Cellular Mechanisms That Cause Suppressed Gamma Interferon Secretion in Endotoxin-Tolerant Mice

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Endotoxin (lipopolysaccharide [LPS]) tolerance is a state of altered immunity characterized, in part, by suppression of LPS-induced gamma interferon (IFN-γ) expression. However, the cellular mediators regulating LPS-induced production of IFN-γ in normal mice and the effect of LPS tolerance on these mediators has not been well characterized. Our studies show that macrophage dysfunction is the primary factor causing suppressed IFN-γ expression in LPS-tolerant mice. Specifically, LPS-tolerant macrophages have a markedly impaired ability to induce IFN-γ secretion by T cells and NK cells obtained from either control or LPS-tolerant mice. However, T cells and NK cells isolated from LPS-tolerant mice produce normal levels of IFN-γ when cocultured with control macrophages or exogenous IFN-γ-inducing factors. Assessment of important IFN-γ-regulating factors showed that interleukin-12 (IL-12) and costimulatory signals provided by IL-15, IL-18, and CD86 are largely responsible for LPS-induced IFN-γ expression in control mice. IL-10 is an inhibitor of IFN-γ production in both the control and LPS-tolerant groups. Expression of IL-12 and the IL-12 receptor β1 (IL-12Rβ1) and IL-12Rβ2 subunits are suppressed in the spleens of LPS-tolerant mice. LPS-tolerant splenocytes also exhibit decreased production of IL-15 and IL-15Rα. However, expression of IL-18 and the B7 proteins CD80 and CD86 are unchanged or increased compared to controls after induction of LPS tolerance. CD28, a major receptor for B7 proteins, is also increased in the spleens of LPS-tolerant mice. Expression of the inhibitory cytokine IL-10 and the IL-10R are sustained after induction of LPS tolerance. These data show that suppression of IFN-γ production in LPS-tolerant mice is largely due to macrophage dysfunction and provide insight into the cellular alterations that occur in LPS tolerance. This study also better defines the factors that mediate LPS-induced IFN-γ production in normal mice.

Endotoxin (lipopolysaccharide [LPS]) is an intrinsic component of the cell walls of gram-negative bacteria. Serious infections with gram-negative bacteria can lead to the development of the sepsis syndrome, a hyperinflammatory condition that is largely precipitated by LPS-induced secretion of proinflammatory cytokines (27, 30). Numerous investigators have reported transient endotoxia in trauma and high-risk surgical patients due to the translocation of enteric bacteria and endotoxin across the gut (3, 4). Exposure of seriously injured patients to LPS may exacerbate the systemic inflammatory response syndrome, a major source of morbidity and mortality in this patient population. The LPS-induced inflammatory response can lead to systemic organ dysfunction and death. Conversely, prior sublethal exposure to LPS results in a state of tolerance to further LPS challenge. LPS tolerance is characterized by decreased production of macrophage-derived cytokines, such as tumor necrosis factor alpha, interleukin-1β (IL-1β), and IL-6, as well as lymphocyte-derived gamma interferon (IFN-γ) (11, 21). LPS tolerance is thought to be an adaptive mechanism designed to protect the host from further inflammatory injury. Whether or not this state of altered immunity leaves the host more susceptible to subsequent infections remains controversial. Some investigators have postulated that the suppressed cytokine response observed in the tolerant host will result in impaired antimicrobial immunity. However, recent reports have shown that LPS-tolerant mice are more resistant to systemic infection with Cryptococcus neoformans or Salmonella enterica serovar Typhimurium (19, 28). LPS tolerance has clinical relevance because the changes in immune function observed in this model parallel those seen following sepsis, major trauma, thermal injury, and high-risk surgery (1, 2, 8). A common feature of all of these conditions is suppression of IFN-γ production (12, 16).

IFN-γ appears to play an important role in the progression of sepsis and systemic inflammatory response syndrome through its ability to amplify the proinflammatory response (10, 24). The expression of IFN-γ is regulated by a complex interaction of macrophage- and lymphocyte-derived cytokines and cell surface proteins. The macrophage-derived cytokines IL-12, IL-15, and IL-18 are known positive regulators of IFN-γ expression (6, 18, 29). These cytokines act synergistically to induce IFN-γ production by T lymphocytes and natural killer (NK) cells. Induction of IFN-γ expression is also mediated through the T-cell receptor complex by interaction with the major histocompatibility complex class II (MHC-II) on antigen-presenting cells (34). The accessory B7 proteins, of which CD80 and CD86 are the best defined, have also been shown to provide costimulatory signals for the induction of IFN-γ expression through interactions with surface CD28 on T cells and NK cells (5). However, the role of these factors in LPS-induced secretion of IFN-γ are not well characterized nor is the effect of LPS tolerance on the expression of these factors well un-
understood. We characterized the role of known IFN-γ-regulating factors in LPS-induced secretion of IFN-γ and determined whether the expression and function of these mediators were altered in LPS tolerance. We report that IL-12 and costimulatory signals from IL-15 and IL-18 as well as the B7 protein CD86 are important mediators of LPS-induced IFN-γ expression by NK cells and T cells. IFN-γ production in response to LPS is independent of MHC-II. LPS tolerance is characterized by macrophage dysfunction and suppressed expression of the cytokines IL-12 and IL-15 but not IL-18. Expression of the B7 proteins CD80 and CD86 are increased in LPS tolerance. LPS-induced IL-10 production is unchanged or increased in LPS-tolerant mice and functions as an inhibitor of LPS-induced IFN-γ expression. These findings extend our current knowledge of the factors that regulate IFN-γ production after LPS challenge and characterize the alterations that occur in LPS tolerance.

