Interferon-γ regulates cellular metabolism and mRNA translation to potentiate macrophage activation

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Interferon-γ (IFN-γ) primes macrophages for enhanced microbial killing and inflammatory activation by Toll-like receptors (TLRs), but little is known about the regulation of cell metabolism or mRNA translation during this priming. We found that IFN-γ regulated the metabolism and mRNA translation of human macrophages by targeting the kinases mTORC1 and MNK, both of which converge on the selective regulator of translation initiation eIF4E. Physiological downregulation of mTORC1 by IFN-γ was associated with autophagy and translational suppression of repressors of inflammation such as HES1. Genome-wide ribosome profiling in TLR2-stimulated macrophages showed that IFN-γ selectively modulated the macrophage translatome to promote inflammation, further reprogram metabolic pathways and modulate protein synthesis. These results show that IFN-γ-mediated metabolic reprogramming and translational regulation are key components of classical inflammatory macrophage activation.

Interferon-γ (IFN-γ) activates innate responses by augmenting inflammatory cytokine and chemokine production, microbial killing and antigen presentation by mononuclear phagocytes such as macrophages1. Immune-cell activation by IFN-γ is entirely dependent on activation of the transcription factor STAT1, which binds to and activates transcription of interferon-stimulated genes (ISGs)2. The direct and rapid activation of ISGs has a key role in IFN-γ-mediated functions2. It has become clear that many IFN-γ activities cannot be explained by the direct effector functions of ISGs, and that important IFN-γ functions are mediated by the cross-regulation of distinct signaling pathways or reprogramming of cell states to alter their responses to extracellular stimuli. For example, IFN-γ augments macrophage cytokine production in response to inflammatory stimuli such as Toll-like receptor (TLR) ligands1 by attenuating signaling via the suppressive transcription factor STAT3 (ref. 3) and by inhibiting expression of the TLR-induced Notch-dependent transcriptional repressors HES1 and HEY1 (ref. 4). In parallel, IFN-γ reprograms the ‘epigenetic landscape’ of macrophages by inducing and priming enhancers to increase transcriptional output in response to TLR signaling5,6. Whether IFN-γ can reprogram macrophage metabolism to alter cell function remains to be elucidated.

The importance of translational control of immune responses is increasingly appreciated2. Increased translation of select cytokine, chemokine and transcription factor mRNAs has been observed after TLR stimulation8,9, and key immune regulators such as I-kBα and IRF7 are under translational control10,11. Selective translational regulation of mRNA transcripts typically occurs at the level of initiation and can be achieved by specific RNA-binding proteins and miRNAs and by modulation of the activity of 5′ cap–binding eukaryotic initiation factor 4E (eIF4E)7. Although eIF4E is a general translation-initiation factor, changes in its activity do not regulate translation globally; instead they selectively affect the translation of a subset of transcripts, including inefficiently translated transcripts with long 5′ untranslated regions (UTRs)7,11,12. eIF4E activity is regulated by MNK kinases and the metabolic checkpoint kinase complex mTORC1 (ref. 7) and thus is responsive to upstream signals that trigger mitogen-activated protein kinase (MAPK) signaling or mTORC1 activity. Type I interferons suppress translation13 by inactivating the translation factor eIF2α (ref. 14) and inducing ISGs that translationally silence viral RNAs15. Interferons can promote the translation of ISGs through various mechanisms16, and IFN-γ suppresses the translation of a small set of mRNAs17. Little is known about the regulation of translation by IFN-γ in immune cells.

The mechanistic target of rapamycin (mTOR) serine-threonine kinase is a component of distinct mTORC1 and mTORC2 complexes, and mTORC1 is an important regulator of mRNA translation18. mTORC1 senses and coordinates cellular responses to the nutrient status of extracellular and intracellular microenvironments and is regulated by growth factors, oxygen and stress, as well as by intracellular amino acid and...
energy levels. mTORC1 activation requires binary inputs from growth factor–induced Akt-mediated signaling that activates mTOR, as well as repletion of intracellular amino acids, which enables the translocation of mTORC1 to lysosomal membranes, where mTOR activation occurs. Under nutrient-replete conditions, mTORC1 promotes anabolic, biosynthetic and proliferative pathways, including protein, lipid and nucleotide synthesis required for cell growth. mTORC1 promotes protein synthesis by phosphorylating and inactivating negative regulators of eIF4E (termed 4E-BPs) and by activating the kinase p70S6K, which phosphorylates ribosome proteins. Understanding of the role of mTORC1 in innate immunity is limited.

In this study we investigated how IFN-γ alters the macrophage cell state to potentiate TLR responses. To maximize the physiological relevance for human inflammatory conditions, we used primary human monocytes and macrophages that have key roles in human inflammatory diseases. We found that IFN-γ regulated TLR2 responses in human macrophages by suppressing MNKs and mTORC1 and by modulating mRNA translation. A genome-wide ribosome-profiling approach showed that translational regulation selectively affected pathways important for cytokine expression, protein synthesis and cell metabolism. Our findings demonstrate an unrecognized function of IFN-γ in reprogramming macrophage metabolism to alter inflammatory responses.

RESULTS
Inhibition of HES1 mRNA translation by IFN-γ
It has been shown that IFN-γ suppresses TLR4-induced expression of HES1 and HEY1, thereby disrupting a feedback inhibitory loop and augmenting production of the inflammatory cytokines interleukin 6 (IL-6) and IL-12 (ref. 4). We tested whether IFN-γ could also repress the induction of HES1 mRNA by TLR2 in primary human macrophages. Stimulation with the TLR2 ligand Pam3CSK4 resulted in HES1 mRNA expression in 5 h, and mRNA abundance increased in a time-dependent manner (Fig. 1a). In contrast to its tenfold repression of TLR4 (lipopolysaccharide)-induced HES1 mRNA expression (Supplementary Fig. 1a), IFN-γ suppression of TLR2-induced HES1 transcript amounts was minimal (Fig. 1a). This observation was consistent among more than 20 human blood donors tested, and no statistically significant difference was apparent when we pooled data from 23 different donors (Supplementary Fig. 1b). In contrast to mRNA regulation, TLR2-induced HES1 protein expression was almost completely abrogated by IFN-γ (Fig. 1b). The inhibitory effect of IFN-γ on TLR2-induced HES1 protein expression became apparent within 4 h of IFN-γ pretreatment and was clearly established after 6 h of priming (data not shown), which is consistent with published studies of IFN-γ priming21. The prominent discrepancy between HES1 mRNA expression and HES1 protein expression suggests that IFN-γ negatively regulates HES1 expression at the protein level.

