Comparison of Methods for Analyzing Human Adipose Tissue Macrophage Content

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Objective: The relationship between inflammation, obesity, and adverse metabolic conditions is associated with adipose tissue macrophages (ATM). This study compared the measurements of human ATM using flow cytometry, immunohistochemistry (IHC), and real-time polymerase chain reaction (RT-PCR) of ATM markers.

Methods: A new software program (AMCounter) was evaluated to help measure ATM using IHC, and this was compared to flow cytometry and RT-PCR.

Results: IHC had good intraindividual reproducibility for total (CD68), proinflammatory (CD14), and anti-inflammatory (CD206) ATM. The AMCounter improved interreader agreement and was more time efficient. Flow cytometry had acceptable intraindividual reproducibility for the percentage of CD68⁺ or CD206⁺, but not for ATMs per gram of tissue. ATMs per gram of tissue was much greater using IHC than flow cytometry. The flow cytometry and IHC measures of ATM from the same biopsies were not correlated. There were statistically significant correlations between RT-PCR CD68 and IHC CD68, CD14, and CD206 ATMs per 100 adipocytes. Also of interest were statistically significant correlations between RT-PCR CD68 and IHC CD68, CD14, and adipose flow cytometry measures of CD68⁺, CD68⁺/CD14⁺, and CD68⁺/CD206⁺ ATMs per gram of tissue.

Conclusions: The AMCounter software helps provide reproducible and efficient measures of IHC ATMs. Flow cytometry, IHC, and RT-PCR measures of adipose inflammation provide somewhat different information.

Introduction

Obesity is linked to adipose tissue inflammation (1-3) with an increase in M1 or proinflammatory adipose tissue macrophages (ATM) and a decrease in M2 or anti-inflammatory ATMs (4). Animal studies have suggested that increased adipose tissue inflammation is a mechanism for the insulin resistance associated with obesity (5-10). We investigated methods to quantify ATMs and, as part of our laboratory protocol, evaluated the reproducibility and comparability of the different approaches.

Immunohistochemistry (IHC) and flow cytometry analyses of ATMs have been used for animal (11,12) and human studies (13,14). The advantages of IHC include the preservation of all cells in the tissue and the ability to store the paraffin-embedded tissue for long periods, allowing for reuse with additional antibodies. However, quantifying ATMs using IHC is time-consuming and requires considerable amounts of tissue. Flow cytometry requires less tissue and can count thousands of cells in a few minutes, and multiple antibodies can be used simultaneously on a single tissue sample. However, flow cytometry requires digestion and extensive handling (potentially resulting in loss of cells), and the sample cannot be saved for future use. Additional problems include macrophage autofluorescence that complicates the analysis of flow cytometry data. We found no reports defining the agreement between IHC and flow cytometry quantification of human ATMs and therefore tested how well these methods compare.

In attempts to reduce the time and resource burden of the IHC method, we collaborated with Biomedical Imaging Resource, Mayo...
Clinic, Rochester, Minnesota, to develop software to automate portions of the IHC analysis. We found that software-assisted IHC quantification of ATM burden offers some advantages and may improve some aspects of the procedure.

Methods

Tissue collection and fat cell size

All protocols were approved by the Mayo Clinic Institutional Review Board, and the volunteers provided written, informed consent. Subcutaneous adipose tissue biopsies were acquired by needle liposuction under sterile conditions using local anesthesia. Biopsies were collected from the abdominal (lateral to the umbilicus) and/or femoral (on the anterior-lateral aspect of the mid-thigh) regions. Fat cell size was measured as previously described (15). The portion of the sample destined for mRNA measurements was immediately frozen in liquid nitrogen and then stored at $-80^\circ$C. The adipose tissue samples that were collected to analyze inter- and intraindividual variations for both the IHC and flow cytometry methods were obtained on two separate occasions approximately 2 weeks apart. To evaluate the intraindividual variability for IHC, we analyzed 38 tissue samples (abdominal and femoral) from 15 individuals collected in duplicate. The analysis of intraindividual variability of flow cytometry was completed using 12 tissue samples from six individuals collected in duplicate. Forty samples were used to analyze reader agreement as well as agreement between manual IHC counting data and AMCounter software data. We used 47 tissues from 27 individuals to compare ATM content between manual IHC counting data and AMCounter software data.