MATERIALS AND METHODS

Reagents. LPS (Escherichia coli serotype 011:B4) and normal goat immunoglobulin G (IgG) were purchased from Sigma Chemical (St. Louis, Mo.). Recombinant IL-12, IL-15, and IL-18, monoclonal anti-CD3ε antibody, and polyclonal antibodies against IL-12, IL-15, IL-18, CD80, and CD86, as well as the CTLA-1g fusion protein, were purchased from R&D Systems (Minneapolis, Minn.). Anti-CD28 antibody was purchased from Caltag Laboratories (Burlingame, Calif.). Polyclonal anti-IL-1 converting enzyme (ICE) p20 was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Anti-MHC-II antibody was purchased from Leinco Technologies (St. Louis, Mo.). Clinical isolates of Pseudomonas aeruginosa and Staphylococcus aureus were obtained from the clinical microbiology laboratory at the Shriners Hospital for Children, Galveston Burns Unit, and were heat-killed at 56°C for 1 h.

Animal model. All studies were approved by the Institutional Animal Care and Use Committee at the University of Texas Medical Branch and met National Institutes of Health guidelines for the use of experimental animals in research. Female, 6- to 8-week-old BALB/c mice (Harlan Sprague Dawley, Indianapolis, Ind.) were used in all studies. Mice were housed in a monitored, light-dark cycled environment and provided standard lab chow and water ad libitum. LPS tolerance was induced by injecting mice intraperitoneally (i.p.) daily for 2 days with LPS (0.4 mg/kg of body weight/mouse in 0.2 ml of normal saline). Control mice received normal saline (0.2 ml) in the same regimen. On day 4, all mice received a challenge dose of LPS (4 mg/kg/mouse; i.p.). Sera and spleens were harvested after LPS challenge for assessment of cytokine expression. All LPS injections were given between 8 a.m. and noon.

Isolation of splenocytes and peritoneal macrophages. Spleens were aseptically harvested from mice and transferred to six-well culture plates containing RPMI 1640 medium supplemented with 10% fetal bovine serum and penicillin (10 U/ml)-streptomycin (10 μg/ml). This medium preparation was used in all experiments. Spleens were minced and passed over a sterile mesh, and erythrocytes were lysed. The remaining cells were resuspended in media and represent the whole spleen mononuclear cell population. Macrophage-depleted splenocytes were prepared by incubating the whole spleen mononuclear cell preparation (10^7 cells/ml) in 75-cm² culture flasks for 16 to 18 h. The nonadherent, macrophage-depleted cell population was harvested, washed, and resuspended in media. T-cell and NK-cell isolation, splenocytes were passed through T-cell enrichment columns and analyzed by flow cytometry after staining with FITC-conjugated anti-CD4 antibody and PE-conjugated anti-CD3 antibody. The percentage of cells staining with each antibody is indicated.

Macrophages (5 x 10^5/well) either in direct contact or separated by Transwells, which are polycarbonate membranes with a 0.4-μm pore size designed to allow passage of soluble factors but prevent direct cell contact (Corning Costar, Cambridge, Mass.). T-NK cells (10^5/well) were cultured with peritoneal

![FIG. 1](https://example.com/figure1.png) Characterization of splenocytes after passage over T-cell enrichment columns. Isolated splenocytes were passaged through T-cell enrichment columns and analyzed by flow cytometry after staining with FITC-conjugated anti-CD14 antibody and PE-conjugated anti-CD3 antibody. The percentage of cells staining with each antibody is indicated.
predicted sizes of the PCR products were 526, 320, 374, and 420 bp for β-actin, IFN-γ, IL-12 p40, and IL-18, respectively.

RPA. RNase protection assay (RPA) was performed using the Ribonuclease Protection Assay system (Pharmingen, San Diego, Calif.) per the manufacturer’s instructions. Briefly, radiolabeled RNA probes were synthesized from DNA template sets using T7 RNA polymerase, 32P-UTP, and pooled nonradio-labeled nucleotides. Isolated mRNAs (10 μg of total RNA/sample) were hybridized with purified riboprobes and subjected to RNase digestion. DNA template sets included probes for the L32 and GADPH (glyceraldehyde-3-phosphate dehydrogenase) housekeeping genes that serve as internal controls. Protected RNA species were separated on 5% polyacrylamide denaturing gels by using 0.5× Tris-borate-EDTA running buffer. Gels were run at 50 W of constant power, and dried and protected fragments were visualized using autoradiography.

Immunoprecipitation and Western blotting. Immunoprecipitation and Western blotting were utilized to determine IL-18 expression by splenic pan-leukocyte preparations. For immunoprecipitation, splenic pan-leukocytes (5 × 106/well) were cultured (37°C, 5% CO2) in six-well plates supplemented with or without LPS (1 μg/ml) for 24 h. Conditioned media were harvested and centrifuged (2,000 × g for 10 min) to remove residual cells. Cells were harvested, washed (three times) with PBS, and disrupted with lysis buffer (62 mM Tris base, 10 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM aprotinin, 1 mM pepstatin, 80 μg of benzamidine/ml, 1% Triton X-100). Protein concentration was determined by the Bradford assay (Bio-Rad). In some studies, protein was harvested directly from whole spleen by homogenizing the tissue in lysis buffer by using a small tissue grinder. Proteins (100 μg/lane) were loaded onto a 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Separated proteins were then transferred (100 V for 1 h at 4°C) to a nitrocellulose membrane (0.2-μm pore size; Bio-Rad) (25 mM Tris, 192 mM glycine, 20% methanol transfer buffer), and processed as described below.

