The role of leptin in the development of pulmonary neutrophilia in infection and Acute Lung Injury

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Online Data supplement

Methods

Human immunohistochemistry

Banked human lung specimens obtained during autopsy were analyzed from patients who died off bacterial (n=3) or influenza A (H1N1) (n=3) pneumonias with ALI/ARDS. Specimens from patients with histologically normal lung tissue without evidence of injury, infection or malignancy (n=3) served as controls. A previously validated protocol for leptin immunostaining was used to identify leptin-positive cells, as we have previously described [1]. Briefly, deparaffinized fixed lung sections (5μm) were stained using a rabbit anti-leptin antibody (1:300; SC-842; Santa Cruz Biotechnology, Santa Cruz, California) and blue substrate kit III (Vector Laboratories, Burlingame, California) Sections were counterstained with Nuclear Fast Red (Vector Laboratories) and mounted. All patient specimens were examined in accordance with an approved Institutional Review Board (IRB) protocol.

Human BAL

Previously reported lavage cell counts, and IgM and protein concentrations [2] were compared to lavage leptin levels, as determined by ELISA (R&D Systems) of the stored samples. IgM levels in BAL fluid samples were determined by ELISA (R&D Systems), and total protein levels by Bradford assay. Differential cell counts were performed on May-Grünwald-Giemsa-stained cytocentrifuged preparations [2]. As a subset of subjects in the original study were treated with activated protein C, we examined only subjects in the control arm of the study who received placebo (IV saline) (n=14).

Murine exposures

Naïve C57Bl/6 mice were anesthetized with inhaled isoflurane (Webster Veterinary, Devens, MA) and received either 50μg pegylated recombinant murine leptin (less than 2EU/ml

endotoxin) [3] in 100µl sterile PBS or 100µl vehicle control (PBS) by oropharyngeal (o.p.) aspiration. This aspiration technique was modified from Lakatos et al [4]. In brief, the anesthetized mouse was placed in an upright position and the tongue was gently extracted from the mouth using blunt forceps. The liquid was pipetted onto the back of the tongue. Respiration was monitored to ensure that the suspension was fully aspirated. The animals were euthanized 6 or 24h after leptin or PBS instillation by pentobarbital (Vortech pharmaceuticals, Dearborn, MI) overdose and exsanguination. Blood, BAL fluid and lung tissue of these mice was collected and processed for analysis of cell counts, cytokine, gene, and protein expression.

Murine influenza A infection was performed as described by Rincon *et al* [5]. Briefly, mice were infected intranasally with sublethal doses ($3x10^3$ EIU) of the A/California/7/2009 H1N1 isolate of the 2009 pandemic H1N1 influenza virus or vehicle (saline) control. Mice were euthanized and examined as above at day 4 post-infection.

Murine bacterial pneumonia infection was performed as previously described [6, 7]. Briefly, mice were inoculated with ~ $1x\ 10^7$ CFU of *E.coli* O6:K2:H1 (ATCC) or vehicle (saline) control with or without 50µg pegylated recombinant murine leptin [3] via o.p. aspiration following brief anesthesia with isoflurane. Mice were euthanized and examined after 24h as described above. *Klebsiella pneumoniae* infection was performed by o.p. instillation of *K. pneumonia* (43816 serotype 2, ATCC, $2x10^3$ CFU) or vehicle (saline) control with or without 50µg pegylated recombinant murine leptin [3]. Mice were euthanized and examined after 24h as described above.

Murine LPS-induced lung injury was performed as previously described [8]. Briefly, Mice were exposed to aerosolized *E. coli* 0111:B4 lipopolysaccharide (LPS; Sigma, St. Louis, MO) using a 3mg/ml solution of LPS in sterile saline nebulized by a Pari LC Plus Reusable Nebulizer with ProNeb Turbo Air Compressor (Pari Respiratory Equipment, Midlothian, VA). The nebulizer was connected, via a central manifold, to a multi-compartment pie-shaped Plexiglas aerosol chamber (modified Tepper box [9]) in which groups of mice were exposed individually but simultaneously to the aerosol for 15min.

Murine immunohistochemistry

Paraffin imbedded murine lungs were cut in 4µm sections and processed for immunohistochemical leptin staining according to Vernooy *et al*. [1]. Immunoreactive epitopes of leptin were exposed by treatment with 0.05% Pronase E in 0.5M Tris/HCl (pH 7.6), and nonspecific binding was blocked with 5% BSA in TBS. Leptin was detected using a rabbit anti-leptin polyclonal Ab (SC-842, Santa Cruz Biotechnology, Santa Cruz, CA). After application of biotin-conjugated goat anti-rabbit IgG Ab (E-0431, DakoCytomation, Glostrup, Denmark) and alkaline phosphatase-labeled avidin–biotin complex (ABC-AP, K-0376, DakoCytomation), enzymatic reactivity was visualized using Blue Substrate Kit III (SK-5300, Vector Laboratories, Burlingame, CA). Sections were counterstained with Nuclear Fast Red (Vector Laboratories) and mounted. Negative controls for nonspecific binding by omitting the primary detecting Ab revealed only nuclear staining in red and no blue staining was observed.

