Interaction between inflammation, coagulation and fibrinolysis during infection

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Chapter 4

Diminished Interferon-γ Production and Responsiveness after Endotoxin Administration to Healthy Humans

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Abstract
To obtain insight in the capacity of the LPS tolerant host to produce IFN-γ and to respond to this cytokine, whole blood was obtained from healthy humans before and 4 hours after intravenous injection of LPS (4 ng/kg) and stimulated ex vivo. LPS exposure in vivo resulted in a diminished capacity to produce IFN-γ upon restimulation with LPS, together with a reduced ability to release the IFN-γ-inducing cytokines interleukin (IL)-12 and IL-18, and with a reduced responsiveness toward these cytokines. In addition, IFN-γ responsiveness was strongly diminished after in vivo LPS exposure as reflected by the facts that blood obtained after LPS injection could not be primed by IFN-γ for LPS-induced tumor necrosis factor-α release, and peripheral blood monocytes could not be stimulated by IFN-γ to upregulate MHC-II expression. Experimentally induced immunoparalysis is associated with strongly reduced IFN-γ production and responsiveness.

Introduction
Endotoxin (lipopolysaccharide, LPS) contained in the outer membrane of gram-negative bacteria, is considered to contribute significantly to the pathogenesis of gram-negative sepsis. When administered intravenously, LPS stimulates the release of cytokines such as Tumor Necrosis Factor (TNF)-α, Interleukin (IL)-6 and IL-8 and causes fever, hypotension, neutrophilia, lymphopenia as well as elevations of cortisol [1]. Upon repeated injection of small quantities of bacterial LPS, animals and humans become transiently refractory to the pyretic, metabolic, and lethal effects of subsequent challenges of LPS. This phenomenon is generally referred to as immunoparalysis or LPS tolerance [2-4]. LPS tolerance is characterized by a reduced capacity of whole blood or peripheral blood monocytes, isolated from patients with gram-negative sepsis or after surgery, to produce pro-inflammatory cytokines upon stimulation with LPS as well as a decrease in major histo-compatibility complex class II (MHC-II) expression on monocytes [2, 3, 5]. Although the diminished monocyte responsiveness during immunoparalysis has received most attention, also granulocytes and lymphocytes have been found to be less reactive upon stimulation with bacterial antigens [2, 6, 7].

Interferon (IFN)-γ is considered to be an important mediator of antibacterial host defense [8]. This cytokine is mainly produced by activated T and natural killer (NK) cells. IFN-γ exerts several immune regulatory activities, including activation of phagocytes, stimulation of antigen presentation by increasing the expression of MHC molecules class I and II on antigen presenting cells (APC's), orchestration of leukocyte-endothelium interactions and stimulation of the respiratory burst [8]. During immunoparalysis the production of IFN-γ is impaired. Indeed, mice exposed to LPS in vivo showed a profoundly reduced capacity to release IFN-γ upon restimulation with LPS [9-11]. Similarly, our laboratory recently reported that T-cells
from healthy humans challenged with LPS intravenously secrete less IFN-γ upon restimulation with specific T-cell agonists [7]. It has been proposed that immunoparalysis may contribute to the enhanced susceptibility to nosocomial infections and late mortality of patients after surgery and patients who survive the initial acute phase of sepsis syndrome [3, 12]. In light of the diminished production capacity of IFN-γ on the one hand, and the potent immunostimulatory properties of this cytokine on the other hand, administration of recombinant IFN-γ has been advocated as a treatment of patients with immunoparalysis. In a pilot study in patients with sepsis and evidence for immunoparalysis, daily subcutaneous injection of IFN-γ restored the TNF-α production capacity of monocytes and enhanced MHC-II expression [13]. The success of such an approach would depend on an intact responsiveness of immune cells toward exogenously administered IFN-γ. In the present study we exposed healthy humans to a single intravenous dose of LPS to induce a transient LPS tolerant state. By using this model, which has been shown previously to be useful in studying mechanisms contributing to immunoparalysis in man [6, 7, 14, 15], we sought to determine (1) to what extent IFN-γ production is impaired in humans with experimentally induced LPS tolerance, and (2) whether blood cells obtained from LPS tolerant humans are able to respond to IFN-γ normally.

