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Rapid induction of expression by LPS is accompanied by favorable chromatin and rapid binding of c-Jun

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ABSTRACT

The response to infection is managed in mammals by a coordinated immune response. Innate responses are rapid and hard wired and have been demonstrated to be regulated at the level of chromatin accessibility. This study examined primary human monocyte responses to LPS as a model of innate responses to bacteria. We utilized inhibitors of chromatin modifying enzymes to understand the inter-relationships of the chromatin complexes regulating transcription. Multiplex digital gene detection was utilized to quantitate changes in mRNA levels for genes induced by LPS. In the first 30 min, genes that were highly induced by LPS as a group exhibited minimal effect of the chemical inhibitors of chromatin modifications. At 60 min, the more highly expressed genes were markedly more inhibitable. The effects of the inhibitors were almost entirely concordant in spite of different mechanisms of action. Two focus groups of genes with either high LPS inducibility at 30 min or high LPS inducibility at 60 min (but not at 30 min) were further examined by ChIP assay. NFκB p65 binding was increased at the promoters of 30- and 60-min highly inducible genes equivalently. Binding of c-Jun was increased after LPS in the 30-min inducible gene set but not the 60-min inducible gene set. H3K4me3 and H4ac were not detectably altered by LPS stimulation. Baseline H3K4me3 and H4ac were higher in the 30-min highly inducible gene set compared to the 60-min highly inducible gene set. NFκB and JNK inhibitors led to diminished H4ac after LPS. The effects of DRB and C646 were greater for LPS-induced *IL6* transcription at 30 min and LPS-stimulated H4ac compared to *TNF* where transcription was largely unaffected by the inhibitors. In conclusion, genes with very rapidly induced expression after LPS exhibited more favorable chromatin characteristics at baseline and were less inhibitable than genes induced at the later time points.

1. Introduction

Organismal responses to pathogens are evolutionarily conserved and the responses are mediated largely by changes in gene expression. Contributions to the inflammatory response include complement activation, coagulation pathway activation, and pain responses (Rankin, 2004). Monocytes and macrophages represent cells that actively participate in the inflammatory response and their responses include an orderly series of changes to gene expression that lead to the elaboration of inflammatory cytokines and changes to the overall metabolism of the cell (Rossol et al., 2011). Toll-like receptors (TLR) represent the major interface between the cell and the inflammatory stimuli. TLR engagement drives a rapid induction of gene expression designed to recruit cells and combat the pathogen. Gram-negative bacteria are responsible for 150,000 cases of sepsis in the USA annually (Martin et al., 2003) and stimulate responses primarily through the TLR4 receptor (Chow et al., 1999; Medzhitov and Janeway, 2002). Understanding the mechanisms central to the response to gram negative bacteria is critical. This study focused on early transcriptional responses to LPS as a TLR4 ligand related to gram negative bacteria.

This topic has been the focus of intense investigation due to its importance in human health. It is known that highly inducible genes are enriched for promoter CpG islands which can contribute to a chromatin conformation favorable for transcription (Thomson et al., 2010; Fenouil et al., 2012). LPS induction of transcription in murine cells is known to occur in waves with early recruitment of NFκB occurring where histone acetylation is high and late recruitment occurring where histone acetylation is low (Saccani et al., 2001). Additional studies have implicated nucleosome repositioning as a key discriminator between early and late induction of expression (Ramirez-Carrozzi et al., 2009; Ramirez-Carrozzi et al., 2006). Long non-coding RNAs may play a role in remodeling of chromatin for the later waves of gene activation (Hu et al., 2016). NFκB and MAP kinases are both central to these chromatin effects but the sequence of events are largely unknown and few studies

Abbreviations: LPS, lipopolysaccharide; ChIP, chromatin immunoprecipitation

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have been performed in primary human monocytes (Mages et al., 2007; O'Neill et al., 2013).

Early-primary (*TNF, CXCL2, PTGS2, IL1B*), late-primary (*CCL5, SAA3, IFNB1*), and secondary (*IL6, IL12B, NOS2, MARCO*) response genes are three waves of gene expression that have been characterized in murine macrophages (Ramirez-Carrozzi et al., 2006; Bhatt et al., 2012; Sen and Smale, 2010). Using high dimensional transcriptional profiling of human leukocytes, temporal dynamic analysis identified fourteen gene expression waves with distinct transcription factors implicated (Nguyen et al., 2011). There is a knowledge gap regarding human monocyte transcriptional dynamics and the interplay of transcription factors and chromatin modifications.

An unusual aspect of transcriptional regulation of highly LPS-inducible genes is the role of pausing. The early-primary genes in mice are characterized by transcriptional pausing of RNA polymerase II. These genes are characterized by a pre-loaded RNA polymerase that has paused approximately 50–80 bp downstream of the transcription start site (Core et al., 2008; Patel et al., 2013). Upon provision of a second signal, elongation can proceed, leading to very rapid kinetics of transcriptional induction. We have recently identified enhancer RNAs mediating pause-release in human monocytes (Shi et al., 2017). The proposed mechanisms that differentiate between the other successive rounds of transcriptional induction are incompletely understood.

