

Genome-wide DNA methylation profiling in rheumatoid arthritis identifies disease-associated methylation changes that are distinct to individual T- and B-lymphocyte populations

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Abbreviations: RA, rheumatoid arthritis; CpG, cytosine-guanine dinucleotide; Th, T-helper; IL, interleukin; 450K array/array, HumanMethylation450 BeadChip; NIMBL, Numerical Identification of Methylation Biomarker Lists; DAVID, database for annotation, visualization and integrated discovery; LINE-1, long interspersed element-1; MGMT, O-6-methylguanine-DNA methyltransferase; CCS, copper chaperone for superoxide dismutase; DUSP22, dual specificity phosphatase 22; GALNT9, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylglucosaminyltransferase 9

Changes to the DNA methylome have been described in patients with rheumatoid arthritis (RA). In previous work, we reported genome-wide methylation differences in T-lymphocyte and B-lymphocyte populations from healthy individuals. Now, using HumanMethylation450 BeadChips to interrogate genome-wide DNA methylation, we have determined disease-associated methylation changes in blood-derived T- and B-lymphocyte populations from 12 female patients with seropositive established RA, relative to 12 matched healthy individuals. Array data were analyzed using NIMBL software and bisulfite pyrosequencing was used to validate array candidates. Genome-wide DNA methylation, determined by analysis of LINE-1 sequences, revealed higher methylation in B-lymphocytes compared with T-lymphocytes ($P \leq 0.01$), which is consistent with our findings in healthy individuals. Moreover, loci-specific methylation differences that distinguished T-lymphocytes from B-lymphocytes in healthy individuals were also apparent in RA patients. However, disease-associated methylation differences were also identified in RA. In these cases, we identified 509 and 252 CpGs in RA derived T- and B-lymphocytes, respectively, that showed significant changes in methylation compared with their cognate healthy counterparts. Moreover, this included a restricted set of 32 CpGs in T-lymphocytes and 20 CpGs in B-lymphocytes (representing 15 and 10 genes, respectively, and including two, *MGMT* and *CCS*, that were common to both cell types) that displayed more substantial changes in methylation. These changes, apparent as hyper- or hypo-methylation, were independently confirmed by pyrosequencing analysis. Validation by pyrosequencing also revealed additional sites in some candidate genes that also displayed altered methylation in RA. In this first study of genome-wide DNA methylation in individual T- and B-lymphocyte populations in RA patients, we report disease-associated methylation changes that are distinct to each cell type and which support a role for discrete epigenetic regulation in this disease.

Introduction

Rheumatoid arthritis (RA) is a systemic autoimmune disease of largely unknown cause that manifests predominantly as chronic inflammation of the synovial joints, leading to progressive joint destruction and functional impairment. It is widely believed that a complex interplay between genetic factors and the environment is integral to the disease process. Epigenetic mechanisms, including DNA methylation, histone modifications and

expression of microRNAs (miRNAs), impact on gene expression without changing the underlying DNA sequence itself.¹ These changes, frequently acting in concert, are thought to represent the likely intermediaries through which the environment can impact on disease, including autoimmune rheumatic diseases.² Indeed, an expanding literature now describes changes to the epigenetic landscape in patients with RA and related conditions (reviewed in refs. 3, 4).

In RA, distinct leukocyte populations, including T- and B-lymphocytes and their subsets, are key players driving

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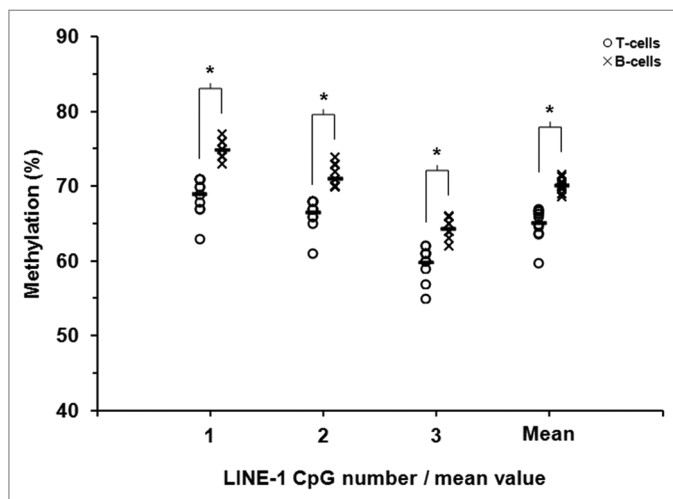


Figure 1. LINE-1 DNA methylation in T-lymphocytes and B-lymphocytes from patients with rheumatoid arthritis. Methylation at three adjacent CpG sites within LINE-1 repetitive sequences was quantified in purified T- and B-lymphocytes from 12 RA patients by sodium bisulfite pyrosequencing. Unfilled circles and crosses represent T-lymphocytes and B-lymphocytes, respectively. The mean levels of methylation for each of the CpG sites are shown by the short black horizontal bars.* $P \leq 0.01$ (Wilcoxon Signed-Rank Test).