For Western blotting, splenocytes were disrupted in lysis buffer and protein content was determined using the Bradford assay (Bio-Rad). In some studies, protein was harvested directly from whole spleen by homogenizing the tissue in lysis buffer by using a small tissue grinder. Proteins (100 μg/lane) were loaded onto a 4 to 20% gradient sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel. Separated proteins were then transferred (100 V for 1 h at 4°C) to a nitrocellulose membrane (0.2-μm pore size; Bio-Rad) (25 mM Tris, 192 mM glycine, 20% methanol transfer buffer), and processed as described below.

Data analysis. For comparisons of data from multiple groups, two-way analysis of variance was performed followed by Student’s t test. A P value of <0.05 was considered significant.

RESULTS

Suppression of IFN-γ production in LPS-tolerant mice is macrophage dependent. The expression of IFN-γ mRNA in mouse spleen and the secretion of IFN-γ protein into mouse sera after LPS challenge were markedly suppressed in LPS-tolerant mice (Fig. 2). Intraperitoneal challenge of control mice with LPS resulted in a marked increase in IFN-γ mRNA expression at 4 h postchallenge as determined by RPA (Fig. 2A). However, IFN-γ mRNA was not significantly induced in the spleens of LPS-tolerant mice challenged with LPS. Assessment of splenic IFN-γ mRNA expression over time using RT-PCR

were harvested from control and LPS-tolerant mice after LPS challenge (100 μg/l.p.) at the time points indicated, and IFN-γ levels were determined using ELISA. n = 6 to 10 mice/group; *, significantly (P < 0.05) greater than LPS-tolerant group. Data shown are means ± standard error of the mean.
PCR showed that IFN-γ expression peaked at 3 h after LPS challenge in control mice and was not detectable within the limits of our assay in LPS-tolerant mice (Fig. 2B). Serum IFN-γ levels peaked at 6 h after LPS challenge in control mice and were markedly decreased in LPS-tolerant mice (Fig. 2C).

Further studies were undertaken to identify the cellular source of IFN-γ in the spleen and determine the effect of LPS tolerance on splenic IFN-γ production by specific lymphocyte populations. In these studies, splenocytes were isolated from control or LPS-tolerant mice 8 h after LPS challenge and IFN-γ production was determined by intracellular staining and flow cytometry (Fig. 3). These studies showed that the majority of IFN-γ producing cells were CD3 negative. In control mice, IFN-γ⁺CD3⁺ cells accounted for 1.1% of total splenocytes and 22.3% of IFN-γ-producing cells, whereas IFN-γ⁺CD3⁻ cells comprised 3.8% of total splenocytes and 77.7% of IFN-γ⁺ cells. Most of the IFN-γ⁺CD3⁻ cells were NK cells (DX5⁺). Specifically, IFN-γ⁺DX5⁻ cells comprised 3.1% of all splenocytes and 61.5% of IFN-γ⁺ cells. Therefore, CD3⁺ and DX5⁻ cells comprised more than 83% of LPS-induced, IFN-γ⁺ cells. In addition, approximately 2% of CD3⁻ cells produced IFN-γ in response to LPS challenge, whereas 74% of DX5⁻ cells were IFN-γ⁺. Induction of LPS tolerance resulted in marked reductions in IFN-γ production by both CD3⁺ and DX5⁻ populations. As a percentage of cells in each population, IFN-γ production was decreased by 83% in the CD3⁺ population and 91% for DX5⁻ cells in LPS-tolerant mice compared to control mice.

In order to identify the cellular source of impaired LPS responsiveness in the tolerant state, isolated splenic T and NK cells were cultured with control or LPS-tolerant peritoneal macrophages and their ability to secrete IFN-γ in response to LPS was determined (Fig. 4). As outlined previously, we utilized a column binding procedure to enrich splenic T and NK cells (Fig. 1). T and NK cells did not secrete IFN-γ in response to LPS when cultured in the absence of macrophages (Fig. 4A). Coculture of T and NK cells isolated from control or LPS-tolerant mice with control peritoneal macrophages resulted in similar levels of IFN-γ secretion that increased in proportion to the number of macrophages added. LPS-induced IFN-γ production by T cells and NK cells isolated from LPS-tolerant mice after incubation with control macrophages did not significantly differ from control T-NK cells incubated with control macrophages (Fig. 4A). However, IFN-γ production by T cells and NK cells obtained from either control or LPS-tolerant mice was decreased by 85 to 90% after coculture with LPS-tolerant macrophages (Fig. 4A). Additional studies were undertaken to determine whether IFN-γ was arising directly from the added peritoneal macrophages. In both control and LPS-tolerant mice, isolated peritoneal macrophages secreted only 2 and 9%, respectively, of the IFN-γ produced by macrophage-T-NK-cell cocultures (Fig. 4B). This does not exclude the possibility that macrophages require the presence of T-NK cells in order to secrete IFN-γ. However, our flow cytometry studies using whole spleen showed that macrophages (CD14⁺) comprised less than 2% of IFN-γ-producing cells after LPS stimulation (data not shown). Macrophage-T-NK cell cocultures obtained from LPS-tolerant mice secreted significantly (P < 0.05) less IFN-γ than control cocultures.

