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

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To obtain insight in the capacity of the lipopolysaccharide (LPS)–tolerant host to produce interferon (IFN)–γ and to respond to this cytokine, whole blood was obtained from healthy humans before and 4 h 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 after restimulation with LPS, together with a reduced ability to release the IFN-γ–inducing cytokines interleukin (IL)–12 and IL-18 and with reduced responsiveness toward these cytokines. In addition, IFN-γ responsiveness was strongly diminished after in vivo LPS exposure, as shown by the fact that blood obtained after LPS injection could not be primed by IFN-γ for LPS-induced tumor necrosis factor–α release and that peripheral blood monocytes could not be stimulated by IFN-γ to up-regulate major histocompatibility complex type II expression. Experimentally induced immunoparalysis is associated with strongly reduced IFN-γ production and responsiveness.

Endotoxin (lipopolysaccharide [LPS]) in the outer membrane of gram-negative bacteria is thought to contribute significantly to the pathogenesis of gram-negative sepsis. When administered intravenously (iv), 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, and elevated cortisol [1]. After 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 proinflammatory cytokines after stimulation with LPS and a decrease in major histocompatibility complex (MHC) class II expression on monocytes [2, 3, 5]. Although the diminished monocyte responsiveness during immunoparalysis has received the most attention, granulocytes and lymphocytes also are less reactive after 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 primarily 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 class I and II molecules on antigen-presenting cells (APCs), orchestration of leukocyte-endothelium interactions, and stimulation of the respiratory burst [8]. During immunoparalysis, IFN-γ production is impaired. Indeed, mice exposed to LPS in vivo have a profoundly reduced capacity to release IFN-γ after restimulation with LPS [9–11]. Similarly, our laboratory recently reported that T cells from healthy humans challenged iv with LPS secrete less IFN-γ after 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 of 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, administration of recombinant IFN-γ has been advocated as a treatment for patients with immunoparalysis.

In a pilot study in patients with sepsis and evidence of immunoparalysis, daily subcutaneous injection of IFN-γ restored the TNF-α production capacity of monocytes and enhanced MHC class 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 iv dose of LPS to induce a transient LPS tolerant state. By using this model, which has been useful for studying mechanisms contributing to immunoparalysis in humans [6, 7, 14, 15], we sought to determine the extent to which IFN-γ production is impaired in humans with experimentally induced LPS tolerance and whether blood cells obtained from LPS-tolerant humans can respond normally to IFN-γ.
Subjects, Materials, and Methods

Human endotoxemia challenge. We studied 15 healthy men (mean age, 22 years; range, 19–29 years). All measurements were done in the first 7 subjects enrolled, except for analysis of MHC class II expression on monocytes, which was done in a subsequent cohort of 8 subjects. All subjects were in good health, as documented by history, physical examination, and hematologic and biochemical screening. They received an iv bolus injection of *Escherichia coli* LPS, lot G (United States Pharmacopeial Convention), for 1 min in an antecubital vein at a final dose of 4 ng/kg of body weight. Heparinized blood for whole blood stimulation and FACScan analysis (flow cytometry) was done immediately before LPS injection and 4 h thereafter.

FACS analysis. We used FACS analysis to determine the number of CD4+/CD8+ lymphocytes and to evaluate MHC class II–specific mean channel fluorescence within the population of CD3+ lymphocytes or as MHC class II–specific mean channel fluorescence within the population of CD14+ monocytes.

