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IL-12 Suppression During Experimental Endotoxin Tolerance: Dendritic Cell Loss and Macrophage Hyporesponsiveness

Maria Wysocka,* Susan Robertson,† Helge Riemann,‡ Jorge Caamano,· Christopher Hunter,† Agnieszka Mackiewicz,* Luis J. Montaner,* Giorgio Trinchieri,§ and Christopher L. Karp†‡

Endotoxin tolerance, the transient, secondary down-regulation of a subset of endotoxin-driven responses after exposure to bacterial products, is thought to be an adaptive response providing protection from pathological hyperactivation of the innate immune system during bacterial infection. However, although protecting from the development of sepsis, endotoxin tolerance also can lead to fatal blunting of immunological responses to subsequent infections in survivors of septic shock. Despite considerable experimental effort aimed at characterizing the molecular mechanisms responsible for a variety of endotoxin tolerance-related phenomena, no consensus has been achieved yet. IL-12 is a macrophage- and dendritic cell (DC)-derived cytokine that plays a key role in pathological responses to endotoxin as well as in the induction of protective responses to pathogens. It recently has been shown that IL-12 production is suppressed in endotoxin tolerance, providing a likely partial mechanism for the increased risk of secondary infections in sepsis survivors. We examined the development of IL-12 suppression during endotoxin tolerance in mice. Decreased IL-12 production in vivo is clearly multifactorial, involving both loss of CD11chigh DCs as well as alterations in the responsiveness of macrophages and remaining splenic DCs. We find no demonstrable mechanistic role for B or T lymphocytes, the soluble mediators IL-10, TNF-α, IFN-αβ, or nitric oxide, or the NF-κB family members p50, p52, or RelB. The Journal of Immunology, 2001, 166: 7504–7513.

Lipopolysaccharide-containing endotoxins, major components of the outer membrane of Gram-negative bacteria, are potent activators of the innate immune system. As such, endotoxins are central to the pathogenesis of Gram-negative sepsis, a state marked by pathological release of proinflammatory mediators, lymphocyte and endothelial cell apoptosis, and disseminated intravascular coagulation (1–4). Despite the fact that Gram-negative sepsis is a major cause of death throughout the world, such deleterious hyperactivation of the innate immune system is an uncommon result of exposure to endotoxins. In part, this is thought to be attributable to the phenomenon of endotoxin tolerance, the transient down-regulation of a subset of endotoxin-driven responses after initial exposure to endotoxin (5, 6). In turn, it recently has become clear that endotoxin tolerance can itself be harmful. A significant percentage of survivors of sepsis have persistent endotoxin tolerance-related alterations in monocyte function. Patients exhibiting this hypoinflammatory state, termed immunological paralysis, have an elevated risk of succumbing to bacterial superinfection before discharge from the hospital (7–9).

Endotoxin tolerance is demonstrable both in vivo and in vitro, at the level of whole organisms as well as in isolated cells. This apparent tachyphylaxis to LPS has been shown to correlate with suppression of proinflammatory cytokine production (5, 6, 10, 11). Monocyte/macrophages have been the prime targets of research in endotoxin tolerance. Tolerance in such cells clearly involves a distinct functional state of activation or differentiation, not a global inhibition of function. Although the production of several proinflammatory cytokines (e.g., TNF-α, IL-1, and IL-6) and antiinflammatory cytokines (e.g., IL-10) is suppressed, the production of other mediators (e.g., IL-1RA) remains unaltered (6, 12). Such observations have led to the concept that endotoxin tolerance represents a reprogramming of macrophages as an adaptive response to bacterial infection (13). It also has become clear that LPS-driven tolerance is but a particular instance of a more general phenomenon of activation-induced reprogramming of monocyte/macrophages: similar effects are seen with other bacterial products (e.g., macrophage-activating lipopeptides, 2 kDa (MALP-2)7) and endogenous proinflammatory mediators (e.g., IL-1 plus TNF-α) (14–16). In addition to monocyte/macrophages, other endotoxin-responsive cells also exhibit endotoxin tolerance. In this regard, the likely relevance of microbial product-induced changes in dendritic cell (DC) function and localization to endotoxin tolerance in vivo recently has become clear (17–19).

IL-12 is an immunoregulatory cytokine that is critical to the orchestration of cell-mediated immune responses in both the innate and adaptive immune systems. Produced largely by monocyte/

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macrophages and DCs, IL-12 is important for protection from diverse bacterial pathogens (20). LPS is a potent inducer of IL-12 secretion. In turn, IL-12 is central to pathological responses to LPS (21, 22). Production of IL-12 recently has been shown to be suppressed during endotoxin tolerance (17). Therefore, the likely relevance of endotoxin tolerance-related dysregulation of IL-12 production to the increased risk of bacterial superinfection in survivors of sepsis has been of considerable interest (17, 19, 23–27).

