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Inhibition of Inflammatory Gene Transcription by IL-10 Is Associated with Rapid Suppression of Lipopolysaccharide-Induced Enhancer Activation

Evan A. Conaway,* Dalila C. de Oliveira,*¹ Christine M. McInnis,*[†] Scott B. Snapper,^{‡,§} and Bruce H. Horwitz*[†]

IL-10 limits the magnitude of inflammatory gene expression following microbial stimuli and is essential to prevent inflammatory disease; however, the molecular basis for IL-10-mediated inhibition remains elusive. Using a genome-wide approach, we demonstrate that inhibition of transcription is the primary mechanism for IL-10-mediated suppression in LPS-stimulated macrophages and that inhibited genes can be divided into two clusters. Genes in the first cluster are inhibited only if IL-10 is included early in the course of LPS stimulation and is strongly enriched for IFN-inducible genes. Genes in the second cluster can be rapidly suppressed by IL-10 even after transcription is initiated, and this is associated with suppression of LPS-induced enhancer activation. Interestingly, the ability of IL-10 to rapidly suppress active transcription exhibits a delay following LPS stimulation. Thus, a key pathway for IL-10-mediated suppression involves rapid inhibition of enhancer function during the secondary phase of the response to LPS. *The Journal of Immunology*, 2017, 198: 2906–2915.

The response of macrophages, even to a single inflammatory stimulus, is remarkably complex. Stimulation of macrophages with LPS rapidly induces the activation of canonical transcription factors, such as NF- κ B and IRF3, in a protein synthesis-independent manner, and this is quickly followed by rapid induction of mRNA for a number of primary response genes (1–3). Following this initial wave, there are subsequent waves of mRNA induction that are sensitive to inhibition of protein synthesis, indicating the secondary nature of the response (4). It was

documented that a significant proportion of secondary response genes are, in fact, responding to the primary induction of IFN- β , but several other factors are involved in driving secondary response genes, including the atypical nuclear I κ B-like molecule I κ B ζ (5–7). Although mRNA induction following LPS-stimulation of macrophages is likely regulated at multiple levels, detailed studies evaluating changes in newly synthesized mRNA strongly suggest that induction of new gene transcription is the driving force behind the observed global alterations in gene expression (1, 2).

Recently, it was demonstrated that LPS-induced transcription in macrophages is associated with enhancer activation (8). Recruitment of stimulus-dependent transcription factors, such as NF- κ B, to genomic sites termed poised enhancers is marked by binding of the pioneer transcription factor PU.1 and monomethylation of histone H3 at lysine 4 (H3K4me1). Activation of these enhancers is associated with increases in acetylation of histone H3 at lysine 27 (H3K27Ac) and increased transcription of *cis*-located genes (8–10). Although there is increased appreciation for the role of enhancer activation in initiating LPS-induced transcription, there is much less known regarding the processes that terminate LPS-induced transcription, and we do not know how factors that limit LPS-induced transcription influence the activation state of LPS-induced enhancers.

One key factor that limits LPS-induced inflammatory gene expression in macrophages is the stimulus-induced production of IL-10, a potent anti-inflammatory cytokine (11). The receptor for IL-10 activates STAT3 through a JAK1-dependent pathway (12). Global profiling experiments comparing mRNA levels in IL-10-deficient macrophages treated with LPS alone or LPS and IL-10 identified a wide range of primary and secondary response genes that are inhibited by IL-10, and studies in STAT3-deficient macrophages indicate that STAT3 is required for IL-10-mediated inhibition (11). Understanding the mechanism of STAT3-mediated inhibition in response to IL-10 has proven enigmatic. In most systems, STAT3 is a transcriptional activator, and there is little evidence that it has direct inhibitory function or binds to regulatory regions of IL-10-inhibited genes (13). This led to the

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E.A.C. designed and performed experiments, collected and analyzed data, and wrote the manuscript; D.C.d.O. and C.M.M. designed and performed experiments and collected and analyzed data; S.B.S. provided critical revision of the manuscript for important intellectual content; and B.H.H. designed experiments, supervised experimentation and data collection, and wrote the manuscript.

The data presented in this article have been submitted to the Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/genbank>) under accession number GSE86170.

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Abbreviations used in this article: BMDM, bone marrow-derived macrophage; ChIP-seq, chromatin immunoprecipitation followed by DNA sequencing; FDR, false discovery rate; H3K4me1, monomethylation of histone H3 at lysine 4; H3K27Ac, acetylation of histone H3 at lysine 27; IP, immunoprecipitation; pre-mRNA, precursor mRNA; RNA-seq, RNA sequencing; WT, wild-type.

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hypothesis that, following IL-10R engagement, STAT3 induces genes that secondarily inhibit inflammatory gene expression. A number of STAT3-induced transcriptional inhibitors have been identified, including *Bcl3* and *Nfil3*; however, studies in genetically deficient macrophages failed to demonstrate that these factors are required for IL-10-mediated inhibition (14, 15). A firm understanding of the kinetics of IL-10-mediated inhibition could contribute significantly to delineating potential inhibitory mechanisms; however, knowledge is lacking in this area.

Inhibitory functions for IL-10 in macrophages have been proposed at the level of transcription, mRNA stability, and translation of individual genes (16–19). With regard to transcription, Aste-Amezaga et al. (20) used nuclear run-on assays to demonstrate that addition of IL-10 1 h prior to LPS stimulation inhibited transcription of *IL12B* in human PBMCs. Murray (16) used RT-PCR of primary transcripts to demonstrate that IL-10 added concomitantly with LPS inhibited transcription of *Il1a*, *Cxcl1*, *Il6*, and *Tnf* in IL-10-deficient bone marrow-derived macrophages (BMDMs). Further, studies of the *Il12b* promoter demonstrated that IL-10 inhibited histone H4 acetylation and prevented PolII association, consistent with inhibition of transcription (21). However, we do not yet understand the kinetics of IL-10-induced transcriptional inhibition or the scope of IL-10-mediated inhibition of the LPS-induced transcriptional program. Further, we do not understand how IL-10 influences the activation status of LPS-induced enhancers (22) and whether suppression of active enhancers by IL-10 occurs in a time frame compatible with suppression of active transcription. Answering these questions would increase our understanding of mechanisms responsible for IL-10-mediated inhibition and potentially lead to novel insights regarding mechanisms that terminate activation of inducible enhancers.

Delineating the kinetics of transcriptional inhibition using assays that measure mRNA is problematic, because the varying stability of mRNA makes it difficult to discern rapid changes in underlying transcriptional rates. Therefore, we used precursor mRNA (pre-mRNA) as a surrogate of transcriptional rate to provide a detailed analysis of the kinetics of IL-10-mediated transcriptional suppression. Further, using an RNA-sequencing (RNA-seq) protocol that allowed us to separately analyze mRNA and pre-mRNA at the global level, we identified two clusters of IL-10-inhibited genes that exhibit different kinetics of suppression and provide a mechanism for these differences. Finally, using chromatin immunoprecipitation followed by DNA sequencing (ChIP-seq), we find that IL-10 induces highly dynamic alterations in enhancer activation that are associated with alterations in transcription of IL-10 targets. These studies significantly increase our understanding of mechanisms that regulate LPS-induced inflammatory gene expression.

Materials and Methods

Experimental animals

All mouse strains were maintained on the 129S6/SvEvTac background. Generation of *Rag2*^{-/-} (wild-type [WT]) and *Il10*^{-/-}*Rag2*^{-/-} (*Il10*^{-/-}, IL-10-deficient) mice was described previously (23). All animal procedures were approved by the Harvard Medical Area Standing Committee on Animals.

