Host-pathogen interactions during (myco)bacterial respiratory tract infections
Wieland, C.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Pulmonary *Mycobacterium tuberculosis* infection in leptin deficient *ob/ob* mice

Catharina W. Wieland
Sandrine Florquin
Edward D. Chan
Jaklien C. Leemans
Sebastiaan Weijer
Annelies Verbon
Giamila Fantuzzi
Tom van der Poll

*Int Immun* 2005; 17: 1399
Abstract

The development of active tuberculosis after infection with *Mycobacterium (M.)* *tuberculosis* is almost invariably caused by a persistent or transient state of relative immunodeficiency. Leptin, the product of the obese-gene (*ob*), is a pleiotropic protein produced mainly by adipocytes and is downregulated during malnutrition and starvation, conditions closely connected with active tuberculosis. To investigate the role of leptin in tuberculosis, we intranasally infected wild-type (Wt) and leptin deficient *ob*/*ob* mice with live virulent *M. tuberculosis*. *Ob*/*ob* mice displayed higher mycobacterial loads in the lungs after 5 and 10 weeks of infection although the difference with Wt mice remained 1 log of *M. tuberculosis* CFU. Nevertheless, *ob*/*ob* mice were less able to form well-shaped granuloma and lung lymphocyte numbers were reduced compared to Wt mice early during infection. In addition, *ob*/*ob* mice had a reduced capacity to produce the protective cytokine interferon γ (IFNγ) at the site of the infection early during infection and upon antigen specific recall stimulation, and showed reduced delayed-type hypersensitivity reaction to intradermal tubercul in purified protein derivative. Leptin replacement restored the reduced IFNγ response observed in *ob*/*ob* mice. Mortality did not differ between *ob*/*ob* and Wt mice. These data suggest that leptin plays a role in the early immune response to pulmonary tuberculosis.
Introduction

*Mycobacterium tuberculosis* and the associated disease tuberculosis are main threats to mankind, with one-third of the world population being infected (1). Over 8 million new cases of tuberculosis and 2 million deaths from this disease occur yearly worldwide (2). The rising incidence of tuberculosis over the last decade has increased the need to define host factors that control resistance to tuberculosis.

The chance of developing active disease after *M. tuberculosis* infection is approximately 10% in a life-time in the non-HIV infected host. The major risk factor for developing active tuberculosis is immunodeficiency. Worldwide, malnutrition and starvation are major causes of immunosuppression and increased susceptibility to infectious diseases like tuberculosis (3-9). Furthermore, wasting has long been recognized as a prominent feature of tuberculosis and it is probably one of the determinants of disease severity and outcome (10-12). Several different aspects of malnutrition can be held responsible for the immunosuppression in malnourished patients, including deficiencies of proteins, selenium, zinc, iron or vitamins (A, C or E) (6, 8, 13, 14). More recently, another possible link between nutrition and immune status has been described: leptin.

Leptin, the 16 kDa protein product of the *obese* (*ob*) gene, is mainly produced by white adipocytes. Leptin belongs to the long-chain helical cytokine family that also includes IL-6 and it signals via a class I cytokine receptor (Ob-Rb). The most important biological properties attributed to leptin are its effects on feeding, metabolism and the neuroendocrine axis (15). In normal mice circulating leptin levels are decreased during prolonged fasting and malnutrition (16). On the other hand, acute and chronic inflammatory conditions are associated with increased circulating leptin levels, anorexia and loss of lean body weight (17-20). Children deficient in leptin have an impaired cell-mediated immune response and an increased incidence of infectious diseases (13, 21). More recently it has been demonstrated that leptin also has a direct effect on T-helper (Th) cells, shifting the immune system towards a Th1 immune response (22-24).

In the *ob/ob* mouse, a mutation of the *ob* gene leads to the formation of a non-functional protein. This leptin deficiency creates a phenotype that closely resembles the neuroendocrine and immune status of wasting (25). *Ob/ob* mice display thymic atrophy, reduced splenic weight, reduced circulating leukocyte numbers and an impaired cellular immune function (9, 22, 23, 26, 27). As a result of their diminished cellular immunity, *ob/ob* mice are protected against experimental autoimmune encephalomyelitis (EAE), an autoimmune disease mediated by auto-reactive T-cells (28).

