Suppression of Interleukin-12 Production by Human Monocytes After Preincubation With Lipopolysaccharide

By Miriam Wittmann, Vivi-Ann Larsson, Petra Schmidt, Gabriele Begemann, Alexander Kapp, and Thomas Werfel

Interleukin-12 (IL-12) is a 75-kD heterodimeric cytokine that plays a crucial role in both the innate and the acquired immune response. The major cells producing IL-12 were found to be macrophages/monocytes, but other cell types have also been reported to produce IL-12. These include dendritic cells, neutrophils, keratinocytes, and murine mast cells.1 The two covalently disulfide-linked N-glycosylated polypeptide chains of approximately 40 kD (p40) and 35 kD (p35) are encoded by two separate genes located on different chromosomes and are independently regulated.1,3 Both subunits have to be produced within the same cell to obtain the biologically active dimer.2 In contrast to p40, p35 is only secreted as part of the p70 heterodimer. The secreted monomeric p40 has no biologic activity, whereas the p40 homodimer p(40)2 binds to the IL-12 receptor, but does not transduce a signal, thus acting as an IL-12 antagonist.6

Although initially described as a cytokine activating cytotoxic lymphocytes spontaneously, the major biological significance of IL-12 secretion lies in its effects on T-helper cells. IL-12 is known to drive Th1 reactions in physiological and pathologic immune responses, mainly mediated by its capacity to stimulate growth and interferon-γ (IFN-γ) production in T cells and natural killer (NK) cells.3,5 Some investigators even consider this cytokine to be an obligatory factor for Th1 generation and proliferation.8,9

In addition to the promotion of Th1 cell development, IL-12 has a broad range of biologic activities including the regulation and proliferation of T and NK cells, the differentiation of CD8+ T cells, and hematopoiesis. Its involvement in autoimmunity has been shown in different diseases. When applied in vivo, IL-12 was shown to enhance the resistance to bacterial and parasitic infections, to promote antitumor immunity, and to influence antiviral responses including human immunodeficiency virus (HIV) in vivo or in vitro. Modulation of IL-12–dependent signaling may provide a therapeutic option for altering the Th1-Th2 balance in allergic and autoimmune diseases, as well as in other conditions.

Bacteria, bacterial products including lipopolysaccharide (LPS), lipoteichoic acid, protein extracts, heat shock proteins, and intracellular parasites have been described to induce production of IL-12 by macrophages/monocytes.5,10,11

The most potent inhibitor of IL-12 synthesis by macrophages/monocytes appears to be IL-10, which acts at the protein, as well as at the mRNA level. IL-10 negatively regulates IL-12p40 expression/accumulation.12,13 Similar to IL-10, addition of prostaglandin E2 (PGE2) to cultures of LPS-stimulated human peripheral blood mononuclear cell (PBMC) downregulates IL-12 production.14 IL-4, IL-13, and transforming growth factor-β (TGF-β) can also suppress the production of IL-12 when added simultaneously with bacterial inducers. In contrast, pretreatment with IL-4 or IL-13 followed by activation with LPS enhances IL-12 production.15

In this study, we show that the well-known induction of IL-12 production with LPS also underlies a complex regulation, with a sequence-dependent reaction to known stimuli. Preincubation with LPS can efficiently inhibit the production of bioactive IL-12 by monocytes stimulated with a combination of IFN-γ or granulocyte-macrophage colony-stimulating factor (GM-CSF) and LPS.

From Hannover Medical University, Department of Dermatology and Allergology, Hannover, Germany.

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Address reprint requests to Miriam Wittmann, MD, Hannover Medical University, Department of Dermatology and Allergology, Ricklinger Str. 5, D-30449 Hannover, Germany.

