Human epigenetic alterations in *Mycobacterium tuberculosis* infection: a novel platform to eavesdrop interactions between *M tuberculosis* and host immunity

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KEY MESSAGES

1. *Mycobacterium tuberculosis* (MTB) infection altered the methylation levels of inflammatory genes in human macrophages.
2. The induced methylations of the host genes were strain- and host-dependent.
3. The methylation profile of active MTB disease, latent MTB infection, and healthy control groups were distinct. These signatures may potentially be further evaluated as biomarkers in the diagnosis of MTB infection in a clinical setting.
4. The study offers new insights into epigenetic changes in modulating the immune response in MTB infections.

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Introduction

DNA methylation regulates many cellular processes of the human immune system, such as cytokine production, anti-inflammatory response, and cell differentiation. A growing number of diseases and disorders are associated with hypermethylation and hypomethylation of DNA, particularly at the promoter region of functional genes. Functionally, methylation of gene promoter regions impedes the binding of transcriptional proteins and results in transcriptional silencing. Increasing scientific attention has focused on promoter hypermethylation in host cells as a result of bacterial invasion.[1-3]

Mycobacterium tuberculosis (MTB) is an intracellular pathogen that can evade host immunity and survive for a long period of time within human macrophages. Successful bacterial clearance/containment depends upon the activation and appropriate regulation of innate and adaptive immunity pathways, in which macrophages play multiple important roles. TLR-2 activates downstream intracellular signals through MyD88 signalling peptide, leading to the rapid production of cytokines, including IL-1β, in MTB infection. Subsequent macrophage activation and production of pro- and anti-inflammatory cytokines recruit inflammatory cells (T cells, neutrophils, and NK cells) to the area of infection and coordinate the inflammatory and adaptive immune response to MTB.

The epigenetic interaction between MTB infection and host immunity has not been studied. This study aimed to (1) generate the methylation profile of human macrophages before and after infection with MTB so as to correlate MTB infection and methylation alterations, (2) investigate MTB strain dependence on the resulting methylation profiles, and (3) investigate the methylation profiles at different stages of progression of MTB infection by studying macrophages from healthy humans, individuals with latent MTB, and patients with active MTB. The induced hypermethylation could influence the immune response such that MTB intracellular survival and/or pathogenesis is facilitated.

Methods

M tuberculosis H37Rv (ATCC 27294) and 15 clinical isolates of MTB were used: three MDR (INHR, RIFR), three sensitive (INHS, RIFS), three Beijing/W, three non-Beijing/W, and three disseminated strains. The phenotypes and genotypes were respectively confirmed by MIC and DTM-PCR method as described.[4]

Ethics approval was obtained from the Joint CUHK-NTEC Clinical Research Ethics Committee. Whole blood specimens (25 mL) were collected from four healthy controls, three individuals with latent MTB, and three patients with active MTB for monocyte isolation and for QuantiFERON TB-Gold Test (to distinguish between healthy and latent individuals) according to the manufacturer's

instructions.

PBMCs were isolated from 20 mL of whole blood using the Ficoll-Hypaque column. The monocytes were purified from PBMC by removal of detached cells. The monocytes were allowed to differentiate into macrophages for DNA harvesting and downstream experiments.

To induce differentiation to macrophages, 1×10^6 THP-1 cells were first treated with 5 ng/ml PMA. Standard MTB strain H37Rv and the 15 clinical isolates were cultured in Middlebrook 7H9 (BD Biosciences, USA) at 37°C, 5% CO_2 until the cultures reached McFarland 1 (about 10^7 CFU/mL). The MTB cells were harvested. The pellet was re-suspended in RPMI medium, and subsequently added to the macrophages. The macrophages were allowed to engulf the bacilli for 2 hours and the excess free-floating bacilli were removed. The bacterial load in the macrophages was determined by plate counts of the lysed macrophages. The number of intracellular bacilli: macrophage ratio was estimated at 3:1 (range, 2.1 to 3.7 in all subsequent experiments). The RPMI medium was replaced once with fresh medium at 24 hours after infection. Control cultures of THP-1 cells or isolated macrophages were set up with identical corresponding treatments but without MTB infection. DNA was harvested at 72 hours for methylation array study.

