Epistasis between MicroRNAs 155 and 146a during T Cell-Mediated Antitumor Immunity

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http://dx.doi.org/10.1016/j.celrep.2012.10.025

SUMMARY

An increased understanding of antitumor immunity is necessary for improving cell-based immunotherapies against human cancers. Here, we investigated the roles of two immune system-expressed microRNAs (miRNAs), miR-155 and miR-146a, in the regulation of antitumor immune responses. Our results indicate that miR-155 promotes and miR-146a inhibits interferon γ (IFNγ) responses by T cells and reduces solid tumor growth in vivo. Using a double-knockout (DKO) mouse strain deficient in both miR-155 and miR-146a, we have also identified an epistatic relationship between these two miRNAs. DKO mice had defective T cell responses and tumor growth phenotypes similar to miR-155−/− mice. Further analysis of the T cell compartment revealed that miR-155 modulates IFNγ expression through a mechanism involving repression of Ship1. Our work reveals critical roles for miRNAs in the reciprocal regulation of CD4+ and CD8+ T cell-mediated antitumor immunity and demonstrates the dominant nature of miR-155 during its promotion of immune responses.

INTRODUCTION

Combating solid tumors remains an enormous challenge for the biomedical community. The need for improved therapies beyond radiation and chemotherapy has become evident, and there is growing interest in optimizing the use of immunotherapy as a treatment option. Among the cell types that hold promising therapeutic potential are T lymphocytes, including CD4+ interferon γ (IFNγ)-expressing Th1 cells and cytotoxic CD8+ T cells, which elicit tumor antigen-specific responses to direct the tumor microenvironment in a manner that restricts or eliminates tumor growth (Dougan and Dranoff, 2008; Dunn et al., 2004; Shiao et al., 2011). However, there remain several aspects of antitumor immunity that are unclear and appear to be governed by complex regulatory systems that have limited this application in the clinic thus far (Zitvogel et al., 2006). Therefore, an improved understanding of the molecular networks that influence T lymphocyte biology in the context of antitumor responses is needed, and this has the potential to improve our ability to manipulate this response in a manner that promotes tumor rejection.

Mammalian miRNAs have recently emerged as important regulators of immune cell development and function and represent a novel layer of control over cellular physiology (O’Connell et al., 2010c), miRNAs are encoded by the genome and their transcription is regulated in a manner similar to other inflammatory protein coding genes, and this can involve such factors as nuclear factor κB (NF-κB) and AP-1 (O’Connell et al., 2007; Taganov et al., 2006; Thai et al., 2007). Following their biogenesis, miRNAs are loaded into the RNA-induced silencing complex and guide this complex to the 3’ UTRs of key target genes, resulting in repressed expression (Filipowicz et al., 2008). In recent years, specific miRNAs have been shown to dramatically impact autoimmune and antimicrobial responses in mammals through their regulation of inflammatory T cells (Lu et al., 2009; O’Connell et al., 2010b; Rodriguez et al., 2007; Thai et al., 2007).

One of the most prominent miRNAs linked to inflammation is miR-155, which is upregulated in both myeloid and lymphoid cells following their activation (Haasch et al., 2002; O’Connell et al., 2007). In the T cell compartment, miR-155 regulates CD4+ regulatory cell fitness through a mechanism involving Socs1 repression (Lu et al., 2009), while also being required for the development of inflammatory Th17 cells during autoimmunity driven by specific tissue antigens (Murugaiyan et al., 2011; O’Connell et al., 2010b). miR-155 has been shown to be necessary for effective vaccination against S. typhimurium and immunity against H. pylori (Oertli et al., 2011; Rodriguez et al., 2007). In contrast to miR-155, miR-146a limits T cell activation and promotes resolution of inflammatory responses. miR-146a−/− mice develop spontaneous autoimmunity and cancer upon aging, and this phenotype involves, among other things, hyperactivation of T cells via derepression of its targets IraK1 and Traf6 (Boldin et al., 2011; Yang et al., 2012; Zhao et al., 2011). Stat1 has also been shown to be a functionally relevant target of miR-146a−/− in T regulatory cells (Lu et al., 2010). Thus, these two miRNAs appear to have opposite impacts on inflammatory responses carried out by T lymphocytes in the contexts of autoimmunity and infection.
To date, little is known about the roles of T cell-expressed miR-155 and miR-146a during antitumor immune responses. To address this, we first tested the ability of miR-155 to mediate antitumor immunity using multiple models of syngeneic solid tumor growth in mice. We found that miR-155/C0 mice permit enhanced growth of transplanted EL4-luc lymphoma and B16 melanoma cells compared to wild-type (WT) controls. This was accompanied by a cell-intrinsic defect in IFN-γ-expressing T cells in tumor-bearing mice. In contrast to miR-155/C0 mice, miR-146a/C0 mice suppressed tumor growth compared to WT controls, and this correlated with an elevated IFN-γ response. Interestingly, mice deficient in both miR-155 and miR-146a, or double-knockout (DKO) mice, had IFN-γ responses resembling those observed in miR-155/C0 mice in vivo. Mechanistically, we found that miR-155 regulates CD4+ T cell expression of IFN-γ through a process involving repression of Ship1. These findings reveal that miRNAs are instrumental in directing CD4+ and CD8+ T cell-mediated antitumor responses, and that miR-155 plays a dominant role compared to miR-146a in the promotion of IFN-γ-expressing T cell development in this context.

