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ORIGINAL ARTICLE

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The effect of type 1 diabetes protection and susceptibility associated HLA class II genotypes on DNA methylation in immune cells

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The HLA region, especially HLA class I and II genes, which encode molecules for antigen presentation to T cells, plays a major role in the predisposition to autoimmune disorders. To clarify the mechanisms behind this association, we examined genome-wide DNA methylation by microarrays to cover over 850,000 CpG sites in the $CD4^+$ T cells and $CD19^+$ B cells of healthy subjects homozygous either for *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6, $n = 14$), associated with a strongly decreased T1D risk, *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2, $n = 19$), or *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8, $n = 17$), associated with a moderately increased T1D risk. In total, we discovered 14 differentially methylated CpG probes, of which 10 were located in the HLA region and six in the *HLA-DRB1* locus. The main differences were between the protective genotype DR2-DQ6 and the risk genotypes DR3-DQ2 and DR4-DQ8, where the DR2-DQ6 group was hypomethylated compared to the other groups in all but four of the differentially methylated probes. The differences between the risk genotypes DR3-DQ2 and DR4-DQ8 were small. Our results indicate that HLA variants have few systemic effects on methylation and that their effect on autoimmunity is conveyed directly by HLA molecules, possibly by differences in expression levels or function.

KEYWORDS
autoimmunity, DNA methylation, HLA, type 1 diabetes

1 | INTRODUCTION

Many polygenic autoimmune diseases have a strong association with the HLA region on chromosome 6, especially the HLA class I and II genes, which encode cell surface molecules used for presenting antigens to immune cells. In type 1 diabetes (T1D), HLA class II genes have been estimated to contribute 40–50% of the genetic risk for the disease.[1,2] In particular, three HLA haplotypes, *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2), *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), and *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), play an important role in T1D risk[1,3,4] as well as many other autoimmune diseases, although they can have opposite effects depending on the disease.[5,6]

In type 1 diabetes, the DR2-DQ6 haplotype confers strong protection against the disease, even when combined with a high-risk haplotype.[1,3] This protection has been reported to cover all stages of disease progression[7] and to protect against T1D during the first five decades of life.[8] On the other hand, the haplotype confers a risk of immune-mediated diseases, such as multiple sclerosis (MS)[9] and narcolepsy with cataplexy.[10] The DR3-DQ2 and DR4-DQ8 haplotypes both confer a risk of T1D[1–4] and celiac disease, where the risk is mainly conferred by the DR3-DQ2 haplotype.[11] Importantly, although DR3-DQ2 and DR4-DQ8 are both associated with T1D risk, they are associated with different first-appearing autoantibodies and different ages at diagnosis,[12–14] and are therefore considered to be associated with different disease etiologies in T1D.

The mechanisms behind the strong association between HLA and autoimmunity are unclear, but might involve their role in antigen presentation. The HLA molecules encoded by different alleles have differences in the structure of their peptide-binding grooves, which affects which molecules can be presented to the immune system. In addition, there are differences in the expression of the different HLA variants,[15–20] which affect the activation of T cells and the strength of the immune response. Genetic variation can also convey its effects by affecting DNA methylation, either in nearby regions (cis-effects) or further away (trans-effects).[21–23] HLA alleles could therefore have wider systemic effects on immune cell function.

Here, we analysed genome-wide DNA methylation in $CD4^+$ T cells and $CD19^+$ B cells from healthy subjects who were homozygous either for DR2-DQ6, DR3-DQ2 or DR4-DQ8. Our aim was to discover whether there are differences in DNA methylation, and therefore gene expression, between the genotypes in cells that express HLA class II genes (in our study, $CD19^+$ B cells) or in cells that closely interact with antigen presenting cells (here, $CD4^+$ T cells). If such methylation differences exist, this would help determine whether the effect of the variants is limited only to the HLA region and the coding part of the sequence or whether they have systemic effects unrelated to antigen presentation. This would shed new light on how the effect of HLA variants on autoimmunity is conveyed.

2 | METHODS AND MATERIALS

2.1 | Study subjects

Our study samples were peripheral blood mononuclear cells (PBMCs) from healthy siblings of children with T1D who had not developed islet autoantibodies. The samples were collected in the context of the Finnish Pediatric Diabetes Register (FPDR)[24] as approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa. FPDR, started in 2002, collects samples and data from children under the age of 16 who have developed diabetes (any type) and from their siblings and family members. For FPDR, written informed consent is given by the parents or legal guardians of the children; in addition, children 10 years or older give informed consent.