Antibody selection

After careful review of the literature and testing of numerous different antibodies, we selected CD68 for total macrophages using IHC. We chose the CD14 receptor as our proinflammatory macrophage marker for both the IHC and flow cytometry experiments because in vitro studies have shown that activation of the CD14 receptor results in a proinflammatory cell signaling cascade (16-18). We selected CD206 for the anti-inflammatory macrophage marker for IHC and flow cytometry. CD206 is a mannose receptor that induces an anti-inflammatory cell signaling cascade following activation (17,19). We initially tested a fluorescein isothiocyanate (FITC) conjugated CD11b antibody for total macrophages by flow cytometry (20) but experienced unacceptable variability, consistent with descriptions on the Purdue Cytometry Discussion List (an online forum for flow cytometry experts) (21) that adipose tissue samples gave excessive autofluorescence in the FITC channel. We changed to the allophycocyanin (APC) conjugated CD68 receptor antibody for flow cytometry to avoid FITC channel autofluorescence and to allow direct comparison with the antigen we selected for IHC. Our strategy for developing the optimal antibody concentration to ensure adequate capture of positive events and to reduce nonspecific staining is provided in the Supporting Information Methods and Figure S1.

IHC

Details of the IHC techniques for sample processing are provided in the Supporting Information Methods. The stained tissue sections were visualized using an Olympus BX43 light microscope (Olympus Scientific Solutions, Waltham, Massachusetts). Ten to twelve randomly selected images per slide were taken at 40× magnification, and two independent observers counted positively stained macrophages, crown-like structures (CLS), and total adipocytes for each field of view. We counted positively stained cells as ATMs if they displayed the known morphological characteristics of macrophages. From this, we derived the number of positively stained cells per 100 adipocytes. All slides were marked with a code rather than the sample identity to ensure the independent observers were blinded to the other reader’s data, the participant, the research protocol, and the biopsy site. Similarly coded quality control slide photographs were included every 30 to 40 samples in the experimental data set to assess whether the readers’ interpretation changed over time.

IHC calculations

Because the data is expressed per 100 adipocytes but adipocyte size may vary between adipose regions and individuals, we also report ATM relative to tissue mass. To estimate the number of ATMs per gram of tissue, we performed a quantitative lipid extraction of adipose and calculated the number of adipocytes per gram of tissue by dividing the lipid content by the average adipocyte size. For the few samples with insufficient tissue to perform a separate lipid extraction, we used the average lipid content of adipose tissue (0.76 g lipid/g tissue) because there was minimal material to sample variability in this value.

Automated IHC software development

The AMCounter automated image analysis program was developed using the Analyze/AVW imaging platform (Biomedical Imaging Resource). Adipocytes and macrophages are automatically segmented and the results presented to the user for approval/modification. The automatic segmentation process takes less than 15 seconds per image on a standard computer workstation. Details of the AMCounter automated image analysis program are provided in the Supporting Information.

IHC automated software program use and statistical analysis

The software places circles to mark and count adipocytes and macrophages. By moving the cursor over a cell, the user can insert new circles at missing sites or delete incorrectly placed circles. The program saves the results and images, including the inserted circles.

Two readers manually counted the number of adipocytes and macrophages from 40 subcutaneous adipose tissue samples (10 images each) and separately using the AMCounter software. Both readers analyzed the same set of images to allow us to assess interreader agreement.

The between-method agreement was calculated with Lin’s concordance correlation coefficient (CCC), which is the product of precision (how far each observation deviates from the best-fit line) and accuracy (how far the best fit deviates from the 45-degree line). The degree of concordance was defined as excellent (0.81-1.00), substantial (0.61-0.80), moderate (0.41-0.60), fair (0.21-0.40), slight (0.00-0.20), and poor (<0.00). For visual assessment, the paired readings from both methods were plotted (for each reader separately) in a scatterplot, and a 45° line through the origin, the concordance line, was drawn.
TABLE 1 Subject characteristics

|                  | Male (n = 13) | Female (n = 36) |
|------------------|--------------|----------------|
| Age (y)          | 41 ± 10      | 37 ± 10        |
| BMI (kg/m²)      | 32 ± 4.5     | 32.1 ± 4.2     |
| Abdominal adipocyte size (µg lipid/cell) | 0.80 ± 0.37 | 0.76 ± 0.29 |
| Femoral adipocyte size (µg lipid/cell)   | 0.78 ± 0.32 | 1.02 ± 0.34 |

Data shown as mean ± SD.

