Effects of IL-18 and IL-10 pre-treatment on the alteration of endogenous cytokines in liver and spleen of mice with experimental endotoxemia

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Mechanisms of interleukin-18 (IL-18) and interleukin-10 (IL-10) in lipopolysaccharide (LPS) induced endotoxemia are not clear; their protective role is being investigated so that they may effectively modulate the host cytokine levels during endotoxemia. The aim of the study was to evaluate protective effects of IL-18 and IL-10 in experimentally induced endotoxemia in mice correlating the changes in tissue anti-oxidant enzymes and circulating cytokines. Liver injury was determined by estimation of serum glutamate oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT), serum nitric oxide (NOx), hepatic anti-oxidant enzyme and cytokine content in LPS (250 µg/kg) induced endotoxemic mice receiving either IL-18 (500 ng/mouse) or IL-10 (600 ng/mouse) treatment. Mice (87% of IL-10 treated and 74% of IL-18 treated) survived when administered prior to LPS challenge. Pre-treatment of mice with either IL-10 or IL-18 followed by LPS, lead to reduction in SGPT and SGOT level, serum NOx, and altered hepatic anti-oxidant enzymes activity and myeloperoxidase activity than the only LPS treated group. Marked reduction in the amounts of LPS-induced hepatic and splenic TNF-α content has been observed after IL-10 pre-treatment. Results suggested that attenuating the induction of TNF-α and IFN-γ and subsequent induction of nitric oxide formation in response to LPS may in part account for efficient protection by IL-18 and IL-10 in the reduction of LPS-induced liver injury.

Keywords: Anti-oxidant enzymes, Cytokine, Endotoxemia, Lipopolysaccharide, Reactive oxygen species

The liver plays an important physiological role in lipopolysaccharide (LPS) detoxification and in particular, hepatocytes are involved in the clearance of endotoxin¹. The liver function test has been recently evaluated, as a potential indicator of bacteremia and it may be useful in detecting sepsis in its early stages². Experimentally endotoxemic mice, although do not completely reproduce all the features of clinical septic shock, the accumulated evidence shows that it reliably mimics gram-negative sepsis³. However, since the development of septic shock occurs due to complex interactions, no adequate results have yet been obtained in human clinical practice⁴. Mice injected with LPS showed a significant increase of reactive oxygen species (ROS) production, tumor necrosis factor-α (TNF-α), and interleukin-1β (IL-1β) release by peritoneal leukocytes⁵. Some anti-oxidants and ROS scavengers exert a protective action against endotoxic shock in rodents by inhibiting TNF-α⁶. Various anti-oxidants with ROS scavenging properties, protected mice against endotoxin mediated organ injury and reduced TNF-α level in blood serum⁷. Significant tissue redox imbalance occurs in both endotoxemia and gram-negative bacterial infection, suggesting that oxidative stress may be an important regulator of inflammatory mediator production during sepsis⁸. Therefore it can be hypothesized that pre-treatment with cytokines may regulate the production of tissue derived pro-inflammatory cytokines during LPS induced endotoxemia. Several observations suggested that ROS and nitrogen intermediates are produced at high concentration in animals challenged with endotoxic shock⁹. Studies have suggested that inflammation and cytotoxicity occur when an imbalance exists between ROS production and anti-oxidant enzyme activity¹⁰.

