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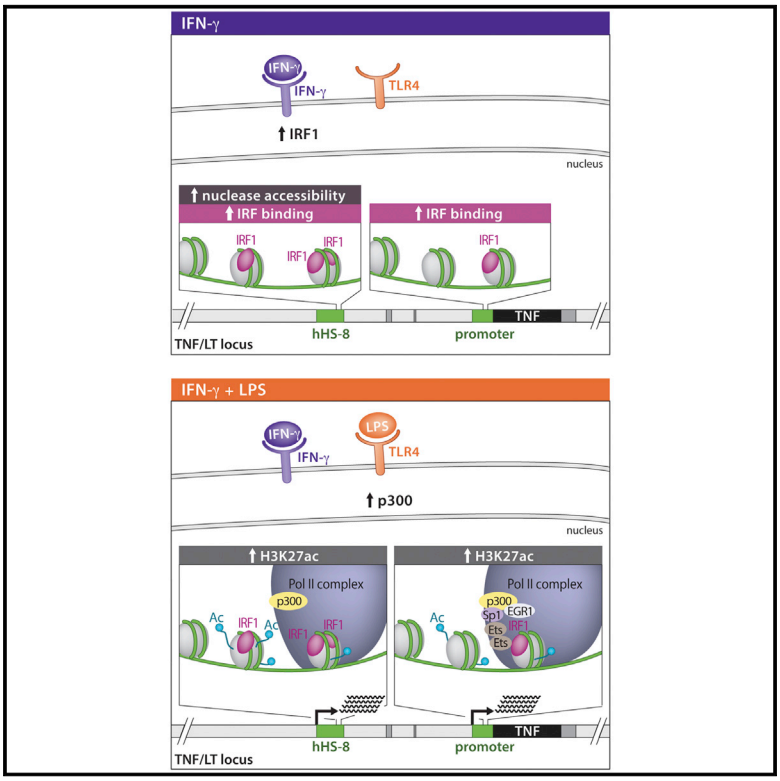
A Distal Locus Element Mediates IFN-γ Priming of Lipopolysaccharide-Stimulated *TNF* Gene Expression

Graphical Abstract



Highlights

IFN-γ priming requires the IRF1-dependent distal *TNF/LT* locus element hHS-8

IFN-γ priming promotes chromatin accessibility and recruitment of IRF1 at hHS-8

IFN-γ priming enriches LPS-stimulated H3K27ac and induces eRNA synthesis at hHS-8

Targeting the hHS-8 IRF1 binding site in vivo with Cas9 abolishes IFN-γ priming

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In Brief

Interferon γ (IFN-γ) priming is a critical immune event that enhances the monocyte and macrophage response, particularly expression of the *TNF* gene, to toll-like receptor (TLR) signaling. Chow et al. demonstrate that IFN-γ priming requires a distal enhancer element within the *TNF/LT* locus, thereby expanding the role of distal regulatory elements in the innate immune response.

Chow et al., 2014, Cell Reports 9, 1718–1728
December 11, 2014 ©2014 The Authors
http://dx.doi.org/10.1016/j.celrep.2014.11.011

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A Distal Locus Element Mediates IFN-γ Priming of Lipopolysaccharide-Stimulated *TNF* Gene Expression

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SUMMARY

Interferon γ (IFN-γ) priming sensitizes monocytes and macrophages to lipopolysaccharide (LPS) stimulation, resulting in augmented expression of a set of genes including *TNF*. Here, we demonstrate that IFN-γ priming of LPS-stimulated *TNF* transcription requires a distal *TNF/LT* locus element 8 kb upstream of the *TNF* transcription start site (hHS-8). IFN-γ stimulation leads to increased DNase I accessibility of hHS-8 and its recruitment of interferon regulatory factor 1 (IRF1), and subsequent LPS stimulation enhances H3K27 acetylation and induces enhancer RNA synthesis at hHS-8. Ablation of IRF1 or targeting the hHS-8 IRF1 binding site in vivo with Cas9 linked to the KRAB repressive domain abolishes IFN-γ priming, but does not affect LPS induction of the gene. Thus, IFN-γ poises a distal enhancer in the *TNF/LT* locus by chromatin remodeling and IRF1 recruitment, which then drives enhanced *TNF* gene expression in response to a secondary toll-like receptor (TLR) stimulus.

INTRODUCTION

Produced by natural killer cells and activated Th1 lymphocytes, interferon γ (IFN-γ) sensitizes circulating monocytes and tissue-resident macrophages, leading to augmentation of macrophage activation after microbial recognition and toll-like receptor (TLR) signaling (Murray, 1988). This phenomenon, known as IFN-γ priming, results in enhanced gene expression of inflammatory cytokines such as tumor necrosis factor (TNF), interleukin 12 (IL-12), and IL-6 (Lorsbach et al., 1993; Ma et al., 1996; Pace et al., 1983; Sanceau et al., 1991). In the case of TNF, de novo transcription of *TNF* is enhanced in human monocytes primed by IFN-γ and then stimulated by lipopolysaccharide (LPS) (Hayes and Zoon, 1993). However, the molecular mechanisms that control IFN-γ priming, and whether these mechanisms are gene specific, are poorly understood.

The *TNF* gene and the genes encoding lymphotoxin-α and lymphotoxin-β (*LTA* and *LTB*) comprise the ~20 kb *TNF/LT* locus region, which lies within the histocompatibility locus on human chromosome 6 and mouse chromosome 17. *TNF* is highly and rapidly expressed in both lymphocytes and monocytes (Goldfeld and Maniatis, 1989; Goldfeld et al., 1990, 1993), and its transcriptional regulation occurs in a cell-type- and inducer-specific manner. Distinct sets of transcription factors and coactivators, including chromatin-modifying enzymes, are recruited to DNA elements in the *TNF* promoter depending on the type of cell and the type of stimulus that is received (Falvo et al., 2000a, 2000b, 2010; Tsai et al., 2000; Tsytsykova and Goldfeld, 2000). Furthermore, the formation of higher-ordered structures, or enhanceosomes, is required for *TNF* gene expression in specific cell types (Tsytsykova and Goldfeld, 2002; Barthel and Goldfeld, 2003). Distal hypersensitive (DH) elements upstream and downstream of the *TNF* transcription start site (TSS) have been identified in the *TNF/LT* locus. A subset of these DH sites also varies by cell type (Barthel and Goldfeld, 2003; Tsytsykova et al., 2007; Taylor et al., 2008; Biglione et al., 2011). For example, DH sites ~9 kb upstream and ~3 kb downstream of the murine gene act as NFATp-dependent enhancers in T cells and participate in activation-induced intrachromosomal interactions with the promoter (Tsytsykova et al., 2007), whereas a myeloid-specific DH site ~7 kb upstream of the TSS functions as a matrix attachment region (Biglione et al., 2011).

In this study, we show that a DH site ~8 kb upstream of the human *TNF* TSS (human hypersensitive site −8 kb [hHS-8]) is required for and mediates IFN-γ-stimulated augmentation of LPS-induced *TNF* gene expression in human monocytes/macrophages. The highly conserved hHS-8 noncoding element exhibits increased nuclease accessibility in response to IFN-γ stimulation, and interferon regulatory factor 1 (IRF1) is recruited. Upon subsequent LPS stimulation of IFN-γ-primed cells, increased acetylation of H3K27 and synthesis of enhancer RNA (eRNA) at hHS-8 occur. IFN-γ priming of *TNF* is abrogated with the ablation of IRF1, disrupting the IRF1 site in reporter assays, or by targeting the IRF1 binding element in hHS-8 with the catalytically inactive form of Cas9 linked to the Krüppel-associated box (KRAB) domain of Kox1 (Margolin et al., 1994; Gilbert et al., 2013) in human monocytic cells. Thus, IRF1 expression and an intact hHS-8 IRF1 binding element are required for IFN-γ priming of *TNF* in vivo.

