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Type I IFN Inhibits Innate IL-10 Production in Macrophages through Histone Deacetylase 11 by Downregulating MicroRNA-145

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Innate immune responses must be tightly regulated to avoid overactivation and subsequent inflammatory damage to host tissue while eliminating invading pathogens. IL-10 is a crucial suppressor of inflammatory responses and its expression is under precise regulation involving complex regulatory networks and multiple feedback loops. MicroRNAs are now emerging as critical regulators in immune response. Our previous work showed that miR-143/145 cluster was markedly downregulated in macrophages upon vesicular stomatitis virus infection. However, the particular role of miR-143/145 cluster in the regulation of innate immune response remains unknown. In this study, we found that miR-143/145 cluster expression was also downregulated dramatically by TLR signals in macrophages, which was dependent on the subsequent type I IFN (IFN-I) production and downstream IFN-I receptor–JAK1–STAT1 signal cascade. Further studies demonstrated that miR-145, but not miR-143, promoted IL-10 expression in TLR4-triggered macrophages through directly targeting the epigenetic histone deacetylase 11. Therefore, we demonstrate that miR-145, downregulated by IFN-I, targets histone deacetylase 11 to promote innate IL-10 expression in macrophages. Our findings suggest a new IFN-I–mediated negative feedback loop in the fine-tuning of innate IL-10 production that creates precise coordination of innate immune responses. The Journal of Immunology, 2013, 191: 3896–3904.

Upon LPS stimulation, the intact production of IL-10 by macrophages requires the activation of both MyD88-dependent and Toll/IL-1R domain-containing adaptor protein inducing IFN-β (TRIF)–dependent signaling cascades. The activation of MyD88-dependent MAPKs (mainly ERK and p38) and NF-κB leads to the initial induction of IL-10, whereas TRIF-dependent production of type I IFNs (IFN-I) and its downstream signaling are essential for sustaining IL-10 production (3, 4). Complex regulatory networks and multiple feedback loops attribute to the precise regulation of innate IL-10 expression. For instance, by targeting MyD88-dependent signaling molecules, IL-10 itself mediates both positive and negative feedback loops to control its own production. IL-10 positive feedback upregulates the expression of tumor progression locus 2, the upstream activator of ERK, and thus provides a positive amplification loop (5). By contrast, IL-10 induces the expression of dual-specificity phosphatase-1, the negative regulator of p38 activation, contributing to a negative feedback loop (6). However, a regulatory network involved in TRIF–IFN-I–dependent IL-10 expression has not yet been described.

Histone deacetylase 11 (HDAC11), the recently identified member of the HDAC family (7), has been shown to function as a crucial epigenetic IL10 gene silencer (8). Through interaction with the distal segment of the promoter of IL10, HDAC11 leads to a less acetylated and more compact chromatin structure in IL10 promoter region with diminished accessibility, and thus blocks the binding of transcription factors necessary for IL10 transcription activation such as STAT3 to silence IL10 expression in mouse and human macrophages. However, to date, there is no report about the regulation of HDAC11 expression during innate immune response.

MicroRNAs (miRNAs) represent a class of highly conserved, small, noncoding RNAs that suppress gene expression by binding to the 3’-untranslational region (3’-UTR) of target mRNAs. miRNAs are key regulators of diverse biological processes, such as development (9), tumorigenesis (10), inflammation, immune response (11–14), and metabolism (15). miR-143 and miR-145 are derived
from a common primary transcript but have no homology with each other. The miR-143/145 cluster is now believed to be multifunctional miRNAs. For instance, both miR-143 and miR-145 act as tumor suppressors to promote cell apoptosis, modulate cell cycle, and thus inhibit tumor cell growth (16, 17); they also function as crucial modulators in smooth muscle cell differentiation and plasticity (18-20); in particular, miR-143 has been described to regulate glucose metabolism (21, 22), and miR-145 has been implicated as a critical proinflammatory molecule in Th2-mediated allergic inflammation (23). Despite that, the roles of miR-143/145 cluster in the regulation of innate immune responses are still largely unknown.

In this study, we focused on the roles of miR-143/145 cluster, the dramatically decreased miRNAs in macrophages upon vesicular stomatitis virus (VSV) challenge as predicted from our miRNA microarray data (24). Besides the RNA virus (VSV)—triggered retinoic acid–inducible gene 1 (RIG-I) signal, we found that TLR signals also significantly downregulated miR-143/145 cluster expression in macrophages, which depends on IFN-I production and subsequent IFN-I receptor (IFNAR)-JAK1–STAT1 signaling pathway. The functional studies demonstrated that miR-145, but not miR-143, promoted IL-10 expression in TLR4-triggered macrophages through directly targeting the epigenetic IL10 gene silencer HDAC11. In this study, we uncover the significance of miR-145 in the regulation of innate immune response. Moreover, our study also suggests an IFN-I–mediated negative regulatory loop in the regulation of innate IL-10 expression. IFN-I, induced by innate signals, feedback decreases miR-145 expression and thus alleviates the repression of its target HDAC11, thereby achieving to the limitation of IL-10 production and contributing to precise coordination of innate immune responses.

