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This information is current as of October 19, 2021.

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J Immunol 2014; 193:3036-3044; Prepublished online 4 August 2014;

doi: 10.4049/jimmunol.1302379

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The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



IFN- γ Primes Macrophage Activation by Increasing Phosphatase and Tensin Homolog via Downregulation of miR-3473b

Cong Wu,^{*,†,1} Yiquan Xue,^{*,1} Pin Wang,^{*,1} Li Lin,[‡] Qiuyan Liu,^{*} Nan Li,^{*} Junfang Xu,[†] and Xuetao Cao^{*,†,‡}

The classical activation of macrophages, one of major innate effector cells, requires IFN- γ pretreatment (priming) and subsequent TLR stimuli (triggering). The priming effect of IFN- γ can promote macrophages to secrete higher level of proinflammatory cytokines but lower level of the anti-inflammatory cytokines, enhancing microbicidal and tumoricidal activity of macrophages. However, the underlying molecular mechanisms for IFN- γ -priming effect on macrophage activation remain to be fully understood. microRNAs (miRNAs) are now emerging as important regulators in immune response, including signaling transduction in immune cell function. In this study, we explored the effect of IFN- γ on miRNA expression profiling in macrophages and tried to identify the definite miRNA involved in the priming effect of IFN- γ . We discovered that miR-3473b, which was significantly downregulated after IFN- γ priming, could attenuate the priming effect of IFN- γ . miR-3473b promoted Akt/glycogen synthase kinase 3 signaling and IL-10 production through directly targeting phosphatase and tensin homolog (PTEN) to suppress activation of macrophages and inflammatory response. Our data indicate that IFN- γ beefs up macrophage innate response and cytotoxicity by downregulating miR-3473b to release PTEN from suppression, and then the increase of PTEN contributes to the full activation of IFN- γ -primed macrophages. Our results provide mechanistic insight to priming effect of IFN- γ on macrophage classical activation by identifying an IFN- γ /miR-3473b/PTEN regulatory loop in the regulation of macrophage function. *The Journal of Immunology*, 2014, 193: 3036–3044.

Interferon- γ is one of the most crucial cytokines in both innate and adaptive immune responses (1), whose expression regulation has already been intensively investigated (2, 3). Under the stress condition or pathogen infection, IFN- γ , mainly produced by NK cells and CD4⁺ T cells (4), can beef up macrophage activation, inducing high level of proinflammatory cytokines and low level of anti-inflammatory cytokines to further promote inflammatory innate response and subsequent immune response, meanwhile enhancing macrophage phagocytic ability and cytolytic potential to eliminate the invading intracellular pathogen (5, 6). This effect of IFN- γ is commonly referred to as priming activity, and the IFN- γ -assisted macrophage activation is called classical activation of macrophages (7).

Macrophages are important immune effector cells whose activation is tightly regulated (8, 9). IL-10 production is one important suppressor loop for macrophage activation in immune response (10). It can prevent overactivation of immune response favorably, but also mediate tumor-educated macrophage dysfunction pathologically. It has been reported that IFN- γ priming could disrupt IL-10 secretion through Akt/glycogen synthase kinase 3 (GSK3) signaling to promote inflammatory response and macrophage activation (11). But the mechanism of IFN- γ mediating the regulation of Akt/GSK3 signaling and IL-10 remains to be fully understood. Also, pretreatment with IFN- γ (IFN- γ priming) could not alter macrophage cytokine secretion before LPS activation. Therefore, it seems that IFN- γ priming makes macrophage prepared for activation. What is the underlying mechanism for IFN- γ -priming effect? It is presumed that IFN- γ priming probably induces some internal changes in macrophages, perhaps at post-transcriptional level and/or epigenetic level, which are responsible for subsequent robust TLR ligand-triggered inflammatory response and classical macrophage activation (12, 13).

MicroRNAs (miRNAs) are conserved small noncoding RNAs that play a critical role in diverse biological processes. They are important posttranscriptional regulators through targeting mRNA 3'-untranslational region (3'-UTR) to induce mRNA degradation (14). Up to now, miRNAs have already been considered as important controllers of immune response (15, 16). Many miRNAs have been identified to regulate signaling transduction in macrophages, like miR-125b (17) and miR-155 (18). And more and more cytokines have been found to function, at least partially, through miRNA. For example, type I IFN downregulates miR-378 to facilitate human NK cell activation (19), and TNF- α induces miR-155 expression in macrophages to promote immune response (20). However, little is known about what kinds of miRNAs could

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Received for publication September 5, 2013. Accepted for publication July 10, 2014.

This work was supported by National Key Basic Research Program of China Grant 2013CB530502, National Natural Science Foundation of China Grants 31390431, 81230074, and 81123006, and National 125 Key Project Grant 2012ZX10002-014.

The sequences presented in this article have been submitted to Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE50569.

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Abbreviations used in this article: BMM, bone marrow-derived macrophage; GSK3, glycogen synthase kinase 3; miRNA, microRNA; PIP3, phosphatidylinositol 3,4,5 triphosphate; PTEN, phosphatase and tensin homolog; siRNA, small interfering RNA; UTR, untranslational region.

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be regulated by IFN- γ and what their roles are in IFN- γ function, like the priming effect on macrophage classical activation, although IFN- γ expression has been proved to be regulated by miR-29 (21).

With the advantage of high-through techniques, such as miRNA sequencing and miRNA microarray, we have explored the miRNA expression profiles in vesicular stomatitis virus-infected macrophages and IFN- α -activated human NK cells and investigated the role of miRNAs in innate antiviral immunity and type I IFN function (18, 19). In this study, we profiled the miRNA expression in bone marrow-derived macrophages (BMMs) by microarrays before and after IFN- γ treatment to identify miRNAs regulated by IFN- γ and involved in IFN- γ -priming activity. We found miR-3473b was significantly downregulated in the IFN- γ -primed macrophages, and overexpression of miR-3473b attenuated priming effect of IFN- γ and the cytotoxicity of TLR-activated macrophages. On the other side, miR-3473b antagomir augmented proinflammatory cytokine production and interrupted anti-inflammatory cytokine production in macrophages, phenocopying the effect of IFN- γ -priming effect. Furthermore, we found miR-3473b could augment Akt/GSK3 phosphorylation, leading to the elevated IL-10 production. Also, PI3K signaling inhibitor or IL-10 deficiency abrogated the effect of miR-3473b on macrophage activation, demonstrating miR-3473b functions through PI3K/Akt/GSK3/IL-10. Moreover, we demonstrated that miR-3473b directly suppressed phosphatase and tensin homolog (PTEN) expression, a known antagonist to PI3K, and PTEN knockdown in macrophages phenocopied the effect of miR-3473b on BMM activation. Therefore, downregulation of miR-3473b expression by IFN- γ pretreatment can increase PTEN expression and consequently suppress the Akt/GSK3/IL-10 feedback inhibitory loop, thus contributing to macrophage classical activation.