In the present study we sought to determine the role of leptin during murine *M. tuberculosis* infection. By making use of *ob/ob* mice, we aimed to mimic a
leptin deficient state with the neuroendocrine and immunological features of wasting. We show that leptin contributes to a protective immune response and therefore leptin might be an important connection between nutritional status and *M. tuberculosis* disease.

**Materials and Methods**

**Mice**

Six- to 8-week-old female 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). Mice were maintained in biosafety level 3 facilities. The Animal Care and Use Committee of the University of Amsterdam approved all experiments.

**Experimental infection**

A virulent laboratory strain of *M. tuberculosis* H37Rv (American Type Culture Collection, Rockville, MA) was grown for 4 days in liquid Dubos medium containing 0.01 % Tween-80. A replicate culture was incubated at 37 °C, harvested at mid-log phase, and stored in aliquots at -70 °C. For each experiment, a vial was thawed and washed with sterile 0.9 % NaCl. Tuberculosis was induced as described previously (29-31). Briefly, mice were anesthetized by inhalation with isoflurane (Abott Laboratories LTD., Kent, United Kingdom) and infected intranasally (i.n.) with 6x10^4 live *M. tuberculosis* H37Rv bacilli in 50 μl saline, as determined by viable counts on Middlebrook 7H11 plates. Groups of eight mice per time-point were sacrificed two, five or ten weeks after infection. Lungs were removed aseptically, and homogenized in 5 volumes of sterile 0.9 % NaCl. 10-fold dilutions were plated on Middlebrook 7H11 agar plates to determine bacterial loads. Colonies were counted after 21 days at 37 °C. Numbers of CFU are provided per g of lungs. In order to check for infection efficacy, four mice per group were sacrificed one day post-infection. For cytokine measurements, lung homogenates were diluted 1:2 in lysis buffer 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; pH 7.4) and incubated on ice for 30 min. Homogenates were centrifuged at 1500 x g at 4 °C for 15 min, and supernatants were sterilized using a 0.22 μm filter (Corning Incorporated, Corning, NY) stored at -20 °C until assays were performed.

**Leptin administration**

Alzet miniosmotic pumps (Durect Corp., Cupertino, CA, model 2004) with an exchange rate of 0.25 μl/h were filled aseptically with either sterile 0.9% NaCl or leptin (PeproTech, Rocky Hill, NJ) at a delivery rate of 5 μg/day. Mice were
anesthetized with Hypnorm® (Janssen Pharmaceutica, Beerse, Belgium) and the pump was implanted s.c.. Four days after implantation, mice were infected i.n. with 5x10^4 CFU *M. tuberculosis* and sacrificed 2 weeks after infection.

**Characterization of inflammatory infiltrates in the lungs**

Pulmonary cell suspensions were obtained by crushing lungs through a 40-µm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described previously (29, 31). Erythrocytes were lysed with ice-cold isotonic NH₄Cl solution (155 mM NH₄Cl, 10 mM KHCO₃, 0.1 mM EDTA, pH 7.4), the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium), and counted by using a hemocytometer. The percentages of macrophages, polymorphonuclear cells (PMNs) and lymphocytes were determined using cytospin preparations stained with haematoxilin and eosin.

**Flow cytometric analysis**

Lung cell suspensions obtained from infected mice were analyzed by flowcytometry using FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) as described previously (29, 31). Cells were brought to a concentration of 4 x 10⁶ cells per mL of FACS buffer (PBS supplemented with 0.5 % BSA, 0.01% NaN₃ and 0.35 mM EDTA). Immunostaining for cell surface molecules was performed for 30 minutes at 4 °C using directly labeled antibodies (abs) against CD3 (CD3-phycoerythrin), CD4 (CD4-CyChrome), or CD8 (CD8-FITC and CD8-peridinin chlorophyl protein). All abs were used in concentrations recommended by the manufacturer (Pharmingen, San Diego, CA). After staining, cells were fixed in 2 % paraformaldehyde, and T cell surface molecules were analyzed within the gate containing CD3⁺ cells.

