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

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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.

Materials and Methods

Mice, cell lines, and reagents

C57BL/6 miR155−/−, CD45.1, and CD90.1 mice were purchased from the Jackson Laboratory, OT-1 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)-IRESCGF (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. Dichloro-fluorescein diacetate (DCFDA), azoxymethane (AOM), and 5-fluorouracil (5-FU) were added at the beginning of the culture. To evaluate the Arginine (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, Kb/OVA tetramer staining, Foxp3 staining, and intracellular IFNγ staining were performed as previously described (32). Samples were conducted on a MACS-Quant 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 450high and SIY-peptide-pulsed eFluor 450low splenocytes were mixed at a ratio of 1:1, and a total of 2 × 10^7 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 CD115+CD11b+Gr1+ or CD115+CD11b+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 mmol/L) were added at the beginning of the culture. To evaluate MDSC tolerogenic activity on in vivo T-cell function, naïve OT-I CD90.1 cells (2 × 10^6 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 × 10^5) 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, Biolegend) and IL6 (40 ng/mL, Biolegend) 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 (Bio-Rad), 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, Biolegend) 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; Dharmacon), 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, Biolegend) and IL6 (40 ng/mL, Biolegend) 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+CD62L+ naïve T cells were selected with a CD4+CD62L+ T-cell isolation kit (Miltenyi Biotec), and injected i.v. at 5 × 10^6 per mouse into CD45.1 mice followed by a s.c. injection of 10^7 LLC1-OVA cells. The conversion of transferred T cells to Foxp3+ cells (CD4+52+) in DLN and spleen from LLC1-OVA tumor-bearing mice were detected by flow cytometer 9 days after tumor cell injection. For 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.
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<sup>−/−</sup>) versus syngeneic, WT (miR155<sup>+/+</sup>) mice. Mice were given AOM and DSS, as previously described, to promote colorectal carcinogenesis (34). Upon AOM and DSS challenge, miR155<sup>−/−</sup> 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<sup>−/−</sup> mice. The multiplicity of colonic neoplasms (number and size of tumors) was also significantly decreased in miR155<sup>−/−</sup> mice. However, there was no significant difference in colon length between untreated WT and miR155<sup>−/−</sup> mice (Fig. 1A). Moreover, WT and miR155<sup>−/−</sup> 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<sup>−/−</sup> mice. MC38 colon cancer cells (Fig. 1B) and LLC1 Lewis lung carcinoma cells (Fig. 1C) were s.c. inoculated into WT or miR155<sup>−/−</sup> mice. Tumors injected into miR155<sup>−/−</sup> 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<sup>−/−</sup> mice (Fig. 1E). However, the sizes of B16-SIY melanoma (expressing SIY antigen) were comparable between the WT and miR155<sup>−/−</sup> 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 phenotype 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<sup>+</sup>), NK cells (NK1.1<sup>+</sup>), or myeloid DCs (CD11b<sup>+</sup>CD11c<sup>+</sup>) in local infiltrates of EG7 (Fig. 2A) or LLC1-OVA (Supplementary Fig. S2A) tumors in miR155<sup>−/−</sup> versus WT mice. Interestingly, remarkably fewer tumor-infiltrating CD8<sup>+</sup>, CD4<sup>+</sup> lymphocytes were found in miR155<sup>−/−</sup> versus WT mice (Fig. 2A and Supplementary Fig. S2A). Although percentages of tumor-infiltrating IFNγ<sup>+</sup> CD8<sup>+</sup> T cells were comparable between groups, tetramer staining showed a greater number of OVA-reactive (tumor-specific) CD8<sup>+</sup> T cells in EG7-bearing (Fig. 2B and C) or LLC1-OVA-bearing (Supplementary Fig. S2B and S2C) miR155<sup>−/−</sup> mice than WT mice. We next examined the cytolytic function of tumor antigen-specific CD8<sup>+</sup> T cells. Target cell lysis in vitro was remarkably improved in DLN of EG7 tumor-bearing miR155<sup>−/−</sup> mice compared with tumor-bearing WT mice (Fig. 2D). To assess the roles of CD4<sup>+</sup>, CD8<sup>+</sup>, and NK cells in the tumor-inhibiting effects observed in miR155<sup>−/−</sup> mice, mice were inoculated with EG7 cells, and subsequently received depleted anti-CD4, anti-CD8<sup>+</sup>, or anti-NK1.1 antibodies against CD4<sup>+</sup> or CD8<sup>+</sup> T cells, or NK cells, respectively. Notably, the tumor-inhibiting advantage of host miR155 deficiency was primarily dependent on CD8<sup>+</sup> cells, but independent of CD4<sup>+</sup> 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 tumor growth. As expected, we found that tumor-associated miR155<sup>−/−</sup> DCs expressed less MHC-II (Supplementary Fig. S3A), and induced less antigen-specific CD8<sup>+</sup> T-cell proliferation compared with WT DCs (Supplementary Fig. S3B). Similarly, tumor-infiltrated miR155<sup>−/−</sup> CD8<sup>+</sup> T cells sorted from LLC1-OVA tumors displayed reduced response to DCs pulsed with OVA-1 peptides in vitro (Supplementary Fig. S3C). In this immune cell–specific context, it is of interest that we observed intrinsic defects in miR155<sup>−/−</sup> tumor–associated DCs and antitumor T cells.

