Host-pathogen interactions during (myco)bacterial respiratory tract infections
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Leptin and host defense against Gram-positive and Gram-negative pneumonia in mice

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Sandrine Florquin
Giamila Fantuzzi
Tom van der Poll

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Abstract

Leptin is a pleiotrophic protein mainly produced by adipocytes that has been implicated as a link between nutritional status and immune function. Severe bacterial infection is associated with elevated plasma levels of leptin. To determine the role of leptin in the host response to bacterial pneumonia leptin deficient ob/ob mice and normal wild-type (WT) mice were intranasally infected with different doses of the Gram-positive pathogen Streptococcus (S.) pneumoniae or the Gram-negative bacterium Klebsiella (K.) pneumoniae. After infection with lower doses of either pathogen ob/ob mice displayed lower pulmonary levels of proinflammatory cytokines, in particular tumor necrosis factor-α and chemokines. However, after infection with a higher dose of S. pneumoniae or K. pneumoniae the lung concentrations of these inflammatory mediators did not differ between ob/ob and WT mice. In addition, the extent and severity of lung inflammation, as assessed by semi-quantitative histopathology, were similar in both mouse strains. Finally, leptin deficiency did not impact on the bacterial outgrowth in the lungs during either Gram-positive or Gram-negative pneumonia irrespective of the infective dose. These data suggest that although leptin may play a modest role in the regulation of inflammation during bacterial pneumonia, it does not contribute to host defense mechanisms that act to limit the outgrowth of S. pneumoniae or K. pneumoniae in the lower airways.
Introduction

Leptin is a 16 kDa pleiotrophic protein encoded by the obese (ob) gene. Leptin belongs to the long-chain helical cytokine family that also includes interleukin (IL-6), IL-2 and IL-15 and is mainly produced by white adipocytes. In the circulation, leptin is bound to plasma proteins or its soluble receptor ObRe. Leptin signals via the long isoform of the leptin receptor ObRb which is present centrally in the hypothalamus. Furthermore, ObRb expression has also been demonstrated in a variety of tissues and cells like lung, pancreas, hematopoietic stem cells, blood mononuclear cells, T and B lymphocytes, endothelial cells and the reproductive organs (1-10).

The most important biological properties attributed to leptin are its effects on feeding, metabolism and the neuroendocrine axis (11, 12). However, several independent studies reported that during the early phase of sepsis, after systemic endotoxin administration and during the acute phase response, circulating leptin levels are elevated in humans and mice (13-19). Furthermore, leptin deficiency has been associated with an increased frequency of infection (6, 20-22).

Recently, Mancuso et al. investigated the role of leptin in host defense against respiratory tract infection in a murine model of Klebsiella (K.) pneumoniae pneumonia (23). Leptin deficient ob/ob mice were found to exhibit a reduced resistance against Klebsiella pneumonia as reflected by an enhanced bacterial outgrowth and a reduced survival. In the current study our primary objective was to determine the role of leptin in the immune response to Gram-positive pneumonia caused by Streptococcus (S.) pneumoniae, the most commonly isolated pathogen in patients with community-acquired pneumonia (24, 25). When we unexpectedly found that ob/ob mice displayed a similar rather than an accelerated outgrowth of pneumococci, we expanded our studies to experiments in which ob/ob mice were infected with K. pneumoniae. We here report, in contrast to the findings by Mancuso et al. (23), that although ob/ob mice show an altered inflammatory response against both Gram-positive and Gram-negative pneumonia, leptin deficiency does not influence bacterial growth in the lungs.

Materials and Methods

Animals

Ten- to 12-week-old male leptin deficient ob/ob mice on a C57Bl/6 background as well as wild-type (WT) C57Bl/6 control mice were obtained from Harlan Nederland (Horst, The Netherlands). The Animal Care and Use Committee of the University of Amsterdam approved all experiments.
Induction of pneumonia

Pneumonia was induced as described previously (26, 27). Briefly, for Gram-positive pneumonia, *S. pneumoniae* serotype 3 (ATCC 6303) were grown for 6 h to mid-logarithmic phase at 37°C using Todd-Hewitt broth (Difco, Detroit, MI). In order to induce Gram-negative pneumonia, *K. pneumoniae* serotype 2 (ATCC 43186) was grown for 5 h to mid-logarithmic phase at 37°C using Tryptic Soy broth (Difco, Detroit, MI). Both strains were harvested by centrifugation at 1500xg for 15 min, and washed twice in sterile isotonic saline. Bacteria were then resuspended in sterile isotonic saline at a concentration of 3x10^3 to 5x10^5 CFUs/50μl, as determined by plating serial 10-fold dilutions on sheep-blood agar plates. Mice were lightly anesthetized by inhalation of isoflurane (Upjohn, Ede, The Netherlands) and 50 μl (containing 3x10^3 to 5x10^5 CFU, depending on the experiment) was inoculated intranasally (i.n.). The infectious doses of *S. pneumoniae* and *K. pneumoniae* respectively were based on our previous investigations (26, 27).

