CD4+ and CD8+ T-Cell-Specific DNA Cytosine Methylation Differences Associated With Obesity

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Objective: Lifestyle factors associated with obesity may alter epigenome-regulated gene expression. Most studies examining epigenetic changes in obesity have analyzed DNA 5-methylcytosine (5mC) in whole blood, representing a weighted average of several distantly related and regulated leukocyte classes. To examine leukocyte-specific differences associated with obesity, a pilot study examining 5mC in three distinct leukocyte types isolated from peripheral blood of women with normal weight and obesity was conducted.

Methods: CD4+ T cells, CD8+ T cells, and CD16+ neutrophils were reiteratively isolated from blood, and 5mC levels were measured across >450,000 CG sites.

Results: Nineteen CG sites were differentially methylated between women with obesity and with normal weight in CD4+ cells, 16 CG sites in CD8+ cells, and 0 CG sites in CD16+ neutrophils (q < 0.05). There were no common differentially methylated sites between the T-cell types. The amount of visceral adipose tissue was strongly associated with the methylation level of 79 CG sites in CD4+ cells, including 4 CG sites in CLSTN7’s promoter, which, this study shows, may regulate its expression.

Conclusions: The methylomes of various leukocytes respond differently to obesity and levels of visceral adipose tissue. Highly significant differentially methylated sites in CD4+ and CD8+ cells in women with obesity that have apparent biological relevance to obesity were identified.

Introduction

Obesity results from many factors, including internal (genetic and epigenetic) and external (lifestyle) influences (1-3). Diet and physical activity are major external factors involved in the pathogenesis of obesity, and they appear to act in part by altering epigenetic programming of gene expression (1,2,4). One such potential change in chromatin structure may occur to the 5’ methylation of DNA cytosine (5-methylcytosine [5mC]), which often results in altered gene expression and alterations to corresponding physiology (5,6). Initial evidence has shown that obesity is associated with altered methylation of specific genes in human tissues (1,7,8), and some are associated with changes in linked gene expression (9). For example, cytosine methylation of the hypoxia inducible factor 3 subunit alpha (HIF3A) gene is associated with BMI (7), as are the leptin (LEP) and adiponectin (ADIPOQ) genes (8).

Most studies examining the association of obesity and DNA cytosine methylation have utilized mixed cell populations, for example, adipose tissue (7,8), skeletal muscle (9), or peripheral blood leukocytes (7,10). However, by the very definition of epigenetics, we expect each cell type within a tissue or organ to have its own distinct DNA methylation profile (11,12). Therefore, analyzing mixed cell types from a tissue together results in a methylation profile that is a weighted average of all included cell types. For example, when the global methylation of both the mixed and individual peripheral leukocytes was examined in relation to obesity, it revealed that there were changes in global methylation only in the B-cell population in individuals with obesity.
and not in the mixed cell type fractions (13). Furthermore, there was no association between obesity and global methylation in the peripheral blood mononuclear cells (PBMCs), which contain the weighted average of methylation levels in T cells, B cells, monocytes, and natural killer cells. This result provides an example of the loss of data in one cell type, B cells, when examining mixed cell types (PBMCs) in relation to obesity (13). The data obtained through analyzing the individual leukocyte types or other individual cell types from tissues (i.e., only differentiated adipocytes from adipose tissue) (14,15) should yield more meaningful and statistically sound information to further an understanding of the role of DNA methylation in obesity-related health risks.

Peripheral blood is undoubtedly the simplest tissue to examine in humans, making it an ideal source of surrogate cell types to assay DNA methylation (16,17). We examined the DNA methylomes of isolated CD4⁺ and CD8⁺ T cells and CD16⁺ neutrophils among women with obesity and normal weight in this pilot study to explore the benefits of utilizing single leukocyte types (details on the isolation of these cells are provided in the online Supporting Information). We recently showed that these three cell types expressed distinctly different levels of the factors controlling DNA methylation and demethylation (18). Furthermore, the distinct biological roles of these three classes of leukocytes led us to hypothesize that (1) there will be differences in DNA methylation that are associated with both obesity and levels of adiposity and that (2) the differences in methylation will be distinct to each of the three classes of leukocytes. We assayed DNA methylation of >450,000 sites in each leukocyte type. Our results identified cell-type-specific differences in DNA cytosine methylation between the women with obesity and with normal weight in both CD4⁺ and CD8⁺ T cells but not in neutrophils. We also identified an association between DNA methylation and the amount of visceral adipose tissue (VAT) in the CD4⁺ T cells, while no associations were found for VAT in the other two cell types.