Altman and Bland (22) approaches were used to assess disagreements between methods and the contribution of bias. For graphical presentation of the agreement, the differences between measurements by the two methods were plotted against their mean (for each reader separately). The graph allows for visual assessment of method agreement and reveals outlying observations.

The intraclass correlation coefficient (ICC) was used to quantify measurement reliability of each method. It was calculated to assess interobserver variability for each method: reader 1 versus reader 2 for manual counting and reader 1 versus reader 2 for software counting.

Flow cytometry and data analysis

Details of the flow cytometry measures are provided in the Supporting Information and Figure S2. All samples were analyzed using FlowJo Version 10 software (FlowJo LLC, Ashland, Oregon). Forward scatter-area (FSC-A) and side scatter-area (SSC-A) gates were initially set based on size and density using control sample tissues. Within this gated population, a second gate was set using live or dead viability stained cells (Apc-Cy7+) versus FSC-A to exclude apoptotic cells. Both gates were then back-gated to our sample of interest. Within the gated populations on the sample of interest, the singlet cell population was selected using FSC-A versus FSC-height. We further gated this population of single cells (as identified above) to CD68+ cells (APC-CD68 vs. FSC-A). Using the CD68+ population, quadrant gating methods were applied to plot for the selected antigens (APC-CD68 vs. PE-CD14 or APC-CD68 vs. PE-Cy7-CD206). The proinflammatory or M1 macrophage population was defined as cells dual-stained with the CD68-APC conjugated antibody and CD14-PE conjugated antibody (CD68+/CD14+). The CD206+ macrophages, or M2 population, was measured as dual-stained CD68-APC and CD206-PE-Cy7 (CD68+/CD206+). To measure the total CD68+ macrophages, we used the number of cells within quadrants which contained the single-stained CD68+ cells and the cells dual-stained for either CD14 or CD206.

All of the flow cytometry results are expressed per gram of tissue, taking into account the number of aliquots utilized per sample run. We also report the percentage of ATMs that were CD14+ and CD206+ and the ratio of CD14+ to CD206+ macrophages.

Real-time polymerase chain reaction

We used the RNeasy Lipid Tissue Mini Kit (#74804, Qiagen, Hilden, Germany) to isolate RNA from adipose tissue samples. The isolated RNA was then reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (#4368813, Applied Biosystems, Foster City, California) into complementary DNA (cDNA) as described by the manufacturer. Real-time polymerase chain reaction (RT-PCR) was performed using TaqMan Gene Expression Assays (Applied Biosystems) (CD68 = Hs00283681_g1, CD14 = Hs002621467_s1, p16 = Hs00923894_m1, and CYCA = Hs09999904_m1) and TaqMan Fast Advanced Master Mix (#4449464, Applied Biosystems) on a Quant thermocycler using “Fast” setting in duplicate. The ΔΔCT method was used to analyze the data, and cyclophilin A was used to normalize samples.

Statistics

Values are provided as average ± SEM. Paired Student t test was used for comparison of results between depots within the same individuals. Univariate regression analyses were used to test for correlations between visit one and visit two for IHC and flow cytometry as well as correlations between the IHC and flow cytometry methods.

TABLE 2 Adipose tissue macrophage content reproducibility

|                  | First biopsy | Second biopsy | Per g tissue | Per 100 adipocytes | Per g tissue | Per 100 adipocytes | r, P | r, P | r, P |
|------------------|--------------|--------------|--------------|--------------------|--------------|--------------------|------|------|------|
| IHC              |              |              |              |                    |              |                    |      |      |      |
| CD68             | 120,408 ± 10,384 | 14.1 ± 1.5 | 130,349 ± 10,098 | 15.4 ± 1.6 | 0.51 | 0.61 | r = 0.63, P = 0.001 | r = 0.73, P = 0.001 |
| CD14             | 43,388 ± 4,939   | 5.5 ± 0.8   | 45,412 ± 4,837   | 5.5 ± 0.7   | 0.92 | 0.93 | r = 0.63, P = 0.001 | r = 0.76, P = 0.0001 |
| CD206            | 119,975 ± 7,223  | 14.0 ± 1.2  | 120,903 ± 7,939  | 13.8 ± 1.0  | 0.93 | 0.87 | r = 0.54, P = 0.006 | r = 0.80, P = 0.0003 |
| Flow cytometry   |              |              |              |                    |              |                    |      |      |      |
| CD68+            | 3182 ± 2772    |            | 2930 ± 2474     | 0.77            |                |                   |      |      |      |
| CD14+/CD68+      | 2514 ± 2319    | 75 ± 11 %   | 2314 ± 1985     | 76 ± 10 %     | 0.78           |                r = 0.40, P = 0.20 | r = 0.58, P = 0.049 |
| CD206+/CD68+     | 2629 ± 2423    | 78 ± 10 %   | 2384 ± 2,154    | 73 ± 24 %     | 0.73           |                r = 0.44, P = 0.16 | r = 0.87, P = 0.0003 |

Data shown as mean ± SD.

