miR-155 augments CD8$^+$ T-cell antitumor activity in lymphoreplete hosts by enhancing responsiveness to homeostatic γ_c cytokines

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Contributed by Thomas A. Waldmann, December 2, 2014 (sent for review October 14, 2014; reviewed by Jianping Huang and Daniel Powell)

Lymphodepleting regimens are used before adoptive immunotherapy to augment the antitumor efficacy of transferred T cells by removing endogenous homeostatic "cytokine sinks." These conditioning modalities, however, are often associated with severe toxicities. We found that microRNA-155 (miR-155) enabled tumor-specific CD8$^+$ T cells to mediate profound antitumor responses in lymphoreplete hosts that were not potentiated by immune-ablation. miR-155 enhanced T-cell responsiveness to limited amounts of homeostatic γc cytokines, resulting in delayed cellular contraction and sustained cytokine production. miR-155 restrained the expression of the inositol 5-phosphatase Ship1, an inhibitor of the serine-threonine protein kinase Akt, and multiple negative regulators of signal transducer and activator of transcription 5 (Stat5), including suppressor of cytokine signaling 1 (Socs1) and the protein tyrosine phosphatase Ptpn2. Expression of constitutively active Stat5a recapitulated the survival advantages conferred by miR-155, whereas constitutive Akt activation promoted sustained effector functions. Our results indicate that overexpression of miR-155 in tumor-specific T cells can be used to increase the effectiveness of adoptive immunotherapies in a cell-intrinsic manner without the need for life-threatening, lymphodepleting maneuvers.

microRNA-155 | adoptive immunotherapy | lymphodepletion | homeostatic cytokines

Adoptive T-cell–based immunotherapy is a potent and effective treatment for patients with advanced cancer (1, 2). Lymphodepleting preconditioning regimens and high-dose interleukin 2 (IL-2) are routinely used to enhance the engraftment and antitumor function of transferred T cells (2). Lymphodepletion enhances T-cell–based immunotherapies by eliminating immunosuppressive CD25$^+$FOXP3$^+$ regulatory T (T_{reg}) cells (3, 4) and activating antigen-presenting cells through Toll-like receptor signaling (5). Perhaps most important, lymphodepletion removes endogenous cellular cytokine sinks, thus increasing the availability of homeostatic cytokines that support the proliferation and function of transferred cells (6).

Mouse studies (7, 8) and retrospective analyses from clinical trials (9) have revealed a strong correlation between the intensity of preconditioning regimens and the efficacy of T-cell therapy. However, toxicities associated with high-intensity regimens can be severe and sometimes lethal (10–12). For instance, patients can experience prolonged leukopenia, increasing the risk for opportunistic infections. Radiation and chemotherapy are also associated with increased incidence of thromboembolic events, including pulmonary venoocclusive disease and thrombotic microangiopathy. Moreover, cortical blindness, a rare toxicity associated with fludarabine, has been reported in a patient receiving lymphodepleting chemotherapy before cell transfer (10).

IL-2 is also provided to convey proliferative and survival signals to transferred cells and enhance their antitumor capacities (1, 2). Similar to lymphodepleting regimens, high-dose IL-2 is associated with substantial toxicities mainly as a result of a capillary leak syndrome, which results in a hypovolemic state and fluid accumulation in the tissues (13).

Because of the potential for severe adverse effects associated with these maneuvers, numerous patients are excluded from T-cell therapies because of advanced age, poor performance status, or a history of cardiovascular and pulmonary diseases. In addition, management of these toxicities requires extensive supportive care and long-term inpatient hospitalization, placing an extraordinary financial burden on patients and the healthcare system. Therefore, the development of novel strategies capable of substituting the need for lymphodepletion and cytokine support while maintaining antitumor efficacy is highly desirable.

MicroRNAs (miRs) are 21–23-base-pair-long noncoding RNAs that modulate gene expression at a posttranscriptional level (14). Recently, microRNA-155 (miR-155) has emerged as a central regulator of immune system homeostasis and inflammatory responses to pathogens and tumors (15, 16). miR-155 is required for plasma cell differentiation (17) and dendritic cell activity (18). It has also been shown to regulate Th1 and Th17 lineage commitment (19). Notably, miR-155 is found to be a key factor regulating T_{reg} cell homeostasis (20). Finally, miR-155 is required for effector CD8$^+$ T-cell immune responses against viruses and cancer (21–23).

In this study, we report that overexpression of miR-155 in tumor-specific CD8$^+$ T cells provides for profound antitumor responses in the absence of lymphodepletion preconditioning and cytokine administration. We found that miR-155 enhanced CD8$^+$ T-cell responsiveness to limited amounts of endogenous homeostatic cytokines by inhibiting the expression of multiple

Significance

We describe here a strategy based on microRNA therapeutics to augment the efficacy of T-cell–based therapies without the requirement of toxic maneuvers such as lymphodepletion preconditioning and the administration of high doses of exogenous cytokines. These findings can lead to the development of safer and more effective T-cell–based therapies for the treatment of patients with advanced cancer.

