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MicroRNA-155 is required for effector CD8+ T cell responses to virus infection and cancer

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SUMMARY

MicroRNAs regulate the function of several immune cells but their role in promoting CD8+ T-cell immunity remains unknown. Here we report that miR-155 is required for CD8+ T-cell responses to both virus and cancer. In the absence of miR-155, accumulation of effector CD8+ T cells was severely reduced during acute and chronic viral infections and control of virus replication was impaired. Similarly, *Mir155*-/- CD8+ T cells were in effective at controlling tumor growth, whereas miR-155 overexpression enhanced the antitumor response. miR-155 deficiency resulted in accumulation of SOCS-1 causing defective cytokine signaling through STAT5. Consistently, enforced expression of SOCS-1 in CD8+ T cells phenocopied the miR-155 deficiency, whereas SOCS-1 silencing augmented tumor destruction. These findings identify miR-155 and its target SOCS-1 as key regulators of effector CD8+ T cells that can be modulated to potentiate immunotherapies for infectious diseases and cancer.

INTRODUCTION

CD8+ T cells are essential effectors in immune responses to intracellular pathogens and cancer (Zhang and Bevan, 2011). Upon stimulation, antigen-specific CD8+ T cells massively expand and differentiate into inflammatory cytokine producing, cytolytic T cells able to eliminate virally infected or transformed cells. As the antigen is cleared, the majority of specific CD8+ effector T cells die (Marrack and Kappler, 2004), whereas only a small number of memory cells survives. The CD8+ T cell response is influenced by a series of costimulatory (and inhibitory) ligands and by multiple soluble mediators such as IL-2 (Boyman and Sprent, 2012). The latter is essential for sustaining an efficient effector response, whereas other cytokines such as IL-7 and IL-15 play crucial roles for the survival of naïve or memory T cells (Cui and Kaech, 2010). Several studies have identified key

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molecular factors involved in the differentiation from naïve to effector CD8+ T cells, but the contribution of microRNAs (miRs) has just begun to be investigated (Almanza et al., 2010).

miRs are a class of small, non-coding RNAs that impart post-transcriptional gene regulation (Bartel, 2004) through several mechanisms including translational repression and mRNA degradation (Djuranovic et al., 2011). They are important in many physiological processes, in carcinogenesis (Calin and Croce, 2006) and in the immune system (Xiao and Rajewsky, 2009). Early studies in mice deficient for Dicer, an RNAse III enzyme important for mature miR production, revealed that miRs are involved in CD4+ T cell differentiation and strongly influence CD8+ T cell responses (Muljo et al., 2005; Zhang and Bevan, 2010). Specific miRs were shown to regulate both lymphocyte development and function. For instance, miR-181a influences thymocyte selection by modulating the expression of molecules involved in TCR signaling (Li et al., 2007). Moreover, the miR-17~92 cluster regulates B cell development (Ventura et al., 2008), autoimmunity and Th1cell differentiation (Jiang et al., 2011; Xiao et al., 2008).

miR-155 is upregulated upon lymphocyte activation (Haasch et al., 2002) to control cell proliferation and differentiation (O'Connell et al., 2008; Turner and Vigorito, 2008). For instance, miR-155 regulates B cell proliferation, malignancy and antibody production, at least in part through inhibition of activation-induced cytidine deaminase and PU.1 expression (Rodriguez et al., 2007; Thai et al., 2007; Vigorito et al., 2007). In CD4+ T cells, miR-155 has been shown to suppress differentiation of naïve cells into Th2 by downregulation of c-Maf, to promote Th17 cell mediated inflammation (Kurowska-Stolarska et al., 2011; O'Connell et al., 2010) and to inhibit IFN-γR expression (Banerjee et al., 2010; Martinez-Nunez et al., 2011). In addition to direct modulation of cytokine receptor expression, miR-155 shapes cytokine signaling in several cell subsets via downregulation of SMAD2 (Louafi et al., 2010) and suppressor of cytokine signaling (SOCS-1) (Lu et al., 2009; O'Connell et al., 2010; Wang et al., 2010). Despite the evidence for an important role of miR-155 in a wide spectrum of immune compartments, it is not known if this miRNA, which is highly expressed in antigen-experienced CD8+ T cells (Salaun et al., 2011), influences CD8+ T cells *in vivo*. In the present study, we have investigated the role of miR-155 during CD8+ T cell responses to viral infection, vaccination and cancer.

RESULTS

CD8+ T cells dynamically regulate miR-155 depending on the magnitude of TCR stimulation or their differentiation state

The strength of TCR signaling has a major impact on the magnitude of CD8+ T cell expansion but not on their differentiation (Zehn et al., 2009). We sought to investigate whether the strength of TCR stimulation affects the expression of miR-155 in CD8+ T cells by transducing human CD8+ T cells with variants of a NY-ESO-1-specific TCR of increasing affinity for its ligand (Derre et al., 2008; Schmid et al., 2010). Comparatively, a mutated low affinity TCR failed to upregulate miR-155 within 48h, whereas TCR variants of higher affinities induced higher levels of miR-155 than the wild type (Figure 1A). Thus, miR-155 expression increased in a TCR affinity-dependent manner in human CD8+ T cells. A similar upregulation was observed for the pri-miR-155 non-coding RNA transcript, BIC (Figure S1). To see if miR-155 was also regulated in an affinity-dependent manner in mouse CD8+ T cells, we activated naïve OT-1 T cells with splenic dendritic cells (DC) loaded with the wild type peptide SIINFEKL (N4) or the weaker altered peptide ligand SIITFEKL (T4) (Daniels et al., 2006). To exclude miR-155 contamination from the DCs, we used *Mir155*-/- DC which retain normal antigen presenting capabilities (O'Connell et al., 2010). Exposure of OT-1 cells to the WT natural peptide resulted in a strong upregulation of miR-155, while a weaker TCR stimulation by the T4 peptide was less effective (Figure 1B). To assess

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miR-155 regulation *in vivo*, we analysed naïve ($CD62L^+CD44^−$), effector ($CD62L^−CD44^+$) and central memory ($CD62L^+CD44^+$) $CD8^+$ T cells following LCMV infection (200 pfu of WE strain). Compared to their naïve counterparts, miR-155 was strongly upregulated in effector cells and to a lower extent in central memory $CD8^+$ T cells 8 days post-infection (Figure 1C). A more detailed kinetics of miR-155 regulation during LCMV infection revealed that numbers of effector cells peaked on day 6, but stayed low in naïve cells (Figure 1D). These results demonstrate that miR-155 is induced in effector $CD8^+$ T cells depending on the strength of stimulation and differentiation.

miR-155 promotes the accumulation of anti-viral effector and central memory $CD8^+$ T cells

To determine the role of miR-155 in activated $CD8^+$ T cells, we monitored the expansion of effector cells following acute LCMV WE strain infection in the presence or absence of miR-155. Percentage, number and phenotype of naïve *Mir155*$^{-/-}$ $CD8^+$ T cells in blood and spleen did not differ from those in wild type mice before infection (Figure S2A and data not shown). In contrast, both percentage and number of total $CD8^+$ T cells as well as virus gp33 tetramer specific $CD8^+$ effector T cells were substantially reduced in spleen and blood of *Mir155*$^{-/-}$ mice at the peak of the response (Figure 2A, B). Following the expansion of $CD44^+$ effector cells in the blood and spleen from days 6 to 8, we observed impaired effector $CD8^+$ cell accumulation in spleen, liver and blood of *Mir155*$^{-/-}$ mice (Figure 2C and data not shown). Despite a defect in the magnitude of effector T cell responses, *Mir155*$^{-/-}$ animals were capable of controlling viral replication and clearing the virus (Figure S2B), as also confirmed by the lack of CD44 upregulation on adoptively transferred naïve LCMV specific P14 T cells (Figure S2C). In line with this result, $CD8^+$ T cells differentiated into phenotypically and functionally cytolytic effector cells similar to wild type cells during LCMV infection (Figure S2A, D, E). Interestingly, circulating T cells in *Mir155*$^{-/-}$ mice exhibited not only a defect in the expansion at the peak of the immune response but also a more rapid contraction compared to wild type animals (Figure 2D). Moreover, $CD127^+CD62L^+KLRG1^−$ memory cells were strongly reduced in the gp33 and np396 $tetramer^+$ $CD8^+$ T cells in blood, liver and spleen of *Mir155*$^{-/-}$ mice three months after infection (Figure 2E, S2F and data not shown). Consistent with these findings, IL-2 production, a hallmark of central memory cells, was strongly diminished in *Mir155*$^{-/-}$ mice after stimulation with gp33 peptide (Figure 2F, S2F). In this immune memory context, it is of interest that we observed a deficient $CD4^+$ effector T cell activation on day 8 of the response in *Mir155*$^{-/-}$ mice (data not shown). Altogether, these results demonstrate that miR-155 is crucial for a robust T cell expansion but not effector functions as well as for a memory phenotype response upon an acute LCMV infection.

