Supplemental Online Material

Immunohistochemistry (IHC) Methods

Adipose tissue samples (~350-400mg) were fixed in 10% zinc formalin (Protocol, Kalamazoo, MI) at room temperature overnight. The next day samples were transferred to a 1% formalin solution for storage if they could not be immediately processed. All samples were paraffin-embedded and sequential 5 micron sections were placed onto slides in the Pathology Research Core at Mayo Clinic, Scottsdale, AZ. The slides were stained at the Pathology Research Core at Mayo Clinic, Rochester, MN. Each sample was stained with a total macrophage and monocyte marker antibody against CD68; an M1 macrophage or pro-inflammatory macrophage marker antibody against CD14 and an M2 macrophage or anti-inflammatory macrophage marker antibody against CD206. The IHC staining procedure was performed on-line using the Leica Bond III Stainer (Leica, Buffalo, IL). The tissue slides were dewaxed using Bond Dewax (Leica, Buffalo, IL). Tissue slides for CD14 and CD68 stain were retrieved for 20 minutes using Epitope Retrieval 2 (Leica, Buffalo, IL) and slides for CD206 stain were retrieved for 20 minutes using Epitope Retrieval 1 (Leica, Buffalo, IL). All primary antibodies were diluted in Bond Antibody Diluent (Leica, Buffalo, IL). The primary antibody CD14 (Sigma-Aldrich, St. Louis, MO) was used at 1:300; CD68 (Clone PG-M1, Dako, Carpinteria, CA) was used at 1:200; and CD206 (Clone 685645, R&D Systems, Minneapolis, MN) was used at 1:200. The detection system used was the Polymer Refine Detection System (Leica, Buffalo, IL). This system includes the hydrogen peroxidase block, secondary antibody polymer, DAB and hematoxylin. Immunostaining visualization was achieved by incubating slides 10 minutes in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1´ Bond Wash Buffer (Leica, Buffalo, IL). Before and after DAB incubation, slides were rinsed in distilled water. Slides were counterstained for 5 minutes using Schmidt hematoxylin and molecular biology grade water (1:1 mixture), followed by several rinses in 1´ Bond wash buffer and distilled water; this is not the hematoxylin provided with the kit. Following completion of the immunochemistry processing the slides were removed from the stainer and rinsed in tap water for 5 minutes. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3

changes of xylene prior to placing a permanent coverslip in xylene-based medium. Tonsil tissue samples were utilized for both positive and negative controls and were treated in the same manner as the adipose tissue samples with the negative control not receiving primary antibody treatment.

Automated IHC Software Development

Novel methods for segmenting the adipocytes and macrophages were implemented. For the adipocyte segmentation the objective was to find all of the bright structures which are separated by thin dark (and sometimes faint) walls. The color RGB microscope image was converted to gray scale image using the following formula:

gray = red * .3 + green * .59 + blue * .11

where red, green and blue represent the individual color bands of the RGB image. The walls of the adipocytes were enhanced by inverting the intensities of the image, applying a sticks filter with an 11x11 kernel where the image intensities are replaced by the maximum sum, and then reinverting the intensities of the image. A histogram of the upper half of the intensity range of the image was then computed and the mode value was determined. The image was thresholded, between 98% of the mode value and the maximum intensity of the image. A chamfer distance transform was then applied to the binary image. The resulting distance transform image was thresholded to separate the adipocytes. Connected component analysis then determined the number and location of the adipocytes.

Given the unique staining characteristics of the macrophages, the macrophage segmentation was performed by subtracting the blue color channel from the red color channel of the RGB microscope image. Thresholding, morphological closing and connected component analysis were applied to obtain the number and location of the macrophages.

Flow Cytometry

For these experiments we collected adipose tissue (250-300mg) and washed the samples with saline to remove excess blood. Digestion of the tissue was performed in a collagenase (Sigma Type

II)/HEPES solution (1mg/ml) at 37º C in a shaking water bath for 10-30 minutes. We did not allow samples to digest for more than 30 minutes to minimize the initiation of apoptosis. The digested tissue was placed into a 6 ml syringe, and filtered through a 250 µM mesh screen into a clean 15 ml conical tube. Tube, syringe, and mesh were washed/rinsed with 2 ml PBS which was also added to the tissue sample. Samples were centrifuged in a refrigerated Beckman Coulter centrifuge model Avanti J-20 XPI using a JS-4.3 swing bucket rotor at 300 x g, 4 °C, for 5 minutes to isolate the stromovascular fraction (SVF). After centrifugation, the fat cake (top layer) was removed from the tissue sample and the supernatant was then aspirated leaving the SVF pellet to be washed with 5 ml PBS, vortexed and the centrifuge step repeated.

