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CD4+ AND CD8+ T CELL-SPECIFIC DNA CYTOSINE METHYLATION DIFFERENCES ASSOCIATED WITH OBESITY

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Abstract

Objective—Lifestyle factors associated with obesity may alter epigenome-regulated gene expression. Most studies examining epigenetic changes in obesity analyze 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 we conducted a pilot study examining 5mC in three distinct leukocyte types isolated from peripheral blood of women of normal and obese weight.

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

Results—19 CG-sites were differentially methylated between women of obese and normal weight in CD4+ cells, 16 CG-sites in CD8+ cells and zero CG-sites in CD16+ neutrophils ($q<0.05$). There were no common differentially methylated sites between the T cells types. The amount of visceral adipose tissue (VAT) was strongly associated with the methylation level of 79 CG-sites in CD4+ cells, including four CG-sites in *CLSTN1*'s promoter, which we show may regulate its expression.

Conclusions—The methylomes of various leukocytes respond differently to obesity and levels of VAT. We identified highly significant differentially methylated sites in CD4+ and CD8+ cells in women of obese weight with apparent biological relevance to obesity.

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Author contributions: LBB, HJP, DBH, and NMH recruited participants and managed study visits. RDL performed the DXA analysis, NMH and HJP isolated cells, NMH performed cellular analyses. AKS and VK analyzed and performed the quality control and normalization of the methylation data, and NMH performed statistical analysis. BGP was involved in the conceptualization and execution of the cell culture study. NMH and RBM conceptualized the overall study and wrote the manuscript with help from AKS and BGP.

Keywords

Epigenetics; Women; Obesity

Introduction

Obesity results from many factors including internal (genetic and epigenetic) and external (lifestyle) influences (1, 2, 3). Diet and physical activity are major external factors involved in the pathogenesis of obesity and 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 (5mC), which often results in altered gene expression and alterations to corresponding physiology (5, 6). Initial evidence shows 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 Alpha Subunit (HIF3A)* gene is associated with BMI (7) as are the *leptin (LEP)* and *adiponectin (ADIOQ)* 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, 16). 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 (17, 18). Thus, 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 are only changes in global methylation in the B cell population in obese individuals and not in the mixed cell type fractions (19). Further, there was no association with obesity and global methylation in the PBMCs, which contains 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 (19). The data obtained through analyzing the individual leukocyte types or other individual cell types from tissues (i.e. only differentiated adipocytes from adipose tissue (20, 21)) 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 (22, 23). We examined the DNA methylomes of isolated CD4+ and CD8+ T cells, and CD16+ neutrophils among women with obesity and women of 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 supplemental text). We recently showed that these three cell types express distinctly different levels of the factors controlling DNA methylation and demethylation (24). Further, 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 (2) the differences in methylation will be distinct to each of the three classes

of leukocyte. 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 obese and normal weight women in both CD4+ and CD8+ T cells, but not in neutrophils. We also identified an association of DNA methylation with the amount of VAT in the CD4+ T cells, while no associations were found for VAT in the other two cell types.

Methods

Study participants

Fourteen normal weight (BMI 18.5 to 24.9 kg/m^2) and eight women with obesity (BMI >30.0 kg/m^2) (age 18–35 years old) were recruited from the Athens, GA 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 as to the design of the study.

The participant's height and weight were obtained by standard protocols and used for the calculation of their BMI (kg/m^2). Body composition was also determined for the participants through duel-energy X-ray absorptiometry (DXA) (Hologic Discovery A, Hologic Inc., Waltham, MA). DXA data was available for N = 13 of the normal weight women and N=7 of the women with obesity.

Cell isolation

10 mL of venous blood samples were collected from all participants after an overnight fast. The samples were stored on ice after collection and processed within four 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. (25). Isolated cells were stored at −80°C in 200 μl of PBS (Phosphate Buffered Saline) until genomic DNA extraction with the DNeasy Kit (Cat # 69506, QIAGEN). The extracted DNA was then quantified using Quant-iT PicoGreen dsDNA assay kit (Cat #P7589, Life Technologies) following manufacture 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 (obese N=8; normal weight N=14), CD8+ T cells (obese N=7; normal weight N=14), and CD16+ neutrophils (obese N=6; normal weight N=12)).

