Mechanism of Endotoxin Desensitization: Involvement of Interleukin 10 and Transforming Growth Factor β

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Summary

Tolerance of monocytes/macrophages to endotoxin (lipopolysaccharide [LPS]) can be induced both in vivo and in vitro by LPS itself. Exposure to LPS, even at a very low dose, induces a downregulation of cytokine response to a second high dose LPS challenge. To learn more about the unknown mechanisms of this phenomenon, we studied the role of antiinflammatory cytokines in this process. Preculture of human peripheral blood monocytes for 24 hours with low concentrations of LPS induced hyporesponsiveness to high-dose LPS rechallenge with respect to tumor necrosis factor (TNF) α and interleukin (IL) 10 but not IL-1RA production. These results suggest that LPS tolerance reflects a functional switch of monocytes rather than a general LPS hyporesponsiveness. IL-10 and transforming growth factor (TGF) β1 showed additive effects in replacing LPS for induction of LPS hyporesponsiveness in vitro. Additionally, neutralizing anti–IL-10 and anti–TGF-β monoclonal antibodies prevented induction of LPS tolerance. In vitro induced LPS tolerance looks like the ex vivo LPS hyporesponsiveness of monocytes from septic patients with fatal outcome: downregulation of LPS-induced TNF-α and IL-10 production but not of IL-1RA secretion. LPS hyporesponsiveness in septic patients was preceded by expression of IL-10 at both the mRNA and protein level. In summary, our data suggests that IL-10 and TGF-β mediate the phenomenon of LPS tolerance in vitro and perhaps in vivo (septic patients), too.

Monocytes and macrophages are highly sensitive for LPS, reacting on encounter with even traces by synthesis of inflammatory mediators. Among them, a network of proinflammatory cytokines is of special importance for the coordinated activation of the immune system in response to microbial invasion. When local infection develops into sepsis, a strong systemic reaction occurs, and cytokines normally restricted to areas of local injury can be detected systemically causing symptoms of septic shock (1). Although whole-body inflammation is frequently initiated by LPS, a single sublethal injection of LPS also renders the host temporarily refractory to subsequent LPS challenge as well as to other inflammatory stimuli. This phenomenon, referred to as endotoxin tolerance may be at least partially due to a reduced capacity of monocytes/macrophages to synthesize cytokines upon reexposure to LPS (2). The phenomenon of in vivo LPS desensitization has been reproduced in vitro using murine or human monocytes/macrophages (3–5). The mechanisms of LPS tolerance are not clear so far. Modulation of LPS-binding sites (CD14), secretion of inhibitory mediators by desensitized monocytes, and an altered intracellular signaling have been discussed (3, 6–8).

Antiinflammatory mediators such as IL-10, TGF-β, or IL-1RA which are produced also by monocytes after LPS con-tact but with delayed kinetics, are thought to be important for downregulating the inflammatory cascade, and they are known to be protective in animal models of sepsis. We wondered whether (a) the production of antiinflammatory mediators is also downregulated in LPS-tolerant monocytes; and (b) the secretion of antiinflammatory mediators, particularly IL-10 and TGF-β, may be involved in the induction of LPS hyporesponsiveness.

We found IL-10 to be also downregulated in the process of LPS desensitization, while production of IL-1RA was not significantly modified. Furthermore, our results indicate that IL-10 and TGF-β are essential factors for the induction of LPS desensitization and that their action is sufficient to establish LPS hyporesponsiveness. Our in vitro model shows parallels to the properties of monocytes from patients suffering from gram-negative sepsis with high late lethality.

Materials and Methods

Cell Culture Techniques. Human PBMC were isolated by Ficoll–Paque density gradient centrifugation and were cultured at a density of 10^6 cells/ml in RPMI-1640 supplemented with 10% (vol/vol) heat-inactivated FCS (both with "low endotoxin certificate," Biochrom KG, Berlin, Germany), 2 mM glutamine, 100

