Compartmentalization of Tolerance to Endotoxin

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Background. Tolerance to endotoxin has been defined and analyzed either entirely in vivo or entirely in vitro. In contrast, the hyporeactivity of circulating leukocytes reported in patients with sepsis, and often referred to as the phenomenon of tolerance to endotoxin, is an ex vivo observation. Therefore, our objective was to set up an ex vivo model of tolerance to endotoxin.

Methods. Mice were injected intravenously with lipopolysaccharide (LPS), and their leukocytes, derived from different compartments, were challenged in vitro with LPS or heat-killed Staphylococcus aureus Cowan I (SAC), for production of tumor necrosis factor (TNF).

Results. Production of TNF was observed in in vitro cultures of bronchoalveolar cells, peritoneal cells, splenocytes, and whole-blood samples harvested from mice 1–3 h after injection of LPS. Bone marrow cells, in contrast, did not release TNF. In parallel, cells acquired tolerance to LPS within 1–3 h after in vivo injection of LPS. A dramatic decrease in the production of TNF, in response to LPS, was observed by use of circulating leukocytes, splenocytes, peritoneal cells, and bone marrow cells 24 h after injection of LPS. In contrast, LPS-induced production of TNF by bronchoalveolar cells was far less reduced. The kinetics of acquisition of tolerance and recovery were different for different compartments. Cross-tolerance with SAC did not parallel the phenomenon of tolerance to endotoxin that was observed by use of LPS.

Conclusion. These data show that tolerance to endotoxin, as monitored by ex vivo analysis, is compartmentalized and that bronchoalveolar cells are less likely than splenocytes, peritoneal cells, and bone marrow cells to develop tolerance to endotoxin.

Tolerance to endotoxin in vivo has been defined in terms of reduced fever in experimental models after repeated injections of bacteria or endotoxin (lipopolysaccharide [LPS]) [1] or in infected patients after a single injection of bacteria or LPS [2, 3]. This finding was further corroborated by the observation of reduced levels of certain circulating cytokines, particularly tumor necrosis factor (TNF), in both tolerized animals and human volunteers [4–6]. Macrophages have been shown to contribute in vivo to tolerance to endotoxin [7], which is consistent with the fact that in vitro cultures of LPS-primed monocytes/macrophages produce diminished levels of TNF and other cytokines on subsequent challenge with LPS [8–11]. Although suggestive, these in vitro experiments, however, are unlikely to accurately reflect the in vivo process in which, for example, glucocorticoids have also been implicated in the etiology of tolerance to endotoxin [12–14].

The capacity of circulating leukocytes to respond to endotoxin is impaired in the case of human sepsis, trauma, major surgery, or any systemic inflammatory response syndrome (SIRS) [15–20]. This immune dysregulation is often referred to as tolerance to endotoxin. An alteration in the NF-κB signalling pathway within such tolerized macrophages and within monocytes from patients with sepsis and patients with trauma has been proposed to be responsible for this effect [21–23]. In contrast to the tolerance to endotoxin defined entirely in vivo or in vitro experimental models, however, analysis of patients has been based on ex vivo experiments. Such studies monitored the consequence of an in vivo insult (e.g., infection, ischemia/reperfusion, blood loss, tissue injury, or endotoxin translocation) in an in vitro culture of isolated leukocytes in the presence of LPS. Although injection of LPS into human volunteers leads to a decreased capacity of circulating mononuclear cells to produce cytokines ex vivo [24–26], the characterization of ex vivo tolerance to endotoxin in animal models has been limited [27, 28].
The hyporeactivity of leukocytes to LPS in patients with SIRS has been mainly demonstrated by use of circulating leukocytes. However, when the cells were derived from other compartments, an enhanced responsiveness was usually observed by use of peritoneal macrophages during infectious peritonitis [29], inflammatory bowel diseases [30], and endometriosis [31, 32] or by use of alveolar macrophages during acute respiratory distress syndrome [33] and after lung irradiation [34].

To further examine the site-specific effects of LPS on macrophages in vivo, we used the mouse model, in which mice were first injected with LPS. The TNF-producing capacity of cells derived from different compartments of mice on ex vivo challenge with either LPS or heat-killed *Staphylococcus aureus* was then examined.