Methods

Study participants

Fourteen women with normal weight (BMI 18.5-24.9 kg/m²) and eight women with obesity (BMI >30.0 kg/m²) (aged 18-35 years old) were recruited from the Athens, Georgia, area. To limit genetic variability, only those women who self-identified as “Caucasian” were selected for this study. The University of Georgia Institutional Review Board approved this protocol, and all subjects provided written informed consent after being made aware of the design of the study.

The participants’ height and weight were obtained by standard protocols and used for the calculation of their BMI (kilograms per meters squared). Body composition of the participants was also determined through dual-energy x-ray absorptiometry (Hologic Discovery A; Hologic Inc., Waltham, Massachusetts). Dual-energy x-ray absorptiometry data were available for 13 of the women with normal weight and 7 of the women with obesity.

Cell isolation

We collected 10 mL of venous blood samples from all participants after an overnight fast. The samples were stored on ice after collection and processed within 4 hours of collection. CD4⁺ T cells, CD8⁺ T cells, and CD16⁺ neutrophils were reiteratively isolated from the whole blood following the protocol published in Hohos et al. (18). Isolated cells were stored at −80°C in 200 μL of phosphate-buffered saline (PBS) until genomic DNA extraction with the DNeasy Kit (category number 69506; QIAGEN, Hilden, Germany). The extracted DNA was then quantified using Quant-IT PicoGreen double-stranded DNA assay kit (category number P7589; Life Technologies, Grand Island, New York) following manufacturer protocol and by NanoDrop. Limitations in cell isolation and DNA yield resulted in a small variation in sample size for the various comparisons (CD4⁺ T cells [obesity, n = 8; normal weight, n = 14], CD8⁺ T cells [obesity, n = 7; normal weight, n = 14], and CD16⁺ neutrophils [obesity, n = 6; normal weight, n = 12]).

DNA methylation analysis

DNA methylation was determined for 61 total samples with the HumanMethylation450 BeadChip (Illumina, San Diego, California). Further details of the analysis are provided in the online Supporting Information.

Methylation and gene expression assay

White blood cells (WBCs) were immediately isolated and cultured in a volume of 2 mL (~500,000 WBCs). 5-azacytidine (5azaC) is an inhibitor of DNA cytosine methyltransferases, and as such, it prevents the de novo methylation of cytosine or its remethylation once methylation is lost; 2μM 5azaC in DMEM was added to samples (control [no drug], n = 6; treatment [5azaC], n = 6) and incubated for 16 hours in a CO₂ incubator. CD4⁺ T cells were isolated as described (24). RNA was extracted, and quantitative real-time polymerase chain reaction assays were performed in a 25-μL reaction using SYBR Green Master Mix (category number ASSMETHL 43677659; Life Technologies) and 4 ng of complementary DNA. All reactions were repeated in triplicate. All data were normalized to the endogenous control 18S mRNA, and then the relative quantity of expression was calculated by the delta delta cycle threshold (ddCT) method. Further details are provided in the online Supporting Information.

Statistics

Differences in biometrical parameters and gene expression were determined by a t test with significance set at P < 0.05. MethLAB version 2.0 (19) was used to test for associations with BMI class in each of the three leukocyte types via linear regressions that modeled the M-values (log(beta-value/(1-beta-value)) as the outcome and the BMI class as a categorial independent variable, or VAT grams as a continuous independent variable, for each CG site on the array. Age was added as a covariate in all regression analyses. Associated sites were considered significant after controlling the false discovery rate with q < 0.05. Functional enrichment analysis was performed using DAVID 6.7 (Leidos Biomedical Research, Inc., Frederick, Maryland) (20,21). Terms were considered enriched in the data set if the EASE score (a modified Fisher exact P value) was < 0.05 and the fold enrichment was >1.5 (21). Further details pertaining to the experimental methods are provided in the online Supporting Information.
### TABLE 1 Differentially methylated sites (DMSs)

