Influence of Glutamine on the Phenotype and Function of Human Monocytes

By Andreas Spittler, Susanne Winkler, Peter Götzinger, Rudolf Oehler, Martin Willheim, Clemens Tempfer, Günter Weigel, Reinhold Fugger, George Boltz-Nitulescu, and Erich Roth

Reduced concentrations of glutamine (GLN) in plasma and skeletal muscle, defective host defense systems, and a diminished expression of the HLA-DR antigen on monocytes are important diagnostic parameters for late post-injury sepsis. In this in vitro study, we investigated whether blood monocyte-derived macrophage antigen expression and function from healthy donors is influenced by GLN. Lowering the GLN concentration in culture medium from 2 mmol/L to 200 μmol/L reduced the expression of HLA-DR by 40% (P < .001) on monocyte-derived macrophages, and decreased tetanus toxoid-induced antigen presentation. In addition, low GLN levels downregulated the expression of intercellular adhesion molecule-1 (ICAM-1/CD54), Fc receptor for IgG (FcRI/CD64), and complement receptors type 3 (CR3; CD11b/CD18) and type 4 (CR4; CD11c/CD18). A correlation was found between the phagocytosis of IgG-sensitized erythrocytes or opsonized Escherichia coli and the decreased expression of FcRRI and CR3. Monocyte expression of CD14, CD71, and FcRRII/CD16 and capacity to phagocytose latex beads were not affected by altering the level of GLN. Depletion of GLN was associated with a significant reduction in cellular adenosine triphosphate (ATP), which may have influenced cell surface marker expression and phagocytosis. It remains to be seen whether these in vitro findings are of clinical significance in the treatment of sepsis. © 1995 by The American Society of Hematology.

Monocytes AND macrophages (Mφ) belong to the mononuclear phagocyte system, are widely distributed in normal and especially inflamed tissue, and play a central role in both specific and nonspecific immunity against bacterial, viral, and fungal infection. Secretory products of monocytes and Mφ include interleukin (IL)-1, IL-3, IL-6, IL-8, IL-10, IL-12, tumor necrosis factor-α (TNF-α), granulocyte-macrophage colony-stimulating factor (GM-CSF), Mφ-CSF, transforming growth factor-β (TGF-β), platelet-activating factor (PAF), prostaglandins, substance P, leukotrienes, proteases, and complement components. During sepsis, a substantial decrease in the number of functioning phagocytes has recently been reported. Monocytes also express major histocompatibility complex (MHC) class II antigens (Ag), which play a crucial role in Ag presentation to T-helper lymphocytes. Recent investigations have shown that changes in MHC class II Ag expression on monocytes are indicative of developing infections after major surgery and that fatality and recovery rates among patients with infection and sepsis are linked to levels of HLA-DR expression.

Glutamine (GLN) is the most abundant amino acid in the blood and in the free amino acid pool of the body. During starvation and catabolic stress after trauma, surgical procedures, or during sepsis and certain cancer diseases, GLN is delivered from skeletal muscle to the gut, liver, kidney, and various cells of the immune system. In these organs, GLN serves as an energy substrate (intestine), acts as a glucose precursor (intestine, liver), counteracts acidosis (kidney), and is possibly responsible for the regulation of intracellular water content in skeletal muscle. Low concentrations of GLN suppress spontaneous transformation in neoplastic NIH-3T3 cells, inhibit mitogen-induced T-cell proliferation, decrease the capacity of murine macrophages to phagocytose opsonized particles, reduce differentiation of B cells into plasma cells, and diminish the generation of lymphokine-activated killer cells.

In addition, GLN influences monocyte antigen expression and function of human monocytes from healthy donors in vitro.

**Materials and Methods**

**Antibodies.** Monoclonal antibody (MoAb) 32.2 against Fc IgG receptor type I (anti-FcγRI/CD64), was obtained from Medarex Inc. (W. Lebanon, NH); MoAb Leu11a against Fc IgG receptor type III (FcγRIII/CD16), anti-complement receptor type 3 (CR3, CD11b/CD18), anti-CR4 (CD11c/CD18), and anti-CD14 were from Becton Dickinson (San Jose, CA); anti–HLA-DR and anti-transferrin receptor/CD71 were from DAKO (Glostrup, Denmark); and anti-intercellular adhesion molecule 1 (ICAM-1/CD54) was purchased from Immunotech (Marseille, France).

