Epigenetics

ISSN: 1559-2294 (Print) 1559-2308 (Online) Journal homepage: http://www.tandfonline.com/loi/kepi20

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To cite this article: David J. Martino, Meri K. Tulic, Lavinia Gordon, Megan Hodder, Tara R. Richman, Jessica Metcalfe, Susan L. Prescott & Richard Saffery (2011) Evidence for age-related and individual-specific changes in DNA methylation profile of mononuclear cells during early immune development in humans, Epigenetics, 6:9, 1085-1094, DOI: 10.4161/epi.6.9.16401

To link to this article: http://dx.doi.org/10.4161/epi.6.9.16401

RESEARCH PAPER

Epigenetics 6:9, 1085-1094; September 2011; © 2011 Landes Bioscience

Evidence for age-related and individual-specific changes in DNA methylation profile of mononuclear cells during early immune development in humans

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Key words: epigenetics, DNA methylation, T cells, immune development, mononuclear cells, allergic disease, cord blood

Environment induced epigenetic effects on gene expression in early life are likely to play important roles in mediating the risk of several immune-related diseases. In order to investigate this fully, it is essential to first document temporal changes in epigenetic profile in disease-free individuals as a prelude to defining environmentally mediated changes. Mononuclear cells (MC) were collected longitudinally from a small number of females at birth, 1 year, 2.5 years and 5 years of age and examined for changes in genome-scale DNA methylation profiles using the Illumina Infinium HumanMethylation27 BeadChip array platform. MC from two males were included for comparative purposes. Flow cytometry was used to define MC cell populations in each sample in order to exclude this as the major driver of epigenetic change. The data underwent quality control and normalization within the R programming environment. Unsupervised hierarchical clustering of samples clearly delineated neonatal MC from all other ages. A further clear distinction was observed between 1 year and 5 year samples, with 2.5 year samples showing a mixed distribution between the 1 and 5 year groups. Gene ontology of probes significantly variable over the neonatal period revealed methylation changes in genes associated with cell surface receptor and signal transduction events. In the postnatal period, methylation changes were mostly associated with the development of effector immune responses and homeostasis. Unlike all other chromosomes tested, a predominantly genetic effect was identified as controlling maintenance of X-chromosome methylation profile in females, largely refractory to change over time. This data suggests that the primary driver of neonatal epigenome is determined in utero, whilst postnatally, multiple genetic and environmental factors are implicated in the development of MC epigenetic profile, particularly between the ages of 1–5 years, when the highest level of inter individual variation is apparent. This supports a model for differential sensitivity of specific individuals to disruption in the developing epigenome during the first years of life. Further studies are now needed to examine evolving epigenetic variations in specific cell populations in relation to environmental exposures, immune phenotype and subsequent disease susceptibility.

Introduction

The clear association between the recent rise of immune diseases and environmental changes associated with 'westernization' and 'urbanization'[1] demonstrates that immune pathways are remarkably susceptible to modern environmental influences. These environmental changes are driving an increasing predisposition to immune dysregulation characteristic of many allergic and autoimmune diseases.[2] In these cases, particularly allergic disease, immunity in the perinatal period is already altered at birth[3-6] highlighting the importance of developmental immune programming. There is growing evidence that inappropriate immune programming may involve variations in specific epigenetic modifications of genes involved in normal immune development.[7-10] These variations can disrupt the pattern of gene expression in functional immune pathways potentially altering immune-phenotypes.[11]

One of the best-studied of these epigenetic modifications is DNA methylation, a change in the methylation of cytosine-5, specifically within CpG dinucleotides, which can regulate gene transcription levels in a stable and heritable manner.[12] DNA methylation profiling is a growing area of research[13] that holds much potential in the field of immune disease, since these modifications are sensitive to the environment and represent an important mechanistic link between immune development, environmental exposures and risk for immune disease.[10,14-17] Although in its infancy in relation to complex disease research, epigenetics provides a new perspective and likely mediator of the majority

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Submitted: 05/02/11; Accepted: 06/20/11
DOI: 10.4161/epi.6.9.16401

of gene:environment interactions. As the molecular mechanisms that underlie epigenetic modification of immune-phenotypes become clearer, it is important to determine the role of these modifications in normal immune development. Epigenetic changes participate in a broad range of innate and adaptive immune functions (reviewed in ref. 18), and several labs are now beginning to characterize 'epigenetic biomarkers' of immune disease. As a prelude to further studies in this area, a more complete understanding of the degree to which normal changes in the epigenome occur during immune development will improve the success of disease-associated markers, particularly in longitudinal contexts.

Several studies have previously investigated how DNA methylation changes with age in blood and tissue samples.[19-22] These studies suggest the genomic distribution and maintenance of 5-methylcytosine are to some extent, determined by genetics, but remarkably subject to 'drift' associated with age, gender and environmental exposures.[19,20] A recent genome-wide analysis of $CD4^+$ and $CD14^+$ immune cells demonstrated clear age-associated changes in DNA methylation occurred independently of relative proportions of leukocyte cell subsets.[23] These rather interesting findings indicate that DNA methylation profiles vary as precursor cells diverge down respective myeloid and lymphoid lineages. Whilst several immune-specific genes have been reported to undergo age-related change in DNA methylation, no studies have focused on the perinatal period as an important time in immune development, and there is a lack of understanding as to which immune pathways are undergoing active regulation by DNA methylation.

The current study was exploratory in nature, although we were particularly interested in identifying any immune-related genes undergoing differential methylation in early development. We utilized clustering and ontology enrichment to assess the extent to which individual methylation profiles change with age, at the genome-scale in longitudinal samples of peripheral blood mononuclear cells. We focused on purified mononuclear cells as these cells provide a degree of enrichment for cell types directly relevant to immune-related disorders, while remaining amenable to practical considerations associated with cohort-based studies. Our data provides novel insights into the primary immune pathways under epigenetic control over the early perinatal period.

