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Long-Term Persistence of Exhausted CD8 T Cells in Chronic Infection Is Regulated by MicroRNA-155

Erietta Stelekati[1], **Zeyu Chen**[1], **Sasikanth Manne**[1], **Makoto Kurachi**[1], **Mohammed-Alkhatim Ali**[1], **Keith Lewy**[1], **Zhangying Cai**[1,2], **Kito Nzingha**[1], **Laura M. McLane**[1], **Jennifer L. Hope**[3,4], **Adam J. Fike**[3], **Peter D. Katsikis**[4], and **E. John Wherry**[1,5,*]

[1]Department of Microbiology and Institute for Immunology, University of Pennsylvania Perelman School Medicine, Philadelphia, PA 19104, USA [2]College of Life Sciences, Peking University, Beijing, China [3]Department of Microbiology and Immunology, Drexel University College of Medicine, University of Pennsylvania, Philadelphia, PA, USA [4]Department of Immunology, Erasmus MC University Medical Center, Rotterdam, the Netherlands

SUMMARY

Persistent viral infections and tumors drive development of exhausted T (T_{EX}) cells. In these settings, T_{EX} cells establish an important host-pathogen or host-tumor stalemate. However, T_{EX} cells erode over time, leading to loss of pathogen or cancer containment. We identified microRNA (miR)-155 as a key regulator of sustained T_{EX} cell responses during chronic lymphocytic choriomeningitis virus (LCMV) infection. Genetic deficiency of miR-155 ablated CD8 T cell responses during chronic infection. Conversely, enhanced miR-155 expression promoted expansion and long-term persistence of T_{EX} cells. However, rather than strictly antagonizing exhaustion, miR-155 promoted a terminal T_{EX} cell subset. Transcriptional profiling identified coordinated control of cell signaling and transcription factor pathways, including the key AP-1 family member Fosl2. Overexpression of Fosl2 reversed the miR-155 effects, identifying a link between miR-155 and the AP-1 transcriptional program in regulating T_{EX} cells. Thus, we identify a mechanism of miR-155 regulation of T_{EX} cells and a key role for Fosl2 in T cell exhaustion.

Graphical abstract

In Brief During persistent viral infections, exhausted T cells (T_{EX}) erode quantitatively and qualitatively and therefore fail to provide protection. Stelekati et al. identified microRNA

*Correspondence: wherry@pennmedicine.upenn.edu.
[5]Lead Contact

SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

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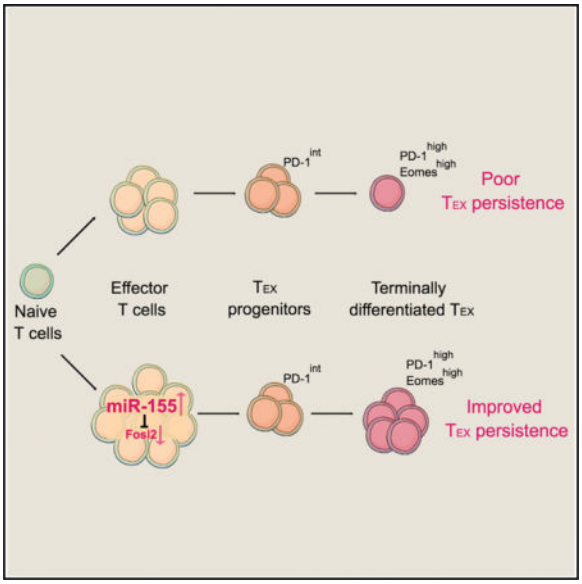
DECLARATION OF INTERESTS

E.J.W. has a patent licensing agreement on the PD-1 pathway.

(miR)-155 as a key molecule that can enhance and sustain T_{EX} responses long-term during chronic viral infection.



INTRODUCTION

Persistent infections and tumors result in the development of dysfunctional T cells, a process driven by chronic antigen stimulation and unresolved inflammation. This T cell exhaustion is characterized by increased expression of inhibitory molecules, reduced cytokine production, an altered transcriptional program, metabolic changes, and failure to persist long-term (Wherry et al., 2003; Wherry and Kurachi, 2015). Although exhausted T (T_{EX}) cells have suboptimal function compared with effector T (T_{EFF}) and memory T (T_{MEM}) cells, T_{EX} cells often establish a host pathogen or host tumor stalemate, partially controlling the pathogen or tumor and/or driving immune selection of viral or tumor variants (Wherry and Kurachi, 2015; Jamieson et al., 2003; Paley et al., 2012). However, in prolonged chronic infections in mice (Wherry et al., 2003; Zajac et al., 1998) and humans (Bengsch et al., 2010; Day et al., 2006; Gruener et al., 2001), T_{EX} cells can quantitatively and qualitatively erode over time, leading to loss of pathogen or tumor containment. Thus, understanding the pathways that underlie the durability and sustainability of T_{EX} cells could have important implications for containing or controlling persistent intracellular pathogens and cancer.

During chronic infections, T_{EX} cells lose proliferative potential and respond poorly to memory self-renewal signals like interleukin-7 (IL-7) and IL-15 (Shin et al., 2007). Human data also support the notion that the durability of T_{EX} cells may be compromised during chronic infections. Therapies (such as antiretroviral therapy for HIV) may increase the proliferative potential of T_{EX} cells; however the half-life of these cells remains substantially reduced compared with healthy CD8 T cells (Hellerstein et al., 1999), demonstrating a pervasive effect on the durability of T cells generated during chronic infection. Similarly, for hepatitis C virus (HCV), prolonged infection is associated with an inability to sustain virus-specific CD8 T cells (Lechner et al., 2000). Checkpoint blockade targeting inhibitory

receptor pathways such as PD-1 can reinvigorate T_{EX} cells (Barber et al., 2006), providing considerable benefits in chronic infection (Trautmann et al., 2006; Velu et al., 2009) and in cancer patients (Brahmer et al., 2012; Topalian et al., 2012). However, recent studies have demonstrated that checkpoint blockade alone does not necessarily improve the durability of "reinvigorated" T_{EX} cells (Pauken et al., 2016). These observations are consistent with the lack of long-term clinical benefits in many patients upon administration of checkpoint inhibitor blockade (Pauken and Wherry, 2015; Sharma and Allison, 2015). Moreover, prolonged chronic infection leads to progressive erosion of T_{EX} cell populations (Paley et al., 2012), and the response to PD-1 blockade also declines over time (Penaloza-MacMaster et al., 2015), indicating a poor ability to sustain T_{EX} cell responses. Thus, understanding the pathways regulating the durability of T_{EX} cells and developing strategies to enhance their persistence could aid in disease control.

We have previously defined subsets of T_{EX} cells based on expression of inhibitory receptors (Blackburn et al., 2009) or the transcription factors T-bet and Eomes (Paley et al., 2012). These data indicate that the T_{EX} cell population is maintained by a proliferative hierarchy of two interrelated subsets. A progenitor subset exists that, in the presence of persisting antigen, gives rise through extensive proliferation to a terminal PD-1^{Hi} subset (Paley et al., 2012). Recent studies have also described subsets based on CXCR5 and TCF-1 expression, with TCF-1 playing a key role in the progenitor subset (He et al., 2016; Im et al., 2016; Leong et al., 2016; Utzschneider et al., 2016; Wu et al., 2016). The terminal $Eomes^{Hi}$ PD-1^{Hi} subset of T_{EX} cells has a short half-life *in vivo*, suggesting constant repopulation from the progenitor pool, but the maintenance of this population remains poorly understood. Both subsets are essential to maintain control of chronic infections (Paley et al., 2012). Because the terminal subset of T_{EX} cells is numerically more abundant, understanding the mechanisms that maintain this population could aid in developing strategies for control of persisting infections and tumors.

Recent studies have focused on the role of inhibitory receptors, cytokine signaling pathways, transcriptional networks, and metabolic changes that regulate T_{EX} cells (Wherry and Kurachi, 2015). The role of non-coding RNAs, however, remains largely unknown. MicroRNAs (miRNAs) regulate many aspects of immune system biology and T cell responses (Mehta and Baltimore, 2016). These small non-coding RNAs can simultaneously downregulate several mRNA targets based on complementarity and, therefore, have a prominent effect on a wide range of biological functions. However, the selective transcription of mRNAs in different cell types results in different availability of target mRNAs. As a result, miRNAs can have diverse effects based on the cell type and cell state examined (Lu et al., 2015). Although there has been considerable effort to understand the role of specific miRNAs in the immune system and, to a lesser extent, in T cells (Moffett et al., 2017), there is little information about the role of specific miRs in T_{EX} cells.

miR-155 has a well-documented role in regulating T cell responses (Lind and Ohashi, 2014). miR-155 enhances CD8 T cell expansion during acute infections and cancer (Gracias et al., 2013; Lind et al., 2013; Lind and Ohashi, 2014; Tsai et al., 2013) and regulates responses to inflammatory and cytokine signals in different settings, such as acutely resolved viral and bacterial infections (Gracias et al., 2013), tumor models (Ji et al., 2015), autoimmunity

(O'Connell et al., 2010), and age-dependent inflammation (Hu et al., 2014). Expression of miR-155 is increased in HIV infection, and higher expression of miR-155 correlates with increased disease (Witwer et al., 2012). Despite this previous work, it is unclear whether miR-155 has a role in T_{EX} cells during chronic infections and, if so, how miR-155 functions in this setting.

