

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

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18

19 **ABSTRACT**

20 DNA methylation is considered to be a relatively stable epigenetic mark. Yet, a growing body of evidence
21 indicates that DNA methylation levels can change rapidly, for example, in innate immune cells facing an
22 infectious agent. Nevertheless, the causal relationship between changes in DNA methylation and gene
23 expression during infection remains to be elucidated. Here, we generated time-course data on DNA
24 methylation, gene expression, and chromatin accessibility patterns during infection of human dendritic
25 cells with *Mycobacterium tuberculosis*. We found that the immune response to infection is accompanied
26 by active demethylation of thousands of CpG sites overlapping distal enhancer elements. However,
27 virtually all changes in gene expression in response to infection occur prior to detectable changes in DNA
28 methylation, indicating that the observed losses in methylation are a downstream consequence of
29 transcriptional activation. Footprinting analysis revealed that immune-related transcription factors (TF),
30 such as NF- κ B/Rel, are recruited to enhancer elements prior to the observed losses in methylation,
31 suggesting that DNA demethylation is mediated by TF binding to cis-acting elements. Collectively, our
32 results show that DNA demethylation is not required for the establishment of the core regulatory program
33 engaged upon infection.

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42 INTRODUCTION

43 Innate immune cells, such as dendritic cells (DCs) and macrophages, are the first mediators recruited in
44 response to an invading pathogen. Upon stimulation, these cells considerably shift their transcriptional
45 program, activating hundreds of genes involved in immune-related processes in a rapid and highly
46 choreographed fashion. This is achieved through the binding of signal-dependent transcription factors
47 (TFs), including NF- κ B/Rel, AP-1, and interferon regulatory factors (IRFs), to gene regulatory regions of
48 the genome where recruitment of various co-activators is initiated [1, 2]. Alterations to the epigenome,
49 such as histone modifications and DNA methylation, are recognized as important permissive or
50 suppressive factors that play an integral role in modulating access of TFs to cis-acting DNA regulatory
51 elements via the regulation of chromatin dynamics. Consequently, changes to the epigenetic landscape are
52 expected to have a significant impact on gene expression.

53
54 Many studies have highlighted the importance of histone modifications in regulating complex gene
55 expression programs underlying immune responses [3, 4]. However, the exact role that DNA methylation
56 plays in innate immune response regulation remains ambiguous. We have previously shown that infection
57 of post-mitotic DCs is associated with an active loss of methylation at enhancers and that such
58 demethylation events are strongly predictive of changes in expression levels of nearby genes [5]. Many
59 other studies correlate these two processes [6-13], but it remains unclear whether altered methylation
60 patterns directly invoke transcriptional modulation or whether such patterns are the downstream
61 consequence of TF binding to regulatory regions. Thus, the causal relationship between changes in DNA
62 methylation and gene expression during infection remains unresolved. To address this question, we
63 characterized in parallel genome-wide patterns of DNA methylation, gene expression, and chromatin
64 accessibility in non-infected and *Mycobacterium tuberculosis* (MTB)-infected DCs at multiple time
65 points. Our results show that the loss of DNA methylation observed in response to infection is not

66 required for the activation of most enhancer elements and that, instead, demethylation is a downstream
67 consequence of TF binding.

68

69 **RESULTS**

70 **Bacterial infection induces stable DNA demethylation at enhancers of dendritic cells**

71 To investigate the relationship between changes in gene expression and DNA methylation in response to
72 infection, we infected monocyte-derived DCs from 4 healthy individuals with a live virulent strain of
73 *Mycobacterium tuberculosis* (MTB) for 2-, 18-, 48-, and 72-hours. At each time-point, we obtained single
74 base-pair resolution DNA methylation levels for over 130,000 CpG sites using a customized capture-
75 based bisulfite sequencing panel (SeqCap Epi, see **Methods**), in matched non-infected and MTB-
76 infected DCs. Our customized SeqCap Epi panel interrogates 33,059 regions highly enriched among
77 putative enhancer elements (58% are associated with the H3K4me1 enhancer mark [14]; **Supplementary**
78 **Figure 1A**), which are the main targets of methylation changes in response to infection [5]. In total, we
79 generated ~717 million single-end reads (mean = 17.5 million reads per sample; **Supplementary Table**
80 **1**), resulting in an average coverage of ~70X per CpG site (**Supplementary Figure 1B**). Methylation
81 values between samples were strongly correlated, attesting to the high quality of the data
82 (**Supplementary Figure 1C**; median r across all samples = 0.94).

83

84 We next assessed temporal changes in methylation levels in response to infection using the DSS software
85 [15]. We defined differentially methylated (DM) CpG sites as those showing a significant difference of
86 methylation between infected and non-infected samples at a False Discovery Rate (FDR) < 0.01 and an
87 absolute mean methylation difference above 10%. Using these criteria, we identified 6,174 DM CpG sites
88 across the time course of infection. Consistent with previous findings [5], the vast majority of changes in
89 methylation (87%) were associated with the loss of DNA methylation in infected cells (**Figure 1A,B**).

