Methods in Molecular Biology (2016) 1433: 143–151
DOI 10.1007/7651_2015_286

Published online: 21 January 2016

Methylation Analysis in Distinct Immune Cell Subsets in Type 1 Diabetes

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Abstract

Epigenetics provides a mechanism in which the environment can interact with the genotype to produce a variety of phenotypes. These epigenetic modifications have been associated with altered gene expression and silencing of repetitive elements, and these modifications can be inherited mitotically. DNA methylation is the best characterized epigenetic mark and earlier studies have examined DNA methylation profiles in peripheral blood mononuclear cells in disease. However, any disease-related signatures identified would just display differences in the relative abundance of individual cell types as each cell subset generates a unique methylation profile. Therefore is it important to identify cell- or tissue-specific changes in DNA methylation, particularly in autoimmune diseases such as type 1 diabetes.

Keywords: Type 1 diabetes, DNA methylation, Cell isolation, Flow cytometry, Magnetic-activated cell sorting, DNA extraction, Illumina HumanMethylation450K BeadChip

1 Introduction

DNA methylation is the most characterized epigenetic modification and is essential for regulating the expression of mammalian genes. It involves the addition of a methyl group to the cytosine in a CpG dinucleotide [1, 2]. DNA methylation has been shown to be tissue-specific [3] and stable for at least 3 years [4]. Epigenetics has been studied in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis as genetic factors cannot fully account for development of these diseases [5]. For example, in type 1 diabetes (T1D), concordance rates in monozygotic twins is ~50 % [6] suggesting a role for non-genetic factors.

We have previously performed an epigenome-wide association study using $CD14^+$ monocytes from T1D-discordant monozygotic twin pair with the Illumina HumanMethylation27K BeadChip [7]. Following on from this, we have extended the study by also isolating $CD19^+$ B cells and $CD4^+$ T cells and the classical subset of monocytes ($CD14^+CD16^-$) from monozygotic twin pairs discordant for T1D. Buccal samples were also collected as they have been

shown to have its own unique methylome [8]. The PBMC layer was harvested through the use of Percoll, a gradient density medium, and the different cell populations were sorted using magnetic-activated cell sorting (MACS). MACS separates different cell populations through use of magnetic beads coated with antibodies against the surface antigen of interest. The cells are then passed through a column in a magnetic field and the cells of interest are retained in the column for a pure population. MACS has been shown to obtain high purities of isolated cell populations and subsequent use of magnetic beads on a single sample did not significantly affect gene expression [9]. The purity of each cell type collected was then assessed using flow cytometry and subsequently, DNA was extracted from the cells and buccal samples. The samples were treated with sodium bisulfite and hybridized onto the beadchips for DNA methylation profiling using Illumina HumanMethylation450K BeadChip. The Illumina Infinium HumanMethylation BeadChip is an example of an array based technology for DNA methylation profiling [10]. Here, we have demonstrated that different cell types ($CD19^+$, $CD14^+CD16^-$, and $CD4^+$) can be isolated from a single blood sample with cell purity over 95 % for epigenetic profiling.

2 Materials

2.1 Cell Isolation

1. 1 M Trisodium Citrate (TNC): Dissolve 29.4 g trisodium citrate in 100 mL of water through a filter.
2. Percoll 1.078 g/mL: 300 mL Percoll (GE Healthcare), 24 mL 10× PBS, 216 mL 1× PBS, 13.8 mL human serum albumin, 7.2 mL 1 M TNC. Store at 4 °C.
3. PBS, 2 mM EDTA (Buffer 2): Add 2 mL 0.5 M EDTA to 500 mL 1× PBS.
4. RPMI: Add 5 mL penicillin streptomycin and 5 mL human serum albumin to 500 mL RPMI 1640 Medium, GlutaMAX™, HEPES (Invitrogen).
5. BD Vacutainer® Sodium Heparin tube with Green Conventional Closure, 10 mL (BD).
6. LS, LD and MS columns (Miltenyi Biotech).
7. MACS stand and separator (Miltenyi Biotec).
8. Pre-separation filters (Miltenyi Biotech).
9. CD19, CD4, CD16, and CD14 MicroBeads, human (Miltenyi Biotech).

