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LET-7 MIRNAS PROGRAM THE FATE OF CD8 T CELLS

A Dissertation Presented

By

ALEXANDRIA WELLS

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DEDICATION

For Mimi

“Good, better, best; Never let it rest; Til the good is better, and the better best”

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ABSTRACT

LET -7 MIRNAS PROGRAM THE FATE OF CD8 T CELLS

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CD8 T cells are the cytotoxic effectors of the adaptive immune response, clearing virally infected and cancerous cells within the host. CD8 T cells acquire their cytotoxic function by differentiating into cytotoxic T lymphocytes (CTLs). Once the CTLs have cleared the antigen, the majority of responding cells will die during contraction; however, a small population of the antigen-specific responders will remain, differentiating into long-lived memory cells that provide potent protection to the host upon re-encounter with the antigen. These differentiation programs often become disturbed in cases of chronic infection and cancer. Instead, CD8 T cells are subverted into the so-called exhausted state, in which cells become functionally inert. As such, the ability to program the differentiation of CD8 T cells into a particular fate is an important therapeutic strategy. We have identified a global post-transcriptional mechanism that determines the fate of differentiating CD8 T cells. Specifically, we have found that the let-7 family of miRNAs is abundantly expressed in naïve CD8 T cells, and that this expression is downregulated upon T cell receptor signaling. The expression of let-7 in naïve CD8 T cells was demonstrated to be necessary and sufficient to maintain the quiescent state of naïve cells. Accordingly, the downregulation of let-7 is necessary for the differentiation of naïve CD8 T cells into cytotoxic T lymphocytes *in vitro*. However, let-7 expression had paradoxical effects on CD8 T cell

function *in vitro* and *in vivo*. Specifically, loss of let-7 expression drives the differentiation of terminal effector CD8 T cells, which in immunosuppressive environments *in vivo*, are diverted into the exhausted state. Accordingly, maintaining let-7 expression restrained terminal effector differentiation, and programmed CD8 T cells to differentiate into highly protective memory cells. These findings may have important implications for the improvement of current immunotherapies targeting CD8 T cell fate.

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CHAPTER 1

INTRODUCTION

1.1 The immune system

The immune system protects the body against infection and cancer. In vertebrates, there are two branches of the immune system that mediate such responses: the innate immune system, and the adaptive immune system. The innate immune system is a relic of evolution from lower organisms and cells of this branch rely on the expression of germline-encoded receptors to recognize conserved molecular patterns associated with multiple pathogens. Cells of the innate immune system respond within minutes of pathogen detection, and contribute to inflammatory responses by producing chemokines and cytokines, and help eliminate pathogens by phagocytosis. Although innate immune responses are essential for the early control of pathogens, the specificity, magnitude, and duration of these responses is limited. On the other hand, the adaptive immune system mounts highly specific, and robust responses that yield lifelong protection to the host (Figure 1.1) (Punt et al., 2019). In fact, the innate immune system is critical in ‘alarming’ the adaptive immune system to the presence of pathogen to ensure effective clearance of the pathogen. Adaptive immune responses are mediated by B and T cells. B cells mature in the bone marrow, and mediate humoral immunity through the production of antibodies. T cells, following maturation in the thymus, aid in pathogen elimination. The function of mature T cells is determined in part by their expression of either the CD4 or CD8 coreceptor. An important function of CD4 T cells is to provide help in the form of cytokine production which contributes to the activation and differentiation of CD8 T cells (Murphy

et al., 2017). CD8 T cells are responsible for the rapid clearance of virally infected and cancerous cells, and store immunological ‘memory’ of these encounters to provide lifelong immunity to the organism. Prior to antigen exposure during such challenges, CD8 T cells are naïve, and lack effector function. Thus, successful protection relies on the appropriate activation and differentiation of CD8 T cells.

1.2 CD8 T cell differentiation

1.2.1 Naive CD8 T cells must be activated to exit the quiescent state

Upon egress from the thymus into secondary lymphoid organs, CD8 T cells are mature, but naïve. Naïve CD8 T cells are maintained in a quiescent state that is characterized by very low rates of proliferation, a ‘quiet’ transcriptional landscape, with no expression of effector molecules, and consequently no cytotoxic function (Surh and Sprent, 2008). Accordingly, these cells have very low metabolic demands, and energy is generated primarily through oxidative phosphorylation (O’Neill et al., 2016). As a direct result of this quiescence, naïve CD8 T cells are small, and round lymphocytes with a nearly absent cytoplasm. The importance of preserving the quiescent state is two-fold; it prevents unsolicited effector activity and maintains a stable population of healthy CD8 T cells in homeostasis. Maintenance of naïve CD8 T cells is achieved through low rates of homeostatic proliferation which is induced in part by the cytokines interleukin-7 (IL-7), and IL-15 (Schluns et al., 2000; Tan et al., 2001; Kimura et al., 2013). IL-7 and IL-15 both signal through heterodimeric cytokine receptors, one subunit of which is the common gamma chain (CD132), while the second subunit is specific to the signaling cytokine. Whereas IL-7 signals exclusively through its own alpha chain (CD127), IL-15 can signal

either through its alpha chain, or the low affinity IL-2 beta chain (CD122) (Sprent and Surh, 2011).

Three signals are required for the activation of naïve CD8 T cells: 1) engagement of the T cell receptor (TCR) with peptide:major histocompatibility class I (MHC-I) complex; 2) co-stimulation; and 3) cytokine signaling. Specifically, CD8 T cells recognize endogenous peptides that are processed, and presented in the context of MHC class I (MHC-I). The CD8 co-receptor binds to an invariant domain on MHC-I, which may serve to stabilize the interaction between the TCR and peptide:MHC complex (Wooldridge et al., 2005). More importantly, this interaction also places the cytoplasmic tail of CD8, which is bound by the kinase Lck, within reach of the immunoreceptor tyrosine-based activation motifs (ITAMs) of CD3, the signaling component of the TCR complex (Marth et al., 1985; Janeway, 1992). Phosphorylation of the CD3 ITAMs by Lck initiates an effective TCR signal. The CD28 co-stimulatory receptor, upon binding of either the B7.1 or B7.2 (CD80 or CD86) ligand, is also phosphorylated by Lck to generate a successful co-stimulation signal (Ledbetter et al., 1990; Holdorf et al., 1999). Several cytokines are important throughout T cell differentiation, with IL-2 being the most critical during the early activation and effector stage of CD8 T cell differentiation (Boyman and Sprent, 2012). This is best exemplified by CD28-mediated upregulation of the high affinity IL-2 receptor (CD25) upon activation (Boyman and Sprent, 2012). Moreover, the distinct signals produced by the TCR and co-stimulation result in the convergence of several transcription factors on the IL-2 promoter to initiate its transcription and production (Jain et al., 1995).

Three important transcription factors involved in the very early activation of CD8 T cells are NF- κ B, NFAT, and AP-1 (Karin et al., 1997; Macian, 2005; Hayden et al.,

2006). As these transcription factors are required to drive transcription of genes immediately after TCR engagement, they must be expressed in naïve cells in an inactive state. Thus, upon CD8 T cell activation, they can be activated and rapidly execute their transcriptional programs. Specifically, NF- κ B is retained in the cytoplasm when bound by its inhibitor, I κ B. TCR signaling results in the phosphorylation of I κ B, which leads to its degradation, freeing NF- κ B to translocate to the nucleus and drive transcription of target genes (Hayden et al., 2006). NFAT is also retained in the cytoplasm by phosphorylation of various serine/threonine residues. Influx of calcium, which is an important signaling event upon TCR engagement, activates calmodulin. Calmodulin then binds and activates calcineurin, a phosphatase that dephosphorylates NFAT, allowing its translocation into the nucleus (Macian, 2005). Finally, MAPK signaling results in the phosphorylation of c-Jun, one subunit of AP-1, in the nucleus. This phosphorylation allows c-Jun to bind its partner c-Fos, thus forming a functional AP-1 complex that can bind DNA and drive transcription (Karin et al., 1997). Importantly, NFAT partners with AP-1 to further amplify T cell activation signals (Jain et al., 1995; Macian, 2005). These transcription factors are responsible for the expression of many other genes needed for T cell activation, in addition to IL-2. Proper regulation of the expression and function of these transcription factors is essential to coordinate productive T cell responses. Some of the consequences of their dysregulation will be discussed later.

1.2.2 CD8 T cell blastogenesis and clonal expansion

The coordinated effects of these three signals result in global changes to the morphology, metabolism, proliferation, and transcriptional activity of the activated CD8 T

cell. The process of acquiring these changes is referred to as blastogenesis. Upon activation, CD8 T cells initiate a growth program that results in the accumulation of cell biomass, particularly in the expanding cytoplasm (Wang et al., 2011). Activation also permits entrance into the cell cycle, which not only facilitates this cell growth, but also rapid proliferation to generate the large numbers of antigen-specific CD8 T cells needed to clear the antigen (Iritani et al., 2002). This proliferative burst is termed clonal expansion, as a responding cell which expresses a TCR specific to a given epitope of the antigen, will only give rise to daughter cells which express the same TCR with the same specificity (Zarozinski and Welsh, 1997; Murali-Krishna et al., 1998). The immediate initiation of cell growth and proliferation programs elicits a dramatic increase in bioenergetic and metabolic demands. To meet these demands, CD8 T cell activation triggers a metabolic switch from oxidative phosphorylation to glycolysis. Although glycolysis is much less efficient than oxidative phosphorylation in terms of ATP production, it results in a high abundance of the biomacromolecular intermediates that are used for DNA and protein synthesis, cell growth and division, and myriad other cellular processes (Warburg, 1956).

The initiation of glycolysis, cell growth, and proliferation is a highly coordinated event, and is mediated largely by the activation of Myc. Myc expression is fully induced by four hours after CD8 T cell activation (Nie et al., 2012), through both TCR and CD28 co-stimulation (Wang et al., 2011, Grzes et al., 2017). Moreover, its expression can be boosted by IL-2 signaling later in blastogenesis (Chou et al., 2014). Myc has previously been demonstrated to direct progression of the cell cycle from the G1 to S phase by inducing expression of G1 cyclins, cyclin dependent kinases, and E2F transcription factors, while simultaneously antagonizing the activity of cell cycle inhibitors (Bretones et al.,

2014). In addition, the expression of glucose transporters, enzymes involved in glycolysis, and protein synthesis enzymes is directly controlled by Myc. While Myc is critical for proliferation and metabolic reprogramming, sustained expression can be toxic to cells (Chang et al., 2000). Thus, Myc expression is transient, and is downregulated by 72 hours after initial TCR stimulation; however, its effects are long-lasting (Chou et al., 2014). This is in part due to the induction of AP-4, a transcription factor that sustains protein synthesis, metabolism, and proliferation, to support the subsequent differentiation into cytotoxic T lymphocytes (CTLs), and the corresponding acquisition of effector function (Chou et al., 2014).

1.2.3 Terminal effector differentiation and the acquisition of cytotoxic function

The majority of CD8 T cells generated during the primary response to antigen will differentiate into cytotoxic T lymphocytes (CTLs), and have fully acquired the ability to kill target cells. CTLs produce cytotoxic granules, which are modified lysosomes that contain effector molecules, primarily perforin, granzyme A, and granzyme B. TCR recognition of the peptide:MHC-I complex presented on the surface of infected or transformed cells will trigger the release of these proteins from the CTL to the target cell. In the CTL, these proteins are contained within modified lysosomes referred to as granules. Restructuring of the secretory machinery in the CTL upon TCR engagement facilitates the transport of these granules directly to the target cell (Stinchombe and Griffiths, 2007). Upon degranulation, perforin generates pores in the membrane of the target cell, allowing the entrance of granzymes. Once inside the target cell, the serine proteases Granzyme A and Granzyme B initiate apoptosis. Granzyme B initiates the intrinsic apoptotic pathway

in two ways: direct cleavage of pro-caspase 3 to activate the proteolytic caspase cascade, and by disrupting the mitochondrial membrane to release cytochrome c. The exact mechanism of Granzyme A-mediated apoptosis is unclear, but likely involves caspase-independent mitochondrial damage (Chowdury and Lieberman, 2008). CTLs also produce cytokines that contribute to the clearance of antigen, namely interferon-gamma (IFN- γ) and tumor necrosis factor- alpha (TNF- α) (Kaech and Wherry, 2007). During viral infection, IFN- γ not only directly prevents viral replication, but also increases expression of MHC-I on target cells to increase detection of infected cells. TNF- α is a pro-inflammatory cytokine that aids in the recruitment and activation of other immune cells to the site of infection to support the CD8 T cell response. If uncontrolled, the CTL response can be extremely devastating. To prevent hyperactivation of the CTL response, activated T cells upregulate the expression of several co-inhibitory receptors, including PD-1, Tim-3, CD160 and 2B4 (Wherry et al., 2007; Blackburn et al., 2009). Although it is known that engagement of these receptors dampens CTL responses, the precise mechanisms of inhibition carried out by each receptor are not fully understood.

The peak of the effector CTL response occurs by seven days post-activation, at which point the majority of the antigen has been cleared (Figure 1.2). In the process of acquiring their effector function, the cells that most efficiently cleared the antigen also acquired several irreversible epigenetic changes which prevents them from differentiating any further. Accordingly, these cells are referred to as terminal effector cells. The resolution of inflammation is characterized by not only the clearance of antigen, but also effector cytokines such as IL-2, and leads to contraction, a massive apoptotic event in which the majority (90-95%) of CTLs die (Kaech and Cui, 2012). Specifically, the cells

cleared during contraction are the terminal effector cells, as they no longer serve a protective purpose to the host. This is another mechanism used to prevent unwarranted inflammation from CTLs which could cause harm to the host if uncontrolled.

1.2.4 Transcriptional regulation of terminal effector differentiation

The magnitude of the terminal effector response is scaled to the severity of the infection (Kaech and Wherry, 2007). Thus, the regulation of terminal effector differentiation and the acquisition of effector function involves a complex transcriptional network that integrates multiple signals to ‘translate’ the degree of inflammation into an appropriate effector response (Kaech and Cui, 2012; Best et al., 2013). The T-box transcription factors T-bet (encoded by *Tbx21*) and Eomesodermin (Eomes), were two of the first transcription factors discovered to regulate effector differentiation (Szabo et al., 2000; Pearce et al., 2003). T-bet and Eomes facilitate the acquisition of effector function by coordinated upregulation of the effector molecules IFN- γ , granzyme B, and perforin. While this is accomplished through partially redundant mechanisms, T-bet and Eomes also control discrete stages of effector differentiation, and thus their expression is somewhat reciprocal (Intlekofer et al., 2007; Kaech and Cui, 2012). T-bet expression peaks early during effector differentiation and is downregulated as CTL differentiation progresses, while Eomes expression peaks later, and is downregulated closer to contraction (Joshi et al., 2011; Kakaradov et al., 2017). This is likely due to TCR-mediated induction of T-bet, while Eomes expression is dependent on IL-2 signaling, and the transcription factor Runx3 (Cruz-Guilloty, 2009; Pipkin et al., 2010). Importantly, the levels of Eomes and T-bet expression are modulated by cytokine signaling, which has important consequences for the

fate of differentiating CD8 T cells. IL-2 signaling amplifies both T-bet and Eomes expression to support CTL differentiation and the acquisition of effector function (Kalia et al., 2009; Pipkin et al., 2010). Alternatively, IL-12 amplifies T-bet and represses Eomes expression to drive cells into the terminal effector state (Takemoto et al., 2006; Joshi et al., 2007). This is accomplished by IL-12-mediated enhancement of mTOR (mammalian target of rapamycin) activity (Rao et al., 2010).

Other transcriptional regulators also make important contributions to terminal effector differentiation. In addition to upregulating Eomes, Runx3 directly regulates granzyme B expression, and synergizes with Eomes and T-bet to promote IFN- γ and perforin expression (Cruz-Guilloty et al., 2009). The transcriptional repressor, Blimp-1 (encoded by the gene *Prdm1*) promotes CTL migration to the site of inflammation, and also drives the expression of effector molecules (Kallies et al., 2009; Kaech and Cui, 2012). The latter role of Blimp-1 is accomplished through redundant functions with T-bet, although deficiencies in T-bet or Blimp-1 alone are sufficient to impair the differentiation of terminal effector cells (Kallies et al., 2009; Xin et al., 2016). The expression of Blimp-1 is driven by the transcription factor IRF4 (Interferon-responsive factor 4), the expression and activity of which is driven by the strength of TCR signaling. Specifically, strong TCR signaling increases IRF4 expression and activity to promote the terminal differentiation of CD8 T cells (Man et al., 2013; Nayar et al., 2014).

Notch is another critical transcriptional regulator of CTL differentiation. Notch expression is upregulated on CD8 T cells by TCR signaling, and by an mTOR-dependent mechanism driven by type I interferons produced during viral infection (Cho et al., 2009; Backer et al., 2014). IL-2 signaling can further amplify Notch expression. Notch-1 was

demonstrated to induce T-bet expression, which in turn further upregulates Notch-1 in a positive feedforward loop that drives transcription of effector molecules (Backer et al., 2014). Additionally, inhibitor of DNA binding 2 (Id2), which functions primarily by preventing E protein transcription factors from binding DNA, promotes terminal effector differentiation by supporting the survival of differentiating cells (Cannarile et al., 2006; Yang et al., 2011; Knell et al., 2013). Consistent with a role in supporting survival, Id2 expression is induced later during the terminal effector differentiation program by IL-2, IL-12, and IL-21 cytokine signaling (Yang et al., 2011). Thus, increased production of these cytokines during the immune response will lead to sustained CTL responses through induction of Id2 (Yang et al., 2011).

In addition to cytokine signaling and mTOR, access to target gene promoters is another important determinant of transcription factor activity, and consequently, expression. In fact, dynamic changes are made to the chromatin landscape of differentiating CD8 T cells and play an important role in modulating the CD8 T cell response (Scott-Browne et al., 2016). What signals determine the extent of the epigenetic changes made during CD8 T cell differentiation remain to be determined. In general, the mechanisms that modulate transcription factor expression and activity to titrate the magnitude of the CTL response according to the demands of the infection are not fully understood. As such, identifying a global regulatory mechanism that ‘translates’ the degree of inflammation by tuning the expression of these transcription factors will be important for better understanding terminal effector differentiation and for identifying new therapeutic targets.

1.2.5 Memory CD8 T cells

Although roughly 90% of responding CD8 T cells die during contraction, approximately 5-10% survive to form the memory CD8 T cell population (Figure 1.2). Memory CD8 T cells are incredibly important as they provide life-long protection to the host (Williams and Bevan, 2007). Accordingly, memory CD8 T cells express high levels of anti-apoptotic proteins, such as Bcl-2, to support both survival through contraction and longevity. These cells ‘remember’ the initial encounter with antigen, such that upon subsequent re-encounter, they will respond with a greater magnitude and more quickly, than cells encountering the antigen for the first time.

The CD8 T cells that will survive contraction to populate the memory pool can be identified during the effector phase of the CD8 T cell response, and are referred to as memory precursor effector cells (MPECs) as they have not yet fully differentiated into memory cells. MPECs are identified by low expression of the terminal effector marker KLRG1, and high expression of the IL-7 receptor (Joshi et al., 2007), as memory cells are dependent on IL-7 signaling to survive. MPECs acquire several molecular, metabolic, and functional alterations as they transition into the memory state. Although all memory cells are characterized by a return to quiescence, the changes acquired are not uniform amongst the differentiating MPECs. The memory CD8 T cell pool is heterogenous and is comprised of four subsets of memory CD8 T cells that can be classified by their anatomic localization, proliferative capacity, effector function, and retained differentiation potential (Gebhardt et al., 2009; Masopust et al., 2010). The most differentiated subset of memory CD8 T cells are tissue resident memory cells which, as their name implies, reside in non-lymphoid peripheral tissues, such as the skin, liver, lung, and white adipose tissue (Masopust et al., 2001). As these cells populate tissues, they serve as a first line of defense and thus retain

high expression of transcripts encoding effector molecules (Gebhardt et al., 2009). Although they proliferate very minimally in homeostasis, they proliferate very rapidly upon restimulation (Schenkel and Masopust, 2014). The least differentiated memory subset, stem cell memory cells, display features of stemness. They have relatively high proliferative capacity, increased self-renewal potential, and importantly, the ability to seed all memory subsets (Gattinoni et al., 2011; Gattinoni et al., 2017).

The two remaining subsets of memory CD8 T cells, effector memory CD8 T cells and central memory CD8 T cells, lie somewhere in between, with effector memory cells being more differentiated, and central memory being less differentiated. These two populations are the best characterized, and their features are broadly used to identify and study memory cells. Accordingly, these two populations, will be the primary focus herein. Consistent with their name, effector memory cells retain higher expression of effector molecules than central memory cells (Sallusto et al., 1999). While effector memory cells circulate throughout the bloodstream, central memory cells home back to secondary lymphoid organs. Thus, central memory cells highly express L-selectin (CD62L) which mediates rolling along the high endothelial venules that lead back to lymph nodes, and the chemokine receptor CCR7, which facilitates lymphocyte migration into, and subsequent retention in secondary lymphoid organs. Although central memory cells have a higher proliferative potential than effector memory cells, both populations undergo low levels of homeostatic proliferation and rely on IL-7 and IL-15 signaling for long term survival (Schluns et al., 2000; Surh et al., 2006). Moreover, both populations of cells will switch their metabolism back to oxidative phosphorylation as there is now a significant decrease in bioenergetic demands, and instead a need to maintain quiescence (van der Windt et al.,

2012). In addition, glutaminolysis has recently been demonstrated to improve the survival of memory precursor cells during the transition into mature memory cells, and their function upon antigen re-encounter (Geiger et al., 2016). Several *in vitro* studies have suggested that memory CD8 T cells also rely on fatty acid beta-oxidation, although it is controversial whether this is consistent *in vivo* (Pearce et al., 2009; van der Windt et al., 2012; Raud et al., 2017).

Despite the many differences between these subsets of memory CD8 T cells, the successful differentiation and maintenance of each population is important for sustaining life-long immunity, yet very little is known regarding this process.

1.2.6 Proposed mechanisms of memory differentiation

In general, all naïve CD8 T cells are considered to have the same potential to become a memory cell, and that memory cells are less differentiated than effector cells (Sallusto et al., 2004; Joshi et al., 2007; Gattinoni et al., 2011). Accordingly, it is likely that over the course of CD8 T cell differentiation, memory potential is lost, rather than gained (Joshi et al., 2007; Kaech and Cui, 2012; Chang et al., 2014; Chen et al., 2018). Although the latter has been proposed to occur through the ‘de-differentiation’ of effector cells, this has only been demonstrated in a limited subset of ‘permissive’ effector cells, and fails to account for the heterogeneity of the memory pool (Akondy et al., 2017; Youngblood et al., 2017). Thus, the two prevailing models of memory CD8 T cell differentiation support the concept that memory potential is lost during CD8 T cell differentiation (Figure 1.3). The first model accounts for the effects compounding signals have on the differentiating CD8 T cell, while the second model accounts for the strength of these signals. The first model

is the progressive differentiation model, and proposes that the accumulation of signals a CD8 T cell receives, either antigenic or cytokine-derived, during differentiation gradually pushes cells into a more terminal effector state, thus diminishing memory potential (Joshi et al., 2007; Sarkar et al., 2008; Gray et al., 2017). Studies in support of this model demonstrate that reducing antigen exposure time drives memory formation (Sarkar et al., 2008), although this could also indicate that the duration of TCR signaling is an important determinant of memory potential. More recently, the importance of signal strength during priming has been incorporated into this model (Figure 1.3) (Daniels and Teixeira, 2015). The second model proposes that both an effector and a memory cell are generated simultaneously from a single primed CD8 T cell via asymmetric division of this cell (Figure 1.3). Upon stimulation by an antigen presenting cell, a T cell will give rise to two daughter cells. The asymmetric division model suggests the daughter cell proximal to the antigen presenting cell is fated to become a terminal effector, as it receives stronger TCR stimulation. The distal daughter cell retains its memory potential, as it receives weaker TCR stimulation (Chang et al., 2007; Chang et al., 2011; Verbist et al., 2016). This model is consistent with reports demonstrating strength of TCR signaling can determine CD8 T cell fate (Chen et al., 2018).

An important discrepancy between these models is whether the timing of signaling, in addition to the accumulation and strength of signal, is relevant to the differentiation of memory CD8 T cells. The progressive differentiation model suggests signaling throughout differentiation is relevant, whereas the asymmetric division model suggests the initial priming event is when the memory fate is specified. However, both models indicate TCR signaling is an important determinant of memory potential, and in reality, there is likely to

be some combination of these proposed mechanisms involved in the generation of such a heterogeneous memory pool.

1.2.7 Transcriptional regulation of memory differentiation

Despite controversy in how and when the memory fate is specified, significant progress has been made in understanding the transcriptional network that is involved in the differentiation and maintenance of CD8 T cell memory. Of particular importance is the transcription factor Tcf1 (encoded by the gene *Tcf7*), which is a downstream component of Wnt signaling. Tcf1 is highly expressed in naïve cells, downregulated upon activation, and then partially re-expressed in memory CD8 T cells (Zhou et al., 2010). The first evidence of a role for Tcf1 in memory cells was demonstrated by constitutive activation of the Wnt signaling pathway, which enhanced memory CD8 T cell differentiation (Gattinoni et al., 2011; Zhou et al., 2010). It was subsequently demonstrated that loss of Tcf1 expression in mature CD8 T cells impaired the maintenance of the memory compartment as a result of diminished CD122 and Bcl-2 expression, and compromised homing to secondary lymphoid organs (Zhou et al., 2010). Tcf1 expression is in part controlled by Foxo1, which also helps drive expression of the genes *Cd127*, *Bcl-2*, *Sell* (encodes CD62L), and *Ccr7*.

Another important function of both Tcf1 and Foxo1 is to promote expression of Eomes. In fact, in addition to its role in effector differentiation, Eomes is critical to the generation of memory cells. Eomes directly upregulates expression of CD122, to support memory CD8 T cell survival. Accordingly, while Eomes-deficient mice generate memory precursor cells in response to viral challenge, they fail to form a mature memory population (Kaech and Cui, 2012). Eomes is not the only transcription factor involved in both effector

and memory differentiation. In fact, Runx3 has also been demonstrated to drive memory formation early during CD8 T cell differentiation (Wang et al., 2018). Although Eomes and Runx3 appear to have dual roles in CD8 T cell differentiation, the majority of transcription regulators involved in terminal differentiation must be actively repressed to generate memory cells. Transcription factors, such as Bcl-6 and Id2, are upregulated in memory cells in part to suppress expression of Blimp-1 and Id3, respectively. Expression of Bcl-6, and Eomes, are upregulated in a STAT3-dependent mechanism downstream of cytokine signaling in the effector to memory transition during contraction (Cui et al., 2011), supporting models in which the memory fate is specified throughout differentiation.

The epigenetic landscape is an additional level of regulation that determines the transcriptional activity during memory CD8 T cell differentiation. Moreover, the enhanced responses of memory cells are proposed to be a result of epigenetic ‘re-wiring’ during the primary response that keeps cells poised to proliferate and produce effector molecules upon antigen re-encounter (Scott-Browne et al., 2016; Akondy et al., 2017; Gray et al., 2017; Youngblood et al., 2017; Yu et al. 2017). However, the transcription factors involved in establishing and maintaining the chromatin landscape of memory CD8 T cells are only beginning to be identified (Pace et al., 2018; Wang et al., 2018). Notably, one of these factors, Runx3, exerts its effects by establishing a chromatin landscape associated with memory cells during TCR stimulation to prevent terminal effector differentiation (Wang et al., 2018), in support of models in which the memory cell fate is programmed during TCR stimulation.

Although the transcriptional regulation of memory differentiation has been well-studied, it is not fully understood. As some of these results suggest, a better understanding

of the transcriptional control of memory differentiation may provide insight into how and when the memory fate is specified.

1.2.8 CD8 T cell exhaustion

The proper differentiation of terminal effector and memory CD8 T cells is intrinsically tied to the ability of these cells to effectively clear the antigen. In cases of chronic infection and cancer, when the antigen fails to be cleared, CD8 T cells are diverted into a compromised state, termed exhaustion (Zajac et al., 1998; Gallimore et al., 1998; Schietinger et al., 2016). Broadly, exhaustion is defined as the loss of effector function, which occurs in a hierarchical manner. First, effector CTLs lose the ability to produce IL-2, proliferate at high rates, and kill target cells, followed by loss of IFN- γ and TNF- α production, and the ability to degranulate, with the most severe form of exhaustion leading to the death of the CD8 T cell (Figure 1.4) Wherry et al., 2003; Wherry, 2011).

Exhaustion is established by the chronic TCR stimulation that results from persistent antigen exposure (Schietinger et al., 2016). This continual TCR signaling drives the upregulation of co-inhibitory receptors, including PD-1, Tim-3, CD160 and 2B4 (Schietinger et al., 2016). Engagement of these inhibitory receptors in immunosuppressive environments maintains exhausted cells in a dysfunctional state (Schietinger and Greenberg, 2014). The best characterized of these receptors is PD-1. The importance of PD-1 in suppressing T cell function was first demonstrated by the spontaneous induction of autoimmunity in PD-1 deficient mice (Nishimura et al., 1996; Nishimura et al., 1998). The ligands for PD-1, PD-L1 and PD-L2, are expressed on a variety of immune and non-immune cells, including cancer cells, in response to interferons produced during the

immune response (Sun et al., 2018). Binding of PD-L1 or PD-L2 to PD-1 was demonstrated *in vitro* to cluster PD-1 with the TCR and Lck, and to recruit the phosphatases SHP-1 and SHP-2 to bind a conserved motif in the intracellular tail of PD-1 (Chemnitz et al., 2004; Sheppard et al., 2004; Yokosuka et al., 2012). This combination of events was thought to bring SHP-1 and SHP-2 into contact with TCR signaling components to dephosphorylate the TCR, and suppress Lck activity, thus dampening TCR signaling (Sheppard et al., 2004; Yokosuka et al., 2012). It has also been suggested that PD-1 signaling not only dampens TCR signals, but also co-stimulatory signaling (Hui et al., 2017; Kamphorst et al., 2017). However, the recent generation of SHP-2-deficient mice demonstrated that SHP-2 is dispensable for PD-1-mediated suppression of T cell responses *in vivo* (Rota et al., 2018). Thus, the exact molecular events driven by PD-1, as well as the other inhibitory receptors, remain to be elucidated.