**Materials and Methods**

**Mice and reagents**

C57BL/6 mice (6–8 wk) were from Joint Ventures Sipper BK Experimental Animal Company (Shanghai, China). IFN regulatory factor 3 (IRF3) knockout mice were obtained from The Jackson Laboratory. All animal experiments were undertaken in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals, with the approval of the Scientific Investigation Board of Second Military Medical University (Shanghai, China). TLR ligands LPS, polyinosinic-polycytidylic acid (PIC), and CpG were described previously (24). The IL-10 promoter was amplified from the mouse genome DNA (premiR-145) and subcloned into the PGL3-enhancer vector (Promega) and subcloned into the PGL3-enhancer vector (Promega) (5). Forward primer and 5′-CGA GCC CGC TTG TAT GTT GTG ACA GCT TGT CTC-3′ for forward primer and 5′-CCC AAC AGG CTT AGA ACT TAC CCT GCT GTG CTG-3′ for reverse primer) and subcloned into the PGL3-enhancer vector (Promega) to generate IL-10 promoter. The IL-10 promoter was amplified from C57BL/6 mouse genome DNA and subcloned into the PGL3-enhancer vector to generate IL-10 pro, as described previously (26). PremiR-143 and premiR-145 were amplified from C57BL/6 mouse genome DNA (premiR-143 forward primer 5′-CGG CAA TTC TGG GTG GGT GAT CTA CAA GAA AGG-3′; premiR-143 reverse primer 5′-CGG CAA TTC TGG GTG GGT GAT CTA CAA GAA AGG-3′; premiR-145 forward primer 5′-CGG CAA TTC TGG GTG GGT GAT CTA CAA GAA AGG-3′; premiR-145 reverse primer 5′-CGG CAA TTC TGG GTG GGT GAT CTA CAA GAA AGG-3′) and subcloned into UBC-ires2-EGFP vector to generate miR-143 and miR-145 encoding plasmids (UBC-134 and UBC-145). The HDAC11 3′-UTR luciferase reporter construct was generated by amplifying the mouse HDAC11 mRNA 3′-UTR sequence or the human HDAC11 mRNA 3′-UTR sequence by PCR and then cloning into pMIR-REPORT Luciferase vector. The miHD11 3′-UTR Δseed or hHD11 3′-UTR Δseed was generated from the mouse HDAC11 3′-UTR or human HDAC11 3′-UTR by deleting the seed sequence of the potential miR-145 binding site. All vectors were verified by sequencing.

**Cell culture and transfection**

HEK293 cell line and murine macrophage cell line RAW264.7 were obtained from American Type Culture Collection and cultured as described previously (27, 28). A total of 1 × 10^6 cells were seeded into 96-well plates and incubated overnight. Jet-PRIME transfection reagent (Polyplus transfection) was used for transfection of plasmids or cotransfection of plasmids and RNAs according to the manufacturer’s instructions. Thioleucylolate-mixed mouse peritoneal exudate macrophages (PEMs) were prepared and cultured as described previously (27, 28). A total of 0.5 ml 2 × 10^5 cells was seeded into 24-well plates, or 2 ml 1 × 10^5 cells were seeded into 6-well plates and incubated overnight and then transfected as described previously (24). The ex vivo generation of human primary macrophages (monocyte-derived macrophages [mo-Mφs]) was performed as follows: CD14+ monocytes were isolated from PBMCs of healthy donors (Blood Center of Shanghai Hospital, Shanghai, China) by magnetic cell sorting using anti-CD14 microbeads (Miltenyi Biotech) and cultured in fresh complete medium supplemented with 20 ng/ml human M-CSF (R&D Systems). Half medium was replaced by fresh complete medium with M-CSF at days 3 and 5. On day 7, mo-Mφs were harvested and seeded into six-well plates (2 ml 1 × 10^6 cells), incubated overnight, and then transfected as described previously (24).

