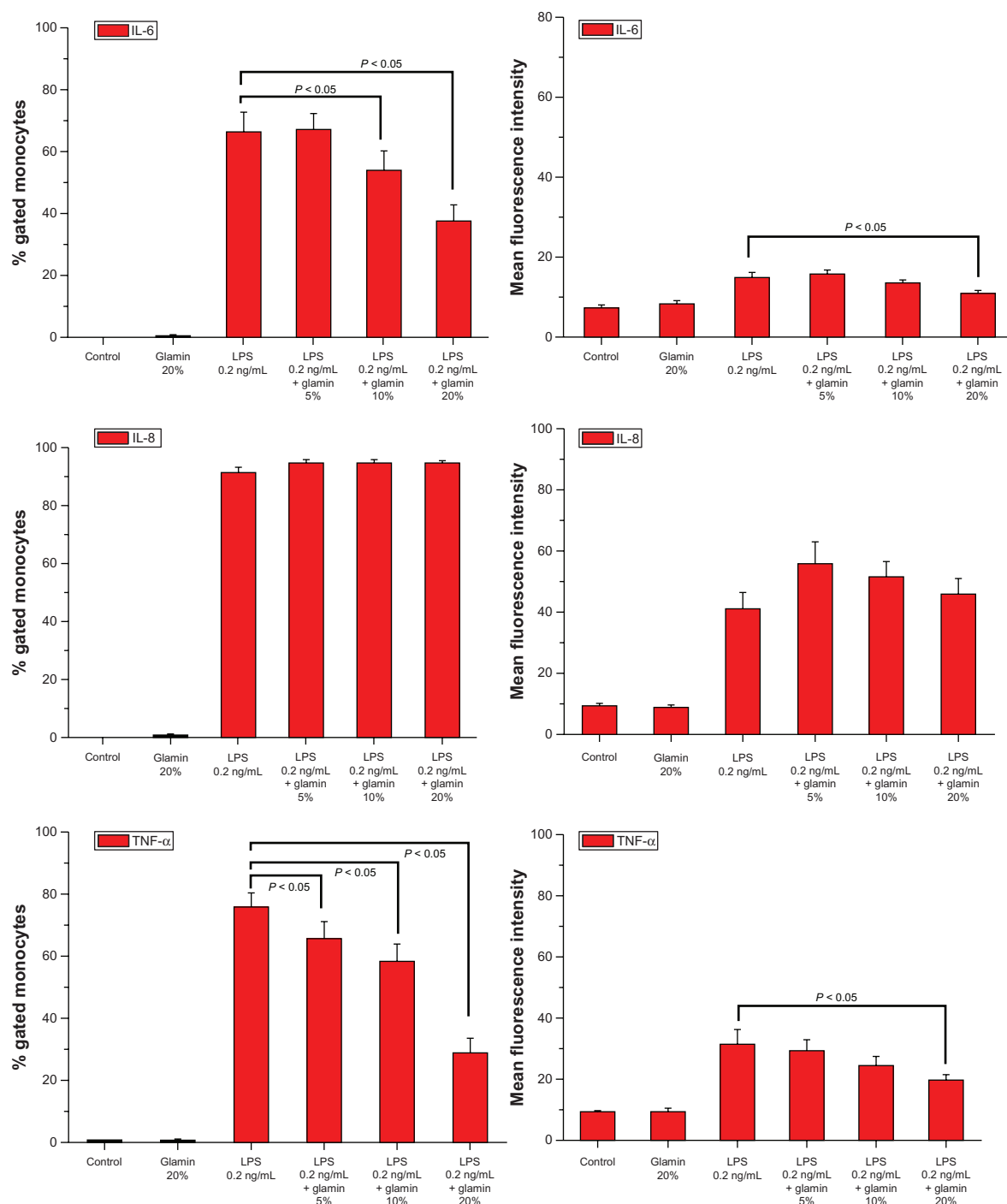


Figure 2 Effect of lipopolysaccharide (LPS) alone and LPS + Glamin® (Fresenius Kabi, Bad Homburg, Germany) at three different concentrations (5%, 10%, 20%) on intracellular interleukin (IL)-6, IL-8, and tumor necrosis factor (TNF)-α expression in monocytes taken from human whole blood measured by flow cytometry.