**MATERIAL AND METHODS**

Male BALB/cJ mice (8–10 weeks old) from either Elevage Janvier or Charles River Laboratories were injected intravenously (iv) with 100 μg of 0111:B4 *Escherichia coli* LPS in 100 μL of saline (0.9% NaCl) or with saline alone. Mice were killed at different intervals, 1–168 h after iv injection of LPS, by cervical dislocation. Peritoneal cells were harvested from the peritoneal cavity by flushing it 3 times with 2.5 mL of cold RPMI 1640 medium (Biowhittaker). Bronchoalveolar cells were harvested by cannulating the trachea and flushing it 10 times with 0.8 mL of cold saline. The splenocyte suspension was prepared by gently teasing the spleen. Bone marrow cells were flushed from the femur by use of RPMI 1640, with a syringe and a 24G needle. Cells were washed, counted, and suspended as follows: 1 × 10⁵ alveolar macrophages/mL, 1.2 × 10⁶ peritoneal cells/mL, 2 × 10⁶ splenocytes/mL, and 2 × 10⁶ bone marrow cells/mL in RPMI 1640 medium supplemented with 1% fetal calf serum (FCS, Valbiotech) and 1 μg/mL indomethacin (Sigma), in a volume of 500 μL/well, in a 24-well plate (Becton-Dickinson Labware). Indomethacin was used to prevent production of prostaglandin, which limits the release of TNF [35]. Cells were cultured for 20 h at 37°C in 5% CO₂, in the absence or presence of 1 μg/mL *E. coli* LPS or heat-killed *S. aureus* Cowan I (SAC; 100 μg/mL) (Pansorbin cells; Calbiochem). Whole blood was diluted 1:4 in the same supplemented culture medium (but without FCS) and was cultured as mentioned above for 20 h.

Experiments with RU486 (Exelgyn) were performed by dissolving it in a mixture of alcohol (0.73%) and mineral oil. It was injected intraperitoneally (ip), at a dose of 10 mg/kg, 24 h before the iv injection of LPS. In other experiments, dexamethasone and betamethasone (Sigma) were dissolved in alcohol and diluted in saline (final alcohol concentration, 3.6%) and were injected iv, at a dose of 2.5 mg/kg, 24 h before the cells were harvested. Control mice received similar injections.

![Figure 1](image-url)  
**Figure 1.** Ex vivo production of tumor necrosis factor (TNF) after injection of lipopolysaccharide (LPS). Mice were intravenously (iv) injected with 100 μg of *Escherichia coli* LPS or with saline, and cells from different compartments were harvested at different times after injection of LPS and were cultured for 20 h. Levels of TNF were assessed in the cell supernatants. Comparisons are with the spontaneous in vitro production of TNF by cells from naive mice. Results are the mean ± SEM of 8–10 experiments. *P < 0.05, **P < 0.01, vs. naive mice.
Figure 2. Ex vivo assessment of tolerance to endotoxin. Mice were intravenously (iv) injected with 100 μg of *Escherichia coli* lipopolysaccharide (LPS) or with saline, and cells from different compartments were harvested at different times after injection of LPS and were cultured for 20 h in the presence of 1 μg/mL *E. coli* LPS. Tumor necrosis factor (TNF) was assessed in the cell supernatants. Comparisons are with the LPS-induced in vitro production of TNF by cells from naive mice. Results are the mean ± SEM of 6–9 experiments and are expressed as the percentage of the LPS-induced responsiveness of cells from naive mice in each experiment (LPS-induced production of TNF varied among the different experiments: 5–27.5 ng/mL TNF for bronchoalveolar cells, 0.8–8.7 ng/mL TNF for peritoneal cells, 369–1095 pg/mL TNF for splenocytes, and 760–5500 pg/mL for bone marrow cells). *P < .05; **P < .01, vs. naive mice.

RESULTS

Ex vivo reflection of LPS-induced cell activation. Cells sampled at different times after the iv injection of LPS were cultured for 20 h in the absence of any activator. As shown in figure 1, 1 h after iv injection of LPS, bronchoalveolar cells, peritoneal cells, and splenocytes produce significantly enhanced amounts of TNF, compared with the cells from naive mice (mice that received no injections of either LPS or saline). The production of TNF by bronchoalveolar and peritoneal cells was still significantly enhanced at 3 h after the injection of LPS. In contrast, bone marrow cells did not display any significant increase in production of TNF. On the contrary, injection of either saline or LPS led to a decrease in the spontaneous production of TNF at 3 h and 24 h after injection. As might be expected, increased production of TNF was also observed in cells obtained from the blood compartment 1 h after injection of LPS (data not shown).