BMDM preparation and stimulation

BMDMs were grown as previously described (24) and split into 24-well plates on the day prior to stimulation. BMDMs were cultured in 500 μ l of 10% FBS DMEM supplemented with penicillin/streptomycin, HEPES, and GlutaMAX at a density of 2.5×10^5 cells per well for RNA extraction or at a density of 1×10^6 cells per well for Western blotting. For stimulation, medium was removed and replaced with fresh medium for unstimulated

samples or medium containing 1 ng/ml LPS from *Escherichia coli* serotype 0127:B8 (Sigma, St. Louis, MO). IL-10 (PeproTech, Rocky Hill, NJ) was used at a final concentration of 1 ng/ml. Actinomycin D (Sigma) was added 2 h after LPS stimulation to a final concentration of 5 μ g/ml.

RNA extraction and RT-PCR

Cell culture medium was aspirated, and cells were lysed in 500 μ l of TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA). RNA was isolated per the manufacturer's instructions with the addition of GlycoBlue Coprecipitant (Thermo Fisher Scientific) and 1-bromo-3-chloropropane (Molecular Research Center, Cincinnati, OH) in lieu of chloroform. cDNA was synthesized from 1 μ g of DNase-treated RNA using random hexamers with TaqMan Reverse Transcription Reagents (Thermo Fisher Scientific). RT-PCR was performed using the StepOnePlus System with TaqMan probes or with SYBR Green Master Mix (both from Thermo Fisher Scientific) and custom primers. Primer sequences are available on request. Expression was normalized to GAPDH, and differences between samples were calculated using the $\Delta\Delta$ cycle threshold method. Fold-change is reported relative to levels observed in untreated macrophages. In graphs of RT-PCR data, each line represents BMDM from an individual mouse; each point on the line represents an individual well. Within the figure legends, *n* indicates the size of the experimental groups in the displayed experiment, and a statement regarding the number of times the experiment was performed is provided.

Western blot

Cells were lysed in 100 μ l of RIPA buffer and 6.5 μ l of lysate loaded onto Novex 4–12% Bis-Tris gels (all from Thermo Fisher Scientific). Gels were transferred to polyvinylidene difluoride membranes and blotted with anti-STAT3 (4904), anti-pSTAT3 (Y705; 9145), and anti-GAPDH (5174; all from Cell Signaling Technology).

ELISA

IL-12 p40 was measured in culture supernatants using capture Ab C15.6 (2 μ g/ml; BD Pharmingen, San Jose, CA) and detection Ab C17-8 (0.5 μ g/ml; Thermo Fisher Scientific). IL-10 was measured using capture Ab JES5-16E3 (4 μ g/ml) and detected using Ab JES5-2A5 (0.5 μ g/ml; both from eBioscience, San Diego, CA). IFN- β was measured with the VeriKine Mouse IFN Beta ELISA Kit, per the manufacturer's instructions (PBL Assay Science, Piscataway, NJ). Each data point represents the results from BMDMs isolated from an individual mouse.

Total RNA-seq

BMDMs were cultured in six-well plates at 1×10^6 cells per well. Cells were rinsed once with PBS, and RNA was isolated following the addition of TRIzol Reagent directly to the plate. RNA was further purified using an RNeasy Lipid Tissue Mini Kit (QIAGEN, Hilden, Germany). rRNA was depleted using a NEBNext rRNA Depletion Kit, and libraries were made with a NEBNext Ultra Directional RNA Library Prep Kit for Illumina (both from New England Biolabs, Ipswich, MA). Samples were sequenced using the Illumina HiSeq or NextSeq Platforms. Data were analyzed as in Gaidatzis et al. (25). Briefly, reads were aligned to mm10 using Rbowtie. Counts were generated using the QuasR package using qCount with only nonoverlapping genes, and differential analysis was carried out using DESeq. To focus on genes that were strongly induced by LPS, we used a false discovery rate (FDR) of 0.1 and only evaluated genes whose intronic signal increased by ≥ 100 -fold. Further, we only evaluated genes that had ≥ 50 reads upon LPS stimulation. For genome level display, one replicate was normalized with deepTools (26) using the size factor generated by DESeq and displayed in the Integrative Genomics Viewer. The heat map was generated in R. All data are accessible through the gene expression omnibus accession number GSE86170.

ChIP-seq

Macrophages were plated at 2×10^7 cells per condition on 15-cm plates and, after stimulation, were fixed for 10 min in 1% formaldehyde. Chromatin was isolated and sheared by sonication (10 cycles, 20 s on, 30 s rest). After aliquoting an input fraction, 4×10^6 cells were incubated overnight at 4°C with Protein A Dynabeads (Thermo Fisher Scientific) precoupled with 5 μ g of anti-H3K27Ac Ab (ab4729; Abcam) or 300 ng of anti-STAT3 Ab (12640; Cell Signaling Technologies). After incubation, the beads were washed sequentially with a low-salt buffer, high-salt buffer, lithium chloride buffer, and TE buffer. DNA-protein complexes were eluted from the beads with 1% SDS TE buffer at 65°C for 15 min. Input and immunoprecipitation cross-links were reversed by overnight incubation at 65°C. DNA was treated with proteinase K and RNase A (Thermo Fisher Scientific) before purification with a QIAquick PCR Purification Kit (QIAGEN).

Immunoprecipitation and input libraries were prepared using a NEB-Next ChIP-Seq Library Prep Master Mix Set for Illumina (New England Biolabs). Samples were sequenced using the Illumina NextSeq platform. Samples were aligned to mm10 using bowtie2 (27), with default parameters and peaks called using MACS2 with the broad flag on for H3K27Ac and H3K4me1. Differential peak calling for H3K27Ac was done with three biological replicates using the DiffBind (28) package and the DESeq algorithm specifying an FDR < 0.1. For genome-level display, one representative replicate's libraries were normalized down to the depth of the least sequenced library and displayed as reads per base pair. STAT3 ChIP-seq data were analyzed in MACS using default parameters. Data were normalized to reads per million for display. For data from Ostuni et al. (8), the untreated H3K4me1 sample was normalized to reads per million. Mean acetylation plots were made using deepTools (26). Plots and statistical analysis in Fig. 6 were done in R. All data are accessible through the gene expression omnibus accession number GSE86170.

Statistical analyses

The Fisher exact test was used to calculate significant overrepresentation of *Irfar1*-dependent genes (Fig. 3D). ANOVA with the Dunnett multiple-comparisons test was used to identify significant differences in IFN- β secretion (Fig. 5B). The Mann-Whitney *U* test was used to identify significant differences in H3K27 enhancer acetylation ratios (Fig. 6A). Two-way ANOVA with the Tukey multiple-comparisons test was used to identify significant differences in *Cxcl2* pre-mRNA (Fig. 8A). For all analyses, $p < 0.05$ was considered statistically significant.

Results

Endogenous IL-10 limits duration of IL-12 transcription

Il12b is an LPS-induced secondary response gene that is strongly inhibited by IL-10. Addition of exogenous IL-10 inhibits *IL12B* transcription in human PBMCs stimulated with LPS, and it inhibits *Il12b* transcription in murine peritoneal macrophages stimulated with LPS and IFN- γ (11, 29). However, the kinetic relationship between expression of endogenous IL-10 and transcription of *Il12b* has not been fully explored. To evaluate this issue, we stimulated WT and IL-10-deficient BMDMs with LPS and evaluated expression of *Il12b* mRNA every 15 min from 0 to 4 h. In WT BMDMs, *Il12b* mRNA was first detected 60 min after stimulation; it increased until reaching a plateau at 120 min that lasted for the duration of the experiment (Fig. 1A). In IL-10-deficient BMDMs, *Il12b* expression appeared to begin at the same time as in WT cells, but rather than reaching a plateau at 2 h, it continued to rise over the course of the next several hours. The difference in *Il12b* mRNA levels between WT and IL-10-deficient BMDMs was reflected in increased IL-12 p40 secretion by IL-10-deficient macrophages (Fig. 1B). These results suggest that endogenously produced IL-10 limits *Il12b* mRNA production and that this phenomenon begins 2 h after LPS stimulation. To determine whether this is consistent with the kinetics of endogenous IL-10 production, we performed an ELISA to measure IL-10 levels within the supernatants of LPS-stimulated WT BMDMs and found that IL-10 was first detected between 1 and 2 h after LPS stimulation, indeed coinciding with the plateau phase of *Il12b* production observed in WT BMDMs (Fig. 1C).