| Cell type/analysis | CG site | Associated gene(s) | 5mC change | P   | Cell type/analysis | CG site | Associated gene(s) | 5mC change | P   |
|--------------------|---------|--------------------|------------|-----|--------------------|---------|--------------------|------------|-----|
| **CD4⁺ T cells (those with obesity vs. normal BMI)** | cg06384413 | LOC404266; HOXB5 | Up | 1.24E-08 | **CD4⁺ T cells (VAT-associated)** | cg020388707 | NGEF | Down | 4.08E-06 |
|        | cg07321536 | LIAS; RPL9 | Up | 7.06E-08 |        | cg143739889 | PEX10 | Down | 4.14E-06 |
|        | cg063524839 | FAM76A | Up | 1.18E-07 |        | cg067466849 | CLDN14 | Down | 4.45E-06 |
|        | cg030567669 | SCAMP1 | Up | 1.22E-07 |        | cg075216689 | MACROD1 | Down | 4.68E-06 |
|        | cg25350057 | GRP177 | Down | 1.31E-07 |        | cg26345916 | n/a | Down | 4.76E-06 |
|        | cg08913530 | C10orf129 | Down | 1.67E-07 |        | cg266399069 | CANCNA1G | Down | 4.79E-06 |
|        | cg17213381 | AGPAT1 | Up | 2.97E-07 |        | cg116434429 | SNORA38; BAT2 | Down | 4.84E-06 |
|        | cg092480079 | MKL2 | Up | 4.65E-07 |        | cg129905759 | KCL4 | Down | 4.87E-06 |
|        | cg122275059 | SLC26A11; SGSH | Up | 5.52E-07 |        | cg024942469 | ALDH3B1 | Down | 4.91E-06 |
|        | cg06090383 | SAP30 | Up | 8.80E-07 |        | cg14559176 | n/a | Down | 5.32E-06 |
|        | cg03704653 | FAM9A | Down | 1.28E-06 |        | cg22614521 | n/a | Down | 5.41E-06 |
|        | cg10318313 | NAPIL4 | Down | 1.29E-06 |        | cg200238819 | LRP1 | Down | 5.48E-06 |
|        | cg15418826 | KIF21A | Up | 1.30E-06 |        | cg243390439 | SPRYD3 | Up | 5.51E-06 |
|        | cg02466749 | FANCC | Up | 1.82E-06 |        | cg119540309 | MYO10 | Down | 5.51E-06 |
|        | cg252919419 | POP1; HRSP12 | Up | 1.86E-06 |        | cg10070328 | n/a | Down | 5.54E-06 |
|        | cg22668822 | UBDT2 | Down | 1.88E-06 |        | cg256498959 | TMEM92 | Down | 5.84E-06 |
|        | cg19180156 | n/a | Down | 1.90E-06 |        | cg18446069 | n/a | Down | 5.92E-06 |
|        | cg077908269 | FADD | Down | 2.08E-06 |        | cg214977809 | WNT5B | Down | 6.30E-06 |
|        | cg276594789 | TRIM65 | Down | 2.11E-06 |        | cg06330289 | n/a | Down | 6.32E-06 |
|        | cg266552599 | TMEM18 | Down | 6.11E-09 |        | cg05312779 | ANPEP | Down | 6.35E-06 |
|        | cg17191443 | MATN4 | Up | 1.21E-07 |        | cg092131249 | IGFBP4 | Up | 6.54E-06 |
|        | cg01419670 | n/a | Down | 2.95E-07 |        | cg145520109 | AFF3 | Down | 6.61E-06 |
|        | cg066543109 | HRNPUL1 | Down | 1.29E-06 |        | cg225129739 | STX1A | Down | 6.67E-06 |
|        | cg110880519 | SLC25A3 | Down | 1.48E-06 |        | cg06815003 | n/a | Down | 6.70E-06 |
|        | cg21579726 | ABT1 | Down | 1.93E-08 |        | cg237124589 | RPH3A | Down | 6.73E-06 |
|        | cg192353079 | IFT122; MBD4 | Down | 6.84E-08 |        | cg01281450 | IFNG | Down | 6.86E-06 |
|        | cg08426200 | AGPHD1 | Down | 2.33E-07 |        | cg018009269 | CLSTN1 | Up | 7.01E-06 |
|        | cg08916477 | n/a | Down | 3.59E-07 |        | cg170282599 | SCARF1 | Down | 7.11E-06 |
|        | cg18449739 | DTX1 | Down | 4.47E-07 |        | cg232797929 | n/a | Down | 7.21E-06 |
|        | cg257322529 | ST6GALNAC4 | Up | 1.25E-06 |        | cg00583861 | n/a | Down | 7.39E-06 |
|        | cg11844737 | BCR | Up | 1.28E-06 |        | cg081512929 | SPEF1 | Up | 7.42E-06 |
|        | cg01059398 | TNFSF10 | Down | 3.95E-07 |        | cg109282579 | MIR449; CDC20B | Up | 7.57E-06 |
|        | cg01560407 | IFTG3 | Up | 9.64E-07 |        | cg05887909 | n/a | Up | 7.83E-06 |
|        | cg16248435 | JARD12 | Up | 1E-06 |        | cg160912929 | C10orf35 | Down | 7.84E-06 |
|        | cg06074534 | ZDHHC7 | Down | 1.31E-06 |        | cg046826899 | SLC38A3 | Down | 8.04E-06 |
|        | cg05942022 | SLCA1 | Down | 1.92E-07 |        | cg240335359 | SHF | Up | 8.06E-06 |
|        | cg033406499 | ZNF60 | Up | 2.05E-07 |        | cg029366799 | n/a | Down | 8.30E-06 |
|        | cg19143282 | CTDP1 | Down | 2.53E-07 |        | cg001231049 | CLSTN1 | Up | 8.32E-06 |
|        | cg14287443 | n/a | Down | 4.27E-07 |        | cg054559719 | DLGAP2 | Down | 8.38E-06 |
|        | cg203290859 | ASXL3 | Down | 6.45E-07 |        | cg019676429 | EPHA10 | Down | 8.46E-06 |
|        | cg196702909 | HDC3; UNC45A | Down | 6.47E-07 |        | cg241389169 | SMTNL2 | Down | 8.48E-06 |
|        | cg12005412 | n/a | Down | 7.98E-07 |        | cg150071239 | FAM109A | Down | 8.51E-06 |
|        | cg26317237 | n/a | Down | 8.42E-07 |        | cg033407679 | PRDM11 | Down | 8.56E-06 |
|        | cg05114959 | n/a | Down | 9.29E-07 |        | cg194231759 | MAP2K | Down | 8.93E-06 |
TABLE 1. (continued).