**Histology and immunohistochemistry**

Lungs were removed 2, 5 or 10 weeks after inoculation with *M. tuberculosis, fixed in* 10 % buffered formaline for 24 h, and embedded in paraffin. Footpads were first fixed in formaline for 48 h followed by decalcification using 12,5% ethylene diamine tetra acetic acid solution (pH 7.4; Merck, Darmstatt, Germany) for 2 weeks. Footpads were then cut longitudinally and embedded in paraffin. Hematoxilin and eosin stained slides were coded and semiquantitatively scored for inflammatory infiltrates and lung granuloma formation by a pathologist. For granulocyte stainings, slides were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by a solution of 0.1% NaN₃/0.03% H₂O₂ (Merck). Slides were then digested by a solution of pepsin 0.25% (Sigma, St. Louis, MO) in 0.01 M HCl. After being rinsed, the sections were incubated in 10% normal goat serum (Dako, Glostrup, Denmark) and then exposed to FITC-labeled anti-mouse Ly-6-G monoclonal antibody (Pharmingen, San Diego, CA). After washes, slides were
incubated with a rabbit anti-FITC antibody (Dako) followed by further incubation with a biotinylated swine anti-rabbit antibody (Dako), rinsed again, incubated in a streptavidine-ABC solution (Dako) and developed using 1% H$_2$O$_2$ and 3.3-diaminobenzidin-tetra-hydrochloride (Sigma) in Tris-HCl. The sections were mounted in glycerin gelatin without counterstaining and analyzed.

**Splenocyte stimulation**

Single cell suspensions were obtained by crushing spleens through a 40-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ) as described (29-31). Erythrocytes were lysed with ice-cold isotonic NH$_4$Cl solution (155 mM NH$_4$Cl, 10 mM KHCO$_3$, 0.1 mM EDTA, pH 7.4); the remaining cells were washed twice with RPMI 1640 (Bio Whittaker, Verviers, Belgium) supplemented with 10% FCS and 1% antibiotic-antimycotic (GibcoBRL, Life Technologies, Rockville, MD). Cells were seeded in 96-well round bottom culture plates at a cell density of 3 x 10$^5$ cells per well in quadruplicate, and stimulated with 20 μg/ml tuberculin purified protein derivative (PPD, Statens Seruminstitut, Copenhagen, Denmark). Supernatants were harvested after 48-h incubation at 37 °C in 5% CO$_2$, and cytokine levels were analyzed by ELISA.

**Delayed-type hypersensitivity (DTH) response to PPD**

To measure DTH responses, we examined swelling responses of footpads in mice according to previously described methods (29). Briefly, Wt and ob/ob mice (n=6 mice per group) were immunized intradermally at the base of the tail with 0.1 mg of heat-killed *M. tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) in 0.1 mL of mineral oil (Sigma). Twelve days after immunization, mice were challenged with 40 μg PPD in saline into one of the hind footpads and in the other with saline alone. Measurements of footpad thickness were performed with a Mitutoyo model 7326 engineer’s micrometer (Mitutoyo MTI Corporation, Aurora, Ill) before, 24 and 48 h after the PPD challenge. The increase in footpad thickness was calculated as the difference in swelling between 0 and 24 or 48 h measurements. Specific DTH reactivity was calculated as the difference between the swelling of the PPD-injected footpads and the swelling of the saline-injected footpads.

**Cytokine and leptin measurements**

Leptin, Interferon (IFN)-γ, Tumor Necrosis Factor-α (TNF), IL-4, IL-10 and IL-6 were measured by ELISA using matched Ab pairs according to the manufacturer’s instructions (R&D Systems Inc., Minneapolis, Minnesota, USA). Detection limits were 40 p/mL for leptin, 63 pg/mL for IFNγ, TNF, IL-4 and IL-10 and 32 pg/mL for IL-6.
**Statistical analysis**

All values are expressed as mean ± SEM. Comparisons were done with Mann-Whitney U tests. For comparison of survival curves, Kaplan-Meier analysis with a log-rank test was used. Values of $P<0.05$ were considered statistically significant.

![Graph](image)

**Figure 1: Pulmonary leptin levels.** Leptin concentrations in lung homogenates of Wt mice 2, 5 and 10 weeks after i.n. infection with $6 \times 10^4$ CFU *M. tuberculosis*. Data are mean ± SEM of 8 mice per time point. *P<0.05, **P<0.01, ***P<0.001 (all versus $t = 0$).