The role of macrophages in LPS-induced IFN-γ secretion was further elucidated using whole-splenocyte cultures as well as macrophage-depleted splenocytes (Fig. 4C). Whole-splenocyte cultures isolated from LPS-tolerant mice secreted significantly (P < 0.05) less IFN-γ into conditioned media than control splenocytes in response to LPS challenge (Fig. 4C). The macrophage dependence of LPS-induced IFN-γ produc-
tion in the spleen was demonstrated by comparing the ability of whole splenic mononuclear cells and macrophage-depleted splenocytes to secrete IFN-γ in response to LPS. In both control and LPS-tolerant mice were cultured in the presence of LPS (100 ng/ml) for 24 h, IFN-γ levels in conditioned media were determined by ELISA. * significantly \((P < 0.05)\) greater than T cells cultured with LPS-tolerant macrophages. (B) Isolated peritoneal macrophages (5 × 10⁴/well in 96-well plates) were cultured with or without isolated splenic T-NK cells (10⁶ cells/well, macrophage-to-T-NK cell ratio of 1:20) for 24 h in the presence of LPS (100 ng/ml). * significantly \((P < 0.05)\) greater than macrophages cultured in the absence of T-NK cells. (C) Whole or macrophage-depleted splenocytes (10⁶ cells/well in 96-well plates) from control and LPS-tolerant mice were cultured with LPS (100 ng/ml) for 24 h, IFN-γ levels were determined by ELISA. * significantly \((P < 0.05)\) greater than macrophage-depleted splenocytes. (D) Splenic T cells and NK cells (10⁶/well) obtained from control and LPS-tolerant mice were cultured in 96-well plates with the indicated factors for 24 h. IL-12 and IL-18 were added at concentrations of 1 and 10 ng/ml, respectively. Immobilized anti-CD3 and anti-CD28 antibodies were added at 10 μg/ml. IFN- γ levels in conditioned media were determined by ELISA. * significantly \((P < 0.05)\) greater than control. For all studies, \(n\) was 6 to 12 wells/group, with cells taken from at least three mice per group. Data shown are means ± standard error of the mean.

**FIG. 4.** LPS-induced IFN-γ production is macrophage dependent. (A) Isolated splenic T cells and NK cells (10⁶ cells/well in 96-well plates) and peritoneal macrophages (macrophage [M₆]-to-T-NK cell ratios) from control and LPS-tolerant mice were cocultured in the presence of LPS (100 ng/ml) for 24 h. IFN-γ levels in conditioned media were determined by ELISA. * significantly \((P < 0.05)\) greater than T cells cultured with LPS-tolerant macrophages. (B) Isolated peritoneal macrophages (5 × 10⁴/well in 96-well plates) were cultured with or without isolated splenic T-NK cells (10⁶ cells/well, macrophage-to-T-NK cell ratio of 1:20) for 24 h in the presence of LPS (100 ng/ml). * significantly \((P < 0.05)\) greater than macrophages cultured in the absence of T-NK cells. (C) Whole or macrophage-depleted splenocytes (10⁶ cells/well in 96-well plates) from control and LPS-tolerant mice were cultured with LPS (100 ng/ml) for 24 h, IFN-γ levels were determined by ELISA. * significantly \((P < 0.05)\) greater than macrophage-depleted splenocytes. (D) Splenic T cells and NK cells (10⁶/well) obtained from control and LPS-tolerant mice were cultured in 96-well plates with the indicated factors for 24 h. IL-12 and IL-18 were added at concentrations of 1 and 10 ng/ml, respectively. Immobilized anti-CD3 and anti-CD28 antibodies were added at 10 μg/ml. IFN-γ levels in conditioned media were determined by ELISA. * significantly \((P < 0.05)\) greater than control. For all studies, \(n\) was 6 to 12 wells/group, with cells taken from at least three mice per group. Data shown are means ± standard error of the mean.

**Induction of IFN-γ by LPS is regulated by multiple cytokines and B7-CD28 interactions.** In order to define factors that mediate LPS-induced IFN-γ production, macrophages and T-NK cells were cocultured either in direct contact or separated from direct contact using Transwells. Transwells are porous membranes that allow the passage of soluble factors such as cytokines but prevent direct cell interaction. The goal of these studies was to determine the importance of direct cell contact in LPS-induced IFN-γ production. Control cells cocultured using Transwells exhibited an 80% decrease in LPS-induced IFN-γ production compared to cells cultured in direct
Cocultures obtained from LPS-tolerant mice also showed a 64% decrease in IFN-γ production when separated by Transwells compared to cells cultured in direct contact. Additional studies were undertaken to elucidate the roles of specific mediators in LPS-induced IFN-γ production. The role of MHC-II in LPS-induced production of IFN-γ was determined by adding antibodies specific for mouse MHC-II in conditioned media to splenocyte cultures that were stimulated with LPS, heat-killed *P. aeruginosa* or heat-killed *S. aureus* during coculture with goat IgG or antibody against mouse MHC-II. Conditioned media were harvested to assess IFN-γ levels by ELISA after 24 h of culture. Addition of anti-MHC-II antibody decreased IFN-γ levels compared to goat IgG. We also investigated the roles of cytokines and B7 proteins in LPS-induced secretion of IFN-γ. Compared to cells cultured without antibodies or with nonspecific goat IgG, addition of anti-IL-10 antibody increased LPS-induced IFN-γ production by 63% (Fig. 5C). However, anti-IL-12 antibody reduced LPS-induced IFN-γ secretion by 84%. Anti-IL-15 antibody alone did not significantly change LPS-induced IFN-γ secretion compared to the control, but addition of anti-IL-18 antibody reduced LPS-induced secretion of IFN-γ by approximately 30%. The combination of anti-IL-12 antibody with either anti-IL-15 or anti-IL-18 antibody reduced IFN-γ to the same level as anti-IL-12 antibody alone (data not shown).
we evaluated levels of IFN-γ-regulating factors in control and LPS-tolerant mice at baseline and after LPS challenge. IL-12 p35 mRNA was constitutively expressed in the spleens of control and LPS-tolerant mice. However, the combination of anti-IL-15 or anti-IL-18 antibody with CTLA-4 Ig fusion protein significantly (P < 0.05) inhibited LPS-induced IFN-γ secretion compared to either cytokine or CTLA-4 fusion protein alone.