In addition, some anti-oxidants and ROS scavengers or anti-oxidant enzymes exerted a protective action against endotoxic shock in rodents
by inhibiting TNF-α. Synthesis of many cytokines is influenced by changes in the cellular oxidant/antioxidant balance. Stimulation of cytokines and chemokines is of pivotal importance in the pathogenesis of sepsis because they are believed to be the main endogenous mediators of organ injury in endotoxic shock. Endotoxia in response to bacterial LPS characterized by the production of inflammatory cytokines such as TNF-α, IL-1 and gamma interferon (IFN-γ) and the release of highly reactive oxygen and nitrogen intermediate which are thought to contribute too much of the end stage tissue damage in this disease. Selective anti-oxidants are, thus, possible candidates for anti-endotoxic clinical trials. Role of IL-18 in liver anti-oxidant response associated with LPS endotoxia is presently unknown. IL-18 deficient mice are resistant to endotoxic induced liver damage but are highly susceptible to endotoxic shock, indicating the prophylactic role of IL-18. Neutralizing IL-18 was accompanied by a decrease in myeloperoxidase (MPO) content in the lung and, reflecting a reduction in neutrophil accumulation. IL-10 has been reported as the primary endogenous modulator of the lethal inflammatory response during sepsis, which drives the compensatory anti-inflammatory response. IL-10 was reported to control IFN-γ and TNF-α production during experimental endotoxia. Thus IL-10 may be one of the best candidates for down regulation of such LPS induced inflammatory reaction. IL-10 has been shown to be crucial in prevention of overproduction of pro-inflammatory cytokine during systemic inflammation. Endogenous IL-10 has been considered as an important anti-inflammatory cytokine controlling both local and systemic acute inflammatory responses, the usefulness of IL-10 in endotoxia aiming to block the production of reactive oxygen species and the release of inflammatory cytokines is under discussion. Many attempts have been made to suppress the actions of these inflammatory cytokines. Despite all efforts, no regimen today seems to be successful in the treatment of septic shock. Therefore, any intervention that inhibited the release of cytokines or neutralized their effect is believed to benefit individuals experiencing septic shock. The present study has been undertaken to determine the role of IL-18 and IL-10 in LPS induced endotoxia and their effects in hepatic anti-oxidant enzyme expression and cytokine level during endotoxia.

**Materials and Methods**

**Mouse model and time schedule**—Male Swiss albino mice (24), 6-8 weeks of age and weighing 20±4 g body weight were purchased from local registered animal suppliers to the Department. All mice were monitored for 5-10 days before being used for experiments and were housed 6 per cage with food and water ad libitum as accredited by the Institutional Animal Ethical Committee. Animal holding rooms were maintained at 21°C-24°C and 40-60% RH with a 12:12 h L:D cycle. Animals were randomly divided into 4 groups composed of 6 mice in each. Control group (Group a) was injected with sterile PBS. Second group (Group b) of mice received either IL-18 (500 ng/mouse) or IL-10 (600 ng/mouse) only. Third group (Group c) received intraperitoneal injections of purified LPS (derived from Escherichia coli O55:B5; Sigma Chemical) at a dose of 250 µg/kg. The fourth group (Group d) was pre-treated with cytokines (IL-18 or IL-10) for three days and then challenged with LPS (250 µg/kg). The animals were sacrificed after 24 h of LPS treatment and blood was collected by cardiac puncture. Serum was promptly prepared at 5000 g and stored at -20°C.

**Measurement of nitric oxide (NOx) concentration from serum**—Serum (50 µl) was incubated in 40 mm Tris (pH 7.9) containing 40 µmol of reduced form of β-nicotinamide adenine dinucleotide phosphate, 40 µmol flavine adenine dinucleotide and 0.05 unit/ml nitrate reductase at 37°C for 15 min. Reduced samples were incubated with an equal volume of Greiss reagent and the absorbance at 550 nm was measured. The total nitrate/nitrite concentration was determined by comparison to a reduced NaNO3 standard curve.

**Measurement of serum glutamatic oxalate transaminase (SGOT) and serum glutamate pyruvate transaminase (SGPT) activity**—Non-hemolyzed serum (0.1 ml) was mixed with 0.5 ml of glutamic-oxaloacetic transaminase substrate and incubated for 1 hr at 37°C. Then 0.5 ml of 2, 4-dinitrophenyl hydrazine solution was added and stood for 15 min at room temperature. Then, 5 ml of 0.4 N NaOH was added mixed and kept at room temperature for 20 min. The intensity of the developed colour was read at 540 nm after setting the instrument at zero density with water. The decrease in density represents the decrease in α-ketoglutarate from which the activity was calculated.
50 mmol/L phosphate buffer (pH 7.4) on ice for 30 sec using a power driven polytron homogenizer. The homogenate was transferred into centrifuge tubes and centrifuged at 9000g at 4°C for 20 min. The supernatant was used to measure anti-oxidant enzymes activity and the amount of protein present.

