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-κB/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

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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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Epi panel interrogates 33,059 regions highly enriched among putative enhancer elements [58% are associated with the H3K4me1 enhancer mark (20); SI Appendix, Fig. S1A], which are the main targets of methylation changes in response to infection (5). In total, we generated ~717 million single-end reads (mean = 17.5 million reads per sample; Dataset S1), resulting in an average coverage of ~70x per CpG site (SI Appendix, Fig. S1B). Methylation values between samples were strongly correlated, attesting to the high quality of the data (SI Appendix, Fig. S1C; median r across all samples = 0.94).

We next assessed temporal changes in methylation levels in response to infection using the DSS software (21). We defined differentially methylated (DM) CpG sites as those showing a significant difference of methylation between infected and noninfected samples at a False Discovery Rate (FDR) < 0.01 and an absolute mean methylation difference above 10%. Using these criteria, we identified 6,174 DM CpG sites across the time course of infection. Consistent with previous findings (5), the vast majority of changes in methylation (87%) were associated with the loss of DNA methylation in infected cells (SI Appendix, Fig. S1A and B).

To evaluate if the losses in methylation induced by MTB infection were specific to DCs, we collected additional methylation profiles on monocyte-derived macrophages from two additional individuals before and after infection with MTB for 20 h. Consistent with our findings in DCs, the vast majority of changes observed in macrophages (~80%) were associated with the loss of DNA methylation in infected cells (SI Appendix, Fig. S2). Moreover, ~30% of the CpG sites changing methylation in response to infection overlapped CpG sites that are also differentially methylated in DCs, which is a 10-fold enrichment compared with random CpG sites from our targeted SeqCap-Epi assay (P = 8.12 × 10^-33; SI Appendix, Fig. S2).

Next, we tested if live bacteria were required to induce the observed changes in DNA methylation. Changes in methylation in response to live and heat-killed MTB were strikingly correlated, particularly at later time-points postinfection (r ≥ 0.84 at 18 h and above; Fig. 1 and SI Appendix, Fig. S3). These results show that DCs do not require exposure to a live pathogen to elicit the overall demethylation response detected in response to infection. Indeed, the stimulation of DCs for 24 h with LPS, which activates Toll-like receptor (TLR) 4, and beta-glucan, a Dectin-1 ligand, and a classical molecule used for trained immunity experiments, is sufficient to induce demethylation at the same CpG sites altered upon MTB infection—albeit at a lower magnitude—suggesting that the activation of single innate immune receptors is sufficient for the induction of active changes in DNA methylation (SI Appendix, Fig. S4). Hierarchical clustering analysis of the DM sites observed when considering samples exposed to either live or heat-killed bacteria showed that >80% of the sites exhibited a gradual loss of methylation over the time course of infection until methylation marks were almost completely erased (DM Cluster 3; Fig. 1 C and D and Dataset S2).

Monocyte-derived DCs do not proliferate in response to infection (5) and, therefore, any observed losses in methylation must occur through an active mechanism involving the ten-eleven translocation (TET) enzymes, a family of enzymes that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) (22). Thus, we used Tet-assisted bisulfite sequencing (TAB-seq) data collected from noninfected DCs (5) to assess if DM sites had significantly different levels of 5hmC compared with non-DM sites. We found that DM sites (Cluster 3) show high levels of 5hmC even before infection (Fig. 1E; 3.2-fold enrichment compared with non-DM sites; Wilcoxon test; P < 1 × 10^-30), suggesting that DM sites are likely prebound by TET enzymes (likely TET2 [23, 24], the most expressed TET enzyme in DCs [SI Appendix, Fig. S5]) and that 5hmC may serve as a stable mark that acts to prime enhancers (25–27).

Up-Regulation of Inflammatory Genes Precedes DNA Demethylation.

We collected RNA-seq data from matched noninfected and infected samples at each time point, for a total of 34 RNA-seq profiles across time-treatment combinations (28) (mean = 42.2 million reads per sample; Dataset S1). The first principal component of the resulting gene expression data accounted for 63% of the variance in our dataset and separated infected and noninfected DCs (SI Appendix, Fig. S6). We found extensive differences in gene expression levels between infected and noninfected DCs: of the 13,956 genes analyzed, 1,987 (14%), 2,041 (14.5%), 4,171 (31.1%), 4,591 (33%), and 5,189 (37%) were differentially expressed (DE) at 2, 18, 48, and 72 h postinfection, respectively (FDR < 0.01 and absolute log2[fold change] > 1; Dataset S3). We also collected RNA-seq data in samples stimulated with heat-inactivated MTB and found that, similar to changes in
methylations, 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. 2A 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; $n = 2,991$) 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. 2B). Gene ontology (GO) enrichment analysis revealed that induced genes were strongly enriched among GO terms directly related to immune function, including defense response (FDR = $1.2 \times 10^{-14}$) and response to cytokine (FDR = $8.2 \times 10^{-10}$), whereas repressed genes were primarily enriched for gene sets associated with metabolic processes (Fig. 2C 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. 1C 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, $P = 3.23 \times 10^{-14}$), intermediate (3.5-fold, $P = 3.59 \times 10^{-25}$), and persistent (3.1-fold, $P = 3.80 \times 10^{-33}$) (Fig. 2D–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. 2E). 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 $|\text{log2FC}| > 1$. Among the set of

**Fig. 2.** (A) Heatmap of differences in expression (standardized logtwofold 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 logtwofold expression changes of genes in each cluster across all time points; shading denotes ±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 log2; 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.
genes associated with both changes in methylation and up-regulated upon infection at any time point, 83.1% show a change in gene expression before any detectable change in methylation. In contrast, only 1.3% (eight genes) show a change in DNA methylation that precedes a statistically detectable change in gene expression (SI Appendix, Figs. S7 and S8), suggesting that no definitive causal relationship between DNA demethylation and gene activation exists.