IFN-γ could downregulate HES1 by either suppressing its synthesis or increasing its degradation. To distinguish between these possibilities, we monitored HES1 half-life in control and IFN-γ–treated macrophages and found that HES1 decay rates were similar in both conditions (Supplementary Fig. 1c). We also found that neither the proteasome inhibitor MG132 nor the autophagosome-lysosome fusion inhibitor Bafilomycin A1 reversed the downregulation of HES1 by IFN-γ (Supplementary Fig. 1d,e), which implied that accelerated

Figure 1 IFN-γ suppresses HES1 translation. (a) Quantitative PCR (qPCR) analysis of HES1 mRNA expression in human primary macrophages cultured with or without IFN-γ (100 U ml−1) for 24 h and then stimulated with Pam3CSK4 (Pam; 10 ng ml−1) for 0–6 h; results are normalized relative to GAPDH mRNA expression. Data are shown as mean and s.d. of triplicate determinants (cumulative results in Supplementary Fig. 1b). (b) Immunoblots of analysis of HES1 in control or IFN-γ–treated macrophages stimulated with Pam (10 ng ml−1) for 0–4 h; p38α serves as a loading control. HES1 was suppressed by 85.5% in six independent experiments (P < 0.0001). (c) Top, representative polysome profiles of control (blue) and IFN-γ–treated (red) macrophages stimulated with Pam (10 ng ml−1) for 4 h. Bottom, the ratio of RNA amounts in IFN-γ–treated conditions relative to those in control conditions. Fractions 3 and 4, individual ribosome 40S and 60S subunits; fraction 5, monomeric 80S ribosomes; fractions 6–8, light polysome fractions; fractions 9–12, heavy polysome fractions. Data are presented as mean and s.d. and were analyzed with one-way analysis of variance (ANOVA); P = 0.1383 for difference between IFN-γ–treated and control conditions. (d) Polysome-shift qPCR analysis of HES1, PABPC1 and ACTB mRNA expression in polysome fractions from c, depicted as the percentage of mRNA in each fraction compared with the total mRNA in all fractions (fractions 1–12). (e) Top, qPCR analysis of PABPC1 mRNA expression in control or IFN-γ–treated macrophages from two independent donors; data are shown as mean and s.d. of triplicate determinants. Bottom, immunoblot analysis of PABPC1 from parallel samples in the same experiments; p38α served as a loading control. PABPC1 was suppressed by 68.75% in pooled data from independent experiments; P = 0.01. Data are representative of >20 (a), 23 (b), 3 (c,d) or 4 (e) independent experiments.
protein degradation was not responsible for the reduced HES1 abundance in IFN-γ-treated cells. This suggested that IFN-γ suppression of translation of HES1 mRNA. We directly tested this hypothesis by using sucrose-gradient centrifugation to track the ribosomal distribution of mRNAs in cytosolic extracts from control and IFN-γ-treated macrophages. The frequency of ribosome binding to an mRNA corresponds to the translation efficiency of each mRNA molecule, and actively translated mRNAs are found in polysome fractions. IFN-γ had no effect on total mRNA amounts associated with heavy polysome fractions (fractions 9–12) and had a minimal suppressive effect on light polysome fractions (fractions 6–8), which indicated that IFN-γ did not globally and nonspecifically suppress translation (Fig. 1c). However, IFN-γ slightly decreased amounts of monomeric 80S ribosomes and correspondingly increased mRNA amounts associated with individual ribosome 40S and 60S subunits relative to amounts in unstimulated cells (Fig. 1c), which suggested that IFN-γ might suppress the initiation of translation (associated with 80S ribosome assembly) of select mRNA transscripts.

A shift in individual mRNAs from heavy polysome to light polysome or monosome fractions indicates decreased translation efficiency. Polysome-shift analysis of mRNAs encoded by specific genes showed that in control TLR2-stimulated macrophages, a substantial fraction of HES1 mRNA resided in heavy polysome fractions (Fig. 1d), indicating efficient translation. In IFN-γ-treated cells, the HES1 mRNA peak shifted to light polysome fractions (Fig. 1d), which indicated decreased translational efficiency. As a positive control for the polysome-shift analysis, we found that mRNA encoded by PABPC1, which is known to be sensitive to translational regulation, was strongly shifted to the light polysome and monosome fractions in IFN-γ-treated macrophages (Fig. 1d). Accordingly, IFN-γ suppressed PABPC1 protein expression without affecting mRNA expression (Fig. 1e). As a specificity control, we found that ACTB mRNA was present in the heavy polysome fractions, and thus was actively translated, regardless of IFN-γ treatment status (Fig. 1d). Collectively, the results showed that IFN-γ had selective effects on the translation efficiency of distinct mRNAs and inhibited the translation of PABPC1 mRNA and HES1 mRNA in primary human macrophages.

IFN-γ attenuates TLR2-induced MAPK-MNK-eIF4E signaling

We wished to test whether IFN-γ inhibits translation by targeting the key regulator of translation-initiation efficiency eIF4E, which is activated after phosphorylation by MAPK-interacting kinases (MNKs)22 (Supplementary Fig. 2a). TLR2 stimulation induced activating phosphorylation of MNK1 and eIF4E, which was substantially but not completely inhibited by IFN-γ at all time points tested (Fig. 2a). These results showed that IFN-γ negatively regulated the activation of TLR2-induced MNK-eIF4E signaling; the functional importance of this suppression was supported by IFN-γ-mediated suppression of translation of the signaling inhibitor I-κBα and the transcription factor IRF8, which was previously shown to be dependent on the MNK-eIF4E pathway9,10 (Fig. 2b and Supplementary Fig. 2b–d). These results suggested that MNKs have a role in promoting the translation of HES1. We tested this notion by inhibiting MNKs or knocking down their expression with short interfering RNAs (siRNAs) and measuring HES1 expression by immunoblot. The MNK inhibitor CGP57380 strongly suppressed HES1 expression in a dose-dependent manner (Fig. 2c) without affecting HES1 mRNA induction (Fig. 2d). Furthermore, relative to amounts in controls, siRNA-mediated knockdown of MNKs (Supplementary Fig. 2e) resulted in diminished phosphorylation of eIF4E and decreased expression of HES1 (Fig. 2e) without a decrease in HES1 mRNA expression (Supplementary Fig. 2f). These data indicate that efficient HES1 translation requires MNK-mediated phosphorylation of eIF4E. To gain insight into how IFN-γ suppresses TLR2-induced MNK phosphorylation, we examined
upstream MAPK activation. IFN-γ attenuated TLR2-induced phosphorylation of p38 and Erk MAPks in primary human macrophages (Fig. 2f), which is consistent with our previous results. These MAPks are dephosphorylated and inactivated by dual-specificity phosphatases (DUSPs); accordingly, IFN-γ increased baseline and TLR2-induced expression of DUSPs 1, 2, 4, 8 and 16 (Fig. 2g) compared with expression in untreated cells. A role for phosphatases in the attenuation of MAPK signaling was further supported by the reversal of IFN-γ-mediated dampening of p38 activation by the phosphatase inhibitor okadaic acid (Supplementary Fig. 2g). Taken together, our results suggest that optimal translation of HES1 mRNA requires phosphorylation of the 5′ mRNA cap-binding protein eIF4E, and that IFN-γ suppresses HES1 translation in part by attenuating TLR2-induced activation of MAPK-MNK-eIF4E signaling.

