Title:
NFκB dynamics determine the stimulus-specificity of epigenomic reprogramming in macrophages

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Summary:
The epigenome defines the cell type, but also shows plasticity that enables cells to tune their gene expression potential to the context of extracellular cues. This is evident in immune sentinel cells such as macrophages, which can respond to pathogens and cytokines with phenotypic shifts that are driven by epigenomic reprogramming\textsuperscript{1}. Recent studies indicate that this reprogramming arises from the activity of transcription factors such as nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB), which binds not only to available enhancers but may produce \textit{de novo} enhancers in previously silent areas of the genome\textsuperscript{2}. Here, we show that NFκB reprograms the macrophage epigenome in a stimulus-specific manner, in response only to a subset of pathogen-derived stimuli. The basis for these surprising differences lies in the stimulus-specific temporal dynamics of NFκB activity. Testing predictions of a mathematical model of nucleosome interactions, we demonstrate through live cell imaging and genetic perturbations that NFκB promotes open chromatin and formation of \textit{de novo} enhancers most strongly when its activity is non-oscillatory. These \textit{de novo} enhancers result in the activation of additional response genes. Our study demonstrates that the temporal dynamics of NFκB activity, which encode ligand identity\textsuperscript{3}, can be decoded by the epigenome through \textit{de novo} enhancer formation. We propose a mechanistic paradigm in which the temporal dynamics of transcription factors are a key determinant of their capacity to control epigenomic reprogramming, thus enabling the formation of stimulus-specific memory in innate immune sentinel cells.
Body Text:

The cellular epigenome, a regulatory network involving chromatin architecture and histone modifications, contains stable, heritable information that determines cell type-specific programs of gene expression\(^4\). At the same time, the epigenome of differentiated cells remains highly plastic\(^5,6\), particularly in immune cells like macrophages. These immune sentinel cells detect and “remember” environmental signals through epigenomic reprogramming in order to coordinate immune responses that are both context and stimulus-appropriate\(^1\). At a molecular level, this reprogramming is initiated by the activity of signal-dependent transcription factors (TFs) such as NFκB\(^7\). In cooperation with pioneer factors such as Pu.1, signal-dependent TFs increase chromatin accessibility and positive regulatory histone marks at previously latent enhancers, thus forming de novo enhancers\(^2,8\). NFκB activated by LPS has been the best-studied TF in this field. However, the degree to which NFκB or other TFs can alter the epigenome in response to different stimuli is not known.

To investigate the stimulus-specificity of de novo enhancer formation, we stimulated bone marrow-derived macrophages (BMDMs) with five well-characterized ligands: TNF (signaling through TNFR), Pam3CSK (TLR1/2), CpG (TLR9), LPS (TLR4), and Poly(I:C) (TLR3). We performed H3K4me1 ChIP-seq to define stimulus-dependent de novo enhancers and identified 3978 regions of the genome that segregated into two clusters. (Fig. 1a). The enhancers in Cluster 1 were most strongly induced by LPS and Poly(I:C) and were enriched for IRF and ISRE motifs (Fig. 1a), consistent with the fact that these stimuli activate IRF3 and type I interferon via the signaling adaptor TRIF\(^9\). In Irf3\(^{-}\)Ifnar\(^{-}\) BMDMs these regions no longer acquired H3K4 methylation in response to LPS and Poly(I:C) (Fig. 1c). Weak induction in response to TNF was consistent with the observation that TNF does not induce IRF3 but IRF1\(^10\).

In contrast, the enhancers in Cluster 2 were highly enriched for NFκB motifs. Surprisingly, although all five stimuli activate NFκB\(^11\), these regions seemed to acquire H3K4me1 in a stimulus-specific manner. We observed that TNF and Poly(I:C) had little effect on these regions, while Pam3CSK, CpG, and LPS produced prominent gains in H3K4me1. These differences were consistent across replicates (Extended Data Fig. 1) and were preserved in Irf3\(^{-}\)Ifnar\(^{-}\) BMDMs (Fig. 1c). Furthermore, 1071 of these regions contained an NFκB-RelA ChIP-seq peak\(^12\) (Fig. 1d). We concluded that these 1071 de novo enhancers were highly likely to be NFκB-driven. A pairwise comparison between samples quantitatively confirmed the stimulus-specificity of these enhancers (Fig. 1e), as the ChIP-seq signals of Pam3CSK, CpG, and LPS were significantly different from TNF or Poly(I:C) (\(p < 10^{-5}\)) in these regions.