In addition to inhibitory receptor engagement, the cytokine milieu of the inflammatory environments associated with chronic viral infections and cancer also suppresses terminal effector cell function. TGF- β and IL-10 are the most prominent cytokines to be implicated in suppressing T cell function. In fact, TGF- β -deficient mice die from uncontrolled inflammation in vital organs (Diebold et al., 1995). TGF- β is produced by several cells in the immunosuppressive environment, including antigen presenting cells and T cells (Flavell et al., 2010; Caja and Vannucci, 2015), and directly inhibits the proliferation and cytotoxic function of CD8 T cells (Thomas and Massague, 2005; Stephen et al., 2014). Innate immune responses are also dampened by TGF- β which can additionally contribute to weakened CD8 T cell responses in the presence of this cytokine (Flavell et al., 2010). Similar to TGF- β , IL-10 is produced by multiple immune cells, including

antigen presenting cells and T cells (Blackburn and Wherry, 2007; Filippi and von Herrath, 2008). IL-10 negatively regulates several features of the CD8 T cell immune response, such that depletion of IL-10 restores CD8 T cell function during chronic infection (Brooks et al., 2006; Ejrnaes et al., 2006). Specifically, IL-10 indirectly contributes to CD8 T cell dysfunction by reducing MHC-II expression on antigen presenting cells to limit CD4 T cell help, and directly by restraining CD8 T cell proliferation and cytokine production (Brooks et al., 2006; Ejrnaes et al., 2006). Interestingly, IL-10 production is increased in exhausted T cells, suggesting that these cells may contribute to their own immunosuppression. It has been hypothesized this may be a preventative mechanism to protect against tissue damage during prolonged immune responses against chronic infection (Blackburn and Wherry, 2007).

The decided impact of inhibitory receptors and suppressive cytokines on the function of CD8 T cells has led to a concerted effort to develop therapies that can overcome these negative signals to revive CD8 T cell function in cancer and chronic infection. The most successful of these so-called immunotherapies are primarily targeted against the inhibitory receptor, PD-1, and its ligand, PD-L1 (Weber, 2010; Brahmer et al., 2012; Topalian et al., 2012). The therapeutic targeting of the PD-1-PD-L1 axis is achieved by blocking antibodies which prevent the receptor-ligand interactions, and is referred to as checkpoint blockade inhibition (CBI) (Weber, 2010; Brahmer et al., 2012; Topalian et al., 2012; Sharma and Allison, 2015). The therapeutic success of CBI against PD-1 guaranteed the two scientists who discovered PD-1 and developed this therapy, Tasuku Honjo and James Allison, the 2018 Nobel Prize in Physiology or Medicine.

However, positive CD8 T cell responses to CBI are not guaranteed, and are not uniform amongst responding CD8 T cells (Blackburn et al., 2008; Paley et al., 2012). It has become evident that the exhausted CD8 T cell pool is not homogenous (Blackburn et al., 2008; Blackburn et al., 2009; Paley et al., 2012). In fact, there are distinct populations of exhausted cells which retain different degrees of effector function, and the potential to be ‘revitalized’ through checkpoint blockade inhibition (Im et al., 2016, Utzschneider et al., 2016; Siddiqui, et al., 2019; Kurtulus et al., 2019). These are likely cells that are at different stages in the gradual acquisition of exhaustion. Moreover, the pattern of inhibitory receptor expression is not uniform across exhausted CD8 T cells, and can group exhausted cells into clusters with different effector functions (Jin et al., 2010; Blackburn et al., 2009; Singer et al., 2016; Chihara et al., 2018). As such, expression of inhibitory receptors does not preclude CD8 T cells to exhaustion, although it is certainly a driving force. Consequently, the better characterizing the distinctions between the terminal effector and exhaustion programs is an active area of research (Singer et al., 2016), and will be important for the improved use of CBI in the clinic.

1.2.9 Transcriptional regulation of CD8 T cell exhaustion

As the exhausted state is established as a result of chronic TCR stimulation, the exhaustion program is driven by dysregulation of many of the transcription factors involved in terminal effector differentiation. As such, two of the first transcription factors identified to promote the exhaustion were T-bet and Eomes (Wherry et al., 2007; Blackburn et al., 2008). In fact, the relative expression of these two transcription factors identified CD8 T cells in different stages of exhaustion (Blackburn et al., 2008; Buggert et al., 2014).

Specifically, high T-bet expression sustained the ‘progenitor’ population of exhausted T cells, and the gradual loss of T-bet expression resulted in the upregulation of Eomes, consequently giving rise to terminally exhausted cells (Kao et al., 2011; Paley et al., 2012; Doering et al., 2012). Blimp-1 was also reported to be highly expressed in exhausted cells, and was demonstrated directly induce inhibitory receptor expression (Shin et al., 2009).

Additionally, upregulation of transcription factors induced by the strength and duration of TCR signaling, such as IRF4 and NFAT, were identified early on to drive the exhaustion program, and were demonstrated to increase expression of inhibitory receptors (Wherry et al., 2007; Martinez et al., 2015; Man et al., 2017). Importantly, in effector cells NFAT forms complexes with the AP-1 transcription factor complex to drive effector gene expression. It was reported that NFAT-AP-1 complexation is disrupted in exhausted cells, and that failure to form this complex allows NFAT to bind alternative regions of DNA, including the promoters of genes such as PD-1 (Martinez et al., 2015). In fact, it was recently demonstrated that in exhausted cells miR-155 targets a component of the AP-1 complex to prevent its expression, and thus contributes to increased transcription by ‘partnerless’ NFAT (Stelekati et al., 2018). However, such defined roles for miRNAs in exhaustion are extremely limited. Similarly, the epigenetic control of transcription factor expression and activity in driving exhaustion has only begun to be explored. As CD8 T cells progress further into the terminal differentiation program, their epigenetic state is ‘rewired’ towards exhaustion (Youngblood et al., 2011). This is mediated by deposition of repressive marks that ‘close off’ of gene accessibility, determining transcriptional programs that are executed, and contributing to the gradual progression of exhaustion (Pauken et al., 2016; Schietinger et al., 2016; Sen et al., 2016). However, the factors which

mediate the epigenetic and transcriptional regulation of exhaustion have not been fully identified. Moreover, how these factors themselves are regulated during the transition into exhaustion is not completely understood.

1.2.10 CD8 T cell differentiation: concluding remarks

The transcriptional network driving CD8 T cell differentiation is clearly very complex and requires tight regulation, as any modification to the timing and magnitude of gene expression can alter the fate of the differentiating T cell. These different populations of CD8 T cells are maintained by distinct transcriptional profiles which are dictated by extracellular signals such as TCR stimulation, co-stimulation, and inflammation. As each CD8 T cell population has different protective capacities, being able to direct cells away from one fate into another has therapeutic implications. However, a major hurdle in implementing such an approach has been to identify global molecular hubs which can simultaneously integrate and translate the multiple signals a T cell receives into a coordinated transcriptional program to control CD8 T cell fate. One less studied mechanism that may provide such global control of CD8 T cell differentiation is post-transcriptional regulation mediated by microRNAs.

1.3 miRNAs and their role in CD8 T cells

1.3.1 miRNA biogenesis and biology

MicroRNAs (miRNAs) regulate multiple important biological processes, including cell differentiation, in a tissue-specific manner through a post-transcriptional mechanism that is based on sequence-specific inhibition of mRNA translation (Fire et al., 1998). The tissue-

specific functions of miRNAs are in part attributed to their genomic organization (Ha and Kim, 2014; Treiber et al., 2018). MiRNA-encoding genes can be located in intergenic regions, in which the upstream promoter exclusively drives expression of the miRNA (Figure 1.5) (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). However, genes encoding miRNAs can also be intronic, in which they are located as introns between exons of another gene, thus sharing the promoter with the flanking gene (Figure 1.5) (Rodriguez, et al., 2004). In this case, the gene encoding the miRNA will be transcribed when the flanking gene is transcribed, and will be spliced out during mRNA processing (Rodriguez, et al., 2004). In both situations (intergenic and intronic) genes encoding miRNAs can be either monocistronic, in which a single miRNA-encoding gene is transcribed by a single promoter, or they can be polycistronic, in which multiple miRNA-encoding genes are clustered together to share a common promoter (Figure 1.5) (Lee et al., 2002; Lagos-Quintana, 2003).

Genes encoding miRNAs are first transcribed as a primary transcript by RNA PolII and PolIII, called primary miRNAs (pri-miRNA), which may vary in size, but are usually very long (Figure 1.6A) (Ha and Kim, 2014). While still in the nucleus, the pri-miRNA is processed into the hairpin-like 60-70 nucleotides (nt) precursor miRNA (pre-miRNA) by the microprocessor, a complex containing the endonuclease Drosha (Lee et al., 2002; Denli et al., 2004). Next, the pre-miRNA is exported from the nucleus to the cytoplasm, where the pre-miRNA stem loop is cleaved by the endonuclease Dicer, generating a mature 21-24 nt double stranded miRNA (Figure 1.6B) (Ha and Kim et al., 2014). A single strand of this mature miRNA is then loaded into the RNA induced silencing complex (RISC), which can prevent protein synthesis either by stalling the ribosomal machinery or by destabilizing

the targeted mRNA (Ha and Kim, 2014). The sequence with which miRNAs bind their RNA targets is known as the “seed sequence”, is typically 6-8 nt long, and is located at the 5’ end of the miRNA. Based on the conservation of this seed sequence, miRNAs are grouped into families, such that all members of one miRNA family have the same target mRNAs. Usually the binding site for the miRNA seed sequence is located in the 3’ untranslated region (UTR) of the target mRNA, although they can be located within the open reading frame (ORF) as well (Lai, 2002).

1.3.2 miRNAs in CD8 T cells

The importance of miRNAs in T cells was first demonstrated through the ectopic expression of various miRNAs during hematopoiesis (Chen et al., 2004). Later, it was found that miRNA depletion achieved by a T cell- specific deletion of Dicer, resulted in severe defects not only in T cell development (Muljo et al., 2005), but also in aberrant proliferation, differentiation, and function of mature peripheral T cells (Zhang and Bevan, 2010). More recently, roles for specific miRNAs have been identified throughout CD8 T cell differentiation (Wu et al., 2007). miR-17-92 was demonstrated to drive CD8 T cell proliferation upon activation, and thus promoted memory differentiation (Wu et al., 2012; Steiner et al., 2011). Several miRNAs have been implicated in exhaustion, including miR-155, and mir-31 (Gracias et al., 2013; Moffett et al., 2017). The latter was reported to control AP-1 expression, thus potentially driving T cell exhaustion by promoting the partnerless NFAT transcription program (Stelekati et al., 2018). Interestingly, NFAT was reported to induce the expression of miR-31 during chronic infection, resulting in the upregulation of several inhibitory molecules (Moffett et al., 2017).

Despite these important studies, the current understanding of miRNA mediated regulation of CD8 T cell differentiation is extremely limited. Moreover, while these studies demonstrate miRNA involvement in single stages of CD8 T cell differentiation, a miRNA family with global regulatory control of CD8 T cell differentiation has yet to be identified. Further, the majority of these studies have focused on miRNAs that are upregulated upon activation, thus miRNA-mediated maintenance of naïve CD8 T cells is similarly lacking.

1.4 let-7 miRNAs

1.4.1 The let-7 miRNA family and its regulation

Let-7 is one of the most highly conserved families of miRNAs in the animal kingdom (Wightman et al., 1993; Use the "Insert Citation" button to add citations to this document. Pasquinelli et al., 2000). Originally identified in *C. elegans*, the let-7 (lethal-7) miRNAs are so named because their deletion in *C. elegans* led to the death of the worm (Reinhart et al., 2000). Having undergone several duplications in evolution, the let-7 family is comprised of multiple paralog genes expressed on different chromosomes, forming the largest miRNA family in mammals (Roush and Slack, 2008). In fact, sequences of mature let-7 miRNAs are often identical although they are derived from different precursors and genes (Bussing et al., 2008). To indicate the differences in sequence across genes, a letter is placed after *let-7* (*i.e.*, *let-7a*, *let-7b*), and a number is placed after this letter to indicate that the same sequence of gene is expressed in multiple genomic locations (*i.e.*, *let-7c-1*, *let-7c-2*). In humans, there are 10 mature let-7 family members generated from 13 precursor sequences, and in mice there are 8 mature family members arising from 11 precursor miRNAs (Bussing et al., 2008; Roush and Slack, 2008).

It has been demonstrated that let-7 miRNAs are involved in multiple biological processes including differentiation, cell death, and metabolism (Bussing et al., 2008; Zhu et al., 2011). Specifically, let-7 miRNAs were identified as potent tumor suppressors that directly target mRNAs of genes involved in the cell cycle and in signal transduction pathways that lead to carcinogenesis (Bussing et al., 2008). Although let-7 miRNAs can be found in many types of cells and tissues, the expression levels vary, revealing complicated regulation. In fact, let-7 miRNA expression is post-transcriptionally regulated by multiple factors that control different stages of let-7 biogenesis. Lin28 and Lin28B are well studied fetal proteins, that block the generation of mature let-7 miRNAs (Piskounova et al., 2008). Both proteins interfere with Dicer processing by binding to a highly conserved sequence within the stem loop of let-7 miRNA precursors and recruiting the terminal uridylyl transferases (TUTases), which uridylate the immature miRNA, allowing the exonuclease Dis3I2 to recognize and degrade it (Heo et al., 2009; Faehnle et al., 2014). This particular mechanism is used to inhibit global let-7 expression during early embryogenesis. Therefore, Lin28-mediated modulation of let-7 miRNA levels has been implicated during early embryogenesis and in establishing the immune responses of neonates (Bussing et al., 2008; Wang et al., 2016).

1.4.2 let-7 miRNAs in the immune system

Interestingly, it has been shown that the let-7 family of miRNAs are the most abundant miRNAs in T cells, and control the differentiation and function of natural killer T cells, an innate-like subset of T cells (Pobezinsky et al., 2015; Yuan et al., 2012). Further, a regulatory role has been suggested for the let-7 miRNAs in controlling the CD4 T helper

response (Polikepahad et al., 2010; Swaminathan et al., 2012; Guan et al., 2013), and in contributing to the function of Tregs (Okoye et al., 2014). A previously published miRNA profile of CD8 T cells reported high levels of let-7a and let-7f expression in naïve cells, and reduced expression in effector CD8 T cells (Wu et al., 2007). However, the extent to which let-7 miRNAs regulate the maintenance, proliferation, differentiation, and function of CD8 T cells has yet to be explored.

1.5 Hypothesis and specific aims

The central hypothesis of this dissertation is that the let-7 miRNAs act as a molecular control hub, regulating the fate of differentiating CD8+ T cells. First, the role of the let-7 miRNAs in maintaining the naïve CD8 T cell state, and promoting the differentiation of terminal effector CD8 T cells will be determined (Figure 1.7). Second, the function of let-7 miRNAs in regulating the differentiation of memory and exhausted CD8 T cells will be defined (Figure 1.7). The research proposed herein is expected to establish the let-7 miRNAs as critical regulators of CD8 T cell immune responses. The elucidation of the let-7-mediated mechanism regulating the maintenance of naïve CD8 T cell quiescence, and the differentiation of terminal effector, memory, and exhausted CD8 T cells will offer new insights into how TCR signaling determines CD8 T cell fate and function. Further, if the central hypothesis is correct, it has the potential to distinguish the let-7 miRNAs as a novel therapeutic target capable of modulating CD8 T cell responses during autoimmunity, infection, and cancer.

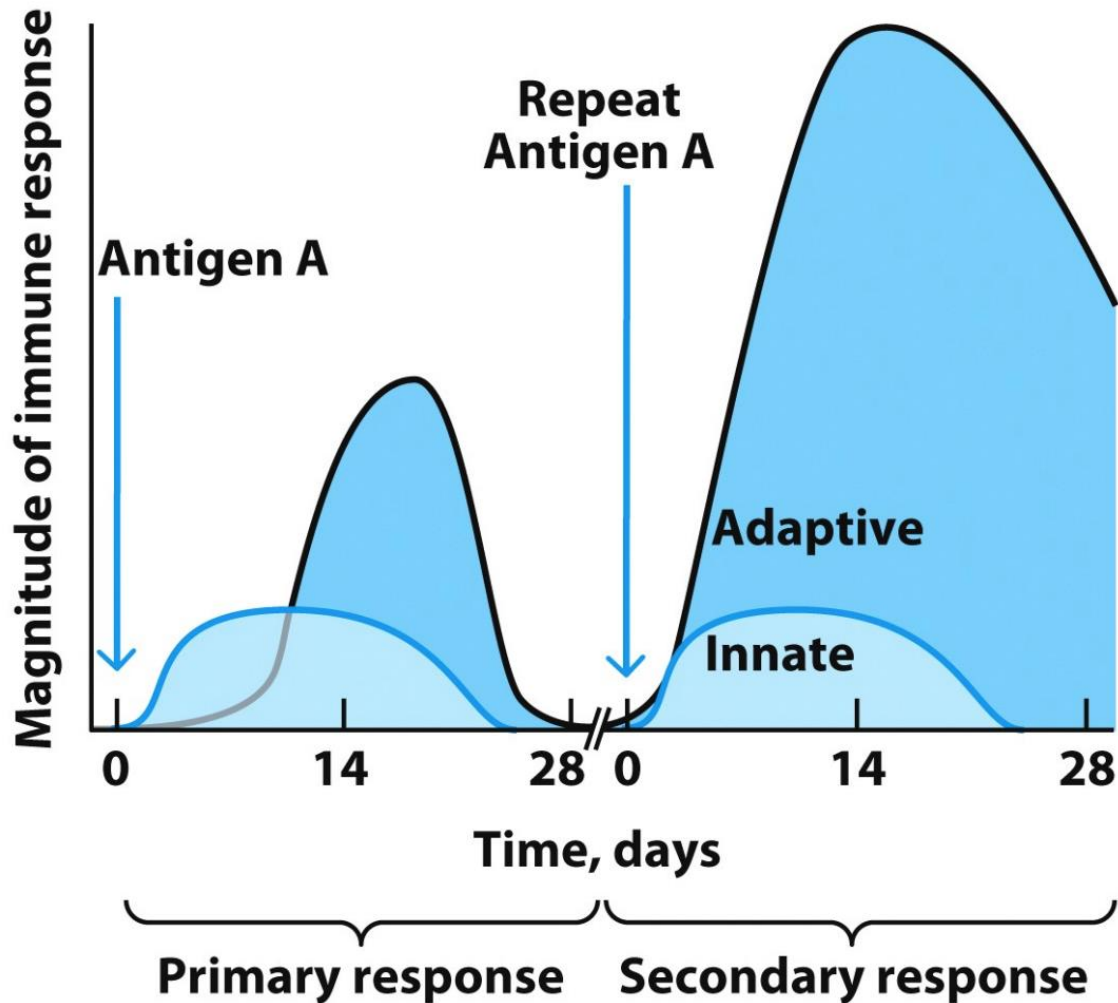


Figure 1-8
Kuby Immunology, Seventh Edition
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Figure 1.1 Magnitude of immune responses

Although the innate immune system responds more quickly than cells of the adaptive immune system, the magnitude of these responses are weaker and fixed. Conversely, the expression of antigen specific receptors and ability to generate immunological memory facilitate the more robust primary and secondary responses yielded by the adaptive immune system. Adapted from *Kuby Immunology*, 7th e.

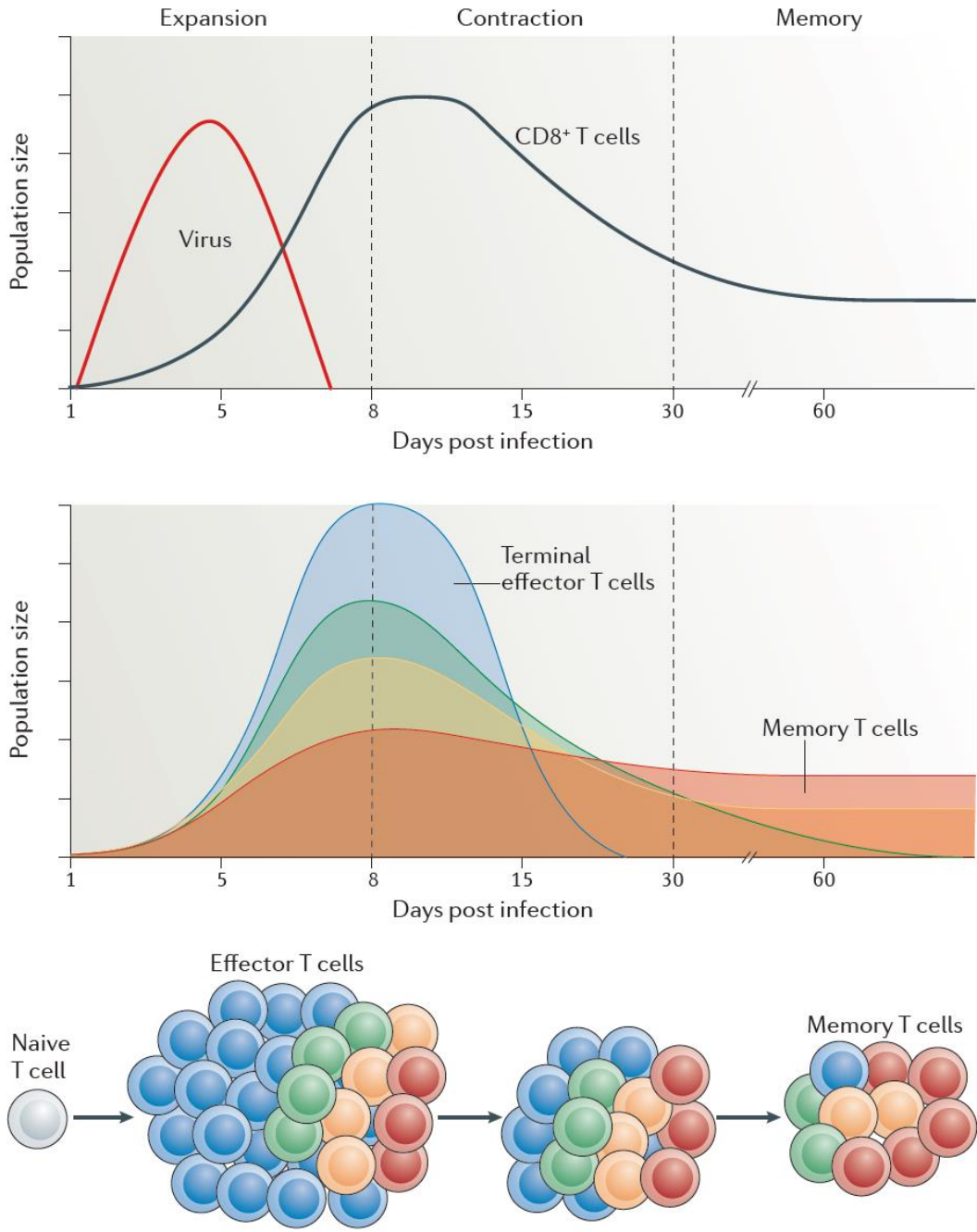


Figure 1.2 Kinetics of the CD8 T cell response

The CD8 T cell response peaks at about 8 days after infection, when the virus or target antigen has been cleared. Following clearance of the antigen, the majority of CD8 T cells die during contraction (top panel). The majority of responding cells are terminal effectors, which do not survive contraction, while a small population of responding cells differentiate into memory cells that survive long term in the host (bottom panel). Adapted from Kaech and Cui, 2012)

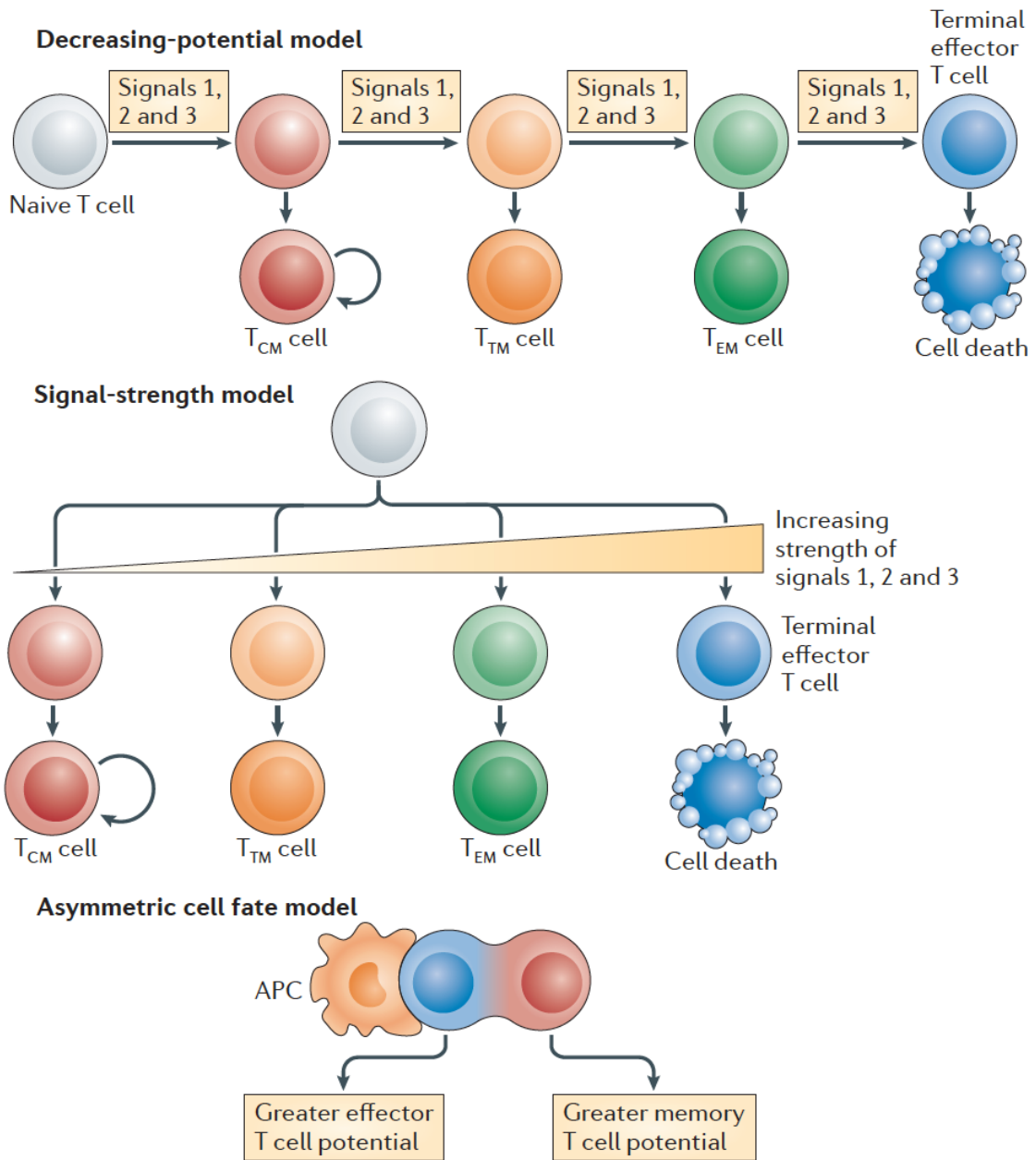


Figure 1.3 Memory differentiation models

The decreasing potential model suggests that with every subsequent signal a CD8 T cell receives, it becomes more terminally differentiated, progressively losing memory potential (top panel). This model has recently been incorporated with the signal strength model, in which increasing strength of signal reduces memory potential by driving terminal effector differentiation (middle panel). The asymmetric fate model suggests that proximity to the antigen presenting cell (APC) determines memory fate, where the distal daughter retains memory potential, and the proximal daughter is fated to become an effector cell (bottom panel). T_{CM}: central memory T cell; T_{TM}: transitional memory T cell; T_{EM}: effector memory T cell. Adapted from Kaech and Cui, 2012.