IFN-γ suppresses mTORC1 activation and downstream function

Given the partial attenuation of MNK activation by IFN-γ but the near-complete suppression of HES1, we hypothesized that additional signaling pathways are involved in the process. A major positive regulator of translation is the mTORC1 complex, which phosphorylates eIF4E-binding proteins (4E-BPs) to release them from eIF4E and thereby promote translation (Supplementary Fig. 2a). Therefore, we tested whether IFN-γ suppresses mTORC1 activity. mTORC1 activity is typically assessed on the basis of phosphorylation of its downstream substrates 4E-BP and p70S6K. As expected, 4E-BP1 was phosphorylated on Thr37 and/or Thr46 in cultured macrophages, consistent with mTORC1 activity maintained by serum growth factors (Fig. 3a). IFN-γ suppressed basal 4E-BP1 phosphorylation (Fig. 3a), and diminished 4E-BP1 phosphorylation was maintained throughout the time course of TLR2 stimulation in IFN-γ-treated macrophages (Fig. 3a).

IFN-γ also suppressed 4E-BP1 phosphorylation in TLR-stimulated human monocyte–derived dendritic cells (Supplementary Fig. 3a). In macrophages IFN-γ suppressed 4E-BP1 phosphorylation about as much as or slightly more than the well-established allosteric inhibitor of mTORC1 rapamycin (Fig. 3b), which only partially inhibits mTORC1 but has strong biological effects related to the suppression of mTORC1 function. The ATP-competitive inhibitors of mTOR kinase activity Torin1 and PP242 suppressed 4E-BP1 phosphorylation more strongly than rapamycin did, as expected. IFN-γ also suppressed phosphorylation of p70S6K, another substrate of mTORC1 that is involved in translation regulation (Fig. 3c). These data show that IFN-γ suppressed the phosphorylation of mTORC1 substrates important in the regulation of translation, and they support the idea of a role for IFN-γ in repressing mTORC1 activity.

mTORC1 suppresses autophagy, a critical cellular process that degrades cytoplasmic proteins and organelles under conditions of nutrient deprivation. Autophagy is characterized by the formation of autophagosomes and generation of the proteolyzed forms of autophagosome markers LC3A and LC3B. We found that IFN-γ induced generation of the proteolyzed isoforms LC3A-II and LC3B-II similarly to rapamycin (Fig. 3d). These results further support the idea that IFN-γ inhibits mTORC1 activity and suggest that one functional outcome of such inhibition is increased autophagy, which promotes microbial killing and antigen presentation. Inhibition of mTORC1 resulted in increased production of inflammatory cytokines (Supplementary Fig. 3b), a finding that supports the notion that attenuation of mTORC1 activity by IFN-γ contributes to the interferon’s activating functions. mTORC1 activation occurs on lysosomal membranes and requires the recruitment of mTORC1 to late endosomes and lysosomes by the Ragulator-Rag complex. We used immunofluorescence microscopy to test whether IFN-γ affected the
localization of mTOR to lysosomes. Control macrophages showed prominent colocalization of mTOR and the lysosome marker LAMP-1 (Fig. 3e). In contrast, IFN-γ-treated macrophages showed clear dissociation of mTOR and LAMP-1 staining (Fig. 3e). Collectively, reduced phosphorylation of 4E-BP1 and p70S6K, enhanced autophagy and disruption of mTOR lysosomal localization led us to conclude that IFN-γ suppresses mTORC1 activity in human macrophages.

The finding that IFN-γ suppressed mTORC1 activity and in parallel suppressed HES1 translation suggested that translation and expression of HES1 might depend on mTORC1 signaling. We tested this by using rapamycin to inhibit mTORC1 and measured HES1 by immunoblot. Rapamycin inhibited TLR2-induced expression of HES1 in a dose-dependent manner (Fig. 3f) but had no effect on HES1 mRNA expression (Supplementary Fig. 3c). Thus, mTORC1 activity is required for the translation of HES1 mRNA into protein. These results link the inhibition of mTORC1 activity by IFN-γ with IFN-γ-mediated suppression of HES1 translation and provide an additional functional outcome of suppression of mTORC1 by IFN-γ.

IFN-γ suppresses localization of mTORC1 to lysosomes

Next we sought to identify mechanisms by which IFN-γ inhibits mTORC1 in human macrophages. mTORC1 activation requires dual inputs, one from the extracellular environment, typically mediated by growth factor–PI3K-Akt–TSC1/2–Rheb signaling, and a second from intracellular nutrient availability, typically conveyed by amino acid–mediated activation of the Ragulator–Rag complex that recruits mTORC1 to lysosomal membranes, where mTOR is activated by the GTP-binding protein Rheb (Supplementary Fig. 3d). We wished to elucidate how IFN-γ suppresses the localization of mTORC1 to lysosomes, as described above. We thus investigated whether IFN-γ regulates the amino acid pathway of mTORC1 activation. The most potent amino acid activator of mTORC1 recruitment is leucine, but mTORC1 activation is also partially dependent on tryptophan and certain additional amino acids. Measurement of intracellular amino acid concentrations by means of ninhydrin reaction–based colorimetric detection analysis showed that intracellular concentrations of most amino acids, including leucine, were not notably diminished, although this technique does not detect tryptophan in our system (data not shown). IFN-γ is a strong inducer of indoleamine 2,3-deoxygenase (IDO), which catalyzes tryptophan degradation and has been shown to deplete extracellular tryptophan and thereby suppress lymphocyte proliferation. We reasoned that IFN-γ might induce sufficient IDO expression in human macrophages to deplete intracellular tryptophan and thereby suppress mTORC1 activity. We observed that IFN-γ strongly induced IDO expression with sustained kinetics in our system (Fig. 4a). Direct measurement of intracellular tryptophan and its catabolites by HPLC followed by mass spectrometry showed a striking depletion of tryptophan (70% decrease after IFN-γ priming) with parallel accumulation of its catabolites in the IDO-mediated degradation pathway (Fig. 4b and Supplementary Fig. 3e,f). To test whether this IDO-mediated depletion of intracellular tryptophan contributes to reduced mTORC1 activity, we treated cells with the IDO inhibitor 1-D-MT and observed them via immunofluorescence microscopy (Fig. 4c). 1-D-MT restored the colocalization of mTOR and LAMP-1 in IFN-γ-treated macrophages (Fig. 4c).
Furthermore, the addition of exogenous tryptophan to IFN-γ-treated cells also partially recovered the lysosomal distribution of mTOR (Fig. 4c), which supported the theory that that IDO-mediated depletion of tryptophan is responsible for IFN-γ-induced mTOR subcellular redistribution, although it is possible that tryptophan metabolites could also contribute to the modulation of mTORC1 activity. To further establish the role of IDO in regulating mTORC1 activity, we used mTORC1-dependent HES1 expression as a downstream readout of mTORC1 signaling. Consistent with the reversal of IFN-γ-mediated suppression of mTORC1 lysosomal localization (Fig. 4c), 1-D-MT reversed IFN-γ-mediated suppression of HES1 expression (Fig. 4d). Consistent with this and with the restoration of lysosomal localization of mTOR (Fig. 4c), tryptophan supplementation partially restored HES1 expression in IFN-γ-treated macrophages in a dose-dependent manner (Fig. 4e). Taken together, these data implicate the depletion of intracellular tryptophan by IDO in IFN-γ-mediated suppression of mTORC1 localization to lysosomes and thus suppression of mTORC1 activity.

