DNA methylation in blood - potential to provide new insights in immune cell biology

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Research article

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Abstract

Background

Epigenetics plays a fundamental role in cellular development and differentiation; epigenetic mechanisms, such as DNA methylation, are involved in gene regulation and the exquisite nuance of expression changes seen in the journey from pluripotency to final differentiation. Thus, DNA methylation has the potential to reveal new insights in to immune cell biology.

Results

We mined publicly available DNA methylation data with a machine-learning approach to identify differentially methylated loci between different white blood cell types. We then interrogated the DNA methylation and mRNA expression of candidate loci in CD4+, CD8+, CD14+, CD19+ and CD56+ fractions from 12 additional, independent healthy individuals (6 male, 6 female). 'Classic' immune cell markers such as CD8 and CD19 showed expected methylation/expression associations fitting with established dogma that hypermethylation is associated with the repression of gene expression. We also observed large differential methylation at loci which are not considered established immune cell markers, and some of these novel loci showed inverse correlations between methylation and mRNA expression (such as PARK2,DCP2).

Conclusions

Our results highlight the value of mining publicly available data, the utility of DNA methylation as a discriminatory marker and the potential value of DNA methylation to provide additional insights into immune cell biology and developmental processes.

Background

Epigenetics refers to the heritable, but reversible, regulation of various genomic functions, including gene expression. It provides mechanisms whereby an organism can dynamically respond to changes in its environment and "reset" gene expression accordingly [1]. Furthermore, these mechanisms play a critical role in development and cell lineage specificity [2]; [3], as highlighted recently when epigenomic profiling revealed a linear differentiation model for memory T-cells [4]. One such epigenetic mechanism is DNA methylation. Methylation of the cytosine nucleotide within CpG dinucleotides in DNA is well documented in humans [5]; [6]. DNA methylation can be developmentally 'hard-wired' (as in the case of imprinting [7]), underpin cell identity (i.e. cell markers of differentiation [8]; [6]) or dynamic and change in response to environmental factors [9]. Therefore, the investigation of an individual's methylation pattern can reveal a lifetime record of environmental exposures as well as potential disease specific marks [10]; [11].

It is well established that epigenetics contributes significantly to the developmental fate of cells and tissues [8]. For instance, epigenetic mechanisms contribute to the differentiation of hematopoietic stem cells from bone marrow [12]; [13]. Importantly, DNA methylation appears to play a crucial role at specific stages along the separation of blood cell lineages (myeloid, lymphoid) and contributes to the establishment and functionality of the final differentiated cell type [14]. Epigenetic marks, including DNA methylation, are increasingly recognised as potential discriminators of cell type [15]. This observation highlights the potential of DNA methylation analyses to uncover 'hidden' biology and, in the case of immunology, to identify previously unrecognised loci that could act as immune cell discriminators in additional to those cell surface markers currently used routinely. Furthermore, such analyses could identify previously unrecognised immune cell populations/sub-types.

DNA methylation as an epigenetic mark is easily quantified and evaluated from blood. Many recent studies using Illumina array technology have made their data publicly available, providing an excellent resource for hypothesis generation and testing *in silica* prior to wet-lab experimentation. We hypothesised that because of its role in differentiation and development DNA methylation could provide new insights into loci that discriminate between immune cell types; these role of these loci in cell discrimination might be previously unrecognised and/or could be harnessed to sort and/or identify potential new cell sub-types. Therefore, we initially performed an *in silico* experiment using data from a study which examined the DNA methylation profile of human white blood cell populations [16]. Reinius *et al.*, investigated DNA methylation in: T cells ($CD8^+$, $CD4^+$); B cells ($CD19^+$); natural killer cells (NK cells; $CD56^+$); monocytes ($CD14^+$); granulocytes (Gran; both $CD16^+$ and $Siglec8^+$ cells); neutrophils (Neu, $CD16^+$), and eosinophils (Eos, $Siglec\text{-}8^+$). The authors identify DNA methylation marks in "classic" immune cell marker loci. Here, we detail an unsupervised analysis approach which, as anticipated, identifies discriminatory DNA methylation marks in 'classic' immune cell markers, but also highlights significant differential methylation in "non-classic" immune markers, and genes for which a role in immune function is yet to be reported.