Results

Technical validation of cell typing and infinium data. In order to explore the epigenetic variations in mononuclear cell fractions with age, we characterized the percentages of the major cell types in samples from all individuals at each age using FACS. The dominant cell fraction at all ages was $CD4^+$ T cells which comprised between 25–31% of MC. This did not vary significantly with age (**Fig. 1**). The proportions of $CD8^+$ T cells and B cells were stable in the postnatal period (with no significant changes between 1 and 5 years of age) although proportions were lower at birth. The proportion of CD14 monocytes was also unchanged between 1 and 5 years but higher at birth (**Fig. 1**). These major cell types (shown in **Fig. 1**) comprise almost 80% of all MC, with the remaining 20% likely to be contaminating neutrophils, platelets and (in a smaller minority) $CD3^+CD4^-CD8^-$ gamma delta T cells.

To validate the performance of the Illumina Infinium Methylation assay four loci were selected from the Infinium data based upon clear differences between birth and 5-year samples. Amplicons were designed for MassArray to cover CpG sites targeted by the Infinium probes. Three of these loci were detected as significantly different by Infinium (positive controls) and one was Included as a negative control. **Figure 2** shows these targets validated independently by Sequenom MassArray.

Consistent levels of overall methylation between birth and 5 years. Longitudinal blood samples were available from 7 individuals for this study, with a combined total of 22 independent samples taken from time-points ranging from birth to 5 years that were used for methylation analysis (summarised in **Sup. Table 1**). Of these, 19 were longitudinal samples taken from females, and 3 male samples were included (two collected longitudinally from one individual and a single sample from one unrelated individual) for comparative purposes. DNA samples were randomised across different arrays but were processed in a single batch. All QC metrics indicated the data was of high quality with no outlying samples (methods section). In order to gauge whether transition from birth to 5 years is associated with an overall increase (or decrease) in DNA methylation levels, or a shift in the overall profile of methylation distribution, we plotted the distribution and calculated a median β value for each time point. There was no significant difference in the median methylation levels in any comparisons by Mann Whitney test (**Fig. 3A**). Closer examination of individuals with repeat data indicated clear age-related changes in methylation levels for a subset of probes over this period, that were not apparent when the population was examined as a whole (**Fig. 3B**).

Clustering of individual genome-scale methylation patterns with age. To examine the relationship between samples according to their methylation profiles, unsupervised hierarchical clustering was performed. Using this exploratory approach, two major branches of the clustering dendrogram were identified (**Fig. 4**).

Cord blood mononuclear cells clearly clustered separately from all other age samples, highlighting the stability of DNA methylation profile at birth across unrelated individuals. The second branch of the dendrogram was further separated between the 1 year and 5 year samples, with the 2.5 year samples variably distributed between the two, indicating a gradual shift in age-related methylation profiles from infancy to early childhood. The rate of change in methylation differed between individuals over the birth to five-year period. For some individuals, the methylation levels at 2.5 year were more similar to 1 year than 5 year samples, whereas others showed the opposite relationship (**Fig. 4**).

Chromosome-specific changes. To examine whether these observations were consistent across the entire genome, we examined the pattern of age-related DNA methylation on each chromosome separately, including the X chromosome. Using the same clustering procedure as above, it was clear the age effect predominated for all autosomal chromosomes, consistent with our observations in the genome as a whole. **Figure 5A** shows

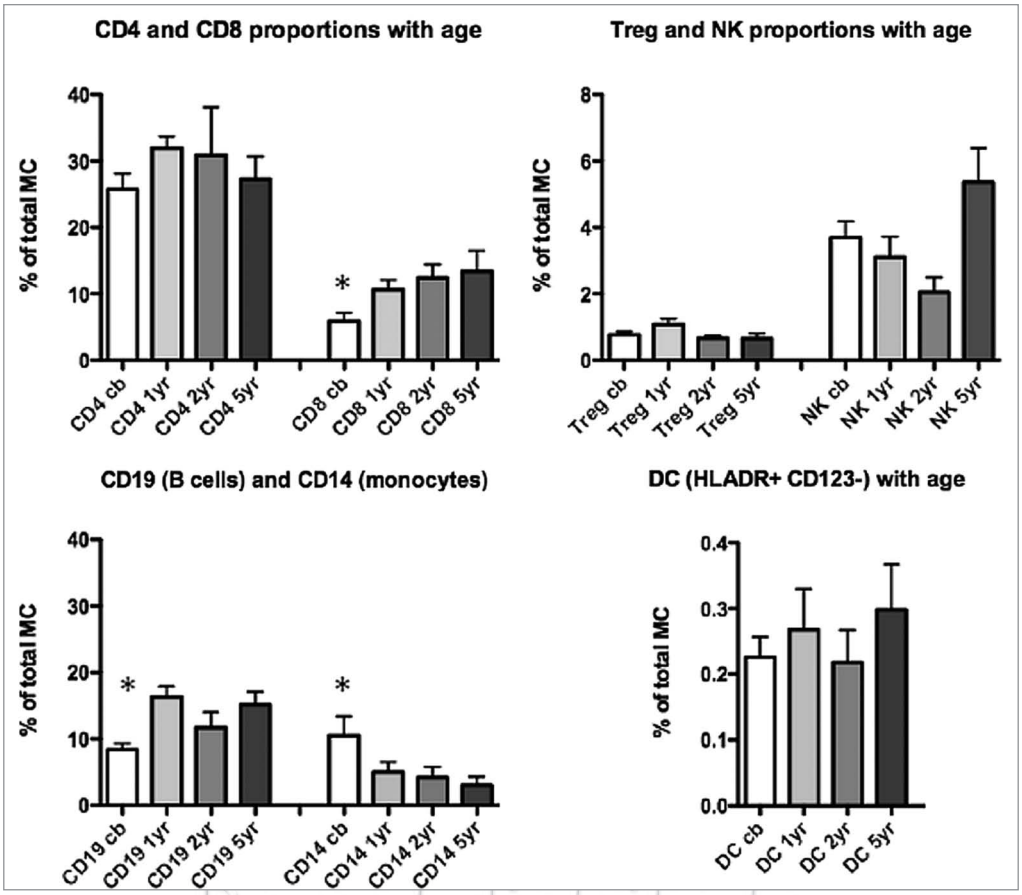

Figure 1. Relative cell subpopulations with age in a mononuclear cell fraction. Mononuclear cells from each individual were stained for flow cytometry. Data is presented as a percentage of parent population with standard error. *p value < 0.05 by unpaired t-test.

clustering according to the probes on chromosome 12 which was chosen as an example due to the high density of probes (1,529 probes) on this chromosome.

