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Am J Reprod Immunol. Author manuscript; available in PMC 2013 July 01.

Published in final edited form as:
Am J Reprod Immunol. 2012 July ; 68(1): 8–27. doi:10.1111/j.1600-0897.2012.01108.x.

Methylome of Fetal and Maternal Monocytes and Macrophages at the Feto-Maternal Interface

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Abstract

Problem—Decidual macrophages (dMϕ) of the mother and placental macrophages (Hofbauer cells, HC) of the fetus are deployed at a critical location: the feto-maternal interface. This study was conducted to compare DNA methylome of maternal and fetal monocytes, dMϕ, and HC, and thereby to determine the immunobiological importance of DNA methylation in pregnancy.

Methods of Study—Paired samples were obtained from normal pregnant women at term not in labor and their own neonates. Maternal monocytes (MM) and fetal monocytes (FM) were isolated from peripheral blood of mothers and from fetal cord blood, respectively. dMϕ and HC were obtained from the decidua of fetal membranes and placenta, respectively. DNA methylation profiling was done using the Illumina Infinium Human Methylation27 BeadChip. Quantitative real-time PCR and western blot were performed for validation experiments.

Results—1) Significant differences in DNA methylation were found in each comparison (MM vs. FM, 65 loci; dMϕ vs. HC, 266 loci; MM vs. dMϕ, 199 loci; FM vs. HC, 1,030 loci). 2) Many of the immune response-related genes were hypermethylated in fetal cells (FM and HC) compared to maternal cells (MM and dMϕ). 3) Genes encoding markers of classical macrophage activation were hypermethylated and genes encoding alternative macrophage activation were hypomethylated in dMϕ and HC compared to MM and FM, respectively. 4) mRNA expressions of DNMT1, DNMT3A, and DNMT3B were significantly lower in dMϕ than in HC. 5) 5-azacytidine treatment increased expression of INCA1 in dMϕ.

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Conflict of Interest
The authors have no financial conflicts of interest.

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Conclusions—The findings herein indicate that DNA methylation patterns change during monocyte-macrophage differentiation at the feto-maternal interface. It is also suggested that DNA methylation is an important component of biological machinery conferring an anti-inflammatory phenotype to macrophages at the feto-maternal interface.

Keywords

Decidua; DNA methylation; DNA methyltransferase; Epigenetics; Epigenome; Hofbauer cell; Placenta; Pregnancy

Introduction

Macrophages play key roles in immune responses against a wide variety of insults such as infection[1–3] and allograft rejection.[4, 5] Human pregnancy is considered an immunologic enigma since the semi-allogeneic fetal graft is accepted and tolerated during the course of pregnancy.[6–8] The mechanisms proposed for immunological tolerance to the fetus include the expression of non-classical HLA molecules on trophoblasts,[9, 10] changes in tryptophan metabolism by indoleamine 2,3-dioxygenase,[11, 12] and regulatory T cells.[13–21] Placental development, ranging from trophoblast invasion to deportation of trophoblast debris into intervillous maternal circulation, is associated with the changes in the frames of feto-maternal immune interaction.[22–27] Therefore, the human placenta is at the epicenter of the feto-maternal interface, and maternal cells of the decidua interact direct with fetal cells, typically trophoblasts, beginning at the time of implantation and continuing throughout pregnancy.[15, 28–31] Maternal decidual macrophages (dMϕ) and fetal placental macrophages (Hofbauer cells, HC), are important cell populations at the feto-maternal interface.[32–39] They interact continuously with potential immunological signals (microbial or danger signals)[40, 41] and cells from the fetus and the mother.[42–49] These two types of macrophages (dMϕ and HC) are known to originate from maternal and fetal blood monocytes although chorionic mesenchymal cells have been proposed as a major source of HC before the formation of villous stromal capillaries.[50, 51] Changes in the immunophenotype, metabolic characteristics, and distribution of peripheral monocytes and dMϕ have been implicated in the pathogenesis of pregnancy disorders such as preeclampsia,[52–56] preterm labor with intact membranes,[57] preterm premature rupture of membranes,[58] fetal systemic inflammation,[2, 59–61] and pyelonephritis.[62] HC have been implicated in the pathogenesis of pregnancy complications[63] and they are activated in the presence of placental lesions consistent with maternal anti-fetal cellular rejection.[64, 65]

DNA methylation is a prototypic example of epigenetic regulation of gene expressions along with small non-coding RNA and histone tail modifications.[66–69] DNA methylation largely involves the cytosine residues of CpG dinucleotides in mammalian cells, and hypermethylation leads to the decreased expression of the genes as seen in several tumor suppressor genes during carcinogenesis.[70, 71] DNA methylation is mediated by a family of DNA methyltransferases (DNMT1, DNMT3A, DNMT3B),[72, 73] and is important in various cellular processes such as differentiation,[74] transcription of genes,[75] and genomic imprinting.[76] Recent studies have demonstrated associations between placental DNA methylation patterns and pregnancy disorders. An analysis of 1,505 CpG sites revealed hypomethylation of 34 loci including the promoter region of TIMP3 in early-onset preeclampsia cases compared to control subjects.[77] It was also shown that hypomethylation of the H19 promoter region is associated with increased H19 expression in the placentas of fetal growth restriction cases.[78] However, considering the heterogeneity of placental cell types and cell type specificity of DNA methylation patterns,[79] the procurement of specific cell populations would be important in the comparative analysis of DNA methylation patterns in the placenta.

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DNA methylation has been shown to be important in the differentiation of hematopoietic progenitor cells, and a subset of genes involved in differentiation is hypomethylated in monocytes and granulocytes during hematopoiesis.[80] Ji et al also have described drastic alterations of DNA methylation with lymphoid and myeloid restriction from their progenitor cells during hematopoiesis.[81] These findings indicate that the changes in CpG methylation are very important in lineage-specific hematopoietic cell differentiation. Tissue macrophages are mainly derived from peripheral blood monocytes to meet the local needs.[82] We hypothesized that distinct changes in DNA methylation occur during differentiation of maternal monocytes (MMo) into dMϕ and during differentiation of fetal monocytes (FMo) into HC, and differential DNA methylation patterns will give clues for further understanding of the immunological characteristics of these cells during human pregnancy.

In the present study, we have compared the DNA methylome of MMo, FMo, dMϕ, and HC using paired sets of samples and thereby to determine the immunobiological importance of DNA methylation in normal pregnancy.

Materials and Methods

Study Design

The study was designed to compare the DNA methylome of MMo, FMo, dMϕ, and HC. The paired samples were obtained from normal pregnant women at term not in labor and their own babies (n=26) enrolled to the bank of biological materials of the Perinatology Research Branch, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institute of Health, USA. Peripheral blood of mothers was collected by venipuncture when admitted for cesarean section, and fetal cord blood was collected from the umbilical vein during cesarean section. The placentas were used to obtain decidual and villous macrophages. The numbers of paired samples used in the different experiments are as follows: Infinium methylation assay (n=6), pyrosequencing (n=6), quantitative RT-PCR (n=5), immunoblotting (n=4), dMϕ culture with 5-azacytidine treatment (n=5), and BrdU assay (n=4). Samples isolated by flow sorting were used for Infinium methylation assay and pyrosequencing, which require more pure cell populations. Faster cell column isolation was used for quantitative RT-PCR, immunoblotting, and cell culture in order to obtain more fresh samples in spite of relatively lower purity. Participating women provided written informed consent, and the collection and use of the samples and clinical data for research purposes were approved by the Institutional Review Boards of Wayne State University and the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, U.S. Department of Health and Human Services.

Isolation of blood monocytes by flow cytometric cell sorting

Peripheral blood mononuclear cells (PBMCs) were isolated from maternal and fetal cord blood in EDTA by discontinuous density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich, St. Louis, MO, USA). PBMCs were stained with phycoerythrin (PE)-Cy7-conjugated anti-CD14, and allophycocyanin (APC)-conjugated anti-CD15 (BD Biosciences, San Jose, CA, USA; for each). CD14+CD15- monocytes were sorted using a FACS Aria™ Cell Sorter (BD Biosciences), while CD14dim+CD15+ neutrophils and the other unstained cells were negatively selected.

