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MicroRNA-155 and MicroRNA-21 Promote the Expansion of Functional Myeloid-Derived Suppressor Cells

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Myeloid-derived suppressor cells (MDSC) are one of the main cell populations that negatively regulate immune responses. However, the mechanism underlying the expansion of MDSC remains unclear. Using miRNA microarray and TaqMan probe–based quantitative RT-PCR assay, we identified microRNA (miR)-155 and miR-21 as the two most upregulated miRNAs during the induction of MDSC from the bone marrow cells by GM-CSF and IL-6. High levels of miR-155 and miR-21 also were detected in bone marrow and spleen MDSC isolated from tumor-bearing mice. Our results also showed that TGF-β promoted the induction of MDSC through upregulating miR-155 and miR-21 expression. Overexpression of miR-155 and miR-21 enhanced whereas depletion of miR-155 and miR-21 reduced the frequencies of cytokine-induced MDSC. Subpopulation analysis indicated that miR-21 and miR-155 induced the expansion of both monocytic and granulocytic MDSC. Furthermore, miR-155 and miR-21 showed a synergistic effect on MDSC induction via targeting SHIP-1 and phosphatase and tensin homolog, respectively, leading to STAT3 activation. Finally, dexamethasone treatment strongly enhanced MDSC expansion through upregulating miR-155 and miR-21 expression, and the effect of dexamethasone on MDSC induction was abolished by depleting cellular miR-155 and miR-21. These results demonstrate a novel miR-155/miR-21–based regulatory mechanism that modulates functional MDSC induction. The Journal of Immunology, 2014, 192: 1034–1043.

Myeloid-derived suppressor cells (MDSC), a heterogeneous population of immature myeloid cells including myeloid progenitors, macrophage precursors, granulocytes, and dendritic cells, have a strong ability to suppress various T cell functions (1, 2). In mice, MDSC are characterized by the coexpression of the cell surface Ags Ly-6C/G and CD11b (2), whereas in humans, MDSC are typically CD11b+CD33+HLA-DR+ (3–5). More recently, MDSC in humans were subdivided into two different subsets: CD11b+CD33+HLA-DR+CD14+ and CD11b+CD15+HLA-DR+CD14+ (2, 6). MDSC in mice can be further subdivided into two different subsets based on Ly-6C/G expression (7, 8). CD11b+Ly-6G+Ly-6Chigh cells have monocytic-like morphology and are referred to as monocytic MDSC (M-MDSC), whereas CD11b+Ly-6G+Ly-6Chigh cells have granulocyte-like morphology and are referred to as granulocytic MDSC (G-MDSC). M-MDSC suppress T cell proliferation by high levels of inducible NO synthase (iNOS) and arginase 1 (ARG1); G-MDSC suppress T cell proliferation mainly through high levels of reactive oxygen species and ARG1 (2, 9). Under pathological conditions such as tumor growth and graft-versus-host disease, the frequency of MDSC increases dramatically, suggesting a critical contribution of MDSC to these immunosuppressive conditions. The activation of MDSC and its protective role against inflammation and autoimmune has been widely observed in various pathophysiological conditions (10–15). Although it is well-known that MDSC can be induced from bone marrow (BM) cells by the combination of GM-CSF and IL-6, the mechanism underlying the expansion of MDSC under either in vitro cytokine stimulation conditions or the in vivo tumor microenvironment remains poorly understood.

The advent of micro RNAs (miRNAs), a class of ~22-nt-long noncoding RNAs, has revealed a new layer of gene regulation in various biological processes (16). In addition to functioning as oncogenes or tumor suppressors (17, 18), miRNAs also play critical roles in modulating host immune systems (19, 20). Through the C/EBP-α–PU.1 pathway, miR-124 was found to promote microglial quiescence and suppress experimental autoimmune encephalomyelitis (21). By targeting phosphatase and tensin homolog (PTEN), miR-21 (22) and miR-214 (23) control the apoptosis of immune cells. In addition, miR-155 is an important factor controlling lymphocyte differentiation and function (24, 25) and has been shown to be a proinflammatory regulator in clinical and experimental arthritis (26), cystic fibrosis (27), and certain autoimmune inflammatory reactions (28). Sonkoly et al. (29) recently reported that miR-155 was overexpressed in patients with atopic dermatitis and modulated T cell–proliferative responses by targeting CTL-associated Ag 4. Uregulation of miR-155 in macrophages also was found to contribute to increased TNF-α production (30). In support of these data, McCoy et al. (31) showed that the induction of miR-155 was inhibited by IL-10 through TLRs. However, although progress has

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K.Z. and C.-Y.Z. designed the research and analyzed data; L.L. and J.Z. contributed equally to this work.