**Catalase activity**—Decomposition of $\text{H}_2\text{O}_2$ due to catalase activity was assayed by the decrease in absorbance of $\text{H}_2\text{O}_2$ at 240 nm. Catalase activity in the cell free homogenate was determined spectrophotometrically by measuring the decrease in $\text{H}_2\text{O}_2$ concentration at 240 nm. At time zero, 1.8 ml of each homogenate was mixed with 0.2 ml of a phosphate buffer containing 10 mmol $\text{H}_2\text{O}_2$. One ml of the mixture was immediately added to a cuvette and placed into a spectrophotometer. Catalase activity was observed via degradation of $\text{H}_2\text{O}_2$ as determined by a decrease in UV light absorbance over time. Measurement of absorbance was taken at 15 sec interval after addition of the homogenate to hydrogen peroxide buffer. Units of catalase activity present in 1 ml of homogenate were calculated$^{31}$.

**Superoxide dismutase (SOD) activity**—Tissue homogenate (100 µl) was mixed with 1.5 ml of a Tris-EDTA-HCl buffer (pH 8.5), then 100 µl of 7.2 mmol/L pyrogallol was added and the reaction mixture was incubated at 25°C for 10 min. The reaction was terminated by the addition of 50 µl of 1mol HCl and measured at 420 nm. One unit was determined as the amount of enzyme that inhibited the oxidation of pyrogallol by 50%. The activity was expressed as U/mg protein$^{35}$.

**Myeloperoxidase (MPO) activity**—MPO activity (as an index of tissue neutrophil content) was assayed spectrophotometrically. For the assay, 150 µl of tissue homogenate was added to a reaction mixture containing 0.8 mmol $\text{H}_2\text{O}_2$, 10 mmol $\text{KH}_2\text{PO}_4$ (pH 6.0) and 0.4 mmol O-dianisidine dihydrochloride. The reaction was performed at 37°C and absorbance was measured spectrophotometrically at 460 nm every 15 sec for 4 min, and the linear portions of the tracing were used for analysis. $\text{H}_2\text{O}_2$ dependent MPO activity was expressed as absorbance/min/g wet weight$^{35}$.

**Cytokines measurements**—For assays done on serum, mice were bled at the indicated times after LPS injection, and the serum was frozen at -80°C until use. Cytometric bead array (CBA) analysis and fluorescence detection by flow cytometry [mouse inflammation arrays (BD Biosciences, San Diego, Calif) specific for IL-6, IL-10, MCP-1, TNF-α, IFN-γ, and IL-12p70] was done on serum, and splenocyte supernatants according to the manufacturer’s instructions. For assays in which cytokine levels were determined directly from spleen and liver homogenates, mice were challenged with LPS and spleen and liver were harvested. Single-cell suspensions were then subjected to four rounds of freeze thaw, and the suspensions cleared by centrifugation. Cleared supernatants were used in CBA analysis to determine cytokine levels as described above. Six mice per treatment group were used per experiment. This sensitive technique allows the detection by flow cytometry of multiple cytokines in small quantities of sample$^{34}$.

**Statistical analysis**—All the experiments were repeated three-times with similar results. The data were represented as mean of the three experiments ± SD. The $P$ value <0.05 was established with one-way ANOVA test for all the experiments. Significant differences of the means between the groups were performed by one-way ANOVA. Scheffe’s F-test was done as post-hoc test for multiple comparisons of means of different groups when significant F value was found$^{35}$.

### Results

**Effect of cytokine pretreatment on LPS induced liver injury**—LPS (250 µg/kg) treated mice showed elevated SGOT (IU/L) level than control. However, pre-treatment of mice with IL-18 followed by LPS injection, significantly decreased the SGOT level in the serum ($P<0.05$) (Table 1). Mice treated with only