90

91 To test if live bacteria were required to induce the observed changes in DNA methylation, we collected
92 similar data on DCs exposed to heat-killed MTB in addition to the live MTB experiments. Changes in
93 methylation in response to live and heat-killed MTB were strikingly correlated, particularly at later time-
94 points post-infection ($r \geq 0.84$ at 18h and above; **Supplementary Figure 2**). These results show that DCs
95 do not require exposure to a live pathogen to elicit the overall demethylation detected in response to
96 infection. Simply, the engagement of innate immune receptors and activation of pathways involved in
97 pathogen sensing and elimination is sufficient to induce methylation shifts. Hierarchical clustering
98 analysis of the DM sites observed when considering samples exposed to either live or heat-killed bacteria
99 showed that >80% of the sites exhibited a gradual loss of methylation over the time course of infection
100 until methylation marks were almost completely erased and that very few changes were detectable at 2
101 hours post-infection (DM Cluster 3; **Figure 1C,D**; **Supplementary Table 2**).

102

103 Monocyte-derived DCs do not proliferate in response to infection [5] and, therefore, any observed losses
104 in methylation must occur through an active mechanism involving the ten-eleven translocation (Tet)
105 enzymes, a family of enzymes that converts 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC)
106 [16]. Thus, we used Tet-assisted bisulfite sequencing (TAB-seq) data collected from non-infected DCs [5]
107 to assess if DM sites had significantly different levels of 5hmC as compared to non-DM sites. We found
108 that DM sites (Cluster 3) show high levels of 5hmC even prior to infection (**Figure 1E**; 3.2-fold
109 enrichment compared to non-DM sites; Wilcoxon test; $P < 1 \times 10^{-16}$), suggesting that DM sites are likely
110 pre-bound by TET enzymes (likely TET2 [17, 18], the most expressed Tet enzyme in DCs
111 (**Supplementary Figure 3**)) and that 5hmC may serve as a stable mark that acts to prime enhancers [19-
112 21].

113

114 **Up-regulation of inflammatory genes precedes DNA demethylation**

115 We collected RNA-seq data from matched non-infected and infected samples at each time point, for a
116 total of 34 RNA-seq profiles across time-treatment combinations (mean = 42.2 million reads per sample;
117 **Supplementary Table 1**). The first principal component of the resulting gene expression data accounted
118 for 63% of the variance in our dataset and separated infected and non-infected DCs (**Supplementary**
119 **Figure 4A**). We found extensive differences in gene expression levels between infected and non-infected
120 DCs: of the 13,956 genes analyzed, 1,987 (14%), 4,371 (31%), 4,591 (33%), and 5,189 (37%) were
121 differentially expressed (DE) at 2, 18, 48, and 72 hours post-infection, respectively (FDR < 0.01 and
122 absolute $\log_2(\text{fold change}) > 1$; **Supplementary Table 3**). We also collected RNA-seq data in samples
123 stimulated with heat-inactivated MTB and found that, similar to changes in methylation, changes in gene
124 expression in response to live and heat-inactivated MTB were strongly correlated ($r \geq 0.94$;
125 **Supplementary Figure 4B**). We next grouped the set of DE genes across the time course (7,457 in total)
126 into 6 distinct temporal expression clusters (**Figure 2A,B**; **Supplementary Table 3**). These clusters cover
127 a variety of differential expression patterns, including genes which show increasing up-regulation over
128 time (DE Cluster 5: Persistent induced; $n = 2,091$) to genes in which the highest levels of expression
129 occur at 2 or 18 hours followed by a decrease towards basal levels (DE Cluster 4: Early induced ($n =$
130 765), and DE Cluster 6: Intermediate induced ($n = 839$), respectively) (**Figure 2B**). Gene ontology (GO)
131 enrichment analysis revealed that induced genes were strongly enriched among GO terms directly related
132 to immune function, including defense response (FDR = 1.2×10^{-11}) and response to cytokine (FDR = 8.2
133 $\times 10^{-12}$), whereas repressed genes were primarily enriched for gene sets associated with metabolic
134 processes (**Figure 2C**; **Supplementary Table 4**).

135

136 We next tested whether genes located near DM sites—particularly focusing on those sites exhibiting a
137 stable loss of methylation (*i.e.*, Cluster 3 in Figure 1C,D)—were more likely to be differentially expressed
138 upon MTB infection relative to all genes in the genome. We found that genes associated with one or more
139 DM sites were strongly enriched among genes that were up-regulated in response to infection, regardless

140 of the time point at which expression levels started to change: early (2.5-fold, $P = 3.23 \times 10^{-11}$),
141 intermediate (3.5-fold, $P = 3.59 \times 10^{-25}$), and persistent (3.1-fold, $P = 3.80 \times 10^{-33}$) (**Figure 2D,E**).

142

143 If demethylation is required for the activation of enhancer elements and the subsequent up-regulation of
144 their target genes, we would expect demethylation to occur *prior* to changes in gene expression; instead,
145 we found the opposite pattern. Among up-regulated genes associated with DM sites ($n = 593$), 37%
146 exhibited at least a two-fold increase in gene expression levels at 2-hours post-infection, although
147 differential methylation did not begin to be detectable until 18-hours post-infection (**Figure 2E**). For only
148 17 genes (less than 3% of all up-regulated genes associated with DM sites), DNA demethylation occurred
149 prior to gene activation (**Supplementary Figure 5**), suggesting that no definitive causal relationship
150 between DNA demethylation and gene activation exists.