**miRNA mimics and inhibitors**

miR-143 mimics and miR-145 mimics (dsRNA oligonucleotides) from GenePhama (Shanghai, China) were used for the overexpression of miR-143 and miR-145 in macrophages. miR-143 inhibitor (anti–miR-143) and miR-145 inhibitor (anti–miR-145) (RNA oligonucleotides with Hairpin structure) from Thermo Scientific Dharamco were used for inhibition of endogenous miR-143 and miR-145 in macrophages. Macrophages described earlier were transfected with RNAs at a final concentration of 10 nM. Negative control mimics (GenePhama) or inhibitor (Thermo Scientific Dharamco) were transfected as matched controls.

**RNA interference**

The STAT1-specific small interfering RNA (siRNA; si-mSTAT1) was 5′-GGG CAG UAA AGU CAG AAA UTT-3′ (sense) and 5′-AUA UCU GAC UUU ACU GUC CTT-3′ (antisense). The HDAC11-specific siRNA (siHD11) was 5′-GGG AUC GCU UUG CUA AAG ATT-3′ (sense) and 5′-UCU UUA GCA AGG UCA CCC CTT-3′ (antisense). The scrambled control RNA sequences were described previously (24). siRNA duplexes were transfected into macrophages at a final concentration of 10 nM as described previously (24).

**RNA quantification**

Total RNA, containing miRNA, was extracted, reverse-transcribed, and real-time PCR was performed as described previously (24). For miRNA analysis, RT primers for miR-143, miR-145, and miR-16 were 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA CTG ACG AGC AT-3′, 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA CTG ACG AGC AT-3′, and 5′-GTC GTA TCC AGT GCA GGG TCC GAG GTA CTG ACG AGC AT-3′, respectively. Quantitative PCR (qPCR) primers were 5′-GCC GTA GAT GAA GCA ACC GCC AA-3′, and 5′-GCC GTA GAT GAA GCA ACC GCC AA-3′. Relative expression level of miRNA was obtained as described previously (24). For miR-143/145 analysis, qPCR primers were 5′-GCT TCC TGA CCT TAG GGA AGG AGG-3′ (forward) and 5′-AGG AGC CAG CAG TCC CTC C-3′ (reverse). For murine hypoxanthine guanine phosphoribosyl transferase (HPRT), IL-10, IL-12p40 mRNA analysis, the primers were 5′-TTG CTC...
GAG ATG TCA TGA AGG A-3′ (HPRT forward) and 5′-TGA GAG ATC ATC TCC ACC AAT AAC TT-3′ (HPRT reverse); 5′- GCT CTT ACT GAC TGG CAT GAG-3′ (IL-10 forward) and 5′-GGC AGC TCT AGG AGC ATG TG-3′ (IL-10 reverse); and 5′-AGA CAT GGA GTC ATA GGC TCT G-3′ (IL-12p40 forward) and 5′-CCA TTT TTC TCC TTC TGG AGC A-3′ (IL-12p40 reverse). For murine HDAC11 mRNA analysis, the primers were 5′-TAT CTG GAG AAG AGC AG-3′ (forward) and 5′-ATG CAA GTT GAG GAT GGA G-3′ (reverse). Data were normalized to the level of HPRT expression in each sample.

ELISA

Into each well of 24-well plates, 0.5 ml of 2 × 10⁵ cells were seeded, incubated overnight, and transfected as described earlier. After 48 h, the cells were stimulated with LPS for indicated time periods. The concentrations of IL-10 and IL-12p70 in culture supernatants were measured with ELISA kits (R&D Systems).

Western blot

The cells were washed twice with cold PBS and lysed with cell lysis buffer (Cell Signaling Technology) supplemented with protease inhibitor mixture (Culbiochem). Protein concentrations of the cell lysis extracts were measured with BCA assay (Pierce, Rockford, IL) and equalized with the extraction reagent. Equal amount of the extracts were loaded and subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and then blotted as described previously (27, 28).

Densitometric analysis

Densitometric analysis was done with Labworks Image Acquisition and Analysis Software (UVP, Upland, CA). The background was subtracted, and the signals of the detected bands were normalized to the amount of loading control bands. The relative values were presented as fold increase over control samples as indicated.

Statistical analysis

Statistical significance was calculated with the two-tailed Student t test, with p < 0.05 considered to be statistically significant.

Results

TLR and RIG-I innate signals downregulate miR-143/145 cluster expression in macrophages

Our previous studies show that miR-146a and miR-155, two of the markedly upregulated miRNAs in macrophages upon VSV infection, play important roles in the regulation of antiviral innate immunity (24, 25). In this study, we selected the miR-143/145 cluster, the dramatically decreased miRNAs in macrophages upon VSV challenge, as our subject of study (Fig. 1A). Compared with the expression of miR-16, the relatively nonaffected miRNA, the abundance of mature miR-143 and miR-145 in macrophages with VSV infection declined constantly and reached to the bottom at the 24-h time point (Fig. 1B), and the magnitude of miR-145 downregulation was much more significant than for miR-143. Besides RIG-I signal, we found that TLR3 and TLR4 signals could also significantly decrease miR-143/145 cluster level in the similar pattern, whereas TLR9 signal could only trigger a slight effect on their expression (Fig. 1C).

