Host miR155 Promotes Tumor Growth through a Myeloid-Derived Suppressor Cell-Dependent Mechanism

Siqi Chen¹, Long Wang², Jie Fan¹, Cong Ye¹, Donye Dominguez¹, Yi Zhang³, Tyler J. Curiel⁵, Deyu Fang⁴, Timothy M. Kuzel¹, and Bin Zhang¹

Abstract

miR155 is a regulator of immune cell development and function that is generally thought to be immunostimulatory. However, we report here that genetic ablation of miR155 renders mice resistant to chemical carcinogenesis and the growth of several transplanted tumors, suggesting that miR155 functions in immunosuppression and tumor promotion. Host miR155 deficiency promoted overall antitumor immunity despite the finding of defective responses of miR155-deficient dendritic cells and antitumor T cells. Further analysis of immune cell compartments revealed that miR155 regulated the accumulation of functional myeloid-derived suppressive cells (MDSC) in the tumor microenvironment. Specifically, miR155 mediated MDSC suppressor activity through at least two mechanisms, including SOCS1 repression and a reduced ability to license the generation of CD4⁺FOXP3⁺ regulatory T cells. Importantly, we demonstrated that miR155 expression was required for MDSC to facilitate tumor growth. Thus, our results revealed a contextual function for miR155 in antitumor immunity, with a role in MDSC support that appears to dominate in tumor-bearing hosts. Overall, the balance of these cellular effects appears to be a root determinant of whether miR155 promotes or inhibits tumor growth.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Corresponding Author: Bin Zhang, Northwestern University, Chicago, IL 60611. Phone: 312-503-2447; Fax: 312-503-0189; E-mail: bin.zhang@northwestern.edu
doi: 10.1158/0008-5472.CAN-14-2331
©2014 American Association for Cancer Research.

Micrornas are evolutionarily conserved small noncoding RNAs that posttranscriptionally modulate the expression of multiple target genes and are hence implicated in a wide series of cellular and developmental processes (1, 2). miR155 is processed from the B-cell integration cluster (BIC), a noncoding transcript primarily upregulated in both activated B and T cells (3) and in monocytes/macrophages upon inflammation (4, 5). Recent gene-targeting studies of miR155 demonstrate a broad role for miR155 in the regulation of both immune cell development and function (6, 7). Indeed, miR155-deficient mice have global immune defects due to defective B- and T-cell immunity and reduced dendritic cell (DC) function. Particularly, miR155-deficient DCs fail to present antigens efficiently (6) and produce cytokines (8), whereas miR155 in CD4⁺ T cells regulates differentiation into the Th1, Th2, and Th17 pathways (6, 9, 10). Furthermore, miR155 is required for CD8⁺ T-cell responses to acute viral and bacterial challenges (11–14). In addition to these immunostimulatory effects, miR155 can also exert some immunosuppressive effects, such as promoting the development (15), or homeostasis and fitness (16) of regulatory T cells (Treg), and expansion of functional myeloid-derived suppressive cells (MDSC; ref. 17). Thus, miR155 could modulate protective immune responses and inflammation through distinct mechanisms.

miR155 dysregulation is closely related to cancer (4). miR155 transgenic mice develop B-cell malignancy (18), and elevated miR155 expression was reported in several types of human B-cell lymphomas (19). A correlation between increased miR155 and development of tumors such as leukemias, glioblastoma, and breast, lung, or gastric cancers has been established recently (20, 21). Therefore, targeting miR155 has been proposed as a promising approach to treat both hematopoietic and solid cancers (22–24). However, the potent immunostimulatory effects of miR155 have also been observed in the context of tumor. Notably, the roles of miR155 in effector CD8⁺ T cells (13, 25), tumor-infiltrating DCs (26, 27), and tumor-associated macrophages (28, 29) can be modulated to potentiate cancer immunotherapies. Thus, when cancer is treated in an immunocompetent host by inhibiting miR155, outcomes are difficult to predict. Importantly, underlying mechanisms of host miR155 in modulating tumor growth are still poorly understood. We show here that host miR155 deficiency hampers the accumulation of functional MDSCs and inducible Treg cells in the tumor microenvironment, thereby promoting antitumor T-cell immunity and retarding tumor growth.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 miR155⁻/⁻, CD45.1, and CD90.1 mice were purchased from the Jackson Laboratory, OT-I Rag1⁻/⁻ and OT-II Rag1⁻/⁻ mice from Taconic, and C57BL/6 miR155⁻/⁻ mice from NCI-Frederick. Dr. Hans Schreiber (University of Chicago)...
provided the MC38, EG7, B16F10, B16-Siy cell lines, anti-Gr1 antibodies (RB6-8C5), and 2C transgenic mice. Murine Lewis Lung Carcinoma (LLC1) cells were purchased from ATCC (CRL-1642). LLC1 cells were infected with MigR1-ovalbumin (OVA)-IRES-eGFP (30), and OVA-expressing cells (LLC1-OVA) were sorted twice based on GFP expression. OVA production was confirmed by ELISA (data not shown). All the cell lines were routinely tested for mycoplasma infections by culture and DNA stain, and maintained in complete medium composed of RPMI 1640 with 5% FBS. All animal experiments were approved by institutional animal use committees of the University of Texas Health Science Center at San Antonio and Northwestern University. The OVA-derived peptide OVA-I (SIINFEKL) was synthesized by GenScript. Dichlorofluorescin diacetate (DCFDA), azoxymethane (AOM), and 5-fluorouracil (5-FU) were purchased from Sigma-Aldrich. Dextran sulfate sodium salt (DSS) was purchased from Affymetrix, Inc. All the mAbs for flow cytometry were purchased from eBioscience and BioLegend. The Annexin V apoptosis detection Kit was from BioLegend. The Kd/OVA tetramers were provided by the National Institutes of Health Tetramer Core Facility. Depleting mAb clone GK1.5 (anti-CD4), clone 53.6.7 (anti-CD8a), and clone PK136 (anti-NK1.1) were purchased from Bio X Cell. Nω-hydroxy-norepinephrine (Nor-NOHA) and arginase I activity kit were purchased from Cayman Chemical Company.