The mechanisms that underlie endotoxin tolerance and immunological paralysis remain unclear. Secretion of soluble mediators, changes in LPS receptor expression or function, alterations in LPS-driven signaling pathways, and primary effects on transcription, mRNA stability, and translational efficiency have all been implicated at one time or another (6, 15, 28, 29). Despite considerable experimental effort, no consensus has been achieved. In part, this may be because of a lack of comparability of models: there are evident species differences, and monocytic cell lines appear to be poor mimics of primary monocytes when it comes to endotoxin tolerance (6). A further reason is that distinct mechanisms appear to be responsible for different aspects of the endotoxin tolerance phenotype, even in single-cell types. For example, although IL-10 and TGF-β are central to TNF-α suppression in tolerant human monocytes (17, 30), these cytokines appear to play no role in endotoxin tolerance-related IL-12 suppression in such cells (17). Finally, the fact that the mechanisms responsible forinducing endotoxin tolerance are not necessarily the same as the mechanisms that are responsible for altered LPS-driven responses during LPS tolerance is poorly appreciated. Thus, although many studies have shown that endotoxin tolerance is associated with alterations in LPS signal transduction at upstream levels (reviewed in Ref. 15), this is entirely compatible with a mechanism involving LPS induction of soluble mediators that, secondarily, lead to such aberrant signal transduction.

In these studies, we explored the mechanisms underlying IL-12 suppression during endotoxin tolerance in a murine system. We demonstrate that decreased IL-12 production in vivo is clearly multifactorial, involving both deletion of splenic CD11c<sup>hi</sup>pDCs as well as alterations in the responsiveness of macrophages and remaining splenic DCs. We find no mechanistic role for B or T lymphocytes, the soluble mediators IL-10, TNF-α, IFN-αβ, or NO, or the NF-κB family members p50, p52, or RelB.

Materials and Methods

**Mice**

Female BALB/c mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). Females of the following strains were purchased from The Jackson Laboratory (Bar Harbor, ME): wild-type C57BL/6 mice; C57BL/6 mice deficient in IL-10 (IL-10<sup>−/−</sup>); C57BL/6-IL-10m1Cgn; C57BL/6 mice deficient in nitric oxide synthase 2 (iNOS<sup>−/−</sup>); C57BL/6-Igh-6tm1Cgn mice deficient in mature B cells (μMT); mice with a targeted disruption of NF-κB p50 (p50<sup>−/−</sup>); C57BL/6,129-NF-κB1tm1Bal; and their wild-type counterparts. BALB/c-IL-10<sup>−/−</sup>, C57BL/6-IL-10<sup>−/−</sup>, C57BL/6-NF-κBp52<sup>−/−</sup>, and C57BL/6-NF-κB RelB<sup>−/−</sup> mice were obtained from Dr. C. Hunter (University of Pennsylvania, Philadelphia, PA); and their wild-type counterparts. BALB/c-IL-10<sup>−/−</sup>, C57BL/6-IL-10<sup>−/−</sup>, C57BL/6-NF-κBp52<sup>−/−</sup>, and C57BL/6-NF-κB RelB<sup>−/−</sup> mice were obtained from Dr. C. Hunter (University of Pennsylvania, Philadelphia, PA). All experiments, mice were used at 5–7 wk of age.

**Reagents**

LPS from *Escherichia coli* serotype 0127:B8 (used unless otherwise noted) and *Salmonella typhimurium* (protein content of both <1%) was purchased from Sigma (St. Louis, MO). Protein-free *E. coli* LPS K235 was generously provided by S. Vogel (Bethesda, MD). Human FLT3L was kindly provided by Immunix (Seattle, WA).

**Induction of LPS tolerance in vivo**

Unless otherwise indicated, mice were injected i.p. with 20 μg of LPS or 0.1 ml of PBS, followed 26 h later by an i.v. injection of 100 μg of LPS. Serum was obtained from blood collected at 1 or 3 h after LPS challenge to assess TNF-α or IL-10 and IL-12 levels, respectively.

**Induction of LPS tolerance in vitro**

Single-cell suspensions from the spleens of two to three mice were cultured in 24-well plates at a density of 3 × 10<sup>5</sup> cells/well under LPS-free conditions in RPMI 1640 supplemented with 10% FCS (HyClone, Logan, UT), 1-glutamine, nonessential amino acids, 1 mM sodium pyruvate, 1 mM HEPES, 2-ME, and penicillin. After 48 h, splenocytes were primed with 100 ng/ml LPS (or mock primed with medium) for 20 h. Cells were subsequently washed twice with PBS and incubated with a second dose of LPS (1 μg/ml) for a further 20 h, after which culture supernatants were harvested for cytokine analysis.