In this study, we investigated whether miR-155 affects CD8 T cell responses to chronic infection and specifically interrogated how miR-155 affects the differentiation of T_{EX} cells. Using T cell adoptive transfers, we demonstrated that genetic deletion of miR-155 reduced CD8 T cell expansion during chronic infection in a dose-dependent manner. In contrast, overexpression of miR-155 in virus-specific CD8 T cells enhanced expansion and long-term persistence during chronic lymphocytic choriomeningitis virus (LCMV) infection. However, this effect during chronic infection was not because miR-155 antagonized exhaustion; rather, miR-155 fostered the development, accumulation, and long-term durability of a large population of terminally differentiated PD-1^{Hi}Eomes^{Hi} T_{EX} cells. Transcriptional profiling revealed several pathways involved in cell differentiation, proliferation, chemotaxis, inflammation, and transcriptional regulation affected by miR-155, including the AP-1 transcription factor pathway. We further demonstrated that the effect of miR-155 on CD8 T cells in chronic infection was reversed by overexpression of the AP-1 family transcription factor Fosl2, demonstrating a key mechanistic link between miR-155 and antagonism of Fosl2 in controlling the accumulation and persistence of terminal T_{EX} cells. Thus, these data support a role for miR-155 in T_{EX} cells through regulation of the AP-1 transcriptional pathway and identify Fosl2 as a key AP-1 family member in exhausted CD8 T cells.

RESULTS

miR-155 Expression Is Elevated in T_{EX} Cells during Chronic Viral Infection

We have previously shown that absence of miR-155 is detrimental to effector CD8 T cell responses during acute infections (Gracias et al., 2013). Here we investigated the role of miR-155 in chronic LCMV infection. We first examined the expression of miR-155 in LCMV-specific CD8 T cells during the development of exhaustion following persisting infection with LCMV clone 13. To this end, we adoptively transferred T cell receptor (TCR) transgenic CD8 T cells specific for the immunodominant LCMV D^b-$gp^{33–41}$ epitope (P14 cells) to congenically distinct naive recipient mice and infected these mice with LCMV clone 13 (Figure S1A). Donor P14 CD8 T cells were sorted, and miR-155 expression was examined by qRT-PCR. Expression of miR-155 was increased ~30-fold in P14 CD8 T cells 8 days post infection (d.p.i.) compared with naive CD8 T cells (Figure S1B), similar to what was observed previously in acute infection (Gracias et al., 2013). However, unlike acute infections, where miR-155 expression declines in memory CD8 T cells (Gracias et al., 2013), miR-155 expression remained elevated 22 and 35 d.p.i. (Figure S1B). These observations suggested a role for miR-155 in developing T_{EX} cells during chronic viral infection. PD-1^{Int} and PD-1^{Hi} subsets of T_{EX} cells were sorted 22 d.p.i. PD-1^{Hi} terminal T_{EX} cells expressed approximately twice as much miR-155 as the PD-1^{Int} progenitor subset (Figure S1B). Thus, miR-155 was highly expressed in T_{EX} cells and associated with more terminal exhaustion.

miR-155 Expression Is Essential for CD8 T Cell Responses during Chronic Viral Infection

To interrogate whether miR-155 regulated T_{EX} cell differentiation, we adoptively transferred congenically marked wild-type (WT), miR-155-haplodeficient (heterozygous [HET]), and miR-155-deficient (knockout [KO]) P14 cells into wild-type recipients and analyzed their response to LCMV clone 13 infection (Figure 1A). miR-155 deficiency conferred a dose-dependent defect on CD8 T cell expansion upon clone 13 infection (Figure 1B), consistent with the role of miR-155 in the expansion of CD8 T cells during acute viral and bacterial infections (Gracias et al., 2013; Lind and Ohashi, 2014; Tsai et al., 2013).

Persistent infections result in the generation of T_{EX} cells that are defined in part by high expression of inhibitory receptors (Blackburn et al., 2009). Therefore, we asked whether miR-155 deficiency altered the expression of inhibitory receptors on developing T_{EX} cells. Because of the dramatically reduced numbers of surviving P14 cells in the absence of miR-155 (KO; Figure 1B), we investigated the phenotype of heterozygous (HET) P14 cells. Reduced miR-155 was associated with decreased expression of PD-1 and CD160 (Figure 1C) on adoptively transferred P14 CD8 T cells. Expression of these inhibitory receptors on the host (WT) CD8 T cells was indistinguishable between the two groups (Figure 1C). These results suggest a CD8 T cell-intrinsic role for miR-155 in promoting exhaustion of CD8 T cells.

miR-155 Overexpression Results in Enhanced Expansion and Long-Term Persistence of CD8 T Cells during Chronic Viral Infection

The decreased CD8 T cell expansion in the absence of miR-155 (Figure 1) suggested that forced expression of miR-155 could promote CD8 T cell responses to a chronic infection. To test this idea, we transduced P14 cells with a retrovirus (RV) vector (MIGR1-GFP) expressing miR-155 and GFP (miR-155 overexpression [OE]) or with an empty MIGR1-GFP vector (control). Transduced P14 CD8 T cells were adoptively transferred into congenically marked recipient mice, followed by infection with clone 13 (Figure 2A). Despite equal transduction efficiency for control and miR-155 OE-transduced cells (Figure 2B), expansion of miR-155 OE cells was significantly greater in both lymphoid (Figures 2C and 2D) and non-lymphoid (Figure S2) tissues. This increased numerical accumulation of miR-155 OE cells was prolonged beyond the first week of infection, and numbers continued to increase between days 9–15, whereas the number of control-transduced cells had begun to decline by day 15. This enhanced expansion also correlated with more miR-155 OE cells 35 d.p.i. (Figures 2C and 2D; Figure S2). Notably, GFP — (i.e., non-transduced) P14 cells were numerically similar between the miR-155 and control groups (Figure 2D). Thus, enforced expression of miR-155 enhanced and prolonged the expansion of antigen-specific CD8 T cells during chronic viral infection.

To investigate how miR-155 affected the long-term durability of CD8 T cells, we examined the persistence of the transduced CD8 T cells long-term. Overexpression of miR-155 resulted in significantly higher numbers of transduced CD8 T cells 60, 90, and 150 d.p.i. (Figure 2E). There was only a minimal decline in the number of transduced CD8 T cells from 1–5 months post infection (Figure 2E), suggesting that miR-155 overexpression not only enhanced expansion but also robustly sustained the persistence of virus-specific CD8 T

cells during chronic viral infection, with ~50- to 100-fold more miR-155-transduced cells present on day 150 compared with control RV-transduced cells.

miR-155 Overexpression Correlates with Higher Expression of Inhibitory Receptors on CD8 T Cells during Chronic Viral Infection

To investigate whether miR-155 altered exhaustion, we analyzed the phenotype of miR-155 OE CD8 T cells compared with control-transduced cells. miR-155 OE led to a progressive increase in the expression of inhibitory receptors (PD-1, CD160, and 2B4) in the spleen (Figure 3A), liver (Figure S3A), and bone marrow (Figure S3B). Furthermore, co-expression of multiple inhibitory receptors was increased in miR-155 OE CD8 T cells (Figure 3B), suggesting a greater number of cells in the terminally exhausted subset driven by miR-155. These differences in inhibitory receptor expression were also apparent when comparing miR-155 OE (GFP+) CD8 T cells with non-transduced (GFP−) CD8 T cells within the same recipients (Figure S4A), excluding environmental differences because of inflammation and/or viral load. Moreover, there were no detectable differences in the viral loads between the two groups of recipient mice 30 d.p.i. (data not shown), likely because of the small numbers of adoptively transferred P14 cells used in this system to avoid immunopathology, altered viral control, or viral escape (Blattman et al., 2009; Frebel et al., 2012; Odorizzi et al., 2015). Thus, these data identify a cell-intrinsic role for miR-155 in regulating the differentiation of T_{EX} cells. To confirm that the effects of miR-155 on CD8 T cell expansion and differentiation were not dependent on the number of transferred P14 cells and/or non-transduced GFP − cells that were co-transferred with the transduced GFP+ cells, we sorted and adoptively transferred different numbers of transduced GFP+ P14 cells. The effects of miR-155 on the expansion (Figure S5A) as well as the phenotype (Figure S5B) of the transferred cells were independent of the number of sorted GFP+ cells transferred. Overall, these data indicate that miR-155 sustained high numbers of T_{EX} cells expressing multiple inhibitory receptors in chronic infection.

Altered Function of CD8 T Cells because of Overexpression of miR-155

T_{EX} cells are characterized by poor function. Specifically, a progenitor pool of T_{EX} cells has moderate cytokine production but weak cytotoxicity. In contrast, a terminal T_{EX} cell population is less efficient in producing interferon γ (IFN-γ) and tumor necrosis factor (TNF) but has slightly elevated cytotoxic potential (Paley et al., 2012). We next investigated whether miR-155 affects effector functions of CD8 T cells during chronic viral infection. miR-155 overexpression reduced the percentages of IFN-γ-and TNF-producing CD8 T cells (Figure 3C). The reduction in cytokine production started with a significant decrease in IFN-γ 15 d.p.i. and progressed with lower TNF production by 35 d.p.i. (data not shown), consistent with a progressive phenotype of exhaustion. However, because of the increased number of miR-155 OE GFP+ cells, the absolute number of cytokine-producing GFP+ cells was increased in miR-155 OE compared with control-transduced cells (Figure S4B). In contrast, miR-155 increased the production of MIP-1α, granzyme B (GzmB; Figure 3D), and degranulation, based on CD107α staining (Figure 3E). These results suggested that miR-155 fostered the development of cells with a functional profile more similar to terminally exhausted T_{EX} cells.

