

# Synergistic Activation of Inflammatory Cytokine Genes by Interferon- $\gamma$ -Induced Chromatin Remodeling and Toll-like Receptor Signaling

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## SUMMARY

Synergistic activation of inflammatory cytokine genes by interferon- $\gamma$  (IFN- $\gamma$ ) and Toll-like receptor (TLR) signaling is important for innate immunity and inflammatory disease pathogenesis. Enhancement of TLR signaling, a previously proposed mechanism, is insufficient to explain strong synergistic activation of cytokine production in human macrophages. Rather, we found that IFN- $\gamma$  induced sustained occupancy of transcription factors STAT1, IRF-1, and associated histone acetylation at promoters and enhancers at the *TNF*, *IL6*, and *IL12B* loci. This priming of chromatin did not activate transcription but greatly increased and prolonged recruitment of TLR4-induced transcription factors and RNA polymerase II to gene promoters and enhancers. Priming sensitized cytokine transcription to suppression by Jak inhibitors. Genome-wide analysis revealed pervasive priming of regulatory elements by IFN- $\gamma$  and linked coordinate priming of promoters and enhancers with synergistic induction of transcription. Our results provide a synergy mechanism whereby IFN- $\gamma$  creates a primed chromatin environment to augment TLR-induced gene transcription.

## INTRODUCTION

Macrophages are innate immune cells important for host defense and are also implicated in the pathogenesis of chronic inflammatory diseases such as rheumatoid arthritis (RA) (Hamilton and Tak, 2009). Macrophages are major producers of inflammatory cytokines that mediate host defense and drive disease pathogenesis. Of these cytokines, tumor necrosis factor (TNF), interleukin-6 (IL-6), and the p40 subunit shared by IL-12 and IL-23 are particularly important in human disease pathogenesis

and have been successfully targeted therapeutically (St Clair, 2009). *TNF*, *IL6*, and *IL12B* (encodes p40) gene expression is strongly induced by microbial products that are sensed by Toll-like receptors (TLRs) and other pattern recognition receptors and by various endogenous inflammatory factors. Mechanisms that regulate TNF, IL-6, and p40 expression are being intensively investigated, because modulation of expression of these genes would have therapeutic utility, especially if achieved in a gene-specific manner (reviewed in Glass and Saijo, 2010; Medzhitov and Horng, 2009; Smale, 2010).

TLR signaling activates key downstream effector molecules to induce target gene expression: NF- $\kappa$ B, mitogen activated protein kinases (MAPKs), and interferon regulatory factors (IRFs) (Takeuchi and Akira, 2010). Canonical NF- $\kappa$ B signaling that activates p65 (RelA) plays a key role in induction of inflammatory cytokine genes, including *TNF*, *IL6*, and *IL12B*. More recently, it has become clear that effective activation of genes by TLR signaling involves overcoming a rate-limiting chromatin barrier imposed by histone-containing nucleosomes that bind DNA (Foster et al., 2007; Ivashkiv, 2013; Smale, 2010; Medzhitov and Horng, 2009; Ramirez-Carrozzi et al., 2009). This barrier is overcome by posttranslational modification of histones, such as acetylation, to “loosen” their interaction with DNA, and by repositioning or removal of nucleosomes by ATP-dependent nucleosome remodeling complexes. In this model, the binding of “signaling transcription factors” such as NF- $\kappa$ B to target genes is determined by the accessibility of chromatin. Thus, the epigenetic landscape or chromatin state at a given gene locus determines the transcriptional output that occurs downstream of a given signal (Natoli et al., 2011).

The chromatin state at gene loci in resting naive macrophages is determined during development by master transcription factors such as PU.1 and C/EBP $\alpha$  that bind to promoters and distal regulatory elements (enhancers) to partially open chromatin and facilitate binding of other factors (Ghisletti et al., 2010; Heinz et al., 2010; Jin et al., 2011; Pham et al., 2012). In resting naive macrophages, cytokine genes are typically characterized by the presence of basal positive histone marks and partial accessibility to nucleases that poise these genes for rapid, albeit transient and

limited, responses to TLR stimulation (Escoubet-Lozach et al., 2011; Medzhitov and Horng, 2009; Ramirez-Carrozzi et al., 2009). TLR signaling further modifies chromatin to increase positive histone marks and remodel nucleosomes (reviewed in Ivashkiv, 2013; Medzhitov and Horng, 2009; Smale, 2012). The primary response gene *Tnf* exhibits accessible chromatin and prebound RNA polymerase II (pol II) stalled just downstream of the transcription start site (TSS), whereas the secondary response genes *Il6* and *Il12b* require de novo protein synthesis and more extensive chromatin remodeling for effective transcription. Induction of these genes also requires activation of enhancers. Although macrophage enhancers have been identified genome-wide based on binding of PU.1 and recruitment of the histone acetyltransferase (HAT) CBP after TLR stimulation (Ghisletti et al., 2010; Heinz et al., 2010; Jin et al., 2011; Pham et al., 2012), little is known about their function or how they are regulated.

Interferon- $\gamma$  (IFN- $\gamma$ ) is a potent macrophage-activating cytokine that activates antimicrobial and antigen-presenting functions and plays a key role in host defense against intracellular pathogens (Schroder et al., 2004). One key function of IFN- $\gamma$ , often termed priming, is to increase inflammatory cytokine production, including TNF, IL-6, and p40, in response to other inflammatory factors such as TLR ligands (Hu and Ivashkiv, 2009). This priming function of IFN- $\gamma$  may be particularly important in pathogenesis of inflammatory diseases such as RA and lupus where macrophages exhibit an IFN and STAT1 signature that contributes to elevated pathogenic cytokine production (Hu et al., 2008a). IFN- $\gamma$  signals via protein tyrosine kinases Jak1 and Jak2 to activate the transcription factor STAT1, which activates expression of numerous interferon-stimulated genes (ISGs) by binding to consensus DNA sequences termed gamma-activated sequence (GAS) elements (Levy and Darnell, 2002; Stark and Darnell, 2012). STAT1 has been traditionally considered as a “signaling transcription factor” that, similar to NF- $\kappa$ B, is transiently recruited to already open chromatin. IFN- $\gamma$  also induces expression of transcription factors such as IRF-1 and IRF-8 that bind to distinct IRF DNA elements and cooperate with STAT1 in the activation of ISGs. *TNF*, *IL6*, and *IL12B* are not directly activated by IFN- $\gamma$  in the absence of a second inflammatory signal (Levy and Darnell, 2002; Stark and Darnell, 2012), do not contain known functional GAS sites in their promoters, are not known to bind STAT1, and thus are not canonical ISGs. Given the absence of known functional GAS sites and STAT1 binding, the mechanism by which IFN- $\gamma$  potentiates TLR-induced *TNF* and *IL6* transcription has been previously attributed to enhancement of TLR signaling (Hu and Ivashkiv, 2009; Schroder et al., 2006). A direct role for IFN- $\gamma$  or STAT1 in regulation of *TNF*, *IL6*, or *IL12B* expression has not been previously described.

In this study, we wished to investigate mechanisms by which IFN- $\gamma$  augments TLR-induced transcription to achieve synergistic activation of cytokine genes such as *TNF*, *IL6*, and *IL12B*. To maximize physiological relevance for human inflammatory conditions, we used primary human monocytes and macrophages that play a key role in human inflammatory diseases. We used gene-specific and genome-wide approaches to find that IFN- $\gamma$  induced stable and coordinated recruitment of STAT1 to enhancers and promoters of genes that are synergistically activated by IFN- $\gamma$  and LPS. STAT1 occupancy was associated with increased histone acetylation and IRF-1 recruitment, activa-

tion of enhancers, and augmented and prolonged recruitment of TLR-induced transcription factors and gene transcription, including transcription of *TNF*, *IL6*, and *IL12B* genes. These results provide a mechanism by which IFN- $\gamma$  augments TLR-induced transcriptional responses by inducing chromatin remodeling at promoters and enhancers and support inhibition of Jak-STAT1 signaling as a therapeutic approach in diseases associated with primed macrophages.