For the immunohistochemistry (IHC) reproducibility, there were 30 samples (abdominal and femoral) from 15 volunteers collected in duplicate 2 to 3 weeks apart. For flow cytometry, there are 12 samples (6 abdominal and 6 femoral) from 6 volunteers collected 2 to 3 weeks apart.
Results

Participant characteristics
The participant characteristics are outlined in Table 1. The study cohort included 49 participants (13 males and 36 females).

Inter- and intraindividual variance in IHC measurements
Subcutaneous abdominal and/or femoral adipose tissue biopsies were collected from 15 participants 2 to 3 weeks apart in order to assess intraindividual variance in macrophage population measured using IHC. The agreements between the total monocyte/macrophage (CD68\(^+\)), CD14\(^+\), and CD206\(^+\) cells for visit one and visit two were judged as good whether expressed per 100 adipocytes or relative to tissue mass (Table 2; representative images shown in Figure 1).

For the total macrophage (CD68) marker, the absolute difference between reader 1 and reader 2 was 8 ± 5 macrophages per 100 adipocytes (mean ± SD, range 2.4-13.9). The absolute difference in CD14\(^+\) macrophages between reader 1 and reader 2 was 3 ± 1 macrophages per 100 adipocytes (mean ± SD, range 1-6). The absolute difference in CD206\(^+\) macrophages between reader 1 and reader 2 was 6 ± 3 macrophages per 100 adipocytes (mean ± SD, range 0.4-11).

We also counted the CLS; there was minimal variation between the two readers and replicate measures, with an average of 1 CLS per 10 fields of view present in CD68 stained slides, 0.07 CLS present in CD14 stained slides, and 0.44 CLS present in CD206 slides. Parenthetically, in our population, there were many samples with no CLS in 10 fields of view. The distribution of the number of CLS per 10 fields of view paired with the number of CD68\(^+\) macrophages per 100 adipocytes for 51 separate biopsies is provided in Supporting Information Figure S3.

Between-method agreement: AMCounter software and manual counting
Between-method agreement was determined using 40 different adipose tissue samples (representative image, Figure 2). For each reader and each antibody, the concordance line plot was constructed separately. The number of macrophages and adipocytes from the same samples by two methods clustered around the line of identity at 45° (Figure 3A-3F).

The between-method comparisons for reader 1 had CCCs of 0.32 for CD68, 0.47 for CD14, and 0.52 for CD206, indicating fair to moderate agreements, respectively. All of the between-method comparisons for reader 2 had CCCs > 0.83 (CD68: 0.90; CD14: 0.88; CD206: 0.83), indicating excellent agreement.
The differences between the methods were plotted against their mean (Figure 3A, 3C, and 3E). For reader 1, more differences were above the "0" line (4:36, 3:37, and 2:38 for CD68, CD14, and CD206, respectively), implying a tendency of reader 1 to count more cells when counting manually than when using the AMCounter. The patterns of reader 2 indicated that manual counts were very similar to the AMCounter software approach.

The interrater ICCs for manual counting were 0.32 for CD68, 0.41 for CD14, and 0.62 for CD206, consistent with the definition of poor between-reader agreement using the manual method (Figure 3A-3F). In contrast, the interrater ICCs calculated for the AMCounter software were 0.88 for CD68, 0.83 for CD14, and 0.84 for CD206, consistent with the definition of excellent between-reader agreement of the AMCounter approach.

### Flow cytometry: intraindividual variance of total macrophage analysis

We tested the reproducibility of total macrophage burden using duplicate abdominal and femoral adipose tissue samples collected from six participants approximately 2 weeks apart. There were no significant differences in the number of APC conjugated CD68+ (total ATMs), CD14+/CD68+ (M1 ATMs), or CD206+/CD68+ (M2 ATMs) per gram of tissue between visit one and visit two (Table 2).