miR-155 Promotes and Sustains T_{EX} Cells in the Absence of CD4 T Cell Help

Depletion of CD4 T cells results in sustained viremia and more severe CD8 T cell exhaustion following clone 13 infection (Matloubian et al., 1994). This prolonged high viral load can erode the T_{EX} cell population over time, reminiscent of poorly sustained T cell responses in humans with uncontrolled long-term HCV or HIV infection (Hellerstein et al., 1999; Lechner et al., 2000). To test whether miR-155 altered T_{EX} cell differentiation in this setting, recipient mice were depleted of CD4 T cells before clone 13 infection, resulting in sustained viremia. miR-155 OE cells persisted at ~40-fold higher numbers compared with control-transduced cells in CD4 depleted (ΔCD4) mice, and this advantage of miR-155 OE cells persisted through at least 90 d.p.i. (Figure 4A). Moreover, miR-155 expression again fostered higher expression of inhibitory receptors and reduced expression of effector-associated (KLRG1) and memory-associated (IL-7Rα) molecules in both CD4-sufficient and ΔCD4 recipients (Figure 4B). Thus, miR-155 promotes the development of a large number of terminally exhausted CD8 T cells that was sustained long-term even in ΔCD4 recipients with a high viral load.

miR-155 Enhances T_{EX} Cell Proliferation and the Terminal T_{EX} Cell Subset while Preserving the Progenitor Pool Long-Term

Because miR-155 expression affected T_{EX} cell differentiation, we next examined the expression of T-bet and Eomes (Paley et al., 2012) as well as TCF-1 (He et al., 2016; Im et al., 2016; Leong et al., 2016; Utzschneider et al., 2016; Wu et al., 2016), which have been implicated in T_{EX} cell subset differentiation. T_{EX} cell populations persist because of a proliferative hierarchy where PD-1^{Int} T-bet^{Hi} progenitor cells proliferate and generate terminally differentiated PD-1^{Hi} $Eomes^{Hi}$ cells (Paley et al., 2012). A role for TCF-1 in this process is now also appreciated. Given the significant increase in persisting T_{EX} cells upon miR-155 overexpression, we asked whether this was a result of a larger T-bet^{Hi} or TCF-1+ progenitor pool because of increased proliferation of PD-1 and/or T-bet defined subsets or as a result of increased persistence of the terminally differentiated PD-1^{Hi} $Eomes^{Hi}$ subset. miR-155 overexpression reduced the expression of TCF-1 and the progenitor TCF-1+Tim-3 − and TCF-1+Ly108+ pool (Figure S4C). Similarly, miR-155 OE cells contained only a small proportion of T-bet^{Hi} cells and mainly consisted of PD-1^{Hi} $Eomes^{Hi}$ cells (Figure 5A), in agreement with the exhausted phenotype described above. However, because of the significantly increased total numbers of miR-155 OE cells, the absolute number of T-bet^{Hi} cells was greater than in the control-transduced population (Figure 5A). We next asked whether miR-155 altered proliferation. The percentage and number of Ki67+ T-bet^{Hi} progenitor T_{EX} subset cells were not altered by miR-155 overexpression (Figure 5B). In contrast, miR-155 enhanced the number of Ki-67+ $Eomes^{Hi}$ cells (Figure 5B). These observations are consistent with the notion that miR-155 increases the generation and/or survival of terminally differentiated $Eomes^{Hi}$ cells without altering the proliferation of the progenitor T-bet^{Hi} pool.

Because inhibitors of the PD-1 pathway can reinvigorate T_{EX} cells by inducing proliferation of progenitor T_{EX} cells, which results in accumulation of terminally differentiated cells (Blackburn et al., 2008; He et al., 2016; Im et al., 2016; Leong et al., 2016; Huang et al., 2017), we tested whether miR-155 OE enhanced the benefit of anti-PDL1 treatment during

chronic LCMV infection. Recipients of miR-155 OE and control P14 cells were treated with anti-PDL1 blocking antibody 22–35 d.p.i. As expected, in anti-PDL1-treated mice, control-transduced P14 cells showed a trend for increased numbers ($p = 0.1$) and had lower inhibitory receptor and increased KLRG1 expression compared with P14 cells from PBS-treated mice (Figure S4D). However, the phenotype of miR-155 OE cells was not significantly altered upon anti-PDL1 treatment (Figure S4D). Although it is possible that longer treatment may benefit miR-155 OE cells or that a local benefit in effector functions occurs, these data suggest that miR-155 OE does not significantly complement the benefit of anti-PDL1 treatments.

Major Transcriptional Changes in T_{EX} Cells because of miR-155 OE

To investigate how miR-155 affects the expansion and differentiation of CD8 T cells during chronic infection, we performed a transcriptome analysis. Recipients of miR-155 OE or control-transduced cells were infected with clone 13. 30 d.p.i., transduced GFP+ P14 cells as well as co-transferred non-transduced GFP- P14 cells were sorted by flow cytometry, and RNA sequencing (RNA-seq) was performed (Figure 6A). GFP+ cells from the control group and GFP − cells from both the control and the miR-155 OE groups served as controls to exclude transcripts that were differentially expressed either because of extrinsic factors (i.e., the environment) or because of the different activation status of the cells at the time of transduction (GFP+ versus GFP−). Pairwise comparisons identified 395 transcripts differentially expressed in miR-155 OE cells with $p < 0.05$ (Figure 6B). Among the 200 transcripts downregulated in miR-155 OE cells, 12 transcripts (Figure 6B) were predicted targets of miR-155 (TargetScan version 7.1). These transcriptionally downregulated miR-155 targets represent 3.4% of all predicted miR-155 targets in our dataset (12 of 354 miR-155 target genes). Because only 1.9% of all transcripts (200 of 10,297 genes) were downregulated in miR-155 OE cells (Figure 6B), this represents a significant enrichment for miR-155 targets being downregulated in miR-155 OE cells (p value of overlap = 0.03). Therefore, we hypothesized that the effect of miR-155 on CD8 T cells responding to chronic infection may involve one or more direct miR-155 targets.

We further investigated the pathways affected by miR-155 and found that several pathways related to responses to cytokines and inflammatory mediators as well as T cell activation were downregulated by miR-155 (Table S1). Gene set enrichment analysis (GSEA) confirmed that several cytokine signaling pathways (TNF, IL-6, IFN-γ, and transforming growth factor β [TGF-β]) were significantly enriched in control-transduced compared with miR-155 OE cells (Figure 6C; Figure S6A). Thus, these data support the idea that miR-155 dampens the response to inflammation, facilitating CD8 T cell persistence in an environment of high inflammatory stress.

We next asked whether these transcriptional changes revealed any common upstream regulators that might act as central “nodes” in the miR-155 effect on T_{EX} cells. Indeed, three transcription factors, *Nfatc2*, *Foxo1*, and *Ets1*, were predicted to be negatively regulated based on the expression of their target genes (Figure S6B). We next constructed a network from the genes differentially regulated by miR-155. This network revealed several cytokine and chemokine receptors (*Il7r*, *Tnf*, *Ccr9*, and *Cxcl10*) as well as transcription factors

(*Prdm1*, *Bcl6*, *Jun*, and *Tcf7*) that were differentially regulated by miR-155 (Figure 6D). These observations suggested that miR-155 regulated T_{EX} cells during chronic infection by modulating responses to cytokines and inflammatory mediators with an effect via several key transcriptional pathways.

The Effect of miR-155 on T_{EX} Cell Differentiation Is Mediated in Part by Targeting the AP-1 Transcription Factor *Fosl2*

The transcriptional pathways inhibited by miR-155 overexpression included several members of the AP-1 pathway, such as *Jun*, *JunB*, *FosB*, and *Fosl2* (Figure 6D; Figure S6B). Among these, *Fosl2* has been previously identified as a direct target of miR-155. The role of *Fosl2* in CD8 T_{EX} cells, however, is unknown. Therefore, we hypothesized that *Fosl2* may play a role in T_{EX} cell differentiation and that the effect of miR-155 on T_{EX} cells could be mediated by targeting *Fosl2*.

To this end, we first investigated the expression of *Fosl2* in T_{EX} cells. *Fosl2* was expressed in T_{EX} cells, but expression was lower in terminally exhausted PD-1^{Hi} T_{EX} cells compared with PD-1^{Int} T_{EX} cells (Figure S7A). These results suggested a *Fosl2* expression pattern in T_{EX} cell subsets that was anti-correlated with miR-155. Furthermore, the pathways of the AP-1 transcription factors *Fosl1* and *Fosl2* were enriched in the transcriptional signatures of control versus miR-155 OE GFP+ CD8 T cells (Figure S7B), supporting the notion that Fosl2 activity was decreased in the presence of miR-155. We hypothesized that the downregulation of Fosl2 by miR-155 OE may increase the activity of nuclear factor activated T cell (NFAT) functioning without canonical AP-1 dimers (“partnerless NFAT”) (Martinez et al., 2015), which induces key T_{EX} cell-associated genes. Indeed, the signature of genes upregulated by partnerless NFAT (Martinez et al., 2015) overlapped with the signature of genes upregulated in miR-155 OE GFP+ compared with control GFP+ CD8 T cells (p value of overlap = 2.64×10^{-10}; Figure S7C). This overlap included genes associated with terminal exhaustion, such as *Prdm1*, *Havcr2*, *Pdcd1*, *Cd200r1*, and *Tigit* (Figure S7C). In addition, the signature of genes downregulated upon binding of partnerless NFAT overlapped with the signature of genes downregulated in miR-155 OE GFP+ compared with control GFP+ CD8 T cells (p value of overlap = 3.93×10^{-10}). These data suggest that miR-155 promotes the transcriptional program of exhaustion by downregulating the AP-1 transcription factor Fosl2 and increasing the activity of partnerless NFAT.