LPS tolerance causes suppressed production of IL-12 and IL-15, but not IL-18 or B7 proteins. In order to identify mechanisms of suppressed IFN-γ production in LPS-tolerant mice, we evaluated levels of IFN-γ-regulating factors in control and LPS-tolerant mice at baseline and after LPS challenge. IL-12 p35 mRNA was constitutively expressed in the spleens of control and LPS-tolerant mice (Fig. 6A). LPS challenge did not significantly change IL-12 p35 expression in either group. In contrast, IL-12 p40 mRNA did not exhibit constitutive expression in either control or LPS-tolerant mice but was induced 1 h after LPS challenge in control mice but not in LPS-tolerant mice. Like IL-12 p35, IL-18 mRNA was constitutively expressed in the spleens of control and LPS-tolerant mice and expression was not significantly changed after LPS challenge in either control or LPS-tolerant mouse (Fig. 6A). IL-10 mRNA was not constitutively expressed but was induced in the spleens of both control and LPS-tolerant mice 4 h after LPS challenge (Fig. 6B). LPS stimulated expression of IL-15 and IFN-γ mRNAs in the spleens of control mice but not in LPS-tolerant mice 4 h after LPS challenge.

Because of its key role in the induction of IFN-γ production, studies were undertaken to better define the effects of LPS tolerance on IL-12 expression (Fig. 7). These studies showed that LPS-induced splenic IL-12 p40 mRNA and serum IL-12 p70 levels were suppressed in LPS-tolerant mice. IL-12 p40 mRNA peaked in the spleens of control mice at 1 h after LPS challenge and was markedly decreased in LPS-tolerant mice (Fig. 7B). IL-12 p70 protein peaked in serum at 3 h after LPS challenge and was markedly increased in LPS-tolerant mice (Fig. 7B). We also performed ex vivo studies to determine the effects of LPS tolerance on IL-12 secretion by isolated peritoneal macrophages. Peritoneal macrophages showed decreased secretion of IL-12 p40 and IL-12 p70 after the induction of LPS tolerance (Fig. 7C and D). Priming of macrophages with IFN-γ augmented LPS-induced secretion of IL-12 p40 and IL-12 p70 in both control and LPS-tolerant macrophages. However, IFN-γ production remained significantly (P < 0.05) lower in LPS-tolerant macrophages than in the control.

Because IL-18 secretion is mediated through complex mechanisms that include intracellular cleavage of pro-IL-18 by ICE, studies were undertaken to further characterize the effect of LPS tolerance on IL-18 synthesis and secretion. IL-18 was constitutively expressed in both control and LPS-tolerant mice and was not suppressed by LPS tolerance (Fig. 8). IL-18 was present in mouse serum prior to challenge with LPS and showed a small, but significant, increase that peaked 1 h after LPS challenge (Fig. 8A). When serum IL-18 levels of control and LPS-tolerant mice were compared, no significant difference was observed at any of the time points studied. IL-18 mRNA was also constitutively expressed in the spleens of control and LPS-tolerant mice (Fig. 8B). LPS treatment increased splenic IL-18 mRNA expression at 1 h after challenge in both control and LPS-tolerant mice, and levels of IL-18 mRNA expression were not different between groups (Fig. 8B). Pro-IL-18 (p25) protein was also present in the spleens of mice prior to and following LPS challenge in both control and LPS-tolerant mice (Fig. 8C). Levels of pro-IL-18 were similar among mice from both groups, and stimulation with LPS did not increase pro-IL-18 levels in the spleens of mice from either group. We did not observe mature IL-18 (p18) in our Western blots of whole mouse spleen. In an ex vivo model, we showed a predominance of p25 in spleen cell lysates (Fig. 9A). Analysis of conditioned media showed a predominance of p18 that was constitutively released by both control and LPS-tolerant splenocytes. Comparison of control and LPS-tolerant splenocytes did not reveal a significant difference in levels of p18 or p25 between the groups. Pro-IL-18 is cleaved by ICE to yield the mature, secreted form of IL-18. We measured ICE levels in splenocytes isolated from control and LPS-tolerant mice (Fig. 9B). Splenocytes obtained from both control and LPS-tolerant mice exhibited constitutive expression of the ICE precursor (p45) as well as the mature component p20. The induction of

![Image](http://iai.asm.org/)

FIG. 6. Cytokine mRNA expression in control and LPS-tolerant mice after LPS challenge. (A) Control and LPS-tolerant mice were challenged with either saline (0.2 ml IP) or LPS (100 μg/mouse; i.p.), and spleens were harvested after 1 h. Total RNA was isolated, and cytokine expression was determined by RPA (DNA template, mCK-2b). (B) Control and LPS-tolerant mice were challenged with LPS as described above. Spleens were harvested 4 h after LPS challenge. RNA was isolated, and cytokine mRNA expression was determined by RPA (DNA template mCK-1). −, saline challenged; +, LPS challenged.
LPS tolerance did not change expression of either p20 or p45 (Fig. 9B).