Clustering on the X chromosome probes revealed three interesting observations: firstly, probes on the X chromosome clustered independently of age; secondly, as expected the male samples clustered independently to females resulting from the presence of X-inactivation specificity in females; and finally, female samples from the same individuals invariably clustered together, rather than with similarly aged samples, possibly reflecting individual-specific patterns of methylation associated with dosage compensation (**Fig. 5B**). This stark contrast between the X chromosome and autosomes, highlights the role of in utero genetic or other factors in determining the methylation profile of the X chromosome, that once established, remains largely refractory to environmentally induced, or age-associated change in the first 5 years of life.

Immune pathways showing significant changes in methylation between birth and 12-months. The period of development shortly after birth is a critical time during which the immune phenotype of the newborn is particularly influenced by signals from the environment. In order to identify the major immune pathways undergoing epigenetic regulation during this period, a moderated t-test[29] was employed to identify probes on the array that change significantly between birth and 12-months. Methylation profiles for all autosomal probes were compared in the relevant samples (n = 6 birth, n = 5 12-month), and a total of 1,030 probe sets (992 genes) were identified as significantly different according to the pragmatic cut-off criteria of FDR-adjusted p value < 0.1,[23] (**Sup. Table 4**). Examination of the log fold change values for this comparison indicated approximately 80% of probes (n = 824) showed a gain in methylation between these ages. Ontology enrichment was performed on the list of significant probes to identify biological themes within the gene list (**Sup. Table 2**). The enrichment analysis indicates the following biological processes undergo a change in epigenetic regulation during the perinatal period: cell surface receptor signalling, cell adhesion, cell communication, protein tyrosine kinase signalling by transmembrane receptors, NFκB/IκB regulation as well as MAPK signalling and regulation of the ERK1 and ERK2 cascades (**Sup. Table 2**). The major molecular functions undergoing epigenetic change were restricted to receptor binding, calcium ion binding and neuropeptide hormonal activity. The ontology analysis indicates the majority of these processes were intrinsic to the plasma membrane. Together the data suggest that the transition away from foetal immunity shortly after birth is accompanied by significant epigenetic changes in cell signalling and communication pathways.

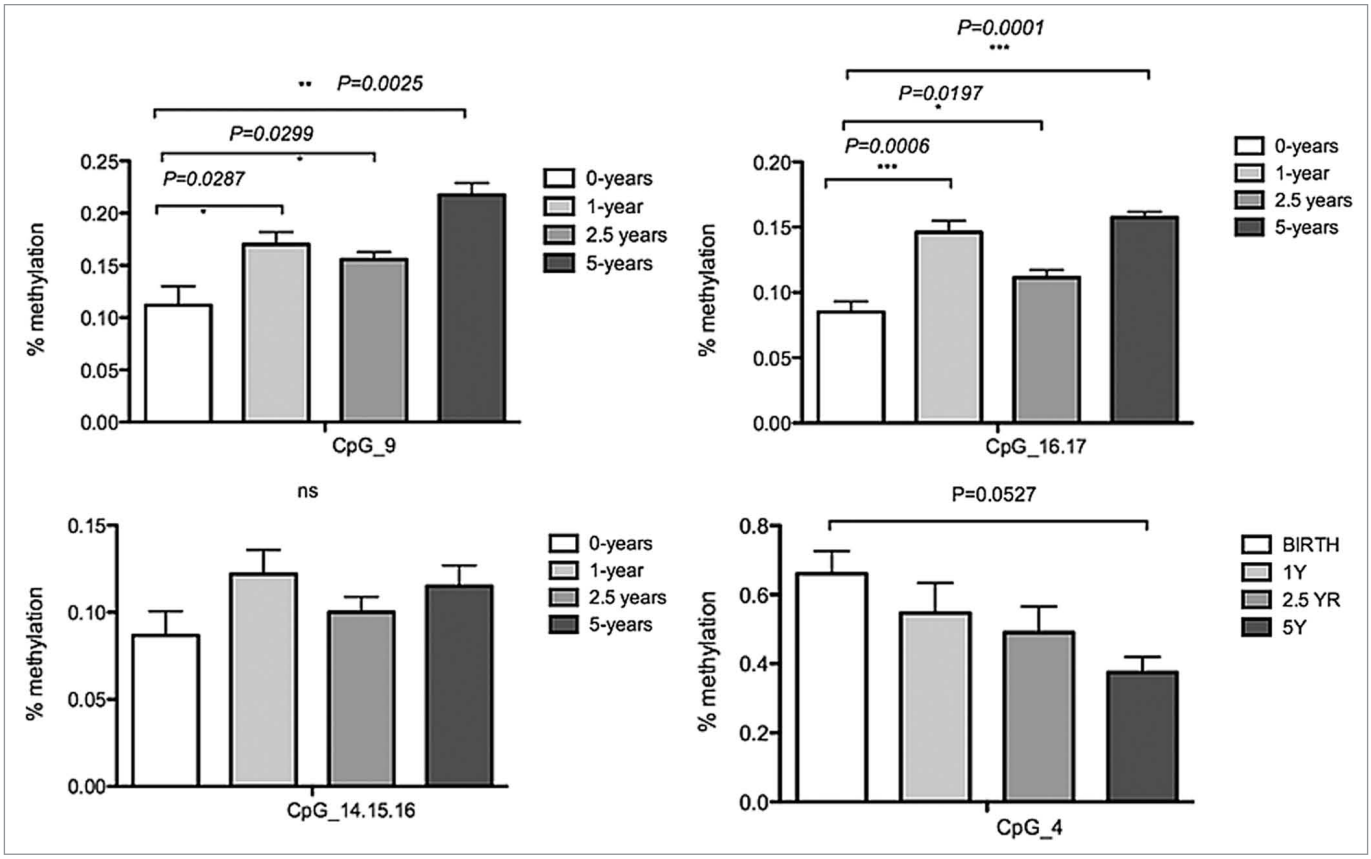

Figure 2. Validation of Infinium genome-wide methylation assay. Four CpG loci were selected based on change from birth to five years and these differences were tested independently MassArray. Data is presented as means with standard deviations and comparisons were made by unpaired t-test.