The observation that secretion of endogenous IL-10 results in a plateau in *Il12b* mRNA levels suggests that the rates of transcription and degradation of *Il12b* are matched or that transcription has ceased, and the remaining *Il12b* message is highly stable. To assess the stability of *Il12b* mRNA, we added the transcriptional inhibitor actinomycin D 2 h after LPS treatment of WT BMDMs and measured the levels of *Il12b* mRNA over the next 2 h. We found very little change in *Il12b* mRNA levels over the course of this experiment, indicating the mRNA was stable and suggesting that transcription of *Il12b* had ceased following expression of IL-10 (Fig. 1D). It was demonstrated that transcriptional activity can be evaluated through measurements of unspliced pre-mRNA, because they typically have a very short

lifespan (16, 30). Therefore, we designed RT-PCR primers that selectively recognized *Il12b* pre-mRNA but not mRNA. As predicted, levels of pre-mRNA fell very rapidly after administration of actinomycin D, indicating that measurement of pre-mRNA was an accurate surrogate for transcription (Fig. 1D). Following LPS stimulation of WT BMDMs, levels of *Il12b* pre-mRNA increased earlier than what we had observed for levels of *Il12b* mRNA but decreased rapidly when *Il12b* mRNA reached the plateau phase (Fig. 1E), concomitant with accumulation of IL-10 within the supernatant of WT BMDMs. In contrast, *Il12b* pre-mRNA levels continued to increase for several additional hours in IL-10-deficient BMDMs (Fig. 1E). These results demonstrate that secretion of endogenous IL-10 rapidly inhibits transcription of *Il12b*.

IL-10 rapidly inhibits active transcription of *Il12b*

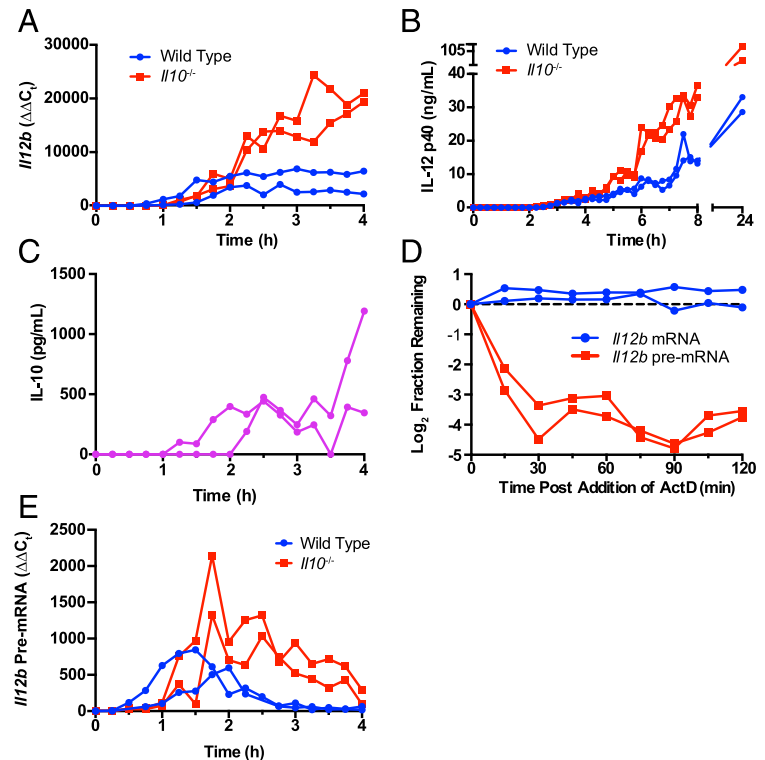
The results above suggest that endogenous IL-10 rapidly suppresses transcription of *Il12b* in WT BMDMs stimulated with LPS. To more accurately assess the kinetics of IL-10-mediated suppression, we added exogenous IL-10 to IL-10-deficient BMDMs at a time of active *Il12b* transcription (1.75 h after LPS stimulation). We observed significant suppression of *Il12b* transcription as early as 15 min after exposure and nearly complete suppression by 30 min (Fig. 2A). Further, we found that addition of IL-10 led to a rapid plateau in levels of *Il12b* mRNA (data not shown). Consistent with these results, the timing of IL-10 addition strongly influenced the accumulation of IL-12 p40 within the culture medium (Fig. 2B). These results indicate that IL-10 rapidly inhibits transcription of *Il12b* in LPS-stimulated BMDMs and that this profoundly influences accumulation of secreted IL-12 p40.

Inhibition of transcription is a central mechanism of IL-10-mediated suppression

To further examine the transcriptional regulation of *Il12b* in response to IL-10 we sequenced ribosome-depleted total RNA from IL-10-deficient macrophages that were left untreated, stimulated with LPS alone for 3.25 h (LPS), stimulated with LPS and IL-10 for 3.25 h (continuous IL-10), or stimulated with LPS for 3.25 h with IL-10 added for the last 30 min (acute IL-10). As expected, although we found no signal in untreated cells, we detected strong signals over the exons of *Il12b* following treatment with LPS (Fig. 3A), indicating the accumulation of spliced mRNA. In addition, we observed signal within the introns of *Il12b* (Fig. 3B), indicating the presence of pre-mRNA and, therefore, active transcription. In the samples treated with LPS and continuous IL-10, we saw no signal in exonic or intronic regions, indicating a lack of mRNA accumulation and lack of active transcription. In contrast, in samples treated with LPS and acute IL-10 (final 30 min of the experiment), although the exonic signal exhibited little change in intensity compared with the samples treated with LPS alone, there was marked inhibition of the intronic signal. This suggests that, consistent with RT-PCR results, IL-10 rapidly inhibits transcription of *Il12b*.

The recognition that RNA-seq of total RNA allowed us to observe rapid alterations in *Il12b* transcription by monitoring pre-mRNA suggested that we could use this technique to evaluate the transcriptional effects of IL-10 at the global level. To accomplish this, we calculated expression levels of mRNA and pre-mRNA for all protein-coding genes across all four conditions (untreated, LPS, continuous IL-10, acute IL-10) using exon-intron split analysis with minor alterations (25). Using this approach, we identified 138 genes whose transcription was increased ≥ 100 -fold (FDR < 0.1) by LPS. We grouped these genes into three clusters using a k-means algorithm (Fig. 3C, 3D). Transcription of genes in

FIGURE 1. IL-10 rapidly inhibits *Il12b* transcription. **(A)** WT and *Il10*^{-/-} BMDMs were stimulated with LPS (1 ng/ml). RNA was harvested every 15 min for 4 h. *Il12b* mRNA was measured by RT-PCR, and expression levels are displayed relative to unstimulated levels using the $\Delta\Delta$ cycle threshold method ($n = 2$). **(B)** WT and *Il10*^{-/-} BMDMs were stimulated with LPS, and supernatants were collected every 15 min for up to 8 h, as well as at 24 h. IL-12 p40 levels were measured by ELISA ($n = 2$). **(C)** IL-10 levels in supernatants of WT BMDMs were measured by ELISA ($n = 2$). **(D)** Actinomycin D (5 μ g/ml) was added to WT BMDMs 2 h after LPS stimulation, and RNA was harvested every 15 min for the ensuing 2 h. *Il12b* mRNA and pre-mRNA were measured by RT-PCR and displayed as the log₂ fraction remaining compared with the time of actinomycin D addition ($n = 2$). **(E)** *Il12b* pre-mRNA was measured by RT-PCR in RNA harvested from WT and *Il10*^{-/-} BMDMs and is displayed relative to the unstimulated levels ($n = 2$). All experiments were performed twice.



