

# Obesity-induced Hyperleptinemia Improves Survival and Immune Response in a Murine Model of Sepsis

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## ABSTRACT

**Background:** Obesity is a growing health problem and associated with immune dysfunction. Sepsis is defined as systemic inflammatory response syndrome that occurs during infection. Excessive inflammation combined with immune dysfunction can lead to multiorgan damage and death.

**Methods:** The authors investigated the influence of a class 1 obesity (body mass index between 30 and 34.9) on immune function and outcome in sepsis and the role of leptin on the immune response. The authors used a long-term high-fat-diet feeding model (12 weeks) on C57Bl/6 mice (n = 100) and controls on standard diet (n = 140) followed by a polymicrobial sepsis induced by cecal ligation and puncture.

**Results:** The authors show that class 1 obesity is connected to significant higher serum leptin levels (data are mean  $\pm$  SEM) ( $5.7 \pm 1.2$  vs.  $2.7 \pm 0.2$  ng/ml; n = 5;  $P = 0.033$ ) and improved innate immune response followed by significant better survival rate in sepsis (71.4%, n = 10 vs. 10%, n = 14;  $P < 0.0001$ ). Additional sepsis-induced increases in leptin levels stabilize body temperature and are associated with a controlled immune response in a time-dependent and protective manner. Furthermore, leptin treatment of normal-weight septic mice with relative hypoleptinemia (n = 35) also significantly stabilizes body temperature, improves cellular immune response, and reduces proinflammatory cytokine response resulting in improved survival (30%; n = 10).

**Conclusions:** Relative hyperleptinemia of class 1 obesity or induced by treatment is protective in sepsis. Leptin seems to play a regulatory role in the immune system in sepsis, and treatment of relative hypoleptinemia could offer a new way of an individual sepsis therapy. (**ANESTHESIOLOGY 2014; 121:98-114**)

**O**VERWEIGHT and obesity are growing health problems, especially in industrialized countries with more than 50% of adults being overweight or obese mostly due to dietary nutrients.<sup>1,2</sup> Obesity is associated with a wide range of comorbidities such as cardiovascular diseases and diabetes mellitus causing an increase of medical costs.<sup>3</sup> Overweight and obesity are classified by using the “body mass index” (BMI), which is a number correlated with the amount of body fat without a direct measurement of fat content.<sup>4</sup> A limitation to the BMI classification system is that it does not reflect body composition or fat mass contribution.

Dietary nutrients also affect the metabolic and endocrine status.<sup>5</sup> Although infections can be associated with a nutritional deficiency, obesity can alter the immune system such that immune dysregulation can occur over the course of time.<sup>6,7</sup> It is known that obesity induces a low-grade chronic inflammation<sup>8</sup> and can alter leukocyte proliferation, activation, and function.<sup>9,10</sup> Immune responses can be enhanced or depressed by dietary lipids, depending on both the

### What We Already Know about This Topic

- Critically ill obese patients do not have increased mortalities

### What This Article Tells Us That Is New

- The relative hyperleptinemia of class 1 obesity or exogenous leptin administration was protective in experimental sepsis
- This suggests leptin is a possible novel therapy for sepsis

concentration and extent of unsaturation of fatty acids. The question of whether these obesity-induced changes improve or worsen the immune response in sepsis has not been fully elucidated and remains controversial.<sup>9,11</sup>

Sepsis is defined as a systemic inflammatory response syndrome that occurs during infection.<sup>12</sup> This response can lead to excessive inflammation, followed by profound immune suppression, multiorgan damage, and death with a mortality rate up to 50%.<sup>13,14</sup> Despite decades of research, effective sepsis therapy is missing and supportive treatment does not

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increase survival rate significantly. However, it is clear that early diagnosis and treatment of sepsis improve outcome.<sup>15</sup> A variety of cytokines and hormones are involved in orchestrating and regulating a sepsis-induced immune response. One of these hormones is leptin, the protein product of the obese gene.<sup>16,17</sup> Secreted by white adipocytes and initially observed as a satiety factor, leptin regulates food intake, body weight (BW), and many other biological effects by its peripheral and central action,<sup>18–21</sup> thus playing a key role in developing obesity.<sup>16</sup> As part of its immune-modulating function, it is able to increase activation and proliferation of monocytes<sup>22</sup> and T-cells<sup>23</sup> and mediates homeostasis in a variety of immune cells.<sup>24</sup> The exact role of leptin in the immune response during sepsis is still unclear because been demonstrated to be either protective,<sup>25–28</sup> have no influence,<sup>29–31</sup> or detrimental.<sup>32</sup>

Using a murine model of sepsis, we investigated the influence of a class 1 obesity on the outcome of sepsis and a possible regulatory and protective function of leptin on the immune response in a time-dependent manner. To simulate a diet-induced class 1 obesity as seen in humans, we used a long-term high-fat-diet (hfd) feeding model (12 weeks) on C57Bl/6 mice followed by polymicrobial sepsis induced by cecal ligation and puncture (CLP). We hypothesized that: (1) a class 1 obesity induced by chronic hfd will enhance survival due to increased leptin serum levels, and (2) leptin treatment of relative hypoleptinemia during sepsis can also improve immune response.

## Materials and Methods

### Animal Studies

All experiments involving animals were performed under protocols approved by the regional animal study committee (Government of Bavaria, Munich, Germany) and are in agreement with the guidelines for the proper use of animals in biomedical research and the guidelines of the European Communities Directive 86/609/EEC regulating animal research or were approved by the Institutional Animal Care and Use Committee of the University of Cincinnati and conformed to National Institutes of Health guidelines. Male C57Bl/6J (wild-type) mice 7 weeks of age were obtained from Janvier Labs (Le Genest Saint Isle, France) and maintained in individually ventilated cages under a 12h light–12h dark cycle under conditions of controlled temperature ( $22^{\circ} \pm 2^{\circ}\text{C}$ ) and relative humidity ( $55 \pm 5\%$ ).

### Feeding of Mice

Mice were maintained *ad libitum* on water and an hfd (E15186-34; Ssniff, Soest, Germany) for 12 weeks to induce class 1 obesity in the animals. The hfd contained mainly saturated fatty acids, and the metabolizable energy was from fat (50.0%), protein (22.0%), and carbohydrate (28.0%). Age-matched C57Bl/6J control mice received water and a control chow diet *ad libitum* for 12 weeks with calories provided by fat (11%), protein (23%), and carbohydrate (65%).

### Cecal Ligation and Puncture

Male mice 19 weeks of age were inflicted with polymicrobial sepsis induced by CLP as described previously.<sup>33</sup> In brief, the CLP operations were always performed between 9:00 AM and 11:59 AM. Mice were anesthetized with a single intraperitoneal injection of pentobarbital (50 mg/kg BW) and buprenorphine (0.3 mg/kg BW). They were allowed to spontaneously breathe room air on an electric heating pad. The skin was shaved and disinfected. After a 1 cm laparotomy (midline incision through the linea alba while avoiding injury to the abdominal vasculature), the latter 80% of the cecum was ligated with a 3-0 silk tie (Catgut, Markneukirchen, Germany) and punctured once on the antimesenteric side with a 23-gauge needle. A small amount of the bowel contents was extruded through the puncture hole to ensure a full-thickness perforation. Care was taken not to obstruct the bowel, and this was tested after the animals' death. The cecum was replaced in its original location, and the midline incision was closed by two-layer suture with 4-0 silk (Catgut). The animals were resuscitated with 1 ml of sterile saline subcutaneously and kept on a heating blanket for 2 h. Sham-treated controls underwent the same surgical procedures (laparotomy and resuscitation), but the cecum was neither ligated nor punctured. After CLP, mice were observed every 24 h for 10 days and monitored for body temperature, BW, and signs of distress. Mice fulfilling fixed criteria for termination during the experiments were killed.

### Leptin Administration (Intraperitoneal)

Recombinant murine leptin (R&D Systems, Minneapolis, MN) was used for intraperitoneal administration.<sup>34</sup> Stock solution was prepared according to the protocol of the manufacturer and stored at  $-80^{\circ}\text{C}$ . After CLP, a leptin dosage of 1  $\mu\text{g/g}$  BW for each injection was performed twice a day until the study termination. This leptin replacement protocol has been shown to achieve normal circulating leptin levels<sup>23</sup> and was chosen to incorporate the range of serum levels measured in humans.<sup>35</sup> As shown in previous experiment, volume-adapted injection of vehicle (intraperitoneal) had no effect on survival and immune response.<sup>27</sup> For dosage-finding experiments, different leptin dosages (0 [= vehicle], 0.2, 1, and 5  $\mu\text{g/g}$  BW) were injected after CLP twice a day until the study termination. For the survival study regarding leptin treatment in control mice, 1  $\mu\text{g/g}$  BW leptin was injected intraperitoneal twice a day until the study termination, whereas the control group received volume-adapted vehicle (intraperitoneal).

### Bronchoalveolar Lavage

Bronchoalveolar lavage was performed 6 and 24 h after CLP. In brief, the trachea was exposed and cannulated with a polyethylene tube connected to a syringe. The lungs were washed by flushing with phosphate buffered saline solution through the tracheal cannula as one 1 ml aliquot and the recovered fluid saved as bronchoalveolar lavage. Protein concentration

of the bronchoalveolar lavage fluid was determined by using a bicinchoninic acid Protein Assay Kit (Pierce, Rockford, IL).

### Flow Cytometry for Surface Staining

Single-cell suspensions of blood were saved in ice-cold Hank buffered salt solution. After washing twice with Hank buffered salt solution, cells from blood, peritoneal lavage fluid, and bronchoalveolar lavage fluid were prepared using standard procedures. Cells were resuspended in fluorescent-activated cell sorting (FACS) buffer (phosphate buffered saline with 1% bovine albumin and 0.1% azide). To avert nonspecific binding to mouse Fc $\gamma$  receptors, cells were blocked with anti-mouse CD16/CD32 (20 min, 1 mg/ml; BD Pharmingen, Franklin Lakes, NJ) and 1% rat serum (Life Technologies, Paisley, United Kingdom) added to the FACS buffer. Cells were stained in a three-color configuration with fluorescein-, phycoerythrin-, or peridinin chlorophyll protein-labeled antibodies. The following antibodies were used: anti-CD4 (clone: RM4-5), anti-NK1.1 (clone: PK136), anti- $\gamma\delta$ TCR (clone: UC7-13D5), anti-TCR $\beta$  (clone: H57-597), anti-CD8 (clone: 53-6.7), anti-CD68 (clone: Y1/82A), anti-Ly6C (clone: RB6-8C5), anti-Ly6G (clone: 1A8), anti-CD45 (clone: 30F-11), and anti-IL-17A (clone: TC11-18H10) (all from BD Biosciences, San Diego, CA). The samples were washed thoroughly with FACS buffer and analyzed using a FACScan flow cytometer and Cell Quest software (BD Biosciences) with standard settings. Ten thousand to 20,000 cells were analyzed from each sample. Mean fluorescence intensity (MFI) was measured by flow cytometry as a marker of cell-surface expression of adhesion molecules.