Peritoneal exudate macrophages were recovered by lavage 3 days after i.p. injection of 2 ml of thioglycollate (Difco, Detroit, MI). Cells were plated in 12-well plates at a density of 2 × 10<sup>5</sup> cells/well, and 4 h later, nonadherent cells were removed by washing. After resting overnight, cells were stimulated with 10 ng/ml LPS for 20 h, washed twice with PBS, and incubated with 10 ng/ml LPS for an additional 20 h, after which culture supernatants were harvested for cytokine analysis.

**Induction of LPS tolerance ex vivo**

Splenocytes from LPS- or PBS-injected mice (2–3 mice/group) were isolated as described above before secondary stimulation with 1 μg/ml LPS. Peritoneal macrophages from LPS- or PBS-injected mice (3–4 mice/group) were recovered by lavage, plated in 24-well plates at a density of 1 × 10<sup>5</sup> cells/well in supplemented RPMI 1640, and allowed to rest for 48 h before stimulation with 1 μg/ml LPS for an additional 20 h.

**Cytokine assays**

IL-12p40, IL-12p70, TNF-α, and IL-10 levels were measured by a two-site radioimmunoassay as described previously (22) with the following Ab pairs: C17.15 and C15.6 (sensitivity 5–10 pg/ml) for IL-12 p40; C18.2 and C17.15 (sensitivity 5–10 pg/ml) for IL-12 p70 (all IL-12 Abs were generated in our laboratory); XT22 (generously provided by Dr. A. Sher, National Institute of Allergy and Infectious Diseases, Bethesda, MD) and polyclonal anti-TNF-α Ab (sensitivity: 100 pg/ml; BD PharMingen, San Diego, CA) for TNF-α; Jese-2A5 (generously provided by Drs. J. Abrams and A. O’Garra, DNAX, Palo Alto, CA) and Jess-16E-5 obtained from BD PharMingen (sensitivity, 10 pg/ml) for IL-10. IL-12 (kindly provided by Genetic Institute, Boston, MA), rTNF-α (Hoffman La Roche, Basel, Switzerland), and rIL-10 (Endogen, Woburn, WA) were used as standards.

**Flow cytometry**

For surface staining, splenocytes obtained from mechanically disrupted spleens were resuspended in PBS containing 0.1% gelatin, and incubated sequentially with anti-Flt3-FITC and anti-CD45-FITC (both from BD PharMingen) for 30 min (all incubations on ice). Cells were washed three times in PBS before analysis. Antihamster IgG-FITC and anti-rat IgG2b-FITC Abs were used as negative controls.

For intracellular IL-12 staining, splenocytes recovered from LPS-challenged mice were cultured for 5 h with monensin (BD PharMingen) followed by surface staining as described above. Cells were then fixed with 4% paraformaldehyde in PBS for 10 min, washed with PBS containing 1% gelatin, and permeabilized with buffer containing 0.1% saponin and 2% gelatin to reduce non-specific binding. Cells were stained with PE-conjugated anti-CD11c, anti-CD11b, and anti-CD11c (BD PharMingen) for 30 min on ice. Cells were washed three times in PBS before analysis.

**Statistical analysis**

Comparisons were analyzed by using the unpaired Student’s t test.
Results

In vivo inhibition of IL-12 in endotoxin tolerance is LPS dose dependent, requiring a higher dose of LPS than TNF-α inhibition

Mice exposed to LPS show a decreased ability to produce TNF-α, IL-12, and other proinflammatory cytokines in response to subsequent LPS exposure (5, 24, 27, 31). In BALB/c mice primed with 1, 5, or 20 μg of LPS and challenged 26 h later with 100 μg of LPS, serum TNF-α levels were decreased by 87–90% compared with nontolerized mice (Fig. 1). Similar treatment of C57BL/6 mice led to a 75–77% decrease in TNF-α production (data not shown). By contrast, priming with 1 μg of LPS did not induce inhibition of IL-12 production on secondary challenge with LPS in either strain of mice, and injection of 5 μg of LPS led to only modest effects (either slight inhibition or enhancement) on IL-12 production (shown for BALB/c in Fig. 1). However, priming with 20 μg of LPS, reproducibly induced significant inhibition of TNF-α, IL-12p40, and IL-12p70 production in both mouse strains (Fig. 1 and data not shown). Serum IL-10 levels increased in an LPS dose-dependent manner, with levels nearly 2-fold those in control mice after priming with 20 μg of LPS (Fig. 1). In all subsequent experiments, a single 20-μg dose of LPS was used for tolerization.