Cluster 1 was strongly inhibited by continuous IL-10 but not by acute IL-10. Transcription of genes in Cluster 2 was strongly inhibited by continuous and acute IL-10. Transcription of genes in

Cluster 3 was induced by IL-10. Excluding genes in Cluster 3, these data indicate that continuous IL-10 inhibits the transcription of the vast majority of LPS-induced genes, whereas acute IL-10 inhibits the transcription of a subset of these genes.

Cluster 1 is enriched for IFN-responsive secondary response genes

We demonstrated markedly different kinetics for the transcriptional response to IL-10 in genes assigned to Clusters 1 and 2. We hypothesized that differential responses could indicate alternative modes of transcriptional regulation. To examine this hypothesis, we scanned the promoters (500 bp upstream of the transcription start site) of all 138 LPS-induced genes for the presence of known transcription factor-binding motifs. Cluster 1 genes were significantly enriched for IRF/ISRE consensus elements, and scanning of genes in Cluster 2 identified strong enrichment for NF- κ B binding without the enrichment for IFN-responsive elements observed in Cluster 1 (Fig. 4).

The observation that promoters for Cluster 1 genes were significantly enriched for IRF/ISRE consensus elements suggested that this cluster could represent IFN- β -induced secondary response genes. To examine this possibility further, we compared the proportion of genes in each cluster whose response to LPS required the presence of *Ifnar1*, based on data presented in Tong et al. (4). We found that Cluster 1 was significantly enriched for genes that required the presence of *Ifnar1* compared with Cluster 2 (class 1 genes [22/86], Cluster 2 genes [0/42], $p < 10^{-4}$, Fig. 3D).

Previous results demonstrate that expression of *Ifnb1* is induced early in the response to LPS, whereas *Ifnb1*-responsive genes exhibit delayed kinetics (5). Therefore, a potential explanation for the failure of acute, but not continuous, IL-10 to inhibit genes in Cluster 1 is that rather than directly inhibiting Cluster 1 genes, IL-10 is required earlier in the time course to inhibit expression of *Ifnb1* itself. To examine this possibility, we evaluated expression of *Ifnb1* and the IFN-induced secondary response gene *Mx1* in LPS-stimulated IL-10-deficient BMDMs. *Ifnb1* mRNA expression

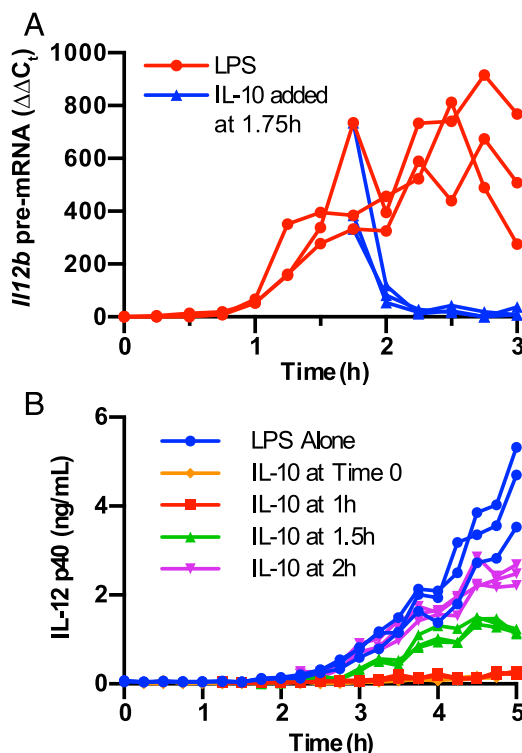


FIGURE 2. Exogenous IL-10 rapidly inhibits *Il12b* transcription. **(A)** *Il10*^{-/-} BMDMs were stimulated with LPS and then treated with IL-10 (1 ng/ml) at 1.75 h or left untreated. RNA was collected every 15 min, and *Il12b* pre-mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. **(B)** *Il10*^{-/-} BMDMs were stimulated with LPS, with or without the addition of IL-10 at the indicated time points. IL-12 p40 levels in the supernatants were measured by ELISA every 15 min ($n = 3$). This experiment was performed once.

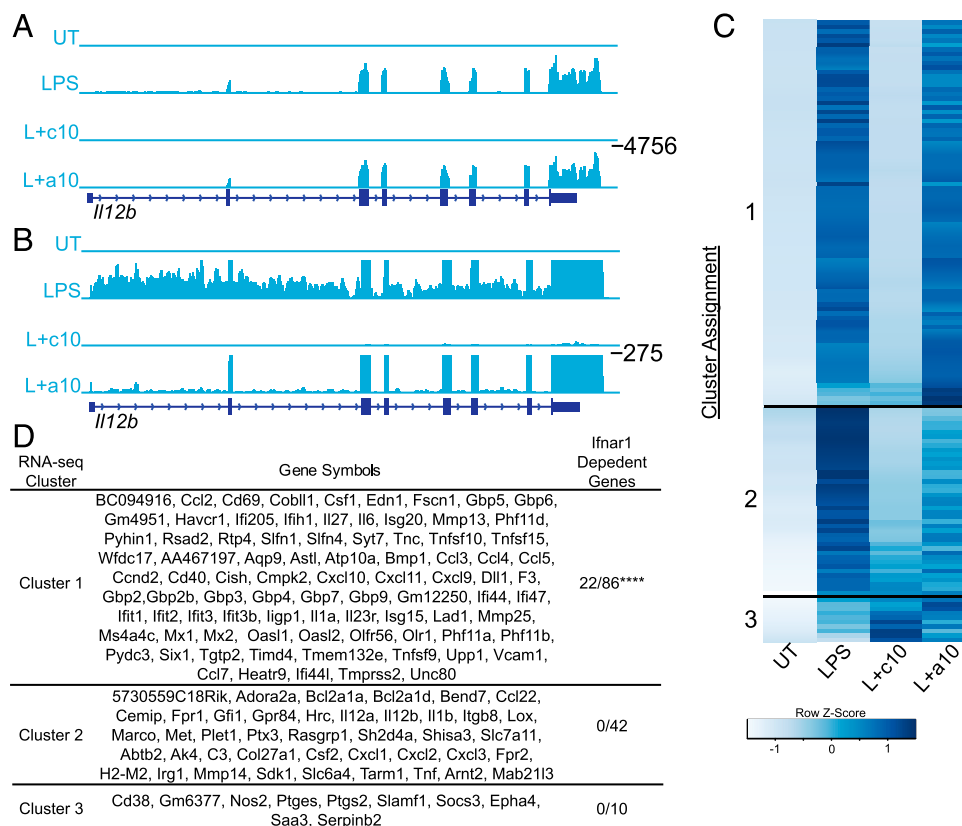


FIGURE 3. Global modulation of LPS-induced gene expression by IL-10. *Il10*^{-/-} BMDMs were left untreated (UT), were stimulated with LPS for 3.25 h (LPS), were stimulated with LPS and IL-10 together for 3.25 h (L+c10), or were stimulated with LPS for 3.25 h with the addition of IL-10 for the last 30 min of the stimulation (L+a10). Total RNA was harvested and sequenced following ribosomal depletion. Normalized distribution of RNA-seq reads is shown at the *Il12b* locus (**A**) and with an expanded y-axis to better demonstrate pre-mRNA (**B**). (**C**) Heat map showing k-means clustering of pre-mRNA signal for the 138 genes induced 100-fold by LPS in *Il10*^{-/-} BMDMs. (**D**) Individual genes that make up each cluster and the number of genes in each cluster deemed *Ifnar1*-dependent by Tong et al. (4). *****p* < 0.0001, Fisher exact test.

peaked 90 min after stimulation before rapidly declining (Fig. 5A). This peak in *Ifnb1* mRNA expression was followed by induction of *Mx1* pre-mRNA (Fig. 5B). Addition of IL-10 at the time of LPS stimulation significantly inhibited expression of *Ifnb1* mRNA and *Mx1* pre-mRNA (Fig. 5A, 5B), as well as secretion of IFN- β into the culture medium (Fig. 5C). In contrast, addition of IL-10 2 h after LPS stimulation (and after the peak of *Ifnb1* mRNA expression) had little effect on transcription of *Mx1* or on the amount of secreted IFN- β (Fig. 5B, 5C). These results strongly suggest that *Ifnb1* itself, rather than an *Ifnb1*-responsive genes, is the primary target for IL-10. Because addition of IL-10 following the peak of *Ifnb1* mRNA expression does not reduce the amount of IFN- β secreted into the culture media, these results explain why addition of IL-10 2 h and 45 min after LPS stimulation (acute IL-10) fails to inhibit transcription of many of the genes in Cluster 1. This is consistent with previous results demonstrating that IL-10 inhibits LPS-induced antiviral activity, presumably IFN- β , but is unable to inhibit gene expression induced in direct response to stimulation with IFN- β (31).

