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Evidence for Postinitiation Regulation of mRNA Biogenesis in Tuberculosis

Hugh Salamon,* Yaming Qiao,† Jeff C. Cheng,‡,1 Ken D. Yamaguchi,* Patricia Soteropoulos,† Michael Weiden,§ Maria Laura Gennaro,†,* and Richard Pine†,*

*Mycobacterium tuberculosis* infection alters macrophage gene expression and macrophage response to IFN-γ, a critical host defense cytokine. However, regulation of these changes is poorly understood. We report discordance of changes in nascent transcript and total nuclear RNA abundance for the transcription factors STAT1 and IRF1, together with lack of effect on their RNA half-lives, in human THP-1 cells infected with *M. tuberculosis* and stimulated with IFN-γ. The results indicate that negative postinitiation regulation of mRNA biogenesis limits the expression of these factors, which mediate host defense against *M. tuberculosis* through the cellular response to IFN-γ. Consistent with the results for STAT1 and IRF1, transcriptome analysis reveals downregulation of postinitiation mRNA biogenesis processes and pathways by infection, with and without IFN-γ stimulation. Clinical relevance for regulation of postinitiation mRNA biogenesis is demonstrated by studies of donor samples showing that postinitiation mRNA biogenesis pathways are repressed in latent tuberculosis infection compared with cured disease and in active tuberculosis compared with ongoing treatment or with latent tuberculosis. For active disease and latent infection donors from two populations (London, U.K., and The Gambia), each analyzed using a different platform, pathway-related gene expression differences were highly correlated, demonstrating substantial specificity in the effect. Collectively, the molecular and bioinformatic analyses point toward downregulation of postinitiation mRNA biogenesis pathways as a means by which *M. tuberculosis* infection limits expression of immunologically essential transcription factors. Thus, negative regulation of postinitiation mRNA biogenesis can constrain the macrophage response to infection and overall host defense against tuberculosis. *The Journal of Immunology*, 2013, 190: 000–000.

Morbidity and mortality from tuberculosis are extremely high, with ~8 million new cases of active disease per year and ~2 million deaths (1); in the absence of effective treatment, mortality is ~50% (2). Increased failure of anti-tuberculous chemotherapy (3, 4) with the emergence of multidrug and extensively drug-resistant strains of *Mycobacterium tuberculosis* has added urgency to the goal of developing effective vaccines and immunotherapies. Macrophages are the immune cells predominantly targeted by *M. tuberculosis*. Bacterial replication occurs in macrophages at two points in the immunologic life cycle of tuberculosis: during the innate immune response to infection (before adaptive immunity forces a transition to latent infection) and during reactivation (when adaptive immunity fails to maintain latent infection; reviewed in 5). Determining effects of *M. tuberculosis* infection on host macrophage gene expression and relating those effects to differences in gene expression between individuals who maintain latent tuberculosis infection (LTBI) and those who develop pulmonary tuberculosis (PTB) should facilitate efforts to apply host immune pressure against tuberculosis.

The interaction between macrophages infected with *M. tuberculosis* and the host immune mediator IFN-γ is a major determinant of host response to *M. tuberculosis* (6). The host transcription factors STAT1 and IRF1 are essential mediators of the response to IFN-γ and of host defense against *M. tuberculosis* (7–10). In humans, mutations in STAT1 confer susceptibility to normally nonpathogenic mycobacterial infections (11, 12); in mice, a deficiency of STAT1 or IRF1 abolishes immune control of *M. tuberculosis* growth, which leads to a fatal fulminant infection rather than a chronic illness with slow disease progression (13, 14). The consequences of deficiencies in these transcription factors emphasize that their regulation is essential for an effective host response to *M. tuberculosis*. Moreover, both are induced by *M. tuberculosis* infection and by IFN-γ stimulation (15–21). IFN-γ induction of STAT1 and IRF1 and *M. tuberculosis* induction of IRF1 are attributable at least in part to increased transcription. However, little is known about whether mechanisms other than regulation of transcription initiation control their expression, or any other transcriptome changes, with or without IFN-γ stimulation in cells infected with *M. tuberculosis*.

Postinitiation steps in mRNA biogenesis (5′ end capping, elongation, splicing, 3′ end cleavage and polyadenylation) allow regulation of gene expression in addition to control of transcription initiation (22–25). Alternative splicing and polyadenylation can determine tissue-specific or signal-mediated levels of transcript isoform expression (reviewed in Refs. 26, 27). For example, in patients with chronic granulomatous disease, increased levels of...
functional NADPH (phagocyte) oxidase as a response to IFN-γ therapy result from mutations that alter CYBB gene exon usage (28, 29). In other examples, stimulation of TLRs by bacteria-derived ligands alters transcripts through effects on alternative splicing and polyadenylation (30–36). With M. tuberculosis infection, alternatively spliced transcripts of IL12Rb2 are produced (37). Even without alternative mRNA processing, changing the rate of a single processing event can control gene expression level, as demonstrated for glucocorticoid-mediated repression of gonadotropin-releasing hormone expression through inhibition of pre-mRNA splicing (38). Thus, postinitiation regulation of mRNA biogenesis might be an important host response to M. tuberculosis infection.

In this study, we characterized expression of genes responsive to M. tuberculosis infection and IFN-γ stimulation and analyzed transcriptome data to better understand the basis for their regulated expression. Data from in vitro infection of THP-1 cells indicated that negative postinitiation regulation of mRNA biogenesis, superimposed on IFN-γ–stimulated activation of transcription, limits increases in STAT1 and IRF1 gene expression. Analysis of transcriptome data demonstrated that downregulation of postinitiation mRNA biogenesis pathways occurs with in vitro infection and distinguishes individuals who develop PTB from those who maintain LTBI.