Results

DNA methylation - discovery

We identified DNA methylation at 1173 CpG sites which clearly differentiated specific immune cell populations using publicly available data from whole blood [16]; hierarchical clustering and principal components analyses provide a visual presentation and highlight that these markers cluster the cell populations in a biologically meaningful way (**Figure 1**). Pathway analyses of the genes to which these 1173 CpG sites mapped strongly supported their discriminatory nature, and, as expected, enrichment for immune cell biological function was observed: enrichment for CD56 (> 79 genes), CD4 (> 68 genes), CD8 (> 34 genes), CD14 (> 69 genes) and CD19 (> 194 genes) was observed. Furthermore, these results suggest that discriminatory CpG marker loci may map to genes with a hitherto unrecognised role in immune cell discrimination and/or function.

This robust differentiation between cell types was explained by non-overlapping sets of CpGs specific for each cell population: CD8+ (n=70); CD4+ (n=96);

CD19+ (n=347); CD56+ (n=112); CD14+ (n=126); Gran (n=128); Neu (n=128), and Eos (n=166). The majority of these sites were relatively hypo-methylated in the cell type of discrimination and hyper-methylated in all other cell populations analysed: CD8+ (46/70), CD4+ (71/96) CD19+ (344/347), CD56+ (111/112), CD14+ (126/126), Gran (94/128), Eos (165/166) with Neu being the exception (33/128). Interestingly, the majority of CpG marker sites identified (~95% of CpGs) mapped to annotated gene loci, with many in regions involved in regulating mRNA expression (e.g. promoters). For each cell type marker the proportion of CpG sites mapping to annotated loci was: CD8+ (62/70), CD4+ (78/96) CD19+ (255/347), CD56+ (99/112), CD14+ (82/126), Gran (102/128), Eos (136/166) and NEU (108/128). For individual marker information including annotation see https://github.com/sirselim/immunecell_methylation_paper_data [DOI: **10.5281/zenodo.336694**].

The largest DNA methylation difference observed was 87% between CD19+ cells against all others. This 87% difference was observed in two genes, *WIPI2* and *CARS2;* while *WIPI2* has a reported role in the immune system [25], no such function has been reported for *CARS2* to our knowledge to date, and neither has been previously considered as a CD19+ discriminatory marker. Ranked by the largest change in methylation the top five CpG sites mapping to annotated loci for each cell type were:

$CD19^+$: 87% (*WIPI2, CARS2*), 83% (*RERE*), 82% (*LOC100129637*), 80% (*POU2F2*)

$CD4^+$: 69% (*CD40LG*), 56% (*PUM1*), 54% (*DCP2, BAG3*), 48% (*SF1*)

$CD8^+$: 67% (*CD8A*), 64% (*CD8A*), 51% (*CD8B*), 49% (*CD8B, CD8A*)

$CD56^+$: 63% (*SLC15A4*), 52% (*RASA3*), 48% (*MAD1L1*), 45% (*KLRB1/CD161*),

43% (*KLRB1/CD161*)

$CD14^+$: 79% (*PARK2*), 70% (*CENPA, PARK2*), 69% (*KIAA0146, FAR1*)

EOS: 73% (*FAM65B*), 72% (*KIAA0317, APLP2*), 70% (*MEF2A, CCDC88A*)

GRAN: 60% (*VPS53, PCYOX1*), 59% (*ARG1*), 58% (*CSGALNACT1*),

56% (*SH3PXD28*)

NEU: 14% (*CUL9*), 12% (*LASP1*), 7% *(GFI1*), 6% (*LRFN1, NFAT5*)