Whole blood stimulation. Blood samples were collected aseptically from healthy human volunteers with a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson). Anticoagulation was done by using LPS-free heparin (Leo Pharmaceutical Products; final concentration, 10 U/mL). Whole blood, diluted 1:2 in sterile and pyrogen-free RPMI 1640 medium (Gibco BRL Life Technologies), was stimulated with different stimuli in sterile polypropylene tubes (Becton Dickinson) for 8 h (for MHC class II expression) or 24 h (for cytokine production) at 37°C with 5% CO2. For these experiments, the polypropylene tubes were prefilled with 1 mL of RPMI containing the appropriate concentrations of the stimuli, after which 1 mL of heparinized blood was added. Tubes were gently mixed and placed in the incubator. The stimuli used were LPS (from *E. coli* serotype O111: B4; 10 ng/mL; Sigma), recombinant human IL-12 (100 ng/mL; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service [CLB]), recombinant human IL-18 (100 ng/mL; CLB), and recombinant human IFN-γ (10 or 100 ng/mL; CLB). In the experiment in which the effect of IFN-γ on LPS-induced TNF-α release was assessed, we added IFN-γ to the blood cultures either concurrently or 4 h before LPS administration. After the incubation, supernatant was obtained after centrifugation and stored at −20°C until ELISAs were performed or blood was used for FACS analysis.

ELISAs. All measurements were done in duplicate by use of specific ELISAs done in accordance with manufacturers’ instructions. The ELISAs and detection limits were as follows: IFN-γ (2 pg/mL; CLB); TNF-α (1.4 pg/mL; CLB); IL-18 (10 pg/mL; Fujisaki Institute, Okayama, Japan), and IL-12 (16.5; R&D Systems).

Statistical analysis. All values are given as mean ± SE. Comparisons were made with the Wilcoxon test. *P* < .05 was considered to be a significant difference.

Results

Clinical and hematologic responses. LPS iv injections were associated with a transient influenza-like syndrome consisting of headache, nausea, myalgia, and chills starting 1–2 h after LPS administration and lasting 2–3 h. In addition, body temperatures increased, peaking 3–4 h after LPS was given (38.3 ± 0.2°C). Table 1 lists the effects of LPS on leukocyte counts and differentials at the time points that whole blood was collected for in vitro stimulation (0 and 4 h). Monocyte and lymphocyte counts strongly decreased after LPS administration. Lymphocytes were decreased in both CD3+/CD4+ and CD3+/CD8+ cells.

Reduced IFN-γ production in LPS-tolerant humans. We first
evaluated 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 restimulation 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, as described elsewhere [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 ($P < .05$, differences between time (T) = 0 and T = 4 h; figure 1).

IFN-γ production is positively controlled by IL-12 and IL-18, and both cytokines 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 blood and were corrected for monocyte counts. Release of both IL-12 and IL-18 were diminished in blood drawn 4 h after LPS injection when compared with blood obtained before LPS administration (both $P < .05$ for differences between T = 0 and T = 4 h; figure 2). Although this finding suggested that impaired IL-12 and IL-18 might contribute to the reduced capacity to produce IFN-γ after restimulation with LPS, the possibility that lymphocytes of LPS-tolerant humans are less responsive to IL-12 and/or IL-18 remained. Therefore, we stimulated whole blood obtained before and 4 h after LPS injection with recombinant IL-12, recombinant IL-18, or both and determined IFN-γ concentrations per 10^6 CD3+/CD4+ and CD3+/CD8+ cells.

Before LPS administration, IL-12 elicited a strong release of IFN-γ that was enhanced by addition of IL-18, but IL-18 alone did not consistently induce IFN-γ secretion (figure 3). At 4 h after LPS injection, the capacity of both CD3+/CD4+ and CD3+/CD8+ cells to release IFN-γ after stimulation with IL-12 with or without IL-18 was strongly reduced ($P < .05$ vs. T = 0). Together with our previous study findings [7], these data suggest that experimentally induced immunoparalysis is associated with a down-regulation of the IFN-γ production capacity by lymphocytes that is independent of the stimulus.
used and that a reduced capacity of monocytes to release IL-12 and IL-18 and an impaired ability of lymphocytes to respond to these cytokines may contribute to this phenomenon.