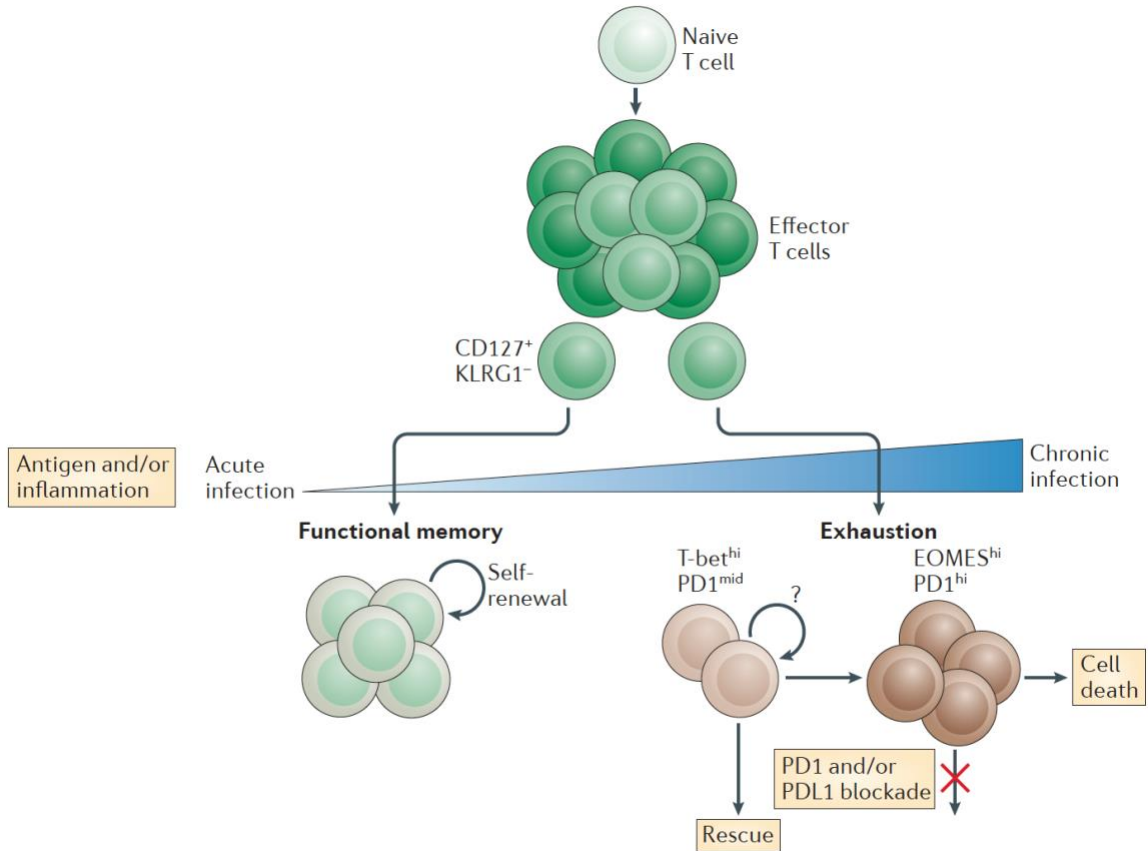


Figure 1.4 Diversion into exhaustion

Persistent antigen exposure diverts effector CD8 T cells into an exhausted, preventing the formation of functional memory cells. The establishment of exhaustion is hierarchical, in which some exhausted cells are able to be rescued (T-bet^{hi}PD-1^{mid}) by checkpoint blockade, and others have reached the terminal exhausted state (Eomes^{hi}PD-1^{hi}), in which cells do not respond to checkpoint blockade and die. Adapted from Wherry and Kurachi, 2015.

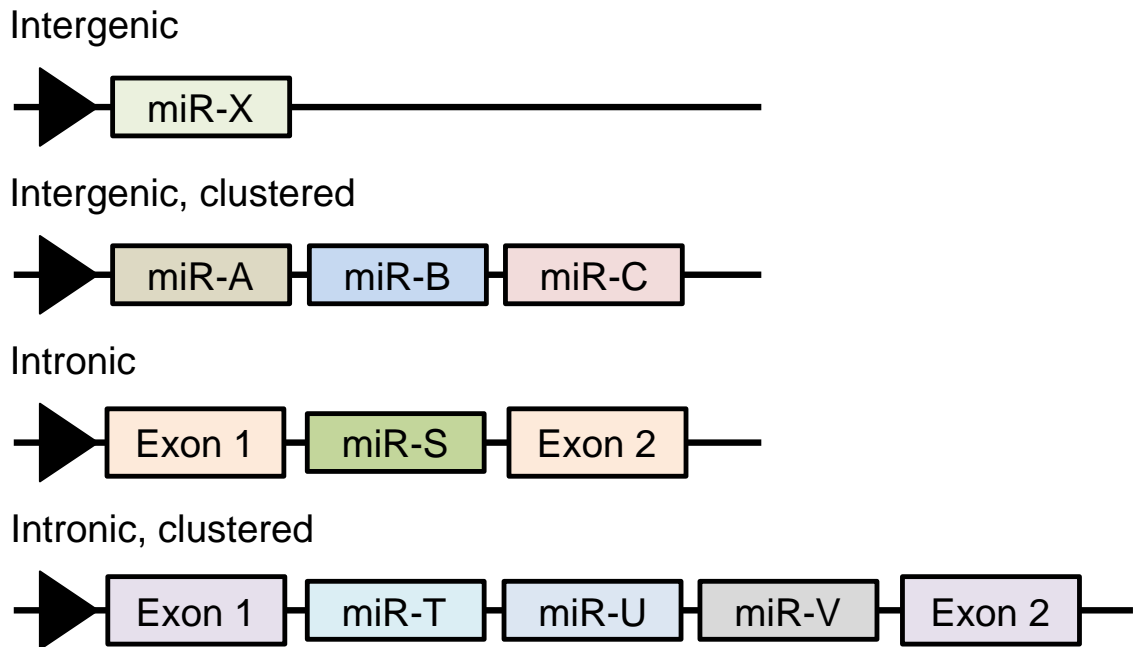


Figure 1.5 Genomic organization of miRNAs

MiRNA genes are usually either intergenic or intronic. Intergenic genes use their own promoters, while intronic genes share promoters with the genes by which they are flanked. Moreover, genes encoding miRNAs can be either monocistronic, encoding a single miRNA, or polycistronic, where several are clustered together. Adapted from Olena and Patton, 2009.

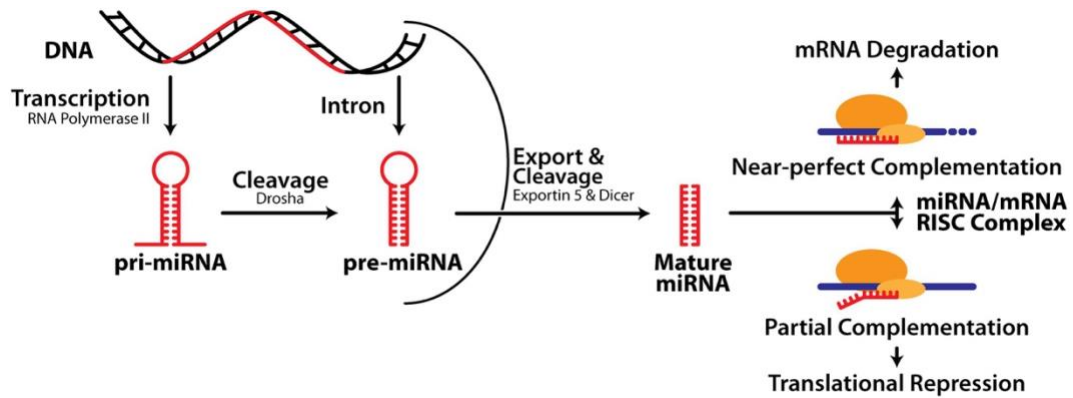


Figure 1.6 miRNA biogenesis and processing

MiRNA genes are transcribed in the nucleus by Polymerase II into a primary miRNA (pri-miRNA) which is then trimmed by Drosha into a precursor miRNA (pre-miRNA). The pre-miRNA is then exported by Exportin 5 into the cytoplasm where the hairpin loop is cleaved by Dicer to generate a mature miRNA. The mature miRNA is then loaded into the RNA-induced silencing complex (RISC) which brings the miRNA to its target transcript and bind as either a perfect match, resulting in mRNA degradation, or as an imperfect match, resulting in translational repression. Adapted from Ryan et al., 2015.

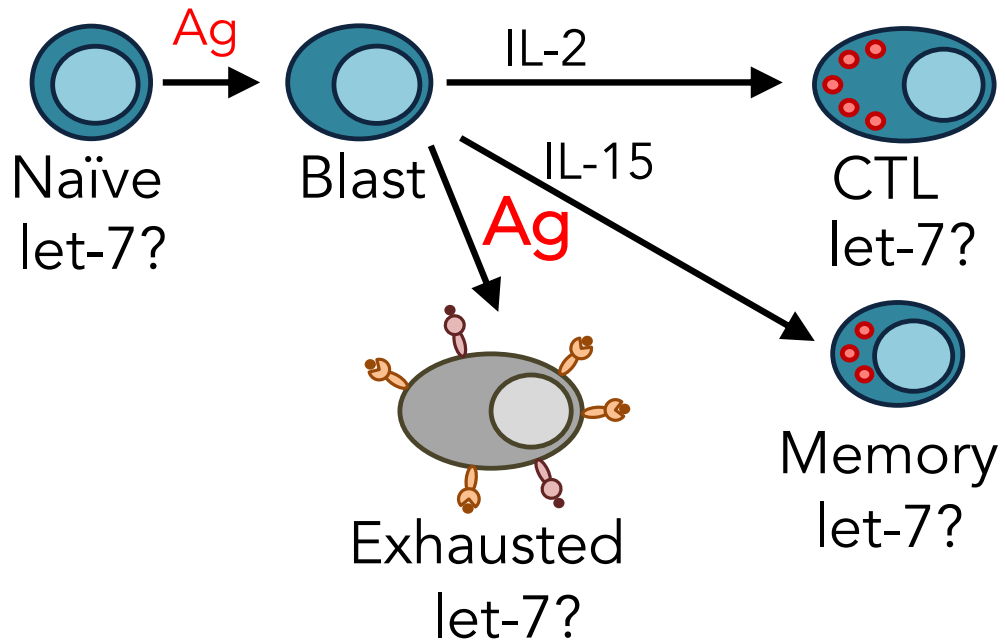


Figure 1.7 Model of specific aims to be tested

The research proposed in this dissertation will assess the role of elt-7 miRNAs in regulating the quiescent state of naïve CD8 T cells, as well as in the differentiation of cytotoxic T lymphocytes (CTLs), memory and exhausted CD8 T cells.

CHAPTER 2

MODULATION OF LET-7 MIRNAS CONTROLS THE DIFFERENTIATION OF EFFECTOR CD8 T CELLS

2.1 Introduction

Maintaining naïve CD8 T cells in a quiescent state is important for their long-term survival and for productive CD8 T cell responses throughout the lifetime of the organism. The maintenance of this population is dependent on very low levels of homeostatic proliferation driven by two signals: low affinity TCR stimulation via self-peptide:MHC-I complexes, and homeostatic cytokine signaling provided by IL-7 and IL-15 (Kirberg et al., 1997; Goldrath and Bevan, 1999; Schluns et al., 2000; Tan et al., 2001). The extent to which cells proliferate in response to these signals must be tightly controlled, as increased rates of homeostatic proliferation can result in the conversion of naïve CD8 T cells (CD44^{lo}C122^{lo}) into cells with a memory phenotype (CD44^{hi}CD122^{hi}), often referred to as ‘virtual memory’ cells (Goldrath and Bevan, 1999; Cho et al., 2000; Hamilton et al., 2006). This is particularly dangerous because these cells may be cross-reactive to self-ligands, and result in the induction of autoimmunity (Ernst et al., 1999; Sprent and Surh, 2011). As such, there are several mechanisms in place that prevent spontaneous activation in naïve CD8 T cells receiving tonic TCR and cytokine signals. This includes retention of the transcription factors NF- κ B, and NFAT, in the cytoplasm and transcriptional control of members of the AP-1 complex (Sprent and Surh, 2011). Moreover, the transcription factor Foxp1 has been demonstrated to play a critical role in the maintenance of quiescence (Feng et al., 2011). However, whether there is a post-transcriptional regulatory mechanism

involved in preventing the spontaneous activation of naïve CD8 T cells remains to be determined.

Antigen recognition through the TCR, licenses CD8 T cells to clonally expand, and acquire effector function, thus differentiating into cytotoxic T lymphocytes (CTLs). It has been suggested that the initial strength of TCR signaling determines the magnitude of the resulting CTL response. Studies supporting this model have demonstrated that stronger TCR stimulation results in heightened proliferation, and enhanced cytotoxic function driven by increased transcription factor expression and activity (Chang et al., 2007; Zehn et al., 2009; King et al., 2012; Verbist et al., 2016). The transcription factor IRF4 is a well characterized interpreter of TCR signal strength. IRF4 expression increases with the strength of TCR stimulation, and results in increased rates of glycolysis, proliferation, and expression of Blimp-1 and T-bet (Man et al., 2013; Yao et al., 2013; Nayar et al., 2014; Nayar et al., 2015). The involvement of post-transcriptional machinery in translating TCR signal strength to scale effector CD8 T cell responses is not known. Further, whether there is conserved molecular machinery involved in both restraining the spontaneous activation of naïve CD8 T cells and in tuning the magnitude of the effector response remains to be determined.

The pattern of high let-7a and let-7f expression in naïve cells, and reduced expression in effector CD8 T cells (Wu et al., 2007), suggests let-7 may regulate elements of naïve and effector CD8 T cells. However, no functional role for the let-7 miRNAs in either of these CD8 T cell populations has been demonstrated. Moreover, the temporal regulation of let-7 expression in the naïve to effector transition has not been addressed. As such, the aim of this study was to better characterize the expression of all let-7 miRNA

family members in naïve and activated CD8 T cells, what signal regulates this expression, and to identify the functional significance of let-7 expression levels in naïve and effector CD8 T cells.

We found that in CD8 T cells high levels of let-7 miRNAs are necessary to maintain the naïve phenotype, while TCR-mediated down-regulation of let-7 levels in activated cells is critical for the effective differentiation and function of CTLs. In fact, experimentally forced let-7 expression severely impairs the proliferation and differentiation of CD8 T cells, while let-7 deficiency significantly enhances the cytotoxic function of CTLs, and consequently immune responses *in vivo*. Given these findings, we propose a model in which let-7 acts as a molecular hub by converting the strength of TCR signaling into the strength of CD8 T cell function.

2.2 Results

2.2.1 let-7 miRNA expression maintains the quiescent state in naïve CD8 T cells

To explore the potential regulatory role of let-7 miRNAs in CD8 T cells, the expression levels of let-7 miRNA family members in naïve and activated CD8 T cells were determined. Two different housekeeping RNAs were used to control for the major changes that occur upon T cell activation. Surprisingly, the initially very high expression of let-7 miRNAs in naïve CD8 T cells was reduced by TCR signaling, and this downregulation was proportional to the strength and duration of TCR-stimulation, regardless of the housekeeping RNA used (Figure 2.1A-C). As a specificity control, the upregulation of miR-17 from the miR-17-92 locus (Figure 2.1D) that is induced upon T cell activation, was

confirmed (Wu et al., 2012; Katz et al., 2014). Taken together, these results suggest that TCR-mediated signaling inhibits the expression of let-7 miRNAs during T cell activation.

To determine the functional significance of let-7 expression in naïve CD8 lymphocytes, we examined CD8 T cells from P14⁺Lin28Tg*Rag2*^{-/-} mice. In this model, T cell-specific expression of the Lin28 protein blocks let-7 biogenesis (Piskounova et al., 2011; Pobezinsky et al., 2015), inhibiting let-7 expression, and P14 is a monoclonal T cell receptor specific to the lymphocytic choriomeningitis virus (LCMV) peptide gp33-41, presented in the context of H-2D^b molecules (Figure 2.2A). In comparison to the P14⁺ wild type counterparts, P14⁺Lin28Tg CD8 T cells were significantly larger in size, with a dramatically increased proportion of Ki67 positive cells (Cuylen et al., 2016) (Figure 2.2B, C). Consistent with the increased frequency of cells entering the cell cycle, P14⁺Lin28Tg mice had a significantly higher frequency of BrdU positive cells in both the spleen and lymph nodes, as compared to wild type P14⁺ mice (Figure 2.2D). In addition, surface expression of T cell activation markers, such as the IL-2 receptor beta-chain (CD122), and CD44 was also increased, while cells remained CD25 negative (Figure 2.2E). Thus, these results suggest that the expression of let-7 miRNAs may maintain the quiescence in naïve CD8 T cells.

2.2.2 let-7 miRNA expression in CTLs affects both the anti-viral and anti-tumor immune responses

TCR stimulation of naïve T cells leads to a rapid loss of the quiescent state and differentiation into effector cells. Given that the expression of let-7 miRNAs, which is critical for the maintenance of naïve CD8 T cells, is inhibited by TCR signaling (Figure

2.1A, B), we hypothesized that the downregulation of let-7 miRNAs in response to TCR stimulation is necessary for the differentiation and function of effector CD8 T cells (Figure 2.3A). To determine whether TCR-mediated downregulation of let-7 miRNAs is required for CD8 T cell differentiation *in vivo*, we analyzed the fate of P14⁺ CD8 T cells with forced let-7 expression in response to acute viral infection with LCMV Armstrong. The doxycycline-inducible let-7g transgene (Zhu et al., 2011) maintains let-7g miRNA expression in lymphocytes in the presence of doxycycline, even after TCR stimulation (Figure 2.3B). Donor CD45.2⁺CD8⁺ T cells from P14⁺ and P14⁺ let-7 transgenic (let-7Tg) mice were adoptively transferred into host congenic wild type CD45.1⁺ mice that were concurrently infected with LCMV, and the differentiation state of P14⁺ cells was assessed seven days post-injection. Interestingly, the recovery of donor CD8 T cells at the peak of the immune response revealed that P14⁺let-7Tg CD8 T cells failed to clonally expand (Figure 2.4A) and lacked KLRG1 expression, an established marker of terminal effector CTLs (Thimme et al., 2005; Joshi et al., 2007; Dominguez et al., 2015) (Figure 2.4B). Furthermore, let-7Tg CTLs had a reduced frequency of IFN- γ ⁺TNF- α ⁺ cytokine double-producing cells, a hallmark of an effective CD8 T cell response (Kaech et al., 2002; Wherry et al., 2004; Williams and Bevan, 2007), while the differentiation of endogenous host-derived CTLs was normal, suggesting a cell-intrinsic mechanism (Figure 2.4B). Importantly, mRNAs of the *Klrg1*, *Ifng* and *Tnfa* genes are not targets of let-7 miRNAs, therefore the reduced frequencies of effector cells generated from let-7Tg CD8 T cells is not simply a result of direct suppression of effector molecule expression. Thus, sustained let-7 expression following TCR activation severely impaired the clonal expansion and differentiation of CTLs in response to viral infection *in vivo*.

As the allogeneic response to foreign MHC elicits a robust CD8 T cell response, we used the P815 mastocytoma, an allogeneic (H-2d haplotype) tumor, to determine whether steady levels of let-7 in T cells would suppress the allogeneic response *in vivo* (Felix and Allen, 2007; Jankovich et al., 2002; Zhan et al., 2000). This response was confirmed to be CD8 T cell dependent, as P14⁺Rag2^{-/-} mice without adoptive transfer of CD8 T cells failed to reject the tumor (Figure 2.4C). When P815 cells were injected into wild type or let-7Tg mice of H-2b haplotype, 60% of let-7Tg mice were unable to effectively respond to, and clear the tumor (Figure 2.4D). Additionally, seven days post-injection, wild type mice retained on average 40 x10⁶ cancer cells in the peritoneal cavity, while let-7Tg mice contained 115 x10⁶ cancer cells (Figure 2.4E). Based on this failure to eliminate P815 tumor cells, we concluded that maintaining let-7 expression during stimulation and differentiation compromised the CD8 T cell response to alloantigen. Taken together, our results demonstrate that downregulation of let-7 expression upon TCR engagement is necessary for the proper proliferation and differentiation of cytotoxic CD8 T cells *in vivo*.

2.2.3 Expression of let-7 miRNAs in activated CD8 T cells inhibits proliferation and the gene expression program responsible for the metabolic switch

To elucidate the underlying mechanisms of let-7 mediated suppression of CD8 T cell responses (Figure 2.4), we first analyzed the impact of let-7 microRNAs on T cell clonal expansion. Sorted naïve (CD44^{lo}CD25⁻) CD8 T cells with different levels of let-7 expression (Figure 2.5A, B) were activated with anti-CD3 mAbs *in vitro* for 3 days. Let-7Tg CD8 T cells proliferated less than their wild type counterparts, while Lin28Tg CD8 T

cells proliferated more rapidly (Figure 2.6A), indicating that let-7 miRNAs negatively regulate clonal expansion of activated CD8 T cells, consistent with the previous observations *in vivo* (Figure 2.4A). Thus, we concluded that TCR-mediated downregulation of let-7 expression is needed for successful proliferation of activated CD8 T cells.

Let-7 miRNAs are well-documented tumor suppressors. It has been shown that let-7 inhibits proliferation in cancer cells by directly targeting the mRNA of genes involved in the regulation of the cell cycle (Johnson et al., 2007). In fact, the expression of some let-7 targets such as phosphatase *cdc25a* (*cdc25a*), kinase *cdk6* (*cdk6*), and cyclin D2 (*ccnd2*), was suppressed in activated let-7Tg CD8 T cells, as compared to Lin28Tg CD8 T cells where it was derepressed (Figure 2.6B). Further, let-7 has been reported to regulate the transcription factor Myc, expression of which is upregulated upon T cell activation and is essential for CD8 T cell proliferation (Best et al., 2013; Iritani et al., 2002; Kim et al., 2009; Nie et al., 2012; Verbist et al., 2016). In fact, Myc expression was regulated by let-7 in activated CD8 T cells (Figure 2.6C). To demonstrate that Myc activity was suppressed by let-7, we also analyzed the expression of a direct transcriptional target of Myc, the transcription factor AP4 (*Tfap4*), which sustains Myc-mediated effects in CD8 T cells during the later stages of differentiation (Chou et al., 2014). Although mRNA of the *Tfap4* gene is not a target of let-7, the expression of *Tfap4* mRNA was significantly reduced in let-7Tg CD8 T cells, and enhanced in Lin28Tg CD8 T cells (Figure 2.6C), suggesting that let-7 regulates Myc activity in CD8 T cells.

Another important function of Myc in activated CD8 T cells is to support the proliferative burst through the metabolic reprogramming of lymphocytes from primarily

oxidative phosphorylation (resting) to glycolysis (activated), as well as through an increase in protein synthesis (Wang et al., 2011). To test whether let-7 miRNAs have an impact on the metabolic switch in activated CD8 T cells through its regulation of Myc, the expression of key glucose transporters, glycolytic enzymes, and protein synthesis enzymes that are directly controlled by Myc in activated CD8 T cells (Wang et al., 2011) was assessed. Strikingly, the expression of all tested targets was suppressed in let-7 transgenic CD8 T cells, and increased in Lin28Tg activated lymphocytes, suggesting that let-7 expression may influence the Myc-dependent metabolic reprogramming of activated CD8 T cells (Figure 2.6D). Taken together, these results demonstrate that let-7 miRNAs control the proliferation of activated CD8 T cells by modulating the expression and activity of genes involved in the regulation of the cell cycle and metabolism.

2.2.4 let-7 expression regulates the function of differentiated CD8 T cells

To identify whether let-7 mediated suppression of CD8 T cell immune responses is due to a failure to acquire effector function in addition to a proliferative defect, we assessed the cytotoxic activity of CTLs generated from P14⁺ wild type, P14⁺let-7Tg, and P14⁺Lin28Tg mice. In fact, expression of the let-7 transgene in P14⁺ CTLs greatly diminished cytotoxic activity (Figure 2.7A). Alternatively, P14⁺Lin28Tg CTLs exhibited enhanced cytotoxicity (Figure 2.7A), which could be reduced by restoring let-7 expression through the induction of the doxycycline-inducible let-7 transgene in P14⁺Let-7TgLin28Tg (4 Tg) CTLs (Figure 2.8A), demonstrating that let-7- deficiency, and not Lin28 overexpression, is responsible for increased cytotoxicity. These results demonstrate that

TCR-mediated downregulation of let-7 microRNAs is necessary for the acquisition of cytotoxic function in differentiating CD8 T cells.

To investigate how let-7 miRNA expression impacts CD8 T cell function, the phenotype of *in vitro* generated effector CTLs from wild type, let-7Tg, and Lin28Tg mice was further examined. Let-7Tg CTLs had less internal complexity based on the intensity of side scatter (SSC) than wild type cells, whereas Lin28Tg CD8 effector cells had significantly greater complexity (Figure 2.7B), suggesting a change in the number of cytotoxic granules. Indeed, let-7Tg CTLs contained fewer Granzyme A and Granzyme B positive granules than wild type CTLs, while Lin28Tg CTLs had more (Figure 2.7C). These results indicated that the expression of let-7 controls the quantity of cytotoxic granules produced during the differentiation of CTLs.

Next, to determine whether let-7 expression in CTLs influences the number of granules by controlling the expression of effector molecules, gene expression of Granzyme A (*Gzma*), Granzyme B (*Gzmb*), and Perforin (*Prf1*), the key cytolytic factors in cytotoxic granules (Chowdury and Lieberman, 2008), was measured. Let-7Tg CTLs, which contained fewer cytotoxic granules, expressed less mRNA for *Gzma*, *Gzmb*, and *Prf1*, as compared to wild type cells, while Lin28Tg CTLs expressed higher levels of these effector molecules (Figure 2.7D). Moreover, the observed changes in mRNA expression of effector molecules, including Interferon-gamma (IFN- γ), were consistent at the protein level (Figure 2.7E, F). Importantly, the induction of let-7 expression in the presence of Lin28 in P14⁺Let-7TgLin28Tg (4 Tg) CTLs reduced the expression of these effector molecules (Figure 2.8B), again demonstrating that the Lin28Tg phenotype is due to let-7- deficiency.

Together, these data indicate that let-7 miRNAs negatively regulate CTL function by preventing the expression of important cytotoxic molecules.

2.2.5 let-7 miRNAs directly target the ‘master regulator’ transcription factor, Eomesodermin during CTL differentiation

To determine the mechanism through which let-7 regulates the differentiation and function of CTLs, we considered the possibility that let-7 miRNAs may directly control the expression of effector molecules. Although typical miRNA binding sites are found in the 3' untranslated regions (UTRs) of mRNA transcripts (Lai et al., 2002; Reinhart et al., 2000; Wightman et al., 1993) we analyzed the full-length mRNA of *Prfl*, *Gzma*, *Gzmb*, and *Ifng*, yet no let-7 binding sites were found. This suggested that let-7 may indirectly regulate the expression of these molecules by controlling more global regulators, such as transcription factors. It is known that the expression of effector molecules and cytotoxic function of CD8 T cells are tightly regulated by a group of transcription factors, including Eomesodermin (Eomes), T-bet, *Zbtb32*, *Runx3*, Notch-1 and Blimp-1 (Backer et al., 2014; Cho et al., 2009; Kallies et al., 2009; Pearce et al., 2003; Szabo et al., 2002; Shin et al., 2017). To determine if these factors are regulated by let-7, expression of *Eomes*, *Tbx21*, *Zbtb32*, *Prdm1*, *Runx3d*, and Notch-1 genes was assessed in CTLs with different levels of let-7. The only transcription factor whose expression on both the mRNA and protein levels was reduced in let-7 transgenic CTLs, and enhanced in Lin28Tg CTLs, was *Eomes* (Figure 2.9A-D). Furthermore, the induction of let-7 expression in the presence of Lin28 in P14⁺Let-7TgLin28Tg (4 Tg) CTLs reduced the expression of *Eomes* (Figure 2.9E), demonstrating that increased expression of *Eomes* in Lin28Tg CTLs is due to the Lin-28-

mediated knockdown of let-7 miRNAs. Of note, we also found that T-bet expression was inversely correlated with *Eomes*, suggesting that an *Eomes* dependent mechanism may control T-bet expression (Figure 2.9A, B). Thus, these results demonstrate that let-7 miRNAs negatively regulate *Eomes* expression.

Next, we sought to determine whether let-7 miRNAs can target *Eomes* mRNA. Interestingly, there is no let-7 binding site located in the 3'UTR of *Eomes*, but rather a conserved binding motif within the open reading frame of *Eomes* mRNA was identified (Figure 2.10A). To determine whether this binding site is functional, the 10-nucleotide mouse *Eomes*-let-7 binding motif was cloned into a dual luciferase vector. The vector was then transfected into NIH 3T3 fibroblasts, which have high endogenous expression of the let-7 family members (Figure 2.10 B, C). This resulted in a significant decrease in luciferase activity, indicating that let-7 can directly bind *Eomes* (Figure 2.10D). When this sequence was mutated, disrupting the let-7 binding site, luciferase activity was restored, confirming that let-7 microRNAs directly target *Eomes* mRNA (Figure 2.10D).

2.2.6 let-7 miRNAs suppress CD8 T cell function through targeting *Eomes* mRNA

To ascertain whether let-7 controlled CD8 T cell differentiation and function through *Eomes*, a loss-of-function approach to knockout *Eomes* expression in Lin28Tg CTLs was used. It was hypothesized that removal of *Eomes* expression would reduce the enhanced cytotoxicity in let-7-deficient cells. T-cell specific deletion of either one or both alleles of *Eomes* in CTLs generated from P14⁺CD4Cre⁺*Eomes*^{fl/wt} Lin28Tg, and P14⁺CD4Cre⁺*Eomes*^{fl/fl}Lin28Tg T cells resulted in gradually decreased levels of *Eomes* (Figure 2.11A). Any residual *Eomes* expression in CTLs derived from

CD4Cre⁺Eomes^{fl/fl}Lin28Tg mice, was attributed to “escapees” of the conditional knockout. The expression of the effector molecules *Gzma*, and *Prfl* was reduced in a manner consistent with the expression of *Eomes*, where loss of *Eomes* ameliorated the enhanced expression of these effector molecules in *Lin28Tg* effector cells (Figure 2.11B, C). Interestingly, the expression of Granzyme B was not affected by loss of *Eomes* expression, suggesting Granzyme B may be more complexly regulated through other *let-7*-dependent, but *Eomes*- independent mechanisms (Figure 2.11D). Ultimately, the loss of *Eomes* expression resulted in a significant reduction of cytotoxic function, even in the absence of *let-7* microRNAs (Figure 2.11E) while restored *Eomes* expression completely rescued both the cytotoxic function and the expression of effector molecules in *Eomes*-deficient *Lin28Tg* CTLs (Figure 2.12A, B). Furthermore, forced expression of the mutated form of *Eomes* (lacking the *let-7* binding motif) in *let-7Tg* CTLs also enhanced the effector phenotype (Figure 2.12C, D). These results demonstrate that *let-7*-mediated suppression of *Eomes* is in part responsible for the compromised differentiation and cytotoxic function of *let-7Tg* CTLs *in vitro*.