Determination of bacterial outgrowth

Six or 48 h after infection, mice were anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium; active ingredients fentanyl citrate and fluanisone) and midazolam (Roche, Meidrecht, the Netherlands) and sacrificed by bleeding out the vena cava inferior. Blood was collected in EDTA containing tubes. Whole lungs were harvested and homogenized in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). CFUs in lungs and blood were determined from serial dilutions of lung homogenates and blood, plated on blood agar plates and incubated at 37°C for 16 h before colonies were counted.

Preparation of lung homogenates for cytokine measurements

For cytokine measurements, lungs were excised, weighed and homogenized in 0.9 % NaCl (1:5, weight/vol). Lung homogenates were then diluted with an equal volume of lysis buffer (pH 7.4) containing 300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% Triton X-100, and Pepstatin A, Leupeptin and Aprotinin (all 20 ng/ml; Sigma, St. Louis, MO) and incubated at 4°C for 30 min. Homogenates were centrifuged at 1500xg at 4°C for 15 min, and supernatants were stored at -20°C until assays were performed.

Assays

Leptin, IL-1β, IL-6, IL-10, Tumor Necrosis Factor α (TNF), cytokine-induced neutrophil chemoattractant (KC) and macrophage inflammatory protein (MIP)-2 were measured using specific ELISAs using high binding ELISA plates (Greiner Bio-One, Frieckenhausen, Germany) according to the manufacturer's instructions (R&D...
Pneumonia in ob/ob mice

Systems, Minneapolis, MN). The detection limits were 40 pg/mL for leptin, 31 pg/ml for IL-6, IL-1β, IL-10 and TNF, 37 pg/ml for KC and 187 pg/ml for MIP-2.

**Histologic examination**

Lungs were removed and fixed in 10 % buffered formalin in PBS for 24 h and embedded in paraffin. Hematoxilin and eosin stained slides were coded and semi-quantitatively scored for inflammatory parameters by a pathologist who was not aware of the origin of the tissue samples. To score lung inflammation and damage, the entire lung surface was analyzed with respect to the following parameters: interstitial inflammation, edema, endothelialitis, bronchitis and pleuritis. Each parameter was graded on a scale of 0 to 4, with 0: absent, 1: mild, 2: moderate, 3: severe and 4: very severe. The total “lung inflammation score” was expressed as the sum of the scores for each parameter, the maximum being 20 (26, 28).

**Statistical analysis**

Data are expressed as mean ± SEM. Two sample comparisons were performed by Mann Whitney U test using GraphPad Prism version 4.00, GraphPad Software (San Diego, CA). Comparisons between multiple groups were performed using Kruskall-Wallis test with Dunn’s Multiple Comparison a post test. P < 0.05 was considered to be statistically significant.

**Results**

**Leptin levels are increased locally during lung infection**

To determine whether leptin is present in the lung during *S. pneumoniae* and *K. pneumoniae* pneumonia, leptin levels were measured in lung homogenates of WT mice 48h after infection (fig. 1). Pulmonary leptin levels were elevated during both *S. pneumoniae* and *K. pneumoniae* pneumonia.

![Figure 1: Lung leptin levels. Leptin concentrations in lung homogenates of WT mice 48h after i.n. infection with different doses of *S. pneumoniae* (A; x10^5) or *K. pneumoniae* (B; x10^5). Data are mean ± SEM of 8 mice per group. *P<0.05, **P<0.01, ***P<0.001, all versus t = 0.](image-url)
Reduced levels of cytokines and chemokines in ob/ob mice during infection

The local production of pro-inflammatory cytokines and chemokines is considered a major host defense mechanism in response to bacteria that invade the respiratory tract (29, 30). We therefore infected ob/ob and WT mice with two doses of either *S. pneumoniae* or *K. pneumoniae* and determined the levels of the cytokines TNF, IL-1β, IL-6 and IL-10 and the chemokines KC and MIP-2 in lung homogenates at 48 h after infection (table I). Ob/ob mice displayed lower lung concentrations of cytokines and chemokines after infection with the lower dose of either *S. pneumoniae* (10^5 CFU) or *K. pneumoniae* (3 x 10^3 CFU) albeit not all differences reached statistical significance (table 1). The most consistent finding was reduced lung TNF levels in ob/ob mice upon infection with low dose *S. pneumoniae* or *K. pneumoniae*. Of note, lung cytokine and chemokine concentrations did not differ between ob/ob and WT mice after infection with higher doses of either *S. pneumoniae* (5 x 10^5 CFU) or *K. pneumoniae* (10^6 CFU) (table I). The pulmonary levels of the anti-inflammatory cytokine IL-10 did not differ between mouse strains in any of the experiments (table I).

