Genome-wide DNA methylation profiling of CD8+ T cells shows a distinct epigenetic signature to CD4+ T cells in multiple sclerosis patients

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Abstract
Background: Multiple sclerosis (MS) is thought to be a T cell-mediated autoimmune disorder. MS pathogenesis is likely due to a genetic predisposition triggered by a variety of environmental factors. Epigenetics, particularly DNA methylation, provide a logical interface for environmental factors to influence the genome. In this study we aim to identify DNA methylation changes associated with MS in CD8+ T cells in 30 relapsing remitting MS patients and 28 healthy blood donors using Illumina 450K methylation arrays.

Findings: Seventy-nine differentially methylated CpGs were associated with MS. The methylation profile of CD8+ T cells was distinctive from our previously published data on CD4+ T cells in the same cohort. Most notably, there was no major CpG effect at the MS risk gene HLA-DRB1 locus in the CD8+ T cells.

Conclusion: CD8+ T cells and CD4+ T cells have distinct DNA methylation profiles. This case–control study highlights the importance of distinctive cell subtypes when investigating epigenetic changes in MS and other complex diseases.

Keywords: Multiple sclerosis, DNA methylation, CD8+ T cells, HLA-DRB1

Findings
Multiple sclerosis (MS) susceptibility is influenced by a combination of genetic factors and environmental exposures. CD4+ T cells have long been favoured as the most important immune cell subset in the pathogenesis of disease, but there is increasing evidence that CD8+ T cells play a substantial role in central nervous system damage (reviewed in [1]). Despite several large genome-wide association studies (GWAS), there remains a large proportion of unexplained heritability in terms of MS risk. Epigenetics can influence the genome without changes to the DNA sequence. Environmental exposures such as smoking and vitamin D levels have been demonstrated to modify epigenetic mechanisms, providing a plausible link between environmental factors and disease [2, 3]. One such epigenetic mechanism is DNA methylation, which is the addition of a methyl group to CpG dinucleotides. We, and others, have used genome-wide DNA methylation technologies to assess differentially methylated regions (DMRs) of CD4+ T cells in MS patients compared to healthy controls [4–6]. We found a striking methylation signal located on chromosome 6p21 with a peak signal at HLA-DRB1, which remained after controlling for background SNP effects, as well as 55 non-HLA CpGs that localise to genes previously linked with MS.

In an effort to determine if these previously identified DMRs were specific to CD4+ T cells, we performed a genome-wide methylation study of CD8+ T cells using the same cohort, workflow and data analysis as described in our previous study [5]. Briefly, DNA from total CD8+ T cells was extracted from 30 MS patients...
and 28 healthy age- and sex-matched controls. The DNA was bisulphite-converted and hybridised to Illumina 450K arrays. Raw fluorescence data were processed using a combination of R/Bioconductor and custom scripts of a total of 442,672 probes representing individual CpG sites that passed quality control (QC) steps. These CpGs were analysed by statistical modelling of methylation levels ($\beta$ values) between MS cases and controls.

Figure 1 shows the genome-wide distribution of differential methylation scores for all CpG sites that passed the nominal $p$ value cut-off of 0.05. We conducted a stepwise prioritisation strategy to extract the most robust CpG loci associated with MS. Based on the criteria of (i) FDR $p < 0.05$ and (ii) $\Delta_{\text{meth}} \geq \pm 0.1$ thresholds, 111 CpGs were extracted. To filter out potential effects of gender and treatment, we performed a subgroup analysis of the methylation statistics as previously described [5]. This process reduced the number of associated CpG sites down to a core panel of 79 (Table 1).

Of the 79 CpGs showing differential methylation in MS patients after filtering, all resided outside the MHC locus on chr 6p21. Of these, 27 were intergenic (34 %), have no gene association, or map to genes of unknown function. Of the remaining 52 loci, 26 % are promoter associated, 9 % are in the 5’UTR, 5 % are in the 1st exon, 20 % are in gene bodies and 8 % are in the 3’UTR. Interestingly, none of these CpGs maps to genes that have previously been reported to have a relationship with MS [7, 8]. There was no overlap between these results and our previous results, and, unlike in CD4+ T cells, there was no gene that contained multiple differentially methylated sites. MORN1 has a single hypermethylated CpG in both CD4+ and CD8+ T cells; however, it was a different site in each study, making it unlikely that this is a significant finding. Our observations are consistent with the recent study by Bos et al., who also identified minimal overlap between the methylation profiles of CD4+ and CD8+ T cells of MS patients [4].