Thus, these data suggest a model in which the *let-7* miRNAs act as a molecular control hub that drives CD8 T cell differentiation and function, in a manner dependent on the magnitude of TCR stimulation (Figure 2.13). Furthermore, we propose that modulation of *let-7* miRNA expression in CD8 T cells provides an exciting, novel therapeutic application to control CTL responses.

2.3 Discussion

This study has identified a critical role for the let-7 miRNAs in regulating the transition between naïve and effector stages of CD8 T cells. Specifically, let-7 expression is high in naïve T cells and, when absent, results in increased proliferation and expression of activation markers, which may suggest a loss of the quiescent phenotype in CD8 lymphocytes. Moreover, the entire family of let-7 miRNAs is downregulated upon antigen stimulation through the TCR, which was demonstrated to be necessary for the successful progression of CD8 T cell differentiation into CTLs. Therefore, these data suggest that let-7 expression keeps CD8 T cells in a naïve state and prevents CTL differentiation, while the magnitude of TCR-mediated downregulation of let-7 expression guides the proliferation, differentiation and the acquisition of effector function of CD8 T cells.

The importance of miRNAs in the regulation of the immune system has been demonstrated through the deletion of the miRNA processing enzyme, Dicer. In fact, it has been shown that Dicer deficiency promotes the differentiation of CD8 T cells into CTLs (Trifiari et al., 2013), suggesting the involvement of specific miRNAs. Let-7 microRNAs, the largest and most abundantly expressed family of miRNAs in CD8 T cells, have been shown to be important in early development, metabolism, and cancer (Zhu et al., 2011; Bussing et al., 2008; Roush and Slack, 2008). Recent studies have also implicated the importance of the let-7 miRNAs in the development, maintenance, and function of the immune system, including T cells (Pobezinsky et al., 2015; Yuan et al., 2012; Okoye et al., 2014). Using both gain-of-function experiments employing the let-7Tg mouse capable of sustaining let-7 expression following TCR activation, and loss-of-function experiments using the Lin28Tg mouse, where the Lin28Tg inhibits let-7 expression, this study has

corroborated these earlier reports, and has identified a novel role for let-7 in CD8 T cell differentiation and function.

Prior to antigen encounter, naïve CD8 T cells are maintained in a quiescent state, in which T cells have no effector function, are metabolically inactive and undergo minimal proliferation. The homeostasis of naïve T cells depends on the balance of two signals, the recognition of low affinity self-ligands by the TCR, and cytokine stimulation (Surh and Sprent, 2011; Kimura et al., 2013). Although it has become clear that the weak recognition of self-ligands is not enough for naïve T cells to lose the quiescent state, the molecular mechanism that prevents the spontaneous activation of T cells under these conditions is not fully understood. Here, the high expression of let-7 microRNAs was demonstrated to be necessary for maintaining the naïve CD8 T cell state. In fact, let-7 ablation in CD8 T cells led to the loss of the quiescent phenotype, as indicated by more active proliferation, an overall increase of cell size, and the upregulation of activation markers such as CD122, and CD44 (Cuylen et al., 2016; Intlekofer et al., 2005). These experiments were conducted using CD8 T cells from P14⁺Lin28Tg*Rag2*^{-/-} mice in order to prevent bystander effects from IL-4- producing PLZF⁺ cells present in Lin28Tg mice (Pobezinsky et al., 2016; Yuan et al., 2012; Weinreich et al., 2010). Although further investigation is needed to distinguish between the let-7-dependent and let-7-independent effects of the Lin28 transgene in naïve CD8 T cells, it can be speculated that high expression of let-7 microRNAs prevents the spontaneous activation of naïve T cells. Furthermore, it will be interesting to explore the precise let-7-mediated regulation of transcription programs, such as Foxp1 (Feng et al., 2011), that may contribute to the control of CD8 T cell quiescence.

Furthermore, we have found that the let-7 mediated “molecular brake” is released upon antigen stimulation, due to the profound downregulation of all members of the let-7 microRNA family in activated CD8 T cells in response to TCR stimulation, in a manner proportional to its strength. Using *in vivo* models to assess both anti-viral and anti-tumor immunity, the importance of let-7 miRNAs in controlling CD8 T cell- mediated immune responses was demonstrated. Let-7Tg CD8 T cells failed to proliferate in response to acute LCMV infection, and the few let-7Tg cells that did respond exhibited a very weak effector phenotype, suggesting that downregulation of let-7 is critical to both the proliferative burst of antigen-specific CD8 T cells upon encounter with viral antigen, and the differentiation of these cells *in vivo*. These results were further bolstered by the failure of let-7Tg mice to reject an allogeneic tumor, the P815 mastocytoma. Altogether, these *in vivo* studies demonstrate the significance of let-7 downregulation in effector CD8 T cells, and suggest a novel level of control of immune responses that can be therapeutically targeted for treatment of infectious diseases, cancer and autoimmunity.

Antigen stimulation of T cells results in the increased biosynthesis that is needed to support the clonal expansion of antigen specific lymphocytes, and ultimately the acquisition of effector function. The hallmark of this process is a metabolic switch from oxidative phosphorylation to aerobic glycolysis (Frauwirth et al., 2002), as well as a concomitant increase in cell size, both of which have been reported to be controlled by Myc (Chou et al., 2014; Wang et al., 2011), the expression of which is rapidly induced upon antigen stimulation of CD8 T cells (Williams and Bevan, 2007; Wang et al., 2011). Presumably due to the pro-apoptotic activity of Myc (Chang et al., 2000; Chou et al., 2014), its expression is transient, eventually receding during the later stages of CD8 T cell

differentiation (Best et al., 2013; Nie et al., 2012). In our study, we have shown that let-7 suppresses the expression of Myc on the mRNA level, and consequently modulates the function of Myc in CD8 T cells, based on the assessment of established Myc targets, supporting previous reports of Myc as a non-canonical target of let-7 miRNAs in cancer cells (Kim et al., 2009). Our results suggest that let-7 likely regulates the metabolic switch in activated CD8 T cells through Myc.

It was also found that the let-7 miRNAs may directly inhibit the proliferation of activated CD8 T cells by suppressing the expression of the cell cycle regulators Cdc25a, Ccnd2 and Cdk6, all of which are known let-7 targets (Johnson et al., 2007). Since these factors have also been described as transcriptional targets of Myc (Bouchard et al., 1999; Galaktinov et al., 1996; Hermeking et al., 2000), the possibility of more complex regulation in which let-7 is not solely responsible for controlling their expression cannot be ruled out.

Moreover, it was demonstrated that let-7 mediated suppression of CD8 T cell immune responses is also due to modulation of effector function. The internal complexity of CTLs generated *in vitro* was diminished in the presence of forced let-7 expression, and it was subsequently determined that this was due to a reduction in the number of cytotoxic granules, as well as in the expression of effector molecules. This led to the conclusion that let-7 functions as a molecular rheostat that quantifies TCR signaling to direct the CTL response upon antigen stimulation, in a similar fashion to other miRNAs, including miR-181 and the miR-17-92 cluster (Heno-Mejia et al., 2013; Li et al., 2007; Wu et al., 2012). These conclusions are supported by a recent study demonstrating that in neonatal mice the residual expression of Lin28B in T lymphocytes skews CD8 T cell differentiation towards an effector phenotype (Wang et al., 2016).

We further wanted to determine the molecular mechanisms through which let-7 acts to inhibit CTL differentiation. *Eomes* was found to be directly regulated by let-7 microRNAs, which can in part explain the block in the differentiation of CD8 T cells with forced let-7 expression. Yet, in contrast to previous studies on the differential roles of *Eomes* and T-bet in governing CD8 T cell function, we have shown that heightened expression of *Eomes* in effector T cells may be more important for effector function than previously thought (Pearce et al., 2003; Intlekofer et al., 2005; Chang et al., 2014). In fact, let-7/ *Eomes*-double deficient CTLs had reduced antigen-specific cytotoxicity *in vitro*. However, this data also suggested that *Eomes* is only a part of this “cytotoxic program”, as deletion of *Eomes* reduced cytotoxicity to the levels exhibited by wild type CTLs, and re-expression of *Eomes* in let-7Tg CTLs only marginally increased cytotoxicity. These results indicate that other let-7- dependent, but *Eomes*- independent mechanisms are involved. More experiments are required to elucidate these *Eomes*- independent mechanisms, and should be enlightening given the suggested redundancy of *Eomes* activity. Additionally, Granzyme A was identified as a probable target of *Eomes*, while *Eomes*-mediated control of Perforin, and IFN- γ expression was confirmed (Pearce et al., 2003). These results are consistent with the enhanced cytotoxicity exhibited by Lin28Tg CD8 T cells with increased *Eomes* expression. The previously reported reciprocal expression between T-bet and *Eomes* (Intlekofer et al., 2005; Chang et al., 2014) was also observed.

These results strongly demonstrate that downregulation of let-7 upon TCR stimulation is a critical process in the determination of the magnitude of the CD8 T cell response *in vivo*, as let-7 miRNAs inhibit proliferation and differentiation by targeting cell cycle regulators, affecting metabolic reprogramming through the suppression of Myc, and

repression of effector function through Eomes dependent and independent mechanisms. Thus, naïve CD8 T cells require let-7 miRNAs to remain quiescent, and only upon antigen stimulation through the TCR can this molecular “brake” be released. Based on these results, we propose that let-7 miRNAs act as a molecular control hub that translates TCR signaling to control CD8 T cell differentiation and function.

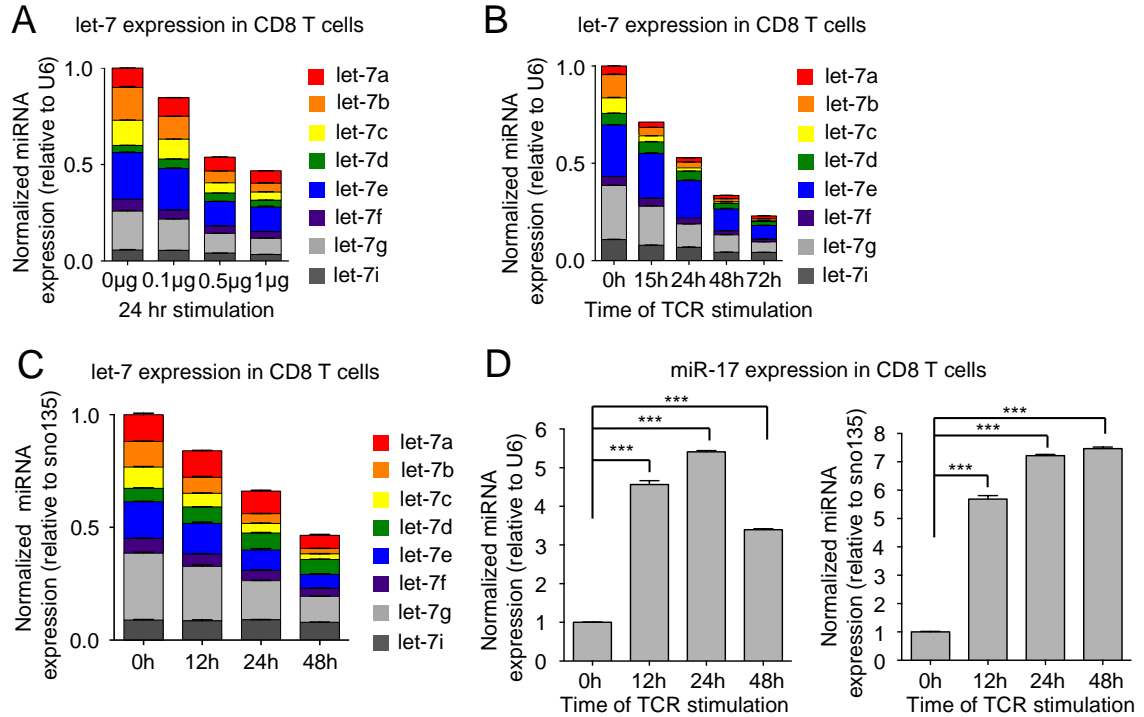


Figure 2.1 let-7 expression is downregulated by TCR signaling

(a) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve CD8 T cells stimulated with plate-bound anti-TCR (μg as indicated) and anti-CD28 ($5 \mu\text{g}$) for 24 hours, presented relative to results obtained for the small nuclear RNA (U6 control) and normalized to the unstimulated ($0 \mu\text{g}$) sample. (b) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve CD8 T cells stimulated with plate-bound anti-TCR ($5 \mu\text{g}$) and anti-CD28 ($5 \mu\text{g}$) over increasing periods of time as indicated, presented relative to results obtained for the small nuclear RNA (U6 control) and normalized to the unstimulated (0h) sample. (c) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve CD8 T cells stimulated with plate-bound anti-TCR β ($10 \mu\text{g}$) and anti-CD28 ($5 \mu\text{g}$) over increasing periods of time as indicated, presented relative to results obtained for the small nucleolar RNA (sno135) and normalized to the unstimulated (0h) sample. (d) Quantitative RT-PCR analysis of miR-17 in naïve CD8 T cells stimulated with plate-bound anti-TCR β ($10 \mu\text{g}$) and anti-CD28 ($5 \mu\text{g}$) over increasing periods of time as indicated, presented relative to results obtained for the small nuclear RNA (U6) (left) or the small nucleolar RNA (sno135) (right) and normalized to the unstimulated (0h) sample. *** $P < 0.001$, compared with 0 hours using two-tailed Student's t -test (d). Data are from one experiment representative of two independent experiments (a, b, c, d; mean and s.e.m. of technical triplicates).

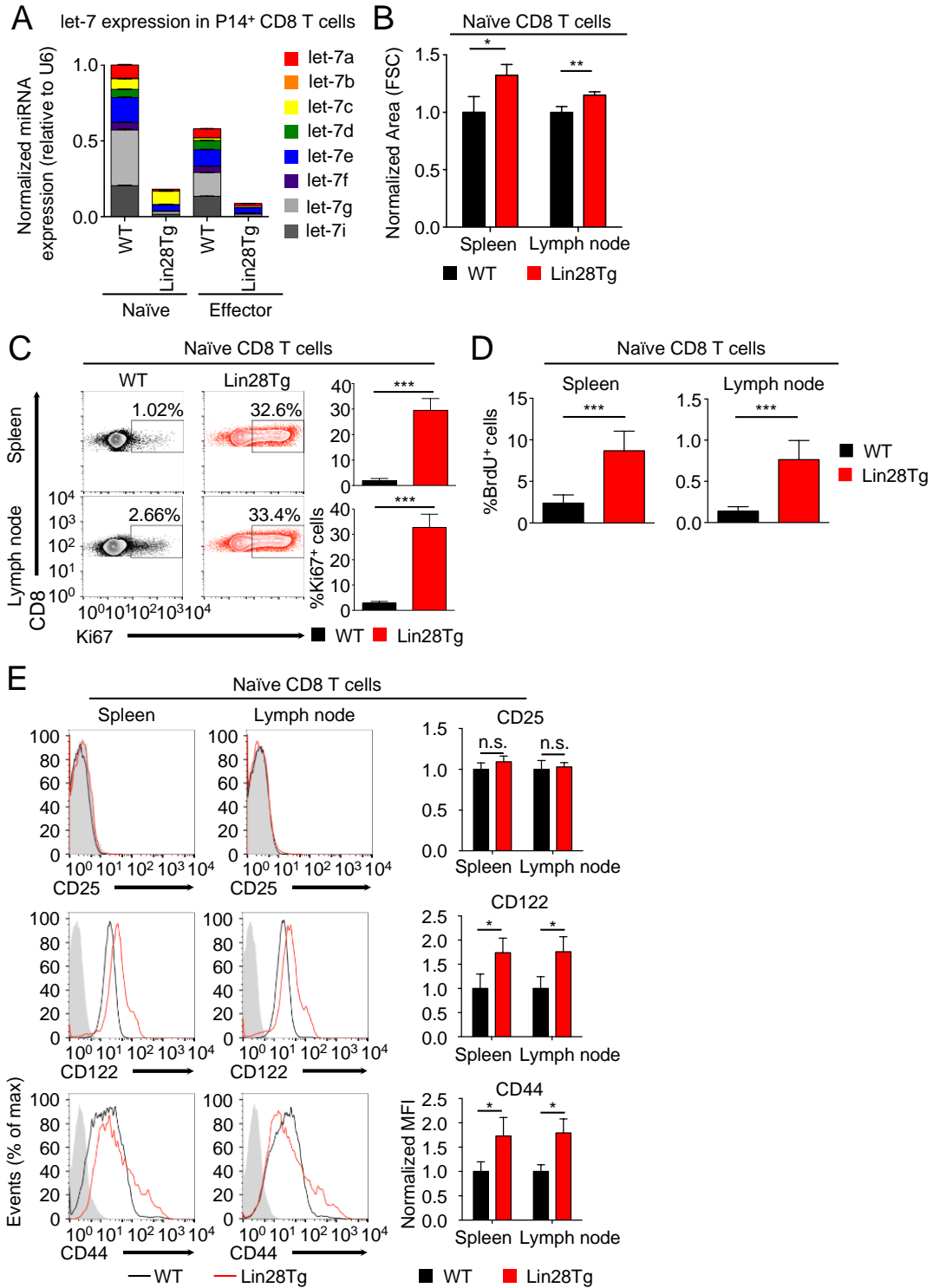


Figure 2.2 let-7 expression is necessary and sufficient to maintain the naïve phenotype of CD8 T cells prior to TCR stimulation

(a) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve and effector (day 5) TCR transgenic CD8 T cells from P14⁺Rag2^{-/-}, and P14⁺Lin28TgRag2^{-/-} mice. (b) Size analysis based on FSC (forward scatter) of naïve CD8 T cells from the spleens and lymph nodes of P14⁺ wild type and P14⁺Lin28Tg mice, both on Rag2^{-/-} background, normalized to wild type. (c) Expression of Ki67 in naïve CD8 T cells from spleens and lymph nodes of P14⁺ wild type and P14⁺Lin28Tg mice, both on Rag2^{-/-} background (left). Quantification of the frequency of Ki67⁺ cells in these populations (right). (d) Frequency of BrdU⁺ cells in naïve CD8 T cells from spleens and lymph nodes of P14⁺ wild type (n=6) and P14⁺Lin28Tg (n=5) mice, both on Rag2^{-/-} background, labeled with BrdU *in vivo*. (e) Surface expression (right) and normalized MFI (left) of CD25, CD44, and CD122 on naïve CD8 T cells from both spleens and lymph nodes of P14⁺ wild type and P14⁺ Lin28Tg mice, both on Rag2^{-/-} background. Gray indicates an isotype control for staining. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test (b, c, d, e) Data are from one experiment representative of three independent experiments (a; mean and s.e.m. of technical triplicates; b, c, d, e; mean and s.e.m. of three experiments).

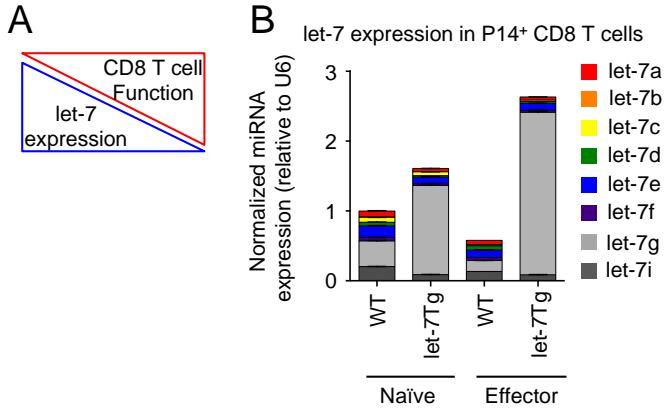


Figure 2.3 Hypothesis and model to test role of let-7 expression in CD8 T cell responses

(a) Schematic representation of the hypothesis that let-7 expression inhibits the differentiation of CD8 T cells. (a) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve and effector (day 5) TCR transgenic CD8 T cells from P14⁺*Rag2*^{-/-}, and P14⁺let-7Tg*Rag2*^{-/-} mice. Data are from one experiment representative of three independent experiments (a; mean and s.e.m. of technical triplicates).

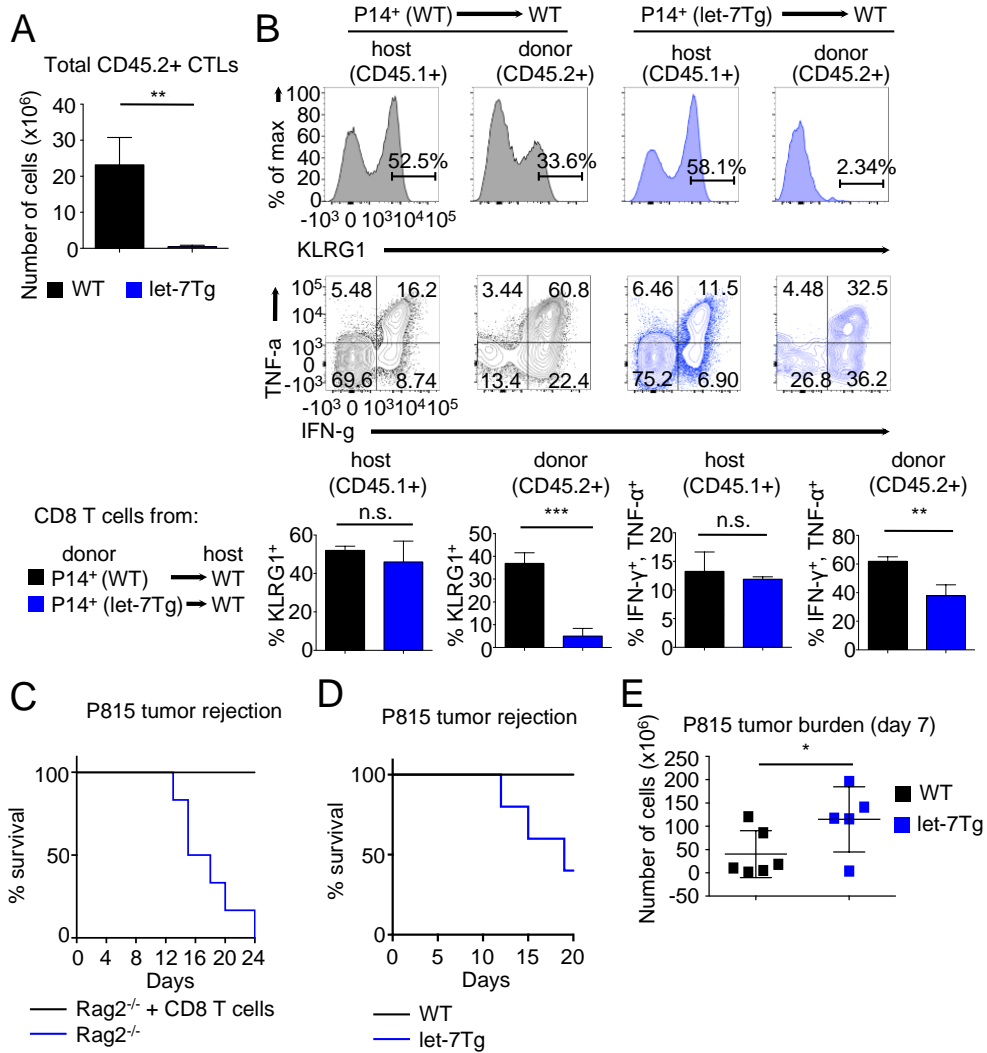


Figure 2.4 Inhibitory role of let-7 miRNA expression in CD8 T cell- mediated responses *in vivo*

(a) Quantification of the number of donor (CD45.2⁺) CD8 T cells from P14⁺ wild type (n=3) or P14⁺let-7Tg mice (n=3) in the spleens of congenic (CD45.1⁺) host mice 7 days after cell transfer and LCMV Armstrong infection. (b) Surface expression of the activation marker KLRG1 on wild type host, and indicated donor cells (top). Expression of IFN- γ , and TNF- α in wild type host, and donor LCMV-specific CD8 T cells from the indicated mice, as determined by re-stimulation with the gp33 peptide, and subsequent intracellular staining (middle). Quantification of the frequency of KLRG1⁺, and IFN- γ ⁺, TNF- α ⁺ populations in wild type host, and donor cells from the indicated mice (bottom). (c) Analysis of the survival of *Rag2*^{-/-} mice injected i.p. with 30X10⁶ P815 cells, which received i.v. adoptive transfer of 10X10⁶ naïve purified CD8 T cells (n=6) or no T cells at all (n=8). (d) Analysis of the survival of wild type (n=5) or let-7Tg (n=5) mice injected i.p. with 30X10⁶ P815 cells. (e) Quantification of the number of P815 tumor cells remaining in the peritoneal cavity 7 days after i.p. injection of 20X10⁶ cells into either wild type (n=6), or let-7Tg mice (n=5). n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test (a, b, e) or one-tailed Student's *t*-test (e). Data are from one experiment representative of three independent experiments (a, b; mean and s.e.m. of three experiments), two experiments (d, e), or one experiment (c).

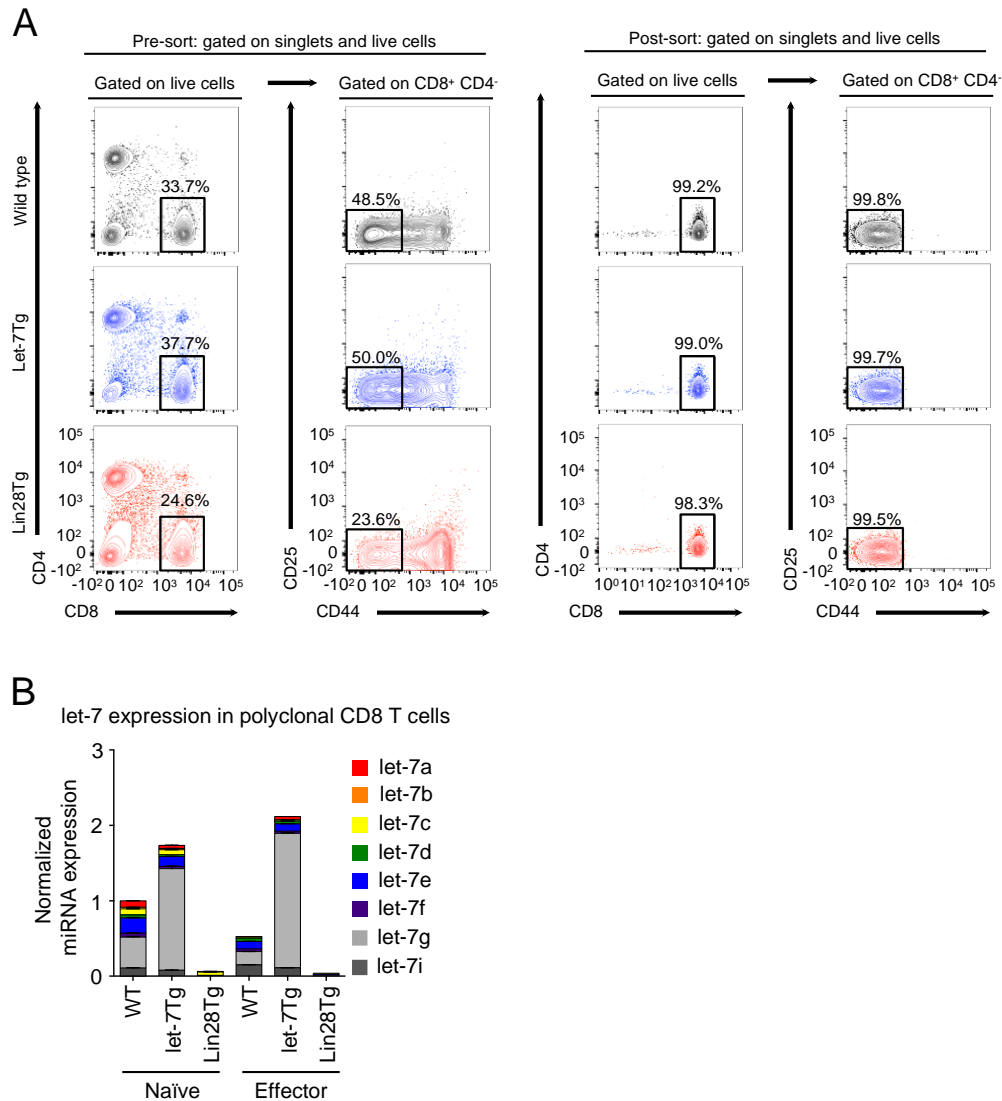


Figure 2.5 Sorting strategy and let-7 expression in polyclonal CD8 T cells

(a) Pre- sort and post- sort purities for naïve CD8 T cells from wild type, let-7Tg, and Lin28Tg mice. Cells were isolated from the lymph nodes of the indicated mice, and enriched for T cells using anti-mouse IgG magnetic beads. Next, to acquire naïve CD8 T cells, lymph node T cells were then stained for CD25, CD44, CD4, and CD8, and sorted as the CD25^{lo}CD44^{lo}CD4⁺CD8⁺ population. (b) Quantitative RT-PCR analysis of individual let-7 miRNAs in naïve and activated (day 5) polyclonal CD8 T cells from wild type, let-7Tg, and Lin28Tg mice. Data are from one experiment representative of three independent experiments (b; mean and s.e.m. of technical triplicates).