U/ml penicillin, and 1 μg/ml streptomycin. Complete medium was essentially free of contaminating endotoxin as indicated by the Limulus assay (Coatest™; Chromogenix AB, Mölndal, Sweden) and the absence of spontaneous TNF-α production (<15 pg/ml per 10^6 cells) by cultured human PBMC. LPS pretreatment in primary culture was performed for 24 h in the presence of 200 pg/ml LPS from Escherichia coli 055:B5 (Sigma Chemical Co., St. Louis, MO). After extensive washing, cells were restimulated in secondary culture with 10 ng/ml LPS for a further 24 h before supernatants were harvested. Neutralizing anti-TGF-β1-3 mAb (Genzyme, Munich, Germany) and anti-IL-10 mAb 19F1 (IgG1) (kindly provided by Dr. J. E. de Vries, DNAX Research Institute, Palo Alto, CA) were added to some cultures as indicated. To simulate the induction of LPS desensitization, cells were treated with recombinant human TGF-β1 (Biermann, Bad Nauheim, Germany) and IL-10 (kindly provided by Dr. de Vries) for 24 h at the indicated concentrations, washed extensively, and stimulated with 10 ng/ml LPS for a further 24 h. For quantification of TNF-α mRNA, secondary culture was done in the presence or absence of 10 ng/ml LPSP for 5 h.

Patients' PBMC were cultured in the presence or absence of 100 ng/ml LPS for 24 h without preculture. In summary, 36 patients suffering from gram-negative sepsis (24 with E. coli and 12 with Pseudomonas species infection) at different stages (early and late phases) were investigated. Sepsis developed on the basis of peritonitis ($n = 19$), pancreatitis ($n = 8$), polytrauma ($n = 8$), and burn ($n = 1$). On the basis of our recent studies (9–11), the patients were divided into three subgroups according to their proportion of HLA-DR+ monocytes in peripheral blood: <30% (fatal outcome: 10 out of 12 patients), 30–45% (fatal outcome: 6 out of 12 patients), >45% (fatal outcome: 3 out of 12 patients). The proportion of HLA-DR+ CD14+ monocytes was determined by two-color flow cytometry as described elsewhere (9, 10).

Cytokine Assays. Cytokine production was measured by ELISAs specific for TNF-α (Medgenix, Ratingen, Germany), IL-10 (Cytoscreen™; Laboserv, Giessen, Germany), and IL-1RA (Quantikine™; Biermann).

Quantification of mRNA Level. Quantitative PCR analyses were performed as described in detail elsewhere (12). Briefly, total RNA was extracted from PBMC. Quantification of TNF-α and IL-10 cDNA derived from this RNA was carried out using multispecific control fragments as an internal standard for competitive PCR (12). First, the various cDNA samples to be compared were equilibrated according to their glyceraldehyde-3-phosphate-dehydrogenase cDNA content. Then, the relative concentration of TNF-α or IL-10 cDNA in each sample was estimated from the concentration of control fragment DNA that achieved equilibrium between its own amplification and that of the target cDNA. The concentrations were expressed in arbitrary units (AU). One AU was defined as the lowest concentration of control fragment that yielded a detectable amplification product given the TNF-α or IL-10 primer pair and PCR conditions used (1 AU = 10^{-9}-fold dilution of control fragment, corresponding to ∼30 molecules) (12).

Results

Regulation of Cytokine Production in LPS Desensitization. Stimulation of PBMC with LPS led to a dose-dependent induction of TNF-α, IL-1RA, and IL-10 during the first 24 h of culture (primary culture) (data not shown). As shown in Fig. 1, presence of LPS during primary culture diminished the capacity to produce both the proinflammatory cytokine TNF-α and the antiinflammatory mediator IL-10 during the
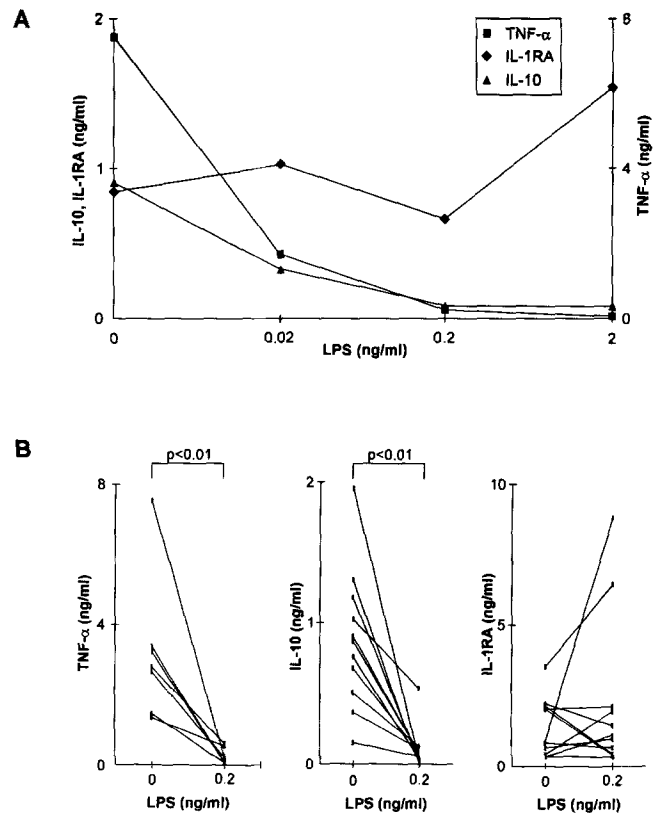