DNA methylation analysis

DNA methylation was determined for 61 total samples with the Illumina HumanMethylation450 Beadchip. Further details of the analysis are provided in the supplemental text.

Methylation and gene expression assay

These studies were approved by the Institutional Review Board at the University of Georgia. White blood cells were immediately isolated and cultured in a volume of 2 ml (~500,000 WBCs). 5azaC is an inhibitor of DNA cytosine methyltransferases and as such prevents the de novo methylation of cytosine or its remethylation once methylation is lost. 2 μM 5azaC in

DMEM was added to samples, N=6 control (no drug) and N=6 treatment (5azaC) and incubated for 16 hr in a CO_2 incubator for 16 hr. CD4+ T cells were isolated as described (25). RNA was extracted and qRT-PCR assays performed in a 25 μl reaction using SYBR green master mix (Life Technologies, Grand Island, NY, USA Cat# 43677659) and 4 ng of cDNA. All reactions were repeated in triplicate. All data was normalized to the endogenous control 18s mRNA and then the relative quantity of expression calculated by the ddCT method. Further details are provided in the supplemental text.

Statistics

Differences in biometrical parameters and gene expression were determined by the Student's t-test with significance set at $p<0.05$. MethLAB (26) 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 categorical independent variable, or VAT g as a continuous independent variable for each CG site on the array. Age was added as a covariate in all regression analysis. Associated sites were considered significant after controlling the false discovery rate with a q-value < 0.05. Functional enrichment analysis was performed using DAVID 6.7 (27, 28). Terms were considered enriched in the data set if the EASE score (a modified fishers exact p-value) was <0.05 and the fold enrichment was >1.5 (28). Further details pertaining to the experimental methods are provided in the supplemental text.

Results

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

Assessment of DNA methylation differences

DNA methylation differences between women classified as obese (BMI ≥ 30 kg/m^2) and normal weight (BMI 18.5 ≤ 24.9 kg/m^2) 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, comparing obese and normal weight individuals. There were 19 significantly Differentially Methylated Sites (DMS) identified in CD4+ T cells ($q < 0.05$), 16 in CD8+ T cells, and zero sites in the CD16+ neutrophils (Table 1).

Among the DMS in the CD4+ T cells, eight 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 the *HOXB5* and *LOC404266* (in the promoter and gene body respectively). This site (cg06384413) and the three sites with the largest mean difference in methylation between the obese and normal BMI women (cg07321536 (LIAS), cg10318313 (NAP1L4), cg25291941 (POP1)) methylation levels are presented as a categorical scatter plot (Figure 1A). Categorical scatter plots are compared for the CD8+ T

cells and the CD16+ neutrophils (Figure 1B and C). Clearly, there is general lack of difference in methylation for these four sites in CD8+ T cells and neutrophils.

Within the significant DMSs in the CD8+ T cells, 10 had decreased methylation and six had increased methylation in the women with obesity. Five of these sites were associated with promoter regions. The most significant DMS ($q < 0.002$) in the CD8+ T cells was cg26655295, which is associated with *TMEM18* in the gene body region. The methylation levels at this site (cg26655295) and the three sites with the largest mean difference in methylation between the women with obese and normal BMIs (cg01059398 (*TNFSF10*), cg19235307 (*IFT122/MBD4*), cg11088051 (*SLC25A3*)) are presented in categorical scatter plots (Figure 2B). For comparison, the methylation data for these sites is presented for the CD4+ T cells and CD16+ neutrophils (Figure 2A, C). None of the significant DMS observed in CD8+ T cells were also DMS in CD4+ T cells or neutrophils. It is worth noting that the cg19235307 site, which showed reduced methylation in the obese CD8+ T cells, is associated with *MBD4*, encoding Methyl-CG Binding Domain 4, DNA Glycosylase. MBD4 has the ability to excise 5mC (29) and its activity is directly involved in the turnover of cytosine methylation, and hence, MBD4 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 19 DMS in CD4+ T cells and the 16 DMS in CD8+ T cells (Figure S1A). CD8+ T cells had much larger differences in the magnitude of methylation change between the two BMI groups, with over 40% of the DMS having at least a 10% difference in methylation. The differences in methylation between the two BMI groups in CD4+ T cells were smaller, with over 70% of the DMS having a difference in methylation between 2.5 to 5%.