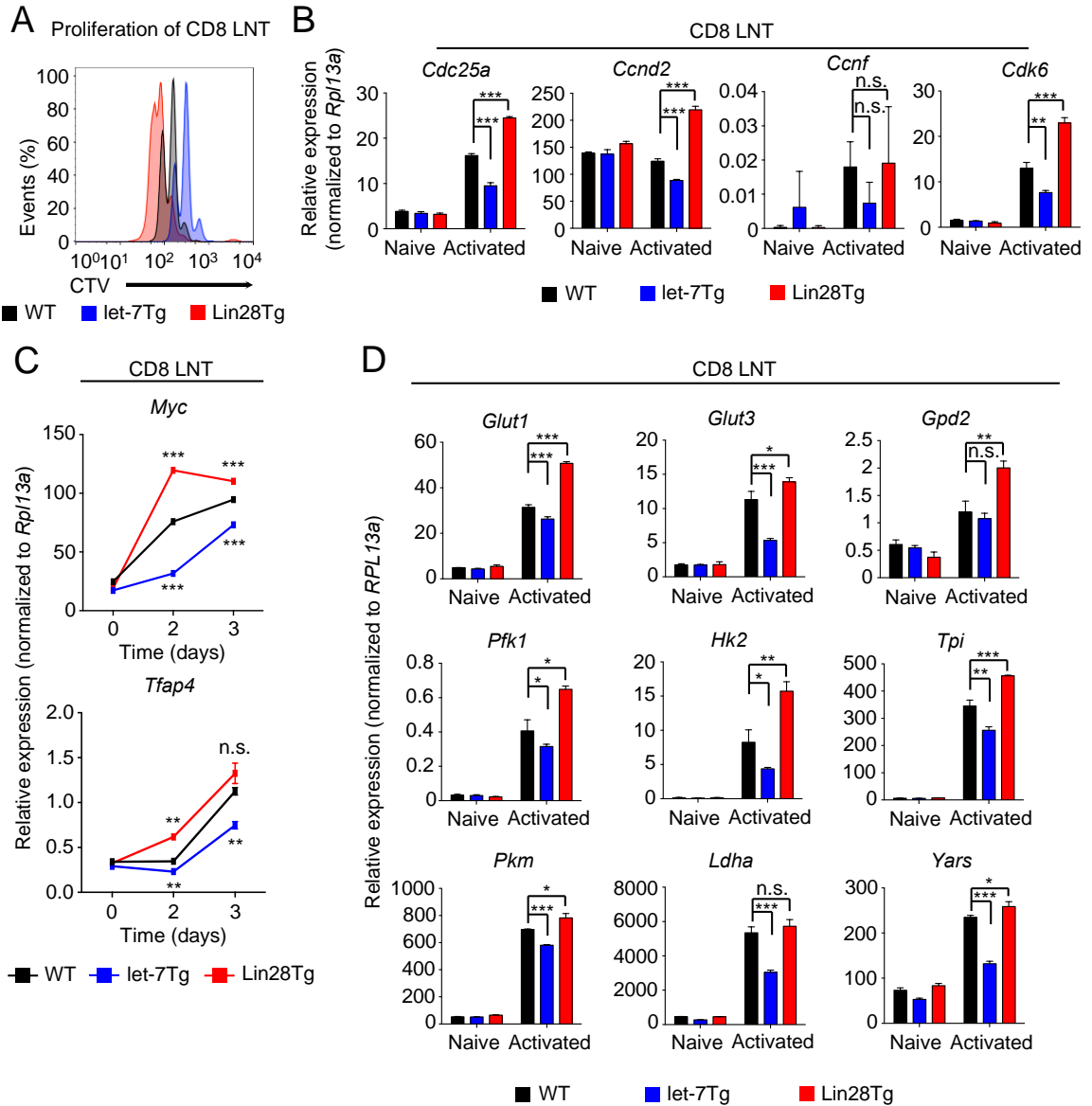


Figure 2.6 let-7 miRNAs suppress the proliferation and metabolism of activated CD8 T cells

(a) Analysis of the proliferation of CellTrace Violet- labeled naïve CD8 T cells from the indicated mice 72 hours after activation with anti- CD3 mAbs. (b) Quantitative RT-PCR analysis of cell cycle regulators: *Cdc25a* (Cell division cycle 25A phosphatase), *Ccnd2* (Cyclin D2), *Ccnf* (Cyclin F), *Cdk6* (Cyclin dependent kinase 6) in naïve and activated wild type, let-7Tg, and Lin28Tg CD8 T cells 3 days after anti-CD3 mAb stimulation, presented relative to the ribosomal protein *Rpl13a*. (c) Quantitative RT-PCR analysis of *Myc* and *Tfap4* (Transcription factor AP-4) in CD8 T cells after stimulation with anti-CD3 mAbs, presented relative to the ribosomal protein *Rpl13a*. Wild type, let-7Tg, and Lin28Tg CD8 T cells were stimulated with anti-CD3 mAbs and differentiated for the indicated time. (d) Quantitative RT-PCR analysis of the expression of genes involved in the metabolic switch: *Glut1* (Glucose transporter 1), *Glut3* (Glucose transporter 3), *Gpd2* (Glycerol-3-phosphate dehydrogenase 2), *Pfk1* (Phosphofructokinase 1), *Hk2* (Hexokinase 2), *Tpi* (Triose phosphate isomerase), *Pkm* (Pyruvate kinase muscle isozyme), *Ldha* (Lactate dehydrogenase A), *Yars* (Tyrosyl-tRNA synthetase) in wild type, let-7Tg, and Lin28Tg CD8 T cells three days after stimulation with anti-CD3 mAbs, presented relative to the ribosomal protein, *Rpl13a*. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test. (a, b, c, d; one experiment representative of three independent experiments (a) or mean and s.e.m. of technical triplicates (b, c, d)).

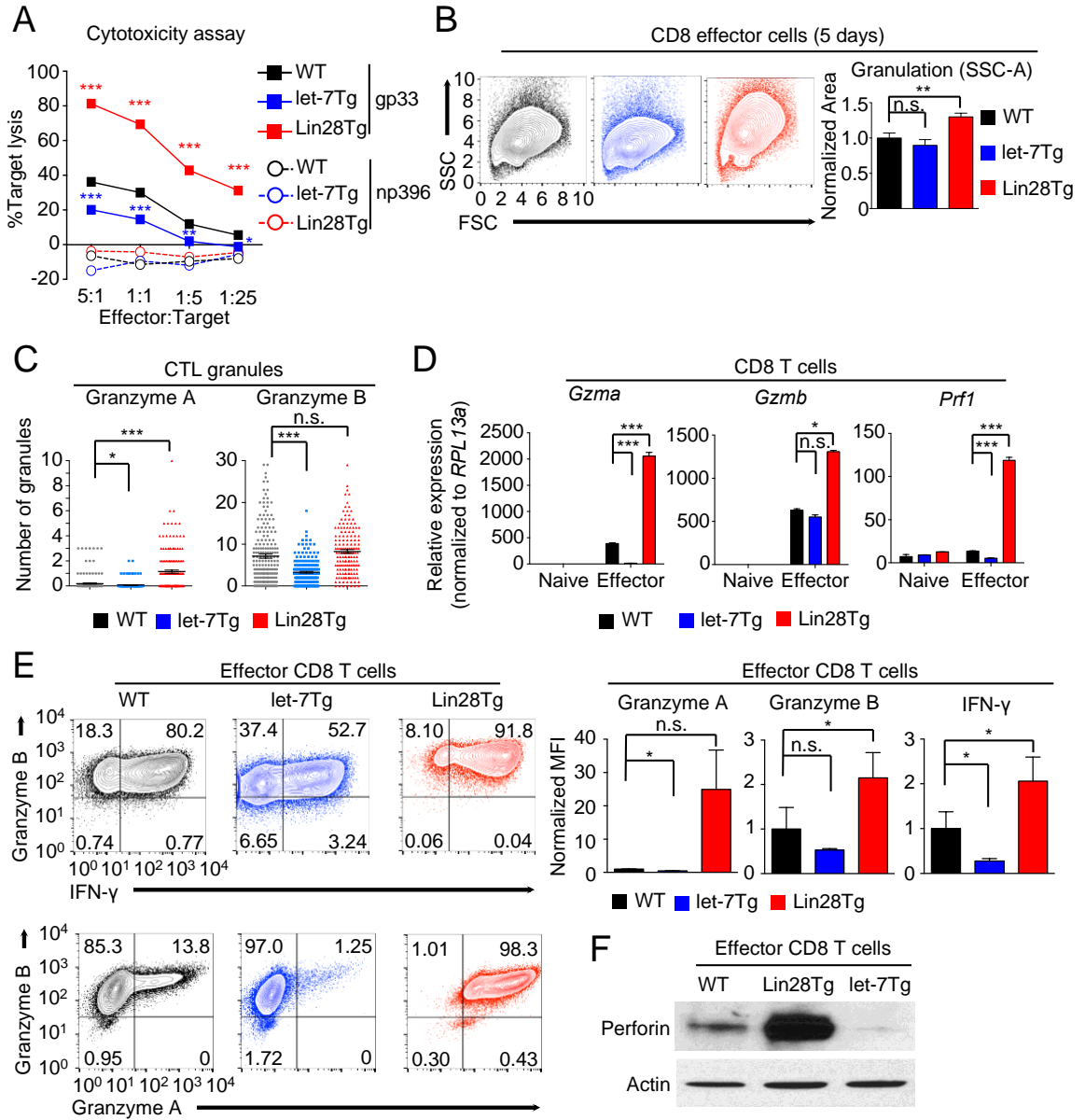


Figure 2.7 let-7 miRNAs negatively regulate differentiation and acquisition of effector function in CTLs

(a) Cytotoxicity assay of differentiated CTLs from P14⁺ wild type, P14⁺let-7Tg, and P14⁺Lin28Tg lymph nodes co-cultured with either LCMV gp33 or LCMV np396 peptide-pulsed splenocytes for 4-5 hours. (b) Analysis of the internal complexity (FSC, forward scatter; SSC, side scatter) of effector (5 days after anti-CD3 mAb stimulation) CD8 T cells generated from wild type, let-7Tg, and Lin28Tg lymphocytes (left) and quantification of SSC of CD8 T cells normalized to wild type. (c) Quantification of Granzyme A and Granzyme B- positive granules in wild type, let-7Tg, and Lin28Tg CTLs via MilliPore Amnis ImageStream. (d) Quantitative RT-PCR analysis of effector molecule mRNA expression: *Gzma* (Granzyme A), *Gzmb* (Granzyme B), *Prfl* (Perforin) in naïve and effector CD8 T cells from wild type, let-7Tg, and Lin28Tg lymph nodes, presented relative to expression of the ribosomal protein *Rpl13a*. (e) Staining (top, middle) and MFI (bottom) of Granzyme B, Interferon- γ , and Granzyme A in wild type, let-7Tg, and Lin28Tg effector CD8 T cells normalized to wild type. (f) Western blot analysis of lysates of wild type, let-7Tg, and Lin28Tg effector CD8 T cells, probed with monoclonal antibodies against Perforin and actin. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test. Data are from one experiment representative of three experiments (a, d, e; mean and s.e.m. of technical triplicates, b, e; mean and s.e.m of three experiments).

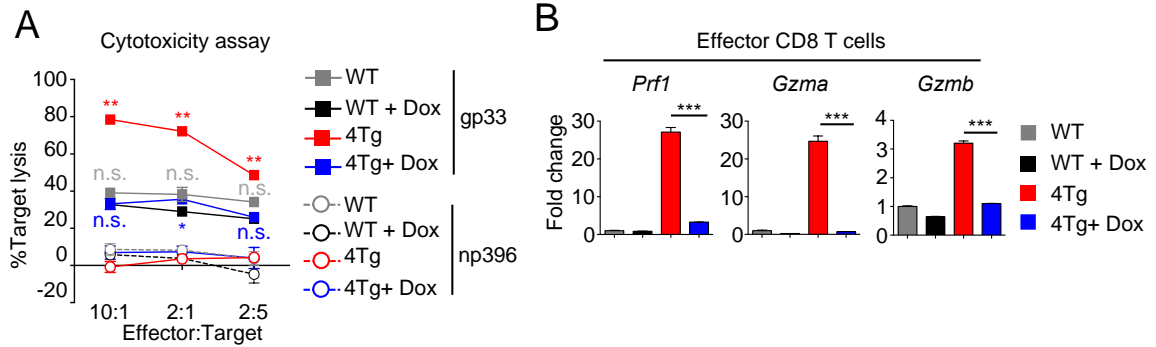


Figure 2.8 Enhanced function of Lin28Tg CTLs is not due to a bystander effect of Lin28 expression

(a) Cytotoxicity assay of differentiated CTLs from P14⁺ wild type (WT), or P14⁺let-7Tg⁺Lin28Tg⁺Rag2^{-/-} (4Tg) lymphocytes co-cultured with either LCMV gp33 or LCMV np396 peptide-pulsed splenocytes for 4-5 hours, either in the presence or absence of doxycycline. (b) Quantitative RT-PCR analysis of effector molecule mRNA expression: *Prf1* (Perforin), *Gzma* (Granzyme A), *Gzmb* (Granzyme B) in effector CD8 T cells from P14⁺ wild type, or P14⁺let-7Tg⁺Lin28Tg⁺Rag2^{-/-} (4Tg) lymph nodes cultured either in the presence or absence of Dox, presented as the fold change in expression, normalized to wild type. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test. Data are from one experiment representative of three experiments (a) or one experiment (b) (a,b; mean and s.e.m. of technical triplicates).

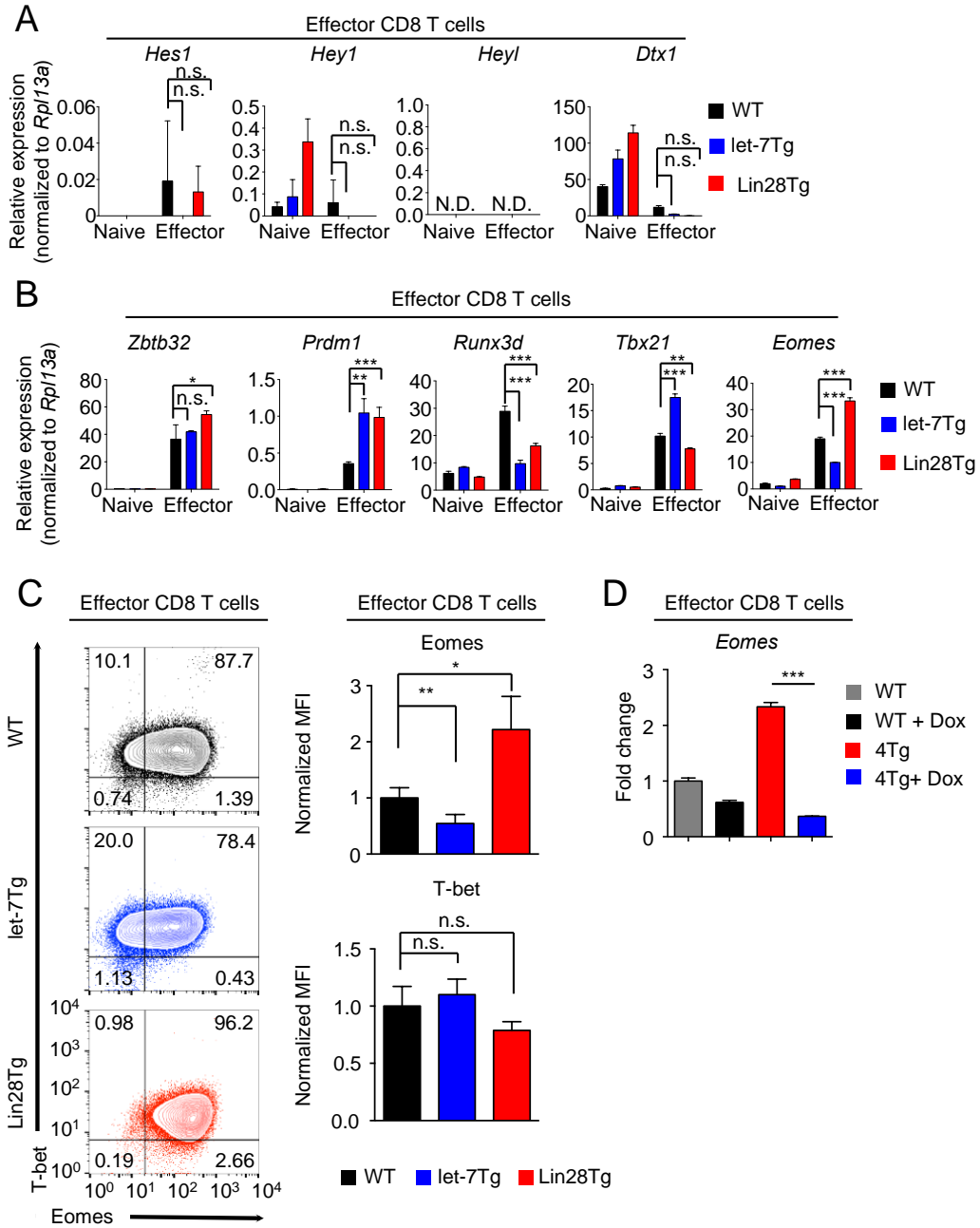


Figure 2.9 let-7 miRNAs regulate Eomes expression in activated CD8 T cells

(a, b) Quantitative RT-PCR analysis of Notch target genes: *Hes1* (Hes family BHLH transcription factor 1), *Hey1* (Hes related family BHLH transcription factor with YRPW motif 1), *Heyl* (Hes related family BHLH transcription factor with YRPW motif-like), *Dtx1* (Deltex 1) (a) and transcription factors that control CD8 T cell differentiation: *Zbtb32* (Zinc finger and BTB domain containing 32), *Prdm1* (Blimp-1), *Runx3d* (Runt related transcription factor 3 distal) *Tbx21* (T-bet), and *Eomes* (b) in naïve and effector (5 days after anti-CD3 mAb stimulation) wild type, let-7Tg, and Lin28Tg CD8 T cells, presented relative to the expression of the ribosomal protein *Rpl13a*. (c) Staining of Eomes and T-bet (left) and MFI of Eomes and T-bet (right) in wild type, let-7Tg, and Lin28Tg effector CD8 T cells, normalized to wild type. (d) Quantitative RT-PCR analysis of *Eomes* (Eomesodermin) mRNA in P14⁺ wild type, or P14⁺let-7Tg⁺Lin28Tg⁺Rag2^{-/-} (4Tg) lymph nodes cultured either in the presence or absence of Dox, presented as the fold change in expression, normalized to wild type. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, compared with wild type using two-tailed Student's *t*-test. Data are from one experiment representative of three independent experiments (a, b, d; mean and s.e.m. of technical triplicates, or three experiments (c)). N.D., not determined.

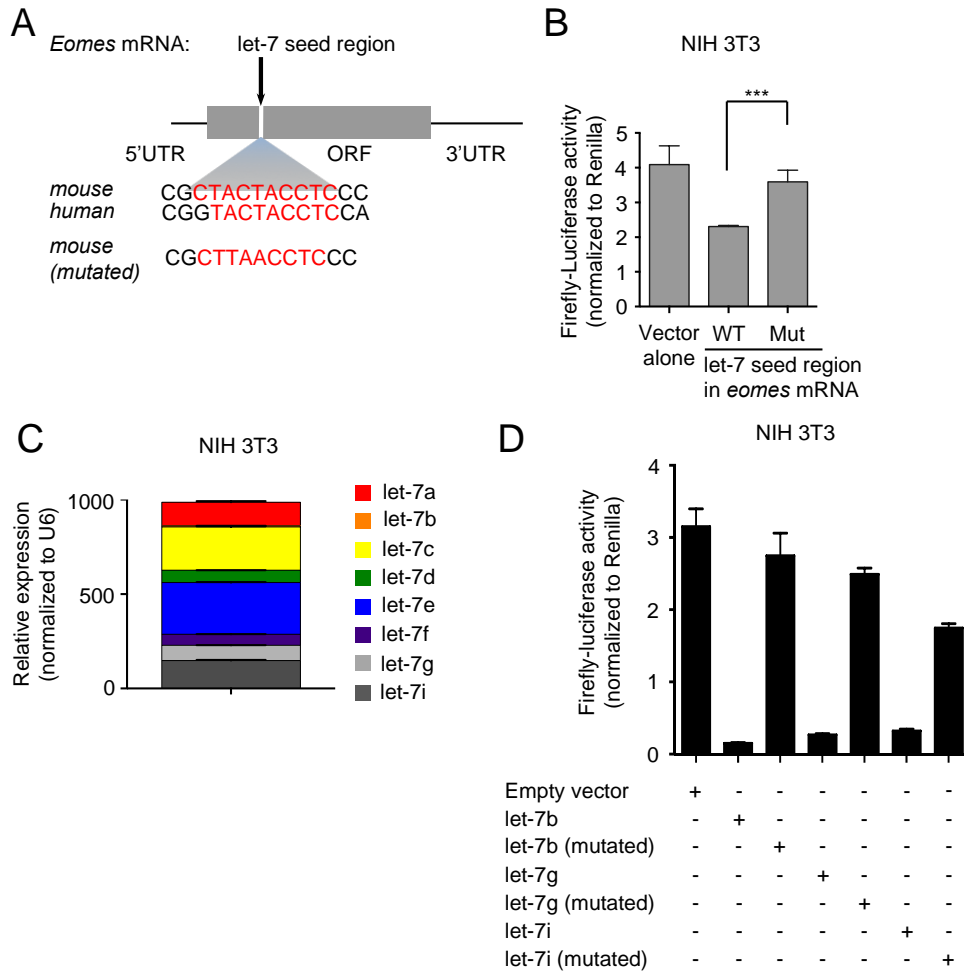


Figure 2.10 let-7 miRNAs directly target the mRNA of *Eomes* in activated CD8 T cells (a) *Eomes* mRNA, including the 5' and 3' untranslated regions (UTR) and the open reading frame (ORF) within which one let-7 binding site was identified (top), sequence conservation between the mouse and human let-7 binding sites of *Eomes* is shown (middle). Mutated sequence of the let-7 binding site in the cDNA of mouse *Eomes* (bottom) used in the luciferase reporter assay. (b) Luciferase reporter assay of let-7 targeting of the *Eomes* ORF in NIH 3T3 fibroblasts transfected with a luciferase reporter containing either the intact or mutated sequence of the let-7 seed region from the mouse *Eomes* ORF; the activity of firefly luciferase was normalized to the Renilla luciferase control. (c) Quantitative RT-PCR analysis of individual let-7 miRNAs in NIH 3T3 fibroblasts, presented relative to results obtained for the small nuclear RNA (U6) (d) Expression analysis of three let-7 family members by luciferase reporter assay. A firefly luciferase reporter containing either intact or mutated let-7b, let-7g, or let-7i antisense seed regions were transfected into NIH 3T3 cells. The activity of firefly luciferase was normalized to the Renilla luciferase control. *** $P < 0.001$, compared with wild type using two-tailed Student's t -test (b). Data are one experiment representative of three independent experiments (b, c, d; mean and s.e.m. of technical triplicates).

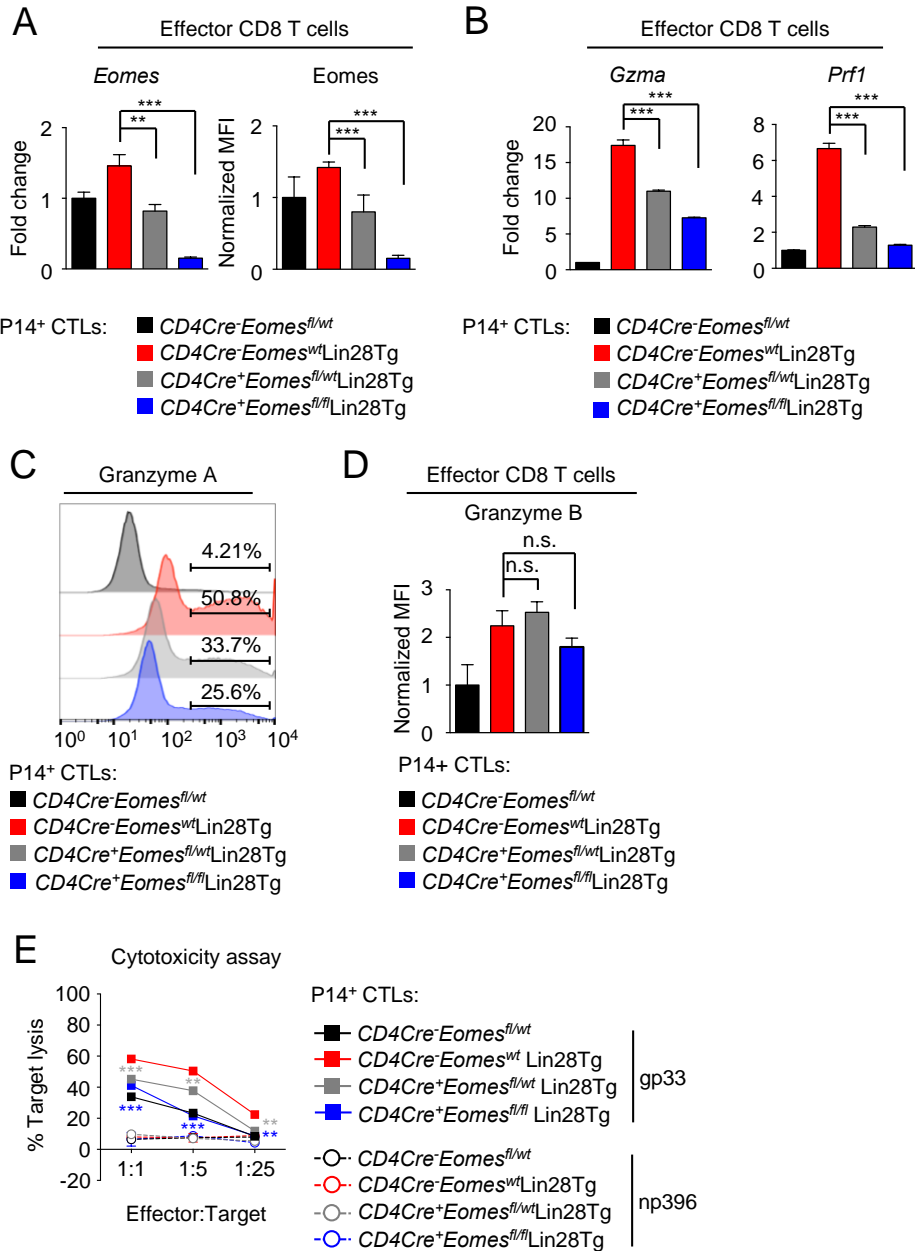


Figure 2.11 let-7 miRNAs control the differentiation of CTLs through Eomes-dependent and independent mechanisms

(a) Quantitative RT-PCR analysis of *Eomes* mRNA, presented as the fold change in expression, normalized to wild type (left) and MFI of Eomes protein expression, normalized to wild type (right), from CTLs generated from P14⁺CD4-Cre *Eomes*^{fl/wt}, P14⁺CD4-Cre *Eomes*^{wt}Lin28Tg, P14⁺CD4Cre⁺ *Eomes*^{fl/wt}Lin28Tg, and P14⁺CD4Cre⁺ *Eomes*^{fl/fl}Lin28Tg CD8 T cells. (b) Quantitative RT-PCR analysis of *Gzma* (Granzyme A) and *Prfl* (Perforin) mRNA in effector CTLs generated from the indicated mice, presented as the fold change in expression, normalized to wild type. (c) Staining of Granzyme A in CTLs generated from the indicated mice. (d) Staining of Granzyme B in CTLs generated from the indicated mice. (e) Cytotoxicity assay demonstrating specific target lysis of differentiated P14⁺ CTLs from the indicated mice, co-cultured with either LCMV gp33 or LCMV np396 peptide-pulsed splenocytes for 4-5 hours. n.s., not significant ($P > 0.05$), ** $P < 0.01$, and *** $P < 0.001$, compared with Lin28Tg (a, b, e) or 4Tg without Dox compared with 4Tg with Dox (d) using two-tailed Student's *t*-test. Data are from one experiment representative of two independent experiments (a, d; mean and s.e.m. of two experiments; b, e; mean and s.e.m. or technical triplicates).