autoimmunity and chronic inflammation. The development and differentiation of the different T- and B-lymphocyte subsets/lineages is tightly regulated, and epigenetic mechanisms including DNA methylation appear to play an essential role in this process.^{5,6} Methylation of the DNA base cytosine in the context of promoter-associated cytosine-guanine dinucleotides (CpGs) is frequently associated with transcriptional repression and gene silencing.⁷ In this context, DNA methylation in T-lymphocytes is known to play an essential role in lineage commitment to the T helper 1 (Th1) and Th2 subsets through regulation of interferon- γ and interleukin-4 expression^{8,9} and the expression of the transcription factors STAT4 and STAT6.^{10,11} Similarly, development of regulatory T-lymphocytes (Treg), essential for immunological self-tolerance,¹² is epigenetically controlled, in this case mediated through the methylation status of the locus encoding the FOXP3 transcription factor that drives Treg differentiation.¹³

Several studies have reported altered levels of global DNA methylation in lymphocytes and peripheral blood mononuclear cells from patients with RA.^{14,15} Moreover, candidate gene studies in RA describe DNA methylation changes in these cells for a number of different genes, including interleukin-6 (*IL6*),¹⁶ *IL10*,¹⁷ *EFNB1*,¹⁸ and *CD40LG*.¹⁹ In patients with other rheumatological conditions, including systemic lupus erythematosus and juvenile idiopathic arthritis, evidence from genome-wide studies indicates distinct methylation changes in multiple genes in T-lymphocytes.^{20,21} To date, however, no systematic genome-wide analysis of DNA methylation in individual T- and B-lymphocyte populations has been described in patients with RA. Indeed, to our knowledge, we were the first to recently characterize intrinsic site-specific differences in genome-wide DNA methylation

between matched pairs of T- and B-lymphocytes from healthy individuals.²²

These observations strongly support the need to interrogate DNA methylation in individual lymphocyte populations that drive disease in RA, an approach that will help establish disease-associated and cell type-specific methylation changes in these patients. Following our recent work examining T- and B-lymphocytes from healthy individuals,²² we now describe genome-wide methylation profiles in T-lymphocyte and B-lymphocyte populations isolated from patients with RA.

Results

Technical validation of array data

High-resolution genome-wide arrays (HumanMethylation450 BeadChip, Illumina Inc.; henceforth referred to as 'array' or '450K array') were used to quantify DNA methylation at over 480,000 unique CpG sites across the genome in T- and B-lymphocyte populations collected from a cohort of 12 RA patients and 11 healthy individuals. A technical validation between array β -values and methylation levels as determined by bisulfite pyrosequencing revealed a strong positive correlation (Spearman's $r = 0.889$, $P < 0.00001$; Fig. S1). However, as in our previous study,²² and as reported by others,²³ pyrosequencing analyses generally reported lower methylation levels than the respective array β -values, in particular across the mid-range of methylation β -values (30–70%).

Global DNA methylation in RA derived T- and B-lymphocytes

Our previous report in healthy individuals revealed a subtle yet statistically significant increase in LINE-1 DNA methylation in B-lymphocytes compared with T-lymphocytes.²² We therefore determined, for this surrogate measure of global methylation, the relationship between these two cell types in patients with RA. As in our previous report, examination of three CpG dinucleotides in the LINE-1 sequence revealed a modest but statistically significant increase in mean global methylation in B-lymphocytes relative to patient matched T-lymphocytes (70.2 vs. 65.2%, $P = 0.0001$, Wilcoxon Signed-Rank Test) (Fig. 1). The observed increase was evident for each of the individual sites examined ($P < 0.0005$ in each case). Assessment of global methylation using a more gene-focused approach by inspecting the average β -value across all array CpGs revealed no significant difference between the cell types, as we had noted in healthy individuals ($\beta = 0.566$ and 0.558 for T- and B-lymphocytes, respectively, $P > 0.1$). Global DNA methylation in RA patients therefore appears to closely reflect our observations in healthy individuals.

Cell type-specific DNA methylation in RA derived T- and B-lymphocytes

Through interrogation of DNA methylation in purified T- and B-lymphocyte populations in healthy individuals, we recently identified 679 individual CpG sites that defined a unique methylation profile that distinguishes between and defines these two lymphocyte types.²² Hierarchical clustering across these 679 sites in T- and B-lymphocytes from RA patients revealed an essentially

identical methylation profile, where samples segregated into two distinct clusters based on cell type (Fig. 2; the reader is also directed to our previous publication for comparison²²). No sites additional to those described above showed methylation differences between the cell types that were unique to RA patients. Overall, these data indicate that intrinsic methylation differences that distinguish T-lymphocytes from B-lymphocytes are recapitulated in RA patients.

T- and B-lymphocytes from RA patients show altered DNA methylation for a restricted and distinct set of CpGs

To identify potential disease-associated methylation changes in RA derived T- and B-lymphocytes, we next compared each of the cell types with their cognate healthy cell counterparts. In this case, we used a series of criteria to filter CpGs in each data set, retaining only those meeting each successive step (Fig. 3). Using an approach that we and others have previously taken,^{22,24} we first removed all non-variable CpG sites from the data set (sites for which methylation β -values in all 23 samples were ≤ 0.2 , or ≥ 0.8). We then directly compared DNA methylation in RA derived T- and B-lymphocytes with their cognate (T or B) counterpart sample from healthy individuals. To account for the variation in β -values observed at individual sites within the RA group, we only considered a CpG to be differentially methylated in this group if six or more of the 12 patients demonstrated a methylation β -value that differed from the mean of the healthy group by two or more times (plus or minus) the standard deviation (step 2, Fig. 3). This approach is similar to one we have used previously to identify disease-associated differentially methylated CpGs.²⁵ By retaining only those sites showing methylation changes in the same direction (hypermethylation or hypomethylation in individual RA samples; step 3, Fig. 3), we identified 509 and 252 CpGs displaying disease-associated methylation differences in RA derived T- and B-lymphocytes, respectively. Moreover, this included a restricted set of 32 and 20 CpGs in T- and B-lymphocytes which displayed larger differences in methylation β -value that were at least 0.2 greater/lower than the average observed in their cognate healthy counterparts and that were distinct to either cell type.