DNA methylation - validation

In order to validate our initial observations from the in silica experiment we selected 11 differentially methylated loci for analysis in 12 independent samples (n=6 female, n=6 male). These DNA methylation sites included 2 discriminatory loci mapping to annotated genes, including the loci with the maximum methylation difference, for each of $CD4^+$ (*CD40LG, DCP2*) , $CD8^+$ (*CD8A, CD248*) , CD19 (*POU2F2, WIPI2*) , $CD56^+$ (*KLRB1, SLC15A5*), $CD14^+$ (*FAR1, PARK2*) , with CD19 included as an additional positive control. We observed a strong agreement with the expected discriminatory patterns of DNA methylation for all loci examined (**Figure 2**).

RNA expression

Given the role that DNA methylation plays in regulation of gene expression we also explored the mRNA levels of the 11 loci from our DNA methylation validation experiment. We investigated gene expression by QRTPCR in the 12 independent samples. A clear differentiation between immune cells types at the gene expression level was observed for *PARK2*, *POU2F2*, *DCP2*, *CD248*, *CD8A*, *SLC15A4*, *CD4A0LG* and *CD19* but not for *FAR1, WIPI2, KLRB1* (**Figure 2**) .

Discussion

DNA methylation is exquisitely placed to reflect a cell's differentiation trajectory. Using publicly available data we identified 1173 unique CpG sites at which DNA methylation discriminated $CD8^+$, $CD4^+$, $CD19^+$, $CD56^+$, and $CD14^+$ cell populations as well as granulocytes, neutrophils, and eosinophils. DNA methylation at two discriminatory CpG loci for each of $CD8^+$, $CD4^+$, $CD19^+$, $CD56^+$, and $CD14^+$ was validated in 12 independent samples.

The majority of the 1173 discriminatory CpG sites mapped to annotated loci, and gene regulatory regions in particular. This suggests that, as expected, DNA methylation is playing a key role in immune cell differentiation and cell-type identification. An important implication of this is that DNA methylation can be utilised to reveal previously unidentified immune cell sub-populations. A good example of this is the transcription factor Foxp3 which plays a key role in the development and function of Treg cells [26]; originally FOXP3 expression was used to identify Treg cells until it was deemed insufficient for the robust identification of suppressive Treg cells [27]; [28]. However, recent work has reported that hypomethylated CpG sites in four regions of *FOXP3*, *CAMTA1* and *FUT7* can be used to distinguish subsets of Tregs from non-regulatory $CD4^+$ T cells [29]. These findings strongly support our view that DNA methylation, and thus loci identified in our study, could be used to inform similar experiments and reveal other drivers of specific immune cell subtypes.

Furthermore, large differences in DNA methylation were observed, and validated, at CpG loci in genes which, while their potential role in immune cell biology has been reported, have not previously been recognised as differentiators of immune cell type, such as *WIPI2 [25]* for $CD19^+$, *SLC15A4 [30]; [31]; [32]* for $CD56^+$ and *PARK2 [33]; [34]* for $CD14^+$ cells. We also identified *POUF2/OCT2* for which a role as a B-cell differentiator was only recently reported [14]. In addition, significant cell type specific changes in DNA methylation were observed, and validated, in genes which, to the best of our knowledge, have no previous reported role in immune biology (*FAR1, CARS2*). Taken together this highlights the significant potential of such analyses to uncover new facets of immunology. Many more additional loci from our in silica analyses showed large differences in DNA methylation, and these warrant further investigation with respect to their roles in immune cell function.

Expression analysis of the genes to which the 11 validated DNA methylation discriminatory loci mapped also revealed discrimination at the mRNA level for *CD248*, and *CD8A* (CD8+), *POU2F2* and CD19 (CD19+), *PARK2* (CD14+), *DCP2 (CD14+*), *SLC15A4 (CD56+*), and *CD40LG* (*CD4+*). There were three genes (*FAR1, WIPI2, KLRB1*) for which this was not observed. One potential explanations is the presence of multiple isoforms per gene, such that the primer/probe combination for the QRTPCR analysis did not target the correct isoform. This possibility warrants further investigation especially given the increasing body of evidence that DNA methylation is an important modulator of alternative splicing [35]; [36]; [37].