## RESULTS

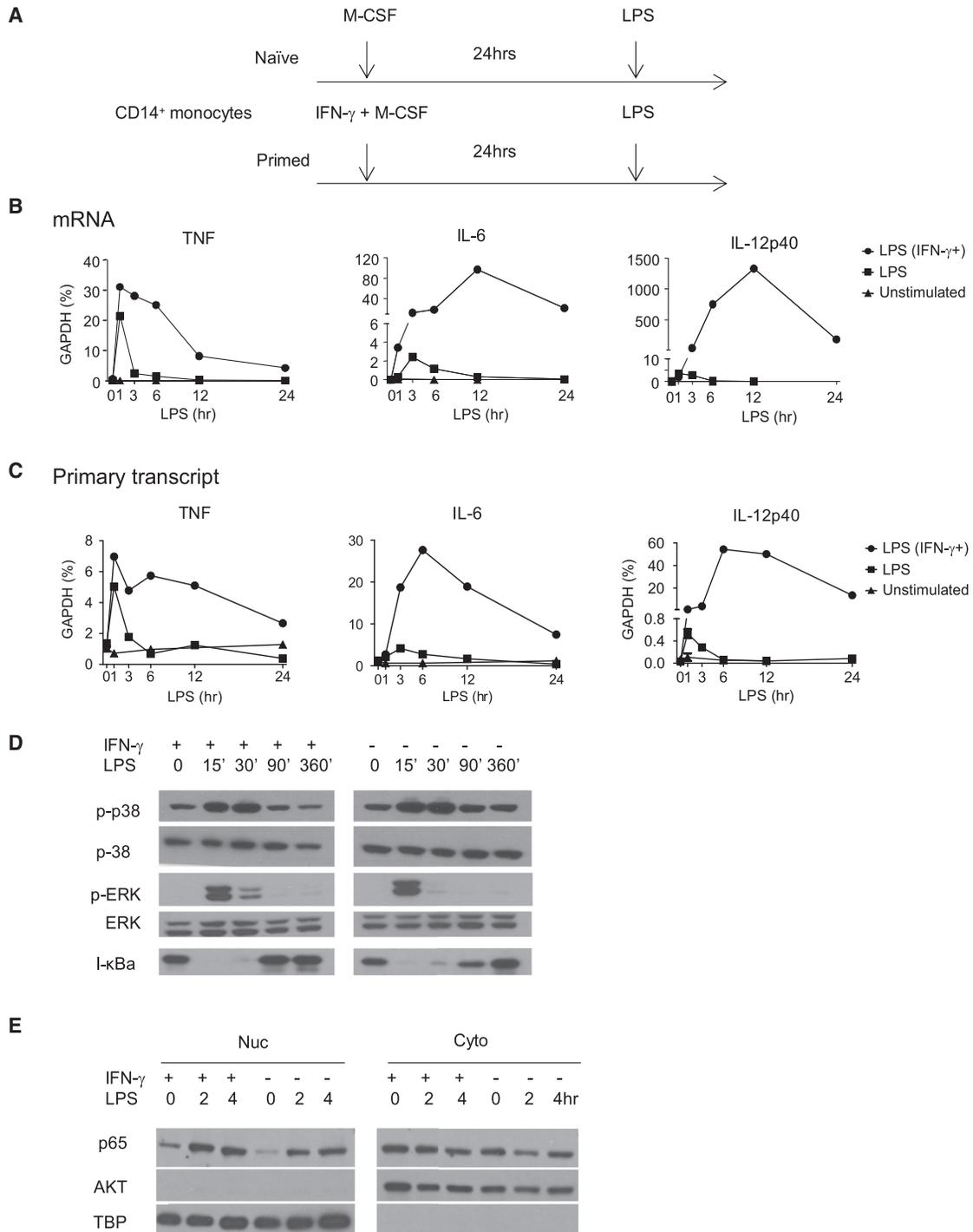
### IFN- $\gamma$ Strongly Increases TLR-Induced Cytokine Gene Transcription but Minimally Affects TLR Signaling

We investigated the mechanisms by which IFN- $\gamma$  augments inflammatory cytokine gene expression in primary human macrophages. Cells were primed with IFN- $\gamma$  for 24 hr, stimulated with LPS, and TLR4-induced gene expression and signaling were analyzed (Figure 1A). As expected, LPS induced transient expression of TNF, IL-6, and IL-12 p40 mRNA in unprimed (naive) resting macrophages, with slower kinetics for the secondary response genes *IL6* and *IL12B* relative to the primary response gene *TNF* (Figure 1B). IFN- $\gamma$  priming alone did not induce expression of these inflammatory cytokines (Figure 1B, “0” time point and data not shown), consistent with previous reports that *TNF*, *IL6*, and *IL12B* are not ISGs (Levy and Darnell, 2002; Stark and Darnell, 2012; and references therein). However, macrophages primed with IFN- $\gamma$  expressed substantially increased amounts of TNF, IL-6, and p40 mRNA with prolonged kinetics after LPS stimulation (Figure 1B). TLR4-mediated induction of TNF, IL-6, and p40 primary transcripts (detected by using intronic PCR primers) was similarly increased in primed human macrophages (Figure 1C), suggesting that IFN- $\gamma$  priming results in increased cytokine gene transcription after TLR stimulation. Priming effects were gene-specific (see Figure S1A available online) and cytokine-specific, because they were not observed with IFN- $\beta$  (Figure S1B).

Previous work with cell lines and murine systems suggested that increased TLR-induced NF- $\kappa$ B and MAPK signaling in IFN- $\gamma$ -activated macrophages contributes to increased inflammatory cytokine gene expression, although these increases in signaling were modest and context dependent (Hu and Ivashkiv, 2009; Schroder et al., 2006). Thus, we investigated the effects of IFN- $\gamma$  priming on TLR4-induced activation of MAPK and NF- $\kappa$ B pathways in human macrophages. Despite the substantial increase in transcription (Figure 1B and 1C), TLR4-induced activation of MAPKs p38 and ERK and of NF- $\kappa$ B signaling, the latter assessed by I- $\kappa$ B $\alpha$  degradation and p65 (RelA) nuclear translocation, was minimally if at all enhanced in IFN- $\gamma$ -primed macrophages (Figure 1D and E). Thus, increased TLR4 signaling cannot explain the increased and sustained transcription of *TNF*, *IL6*, and *IL12B* in primed human macrophages. These results suggest that transcriptional output downstream of canonical TLR-induced signaling pathways is regulated by IFN- $\gamma$  in a gene-specific manner, possibly at the chromatin level.

### Increased Chromatin Accessibility and Recruitment of Transcription Factors in IFN- $\gamma$ -Primed Macrophages

Induction of cytokine gene expression downstream of canonical TLR signaling is constrained by a chromatin barrier whose



**Figure 1. IFN- $\gamma$  Increases TLR-Induced Cytokine Gene Transcription but Not TLR Signaling**

(A) Experimental design. Human macrophages were cultured  $-/+$  IFN- $\gamma$  (100 U/ml) and then stimulated with LPS (20 ng/ml).

(B) mRNA of the indicated genes was measured by quantitative PCR (qPCR) and normalized relative to GAPDH mRNA.

(C) Primary transcripts were measured by qPCR.

(D and E) Immunoblot of whole cell (D) or nuclear (E) lysates. Abbreviations are as follows: Nuc, nuclear; Cyto, cytoplasmic lysate. Data are representative of at least three experiments (B-E).

remodeling is required for expression of many TLR-inducible genes (Ivashkiv, 2013; Medzhitov and Horng, 2009; Natoli et al., 2011; Smale, 2010). We began to investigate the possibility

that IFN- $\gamma$  priming overcomes this chromatin barrier by using chromatin immunoprecipitation (ChIP) assays to measure occupancy of general and specific transcription factors at the TNF,

IL-6, and IL-12 p40 promoters. RNA polymerase II (pol II) recruitment was transiently increased at all three promoters by LPS stimulation of naive cells (Figure 2A), which was consistent with the kinetics of transcription. Pol II recruitment was enhanced and prolonged at all three gene loci after LPS stimulation of IFN- $\gamma$ -primed macrophages relative to naive macrophages (Figure 2A). The kinetics of pol II recruitment paralleled the kinetics of RNA induction observed in Figures 1B and 1C. Lack of pol II occupancy at the silent hemoglobin (*HBB*) promoter and decreased occupancy at *IL10* served as negative controls (Figure 2A; data not shown). A similar pattern of increased and prolonged occupancy at TNF, IL-6, and p40 promoters after TLR4 stimulation of IFN- $\gamma$ -primed macrophages was observed with TATA binding protein (TBP, a component of the transcriptional initiation complex) (Figure 2B). These results suggest that IFN- $\gamma$  increases TLR4-induced gene expression by enhancing formation of pol II-containing transcription initiation complexes at cytokine gene promoters.

Pol II recruitment is facilitated by TLR-induced transcription factors such as NF- $\kappa$ B and C/EBP $\beta$  (Smale, 2010). IFN- $\gamma$  priming resulted in substantially increased and prolonged recruitment of p65 at TNF and IL-6 promoters after TLR4 stimulation (Figure 2C), despite comparable overall nuclear p65 amounts in naive and primed TLR-stimulated cells (Figure 1E). As NF- $\kappa$ B is considered a “signaling transcription factor” that is recruited to open chromatin in an acute stimulation setting (Ivashkiv, 2013; Sacconi et al., 2002; Weinmann et al., 2001), these results suggest that IFN- $\gamma$  increases opening of chromatin to facilitate p65 recruitment. Enhanced recruitment of C/EBP $\beta$  in IFN- $\gamma$ -primed macrophages was also observed (Figure 2D). The possibility that IFN- $\gamma$  priming increases chromatin accessibility was further tested with restriction enzyme accessibility assays (REAs). IL-6 promoter accessibility was increased by IFN- $\gamma$  priming and further increased after LPS stimulation (Figure 2E). Collectively, the results suggest that IFN- $\gamma$  promotes chromatin remodeling to increase chromatin accessibility, recruitment of TLR-induced transcription factors, and transcription at inflammatory cytokine genes.

