doi:10.1093/jmcb/mjs044
Published online July 24, 2012

Letter to the Editor

Re-polarization of tumor-associated macrophages to pro-inflammatory M1 macrophages by microRNA-155

Dear Editor,

A significant macrophage population has been detected within almost all human and mouse tumors. These tumor-associated macrophages (TAMs) are critical regulators of the tumor microenvironment and directly affect multiple steps in tumor development, including the growth, survival, invasion, and metastasis of tumor cells. Macrophages are highly versatile, multifunctional cells, and their phenotype closely depends on the physiological or pathological context. In response to microenvironmental signals, macrophages undergo different activation, including the 'classic' pro-inflammatory phenotype (also called M1) and the 'alternative' activated M2 polarization associated with an anti-inflammatory profile (Mosser, 2003). Classically activated M1 macrophages and alternatively activated M2 macrophages represent two extreme macrophage phenotypes. TAMs closely resemble 'alternative' (M2) macrophages, which produce high amounts of interleukin (IL)-10 but not IL-12, express scavenger receptors, and exhibit anti-inflammatory and tissue repair functions (Sica et al., 2008). In contrast, M1 macrophages, activated by interferon (IFN)-γ or other microbial products, produce large amounts of pro-inflammatory cytokines, express high levels of major histocompatibility complex molecules, and are potent killers of pathogens and tumor cells (Mills et al., 2000). The molecular mechanisms underlying TAM polarization to different phenotypes are the focus of intense investigation. Here, we present the first evidence that microRNA-155 (miR-155) is akey molecule controlling macrophage polarization. We found that the overexpression of miR-155 could re-program anti-inflammatory, pro-tumorial M2 TAMs to pro-inflammatory, antitumor M1 macrophages.

Given the fact that the discovery of miRNAs, a class of ~22-nucleotide-long non-coding RNAs, has revealed a new layer of gene regulation in almost every aspect of the biological process, including immune systems (Lodish et al., 2008), it is logic to hypothesize that miRNAs are involved in modulating macrophage polarization. To identify alterations in the miRNA expression profile during macrophage polarization, we prepared standard M1 and M2 phenotype macrophages. In this experiment, bone marrow-derived macrophages (BMDMs) were isolated from BALB/c mice and treated with lipopolysaccharide (LPS) and IFN-γ (for M1 polarization) or IL-4 (for M2 polarization) (Pelegrin and Surprenant, 2009). We used tumour necrosis factor-alpha (TNF-α), Nos2, and IL-12 as markers for the M1 phenotype and four other proteins, Arg1, Ym1, Msr2, Fizz1, and IL-10, as markers for the M2 phenotype. As shown in Supplementary Figure S1A, the marker gene assay clearly indicated that LPS and IFN-γ induced BMDMs to the M1 phenotype, whereas IL-4 induced BMDMs to the M2 phenotype. BMDMs treated with LPS/IFN-γ had significantly higher TNF-α and Nos2 levels but lower levels of Arg1, Ym1, Msr2, and Fizz1 than IL-4-treated BMDMs. As shown in Supplementary Figure S2B and C, the enzyme-linked immunosorbent assay (ELISA) indicated that BMDMs treated with LPS/IFN-γ had a significantly higher IL-12 level but lower IL-10 level than IL-4-
treated BMDMs. Employing a low-density miRNA microarray assay, we compared the miRNA expression profiles in BMDMs, LPS/IFN-γ-induced M1, and IL-4-induced M2 macrophages (Supplementary Table S1). The microarray data showed a dramatic differential expression of miRNAs in M1 and M2 macrophages (Figure 1A). In particular, when BMDMs were induced to M1 macrophages, a panel of miRNAs, including miR-155, miR-147, miR-214, and miR-455, were significantly up-regulated, whereas the up-regulation of these miRNAs was not observed when BMDMs were induced to the M2 phenotype. TaqMan probe-based quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) assays further showed that, among the miRNAs we tested, miR-155 was the most up-regulated (>120-fold) when BMDMs polarized to the M1 phenotype (Figure 1B).