The filtered medium from the infected THP-1 differentiated macrophages was analysed for TNF-alpha and IL-1-beta by the Luminex System using the multiplex cytokine assay (Merck Millipore, USA). Results of each cytokine level are expressed as picograms per mL of the filtered medium.

The Human Inflammatory Response Methyl-Profiler DNA Methylation PCR Arrays (SABiosciences, Germany) examined the promoter methylation status of a panel of 24 genes whose involvement in inflammation has been well documented in anti- or pro-inflammatory responses. DNA extracts from macrophages were processed according to the instructions of the manufacturer. DNA was quantified by real-time PCR in each individual enzyme reaction using primers that flanked a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA were subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt method.

Methylated DNA samples from macrophages were extracted following the instructions of the MethylMiner Methylated DNA Enrichment Kit (Applied Biosystems, USA). The Methylated DNA immunoprecipitation–isolated DNA was amplified by GenomePlex Whole Genome Amplification Kit (Sigma, USA) and was then consecutively labelled using the Genomic DNA Enzymatic Labeling Kit. Cy5-labeled methylated DNA and Cy3-labelled input genomic DNA were competitively hybridised onto Agilent Human CpG Island Microarray (consists of 237 220 probes to cover 27 800 CpG island sequences in the human genome), using Oligo aCGH/ChIP-on-chip Hybridization Kit, according to the manufacturer's instructions. The data were normalised to baseline, by dividing Cy5 signal (methylated fragment) by Cy3 signal (total genomic DNA), $\log_2$ transformed and normalised for all the arrays. Data are represented as $\log_2$ (methylated DNA/total genomic DNA) after inter-array normalisation for each probe of the array. Higher $\log_2$ ratios corresponded to higher methylation levels. An ANOVA tool was used to calculate the fold change in methylation for each probe between samples. Signal comparison between macrophages before and after MTB infection, between different strains of MTB, with and without inhibitor treatments, would yield the methylation alteration profiles. Differential methylation with $\log_2 \geq 2$ (~4-fold) signal ratios was used as a cut-off to identify hyper/hypo-methylations.

The relative fractions of methylated and unmethylated DNA were determined using the integrated Excel-based templates downloaded from http://www.sabiosciences.com/dna_methylation_data_analysis.php. The methylated rate was calculated by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt values of the EpiTect Methyl II PCR Array results. Clustering analysis of methylation levels was performed using online software of the Qiagen website (http://www.sabiosciences.com/dna_methylation_heatmap.php). For the Agilent Human CpG Island Microarray, the array images were digitalised by Agilent Feature Extraction 11.0 (Agilent Technologies, Santa Clara [CA], USA). Data obtained from Feature Extraction were imported to Partek Genomic suite 6.5 for further normalisation and ANOVA computation. The significant features ($P<0.05$) annotated with genes or genes' promoter regions were extracted and imported to Genespring 12.5 for pathway analysis. Comparisons between groups were performed using Mann-Whitney *U* test or Kruskal-Wallis test as appropriate. A P value of ≤0.05 was considered statistically significant.

Results

MTB (H37Rv) infection altered the methylation profile of THP-1 macrophages

H37Rv infection altered the methylation profile of THP-1 macrophages and at 72 hours post-infection: the hypermethylated levels of IL12B, IL4R and the intermedium methylated level of CCL25, IL4R and IL13Ra were noted (Fig 1). The individual promoter methylation levels were compared with those of clinical strains in a later section of the results.