To directly test the causal connection between Fosl2, exhaustion, and miR-155, we asked whether overexpression of Fosl2 could reverse the effect of miR-155. We co-transduced P14 CD8 T cells with an miR-155 OE RV (with the GFP reporter) and an RV overexpressing Fosl2 (Fosl2-OE RV with the violet excited protein [VEX] reporter). Because only the coding region of Fosl2 was introduced, Fosl2 expressed from the RV is not subjected to regulation by miR-155 targeting the Fosl2 3′ UTR. P14 cells transduced either with miR-155-GFP or with miR-155-GFP and control-VEX (empty-VEX [eVEX]) expanded more and expressed more inhibitory receptors (Figure 7A), in agreement with the results above. Overexpression of Fosl2 resulted in greater expansion of transduced P14 cells and decreased co-expression of CD160 and PD-1 (Figure 7B). These results demonstrate a role for the AP-1 transcription factor Fosl2 in T_{EX} cell differentiation.

Because miR-155 represses Fosl2 by directly binding to the 3′ UTR (Hu et al., 2014), and expression of Fosl2 was decreased upon miR-155 overexpression (Figure 6B), we hypothesized that the miR-155 effect would be reversed by overexpression of Fosl2. Enforced expression of non-repressible Fosl2 in the presence of miR-155 OE completely reversed the effect of miR-155 on inhibitory receptor expression and fostered higher IL-7Rα compared with miR-155-GFP single-transduced cells (Figure 7C). These results demonstrate that enforced expression of Fosl2 antagonizes the effect of miR-155, suggesting that inhibition of the AP-1 pathway is a mechanism by which miR-155 promotes exhaustion and long-term persistence of CD8 T cells during chronic infection.

DISCUSSION

In this study, we identified a role for miR-155 in regulating CD8 T cell exhaustion during chronic viral infection. miR-155 was highly expressed by terminally exhausted CD8 T cells during chronic infection. Enhanced expression of miR-155 promoted long-term persistence of mainly the terminal T_{EX} cell subset by preserving the progenitor pool and increasing the generation and durability of terminally differentiated PD-1^{Hi} $Eomes^{Hi}$ T_{EX} cells. miR-155 overexpression induced prominent transcriptional changes in CD8 T cells responding to chronic infection, affecting several key pathways. Among these, we identified a connection between miR-155 and the AP-1 transcriptional pathway in T_{EX} cells through Fosl2.

Transcriptional profiling revealed several pathways altered because of miR-155 overexpression. These pathways included key inflammatory circuits as well as TCR signaling molecules. Although inflammatory cytokines are essential for optimal T cell priming, persistent inflammation, and particularly type I IFN signaling during chronic infection, are detrimental for T cell differentiation (Stelekati et al., 2014; Teijaro et al., 2013; Wilson et al., 2013). We have previously shown that miR-155 tunes the response of T_{EFF} cells to type I IFN by inhibiting the phosphorylation of STAT1 (Gracias et al., 2013). Our transcriptional profiling here suggests that miR-155 reduces inflammatory cytokine signaling (TNF, IL-6, IFN-γ, and TGF-β) and shields T_{EX} cells from the detrimental effects of persistent inflammation. On the other hand, it has been shown that miR-155 enhances the responsiveness of tumor-specific CD8 T cells to common γ-chain cytokines and subsequent phosphorylation of STAT5 (Ji et al., 2015), suggesting that multiple context-dependent mechanisms may exist for miR-155 to enhance CD8 T cell responses. Although our observations suggest a connection between miR-155 and inflammatory and TCR signaling, precisely how miR-155 integrates these events in T_{EX} cells remains to be determined.

In addition to these pathways, transcriptional profiling also identified control of proliferation as a major change associated with miR-155. These transcriptional data are consistent with Ki67 staining and support a role for miR-155 in sustaining proliferation. Specifically, miR-155 fostered greater expansion of CD8 T cells and increased the numbers of T_{EX} cell progenitors and Ki67+ terminal T_{EX} cells. Thus, these data suggest that miR-155 enhances the number of T_{EX} cell progenitors while increasing the durability of terminal T_{EX} cells. The increased Ki67 in the terminal T_{EX} cells might suggest a role for miR-155 in increasing the fraction of T_{EX} cells that survive the proliferative event in progenitor to terminal T_{EX} cell conversion. This may occur by increasing the frequency of this conversion without

eroding the progenitor pool or perhaps by directly inducing proliferation in the terminal subset. Thus, miR-155 represents a circuit in the regulation of the balance and sustainability of T_{EX} cell subsets.

Our studies identified a mechanism for the AP-1 pathway in regulating T_{EX} cells. The AP-1 transcription factor complex consists of heterodimers between members of the Jun family with members of the Fos family or with members of the basic leucine zipper ATF-like transcription factor (BATF) family. Fos family members compete with BATF family members for binding to Jun, JunB, and JunD (Murphy et al., 2013). AP-1 heterodimers can then interact with other transcription factors, including NFATs (Martinez et al., 2015) or IRF4 (Murphy et al., 2013). BATF promotes T cell exhaustion, perhaps by interfering with canonical AP-1 function (Quigley et al., 2010). Moreover, at least one key function of canonical Fos/Jun AP-1 dimers is to interact with NFAT1 and NFAT2 and ensure the transcriptional program of functionality. Indeed, partnerless NFAT working in the absence of optimal AP-1 can drive T_{EX} cell gene expression (Martinez et al., 2015). Here we show that Fosl2 antagonizes the effects of miR-155, suggesting a link in T_{EX} cells between miR-155 and the AP-1 transcriptional pathway through Fosl2. miR-155 directly represses Fosl2 by binding to the $3^{'}$ UTR (Hu et al., 2014). By reducing the levels of Fosl2 in T_{EX} cells, miR-155 may increase the availability of Jun family members for binding to BATF or other partners. Another possibility is that, by downregulating Fosl2, miR-155 decreases the abundance of canonical AP-1 dimers and increases the binding of partnerless NFAT, which, in turn, promotes exhaustion (Martinez et al., 2015). However, we also observed decreased NFAT1 (NFATc2) upon miR-155 overexpression, suggesting perhaps an altered balance of NFAT1 and NFAT2 in addition to Fosl2 regulation. Interrogating the effects of altering the balance of AP-1 family members as well as other partner transcription factors, such as NFATs and IRF4, will be an important future goal.

Overall, these data identify a key role for miR-155 in CD8 T cell exhaustion. Through the study of the effects of miR-155, we reveal two concepts with potential clinical implications. First, we identify a key role for a member of the AP-1 family, Fosl2, in regulating T cell exhaustion, suggesting that manipulating the balance of AP-1 family members could be used to tune T cell exhaustion clinically. Second, we reveal an opportunity to sustain T_{EX} cell populations long-term and increase the durability of this critical cell type through miR-155. Future work exploring the manipulation of miR-155 could lead to approaches that promote long-term persistence T_{EX} cell populations or adoptively transferred T cells in chronic infection or cancer.

EXPERIMENTAL PROCEDURES

Mice

Six- to eight-week-old male C57BL/6 (CD45.2) and C57BL/6 Ly5.2CR (CD45.1) mice were purchased from National Cancer Institute (NCI). miR-155-deficient (*bic/miR-155*$^{-/-}$) mice were purchased from The Jackson Laboratory. TCR-transgenic mice specific for the immunodominant LCMV D^{b}-$gp^{33–41}$ epitope (P14 mice) on the C57BL/6 background were bred at the University of Pennsylvania. Animal procedures were performed according to Institutional Animal Care and Use Committee guidelines for the University of Pennsylvania.

Viral Infection and Calculation of Viral Loads

Mice were infected intravenously (i.v.) with 4×10^6 plaque-forming units (PFUs) LCMV clone 13. For depletion of CD4 T cells, recipient mice were injected twice with 200 μg anti-CD4 (GK1.5, Bio X Cell) intraperitoneally (i.p.) at −1 day and +1 day of LCMV infection. For calculation of viral loads, tissues were homogenized, and 10-fold dilutions were incubated on adherent Vero cells for 1 hr. Cells were overlaid with a 1:1 mixture of medium and 1% agarose and cultured for 4 days. Plaques (PFUs) were counted after overlaying with a 1:1:20 mixture of medium:1% agarose:neutral red for 16 hr.

Retroviral Experiments and Adoptive Transfers

miR-155 cDNA was cloned into the murine stem cell virus (MSCV)-internal ribosomal entry site (IRES)-GFP plasmid as described previously (Gracias et al., 2013). The mouse Fosl2 coding sequence without the 3′ UTR was cloned into the MSCV-IRES-VEX plasmid. Empty MSCV-IRES-GFP or MSCV-IRES-VEX plasmids were used as controls (Gracias et al., 2013). RV supernatants were produced by transfecting 293T cells with an RV expression plasmid and a pCl-Eco packaging plasmid using Lipofectamine 3000 (Invitrogen). For RV transduction, CD8 T cells were purified from spleens of P14 mice using the EasySep Mouse CD8+ T Cell Isolation Kit (STEMCELL Technologies) and stimulated *in vitro* for 20 hr with 1 μg/mL soluble anti-CD3 (145–2C11, BioLegend), 0.5 μg/mL anti-CD28 (37.51, BioLegend), and 100 U/mL recombinant human IL-2 (PeproTech) as described previously (Kurachi et al., 2017). Activated P14 cells were transduced with RV in the presence of Polybrene (0.5 μg/mL) during spin infection (2,000 × *g* for 60 min at 30°C), as described previously (Bengsch et al., 2016; Kurachi et al., 2017). Transduced P14 cells were adoptively transferred i.v. into recipient mice that were infected 24 hr earlier.

