IL-12, IL-18 and IFN-gamma in the immune response to bacterial infection
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Citation for published version (APA):
Lauw, F. N. (2000). IL-12, IL-18 and IFN-gamma in the immune response to bacterial infection
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Reduction of Th1, but not Th2, cytokine production by lymphocytes after in vivo exposure of healthy subjects to endotoxin

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Endotoxin (LPS) tolerance is characterized by a reduced capacity of monocytes to produce proinflammatory cytokines upon restimulation in vitro. To determine whether LPS exposure induces a change in lymphocyte cytokine production and whether this results in a shift in the Th1/Th2 balance, whole blood obtained from 7 healthy subjects before and after an i.v. injection of LPS (4 ng/kg), was stimulated in vitro with the T cell stimuli anti-CD3/CD28 or Staphylococcal Enterotoxin B. Whole blood production of Th1 cytokines IFN-γ and IL-2 was markedly reduced at 3 and 6 h, while the production of Th2 cytokines IL-4 and IL-5 was not influenced or slightly increased. The IFN-γ/IL-4 ratio was strongly decreased at 6 h. Serum obtained after LPS exposure could slightly inhibit the release of IFN-γ, while increasing IL-4 production during stimulation of blood drawn from subjects not previously exposed to LPS. Normal serum also inhibited IFN-γ production, albeit to a lesser extent. LPS exposure influences lymphocyte cytokine production, resulting in a shift towards a Th2 type cytokine response, an effect that in part may be mediated by soluble factors present in serum after LPS administration in vivo.
Endotoxin (lipopolysaccharide, LPS), a component of the outer cell membrane of gram-negative bacteria, is considered to be a central mediator in the pathogenesis of gram-negative sepsis. Intravenous injection of LPS to healthy humans not only initiates a cascade of inflammatory pathways, but also induces a temporary refractory state, generally referred to as LPS tolerance (10, 28). LPS tolerance is characterized by decreased production of tumor necrosis factor α (TNF), interleukin-1β (IL-1β), IL-6 and IL-10, with concurrently increased production of IL-1 receptor antagonist (IL-1ra), upon ex vivo restimulation of whole blood or peripheral blood mononuclear cells (PBMC's) with LPS. The same alterations in the capacity to produce cytokines have been found in whole blood or monocytes isolated from sepsis patients (8, 13, 21, 29) or from patients after surgery (14). Therefore, LPS tolerance can be considered an adaptive host immune response rather than a generalized hyporesponsiveness, which is not specific for prior exposure to LPS.

Most research on LPS tolerance focused on the reduced reactivity of monocytes. Only recently it has become apparent that other cell types may also display a reduced responsiveness during in vitro stimulation. Indeed, neutrophilic granulocytes isolated from patients with sepsis, produced less IL-1β and IL-8 upon ex vivo stimulation with LPS (15, 18). The effects of endotoxemia on the production of cytokines by T lymphocytes are unknown. It has been reported that major surgery results in a severe defect in T cell proliferation and cytokine secretion (6, 12). In addition, surgical stress may induce a shift in the T helper 1 (Th1)/Th2 balance towards a Th2 type immune response (6, 24). A recent study in mice indicated that in vivo administration of LPS may result in a reduced ability of splenocytes to produce the T cell cytokines IL-2, IL-4 and interferon-γ (IFN-γ) upon ex vivo stimulation with concanavalin A (ConA), as reflected by a diminished capacity to accumulate mRNA encoding these cytokines (5). Therefore, in the present study we sought to determine whether LPS exposure in healthy subjects induces a change in cytokine production by lymphocytes, and whether this results in a shift in the Th1/Th2 balance, as indicated by the production of Th1 and Th2 type cytokines during in vitro whole blood stimulation with T cell stimuli.
Materials and methods

Study design
Seven healthy male volunteers (mean age 21, range 19-25 years) were admitted to the Clinical Research Unit of the Academic Medical Center. Written informed consent was obtained from all study subjects. The study was approved by the research and ethical committees of the Academic Medical Center. Medical history, physical and routine laboratory examination, chest X-ray and electrocardiogram of all volunteers were normal. Each volunteer received an intravenous bolus injection of *Escherichia coli* LPS, lot G (United States Pharmacopeial Convention, Rockville, MD) administered over one minute in an antecubial vein at a dose of 4 ng/kg. Heparinized blood for in vitro stimulation and FACScan analysis was obtained directly before LPS injection (t= 0 h) and at 3, 6 and 24 h thereafter. In addition, blood was collected in vacutainer tubes and after clotting, serum was collected after centrifugation (10 min. at 1600 x g at 4°C) and stored at -20°C.