Impaired ability of splenocytes and peritoneal macrophages from LPS-treated mice to produce IL-12 in response to secondary LPS exposure in vitro

The pattern of cytokines secreted ex vivo by splenocytes from LPS-primed mice closely resembled that observed in vivo in LPS-tolerized mice. Splenocytes from BALB/c mice sensitized in vivo with LPS produced 47% less IL-12p40 and 75% less IL-12p70 and TNF-α after secondary in vitro stimulation with LPS, compared with mock-tolerized mice (Fig. 2A). Similarly, IL-10 secretion was increased ~2.5-fold in splenocytes from BALB/c mice sensitized in vivo with LPS, suggesting a potential role for IL-10 in controlling IL-12 production in this model.

By contrast, the ex vivo production of IL-10 by peritoneal macrophages from LPS-primed mice decreased in parallel with IL-12p40 and TNF-α production (Fig. 2B). IL-12p70 production by peritoneal macrophages was undetectable in these experiments, as they had not been pretreated with IFN-γ, a necessary requirement for the production of IL-12p70 by these cells (20).

In addition to providing a cellular counterpart for the in vivo data, these results suggest that IL-10 production is differentially regulated in disparate cell types in response to LPS. They also suggest that cells other than macrophages are likely to be responsible for augmented IL-10 levels in the serum of LPS-tolerized mice.

Down-modulation of IL-12 in LPS-tolerized mice does not require IL-10, NO, TNF-α, IFN-αβ, or T and B cells

IL-10−/− mice could not be primed with our standard tolerogenic dose of 20 μg of LPS, because doses higher than 5 μg are lethal to these mice (31). Thus, IL-10−/− C57BL/6 and IL-10−/− BALB/c mice were primed and challenged with 1 and 5 μg of LPS, respectively, according to a previously published protocol (31). This resulted in a 2-fold decrease in TNF-α levels in both strains of mice (data not shown). Serum levels of IL-12p40 and

![FIGURE 1.](http://www.jimmunol.org/) Higher doses of LPS are required to induce IL-12 than TNF-α inhibition during in vivo endotoxin tolerance. BALB/c mice were injected i.p. with 1, 5, or 20 μg of LPS (or 100 μl of PBS) and challenged i.v. 26 h later with 100 μg of LPS. Serum was harvested from blood collected 1 h (TNF-α, IL-10) and 3 h (IL-12) after challenge. Data represent means (+SD) of three mice per group and are from a single experiment that is representative of an n of 3. *, p < 0.001, compared with mock priming.
IL-12p70 were, on average, 5-fold higher than in control wild-type mice in the absence of LPS priming. Such IL-12 levels were either not affected or only slightly decreased (10–15%) by sensitization with LPS at these doses (data not shown), similar to data reported in Fig. 1 for BALB/c mice. Thus, in vitro experiments were conducted in which splenocytes from IL-10−/− and wild-type C57BL/6 mice were primed with 100 ng/ml LPS for 20 h, followed by a challenge dose of 1 μg/ml LPS for an additional 20 h. Notably, this priming dose reproducibly induces IL-12 suppression in the splenocytes of all mouse strains tested to date (data not shown). LPS priming of IL-10−/− splenocytes led to 90% inhibition of IL-12p40, IL-12p70, and TNF-α production in response to secondary LPS challenge (Fig. 3A). Similarly, wild-type C57BL/6 splenocytes demonstrated an 80% decrease in IL-12 production.

**FIGURE 3.** Down-regulation of IL-12 during endotoxin tolerance does not depend on IL-10, NO, TNF-α, IFN-αβ, or T or B cells. A, Splenocytes were isolated from IL-10−/− and wild-type (C57BL/6) mice, stimulated in vitro for 20 h with 100 ng/ml LPS, washed twice with PBS, and incubated for an additional 20 h with 1 μg/ml LPS. Neutralizing Abs to IL-10 and normal rat IgG (cIg) were used at a final concentration of 10 μg/ml. Data represent means (± SD) of three independent experiments. B, Splenocytes isolated from PBS- or LPS (20 μg)-primed iNOS−/− or wild-type (C57BL/6) mice, restimulated in vitro with 1 μg of LPS (■) or medium (○) as in Fig. 2. Data are from a single experiment, representative of two performed. C, TNF-α receptor-deficient (TNF-αR2−/−) and wild-type (129/C57BL/6) mice were injected i.p. with 20 μg of LPS (■) or 100 μl of PBS (○) and challenged i.v. 26 h later with 100 μg of LPS. Data represent means (± SD) of five mice per group from a single experiment representative of two performed. Serum was collected 1 and 3 h after challenge to measure TNF-α and IL-12 production, respectively, in C, D, and E. ■, LPS primed; ○, PBS primed. *, p < 0.001; **, p < 0.01; and ***, p < 0.05, compared with mock priming.
Consistent with reported in vivo data (32), IL-10−/− splenocytes produced higher levels of IL-12 in response to LPS than did splenocytes from wild-type controls. Although splenocytes from wild-type mice produced increased levels of IL-12 and TNF-α when endogenous IL-10 was neutralized with Ab, genetic deletion or Ab-mediated neutralization of IL-10 did not prevent the development of endotoxin tolerance-mediated inhibition of these cytokines (Fig. 3A).