Isolation of tissue macrophages by flow cytometric sorting

For isolation of dMϕ, decidual tissue (7 gm), procured by scraping the decidual side of the chorion and followed by mincing with a razor blade, was incubated in 50 ml of RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% FBS and Dispase (2 mg/ml; Invitrogen) at

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37 °C for 30 min with shaking (175 rpm). Then the samples were centrifuged at 600 × g for 5 min, and the pellets were resuspended and incubated in 50 ml of RPMI 1640 containing type IV collagenase (2 mg/ml; Worthington Biochemical Corporation, Lakewood, NJ, USA), hyaluronidase (2 mg/ml; Sigma-Aldrich), and DNase I (50 μg/ml; Roche, Mannheim, Germany) for 30 min at 37°C with shaking. The digest was homogenized using a syringe with a 20-gauge needle, and incubated for an additional 30 min at 37°C with shaking (175 rpm). After filtering through the gauze and 100-micron nylon mesh, the digests were washed 2 times with DPBS and the cell numbers were counted. Antibody labeling and cell sorting were performed as described above for blood monocytes.

For isolation of HC, the basal plate, the chorionic plate, and grossly visible blood vessels were removed from the placental tissue. After 3 washes with DPBS, 7 gm of villous tissues were used for a four-stage digestion protocol to isolate single cells from villous tissue.[83] First, 50 ml of collagenase type 1A (0.624 mg/ml; Sigma-Aldrich) solution containing 0.684 mg/ml of hyaluronidase (Sigma-Aldrich), 0.12 mg/ml of DNase I (Roche), and 1 mg/ml of BSA (Sigma-Aldrich) in DPBS was added to the tissue. Following incubation for 6 min at 37°C with shaking (175 rpm), the tubes were kept on ice for 5 min and supernatant was discarded. For the second stage, 50 ml of trypsin solution (0.25 %, Invitrogen) containing 0.12 mg/ml of DNase I (Roche) and 0.5 mM of EDTA (pH 8.0, Invitrogen) were added and incubated at 37°C for 30 min with shaking (175 rpm). The tissue digest was centrifuged at 600×g for 5 min, and the pellet was then incubated with collagenase type IV (2 mg/ml; Worthington) in 50 ml of RPMI 1640 (10% FBS without antibiotics) containing 2 mg/ml of hyaluronidase (Sigma-Aldrich), and 50 μg/ml of DNase I (Roche) at 37°C for 30 min with shaking (175 rpm). The tissue digest was centrifuged at 600×g for 5 min and the supernatant was removed. Finally, collagenase type IV treatment was repeated. Following the digestion process, tissue digest was homogenized using a syringe with a 20-gauge needle, and incubated for an additional 30 min at 37°C with shaking. This digest was filtered and washed according to the same procedure used for decidual cell isolation as described above. Antibody labeling and cell sorting were performed as described above for blood monocytes.

Monocyte and macrophage isolation using a separation column

Monocytes and macrophages were isolated using cell separation columns. PBMCs of maternal blood and fetal cord blood were isolated by discontinuous density gradient centrifugation using Histopaque 1077 (Sigma-Aldrich). After lysing the red blood cells with sterile 0.2 % NaCl (pH 6.75) for 45 s, the cells were restored with sterile 1.6 % NaCl (pH 6.75) and centrifuged at 300 ×g for 5 min. Monocytes were isolated using Monocyte Isolation Kit II and MS columns (Miltenyi Biotech Inc., Auburn, CA, USA) according to the manufacturer's instructions. A small fraction of isolated monocytes were stained with APC-conjugated CD14 for the flow cytometric assessment of purity.

For isolation of dMϕ and HC, isolated single cells obtained by the protocols described above were incubated with sterile 0.2 % NaCl (pH 6.75) for 45 s and restored with sterile 1.6 % of NaCl (pH 6.75). Following removal of dead cells using a Dead Cell Removal Kit (Miltenyi Biotech), macrophages were isolated by CD14 microbeads and LS columns (Miltenyi Biotech). A small fraction of isolated macrophages were stained with APC-conjugated CD14 for flow cytometry.

Confirmation of isolated monocytes and macrophages

Each population of positively sorted cells was reanalyzed by flow cytometry to evaluate the purities of monocytes and macrophages. For visualization of the sorted CD14+ monocytes and macrophages in a few representative cases, positively selected PE-Cy7-conjugated anti-CD14+ cells were smeared on silanized slides, and mounted with Prolong Gold Antifade

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Reagent with DAPI (Invitrogen). The cells were examined using a Leica TCS SP5 spectral confocal system (Leica Microsystems, Wetzler, Germany).

Infinium methylation assay

Genomic DNA of sorted MMo, FMo, dMϕ, and HC from women at term not in labor (n=6) was isolated using a QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA). DNA (500 ng) samples were bisulfite modified using an EZ DNA Methylation Kit (Zymo Research, Irvine, CA, USA), and were hybridized with the Illumina Human Methylation27 BeadChip (Illumina, Inc., San Diego, CA, USA), according to the manufacturer's instructions. After hybridization, allele-specific single-base extension and x-staining were performed on the BeadChip, and fluorescent signals were acquired with the Bead Array Reader (Illumina). The data from these images were analyzed using BeadStudio Methylation Module (Illumina), and the relative level of methylation (β) was calculated as the ratio of methylated-locus signal to total locus signal intensity.

Bioinformatics analysis

The relative level of methylation (β value) ranges between 0 and 1 with values close to 0 indicating no methylation and values close to 1 indicating presence of methylation. The raw data was quantile normalized[84] among the 24 samples and differential methylation was tested using a paired moderated t-test.[85] A given locus was deemed significant if its False Discovery Rate[86] adjusted p-value (called q-value) was less than 0.05 and the difference in average β values between the two groups was 0.2 β units or more. Differentially methylated gene listswere interpreted using Gene Ontology (GO) terms explaining that they enrich using an over-representation analysis[87] implemented in the Bioconductor (www.bioconductor.org) package GOStats.[88] All analyses were performed using the R statistical language (www.r-project.org).

Bisulfite DNA pyrosequencing

Based on the results obtained from the Infinium Methylation Assay, the methylation status of LAG3, INCA1, and IL1B promoter regions was validated by bisulfite pyrosequencing. Genomic DNA (500 ng) samples of MMo, FMo, dMϕ, and HC from an independent group of pregnant women at term not in labor (n=6) were treated with bisulfite, and the samples were purified using Zymogen DNA columns (Zymo Research). Bisulfite-treated DNA was eluted in 20 μl of TE buffer (pH 8.0), and 1 μl was used for each PCR. PCR was performed with 0.2 μM of forward and reverse primers, and the PCR product was immobilized to Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden). The Sepharose beads containing the immobilized PCR product were purified, washed and denatured using a 0.2 M NaOH solution and rewashed using the Pyrosequencing Vacuum Prep Tool (Qiagen), according to the manufacturer's instructions. For pyrosequencing, 0.2 μM of sequencing primers was annealed to the purified single-stranded DNA, and 10 μl of the PCR products were sequenced by Pyrosequencing PSQ96 HS System (Biotage AB, Uppsala, Sweden), according to the manufacturer's instructions. The methylation status of each locus was analyzed individually as a T/C SNP using QCpG software (Qiagen).

Real-time quantitative RT-PCR

The total RNA of monocytes and macrophages (n=5) was isolated using the RNeasy Mini Kit (Qiagen). Reverse transcription was done using the orImpromII Reverse Transcription System (Promega, Madison, WI, USA). All PCR analyses were carried out using TaqMan assays (Applied Biosystems, Foster City, CA, USA) for DNMT1 (Hs00154749_m1), DNMT3A (Hs01027166_m1), DNMT3B (Hs00171876_m1), INCA1 (Hs01652223_m1),

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and RPLPO (4326314E). PCR reactions were done using the 7500 Fast Real-Time PCR System (Applied Biosystems).