To explore the exact signaling pathway involved for the decrease of miR-143/145 cluster expression by the earlier innate signals, we applied specific kinase inhibitors, including inhibitors of NF-kB, ERK, JNK, and p38 and inhibitors of AKT and GSK-3α/β, and interestingly found that none of those inhibitors could attenuate the downregulation of miR-145 by TLR4 stimulation (Fig. 1D). The data suggest that activation of Myd88–NF-kB–MAPK or P38–AKT pathway is irrelevant to the downregulation of miR-143/145 cluster expression during innate response.

We next tested the TRIF–IRF3–IFN-β/α pathway. Interestingly, we found that the amount of IFN-β production was positively correlated with the decrease of miR-143/145 cluster expression in macrophages upon TLR3, TLR4, TLR9 stimulation or VSV infection (Fig. 1E), suggesting that IFN-β production induced by TLRs or RIG-I signal might be responsible for the downregulation of miR-143/145 cluster expression by innate signals.

Innate signal-triggered downregulation of miR-143/145 cluster depends on IFN-I production and its downstream JAK1-STAT1 signal cascade

To verify the role of IFN-I in the downregulation of miR-143/145 cluster in TLR-triggered macrophages, macrophages from IFN3 or IFNAR-deficient mice were prepared and stimulated with LPS. Then we found that IRF3 or IFNAR-deficiency could significantly decrease miR-143 and miR-16, shown as positive control. (B and C) PEMs were infected with VSV (MOI = 10) (B) or challenged with LPS (100 ng/ml), PIC (10 μg/ml), or CpG-ODG (2 μM) (C) for indicated times. The expression of mature miR-143, miR-145, and miR-16 were measured by qPCR and normalized to the expression of U6 in each sample. (D) PEMs were pretreated with NF-κB inhibitor pyrrolidinedithiocarbamic acid (100 μM), ERK inhibitor PD98059 (10 μM), p38 inhibitor SB203580 (5 μM), or JNK inhibitor SP600125 (10 μM), respectively, or pretreated with AKT inhibitor 124008 (1 μM) or GSK-3α/β inhibitor SB216763 (10 μM) for 30 min, and then stimulated with LPS (100 ng/ml) for 24 h. Mature miR-145 was measured by qPCR. (E) A total of 0.5 ml of 4 × 10⁶ PEMs was stimulated with LPS (100 ng/ml), PIC (10 μg/ml), CpG-ODG (2 μM), or infected with VSV (MOI 10) for indicated times. IFN-β in supernatant was measured by ELISA. Data are shown as mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, p = NS.
production are responsible for the downregulation of the miR-143/145 cluster in TLR4-triggered macrophages. Furthermore, detection of miR-143/145 cluster primary transcript indicates that IFN-I mediates the downregulation of miR-143/145 cluster mainly at the transcriptional level, as pri-miR-143/145 cluster decreased dramatically upon rmlIFN-β treatment, with the time phase earlier than that of mature ones (Fig. 2C).

Next, we applied cycloheximide, the protein biosynthesis inhibitor, to test whether the regulation of miR-143/145 cluster mediated by IFN-I depends on new protein synthesis. As shown in Fig. 2D, pretreatment with cycloheximide did not rescue the downregulation of mature miR-145 triggered by IFN-β, suggesting that the decrease of miR-143/145 cluster is directly induced by IFN-I signal. Furthermore, to clarify the detailed signal pathway, we applied specific JAK1 and STAT1 inhibitors to determine whether the cardinal pathway that transmits IFN signals is getting involved. As shown in Fig. 2E, pretreatment with ruxolitinib or fludarabine completely disrupted the decrease of miR-145 by IFN-β. In addition, siRNA-mediated specific STAT1 knockdown also attenuated the decrease of miR-145 expression upon IFN-β stimulation (Fig. 2F), which was consistent with the data of fludarabine. These data suggest that the downregulation of miR-143/145 cluster by IFN-I depends on the JAK1-STAT1 cascade.