Intrinsic expression of miR-155 in $CD8^+$ T cells promotes proliferation and limits apoptosis of effector $CD8^+$ T cells

To investigate whether the defective expansion of $CD8^+$ T cells was cell-intrinsic, we cocultured naïve wild type and congenic *Mir155*$^{-/-}$ OT-1 $CD8^+$ T cells together with peptide pulsed dendritic cells and analysed the OT-1 cell ratio. After 5 days, wild type $CD8^+$ T cells outnumbered *Mir155*$^{-/-}$ cells and the abundance of dead cells was strongly increased among *Mir155*$^{-/-}$ T cells (Figure 3A). To assess these parameters *in vivo*, we co-transferred equal numbers of congenic polyclonal wild type and *Mir155*$^{-/-}$ $CD8^+$ T cells into either wild type or *Mir155*$^{-/-}$ hosts, which were then infected with LCMV. Despite the initial low frequency of wild type $CD8^+$ T cells transferred in miR-155 ablated hosts (about 1% of $CD8^+$ T cells in blood before infection), these cells expanded to about 30% of the $CD8^+$ T cells at the peak of the response. In contrast, the frequency of *Mir155*$^{-/-}$ $CD8^+$ T cells transferred into wild type hosts decreased upon infection (Figure 3B), clearly demonstrating a stronger response of wild type compared to *Mir155*$^{-/-}$ $CD8^+$ T cells. When Rag2 and common γ chain (γc) deficient hosts were engrafted with a 1:1 mix of wild type and *Mir155*$^{-/-}$ splenocytes,

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both populations reached similar frequencies after 2 months, indicating comparable homeostatic expansion (Figure 3C). However, following LCMV infection, wild type T cells again showed an advantage in expansion over their *Mir155*$^{-/-}$ counterparts. To determine the basis for the impaired accumulation of virus-specific CD8$^+$ T cells in the absence of miR-155, LCMV infected wild type and *Mir155*$^{-/-}$ mice were pulsed with BrdU and proliferation and apoptosis were measured 4h later. We found that the proliferation of *Mir155*$^{-/-}$ CD44$^+$ effector CD8$^+$ T cells was decreased compared to wild type cells 6 days after infection (Figure 3D). Additionally, the frequency of proliferating Ki67$^+$ cells within the CD44$^+$CD62L$^-$ effector CD8$^+$ T cells was reduced in *Mir155*$^{-/-}$ mice (Figure 3E). Finally, we observed an increased frequency of AnnexinV$^+$ apoptotic cells in *Mir155*$^{-/-}$compared to wild type effector CD8$^+$ T cells 7 days after infection (Figure 3F). Altogether, these data demonstrate a cell-intrinsic role of miR-155 in the proliferation and survival of effector CD8$^+$ T cells in response to LCMV infection but not for homeostatic expansion in lymphopenic hosts.

miR-155 is crucial for effector CD8$^+$ T cell accumulation and virus control in chronic LCMV infection

Based on the strong impairment of effector CD8$^+$ T cell accumulation in low dose LCMV infection, we asked how *Mir155*$^{-/-}$ mice would respond to high dose and long-lasting antigen exposure, which characterizes chronic infections and cancer. Mice were inoculated with 2×10^6 pfu of LCMV clone 13, causing a chronic infection for several weeks (Moskophidis et al., 1993; Salvato et al., 1991). While wild type mice mounted a robust effector CD8$^+$ T cell response with high percentages of CD44$^+$CD62L$^-$ effector cells that were maintained overtime, *Mir155*$^{-/-}$ mice progressively lost effector CD8$^+$ T cells (Figure 4A). Interestingly, the remaining CD44$^+$ cells in spleen showed high CD127 and CD62L expression, reminiscent of a memory phenotype (Figure 4B). Percentages and numbers of gp33 tetramer positive cells were also strongly decreased in deficient mice 5 weeks and 3 months post infection (Figure 4B, C and data not shown). At this time, we could not detect cells capable of producing effector cytokines in response to a cocktail of LCMV peptides in miR-155 ablated mice, confirming the loss of most virus specific *Mir155*$^{-/-}$ CD8$^+$ T cells, and ruling out TCR downregulation that may appear as tetramer negative T cells (Figure 4E and data not shown). Whereas about 50% of wild type cells remained positive for PD-1, associated with T cell exhaustion, PD-1 was barely detectable on *Mir155*$^{-/-}$ CD8$^+$ T cells 5 weeks upon infection (Figure 4C). Importantly, virus titers were elevated five weeks and two months post infection in miR-155 ablated mice (Figure 4D and data not shown). Finally, wild type but not miR-155 ablated mice showed symptoms of immunopathology such as shivering, hunching and weight loss, suggesting a lower inflammatory response in the absence of miR-155 (Figure 4F and data not shown). These data demonstrate an important role of miR-155 in maintenance and survival of CD8$^+$ effector T cells as well as virus control in chronic virus infections.

miR-155 expression in CD8$^+$ T cells is crucial for efficient immunization and cancer immunotherapy

Because we observed an important role of miR-155 to sustain CD8$^+$ T cell responses to chronic infection, we looked at the impact of miR-155 on CD8$^+$ T cell dependent anti-tumor immunity, which requires robust CD8$^+$ T cell responses. We examined the role of miR-155 in CD8$^+$ T cells for vaccination, a clinically relevant setting characterized by limited adjuvant-induced inflammation. Polyclonal and OVA-specific OT-1 CD8$^+$ T cells (either wild type or *Mir155*$^{-/-}$) were cotransferred into wild type mice before immunization with OVA peptide adjuvanted with IFA and CpG-ODNs. While the ratio of wild type OT-1 to polyclonal cells strongly increased following immunization, there was only a minor increment in the ratio of *Mir155*$^{-/-}$ OT-1 to polyclonal wild type cells (Figure 5A). We next

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cotransferred OT-1 cells from both wild type and *Mir155*$^{-/-}$ backgrounds into wild type mice before immunization. Wild type and *Mir155*$^{-/-}$ cells were found in similar proportions indicating a comparable survival after adoptive transfer. Following immunization, however, wild type cells accumulated more efficiently than *Mir155*$^{-/-}$ cells (Figure 5B), whereas upregulation of CD44 and the proportion of cells producing IFN-γ were comparable (Figure S3A, B). Now we were interested, if miR-155 would also be critical for tumor control by CD8$^+$ T cells and engrafted B16 melanoma upon transfer of naïve wild type or *Mir155*$^{-/-}$ OT-1 T cells and therapeutic vaccination one week later.

Compared to wild type, *Mir155*$^{-/-}$ CD8$^+$ T cells were less effective in inhibiting tumor growth and ensuring the survival of tumor-challenged mice (Figure 5C, D). Given the inability of T cells to expand and control tumor growth in the absence of miR-155, we hypothesized that enforcing miR-155 expression would augment the anti-tumor activity of CD8$^+$ T cells. Pmel-1 TCR transgenic CD8$^+$ T cells specific for the melanoma antigen gp100 were transduced with a retrovirus encoding miR-155 or scrambled miR and adoptively transferred into tumor-bearing mice in conjunction with gp100 vaccination and IL-2. There were no phenotypic differences between control and miR-155 overexpressing T cells prior to adoptive transfer (Figure S3C). However, overexpression of miR-155 greatly enhanced the anti-tumor responses compared to scrambled miR control (Figure 5E). Notably, 80% of mice receiving miR-155 transduced T cells survived for over 60 days, whereas all mice treated with control cells or left untreated had to be euthanized after less than 40 days due to tumor size (Figure 5F). Taken together, these results show that upon vaccination, *Mir155*$^{-/-}$ CD8$^+$ T cells differentiated into effector cells but failed to accumulate in normal numbers. Consequently, the anti-tumor response was strongly dependent on miR-155 and could be therapeutically boosted by enforced miR-155 expression.