151

152 To confirm that our findings were generalizable to other innate immune cell types and pathogenic
153 infections, we performed a separate time-course analysis of differential methylation in Salmonella-
154 infected macrophages from one additional donor over six time-points (**Supplementary Table 1**). We
155 discovered hundreds of CpG sites that exhibited a progressive loss of methylation over the time course of
156 infection, corroborating our findings in MTB-infected DCs (**Figure 3A**). To assess whether
157 demethylation arises after the activation of associated enhancers, we collected ChIP-seq data for
158 acetylation of histone 3 lysine 27 (H3K27ac) at 2-hours post-infection, as changes in DNA methylation
159 have yet to occur at this point. We found that the deposition of activating H3K27ac marks preceded
160 demethylation at these CpG sites (**Figure 3B**). Moreover, using previously published RNA-seq
161 expression data from Salmonella-infected macrophages [22], we found that most genes associated with
162 these sites were up-regulated at 2-hours post-infection (**Figure 3C**), prior to any changes in methylation.
163 Collectively, these findings indicate that DNA demethylation is not required for the activation of most

164 enhancer elements and that the vast majority of methylation changes induced by infection are a
165 downstream consequence of transcriptional activation.

166

167 **The binding of most infection-induced TFs does not require active demethylation**

168 We next asked whether MTB-induced gene expression changes were associated with changes in
169 chromatin accessibility. To do so, we profiled regions of open chromatin in non-infected and infected
170 DCs at the same time-points (plus one additional time-point at 24 hours) using ATAC-seq [23]. Overall,
171 we found that the response to MTB infection was accompanied by an increase in chromatin accessibility
172 across regulatory regions associated with genes up-regulated upon MTB infection, regardless of their
173 expression profiles (**Figure 4A**). Interestingly, most increases in chromatin accessibility were observed at
174 later stages of infection, suggesting that the activation of early response genes does not require significant
175 modifications to the chromatin structure.

176

177 To investigate the relationship between DNA methylation and TF occupancy, we performed TF
178 footprinting analysis on our target regions (*i.e.*, the set of putative enhancers tested for dynamic DNA
179 methylation). We classified target regions as “hypomethylated regions” ($n = 1,877$) or “non-differentially
180 methylated regions” (non-DMRs) ($n = 31,182$) according to whether or not these regions overlap DM
181 CpG sites (from differential methylation Cluster 3, specifically). We found that hypomethylated regions
182 were significantly enriched for the binding of immune-related TFs relative to regions exhibiting
183 consistent methylation levels. These immune-related TFs include several master regulators of the innate
184 immune response, such as NF- κ B/Rel family members NFKB1 (up to 4.6-fold enrichment across the time
185 course ($FDR = 4.78 \times 10^{-29}$)) and RELA (up to 3.6-fold enrichment across the time course ($FDR = 1.95 \times$
186 10^{-18})) **Figure 4B; Supplementary Table 5**.

187

188 We next used CentriDual [5] to test for differential binding of TFs between non-infected and infected
189 samples, specifically focusing on the set of TF family members known to orchestrate innate immune
190 responses to infection (*i.e.*, NF- κ B/Rel, AP-1, STATs, and IRFs). We found increased binding at NF-
191 κ B/Rel binding motifs starting at 2-hours post-infection, despite the fact that no changes in methylation
192 were observed at such early time points ($P = 0.002$; **Figure 4C**; **Supplementary Table 5**; see **Methods**).
193 A similar pattern was observed for AP-1 ($P = 0.01$; **Supplementary Figure 6**). These data show that,
194 while demethylated regions overlap areas bound by immune-induced TFs, the binding of these TFs occurs
195 prior to DNA demethylation.

196

197 Although demethylation does not appear to be required for the binding of key TFs involved in regulation
198 of innate immune responses, it is plausible that the removal of methylation marks at DM sites might
199 enable occupancy of methylation-sensitive factors at later time points [24-26]. In support of this
200 hypothesis, we found that, at later time-points (18 hours and above), there was a stronger enrichment for
201 the binding of TFs that preferentially bind to unmethylated motifs (or “methyl-minus” as defined by Yin
202 *et al.* [24]) within hypomethylated regions (up to 1.7-fold enrichment; χ^2 -test; $P = 4.14 \times 10^{-34}$; **Figure**
203 **4D**; see **Methods**). Collectively, these results suggest that, although demethylation is likely not required
204 for the engagement of the core regulatory program induced early after infection, it might play a role in
205 fine-tuning the innate immune response by facilitating the binding of salient methyl-sensitive TFs that
206 mediate later immune responses.

207

208 **DISCUSSION**

209 In this study, we generated paired data on DNA methylation, gene expression, and chromatin accessibility
210 in non-infected and MTB-infected DCs at multiple time-points. Our results show that bacterial infection
211 leads to marked remodeling of the methylome of phagocytic cells (both DCs and macrophages), with
212 several thousand CpG sites showing stable losses of methylation via active DNA demethylation.