### IFN- $\gamma$ Increases Histone Acetylation and CBP/p300 Recruitment

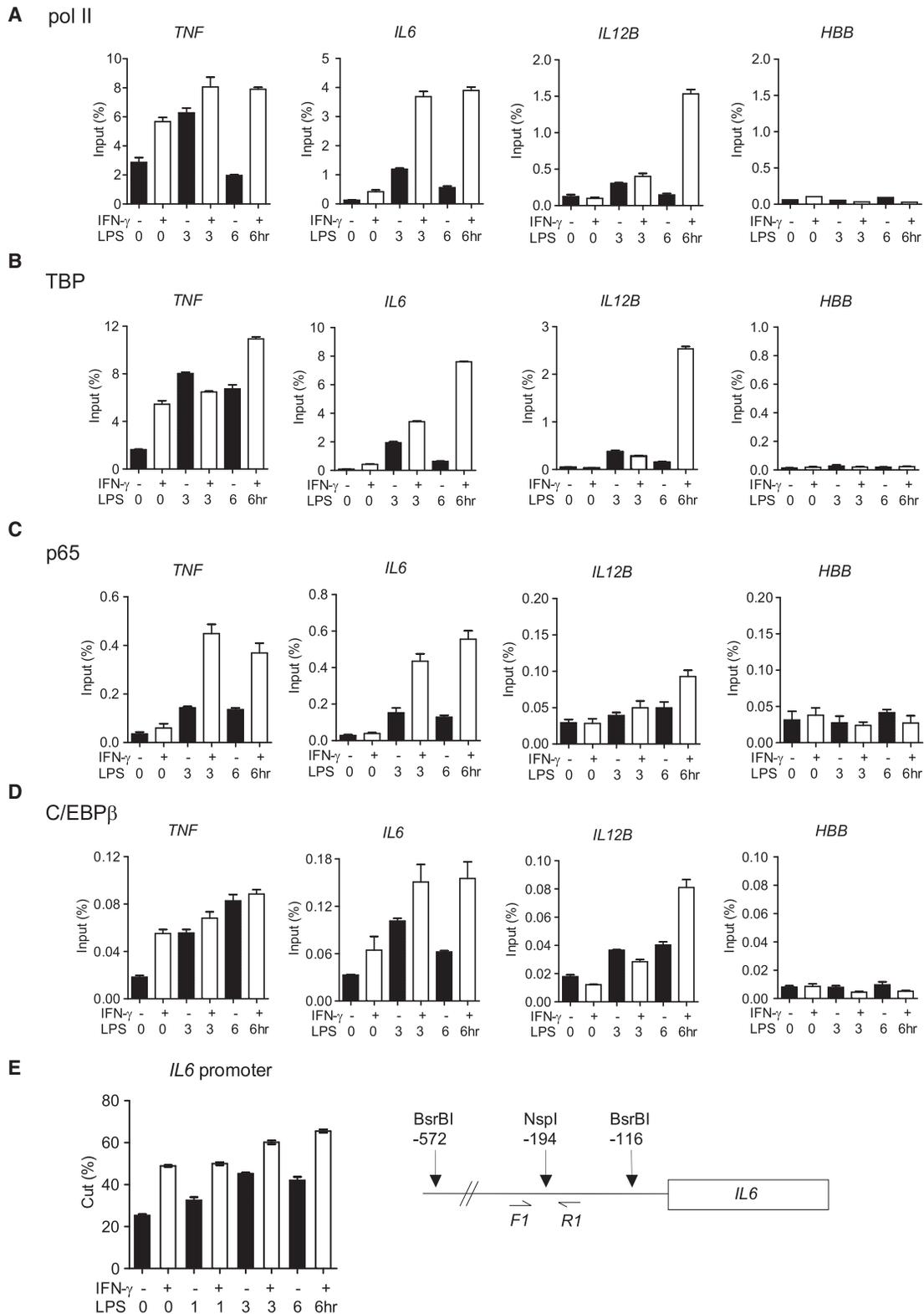
Chromatin accessibility is increased by acetylation of histones by HATs such as CBP or p300 and by ATP-dependent nucleosome remodeling complexes (Suganuma and Workman, 2011). We next investigated the effects of IFN- $\gamma$  priming on histone acetylation at the *TNF*, *IL6*, and *IL12B* loci. Histone 4 acetylation (H4-Ac) increased after IFN- $\gamma$  priming and further increased after TLR4 stimulation, such that H4-Ac was higher in LPS-stimulated primed macrophages than in LPS-stimulated resting macrophages (Figure 3A). In contrast, IFN- $\beta$  did not increase basal or LPS-induced H4-Ac at the same gene loci (Figure S1C), which was consistent with the lack of increased transcription (Figure S1B). We then evaluated the genome-wide effect of IFN- $\gamma$  priming on histone acetylation by using ChIP coupled with high-throughput sequencing (ChIP-seq) to map H3K27-Ac, a histone mark for active enhancers and promoters (Buecker and Wysocka, 2012; Natoli et al., 2011). Analysis with ChIPseeqer (Giannopoulou and Elemento, 2011) showed comparable peak numbers ( $p < 10^{-15}$ , FDR <

0.05) among the four analyzed conditions (Figures S2A and S2B). However, IFN- $\gamma$  priming induced a change in the distribution of H3K27-Ac tag densities at individual gene loci (Figure 3B; Figure S2C). IFN- $\gamma$  priming had a much more pervasive effect on genomic distribution of H3K27-Ac peaks than LPS stimulation (Figure 3B), suggesting that IFN- $\gamma$  can broadly alter macrophage epigenetic landscape and thereby influence gene expression in response to subsequent stimuli. Figure 3C shows gene tracks of ChIPseq-derived H3K27-Ac tag density at the *IL6* and *TNF* loci. Consistent with Figure 3A, IFN- $\gamma$  priming alone increased H3K27 acetylation at IL-6 and TNF promoters, and H3K27-Ac peaks were highest in IFN- $\gamma$ -primed macrophages that were stimulated with LPS. This increased histone acetylation was associated with increased recruitment of the HATs p300 and CBP (Figure 3D and 3E). Collectively, the results suggest that IFN- $\gamma$  stably “marks” gene promoters by increasing histone acetylation, thereby initiating priming-mediated chromatin remodeling prior to LPS stimulation. Histone acetylation is then superinduced upon activation of TLR4 signaling, with concomitant increased gene transcription.

### IFN- $\gamma$ Primes Activation of Distal Regulatory Elements

Distal regulatory elements such as enhancers are characterized by DNaseI hypersensitivity, H3K27-Ac, and binding of p300/CBP (Buecker and Wysocka, 2012; De Santa et al., 2010; Djebali et al., 2012; Bernstein et al., 2012). IFN- $\gamma$  induced broad H3K27-Ac peaks 25 kb, 50 kb, and 65 kb upstream of the *IL6* TSS that were more pronounced in the IFN- $\gamma$  + LPS condition and overlapped with DNaseI hypersensitivity sites (Figure 3C, track 5; DNaseI hypersensitivity data obtained from ENCODE/University of Washington [Thurman et al., 2012]); these upstream sites were not enriched in H3K4me3 promoter marks (data not shown) and did not overlap with any annotated genes. This suggests that IFN- $\gamma$  and LPS activate upstream enhancers, with greatest activation in the IFN- $\gamma$  + LPS condition. Activated macrophage enhancers are characterized by increased recruitment of CBP, NF- $\kappa$ B p65, and transcription of enhancer RNA (eRNA) (De Santa et al., 2010; Escoubet-Lozach et al., 2011; Ghisletti et al., 2010). Concordantly, the DNA region 25 kb upstream of the *IL6* TSS showed increased recruitment of CBP and p65 in IFN- $\gamma$ -primed LPS-stimulated macrophages (Figure 4A). There was a concomitant IFN- $\gamma$ -mediated superinduction of putative enhancer transcripts in this region (Figure 4B).

We also analyzed regulation of a previously identified LPS-activated enhancer 10 kb upstream of the mouse *Il12b* TSS (Zhou et al., 2007) that is conserved and corresponds to a DNaseI hypersensitive site in humans (Thurman et al., 2012). Although H3K27-Ac marks in ChIP-seq experiments were weak at the time point tested, ChIP-PCR showed clear superinduction of H4-Ac at the -10 kb *IL12B* enhancer in IFN- $\gamma$ -primed LPS-stimulated macrophages, with concomitant increased recruitment of CBP, p65, and C/EBP $\beta$ , and of eRNA transcription (Figures 4C and 4D). Thus, IFN- $\gamma$  priming results in superactivation of a bona fide enhancer on subsequent LPS challenge. Collectively, the results suggest that IFN- $\gamma$  primes enhancers for stronger activation in response to TLR stimulation. Genome-wide, IFN- $\gamma$  priming induced approximately 5,000



**Figure 2. IFN- $\gamma$  Priming Increases TF Recruitment and Chromatin Accessibility**

(A–D) ChIP assays. Primed (white bars) and unprimed (black bars) macrophages were stimulated with LPS (20 ng/ml) and the occupancy of RNA polymerase II (Pol II) (A), TBP (B), NF- $\kappa$ B p65 (C), and C/EBP $\beta$  (D) at the promoters of the indicated genes was assessed by ChIP.

(E) Restriction enzyme accessibility assay (REA) with primer set F1 and R1 to measure cutting at NspI site in *IL6* promoter. Data shown are representative of at least three experiments (A–E).

new H3K27-Ac peaks in distal and intergenic regions (defined as 2–50 kb or >50 kb relative to TSS, respectively) (Figure 3B; Figure S2C, upper table), with an additional 6,000 unique H3K27-Ac peaks in LPS-stimulated IFN- $\gamma$ -primed cells (Figure S2C, lower table). This suggests that IFN- $\gamma$  alters the regulatory and enhancer landscape to modulate macrophage responses to inflammatory stimulation.

#### Recruitment of STAT1 to Inflammatory Cytokine Loci

*TNF*, *IL6*, and *IL12B* are not classical ISGs and previous analysis of their promoters did not reveal functional GAS elements or STAT1 binding (Levy and Darnell, 2002; Stark and Darnell, 2012). Nonetheless, because STAT1 is a critical mediator of IFN- $\gamma$  responses and recruits CBP/p300 to increase histone acetylation (Levy and Darnell, 2002; Stark and Darnell, 2012), we tested whether STAT1 was recruited to *TNF*, *IL6*, and *IL12B* promoter and putative enhancer elements by IFN- $\gamma$  priming. Surprisingly, we observed STAT1 recruitment to these promoters and also to enhancers (Figure 5A); this finding was confirmed by using three different STAT1 antibodies (data not shown). There were several salient features to the pattern of STAT1 occupancy: (1) IFN- $\gamma$ -induced transient early recruitment similar to that observed at many classic ISGs (columns 1–3). (2) IFN- $\gamma$  priming resulted in late phase sustained STAT1 occupancy (column 7) that coincides with high STAT1 expression (Hu et al., 2002). (3) Transient induction after LPS stimulation related to autocrine IFN- $\beta$  that activates the ISGF3 complex comprised of STAT1, STAT2, and IRF9 that binds to distinct ISRE DNA elements (STAT2 and IRF9 recruitment are shown in Figure S3A and S3B). (4) Increased occupancy in IFN- $\gamma$ -primed macrophages relative to naive macrophages after LPS stimulation (columns 8 and 9 versus 5 and 6).