Ex vivo reflection of in vivo–induced tolerance to LPS. Mice were injected with either LPS or saline, and cells were obtained from the different compartments at various times. These cells were then cultured in the presence of LPS, and the level of TNF in the culture supernatant was detected 20 h later. As shown in figure 2, in all the cell types tested, iv injection of saline had a marginal effect on the LPS-induced production of TNF. In contrast, as soon as 1 h after injection of LPS, a marked reduction in LPS-induced production of TNF was observed in peritoneal cells and bone marrow cells. The same observation occurred with splenocytes 3 h after iv injection of LPS. The phenomenon was particularly marked when cells had been sampled 24 h after injection of LPS. In contrast, injection of alcohol, with or without mineral oil. Sham-operated and adrenalectomized BALB/c mice (Elevage Janvier) were studied 2 weeks after surgery.

Supernatants were harvested after 20 h of cell culture, were centrifuged, and were frozen at −30°C until they were assayed. TNF was measured by use of a specific ELISA (Duo Set; R&D System), in accordance with the manufacturer’s instructions. Statistical analysis was performed by use of the Mann-Whitney U test.
of LPS had a marginal effect on the capacity of alveolar cells to produce TNF in response to LPS in vitro, although the weak decrease observed 3 h after injection of LPS reached statistical significance.

We next investigated the kinetics of the recovery from tolerance to LPS. As shown in figure 3, the capacity of splenocytes to respond to LPS in LPS-injected mice was restored at 72 h after injection of LPS, whereas, for bone marrow cells, it required 144 h. Finally, reduced peritoneal cell responsiveness to in vitro challenge with LPS was still observed 168 h after injection of LPS.

Ex vivo LPS-induced cross-tolerance to SAC. Since the phenomenon of cross-desensitization has been reported, we were interested in examining the ability of cells from the various compartments of LPS-primed mice to secrete TNF after an in vitro challenge with heat-killed SAC. As shown in figure 4, a slight decrease in the capacity to produce TNF was observed from splenocytes at 3 h and 24 h after injection of LPS, compared with splenocytes from either mice injected with saline or naive mice. At 24 h after injection of LPS, the capacity of bone marrow cells to respond to SAC was also altered. The responsiveness of splenocytes and bone marrow cells to ex vivo challenge with LPS was recovered 48 h after injection of LPS (data not shown).

Effect of in vitro stimulation by LPS/SAC on cell viability. Cells were analyzed for their viability after 20 h of culture in the presence or absence of LPS/SAC, by use of the tetrazolium salt colorimetric method. These cells were derived from the different compartments at 1 h and 24 h after injection of LPS or saline. Viability was comparable between cells from the different compartments (data not shown). These results exclude cell death as a possible explanation for the differences in production of TNF.

Ex vivo analysis of LPS-induced tolerance within the blood compartment. Because, in most human studies, the alteration of LPS responsiveness is analyzed by use of circulating blood leukocytes, we investigated the responsiveness of these cells in our mouse model. As shown in figure 5, injection of saline enhanced the in vitro response to LPS, compared with that in naive mice. In contrast, at 3 h after injection of LPS, there was a dramatic reduction in the production of TNF that lasted until the 72-h time point. The response to SAC was also reduced at 1 h and 3 h after injection of LPS and returned to normal values (i.e., those for naive mice) by 24 h, although, by 72 h, the levels of TNF remained lower than those of the corresponding cells from saline-injected mice.

Involvement of glucocorticoids in ex vivo analysis of tolerance to endotoxin. Since peritoneal and bone marrow cells appeared to be the most sensitive to tolerization, we investigated the role of glucocorticoid involvement in the in vivo desensitization of these cells to LPS. The injection of synthetic glucocorticoids 24 h before in vitro culture did not lead to a significant reduction in the capacity of these cells to produce TNF in response to challenge with LPS (table 1). RU486, a glucocorticoid receptor antagonist, did not prevent LPS-induced tolerization (data not shown). Furthermore, ex vivo analysis of production of TNF

![Figure 3](image-url) Ex vivo stability of lipopolysaccharide (LPS)–induced tolerance in vitro: recovery of LPS responsiveness of leukocytes from different compartments after intravenous (iv) injection of 100 μg of Escherichia coli LPS. Cells were harvested at different times after injection of LPS and were cultured for 20 h in the presence of 1 μg/mL E. coli LPS. Levels of tumor necrosis factor were assessed in the cell supernatants. Results are the mean ± SEM of 3–8 experiments and are expressed as the percentage of the LPS-induced responsiveness of cells from naive mice in each experiment.
Figure 4. Cross-tolerance to Staphylococcus aureus Cowan I (SAC). Mice were intravenously (iv) injected with 100 μg of Escherichia coli lipopolysaccharide (LPS) or with saline, and cells from different compartments were harvested at different times after injection of LPS and were cultured for 20 h in the presence of 100 μg/ml SAC. Levels of tumor necrosis factor (TNF) were assessed in the cell supernatants. Comparisons are with the SAC-induced in vitro production of TNF by cells of naive mice. Results are the mean SEM of 6–9 experiments and are expressed as the percentage mean of the SAC-induced responsiveness of cells from naive mice in each experiment (SAC-induced production of TNF varied among the different experiments: i.e., 7.6–27.7 ng/ml TNF for bronchoalveolar cells, 2.8–36.0 ng/ml TNF for peritoneal cells, 245–9180 pg/ml TNF for splenocytes, and 0.9–34.4 ng/ml for bone marrow cells). *P < .05 vs. naive mice.