Using GSEA with WebGestalt, our patient cohort did not have prominent pathways in the KEGG Pathway analysis or disease association analysis. The most significant promoter associated with differential methylation was the ferritin light chain ($FTL$) gene. The MS cohort displayed decreased methylation at this CpG locus compared to controls. The gene’s biological function is cation transport. One of the statistically significant genes, $ERG$ (ETS-related gene), had a single hypermethylated CpG in the MS cohort compared to controls. $ERG$ is a member of the transcription factor family involved in activities such as cell proliferation, differentiation,
| Probe ID | CHR  | Position | Gene  | Feature | Median (case) | Median (control) | Δ meth | p value  |
|---------|------|----------|-------|---------|---------------|------------------|--------|----------|
| cg03431738 | 21   | 40031295 | ERG 5′ UTR | 0.81 | 0.68 | 0.13 | 0.004033 |
| cg12026095 | 19   | 49468461 | FTL TSS200 | 0.30 | 0.49 | -0.18 | 0.004033 |
| cg26228123 | 14   | 73392919 | DCAF4 TSS200 | 0.09 | 0.20 | -0.11 | 0.004033 |
| cg10478035 | 13   | 80919503 | - | - | 0.75 | 0.64 | 0.11 | 0.004033 |
| cg04474988 | 10   | 131770171 | - | - | 0.34 | 0.46 | -0.11 | 0.03549 |
| cg25152348 | 22   | 50946712 | NCAPH2 1st exon | 0.30 | 0.47 | -0.17 | 0.03549 |
| cg08206623 | 11   | 2907334 | CDKN1C TSS1500 | 0.29 | 0.44 | -0.15 | 0.004033 |
| cg13738615 | 9    | 109624741 | ZNF462 TSS1500 | 0.18 | 0.31 | -0.13 | 0.004033 |
| cg10478035 | 13   | 80919503 | - | - | 0.75 | 0.64 | 0.11 | 0.004033 |
| cg04474988 | 10   | 131770171 | - | - | 0.34 | 0.46 | -0.11 | 0.03549 |
| cg25152348 | 22   | 50946712 | NCAPH2 1st exon | 0.30 | 0.47 | -0.17 | 0.03549 |
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apoptosis and inflammation. FTL is a component of ferritin, and defects in this subunit are associated with other neurodegenerative diseases where mutations result in accumulation of iron in the brain [9]. Relapsing–remitting multiple sclerosis (RRMS) patients have increased iron deposits in their grey matter as compared to healthy controls; thus, misregulation of FTL could be important in disease pathology [10, 11]. Mutations in DCAF4 are associated with leucocyte telomere length, and there is evidence that shortened telomere length in

