Malnutrition compromises immune function, reducing resistance to infection. We examine whether the decrease in leptin induced by starvation increases susceptibility to lipopolysaccharide (LPS)- and tumor necrosis factor (TNF)-induced lethality. In mice, fasting for 48 hours enhances sensitivity to LPS. Decreasing the fasting-induced fall in leptin by leptin administration markedly reduced sensitivity to LPS. Although fasting decreases basal leptin levels, LPS treatment increased leptin to the same extent as in fed animals. Fasting increased basal serum corticosterone; leptin treatment blunted this increase. Fasting decreased the ability of LPS to increase corticosterone; leptin restored the corticosterone response to LPS. Serum glucose levels were decreased in fasted mice and LPS induced a further decrease. Leptin treatment affected neither basal glucose nor that after LPS. LPS induced a fivefold greater increase in serum TNF in fasted mice, which was blunted by leptin replacement. In contrast, LPS induced lower levels of interferon-γ and no differences in interleukin-1β in fasted compared to fed animals; leptin had no effect on those cytokines. Furthermore, fasting increased sensitivity to the lethal effect of TNF itself, which was also reversed by leptin treatment. Thus, leptin seems to be protective by both inhibiting TNF induction by LPS and by reducing TNF toxicity. (Am J Pathol 2000, 156:1781–1787)

Leptin plays a crucial role in the homeostasis of body weight by regulating food intake and energy expenditure. Circulating leptin levels are directly related to adipose tissue mass. High leptin levels signal the presence of sufficient energy stores to sites in the central nervous system, which respond by reducing appetite and increasing energy expenditure to prevent obesity. In addition, leptin can promptly signal the shift between sufficient and insufficient energy stores. For example, leptin levels fall rapidly with the onset of starvation, disproportionally to changes in adipose tissue mass. The fall in leptin levels is a signal for the brain to initiate the adaptative responses to starvation. The adaptation of the organism to starvation is characterized by metabolic and endocrine changes, which include suppression of reproductive and thyroid function and stimulation of the hypothalamus-pituitary-adrenal (HPA) axis. Preventing the starvation-induced fall in leptin with exogenous leptin administration substantially blunts the changes in gonadal, adrenal, and thyroid axes in male mice and prevents the starvation-induced delay in ovulation in female mice. In ob/ob mice, leptin deficiency leads to a complex syndrome characterized by most of the signs and symptoms of early starvation, such as abnormal reproductive function, hormonal abnormalities, and decreased activity. Therefore ob/ob mice seem to exist in a state of perceived starvation and as a consequence, they became obese with free access to food.

Malnutrition is known to induce a state of immunodeficiency and to predispose to death from infectious diseases. Starvation suppresses immunity, particularly T-lymphocyte responses, and decreases resistance to infection. Leptin has been shown to have a direct effect on T lymphocytes, enhancing T-helper (Th) allogenerative response, polarizing Th cells toward a Th1 phenotype. Exogenous leptin administration has also been shown to reverse the inhibitory effect of starvation on the development of a delayed-type hypersensitivity reaction. It has recently been shown that exogenous administration of leptin during fasting protects mice from the lymphoid atrophy associated with starvation, indicating a role for leptin in the immune dysfunction of starvation.

Leptin levels are acutely increased by inflammatory stimuli such as lipopolysaccharide (LPS) and turpentine and by cytokines, indicating that leptin induction is part of the acute phase response to inflammation. Furthermore, the increase in leptin production during local and systemic inflammation is absent in interleukin (IL)-1β-deficient mice. Thus, during inflammation leptin expression is regulated in a manner similar to the cytokine response to infection and injury. In addition, both the structure of leptin and that of its receptor suggest that leptin might be classified as a cytokine. The leptin receptor is homologous to the gp-130 signal-transducing sub-
unit of the IL-6-type cytokine receptors,\textsuperscript{15–17} and the secondary structure of leptin itself has similarities to the long-chain helical cytokine family, which includes IL-6, IL-11, ciliary neurotrophic factor (CNTF), and leukemia inhibitory factor (LIF).\textsuperscript{18} Moreover, in obese, leptin-deficient ob/ob mice there is increased susceptibility to LPS- and tumor necrosis factor (TNF)-induced lethality,\textsuperscript{19,20} suggesting that a functional leptin system may represent a protective component of the host response to inflammation.

Based on these observations, we hypothesized that the decrease in leptin levels that accompanies starvation may contribute to the increased susceptibility to lethal infections that occurs during starvation. Here we demonstrate that starvation increases the mortality induced by LPS and TNF and that this increase can be prevented by treatment with exogenous leptin.