The effect of LPS tolerance on expression of the B7 proteins CD80 and CD86 was determined using flow cytometry either prior to or 4 h after LPS challenge. B7 protein expression by splenic macrophages (CD14^+), B lymphocytes (CD19^+), and T lymphocytes (CD3^+) was ascertained. The data presented are representative of three separate analyses. Among the macrophage population, the percentage of CD14^+CD80^+ cells was not different when comparing the control and LPS-tolerant groups either prior to or after LPS challenge (Fig. 10). In both groups, approximately 65% of CD14^+ cells expressed cell surface CD80. However, the level of CD80 expression per cell as indicated by mean fluorescence intensity (MFI) was increased in CD14^+ cells from LPS-tolerant mice at baseline and following LPS stimulation by more than onefold compared to that of controls. Among T cells, the percentage of cells expressing CD80 in the LPS-tolerant group was approximately 50%, and the MFI was elevated by more than onefold prior to LPS challenge. However, after LPS stimulation, the percentage of T cells expressing CD80 and MFI in the LPS-tolerant group was not different from the control. The percentage of CD14^+ cells expressing CD80 increased in both groups 4 h after LPS challenge and was not significantly different between groups either before or after LPS challenge. However, the MFI for CD80 was increased by approximately twofold and the MFI was elevated by more than onefold in LPS-tolerant mice both before and after LPS challenge (Fig. 11). The percentage of B cells from LPS-tolerant mice expressing CD80 was not different from the controls. However, the MFI was increased by more than twofold at baseline and by approximately 60% after LPS challenge in the LPS-tolerant group compared to the control. T cells from LPS-tolerant mice exhibited an increase in both the percentage of cells expressing CD80 and in MFI both at baseline and after LPS challenge.

LPS tolerance causes decreased expression of IL-12R and IL-15R. The effect of LPS tolerance on splenic cytokine receptor mRNA expression was determined by RPA 4 h after LPS challenge (Fig. 12). IL-10 receptor (IL-10R) and IL-12Rβ1 mRNAs were constitutively expressed in the spleens of both control and LPS-tolerant mice (Fig. 12A). LPS challenge did
not change IL-10R mRNA expression in either group, but LPS stimulation increased expression of IL-12Rβ1 mRNA to a greater level in control mice than in LPS-tolerant mice. Unlike IL-12Rβ1, IL-12Rβ2 mRNA was not constitutively expressed in control spleen but was constitutively expressed in the spleens of LPS-tolerant mice. Challenge of control mice with LPS induced a greater level of IL-12Rβ2 expression than in LPS-tolerant mice. Both IFN-γRα and IFN-γRβ mRNA were constitutively expressed in the spleens of both groups; their levels were not increased by LPS stimulation nor were their
pressed in the LPS-tolerant group compared to the control groups, but induction of IL-15R mRNA was constitutively expressed by splenocytes from both groups. Splenic IL-2R challenge nor were there significant differences between groups. IL-15R subunit, the second component of the functional IL-15R, was also constitutively expressed by splenocytes from both groups, but levels were not increased by LPS challenge nor were there significant differences between groups. Splenic IL-2Rα levels were significantly lower in LPS-tolerant mice compared to controls after LPS challenge, but IL-2Rβ and γc were strongly expressed in both groups. Expression of CD28 by splenocytes from control and LPS-tolerant mice was determined by flow cytometry (Fig. 13). The percentage of cells expressing CD28 was not different between groups either prior to or after LPS challenge. However, the MFI was increased by nearly twofold prior to LPS challenge and by approximately 70% after LPS challenge in the LPS-tolerant group.

**Splenocytes from LPS-tolerant mice remain responsive to exogenous IFN-γ-inducing factors.** Because LPS tolerance is associated with down-regulation of some IFN-γ-inducing cytokines and their receptors, we examined the abilities of normal and LPS-tolerant splenocytes to respond to exogenously applied IFN-γ-regulating factors. Addition of antibody to IL-10 significantly (P < 0.05) increased IFN-γ production after LPS challenge in both control and LPS-tolerant mice compared to cells cultured with LPS alone. Treatment of splenocytes from LPS-tolerant mice with anti-IL-10 antibody returned LPS-induced IFN-γ production to levels observed in control splenocytes challenged with LPS alone. Addition of IL-12 or IL-15 also significantly (P < 0.05) increased LPS-induced secretion of IFN-γ in both control and LPS-tolerant mice compared to splenocytes cultured with LPS alone. Interestingly, LPS-tolerant splenocytes were more responsive to stimulation with LPS plus exogenous IL-12 than control splenocytes. Stimulation with LPS and exogenous IL-18 did not significantly change IFN-γ secretion compared to cells challenged with LPS alone while treatment with anti-CD28 antibody increased LPS-induced secretion of IFN-γ in the LPS-tolerant group but not in the control group compared to cells challenged with LPS alone.

**DISCUSSION**

LPS tolerance is characterized, in part, by suppressed expression of macrophage-, NK-cell-, and T-cell-derived cytokines. Decreased production of IFN-γ, a cytokine derived primarily from T cells and NK cells, has been previously described for LPS-tolerant mice and humans (2, 17) and is consistent with our observations. However, the cellular alterations that cause decreased IFN-γ production following induction of LPS tolerance are not well understood. In our studies, modified macrophage function was the primary factor resulting in suppressed IFN-γ secretion in LPS-tolerant mice. LPS-tolerant macrophages had a reduced ability to stimulate IFN-γ production by T cells and NK cells obtained from either control or LPS-tolerant mice, whereas T cells and NK cells isolated from LPS-tolerant mice responded normally to control macrophages (Fig. 2). In addition, T cells and NK cells obtained from LPS-tolerant mice secreted IFN-γ at levels that were comparable to those of control cells in response to exogenously added inducers of IFN-γ, such as IL-12 and IL-18, and at higher levels in response to the polyclonal T-cell activator anti-CD3 antibody. We also demonstrated that NK cells, followed by T cells, comprise the most abundant population of cells in the spleen that produce IFN-γ in response to LPS (Fig. 3). T cells and NK cells accounted for more than 83% of splenic IFN-γ-producing cells. The induction of LPS tolerance caused marked suppression of IFN-γ production by both cell types. Our results also showed that macrophages produce small amounts of IFN-γ in response to LPS (Fig. 4) and are likely to account for some of the remaining IFN-γ-producing cells in the spleen. However, our flow cytometry studies showed that macrophages comprise less than 2% of cells that produce IFN-γ in the mouse spleen.