213 Strikingly, in our experiment, virtually all changes in gene expression in response to infection occurred
214 prior to detectable alterations in DNA methylation, suggesting that the observed demethylation is a
215 downstream consequence of TF binding and transcriptional activation. We note, however, that our
216 bisulfite sequencing data does not allow us to distinguish between 5mC and 5hmC. Thus, it is possible
217 that the gain of 5hmC in DM sites, which do not show a loss of 5mC at 2-hours post-infection, precedes
218 the activation of certain enhancers, as was recently suggested in T cells [8].

219
220 The observed changes in methylation most likely occur via TET2-mediated active demethylation, as
221 previously shown [5, 17, 27]. Consistent with this hypothesis, we found that CpG sites that lose
222 methylation upon infection display high levels of 5hmC at baseline, suggesting that these regions are
223 actively bound by TET2 even prior to infection. Moreover, *TET2* is strongly upregulated 2 hours after
224 infection (~2.5 fold; **Supplementary Figure 7**). 5hmC could be a stable intermediate that serves as an
225 epigenetic priming mark, ensuring the rapid response of DCs against infection [19-21, 27-30]. Further
226 studies are necessary to investigate the functional relevance of 5hmC in the induction of inflammatory
227 genes during infection.

228
229 Using footprint analysis, we show that NF- κ B/Rel, a master regulator of inflammation, is recruited to
230 hypomethylated regions as soon as 2-hours post-infection. This finding is consistent with ChIP-seq data
231 collected from macrophages stimulated with Kdo2-Lipid A (KLA), a highly specific TLR4 agonist, which
232 shows that the NF- κ B subunit p65 is rapidly recruited to enhancer elements within one hour post-
233 stimulation [31]. We hypothesize that the rapid binding of NF- κ B, and of other immune-induced TFs,
234 instigates chromatin opening which is then followed by the recruitment of histone acetyltransferase p300
235 and the subsequent deposition of activating H3K27ac marks in these regions [32]. Interestingly, p300 can
236 acetylate TET2, conferring enhanced enzyme activity [33], which might account for the eventual loss of
237 DNA methylation in response to infection.

238

239 Our results indicate that most changes in gene expression that occur in response to infection are
240 independent of DNA demethylation, further supporting a lack repressive capacity of DNA methylation
241 [34]. Notably, for only 17 genes—out of thousands of differently expressed genes in response to MTB
242 infection—there is evidence that DNA demethylation occurred prior to gene activation. Similar to
243 previous findings [27, 35-40], our results further reinforce the idea that site-specific regulation of DNA
244 demethylation is mediated by TFs that bind to cis-acting sequences. Interestingly, several recent reports
245 have shown that other epigenetic modifications, such as the H3K4me1 enhancer mark, have a similar
246 passive regulatory function [41-43]. However, our results do not exclude the possibility that
247 demethylation might be necessary for the binding of a second wave of TFs that only play a role at later
248 stages of infection (18 hours post-infection or later). In agreement with this hypothesis, we observed a
249 significant enrichment of binding of TFs known to preferentially bind unmethylated CpGs in
250 hypomethylated regions, primarily at later stages post-infection. Ultimately, this suggests that DNA
251 demethylation is not a key regulatory mechanism of early innate immune responses but that it could still
252 play a role in fine-tuning later innate immune responses by facilitating the binding of methylation-
253 sensitive TFs at enhancers.

254

255 After an infection is cleared, TFs are expected to unbind, and gene expression as well as DNA
256 methylation levels are anticipated to return to basal state. However, our 72-hour time course study of
257 DNA methylation shows that levels of methylation at DM sites gradually decrease with time post-
258 infection and never revert back to higher levels. Interestingly, this pattern is also observed for genes in
259 which the largest fold changes in gene expression occur at earlier time points. Thus, we speculate that
260 demethylation in response to infection could have a specific biological role in innate immune memory
261 [44-47], and that regions that stably lose methylation may act as primed enhancers, potentially allowing
262 for a faster response to a secondary infection.

263

264 **METHODS**

265 **Biological material and sequencing libraries.** Buffy coats from healthy donors were purchased from
266 Indiana Blood Center and all participants signed a written consent. The ethics committee at the CHU
267 Sainte-Justine approved the project (protocol #4023). Peripheral blood mononuclear cells (PBMCs) were
268 obtained by centrifugation on Ficoll-Paque, and monocytes were isolated by positive selection with CD14
269 magnetic beads (Miltenyi Biotec). Monocytes were differentiated into either DCs by adding rhIL-4 (20
270 ng/mL; Shenandoah Biotechnology, Inc) and rhGM-CSF (20 ng/mL; R&D Systems Inc.) or macrophages
271 by adding rhM-CSF (20ng/mL; R&D Systems Inc.) in the cell culture medium.

272

273 DCs were infected with MTB for 2, 18, 48, and 72 h at a multiplicity of infection (MOI) of 1:1 or with
274 heat-killed MTB at MOI of 5:1, as this MOI induces virtually the same transcriptional response at all four
275 time points compared to that observed with live MTB (**Supplementary Figure 2**). Macrophages were
276 infected with *Salmonella typhimurium* as previously described [22]. Briefly, macrophages were infected
277 at MOI of 10:1 for 2 hours, washed, and cultured for 1 hour with 50µg/ml gentamycin, then washed again
278 and cultured in complete medium with 3µg/ml gentamycin for an additional 2, 4, 8, 12, 24 or 48 h, the
279 time points we refer to in the main text.