\textbf{Materials and Methods}

\textbf{Animals and Treatments}

Five- to 6-week-old female C57Bl/6 mice, weighing approximately 20 g, were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were fed, fasted, or fasted and treated with leptin for 48 hours. Body weight and water intake were recorded daily at 9:00 a.m. Water intake was evaluated per cage with each cage containing five mice. Murine leptin (Amgen, Thousand Oaks, CA) was given intraperitoneally (i.p.) twice a day (9:00 a.m. and 6:00 p.m.) at the dose of 1 \( \mu \)g/body weight. This dose of leptin has been shown to produce a concentration of leptin 6 and 12 hours after injection similar to fed controls.\textsuperscript{4} This protocol has previously been used to study the role of leptin in the physiology of starvation.\textsuperscript{4} After 48 hours, at 9:00 a.m., mice were administered LPS (Difco Laboratories, Detroit, MI), i.p. at 7.5, 15, or 25 mg/kg or recombinant murine TNF (Genentech Inc., So. San Francisco, CA), intravenously (i.v.) at 0.5 mg/kg. Control mice received i.p. or i.v. saline. Survival was monitored for up to 6 days. For TNF and corticosterone determination blood was collected from the retroorbital plexus under halothane anesthesia 2 hours after LPS. For leptin determination, blood was collected 8 hours after LPS. Control mice received saline. The Animal Studies Committee of the Veterans Affairs Medical Center, San Francisco, approved these studies.

\textbf{Cytokine Measurements}

TNF-\( \alpha \), IL-1\( \beta \), and interferon (IFN)-\( \gamma \) were measured using DuoSet enzyme-linked immunosorbent assay kits specific for murine cytokines (Genzyme Diagnostic, Cambridge, MA) following the manufacturer’s instructions.

\textbf{Other Assays}

Serum leptin levels were measured using an enzyme-linked immunosorbent assay kit specific for mouse leptin (Linco Research, Inc., St. Charles, MO). Serum corticosterone was measured using a kit from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). Serum glucose was measured using a kit from Sigma Chemical Co. (St. Louis, MO).

\textbf{Statistical Analysis}

Data are expressed as the mean \( \pm \) SEM. Analysis of variance with Bonferroni as post hoc test was used for comparison among three groups. Student’s \( t \)-test was used for comparison between two groups. Statistical significance for lethality rates was determined by Fisher’s exact test.

\textbf{Results}

\textbf{Fasting Increases Susceptibility to LPS-Induced Lethality}

The effect of acute starvation (48 hours) with or without exogenous leptin administration on body weight and water intake is shown in Table 1. Depriving mice of food for 48 hours caused a fall in body weight (17%) and dramatically reduced water intake (61% and 68% between 0 to 24 and 24–48 hours, respectively). Serum leptin levels decrease during fasting.\textsuperscript{4} To increase the leptin levels in fasted mice, we administered exogenous leptin following a protocol previously used to study the role of leptin in the physiology of starvation (1 \( \mu \)g/g body weight, i.p. twice a day for 2 days).\textsuperscript{4} Leptin treatment of fasted mice did not affect the decreases in either body weight or in water intake (Table 1).

To study the effect of short-term starvation on LPS-induced lethality, fed mice or fasted mice were challenged with different doses of LPS. As shown in Figure 1, fasting marked increases the sensitivity to LPS toxicity. A dose of LPS of 7.5 mg/kg that did not cause lethality in fed mice induced 60% mortality in fasted mice. A dose of 15 mg/kg of LPS caused 100% lethality in fasted mice compared to 20% mortality in fed mice. Because mice injected with LPS (15 mg/kg) became anorectic, to exclude the possibility that fasted mice subsequently treated with LPS died of starvation, a fasted group of mice were treated with saline and kept fasted. No deaths

\textbf{Table 1. Body Weight and Water Intake in Fed, Fasted, and Leptin-Treated Fasted Mice}

