Supplemental Material

Weight Loss Decreases Inherent and Allergic Methacholine Hyperresponsiveness in Mouse Models of Diet-Induced Obese Asthma

Jennifer L. Ather, Michael Chung, Laura R. Hoyt, Matthew J. Randall, Anna Georgsdottir, Nirav A. Daphtary, Minara I. Aliyeva, Benjamin T. Suratt, Jason H.T. Bates, Charles G. Irvin, Sheila R. Russell, Patrick M. Forgione, Anne E. Dixon, and Matthew E. Poynter

Materials and Methods

Mice. Mice were purchased from The Jackson Laboratory and were then housed in an American Association for the Accreditation of Laboratory Animal Care (AALAC)-approved facility at the University of Vermont, maintained on a 12h light/dark cycle, and provided food and water ad libitum. Low-fat diets (D12450B) and high-fat diets (D12492) were purchased from Research Diets (New Brunswick, NJ) and stored under refrigerated conditions. Food in cages was replaced twice each week. Protocols were approved by the University of Vermont Institutional Animal Care and Use Committee. Mice were euthanized with sodium pentobarbital (150 mg/kg by i.p. injection; Wilcox Pharmacy, Rutland, VT).

Model of obese inherent asthma. Twenty-week old male C57BL/6J mice were purchased from The Jackson Laboratory having been maintained on food containing 10% fat (low-fat diet; LFD) or 60% fat (high-fat diet; HFD) from 6 weeks of age. Mice were acclimated at UVM for a week following shipping and were maintained on their same food or subjected to dietary or surgical weight loss regimens.

House dust mite (HDM) extract model of allergic asthma. Sixteen-week old male C57BL/6J mice maintained on LFD or HFD since 6 weeks of age were purchased from The Jackson Laboratory and allergen-sensitized by the intranasal instillation of 1 μg (by protein) HDM extract (Greer, Lenoir, NC) in 40 μl saline twice a week for 4 weeks. Mice remained on their respective diets and HDM exposure regimens until approximately 20 weeks of age. Groups of mice were then maintained on their same diet or were subjected to the dietary weight loss regimen for 3 weeks, during which time they received no intranasal HDM exposures. To elicit an allergic asthma-like exacerbation, approximately 23-week-old mice were challenged intranasally 4 days in a row with 10 μg HDM extract in 40 μl saline and analyzed 1 day following the fourth challenge.

Mouse model of bariatric surgery-induced weight loss. Mice were fasted overnight and the following day were placed under stable anesthesia using isoflurane/O_2, the abdomen was shaved and sterilized, and buprenorphine was administered to mice atop a heating pad. After the stomach was externalized, the gastrosplenic ligament connecting the left superior stomach to the spleen was divided using electrocautery, thereby freeing the gastric fundus from the surrounding tissue. The right and left inferior poles of the stomach were then extended downward with forceps. Using a Ligaclip Clip Applier (Ethicon, Somerville, NJ), a 10-mm clip was placed horizontally from the other side of the stomach, thereby excluding the entire inferior aspect of the stomach and creating the gastric sleeve. The excluded part of the stomach was then excised with scissors and the cut edges of the stomach sterilized with 70% alcohol solution. Similar to other previous reports (1-3), this eliminated more than 80% of stomach volume while maintaining gastric and duodenal continuity of the GI tract. The stomach was then returned to the abdomen and the abdomen closed with a running 6-0 vicryl stitch and the

skin was then closed by interrupted suturing using 6-0 nylon monofilament. One ml subcutaneous saline (warmed) was provided for fluid maintenance. Mice were monitored continuously until ambulatory and were monitored daily for food intake and body weight. Carprofen analgesic was given daily for 3 days or as needed beyond that time.

Mouse model of diet-induced weight loss. Obese mice were switched from a diet in which 60% of the calories originate from fat to a diet in which the fat content is only 10% of the total calories. Mice were maintained on the 10% fat diet a time sufficient to induce significant and substantial weight loss compared to the matched high-fat diet-fed mice that continued to gain weight, which was at least 2 weeks.