RESULTS

Enhanced Growth of Syngeneic Tumors in miR-155/C0 Mice

To assess the impact of miR-155 on solid tumor growth, we administered 2 × 10^6 syngeneic EL4-luc lymphoma cells subcutaneously into WT or miR-155/C0 mice and monitored tumor growth over a time course. Although the tumor sizes were similar between the groups by day 9 post-injection, substantial differences in tumor diameters and weights were observed by day 12, with tumors growing much larger in miR-155/C0 versus WT mice (Figures 1A and 1B). Because the tumor cells express luciferase, we also found that luciferase activity correlated with tumor size using Xenogen whole animal imaging (Figures 1C and S1). Of note, we did not observe a luciferase signal from locations other than the site of tumor injection, suggesting that the tumor cells had not metastasized. Next, we challenged both groups of mice with two other types of syngeneic tumors, B16-F1 or B16-F10 melanoma cells, and once again observed increased tumor growth in miR-155/C0 versus WT mice (Figures 1D, 1E, and 1F). Of note, the differences in B16 tumor growth between the genotypes were not as dramatic as those observed with the EL4-luc
tumors, which is probably a consequence of differences in tumor immunogenicity.

Hematoxylin and eosin (H&E)-stained tumor tissue sections revealed significant necrosis in tumors growing in WT mice, while those growing in miR-155−/− mice had few of these features (Figure 1G). Furthermore, we observed elevated numbers of tumor infiltrating lymphocytes (TILs) in both EL4-luc and B16-F10 tumors growing in WT compared to miR-155−/− hosts (Figures 1G and 1H). These observations indicate that miR-155−/− mice have a defect in limiting syngeneic tumor growth, and this correlates with a reduced antitumor immune response.

Defective IFNγ+CD4+ T Cell Development in miR-155−/− Tumor-Bearing Mice Occurring through a CD4+ T Cell-Intrinsic Mechanism

miR-155 is expressed predominately in the immune system following cellular activation, and has been implicated in directing antigen-specific responses and lineage skewing by lymphocytes in vivo. Because IFNγ-expressing T cells are instrumental in repressing tumor growth (Ikeda et al., 2002; Jiang et al., 2011; Maekawa et al., 1988; Muranski et al., 2011; Yim et al., 1999), we assayed the amount of CD4+ IFNγ-expressing T cells in WT compared to miR-155−/− tumor-bearing mice. In both the spleens and lymph nodes of mice with EL4-luc tumors, a lack of miR-155 led to significant reductions in both the percentage and absolute number of IFNγ-producing CD4+ T cells compared to WT controls (Figures 2A, 2B, and S2). Of note, we only observed significant differences in the total number of IFNγ+CD4+ splenic T cells in WT versus miR-155−/− mice after administration of the tumor (Figure 2C). Further demonstrating a defective tumor immune response, we found that transfer of total splenocytes from WT, but not miR-155−/−, tumor-bearing mice protected naive WT mice from a primary tumor challenge (Figure S3).