Summary data for the 32 and 20 CpGs identified according to the criteria described above as differentially methylated in T- and B-lymphocytes respectively are presented in Table 1 (a

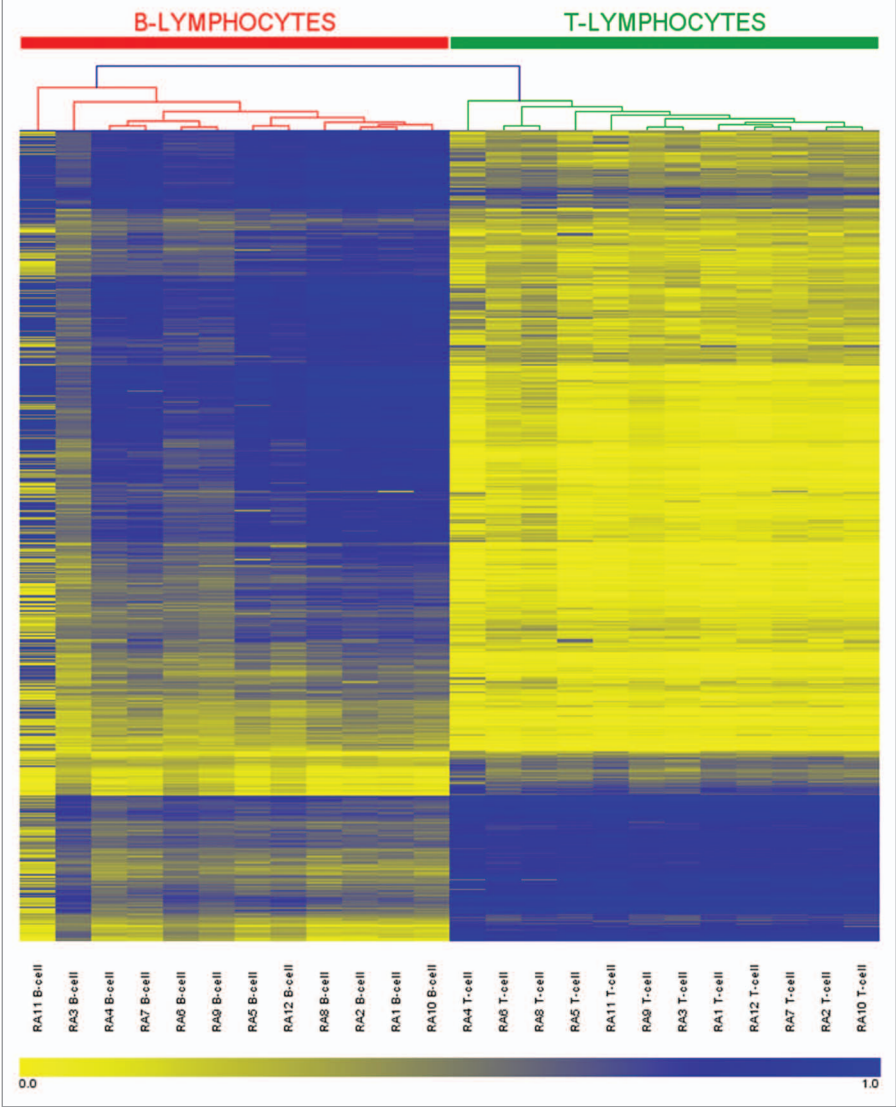


Figure 2. DNA methylation heatmap for the 679 CpG methylation signature in RA derived T- and B-lymphocytes. CpGs presented in the heatmap were described in our previous work (22) and represent those sites which define a unique methylation signature distinguishing healthy T-lymphocytes from B-lymphocytes. Each row represents an individual CpG, ordered according to the clustering output in healthy individuals (22), and each column a different sample (listed beneath the heatmap). Color gradation from yellow to blue represents low to high DNA methylation respectively, with β -values ranging from 0 (no methylation; yellow) to 1 (complete methylation; blue). Intrinsic methylation differences previously observed between the cell types in healthy individuals are accurately preserved in RA patients.

complete list and annotation for the CpGs/genes is provided in Table S1). Of the 32 CpGs identified in T-lymphocytes, approximately equal numbers were hypermethylated as were hypomethylated. Conversely, all 20 CpGs identified in B-lymphocytes were hypomethylated in RA relative to their corresponding healthy counterparts. In both cell types approximately half of the CpGs identified were associated with known genes (18/32 and 10/20, T- and B-lymphocytes respectively). The majority of CpGs identified in T-lymphocytes were located within a CpG island or the surrounding island shores and shelves (19; 59.4%). In contrast, fewer than half (8/20) of the CpGs identified in B-lymphocytes