Author contributions: Y.J., C.D.S., T.A.W., N.P.R., and L.G. designed research; Y.J., C.W., Z.Y., J.H., S.G., N.V.H., W.G.T., D.C.P., Z.F., M.S., R.R., D.C., C.A.K., and L.G. performed research; Y.J. and L.G. analyzed data; and Y.J. and L.G. wrote the paper.

Reviewers: J.H., University of Florida; and D.P., University of Pennsylvania.

The authors declare no conflict of interest.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1422916112/-/DCSupplemental.

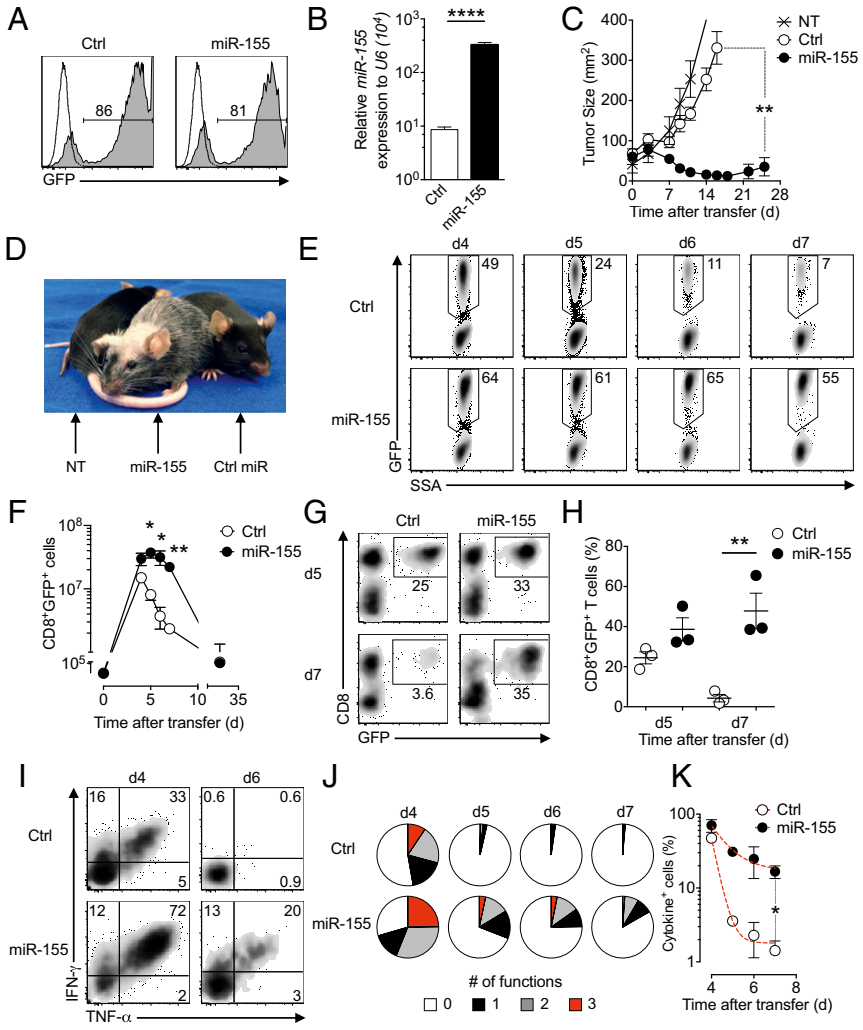

Fig. 1. miR-155 enhances CD8$^+$ T-cell antitumor response by augmenting CD8$^+$ T-cell expansion and effector function in the absence of lymphodepletion and cytokine support. (*A*) Frequency of GFP$^+$ cells (numbers above bracketed lines) among pmel-1 CD8$^+$ T cells transduced with retrovirus expressing miR-155-GFP (miR-155) or GFP alone (Ctrl). (*B*) RT-PCR analysis of miR-155 expression in cells transduced as in *A*. (*C*) Tumor size of C57BL/6 mice bearing B16 tumors established for 10 d receiving 5×10^6 miR-155 or Ctrl cells in conjunction with gp100-VV. NT, no treatment. (*D*) Vitiligo of C57BL/6 hosts receiving no cells, Ctrl miR, or miR-155 cells. (*E* and *F*) Flow cytometry (*E*) and numbers (*F*) of splenic T cells after adoptive transfer of 10^6 miR-155 or Ctrl cells into C57BL/6 mice infected with gp100-VV, assessed 4–35 d after infection. Numbers adjacent to outlined areas indicate percentage of GFP$^+$ T cells after gating on CD8$^+$ cells. (*G* and *H*) Flow cytometry (*G*) and percentage (*H*) of transferred T cells within the tumor mass after adoptive transfer of 10^6 miR-155 or Ctrl cells into tumor-bearing mice infected with gp100-VV, assessed day 5 and day 7 after infection. Numbers adjacent to outlined areas indicate percentage of CD8$^+$GFP$^+$ T cells. (*I*) Intracellular cytokine staining of GFP$^+$ CD8$^+$ T cells isolated from the spleen after the adoptive transfer of miR-155 or Ctrl cells. Numbers in quadrants indicate percentage of IFN-γ^+, TNF-α^+, and IFN-γ^+ TNF-α^+ after gating on GFP$^+$ CD8$^+$ cells. (*J*) Pie charts depicting the quality of the cytokine response in GFP$^+$ CD8$^+$ T-cell of three mice, as determined by the Boolean combination of gates identifying IFN-γ^+, IL-2$^+$, and TNF-α^+ cells. (*K*) Percentage of cytokine-producing cells in the spleen of mice transferred with miR-155 or Ctrl cells. *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$ (a two-tailed Student *t* test). Data are representative of two independent experiments [error bars (*B*), SEM of three samples; error bars (*C, D*), SEM of four two five samples; error bars (*F*, *H*, and *K*), SEM of three to four samples].