280

281 DNA from DCs was extracted using the PureGene DNA extraction kit (Gentra Systems). DNA from
282 macrophages was extracted using the DNeasy Blood and Tissue Kit (Qiagen). RNA was extracted using
283 the miRNeasy mini kit (Qiagen). RNA quality was evaluated with the 2100 Bioanalyzer (Agilent
284 Technologies) and only samples with no evidence of RNA degradation (RNA integrity number > 8) were
285 kept for further experiments. RNA-seq libraries were prepared using the TruSeq RNA Sample Prep Kit
286 v2, as per the manufacturer's instructions.

287

288 ATAC-seq libraries were generated from 50,000 cells, as previously described [23]. We collected ChIP-
289 seq data for the H3K27ac histone mark in non-infected and *Salmonella*-infected macrophages as
290 previously described [5]. Sequencing was performed using the Illumina HiSeq 2500, as per the
291 manufacturer's instructions.

292

293 **SeqCap Epi library preparation and sequencing.**

294 Libraries were generated with KAPA Library Preparation Kit for Illumina Platforms (KAPA Biosystems),
295 as per the manufacturer's instructions. Briefly, genomic DNA was fragmented to 100-300 bp with an S2
296 sonicator (Covaris). Fragments were then end-repaired, A-tailed, and ligated with methylated sequencing
297 adapters. Between every enzymatic step, libraries were purified using AMPure beads (Agencourt). After
298 ligation, in addition to the AMPure bead purification, a DUAL-SPRI size selection was performed to
299 further select for fragments with adapters in the window of 200-400 bp. Sodium bisulfite conversion was
300 performed with EZ DNA Methylation Lightning Kit (Zymo Research), and libraries were amplified using
301 KAPA Hifi HotStart Uracil Tolerant Enzyme (KAPA Biosystems). Library quality was assessed by 2100
302 Bioanalyzer (Agilent Technologies). Samples showing the desired profile were pooled together in equal
303 mass according to Qubit quantification. We then performed a hybridization using the SeqCap Epi kit
304 (Roche NimbleGen). The sample pool, indexes corresponding to the sequences of the adapters used for
305 library preparation, and repetitive DNA (C_{0t}) were desiccated and then incubated in hybridization buffer
306 with a set of customized probes for 72 hours to select and sequence target regions only. Specifically,
307 DNA methylation data was collected for 33,059 target regions spanning >130,000 CpG sites (mean length
308 = 300 bp; mean number of CpG sites = 5), which is less than 1% of the ~28 million CpGs contained in the
309 human genome. These regions were primarily comprised of MTB-induced differentially methylated
310 regions identified at 18 hours post-infection using whole-genome bisulfite sequencing, as well as other
311 distal regulatory elements in DCs where changes in DNA methylation have been shown to be most likely
312 to occur (**Supplementary Figure 1A**) [5]. Moreover, these candidate regions were nearby differentially

313 expressed genes in response to MTB at 18 hours. Probes targeting a two kilobase region between
314 coordinates 4500 and 6500 bp of the lambda genome (NC_001416.1) were also included in the SeqCap
315 Epi design, as a control for bisulfite conversion efficiency. Sequencing was performed using the Illumina
316 HiSeq 2500, as per the manufacturer's instructions.

317

318 **SeqCap Epi data processing and differential methylation analysis.** Adaptor sequences and low-quality
319 score bases (Phred score < 20) were first trimmed using Trim Galore
320 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The resulting reads were mapped to
321 the human reference genome (GRCh37/hg19) and lambda phage genome using Bismark [48], which uses
322 Bowtie 2 [49] and a bisulfite converted reference genome for read mapping. Only reads that had a unique
323 alignment were retained. Methylation levels for each CpG site were estimated by counting the number of
324 sequenced C ('methylated' reads) divided by the total number of reported C and T ('unmethylated' reads)
325 at the same position of the reference genome using Bismark's methylation extractor tool. We performed a
326 strand-independent analysis of CpG methylation where counts from the two Cs in a CpG and its reverse
327 complement (position i on the plus strand and position $i+1$ on the minus strand) were combined and
328 assigned to the position of the C in the plus strand. To assess MethylC-seq bisulfite conversion rate, the
329 frequency of unconverted cytosines (C basecalls) at lambda phage CpG reference positions was
330 calculated from reads uniquely mapped to the lambda phage reference genome. Overall, bisulfite
331 conversion rate was >99% in all of the samples (**Supplementary Table 1**).

332

333 In DCs, differentially methylated (DM) CpG sites at each time point following MTB infection were
334 identified using the R package DSS [15]. We used a linear model with the following design: *DNA*
335 *methylation* ~ *Donor* + *Infection*, which allowed us to consider the paired nature of the experiment and
336 capture the effects of infection on DNA methylation observed within donors. We considered a CpG site
337 as differentially methylated if statistically supported at a False Discovery Rate (FDR) < 0.01 and an

338 absolute mean methylation difference above 10%. Only CpG sites that had a coverage of at least 5X in
339 each of the samples were included in the analysis (103,649 in total).