### Oxidative Burst and Activation of Neutrophils

For determination of neutrophil activation and hydrogen peroxide production, blood was collected by cardiac puncture and 5 to 6 blood droplets saved in ice-cold Hank buffered salt solution. Cells from blood and peritoneal lavage fluid were washed twice with ice-cold Hank buffered salt solution. In parallel experiments, cells were incubated at 37°C for 15 min and then activated for another 15 min with 10<sup>-5</sup> M of the chemotactic tripeptide formyl-methionyl-leucyl-phenylalanine. Activation was stopped by putting cells on ice. Fc $\gamma$  receptors were blocked by anti-mouse CD16/CD32 (20 min; 1 mg/ml; BD Pharmingen), and cells were incubated with anti-mouse Gr1 (clone RB6-8C5; BioLegend, San Diego, CA) as well as PE anti-mouse CD11b (clone M1/70; BioLegend) to identify granulocytes. After washing with phosphate buffered saline, cells were analyzed by flow cytometry.

### Body Temperature

Body temperature was measured rectally using a Greisinger GTH1170 thermometer (Fa. Greisinger, Regenstauf, Germany).

### Cytokine and Leptin Measurement

Peritoneal fluid was harvested from mice after aseptic preparation of the abdominal wall, followed by injection of 9 ml of sterile saline into the peritoneal cavity and aspiration of peritoneal fluid. Whole blood was collected by sterile, percutaneous cardiac puncture and placed in lithium-heparin plasma separator tubes (BD Biosciences). Serum was obtained from blood samples after centrifugation (at 10,000g for 10 min) and stored at -20°C until analysis. Interleukin (IL)-6, IL-10, IL-1 $\beta$ , tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), monocyte-inhibitory protein-2 (MIP-2), and leptin levels in the peritoneal fluid and serum were analyzed using enzyme-linked immunosorbent assay kits according to the protocol of the manufacturer (PeproTech, Hamburg, Germany) with a sensitivity of 62, 47, 63, 16, 16, and 20 pg/ml, respectively.

### Leukocytes Counts

Numbers of leukocytes from blood and peritoneal lavage fluid were measured using a Sysmex KX-21N cell counter (Sysmex, Lincolnshire, IL).

### Colony-forming Units

Bacterial counts were performed on aseptically harvested blood by cardiac puncture and peritoneal lavage fluid both collected 6 h after CLP. Samples were serially diluted in sterile saline and cultured on tryptic soy agar pour plates. Plates were incubated at 37°C for 48 h, and colony-forming unit counts were performed.

### Organ Histology

Organ tissues were fixed in 5% neutral-buffered formalin (Sigma-Aldrich, Hamburg, Germany) and then embedded in paraffin for light microscopy. Sections were stained with hematoxylin and eosin for histological examination in an investigator-blinded manner.

### Statistical Analysis

GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA) was used for statistical analyses. Quantitative data are presented as mean  $\pm$  SEM. Survival probabilities were graphically assessed by the Kaplan-Meier method, and statistical comparisons were performed using the log-rank test for survival. Data were tested for normal distribution using the Kolmogorov-Smirnov test. The nature of hypothesis testing was two-tailed. For simple comparisons of two groups (hfd and control groups) at the starting timepoint to define baseline observations ("before" data), a two-tailed Student *t* test for unpaired samples was used. For comparisons of body temperature between groups over 10 days or 7 days survival, a two-way ANOVA for repeated measurements was used followed by a *post hoc* Bonferroni test. For comparisons of more than two groups concerning the influence of hfd on immune response parameters, a two-way ANOVA for unmatched samples was used followed by analyses between groups or within groups over

time using a *post hoc* Bonferroni test. Comparisons of more than two groups concerning the influence of leptin treatment or different leptin dosages on immune response parameters, a two-way ANOVA for unmatched samples was used followed by analyses between groups or within groups over time using a *post hoc* Bonferroni test. Analysis of the leptin-dosage data concerning IL-6, IL-10, IL-17 production and colony-forming units using one group of mice (controls) at one single timepoint (6 h after CLP) were done by using a one-way ANOVA for unmatched samples followed analyses between different dosages using a *post hoc* Bonferroni test. A *P* value of 0.05 or less was considered as statistically significant.

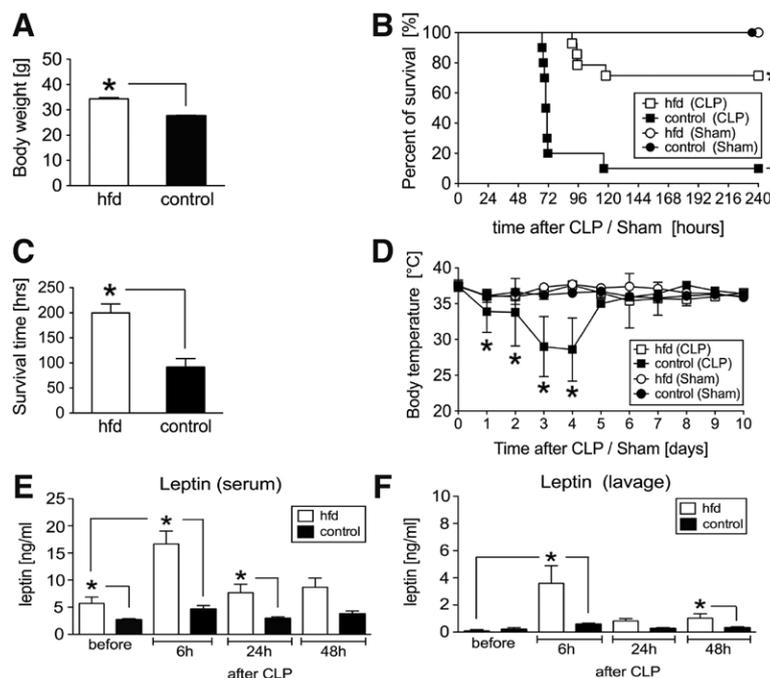
## Results

### Hfd Mice Show Improved Outcome after CLP

To simulate a diet that would induce an obesity as observed in humans, normal wild-type mice (ca.  $17.9 \pm 0.6$  g BW;

$n = 40$ ) were fed an hfd (50% fat) for 12 weeks. These mice gained significantly more BW (25%) within 12 weeks of feeding as compared with the BW of control group with normal standard diet (11% fat) resulting in a significant higher BW ( $34.4 \pm 0.5$  g;  $n = 55$ ;  $P < 0.0001$ ) than the controls ( $27.7 \pm 0.2$  g;  $n = 60$ ) (fig. 1A). We conclude that 12 weeks after initiation of the diet, the weight gain of 25% is equivalent to an increase of BMI in a normal person generating an obesity class 1.

We first determined the impact of an hfd-induced obesity in the outcome of polymicrobial sepsis. CLP was performed using hfd and control mice. As shown in figure 1B, hfd mice had a significant increase in survival rate (71.4%;  $n = 14$ ;  $P < 0.0001$ ) as compared with the survival rate in control mice with standard diet (10%;  $n = 10$ ). Sham mice of both hfd ( $n = 3$ ) and control groups ( $n = 3$ ) showed a survival rate of 100%. Consistent with these survival rates, the mean survival time was increased in hfd mice ( $199.9 \pm 17.6$  h;  $n = 14$ ;  $P = 0.0003$ ) as compared with the mean survival time



**Fig. 1.** High-fat-diet (hfd) contributed to survival, body temperature, and leptin levels after cecal ligation and puncture (CLP)-induced sepsis. (A) Male C57Bl/6 mice (7 weeks old,  $17.9 \pm 0.6$  g body weight,  $n = 40$ ) receiving hfd gained a significant higher body weight within 10–12 weeks ( $34.4 \pm 0.7$  g,  $n = 55$ ,  $*P < 0.0001$  of unpaired *t* test) as compared with the body weight of control group with standard diet ( $27.7 \pm 0.2$  g,  $n = 60$ ). (B) Survival after CLP in male control mice ( $n = 10$ ), hfd mice ( $n = 14$ ), and Sham mice of both groups (hfd:  $n = 3$ , controls:  $n = 3$ ) monitored for 10 days. The survival curve combines data from two independent experiments, each of which gave similar results;  $*P < 0.0001$  of log-rank test as compared with the control group. (C) Mean survival time of hfd ( $n = 14$ ) and control group ( $n = 10$ ) after CLP (combined data from two independent experiments, each of which gave similar results);  $*P = 0.0003$  of unpaired *t* test. (D) Body temperature as independent predictor of mortality was decreased in control mice ( $n = 10$ ) after CLP as compared with that of hfd mice ( $n = 14$ ) and Sham mice (hfd:  $n = 3$ , controls:  $n = 3$ );  $*P < 0.05$  of two-way ANOVA for repeated measurements followed by a *post hoc* Bonferroni test. (E, F) Serum and peritoneal lavage fluid were collected before (hfd:  $n = 5$ , controls:  $n = 5$ ), 6 h (hfd:  $n = 14$ , controls:  $n = 19$ ), 24 h (hfd:  $n = 14$ , controls:  $n = 23$ ), and 48 h (hfd:  $n = 10$ , controls:  $n = 11$ ) after CLP (combined data from several independent experiments). Serum and lavage levels of leptin were determined by enzyme-linked immunosorbent assay;  $*P < 0.05$  of unpaired *t* test for comparison of baseline levels between two groups (hfd and control) and of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time. All values are expressed as mean  $\pm$  SEM.

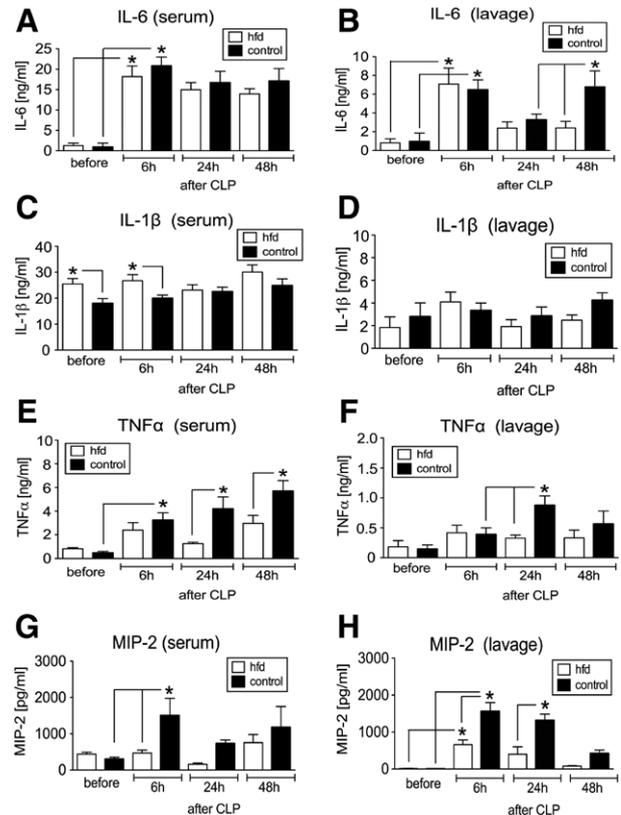
in control mice ( $91.3 \pm 17.2$  h;  $n = 10$ ) (fig. 1C). There was no significant change in BW in either group. In contrast, body temperature, an independent predictor of mortality,<sup>36</sup> was significantly decreased in control mice within the first 4 days after CLP ( $n = 10$ ;  $P = 0.019$ ) as compared with that in the hfd group ( $n = 14$ ) and both sham groups (hfd:  $n = 3$ , controls:  $n = 3$ ) (fig. 1D). The hfd group showed significant higher serum leptin levels before CLP as compared with the leptin levels in the control group ( $5.7 \pm 1.2$  vs.  $2.7 \pm 0.2$  ng/ml;  $n = 5$  per group;  $P = 0.033$ ) (fig. 1E). Although low levels of leptin could be detected in peritoneal lavage of both groups before sepsis induction ( $0.1 \pm 0.1$  vs.  $0.2 \pm 0.1$  ng/ml;  $n = 5$  per group), 6 h after CLP there was a significant increase in leptin levels in both serum ( $16.7 \pm 2.4$  ng/ml;  $n = 14$ ;  $P = 0.019$ ) and peritoneal lavage ( $3.6 \pm 1.3$  ng/ml;  $n = 14$ ;  $P = 0.003$ ) in the hfd group (fig. 1F). After 24 h ( $n = 14$ ;  $P = 0.0007$ ) and 48 h ( $n = 10$ ;  $P = 0.006$ ), these levels decreased again but were still higher as compared with that in the control group. In the control group, no increase in leptin could be detected in both serum and peritoneal lavage 6 h ( $n = 19$ ), 24 h ( $n = 23$ ), and 48 h ( $n = 11$ ) after CLP.

