Obesity is associated with neutrophil dysfunction and attenuation of murine acute lung injury

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Online Data Supplement

Lipopolysaccharide-induced Lung Injury

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 (E1)) in which groups of mice were exposed individually but simultaneously to the aerosol for 15 minutes. Saline controls were performed using a similar approach. Both lean and obese mice (3-4 of each) were exposed simultaneously in the aerosol chamber in each experiment, and each experiment was repeated 2-3 times. The animals were euthanized 2 or 24h later by pentobarbital overdose. Blood was collected via cardiac puncture into a syringe containing EDTA and 1x protease inhibitor cocktail (p-8340, Sigma). Aliquots were analyzed for cell count and differential using an Advia 120 Hematology Analyzer with veterinary software (Bayer, Tarrytown, NY), and serum from the remaining blood was frozen for later analysis. For bronchoalveolar lavage, a midline neck incision was performed and an 18G catheter (Baxter Health Care) was inserted into the trachea and secured with suture. Lungs were lavaged with 1mL of chilled 0.1% BSA in PBS with 1x protease inhibitor cocktail. Bronchoalveolar lavage was spun for 6 minutes at 500*g* and the resulting supernatant was aspirated and snap-frozen for later analysis, while the cell pellet was resuspended in 5% BSA in PBS and analyzed for cell count and differential using the Advia 120. In some experiments, lavaged and exsanguinated lungs were snap-frozen for later analysis by myeloperoxidase assay (Life Technologies, Grand Island, NY). In other experiments whole lungs were fixed for histology in 4% PFA by gravity-instillation through a tracheal catheter, paraffin-embedded, and 5um sections were mounted on slides for examination using light

microscopy after H&E staining. Images were obtained by the Olympus BX50 light microscope with an Optronics Magnafire digital camera.

Determination of Cytokine Levels

Cytokine levels in mouse plasma and bronchoalveolar lavage supernatants were assayed using a Bio-Plex suspension array system (Bio-Rad, Hercules, CA) as follows. Milliplex kits containing beads and antibodies recognizing 4 cytokines/chemokines (IL-6, KC, TNF-α, MIP-2, and MCP-1) were from Millipore (Billerica, MA). All assays were performed in duplicate according to manufacturer's instructions. Briefly, 25µl of undiluted culture supernatant, standard (in culture media), or culture media (background) was added to each well of a pre-wet 96-well vacuum filter plate. 25µl of assay buffer plus 25µl of antibody-conjugated beads were added to each well and the plates were covered, shaken vigorously for 1 minute on an IKA (Wilmington, NC) MTS 2/4 digital microtiter plate shaker and then moderately shaken for 2 hours at room temperature. After washing using a Bio-Rad (Hercules, CA) Bio-Plex Pro II wash station, 25µl of biotinylated detection antibodies were added to the appropriate wells for 1 hour followed by addition of 25µl of streptavidin-phycoerythrin to all wells for 30 minutes. The wells were washed and the beads were resuspended in 125µl sheath fluid. Data were acquired using the Bio-Rad Bio-Plex suspension array system and Bio-Plex Manager 6.0 software. Fluorescence intensity of the background was subtracted from the values for each sample and standard for each specific bead. Standard curves were generated from 4-fold dilutions of standards provided in the Milliplex kits, which were analyzed using 5-place logistic regression from standards within 70-130% of the expected values. Upper levels of quantitation and lower levels of quantitation were calculated by the Bio-Plex Manager software. Reported concentrations are in pg/ml.

Determination of Plasma LDL Cholesterol Levels

Total plasma cholesterol concentration in uninjured mice was first determined by a micro-enzymatic method. Total cholesterol reagent (Cholesterol HP, Roche Diagnostics) was added to dilutions of plasma in a microtiter plate and the OD at 546nm was measured using a Genios microtiter plate reader (Tecan). Plasma samples were then diluted with cold phosphate buffered saline, centrifuged at 10,000xg for 2 min at 4°C, and the supernatant (containing approximately 15μg cholesterol in 60μl) was analyzed by FPLC with online mixing of the column effluent with enzymatic reagent (Cholesterol Liquid Stable, Thermo Electron) to determine LDL content, as previously described (E2).

Statistical Analysis

Correlations between weight and bronchoalveolar lavage neutrophil levels, as well as covariates that might affect bronchoalveolar lavage neutrophil levels, including age, and weight, were analyzed by linear regression using STATA 10.0 (College Station, TX). Covariates significant at < 0.1 by univariate analysis were included in the final model. All other data were analyzed with the Student's or Welch's t-test, using Prism 5 software (GraphPad Sofware, La Jolla, CA). An F-test was used to analyze whether groups exhibited equal variances, and the Welch's t-test was used in lieu of the Student's t-test if the variances were un-equal. Differences between groups were reported as reaching statistical significance when $p < 0.05$. Results are reported with SEM in the case of t-test analysis, and with 95% confidence intervals for linear regressions.