Conclusions

In summary, this study highlights the value of mining publicly available data, the utility of DNA methylation as a discriminatory marker, the potential value of DNA methylation to provide additional insights into immune cell biology and developmental processes, and the tantalising possibility that DNA methylation can be harnessed to reveal currently unrecognised/undistinguishable immune cell sub-types.

Methods

Samples:

Ethics was obtained from, and all experimental protocols were approved by, The Health and Disability Ethics Committee NZ (HDEC, 15/NTB/153). All methods were carried out in accordance with relevant guidelines and regulations. Written, informed consent was obtained from all participants who were all over 18 years of age at the time of collection. Blood from 12 healthy individuals (n=6 male, n=6 female), was collected into sterile K2 EDTA vacutainers (BD Biosciences), and the buffy coat isolated. Peripheral blood mononuclear cells (PBMCs) were Fc receptor blocked, labelled with fluorescent antibodies specific for: CD3 (OKT3), CD4 (OKT4), CD8 (HIT8a), CD14 (HCD14), CD19 (HIB19) and CD56 (HCD56; all antibodies were from Biolegend) and dead cells were identified by DAPI exclusion. $CD4^+$, $CD8^+$, $CD14^+$, $CD19^+$ and $CD56^+$ fractions were collected (Influx cell sorter, BD Biosciences) directly into ice-cold FACS buffer, immediately frozen on dry ice and stored at –80°C.

DNA and RNA extraction.

Both nucleic acids were extracted simultaneously using a Qiagen All prep DNA/RNA kit as per the manufacturers protocol. High quality genomic DNA and RNA were obtained, with RNA RIN $\geq$ 7.5.

DNA methylation Analysis

Public Data. Publicly available methylation data was obtained from MARMAL-AID [17].

GLMnet penalised ridge-regression mixed with lasso in an elastic-net framework was used as implemented via the R package glmnet [18] to explore methylation association between each of the cell-types (CD8+, CD4+, CD19+, CD14+, CD56+, Neutrophils, Eosinophils, Granulocytes, as well as combinations of cell populations, PBMC and whole blood). The number of variables (~450,000 CpG sites) far outweighs the number of cell-types, as such it is accepted that conventional statistical analysis procedures that test each CpG within an independent regression model suffer from multiple testing burden and reduced statistical power. To overcome this issue we choose to use the penalised regression procedures of GLMNet, which tests all markers simultaneously, i.e. in a single regression model. GLMNet was specifically designed to overcome issues of large variable number (k) and small sample size (n) and has been successfully applied to several genome-wide association studies of SNPs [19]; [20]; [21] and recently methylation [22]. We have previously developed and report on this method in detail to identify aging associated DNA methylation loci [23]. The FactoMineR package [24] was used for PCA analysis. All analyses were performed in R 3.5.2.

Pyrosequence analysis: DNA methylation pyrosequencing was designed and performed by EpigenDX (USA), who were provided with the Illumina probe information.

Gene Expression Analysis: 150ng total RNA was reverse transcribed using VILO Superscript (Thermo Fischer). QRTPCR was performed in triplicate on 7ng cDNA using TaqManGene expression assays (*CD40LG* Hs00163934_m1, *DCP2* Hs00400339_m1, *WIPI2* Hs01093807, *POUF2* Hs00922179_m1, *CD19* Hs01047413_g1, *CD8A* Hs01555594_g1, *CD248* Hs005 Gene expression was normalised against the non-variable (data not shown) endogenous control genes, *GAPDH* and *GUSB,* using the ΔCt method ($Ct_{candidate}$-$MeanCt_{controls}$).