Whole blood stimulation
Heparinized blood, diluted 1:1 in pyrogen-free RPMI 1640 (Bio Whittaker, Verviers, Belgium), was stimulated for 24 h at 37°C in the presence or absence of the T cell stimuli anti-(α)CD3/αCD28 (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam, the Netherlands (CLB); final concentration 1:1000 both) or the superantigen Staphylococcal Enterotoxin B (SEB) (Sigma, St. Louis, MO; 1 μg/ml). After the incubation, supernatant was collected after centrifugation and stored at -20°C until assays were performed.

In a separate series of experiments, serum obtained from the 7 volunteers before (normal serum) and 3 h after in vivo exposure to LPS (post-LPS serum) was pooled and subsequently incubated for 24 h at 37°C with whole blood obtained from 6 other healthy donors (who were not exposed to LPS) in the presence or absence of αCD3/ αCD28. Whole blood was collected aseptically using a sterile collecting system consisting of a butterfly needle connected to a syringe (Becton Dickinson & Co, Rutherford, NJ). Anticoagulation was obtained using endotoxin-free heparin (Leo Pharmaceutical Products B.V., Weesp, the Netherlands; final concentration 10 U/ml). In these experiments, whole blood was diluted 1:1 in pyrogen free RPMI containing different dilutions of pooled normal or post-LPS serum (final concentrations 1-20 %). After the incubation, plasma was prepared by centrifugation and stored at -20°C until assays were performed.

FACScan analysis
PBMC’s were isolated by Ficoll-Hypaque density gradient centrifugation (Ficoll Paque, Pharmacia Biotech, Uppsala, Sweden) at room temperature for 20 minutes at 1000 x g.
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PBMC’s were collected in the inter-phase, washed twice with PBS and resuspended in FACS buffer (PBS supplemented with 0.01% (w/v) Na$_3$NO$_2$, 0.5% (w/v) BSA and 0.3 mM EDTA). For staining, 0.5 x 10$^6$ cells/ tube were incubated with the following mouse monoclonal Abs: Cy-Chrome5-labeled anti-CD3 (Immunotech, Marseille, France) and phycoerythrin-labeled anti-CD4 (Immunotech) or isotype controls (Immunotech). Lymphocytes were gated by forward and site scatter and 5,000 cells were counted.

Assays
IFN-γ and IL-4 (both CLB; detection limit 4 and 1.2 pg/ml respectively), IL-2 (R&D Systems, Abingdon, United Kingdom) and IL-5 (Medgenix, Fleurus, Belgium; 8.2 pg/ml) were measured by ELISA’s according to the instructions of the manufacturer. Leukocyte counts and differentials were determined in K$_3$-EDTA-anticoagulated blood using flow cytometry.

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 response to LPS
Intravenous injection of LPS was associated with transient influenza-like symptoms, including headache, nausea, myalgia and chills, starting 1-2 h after LPS administration, and lasting no longer than 3-4 h. In addition, a rise in body temperature was recorded, peaking at 3-4 h after LPS (38.8 ± 0.2°C; P < 0.05).

Table 1. Effect of LPS administration in vivo on cell counts and differentials

<table>
<thead>
<tr>
<th>Time after LPS</th>
<th>Leukocytes</th>
<th>Lymphocytes</th>
<th>% of CD3+/CD4+</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>5.00 ± 0.50</td>
<td>1.67 ± 0.12</td>
<td>0.79 ± 0.09</td>
</tr>
<tr>
<td>3</td>
<td>3.99 ± 0.57*</td>
<td>0.36 ± 0.03*</td>
<td>0.14 ± 0.03*</td>
</tr>
<tr>
<td>6</td>
<td>10.77 ± 0.91*</td>
<td>0.28 ± 0.02*</td>
<td>0.07 ± 0.02*</td>
</tr>
<tr>
<td>24</td>
<td>11.73 ± 0.46*</td>
<td>1.62 ± 0.11</td>
<td>0.77 ± 0.07</td>
</tr>
</tbody>
</table>