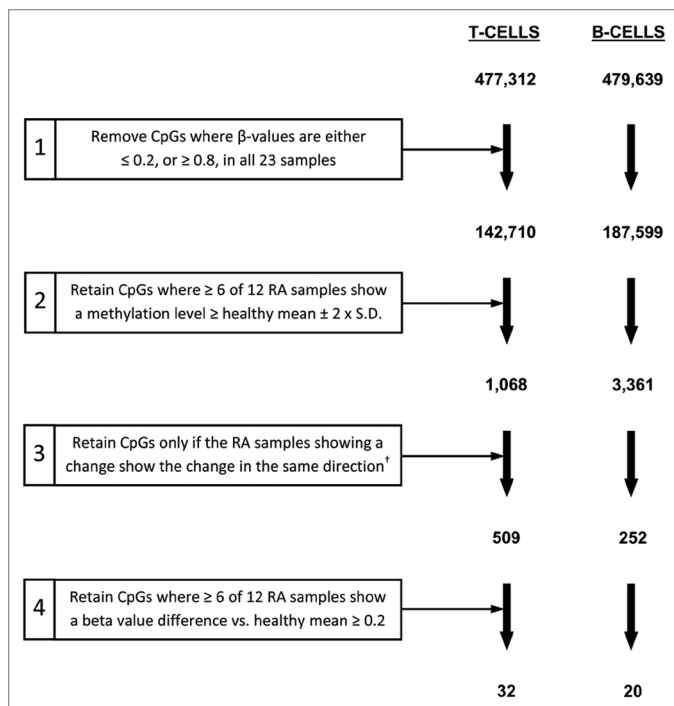


Figure 3. Filtering criteria for identification of CpGs differentially methylated between RA patients and healthy individuals. For T-lymphocytes and B-lymphocytes separately, the starting number of CpGs (477,312 and 479,639 for T- and B-lymphocytes respectively) was derived through the removal of unreliable sites (CpGs with detection p-values > 0.05) and those with missing β -values, as described in the Methods. Numbers indicate the number of CpGs remaining at each step.† Refers to CpGs for which all RA samples showing a change from the healthy mean \pm two-times the SD in the preceding step were either hypermethylated or hypomethylated in RA.

were associated with a CpG island or surrounding regions. Four of the candidate sites identified were common to both cell types, of which two were associated with known genes (*MGMT* [O-6-methylguanine-DNA methyltransferase] and *CCS* [Copper chaperone for superoxide dismutase]).

Hierarchical clustering based on the 32 and 20 candidate CpGs identified in T- and B-lymphocytes respectively was suggestive of patients and healthy controls clustering independently, although perfect segregation of the two groups was not observed (Fig. S2). While this may reflect the absolute numbers used in the analysis, an interesting observation was the identification of a cluster of six RA patients where methylation across the 32 CpG candidates in T-lymphocytes was distinct from the other six patients and healthy controls. However, examination of known clinical characteristics in these patients, including measures of systemic inflammation, disease activity and functional outcome, did not reveal any differences relative to these measures in the other six patients.

Independent validation of candidate CpGs

To confirm the altered methylation of candidate CpGs identified in our genome-wide approach, we selected eight individual sites, four of these from the T-lymphocyte candidates (*ARSB*, *DUSP22*, *GALNT9*, and *MGMT*) and four from the

B-lymphocyte candidates (*ADAMTS17*, *ASB1*, *BARX2* and *MGMT*), for independent validation by bisulfite pyrosequencing. We specifically focused on CpGs that were associated with a gene and/or CpG island and its surrounding region (assay details are provided in Table S2). As presented in Figure 4, the individual RA samples that were identified as differentially methylated for each of the candidate sites by array analysis (filled circles) were confirmed by pyrosequencing. In each case, the same individual RA samples showed methylation changes consistent with those at the array-interrogated site (filled circles in the pyrosequencing data indicate the same individual samples denoted by filled circles in the array data).

Pyrosequencing analysis also revealed that for both *DUSP22* (dual specificity phosphatase 22; two sites) and *GALNT9* (UDP-N-acetyl- α -D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 9; four sites), additional CpGs juxtaposed to the array-identified site also showed methylation changes in RA patients that were consistent with those observed at the array site (hypermethylation for *DUSP22* and hypomethylation for *GALNT9*; Fig. 5). In addition to the validated candidates described, we also sought to examine by pyrosequencing several other array-identified candidate CpGs, but in these cases noted the presence of single nucleotide polymorphisms at the interrogated site. Validation of multiple candidates reinforces the robustness of the array data and our approach to identify sites differentially methylated in T- and B-lymphocytes derived from patients with RA.

Discussion

Changes to the epigenetic landscape, as defined by DNA methylation, histone modifications and the expression of miRNAs, are apparent in multiple human diseases. Indeed these changes encompass a broad spectrum of autoimmune disorders such as RA, a disease in which lymphocytes and other immune cells play an essential role in autoimmunity and chronic inflammation. In RA, investigations of DNA methylation have typically analyzed global methylcytosine levels or have adopted candidate gene approaches to examine synovial joint cells or cells isolated from blood.^{14-19,26,27} More recently, several groups have used genome-wide arrays to examine DNA methylation in RA synovial fibroblasts^{28,29} or the mononuclear cell fraction extracted from whole blood.³⁰ However, and to our knowledge, no array-based studies have examined individual lymphocyte populations. We now report a systematic genome-wide interrogation of DNA methylation in highly purified T- and B-lymphocyte populations from patients with RA. Through comparison with methylation levels determined in their equivalent healthy counterparts we have identified and validated a restricted set of CpGs that show distinct methylation differences in T- and B-lymphocytes from patients with RA.