To gain further insight into the binding of STAT1, we performed ChIP-seq analysis. IFN- $\gamma$  priming resulted in a dramatic increase in STAT1 binding sites that were apparent 24 hr after IFN- $\gamma$  stimulation (Figure S4A: from 1,188 peaks in untreated to 37,230 peaks in IFN- $\gamma$ -primed samples;  $p < 10^{-15}$ , FDR < 0.008 for the stimulated conditions). IFN- $\gamma$  induced dramatically more STAT1 peaks than did LPS, and most STAT1 peaks were maintained or increased after LPS stimulation in IFN- $\gamma$ -primed cells (Figure 5B; Figure S4A). Similar to ChIP-qPCR, ChIP-seq (Figures 5C and 5D) revealed that IFN- $\gamma$  priming induced sustained late phase STAT1 occupancy peaks at *IL6* and *TNF* loci (track 2 versus track 1); STAT1 peaks occurred coordinately in promoter, intronic, and distal regions. Several new peaks were observed in distal and intronic regions solely after LPS stimulation of IFN- $\gamma$ -primed macrophages (track 4). The distal and intronic STAT1 peaks aligned with valleys in the broad H3K27-Ac peaks (track 5) and with DNaseI hypersensitivity sites (track 6). This corresponds to a well-established binding pattern of a transcription factor to a nucleosome-depleted regulatory region (Neph et al., 2012), further supporting the notion that these sites correspond to regulatory elements. Collectively, the results show that IFN- $\gamma$  induces recruitment of STAT1 to regulatory regions of *IL6* and *TNF* that is associated with histone acetylation, and support the idea that IFN- $\gamma$  primes promoters and enhancers to allow increased gene expression on subsequent LPS challenge.

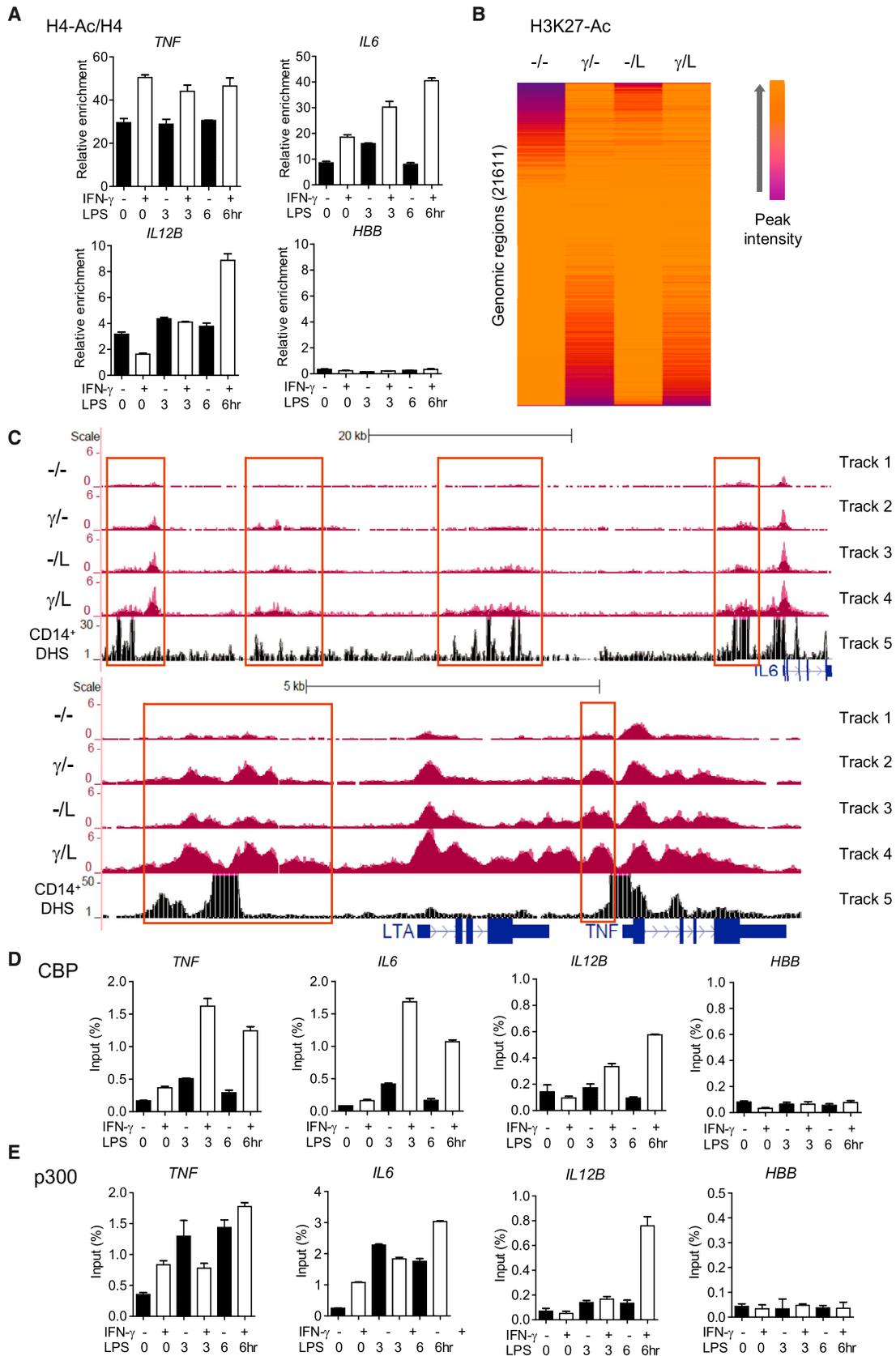
#### Genome-wide Association of STAT1 Binding and H3K27 Acetylation

We next examined the genome-wide relationship between IFN- $\gamma$  priming-induced STAT1 and H3K27-Ac peaks (Figure 5E). Although the number of STAT1 peaks varied among the four conditions tested, more than one third of STAT1 peaks were associated with H3K27-Ac peaks in all four conditions; this association is significant ( $Z$  score > 450 in stimulated conditions) and consistent with the role of STAT1 as a recruiter of HATs (Levy and Darnell, 2002; Stark and Darnell, 2012; Vahedi et al., 2012). Conversely, only 2.0% of H3K27-Ac peaks (which were comparable in number in all four conditions) associated with STAT1 peaks in resting macrophages, but this increased to 14.3% in LPS-stimulated cells and to 32.1% in IFN- $\gamma$ -primed LPS-stimulated macrophages (Figure 5E). Concordantly, genome-wide analysis showed peak H3K27 acetylation occurred within 500 bp of STAT1 peak summits (Figure 5F). The highly statistically significant genome-wide correlation of IFN- $\gamma$ -induced STAT1 recruitment with increased H3K27 acetylation suggests that IFN- $\gamma$  primes regulatory elements via recruitment of STAT1 and associated HATs that promote histone acetylation.