Targeting of SOCS-1 by miR-155 in effector CD8$^+$ T cells enables cytokine responsiveness and accumulation

miR-155 has been shown to regulate γc-chain cytokine signaling by targeting SOCS-1 expression (D'Souza and Lefrancois, 2003; Lu et al., 2009; Wang et al., 2010). We assessed SOCS-1 regulation in splenic effector CD44$^+$CD62L$^-$CD8$^+$ T cells during the response to acute LCMV infection of wild type and *Mir155*$^{-/-}$ mice. We found that both wild type and *Mir155*$^{-/-}$ CD8$^+$T cells downregulated SOCS-1 on days 6 and 8 compared to CD62L$^+$CD44$^-$ naïve CD8$^+$ T cells from non-infected mice (Figure 6A). To more directly test if SOCS-1 was regulated by miR-155, we measured SOCS-1 mRNA in wild type and *Mir155*$^{-/-}$ CD8$^+$ T cells as well as in cells overexpressing miR-155 or scrambled control miR. We found that the amounts of SOCS-1 transcripts were inversely related to the cellular content of miR-155, with the highest concentration of SOCS-1 in *Mir155*$^{-/-}$ cells and the lowest in miR-155 transduced cells (Figure 6B). These results were further confirmed at the protein level, indicating that miR-155 is a critical regulator of SOCS-1 translation in CD8$^+$ T cells (Figure 6C). To test whether the loss of miR-155 impaired γc chain cytokine signaling in CD8$^+$ T cells by upregulating SOCS-1, we compared STAT5 phosphorylation in response to IL-2, IL-7 or IL-15 in wild type and *Mir155*$^{-/-}$ cells. Stimulation of naïve and effector CD8$^+$ T cells isolated 8 days after LCMV infection resulted in a limited phosphorylation of STAT5 in miR-155 ablated cells, demonstrating an impaired cytokine signaling (Figure 6D). Diminished STAT5 phosphorylation was not due to differential expression of the cytokine receptor chains CD25, CD122, CD127 or CD132 (Figure S4). To further investigate whether the impaired cytokine signaling was dependent on the higher SOCS-1 concentration in *Mir155*$^{-/-}$ CD8$^+$ T cells, we transduced wild type and *Mir155*$^{-/-}$ CD8$^+$ T cells with control or shSOCS-1 lentivirus. Although *in vitro* activation of T cells diminished the impact of miR-155 on cytokine signaling, we consistently detected a rescue of pSTAT5 generation in shSOCS-1 transfected *Mir155*$^{-/-}$ cells (Figure 6E). Interestingly, baseline pSTAT5

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expression was already higher in wild type than in *Mir155*$^{-/-}$ cells without additional IL-2 stimulation. The difference between wild type and *Mir155*$^{-/-}$ cells was still apparent with intermediate, but disappeared with high IL-2 concentrations, demonstrating that saturating amounts of IL-2 overcome the miR-155 and SOCS-1 dependent inhibition of cytokine signaling, as shown for regulatory T cells (Lu et al., 2009). Together, these results demonstrate a dynamic and differentiation-dependent regulation of SOCS-1 during the response to LCMV and suggest that *Mir155*$^{-/-}$ CD8^{+} T cells have impaired cytokine signaling due to increased SOCS-1.

SOCS-1 restrains CD8^{+} T cell responses to virus and cancer

To test whether increased SOCS-1 expression recapitulated the impaired antigen-driven expansion of *Mir155*$^{-/-}$ CD8^{+} T cells, SOCS-1 transgenic or wild type P14 CD8^{+} T cells were adoptively transferred into congenic mice prior to infection with LCMV WE strain. The expansion of SOCS-1 transgenic P14 T cells in blood and spleen was reduced compared to P14 wild type cells (Figure 7A and data not shown). Whereas effector phenotype, granzyme B and cytokine production were not impaired (Figure S5A-C), we detected enhanced apoptosis of SOCS-1 overexpressing cells (Figure 7B), thus phenocopying *Mir155*$^{-/-}$ CD8^{+} T cells (Figures 2, 3 and S2). To test if suppression of SOCS-1 could be therapeutically exploited to enhance the CD8^{+} T cell anti-tumor response Pmel CD8^{+} T cells transduced with shSOCS-1 were adoptively transferred into tumor-bearing mice. SOCS-1 depletion by the construct was verified by Immunoblot analysis (Figure S5D). An increased expansion of cells expressing shSOCS-1 was detected in the spleen on day 4 compared to control (Figure 7C), associated with profound tumor regression in mice that received shSOCS-1 transduced cells compared to untreated mice or mice treated with control cells (Figure 7D). Together, these results demonstrate that SOCS-1 is negatively regulating the effector CD8^{+} T cell response to virus and cancer and highlight the importance of SOCS-1 downregulation by miR-155 for efficient CD8^{+} T cell responses.

DISCUSSION

We have shown that miR-155 expression is essential for optimal CD8^{+} T cell responses towards virus infection, vaccination and cancer. Interestingly, the phenotype of *Mir155*$^{-/-}$ CD8^{+} T cells was similar to Dicer deficient CD8^{+} T cells (Zhang and Bevan, 2010), suggesting that miR-155 is an important, likely non-redundant miRNA for CD8^{+} effector T cells. Recently, we and others found differential miR-155 expression associated with discrete differentiation stages in CD8^{+} T cells (Almanza et al., 2010; Salaun et al., 2011; Wu et al., 2007). Here, we showed that miR-155 is highly upregulated in effector CD8^{+} T cells responding to viral infection but at intermediate concentration in memory cells. This dynamic modulation of miR-155 raises the question of the nature of the regulating factors that are involved. miR-155 is induced by NF-kappaB dependent factors (Kluiver et al., 2007) and AP-1 downstream of B and T cell receptors (Haasch et al., 2002; Yin et al., 2008). Interestingly, we observed that miR-155 expression was proportional to the strength of TCR signaling, suggesting that miR-155 provides competitive fitness to the most avid antigen-specific CD8^{+} T cells.

Whereas others reported a disadvantage of *Mir155*$^{-/-}$ naive CD8^{+} T cells in bone-marrow chimeras (Lu et al., 2009), we found that expansion and long term survival of naïve *Mir155*$^{-/-}$ CD8^{+} T cells transferred into lymphopenic hosts were not different from wild type cells. miR-155 was crucial for effector CD8^{+} T cell proliferation and survival during the peak of LCMV infection, but it did not influence cell survival upon antigen clearance. In contrast to the reported role of miR-155 in other immune cells, CD8^{+} T cells were not affected in effector functions such as killing or cytokine production and miR-155 deleted mice readily cleared low doses of LCMV. Interestingly, we detected an impaired generation

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of virus-specific central memory CD8+ T cells as most *Mir155-/-* cells displayed a terminally differentiated phenotype. Whether this was due to a lack of CD4+ T cell help (Janssen et al., 2003; Shedlock and Shen, 2003), lower cytokine signaling due to higher SOCS-1 expression and/or additional miR-155 targets will be subject of further investigation. In contrast to low-dose LCMV infection, we found that virus-specific *Mir155-/-* effector cells disappeared during chronic LCMV infection using high doses of clone 13. Under these conditions, wild type CD8+ T cells undergo progressive attrition and display an 'exhausted' phenotype with impaired effector functions and high PD-1 expression (Jin et al., 2010; Mueller and Ahmed, 2009; Wherry et al., 2003). We hypothesize that miR-155 is key for the survival of effector CD8+ T cells in conditions of long-term exposure to antigen and inflammation as found in chronic viral infections or cancer. Consistent with the impaired virus control, lack of miR-155 protected from LCMV-induced severe immunopathology, which is in line with reports demonstrating a pivotal role of miR-155 in autoimmune inflammation (Murugaiyan et al., 2011; O'Connell et al., 2010). With regard to T cell survival, miR-155 was shown to inhibit caspase 3 activity in Jurkat T cells (Ovcharenko et al., 2007), and FADD expression in macrophages (Tili et al., 2007). Whether the increased apoptosis of *Mir155-/-* CD8+ T cells was due to such mechanisms remains to be determined.

In line with our results in virus infection, we demonstrate a central role for miR-155 in tumor specific CD8+ T cells. First, the efficient accumulation of CD8+ T cells by vaccination with adjuvanted peptide required intrinsic miR-155 function, recapitulating our observations in LCMV infection. More importantly, *Mir155-/-* effector cells were severely impaired in their ability to curb tumor growth. Since these cells acquired full effector functions independently of miR-155, the different tumor control was likely due to defect in the magnitude of tumor-specific T cells, or to higher susceptibility to the suppressive tumor-microenvironment (Klebanoff et al., 2011). Conversely, overexpression of miR-155 greatly increased tumor killing by wild type CD8+ T cells. This indicates that tumor specific CD8+ T cell activity is directly dependent on miR-155 expression.