Address correspondence and reprint requests to Dr. Ke Zen and Dr. Chen-Yu Zhang, Jiangsu Engineering Research Center for MicroRNA Biology and Biotechnology, State Key Laboratory of Pharmaceutical Biotechnology, School of Life Sciences, Nanjing University, Nanjing, Jiangsu 210093, China. Email addresses: kzen@nju.edu.cn (K.Z.) and cyzhang@nju.edu.cn (C.-Y.Z.).

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Abbreviations used in this article: ARG1, arginase 1; BM, bone marrow; Dax, dexamethasone; G-MDSC, granulocytic myeloid-derived suppressor cell; iNOS, inducible NO synthase; LLC, Lewis lung cancer; MDSC, myeloid-derived suppressor cell; miR or miRNA, microRNA; M-MDSC, monocytic myeloid-derived suppressor cell; ORF, open reading frame; pSTAT3, phosphorylated STAT3; PTEN, phosphatase and tensin homolog; qRT-PCR, quantitative RT-PCR; sRNA, small interfering RNA.
been made in exploring miRNA functions in the host immune system, it remains unknown whether or how miRNAs modulate the differentiation and phenotypic switching of myeloid-derived leukocytes.

In this study, we used miRNA microarrays and TakMan probe-based quantitative RT-PCRs (qRT-PCRs) to demonstrate that miR-155 and miR-21 are two critical molecules regulating the induction of functional MDSC. A striking elevation of miR-155 and miR-21 expression levels was observed in both G-MDSC and M-MDSC directly derived from BM cells that were stimulated with GM-CSF and IL-6 as well as in MDSC from tumor-bearing mice. Dexamethasone (Dex), which has been shown to promote the expansion of MDSC, strongly upregulated miR-155 and miR-21 expression in BM cells, and the effect of Dex on expanding MDSC was abolished by depleting miR-155 and miR-21. Our results further suggest that by targeting SHIP1 and PTEN, respectively, miR-155 and miR-21 have a synergistic effect on increasing STAT3 activity and MDSC expansion. This study provides a novel miR-155/miR-21–based regulatory mechanism for the regulation of MDSC expansion and a potential therapeutic target for diseases associated with inflammation and autoimmunity.

**Materials and Methods**

**Reagents and Abs**

Murine IL-6, GM-CSF, and TGF-β1 cytokines were from PeproTech (Rocky Hill, NJ). Con A and Dex were from Sigma-Aldrich (St. Louis, MO). Anti-PTEN, anti-STAT3, and anti–phosphorylated STAT3 (pSTAT3) Abs were purchased from Cell Signaling Technology (Beverly, MA). Anti–SHIP-1 and GAPDH Abs were from Santa Cruz Biotechnology (Santa Cruz, CA). PE-conjugated anti-mouse CD11b and PerCP/Cy5.5 anti-mouse Gr-1 were purchased from BioLegend (San Diego, CA). FITC-conjugated rat anti-mouse CD4, CD8a, allophycocyanin-conjugated anti-mouse Ly6G, and Alexa Fluor 488–conjugated anti-mouse Ly-6C were purchased from BD Biosciences (Franklin Lakes, NJ). miR-155 agonir, miR-155 antagonist, miR-21 agonir, miR-21 antagonist, and normal controls for agomirs and antagomirs were purchased from Ribobio (Guangzhou, China). Lentivirus-expressing PTEN open reading frame (ORF), SHIP-1 ORF, PTEN small interfering RNA (siRNA) expression, or SHIP-1 siRNA were from GeneCopoeia (Guangzhou, China).