Materials and Methods

Mice and reagents

Female C57BL/6 mice (4–6 wk), BALB/c mice, and nude mice (6–8 wk) were purchased from Joint Ventures Sipper BK Experimental Animal (Shanghai, China). All animal experiments were performed 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). LPS (*Escherichia coli*, O26:B6) was purchased from Sigma-Aldrich (St. Louis, MO). Recombinant mouse M-CSF, IFN- γ , and recombinant human M-CSF, IFN- γ were from R&D Systems (Minneapolis, MN). Annexin V FITC and propidium iodide were from Invitrogen. Abs specific to Akt, PTEN, p-Akt, and p-GSK3 were from CST. PI3K inhibitor GDC0941 was purchased from Selleckchem. Akt inhibitor 124008 was from Calbiochem. F4/80-PerCP, CD11b-allophycocyanin, CD80-PE, CD86-PE, CD40-PE, and Ia^b-PE were from BD Pharmingen (San Diego, CA).

Cell culture and transfection

HEK293T and EL4 cells were obtained from American Type Culture Collection and cultured at 37°C with 5% CO₂ in DMEM or RPMI 1640 supplemented with 10% FBS. BMMs were generated, as previously described, with moderate modification (22). Briefly, bone marrow progenitors were cultured for 72 h in DMEM supplemented with 10% FBS and 20 ng/ml murine M-CSF. Nonadherent cells were gently removed; the remaining adherent cells were cultured for an additional 3 d. A total of 4×10^5 cells was seeded into each well of 24-well plates, or 1×10^6 cells were seeded into each well of 6-well plates and incubated overnight and then transfected, according to the manufacturer's instruction, for 24 h before use.

miRNA microarray assay

The miRNA microarray assay was conducted by LC Sciences. In brief, the assay was performed on 2–5 μ g total RNA samples from normal BMMs (labeled by Cy3) and BMMs primed by IFN- γ (100 U/ml) for 4 h (labeled by Cy5). The small RNAs (<200 nt) were 3' extended with apolyadenylate tail using polyadenylate (poly A) polymerase and then ligated to an

oligonucleotide tag for later staining. Hybridization was done overnight on a μ Parafluo microfluidic chip using a microcirculation pump (Atactic Technologies). After hybridization, images were collected and quantified. The microarray data had been deposited on Gene Expression Omnibus public database (<http://www.ncbi.nlm.nih.gov/geo/>) under the accession number GSE50569.

RNA quantification

Total RNA containing miRNA was extracted and reverse transcribed, and quantitative real-time PCR was performed, as described previously (18). For miRNA analysis, reverse transcription primers for mmu-miR-3473b were 5'-GTC GTA TCC AGT GCA GGG TCC GAG GTA TTC GCA CTG GAT ACG ACC TGA GC-3'. Real-time PCR primers were 5'-ATA ATG GGG CTG GAG AGA TG-3' (forward) and 5'-GTG CAG GGT CCG AGG T-3' (reverse). U6 small nuclear RNA was quantified using its reverse primer for reverse transcription reaction and its forward and reverse primers for quantitative PCR, which were 5'-CTC GCT TCG GCA GCA CA-3' (forward) and 5'-AAC GCT TCA CGA ATT TGC GT-3' (reverse). The relative expression level of miRNAs was normalized to that of internal control U6 by using $2^{-\Delta\Delta C_t}$ cycle threshold method and then multiplied with a certain value (23).

RNA interference, miRNA mimics, and antagomir

miR-3473b mimic (dsRNA oligonucleotides) and miR-3473b antagomir (single-stranded chemically modified oligonucleotides) from GenePharma (Shanghai, China) were used for the overexpression and inhibition of miR-3473b in murine macrophages, respectively (18). Macrophages described above were transfected with miR-3473b mimic or miR-3473b antagomir at a final concentration of 10 nM, and negative control mimic or control

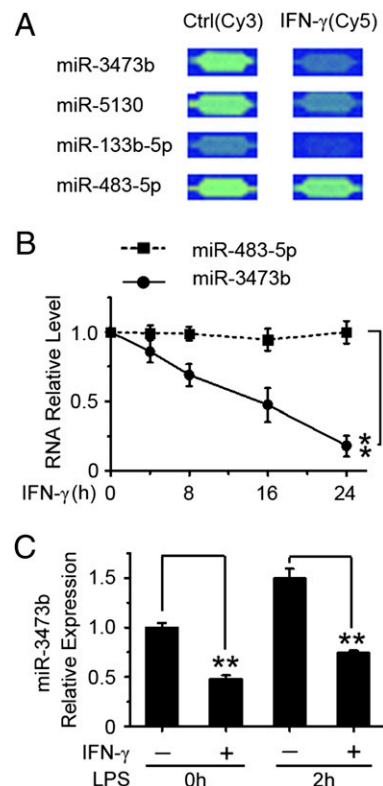


FIGURE 1. IFN- γ stimulation suppresses the expression of miR3473b in macrophages. **(A)** Microarray analysis of miRNA expression profile in mouse BMMs stimulated with IFN- γ for 4 h (labeled with Cy5) or not (labeled with Cy3). The top five miRNAs were selectively listed and presented on the basis of the array image, as well as one relatively non-affected miRNA. **(B)** Quantitative PCR analysis was used to test the miRNA microarray result. Relative expression of miR-3473b in BMMs treated with IFN- γ (100 U/ml) for the indicated time was assessed by quantitative PCR. **(C)** BMMs were stimulated with LPS after IFN- γ priming for 4 h. Relative expression of miR-3473b was assessed by quantitative PCR assay. Data are shown as mean \pm SD of three independent experiments. ** $p < 0.01$.