Immunoblotting

Total proteins from monocytes and macrophages (n=4) were isolated using RIPA buffer (Sigma-Aldrich) containing a protease inhibitor cocktail (Roche). Twenty μg of proteins were subjected to 10 % SDS-polyacrylamide gel electrophoresis, and electro-transferred onto nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline containing 0.1% Tween 20, and probed overnight at 4°C with a rabbit polyclonal anti-INCA1 (1:500 dilution; ProSci Inc, Poway, CA, USA) or a mouse monoclonal anti-β-actin (1:5,000 dilution; Sigma-Aldrich). Horseradish peroxidase-conjugated anti-rabbit or mouse IgG (Vector Laboratories, Burlingame, CA, USA) was used as a secondary antibody, and signals were detected by chemiluminescence using ChemiGlow West Reagents (Alpha Innotech Corporation, San Leandro, CA, USA).

Decidual macrophage culture with 5-Azacytidine treatment and BrdU incorporation assay

The dMϕ (n=5) were plated in 6-well plates at the density of 5×10^5 cells per well in RPMI1640 (Invitrogen) media supplemented with 10 % FBS and antibiotics. The cells were treated with 5-azacytidine (Sigma-Aldrich) at the concentration of 5, 10, or 50 μM for 3 days. The cells were then pulsed with BrdU (10 μM) for 1 h, and stained using a BrdU Flow Kit (BD Bioscience), according to the manufacturer's instructions. Briefly, the cells were fixed and permeabilized with BD Cytofix/Cytoperm buffer, and incubated with BD Cytoperm Plus buffer. The cells were then fixed again with BD Cytofix/Cytoperm Buffer, followed by DNase (0.3 mg/ml) treatment for 1 h at 37°C. After staining with FITC-conjugated anti-BrdU antibody, the cells were analyzed for BrdU uptake using BD LSR II flow cytometry (BD Biosciences) equipped with BD FACSDiva software version 6.0. Total events numbered 3,000.

Statistical analysis

Wilcoxon signed rank tests for related variables and the Mann-Whitney U test for independent variables were performed using the SPSS version 15.0 (SPSS Inc, Chicago, IL, USA). All p-values were two-sided, and a value of $p<0.05$ was considered to be statistically significant.

Results

Purity of isolated monocytes and macrophages

The purity of monocytes and macrophages isolated by cell sorter for microarray and pyrosequencing was higher than 60.0% in all samples analyzed (Figure 1). The median of purity was 99.4% (96.5–100%) for MMo, 98.8% (98.4–99.3%) for FMo, 93.7% (60.0–99.3%) for dMϕ, and 87.5% (69.4–92.3%) for HC, respectively. The median of purity for monocytes and macrophages isolated by MACS column for qPCR, immunoblot, and decidual cell culture was 90.6% (76.7–94.5%) for MMo, 79.1% (46.9–93.3%) for FMo, 70.8% (52.5–85.7%) for dMϕ, and 73.9% (51.1–93.9%) for HC, respectively.

DNA methylation patterns: maternal vs. fetal monocytes and decidual macrophages vs. Hofbauer cells

The majority of 27,551 annotated loci on the Illumina Infinium Methylation bead-array showed an absence of methylation (Figure 2A: the left peak near 0.0), with about 9% of all loci showing a methylation level β above 0.8 in each sample (Figure 2A: the right peak). An unsupervised analysis of methylation levels showed clustering of the samples according to

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the cell populations studied: MMo, FMo, dMϕ, and HC (Figure 2). The basic comparisons of the array data were done according to the host type (Maternal vs. Fetal) and the cell type (Monocytes vs. Macrophages) (Figure 3). The data set is available in www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=tlyrpmsaqwkqwpe&acc=GSE31680.

In the comparisons between the mother and the fetus, 65 loci were differentially methylated between MMo and FMo, whereas 266 loci were differentially methylated between dMϕ and HC. Among the 65 differentially methylated loci, 25 loci and 40 loci were hyper- and hypo-methylated in MMo compared to FMo. Interestingly, most of the immune response-related genes such as SP2, LAG3, PGLYRP2, GBP1, and CX3CL1 were hypermethylated in FMo. When the methylation patterns of dMϕ and HC were compared, 145 loci were hypermethylated in dMϕ and 121 loci were hypermethylated in HC. Many of the immune response-related genes such as EDG6, ADA, PGLYRP1, CST7, TRAF1, IL1B, PTGDR, LAG3, and CD79A were found to be hypermethylated in HC. Other differentially methylated loci included the genes related to cell cycle and apoptosis such as INCA1, CASP8, and AIM2 (Table I). Of note, INCA1 and LAG3 were also among those 15 loci whose differential methylation was found both in the comparisons between MMo and FMo and between dMϕ and HC (Table II).

DNA methylation patterns: maternal monocytes vs. decidual macrophages and fetal monocytes vs. Hofbauer cells

In the comparisons between blood monocytes and tissue macrophages, 199 loci were differentially methylated between MMo and dMϕ, and 1,030 loci were differentially methylated between FMo and HC (Table I). Of note, the majority [160/199 (80%)] of the differentially methylated loci between MMo and dMϕ were also differentially methylated between FMo and HC (Table II).

Gene Ontology analysis of differentially methylated genes revealed enrichment of 215 biological processes for MMo vs. dMϕ comparison and 52 processes for FMo vs. HC (Table III). Most of the biological processes enriched in comparison between FMo vs. HC (51/52) were also enriched in comparison between MMo and dMϕ, and they were related to the variable aspects of immune responses, complement activation, cell adhesion, and angiogenesis. The biological processes enriched in the comparison between MMo and dMϕ included ion homeostasis, coagulation, and aminoglycan metabolic processes.

In the comparison between MMo vs. dMϕ, the genes encoding markers of classical macrophage activation such as TLR9, IL1B, IL12RB2, CD48, and FGR were hypermethylated in dMϕ, while those of alternative macrophage activation markers such as CCL13, CCL14, A2M, HNMT, and IL10 were hypomethylated. The results suggested that the anti-inflammatory property of human dMϕ is partly associated with its DNA methylation pattern. The comparison of FMo vs. HC also demonstrated hypermethylation of the genes encoding markers of classical macrophage activation such as TLR9, IL1B, IL12RB2, CD48, and FGR in HC, whereas the genes encoding alternative macrophage activation markers such as CCL2, CCL13, CCL14, CD209, and A2M were hypomethylated in HC.

Of note, two key enzymes, DNMT1 and DNMT3B, involved in DNA methylation were also among the differentially methylated loci between FMo and HC.

Confirmation of CpG methylation of specific genes: LAG3, INCA1, IL1B

Three genes (LAG3, INCA1, IL1B) were selected for further validation of the methylation status in each type of cells. LAG3 and INCA1 were differentially methylated between maternal and fetal cells (MMo vs. FMo and dMϕ vs HC; Table IIA), and IL1B was found to be differentially methylated between the blood monocyte and the tissue macrophage both in

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the mother and the fetus (Table IIB). Three CpG sites of each gene for bisulfite pyrosequencing were chosen from the region interrogated by methylation microarray, and the sequencing results of the three genes correlated with the microarray data. As shown in Figure 4A, the methylation percentages of the LAG gene in MMo and dMϕ were lower than those in FMo and HC, respectively ($p<0.05$ for each). The median of methylation percentages was 59.5% for MMo, 82.8% for FMo, 60.2% for dMϕ, and 82.0% for HC. The degree of methylation across three CpG sites of the INCA1 promoter region was also confirmed to be higher in MMo (61.3%) than in FMo (45.3%), and in dMϕ (35.1%) than in HC (8.7%), respectively ($p<0.05$ for each, Figure 4B). For IL1B, which was found to be differentially methylated between monocyte and macrophage in the microarray analysis, the median of methylation percentages was 31.1% for MMo, 51.9 % for dMϕ, 30.4% for FMo, and 69.4% for HC, also confirming the microarray data (Figure 4C).

DNMT expression in blood monocytes, decidua, and chorionic villi

As DNMT1 and DNMT3B were among the differentially methylated loci between FMo and HC in the microarray analysis and as they are major methyltransferases, we evaluated mRNA expression levels of DNA methyltransferases (DNMT), DNMT1, DNMT3A, and DNMT3B by quantitative RT-PCR using RNA samples from MMo, FMo, dMϕ, and HC. mRNA expressions of DNMT1, DNMT3A, and DNMT3B were significantly lower in dMϕ when compared to MMo and HC. DNMT1 mRNA expression in MMo was higher than that of FMo ($p<0.05$ for each, Figure 5). The data suggested that the differences in the methylation patterns of the cells tested are partly associated with the expression of DNMTs.

