

# Gene activation precedes DNA demethylation in response to infection in human dendritic cells

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DNA methylation is considered to be a relatively stable epigenetic mark. However, a growing body of evidence indicates that DNA methylation levels can change rapidly; for example, in innate immune cells facing an infectious agent. Nevertheless, the causal relationship between changes in DNA methylation and gene expression during infection remains to be elucidated. Here, we generated time-course data on DNA methylation, gene expression, and chromatin accessibility patterns during infection of human dendritic cells with *Mycobacterium tuberculosis*. We found that the immune response to infection is accompanied by active demethylation of thousands of CpG sites overlapping distal enhancer elements. However, virtually all changes in gene expression in response to infection occur before detectable changes in DNA methylation, indicating that the observed losses in methylation are a downstream consequence of transcriptional activation. Footprinting analysis revealed that immune-related transcription factors (TFs), such as NF- $\kappa$ B/Rel, are recruited to enhancer elements before the observed losses in methylation, suggesting that DNA demethylation is mediated by TF binding to cis-acting elements. Collectively, our results show that DNA demethylation plays a limited role to the establishment of the core regulatory program engaged upon infection.

DNA methylation | epigenetic | immune responses | tuberculosis | dendritic cells

Innate immune cells, such as dendritic cells (DCs) and macrophages, are the first mediators recruited in response to an invading pathogen. Upon stimulation, these cells considerably shift their transcriptional program, activating hundreds of genes involved in immune-related processes in a rapid and highly choreographed fashion. This is achieved through the binding of signal-dependent transcription factors (TFs), including NF- $\kappa$ B/Rel, AP-1, and IFN regulatory factors (IRFs), to gene regulatory regions of the genome where recruitment of various coactivators is initiated (1, 2). Alterations to the epigenome, such as histone modifications and DNA methylation, are recognized as important permissive or suppressive factors that play an integral role in modulating access of TFs to cis-acting DNA regulatory elements via the regulation of chromatin dynamics.

Many studies have highlighted the importance of histone modifications in regulating complex gene expression programs underlying immune responses (3, 4). However, the exact role that DNA methylation plays in innate immune response regulation remains ambiguous. We have previously shown that infection of postmitotic DCs is associated with an active loss of methylation at enhancers and that such demethylation events are strongly predictive of changes in expression levels of nearby genes (5). Many other studies correlate these two processes (6–13), but it remains unclear whether altered methylation patterns directly invoke transcriptional modulation or whether such patterns are the downstream consequence of TF binding to regulatory regions. Thus, the causal relationship between changes in DNA methylation and gene expression during infection remains unresolved. To address this question, we characterized in parallel genome-wide patterns of DNA methylation, gene expression, and chromatin accessibility in

noninfected and *Mycobacterium tuberculosis* (MTB)-infected DCs at multiple time points. Our results show that the loss of DNA methylation observed in response to infection is not required for the activation of most enhancer elements and that, instead, demethylation is a downstream consequence of TF binding.

## Results

**Bacterial Infection Induces Stable DNA Demethylation at Enhancers of Dendritic Cells.** We infected monocyte-derived DCs from four healthy individuals with a live virulent strain of *M. tuberculosis* (MTB) for 2, 18, 48, and 72 h. We chose to work with DCs because they play an essential, nonredundant role in protective immunity to TB (14). In the absence of DCs, CD4<sup>+</sup> T cell responses are impaired and bacterial load is uncontrolled (15–17), arguing for an important role for DCs in resistance to mycobacteria infections (18). At each time-point, we obtained single base pair resolution DNA methylation levels for over 130,000 CpG sites using a customized capture-based bisulfite sequencing panel (SeqCap Epi, ref. 19; see *Materials and Methods*), in matched noninfected and MTB-infected DCs. Our customized SeqCap

## Significance

Immune response to infection is accompanied by active demethylation of thousands of CpG sites. Yet, the causal relationship between changes in DNA methylation and gene expression during infection remains to be elucidated. Here, we investigated the role of DNA methylation in the regulation of innate immune responses to bacterial infections. We found that virtually all changes in gene expression in response to infection occur prior to detectable alterations in the methylome. We also found that the binding of most infection-induced transcription factors precedes loss of methylation. Collectively, our results show that changes in methylation are a downstream consequence of transcription factor binding, and not essential for the establishment of the core regulatory program engaged upon infection.