Again the supernatant was aspirated leaving the SVF pellet, which was re-suspended in 500 µL 1X ACK (Ammonium Chloride/Potassium Bicarbonate/EDTA), a red blood cell lysis buffer. The SVF pellet was suspended in ACK buffer at room temperature for 3-5 minutes with occasional agitation. Following this incubation, the ACK buffer was neutralized with 5 ml fluorescence activated cell sorting (FACS) buffer (1X PBS, .05% NaN_3, and .5% BSA), vortexed and centrifuged as before. The aspiration step was repeated and the SVF pellet was then re-suspended in 2 ml cold FACS buffer vortexed and centrifuged using the same settings and time as stated above. Following the FACS buffer wash, samples were re-suspended in 1.2 ml of FACS buffer, vortexed, and 300 µL were aliquoted into 4 separate flow cytometry tubes (5ml polystyrene round bottom; Falcon 35-2058). Tubes were centrifuged again for 5 minutes same settings as before. Once centrifuged, the tubes were “dumped” to remove supernatant (macrophages will cling to bottom of the tubes) and incubated in 100ul Hu FcR Binding Inhibitor (eBioscience, San Diego, CA) mixture (1:4 Hu FcR Inhibitor:FACS) on ice for ten minutes. All tubes and reagents were kept on ice in an effort to prevent the macrophages from adhering to the plastic tubes. Four flow cytometry tubes were labeled and used for staining positive controls. Positive controls tubes were prepared with BD CompBeads (particles set #552843 BD Biosciences) following product direction (1 drop of negative control and Anti-Mouse Ig, per control tube). Following the 10-minute incubation for the blocking step, the samples and control compensation beads were incubated with their corresponding

antibodies in the dark, on ice for 20 minutes. Set-up for the tissues samples were as follows: tube 1: NIL sample without antibodies; tube 2: IgG mouse Isotype negative control; tube 3: our sample of interest with staining antibodies; tube 4: viability stain (APC-Cy7) control. Set-up for the compensation beads as positive controls was completed using one antibody per control tube. The following antibodies were used in each experimental tube: FITC conjugated CD11b (BD Bioscience), PE conjugated CD14 (BD Bioscience), PE-Cy7 conjugated CD206 (eBioscience), APC conjugated CD68 (Biolegend, San Diego, CA), APC-Cy7 conjugated viability dye (BD Bioscience).

Following the antibody incubation, 500 µL of FACS buffer was added to all samples and control bead tubes, followed by vortexing and centrifuging at 300 x g, 4 °C for 5 minutes. Following centrifugation, tubes were "dumped" and another 500 µL FACS buffer was added to all tubes, followed by vortexing, and centrifuging using same time and settings stated above. These steps were repeated two more times. All samples were then fixed with 300 µL of BD Stabilizing Fixative (BD Sciences) and transferred to the Flow Cytometry Research Core where they were analyzed on a BDFACSCanto machine as single-cell suspension of mononuclear cells.

A tissue sample without any antibodies or stains was run to determine the auto-fluorescence of each sample. In addition, we used a tissue sample stained with IgG controls for each conjugated antibody to test for non-specific staining. An APC-Cy-7 conjugated viability dye was used to exclude apoptotic cells.

Flow Cytometry Protocol Development

We performed an antibody titration to identify the optimal antibody concentration to ensure adequate capture of positive events and to reduce non-specific staining. For each antibody (CD68, CD11b, CD206, CD14) we tested volumes of 5, 2, 1, 0.5 and 0.3 µL. To analyze our results we calculated the staining index for each volume of antibody using the median intensity for both the positive and negative populations as well as the standard deviation between the two. Staining index = $(\text{Median}_{pos} - \text{Median}_{neg})/2*\text{Standard deviation}$. The staining index was plotted against the antibody volume to determine our optimal antibody concentration (Supplemental figure 1). We found that a 2.5 µL volume

provided the best results for the CD68, CD11b and CD14 antibodies, whereas a 1 μL volume was optimal for the CD206 antibody.