Materials and Methods

Cell growth and infections

All manipulations with viable M. tuberculosis were performed under biosafety level 3 containment. M. tuberculosis TN931, a prevalent, drug-sensitive clinical isolate of the C strain from the 1990–1994 New York City tuberculosis outbreak (39), was obtained from the Public Health Research Institute Tuberculosis Center. The human monocytic cell line THP-1 was obtained from American Type Culture Collection. The bacteria and cells were maintained, and THP-1 cells were differentiated and infected, as described previously (17). Differentiated THP-1 cells model human alveolar macrophages, as judged by a variety of criteria (17, 20, 40, 41). Three days after infection, infected cells and parallel cultures of uninfected cells were left untreated or were treated for 2 h with IFN-γ (Peprotech, Rocky Hill, NJ) at 1 ng/ml. In some experiments, actinomycin D (Calbiochem, La Jolla, CA) was then added to a concentration of 10 ng/ml, and cells were differentiated and infected, as described previously (17). Differentiated THP-1 cells model human alveolar macrophages, as judged by a variety of criteria (17, 20, 40, 41). Three days after infection, infected cells and parallel cultures of uninfected cells were left untreated or were treated for 2 h with IFN-γ (Peprotech, Rocky Hill, NJ) at 1 ng/ml. In some experiments, actinomycin D (Calbiochem, La Jolla, CA) was then added to a concentration of 10 ng/ml, and cells were harvested at various times thereafter to determine transcript half-life. The titer of the inoculum on the day of infection and the presence of intracellular bacteria on the day of harvest were confirmed by plating to determine CFUs.

Cell fractionation and recovery of RNA

All steps were performed at 0–4°C. Cells were scraped from flasks and collected by centrifugation at 400 × g for 5 min. To isolate total cellular RNA, cell pellets were extracted immediately. Alternatively, nuclear and cytoplasmic fractions were prepared as described previously (20). Cytoplasmic RNA was extracted, and the nuclei were suspended in nucleic run-on buffer containing 0.1% NP-40 and lacking nucleotide triphosphates. Nuclei then were used in the nuclear run-on assay (below) or were further purified by sedimentation through a cushion of 60% glycerol in the same buffer (42) before extraction of RNA. RNA was recovered using TRI-Reagent (Molecular Resource Center) as recommended by the vendor.

Nuclear run-on assay

Nascent transcript abundance was determined as described previously (21). Signal from hybridization to pGem1 provided a negative control for specificity and for background correction. Signal from hybridization to human GAPDH provided a positive control and an internal standard for normalization. Probes for GAPDH, IRF1, ISG15, and STAT1 were described previously (15, 16, 20, 43). All genes are denoted by Human Genome Organization official gene symbols. Data are from six independent experiments. Quantification of nascent transcript abundance is shown as the average of fold induction relative to expression in uninfected, unstimulated cells. Fold induction is used to allow comparison among genes and among experimental conditions. Error bars for each perturbation (M. tuberculosis infection, IFN-γ stimulation, or both) represent ± SEM. Statistical significance is taken as p < 0.05 based on a two-tailed Student t test.

Quantitative RT-PCR

Reverse transcription reactions, quantitative PCR reactions using molecular beacons for amplicon detection, and calculation of target abundance were performed as described previously for IRF1. The results were normalized to the level of GAPDH exon 9 for the respective samples (21). Mock reverse transcription samples for each gene were prepared to demonstrate genome DNA contamination. The specificity of assays for introns and exon junctions was confirmed with genomic DNA and cDNA templates. Data are from four to six independent experiments; not all genes were assayed in some experiments. RNA half-life was calculated as the average of values determined from exponential decay curves for individual experiments (n = 2–4). Error bars represent ± SEM for (n > 2) or the range of values (for n = 2). Statistical significance for data sets with n ≥ 3 was taken as p < 0.05 based on a two-tailed Student t test.

Transcriptome analysis

Gene expression profiles for THP-1 cells were determined for four replicate experiments that each included all four experimental conditions (control, M. tuberculosis infection, IFN-γ stimulation, infection and stimulation) using Affymetrix Human Genome U133A 2.0 Gene Chips, following the vendor’s protocols for cRNA preparation, labeling, hybridization, and scanning. Quantification of probe expression levels was based on the “preferred methods” of Choe et al. (44). This analysis included Microarray Suite 5 (Affymetrix) for background correction, global scale to 500 for probe-level normalization, perfect match minus mismatch hybridization specificity correction, robust multiaarray analysis’ median polish for gene expression summary calculation, GAPDH expression for gene expression normalization, and Loess normalization for intensity skew correction. The use of GAPDH as an internal standard for expression level normalization was empirically validated based on 60 different segments of the GAPDH gene. The signal varied among the probes, but was comparable under all conditions for each one (data not shown). These data are publicly available (Gene Expression Omnibus accession number GSE17477, http://www.ncbi.nlm.nih.gov/geo/).

For gene ontology (GO) annotation analysis of gene expression, we first identified regulated genes based on statistically significant differential expression in comparisons of gene expression between each perturbation and uninfected, unstimulated cells. A t test with a dynamic threshold (Cyber-T) (45) was used to identify differentially expressed genes (p < 0.05 or p < 0.01). A more stringent threshold for differential expression was set using a permutation-based implementation to calculate false discovery rate (p < 0.1). Fisher exact test was used to assess the statistical significance (p < 0.05) for the proportion of regulated genes having particular combinations of annotations taken from the GO database (http://www.geneontology.org/) (46) among downregulated genes in comparison with the overall proportion of genes having those annotations among those probed.