**Animals**

Animal maintenance and experimental procedures were carried out in accordance with the U.S. National Institutes of Health Guidelines for Use of Experimental Animals and approved by the Medicine Animal Care Committee of Nanjing University (Nanjing, China). Eight-week-old male C57BL/6 mice (Nanjing University Animal Center) were used in this study. To establish tumor, 10^6 Lewis lung cancer (LLC) cells were s.c. injected into the left armpits of the animals. To obtain BM-derived MDSC, previously described methods were used (32). In brief, cells were plated into dishes using RPMI 1640 medium supplemented with 2 mM L-glutamine, 10 mM HEPES, 20 μM 2-ME, 150 mM streptomycin, 200 μM penicillin, and 10% FBS and stimulated with combinations of GM-CSF (40 ng/ml) and IL-6 (40 ng/ml). Cells were cultured at 37°C in 5% CO₂–humidified atmosphere for 4 d.

**Cell transfection**

BM cells were cultured on 6-well plates and transfected in the following day. For miRNA overexpression or knockdown, 100 pmol individual miRNA agomir or antagonist was used. Oligonucleotides with random sequence served as negative controls for miRNA agonirs or antagonirs. For overexpression or knockdown of SHIP-1 or PTEN, lentivirus vector expressing ORF or siRNA of SHIP and PTEN were used. During transfection, the viral suspensions were mixed with Polybrene (Sigma-Aldrich) to a final concentration of 5 μg/ml and used for infection of BM-MDSC. Infection was performed in 6-well plates (5 × 10^6 cells/well) after a 12-h incubation at 37°C and 5% CO₂, and the cells were washed and resuspended in RPMI 1640 medium. Cells were harvested 72 h posttransfection for Western blotting, flow analysis, and miRNA quantification. SHIP-1 siRNA sequence was 5′-GCATATCCTGCATACAGCT-3′, and PTEN siRNA sequence was 5′-TGTCTCGTGCTTACTCTT-3′.

**Quantitative RT-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen). Quantitative RT-PCR was carried out using TaqMan probes (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Briefly, total RNA was reverse transcribed to cDNA using avian myeloblastosis virus reverse transcriptase (TaKaRa) and a stem-loop reverse transcriptase primer. Real-time PCR was performed using a TaqMan PCR kit on an Applied Biosystems 7300 Sequence Detection System. All reactions were run in triplicate. After the reaction, the Ct values were determined using fixed threshold settings. In the experiments presented in this paper, miRNA expression in cells is normalized to U6 small noncoding RNA. For mRNA quantification, total RNA was reverse transcribed to cDNA using oligo(dT). Real-time qPCR was performed using SYBR Green normalized to GAPDH. The following PCR primers were used: iNOS forward, 5′-CCAGCGCTCTCACCCTGTTCC-3′; iNOS reverse, 5′-CTCTGGAGGTGTGACACAAAGG-3′; ARG1 forward, 5′-CTCCAAGGCAAGTCTTAGAG-3′; ARG1 reverse, 5′-AGGAAGCTGCTATGGGACATC-3′; GAPDH forward, 5′-TGAAGCAAGCATTGAGG-3′; and GAPDH reverse, 5′-CAGAGGTTGGAAG AGTGAG-3′.

**Flow cytometry**

Flow cytometry was conducted using a BD FACSCalibur device and analyzed with FCS express V3. After washing with Hank’s buffer devoid of Ca²⁺ and Mg²⁺ (HBSS), 5 × 10^5 cells from BM, spleen, and blood were blocked in 100 μl 1% BSA at 4°C for 30 min. CD11b, Gr-1, CD4, CD8, Ly-6G, and Ly-6C Abs were added for incubation in another 30 min at 4°C.

**Western blot analysis**

BM cells were lysed in a buffer containing 1% Triton X-100, a protease inhibitor mixture and PMSF. In addition, to keep protein’s phosphorylation, sodium vanadate and sodium fluoride were added when extracted protein for detecting protein phosphorylation change. Then we used PTEN, STAT3, phospho-STAT3, and SHIP-1 Abs in the following SDS-PAGE and Western blot detection. The intensity of each protein band was quantified using the BandScan software (Glyko) and normalized against GAPDH in the same samples blotted by anti-GAPDH Abs.

**MDSC sorting and T cell proliferation**

To obtain high-purity MDSC of BM and spleen, cell isolation kit was used (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions. For T cell suppression assay, the splenocytes were separated with lymphocyte separation medium. Then CD4⁺ or CD8⁺ T cells from the spleens were isolated via negative selection by using CD4⁺ or CD8⁺ T cell isolation kit (Miltenyi Biotec). CD4⁺ or CD8⁺ T cells were labeled with CFSE, according to the manufacturer’s instructions (Invitrogen). CFSE-labeled CD4⁺ or CD8⁺ T cells were stimulated with Con A (Sigma-Aldrich), and T cells were cocultured at 2:1, 4:1, 10:1, or 100:1 ratio with BM-MDSC in 96-well flat-bottom plates. On the fourth day, cells were analyzed by flow cytometry.