| Cell type/analysis | CG site    | Associated gene(s) | 5mC change | P     | Cell type/analysis | CG site    | Associated gene(s) | 5mC change | P     |
|--------------------|------------|--------------------|------------|-------|--------------------|------------|--------------------|------------|-------|
| cg24551579a        | CLSN1      | Up                 | 9.88e-07   |       | cg23400715         | FAM19A5    | Down               | 9.10e-06   |       |
| cg22053720          | PTK7       | Down               | 1.17e-06   |       | cg11679124         | FRMD4A     | Down               | 9.18e-06   |       |
| cg25133192a         | DHX9       | Up                 | 1.33e-06   |       | cg04486919         | MA3D1L1    | Down               | 9.25e-06   |       |
| cg01543179          | NKX3-1     | Up                 | 1.40e-06   |       | cg13576552         | n/a        | Up                 | 9.63e-06   |       |
| cg23936609          | BRD4       | Down               | 1.50e-06   |       | cg01161042         | ZFYVE28    | Down               | 9.74e-06   |       |
| cg02835977          | n/a        | Down               | 2.27e-06   |       | cg23673974         | TKBKP1     | Down               | 9.74e-06   |       |
| cg09082287          | DNAJC6     | Up                 | 3.08e-06   |       | cg18431489         | TNXB       | Down               | 1.00e-05   |       |
| cg18803110          | PRKCZ      | Down               | 3.55e-06   |       | cg01312828         | n/a        | Down               | 1.01e-05   |       |
| cg17177074          | CASZ1      | Down               | 3.86e-06   |       | cg16630259         | WIPF2      | Down               | 1.02e-05   |       |
| cg22221131          | RNASEH2B   | Up                 | 3.88e-06   |       | cg04527989         | PTCD2; MRPS27 | Down               | 1.04e-05   |       |
| cg01447854          | OBSCN      | Down               | 3.90e-06   |       | cg139322865        | n/a        | Down               | 1.05e-05   |       |
| cg07442105          | n/a        | Down               | 4.02e-06   |       | cg07873325a        | KRCC1      | Down               | 1.07e-05   |       |
| cg19858017a         | CLSN1      | Up                 | 4.04e-06   |       | cg27166993a        | LGR5       | Up                 | 1.11e-05   |       |