IL-10 rapidly inhibits LPS-induced enhancer activation of genes in Cluster 2

It was shown that LPS induces H3K27 acetylation of a group of poised enhancers marked by H3K4me1 following stimulation of BMDMs, but it is not known whether IL-10 inhibits LPS-induced transcription by interfering with LPS-induced enhancer acetylation (8). Further, because we showed above that IL-10 rapidly suppresses LPS-induced transcription of genes in Cluster 2, we

were interested in determining whether rapid inhibition of transcription was associated with rapid suppression of enhancer acetylation. To study the effect of IL-10 on LPS-induced enhancer acetylation, we performed ChIP-seq for H3K27Ac in IL-10-deficient macrophages. We found that treatment with LPS for 3.25 h significantly induced 8417 acetylation peaks (FDR < 0.1). To identify acetylation peaks in proximity to LPS-induced genes, we filtered on LPS-induced H3K27Ac peaks that were located within 50 kb upstream of the transcriptional start site, as well as within 50 kb downstream of the transcription termination site of Cluster 1 and 2 genes. We found that many of these peaks overlapped with sites of H3K4me1 defined by Ostuni et al. (8), indicating that these peaks likely represented bona fide enhancers (data not shown). We found that 49 of 86 genes in Cluster 1 were associated with at least one LPS-induced acetylation peak, and 34 of 42 genes in Cluster 2 were associated with at least one LPS-induced peak. Next, we compared the magnitude of these H3K27Ac peaks in BMDMs stimulated with LPS alone with those stimulated with LPS and continuous IL-10 or with LPS and acute IL-10. Treatment with continuous IL-10 markedly reduced H3K27Ac at peaks associated with genes from Clusters 1 and 2 (Fig. 6A). However, following treatment with acute IL-10, H3K27Ac was significantly lower at peaks associated with genes from Cluster 2 than at peaks associated with genes from Cluster 1 (*p* < 0.01, Mann-Whitney *U* test, Fig. 6A). Examples of Cluster 1 and Cluster 2 enhancers are shown in Fig. 6B and 6C, respectively. Interestingly, this analysis identified a peak inhibited by acute IL-10 that encompasses a DNase hypersensitivity site 10 kb upstream of *Il12b* that was

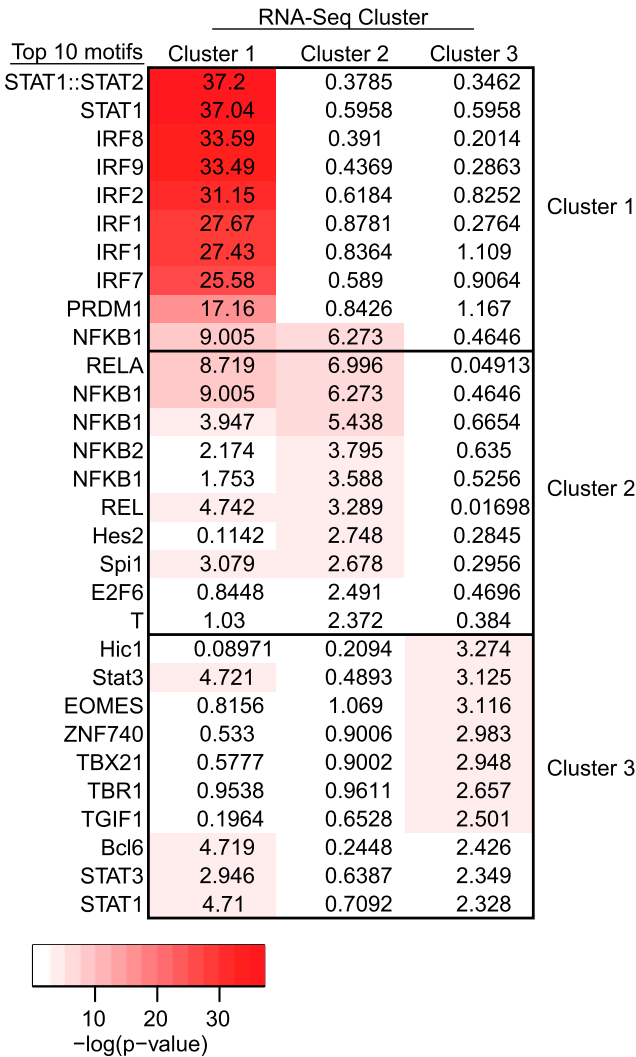


FIGURE 4. Transcription factor binding motifs in LPS-induced promoters. PSCAN analysis of promoters for LPS-induced genes clustered as in Fig. 3C. The top 10 motifs for each cluster are shown on the left. Columns represent enrichment p values for each motif organized by cluster (top). The color intensity is proportional to the negative log of the p value.

previously demonstrated in a reporter assay to exhibit enhancer activity (Fig. 6C) (22). These data indicate that IL-10 can rapidly suppress enhancers associated with genes in Cluster 2, suggesting that the ability of IL-10 to rapidly suppress transcription of genes in Cluster 2 may be based on suppression of enhancer function.

IL-10-mediated inhibition of Cluster 2 genes is not associated with direct STAT3 binding

STAT3 is required for IL-10-mediated inhibition of LPS-induced inflammatory gene expression (11). However, the function of STAT3 in the inhibitory process is not clear. STAT3 is a transcriptional activator, and binding sites for STAT3 in the proximity of genes inhibited by IL-10 have not been identified (13). This led to the hypothesis that STAT3-dependent induction of inhibitory factors mediates the inhibitory function of IL-10. However, the rapidity with which IL-10 is able to inhibit genes in Cluster 2 argues that dependence on new protein synthesis is unlikely, although certainly not impossible. Therefore, we sought to consider alternative mechanisms that might explain rapid STAT3-mediated inhibition. One possibility is that STAT3 binding directly suppresses activation of enhancers associated with genes in Cluster 2. To address this possibility, we performed ChIP-seq with an anti-

STAT3 Ab to identify STAT3 binding sites. After treatment of IL-10-deficient BMDMs with IL-10 alone for 30 min, we identified 31 STAT3-binding peaks within 50 kb upstream of the top 138 LPS-induced genes; strikingly, we found an additional 54 peaks within these regions when BMDMs were stimulated with LPS prior to the addition of IL-10. This indicates that prior LPS treatment reveals IL-10-induced STAT3 binding sites that were not present in unstimulated cells. Interestingly, virtually all identified STAT3 binding sites were located within H3K4me1 peaks, suggesting that STAT3 binds to enhancers. As anticipated, we found strong STAT3 binding near Cluster 3 genes induced by IL-10, such as *Socs3* (Fig. 7, lower left panel), and IL-10 induced an increase in average mean acetylation at STAT3 binding sites associated with genes in Cluster 3, consistent with enhancer activation (Fig. 7, lower right panel). Interestingly, prior LPS treatment

