Impaired Alveolar Macrophage Response to *Haemophilus* Antigens in Chronic Obstructive Lung Disease

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Online Data Supplement

Recruitment of subjects – All study procedures received prior Institutional Review Board approval. All participants were at least 30 years of age. After informed consent, volunteers were screened for inclusion into one of three groups: Group 1 - ex-smokers with COPD; Group 2 - ex-smokers without COPD; Group 3 - non-smokers. All underwent clinical assessment, routine spirometry and chest x-rays. All participants had expired breath carbon monoxide of $\leq$0.02ppm (Vitalograph Breath CO Monitor, Lenexa, KS).

For Group 1 (ex-smokers with COPD), exclusion criteria included a forced expiratory volume at one second (FEV_1) <35%, hypercapnia and serious co-morbid diseases that would render bronchoscopy unsafe. Inclusion criteria for Group 1 were: 1) presence of chronic bronchitis by history and/or presence of emphysema by chest x-ray or CT; 2) $\geq$20 pack years of cigarette smoking, but cessation of smoking for at least one year prior to enrollment; 3) absence of other lung disease, including asthma and bronchiectasis; 4) chest x ray findings that were either normal or compatible with COPD, but detected no other disease; 5) evidence of obstructive airway disease on spirometry; 6) non-atopic by history; 7) <15% bronchodilator response with inhaled albuterol on spirometry; 8) no antibiotic or steroid use for four weeks preceding enrollment.

Inclusion criteria for Group 2 (ex-smokers without COPD) were the same as for Group 1, except for absence of lung disease by clinical evaluation, normal chest x-ray and normal spirometry. Participants of Group 3 (healthy non-smoking controls) met all the inclusion criteria of Group 2, except that all were non-smokers, defined as less than five cumulative pack years.

Purification of human alveolar macrophages – All participants underwent bronchoalveolar lavage (BAL) to obtain alveolar macrophages within 72 hours of blood monocyte purification.

Conscious sedation with midazolam and fentanyl was administered and topical anesthesia (4% cocaine) was applied to the nasal mucosa. A sequential two-scope procedure was employed. The first bronchoscope was used to anesthetize the glottis and a second bronchoscope, with an uncontaminated channel, was used to lavage the right middle lobe three times with 50 ml of sterile saline. Human alveolar macrophages were purified from BAL suspensions by centrifugation and seeded onto 96 well tissue culture plates (10^5 cells/ml). After incubation (24 hours), non-adherent cells were removed. Remaining alveolar macrophages were consistently 98-100% esterase-positive (E1,E2).

Macrophage-NTHI antigen incubation - Human alveolar and blood macrophages were incubated with each NTHI outer membrane constituent (0.1-10 ug/ml; 8-48 hours), as previously described (E3). No loss of cell viability was detected by live-dead cell assay (Molecular Probes Inc., Eugene, OR) or trypan blue exclusion. Cells of each donor were treated with each bacterial constituent in the same experiment for assay of each elicited cytokine. All experiments included control wells treated with *E. coli* K235 LPS (1μg/ml) (Sigma Chemical Co., St. Louis MO) or with buffer diluents of each antigen. Cell supernatants were harvested, centrifuged and separated from pelleted cells, before being frozen. All studies were done using strictly pyrogen-free reagents and pipette tips (E3).

Cytokine analysis – Cytokine concentrations were determined by multianalyte microsphere assay, analyzed by flow cytometry (E4). Capture and detection antibody pairs directed against different noncompeting epitopes of their respective cytokine and recombinant protein standards for IL-1β, TNF-α, IL-8, IL-12 (R&D Systems (Minneapolis, MN) and IL-10 (BD Pharmingen

(San Diego, CA) were covalently coupled to Multi-Analyte carboxylated microspheres (Luminex Corp., Austin, TX), each set of which has a unique proportion of red and orange fluorescent dyes, through free amino groups with carbodiimide and N-hydroxysuccinimide. Cytokine capture antibodies were each coupled to a different microsphere bead set. A mixture of water-soluble 24 mM 1-Ethyl-3-(3 Dimethylaminopropyl)-Carbodiimide Hydrochloride (EDC) and 20 mM N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce Biotechnology, Rockford, IL) was used to activate free carboxyl groups on the beads. After activation, beads were washed in 50 mM 2-(N-morpholino) ethanesulfonic acid (MES, Pierce) and the antibody to be coupled, dialyzed into MES, was immediately added (250 ug/ml). After overnight rotation at ambient temperature, the beads were washed and resuspended in PBS-TBN (phosphate buffered saline, pH 7.4, containing 0.02% Tween 20, 0.1% bovine serum albumin, and 0.02% sodium azide, (Sigma Chemical Co., St. Louis, MO) at 8 x 10^6/ml.

Multiplexed assays were performed in 96-well microtiter plates (Multiscreen HV plates, Millipore, Billerica, MA) with PVDF membranes, prewetted and washed with PBS-TBN, using a Tecan Genesis liquid handling robot (Research Triangle Park, NC) for all manipulations of the microtiter plate. Bead sets coated with capture antibody were diluted in PBS-TBN and pooled. 1000 beads from each set were added per well. Recombinant protein standards were titrated from 33,333 to 0.56 pg/ml using 3-fold dilutions in PBS-TBN. Samples were diluted 4 fold in PBS-TBN and standards and samples were added in 50 ul volumes to wells containing beads. Plates were incubated at ambient temperature for 20 minutes on a rocker, washed twice with PBS using a vacuum manifold to aspirate. Biotinylated detection antibodies for each cytokine, diluted in PBS-TBN, were added in 50 ul volumes and incubated for 20 min. before the beads were washed with PBS. The appropriate detection antibody concentration, defined as the lowest titer that still

saturated the standard curve, was predetermined for each detection antibody and capture bead set. 50 ul of phycoerythrin-conjugated streptavidin (Caltag Laboratories, Burlingame, CA) was added to each well and the plates were incubated and washed as before. The beads were resuspended in 150 ul of PBS-TBN and analyzed by flow cytometry (Luminex 100, Luminex Corp).

Cytokine data analysis - Samples were measured in duplicate and blank values were subtracted from all readings. A log log regression was calculated (Microsoft Excel, Microsoft, Redmond, WA) using the bead mean fluorescence intensity (MFI) values for each concentration of recombinant protein standard. Points below detection limits or above saturation were excluded from the curve. Sample cytokine concentrations were calculated from MFIs of beads by interpolating the resulting best fit line. Samples with values above the standard curve were further diluted and reanalyzed. Final concentrations were corrected for differences in macrophage numbers between experiments before statistical analysis.

References:

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