Material and methods

**Human endotoxemia challenge**

In total 15 healthy males (mean age: 22 years, range: 19-29) were studied. All measurements were done in the first 7 subjects enrolled, except for analyzing MHC-II expression on monocytes which was done in a subsequent cohort of 8 subjects. The research and ethical committees of the Academical Medical Center approved the study. Written informed consent was obtained from all study subjects. All subjects were in good health, as documented by history, physical examination, and hematological and biochemical screening. They received an intravenous bolus injection of *Escherichia coli* LPS, lot G (United States Pharmacopeial Convention, Rockville, MD), over one minute in an antecubital vein at a final dose of 4 ng/kg bodyweight. Heparinized blood for whole blood stimulation and FACScan analysis was obtained directly before LPS injection and 4 hours thereafter.

**FACS analysis**

FACS analysis was used to determine the number of CD4+/CD8+ lymphocytes and to evaluate MHC-II (HLA-DR,DP,DQ) expression on monocytes. For FACS analysis erythrocytes were lysed with ice-cold isotonic NH₄CL solution (155 mMol/l NH₄CL, 10 mMol/l KHCO₃, 0.1 mMol/l EDTA, pH 7.4) for 10 minutes. Incubations for FACScan analysis were performed in 96-well V-shaped micro plates (Greiner B.V., Alphen a/d Rijn, the Netherlands). For staining, 3x10⁶ cells/well were incubated with the following mouse anti–human monoclonal antibodies: FITC-labeled anti-CD4, PE-labeled anti-CD8, chyochrome labeled anti-CD3, FITC-labeled anti-HLA-DR,DP,DQ
and APC-labeled anti-CD14 (1:100; all Pharmingen, San Diego, CA). The appropriate isotype antibody controls (Pharmingen) were included in all experiments. Cells were incubated on ice for 30 min, and washed twice with cold FACS buffer (PBS supplemented with 0.01% NaN₃, 0.5% Bovine Serum Albumen (BSA) and 0.3 mM EDTA) and resuspended in FACS buffer. Lymphocytes and monocytes were gated by forward scatter and side scatter using a FACScan (Becton Dickinson, Franklin Lakes, NJ) and 5000 cells were counted. Results are given as percentage CD4⁺ or CD8⁺ cells within the population of CD3⁺ lymphocytes, or as MHC-II specific mean channel fluorescence within the population of CD14⁺ monocytes.

**Whole blood stimulation**

Blood was collected aseptically from healthy human volunteers using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson & Co, Rutherford, NY). Anticoagulation was obtained using LPS-free heparin (Leo Pharmaceutical Products B.V., Weesp, the Netherlands; final concentration 10 U/ml). Whole blood, diluted 1:2 in sterile and pyrogen-free RPMI-1640 (GibcoBRL, Life Technologies Inc, Grand Island, NY) was stimulated for 8 hours (for MHC-II expression) or 24 hours (for cytokine production) at 37°C and 5% CO₂ with different stimuli in sterile polypropylene tubes (Becton Dickinson & Co, Rutherford, NY). For these experiments, polypropylene tubes were prefilled with 1 ml RPMI containing the appropriate concentrations of the stimuli, after which 1 ml heparinized blood was added. Tubes were gently mixed and placed in the incubator. The stimuli used were LPS (from *Escherichia coli* serotype 0111:B4; Sigma, St Louis, MO; 10 ng/ml), recombinant human IL-12 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, the Netherlands, 100 ng/ml), recombinant human IL-18 (CLB, 100 ng/ml) and recombinant human IFN-γ (CLB, 10 ng/ml or 100 ng/ml). In the experiment in which the effect of IFN-γ on LPS induced TNF-α release was assessed, IFN-γ was added to the blood cultures either concurrently or 4 hours prior to LPS. After the incubation, supernatant was obtained after centrifugation and stored at -20°C until ELISA’s were performed or blood was used for FACS analysis.