340

341 To identify DM sites that show a stable loss of methylation (as Cluster 3 DM sites in DCs) in *Salmonella*-
342 infected macrophages using one individual, we performed a hierarchical clustering analysis on sites that
343 specifically: (i) do not change methylation at 2 hours ($|\text{methylation difference}| < 10\%$), and (ii) lose
344 methylation at 48 hours (methylation difference $< -40\%$).

345

346 **5hmC enrichment at DM sites.** To calculate the enrichment of 5-hydroxymethylcytosine (5hmC) at DM
347 CpG sites (Clusters 1, 2 and 3), we compared the distribution of 5hmC levels in non-infected DCs
348 between DM and non-DM sites. Since non-DM sites have lower overall levels of baseline methylation
349 than DM sites (**Supplementary Figure 8A**), we performed similar enrichment analysis by using a
350 random set of non-DM sites that matches the distribution of methylation found in non-infected samples
351 within each set of DM sites (**Supplementary Figure 8B**). Each random set contains the same number of
352 CpG sites as those identified within each DM cluster.

353

354 **RNA-seq data processing and identification of differentially expressed genes.** Read count estimates
355 per gene were obtained using the alignment-free method Kallisto [50]. For all downstream analyses, we
356 excluded non-coding and lowly-expressed genes with an average read count lower than 10 in all of the
357 samples, resulting in 13,955 genes in total. The R package DESeq2 [51] was used to identify differences
358 in expression levels between non-infected and infected samples at each time point. Nominal p-values
359 were corrected for multiple testing using the Benjamini-Hochberg method [52]. The complete list of
360 differentially expressed genes can be found in Supplementary Table 3.

361

362 **Gene set enrichment analysis.** We used ClueGO [53] at default parameters to test for enrichment of
363 functionally annotated gene sets among differentially expressed genes. The results for these enrichment
364 analyses are reported in Supplementary Table 4. Enrichment p-values were based on a hypergeometric
365 test using the set of 13,955 genes as background. Benjamini-Hochberg method was applied for multiple
366 testing correction.

367
368 **ChIP-seq data processing and tag density profiles.** ChIP-seq reads were trimmed for adapter sequences
369 and low-quality score bases using Trim Galore. The resulting reads were mapped to the human reference
370 genome using Bowtie 2 with the following option: -N 1. Only reads that had a unique alignment were
371 retained, and PCR duplicates were further removed using Picard tools
372 (<http://broadinstitute.github.io/picard/>). Tag density profiles for chromatin modifications and genome
373 accessibility patterns around regions of interest were accomplished with ngs.plot package [54] using
374 default parameters.

375
376 **ATAC-seq data processing and TF footprinting analysis.** ATAC-seq reads were trimmed for adapter
377 sequences and low-quality score bases and were mapped to the human reference genome. Mapping was
378 performed using BWA-MEM [55] in paired-end mode at default parameters. Only reads that had a unique
379 alignment (mapping quality > 10) were retained. TF footprinting analyses were performed as previously
380 described, using the Centidual algorithm [5] and JASPAR annotated human TF binding motifs (2018
381 release) [56]. For each of the actively bound TFs in DCs (241 in total; FDR < 0.05 at 18 hours post-
382 infection; **Supplementary Table 5**), we first trained Centidual assuming that the footprint was bound in
383 the two conditions. Then, we fixed the model parameters and generated a likelihood ratio and posterior
384 probability π_{it} for each condition t separately and for each site l . To detect if the footprint was more
385 active in one of the two conditions, we fit a logistic model that included an intercept for each condition (
386 α and δ), the PWM effect β , and PWM times the treatment effect γ :

$$\log\left(\frac{\pi_t}{1-\pi_t}\right) = \alpha \times (1 - I_t) + \beta \times \text{PWMscore}_t + \delta \times I_t + \gamma \times (I_t \times \text{PWMscore}_t)$$

387

388 where I_t is an indicator variable that takes the value 1 if $t = \text{“treatment”}$ and 0 if $t = \text{“control”}$. We then

389 calculated a Z-score for the interaction effect γ , corresponding to the evidence for condition-specific

390 binding. ATAC-seq samples were down-sampled to obtain similar number of reads between NI and HI

391 samples at each time-point. We used a window size of 300 bp on either side of the motif match, and

392 reads with fragment lengths [40, 140] and [141, 600] bp for footprinting analyses.

393

394 To test for differential binding of immune-related TFs (NF- κ B/Rel, AP-1, STATs, and IRFs) between

395 non-infected and infected samples, we compared the intensity of the Tn5 sensitivity-based footprint

396 across all matches to motifs of TFs that belong to each family in the hypomethylated regions.