DMSs (q < 0.05) between women classified as having obesity and those classified as having normal BMI in CD4+ T cells and CD8+ T cells, as well as VAT-associated DMSs in CD4+ T cells, associated genes, direction of 5mC change, and P values, are listed.

a CG sites associated with promoter region.

n/a = not associated with gene.

Results

The women with obesity and normal weight differed in their body weight, BMI, percent body fat, amount of VAT, and VAT normalized to body weight (P < 0.05) (Supporting Information Table S1). Anthropometric data describing the subgrouping of these women used for the VAT and DNA methylation analysis are provided in Supporting Information Table S2.

Assessment of DNA methylation differences

DNA methylation differences between women with obesity (BMI ≥ 30) and with normal weight (BMI 18.5 ≤ 24.9) for all 485,000 sites on the methylome array were analyzed for each of the three leukocyte types assayed, CD4+ T cells, CD8+ T cells, and CD16+ neutrophils. There were 19 significantly differentially methylated sites (DMSs) identified in CD4+ T cells (q < 0.05), 16 in CD8+ T cells, and 0 sites in the CD16+ neutrophils (Table 1).

Among the DMSs in the CD4+ T cells, 8 had decreased methylation and 11 had increased methylation in the women with obesity. Additionally, eight of the DMSs were associated with promoter regions. The most significant DMS (q < 0.005) was cg06384413, which is physically associated with both homeobox B5 (HOXB5) and LOC402466 (in the promoter and gene body, respectively). The methylation levels of this site (cg06384413) and the three sites with the largest mean difference in methylation between the women with obesity and those with normal BMI (cg01059398 [TNF superfamily member 10, TNFSF10], cg1923507 [trafflagellar transport 12, JFT122/methyl-CpG binding domain 4, DNA glycosylase, MBDA4], and cg11088051 [solute carrier family 25 member 3, SLC25A3]) are presented in categorical scatterplots (Figure 2B). For comparison, the methylation data for these sites are presented for the CD4+ T cells and CD16+ neutrophils (Figure 2A and 2C). None of the significant DMSs observed in CD8+ T cells was also a DMS in CD4+ T cells or neutrophils. It is worth noting that the cg1923507 site, which showed reduced methylation in the CD8+ T cells of the women with obesity, is associated with MBDA4, which has the ability to excise 5mC (22), and its activity is directly involved in the turnover of cytosine methylation; therefore, MBDA4 may participate directly in the methylome response to physiological changes such as obesity.

The absolute difference in methylation between the two groups was calculated for the nineteen DMSs in CD4+ T cells and the 16 DMSs in CD8+ T cells (Supporting Information Figure S1A). CD8+ T cells had much larger differences in the magnitude of methylation change between the two BMI groups, with more than 40% of the DMSs having at least a 10% difference in methylation. The differences in methylation in CD4+ T cells between the two BMI groups were smaller, with more than 70% of the DMSs having a difference in methylation between 2.5% and 5%.

Twelve of the nineteen (63.2%) DMSs in CD4+ T cells were located in CG islands (CGIs), while eleven of the sixteen (68.8%) DMSs in the CD8+ T cells were located in the flanking regions of...
CGIs, with very few within the island themselves. Only a small percentage of the DMSs were located outside of a CGI and immediate flanking regions in the open sea, including three sites in CD4^+ T cells and four sites in CD8^+ T cells (Supporting Information Figure S1B).