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Cytokines and reagents. All cytokines were used as purified recombinant human preparations. Human IFN-γ was kindly provided by Dr Karl Thomae GmbH (Biberach an der Riss, Germany). LPS was derived from Escherichia coli (E coli) Serotype 055:B5 (Sigma, Deisenhofen, Germany), GM-CSF (Genzyme, Rüsselsheim, Germany) anti-IL-4 antibody useful for neutralization of human IL-4 bioactivity with a low endotoxin level (R&D Systems, Wiesbaden, Germany), neutralizing rat anti-human IL-10 monoclonal antibody (MoAb) with a low endotoxin level (PharMingen, Hamburg, Germany), neutralizing anti-human tumor necrosis factor-α (TNF-α) antibody with a low endotoxin level (R&D Systems), indomethacin (Serva, Braunschweig, Germany), N-methyl-L-arginine (NMLA) (Sigma); actinomycin D (Sigma), paclitaxel (PTXL; Taxol) from Taxus barcata, Bristol-Myers Squibb, New York, U.S.A. (Boehringer-Mannheim). To control for DNA contamination, cDNA synthesis was performed in the absence of RT. First-strand cDNA was diluted and labeled with dig-NTP (PCR-enzyme–linked immunosorbent assay [ELISA], Dig-Labelling kit; Boehringer-Mannheim) at a final concentration of 2 µg/mL (monoclonal mouse anti-human IL-12 [p40/p70]; this antibody reacts with human IL-12/p40 monomer and with the p70 heterodimer, but not with the p35 monomer; monoclonal rat anti-human-IL-6; PharMingen). After subsequent washings in permeabilization buffer, cells were resuspended and measured in PBS by flow cytometric analysis. Expression of surface antigens on monocytes was assessed using labeled CD14 (Coulter-Immunotech, Hamburg, Germany) and Stratagene (La Jolla, CA, U.S.A.) antibodies. Samples were analyzed with CellQuest software (Becton Dickinson). To facilitate diffusion of Ab through the cell membranes, cells were permeabilized in PBS with 0.1% saponin (Riedel de Haen, Seelze, Germany) for 15 minutes. Thereafter, pretitrated cytokine-specific MoAb diluted in the permeabilization buffer (PBS-saponin) were added and incubated for 45 min at 4°C. The PE-conjugated cytokine specific MoAb and IgG1 isotype control MoAb were used at final concentrations of 2 µg/mL (monoclonal mouse anti-human IL-12 [p40/p70]; this antibody reacts with human IL-12/p40 monomer and with the p70 heterodimer, but not with the p35 monomer; monoclonal rat anti-human-IL-6; PharMingen). After subsequent washings in permeabilization buffer, cells were resuspended and measured in PBS by flow cytometric analysis. Expression of surface antigens on monocytes was assessed using labeled CD14 (Coulter-Immunotech, Hamburg, Germany) and CD54 (anti-intercellular adhesion molecule [ICAM]-1; Coulter-Immunotech) antibodies. Samples were analyzed on a FACSscan flow cytometer (Becton Dickinson, Heidelberg, Germany). Results were analyzed using Cellquest software (Becton Dickinson).

Cytokine determinations. IL-12p70 ELISA was performed using an IL-12p70 detection kit (R&D Systems), which recognizes only the IL-12p70 heterodimer. The same samples were analyzed in another ELISA specific for p40 (R&D Systems) to determine the amount of non-p70 bound-p40. IL-10 production was measured by ELISA (Labserv, Giessen, Germany).

RESULTS

Dose-dependent suppression of IL-12 production by preincubation with LPS. Stimulation of monocytes with 300 U/mL IFN-γ 2 hours before addition of 10 to 50 ng/mL of LPS
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...ng/mL LPS were used, P < .005 for 10 ng/mL LPS).

Fig 1. Effects of LPS preincubation on intracellular IL-12 (p40/p70) production by human monocytes. Human monocytes were stimulated with 300 U/mL IFN-γ and 50 ng/mL LPS for 24 hours. Preincubation was performed as indicated in Materials and Methods using 50 and 10 ng/mL LPS, respectively. The percentages of monocytes positive for IL-12 (p40/p70) or IL-6, as detected by flow cytometric measurement, are shown. Results are given as mean ± standard error of mean (SEM) of 13 different donors. The downmodulation of IL-12 by preincubation with LPS was significant (P < .0001 when 50 ng/mL LPS were used, P < .005 for 10 ng/mL LPS).