Assessment of airway responsiveness to methacholine. Responsiveness to inhaled methacholine was assessed in closed-chested mice. Mice were anaesthetized with i.p. sodium pentobarbital (90 mg/kg), the trachea was cannulated, the mice were connected to a flexiVent™ computer controlled small animal ventilator (SCIREQ, QC, Canada), and the mice were ventilated at 200 breaths/minute with a 0.25 ml tidal volume, as previously described (4-6). Next, the mice were paralyzed with an i.p. injection of pancuronium bromide i.p. (0.8 µg/kg). The animals were stabilized over about ten minutes of regular ventilation at a positive end-expiratory pressure (PEEP) of 3 cmH_2O. A standard lung volume history was then established by delivering two total lung capacity maneuvers (TLC) to a pressure limit of 25 cmH_2O and holding for three seconds. Next, two baseline measurements of respiratory input impedance (Zrs) were obtained. This was followed by an inhalation of aerosolized PBS (control) for 10 seconds, achieved by an in-line piezo electric nebulizer (Aeroneb, Aerogen, Galway, Ireland). Zrs was then measured every 10 seconds for 3 minutes (18 measurements of Zrs in total). This complete sequence of maneuvers and measurements was then repeated for aerosol

exposures to three ascending doses of aerosolized methacholine (12.5, 25, and 50 mg/ml). Data were fit to the single-compartment model (7) to provide values for resistance (R), reflecting constriction in the lungs, and elastance (E), reflecting the elastic rigidity of the lungs. Data were also fit to the constant phase model to provide values reflecting airway resistance (R_N), tissue damping (G), and tissue resistance (H). R, E, R_N, G, and H were calculated for each mouse by averaging three measurements at each methacholine dose: the peak value and those immediately preceding and following it. The average values (± SEM) of R, E, R_N, G, and H in each of the mouse groups, at each incremental methacholine dose, are reported (n=8-10mice/group).

Serum collection and analysis. Following euthanasia at the completion of flexiVent analysis, approximately 300 µl of blood was collected via cardiac puncture of the right ventricle using a 25g needle attached to a 1 ml syringe, transferred into serum separator tubes (Becton Dickinson, Franklin Lanes, NJ), centrifuged, and serum was kept frozen at -80ºC. Serum was analyzed by custom Luminex-based multiplex assays (EMD Millipore, Billerica, MA) for mouse amylin, GLP-1, ghrelin, IL-6, TNF, PP, PYY, leptin, MCP-1, adiponectin, and SAA3, according to manufacturer instructions. Serum HDM-specific IgG1 and IgG2c were measured by ELISA, as previously described (8) except that HDM extract was used to coat the plates and serum was used over a series of eight 4-fold dilutions starting at 1:20.

Bronchoalveolar lavage (BAL) collection and processing. Following blood collection, lungs were lavaged with 1 mL DPBS (Sigma-Aldrich, St. Louis, MO). The BAL fluid was centrifuged at 400 x *g*, the supernatant was collected and frozen. Total cell counts in the resuspended pellet were enumerated using an Advia 120 Hematology System (Bayer HealthCare, Leverkusen, Germany). Differential analysis was performed by H&E stain of cytospins and

counting approximately 200 cells per slide. Mouse amylin, GLP-1, ghrelin, IL-6, TNF, PP, PYY, leptin, MCP-1, adiponectin, and SAA3 were analyzed from BAL fluid by custom Luminex-based multiplex assays (EMD Millipore) according to manufacturer instructions.

Analysis of cytokines in lung tissues. Following the collection of BAL fluid, lungs were dissected, ground to a fine powder using a liquid nitrogen-chilled mortar and pestle, and an aliquot was subsequently resuspended and vortexed in 400 µL of saline. The suspensions were transferred into separate QiaShredder spin columns (Qiagen, Valencia, CA) and centrifuged at 13,000 x *g* at 4ºC for 10 minutes. Flow-throughs were then transferred to clean microcentrifuge tubes, while avoiding the pellet on the bottom, and were stored at -80ºC until analysis. Following thawing on ice, total protein concentrations were assessed using a Bradford assay (Bio-Rad, Hercules, CA), and relative cytokine concentrations were enumerated using ELISAs for mouse IL-5, IL-6, IL-13, IL-17A, and IFNγ (R&D Systems, Minneapolis, MN), as well as TNF (BD Biosciences, San Jose, CA), according to manufacturer's instructions.

In vitro antigen restimulation and cytokine quantitation. Following collection of lungs, spleens were harvested, mechanically disrupted, and splenocytes were isolated using Lymphocyte Separation Media (MP Biomedicals, Solon, OH), as previously described (9). Cells were counted with an Advia 120 Hematology System and $4x10^6$ cells/ml were cultured in RPMI-1640 supplemented with 5% FBS (Cell Generation, Fort Collins, CO), 2500 µg/ml glucose, 2 mM L-glutamine, 10 µg/ml folic acid, 1 mM sodium pyruvate, 50 µM 2-mercaptoethanol, 100U/ml penicillin, and 100 µg/ml streptomycin and treated with 15 µg/ml HDM extract (Greer). Supernatants were collected after 96 hours of incubation at 37°C in 5% CO_2. Analysis of

cytokine content from cell supernatants was performed using ELISA kits for IL-5, IL-13, IL-17A, and IFNγ (R&D Systems, Minneapolis, MN).