Increased expression of INCA1 mRNAby 5-azacytidine

Based on the microarray and pyrosequencing data, we further studied the effects of 5-azacytidine induced demethylation on the mRNA expression of INCA1 (inhibitor of CDK interacting with cyclin A1). INCA1 was hypermethylated in MMo or dMϕ and hypomethylated in FMo or HC. Quantitative RT-PCR and immunoblotting were used to confirm expression levels of INCA1 in monocytes and macrophages. INCA1 mRNA expression was significantly down-regulated in dMϕ compared to HC ($p<0.05$); however, there was no significant difference between MMo and FMo (Figure 6A). This seemed to be due to a relatively small difference in the extent of DNA methylation between MMo and FMo. INCA1 protein expression was also significantly lower in dMϕ than in HC as well as in MMo than FMo (Figure 6B).

The treatment of isolated dMϕ with 5-azacytidine (10 nM) for 3 days significantly increased INCA1 mRNA expression by 3.1 fold ($p<0.05$, Figure 6C). Because INCA1 is an inhibitor of cell cycle progression,[89, 90] we further studied the effect of the INCA1 methylation on the proliferation of macrophages by BrdU labeling. Following the treatment with 5-azacytidine for 3 days, the dMϕ were pulsed with BrdU. Flow cytometric evaluation demonstrated decreased BrdU labeling in dMϕ with 5-azacytidine treatment in a dose-dependent manner ($p<0.05$, Figure 6D).

Discussion

Monocytes and macrophages are important in the immunological interaction between the mother and the fetus.[91, 92] We investigated the methylome of blood monocytes and tissue macrophages at the feto-maternal interface. The principal findings of this study are: 1) There are distinct differences in the DNA methylation patterns between maternal cells and fetal cells (MMo vs. FMo; dMϕ vs. HC), and the majority of differentially methylated genes related to immune response are hypermethylated in the fetal cells; 2) The methylation patterns of monocytes and macrophages are also quite different (MMo vs. dMϕ; FMo vs.

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HC), and the differential methylation pattern is more prominent between FMo and HC; 3) Gene Ontology analysis of differentially methylated genes demonstrates significant enrichment of many biological processes associated with immune responses in monocyte vs. macrophage comparisons; 4) Pyrosequencing confirmed microarray results of three genes tested (LAG3, INCA1, IL1B); 5) mRNA expression of DNMT1, DNMT3A, and DNMT3B was significantly lower in dMϕ compared to HC; and 6) Treatment of dMϕ with 5-azacytidine significantly increased mRNA expression of INCA1 with a concomitant decrease in BrdU labeling.

DNA hypermethylation in fetal monocytes relevant to their impaired immune responses

Hypermethylation of the genes related to immune response in FMo compared to MMo indicates that DNA methylation contributes to decreased immune response capacity of FMo compared to MMo. The results are quite consistent with the gene expression data in a recent transcriptome study of adult peripheral monocytes and umbilical cord blood monocytes. Jiang et al have shown differential increases in gene expression between adult monocytes and cord blood monocytes activated by lipopolysaccharides including cytokines, chemokines, transcription factors, signal transduction, apoptotic regulation, and cell structure.[93] A previous study on relatively impaired responses of neonatal monocytes and macrophages to multiple Toll-like receptor ligands also supports immature functional capacity of neonatal monocytes compared to that of adult monocytes.[94] An additional possible explanation for differential methylation between FMo and MMo may be age-related DNA methylation changes.[95] The CD34+ hematopoietic progenitor cells from adult peripheral blood showed differential methylation patterns compared to those from cord blood[80] and age-related DNA methylation changes are tissue-specific.[95]

DNA methylation during monocyte-macrophage differentiation

The present study clearly demonstrates distinct changes of DNA methylation during monocyte-macrophage differentiation. The differences are more profound between FMo and HC. The dMϕ and HC are deployed at the immunologically sensitive location: the feto-maternal interface where an enhanced pro-inflammatory response can be detrimental to the maintenance of pregnancy. Therefore, hypermethylation of certain genes encoding classical activation markers and hypomethylation of a subset of gene-encoding alternative activation markers of macrophages in dMϕ compared to MMo and in HC compared to FMo are biologically quite relevant. DNA methylation seems to contribute to the anti-inflammatory phenotype of dMϕ and HC. Gustafsson et al compared the transcriptome of CD14+ macrophages isolated from first trimester decidual tissue and peripheral blood monocytes. Their study also has shown that the expression of the genes encoding alternative macrophage activation markers such as CCL18 and CD209 is higher in dMϕ. Therefore, dMϕ displays an anti-inflammatory phenotype compared to maternal blood monocytes.[96] Of note, their observation of increased expression of alpha-2-macroglobulin (A2M) in dMϕ is consistent with the hypomethylation of this gene in this study, whereas decreased expressions of asialoglycoprotein receptor 2 (ASGR2) and intercellular adhesion molecule 3 (ICAM3) are relevant with the hypermethylation of these genes in dMϕ. In addition to the differential methylation of CpG sites, the M2-macrophage phenotype is epigenetically regulated by chromatin remodeling following reciprocal changes in histone H3 lysine-4 and histone H3 lysine-27 methylation.[97] Our data is also consistent with recent observations on the expression of selected molecules associated with macrophage polarization in HC.[98, 99] Studies have shown that M2 markers such as DC-SIGN, CD163, and mannose receptor/CD206 are detectable in HC but not in M1 markers (CX3CR1, IL7R, CCR7). Expression of different combinations of M2 markers in dMϕ and HC which do not precisely match the transcriptional profile of in vitro generated M2 macrophages also suggests the potential

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importance of the tissue microenvironment during monocyte to macrophage differentiation.[100, 101]

A more profound difference in the DNA methylome between FMo and HC than between MMo and dMϕ has biological implications. The origin of placental HC has been controversial. It has been proposed that they derive from chorionic mesenchymal cells in the villous stroma before the formation of villous capillaries,[50, 51] while mobilization of blood monocytes becomes the main route after the development of capillaries[102] Therefore, HC might represent a heterogeneous population of cells in the context of histogenesis. Our observation of transdifferentiation of myofibroblasts into macrophages in the chorionic mesoderm of the reflected chorion also supports that HC can differentiate from chorionic mesenchymal cells.[103] Furthermore, HC can normally proliferate, or in response to biological stimuli such as inflammation[64, 104] and pro-inflammatory cytokines such as IL-6, would confer more chances of DNA methylation by changing gene expression patterns and increasing expression of methyltransferase.[105] Hypomethylation of INCA1 in HC compared to dMϕ may also in turn contribute to the difference in the proliferative potential of these fetal and maternal cells.

Differential regulation of DNA methyltransferase expression

This study also reports an interesting feature of the regulation of DNA methyl transferase expression. DNMT1 and DNMT3B were among differentially methylated loci between FMo and HC, and their mRNA expressions in dMϕ were lower than those in MMo and HC, which in turn could modify overall methylation patterns of the cells studied. DNMTs have two types of actions. DNMT1 is responsible for the maintenance of DNA methylation status, while DNMT3A and DNMT3B are important for de novo methylation.[72, 73] DNMT1 is expressed ubiquitously whereas DNMT3A and DNMT3B have tissue-specific expression patterns.[106] Promoter methylation of DNA methyltransferases themselves may have a broader impact on methylation patterns, and hypomethylation of DNMT3B was demonstrated in glial tumors.[107] We also demonstrated that differential DNA methylation patterns have biological significance by showing the effects of INCA1 in dMϕ.