This study included *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6) homozygotes ($n = 14$) with strongly decreased T1D risk, and *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2) homozygotes ($n = 19$), and *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8) homozygotes ($n = 17$) with increased T1D risk. Due to the rarity of DR2-DQ6 homozygotes in FPDR, all available *DQB1*06:02* homozygote samples from healthy autoantibody negative subjects were included in the study, and then samples of the other genotype groups (DR3-DQ2 and DR4-DQ8) were chosen to match the DR2-DQ6-group based on age and sex. The mean ages (± SD) in the three HLA groups (DR2-DQ6, DR3-DQ2, and DR4-DQ8) were 15.0 (± 8.3), 11.1 (± 5.6), and 11.8 (± 7.9), respectively; their male-to-female ratios were 8/6, 9/10, and 11/6, respectively.

2.2 | HLA genotyping

The HLA class II genotypes of the FPDR samples were analysed for the *HLA-DR/DQ* haplotypes relevant to T1D risk using sequence-specific oligonucleotide probes. The genotyping was done stepwise, starting with defining the *DQB1* alleles, including probes for the *DQB1*02*, **03:02*, **06:02/3*, and **06:03/4* alleles. The analysis was then expanded to the *DQA1* locus for alleles **02:01*, **03*, and **05*. When relevant, a *DRB1* locus was also examined for the selection of *DRB1*04* alleles. This method has been previously described.[25]

2.3 | Autoantibody analysis

The samples were screened for T1D-associated insulin autoantibodies (IAA), glutamic acid decarboxylase 65 (GADA), and islet antigen-2 (IA–2A) with specific radiobinding assays, as previously described.[26]

2.4 | Isolation of $CD4^+$ T cells and $CD19^+$ B cells from PBMC

PBMCs were isolated from lithium heparin blood using Ficoll Paque Plus density gradient centrifugation and then stored in a viable state at −135°C until analysis. Frozen PBMC samples were thawed in a 37°C water bath and washed with prewarmed Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with CTL Anti-Aggregate Wash™ 20× Solution (ImmunoSpot, Cleveland, Ohio, USA). $CD4^+$ T cells and $CD19^+$ B cells were then isolated consecutively from the PBMC samples using the Dynabeads™ CD4 Positive Isolation Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), Dynabeads™ CD19 Pan B (Thermo Fisher Scientific), and DETACHaBEAD™ CD19 Kit (Thermo Fisher Scientific), according to the manufacturer's instructions. After isolation, the cells were lysed with Buffer RLT Plus (Qiagen, Hilden, Germany), supplemented with 1% 2-Mercaptoethanol (Thermo Fisher Scientific), and stored at −80°C.

2.5 | Isolation of genomic DNA from cell subsets

DNA was extracted from the frozen cell lysates with the AllPrep DNA/RNA/miRNA Universal Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After extraction, the DNA concentrations were measured with an Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific) using the Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific).

2.6 | Sample pooling for methylation analysis

For the DNA methylation analysis with the Infinium MethylationEPIC microarray, the individual DNA samples were pooled into 11 samples, with 4–5 samples per pooled sample. The original 50 samples were assigned to pools based on age and sex to ensure that the age and sex distributions were as similar as possible among the pooled samples (Table 1).

For the pooling to be successful, it was important to ensure that equal amounts of DNA from individual samples were pooled. The DNA concentrations were measured using the Invitrogen Qubit 4 Fluorometer (Thermo Fisher Scientific) and Qubit dsDNA BR Assay Kit (Thermo Fisher Scientific). When pooling, the stock samples were first carefully homogenised by pipetting. Then, samples were diluted into 7 ng/μL concentration and, after dilution, DNA concentrations were measured twice with Qubit. If the DNA concentrations were 7 ± 0.5 ng/μL, the samples were pooled, and if not, the concentrations were adjusted and measured again. Similar pooled samples were created for both the $CD4^+$ T cell and the $CD19^+$ B cell samples.

2.7 | DNA methylation analysis with the Infinium MethylationEPIC microarray

DNA methylation was examined in $CD4^+$ T cells and $CD19^+$ B cells using 11 pooled samples per cell subset.

TABLE 1 Pooled samples.