Pathways Enrichment: Functional enrichment was performed in the ToppGene Suite webserver (https://toppgene.cchmc.org/) using the ToppFun function. Bonferroni adjusted correction was used in the reporting of all pathways results (adjusted P<0.05).

Declarations

Ethics approval and consent to participate: Ethics was obtained from, and all experimental protocols were approved by, The Health and Disability Ethics Committee NZ (HDEC, 15/NTB/153). All methods were carried out in accordance with relevant guidelines and regulations. Written, informed consent was obtained from all participants who were all over 18 years of age at the time of collection.

Consent for publication: Not applicable.

Availability of data and materials: All data and results are available via GitHub (https://github.com/sirselim/immunecell_methylation_paper_data)[DOI: **10.5281/zenodo.336694**].

Competing interests: The authors declare that they have no competing interests.

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Authors' Contributions: MB and DMC conceptualised the research and designed the experiment. AC isolated PBMCs and performed cell-sorting. JC and DMC extracted DNA and RNA from cell populations, performed QC and JC ran real-time qrtPCRs. MB and DMC performed the statistical analysis and data interpretation. MB, DMC and AC wrote the manuscript. All authors contributed to manuscript revision and final proofreading, and approved the final manuscript.

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Figures

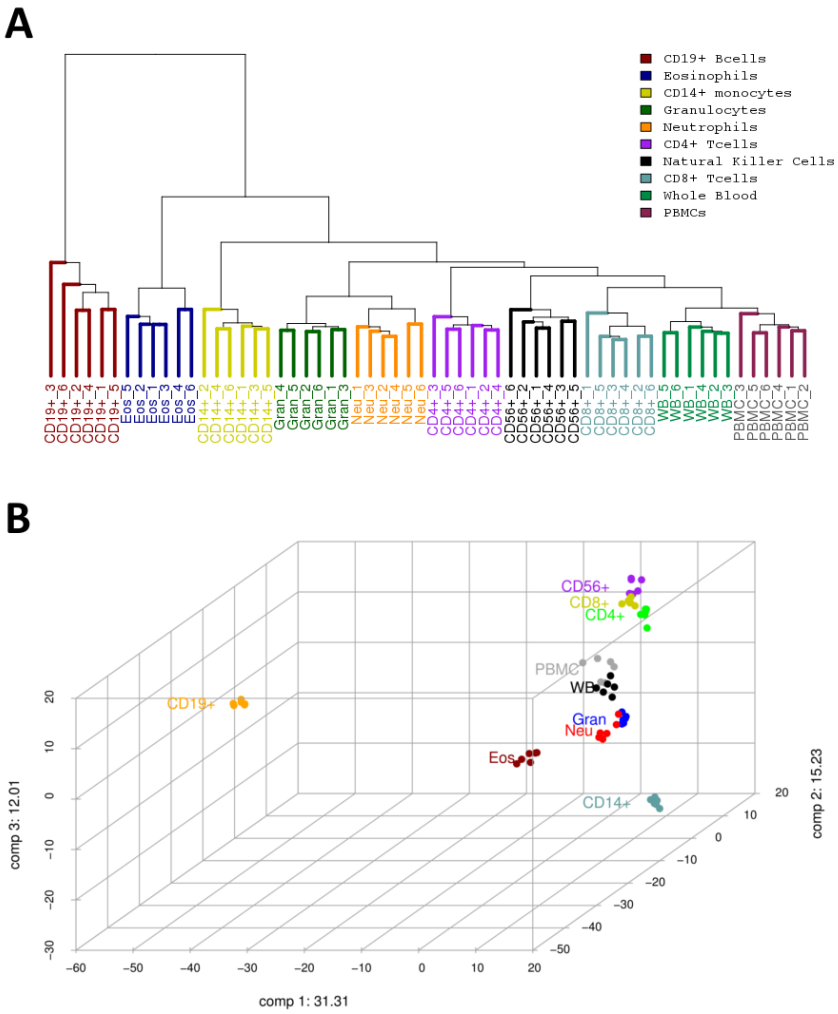

Figure 1

Demonstration of immune cell population discrimination using sets of identified epigenetic markers (CpGs). A) Hierarchical clustering of all 1173 identified probes demonstrates perfect separation of present cellular populations. B) 3D Scatter-plot of the top 3 principal components shows excellent discrimination based on methylation profile. The top 3 components have been plotted, x-axis component 1 (31.31% variance), y-axis component 3 (12.01% variance), and z-axis component 2 (15.23%).