Functional enrichment analysis found that 11 out of 19 and 8 out of 19 of the DMSs were associated with transcription factor-binding sites for interferon regulatory factor 2 (IRF2) and IRF1, respectively (Table 2). Recent evidence has shown that IRF1 was more highly expressed in PBMCs of children and adolescents with obesity, while after 18 months of a decreased BMI, the expression of both IRF1 and IRF2 was significantly decreased (23), suggesting a potential altered role of these transcriptional regulators in obesity.

DNA methylation levels correlated with VAT
The methylation of 79 CG sites in CD4^+ T cells was significantly associated with the amount of VAT (q < 0.05) (Table 1; Supporting Information Figure S3). None of these sites in CD8^+ T cells or CD16^+ neutrophils was significantly (i.e., q < 0.05) associated with the amount of VAT (Supporting Information Figure S3), and none of these sites was differentially methylated between the women with obesity and those with normal BMI in neutrophils, CD4^+ T cells, or CD8^+ T cells. Gene function enrichment analysis of the genes containing these 79 DMSs in CD4^+ T cells revealed genes related to phosphate metabolism, phosphorylation, negative regulation of signal transduction and cell communication, and intracellular transport (Figure 3).
Figure 2 Genes with the largest DMSs in CD8$^+$ T cells between the women with obesity and those with normal BMI. The most significantly associated DMSs between the women with obesity and those with normal BMI in the CD8$^+$ T cells (cg26655295), as well as the DMSs with the largest mean methylation difference between the two BMI groups (cg01059398, cg19235307, and cg11088051) in the CD8$^+$ T cells, are presented as categorical scatterplots with the bar representing the mean. (A) CD4$^+$ T cells, (B) CD8$^+$ T cells, and (C) CD16$^+$ neutrophils. *p < 0.05.

TABLE 2 Functional enrichment analysis of DMSs between women with obesity and normal BMI in CD4$^+$ T cells

| Enriched TFBS | Number of genes | Percent of gene list | P     | Fold enrichment |
|---------------|-----------------|----------------------|-------|-----------------|
| IRF2 sites    | 11              | 57.9%                | 0.0099| 2.02            |
| IRF1 sites    | 8               | 42.1%                | 0.022 | 2.41            |

Functional enrichment analysis performed for University of California Santa Cruz (UCSC) transcription factor-binding sites (TFBS) with associated genes of DMSs in obesity in CD4$^+$ T cells. P value listed is an EASE score, a modified Fisher exact P value, and terms considered enriched at P < 0.05 (21). Magnitude of enrichment of UCSC transcription factor term to total genes in human genome is listed as fold enrichment value (21). Fold enrichment values of greater than 1.5 and lower EASE scores are considered enriched in data set (21).
Of the 79 DMS CG sites that were identified in CD4+ T cells, 61 displayed decreasing methylation with an increasing amount of VAT. Twenty-six of these sites were associated with enhancer regions and five with promoter regions. Many of the sites were either in a CGI or in the flanking regions (Supporting Information Figure S1C), although more sites were identified in the open sea than the sites with differential methylation between the obesity and normal-BMI groups in either of the two T-cell types. One gene, cal-syntenin 1 (CLSTN1), had four CG sites that had increased methylation with the amount of VAT (Supporting Information Figure 4A). The methylation levels for these four sites in CLSTN1 were not significantly associated with VAT levels in CD8+ T cells or CD16+ neutrophils (Supporting Information Figure 4B-4C).

Validating a role for DNA methylation changes in gene expression

To examine the role methylation plays in the general expression of several key genes of interest, we directly tested the role of methylation in CLSTN1, which contained four DMSs associated with VAT, in gene regulation in CD4+ T cells. We also examined four additional genes with significant DMSs (between the women with obesity and those with normal BMI) or those associated with VAT in the CD4+ T cells, genes whose CG sites showed the largest DNA methylation changes, or genes that were related to a pathway of interest (Table 3). The expression of CLSTN1 transcript increased significantly in the 5azaC-treated CD4+ T cells compared with those cells cultured with mock drug treatment (Table 3; Supporting Information Figure S2). Transcript levels for the other four genes assayed were not significantly altered in response to 5azaC treatment (Table 3).