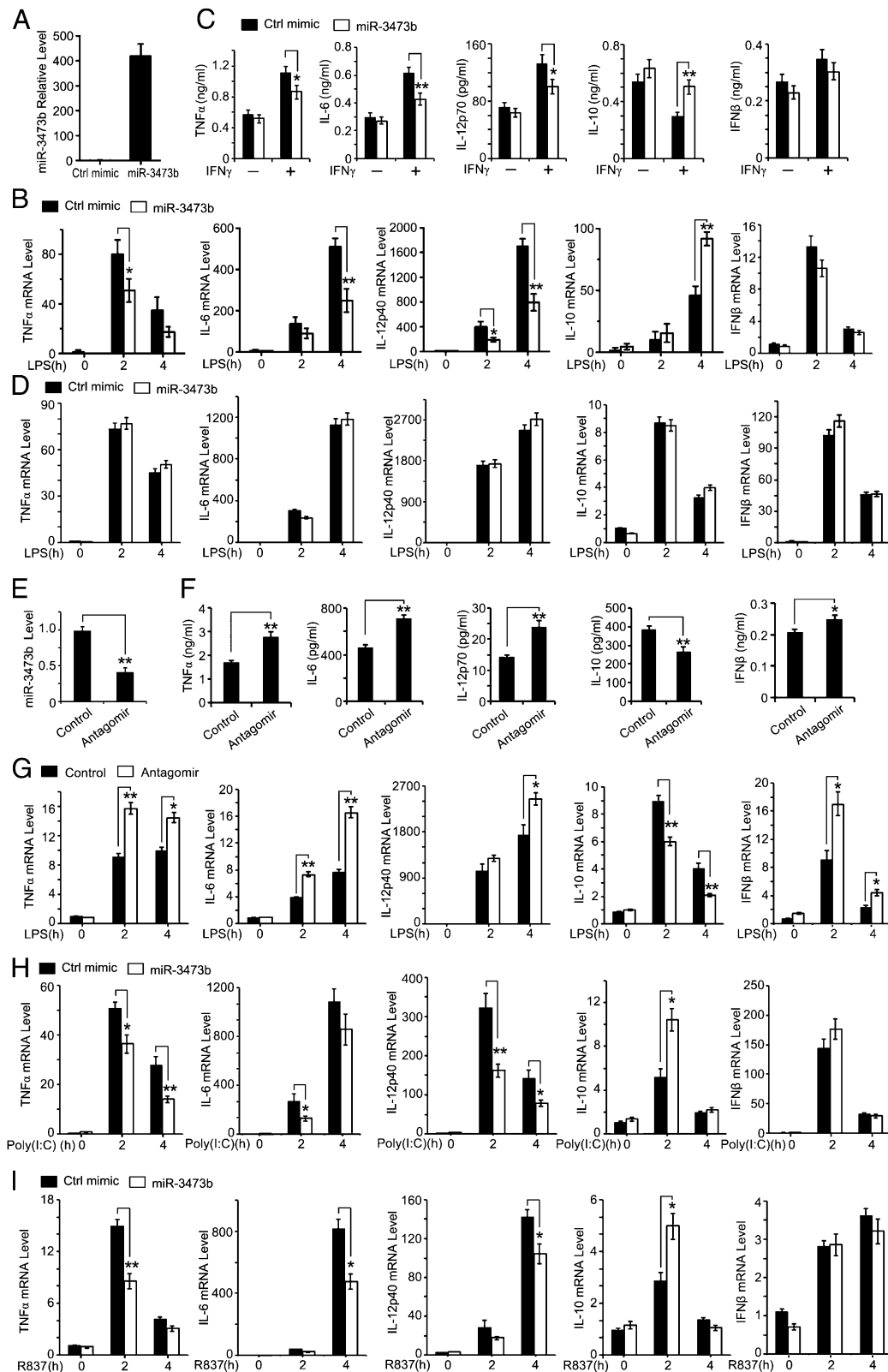


FIGURE 2. miR-3473b inhibits IFN- γ priming effect on macrophage activation. **(A)** BMMs were transiently transfected with control mimic or miR-3473b mimic at a final concentration of 10 nM. After 24 h, the expression of miR-3473b was measured by quantitative PCR and normalized to the expression of internal control U6 in each sample. **(B)** BMMs transfected with control mimic or miR-3473b mimic for 24 h were pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with LPS (50 ng/ml) for the indicated time. The expression of TNF- α , IL-6, IL-12, IL-10, and IFN- β mRNAs was measured by quantitative PCR and normalized to the expression of β -actin. **(C)** BMMs were transfected with control mimic or miR-3473b mimic. After 24 h, these transfected BMMs were pretreated with IFN- γ (100 U/ml) for 4 h or not and then stimulated with LPS (50 ng/ml) for 6 h. TNF- α , IL-6, IL-12, IL-10, and IFN- β in supernatants were measured by ELISA. **(D)** BMMs transfected with miR-3473b mimic for 24 h were stimulated with LPS (50 ng/ml) for the indicated time. The expression of TNF- α , IL-6, IL-12, IL-10, and IFN- β mRNAs was measured by quantitative PCR. **(E–G)** BMMs transfected with miR-3473b antagomir for 24 h (10 nM) were pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with LPS (50 ng/ml) for (Figure legend continues)

antagomir was transfected as their matched control, respectively. The sense and antisense strands of murine PTEN small interfering RNA (siRNA) were 5'-AGA GAU CGU UAG CAG AAA CTT-3' (sense) and 5'-GUU UCU GCU AAC GAU CUC UTT-3' (antisense). The scrambled control RNA sequences were described previously (18). siRNA duplexes were transfected into BMMs at a final concentration of 10 nM.

Assay of luciferase reporter gene expression

The PTEN 3'-UTR luciferase reporter construct was made by amplifying the mouse PTEN mRNA 3'-UTR sequence by PCR and cloned into pGL3-promoter construct (Promega, Madison, WI). HEK293 cells were cotransfected with 80 ng luciferase reporter plasmid, 40 ng pRL-TK-*Renilla*-luciferase plasmid (Promega), and indicated RNAs (final concentration: 20 nM) using JetPRIME transfection reagents (PolyPlus Transfection, Illkirch, France). After 24 h, normalized luciferase activities were obtained using the Dual-Luciferase Reporter Assay System (Promega), according to the manufacturer's instruction. Data were normalized for transfection efficiency by dividing firefly luciferase activity with that of *Renilla* luciferase.

Immunoblot

Cells were lysed with M-PER Protein Extraction Reagent (Pierce) supplemented with protease inhibitor mixture, and protein concentrations of the extracts were measured by bicinchoninic acid assay (Pierce). A total of 50 μ g cell lysates was fractionated by NaDodSO₄-PAGE (SDS-PAGE), transferred onto nitrocellulose membranes, and then blotted.

Flow cytometry

Cells were stained using indicated Abs. Flow cytometric analysis was performed on a FACS LSRII with FACS Diva software (BD Biosciences), and the data were analyzed with FlowJo.

Experiments for antitumor effect in vitro

For assay of macrophage cytotoxicity, 2×10^5 BMMs expressing either negative control mimic or miR-3473b were stimulated with LPS (50 ng/ml) for 4 h, and then 4×10^5 EL4 cells were added to each well. EL4 cell apoptosis was measured 16 h later by staining cells with annexin V and propidium iodide.

Statistical analysis

Statistical significance was determined by pairing two-tailed Student's *t* test, with a *p* value <0.05 considered to be statistically significant.

Results

miR-3473b expression in macrophages is downregulated by IFN- γ priming

To investigate what kinds of miRNAs were regulated in macrophages by IFN- γ priming, we explored the expression profile of miRNAs with microarray technique in BMMs before and after IFN- γ treatment. After further validation by quantitative PCR, we found many miRNAs were downregulated in BMMs by IFN- γ priming, indicating IFN- γ probably promotes protein synthesis at posttranscriptional level and prepares macrophages for activation. Among the top three miRNAs with most differential expressions after IFN- γ priming, we found miR-3473b, which has a relative high expression level in resting BMMs before IFN- γ treatment, decreases significantly after IFN- γ treatment (Fig. 1A). With quantitative PCR assay, we explored the dynamic expression of miR-3473b in the process of IFN- γ priming. Compared with miR-483-5p with relative stable expression level, miR-3473b expression continuously decreased after IFN- γ treatment (Fig. 1B). Then we explored whether IFN- γ priming could also suppress