**Results**

*Leptin levels are increased locally during *M. tuberculosis* infection*

To determine whether leptin is present locally during murine *M. tuberculosis* infection, leptin concentrations were measured in lung homogenates of Wt mice. Lung tuberculosis was associated with a transient increase in pulmonary leptin levels, peaking after 2 weeks (fig. 1).

*Increased bacterial loads in lungs of *ob/ob* mice late in infection*

Twenty-four hours after infection, lungs of Wt and *ob/ob* mice contained equal numbers of viable mycobacteria ($4.0 \pm 1.2 \times 10^4$ CFU/g lung and $6.5 \pm 1.8 \times 10^4$ CFU/g lung respectively; figure 2). During the first 5 weeks of infection, mycobacteria grew exponentially in lungs of both mouse strains, albeit with different kinetics. After 2 weeks of infection, the bacterial load in lungs of *ob/ob* mice was modestly but significantly lower than in Wt mice. However, thereafter the bacterial loads increased faster in *ob/ob* mice than in Wt mice. After 5 weeks bacterial counts reached their highest levels in Wt mice, which did not increase further until 10 weeks. Although the bacterial burden in the lungs of *ob/ob* mice did not reach such a plateau, the difference present at 5 weeks post-infection did not further increase thereafter.
Survival
To investigate the role of leptin in the outcome of tuberculosis, Wt and ob/ob mice were monitored during a period of 28 weeks after infection (Figure 3). Consistent with the resistant C57BL/6 genetic background, few Wt mice (2/14) died during this follow up. Although ob/ob mice displayed an increased mortality (5/12), the difference with Wt mice did not reach statistical significance (P=0.1).

![Figure 2: Enhanced mycobacterial outgrowth in lungs of ob/ob mice. Wt (closed symbols) and ob/ob mice (open symbols) were i.n. infected with 6 x 10^4 CFU M. tuberculosis H37Rv, and mycobacterial loads were determined in lungs on day 1 and 2, 5 and 10 weeks afterwards. Data are mean ± SEM of 7-8 mice per group at each time point (except for day 1, n = 4 per group). *P< 0.05 versus Wt mice.](image)

![Figure 3: Survival. Survival of Wt (n = 14; closed symbols) and ob/ob mice (n = 12; open symbols) after i.n. infection with M. tuberculosis. The difference between groups was not significant (P = 0.10).](image)

Altered pathology in leptin deficient mice: Infiltration of granulocytes
The effect of leptin deficiency on the histopathology of lungs from M. tuberculosis-infected mice was assessed at 2, 5 and 10 wks post-infection. Two weeks after inoculation, the lungs of Wt mice displayed small lymphocytic granulomas generally located around small bronchi and vessels (Figure 4A). Ob/ob mice also exhibited a strong inflammatory infiltrate but, in contrast to the lymphocytic granuloma seen in Wt mice, it primarily consisted of polymorphonuclear cells (PMNs) (Figure 4B). This difference was more striking 5 weeks after infection (Figures 4C and D). Although the inflammation had become more diffuse in both groups at that time point, the infiltrate in Wt mice still consisted primarily of lymphocytes, whereas the infiltrate in the lungs of ob/ob mice was dominated by PMNs. Furthermore, at this time, edema and pleuritis were more pronounced in ob/ob than Wt mice. At 10 weeks post-infection, no clear differences in histopathology were detected between Wt and ob/ob mice any longer. Nevertheless, although not as apparent as
at the earlier time points studied, pulmonary infiltrates consisted of more PMNs in the lungs of ob/ob mice than Wt mice (Figures 4E and F).

Impaired lymphocyte recruitment to lungs of ob/ob mice
To analyze leukocyte influx in a more quantitative way, cells infiltrating the lungs were isolated, counted and differentiated by performing a modified hematoxilin and eosin staining on cytopsin preparations (Table I). An increase in cell numbers
during the infection was observed in both groups and no difference in total cell numbers between Wt and \textit{ob/ob} mice was observed at the different time-points of infection. Similar to our analysis of the pathological slides, differential cell counts revealed that the infiltrate in the lungs of \textit{ob/ob} mice was composed of significantly less lymphocytes compared to Wt mice at all time-points after infection. This lack in lymphocytes was compensated by an increase in the number of PMNs. No differences in lung macrophage counts were observed between groups.