The transcriptomes of samples from clinical studies were obtained from Gene Expression Omnibus—GEO GSE19491 (47), GSE19439 (48), and GSE11199 (49). The first two studies included comparison of whole blood from LTBI and PTB donors. The third study included comparison of monocyte-derived macrophages from LTBI and cured PTB donors. The publishing authors’ normalized expression values were used.

For pathway analysis of data from in vitro infection and from the clinical studies, the significance of gene sets defined by Reactome pathway annotations (http://www.reactome.org/) (50, 51) was evaluated using the Coincident Extreme Ranks in Numerical Observations (CERNO) method (52). The CERNO test uses the rank order of significance for differential expression of the genes in a pathway to determine significance for the pathway annotation without a threshold on expression differences for individual genes. A nested CERNO testing routine was applied (53). Benjamini-Hochberg false discovery rates (54) were calculated for the entire collection of gene sets, but for each sample comparison separately. Eighteen postinitiation mRNA biogenesis Reactome gene sets were identified with at least three representative genes on the Affymetrix Human Genome U133A 2.0 and the Agilent-04850 Whole Human Genome Microarray. A one-sided Student t test was performed to test whether individual genes were downregulated. Statistical analysis methods not otherwise cited were performed in the R programming environment (55).

Results

Differential induction of nascent transcripts and total nuclear RNA for both STAT1 and IRF1

Comparing induction at different points in the course of gene expression will reveal the presence of regulated steps. To set a baseline for identifying postinitiation regulation, we first measured the levels of nascent transcripts for STAT1 and IRF1, two central regulators of
response to *M. tuberculosis* infection and to IFN-γ stimulation. *M. tuberculosis* infection and IFN-γ stimulation each induced expression of the two genes (Fig. 1A, 1B). When infected cells were also stimulated with IFN-γ, the increases in nascent transcripts for STAT1 and IRF1 were 2–3-fold greater than the sum of the responses to each perturbation alone; therefore, a synergistic response occurs. To test for specificity in the effects of *M. tuberculosis* infection on the response to IFN-γ, we measured transcription of ISG15 because it is induced by *M. tuberculosis*, but not by IFN-γ (15, 20). In contrast to STAT1 and IRF1, the level of ISG15 nascent transcripts was similar with and without IFN-γ stimulation of infected cells (Fig. 1A, 1B). These data demonstrate specific synergistic induction of STAT1 and IRF1 nascent transcripts with IFN-γ stimulation of *M. tuberculosis*-infected cells.

If regulation were only at the level of transcription initiation, the synergistic induction of nascent transcripts for STAT1 and IRF1 would lead to comparable increases in the nuclear and cytoplasmic pools of the corresponding mRNAs. This was the case with *M. tuberculosis* infection alone, because the levels of STAT1, IRF1, and ISG15 increased in total nuclear RNA as much as in nascent transcripts (compare Fig. 1B to 1C; the ratio is shown in Fig. 1D). IFN-γ–mediated induction of STAT1 in total nuclear RNA and in nascent transcripts was also similar. However, induction of IRF1 in total nuclear RNA was 2.5-fold greater than in nascent transcripts, indicating that induction of nascent transcripts and additional positive regulation determine the expression of IRF1 in response to IFN-γ stimulation. In sharp contrast, when cells were infected with *M. tuberculosis* and stimulated with IFN-γ, the ratio of the total nuclear RNA to nascent transcript for both STAT1 and IRF1 was one fourth the ratio in uninfected cells stimulated with IFN-γ (Fig. 1B–D). ISG15 showed less of an effect. These results suggest that expression of STAT1 and IRF1 is subject to gene-specific negative regulation that limits their induction by IFN-γ in infected cells. Comparing induction of polyA+ mRNA to nascent transcripts also revealed the limit on expression of STAT1 and IRF1 in *M. tuberculosis*–infected cells stimulated with IFN-γ (data not shown). The reduced induction of STAT1 and IRF1 associated with IFN-γ stimulation of infected cells was specific for *M. tuberculosis*, because it was not observed in cells infected with the nonpathogenic *Mycobacterium bovis* BCG (Supplemental Fig. 1). Thus, in cells infected with *M. tuberculosis*, regulation in addition to initiation of transcription controls IFN-γ–stimulated STAT1 and IRF1 expression.

**Postinitiation regulation of response to ** *M. tuberculosis* and IFN-γ

The differentials between induction in total nuclear RNA and in nascent transcripts for both STAT1 and IRF1 seen with IFN-γ stimulation of *M. tuberculosis*–infected cells could result from increased RNA turnover, negative postinitiation regulation of mRNA biogenesis, or both. We first addressed the turnover hypothesis by measuring the half-life of exon and intron sequences for STAT1 and IRF1 in total nuclear RNA. We found no significant difference in turnover under any condition (Fig. 2), suggesting that neither