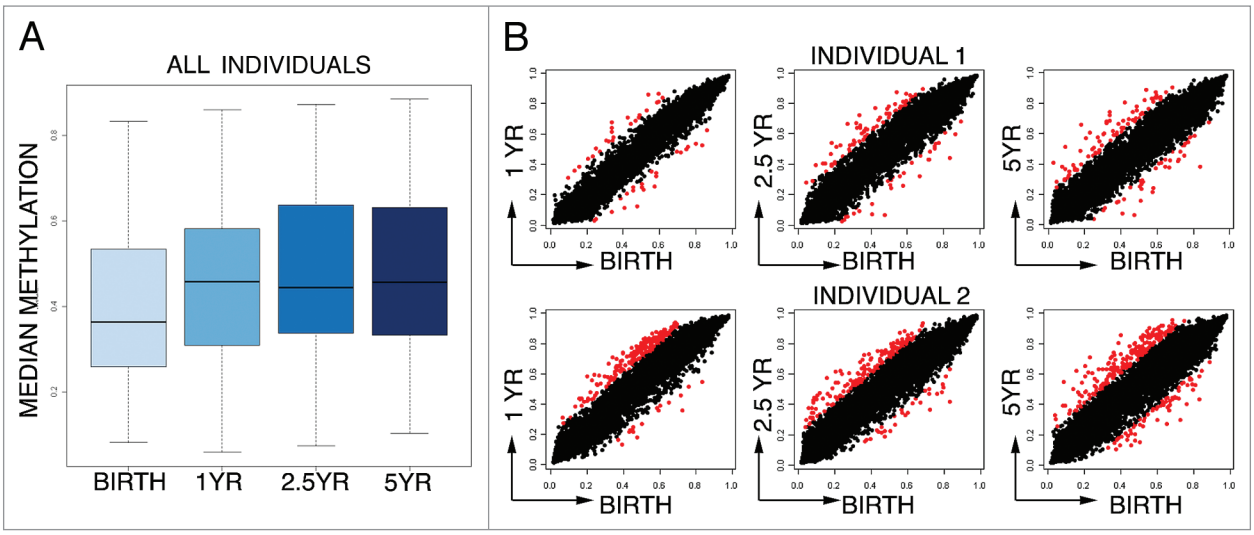

Figure 3. Global versus individual-specific change in methylation with age. Despite a trend for increasing median methylation levels for all Infinium probes, there was no statistical evidence of an age-dependent effect in the entire cohort (A). In contrast, substantial individual-specific differences were apparent for a subset of probes (B). Probes with a beta-value differences of 0.2 are shown in red on the scatterplots. This cut-off has been shown in previous studies to represent a biologically meaningful change.[42]

Immune pathways showing significant changes in methylation during postnatal life. During early childhood immune-phenotypes consolidate with age with respect to patterns of immune responses to allergens and infectious antigens. In order to identify methylation events associated with this, the moderated t-test was employed to identify probes significantly

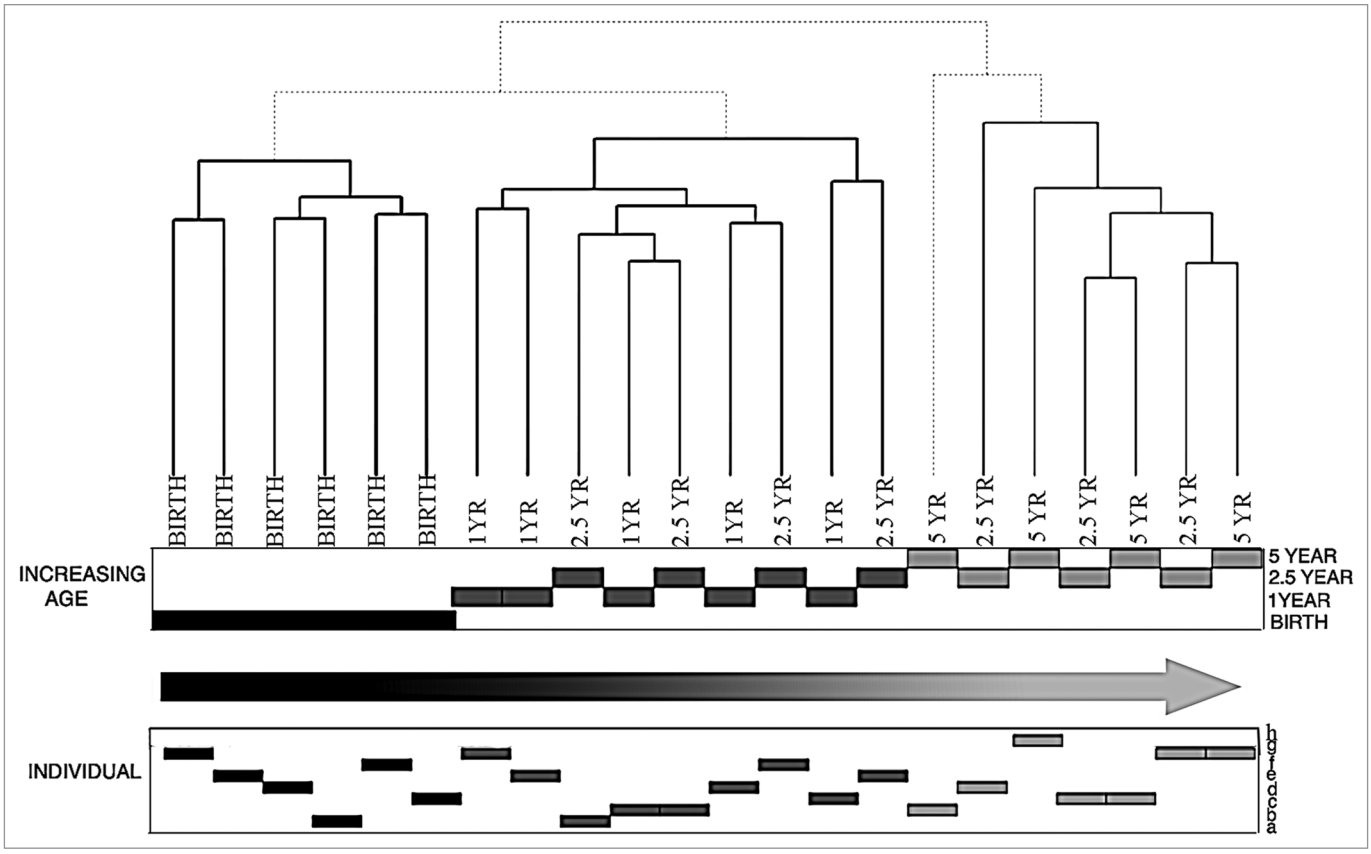

Figure 4. Clustering of different aged samples by autosomal probes. Unsupervised hierarchical clustering was employed to visualize relationships between samples according to methylation profiles.