Our results expand the functional role of distal regulatory elements in the innate immune response to IFN-γ priming and

highlight the potential of CRISPR/Cas9 technology as a tool for interrogating the function of distal regulatory elements in human hematopoietic cells.

RESULTS

IFN-γ Promotes Chromatin Accessibility at hHS-8 in the *TNF/LT* Locus

As a single stimulus, LPS significantly induced TNF mRNA levels, whereas IFN-γ alone was not sufficient to induce *TNF* gene expression in human monocytic THP-1 cells (Figure 1A). However, priming of cells by pretreatment with IFN-γ for 2 hr before LPS stimulation significantly enhanced TNF mRNA levels compared with stimulation by LPS alone (Figure 1A). This observation supported our hypothesis that IFN-γ poises the *TNF* gene for enhanced transcription in response to LPS by stimulating chromatin remodeling at the *TNF/LT* locus.

In order to test this idea, we performed a DNase I hypersensitivity assay (DHA) comparing the landscape and intensity of hypersensitive sites across the *TNF/LT* locus in IFN-γ-treated and -untreated THP-1 cells. IFN-γ treatment of THP-1 cells promoted chromatin accessibility at a DH site located ~8 kb upstream of the *TNF* TSS (hHS-8), as evidenced by IFN-γ-dependent enhancement of the DNase I-generated band corresponding to hHS-8 (Figure 1B, compare lane 4 with lane 8). Thus, in a population of unstimulated human monocytic cells, hHS-8 was constitutively present, and upon IFN-γ treatment, the proportion of cells in which hHS-8 became accessible to DNase I increased. We also observed a smaller increase in DNase I cleavage at the *TNF* promoter as compared with hHS-8 in response to IFN-γ (Figure 1C, compare lane 4 with lane 8).

To extend our findings to human primary cells, we examined the effects of IFN-γ priming on monocyte-derived macrophages (MDMs) and confirmed that IFN-γ pretreatment significantly enhanced TNF mRNA levels as compared with stimulation by LPS alone (Figure 1D). Similar to what we observed in THP-1 cells, IFN-γ treatment increased DNase I cleavage at hHS-8 in primary human MDMs (Figure 1E, compare lane 3 with lane 6). Furthermore, in both cell types, IFN-γ priming prior to LPS stimulation led to enhanced DNase I cleavage as compared with LPS stimulation alone at hHS-8 (compare lanes 12 and 16 of Figure 1B, and lanes 9 and 12 of Figure 1E). An examination of data from the ENCODE database (Thurman et al., 2012) revealed that a constitutive DH site ~8 kb upstream of the *TNF* TSS was present in resting primary human monocytes in this data set (Figure S1), confirming our detection of a DH site at this location in resting monocytic cells. In contrast to THP-1 cells, we observed no change in DNase I cleavage at the *TNF* promoter upon IFN-γ stimulation (Figure 1F, compare lane 3 with lane 6). The restriction sites and probe positions for DHAs of both hHS-8 and the *TNF* promoter are shown in Figure 1G.

A decrease in total H3 levels is generally reflective of enhanced chromatin accessibility (Reinke and Hörz, 2004). In order to confirm chromatin remodeling of the *TNF/LT* locus after IFN-γ stimulation, we next measured total H3 levels at hHS-8 and the *TNF* promoter by chromatin immunoprecipitation (ChIP) analysis in THP-1 cells and primary human MDMs under the same conditions as used for the DHA. Consistent with the DHA findings, we observed a significant reduction in total H3 levels at hHS-8 in response to IFN-γ in both THP-1 cells (Figure 1H) and primary MDMs (Figure 1I), but not at the *TNF* promoter (Figures 1H and 1I). Furthermore, LPS alone and IFN-γ + LPS stimulation caused a significant reduction of total H3 levels at both hHS-8 and the *TNF* promoter in both the THP-1 cells and MDMs (Figures 1H and 1I). Taken together, these findings show that sole stimulation with IFN-γ remodels the *TNF/LT* locus at the distal DNA element hHS-8 to increase nuclease accessibility, consistent with hHS-8 playing a role in IFN-γ-mediated *TNF* transcriptional augmentation. In the case of LPS, nuclease accessibility is increased after stimulation at both the *TNF* promoter and hHS-8.

IRF1 Binds to hHS-8 in an IFN-γ-Inducible Manner

IFN-γ is a potent inducer of the transcription factor IRF1 (Pine et al., 1990). An examination of the *TNF/LT* locus for sequences resembling the IRF consensus binding sequence 5′-AANNGAAANGAA-3′ (Tamura et al., 2008) revealed putative IRF sites in both the *TNF* promoter and hHS-8 (Figure 2A). To determine whether IRF1 is capable of binding to these sequences, we first performed a quantitative DNase I footprinting analysis with recombinant IRF1 (rIRF1). We found that the protein binds to the *TNF* proximal promoter at the 5′ boundary of the predicted site, which lies within a composite binding site of the *TNF* enhanceosome that also binds Sp1, Egr1, NFATp, and Ets in a cell-type-specific manner (Tsai et al., 2000; Figure 2B). Moreover, rIRF1 also binds to hHS-8 at an IRF binding motif containing three 5′-GAAA-3′ motifs (Figure 2C). Using ChIP, we confirmed that IRF1 binds to hHS-8 in vivo in primary human MDMs and that its binding is significantly enhanced upon IFN-γ stimulation in vivo. By contrast, IRF1 recruitment to the promoter was minimal. After LPS treatment of IFN-γ-primed primary human MDMs, IRF1 recruitment to the *TNF* promoter increased and IRF1 binding at hHS-8 declined, but IRF1 binding at hHS-8 remained significantly elevated as compared with results obtained under nonstimulated conditions (Figure 2D).

In previous comparative analyses of *TNF* noncoding sequences 5′ of the TSS in the primate lineage, we delineated phylogenetic footprints that matched and were predictive of important *TNF* regulatory elements (Leung et al., 2000; Baena et al., 2007). Here, when we specifically focused on the hHS-8 ~50 bp IRF1 binding element and compared it with corresponding sequences in the primate lineage and with the murine sequence, we found that the core 5′-GAAA-3′ motifs were completely conserved in the primate lineage representatives down to *Callithrix jacchus*, the common marmoset (Figure 2E). Furthermore, even the differences observed between the mouse and human sequences did not impact IRF1 binding to the site (Figure S2A). Thus, the exquisite level of sequence conservation of the IRF1 phylogenetic footprint in hHS-8 strongly suggested that there is an important function related to the conservation of these specific sequences. Notably, when we examined the sequence conservation of the entire 1.3 kb hHS-8 element, we found it to be ~70% conserved between human and mouse (Figure S2B), further supporting the notion that it plays an important role in *TNF* gene regulation.