12 of the 19 (63.2%) DMS in CD4+ T cells were located in CG islands (CGI), while 11 of the 16 (68.8%) DMS in the CD8+ T cells were located in the flanking regions of CGIs and very few within the island themselves. Only a small percentage of the DMS were located outside of a CGI and immediate flanking regions in the open sea, including 3 sites in CD4+ T cells and 4 sites in CD8+ T cells (Figure S1B).

Functional enrichment analysis identified 11/19 and 8/19 of the DMS were associated with transcription factor binding sites for Interferon Regulatory Factor 2 (IRF2) and Interferon Regulatory Factor 1 (IRF1) respectively (Table 2). Recent evidence has shown that IRF1 is 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 is significantly decreased (30), suggesting a potential altered role of these transcriptional regulators with obesity.

DNA methylation levels correlated with VAT

The methylation of 79 CG sites in CD4+ T cells were significantly associated with the amount of VAT (q-value<0.05) (Table 1, Figure S3). None of these sites in CD8 T cells or CD16 neutrophils were significantly (i.e., q-value < 0.05) associated with the amount of VAT (Figure S3) and none of these sites were differentially methylated between the women of obese and normal BMI in neutrophils, CD4+ T cells, or CD8+ T cells. Gene function enrichment analysis of the genes containing these 79 DMS in CD4+ cells revealed genes

related to phosphate metabolism, phosphorylation, negative regulation of signal transduction and cell communication, and intracellular transport (Figure 3).

Of the 79 DMS CG sites that were identified in CD4+ cells, 61 displayed decreasing methylation with increasing amount of VAT. 26 of these sites were associated with enhancer regions and 5 with promoter regions. Many of the sites were either in a CGI, or in the flanking regions (Figure S1C), although more sites were identified in the open sea than the sites with differential methylation between the obese and normal BMI groups in either of the two T cell types. One gene, *CLSTN1*, had four CG sites that increased with the amount of VAT (Figure 4A). The association between VAT and the methylation levels for these four sites in *CLSTN1* are not significantly associated with VAT levels the CD8+ T cells or CD16+ neutrophils (Figure 4B–C).

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,* that contained four DMS associated with VAT, in gene regulation in CD4+ T cells. We also examined four additional genes with significant DMS between the women with obese and normal BMI or associated with VAT in the CD4+ T cells, genes whose CG sites showed the largest DNA methylation changes or were related to a pathway of interest (Table 3). The expression of *CLSTN1* transcript increased significantly in the 5azaC treated CD4+ T cells as compared to those cells cultured with mock drug treatment (Table 3, 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 of 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 upon our observation these cells express distinctly different levels of the factors controlling cytosine methylation and de-methylation (24). 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 (Summarized in Figure 5). To our knowledge, only two prior studies have been performed examining DNA methylation differences in obesity in single leukocyte types. One study examined global 5mC levels in different peripheral leukocytes and found there are only obesity related differences in the B cells (19). 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 manuscript, the CD4+ T cells 5mC profile was examined in a mixed population of adults (31). Eight DMS are correlated with obesity and five with waist circumference (a measure of central adiposity). However, none of the DMS identified in this study were 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 (31), and they looked for associations with BMI as a continuous variable, where we used BMI as a categorical

variable. None-the-less, 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). Thus, the DNA methylation differences observed occurred before the development of associated comorbidities and thus, the DMS we found were only associated 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 that have developed such comorbidities that might impact the DMS of other genes (32, 33).

Associations with VAT

Increased levels of VAT are associated with chronic low-grade inflammatory state (34, 35). BMI is a height to weight ratio and does not provide information about percent body fat or adipose tissue distribution (36). Thus, our analysis of 5mC between the obese and normal BMI groups may have missed relationships with VAT. VAT experiences changes to its cellular makeup with increasing adiposity as well as releasing a milieu of cytokines that affect the overall inflammatory state involving both CD4+ and CD8+ T cells (3), which likely effects their status in peripheral blood. Neutrophils are also involved in the changes that occur in obese VAT, recruiting greater numbers macrophages to the tissue and further promoting the inflammatory state (37). 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 of 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 associated with changes in DNA methylation in the CD4+ T cells. The same explanations may be applied as to 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 as CD8+ T cells have been shown to be involved in the early stages of increased adiposity, where they infiltrate VAT, before the increased adipose macrophage recruitment (38).