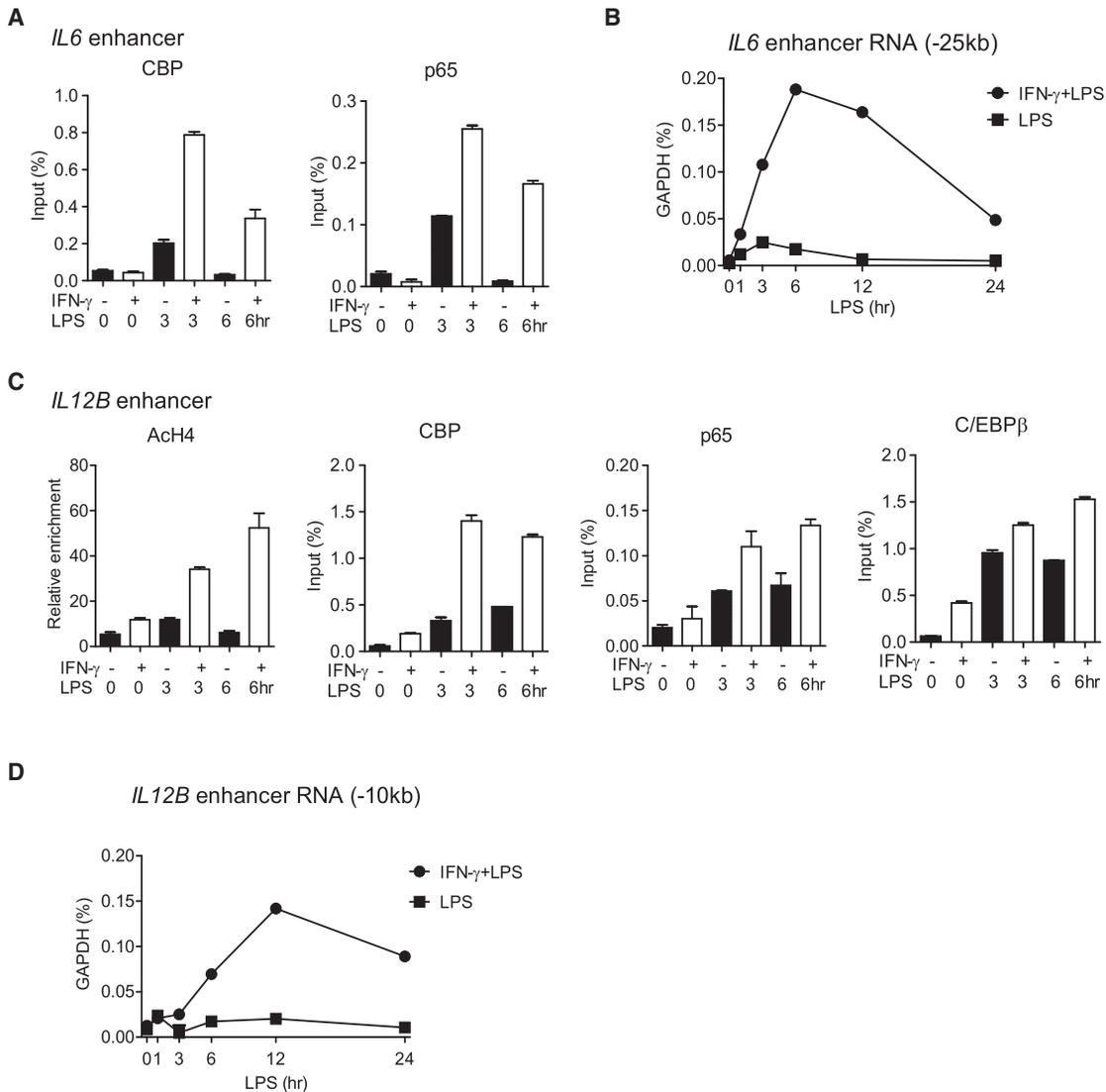
#### Genomic Profile of STAT1 Binding

We further analyzed genome-wide STAT1 binding to gain insights into its biological function and mechanisms of priming. IFN- $\gamma$  priming induced a massive increase in STAT1 binding peaks at both promoters and distal regions (Figures S4A and S4B, column A; Figure S4C). LPS-stimulated IFN- $\gamma$ -primed cells exhibited unique STAT1 peaks compared to primed cells or LPS-stimulated naive cells, especially in introns. Induction of unique peaks in LPS-stimulated IFN- $\gamma$ -primed cells (Figure S4B, column B; Figure S4C) suggests synergistic activation of regulatory regions. Motif analysis to identify transcription factor binding motifs within a 200 bp window relative to the summit of STAT1 peaks revealed the expected enrichment of GAS elements (consensus binding motifs for STAT1 dimers) (Figure 6A). However, consistent with previous literature, GAS sequences were not present at *IL6* or *TNF* promoters. In contrast, GAS-like sequences were present under STAT1 peaks at distal regulatory elements upstream of *IL6* (Table S1), and also upstream of *IL12A* and *EGR3*, genes that were also synergistically activated by IFN- $\gamma$  and LPS (Table S1). GAS sequences derived from upstream STAT1 peaks in the *IL6* locus and the *IL12A* locus sequence showed strong and specific binding to the classic STAT1-binding hSIE oligonucleotide (Hu et al., 2002) (Figure S4D). The *EGR3* locus GAS-like sequence showed weak and inconsistent binding activity, whereas three additional oligonucleotides including a TNF promoter-derived sequence did not show DNA-binding activity. Collectively, the results suggest that in IFN- $\gamma$ -primed macrophages STAT1 binds to its cognate GAS sequence at sites such as upstream regulatory elements at the *IL6* locus but that binding of STAT1 at other peaks may reflect indirect or cooperative binding with other proteins.

Additional bioinformatic analysis revealed that STAT1 peaks in IFN- $\gamma$ -primed macrophages were also enriched in motifs that represent binding sites for the transcription factors PU.1, IRF, ISGF3, NF- $\kappa$ B, C/EBP $\beta$ , AP-1, and ERG (Figure S4E), which is characteristic of activated enhancers in macrophages (Escoubet-Lozach et al., 2011; Ghisletti et al., 2010; Heinz



(legend on next page)



**Figure 4. IFN- $\gamma$  Activates Upstream Enhancers at *IL6* and *IL12B* Loci**

(A and B) Analysis of region 25 kb upstream of *IL6* TSS containing H3K27-Ac and DNaseI hypersensitivity peaks shown in Figure 3C. (A) ChIP assays for recruitment of CBP and p65 to *IL6* putative enhancer under indicated conditions. (B) qPCR analysis of eRNA transcripts.

(C and D) Analysis of *IL12B* -10 kb enhancer (Zhou et al., 2007). (C) ChIP assays for H4-Ac, CBP, p65, and C/EBP $\beta$ . (D) qPCR analysis of eRNA transcripts. Results are representative of at least three independent experiments.

et al., 2010; Natoli et al., 2011; Pham et al., 2012). Consistent with the motif analysis, about two thirds of STAT1 peaks overlapped with previously reported PU.1 peaks in human macrophages (Figure S4F) (Pham et al., 2012). Greater than 97% of STAT1 peaks corresponded to DNaseI hypersensitive sites (Figure S4G) (Thurman et al., 2012), further supporting the notion that STAT1

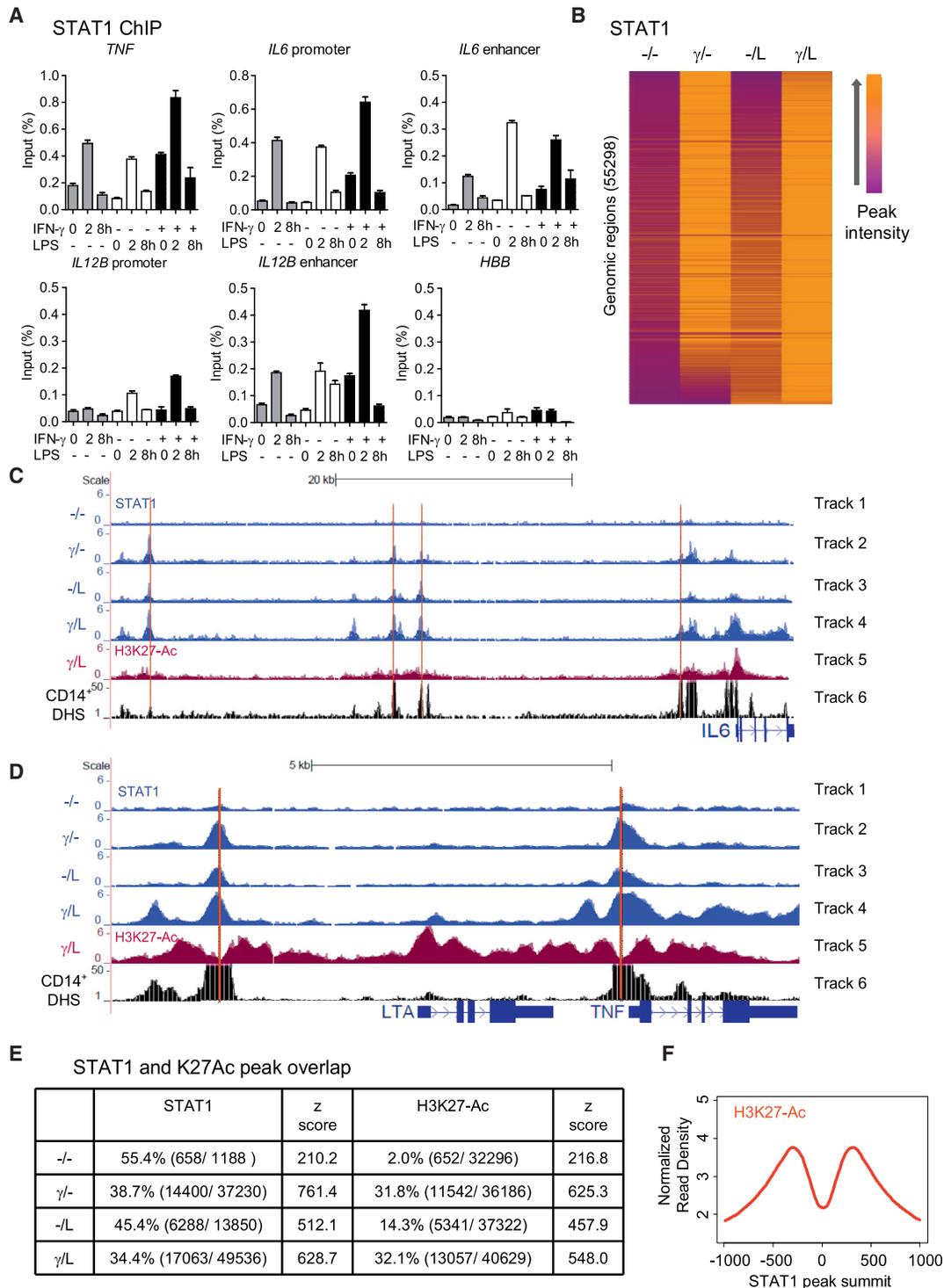
is recruited to regulatory elements. To gain additional insight into STAT1 binding targets, we compared the STAT1 cistrome in IFN- $\gamma$ -primed macrophages, which express elevated amounts of STAT1 and various IFN- $\gamma$ -inducible genes, with the STAT1 cistrome in macrophages acutely stimulated with IFN- $\gamma$  for 30 min (which induces nuclear translocation of latent STAT1 prior to

**Figure 3. IFN- $\gamma$  Increases Histone Acetylation and CBP/p300 Recruitment to Promoters**

(A, D, E) ChIP analysis. Primed (white bars) and unprimed (black bars) macrophages were stimulated with LPS (40 ng/ml) and the amounts of H4-Ac (A), CBP (D), and p300 (E) at promoters of indicated genes were assessed by ChIP. H4 acetylation was normalized to total H4.

(B) Heatmap of H3K27-Ac peaks. Each row on the vertical axis corresponds to a genomic region of H3K27-Ac, and the gradation from purple to orange represents increased H3K27-Ac tag density. Peaks are sorted based on the ratio of H3K27-Ac in ( $\gamma$ -/-) relative to (-/-).

(C) Read density of H3K27-Ac tags at the *IL6* (upper panel) and *TNF* (lower panel) loci (tracks 1–4). Track 5 shows DNase I hypersensitivity peaks from the ENCODE database (Thurman et al., 2012). Red boxes enclose inducible H3K27-Ac peaks. Data are representative of at least three experiments.



**Figure 5. IFN- $\gamma$  Priming Induces STAT1 Recruitment and Associated Histone Acetylation at *TNF*, *IL6*, and *IL12B* Loci and Genome-wide**

(A) ChIP-qPCR analysis of recruitment of STAT1 to indicated promoters or putative enhancers.

(B) Heatmap of STAT1 peaks (rows).

(C and D) Read density for STAT1 ChIP-seq library at the *IL6* (C) and *TNF* loci (D) (tracks 1–4). Track 5 shows H3K27-Ac peaks and track 6 shows DNase I hypersensitive sites (Thurman et al., 2012). The red lines mark examples of STAT1 peaks that align with valleys in H3K27-Ac peaks and with DNase I hypersensitive sites.