with peritoneal macrophages from sham-operated and adrenalectomized mice (data not shown) showed a similar hyporesponsiveness to in vitro challenge with LPS, suggesting no involvement of glucocorticoids in this phenomenon.

DISCUSSION

Systemic administration of endotoxin activates cells in many compartments, in terms of activation of NF-κB [36], production of cytokines [37, 38], and expression of cytokine receptors [39]. Our ex vivo analysis confirms these observations for bronchoalveolar cells, peritoneal cells, and splenocytes and suggests that bone marrow cells do not contribute significantly to the production of TNF. However, a markedly enhanced production of interleukin (IL)–6 by cells from all 4 compartments was observed 1 h and 3 h after injection of LPS (data not shown). The ex vivo production of TNF by murine alveolar macrophages is in contrast with the absence of production of TNF by alveolar macrophages in human volunteers injected with LPS [40]. Nonetheless, it is in agreement with the fact that systemic administration of LPS in animal models leads to activation of NF-κB in the lungs [36], local cytokine production [41], and lung injury [42].

Soon after primary exposure to LPS, cells acquired tolerance to endotoxin. This was evidenced by the decreased capacity of splenocytes, peritoneal cells, and bone marrow cells to respond to in vitro challenge with LPS. The kinetics, however, were found to vary. Peritoneal and bone marrow cells were tolerized within 1 h, whereas splenocytes acquired their tolerance 3 h after injection of LPS. Interestingly, bronchoalveolar cells maintained their sensitivity to LPS throughout the course of the experiment. Only a weak, decreased reactivity was noticed after 3 h. This finding suggests that cells from the lung compartment could escape the tolerization process. If this observation could be confirmed in other experimental settings, it would explain why the lungs are a major place of inflammation during infection. Indeed, preliminary experiments suggest that in vitro induction of tolerance to endotoxin is markedly lower with alveolar macrophages than with peritoneal macrophages. This observation is in agreement with prior reports stating that, instead of inducing tolerance, repeated exposure to LPS leads to chronic inflammation of the lungs in mice [43, 44].
Figure 5. Ex vivo tolerance to endotoxin in the blood compartment. Mice were intravenously (iv) injected with 100 μg of Escherichia coli lipopolysaccharide (LPS) or with saline, and whole-blood samples were harvested at different times after injection of LPS and were cultured for 20 h in the presence of 1 μg/mL E. coli LPS (A) or 100 μg/mL Staphylococcus aureus Cowan I (SAC) (B). Levels of tumor necrosis factor (TNF) were assessed in the cell supernatants. Comparisons are with the in vitro production of TNF by naive mice. Results are the mean ± SEM of 3 experiments and are expressed as the percentage of the responsiveness of cells from naive mice (i.e., 290–480 pg/mL TNF for LPS and 500–950 pg/mL TNF for SAC). *P < .05; ** P < .01 vs. naive mice.

thermore, systemic administration of LPS to human volunteers has been demonstrated to prime alveolar macrophages for increased in vitro production of TNF and other inflammatory mediators, in response to LPS [45]. Our observation therefore highlights a role for tissue localization in defining endotoxin-derived tolerance. For example, mouse alveolar macrophages fail to produce IL-10 in response to LPS, which is in contrast with other macrophage populations [46]. In a similar vein, human monocytes fail to produce nitric oxide in response to LPS, which also is in contrast with other macrophage populations [47]. Furthermore, cells in the environment could account for the differences in macrophage response to LPS. The distinct kinetics of recovery from tolerance to endotoxin observed between splenocytes, peritoneal cells, and bone marrow cells further underscores the significance of compartmentalization. In accordance with our present findings, Flohé et al. [48] showed, in a model of hemorrhagic shock–induced endotoxin hyporeactivity, that LPS-induced production of TNF was reduced in splenocytes, bone marrow cells, and blood cells.