#### Hfd Improves Proinflammatory Immune Response

A significant increase in IL-6 levels was observed 6 h after CLP in serum ( $18.2 \pm 2.6$  and  $20.8 \pm 2.2$  ng/ml;  $P = 0.004$ ) (fig. 2A) and peritoneal lavage ( $7.1 \pm 1.7$  and  $6.5 \pm 1.1$  ng/ml;  $P = 0.008$ ) in both hfd ( $n = 14$ ) and control groups ( $n = 19$ ) (fig. 2B). This increase was followed by a decrease after 24 h in both groups (hfd:  $n = 14$ ; control:  $n = 23$ ). In the peritoneal lavage, IL-6 levels of the hfd group were lower 24 and 48 h after CLP as compared with IL-6 levels in controls. Interestingly, we detected a second significant increase in peritoneal lavage of the control group ( $6.8 \pm 1.7$  ng/ml;  $n = 11$ ) 48 h after CLP as compared with that in the hfd group ( $2.4 \pm 0.7$  ng/ml;  $n = 10$ ;  $P = 0.044$ ).

The hfd group showed significantly higher serum IL-1 $\beta$  levels before sepsis ( $25.5 \pm 2.1$  ng/ml;  $n = 5$ ;  $P = 0.026$ ) as compared with that in the controls ( $18.1 \pm 1.8$  ng/ml;  $n = 5$ ) (fig. 2C). This difference remained significant 6 h after CLP (hfd:  $26.7 \pm 2.4$  ng/ml,  $n = 14$  vs. control:  $19.9 \pm 1.3$  ng/ml,  $n = 23$ ;  $P = 0.011$ ) with no significant differences at the 24 h (hfd:  $n = 14$ ; control:  $n = 23$ ) and the 48 h timepoint (hfd:  $n = 10$ ; control:  $n = 11$ ). Peritoneal IL-1 $\beta$  levels of both groups showed no significant changes at the 6 h (hfd:  $n = 14$ ; control:  $n = 19$ ), 24 h (hfd:  $n = 14$ ; control:  $n = 23$ ), and 48 h timepoint (hfd:  $n = 10$ ; control:  $n = 11$ ) (fig. 2D).

Tumor necrosis factor- $\alpha$  serum levels increased 6 h after CLP in both groups (hfd:  $n = 14$ ; control:  $n = 19$ ), in the controls even significantly ( $3.2 \pm 0.6$  ng/ml;  $P = 0.006$ ). TNF $\alpha$  serum levels of controls trended higher after CLP reaching significant higher levels 24 h (control:  $4.2 \pm 1.0$  ng/ml,  $n = 23$  vs. hfd:  $1.3 \pm 0.1$  ng/ml,  $n = 14$ ;  $P = 0.008$ ) and 48 h (control:  $5.7 \pm 0.9$  ng/ml,  $n = 11$  vs. hfd:  $3.0 \pm 0.7$  ng/ml,  $n = 10$ ;  $P = 0.023$ ) after CLP as compared with that in the hfd group (fig. 2E). In addition, we detected a significant peak of TNF $\alpha$



**Fig. 2.** Proinflammatory cytokine response after cecal ligation and puncture (CLP). Serum and peritoneal lavage fluid were collected from high-fat-diet (hfd) and control mice before (hfd:  $n = 5$ , controls:  $n = 5$ ), 6 h (hfd:  $n = 14$ , controls:  $n = 19$ ), 24 h (hfd:  $n = 14$ , controls:  $n = 23$ ), and 48 h (hfd:  $n = 10$ , controls:  $n = 11$ ) after CLP (combined data from several independent experiments). Serum and peritoneal lavage fluid levels of (A, B) interleukin (IL)-6, (C, D) IL-1 $\beta$ , (E, F) tumor necrosis factor- $\alpha$  (TNF $\alpha$ ), and (G, H) monocyte-inhibitory protein-2 (MIP-2) were determined by enzyme-linked immunosorbent assay. All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of unpaired  $t$  test for comparison of baseline levels between two groups (hfd and control) and of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time.

at the 24 h timepoint in peritoneal lavage of the control group reaching significantly higher levels as compared with that in the hfd group (control:  $0.9 \pm 0.2$  ng/ml,  $n = 23$  vs. hfd:  $0.3 \pm 0.1$  ng/ml,  $n = 14$ ;  $P = 0.007$ ), whereas the TNF $\alpha$  levels of the hfd group did not change within the first 48 h (6 h:  $n = 14$ ; 24 h:  $n = 14$ ; and 48 h:  $n = 10$ ) (fig. 2F).

Levels of monocyte-inhibitory protein-2 in the control group were significantly higher in both serum and peritoneal lavage ( $1,506 \pm 471$  pg/ml;  $n = 19$ ;  $P = 0.045$  and  $1,561 \pm 233$  pg/ml;  $n = 19$ ;  $P = 0.003$ ) 6 h after CLP as compared with that in the hfd group ( $467 \pm 86$  pg/ml;  $n = 14$  and  $658 \pm 125$  pg/ml;  $n = 14$ ). At the 24 h timepoint, MIP-2 levels were still higher in serum of the control group ( $727 \pm 101$  pg/ml; pg/ml;  $n = 23$ ) and significantly higher in the peritoneal lavage fluid ( $1,312 \pm 170$  pg/ml;  $n = 23$ ;  $P = 0.002$ ) as compared

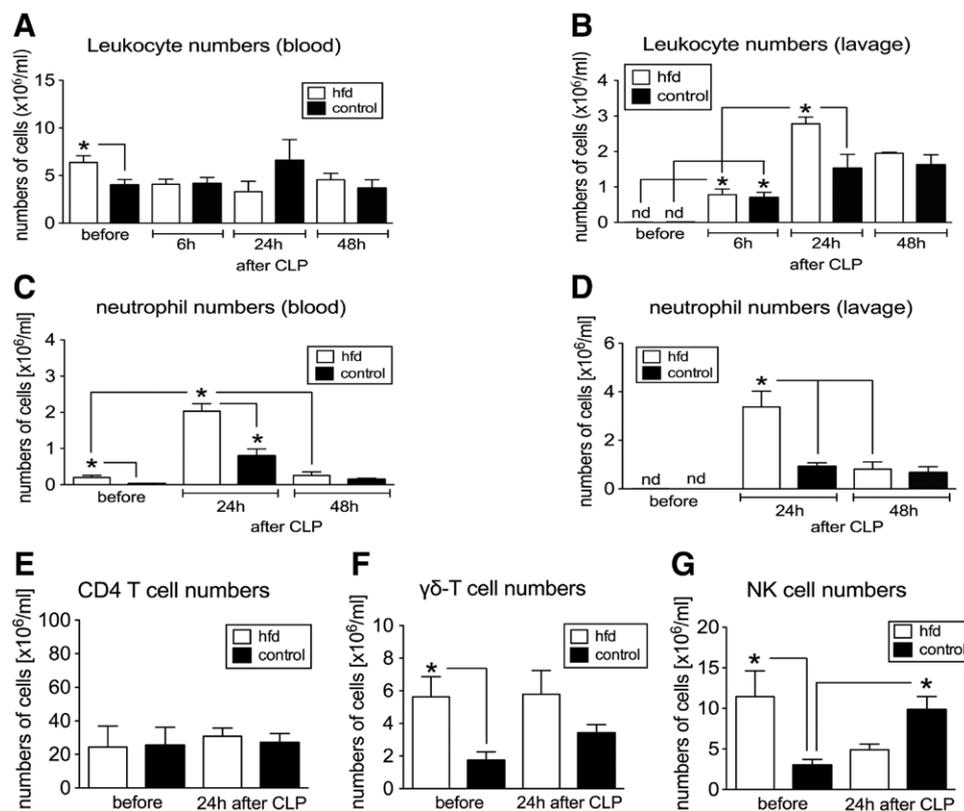
with that in the hfd group (serum:  $158 \pm 40$  pg/ml;  $n = 14$  and lavage:  $398 \pm 206$  pg/ml;  $n = 14$ ) (fig. 2, G and H). After 48 h, serum MIP-2 levels of the controls were still higher as compared with that of the hfd group ( $1,181 \pm 572$  pg/ml;  $n = 11$  vs.  $755 \pm 220$  pg/ml;  $n = 10$ ). In the peritoneal lavage fluid, MIP-2 decreased in both groups 48 h after CLP, but was still higher in the controls ( $419 \pm 95$  pg/ml;  $n = 11$  vs.  $78 \pm 12$  pg/ml;  $n = 10$ ).

### Hfd Increases Circulating and Recruited Leukocyte and Neutrophil Numbers

To determine whether chronic hfd influences the immune system by modifying numbers of circulating or recruited leukocytes, we measured leukocytes counts in blood and peritoneal lavage fluid before and after CLP. Blood-circulating leukocyte numbers were significantly higher before sepsis in the hfd group as compared with that in the controls (hfd:  $6.4 \pm 0.7 \times 10^6$ /ml,  $n = 5$  vs. control:  $4.0 \pm 0.5 \times 10^6$ /ml;  $n = 5$ ;  $P = 0.032$ ) (fig. 3A). Leukocyte numbers decreased in the

hfd group 6 h after CLP ( $4.1 \pm 0.5 \times 10^6$ /ml;  $n = 10$ ). There were no significant changes in blood counts after CLP in the controls at 6 h ( $n = 15$ ), 24 h ( $n = 5$ ), and 48 h ( $n = 5$ ) after CLP. At the side of infection (intraperitoneal), there were significant increases in both groups after 6 h (hfd:  $n = 10$ ,  $P = 0.048$ ; control:  $n = 15$ ,  $P = 0.009$ ) and in the hfd group again 24 h after CLP ( $2.8 \pm 0.2 \times 10^6$ /ml;  $n = 5$ ;  $P = 0.003$ ) (fig. 3B). Interestingly, peritoneal levels of leukocytes in the hfd group were significant higher ( $P = 0.034$ ) 24 h after CLP as compared with that in controls ( $1.5 \pm 0.4 \times 10^6$ /ml;  $n = 5$ ). After 48 h, leukocyte numbers of the hfd group decreased to similar levels ( $1.9 \pm 0.03 \times 10^6$ /ml;  $n = 4$ ) as in the controls ( $1.6 \pm 0.3 \times 10^6$ /ml;  $n = 8$ ).