(E) Genome-wide evaluation of the overlap between STAT1 and H3K27-ac peaks.

(F) Average binding profile for H3K27-Ac after IFN- $\gamma$  priming, centered around STAT1-binding peaks across a 2000 bp region. Data are representative of two (B–F) or three (A) experiments.

substantial induction of ISGs). Acute IFN- $\gamma$  stimulation induced a smaller number of STAT1 peaks (13,700) that were predominantly contained within the larger group of STAT1 peaks observed after priming and within DNaseI hypersensitive sites (Figure S4H). Acute stimulation induced STAT1 peaks at far upstream elements at the *IL6* locus, whereas priming induced a broader pattern of binding that also included the promoter and a proximal upstream peak (Figure S4I). In contrast, acute and prolonged IFN- $\gamma$  stimulation induced comparable STAT1 occupancy at classic ISGs *CXCL9* and *CXCL10* (Figure S4I). Notably, STAT1 binding at *CXCL10* and the *IL6* upstream regions (where STAT1 peaks were detected after 30 min IFN- $\gamma$  stimulation) was independent of de novo protein synthesis, while binding at the *IL6* promoter (detected predominantly only in primed cells) required de novo protein synthesis (Figure S4K). Taken together, the ChIPseq and electrophoretic mobility shift assay (EMSA) results suggest direct binding of STAT1 to GAS elements present in ISGs and upstream *IL6* enhancers, with a broadening of the genomic profile of STAT1 binding over time during IFN- $\gamma$ -mediated priming that may reflect association with additional IFN- $\gamma$ -induced proteins and indirect or cooperative binding to alternative sites; the broader pattern of STAT1 occupancy in primed macrophages could not be explained solely by increased STAT1 expression (Figure S4J; data not shown).

### Coordinate Recruitment of STAT1 to Enhancers and Promoters

Coordinate activation of promoters and enhancers is associated with increased gene induction in other systems (reviewed in Spitz and Furlong, 2012). We found that a much larger number of genes demonstrated coordinate STAT1 occupancy of enhancers and promoters at individual gene loci in IFN- $\gamma$ -primed relative to naive LPS-stimulated cells (Figure 6B; Figure S5A). This suggests that LPS stimulation resulted in STAT1 recruitment to only promoters of certain genes in unprimed cells but in coordinate recruitment to promoters and intronic or distal regulatory elements in IFN- $\gamma$ -primed cells. In line with this notion, out of the 3,064 genes with STAT1 recruitment to only promoters in naive cells after LPS-stimulation, 702 genes showed coordinate recruitment of STAT1 to promoters and enhancers after LPS stimulation in primed cells (Figure 6C). This group of genes was enriched in immune functions and regulation of cytokine production (Figure 6C; Figures S5B and S5C) and included genes synergistically activated by IFN- $\gamma$  and TLR signaling in our previous microarray analysis (Hu et al., 2007).

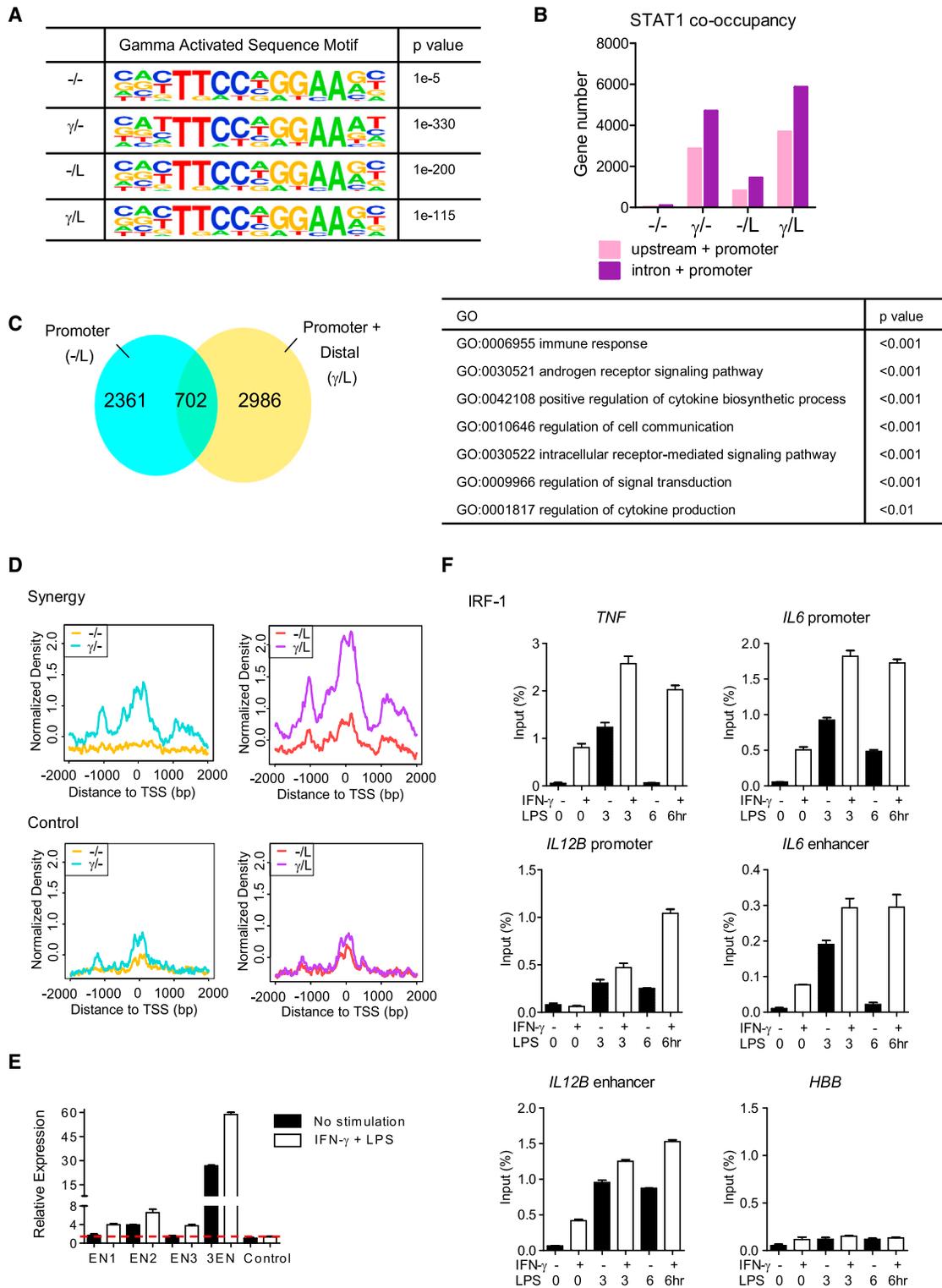
To further correlate the pattern of STAT1 binding with gene expression, we plotted the normalized read density of STAT1 binding around genes that are synergistically activated by IFN- $\gamma$  priming and TLR stimulation (Hu et al., 2007), similar to the pattern of induction of *TNF*, *IL6*, and *IL12B* (Table S2). IFN- $\gamma$  induced coordinate STAT1 occupancy at the promoter, distal upstream, and intronic regulatory elements in these synergistically activated genes (Figure 6D, upper left panel), and the intensity of STAT1 binding increased after LPS stimulation (Figure 6D, upper right panel). In contrast, there was no clear difference in STAT1 binding intensity between primed and unprimed conditions in the control set of genes (Table S2), the LPS-induced transcription of which was not augmented by IFN- $\gamma$  priming (Figure 6D, bottom panels). Thus, coordinate binding

of STAT1 to proximal and distal regulatory regions is associated with synergistic induction of gene expression by IFN- $\gamma$  and TLR signaling. The function of STAT1-binding distal regulatory elements was further supported by reporter gene assays that demonstrated IFN- $\gamma$ - and LPS-inducible enhancer function for three out of four *IL6* distal elements and for distal elements from the *IL12A* and *EGR3* loci (genes that were also synergistically induced by IFN- $\gamma$  and TLRs) (Table S3; Figure S5D). Subcloning of all three functional *IL6* enhancers into one reporter plasmid resulted in substantially increased enhancer activity (Figure 6E), further supporting the notion that these elements can act together to drive gene expression. Overall, the results suggest that IFN- $\gamma$ -induced coordinate priming of multiple regulatory elements at a gene locus is linked with increased transcription on subsequent TLR stimulation.

We next tested the possibility that, in addition to increasing H-Ac, STAT1 could enhance the function of regulatory elements by cooperating with or enhancing recruitment of additional transcription factors, even before TLR stimulation. IRF-1 has not been previously implicated in induction of *TNF* or *IL6* but is induced by IFN- $\gamma$  and cooperates with STAT1 in other systems. Binding sites for IRFs were enriched under STAT1 peaks (Figure S4E), and IFN- $\gamma$  alone induced sustained recruitment of IRF-1 to *TNF*, *IL6*, and *IL12B* loci, including *TNF* and *IL6* promoters that do not contain GAS sites but do contain IRF-like sites (Figure 6F). IRF-1 recruitment to these loci was increased and remained stable after TLR stimulation. ChIP-seq analysis showed that IRF1 binding largely colocalized with STAT1 binding, including at the *TNF* and *IL6* promoters (Figures S5E and S5F). Furthermore, we confirmed a previous report (Chatterjee-Kishore et al., 2000) that IRF-1 and STAT1 proteins interact by using coimmunoprecipitation assays in IFN- $\gamma$ -primed macrophages (Figure S5G). These results, together with the genome-wide enrichment of IRF sites under STAT1 peaks in primed macrophages (Figure S4E), suggest that interactions with IRF-1 (and likely other proteins) can facilitate indirect recruitment of STAT1 to non-GAS sites. The sustained occupancy of regulatory elements by STAT1 and IRF-1 in IFN- $\gamma$ -primed macrophages and associated histone acetylation can contribute to the prolonged kinetics of gene transcription on subsequent TLR challenge.