Relevance of DMS to gene expression

We identified four CG sites within the gene *CLSTN1, calsyntenin 1,* with methylation levels correlating with the amount of VAT in CD4+ T cells, all located just upstream of the TSS. We showed that when DNMT-dependent re-methylation of hemi-methylated DNA was inhibited, there was an increase in *CLSTN1* gene expression in CD4+ T cells, suggesting the DNA methylation is involved in regulating *CLSTN1* expression (Table 3, Figure S2). We examined 4 other genes with only one DMS each, but DNMT inhibition resulted in no detectable changes in their transcript levels (Table 3, Figure S2). Most work on *CLSTN1* has been performed in brain, where the gene has been shown to be involved in the trafficking of the amyloid precursor protein and the pathogenesis of Alzheimer's disease (39). However,

there is also evidence of this protein having an effect in CD4+ T cells, as in some patients with acute myeloid leukemia (AML), CLSTN1 peptides are able to stimulate CD4+ T helper cells (40).

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 is increased expression of *CLSTN1* in the kidneys (41). In the subcutaneous adipose tissue of morbidly obese women, there is increased expression of *CLSTN1* (42). Finally, in PBMCs, the differential methylation of one CG site in *CLSTN1* was observed between obese and normal weight participants, between obese and successful weight loss maintainers, and between normal weight and successful weight loss maintainers (43), but 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.

Conclusions

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 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

ADIPOQ	adiponectin
5mC	5-methylcytosine
BMI	body mass index

CG	cytosine guanine dinucleotide
CGI	GC island
CLSTN1	Calsyntenin 1
DMS	differentially methylated site
DNMT	DNA methyl transferase
HIF3A	Hypoxia Inducible Factor 3 Alpha Subunit
HOXB5	homeobox 5
IFN-γ	interferon gamma
IFT112	intrflagellar transport 112
IRF1	interferon regulatory factor 1
IRF2	interferon regulatory factor 2
LEP	Leptin
LIAS	Lipoic Acid Synthetase
MBD4	Methyl-CG Binding Domain 4, DNA Glycosylase
NAP1L4	Nucleosome Assembly Protein 1 Like 4
PBMCs	peripheral blood mononuclear cells
POP1	POP1 Homolog, Ribonuclease P/MRP Subunit
qRT-PCR	quantitative real-time polymerase chain reaction amplification of reverses transcribed cDNA
SLC25A3	Solute Carrier Family 25 Member 3
T2D	type 2 diabetes
TMEM18	transmembrane protein 18
TNFα	tumor necrosis factor alpha
TNFSF10	Tumor Necrosis Factor Superfamily Member 10
TSS	transcription start site
VAT	visceral adipose tissue
WBC	white blood cells

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- Epigenome regulated gene expression is influenced by factors associated with obesity.
- Most DNA methylation studies in obesity are performed on mixed cell types, while DNA methylation is a cell type specific modification.
- This study assesses DNA methylation in women with obesity showing cell type specific methylation differences in CD4+ and CD8+ T cells, but not CD16+ neutrophils.