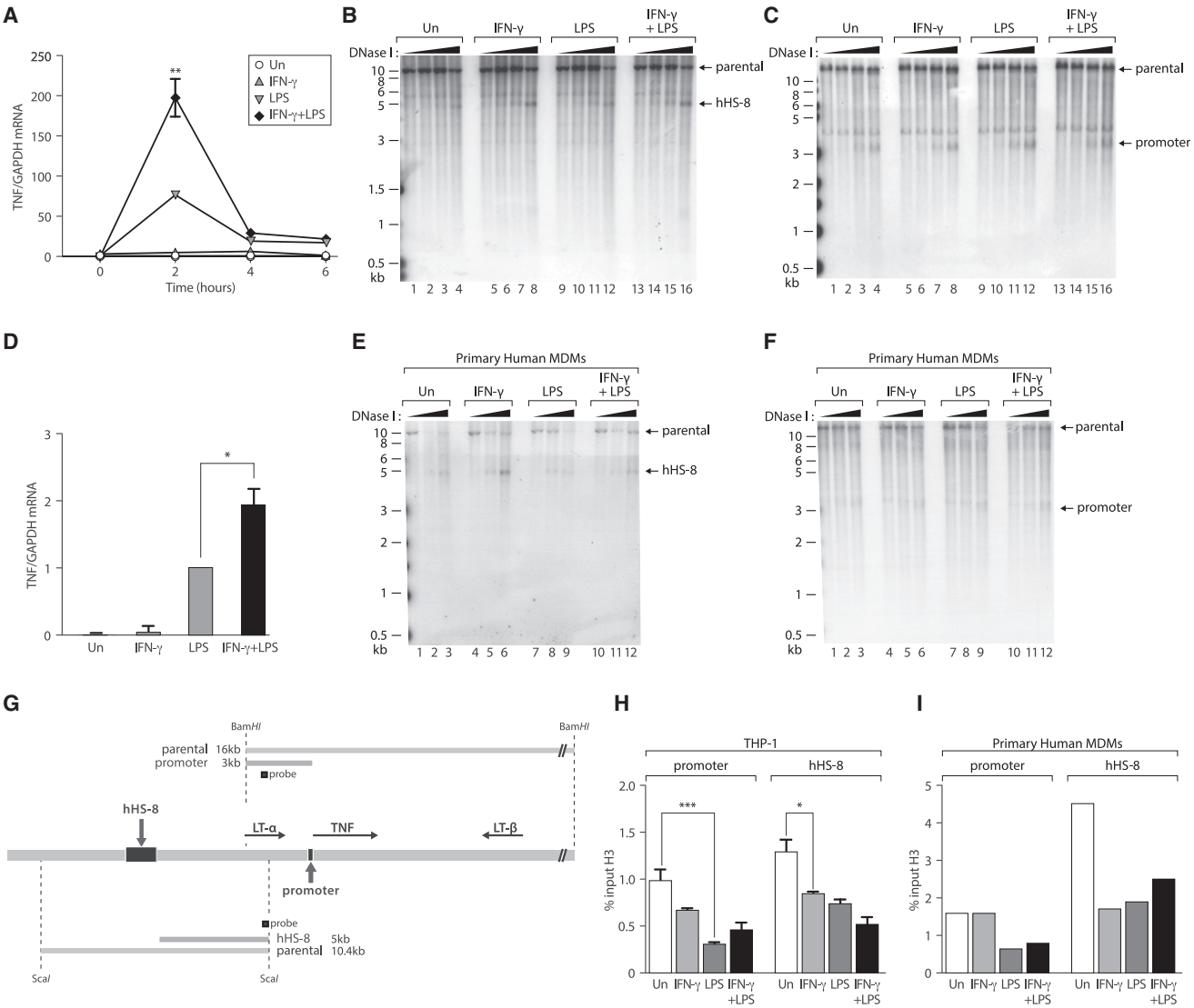

Figure 1. IFN-γ Priming Promotes Chromatin Accessibility at hHS-8 in the *TNF/LT* Locus

(A) IFN-γ priming enhances TNF mRNA levels in THP-1 cells stimulated with LPS. Cells were stimulated with IFN-γ alone for 3 hr, LPS alone for 1 hr, and both IFN-γ and LPS (IFN-γ for 2 hr followed by LPS for 1 hr). TNF mRNA levels were measured after LPS stimulation by qPCR. **$p \leq 0.01$; data are represented as mean ± SEM.

(B and C) IFN-γ increases the chromatin accessibility of hHS-8. DHAs using the restriction enzyme ScaI (B) and BamHI (C) allowed for examination of hHS-8 and the *TNF* promoter, respectively, in resting and IFN-γ-treated THP-1 cells.

(D) IFN-γ priming enhances TNF mRNA levels in primary human MDMs stimulated with LPS. MDMs were stimulated as in (A) and RNA was collected 1 hr after LPS stimulation. Data from three separate donors; *$p \leq 0.05$; data are represented as mean ± SEM.

(E and F) DHAs were performed in resting and IFN-γ-treated primary human MDMs as in (B) and (C).

(G) Map of the human *TNF/LT* locus. DH sites and positions, and directions of transcription of the *TNF*, *LTA*, and *LTB* genes are shown. The positions of the parental ScaI, parental BamHI, and DNase I digestion products for the DHAs are indicated.

(H and I) IFN-γ and LPS decrease nucleosome occupancy at the *TNF* promoter and hHS-8. ChIP using THP-1 cells (H) and primary human MDMs (representative donor, I) measures nucleosome occupancy (total H3 levels) at both the *TNF* promoter and hHS-8. IFN-γ alone decreased total H3 levels at the *TNF* promoter (albeit not significantly; $p = 0.054$) in THP-1 cells; this was not repeated in the MDM donor. Data from three separate experiments; *$p \leq 0.05$, ***$p \leq 0.001$; data are represented as mean ± SEM.

See also Figure S1.

IRF1 Is Required for Enhanced TNF Expression in IFN-γ-Primed Monocytes and Macrophages

IRF1 is a member of the nine-member IRF family of transcription factors, all of which share a cognate binding motif (Tamura et al., 2008). Although transcription of IRF1, IRF8, and IRF9 is induced by IFN-γ treatment, IRF1 is thought to be the dominant IFN-γ-inducible IRF family member (Tamura et al., 2008). To test for a specific and nonredundant functional role of IRF1

in IFN-γ-induced enhancement of TNF expression, we examined bone-marrow-derived macrophages (BMDMs) from wild-type and IRF1-deficient (*Irf1*$^{-/-}$) mice (Figures 2F and 2G). BMDMs from wild-type control mice responded to IFN-γ priming and secreted significantly higher levels of TNF protein after LPS stimulation as compared with cells stimulated with LPS alone (Figures 2F and 2G). By contrast, whereas LPS-induced TNF protein production in *Irf1*$^{-/-}$ BMDMs was similar to protein levels in wild-type BMDMs, priming by IFN-γ pretreatment was eliminated in the IRF1-deficient cells.

To extend this finding to human monocytic cells, we next constructed lentiviral expression vectors encoding a short hairpin RNA (shRNA) targeting IRF1 transcripts or a nonspecific control shRNA, and demonstrated that IFN-γ-induced IRF1 mRNA levels were significantly inhibited in the cells carrying the shRNA targeting IRF1 (Figure 2H). We then tested the ability of IFN-γ priming to prime LPS-induced TNF mRNA expression in the IRF1-deficient cells and found that IFN-γ priming was abrogated, whereas LPS induction of *TNF* transcription was not affected (Figure 2I). These experiments thus demonstrated that (1) IRF1 is significantly recruited to the highly conserved hHS-8 element upon sole IFN-γ stimulation in vivo, but is only minimally recruited to the human *TNF* promoter, and (2) IRF1 is necessary for IFN-γ priming of LPS-stimulated *TNF* gene expression in both murine macrophages and human monocytic cells, and thus other IRF family members cannot compensate for its loss in IFN-γ priming of TNF.

hHS-8 Functions as an IFN-γ-Inducible, IRF1-Dependent Enhancer of *TNF* Gene Expression