Pool	HLA group	Samples per pool	Age (years)	Sex (M/F)
1	DR2-DQ6	5	13.8 ± 9.3	3/2
2	DR2-DQ6	5	15.3 ± 7.9	3/2
3	DR2-DQ6	4	16.4 ± 9.6	2/2
4	DR3-DQ2	4	10.4 ± 8.0	2/2
5	DR3-DQ2	5	12.1 ± 7.2	2/3
6	DR3-DQ2	5	10.9 ± 4.6	2/3
7	DR3-DQ2	5	11.0 ± 4.6	3/2
8	DR4-DQ8	4	11.5 ± 8.8	2/2
9	DR4-DQ8	4	11.4 ± 7.6	3/1
10	DR4-DQ8	4	10.2 ± 5.5	3/1
11	DR4-DQ8	5	13.7 ± 10.8	3/2

First, the genomic DNA (315 ng) was bisulfite converted using an EZ-96 DNA Methylation Kit (Zymo Research Corporation, Irvine, California, USA) according to the manufacturer's protocol. The genome-wide DNA methylation was analysed using the Infinium MethylationEPIC BeadChip (Illumina, San Diego, California, USA), according to the manufacturer's protocol, and imaged using the iScan System (Illumina, San Diego, California, USA). Quality control (QC) analysis was performed using the Genome Studio v2011.1 software (Illumina, San Diego, California, USA). The bisulfite conversion of the pooled samples, the DNA methylation analysis with Infinium MethylationEPIC BeadChip, and the QC data analysis with Genome Studio v2011.1 were carried out at the Mutation Analysis Facility (MAF) in Karolinska University Hospital (Stockholm, Sweden).

2.8 | Statistical analysis of the microarray results

For additional QC, preprocessing, and downstream analysis of the data, an open-source SeSAMe package[27] (version 1.16.1) was used in R (version 4.2.3). As QC, the signal detection success rates were investigated, and all arrays demonstrated the success rate higher than 98.3%. As recommended for the Infinium MethylationEPIC BeadChip, the preprocessing comprised a quality mask, channel inference, dye bias correction, detection *p*-value call, and background subtraction. CpG probes with missing levels were excluded before the differential methylation tests. $P < 9.42 \times 10^{-8}$ and a more than 10% difference in the β values were considered significant. It is expected that about 3% of CpG probes have more than 90% of statistical power with adequate control of the false-positive rate for 11 total samples.[28]

3 | RESULTS

We used an Infinium MethylationEPIC microarray to examine methylation in over 850,000 CpG sites in the $CD4^+$ T cells and $CD19^+$ B cells of healthy subjects with HLA genotypes associated with either decreased or increased T1D risk. We compared methylation between three groups: DR2-DQ6 homozygotes ($n = 14$), DR3-DQ2 homozygotes ($n = 19$), and DR4-DQ8 homozygotes. The analysis was performed using pooled samples (Table 1). After quality checks, there were 754,174 CpG probes in $CD4^+$ T cells and 755,381 CpG probes in $CD19^+$ B cells considered for methylation analysis.

Since DR2-DQ6 is strongly protective of T1D, whereas both the DR3-DQ2 and DR4-DQ8 genotypes are associated with an increased risk of T1D, we first compared methylation between DR2-DQ6 and a combined group of DR3-DQ2 and DR4-DQ8. We discovered six differentially methylated CpG probes (DMP) that were statistically significant in both cell subsets. In addition, we discovered three $CD4^+$ specific DMPs and one $CD19^+$ specific DMP (Figure 1A and Table 2). Eight of the 10 CpG probes were located in the HLA region close to or within *HLA-DRB1* and *HLA-DRB6*.

The DR3-DQ2 and DR4-DQ8 groups are both T1D risk genotypes, but they are associated with different T1D endotypes and are considered to reflect different disease etiologies. Therefore, we also compared DR2-DQ6 separately to the other genotypes and DR3-DQ2 and DR4-DQ8 to each other. When DR2-DQ6 was compared to DR3-DQ2, eight of the DMPs discovered earlier remained significant, and we discovered three novel DMPs: cg07180897 (*HLA-DQB2*), cg24668570 (*KNDC1*), and cg27576367 (*GADD45A*; Figure 1B and Table 2). Between the DR2-DQ6 and DR4-DQ8 groups, there were six DMPs (Figure 1C and Table 2), which were all statistically significant in the comparison of DR2-DQ6 and DR3-DQ2+DR4-DQ8.

There were only two DMPs between the DR3-DQ2 and DR4-DQ8 groups in the $CD19^+$ B cells and none in the $CD4^+$ T cells. The two DMPs were located in a region containing the *HLA-DQB2* and *HLA-DQB3* loci (Figure 1D and Table 2).

In addition to DMP analysis, differentially methylated region (DMR) -analysis was conducted as well. However, in general the DMP and DMR analyses highlighted the same genes and regions (Tables S1–S7). There were no significant probes in the DMR analysis when the DR3-DQ2 and DR4-DQ8 groups were compared in $CD4^+$ T cells.

In general, the main DNA methylation differences were between the DR2-DQ6 and the combined groups of DR3-DQ2 and DR4-DQ8. The differences were located primarily in the HLA region, especially the *HLA-DRB1* locus. All significant reported CpG probes had at least a 20% difference in methylation between the compared groups; the DR2-DQ6 group was hypomethylated compared to the other genotypes in all but four CpG probes (Table 2 and Figure 2).