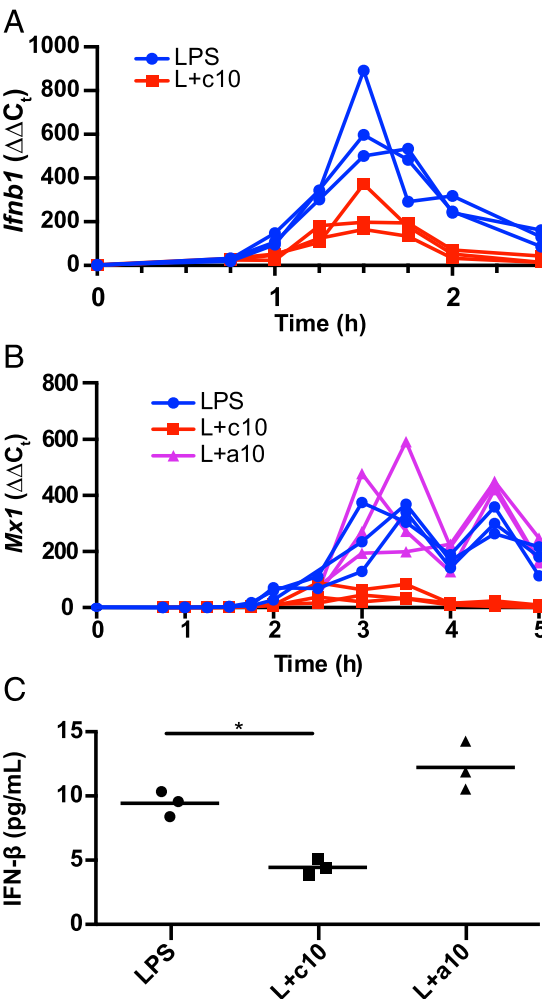
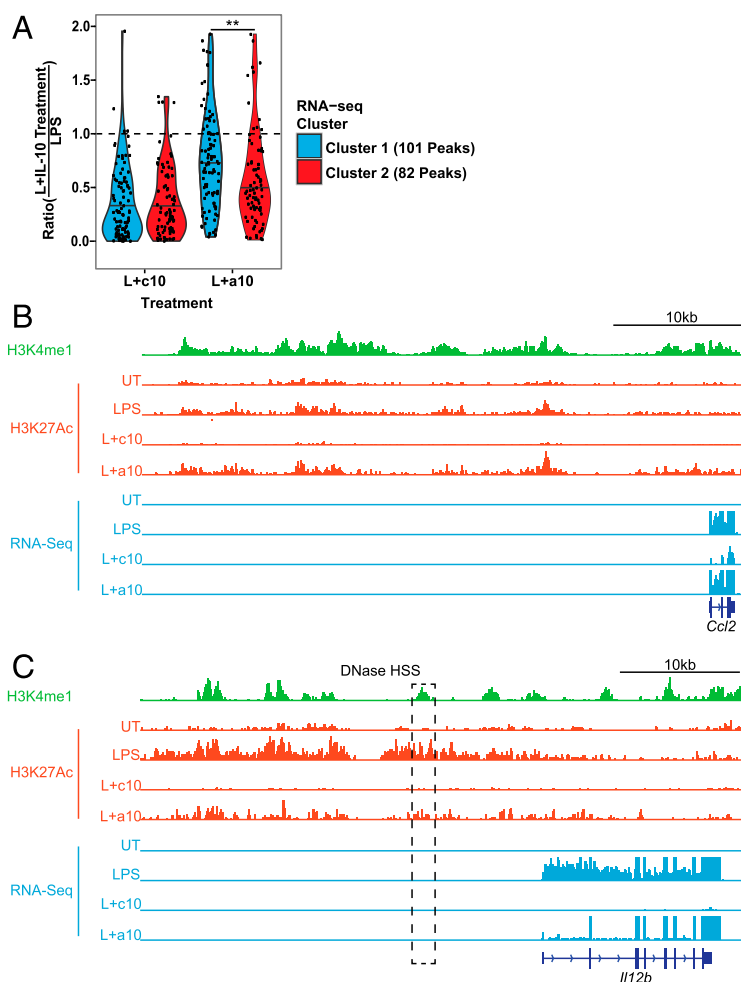


FIGURE 5. IL-10 inhibits IFN- β production. **(A)** *Il10*^{-/-} BMDMs were stimulated with LPS or with LPS and IL-10 (L+c10). RNA was harvested at the indicated time points, and *Ifnb1* mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. **(B)** *Il10*^{-/-} BMDMs were stimulated with LPS, stimulated with LPS and IL-10 (L+c10), or stimulated with LPS with the addition of IL-10 2 h after LPS stimulation (L+a10). RNA was harvested at the time points indicated, and *Mx1* mRNA was measured by RT-PCR ($n = 3$). This experiment was performed three times. **(C)** *Il10*^{-/-} BMDMs were stimulated with LPS for 4 h (LPS), stimulated with LPS and IL-10 for 4 h (L+c10), or stimulated with LPS for 4 h with addition of IL-10 for the last 2 h (L+a10). Culture supernatants were harvested and analyzed for IFN- β protein secretion by ELISA. This experiment was performed once. * $p < 0.05$, ANOVA, with Dunnett multiple-comparisons test ($n = 3$).

FIGURE 6. IL-10 causes rapid changes in enhancer H3K27Ac status. *Il10*^{-/-} BMDMs were left untreated (UT) or were stimulated with LPS for 3.25 h (LPS), stimulated with LPS and IL-10 for 3.25 h (L+c10), or stimulated with LPS for 3.25 h with addition of IL-10 for the last 30 min of the stimulation (L+a10). Chromatin was harvested, and ChIP-seq was performed with an Ab specific for H3K27Ac. **(A)** The H3K27Ac signal intensity was determined for each LPS-induced acetylation peak within 50 kb upstream or downstream of Cluster 1 and Cluster 2 genes, and a ratio between the signal with LPS alone and with continuous or acute IL-10 treatment was calculated for each peak. These ratios are represented on a violin plot where each dot represents an individual enhancer, and the width of the contours represents a smoothed density of these values. The horizontal line within each violin plot indicates the median value. Representative locus shown for Cluster 1 gene *ccl2* **(B)** and Cluster 2 gene *Il12b* **(C)**. H3K4me1 ChIP-seq data from Ostuni et al. (8) normalized to reads per million (green). H3K27Ac ChIP-seq data from macrophages treated as in (A) normalized to the depth of the least-sequenced library (red). Normalized RNA-seq data from macrophages treated as in Fig. 3C (blue). The boxed region in (C) represents the *Il12b* -10 kb enhancer described by Zhou et al. (22). ***p* < 0.01, Mann-Whitney *U* test.



revealed STAT3 binding sites near Cluster 1 genes that were not present with IL-10 treatment alone, and these sites also demonstrated an increase in mean acetylation in response to IL-10, although these peaks had a much weaker STAT3 signal relative to Cluster 3 gene peaks (Fig. 7). Lastly, IL-10 had little effect on mean acetylation at STAT3 binding peaks located within the 50 kb upstream of genes in Cluster 2, and STAT3 binding sites were not identified in the 50 kb upstream of *Il12b* (Fig. 7). Thus, there is little evidence that STAT3 binding directly suppresses activation of LPS-induced enhancers. These results raise the possibility that IL-10-induced STAT3 may have an inhibitory function that does not depend on direct DNA binding.