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...similarity of results obtained using GM-CSF instead of IFN-γ. In unstimulated cells, IL-12/p70 was undetectable with the ELISA used. LPS alone (ie, without a priming dose of IFN-γ or GM-CSF) did not induce any detectable IL-12 response on the protein level, as has been shown by others.3

Preexposure of monocytes to LPS before the stimulation procedure (designated as “preincubation” in this report) significantly decreased the production of bioactive IL-12. As shown in Fig 1, preincubation with 50 ng/mL LPS suppressed intracellular IL-12 production to nearly undetectable levels. A clear dose dependency of this effect was evident (Figs 1 and 2), with a marked suppression of intracellular IL-12 production using LPS doses down to 1 ng/mL. A total of 0.1 ng/mL LPS still caused a reduction of more than 50%, while 0.01 ng/mL LPS did not alter IL-12 production as compared with the positive control (ie, “stimulation” without “preincubation”).

Suppression was not due to downmodulation of the CD14 antigen as determined by flow cytometric analysis of receptor expression after LPS preincubation (81.6%; standard deviation [SD], 11.4%; n = 3, of gated cells stained positive) as compared with LPS-free medium (86.0%; SD, 4.42%; n = 3).

In this experimental setting, suppression was selective for IL-12, as IL-6 downregulation was not seen under the described experimental conditions. Figures 1 and 2 show the data obtained for IL-12 (p40/p70) in comparison to IL-6. A general state of hyporesponsiveness to the priming signal can be ruled out, as the upregulation of CD54 induced by IFN-γ was even further increased in cells preincubated with LPS (Fig 3).

LPS preincubation did not exclusively interact with IFN-γ-induced signal cascade. With GM-CSF as a priming signal, similar results were obtained: LPS preincubation-induced suppression of IL-12 (p40/p70) on monocytes primed with 100 ng/mL GM-CSF instead of IFN-γ was over 95% in 2 experiments.

A dose-dependent reduction of IL-12/p70 secretion was observed in supernatants of monocytes, which had been preincubated with LPS in varying concentrations before stimulation (Fig 4).

On the mRNA level, a dose-dependent suppression could be seen for IL-12/p40 and p35 accumulation when 10 ng/mL to 100 ng/mL LPS were used (Fig 5 shows a representative experiment).

The LPS-induced IL-12 downregulation was already detectable after a “preincubation” period of only 2 hours and could still be observed when supernatants were collected 48 hours after the stimulation procedure, indicating that recovery is not fast. In some experiments, monocytes were preincubated for 24 hours with LPS, washed, and incubated for another 6 to 48 hours before stimulation with IFN-γ/LPS. In these experiments, the suppression was still the same, showing that LPS-induced IL-12 hyporesponsiveness is long-lasting (data not shown).

Both IL-12 subunits are suppressed by LPS preincubation.

Figures 4 and 6 show that p40 suppression was in parallel to p70. p40 production after stimulation without preincubation with LPS was 25-fold to 40-fold higher than p70 production (as has been described by others1,10,13).

As shown in Figs 5 and 7, a clear reduction of IL-12/p40 and p35 mRNA accumulation was observed on stimulation with IFN-γ/LPS for 5 hours after LPS preincubation. A marked suppression of p40 and p35 mRNA was seen in LPS preincubated and GM-CSF/LPS–stimulated monocytes as compared with monocytes stimulated with GM-CSF/LPS alone (data not shown). Relative quantities of both subunits were determined by PCR-ELISA. A significant reduction 5 hours after stimulation is shown in Fig 8. A significant inhibition was still observed for both subunits 24 hours after stimulation (data not shown).

Kinetic experiments showed a peak in IL-12/p40 mRNA accumulation between 5 and 8 hours after stimulation with IFN-γ/LPS (Fig 8). LPS-pretreated monocytes reacted to IFN-γ/LPS stimulation with a maximal IL-12/p40 mRNA accumulation between 5 and 8 hours as well, although on a lower absolute level (Fig 8). Using actinomycin D (5 µg/mL), which blocks transcription, we could not detect any difference in RNA stability between LPS pretreated and nonpretreated monocytes: in both experimental settings, a marked decline of mRNA specific for IL-12/p40 was observable 6 to 8 hours after addition of actinomycin D.