IFN-γ suppresses mTORC1 activation by extracellular cues

The inhibition of lysosomal localization of mTOR by IFN-γ was not complete (Fig. 3c) and was only partially reversed by 1-D-MT or Trp (Fig. 4), which suggested that IFN-γ also inhibits another pathway required for mTORC1 activity. Thus we considered the possibility that IFN-γ also suppresses PI3K-Akt-TSC signaling (Supplementary Fig. 3d). PI3K-Akt-TSC signaling is a major regulator of mTORC1 activity in response to extracellular factors, such as growth factors, and is also activated by inflammatory stimuli such as TLR ligands. We found that TLR2-induced Akt phosphorylation was attenuated by IFN-γ (Fig. 5a). The PI3K inhibitor LY294002 abrogated Akt phosphorylation, which confirmed that this inducible phosphorylation was due to PI3K activation (Fig. 5a). To corroborate the suppression of Akt signaling by IFN-γ, we measured the downstream induction of β-catenin, which is dependent on Akt-GSK3 signaling. The accumulation of β-catenin induced by TLR2 signaling was effectively inhibited by IFN-γ, which further supported the idea that IFN-γ inhibits Akt signaling (Fig. 5b). Akt activates mTORC1 by phosphorylating and thereby deactivating the negative regulator TSC2. Consistent with the suppression of Akt signaling, IFN-γ also suppressed TSC2 phosphorylation (Fig. 5c). These results indicated that IFN-γ attenuates TLR2-activated signals that lead to Rheb-mediated activation of mTORC1 (Supplementary Fig. 3d). We also tested whether IFN-γ could inhibit signaling by growth factors that maintain basal mTORC1 activity in macrophages by stimulating Akt...
activity. First, we confirmed that serum components and macrophage colony-stimulating factor (M-CSF) were key factors for maintaining basal mTORC1 activity in human macrophages (Supplementary Fig. 4a,b). M-CSF is known to promote Akt signaling. Although basal Akt phosphorylation was difficult to detect because of the limited sensitivity of immunoblotting, we found that IFN-γ effectively blocked M-CSF–induced Akt phosphorylation in macrophages that had been starved of serum and M-CSF for 4 h (Fig. 5d). These results indicated that IFN-γ suppresses the activation of mTORC1 by extracellular factors by suppressing the activation of Akt.

We investigated mechanisms by which IFN-γ suppresses growth factor–mediated Akt signaling in macrophages. We found that IFN-γ suppressed the expression of M-CSF receptor (M-CSFR (CSFR1)) mRNA and protein (Fig. 5e), which corroborated published results31. Decreased M-CSFR expression could explain diminished Akt activation by M-CSF and had a minimal effect on cell survival under IFN-γ–primed conditions (Supplementary Fig. 4c). IFN-γ–mediated suppression of TLR2–induced Akt activation was mediated by an okadaic acid–sensitive phosphatase (Supplementary Fig. 2g) but was not associated with changes in the phosphatases SHP or PTEN, which regulate this pathway (data not shown); future work will be needed to determine how IFN-γ suppresses TLR2–induced Akt phosphorylation. To investigate mechanisms by which IFN-γ suppresses M-CSFR expression, we tested the role of Myc, which has been shown to promote mTORC1 activity in lymphocytes, although the underlying mechanisms have not been fully clarified. We found that Myc and M-CSFR expression were induced during cell culture with M-CSF (Fig. 5f,g), and inhibition of Myc with the compound 10058–F4 suppressed the induction of M-CSFR expression (Fig. 5g), suggesting that Myc is required for M-CSFR expression and mTORC1 activity in macrophages. Consistent with this notion, inhibition of Myc effectively suppressed 4E-BP phosphorylation (Fig. 5h), as well as the downstream induction of mTORC1–dependent HES1 expression (Supplementary Fig. 4d); similar results were obtained when Myc expression was knocked down with siRNA (Supplementary Fig. 4e).

IFN-γ suppressed Myc expression (Fig. 5f,i). These results suggest that IFN-γ suppresses expression of the transcription factor Myc to downregulate M-CSFR expression and downstream activity of mTORC1, and they identify the regulation of Myc as a link between IFN-γ and cell metabolism. Collectively, the results indicate that IFN-γ suppresses Akt signaling and thereby attenuates signals from extracellular factors that are required for mTORC1 activity (Supplementary Fig. 3d). This attenuation of signaling, in concert with decreased recruitment of mTORC1 to lysosomal membranes (Fig. 3e), could explain the strong inhibition of mTORC1 activity by IFN-γ.