Cluster 2 gene *Cxcl2* is inhibited during the secondary response phase to LPS

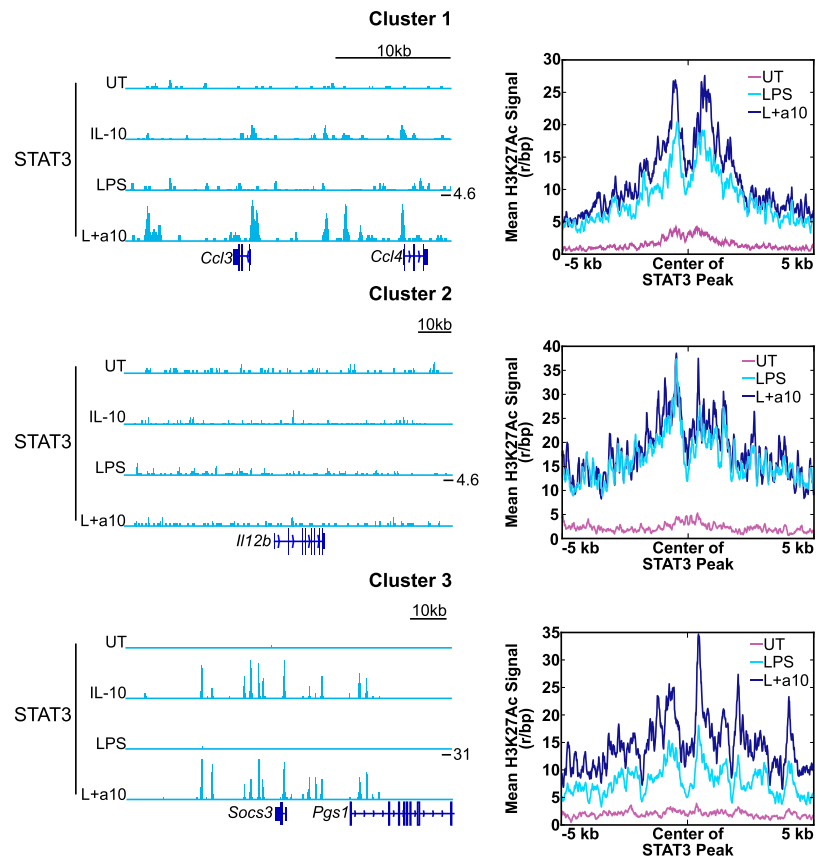
Inspection of genes in Cluster 2 revealed many, including *Il12b*, that were previously characterized as secondary response genes in LPS-stimulated BMDMs. However, there were also many genes that have been characterized as primary response genes (*Cxcl1*, *Cxcl2*) (Fig. 3D) (4). Because we had only examined the transcriptional effects of IL-10 at relatively late time points (105 and 165 min following LPS stimulation), we wondered whether the ability of IL-10 to rapidly inhibit transcription was operational at an early time point following LPS stimulation. To evaluate this issue, we compared induction of *Cxcl2* transcription in IL-10-deficient macrophages stimulated with LPS alone, or LPS and IL-10, or stimulated with IL-10 for 1 h prior to stimulation with LPS. As predicted, LPS rapidly induced *Cxcl2* pre-mRNA within 1 h of stimulation. Surprisingly, addition of IL-10 at

the time of LPS stimulation or addition 1 h prior to LPS stimulation had little influence on *Cxcl2* pre-mRNA at 1 h post-LPS stimulation, although significant suppression was observed at 2 h post-LPS stimulation (Fig. 8A). This difference was not caused by an inability of macrophages to respond to IL-10 prior to LPS stimulation because similar induction of STAT3 Y705 phosphorylation was observed when cells were treated with IL-10 in parallel with LPS or at later time points (Fig. 8B). These results strongly suggest that IL-10 interferes with a process that is unique to the secondary phase of the response to LPS.

Discussion

We investigated the kinetics of IL-10-mediated inhibition of LPS-induced gene expression. We found that IL-10 rapidly inhibits LPS-induced transcription of *Il12b* in WT cells and that addition of IL-10 to IL-10-deficient macrophages leads to the rapid termination of transcription. Using a novel approach to evaluate global changes in transcription of LPS-induced genes, we found that, although administration of IL-10 at the time of LPS stimulation of IL-10-deficient BMDMs had broad inhibitory effects, only a subset of these genes (Cluster 2) was rapidly inhibited when IL-10 was given 2.75 h after LPS stimulation. Rapid inhibition of transcription of genes in Cluster 2 was accompanied by rapid inactivation of putative *cis*-acting enhancer-like elements, suggesting that IL-10 was acutely influencing enhancer function. Taken together, these results indicate that rapid inhibition of enhancer function may be a key mechanism of IL-10-mediated inhibition.

FIGURE 7. STAT3 binding is associated with gene induction. Left, *Il10*^{-/-} BMDM were left untreated (UT) or were stimulated with IL-10 for 30 min (IL-10), stimulated with LPS for 3.25 h (LPS), or stimulated with LPS for 3.25 h with addition of IL-10 for the last 30 min of the stimulation (LPS+a10). Chromatin was harvested, and ChIP-seq was performed for STAT3. STAT3 ChIP-seq signal in reads per million shown for representative locus for Cluster 1 genes *Ccl3* and *Ccl4* (top panel), Cluster 2 gene *Il12b* (middle panel), and a Cluster 3 gene *Socs3* (bottom panel). Y-axis maximum for each locus marked in L+a10 track. Right, Mean H3K27Ac ChIP-Seq signal intensities from *Il10*^{-/-} BMDMs treated as in Fig. 6 for STAT3 peak centers identified within 50 kb upstream of genes in each cluster. Signal is normalized by library size to reads per base pair.



It was demonstrated previously that there is a delay in *Il12b* expression following LPS stimulation of WT BMDMs (21), but we were quite surprised to find that the period of active *Il12b* transcription was extremely short due to rapid inhibition by endogenously produced IL-10. Despite the short period of active transcription, *Il12b* mRNA remained present for hours after transcription had terminated, and small differences in the timing of addition of exogenous IL-10 led to large differences in the amount of IL-12 p40 protein measured in the supernatant 24 h after stimulation. Because we expect that similar kinetics will be observed in human cells, these results emphasize that relatively small variations in the timing of induction of *Il12b* transcription or production of IL-10 among individuals could lead to quite large differences in overall levels of IL-12 p40 secretion and biological function. Whether this is an important factor controlling differences in immune and inflammatory responses among individuals remains to be determined.

Although global induction of LPS-induced genes has been widely evaluated (2, 4, 11), the results presented in this article using exon-intron split analysis of total RNA-seq data provide a unique perspective on the kinetics of transcription termination that cannot be fully appreciated from evaluation of mRNA levels alone. This simple method to estimate transcription rates relies on sequencing of ribosome-depleted total RNA and does not require purification of chromatin, as is required for nascent transcript RNA-seq, or IP of chromatin that is required for PolII ChIP-seq. Further, this method simultaneously provides quantification of mRNA and relative rates of active transcription. Application of this technique to other systems where strict temporal regulation of gene expression is critical could reveal dynamic regulation that has not been observed previously.

Use of the exon-intron split analysis allowed us to determine that, although transcription of virtually all LPS-induced genes is inhibited when IL-10 is added at the time of LPS stimulation,

transcription of only a subset is rapidly inhibited when IL-10 is administered to IL-10-deficient macrophages 2 h and 45 min following LPS stimulation (Cluster 2). Although we do not yet understand the biochemical basis for IL-10-mediated inhibition, we demonstrated the rapid loss of H3K27 acetylation at LPS-induced enhancer elements associated with Cluster 2 genes following IL-10 treatment. This suggests that the mechanism of IL-10-mediated inhibition involves rapid modulation of LPS-induced enhancer function. It was shown previously that IL-10 can reduce total histone 4 acetylation at the *Il12b* promoter and potentially enhance the function of HDACs, but the exact nature of IL-10-mediated alterations in chromatin acetylation have not been described at the genome-wide level (21). Interestingly, our observation that IL-10 reverses LPS-induced H3K27 acetylation at many putative enhancer elements raises the possibility that previous genome-wide evaluations of LPS-induced enhancer elements may have significantly underestimated their number, because these studies were performed on WT macrophages at time points where one would predict the presence of significant amounts of IL-10 in the culture supernatant (8). Although we believe that rapid modulation of enhancer function by IL-10 is a key pathway mediating suppression of genes in Cluster 2, an important limitation to these conclusions is the variability noted in the response of genes in this cluster to IL-10. This variability may indicate the presence of alternative mechanisms of suppression for selected genes in this cluster.