\textit{Impaired T-cell activation in leptin deficient mice}

To obtain more insight in the T-cell subsets of the pulmonary infiltrates, we used flow cytometry to examine the phenotype of CD3$^+$ cells within whole lung cell suspensions (Table II). At 2 and 5 weeks post-infection, the proportions of CD4$^+$ and CD8$^+$ cells within the CD3$^+$ population were similar in Wt and \textit{ob/ob} mice. At 10 weeks, \textit{ob/ob} mice displayed a lower percentage of CD8$^+$ and a higher percentage of CD4$^+$ cells when compared to Wt mice. To evaluate the activation status of T cells in the lungs, we analyzed CD69 surface expression on CD3$^+$/CD4$^+$ and CD3$^+$/CD8$^+$

<table>
<thead>
<tr>
<th>Lung</th>
<th>Cells x 10$^4$</th>
<th>% Més</th>
<th>% PMNs</th>
<th>% Lymphocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{2 wk postinfection}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>873 ± 238</td>
<td>17.4 ± 1.6</td>
<td>39.2 ± 1.6</td>
<td>43.1 ± 1.5</td>
</tr>
<tr>
<td>\textit{ob/ob}</td>
<td>561 ± 123</td>
<td>17.4 ± 1.7</td>
<td>49.1 ± 1.1***</td>
<td>33.8 ± 2.1***</td>
</tr>
<tr>
<td>\textit{5 wk postinfection}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>2004 ± 648</td>
<td>14.5 ± 1.5</td>
<td>39.4 ± 1.4</td>
<td>50.7 ± 5.6</td>
</tr>
<tr>
<td>\textit{ob/ob}</td>
<td>1622 ± 4261</td>
<td>13.6 ± 1.2</td>
<td>52.5 ± 2.3**</td>
<td>33.9 ± 2.3**</td>
</tr>
<tr>
<td>\textit{10 wk postinfection}</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wt</td>
<td>1479 ± 1277</td>
<td>23.7 ± 2.6</td>
<td>36.3 ± 2.1</td>
<td>40.0 ± 2.3</td>
</tr>
<tr>
<td>\textit{ob/ob}</td>
<td>1729 ± 2238</td>
<td>20.2 ± 2.0</td>
<td>42.0 ± 6.1*</td>
<td>31.9 ± 2.6*</td>
</tr>
</tbody>
</table>

\textit{Table I: Shift in pulmonary cellular composition of infected \textit{ob/ob} mice.} Leukocytes in lungs of Wt and \textit{ob/ob} mice 2, 5 or 10 weeks after i.n. infection with \textit{M. tuberculosis}. Data are mean ± SEM of 7-8 mice per group at each time point. *P<0.05, **P<0.01, ***P<0.001 (all versus Wt mice at the same time point).

<table>
<thead>
<tr>
<th>CD4$^+$</th>
<th>CD4$^+$/CD69$^+$</th>
<th>CD8$^+$</th>
<th>CD8$^+$/CD69$^+$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wt</td>
<td>29.0 ± 2.9</td>
<td>18.0 ± 2.8</td>
<td>51.6 ± 3.1</td>
</tr>
<tr>
<td>\textit{ob/ob}</td>
<td>23.8 ± 3.7</td>
<td>7.6 ± 2.7*</td>
<td>56.7 ± 5.1</td>
</tr>
<tr>
<td>Wt</td>
<td>47.5 ± 3.0</td>
<td>21.9 ± 1.7</td>
<td>43.4 ± 2.6</td>
</tr>
<tr>
<td>\textit{Ob/ob}</td>
<td>47.5 ± 3.0</td>
<td>15.8 ± 3.1*</td>
<td>38.5 ± 2.2</td>
</tr>
<tr>
<td>Wt</td>
<td>47.5 ± 3.0</td>
<td>32.2 ± 3.1</td>
<td>40.3 ± 3.0</td>
</tr>
<tr>
<td>\textit{ob/ob}</td>
<td>59.4 ± 1.5*</td>
<td>38.0 ± 1.5</td>
<td>27.3 ± 1.8**</td>
</tr>
</tbody>
</table>