LPS-induced IL-12 suppression is not due to enhanced endogenous production of IL-10, IL-4, PGE2, TNF-α, or nitric oxide (NO). IL-10, IL-4, and PGE2 have been shown to downregulate IL-12 production under defined experimental conditions. We therefore tested whether endogenous production of these mediators might be responsible for the LPS-induced IL-12 downregulation using indomethacin or neutralizing antibodies for IL-4 and IL-10, respectively. The downregulatory effect of LPS remained stable in the presence of neutralizing anti–IL-10, anti–IL-4 antibodies, and the prostaglandin synthesis inhibitor, indomethacin, respectively, during preincubation with a secretion of IL-12 diminished to nearly undetectable levels. Figure 6 summarizes the amounts of IL-12 in the supernatants as determined by an ELISA specific for p70 and...
p40, respectively. Figure 9 shows the results obtained for intracellular cytokine staining.

The LPS preincubation induced effect could be seen on monocytes obtained via adherent step, as well as on highly purified monocytes using Dynabeads.

Using an ELISA specific for IL-10, it could be shown that IL-10 production increased after LPS/IFN-γ stimulation. LPS preincubation did not, however, further induce IL-10 secretion (data not shown).

NO and TNF-α have both been implicated in IL-12 suppression in the murine system. The NO-synthase inhibitor NMLA (Fig 6), as well as a neutralizing anti-human TNF-α antibody, had no effect on the reduction of IL-12 on preincubation with LPS. The mean IL-12 (p40/p70) inhibition seen with LPS preincubation in the presence of 5 µg/mL anti-human TNF-α neutralizing antibody was 98.7% (±1.3% SD) as compared with 99.4% (±0.9% SD) inhibition without the antibody, as determined in 3 independent experiments by intracellular cytokine staining.

Suppression of IL-12 production is induced by PTXL preincubation on intracellular level, but not on mRNA level. The ability of PTXL, known to stimulate monocytes via a similar intracellular transduction pathway as LPS, to downregulate IL-12 production was tested in some experiments in parallel to LPS. In contrast to LPS, we detected no downregulation of p40 and p35 mRNA neither 5 nor 24 hours after the stimulation procedure (Fig 7). On the intracellular level, however, we detected a marked downregulation of IL-12 (p40/p70) (Fig 10) using 5 to 20 µmol/L PTXL. IL-6 production remained stable (Fig 10) when monocytes were preincubated with the same doses of PTXL before IFN-γ/LPS stimulation. Cells were shown to survive the stimulation procedure in separate experiments (data not shown).

**DISCUSSION**

In the present study, we provide evidence that the important proinflammatory and immunoregulatory cytokine, IL-12, can...
be selectively downregulated by preincubation with LPS before stimulation with IFN-γ or with GM-CSF and LPS.

Endotoxic LPS, a major component of the outer membrane of gram-negative bacteria, is known to activate the immune system by inducing the release of inflammatory mediators. Monocytes/macrophages require a priming “signal” before LPS stimulation for the optimal production of proinflammatory mediators. Previously, it was described that IFN-γ efficiently primes monocytes for IL-12 production in response to LPS, which could be confirmed in this study.

LPS can induce a state of hyporesponsiveness to its own effects, a phenomenon mediated by monocytes/macrophages. An LPS-induced “tolerance” has been described for TNF, IL-6, IL-8, IL-10, NO, and other cytokines, characterized by a diminished synthesis of these mediators by monocytes. This phenomenon has been extensively studied in various animal species and cell types in vitro and in vivo, but the cellular and molecular changes that contribute to this kind of induced hyporesponsiveness are not fully understood. It has been demonstrated that the sequence, amount, and duration of stimuli given is crucial in this context. LPS-induced tolerance appears to be a complex process and may vary considerably in different experimental models. The ability of LPS to inhibit subsequent induction of IL-12p70 is a new finding. This may be of particular importance because of the crucial role of IL-12 in acute bacterial infections, in autoimmune disorders, and in the development of specific immunity against a number of intracellular pathogens. Moreover, our findings point to the necessity to deplete LPS from reagents in studies, which address the regulation of IL-12.

In further experiments, we examined whether endogenous production of known IL-12 antagonists play a causal role in LPS-mediated IL-12 suppression.

The most potent inhibitor of IL-12 synthesis by monocytes/macrophages appears to be IL-10, which acts at the protein as well as at the mRNA level. IL-10 itself can be affected by LPS-induced hyporesponsiveness.

Randow et al have identified IL-10 and TGF-β as an endogenous mediator for LPS-mediated TNF downregulation. In contrast, we did not observe an enhancement of IL-12 production by neutralization of endogenously produced IL-10 in our experimental setting.