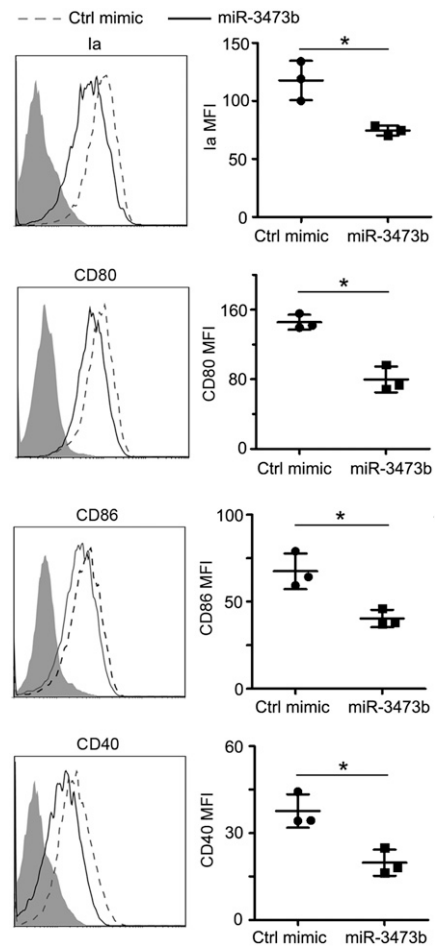


FIGURE 3. miR-3473b suppresses the expression of class II MHC molecule (Ia) and costimulatory molecules in macrophages. BMMs were transfected with control mimic or miR-3473b mimic. After 24 h, these transfected BMMs were pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with LPS (50 ng/ml) for 24 h. The surface expression of Ia, CD80, CD86, and CD40 was analyzed by FACS. Mean fluorescence intensity (MFI) is shown on the right. Data are shown as mean \pm SD of three independent experiments. **p* < 0.05.

miR-3473b expression in LPS-activated BMM, and our data in Fig. 1C showed that miR-3473b was downregulated by IFN- γ in BMMs before and after LPS stimulation, indicating miR-3473b takes part in IFN- γ -assisted macrophage activation.

miR-3473b suppresses classical macrophage activation

To identify whether miR-3473b could affect macrophage classical activation, we transfected BMMs with miR-3473b mimic to rescue the downregulation of miR-3473b by IFN- γ treatment (Fig. 2A) and then examined the cytokine production by BMMs after LPS stimulation. We found that the ectopic expression of miR-3473b inhibited the proinflammatory cytokine expression of TNF- α , IL-6, and IL-12 in IFN- γ -primed LPS-activated BMMs. On the contrary, anti-inflammatory cytokine IL-10 was increased by transfection of miR-3473b mimic, at both mRNA and protein levels (Fig. 2B, 2C). However, in LPS-triggered BMMs without

the indicated time. The expression of miR-3473b (E) and TNF- α , IL-6, IL-12p40, IL-10, and IFN- β mRNAs (G) was measured by quantitative PCR. TNF- α , IL-6, IL-12, IL-10, and IFN- β in supernatants after 6-h LPS stimulation were measured by ELISA (F). (H and I) BMMs transfected with miR-3473b mimic for 24 h were pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with poly(I:C) (20 μ g/ml) (H) or R837 (20 μ g/ml) (I) for the indicated time. The expression of TNF- α , IL-6, IL-12, IL-10, and IFN- β mRNAs was measured by quantitative PCR. Data are shown as mean \pm SD of three independent experiments. **p* < 0.05, ***p* < 0.01.

IFN- γ priming, overexpression of miR-3473b had no effect (Fig. 2D), probably because miR-3483b has already been maintained at a high level in BMMs without IFN- γ priming. Therefore, we used miR-3473b antagomir to perform loss of function in BMMs without IFN- γ priming (Fig. 2E). As shown in Fig. 2F and 2G, miR-3473b antagomir augmented proinflammatory cytokine production but impaired IL-10 production at both mRNA and protein levels, mimicking the effect of IFN- γ priming. Also, to explore whether the effect of miR-3473b confines to LPS-mediated macrophage activation, we used another two TLR agonists, poly(I:C) for TLR3 and R837 for TLR7/8, to stimulate BMMs. We found miR-3473b could also antagonize IFN- γ -priming effect in these activated BMMs (Fig. 2H, 2I).

With flow cytometry analysis, we also evaluated the surface molecule expression of BMMs and found miR-3473b could decrease functional molecule expressions, such as MHC II molecule (Ia) and costimulatory molecules (CD80, CD86, and CD40) (Fig. 3), further indicating that macrophage classical activation is suppressed by miR-3473b. Therefore, our data demonstrate that miR-3473b, which is markedly downregulated by IFN- γ priming, can attenuate classical activation of macrophages, functioning as

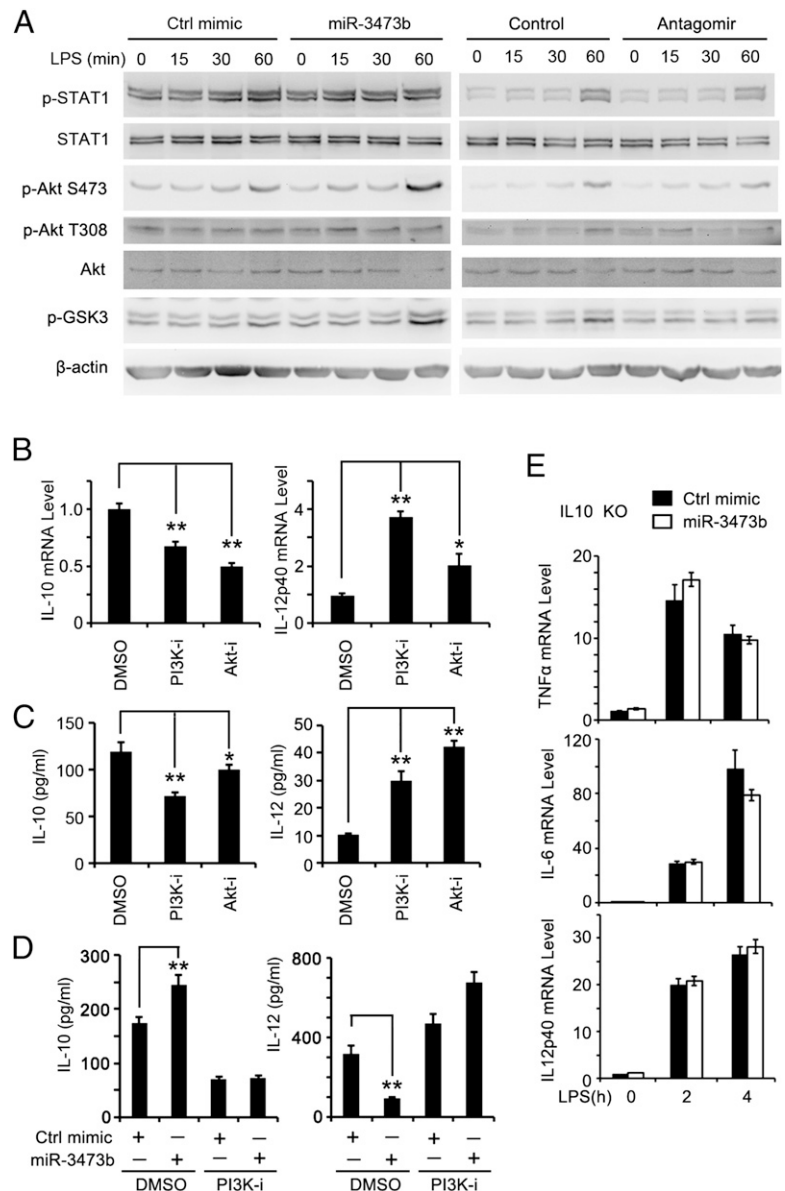
a negative regulator of inflammatory response and macrophage classical activation. Therefore, IFN- γ can promote macrophage activation and proinflammatory cytokine production, to a large extent, through decreasing miR-3473b expression in macrophages.