**ELISA’s**

All measurements were done in duplicate using specific ELISA’s. The following ELISA’s were used according to the instructions of the manufacturers (with detection limits in pg/ml): IFN-γ; CLB; 2) TNF-α (CLB; 1.4), IL-18 (Fujisaki institute, Okayama, Japan; 10) and IL-12 (R&D systems, Abington, UK; 16.5).

**Statistical analysis**

All values are given as mean ± SE. Comparisons were done using the Wilcoxon test. P<0.05 was considered to represent a significant difference.
Results

Clinical and hematological responses

Intravenous injection of LPS was associated with a transient influenza-like syndrome, consisting of headache, nausea, myalgia and chills, starting at 1-2 hours after LPS administration, and lasting 2-3 hours. In addition, a rise in body temperature was recorded, peaking at 3-4 hours after LPS (38.3 ± 0.2 °C). Effects of LPS on leukocyte counts and differentials at the time points at which whole blood was collected for in vitro stimulation (0 and 4 h) are listed in table 1. Monocyte and lymphocyte counts strongly decreased after LPS administration. The decrease of lymphocytes consisted of decreases in the number of both CD3⁺/CD4⁺ and CD3⁺/CD8⁺ cells.

Table I

<table>
<thead>
<tr>
<th>(x10⁶/ml)</th>
<th>T0h</th>
<th>T24h</th>
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<tbody>
<tr>
<td>Monocytes</td>
<td>0.38 ± 0.01</td>
<td>0.18 ± 0.06*</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>1.75 ± 0.14</td>
<td>0.47 ± 0.07*</td>
</tr>
<tr>
<td>CD3⁺/CD4⁺</td>
<td>0.96 ± 0.13</td>
<td>0.25 ± 0.04*</td>
</tr>
<tr>
<td>CD3⁺/CD8⁺</td>
<td>0.64 ± 0.06</td>
<td>0.19 ± 0.03*</td>
</tr>
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</table>

Effect of LPS administration in vivo on monocyte and lymphocyte counts. Values are mean ± SE of 7 healthy subjects. LPS (4ng/kg) was given as an i.v. bolus injection at T=0 h. Analysis was performed by flow cytometry and FACSscan analysis. Data are expressed as cell counts *P<0.05 versus T=0

Reduced IFN-γ production in LPS tolerant humans

First, we wished to evaluate the extent to which IFN-γ production is disturbed in humans exposed to LPS in vivo. For this we compared IFN-γ release in whole blood, drawn directly before and 4 h after LPS injection, and restimulated with LPS. Since the numbers of lymphocytes, the main producers of IFN-γ in whole blood, changed after LPS administration (table 1), IFN-γ concentrations were corrected for the number of CD3⁺/CD4⁺ and CD3⁺/CD8⁺ cells according to previously described methods [7]. We found that LPS exposure in vivo resulted in a strongly diminished IFN-γ production capacity by CD3⁺/CD4⁺ and CD3⁺/CD8⁺ cells in whole blood stimulated with LPS (both P < 0.05 for the difference between t=0 and t=4 h; Figure 1). The production of IFN-γ is positively controlled by IL-12 and IL-18, cytokines that both are mainly monocyte/macrophage derived [16]. Whereas IL-12 is the most potent inducer of IFN-γ synthesis, IL-18 can synergistically enhance IL-12 induced IFN-γ release. To investigate whether the impaired LPS-induced IFN-γ release could be related to a reduced production of IL-12 and IL-18 as a consequence of monocyte anergy, the concentrations of these cytokines were measured in LPS stimulated whole
Figur ee  1 :  Reduced  IFN-γ  production  capacit y  afte r  in  vivo  LP S  exposu re. Whole  bloo d,  obtained  before  and  4 h  after  LP S  injection,  was  stimulated  in  vitro  for  24 h  at 37°C  with  LP S  (10  ng/ml).  Data  are  expressed  as  mean  (±SE)  pg  per  10⁶  CD3⁺/CD4⁺  cells  (A)  or  as  pg  per  10⁶  CD3⁺/CD8⁺  cells  (B).  *P<0.05  versus  T=0.