Figure 1. LPS desensitization diminishes TNF-α and IL-10 synthesis but not IL-1RA synthesis. 1 million PBMC/ml were treated with the indicated amounts of LPS for 24 h. After extensive washing, cells were restimulated with 10 ng/ml LPS for a further 24 h. Supernatants were harvested and cytokines were determined by specific ELISAs. (A) One representative dose–response curve out of four independent experiments is shown. (B) Results of independent experiments ($n = 7$ for TNF-α, $n = 11$ for IL-10 and IL-1RA) using different donors are summarized. Statistical analysis was done by Mann-Whitney-Wilcoxon test.

secondary culture with LPS (second 24 h). The degree of inhibition depended on the amount of LPS used in primary culture. Fig. 1 B shows great interindividual and interexperimental variability in the amounts of TNF-α and IL-10 synthesized by non–LPS-pretreated cells and the profound downregulation of production after pretreatment with 200 pg/ml LPS ($p < 0.01$, Mann-Whitney-Wilcoxon test). Independent of the highly variable capacity of non–LPS-pretreated cells to produce these two cytokines, all but one of the LPS-pretreated cultures were desensitized to the same degree. In contrast to the results obtained for TNF-α and IL-10, synthesis of IL-1RA in LPS-pretreated cells was not significantly influenced (Fig. 1). Essentially the same pattern of regulation was found for different lengths of secondary culture (4–48 h) and for varying amounts of LPS (1–100 ng/ml) used for restimulation (data not shown). The high variability in the amounts of IL-1RA produced in non–LPS-pretreated cells is very well conserved in LPS-pretreated cells (Fig. 1 B). Although in rare cases a marginal reduction of IL-1RA production was found, no significant difference or even a considerable priming was seen in most experiments.

Recently, it has been shown that LPS desensitization is

established at the pretranslational stage (5, 7, 13). We reproduced this result for TNF-α (data not shown) and semi-quantified mRNA levels. Untreated PBMC contained some TNF-α mRNA that was halved in LPS-pretreated cells after LPS-free secondary culture. Non–LPS-pretreated cells responded to LPS in secondary culture by increasing their amount of TNF-α mRNA about fivefold, while rechallenge of LPS-pretreated cells did not increase their TNF-α mRNA.

Prevention of LPS Desensitization by Neutralization of Endogenous TGF-β and IL-10. Requirement of active protein biosynthesis for establishment of the desensitized stage (5, 7, 13) caused us to study whether cytokines, particularly IL-10 and TGF-β, known for their cytokine synthesis–inhibiting activity and produced during primary culture, may be involved in downregulation of TNF-α production after LPS rechallenge (14, 15). Neutralization of TGF-β during primary culture led to a slightly increased TNF-α synthesis in secondary culture compared with desensitized control cells

Figure 2. Neutralization of IL-10 and TGF-β during primary culture prevents establishment of LPS desensitization, while addition of IL-10 and TGF-β1 induces LPS hyporesponsiveness. PBMC were cultured for 24 h in the presence (*A*) or absence (*B* and *C*) of 200 pg/ml LPS and neutralizing anti-TGF-β and anti–IL-10 mAbs (*A*) or TGF-β1 and IL-10 (*B* and *C*) as indicated. Controls (naive cells) were cultured without LPS. After washing, cells were restimulated with 10 ng/ml LPS for a further 24 h. Cytokines in supernatants were determined by ELISAs. Amounts of TNF-α and IL-10 produced in secondary culture by naive cells from different donors in four independent experiments ranged from 1,346 to 6,250 pg/ml and 152 to 1,956 pg/ml, respectively. The relative production of TNF-α and IL-10 (mean and SE) in comparison to that of naive PBMC is shown.