We next assessed the level of miR-155 in macrophages after the dynamic process of macrophage re-polarization. Macrophages with the M1 or M2 phenotypes were re-polarized to the M2 or M1 phenotype by treatment with LPS/IFN-γ or IL-4, respectively. The marker gene assays clearly showed that M2 macrophages could be re-polarized to M1 macrophages by LPS and IFN-γ (Supplementary Figure S1D–F), whereas M1 macrophages could be re-polarized to M2 macrophages by IL-4 (Supplementary Figure S1G–I). Interestingly, the miR-155 levels in macrophages were strikingly decreased following macrophage M1-to-M2 re-polarization (Figure 1C) but elevated during macrophage M2-to-M1 re-polarization (Figure 1D). The results suggest that miR-155 may play a critical role in promoting macrophage polarization to the M1 phenotype.

The role of miR-155 in macrophage polarization was further explored. Through depleting miR-155 in M1 macrophages using an anti-miR-155 antisense oligonucleotide (Supplementary Figure S2A), we successfully re-polarized macrophages

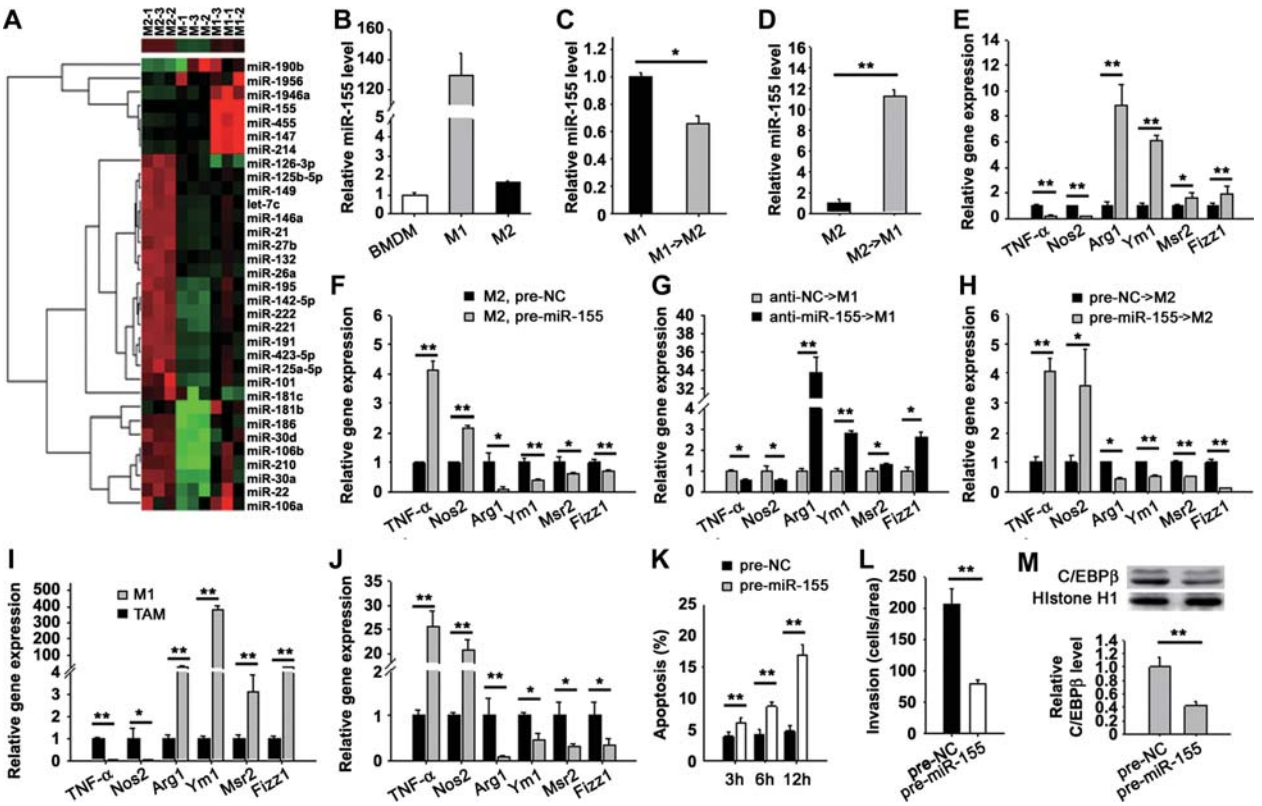

Figure 1 Differential expression of miR-155 during macrophage polarization and its role in re-polarizing TAMs to M1 macrophages. (**A**) The miRNA expression profile of macrophages after M1 or M2 polarization. Microarray assays were performed three times. (**B**) qRT-PCR assays confirmed that miR-155 was the most up-regulated miRNA during the M1 polarization of BMDMs. (**C**) miR-155 levels in macrophages following M1-to-M2 re-polarization by IL-4 for 10 h. (**D**) miR-155 levels in macrophages following M2-to-M1 re-polarization by LPS and IFN-γ for 10 h. (**E**) Re-polarization of M1 macrophages to M2 macrophages by depleting miR-155. (**F**) Re-polarization of M2 macrophages to M1 macrophages through the overexpression of miR-155. (**G**) Macrophages were transfected with an anti-miR-155 ASO or a control oligonucleotide and then stimulated with LPS and IFN-γ for M1 