Flow Cytometry and Cell Sorting

Surface staining was performed in PBS containing 2% fetal bovine serum (FBS) and 0.02% sodium azide using antibodies against CD8 (53–6.7, BioLegend), CD45.1 (A20, BioLegend), CD45.2 (104, BioLegend), CD44 (IM7, BioLegend), KLRG1 (2F1/KLRG1), CD127 (A7R34, BioLegend), CD62L (MEL-14, BioLegend), CD27 (LG.7F9, BioLegend), PD-1 (RMP1–30, BioLegend), CD160 (7H1, BioLegend), and 2B4 (eBio244F4, eBioscience). For staining for intracellular proteins, cells were fixed with fixation/permeabilization buffer (eBioscience) and stained in permeabilization buffer (eBioscience) with antibodies against Eomes (Dan11mag, eBioscience), Ki67 (16A8, BioLegend), T-bet (4B10, BioLegend), and GFP (FM264G, BioLegend). Intracellular cytokine staining was performed after 5 hr *ex vivo* stimulation with $gp^{33–41}$ peptide in the presence of GolgiPlug (Becton Dickinson), GolgiStop (BD), and anti-CD107a (1D4B, BioLegend) using antibodies against TNF-α (MP6-XT22, BioLegend), IFN-γ (XMG1.2, BD Pharmingen), GzmB (GRB17, Life Technologies), MIP-1α (IC450P, R&D Systems), and GFP (FM264G, BioLegend). Cells were analyzed using an LSRII (BD Biosciences) and FlowJo software (Tree Star).

GFP+ and GFP− P14 cells were sorted from splenocytes after surface staining with CD8 (53–6.7, BioLegend), CD45.1 (A20, BioLegend), CD45.2 (104, BioLegend), and CD44

(IM7, BioLegend) for 30 min on ice. Cell sorting was performed on Aria II (BD Biosciences).

qRT-PCR

Total RNA was isolated using the miRNeasy mini kit (QIAGEN). Mouse miR-155 and snoRNA-429 (as an endogenous control) primers were from TaqMan (ABI Biosciences). qRT-PCR was performed using iTaq Universal SybrGreen Supermix from Bio-Rad on the ViiA 7 real-time PCR system according to the manufacturer's instructions.

RNA-Seq

RNA from sorted cells was isolated using RNA Plus Micro Kit (QIAGEN). Libraries were prepared using the SMART er Ultra Low RNA Kit (Clontech Laboratories) at the Next Generation Sequencing Core Facility at the University of Pennsylvania (https://ngsc.med.upenn.edu/#/). Sequenced libraries were mapped to the mm9 reference genome using RNA-seq unified mapper (RUM) (Grant et al., 2011). Read counts for NCBI Reference Sequence Database (RefSeq) transcripts were processed with Deseq2 (Love et al., 2014). GSEA (Subramanian et al., 2005) was performed using MSigDb (v5.1). The R package "pheatmap" was used to create heatmaps. Significant genes were analyzed with Metascape (http://metascape.org) for functional enrichment using ontology terms collected from Gene Ontology (http://geneontology.org) for biological processes. The upstream networks were generated through the use of QIAGEN's Ingenuity Pathway Analysis (IPA; QIAGEN, Redwood City; https://www.qiagen.com/ingenuity).

Statistical Analysis

Normal distribution was tested using the D'Agostino and Pearson normality test. For normally distributed samples, statistical significance was calculated using unpaired two-tailed t test or one-way ANOVA (with Tukey's multiple comparisons test), as indicated. For non-normally distributed samples or samples too small to test for normal distribution, non-parametric Mann-Whitney test or Kruskal-Wallis test (with Dunn's multiple comparisons test) was performed, as indicated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- miR-155 enhances long-term persistence of exhausted CD8 T cells
- miR-155 promotes an exhausted phenotype in CD8 T cells during chronic infection
- Overexpression of Fosl2 reduces exhaustion of CD8 T cells during chronic infection
- Fosl2 overexpression antagonizes the effect of miR-155 overexpression

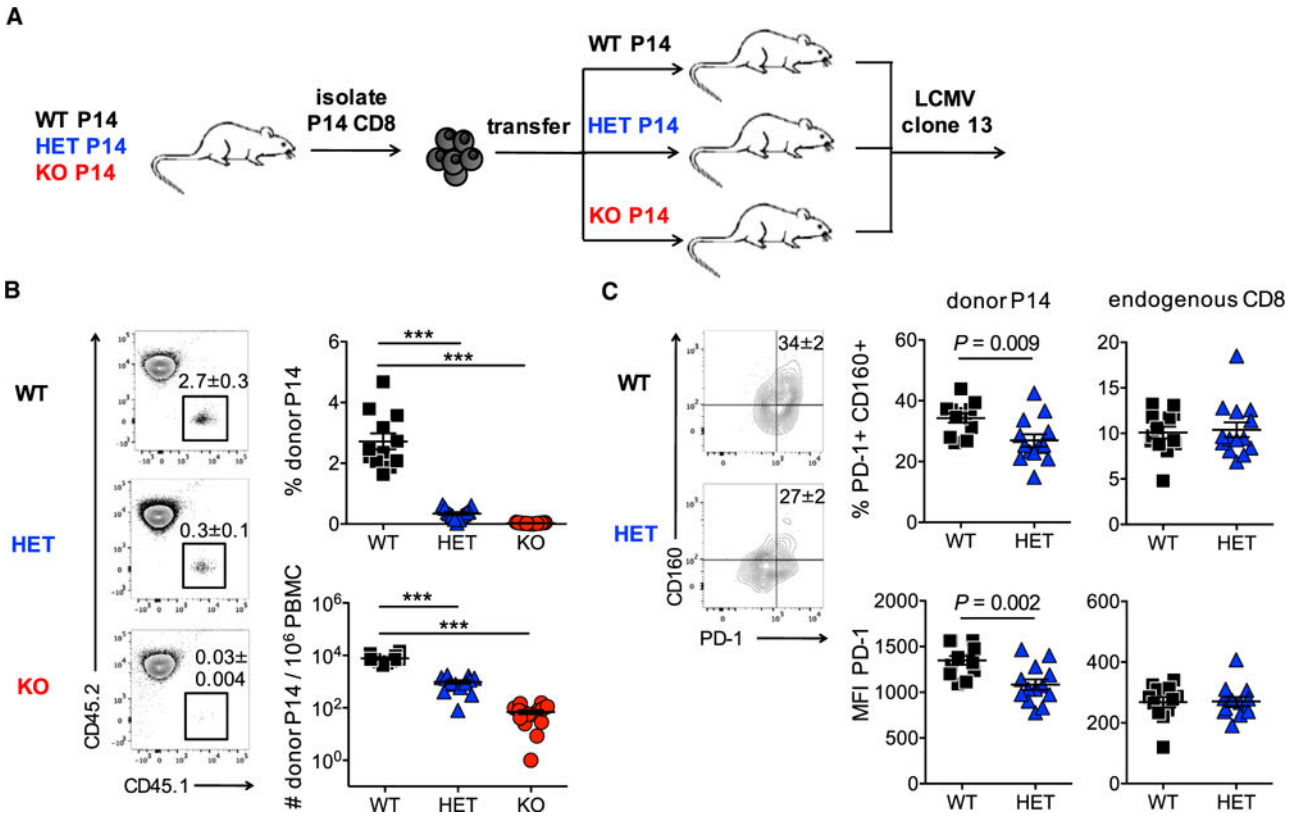

Figure 1. miR-155 Is Essential for the Expansion of CD8 T Cells during Chronic Infection

10^3 CD8 T cells from naive wild-type (WT, n = 12), miR-155 heterozygous (HET, n = 13), or miR-155-deficient (KO, n = 15) P14 CD45.1+ mice were adoptively transferred into congenically distinct CD45.2+ recipient mice, followed by infection with LCMV clone 13.

(A) Experimental design.

(B) Donor P14 cells were analyzed in peripheral blood at 9 d.p.i. ***$p < 0.001$; one-way ANOVA with Tukey's multiple comparisons test.

(C) The phenotype of donor P14 cells (gated on CD45.1+) was analyzed in blood 9 d.p.i. Significance was calculated with unpaired t test.

Each data point represents an individual mouse. Representative results of two independent experiments are shown. The error bars represent the SEM.