negative regulators of Akt and signal transducer and activator of transcription 5 (Stat5) signaling, including the inositol 5-phosphatase Ship1, suppressor of cytokine signaling 1 (Socs1), and the protein tyrosine phosphatase Ptpn2. These findings indicate that miR-155 can be used to augment the efficacy of T-cell–based therapies in a cell-intrinsic manner, providing an innovative approach to circumvent life-threatening maneuvers such as lymphodepleting preconditioning and high-dose cytokine support.

Results

miR-155 Enhances CD8$^+$ T-Cell Antitumor Responses in the Absence of Lymphodepletion and Cytokine Support. We and others have shown that miR-155 is required for mounting CD8$^+$ T-cell effector responses against tumors (21, 23). We sought to determine whether overexpression of miR-155 could enhance the efficacy of T-cell therapy in lymphoreplete hosts without cytokine support. We transduced pmel-1 T-cell receptor transgenic CD8$^+$ T cells, which recognize the melanocyte differentiation antigen gp100, with a γ-retroviral vector encoding miR-155-GFP (miR-155) or GFP alone as control (Ctrl). T cells were efficiently transduced, as revealed by the high frequency of cells expressing GFP (Fig. 1*A*) and the expression levels of *miR-155* (Fig. 1*B*). We transferred these cells into C57BL/6 mice bearing a s.c. established B16 melanoma in conjunction with a recombinant vaccinia virus encoding gp100 (gp100-VV), which is essential to induce tumor regression in this model (Fig. S1) (24). Remarkably, we found that miR-155 cells were capable of inducing profound tumor destruction in the absence of lymphodepletion, whereas control cells were

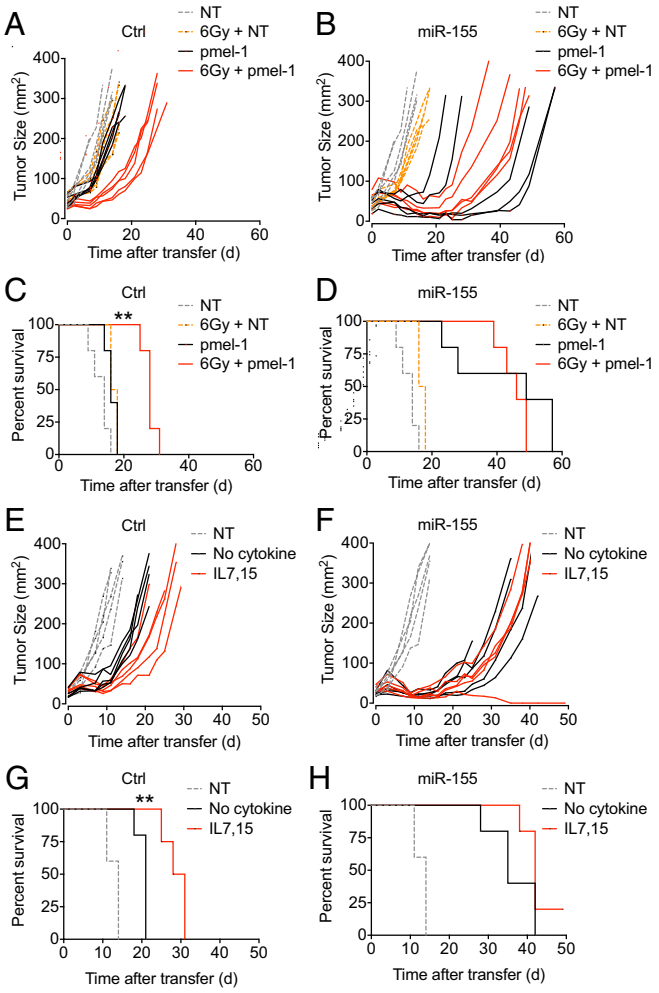

Fig. 2. miR-155 antitumor activity is not enhanced by lymphodepletion or exogenous homeostatic γc cytokines. (*A* and *B*) Tumor size of C57BL/6 mice bearing B16 tumors established for 10 d receiving 5×10^6 Ctrl (*A*) or miR-155 (*B*) cells in conjunction with gp100-VV with/without a sublethal dose of 6-Gy lymphodepletion. (*C* and *D*) Survival of mice treated with Ctrl (*C*) or miR-155 (*D*) cells described in *A* and *B*. (*E* and *F*) Tumor size of C57BL/6 mice bearing B16 tumors established for 10 d receiving 5×10^6 Ctrl (*E*) or miR-155 (*F*) cells in conjunction with gp100-VV with/without a high dose of IL-7 and IL-15 administration. (*G* and *H*) Survival of mice treated with Ctrl (*G*) or miR-155 (*H*) cells described in *E* and *F*. NT, no treatment. $^{**}P < 0.01$ [A Log-rank (Mantel-Cox) test]. Data are representative of two independent experiments (error bars, SEM of five samples).