blood,  and  corrected  for  monocyte  counts.  Both  IL-12  and  IL-18  release  were  diminished  in  blood  drawn  4 h  after  LP S  injection  when  to  compared  to  their  release  in  blood  obtained  before  LP S  administration  (both  P<0.05  for  the  difference  between  t=0  and  t=4 h;  Figure  2).  Although  this  finding  suggested  that  impaired  IL-12  and  IL-18  contribution  may  contribute  to  the  reduced  capacity  to  produce  IFN-γ  upon  restimulation  with  LP S,  the  possibility  that  lymphocytes  of  LP S  tolerant  humans  are  less  responsive  to  IL-12  and/or  IL-18  remained.  We  therefore  stimulated  whole  blood  obtained  before  and  4 h  after  LP S  injection  with  recombinant  IL-12  and/or  recombinant  IL-18  and  determined  IFN-γ  concentrations  per  10⁶  CD3⁺/CD4⁺  and  CD3⁺/CD8⁺  cells.  Before  LP S  administration,  IL-12  elicited  a  strong  release  of  IFN-γ,  which  was  enhanced  by  addition  of  IL-18;  IL-18  alone  did  not  consistently  induce  IFN-γ  secretion  (Figure  3).

Figure  2 :  Reduced  IL-12  and  IL-18  production  capacit y  after  in  vivo  LP S  exposu re. Whole  bloo d,  obtained  before  and  4 h  after  LP S  injection,  was  stimulated  in  vitro  for  24 h  at 37°C  with  LP S  (10  ng/ml).  IL-12  concentrations  (A)  and  IL-18  concentrations  (B)  are  expressed  as  pg  per  10⁶  monocytes  (mean  ±SE).  * P<0.05  versus  T=0.
Figure 3: Diminished IL-12 and IL-18 induced IFN-γ production after in vivo LPS exposure.
Whole blood, obtained before and 4 h after LPS injection, was stimulated in vitro for 24 h at 37°C with IL-12 (100 ng/mL), IL-18 (100 ng/ml) or their combination. Data (mean ± SE) are expressed as pg per 10^6 CD3^+/CD4^+ cells (A) or as pg per 10^6 CD3^+/CD8^+ cells (B). * P<0.05 versus T=0.

Four h after LPS injection, the capacity of both CD3^+/CD4^+ and CD3^+/CD8^+ cells to release IFN-γ upon stimulation with IL-12 with or without IL-18 was strongly reduced (P < 0.05 versus t=0). Together with our previous study [7], these data suggest that experimentally induced immunoparalysis is associated with a downregulation of the IFN-γ production capacity by lymphocytes which is independent of the stimulus used, and that a reduced capacity of monocytes to release IL-12 and IL-18, as well as an impaired ability of lymphocytes to respond to these cytokines may both contribute to this phenomenon.

Reduced IFN-γ responsiveness in LPS tolerant humans
We next wished to investigate whether monocytes from humans in a LPS tolerant state demonstrated an altered responsiveness toward exogenous IFN-γ. For this purpose we first evaluated the capacity of IFN-γ to prime for LPS-induced TNF-α production. In the experiments in which the effect of IFN-γ on LPS stimulated TNF-α release was determined, LPS was added simultaneously with IFN-γ (Figure 4A) or 4 h after IFN-γ (Figure 4B). In blood obtained before LPS injection, IFN-γ enhanced LPS induced TNF-α secretion irrespective of when it was added to the whole blood cultures (P< 0.05 versus LPS alone). Four h after LPS injection, the capacity of monocytes to release TNF-α upon restimulation with LPS was profoundly diminished, confirming earlier reports [14, 15]. More importantly, at that time point of LPS tolerance, neither concurrent nor preincubation with IFN-γ was able to increase LPS-induced TNF-α concentrations (both P< 0.05 versus t=0).
To obtain further evidence that monocytes are relatively unresponsive to IFN-γ after in vivo exposure to LPS, we next evaluated the capacity of IFN-γ to upregulate the expression of MHC-II on circulating monocytes (Figure 5). First we assessed that LPS injection into healthy humans was associated with a downmodulation of MHC-II expression on peripheral blood monocytes in vivo (P < 0.05 for the difference between t=0 and 4 h; Figure 5A). Before LPS injection, stimulation with IFN-γ strongly increased monocyte expression of MHC-II (P < 0.05 versus RPMI control; Figure 5B). Four hours after LPS injection, the capacity of IFN-γ to upregulate MHC-II expression was strongly diminished (P < 0.05 versus t=0), although monocyte MHC-II expression was restored to that measured on monocytes obtained before LPS injection and incubated without stimuli.