Our investigation of individual lymphocyte populations in patients with RA is an extension of previous studies in which we specifically sought to define intrinsic methylation differences between highly purified T- and B-lymphocyte populations

Table 1. Summary characteristics for the differentially methylated CpGs identified in RA derived T-lymphocytes and B-lymphocytes.*

	T-lymphocyte CpG candidates	B-lymphocyte CpG candidates	All differentially methylated CpGs
CpGs differentially methylated†	32	20	52
<i>In CpG islands</i>	6 (18.8)	1 (5.0)	7 (13.5)
<i>In CpG island shores</i>	10 (31.3)	2 (10.0)	12 (23.1)
<i>In CpG island shelves</i>	3 (9.4)	5 (25.0)	8 (15.4)
<i>Non-island associated CpGs</i>	13 (40.6)	12 (60.0)	25 (48.1)
Hyper/Hypomethylated CpGs	17/15	0/20	17/35
CpGs associated with a gene‡	18 (56.3)	10 (50.0)	28 (50.0)
<i>In TSS200/TSS1500</i>	7 (38.9)	2 (20.0)	9 (32.1)
<i>In 5' UTR/1st Exon</i>	1 (5.6)	0 (0.0)	1 (3.6)
<i>In Gene body</i>	8 (44.4)	7 (70.0)	15 (53.6)
<i>In 3' UTR</i>	2 (11.1)	1 (10.0)	3 (10.7)
CpGs associated with a promoter	12 (37.5)	2 (10.0)	14 (26.9)
Unique genes represented	15	10	23‡

Unless otherwise indicated, all figures are the number (%); * Differentially methylated CpGs were identified according to the criteria described in the Results section and **Figure 3**; † Annotation describing CpG island, shore and shelf status, and genetic location relative to defined gene regions, is derived from the University of California at Santa Cruz (UCSC) database; ‡ Two genes were common to both the T-lymphocyte and B-lymphocyte candidate sites; Abbreviations: TSS200, 200-bp block upstream of the transcription start site; TSS1500, 1500-bp block upstream of the transcription start site; 5' UTR, 5' untranslated region; 3' UTR, 3' untranslated region.

derived from healthy individuals.²² For those cell populations we described a unique methylation profile that was consistent across individuals and which distinguished the two cell types from each other. This enabled us to investigate DNA methylation in these cells derived from RA patients with a priori knowledge of the differences defining each lymphocyte type. Strikingly, and as illustrated by clustering and heatmap analysis, we found that an almost identical methylation profile to that we initially reported in healthy individuals²² was apparent in purified T- and B-lymphocyte populations from RA patients. However, we did not identify any further changes in methylation between these two cell populations (T-lymphocytes relative to B lymphocytes) that were unique to RA patients i.e. disease-associated. This is supported by our additional finding that methylation within LINE-1 sequences, as a surrogate measure for global methylation, was no different between patients and healthy individuals, for either cell type. The differences observed between the two cell types in RA therefore reflect methylation differences that are intrinsic to the cellular phenotypes (rather than those that are associated with the disease). Similarly, work from other groups has demonstrated that the major blood-derived leukocyte populations show broad-scale differences in methylation in healthy individuals.³¹

T- and B-lymphocytes are known to play an important role in autoimmunity and the chronic inflammation associated with RA, including, for example, the production of inflammatory cytokines and other mediators, reciprocal interactions with immune and synovial cells, and in the case of B-lymphocytes, antigen presentation and release of autoantibodies (reviewed in refs. 32 and 33). Through the analysis of these specific cell populations in RA patients and healthy individuals, the principle finding of our study was the identification and subsequent validation

of methylation differences at multiple individual CpG sites that were disease-associated and that were distinct to either the T- or B-lymphocyte populations relative to their corresponding healthy counterparts (32 and 20 CpGs in each cell type, respectively). These sites represented 15 genes in T-lymphocytes and 10 genes in B-lymphocytes, and included two genes that were common to both cell types (*MGMT* and *CCS*).

Among the CpGs identified as differentially methylated in RA derived T-lymphocytes, six sites within the gene coding for dual specificity phosphatase 22 (*DUSP22*) (four identified by array and a further two by pyrosequencing) were found to be hypermethylated in RA patients. These sites demonstrated increased methylation in the majority of patients and were associated with a CpG island/shore. *DUSP22* is a protein tyrosine phosphatase that, among other actions, has been shown to negatively regulate STAT3 and the IL-6/STAT3-mediated signaling pathway.³⁴ IL-6 is a pivotal cytokine in RA which contributes to autoimmunity, chronic inflammation and joint destruction and the inhibition of which is an effective treatment in this disease.³⁵ The expression and activation of STAT3 in synovial tissues and circulating lymphocytes is also elevated in RA patients.³⁶⁻³⁸ A recent study has also described a STAT3-inducible gene expression signature in T-lymphocytes from patients with RA.³⁹ Interestingly, altered methylation of *DUSP22* has recently been reported in CD4⁺ T-lymphocytes from patients with the autoimmune condition Sjögren's syndrome.⁴⁰ Our data suggest a possible impact of epigenetic alterations in *DUSP22* that may influence its role as a negative regulator of the IL-6/STAT3-mediated signaling pathway. We also identified, through the use of array and pyrosequencing analysis, multiple sites within *GALNT9* that were consistently hypomethylated in T-lymphocytes from RA patients. The protein product of this gene is one of several glycosyltransferases that are