**Cell isolation and culture.** Heparinized whole blood (500 mL) was collected from healthy donors and separated by Ficoll-Paque density gradient centrifugation, and monocytes were isolated from peripheral blood mononuclear cells (PBMC) by centrifugal elutriation. Cell viability, assessed by trypan blue exclusion, varied between 95% and 98%. About 85% to 95% of cells were scored as monocytes, judged by typical morphology of labeled Giemsa-stained cytopsin preparations, nonspecific esterase type-1 staining, and expression of CD14. Of the remaining cells, 4% to 7% were CD16+, 3% to 6% CD3+, and 1% to 4% granulocytes. To prevent cell adhesion, and thus possible activation, monocytes were incubated in teflon-fluorinated ethylene propylene bottles (Nalgene Labware, Rochester, NY). Monocytes from healthy donors were cultured for 7 days in GLN and phenol red-free RPMI 1640 medium (BioWhittaker, Walkervil-
ersville, MD) supplemented with various concentrations of GLN, 100 U/mL penicillin, 100 µg/mL streptomycin (all from Flow Laboratories, Irvine, UK), and 10% heat-inactivated fetal calf serum (FCS; GIBCO Ltd, Paisley, UK). The FCS-supplemented culture medium was tested for endotoxin by the Limulus amebocyte lysate assay, and a concentration less than 0.2 ng/mL was found. The cell culture medium was changed after 3.5 days.

**Immunofluorescence and flow cytometric analysis.** Cells were harvested, washed, and resuspended in phenol red-free Hank's balanced salt solution (HBSS) containing 0.3% bovine serum albumin (BSA) and 0.1% NaN₃. After preincubation of 5 × 10⁵ cells for 30 minutes on ice with 20% human AB serum, cells were washed and then incubated with 50 µL of MoAb dilution for 30 minutes on ice. Cells were then washed and treated for a further 30 minutes with 50 µL of fluorescein isothiocyanate (FITC)-labeled sheep F(ab')₂ fragments of goat anti-mouse IgG and IgM antibodies (An der Grub, Vienna, Austria). Cells were washed three times and resuspended in 300 µL of HBSS supplemented with 1 µg/mL propidium iodide to allow exclusion of dead cells. The viability of cultured monocytes ranged between 75% and 90% and was independent of the GLN concentration. At least 10⁵ cells were analyzed on a FACScan (Becton Dickinson, San Jose, CA). The data were collected with 4 decade logarithmic amplification and expressed as arbitrary units of mean channel fluorescence (MCF). During this study, identical instrument settings were used.

**Antigen-presenting capacity.** Monocytes were harvested after 7 days in culture, irradiated (0.06 Gy for 6 minutes), stimulated with optimal concentration of tetanus-toxoid (established in preliminary experiments), and cultured for 24 hours in the above-indicated tissue culture medium.³³ Donor lymphocytes were then added, and the cell preparation was incubated in 2 mmol/L GLN for 4 days. Eight hours before freezing (−20°C), cells were pulsed with ³H-thymidine (1 µCi per well). Freeze-thawed cells were harvested onto glass fiber filters with a semiautomatic cell harvester (Wallac, Turku, Finland). Filters were dried, and the incorporated activity was measured using a Betaplate liquid scintillation counter (LKB Wallac, Pharmacia, Turku, Austria).

**Phagocytosis.** Ox erythrocytes (Ox; obtained from University of Veterinary Medicine, Vienna, Austria) were washed three times in saline and optimally sensitized with goat IgG anti-Eo antibodies (Eo-IgG; purchased from Cappel Labs, Downingtown, PA). Monocytes and Eo-IgG were mixed, centrifuged, and incubated for 40 minutes at 37°C. Nonphagocytosed Eo-IgG were lysed, and the percentage of phagocytic cells was estimated by light microscopy. Immunoglobulin-opsonized and FITC-conjugated Escherichia coli (Phagest; Orpegen, Heidelberg, Germany) was additionally opsonized with component-containing autologous serum. Cells (2 × 10⁵) and bacteria (2 × 10⁶) were incubated for 10 minutes at 37°C, washed, quenched, and fixed according to the manufacturer's procedure. The percentage of phagocytic cells and the MCF were determined by FACScan analysis.³⁴ FITC-conjugated latex beads (Fluoresbrite plain YG, 0.75 µm in ⌀) were obtained from Polysciences Inc (Warrington, PA). Monocytes (2 × 10⁵) and beads (8 × 10⁵) were incubated for 1 hour at 37°C, shifted over FCS, centrifuged, washed, and analyzed by fluorescence-activated cell sorting (FACS).