Analysis of cells by flow cytometry

All samples were initially incubated with 2.4G2 to block antibody binding to Fc receptors. Single-cell suspensions were stained with 1 μg of relevant mAbs and then washed twice with cold PBS. Reactive oxygen species (ROS) detection by DCFDA staining was conducted as described by Marigo and colleagues (31). The Annexin V staining, Kd/OVA tetramer staining, Foxp3 staining, and intracellular IFNγ staining were performed as previously described (32). Samples were conducted on a MACS-Quanta Analyzer (Miltenyi Biotec), and data were analyzed with FlowJo software.

In vivo killing assay

Analysis of tumor antigen-specific effector CTL activity in vivo was performed as previously described (32). Briefly, OVA-I-SIINFEKL peptide-pulsed eFluor 450+ and SIY-peptide-pulsed eFluor 450low splenocytes were mixed at a ratio of 1:1, and a total of 2 × 10⁶ cells were injected i.p. into recipient animals. Draining lymph nodes (DLN) and spleen were then harvested 24 hours after adoptive transfer, and eFluor 450 fluorescence intensity was analyzed by flow cytometry.

MDSC suppressive assay

Splenic MDSCs from tumor-bearing wild-type (WT) or mir155−/− mice were selected with CD11b MicroBeads (Miltenyi Biotec), and tumor-infiltrating CD11b−CD11c+Gr1− MDSCs were sorted by a BD FACSAria cell sorter from LLC1-bearing mice. MDSCs were added at different ratios to OT-I or 2C splenocytes stimulated with 0.5 μg/mL OVA-I or SIY peptides for 3 days, and [3H] thymidine uptake was measured. For experiments that examined the effect of arginase inhibitors, nor-NOHA (Nω-hydroxyl-nor-L-arginine, 0.5 mM) were added at the beginning of the culture. To evaluate MDSC tolerogenic activity in vivo T-cell function, naive OT-I CD90.1 cells (2 × 10⁶ per mouse) were transferred to CD90.2 congenic recipients, which were s.c. immunized. 2 days later, with 10 μg OVA-I peptides in incomplete Freund’s adjuvant (IFA). MDSCs (2 × 10⁶) from MC38 tumor-bearing WT or mir155−/− mice, either pulsed or not with OVA-I peptides, were transferred on the same day of the immunization. DLNs were collected 10 days after immunization and stimulated with 0.5 μg/mL OVA-I in vitro for 3 days to measure T-cell proliferation by [3H] thymidine uptake and IFNγ-secreting CD8+ T cells by flow cytometry.

Arginase activity

Arginase activity was measured in cell lysates using the commercially available QuantiChrom Arginase Assay Kit (BioAssay Systems) according to the manufacturer’s instructions.

Bone marrow–derived MDSC generation

Tibias and femurs from C57BL/6 mice were removed using sterile techniques, and bone marrow (BM) cells were flushed. To obtain BM-derived MDSCs, cells were cultured with GM-CSF (40 ng/mL Biologend) and IL6 (40 ng/mL Biologend) for 4 days. BM-derived MDSCs were selected using CD11b or Gr1 MicroBeads (Miltenyi Biotec).

RNA extraction and real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer’s instructions. mir155 expression was detected by a TaqMan MicroRNA Assay kit (Applied Biosystems). The cDNA synthesis was performed using SuperScript One-Step RT-PCR (Invitrogen). Quantitative real-time PCR was used to quantify a series of MDSC-associated genes by SYBR Green (BioRad), and relative abundance of each mRNA was normalized to GAPDH mRNA.

Transfection of BM-derived MDSCs

The transfection of primary BM cells was performed according to the instructions of the manufacturer (AMAXA). BM cells were treated with GM-CSF (40 ng/mL; Biologend) for 24 hours, followed by the transfection with 1 μmol/L pre-miR155/BIC (P-MDSC; Ambion), 2 μmol/L mir155 inhibitor miRNA (I-MDSC; Biolegend), or control oligonucleotides (C-MDSC; Dharmacon) by AMAXA. For knockdown of SOCS1, specific and respective control siRNAs used for transfection were from Santa Cruz Biotechnology. To recover, cells were cultured for additional 72 hours in the presence of GM-CSF (40 ng/mL; Biologend) and IL6 (40 ng/mL; Biologend) after transfection. After selection with CD11b or Gr1 MicroBeads (Miltenyi Biotec), these GM-CSF and IL6-conditioned BM-derived MDSCs were tested for suppressive assay.

Treg induction

Splenic WT or mir155−/− CD4+CD25+ naïve T cells were selected with a CD4+CD25+ T-cell isolation kit (Miltenyi Biotec), and injected i.v. at 5 × 10⁶ per mouse into CD45.1 mice followed by a s.c. injection of 10⁶ LLC1-OVA cells. The conversion of transferred T cells to Foxp3+ cells (CD45.2+ ) in DLN and spleen from LLC1-OVA tumor-bearing mice were detected by flow cytometer 9 days after tumor cell injection. MDSC-mediated Treg induction, splenic WT and mir155−/− Gr1+ CD11b+ MDSCs from LLC1 tumor-bearing mice were cocultured with OT-II splenocytes at a 1:4 ratio for 5 days in the absence or presence of 2 ng/mL TGFβ, and induced CD25+ Foxp3+ cells among total CD4+ T cells were subsequently determined by flow cytometry.
AOM and DSS treatment

For the colitis-associated colon cancer model, mice were given 10 mg/kg AOM via i.p. injection. One week later, 2.5% DSS was given in the drinking water for 7 days followed by 14 days of normal water for a total of three cycles. Colonos were harvested, flushed of feces, cut longitudinally, and fixed in 10% buffered formalin overnight. The colons were scored with the aid of a magnifier for the number of colonic neoplasms to determine the incidence (number of animals with at least one tumor) and multiplicity (number of tumors per animal) of neoplasms. Tumor area was also evaluated based on length and width.