To determine whether hHS-8 can function as an IFN-γ-inducible enhancer element, we inserted the 1.3 kb hHS-8 element upstream of the *TNF* promoter in a luciferase reporter construct and compared its transcriptional activity with the activity of a reporter construct containing only the human *TNF* promoter (with sequences up to 982 bp upstream of the *TNF* TSS). As shown previously (Tsai et al., 2000), the human *TNF* promoter alone is LPS inducible (Figure 3A). Consistent with our findings for the endogenous *TNF* gene (see Figure 1), sole treatment with IFN-γ did not activate expression of the *TNF* promoter-reporter construct (Figure 3A). Furthermore, IFN-γ priming did not enhance LPS-induced transcriptional activity (Figure 3A, $p = 0.653$). Thus, the *TNF* promoter alone was not sufficient to mediate IFN-γ priming of transcription. By contrast, when the 1.3 kb hHS-8 sequence was inserted upstream of the *TNF* promoter, we found a significant enhancement of IFN-γ-primed, LPS-stimulated reporter expression (Figure 3A, $p = 0.001$). Strikingly, the introduction of mutations that disrupt IRF1 binding within the context of the otherwise isogenic 1.3 kb hHS-8 element (Figure 3B) completely abolished IFN-γ priming of LPS-driven *TNF* promoter activity ($p = 0.001$), but did not impair LPS induction of the gene (Figure 3A, $p = 0.222$). We also note that consistent with the regulation of the endogenous gene in monocytic cells (Figures 1A and 1D), sole treatment with IFN-γ did not activate expression of the wild-type *TNF* promoter + hHS-8-reporter construct (Figure 3A). These data thus demonstrate that the *TNF* promoter alone is unable to drive enhanced transcription in response to IFN-γ priming, but gains this capacity when linked to the IRF1-dependent inducible hHS-8 regulatory element. This is in contrast to the *IL12A* promoter, which contains an IRF1 binding site and is sufficient for IFN-γ priming of LPS-driven transcriptional activation (Liu et al., 2003).

IFN-γ Priming Enhances H3K27 Acetylation at hHS-8 upon LPS Stimulation

In addition to increased chromatin accessibility, activated enhancers are associated with enrichment in H3K27ac levels (Calo and Wysocka, 2013). Therefore, we next investigated whether H3K27ac levels were altered at hHS-8 or the *TNF* promoter during IFN-γ priming. At the *TNF* promoter, although we saw no change in H3K27ac levels in response to IFN-γ alone, H3K27ac enrichment increased dramatically after the single LPS stimulus (Figure 3C), and there was no further enhancement in H3K27ac levels in response to IFN-γ priming followed by LPS stimulation (Figure 3C). By contrast, although solitary LPS or IFN-γ treatment of THP-1 cells did not cause an increase in H3K27ac levels at hHS-8, H3K27ac enrichment increased significantly at hHS-8 in cells that had first been primed with IFN-γ and then stimulated with LPS (Figure 3C). We note that the acetyltransferases CBP/p300 are inducibly recruited to the *TNF* promoter after LPS stimulation (Tsai et al., 2000), and that IFN-γ stimulation alone is not sufficient to induce p300 recruitment to hHS-8, which requires LPS stimulation (Figure S3), consistent with the pattern of enhanced H3K27ac levels after dual IFN-γ and LPS treatment.

eRNA Is Synthesized at hHS-8 during IFN-γ Priming

Since eRNA production is associated with functional enhancer elements (Jiao and Slack, 2014), we next investigated whether hHS-8 eRNA was transcribed during IFN-γ priming and LPS stimulation of THP-1 cells and primary human MDMs. In both cell types, similar to our findings with *TNF* gene transcription initiated at the promoter, IFN-γ as a single stimulus did not induce transcription of hHS-8 eRNA (Figures 3D and 3E). However, IFN-γ priming of THP-1 cells prior to LPS stimulation significantly enhanced hHS-8 eRNA synthesis as compared with stimulation by LPS alone (Figure 3D), and a similar effect was seen in MDMs from a representative donor (Figure 3E).

hHS-8 Is Required for IFN-γ Augmentation of *TNF* Gene Expression In Vivo

Finally, to demonstrate the functional role of hHS-8 in IFN-γ priming within the endogenous chromatin environment of the *TNF/LT* locus, we employed CRISPR/Cas9 technology. We used the catalytically "dead" version of codon-optimized Cas9 (dead Cas9 or dCas9) linked to the KRAB repressive domain (Gilbert et al., 2013) to specifically target the IRF1 binding element within hHS-8. We modified the lentiCRISPR lentiviral vector developed by Shalem et al. (2014) to encode the far-red reporter E2-Crimson, and made nucleotide changes to the Cas9 sequence to introduce the D10A and H840A mutations to generate dCas9. To enhance the targeting strategy, we incorporated two human pol III promoters into this lentivirus to drive expression of two unique guide RNAs in order to cover the entire 50 bp IRF1 binding element (CRISPR-hHS-8). As a positive control, we designed a lentivirus encoding two guide RNAs directed against the TATAA box and

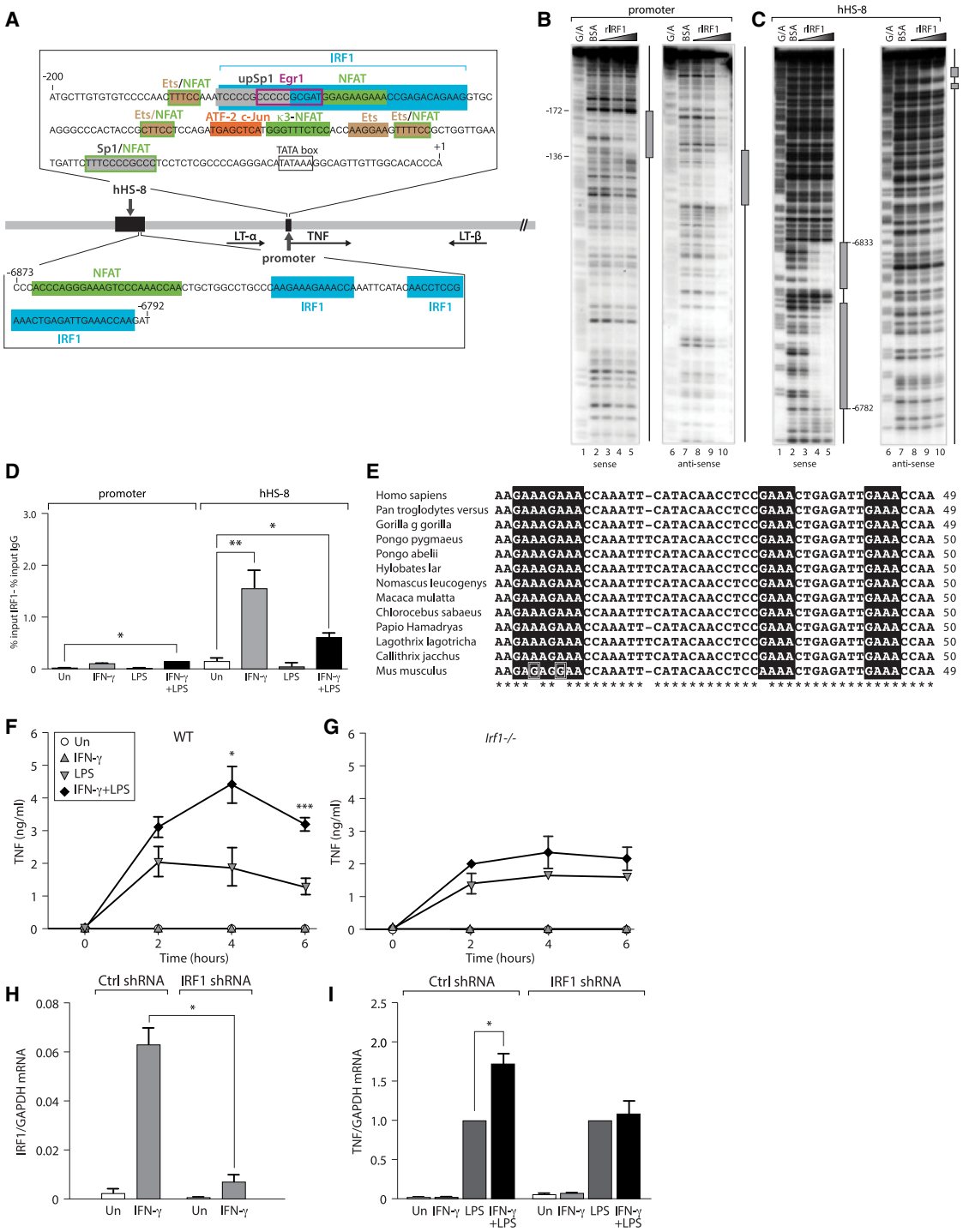

Figure 2. IRF1 Binds to hHS-8 in an IFN-γ-Inducible Manner

(A) *TNF/LT* locus with partial sequences and binding-site positions of transcription factors for both the *TNF* promoter and hHS-8.