4 | DISCUSSION

Due to the importance of HLA genes and their alleles in autoimmune diseases, the effects and differences between HLA alleles have been examined in many ways. However, most research has focused on the structure of HLA molecules, their peptide repertoire, or their cognate

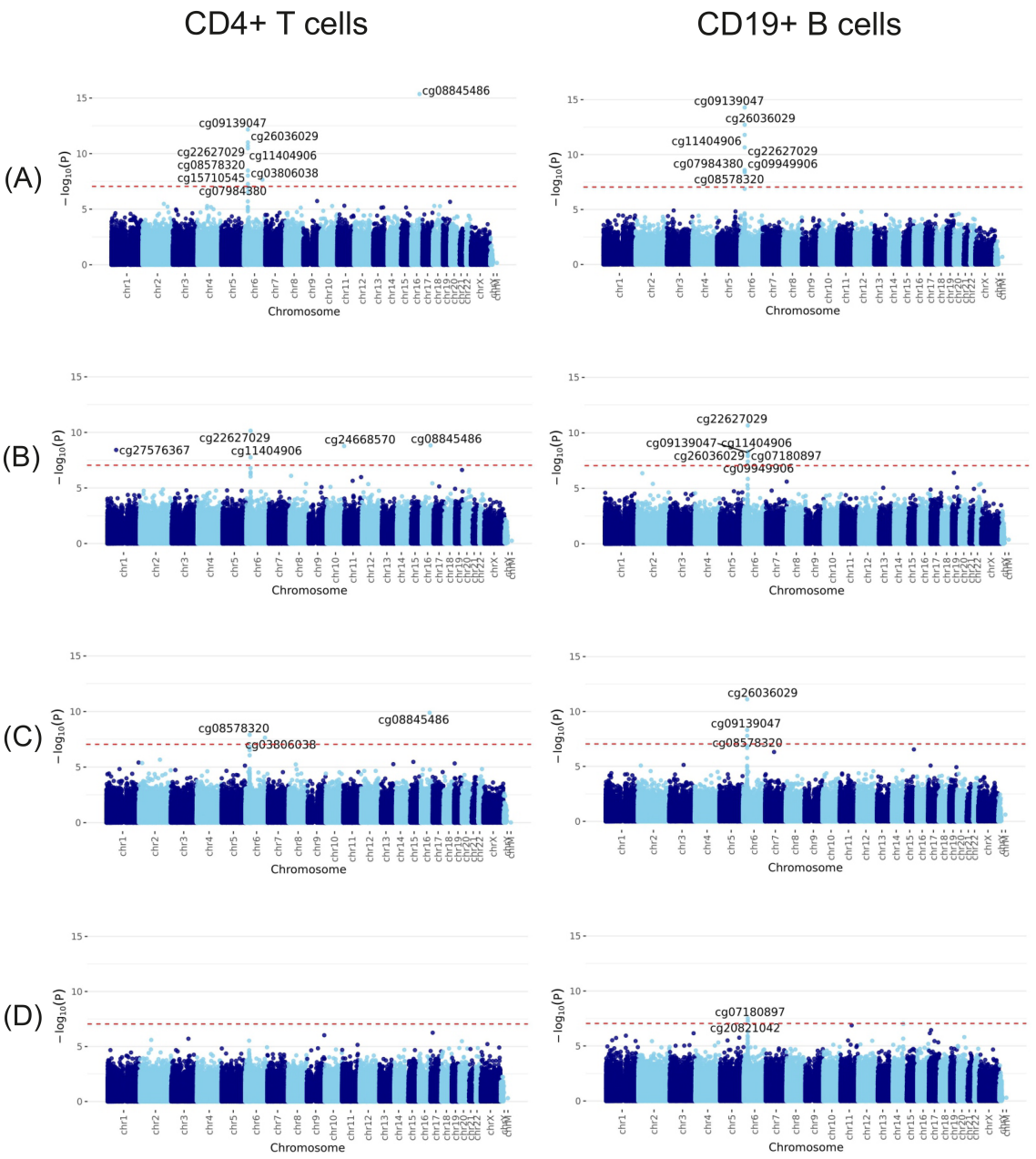

FIGURE 1 Manhattan plots showing differentially methylated CpG probes (DMP) in $CD4^+$ T cells (figures on the left) and $CD19^+$ B cells (figures on the right) between (A) DR2-DQ6 and the combined group of DR3-DQ2 and DR4-DQ8 (9 and 7 DMPs), (B) between the DR2-DQ6 and DR3-DQ2 groups (5 and 6 DMPs), (C) between the DR2-DQ6 and DR4-DQ8 groups (3 and 3 DMPs), and (D) between the DR3-DQ2 and DR4-DQ8 groups (0 and 2 DMPs). The red horizontal line marks the threshold for genome-wide significance ($P < 9.42 \times 10^{-8}$). In all comparisons, the significant differentially methylated CpG probes were located mainly on chromosome 6 and within the HLA region. The Manhattan plots were created with SeSAMe package (version 1.16.1) used in R (version 4.2.3). The final figure was produced with CorelDRAW 2019 (21.2.0.706; Alludo, Ottawa, Canada).