### Primed State Is Partially Stable but Newly Sensitive to Jak and BET Inhibitors

Primed human macrophages exhibit ongoing Jak signaling, STAT1 tyrosine phosphorylation, and high STAT1 expression (Hu et al., 2002). Because genetic approaches were not feasible, we tested the function of Jak-STAT1 signaling in primed cells by using the Jak inhibitor tofacitinib (also known as CP-690550 [CP]). As expected, addition of CP resulted in a time-dependent decrease in STAT1 tyrosine phosphorylation (Figure 7A). Concordantly, STAT1 and CBP occupancy at *TNF*, *IL6*, and *IL12B* loci was decreased, although the decrease in CBP occupancy was not complete (Figures 7B and 7C). IRF-1 occupancy was also diminished after inhibition of Jak signaling (Figure 7D), suggesting a role for STAT1 in maintaining IRF-1 occupancy. Furthermore, addition of CP immediately before TLR4 stimulation partially but substantially suppressed TLR4-mediated activation of *TNF*, *IL6*, and *IL12B* in IFN- $\gamma$ -primed macrophages



**Figure 6. Genome-wide Analysis of STAT1 Binding and Association with Synergistically Activated Genes**

(A) Genome-wide assessment of the enrichment of GAS motifs under STAT1 binding peaks with HOMER.

(B) IFN- $\gamma$  induces coordinate binding of STAT1 to promoters and upstream or intronic enhancers (pink and purple bars, respectively).

(C) A group of 702 genes, depicted in green in the overlap region, bind STAT1 only at promoters only in LPS-stimulated naive cells but binds STAT1 at both promoters and distal regulatory elements in LPS-stimulated IFN- $\gamma$ -primed cells. GO analysis of these 702 genes is depicted on the right.

(legend continued on next page)

(Figure 7E, lower panels), although Jak inhibition actually super-induced LPS-mediated expression of *TNF*, *IL6*, and *IL12B* in naive macrophages, as expected (Pattison et al., 2012) (Figure 7E, upper panels). These results support a role for Jak-STAT1 signaling and associated chromatin changes in enhanced TLR-induced cytokine gene expression, although the partial attenuation of CBP occupancy and cytokine gene expression is suggestive of a stable epigenetic mechanism that persists after signaling is terminated.

Similar to CP, we found that the compound I-BET, which blocks the recruitment of BET proteins to acetylated histones and thereby suppresses aspects of H-Ac-dependent gene transcription (Nicodeme et al., 2010), partially suppressed LPS-induced *TNF* and *IL6* activation in primed macrophages, but not in naive macrophages (Figure 7F). Lack of suppression of *IL6* in naive human macrophages reflects a context and activation state-dependent effect that differs from findings in mouse macrophages (Nicodeme et al., 2010), which we reproduced (data not shown). These results support a functional role for the increased histone acetylation in primed macrophages in augmenting cytokine gene expression and have therapeutic implications for suppressing cytokine production in diseases associated with primed macrophages.

## DISCUSSION

An important function of IFN- $\gamma$  is to prime macrophages for synergistic transcription of inflammatory cytokine genes upon subsequent stimulation with inflammatory factors such as TLR ligands (Hu and Ivashkiv, 2009). In this study, we found that in human primary macrophages, IFN- $\gamma$  stably and coordinately primed proximal and distal regulatory elements (promoters and enhancers) genome-wide by inducing sustained transcription-factor occupancy by STAT1 and IRF-1 and associated histone acetylation. This priming or opening of chromatin resulted in greatly augmented and prolonged recruitment of additional transcription factors and pol II after TLR stimulation and increased transcription of genes, including the key pathogenic cytokine genes *TNF*, *IL6*, and *IL12B*. Primed TLR-induced cytokine transcription was preferentially sensitive to inhibition of Jak kinases and BET proteins. Our results provide a priming mechanism whereby IFN- $\gamma$  removes a rate-limiting chromatin barrier and creates a primed chromatin environment that increases and prolongs transcriptional responses to TLR-induced signals. The results help explain synergistic activation of inflammatory cytokine genes such as *TNF*, *IL6*, and *IL12B* by IFN- $\gamma$  and TLRs and also suggest therapeutic approaches to preferentially suppressing primed inflammatory cytokine production in diseases such as RA that are characterized by IFN-STAT1-activated macrophages (Ivashkiv and Hu, 2003).

The importance of IFN- $\gamma$  as a macrophage-activation factor and synergistic activation of inflammatory cytokine production

by IFN- $\gamma$  and microbial products has been appreciated for more than 25 years (Adams and Hamilton, 1987). A multitude of molecular mechanisms that enhance TLR signaling have been proposed to explain synergistic induction of cytokine transcription by IFN- $\gamma$  and TLRs (Hu and Ivashkiv, 2009; Schroder et al., 2006). However, the typically modest augmentation of TLR signaling by IFN- $\gamma$  suggests that signaling does not represent the sole or major rate-limiting step that determines the magnitude of synergistic downstream gene transcription and cannot explain why synergy occurs in a gene-specific manner. Our results help resolve this conundrum by suggesting a model whereby IFN- $\gamma$  and TLRs act on two different rate-limiting steps that determine transcriptional output—IFN- $\gamma$  on removing a chromatin barrier that limits upstream signals and TLRs on providing positive signals required for gene induction. This model is consistent with emerging insights that chromatin is an important and regulated barrier that serves as a rheostat to control amplitude of transcriptional responses to inflammatory signals (Ivashkiv, 2013; Natoli et al., 2011). Because IFN- $\gamma$  priming modifies chromatin in a gene-specific manner, likely driven by specific STAT1 recruitment, this model can also explain gene-specific synergistic induction of a subset of TLR-inducible genes.

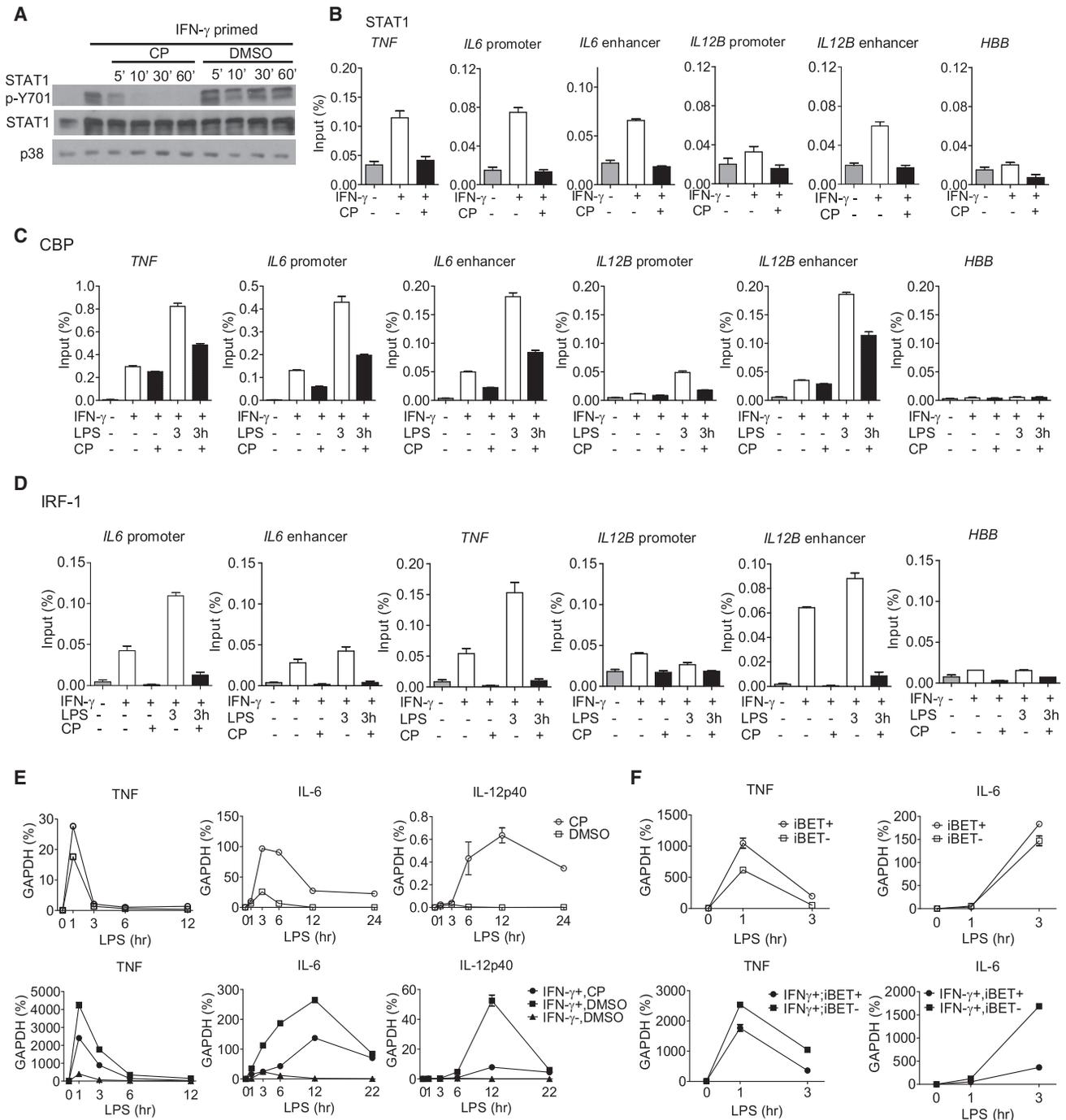
Previous work has focused on the epigenetic landscape established during macrophage development and its remodeling during acute inflammatory stimulation (Ivashkiv, 2013; Medzhitov and Hornig, 2009). Our work highlights the importance of changes in macrophage chromatin states induced by polarizing cytokines such as IFN- $\gamma$  and extends emerging concepts about how environmental cues can alter mature macrophages to determine subsequent transcriptional responses in a gene-specific manner. Chromatin can be remodeled to facilitate gene activation, as described herein, or closed to silence inflammatory gene expression, as has been described in endotoxin tolerance (Chen and Ivashkiv, 2010; Foster et al., 2007; Park et al., 2011). Chromatin state appears to represent a rate-limiting step that determines the magnitude and qualitative nature of gene responses to canonical signals, and changes in chromatin state can reprogram macrophages for altered responses to environmental cues.