(B) rIRF1 binds to the *TNF* promoter. Quantitative DNase I footprinting analysis of the *TNF* promoter (−200 to +1) and increasing concentrations of rIRF1. Sense and antisense strand with G+A ladder and BSA control. Bars mark areas of rIRF1 binding at −172 to −136.

(C) rIRF1 binds to hHS-8. Quantitative DNase I footprinting analysis of hHS-8 (−7031 to −6782) was performed as in (B). Bars mark areas of rIRF1 binding at −6833 to −6782.

(D) IRF1 is recruited to hHS-8 in an IFN-γ-inducible manner. ChIP using primary human MDMs and analyzing IRF1 recruitment to the *TNF* promoter and hHS-8. Data from three separate donors; *$p \leq 0.05$, **$p \leq 0.01$; data are represented as mean ± SEM.

(E) IRF1 binding sites in hHS-8 are highly conserved in all of the primate species examined. Critical 5′-GAAA-3′ motifs for IRF1 binding are highlighted.

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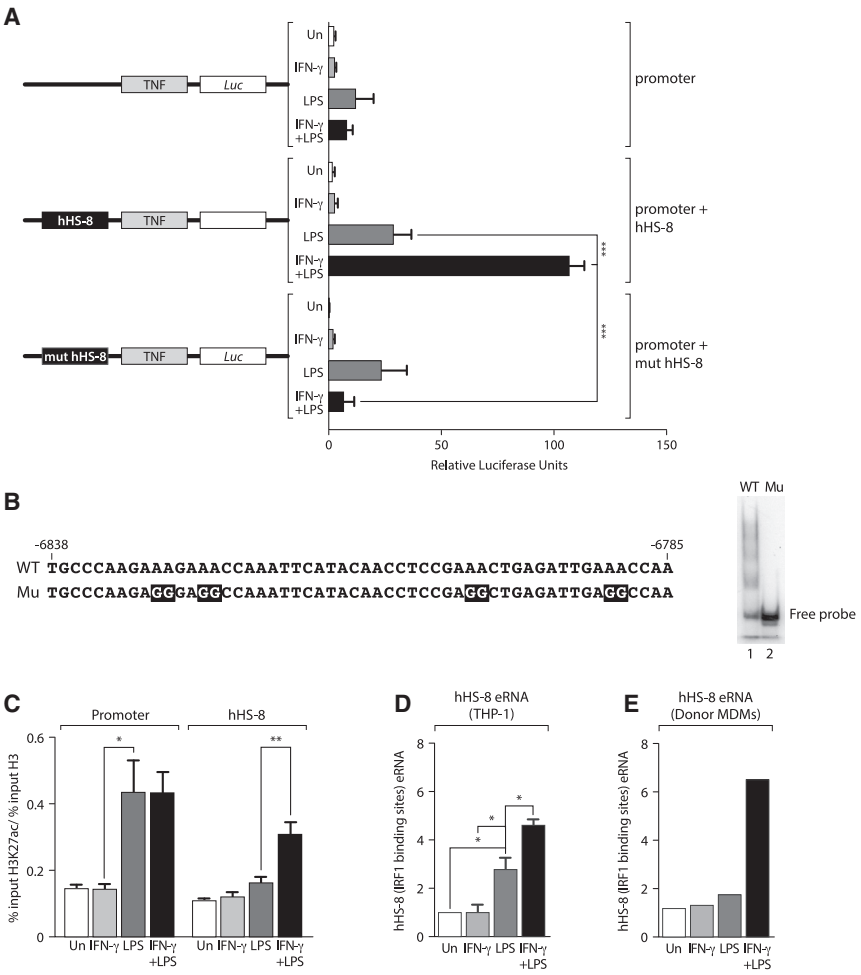

Figure 3. hHS-8 Functions as an IFN-γ-Inducible, IRF1-Dependent Enhancer of *TNF* Gene Expression

(A) Disruption of IRF1 binding to hHS-8 abolishes inducible enhancer function and thus enhanced *TNF* gene expression induced by IFN-γ priming. Constructs using the pGL3-Basic luciferase vector were transfected into J774 cells and stimulated with IFN-γ alone for 8 hr, LPS alone for 6 hr, and both IFN-γ and LPS (IFN-γ for 2 hr followed by LPS for 6 hr). TNF, the *TNF* promoter; hHS-8, the entire sequence of hHS-8 (1,250 bp); muthHS-8, hHS-8 with mutations that disrupt IRF1 binding. Data from three separate experiments; ***p ≤ 0.001; data are represented as mean ± SEM.

(B) Nucleotide changes in the critical 5′-GAAA-3′ motifs disrupt rIRF1 binding. An electrophoretic mobility shift assay was performed with rIRF1 and wild-type and mutant radiolabeled P^{32} oligonucleotides (sequences for positions −6838 to −6785).

(C) Activation of hHS-8 enhancer function corresponds with increased H3K27ac prevalence at hHS-8. ChIP analyzing H3K27ac prevalence was performed with THP-1 cells and analyzing H3K27ac prevalence at the *TNF* promoter and hHS-8. Data from three separate experiments; *p ≤ 0.05; data are represented as mean ± SEM.

(D and E) IFN-γ + LPS induces hHS-8 eRNA transcription. hHS-8 eRNA (RNA sequence containing IRF1 binding sites) were measured after LPS stimulation in THP-1 cells (D) and primary human MDMs (representative donor, E). Data from three separate experiments; *p ≤ 0.05; data are represented as mean ± SEM.

See also Figure S3.

a core Sp1 site within the *TNF* core promoter (CRISPR-TNFp). As a negative control, we generated a lentivirus encoding two guide RNAs that contain at least two mismatches with any human genomic sequence and for which the closest genomic targets lack the required NGG protospacer adjacent motif (PAM) at their 3′ ends (CRISPR-Ctrl; sequences and strategy are shown in Figure S4 and Table S1). After transducing THP-1 cells with each lentivirus, we enriched for E2-Crimson+ cells by two rounds of sorting, which resulted in >95% E2-Crimson+ cells at the time of experimental analysis (Figure 4A).

Consistent with the requirement of the TATAA box and the core *TNF* promoter Sp1 site for LPS-driven *TNF* transcription (Goldfeld et al., 1990; Falvo et al., 2000a), LPS-stimulated *TNF* gene expression was ablated (>98%) in the CRISPR-*TNF*p cells as compared with the CRISPR-Ctrl cells (Figure 4B, left). Furthermore, we showed that *TNF* transcriptional repression was specific to the *TNF* gene, since induction of IL-6 mRNA synthesis by LPS was highly inducible in both the CRISPR-Ctrl and CRISPR-*TNF*p cells (Figure 4B, right).

We then examined the impact of targeting the hHS-8 IRF1 binding element with dCas9-KRAB upon IFN-γ priming of LPS-stimulated *TNF* gene transcription. As shown in the left panel of Figure 4C, there was no difference between the CRISPR-Ctrl and CRISPR-hHS-8 cells in their transcriptional

(F and G) Enhanced TNF expression induced by IFN-γ priming is abrogated in IRF1-deficient murine BMDMs. Wild-type (F) and *Irf1*$^{-/-}$ (G) BMDMs were stimulated, and TNF protein levels in supernatants were measured by ELISA after LPS stimulation. Data from three separate experiments, each with n = 3; *p ≤ 0.05, ***p ≤ 0.001; data are represented as mean ± SEM.