Neutrophils form the main subgroup (60 to 70%) of leukocytes and belong to the first line of defense in fighting against bacterial infections.<sup>37</sup> We postulated that this group of immune cells was key in the better outcome of the hfd group during sepsis. We found that serum numbers of neutrophils were significantly higher in the hfd



**Fig. 3.** Cellular immune response after cecal ligation and puncture (CLP)-induced sepsis. (A, B) Blood and peritoneal lavage fluid were collected from high-fat-diet (hfd) and control mice before ( $n = 5$  per group), 6 h (hfd:  $n = 10$ , control:  $n = 15$ ), 24 h (hfd:  $n = 8$ , control:  $n = 5$ ), and 48 h ( $n = 5$  per group) after CLP. Leukocyte numbers of hfd and control were enumerated in blood and peritoneal lavage fluid before and after CLP. (C, D) Blood and peritoneal lavage fluid were collected from hfd and control mice before ( $n = 5$  per group), 24 h ( $n = 14$  per group), and 48 h ( $n = 5$  per group) after CLP. Neutrophil numbers in serum and peritoneal lavage fluid of hfd and control mice were enumerated by flow cytometric analysis (combined data from three independent experiments). (E) Peritoneal CD4 T cells, (F)  $\gamma\delta$ -T cells, and (G) natural killer (NK) cells of hfd and control mice were enumerated before (hfd:  $n = 5$ , control:  $n = 5$ ) and 24 h after CLP (hfd:  $n = 9$ , control:  $n = 10$ ) by flow cytometric analysis (combined data from three independent experiments). All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of unpaired *t* test for comparison of baseline levels between two groups (hfd and control) and of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time. nd = not detectable.

group ( $0.20 \pm 0.06 \times 10^6/\text{ml}$ ;  $n = 5$ ;  $P = 0.011$ ) before inducing sepsis as compared with that in the control mice ( $0.03 \pm 0.01 \times 10^6/\text{ml}$ ;  $n = 5$ ) (fig. 3C). In peritoneal lavage, no neutrophils could be detected in either group before CLP-induced sepsis ( $n = 5$  per group) (fig. 3D). Neutrophil numbers of the hfd group showed a significant increase in serum ( $2.0 \pm 0.2 \times 10^6/\text{ml}$ ;  $n = 14$ ;  $P = 0.0003$ ) and peritoneal lavage ( $3.4 \pm 0.7 \times 10^6/\text{ml}$ ;  $n = 14$ ;  $P = 0.017$ ) 24 h after CLP-induced sepsis reaching significant higher levels as compared with that in the controls (serum:  $0.8 \pm 0.2 \times 10^6/\text{ml}$ ,  $n = 14$ ; lavage:  $0.9 \pm 0.2 \times 10^6/\text{ml}$ ,  $n = 14$ ;  $P = 0.001$ ). Neutrophil numbers of both groups decreased after 48 h to a similar level in serum (hfd:  $0.3 \pm 0.1 \times 10^6/\text{ml}$ ,  $n = 5$  and control:  $0.2 \pm 0.1 \times 10^6/\text{ml}$ ,  $n = 5$ ) and peritoneal lavage (hfd:  $0.8 \pm 0.3 \times 10^6/\text{ml}$ ,  $n = 7$  and control:  $0.6 \pm 0.2 \times 10^6/\text{ml}$ ,  $n = 9$ ).

We further assessed neutrophil activation and functionality. Although we observed an increase in CD11b expression 24 h ( $n = 14$  per group) and 48 h ( $n = 5$  per group) after CLP in blood and peritoneal lavage fluid of hfd and control groups, there were no significant differences between both groups (data not shown). Spontaneous oxidative burst activity in blood neutrophils isolated from hfd mice and control mice increased significantly 24 h after CLP in both groups (hfd group:  $295 \pm 22$  MFI,  $n = 5$  vs.  $447 \pm 23$  MFI,  $n = 14$ ,  $P = 0.002$  and control group:  $341 \pm 15$  MFI,  $n = 5$  vs.  $447 \pm 16$  MFI,  $n = 14$ ,  $P = 0.002$ ) (data not shown). After 48 h, the oxidative burst activity in both the hfd and control groups increased again (hfd:  $515 \pm 94$  MFI,  $n = 5$  and control:  $625 \pm 79$  MFI,  $n = 5$ ). However, there were no differences between the two groups at any timepoint. Peritoneal neutrophils of the hfd group demonstrated a slight increased trend of spontaneous oxidative burst activity between 24 and 48 h after CLP ( $517 \pm 15$  MFI,  $n = 14$  vs.  $606 \pm 73$  MFI;  $n = 5$ ), whereas we found a significant increase of spontaneous oxidative burst activity in the controls ( $703 \pm 64$  MFI,  $n = 14$  vs.  $570 \pm 17$  MFI;  $n = 5$ ;  $P = 0.026$ ) (data not shown). After stimulation with formyl-methionyl-leucyl-phenylalanine, no further increase could be detected in blood or peritoneal neutrophils of either group 24 h ( $n = 14$  per group) and 48 h after CLP ( $n = 5$  per group). Finally, we did not observe any changes in hemoglobin, hematocrit levels, and platelet numbers after CLP in hfd (6 h:  $n = 10$ , 24 h:  $n = 8$ , and 48 h:  $n = 5$ ) and control groups (6 h:  $n = 15$ , 24 h:  $n = 5$ , and 48 h:  $n = 5$ ) (data not shown).

### Hfd Influences Cellular Immune System

We next investigated the possible differences in different T-cell subtypes between both groups at the side of infection (intraperitoneal) before and 24 h after CLP-induced sepsis. Numbers of CD4 T cells in peritoneal lavage did not differ between both groups before CLP (hfd:  $n = 5$ , control:  $n = 5$ ) or 24 h after CLP (hfd:  $n = 9$ , control:  $n = 10$ ) (fig. 3E).

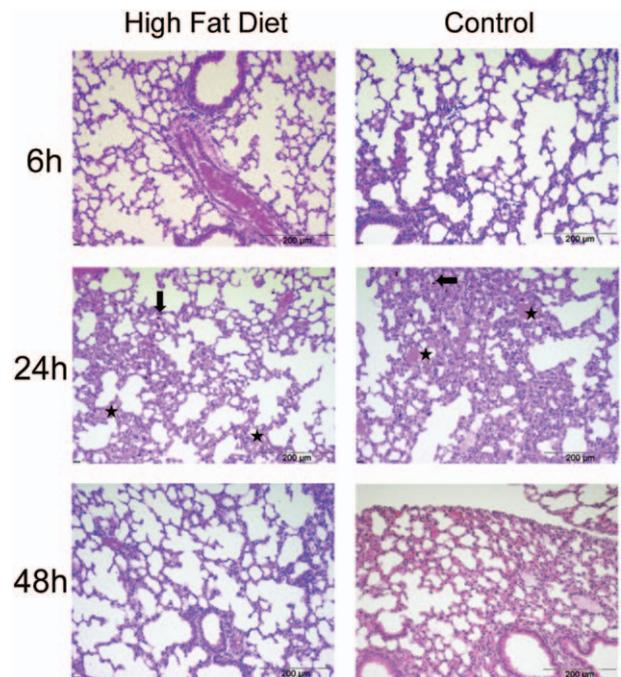
In contrast, we detected a significant higher amount of  $\gamma\delta$ -T cells in the hfd group before sepsis ( $5.6 \pm 1.2 \times 10^6/\text{ml}$ ;  $n = 5$ ;  $P = 0.041$ ) as compared with that in the control group ( $1.7 \pm 0.5 \times 10^6/\text{ml}$ ;  $n = 5$ ) (fig. 3F). After induction of sepsis,

$\gamma\delta$ -T cells in the hfd group showed no changes 24 h after CLP ( $5.8 \pm 1.5 \times 10^6/\text{ml}$ ;  $n = 9$ ), whereas  $\gamma\delta$ -T cell numbers of the control mice slightly increased ( $3.4 \pm 0.5 \times 10^6/\text{ml}$ ;  $n = 10$ ).

Interestingly, hfd mice showed a significant higher amount of natural killer (NK) cells in peritoneal lavage before induction of sepsis ( $11.4 \pm 3.2 \times 10^6/\text{ml}$ ;  $n = 5$ ;  $P = 0.023$ ) as compared with that in the controls ( $3.0 \pm 0.7 \times 10^6/\text{ml}$ ;  $n = 5$ ) (fig. 3G). Numbers of NK cells showed a decrease 24 h after CLP in the hfd group ( $4.9 \pm 0.7 \times 10^6/\text{ml}$ ;  $n = 9$ ), whereas there was a significant increase in the number of NK cells in control mice ( $9.9 \pm 1.6 \times 10^6/\text{ml}$ ;  $n = 10$ ;  $P = 0.018$ ) generating higher numbers at this timepoint as compared with that in the hfd group.

We corroborated our findings with histopathological examinations of lung and liver in both groups. The organs of the hfd group generally showed the beginning of a fatty degeneration in both liver and lung. Six hours after CLP, we did not detect evidence of an inflammatory process in the lungs of both groups (fig. 4). Twenty-four and 48 h after CLP, we found interstitial and alveolar edema and alveolar macrophages more often and distinct in the control group as compared with that in the hfd group. In contrast, the liver showed no differences within the two groups at the early timepoints observed (data not shown).

The inability of the control group to increase leptin levels in sepsis together with the observed decrease in body temperature, increased levels of IL-6 and TNF- $\alpha$ , and reduced numbers of recruited leukocytes led us to postulate whether



**Fig. 4.** Lung histopathology. Hematoxylin and eosin-stained lung sections from high-fat-diet and control mice at different timepoints (6, 24, and 48 h) after cecal ligation and puncture-induced sepsis. Images are shown at a  $\times 100$  magnification. Arrows: alveolar macrophages; stars: alveolar edema.

increased leptin levels were a possible reason for better survival in the hfd group. To test this hypothesis, we injected leptin (1  $\mu\text{g/g}$  BW, intraperitoneal) twice a day starting after inducing sepsis.

### **Leptin Treatment Increases Leptin Levels and Body Temperature in Sepsis**

Leptin treatment showed no effect on BW in both groups within 48 h after CLP (6 h:  $n = 15$ , 24 h:  $n = 15$ , and 48 h:  $n = 5$ ) (data not shown). In contrast, body temperature increased significantly 6 h after CLP in the controls ( $35.5^\circ \pm 0.1^\circ\text{C}$ ;  $n = 25$ ;  $P = 0.001$ ) as well as in the hfd group ( $35.9^\circ \pm 0.2^\circ\text{C}$ ;  $n = 20$ ;  $P = 0.008$ ) due to leptin treatment ameliorating the significant decrease seen in both groups 6 h after CLP without leptin treatment (hfd:  $32.7^\circ \pm 1.2^\circ\text{C}$ ,  $n = 10$  and control:  $32.7^\circ \pm 1.0^\circ\text{C}$ ;  $n = 14$ ;  $P < 0.001$ ) (fig. 5A). Leptin treatment significantly increased body temperature in the control group 24 h ( $n = 14$ ,  $P = 0.009$ ) and 48 h ( $n = 5$ ,  $P = 0.013$ ) after CLP to values in the same range as in the hfd group (24 h:  $n = 14$ , 48 h:  $n = 5$ ) abolishing the temperature decrease in the controls observed in the earlier experiments (6 h:  $n = 14$ , 24 h:  $n = 34$ , and 48 h:  $n = 12$ ). In addition, we analyzed the changes in body temperature between the timepoints observed. We found significant leptin-dependent changes 24 h ( $P = 0.012$ ) and 48 h ( $P = 0.018$ ) after CLP not only in the control group but also in the hfd group ( $P = 0.007$ ) (fig. 5B). Negative decreases seen before in both groups were reduced at the 6 h timepoint (hfd:  $n = 20$ , control:  $n = 25$ ) or even converted to increases at the later timepoints (24 h:  $n = 14$  per group and 48 h:  $n = 5$  per group) due to leptin treatment.