The accumulation of effector CD8+ T cells is influenced by the cytokine milieu, e.g. their initial expansion is promoted by γc cytokines, which signaling is regulated by SOCS-1 (Cornish et al., 2003). More specifically, CD8+ effector cell accumulation in the LCMV response is reduced if cells lack γc signaling (Decaluwe et al., 2010). Interestingly, IL-2 was found to be critical for the maintenance of effector CD8+ T cells in chronic LCMV infections (Bachmann et al., 2007). Here we show that miR-155 enhances cytokine signaling in CD8+ T cells by targeting SOCS-1. Consequently, *Mir155-/-* naïve and effector CD8+ T cells failed to mount physiologic levels of pSTAT5 in response to γc cytokines. In line with *in vitro* experiments using CD4+ T cells (Lu et al., 2009), we observed strong and transient *in vivo* downregulation of SOCS-1 mRNA in virus-specific CD8+ T cells at day 6 of the response. Interestingly, this occurred in a partially miR-155 independent manner, whereas SOCS-1 protein concentrations of naïve and effector CD8+ T cells were found to be clearly dependent on miR-155. Moreover, we did not detect an impact of miR-155 on pSTAT5 in response to IL-2 in the early effector response *in vivo* as well as after *in vitro* priming (unpublished data), whereas the effect was pronounced in naïve and late effector cells. This suggests a miR-155 independent mechanism allowing full cytokine signaling early in the effector response, whereas miR-155 is modulating cytokine signaling in naïve and late effector cells, promoting their accumulation and survival. Interestingly, at the peak of the response, effector CD8+ T cells are reportedly dependent on IL-2 and IL-15 for sustained expansion (D'Souza and Lefrancois, 2003; Sanjabi et al., 2009). We were able to confirm a SOCS-1 dependent inhibition of cytokine signaling in *Mir155-/-* T cells by transduction with a shSOCS-1 lentivirus in *in vitro* experiments. Although the formal proof that loss of SOCS-1 in *Mir155-/-* CD8+ T cells *in vivo* would restore their function remains to be shown, the observed rescue of STAT5 phosphorylation in *Mir155-/-* cells back to wild type levels

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suggests that suppression of SOCS-1 in wild type but not *Mir155*$^{-/-}$ T cells caused the differences in pSTAT5 signaling. This hypothesis was further supported by our observation that virus specific, SOCS-1 transgenic CD8$^+$ T cells fully differentiated but failed to accumulate to normal numbers at the peak of the LCMV response, which mirrors the phenotype of *Mir155*$^{-/-}$ T cells and identifies SOCS-1 as an important regulator of CD8$^+$ T cell responses *in vivo*. Conversely, suppression of SOCS-1 in tumor specific cells increased the accumulation of transferred cells and subsequently was highly therapeutic in limiting growth of established melanoma. Thus, although a negative role for SOCS-1 in CD8$^+$ T cell responses has been suggested before (Chong et al., 2003; Cornish et al., 2003; Davey et al., 2005; Marine et al., 1999; Palmer and Restifo, 2009), we here demonstrate a cell-intrinsic role of SOCS-1 in responses to virus and cancer. Our results of the highly dynamic regulation of SOCS-1 expression *in vivo* and the strong impact of SOCS-1 alterations in responses to virus and tumor suggests that a major part of the effects caused in CD8$^+$ T cells by miR-155 deletion are due to increased SOCS-1 expression.

In summary, the results presented here identified a crucial cell-intrinsic role of miR-155 and its target SOCS-1 in effector CD8$^+$ T cells, and demonstrated that this miRNA is required for an optimal CTL response to both virus and tumor. Moreover, miR-155 overexpression in tumor specific CD8$^+$ T cells substantially increased their potency, thus providing strong evidence for a clinical potential in the context of therapeutic adoptive T cell transfer.

EXPERIMENTAL PROCEDURES

Mice

C57BL/6 mice were from Harlan. *Mir155*$^{-/-}$ and *Rag2*$^{-/-}$*Il2rg*$^{-/-}$, TCR transgenic OT-I and P14 mice bearing a transgenic TCR specific for $K^b/OVA_{257-264}$ or LCMV D^b/GP_{33-41} MHC-I/peptide complexes, respectively, were from Jackson and were bred under specific pathogen free conditions. P14xSOCS-1 transgenic (Seki et al., 2007) and Pmel-1 transgenic mice expressing a TCR specific for an H-2D^b-restricted CD8$^+$ T cells epitope from the murine melanoma tumor antigen $gp100_{25-33}$ or human $gp100_{25-33}$ have been described (Overwijk et al., 2003). All animal experiments were conducted with the approval of the Lemanic Animal Facility Network (RESAL), NCI (protocol SB-126) or NIAID (protocol LI-10) Animal Use and Care Committees.

Quantitative PCR

Total RNA from subsets was extracted with the miRVana kit (Ambion), and mature microRNAs (miR-155 and controls RNU44 and snoRNA202) were reverse transcribed with TaqMan RT MicroRNA kit (Applied Biosystems) and amplified using Universal Fast Start Rox Probe Master Mix (Roche) and microRNA assay kits in 384 well plates (Applied Biosystems) on an ABI Prism 7900 HT device (Applied Biosystems). RT-PCR for *Socs1* was performed with primers from Applied Biosystems. Gene expression was calculated relative to G6PDX.

In vitro priming with dendritic cells

T cells were purified from lymphoid tissues using anti-CD8α beads (Miltenyi) and cocultured with peptide pulsed *Mir155*$^{-/-}$ DC. In competition experiments, equal numbers of WT and *Mir155*$^{-/-}$ OT-1 CD8$^+$ T cells were cocultured with WT DC.

Differential stimulation of human CD8$^+$ T cells and microarray

Human T cells transduced with NY-ESO-1 TCR variants (Schmid et al., 2010) were stimulated with 0.01μg/ml (for miR-155 quantification) or 0.002 μg/ml (for BIC microarray) NY-ESO-$1_{157-165}$ multimer. BIC quantification was done by Miltenyi Biotec.

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LCMV infection

Mice were infected intravenously with 200 pfu of the WE strain or 2×10^6 pfu clone 13 of LCMV. Cell counts in blood were performed with TrueCount tubes according to the manufacturer's instructions (BD). Viral titrations were performed by a standard plaque assay.

Cytokine and cytotoxicity assays

Splenocytes were stimulated with indicated peptides or a mix of LCMV peptides (gp33, gp70, gp92, gp118, gp276, np166, np205, np235, np396) at 5 μM each or PMA (50 ng/mL) and Ionomycin (500 ng/mL) in the presence of GolgiStop (BD Pharmingen) for analysis in flow cytometry. Cytolytic activity was assessed using the chromium release assay with EL4 pulsed with 1 μM $GP_{33\text{-}41}$ peptide and measured with a TopCount reader (Canberra Packard).

Adoptive cell transfers

Before LCMV infection, $CD8^+$ T cells were purified by magnetic beads sorting (Miltenyi Biotec) and 1×10^6 purified $CD8^+$ T cells were injected into the tail vein. In the P14xSOCS-1 experiments, 3×10^4 cells were injected normalized to gp33 tetramer positive populations. For vaccination and tumor challenge, OT-1cells were purified and 1×10^6 cells were injected intravenously three days before subdermal injection of 1×10^5 B16 melanoma cells expressing OVA. 7 days later, mice were vaccinated s.c. with 25 μg $OVA_{257\text{-}264}$ peptide and 50 μg CPG-ODN in PBS.

T cell transduction and transfers into tumor bearing mice

Pmel $CD8^+$ T cells were stimulated with anti-CD3 (2μg/ml), anti-CD28 (1μg/ml) for 24h, transduced with retrovirus expressing miR-155 or scrambled miR and expanded for 4 days. To knock down SOCS-1, pmel $CD8^+$ T cells were transduced 2 days after stimulation with lentivirus expressing shSOCS-1 or empty vector and selected in puromycin from days 4-7 prior to transfer into tumor-bearing hosts. 10^7 cells (miR-155 experiments) or 10^6 (shSOCS-1 experiments) were injected i.v. into mice bearing B16 tumor in conjunction with 2×10^7 pfu rvvhgp100 and IL-2 (6X6e4 cu). In shSOCS-1 experiments, mice were sublethally irradiated prior to cell transfer. Tumor volumes were plotted as the product of perpendicular diameters. For rescue of pSTAT5 in miR-155 cells, wild type and *Mir155*$^{-/-}$ $CD8^+$ T cells were transduced with lentivirus encoding shSOCS-1 or control sh and cultured for one week with IL-2 or IL-15 (R&D systems, 20 ng/ml) before cytokine stimulation (vector sequences upon request).

Flow Cytometry and pSTAT5 measurements

Cells were stained with antibodies of the indicated specificities (eBioscience) or H-2 D^b/ peptide-loaded MHC. PD-1 PE-Cy7 antibody was from Biolegend. For detection of pSTAT5, cells were stimulated with indicated cytokines (R&D systems) for 18 min and fixed with 0.5% formaldehyde in PBS for 15 min. Upon washing in medium containing 10% FCS, cells were permeabilized in 80% methanol for 20 min, washed and stained with pSTAT5 antibody (BD biosciences) and additional markers (eBiosciences). Labeled cells were analysed on LSR-II (Becton Dickinson).

Immunoblot

Cells were lysed (Cell Signaling Technologies) and immunoblotting was performed using Bio-Rad TGX reagents and protocols on nitrocellulose paper, incubated with antibodies against SOCS-1 (Lifespan Biosciences (Figure 6C) or Imgenex (Figure S6)) and with appropriate HRP-conjugated secondary antibodies (Cell Signal Technologies). Blots were

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developed using chemiluminescence (Pierce), gel images were captured with Gel Doc XRS (Bio-Rad) and densitometry evaluated using Quantity One software (Bio-Rad).