**Determination of intracellular adenosine triphosphate (ATP).** At the end of the incubation period, cells were harvested. Cell aliquots (10⁵) were centrifuged and lysed by the addition of 250 µL of 0.5 mol/L HClO₄. The pH was then neutralized with 1 mol/L K₂CO₃, and the lysates were transferred into cups and centrifuged. Supernatants were then analyzed for ATP content.³⁵ ATP was measured by bioluminescence using an ATP-monitoring reagent (BioOrbit, Turku, Finland) and a Luminometer (Berthold GmbH & Co, Bad Wildbach, Germany).

**RESULTS**

**Influence of GLN on HLA-DR antigen expression and antigen-presenting capacity.** Monocytes were cultured for 7 days with various concentrations of GLN and harvested, and the expression of HLA-DR was analyzed by flow cytometry. After this incubation period, they may be considered as monocyte-derived Mø. The basal level of HLA-DR expression was significantly inhibited at 0.05 to 1 mmol/L GLN (Fig 1). The capacity of monocyte-derived Mø to present tetanus-toxoid to T helper lymphocytes, as determined by ³H-thymidine uptake, was also diminished in a concentration-dependent manner.

**Effects on FcγRI/CD64 expression and phagocytosis of IgG-sensitized ox Eo.** The expression of the high affinity receptor for IgG (FcγRI) on monocytes cultured with low concentrations of GLN (0.05 to 0.3 mmol/L) was significantly downregulated (Fig 2). Furthermore, the capacity of peripheral blood monocytes, cultured with low concentrations of GLN (0.05 to 0.6 mmol/L), to phagocytose Ox-IgG was significantly reduced.

**Effects of GLN concentrations on the expression of complement receptors and phagocytosis of opsonized E coli.** CR3 (Mac-1, CD11b/CD18) is relatively well expressed on resting human blood monocytes, and low concentrations of GLN significantly suppressed CR3 expression (Fig 3). Furthermore, the percentage of monocyte-derived Mø ingesting opsonized E coli was slightly diminished.

**Influence on the expression of other cell surface markers.** The results depicted in Table 1 show that monocytes cultured...
Monocytes were cultured for 7 days with the indicated concentrations of GLN, and the expression of CD64 (MCF) was determined by light microscopy. Data with monocytes from five apparently healthy donors represent the mean ± SD. Statistically significant decrease in comparison to 2 mmol/L GLN, Student’s t-test: *P < .05, **P < .01, ***P < .001.

The capacity of monocyte-derived Mφ to ingest FITC-conjugated latex beads varied between 88% and 91%, and in contrast to FcyR- or CR3-mediated phagocytosis, their phagocytic properties were independent of GLN concentration (Table 2).

Dependence of cellular ATP levels on GLN levels. As shown in Fig 4, the ATP levels decreased significantly when monocytes were cultured with 0.2 or 0.05 mmol/L GLN. The mean 100% ATP content per 10⁶ cells in four experiments was 2.695 ± 0.52 nmol/L.