| Table 1 MS-associated CpGs in CD8+ T cells (Continued) |
|----------------------------------------------------------|
| Probe ID on 450K chip | Chromosome | Gene annotated to probe | Differential-methylated score | p value for specified probe in CD8+ T cells |
|------------------------|------------|-------------------------|-----------------------------|------------------------------------------|
| cg12093775             | 13         | 112548065               | -                           | 0.15                                    | 0.26 | −0.11 | 0.000358 |
| cg12787323             | 10         | 119494959               | -                           | 0.16                                    | 0.27 | −0.11 | 0.000433 |
| cg22792862             | 14         | 67827087                | EIF2S1 1st exon             | 0.23                                    | 0.38 | −0.15 | 0.000433 |
| cg08969532             | 10         | 99790438                | CRTAC1 1st exon             | 0.05                                    | 0.15 | −0.10 | 0.000433 |
| cg18185028             | 3          | 154042079               | DHX36 1st exon              | 0.30                                    | 0.41 | −0.11 | 0.000358 |
| cg23059965             | 19         | 50655862                | C19orf41 3′UTR              | 0.81                                    | 0.70 | 0.11  | 0.000433 |
| cg02192678             | 8          | 1495185                 | DLGAP2 5′UTR                | 0.78                                    | 0.68 | 0.11  | 0.000433 |
| cg02976009             | 6          | 32068226                | TNXB 5′UTR                  | 0.71                                    | 0.59 | 0.12  | 0.03549  |
| cg18073471             | 4          | 81119198                | PRDM8 Body                  | 0.18                                    | 0.29 | −0.11 | 0.03549  |
| cg00945810             | 7          | 814391                  | HEATR2 Body                 | 0.67                                    | 0.56 | 0.11  | 0.03549  |
| cg04875614             | 4          | 2008706                 | WHSC2 Body                  | 0.80                                    | 0.69 | 0.10  | 2.34E-05 |
| cg266920627            | 1          | 7319248                 | CAMTA1 Body                 | 0.75                                    | 0.63 | 0.12  | 0.000433 |
| cg26647242             | 2          | 30040525                | ALK Body                    | 0.78                                    | 0.67 | 0.11  | 0.000433 |
| cg04605816             | 20         | 62092443                | KCNQ2 Body                  | 0.83                                    | 0.71 | 0.12  | 0.000433 |
| cg10944063             | 2          | 120233706               | SCTR Body                   | 0.58                                    | 0.46 | 0.12  | 0.000433 |
| cg14595269             | 7          | 151216272               | RHEB Body                   | 0.14                                    | 0.24 | −0.10 | 2.34E-05 |
| cg23720125             | 5          | 177097760               | LOC202181 Body              | 0.85                                    | 0.73 | 0.12  | 0.000433 |
| cg20047661             | 3          | 51976883                | RRP9 TSS1500                | 0.64                                    | 0.52 | 0.11  | 0.000433 |
| cg07925549             | 12         | 52828840                | KRT75 TSS1500               | 0.75                                    | 0.63 | 0.12  | 0.03549  |
| cg06697094             | 17         | 54911185                | DGKE TSS1500                | 0.16                                    | 0.28 | −0.12 | 0.03549  |
| cg18789663             | 1          | 24268591                | PLD5 TSS1500                | 0.09                                    | 0.20 | −0.11 | 0.03549  |
| cg03468541             | 14         | 89029199                | ZC3H14 TSS200               | 0.17                                    | 0.30 | −0.13 | 0.000433 |
| cg13526221             | 8          | 987389                  | -                          | 0.79                                    | 0.69 | 0.11  | 0.000433 |
| cg03313895             | 4          | 24803042                | -                          | 0.05                                    | 0.54 | 0.10  | 0.03549  |
| cg19442593             | 2          | 26252851                | -                          | 0.85                                    | 0.74 | 0.11  | 0.000433 |
| cg04851089             | 6          | 28953923                | -                          | 0.39                                    | 0.54 | −0.15 | 0.000433 |
| cg24520975             | 6          | 31651362                | -                          | 0.86                                    | 0.75 | 0.11  | 0.03549  |
| cg01932076             | 21         | 47394659                | -                          | 0.18                                    | 0.30 | −0.12 | 2.34E-05 |
| cg17555825             | 5          | 76924190                | -                          | 0.16                                    | 0.26 | −0.10 | 0.03549  |
| cg23154781             | 15         | 80634195                | -                          | 0.81                                    | 0.69 | 0.12  | 0.000433 |
| cg00792513             | 6          | 100066698               | -                          | 0.34                                    | 0.47 | −0.14 | 0.03549  |
| cg23708569             | 14         | 106058450               | -                          | 0.63                                    | 0.51 | 0.13  | 2.34E-05 |
| cg09579989             | 12         | 110685438               | -                          | 0.81                                    | 0.71 | 0.10  | 0.03549  |
| cg12077664             | 12         | 125145446               | -                          | 0.78                                    | 0.64 | 0.14  | 0.000358 |
| cg24824082             | 2          | 133030701               | -                          | 0.24                                    | 0.35 | −0.11 | 0.000358 |

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leucocytes is associated with other neurodegenerative diseases, such as Parkinson and Alzheimer's disease [12–14]. In addition, one study found a shortened telomere length in primary progressive MS patients, with no correlation between RRMS and differing telomere length has been established [15].