Leptin treatment led to significant increases of serum (fig. 5C) and peritoneal leptin levels (fig. 5D) in controls ( $35.8 \pm 3.3$  ng/ml,  $n = 9$ ,  $P < 0.001$  and  $8.4 \pm 2.1$  ng/ml;  $n = 9$ ,  $P < 0.001$ ) and even increases in the hfd group ( $30.6 \pm 4.6$  and  $5.6 \pm 1.0$  ng/ml;  $n = 9$  per group) as early as 6 h after CLP. In addition, serum and peritoneal lavage leptin levels of the control group were significantly increased 24 h after CLP ( $5.7 \pm 1.2$  ng/ml,  $n = 9$ ,  $P = 0.004$  and  $0.8 \pm 0.2$  ng/ml,  $n = 9$ ,  $P = 0.003$ ) and peritoneal lavage leptin levels 48 h after CLP ( $1.2 \pm 0.6$  ng/ml,  $n = 5$ ,  $P = 0.043$ ) due to leptin treatment. The hfd group showed only an additional increase in peritoneal lavage 48 h after CLP due to leptin treatment ( $n = 5$ ).

### **Leptin Influences Proinflammatory Cytokines in Sepsis**

In serum, leptin treatment decreased IL-6 levels of the hfd group ( $n = 9$ ) within 6 h as compared with that in the control group ( $n = 9$ ) (fig. 5E). Levels were reduced in both groups (hfd and control groups) down to a similar level 24 h ( $11.0 \pm 0.8$  vs.  $10.2 \pm 0.6$  ng/ml;  $n = 9$  per group) and 48 h ( $10.4 \pm 2.0$  vs.  $12.2 \pm 1.3$  ng/ml;  $n = 5$  per group) after CLP. At the side of infection, leptin treatment decreased IL-6 levels significantly in both groups (hfd:  $1.6 \pm 0.4$  ng/ml,  $n = 9$ ,  $P = 0.039$  and control:  $2.8 \pm 0.5$  ng/ml,  $n = 9$ ,  $P = 0.034$ ) as early as 6 h after CLP (fig. 5F). At the 24 h ( $n = 9$  per group)

and 48 h timepoint ( $n = 5$  per group), there was a further decrease detectable in both groups. The second peak of IL-6 in the control group at the 48 h timepoint seen in the experiments before ( $n = 11$ ) was impressively abolished to a level comparable with the level in hfd group (hfd:  $0.8 \pm 0.7$  ng/ml,  $n = 5$  and control:  $1.1 \pm 0.5$  ng/ml;  $n = 5$ ,  $P = 0.011$ ). Leptin treatment only led to an increase in IL-1 $\beta$  serum levels of the control group 6 h after CLP ( $25.8 \pm 1.2$  ng/ml;  $n = 9$ ) comparable with the levels of the hfd group ( $26.4 \pm 2.2$  ng/ml;  $n = 9$ ) abolishing the significant difference seen before at this timepoint (data not shown). No further leptin-dependent changes were detectable at the later timepoints.

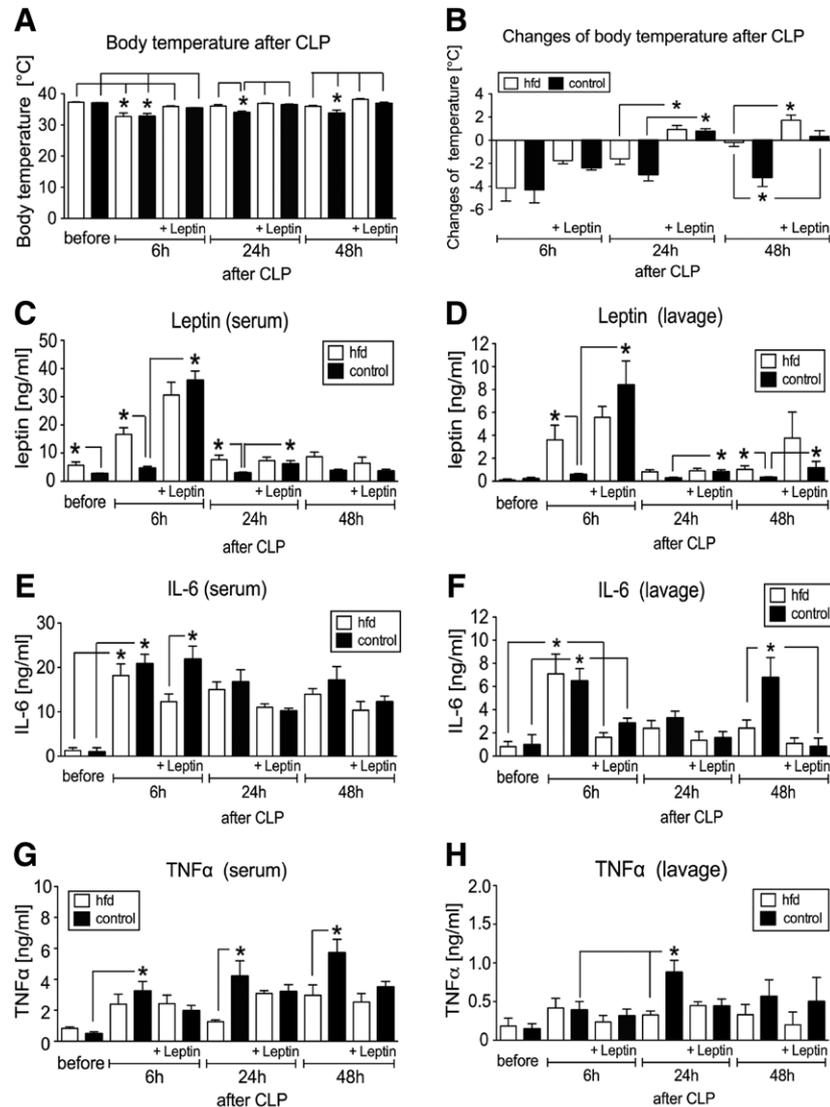
Leptin treatment reduced TNF $\alpha$  serum levels of the control group 6 h ( $2.0 \pm 0.4$  ng/ml;  $n = 9$ ), 24 h ( $3.2 \pm 0.5$  ng/ml;  $n = 9$ ), and 48 h after CLP ( $3.5 \pm 0.4$  ng/ml;  $n = 5$ ) abolishing the continuous increase observed before (fig. 5G). At the same time, leptin increased TNF $\alpha$  levels in the hfd group at the 24 h timepoint bringing both groups to a similar level ( $3.1 \pm 0.2$  ng/ml;  $n = 9$ ). The peak of TNF $\alpha$  seen in the control group at the side of infection 24 h after CLP was also abolished by leptin treatment ( $0.4 \pm 0.1$  ng/ml;  $n = 9$ ) (fig. 5H). Apart from that, TNF $\alpha$  levels in peritoneal lavage fluid showed no further differences in both groups due to leptin treatment at the 24 h (hfd:  $n = 9$  per group) and 48 h timepoint ( $n = 5$  per group). Finally, leptin treatment had no effect on MIP-2 levels of the hfd and the controls 6 h ( $n = 9$  per group), 24 h ( $n = 9$  per group), and 48 h ( $n = 5$  per group) after CLP.

### **Leptin Increases Neutrophil Numbers in Sepsis**

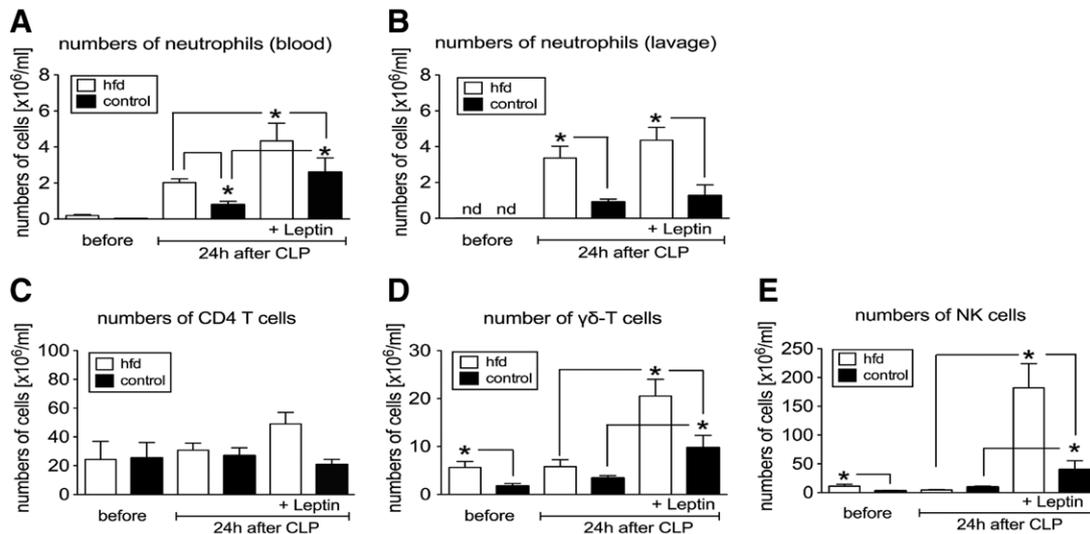
In both hfd and control groups, we did not observe differences in leukocyte numbers after leptin treatment in both serum and peritoneal lavage (data not shown). In contrast, leptin treatment led to a significant increase in neutrophil numbers in blood 24 h after CLP in both hfd ( $4.3 \pm 1.0 \times 10^6$ /ml;  $n = 4$ ,  $P = 0.022$ ) and controls ( $2.2 \pm 1.0 \times 10^6$ /ml;  $n = 5$ ,  $P = 0.004$ ) (fig. 6A), whereas neutrophil numbers at the side of infection were unaffected (hfd:  $n = 4$ , control:  $n = 5$ ) (fig. 6B). In the next experiments, we determined whether leptin treatment influences neutrophil activation or function. Leptin treatment had no effect on neutrophil activation in both groups ( $n = 5$  per group). In addition, although leptin treatment slightly decreased oxidative burst in serum neutrophils of the hfd group 24 h after CLP, leptin treatment showed no further effects on activity of neutrophils in both groups ( $n = 5$  per group) (data not shown).