We also measured the auto-fluorescence of each sample by using a negative control tissue sample that was not treated with any antibodies or stains. There was a large amount of auto-fluorescence, with an average of 40 ± 22% of cells in the forward scatter (FSC) and side scatter (SSC) ranges we selected for macrophages displaying auto-fluorescence tendencies or high levels of background. While the auto-fluorescence was present in all channels it was most pronounced in the FITC channel and we therefore avoided using this channel.

Lastly, we utilized IgG isotype controls for each antibody to assess non-specific staining in our samples. We found an average of 0.4 ± 12.5% of total macrophages were positive for IgG non-specific staining.

Our gating strategy was as follows:

We started with the forward scatter (FSC) and side scatter (SSC) to sort the cells based on size and density into those that were consistent with macrophages (Supplemental figure 2A and B - total gated macrophage burden). We then used the live-dead marker APC-Cy7 and FSC to identify the live cells (Supplemental figure 2C). With the live cells identified we back gate into the tube of interest (Supplemental figure 2D). Within the live cell population, additional gating was done to include only single cells using FSC-area and FSC-height to exclude doublets (Supplemental figure 2E). We used the single cell population and applied a quadrant gating method to identify cells that stain for APC (CD68). The subsequent gates were done from the $CD68^+$ cell gate that we gated from the single cell gate. We applied a quadrant gating method to identify cells that stain for APC (CD68) alone in the right lower quadrant (Supplemental figure 2F) or both APC and PE (CD14) in the right upper quadrant (Supplemental figure 2G). We used the same quadrant gating method to identify cells that stain for APC (CD68) alone in the right lower quadrant or both APC and Cy7 (CD206) in the right upper quadrant (Supplemental figure 2H).

Supplemental Figure S1. A range of antibody concentrations were used to determine the staining index of each antibody. Staining index of CD68-APC (A), CD14-PE (B), and CD206-PE-Cy7 (C).

Supplemental Figure S2. Representative images of flow cytometry data analysis using the FlowJo software. This was our sequential gating strategy for flow cytometry measures of human adipose tissue macrophage populations. A - Control tube with forward scatter area (FSC-A) vs. side scatter area (SSC-A) back-gated to sample in panel B; B - Actual sample with FSC-A and SSC-A; C - Live cell gate done on Control tube (panel A); D – Live cell back-gate from sample in panel B; E - Singlets gate on panel D from live cell gate; F - CD68(+) cell gate set from live cells in panel E; G – Cells of macrophage size that are CD68+ (Q3) & double stained CD68+/CD14+ (Q2) from panel F; H - Cells of macrophage size that are CD68+ (Q7) & double stained CD68+/CD206+ (Q6) from F.

Supplemental Figure S3. Results from 51 immunohistochemistry adipose samples where $CD68^{+}$ macrophages were counted (CD68 ATM/100 adipocytes - blue bars) and the number of crown-like structures in the same 10 random fields were counted (CLS – adjacent red bars). Only 16 of the 51 samples had any CLS found in the 10 random fields.