T-cell receptors. Our aim was to examine whether the autoimmunity-associated HLA variants have wider effects in immune cells or if their effect is centred on the HLA region. For this, we analysed genome-wide DNA methylation in $CD4^+$ T cells and $CD19^+$ B cells from healthy subjects and compared CpG methylation between three different T1D-associated genotypes: *DRB1*15-DQA1*01-DQB1*06:02* (DR2-DQ6), associated with a strongly decreased T1D risk, *DRB1*03-DQA1*05-DQB1*02* (DR3-DQ2), and *DRB1*04:01-DQA1*03-DQB1*03:02* (DR4-DQ8), associated with a moderately increased T1D risk.

In total, we discovered 14 differentially methylated CpG probes in all comparisons. Ten of the 14 probes were in the HLA region, indicating that the effects of the HLA

TABLE 2 All statistically significant differentially methylated CpG probes in all comparisons.

Comparison	Cell subset	CpG probe	Difference	*P* value	Gene	Chromosome	Location	Strand	Methylation
DR2-DQ6 vs. DR3-DQ2 + DR4-DQ8	CD4	cg22627029	−0.88	1.95×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32,552,839	−	hypermethylated
	CD19	cg22627029	−0.93	2.19×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32,552,839	−	hypermethylated
	CD4	cg07984380	−0.27	5.44×10^{-8}	*HLA-DRB1*	6	32,579,243	−	hypermethylated
	CD19	cg07984380	−0.21	2.54×10^{-9}	*HLA-DRB1*	6	32,579,243	−	hypermethylated
	CD4	cg11404906	0.76	3.42×10^{-11}	*HLA-DRB1*	6	32,583,973	+	hypomethylated
	CD19	cg11404906	0.72	1.61×10^{-12}	*HLA-DRB1*	6	32,583,973	+	hypomethylated
	CD4	cg08578320	0.82	3.38×10^{-9}	*HLA-DRB1*	6	32,584,263	−	hypomethylated
	CD19	cg08578320	0.81	4.08×10^{-9}	*HLA-DRB1*	6	32,584,263	−	hypomethylated
	CD4	cg09139047	0.88	6.81×10^{-13}	*HLA-DRB1*	6	32,584,266	−	hypomethylated
	CD19	cg09139047	0.90	5.09×10^{-15}	*HLA-DRB1*	6	32,584,266	−	hypomethylated
	CD19	cg09949906	0.85	3.25×10^{-9}	*HLA-DRB1*	6	32,584,574	+	hypomethylated
	CD4	cg26036029	0.24	9.79×10^{-12}	*HLA-DRB1*	6	32,584,667	−	hypomethylated
	CD19	cg26036029	0.37	1.93×10^{-13}	*HLA-DRB1*	6	32,584,667	−	hypomethylated
	CD4	cg15710545	−0.40	9.69×10^{-9}	*HLA-DRB1; HLA-DQA1*	6	32,610,338	−	hypermethylated
	CD4	cg03806038	0.59	2.23×10^{-8}	*TAB2*	6	149,317,906	+	hypomethylated
	CD4	cg08845486	0.92	4.37×10^{-16}	*NOL3; E2F4*	16	67,183,822	+	hypomethylated
DR2-DQ6 vs. DR3-DQ2	CD4	cg27576367	0.89	3.80×10^{-9}	*GADD45A*	1	67,685,889	+	hypomethylated
	CD4	cg22627029	−0.89	7.09×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32,552,839	−	hypermethylated
	CD19	cg22627029	−0.93	2.19×10^{-11}	*RNU1-61P; HLA-DRB6*	6	32,552,839	−	hypermethylated
	CD4	cg11404906	0.76	1.76×10^{-8}	*HLA-DRB1*	6	32,583,973	+	hypomethylated
	CD19	cg11404906	0.73	5.19×10^{-9}	*HLA-DRB1*	6	32,583,973	+	hypomethylated
	CD19	cg09139047	0.89	5.99×10^{-9}	*HLA-DRB1*	6	32,584,266	−	hypomethylated
	CD19	cg09949906	0.88	3.85×10^{-8}	*HLA-DRB1*	6	32,584,574	+	hypomethylated
	CD19	cg26036029	0.37	1.25×10^{-8}	*HLA-DRB1*	6	32,584,667	−	hypomethylated
	CD19	cg07180897	−0.43	1.11×10^{-8}	*HLA-DQB2*	6	32,761,354	−	hypermethylated
	CD4	cg24668570	0.83	1.70×10^{-9}	*KNDC1*	10	133,160,275	−	hypomethylated
	CD4	cg08845486	0.92	1.48×10^{-9}	*NOL3; E2F4*	16	67,183,822	+	hypomethylated