changing from 12-months (n = 5) to 5 years of age (n = 4). Using the same criteria as before (adjusted p-Val < 0.1), 231 probes (231 genes) were significantly modulated during postnatal development (**Sup. Table 4**). Approximately 74.5% of probes (n = 172) showed a gain in methylation between these ages. A comparison of the neonatal and postnatal gene lists revealed that 40 genes were identified in both comparisons. Ontology enrichment of this list of 231 significant genes indicates the following biological processes are developmentally regulated from infancy through to early childhood: inflammatory responses, humoral immunity including complement and immunoglobulin development, somatic recombination of immune receptors, patterning of effector immune responses, cell to cell signalling and cellular homeostasis (**Sup. Table 3**). Interestingly, we also observed enrichment for genes involved in heart development. The top enriched molecular functions included cytokine activity, chemokine receptor activity and structural constitution of muscle. These processes were localized to the extracellular compartments. Collectively the data provides unique insights into the methylation changes surrounding the patterning of effector immune responses during early childhood.

Geneset enrichment analysis. In order to validate the gene ontology and formally test the hypothesis that immune pathways are subject to temporal variations in DNA methylation, a gene sets test was employed. Cord blood samples (n = 6) and five-year samples (n = 5) were combined in a two-class unpaired comparison of canonical pathways. Immune-related pathways identified as differentially methylated included the complement and coagulation cascade, JAK-STAT signalling pathway, the high affinity IgE recetor pathway, immune signalling pathway and cell-cell adhesion, providing a good level of agreement with the gene ontology. A complete list of the pathways identified is provided in **Supplemental Table 5**, and visual representation of the pathways are provided in **Supplemental Figure 1**.

Validation of candidate genes. Next we validated the observations from the Infinium data using an independent technology. A small number of genes showing statistically significant coordinated change in methylation between birth and five years, with biological functions relevant to immunity were selected for validation by MassArray. Amplicons were designed to the promoter regions of three candidate genes *CALCA, DNTT* and *CHRNE*. Several replicate measurements were performed at each age and between groups comparisons were made using the unpaired t-test. Data QC was performed in the Epityper software. Any CpGs with more than 70% of data missing across all cases were excluded from the analysis, as were individual cases with more than 40% missing data. No individual CpG units containing primer sequences were detected. The results are presented in **Table 1**. All amplicon sequences and alignments are provided as **Supplemental Material**.

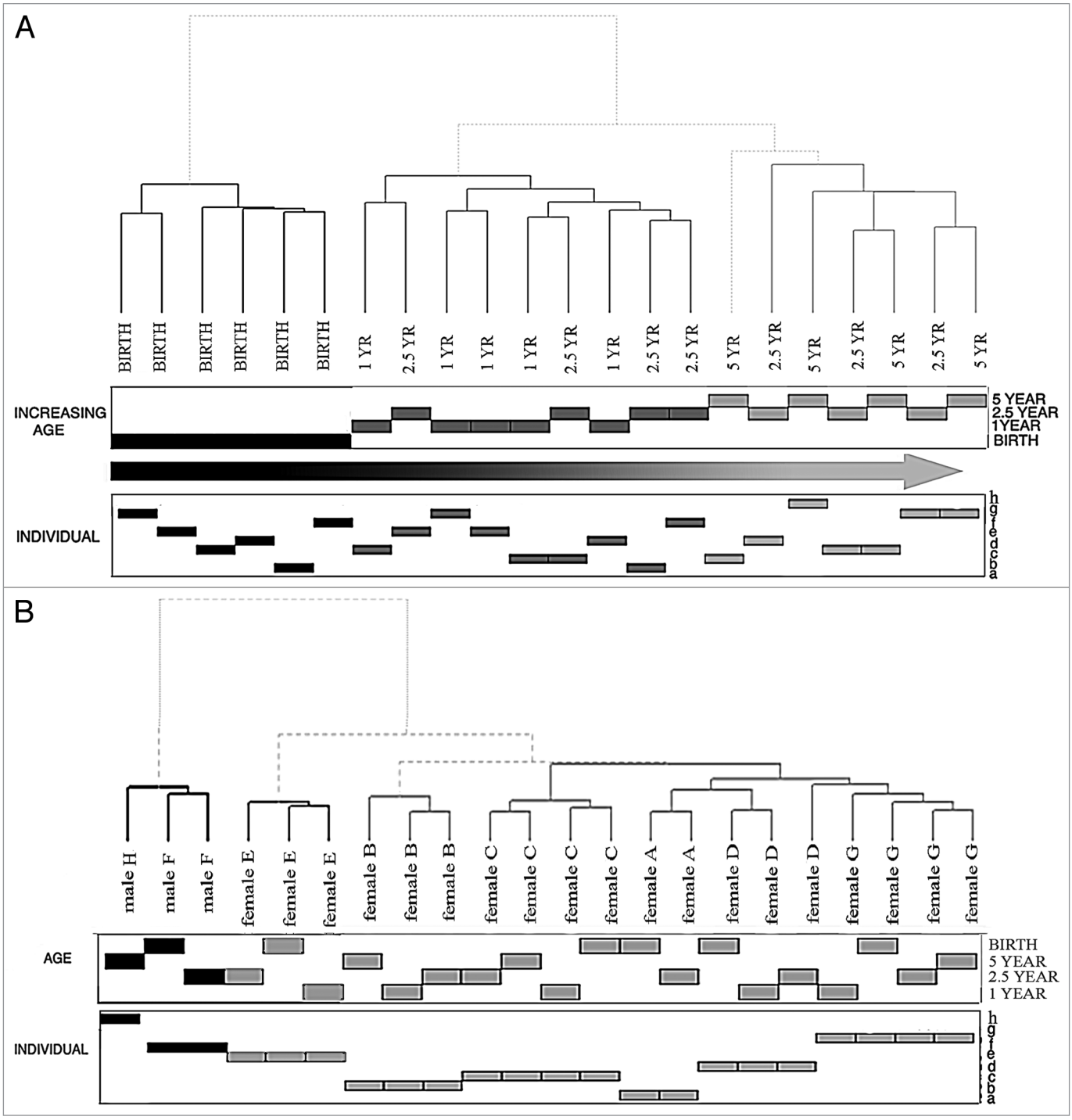

Figure 5. Clustering of samples on specific chromosomes and X chromosome. The age-dependent change in methylation was found to be consistent across all autosomal chromosomes. (A) shows clustering by probes on chromosome 12. (B) shows clustering on the X chromosome resulted in individual samples grouping together. Male controls are shaded.