These differences would be difficult to explain if NFκB were a binary on-off switch, but NFκB is in fact activated with complex, stimulus-specific temporal dynamics\(^11,13,14\). Using live-cell microscopy of macrophages from mVenus-RelA mice\(^3\), we characterized the single-cell dynamics of NFκB p65 in response to all five ligands (Fig. 1f). We have previously identified six essential features of NFκB dynamics that function as “code words” to encode ligand identity and dose\(^3\). We correlated mean H3K4me1 counts in the NFκB-driven enhancers with these six features: duration, early vs late activity, oscillatory power, peak amplitude, activation speed, and total activity (Extended Data Fig. 2). We found
that oscillatory power \((r = -0.95)\), total activity \((r = 0.77)\), and peak amplitude \((r = 0.78)\) were correlated with the capacity of a given stimulus to form de novo enhancers (Fig. 1g).

We hypothesized that temporal patterns of NFκB activity might regulate its interaction with chromatin. Crystallographic studies imply that stable NFκB-DNA binding requires the DNA to be nucleosome-free, because NFκB dimers embrace the DNA double helix circumferentially\(^\text{15,16}\) (Fig. 2a).

However, NFκB is capable of at least transiently interacting with nucleosomal DNA\(^\text{17}\), and can displace nucleosomes in cooperation with pioneer factor Pu.1\(^\text{2}\) or remodeling machinery such as SWI/SNF complexes\(^\text{18}\). Furthermore, the DNA-histone interface is not static but is composed of low-affinity interactions that promote spontaneous disassociation or “breathing”\(^\text{19}\). Thus, successive disruptions of DNA-histone contacts by NFκB may displace the nucleosome (Fig. 2b), and be followed by binding of lineage-determining TFs such as Pu.1 and the deposition of histone modifications marking the region as a de novo enhancer\(^\text{2}\).

This provided the mechanistic basis for a multi-step model describing how dynamical NFκB activity might affect chromatin. We constructed a series of 14 Hill equations describing the competition between NFκB and histone for interacting with DNA (Fig. 2c) based on the number of contact points in the histone-DNA crystal structure\(^\text{20}\). Relative rates of nucleosome wrapping and unwrapping were based on available biophysical data\(^\text{21}\). Using measured single-cell NFκB activities (Fig. 1e) as inputs, the model simulations reproduced the differences in experimental ChIP-seq data (Fig. 2d-2e and Extended Data Fig. 3a) across a range of parameter values (Extended Data Fig. 4).

We used the model to investigate which features of NFκB dynamical activity were the key determinants of chromatin state. We examined the contribution of the three features most highly correlated with the ChIP-seq data (Fig. 1g): oscillations, amplitude, and total activity. We compared oscillatory vs. non-oscillatory activity while holding amplitude and total activity constant, and the model predicted that a non-oscillatory dynamic produces a two-fold greater chromatin accessibility than an oscillatory dynamic (Fig. 2f). NFκB activity must have a minimal amplitude (Fig. 2g) and extend for a minimal duration (Fig. 2h) to open chromatin. Above these thresholds, non-oscillatory NFκB has greater capacity to open chromatin than oscillatory NFκB for any given value of amplitude or duration. These simulations indicated that dynamic features of NFκB, especially the presence or absence of oscillations, determine whether it preserves or alters the chromatin state.

To test this prediction, we generated a knockout mouse in which NFκB dynamics are perturbed. In response to TNF, NFκB rapidly induces expression of Nfkbia, whose gene product is the negative regulator IκBα\(^\text{22}\) (Fig. 3a) and mediates oscillatory behavior of NFκB. As IκBα knockout mice are embryonic lethal due to chronic hyperinflammation\(^\text{23}\), we bred the Nfkbia\(^{-}\) allele into a Rel\(^{-}\)Tnf\(^{-}\)Nfkbie\(^{-}\) background, enabling the isolation of BMDMs from adult IκBα\(^{-}\) mice.