Proliferation and Apoptosis assay

Mice were injected i.p. with 200 μg BrdU and proliferation was measured in spleen cells 1h (P14xSOCS-1 experiments) or 4h (miR-155 experiments) later by staining with anti-BrdU and anti-ki67^{+} antibodies. Apoptotic cells were detected by staining splenocytes for AnnexinV upon 1h *in vitro* incubation at 37 °C (all BD Pharmingen).

Statistics

The two-tailed student's T test was used to compare two groups, multiple groups' comparisons were performed with one way analysis of variance (ANOVA) corrected with Bonferroni's multiple comparison test factor. Statistical survival differences in the tumor experiments were analyzed with the Log-Rank test. Statistical significance is displayed as *: p<0,05, **: p<0,01, ***: p<0,001. Data are displayed as Mean and standard error of mean (SEM) if not indicated otherwise.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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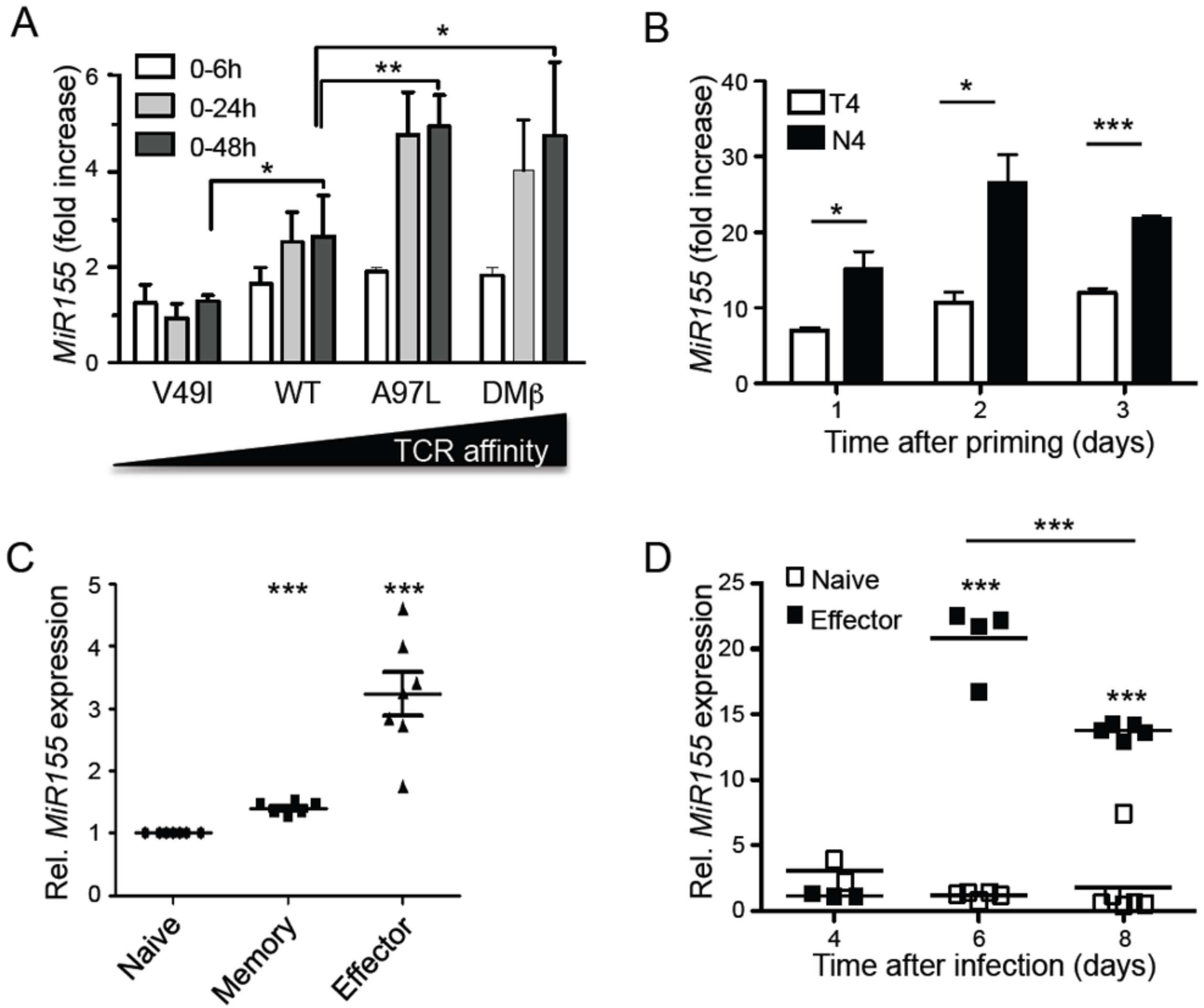

Figure 1. miR-155 expression is regulated at various stages of CD8$^+$ T cell differentiation in a TCR- affinity dependent manner

(A) Quantification of miR-155 in human CD8$^+$ T cells transduced with TCRs of increasing affinity following TCR stimulation with multimers (N=3 experiments) as fold increase relative to unstimulated clones. (B) miR-155 expression of naïve mouse OT-1 T cells stimulated with splenic dendritic cells pulsed with the natural SIINFEKL (N4) or weaker SIITFEKL (T4) altered peptide ligand as relative to day 0 unstimulated cells. Data are representative for triplicates in one out of two experiments. (C) miR-155 concentrations in naïve, central memory and effector CD8$^+$ T cells sorted at day 8 after LCMV WE infection as fold change relative to naïve cells. (D) Relative miR-155 expression in splenic naive and effector CD8$^+$ T cells sorted from LCMV infected mice. Symbols represent individual mice and the line is the mean +/- SEM. Data are representative for 2 independent experiments. For human BIC expression please also see Figure S1.

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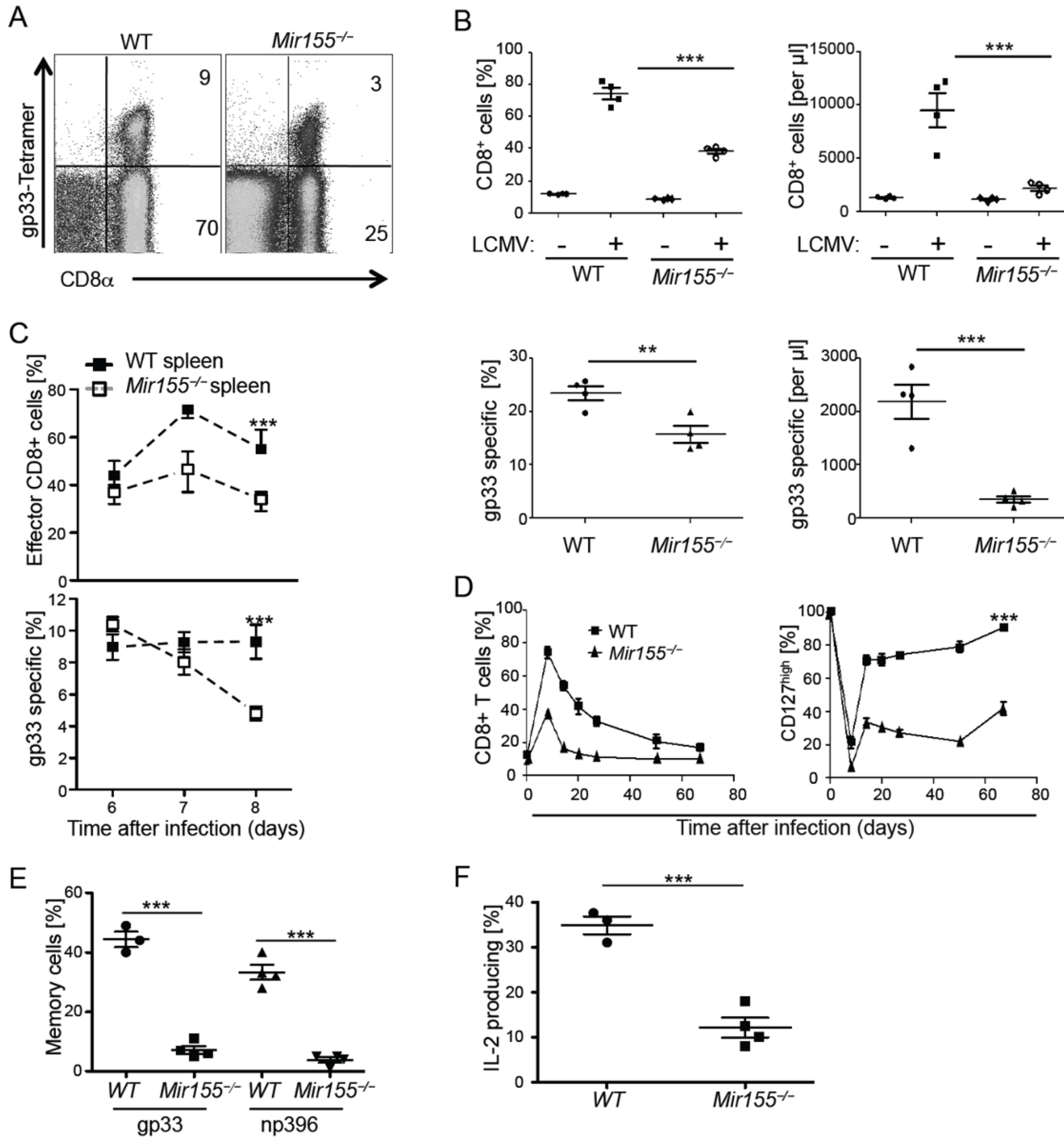