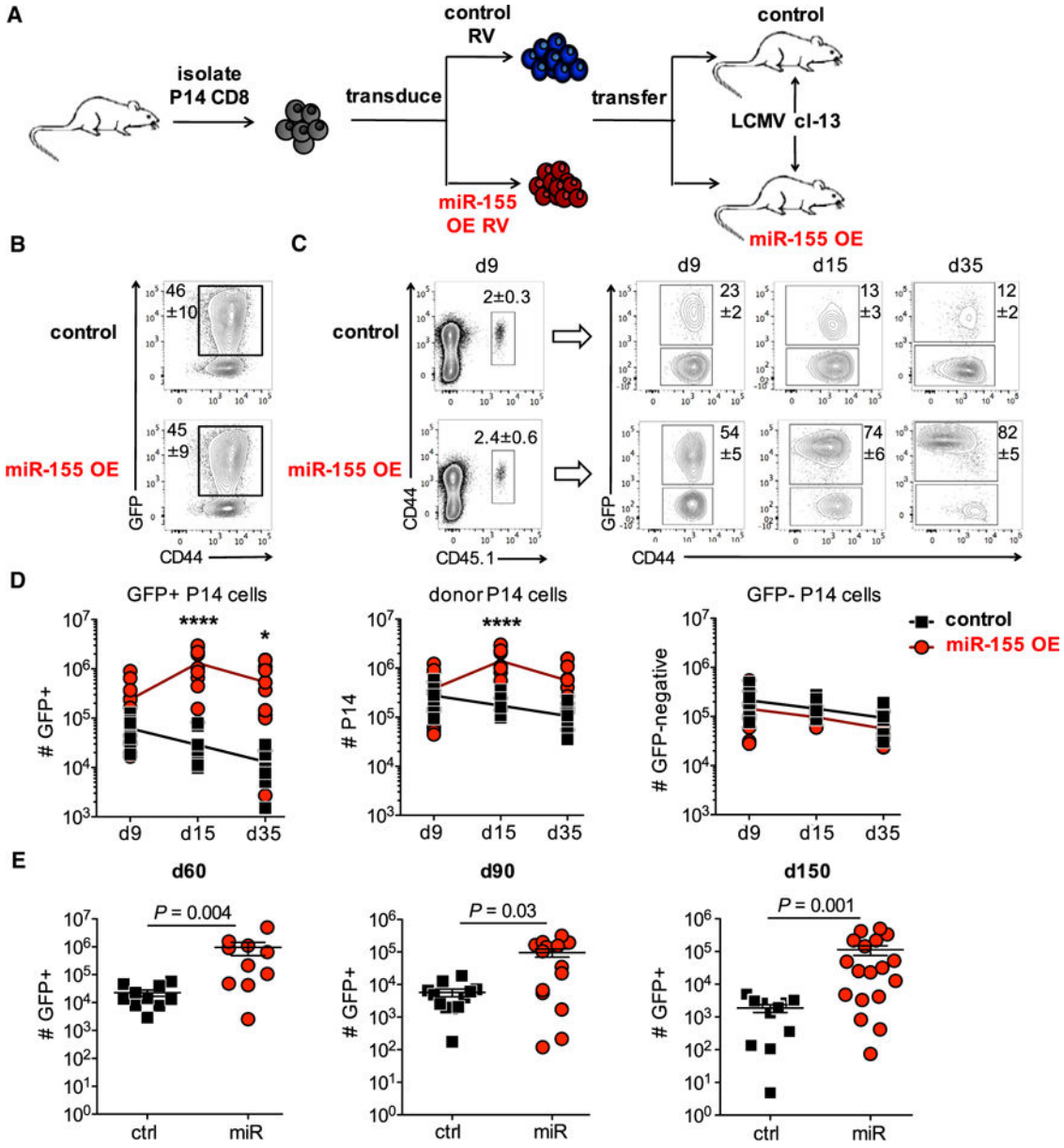

Figure 2. miR-155 Enhances and Prolongs the Expansion of CD8 T Cells during Chronic Viral Infection

CD8 T cells were purified from the spleens of congenically marked (CD45.1) P14 mice, transduced with control (ctrl)-GFP RV or miR-155 OE-GFP RV (miR), and adoptively transferred to CD45.2+ recipient mice that were infected 24 hr earlier with LCMV clone 13.

(A) Experimental design.

(B) Transduction efficiency was measured after culturing the CD8 T cells *in vitro* for 24 hr post-transduction. Representative plots show GFP expression on CD8 T cells.

(C) Spleens of recipient mice were isolated 9, 15, and 35 d.p.i., and transduced donor cells were identified based on CD45.1 and GFP expression.

(D) Absolute number of transferred GFP+ P14 cells, total donor P14 cells, and GFP− P14 cells were determined in spleens of recipient mice 9, 15, and 35 d.p.i. ****$p < 0.0001$, *$p < 0.05$; significance was calculated by two-way ANOVA.

(E) Absolute numbers of GFP+ donor P14 cells determined in spleens of recipient mice 60 ($n = 10$), 90 ($n > 12$), and 150 ($n > 11$) d.p.i. Significance was calculated by Mann-Whitney test.

Each data point represents an individual mouse. Shown are representative results of at least two independent experiments with a total of at least 10 mice per group. The error bars represent the SEM.

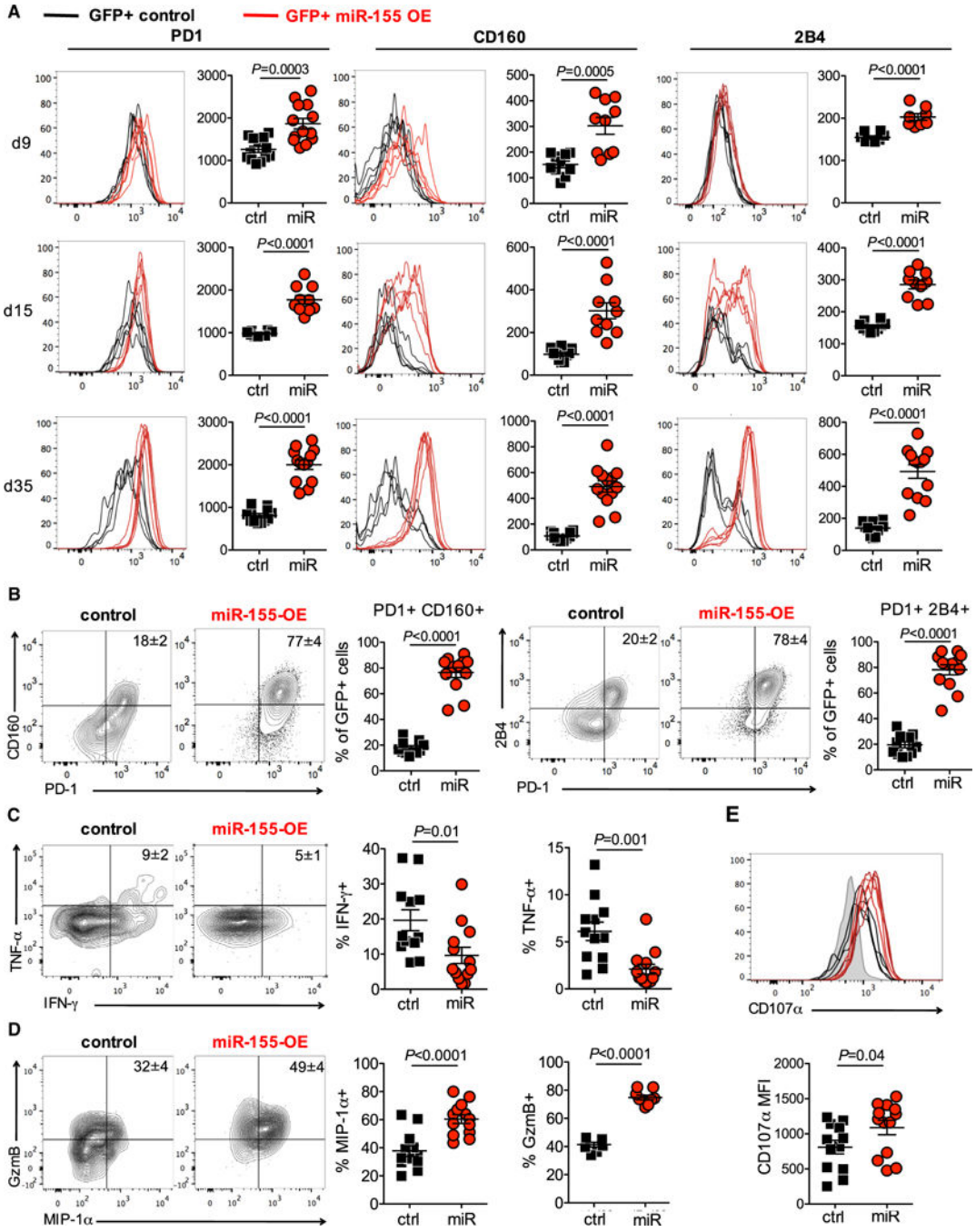

Figure 3. miR-155 Promotes the Phenotype of Exhaustion on CD8 T Cells during Chronic Viral Infection

CD45.1+ P14 CD8 T cells were transduced with either control-GFP RV or miR-155 OE-GFP RV (miR) and adoptively transferred to CD45.2+ recipient mice that were infected 24 hr earlier with LCMV clone 13.

(A) Expression of the inhibitory receptors PD-1 (n > 10), CD160 (n > 10), and 2B4 (n > 8) was analyzed on donor GFP+ CD8 T cells in the spleens of recipient mice 9, 15, and 35 d.p.i. Significance was calculated by unpaired t test.

(B) Co-expression of PD-1 and CD160 (left) and PD-1 and 2B4 (right) on donor GFP+ CD8 T cells in spleens 35 d.p.i. (n > 12). Each data point represents an individual mouse. Representative results of at least two independent experiments are shown. Significance was calculated by Mann-Whitney test (PD-1+CD160+) or unpaired t test (PD-1+2B4+).
(C) Production of IFN-γ and TNF by donor GFP+ CD8 T cells was measured by flow cytometry. Significance was calculated by Mann-Whitney test (n > 12).
(D) Production of MIP-1α (n > 12) and GzmB (n > 8) by donor GFP+ CD8 T cells was measured by flow cytometry. Significance was calculated by unpaired t test.
(E) Degranulation was measured by staining for CD107a. Black lines, control transduced GFP+ P14 cells; red lines, miR-155 OE GFP+ P14 cells; gray line, unstimulated control. Significance was calculated by Mann-Whitney test (n > 12).
Representative plots are gated on GFP+ CD45.1+ CD8 T cells. Each data point represents an individual mouse. Representative results of at least three independent experiments are shown. The error bars represent the SEM.