<table>
<thead>
<tr>
<th>Groups</th>
<th>Serum glutamate oxalate transaminase activity (U/min/L)</th>
<th>Serum glutamate pyruvate transaminase activity (U/min/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>38.33 ± 3.48</td>
<td>26.89 ± 0.70</td>
</tr>
<tr>
<td>LPS alone</td>
<td>84.38 ± 5.23</td>
<td>57.77 ± 6.23</td>
</tr>
<tr>
<td>IL-10 alone</td>
<td>40.91 ± 0.40</td>
<td>35.99 ± 0.63</td>
</tr>
<tr>
<td>IL-10+LPS</td>
<td>45.22 ± 2.92</td>
<td>30.30 ± 1.26</td>
</tr>
<tr>
<td>IL-18 alone</td>
<td>67.91 ± 3.16</td>
<td>45.33 ± 1.15</td>
</tr>
<tr>
<td>IL-18 +LPS</td>
<td>56.07 ± 2.39</td>
<td>37.00 ± 0.11</td>
</tr>
</tbody>
</table>

All values are significant at the 0.05 level in the population mean.
IL-18 (500 ng/mouse) also showed higher SGOT value than the control. A significant increase in the SGPT level was observed from control after LPS administration. However, pre-treatment of mice with IL-18 followed by LPS injection, lead to reduction in SGPT level to than the LPS treated group ($P<0.05$) (Table 1). Conversely administration of IL-10 (600 ng/mouse) to mice with endotoxin confers significant liver protection as evident by decreased SGOT level than the LPS challenged mice ($P<0.05$) (Table 1). A significant increase in SGPT level was found from control ($P<0.05$) in LPS treated mice. However, pre-treatment of mice with IL-10 followed by LPS, lead to reduction in SGPT level than the LPS treated group (Table 1).

**Effect of cytokine pre-treatment on LPS induced NOx release**—Stimulation of LPS leads to significant NOx ($\mu$mol/L) production compared to control. NOx content of serum from IL-18 pre treated mice showed a reduction from that of LPS challenged mice, however unable to reduce towards basal level as that of control. IL-10 treatment before LPS challenge also showed significant decreased serum NOx level, than the LPS challenged mice ($P<0.05$) (Fig. 1).

**Mouse lethality study**—Significant protection was observed when IL-10 (600 ng/mouse) and IL-18 (500 ng/mouse) was given separately prior to LPS (250 µg/kg). The present results showed that only 67% of mice survived after only LPS injection, 74% of mice survived after IL-18 treatment and 93% of animals survived after IL-10 treatment, where as 87% of IL-10/IL-18 treated mice survived when IL-10/IL-18 was administered for 3 days prior to LPS challenge ($P<0.005$) (Fig. 2).

**Effect of cytokine pre-treatment on LPS induced hepatic catalase activity**—Mice injected with the LPS (250 µg/kg) showed a significant increased ROS production as evident by higher catalase activity compared to control. However, treatment with IL-18 prior to LPS challenge showed a significant decline in hepatic catalase in mice than the LPS treated mice ($P<0.05$). Hepatic catalase activity was also decreased in the IL-10 pre-treated followed by LPS injected mice than the LPS treated group (Table 2).

**Effect of cytokine pre-treatment on LPS induced hepatic SOD activity**—Results showed that LPS stimulation induces an enhanced hepatic SOD activity that was reduced in case of IL-18 pre-treatment followed by LPS challenge. The LPS induced elevated hepatic SOD activity was also significantly reduced after IL-10 pre-treatment ($P<0.05$) (Table 2).

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### Table 2—Effect of IL-10 and IL-18 treatment on LPS induced hepatic antioxidant enzymes activity

<table>
<thead>
<tr>
<th>Groups</th>
<th>Catalase activity (U/mg of tissue protein)</th>
<th>Superoxide dismutase activity (U/mg of tissue protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.55±0.021</td>
<td>6.12±0.17</td>
</tr>
<tr>
<td>LPS alone</td>
<td>12.63±0.646</td>
<td>11.14±0.40</td>
</tr>
<tr>
<td>IL-10 alone</td>
<td>3.07±0.81</td>
<td>6.99±0.20</td>
</tr>
<tr>
<td>IL-10 +LPS</td>
<td>4.89±0.22</td>
<td>7.62±0.06</td>
</tr>
<tr>
<td>IL-18 alone</td>
<td>6.87±0.47</td>
<td>8.87±0.17</td>
</tr>
<tr>
<td>IL-18 +LPS</td>
<td>6.8±0.47</td>
<td>8.26±0.19</td>
</tr>
</tbody>
</table>

All values are significant at the 0.05 level in the population mean.
Effect of cytokine pre-treatment on LPS induced hepatic MPO activity—Increased hepatic MPO activity (change in optical density/min.mg tissue protein) was found in LPS stimulated mice compare to control. In the contrary, pre-treatment of mice with IL-18 lead to reduced MPO content in the liver even after LPS administration. Whereas, pre-treatment of mice with IL-10 followed by LPS administration, showed reduced liver MPO content than the LPS challenged group (P<0.05) (Fig. 3).