(H) IRF1 mRNA levels induced by IFN-γ are silenced by IRF1 shRNA. THP-1 cells that constitutively express lentivirally delivered shRNA targeting IRF1 or control shRNA encoding a scrambled sequence were stimulated with IFN-γ alone for 3 hr. Data from three separate experiments; *p ≤ 0.05; data are represented as mean ± SEM.

(I) Enhanced *TNF* gene expression induced by IFN-γ priming is abrogated in human monocytes where IRF1 expression is silenced. THP-1 cells expressing IRF1 and control shRNA were stimulated, and TNF mRNA levels were measured (shown relative to LPS values). Data from three separate experiments; *p ≤ 0.05; data are represented as mean ± SEM.

See also Figure S2.

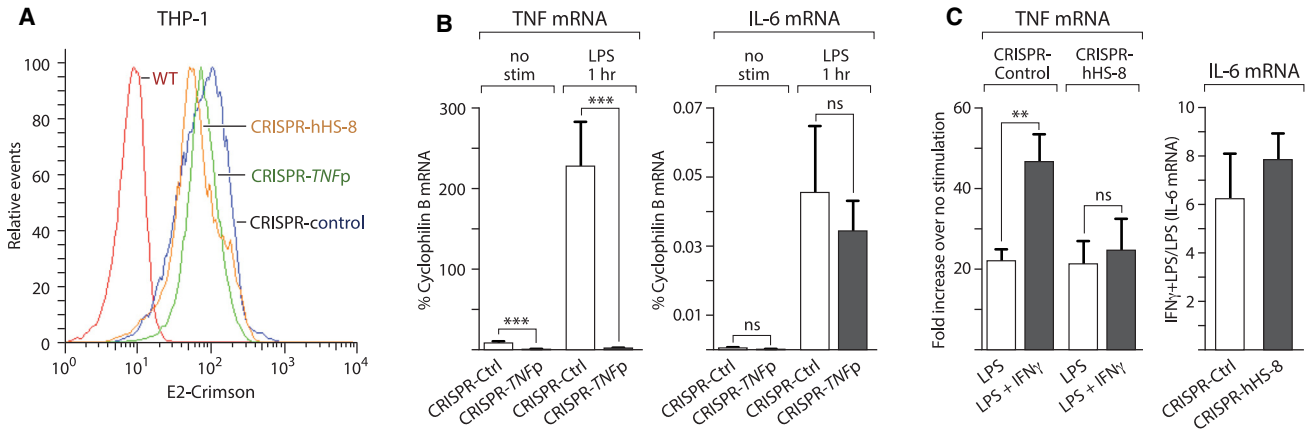

Figure 4. hHS-8 IRF1 Binding Sites Are Required for IFN-γ Priming of *TNF* Gene Expression In Vivo

(A) Flow-cytometry data demonstrating that >95% of THP-1 cells were successfully transduced with the CRISPR-Ctrl, CRISPR-*TNF*p, and CRISPR-hHS-8 lentiviruses at the time of experimental analysis.

(B) Targeting of the *TNF* promoter with dCas9-KRAB. CRISPR-Ctrl, and CRISPR-*TNF*p THP-1 cells was mock stimulated or stimulated with LPS for 1 hr, and TNF and IL-6 mRNA were quantitated after normalization to the housekeeper cyclophilin B. Data from at least three independent experiments are shown; ***$p \leq 0.001$; data are represented as mean ± SD.

(C) Targeting hHS-8 with dCas9-KRAB blocks priming of *TNF*. CRISPR-Ctrl and CRISPR-hHS-8 THP-1 cells were mock stimulated, stimulated with LPS for 1 hr, or stimulated with IFN-γ for 2 hr and LPS for 1 hr. For analysis of *TNF* expression, data are presented as fold inductions over unstimulated TNF mRNA levels to control for baseline constitutive *TNF* transcription in THP-1 cells. For analysis of *IL6* expression, data are presented as fold induction of primed versus nonprimed conditions due to the absence of detectable IL-6 transcripts in the absence of stimulation. Data from three independent experiments are presented; **$p < 0.01$; data are represented as mean ± SD.

See also Figure S4.

response to LPS, and the gene was highly inducible in both, indicating that the dCas9-KRAB fusion did not have a general repressive effect upon *TNF* activation in a stimulus-independent manner. However, when the CRISPR-hHS-8 cells were primed with IFN-γ prior to LPS stimulation, augmentation of *TNF* gene expression was abolished, but it proceeded normally in the CRISPR-Ctrl cells (Figure 4C, left). As a control for specificity, we also examined IFN-γ priming of the endogenous *IL6* gene in the CRISPR-hHS-8 cells and found that IFN-γ priming of *IL6* gene transcription was not affected in the CRISPR-hHS-8 cells (Figure 4, right), indicating that loss of IFN-γ priming at the *TNF/LT* locus was specific and not due to off-target effects of the hHS-8 guide RNAs. These findings provide a fundamental functional demonstration that hHS-8 is required for IFN-γ priming of LPS-induced *TNF* gene expression in vivo.

DISCUSSION

We have demonstrated that IFN-γ priming of *TNF* requires an exquisitely conserved distal regulatory element, hHS-8, which lies ~8 kb upstream of the *TNF* TSS. Upon exposure to IFN-γ, hHS-8 becomes more accessible to DNase I and IRF1 is recruited. Once the LPS signal occurs, levels of H3K27ac are enriched and eRNA is transcribed, which corresponds to augmented *TNF* gene expression. Both ablation of IRF1 in murine macrophages or human monocytic cells and targeting of the endogenous IRF1 binding element in hHS-8 with a dCas9-KRAB fusion protein in human monocytic cells abolish IFN-γ priming of LPS-stimulated *TNF* transcription. Thus, a combination of IFN-γ-induced chromatin accessibility and IRF1 binding at the distal hHS-8 enhancer poise the *TNF/LT* locus for augmented *TNF* gene expression in response to the TLR signal. These experiments thus provide a fundamental functional demonstration that a distal regulatory element enhances expression of a specific gene during classical macrophage activation.

A major question regarding the epigenetic and transcriptional mechanisms underlying IFN-γ priming at the *TNF/LT* locus is: Are these mechanisms unique to *TNF* or are they (or similar mechanisms) involved in the priming of other inflammatory genes? In contrast to our finding that a distal enhancer element is necessary for priming of *TNF*, it was previously shown that the *IL12A* promoter is sufficient for IFN-γ priming of LPS-induced *IL12A* gene expression (Liu et al., 2003), and the *IL6* promoter is sufficient for IFN-γ priming of *IL6* in response to stimulation by TNF (Sancéau et al., 1995).

In a recent study, Qiao et al. (2013) showed that IFN-γ + M-CSF treatment of primary human monocytes for 24 hr led to increased histone acetylation and STAT1 recruitment at the promoters and distal sites upstream of the *IL6*, *IL12B*, and *TNF* genes as compared with M-CSF treatment alone. They also found that subsequent LPS activation after IFN-γ + M-CSF pretreatment led to enhanced H3K27ac enrichment at the promoters and distal sites upstream of the *IL6*, *IL12B*, and *TNF* genes. They reported synthesis of eRNA at the *IL6* and *IL12* upstream sequences; however, they did not examine the regions upstream of the *TNF* gene for eRNA production, IRF1 binding, or enhancer function. The authors mined the ENCODE database (http://genome.ucsc.edu/ENCODE/) and found that DNase I hypersensitive sites identified in $CD14^+$ human monocytes corresponded to the general upstream regions where increased

histone acetylation and STAT1 recruitment were detected by ChIP sequencing. This led them to conclude that these distal noncoding elements are involved in IFN-γ augmentation of LPS-induced gene expression, although no functional analyses linking epigenetic modifications and changes in transcription factor recruitment to gene regulation were shown (Qiao et al., 2013). Indeed, only marginal (and nonsignificant) enhancement of SV40 promoter-driven expression by upstream sequences from the *IL6* and *IL12B* loci in reporter assays in response to IFN-γ + LPS activation was observed.