We examined the dynamics of NFκB in IκBα\(^{-}\) BMDMs by crossing these mice with mVenus-RelA knock-in mice and performing live cell imaging of BMDMs stimulated with TNF. We observed that knockout of IκBα significantly disrupted NFκB dynamics (Fig. 3b). We quantified the differences in the
distribution of single cell dynamic features by Kolmogorov–Smirnov (K-S) test (Fig. 3c, Extended Data Fig. 5a) and found that the greatest dynamic difference between IxBα−/− and WT was a loss of oscillatory activity, with a K-S test statistic (D) of 0.85, corresponding to a p-value < 10−16. The other key dynamic features were either unaffected, or in the case of activation speed (D = 0.66) and early-vs-late activity (D = 0.52) would intuitively favor NFκB activity in WT cells. In addition, we calculated the area under the NFκB activity curve at the time points used in subsequent experiments and found no difference (Extended Data Fig. 5b). Based on single-cell microscopy measurements, we concluded that the primary impact of IxBα knockout was loss of oscillations.

To profile the chromatin state, we stimulated BMDMs from IxBα−/− and littermate controls with TNF and performed ATAC-seq at two, four, and eight hours. This was followed by a 16-hour washout period, and a final time point was collected after washout (Extended Data Fig. 6a). We identified 1443 genomic regions that demonstrated TNF-inducible chromatin accessibility in either genotype. Of these, 332 were differentially inducible between control and IxBα−/−. Strikingly, 97% of these regions (n=322) had greater chromatin accessibility in the knockout than control (Fig. 3d). These differentially inducible regions were strongly enriched for NFκB motifs (Fig. 3e), and 311 of 322 overlapped with a RelA ChIP-seq peak (Extended Data Fig. 6c). Differentially inducible regions were more likely than constitutively accessible regions to fall in intergenic portions of the genome (Extended Data Fig. 6b), suggesting that they tend to function as cis-acting enhancer elements near key innate immune genes such as Ccl5 (Fig. 3f), which has previously been shown to require chromatin remodeling for full induction12.

Our model predicted that chromatin accessibility is primarily determined by whether NFκB is oscillatory or non-oscillatory within a single cell. We therefore considered that the magnitude of ATAC-seq signal can be interpreted as the proportion of cells in a sample in which a particular region of DNA is accessible. By microscopy, 87% of IxBα−/− cells have non-oscillatory NFκB, compared to 25% in WT cells. This was similar to the magnitude of ATAC-seq differences between IxBα−/− and control. For example, at an intergenic peak on chromosome 15, 67% of the cells in IxBα−/− were accessible, compared to 22% of cells in the control (Fig. 3g).

To investigate more definitively that the negative feedback function of IxBα rather than its basal activity is critical for the observed effects, we utilized a recently described IxBαΔBxB mutant in which NFκB-binding sites in the promoter of the Nfkbia gene are disrupted24 (Extended Data Fig. 7a). In this model, basal IxBα expression is preserved, and the mice live into adulthood without requiring compound suppressor mutations. We confirmed that upon TNF stimulation IxBαΔBxB BMDMs activate NFκB in a non-oscillatory manner (Extended Data Fig. 7b). ATAC-seq analysis of TNF-stimulated WT vs IxBαΔBxB BMDMs recapitulated our findings in the IxBα−/− system, with 131 genomic regions demonstrating greater gain of chromatin accessibility in the mutant compared to WT (Extended Data Fig. 7c). These regions were enriched for NFκB motifs, and 90% overlapped with a RelA ChIP-seq peak (Extended Data Fig. 7d-7e). Taken together, the ATAC-seq data from both IxBα−/− and IxBαΔBxB experimental models indicated...
that loss of inducible negative feedback in the NFκB signaling system, which results in a loss of
oscillations, results in greater chromatin accessibility.

Next, we examined whether regions with differentially inducible chromatin accessibility acquire
the corresponding histone mark of enhancers. We performed H3K4me1 ChIP-seq in TNF-stimulated
control and IκBα−/− BMDMs and found that in the 322 differentially inducible ATAC-seq regions there was
also a greater gain of H3K4me1 signal in IκBα−/− than control (Fig. 3h). Notably, these histone marks
persisted even after a 16-hour washout. This suggests that chromatin opening facilitated by NFκB may be
transient but leads to durable H3K4 methylation even after the stimulus is removed, marking the region as
a de novo enhancer and reprogramming the epigenome.