(Fig. 2 *A*). Blocking of endogenous IL-10 by a neutralizing mAb was even more effective, leading to a fourfold increased TNF-α secretion. However, neutralization of both cytokines was necessary to prevent completely the LPS-induced LPS tolerance (Fig. 2 *A*). Addition of these mAbs to LPS-free primary cultures caused no modification of TNF-α expression in secondary cultures, supporting the specificity of their effects (data not shown). Furthermore, LPS desensitization was not prevented by addition of these mAbs to secondary culture (data not shown), thus excluding the possibility of remaining traces of antibodies from primary culture being responsible for the elevated TNF-α synthesis.

Mimicry of LPS Desensitization by TGF-β1 and IL-10. Next, we wondered whether TGF-β and IL-10 are essential factors for establishment of LPS desensitization or if these cytokines could even replace LPS during primary culture. Cells treated for 24 h with IL-10 and TGF-β1, washed extensively, and stimulated with LPS showed a strongly diminished IL-10 and TNF-α synthesis similar to the level reached by LPS desensitization (Fig. 2, *B* and *C*). IL-10 pretreatment of monocytes inhibited significantly the production of both TNF-α and IL-10. While TGF-β1 by itself preferentially downregulated IL-10 expression, IL-10 more strongly inhibited TNF-α production. Regarding IL-10 production, TGF-β and IL-10 acted in an additive manner.

LPS Hyporesponsiveness in Septic Disease. Recently, we and others (10, 11, 16) described an impaired capacity of monocytic LPS-induced TNF-α, IL-1β, and IL-6 production in a subgroup of septic patients with high lethality. The functional deactivation of patients' monocytes was closely related to the downregulation of monocytic HLA-DR antigen expression (10, 11).

To test the possibility of a relationship between "classical" LPS tolerance and the phenomenon of LPS hyporesponsiveness in patients suffering from fatal sepsis, we looked for similarities between both phenomena. As shown in Fig. 3, the 24-h supernatants of monocytes from patients with low monocytic HLA-DR expression (and high lethality) contained much lower amounts of TNF-α and IL-10 compared with control subjects, while their IL-1RA content was not significantly different.