High concentrations of NO inhibit IL-12 synthesis (33, 34). Controversy surrounds the issue of NO regulation in LPS-tolerized cells (25, 35–38). In our hands, NO production by BALB/c splenocytes and peritoneal macrophages was inhibited after either in vivo or in vitro sensitization with LPS (data not shown), indicating that NO synthase activity is suppressed during LPS tolerance and arguing against a role for NO in IL-12 inhibition in this model. Indeed, ex vivo experiments with splenocytes from iNOS−/− mice revealed that LPS priming leads to secondary suppression of IL-12 and TNF-α production in these mice (Fig. 3B).

TNF-α recently has been shown to exert an inhibitory effect on IL-12 production (39, 40). Although itself a target of inhibition during endotoxin tolerance, TNF-α is produced earlier than IL-12 and thus might be of mechanistic importance in endotoxin tolerance-driven inhibition of IL-12 (22). However, LPS-primed TNF-αR55p75−/− mice produced decreased serum levels of IL-12 and TNF-α in response to secondary LPS exposure (Fig. 3C), suggesting that TNF-α controls neither itself nor IL-12 production in LPS-induced tolerance.

IFN-αβ inhibits IL-12 production stimulated by bacterial and viral infection. In vitro data suggest inhibition of IL-12 production by both monocyte/macrophages and DCs (Refs. 41–44; C. L. Karp, unpublished observations). IFN-αβ production also is triggered by LPS (45). However, mice with a genetic disruption in the IFN-αβ receptor (IFN-αβR−/−) produced decreased levels of IL-12 and TNF-α in response to secondary LPS exposure (Fig. 3D), suggesting the lack of a significant role for IFN-αβ in inhibiting the production of these proinflammatory cytokines in this model.

Finally, both T cell-deficient (nude) and B cell-deficient (μMT) mice demonstrated decreased IL-12 and TNF-α production in response to secondary LPS challenge (Fig. 3E), indicating that inhibition of these cytokines in this model of experimental endotoxin tolerance is independent of T and B cells and of downstream factors produced by these cells.

**FIGURE 4.** Induction of LPS tolerance in NF-κBp50−/− mice in vivo. NF-κBp50−/− mice and their wild-type counterparts were subjected to LPS tolerance as described in the legend to Fig. 3. Serum was collected at 1 and 3 h post-challenge to assess TNF-α and IL-12 levels, respectively. Data represent means (+SD) of six mice per group in two independent experiments. ■, LPS primed; □, PBS primed. *, p < 0.001; **, p < 0.01, compared with mock priming.

**Wild-type**

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NF-κB p50, p52, and RelB family members are not responsible for LPS-induced tolerance

In vitro studies have suggested that formation of transcriptionally inactive NF-κB p50 homodimers may be responsible for endotoxin tolerance in some systems (46, 47). Most recently, Bohuslav et al. reported that peritoneal macrophages from mice deficient in the NF-κB family member p50 (p50−/−) were incapable of undergoing endotoxin tolerance-mediated suppression of TNF-α production (48). To address the role of this transcription factor in IL-12 suppression during LPS tolerance, p50−/− mice were injected with LPS in vivo. Surprisingly, LPS-primed p50−/− mice (along with wild-type controls) exhibited decreased serum levels of IL-12 and TNF-α in response to LPS challenge (Fig. 4). Of note, p50−/− mice produced significantly more TNF-α than wild-type mice, suggesting a basal role for the p50 in TNF-α regulation.

Given these findings, we subsequently characterized the ability of splenocytes and macrophages from p50−/− mice to undergo endotoxin tolerance induction in vitro. Splenocytes from p50−/− mice also were tolerizable for IL-12 and TNF-α production; again, the overall production of TNF-α by p50−/− splenocytes was higher than that of wild-type splenocytes (Fig. 5A). Finally, and in direct contrast to the findings of Bohuslav, et al. (48), we found that elicited peritoneal macrophages from p50−/− mice were indeed capable of endotoxin tolerance-mediated inhibition of both IL-12 and TNF-α production. Priming of macrophages with 10 ng/ml LPS from E. coli strain 0127:B8 consistently induced inhibition of IL-12 and TNF-α production in response to secondary LPS exposure (Fig. 5B). This inhibition of IL-12 and TNF-α production by p50−/− and wild-type macrophages was independent of the source of LPS used for priming and challenge, as similar data were obtained with LPS from S. typhimurium or with protein-free, phenol/water-extracted LPS from E. coli strain K3562 (Ref. 49; data not shown). Because of the surprising nature of these findings, all mice used in these studies were carefully genotyped.