Next, we wanted to determine if this defective CD4+ T cell response was due to cell-intrinsic or cell-extrinsic pathways regulated by miR-155. To make this assessment, 4 × 10^6 naive miR-155−/− or WT CD45.2+ CD4+ T cells were transferred into sublethally irradiated CD45.1+ WT recipients and 5 × 10^6 B16-F10 tumor cells injected the next day. Consistent with miR-155 playing a CD4+ T cell-intrinsic role, the transferred CD45.2+ miR-155−/− CD4+ T cells exhibited defective IFNγ responses compared to CD45.2+ WT CD4+ T cells in the tumors of CD45.1+ WT recipient mice (Figures 2D and 2E). To corroborate these findings, the reverse experiment was performed by adoptively transferring 4 × 10^6 naive CD45.1+ CD4+ WT T cells into sublethally irradiated CD45.2+ WT or miR-155−/− mice 1 day before challenging these mice with B16-F10 cells. Another group of CD45.2+ miR-155−/− mice received 4 × 10^6 naive CD45.2+ miR-155−/− CD4+ T cells following irradiation, and served as a control group. The transferred WT T cells readily differentiated into IFNγ+ expressing cells both in the spleens (Figures 2F, 2G, and 2H) and the tumors (Figures 2F and 2I) of miR-155−/− mice. This significantly increased the percentage of IFNγ+ expressing cells within the tumors, but did not reach WT levels (Figure 2J). Furthermore, tumors in miR-155−/− mice having received WT CD4+ T cells trended toward being smaller than tumors growing in miR-155−/− mice that received miR-155−/− CD4+ T cells (Figure 2K). Cellular engraftment and IFNγ+CD4+ T cell differentiation (by the transferred WT T cells) was more robust in miR-155−/− versus WT recipients (Figures 2F, 2G, and 2H), possibly due to an increased availability of T cell niches in mice lacking miR-155. These findings, and similar observations with EL4-luc tumor cells (Figure S3), indicate that miR-155 plays a cell-intrinsic role during the formation of IFNγ-expressing CD4+ T cells in response to solid tumor growth.

miR-155 Is Required for Accumulation of IFNγ+CD8+ T Cells in Tumors, and This Also Occurs through a CD8+ T Cell-Intrinsic Mechanism

The immune response against solid tumors involves multiple cell types, including both CD4+ and CD8+ T cells. Although we have found a clear T cell-intrinsic function for miR-155 in the promotion of IFNγ responses by CD4+ T cells in response to solid tumors, a role for miR-155 in CD8+ T cell biology during antitumor immunity has not been reported. Thus, we first examined IFNγ expression by CD8+ T cells following their activation in vitro, and observed defective IFNγ mRNA levels in the absence of miR-155 (Figure 3A). Next, we assessed the CD8+ TIL population in tumors growing in WT versus miR-155−/− mice, and observed a reduction in the percentage of IFNγ+CD8+ T cells among total CD8+ T cells in the absence of miR-155 (Figure 3B). Adoptively transferred miR-155−/− CD45.2+ CD8+ T cells were significantly defective in accumulating and producing IFNγ in tumors growing in CD45.1+ WT mice, indicating a cell-intrinsic role for miR-155 in CD8+ T cells (Figure 3C). Furthermore supporting a CD8+ T cell-intrinsic role for miR-155, transfer of WT CD45.1+CD8+ T cells into sublethally irradiated CD45.2+ WT or miR-155−/− hosts followed by a tumor challenge revealed that CD8+ T cells could still mediate strong IFNγ responses in a miR-155−/− environment (Figures 3D and 3E). miR-155−/− mice receiving WT CD8+ T cells also tended to have reduced tumor weights compared with those receiving miR-155−/− CD8+ T cells (Figure 3E). Thus, miR-155 functions within both CD4+ and CD8+ T cells to promote IFNγ-dependent antitumor immunity.

miR-155 Plays a Dominant Role Compared to miR-146a during T Cell-Mediated Tumor Immunity

Like miR-155, miR-146a is also expressed in activated T cells. However, in contrast to miR-155, miR-146a−/− T cells have been reported to be hyperactivated during acute and chronic immune responses (Yang et al., 2012). Therefore, we tested whether miR-146a−/− mice have enhanced antitumor immunity compared to WT mice. miR-146a−/−, miR-155−/−, and WT mice were inoculated subcutaneously with 1 × 10^6 B16-F10 cells and tumor growth was monitored over a time course (Figure 4A). Interestingly, tumors grew at a reduced rate in the absence of miR-146a compared to WT mice, while once again they grew larger in miR-155−/− mice, suggesting that these miRNAs play opposing roles during antitumor responses.

To examine the cross-regulation of tumor immunity by miR-155 and miR-146a, we created mice deficient in both miRNAs (Figure 4B). Using these mice, we assessed whether these opposing phenotypes would be canceled out, or if one of these two miRNAs plays a dominant role. DKO mice were viable and...
Figure 2. miR-155 Promotes IFN-γ+CD4+ T Cell Formation in Tumor-Bearing Mice through a CD4+ T Cell-Intrinsic Mechanism

(A) IFN-γ expression by CD4+ T cells from the spleens of EL4-luc tumor-bearing WT or miR-155−/− mice following 12 days of tumor growth.