Values are mean ± SE of 7 healthy subjects. LPS (4 ng/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 or percentage positive cells within the lymphocyte population. * indicates P < 0.05 versus baseline by Wilcoxon test.
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Effects of LPS on lymphocyte counts

Effects of LPS on leukocyte counts and differentials at time points at which whole blood was collected for in vitro stimulation are listed in Table 1. After an initial decline, leukocyte counts strongly increased after LPS administration, and remained high until 24 h. Lymphocyte counts strongly decreased after LPS administration with lowest cell numbers after 6 h, returning to baseline after 24 h. This decrease in lymphocytes was associated with a decrease in the number of CD3+/CD4+ cells with lowest cell counts at 6 h.

![Graphs showing cytokine production](image)

**Figure 1.** Effect of LPS exposure on Th1 (IFN-γ and IL-2) and Th2 (IL-4 and IL-5) cytokine production. Whole blood, obtained from 7 healthy subjects before and at different time points after LPS injection (4 ng/kg), was stimulated in vitro for 24 h at 37°C with αCD3/αCD28 (1:1000) or SEB (1 μg/ml). Data are mean ± SE, and are expressed as cytokine production per 10^6 CD3+/CD4+ lymphocytes. * indicates P < 0.05 versus baseline by Wilcoxon test.

In vitro cytokine production by whole blood after in vivo LPS injection

Since the number of peripheral blood Th cells changed after LPS injection, cytokine production was corrected for the number of CD3+/CD4+ lymphocytes present at the selected time points, and expressed per 10^6 CD3+/CD4+ cells. IFN-γ and IL-2 were measured as Th1 cytokines, while IL-4 and IL-5 were measured as Th2 cytokines. Incubation of whole blood without stimulus did not result in detectable cytokine levels. After stimulation with αCD3/αCD28 or SEB, high levels of IFN-γ and IL-2, and low levels of IL-4 and IL-5 were found (Fig. 1). The capacity of whole blood to produce IFN-γ after stimulation with αCD3/αCD28 or SEB was markedly reduced at 3 h and 6 h after in vivo
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exposure to LPS. Also, SEB-induced IL-2 production was strongly decreased. In contrast, αCD3/αCD28-stimulated IL-4 production was slightly increased although this difference was not significant. The capacity to produce IL-5 after stimulation with αCD3/αCD28 and SEB increased after LPS injection, peaking at 6 h.

To determine whether LPS exposure induced a shift in the Th1/Th2 balance, we calculated the ratio between IFN-γ, the prototypic Th1 cytokine, and IL-4, the prototypic Th2 cytokine, production at the different timepoints. The IFN-γ/IL-4 ratio strongly decreased after restimulation in vitro with either αCD3/αCD28 or SEB, with lowest ratio at 6h after LPS injection (Fig. 2).

**Figure 2.** Effect of LPS exposure on the IFN-γ/IL-4 ratio during in vitro stimulation. Ratio between IFN-γ, as the prototypic Th1, and IL-4, as the prototypic Th2 cytokine, was calculated after in vitro whole blood stimulation with αCD3/αCD28 or SEB after LPS exposure in vivo. Data are mean ± SE. * indicates P < 0.05 versus baseline by Wilcoxon test.

Effect of serum obtained after in vivo exposure to LPS on cytokine production by normal whole blood