\textit{Table II: Effect of leptin deficiency on T cell subsets in lungs during tuberculosis.} T cell subsets in lungs of mice infected with \textit{M. tuberculosis} 2, 5 and 10 weeks post-infection. Lung cells were collected and FACS was performed as described in the Methods section. Results are presented as % CD4$^+$, CD8$^+$ and CD69$^+$ within the CD3$^+$ lymphocyte population. Data are mean ± SEM of 7 to 8 mice per group at each time point. *P<0.05, **P<0.01, ***P<0.001 (all versus Wt mice at that time point).
CD8' cells throughout the infection. Although the percentages of CD69' T cells increased over time in both mouse strains, CD3'/ CD4' and CD3'/ CD8' cells of ob/ob mice were less activated as reflected by a reduced expression of CD69 relative to Wt mice. Together these data suggest that ob/ob mice recruited less lymphocytes to the site of the infection during tuberculosis and that these recruited T cells were less activated. Particularly, during the early phases of the infection this reduced lymphocyte recruitment involved both CD4' and CD8' T cells.

Reduced IFNγ levels in lungs of ob/ob mice
IFNγ is a key cytokine in the protective immune response to M. tuberculosis infection. Therefore, we measured IFNγ concentrations in lung homogenates of infected mice (Figure 5). Although IFNγ levels increased during infection in both groups, ob/ob mice had strongly reduced lung IFNγ levels at all time points examined (P < 0.05 versus Wt mice). In contrast, the concentrations of IL-4, TNF, IL-6 and IL-10 in lung homogenates did not differ significantly between the mouse strains at any time point (data not shown).

Figure 5: Reduced IFNγ levels in lungs of ob/ob mice. IFNγ concentrations in lung homogenates of Wt (filled bars) and ob/ob mice (open bars) 2, 5 and 10 weeks after infection with M. tuberculosis. Data are mean ± SEM of 7-8 mice per group at each time point. *P < 0.05 versus Wt mice.

Figure 6: Reduced IFNγ response to tuberculosis specific antigen by splenocytes from infected ob/ob mice. Splenocytes were isolated from Wt (filled bars) and ob/ob mice (open bars) 2, 5 and 10 weeks after infection with M. tuberculosis and stimulated with PPD for 48h. IFNγ was measured in culture supernatants. Data are mean ± SEM of 7-8 mice per group at each time point. *P < 0.05 versus Wt mice.
Reduced IFN\(\gamma\) production upon ex vivo stimulation of splenocytes

Next we determined the capacity of splenocytes obtained from infected mice to respond to a recall antigen. Therefore, splenocytes were harvested at 2, 5 and 10 weeks after infection and stimulated with PPD, after which IFN\(\gamma\) and IL-4 were measured in the supernatant. Splenocytes from infected ob/ob mice produced significantly less IFN\(\gamma\) at 2 and 5 wks after infection (Figure 6). This difference had fully disappeared at 10 weeks post-infection. IL-4 levels were elevated in supernatants from ob/ob splenocytes compared to splenocytes from WT mice, but this difference was not statistically significant (data not shown).

Figure 7: DTH response. Wt (filled bars;A) and ob/ob mice (open bars;B) were immunized with heat-killed M. tuberculosis and challenged in one hind footpad with PPD and in the other footpad with saline. Footpad swellings were measured 0 and 24 h following the challenge and calculated as described in methods (C). Data mean ± SEM of 8 mice per group. Representative section of the footpad of a Wt (A) mouse 48h after a DTH reaction showing a diffuse and dense inflammatory infiltrate consisting of mononuclear cells and neutrophils. In the footpad of the ob/ob (B) mouse, the inflammatory reaction was similar but reduced. H&E staining, magnification x10.
Reduced DTH reaction to M. tuberculosis specific antigen in ob/ob mice

The recruitment of leukocytes into inflamed areas is critical for the development of DTH responses. To investigate leukocyte recruitment during a DTH reaction against M. tuberculosis-specific antigens, mice were immunized and subsequently challenged in one footpad with PPD and with saline in the other. Both Wt and ob/ob mice showed significant footpad swelling after the challenge. At 24 and 48 h after PPD challenge, the increase in footpad thickness was strongly reduced in ob/ob mice compared to Wt mice (Figure 7C; not shown for 48 h). Histological analysis confirmed that the DTH reaction to PPD was strongly reduced in ob/ob mice. The footpads of Wt mice showed a diffuse and dense infiltrate consisting primarily of mononuclear cells and PMNs (Figure 7A). In the ob/ob mice the inflammatory infiltrate was reduced and limited to the subcutis and in addition, edema was almost absent (Figure 7B).