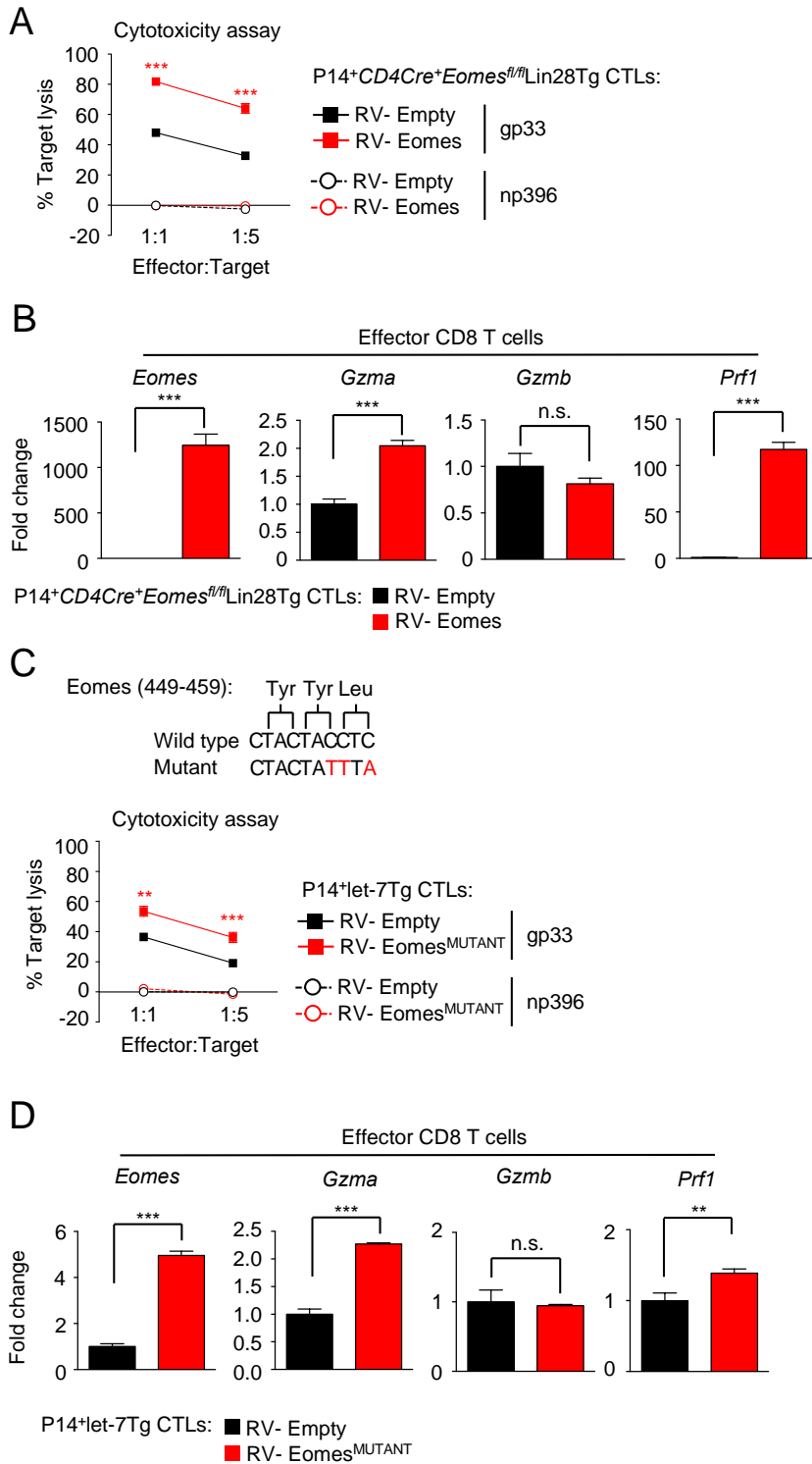


Figure 2.12 Re-expression of Eomes enhances cytotoxic function of CTLs

(a) Cytotoxicity assay demonstrating specific target lysis of LCMV gp33 or LCMV np396 peptide-pulsed splenocytes by differentiated P14⁺CD4Cre⁺Eomes^{fl/fl}Lin28Tg CTLs transduced with the indicated virus, cells were co-cultured for 4-5 hours. (b) Quantitative RT-PCR analysis of *Eomes* (Eomesodermin), *Gzma* (Granzyme A), *Gzmb* (Granzyme B), and *Prfl* (Perforin) expression in CTLs described in a, presented as the fold change in expression, normalized to RV-Empty. (c) Visualization of the mutations made to the let-7 binding motif in mouse Eomes ORF to generate the RV-Eomes^{MUTANT} (top), and cytotoxicity assay demonstrating specific target lysis of differentiated P14⁺let-7Tg CTLs transduced with the indicated virus, co-cultured with either LCMV gp33 or LCMV np396 peptide-pulsed splenocytes for 4-5 hours (bottom). (d) Quantitative RT-PCR analysis of *Eomes* (Eomesodermin), *Gzma* (Granzyme A), *Gzmb* (Granzyme B), and *Prfl* (Perforin) in effector CTLs from c, presented as the fold change in expression, normalized to RV-Empty. n.s., not significant ($P > 0.05$), ** $P < 0.01$, *** $P < 0.001$, RV-Eomes or RV-Eomes^{MUTANT} compared with RV-Empty using two-tailed Student's *t*-test (a, b, c, d). Data are from one experiment representative of two independent experiments (a, b, c, d; mean and s.e.m. of technical triplicates).

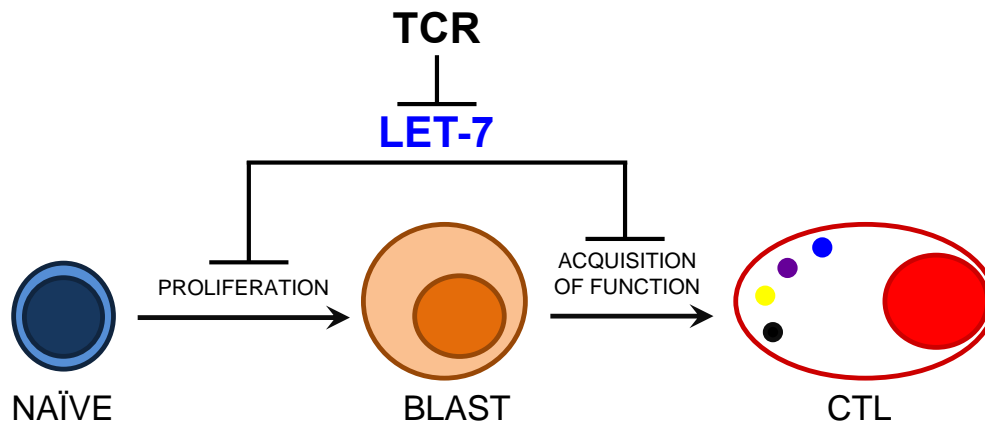


Figure 2.13 Model of let-7- mediated regulation of CD8 T cell differentiation

(a) Upon TCR stimulation, let-7 miRNAs are rapidly down-regulated, in order to release the 'molecular brake' let-7 places on CD8 T cell differentiation in naïve cells. Our study suggests that let-7 miRNAs inhibit clonal expansion and metabolic reprogramming of activated CD8 T cells through inhibition of Myc and other cell cycle controlling factors. Further, our data indicates let-7 also suppresses the acquisition of effector function of CTLs through the direct targeting of Eomes mRNA.

CHAPTER 3

LET-7 MIRNAS IN CD8 T CELLS PROMOTE MEMORY FORMATION BY RESTRAINING TERMINAL DIFFERENTIATION AND EXHAUSTION

3.1 Introduction

Antigen stimulation licenses CD8 T cells to rapidly differentiate into cytotoxic T lymphocytes (CTLs), the majority of which die during contraction following clearance of the antigen, while the surviving cells differentiate into highly protective long-lived memory cells (Kaech and Cui, 2012). Although terminal effector CTLs provide robust cytotoxic responses against the antigen, they do not contribute to the memory pool, and are cleared during contraction (Schluns et al., 2000; Kaech et al., 2003). In fact, as CD8 T cells progress further into the terminal effector program, they steadily lose the potential to differentiate into memory cells (Joshi et al., 2007; Kaech and Cui, 2012; Chang et al., 2014; Gray et al., 2017; Chen et al., 2018). Moreover, terminal effector cells express high levels of co-inhibitory receptors, such as PD-1, Tim-3, CD160, and 2B4, to prevent hyperactivation, and to scale the effector response to the demands of the infection (Wherry et al., 2007; Doering et al., 2012; Paley et al., 2012; Singer et al., 2016). However, in chronic infections and cancer, persistent antigen exposure results in sustained, and often heightened expression of inhibitory receptors, making the terminal effector population vulnerable to exhaustion (Wherry et al., 2007; Doering et al., 2012). Engagement of these receptors in immunosuppressive settings, such as the tumor microenvironment, re-routes terminal effector cells into an unresponsive, exhausted state (Wherry and Kurachi, 2015). Additionally, the chronic TCR stimulation which results from persistent antigen exposure

drives cells into a dysfunctional state by establishing irreversible epigenetic modifications (Schieter et al., 2016; Yu et al., 2017).

Interestingly, it was recently demonstrated that memory cells confer robust anti-tumor protection (Geiger et al., 2016; Sahin et al., 2017; Herndler-Brandstetter et al., 2018). Although the exact mechanisms for these enhanced responses are not known, it is perhaps due to low inhibitory receptor expression and the enhanced survival program in memory CD8 T cells (Kaech et al., 2002), rendering these cells impervious to the immunosuppressive environments established during chronic infection and cancer. Thus, a more complete understanding of the molecular mechanisms that restrain CD8 T cell terminal differentiation, and in turn preserve memory potential in these cells is warranted, and would have therapeutic implications for both preventing exhaustion and promoting memory formation in immunosuppressive settings. As the differentiation of terminal effector and memory cells is regulated at multiple levels, it is likely that a global molecular hub which can regulate multiple cellular processes controls terminal effector and memory differentiation. Post-transcriptional regulation mediated by miRNAs provides robust sequence-specific regulation of gene expression in a tissue and context-dependent manner. Accordingly, miRNAs are strong candidates for controlling the differentiation of terminal effector and memory CD8 T cells.

In fact, the involvement of miRNAs in terminal effector and memory CD8 T cell differentiation has been demonstrated, although the extent of their involvement is not fully understood. While roles for miR-31 (Moffett et al., 2017) and the miR-23-27-24 cluster (Cho et al., 2016) have been suggested, the miRNA most extensively studied in terminal effector and memory differentiation is miR-155 (Gracias et al., 2013; Lind et al., 2013;

Stelekati et al., 2018). Mir-155 expression is very dynamic in different CD8 T cell populations. It is expressed at low levels in naïve cells, is upregulated in terminal effector cells, becomes highly expressed in exhausted CD8 T cells, and is lowly expressed in memory CD8 T cells (Gracias et al., 2013). A functional role for miR-155 in effector cells was identified by the compromised the effector CD8 T cell response in miR-155-deficient mice, which was attributed to an impairment in CD8 T cell proliferation and expansion (Gracias et al., 2013; Lind et al., 2013). Consistent with high expression of miR-155 in exhausted cells, overexpression of miR-155 also compromised the proliferation and function of terminal effector CD8 T cells (Gracias et al., 2013; Stelekati et al., 2018). This was demonstrated to be a result of miR-155-mediated suppression of the Fos12 component of the AP-1 transcription factor complex (Stelekati et al., 2018). Interestingly, miR-155 expression was also demonstrated to be important for the long-term survival of exhausted CD8 T cells (Stelekati et al., 2018). The miR-17-72 cluster has also been demonstrated to modulate CD8 T cell differentiation. Similar to miR-155, the miR-17-92 cluster is strongly upregulated in effector cells and downregulated in memory cells (Wu et al., 2012). This miRNA cluster was demonstrated to support the proliferation of effector CD8 T cells by enhancing mTOR activity (Wu et al., 2012). Accordingly, failure to downregulate the miR-17-92 cluster impaired memory formation, due to direct targeting of *Id2* and *Bcl-2* mRNAs (Wu et al., 2012; Khan et al., 2013).

These studies provide strong, albeit limited, evidence that temporal modulation of miRNA expression has important consequences for CD8 T cell differentiation. Whether other miRNAs are involved in this process, and to what extent remains to be determined. Moreover, these studies have primarily focused on miRNAs which are upregulated during

activation, which may be a potential limitation in fully understanding the positive regulation by miRNAs during memory differentiation. We have previously demonstrated that the let-7 miRNAs are downregulated upon TCR stimulation as let-7 expression suppresses CTL differentiation (chapter 2; Wells et al., 2017). This study identified a surprising role for the let-7 miRNAs in determining the fate of CD8 T cells, and in doing so, we also describe a critical paradox in the study of CD8 T cell differentiation, where the *in vitro* function of CTLs does not necessarily predict the performance of cells *in vivo*. Specifically, it was determined that the absence of let-7 miRNAs drove cells into a terminal effector state characterized by high expression of inhibitory receptors. Accordingly, upon introduction into the immunosuppressive environment, these cells became functionally exhausted. Conversely, overexpression of let-7 improved tumor rejection due to an enhanced memory differentiation. This study has identified an important role for the let-7 miRNAs in restraining terminal differentiation to maintain memory potential in differentiating CD8 T cells.

3.2 Results

3.2.1 let-7 expression in CTLs enhances tumor rejection

To test the superior function of let-7-deficient CTLs in anti-tumor responses, B16F10 mouse melanoma cells were genetically engineered using a retroviral vector which contains an IRES-GFP reporter, to express the gp33-41 peptide (Figure 3.1A, referred to as B16^{gp33}) from the lymphocytic choriomeningitis virus (LCMV). Thus, tumor-specific lysis can be assessed using P14 TCR transgenic CD8 T cells which recognize this peptide in the context of MHC-I (H-2D^b). P14 CTLs were generated from WT, Lin28Tg, or let-

7Tg mice and the cytotoxic activity against B16^{gp33} cells was assessed *in vitro*. Lin28Tg CTLs killed B16^{gp33} cells with a much greater efficacy than WT CTLs, while let-7Tg CTLs demonstrated the least cytotoxic function (Figure 3.1B). These results indicated that P14 CTLs can recognize B16^{gp33} tumor cells, and are consistent with the previously observed let-7-mediated control of *in vitro* cytotoxic function (Figure 2.7 A; Wells et al., 2017). However, upon adoptive transfer into B16^{gp33} tumor-bearing mice, Lin28Tg CTLs completely failed to control tumor growth. Rather, quite surprisingly, let-7Tg CTLs provided the most robust tumor protection *in vivo*, such that mice which received let-7Tg CTLs were the only mice that survived the study (Figure 3.2A-C). Importantly, tumors that did grow in these mice were found to be GFP-, indicating that outgrowth was due to a loss of gp33 expression (Figure 3.2D). Moreover, in a metastatic model of B16^{gp33}, transfer of let-7Tg CTLs significantly reduced metastatic burden in the lung, while Lin28Tg CTLs conferred no improvement to the anti-tumor response (Figure 3.2E). Altogether, these results demonstrate that let-7Tg, rather than Lin28Tg CTLs improve tumor rejection, despite opposing cytotoxicity *in vitro*.

3.2.2 Molecular characterization of let-7Tg and Lin28Tg CTLs

To explain the paradox between the *in vitro* and *in vivo* performances of Lin28Tg and let-7Tg CTLs, the transcriptomes of CTLs were generated for five days *in vitro* in the presence of IL-2, were analyzed. When compared to wild type CTLs, let-7Tg and Lin28Tg CTLs, differentially expressed 502 genes and 876 genes, respectively (Figure 3.3A). To characterize the signature of let-7Tg and Lin28Tg CTLs, their transcriptomes were analyzed for signatures of memory or terminal effector CD8 T cells (Kaech et al., 2002;

Doering et al., 2012). Gene set enrichment analysis revealed that let-7Tg CTLs express a memory signature, indicated by upregulation of genes highly expressed in memory cells generated in response to acute viral infection with LCMV Armstrong. Conversely, the gene signature of Lin28Tg CTLs is associated with genes expressed in terminal effector cells generated during chronic infection with LCMV clone 13, which results in persistent antigen exposure (Figure 3.3B) (Doering et al., 2012).

Specifically, let-7Tg CTLs preferentially expressed memory markers such as the fate-specifying transcription factors *Id3*, *Tcf7* (gene that encodes Tcf1) and *Foxo1* (Ji et al., 2011; Zhou et al., 2010). Let-7Tg CTLs upregulated expression of the secondary lymphoid homing receptors CD62L (encoded by the gene *Sell*) and *Ccr7*, which facilitate travel of memory CD8 T cells to the spleen and lymph nodes, the primary niches for memory CD8 T cells (Kaech et al., 2003; Sallusto et al., 2004). Expression of the receptor for IL-7 (CD127), an important cytokine for memory CD8 T cell homeostasis was also increased in let-7Tg CTLs (Schluns et al., 2000; Kaech et al., 2003; Sallusto et al., 2004). Further, let-7Tg CTLs increased expression of the anti-apoptotic protein Bcl-2 which helps promote the survival and longevity of memory CD8 T cells (Schluns et al., 2000; Hand et al., 2010) (Figure 3.3C). These results are particularly significant as let-7Tg CTLs were generated in conditions which do not typically support expression of memory-associated genes, suggesting let-7 miRNA expression maintains memory fate potential.

In contrast, Lin28Tg CTLs expressed high levels of *Id2*, and *Ikzf2* (gene that encodes Helios), two transcription factors associated with terminal differentiation and exhaustion (Cannarile et al., 2006; Doering et al., 2012). This is in addition to Eomes, which has been demonstrated to be regulated by let-7 (Figure 2.9A, B), and is highly

expressed in exhausted cells (Doering et al., 2012). Accordingly, expression of the inhibitory receptors Tim-3 (encoded by the gene *Havcr2*), PD-1 (encoded by the gene *Pdcd1*), CD160, and 2B4 (encoded by the gene *Cd244*) was upregulated in Lin28Tg CTLs (Wherry et al., 2007) (Figure 3.3D). Expression of the TNF costimulatory receptor 4-1BB (encoded by the gene *Tnfrsf9*) was also increased, consistent with its expression on strongly activated cells (Williams et al., 2017). Interestingly, OX-40 (encoded by the gene *Tnfrsf4*), another TNF costimulatory receptor, was downregulated (Figure 3.3D). This suggested the expression and function of these receptors in terminal effector differentiation may be more complex than is currently appreciated (Williams et al., 2017). Expression of Wnt10b (Trischler et al., 2016) was also elevated in Lin28Tg CTLs, in addition to the suppressive cytokine IL-10 (Brooks et al., 2006; Ejrnaes et al., 2006), which is a direct let-7 target (Figure 3.3D, E). These data suggest that the absence of let-7 miRNAs leads to the terminal differentiation, and potentially the exhaustion, of CD8 T cells.

It is well established that terminal effector CD8 T cells are dependent on glycolysis to meet their high bioenergetic demands, and that the transition of memory cells back to a quiescent state is accompanied by reduced usage of glycolysis (Frauwirth et al., 2002; Wang et al., 2011; van der Windt and Pearce, 2012; O'Sullivan et al., 2018). To determine the extent to which the metabolism of let-7Tg and Lin28Tg CTLs reflects this feature of memory and terminal effector CD8 T cells, glycolytic usage was assessed. As glycolysis results in the release of lactate and H⁺, which acidify the cell culture media, the rate of glycolysis can be assessed by measuring the extracellular acidification rate (ECAR). In fact, let-7 regulated the extent to which CTLs used glycolysis, in a manner representative of differentiation into either a terminal effector, or memory state (Figure 3.3F). Ultimately,

these data strongly suggest that let-7 miRNAs control the differentiation of memory and terminal effector CD8 T cells.

3.2.3 Maintenance of let-7 expression during activation supports memory T cell differentiation

To directly assess the impact of the let-7 miRNAs in the generation of CD8 T cell memory, naïve P14⁺ CD8 T cells from WT, let-7Tg or Lin28Tg mice were adoptively transferred into congenic CD45.1⁺ hosts, which were then subsequently infected with *Listeria monocytogenes* expressing the LCMV peptide gp33 (LM-33) (Manjunath et al., 2001; Shen et al., 1998). At the peak of the effector response (day nine), there was a higher frequency of memory precursor (CD44⁺CD62L^{hi}) P14 cells in the spleens of mice which received let-7Tg CD8 T cells, while nearly all donor Lin28Tg cells were effector CTLs (CD44⁺CD62L^{lo}). These results are further supported by the distribution of KLRG1/CD127 expression on P14 cells (Figure 3.4A) (Kaech et al., 2003; Joshi et al., 2007). Moreover, by day thirty, the few donor Lin28Tg cells that survived contraction retained an effector phenotype (CD44⁺CD62L^{lo}) (Figure 3.4B). Consistent with previously described data, the maintenance of let-7 expression greatly inhibited the potential for let-7Tg P14 cells to proliferate and expand *in vivo* (Figure 3.4B) (Chapter 2; Wells et al., 2017), making long term analysis of this population difficult. To overcome this limitation, we used an established *in vitro* system for generating memory CD8 T cells in the presence of the memory cytokine IL-15 (Manjunath et al. 2001). As expected, WT CTLs upregulated the expression of *Tcf7*, *Sell*, *Ccr7*, and *CD127* only in the presence of IL-15, whereas let-7Tg CTLs displayed an enhanced memory phenotype regardless of the use of IL-2 or IL-

15 (Figure 3.5A). Although IL-15 reduced expression of *Id2*, *Havcr2*, *Pdcd1*, and *Cd244* in Lin28Tg CTLs, the presence of IL-15 was not sufficient to induce expression of memory markers in Lin28Tg CTLs (Figure 3.5A). Thus, while IL-15 reduced exhaustion, it was unable to initiate the memory differentiation program in Lin28Tg CTLs. In addition, an *in vitro* assay which assesses T cell survival through immediate withdrawal of IL-2, demonstrated that let-7 expression determines CTL viability during cytokine withdrawal (Figure 3.5B) (Geiger et al., 2016). Ultimately, these studies indicate that let-7 programs the fate of the memory CD8 T cell population.

It has been suggested that cues essential for the formation of memory cells occur throughout CD8 T cell differentiation (Badovinac et al., 2005; Kolumam et al., 2005; Joshi et al., 2007; Harty and Badovinac, 2008; Araki et al., 2009; Kaech and Cui, 2012). However, several reports also suggest that the memory fate is established during early activation (Kaech and Ahmed, 2001; Lauvau et al., 2001; Chang et al., 2007; King et al., 2012; Kakaradov et al., 2016; Verbist et al., 2016; Wang et al., 2018). To determine if the timing of let-7 expression was important for its enhancement of memory formation, let-7 expression was induced periodically during CTL differentiation using the doxycycline-inducible transgene (Figure 3.5C). As previously demonstrated (Figure 3.3C), maintaining let-7 expression for all five days of CTL differentiation resulted in high levels of *Tcf7*, *Sell*, and *Ccr7* expression, and downregulation of *Id2*, *Cd244*, and *Havcr2* (Figure 3.5C). However, limiting let-7 overexpression to the first 48 hours of stimulation, also resulted in heightened expression of memory markers, accompanied by the downregulation of genes that contribute to terminal differentiation (Figure 3.5C). Consequently, inducing let-7 expression after the first 48 hours of TCR stimulation was insufficient to enhance the

memory phenotype, and to downregulate expression of the terminal effector genes *Id2* and *Havcr2* to the same extent as cells with high levels of let-7 expression during activation (Figure 3.5C). Taken together, these results demonstrate that let-7 expression, specifically within the first few hours of activation, is necessary and sufficient for the generation of memory CD8 T cells. Additionally, this data provides a molecular mechanism to support earlier studies illustrating that the memory fate is established during antigen stimulation (Kaech and Ahmed, 2001; Lauvau et al., 2001; Chang et al., 2007; King et al., 2012; Kakaradov et al., 2016; Verbist et al., 2016; Wang et al., 2018).

3.2.4 Downregulation of let-7 miRNAs leads to terminal differentiation

Our previous data demonstrated that the maintenance and quiescence of naïve CD8 T cells is negatively impacted by the absence of the let-7 miRNAs (Figure 2.2; Wells et al., 2017). To eliminate the possibility that the dysfunction of Lin28Tg CTLs is due to altered homeostasis of naïve CD8 T cells, mice expressing Lin28 with a GFP reporter downstream of a loxP flanked STOP cassette knocked into the Rosa26 locus ($R26^{STOP-Lin28-GFP}$) (Figure 3.6A), were crossed with mice that express Cre recombinase under the control of the granzyme B promoter ($Gzmb^{Cre+}$), such that only activated T cells will express Lin28 following Cre-mediated excision of the STOP cassette ($Gzmb^{Cre+}R26^{STOP-Lin28-GFP}$). These mice were then inoculated subcutaneously with the MC57 fibrosarcoma, which is controlled in healthy syngeneic C57BL/6 mice (Kundig et al., 1995; Schmits et al., 1996). Wild type mice were able completely clear the tumor by 13 days after the initial implantation (Figure 3.7A). However, this tumor rejection was compromised by depleting let-7 miRNAs in responding CD8 T cells (Figure 3.7A). These results demonstrate that loss

of let-7 expression upon activation is responsible for the dysfunction of Lin28Tg CTLs *in vivo*.

We then hypothesized that the immunosuppressive tumor microenvironment engages the inhibitory receptors that are highly expressed on Lin28Tg CTLs (Figure 3.3D), thus driving dysfunction in let-7-deficient CTLs. To test this hypothesis, anti-PD-L1 was administered to EL-4^{gp33} tumor bearing mice upon transfer of Lin28Tg CTLs. Blocking engagement of the PD-1 inhibitory receptor via treatment with anti-PDL1 antibodies fully rescued Lin28Tg CTL function, enhancing anti-tumor responses to the level of let-7Tg CTLs (Figure 3.6B-D), indicating that Lin28Tg CTL dysfunction is due to functional exhaustion in the tumor microenvironment.

To determine if the duration of let-7 downregulation is an important component in re-directing differentiating CTLs to a terminal effector fate, Lin28Tg^{GFP} mice were crossed to mice with a doxycycline inducible Cre (iCre⁺R26^{STOP-Lin28-GFP}) (Figure 3.6B), such that permanent Lin28 expression can be induced at any point during CTL differentiation. Interestingly, regardless of how long let-7 expression was depleted, expression of the terminal effector genes *Id2*, *Cd244*, *Havcr2*, and *Pdcd1* was upregulated, while expression of memory phenotypic markers (*Tcf7*, *Sell*, *Ccr7*) were reduced (Figure 3.7E). These results indicate that loss of let-7 expression drives cells into the terminal effector program, at the expense of memory potential, independent of the duration of let-7 depletion during differentiation.

3.2.5 let-7 miRNAs regulate terminal differentiation through a complex transcriptional mechanism

Finally, to uncover the molecular machinery let-7 regulates to restrain terminal differentiation, we identified five genes that are direct let-7 targets (Figure 3.8A). These genes were also selected because they have been implicated in transcriptional regulation. All five candidates were individually overexpressed in let-7Tg CD8 T cells to assess the extent to which each gene could drive terminal differentiation in memory-like CD8 T cells.

The first candidate, *Nupr1*, has a single eight-nucleotide let-7 binding site in its 3' untranslated region (UTR), and has been demonstrated to be involved in various cellular stress responses. (Malicet et al., 2005; Malicet et al., 2006; Syn et al., 2017; Santofimia-Castano et al., 2018). Consistent with no known function of *Nupr1* in CD8 T cells, overexpression of *Nupr1* had no effect in reversing the memory phenotype of let-7Tg CTLs (Figure 3.8B). The second candidate, *Helios*, has two seven-nucleotide let-7 binding sites in the 3' UTR. *Helios* is a transcription factor frequently identified in gene profiles from exhausted and terminally differentiated CD8 T cells (Doering et al., 2012). While a specific role for *Helios* in driving terminal effector cell differentiation has yet to be determined, *Helios* has been demonstrated to support an immunosuppressive subset of T cells known as regulatory T cells (Tregs). Specifically, *Helios* is important for the development of Tregs in the thymus, and its expression is necessary to mediate the inhibitory functions of Tregs in the periphery (Thornton et al., 2010; Kim et al., 2015). However, despite being highly expressed in exhausted CD8 T cells, its re-expression into let-7Tg CD8 T cells had no significant impact during differentiation (Figure 3.8C).

The expression of the third candidate, *Hmga1*, has a single seven-nucleotide let-7 binding site in the 3' UTR. *Hmga1* has been demonstrated to be induced by TCR signaling, and consequently in driving expression of IL-2 and IFN- γ (Shannon et al., 1998; Chau et

al., 2005), suggesting its involvement in terminal effector differentiation (Figure 3.9A, B). Interestingly, while overexpression of Hmga1 was sufficient to modulate expression of inhibitory receptors, it had no effect on the production of IFN- γ (Figure 3.9A). Thus, although Hmga1 is involved in the regulation of terminal effector differentiation, its exact function is still unclear.

Of the identified targets, the strongest candidate was Eomes, as it has already been demonstrated to be a functional target of let-7 due to a unique 10-nucleotide let-7 binding site in the open reading frame of the RNA. In addition, high levels of Eomes expression is associated with terminal effector differentiation, and may control a unique gene program to drive this state during chronic viral infection (Doering et al., 2012). Moreover, Eomes has been identified as a marker of the most severe stage of exhaustion *in vivo* (Wherry, 2011). We found that overexpression of Eomes resulted in the upregulation of Tim-3, and modest upregulation of 2B4, while CD160 expression remained unchanged (Figure 3.9C, D). Eomes overexpression downregulated the memory marker CD62L, although this was not consistent at the RNA level (Figure 3.9C, D). To take the opposite approach, and assess if removal of Eomes from Lin28Tg cells could restrain terminal differentiation, CTLs generated from Lin28Tg mice with a T cell-specific deletion of Eomes (CD4Cre⁺Eomes^{fl/fl}Lin28Tg) were analyzed. While deletion of Eomes in Lin28Tg CTLs had no effect on the expression of CD62L, PD-1, 2B4, or CD160, the expression of both Tim-3, and IL-10 was significantly reduced (Figure 3.9E). These results demonstrate that Eomes contributes to the terminal effector differentiation program by regulating expression of Tim-3 and IL-10.