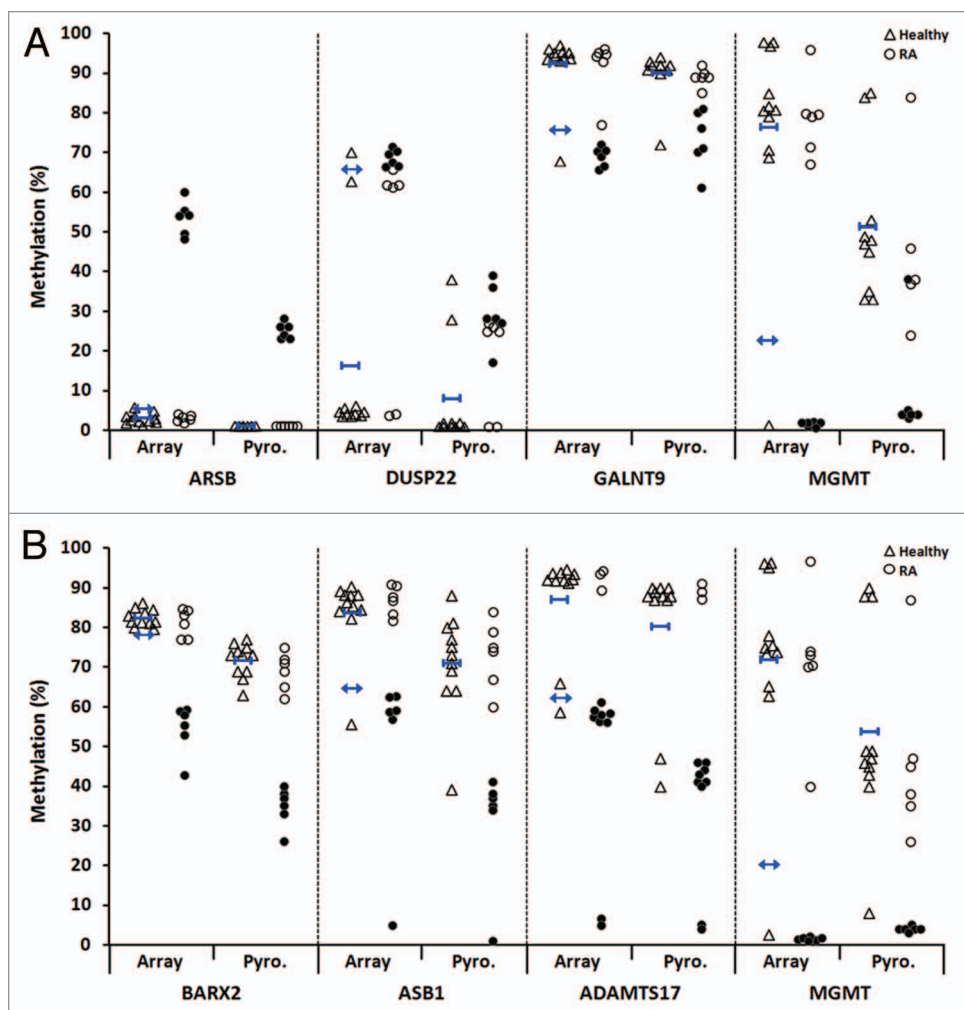


Figure 4. Validation by bisulfite pyrosequencing of differentially methylated CpG candidates in RA derived T- and B-lymphocytes. For each cell type, four candidate CpGs that were differentially methylated between RA patients and healthy individuals were selected. This included two sites each that were hypermethylated (*ARSB* and *DUSP22*) and hypomethylated (*GALNT9* and *MGMT*) in RA derived T-lymphocytes (A), and included four hypomethylated sites in B-lymphocytes (B). In each plot, unfilled triangles indicate healthy individuals and where the mean methylation and two-times the SD of the mean are indicated for the array data by short blue horizontal bars with vertical stops and with arrowheads, respectively. RA patients are indicated by circles, where filled circles in the array data indicate the individual samples that were identified as differentially methylated relative to healthy individuals (as defined by the criteria in Fig. 3). These same samples are also indicated by filled circles in the pyrosequencing data. Abbreviations: Pyro., bisulfite pyrosequencing.

essential in the first step of mucin biosynthesis,⁴¹ large glycosylated proteins that have been implicated in RA pathogenesis.^{42,43} We recognize however, that further investigations of these and other candidates in independent patient cohorts is warranted.

Although a number of different genes have been reported to show methylation changes in RA derived leukocytes,¹⁶⁻¹⁹ to our knowledge we are the first to examine and report DNA methylation changes in RA derived B-lymphocytes. In these cells, all of the differentially methylated CpGs we identified were hypomethylated in six or more of the RA patients and relative to their equivalent healthy counterparts. Interestingly, of the 10 sites that were associated with specific genes, seven were located within the gene body (intragenic), a region in which DNA methylation

shows positive correlation with transcription levels in B-lymphocytes and other cell types.^{44,45} In a similar manner, the majority of gene-associated hypomethylated sites we identified in RA derived T-lymphocytes were also located within the gene body. However, and in contrast to these findings, nearly all of the sites identified as hypermethylated in RA patients (15 of 17; all in T-lymphocytes) were located either within a CpG island – regions of CpG-rich DNA that co-localize with 40–60% of gene promoters^{46,47} – or, more frequently, within the regions surrounding these islands, termed CpG island shores and shelves, which may be important in the context of gene regulation.⁴⁸ Thus, in RA derived T- and B-lymphocytes, hypermethylation was mostly observed at CpG island-associated sites, whereas hypomethylation occurred most frequently at CpGs within gene bodies. It is interesting to note that a similar predominance of differentially methylated CpG loci located within gene bodies was recently observed in joint derived cells from patients with RA.²⁸