almost completely ineffective (Fig. 1*C*). Similar findings were observed using scrambled miR-GFP (Ctrl miR) as control, excluding potential nonspecific effects resulting from the displacement of endogenous miRNAs by overexpressed miR-155 (Fig. S2). With the exception of autoimmune vitiligo (Fig. 1*D*), a well-characterized on-target toxicity (24), there were no discernable acute adverse events in mice receiving miR-155 cells (Fig. S3*A*). Because miR-155 dysregulation has been linked to tumor development (25), we monitored mice engrafted with miR-155 or Ctrl cells during a period of up to 747 d. There were no differences in the incidence of cellular transformation in mice receiving miR-155 compared with Ctrl cells (Fig. S3*B*).

To investigate the cellular basis behind the enhanced antitumor activity of miR-155 cells, we measured the expansion and function of adoptively transferred miR-155 or Ctrl cells after gp100-VV vaccination. Ctrl cells displayed a prototypical response characterized by a rapid expansion phase that peaked 4 d after immunization, followed by a profound contraction, which resulted in a small fraction of surviving cells by day 7 (Fig. 1 *E* and *F*). Although miR-155 cells expanded similarly, their contraction was substantially delayed (Fig. 1 *E* and *F*). Similar findings were also observed within the tumor mass (Fig. 1 *G* and *H*). Notably, we did not observe differences in cell numbers at the memory phase of the immune response, indicating that miR-155 overexpression did not result in unrestrained expansion (Fig. 1*F*). Strikingly, miR-155 cells also exhibited increased polyfunctionality and sustained ability to secrete IL-2, TNF-α, and IFN-γ overtime compared with Ctrl cells, which rapidly became functionally exhausted after the peak of the immune response (Fig. 1 *I*–*K*). These findings indicate that miR-155 augments $CD8^+$ T-cell antitumor responses by enhancing both cell survival and effector function in the absence of lymphodepletion.

miR-155 Enhances T-Cell Function Under Conditions of Limited Cytokine Availability. We sought to determine whether the antitumor activity of miR-155 cells could be further enhanced in tumor-bearing hosts rendered lymphopenic by 6 Gy total body irradiation. Consistent with previous findings (6), this preconditioning regimen potentiated the therapeutic efficacy of Ctrl pmel-1 cells, resulting in prolonged survival of tumor-bearing mice (Fig. 2 *A* and *C*). Surprisingly, miR-155 cells displayed similar antitumor efficacy in lymphoreplete and lymphodepleted hosts (Fig. 2 *B* and *D*), although vitiligo was less pronounced in the lymphoreplete setting (Fig. S4).

Because miR-155 is induced on T-cell activation (21), there was the possibility that the activating cues present in lymphodepleted hosts could up-regulate endogenous miR-155 levels in Ctrl cells, thus enhancing their antitumor activity, whereas the