**Statistical analysis**

All data from Western blot and semiquantitative RT-PCR analysis are representative of at least three independent experiments. Data shown are presented as mean ± SD of at least three independent experiments; differences are considered statistically significant at p < 0.05 by Student t test.

**Results**

Strong elevation of miR-155 and miR-21 levels in MDSC induced from BM cells

To identify the miRNAs that have a potential role in modulating MDSC induction, we used miRNA microarrays to monitor the changes in the miRNA profile in BM cells during the course of MDSC induction. First, freshly isolated mouse BM cells were treated with GM-CSF and IL-6 (40 ng/ml each) for 4 d to allow MDSC induction. As shown in Fig. 1, we assayed MDSC using biomarker proteins such as Gr-1 and CD11b, and the results showed that Gr-1⁺CD11b⁺ MDSC were effectively induced from BM cells treated with GM-CSF and IL-6 (Fig. 1A). T cell proliferation assays also showed that MDSC induced from BM cells (BM-MDSC) strongly suppressed T cell proliferation (Fig. 1B). In
addition, compared with untreated BM cells, BM-MDSC contained significantly higher mRNA levels of two MDSC functional markers, iNOS and ARG1 (Fig. 1C) (2, 9). Microarray data demonstrated that BM-MDSC had a significantly altered miRNA profile compared with untreated BM cells (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=qnehcmmclbyzvar&acc=GSE50914). There were 45 miRNAs that were upregulated and 17 miRNAs that were downregulated more than 2-fold. Among these miRNAs, miR-155 and miR-21 had the highest upregulation after cytokine induction (Fig. 1D). This result was validated by TaqMan probe-based qRT-PCR analysis (Fig. 1E). The levels of miR-155 and miR-21 in BM-MDSC during the course of MDSC induction were further monitored. As shown in Fig. 1F, the levels of miR-155 and miR-21 in GM-CSF/IL-6-treated BM cells increased in a time-dependent manner compared with those in nontreated BM cells. Because BM cells are generally differentiated into both CD11b+ Ly6GhighLy6CloMonocytic and CD11b+Ly6GlowLy6Chigh Granulocytic MDSC after treating with GM-CSF and IL-6, we next sorted out these two MDSC fractions and determined their respective levels of miR-155 and miR-21. As shown in Fig. 1G, Gr-1+CD11b+ MDSC were sorted into Ly-6C+ Monocytic and Ly-6G+ Granulocytic MDSC, two major MDSC fractions, from the BM cells. The sorting results suggest that the majority of Gr-1+CD11b+ cells (>85% of total marker-positive cells) that were selected in flow cytometry analysis were MDSC of monocytic or granulocytic origin. Compared with freshly isolated BM cells,
both granulocytic and monocytic MDSC had significantly higher levels of miR-155 and miR-21.

MDSC derived from tumor-bearing mice also contain high levels of miR-155 and miR-21

Although all MDSC have a shared function to suppress T cell proliferation, they are a highly heterogeneous cell population and can be derived from different sources through different induction pathways. It is well known that MDSC are induced under tumor conditions and contribute to tumor immune evasion (8). To test whether MDSC derived from tumor conditions also contain higher levels of miR-155 and miR-21, we isolated MDSC from the BM, spleen, and tumor tissue of C57BL/6 mice–bearing LLC and compared the miR-155 and miR-21 levels with those of BM and peripheral monocytes or neutrophils from control mice. Gr-1+CD11b+ MDSC were sorted out from the BM, spleen, and tumor tissue in mice that had been implanted with LLC tumor cells for 3 wk (Fig. 2A, 2B). As shown in Fig. 2C, the levels of miR-155 and miR-21 were significantly higher in MDSC derived from the BM, spleen, or tumor tissue in tumor-bearing mice than those in control mice. In a similar fashion, we further sorted Gr-1+CD11b+ MDSC from tumor-bearing mice into Ly-6C+ monocytic and Ly-6-G+ granulocytic MDSC and found that both MDSC fractions had higher expression levels of miR-155 and miR-21 compared with control BM and peripheral monocytes or neutrophils (Fig. 2D).