Restoration of reduced IFNγ response by leptin replacement

Ob/ob mice intrinsically exhibit lower peripheral blood leukocyte counts, thymic atrophy and reduced T cell responses. To study if the reduced immune response observed in ob/ob mice during M. tuberculosis infection were due to the natural defects of ob/ob mice or a direct cause of leptin deficiency, we treated ob/ob mice with exogenous leptin (continuous delivery; 5 µg/day), four days before and during the first two weeks of infection. Leptin administration to ob/ob mice increased the reduced lung IFNγ concentration and the diminished IFNγ release after antigen-specific stimulation of splenocytes to levels found in normal Wt mice (fig. 8). Leptin administration did not influence the number of M. tuberculosis CFU at this early time point.

![Figure 8: Leptin administration to ob/ob mice restores the IFNγ response.](image-url)

*P< 0.05 and **P<0.01 versus Wt mice.
Discussion

Malnutrition is an important cause of immunosuppression worldwide. The effects of nutritional deficiencies on tuberculosis could result from the impairment of several important effector mechanisms of the immune system, including cellular immunity (4, 8, 13, 22, 32). In \textit{M. tuberculosis} infection, immunosuppression can cause loss of infection control leading to active disease. Although starvation results in deficiencies of several important nutrients (such as vitamins, zinc, etc.) that influence immune function, another factor influencing both immunity and nutritional status is leptin. Leptin, an adipocyte-derived protein of the long-chain helical cytokine family, has been proposed to act as a link between nutritional status and immune function (13, 22, 33). Furthermore, leptin has multiple biological effects on nutritional status, metabolism and the neuroendocrine axis (33). We here demonstrate that leptin-deficient \textit{ob/ob} mice have a moderately impaired immune response to pulmonary infection with \textit{M. tuberculosis} as reflected by a modestly enhanced outgrowth of mycobacteria, a reduced capacity to produce IFN\textgamma; at the site of the infection early during infection and upon antigen specific recall stimulation, and a disturbance of the composition of the inflammatory infiltrate in the lungs characterized by less well shaped granulomas, reduced lymphocyte and higher PMN numbers. Nonetheless, these alterations in host defense did not lead to a reduction in survival. In line, at 10 weeks post-infection, the immune response of the \textit{ob/ob} mice seem to catch up with that observed in Wt mice. Altogether our data suggest that \textit{ob/ob} mice display a transiently reduced host defense against pulmonary tuberculosis.

To our knowledge, three earlier studies investigated a possible correlation between plasma leptin levels and the presence of tuberculosis and/or disease severity in humans (34-36). One study reported lower plasma leptin concentrations in tuberculosis patients than in controls (35), whereas a second study found higher leptin levels in 8 female (but not in 22 male) patients suffering from active tuberculosis (34). The third study did not include a control group and found that during therapy, leptin levels were solely dependent on body fat mass (36). Van Crevel et al. (35) hypothesize that wasting, the inflammatory response and decreased energy-intake during disease lead to low plasma leptin levels, which then results in reduced cellular immunity and subsequently increased disease severity. This hypothesis is, in part, supported by our current investigation using leptin-deficient mice. However, in contrast to the data on plasma levels in chronically ill patients, in our murine model of tuberculosis local lung leptin levels were elevated during disease. Of note, at 10 weeks post-infection, Wt mice did not yet suffer from wasting.

Little is known about the role of leptin in animal models of infectious diseases. One study using \textit{ob/ob} mice investigated the consequences of leptin deficiency in
*M. tuberculosis* infection in *ob/ob* mice

*Klebsiella pneumoniae* induced pulmonary infection (37). In this acute gram-negative pneumonia, *ob/ob* mice demonstrated a much clearer exhibited enhanced bacterial outgrowth and reduced survival; similar to our study, local lung leptin levels were elevated in Wt mice.