(B) Results from (A) are shown graphically for multiple mice (n = 5).

(C) Number of IFN-γ+CD4+ T cells in the spleens of WT or miR-155−/− mice with and without B16-F10 tumors for 14 days.

(D) FACS plots (gated on CD4+ T cells) showing IFN-γ expression by transferred WT or miR-155−/− CD45.2+ CD4+ T cells in the tumors of WT CD45.1+ B16-F10 tumor-bearing mice.

(E) Graphs from multiple mice in (D) (n = 5).

(F) FACS plots showing IFN-γ expression by transferred WT CD45.1+ CD4+ T cells in the spleens (top) or tumors (bottom) of WT or miR-155−/− CD45.2+ B16-F10 tumor bearing mice. Transferred miR-155−/− CD4+ cells are CD45.2+ and all plots are gated on CD4+ T cells.

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fertile and did not exhibit obvious gross abnormalities by the age of 8 weeks. The absence of both miR-155 and miR-146a expression was confirmed by quantitative PCR (qPCR) using RNA from in vitro activated CD4+ T cells (Figure 4C). When these mice and the relevant control groups were challenged with 1 × 106 B16-F10 tumor cells, there was a consistent trend toward increased tumor growth in DKO compared to WT mice, similar to tumors growing in miR-155−/− mice (Figures 4D and 4E). Again, miR-146a−/− mice restricted tumor growth compared to WT mice.

IFNγ+CD4+ T cell numbers in the spleens of both DKO and miR-155−/− tumor-bearing mice were significantly reduced compared to WT mice, while miR-146a−/− mice had the highest levels (Figure 4F). This same trend was also observed when CD45.2+ WT, miR-155−/−, miR-146a−/−, or DKO CD4+ T cells were transferred into CD45.1+ WT mice followed by 15 days of tumor growth, consistent with these miRNAs playing CD4+ T cell intrinsic roles to regulate IFNγ production (Figures 4G and 4H).

Upon analyzing single cell tumor suspensions by fluorescence-activated cell sorting (FACS), miR-155−/− and DKO mice had reduced percentages of cells in the forward scatter-side scatter leukocyte gate versus those observed in WT mice, and this cellular compartment contained CD3+CD4+ and CD3+CD8+ TILs (Figure 5A). Alternatively, increased percentages of TILs were observed in tumors from miR-146a−/− compared to WT mice (Figure 5A). The percentages of IFNγ+CD4+ and IFNγ+CD8+ T cells among total CD4+ or CD8+ TILs, respectively, were reduced in tumors growing in miR-155−/− and DKO mice, and marginally increased in tumors from miR-146a−/− animals (Figures 5B and 5C). Upon FACS sorting CD3+CD4+ or CD3+CD8+ TILs from the tumors, we recovered fewer numbers of total and IFNγ+CD4+ and IFNγ+CD8+ T cells from tumors growing in miR-155−/− and DKO mice compared to WT mice, while miR-146a−/− mice had elevated amounts (Figures 5D and 5E). Furthermore, miR-155 and miR-146a were expressed in both CD4+ and CD8+ TILs (Figure S4). Together, these observations indicate that miR-155 plays dominant, T cell intrinsic roles in promoting antitumor responses by both CD4+ and CD8+ T cells.

miR-155 Targets the IFNγ Regulator Ship1 in T Cells

Because we found that miR-155 functions in CD4+ T cells during the antitumor response, we FACS sorted CD4+ T cells from the spleens of miR-155−/− and WT tumor-bearing mice and analyzed gene expression differences to obtain mechanistic insight into its impact on IFNγ expression. Derepression of Ship1, a previously identified target of miR-155 in myeloid cells (O’Connell et al., 2009), was observed by qPCR in cells lacking miR-155 (Figure 6A). BIC, the noncoding RNA that produces miR-155, was assayed as a control and not detected in miR-155−/− cells. To assay Ship1 expression at the protein level in miR-155−/− CD4+ T cells, western blotting was performed using lysates prepared from naive WT or miR-155−/− T cells that had been activated with αCD3 and αCD28 antibodies for 96 hr (Figure 6B). Elevated Ship1 protein concentrations were observed in miR-155−/− T cells under these conditions. Next, we extended our analysis to activated DKO CD4+ T cells and again found enhanced expression of Ship1 at the protein and mRNA levels in both miR-155−/− and DKO CD4+ T cells compared to WT and miR-146a−/− cells (Figures 6C and 6D). Importantly, levels of Ship1 inversely correlated with the expression of IFNγ in WT, miR-155−/−, and DKO T cells (Figure 6E). Of note, there was little impact by the different miRNA deficiencies on CD4+ T cell growth in vitro (Figure S5), and activated miR-146a−/− CD4+ T cells still produced elevated levels of IFNγ even after CD25 T regulatory cells were depleted (Figure S6).