Knockdown of miR-145 suppresses IL-10 expression but promotes IL-12 expression in TLR4-triggered macrophages

To further investigate the roles the miR-143/145 cluster might play in macrophages with TLR4 ligation, we next applied inhibitors of miR-143 and miR-145 to examine the effect of miR-143/145 cluster on the expression of the signature molecules produced by TLR4-activated macrophages, including proinflammatory cytokines (TNF-α, IL-6, and IL-12), IFN-I (IFN-β), anti-inflammatory cytokine (IL-10), chemokines (CCL3 and CCL5), and inducible NO synthase. As shown in Fig. 3A, transfection of anti–miR-143 or anti–miR-145 significantly decreased miR-143 or miR-145 expression level, respectively, and inhibition of one of the two miRNAs had no effect on the other miRNA expression. Based on the inhibition experiments, we found that knockdown of miR-145, but not miR-143, led to repression of IL-10 expression and promotion of IL-12 expression in TLR4-triggered macrophages at both the mRNA and the protein level (Fig. 3B, 3C). However, neither miR-143 nor miR-145 inhibition had a significant effect on the expression of the other products described earlier (data not shown).

Ectopic miR-145 expression promotes IL-10 expression at transcription level

To further confirm the biological function of miR-145 in TLR4-triggered macrophages described earlier, we next transfected miR-143 mimics or miR-145 mimics into macrophages, respectively (Fig. 4A), and then found that overexpression of miR-145, but not miR-143, promoted TLR4-triggered IL-10 production and simultaneously suppressed IL-12p70 production (Fig. 4C). As to the mRNA level, however, IL-10 mRNA level was upregulated in LPS-stimulated macrophages with ectopic miR-145, whereas IL-12p40 mRNA expression unexpectedly made no difference (Fig. 4B).

To address whether miR-145 influences transcription activity of the Il10 and/or Il12p40 promoter, we cotransfected RAW264.7 cells with a reporter gene containing the Il10 or Il12p40 promoter fused to a luciferase gene (Il10 pro or Il12b pro) and simultaneously plasmid encoding miR-145 (UBC-145), and then stimulated them with LPS. There was a significant elevation of luciferase activity in cells transfected with Il10 reporter gene (Il10 pro) and UBC-145, no matter with or without LPS stimulation. On the contrary, luciferase activity in cells transfected with Il12p40 pro and UBC-
miR-145 had no change (Fig. 4D). Taken together, the results presented earlier demonstrated that miR-145 promoted \textit{Il10} expression by markedly enhancing the transcription activity of \textit{Il10} gene, and the alteration in IL-12 production might be the secondary effect.

\textbf{miR-145 targets HDAC11}

As we know, after TLR stimulation, activation of ERK (29, 30) or p38 (31, 32) kinase in the MAPK cascade, NF-\textit{k}B (33), as well as GSK-3\textit{a/\beta} (34) in the PI3K/AKT pathway, modulates IL-10 expression in macrophages. However, we detected little effect of miR-145 overexpression on phosphorylation of the kinases described earlier (Fig. 5A), suggesting that the target(s) of miR-145 might be the molecule(s) located downstream of those kinases, perhaps mainly in the nucleus. Up to now, molecules in the nucleus, such as the epigenetic \textit{Il10} gene silencer HDAC11, transcription factors MHC CIITA in mouse dendritic cells (35), STAT1 in human monocytes (36), and B cell lymphoma 3 in mouse macrophages (37), have been shown to negatively regulate the expression of IL-10. To identify the molecular target(s) of miR-145 in the modulation of IL-10 production in macrophages triggered by LPS stimulation, the computational prediction software miranda (http://www.microrna.org) for prediction of miRNA targets was next used. Among the known nuclear negative regulators of \textit{Il10} expression, we found that only mouse HDAC11 had one putative miR-145 target site (Fig. 5B, left panel). In addition, the HDAC11 phenotype presented in the previous study (8) was consistent with that of miR-145. So we predicted that HDAC11 might be the most potential target of miR-145.

To certify the possibility that HDAC11 was regulated post-transcriptionally by miR-145, we generated reporter plasmid by cloning 1.4 kb of the 3'UTR from mouse HDAC11 to the 3'UTR region of firefly luciferase gene. By cotransfecting HEK293 cells with reporter gene with mHD11–3'UTR and plasmid encoding miR-145, we observed that, instead of miR-143, miR-145 significantly decreased the luciferase activity of reporter gene with mHD11–3'UTR. However, the effect of miR-145 on the luciferase activity disappeared when reporter plasmid containing mHD11–3'UTR with seed deleted mutant (deletion of the corresponding target site of miR-145 seed region in mHD11–3'UTR) was applied (Fig. 5B, right panel). Furthermore, compared with miR-143, miR-145 overexpression in RAW264.7 cells led to decreased HDAC11 protein level (Fig. 5C, right panel), whereas no alteration in the HDAC11 mRNA level (Fig. 5C, left panel) suggested that miR-145 regulates HDAC11 expression via inhibition of HDAC11 mRNA translation. Conversely, we applied the miR-145 inhibitor (anti-miR-145) to specifically inhibit endogenous miR-145 in mouse PEMs and found that HDAC11 protein level increased upon miR-145 inhibition (Fig. 5D), which further confirms that HDAC11 is a target of miR-145.
target site, as miR-145–mediated inhibition of luciferase activity of reporter gene with hHD11–3'-UTR was completely disrupted upon seed deleted mutation (Fig. 5E, right panel). More importantly, we also applied human primary mo-MØs and evaluated HDAC11 protein level upon ectopic miR-145 expression. The repressed HDAC11 protein expression in miR-145–overexpressed mo-MØs (Fig. 5F) further confirms that human HDAC11 is targeted by miR-145. The same results were also obtained in human monocytic cell line THP1 cells (data not shown).