To study whether soluble factors present in serum play a role in the effects on Th1 and Th2 cytokine production, serum was collected before and after LPS exposure and added during stimulation with αCD3/αCD28 of whole blood, drawn from subjects not previously exposed to LPS in vivo. Normal serum inhibited the production of IFN-γ induced by αCD3/αCD28 in a dose-dependent manner (P < 0.05), while IL-4 production was not changed (Fig. 3). Compared with levels obtained after stimulation with similar concentrations of normal serum, serum obtained 3 h after in vivo exposure to LPS slightly reduced αCD3/αCD28-induced IFN-γ production when added at a concentration of 1% (P < 0.05). In contrast, post-LPS serum at 1% and 10% increased the production αCD3/αCD28-induced IL-4 production compared to normal serum (both P < 0.05). The effect of post-LPS serum on IFN-γ and IL-4 production did not differ from normal serum when added at 20%. The IFN-γ/IL-4 ratio after whole blood stimulation with αCD3/αCD28 was 370.5 ± 95.9 when no serum was added. Addition of normal serum caused a dose-dependent decrease in the IFN-γ/IL-4 ratio (P < 0.05) (Fig. 3). Serum obtained at 3 h after LPS injection caused a further decrease in the IFN-γ/IL-4 ratio compared with normal serum (P < 0.05 when added at 1%). IL-2 production was not changed by the addition of either normal or post-LPS serum, while IL-5 production was influenced by normal and post-LPS serum in a similar manner as IL-4 production (data not shown).
LPS tolerance is characterized by a reduced responsiveness of monocytes isolated from healthy subjects after LPS exposure or from sepsis patients, upon restimulation in vitro. In the present study we sought to determine whether LPS exposure in vivo induces a change in the capacity of lymphocytes to produce cytokines, and whether this results in a shift in the Th1/Th2 balance. We found that after LPS injection into healthy subjects, stimulation of whole blood in vitro with specific T cell stimuli resulted in a markedly decreased production of the Th1 cytokines IFN-γ and, partly, IL-2, while the production of the Th2 cytokines IL-4 and IL-5 was not influenced or slightly increased. Consequently, the Th1/Th2 balance was shifted towards a Th2 type cytokine response. Addition of serum obtained after LPS exposure to normal blood could in part mimic the LPS tolerant state found after direct αCD3/αCD28 stimulation of post-LPS blood.

CD4+ T helper cells can be divided into Th1 and Th2 cells, that can be distinguished by the pattern of cytokine production upon ex vivo stimulation (20). Th1 cells produce cytokines like IFN-γ, IL-2 and TNF, while Th2 cells secrete IL-4, IL-5 and IL-10. In the present study, we chose to measure IFN-γ, IL-2, IL-4 and IL-5 since, unlike TNF and IL-10, they are produced predominantly or exclusively by lymphocytes (20). LPS

Discussion

Figure 3. The effect of serum obtained after LPS exposure on IFN-γ and IL-4 production (A) and IFN-γ/IL-4 ratio (B) compared to normal serum. Serum obtained from 7 healthy subjects directly prior to (normal serum) and 3 h after (post-LPS serum) an i.v. injection of LPS (4 ng/kg) was pooled and added to whole blood obtained from subjects (n=6) not previously exposed to LPS, which was then stimulated with αCD3/αCD28 for 24 h at 37°C. Data (mean ± SE) are expressed as percentage change relative to incubation of whole blood with αCD3/αCD28 only (i.e. without serum added). IFN-γ concentrations after whole blood stimulation with αCD3/αCD28 were 17.2 ± 4.3 ng/ml, IL-4 levels were 49.4 ± 10.0 pg/ml. * P < 0.05 versus no serum added. * indicates P < 0.05 versus normal serum.
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administration induced a strong decrease in the number of CD3+/CD4+ lymphocytes. Therefore, the cytokine production by whole blood was expressed as nano- or picograms per 10^6 CD3+/CD4+ lymphocytes present in blood at the selected timepoints. Hence, the decrease in cell numbers can not explain the observed changes in cytokine production. We used whole blood stimulation rather than stimulation of isolated cells, since the former system is considered to mimic in vivo conditions best, with hormones, cytokines and other soluble factors able to influence cytokine production present (25).

Results of our study are consistent with data reported on lymphocyte function after major surgery. Surgical stress results in a decreased T cell effector function, associated with a reduced capacity to produce Th1 cytokines, and a severe defect in T cell proliferation (6, 12). In addition, major trauma has been reported to result in a shift towards a Th2 cytokine response (24). Until recently it was thought that LPS does not have an effect on T lymphocyte function. However, a study in mice demonstrated that LPS administration results in activation of CD4+ T cells, as measured by the expression of T cell activation markers (5). Also, LPS injection was associated with a diminished capacity of splenocytes to accumulate mRNA encoding IL-2, IL-4 and IFN-γ upon in vitro stimulation with ConA.