IFN-γ expression is induced by a variety of factors and is regulated through a complex interaction between cytokines, accessory molecules, and the T-cell receptor complex (5, 6, 18, 29, 34). IL-12, IL-15, and IL-18 are macrophage-derived cytokines that act synergistically to induce IFN-γ secretion by T cells and NK cells (6, 18, 29). Activation of the T-cell receptor complex by antigen-laden MHC-II or of CD28 by B7 proteins has also been shown to be a stimulus for the synthesis and secretion of IFN-γ (5, 34). However, the roles of these factors...
in LPS-induced production of IFN-γ have not been well defined. Results from our studies show that IL-12, IL-15, and IL-18 play functional roles in LPS-induced production of IFN-γ, with IL-12 being the predominant factor. Specifically, treatment of normal mouse splenocytes with antibodies to IL-12 almost completely abrogated LPS-induced IFN-γ production (Fig. 5). Anti-IL-18 antibody also significantly lowered IFN-γ expression but to a lesser extent than anti-IL-12 antibody. Antibodies against IL-15 did not inhibit LPS-induced IFN-γ production when added alone but, like anti-IL-18 antibody, potentiated the inhibitory effect of CTLA-4 Ig. The results of our studies also demonstrated that IFN-γ expression in response to LPS is independent of MHC-II. However, B7-CD28 interactions appear to partly mediate LPS-induced production of IFN-γ. These findings extend previous investigations (20) showing that LPS-induced T-cell proliferation is
MHC-II unrestricted but dependent on B7 interactions. Our study shows that CD86 plays a more functional role in LPS-induced IFN-γ production than CD80 (Fig. 5). Mattern et al. (20) reported that CD80 was a primary cofactor for LPS-induced T-cell proliferation. These findings suggest that the B7 proteins regulate different, but complementary, aspects of T-cell activation.

The primary goal of these studies was to define mechanisms by which LPS tolerance suppresses IFN-γ production. Therefore, we examined the effects of LPS tolerance on known IFN-γ-regulating factors. As described above, IFN-γ expression was markedly suppressed at the transcriptional level in splenocytes from LPS-tolerant mice (Fig. 6). However, expression of the IFN-γRα and IFN-γRβ subunits were not affected.

FIG. 11. Effect of LPS tolerance on CD86 expression by mouse splenocytes. Splenocytes were harvested from control or LPS-tolerant mice either prior to or 4 h after LPS (100 μg; i.p.) challenge, and CD86 expression was determined by flow cytometry. Unseparated, total splenocytes were stained with PE-conjugated anti-CD86 antibody and FITC-conjugated anti-CD14, -CD19, or -CD3 antibody. Data are expressed as the percentage of each subpopulation staining positively for CD86 and the mean fluorescence intensity (MFI) of CD86 staining.
showing that LPS-induced expression of both the IL-12R 

tolerant groups, and LPS-induced expression was not affected

p35 was constitutively expressed in both the control and LPS-
due primarily to suppression of IL-12 p40 transcription. IL-12 
promotion of LPS tolerance. The decrease in IL-12 production was 
demonstrated markedly decreased production of IL-12 after induc-
(Fig. 12). In agreement with previous studies (2, 15), we dem-
strated markedly decreased production of IL-12 after induc-
ition of LPS tolerance. The decrease in IL-12 production was 
due primarily to suppression of IL-12 p40 transcription. IL-12 
p35 was constitutively expressed in both the control and LPS-
tolerant groups, and LPS-induced expression was not affected 
by LPS tolerance. Our studies also extend a prior report (2) by 
showing that LPS-induced expression of both the IL-12Rβ1 
and IL-12Rβ2 subunits was decreased, but not ablated, in the 
LPS-tolerant state. However, T cells obtained from LPS-toler-
ant mice appear to be equally as responsive to exogenous IL-12 
as control T cells. These data confirm normal T-cell function 
and further support the hypothesis of impaired macrophage function 
as the source of suppressed IFN-γ production in LPS 
tolerance. Because T cells from LPS-tolerant mice respond 
normally to control macrophages and to exogenously applied 
cytokines and costimulatory factors, signal transduction path-
ways leading to IFN-γ induction must remain intact. IL-12R 
mRNA levels were measured in total splenocytes, in which 
there are multiple IL-12R-expressing cell types, such as T cells 
(33), NK cells (32) and dendritic cells (22). Because we do not 
know the relative levels of IL-12R expression by each popula-
tion, we cannot exclude the possibility that some IFN-γ-pro-
ducing cells may have normal or increased levels of IL-12R 
expression. Likewise, IL-12 can induce its own receptor (32), 
so it is possible that suppressed IL-12 expression by LPS-
tolerant macrophages may lead to decreased IL-12R expres-
sion on T cells and NK cells. Application of exogenous IL-12 
may be able to restore IL-12R expression on splenocytes from 
LPS-tolerant mice. However, further studies will need to be 
performed to characterize IL-12R regulation and the effect of 
LPS tolerance.

Like IL-12, LPS-induced expression of IL-15 and the IL-
15Rα subunit were transcriptionally suppressed in LPS-toler-
ant splenocytes (Fig. 6 and 12). Also, addition of exogenous 
IL-15 in combination with LPS resulted in the restoration of 
IFN-γ production to control levels (Fig. 14). In contrast, IL-18 
expression was unchanged in LPS-tolerant mice compared to 
controls. IL-18 and ICE, an important factor in the processing 
of IL-18, were constitutively expressed at similar levels in both 
the control and LPS-tolerant groups. Addition of exogenous 
IL-18 in combination with LPS had no effect on IFN-γ pro-
duction compared to splenocytes challenged with LPS alone in 
either group. Together, these findings suggest that suppression 
of IL-12 and IL-15, but not IL-18, contributes to the decreased 
IFN-γ production observed in LPS-tolerant mice.