Analysis of mice rendered genetically deficient for the NF-κB family members p52 and RelB, failed to implicate these transcription factors in LPS-induced suppression of IL-12 and TNF-α as well, as splenocytes from both knockout strains were able to undergo endotoxin tolerance induction in vitro (Fig. 5C). Interestingly, LPS-stimulated splenocytes from p52−/− mice produced significantly more IL-12p40 and IL-12p70 than wild-type mice, suggesting a regulatory role for p52 in IL-12 production in response to LPS.
LPS-dependent depletion of CD11chigh DCs leads to decreased IL-12 production

It previously has been shown that LPS not only induces maturation and migration of splenic DCs, but also triggers apoptosis in those cells (19, 50, 51). These data, together with the fact that DCs are major producers of IL-12 (52–54), have suggested that endotoxin tolerance-related decreases in IL-12 levels in serum and in the supernatants of splenocytes predominantly reflect loss of splenic DCs rather than the induction of a tolerant state in these cells. Flow cytometric analysis was used to examine the effect of LPS on splenic populations of DCs and macrophages. Such analysis revealed a profound decrease in the number of CD11chigh DCs in the spleens of mice primed with LPS 26 h earlier (Fig. 6A). A particularly dramatic loss of splenic DCs was seen in FLT3L-treated...
mice primed with LPS (Fig. 6B). FLT3L, which stimulates production of DCs de novo from precursors (55, 56), increased the number of DCs in spleen but did not prevent the rapid disappearance of such CD11chigh cells from spleens after in vivo administration of LPS. This correlated with markedly decreased production of IL-12 and TNF-α ex vivo by LPS-stimulated splenocytes (data not shown).

Flow cytometric analysis of splenocytes from LPS- or PBS-primed, FLT3-treated mice further confirmed a marked reduction in the number of DCs able to produce IL-12 in LPS-primed mice. Whereas 1.7% of cells from the spleens of PBS-primed mice stained for both CD11c and IL-12p40 in response to LPS challenge, only 0.3% of cells from the spleens of LPS-primed mice were double positive (Fig. 7, top). Endotoxin priming thus leads to loss of splenic DCs as well as suppression of the IL-12 productive capacity of remaining DCs.

By 3 days after priming, the numbers of CD11c^high^ cells gradually increased. Similarly, splenocytes stimulated in vitro 3 days after in vivo priming produced increasing amounts of IL-12. By day 10, there was no difference between naive and LPS-primed mice in either the numbers of splenic DCs or the levels of IL-12 detected in supernatants of splenocytes secondarily stimulated with LPS (data not shown).

Consistent with previous findings (51), an increased number of CD11b^high^ macrophages was observed in the spleen and peritoneal cavity after LPS priming (Fig. 8). No significant intracellular staining for IL-12 was observed in CD11b^high^ cells, likely because of the poor response of this population to LPS (53) and the low threshold of sensitivity of the FACS assay (Fig. 7, bottom).

**Discussion**
The mechanisms underlying endotoxin tolerance remain poorly understood, despite the undoubted biological importance of the phenomenon as well as the considerable experimental effort that has been expended in trying to understand it. We recently reported that the central immunoregulatory cytokine IL-12 undergoes potent endotoxin tolerance-mediated suppression in human monocyte/macrophages and DCs (17). To probe the mechanisms responsible for IL-12 suppression during endotoxin tolerance, we turned to in vivo, ex vivo, and in vitro murine models to take advantage of the availability of mice with genetic deficiencies of putative
mediators of endotoxin tolerance. Despite variation in experimental protocols, we were able to confirm previous reports of IL-12 suppression after priming doses of LPS in mice (19, 24, 27). Such down-regulation of IL-12 production in vivo is LPS dose dependent; a higher (single) priming dose of LPS is required to inhibit IL-12 than to inhibit TNF-α. Our major findings with these models are 2-fold. First, IL-12 suppression appears to be a function both of splenic DC loss and of altered monocyte/macrophage and residual DC function. Second, by using knockout mice, we find no mechanistic role for lymphocytes, the soluble mediators IL-10, TNF-α, IFN-αβ, or NO, or the NF-κB family members p50, p52, or RelB in endotoxin tolerance-mediated suppression of IL-12. These latter findings include data that are both confirmatory of (for IL-10; Ref. 30) and in direct opposition to (for NF-κB p50; Ref. 48) previously reported observations.