### **Leptin Affects Cellular Immune Response**

Leptin treatment increased numbers of peritoneal CD4 T cells in the hfd group 24 h after CLP ( $49.1 \pm 8.0 \times 10^6$ /ml;  $n = 4$ ) being higher as compared with that in the control group ( $20.9 \pm 3.6 \times 10^6$ /ml;  $n = 5$ ) which showed no leptin-dependent effects (fig. 6C). Interestingly, leptin induced a significant increase of  $\gamma\delta$ -T cells in both groups 24 h after sepsis (hfd:  $20.5 \pm 3.5 \times 10^6$ /ml,  $n = 4$ ,  $P < 0.001$  and controls:  $9.8 \pm 2.5 \times 10^6$ /ml,  $n = 5$ ,  $P = 0.002$ ) (fig. 6D). In addition,



**Fig. 5.** Influence of intraperitoneal leptin treatment (1 μg/g body weight, twice a day) starting after cecal ligation and puncture (CLP)-induced sepsis on body temperature, leptin levels, and proinflammatory cytokine levels of high-fat-diet (hfd) and control mice. (A) Body temperature was measured rectally 6, 24, and 48 h after conducting CLP and leptin treatment in hfd (6 h: n = 20, 24 h: n = 14, and 48 h: n = 5) and control mice (6 h: n = 25, 24 h: n = 14, and 48 h: n = 5) and compared with that in the untreated hfd (before: n = 5, 6 h: n = 10, 24 h: n = 30, and 48 h: n = 12) and control group (before: n = 5, 6 h: n = 14, 24 h: n = 30, and 48 h: n = 12) (combined data from several independent experiments). (B) Changes in body temperatures between the observed timepoints (6, 24, and 48 h) after conducting CLP and leptin treatment in hfd (6 h: n = 20, 24 h: n = 14, and 48 h: n = 5) and control mice (6 h: n = 25, 24 h: n = 14, and 48 h: n = 5) and compared with that in the untreated hfd (before: n = 5, 6 h: n = 10, 24 h: n = 30, and 48 h: n = 12) and control group (before: n = 5, 6 h: n = 14, 24 h: n = 30, and 48 h: n = 12) (combined data from several independent experiments). (C, D) Serum and peritoneal lavage fluid were collected 6, 24, and 48 h after CLP and leptin treatment from hfd (6 h: n = 9, 24 h: n = 9, and 48 h: n = 5) and control mice (6 h: n = 9, 24 h: n = 5) and compared with the untreated hfd (before: n = 5, 6 h: n = 14, 24 h: n = 14, and 48 h: n = 10) and control group (before: n = 5, 6 h: n = 19, 24 h: n = 23, and 48 h: n = 11) (combined data from several independent experiments). The serum and lavage levels of leptin were determined by enzyme-linked immunosorbent assay. In addition, the serum and lavage levels of (E, F) interleukin-6 (IL-6) and (G, H) tumor necrosis factor-α (TNFα) were determined 6, 24, and 48 h after CLP and leptin treatment from hfd (6 h: n = 9, 24 h: n = 9, and 48 h: n = 5) and control mice (6 h: n = 9, 24 h: n = 9, and 48 h: n = 5) and compared with that in the untreated hfd (before: n = 5, 6 h: n = 14, 24 h: n = 14, and 48 h: n = 10) and control group (before: n = 5, 6 h: n = 19, 24 h: n = 23, and 48 h: n = 11). The serum and lavage levels of leptin were determined by enzyme-linked immunosorbent assay (combined data from several independent experiments). All values are expressed as mean ± SEM; \*P < 0.05 of unpaired *t* test for comparison of baseline levels between two groups (hfd and control) and of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time.



**Fig. 6.** Influence of intraperitoneal leptin treatment (1  $\mu\text{g/g}$  body weight, twice a day) on cellular immune response after cecal ligation and puncture (CLP)-induced sepsis. (A, B) Blood and peritoneal lavage fluid were collected 24 h after CLP and leptin treatment from high-fat-diet (hfd) ( $n = 4$ ) and control mice ( $n = 5$ ). Neutrophil numbers in blood and peritoneal lavage fluid were enumerated by flow cytometric analysis and compared with that in the untreated hfd and control groups before ( $n = 5$  per group) and 24 h after CLP ( $n = 14$  per group). Peritoneal lavage fluid was collected 24 h after CLP and leptin treatment from hfd ( $n = 4$ ) and control mice ( $n = 5$ ). (C) CD4 T cells, (D)  $\gamma\delta$ -T cells, and (E) natural killer (NK) cells were enumerated by flow cytometric analysis and compared with that in the untreated hfd and control groups before ( $n = 5$  per group) and 24 h after CLP ( $n = 14$  per group). All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of unpaired  $t$  test for comparison of baseline levels between two groups (hfd and control) and of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time. nd = not detectable.

the numbers of  $\gamma\delta$ -T cell levels of the hfd group were significantly higher ( $P = 0.037$ ) as compared with that of controls due to leptin treatment. We also detected a leptin-dependent significant increase in NK cells 24 h after CLP in both hfd ( $182.2 \pm 41.6$ ;  $n = 4$ ,  $P = 0.002$ ) and control groups ( $39.9 \pm 15.5 \times 10^6/\text{ml}$ ;  $n = 5$ ,  $P = 0.008$ ) (fig. 6E). Again, NK cells of the hfd group were higher at this timepoint as compared with that in the controls ( $P = 0.010$ ).

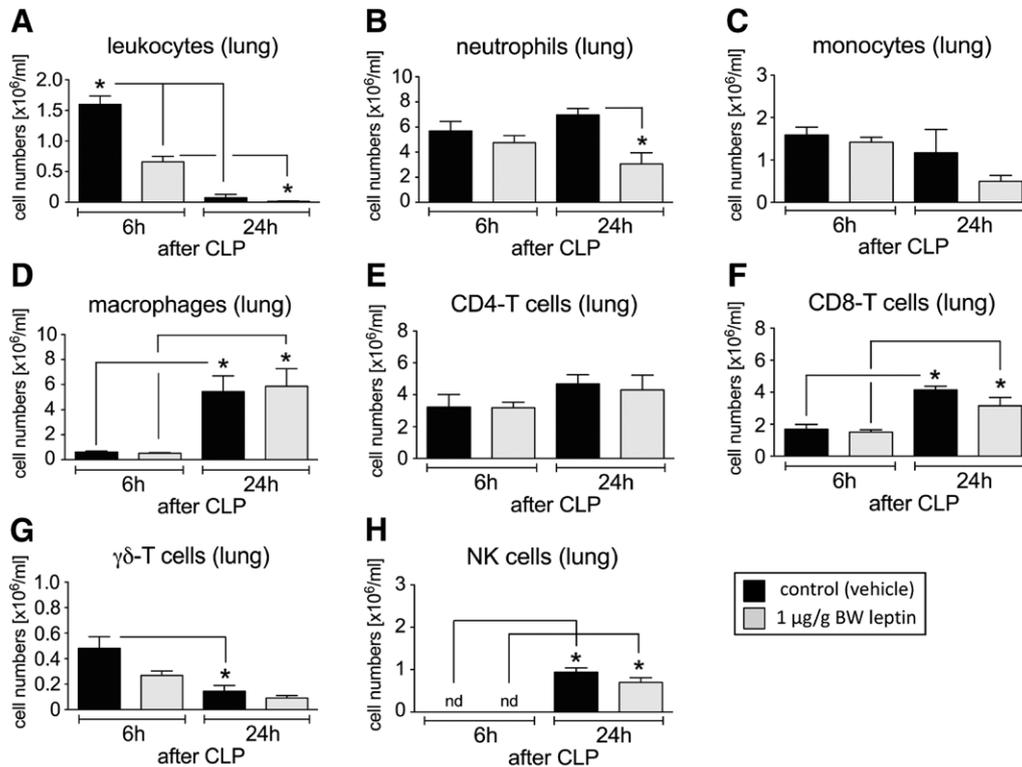
#### Leptin Influences Cellular Immune Response in the Lung

The lung is a key organ at risk for tissue injury during sepsis. In our sample cohorts, we observed increased tissue injury in control mice as compared with the tissue injury in the hfd group (fig. 4). Accordingly, we next determined whether leptin could affect the lung leukocyte numbers during sepsis. Six hours after CLP, leptin treatment decreased leukocyte numbers in the lung significantly ( $0.66 \pm 0.09 \times 10^6/\text{ml}$ ;  $n = 5$ ;  $P = 0.008$ ) as compared with that in the vehicle controls ( $1.60 \pm 0.14 \times 10^6/\text{ml}$ ;  $n = 5$ ) (fig. 7A). Numbers of neutrophils (fig. 7B), monocytes (fig. 7C), macrophages (fig. 7D), CD4 T cells (fig. 7E), CD8-T cells (fig. 7F), and  $\gamma\delta$ -T cells (fig. 7G) showed no differences, whereas NK cells were not detectable in both at this early timepoint ( $n = 5$  per group) (fig. 7H). In both groups, leukocyte numbers ( $0.02 \pm 0.01 \times 10^6/\text{ml}$ ;  $n = 4$ ,  $P = 0.016$  and  $0.07 \pm 0.05 \times 10^6/\text{ml}$ ;  $n = 4$ ,  $P = 0.016$ ) and  $\gamma\delta$ -T cell numbers ( $0.14 \pm 0.02 \times 10^6/\text{ml}$ ;  $n = 4$  and  $0.09 \pm 0.01 \times 10^6/\text{ml}$ ;  $n = 4$ ,  $P = 0.032$ ) were decreased 24 h after CLP.

Neutrophils were also reduced due to leptin treatment 24 h after CLP ( $3.1 \pm 0.9 \times 10^6/\text{ml}$ ;  $n = 4$ ) reaching significant lower levels ( $P = 0.01$ ) as compared with that in the vehicle-treated controls ( $7.0 \pm 1.0 \times 10^6/\text{ml}$ ;  $n = 4$ ). We further observe that leptin treatment decreased monocytes ( $0.5 \pm 0.1$  vs.  $1.2 \pm 0.6 \times 10^6/\text{ml}$ ;  $n = 4$ ) at the 24 h timepoint. Although there was no change in CD4 T cell numbers detectable, macrophage (vehicle control:  $5.4 \pm 1.2 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P = 0.003$  and leptin:  $5.9 \pm 1.4 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P = 0.004$ ), CD8 T cell (vehicle control:  $4.1 \pm 0.2 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P = 0.016$  and leptin:  $3.2 \pm 0.5 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P = 0.032$ ), and NK cell numbers (vehicle control:  $0.94 \pm 0.01 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P < 0.001$  and leptin:  $0.7 \pm 0.01 \times 10^6/\text{ml}$ ,  $n = 4$ ,  $P = 0.008$ ) increased significantly in both groups without any leptin-dependent differences.

#### Leptin-dosage Dependency of Immune Response and Bacterial Load in Sepsis

We next determined whether the leptin-dependent effects demonstrated any leptin-dosage dependency. Control mice were treated with leptin after CLP using four different concentrations: 0 (vehicle), 0.2, 1, and 5  $\mu\text{g/g}$  BW (intra-peritoneal; two times daily) to investigate influences on the cellular immune response at the side of infection. We could not detect dosage-dependent changes in numbers of leukocytes, neutrophils, monocytes, CD8-T cells,  $\gamma\delta$ -T cells, and NK cells at the 6 h timepoint ( $n = 5$  per group) (fig. 8). The lowest leptin dosage led to an increase in macrophages