It was of interest to follow the time kinetics for acquisition
of tolerance to endotoxin in the blood compartment, because this is the main compartment that is studied in patients with sepsis and patients with SIRS. Surprisingly, although injection of saline had minimal effect on the production of LPS-induced TNF by the cells from the 4 other compartments studied, there was enhanced production observed in the whole-blood assays. This alteration of the blood leukocyte responsiveness may be a reflection of the consequence of stress that subsequent to the manipulation of the mice required for the injection. It is well known that various types of stress can affect the capacity of leukocytes to respond normally [49, 50]. Injection of LPS led to a dramatic decrease in the capacity of blood leukocytes to respond to LPS. This may be partially explained by a decreased frequency of circulating monocytes after injection of LPS. However, the number of circulating monocytes is known to be restored by 24 h after the injection of LPS [51]. In addition, the decreased effect on SAC-induced production of TNF suggests that the hyporeactivity of the blood compartment to LPS cannot be explained solely by a decreased number of circulating monocytes. Indeed, the hyporeactivity of circulating leukocytes after an injection of LPS reflects the data that have been frequently reported from whole-blood assays performed by use of samples from patients with sepsis [18, 52, 53]. The discrepancy between the lack of reactivity of circulating cells to LPS and that to SAC suggests that in vivo–acquired cross-tolerance may not affect all cells in a similar fashion.

Although tolerance to endotoxin has long been thought to be specific to LPS, it is now recognized that this is not the case [54]. Recent experimental models of tolerance to endotoxin have demonstrated a cross-tolerance with whole bacteria [11, 55, 56], bacterial toxin [57], and Toll-like receptor (TLR) ligands [56, 58, 59]. Hyporeactivity of circulating leukocytes has been demonstrated not only with LPS but also with Streptococcus pyogenes [60], S. aureus [61, 62], and superantigens [16, 24, 63], in patients with sepsis, patients undergoing surgery, or human volunteers receiving an injection of LPS. However, it has been shown that pretreatment of mouse peritoneal macrophages with LPS primed the cells to a further-enhanced production of TNF induced by heat-killed S. aureus [64], and we have shown that circulating leukocytes from resuscitated patients after cardiac arrest [65] and from patients with trauma [66] had an ability to produce TNF in response to heat-killed S. aureus that was similar to that of healthy control subjects. The reduced capacity to produce TNF in response to SAC was only observed among splenocytes and bone marrow cells. The decrease, however, was not as dramatic as that observed by use of LPS. Furthermore, the kinetics were quite different for bone marrow cells that were fully reactive to SAC at 1 h and 3 h after injection of LPS, whereas the same cells were already profoundly affected, in terms of responsiveness to LPS. These data establish a dissociation between tolerance to endotoxin and the cellular reactivity to both stimuli. This is confirmed in the blood compartment, for which the reduced capacity to respond to SAC did not parallel that observed by use of LPS. Our previous observation in patients with cardiac arrest and patients with trauma led us to suggest that the down-regulation of the pathways initiated by LPS (i.e., TLR4) and that initiated by SAC are different [65, 66]. This was also illustrated by the fact that LPS did not activate NF-κB in leukocytes from patients with trauma, whereas SAC did [23]. It would be of interest to confirm, in our model, the differential activation of NF-κB by LPS versus that by SAC.

Since glucocorticoids have been shown to contribute to the phenomenon of in vivo tolerance to endotoxin [12–14], we examined whether the ex vivo observation could be linked to the enhanced circulating glucocorticoids occurring after injection of LPS. Using RU486, a glucocorticoid receptor antagonist that has been shown to increase LPS-induced lethality [67], we did not see any effect on our ex vivo analysis. In addition, the injection of 2 different synthetic glucocorticoids and the use of adrenalectomized mice did not reveal any role of glucocorticoids in our experimental model. Although these observations are surprising, they suggest that our ex vivo model may be closer to the in vitro tolerance model than to the in vivo one.

In conclusion, our ex vivo model of tolerance to endotoxin revealed that bronchoalveolar cells are less sensitive to the phenomenon, even though they are activated to produce TNF in response to a systemic injection of LPS. In contrast, bone marrow cells that do not produce TNF after LPS has been delivered in vivo are dramatically tolerized. Each compartment behaves independently, and the model reveals that cross-tolerance, which exists between LPS and SAC, is transient, and, when it has been addressed, it may explain the discrepancy in the literature.

### References


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<th>Table 1. Production of tumor necrosis factor (TNF) by peritoneal macrophages and bone marrow cells recovered 24 h after intravenous injection of coccidioides and exposed to in vitro activation of lipopolysaccharide (1 μg/mL).</th>
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NOTE. Data are from 3 experiments.


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