We analysed each CpG site separately, and considered them together. Of the 6 positive control CpG units tested for *CALCA*, 4 contained individual CG dinucleotides, and the remaining 2 units represented averages of multiple adjacent CpGs. Of the 6 measurements, a between four and five of these units were significantly different at all ages (**Table 1**). As a cumulative score, we found an age-dependent increase in the total methylation at these CpGs from birth to five years (**Fig. 6A**), consistent with the microarray data.

A total of 5 units were analysed in the negative control amplicon to determine the rate of false-negatives. Of these, 3 contained individual CG dinucleotides and the remaining two units

Table 1. Sequenom mass array validation

		Birth			1-year				2.5-years				5-years			
		% Methylation	SEM	N	% Methylation	SEM	N	p value (CB vs 1yr)	% Methylation	SEM	N	p value (CB vs 2.5yr)	% Methylation	SEM	N	p value (CB vs 5yr)
Amplicon 1 *positive controls*	Calca1_CpG_1.2	24.17%	0.05	6	33.80%	0.05	5	***0.0137***	34.00%	0.03	7	***0.0011***	39.75%	0.02	4	***0.0005***
	Calca1_CpG_8	8.50%	0.02	6	16.00%	0.04	5	***0.0024***	14.00%	0.02	7	***0.003***	21.00%	0.02	4	***<0.001***
	Calca1_CpG_9	11.20%	0.04	5	17.00%	0.03	5	***0.0287***	15.57%	0.02	7	***0.0299***	21.75%	0.02	4	***0.0025***
	Calca1_CpG_10	14.67%	0.05	6	17.40%	0.03	5	0.2987	17.00%	0.03	7	0.3069	17.75%	0.01	4	0.2273
	Calca1_CpG_13	9.67%	0.03	6	16.20%	0.05	5	***0.0178***	11.86%	0.02	7	0.1344	17.00%	0.02	4	***0.0031***
	Calca1_CpG_16.17	8.50%	0.02	6	14.60%	0.02	5	***0.0006***	11.14%	0.02	7	***0.0197***	15.75%	0.01	4	***0.0001***
	Amplicon Average	12.78%			19.17%			0.1279	17.26%			0.317	22.17%			***0.0586***
Amplicon 2 *negative controls*	Calca3_CpG_1	4.17%	0.04	6	6.40%	0.03	5	0.3607	4.14%	0.03	7	0.9912	7.25%	0.02	4	0.225
	Calca3_CpG_2	20.33%	0.07	6	25.20%	0.07	5	0.2871	22.43%	0.05	7	0.5343	26.75%	0.06	4	0.1659
	Calca3_CpG_4.5.6.7.8	8.33%	0.04	6	12.60%	0.04	5	0.119	10.14%	0.04	7	0.4115	10.50%	0.02	4	0.3664
	Calca3_CpG_14.15	8.67%	0.03	6	12.20%	0.03	5	0.111	10.00%	0.02	7	0.4281	11.50%	0.02	4	0.1931
	Calca3_CpG_18	25.33%	0.06	6	34.20%	0.09	5	0.0693	30.29%	0.06	7	0.1614	35.00%	0.06	4	***0.0321***
	Amplicon Average	13.37%			18.12%			0.08074	15.40%			0.6424	18.20%			***0.3958***
Amplicon 3	DNTT.2_CpG_1.2	76.83%	0.15	6	63.60%	0.10	5	0.1373	62.71%	0.15	7	0.1272	58.25%	0.09	4	***0.0061***
	DNTT.2_CpG_3.4	79.50%	0.16	6	65.40%	0.14	5	0.1561	65.43%	0.14	7	0.1156	61.50%	0.08	4	***0.00707***
	DNTT.2_CpG_9.10	68.33%	0.11	6	59.00%	0.09	5	0.1604	61.71%	0.14	7	0.3672	58.75%	0.09	4	0.1779
Amplicon 4	DNTT.1_CpG_4.5	70.17%	0.06	6	62.80%	0.03	5	***0.0425***	71.60%	0.05	5	0.7014	64.50%	0.05	4	0.1798
	Amplicon Average	73.71%			62.70%			***0.0102***	65.36%			0.0527	60.75%			***0.0052***
Amplicon 5	Chrne.1_CpG_2	73.80%	0.16	5	68.33%	0.13	3	0.6381	60.75%	0.19	4	0.2971	58.50%	0.11	2	0.278
	Chrne.1_CpG_4	66.00%	0.15	5	54.67%	0.15	3	0.3359*	49.00%	0.15	4	0.1328	37.50%	0.06	2	***0.0527***
	Chrne.1_CpG_5	26.00%	0.15	5	17.00%	0.12	3	0.4256	12.75%	0.12	4	0.2044	9.50%	0.06	2	0.2193
	Amplicon Average	55.27%			46.67%			0.7074	40.83%			0.5238	35.17%			0.3826

represented averages of multiple adjacent CpGs. We found methylation at only 1 of these CpG units to be significantly different in the birth vs. five-year comparison only.

Two amplicons were designed for *DNTT* and together these covered 4 units containing 8 CpGs. Methylation at two units were significantly different between birth and five years, and one unit was different from birth to 12-months. We observed an age-dependent decrease in *DNTT* methylation across these CpGS from birth to five years (**Fig. 6B**).

Three individual CpG units were analysed for CHRNE but none were significantly different for any of the comparisons. There was a trend for decreasing methylation in all individual from birth to five years (**Fig. 6C**) paralleling the direction of change noted in the array analysis.