2.2 Flow Cytometry

1. FITC conjugated monoclonal mouse anti-human CD14, clone MφP9 (BD Biosciences).
2. PE conjugated monoclonal mouse anti-human CD16, clone B73.1/leu11c (BD Biosciences).
3. PerCP-Cy5.5 conjugated monoclonal mouse anti-human CD64, clone 10.1 (BD Biosciences).
4. PE-CY7 conjugated monoclonal mouse anti-human CD45, clone HI30 (Invitrogen).
5. FITC conjugated monoclonal mouse anti-human CD4, clone M-T466 (Miltenyi Biotec).
6. PE conjugated monoclonal mouse anti-human CD19, clone LT19 (Miltenyi Biotec).
7. Falcon® 5 mL Round Bottom Polystyrene Test Tube (Corning).
8. Anti-Mouse Ig, κ/Negative Control (FBS) Compensation Particles Set (BD Bioscience).
9. FITC conjugated monoclonal mouse anti-human Ig, κ light chain, clone TB28-2 (BD Biosciences).
10. PE conjugated monoclonal mouse anti-human Ig, κ light chain, clone TB28-2 (BD Biosciences).
11. PerCP-Cy™5.5 conjugated mouse IgG1 κ isotype control (BD Biosciences).
12. PE-Cy® 7 conjugated mouse IgG1 (Invitrogen).
13. FITC conjugated mouse IgG1 (Miltenyi Biotech).
14. PE conjugated mouse IgG1 (Miltenyi Biotech).
15. BD FACSCanto II Flow Cytometer (BD).

2.3 DNA Extraction and Sodium Bisulfite Treatment

1. QIAamp DNA Blood Mini Kit (250) (Qiagen).
2. RNAProtect (Qiagen).
3. Qubit fluorometer (Invitrogen).
4. Qubit dsDNA assay kit (Invitrogen).
5. Qubit assay tubes (Invitrogen).
6. Gentra Puregene Buccal Cell Kit (100) (Qiagen).
7. EZ DNA Methylation kit (Zymo Research).

2.4 Agarose Gel Electrophoresis

1. Gel tank (Bio-Rad).
2. UltraPure agarose (Invitrogen).
3. TBE buffer 10×: Dissolve 108 g tris base, 51 g boric acid, and 40 mL 0.5 M EDTA into 500 mL distilled water.
4. Ethidium bromide solution (Sigma Aldrich).

3 Methods

Keep Buffer 2 and cells on ice unless otherwise specified. Centrifuge sample with brakes unless otherwise specified.

3.1 Peripheral Blood Mononuclear Cell (PBMC) Extraction

1. Collect 50 mL of blood in sodium heparin tubes and dilute 1:1 with RPMI. Leave the sample rolling overnight at room temperature (*see* **Note 1**).
2. Carefully pipette 20–25 mL of the diluted blood onto 12.5 mL Percoll 1.078 g/mL in four 50 mL tubes.
3. Centrifuge the sample for 20 min, at 800 × *g*, with no brake at 20 °C. After centrifugation, the sample will split into different layers (Fig. 1).
4. Remove most of the plasma layer and harvest the PBMC ring into two 50 mL tubes. Wash with the PBMC ring with Buffer 2, centrifuge at 550 × *g* for 8 min at 20 °C.
5. Pool the cell pellets into one 50 mL tube and fill the tube with Buffer 2. Centrifuge at 550 × *g* for 8 min at 4 °C.
6. Resuspend the cells in 10 mL Buffer 2. With an aliquot of cell solution, mix 1:1 with trypan blue. Count cells using a haemocytometer.
7. Add up to 45 mL of Buffer 2 to the 50 mL tubes and spin at 550 × *g* for 6 min at 4 °C.

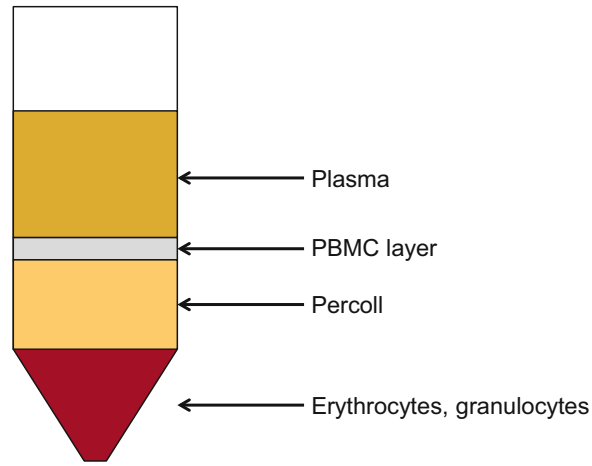

Fig. 1 Extraction of PBMCs on a Percoll gradient. PBMCs are separated from blood using centrifugation on Percoll. The diluted blood is overlayed onto Percoll in a 50 mL Falcon tube. Centrifugation will result in the formation of different layers. The different layers that will be observed from *top* to *bottom* are as follows: plasma, PBMC layer, Percoll, and erythrocytes and granulocytes. The PBMC layer is then harvested and washed