TABLE 2 (Continued)

Comparison	Cell subset	CpG probe	Difference	*P* value	Gene	Chromosome	Location	Strand	Methylation
DR2-DQ6 vs. DR4-DQ8	CD4	cg08578320	0.83	1.26×10^{-8}	*HLA-DRB1*	6	32,584,263	−	hypomethylated
	CD19	cg08578320	0.85	1.64×10^{-8}	*HLA-DRB1*	6	32,584,263	−	hypomethylated
	CD19	cg09139047	0.90	4.81×10^{-9}	*HLA-DRB1*	6	32,584,266	−	hypomethylated
	CD19	cg26036029	0.38	7.78×10^{-12}	*HLA-DRB1*	6	32,584,667	−	hypomethylated
	CD4	cg03806038	0.62	2.28×10^{-8}	*TAB2*	6	149,317,906	+	hypomethylated
	CD4	cg08845486	0.92	1.29×10^{-10}	*NOL3; E2F4*	16	67,183,822	+	hypomethylated
DR3-DQ2 vs. DR4-DQ8	CD19	cg20821042	−0.32	5.46×10^{-8}	*HLA-DQB3; MIR3135B*	6	32,741,382	−	hypermethylated
	CD19	cg07180897	0.41	3.13×10^{-8}	*HLA-DQB2*	6	32,761,354	−	hypomethylated

variants are strongly localised to the HLA region, especially the *HLA-DRB1* locus. In previously published research, genetic variation in the HLA region has also been associated with trans-effects,[29,30] but most of the discovered expression-quantitative loci (eQTL) have been cis-acting. When Houtman et al.[31] compared the transcriptome in $CD4^+$ and $CD8^+$ T cells and $CD14^+$ monocytes between different *DRB1* alleles, they found that the *HLA-DRB* and *HLA-DQ* genes were differentially expressed between the allele carriers but found no expression differences outside the HLA region. Similarly, when Kindt et al.[32] compared genome-wide DNA methylation in cord blood samples between DR3-DQ2 and DR4-DQ8 carriers, they found 196 differentially methylated CpG probes, 181 on chromosome 6, and 41 probes in a region spanning *HLA-DRA* to *HLA-DQB2*. These results, along with ours, seem to indicate that the effect of HLA variants on gene expression is primarily focused on the HLA region.

The effects of HLA alleles specifically on HLA gene expression have been studied more comprehensively. A majority of the DMPs we discovered were between the protective DR2-DQ6 and the combined group of risk genotypes DR3-DQ2 and DR4-DQ8. In both cell subsets, there were seven differentially methylated CpG probes in the *DRB1* or *DRB6* loci or near them. In the *DQA1*[15–20] and *DQB1* loci,[16,20,33] alleles have been shown to be differentially expressed. Differences in promoter methylation between alleles of *DQA1* have also been reported, although no correlation between promoter methylation and allele expression was seen.[19,34] *DRB1* alleles seem to have effects both on *DRB1* expression and on other HLA loci,[31,35–38] and *DRB1* expression also seems to be regulated by DNA methylation.[39–42]

In earlier studies, DNA methylation changes in *HLA-DRB1* region have been associated with autoimmune diseases such as psoriasis,[41] MS disease,[40] systemic lupus erythematosus (SLE)[43] and narcolepsy with cataplexy[44] and, at least in MS disease, this association seems to be mediated by the *DRB1*15:01* variant.[40,42] Although hypomethylation of the region seems to associate with *DRB1* expression,[41,42] otherwise there has been fairly limited research about its functional effects. When Miller et al. discovered SLE associated methylation changes in *HLA-DRB1* in $CD8^+$ T cells, they examined how these cells responded to stimulation.[43] They observed that IFNa stimulation upregulated the *DRB1* expression in $CD8^+$ T cells from SLE patients, but not from control subjects and that these IFNa stimulated $CD8^+$ T cells were then able to activate $CD4^+$ T cells.[43] Miller et al. concluded that $CD8^+$ T cells from SLE patients were epigenetically primed to respond to IFNa stimulation.[43] Already in 2012 Miao et al. reported differences in histone modifications