Neutrophil chemotaxis

Neutrophil chemoattractant response to IL-8, KC (both R&D Systems), or recombinant leptin [3] was examined using a 48-well modified Boyden chamber (Neuroprobe Gaithersburg, MD) as previously described [10] with or without pre-incubation of the neutrophils for 30 minutes

at 37ºC with PI3K- (50μM) or JAK2-inhibitor (100μM) (Calbiochem). Briefly, isolated human peripheral blood neutrophils and murine bone marrow neutrophils were isolated as described above, and resuspended at 3.0 x 10^6 cells/ml in H/H buffer (1x HBSS, 2mg/ml BSA, 10mM HEPES, 1mM $CaCl_2$ and 1mM $MgCl_2$) (murine neutrophils) or 1x HBSS supplemented with 1mM $CaCl_2$ and 1mM $MgCl_2$ (human neutrophils). Chemoattractants in the appropriate buffer were added to the lower chambers of the apparatus. Polycarbonate membranes with 8μm (in the case of human neutrophils) or 5μm (in the case of murine neutrophils) pores (Neuroprobe, Gaithersburg, MD) were placed between the upper and lower chambers, and 50μl of cells were then added to the upper chambers. Cells were allowed to migrate for 30min at 37°C with 5% CO_2, before the membrane was removed, gently scraped, and stained using the Diff-Quik system (Hema3, Fisher Scientific, Middletown, VA). Three random fields of each well were scored using light microscopy (40x) to count the membrane bound intact cells. It is worth noting that pegylated leptin (MW 56 kDa) was used in our experiments in place of native leptin (MW 16 kDa) in order to improve the cytokine's stability. Thus, the concentrations used in this experiment (10 to 1000 ng/mL) correspond to significantly lower levels of native leptin (2.9 to 285.7 ng/mL or 0.179 nM – 17.9 nM), which are well within physiologically relevant range of this cytokine [11].

Murine albumin western blot analysis

Murine alveolar lavage supernatant (2.5ul) was loaded and separated on a polyacrylamide gel, followed by transfer to a 0.45μm nitrocellulose membrane (Bio-Rad) by electroblotting. The membrane was blocked for 1h at room temperature in 5% (w/v) nonfat, dried milk diluted in TBS-Tween20 (0.05%). Nitrocellulose blots were washed in TBS-Tween20 (0.05%) followed by 1hr incubation at room temperature, with primary antibody (goat anti-mouse Albumin; 1/5000; Immunology Consultants Laboratory Inc., Portland, OR Immunology Consultants

Laboratory Inc). After three washes of 15min each, the blots were probed with horseradish peroxidase-conjugated anti-goat antibody (1/10,000; Jackson Immunology Research, West Grove, PA) and visualized by chemiluminesence using Supersignal® West Pico Chemiluminescent Substrate (Pierce Biotechnology, Rockford, IL) according to the manufacturer's instructions and exposed to film.

Murine cytokine analysis

Macrophage Inflammatory Protein (MIP)-2 concentrations in BAL were analyzed by ELISA (CXCL-2/MIP-2 DuoSet, R&D Systems), performed according to manufacturer's protocol. Murine IL-1β, IL-6, KC, MCP-1, G-CSF and TNF-α levels in BAL supernatants were assessed using a Bio-Plex suspension array system (Bio-Rad, Hercules, CA), as previously described [8]. BAL leptin concentrations were measured with a murine leptin ELISA (Quantikine, R&D Systems) according to manufacturer's protocol.

Quantitative RT-PCR

Frozen mouse lung samples, obtained as detailed above, were pulverized using chilled mortars and pestles. Transcription of leptin and GAPDH was assessed using a Bio-Rad quantitative PCR system following mRNA isolation and cDNA preparation, as described previously [10]. The following primer sequences were used: for leptin TGCACACCAAAACCCTCATCA and TCATTGGCTATCTGCAGCAC; and GAPDH TaqMan Gene expression assay, AOD, (Applied Biosystems). Leptin levels were analyzed using the cycle threshold (ΔΔCt) method and normalized to GAPDH. Tissue leptin mRNA expression for injured lungs was reported as the fold increase over uninjured mouse lung tissue expression.