To determine whether tumor relevant cytokines induce MDSC expansion through upregulating miR-155 and miR-21, the BM was isolated, treated with TGF-β, GM-CSF, or IL-6. The levels of Gr-1+CD11b+ MDSC during the course of BM induction were further monitored. As shown in Fig. 2E, TGF-β can effectively promote Gr-1+CD11b+ MDSC expansion in a time-dependent manner compared with BM cells treated with GM-CSF and IL-6 (cytokine). The levels of miR-155 and miR-21 were assessed 2 d after GM-CSF, IL-6, or TGF-β treatment. As can be seen in Fig. 2F, TGF-β also significantly upregulated the levels of two miRNAs compared with BM cells treated with GM-CSF and IL-6. In addition, TGF-β–treated BM-MDSC contained significantly higher mRNA levels of iNOS and ARG1 compared with BM-MDSC cells without TGF-β treatment (Fig. 2G). T cell proliferation assays further demonstrated that TGF-β–treated BM-MDSC strongly suppressed T cell proliferation (Fig. 2H).

miR-155 and miR-21 synergistically enhance MDSC expansion via targeting SHIP-1 and PTEN, respectively

To characterize the effect of miR-155 and miR-21 on MDSC induction, we transfected BM cells with miRNA agomirs or antagonirs to overexpress or deplete, respectively, miR-155 and miR-21 after 2 d of GM-CSF and IL-6 treatment (Supplemental Fig. 1). We then monitored the induction of MDSC after additional 3 d. As shown in Fig. 3A, the flow cytometry results clearly show that the overexpression of miR-155 or miR-21 increases the induction of MDSC from BM cells treated with GM-CSF and IL-6, whereas the depletion of miR-155 or miR-21 reduces the induction of MDSC. When miR-155 and miR-21 were overexpressed or depleted in BM cells at the same time, the differentiation of MDSC was induced or reduced, respectively, to a greater degree than when the individual miRNAs were overexpressed or depleted, suggesting a synergistic effect of miR-155 and miR-21 to regulate MDSC generation. To test whether miR-155 and miR-21 modulate the expansion of MDSC in a different manner, we further test the levels of the granulocytic- and the monocytic-like cells 3 d after transfection. As shown in Fig. 3B, miR-21 and miR-155 can both modulate the expansion of the granulocytic- and monocytic-like MDSC. In addition, we also found that an overexpression or depletion of miR-155 and miR-21 levels increased or decreased, respectively, the levels of iNOS and ARG1 mRNA in BM cells 3 d after transfection (Fig. 3C). Given that the T cell proliferation assay showed that MDSC induced by miR-155 and miR-21 overexpression strongly suppressed the proliferation of CD4+ and CD8+ T cells in a ratio-dependent manner (Fig. 3D, 3E), the miR-155/21–induced MDSC are fully functional.

Previous studies showed that miR-155 and miR-21 were involved in various biological processes such as immune response and tumorigenesis by their targeting of SHIP-1 and PTEN, respectively (22, 26). To determine whether the synergistic effect of miR-155 and miR-21 to promote MDSC expansion is through the targeting of SHIP-1 and PTEN, we assessed the correlation of the levels of SHIP-1, PTEN, STAT3, pSTAT3, the frequencies of MDSC, and the levels of miR-155 and miR-21. First, we treated mouse BM cells with GM-CSF and IL-6 for 4 d to induce BM-MDSC. As shown in Fig. 4A, the levels of SHIP-1 and PTEN in cytokine-treated cells (induced) were significantly lower compared with those in untreated cells (fresh). In contrast, the level of pSTAT3 in cytokine-treated cells was strongly elevated compared with nontreated cells, whereas the total amount of STAT3 in both cells was nearly the same. These data are in agreement with the previous observation that the reduction of SHIP-1 and PTEN levels results in an increase in STAT3 activity, leading to MDSC expansion (33, 34). Next, we sorted MDSC from tumor-bearing mice and compared their PTEN, SHIP-1, pSTAT3, and STAT3 levels with those in peripheral leukocytes from control mice. A similar reduction of SHIP-1 and PTEN levels but an increase in pSTAT3 levels was observed in MDSC from tumor-bearing mice (Fig. 4B, 4C). In addition, TGF-β–treated BM-MDSC contain lower PTEN and SHIP-1 but higher pSTAT3 compared with BM-MDSC without TGF-β treatment (Fig. 4D). By overexpressing or depleting miR-155 and miR-21 in cytokine-treated BM cells using miRNA agomirs (Fig. 4E) or antagonirs (Fig. 4F), we showed inverted correlations between miR-155 and SHIP-1 and between miR-21 and PTEN, confirming SHIP-1 and PTEN as target genes of miR-155 and miR-21, respectively. As shown in the figure, the reduction of SHIP-1 or PTEN by the overexpression of miR-155 or miR-21, respectively, significantly increased the pSTAT3 level. In contrast, the depletion of miR-155 or miR-21 resulted in an increase in SHIP-1 or PTEN levels, respectively, leading to a decrease in pSTAT3 levels. Interestingly, as the downregulation of SHIP-1 and PTEN can both lead to activation of STAT3, which promotes MDSC expansion (35), targeting SHIP-1 by miR-155 and PTEN by miR-21 supports a synergistic effect of these two miRNAs to enhance STAT3 activity and MDSC production.