A caveat associated with our findings is that they are based on a relatively small group of patients. However, all patients were female Caucasians with seropositive established disease, were all treated with methotrexate and were all non-smokers, which mitigates against the influence of potentially confounding variables, such as race, gender and smoking that have previously been associated with DNA methylation.⁴⁹⁻⁵² Indeed, despite some variation in age, disease duration and the specific treatments received, the methylation changes at candidate CpGs did not appear to be related to these factors. Rather, candidate CpGs showed consistent methylation changes across multiple patient samples suggesting that the observed changes were indeed disease-related. To our knowledge, established relationships between the co-medications patients were receiving and effects on genome-wide DNA methylation have not been described. However, we are mindful that these medications and perhaps other unknown confounders may influence our findings. The findings from our work will require independent verification in larger patient cohorts, and perhaps to be extended to include specific lymphocyte subsets.

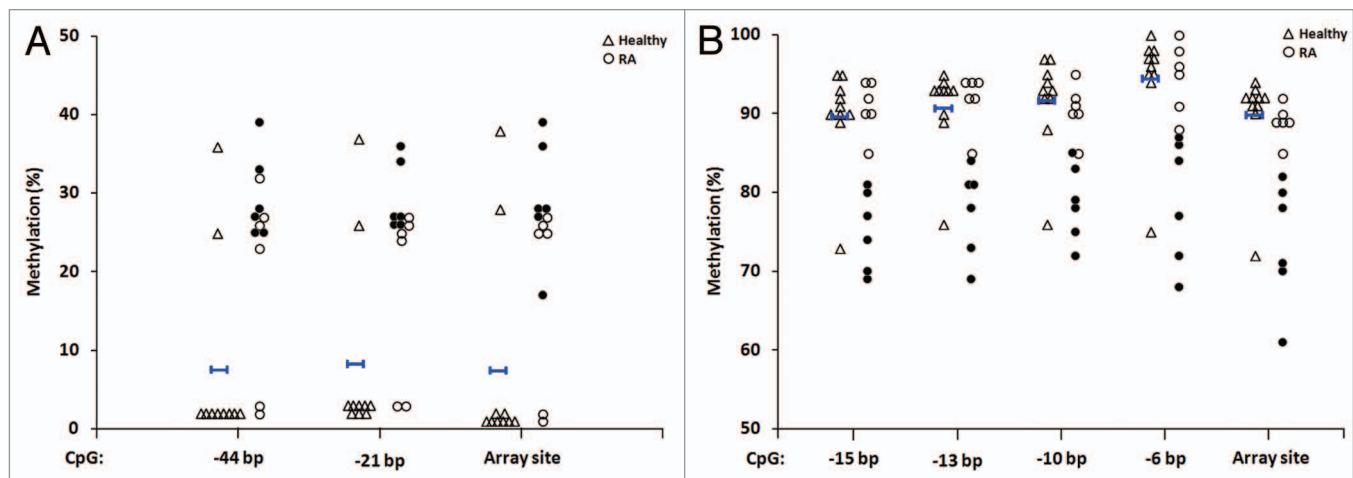


Figure 5. Identification of additional differentially methylated CpGs for two array-identified candidates in RA derived T-lymphocytes. Shown are additional CpGs adjacent to the array-identified candidate site for DUSP22 (two additional sites) (A) and GALNT9 (four additional sites) (B) that were also found to be differentially methylated in RA patients relative to healthy individuals, as determined by pyrosequencing. Negative base pair numbers on the x-axis indicate bases upstream of the array CpG site. In each plot, unfilled triangles indicate healthy individuals and where the mean methylation is indicated by short blue horizontal bars with vertical stops. RA patients are indicated by circles, where filled circles for the array site indicate the individual samples that were identified as differentially methylated relative to healthy individuals (as defined by the criteria in Fig. 3). These same samples are also indicated by filled circles in each of the adjacent CpGs presented. Abbreviations: bp, base pairs.

In summary, and by focusing on individual lymphocyte populations in this and our previous study,²² we have identified intrinsic methylation differences between T- and B-lymphocytes that are consistent in both RA patients and healthy individuals. Moreover, using a genome-wide approach we report in patients with RA evidence of disease-associated aberrations of DNA methylation at multiple CpG loci that are distinct to either the T- or B-lymphocyte populations. These findings will form the basis for further investigations by us and other research groups to further explore the role of DNA methylation and other putative epigenetic changes in the genesis, progression and clinical management of RA.

Patients and Methods

Patients

Peripheral blood samples were collected from 12 Caucasian female patients with seropositive established RA (disease duration ≥ 5 y) and from 12 healthy Caucasian females who served as a control group. All RA patients satisfied the 1987 American College of Rheumatology revised criteria set for the classification of RA³³ and were being treated with methotrexate as monotherapy ($n = 5$) or in combination with sulphasalazine ($n = 6$) or hydroxychloroquine ($n = 1$). Six patients were receiving non-steroidal anti-inflammatory drugs and three were taking analgesics. A number of patients were also receiving medication for co-morbid diseases, including hypertension ($n = 4$), hypercholesterolemia ($n = 2$), type II diabetes ($n = 2$), anemia ($n = 1$) and osteoporosis ($n = 1$). Five patients had no documented co-morbidities and no patients had been diagnosed with cancer at the time of recruitment. The average age and disease duration of patients was 64.6 ± 7.5 y and 15.8 ± 10.7 y (mean \pm SD), respectively. Health status

for the control group was established using a short questionnaire, excluding any individuals reporting inflammatory or musculoskeletal-related conditions. The average age for the healthy control group was 52.6 ± 4.9 y (mean \pm SD). This was significantly lower than the average age of the RA group ($P < 0.01$, Student's *t* test). All subjects were non-smokers. Subjects were recruited at the Haywood Rheumatology Centre in Stoke-on-Trent, UK and written informed consent was obtained in all cases. The study was approved by the East Midlands (Derby) Research Ethics Committee.