IL-4 is another antagonist of proinflammatory monokines, which has been shown to downregulate IL-12, as well as the expression of CD14 in normal human monocytes. Similar to IL-10, the addition of neutralizing anti–IL-4 antibody to monocyte cell cultures failed to overcome the LPS-mediated suppression of IL-12 release. Moreover, LPS-induced IL-12 downregulation was also observed in highly purified monocytes.
containing only small amounts of potentially IL-4 producing lymphocytes, which suggests that T-cell–derived IL-4 is not causal for the observed effects.

Recently, it was reported that PGE₂ is a potent downregulator of IL-12p40 subunit production by LPS-stimulated monocytes in whole blood cultures. Both IL-12 and PGE₂ are secreted by monocytes, macrophages, and other antigen presenting cells (APC) in response to a variety of compounds, including bacterial products. Indomethacin did not revert the suppressive effect of LPS, which excludes a role of endogenously produced PGE₂ in our experiments. This is in accordance with the studies by Bodgan et al, who could not define a role of PGE₂ in LPS-mediated suppression of IFN-γ-induced reactive nitrogen intermediates (RNI) release.

It is well known that LPS can induce the expression of the TNF receptor on murine macrophages, as well as the secretion of TNF protein, suggesting that LPS effects are in part mediated by the autocrine action of TNF-α. IL-12p40 gene expression in murine macrophages has been shown to be also regulated by NO. It has been discussed that endogenous NO is involved in LPS-induced desensitization of mouse macrophages. In the murine system, both TNF-α and...
LPS-induced cytokine downregulation may function in at least 2 different ways in monocytes. LPS preincubation may either induce a general "hyporesponsiveness/""desensitization" affecting a broad range of proinflammatory responses or it may deviate the monocyte in a specific way. Bogdan et al.20 pointed to the difference between LPS hyporesponsiveness in which monocytes/macrophages exposed to a primary dose of LPS become refractory to a challenge dose of LPS, and the suppressive activity that LPS exerts on certain mediators, such as NO. The data presented in this report point to a restricted modulation of monocyte functions.

In our experimental setting, LPS pretreatment did not result in a complete desensitization of monocytes, as IL-6 was not affected by LPS-mediated downregulation. This is in accordance with an in vivo study of Mackensen et al.22 who found a marked downregulation of TNF, IL-8, G-CSF, M-CSF, but not of IL-6 after repeated injections of LPS. Mengozzi et al.23 also demonstrated IL-6 to be unaffected by LPS tolerance in human monocytes. In different experimental settings, however, others found IL-6 to be affected by downregulation in vitro24 and in vivo.25

A general state of hyporesponsiveness to the priming signal can also be ruled out, as the upregulation of CD54 on IFN-γ priming was increased in monocytes preincubated with LPS. Furthermore, we were able to show that LPS preincubation did not interfere exclusively with IFN-γ signal transduction pathways, as the same effect on IL-12 expression was observable when GM-CSF instead of IFN-γ was used as a priming signal.

Enhanced expression of IL-12p40 leads to formation of the p40 homodimer, which has been shown in a murine cell system to antagonize IL-12 activities.36 Experiments with recombinant (p40)2 indicate that an about 10-fold to 100-fold excess of IL-12(p40)2 over IL-12 is required to achieve a 50% to 90% inhibition of the effects of IL-12 on Th1 cells.1 In accordance with the findings of others,3,13 we found that both subunits of IL-12 are inducible by stimulation with IFN-γ/LPS and that both are downregulated on LPS preincubation. We found that p40 paralleled the downregulation of p70 protein. On the mRNA level, a reduction of both p35 and p40 accumulation was observed, which confirms very recent data by Karp et al.37 With the methodical approach performed, there is no indication for this mRNA reduction to be regulated on the level of mRNA stability.

We used amplification by RT-PCR to facilitate detection of the mRNA species, arguing that although RT-PCR is a nonquantitative method, large differences in the intensity of the bands obtained with the same primer set should nonetheless reflect significant differences in the expression levels of the amplified mRNA. We have checked to be within the linear range with the cycle numbers performed. Using the PCR-ELISA, our main point was to depict differences between two samples within the same experiment. With this semiquantitative method, absolute mRNA amounts were not determined, as a competitive PCR was not performed.