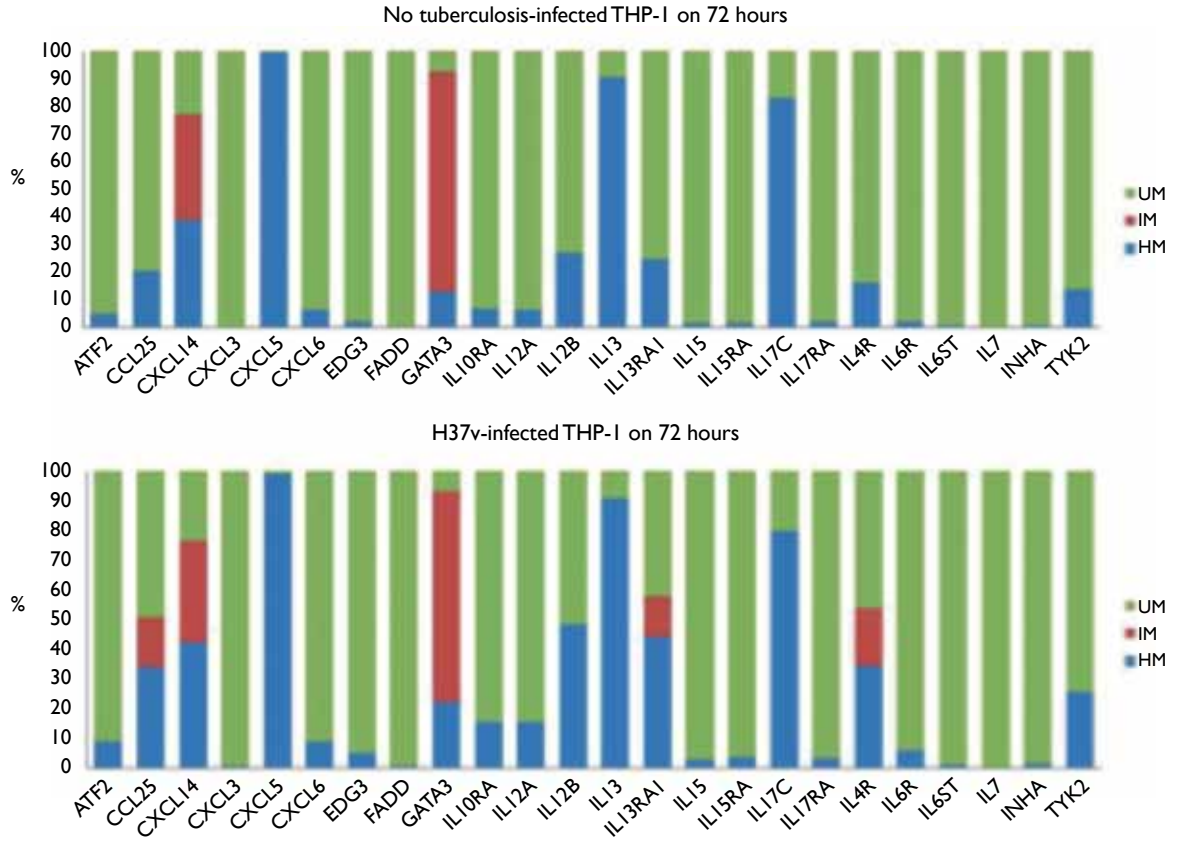

FIG 1. Graphical representation of methylation levels of 24 inflammatory genes in THP -1 macrophages at 72 hours with or without infection with H37Rv*

* Percentages of different methylated fraction of DNA are used to interpret gene methylation status. UM denotes the fraction of input genomic DNA containing no methylated CpG sites, IM intermedium methylated CpG sites, and HM hypermethylated CpG sites in the amplified region of the targeted gene

Methylation profile of different clinical MTB strains on THP-1 macrophages

The level of hypermethylation for each gene from the four groups of strains, namely Beijing/W, non-Beijing/W, strains from disseminated disease, and H37Rv standard strain, were compared. Genes that demonstrated significant difference in methylation levels between these groups are shown in Fig 2. The hypermethylation level of the genes that were significantly different included those of IL17RA ($P<0.027$), IL15RA ($P<0.024$), IL4R ($P<0.024$), IL6R ($P<0.024$), and IL6ST ($P<0.024$) in Beijing/W strains and IL17RA ($P<0.027$), IL6R ($P<0.024$), and IL6ST ($P<0.024$) in non-Beijing/W strains, compared with uninfected macrophages. Only the hypermethylation level of IL4R ($P<0.009$) in THP-1 macrophages showed significant difference in infection with Beijing/W and non-Beijing/W strains. MTB strain from patients with pulmonary disease versus disseminated disease induced significantly higher hypermethylation of IL12A ($P<0.048$) and IL7 ($P<0.048$), and of IL13RA1 ($P<0.048$) in THP-1 infected macrophages. No significant difference was noted between sensitive and resistant MTB isolates (data not shown).