Figure 2. miR-155 is required for optimal effector CD8+ T cell accumulation and memory cell differentiation during acute LCMV infection

(A) Splenocytes from day 8 infected wild type (WT) and *Mir155-/-* mice were stained with K^b/LCMV gp33 tetramers and anti-CD8α. (B) Blood percentages and numbers of CD8+ (upper panels) and gp33 tetramer+ cells (lower panels) on day 8 of infection. (C) Percentages of CD44^high CD62L^low effector CD8+ T cells gated on lymphocytes in wild type (WT) and *Mir155-/-* spleen cells (upper) and of gp33 tetramer+ cells within the CD8+ T cells (lower panel) at days 6 to 8. (D) Percentages of total CD8+ T cells (left) and CD127+ cells within tetramer gp33+ CD8+ cells (right panel) in blood at given time points. (E) Percentage of liver CD127^high CD62L^high tetramer gp33 and np396+ memory cells and (F) IL-2 production upon gp33 peptide restimulation of splenocytes within IFN-γ postitive CD8+ T cells at 3 months past infection. Symbols represent individual mice, and the line is the mean +/- SEM. Representative results of one out of four (A-C) to two (D-F) independent experiments with three to five mice are pictured. Please also see Figure S2.

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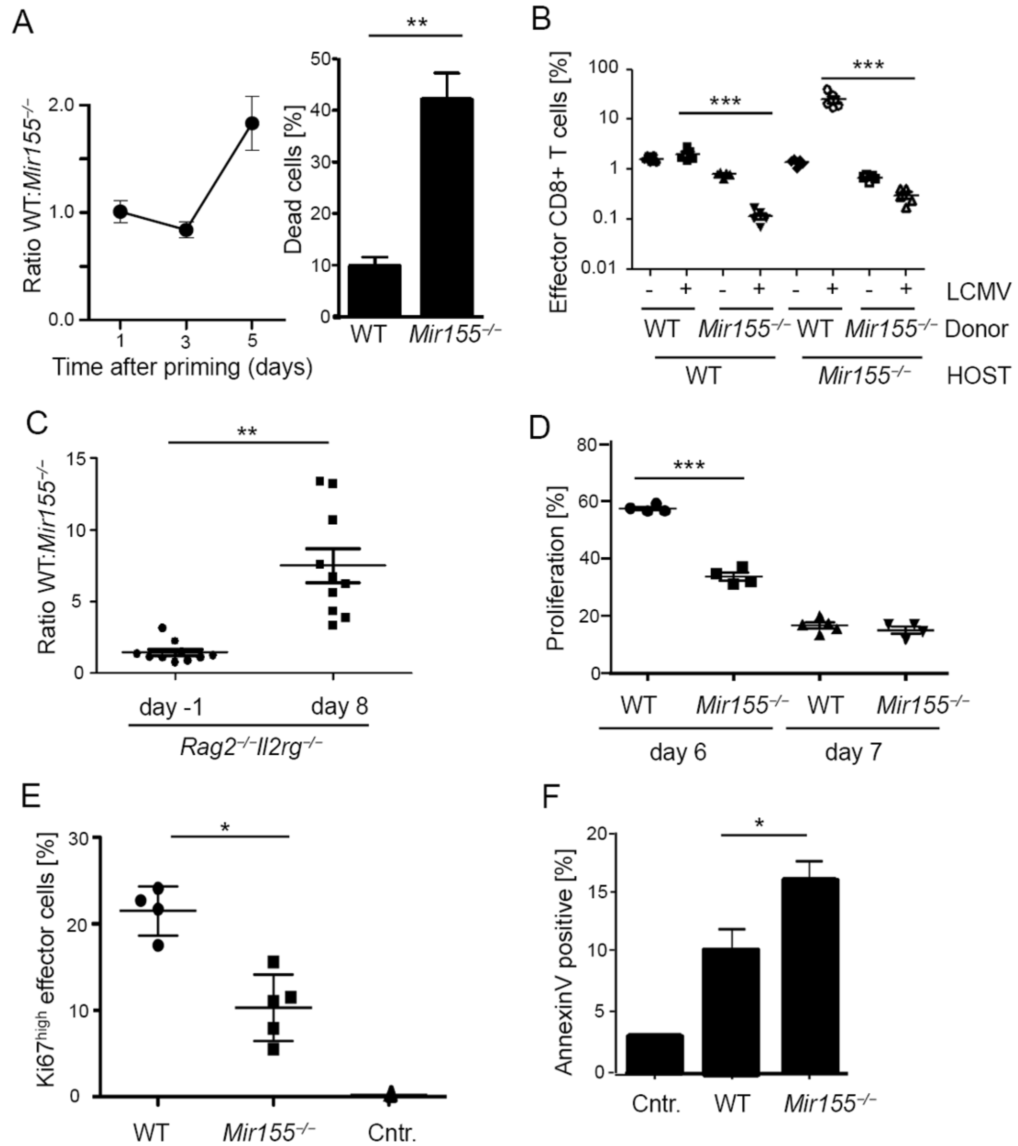

Figure 3. A cell intrinsic role for miR-155 in promoting effector CD8^{+}T cells

(A) Congenically marked wild type (WT) and *Mir155*$^{-/-}$ OT-1 cells were competitively cocultured with peptide pulsed dendritic cells and the ratio of populations is pictured at indicated time points (left). On day 5, percentage of trypan blue cells harvested from either WT or *Mir155*$^{-/-}$ cultures was counted (right graph). Pooled data from three representative experiments are pictured. (B) CD8^{+} T cells from WT and *Mir155*$^{-/-}$ mice were cotransferred into WT or deficient hosts before LCMV WE infection and percentages in blood at day 8 were measured. (C) A 1:1 mix of WT and *Mir155*$^{-/-}$ splenocytes was adoptively transferred into Rag2 and IL2Rγ double deficient mice which were infected with LCMV WE two months after transfer. CD8^{+} effector T cell ratios at days -1 and 8 post infection are pictured. (D) Proliferating BrdU positive splenic CD44^{high} CD8^{+} effector T cells at days 6 and 7 post infection. (E) At the same time, cells were stained for the proliferation marker Ki67 (day7) and (F) apoptotic cells were identified by AnnexinV staining. Symbols represent individual mice, and the line is the mean +/- SEM. Representative results from two (B, C) to three (D-F) experiments are pictured.