Discussion

We examined DNA cytosine methylation differences as a function of BMI in women with normal weight and obesity and as a function of VAT mass among CD4+ T cells, CD8+ T cells, and CD16+ neutrophils. We were exploring the idea that the machinery regulating DNA methylation in the various leukocyte types responded differently to obesity following our observation that these cells expressed distinct levels of the factors controlling cytosine methylation and demethylation (18). We identified CG sites with altered methylation levels associated with BMI in CD4+ and CD8+ T cells and associated with VAT mass in CD4+ T cells (Figure 5). To our knowledge, only two prior studies have examined DNA methylation differences in obesity in single leukocyte types. One study examined global 5mC levels in different peripheral leukocytes and found that there were obesity-related differences only in the B cells (13). We also did not observe global methylation changes in the three leukocyte types assayed in our study, suggesting that the methylation changes associated with obesity in the two T-cell types are site specific. In the other study, the CD4+ T-cell 5mC profile was examined in a mixed population of adults (24). Eight DMSs were correlated with obesity and five with waist circumference (a measure of central adiposity). However, none of the DMSs identified in this study was also identified in our analysis of CD4+ T cells. This may be because only women were examined in our study, while both sexes were included in the previous study (24). In addition, the previous study looked for associations with BMI as a categorical variable, while we used BMI as a categorical variable. Nonetheless, this previous data and ours support the idea that 5mC levels in CD4+ T cells, in particular, respond to obesity.

It is important to consider that the women with obesity included in our study had no metabolic comorbidities of obesity and were overall healthy women (self-identified). Therefore, the DNA methylation differences observed occurred before the development of associated comorbidities, and thus the DMSs we found were associated only with increased adiposity. The inclusion of only self-identified healthy women with obesity in this study is important to consider when comparing DNA methylation to individuals who have developed comorbidities that might impact the DMSs of other genes (25,26).

Associations with VAT

Increased levels of VAT are associated with a chronic low-grade inflammatory state (27,28). BMI is a height-to-weight ratio and does not provide information about percent body fat or adipose tissue distribution (29). Therefore, our analysis of 5mC between the women with obesity and those with normal BMI may have missed
relationships with VAT. VAT experiences changes to its cellular makeup with increasing adiposity as well as releases a milieu of cytokines that affect the overall inflammatory state involving both CD4\(^+\) and CD8\(^+\) T cells (3), which likely affects their status in peripheral blood. Neutrophils are also involved in the changes that occur in VAT in obesity, recruiting greater numbers of macrophages to the tissue and further promoting the inflammatory state (30). We only identified DNA methylation correlating with the amount of VAT in the CD4\(^+\) T cells. The 79 sites at which methylation levels correlated to the amount of VAT were unique to this analysis, showing no overlap with the site differences observed between the women with obesity and those with normal weight. Interestingly, when using the amount of VAT per gram of body fat or body mass, we did not observe any associations with methylation changes (data not shown). This suggests that the amount of VAT, regardless of total body mass or adiposity, has an impact at the molecular level and is associated with changes in DNA methylation in the CD4\(^+\) T cells. The same explanations may be applied to explain why we did not observe differences in the neutrophils. They have low levels of CG methylation, low levels of machinery for methylation and demethylation, and very short half-lives relative to T cells. We might have expected to see the correlation with DNA methylation and VAT in the CD8\(^+\) T cells, especially because CD8\(^+\) T cells have been shown to be involved in the early stages of increased adiposity, in which they infiltrate VAT before the increased adipose macrophage recruitment (31).