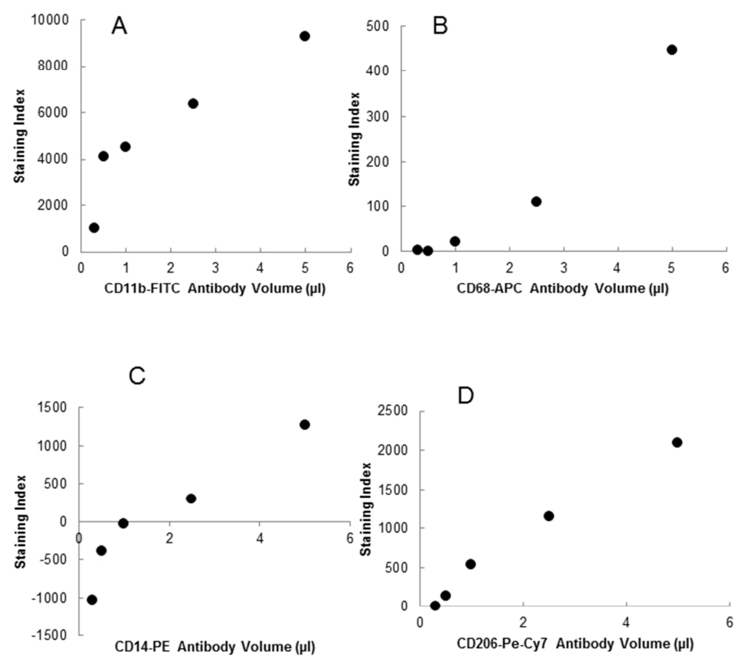

Figure S1

254x190mm (96 x 96 DPI)

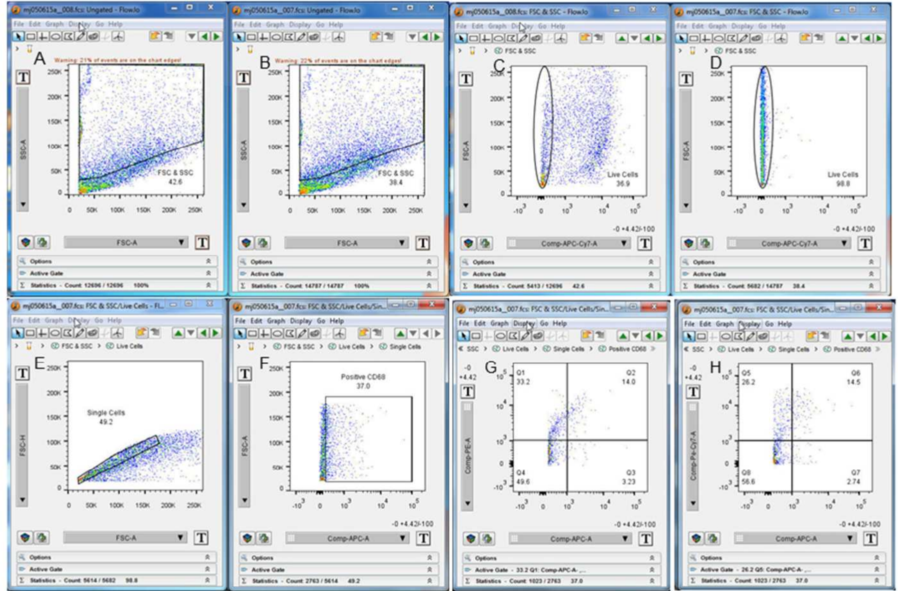

Figure S2

254x190mm (96 x 96 DPI)

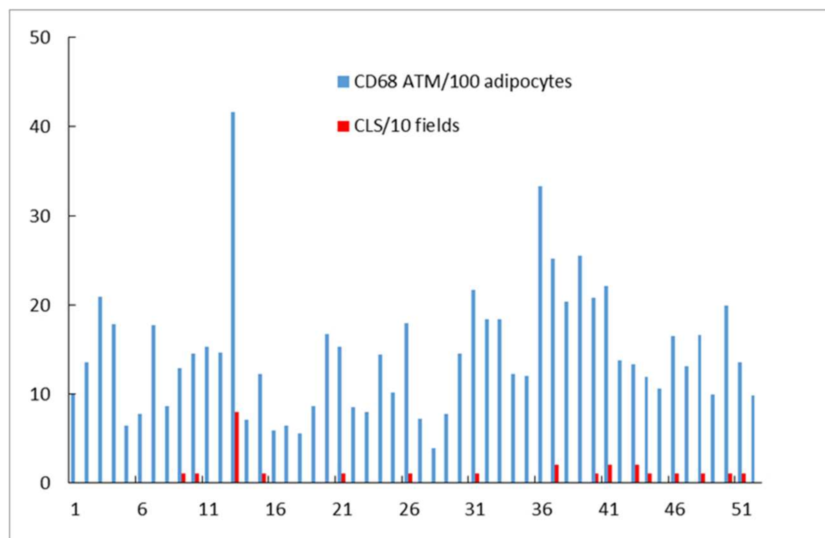

Figure S3

254x190mm (96 x 96 DPI)