Notes: Figure shows the portion (%) of cytokine-producing CD14⁺ cells and mean fluorescence intensity, representing the intracellular cytokine expression of each monocyte. N = 10 experiments per group. Data values are mean ± standard error of the mean; $P < 0.05$ versus LPS.

cells and the MFIs were investigated. For arginine, glycine, and glycyl-glutamine, both the portion of CD14⁺ cells and the MFI of IL-6, IL-8, and TNF-α were not significantly affected in LPS-stimulated human monocytes, even after incubation with varying elevating concentrations

of added arginine, glycine, and glycyl-glutamine 2 and 5 mM; data for arginine and glycyl-glutamine not shown, data for glycine are demonstrated in Figure 1. Arginine did not alter production of IL-6 (LPS: 66.2% ± 2.8%; LPS + arginine 5 mM: 63.6% ± 3.2%), IL-8 (LPS:

91.2% $\pm$ 1.8%; LPS + arginine 5 mM: 92.06% $\pm$ 2.0%), or TNF- α (LPS: 76.4% $\pm$ 4.8%; LPS + arginine 5 mM: 78.0% $\pm$ 4.2%) in LPS-stimulated human monocytes. Similarly, glycyl-glutamine did not affect production of IL-6 (LPS: 62.2% $\pm$ 6.8%; LPS + glycyl-glutamine 5 mM: 61.4% $\pm$ 6.2%), IL-8 (LPS: 94.2% $\pm$ 2.8%; LPS + glycyl-glutamine 5 mM: 94.4% $\pm$ 2.0%), or TNF- α (LPS: 83.4% $\pm$ 2.8%; LPS + glycyl-glutamine 5 mM: 83.2% $\pm$ 3.2%) in LPS-stimulated human monocytes. When assessing the MFI for IL-6, IL-8, and TNF- α , the results for arginine and glycyl-glutamine were comparable with the data analyses of the percentage of cytokine-positive cells.

In contrast, the amino acid/dipeptide solution Glamin was found to strongly inhibit IL-6 and TNF- α expression of human monocytes stimulated with LPS in a dose-dependent manner by downregulation of both CD14⁺ cytokine-producing cells and intracellular, monocyte cytokine expression.

CD14⁺ IL-6-producing cells were strongly suppressed, from 67.2% $\pm$ 4.8% (LPS) to 53.2% $\pm$ 5.8% (Glamin 10%) and 38.4% $\pm$ 4.2% (Glamin 20%). The MFI for IL-6 was significantly reduced, from 14.2 $\pm$ 1.8 (LPS) to 10.2 $\pm$ 1.0 (Glamin 20%) (Figure 2). Additionally, CD14⁺ TNF- α -producing cells were strongly inhibited, from 75.2% $\pm$ 4.2% (LPS) to 65.2% $\pm$ 4.2% (Glamin 5%), 57.2% $\pm$ 4.5% (Glamin 10%), and 28.4% $\pm$ 3.2% (Glamin 20%). The MFI for TNF- α was significantly suppressed, from 30.8% $\pm$ 3.8% (LPS) to 19.2% $\pm$ 1.3% (Glamin 20%) (Figure 2).

However, Glamin did not affect either portion of CD14⁺ IL-8 producing cells nor the MFI for IL-8 in the present ex vivo sepsis model (Figure 2).

These data suggest a summing effect of arginine, glycine, and glutamine in the combined amino acid solution Glamin, leading to an additive, exponential reduction in IL-6 and TNF- α production in septic conditions (Figure 2).

Discussion

Septic shock is the most common cause of death in intensive care units; approximately 50% of patients admitted with septic shock die of refractory hypotension or progressive multiorgan failure.^{53,54} Excessive release of proinflammatory cytokines such as TNF- α or IL-6/8 produced from LPS-activated monocytes/macrophages and T cells, is the key mediator in the early phase of septic shock.^{55–57} Several therapeutic trials^{58–63} have been performed in septic patients, but until now these studies have all failed to show clinical efficacy. Antibodies to endotoxin have been prepared to counteract and block the primary agents responsible for the inflammation, and therapies against cytokines have been developed.^{58–63} However, although these

antibodies have been effective in animal models, they have not had the potency to reduce mortality in humans. Recent studies have shown that a supply of amino acids in pharmacological quantities was able to reduce mortality in intensive care unit patients, possibly via an influence on monocyte phenotype and function.^{64,65} Studies on the mechanisms of amino acid-mediated reduction of septic mortality have provided compelling evidence for amino acid-induced suppression of proinflammatory cytokines in monocytes being an important part of the innate immune system.

Therefore, our investigation set out to analyze the synthesis of the proinflammatory cytokines IL-6, IL-8, and TNF- α in LPS-stimulated human monocytes influenced by different concentrations of the amino acids arginine, glycine, glycyl-glutamine, and a combination of all of these. As high values of IL-6, TNF- α , and IL-8 are associated with a poor outcome in septic patients, downregulation of cytokine formation by amino acids could have positive effects on morbidity and mortality of patients with sepsis.