**Reduced IFN-γ responsiveness in LPS-tolerant humans.** We next investigated whether monocytes from humans in an 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, regardless of when it was added to the whole blood cultures (P < .05 vs. LPS alone). At 4 h after LPS injection, the capacity of monocytes to release TNF-α after restimulation with LPS was profoundly diminished, confirming earlier reports [14, 15]. Of more importance, 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 < .05, vs. 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 up-regulate the expression of MHC class II on circulating monocytes (figure 5). First we assessed that LPS injection into healthy humans was associated with a down-modulation of MHC class II expression on peripheral blood monocytes in vivo (P < .05 for the difference between T = 0 and T = 4 h; figure 5A). Before LPS injection, stimulation with IFN-γ strongly increased monocyte expression of MHC class II (P < .05, vs. RPMI control; figure 5B). At 4 h after LPS injection, the capacity of IFN-γ to up-regulate MHC class II expression was strongly diminished (P < .05, vs. T = 0), although monocyte MHC class II expression was restored to that measured on monocytes obtained before LPS injection and incubated without stimuli.

**Discussion**

Injection of low-dose LPS represents a reproducible model in which 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 after restimulation. As such, the human endotoxemia model is suitable for study of mechanisms that contribute to the phenomenon generally referred to as immunoparalysis or LPS tolerance, which is 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 of immunoparalysis, IFN-γ is a major activator of monocytes. Here we demonstrate that immunoparalysis in humans is induced by low-dose LPS injection and is associated with a reduced capacity of whole blood to release IFN-γ after 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 up-regulation of MHC class II expression, was diminished in LPS-tolerant humans.

Our laboratory recently demonstrated that experimental immunoparalysis in healthy humans results in diminished ability of peripheral blood lymphocytes to release IFN-γ after stimulation with the T cell agonists staphylococcal enterotoxin B

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**Table 1.** Effect of lipopolysaccharide (LPS) administration in vivo on monocyte and lymphocyte counts by time (T) after administration.

<table>
<thead>
<tr>
<th>Cell type</th>
<th>T = 0 h</th>
<th>T = 4 h</th>
</tr>
</thead>
<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>
</tbody>
</table>

NOTE. Data are ×10⁶ cells/L (mean ± SE) of 7 healthy subjects. LPS (4 ng/kg) was given as an intravenous bolus injection at T = 0 h. Analysis was performed by flow cytometry and FACScan analysis.

* P < .05, vs. T = 0.
or anti–CD3/CD28 [7]. The present investigation extends these findings to an impaired IFN-γ secretion induced by reexposure of whole blood to LPS. Similar to our earlier study [7], we expressed IFN-γ concentrations per 10^6 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] 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).

IFN-γ production 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 after stimulation with LPS and by an impaired ability of blood cells to produce IFN-γ after stimulation with recombinant IL-12 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 in the context of recent mouse studies. In accordance with our study results, LPS-tolerant mice had a profoundly reduced ability to release IFN-γ into their circulation after repeated challenges with LPS. This 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 over 2 days produced similar concentrations of IFN-γ after stimulation with IL-12 and IL-18, compared with T and NK cells from nontolerant control mice [10]. Species differences and differences in experimental approaches probably account for the 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 another study, preincubation of human monocytes with LPS resulted in a reduced capacity to release IL-12 after incubation with LPS and IFN-γ [17]. 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, compared with patients with an uneventful recovery after surgery [18].

Immunoparalysis is associated with a reduced expression of MHC class II on circulating monocytes [13, 19]. Recent studies indicate that such decreased MHC class II expression coincides with a reduced antigen-presenting capacity [20, 21]. Decreased antigen-presenting capacity, together with monocyte deactivation, are thought 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 to the more traditional antiinflammatory strategies, to treat patients with sepsis. In one immunostimulatory study involving 9 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 class II expression [13]. These clinical data are 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 demonstrated that iv injection of LPS results in a down-regulation of monocyte MHC class II expression, mimicking the situation found in patients with sepsis. Incubation of whole blood drawn before LPS injection with recombinant IFN-γ profoundly up-regulated monocyte MHC class 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 class II expression (although MHC class 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. Here, we demonstrate that experimentally induced immunoparalysis, resulting from a single iv injection of LPS into healthy humans, is associated with a reduced capacity of whole blood to release IFN-γ after 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 after restimulation with LPS and a diminished surface expression of MHC class II, could not or could only partially be restored by addition of recombinant IFN-γ. The diminished IFN-γ responsiveness may influence the design of immunostimulatory trials in patients with immunoparalysis.

References