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The authors declare no conflict of interest.

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Data deposition: Data generated in this study have been deposited in the NCBI Gene Expression Omnibus (GEO; <https://www.ncbi.nlm.nih.gov/geo/>) under accession numbers GSE116406 (ATAC-seq), GSE116411 (ChIP-seq), GSE116405 (RNA-seq), and GSE116399 (SeqCap Epi).

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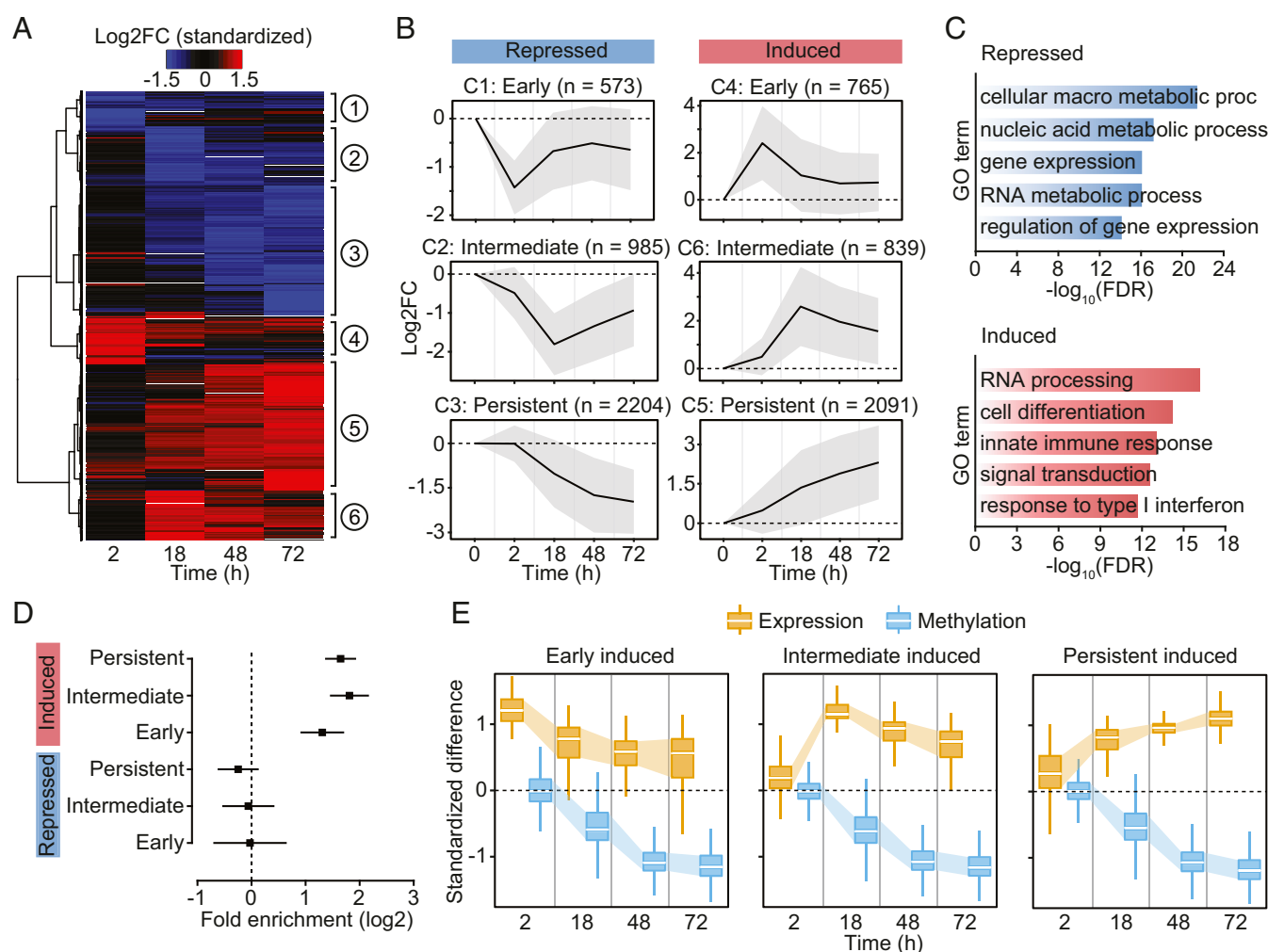