Effect of IL-10 and IL-18 pre-treatment on LPS induced TNF-α alteration in liver and spleen—Alteration in the production of TNF-α in liver and spleen was studied since these organs have thought to be damaged by TNF-α during endotoxemia. In vivo administration of IL-10 showed inhibition in the release of TNF-α from liver and protects mice from LPS induced lethality. Administration of IL-10 prior to LPS challenge (P<0.05) also showed significant reduction in TNF-α level in the liver as well as in spleen.

Consequently, pre-treatment of mice with IL-18 dose not fully neutralized the LPS induced TNF-α production in liver. IL-18 pre-treatment also reduces the TNF-α level in the spleen 24 h after LPS challenge (P<0.05). This present results also showed that systemic endotoxemia triggers the release of TNF-α in the liver with respect to control but no such effect observed in the spleen (Fig. 4).

Effect of IL-10 and IL-18 pre-treatment on LPS induced IFN-γ level in liver and spleen—Systemic IL-10 treatment reduced LPS induced IFN-γ release in the liver, as LPS induced group showed much higher liver IFN-γ level (P<0.05). However, in the spleen such significant difference has also been found in the IL-10 pre-treated followed by LPS challenged group than the only LPS challenged mice (P<0.05). Results also showed reduced IFN-γ in the spleen of the mice received IL-18 treatment prior to LPS challenge and the decrement was more prominent in the liver (P<0.05), where LPS challenged liver showed much elevated level of IFN-γ (Fig. 5).

Effect of IL-18 pre-treatment on LPS induced IL-10 alteration in liver and spleen—Administration of IL-18 did not induce much alteration in splenic IL-10 level among only IL-18 treated, IL-18 pre- treated followed by LPS and only LPS challenged group of mice, however, hepatic IL-10 content was not significantly reduced in IL-18 pre-treated than in the liver of endotoxemic mice. LPS treatment leads to higher hepatic IL-10 content as compare to control (P<0.05) (Fig. 6).

![Fig. 3](image-url) The effect of IL-10 and IL-18 treatment on LPS induced changes in hepatic neutrophil infiltration quantified by myeloperoxidase (MPO) activity [Values are mean ± SD of triplicate experiment and are significant at 0.05 level in the population mean n=6]

![Fig. 4](image-url) The effect of IL-10 (A) and IL-18 (B) pre-treatment on LPS induced TNF-α alteration in liver and spleen [Treatment with IL-10 (panel A) reduces IFN-γ level compared to IL-18 treated mice (panel B). [Values are mean ± SD of triplicate experiment and are significant at 0.05 level in the population mean n=6]
Discussion

Since IL-18, a major regulating molecule of LPS induced endotoxic shock spans from controlling the secretion of pro-inflammatory cytokines to rescuing mice from lethal endotoxemia, and IL-10 an anti-inflammatory cytokine, the roles of IL-18 and IL-10 pre-treatment in LPS induced endotoxic shock and liver injury were investigated using Swiss albino mice. In the present study liver injury during experimental endotoxemia could be lowered by both IL-18 and IL-10 pre-treatment and to some extent give protection. IL-18/IL-10 treatment showed lower SGOT and SGPT activity as compared to endotoxic group. This in vivo study suggests that IL-18/IL-10 has an essential role in liver functions and the level of IL-18/IL-10 levels may be of value as a marker for the outcome of septic shock. However, IL-18 alone group showed no such significant alteration in liver injury. This present report showed that liver injury might be mediated by TNF-α, IFN-γ and IL-10 since LPS sensitized mice showed elevated hepatic TNF-α, IFN-γ and IL-10 which also supports previous reports. The results of the present study also demonstrated that endogenously produced NO may causes hepatocellular damage by stimulating the expression of TNF-α.