Limitations of this study

There are limitations to this study. First, the samples used for the analysis in the present study were obtained from normal pregnant women at term, and the data related to pathologic pregnancies are not available. Intra-amniotic infection by microbial invasion of the amniotic cavity is a leading cause of preterm birth[108–111] and elicits a robust acute inflammatory response of the fetus.[2, 60, 61, 112–120] Furthermore, the process is associated with an elevation of the amniotic fluid concentrations of cytokines and chemokines such as macrophage migration inhibitory factor and monocyte chemotactic protein-1.[2, 121–123] The fetal chorionic mesenchymal cells also acquire macrophage phenotype in the presence of intra-amniotic infection and chorioamnionitis.[103] Therefore, the biological significance of DNA methylation of fetal and maternal monocytes and macrophages during intra-amniotic infection needs to be further studied. The data also might not represent the methylome of dMϕ or HC in the early gestational period. It is well-known that the number of HC decreases with advancing gestation.[124] Considering the duration of gestation, it is very likely that methylation patterns of macrophages change across gestation. Houser et al have recently demonstrated that there are two different populations of CD14+ macrophages according to the expression level of CD11c (high vs. low) in the first trimester decidual tissues with different functional implications in lipid metabolism, inflammation, and tissue growth.[125] Therefore, it is possible that different subpopulations of macrophages were analyzed together in our analysis.

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Conclusions

We report the DNA methylome of maternal and fetal monocytes and macrophages at the feto-maternal interface for the first time. The distinct nature of DNA methylation patterns in each subset of cells strongly suggests that epigenetic regulation of multiple genes is at the core of functional diversification of the critical players in maternal and fetal immune interaction during pregnancy. Particularly, DNA methylation seems to be a part of the machinery conferring an anti-inflammatory phenotype to the macrophages at the feto-maternal interface.

Acknowledgments

This work was supported in part by the Perinatology Research Branch, Division of Intramural Research, *Eunice Kennedy Shriver* National Institute of Child Health and Human Development, National Institutes of Health, Department of Health and Human Services.

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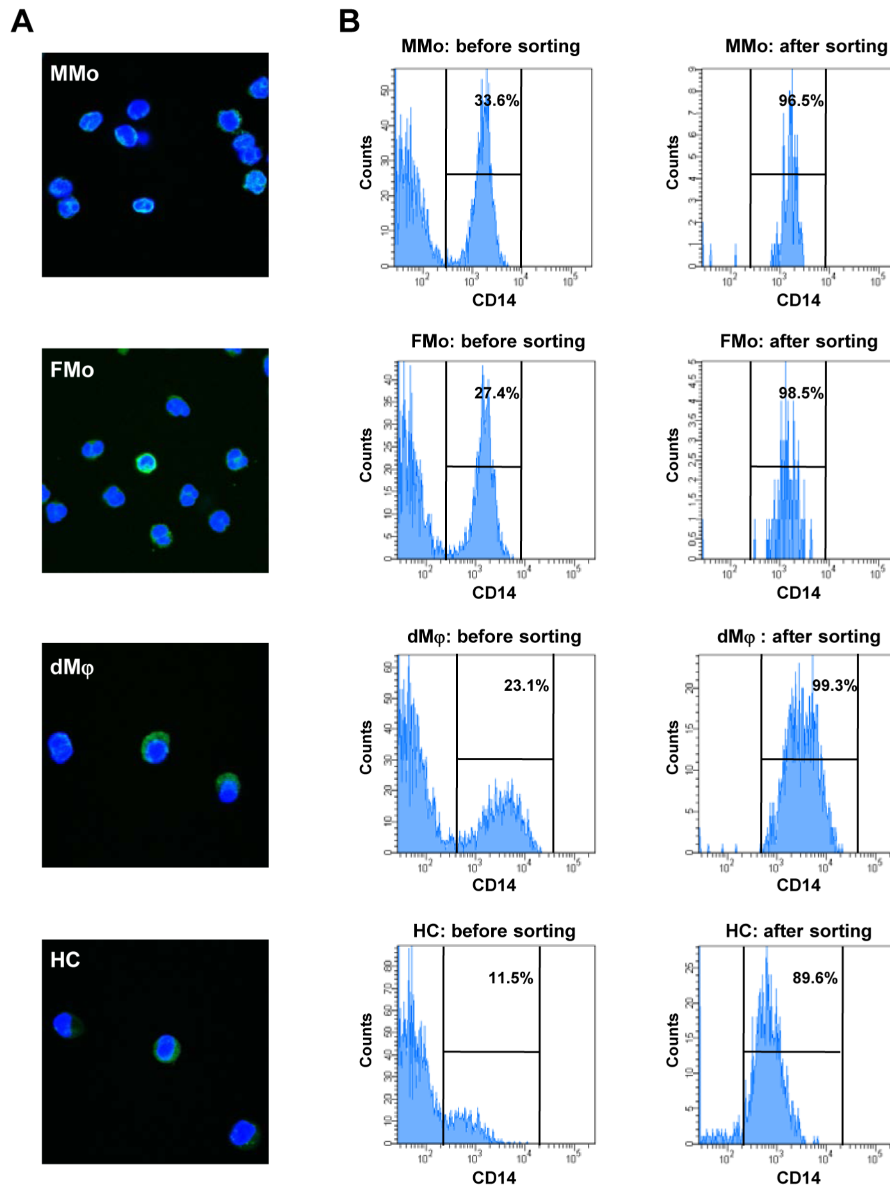

Figure 1.

Identification of isolated monocytes and macrophages. (A) Immunoflourescent staining of CD14 (green) positive sorted maternal monocytes (MMo), Fetal monocytes (FMo), decidual macrophages (dMϕ) and Hofbauer cells (HC). The nuclei are stained with DAPI (blue). Sorted cells show mononuclear morphology. (B) The purity of isolated cells for methylation microarray by flow sorting was more than 80 % in each of MMo, FMo, dMϕ, and HC.

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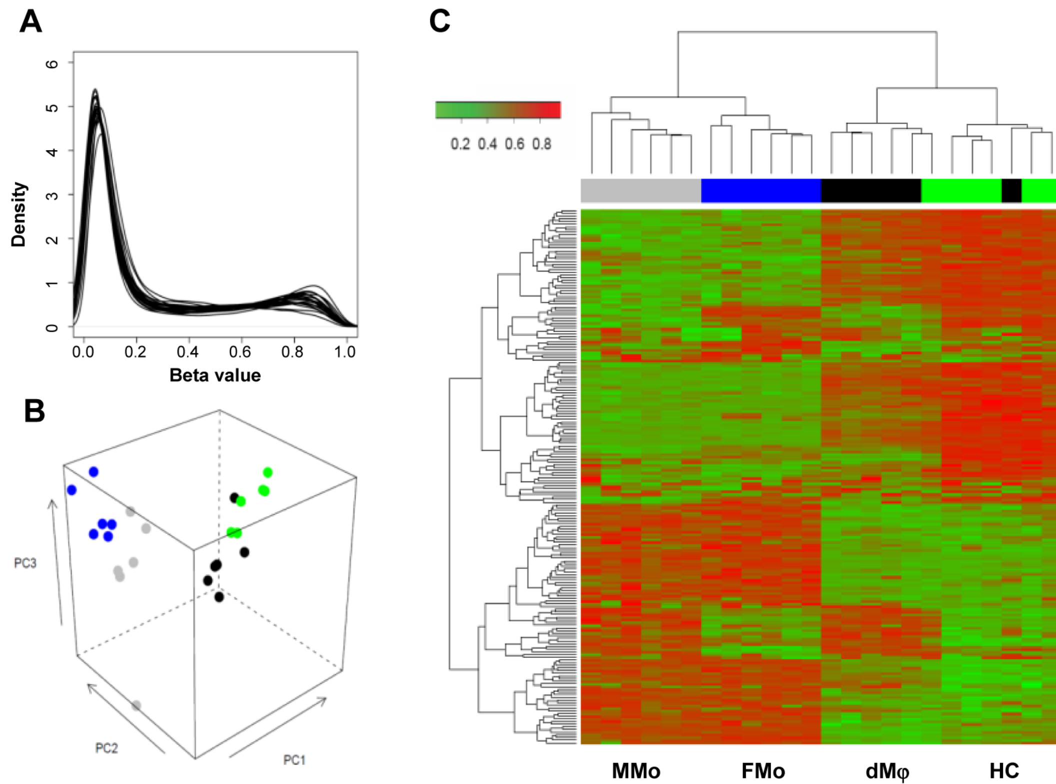

Figure 2.