polarization. (**H**) Macrophages were transfected with pre-miR-155 prior to IL-4-mediated M2 polarization. (**I**) The M2-like phenotype of TAMs isolated from mice implanted with S-180 tumors. (**J**) The promotion of TAM re-polarization to the M1 phenotype by miR-155. (**A**–**J**) The data are expressed as the means ± SEM of three independent experiments. (**K**) LLC cell apoptosis was assessed with the FITC-Annexin V assay after 3, 6, and 12 h co-cultured with TAM. (**L**) The invasion of LLC cells after 48 h co-culture with TAM cells in a modified chamber without direct cell-to-cell contact. (**M**) Western blot analysis of C/EBP-β protein level in TAMs. (**K** and **L**) The data are presented as the mean ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$.

from the M1 phenotype to the M2 phenotype, in which the M1 marker TNF-α, Nos2, and IL-12 were down-regulated and the M2 markers Arg1, Ym1, Msr2, Fizz1, and IL-10 were up-regulated (Figure 1E and Supplementary Figure S2B and C). In contrast, when miR-155 was overexpressed in M2 macrophages (Supplementary Figure S2D), they re-polarized toward the M1 phenotype, in which TNF-α, Nos2, and IL-12 were significantly up-regulated and Arg1, Ym1, Msr2, Fizz1, and IL-10 were down-regulated (Figure 1F and Supplementary Figure S2E and F). In addition, when macrophages were transfected with an anti-miR-155 ASO (Supplementary Figure S2G) and then treated with LPS and IFN-γ, the M1 polarization induced by LPS and IFN-γ was inhibited (Figure 1G and Supplementary Figure S2H and I), suggesting that the promotion of macrophage M1 polarization by LPS and IFN-γ might act through the up-regulation of the miR-155 level. In contrast, when macrophages were transfected with pre-miR-155 prior to IL-4-mediated M2 polarization, the elevation of miR-155 in macrophages (Supplementary Figure S2J) caused a reduction of macrophage M2 polarization induced by IL-4 (Figure 1H and Supplementary Figure S2K and L).