![FIGURE 1. Effects of *M. tuberculosis* infection and IFN-γ stimulation on nascent and total nuclear RNA. THP-1 cells were differentiated and infected with *M. tuberculosis* or stimulated with IFN-γ, or both. Next, nascent transcripts were measured using the nuclear run-on assay, or total nuclear RNA was extracted and quantified by quantitative RT-PCR. (A) The hybridization results for nuclear run-on assays were imaged and quantified using a phosphorimager. The figure is a composite of contiguous portions from a single phosphorimager exposure that included the membranes for all four conditions in a representative experiment. (B) Nascent transcript measurement is shown as average fold induction ± SEM for six replicate experiments. (C) Total nuclear RNA abundance is shown as average fold induction ± SEM for four to six replicate experiments as for (B). (D) The ratios of the abundance of total nuclear RNA relative to the level of nascent transcripts were calculated from the averages for each. Error bars represent ± SEM. Statistically significant differences (*p < 0.05) are indicated compared with control (†), compared with *M. tuberculosis* (*), and compared with IFN-γ (▲).
degradation nor nuclear export were affected. We next determined that the induction of poly-A⁺ nuclear RNA was proportional to the induction of total nuclear RNA for both STAT1 and IRF1 (Supplemental Fig. 2), suggesting that cleavage and polyadenylation were not regulated for either gene. Moreover, the half-lives of the total cellular poly-A⁺ RNAs were comparable in uninfected and infected cells with and without IFN-γ stimulation for each of the two genes (Supplemental Fig. 3), indicating that the overall turnover of those transcripts is also not regulated in response to these perturbations. Because regulation of neither turnover nor final maturation explains differences between induction of nascent transcripts and total nuclear RNA, we interpret the data as indicating that STAT1 and IRF1 expression is limited at a postinitiation step in mRNA biogenesis (i.e., before a mature mRNA transcript is produced).

Control of transcript elongation is one mechanism for postinitiation regulation of mRNA biogenesis. Therefore, we considered whether the observed limited induction of STAT1 and IRF1 in M. tuberculosis–infected cells stimulated with IFN-γ was caused by downregulation of elongation. When we assayed introns throughout the STAT1 and in IRF1 transcripts, each target exhibited the induction caused by M. tuberculosis infection, IFN-γ stimulation, or both, that was shown by the exon measurements (compare Fig. 1C and Fig. 3, left panel). Little or no change occurred in the ratio of STAT1 intron 2 to intron 22 or in the ratio of IRF1 intron 1 to intron 9 under any condition (Fig. 3, right panel). Thus, regulated elongation contributes to neither positive postinitiation regulation of IRF1 by IFN-γ nor negative postinitiation regulation of STAT1 and IRF1 in cells infected with M. tuberculosis and stimulated with IFN-γ.

To assess the possibility that regulated splicing accounts for the observed differences between induction of nascent and total nuclear RNA for STAT1 and for IRF1, we measured the abundance of exon-exon junctions and of the respective introns for both transcripts. Because the ratio of spliced to unspliced transcripts reflects the rate of each splice, a change in gene expression owing to a regulated splice would alter the ratio. For STAT1, the ratios of the spliced exon 2:3 junction compared with intron 2 and of the spliced exon 22:23 junction compared with intron 22 were similar in uninfected, unstimulated cells, and they decreased similarly with M. tuberculosis infection, IFN-γ stimulation, and stimulation of infected cells (Fig. 4A, 4B). In contrast, analysis of IRF1 splices showed that the ratios between the spliced exon 1:2, 4:5, and 9:10

![FIGURE 3](http://www.jimmunol.org/) Effects of M. tuberculosis infection and IFN-γ stimulation on STAT1 and IRF1 transcript elongation. Introns sequences in total nuclear STAT1 and IRF1 transcripts were assayed. The average fold-induction of each target region is shown. Error bars represent ± SEM for each average and for the ratios. (A) STAT1 intron 2 and intron 22. (B) IRF1 intron 1 and intron 9. Statistically significant differences (p < 0.05) are indicated compared with control (†), compared with M. tuberculosis (‡), and compared with IFN-γ (§).

![FIGURE 4](http://www.jimmunol.org/) Effects of M. tuberculosis infection and IFN-γ stimulation on spliced and unspliced STAT1 and IRF1 transcripts. Exon junction and intron sequences in total nuclear STAT1 and IRF1 transcripts were assayed. The average fold-induction of each target region is shown. Error bars represent ± SEM for each average and for the ratios. (A) STAT1 exon 2-3 junction and intron 2. (B) STAT1 exon 22-23 junction and intron 22. (C) IRF1 exon 1-2 junction and intron 1. (D) IRF1 exon 4-5 junction and intron 4. (E) IRF1 exon 9-10 junction and intron 9. Statistically significant differences (p < 0.05) are indicated compared with control (†), compared with M. tuberculosis (‡), and compared with IFN-γ (§).
The finding that induction of STAT1 and IRF1 is limited by negative postinitiation regulation in *M. tuberculosis*–infected cells stimulated with IFN-γ suggests that the processes and pathways involved may be downregulated. To test this possibility, we analyzed gene expression profiles (Gene Expression Omnibus accession number GSE17477) for differentiated THP-1 cells that were uninfected or infected with *M. tuberculosis* with and without IFN-γ stimulation. We found that among all regulated genes the number induced was much greater than the number repressed under all conditions tested (Fig. 5A). The same result was obtained in IFN-γ–treated cells with genes annotated for mRNA processing in GO (46) (Fig. 5B). In contrast, more of those genes were repressed than induced in cells that were infected with *M. tuberculosis* with and without IFN-γ stimulation (Fig. 5B). Thus, the effect of *M. tuberculosis* infection was different for expression of genes annotated for mRNA processing than for the overall transcriptome. Moreover, mRNA processing genes were overrepresented among all the downregulated genes when various stringency criteria were used for defining regulated genes (see Materials and Methods). Among the 14 mRNA processing genes deemed regulated under the more stringent criteria, four were responsive to infection alone, and the other 10 were downregulated only in cells that were infected and stimulated with IFN-γ (Table I). Thus, the transcriptome analysis demonstrated a statistically significant downregulation of mRNA processing genes consistent with limited induction of STAT1 and IRF1 expression owing to postinitiation downregulation when cells infected with *M. tuberculosis* are stimulated with IFN-γ.