Tumor challenge and treatments

B16F10, B16-SIY, LLC1, LLC1-OVA, MC38, or EG7 cells (1 × 10^6) in suspension were injected s.c. For MDSC depletion, 3 days after tumor cell injection, mice were injected i.p. by 5-FU (50 mg/kg) or anti-Gr1 antibodies (RB6-8C5, 200 μg) once every 4 days. Depletion of CD4+ T cells, CD8+ T cells, or natural killer (NK) cells was achieved by twice a week i.p. injection of depleting mAb clone GK1.5 (anti-CD4, 200 μg), clone 53.6.7 (anti-CD8α, 200 μg) or clone PK136 (anti-NK1.1, 200 μg) starting one day before tumor challenge. Flow cytometry confirmed depletion efficiency of target cells for 3 days following injections. For adoptive transfer of MDSCs, splenic G1+ CD11b+ MDSCs from tumor-bearing WT or miR155+/− mice were injected i.v. at 5 × 10^6 per mouse into LLC1-bearing mice at days 7 and 14. For adoptive transfer of Tregs (33), splenic CD4+CD25+ Treg cells were selected with a CD4+CD25+ regulatory T-cell isolation kit (Miltenyi Biotec) from WT or miR155+/− mice, and i.v. injected at 2 × 10^6 per mouse into LLC1-bearing mice on days 7, 14, and 20. The size of tumor was determined at 2- to 3-day intervals. Tumor volumes were measured along 3 orthogonal axes (a, b, and c) and calculated as abc/2.

Statistical analysis

Mean values were compared using an unpaired Student t test. Probability values >0.05 were considered nonsignificant.

Results

Host miR155 promotes tumor growth

The immunoregulatory role of miR155 has been well documented in numerous experimental settings. However, the specific contributions of endogenous miR155 to antitumor immunity and tumorigenesis are poorly understood. We compared chemically-induced tumor and transplantable tumor growth in miR155-deficient (miR155−/−) versus syngeneic, WT (miR155+/+) mice. Mice were given AOM and DSS, as previously described, to promote colorectal carcinogenesis (34). Upon AOM and DSS challenge, miR155−/− mice exhibited less acute body weight loss comparable with WT mice (data not shown). AOM and DSS produced colonic tumors in all 8 WT mice, but in 3 of 8 miR155−/− mice. The multiplicity of colonic neoplasms (number and size of tumors) was also significantly decreased in miR155−/− mice. However, there was no significant difference in colon length between untreated WT and miR155−/− mice (Fig. 1A). Moreover, WT and miR155−/− mice given AOM alone or DSS alone had no macroscopic colonic tumors (data not shown). We next studied the role of host miR155 on transplantable tumor growth in miR155−/− mice. MC38 colon cancer cells (Fig. 1B) and LLC1 Lewis lung carcinoma cells (Fig. 1C) were s.c. inoculated into WT or miR155−/− mice. Tumors injected into miR155−/− mice exhibited delayed growth compared with those in control mice (Fig. 1B and C). In addition, miR155 deficiency was also effective in inhibiting the growth of immunogenic LLC1-OVA (Fig. 1D). Similarly, the growth of lymphoma EG7 (expressing OVA antigen) tumors was inhibited in miR155−/− mice (Fig. 1E). However, the sizes of B16-SIY melanoma (expressing SIY antigen) were comparable between the WT and miR155−/− mice at multiple time points (Supplementary Fig. S1A), suggesting that the role of host miR155 in tumor growth is tumor-dependent.

Host miR155 deficiency enhances antigen-specific antitumor T-cell immunity

Given the importance of miR155 in immune regulation, we next examined the phenomenon and cytokine profile of tumor-infiltrating immune cells in tumor-bearing mice. At 19 days after tumor inoculation, we found no significant alterations in the percentages of B cells (CD19+), NK cells (NK1.1+), or myeloid DCs (CD11b+CD11c+) in local infiltrates of EG7 (Fig. 2A) or LLC1-OVA (Supplementary Fig. S2A) tumors in miR155−/− versus WT mice. Interestingly, remarkably fewer tumor-infiltrating CD8+, CD4+ lymphocytes were found in miR155−/− versus WT mice (Fig. 2A and Supplementary Fig. S2A). Although percentages of tumor-infiltrating IFNy+ CD8+ T cells were comparable between groups, tetramer staining showed a greater number of OVA-reactive (tumor-specific) CD8+ T cells in EG7-bearing (Fig. 2B and C) or LLC1-OVA–bearing (Supplementary Fig. S2B and S2C) miR155−/− mice than WT mice. We next examined the cytolytic function of tumor antigen-specific T cells. Target cell lysis in vivo was remarkably improved in DLN of EG7 tumor-bearing miR155−/− mice compared with tumor-bearing WT mice (Fig. 2D). To assess the roles of CD4+, CD8+, and NK cells in the tumor-inhibiting effects observed in miR155−/− mice, mice were inoculated with EG7 cells, and subsequently received depleting anti-CD4, anti-CD8α, or anti-NK1.1 antibodies against CD4+ or CD8+ T cells, or NK cells, respectively. Notably, the tumor-inhibiting advantage of host miR155 deficiency was primarily dependent on CD8+ cells, but independent of CD4+ cells or NK cells (Fig. 2E). These data suggest that loss of miR155 expression in mice results in the enhanced antitumor T-cell immunity that contributes to the inhibition of immunogenic tumor growth.