Methylation profile of human macrophages in different patient cohorts

The methylation level in human macrophages of the active MTB disease, latent MTB infection, and healthy control cohorts was examined using Human Inflammatory Response Methyl-Profiler DNA Methylation PCR Array. Significant DNA hypomethylation of FADD ($P<0.023$) and IL17RA ($P<0.049$) was noted in the active MTB disease cohort. Methylated DNA immunoprecipitation in combination with CpG island arrays was performed to characterise at high resolution the DNA

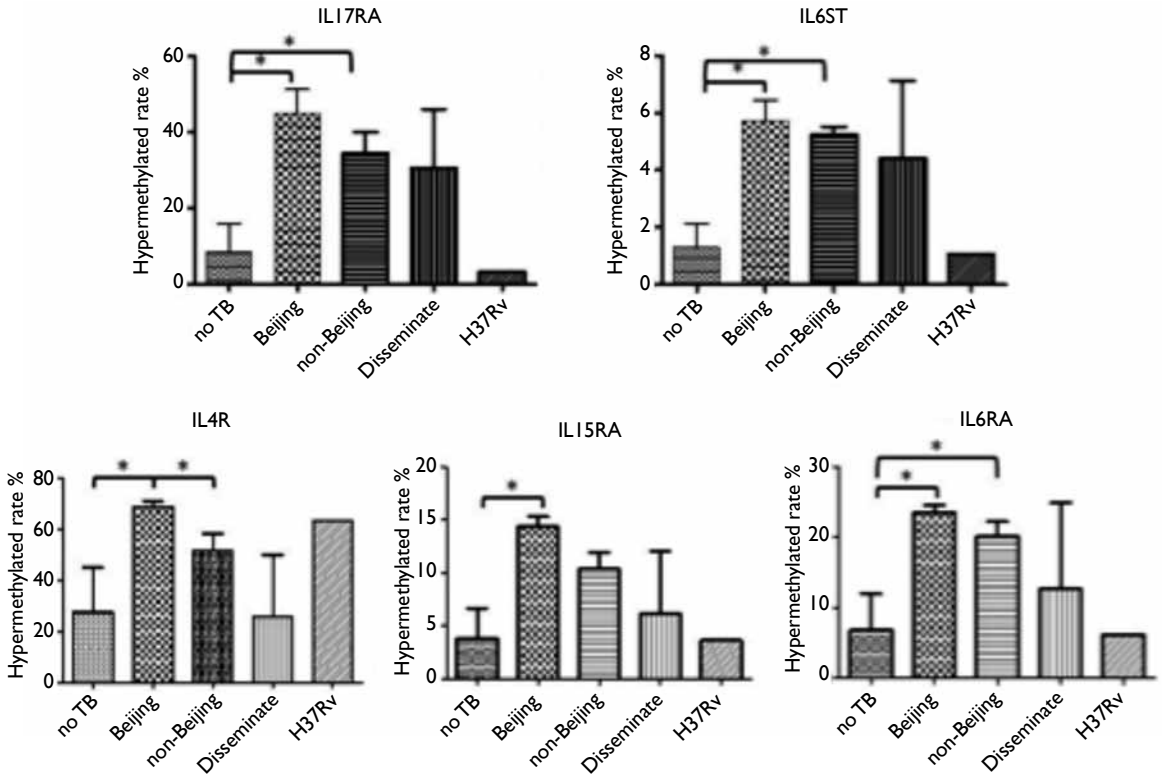

FIG 2. Hypermethylation level in THP-1 macrophages infected with different clinical *Mycobacterium tuberculosis* (MTB) strains, H37Rv-infected, and controls of uninfected THP-1 macrophages. The hypermethylated rate is calculated by comparing the amount in each digest with that of a mock (no enzymes added) digest using the ΔCt values of the EpiTect Methyl II PCR Array