DISCUSSION

Monocytes are circulating blood precursors of tissue Mφ and play a central role in the immune response. In the present study, freshly isolated peripheral blood monocytes were cultured for 7 days in the presence of various concentrations of GLN. After this incubation period, they resemble Mφ in many respects and are, therefore, termed monocyte-derived Mφ. Monocytes and Mφ express cell surface antigens with a wide variety of functions. The gene products HLA-DR, DQ, and DP of the MHC class II complex are essential in presentation of intracellularly processed antigens to CD4⁺ T cells. Cell surface markers such as FcyRI/CD64, FcyRII/CD32, FcεRI/CD89, FcεRII/CD23, FcγRI, and receptors for complement (CR1/CD35, CR3/CD11b, and CR4/CD11c) are expressed by monocytes, and some of these structures are important in the internalization and destruction of opsonized particles. Other molecules, such as the receptors for FcyRIII/CD16 and transferrin/CD71, are under resting conditions poorly expressed on blood monocytes. Monocytes and Mφ also constitutively express the CD14 antigen, which is a differentiation marker. Furthermore, the CD14 antigen binds to the lipid A moiety of bacterial lipopolysaccharide (LPS) and LPS-binding protein, playing an important role in cell activation and stimulation for cytokine production (eg, TNF-α, IL-1) ²⁰

The current study shows that both the expression of various cell surface molecules and the function of monocytes are also regulated by GLN. The expression of HLA-DR, FcyRI/CD64, CR3 (CD11b/CD18), and CR4 (CD11c/CD18) antigens were downregulated by GLN in a concentration-dependent manner, whereas the expression of CD14, CD71, and FcyRIII/CD16 was GLN-independent.

Decreasing the concentration of GLN from 2 mmol/L (normally used for in vitro cell culture) to 0.5 μmol/L reduced HLA-DR expression by 58%. A significant decrease in HLA-DR expression was also seen on lowering the GLN concentration from 600 μmol/L (physiologic range of GLN in the plasma) to 200 μmol/L (plasma concentration found in critically ill patients). Interestingly, reduced HLA-DR expression was paralleled by the diminished capacity of monocytes to present tetanus toxoid Ag to T cells.

Previous in vitro studies have shown that GLN at 300
GLN, by GLN are not yet clear. Studies on monocytes from patients with reduced plasma GLN and after GLN substitution on monocytes was also significantly downregulated by low cell proliferation and modifies Ag expression and function of involved in the costimulation of resting T cells. In the present effect of GLN on intracellular ATP levels has already de- relevance.

will determine whether these in vitro results have clinical data show that monocytes cultivated with a reduced amount of GLN have low ATP levels. Depletion of ATP levels can metastases performed with lymphocytes and Mφ have shown that these cells have a high GLN use GLN

of GLN may additively influence the capacity of monocytes to present various antigens to lymphocytes by reducing HLA-DR expression and lymphocyte proliferation. Metabolic investigations performed with lymphocytes and Mφ have shown that these cells have a high GLN use GLN which are required for the synthesis of nucleic acids. Our show that monocytes cultivated with a reduced amount of GLN have low ATP levels. Depletion of ATP levels can alter the organization of the cytoskeleton? which possibly affects antigen expression and phagocytosis. A protective effect of GLN on intracellular ATP levels has already described in endothelial cells during oxidant injury.25 However, the exact mechanism by which GLN depletion reduces T-cell proliferation and modifies Ag expression and function of monocytes and whether cytokine production is modulated by GLN are not yet clear. Studies on monocytes from patients with reduced plasma GLN and after GLN substitution will determine whether these in vitro results have clinical relevance.

ICAM-1/CD54, the receptor for lymphocyte function-associated antigen-1 (LFA-1) on antigen-presenting cells, is involved in the costimulation of resting T cells. In the present study, the constitutive expression of this accessory molecule on monocytes was also significantly downregulated by low