With the mechanisms underlying endotoxin tolerance remaining obscure even after reductive approaches involving single-cell types in vitro, the analysis of such phenomena in vivo adds an unavoidable further layer of complexity. The origin of the cytokines present in serum after LPS challenge presumably represents an integration of total body cytokine production, but the actual origin of the cytokines produced is unclear. In the experiments reported here, serum cytokine levels closely paralleled splenocyte origin of the cytokines produced. The discrepancies between spleen and serum on the one hand and purified, elicited peritoneal macrophages on the other are reported here, serum cytokine levels closely paralleled splenocyte responses. The discrepancies between spleen and serum on the one hand and purified, elicited peritoneal macrophages on the other are instructive. Like purified human monocyte/macrophages (but unlike murine serum and splenocytes): 1) IL-10 production by peritoneal macrophages was suppressed by LPS priming; and 2) IL-12p70 in such cells was not inducible without IFN-γ priming. However, peritoneal macrophages clearly undergo endotoxin tolerance-mediated suppression of IL-12 production.

In the spleen, in vivo priming with LPS led to an increase in macrophage numbers, but IL-12 production (even of the p40 subunit) by such cells was not easily demonstrable, as previously reported by Reis e Sousa et al. (54). However, splenic DC numbers plummeted after LPS priming. Of note, the kinetics of splenic DC repopulation closely paralleled that of increasing IL-12 production by splenic leukocytes after secondary stimulation ex vivo. IL-12 production by residual DCs was also significantly impaired. These findings integrate data previously reported in human and murine systems. By using blood-derived human DCs, we and others have reported endotoxin tolerance-mediated suppression of IL-12 production in vitro (17, 57). Langenkamp et al. (18) have referred to this as an exhaustion of cytokine production, pointing out the likely role of a defined temporal pattern of cytokine release by DCs after microbial stimulation in the maintenance of immune response class homeostasis. In none of these studies was death of DCs seen. However, in vivo exposure to LPS has been shown to trigger apoptotic death of DCs (not, e.g., simply down-regulation of identifying surface markers) in the murine spleen (19, 50, 51). In our hands, the process leading to DC disappearance is very rapid, with marked depletion within 24 h after LPS administration. Although FLT3L treatment of mice increased baseline numbers of DCs in the spleen, it did not protect these cells from the effects of LPS. Whether the differences in DC fate in these various studies is attributable to fundamental differences between human and murine DCs or, more likely, is attributable to the biological context of an intact splenic microenvironment in vivo (19) remains to be seen.

The ability of isolated cell populations to undergo endotoxin tolerance suggests that, if soluble mediators are mechanistically important in the genesis or maintenance of this state, they can be autocrine. The relevant pathways in vivo may be quite different, however, as suggested by the relative resistance of adrenalectomized mice to the induction of endotoxin tolerance (58) as well as the critical effects of the splenic microenvironment on DC "paralysis" attributable to microbial stimuli other than LPS (19). Thus, we examined the role of various known soluble inhibitors of IL-12 in endotoxin tolerance in vivo in knockout mice. T and B lymphocytes produce multiple IL-12 down-regulatory mediators. However, experiments with T and B cell-deficient mice suggested that such cells are unnecessary for the induction of endotoxin tolerance-mediated suppression of IL-12 in vivo. IL-10 is a prime autocrine and paracrine regulator of IL-12 production. In human monocyte/macrophages, neutralization of IL-10 abolates endotoxin tolerance-mediated inhibition of TNF-α (17, 30) though not IL-12 (17). Replicating previously published data (31), we found that IL-10−/− mice undergo tolerance for TNF-α production both in vivo and in vitro. The LPS doses required for in vivo induction of tolerance for IL-12 production are lethal for IL-10−/− mice. However, such tolerance is exhibited by the splenocytes of such mice and is easily demonstrated in the presence of Ab-mediated neutralization of IL-10 in wild-type splenocytes. Increased levels of IL-10 were seen after LPS priming in serum and in the supernatants of splenocytes, but not in those from peritoneal macrophages, suggesting that cells other than macrophages may be a significant source of IL-10 in vivo. The inverse correlation between serum IL-12 and IL-10 levels in LPS-tolerized mice suggest tight, reciprocal regulation of the production of these cytokines, as has been previously reported in vitro studies (13, 59, 60). Further, splenocytes from IL-10−/− mice exhibit enhanced baseline production of IL-12 in response to LPS, clearly indicating a tonic down-regulatory role for IL-10 in IL-12 release.