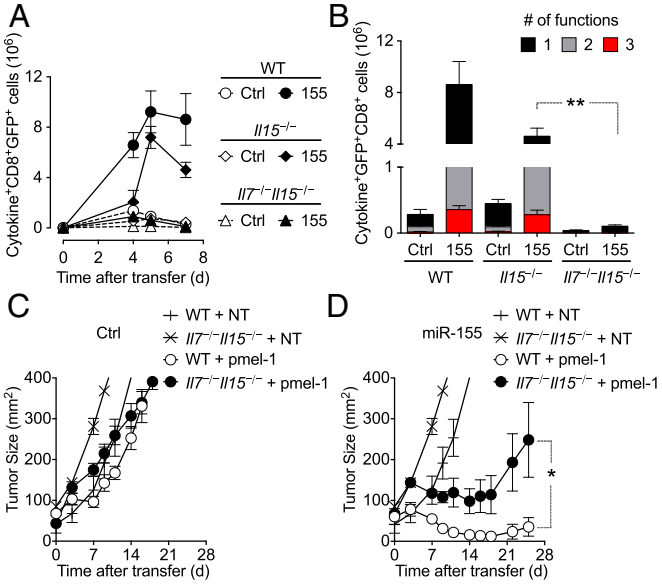

Fig. 3. Endogenous IL-7 and IL-15 are required to support the enhanced functionality of miR-155 overexpressing cells. (*A*) Numbers of cytokine producing $CD8^+$ GFP^+ cells isolated from the spleen of WT, *Il15*$^{-/-}$, and *Il7*$^{-/-}$ *Il15*$^{-/-}$ hosts after the adoptive transfer of Ctrl or miR-155 cells in conjunction with gp100-VV at indicated points after infection. (*B*) Numbers of the polyfunctional $CD8^+GFP^+$ cells isolated from the spleen of WT, *Il15*$^{-/-}$, and *Il7*$^{-/-}$ *Il15*$^{-/-}$ hosts after the adoptive transfer of Ctrl or miR-155 cells in conjunction with gp100-VV at day 7 after infection. (*C* and *D*) Tumor size of WT or *Il7*$^{-/-}$ *Il15*$^{-/-}$ mice bearing B16 tumors established for 10 d receiving 2×10^6 Ctrl (*C*) or miR-155 (*D*) cells in conjunction with gp100-VV. NT, no treatment. $^{*}P < 0.05$, $^{**}P < 0.01$ (a two-tailed Student *t* test). Data are representative of two independent experiments [error bars (*A* and *B*), SEM of three to four samples; error bars (*C* and *D*), SEM of four to five samples].

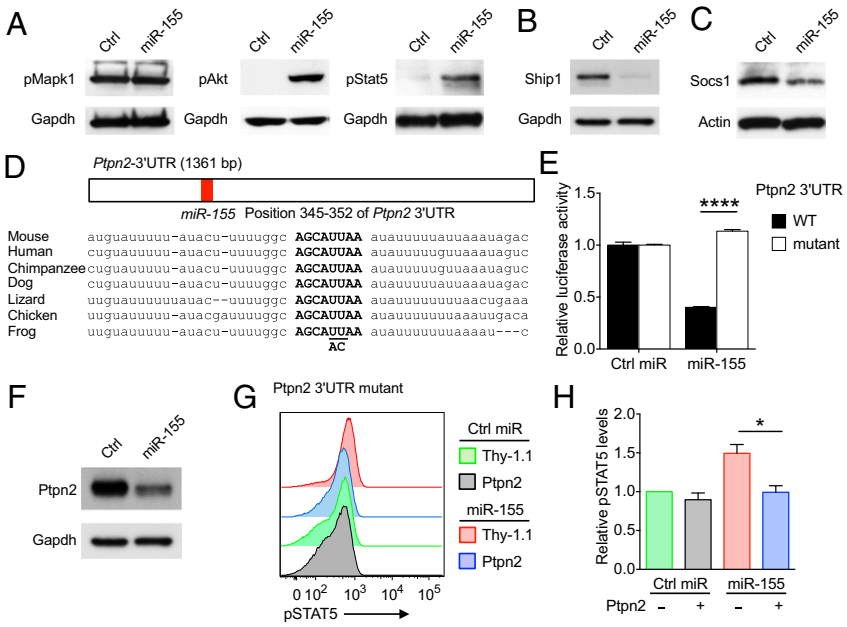

Fig. 4. miR-155 inhibits multiple negative regulators of Akt and Stat5 signaling. (*A*–*C*) Immunoblot analyses of miR-155 or Ctrl cells, probed with anti-pMapk1 (*A*, *Left*), anti-pAkt (*A*, *Middle*), anti-pStat5 (*A*, *Right*), anti-Ship1 (*B*), and anti-Socs1(*C*). Gapdh (*A* and *B*) and β-actin (*C*) were used as loading controls. (*D*) Multiple species sequence alignment of the Ptpn2 3′ UTR, including the predicted miR-155 target site sequence (bold). Mutations of the miR-155 target sites are shown below. (*E*) HEK293T cells were cotransfected with WT or mutated Ptpn2 3′ UTR and miR-155 mimic or control scrambled miR mimic and assessed for luciferase activity 24 h after transfection. (*F*) Immunoblot analyses of cells prepared as in *A*, probed with anti-Ptpn2 and anti-Gapdh. (*G* and *H*) Flow cytometry (*G*) and relative levels of pStat5 (*H*) in $CD8^+$ T cells coexpressing miR-155/scrambled miR and Ptpn2/Thy-1.1. $*P < 0.05$, $****P < 0.0001$ (a two-tailed Student *t* test). Data are representative of two independent experiments [error bars (*E*), SEM of three to four samples] or four independent experiments (*G* and *H*).

efficacy of miR-155 cells could not be further boosted in the setting of lymphodepletion because overexpression of miR-155 had driven the biological activity to plateau. However, lymphodepletion preconditioning did not cause up-regulation of endogenous miR-155 (Fig. S5*A*). In contrast, miR-155 levels increased in miR-155 cells in the setting of lymphodepletion, a finding consistent with the notion that lymphocyte activation augments transgene expression (26).

A major mechanism behind the increased efficacy of tumor-specific T cells in the lymphopenic environment is the removal of $CD4^+$ T_{reg} cells (3, 4). We therefore hypothesized that miR-155 might enable $CD8^+$ T cells to overcome T_{reg} cell suppression. However, in our model system, $CD4^+$ T cells appeared to play a minor role, as pmel-1 cells demonstrated similar antitumor responses in wild-type (WT) or *Cd4*$^{-/-}$ hosts (Fig. S5*B*).