Figures

Figure S1

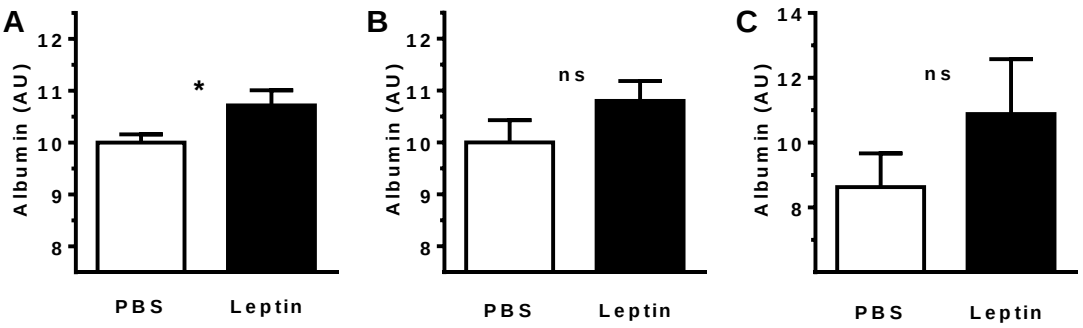

Figure S1. Airspace albumin levels are increased following leptin instillation. Albumin levels were determined in BAL samples at 24h after o.p. aspiration of control (PBS) or recombinant pegylated leptin (50 µg) in uninjured mice (**A**) and in *E. coli* pneumonia (**B**) and *K. pneumoniae* (**C**) infected mice. n=3 in both groups of uninfected mice. n=4 in the control group and pegylated leptin treated group of *E. coli* pneumonia infected mice. n=8 in both groups of *K. pneumoniae* infected mice. Data are presented as mean ± SEM. * $p \leq 0.05$ compared to PBS instilled controls.

Figure S2

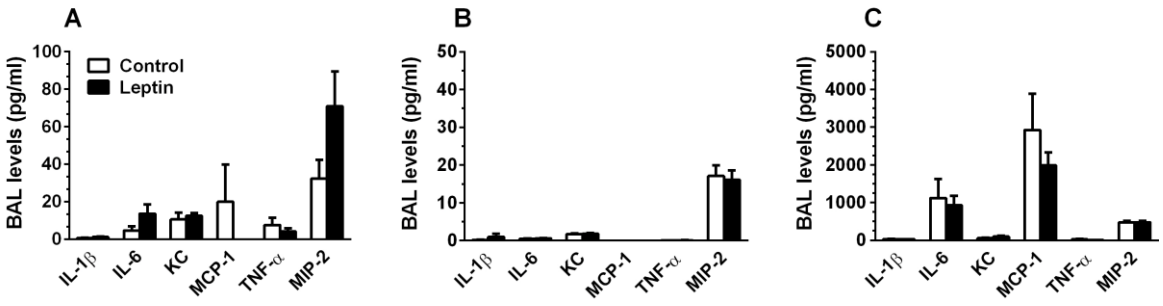

Figure S2. BAL inflammatory cytokine levels remain unchanged after leptin aspiration in uninjured mice as well as in mice with *E. coli* pneumonia. Brochoalveolar lavage cytokine levels were determined in response to leptin airway instillation in uninjured mice at 6h (**A**) and 24h (**B**) after leptin instillation, as well as 24h after *E. coli* infection and leptin instillation (**C**). No significant differences were observed between control and leptin-treated mice. n=5 in all groups. Data are presented as mean ± SEM.

Figure S3

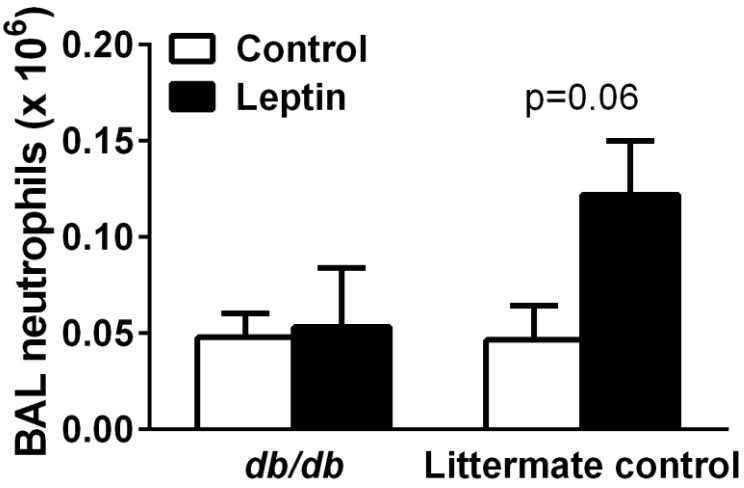

Figure S3. Oropharygeal instillation of leptin fails to recruit neutrophils to the airspace in leptin receptor-deficient mice. Total neutrophil counts in brochoalveolar lavage fluid were determined at 24h after o.p. aspiration of control (PBS) or recombinant pegylated leptin (50 µg) in leptin receptor-deficient (*db/db*) mice and their heterozygous littermates. While no difference in BAL neutrophil levels were found between leptin and PBS treated mice, a trend (p=0.06) toward higher neutrophils was noted in leptin-treated heterozygous littermates. n=5 in all groups. Data are presented as mean ± SEM**.**

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