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+/CD11b+ MDSCs were consistently decreased in LLC1-OVA–bearing miR155−/− mice in comparison with WT mice (Fig. 3A and B). Further analysis revealed significant reductions in the percentage of CD11b+Gr1+ cells from miR155−/− 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−/− 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+Gr1+ cells between tumor-free miR155−/− and WT mice (data not shown). MDSC consists of ly6G−ly6Chigh (monocytic) and ly6G+ly6Clow (granulocytic) subpopulations (31, 35). Of note, the preferential reduction of the splenic (Supplementary Fig. S4B and S4C) and tumor-infiltrating (Fig. 3D) monocytic ly6G−ly6Chigh subset was observed in LLC1-OVA–bearing miR155−/− 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−/− 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−/− 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−CD11b− MDSCs in WT populations (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+ cells from MC38-bearing mice and cocultured with OT-I splenocytes. Notably, the miR155−/− MDSC appreciably lost their capacity to suppress proliferation of
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...
hematopoietic organs such as the BM and possibly spleen (at least in mice; refs. 40–42). GM-CSF alone (43) or the combination of GM-CSF plus IL6 (44) has been used successfully to generate MDSCs in short-term culture in vitro from BM precursor cells. Interestingly, GM-CSF alone upregulated miR155 expression during the induction of BM-derived MDSCs. Moreover, a combination of GM-CSF and IL6 induced higher levels of miR155 expression (Supplementary Fig. S5A). We next analyzed whether miR155 affected cytokine-induced MDSC function as observed in tumor-bearing mice. As shown in Supplementary Fig. S5B, miR155+−/− MDSCs failed to suppress antigen-specific T-cell proliferation in vitro compared with WT MDSCs. To examine further the functional contribution of miR155 expression to the immunoregulatory activity of MDSCs, we transfected BM cells with a miR155-specific inhibitor or a respective pre-miR155 (precursor) and analyzed the proliferative capacity of antigen-specific T cells in the presence of cytokine-induced MDSCs (Supplementary Fig. S5C). To this end, pre-miR155, miR155 inhibitor, or control-transfected MDSCs were cocultured with responder T cells at different ratios. As expected, control transfection in MDSCs did not alter their suppressive capacity. In sharp contrast, treatment with miR155 inhibitors abrogated MDSC suppressive activity. Consistent with these, overexpression of miR155 resulted in stronger suppression of T-cell proliferation versus control-transfected MDSCs.

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+−/− 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 downregulated, whereas socs1 and shpi1 were upregulated in miR155+−/− 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

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**Figure 3.** miR155 is required for MDSC accumulation in the tumor microenvironment. A, the percentage of tumor-infiltrating Gr1+CD11b+ MDSCs was determined by flow cytometry from LLC1-OVA tumor-bearing mice. B, the absolute number of tumor-infiltrating MDSCs (∼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+Ly6G−Ly6C+ (granulocytic) and CD11b−Ly6G+Ly6C+ (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 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-1 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-1 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+ T cells as determined by flow cytometry are summarized. F, real-time quantitative RT-PCR analysis of different gene expression in WT and miR155−/− mice. G, ROS production was measured with DCFDA staining by flow cytometry and summarized within the granulocytic and monocytic tumor-infiltrating 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. 

Figure 4.

miR155 targets SOCS1 in MDSCs

We initially confirmed that splenic CD11b+ cells from tumor-bearing mice had higher levels of miR155 expression than those counterparts from tumor-free mice (Fig. 5A), whereas no detectable miR155 expression was found in tumor-bearing miR155−/− mice (Fig. 5B), suggesting a link between miR155 upregulation and MDSC suppressive function.
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+Gr1+ 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 Gr1+ CD11b+ cells from WT naïve 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−/− Gr1+ CD11b+ cells from naïve mice and LLC1 tumor-bearing mice (n = 5). D, to define the function of SOCS1 within Gr1+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 means ± 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−/− 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−/− mice. However, the prevalence of MDSCs and Tregs was similar in tumor-free WT versus miR155−/− 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−/− 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 antitumor 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).
缺陷免疫刺激活动观察到miR155在肿瘤生长中的作用，正如我们在研究中所示。尽管存在MDSC和Treg细胞等细胞亚群在肿瘤中的角色，miR155在效应T细胞中的作用被分析，但没有分析其他不同的细胞。在这方面的先例（25）关注的是内在生长差异，没有考虑到其他细胞的作用，平衡这些效应可能会使miR155的调节作用一个免疫细胞类型可以对抗其他不同细胞类型的作用。

*mIR155表达由MDSCs促进肿瘤生长。A，WT或mIR155−/−小鼠被皮下注射10^6 LLC1肿瘤细胞。三天后，小鼠被皮下注射5-FU或PBS（对照）；A）或Anti-Gr1抗体（B）每4天注射一次。MDSCs depletion by 5-FU or anti-Gr1 depleting antibodies in vivo was determined by flow cytometry and summarized (n = 5). Tumor volume was measured and plotted at indicated times. NS, not significant. C, splenic Gr1^+ CD11b^+ MDSCs from tumor-bearing WT or miR155−/− mice were injected i.v. into LLC1-bearing mice at days 7 and 14. Mice receiving PBS without MDSCs were controls. D, MDSC depletion by 5-FU or anti-Gr-1 depleting antibodies in vivo was determined by flow cytometry and summarized (n = 5). Tumor volume was measured and plotted at indicated times. NS, not significant.

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 hosts. Moreover, intact suppressive activity of miR155-deficient Tregs was observed, consistent with previous results (15, 16). In addition to the direct inhibition 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 Treg cells resulted in increased SOCS1 expression accompanied by impaired activation of STAT3 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. Overexpression 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 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.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Chen, T.J. Curlot, D. Fang, T.M. Kuzel, B. Zhang

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References

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