Analogous results were obtained when the transcriptome results were analyzed using a different annotation knowledge base focused on pathways (Reactome; http://www.reactome.org) (50, 51) and a different statistical approach (CERNO, see Materials and Methods) (52, 53) (Fig. 6 and Supplemental Fig. 4). Of 18 Reactome pathways pertaining to postinitiation mRNA biogenesis, 16 were significantly downregulated in infected cells (Fig. 6A and Supplemental Fig. 4A). Significant downregulation occurred for 17 of these pathways in cells that were infected and stimulated with IFN-γ compared with cells that were only IFN-γ stimulated (Fig. 6B and Supplemental Fig. 4B). The analyses of transcriptome databases based on annotations for process and for pathways, taken together with the results for expression of STAT1 and IRF1, identify negative postinitiation regulation of mRNA biogenesis as a factor in an essential immunologic response to IFN-γ stimulation of *M. tuberculosis*–infected cells.

### Table 1. mRNA processing genes downregulated by *M. tuberculosis* infection with or without IFN-γ stimulation

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Regulation</th>
<th>Biologic Process</th>
</tr>
</thead>
<tbody>
<tr>
<td>HNRPA0</td>
<td>M. tuberculosis = IFN-γ</td>
<td>Nuclear mRNA splicing, via spliceosome</td>
</tr>
<tr>
<td>LSM5</td>
<td>M. tuberculosis = IFN-γ</td>
<td>Spliceosome assembly</td>
</tr>
<tr>
<td>NUDT21</td>
<td>M. tuberculosis = IFN-γ</td>
<td>mRNA 3’ end formation</td>
</tr>
<tr>
<td>SFRS11</td>
<td>M. tuberculosis = IFN-γ</td>
<td>mRNA splicing</td>
</tr>
<tr>
<td>SNRNP25</td>
<td>M. tuberculosis + IFN-γ</td>
<td>mRNA splicing, mRNA 3’ end formation</td>
</tr>
<tr>
<td>CPSF1</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Spliceosome assembly</td>
</tr>
<tr>
<td>GEMIN4</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Spliceosome assembly</td>
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<tr>
<td>NHP2L1</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Spliceosome assembly</td>
</tr>
<tr>
<td>RBM5A</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Exon junction complex formation</td>
</tr>
<tr>
<td>SF1</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Pre-mRNA 3’ splice site recognition, spliceosome assembly</td>
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<td>SFRS14</td>
<td>M. tuberculosis + IFN-γ</td>
<td>mRNA splicing</td>
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<td>SNRPN</td>
<td>M. tuberculosis + IFN-γ</td>
<td>snRNP assembly</td>
</tr>
<tr>
<td>TARDBP</td>
<td>M. tuberculosis + IFN-γ</td>
<td>Alternative mRNA splicing</td>
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</tbody>
</table>

*Human Genome Organization Gene Nomenclature Committee official symbol.*

*Perturbations that caused downregulation: M. tuberculosis = IFN-γ (downregulated by infection with or without IFN-γ stimulation); M. tuberculosis + IFN-γ (downregulated only by infection and IFN-γ stimulation).*

*Biological process annotations from gene ontology.*
Pulmonary tuberculosis is associated with downregulation of postinitiation mRNA biogenesis pathways

To investigate the clinical relevance of the above results from in vitro infection of macrophage-like THP1 cells, we analyzed transcriptome data obtained from clinical samples. In two studies comparing whole blood from LTBI and PTB donors (47, 48), the analysis revealed that PTB was associated with significant downregulation of postinitiation mRNA biogenesis pathways (Supplemental Fig. 4C, 4D). Moreover, PTB donors also exhibited this downregulation in longitudinal comparisons with gene expression data from samples collected 2 and 12 mo after initiation of antituberculous treatment (Supplemental Fig. 4E, 4F). In a study comparing monocyte-derived macrophages from LTBI donors and cured PTB donors (49), significant downregulation for 10 of the 18 postinitiation mRNA biogenesis pathways was associated with LTBI (Supplemental Fig. 4G). Thus, the negative postinitiation regulation of mRNA biogenesis described above as an effect of in vitro infection is also identified as a difference associated with infection/disease status in vivo.

We next considered the relationship between the expression of individual genes and the disease-stage–specific effects on postinitiation mRNA biogenesis pathways. Although the data from the two studies of PTB and LTBI donors come from different patients in different geographic regions measured on different gene expression platforms (Affymetrix versus Agilent), differential expression of individual transcripts was significantly similar in the two studies (Fig. 7). The significance of the correlation provides evidence that PTB is associated with specific changes in the expression of particular genes that mediate postinitiation mRNA biogenesis. Moreover, querying for mRNA postinitiation biogenesis genes differentially expressed at a nominal p, 0.05 level revealed nine genes common across sample comparisons in four independent studies involving three distinct biologic systems (THP-1 cells infected in vitro, two studies of whole blood comparing LTBI and PTB donors, and a study comparing monocyte-derived macrophages from LTBI donors and cured PTB donors).