Similar histopathology in infected lungs from WT and ob/ob mice

To further evaluate the role of leptin in lung inflammation, histological slides prepared from lungs harvested 48 h after infection were scored for signs of pulmonary inflammation. Ob/ob and WT mice demonstrated no differences in lung inflammation scores after infection with the different inocula of *S. pneumoniae* or *K. pneumoniae* (table II). Figure 2 shows representative slides of WT (fig. 2 A and

<table>
<thead>
<tr>
<th>TNF</th>
<th>IL-1β</th>
<th>IL-6</th>
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<tr>
<td>WT</td>
<td>ob/ob</td>
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<tr>
<td>5 x 10^5 CFU</td>
<td><em>S. pneumoniae</em></td>
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<tr>
<td>S. pneumoniae 1 x 10^6 CFU</td>
<td>0.9±0.4</td>
<td>0.02±0.02**</td>
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<th>MIP-2</th>
<th>IL-10</th>
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<td>WT</td>
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<tr>
<td>5 x 10^5 CFU</td>
<td><em>S. pneumoniae</em></td>
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</tr>
<tr>
<td>K. pneumoniae 3 x 10^5 CFU</td>
<td>13.8±4.2</td>
<td>5.1±1.7</td>
</tr>
<tr>
<td>K. pneumoniae 3 x 10^5 CFU</td>
<td>10.8±2.1</td>
<td>4.7±0.7*</td>
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</table>

Table I: Lung cytokine and chemokine levels 48 h post-infection. T and ob/ob mice were infected with indicated inocula of *S. pneumoniae* and *K. pneumoniae*. After 48 h, mice were sacrificed, lungs were removed and TNF, IL-1β, IL-6, KC, MIP-2 and IL-10 and were determined using ELISA (ng/mL). Data are means ± SEM, n = 8 mice per group; *P<0.05, **P<0.01 versus WT.
Figure 2: Lung histopathology. Representative histologic sections of lungs of WT (A, C) and ob/ob (B, D) mice sacrificed 48 h after infection with either 1x10⁵ S. pneumoniae (A, B) or 3x10³ K. pneumoniae (C, D). Hematoxillin & eosin staining, magnification x20.

C) and ob/ob (fig. 2 B and D) mice infected with either 1x10⁵ CFU of S. pneumoniae (fig. 2 A and B) or 3x10³ CFU of K. pneumoniae (fig. 2 C and D). In both types of pneumonia, pleuritis and interstitial inflammation were predominant features of pneumonia and the mice displayed signs of inflammation of the small vessels and bronchi of the lung. WT and ob/ob mice did not differ in the percentage of mice presenting with areas of confluent pneumonia in any of the experiments (data not shown).

<table>
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<tr>
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<th>WT</th>
<th>ob/ob</th>
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<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>5 x 10⁵ CFU S. pneumoniae</td>
<td>8.0 ± 2.0</td>
<td>10.2 ± 2.2</td>
</tr>
<tr>
<td>3 x 10³ CFU K. pneumoniae</td>
<td>3.9 ± 0.5</td>
<td>4.8 ± 0.6</td>
</tr>
<tr>
<td>1 x 10⁴ CFU K. pneumoniae</td>
<td>5.6 ± 1.1</td>
<td>5.8 ± 0.5</td>
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Table II: Lung inflammation scores 48 h after infection. WT and ob/ob mice were infected with the indicated inocula of S. pneumoniae and K. pneumoniae. After 48h mice were sacrificed, lungs were removed; hematoxillin/eosin stained slides were prepared and scored (see Methods for details). Data are means ± SEM, n = 8 mice per group. Differences between groups were not significant.

Leptin deficiency does not influence bacterial outgrowth

To study whether leptin deficiency interferes with the outgrowth of respiratory pathogens during bacterial pneumonia, we determined the number of CFU in lungs of WT and ob/ob mice obtained 48h after infection with 10⁵ or 5x10⁵ S. pneumoniae or with 3x10³ or 10⁴ K. pneumoniae. In none of these experiments
differences were found between ob/ob and WT mice (fig. 3). In addition, the percentage of mice with positive blood cultures was not different between groups although ob/ob mice tended to have fewer positive blood cultures after infection with the lower S. pneumoniae dose (fig. 3). To examine whether leptin deficiency impacts on the very early immune response to respiratory pathogens we infected ob/ob and WT mice with $1 \times 10^5$ CFU S. pneumoniae and $3 \times 10^3$ CFU K. pneumoniae and prepared homogenates from lungs harvested 6h post infection. Again no differences in pulmonary bacterial loads were detected between WT and ob/ob mice (data not shown).