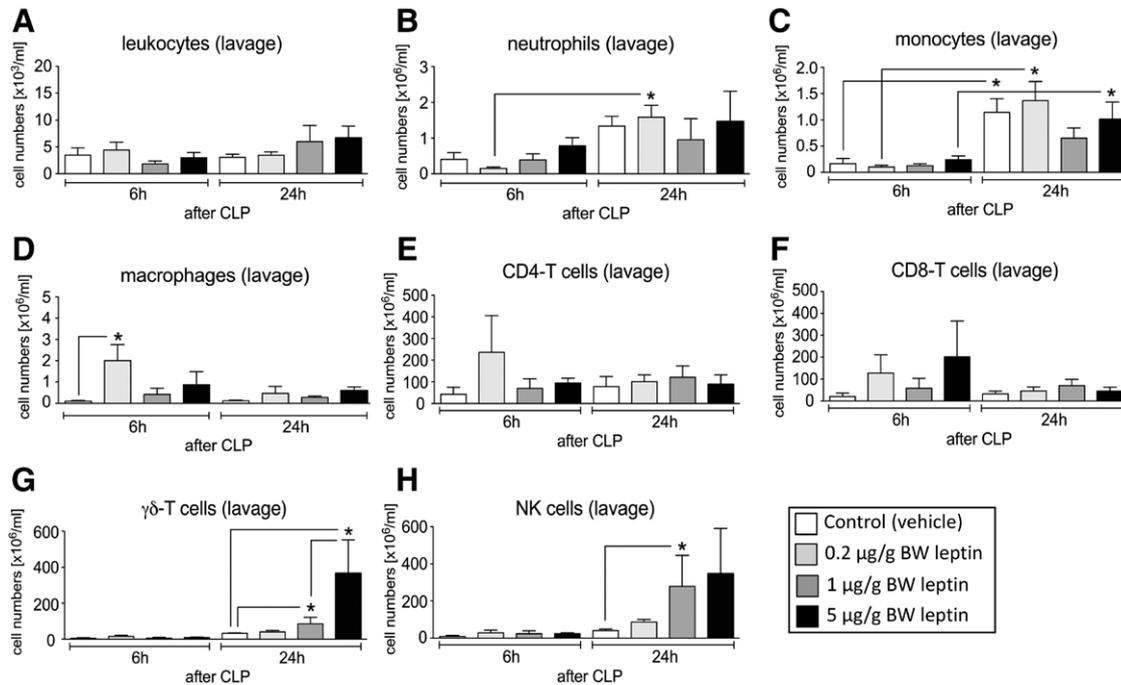
**Fig. 7.** Influence of intraperitoneal leptin treatment (1  $\mu\text{g/g}$  body weight [BW], twice a day) on cellular immune response in the lung after cecal ligation and puncture (CLP)-induced sepsis. Bronchoalveolar lavage fluid from leptin-treated and vehicle (sodium chloride 0.9%) injected control mice was collected 6h ( $n = 5$  per group) and 24h ( $n = 4$  per group) after CLP. Numbers of (A) leukocytes, (B) neutrophils, (C) monocytes, (D) macrophages, (E) CD4 T cells, (F) CD8-T cells, (G)  $\gamma\delta$ -T cells, and (H) natural killer (NK) cells were enumerated by flow cytometric analysis. All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time. nd = not detectable.

( $2.0 \pm 0.8 \times 10^6/\text{ml}$ ;  $n = 5$ ,  $P = 0.036$ ) (fig. 8D) and CD4 T cell numbers ( $236 \pm 170 \times 10^3/\text{ml}$ ;  $n = 5$ ) (fig. 8E). Interestingly,  $\gamma\delta$ -T cells (fig. 8G) and NK cells (fig. 8H) showed leptin dosage-dependent increases in cell numbers at the 24h timepoint ( $n = 3$  per group,  $P = 0.048$  and  $P = 0.035$ ) confirming the significant increase in cell numbers shown before using 1  $\mu\text{g/g}$  BW leptin (fig. 6, D and E).

The different leptin dosages used had also no influence on IL-6 serum levels at the 6h timepoint ( $n = 4$  per group) known to be predictive for outcome and survival (fig. 9A). Interestingly, IL-10 levels increased in serum ( $17.4 \pm 0.3 \text{ ng/ml}$ ;  $n = 4$ ;  $P = 0.029$ ) using the lowest leptin concentration (0.2  $\mu\text{g/g}$  BW) and continuously decreased again with increasing leptin dosages still reaching higher levels as compared with that in the vehicle control ( $3.3 \pm 0.2$  and  $0.8 \pm 0.1 \text{ ng/ml}$ ;  $n = 4$  per group) (fig. 9B). According to these data, the IL-6/IL-10 ratio correlated with a poor outcome if increased<sup>38</sup> was highest in controls without leptin. Treatment with the lowest leptin dosage resulted in the lowest IL-6/IL-10 ratio as compared with the ratio in the untreated group ( $P = 0.002$ ) which is continuously increasing with increasing leptin dosages (fig. 9C). At the site of infection, there was also no leptin-dependent influences on IL-6 levels detectable 6h after CLP ( $n = 4$  per

group) (fig. 9D). IL-10 levels increased in peritoneal lavage ( $2.3 \pm 0.6 \text{ ng/ml}$ ;  $n = 4$  per group) using the lowest leptin concentration (0.2  $\mu\text{g/g}$  BW) and continuously decreased again with increasing leptin dosages ( $n = 4$  per group) (fig. 9E). The IL-6/IL-10 ratio was lowest in the group treated with the lowest leptin dosage (fig. 9F).

The proinflammatory cytokine IL-17 is shown to increase during sepsis and to be associated with bacterial clearance.<sup>39</sup> To determine whether leptin has any influence on IL-17 production, we measured numbers of IL-17-producing cells by FACS analysis at the site of infection. Interestingly, 6h after CLP, we found increased IL-17 production in peritoneal T cells ( $631 \pm 335 \times 10^3/\text{ml}$ ;  $n = 4$ ) (fig. 9G),  $\gamma\delta$ -T cells ( $28 \pm 7 \times 10^3/\text{ml}$ ;  $n = 4$ ,  $P = 0.044$ ) (fig. 9H), and NK cells ( $57 \pm 17 \times 10^3/\text{ml}$ ;  $n = 4$ ,  $P = 0.036$ ) (fig. 9I) after leptin treatment with the lowest dosage as compared with that in the vehicle control ( $n = 4$  per group), whereas the higher dosages did not lead to significant different numbers as compared with that in the controls ( $n = 4$  per group). We also found a higher bacterial load in serum connected to the lowest leptin dosage 6h after CLP ( $n = 4$ ) (fig. 9J). With increasing leptin dosages, bacteremia decreased significantly ( $n = 4$  per group,  $P = 0.032$ ). In agreement with this, a similar trend was also observed in the peritoneal lavage fluid ( $n = 4$  per group) (fig. 9K).



**Fig. 8.** Influence of intraperitoneal leptin treatment using different dosages on cellular immune response in the peritoneum after cecal ligation and puncture (CLP)-induced sepsis. Control mice were treated with vehicle (sodium chloride 0.9%, intraperitoneal,  $n = 8$ ) or 0.2, 1, or 5  $\mu\text{g/g}$  body weight (BW) leptin (intraperitoneal;  $n = 8$  per group) twice a day starting after CLP. Peritoneal lavage fluid was collected 6 h ( $n = 5$  per group) and 24 h ( $n = 3$  per group) after CLP. Numbers of (A) leukocytes, (B) neutrophils, (C) monocytes, (D) macrophages, (E) CD4 T cells, (F) CD8-T cells, (G)  $\gamma\delta$ -T cells, and (H) natural killer (NK) cells were enumerated by flow cytometric analysis. All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of two-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups or within groups over time.

According to these data, the lowest leptin dosage seemed to lead not only to a better IL-6/IL-10 ratio but also to higher IL-17 levels and higher bacteremia. The highest leptin dosage increased  $\gamma\delta$ -T cells and NK cells but was connected to lowest IL-6/IL-10 ratio. Thus, the dosage used in our previous experimental settings (1  $\mu\text{g/g}$  BW) seems to combine positive influence on cellular and cytokine response and less negative influences as seen in IL-17 production and bacterial load.

Finally, we investigated whether intraperitoneal leptin treatment used in the initially used dosage (1  $\mu\text{g/g}$  BW) had any influence on survival in control mice after CLP (fig. 10A). We found a three-fold increase of survival rate (30%;  $n = 10$ ) due to leptin treatment within 7 days of observation as compared with the survival rate of the control mice treated with volume-adapted vehicle (8.3%;  $n = 12$ ). Interestingly, body temperature of the leptin-treated group did not decrease at days 3 ( $n = 6$ ,  $P = 0.005$ ) and 4 ( $n = 3$ ,  $P = 0.047$ ) as compared with that in the controls (day 3:  $n = 5$ , day 4:  $n = 3$ ) but showed a stable course over the observed time period as we found in the hfd group before (fig. 10B).

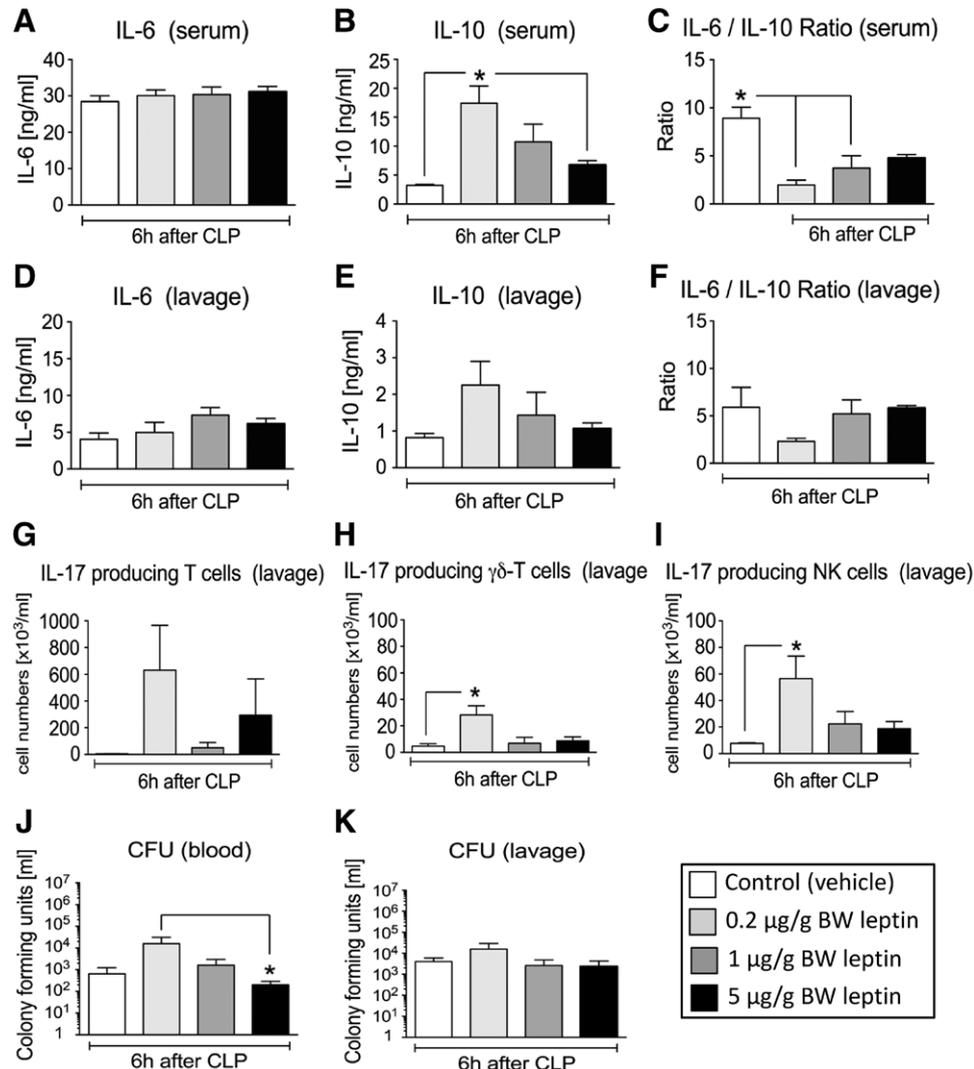
## Discussion

Our data indicate that a class 1 obesity is connected to hyperleptinemia and improved survival using a murine model of

sepsis. In addition, sepsis-induced leptin increases lead to a stable body temperature and effective immune response in a time-dependent and beneficial manner. Furthermore, leptin treatment of a relative hypoleptinemia in sepsis stabilizes body temperature, enhances cellular immune response, and reduces an overwhelming proinflammatory cytokine response being reflected in increased survival.