To test the functional impact of elevated Ship1 expression (as observed in the absence of miR-155) on IFNγ levels, we utilized small hairpin RNAs (shRNAs) to knock down Ship1 expression in activated WT, miR-155−/−, miR-146a−/−, or DKO CD4+ T cells. We found that in T cells of all genotypes tested, reductions in Ship1 using either of two different shRNAs resulted in increased expression of IFNγ mRNA compared to cells given a scrambled control vector (Figure 6F). Knockdown by the shRNA was confirmed by western blotting against Ship1 (Figure 6G). Expression of a Ship1 shRNA in activated CD4+ T cells also increased production of IFNγ at the protein level as determined by ELISA (Figure 6H). Retroviral transduction of CD4+ T cells was approximately 50% in all cases as determined by FACS to identify GFP+ cells. Next, we also assayed Ship1 protein levels in CD8+ T cells from the different groups. Like CD4+ T cells, Ship1 was also elevated in miR-155−/− and DKO compared to WT and miR-146a−/− CD8+ T cells (Figure 6I). Taken together, these findings indicate that Ship1 is repressed by miR-155 in both CD4+ and CD8+ T cells. Furthermore, miR-155 promotes IFNγ expression by CD4+ T cells through a mechanism involving repression of Ship1. However, the partial recovery of the IFNγ phenotype following Ship1 knockdown indicates that additional targets of miR-155 are also involved in this phenotype.

DISCUSSION

MicroRNA-155 has quickly emerged as an important promoter of inflammatory responses, with a clear connection to autoimmune disease. Furthermore, miR-155 is overexpressed in a variety of tumor cell types and can promote tumor growth in many cases (Bakirtzi et al., 2011; Chang et al., 2011; Han et al., 2012; Philippidou et al., 2010; Segura et al., 2010; Volinia et al., 2006; Zheng...
et al., 2012). Thus, it has been suggested that therapeutic inhibition of miR-155 may be a strategic means to treat autoimmunity or cancer. However, in the current study, we found that transferred syngeneic tumors grew substantially larger in mice genetically deficient in miR-155. Despite the tumors being weakly immunogenic, defects in the antitumor immune response were observed in the absence of miR-155, demonstrating a protective role for this miRNA in immune cells in the context of a tumor challenge. Consequently, clinical approaches aimed at targeting miR-155 within tumor cells themselves could have deleterious consequences if unintended repression of miR-155 occurs in immune cells. One could imagine this to be highly possible because solid tumors are physically associated with TILs. Furthermore, inhibition of miR-155 to treat autoimmunity might also block protective functions mediated by miR-155, including tumor immunosurveillance. Thus, our results clearly indicate the need for highly specific and targeted approaches to modulating miR-155 levels in the treatment of human disease.

There has been much consideration for using miR-155 as a biomarker of disease type and severity in human cancers where it is commonly overexpressed. Several instances of increased miR-155 levels correlating with more aggressive tumors with poor clinical outcomes have been reported (Chang et al., 2011; Han et al., 2012). However, a recent study looking at human melanoma patients found that increased miR-155 expression correlated with an improved prognosis (Segura et al., 2010). Although the study did not analyze distinct cellular subsets within the tumor, one could speculate that the increased miR-155 expression was a consequence of enhanced accumulation of immune cells within the tumor. Based upon our results here, it may prove valuable to
carefully assess whether overexpression of miR-155 is occurring in tumor cells or in TILs that are actively fighting the tumor. This may give a more accurate assessment of whether increases in miR-155 are protective or deleterious.