Given that our SeqCapEpi panel mostly interrogates enhancer elements, we cannot exclude the possibility that rapid changes in methylation nearby early response genes may occur in their promoter region. Thus, we performed whole-genome bisulfite sequencing (WGBS) on three of our samples in matched noninfected and MTB-infected DCs (2 h postinfection). In total, we generated ~1.4 billion paired-end reads, resulting in an average coverage of ~5x per CpG site. Using these data, we found no evidence that promoter regions of differentially expressed genes—regardless of their expression dynamics—significantly changed methylation levels in response to infection compared with nondifferentially expressed genes (SI Appendix, Fig. S9). This finding recapitulates what we have previously reported at 18 h postinfection, where promoter regions appeared to be largely refractory to methylation status changes in response to infection (5).

To confirm that our findings were generalizable to other innate immune cell types and pathogenic infections, we performed a separate time-course analysis of differential methylation in Salmonella-infected macrophages from one additional donor over six time points (Dataset S1). We discovered hundreds of CpG sites that exhibited a progressive loss of methylation over the time course of infection, corroborating our findings in MTB-infected DCs (Fig. 3A). To assess whether demethylation arises after the activation of associated enhancers, we collected ChIP-seq data for acetylation of histone 3 lysine 27 (H3K27ac) at 2 h postinfection, as changes in DNA methylation have yet to occur at this point (29). We found that the deposition of activating H3K27ac marks preceded demethylation at these CpG sites (Fig. 3B). Moreover, using previously published RNA-seq expression data from Salmonella-infected macrophages (30), we found that most genes associated with these sites were upregulated at 2 h postinfection (Fig. 3C), before any changes in methylation. Collectively, these findings indicate that DNA demethylation is not required for the activation of most enhancer elements and that the vast majority of methylation changes induced by infection are a downstream consequence of transcriptional activation.

The Binding of Most Infection-Induced TFs Does Not Require Active Demethylation. We next asked whether MTB-induced gene expression changes were associated with changes in chromatin accessibility. To do so, we profiled regions of open chromatin in noninfected and infected DCs at the same time points (plus one additional time point at 24 h) using ATAC-seq (31, 32). We note, however, two limitations of our bisulfite sequencing data. For visualization purposes, we also show the 0 h time point, where we expect no changes in methylation. (B) Composite plots of patterns of H3K27ac ChIP-seq signals ±5 kb around the midpoints of hypomethylated sites (x-axis) in macrophages at 2 h postinfection with Salmonella. (C) Distribution of logtwofold expression changes (between noninfected and Salmonella-infected macrophages at 2 h) for genes associated with DM sites in A (n = 269).

Discussion

Our results show that bacterial infection leads to marked remodeling of the histome of phagocytic cells. Strikingly, virtually all changes in gene expression in response to infection occurred before detectable alterations in DNA methylation, suggesting that the observed demethylation is a downstream consequence of TF binding and transcriptional activation. This pattern holds true genome-wide as well as when focusing the analyses to genes known to be associated with immunity to TB (SI Appendix, Figs. S11 and S12). We note, however, two limitations of our bisulfite sequencing data.

To investigate the relationship between DNA methylation and TF occupancy, we performed TF footprinting analysis on our target regions (i.e., the set of putative enhancers tested for dynamic DNA methylation). We classified target regions as “hypomethylated regions” (n = 1,877) or “non-differentially methylated regions” (non-DMRs) (n = 31,182) according to whether or not these regions overlap DM CpG sites (from differential methylation Cluster 3, specifically). We found that hypomethylated regions were significantly enriched for the binding of immune-related TFs relative to regions exhibiting consistent methylation levels. These immune-related TFs include several master regulators of the innate immune response, such as NF-xB/Rel, AP-1, STATs, and IRFs. We found increased binding at NF-xB/Rel binding motifs starting at 2 h postinfection, despite the fact that no changes in methylation were observed at such early time points (P = 0.002; Fig. 4C and Dataset S5; see Materials and Methods). A similar pattern was observed for AP-1 (P = 0.01; SI Appendix, Fig. S10). These data show that, while demethylated regions overlap areas bound by immune-induced TFs, the binding of these TFs occurs before DNA demethylation.

Although demethylation does not appear to be required for the binding of key TFs involved in regulation of innate immune responses, it is plausible that the removal of methylation marks at DM sites might enable occupancy of methylation-sensitive factors at later time points (33–35). In support of this hypothesis, we found that, at later time points (18 h and above), there was a stronger enrichment for the binding of TFs that preferentially bind to unmethylated motifs (or “methyl-minus” as defined by Yin et al. [33]) within hypomethylated regions (up to 1.7-fold enrichment; y2-test; P = 4.14 × 10−32; Fig. 4D; see Materials and Methods). Collectively, these results suggest that, although demethylation is likely not required for the engagement of the core regulatory program induced early after infection, it might play a role in fine-tuning the innate immune response by facilitating the binding of salient methyl-sensitive TFs that mediate later immune responses.
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 5mC in DM sites, which do not show a loss of 5mC at 2 h post-infection, 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 also noticed 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 data cannot 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 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 CleaveGO (59) to test for enrichment of functionally annotated gene sets among differentially expressed genes. TF footprinting analyses were performed using the Centidual algorithm (5) and JASPAR annotated human TF binding motifs (60).

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