Our findings identify several complementary components of IFN- $\gamma$ -mediated priming that can cooperate to enhance transcription. First, IFN- $\gamma$  primed individual proximal and distal regulatory elements by inducing binding of STAT1, IRF-1, and associated histone acetylation. Although the quantitative changes in histone acetylation at many individual elements were modest, priming resulted in the induction of new peaks that could enhance transcription. Second, the binding of STAT1 and IRF-1 was stable and sustained at later time points and was associated with prolonged occupancy by CBP/p300 and histone acetylation after TLR stimulation that could extend the kinetics of cytokine gene transcription. Third, IFN- $\gamma$  induced coordinate priming of proximal, upstream, and intronic

(D) STAT1 binds coordinately to distal and promoter regions of genes induced synergistically by IFN- $\gamma$  and LPS. Average binding profile of STAT1 is plotted across a 4 kb region centered on the TSS. Upper panels correspond to genes synergistically induced by IFN- $\gamma$  and LPS, whereas lower panels correspond to control genes that were not induced.

(E) Reporter gene assays in RAW264.7 cells to measure enhancer function of the three STAT1-binding enhancers upstream of *IL6* (corresponding to EN1, EN2, and EN3 in Figure S5D); in 3EN the three enhancers were tested in tandem. Data is representative of three experiments.

(F) ChIP-qPCR analysis of IRF-1 recruitment to indicated gene promoters or putative enhancers.



**Figure 7. Primed Cytokine Transcription Is Preferentially but Partially Sensitive to Inhibition of Jaks and BET Proteins**

(A–E) Macrophages were primed with IFN- $\gamma$  (100 U/ml) for 24 hr and then treated with Jak inhibitor CP-690550 (CP) (10  $\mu$ M) or DMSO vehicle control. (A) STAT1 tyrosine phosphorylation was measured by WB. (B) STAT1 binding at the indicated gene loci 3 hr after adding CP was measured by ChIP-qPCR. (C and D) ChIP-qPCR analysis of CBP (C) and IRF-1 (D); CP was added 1 hr prior to LPS. (E) RT-qPCR analysis of TNF, IL-6, and IL-12 p40 mRNA normalized relative to GAPDH mRNA.

(F) RT-qPCR analysis of TNF, IL-6, and IL12 p40 mRNA. iBET (5  $\mu$ M) or DMSO vehicle control was added 0.5 hr prior to LPS (20 ng/ml). Results are representative of at least three independent experiments.

regulatory elements at individual gene loci that are synergistically activated by IFN- $\gamma$  and TLRs. These three components of priming stably remove rate-limiting steps of chromatin remodeling at

both promoters and enhancers and thus prepare primed macrophage gene loci for enhanced TLR-induced transcriptional responses. In addition, priming of enhancers led to increased

TLR-induced transcription of eRNA, which could further enhance cytokine gene transcription.

The IFN- $\gamma$ -induced altered epigenetic state was stable in the presence of cytokine and was at least partially maintained after IFN- $\gamma$  signaling was terminated. These results, together with two recent reports showing a role for STAT1 in formation of active enhancers in T cells and activation of latent enhancers in mouse macrophages (Ostuni et al., 2013; Vahedi et al., 2012), suggest that the traditional view that cytokine signaling by the Jak-STAT pathway functions mainly to transiently induce gene expression mediated by transient STAT activation (reviewed in Levy and Darnell, 2002; Stark and Darnell, 2012) is incomplete. Instead, cytokine-activated transcription factors such as STATs might not function solely as “signaling transcription factors,” whose function is constrained by the chromatin environment, but can also initiate chromatin remodeling to alter epigenetic states.

Previous investigation of IFN- $\gamma$ -induced transcriptional responses has focused on canonical ISGs activated by direct binding of STAT1 to gene promoters. Induction of canonical ISGs does not appear to require a second signal, although transcription might depend upon cooperation of STAT1 with additional transcription factors, which are expressed in the cell or induced by IFN- $\gamma$  (Levy and Darnell, 2002; Stark and Darnell, 2012). Our work highlights the existence of a different subset of IFN- $\gamma$ -regulated genes, which includes *TNF*, *IL6*, and *IL12B*, that bind STAT1 in a sustained manner but do not activate transcription until cells receive a second exogenous signal. In the case of inflammatory cytokine genes, this second signal is provided by TLR-mediated activation of NF- $\kappa$ B and other factors that can cooperate with STAT1 to activate transcription. Genome-wide analysis indicated that only 7.5% of genes that bound STAT1 after IFN- $\gamma$  priming were transcriptionally active at that time point (Y.Q., data not shown). This suggests that IFN- $\gamma$  priming marks a large number of genes with STAT1 and associated H-Ac not necessarily to drive ongoing transcription but to “mark” genes to imbue a capacity for altered gene responses to various subsequent environmental stimuli. The nature of the second signal and how it interacts with the primed chromatin state and transcription factors bound to regulatory regions at specific gene loci would determine the ensuing pattern of gene expression.

In IFN- $\gamma$ -primed macrophages, STAT1 was predominantly targeted to preexisting poised regulatory elements, as defined by DNase I hypersensitivity and PU.1 binding. The primed STAT1 cistrome encompassed STAT1 sites bound after acute IFN- $\gamma$  stimulation and sites that contain GAS motifs, including enhancers upstream of synergistically activated genes such as *IL6*. EMSA assays supported direct binding of STAT1 to these enhancers. However, the primed STAT1 cistrome also showed a more extended binding pattern, which included binding to sites that do not contain clear-cut GAS sequences, such as the *TNF* and *IL6* promoters. We have not definitively excluded a role for increased STAT1 expression in extending the genomic profile of STAT1 binding. However, colocalization of STAT1 and IRF-1 peaks, STAT1-IRF-1 coimmunoprecipitation, and enrichment of IRF motifs under STAT1 peaks suggests a role for interaction of STAT1 with IRF-1 and possibly other IRFs and ISGs in expanding the STAT1 cistrome. This notion was further

supported by evidence that binding of STAT1 to the *IL6* promoter that does not contain a canonical GAS site required de novo protein synthesis, whereas binding to GAS sites in classical ISGs did not. Coordinate and possibly cooperative binding of STAT1 and IRFs was also suggested by Ostuni et al., (2013), in that case at latent enhancers, and is in line with established models of cooperative function of transcription factors at regulatory elements (Ghisletti et al., 2010; Natoli et al., 2011; Spitz and Furlong, 2012).

In summary, our study shows that IFN- $\gamma$  alters the epigenetic landscape of human macrophages by priming TSS-proximal and -distal regulatory elements to reprogram subsequent responses to environmental cues, including synergistic cytokine transcription after inflammatory challenge. These results alter our view about mechanisms of cytokine action during cell activation and suggest new therapeutic approaches that may be less toxic and more specific by selectively targeting priming mechanisms while leaving residual TLR functions intact for host-defense functions.

## EXPERIMENTAL PROCEDURES

### Cell Culture

CD14<sup>+</sup> human monocytes were purified by positive selection with anti-CD14 beads (Miltenyi Biotec) from peripheral blood mononuclear cells obtained from the New York Blood Center as previously described (Hu et al., 2002) with a protocol approved by the Hospital for Special Surgery Institutional Review Board. Monocytes were cultured in RPMI1640 (Invitrogen) supplemented with 10% defined FBS (HyClone) and 10 ng/mL M-CSF (Peprotech).

### Reagents

LPS was purchased from Sigma (L-2360). The JAK inhibitor CP-690550 (Tofacitinib) was from Reagents Direct (Cat# 59-W26).

### Chromatin Immunoprecipitation and ChIP-Seq

Details of ChIP and ChIP-seq experiments are provided in the [Supplemental Experimental Procedures](#). For ChIP-seq experiments, sequence tags were mapped to the current human reference sequence (GRCh37/hg19) with Bowtie with default parameters, and clonal reads were removed from further analysis. More than 100 million nonclonal mapped tags were obtained for each condition. ChIPseeqer (Giannopoulou and Elemento, 2011) was used for peak detection, annotation, comparison between different lists of peaks, pathway analysis, and peak clustering. Peak calling was normalized to input DNA sequencing data, with  $p < 10^{-15}$  and fold induction  $>2$  unless otherwise indicated. Two to three biological replicates were performed in the ChIP-seq experiments, and scatterplots and Pearson correlation coefficients between peak heights in biological replicates are shown in Figures S2D and S4L. ChIP-seq data were deposited in the GEO database with accession number GSE43036. Motif analysis was performed with HOMER (Heinz et al., 2010).

### Immunoblotting

Protein extracts were fractionated by SDS-PAGE, transferred to polyvinylidene fluoride membranes (Millipore), and incubated with the antibodies described in [Supplemental Experimental Procedures](#).

### Quantitative Real-Time PCR

RNA was extracted with the RNeasy Mini Kit (QIAGEN) and reverse-transcribed with the First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was performed in triplicate with an ABI7500 thermal cycler. Primer sequences are provided in the [Supplemental Information](#).

### Reporter Gene Assay

Transient transfections of RAW264.7 cells were performed in duplicate with Lipofectamine PLUS (Invitrogen). The genomic regions of interest (Table S3) were cloned into the pGL3 reporter vector (Promega), and luciferase

assays were performed with the Dual-Luciferase® Reporter Assay System (Promega).

#### ACCESSION NUMBERS

ChIP-seq data were deposited in the GEO database with accession number GSE43036.

#### SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures, four tables, and Supplemental Experimental Procedures and can be found with this article online at <http://dx.doi.org/10.1016/j.immuni.2013.08.009>.

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