397 Specifically, the following motif IDs (and corresponding names) were aggregated to their respective TF

398 family:

399

NF- κ B/Rel		AP-1		STATs		IRFs	
MA0105.3	NFKB1	MA0833.1	ATF4	MA0137.1	STAT1	MA0050.2	IRF1
MA0105.2	NFKB1	MA0834.1	ATF7	MA0137.2	STAT1	MA0050.1	IRF1
MA0105.1	NFKB1	MA0462.1	BATF::JUN	MA0137.3	STAT1	MA0051.1	IRF2
MA0105.4	NFKB1	MA0835.1	BATF3	MA0517.1	STAT1::STAT2	MA1418.1	IRF3
MA0778.1	NFKB2	MA0476.1	FOS	MA0144.2	STAT3	MA1419.1	IRF4
MA0101.1	REL	MA0099.3	FOS::JUN			MA1420.1	IRF5
MA0107.1	RELA	MA1126.1	FOS::JUN(var.2)			MA0772.1	IRF7
MA1117.1	RELB	MA1134.1	FOS::JUNB			MA0652.1	IRF8
		MA1141.1	FOS::JUND			MA0653.1	IRF9
		MA1127.1	FOSB::JUN				
		MA1135.1	FOSB::JUNB				
		MA1136.1	FOSB::JUNB(var.2)				
		MA0477.1	FOSL1				
		MA1128.1	FOSL1::JUN				
		MA1129.1	FOSL1::JUN(var.2)				
		MA1137.1	FOSL1::JUNB				
		MA1142.1	FOSL1::JUND				
		MA1143.1	FOSL1::JUND(var.2)				
		MA0478.1	FOSL2				
		MA1130.1	FOSL2::JUN				
		MA1131.1	FOSL2::JUN(var.2)				
		MA1138.1	FOSL2::JUNB				
		MA1139.1	FOSL2::JUNB(var.2)				
		MA1144.1	FOSL2::JUND				
		MA1145.1	FOSL2::JUND(var.2)				
		MA0655.1	JDP2				
		MA0656.1	JDP2(var.2)				
		MA0488.1	JUN				
		MA0489.1	JUN(var.2)				
		MA1132.1	JUN::JUNB				
		MA1133.1	JUN::JUNB(var.2)				
		MA0490.1	JUNB				
		MA1140.1	JUNB(var.2)				
		MA0491.1	JUND				

400

401 To test for enrichment of binding of methylation-sensitive (“methyl-minus”) TFs in hypomethylated
 402 regions, we compared the proportion of regions that overlap well-supported footprints (posterior
 403 probability > 0.99) of “methyl-minus” TFs reported in Yin *et al.* [24]) among non-DMRs and
 404 hypomethylated regions (with 250-bp flanking the start and end). The list of motif IDs (and
 405 corresponding names) that were included in the analysis are shown below:

MA0018.1	CREB1	MA0495.2	MAFF	MA0823.1	HEY1
MA0018.3	CREB1	MA0511.2	RUNX2	MA0830.1	TCF4
MA0028.2	ELK1	MA0526.1	USF2	MA0831.1	TFE3
MA0058.1	MAX	MA0526.2	USF2	MA0831.2	TFE3
MA0058.3	MAX	MA0636.1	BHLHE41	MA0834.1	ATF7
MA0059.1	MAX::MYC	MA0638.1	CREB3	MA0835.1	BATF3
MA0062.1	GABPA	MA0640.1	ELF3	MA0839.1	CREB3L1
MA0093.2	USF1	MA0641.1	ELF4	MA0871.1	TFEC
MA0095.1	YY1	MA0649.1	HEY2	MA1126.1	FOS::JUN(var.2)
MA0095.2	YY1	MA0663.1	MLX	MA1127.1	FOSB::JUN
MA0099.3	FOS::JUN	MA0664.1	MLXIPL	MA1128.1	FOSL1::JUN
MA0104.4	MYCN	MA0736.1	GLIS2	MA1129.1	FOSL1::JUN(var.2)
MA0136.2	ELF5	MA0749.1	ZBED1	MA1130.1	FOSL2::JUN
MA0149.1	EWSR1-FL11	MA0750.1	ZBTB7A	MA1131.1	FOSL2::JUN(var.2)
MA0156.2	FEV	MA0750.2	ZBTB7A	MA1134.1	FOS::JUNB
MA0464.2	BHLHE40	MA0757.1	ONECUT3	MA1135.1	FOSB::JUNB
MA0470.1	E2F4	MA0759.1	ELK3	MA1136.1	FOSB::JUNB(var.2)
MA0473.1	ELF1	MA0761.1	ETV1	MA1137.1	FOSL1::JUNB
MA0473.2	ELF1	MA0762.1	ETV2	MA1138.1	FOSL2::JUNB
MA0474.2	ERG	MA0763.1	ETV3	MA1139.1	FOSL2::JUNB(var.2)
MA0475.1	FLI1	MA0764.1	ETV4	MA1141.1	FOS::JUND
MA0475.2	FLI1	MA0765.1	ETV5	MA1142.1	FOSL1::JUND
MA0476.1	FOS	MA0772.1	IRF7	MA1143.1	FOSL1::JUND(var.2)
MA0477.1	FOSL1	MA0821.1	HES5	MA1144.1	FOSL2::JUND
MA0478.1	FOSL2	MA0822.1	HES7	MA1145.1	FOSL2::JUND(var.2)

406

407

408 **Relationship between gene expression and chromatin accessibility.** Peaks were first called on ATAC-

409 seq using the MACS2 software suite [57] with the added parameters: -g hs -q 0.05 --broad --nomodel --

410 extsize 200 --nolambda. All peaks from each sample were then merged to provide one set of combined

411 peaks. To count the number of reads overlapping peaks, we used featureCount (from the subread

412 package) [58] with the following option: -p -P. For all downstream analyses, we excluded low-count