## Influence of Obesity

The association between increased BW and outcome among critically ill patients remains ambiguous. Obesity is an independent risk factor of mortality in sepsis,<sup>40–42</sup> although there are studies demonstrating decreased mortality<sup>43,44</sup> and no association.<sup>45</sup> Morbid obesity leads to reduced intracellular bacterial killing capacity and reduced cellular immune function. In contrast, Mica *et al.*<sup>46</sup> showed in patients with septic trauma that a higher BMI was connected to a reduced incidence of sepsis. Thus, it seems to be important to make differences of the obesity class and its role in sepsis: overweight/class 1 obesity seems to improve immune function due to hyperleptinemia and enhanced cellular immune system but without obesity-dependent comorbidities. Higher classes of obesity diminish immune function due to impaired immune cell function<sup>8</sup> and existing comorbidities being responsible for increased organ damage and mortality.

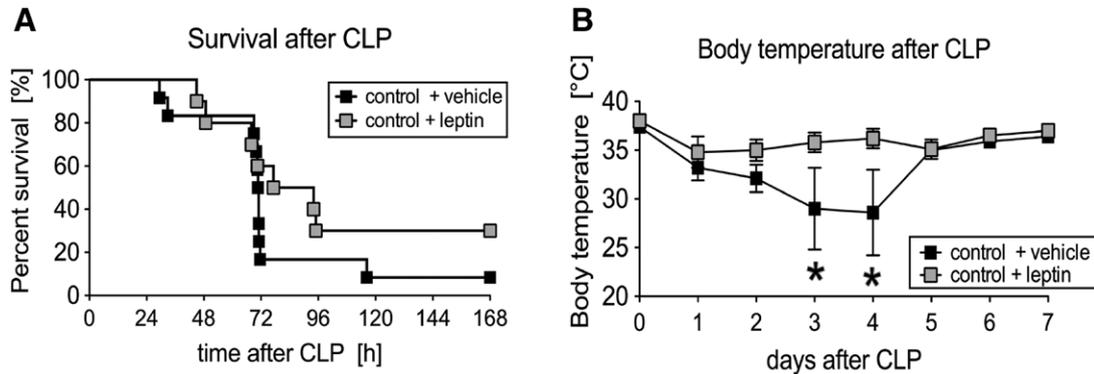


**Fig. 9.** Influence of intraperitoneal leptin treatment using different dosages on cytokine levels, interleukin (IL)-17–producing cells, and bacterial load after cecal ligation and puncture (CLP)–induced sepsis. Control mice were treated with vehicle (sodium chloride 0.9%, intraperitoneal,  $n = 4$ ) or 0.2, 1, or 5  $\mu\text{g/g}$  body weight (BW) leptin (intraperitoneal,  $n = 4$  per group) directly after CLP. Serum and peritoneal lavage fluid were collected 6h after CLP. IL-6 and IL-10 were measured and the IL-6/IL-10 ratio was determined in (A–C) serum and (D–F) peritoneal lavage fluid 6h after CLP and treatment with the different leptin dosages. IL-17 production of (G) T cells, (H)  $\gamma\delta$ -T cells, and (I) natural killer (NK) cells 6h after CLP and treatment with the different leptin dosages was determined in peritoneal lavage fluid by flow cytometric analysis. Colony-forming units (CFU) in (J) blood and (K) peritoneal lavage fluid were determined 6h after CLP and treatment with the different leptin dosages. All values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of one-way ANOVA for unmatched samples followed by a *post hoc* Bonferroni test for analyses between more than two groups.

Because hfd changes bowel microflora and can induce inflammation by increasing endotoxin levels in the intestinal lumen,<sup>47</sup> the hfd group should develop higher mortality as compared with the mortality in lean controls. In contrast to this, we observed the opposite effect and could not detect different bacterial loads 24h after CLP (data not shown). Another criterion for better survival of the obese mice could be the different size of the cecum. The cecum of the hfd group was smaller as compared with the cecum of controls and might that is why induce only a less severe inflammation. Nevertheless, after macroscopic preparation, the cecum of both groups looked equal according to swollen

tissue, necrosis, abscess formation, and affected intestine tissue. Taken together with equal IL-6 serum levels and equal amount of bacterial load, we assume the severity of the induced infection to be equal in both groups.

Although leptin acts mainly in the central nervous system, it also exerts peripheral biological effects.<sup>48</sup> Most of the immune cells express the long-form leptin receptor and it is known that leptin affects these immune cells.<sup>23,24</sup> Components of the first line of defense are neutrophils, NK cells, and  $\gamma\delta$ -T cells. As reported previously,  $\gamma\delta$ -T cells can reduce organ injury and mortality in sepsis.<sup>49</sup> Higher NK cell counts were connected to improved survival of patients



**Fig. 10.** Influence of intraperitoneal leptin treatment (1  $\mu\text{g/g}$  body weight, twice a day) on survival and body temperature after cecal ligation and puncture (CLP)-induced sepsis. (A) Survival after CLP in control mice receiving volume-adapted vehicle (sodium chloride 0.9%,  $n = 12$ ) or leptin treatment ( $n = 10$ ) intraperitoneal and monitored for 7 days. Analysis by log-rank test. (B) Body temperature as independent predictor of mortality did not decrease in control mice receiving leptin treatment after CLP compared with that in the vehicle-treated control. Values are expressed as mean  $\pm$  SEM; \* $P < 0.05$  of two-way ANOVA for repeated measurements followed by a *post hoc* Bonferroni test for analyses within groups over time.

with severe sepsis.<sup>50</sup> However, NK cells can amplify the immune response, which can lead to organ dysfunction. Leptin directly influences differentiation, proliferation, and function of NK cells.<sup>51</sup> Caspar-Bauguil *et al.*<sup>52,53</sup> demonstrated NK and  $\gamma\delta$ -T cells in adipose tissue and showed that their contents were directly dependent on leptin. Furthermore, a correlation between leptin and leukocytes has been reported in humans.<sup>54</sup> This is consistent with a study on obese subjects showing increased numbers of leukocytes and lymphocytes.<sup>6</sup> In contrast, in leptin-receptor-deficient mice, NK cells were diminished,<sup>55</sup> leptin-deficiency was associated with decreased lymphocyte numbers.<sup>56</sup> In this study, leptin treatment increased cell numbers of the neutrophils at the side of infection. Neutrophils are critical for the immune response because they curtail bacterial dissemination to circulation and a deficiency is linked to increased severity of bacterial infections.<sup>57</sup> Craciun *et al.*<sup>58</sup> found that increasing the neutrophils at the side of infection at an early timepoint of sepsis improved survival. However, an overproduction of cytokines such as TNF- $\alpha$  can inhibit neutrophil migration.<sup>59,60</sup> This fact confirms our results showing increasing TNF- $\alpha$  levels in sepsis combined with diminished neutrophil numbers in situations of relative hypoleptinemia. In contrast, leptin as a chemoattractant for neutrophils<sup>61</sup> together with reduced TNF- $\alpha$  and IL-6 levels enabled the hfd group to recruit neutrophils into the peritoneum early and more effective. In our study, obesity-induced hyperleptinemia was connected to increased neutrophils, NK cell, and  $\gamma\delta$ -T cell numbers, and leptin treatment led to dosage-dependent additional increases while other immune cells were unaffected. Therefore, obesity-induced hyperleptinemia seems to improve cellular immune response by increasing numbers of immune cells leading to a more powerful immune system. A more robust immune response improves local containment of bacterial products and prevents an overwhelming and uncontrolled immune response later on.

In contrast to our studies, Kaplan *et al.*<sup>62</sup> showed that short-time feeding (3 weeks) of an hfd led to an increased mortality within the first 30 h of sepsis. However, the BW of their hfd group still corresponded to a normal-weight BMI. Nevertheless, they found less lung injury and reduced plasma IL-6 and TNF- $\alpha$  levels combined with increased leptin levels within the first 18 h of sepsis in their hfd group comparable with our results. A possible explanation of the different survival seen might be the short-time feeding and the severity of the model. Within 3 weeks of feeding, there might be not enough time to develop leptin-dependent protection on the immune system leading to improved immune response.

#### Effects of Hyperleptinemia and Leptin Treatment in Sepsis

The inability to increase leptin in sepsis was connected to profound hypothermia. Although fever is a main feature in sepsis and enhances the function of several immune cells, the failure to mount a febrile response or hypothermia is connected to worse outcome.<sup>63,64</sup> Although IL-6<sup>65</sup> and TNF- $\alpha$  are involved in increasing body temperature in sepsis, hypothermia is not linked with decreased cytokine levels.<sup>64</sup> Because IL-6 and TNF- $\alpha$  levels did not differ at the earlier timepoints between both groups, these cytokines do not seem to be the main mediator for hypothermia. However, leptin regulates body temperature by its central action *via* IL-1.<sup>66</sup> Absence of leptin can lead to hypothermia as shown in leptin-deficient ob/ob mice,<sup>20</sup> and hypoleptinemic states are associated with increased risk of infection.<sup>67</sup> The administration of leptin increased body temperature in ob/ob mice.<sup>68</sup> Thus, the inability of the control group to increase leptin in sepsis might be a possible explanation for the hypothermia in sepsis. Increased leptin levels due to treatment generated sufficient leptin levels to improve body temperature and prevent hypothermia in sepsis.

In general, sepsis induces a proinflammatory cytokine response, but severity of immune response can lead to an

overwhelming and uncontrolled cytokine release being responsible for organ damage and mortality.<sup>69</sup> IL-1 $\beta$  and TNF- $\alpha$  are important mediators of the systemic host response to sepsis.<sup>70</sup> However, higher TNF- $\alpha$  levels are linked to organ damage and cell necrosis.<sup>71</sup> In our experiments, the control group developed an enhanced proinflammatory response. In contrast, obesity-induced hyperleptinemia led to an early but time-limited cytokine peak. The fact that leptin has a protective role against, for example, TNF- $\alpha$  in systemic inflammatory responses<sup>72</sup> fits to the diminished TNF- $\alpha$  response due to both hfd and leptin treatment. In addition, leptin directly increased IL-10 levels dose dependently. For that, we suggest that leptin has a key regulatory function by controlling the duration and extent of the immune response.

There is still the question which dosage of leptin should be used. Although a low dosage improves macrophages, CD4 T cells, and the anti-inflammatory action, it also promotes IL-17 production with a negative effect on bacterial burden. In contrast, although the highest leptin concentration was connected to less IL-17 and lowest bacterial burden, it had only minor effects on IL-10. The initially used leptin dosage represents a compromise of cellular and cytokine-dependent improvement and possible negative side effects and offers an appropriate way of treatment confirmed by an increased survival. In a previous work, we showed that intracerebroventricular administration of leptin increased survival in sepsis.<sup>27</sup> Because both ways of leptin treatment (intraperitoneally and intracerebroventricularly) induced the same increase in survival, we might prove here that peripheral leptin treatment influences immune response in two ways: peripherally by affecting immune cell numbers and the cytokine response and centrally by affecting body temperature, the course of immune response, and survival by central nervous system-specific pathways.

Taken together, our model of class 1 obesity reflects hyperleptinemia and an improved cellular immune system connected to time-limited immune response and better survival. Relative hyperleptinemia whether class 1 obesity or treatment related acts protective in sepsis by improving both the peripheral cellular and cytokine response and the central leptin-dependent signalling. In conclusion, leptin seems to play a regulatory role in the immune system in sepsis and treatment of relative hypoleptinemia could offer a new way of an individual sepsis therapy.

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### Competing Interests

The authors declare no competing interests.

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