Overexpression of miR-3473b inhibits Akt/GSK3 activation in IFN- γ -primed macrophages

Next, we investigated the underlying mechanism for the role of miR-3473b in IFN- γ -priming effect. Many functions of IFN- γ have been ascribed to STAT1-mediated induction of immune effector genes, so we investigated whether miR-3473b could affect STAT1 signaling. We observed little influence of miR-3473b on the phosphorylation and expression of STAT1 (Fig. 4A, left). This indicates that miR-3473b does not function through STAT1 signaling.

Upon TLR ligand activation, IL-10 is induced through Akt/GSK3 signaling as a negative regulatory loop for inflammatory response. It has been reported that IFN- γ priming disrupts IL-10-induced feedback inhibitory loop through suppressing GSK3 phosphorylation (11). Therefore, we checked Akt/GSK3 signaling in LPS-activated macrophages. Immunoblot results showed that

FIGURE 4. miR-3473b inhibits macrophage activation through promoting PI3K/Akt/IL-10 negative regulatory loop. **(A)** (Left) BMMs were transfected with miR-3473b mimic or its control, and 24 h later, pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with LPS (50 ng/ml) for the indicated time. (Right) BMMs were transfected with miR-3473b antagomir or its control, and 24 h later, stimulated with LPS (50 ng/ml) for the indicated time. Cell extracts were analyzed by immunoblot for the phosphorylation of STAT1, Akt, and GSK3. Similar results were obtained in three independent experiments. **(B and C)** BMMs were preincubated with DMSO, 1 μ M PI3K inhibitor (PI3K-i) GDC0941, or 1 μ M Akt inhibitor (Akt-i) for 1 h and then stimulated with 50 ng/ml LPS for 4 h. The expression of IL-10 and IL-12 was measured by quantitative PCR at mRNA level (B), or 5 h after LPS stimulation the supernatants were analyzed by ELISA (C). **(D)** BMMs transfected with miR-3473b mimic for 24 h were treated with IFN- γ (100 U/ml) for 4 h, and then, after being preincubated with DMSO or 1 μ M PI3K inhibitor (PI3K-i) GDC0941 for 1 h, stimulated with LPS (50 ng/ml) for 5 h. IL-12 and IL-10 in the supernatants were measured by ELISA. **(E)** BMMs from IL-10-deficient mice were transfected with miR-3473b mimic. After 24 h, cells were pretreated with IFN- γ (100 U/ml) for 4 h and then stimulated with LPS (50 ng/ml) for the indicated time. The expression of TNF- α , IL-6, and IL-12p40 mRNAs was measured by quantitative PCR. Data are shown as mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01.



overexpression of miR-3473b in IFN- γ -primed BMMs increased the phosphorylation level of both Akt and GSK3 after LPS stimulation, whereas miR-3473b antagomir had the opposite effect (Fig. 4A, right). We then investigated whether the Akt signaling could affect the pro- and anti-inflammatory cytokine production in BMM activation. Inhibition of PI3K or Akt using pharmacological inhibitors resulted in reduced production of IL-10 but enhanced production of IL-12 at both mRNA and protein levels (Fig. 4B, 4C), which phenocopied IFN- γ -priming effect, indicating that IFN- γ functions through inhibiting PI3K/Akt signaling.

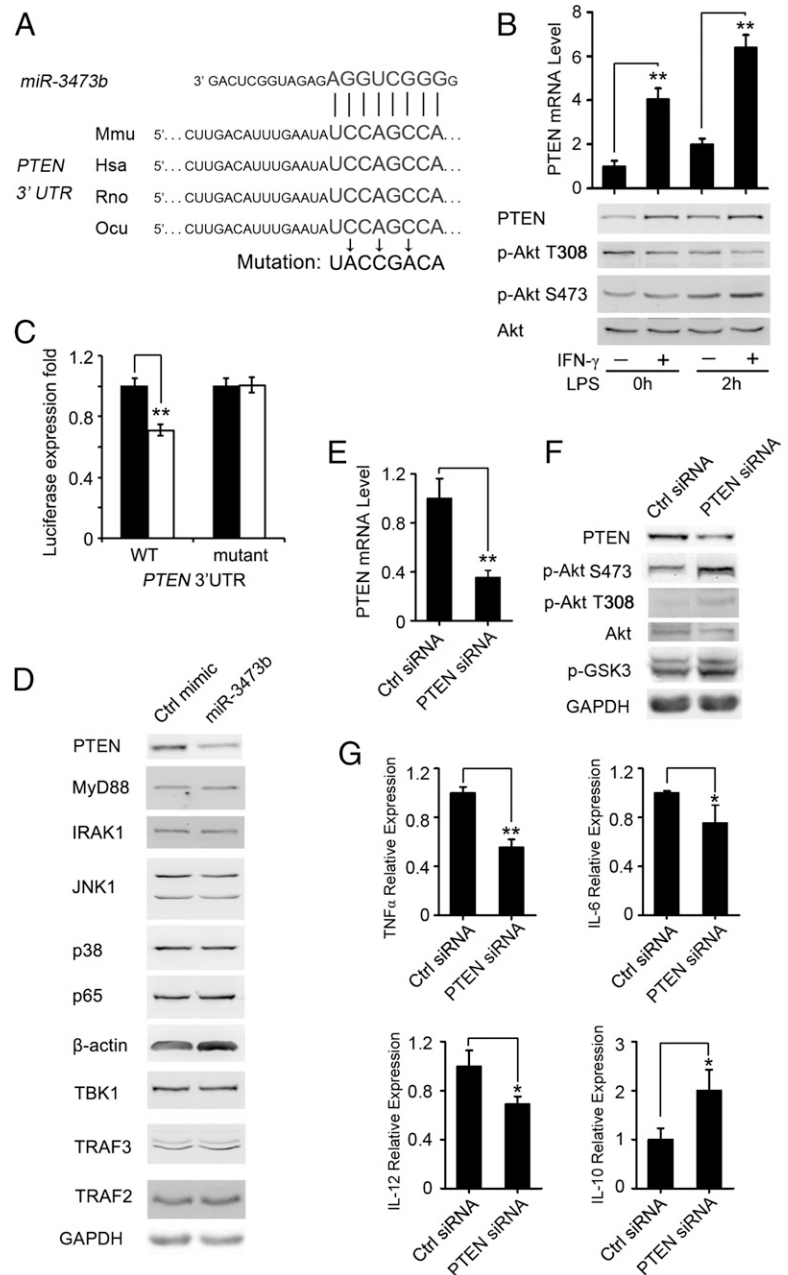
To investigate whether miR-3473b functions through PI3K/Akt/IL-10 inhibitory loop, we used PI3K inhibitor GDC0941 to pretreat the cells and found PI3K inhibition abolished the effect of miR-3473b on cytokine production (Fig. 4D), indicating miR-3473b functions through PI3K signaling. Then we used BMMs from IL-10-deficient mice to explore whether miR-3473b activity depends on IL-10. As shown in Fig. 4E, IL-10 deficiency abolished the effect of miR-3473b on cytokine production.