413 peaks with an average read count lower than 10 across all samples, resulting in 79,282 peaks in total. We

414 then plotted the distribution of changes in Tn5 accessibility (between non-infected and MTB-infected

415 DCs across the five time-points of infection (2, 4, 18, 24, 48, and 72 hours)) for the top 25% most

416 variable peaks associated with DE genes in each cluster. The DE genes associated with the selected peaks

417 represent ~50% of the total genes within each of the DE clusters: (i) Early induced: 418/765 = 55%; (ii)

418 Intermediate induced: 418/839 = 49%; and (iii) Persistent induced: 1083/2091 = 52%.

419

420 DATA ACCESS

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

424

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431

432 COMPETING INTEREST STATEMENT

433 The authors declare no competing financial interests.

434

435 FIGURE LEGENDS

436 **Figure 1.** (A) Barplots showing the number of differentially methylated (DM) CpG sites identified at a
437 $|\text{methylation difference}| > 10\%$ and $\text{FDR} < 0.01$ (y-axis) at each time point after MTB infection (2, 18, 48,
438 and 72 hours (h)) (x-axis). (B) Distribution of differences in methylation between infected and non-
439 infected cells at DM sites, at each time point. (C) Heatmap of differences in methylation constructed
440 using unsupervised hierarchical clustering of the 4,578 DM sites (identified at any time point using live
441 and heat-inactivated MTB-infected samples combined; y-axis) across four time points after infection,
442 which shows three distinct patterns of changes in methylation. (D) Mean differences in methylation of
443 CpG sites in each cluster across all time points; shading denotes ± 1 standard deviation. For visualization

444 purposes, we also show the ‘0h’ time point, where we expect no changes in methylation. (E) Boxplots
445 comparing the distribution of 5hmC levels in non-infected DCs between non-DM and DM sites (Cluster
446 3).

447

448 **Figure 2.** (A) Heatmap of differences in expression (standardized log₂ fold changes) constructed using
449 unsupervised hierarchical clustering of the 7,457 differentially expressed genes (identified at any time
450 point using cutoffs of $|\log_2\text{FC}| > 1$ and $\text{FDR} < 0.01$; y-axis) across four time points after MTB infection
451 results in six distinct patterns of changes in expression. (B) Mean log₂ fold expression changes of genes
452 in each cluster across all time points; shading denotes ± 1 standard deviation. For visualization purposes,
453 we also show the ‘0h’ time point, where we expect no changes in expression. (C) Gene ontology
454 enrichment analyses among genes that are repressed or induced in response to MTB infection. (D)
455 Enrichment (in log₂; x-axis) of differentially expressed genes associated with differentially methylated
456 CpG sites (Cluster 3). Error bars show 95% confidence intervals for the enrichment estimates. (E)
457 Boxplots showing the distribution of standardized differences in methylation of DM sites in Cluster 3
458 (blue) along with the corresponding standardized differences in expression of the associated genes
459 (orange), across all time points.

460

461 **Figure 3.** (A) Mean differences in methylation (y-axis) in CpG sites that show stable losses of
462 methylation (similar to Cluster 3 DM sites in Figure 1C,D; $n = 453$) in *Salmonella*-infected macrophages,
463 across six time points after infection (2, 4, 8, 12, 24, and 48 hours (h); x-axis). Shading denotes ± 1
464 standard deviation. For visualization purposes, we also show the ‘0h’ time point, where we expect no
465 changes in methylation. (B) Composite plots of patterns of H3K27ac ChIP-seq signals ± 5 kb around the
466 midpoints of hypomethylated sites (x-axis) in macrophages at 2 hours post-infection with *Salmonella*. (C)
467 Distribution of log₂ fold expression changes (between non-infected and *Salmonella*-infected macrophages
468 at 2 hours) for genes associated with DM sites in Figure 3A ($n = 269$).

469

470 **Figure 4.** (A) Boxplots showing the distribution of log₂ fold changes in chromatin accessibility between
471 non-infected and MTB-infected DCs across the five time points of infection (2, 4, 18, 24, 48, and 72
472 hours) for open chromatin regions associated with the three classes of induced genes described in Figure
473 2A,B. (B) TF binding motifs for which the number of well-supported footprints (posterior probability >
474 0.99) within hypomethylated regions were enriched (FDR < 0.01) relative to non-DMRs (with 250-bp
475 flanking the start and end) in MTB-infected DCs. The enrichment factors (x-axis) are shown in a log₂
476 scale and error bars reflect the 95% confidence intervals. A complete list of all TF binding motifs for
477 which footprints are enriched within hypomethylated regions can be found in Supplementary Table 5. (C)
478 Barplots showing significant differences in TF occupancy score predictions for NF-κB/Rel motifs
479 between MTB-infected and non-infected DCs ($Z_{MTB} - Z_{NI}$; y-axis; see **Methods**) across all time points (x-
480 axis). A positive Z-score difference indicates increased TF binding in hypomethylated regions after MTB
481 infection. (D) Proportion of regions that overlap a methylation-sensitive (“methyl-minus”; reported in Yin
482 *et al.* [24]) TF footprint (y-axis) observed among non-DMRs and hypomethylated regions (or hypo-
483 DMRs; see **Methods**).

484

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