<table>
<thead>
<tr>
<th></th>
<th>Fed</th>
<th>Fasted</th>
<th>Fasted + leptin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>Initial</td>
<td>Final</td>
<td>Final 18.2</td>
</tr>
<tr>
<td></td>
<td>17.9 ( \pm ) 0.8</td>
<td>17.6 ( \pm ) 0.4</td>
<td>18.3 ( \pm ) 0.5</td>
</tr>
<tr>
<td>Water intake (ml/ mouse)</td>
<td>14.6 ( \pm ) 0.4*†</td>
<td>15.0 ( \pm ) 0.6*†</td>
<td></td>
</tr>
<tr>
<td>0–24 hours</td>
<td>4.4 ( \pm ) 0.3</td>
<td>1.7 ( \pm ) 0.4</td>
<td>2.3 ( \pm ) 0.5</td>
</tr>
<tr>
<td>24–48 hours</td>
<td>4.1 ( \pm ) 0.3</td>
<td>1.3 ( \pm ) 0.1</td>
<td>1.6 ( \pm ) 0.3</td>
</tr>
</tbody>
</table>

For body weight, data are means \( \pm \) SEM (n = 5 for each group). \( *P < 0.05; \) final versus initial body weight.

For water intake, data represent means \( \pm \) SD of two experiments.

\( P < 0.05 \) versus fed (final body weight) (by analysis of variance); for water intake, data represent means \( \pm \) SD of two experiments.
were observed in the fasted group during a period of up 
to 6 days of fasting.

Exogenous Leptin Protects Fasted Mice from 
LPS-Induced Lethality

Food deprivation for 48 hours reduced serum leptin lev-
els from 6.3 ± 0.8 to 1.3 ± 0.3 ng/ml (Table 2). A single 
i.p. injection of 1 µg/g body weight of leptin has been 
previously shown to produce concentrations of leptin at 6 
and 12 hours after injection similar to fed controls.4 We 
measured leptin levels in the serum of fasted mice 
treated with leptin 23 hours after the last leptin injection 
and leptin levels were still higher than in fasted mice 
(1.3 ± 0.3 versus 2.7 ± 0.4 ng/ml).

We next determined whether leptin deficiency second-
ary to fasting contributes to the increased susceptibility to 
endotoxic shock. To test this possibility, mice were fed, 
fasted, or fasted and leptin treated for 48 hours. Mice 
were subsequently injected with a dose of LPS of 15 
mg/kg and survival was monitored up to 6 days. As 
expected, this dose of LPS resulted in 100% mortality in 
fasted mice (Figure 2). In contrast, 73% of the fed mice 
survived. Exogenous leptin replacement during the pe-
riod of food deprivation was protective, increasing survival 
(31% survival). Thus, decreasing the fasting-induced fall in 
leptin with exogenous leptin administration substantially 
blunts the increase in sensitivity to LPS toxicity.

Table 2. Serum Leptin Levels after LPS in Fed, Fasted and 
Leptin-Treated Fasted Mice

<table>
<thead>
<tr>
<th>Leptin (ng/ml)</th>
<th>Basal</th>
<th>LPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>6.2 ± 0.61</td>
<td>16.6 ± 2.92</td>
</tr>
<tr>
<td>Fasted</td>
<td>1.3 ± 0.3</td>
<td>14.1 ± 4.14</td>
</tr>
<tr>
<td>Fasted + leptin</td>
<td>2.7 ± 0.4*</td>
<td>15.3 ± 4.7†</td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 5 for each group). 
*P < 0.05, †P < 0.001, versus fasted basal (by Student’s t-test). 
*P < 0.05, †P < 0.001, versus fasted (by Student’s t-test).

Because LPS has been shown to acutely increase 
leptin levels, we next measured serum leptin levels after 
LPS treatment. Mice were fed, fasted, or fasted and 
treated with leptin (twice a day, 1 µg/g body weight, i.p.) 
for 48 hours. Mice were then challenged with LPS or 
saline and 8 hours later serum leptin levels were deter-
mimed. As shown in Table 2, despite the different basal 
levels due to different nutritional status, leptin levels were 
induced by LPS to similar levels in fasted, leptin-replaced 
fasted, or fed mice (Table 2). These data suggest that, in 
mice, basal leptin levels, rather than the levels induced 
by LPS, are crucial in determining sensitivity to LPS.

Serum Glucose and Corticosterone Levels after 
LPS Administration

Endotoxemia and fasting are both conditions character-
ized by reduced serum glucose levels. To evaluate the 
possibility that a more profound hypoglycemia might oc-
cur in fasted mice treated with LPS, we measured serum 
glucose levels before and after LPS in the different nutri-
tional conditions. As expected, depriving mice of food for 
48 hours caused a decrease in basal glucose levels from 
138 to 63 mg/dl (Table 3). As previously reported,4 lep-
tin treatment of fasted mice did not alter the basal levels of 
glucose. After LPS administration, a profound decrease 
in blood glucose was observed in fed mice; glucose 
levels after LPS were somewhat lower in fasted mice. 
Leptin treatment gave intermediate levels of glucose after 
LPS (Table 3).