Type I IFNs are potent inhibitors of IL-12 production in human and murine systems (Refs. 42–44; C. L. Karp, unpublished observation). Indeed, exogenous IFN-α has been shown to protect against endotoxin toxicity in mice (61). However, our findings with IFN-αβR−/− mice indicate that type I IFNs are not important in IL-12 suppression during endotoxin tolerance. Studies with TNFR−− and iNOS−/− mice similarly were negative. One notable finding of these studies, unlike experiments with human monocyte/macrophages (17), is that none of them functionally distinguished IL-12 from TNF-α regulation. However, such in vivo experiments have clear limitations. First, it is unclear which cells are primarily responsible for the serum cytokines being measured. It is possible, e.g., that IFN-αβ is important mechanistically in the induction of tolerance in peritoneal macrophages, but that serum cytokines derive largely from splenic DCs (hepatic macrophages, intravascular neutrophils; Ref. 62) that have no such dependence on IFN-αβ. Second, there may be extensive redundancy in mechanism; single-knockout mice may be insufficient.

Recently, two potential mechanistic explanations for endotoxin tolerance have emerged in the literature: alterations in NF-κB family member activity (46–48) and down-regulation of surface expression of Toll-like receptor 4 (TLR4; Refs. 16 and 29). The NF-κB/Rel family of transcription factors play important roles in proinflammatory cytokine production. Five mammalian family members are known: p50, p65 (RelA), p52, c-Rel, and RelB. DNA binding by family members occurs after homo- or heterodimeric complex formation, and/or higher-order complex formation with other transcription factors. Although suppressed transcription of TNF-α has been associated with increased nuclear mobilization of transcriptionally inactive NF-κB p50 homodimers in some murine and human cell lines (46, 47), endotoxin tolerance has largely seemed to correlate with inhibition of NF-κB nuclear translocation in primary human and murine monocyte/macrophages (63, 64). However, Bohuslav et al. (48) have reported that endotoxin tolerance (as measured by the suppression of TNF-α production) is not

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inducible in peritoneal macrophages derived from mice with a genetic deletion of NF-κB p50. We are unable to replicate these findings. We provide data demonstrating that: 1) the peritoneal macrophages and splenocytes of such mice are unaltered in their ability to undergo endotoxin tolerance-mediated suppression of both TNF-α and IL-12; and 2) suppression of TNF-α and IL-12 production in response to systemic LPS challenge is part of the in vivo phenotype of endotoxin tolerance in such mice. Inhibition occurred regardless of the source (or purity) of the LPS used. Consistent with the finding that p50 homodimer overexpression leads to the inhibition of TNF-α gene expression (48), we found that p50/p50 mice produce more TNF-α (though not IL-12) in response to LPS than their wild-type counterparts, suggesting a tonic regulatory role for p50 in TNF-α gene expression. This several-fold increase in TNF-α production was seen in serum and in the supernatants of splenocytes stimulated with LPS. However, in accord with previous studies, TNF-α levels produced by p50/p50 peritoneal macrophages stimulated in vitro with LPS were not dramatically increased (46, 65). Because LPS stimulation induces TNF-α production in both macrophages and DCs, our results support the possibility that the activation of NF-κB p50 is differentially regulated in these different cell types. Studies with mice deficient in other subunits of the NF-κB transcription factors family also have supported this notion (66–68). Splenocytes from p52−/−, though not RelB−/−, mice produced increased levels of IL-12 in response to LPS compared with wild-type splenocytes, suggesting a regulatory role for pathways involving p52 in limiting IL-12 production similar to that suggested by the experiments in IL-10 knockout mice. Nevertheless, splenocytes from p52−/− and RelB−/− mice clearly undergo endotoxin tolerance-mediated inhibition of TNF-α and IL-12 production.

The most recent candidate mechanism for endotoxin tolerance focuses on TLR expression. The endotoxin-tolerant state is known to be associated with alterations in LPS signal transduction at upstream levels (reviewed in Ref. 15). Before identification of the mammalian TLRs (69, 70), down-regulation of surface expression of the LPS-binding receptor CD14 was investigated and excluded as an underlying mechanism for tolerance (6, 71). With recognition of the central role of TLR4 in LPS signaling, data have been presented suggesting that down-regulation of surface expression of TLR4 (or of surface expression of TLR4/MD-2 complexes) may be responsible for LPS tolerance (16, 29, 72). We report no data bearing directly on this assertion in the present paper. However, these data are somewhat difficult to relate causally to the fact that only a subset of LPS-driven responses are suppressed during endotoxin tolerance. Furthermore: 1) such data, derived with a mAb of unclear specificity, are at variance with TLR4 mRNA data (15, 29); 2) LPS sensitization leads to down-regulation of TLR2-driven signaling in the absence of apparent effects on TLR2 expression, something mirrored by TLR2-mediated tolerance induction itself (16); and 3) TLR4 overexpression fails to obviate the induction of endotoxin tolerance (S. Vogel, unpublished observations). It would appear that the mechanisms that underlie endotoxin tolerance-mediated alterations in cytokine production will bear further investigation.
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