Previous studies have demonstrated the involvement of miR155 in the DCs (26, 27) and T cells (13, 25) in controlling immune cell functions. Here, we showed the inhibitory advantage of host miR155 deficiency in the context of tumor-bearing mice, which was independent of CD4+ cells or NK cells. Moreover, tumor growth inhibition by host miR155 deficiency was strongly dependent on CD8+ cells, but independent of CD4+ cells or NK cells (Fig. 2E). These data suggest that loss of miR155 expression in mice results in the enhanced antitumor T-cell immunity that contributes to the inhibition of immunogenic tumor growth.

miR155 is required for MDSC accumulation in tumor-bearing mice

Although above data suggest a cell-intrinsic role of miR155 in tumor-associated DCs and antitumor T cells, host miR155 deficiency promoted overall antitumor T-cell immunity and inhibited tumor growth. In search of a cellular mechanism for the miR155-mediated tumor-promoting effect, we investigated...
the well-defined immunosuppressive immune cell subsets in tumor, including MDSCs and Tregs. We observed that intratumoral Gr1<sup>+</sup>CD11b<sup>+</sup> MDSCs were consistently decreased in LLC1-OVA-bearing miR155<sup>−/−</sup> mice in comparison with WT mice (Fig. 3A and B). Further analysis revealed significant reductions in the percentage of CD11b<sup>+</sup>Gr1<sup>+</sup> cells from miR155<sup>−/−</sup> mice compared with WT controls in spleen and peripheral blood (Fig. 3C). We also tested other tumor models, including EL4, B16F10, and LLC1, and found that miR155<sup>−/−</sup> mice have much fewer splenic MDSCs than WT mice (Supplementary Fig. S4A). These results confirmed that miR155 is required for MDSC accumulation under tumor-bearing conditions because no significant differences were noted in the percentages of splenic CD11b<sup>+</sup>Gr1<sup>+</sup> cells between tumor-free miR155<sup>−/−</sup> and WT mice (data not shown). MDSC consists of ly6G<sup>−</sup>/ly6C<sup>high</sup> (monocytic) and ly6G<sup>−</sup>/ly6C<sup>low</sup> (granulocytic) subpopulations (31, 35). Of note, the preferential reduction of the splenic (Supplementary Fig. S4B and S4C) and tumor-infiltrating (Fig. 3D) monocytic ly6G<sup>−</sup>/ly6C<sup>high</sup> subset was observed in LLC1-OVA-bearing miR155<sup>−/−</sup> mice compared with WT mice. These results suggest miR155 is required for tumor-associated MDSC accumulation particularly with a monocytic phenotype. To dissect the role of miR155 further in regulating MDSC accumulation, we stained with Ki67 (Fig. 3E) and Annexin V (Fig. 3F) to test the proliferative ability and apoptotic status of MDSCs within the tumor microenvironment, respectively. No significant differences in both granulocytic and monocytic MDSC subsets were found between WT mice and miR155<sup>−/−</sup> mice.

Given the critical function of miR155 in promoting myeloid lineage commitment in hematopoietic stem cells and myeloid progenitors (36), we asked whether MDSC differentiation requires miR155. To evaluate differentiation of myeloid cells in the presence of tumor-derived factors, BM cells from miR155<sup>−/−</sup> mice and their WT littermates were cultured with GM-CSF for 5 days in tumor cell-conditioned medium (TCM). As expected, tumor-derived factors significantly reduced the differentiation of DCs and macrophages and increased the generation of Gr1<sup>−</sup>CD11b<sup>−</sup> MDSCs in WT populations (Fig. 3G), consistent with previous observation (37). In contrast, TCM failed to inhibit the differentiation of myeloid progenitor cells appreciably from miR155<sup>−/−</sup> mice (Fig. 3G), suggesting an important role of miR155 in MDSC differentiation in the tumor microenvironment.

miR155 is required for MDSC suppressive function during tumor growth

To determine whether miR155 is required for MDSC suppressive function, we purified CD11b<sup>+</sup> cells from MC38-bearing mice and cocultured with OT-I splenocytes. Notably, the miR155<sup>−/−</sup>-MDSC appreciably lost their capacity to suppress proliferation of
Host miR155 Promotes Tumor Growth

antigen-specific T cell in vitro, whereas WT MDSC remained strongly suppressive (Fig. 4A). To corroborate these findings further, splenic MDSCs were isolated from EG7 (Fig. 4B) or B16-SIY (Supplementary Fig. S1B) tumor–bearing WT or mir155−/− mice. As expected, mir155−/− MDSCs were unable to inhibit antigen-specific T-cell proliferation in vitro compared with WT MDSCs. Emerging data show that the degree of immunosuppression varies among populations of MDSCs isolated from different organs, and intratumoral MDSCs are the most immunosuppressive (38). In the LLC1 tumor model, CD115 acts as a function marker for MDSCs (39). Thus, we sorted CD115+ CD11b+ cells and CD115− CD11b+ cells from LLC1 tumor tissues, and compared their suppressive activity between WT and mir155−/− mice (Fig. 4C). Consistent with previous results, intratumoral WT CD115+ CD11b+ cells but not CD115− CD11b+ cells were inhibitory. In contrast, mir155−/− CD115+ CD11b+ cells were unable to suppress T-cell proliferation (Fig. 4B). To evaluate MDSC tolerogenic activity on antigen-specific CD8+ T cells in vivo, MDSCs from tumor-bearing WT or mir155−/− mice, either pulsed or not with OVA-I peptides, were transferred on the same day of the immunization. DLNs were collected 10 days after immunization and stimulated in vitro to measure T-cell proliferation (Fig. 4D) and enumerate CD8+ T cells producing IFN-γ (Fig. 4E). Both the number of transferred CD90.1+ cells and number of IFN-γ-secreting CD8+ T cells in DLNs were significantly reduced in mice that received MDSCs derived from WT tumor–bearing mice, but not mir155−/− tumor–bearing mice.