The mRNA of the final candidate, Arid3a, contains five unique let-7 binding sites, three of which are in the 3'UTR, and two of which are in the open reading frame. This high number of let-7 binding sites indicative of functional suppression. In fact, Arid3a has previously been demonstrated to be a functional target of let-7 in fetal B cell development (Zhou et al., 2015), and in the regulation of specialized thymus-resident B cells in the adult organism (Xiao et al., 2018). Constitutive expression of Arid3a in B cells results in increased proliferation and autoantibody production, suggesting that in this population of immune cells its regulation is critical (Ratliff et al., 2014). However, there is no known function of Arid3a in CD8 T cells. When overexpressed in let-7Tg CTLs, Arid3a increased the frequency of cells expressing Tim-3, 2B4, and CD160, and the expression of PD-1 more than doubled (Figure 3.9F, G). Conversely, Arid3a expression reduced the frequency of CD62L⁺ cells, while also downregulating the expression of *Tcf7* and *Ccr7* mRNA (Figure 3.9F, G). These results suggest that Arid3a may be another important contributing factor to let-7 mediated regulation of terminal effector differentiation.

Together, these results suggested that while Arid3a, Eomes, and Hmgal may contribute to the establishment of the terminal differentiation program, thus preventing memory formation, they are insufficient to do so independently. We speculate that a synergistic network of key factors is involved, and that the let-7 miRNAs act as a global regulator to suppress this network, thereby restraining terminal differentiation and in turn promoting the generation of memory CD8 T cells. Ultimately, this study identified let-7 miRNAs as a unique therapeutic tool, where both its overexpression and deletion, when in combination with checkpoint blockade inhibitors, improve distinct components of CD8 T cell immunity.

3.3 Discussion

This study has identified a novel post-transcriptional mechanism involved in terminal effector and memory differentiation. Specifically, these results provide compelling data demonstrating an important role for the let-7 miRNAs suppress a complex transcriptional network to restrain terminal differentiation and thus preserve the memory potential of CD8 T cells. This was demonstrated primarily through the use of an aggressive B16 melanoma cancer model, in which expression of let-7Tg CTLs provided the most robust anti-tumor protection. However, if aided by checkpoint blockade inhibition, Lin28Tg terminal effector cells also efficiently eliminated tumors. These results directly implicate the let-7 miRNAs as a unique therapeutic tool.

Transcriptome analysis revealed let-7Tg CTLs were highly enriched for the memory phenotype (Kaech et al., 2002). A functional role for let-7 miRNAs was in maintaining the memory phenotype was demonstrated in an *in vitro* memory differentiation model (Manjunath et al., 2001). Overexpression of let-7 miRNAs supported memory differentiation, even in conditions used to generate effector cells. On the other hand, Lin28Tg failed to generate memory cells, despite being cultured in memory-differentiating conditions. These data demonstrate that the let-7 miRNAs are sufficient to program cells into the memory fate, and that loss of let-7 expression irreparably damages the ability to initiate the memory differentiation program. Moreover, let-7 expression improved the survival of CD8 T cells upon IL-2 withdrawal. Accordingly, it will be important to follow up on the expression and function of pro- and anti-apoptotic proteins, including Bcl-2, that may contribute to the enhanced survival of let-7Tg CTLs. These results additionally

suggest that let-7 expression may be a contributing factor for the long-term maintenance of memory CD8 T cells. The let-7-mediated inhibition of proliferation prevented the investigation of the let-7 miRNAs in long-lived memory populations. As such, the role of let-7 miRNAs in the maintenance of memory CD8 T cells was not addressed. From these observations, it can be speculated that high let-7 expression may facilitate the maintenance of memory CD8 T cells by enhancing survival and reducing proliferation to preserve quiescence. This would be consistent with a role for the let-7 miRNAs in maintaining the quiescent state in naïve CD8 T cells (Chapter 2; Wells et al., 2017).

The finding that let-7 expression during the first 48 hours of antigen stimulation is sufficient to generate cells with a memory phenotype provides critical insight into the mechanism which specifies the memory fate. Specifically, these results present evidence demonstrating that the most critical signal to generate memory cells is received during TCR stimulation. Based on this, and previous observations that let-7 expression is TCR-dependent (chapter 2), it can be speculated that either weaker or shorter TCR signaling will yield a more plastic, stem-like CD8 T cell population with increased memory potential by keeping let-7 expression relatively high. Importantly, these results provide a molecular mechanism supporting studies which report a TCR-dependent model of memory differentiation (Chang et al., 2007; Zehn et al., 2009; King et al., 2012; Verbist et al., 2016).

The data presented here also define a role for the let-7 miRNAs in terminal effector differentiation, and exhaustion, which was first identified by transcriptome analysis. Lin28Tg CTLs were highly enriched for a terminal effector/ exhausted signature. Expression of transcription factors, inhibitory receptors, and cytokines associated with terminal effector cells were all upregulated in Lin28Tg CTLs (Doering et al., 2012), with

the exception of the co-stimulatory receptors, of which OX-40 was upregulated in let-7Tg CTLs. It has primarily been thought that 4-1BB and OX-40 have similar functions in CD8 T cell activation and effector differentiation (Croft et al., 2014). There is direct evidence that 4-1BB identifies exhausted cells (Williams et al., 2017; Horton et al., 2018), while such a role for OX-40 has not been as clearly defined (Croft et al., 2014). This data may indicate divergent roles for these two co-stimulatory receptors in effector and memory formation. Further elucidation of the distinct functions of costimulatory receptors belonging to the TNF receptor superfamily may thus have important implications for their therapeutic applications.

This study also provides mechanistic insight into the establishment of exhaustion. Shortening the duration of let-7 depletion via excision of the STOP cassette from iCreR26^{STOP-Lin28-GFP} CTLs progressively later in differentiation, demonstrated that the terminal effector program can be established at any point upon depletion of let-7 miRNAs, independent of the duration of let-7 depletion. Importantly, this came at the expense of memory differentiation, consistent with our data demonstrating Lin28Tg CTLs are unable to initiate the memory program. If extrapolated, this data could indicate that at any point during CD8 T cell differentiation, strong TCR signals can drive CD8 T cells into the terminal effector fate, and divert CD8 T cells away from the memory fate.

Although a single target responsible for the let-7-mediated restraint of terminal effector differentiation was not identified, several of the tested candidates did demonstrate moderate contributions to this process. Whether this is their primary function in CD8 T cells remains to be determined. Interestingly, every candidate gene which drove the effector phenotype in let-7Tg CTLs most significantly modulated Tim-3 expression. This

supports recent studies that have identified Tim-3 as having a more significant function in the differentiation of terminal effector cells, and their progression into exhaustion, than was originally appreciated (Jin et al., 2010; Singer et al., 2016; Kurtulus et al., 2019).

It is improbable that let-7 would target one factor to restrain the entire terminal effector differentiation program. Let-7 is a very ancient miRNA, and accordingly its targets are numerous and highly conserved (Roush and Slack, 2008). Accordingly, let-7 and its targets would have co-evolved during the acquisition of the adaptive immune system, and there may be functional redundancies amongst its targets. Moreover, these candidates may need to cooperate together to exert their functions, which could be determined by combinatorial overexpression. Additionally, as cells enter into the exhaustion program, the epigenetic landscape is reconfigured (Schietinger et al., 2016; Yu et al., 2017). Further, the plasticity of memory CD8 T cell responses is associated with a ‘poised’ chromatin state (Gray et al., 2017; Akondy et al., 2017; Wang et al., 2018). Let-7 may play an important role in shaping the epigenetic landscape of differentiating CD8 T cells, and differing chromatin states in let-7Tg and Lin28Tg CTLs may mask the extent to which the tested candidates are involved in CD8 T cell differentiation. Thus, determining the chromatin landscapes of let-7Tg and Lin28Tg CTLs, and the role of let-7 miRNAs in regulating epigenetic modifications will be important.

Future studies to determine the physiological expression of let-7 miRNAs in memory, terminal effector, and exhausted CD8 T cells will further elucidate their function in these populations. Asymmetric distribution of let-7 during antigen stimulation may explain the high let-7 expression required for memory cell differentiation, and the low let-7 expression required for terminal effector differentiation. This would also provide a

molecular mechanism for the asymmetric division model of memory differentiation. Specifically, we would hypothesize the proximal daughter cell becomes a terminal effector cell due to downregulation of let-7 driven by stronger TCR stimulation. Conversely, the distal daughter CD8 T cell differentiates into a memory cell as it receives weaker TCR stimulation, and would thus retain let-7 expression (Chang et al., 2007; Verbist et al., 2016). In regards to exhaustion, progressive downregulation of let-7 either by repeated TCR stimulation due to persistent antigen load might provide a mechanism for the gradual ‘acquisition’ of exhaustion (Paley et al., 2012). Determining the direct mechanism by which TCR signaling regulates let-7 expression will be critical to the implementation of the let-7 miRNAs as a therapeutic tool.

Differences in the temporal requirements for memory and terminal effector differentiation may be indicative of a central strategy used to generate effective CD8 T cell responses. Memory cells provide lifelong immunity, and may populate the effector population as it becomes exhausted during chronic infection and cancer (Im et al., 2016, Utzschneider et al., 2016; Siddiqui et al., 2019; Kurtulus et al., 2019). Specifying cells to the memory fate early during activation may be a mechanism by which to prioritize and guarantee their generation. The ability to then initiate the terminal effector differentiation program any point thereafter would allow the magnitude of the effector response to be scaled according to the severity of the infection. Thus, the persistence of antigen would determine the extent to which these cells are driven into the terminal effector program toward exhaustion (Wherry, 2011; Schietinger and Greenberg, 2014). This might further suggest that exhaustion may not be a negative bystander effect, but rather an intentional mechanism used to indirectly boost immune responses, supporting hypotheses which

propose CD8 T cell exhaustion is a strategy used by the immune system to drive neoantigen generation, and solicit ‘fresh’ and clonally diverse CD8 T cells to the tumor and site of infection to aid in clearing the unhealthy cells (Vezys et al., 2006; Wherry, 2011; Schietinger and Greenberg, 2014; Schietinger et al., 2016). This would also support evidence that exhaustion is an intentional program which aids in viral clearance, and prevents tissue damage during prolonged T cell responses (Vezys et al., 2006; Paley et al., 2012; Cornberg et al., 2013).

The data presented here suggests immense potential for manipulating let-7 miRNAs as a therapeutic approach. A mechanism by which to modulate let-7 expression could feasibly be integrated into the *in vitro* expansion phase of adoptive T cell therapy, and during CAR-T cell generation (Rosenberg and Restifo, 2015; Ramos et al., 2016). Moreover, these results may have implications for the improvement of vaccination strategies which rely on the formation of memory cells. Additionally, suppressing let-7 miRNAs expression may be an effective therapeutic strategy in combating autoimmunity by intentionally ‘exhausting’ these autoreactive cells to inhibit their effector function against healthy host cells.

Ultimately, this study has established a global role for the let-7 miRNAs in regulating CD8 T memory and terminal effector cell differentiation, and has identified the let-7 miRNAs as a unique therapeutic target for modulating CD8 T cell responses during chronic infection, cancer, and autoimmunity.

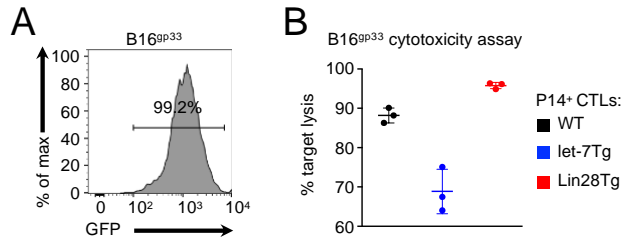


Figure 3.1 Generation of an antigen-specific tumor model

(a) GFP expression on B16^{gp33} tumor cells which have transduced to express the LCMV gp33 antigen in an IREs-GFP retroviral vector. (b) Cytotoxicity assay of differentiated CTLs from P14⁺ wild type, P14⁺let-7Tg, and P14⁺Lin28Tg lymph nodes co-cultured with B16^{gp33} tumor cells for 14-16 hours. Data are from one experiment representative of two experiments (b; mean and s.e.m. of technical triplicates).

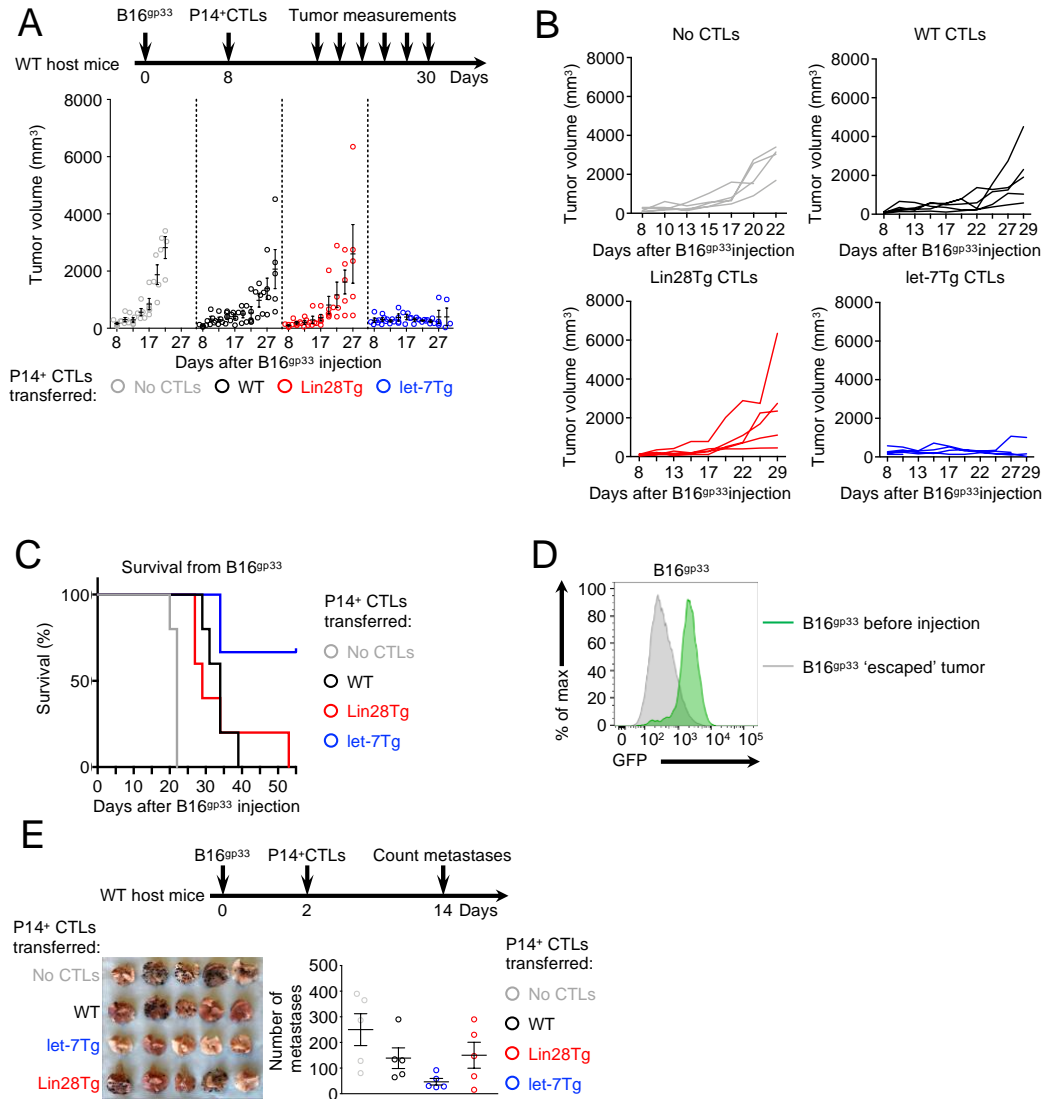


Figure 3.2 let-7 miRNA expression dramatically improves anti-tumor CTL responses *in vivo*

(a, b) Tumor growth curves in mice inoculated s.c. with 0.25×10^6 B16^{gp33}. Tumor-bearing mice received adoptive transfer of 1.5×10^6 CTLs generated from either P14+WT (n=5), P14+let-7Tg (n=5), P14+Lin28Tg (n=5) lymphocytes, or no CTLs at all (n=5). (c) Analysis of the survival of B16^{gp33} tumor bearing mice, which were received adoptive transfer of 1.5×10^6 CTLs generated from either P14+WT (n=5), P14+let-7Tg (n=5), P14+Lin28Tg (n=5) lymphocytes, or no CTLs at all (n=5). (d) GFP expression on B16^{gp33} tumor cells before injection into the host mouse and after cells were isolated from a mouse which received let-7Tg CTLs, and demonstrated tumor growth until the end point of the study. (e) Image (left) and quantification (right) of the number of metastatic nodes in the lungs of mice which received 0.2×10^6 B16^{gp33} tumor cells i.v. and received 1.5×10^6 CTLs generated from either P14+WT (n=5), P14+let-7Tg (n=5), P14+Lin28Tg (n=5) lymphocytes, or no CTLs at all (n=5). Data are from one experiment representative of two experiments (a,e; mean and s.e.m. of biological replicates (n=5)).

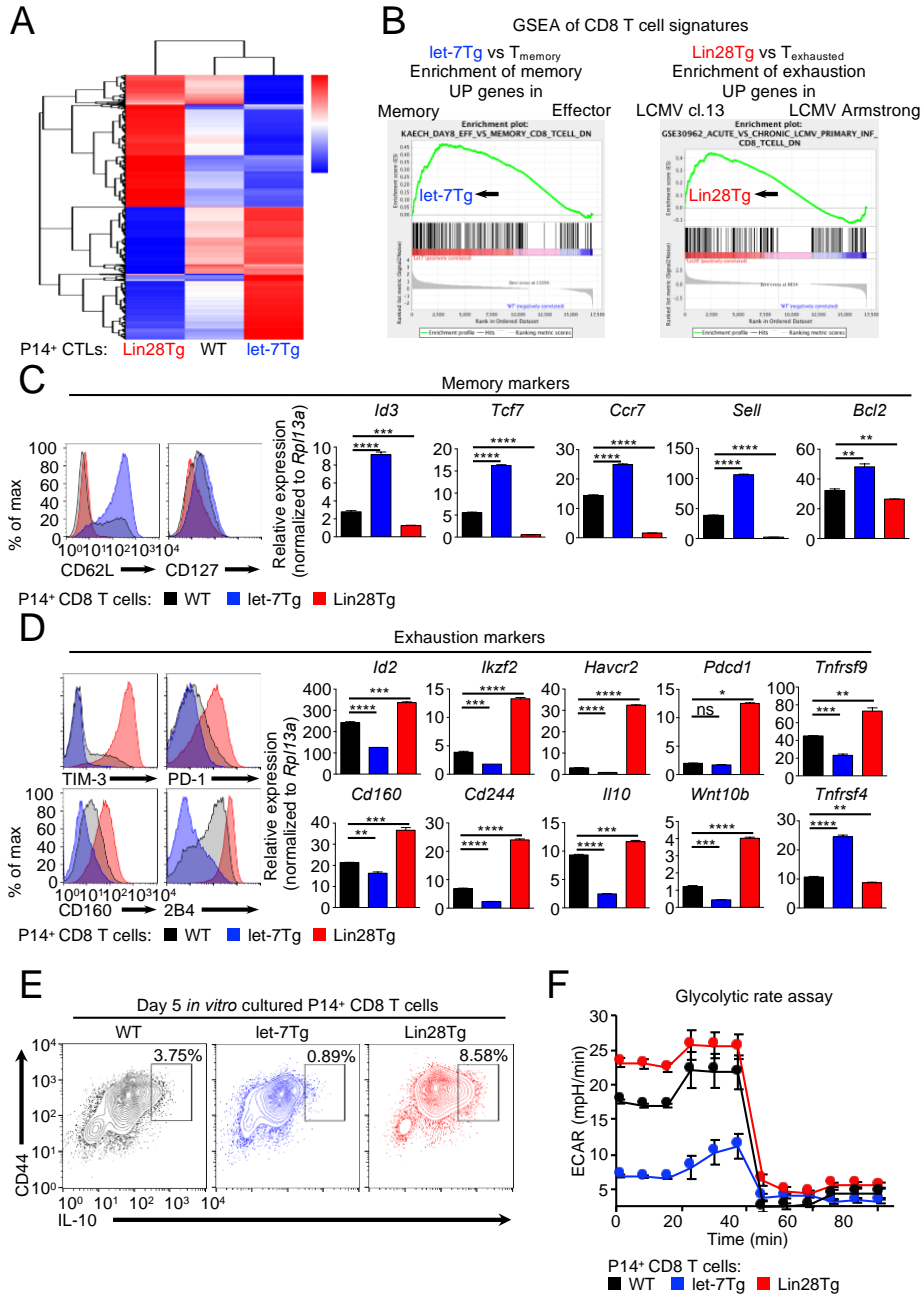


Figure 3.3 Molecular characterization of let-7Tg and Lin28Tg CTLs generated *in vitro*

(a) Heat map illustrating differential gene expression between P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs. (b) Gene set enrichment analysis of the let-7 transcriptome aligned to the transcriptome of memory CD8 T cells generated in response to acute viral infection (left) and the Lin28Tg transcriptome aligned to the transcriptome of exhausted CD8 T cells generated in response to chronic viral infection. (c) Staining of CD62L and CD127 on P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs (left) and quantitative RT-PCR analysis of mRNA expression: *Id3*, *Tcf7* (Tcf1), *Ccr7*, *Sell* (Cd62L), and *Bcl-2* in P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs presented relative to expression of the ribosomal protein *Rpl13a* (right). (d) Staining of TIM-3, PD-1, CD160, and 2B4 on P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs (left) and quantitative RT-PCR analysis of mRNA expression: *Id2*, *Ikzf2* (Helios), *Havcr2* (TIM-3), *Pdcd1* (PD-1), *Tnfrsf9* (4-1BB), *Cd160*, *Cd244* (2B4), *Il10*, *Wnt10b*, and *Tnfrsf4* (OX-40) in P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs presented relative to expression of the ribosomal protein *Rpl13a* (right). (e) Intracellular staining of IL-10 in P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs. (f) Glycolytic rate assay of P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, **** $P < 0.001$ compared with wild type using two-tailed Student's *t*-test (c, d). Data from one experiment (a, b; mean of technical triplicates) or from one experiment representative of three experiments (c, d; mean and s.e.m. of technical triplicates).

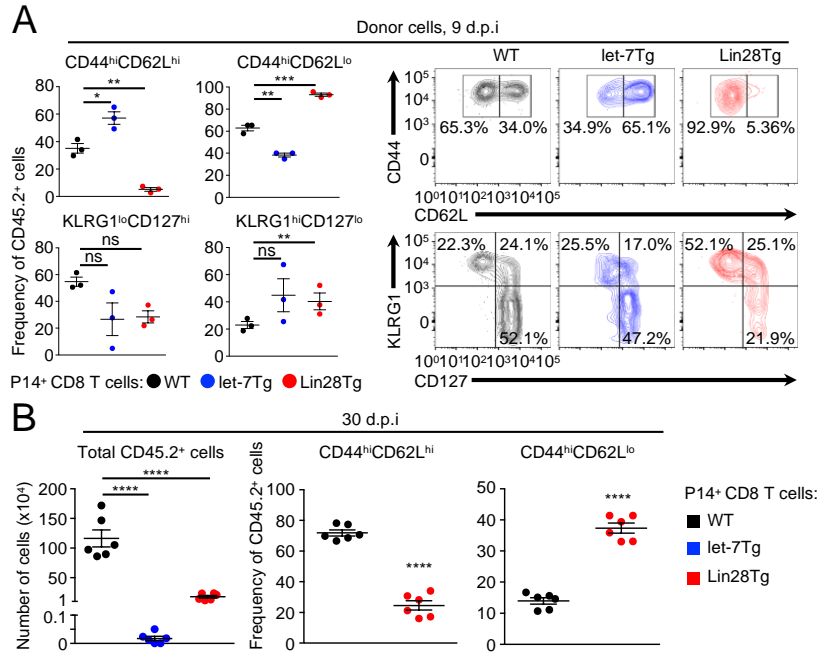


Figure 3.4 let-7 miRNAs enhance the memory precursor phenotype during the effector response to LM-33 *in vivo*

(a) Quantification of the frequency and staining of CD44^{hi}CD62L^{hi}, CD44^{hi}CD62L^{lo} (top), KLRG1^{lo}CD127^{hi}, KLRG1^{hi}CD127^{lo} (bottom) populations in the spleens of CD45.1+ host mice 9 days after adoptive transfer of 2×10^4 naïve CTLs from the indicated mice prior to infection with LM-33 (bottom left) ($n=3$). (b) Quantification of the number of donor (CD45.2+) CD8 T cells from P14 wild type ($n=6$), P14 let-7Tg mice ($n=6$), or P14 Lin28Tg mice in the spleens of congenic (CD45.1+) host mice 30 ($n=3$) and 31 ($n=3$) days after cell transfer and LM-33 infection (left). Quantification of the frequency of CD44^{hi}CD62L^{hi}, CD44^{hi}CD62L^{lo} populations in the spleens of mice 30-31 days after cell transfer and LM-33 infection (right). n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, **** $P < 0.001$ compared with donor WT P14 cells using two-tailed Student's *t*-test (c, d). Data are from one experiment (a, b; mean and s.e.m. of biological replicates).

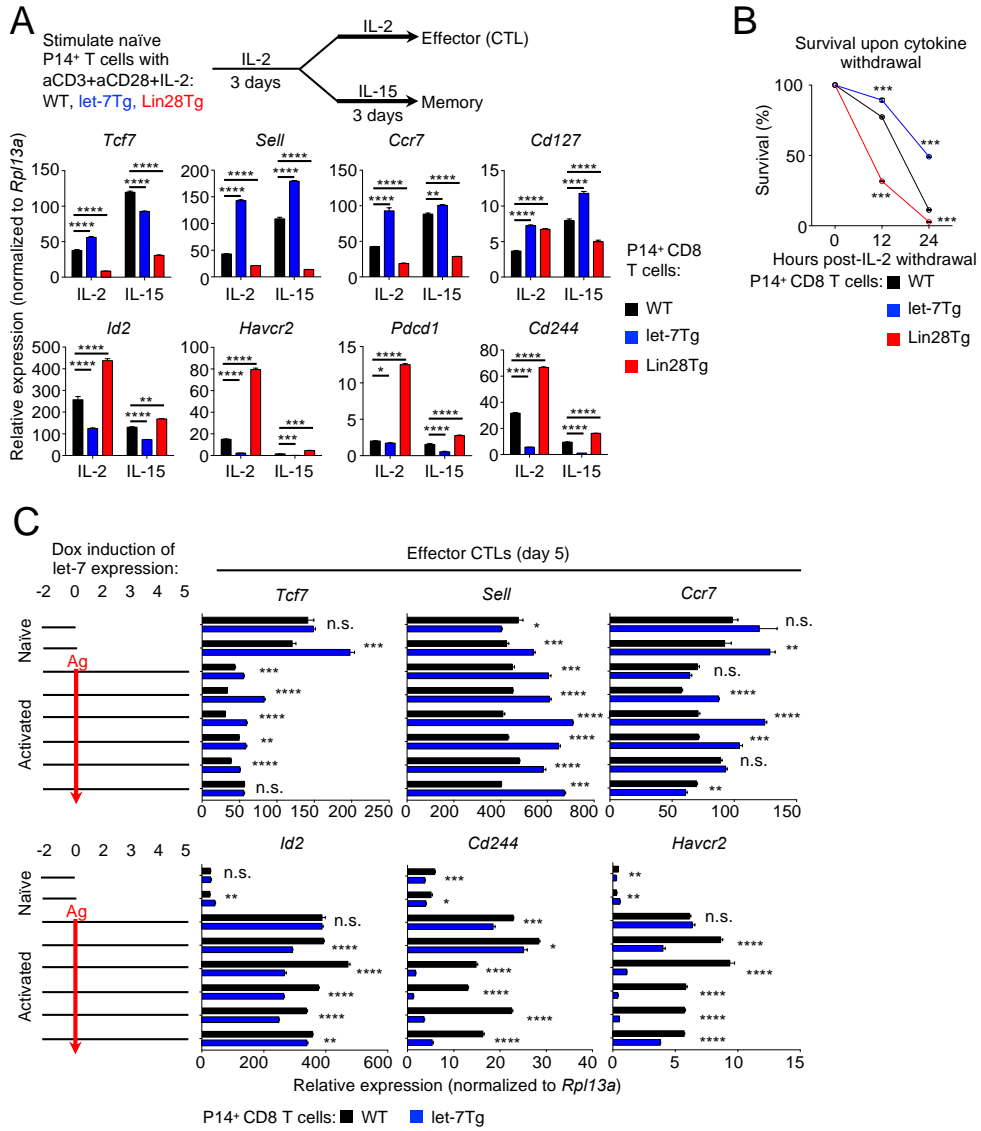


Figure 3.5 Maintenance of let-7 expression during activation supports memory T cell differentiation

(a) Quantitative RT-PCR analysis of mRNA expression presented relative to expression of the ribosomal protein *Rpl13a*: *Tcf7* (Tcf1), *Sell* (CD62L), *Ccr7*, *Cd127*, *Id2*, *Havcr2* (TIM-3), *Pdcd1* (PD-1), and *Cd244* (2B4) in P14 WT, P14 let-7Tg, and P14 Lin28Tg CTLs stimulated and cultured for three days in IL-2, and for an additional three days in either IL-2 or IL-15. (b) Analysis of survival 0, 12, and 24 hours after IL-2 withdrawal of P14 WT, P14 let-7Tg, P14 Lin28Tg CTLs which had been cultured in IL-2 for five days. (c) Quantitative RT-PCR analysis of mRNA expression: *Tcf7* (Tcf1), *Sell* (CD62L), *Ccr7*, *Id2*, *Havcr2* (TIM-3), and *Cd244* (2B4) in P14 WT and P14 let-7Tg CTLs which received doxycycline either prior to stimulation or during the culture, as indicated (left, blue boxes), presented relative to the expression of the ribosomal protein *Rpl13a*. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, **** $P < 0.001$ compared with WT P14 cells from matched culture conditions using two-tailed Student's *t*-test (a, b, c). Data are from one experiment representative of two experiments (a, b, c; mean and s.e.m. of technical triplicates).