Whereas miR-155 plays a host protective role against solid tumor growth, miR-146a appears to limit immunity against the same tumor type. We provide evidence that these contrasting roles are the consequence of reciprocal effects by these miRNAs.
on the tumor accumulation of IFNγ expressing cells, including CD4+ and CD8+ T lymphocytes, which are critical mediators of antitumor immunity. These findings demonstrate that miRNAs in the immune system can play opposing roles in the regulation of a given phenotype. Thus, it is plausible that sets of miRNAs have evolved to provide balance to specific aspects of mammalian immunity, as has been proposed in stem cells (Melton et al., 2010). Consequently, we tested whether miR-155 and miR-146a function to provide immunological balance, or if one of these miRNAs has a dominant effect on IFNγ+ T cell formation and antitumor immunity. Using DKO mice, we determined that loss of miR-155 is largely epistatic to a deficiency in miR-146a in the tumor immunity. The enhanced antitumor response observed in miR-146a−/− mice was not only dependent upon miR-155 but was also worse than that observed in WT mice when miR-155 was also genetically absent. Therefore, our results indicate that miR-155 plays a dominant role, compared to miR-146a, in this context.

Although studies carried out by our lab and others have provided evidence that these miRNAs oppose one another within T cells, it is probable that miR-155 and miR-146a impact antitumor immune responses by also acting in non-T cell types, such as macrophages, dendritic cells, and natural killer cells, where they have been shown to be expressed and to impact inflammatory responses (Boldin et al., 2011; Cubillos-Ruiz et al., 2012; O’Connell et al., 2007; Trotta et al., 2012). Once conditional knockout mice are available for miR-155 and miR-146a, studies can be carried out to specifically test the relative contributions of these miRNAs to the functions of distinct cell types that drive tumor immunity, and this will shed additional light on the cellular basis of the observed epistasis.

In an effort to unravel the molecular basis for miR-155’s function in the T cell compartment, we found that miR-155 target Ship1 to be part of the connection between miR-155 and IFNγ expression by CD4+ T cells. Ship1 is a phosphatase that negatively regulates cytokine signaling via repression of the PI3K pathway (Kerr, 2011). A recent study looking at deletion of Ship1 specifically in CD4+ T cells using a CD4-CRE mouse strain to inhibit NF-κB following T cell receptor engagement as a result of derepression of its targets IRAK1 and TRAF6 (Yang et al., 2012). This causes increased expression of IFNγ by effector T cells deficient in miR-146a. Consequently, it is possible that the elevated levels of Ship1 (Figure 5) and Socs1 (Lu et al., 2009) that are observed in miR-155−/− T cells act to inhibit NF-κB activation, as they have been shown to do in other cell types (Gabhann et al., 2010; Serezani et al., 2011; Strebovsky et al., 2011). This would negate the enhanced T cell activation observed in the absence of miR-146a alone. A careful dissection of these signaling pathways in the context of the different miRNA deficiencies will be an important future endeavor.

Taken together, our study identifies a protective role for miR-155, and an inhibitory function for miR-146a, during antitumor immune responses, and argues for the importance of developing highly specific methods of modulating miRNAs when such approaches are used to combat cancer or autoimmunity. Additionally, by combining approaches that enhance miR-155 and/or repress miR-146a levels in T lymphocytes with tumor vaccines or adoptive cell transfer therapies, one might achieve increased therapeutic efficacy in the clinic. Further studies will also be necessary to determine if miR-155 or miR-146a impact tumor metastasis in addition to modulating tumor growth. Finally, the importance of miR-155 in regulating IFNγ+ T cell responses during tumor immunity is highlighted by the finding that a miR-155 deficiency is epistatic to a loss of miR-146a in this cellular compartment.

**EXPERIMENTAL PROCEDURES**

**Mice**

All mice were on a C57BL6 genetic background and housed in the animal facility at the University of Utah. Experiments were approved by the

**Figure 5. Dominant Role for miR-155 versus miR-146a during T Cell-Mediated Antitumor Immune Responses**

(A) Representative FACS plots showing the percentage of tumor infiltrating CD3+CD4+ and CD3+CD8+ TILs in WT, miR-155−/−, miR-146a−/−, and DKO mice (n = 5).

(B–E) Representative FACS plots showing the percentage of tumor infiltrating CD3+CD4+ and CD3+CD8+ TILs from the indicated genotypes (n = 5). The number (per gram of tumor) of IFNγ+/− and IFNγ−/− CD3+CD4+ (D) or CD3+CD8+ (E) TILs from tumors growing in the different genotypes is shown. Data represent two independent experiments. Data are presented as ± SEM. See also Figure S4.
Figure 6. Ship1 Is a Target of miR-155 in T Lymphocytes

(A) Expression levels of Ship1 mRNA and BIC noncoding RNA were determined in CD4+ T cells purified from the spleens of tumor bearing mice WT and miR-155−/− mice (n = 4).