FIGURE 6. Postinitiation mRNA biogenesis Reactome pathways and genes are downregulated by M. tuberculosis infection of THP-1 cells. Eighteen pathways were tested. The pathways shown are significant for the indicated comparisons (A, B) based on a false discovery rate criterion of 0.05 for the CERNO test results. The pathway tests were based on the rank order of differential expression for all genes in a pathway (among all transcript measurements of all named genes targeted by the gene expression platform). The top matrix depicts the membership of genes (columns) in pathways (rows) with red squares. The rows and columns of the top matrix are sorted to bring together similar pathway membership patterns. Genes shown are members of one or more pathways that exhibited differential expression for the indicated comparison (one-sided Student t test to assess gene downregulation; p < 0.05 unless noted otherwise). The lower matrix is a heat map of expression for each of the significantly regulated genes in each of the samples that were compared. The heat map shows gradations from higher to lower expression as yellow to blue. The decrease in expression (blue) is evident for infected cells. Gene symbols are shown with the NCBI Gene IDs. (A) Sixteen pathways were downregulated when comparing infected THP-1 cells with uninfected cells. (B) Seventeen pathways were downregulated when comparing THP-1 cells infected with M. tuberculosis and stimulated with IFN-γ with uninfected, IFN-γ–stimulated THP-1 cells.
negative postinitiation regulation in vitro limits the expression of these genes induced by IFN-γ stimulation of infected cells. In agreement with the molecular data interpretation, analysis of biologic process and pathway annotations identified significant effects on genes annotated for postinitiation mRNA biogenesis among genes downregulated by M. tuberculosis infection with or without IFN-γ stimulation in vitro. Moreover, analysis of publicly available transcriptome data from clinical studies identified reduced expression of postinitiation mRNA biogenesis pathways with PTB compared with LTBI, PTB compared with treated PTB, and LTBI compared with cured PTB. These data demonstrate that M. tuberculosis infection licenses novel responses to IFN-γ that limit induction of STAT1 and IRF1 through negative postinitiation regulation. This limit to induction constitutes a new constraint on IFN-γ-induced gene expression in macrophages. Moreover, the limit on induction of STAT1 and IRF1, and perhaps other genes, may be associated with clinically relevant host response to M. tuberculosis, particularly PTB, inasmuch as postinitiation mRNA biogenesis pathways are downregulated in PTB compared with LTBI or to treated PTB, and in LTBI compared with cured PTB.

The results from both in vitro infection and clinical samples help elucidate the consequences of M. tuberculosis infection for postinitiation regulation of mRNA biogenesis. For in vitro infection, the comparison of different transcript pools for STAT1, IRF1 and several other immune response genes (e.g., CCL2, CXCL10, FCGR1, and MX1; Y. Qiao and R. Pine, unpublished observations) provided evidence that negative postinitiation regulation does not alter expression uniformly, because these genes exhibited various ratios for induction in total nuclear RNA relative to nascent transcripts. Moreover, the transcriptome analyses indicated the involvement of a specific subset of significantly downregulated genes associated with postinitiation mRNA biogenesis. Nine genes were found common to the in vitro and in vivo results, and the two comparisons of PTB to LTBI transcriptomes from blood demonstrated similar extents of differential expression for individual transcripts. Thus, the consequences of M. tuberculosis infection for postinitiation regulation of mRNA biogenesis are gene specific.

The negative postinitiation regulation that limits STAT1 and IRF1 expression is not merely an inhibition of a positive response, for at least two reasons. First, IFN-γ stimulation caused an increase in the ratio of spliced to unspliced IRF1 nuclear RNA that was similar in uninfected and infected cells, indicating that positive postinitiation regulation of IRF1 by IFN-γ still occurs in cells infected with M. tuberculosis. The positive effect could be related to the presence of exonic splicing enhancer sequences in the IRF1 transcript (J.C. Cheng and R. Pine, unpublished observations), strongly suggesting that IFN-γ mediates posttranslational regulation of SR family proteins that act at such sites. Second, IFN-γ stimulation did not cause positive postinitiation regulation of STAT1 expression, yet STAT1 is subject to the negative regulation. Taken together, the results reveal the simultaneous occurrence of a positive postinitiation response to IFN-γ and a distinct negative postinitiation response for these two genes in cells that are also infected with M. tuberculosis.

To our knowledge, the case reported above for STAT1 and IRF1 is the first example for the immune response in which downregulation of genes in postinitiation mRNA biogenesis pathways is associated with negative postinitiation regulation of target gene expression. A parallel occurs in the neuroendocrine system, where a glucocorticoid-mediated decrease in expression of the mRNA processing factor NOVA1 decreases the splicing of gonadotropin-releasing hormone pre-mRNA to reduce the level of the mature mRNA in the hypothalamus (38). The negative postinitiation

Discussion

When M. tuberculosis infects macrophages, complex changes occur in host gene expression. Infection increases host production of IFN-γ, which then alters macrophage gene expression. Infection also affects the macrophage response to IFN-γ. To understand the consequences for regulation of host cell gene expression, we characterized the induction of STAT1 and IRF1, transcription factors that are key to the cellular IFN-γ response and to host defense against the pathogen, and we analyzed transcriptome data obtained from in vitro and in vivo infection. These studies revealed negative postinitiation regulation of mRNA biogenesis as a novel way in which M. tuberculosis alters macrophage gene expression. With IFN-γ stimulation of cells infected with M. tuberculosis, but not with IFN-γ stimulation alone or with infection alone, less induction of STAT1 and IRF1 transcripts occurred in total nuclear RNA than in nascent RNA, and no difference occurred in transcript turnover. These results strongly suggest that comparing LTBI and PTB donors from different regions using different platforms, and monocyte-derived macrophages from LTBI and cured donors: RNPS1, HNRNPA0, HNRNPU1L1, COBRA1, GTF2H4, CPSF1, NHP2L1, SNRNP70, TCEB1. The existence of a core set of regulated genes and the correlation for differential gene expression in data from independent studies of PTB and LTBI donors provide strong support for the clinical relevance of decreased gene expression related to postinitiation mRNA biogenesis.