methylation, changes in gene expression in response to live and heat-inactivated MTB were strongly correlated ( $r \geq 0.94$ ; *SI Appendix, Fig. S6B*). We next grouped the set of DE genes across the time course (7,457 in total) into six distinct temporal expression clusters (Fig. 2 *A* and *B* and *Dataset S3*). These clusters cover a variety of differential expression patterns, including genes which show increasing up-regulation over time (DE Cluster 5: Persistent induced;  $n = 2,091$ ) to genes in which the highest levels of expression occur at 2 or 18 h followed by a decrease toward basal levels (DE Cluster 4: Early induced [ $n = 765$ ], and DE Cluster 6: Intermediate induced [ $n = 839$ ], respectively) (Fig. 2*B*). Gene ontology (GO) enrichment analysis revealed that induced genes were strongly enriched among GO terms directly related to immune function, including defense response ( $\text{FDR} = 1.2 \times 10^{-11}$ ) and response to cytokine ( $\text{FDR} = 8.2 \times 10^{-12}$ ), whereas repressed genes were primarily enriched for gene sets associated with metabolic processes (Fig. 2*C* and *Dataset S4*).

We next tested whether genes located near DM sites—particularly focusing on those sites exhibiting a stable loss of methylation (i.e., Cluster 3 in Fig. 1 *C* and *D*)—were more likely to be differentially expressed upon MTB infection relative to all

genes in the genome. We found that genes associated with one or more DM sites were strongly enriched among genes that were up-regulated in response to infection, regardless of the time point at which expression levels started to change: early (2.5-fold,  $P = 3.23 \times 10^{-11}$ ), intermediate (3.5-fold,  $P = 3.59 \times 10^{-25}$ ), and persistent (3.1-fold,  $P = 3.80 \times 10^{-33}$ ) (Fig. 2 *D* and *E*).

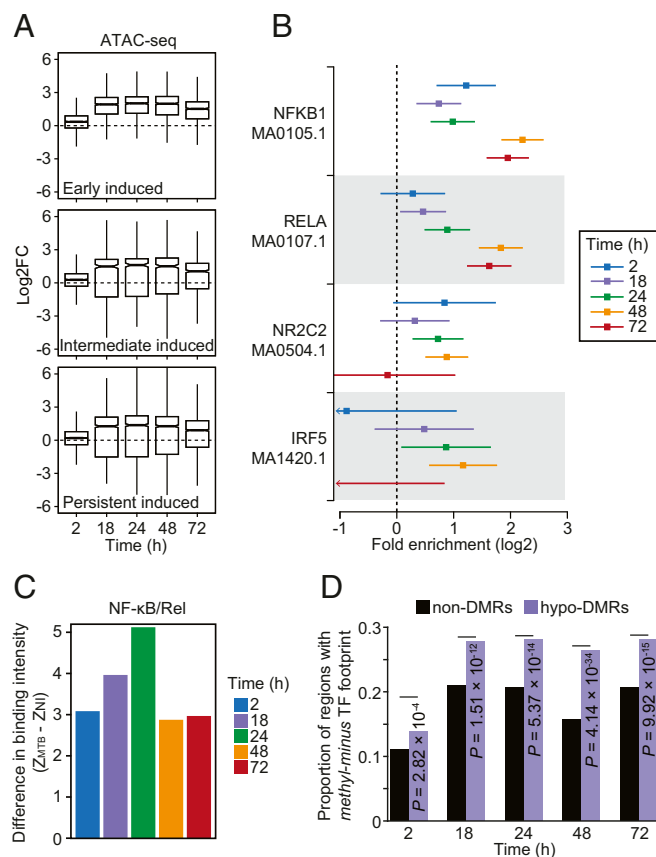
If demethylation is required for the activation of enhancer elements and the subsequent up-regulation of their target genes, we would expect demethylation to occur prior to changes in gene expression; instead, we found the opposite pattern. Among up-regulated genes associated with DM sites ( $n = 593$ ), 37% exhibited at least a twofold increase in gene expression levels at 2 h postinfection, although differential methylation did not begin to be detectable until 18 h postinfection (Fig. 2*E*). To better delineate the relationship between changes in DNA methylation and changes in gene expression, we collected data from three individuals at additional early/intermediate time points—4, 5, and 6 h postinfection. Again, we did not detect changes in DNA methylation until after 6 h postinfection (*SI Appendix, Fig. S7*). However, by 6 h, 5,110 genes are already differentially expressed at a stringent FDR of 1% and  $|\log_2\text{FC}| > 1$ . Among the set of