Figure 4: Absence of the priming effect of IFN-γ on LPS-induced TNF-α release after in vivo LPS exposure. Whole blood, obtained before and 4 h after LPS injection, was stimulated in vitro for 24 (A) or 20 h (B) at 37°C with LPS (10 ng/ml) in the presence or absence of IFN-γ (10 ng/ml). IFN-γ was added to blood simultaneously with LPS (A) or 4 h before adding LPS (B). Data are mean ± SE. ** P<0.05 vs. LPS, T=0. *P<0.05 versus T=0.

Figure 5: Effect of LPS exposure on monocyte MHC-II expression. (A) MHC-II expression on circulating monocytes before and 4 h after LPS injection. (B) Whole blood, obtained before and 4 h after LPS injection, was incubated in vitro with IFN-γ (100 ng/ml) or without IFN-γ for 8 h at 37°C. Data (mean ± SE) are expressed as mean cell fluorescense intensity (MFI) of MHC-II expression on CD14+ monocytes. ** P<0.05 versus no IFN-γ. ** P<0.05 vs. IFN-γ , *P<0.05 versus T=0.
Discussion

Intravenous injection of low dose LPS represents a reproducible model to study the early responses to an acute bacterial challenge in humans [1]. Shortly after the initial proinflammatory phase, LPS administration results in a transient refractory state in which blood cells are relatively deficient in responding to LPS and other agonists upon restimulation. As such the human endotoxemia model is suitable to study mechanisms that contribute to the phenomenon generally referred to as immunoparalysis or LPS tolerance, frequently observed in patients with sepsis or after surgery or trauma. IFN-γ deficiency may play an essential role in the pathophysiology of immunoparalysis. Indeed, whereas monocyte deactivation is a hallmark feature of immunoparalysis, IFN-γ is known as a major activator of monocytes. We here demonstrate that immunoparalysis induced by low dose LPS injection in humans is associated with a reduced capacity of whole blood to release IFN-γ upon restimulation with LPS, confirming recent studies in LPS tolerant mice [9-11]. In addition, the responsiveness of monocytes to recombinant IFN-γ, as measured by priming for LPS-induced TNF-α production and upregulation of MHC-II expression, was diminished in LPS tolerant humans.

Our laboratory recently demonstrated that experimental immunoparalysis in healthy humans results in a diminished ability of peripheral blood lymphocytes to release IFN-γ after stimulation with the T cell agonists staphylococcal enterotoxin B or anti-CD3/CD28 [7]. The present investigation extends these findings to an impaired IFN-γ secretion induced by re-exposure of whole blood to LPS. Like in our earlier study [7], we expressed IFN-γ concentrations per 10⁶ CD3+/CD4+ or CD3+/CD8+ cells, the main producers of IFN-γ in blood. Hence, the transient lymphocytopenia observed after LPS injection cannot explain the reduced IFN-γ production. We used whole blood to investigate the LPS tolerant state in this and previous studies [7,15], in order to minimize artifacts secondary to isolation of cells and to enable investigation of the responsiveness of cells in a setting that is likely to have more physiologic relevance (i.e. in the presence of all blood cell types and plasma proteins).