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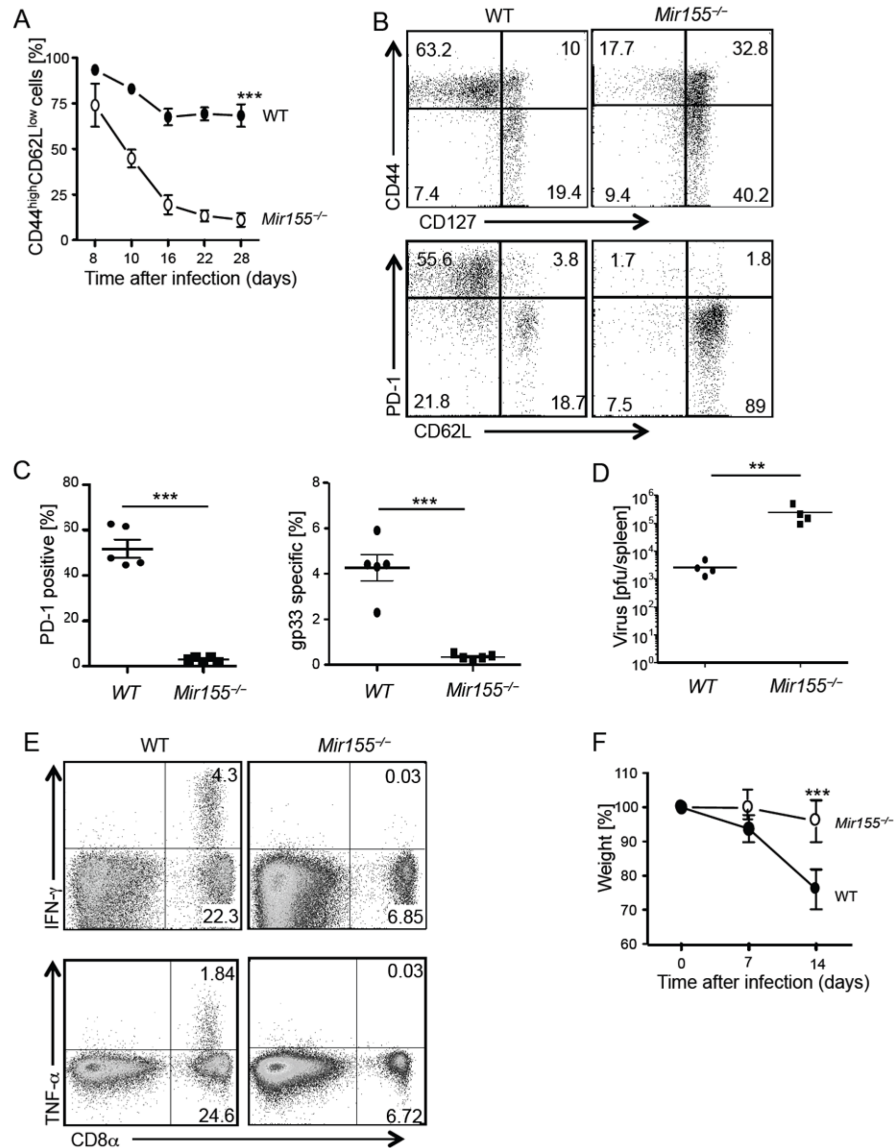

Figure 4. miR-155 drives survival of effector cells and sustains the anti-viral response in a chronic LCMV infection

Wild type (WT) and *Mir155*$^{-/-}$ mice were infected with LCMV clone 13 and phenotype and expansion of effector cells was monitored by flow cytometry. (A) Effector CD44^{high}CD62L^{low} within blood CD8^{+} T cells at indicated time points. (B, C) At five weeks, spleenic CD8^{+} cells were analysed for the indicated activation markers and gp33 tetramer^{+} cells shown as (B) flow cytometry dot blots from representative mice and (C) graph from one representative out of three experiments. (D) Virus titer and (E) cytokine response upon stimulation with a peptide cocktail was determined in the blood at 2 and 3 months after infection, respectively. (F) Weight of mice was monitored the first 2 weeks post-infection. Symbols represent individual mice, and the line is the mean (C, D). Error bars are given as +/- SEM. Shown are representative results from one out of two experiments with three to five mice per group.

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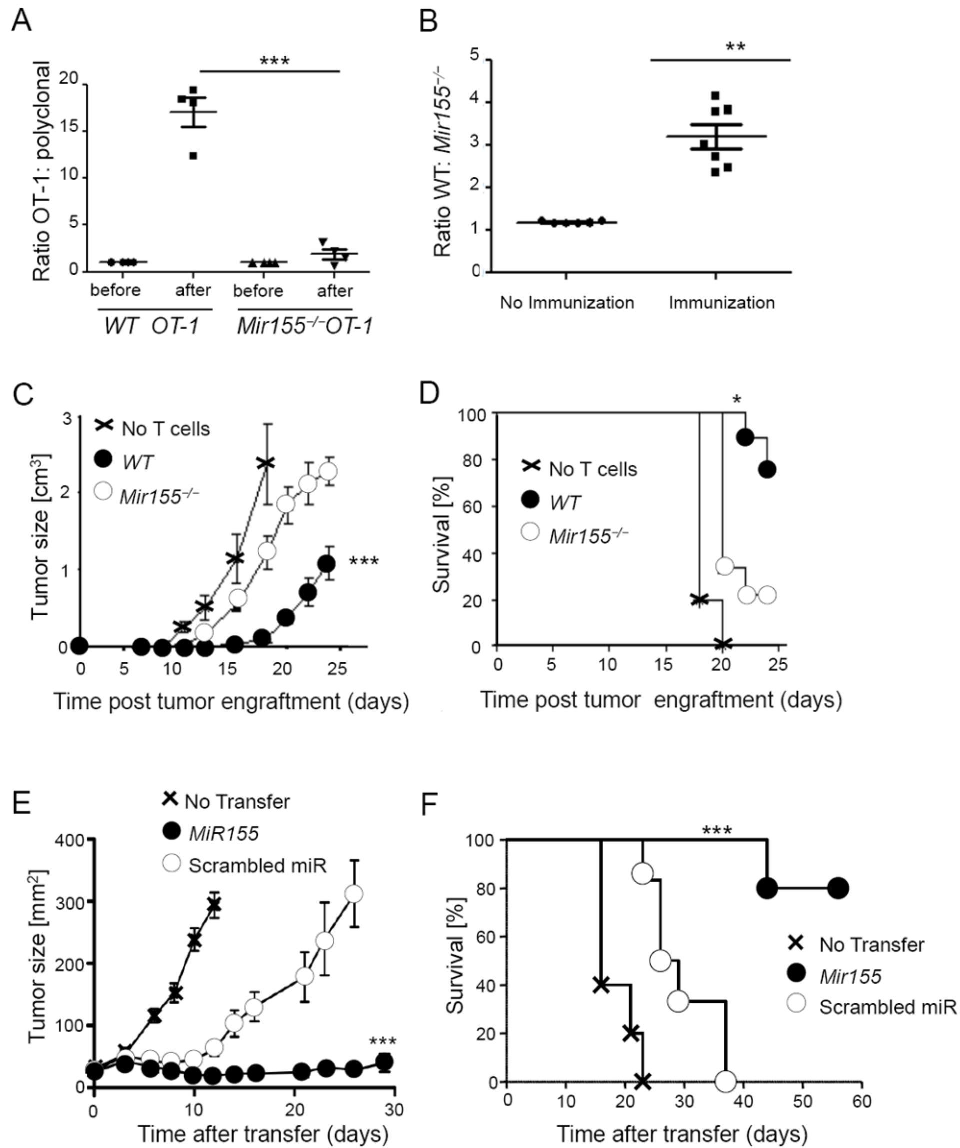

Figure 5. miR-155 is crucial for the CD8$^+$ T cell response to peptide vaccination and tumor challenge

(A) Congenically marked OT-1 and polyclonal CD8$^+$ T cells from wild type (WT) or *Mir155*$^{-/-}$ backgrounds were transferred into WT hosts, which were immunized with OVA peptide and CpG in IFA. Shown is the ratio of antigen specific versus polyclonal cells in the draining lymph nodes 4 days later. Symbols represent individual mice, and the line is the mean +/- SEM. (B) Ratio of blood WT and *Mir155*$^{-/-}$ OT-1 CD8+ T cells cotransferred into WT hosts, which were immunized as in (A) on day 7. Symbols represent individual mice, and the line is the mean +/- SEM. Data from 1 out of 2 (A) to 3 (B) independent experiments are pictured. (C, D) Upon adoptive transfer of WT or *Mir155*$^{-/-}$ OT-1 cells and immunization, mice were engrafted with B16 melanoma cells expressing OVA. Tumor growth (C) and survival (D) are pictured (n=5-9 mice per group, data from 1 out of 2 independent experiments) displayed as mean +/- SEM. (E, F) TCR transgenic pmel CD8$^+$ T overexpressing miR-155 or control scrambled miR were transferred into B16 tumor-bearing mice and (E) tumor growth and (F) survival of mice are shown from one representative out

of two experiments with five to eight mice per group displayed as mean +/- SEM. Please also see Figure S3.