The negative postinitiation regulation that limits STAT1 and IRF1 expression is not merely an inhibition of a positive response, for at least two reasons. First, IFN-γ stimulation caused an increase in the ratio of spliced to unspliced IRF1 nuclear RNA that was similar in uninfected and infected cells, indicating that positive postinitiation regulation of IRF1 by IFN-γ still occurs in cells infected with M. tuberculosis. The positive effect could be related to the presence of exonic splicing enhancer sequences in the IRF1 transcript (J.C. Cheng and R. Pine, unpublished observations), strongly suggesting that IFN-γ mediates posttranslational regulation of SR family proteins that act at such sites. Second, IFN-γ stimulation did not cause positive postinitiation regulation of STAT1 expression, yet STAT1 is subject to the negative regulation. Taken together, the results reveal the simultaneous occurrence of a positive postinitiation response to IFN-γ and a distinct negative postinitiation response for these two genes in cells that are also infected with M. tuberculosis.

To our knowledge, the case reported above for STAT1 and IRF1 is the first example for the immune response in which downregulation of genes in postinitiation mRNA biogenesis pathways is associated with negative postinitiation regulation of target gene expression. A parallel occurs in the neuroendocrine system, where a glucocorticoid-mediated decrease in expression of the mRNA processing factor NOVA1 decreases the splicing of gonadotropin-releasing hormone pre-mRNA to reduce the level of the mature mRNA in the hypothalamus (38).
regulation identified by our study is layered over synergistic transcriptional activation of STAT1 and IRF1 expression as a response to M. tuberculosis infection and IFN-γ stimulation. Limiting the consequences of synergistic transcriptional activation can account for the comparable induction of IRF1 mRNA in uninfected and M. tuberculosis–infected cells stimulated with IFN-γ (21, 56). Many studies have examined changes in macrophage gene expression caused by M. tuberculosis and other mycobacteria (17, 19, 20, 56–64). Effects of infection were described for RNA stability, total RNA abundance, nascent transcript levels, promoter activity, and epigenetic modifications, but changes in nascent and total transcript abundance were not compared. In this study, comparison of target gene expression at different levels, such as total nuclear RNA relative to nascent transcripts or spliced relative to unspliced, allowed detection of posttranscriptional regulation.

This report opens new avenues to investigate the ability of M. tuberculosis to persist or grow despite ongoing macrophage response to IFN-γ. Future studies will further define the mechanisms that affect host defense by limiting expression of STAT1 and IRF1. For example, detailed characterization of RNA polymerase II distribution on the STAT1 and IRF1 genes might discover local fluctuations in elongation that were not discovered by comparing the abundance of one upstream and one downstream intron in total nuclear RNA for each transcript. Regulation of 3′ transcript cleavage and polyadenylation also needs to be addressed. While characterizing mechanisms for the newly discovered negative regulation that limits macrophage response to IFN-γ, it will be important to determine whether manipulating mRNA processing can restore an unconstrained macrophage response to IFN-γ and thereby impede M. tuberculosis infection. Moreover, the discovery that PTB downregulates posttranscriptional mRNA biogenesis pathways indicates a need to better understand how that effect influences the course of infection.

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Disclosures

The authors have no financial conflicts of interest.

References


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**Supplementary Figure Legends**

**Figure S1.** Effects of *M. bovis* BCG infection and IFNγ stimulation on nascent and cellular poly-A+ RNA for STAT1 and IRF1. THP-1 cells were differentiated, infected with *M. bovis* BCG (as described for *M. tb*) and/or stimulated with IFNγ, and then nascent transcripts were measured using the nuclear run-on assay, or cellular RNA was extracted and poly-A+ transcripts were quantified by qRT-PCR. The ratio of the fold-change in the abundance of the poly-A+ RNA relative to the fold-change in the level of nascent transcripts is shown for one of two independent experiments that gave similar results.

**Figure S2.** Effects of *M. tuberculosis* infection and IFNγ stimulation on total and poly-A+ nuclear RNA for STAT1 and IRF1. THP-1 cells were differentiated, infected with *M. tb* and/or stimulated with IFNγ, and then total nuclear RNA was extracted and quantified by qRT-PCR using random nonamer or oligo-dT(15) primers for reverse transcription. The ratio of the fold-change in the level of poly-A+ nuclear RNA relative to the fold-change in the level of total nuclear RNA is shown for the averages of each from 4-6 replicate experiments.