There were no significant correlations between the duplicates of these measures per gram of tissue. However, for replicate data between the first and second biopsies, there were statistically significant correlations between the percentage of CD68+ cells that stained positive for both CD68 and CD14 and that stained positive for both CD68 and CD206 (Table 2). There was not a significant correlation between the ratio of CD14+/CD68+ (M1 ATMs) to CD206+/CD68+ (M2 ATMs) for the first and second biopsies ($r = 0.29, P = 0.35$).

### IHC and flow cytometry comparison

Forty-seven adipose tissue samples (abdominal and femoral) from 24 participants were used to analyze the agreement between the IHC and flow cytometry methods. As presented in Table 2, the estimated numbers of ATMs were markedly greater with IHC than flow cytometry. There was not a statistically significant association between the number of IHC CD68+ ATMs per gram of tissue and the number of CD68+ cells per gram of tissue measured by flow cytometry ($r = 0.20, P = 0.18$) or between the IHC CD14+ ($r = 0.18, P = 0.24$) or CD206+ ($r = 0.04, P = 0.77$) ATMs per gram of tissue and the number of CD68+/CD14+ and CD68+/CD14+ cells per gram of tissue, respectively, measured by flow cytometry. Likewise, there were no statistically significant correlations between the IHC and flow cytometry methods for the CD14+ percentage of CD68+ ATMs or for the CD206+ percentage of CD68+ ATMs.

### RT-PCR comparison to IHC and flow cytometry

Forty-three adipose tissue samples (abdominal and femoral) from 21 participants were used to analyze the agreement between the RT-PCR, IHC, and flow cytometry methods. There were significant correlations between RT-PCR CD68 and IHC CD68 ($r = 0.36, P = 0.017$), CD14 ($r = 0.58, P < 0.0001$), and CD206 ATMs ($r = 0.56, P = 0.0001$) per 100 adipocytes. These relationships between RT-PCR data and IHC were less strong when IHC data was expressed per gram of tissue (CD14: $r = 0.46, P = 0.002$, CD206: $r = 0.29, P = 0.05$, CD68: $r = 0.13, P = 0.40$).

Adipose total macrophage burden per gram of tissue as assessed by flow cytometry CD68 was also significantly related to RT-PCR CD68+ ($r = 0.43, P = 0.004$). The macrophage content per gram of tissue by flow cytometry of CD68+/CD14+ ($r = 0.43, P = 0.004$) and CD68+/CD206+ ($r = 0.48, P = 0.001$) was likewise correlated with RT-PCR CD14 and CD206 mRNA.

### Discussion

An important aspect of adipose inflammation is the burden of pro-and anti-inflammatory macrophages (23-26). In the process of establishing our laboratory standards for the measurement of ATMs, we evaluated the reproducibility and comparability of IHC and flow cytometry methods for human tissue. Several issues arose that seem not to have been addressed in the literature. We also tested an image analysis software program that we commissioned in hopes it would
reduce the burden of manual image analysis. We found that (1) IHC is reproducible in measuring ATM populations within the same individual; (2) the AMCounter software allows for better agreement between readers than the traditional manual read method and saves time; (3) there is not good agreement between the IHC and flow cytometry methods; and (4) the expression of CD68, CD14, and
CD206 mRNA in adipose tissue can predict some of the inter-individual variability in CD68⁺, CD14⁺, and CD206⁺ ATMs measured using either IHC or flow cytometry.

The test-retest differences in IHC ATM measurements for the same individuals biopsied twice with no intervention suggest that IHC quantification of ATMs is reproducible, but to do so is tedious and time intensive. The Biomedical Imaging Resource staff developed a semi-automated software approach to quantify the ATMs that improves the between-reader agreement and reduces time needed from 8 to 10 minutes for one slide with 10 pictures for purely manual reading to 5 to 8 minutes using AMCounter.

The agreement (CCC) between relatively novice readers was variable using the manual counting method but much better when using the software program (Figure 3). This difference may relate to the need to manually track the number of adipocytes and positively stained cells on each slide and enter this data into spreadsheets; both tasks are automatically tracked with AMCounter. Another factor may be that different users do not always agree on what constitutes a positively stained, nucleated macrophage when counting manually. This selection bias is unavoidable because different readers count according to their judgment of whether the cell has the morphology of a macrophage, which may also change over time—thus the importance of double reading and quality control slides for longitudinal data integrity. The software selects macrophages and adipocytes based on the intensity of color and contrast to the surrounding tissue, which should allow better consistency over time. To examine how many CD68⁺ cells did not have macrophage morphology, we re-reviewed a subset of 20 IHC samples and counted all CD68⁺ cells, sorting them into macrophage and non-macrophage appearing cells. We found 16.6 ± 5.3 CD68⁺ macrophages per 100 adipocytes and 9.3 ± 2.7 CD68⁺ cells that did not have the appearance of macrophages per 100 adipocytes. Providing this is a representative sample, it suggests that the well-known expression of CD68 in other immune cells in adipose tissue can be significant. Finally, the AMCounter program saves a permanent photographic record with inserted circles and a corresponding spreadsheet, allowing investigators to retrieve images and determine the reasons for discrepancies between users.