Knockdown of HDAC11 promotes IL-10 production in TLR4-triggered macrophages

As shown in Fig. 6A, siRNA specific to HDAC11 significantly inhibited the expression of HDAC11. Consistent with the report (8), knockdown of HDAC11 expression mimicked the phenotype of miR-145 overexpression both at the mRNA level (Fig. 6B) and the protein level (Fig. 6C), including increased IL-10 expression, decreased IL-12 production, and no change in other signature molecules (data not shown).

After LPS stimulation, as described in Fig. 1A, the expression of miR-145 in macrophages declined in a time-dependent manner. Therefore, we intended to figure out dynamic change of HDAC11 mRNA and protein level in macrophages upon LPS challenge. As shown in Fig. 6D, after TLR4 ligation, HDAC11 mRNA level decreased immediately at first, then later on increased constantly. The similar kinetic change was exhibited in HDAC11 protein level (Fig. 6E). Of note, despite the relatively lower HDAC11 mRNA level (constant lower than the initial level at 0 time point in 24 h) after LPS treatment, HDAC11 protein level significantly increased at the late-phase time (12–24 h) with higher amounts compared with the initial level, suggesting the posttranscriptional regulation of HDAC11 expression exists in the late phase. In addition, the same kinetic change also occurred in HDAC11 protein level in macrophages treated with miIFN-β (Fig. 6F). Therefore, the dynamic increase of HDAC11 protein happened to be in line with the kinetic decrease of miR-145, further confirming that HDAC11 is a target of miR-145.

In conclusion, we demonstrate that miR-145 targets HDAC11 to promote innate IL-10 expression in macrophages, and IFN-I limits IL-10 production through suppressing miR-145–mediated inhibition of HDAC11. Moreover, we describe a negative feedback loop mediated by IFN-I in the fine-tuning of innate IL-10 expression, as shown in Fig. 7.

Discussion

To date, there are two negative feedback loops described in the negative regulation of innate IL-10 production. Except for the one mediated by IL-10 itself, the other one is mediated by IFN-γ. Through directly blocking TLR4-triggered MAPK activation, IFN-γ inhibits ERK- and p38-dependent IL-10 production (34). In contrast, through antagonizing PI3K-AKT activation, IFN-γ inhibits TLR-induced IL-10 production by suppressing the binding of AP-1 to the Il10 promoter (34). In this article, we present a third negative feedback loop mediated by IFN-I in the regulation of IL-10 production. Based on what we found, the particular event involved is, the inducible IFN-I upon innate signals inhibits the transcription of the miR-145 gene, which leads to the decrease of miR-145, and thus alleviates the repression of its target HDAC11. Ultimately, the increased HDAC11 ceases IL-10 production through its epigenetic inhibition of the Il10 gene. For the first time, to our knowledge, we describe IFN-I–mediated negative feedback loop in fine-tuning of innate IL-10 production, achieved by suppressing miR-145–mediated inhibition of HDAC11.

As shown in Fig. 5E (left panel), a potential miR-145 target site (matched with miR-145 seed region) existed in hHDAC11 3'-UTR, which indicates that miR-145 might also target human HDAC11. We next generated reporter plasmid containing hHD11–3'-UTR and the corresponding seed deleted mutant (hHD11–3'-UTR Δseed), and performed the luciferase gene reporter experiment as described in Fig. 5B. The results demonstrated that miR-145 directly targeted human HDAC11 through the potential miR-145
miR-145 INCREASES IL-10 BY TARGETING HDAC11