By contrast, we saw enhanced H3K27 acetylation at hHS-8 only after IFN-γ + LPS stimulation, and not after IFN-γ treatment alone. Furthermore, we observed that hHS-8 dramatically and significantly enhanced *TNF* promoter-driven reporter expression in response to IFN-γ + LPS versus LPS alone, and conferred a priming capacity to the otherwise "nonprimable" *TNF* promoter (Figure 3A). Moreover, disruption of the IRF1 binding element in hHS-8 abolished the ability of this 1.3 kb enhancer to augment *TNF* promoter-driven reporter expression. Finally, precise targeting of dCas9-KRAB to the hHS-8 IRF1 binding element within the endogenous chromatin environment inhibited IFN-γ priming of LPS-induced *TNF* gene expression, clearly demonstrating the importance of this upstream region for priming of this critical inflammatory gene.

Thus, although the TNF hHS-8 functions as an essential priming enhancer element, to date, the sites upstream of *IL6* and *IL12B* have not been demonstrated to have a functional role in the regulation of these genes. It will be of interest to determine in future studies whether the regions upstream of the *IL6* and *IL12B* genes identified by Qiao et al. (2013) play a role in IFN-γ priming of LPS-induced gene expression at these loci, or whether the epigenetic and other changes seen at these sites in response to IFN-γ stimulation are bystander marks of a localized, "primed" chromatin environment.

Our data suggest the possibility that IRF1 may function at hHS-8 as a "pioneer factor" (Zaret and Carroll, 2011) for enhanced *TNF* gene expression in primed monocytes and macrophages. In this scenario, IRF1 binding to hHS-8 would promote increased DNase I accessibility at hHS-8 and the recruitment of chromatin-remodeling complexes and additional factors that poise this element for rapid activation in response to the LPS signal. Indeed, several studies have identified a class of enhancers, termed "poised enhancers," that are linked to inactive genes and are distinguished by the absence of H3K27ac (Zentner et al., 2011; Creyghton et al., 2010; Rada-Iglesias et al., 2011; Cotney et al., 2012). When activated, these poised enhancers become enriched in H3K27ac (Rada-Iglesias et al., 2011). This is reminiscent of the LPS-dependent acetylation of hHS-8 that we observed upon LPS stimulation of IFN-γ-primed cells. We note that IRF1 was previously shown to recruit CBP/p300 (Marsili et al., 2004) and PCAF (Masumi et al., 1999). We imagine that upon IFN-γ stimulation of monocytes/macrophages, there is an increase in the percentage of cells in the sample population in which hHS-8 is "open" and associated with IRF1, resembling a poised enhancer ready for activation. Indeed, IRF1 could function as a beacon for enhanced recruitment of CBP/p300 to hHS-8 following IFN-γ + LPS stimulation, leading to H3K27 acetylation and the commissioning of hHS-8 as an active enhancer to augment transcription of *TNF*.

Finally, by demonstrating that specific targeting of dCas9-KRAB to the IRF1 binding element in hHS-8 within its endogenous chromatin environment abrogates IFN-γ augmentation of LPS-induced *TNF* transcription in human monocytic cells, we have confirmed that hHS-8 is required for priming of this critical early response gene during classical macrophage activation.

Our data also suggest that applying dCas9-KRAB technology to the functional interrogation of global data sets such as ENCODE would be of particular value. Furthermore, by probing the function of distal elements linked to specific genes, we can achieve a fundamental understanding of the role of long-range interactions in controlling cell-type- and/or stimulus-specific gene expression.

EXPERIMENTAL PROCEDURES

Cell Culture and Stimulations

THP-1 cells were maintained in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). J774 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. For primary human MDMs, enriched populations of human monocytes were isolated from healthy human donor buffy coats using a $CD14^+$ positive selection kit (STEMCELL Technologies). MDMs were obtained after 6 days of culture in RPMI-1640 medium supplemented with 5% human serum AB (GemCell) and GM-CSF (50 ng/ml; Peprotech). Cells were treated with IFN-γ (100 ng/ml; R&D Systems) and LPS (100 ng/ml; Sigma-Aldrich, *E. coli* O111:B4).

RNA Extraction and Real-Time Quantitative RT-PCR

Total RNA was extracted from cells with the use of the QuickRNA Mini kit (Zymo) and treated with the Turbo DNA-free kit (Invitrogen). cDNA was synthesized from total RNA with M-MLV reverse transcriptase (Invitrogen) and 20-residue oligo (dT; Invitrogen). eRNA was synthesized from total RNA with hHS-8 eRNA reverse primer. TNF mRNA levels were measured by the change-in-threshold (ΔΔCt) method based on real-time quantitative PCR (qPCR) in an iCycler iQ (Bio-Rad) with SyberGreen Master Mix (Invitrogen) and primers recognizing exon 4 and exon 3 of the human *TNF* gene, the human *GAPDH* gene, and the human *IRF1* gene. Primers used for ChIP and cDNA measurements by real-time qPCR are shown in Table S1.

DHAs

DHAs were performed using both THP-1 cells and primary human MDMs. MDMs were detached from the culture surface using TrypLE (Life Technologies). Cells were harvested, washed with PBS, and resuspended in RSB buffer (10 mM Tris-HCl [pH 7.4], 10 mM NaCl, and 3 mM $MgCl_2$). Cells were lysed with lysis buffer (0.5% NP-40 in 1× RSB buffer) on ice for 5 min. Resuspended nuclei in RSB buffer were treated with DNase I (40 ng/μl) at 37C for 5 min. DNase I activity was quenched upon addition of a stop solution (0.6 M NaCl, 20 mM Tris-HCl [pH 8.0], 10 mM EDTA, and 1% SDS). Samples were treated with Proteinase K at 56°C overnight (O/N). DNA was digested with ScaI and BamHI restriction enzymes and analyzed by Southern blotting using a radiolabeled P^{32} probe corresponding to the coding region of *LTA*, with 10 μg of DNA used for each lane.

ChIP

ChIP assays were performed with anti-IRF1 (H-205; Santa Cruz Biotechnology), Rb IgG (Diagenode), anti-H3K27me3 (C36B11; Cell Signaling Technology), anti-H3K27ac (Active Motif), and anti-H3 (Abcam). THP-1 cells and primary human MDMs were treated (IFN-γ 100 ng/ml, LPS 100 ng/ml), fixed with 10% formaldehyde for 15 min, treated with 2.5 M glycine for 5 min, harvested, washed with PBS, lysed with 0.25% Triton X-100 and 0.5% NP-40 for 5 min, centrifuged at 1,200 rpm for 10 min, resuspended in 1% SDS lysis buffer, and sonicated for 5 min for four cycles in a Biorupter. Sonicated DNA

was set up for immunoprecipitation O/N and DNA-protein complexes were recovered by adding Protein A/G Plus Agarose Beads (Thermo Scientific) for 3 hr. Samples were washed six times with 1 ml of wash buffer and treated with proteinase K at 65°C O/N. Samples were treated with phenol/chloroform before O/N ethanol precipitation. DNA fragments for IRF1 recruitment were analyzed by real-time qPCR with SYBER Green Master Mix (Invitrogen) and primer sets for regions −244 to −82 (promoter) relative to the *TNF* TSS, and −6842 to −6737 (hHS-8) relative to the *TNF* TSS (Table S1). Rb IgG percent input values were subtracted from IRF1 percent input values. DNA fragments for H3K27me3 and H3K27ac analysis were analyzed by real-time qPCR using Jumpstart *Taq* ReadyMix for Quantitative PCR (Sigma-Aldrich) and primer/probe sets for the *TNF* promoter and hHS-8 (Table S1). H3K27me3 and H3K27ac percent input values were normalized to H3 percent input values.