Interestingly, we did not see a cluster of differentially methylated CpGs within HLA-DRB1 as seen in CD4+ T cells [5]. It is well known that the HLA region is notoriously difficult to investigate with many molecular techniques due to increased genetic variation. To minimise the possibility that our observed methylation profile was due to the probes in this region not meeting QC, we used targeted pyrosequencing on available case and control DNA samples. This assay covered seven of the ten differentially methylated CpGs identified in our previous study, but due to high sequence variability, only five of the seven sites returned data. We calculated the median beta values across the five CpG sites using the K–S test. Results showed that the median methylation level in the cases (median = 3.6) and controls (median = 3.6) was not significantly different ($p = 0.72$). This supports a conclusion that any false positive signals exclusively due to SNP effects would be subsequently identified by genotyping at the key loci. In support of this notion, pyrosequencing of the key HLA-DRB1 locus did not alter our array-based findings. Additionally, we did not observe a signal at the HLA-DRB1 locus in CD8+ T cells but did in CD4+ T cells, providing further support that SNPs are not influencing the findings at this locus.

One important consideration of our study is that the patients were being, or had been, treated with various immunomodulatory therapies at the time of recruitment. In particular, eight patients were being treated with fingolimod, which prevents CD4+ lymphocyte egress from lymphoid tissue. As part of our analysis, we stratified our case–control analysis based on treatment groups in an effort to determine whether overall differential methylation signal may be confounded. None of the patient treatment groups shows a distinct methylation signature, including fingolimod (data not shown), which supports the notion that the small number of treated patients in our cohort is not affecting our results. We do note that this does not necessarily mean that fingolimod is not acting on the methylome, but we can conclude that the small number of patients being treated with fingolimod in our study is not confounding the findings. Future studies will benefit from treatment-naïve patients or will be limiting the study to patients on a particular treatment group.

In this study, we identified 79 CpGs showing minor association with MS. None of these hits was observed in the CD4+ T cells from the same cohort, including the major CD4+ DMR at HLA-DRB1. All genome-wide DNA methylation studies to date have used relatively small sample sizes. This has resulted in identification of large-effect regions only. Large-scale studies are needed to identify minor-effect DMRs. Future studies should also examine the functional consequences of these changes through transcript analysis. Primarily, the results of this study highlight the need to focus on individual cell types when assessing DNA methylation associated with MS susceptibility.

**Ethics statement**

The Hunter New England Health Research Ethics Committee and University of Newcastle Human Ethics committee approved this study (05/04/13.09 and H-505-0607, respectively). MS patients gave written and verbal consent. The Australian Red Cross Blood Service ethics committee approved the use of blood from healthy donors.

**Abbreviations**

DMR: differentially methylated region; DNA: deoxyribonucleic acid; FDR: false discovery rate; GSEA: gene set enrichment assay; GWAS: genome-wide association study; MHC: major histocompatibility complex; MS: multiple sclerosis; QC: quality control; SNP: single nucleotide polymorphism.

**Competing interests**

Dr Lechner-Scott's institution receives non-directed funding as well as honoraria for presentations and membership on advisory boards from Sanofi Aventis, Biogen Idec, Bayer Health Care, Merck Serono, Teva and Novartis Australia.

**Authors' contributions**

VEM performed experiments, was involved in interpretation of the data, wrote the manuscript and revised all versions of the manuscript. MCG contributed to the original study design, performed experiments, and contributed to the first draft of the manuscript. RAL and MCB performed data analysis, interpretation of the data, and critically reviewed the manuscript. KS performed experiments and critically reviewed the manuscript. LT contributed to initial study design and critically reviewed the manuscript. JLS and RJS initiated and designed the original study, they critically reviewed the manuscript and are responsible for the infrastructure in which the study was conducted. JLS supervised all aspects of the study. All authors read and approved the final manuscript.

**Acknowledgements**

This study was supported by the John Hunter Charitable Trust. Rodney Lea, Vicki Maltby and Katherine Sanders are supported by fellowships from Multiple Sclerosis Research Australia. We would like to thank the MS patients and clinical team at the John Hunter Hospital MS clinic who participated in this study and the Australia Red Cross Blood Service for providing healthy control samples. We also acknowledge the Analytical Biomolecular Research Facility at the University of Newcastle for flow cytometry support, EpigenDx for pyrosequencing and the
Australian Genome Research Facility for performing the bisulfite conversions and hybridisations to the Illumina 450K arrays.

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Received: 30 August 2015 Accepted: 29 October 2015

Published online: 05 November 2015

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