The activation of the HPA axis is an important protec-
tive response to infection and inflammation and is also 
part of the neuroendocrine response to starvation.5,21 
Corticosterone levels rise during starvation and leptin has 
been shown to blunt that increase.4 We therefore mea-
sured serum corticosterone levels. As reported previ-
ously,4 we also found that fasted mice display high basal 
levels of serum corticosterone compared to fed mice and 
exogenous leptin treatment blunted the fasting-induced
rise in basal serum corticosterone levels by 30% (Table 3). After LPS administration, a marked increase in serum corticosterone was observed in fed mice, which resulted in a difference between basal and induced levels (Δ, the difference between two sets of mice, fed and fed-treated with LPS) of 378 ng/ml. Less induction of corticosterone was observed in fasted mice (Δ = 191 ng/ml), suggesting that fasting affects the responsiveness of the HPA axis to LPS. When animals were treated with leptin during fasting, there was a more marked increase in corticosterone after LPS administration, resulting in a Δ of 438 ng/ml, comparable to that which occurred in fed mice.

Circulating Cytokine Levels after LPS Administration

The systemic release of cytokines mediates the endotoxic shock syndrome. We therefore assessed whether the increased sensitivity to LPS lethality due to fasting was associated with increases in TNF, IFN-γ, or IL-1β induction. As shown in Figure 3, TNF induction by LPS was found to be approximately fivefold higher in fasted than in fed mice. Moreover, endogenous leptin replacement inhibited this marked rise in TNF levels after LPS administration in fasted mice. In contrast, LPS induced lower levels of IFN-γ in both fasted and fasted leptin-treated mice compared to fed mice (Table 4). No significant differences were observed in IL-1β levels among the three groups (Table 4). TNF, IFN-γ, and IL-1β were undetectable in the serum of mice that were fed, fasted, or fasted and treated with leptin injected with saline.

Exogenous Leptin Protects Fasted Mice from TNF-Induced Lethality

The increase in TNF production during fasting may contribute to the greater LPS toxicity seen in fasted animals. Significantly, this increase in the induction of TNF during fasting seems to be, at least in part, prevented by leptin replacement (Figure 3). Because genetic leptin deficiency has previously been shown to increase sensitivity to the lethal effect of TNF,20 we therefore next assessed whether fasting would exacerbate TNF toxicity. A dose of mTNF of 500 μg/kg caused 55% lethality when administered to fed mice (Figure 4). This same dose resulted in 100% lethality in mice fasted for 48 hours. Most importantly, leptin replacement reversed the increase in TNF toxicity induced by fasting, reducing the lethality to levels comparable to fed mice (Figure 4).

Discussion

Starvation has long been known to induce a state of immunodeficiency, characterized by disproportionate loss of lymphoid tissue, impaired cell-mediated immunity, and increased susceptibility to infectious disease.7,22 Here we show that short-term starvation sensitizes mice

Table 3. Serum Corticosterone Levels after LPS in Fed, Fasted and Leptin-Treated Fasted Mice

<table>
<thead>
<tr>
<th></th>
<th>Glucose (mg/dl)</th>
<th>Corticosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Basal</td>
<td>LPS</td>
</tr>
<tr>
<td>Fed</td>
<td>138.0 ± 5.8*</td>
<td>50.7 ± 2.5**</td>
</tr>
<tr>
<td>Fasted</td>
<td>62.6 ± 1.6</td>
<td>41.2 ± 2.6†</td>
</tr>
<tr>
<td>Fasted + leptin</td>
<td>67.8 ± 0.8</td>
<td>45.0 ± 1.3†</td>
</tr>
</tbody>
</table>

Δ represents the difference between the corticosterone levels measured in the two sets of mice (saline and LPS treated).

Data are means ± SEM (n = 5 for each group).

*P < 0.05, †P < 0.001 versus fed basal (by Student’s t-test).

**P < 0.05 versus fed basal (by Student’s t-test).

Table 4. Serum IFN-γ and IL-1β Levels after LPS in Fed, Fasted and Leptin-Treated Fasted Mice

<table>
<thead>
<tr>
<th></th>
<th>IFN-γ (ng/ml)</th>
<th>IL-1β (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fed</td>
<td>5.8 ± 0.3</td>
<td>170 ± 54</td>
</tr>
<tr>
<td>Fasted</td>
<td>3.5 ± 0.7†</td>
<td>231 ± 70</td>
</tr>
<tr>
<td>Fasted + leptin</td>
<td>2.3 ± 0.4†*</td>
<td>251 ± 70</td>
</tr>
</tbody>
</table>

Data are means ± SEM (n = 5 for each group).