(B) Expression of Ship1 was quantified in activated WT and miR-155−/− CD4+ T cells by western blotting. Data from two WT and two miR-155−/− mouse T cell donors are shown.

(C and D) Expression of Ship1 in sCD3 and sCD28 activated CD4+ splenic T cells from WT, miR-155−/−, miR-146a−/−, and DKO mice was assayed by western blotting and qPCR.

(E) Expression of IFNγ mRNA in the same cells from (D) is shown (n = 2).

(F) sCD3 and sCD28 antibody activated CD4+ splenic T cells from WT, miR-155−/−, miR-146a−/−, and DKO mice were transduced with a control or one of two different Ship1 shRNA producing retroviral vectors after 24 hr of activation. Expression of Ship1 and IFNγ mRNA levels were assayed by qPCR after 72 hr of knockdown. Data are presented as the ratio of expression in the shRNA versus control (scrambled) conditions. The average knockdown of Ship1 or increase in IFNγ for all genotypes is shown on the right.

(G) Knockdown of Ship1 in the different cell types by the Ship1 shRNA was determined by western blotting.

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subjected to a spin infection using retrovirus medium at 2,500 rpm at the University of Utah. Other antibodies used for FACS include...

Tumor Challenges and Harvests
To create syngeneic subcutaneous tumors in mice, either EL4-luc lymphoma, B16-F10 melanoma, or B16-F1 melanoma cells were injected into the rear flanks of mice. The sites of tumor cell administration were shaved and cleaned before injection. Tumor growth was monitored over a time course by measuring tumor diameter. For analyses at the end of the time course, mice were euthanized and their tumors, spleens, and lymph nodes were removed and processed for FACS or histology. Following dissection, the tumors were weighed, minced into small pieces using a razor blade, and subsequently digested using Accumax. After the enzymatic digestion, the tumor cells were washed before further analysis. In some experiments, TILs were purified using FACS.

Adoptive Transfer of CD4+ and CD8+ T Cells
For T cell transfer experiments, CD4+ or CD8+ T cells were purified from naïve mice (see below) and the indicated amounts of T cells were injected intravenously into recipient mice 1 day before tumor administration. In some experiments, recipients were first irradiated with 500 Rads using an X-ray source before receiving T cells. To distinguish between donor and recipient T cells, CD45.1 and CD45.2 congenic mouse strains were used when possible.

T Cell Isolation and Retroviral Infections
T cells were purified from red blood cell-lysed splenocytes using the MACS CD4+ or CD8+ T cell isolation kit (negative selection) from Miltenyi. Purity was assessed by FACS and routinely reached 90%–95%. To create replication-deficient murine stem cell virus-based retroviral particles carrying the Ship1 or a scrambled control shRNA sequence, 293T cells were transfected with the MGP backbone and pC-Lo-C-ECO packaging plasmids and retrovector-containing supernatant was recovered after 48 hr. The shRNA Ship1 expression vector has been described previously (O’Connell et al., 2009). For retroviral transduction of CD4+ T cells, the cells were stimulated with sCD3 (3 μg/ml) and sCD28 (2 μg/ml) for 24 hr, subjected to a spin infection using retrovirus medium at 2,500 rpm at 30°C for 1.5 hr, then brought up in fresh activation medium for another 72 hr. Cellular infection was determined by microscopy or FACS to identify GFP+ cells.

Intracellular Staining and FACS
Intracellular staining was performed as described previously (O’Connell et al., 2010b). In short, 1 x 10^6 splenocytes, lymph node cells, or tumor suspension cells were restimulated with phorbol myristate acetate and ionomycin for 4 hr in the presence of Golgi Plug. Cells were next surface stained with sCD4 or sCD8 antibodies and stained with CD3, and sCD4 or sCD8 fluorochrome-conjugated antibodies, and cellular populations were sorted using a FACS Aria II in the Flow Cytometry Core Facility at the University of Utah. Other antibodies used for FACS include sCD45.1 and sCD45.2.

Western Blotting, ELISA, and qPCR
Western blotting using cellular extracts from T lymphocytes was performed using standard protocols. Antibodies against mouse Ship1 and β-actin were obtained from Santa Cruz Biotechnology. For qPCR, RNA was extracted using the RNeasy or miRNeasy kits from Qiagen according to manufacturer’s instructions. Following cDNA synthesis using total RNA, SYBRgreen-based qPCR was performed with gene-specific primers and the Roche Light Cycler 480. Primer sequences are available upon request. For detection of mature miRNAs 155 and 146a, or 5S ribosomal RNA, reagents and protocols from Exiqon were utilized. The ELISA assay used to quantify mouse IFNγ concentrations was obtained from ebioscience and performed using the manufacturer’s suggested protocol.