Transcription of these LPS-inducible genes can be followed by a refractory phase known as endotoxin tolerance (Biswas and Lopez-Collazo, 2009; Draisma et al., 2009). The mechanism of endotoxin tolerance is also regulated largely at the level of chromatin modifications although altered signaling molecules are also important (Biswas and Lopez-Collazo, 2009; Chen and Ivashkiv, 2010; Adib-Conquy and Cavaillon, 2002; Blackwell et al., 1997a,b; Rajaiah et al., 2013). This supports further study of the chromatin landscape regulating expression because endotoxin tolerance can be both protective and pathologic. Both type I and type II interferons can reset the permissive transcriptional environment in endotoxin tolerant cells and both are believed to function at the level of chromatin remodeling (Chen and Ivashkiv, 2010; Shi et al., 2015). Therefore, histone modifications and chromatin more generally appear to be strongly implicated in the regulation of transcriptional response to LPS. While much has been learned regarding the transcriptional regulation of genes downstream of TLR4 engagement by LPS, little is understood regarding the distinction between the different waves of transcriptional responses and the role of histone modifications in the regulation of expression, particularly in human monocytes. We therefore undertook a high dimensional analysis of transcriptional responses to LPS, a cell wall product of gram negative bacteria, using specific inhibitors to define the role of chromatin modifying enzymes.

2. Methods

2.1. Cells and inhibitors

Primary monocytes were obtained from a campus core facility under an IRB-approved protocol. Bead purification, using a method that leaves the monocytes untouched, was utilized (Dynabeads Untouched Monocyte Kit). Monocytes were treated with the indicated inhibitors for 20 min prior to stimulation. Two time points were analyzed: 30 min and 60 min after LPS for the transcriptional analysis. The chromatin modifying enzyme inhibitors utilized included C646 (Santa Cruz Biotechnology, Dallas, Texas) which was used at 20 μM. It is an inhibitor of CBP/p300 induced histone acetylation (Bowers et al., 2010). The inhibitor iBET151 (Life science Research, Billerica, Massachusetts) was used at 20 μM as a specific inhibitor of BRD3/4, important for transcriptional activation and elongation at paused genes (Yang et al., 2005). DRB (Sigma-Aldrich, Allentown, PA) was used at 40 μM as an inhibitor of elongation regulating eviction of NELF via P-TEFb (Yankulov et al., 1995). JSH-23 (Santa Cruz Biotechnology) was used at 20 μM as a specific inhibitor of NFκB translocation (Shin et al., 2004) and SP600125 (Calbiochem, Darmstadt, Germany) was used at 10 μM an inhibitor of the JNK MAP kinase.

2.2. Transcriptional analysis

Two independent replicates were utilized and averaged for the results presented. RNA was harvested using Directzol (Zymo Research, Irvine, CA). Detection utilized a multiplex digital approach without PCR amplification to detect changes in gene expression with high fidelity and without amplification artifact. The nCounter Human Immunology v2 panel (Nanostring, Seattle, WA) was utilized for profiling on the nCounter SPRINT Profiler. A key advantage of this methodology is that there is no amplification step, which eliminates artifact from primer efficiency. Thus, cross comparisons across different mRNAs have high fidelity. The heat maps utilized a curated set of genes with > 1.5 fold change.

2.3. ChIP assays

ChIP experiments were carried out as previously described (Garrett et al., 2008a,b). Briefly, five to ten million cells in each condition were prepared for the chromatin immunoprecipitation (ChIP) assays following the protocol from Upstate Biotechnology (Lake Placid, NY) with some modifications. Cells were treated with 1% formaldehyde for 10 min at room temperature to crosslink. Lysed cells were sonicated and immunoprecipitated overnight at 4 °C. Antibody-bound complexes were collected with a slurry of protein A (Invitrogen, Carlsbad, CA), washed extensively and immune complexes eluted. DNA was extracted by phenol-chloroform after reverse-crosslinking for 6 h at 65 °C and after protein removal by proteinase K (200 μg/ml, Roche) treatment in the presence of 20 μg/ml glycogen. DNA was finally RNase treated (40 μg/ml, Roche) for 30 min at 37 °C and quantitated before analyses. Antibodies utilized included those to: c-Jun (Santa Cruz Biotechnology, Santa Cruz, CA), NFκB p65 (Santa Cruz Biotechnology), H2BK120ub (Cell Signaling Technology, Danvers, MA), H4ac (Merck Millipore, Billerica, MA), and H3K4me3 (Active Motif, Carlsbad, CA). The GST antibody (Invitrogen, Camarillo, CA) was used as a negative control for all ChIP assays. Cells were harvested at 30 min after LPS stimulation. The primers for the ChIP assays are listed below:

Primer name	Sequence
CCL20-Promoter	FWD: 5′- CTCCTTGACTGGTTCTGGAAAG −3′
	Probe: 5′-/56-AGGAGTTCTGGAATGTTCCTGTGTGG/36-TAMSp/-3′
	REV: 5′- AGAGGATTAACAGCGATACAAAGG −3′
CCL3-Promoter	FWD: 5′- TGACAGCATCACTACGCTTAAA −3'
	Probe: 5′-/56-FAM/CATCCGCCAGGGCTGCCTATAA/36-TAMSp/-3′
	REV: 5′- TCTGAAACCAGCTCTCCTCT −3'
CCL4-Promoter	Fwd: 5′- GAGCAGCACAGTTCTTGTCTA −3′
	Probe: 5′-/56-FAM/AGCCACTTGTAGCAGGTGTGAACA/36-TAMSp/-3′
	REV: 5′- GGGAAGTGGTACAGCCAAA −3′
PTGS2-promoter	FWD: 5′- GGCGGAAAGAAACAGTCATTTC −3′
	Probe: 5′-/56-FAM/AAGGTTCTCTCGGTTAGCGACCAA/36-TAMSp/-3′
	REV: 5′- CGCTCACTGCAAGTCGTAT-3′