**Figure S3.** Effects of *M. tuberculosis* infection and IFNγ stimulation on STAT1 and IRF1 transcript half-life. THP-1 cells were infected with *M. tuberculosis* and/or stimulated with IFNγ, and then treated with actinomycin D for various times. Cellular RNA was extracted and assayed for poly-A+ STAT1 and IRF1 transcripts. The half-life of each was calculated. The averages and standard deviations from three or four replicate experiments are shown.
Figure S4. Post-initiation mRNA biogenesis Reactome pathways are down-regulated by \textit{M. tuberculosis} infection. Eighteen pathways were tested. The pathways shown are significantly different for the indicated comparisons (A-G) based on a false discovery rate (FDR) criterion of 0.05 for the CERNO test results. The pathway tests were based on the rank order of differential expression for all genes in a pathway (among all transcript measurements of all named genes targeted by the gene expression platform). The top matrix depicts the membership of genes (columns) in pathways (rows) with red squares. The rows and columns of the top matrix are sorted to bring together similar pathway membership patterns. Genes shown are members of one or more pathways that exhibited differential expression for the indicated comparison (one-sided Student’s T-test to assess down-regulation, \( p < 0.05 \) unless otherwise noted). The lower matrix is a heatmap of expression for each of the significantly regulated genes in each of the samples that were compared. The heatmap shows gradations from higher to lower expression as yellow to blue. Gene symbols are shown with the NCBI Gene IDs and the probe (or probe set) identifiers that measured significant differential expression. \textit{M. tuberculosis} is associated with significance for various of the tested pathways as follows: A) 16 pathways were down-regulated when comparing \textit{M. tuberculosis}-infected THP-1 cells to uninfected cells; B) 17 pathways were down-regulated when THP-1 cells infected with \textit{M. tuberculosis} and stimulated with IFN\( \gamma \) were compared to uninfected, IFN-\( \gamma \)-stimulated THP-1 cells; C) four pathways were down-regulated when comparing blood from PTB donors to blood from LTBI donors from London; D) eight pathways were down-regulated when comparing blood from PTB donors to blood from LTBI donors from The Gambia (genes shown for t-test \( p \)-value < 0.001); E) seven pathways were down-regulated when comparing infected (pretreatment) patients to patients after two months of treatment (based on the results of one-sided paired T-tests for increase in expression associated
with treatment); F) 15 pathways were down-regulated when comparing infected (pretreatment) patients to patients after twelve months of treatment (based on the results of one-sided paired T-tests for increase in expression associated with treatment); and G) 10 pathways were down-regulated when comparing monocyte-derived macrophages (MDM) from LTBI patients to MDM from cured PTB patients.
**Figure S1**

The image shows a bar chart illustrating the ratio of total polyA+ nascent RNA for STAT1 and IRF1 under different conditions: BCG and/or IFNγ treatment. The chart compares the following conditions:

- **- BCG - IFNγ**: Represented by a white bar.
- **+ BCG - IFNγ**: Represented by a light gray bar.
- **+ IFNγ**: Represented by a medium gray bar.
- **+ BCG + IFNγ**: Represented by a black bar.

The y-axis represents the total polyA+ nascent RNA ratio, ranging from 0 to 5. The x-axis is divided into two sections: STAT1 and IRF1. The bars indicate the relative expression levels under each condition, with higher bars suggesting a greater ratio.
Figure S2

![Graph showing poly-A+ nuclear RNA to total nuclear RNA ratio with TB and IFNγ treatments.](image)

- TB
- IFNγ

+ TB
+ IFNγ

+ TB + IFNγ
Figure S3

The figure shows the total poly-A+ RNA half-life (hours) for STAT1 and IRF1 under different conditions: -TB, +TB, -IFNγ, +IFNγ, and +TB + IFNγ. The bar chart indicates that the half-life is significantly shorter under the +IFNγ condition compared to the other conditions.
Figure S4

A.

B.

Processing of capped introns/Pre-mRNA REACT_1769
SLBP: Independent Processing of Mature Pre-mRNA REACT_185
SLBP: Dependent Processing of Replication-Dependent Histoene Pre-mRNAs REACT_1362
mRNA 3’end processing REACT_1849
RNA Polymerase II Transcription Termination REACT_894
Chlorination of Growing Transcript in the Termination Region REACT_387
mRNA Capping REACT_5470
RNA Pol II CTD phosphorylation and interaction with CE REACT_6237
RNA Pol II CTD phosphorylation and interaction with CE REACT_6273
Formation of the Early Elongation Complex REACT_646
Formation of RNA Pol II elongation complex REACT_1845
RNA Polymerase II Transcription Elongation REACT_833
mRNA Splicing - 3’End Pathway REACT_1893
Processing of capped introns/Containing Pre-mRNA REACT_125
mRNA Splicing REACT_1575
mRNA Splicing - Major Pathway REACT_1487

Sample Class | Infection | Stimulation
--- | --- | ---
TB-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated
BM6-infected | M1-infected | Untreated

Processing of introns/Pre-mRNA REACT_1096
Processing of capped introns/Pre-mRNA REACT_1768
SLBP: Independent Processing of Mature Pre-mRNA REACT_185
SLBP: Dependent Processing of Replication-Dependent Histoene Pre-mRNAs REACT_1362
mRNA 3’end processing REACT_1849
RNA Polymerase II Transcription Termination REACT_894
Chlorination of Growing Transcript in the Termination Region REACT_387
mRNA Capping REACT_5470
RNA Pol II CTD phosphorylation and interaction with CE REACT_6237
RNA Pol II CTD phosphorylation and interaction with CE REACT_6273
Formation of the Early Elongation Complex REACT_646
Formation of RNA Pol II elongation complex REACT_1845
RNA Polymerase II Transcription Elongation REACT_833
mRNA Splicing - 3’End Pathway REACT_1893
mRNA Splicing - Major Pathway REACT_1487
mRNA Splicing - Minor Pathway REACT_1755

Sample Class | Infection | Stimulation
--- | --- | ---
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
BM6-infected | IFNr | Untreated
mRNA Splicing - Minor Pathway REACT_1753
Processing of Capped Intron-Containing Pre-mRNA REACT_125
mRNA Splicing REACT_1735
mRNA Splicing - Major Pathway REACT_467
Formation of the Early Elongation Complex React_846
Formation of RNA Pol II elongation complex React_1045
RNA Polymerase II Transcription Elongation React_833
mRNA Splicing - Minor Pathway React_1783
mRNA 3-end processing React_1849
RNA Polymerase II Transcription Termination React_884
Cleavage of Growing Transcript in the Termination Region React_387
mRNA Splicing - Major Pathway React_467
Processing of Capped Intron-Containing Pre-mRNA React_125
mRNA Splicing React_1735