Relevance of DMSs to gene expression
We identified four CG sites within the gene CLSTN1 with methylation levels correlating with the amount of VAT in CD4\(^+\) T cells, all located just upstream of the transcription start site. We showed that when DNA methyltransferase-dependent remethylation of hemimethylated DNA was inhibited, there was an increase in CLSTN1 gene

Figure 4 Methylation levels of the four CG sites associated with the amount of VAT in CD4\(^+\) T cells in the CLSTN1 gene. Methylation level (beta values: 0% methylated; 1 = 100% methylated) and the amount of VAT are plotted for the four DMSs in CLSTN1. (A) These DMSs were positively correlated with the amount of VAT in only CD4\(^+\) T cells. To show the lack of association in the other two cell types, the relationship between methylation level and VAT is presented for (B) the CD8\(^+\) T cells and (C) the CD16\(^+\) neutrophils. P-values (unadjusted) and q values (adjusted P value after correcting for multiple testing) are shown.
expression in CD4$^+$ T cells, suggesting that DNA methylation is involved in regulating CLSTN1 expression (Table 3; Supporting Information Figure S2). We examined four other genes with only one DMS each, but DNA methyltransferase inhibition resulted in no detectable changes in their transcript levels (Table 3; Supporting Information Figure S2). Most work on CLSTN1 has been performed in the brain, where the gene has been shown to be involved in the trafficking of the amyloid precursor protein and the pathogenesis of Alzheimer disease (32). However, there is also evidence that this protein has an effect in CD4$^+$ T cells, as in some patients with acute myeloid leukemia, CLSTN1 peptides were able to stimulate CD4$^+$ T helper cells (33). Other studies have suggested that CLSTN1 may be relevant to diet and obesity. In rats fed a low-protein diet supplemented with vitamin D, there was increased expression of CLSTN1 in the kidneys (34). In the subcutaneous adipose tissue of women with morbid obesity, there was increased expression of CLSTN1 (35). Finally, in PBMCs, the differential methylation of one CG site in CLSTN1 was observed between participants with obesity and those with normal weight, between weight-loss maintainers with obesity and successful weight-loss maintainers, and between weight-loss maintainers with normal weight and successful weight-loss maintainers (36); however, this was not one of the DMSs we observed. Together, all these data suggest that CLSTN1 methylation levels are increased with increasing amounts of VAT, appear to regulate CLSTN1 transcript expression, and are important to increased adiposity and obesity.

**Conclusion**

In our exploratory study, we identified statistically significant differences in DNA methylation in CD4$^+$ and CD8$^+$ T cells in women with obesity and in CD4$^+$ T cells with increasing amounts of VAT. The differences observed were unique to each cell type and revealed no overlap in methylation changes between the different analyses. The data herein provide evidence of the advantages of examining physiologically induced changes in DNA methylation in single cell types. Neutrophils are the majority cell type in WBCs, and we observed no methylation differences in this cell type. If we had performed these experiments in total peripheral WBCs, the statistically

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**TABLE 3** Effect of 5azaC on gene expression in CD4$^+$ T cells

| Gene  | DMSs associated with phenotype | q for association with phenotype | DMS gene region location | Direction of methylation change | Effect of 5azaC on gene expression | P for 5azaC treatment |
|-------|-------------------------------|---------------------------------|--------------------------|---------------------------------|-----------------------------------|----------------------|
| IFNG  | 1                             | 0.045                           | 3’UTR                    | Decreases with increasing VAT    | No effect                         | 0.35                 |
| CLSTN1 | 4                             | 0.034 0.045                     | TSS200 TSS200            | Increases with increasing VAT    | Increased                         | 0.037                |
| NAP1L4 | 1                             | 0.043                           | TSS1500                  | Decreased in obesity            | No effect                         | 0.33                 |
| POP1  | 1                             | 0.048                           | TSS1500/1st exon         | Increased in obesity            | No effect                         | 0.15                 |
| LIAS  | 1                             | 0.011                           | TSS1500                  | Increased in obesity            | No effect                         | 0.41                 |

Five genes chosen to determine whether DNA methylation is involved in regulation of their expression in CD4$^+$ T cells are listed. One-tailed t test performed between control and 5azaC samples at 24 hours. Significance set to P < 0.05 (bold), and P values for this analysis are listed. UTR, untranslated region; TSS, transcription start site.
significant cell-type-specific differences in the T cells we observed would likely have been obscured by the heavily weighted lack of change in the methylation profile of neutrophils.

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