We also show that IL-10 plays an inhibitory role in LPS-
induced IFN-γ secretion. This finding is consistent with previ-
ous reports of IL-10-mediated inhibition of a variety of im-
mune responses (9, 13, 31). LPS was a potent stimulus for 
IL-10 expression in both control and LPS-tolerant mice (Fig. 6) 
and that anti-IL-10 antibody enhanced LPS-induced IFN-γ 
production in both groups (Fig. 5 and 14). We also observed 
constitutive expression of IFN-10R at similar levels in mice from 
both groups and that expression of IL-10R was not changed by 
LPS challenge (Fig. 12). Our studies extend prior findings by 
better defining the role of IL-10 in IFN-γ suppression and 
characterizing the effects of LPS tolerance on IL-10 and IL-
10R expression. Specifically, expression and function of IL-10 
remains intact after induction of LPS tolerance. In contrast, 
the production of IL-12 and IFN-γ is suppressed. This obser-
vation suggests that different pathways mediate LPS-induced 
expression of IL-10 and IL-12 and that the IL-10-regulating 
pathways remain intact in this model. Recent studies have 
demonstrated down-regulation of TLR4, an important early 
activator of LPS-induced signal transduction (25). In addition, 
decreased mitogen-activated protein kinase phosphorylation 
and impaired translocation of the transcription factor nuclear 
factor κB (NF-κB) in LPS tolerance have been demonstrated 
(21). Transcription of the IL-12 and IL-15 genes, both of which 
are suppressed in LPS tolerance and are key factors in LPS-
induced expression of IFN-γ, are highly dependent on activa-
tion of the NF-κB pathway. The persistence of IL-10 expres-
sion in LPS-tolerant mice suggests that IL-10 gene expression 
is independent of TLR4 activation and NF-κB nuclear trans-
location. Further studies are needed to characterize factors 
that are important in LPS-induced expression of IL-10.

In the present study, expression of the B7 proteins CD80 
and CD86 were unchanged or increased among splenic mac-
rophages, B cells, and T cells from LPS-tolerant mice (Fig. 10 
and 11). This finding confirms the observation of Wolk et al. 
(31), who showed increased CD80 expression by LPS-tolerant 
human monocytes. Our study extends this finding by showing 
that T cells and B cells from LPS-tolerant mice also exhibit

FIG. 12. Effect of LPS tolerance on cytokine receptor expression. 
Control and LPS-tolerant mice were challenged with saline (0.2 ml/ 
mouse) or LPS (100 μg/mouse). Splenic RNA was harvested 4 h after 
challenge, and cytokine receptor mRNA levels were determined by 
RPA. Shown are results obtained with DNA template sets mCR-3 (A) 
and mCR-1 (B).
increased CD80 expression. In contrast to their report, we demonstrate that CD86 expression is increased in macrophages as well as T cells and B cells from LPS-tolerant mice. We examined B7 expression on T cells and B cells as well as the CD14^+ population because reports indicate that B7 proteins expressed by activated lymphocytes as well as antigen presenting cells provide costimulatory signals for activation of cellular immune responses (7, 23). Likewise, levels of CD28, a major receptor for B7 proteins, were increased on T cells isolated from the spleens of LPS-tolerant mice (Fig. 13). However, the functional significance of this finding is not known. A recent study showed that the inhibitory effect of IL-10 on T-cell function was mediated, in part, by inhibition of CD28 phosphorylation and activation (14). Our studies clearly show that

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**FIG. 13.** CD28 expression is increased in LPS-tolerant mice. Splenocytes were harvested from control and LPS-tolerant mice either prior to or 4 h after LPS (100 µg/mouse) challenge, and CD28 levels were determined by flow cytometry. Unseparated total splenocytes were stained with PE-conjugated anti-CD28 antibody and FITC-conjugated anti-CD3 antibody. Data are presented as the percentage of CD3^+ cells expressing CD28 and the mean fluorescence intensity (MFI) of CD28 staining.

**FIG. 14.** Response of control and LPS-tolerant splenocytes to exogenous IFN-γ-regulating factors. Splenocytes (10⁶/well) were isolated from control or LPS-tolerant mice and cultured with LPS (100 ng/ml) and exogenous IFN-γ-regulating factors. IL-12 was added at 1 ng/ml. IL-15 and IL-18 were added at 10 ng/ml. Anti-IL-10 and anti-CD28 antibodies were added at 1 and 10 µg/ml, respectively. Conditioned media were harvested after 24 h, and IFN-γ levels were determined by ELISA. *, significantly (P < 0.05) different from splenocytes cultured with LPS alone; n = 4 per group. Data shown are means ± standard error of the mean.
IL-10 plays an inhibitory role in LPS tolerance. Whether or not the effect of IL-10 in this model is mediated through inhibition of CD28 remains to be determined. An additional factor that may be functional in LPS tolerance is CTLA-4. CTLA-4, which is expressed primarily on activated T cells, binds B7 proteins but, in contrast to CD28, serves as an inhibitor of many T-cell functions (26). The potential role of CTLA-4 in the immunological alterations observed in LPS tolerance remains to be determined.

In conclusion, our results indicate that suppression of IFN-γ production in LPS-tolerant mice is macrophage dependent and that T-cell and NK-cell function appears to be normal. Suppressed expression of the IFN-γ-inducing factors IL-12 and IL-15, but not IL-18 or B7 proteins, are key elements in the impaired IFN-γ production associated with LPS tolerance.

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REFERENCES