Quantitative RT-PCR. Total RNA was extracted from an aliquot of the snap-frozen and pulverized lung lobes using the PrepEase RNA Isolation kit (USB Corp., Cleveland, OH) and reverse transcribed to cDNA using the iScript kit (Bio-Rad, Hercules, CA). Primers were designed for mouse *Muc5ac* (5'-CCATGCAGAGTCCTCAGAACAA-3' and 5'-TTACTGGAAAGGCCCAAGCA-3'), *Gob5* (*Clca1*) (5'-AAGCAAACCACTCCCATGAC-3; and 5'-TGCGAAAGCATCAACAAGAC), *Acta2* (5'-TGTGCTGGACTCTGGAGATG-3' and 5'-GAAGGAATAGCCACGCTCAG-3'), *Il6* (5'-CCGGAGAGGAGACTTCACAG-3' and 5'-GAGCATTGGAAATTGGGGTA-3'), *Myh11* (5'-CTCTGGCCTCTTCTGTGTGG-3' and 5'-TCTTTCTTGCCCTTGTGGGA-3'), *CCL2* (*MCP-1*) (5'-GAGCTACAAGAGGATCACCAGCA-3' and 5'-GTTCTGATCTCATTTGGTTCCGATCC-3'), *Ntrk2* (5'-CGTTGACCCGGAGAACATC-3' and 5'-AAACTTTAAGCCGGAATCCAC-3'), *Cdf* (5'-GGAGTGACGGATGACGACTC-3' and 5'-CATGGTACGTGCGCAGATTG-3'), *Saa3* (5'-CAGGATGAAGCCTTCCATTG-3' and 5'-CATGACTGGGAACAACAGGA-3'), and *Tnfrs1b* (5'-TGCCAGCTCCAGGATTTCAG-3' and 5'-CTCAGCCCTCACTTGACCTG-3') using NCBI Primer-BLAST and synthesized by Integrated DNA Technologies (Coralville, IA). Quantitative RT-PCR was performed using SYBR Green Supermix (Bio-Rad) and normalized separately to *Gapdh* (5'-ACGACCCCTTCATTGACCTC-3' and 5'-TTCACACCCATCACAAACAT-3') and *Actb* (5'-TCCTTCGTTGCCGGTCCACA-3' and 5'-CGTCTCCGGAGTCCATCACA-3') using the $\Delta\Delta C_T$ method, as previously described (32). The choice of housekeeping gene used for normalization had no influence on the overall results.

Measurement of fecal bacterial diversity. From fecal pellets collected on the day of the final HDM challenge from mice exposed to the allergic asthma model, bacterial DNA was extracted (Qiagen) and subjected to quantitative PCR analysis of the 16S ribosomal RNA (rRNA) present in all eubacteria as well as the 16S rRNA present specifically in the flora of the mouse gastrointestinal tract, including the phyla *Bacteroidetes* (*Bacteroides,* mouse intestinal *Bacteroides*), *Fermicutes* (*Clostridium perfiringens*, *Eubacterium rectale/Clostridium coccoides*, and *Lactobacillus/Lactococcus*), and *Proteobacteria* (*Enterobacteriaceae*, *Salmonella*, and *Helicobacter pylori*) using primers described previously (10). Standards were generated from the pooled products of mixed mouse fecal samples following 20 rounds of PCR, which were subsequently used to establish standard curves for each of the primer reactions over a range of 4-fold dilutions between 1:100 and 1:400,000. Cycle threshold (Ct) values for each PCR reaction were applied to the respective standard curve, run on each plate on a Bio-Rad CFX96 or Chromo4 96-well quantitative real-time PCR detection system. PCR product abundance was log-transformed and the average abundance in the low-fat diet group was used to normalize relative abundance from each animal, values that were then used to calculate the mean ± SEM for each bacterial 16S rRNA in each mouse group. The average relative abundance of specific 16S rRNAs of each plyla in each mouse were used to calculate the overall *Bacteroidetes, Fermicutes,* and *Proteobacteria* abundance, from which the *Fermicutes:Bacteroidetes* ratios were calculated.

Statistical analyses. Data were analyzed by two-tailed unpaired t test, one-way ANOVA or two-way ANOVA and Tukey post hoc test using GraphPad Prism 6 for Windows (GraphPad Software, Inc, La Jolla, CA). Data are presented as mean ± SEM. A p value <0.05 was

considered statistically significant. Unless otherwise indicated in the figure legends, * = $p \leq 0.05$; ** = $p \leq 0.01$; *** = $p \leq 0.001$; **** = $p \leq 0.0001$.

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