Our goal was to develop a reliable and reproducible flow cytometry protocol for quantifying ATMs because we did not know whether IHC or flow cytometry would better suit our needs. To our surprise, we found poor agreement between the two methods in terms of ATMs per gram of tissue, although we had only 12 replicate samples and may have missed a modest correlation. We hypothesize that this lack of agreement is due to the variability in macrophage recovery from tissue during the digestion and processing for flow cytometry. In support of this hypothesis, there was good reproducibility of flow cytometry for measuring the percentage of CD68⁺ cells that were also positive for CD14 or CD206 even with only 12 replicate samples. Another possible explanation for the lack of agreement between IHC and flow cytometry is that the two methods use different antibody clones to detect the CD68, CD14, and CD206 antigens. Unfortunately, the antibodies used for flow cytometry cannot be used for IHC, which prevented us from determining how much of the disagreement was due to this factor. This cannot explain the suboptimal reproducibility of some aspects of flow cytometry, however. An advantage of flow cytometry is the opportunity to quickly analyze a more comprehensive list of immune cells of adipose tissue. It is possible that different gating strategies would improve the agreement between flow cytometry and immunohistochemistry measures of ATMs. To that end, the Supporting Information includes the fcs data files and immunohistochemistry data from 20 of the samples we used for comparison so that interested investigators can apply their own flow cytometry approaches to our data set.

We found positive correlations between RT-PCR measures of CD68, CD14, and CD206 expression and direct ATM measures from both IHC and flow cytometry. This is reassuring in that it suggests all three measures provide some common information. However, even the best RT-PCR association could explain only 34% of the variance in the direct measure of ATM burden. This is not surprising given that cells other than macrophages can express these three receptors. The flow cytometry gating strategy we use to sort for macrophage size, live, single cells excludes other CD68 positive cells. When we reanalyzed some of our data, counting all CD68 + cells irrespective of the FSC-A and SSC-A, there were approximately five times more CD68 + cells than if we counted only live singlet cells without gating on FSC and SSC. Thus, while RT-PCR may be adequate to distinguish group differences in adipose inflammatory burden, it is probably insufficiently specific to be valuable as a measure of individual differences.

We suggest there is a population of macrophages that stain positively for both pro- (CD14⁺) and anti-inflammatory (CD206⁺) markers, as indicated by the ratio of CD14⁺ to CD206⁺ macrophage burden quantified via the flow cytometry. These findings are in agreement with recent studies using immunofluorescent techniques to show that both CD14⁺ and CD206⁺ receptors can be present within the same cell (27). In addition, recent studies have also found that both CD14⁺ and CD206⁺ macrophages are present within CLS (28), indicating greater inflammation in animal models (29). These findings suggest that the inflammatory story is not as simple as pro- versus anti-inflammatory macrophage burden. It may be that total macrophage burden, regardless of specific receptor makeup, is a good measure of adipose inflammation. Further studies of human samples should be conducted to determine if indeed CD14⁺ and CD206⁺ macrophage receptors coexist within the same cell and/or CLS.

Although our data suggests that IHC is a reliable way to measure ATMs and that the AMCounter may be more reproducible and efficient, there are some remaining questions. For example, we do not know whether our results apply only to adipose tissue that has been obtained via needle aspirate or whether they would also apply to samples collected via surgical excision. Our data also suggests that flow cytometry measures of ATMs are better suited to understanding the relative distribution of different kinds of macrophages rather than the total macrophage burden per se. These observations have the potential to help investigators better select their measures of human adipose inflammation depending on their goals.

Acknowledgments

We would like to thank the personnel of the Mayo Clinic Clinical Research and Trials Unit, the Mayo Clinic Rochester Pathology Research Cores, and the Flow Cytometry and Cell Sorting Microscopy team of the Cell Analysis Core, as well as Ms Monica Davis for editorial assistance and our volunteers.

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