FIGURE 5. miR-145 targets HDAC11. (A) PEMs were transfected with Ctrl or miR-145 mimics for 48 h and then stimulated with LPS (100 ng/ml) for 0–60 min. For immunoblot analysis of phosphorylated ERK1/2, phosphorylated p38, phosphorylated IκBα, phosphorylated Akt, or phosphorylated GSK-3β in lysates of these samples, β-actin protein served as the loading controls. Data are representative of three experiments. (B) Mouse HDAC11 might be molecular target of mmu-miR-145. Shown is a sequence alignment of miR-145 and its target site in 3′-UTR of Hdac11, which was downloaded from miRanda (http://www.microrna.org; left panel). Luciferase activity of lysates of HEK293 cells cotransfected with plasmid containing firefly luciferase reporter gene plus mHdac11 3′-UTR or mHdac11 3′-UTR Δseed, and UBC plasmid encoding miR-145 (UBC-145) or miR-143 (UBC-143) or empty plasmid (UBC), then analyzed after 36 h. Firefly luciferase activity was measured and normalized to Renilla luciferase activity (right panel). (C) qPCR analysis of mHdac11 RNA (left panel) and immunoblot analysis of HDAC11 protein (right panel) in RAW264.7 cells transfected with plasmids described in (B) as UBC-145, UBC-143, or UBC for 36 h. (D) Immunoblot analysis of HDAC11 protein in mouse PEMs transfected with Ctrl inhibitor, anti–miR-145, or anti–miR-143 as described in Fig. 3. (E) hsa-miR-145 might target human HDAC11. Shown is a sequence alignment of miR-145 and its potential target site in 3′-UTR of HDAC11 (left panel). Luciferase activity of lysates of HEK293 cells cotransfected with plasmid containing reporter gene plus hHdac11 3′-UTR or hHdac11 3′-UTR Δseed, and UBC-145 or UBC-143 or UBC, as described in (B) (right panel). Data in (B) and (E) are shown as mean ± SD. n = 6 of one representative experiment. Similar results were obtained in three independent experiments. *p < 0.01. (F) Immunoblot analysis of HDAC11 protein in human primary mo-Mφ transfected with Ctrl, miR-145, or miR-143 mimics as described in Fig. 4. β-Actin (C, D) or GAPDH (F) served as the loading controls. Bar graphs in (C), (D), and (F) indicated densitometric quantification of HDAC11 protein levels relative to the corresponding β-actin or GAPDH expression, respectively. Data in (C), (D), and (F) are representative of three experiments.

Professional phagocytes such as macrophages phagocytose apoptotic cells to prevent inflammation, and during the phagocytosis of apoptotic cells, a large amount of IL-10 is produced by macrophages to ensure cellular homeostasis (38). According to the fact that miR-145 can promote apoptosis as a tumor suppressor, the promotion of IL-10 expression exerted by miR-145 in our case might be the second effect in response to apoptotic cells induced by miR-145 overexpression. The concerned possibility can be excluded by the following three proofs. First, we found that apoptosis barely occurred in macrophages with ectopic miR-145, and there was no significant difference in comparison with the control group or miR-143 overexpression group (data not shown). Second, the data from previous studies (39, 40) indicate that the activities of p38 and ERK MAPK are crucial for apoptotic cell–induced IL-10 transcription and protein production; and as shown in Fig. 4A, we did not detect significant activation of p38 or ERK in macrophages with ectopic miR-145 (at 0 time point). Finally, the data from inhibition of endogenous miR-145 conversely support the conclusion that miR-145 promotes IL-10 expression in a direct manner.

Chromatin accessibility in genes is influenced by the acetylation status of their promoters. Generally speaking, histone acetylation is associated with transcriptionally active chromatin, whereas histone deacetylation mediated by HDAC proteins leads to an inactive chromatin (41). HDAC11, the recently identified member of the HDAC family, has been identified as an epigenetic modifier (8). Its involvement in the regulation of HDAC11 gene transcription is well established. However, little is known about the regulation of HDAC11 expression during its fine-tuning of IL-10 expression. To our knowledge, our study first identified that HDAC11 was negatively regulated by miR-145 at the posttranscriptional level. In addition, we found that HDAC11 was deceased immediately in response to TLR4 ligation at both the mRNA and the protein level, which was comprehensible because its elimination and accompanying high acetylation status of Il10 promoter were required for transcription activation of Il10.
are representative of three experiments.

In addition to the production of proinflammatory cytokines and IFN-I (1), which alleviates the epigenetic inhibition of the Il10 gene. The innate signals also suppress the expression of HDAC11 in an unknown manner (3). Ultimately, the increased amounts of HDAC11 result in the cessation of IL-10 expression through its epigenetic inhibition of Il10 gene (5). Of note, in early phase, innate signals also suppress the expression of HDAC11 in an unknown manner (?), which alleviates the epigenetic inhibition of the Il10 gene. The early phase and the late phase of the innate response are indicated as open and solid bars, respectively.

miRNAs have been thought to target multiple targets. A single miRNA might tune thousands of genes expression by direct or indirect effects (42). In our study, among the known nuclear negative regulators of IL-10 expression, we fortunately identified HDAC11 as a critical target of miR-145, but we are not able to exclude the possibility that other unknown nuclear negative regulator(s) might also be involved as the target(s) of miR-145. Thus, much more work needs to be done for unveiling other targets of miR-145 in the fine-tuning of IL-10 expression.