**Genome-wide analysis of translational regulation by IFN-γ**

The MNK-eIF4E and 4EBP-eIF4E pathways that were suppressed by IFN-γ selectively regulate translation7,10–12, and translational regulation is important for inflammatory responses7. To address the broad functional role of IFN-γ–regulated translation in inflammatory responses in primary human macrophages, we used a genome-wide approach combining high-throughput RNA sequencing with ribosome profiling. Ribosome profiling provides a snapshot of the translome by quantifying ribosome-protected RNA fragments (RPFs), the frequency of which on each transcript reflects the translation rate of the corresponding mRNA12. We quantified and compared the abundance of mRNA (in counts per million reads (c.p.m.)) and actively translated mRNA (RPF (c.p.m.)) in TLR2–activated macrophages that had been treated with or without IFN-γ in two biological replicates (Supplementary Fig. 5a,b and Online Methods). For each of 9,290 genes that met stringency cutoffs (Online Methods), we calculated the translational efficiency (TE) by dividing the change in RPF reads by the change in c.p.m.; this identified changes that could be attributed solely to changes in translation rate. As expected, IFN-γ treatment altered the abundance of multiple mRNA transcripts (Fig. 6a). IFN-γ also induced significant changes in TE in almost 1,000 genes (Fig. 6a and Supplementary Fig. 5c), of which 396 were strongly affected (greater than twofold change in TE). In contrast to the consistent translational repression of most mRNAs after
complete inhibition of mTOR\textsuperscript{23}, the effects of IFN-\(\gamma\) on translation were bidirectional, and IFN-\(\gamma\) increased and decreased the TE of similar numbers of genes (Fig. 6a and Supplementary Fig. 5c). Thus, ribosome profiling revealed an unappreciated role of IFN-\(\gamma\) in modulating the translation of mRNA at a genome-wide level.

We wished to confirm our genome-wide findings by analyzing individual genes and to corroborate regulation by IFN-\(\gamma\) at the level of mRNA expression (c.p.m.), translation (TE) and RPF reads; changes in RPF reads reflect a translation (TE) and RPF reads; changes in RPF reads reflect a change in mRNA expression which is consistent among biological replicates and among the combined data set (Fig. 6d).

 Ingenuity Pathway Analysis of ribosome profiling data (RPF analysis) showed highly statistically significant regulation of well-known IFN-\(\gamma\)-mediated pathways important in immune responses such as antigen presentation (Fig. 7a and Table 1). The patterns of pathway enrichment were consistent among biological replicates and among the combined data set (Supplementary Fig. 6a). These results are in accord with a

**Table 1** IPA canonical pathways regulated by IFN-\(\gamma\)

| Canonical pathway | Uregulated | Downregulated | Not detected | Total |
|------------------|------------|---------------|--------------|-------|
|EIF2 signaling    | 9.9        | 79.1          | 11            | 172   |
|Antigen-presentation pathway | 70.3 | 13.5 | 16.2 | 37 |
|tRNA charging     | 2.6        | 92.1          | 5.3           | 38    |
|Purine nucleotide synthesis | 0     | 90.9          | 9.1           | 11    |
|Regulation of eIF4 and p70S6K | 12.7 | 71.1 | 16.2 | 142 |
|Crosstalk between dendritic cells and NK cells | 41.6 | 12.4 | 46.1 | 89 |
|mTOR signaling    | 13.7       | 63.2          | 23.1          | 182   |

Ingenuity Pathway Analysis (IPA) of canonical pathways most enriched at the level of ribosome footprint frequency (RPF), ranked by \(P\) value (Fig. 7a). Data were generated from a merged data set from two biological replicates.
Figure 8 IFN-γ downregulates miRNAs that target translationally upregulated genes. (a) Scatter plot showing normalized miRNA abundance of all conditions analyzed (x-axis) and changes in miRNA expression induced by IFN-γ (y-axis). Statistical analysis was done with edgeR; the top 12 most significantly regulated genes are marked in red (*P* values in Table 2). (b) Potential target mRNAs of miR-146b-3p that were translationally upregulated by IFN-γ. Asterisks indicate the 3’ UTR sequence complementary to the seed sequence of miR-146b-3p. Data shown were generated from a merged data set from two biological replicates.

feed-forward loop described for other systems whereby proteins important for translation are themselves regulated at the translational level^{15}. Thus, translational dampening of eIF4 pathway components probably cooperates with signaling attenuation (Figs 2 and 3) to regulate eIF4 function in IFN-γ–treated macrophages. We found the suppression of tRNA charging to be particularly striking, as translation of 29 out of 36 detected aminoacyl tRNA synthetases was suppressed (Supplementary Fig. 6b). This included leucyl-tRNA synthetase (LARS), a direct amino acid sensor that recruits mTORC1 to the lysosomal surface and mediates its leucine-dependent activation^{15}. Suppression of LARS expression was confirmed by immunoblot (Fig. 7b). Decreased LARS expression would contribute to the decreased mTORC1 localization to lysosomal membranes shown in Figure 4. Collectively, our ribosome-profiling results suggested that the cellular translational machinery might represent an unrecognized target for negative regulation by IFN-γ.

We next addressed the question of whether IFN-γ–induced changes in TE generally reinforce or oppose and modulate the overall IFN-γ response, as measured in an integrated manner by RPF reads. We analyzed the relationship between changes in TE and changes in RPF reads (Fig. 7c). Overall, the correlation between these two values was low (Pearson correlation coefficient $R = 0.27$, $R^2 = 0.072$), which suggests that at the genome-wide level, regulation of translation modulates and fine-tunes the transcriptional response to IFN-γ. Consistent with this notion, of the genes with decreased RPF reads and significant changes in TE, 37% had a discordant increase in TE (Fig. 7c). In contrast, of genes with increased RPF reads, the majority (77%) had a concordant increase in TE (Fig. 7c). Thus, IFN-γ–mediated increases in TE tended to reinforce and augment the upregulation of gene expression. To analyze the function of genes regulated at the level of TE, we selected highly regulated genes (*z*-score $= \pm 1.5$ in a pooled data set) and stratified them into functional subcategories on the basis of gene ontology (Supplementary Fig. 7a,b). This analysis showed that genes related to metabolic processes were the most enriched among translation–regulated genes; immune genes were enriched as well (Supplementary Fig. 7; gene lists are shown in Supplementary Tables 1 and 2, and representative genes are shown in Fig. 7d). Genes encoding inflammatory cytokines and chemokines such as IL-6, TNF, LTA, LTβ, CXCL2 and CXCL3 were included in the immune-gene group with increased TE (Fig. 7d). This suggested that translational regulation contributes to the described augmentation of TLR-induced inflammatory responses by IFN-γ. Pathway analysis showed that metabolic-process genes whose translation was increased were strongly associated with oxidative phosphorylation and mitochondrial pathways, that genes whose translation was decreased were associated with stress pathways, and that both sets of genes were associated with growth-factor pathways (Supplementary Fig. 8a).

Genes encoding RNA modification factors and transporters of amino acids and other metabolites were among the genes most strongly downregulated by IFN-γ at the level of TE (Fig. 7d); additional investigation is required to discern the functional effects of translational regulation of these genes. Thus, we determined that IFN-γ regulates macrophase metabolism at the translational level. In summary, our genome-wide ribosome-profiling analysis indicated that IFN-γ changes the translational landscape of human macrophages, probably contributing to the inflammatory response and metabolic reprogramming.