3.2 Isolating B Cells

1. Resuspend the cell pellet in 80 μL Buffer 2 per 10 million cells. Then add 20 μL of CD19 MB per 10 million cells.
2. Incubate for 15 min at 4 °C. Add 2 mL of Buffer 2 to cells and spin at 300 × *g* for 6 min at 4 °C.
3. Whilst waiting for the samples to finish spinning, wash an LS column with 3 mL Buffer 2 through a pre-separation filter.
4. Resuspend the cells in 500 μL of Buffer 2 and apply the cell suspension to the column through the pre-separation filter.
5. Add 3 mL of Buffer 2 to the column three more times (*see* **Note 2**). Remove the column from the magnet and add 5 mL of Buffer 2 to the column.
6. Use the plunger to flush out the purified cells. Centrifuge the flow through and purified cells at 300 × *g* for 6 min at 4 °C. Wash an MS column with 500 μL of Buffer 2.
7. Decant the supernatant from the $CD19^-$ tube and leave on ice to one side.
8. Resuspend the $CD19^+$ fraction in 500 μL of Buffer 2 and add to the MS column (*see* **Note 3**).
9. Add 500 μL of Buffer 2 to column three times. Remove column from the magnet and add 2 mL of Buffer 2 to the column.
10. Use the plunger to flush out the purified cells and perform a cell count.
11. Discard the flow through (second $CD19^-$ fraction).

3.3 Isolating Monocytes

1. Resuspend the remaining cell pellet to one side (first $CD19^-$ fraction) in 50 μL Buffer 2 per 50 million cells. Add 50 μL of CD16 MB per 50 million cells.
2. Incubate for 30 min at 4 °C. Add 2 mL of Buffer 2 to cells. Spin at 300 × *g* for 6 min at 4 °C.
3. Wash an LD column with 2 mL Buffer 2. Resuspend the cells in 500 μL of Buffer 2 and apply the cell suspension to the column.
4. Add 1 mL of Buffer 2 to column twice.
5. Collect the flow through and perform a cell count. Then spin at 300 × *g* for 6 min at 4 °C.
6. Resuspend the pellet in 80 μL Buffer 2 per 10 million cells. Add 20 μL of CD14 MB per 10 million cells.
7. Incubate for 15 min at 4 °C. Add 2 mL of Buffer 2 to cells. Spin 300 × *g* for 6 min at 4 °C.
8. Wash an MS column with 500 μL Buffer 2. Resuspend the cells in 500 μL of Buffer 2 and apply the cell suspension to the column.
9. Add 500 μL of Buffer 2 to column three times. Remove the column from the magnet and add 2 mL of Buffer 2 to column.

10. Use the plunger to flush out the purified cells and perform a cell count.
11. Collect flow through and perform a cell count. Spin at 300 × *g* for 6 min at 4 °C.

3.4 Isolating T Cells

1. Resuspend the cell pellet in 80 μL Buffer 2 per 10 million cells. Add 20 μL of CD4 MB per 10 million cells.
2. Incubate for 15 min at 4 °C. Add 2 mL of Buffer 2 to cells. Spin at 300 × *g* for 6 min at 4 °C.
3. Wash MS column with 500 μL Buffer 2. Resuspend cells in 500 μL of Buffer 2. Apply cell suspension to the column.
4. Add 500 μL of Buffer 2 to column three times. Remove the column from the magnet and add 2 mL of Buffer 2 to the column.
5. Use the plunger to flush out the purified cells. Perform a cell count.

3.5 Flow Cytometry

1. Add an aliquot from each cell type ($CD19^+$, $CD14^+CD16^-$, and $CD4^+$) to separate 5 mL FACS tubes. Spin down at 300 × *g* for 6 min at 4 °C (*see* **Note 4**).
2. Resuspend in 100 μL of Buffer 2.
3. Add antibodies (Table 1) (*see* **Note 5**). Vortex tubes and incubate for 10 min at 4 °C.
4. Wash with 2 mL Buffer 2 then centrifuge at 300 × *g* for 6 min at 4 °C.
5. Resuspend in 500 μL Buffer 2.
6. Set up single color controls with the compensation beads set by adding 100 μL of Buffer 2 into a new tube for each staining

Table 1
Antibody panel for the individual cell types

Monocytes		
CD14	FITC	20 μL
CD16	PE	20 μL
CD64	PerCP-Cy5.5	5 μL
CD45	PE-CY7	5 μL
T cells		
CD4	FITC	10 μL
B cells		
CD19	PE	10 μL

Table 2
Setting up the FMO controls for analyzing monocytes

	FITC	PE	PerCP-Cy5.5	PE-CY7
FITC	–	✓	✓	✓
PE	✓	–	✓	✓
PerCP-Cy5.5	✓	✓	–	✓
PE-CY7	✓	✓	✓	–

The tubes are represented by the first columns (*bold*). The rest of the columns specify which antibody to add in a tube. For example, the FITC FMO will include the PE, PerCP-Cy5.5, and PE-CY7 but not the FITC antibody