IFNγ is the key protective cytokine in host defense against *M. tuberculosis* and IFNγ gene-deficient mice succumb to experimentally induced tuberculosis (38, 39). IFNγ is an important regulator of the cellular immune response: it directly activates macrophages and enables them to kill ingested pathogens, including *M. tuberculosis*. Two mechanisms by which IFNγ activates macrophages are production of Nitric Oxide by inducible nitric oxide synthase 2 or the induction of LRG-47, a p47 GTPase which is associated with autophagy (40-42). We found reduced lung IFNγ concentrations at all time points studied after infection. Interestingly, this difference in local IFNγ only resulted in a modest difference in bacterial load in the lungs. During lung tuberculosis, IFNγ is produced by CD4+ and CD8+ T cells as well as alveolar macrophages (43, 44). In our study, the number of CD4+ and CD8+ lymphocytes as well as their activation status was reduced in the lungs of *ob/ob* mice, which may, at least in part, explain the lower IFNγ levels. These results point to a decrease in a locally effective Th1 response in *ob/ob* mice following infection. In the spleen, the Th1 response was also impaired in infected *ob/ob* mice: recall antigen stimulation using PPD revealed that splenocytes from leptin-deficient mice failed to secrete significant amounts of IFNγ following antigen challenge in vitro 2 and 5 weeks after infection. These data suggest that leptin deficiency resulted in a defective priming of naïve and memory T-cells by *M. tuberculosis* in vivo in the spleen. Interestingly, at 10 weeks post-infection, PPD stimulation of splenocytes did not result in a significant difference in the release of IFNγ; hence the T cell response appeared to have caught up. Altogether these data suggest that *ob/ob* mice have a reduced capacity to produce the protective cytokine IFNγ early during tuberculosis, which is compensated for later in infection. Granulomas are well-organized structures composed of aggregated macrophages, lymphocytes and epithelioid cells, known to wall off the infectious site and prevent dissemination (45). Our data demonstrate that *ob/ob* mice are less able to develop well-formed granulomas during experimental tuberculosis, which was associated with lower lymphocyte and higher PMN numbers in infected lungs. Conceivably, the increased influx of PMNs into the site of infection represents a compensatory response due to diminished lymphocyte recruitment. Indeed, a similar reaction was observed previously in CD44 gene deficient mice (29). In order to measure the effect of leptin deficiency on the memory T cell responses most likely occurring in *M. tuberculosis* infected mice, we induced a DTH reaction using heat-killed H37Ra as the immunizing agent and PPD as recall antigen. It has been reported earlier that leptin-deficient mice display a defective DTH reaction to different antigens.
In line, we found reduced footpad swelling and cellular infiltration in immunized ob/ob mice after subcutaneous injection of PPD revealing that the early memory T cell response is most likely impaired in *M. tuberculosi* infected ob/ob mice.

Investigations on the role of leptin in disease using ob/ob mice, are hampered by the complex phenotype of these animals; they are obese, hyperglycemic, overproduce glucocorticoids, suffer from thymic atrophy and have lower peripheral blood leukocyte counts. In the context of malnutrition, it should be noted that these mice are overfed and not starved. Nevertheless, the ob/ob phenotype resembles several phenotypic aspects of malnourished humans including thymic atrophy, decreased DTH and reduced T cell responses (7). Exogenous leptin replacement is the only option to establish whether our observations in ob/ob mice are solely due to leptin deficiency and not to the other phenotypic changes. We therefore implanted osmotic pumps s.c. in mice delivering continuously 5 μg of leptin per day (46) starting 4 days before infection until 2 weeks after infection when the animals were sacrificed. Leptin administration increased the impaired IFNγ response to the response measured in normal Wt mice. These data support the notion that the absence of the stimulatory effect of leptin on cellular and IFNγ responses is likely responsible for the increased susceptibility of ob/ob mice to *M. tuberculosi*.

In this study, we show that leptin plays a role in the early immune response to pulmonary infection with *M. tuberculosi* most likely by mediating an effective IFNγ driven Th1 response, adequate lymphocyte trafficking and granuloma formation.

**Acknowledgments**

We thank Ingvild Kop, Joost Daalhuisen and Marieke ten Brink for expert technical assistance and Nike Claessen for the immunohistochemical stainings.

**References**