**Fig. 2.** (A) Heatmap of differences in expression (standardized log<sub>2</sub> fold changes) constructed using unsupervised hierarchical clustering of the 7,457 differentially expressed genes (identified at any time point) across four time points after MTB infection. (B) Mean log<sub>2</sub> fold expression changes of genes in each cluster across all time points; shading denotes  $\pm 1$  SD. For visualization purposes, we also show the 0 h time point, where we expect no changes in expression. (C) Gene ontology enrichment analyses among genes that are repressed or induced in response to MTB infection. (D) Enrichment (in log<sub>2</sub>; x-axis) of differentially expressed genes associated with differentially methylated CpG sites (Cluster 3). Error bars show 95% confidence intervals for the enrichment estimates. (E) Boxplots showing the distribution of standardized differences in methylation of DM sites in Cluster 3 (blue) along with the corresponding standardized differences in expression of the associated genes (orange), across all time points.







**Fig. 4.** (A) Boxplots showing the distribution of logtwofold changes in chromatin accessibility between noninfected and MTB-infected DCs across the five time points of infection (2, 4, 18, 24, 48, and 72 h) for open chromatin regions associated with the three classes of induced genes described in Fig. 2 A and B. (B) TF binding motifs for which the number of well-supported footprints (posterior probability > 0.99) within hypomethylated regions (i.e., the combined set of DM sites for all four time-points) were enriched (FDR < 0.01) relative to non-DMRs (with 250 bp flanking the start and end) in MTB-infected DCs. The enrichment factors (x-axis) are shown in a log2 scale and error bars reflect the 95% confidence intervals. A complete list of all TF binding motifs for which footprints are enriched within hypomethylated regions can be found in [Dataset S5](#). (C) Barplots showing significant differences in TF occupancy score predictions for NF-κB/Rel motifs between MTB-infected and noninfected DCs ( $Z_{MTB} - Z_{NI}$ ; y-axis; see *Materials and Methods*) across all time points (x-axis). A positive Z-score difference indicates increased TF binding in hypomethylated regions after MTB infection. (D) Proportion of regions that overlap a methylation-sensitive ("methyl-minus"; reported in Yin et al. [33]) TF footprint (y-axis) observed among non-DMRs and hypomethylated regions (or hypo-DMRs; see *Materials and Methods*).

First, there might be subtle changes in methylation that occur at early time points that we cannot detect given our small sample sizes, or changes in methylation that occur in regions not covered by our targeted array. Second, our data do not allow us to distinguish between 5mC and 5hmC. Thus, it is possible that the gain of 5hmC in DM sites, which do not show a loss of 5mC at 2 h postinfection, precedes the activation of certain enhancers, as was recently suggested in T cells (8). In *SI Appendix, Fig. S13*, we provide a schematic representation of our proposed model that links changes in DNA methylation with changes in gene expression in the context of an innate immune response.

The observed changes in methylation most likely occur via TET2-mediated active demethylation, as previously shown (5, 23, 36). Consistent with this hypothesis, we found that CpG sites that lose methylation upon infection display high levels of 5hmC at baseline, suggesting that these regions are actively bound by

TET2 even before infection. Moreover, TET2 is strongly up-regulated 2 h after infection (~2.5 fold; *SI Appendix, Fig. S14*). 5hmC could be a stable intermediate that serves as an epigenetic priming mark, ensuring the rapid response of DCs against infection (25–27, 36–39). Interestingly, albeit not significant, we noticed a clear trend toward higher levels of 5hmC among early induced genes compared with later induced genes ( $P = 0.1$ , *SI Appendix, Fig. S15*), suggesting that 5hmC could be particularly important for the up-regulation of early response genes.