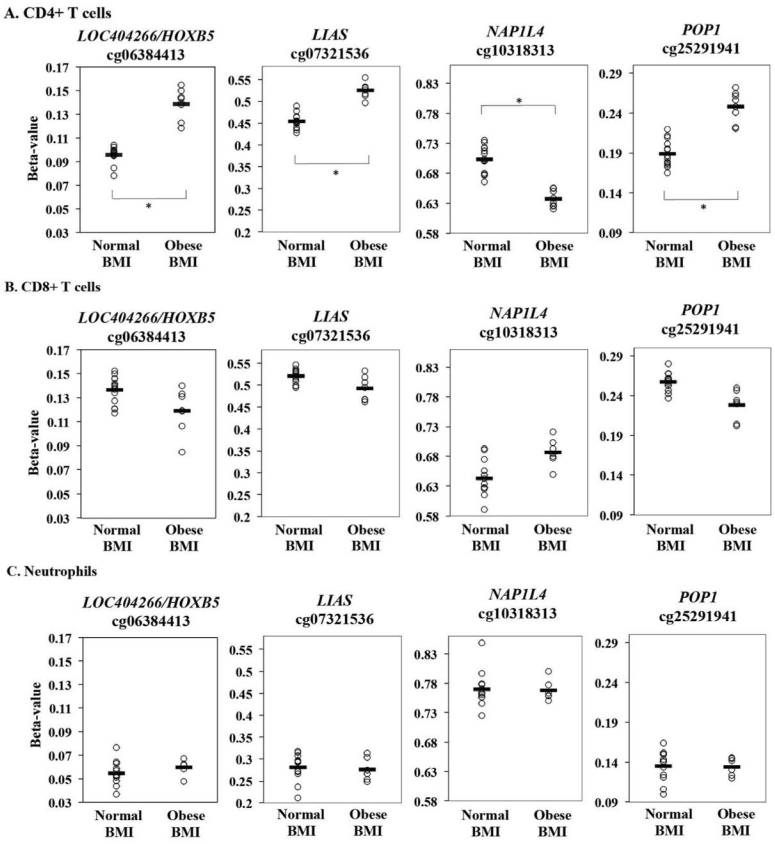

Figure 1. Genes with the greatest DMS in CD4+ T cells between the obese and normal BMI women

The most significantly associated DMS between the obese and normal BMI women in the CD4+ T cells (cg06384413), and the DMS with the largest mean methylation difference between the two BMI groups (cg07321536, cg10318311, cg25291941) in the CD4+ T cells are presented as categorical scatter plots with the bar representing the mean. A. CD4+ T cells, B. CD8+ T cells, C. CD16+ neutrophils. * q<0.05.

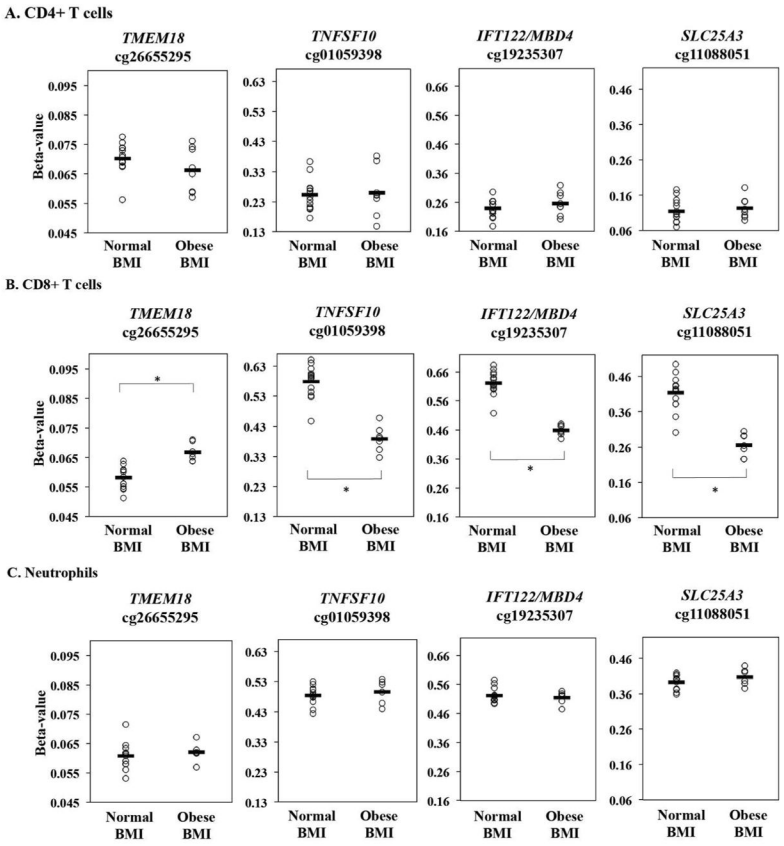

Figure 2. Genes with the greatest DMS in CD8+ T cells between the obese and normal BMI women

The most significantly associated DMS between the obese and normal BMI women in the CD8+ T cells (cg26655295), and the DMS with the largest mean methylation difference between the two BMI groups (cg01059398, cg19235307, cg11088051) in the CD8+ T cells are presented as categorical scatter plots with the bar representing the mean. A. CD4+ T cells, B. CD8+ T cells, C. CD16+ neutrophils. * $q<0.05$.