We next evaluated whether miR-155 could re-polarize pro-tumorial M2 TAMs into antitumor M1 phenotype macrophages. In this experiment, TAMs were isolated from mice implanted with an S-180 tumor and marker gene assays and ELISA assay indicated that these TAMs were macrophages with the M2-like phenotype (Figure 1I, and Supplementary Figure S3A and B). TaqMan probe-based qRT-PCR assays showed that the miR-155 level in the TAMs was markedly lower than in M1 macrophages (Supplementary Figure S3C). However, when the miR-155 level in TAMs was significantly increased via transfection with a miR-155 mimic (Supplementary Figure S3D), the TAMs were re-polarized into the M1 phenotype (Figure 1J and Supplementary Figure S3E and F).

To determine whether the TAMs that are 're-educated' by miR-155 have the tumor-killing function possessed by M1 macrophages, we transfected TAMs isolated from S-180 tumor-implanted mice with pre-miR-155 or control oligonucleotide

and then incubated these 'modified' TAMs with Lewis lung carcinoma (LLC) cells. LLC cells were labeled with DiI-16 and co-cultured 1:1 with TAM cells that had been transfected with pre-NC or pre-miR-155. The cells were then labeled with FITC-Annexin V (Invitrogen) to assess apoptosis by fluorescence-activated cell sorting analysis (Becton Dickinson). Tumor cell invasion was measured using an artificial basement membrane (Hagemann et al., 2006). Compared with TAMs transfected with a control oligonucleotide, TAMs overexpressing miR-155 significantly decreased tumor cell survival (Supplementary Figure S4A), promoted tumor cell apoptosis (Figure 1K and Supplementary Figure S4B), and inhibited tumor cell invasion (Figure 1L and Supplementary Figure S4C).

Accumulating evidence has shown that macrophage programming and phenotypic switch is regulated by different signal molecules, cytokines, and inflammatory factors. Recent studies showed that miR-155-regulated inflammatory cytokine production in TAMs or proliferation, survival and cell activation in hematopoietic cells via targeting C/EBP-β (He et al., 2009), a critical negative regulator of macrophage programming and activation. Compared with TAMs transfected with a control oligonucleotide, TAMs overexpressing miR-155 significantly reduced the C/EBP-β protein levels but recover the anti-tumor activity of TAMs (Figure 1M). Thus, it is possible that miR-155 promotes the M1 polarization of macrophages through suppressing C/EBP-β signaling cascade. Previous studies by O'Connell et al. (2009), Martinez-Nunez et al. (2011), and Sierra-Filardi et al. (2011) showed that miR-155 could target SH2-containing inositol-5′-phosphatase 1, IL13Rα1, or SMAD2/3 during the regulation of macrophage programming and activation. It is also possible that miR-155 promotes the M1 polarization of macrophages through suppressing the signaling cascade mediated by these molecules. Although the mechanism by which miR-155 governs macrophage M1 polarization remains unknown at this stage, several lines of evidence suggest that miR-155 is not only an M1 phenotype indicator of macrophages but also a critical molecule causing macrophage M1 polarization. First, among all miRNAs we tested, miR-155 had the largest increase when macrophages polarized to the M1 phenotype. In contrast, when macrophages were re-polarized from the M1 to the M2 phenotype, the miR-155 level in the macrophages was reduced. Second, directly overexpressing miR-155 in M2 macrophages or M2-like TAMs, induced macrophage M1 polarization, whereas depleting miR-155 resulted in an inhibition of macrophage M1 polarization induced by LPS and IFN-γ. Finally, the overexpression of miR-155 could re-polarize the anti-inflammatory, tumor-supporting TAMs into pro-inflammatory, tumor-killing M1-like macrophages. Taken together, our results collectively demonstrate a novel miR-155-mediated mechanism for promoting the M1 phenotype of macrophage polarization. Because miR-155-modified TAMs can regain tumor-killing capacity, this study provides a potential therapeutic approach in cancer treatment.

[This work was supported by grants from the National Basic Research Program of China (2012CB517603 and 2011CB50480 3863) and the National Natural Science Foundation of China (30988003, 30890 044, 30771036, 30772484, 30725008, 308 90032, 31071232, 31000323, 90608010, and 90813035).]

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