To further determine whether SHIP-1 and PTEN are involved in modulating MDSC induction, we directly increased or decreased the levels of SHIP-1 and PTEN in BM cells treated with GM-CSF and IL-6 and then determined the frequencies of MDSC. In this experiment, GM-CSF/IL-6–induced BM-MDSC were transfected with SHIP-1–expressing lentivirus (SHIP-1 ORF), PTEN-expressing lentivirus (PTEN ORF), SHIP-1 siRNA-expressing lentivirus (SHIP-1 siRNA), or PTEN siRNA-expressing lentivirus (PTEN siRNA). The results (Fig. 4G, 4H) clearly indicate that SHIP-1 and PTEN expression were strongly increased by transfection with the SHIP-1 ORF and PTEN ORF but were decreased by transfection with SHIP-1 siRNA and PTEN siRNA, respectively. As shown in Fig. 4I and 4J, SHIP-1 and PTEN were negative regulators of MDSC expansion induced by GM-CSF and IL-6, and their effects on MDSC production are also synergistic. Because the promoting effects of miR-155 and miR-21 on MDSC induction were abolished by the overexpression of SHIP-1 and PTEN, respectively, the results suggest that miR-155 and miR-21 promote MDSC induction.
by targeting SHIP-1 and PTEN. Detection of the mRNA levels of iNOS and ARG1, two functional markers for MDSC, also confirmed that MDSC induction is promoted by miR-155 and miR-21 through the targeting of SHIP-1 and PTEN, respectively (Fig. 4K). As seen in the figure, miR-155 and miR-21 agomirs, as well as SHIP-1 and PTEN siRNAs, strongly increased the levels of iNOS and ARG1 mRNA in induced MDSC, whereas miR-155 and miR-21 antagonirs, as well as SHIP-1 and PTEN ORFs, significantly decreased iNOS and ARG1 mRNA levels.