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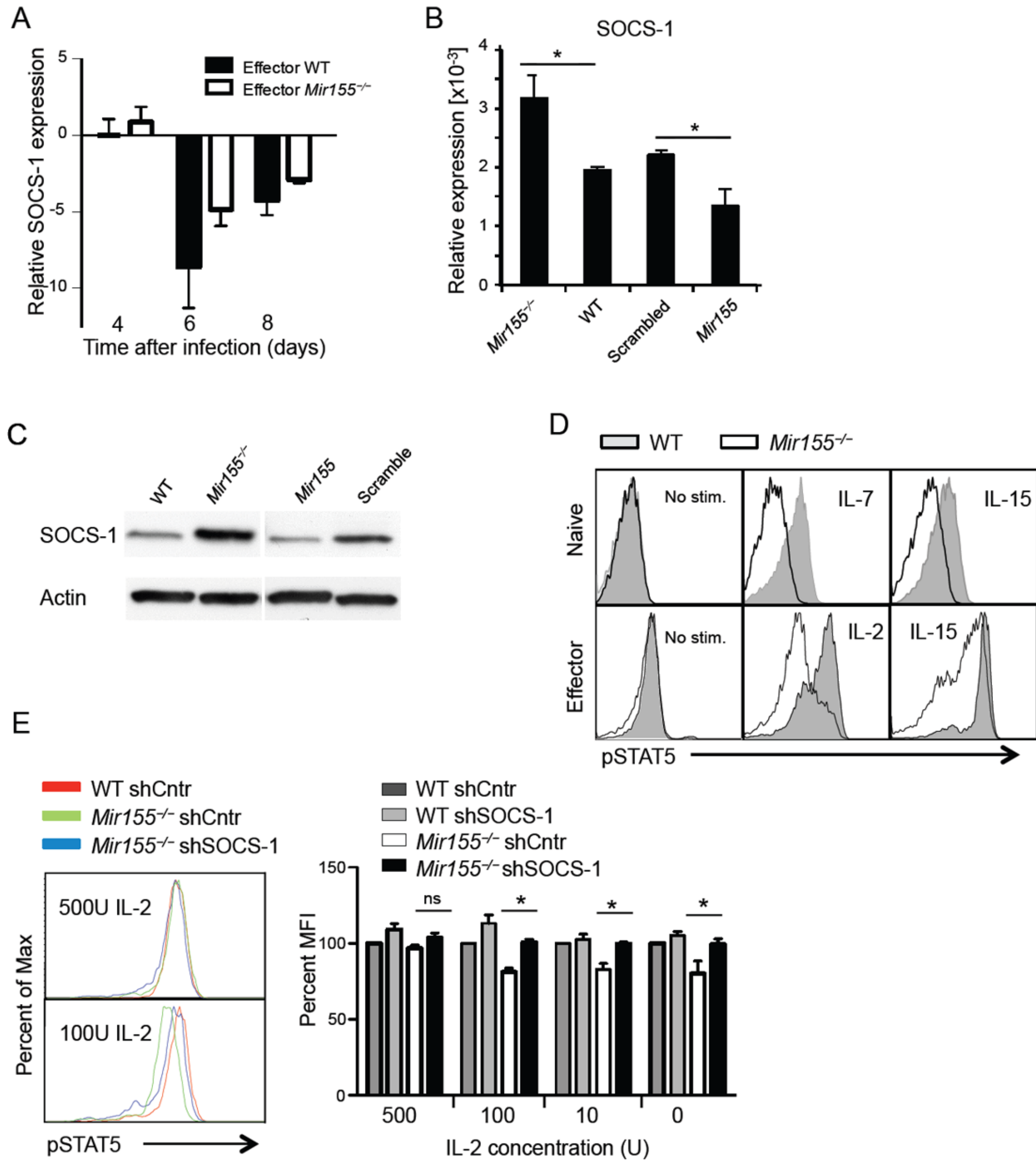

Figure 6. SOCS-1 and miR-155 modulate the antiviral CD8$^+$ T cell response and cytokine signaling

(A) SOCS-1 mRNA concentrations were measured upon LCMV WE infection in purified effector (CD44^{high}CD62L^{low}) wild type (WT) and *Mir155*$^{-/-}$ splenic CD8$^+$ T cells by qPCR relative to naïve CD8$^+$ T cells from non-infected mice. (B, C) Regulation of SOCS-1 by miR-155 in naïve CD8$^+$ T cells from WT and miR-155 deleted mice as well as after retroviral transfection with miR-155 overexpressing or control vectors was tested by (B) qPCR (shown as relative to β-actin) and (C) Immunoblot. (D) Naïve or effector T cells from LCMV infected mice were stimulated with indicated cytokines and pSTAT5 was measured by flow cytometry. (E) WT and *Mir155*$^{-/-}$ T cells were transduced with control or shSOCS-1 lentivirus and pSTAT5 response to IL-2 is shown. The table gives the percentages of MFI normalized to the MFI measured in wild type sh-control cells set to 100%. Representative data from two (A-C) to three (D, E) experiments are pictured as mean +/- SEM. Please also see Figure S4.

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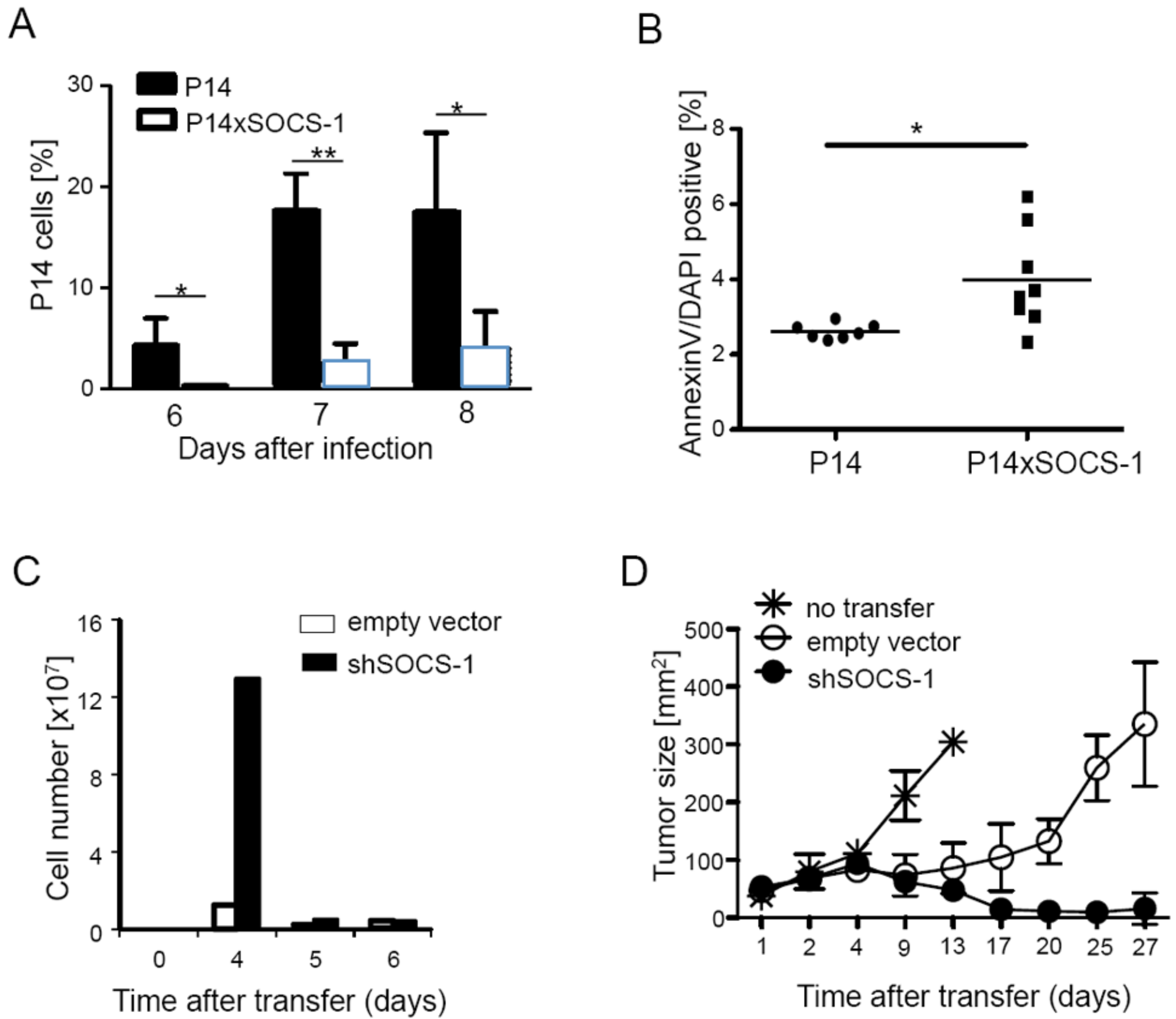

Figure 7. SOCS-1 limits the CD8$^+$ T cell response to virus and cancer

(A) TCR transgenic P14 CD8$^+$ T cells overexpressing SOCS-1 (P14×SOCS-1) or not were adoptively transferred before LCMV WE infection. Data show the percentage of transferred cells in the lymphocyte gate at days 6, 7 and 8 post infection as mean +/- SEM; (B) Apoptotic cells within P14 T cells 7 days upon infection. Symbols represent single mice and the line is the mean. (C, D) TCR transgenic pmel CD8$^+$ T cells were transduced with a retrovirus encoding for a scrambled control or shSOCS-1 mRNA and adoptively transferred into tumor bearing mice. (C) Absolute numbers of donor CD8$^+$ T cells were determined in spleen at days 4 to 6 after adoptive transfer and (D) tumor size of mice was monitored. Data are from one representative out of two independent experiments with two (C) to five mice (D) per group and displayed as mean +/- SEM. Please also see Figure S5.