Purification of T- and B-lymphocyte populations

Peripheral blood mononuclear cells were isolated from whole blood using density-gradient centrifugation (Histopaque-1077, Sigma-Aldrich), following which CD3⁺ T-lymphocytes and CD19⁺ B-lymphocyte populations were purified by positive selection using anti-CD3 and anti-CD19 magnetic microbeads, respectively, and according to the manufacturer's instructions (MiniMACS[®] Separation System; Miltenyi Biotec). Isolated cell populations were lysed, homogenized (QIASHredder spin columns; Qiagen) and archived prior to DNA extraction. An aliquot of each purified T- and B-lymphocyte population was stained with either an anti-CD3 or anti-CD20 phycoerythrin-labeled IgG monoclonal antibody (Miltenyi Biotec), respectively, for assessment of cell purity by flow cytometry, as we have previously described.²² T-lymphocyte and B-lymphocyte purities were $99.1 \pm 0.1\%$ and $89.5 \pm 11.3\%$ respectively for RA patients, and $99.5 \pm 0.4\%$ and $90.9 \pm 9.5\%$ respectively for the healthy control group (mean \pm SD).

Genome-wide DNA methylation profiling

Genome-wide DNA methylation profiling was performed using Infinium-based HumanMethylation450 BeadChips (Illumina Inc.) that interrogate over 480 000 unique CpG sites across the genome (99% of RefSeq genes).⁵⁴ Genomic DNA was

first extracted from cell lysates using an AllPrep DNA/RNA Mini kit (Qiagen). Subsequently, T- and B-lymphocyte DNA from the 12 RA patients and 12 healthy subjects (48 samples in total) were sodium bisulfite converted and hybridized to arrays according to Illumina recommended protocols (performed by Gen-Probe Life Sciences Ltd) that we have previously described.²² Methylation at individual CpGs is reported as a methylation β -value,⁵⁴ which falls on a continuous linear scale ranging from 0 (unmethylated) to 1 (completely methylated).

Validation by sodium bisulfite pyrosequencing

Validation of array data and independent quantification of methylation at candidate CpG loci was performed by sodium bisulfite pyrosequencing. To increase template quantity for pyrosequencing assays, we first performed whole genome amplification of bisulfite-converted DNA according to conditions we have recently described.²² Thereafter, amplicons containing CpGs of interest were prepared by a touchdown PCR method⁵⁵ using forward, reverse and sequencing primers specific for bisulfite-converted DNA (designed using PyroMark Assay Design software (Qiagen) and purchased from Biomers.net) (assay details are provided in Table S2). Pyrosequencing assays were run on a PyroMark Q24 instrument according to the manufacturer's instructions (Qiagen), and in each case included at least one control dispensation to confirm sequence identity and completeness of bisulfite conversion. Pyrograms were analyzed using PyroMark Q24 software (v 2.0.6., build 20; Qiagen).

Data analysis

Initial processing, probe type correction and assessment of array data was conducted using NIMBL software.⁵⁶ To adjust for the reported difference in sensitivity between the two probe types on the arrays, NIMBL was used to perform 'peak-based correction' according to Dedeurwaerder et al.⁵⁷ All comparative analyses were conducted on peak-based corrected β -values. The Illumina 450K array allows parallel assessment of 12 samples reducing within-array variability. However, the effect of batch (array-to-array) variation on Illumina 450K arrays is still debated and a consensus on an appropriate method to "normalize" data has not

been reached.⁵⁸⁻⁶⁰ In an attempt to best control for batch-effects, array experiments were conducted with block-randomization such that six RA and six healthy samples were present on each of the arrays. Each array passed quality control assessment based on the performance of internal array controls, and the distribution of β -values across all array CpGs was found to be similar in each of the T- and B-lymphocyte samples. Subsequent to array hybridization, it was necessary to exclude one healthy individual who no longer satisfied the clinical exclusion criteria. All analyses were thus conducted with a revised group of 11 healthy subjects. For each of the T- and B-lymphocyte data sets separately, and as we have previously described,²² we excluded all CpGs for which one or more of the 23 samples displayed detection P values > 0.05 (indicating an unreliable site) or presented with missing β -values. In performing this step, we excluded 1.7% and 1.1% of array CpGs from the T-lymphocyte and B-lymphocyte data sets and therefore retained 477,312 and 479,639 CpGs, respectively. Criteria for identification of CpGs displaying altered methylation between RA patients and healthy subjects for each cell type are described in the Results. Hierarchical clustering using Euclidian distance and average linkage criteria was performed using Genesis software (v1.7.6)⁶¹ and statistical analyses were performed using NCSS 2000 (NCSS LCC). P values < 0.05 were considered significant.

Conflict-of-interest and financial disclosure statement

All listed authors (JRG, RDE, NBN, KEH, JCP, PTD, AAF, DLM and WEF) have no conflicts-of-interest to declare, and have no relevant financial relationships to disclose.

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Supplemental Materials

Supplemental materials may be found here:
www.landesbioscience.com/journals/epigenetics/article/29718

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