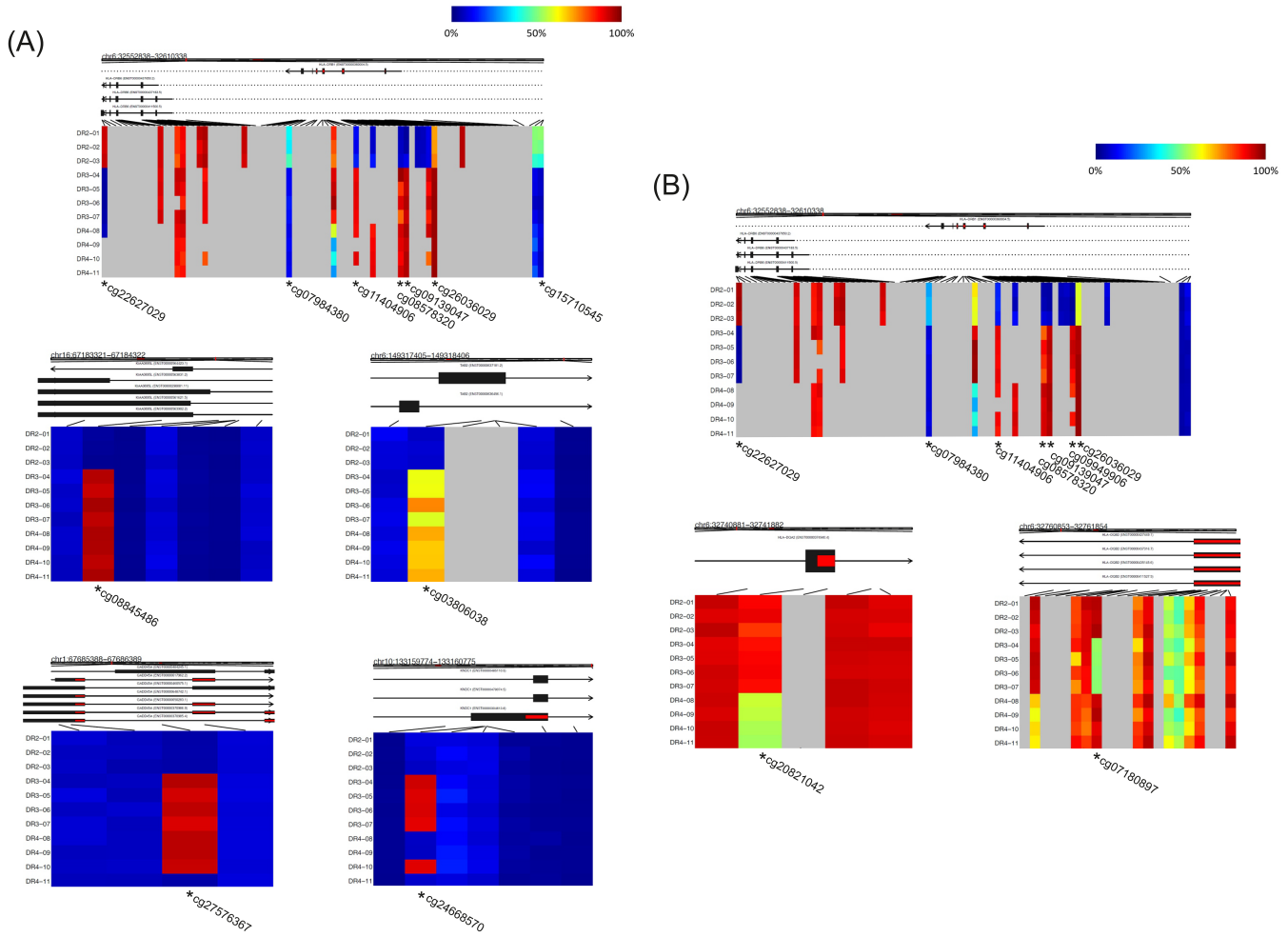

FIGURE 2 Heatmaps showing the DNA methylation levels in the pooled samples in differentially methylated CpG probes in (A) $CD4^+$ T cells and (B) $CD19^+$ B cells. The DNA methylation β values of each CpG probe and each sample pool are represented by a colour scale, where red indicates a high methylation level and blue a low methylation level. Grey indicates that the β value was masked due to low signal intensities. Each row represents one sample pool, with the names of the pooled samples shown on the left. The differentially methylated probes are marked with an asterisk below the heatmap along with the probe IDs. The genomic location of the probes is marked above the heatmaps. The plots were created with SeSAMe package (version 1.16.1) used in R (version 4.2.3). The final figure was produced with CorelDRAW 2019 (21.2.0.706; Alludo, Ottawa, Canada).

in upstream regions of *HLA-DRB1* and *-DQB1* in monocytes extracted from T1D patients and they also reported increased expression of *DRB1* and *DQB1* in response to interferon stimulation in these cells.[45] However, neither Miao et al. or Miller et al. analysed the effect of genetic variation.[43,45]

Interestingly, the effect of genetic variation on gene expression in the HLA region seems to depend on both cell subset and cell state.[46–48] Gutierrez-Arcelus et al.[46] reported 182 sites in the HLA region and 15 in the *HLA-DQB1* locus, where expression changed in an allele-specific manner when the $CD4^+$ T cells were stimulated.