Regulation of MNKs and mTORC1 can explain the downregulation of translation by IFN-γ. We wished to investigate distinct mechanisms that might underlie the increased TE of some mRNAs, such as *IL6* and *TNF*. IFN-γ modestly and transiently decreased phosphorylation of the translation factor eIF2α (Supplementary Fig. 8b), which increases translation and could contribute to global maintenance of translation in IFN-γ–treated cells, and possibly to enhanced translation of specific transcripts. However, as the regulation of eIF2α was modest, we tested the non–mutually exclusive hypothesis that IFN-γ could increase the TE of select mRNAs by downregulating the expression of miRNAs that suppress the translation of these transcripts. We carried out global profiling of miRNA expression in human primary macrophages with miRNA-Seq. We found that 20 miRNAs were significantly induced by TLR2 stimulation in two independent experiments, including known regulators of inflammatory responses miR-155, miR-146a, miR-9 and miR-132 (Supplementary Table 3). IFN-γ suppressed the expression of 54 miRNAs by at least twofold in TLR2-stimulated macrophages (Fig. 8a, Supplementary Table 3).

Table 2 miRNAs suppressed by IFN-γ

| miRNA          | Expression (log₂ fold) | *P* value | FDR   |
|----------------|------------------------|-----------|-------|
| hsa-miR-146b-3p| –2.56                  | 8.35 × 10⁻⁷| 0.0005|
| hsa-miR-4662a-5p| –2.70                  | 1.71 × 10⁻⁶| 0.0005|
| hsa-miR-909b-5p| –2.27                  | 3.89 × 10⁻⁶| 0.0008|
| hsa-miR-146b-5p| –2.46                  | 3.76 × 10⁻⁶| 0.0050|
| hsa-miR-654-3p| –2.66                  | 8.75 × 10⁻⁴| 0.0559|
| hsa-miR-125a-5p| –1.56                  | 8.96 × 10⁻⁴| 0.0559|
| hsa-let-7e-3p| –1.98                  | 1.30 × 10⁻³| 0.0653|

miRNAs most significantly suppressed by IFN-γ in TLR-stimulated macrophages (*P* < 1.5 × 10⁻⁵, FDR < 0.0653). We calculated expression (log₂ fold) as the relative miRNA expression by comparing IFN-γ–treated and control conditions; expression (log₂ fold) = log₂(IFN-γ/control). Statistical analysis was done with edgeR.
and data not shown). IFN-γ suppressed the expression of members of the let-7 miRNA family (Fig. 8a and Supplementary Table 4), which suppress inflammation by directly targeting ILE mRNAs. This observation supports the hypothesis that IFN-γ augments the translation of inflammatory cytokines by suppressing miRNA expression. To analyze whether downregulation of additional miRNAs could contribute to increased TE of select transcripts, we integrated miRNA and ribosome profiling data to identify potential miRNA targets among all mRNAs with increased TE (Fig. 6a) on the basis of complementarity of 3' UTR sequences to miRNA seed sequences. We focused on miRNAs most significantly (P < 1.3 × 10⁻⁴, false discovery rate < 0.0653) downregulated by IFN-γ in TLR2-stimulated macrophages (Fig. 8a and Table 2). Of these miRNAs, a majority had potential target mRNAs with increased TE; miR-146b-3p, a TLR2-induced miRNA that was suppressed sixfold by IFN-γ, had several potential target mRNAs whose TE was increased by IFN-γ (Fig. 8b). Taken together, the results suggest that suppression of miRNA expression by IFN-γ may contribute to increased translation of certain mRNAs. Overall the results suggest that IFN-γ regulates human macrophage metabolism and mRNA translation in an integrated manner to specify an activated phenotype (Supplementary Fig. 8c).

**DISCUSSION**

The profound activating effects of IFN-γ on macrophages have been predominantly attributed to the transcriptional activation of ISGs, and its augmentation of inflammatory responses is attributed to signaling crosstalk and chromatin remodeling. In this study we found that IFN-γ altered macrophage metabolism by suppressing the central metabolic regulator mTORC1 and selectively altered mRNA translation to promote inflammation, further reprogram metabolic pathways and modulate cellular translational machinery. IFN-γ induced translational reprogramming by targeting mTORC1 and MNK pathways that are upstream of eIF4E, a factor that selectively regulates translation. Conversely, genome-wide analysis showed that translational regulation modulated the expression of genes important in metabolism. Thus, these newly described IFN-γ functions are tightly integrated to specify an activated macrophage phenotype. Inhibition of mTORC1 by pharmacological or genetic means has been shown to promote inflammation by suppressing STAT3 activity and to augment microbial killing and antigen presentation, in part by increasing autophagy. Thus, our findings provide insights into mechanisms by which physiological regulation of mTORC1 and translation by IFN-γ can contribute to hallmark IFN-γ functions such as boosting inflammatory responses and antimicrobial mechanisms. These findings also advance our understanding of the reprogramming of cell states by IFN-γ and add metabolism and translation to the range of affected functions.

The importance of cellular metabolism is well established for adaptive immunity and is emerging for innate immunity. Published work on innate immune cells has focused on TLR responses, which trigger a switch to aerobic glycolysis. Full inflammatory activation of macrophages by TLR ligands requires IFN-γ (refs. 1,43), and our signaling results and genome-wide analysis of translational regulation suggest that extensive metabolic reprogramming contributes to IFN-γ-mediated polarization of macrophages, which affects the way in which macrophages respond to subsequent environmental challenges.

IFN-γ suppressed mTORC1 in a physiological cell-culture model involving primary human macrophages that modeled the well-known ‘priming’ effects of IFN-γ, which enhance responses to inflammatory factors and increase microbial killing. Physiological regulation of mTORC1 by IFN-γ in human macrophages was partial but similar to that achieved by the allosteric mTORC1 inhibitor rapamycin. The degree of mTORC1 inhibition achieved by rapamycin, and thus IFN-γ, has potent effects on immune responses and has been shown to augment innate responses in vitro and in vivo in mice and humans, in part by augmenting inflammatory cytokine production and M1 polarization. Furthermore, partial mTORC1 suppression has been shown to enhance the host defense against Legionella pneumophila and pathogenic Shigella, in the latter case by augmenting autophagic clearance mechanisms. This work supports the biological importance of the newly described regulation of mTORC1 by IFN-γ and helps link our findings with canonical IFN-γ functions. Overall, our results are in accord with literature suggesting a suppressive role for mTORC1 in innate immunity. However, as a negative feedback loop induced by constitutive mTORC1 activity in TSC1-knockout mice can augment cytokine production, and as mTOR has been linked to trained innate immunity, the function of mTORC1 in innate immunity is context dependent and is likely to be determined by the absence or presence of an IFN-γ response and concomitant ISG expression.