**Dex promotes MDSC expansion via upregulating miR-155 and miR-21**

As an immune suppressor, Dex and other glucocorticoid analogs have been used widely to treat various active inflammation and
FIGURE 3. miR-21 and miR-155 synergistically promote the accumulation and function of MDSC. (A) BM was isolated and treated with GM-CSF and IL-6; miR-21 agomir (miR-21), miR-155 agomir (miR-155), miR-21 antagonist (miR-21 ASO), miR-155 antagonist (miR-155 ASO), and scrambled oligonucleotides were transfected on the second day after induction. Gr-1+CD11b+ MDSC were evaluated by flow cytometry after 3 d. The percentage of each treated group was normalized to fresh BM-MDSC, and the level was set as 0. (B) The subpopulations of Gr-1+CD11b+ MDSC. One representative of three independent experiments was shown. The lower panel represents the statistical analysis results. The percentage of each treated group was normalized to fresh BM-MDSC, and the level was set as 0. (C) The relative levels of iNOS and ARG1 mRNA in BM-MDSC detected by qRT-PCR after 3 d of transfection. (D) Comparison of MDSC activity between miR-21– and miR-155–transfected cells and controls. CFSE-labeled T cells were cocultured with BM-MDSC at 2:1 ratio. (E) Comparison of MDSC activity between miR-21– and miR-155–transfected cells at different T cell/MDSC ratio. Triplicate assays were conducted for each experiment. Data are expressed as mean ± SD. *p < 0.05, **p < 0.01.
PTEN and SHIP-1 are targets of miR-21 and miR-155, respectively, and synergistically regulate the accumulation and function of MDSC via the STAT3 pathway. (A) BM-MDSC induced by GM-CSF and IL-6 for 4 d. PTEN, SHIP-1, pSTAT3, STAT3, and GAPDH protein levels were detected in induced BM-MDSC (Induced) and fresh BM (Fresh). (B) Comparison of PTEN, SHIP-1, pSTAT3, STAT3, and GAPDH (Figure legend continues)
autoimmune diseases (36, 37). A previous study by Varga et al. (38) suggested that the immunosuppressive effect of Dex might be because of its ability to expand MDSC. By treating BM cells with Dex, we confirmed that Dex strongly promotes the generation of MDSC, and more intriguingly, the combination of cytokines (GM-CSF and IL-6) and Dex had a synergistic effect on the promotion of MDSC and its subpopulation production (Fig. 5A, 5B). To test whether miR-155 and miR-21 play a role in Dex-mediated MDSC expansion, we assessed the levels of miR-155 and miR-21 in BM cells treated with or without cytokines and Dex. As shown in Fig. 5C, Dex treatment strongly increased the levels of miR-155 and miR-21 and, in a similar manner, Dex and cytokines had a synergistic effect on enhancing miR-155 and miR-21 expression. In addition, treatment with both Dex and cytokines (GM-CSF and IL-6) more significantly increased the levels of iNOS and ARG1 mRNA in induced MDSC compared with cytokine treatment alone (Fig. 5D). T cell proliferation assays also confirmed that the MDSC induced by the combination of Dex and cytokines were functionally active (Fig. 5E). In support of the hypothesis that Dex promotes MDSC expansion through the upregulation of miR-155 and miR-21, we showed that MDSC induction by Dex was largely abolished by depleting miR-155 and miR-21 in BM cells (Fig. 5F). T cell proliferation assays confirmed that depleting miR-155 and miR-21 also inhibits the functions of MDSC induced by Dex (Fig. 5G).

**Discussion**

miRNAs have been implicated in the regulation of maturation, activation, proliferation, and differentiation of myeloid cells (39, 40). miRNA dysregulation can lead to various pathological disease including inflammation and cancer (41). MDSC are one of the major cell components linking inflammation and cancer (6). Recent studies have demonstrated that miRNAs may play a critical role in modulating the induction and function of MDSC. For example, miR-494 (34), miR-223 (42), miR-17-5p, and miR-20a (43) have been reported to be involved in the activity of tumor-expanded MDSC. miR-223 (42) and miR-20a (43) alleviated the immunosuppressive potential of MDSC by targeting MEF2C and STAT3 expression, respectively, whereas miR-494 (34) promoted the accumulation and functions of tumor-expanded MDSC via targeting PTEN. The differential expression of miRNAs in MDSC compared with BM cells, however, has not been characterized at genome-wide level.

The present study attempts to identify the key miRNAs that modulate the induction, expansion, and immune-suppressive function of MDSC. Our results indicate that miR-155 and miR-21 are both markedly upregulated in MDSC and play a significant role in promoting the induction of functional MDSC. We also demonstrate that the expressions of miR-155 and miR-21 are regulated by tumor-associated factor TGF-β and immunosuppressant Dex. miR-21 and miR-155 are well-known oncogenic miRNAs (26, 44). Increasing numbers of targets for miR-21 and miR-155 have been verified, and most of them are tumor suppressors. miR-21 and miR-155 also have been implicated in the differentiation and activation of cells of both innate and adaptive immune systems. They contribute to decrease antitumor immunity and enhance cell proliferation and oncogenesis through STAT transcription factors or AKT and TGF-β signaling pathways. This feature coincides with the immune suppression function of MDSC. Several studies have shown that the expansion and suppressive function of MDSC is mediated via the STAT pathway (45).