miR155 is upregulated and functions in cytokine-induced MDSCs

It is generally accepted that MDSCs are elicited by tumor-derived factors (e.g., GM-CSF, IL6) from precursors present in...
miR155 is required for MDSC accumulation in the tumor microenvironment. A, the percentage of tumor-infiltrating Gr1<sup>+</sup>CD11b<sup>−</sup> MDSCs was determined by flow cytometry from LLC1-OVA tumor-bearing mice. B, the absolute number of tumor-infiltrating MDSCs (n = 3). C, the percentage of MDSCs in spleen (n = 9) and peripheral blood (n = 5) from LLC1-OVA tumor-bearing mice is summarized. D, percentage of CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>dim</sup> (granulocytic) and CD11b<sup>+</sup>Ly6G<sup>−</sup>Ly6C<sup>high</sup> (monocytic) MDSCs is indicated within plots and summarized (n = 9). Flow cytometry analysis of expression of Ki-67 (E) and Annexin V (F) on both granulocytic and monocytic tumor-infiltrating MDSCs (n = 6). G, BM cells were cultured with GM-CSF and IL4 for 5 days in complete culture medium or in the TCM. The cell phenotypes were examined by flow cytometry. Data are given as mean ± SEM. *P < 0.05; **P < 0.01; ***P < 0.001. Data are representative of two independent experiments.

miR155 deficiency downregulates tumor-associated MDSC suppressive pathways

To identify the factors by which miR155 regulated MDSCs, we analyzed gene expression profiles in WT versus miR155<sup>−/−</sup> MDSCs from LLC1-OVA–bearing mice. We used real-time PCR to evaluate mRNA levels of genes related to tumor angiogenesis, immune responses, and immune suppression (Fig. 4F). We found that mmp9, vegf, inos, and arg1 were down regulated, whereas socs1 and ship1 were upregulated in miR155<sup>−/−</sup> MDSCs. On the basis of previous observations (45–47), VEGF and MMP-9 expressed by MDSCs contribute to the proangiogenic tumor microenvironment. Thus, our results raised the possibility that miR155 expressed by MDSCs could promote tumor growth by stimulating tumor angiogenesis. Because inducible nitric oxide synthase

Chen et al.
miR155 is required for MDSC suppressive function during tumor growth. A, suppressive activity of MDSCs from MC38-bearing WT versus miR155−/− mice. Splenic Gr1+CD11b+ MDSCs from either MC38-bearing WT or miR155−/− mice were added at different ratios to OT-I splenocytes stimulated with OVA-I peptides for 3 days, and [3H] thymidine uptake was measured. The suppressive activities of tumor-infiltrating MDSCs from EG7-bearing (B) or LLC1-bearing (C) WT and miR155−/− mice were assessed in a similar manner as described in A. D, to evaluate MDSC tolerogenic activity on in vivo T-cell function, naive OT-I CD90.1 cells were transferred to CD90.2 congenic recipients, which were s.c. immunized, 2 days later, with OVA-I peptides in IFA. MDSCs from MC38 tumor-bearing WT or miR155−/− mice, either pulsed or not with OVA-I peptides, were transferred on the same day of the immunization. DLNs were collected 10 days after immunization and stimulated with OVA-I in vitro to measure T-cell proliferation by [3H] thymidine uptake. E, frequencies of CD8+CD90.1 cells and IFN-γ-secreting CD8+ T cells as determined by flow cytometry are summarized. F, real-time quantitative RT-PCR analysis of different gene expression in WT and miR155−/− MDSCs from LLC1-OVA-bearing mice (n = 5-14). G, ROS production was measured with DCFDA staining by flow cytometry and summarized within the granulocytic and mononuclear cell gate in both WT and miR155−/− MDSCs. H, arginase I activity of WT versus miR155−/− MDSCs. I, arginase I inhibitor nor-NOHA was able to blunt the suppressive activity of WT MDSC but not miR155−/− MDSCs. All samples had MDSC, and the ratio of T cell/MDSC was 2:1.
and MDSC induction upon tumor-bearing conditions. Interestingly, we detected enhanced levels of socs1 in miR155−/− MDSCs compared with WT MDSCs in the tumor-bearing mice but not tumor-free mice (Fig. 5C). To test the functional consequence of elevated socs1 expression (as observed in the absence of miR155) on MDSC suppressive activity, we utilized siRNAs to knock down socs1 expression in activated miR155−/− MDSCs. We found that socs1 knockdown by specific siRNAs completely restored suppressive activity of miR155−/− MDSCs compared with cells given a scrambled control (Fig. 5D). Thus, miR155 targets SOCS1 to regulate the suppressive function of MDSCs.