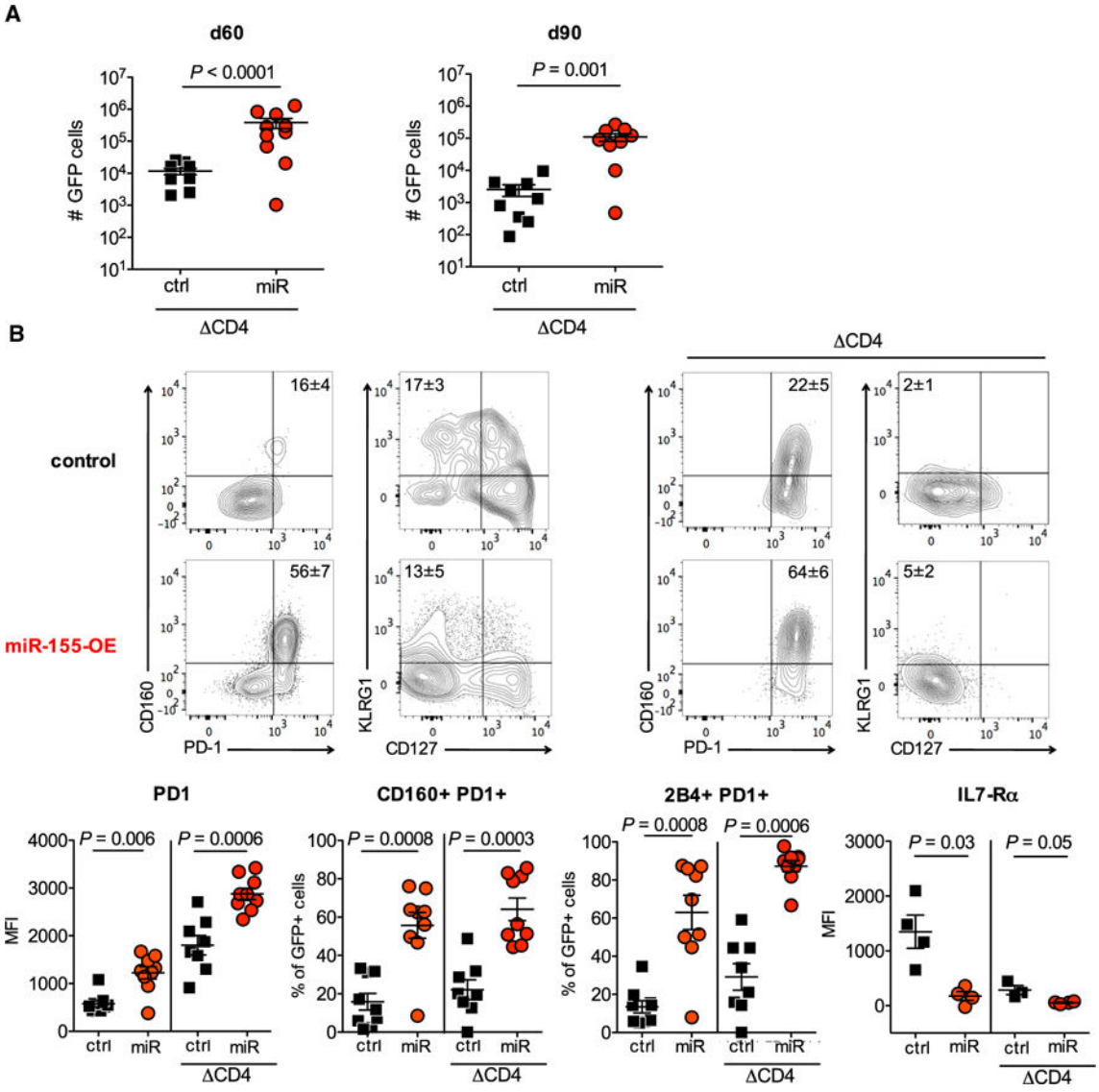

Figure 4. miR-155 Promotes a Terminally Exhausted T_{EX} Cell Subset without CD4 T Cell Help during Chronic Viral Infection

CD45.1+ P14 CD8 T cells were transduced with either control-GFP RV or miR-155 OE-GFP RV and adoptively transferred to CD45.2+ recipient mice that were infected 24 hr earlier with LCMV clone 13 and treated (ΔCD4) with anti-CD4 or left untreated.

(A) Absolute number of GFP+ donor CD8 T cells in spleens 60 (n = 10) and 90 (n = 9) d.p.i. in mice treated with anti-CD4 (ΔCD4).

(B) Phenotype of GFP+ donor CD8 T cells in spleens 90 d.p.i.

Representative plots are gated on GFP+ CD45.1+ CD8 T cells. Each data point represents an individual mouse. Significance was calculated by Mann-Whitney test. Representative results of two independent experiments are shown. The error bars represent the SEM.

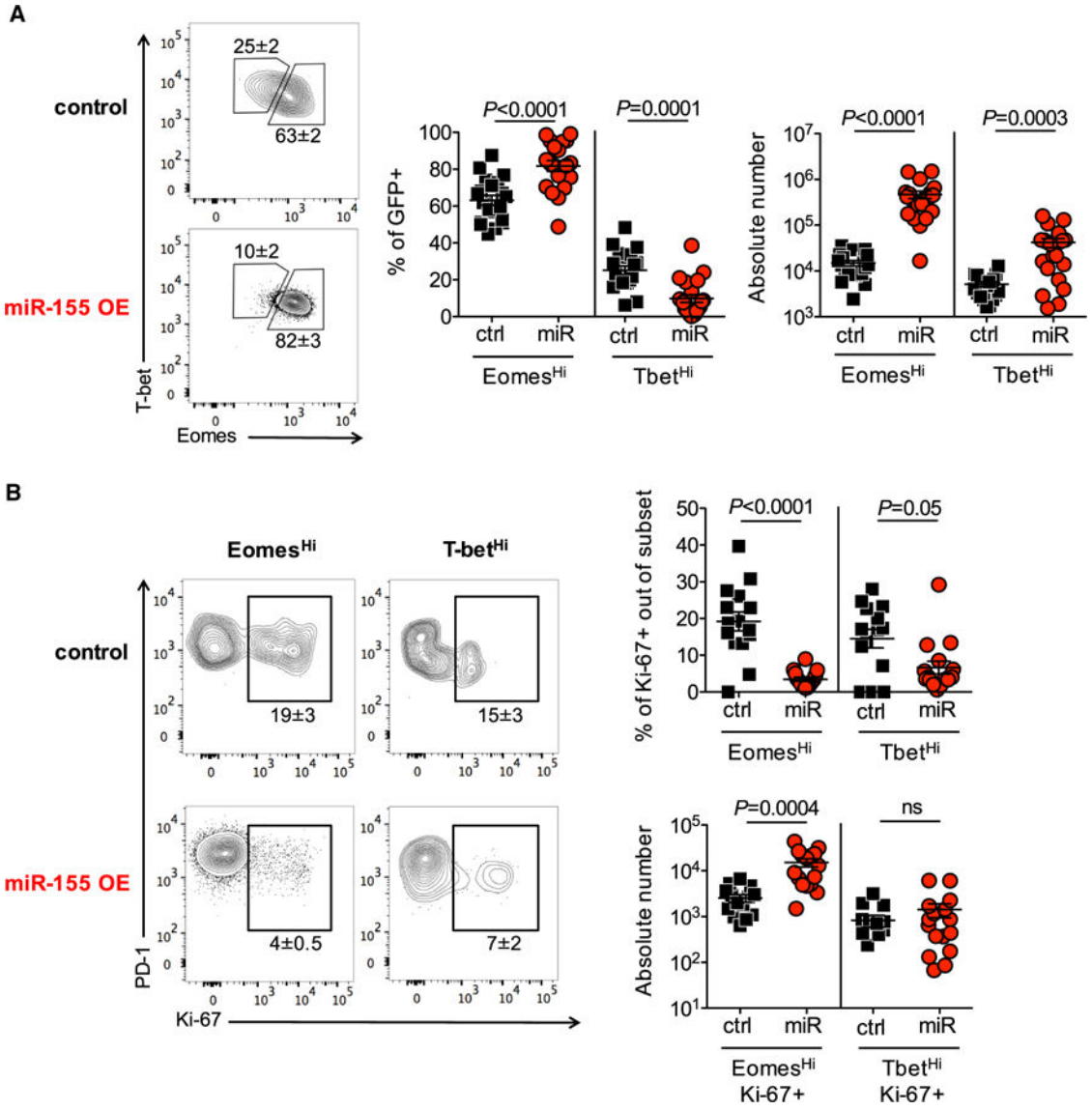

Figure 5. miR-155 Increases CD8 T Cell Proliferation while Preserving the T_{EX} Cell Progenitor Pool Long-Term

CD45.1+ P14 CD8 T cells were transduced with either control-GFP RV (control) or miR-155 OE-GFP RV and adoptively transferred to CD45.2+ recipient mice that were infected 24 hr earlier with LCMV clone 13.

(A) Expression of T-bet and Eomes was analyzed on donor GFP+ CD8 T cells in the spleens of recipient mice 35 d.p.i. (n > 20). Representative plots are gated on GFP+ P14 cells.

(B) Expression of Ki-67 in $Eomes^{Hi}$ and $T\text{-}bet^{Hi}$ donor GFP+ CD8 T cells was analyzed in the spleens of recipient mice 35 d.p.i. (n > 14). Representative plots are gated on $Eomes^{Hi}$ or $T\text{-}bet^{Hi}$ GFP+ P14 cells.

Each data point represents an individual mouse. Representative results of four independent experiments are shown. Significance was calculated by unpaired t test (for $Eomes^{Hi}$ cells, normally distributed samples) or Mann-Whitney test (for $Tbet^{Hi}$ cells, non-normally distributed samples). The error bars represent the SEM.