Investigation of translational regulation by interferons has focused predominantly on type I interferons, which globally suppress translation by suppressing eIF2α and by direct effects of ISGs. Regulation of translation by IFN-γ has been investigated primarily in cell-line models of cellular transformation. In transformed cells, IFN-γ, and also type I interferon, directly and transiently activates Akt-mTORC1 and MNK-eIF4E signaling, which results in increased ISG mRNA translation and thus augmentation of the early phase of the interferon response. In contrast, we did not observe the early phase of Akt or MNK activation by IFN-γ in primary human macrophages; instead, IFN-γ suppressed Akt-mTORC1 and MNK-eIF4E at later time points by indirect mechanisms such as inhibition of M-CSFR expression. Thus, the effects of IFN-γ on Akt-mTORC1 and MNK-eIF4E signaling are dependent on cell context and increase with the duration of IFN-γ exposure. A contrast between the translational effects of IFN-γ and those of type I interferons is the selective regulation of translation of only a subset of expressed mRNAs by IFN-γ, which may be explained by selective regulation of translation by eIF4E. In contrast, suppression of eIF2α by type I interferons has more global effects. A modest increase in eIF2α activity by IFN-γ might contribute to enhanced translation of specific transcripts, as could downregulation of miRNAs that suppress translation. Overall our work establishes mechanisms by which IFN-γ downregulates translation and provides insights into mechanisms by which IFN-γ might augment the translation of select mRNAs.

This study describes the first (to our knowledge) comprehensive genome-wide translational-profiling analysis in primary immune cells and in terminally differentiated nonproliferating cells. Analysis of genome-wide data revealed several functions for IFN-γ-mediated translational regulation in macrophages. First, IFN-γ-induced changes in TE were superimposed on changes in mRNA abundance to selectively modulate the expression of different gene sets. Second, regulation of TE potentiated inflammatory activation by augmenting the production of inflammatory proteins (TNF, IL-6 and lymphotokins) while decreasing the production of anti-inflammatory feedback inhibitors (IL-10 and HES1). Third, by suppressing the expression of components of the translational machinery, IFN-γ could potentiate proposed ‘translational skewing’ toward highly expressed transcripts that favors host defense, although our genome-wide analysis showing selective translational regulation suggested that the translational-skewing model might need to be refined to take into account...
properties of specific transcripts. Fourth, translational regulation by IFN-γ broadly affected proteins in metabolic pathways. Fifth, IFN-γ-mediated translational downregulation of multiple tRNA synthetases might have important consequences for macrophage activation, as these proteins have various noncanonical functions independent of their aminocyl transferase activity.48

In summary, the present study shows that IFN-γ regulates mTORC1 and mRNA translation in an integrated manner in human macrophages. This regulation and associated changes in intracellular metabolism are linked with key IFN-γ functions such as potentiation of inflammatory activation and autophagy, which is related to microbial killing. These findings extend our understanding of the role of metabolic regulation in innate immune cell activation, identify new functions for translational regulation by IFN-γ and suggest approaches for targeting metabolic pathways and translation factors to modulate macrophage activation and function.

METHODS

Methods and any associated references are available in the online version of the paper.

Accession codes. GEO: ribosome profiling, RNA-Seq and miRNA-Seq data, GSE66810.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

X.S. designed and conducted experiments, analyzed data and prepared the manuscript; Y.Y. performed polysome-profiling and ribosome-profiling experiments and analyzed data; Y.Z. analyzed ribosome-profiling, RNA-Seq and miRNA-Seq data; E.G.G. analyzed ribosome-profiling and RNA-Seq data, GSE66810.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell culture. Primary human CD14+ monocytes were isolated from buffy coats purchased from the New York Blood Center with anti-CD14 magnetic beads (Miltenyi Biotec) as described; the protocol used was approved by the Hospital for Special Surgery Institutional Review Board. Monocytes were cultured in RPMI 1640 medium (Invitrogen) supplemented with 10% (vol/vol) FBS (Hyclone) and M-CSF (20 ng/ml). IFN-γ (100 U/ml) was added at the initiation of cultures, and cultures were maintained for at least 24 h before subsequent stimulation or collection of cells.

Reagents and antibodies. Human IFN-γ and S7 micrococcal nuclease were from Roche, Human M-CSF was from Peprotech, Pam3CSK4 was from EMCD Microcollections and Ultrapure Escherichia coli lipopolysaccharide was from Invivogen. MG132, CGP57380, rapamycin and PP242 were from Calbiochem. Cycloheximide, Bafilomycin A1, 1-methyl-D-tryptophan (1-D-MT), Cycloheximide, Bafilomycin A1, 1-methyl-D-tryptophan (1-D-MT), tetrodotoxin and 10058-F4 were from Sigma. Torin 1 was from R&D Systems. LYS 294002 was from EMD Millipore. Antibodies to the following proteins were purchased from Cell Signaling: mTOR (2983), p-TSC2(Thr1462) (3617), TSC2 (2855), p-Akt (Ser473) (4060), p-p70S6K(Thr421/Ser424) (9204), 4E-BP1 (9644), p-E-BP1(Thr37/46) (2585), LCA3 (4599), LC3B (3868), b-actin (9242), Mnk1(12195), p-Mnk1(Thr197/202) (2111), eIF4E (9742), p-eIF4E(Ser209) (9741), p-p38(Thr180/Tyr182) (9219), p-Erk1/2(Thr202/Tyr204) (9101), Erk 1/2 (9102), β-catenin (9622) and M-CSF (3152). Antibodies to p38 (sc-535), HES1 (sc-25392), TRP (sc-204), Lamp-1 (sc-20011) and Myc (sc-764) were from Santa Cruz Biotechnology. Antibodies to PABPC1 (ab16251), β-tubulin (ab11307) and leucyl-tRNA synthetase (ab31534) were from Abcam. A mIR/vana miRNA isolation kit was purchased from Ambion/Life Technologies.

RNA extraction and quantitative PCR. Total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen), and 500 ng of total RNA was reverse transcribed with the RevertAid First Strand cDNA Synthesis Kit (Fermentas). Real-time PCR was done in triplicate with Fast SYBR Green Master Mix and a 7500 Fast Real-time PCR system (Applied Biosystems).