Table 1. Effects of GLN on Antigen Expression by Monocyte-Derived Macrophages

<table>
<thead>
<tr>
<th>GLN (mmol/L)</th>
<th>CD11c/CD18</th>
<th>CD54</th>
<th>CD14</th>
<th>CD71</th>
<th>CD16</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>753.5 ± 19.8</td>
<td>145.1 ± 13.1</td>
<td>745.9 ± 59.8</td>
<td>32.8 ± 0.5</td>
<td>16.3 ± 1.9</td>
</tr>
<tr>
<td>1.0</td>
<td>714.6 ± 24.2</td>
<td>135.4 ± 11.3</td>
<td>728.9 ± 35.5</td>
<td>30.8 ± 0.3</td>
<td>15.1 ± 0.5</td>
</tr>
<tr>
<td>0.6</td>
<td>693.3 ± 39.1</td>
<td>127.6 ± 11.2</td>
<td>786.4 ± 50.1</td>
<td>33.6 ± 3.9</td>
<td>14.7 ± 0.3</td>
</tr>
<tr>
<td>0.3</td>
<td>669.5 ± 37.1*</td>
<td>120.7 ± 10.1</td>
<td>782.8 ± 35.1</td>
<td>35.4 ± 3.5</td>
<td>15.7 ± 0.7</td>
</tr>
<tr>
<td>0.2</td>
<td>601.6 ± 32.5t</td>
<td>116.2 ± 10.3*</td>
<td>792.6 ± 86.2</td>
<td>32.9 ± 3.5</td>
<td>15.2 ± 0.7</td>
</tr>
<tr>
<td>0.1</td>
<td>542.8 ± 22.5t</td>
<td>111.4 ± 8.8*</td>
<td>826.5 ± 81.7</td>
<td>30.3 ± 0.4</td>
<td>16.3 ± 2.2</td>
</tr>
<tr>
<td>0.05</td>
<td>465.4 ± 58.3t</td>
<td>91.4 ± 4.4t</td>
<td>784.0 ± 68.8</td>
<td>30.9 ± 1.3</td>
<td>14.3 ± 1.1</td>
</tr>
</tbody>
</table>

* Data from three to five experiments, with cells cultured for 7 days with the indicated concentrations of GLN, represent the MCF intensity ± SD. Statistically significant decreases are by Student’s t-test.
† P < .05.
‡ P < .01.

μmol/L reduced the mitogen-induced T-lymphocyte proliferation to 20%, and at 50 μmol/L, to 50%.8 Therefore, in vivo GLN may additively influence the capacity of monocytes to present various antigens to lymphocytes by reducing HLA-DR expression and lymphocyte proliferation. Metabolic investigations performed with lymphocytes and Mφ have shown that these cells have a high GLN use rate.21,22 GLN is responsible for energy generation and provides intermediates in the biosynthesis of purine and pyrimidine nucleotides, which are required for the synthesis of nucleic acids. Our data show that monocytes cultivated with a reduced amount of GLN have low ATP levels. Depletion of ATP levels can alter the organization of the cytoskeleton,23,24 which possibly affects antigen expression and phagocytosis. A protective effect of GLN on intracellular ATP levels has already described in endothelial cells during oxidant injury.25 However, the exact mechanism by which GLN depletion reduces T-cell proliferation and modifies Ag expression and function of monocytes and whether cytokine production is modulated by GLN are not yet clear. Studies on monocytes from patients with reduced plasma GLN and after GLN substitution will determine whether these in vitro results have clinical relevance.

ICAM-1/CD54, the receptor for lymphocyte function-associated antigen-1 (LFA-1) on antigen-presenting cells, is involved in the costimulation of resting T cells. In the present study, the constitutive expression of this accessory molecule on monocytes was also significantly downregulated by low

concentrations of GLN. The expression of CD14, the LPS-binding protein receptor on the monocytes, and CD71, the transferrin receptor, was not affected by lowering the GLN concentrations.

Depletion of GLN was correlated with altered phagocytic ability of monocytes. Low GLN concentrations led to a downregulation of the markers responsible for the phagocytic capacity of monocytes, such as the high affinity receptor for IgG (FcγRI/CD64) and CR3 (CD11b/CD18). Previous in vitro studies revealed that the ability of murine peritoneal Mφ to phagocytose 125I-labeled yeast cell walls (Zymosan) or 51Cr-labeled sheep red blood cells is dependent on the concentration of GLN.8,5 These results partly confirm our findings. The phagocytosis of IgG sensitized ox Eo was significantly reduced under lowered GLN concentrations. There was also a tendency of diminished phagocytosis of opsonized E coli. However, in contrast to the results obtained with murine Mφ, in our study, the phagocytosis of latex particles by monocyte-derived Mφ was not influenced by GLN.