We also compared the DR3-DQ2 and DR4-DQ8 genotypes to each other. Both are T1D risk genotypes, but they seem to be associated with different T1D endotypes associated with differences in relation to the first appearing autoantibodies, age at diagnosis, and environmental factors.[49] Methylation differences between the genotypes could explain the mechanisms behind these associations, but we discovered only two differentially methylated CpG probes in $CD19^+$ B cells and none in $CD4^+$ T cells: cg20821042 situated in the proximal promoter of *HLA-DQB2* and cg07180897 in the *HLA-DQB2* gene body. These two DMPs were originally reported by Kindt et al.[32] We discovered only two DMPs between DR3-DQ2 and DR4-DQ8, whereas Kindt et al. discovered 196 probes, which is most likely due to our smaller sample size.

Kindt et al.[32] observed that the DR3-DQ2 carriers had reduced HLA-DR protein expression on the surface of

$CD14^+$ monocytes extracted from cord blood when compared to DR4-DQ8 carriers.[32] A similar effect was seen in the plasmacytoid dendritic cells (pDCs) extracted from PBMC samples from adolescents. There is also other genetic variation in the HLA region that has been associated with effects on DQ-surface expression.[50–53] Changes in DQ or DR expression could impact immune responses and predispose individuals to autoimmune diseases.[54,55]

Taken together these results indicate that there may be a significant difference in how the immune cells from DR2-DQ6 carriers respond to stimulation, compared to DR3-DQ2 or DR4-DQ8 carriers, and that this difference may be mediated by DNA methylation. Results from Kindt et al. also point to difference in DR expression between DR3-DQ2 and DR4-DQ8 carriers.[32] These differences could lead to variance in immune responses against pathogens or to gut microbiota. In type 1 diabetes virus infections are associated with disease development, especially in DR4-DQ8 carriers.[56] Lately there has also been studies showing that genetic risk to T1D is associated with changes in gut microbiome[57] and that they affect how probiotics colonise the infant gut.[58] It is not surprising therefore, that in type 1 diabetes, HLA haplotypes are associated with autoantibody seroconversion, that is, the beginning of the beta cell specific autoimmunity, rather than the disease progression and clinical diagnosis.[59]

One of the strengths of our study is that the analysis was performed using sorted immune cells instead of heterogeneous samples, such as whole blood or PBMCs. We also examined methylation genome-wide. The Infinium MethylationEPIC Beadchip includes over 850,000 CpG sites throughout the genome, including enhancer regions, promoters, CpG islands, and gene bodies, giving an overview of the entire genome. It is noteworthy that some CpG probes failed in the HLA region; that is, the Illumina probes could not hybridise to their sites. This could be due to the genomic polymorphism of the region; such probes were carefully masked when the results were analysed to avoid erroneous reporting of their methylation levels.[27] Because of this, there might be methylation differences that we were unable to observe. In future studies, sequencing-based approach would be preferable when analysing the HLA region for more comprehensive results. We used sample pooling with the effect that individual methylation levels in the pool could not be measured; instead, we obtained a more stable average estimate of the methylation level in the pooled samples.

In conclusion, the methylation differences now reported were mainly between the DR2-DQ6 genotype and the combined group of DR3-DQ2 and DR4-DQ8 genotypes. The differences were located primarily in the HLA region, and especially the *HLA-DRB1* locus. These could indicate functional differences in HLA gene expression.

AUTHOR CONTRIBUTIONS

Jorma Ilonen, Johanna Lempainen, Sirpa Pahkuri, Shintaro Katayama, and Juha Kere designed the study. Mikael Knip provided the samples and clinical information on the study children. Jorma Ilonen provided the genotype data. Sirpa Pahkuri, Milla Valta, and Lucas Nygård performed sample processing. Shintaro Katayama performed the data analysis. All the authors interpreted the results. Sirpa Pahkuri drafted the manuscript. All authors revised the manuscript and approved the final version.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in ArrayExpress database at https://www.ebi.ac.uk/biostudies/arrayexpress, under the accession number E-MTAB-13654.

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