Discussion

This study characterizes the dynamics of DNA methylation during a key period of immune development. The major observations from this study support a role for environment and individual-specific factors in driving epigenetic changes in immune pathways over this period. The FACS analysis argues that these changes cannot be entirely attributed to changes in the major MC subtypes per unit volume of blood, but reflect changes in cellular pathways during immune maturation. This is with the exception of cord blood samples, which showed differences in the percentage of CD14 positive cells, $CD8^+$ and $CD19^+$ cells. The extent to which this would account differences in overall DNA methylation patterns is unknown; however we speculate the effect would be marginal considering these cell types comprise a maximum 15–20% of a mononuclear cell fraction.

Consistent with other published observations[20,21,33] we found the distribution of methylation marks in the autosomal genome to vary with age, however, our study is unique in that it focuses on early life, and therefore the dynamic changes seen here reflect pathways undergoing maturation, rather than methylation events associated with ageing and cellular senescence. However there are likely to be common pathways and it is notable that we did detect an overlap of at least 20 genes in our analyses, with age-related genes identified in a recent publication using a different platform,[34] and these appear to be common to earlier studies in reference 23 and 33, including MYOD1, ACRV1, SOD3 and CARD family members.

The methylation profile of foetal immune cells differed markedly relative to other ages, reflecting the unique immunological demands of the in utero environment. This particular pattern of DNA methylation is likely to represent both a lack of antigen experience and adaptive mechanisms designed for symbiosis with the maternal immune system. This is consistent with the well documented differences in neonatal immunity spanning a broad range of immune functions,[35] and likely to reflect known differences in cord blood T-cell phenotypes,[36] differences in the ratio

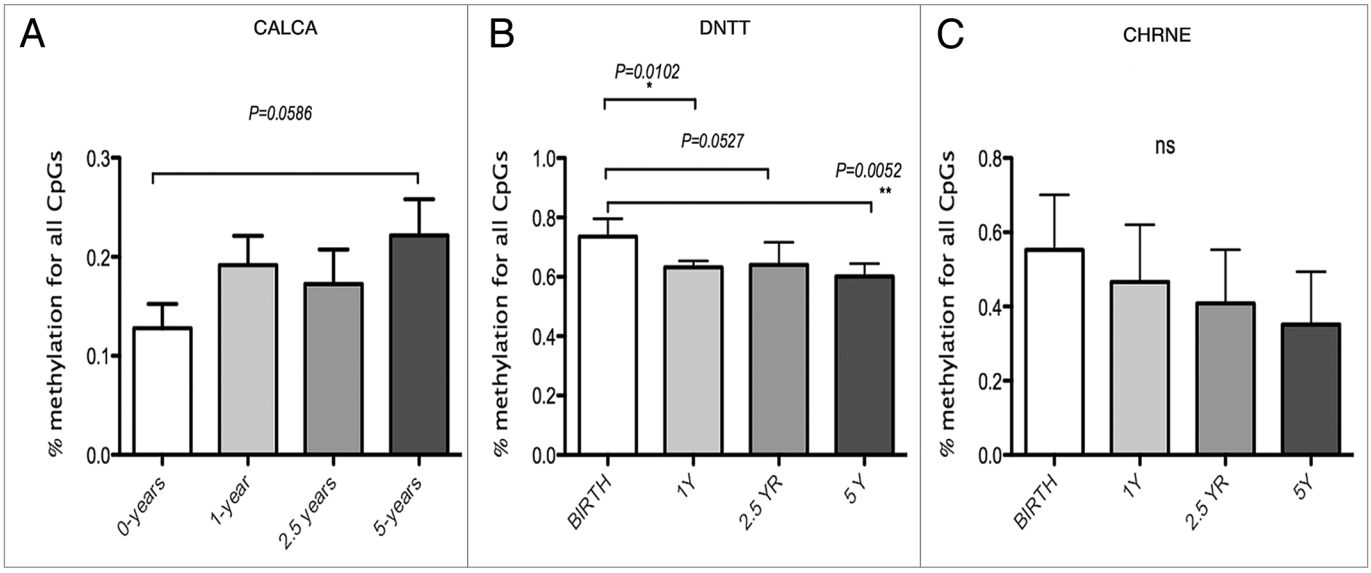

Figure 6. Cumulative change in percent CpG methylation from birth to five years. Data is presented as average percent methylation across all CpG units measured. Statistical comparisons made by t-test. Standard errors are shown on graph. *ns* = not significant.

of CD45RA/RO, reduced expression of HLA-DR[37] and high levels of $CD34^+$ progenitors. We identified the most significantly changing probes between foetal and 1-year samples as a proxy marker of cellular immune pathways that transition after birth. Ontology analysis indicates that these probes were enriched for signaling pathways initiated at the plasma membrane, a finding likely to reflect innate and adaptive immune pathways responsive to the new antigen-rich environment.[38]

In the postnatal period of immune development, the age-dependent change in DNA methylation was subject to individual variation, reflecting differences in host patterns of immune responses, under the influence of multiple environmental and genetic factors. Ontology enrichment of variable probes over this period gave a strong signal for immune-cell receptor recombination as well as patterning of effector responses, establishment of homeostasis, complement and immunoglobulin development. We validated two genes with relevant functions that exhibit an age-dependent change in DNA methylation over this early period of immune development. DNTT (deoxynucleotidyltransferase) encodes a template-independent DNA polymerase with restricted expression in pre-B and pre-T lymphocytes during early differentiation, where it generates antigen receptor diversity by re-arranging immunoglobulin heavy chains and T cell receptor gene segments.[39] CALCA encodes the peptide hormones calcitonin and katacalcin which function in calcium regulation and phosphorous metabolism.[40]

Clustering on the X-chromosome indicated a strong genetic contribution in determining the pattern of X-inactivation in females, which was largely refractory to change over time. This supports previous data of variability in methylation profile of the inactive X chromosome in females,[41] but is the first report demonstrating stability of this variability over time.

Despite these novel observations this study was limited by the small number of individuals with longitudinal data. Importantly, our analyses were carried out retrospectively, once the lack of symptoms common in immune related disorders known to develop prior to this age had been confirmed. Whilst the primary aim of this paper was to investigate temporal changes in DNA methylation, the implications here suggest pathways undergoing regulation of gene expression, a process we have yet to demonstrate. Bearing this in mind, this study will provide a solid foundation on which to carry out further extensive analyses of DNA methylation profile in larger numbers of 'proven' disease free' cases to further define the range of variation in immune cell epigenetic maturation, and will also inform case: control studies aimed at investigating the association of specific epigenetic changes associated with immune-related disorders.