LPS-induced IL-12 suppression was not due to downmodulation of the LPS receptor, CD14. This is in accordance with published data.21,26,38 Other investigators were able to detect an LPS-induced downmodulation of CD14 expression.23 Differences may be due to different stimulation schemes and incubation times, as well as different sources of monocytes.

Furthermore Fahmi et al.39 who examined the LPS-binding sites in LPS-tolerant mouse macrophages, found no correlation between desensitization of macrophages to endotoxin effects and downregulation of LPS-binding sites. They concluded that downregulation of LPS receptors is not a prerequisite for the induction of LPS tolerance, and that different LPS substructures can downregulate differently the various responses of the cells to LPS, thus suggesting that different pathways are operative for induction of endotoxin tolerance.

LPS and the antitumor agent, PTXL, induce similar responses in murine macrophages, although there are no obvious structural similarities between the 2 agents. Like LPS, PTXL provides a “second” signal for macrophage activation to tumoricidal activity.40 PTXL-induced macrophage stimulation requires an LPS-responsive genetic background41 (most prob-
ably the wild-type Tlr-4 (LPS) receptor and PTXL-induced macrophage stimulation could be blocked by well-characterized LPS antagonist. These data imply an intimate association of LPS and PTXL signaling mechanisms leading to macrophage activation.

PTXL interacts with the cytoskeleton by binding on β-tubulin and stabilizing microtubules. Probably other molecular targets and signaling mechanisms of PTXL are involved in the reaction by which PTXL mimics LPS effects in macrophages. In our study, we found a PTXL-induced IL-12 downregulation on the protein, but not on the mRNA level. This indicates that downregulation of IL-12 is a complex process.

The herein described suppression of IL-12 production by preincubation with LPS seems to be specific for LPS and cannot be completely mimicked by PTXL, despite similar signaling mechanisms. Because different substructures of LPS can have different downregulatory effects on macrophages (at least in murine models), the identification of the responsible “active side” in LPS-induced IL-12 suppression should be addressed in further studies.

Sutterwala et al have recently demonstrated that ligation of phagocytic receptors like the scavenger and Fcγ receptor may be an important downmodulator of IL-12 mRNA synthesis and protein secretion. In addition, we could recently show that the anaphylatoxin C5a leads to a marked downregulation of IL-12 in IFN-γ–primed monocytes. In conclusion, the clearance of extracellular bacteria or opsonized particles, the contact of monocytes/macrophages to C5a, or a LPS-induced state of hyporesponsiveness may contribute to the cessation of IL-12 production. This downmodulation can potentially influence the development of immunity to both extracellular and intracellular pathogens.

LPS can act as a bifunctional modulator on IL-12 production, exerting either enhancing or suppressive effects, depending on its concentration, sequence, and duration of its addition with

Fig 9. Effect of neutralizing anti–IL-10 and anti–IL-4 antibody on intracellular IL-12 (p40/p70) staining of human monocytes pretreated with LPS or medium. Immunofluorescence was performed with human monocytes stimulated as described for 24 hours. The binding of phycoerythrin (PE)-labeled mouse anti-human IL-12 (p40/p70) is shown (vertical; horizontal: forward scatter). Quadrants were set according to the isotype-matched controls. The percentages of cells in the corresponding quadrants are given. (Left) Stimulation; (middle) preincubation with 50 ng/mL LPS; (right) preincubation with 50 ng/mL LPS and neutralizing IL-10 antibody or neutralizing IL-4 antibody, respectively.

Fig 10. Effects of PTXL preincubation on the intracellular IL-12 (p40/p70) production of human monocytes. Monocytes were preincubated with medium, LPS (50 ng/mL), or PTXL (10 μmol/L) for 24 hours. Subsequently, cells were stimulated with 300 U/mL IFN-γ and 50 ng/mL LPS for another 24 hours. The percentage of monocytes positive for IL-12 (p40/p70) or IL-6 as detected by flow cytometric measurement are shown. Results are given as mean ± SEM of 5 independent experiments, performed with cells from different donors.
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respect to costimulators. The complete elucidation of the cellular events induced by LPS will aid in understanding inflammatory processes and may potentially lead to novel therapeutic strategies in sepsis, shock, allergic, and autoimmune diseases.

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