SHIP-1 functions as a negative regulator of myeloid cell proliferation and survivals. Its expression is restricted in hematopoietic cells (46, 47). PTEN is a well-known tumor suppressor gene through its phosphatase protein product action (48). SHIP-1 and PTEN have been reported previously as the targets of miR-155 (26, 49) and miR-21 (20), respectively. The present study confirmed that miR-155 and miR-21 target these two essential molecules accordingly during the induction of functional MDSC. Because downregulation of either SHIP-1 or PTEN led to the increase of STAT3 activation, a main signaling in promoting MDSC production (50, 51), miR-155 and miR-21, through downregulation of respective SHIP-1 and PTEN signaling, would play a synergistic role in promoting the induction of functional MDSC. Our data clearly point to the synergistic effect of miR-155 and miR-21 on enhancing functional MDSC induction. Overexpression or depletion of both miRNAs showed an additional effect on enhancing or suppressing MDSC induction compared with overexpression or depletion of individual miRNAs, respectively (Fig. 2). A similar additional effect on modulating MDSC induction also was observed when directly knocking down or overexpressing both SHIP-1 and PTEN compared with knocking down or overexpressing individual protein (Fig. 3). Of course, although our data suggest that SHIP-1 and PTEN are two main protein molecules modulated by miR-155 and miR-21 during MDSC induction, the biology of mRNA signaling in functional MDSC induction is likely to be more complex. At this stage, we cannot exclude the involvement of the proteins other than SHIP-1 and PTEN, or even the miRNAs other than miR-155 and miR-21 in regulation of functional MDSC induction.

As an effective immune suppressor, Dex has been used widely to control active inflammation and autoimmune responses, such as allergies, asthma, autoimmune diseases, and sepsis. It is generally believed that Dex executes its effects by upregulating the expression of anti-inflammatory proteins or downregulating the expression of proinflammatory proteins (37). Although efforts have been made to identify these important genes and pathways involved in the anti-inflammatory effect, the mechanism underneath the immune-suppressive effect of Dex is not completely understood. Recently Varga et al. (35) reported that Dex can promote MDSC expansion, suggesting that Dex-induced MDSC may contribute to certain
immune suppressive effect of Dex. Glucocorticoids also have been shown to suppress the immune responses through modulating the expression of miRNAs, including miR-29, miR-27, miR-15a∼16, miR-34a, miR-150, let-7, miR-155, miR-181, miR-182, miR-21, miR-221/222, and miR-17∼92 (52, 53). Our results not only confirmed the effect of Dex on inducting MDSC but also showed that upregulation of miR-155 and miR-21 by Dex could serve as molecular basis underlying the expansion of MDSC by Dex treatment. Because the rapid elevation of cellular miR-155 and miR-21 levels is tightly linked to the expansion of MDSC, the levels of miR-155 and miR-21 may serve as an early indicator for predicting the responses of patients to glucocorticoid therapy.

To our knowledge, the data from this study provide the first evidence that miR-155 and miR-21 can promote MDSC expansion via targeting SHIP-1 and PTEN, respectively, during BM-MDSC induction by GM-CSF and IL-6. As illustrated in the working model (Fig. 5H), the targeting of SHIP-1 by miR-155 and PTEN by miR-21 synergistically contributes to the increase in STAT3 activity, which leads to the expansion of MDSC. Our results also demonstrated that upregulation of miR-155 and miR-21 by Dex play a key role in Dex-mediated MDSC induction. Dex strongly increased the level of both miR-155 and miR-21 and thus has a strong effect on promoting MDSC expansion. Because a synergistic effect between Dex and cytokine (GM-CSF and IL-6) was observed during MDSC induction, the mechanism underlying the upregulation of miR-155 and miR-21 by Dex or cytokines might be different. By identifying a miR-155/miR-21–based mechanism that governs MDSC expansion, our study also provides potential novel targets for controlling body inflammation and autoimmune activity.

Disclosures
The authors have no financial conflicts of interest.

References
Supplementary Figure S1. Effectively overexpressing or depleting miR-155 and miR-21 in BM cells by transfection of miRNA agomir or antagonir (ASO), respectively. BM cells were transfected with miRNA agomirs (miR-21, miR-155 and scrambled control RNA) (A) or miRNA antagonirs (miR-21 ASO, miR-155 ASO and scrambled control RNA) (B). The levels of miR-21 and miR-155 were evaluated by qRT-PCR after three days of cytokine treatment. *, $P<0.05$; **, $P<0.01$. 

* Updated in response to reviewer feedback.*