Lymphodepletion also increases the availability of homeostatic cytokines to adoptively transferred T cells by removing endogenous cytokine sinks (6). We sought to determine whether miR-155 enhanced $CD8^+$ T-cell responsiveness to cytokines in lymphoreplete hosts, which have limited availability of homeostatic cytokines. We transferred miR-155 or Ctrl pmel-1 cells into B16 tumor-bearing mice, together with gp100-VV, in the presence or absence of high doses of the homeostatic cytokines IL-7 and IL-15. Consistent with previous data (6), provision of these cytokines increased the therapeutic efficacy of Ctrl cells and improved animal survival (Fig. 2 *E* and *G*). In sharp contrast, we did not observe additional benefits in mice receiving miR-155 cells (Fig. 2 *F* and *H*), suggesting the limited amounts of homeostatic cytokines available in lymphoreplete hosts are sufficient to promote the potent immune response mediated by miR-155 cells.

To test whether homeostatic cytokines were required for the enhanced functionality of miR-155 cells, we evaluated the kinetic of the immune response in mice deficient in IL-7 and IL-15. We transferred miR-155 or Ctrl cells in conjunction with gp100-VV into WT, *Il15*$^{-/-}$, and *Il7*$^{-/-}$ *Il15*$^{-/-}$ hosts and enumerated the number of cytokine-producing pmel-1 cells. Recapitulating earlier findings, overexpression of miR-155 increased the number of functional cells compared with Ctrl in WT recipients. However, the number of functional miR-155 cells was reduced in *Il15*$^{-/-}$ hosts and was more severely decreased in mice lacking both homeostatic cytokines (Fig. 3*A*). Moreover, the sustained polyfunctionality observed in miR-155 cells in WT hosts was severely impaired in *Il7*$^{-/-}$ *Il15*$^{-/-}$ mice (Fig. 3*B*). To determine whether IL-7 and IL-15 were essential for the increased antitumor response of miR-155 cells, we transferred miR-155 or Ctrl pmel-1 cells in conjunction with gp100-VV into WT or *Il7*$^{-/-}$ *Il15*$^{-/-}$ mice bearing B16 melanoma. We found that the antitumor efficacy of miR-155 cells was significantly decreased in *Il7*$^{-/-}$ *Il15*$^{-/-}$ mice, indicating that benefit provided by miR-155 was dependent on homeostatic cytokine signaling (Fig. 3 *C* and *D*). Taken together, these results suggest that the presence of homeostatic γ_c cytokines is essential for the enhanced activity of miR-155 cells.

miR-155 Inhibits Multiple Negative Regulators of Akt and Stat5 Signaling. We sought to dissect the molecular mechanisms behind the increased efficacy of miR-155 cells in conditions where homeostatic cytokines are limiting. Because miR-155 does not affect the expression of the IL-15 and IL-7 receptor complexes (21), we focused on downstream signaling. It is well established that γ_c cytokines primarily signal through three pathways: Mapk, Jak3/Stat5, and PI3K/Akt (27). To determine whether any of these pathways were modulated by miR-155 overexpression, we evaluated the amount of phosphorylated Mapk1, Akt, and Stat5 in miR-155 and Ctrl cells. Strikingly, we detected increased pAkt and pStat5 in miR-155 cells, but no major differences in pMapk1 (Fig. 4*A*). It has been recently reported that Stat1 signaling is enhanced in $CD8^+$ T cells lacking miR-155 (22); however, we did not observe noticeable differences in pStat1 levels in miR-155 compared with Ctrl cells (Fig. S6). Ship1, a negative regulator of Akt signaling, is an established miR-155 target (28). Consistently, we detected decreased levels of Ship1 in miR-155 compared with Ctrl cells, thus providing a mechanistic basis for the observed enhancement in Akt activity (Fig. 4*B*). Previously, we have demonstrated that miR-155 regulates Stat5 signaling by targeting the suppressor of cytokine signaling, Socs1 (21). Here, we noted that Socs1 levels were decreased in miR-155 cells (Fig. 4*C*).

Bioinformatic analysis by TargetScan predicted an evolutionarily conserved miR-155 binding site at the 3′ UTR region of Ptpn2 (Fig. 4*D*). Ptpn2 is a negative regulator of Jak3/Stat5 signaling that has been shown to restrain homoeostatic T-cell responses (29). Furthermore, knockdown of *PTPN2* in human T-cell acute lymphoblastic leukemia increased the proliferation and cytokine sensitivity of leukemic cells (30), a phenotype