In the present study we found that LPS exposure in vivo results in a decreased capacity of lymphocytes to produce Th1 cytokines, with a shift towards a Th2 type cytokine response. The Th1/Th2 balance plays a critical role in the outcome of several infectious and autoimmune diseases (22). A Th1 mediated response is known to enhance cell-mediated immunity, while a Th2 type response in associated with humoral immunity (1). Our data suggest that during systemic infection, a shift towards a Th2 type cytokine response occurs, which may result in a defect in cell-mediated immunity. In addition, since IFN-γ is a major activator of monocyte functions (2), our data suggest that the reduced capacity of T cells to produce IFN-γ may contribute to the diminished monocyte responsiveness during endotoxemia and sepsis. Indeed, treatment of septic patients with IFN-γ has been found to restore monocyte LPS-induced TNF production ex vivo (7).

Some differences were seen between the effects of αCD3/αCD28- or SEB-stimulated cytokine production, in particular on IL-2 production. It is conceivable that differences in the mechanisms by which αCD3/αCD28 and SEB activate T cells contribute to this discrepancy. Indeed, crosslinking of CD3 and CD28 results in direct T cell activation, which is independent of the presence of antigen-presenting cells (APC’s). SEB, a product of Staphylococcus aureus, is a superantigen which requires binding to both an APC and a T cell to induce T cell stimulation. By binding to the MHC class II peptide of the APC, SEB can bind to the Vβ region of the T cell receptor, resulting in polyclonal T cell activation (16).

Previous studies have tried to elucidate the mechanisms which contribute to the development of a LPS refractory state of monocytes. After LPS injection into healthy
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subjects, the capacity of whole blood to produce monocyte-derived proinflammatory cytokines diminishes strongly (28). This effect is partly mediated by soluble mediators produced within 2 h after LPS administration, since stimulation of normal whole blood with serum obtained after LPS exposure, could in part mimic the LPS tolerant state (28). It has been reported that septic serum inhibits TNF production by whole blood stimulated with *Escherichia coli* (26). Also, plasma obtained from patients with meningococcal septic shock, strongly inhibits LPS-induced activation of normal human monocytes, with an important role of IL-10 in the decreased monocyte responsiveness (4). Given these data and considering that LPS can not influence lymphocyte function directly, we found it of interest to evaluate the role of soluble factors induced by LPS in the observed alterations in lymphocyte cytokine secretion. Addition of serum obtained after LPS exposure in vivo could qualitatively mimic the effects found on the production of lymphocyte-derived cytokines after direct stimulation of post-LPS blood with T cell stimuli. Hence, these data suggest that these changes likely occur in an indirect way, possibly via soluble factors produced after LPS exposure. Recently, it has been described that increased expression of the p50 subunit of NFκB is involved in the suppression of TNF production in LPS-tolerant monocytes/macrophages (3). Which intracellular effect mediates the change in lymphocyte cytokine production remains to be established.

Interestingly, normal serum dose-dependently inhibited αCD3/αCD28-induced IFN-γ production, while not influencing IL-4 production, thus resulting in a decrease in the IFN-γ/IL-4 ratio. These data suggest that normal serum contains soluble factors which direct the immune response towards a Th2 type cytokine response, and that LPS exposure increases the concentration and/ or activity of these or other factors in serum. However, since the effects of 20% normal and post-LPS serum on lymphocyte cytokine release were similar, the LPS effect apparently is overruled when serum is added in higher amounts, i.e. when the physiologically present “Th1 inhibitory factors” are added in relatively high concentrations. At present it remains speculative which serum factors may be involved in inhibition of a Th1 cytokine response, although the IL-12p40 homodimer and the recently identified IL-18 binding protein seem to be conceivable candidates (9, 17, 23). IL-12p40 homodimers function as an IL-12 receptor antagonist, thereby inhibiting the Th1 driving cytokine IL-12, and IL-12p40 homodimer levels increase after an LPS challenge (11). IL-18 binding protein can be considered a soluble IL-18 decoy receptor which reduces the biological availability of IL-18, an important cofactor for IFN-γ production and IL-12-driven Th1 development (19, 23, 27).

LPS injection into normal humans alters the profile of cytokines released by activated T cells, which is associated with a shift towards a Th2 type cytokine response. Serum obtained after LPS exposure could qualitatively reproduce these changes during stimulation of normal blood, suggesting that soluble factors in serum contribute to this effect. Further
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studies are required to identify which soluble factors and which intracellular pathways are involved in LPS-induced changes in lymphocyte cytokine production.

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