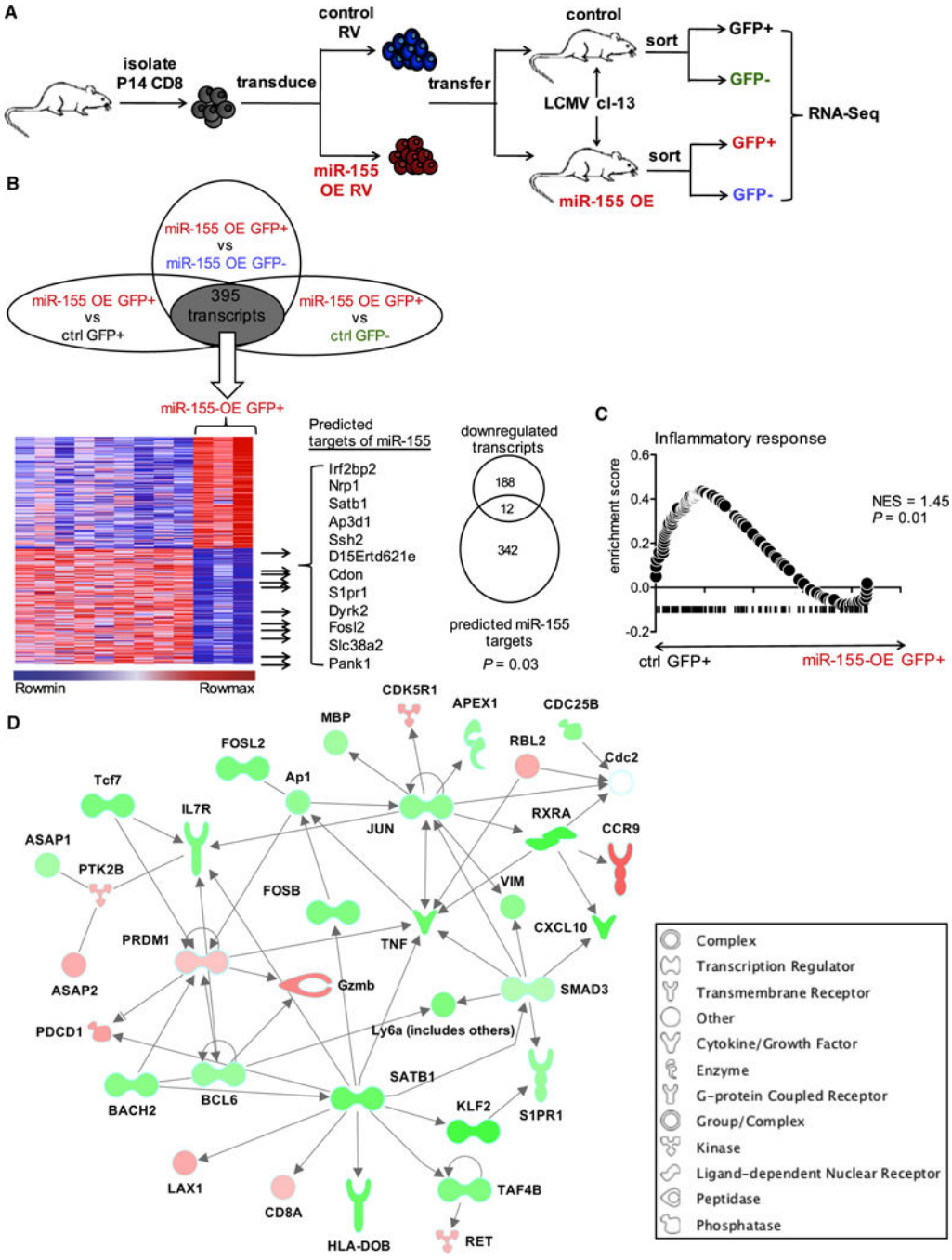

Figure 6. Overexpression of miR-155 Leads to Major Transcriptional Changes

CD45.1+ P14 CD8 T cells were transduced with either control-GFP RV or miR-155 OE-GFP RV and adoptively transferred to CD45.2+ recipient mice that were infected 24 hr earlier with LCMV clone 13. GFP+ as well as GFP− donor P14 CD8 T cells were isolated 30 d.p.i. by flow cytometry, and RNA-seq was performed.

(A) Experimental design.

(B) Heatmap showing the transcripts that were differentially expressed ($p < 0.05$) in miR-155 OE GFP+ cells compared with control GFP+ cells and GFP− cells, identified by

pairwise comparisons using DESeq2. The Venn diagram shows the overlap between transcripts downregulated in miR-155 OE GFP+ cells and predicted targets of miR-155 that were expressed in our dataset.

(C) Enrichment of a gene set of inflammation (HALLMARK_INFLAMMATORY_RESPONSE) in control GFP+ versus miR-155 OE GFP+ cells analyzed by GSEA.

(D) Network constructed using the 395 transcripts in (B) using IPA. Green represents downregulation in miR-155 OE cells compared with control GFP+ cells; red represents upregulation.

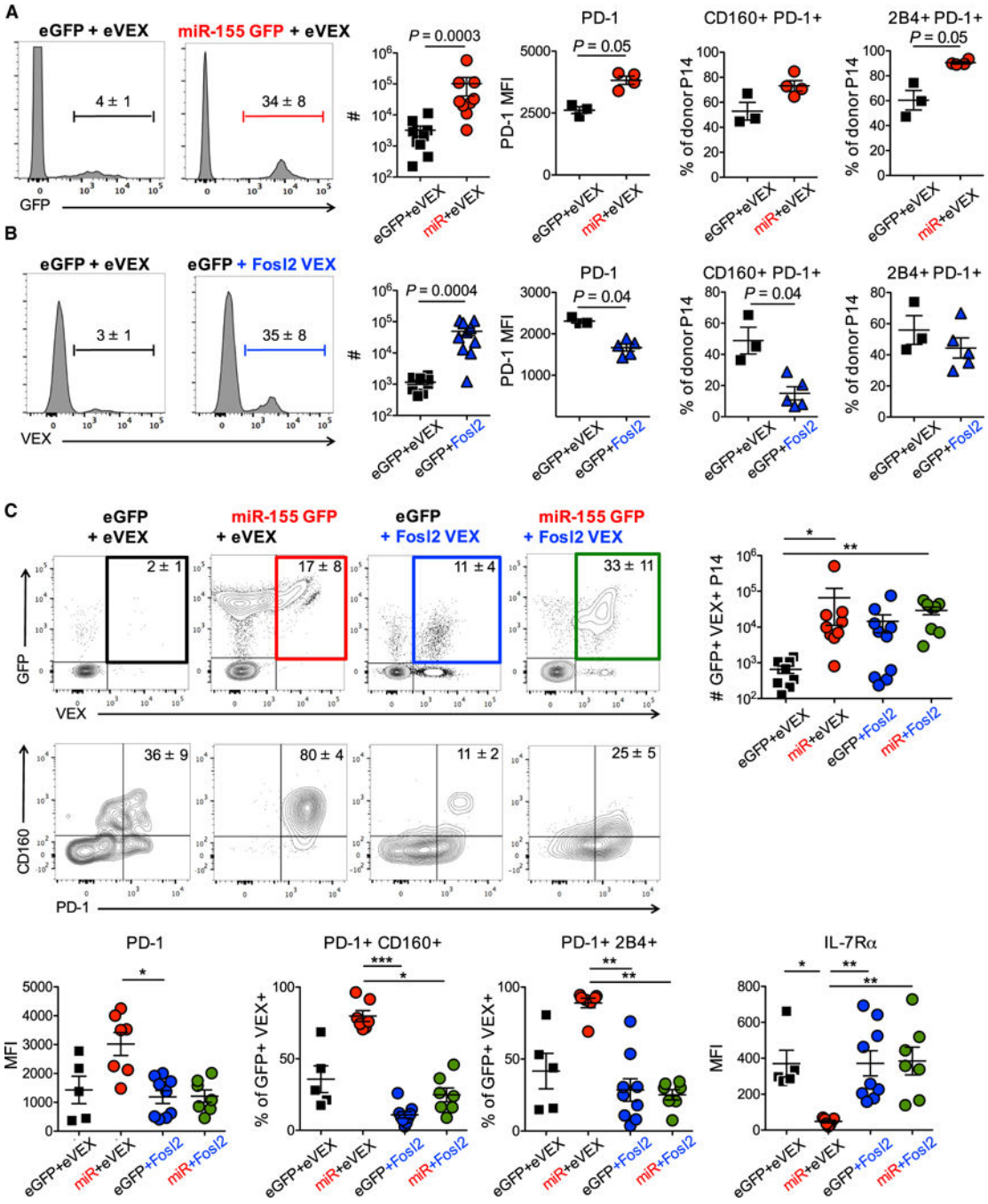

Figure 7. Overexpression of Fosl2 Reverses the Effect of miR-155 Overexpression

CD45.1+ P14 CD8 T cells were co-transduced with a combination of two of the following RVs: empty-GFP (eGFP), empty-VEX (eVEX), miR-155 OE-GFP (miR), and Fosl2-OE-VEX (Fosl2) and adoptively transferred to CD45.2 recipient mice that were infected 24 hr earlier with LCMV clone 13.

(A) Absolute numbers and phenotype of cells overexpressing miR-155 (identified as GFP+) were analyzed 30 d.p.i. in the spleen.

(B) Absolute numbers and phenotype of cells overexpressing Fosl2 (identified as VEX+) were analyzed 30 d.p.i. in the spleen.
(C) Absolute numbers and phenotype of cells overexpressing both miR-155 and Fosl2 (identified as GFP+VEX+) were analyzed 30 d.p.i. in the spleen. The numbers in the flow plots indicate the mean ± SEM for all mice included in the analysis.
Each data point represents an individual mouse. Representative data from three independent experiments with at least 5 mice per group are shown. Significance was calculated with Mann-Whitney test (for A and B) or with Kruskal-Wallis test with Dunn's multiple comparisons test (for C). Primary data are available at https://data.mendeley.com/datasets/b7hkyjhvd6/1.