closely resembling the effects of miR-155 overexpression in our model. Thus, we sought to test whether miR-155 directly regulates Ptpn2. We transfected HEK293T cells with luciferase reporter constructs containing the *Ptpn2* 3′ UTR with an intact or mutated miR-155 binding sequence together with miR-155 or a Ctrl miR. Cotransfection of *Ptpn2* 3′ UTR with miR-155 resulted in a significant repression of reporter activity, whereas mutation of the miR-155 seed-binding site abolished this repression (Fig. 4*E*). These findings indicate that miR-155 directly regulates *Ptpn2* expression. Next, we evaluated Ptpn2 protein levels in miR-155 cells. Consistently, we detected a substantial decrease of Ptpn2 by Western blot (Fig. 4*F*), indicating that Ptpn2 is targeted by miR-155. To determine whether down-regulation of Ptpn2 was relevant to the enhanced Stat5 signaling mediated by miR-155 overexpression, we measured pStat5 in miR-155 cells in the presence of ectopic expression of Ptpn2. Overexpression of Ptpn2 significantly reduced pStat5 levels in miR-155 cells (Fig. 4 *G* and *H*), suggesting that modulation of Ptpn2 contributes to the functional advantage mediated by miR-155. Altogether, these findings reveal that miR-155 enhances Akt and Stat5 signaling in a cell-intrinsic manner by repressing multiple negative regulators of these pathways in $CD8^+$ T cells.

Akt and Stat5 Signaling Contribute to the Functional Advantage Conferred by miR-155. Having demonstrated that miR-155 promoted Akt and Stat5 signaling, it remained to be determined whether either or both pathways contribute to the enhanced $CD8^+$ T-cell functionality mediated by miR-155 overexpression. We cotransduced pmel-1 cells with Ctrl or miR-155, together with a constitutively active form of Akt (AktCA) or Stat5 (Stat5aCA) (Fig. 5*A*), and tracked the fate of double-transduced cells by monitoring the reporter markers GFP (miR-155 or Ctrl) and Thy1.1 (AktCA, Stat5aCA, or Ctrl) after adoptive transfer into WT hosts in conjunction with gp100-VV (Fig. 5*A*). Because Stat5 signaling has been shown to induce miR-155 (31), we first tested the expression of miR-155 in Stat5aCA cells. We found that although Stat5aCA up-regulated miR-155 expression, its levels were far below the amounts detected in miR-155 cells (Fig. S7). Importantly, AktCA did not influence the endogenous expression of miR-155 (Fig. S7). Next, we evaluated the effects of AktCA and Stat5aCA on miR-155 cells by assessing the numbers and function of transduced cells 7 d after transfer, the time at which the differences between miR-155 and Ctrl cells are most prominent (Figs. 1 *E–K* and 3 *A* and *B*). Overexpression of Stat5aCA, but not AktCA, reduced the differences in cell numbers between miR-155 and Ctrl cells, as demonstrated by percentage and ratios, indicating functional redundancy between miR-155 and Stat5aCA in delaying contraction kinetics (Fig. 5 *B* and *C*). In contrast, overexpression of AktCA significantly reduced the differences in cell functionality between miR-155 and Ctrl cells, suggesting miR-155 promoted sustained cytokine production through the activation of Akt (Fig. 5 *D* and *E*). Altogether, these findings indicated that Stat5 and Akt play non-redundant and distinctive roles in driving miR-155-mediated numeric and functional advantages.

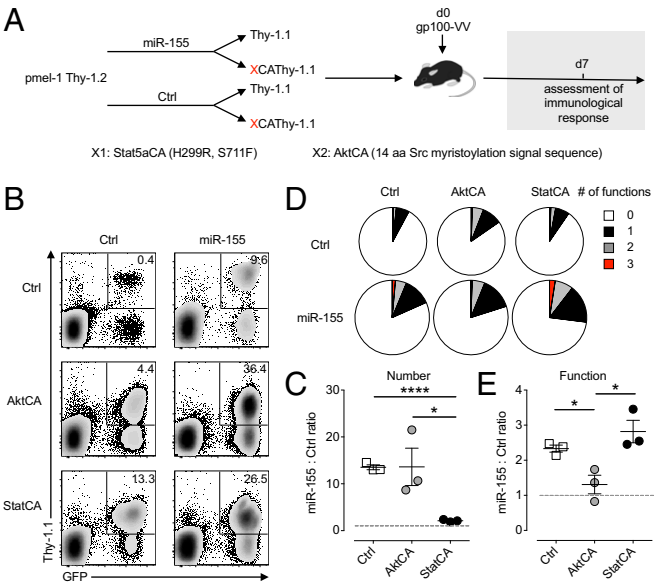

Fig. 5. Akt and Stat5 signaling contribute to the functional advantage conferred by miR-155. (*A*) Experimental layout used to dissect the distinctive contribution of Stat5 or Akt signaling in miR-155 cells. (*B*) Flow cytometry of splenic T cells after adoptive transfer of 3×10^5 FACS sorted pmel-1 $CD8^+GFP^+Thy\text{-}1.1^+$ T cells (transduced as in *A*) into C57BL/6 mice infected with gp100-VV, assessed at day 7 after infection. Numbers adjacent to outlined areas indicate percentage of $GFP^+Thy\text{-}1.1^+$ T cells after gating on $CD8^+$ cells. (*C*) Ratio of miR 155 to Ctrl cells cotransduced with Stat5aCA, AktCA, or Ctrl Thy-1.1 isolated from the spleen of C57BL/6 mice, as described in *B*. (*D*) Pie charts depicting the quality of the cytokine response in $CD8^+GFP^+Thy\text{-}1.1^+$ T cells treated as in *B*. (*E*) Ratio of miR 155 to Ctrl cytokine producing cells from pmel-1 $CD8^+GFP^+Thy\text{-}1.1^+$ T treated as in *B*. $^*P < 0.05$, $^{****}P < 0.0001$ (a two-tailed Student *t* test). Data are representative of two independent experiments [error bars (*C* and *E*), SEM of three to four samples).