Because histone methylation is more durable and indicative of enhancer function, we analyzed
the H3K4me1 ChIP-seq data independently and identified 2081 regions that acquired more H3K4
methylation in IκBα−/− than control (Fig. 4a). These differentially induced, dynamics-dependent de novo
enhancers persisted after the TNF stimulus was washed out, and they were strongly enriched for NFκB
motifs (Fig. 4b). We then asked whether these regions, which are dependent on non-oscillatory NFκB in
the IκBα−/− system, corresponded to the stimulus-specific NFκB-driven de novo enhancers in WT BMDMs
(Fig. 1d). We found that there was a highly significant overlap (p = 10 e-45), and the inducible ChIP-seq
signal was consistently greater when NFκB dynamics were non-oscillatory rather than oscillatory, whether
by genetic perturbation or by stimulus-specific signaling mechanisms (Fig. 4c).

Next, we asked whether these NFκB dynamics-dependent enhancers had a functional role in
macrophage gene expression. We hypothesized that de novo enhancers would alter transcriptional
responses to subsequent stimulation. We primed control and IκBα−/− BMDMs with TNF for eight hours
followed by 16-hour washout as before, then re-stimulated with secondary TNF over eight hours (Fig. 4d).
We performed RNA-seq in the basal (untreated) condition and at zero, one, three, and eight hours of
secondary TNF stimulation. We explored the relationship between differentially inducible enhancers and
gene expression using two approaches. First, using a peak-centric approach, we linked the 2081
enhancers to their nearest expressed genes, removed duplicates, and identified three distinct patterns of
expression for the 1511 genes. Cluster 1 and 2 genes were not TNF-responsive in either condition,
reflecting an intrinsic limitation of this approach when enhancers often regulate much more distant
genes. Despite this limitation, 58% of nearest genes were both TNF-responsive and more strongly
induced in IκBα−/− BMDMs (Fig. 4e Cluster 3). Many of these genes were not induced in controls at all. The
differentially induced genes were enriched for ontology terms “Immune system process” and
“Inflammatory process” (Fig. 4f).

To corroborate the results from the peak-centric analysis, we also examined our data using a
gene-centric approach. From the RNA-seq dataset we identified 1958 TNF-inducible genes, 482 of which
were differentially regulated in IκBα−/− versus control (Extended Data Fig. 8a-8b). For each gene, we
measured the genomic distance to the nearest differentially inducible H3K4me1 ChIP-seq region. We
found that differentially inducible genes were significantly closer to differentially inducible enhancers (p =
1.13 e-9) than genes that were not differentially inducible (Extended Data Fig. 8c-8d). Thus, both analytical approaches indicated that NFκB dynamics-dependent de novo enhancers play a functional role in differentially regulating gene expression response to secondary TNF.

The dynamics-dependent gene expression program included Nos2, Mmp2, and Mmp9, which are well-defined markers of classical macrophage activation26, as well as Acs11, which plays a role in the pathogenesis of atherosclerosis27 (Fig. 4g). Each of these genes had a nearby enhancer that gained more H3K4me1 signal in the presence of non-oscillatory NFκB, whether in the IkBa-/- system or in WT BMDMs stimulated with different ligands (Fig. 4h). These specific examples further suggested that de novo enhancers formed by non-oscillatory NFκB regulate genes involved in macrophage activation.

In summary, our results indicate that NFκB dynamics, particularly whether it is oscillatory or non-oscillatory, determine its capacity to reprogram the macrophage epigenome. We show with a mathematical model how biophysical principles governing nucleosome dynamics might decode stimulus-specific NFκB dynamical features. The role of temporal dynamics may thus complement the structure-function model in which pioneering TFs access nucleosomal DNA based on their recognition of partially exposed DNA motifs28. More broadly, our findings imply that stimulus-specific temporal dynamics of TF activity may result in stimulus-specific memory in macrophages. In response to some stimuli, immune sentinel cells activate oscillatory NFκB, which is sufficient for gene expression but does not produce de novo enhancers. In response to other stimuli, cells activate non-oscillatory NFκB, which activates a comparable gene expression program29 while also altering the epigenome, changing the phenotypic state of the cell and its response to subsequent stimuli. While further work will be needed to determine the physiological functions of NFκB dynamics-dependent de novo enhancers, our study establishes a mechanistic paradigm of TF temporal dynamics being a key determinant for driving epigenetic reprogramming.