DNase I Footprinting Assay

Radiolabeled P^{32} fragments of the *TNF* promoter (−200 to +1) and hHS-8 (−7031 to −6782) regions were incubated with recombinant IRF1 protein (Abcam) and treated with diluted DNase I at room temperature for 5 s before enzyme activity was quenched with a stop solution (0.13 mM EDTA, 0.5% SDS, and tRNA). Samples were treated with phenol chloroform and DNA was precipitated O/N at −20°C. G+A ladder was treated with 4% formic acid, radiolabeled with P^{32}, treated with 1 M piperidine, and precipitated with n-butanol. DNA fragments were separated by electrophoresis on an 8% sequencing gel.

Mice

C57BL/6J mice and B6.129S2-*Irf1*^{tm1Mak}/J mice (6–8 weeks old) were purchased from The Jackson Laboratory. Experimental procedures were done in accordance with the Institutional Animal Care and Use Committee and the Harvard Medical Area Standing Committee on Animals.

Isolation, Culture, and Stimulation of Murine BMDMs

For the generation of murine BMDMs, bone marrow cells of wild-type or *Irf1*$^{-/-}$ mice (6–8 weeks old) were cultured in DMEM supplemented with 10% FBS, 10% L929 cell conditioned medium (LCCM), 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM L-glutamine. Cells were fed on day 5 and media were changed on day 7, 3 hr before mIFN-γ (100 ng/ml; R&D Systems) and LPS (100 ng/ml; Sigma-Aldrich, *E. coli* O111:B4) treatment. Supernatant was collected 2, 4, and 6 hr after treatment.

shRNA

The lentiviral plasmid pLKO.1 expressing shRNA targeting human IRF1 was purchased from the RNAi Consortium (TRC) Lentiviral shRNA Library (Thermo Scientific). Clone TRCN0000014668 with a target sequence of 5′-CGTG TGGATCTTGCCACATTT-3′ was validated in our laboratory. Control shRNA encodes a scrambled sequence. Lentiviruses encoding shRNA sequences were generated by transfecting the packaging cell line HEK293T with the shRNA-encoding pLKO.1 plasmids in combination with the packaging plasmid psPAX2 and the envelope plasmid pMD2.G using Effectene transfection reagent (QIAGEN). Supernatants were collected 48 hr posttransfection, clarified by centrifugation, and stored at −80°C. THP-1 cells were transduced with the lentiviral particles by culturing the cells with supernatants from the virus-producing cells in the presence of 8 μg/ml polybrene (Millipore) and spinoculation for 2 hr at 2,000 rpm. Successfully transduced cells were selected and expanded by treatment with 0.8 μg/ml puromycin.

Electrophoretic Mobility Shift Assay

Radiolabeled ^{32}P oligonucleotides were added to THP-1 nuclear extracts or recombinant IRF1 protein (Abcam) in a binding buffer solution (10 mM Tris-HCl [pH 7.5], 53 mM NaCl, 1 mM dithiothreitol, 0.01% Nonidet-P40, 5% glycerol, and 0.05 μg/μl of double-stranded poly(dI-dC)) at room temperature for 30 min. In supershift experiments, samples were incubated with 2 μg of anti-IRF1 (H-205; Santa Cruz Biotechnology). Protein-DNA complexes were separated by electrophoresis on a 5% PAAG gel.

Sequencing

Cell lines and samples of blood or DNA from representative individuals of the primate species and subspecies were procured as previously described (Leung et al., 2000; Baena et al., 2007). Genomic DNA was isolated using the QIAamp DNA Blood Kit (QIAGEN). Sequence alignments were performed using ClustalW2 multiple sequence alignment provided by EMBL-EBI.

Plasmids

Construction of the *TNF* promoter-driven luciferase reporter was previously described (Tsai et al., 2000). The *TNF* promoter with hHS-8 plasmid was constructed by inserting nucleotides −7,833 to −6,583 relative to the *TNF* TSS into the *TNF* promoter-driven luciferase reporter construct using MluI and NheI restriction enzyme sites. The *TNF* promoter with mutated hHS-8 plasmid was constructed by circular site-directed mutagenesis.

Luciferase Reporter Assay

J774 cells were transfected with luciferase reporter constructs using an Effectene Transfection Reagent Kit (QIAGEN). Cells were treated with mIFN-γ (100 ng/ml; R&D Systems) and LPS (100 ng/ml, Sigma-Aldrich, *E. coli* O111:B4). Luciferase assays were performed 8 hr after treatment under the Dual Luciferase Reporter Assay System (Promega) using a Dynex luminometer and *Renilla* luciferase (pRL-TK) as a control.

CRISPR/dCas9 Analysis

The plasmids pCas9_GFP (Addgene plasmid 44719, deposited by Kiran Musunuru) and LentiCRISPR (Addgene plasmid 49535, deposited by Feng Zhang) were obtained from Addgene. D10A and H840A substitutions were introduced into the Cas9 coding region of pCas9_GFP by overlapping PCR in order to generate the catalytically inactive dCas9 as described previously (Qi et al., 2013). The KRAB coding sequence was ordered as a gBlock fragment from Integrated DNA Technologies and cloned in-frame at the 3′ end of the dCas9 coding sequence. After the E2-Crimson coding sequence (Clontech), preceded by the P2A self-cleaving peptide DNA sequence, was substituted for the 2A-puromycin resistance gene in LentiCRISPR, the dCas9-KRAB sequence was amplified and substituted for Cas9 in LentiCRISPR upstream and in-frame with the 2A-E2-Crimson sequence. To generate guide RNA, we first cloned a cassette containing the tracr RNA sequence from LentiCRISPR followed by a TTTTTTT termination signal and the 98 bp H1 promoter sequence into plasmid pSP73 (Promega). This plasmid was used as a template with the primers shown in Table S1 to create individual PCR products consisting of a BsmBI site-20bp target#1-tracr-term-H1pro-20bp target#2-BsmBI site. This was then cloned into the BsmBI sites of the modified lentiCRISPR vector, with the cassette placed after the U6 promoter and before the tracr-term sequence already present in the vector. The sequences in the *TNF* promoter and hHS-8 that were targeted with this dual guide RNA vector system are shown in Figure S4. For the control lentivirus, we used the 20 bp targets 5′gttcgtgtcgtcgtgtctta-3′ and 5′gaatctagcggtctgacatt-3′ because these sequences have at least two mismatches with any 20 bp sequence in the human genome, and the closest matches in the human genome do not possess the 5′-NGG-3′ PAM sequence required for full Cas9 binding.

The CRISPR/dCas9-KRAB lentiviruses were prepared and THP-1 cells were transduced as described above for the shRNA lentiviruses, except that virus-containing medium was centrifuged over 20% sucrose at 11,500 rpm for 4 hr to increase the lentiviral concentration prior to spinoculation. After expansion of transduced cells, E2-Crimson+ cells were enriched by fluorescence-activated cell sorting at two time points over the course of 3 weeks. For experimental analysis, cells were seeded at 5 × 10^5 cells/ml and stimulated with IFN-γ and LPS, and RNA isolation, cDNA synthesis, and qPCR were performed as described above using primer sets for cyclophilin B, TNF, and IL-6 as shown in Table S1.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2014.11.011.

ACKNOWLEDGMENTS

We are indebted to Laurens Kruidenier and David Tough for stimulating discussions and experimental suggestions. We thank Alla Tsytsykova and Shahin

Ranjbar for critical experimental advice and discussions. We also thank James Falvo for helpful discussions. This work was supported by grants from GlaxoSmithKline and the NIH/NIGMS (GM076685) to A.E.G.

Received: March 22, 2014
Revised: October 16, 2014
Accepted: November 6, 2014
Published: December 4, 2014

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