Immunofluorescence staining. Primary human macrophages were cultured on coverslips coated with poly-D-lysine (BD Biosciences Discovery Labware) for 24 h in the presence or absence of IFN-γ (100 U/ml). Cells were fixed with 2% formaldehyde for 15 min at room temperature and were permeabilized with 0.1% Tween-20 at room temperature for 10 min. 5% goat serum (sc-2043; Santa Cruz) was used for blocking at 37 °C for 1 h, and then cells were stained with rabbit antibody to mTOR (2983 (7C10); Cell Signaling) and mouse antibody to Lamp-1 (1-HA3 sc-2001; Santa Cruz Biotechnology) simultaneously in a 4 °C cold room for at least 12 h. Secondary Alexa Fluor 488 goat anti-rabbit (Invitrogen) and Alexa Fluor 594 goat anti-mouse (Invitrogen) were then used to detect mTOR and LAMP-1 primary antibodies, respectively. Coverslips were mounted with Vectashield mounting medium (Vector Laboratories), and images were obtained with a Nikon Eclipse Microscope.

CBA assay. Culture supernatants of primary human macrophages as indicated in figure legends were collected and processed immediately or snap-frozen on dry ice for storage. Supernatant concentrations of secreted cytokines TNF, IL-6 and IL-10 were measured with the BD CBA Human Inflammatory Cytokines Kit according to the manufacturer’s instructions. Data were acquired on a BD FACSCanto flow cytometer, and results were analyzed with FCAP Array software.

HPLC–mass spectrometry analysis. For measurement of intracellular tryptophan and related metabolites, samples were processed and analyzed by the Donald B. and Catherine C. Marron Cancer Metabolism Center at the Memorial Sloan Kettering Cancer Center. We collected 10 million cells for each condition and quenched metabolism with 1 ml of 80% methanol supplemented with 2 μM D5-2HG. Protein was precipitated and removed by centrifugation. The samples were analyzed with an Agilent 6230 time-of-flight liquid chromatography–mass spectrometry instrument.

Ribosome profiling. To obtain enough material to generate high-quality ribosome footprints, we pooled human primary macrophage lysates from different blood donors (four donors for replicate 1 and six donors for replicate 2; Supplementary Fig. 5a). Control or IFN-γ–treated human primary macrophages were stimulated for 4 h with Pam3CSK4 (10 ng/ml) and then treated with cycloheximide (100 µg/ml) for 7 min. We used the same pools of lysates for both RPFs and RNA library construction. We followed a published protocol for ribosome profiling49, with the following modifications: S7 micrococcal nuclease (120 U/ml) was used to generate mRNA-associated monosomes, and the monosomes were separated on a sucrose-density gradient, with a subsequent sucrose-cushion purification step to minimize contamination with other protein-RNA complexes. A modified reverse-transcription primer

Preparation of nuclear extracts. Pefabloc (Roche, 0.5 mg/ml) was added to cells 15 min before cell collection. We obtained cytoplasmic and nuclear extracts by incubating cells in Buffer A (10 mM HEPES, pH 7.9, 1.5 mM MgCl2, 10 mM KCl, 1 mM DTT, supplemented with protease inhibitor cocktail (Roche, 1×)) for 15 min on ice. The plasma membrane was then solubilized by incubation with NP-40 (0.2%) for 2 min. The nuclear pellet was obtained by centrifugation at 10,000g for 30 s; supernatants corresponded to cytoplasmic lysates. We obtained nuclear lysates by directly lysing nuclear pellets in 1× SDS-PAGE loading buffer.

Immunoblotting. All samples for immunoblotting were denatured at 95 °C for at least 10 min. Denatured cell lysates were fractionated on 7.5%, 10% or 12% polyacrylamide gels with SDS-PAGE and transferred to polyvinylidene difluoride membranes for probing. Western Lightning Plus–ECL Enhanced Chemiluminescence Substrate (PerkinElmer) was used for detection. Images shown in figures were derived from one gel. In Figure 3h, intervening lanes were spliced out, and the white vertical line indicates lanes that were noncontiguous on the gel.

RNA interference. Immediately after isolation, primary human monocytes were nucleofected with On-Target plus SMARTpool siRNA purchased from Dharmacon Inc. (Lafayette, Colorado, USA) specific for MNK1, MNK2 or Myc. Non-targeting siRNA 5 was used as a control. Human Monocyte Nucleofector buffer (Lonza Cologne, Cologne, Germany) and the AMAXA Nucleofector System program Y001 for human monocytes were used according to the manufacturers’ instructions.
The human genome sequence hg19 (Home_Sapiens.GRCh37.75.gtf) was downloaded from the Archive Ensembl website (http://feb2014.archive.ensembl.org/downloads.html). A .gtf file that contained only protein-coding genes was extracted on the basis of features defined in the original .gtf file. Before alignment, the first seven degenerate nucleotides were trimmed from the 5′ end, and the polyA adaptor at the 3′ end was removed by Clip; low-quality reads were filtered out (mean quality score of <20). rRNA contamination of RPFs was removed by Bowtie2 with the default setting. Both RPF and RNA-Seq reads were aligned to the genome sequence with TopHat2. The BAM files generated from TopHat2 were annotated with HiTseq. Here we analyzed only protein-coding genes guided by the .gtf file described above. Genes that had fewer than 128 combined reads in two RNA libraries (control plus IFN-γ–primed condition) of each biological replicate were removed for the reasons previously discussed32,33. Reads that had multiple alignments were filtered out, and the c.p.m. value was calculated as c.p.m. = 10^6(Ci/N), where Ci is the number of reads mapped to exons of gene i and N is the number of mapped reads in the entire library, including multiple aligned reads. Data sets from each replicate were either analyzed individually or merged by summing the c.p.m. value for each condition. TE was calculated as the ratio of RPF to RNA (TE = RPF/RNA).

miRNA sequencing and data analysis. Control or IFN-γ–primed human primary macrophages were stimulated for 4 h with Pam3CSK4 (10 ng/ml) and then treated with cycloheximide (100 μg/ml) for 7 min. Cell pellets were lysed in polysome lysis buffer (20 mM Tris, pH 8.0, 140 mM NaCl, 15 mM MgCl2, 100 μg/ml cycloheximide, 0.5% Triton X-100 and protease inhibitor cocktail (Roche, 1×)) and then passed five to six times through a 26-gauge needle with a 1-ml syringe. Cell lysates were gently laid onto 10%–50% sucrose-density gradients. Gradients were centrifuged in an SW41 ultracentrifuge rotor at 35,000 r.p.m. for 2 h.

Statistics. Student’s t-test was used to analyze differences in experiments with two conditions; one-way ANOVA followed by Bonferroni’s multiple-comparison post-test was used for experiments with more than two conditions. Statistical analyses were done with GraphPad Prism 5.

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