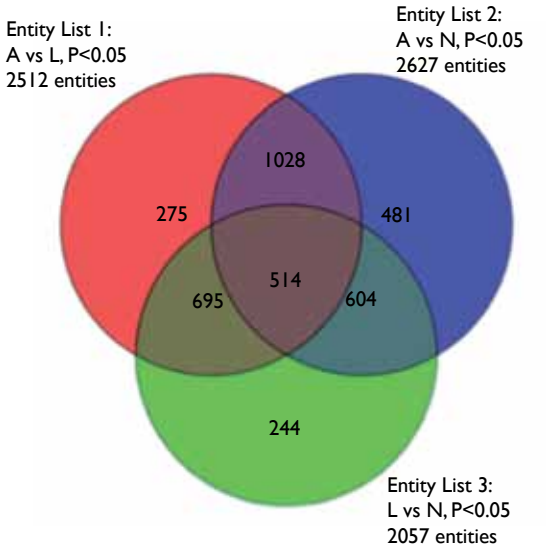

FIG 3. Venn diagram illustrates groups of genes that are differentially methylated when two of the three groups are compared using one-way ANOVA (P<0.05). The red area represented the genes differentially expressed between active and latent *Mycobacterium tuberculosis* (MTB) groups, whereas the blue area between active MTB and healthy control groups and the green area between latent MTB and healthy control groups.

methylation changes that occurred in the genomes of the three cohorts. A Venn diagram illustrates the groups of genes that are differentially methylated comparing two of the three groups (P<0.05, Fig 3). Potentially these may provide specific signatures to distinguish these cohorts for clinical diagnosis.

Discussion

According to the pathway analysis, some biological process in MTB infection was affected by the altered methylation level of different MTB cohorts. Differences between active and latent MTB groups included membrane change through fatty acid and glycan metabolite, signal transduction through GPCR, Notch and Wnt, as well as biological processes such as apoptosis and cell cycle. Changes in the immune response signalling pathway were highly associated with active MTB group, compared with healthy controls. However, difference between the latent MTB group and healthy controls was few. The IL17 signalling pathway was shown to contribute to the inflammatory response against primary MTB infection. MTB infection in active and latent MTB groups could suppress the inflammatory reaction by enhancing the hypermethylated level of IL17RA

and downregulating that of IL17D, compared with healthy controls. Only the hypermethylated level of IL17RD and IL17C was differentially expressed between the active and latent MTB groups. The mechanisms of methylation in IL17 family associated with MTB infection warrant further study.

IL6 plays a key role in MTB infection. MTB regulates host IL6 production to inhibit type I interferon signalling. MTB infection induced IL6R promoter partial methylation in the macrophages, but how the methylated epigenomic modification affects IL6R expression and function remains elusive. In addition, IL-6 is one of the major cytokines responsible for differentiating T-helper lymphocytes into Th17 cells, further confirming the interrelationship and importance of these two pathways in MTB infection.

DNA methylation has been the most studied epigenetic alteration and an important biomarker in cancer diagnosis, but little is known about its role in MTB infection. To investigate DNA methylation changes associated with MTB infection, the common methylation profile of infection with different clinical MTB strains in different MTB hosts was studied. The results provide insight into the molecular mechanisms and biological pathways that underlie MTB infection and correlate CpG island methylation status with anti- or pro-inflammatory responses.

Our data confirm the occurrence of DNA hypermethylation of inflammatory genes in different clinical MTB strains and MTB groups, and these profiles may be biosignatures that signify latency or disease. Our studies also lead to the discovery of novel methylated genes that could be implicated in MTB infection.

The hypothesis that MTB infection can alter the methylation profile of the host cells is proven. The methylation profiles of varying factors, including the duration of infection, strain type, and host type were obtained. Our study proved that the induced methylation profiles are strain-dependent and host-dependent. The resulting methylation-profiles will enhance our understanding of MTB and human immunity, substantiate potential strain-dependence and host-dependence on the outcome of infection, and aid future drug design. There is potential application of the methylation profile as a biomarker in the diagnosis of MTB infections.

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