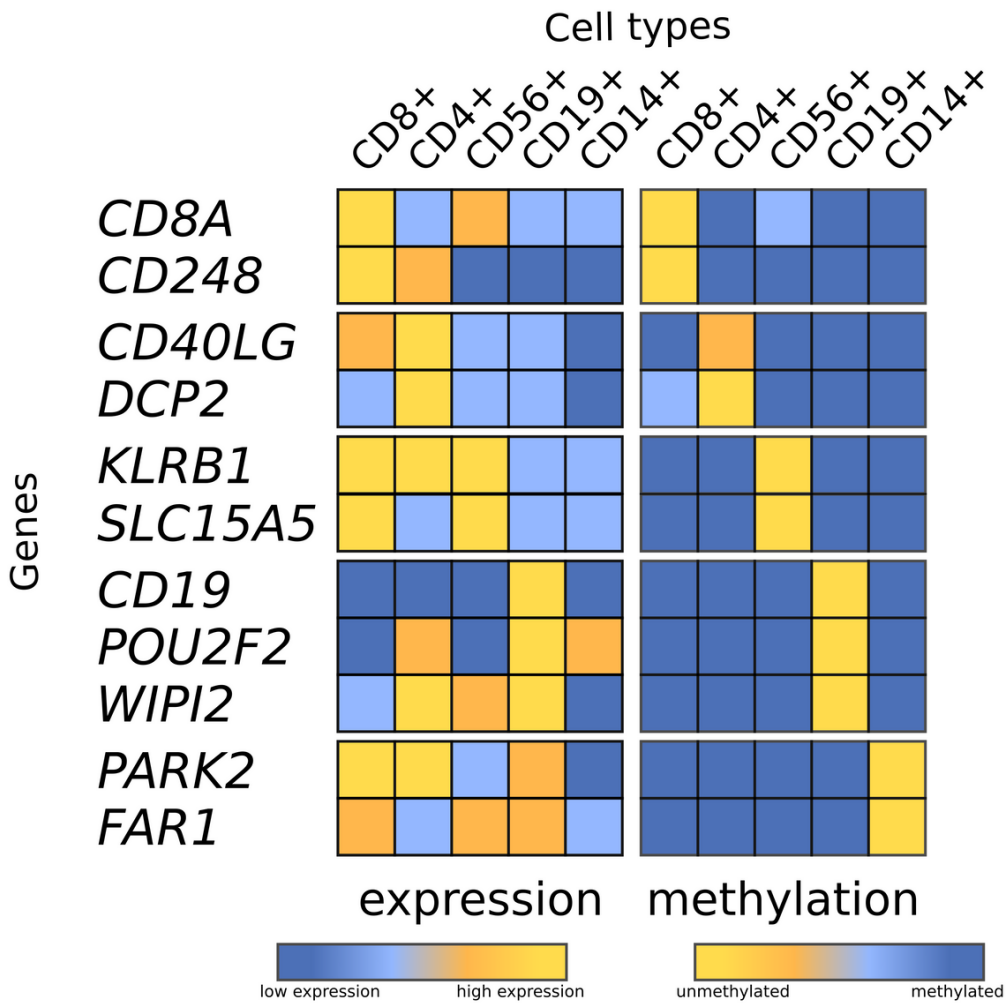

Figure 2

Methylation and gene expression heatmaps for all 11 genes investigated. Expression and methylation measures were split into quartiles and their levels coloured accordingly.