References:

E1. Rudmann DG, Moore MW, Tepper JS, Aldrich MC, Pfeiffer JW, Hogenesch H, Tumas DB. Modulation of allergic inflammation in mice deficient in tnf receptors. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1047-1057.

E2. Garber DW, Kulkarni KR, Anantharamaiah GM. A sensitive and convenient method for lipoprotein profile analysis of individual mouse plasma samples. *J Lipid Res* 2000;41:1020-1026.

Figure Legend:

Figure E1: Histological evidence of lung inflammation and injury is reduced in obese mice following LPS exposure. Shown are LPS-injured mouse lungs (24h) from db/db obese (**A/E**) and diet-induced obese (**C/G**) mice, as well as injured lean mice from both obesity models (db/db **B/F**; diet induced **D/H**). Lower panels represent higher magnification views of the selected areas. Representative images are shown from lean (n=3) and obese mice (n=3). Lungs are 4% PFA inflation-fixed and H&E stained before imaging (see Methods). Magnification **A-D** 200x; **E-H** 400x.

Figure E2: Lung myeloperoxidase content following lung injury is decreased in db/db obese mice compared to leans, yet no difference is seen between diet-induced obese and lean mice. Whole lung myeloperoxidase (MPO) content in lean compared to genetically obese (db/db) and diet-induced obese mice was determined in perfused, lavaged lungs 6h and 24h after nebulized LPS exposure. Lungs from uninjured mice from all four groups were similarly examined. n=3 mice/condition. * $p<0.05$, ** $p<0.01$, ns = not significant.

Figure E3: Lung cytokine response 6h after LPS injury is normal in obese mice. Bronchoalveolar lavage cytokine (**A/C**) and neutrophil (**B/D**) levels 6h after nebulized LPS exposure in lean compared to genetically obese (db/db) and diet-induced obese mice were determined by Bio-Plex and cell counter, respectively. n=6 mice/condition. * $p<0.05$, ns = not significant.

Figure E4: Obesity impairs neutrophil chemotaxis. Chemotaxis of density centrifugation-isolated mature bone marrow neutrophils from genetically obese (db/db) (**A**) and diet-induced obese (**B**) mice was compared to lean controls using a modified Boyden chamber with varying concentrations of KC. Membrane counts were expressed as total neutrophils migrated per field for each experiment. Three separate experiments were performed on both db/db and diet-induced obese mouse isolated neutrophils and respective controls.

Figure E5: Adoptive transfer of obese versus lean mouse neutrophils leads to reduced airspace neutrophilia following lung injury. Neutrophil adoptive transfer was performed on lean recipient mice using bone marrow neutrophils isolated from genetically obese (db/db) vs. lean (heterozygous littermate control) mice (**A**), and diet-induced obese (60% fat diet) vs. lean (10% fat diet) mice (**B**) (see Methods). Following transfer, acute lung injury was induced in the recipient mice by LPS inhalation 24h prior to determining bronchoalveolar lavage neutrophil levels by cell counter. n=8 mice/condition from 3 separate experiments. *$p<0.05$.

Figure E6: Obesity is associated with decreased neutrophil surface expression of CXCR2. Cell surface levels of CXCR2 were determined on mature bone marrow neutrophils isolated from genetically obese (db/db) (**A**) and diet-induced obese (**B**) mice and compared to lean controls using flow cytometry. Representative cytometric histograms are presented from three separate experiments on both db/db and diet-induced obese mice and respective controls.

Figure E7: Airspace IL-6 and MCP-1 response 24h after LPS injury is impaired in obese

mice. Bronchoalveolar lavage cytokine (**A/C**) and neutrophil (**B/D**) levels 24h after nebulized LPS exposure in lean compared to genetically obese (db/db) and diet-induced obese mice were determined by Bio-Plex and cell counter, respectively. n=6 mice/condition. * $p<0.05$, ** $p<0.01$, ns = not significant.

Figure E8: Baseline plasma cytokine levels are normal in obese mice. Plasma cytokine levels in lean compared to genetically obese (db/db) (**A**) and diet-induced obese (**A**) mice were determined by Bio-Plex. n=8 mice/condition. nd = none detected.

Figure E9: Both db/db and diet-induced obese mice demonstrate elevated plasma levels of LDL compared to lean controls. Plasma LDL levels from uninjured genetically obese (db/db) and diet-induced obese mice and their lean controls were determined by FPLC. n=3-5/group. * $p<0.03$; ** $p<0.001$.