miR155 is required for MDSC-mediated Treg induction

Because fewer tumor-infiltrating CD4+Foxp3+ Tregs were found in tumor-bearing miR155−/− mice than in WT mice (Fig. 6A and Supplementary Fig. S6), we tested a role for miR155 in Treg in immune suppression. As shown in Supplementary Fig. S7A, Tregs from either WT or miR155−/− mice potently suppressed proliferation of CD4+ T cells in vitro, consistent with previous results (15, 16). Moreover, miR155−/− Tregs from tumor-bearing mice had similar levels of CD39, CD73, CTLA-4, GITR, CD44, and CD62L expression compared with WT Treg (Supplementary Fig. S7B). To test further the tumor-promoting role of Tregs, we performed adoptive transfer of WT or miR155−/− Tregs into LLC1-bearing mice. There was a subtle difference in tumor growth between mice receiving WT and miR155−/− Tregs (Supplementary Fig. S7C). In addition, the role of miR155 in tumor-mediated conversion of Tregs was also evaluated. We found similar conversion of CD4+ Foxp3+ cells in spleen and DLNs after transfer of WT or miR155−/− CD62L+CD4+ naive T cells into the tumor-bearing mice (Fig. 6B). Thus, these data exclude a direct contribution of miR155 to Treg-mediated suppressive function and tumor promotion, and tumor-mediated conversion of Tregs.

MDSCs induce Treg expansion in tumor-bearing mice (39, 49). To determine whether miR155 mediates MDSC-mediated Treg induction, miR155−/− or WT MDSCs were cultured with OT-II T cells plus OVA-II peptides. As expected, MDSCs were ineffective to induce antigen-specific Treg in the absence of TGFβ, but decreased Treg cell induction was observed when comparing miR155−/− with WT MDSCs in the presence of TGFβ (Fig. 6C), indicating a role for miR155 in MDSC-mediated Treg induction.

miR155 expression by MDSC facilitates tumor growth

As miR155 was required for MDSC accumulation and function, we tested whether miR155 promoted tumor growth in an MDSC-dependent manner. We performed MDSC depletion in WT and miR155−/− mice using either 5-FU (Fig. 7A) or depleting anti-Gr1 antibodies (Fig. 7B) after LLC1 tumor challenge. Consistent with prior published data (50, 51), both 5-FU and anti-Gr1 efficiently depleted CD11b+Gr1hi populations, especially the Gr1hi population within tumor-bearing mice (Fig. 7A and B). Importantly, MDSC depletion greatly inhibited tumor growth in WT mice, indicating a tumor-promoting role for MDSCs. By contrast, MDSC depletion minimally affected tumor growth in miR155−/− mice compared with WT mice at later time points (starting from day 22; Fig. 7A and B). Moreover, adoptive transfer of WT MDSCs into miR155−/− mice resulted in faster tumor growth than transfer of miR155−/− MDSCs (Fig. 7C), further consistent with the direct role of miR155 on MDSCs in tumor growth. We did not expect miR155−/− MDSCs to have migration defects. This notion is supported by the fact that miR155−/− MDSCs displayed equal ability to traffic to the tumor site as WT MDSC (Fig. 7D), excluding the possibility that miR155−/− MDSC may not reach the tumor to

Figure 5. miR155 targets socs1 within MDSCs. A and B, miR155 expression was measured by the real-time quantitative RT-PCR in splenic Gr1hi CD11b+ cells from WT naive mice, LLC1 tumor-bearing WT mice (A), and miR155−/− tumor-bearing mice (n = 3; B). C, Socs1 gene expression was measured by the real-time quantitative RT-PCR in splenic WT or miR155−/− Gr1hi CD11b+ cells from naive mice and LLC1 tumor-bearing mice (n = 5). D, to identify the function of SOCS1 within Gr1hi CD11b+ MDSC, miR155−/− MDSCs were transfected with siRNAs against SOCS1 or control oligos, and WT MDSCs were also transfected with control oligos by AMAXA. MDSCs 48 hours after transfection were added at different ratios to OT-I splenocytes stimulated with OVA-I peptides for 3 days, and [3H] thymidine uptake was measured. Data are given as mean ± SEM. Data are representative of two independent experiments. *, P < 0.05; **, P < 0.01.
exert their effect. These results indicate that miR155 expression is required for MDSCs to facilitate tumor growth.

**Discussion**

miR155 is required for development and function of both innate and adaptive immune cells, and is thought to be largely immune stimulatory (52–54). However, to our surprise, chemically-induced tumor incidence and transplanted tumor growth were decreased in miR155/C0/C0 mice. This was associated with a number of immune phenotypic and functional alterations. MDSCs and Treg cells are important immunosuppressive cells in the tumor microenvironment. Notably, there were significantly more MDSCs and Treg cells in tumor-bearing WT mice than in tumor-bearing miR155/C0/C0 mice. However, the prevalence of MDSCs and Tregs was similar in tumor-free WT versus miR155/C0/C0 mice. Our results indicate that miR155 could regulate the development of MDSCs and Treg cells in the context of tumor, and in turn affect antitumor immune responses. In line with our observations, a recent study showed that miR155 was upregulated in cytokine-induced MDSCs from BM cultures and spleen MDSCs isolated from tumor-bearing mice, and promoted expansion of functional MDSCs (17). However, it remained unclear whether miR155 mediates inhibition of tumor growth in a MDSC-dependent manner despite its defined immune-stimulatory functions. In addition to regulating immunosuppressive factors, we do not rule out the contribution of miR155 to tumor immunity through other immune components. Indeed, we observed the intrinsic defects in miR155/C0 tumor–associated DCs and antitumor T cells. Consistent with this concept, miR155 is required for activation of tumor-associated DCs (26, 27) and effector CD8+ T cell (13, 25) responses to cancer. Moreover, ectopic miR155 expression repolarized protumoral M2 macrophages toward an anti-tumor M1 phenotype (27), and increasing miR155 levels in tumor-associated DCs by miRNA mimetics increased antitumor responses (26). We do not exclude, however, that miR155 insufficiency in other immune compartments may have similar protumoral effects, as recently proposed for NK cells (55).