Methylation microarray analysis. (A) The distribution of loci methylation β values is shown for each of the 24 samples. The peaks near β=0 illustrates that most of loci are un-methylated in each sample, while the peaks near β=1 show that there is a sizable portion of loci with high methylation β values in each sample. A principal component analysis (PCA) plot (B) and a heat map (C) generated with data from 6 pairs of maternal monocytes (MMo, gray), fetal monocytes (FMo, blue), decidual macrophages (dMϕ, black) and Hofbauer cells (HC, green) show a clear segregation among the cell groups. The PCA plot (B) uses the data from all loci, while the loci chosen for the heat map (C) are the top 200 ones varying the most across all 24 samples (unbiased filtering) as described elsewhere.[126]

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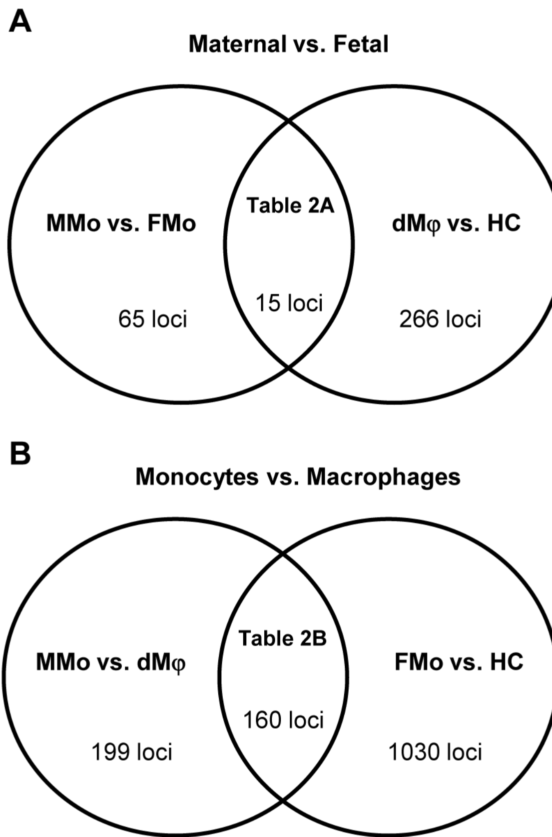

Figure 3.

Venn diagram summary of differentially methylated loci between the groups. Differentially methylated loci were using a FDR cut-off of 5% and average DNA methylation difference ($\Delta\beta$)>0.2. (A) Division of maternal vs. fetal groups. 65 loci were differentially methylated between maternal monocytes (MMo) and fetal monocytes (FMo), and 266 loci were differentially methylated between decidual macrophages (dMϕ) and Hofbauer cells (HC). Fifteen loci were shared between each comparison. (B) Division of blood monocytes vs. tissue macrophages. One hundred ninety-nine loci were differentially methylated between MMo and dMϕ, and 1,030 loci were listed for differentially methylated genes between FMo and HC. One hundred sixty loci were overlapped between each comparison.

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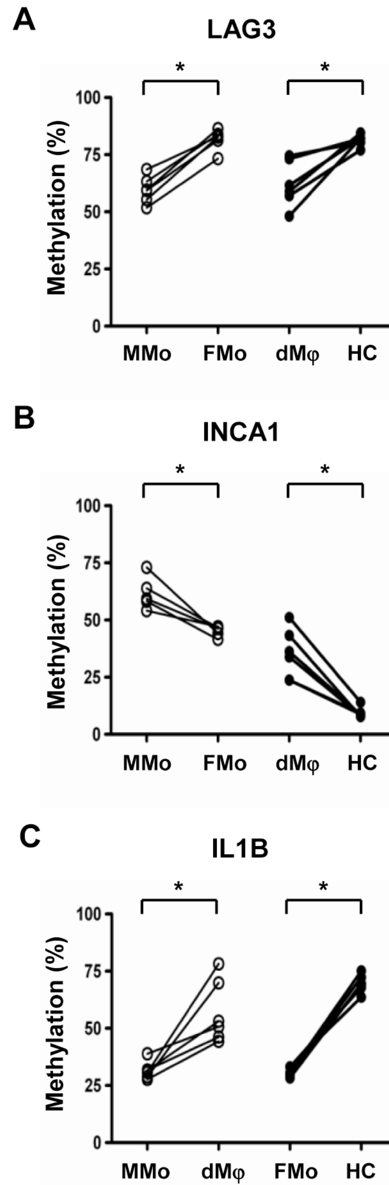

Figure 4.

Bisulfite pyrosequencing results of (A) LAG3, (B) INCA1 and (C) IL-1β in 6 samples of genomic DNAs from maternal monocytes (MMo), fetal monocytes (FMo), decidual macrophages (dMϕ) and Hofbauer cells (HC). Bisulfite sequencing was performed across 3 CpG sites of each gene chosen from the region interrogated by methylation microarray, and the average percentages of methylation are well-correlated with microarray data. *$p<0.05$.

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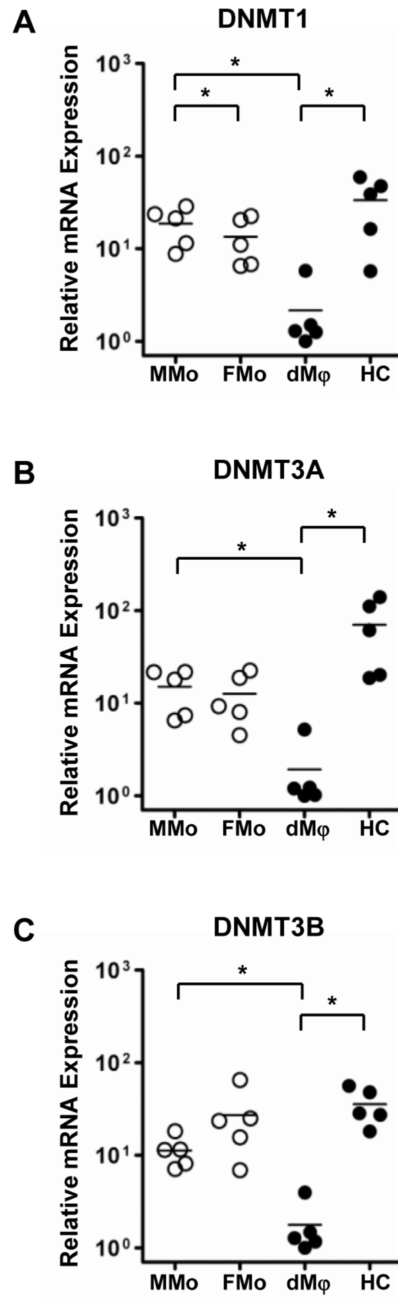

Figure 5.
mRNA expressions of DNA methyltransferases by qRT-PCR show distinguished patterns in maternal monocytes (MMo), fetal monocytes (FMo), decidual macrophages (dMϕ), and Hofbauer cells (HC). The DNMTs mRNA expressions were normalized on the content of RPLPO for each. *p<0.05, n=5.

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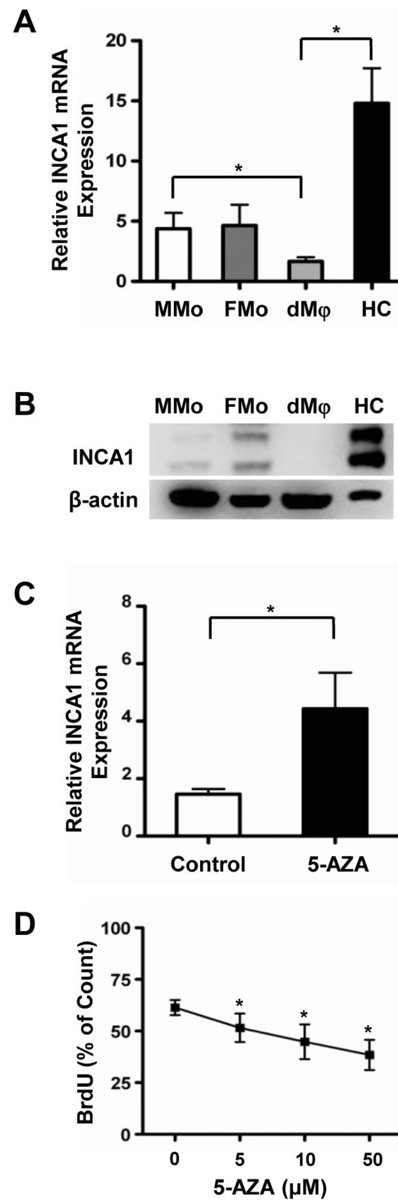

Figure 6.