Concerning IL-10, in septic patients expressing low or intermediate levels of monocytic HLA-DR antigen (<45% HLA-DR[+] monocytes), two distinct functional states of monocytes were observed: (*a*) Many of these patients (19 out of 24) showed significant IL-10 production within 4 h after LPS stimulation in vitro (data not shown), while normally IL-10 is not detected before 10–12 h. This suggests in vivo priming for early onset of IL-10 secretion. This view is supported further by the detection of IL-10 mRNA in freshly isolated monocytes in 18 out of 24 patients with <45% HLA-DR[+] monocytes. In contrast, IL-10 mRNA was rarely (3 out of 12) or not (0 out of 12) detectable in septic patients with >45% HLA-DR[+] monocytes or healthy donors, respectively. Thus, the early secretion of IL-10 after LPS stimulation may result in downregulation of TNF-α synthesis and self-limiting IL-10 production, which was measured 24 h later. (*b*) The absence of early IL-10 production and the low levels
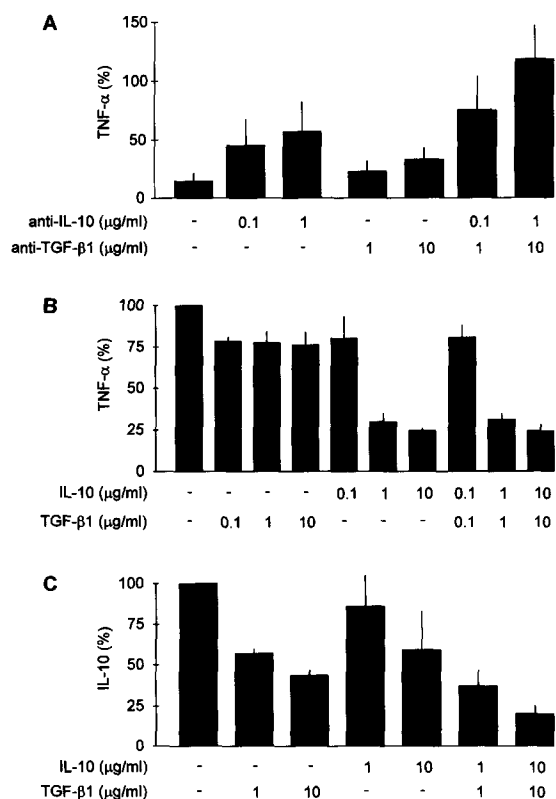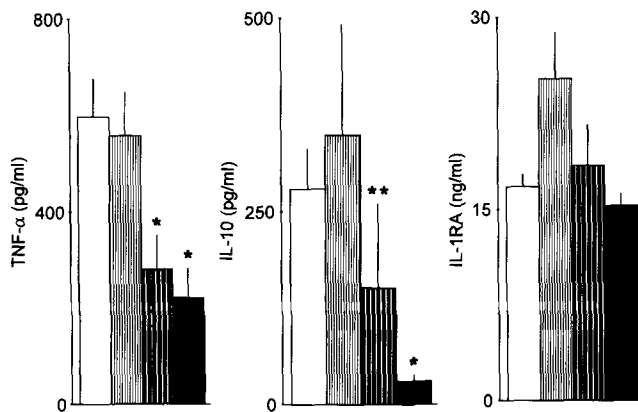
Figure 3. Downregulation of TNF-α and IL-10 but not of IL-1RA production by monocytes from patients with fatal sepsis. PBMC from 36 patients suffering from gram-negative sepsis were stimulated for 24 h with 100 ng/ml LPS. Cytokine concentrations in supernatants were determined by specific ELISAs. Septic patients were divided into three groups (12 patients in each group) according to their monocytic HLA-DR antigen expression (healthy donors: 80–95% [☐]). Low HLA-DR antigen expression (group I: HLA-DR$^+$CD14$^+$ <30% [■] or group II: HLA-DR$^+$CD14$^+$ 30–45% [▦]) was related to high Lethality (10 out of 12 = 84% and 6 out of 12 = 50%, respectively), while relatively high HLA-DR antigen expression (group III: HLA-DR$^+$CD14$^+$ >45% [▨]) was found in the patients' group with low lethality (3 out of 12 = 25%). Mean values and SE are shown. *p <0.01 vs probands; **p <0.05 vs probands.

of both IL-10 and TNF-α after 24 h in 5 out of 24 patients indicate a "completely desensitized" state. This suggests that desensitization had occurred already in vivo.

Discussion

Excessive production of TNF-α and other proinflammatory mediators after stimulation by LPS may result in fever, intravascular coagulation, and lethal septic shock. An efficient way of preventing the whole-body inflammation may be desensitization of monocytes/macrophages to LPS. We have further characterized the phenomenon of LPS tolerance and analyzed the mechanisms involved in the induction of desensitization. Our results may be summarized as follows: (a) In addition to the well-established downregulation of TNF-α production, LPS tolerance also switched off IL-10 production, while secretion of IL-1RA, another antiinflammatory cytokine, was not significantly influenced. This suggests that LPS tolerance is not a phenomenon of general hyporesponsiveness. (b) IL-10 seems to be an essential endogenous mediator that induces the state of LPS tolerance because IL-10 replaced LPS and anti–IL-10 mAb prevented the LPS-mediated induction of LPS tolerance in an additive manner with TGF-β1 and anti–TGF-β mAb, respectively. (c) Fatal sepsis is associated with a state of monocytic LPS hyporesponsiveness similar to the phenomenon of LPS tolerance, i.e., normal expression of IL-1RA and reduced synthesis of TNF-α and IL-10.