Using footprint analysis, we show that NF-κB/Rel, a master regulator of inflammation, is recruited to hypomethylated regions as soon as 2 h postinfection. This finding is consistent with ChIP-seq data collected from mouse macrophages stimulated with Kdo2-Lipid A (KLA), a highly specific TLR4 agonist, which shows that the NF-κB subunit p65 is rapidly recruited to enhancer elements within 1 h poststimulation (40). We hypothesize that the rapid binding of NF-κB, and of other immune-induced TFs, instigates chromatin opening which is then followed by the recruitment of histone acetyltransferase p300 and the subsequent deposition of activating H3K27ac marks in these regions (41). Interestingly, p300 can acetylate TET2, conferring enhanced enzyme activity (42), which might account for the eventual loss of DNA methylation in response to infection. Incorporating time-course ChIP-Seq data for NF-κB (or other immune-induced TFs) with methylation and gene expression data will be an important next step to validate the link between TF binding, gene activation, and losses in DNA methylation.

Our results indicate that most changes in gene expression that occur in response to infection are independent of DNA demethylation, further supporting a lack of repressive capacity of DNA methylation (43). Similar to previous findings (36, 44–49), our results further reinforce the idea that site-specific regulation of DNA demethylation is mediated by TFs that bind to cis-acting sequences. Interestingly, several recent reports have shown that other epigenetic modifications, such as the H3K4me1 enhancer mark, have a similar passive regulatory function (50–52). However, our results do not exclude the possibility that demethylation might be necessary for the binding of a second wave of TFs that only play a role at later stages of infection. In agreement with this hypothesis, we observed a significant enrichment of binding of TFs known to preferentially bind unmethylated CpGs in hypomethylated regions, primarily at later stages postinfection. Ultimately, this suggests that DNA demethylation is not a key regulatory mechanism of early innate immune responses but that it could still play a role in fine-tuning later innate immune responses by facilitating the binding of methylation-sensitive TFs at enhancers. This conclusion should be further validated on a cellular system where one can prevent demethylation to occur upon infection (e.g., by using TET2-deficient cells) to study the downstream impact of such changes in the overall immune response.

After an infection is cleared, TFs are expected to unbind, and gene expression as well as DNA methylation levels are anticipated to return to basal state. However, our 72-h time course study of DNA methylation shows that levels of methylation at DM sites gradually decrease with time postinfection and never revert back to higher levels. Thus, we speculate that demethylation in response to infection could have a specific biological role in innate immune memory (53–56), and that regions that stably lose methylation may act as primed enhancers, potentially allowing for a faster response to a secondary infection.

## Materials and Methods

Details of the experimental and statistical procedures can be found in *SI Appendix, SI Materials and Methods*. Buffy coats from healthy donors were purchased from Indiana Blood Center and all participants signed a written consent. The ethics committee at the CHU Sainte-Justine approved the project (protocol #4023). Blood mononuclear cells from each donor were isolated by Ficoll-Paque centrifugation and blood monocytes were purified from peripheral blood mononuclear cells (PBMCs) by positive selection with magnetic CD14 MicroBeads (Miltenyi Biotec). Monocytes were then derived into DCs (5) or macrophages (30) and subsequently infected with MTB or

*Salmonella typhimurium*. RNA-seq libraries were prepared using the TruSeq RNA Sample Prep Kit v2. ATAC-seq libraries were generated from 50,000 cells, as previously described (32). SeqCap Epi and whole-genome bisulfite sequencing libraries were generated using the KAPA Library Preparation Kit. Bisulfite sequencing reads were mapped to the human reference genome using Bismark (57), and MTB-induced differences in methylation were identified using the R package DSS (21), which implements the BSmooth smoothing method (58). We used ClueGO (59) to test for enrichment of functionally annotated gene sets among differentially expressed genes. TF

footprinting analyses were performed using the Centridal algorithm (5) and JASPAR annotated human TF binding motifs (60).

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