Within the 79 sites with methylation levels associated with the amount of VAT in CD4+ T cells				
Enriched GO: Biological Process	Number of genes	Percent of gene list	p-value	Fold Enrichment
GO:0006468 protein amino acid phosphorylation	7	12.07	0.01	3.74
GO:0006796 phosphate metabolic process	8	13.79	0.015	2.93
GO:0006793 phosphorus metabolic process	8	13.79	0.015	2.93
GO:0016310 phosphorylation	7	12.07	0.02	3.12
GO:0009968 negative regulation of signal transduction	4	6.90	0.02	6.44
GO:0010648 negative regulation of cell communication	4	6.90	0.03	5.74
GO:00460907 intracellular transport	6	10.34	0.03	3.25

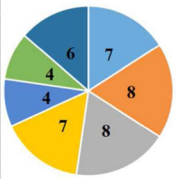

Figure 3. Functional enrichment analysis of CG sites with methylation levels correlating with the amount of VAT in CD4+ T cells

Functional enrichment analysis was performed for GO: biological processes with the associated genes of the sites with methylation levels correlating with VAT in CD4+ T cells. The p-value listed is an EASE score, a modified fisher exact p-value, terms were considered enriched at $p<0.05$ (28). The magnitude of enrichment of the biological process term to the total genes in the human genome is listed as the fold enrichment value (28). Fold enrichment values of greater than 1.5 and lower EASE scores are considered enriched in the data set (28).

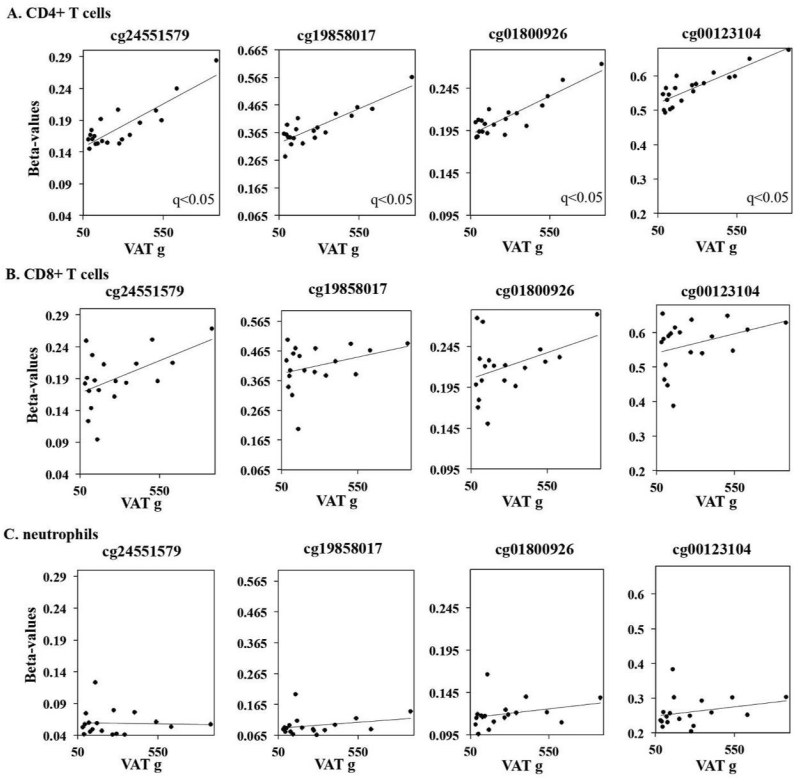

Figure 4. Methylation levels of the four CG sites associated with the amount of VAT in CD4+ T cells in the *CLSTN1* gene

The methylation level (beta values, 0: 0% methylated, 1:100% methylated) and the amount of VAT are plotted for the four DMS in *CLSTN1.* These DMS were positively correlated with the amount of VAT in only CD4+ T cells (A). To show the lack of association in the other two cell types the relationship between methylation level and VAT is presented for the CD8+ T cells (B) and the CD16+ neutrophils (C). p-values (unadjusted) and q-values (adjusted p-value after correcting for multiple testing) are shown.