†P < 0.01 versus fed (by analysis of variance).

‡P < 0.001 versus fed (by analysis of variance).
leptin treatment during fasting on TNF-induced lethality. Mice were fed, fasted, or fasted and treated with leptin for 48 hours. Fasted mice treated with leptin were administered leptin i.p., 1 μg/g body weight, twice a day for 2 days. Fasted or fed mice received saline. Mice were then challenged with mTNF (0.5 mg/kg, i.v.). Mortality was assessed daily for 6 days. In contrast, no differences were observed in IL-1β and IFN-γ levels of IFN-γ measured in fasted mice might also reflect alterations in lymphocyte populations because T lymphocytes are an important source of IFN-γ and are depleted during fasting. Interestingly, it has recently been shown that prevention of lymphocyte apoptosis is associated with improved survival in a murine model of sepsis, suggesting a critical role of the lymphocyte in resolving severe infection. It has been suggested that the significant decrease in the number of lymphocytes which occurs in septic and endotoxic shock, will impact multiple facets of the immunological response and may lead to uncontrolled inflammatory response and death. The profound lymphopenia of fasted mice might therefore substantially contribute to their increased susceptibility to infection and inflammation and leptin could confer protection by preventing fasting-induced lymphopenia.

A decrease in serum glucose is among the metabolic changes occurring during starvation and is also a component of the acute phase response. LPS further reduced glucose levels in fasted mice and the levels were lower than those in LPS-treated fed animals. However, leptin treatment during fasting did not affect either the basal glucose levels, as previously reported, or significantly reverse the further decrease in the glucose levels after LPS. It seems therefore that the protective effect of leptin on LPS lethality is not mediated by the hypoglycemic response.

During the acute phase response, the activation of the HPA axis results in an increase in glucocorticoids, which attenuates the inflammatory reaction by exerting a negative feedback on TNF production after LPS and also protects against TNF toxicity. Several lines of evidence suggest a regulatory loop between HPA axis and circulating leptin. Leptin deficiency, as observed in ob/ob mice, results in chronic HPA axis activation, which is reversed by leptin treatment. In addition, leptin administration substantially prevents the activation of the HPA.
axis in response to stress or fasting. Furthermore, in mice, adrenalectomy decreases basal leptin levels and corticosterone replacement therapy restores circulating leptin to physiological levels. Here we show that fasted mice have an impaired HPA axis activation after LPS administration, suggesting that fasting might affect the responsiveness of the HPA axis to LPS. As previously reported, leptin administration during fasting reduced the basal serum corticosterone levels in fasted mice; in addition we now show that leptin restores the HPA axis activation in response to LPS in fasted mice to levels comparable to that observed in fed animals. It is possible that the restored HPA axis response contributes to the protective effect of leptin in fasted animals.

Interestingly, α-MSH, a pro-opiomelanocortin-derived peptide, is an anti-inflammatory agent which, like leptin, is down-regulated by fasting. A link between leptin and the melanocortin system has been suggested and leptin has been demonstrated to stimulate pro-opiomelanocortin expression. Therefore the protective effect of leptin during starvation, might, at least to some extent, be mediated by α-MSH.

We also have shown that fasting increases sensitivity to TNF lethality, indicating that the effect of fasting on LPS toxicity also occurs downstream to TNF production. Importantly, leptin replacement reversed the increase in TNF toxicity secondary to fasting. Therefore, the protective effect of leptin on TNF toxicity is also likely to be downstream to TNF suggesting that leptin exerts its anti-inflammatory effects both by decreasing TNF induction by LPS and also by protecting against the TNF-induced inflammatory cascade. The protective effect of leptin on TNF toxicity in starved mice might involve TNF-induced cytokine production. For example, IL-1 contributes to TNF lethality and cross-regulation exists between leptin and TNF toxicity.

In summary, we have shown that fasting increases susceptibility to LPS and leptin administration can inhibit this increase. The ability of leptin to inhibit TNF production and TNF toxicity could partially account for the protective effect of leptin on LPS toxicity in fasted mice. These data indicate that leptin represents a link between nutritional status and immune response. Our findings suggest that the decrease in leptin that occurs during food deprivation could contribute to the increased morbidity and mortality of infections in malnourished patients and raises the possibility that leptin treatment may be beneficial in malnourished patients at high risk for sepsis.

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Leptin and the Effect of Fasting on Endotoxic Shock

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