Table 2. Effects of GLN on Phagocytosis of FITC-Conjugated Latex Beads by Monocyte-Derived Macrophages

<table>
<thead>
<tr>
<th>GLN (mmol/L)</th>
<th>Mean ± SD</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>538.1 ± 66.8</td>
<td>91</td>
</tr>
<tr>
<td>1.0</td>
<td>540.2 ± 22.9</td>
<td>89</td>
</tr>
<tr>
<td>0.6</td>
<td>565.7 ± 61.4</td>
<td>91</td>
</tr>
<tr>
<td>0.3</td>
<td>588.8 ± 29.7</td>
<td>90</td>
</tr>
<tr>
<td>0.2</td>
<td>546.7 ± 83.1</td>
<td>88</td>
</tr>
<tr>
<td>0.1</td>
<td>562.3 ± 27.4</td>
<td>89</td>
</tr>
<tr>
<td>0.05</td>
<td>553.7 ± 20.1</td>
<td>88</td>
</tr>
</tbody>
</table>

* Data from four experiments, with cells cultured for 7 days with the indicated concentrations of GLN, represent the MCF intensity ± SD and percentage of phagocytic cells.

Fig 4. Influence of GLN on cellular ATP levels. Monocytes were cultivated for 7 days with the indicated concentrations of GLN. The value obtained with cells cultured in 2 mmol/L GLN was arbitrarily considered as 100%. Data from duplicates of four experiments are presented as the mean ATP content per 10⁶ cells ± SD. Statistically significant decreases in comparison with 0.6 mmol/L GLN, Student’s t-test: *P < .05, **P < .01.
The clinical relevance of a GLN deficiency is well documented. Low plasma GLN concentrations are indicative for a bad prognosis in catabolic patients. In a recent report, it was suggested that a decrease in plasma GLN concentration may contribute to the injury-induced impairment of immune function after major burn injury. An increasing number of experimental and clinical studies have documented the therapeutic efficacy of GLN-enriched nutrition support. The administration of GLN-supplemented parenteral nutrition after bone marrow transplantation diminished the incidence of clinical infection and microbial colonization and shortened the hospital stay of the patients. However, no immunologic monitoring was performed in this study, and it is not clear whether GLN improved the immunologic and/or the nutritional metabolic state. The present report documents that low GLN levels reduce the HLA-DR expression under in vitro conditions. A reduced HLA-DR expression was described in septic patients after trauma and in patients with inflammatory bowel disease. Prognostic relevance of HLA-DR expression on monocytes in septic patients has been described, where a reduction of HLA-DR expression of more than 70% correlates with patient mortality. However, in these clinical studies, the plasma GLN levels were not monitored, and therefore, we can only hypothesize that low plasma GLN concentrations may have contributed to the reduced HLA-DR expression on monocytes in these patients.

LPS is one of the main mediators in sepsis and is known to influence monocyte function both in vivo and in vitro. In our experimental system, LPS levels in the culture medium were below 0.2 ng/mL. Though several investigators consider these LPS levels as LPS-free, effects on specific functions of monocytes have also been reported at levels of LPS between 10 and 100 pg/mL, eg, inhibition of interferon-gamma–induced Fc receptor expression and of respiratory burst capacity. Contradictory effects of LPS on HLA-DR expression on monocytes have been described. In one study, a downregulation of monocyctic HLA-DR expression was revealed after stimulation with LPS concentrations up to 1 µg/mL, whereas another study described an upregulation of HLA-DR expression. In our opinion, the concentrations of LPS monitored in our culture medium are unlikely to influence the expression of cell surface markers, but a contributory role of LPS cannot entirely be excluded. Future experiments performed with different concentrations of GLN and exogenous LPS should clarify whether there is a combined or adverse effect of these two mediators.

In conclusion, our data show for the first time that the expression of various antigens on human monocytes from apparently healthy volunteers is dependent on the GLN concentration in cell culture. These findings may be of clinical relevance, because monocytes from critically ill patients have both low GLN levels and a reduced expression of HLA-DR. However, the in vivo findings of low GLN and decreased HLA-DR expression may also be the coincidental result of some other perturbation, rather than evidence of a cause-and-effect relationship. Our in vitro study shows that depletion of GLN correlates with altered phagocytic ability of monocytes and suggests a possible relationship between GLN depletion and decreased expression of certain cell surface antigens on monocytes.

REFERENCES
Influence of glutamine on the phenotype and function of human monocytes

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