Although evidence from the exon split analysis suggests rapid inhibitory function for IL-10, we were quite surprised that addition of IL-10 at the time of LPS stimulation did not inhibit the initial transcription of an early response gene, *Cxcl2*, despite clear evidence for suppression at later time points. Although we initially considered the possibility that this was evidence for a delayed effect of IL-10, we do not believe that this is the case, because

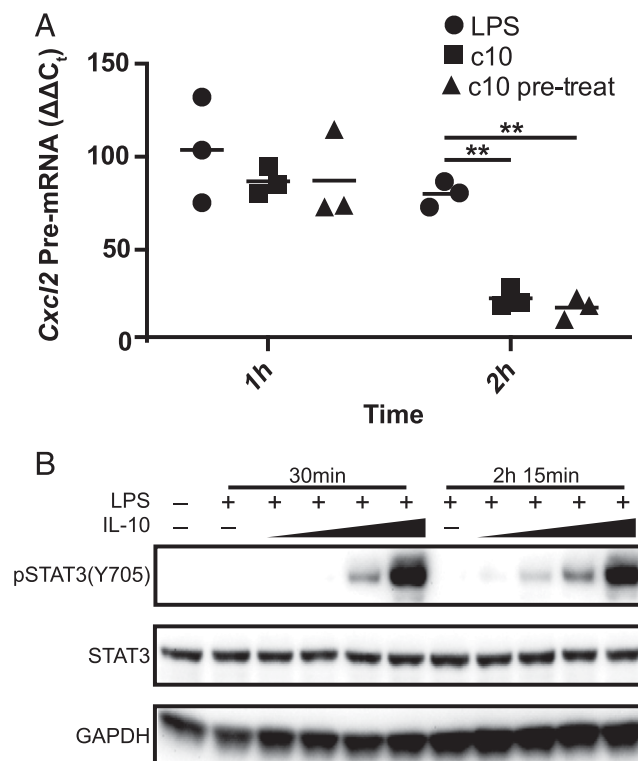


FIGURE 8. The ability of IL-10 to inhibit *Cxcl2* transcription exhibits a delay following LPS stimulation. **(A)** *Il10*^{-/-} BMDMs were stimulated with LPS alone, treated with IL-10 concurrently with LPS stimulation, or pretreated with IL-10 for 1 h prior to LPS stimulation. RNA was harvested at 1 and 2 h after LPS stimulation. *Cxcl2* pre-mRNA levels were measured by RT-PCR and are displayed relative to levels in unstimulated cells. This experiment was performed three times. **(B)** *Il10*^{-/-} BMDMs were stimulated with LPS for 30 min and 2.25 h, with or without addition of IL-10 in increasing doses of 0.0625, 0.25, 1, and 4 ng/ml for the last 30 min of the experiment. Cell extracts were prepared and assayed for pSTAT3 (Y705) or total STAT3 by Western blot. This experiment was performed twice. ***p* < 0.01, two-way ANOVA with Tukey multiple-comparisons test (*n* = 3).

administration of IL-10 1 h prior to LPS stimulation did not reduce the time to the point where inhibition was first observed. This delay was not based on a requirement for LPS to induce components of the IL-10R apparatus, because exogenous IL-10 activated STAT3, even in the absence of prior LPS stimulation. Thus, we prefer the possibility that IL-10 is unable to inhibit the first wave of LPS-induced gene expression but rather functions, at least in part, by inhibiting an LPS-induced positive regulator that is required for the induction of a subset of delayed-response genes and the sustained expression of immediate-response genes. One potential candidate for this positive regulator is IκBζ, which is induced in response to LPS and is required for LPS-induced expression of *Il12b* (7). Interestingly, it was reported that IκBζ interacts with activated STAT3 (32), but it remains to be determined whether this interaction is necessary for IL-10-mediated suppression.

It is tempting to speculate that genes inhibited by continuous, but not acute, IL-10 (Cluster 1) are inhibited more slowly than genes in Cluster 2; however, we believe that our data support a model in which a substantial proportion of genes in Cluster 1 are in fact responding to secreted IFN-β, because Cluster 1 is strongly enriched for LPS-induced genes that require the presence of the type-1 IFN-β (4). Although we confirmed that IFN-β is inhibited by IL-10, peak expression of IFN-β occurs at 90 min, thus explaining why IL-10 administration at the time of stimulation,

but not after 2 h and 45 min, inhibits Cluster 1 genes. Interestingly, previous work suggested that LPS-induced IFN-β expression may have a role in the regulation of IL-10 itself (33). However, it is important to note that IFN-β priming of IL-10 expression cannot explain the results obtained in these experiments, because the BMDMs used in these experiments lack endogenous IL-10. An important caveat to our conclusions is that select secondary response genes within Cluster 1 are independent of IFN-β; therefore, inhibition of *Ifnb1* expression is unlikely responsible for IL-10-mediated inhibition of all genes within Cluster 1. However, because several of the IFN-β-independent secondary response genes identified in Cluster 1, including IL-6 and IL-27, are TRIF dependent, it may be possible that a common IL-10-mediated pathway that inhibits TRIF function plays an important role in inhibiting genes in this cluster (4). Identifying the IL-10-responsive mediators of these effects could have important implications for understanding the overall regulation of the response to LPS.

It is somewhat paradoxical that genes in Cluster 1 only exhibit responsiveness to IL-10 suppression at times points prior to robust secretion of endogenous IL-10 by LPS-stimulated macrophages. However, there are multiple sources of IL-10 in vivo, including regulatory T cells, and it has been demonstrated that non-macrophage sources of IL-10 are essential to prevent inflammatory diseases, such as inflammatory bowel disease (34). Although it is difficult to completely define when a macrophage is first exposed to IL-10 in vivo, we believe that it is plausible that macrophages at sites of pathogen interface, such as the intestine, are exposed to IL-10 at varying times relative to the receipt of an inflammatory stimuli. The critical window of IL-10 responsiveness for genes in Cluster 1, revealed by our study, could have a central role in defining how macrophages respond based on temporal variability in receipt of the IL-10 signal.

What then have we learned regarding the mechanism of IL-10-mediated inhibition? Results from this study suggest that inhibition is based on the rapid suppression of active transcription and deacetylation of LPS-induced enhancer-like elements. This mechanism extends to most LPS-induced genes but is not operational without prior LPS stimulation. The rapid nature of IL-10-mediated suppression would seem to indicate that STAT3-driven induction of a secondary transcriptional inhibitor is unlikely (although certainly not impossible), given that inhibition of active transcription can be observed within 15 min of IL-10 treatment. Although experiments using the protein synthesis inhibitor cycloheximide suggested that IL-10-mediated inhibition requires new protein synthesis, in our hands cycloheximide treatment rapidly inhibits ongoing transcription of *Il12b* (data not shown), making it quite difficult to discern any additional inhibitory effects of IL-10. Thus, we believe that the question of whether IL-10-mediated inhibition requires new protein synthesis remains open. Alternative possibilities to explain IL-10-mediated inhibition that could potentially function on the more rapid time scale observed in this study include the induction of an inhibitory RNA species or a direct inhibitory function for STAT3. However, STAT3 ChIP-seq failed to uncover IL-10-induced STAT3 binding sites associated with suppression of LPS-induced enhancer activation. Thus, further directed experiments geared toward understanding IL-10-mediated signaling pathways that rapidly inhibit transcription are required. Delineating these mechanisms could have important implications for understanding the basis for inflammatory disease, as well as the development of novel therapeutics.

Disclosures

The authors have no financial conflicts of interest.

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