Differential INCA1 expression in monocytes or macrophages by function of DNA methylation. (A) qRT-PCR of INCA1 transcripts of maternal monocytes (MMo), fetal monocytes (FMo), macrophages from decidua (dMϕ), and Hofbauer cells (HC). mRNA expression of INCA1 is significantly down-regulated in dMϕ than in HC. The INCA1 mRNA expressions were normalized on the content of RPLPO. *p<0.05, n=5. (B) Western blot of INCA1 of total proteins from columned MMo, FMo, dMϕ, and HC shows that protein expression of INCA1 is higher in HC than in dMϕ, n=4. (C) qRT-PCR of INCA1 mRNA expression following treatment of 10 μM of 5-Azacytidine for 3 days to dMϕ. mRNA expression of INCA1 was increased in dMϕ exposed to 5-Azacytidine. The INCA1 mRNA expressions were normalized on the content of RPLPO. *p<0.05, n=5. (D) BrdU pulse labeling detection in 5-Azacytidine treated dMϕ. After treatment of 5-Azacytidine (0, 5, 10, or 50 μM), macrophages were pulsed with BrdU (10 μM) for 1 h, and stained with anti BrdU antibody. The percentages of BrdU positive cells were measured by FACS. BrdU

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labeling decreased with 5-azacytidine treatment in a dose-dependent manner. *$p < 0.05$ vs. control (0 μM), n=4.

Table I

Top 30 differentially methylated loci in each comparison

MMo *vs.* FMo				dMϕ vs. HC			
Gene	***q*-value**	**Difference**	**Direction**	**Gene**	***q*-value**	**Difference**	**Direction**
CD59	2.24E-04	0.49	FMo	LACTB	4.98E-03	0.24	dMϕ
FLJ10945	2.24E-04	0.29	FMo	S100A2	4.98E-03	0.32	HC
LTBR	3.49E-04	0.29	FMo	PSTPIP1	5.42E-03	0.25	HC
PRKG2	6.95E-04	0.57	FMo	OXCT2	5.42E-03	0.31	HC
CETP	6.95E-04	0.34	FMo	CMTM5	5.42E-03	0.37	dMϕ
ECEL1	7.25E-04	0.46	MMo	LTBP3	6.66E-03	0.30	HC
FLJ10945	7.25E-04	0.32	FMo	SLCO4A1	6.66E-03	0.32	HC
SYDE1	1.06E-03	0.24	FMo	HOXB1	6.66E-03	0.40	dMϕ
OLFML2A	1.16E-03	0.44	FMo	CDH10	6.66E-03	0.22	dMϕ
ECEL1	1.20E-03	0.39	MMo	CDH9	6.66E-03	0.21	dMϕ
KLHDC7B	1.82E-03	0.28	MMo	ADAMTS13	6.66E-03	0.29	dMϕ
C21orf84	1.82E-03	0.25	FMo	KRTHB6	7.03E-03	0.32	dMϕ
MPI	3.40E-03	0.22	FMo	IL22RA1	7.10E-03	0.32	dMϕ
KCNAB3	3.40E-03	0.74	MMo	ABCC13	7.92E-03	0.22	dMϕ
LAG3	3.40E-03	0.38	FMo	SMPD3	7.92E-03	0.22	dMϕ
EDARADD	5.16E-03	0.43	FMo	TSPYL5	7.92E-03	0.29	HC
MSRB2	5.16E-03	0.26	FMo	TFAP2E	7.92E-03	0.27	dMϕ
POU3F1	5.43E-03	0.39	MMo	VDAC1	7.92E-03	0.22	dMϕ
AKR1C3	5.93E-03	0.33	FMo	LRRC4	7.92E-03	0.27	dMϕ
ZNF710	7.20E-03	0.29	MMo	DUOX2	7.92E-03	0.30	dMϕ
WT1	7.20E-03	0.27	MMo	SPARCL1	7.92E-03	0.20	dMϕ
RUNX2	7.54E-03	0.29	FMo	TNFRSF9	7.92E-03	0.29	HC
NAV1	7.54E-03	0.42	MMo	FRMPD2	7.92E-03	0.36	dMϕ
TFAP2E	7.64E-03	0.22	MMo	ZNF385	8.45E-03	0.29	HC
SDS	7.64E-03	0.32	FMo	SUSD2	8.52E-03	0.25	dMϕ
RRP22	7.78E-03	0.25	MMo	INCA1	8.52E-03	0.31	dMϕ
SNTB1	8.01E-03	0.26	FMo	ACTN3	8.84E-03	0.30	dMϕ
ACTN3	8.01E-03	0.30	MMo	SLC4A3	9.55E-03	0.27	dMϕ

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MMo *vs*. FMo				dMϕ vs. HC			
Gene	***q*-value**	**Difference**	**Direction**	**Gene**	***q*-value**	**Difference**	**Direction**
CHRNE	9.41E-03	0.33	FMo	CPZ	9.55E-03	0.42	HC
GUCY1B2	1.00E-02	0.40	FMo	C15orf2	1.03E-02	0.24	dMϕ

MMo *vs*. dMϕ				FMo vs. HC			
Gene	***q*-value**	**Difference**	**Direction**	**Gene**	***q*-value**	**Difference**	**Direction**
LOC283487	5.27E-09	0.56	MMo	LOC283487	1.39E-09	0.61	FMo
CCL14	3.96E-08	0.45	MMo	CLDN15	4.41E-09	0.59	HC
ROBO4	9.56E-08	0.45	dMϕ	FLJ38159	4.41E-09	0.61	FMo
LMO2	9.56E-08	0.55	dMϕ	CCL14	4.64E-08	0.42	FMo
ATF5	9.56E-08	0.66	MMo	CDKN2B	1.11E-07	0.68	HC
FLJ38159	1.27E-07	0.47	MMo	GIMAP5	1.17E-07	0.50	FMo
NUMA1	4.16E-07	0.56	MMo	BIRC4BP	1.31E-07	0.45	FMo
ICAM3	4.16E-07	0.48	dMϕ	ICAM3	1.43E-07	0.51	HC
CCDC57	4.16E-07	0.64	MMo	CDKN2B	1.43E-07	0.63	HC
A2M	4.16E-07	0.34	MMo	CTNND1	1.56E-07	0.42	FMo
CLDN15	5.33E-07	0.40	dMϕ	ATF5	2.50E-07	0.59	FMo
KCNE1	7.14E-07	0.35	dMϕ	TLR9	2.55E-07	0.65	HC
GPR21	7.14E-07	0.51	dMϕ	HTR2B	2.70E-07	0.38	FMo
ABI3	7.30E-07	0.56	MMo	KLHDC7B	2.87E-07	0.50	HC
TLR9	7.38E-07	0.60	dMϕ	GPR21	2.87E-07	0.53	HC
HTR2B	1.12E-06	0.35	MMo	LTBR	2.87E-07	0.43	FMo
OGFR	1.12E-06	0.40	dMϕ	IL1B	2.87E-07	0.56	HC
BIRC4BP	1.25E-06	0.36	MMo	C6orf188	3.18E-07	0.29	FMo
HYAL2	3.09E-06	0.27	MMo	SORBS3	3.38E-07	0.40	FMo
PTPRCAP	3.09E-06	0.49	dMϕ	LMO2	3.75E-07	0.45	HC
NFE2	3.09E-06	0.43	dMϕ	SLC39A2	3.75E-07	0.30	FMo
CDKN2B	3.45E-06	0.49	dMϕ	ITGB2	3.79E-07	0.36	HC
ABI3	5.81E-06	0.52	MMo	NUMA1	4.52E-07	0.52	FMo
SORBS3	6.84E-06	0.32	MMo	CTSZ	4.52E-07	0.54	FMo
ARL4	7.33E-06	0.27	MMo	CD6	4.61E-07	0.83	HC
XLKD1	7.33E-06	0.31	MMo	CSF3R	5.76E-07	0.52	HC