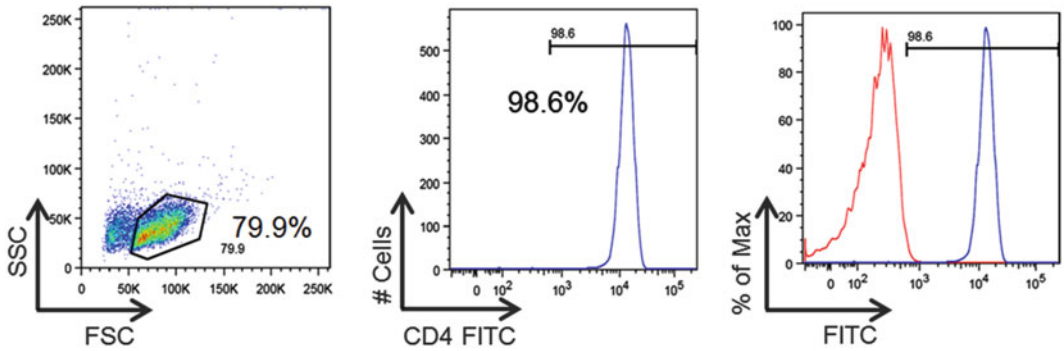

Fig. 2 Flowjo analysis of $CD4^+$ cells. Representative FACS results for purified $CD4^+$ cells. $CD4^+$ cells are stained with FITC conjugated antibodies (*dark blue*). 10,000 events are recorded in the initial gate. Mouse anti-IgG FITC antibody is also used to stain the cells to establish a negative control (*light blue*). Purity for all samples will be over 95 %

antibody. Then add a drop of the negative control and anti-mouse Ig K beads to each tube. Add the same volume of antibodies indicated in Table 1 to the tubes.

7. For setting up isotype controls, use the same volume of antibodies as in Table 1.
8. For setting up fluorescence-minus-one (FMOs) controls, *see* Table 2.
9. Use the BD Canto II instrument to assess the purity of each cell type (Fig. 2).

3.6 DNA Extraction and Sodium Bisulfite Treatment

1. Split each isolated cell type into 2 eppendorf tubes.
2. Spin all at 14,000 rpm for 2 min at RT.
3. Add 200 µL PBS and 200 µL AL lysis buffer to one pellet for DNA extraction, vortex then store at −20 °C.

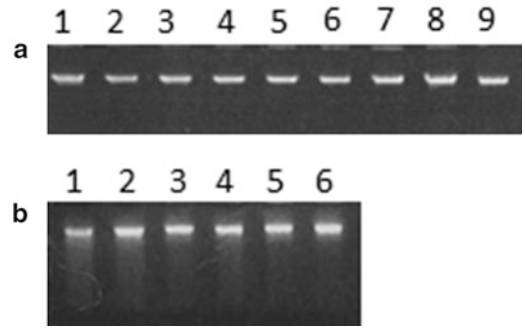

Fig. 3 Quality control for DNA extraction. DNA is measured using a Qubit instrument and 100 ng DNA from purified cells and buccal samples are run on a 2 % agarose gel to assess any degradation. The gels are viewed using a UV gel imager. (**a**) DNA from purified cells, *lanes 1*, *4*, and *7* are DNA from $CD14^{+}CD16^{-}$ cells. *Lanes 2*, *5*, and *8* are DNA from $CD4^{+}$ cells. *Lanes 3*, *6*, and *9* are DNA from $CD19^{+}$ samples. (**b**) DNA from buccal brushes from different individuals

4. Resuspend the remaining pellet in 300 μL RNAprotect, vortex and store at −80 °C for future RNA extraction.
5. Extract DNA from cells using QIAamp DNA Blood Mini Kit.
6. Measure DNA concentration using Qubit.
7. Run 100 ng DNA on a 2 % agarose gel and visualize on a UV transilluminator (Fig. 3).
8. Extract DNA from the buccal brushes using the Gentra Puregene Buccal cell kit according to manufacturer's instructions. Measure the DNA concentration with the Qubit instrument.
9. 500 ng DNA is ready to be sent to a genome center to be treated with sodium bisulfite and then hybridized onto the Infinium HumanMethylation450K BeadChip.

4 Notes

1. Samples are taken at any point in the day and therefore are left rolling overnight in order to standardize the protocol.
2. Wait for the 3 mL of Buffer 2 to flow through the column completely before the addition of another 3 mL.
3. There is no need for pre-separation filters from then on. Sorting cells from PBMCs may clog up the column while positively selecting for CD19 cells.
4. Whilst decanting the supernatant from the 5 mL FACS tubes, blot onto absorbent paper.
5. Sample gating and background signal were determined using unstained, isotype, and fluorescence-minus-one (FMO) controls.

Acknowledgement

This work was supported by JDRF and BLUEPRINT EU-FP7.

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