Discussion

In this study, we report a strategy to eliminate the need for preparative lymphodepleting regimens as well as the administration of exogenous cytokines in adoptive immunotherapy. Overexpression of miR-155 in tumor-specific $CD8^+$ T cells enhanced their responsiveness to limited amounts of cytokines present in lymphoreplete hosts in a cell-intrinsic manner, resulting in increased survival and sustained functionality. These findings have parallels in $CD4^+$ T cells, where miR-155 is essential for long-term maintenance of T_{reg} cells in lymphoreplete, but not lymphodepleted, mice (20). Other strategies to potentiate adoptive T-cell therapy in the absence of lymphodepletion have been recently proposed, such as PD1 blockade in the presence of IL-2/anti-IL-2 antibody complexes (32) or overexpression of IL-12 in tumor-reactive T cells (33). However, it is unclear whether such approaches can replace both lymphodepletion and cytokine support. For instance, overexpression of IL-12 in pmel-1 cells could not substitute the requirement for lymphodepletion in this model of T-cell therapy (34). Here, we demonstrate that total body irradiation preconditioning or high-doses of exogenous cytokines could not increase the treatment efficacy of miR-155 cells, indicating that overexpression of miR-155 is sufficient in surrogating the therapeutic advantages conveyed by these regimens. Although miR-155 overexpression promoted vitiligo, the severity of this adverse effect was significantly reduced compared with Ctrl cells transferred into lymphodepleted hosts, making this therapeutic strategy even more attractive.

By dissecting the molecular mechanism behind the activity of miR-155 overexpression, we discovered that miR-155 enhanced Akt and Stat5 signaling by suppressing multiple negative regulators of these pathways. We confirmed that miR-155 targets the Akt inhibitor, Ship1, as well as Socs1, a well-established Stat5 inhibitor. Moreover, we identified Ptpn2 as a novel target of miR-155 and demonstrated that down-regulation of this molecule is relevant to the modulation of Stat5 signaling mediated by miR-155. Intriguingly, increased Stat5 signaling appears to contribute primarily to cell survival, whereas enhanced Akt signaling seems mainly responsible for sustained functionality mediated by miR-155 overexpression. Enhancing the activity of both Akt and Stat5 by modulating miR-155 expression provides advantages over strategies relying on constitutive activation of these two

pathways, as it allows fine-tuning of multiple cellular processes by targeting numerous molecules without the negative effects that can be associated with a strong constitutive activation of a single pathway. For instance, constitutive Akt activation can drive cells toward terminal differentiation, impairing their long-term survival (35). This represents a clear disadvantage, as persistence of transferred cells correlates to antitumor responses in preclinical mouse models (36, 37) and clinical trials (9, 38).

Although we did not observe increase rates of malignant transformation of miR-155 cells (Fig. S3*B*), the potential oncogenicity of miR-155 warrants cautions and might require the introduction of suicide genes to enable the elimination of transferred cells if transformation occurs (39). Alternatively, inducible promoters might be used to control the expression of miR-155. For example, an NFAT-responsive promoter has been successfully used to limit the toxicities associated with constitutive expression of IL-12 (40).

Lymphodepleting preconditioning regimens and administration of high doses of cytokines are currently used in the clinic to augment the therapeutic efficacy of T-cell therapies (1, 2), but the toxicities associated with these maneuvers and the high costs of patient supportive care prevent access and widespread adoption of these treatments worldwide. Moreover, the requirement of preparative therapies before T-cell transfer excludes the possibility of multiple rounds of T-cell administration at short intervals, as repeated rounds of preconditioning treatments are not feasible. Enforced expression of miR-155 in tumor-reactive T cells would not only overcome the requirement for use of nonspecific modalities such as chemotherapy or total body irradiation, with associated life-threatening adverse effects, but also might allow short-term repetitive T-cell administration to achieve optimal therapeutic outcomes.

Materials and Methods

All mouse experiments were approved by the National Cancer Institute Animal Use and Care Committee. Real-time RT-PCR, immunoblot, retrovirus production, transduction of $CD8^+$ T cells, adoptive cell transfer, infection, and tumor treatment were previously described (21, 37). Luciferase reporter assay was performed using LightSwitch Luciferase Assay Kit (Switchgear Genomics) according to the manufacturer's protocol. Detailed information about mice, experimental procedures and statistical analyses can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank A. Mixon and S. Farid for flow cytometry sorting. We thank Dr. Hand for providing the AktCA and Stat5aCA constructs. This work was supported by the Intramural Research Program of the National Cancer Institute, Center for Cancer Research, National Institutes of Health.

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