Taken together, these results indicate that miR-3473b inhibits innate inflammatory responses and macrophage classical activation through promoting Akt/GSK3/IL-10 inhibitory loop.

PTEN is directly targeted by miR-3473b in macrophages

We then investigated the possible target of miR-3473b that could modulate PI3K/Akt signaling. As our data revealed that total Akt protein level was not affected by miR-3473b (Fig. 4A), so we speculated that the target of miR-3473b should be upstream of Akt phosphorylation in PI3K/Akt signaling or be suppressor of PI3K/Akt signaling. Using web-based TargenScan prediction, we scanned the list of genes in mouse genome whose 3'-UTRs contained conserved potential miR-3473b binding sites and found phosphatase PTEN (Fig. 5A), which is an antagonist to PI3K signaling through dephosphorylating phosphatidylinositol 3,4,5 triphosphates (PIP3) to phosphatidylinositol 4,5-bisphosphates (24). In addition, we found that PTEN was upregulated by IFN- γ priming in BMMs with or without LPS stimulation (Fig. 5B),

FIGURE 5. miR-3473b functions through directly targeting PTEN. (A) Predicted duplex formation between miR-3473b and mouse PTEN 3'-UTR along with the mutant sequence used in the luciferase reporter assay, and the sequences of the miR-3473b binding site within the PTEN 3'-UTR of human (Hsa), rat (Rno), and rabbit (Ocu). (B) BMMs pretreated with or without IFN- γ (100 U/ml) for 4 h were stimulated with LPS (50 ng/ml) for the indicated time; the relative level of PTEN mRNA was measured by quantitative PCR (upper); and the indicated protein level was measured by immunoblot (lower). (C) Luciferase reporter assays with miR-3473b and the PTEN 3'-UTR. Assays were performed by cotransfection of miR-3473b with a luciferase reporter gene linked to the mouse wild-type PTEN 3'-UTR (WT) or the mutant 3'-UTR (mutant); after 32 h, firefly luciferase activity was measured and normalized by *Renilla* luciferase activity. Data are the mean with SD of three independent experiments. (D) Immunoblot of the indicated proteins in BMMs infected with miR-3473b mimic for 36 h. (E) BMMs were transfected with negative control siRNA and PTEN siRNA; 24 h later, PTEN gene expression was analyzed by quantitative PCR. (F) Immunoblot analysis of Akt and GSK3 phosphorylation in PTEN-silenced BMMs stimulated with LPS (50 ng/ml) for 1 h. (G) BMMs were transfected with control siRNA or PTEN siRNA, and then after 24 h cells were stimulated with LPS for 3 h. The expression of TNF- α , IL-6, IL-10, and IL-12p40 was measured by quantitative PCR. Data are shown as mean \pm SD of three independent experiments. * p < 0.05, ** p < 0.01.



which was negatively correlated with miR-3473b level (Fig. 1C). To verify the possibility that PTEN was directly regulated by miR-3473b, we cloned wild-type 3'-UTR sequence of PTEN gene and its miR-3473b binding site mutant sequence, inserted it into luciferase reporter vectors, and then cotransfected with miR-3473b mimic or control mimic to check the luciferase activity. We found that miR-3473b mimic markedly decreased the relative luciferase level compared with control mimic, whereas miR-3473b binding site mutant abolished this effect (Fig. 5C). Then we tested whether miR-3473b could suppress endogenous PTEN protein expression in macrophages. As revealed by our immunoblot assay, transfection of miR-3473b mimic decreased PTEN expression in BMMs (Fig. 5D). Therefore, our data demonstrated PTEN was the direct target of miR-3473b in macrophages. However, we did not identify any component genes in TLR signaling pathway having a potential miR-3473b binding site, but we still checked the expression of major TLR pathway components by immunoblot in BMMs with miR-3473b overexpression, showing no obvious changes by miR-3473b (Fig. 5D).

Next, we knocked down the expression of PTEN using RNA interference (Fig. 5E, 5F) and examined the effect on Akt/GSK3 signaling. We found increased phosphorylation of Akt and GSK3 by PTEN siRNA (Fig. 5F). Then we examined pro- and anti-inflammatory cytokine production, and found, similar to miR-3473b mimic transfection, PTEN siRNA resulted in higher IL-10 production and lower expression of TNF- α , IL-6, and IL-12 (Fig. 5G).

These results show that miR-3473b functions through directly targeting PTEN to promote Akt/GSK3 signaling and IL-10 production, finally suppressing the inflammatory innate response of classically activated macrophages.

miR-3473b inhibits tumoricidal capacity of macrophages

Besides high level of proinflammatory cytokine production, IFN- γ -primed classically activated macrophages exert more strong microbicidal and tumoricidal capacity. To assess the biological functional outcome of miR-3473b on macrophages, we checked whether miR-3473b could affect cytolytic function of IFN- γ -assisted activated macrophages. We cocultured EL4 tumor cells with LPS-activated BMM-transfected miR-3473b mimic or control mimic for 16 h. We found BMMs with miR-3473b overexpression were less efficient to induce tumor cell apoptosis (Fig. 6), suggesting miR-3473b could suppress tumor-suppressing capacity of activated macrophages.

Together, our data demonstrated that miR-3473b directly targets PTEN 3'-UTR, enhancing Akt/GSK3 signaling and IL-10 production to suppress the priming effect of IFN- γ on macrophage classical activation and function (Fig. 7).

Discussion

IFN- γ is one of the most important mediators of immunity and inflammation and plays a key role in macrophage activation, inflammation, host defense against intracellular pathogens, Th1 cell response, and tumor surveillance. In this study, we demonstrated that IFN- γ priming of macrophages could downregulate the expression of miR-3473b in macrophages and ectopic expression of miR-3473b could abrogate the priming effect of IFN- γ on macrophage activation, resulting in lower proinflammatory cytokine and costimulatory molecule expression and higher IL-10 expression. Furthermore, we found that miR-3473b could increase phosphorylation of Akt and GSK3, enhancing the inhibitory feedback loop of Akt/GSK3/IL-10 through directly targeting PTEN, the antagonist of PI3K. PTEN knockdown with siRNA phenocopied miR-3473b function in diminishing inflammatory

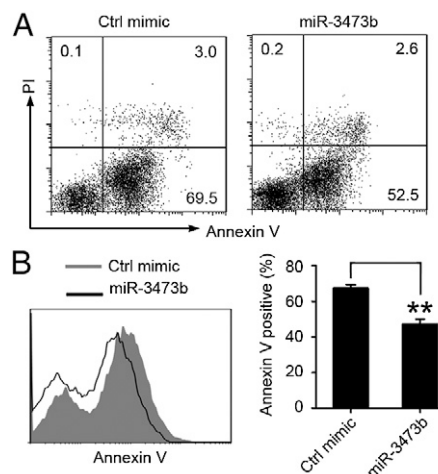


FIGURE 6. miR-3473b suppresses cytotoxicity of LPS-activated macrophages. (A) EL4 cells were cocultured for 16 h with control or miR-3473b-overexpressing macrophages pretreated with IFN- γ (100 U/ml) for 4 h and then activated with LPS (50 ng/ml) for 1 h; the apoptosis of EL4 cells was assessed by annexin V and propidium iodide stain. (B) The percentage of annexin V-positive EL4 cells after 16 h of coculture with control or miR-3473b-overexpressing macrophages, as in (A). A representative flow cytometry plot is shown on the left. Data are expressed as mean \pm SD of three independent experiments. ****** p < 0.01.

response. Therefore, we proposed that IFN- γ exerts its priming activity, to a large extent, through downregulating miR-3473b to promote PTEN expression and consequently interrupt Akt/GSK3/IL-10 feedback inhibitory loop (Fig. 7). As shown in Fig. 1C, LPS treatment increases miR-3473b expression in macrophages, which could be a negative feedback regulatory loop in macrophage activation and inflammatory response, whereas IFN- γ priming could attenuate this negative loop through suppressing miR-3473b expression.