The production of IFN-γ is regulated by a number of macrophage derived cytokines, of which IL-12 is the most potent IFN-γ inducer. Although IL-18 by itself does not stimulate the synthesis of IFN-γ, it synergistically enhances IL-12 induced IFN-γ secretion [16]. In the present study, the diminished IFN-γ production was accompanied by a reduced capacity to release the IFN-γ inducing cytokines IL-12 and IL-18 upon stimulation with LPS, and by an impaired ability of blood cells to produce IFN-γ upon stimulation with recombinant IL-12 and/or IL-18. These findings suggest that both a reduced production of IL-12 and IL-18, and a reduced responsiveness to IL-12 and IL-18 may contribute to the diminished IFN-γ production capacity. Our observations in humans should be viewed upon in the context of recent mouse studies. In accordance with our study, LPS tolerant mice were reported to have a profoundly reduced ability to release IFN-γ into their circulation upon repeated challenges with
LPS, which was accompanied by acquired defects in both the production of and the responsiveness to IL-12 [11]. In other studies, the production of IL-12 was only modestly attenuated in LPS tolerant mice, whereas IL-18 release was unaffected [9, 10]. Furthermore, T and NK cells isolated from mice rendered tolerant to LPS by daily injection of LPS during two days, produced similar concentrations of IFN-γ upon stimulation with IL-12 and IL-18 when compared with T and NK cells from non-tolerant control mice [10]. Likely, species differences and differences in experimental approaches may account for this discrepancies. Nonetheless, the majority of data point to a role for impaired IL-12 synthesis and IL-12 responsiveness in IFN-γ deficiency during immunoparalysis. In accordance, preincubation of human monocytes with LPS resulted in a reduced capacity to release IL-12 after incubation with LPS and IFN-γ [17]. Interestingly, a reduced IL-12 production capacity may contribute to an enhanced susceptibility to postoperative sepsis. Indeed, monocyte IL-12 secretion was significantly impaired before elective surgery in patients who developed sepsis postoperatively when compared to patients with an uneventful recovery after surgery [18].

Immunoparalysis is associated with a reduced expression of MHC-II on circulating monocytes [13, 19]. Recent studies have indicated that such decreased MHC-II expression coincides with a reduced antigen presenting capacity [20, 21]. Decreased antigen-presenting capacity together with monocyte deactivation are considered to play an important role in the immune dysfunction that accompanies critical illness [12, 22], and in the development of secondary nosocomial infections [3]. This has led to immunostimulatory approaches, rather than the more traditional anti-inflammatory strategies, to treat patients with sepsis. In one such immunostimulatory study, involving nine septic patients with evidence of monocyte deactivation, daily subcutaneous injection of recombinant IFN-γ restored the impaired TNF-α production capacity of monocytes and enhanced MHC-II expression [13]. These clinical data were in accordance with in vitro data, demonstrating a positive effect for IFN-γ on the diminished TNF-α production by LPS-desensitized monocytes [23, 24]. In the present investigation we demonstrate that intravenous injection of LPS results in a downregulation of monocyte MHC-II expression, mimicking the situation found in patients with sepsis. Incubation of whole blood drawn before LPS injection with recombinant IFN-γ profoundly upregulated monocyte MHC-II expression, and primed monocytes for LPS-induced TNF-α release. IFN-γ had markedly less effect on blood obtained 4 h after LPS administration. Indeed, at this time point IFN-γ failed to enhance LPS-induced TNF-α secretion, and only modestly increased monocyte MHC-II expression (although MHC-II levels were restored to levels detected on unstimulated monocytes before LPS injection). These data establish that at least in our model of experimental immunoparalysis in healthy humans IFN-γ responsiveness is strongly diminished.
Deficient IFN-γ production has been implicated as an important phenomenon in the pathogenesis of immunoparalysis in general and in the development of monocyte anergy in particular. We here demonstrate that experimentally induced immunoparalysis, resulting from a single intravenous injection of LPS into healthy humans, is associated with a reduced capacity of whole blood to release IFN-γ upon restimulation with LPS, and that this impaired IFN-γ production at least in part is caused by a diminished capacity of monocytes to produce IL-12 and IL-18 and by a reduced ability of T cells to respond to IL-12 and IL-18. Monocyte anergy, reflected by a reduced capacity to produce TNF-α upon restimulation with LPS and a diminished surface expression of MHC-II, could not or only partially be restored by addition of recombinant IFN-γ. The occurrence of diminished IFN-γ responsiveness may influence the design of immunostimulatory trials in patients with immunoparalysis.

References