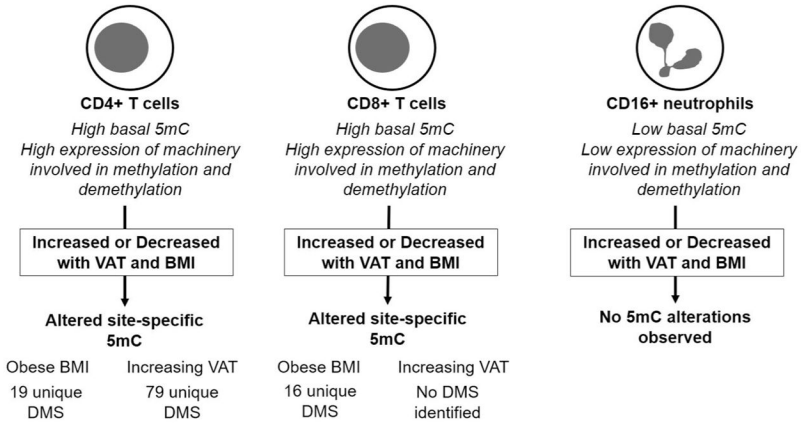

Figure 5. Summary of cell type specific DNA methylation differences in different leukocyte types

Summary figure showing cell type specific differences in CD4, CD8, and CD16 cells relating to DNA methylation (from references 52 and 82 in supplemental text) and the DMS in each cell type related to BMI or VAT identified in the current study.

Table 1

Differentially Methylation Sites

Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value	Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value
CD4+ T cells/obese vs. normal BMI	cg06384413	LOC404266; HOXB5	Up	1.24E-08	**CD4+ T cells/VAT**	cg20388707	NGEF	Down	4.08E-06
	cg07321536*	LIAS; RPL9	Up	7.06E-08		cg14373988*	PEX10	Down	4.14E-06
	cg06352483*	FAM76A	Up	1.18E-07		cg06745684*	CLDN14	Down	4.45E-06
	cg03056766*	SCAMP1	Up	1.22E-07		cg07521668	MACROD1	Down	4.68E-06
	cg25350057	GPR177	Down	1.31E-07		cg26345916	n/a	Down	4.76E-06
	cg08913530	C10orf129	Down	1.67E-07		cg26639906	CANCNA1G	Down	4.79E-06
	cg17213381	AGPAT1	Up	2.97E-07		cg11643442*	SNORA38; BAT2	Down	4.84E-06
	cg09248007*	MKL2	Up	4.65E-07		cg12990575	KCL4	Down	4.87E-06
	cg12227505*	SLC26A11; SGSH	Up	5.52E-07		cg02494246*	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	NAP1L4	Down	1.29E-06		cg20029881	LRP1	Down	5.48E-06
	cg15418826	KIF21A	Up	1.30E-06		cg24339043*	SPRYD3	Up	5.51E-06
	cg02466749	FANCC	Up	1.82E-06		cg11954030	MYO10	Down	5.51E-06
	cg25291941*	POP1; HRSP12	Up	1.86E-06		cg10070328	n/a	Down	5.54E-06
	cg22068822	UBTD2	Down	1.88E-06		cg25649895	TMEM92	Down	5.84E-06
	cg19180156	n/a	Down	1.90E-06		cg18446069	n/a	Down	5.92E-06
	cg07790826*	FADD	Up	2.08E-06		cg21497780	WNT5B	Down	6.30E-06
	cg27659478*	TRIM65	Down	2.11E-06		cg06330289	n/a	Down	6.32E-06
CD8+ T cells/obese vs. normal BMI	cg26655295*	TMEM18	Up	6.11E-09		cg05312779	ANPEP	Down	6.35E-06
	cg17191443	MATN4	Up	1.21E-07		cg09213124*	IGFBP4	Up	6.54E-06
	cg01419670	n/a	Down	2.95E-07		cg14552010	AFF3	Down	6.61E-06
	cg06544310*	HRNPUL1	Down	1.29E-06		cg22512973	STX1A	Down	6.67E-06
	cg11088051*	SLC25A3	Down	1.48E-06		cg06815003	n/a	Down	6.70E-06
	cg21579726	ABT1	Down	1.93E-08		cg23712458	RPH3AL	Down	6.73E-06
	cg19235307*	IFT122; MBD4	Down	6.84E-08		cg01281450	IFNG	Down	6.86E-06
	cg08426200	AGPHD1	Down	2.33E-07		cg01800926*	CLSTN1	Up	7.01E-06