As IFN- γ has been demonstrated to have a profound effect in the immune system (25, 26), both innate and adaptive immunity,

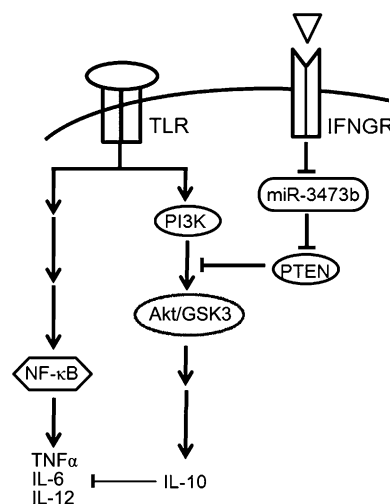


FIGURE 7. A working model depicting IFN- γ -priming effect on macrophage activation through downregulating miR-3473b. Innate signals, like TLRs, directly induce the production of proinflammatory cytokines mainly through NF- κ B activation, which eventually leads to macrophage activation; meanwhile, TLR signaling also triggers a negative feedback loop through PI3K/Akt-mediated IL-10 production. Our work demonstrated that, in IFN- γ -primed macrophages, miR-3473b is downregulated to release its target PTEN, which then inhibits PI3K/Akt/IL-10-negative loop, finally promoting inflammatory response and classical activation of macrophages.

miR-3473b, which is highly expressed in macrophages, may be also involved in other functions related to IFN- γ , such as regulating Th1 cell response. IFN- γ mainly triggers Jak/STAT signaling to induce the expression of immune effector genes. In our experiments, we found that miR-3473b had no effect on STAT1 phosphorylation and STAT1 expression, but it can affect Akt signaling through PTEN, which is a cross-talking mechanism between IFN- γ signaling and PI3K/Akt signaling at miRNA levels.

Priming of TLR responses by IFN- γ greatly augments TLR-induced expression of inflammatory mediators and profoundly affects biological outcomes of innate immunity and inflammation. The underlying mechanisms should include the enhancement of positive signaling and suppression of feedback inhibition. Some studies show that, in liver ischemic/reperfusion injury, PTEN can promote local macrophage activation, proinflammatory cytokine expression, and neutrophil accumulation through suppressing Akt/ β -catenin-mediated NF- κ B inhibition and promoting Foxo1-mediated NF- κ B activation (27, 28). Also, previous studies have shown that PI3K/Akt signaling could directly regulate MAPK activation (29) and GSK3 could directly crosstalk with NF- κ B activation triggered by TLR agonists (30). Therefore, down-regulating miR-3473b by IFN- γ priming to suppress PI3K/Akt/GSK3 signaling may also directly regulate TLR-triggered MAPK and NF- κ B signaling to beef up macrophage activation.

PTEN is a key negative regulator of PI3K/Akt signaling pathway; the principal catalytic function of PTEN is to dephosphorylate PIP3, which is a potent activator of 3 phosphoinositide-dependent kinase and Akt (31). As the unique PIP3 phosphatase, PTEN plays important roles in tumor suppression, and Akt signaling drives cell survival, cell proliferation, angiogenesis, and cellular metabolism by phosphorylating downstream signaling (24). PTEN/PI3K/Akt/mTOR pathway can influence key steps in metabolic pathways during cell proliferation and tumorigenesis. Therefore, as a regulator of PTEN and PI3K/Akt signaling, miR-3473b may also participate in tumorigenesis, which needs to be verified in future research.

Although we identified miR-3473b as a negative regulator of IFN- γ -assisted macrophage classical activation and its downregulation by IFN- γ contributes to priming effect of IFN- γ through derepressing PTEN expression, we could not exclude the possibility that other miRNAs, especially those downregulated ones, may participate in the regulation of macrophage activation or feedback regulate IFN- γ signaling. We also explored the expression profile of some reported miRNAs involved in macrophage activation and inflammatory response in our microarray data of IFN- γ priming, like miR-125b (17) and miR-155 (20), whose expression could be regulated by TLR signal and TNF- α . We found their expressions were not changed by IFN- γ treatment alone (data not shown), so we propose that miRNAs, induced by macrophage activation signals such as TLR signal or TNF- α , might be also involved in macrophage activation, but may not participate in IFN- γ priming effect.

In conclusion, our data suggest that IFN- γ promotes PTEN expression by suppressing miR-3473b to impair Akt/GSK3/IL-10 negative feedback loop, promoting macrophage classical activation and inflammatory response. Our work proposed IFN- γ regulated miR-3473b as a positive regulator of PI3K/Akt signaling and a negative regulator of inflammatory response and macrophage classical activation through directly targeting PTEN, which may have some potential relevance to clinical diseases involving tumor-educated macrophage dysfunction or PTEN/Akt/GSK3 signaling disorders, and also autoimmune diseases involving IFN- γ activity.

Disclosures

The authors have no financial conflicts of interest.