**Figure 6.**

miR155 is required for MDSC-mediated Treg induction. A, representative dot plots of Foxp3 expression in LLC1-OVA tumor–infiltrating CD4+ cells. The percentage of Foxp3+ cells is indicated within plots and summarized (n = 5). B, WT or miR155−/−CD4+CD62L+ naive T cells were transferred into CD45.1 mice, followed by a s.c. injection of LLC1-OVA cells. The conversion of transferred T cells to Foxp3+ cells (CD45.2+) in DLN and spleen from LLC1-OVA tumor–bearing mice was detected by flow cytometry 9 days after tumor cell injection. The levels of converted Foxp3 expression were determined by mean fluorescent intensity (MFI). Endogenous Foxp3+ cells (CD45.1+) from host mice are shown as controls. C, WT and miR155−/−MDSCs from LLC1 tumor–bearing mice were cocultured with OT-II splenocytes at a 1:4 ratio for 5 days in the absence or presence of TGFβ, and induced CD25+Foxp3+ cells among total CD4+ T cells were subsequently determined by flow cytometry. Data are representative of two independent experiments. *** P < 0.001.
Notably, our results on host miR155 deficiency and tumor growth differ from a recent study (25) using the EL4 tumor model. Reasons for this discrepancy could include differences in the tumor cell lines that could alter the accumulation of distinct immune cell subsets in the tumor microenvironment. We also used EG7 cells expressing the surrogate antigen OVA, rather than parental (OVA-negative) EL4 cells in the prior report (25). These immune differences could alter in vivo outcomes. Finally, given that miR155 regulation of one immune cell type can antagonize the function of other cells, the balance of these effects may determine whether miR155 is beneficial or detrimental to tumor growth. In this regard, the prior study (25) focused on the intrinsic role of miR155 in effector T cells, but did not analyze other distinct cellular subsets within the tumor such as MDSCs and Treg cells that promote tumor growth, as we showed in our study. Despite defects in immunostimulatory activities observed in miR155−/− effector T cells and DCs, they are still able to mount antitumor responses. Increased miR155 could play a critical role in balancing anti- and protumor immune components within the tumor. In a given tumor model system, miR155 could preferentially promote MDSCs and Treg cells before potent antitumor T-cell immunity is established. Furthermore, the extent and regulation of tumor-induced immunosuppression including MDSC and Tregs could vary in different tumor types and/or tumor stages. In support, we showed that host miR155 deficiency inhibited the growth of MC38 and LLC1 tumors rather than B16 tumors. Thus, it is likely that miR155 plays dominant, MDSC-intrinsic roles in impairing antitumor T-cell immunity in these tumor models. Our data suggest that the immune regulation of miR155 is highly context-dependent, and varies in the presence of different cells, phases of immune responses, and tumor model systems. Our studies highlight the importance of evaluating the intrinsic contribution of miR155 carefully in major immune cell subsets, where miR155 could be either protective or deleterious to antitumor immunity.
Although miR155 is required for Treg cell homeostasis in the presence of limiting amounts of IL2, it is dispensable in non-competitive lymphopenic settings (16). Indeed, we showed no impaired ability of miR155-deficient T cells to induce Foxp3 in tumor-bearing mice. Moreover, intact suppressive activity of miR155-deficient Tregs was observed, consistent with previous results (15, 16). In addition to the direct inhibiton of T-cell proliferation, MDSCs can induce Treg expansion in tumor-bearing mice. Considering the importance of miR155 for functional MDSC development, we tested whether miR155 is required for MDSC-mediated Treg induction. It appears that loss of miR155 results in the reduced accumulation of MDSCs that not only can inhibit clonal expansion of activated effector T cells but also induce tumor-specific Tregs to establish and maintain T-cell suppression in tumor-bearing mice. Therefore, our results indicate that miR155 is likely involved in a close interaction of MDSCs and Treg development during tumor progression.

In an effort to unravel the molecular basis for miR155’s function in the MDSCs, we found the miR155-targeted SOCS1 to retain the suppressive activity of MDSCs. SOCS1 is defined as an important mechanism for the negative regulation of the cytokine-JAK-STAT pathway (56). Several studies have demonstrated that the expansion and suppressive function of MDSC is mediated via the STATs (40–42). A recent study reported that miR155 deficiency in T cells resulted in increased SOCS1 expression accompanied by impaired activation of STAT5 transcription factor in response to limiting amounts of IL2, and suggested that Foxp3-dependent regulation of miR155 maintains competitive fitness of Treg cells by targeting SOCS1 (16). In line with these findings, our SOCS1 shRNA experiments showed that defective suppressive activity by miR155+/− MDSCs could be complemented by knockdown of SOCS1 expression, which was elevated in these MDSCs. Our data indicate that SOCS1 could impair the suppressive function of MDSCs when miR155 is absent, and at least partially explain why miR155 helps maintain MDSC activity. We also noted increased SHIP-1 expression in miR155−/− MDSCs. Interestingly, SHIP-1 was recently reported as a target of miR155 specifically in MDSC expansion (17), consistent with the previous observation that myeloid-specific ablation of SHIP resulted in an increase in MDSC numbers (57). However, the prior study did not examine the importance of MDSC SHIP status in tumor growth (17). To our knowledge, our data clearly provide the first evidence that cell-intrinsic MDSC miR155 is required for MDSCs to facilitate tumor growth, using both adoptive transfer and MDSC depletion analyses. We showed inverse correlations between MDSC SHIP-1/SOCS1 and miR155, suggesting both SHIP-1 and SOCS1 as target genes of miR155 during functional MDSC generation. As downregulation of either SHIP-1 or SOCS1 could increase STAT3 activation (17, 58), which promotes functional MDSC expansion (37, 45), targeting both SHIP-1 and SOCS1 by miR155 would enhance STAT3 activity and MDSC accumulation. However, the biology of miRNA signaling in MDSC development is likely to be more complex. At this stage, we cannot exclude the involvement of additional targets other than SHIP-1 and SOCS1 or even miRNAs other than miR155 in regulation of functional MDSC induction.