The present results suggested that IL-10 pre-treated mice were protected from LPS induced death. However, treatment of mice with IL-18 showed no significant alteration in hepatic catalase activity. Although excessive ROS production as induced by endotoxin can lead to inflammation and liver cytotoxicity, small alteration in ROS generation and induction of catalase activity can result in the activation of various signaling pathways resulting in cytokine gene expression. No desired results were obtained in hepatic SOD activity in either cytokine (IL-18 or IL-10) pre-treated or LPS treated mice. Stimulation of LPS may cause profuse PMN recruitment in the liver as evident by hepatic MPO content. However, mice receiving either IL-18 or IL-10 prior to LPS challenge, showed reduced PMN infiltration in the liver as evident by decreased hepatic MPO content in those mice. The present result provide experimental demonstration that IL-10 pre-treatment reduces the consequences of septic shock induced by LPS by decreasing the production of TNF-α, IFN-γ in the liver and thereby limiting tissue inflammation (less recruitment of PMN) and end organ damage. Several inflammatory cytokines (such as IFN-γ) were over-produced during endotoxic shock and protect mice by modulating IFN-γ production indicating that IL-18 is a negative regulator for this cytokines. Reduced splenic TNF-α was obtained in IL-18 pre-treated mice during endotoxemia, which also subsequently supported the study that IL-18 treatment may reduce the TNF-α especially during.
Sepsis. Suppression of LPS induced TNF-α level in spleen and liver by IL-10 and the parallel increase in TNF-α after IL-18 pre-treatment is not unique. IL-10 was administered for 3 days which lead to enhanced TNF-α level in spleen of mice 24 h after LPS challenge. These studies may suggest a regulatory role of endogenous IL-10 in regulating TNF-α levels in spleen and liver. This elevated IL-10 in spleen may lead to reduced IFN-γ level in spleen. Reduced IFN-γ in spleen of IL-18 pre-treated mice was also detected. It was found that systemic IL-10 treatment decreased the LPS induced IFN-γ release in the liver and spleen. Suppression of the production of IFN-γ in the liver may suggest a sort of protection to these organs are thought to be damaged by IFN-γ during sepsis. Therefore LPS induced IFN-γ in liver was down regulated by IL-10. Previous reports suggest that administration of IL-18 did not induce elevation of serum IL-10 level in mice. Interestingly, insignificant reduction was observed in hepatic IL-10 level in the IL-18 pre-treated mice, before LPS challenge. This in vivo study strongly suggest that attenuation of the induction of cytokine TNF-α and IFN-γ, subsequent induction of NO formation in response to LPS may in part account for clinical efficacy of IL-18 or IL-10 in the treatment of LPS induced inflammatory disease. IL-18 or IL-10 is effective in diminishing the LPS induced tissue-formation of TNF-α and IFN-γ, in this in vivo model of endotoxemia. Elevated splenic IL-10 content in the IL-18 pre-treated mice was obtained, indicating activation of TH2 cells in the spleen compared to liver. This elevated IL-10 in spleen may lead to inhibition of TH1. Reduced IFN-γ in the spleen of IL-18 pre-treated mice may indicate reduced proliferation of TH1 in spleen. Since IL-18 pre-treatment lead to reduced IFN-γ level in spleen, inhibition of TH2 by the lower level of IFN-γ is not possible; rather TH2 cell are activated in spleen of IL-18 pre-treated mice. Here the results demonstrated that pre treatment with IL-18 or IL-10 protects mice from LPS-endotoxemia via both cytokine and liver anti-oxidant dependant pathways. However, the usefulness of protective strategies in sepsis aiming to block the activity of several mediators by IL-18 or IL-10 in the cascade of LPS induced immune reactions both at the cellular and molecular level needs further investigations. Knowledge regarding the source, kinetics of production, synergism, and regulation of cytokines will be of value in the design of novel therapies. The present study supports and extends the findings of others and provides a basis to pursue these questions in greater detail.

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References