Methods

Sample collection. Whole blood samples were collected at birth, 1 year, 2.5 years and 5 years of age. All children were healthy with no evidence of any immune disorders including allergic diseases or sensitization (as confirmed on allergy testing at 1, 2.5 and 5 years of age). Given the previously documented differences in epigenetic profile between males and females, the majority of subjects in this study were female, with a small number of males include for comparative purposes (**Sup. Table S1**).

Cord blood (CB) was collected at the delivery of each infant. All had uncomplicated pregnancies and deliveries. The surface of the placenta was carefully cleaned of all macroscopic blood, then swabbed down with 70% alcohol three times and allowed to dry. The major placental vessels were then cannulated using a 19 gauge needle and blood drawn into pre-heparinised syringes within minutes of delivery. The cord blood was then placed immediately into an equivolume of RPMI (Roswell Park Memorial Institute) culture medium (Gibco BRL Life Technologies, New York, USA) for transport. Children attended clinical follow-up visits at

1 year, 2.5 years and 5 years of age. Peripheral blood samples were collected at each visit by routine venipuncture of a cubital fossa vein. Mononuclear cells (MC) were isolated by Ficoll-Hypaque gradient centrifugation and cryopreserved (in 7.5% DMSO) for future use using established techniques.[24] All samples were transported at room temperature, and processed within 8 hours, and as approved by the Princess Margaret Hospital Ethics Committee.

Clinical characteristics. At each visit, parents completed questionnaires to confirm that there was no evidence of immune disease, including symptoms of allergic disease (eczema, food allergy, allergic rhinitis or asthma). The children had allergy skin prick tests at each visit to exclude sensitization.

Flow cytometry to examine MC fractions at each age. To examine the different mononuclear cell (MC) fractions at each age, 1 x 10^6 cells were stained with each of the three antibody cocktail panels to phenotype (i) monocytes and dendritic cells (DCs), (ii) T and B cells and (iii) regulatory and natural killer T cells. Monocyte and DC panel consisted of antibodies directed against CD14 APCCy7 (1:100), HLADR-APC (1:50), Lin1-FITC (1:200) and CD123-PerCPCy5.5 (1:200). Monocytes were defined as $CD14^+$ and conventional DCs as $HLADR^+CD123^-$. Panel 2 stained for T and B cells using CD3-APCH7 (1:20), $CD4^-$ APC (1:20), CD8-FITC (1:40) and CD19-PE (1:5). Effector T cells were $CD3^+CD4^+$, cytotoxic T cells $CD3^+CD8^+$ and B cells $CD19^+$. The last panel stained for Tregs and NK cells using CD4-APC (1:20), CD25-PerCPCy5.5 (1:20), CD127-PE (1:20) and CD56-FITC (1:200). Tregs were defined as $CD4^+CD25^+CD127^-$/lo and NK cells as $CD56^+$. Samples were incubated with each of the antibody cocktails (or concentration-matched isotype controls) for 30 minutes in the dark at 4°C. All antibodies were purchased from Becton Dickinson (BD) Biosciences (San Diego, USA) and staining was performed in round-bottom 96-well plates. Gates were set by using isotype-matched controls and BD CompBeads®. Cell types were enumerated on a 6-colour laser BD LSR II flow cytometer. Fifty thousand events were acquired for each panel. The data were analyzed using FlowJo software (Tree Star, Ashland, USA) and expressed as percentage of total MC population.

Genome-scale DNA methylation profiling. Genomic DNA was isolated from the MC sub-populations using QIAGEN DNAeasy columns according to manufacturer's instructions (QIAGEN, Australia), and DNA was processed using the Methyl Easy™ bisulphite modification kit (Human Genetic Signatures, Sydney, Australia), according to the manufacturer's instructions. DNA methylation profiling was carried out using The Illumina Infinium HumanMethylation27 BeadChip platform with hybridization and scanning carried out as per manufacturer's instructions at Service XS (Leiden, The Netherlands).

Methylation array analysis. Bisulphite-converted genomic DNA was analysed using Illumina's Infinium Human Methylation27 BeadChip Kit. The BeadChip contains 27,578 CpG loci covering more than 14,000 human RefSeq genes at single-nucleotide resolution. Raw data was exported from BeadStudio.

Statistical analysis. Data was analysed using the statistical programming language R (http://cran.r-project.org/index.html) with packages from Bioconductor.[25] Data quality was confirmed using *arrayQualityMetrics*,[26] which produces a number of diagnostic plots to assess reproducibility, identifier apparent outlier arrays and compute measures of signal-to-noise. All twenty-two samples passed quality control. All probes overlapping with SNPs were removed (a total of 67).[27] All probes on the X and Y chromosomes were removed. The *lumi*[28] package was used to calculate the log2 ratio for methylated probe intensity to unmethylated probe intensity, the M-value. These probes underwent colour-adjustment, background-correction and quantile-normalization. A total of 119 probes for which the detection p value was > 0.05 in all samples were excluded from analysis. This reduced the number of probes to 26,303 (13,820 genes). This set of M-values were subsequently passed to *limma*[29] for differential methylation analysis. A linear model was fitted for all comparisons. Differentially methylated probes were determined using the Benjamini and Hochberg adjusted p-value with a cut-off of < 0.1. The M-values were converted to methylation (beta) values for all further analysis. The data underwent unsupervised hierarchical clustering analysis with the Euclidean distance and complete linkage algorithm, and a heatmap with associated dendrogram was created using gplot[30]s A hypergeometric test was carried out to compute p-values for over or under-representation of gene ontologies.[31] Genesets analysis was carried out using *GSA*.[32] Briefly, cord blood and five year samples were combined for a two-class unpaired comparison between the canonical pathways collection hosted by the Broad Institute (http://www.broadinstitute.org/gsea/msigdb/index.jsp). The cases and variables was permuted 1,000 times to estimate the null distribution and differentially methylated genesets were called at the FDR < 0.01 cutoff.

Note

Supplemental materials can be found at:
www.landesbioscience.com/journals/epigenetics/article/16401

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