miR155 expression is controlled by a wide range of inflammatory factors, and transgenic overexpression of miR155 results in cancer (18). Being oncogenic and pertinent to inflammation, miR155 is considered as prototypical microRNA bridging inflammation and cancer development (4, 59). In support, we found that miR155 deficiency inhibited carcinogenesis in the AOM and DSS-induced colorectal cancer model. MiR155 deficiency could reduce colon inflammation that is known to drive carcinogenesis in this model (60). Moreover, miR155 might promote tumor growth in an intrinsic manner as this is an induced and not transplanted model. Nevertheless, miR155 positively regulates myeloid cell development by acting on BM progenitors during inflammatory stress. Particularly, our and other data (17) show that miR155 is upregulated in MDSC either from tumor-bearing hosts or generated from BM progenitors by GM-CSF and IL6. Over-expression of miR155 enhanced, whereas depletion of miR155 reduced the suppressive function of cytokine-induced MDSCs. Moreover, MDSC accumulation was impaired in tumor-bearing mice lacking miR155 and miR155-deficient MDSCs failed to inhibit T-cell functions. Thus, the induction of MDSC by proinflammatory mediators led to the novel hypothesis that inflammation promotes the accumulation of functional MDSC by increased miR155 that downregulates immune surveillance and antitumor immunity, thereby facilitating tumor growth. MDSCs also promote tumor progression through immune mechanisms. Their release of MMP-9 and VEGF contributes to tumor angiogenesis. Given the decreased production of MMP-9 and VEGF from miR155−/− MDSCs, further studies will determine whether miR155 mediates MDSC-dependent tumor angiogenesis.

Extensive evidence indicates that miR155 functions as an oncomiR in many solid as well as hematologic tumors, and it is often associated with poor prognosis (61, 62). Thus, it has been suggested that therapeutic inhibition of miR155 could be an effective strategy to treat cancer (22–24). However, in these studies, the contributions of immune regulation by miR155 to tumor progression were unappreciable (23, 63, 64). Although our data expand the role of miR155 to MDSC-mediated tumor protection, the cancer cell-intrinsic roles of miR155 in both immune and nonimmune conditions need further investigation. On the other hand, miR155 activation in effector T cells and DCs boosts antitumor immunity, demonstrating a potential beneficial role for this miRNA during tumor progression. In this regard, besides its oncogenic activity, miR155 functions as a cell context-dependent "immunomiR" in orchestrating protumor or antitumor immune responses. Thus, our results suggest additional investigations before considering miR155 manipulation for cancer therapy. For example, cell-specific targeting of miR155 and consideration of tumor effects on miR155-mediated outcomes merit additional attention.

In summary, we investigated the role of host miR155 in AOM and DSS-induced colon carcinogenesis and multiple transplantable tumor models. Our study identified a crucial cell-intrinsic role of miR155 and its target SOCS1-1 in MDSCs and demonstrated that this miRNA is required by MDSCs to limit antitumor T-cell immunity. Despite the evidence for an established role of miR155 in effector T cells and DCs, this miRNA is closely linked to the development of MDSCs and Treg cells, triggers tumor immune suppression, and thereby facilitates tumor growth. Our data indicate that the biologic activities of miR155 are highly cell context-dependent, including tumor dependent. Further studies will also be necessary to determine if host miR155 affects tumor angiogenesis and metastasis.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Chen, Y. Zhang, T.J. Curiel, D. Fang, B. Zhang

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Chen, L. Wang, J. Fan, D. Fang, T.M. Kuzel, B. Zhang

Writing, review, and/or revision of the manuscript: S. Chen, Y. Zhang, T.J. Curiel, D. Fang, T.M. Kuzel, B. Zhang

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): L. Wang, J. Fan, C. Ye, D. Dominguez, D. Fang, T.M. Kuzel, B. Zhang

Study supervision: D. Fang, B. Zhang

References


Acknowledgments

The authors thank the National Institutes of Health Tetramer Facility for providing the R6Kova tetramers.

Grant Support

This research was in part supported by NIH grant CA149669, Ovarian Cancer Research Foundation Funds (UT/UTHSC/01.2011), the Northwestern University BILHCC Flow Cytometry Facility, Cancer Center Support Grants (NCI CA065533 and CA045174), the Owens Foundation, and the Skinner Endowment at the Holly Beach Public Library.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 13, 2013; revised November 4, 2014; accepted November 18, 2014; published OnlineFirst December 10, 2014.
Host miR155 Promotes Tumor Growth

Host miR155 Promotes Tumor Growth through a Myeloid-Derived Suppressor Cell–Dependent Mechanism

Siqi Chen, Long Wang, Jie Fan, et al.

*Cancer Res* 2015;75:519-531. Published OnlineFirst December 10, 2014.

Updated version

Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-2331

Supplementary Material

Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/12/10/0008-5472.CAN-14-2331.DC1

Cited articles

This article cites 64 articles, 31 of which you can access for free at:
http://cancerres.aacrjournals.org/content/75/3/519.full#ref-list-1

Citing articles

This article has been cited by 4 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/75/3/519.full#related-urls

E-mail alerts

Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions

To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/75/3/519.
Click on “Request Permissions” which will take you to the Copyright Clearance Center's (CCC) Rightslink site.