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Author contributions:
QC, SO, AA, and BT performed the experiments. QC, SO, KS, RS, and AA analyzed the data. KS and BT developed the mathematical model. QC and AH wrote the manuscript with input from KS. All authors reviewed the manuscript. AH coordinated and funded the work.
Bibliography


Figure 1: NFκB-driven de novo enhancers are stimulus-specific and correlate to dynamic features of NFκB activity.

a) Heat map of H3K4me1 ChIP-seq inducible peaks from BMDMs stimulated with five ligands for eight hours, unsupervised K-means clustering. b) Known transcription factor motifs with greatest enrichment in Cluster 1 and Cluster 2 peaks. c) Heat map of H3K4me1 ChIP-seq in Ifnar<sup>-/-</sup> BMDMs, using same clusters as panel (a). d) Heat map of subset of Cluster 2 peaks that overlap with a RelA binding event by ChIP-seq. e) Heat map of matrix of p-values between ChIP-seq counts in panel (d), by two-tailed t-test between pairs of conditions. f) Heat maps of NFκB activity in single cells by live cell microscopy of mVenus-RelA BMDMs, showing nuclear abundance of NFκB in response to five stimuli. g) Bar graph of correlations (absolute value) between mean ChIP-seq counts in panel (d) and the six key features of NFκB dynamics<sup>3</sup> (see also Extended Data, Fig. 2).
Mathematical model predicts epigenetic response to distinct dynamic features of NFκB.

Figure 2: Mathematical model predicts epigenetic response to distinct dynamic features of NFκB. 

a) Crystal structures of nucleosomal DNA (PDB 1F66) vs. NFκB-bound DNA (PDB 1VKX), where p65:p50 NFκB dimer is in green. b) Schematic of model illustrating NFκB-driven displacement of nucleosome. c) Multi-step model with 14 steps to complete nucleosome unwrapping, each expressed as a Hill function. d) Heat maps of simulations of chromatin opening in response to different stimuli, using single cell trajectories from microscopy data as input. e) Model simulation vs. ChIP-seq data. Mean ChIP-seq counts from Fig. 1a Cluster 2, background-subtracted and scaled to maximum signal (LPS stimulation). Model simulations are mean of maximum E0 fraction per cell (cf. Extended Data Fig 3a), scaled to LPS condition. f) Model simulation of predicted chromatin accessibility comparing oscillatory vs. non-oscillatory input activities. g-h) Model simulation of predicted chromatin opening across a range of amplitudes and durations.
Figure 3: IкBα knockout abolishes NFκB oscillations, increasing chromatin accessibility and de novo enhancer formation. a) Schematic of IкBα as key regulator of NFκB oscillations. b) Heat map of single cell NFκB activity by microscopy comparing TNF response in WT vs. IкBα−/− macrophages. c) Bar graph of K-S test statistic for difference in distribution of six key signaling features and areas under NFκB activity curve, comparing IкBα−/− and WT. d) Heat map of ATAC-seq signal at 322 genomic regions that are TNF-inducible and differential between IкBα−/− and control. “Wash” = 16h washout. e) Known transcription factor motifs with greatest enrichment in differentially inducible ATAC-seq regions. f) Genome browser tracks for representative differentially inducible ATAC-seq regions, two replicates per time point. g) Percentage of cells with non-oscillatory NFκB dynamics by microscopy, compared with relative percentage of cells with accessible chromatin at Chr15 intergenic peak by ATAC-seq. h) Heat map of H3K4me1 ChIP-seq signal over the 322 regions defined as differentially inducible by ATAC-seq. “Wash” = 16h washout.
**Figure 4: NFκB dynamics-dependent enhancers are associated with dynamics-dependent gene expression.** a) Heat map of H3K4me1 ChIP-seq at 2081 regions that are TNF-inducible and differential between IκBα− and control, i.e. dynamics-dependent enhancers. “Wash” = 16h washout. b) Known transcription factor motifs with greatest enrichment in dynamics-dependent enhancers. c) Heat map of H3K4me1 signal after 8h stimulation at regions that overlap between Fig. 4a and Fig. 1d (n=211, p for overlap=10 e-45). d) Schematic of RNA-seq experiment. e) Heat map showing expression of genes closest to dynamics-dependent enhancers, where Cluster 3 exhibits differential gene expression between IκBα− and control. f) Top biological process ontology terms for genes in Cluster 3 of Fig. 4e. g) Examples of genes differentially induced between IκBα− and control, average and standard deviation of two replicates. h) Genome browser tracks of differentially inducible H3K4me1 peaks near differentially inducible genes, showing TNF-stimulated IκBα− vs. control and stimulus-specific response in WT BMDMs. More darkly shaded tracks indicate non-oscillatory NFκB conditions.