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Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value	Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value
	cg08916477	n/a	Down	3.59E-07		cg17028259	SCARF1	Down	7.11E-06
	cg18449739	DTX1	Down	4.47E-07		cg23279792	n/a	Down	7.21E-06
	cg25732252*	ST6GALNA C4	Up	1.25E-06		cg00583861	n/a	Down	7.39E-06
	cg11844737	BCOR	Up	1.28E-06		cg08151292	SPEF1	Up	7.42E-06
	cg01059398	TNFSF10	Down	3.95E-07		cg10928257*	MIR449; CDC20B	Up	7.57E-06
	cg01560407	ITFG3	Up	9.64E-07		cg05897809	n/a	Up	7.83E-06
	cg16248435	JARID2	Up	1E-06		cg16091292	C11orf35	Down	7.84E-06
	cg06074534	ZDHHC7	Down	1.31E-06		cg04682699	SLC38A3	Down	8.04E-06
CD4+ T cells/VAT	cg05942022	SLC2A1	Down	1.92E-07		cg24033558	SHF	Up	8.06E-06
	cg03340649*	ZNF660	Up	2.05E-07		cg02936679	n/a	Down	8.30E-06
	cg19143282	CTDP1	Down	2.53E-07		cg00123104*	CLSTN1	Up	8.32E-06
	cg14287443	n/a	Down	4.27E-07		cg05455971	DLGAP2	Down	8.38E-06
	cg20329085*	ASXL3	Up	6.45E-07		cg01967642	EPHA10	Down	8.46E-06
	cg19670290*	HDDC3; UNC45A	Down	6.47E-07		cg24138916	SMTNL2	Down	8.48E-06
	cg12005412	n/a	Down	7.98E-07		cg15007123	FAM109A	Down	8.51E-06
	cg26317237	n/a	Down	8.42E-07		cg03470671	PRDM11	Down	8.56E-06
	cg05114959	n/a	Down	9.29E-07		cg19423175	MAP2K	Down	8.93E-06
	cg24551579*	CLSNT1	Up	9.88E-07		cg23400715	FAM19A5	Down	9.10E-06
	cg22053720	PTK7	Down	1.17E-06		cg11679124	FRMD4A	Down	9.18E-06
	cg25133192*	DHX9	Up	1.33E-06		cg04486919	MAD1L1	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	TBKBP1	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		cg13932865	n/a	Down	1.05E-05
	cg07442105	n/a	Down	4.02E-06		cg07873325*	KRCC1	Down	1.07E-05

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Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value	Cell Type/Analysis	CG sites	Associated Genes	5mC Change	p-value
	cg19858017*	CLSNT1	Up	4.04E-06		cg27166993*	LGR5	Up	1.11E-05

DMS ($q < 0.05$) between the obese and normal BMI classified women in CD4+ T cells and CD8+ T cells and the VAT associated DMS in CD4+ T cells, associated genes, direction of 5mC change and p-values are listed. n/a: not associated with gene. CG sites marked with * designate sites associated with the promoter region.

Table 2

Functional enrichment analysis of DMS between obese and normal BMI women in CD4+ T cells

Enriched TFBS	Number of genes	Percent of gene list	p-value	Fold Enrichment
IRF2 sites	11	57.9%	0.0099	2.02
IRF1 sites	8	42.1%	0.022	2.41

Functional enrichment analysis was performed for UCSC transcription factor binding sites with the associated genes of the DMS in obesity in the CD4+ T cells. The p-value listed is an EASE score, a modified fisher exact p-value, and terms were considered enriched at $p<0.05$ (28). The magnitude of enrichment of the UCSC transcription factor term to the total genes in the human genome is listed as the fold enrichment value (28). Fold enrichment values of greater than 1.5 and lower EASE scores are considered enriched in the data set (28).

Table 3

Effect of 5azaC on gene expression in CD4+ T cells

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

The five genes chosen to determine if DNA methylation is involved in the regulation of their expression in CD4+ T cells are listed. A one-tailed t-test was performed between control and 5azaC samples at 24 hours. Significance was set to $p<0.05$ (bold), and the p-values for this analysis are listed.