References

- Young, H. A., and J. H. Bream. 2007. IFN-gamma: recent advances in understanding regulation of expression, biological functions, and clinical applications. *Curr. Top. Microbiol. Immunol.* 316: 97–117.
- Gomez, J. A., O. L. Wapinski, Y. W. Yang, J. F. Bureau, S. Gopinath, D. M. Monack, H. Y. Chang, M. Brahic, and K. Kirkegaard. 2013. The NeST long ncRNA controls microbial susceptibility and epigenetic activation of the interferon- γ locus. *Cell* 152: 743–754.
- Aune, T. M., P. L. Collins, S. P. Collier, M. A. Henderson, and S. Chang. 2013. Epigenetic activation and silencing of the gene that encodes IFN- γ . *Front. Immunol.* 4: 112.
- Absher, D. M., X. Li, L. L. Waite, A. Gibson, K. Roberts, J. Edberg, W. W. Chatham, and R. P. Kimberly. 2013. Genome-wide DNA methylation analysis of systemic lupus erythematosus reveals persistent hypomethylation of interferon genes and compositional changes to CD4+ T-cell populations. *PLoS Genet.* 9: e1003678.
- Mosser, D. M., and J. P. Edwards. 2008. Exploring the full spectrum of macrophage activation. *Nat. Rev. Immunol.* 8: 958–969.
- Raetz, M., S. H. Hwang, C. L. Wilhelm, D. Kirkland, A. Benson, C. R. Sturge, J. Mirpuri, S. Vaishnav, B. Hou, A. L. Defranco, et al. 2013. Parasite-induced TH1 cells and intestinal dysbiosis cooperate in IFN- γ -dependent elimination of Paneth cells. *Nat. Immunol.* 14: 136–142.
- Murray, P. J., and T. A. Wynn. 2011. Protective and pathogenic functions of macrophage subsets. *Nat. Rev. Immunol.* 11: 723–737.
- Lawrence, T., and G. Natoli. 2011. Transcriptional regulation of macrophage polarization: enabling diversity with identity. *Nat. Rev. Immunol.* 11: 750–761.
- Yue, S., J. Rao, J. Zhu, R. W. Busuttill, J. W. Kupiec-Weglinski, L. Lu, X. Wang, and Y. Zhai. 2014. Myeloid PTEN deficiency protects livers from ischemia reperfusion injury by facilitating M2 macrophage differentiation. *J. Immunol.* 192: 5343–5353.
- Saraiva, M., and A. O'Garra. 2010. The regulation of IL-10 production by immune cells. *Nat. Rev. Immunol.* 10: 170–181.
- Hu, X., P. K. Paik, J. Chen, A. Yafilina, L. Kockeritz, T. T. Lu, J. R. Woodgett, and L. B. Ivashkiv. 2006. IFN-gamma suppresses IL-10 production and synergizes with TLR2 by regulating GSK3 and CREB/AP-1 proteins. *Immunity* 24: 563–574.
- Chen, J., and L. B. Ivashkiv. 2010. IFN- γ abrogates endotoxin tolerance by facilitating Toll-like receptor-induced chromatin remodeling. *Proc. Natl. Acad. Sci. USA* 107: 19438–19443.
- Bowdridge, S., and W. C. Gause. 2010. Regulation of alternative macrophage activation by chromatin remodeling. *Nat. Immunol.* 11: 879–881.
- O'Neill, L. A., F. J. Sheedy, and C. E. McCoy. 2011. MicroRNAs: the fine-tuners of Toll-like receptor signalling. *Nat. Rev. Immunol.* 11: 163–175.
- Bai, Y., C. Qian, L. Qian, F. Ma, J. Hou, Y. Chen, Q. Wang, and X. Cao. 2012. Integrin CD11b negatively regulates TLR9-triggered dendritic cell cross-priming by upregulating microRNA-146a. *J. Immunol.* 188: 5293–5302.
- Gracias, D. T., E. Stelekati, J. L. Hope, A. C. Boesteanu, T. A. Doering, J. Norton, Y. M. Mueller, J. A. Fraietta, E. J. Wherry, M. Turner, and P. D. Katsikis. 2013. The microRNA miR-155 controls CD8(+) T cell responses by regulating interferon signaling. *Nat. Immunol.* 14: 593–602.
- Chaudhuri, A. A., A. Y. So, N. Sinha, W. S. Gibson, K. D. Taganov, R. M. O'Connell, and D. Baltimore. 2011. MicroRNA-125b potentiates macrophage activation. *J. Immunol.* 187: 5062–5068.
- Wang, P., J. Hou, L. Lin, C. Wang, X. Liu, D. Li, F. Ma, Z. Wang, and X. Cao. 2010. Inducible microRNA-155 feedback promotes type I IFN signaling in antiviral innate immunity by targeting suppressor of cytokine signaling 1. *J. Immunol.* 185: 6226–6233.
- Wang, P., Y. Gu, Q. Zhang, Y. Han, J. Hou, L. Lin, C. Wu, Y. Bao, X. Su, M. Jiang, et al. 2012. Identification of resting and type I IFN-activated human NK cell miRNomes reveals microRNA-378 and microRNA-30e as negative regulators of NK cell cytotoxicity. *J. Immunol.* 189: 211–221.
- O'Connell, R. M., K. D. Taganov, M. P. Boldin, G. Cheng, and D. Baltimore. 2007. MicroRNA-155 is induced during the macrophage inflammatory response. *Proc. Natl. Acad. Sci. USA* 104: 1604–1609.
- Ma, F., S. Xu, X. Liu, Q. Zhang, X. Xu, M. Liu, M. Hua, N. Li, H. Yao, and X. Cao. 2011. The microRNA miR-29 controls innate and adaptive immune responses to intracellular bacterial infection by targeting interferon- γ . *Nat. Immunol.* 12: 861–869.
- Krausgruber, T., K. Blazek, T. Smallie, S. Alzabin, H. Lockstone, N. Sahgal, T. Hussell, M. Feldmann, and I. A. Udalova. 2011. IRF5 promotes inflammatory macrophage polarization and TH1-TH17 responses. *Nat. Immunol.* 12: 231–238.
- Taganov, K. D., M. P. Boldin, K. J. Chang, and D. Baltimore. 2006. NF-kappaB-dependent induction of microRNA miR-146, an inhibitor targeted to signaling proteins of innate immune responses. *Proc. Natl. Acad. Sci. USA* 103: 12481–12486.
- Manning, B. D., and L. C. Cantley. 2007. AKT/PKB signaling: navigating downstream. *Cell* 129: 1261–1274.
- Petro, M., D. Kish, O. A. Guryanova, G. Ilyinskaya, A. Kondratova, R. L. Fairchild, and A. V. Gorbachev. 2013. Cutaneous tumors cease CXCL9/Mig production as a result of IFN- γ -mediated immunoeediting. *J. Immunol.* 190: 832–841.
- da Silva, H. B., E. M. de Salles, R. H. Panatieri, S. B. Boscardin, S. M. Rodríguez-Málaga, J. M. Alvarez, and M. R. D'Império Lima. 2013.

- IFN- γ -induced priming maintains long-term strain-transcending immunity against blood-stage *Plasmodium chabaudi* malaria. *J. Immunol.* 191: 5160–5169.
27. Kamo, N., B. Ke, R. W. Busuttil, and J. W. Kupiec-Weglinski. 2013. PTEN-mediated Akt/ β -catenin/Foxo1 signaling regulates innate immune responses in mouse liver ischemia/reperfusion injury. *Hepatology* 57: 289–298.
 28. Ke, B., X. D. Shen, N. Kamo, H. Ji, S. Yue, F. Gao, R. W. Busuttil, and J. W. Kupiec-Weglinski. 2013. β -catenin regulates innate and adaptive immunity in mouse liver ischemia-reperfusion injury. *Hepatology* 57: 1203–1214.
 29. Medina, E. A., I. R. Morris, and M. T. Berton. 2010. Phosphatidylinositol 3-kinase activation attenuates the TLR2-mediated macrophage proinflammatory cytokine response to *Francisella tularensis* live vaccine strain. *J. Immunol.* 185: 7562–7572.
 30. Martin, M., K. Rehani, R. S. Jope, and S. M. Michalek. 2005. Toll-like receptor-mediated cytokine production is differentially regulated by glycogen synthase kinase 3. *Nat. Immunol.* 6: 777–784.
 31. Song, M. S., L. Salmena, and P. P. Pandolfi. 2012. The functions and regulation of the PTEN tumour suppressor. *Nat. Rev. Mol. Cell Biol.* 13: 283–296.