![Figure 3: Bacterial outgrowth.](image)

**Figure 3: Bacterial outgrowth.** WT and ob/ob mice were inoculated with $5 \times 10^5$ or $10^5$ CFU of S. pneumoniae or $3 \times 10^3$ or $10^4$ CFU K. pneumoniae and bacterial outgrowth in lungs was determined 48 h later. Data represent mean ± SEM of 8 mice per group, 48h after infection. Differences between groups were not significant.

**Discussion**

Leptin is a multifunctional protein that exerts its main effects on food regulation and basal metabolism. More recent research focused on the effects of leptin on the immune system. It has been hypothesized that leptin deficiency caused by malnutrition as well as leptin resistance caused by obesity can lead to an altered inflammatory response, reduced Th1 responses and possibly reduced protection against infection (31, 32). One study indeed demonstrated that children deficient in leptin exhibit an impaired cell-mediated immune response and an increased incidence of infectious diseases (20). In line, we recently demonstrated a role for leptin in the protective immune response to experimentally induced lung tuberculosis in mice (33).

Little is known about the role of leptin in bacterial infections. Mancuso et al. reported about the role of leptin in K. pneumoniae induced pulmonary infection using ob/ob mice (23). In this acute Gram-negative pneumonia, ob/ob mice exhibited an enhanced bacterial outgrowth and a reduced survival; similar to our study local lung leptin leptin levels were elevated in WT mice. The diminished
Pneumonia in \textit{ob/ob} mice

Resistance against \textit{Klebsiella} pneumonia was accompanied by an intrinsically impaired phagocytosis capacity of \textit{ob/ob} alveolar macrophages (23) and neutrophils (34). These data contrast with the results presented here. Our primary goal was to determine the contribution of leptin to the protective immune response to Gram-positive pneumonia caused by \textit{S. pneumoniae}. When we inoculated mice with several infectious doses, including very low doses ($\text{10}^4$ CFU) that were effectively cleared (data not shown), we did not detect differences in the outgrowth of pneumococci in the lungs of \textit{ob/ob} and WT mice. We then evaluated the possibility of a pathogen specific role for leptin in host defense against bacterial pneumonia and infected mice with \textit{K. pneumoniae} and again did not detect differences in bacterial loads in lungs of \textit{ob/ob} and WT mice. This is remarkable since we used the same \textit{K. pneumoniae} strain (serotype 2, ATCC 43816) and the same dose range as described in the study by Mancuso and coworkers (23). A few differences in study design between this latter investigation and our study should be noted. Firstly infection was accomplished by slightly different routes (intratracheal versus intranasal). One can argue that \textit{ob/ob} mice are less capable of inhaling pathogens during the intranasal inoculation procedure because of their high body fat mass, possibly obstructing the airways; as a consequence the effective infectious dose could be lower in \textit{ob/ob} mice. To rule this out, we compared the efficacy of the infection by measuring the bacterial loads 1h after intranasal inoculation of \textit{K. pneumoniae} and found no differences between \textit{ob/ob} and WT mice (data not shown). Secondly, the age of the mice was not stated in the publication by Mancuso et al. (23). It is known that defense against pulmonary infections is in part dependent on the age of the host and differences in age of the mice studied therefore might impact on study outcomes (older mice are less susceptible to infections than very young mice) (35).

We investigated the role of leptin in the inflammatory response to respiratory pathogens by measuring cytokine and chemokine concentrations in lung homogenates of mice infected with \textit{S. pneumoniae} or \textit{K. pneumoniae}. Indeed, \textit{ob/ob} mice displayed lower concentrations of TNF (and several other proinflammatory mediators) in their lungs after infection with the lower doses of either \textit{S. pneumoniae} or \textit{K. pneumoniae}. At higher infectious doses these differences disappeared suggesting that leptin plays a limited role in the cytokine response to bacterial pneumonia that is dependent on the severity of the challenge delivered to the airways. Of note, Mancuso and coworkers did not find differences in TNF or MIP-2 in lungs of \textit{ob/ob} and WT mice 48h after infection with \textit{K. pneumoniae} (23). These authors infected mice with $5 \times 10^3$ CFU intratracheally, whereas we inoculated mice with $3 \times 10^3$ CFU or $1 \times 10^4$ CFU intranasally. Hence, the cytokine data presented here and by Mancuso et al. (23) do not contradict each other.
The studies presented here point to a limited role of leptin the inflammatory response to bacterial pneumonia caused by either a common Gram-positive or a common Gram-negative respiratory pathogen. Leptin deficiency does not appear to impact on host defense mechanisms important for inhibiting the growth of S. pneumoniae or K. pneumoniae in the lower airways.

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References