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MMo *vs*. dMϕ				FMo vs. HC			
Gene	***q*-value**	**Difference**	**Direction**	**Gene**	***q*-value**	**Difference**	**Direction**
GIMAP5	9.26E-06	0.34	MMo	CD6	6.49E-07	0.74	HC
RNASE1	9.38E-06	0.51	MMo	KCNE1	8.18E-07	0.33	HC
CTNND1	1.20E-05	0.29	MMo	CBFA2T3	8.25E-07	0.55	HC
GPR92	1.32E-05	0.47	MMo	RNF36	1.29E-06	0.34	FMo

*Difference represents Δβ. q-values represent the False Discovery Rate adjusted p-values

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Table II

Differentially methylated loci between maternal and fetal cells and between monocytes and macrophages

(A) Maternal *vs*. Fetal		(B) Monocyte (Mo) *vs*. Macrophage (Mϕ)					
Gene	**Direction**	**Gene**	**Direction**	**Gene**	**Direction**	**Gene**	**Direction**
ACTN3	Maternal	A2M	Mo	IL32	Mo	FAM83F	Mϕ
DUOX2	Maternal	ABI3	Mo	KRT23	Mo	FCGR3B	Mϕ
INCA1	Maternal	ABI3	Mo	LILRB5	Mo	FGR	Mϕ
PLEK2	Maternal	ANKRD9	Mo	LOC283487	Mo	FLJ37396	Mϕ
POU3F1	Maternal	ARL4	Mo	LOC92689	Mo	FXYD1	Mϕ
TFAP2E	Maternal	ATF5	Mo	MEFV	Mo	GNLY	Mϕ
TMEM102	Maternal	BIRC4BP	Mo	MGC4618	Mo	GPR21	Mϕ
WT1	Maternal	BLNK	Mo	NUMA1	Mo	GPR21	Mϕ
ACOT11	Fetal	C1QA	Mo	NYX	Mo	GSG1	Mϕ
AKR1C3	Fetal	C1QA	Mo	PIB5PA	Mo	GUCY1B2	Mϕ
CHRNE	Fetal	C1QC	Mo	PIP	Mo	HP	Mϕ
EDARADD	Fetal	C1QC	Mo	PLEKHG5	Mo	ICAM2	Mϕ
FLJ46365	Fetal	C1S	Mo	PVALB	Mo	ICAM3	Mϕ
LAG3	Fetal	C20orf55	Mo	RNASE1	Mo	IL12RB2	Mϕ
PRKG2	Fetal	C3	Mo	RNASE1	Mo	**IL1B**	Mϕ
		C6orf188	Mo	RNF36	Mo	KCNE1	Mϕ
		CCDC57	Mo	RUFY3	Mo	LAIR2	Mϕ
		CCL13	Mo	SDC4	Mo	LCK	Mϕ
		CCL14	Mo	SLC7A7	Mo	LMO2	Mϕ
		CCL5	Mo	SN	Mo	LMO2	Mϕ
		CD163	Mo	SNX8	Mo	LOC144501	Mϕ
		CD226	Mo	SORBS3	Mo	LOC339789	Mϕ
		CD300LG	Mo	TADA3L	Mo	LTBP3	Mϕ
		CD4	Mo	TBC1D14	Mo	MFAP4	Mϕ
		CEBPG	Mo	TIMD4	Mo	MGC23244	Mϕ
		CFLAR	Mo	TNFSF12-TNFSF13	Mo	MKNK2	Mϕ
		CMKLR1	Mo	WDR58	Mo	MPHOSPH9	Mϕ

(A) Maternal *vs*. Fetal				(B) Monocyte (Mo) *vs*. Macrophage (Mϕ)			
Gene	**Direction**	**Gene**	**Direction**	**Gene**	**Direction**	**Gene**	**Direction**
		CTNNAL1	Mo	XLKD1	Mo	NFE2	Mϕ
		CTNND1	Mo	ASGR2	Mϕ	NKG7	Mϕ
		CTSK	Mo	AZU1	Mϕ	NR1I2	Mϕ
		CTSZ	Mo	BRE	Mϕ	OGFR	Mϕ
		DCHS1	Mo	C10orf91	Mϕ	P2RX1	Mϕ
		DIO1	Mo	C12orf25	Mϕ	P2RY2	Mϕ
		EIF2C4	Mo	CBFA2T3	Mϕ	PLAC8	Mϕ
		ENG	Mo	CCIN	Mϕ	PPP2R4	Mϕ
		FCGR2A	Mo	CD48	Mϕ	PRR5	Mϕ
		FCGR3A	Mo	CD5	Mϕ	PRTN3	Mϕ
		FGD4	Mo	CD6	Mϕ	PTPN7	Mϕ
		FLJ20581	Mo	CD6	Mϕ	PTPRCAP	Mϕ
		FLJ38159	Mo	CDKN2B	Mϕ	PTPRCAP	Mϕ
		FOLR2	Mo	CDKN2B	Mϕ	ROBO4	Mϕ
		GABARAP	Mo	CDKN2B	Mϕ	RUNX3	Mϕ
		GAS2L1	Mo	CDKN2B	Mϕ	S100A5	Mϕ
		GIMAP5	Mo	CDKN2B	Mϕ	SCNN1A	Mϕ
		GNA13	Mo	CDKN2B	Mϕ	SERPINF1	Mϕ
		GNPDA1	Mo	CLDN15	Mϕ	SLC45A1	Mϕ
		GPNMB	Mo	CLEC3B	Mϕ	SPN	Mϕ
		GPR92	Mo	CRHBP	Mϕ	TBC1D10C	Mϕ
		GSN	Mo	CRHBP	Mϕ	TBC1D10C	Mϕ
		HAMP	Mo	CSF3R	Mϕ	TLR9	Mϕ
		HMOX1	Mo	CST7	Mϕ	TMC6	Mϕ
		HNMT	Mo	F12	Mϕ	TSPAN32	Mϕ
		HTR2B	Mo	F2RL2	Mϕ	VAV1	Mϕ
		HYAL2	Mo				

Bold: loci chosen for follow-up study by pyrosequencing.

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Table III

Top 20 biological processes enriched in monocyte vs. macrophage comparison

	Biologic Process	# Enriched Genes/# Genes in GO	q-value
MMo *vs.* dMϕ	defense response	34/477	3.80E-14
	immune response	32/522	1.46E-11
	immune system process	35/750	1.48E-09
	response to wounding	25/389	2.92E-09
	inflammatory response	21/268	3.25E-09
	acute inflammatory response	12/65	4.05E-09
	response to external stimulus	29/615	5.44E-08
	response to stimulus	56/2024	5.93E-08
	response to stress	40/1142	7.34E-08
	regulation of response to stimulus	16/215	1.16E-06
	positive regulation of immune system process	12/130	7.32E-06
	regulation of immune response	12/130	7.32E-06
	extracellular region	43/1425	1.26E-05
	leukocyte mediated immunity	10/87	1.10E-05
	cell activation	15/233	1.70E-05
	regulation of leukocyte mediated immunity	7/36	2.08E-05
	positive regulation of response to stimulus	11/121	2.32E-05
	lymphocyte mediated immunity	9/78	3.61E-05
	activation of immune response	8/58	3.75E-05
	immune effector process	11/130	4.05E-05
FMo *vs.* HC	immune response	76/522	8.10E-09
	defense response	71/477	8.10E-09
	immune system process	94/750	4.16E-08
	response to wounding	58/389	3.35E-07
	response to external stimulus	79/615	3.35E-07
	inflammatory response	45/268	5.33E-07
	acute inflammatory response	18/65	2.29E-05
	response to stimulus	179/2024	1.09E-04
	activation of plasma proteins during acute inflammatory response	12/33	1.09E-04
	humoral immune response	16/61	1.95E-04
	complement activation	11/32	5.26E-04
	innate immune response	22/118	8.34E-04
	cell adhesion	64/572	8.36E-04
	biological adhesion	64/572	8.36E-04
	activation of immune response	14/58	1.83E-03
	complement activation, classical pathway	9/25	2.00E-03
	positive regulation of immune response	17/83	2.03E-03
	immune effector process	22/130	2.75E-03
	positive regulation of response to stimulus	21/121	2.75E-03

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Biologic Process	# Enriched Genes/# Genes in GO	q-value
leukocyte mediated immunity	17/87	3.14E-03