Both in vitro and in vivo studies have described the crucial roles of miR-145, as well as miR-143, in smooth muscle cell plasticity (18–20). Vascular smooth muscle in miR-145 knockout mice display impaired contractile capabilities (20) and poor responsiveness to vascular injury (19), and thus create a vulnerable cardiovascular system. Given that conventional miR-145 knockout might have biased effects on the in vivo importance of miR-145 in immune response, macrophage-specific miR-145 conditional knockout mice would be more preferable in our future in vivo study. Notably, the previous work presented by Collison et al. (23) describes the roles of miR-145 in the regulation of the airways allergic inflammation. In their study, an experimental mouse model that mimics hallmark features of allergic asthma was used. By inhibition of miR-145 with antagonirs in vivo, Collison et al. (23) found that miR-145 served as a critical proinflammatory molecule in Th2-mediated allergic inflammation. On the contrary, our work suggests that miR-145 functions as an anti-inflammatory molecule in macrophage-involved immune defense through targeting the epigenetic Il10 gene silencer HDAC11. Distinct targets or targetome of miR-145 in different cell types or cell statuses might contribute to the distinct functions and even opposite roles in different physiological or pathological circumstances. In addition, the whole-body inhibition of miR-145 with antagonirs suggests a global effect of all cells in vivo, but not the effect of individual cell type. For our study, the in vivo effect of miR-145 in macrophage-involved immune defense should be further investigated in our future work.

The immunomodulatory functions of IFN-I have been increasingly attracting attention in recent years (43–45). One manifestation of their immunomodulatory potential is that IFN-I promotes the expression of IL-10, which has been shown in both mouse and human APCs (46, 47). The involved mechanisms seem diverse depending on the responding cell types and the stimuli used. IFN-α–induced activation of STAT1 and IRF1 is responsible for the production of IL-10 by human monocytes (46), whereas IFN-β stimulates IL-10 production in human dendritic cells by activating JAK1 and PI3K pathways (47). In this article, we proposed a regulatory network involved in IFN-I–dependent IL-10 expression. IFN-I promotes the expression of IL-10, representing the direct, rapid, feed-forward motif. Concomitantly, IFN-I suppresses miR-145–mediated inhibition of HDAC11 to limit IL-10 production, representing the indirect, slow, feed-backward motif. Thus, activation of both motifs is required for IFN-I to fine-tune IL-10 production that gives rise to precise coordination of innate responses. Moreover, perhaps the strategies that target the miR-145–HDAC11 regulatory elements can be used to modulate therapeutic

FIGURE 6. Knockdown of HDAC11 promotes IL-10 production in TLR4-triggered macrophages. (A) PEMs (1 × 10⁵) were transfected with Ctrl RNA or HDAC11 siRNA as indicated at a final concentration of 10 nM. After 48 h, HDAC11 and β-actin were detected by immunoblot. (B) PEMs were transfected as indicated in (A). Forty-eight hours later, PEMs were stimulated with LPS (100 ng/ml) for indicated times. Expression of IL-10 mRNA (upper panel) and IL-12p40 mRNA (lower panel) was measured by qPCR. (C) A total of 0.5 ml of 3 × 10⁵ PEMs was transfected as described in (A). Forty-eight hours later, PEMs were stimulated by LPS (100 ng/ml) for 24 h. IL-10 (upper panel) and IL-12p70 (lower panel) in supernatants were measured by ELISA. Data in (B) and (C) are shown as mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01.

FIGURE 7. A model depicting IFN-I–mediated negative feedback loop in the regulation of innate IL-10 expression. Immune signals, like TLR or RIG-I signal, directly induce the expression of IL-10 (1) in macrophages, in addition to the production of proinflammatory cytokines and IFN-I (5). The inducible IFN-I feedback initiates the secondary induction of IL-10 production as reported earlier (2). Concomitantly, IFN-I inhibits the transcription of miR-145 gene in a JAK1-STAT1–dependent manner (3), which leads to the decrease of mature miR-145 in late phase, and thus alleviates the repression of its target HDAC11 as we observed (4). Ultimately, the increased amounts of HDAC11 result in the cessation of IL-10 expression through its epigenetic inhibition of Il10 gene (5).
efficacy of IFN-I as anti-inflammatory drugs in inflammatory and autoimmune diseases.

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