Several reports on soluble factors mediating LPS desensitization indirectly support our data on the involvement of IL-10 in this process (6, 7). Inhibition of cyclooxygenase by indo-

methacin was found to interfere with establishment of LPS desensitization, while on the other hand, PGE$_2$ (a major product of this pathway and known to negatively regulate TNF-α synthesis) on its own was unable to induce hyporesponsiveness (7). Recently, we reported that PGE$_2$ or prostacyclin considerably increased the LPS-induced IL-10 secretion, while it had no effect in the absence of LPS (17). Thus, the inhibition of LPS desensitization by indomethacin could be mediated indirectly via diminished IL-10 production.

Concerning the central position of IL-10 in the regulatory network leading to LPS desensitization, it is surprising to find IL-10 as downregulated as TNF-α. However, inhibition of IL-10 production by itself is well established (14). Obviously, IL-10 is essential for induction but not for maintenance of LPS hyporesponsiveness because neutralization of IL-10 in primary but not in secondary culture reestablished TNF-α expression. IL-10 seems to mediate its long-lasting effect on TNF-α expression via induction of another protein since its downstream signaling is inhibited by cycloheximide (15).

The mechanism of TGF-β action on LPS desensitization is not so clear. Whereas neutralization of endogenous TGF-β improved TNF-α secretion after LPS pretreatment, addition of exogenous TGF-β1 alone to naive monocytes did not induce LPS hyporesponsiveness. This observation suggests the existence of endogenously produced TGF-β that is not sufficient to induce LPS desensitization in the absence of IL-10. In fact, PBMC constitutively express TGF-β mRNA (data not shown).

The induction of LPS desensitization is a highly organized process that involves de novo protein synthesis (7). But even the desensitized monocyte/macrophage reflects not simply a deactivated cell since it is able to produce regulatory mediators such as G-CSF (5) or IL-1RA (this work).

It is well established that inflammatory mediators, particularly TNF-α, play an important role in the pathogenesis of septic disease (1). However, the course of septic disease seems to reflect a biphasic inflammatory response. The initial hyperinflammatory phase, which induces shock, etc., may switch into a hypoinflammatory state. In fact, in the subgroup of patients with strongly reduced proportions of HLA-DR$^+$ monocytes, we observed diminished TNF-α and IL-10 production after LPS stimulation, while IL-1RA secretion was not influenced. In light of our analogous results on in vitro–desensitized PBMC, it is tempting to speculate on similar mechanisms for both phenomena. The first contact with LPS should lead to induction of early inflammatory and delayed antiinflammatory cytokine production. After the antiinflammatory response, a long-lasting state of hyporesponsiveness may be established.

Induction of LPS tolerance has been suggested as an alternative therapeutic strategy for septic disease. Animal experiments (18) and recent human studies on the effects of single low dose LPS administration (19, 20) have demonstrated the efficacy of such an approach. To prevent undesired side effects, the use of modified nontoxic LPS analogues was postulated as an alternative strategy (5). Our data suggest that IL-10 may replace LPS or LPS derivatives for induction of LPS tolerance in vivo.

IL-10 has turned out to be a potent antiinflammatory

cytokine, and it has protected animals against lethal LPS administration (21). However, our data suggest that timing of IL-10 administration will strongly influence its efficacy. Administration before onset of septic shock may be successful, but it will probably fail in established sepsis, as have other antiinflammatory therapeutic strategies (e.g., anti-TNF mAb) that worked well in animal models but failed in controlled human trials (22–24). The reason for this discrepancy might be that many patients who survived the acute phase of septic shock because of modern intensive care died from multiple-organ failure and persistent infections weeks or even months

later in the intensive care unit. Late mortality in septic disease is associated with a low level of monocytic HLA-DR expression and LPS hyporesponsiveness (Fig. 3) (10, 11). Theoretically, IL-10 is not expected to have any protective effect on this situation. On the other hand, immunostimulatory strategies (such as administration of IFN-γ, GM-CSF, anti–IL-10 mAb, and inhibitors of PGE synthesis) may be useful to break monocytic hyporesponsiveness during the late (hypo-inflammatory) phase but are dangerous during the early (hyper-inflammatory) phase because they are able to magnify the overshooting inflammatory reaction.

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