Our data could not detect any amino acid-dependent regulation of IL-6, IL-8, or TNF- α in the absence of LPS stimulation. These results are in line with clinical data demonstrating unaltered cytokine levels after glutamine administration and emphasize again the innocuous impact of amino acid administration on monocytes.⁶⁶

Further, our results demonstrate that Glamin – a proprietary amino acid solution containing arginine, glycine, and glycyl-glutamine – is effective in significantly suppressing the production of TNF- α , IL-6, and IL-8 in human monocytes in a dose-dependent manner. Using flow cytometry, we demonstrated that the MFI – which shows the amount of cytokine produced per cell, as well as the portion of cytokine-producing monocytes in relation to the total number of monocytes – was positively affected by Glamin in the same way. These data are in line with other trials demonstrating immunomodulatory effects in clinical trials by infusion solution for parenteral nutrition containing essential and nonessential amino acids covered in Glamin.^{42,67} However, other studies could not show reduced blood mononuclear cell cytokine release in endotoxemia^{68,69} – but it should be noted that these studies were performed with cells that had passed through one or more steps of purification. Using this method, it is not possible to determine which specific cell population in whole blood is responsible for cytokine production. In contrast, our investigation used whole blood, which retains all blood components, including serum, and maintains the in vivo ratios of the cells and non-cellular components.³⁴ This biological system permits interactions between the

normal blood constituents and mimics the physiological *in vivo* situation. Further, by using flow cytometry, it is possible to reveal and analyze responses at the level of the single cell in the context of unselected cellular backgrounds. Flow cytometric methods are based on the direct detection of intracellular cytokine expression with fluorochrome-conjugated anti-cytokine antibodies after short periods (3 to 6 hours) of activation.

This is also in contrast to O’Riordain et al’s study,⁶⁹ which used long, unphysiological incubation periods (of up to 24 hours) with very high concentrations of LPS that did not mimic septic conditions. Our previous investigations showed a significant correlation between cytokine expression and LPS dose, with a median effective dose of 0.2 ng mL⁻¹ (ie, achieving 50% stimulation of cytokine expression) (data not shown).^{52,70,71} For this reason, and taking into account Opal et al’s measurement of the median endotoxin level in patients with sepsis as 0.3 ng/mL,⁷² we used the median effective dose of 0.2 ng mL⁻¹ to mimic septic conditions.

In contrast to Glamin incubation, LPS stimulation in combination with administration of an individual amino acid (glycine, arginine) or the dipeptide glycyl-glutamine resulted in unaltered TNF- α , IL-6, and IL-8 levels of LPS-incubated monocytes. The impact of glycine on cytokine production and release has been described earlier in endotoxin or shock models of the liver^{20,73} and the musculoskeletal system,⁷⁴ demonstrating that glycine pretreatment prevents TNF- α and IL-6 mRNA induction during sepsis.¹⁴ However, these trials only investigated regional tissue cytokine concentration and not systemic intracellular cytokine production of monocytes in whole blood. Having protective effects on hepatic Kupffer cells, glycine is indirectly able to downregulate regional expression of proinflammatory cytokines in the liver without having any negative impact on the complete organism.⁷⁵

Vincent et al have suggested that the intracellular intake of glutamine is improved by the simultaneous administration of glycine.⁴¹ This suggests that critically ill patients might benefit more from the administration of glycyl-glutamine than from glycine. However, in our study, glycyl-glutamine was unable to attenuate the LPS-induced cytokine expression of human monocytes. The literature indicates that glycyl-glutamine infusion can reduce immunosuppression; however, these trials could only demonstrate a glycyl-glutamine-mediated restoration of anti-inflammatory cytokine production after LPS stimulation, not a significant downregulation of proinflammatory mediators after experimental sepsis.² A further explanation for our divergent results in relation to glycyl-glutamine-induced regulation

of proinflammatory cytokines compared with the literature might be that most previous trials reporting positive effects on cytokine expression investigated trauma patients rather than septic conditions, the focus of our study.^{2,47,76}

In line with data from previous studies,^{4,7,77} arginine alone did not modify the production of any of the proinflammatory cytokines tested in our study. According to results from both other studies and the study described here, arginine does not play a decisive role in immunomodulation during sepsis, although it is used as a carrier substance for glutamine as a dipeptide for parenteral infusion.

Conclusion

Monocytes, a major source of proinflammatory cytokines, are an important part of the innate immune system. Using flow cytometry, we were able to demonstrate that Glamin, a proprietary combination of arginine, glycine, and glycyl-glutamine, has a strong immunomodulating effect in an *ex vivo* whole-blood setting by inhibiting, in particular, the production of TNF- α and IL-6 in LPS-stimulated human monocytes in a dose-dependent manner. The administration of the amino acids arginine and glycine or the dipeptide glycyl-glutamine individually did not modify the production of any of the proinflammatory cytokines tested in our study.

The results of this investigation suggest that the well-known anti-inflammatory and immunomodulating effects of arginine, glycine, and glycyl-glutamine individually do not suffice to significantly downregulate the expression of proinflammatory cytokines in LPS-stimulated human monocytes. However, in combination, these three substances potentiated the anti-inflammatory impact, leading to marked suppression of TNF- α , IL-6, and IL-8 in an experimental model of sepsis.

Disclosure

The authors report no conflicts of interest in this work. The Ethics Committee of the Martin Luther University Halle-Wittenberg approved the investigation reported here. The study complied with the code of ethics of the World Medical Association (Declaration of Helsinki). This work has not been published previously, is not under consideration for publication elsewhere, is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and will not be published elsewhere in the same form, in English or in any other language. This study was financially supported by grants from the German Research Foundation (DFG SCHM2641/2-1).

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