Histopathology
The tumors were dissected from the respective hosts and fixed with 10% formalin for at least 48 hr at room temperature. After fixation, the tumors were bisected across a maximum dimension and processed for paraffin embedding. Next, 5 mm-thick tissue sections were cut from paraffin blocks and stained with H&E per standard H&E protocol. The histopathological analysis was performed by a board-certified pathologist, and the images were taken using an Olympus BX41/DP72 microscope/camera. The magnification of the objective lens for each image is provided in the figures.

Statistical Analysis
A Student’s t test was performed to determine statistical significance.

SUPPLEMENTAL INFORMATION
Supplemental Information includes six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.10.025.

ACKNOWLEDGMENTS
We would like to thank Allan Bradley for generously providing miR-155−/− mice, Lili Yang for providing the EL4-luc cells, and Glenn Dranoff for the B16-F10 cells. D.B. is a scientific advisor to Regulus Therapeutics, a company devoted to microRNA therapeutics. R.M.O. is supported by the National Institutes of Health grant SR00HL102229-04.

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Supplemental Information

Figure S1. Quantification of Tumor Imaging Data, Related to Figure 1
Luminescent signals from EL4-luc tumors growing in WT or miR-155−/− mice are quantified using Xenogen living image software. * denotes a p value less than 0.05. Data are presented as ±SEM.
Figure S2. Percentage and Number of IFNγ+CD4+ T Cells in the Lymph Nodes of EL4 Tumor-Bearing WT and miR-155−/− Mice, Related to Figure 2
(A) IFNγ expression by CD4+ T cells from the lymph nodes (LNs) of EL4-luc tumor bearing WT or miR-155−/− mice following 12 days of tumor growth.
(B) Results are shown graphically for multiple mice (n = 5) on the right. * denotes a p value less than 0.05. Data are presented as ±SEM.
Figure S3. Tumor Educated miR-155−/− Splenocytes Fail to Protect Naive Mice from a Tumor Challenge, and Adoptive Transfer of WT CD4+ T Cells into miR-155−/− Mice Restores IFNγ Expression by CD4+ T Cells in the Spleens of EL4-luc Tumor Bearing Mice, Related to Figure 2

(A) WT or miR-155−/− mice were subcutaneously challenged with EL4-luc tumor cells for 14 days. Splenocytes were recovered from these two groups and 25 x 10^6 of these cells were injected i.v. into naive WT recipient mice who were subsequently inoculated with EL4-luc tumor cells. A control group not receiving splenocytes was also given tumor cells.

(B and C) Tumor diameters over the 11-day timecourse (B) and final tumor weights (C) are shown graphically.

(D and E) Purified WT CD4+ CD45.1+ cells used for transfer (D) and representative FACS plots showing the adoptively transferred WT CD45.1+CD4+ T cells expressing IFNγ in the spleens of WT and miR-155−/− EL4-luc tumor bearing mice (E) are shown.

(F) Expression of IFNγ in the splenic CD4+ T cell compartment in EL4-luc tumor bearing mice receiving WT CD4+ T cells is shown on the right (n = 5). * denotes a p value less than 0.05. Data are presented as ± SEM.
Figure S4. miR-155 and miR-146a Are Expressed in CD4+ and CD8+ TILs, Related to Figure 5

Expression of miR-155 and miR-146a in sorted CD4+ and CD8+ TILs from WT or the indicated miRNA knockout mice was determined using qPCR.
Figure S5. Proliferation of Activated WT, miR-155−/−, miR-146a−/−, or DKO CD4+ T Cells, Related to Figure 6

WT, miR-155−/−, miR-146a−/−, and DKO CD4+ T cells were stimulated with αCD3 and αCD28 and their cell numbers were determined daily for 96 hr (n = 2). Data are presented as ±SEM.
Figure S6.  *miR-146a*⁻/⁻ CD25-Depleted CD4+ T Cells Express Higher Levels of IFNγ Compared to WT CD4+ T Cells Following Their Activation In Vitro in Figure 6

(A) FACS plots showing sorted CD25-CD4+ T cells from WT or *miR-146a*⁻/⁻ mice.
(B) qPCR and ELISA was performed to assay IFNγ expression in the sorted cell populations. Data are presented as ±SEM.