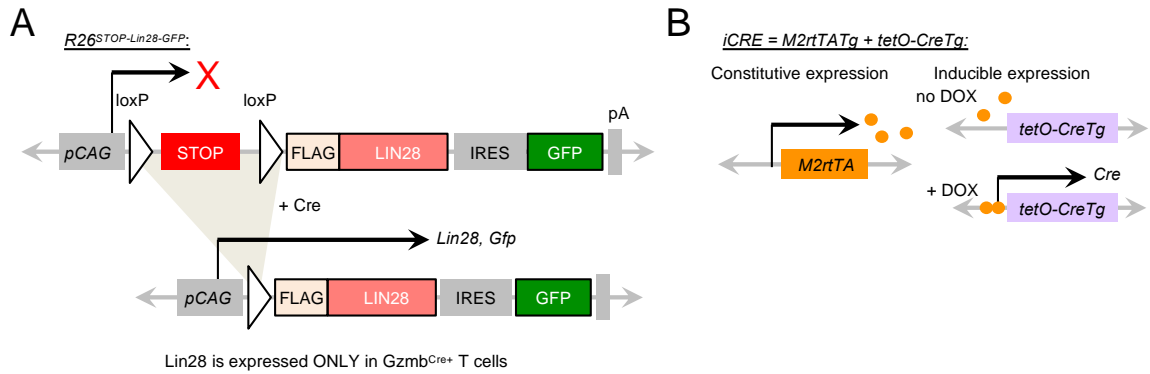


Figure 3.6 Genetic schematics of $R26^{STOP-Lin28-GFP}$ and $iCre$ mice

(a) Cre deletes the STOP cassette to induce the expression of Lin28 and the GFP reporter.
 (b) Doxycycline- inducible Cre ($iCre$) is only expressed when doxycycline is present, such that when crossed with $R26^{STOP-Lin28-GFP}$ mice, cells will only express Lin28 and GFP in the presence of doxycycline.

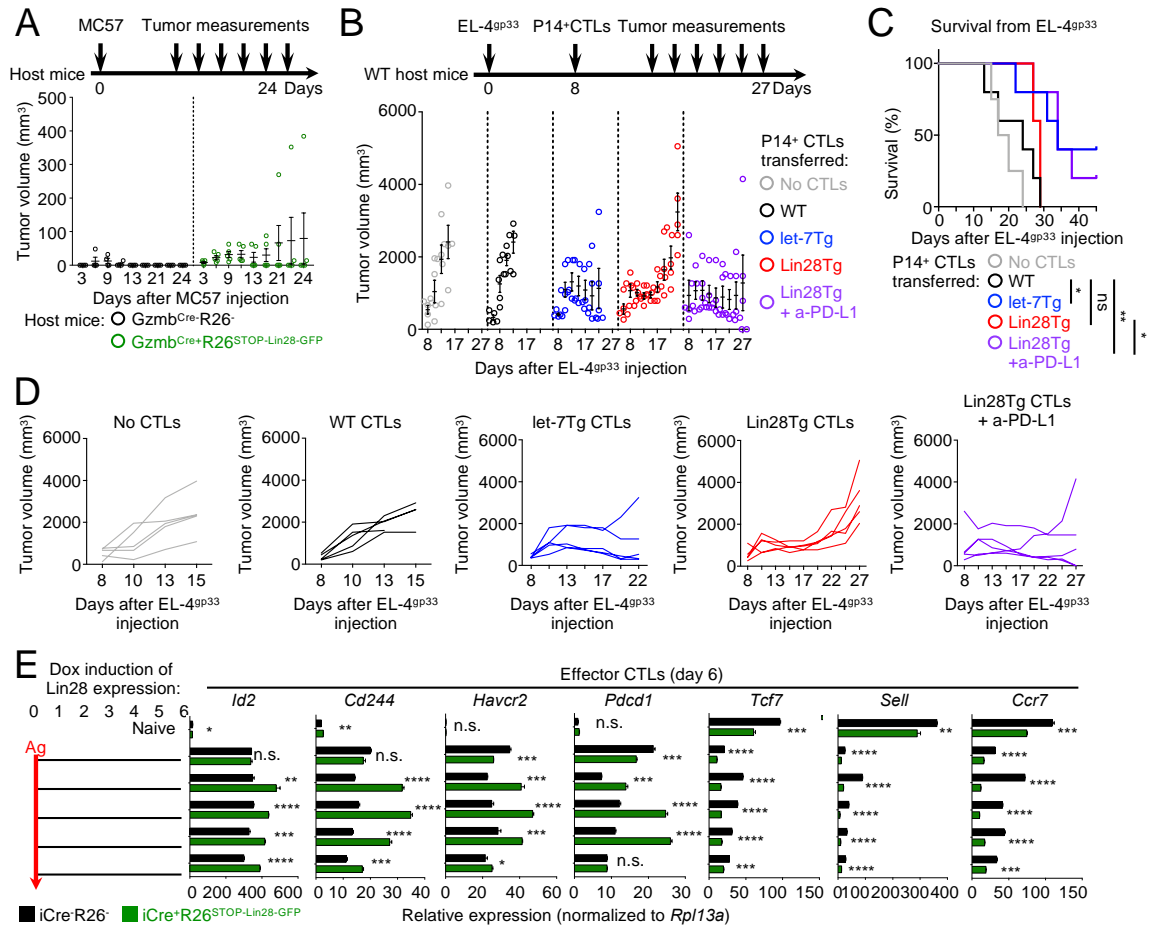


Figure 3.7 Downregulation of let-7 miRNAs after activation leads to terminal differentiation

(a) Tumor growth curves of MC57 (1×10^6 , s.c.) in Gzmb^{Cre}R26⁻ (WT) (n=3) or Gzmb^{Cre}+R26^{STOP}-Lin28-GFP (n=3) mice. (b, d) Tumor growth curves of EL-4^{gp33} (0.25×10^6) tumors in host mice that received adoptive transfer of 1.5×10^6 CTLs generated from P14 WT (n=5), P14 let-7Tg (n=5), P14 Lin28Tg (n=5), P14 Lin28Tg+anti-PD-L1 (n=5), or no CTLs (n=5) at all. Mice which received anti-PD-L1 antibodies, were first treated (i.p.) immediately upon CTL adoptive transfer, and were injected (i.p.) every two days a total of four injections had been received. (c) Survival analysis of the mice in (b, d). (e) Quantitative RT-PCR analysis of mRNA expression presented relative to the expression of the ribosomal protein *Rpl13a*: *Id2*, *Cd244* (2B4), *Havcr2* (TIM-3), *Pdccl1* (PD-1), *Tcf7* (Tcf1), *Sell* (CD62L), *Ccr7* in iCreR26⁻ (WT) and iCreR26^{STOP}-Lin28-GFP CTLs which received doxycycline for 48 hours at the indicated times (start of the green bar). The green bar represents Lin28 expression, rather than doxycycline treatment time. n.s., not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.001$ compared with EL-4^{gp33} tumor bearing mice receiving P14 WT CTLs (c) or iCreR26⁻ CTLs (e) using two-tailed Student's *t*-test (c, e). Data are from one experiment (a, b, c, d (b; mean and s.e.m. of biological replicates)) or one experiment representative of two independent experiments (e; mean and s.e.m. of technical replicates).

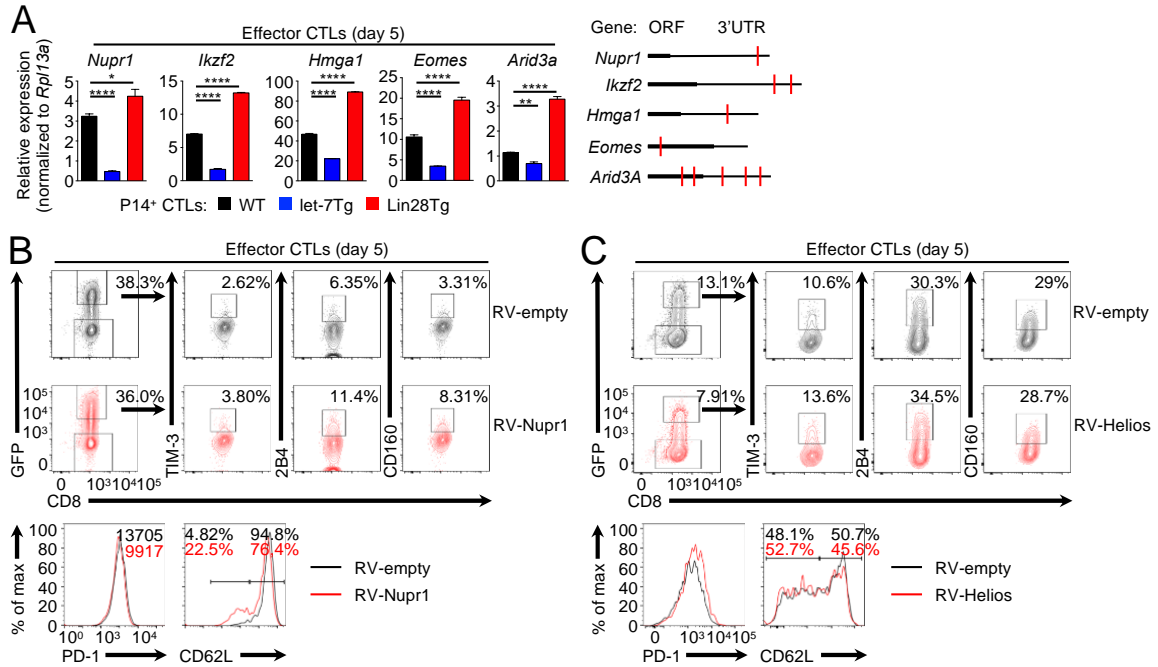


Figure 3.8 *let-7* miRNAs target several genes, but do not functionally regulate *Nupr1* or *Ikzf2* in CD8 T cells

(a) Quantitative RT-PCR analysis of mRNA expression presented relative to expression of the ribosomal protein *Rpl13a*: *Nupr1* (Tcf1), *Ikzf2* (Helios), *Hmga1*, *Eomes*, *Arid3a*, in P14 WT, P14 *let-7*Tg, and P14 Lin28Tg CTLs (left). Schematics indicating the number and locations of *let-7* binding sites in the tested candidate genes (right). (b, c) Staining of TIM-3, 2b4, CD160, PD-1 and CD62L on P14 *let-7*Tg CD8 T cells transduced with either RV-empty or RV-*Nupr1* (b) or RV- empty or RV-*Helios* (c) which expresses a GFP reporter. Frequencies of protein-expressing cells (GFP, TIM-3, 2B-4, CD160, CD62L), cells with no protein expression (CD62L) and MFI of protein expression (PD-1) are indicated. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, **** $P < 0.0001$ compared with P14 WT CTLs using two-tailed Student's *t*-test (a). Data are from one experiment representative of two experiments.

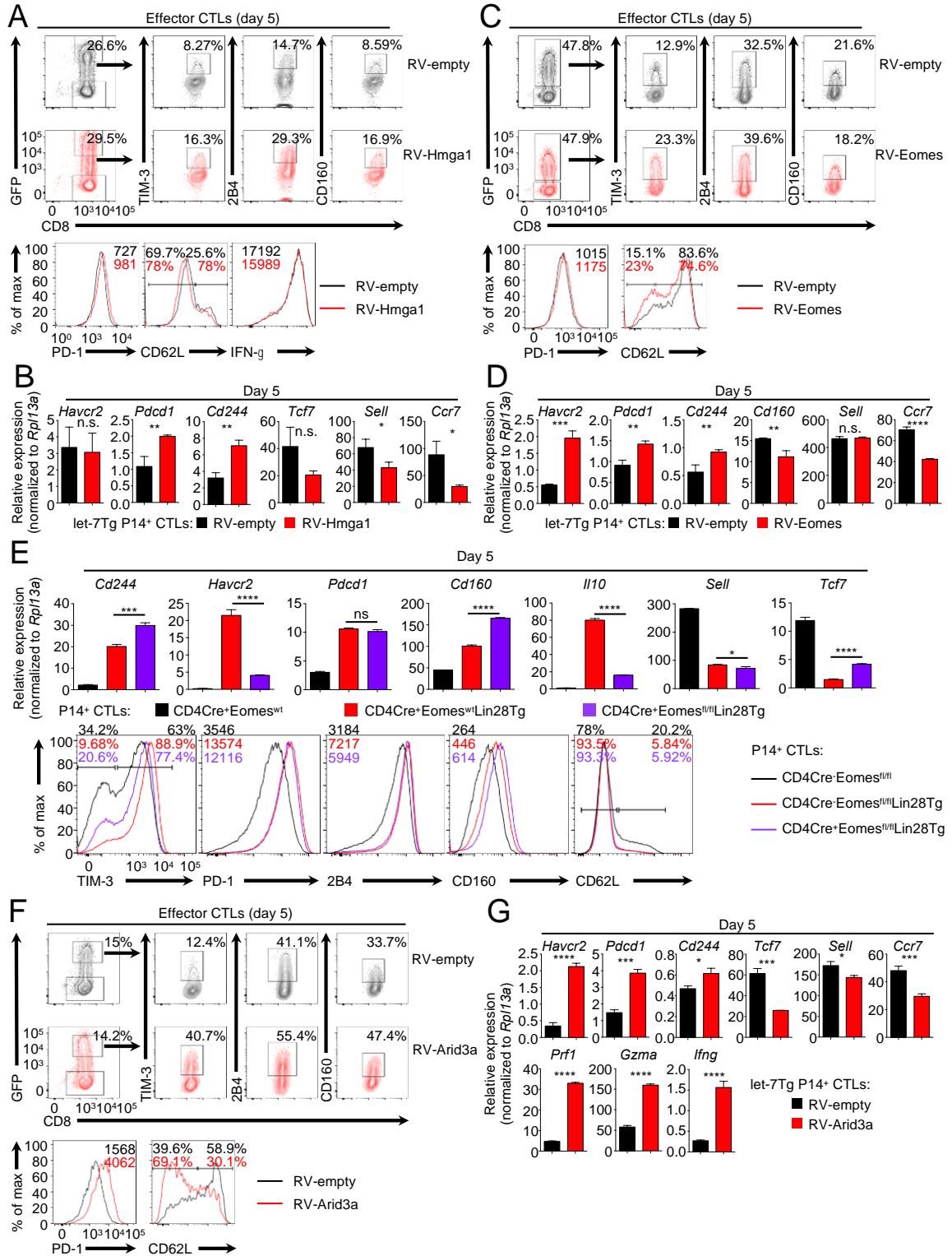


Figure 3.9 let-7 miRNAs regulate terminal differentiation through a complex transcriptional mechanism involving *Hmgal1*, *Eomes*, and *Arid3a*

(a) Staining of TIM-3, 2B4, CD160, PD-1, and CD62L and intracellular staining of IFN- γ on P14 let-7Tg CD8 T cells transduced with either RV-empty or RV-Hmgal1 which expresses a GFP reporter. Frequencies of protein-expressing cells (GFP, TIM-3, 2B-4, CD160, CD62L), cells with no protein expression (CD62L) and MFI of protein expression (PD-1, IFN- γ) are indicated. (b) Quantitative RT-PCR analysis of mRNA expression: *Havcr2* (TIM-3), *Pdcd1* (PD-1), *Cd244* (2B4), *Tcf7* (Tcf1), *Sell* (CD62L), and *Ccr7* in sorted GFP+ let-7Tg CD8 T cells transduced with either RV-empty or RV-Hmgal1, presented relative to the expression of the ribosomal protein *Rpl13a*. (c) Staining of TIM-3, 2B4, CD160, PD-1, and CD62L on P14 let-7Tg CD8 T cells transduced with either RV-empty or RV-Eomes which expresses a GFP reporter. Frequencies of protein-expressing cells (GFP, TIM-3, 2B-4, CD160, CD62L), cells with no protein expression (CD62L) and MFI of protein expression (PD-1) are indicated. (d) Quantitative RT-PCR analysis of mRNA expression: *Havcr2* (TIM-3), *Pdcd1* (PD-1), *Cd244* (2B4), *Cd160*, *Sell* (CD62L), and *Ccr7* in sorted GFP+ let-7Tg CD8 T cells transduced with either RV-empty or RV-Eomes, presented relative to the expression of the ribosomal protein *Rpl13a*. (e) Quantitative RT-PCR of mRNA expression: *Cd244* (2B4), *Havcr2* (TIM-3), *Pdcd1* (PD-1), *Cd160*, *Il10*, *Sell* (CD62L), and *Tcf7* (Tcf1) in CTLs generated from P14 *CD4-Cre⁻Eomes^{fl/fl}*, P14 *CD4-Cre⁻Eomes^{fl/fl}Lin28Tg*, P14 *CD4Cre⁺Eomes^{fl/fl}Lin28Tg* mice (top). Staining of TIM-3, PD-1, 2B4, CD160, and CD62L on CD8 T cells from the indicated mice (bottom). (f) Staining of TIM-3, 2B4, CD160, PD-1, and CD62L on P14 let-7Tg CD8 T cells transduced with either RV-empty or RV-Arid3a which expresses a GFP reporter. Frequencies of protein-expressing cells (GFP, TIM-3, 2B-4, CD160, CD62L), cells with no protein expression (CD62L) and MFI of protein expression (PD-1) are indicated. (g) Quantitative RT-PCR analysis of mRNA expression: *Havcr2* (TIM-3), *Pdcd1* (PD-1), *Cd244* (2B4), *Tcf7* (Tcf1), *Sell* (CD62L), *Ccr7*, *Prfl* (perforin), *Gzma* (Granzyme A), and *Ifng* in sorted GFP+ let-7Tg CD8 T cells transduced with either RV-empty or RV-Arid3a, presented relative to the expression of the ribosomal protein *Rpl13a*. not significant ($P > 0.05$), * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, **** $P < 0.001$ compared with GFP+ let-7Tg CTLs transduced with RV-empty (a, b, c, d, f, g) or CTLs generated from P14 *CD4-Cre⁻Eomes^{fl/fl}* mice using two-tailed Student's *t*-test. Data are from one experiment representative of two experiments (b, d, e, f, g; mean and s.e.m of technical triplicates).

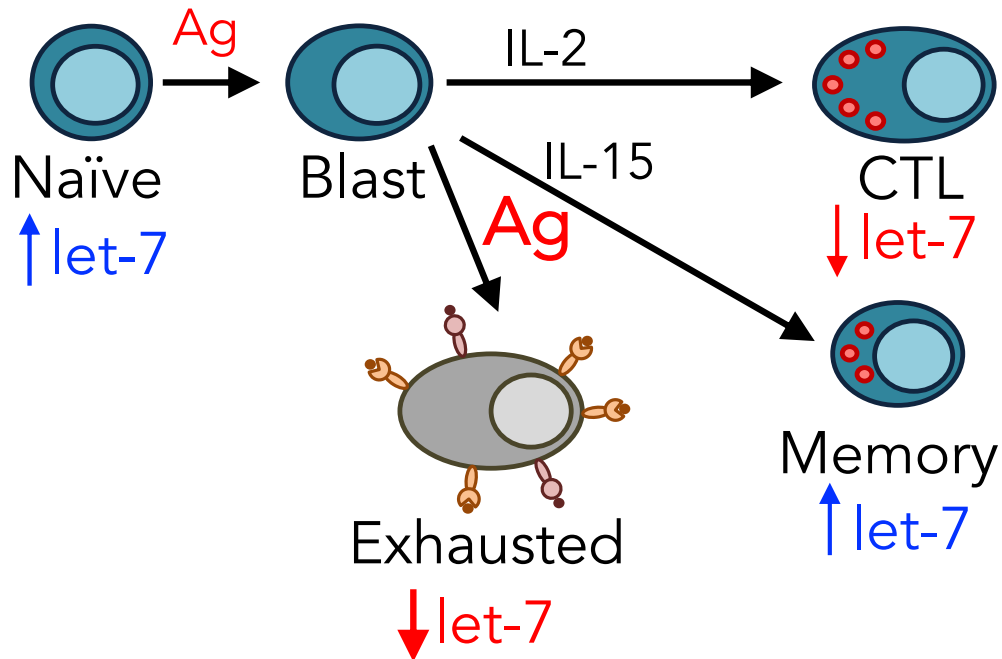


Figure 3.10 let-7 miRNAs program CD8 T cell fate

Model illustrating how let-7 miRNAs program the fate of CD8 T cells, where high let-7 expression is necessary to maintain the quiescent state of naïve cells and promote memory differentiation. Conversely, let-7 miRNAs must be moderately downregulated to produce effective terminal effector CTL differentiation. It is important that let-7 downregulation is moderate, as severe downregulation leads to exhaustion.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

Taken together, the results presented in this dissertation demonstrate that the let-7 miRNAs are a molecular control hub that regulate the fate of CD8 T cells. First, it was determined that let-7 miRNAs are very highly expressed in naïve CD8 T cells and that their expression is downregulated through TCR signaling, in which both the duration and strength of signal control the expression of let-7. The high expression of let-7 miRNAs is necessary to maintain the quiescence of naïve CD8 T cells, as removal of let-7 expression in this population resulted in increased proliferation and expression of activation markers. Moreover, the downregulation of let-7 expression in activated cells is necessary for the acquisition of effector function, as overexpressing let-7 throughout CTL differentiation severely impaired the response to viral infection and the clearance of an allogeneic tumor. Specifically, the failure to downregulate let-7 impaired proliferation and important metabolic changes that occur during blastogenesis, likely through its regulation of Myc. Later in CTL differentiation, the downregulation of let-7 was necessary to express Eomes and acquire effector function. In fact, Eomes was identified as a novel direct target of let-7. These data suggested that let-7 tunes the magnitude of CTL responses according to the strength of TCR signaling, where weak TCR signaling yields mild CTL responses due to retention of let-7 miRNA expression, and strong TCR signaling generates robust CTL responses as a result of strong downregulation of let-7 (Figure 2.13).

Further, a role for the let-7 miRNAs in regulating terminal effector and memory CD8 T cell differentiation was identified through the use of both bacterial infection and tumor models. Contrary to the cytotoxicity observed *in vitro*, let-7-deficiency did not improve anti-tumor CTL responses. Rather, CTLs in which let-7 was overexpressed mediated the most efficient tumor rejection. Molecular characterization of these cells revealed that overexpression of let-7 restrained the terminal differentiation program, which allowed these cells to differentiate into the memory fate. Consequently, depletion of let-7 miRNAs pushed CTLs into a terminal effector state, which became functionally exhausted in the context of the immunosuppressive tumor microenvironment. Accordingly, the function of let-7-deficient CTLs was rescued through the use of checkpoint blockade inhibition. Although the exact let-7-mediated molecular mechanism was not fully characterized, several genes that let-7 targets to restrain terminal differentiation and preserve memory CD8 T cells were identified. Each of these factors likely contributes to a complex and synergistic transcriptional network regulated by let-7 (Figure 3.10).

4.2 Future directions

Much remains to be determined to better understand the role of the let-7 miRNAs in controlling CD8 T cell fate. There are several important points which remain to be addressed, including: (1) understanding better the mechanisms contributing to the paradoxical functions observed between the 2-dimensional *in vitro* setting and in 3-dimensional *in vivo* environments; (2) determining the function of Arid3a and Hmgal1 in CD8 T cells, which should further illuminate the importance of their regulation by let-7; (3) testing the function of the let-7 miRNAs in memory differentiation *in vivo* and how this

let-7-mediated mechanism fits into the proposed models of memory differentiation; (4) extrapolating upon this data to establish a role for the let-7 miRNAs in the differentiation and function of CD4 T cells; (5) identifying the molecular mechanism by which TCR signaling regulates the expression of the let-7 miRNAs.

4.2.1 Characterizing the *in vitro* versus *in vivo* paradox

The dramatic differences in the function of Lin28Tg and let-7Tg CTLs *in vitro* versus *in vivo* raises important questions regarding not only the method by which cells are cultured on 2-D plastic, but also the mechanism required to successfully transduce inhibitory signals to CD8 T cells. It is not known if the engagement of inhibitory receptors and immunosuppressive cytokine signaling alone are responsible for the exhaustion of CD8 T cells, or if there are other environmental factors which are necessary for these signals to establish T cell exhaustion. It has been proposed that the stiff substrates drive cells into a more terminal effector state (O'Connor et al., 2012; Saitakis et al., 2017). Moreover, the inability to rescue exhausted CD8 T cells via checkpoint blockade immunotherapy has been documented (Wei et al., 2018). These two findings suggest that the ECM may have a previously unappreciated role in mediating signals through inhibitory receptors to promote exhaustion.

In fact, we attempted to address the role of the ECM in driving CD8 T cell exhaustion by using tunable hydrogels, in which the gel can be manipulated to mimic the rigid ECM stiffnesses associated with the tumor microenvironment. Specifically, let-7Tg, Lin28Tg, and WT P14 CTLs were encapsulated in gels of either low or high stiffness, and the expression of inhibitory receptors was assessed, hypothesizing that high stiffness ECM

would increase expression of inhibitory receptors. However, encapsulation of activated CD8 T cells in the gels resulted in cell death, inhibiting the study of the ECM in contributing to CD8 T cell exhaustion (Figure 4.1). This does not mean that the ECM is not an important factor in establishing exhaustion or in the transduction of inhibitory signals, thus further investigation is warranted. Encapsulating T cells in a simpler gel system, such as poly(NIPAAm) may be useful to initially establish a role for a 3-dimensional environment in promoting inhibitory receptor signaling (Haq et al., 2017). In this situation, performing an *in vitro* cytotoxicity assay in the gel versus on tissue culture plastic may be informative. If 3-dimensional interactions are necessary to transmit signals through the inhibitory receptors, it would be hypothesized that the high cytotoxic function of Lin28Tg CTLs against cancer cells on 2-dimensional plastic, would be reduced in the gel and more accurately represent the *in vivo* function of these cells.

4.2.2 Identify the role of Hmga1 and Arid3a in CD8 T cell differentiation

An important outcome of this research is the identification of the novel let-7 targets, Eomes, Hmga1, and Arid3a, which control the differentiation of CD8 T cells. As two of these factors, Hmga1 and Arid3a, have either been poorly characterized, or have no identified role in the differentiation of CD8 T cells, it will also be interesting to characterize their function in CD8 T cells independent of let-7. This can be accomplished by both gain of function and loss of function approaches. As this study focused only on the phenotype of *in vitro* differentiated cells transduced with cDNA that encodes these two factors, it will be very important to determine how they impact the function of CD8 T cells. The retroviruses used in this dissertation can be used to overexpress Hmga1 and Arid3a in CD8

T cells, that are transferred into mice which are subsequently infected with LM-33. The phenotype of donor cells overexpressing either Hmga1 or Arid3a can be assessed over time by analyzing circulating cells in the blood. This will provide insight into the differentiation of these cells *in vivo*, hypothesizing that overexpression of these factors would lead to the terminal differentiation of donor cells, and thus the inability to generate a robust long-lived memory population. At the end of the study, secondary lymphoid organs can be analyzed further to better characterize how memory CD8 T cell differentiation was affected by overexpression of these genes. The tumor models generated in this dissertation will also be useful in characterizing the function of Hmga1 and Arid3a in establishing the exhausted state in CD8 T cells. By monitoring tumor growth in mice with solid tumors which receive transfer of CTLs transduced with either of these factors, whether Hmga1 and Arid3a alone are sufficient to establish exhaustion *in vivo* can be determined. Moreover, loss of function approaches will also provide insight into the role of these factors in CD8 T cell differentiation. By introducing shRNAs against these factors into differentiating CD8 T cells, one would hypothesize memory differentiation might be enhanced. This can be tested *in vivo* again by transfer of CD8 T cells expressing these shRNAs into mice which are then infected with LM-33. It is important to note that *in vitro*, Hmga1, Arid3a, and even Eomes, only partially contributed to the terminal differentiation phenotype, with neither factor being able to entirely recapitulate the phenotype observed in Lin28Tg CTLs. Thus, it will also be interesting to introduce these factors into CD8 T cells in combination. This will provide insight into their cooperation and may explain why each factor individually only moderately skewed the differentiation of CD8 T cells.

The data presented in this dissertation suggests there is a role for Eomes, Hmga1, and Arid3a in directing differentiating CD8 T cells toward the terminal effector fate. To better understand how these transcriptional regulators accomplish this in an unbiased approach, performing ATAC-seq and ChIP-seq will generate global maps of the genetic targets of these factors. Moreover, RNA-seq can be performed on CD8 T cells in which these factors are either overexpressed or knocked down to identify which genes targeted by these factors are functionally relevant to CD8 T cell differentiation.

4.2.3 Further characterization of let-7 miRNAs in memory differentiation

Several challenges were encountered in attempting to study the role of the let-7 miRNAs in memory P14 T cell differentiation *in vivo* through the adoptive transfer of naive let-7Tg CD8 T cells. Specifically, donor let-7Tg P14 T cells could not be identified in host spleens 30 days after vaccination with gp33, poly(I:C) and anti-CD40 monoclonal antibody (Figure 4.2A). Cells were properly activated as they could be recovered in equal numbers to WT P14 cells 48 hours after activation by LM-33 infection (Figure 4.2B). To overcome the let-7-mediated block on proliferation, 1×10^6 naïve let-7Tg P14 T cells, as opposed to 2×10^4 , were transferred to host mice prior to LM-33 infection, but only resulted in a mild improvement in cell recovery (Figure 4.2C). To address the possibility that let-7Tg P14 T cells preferentially become tissue-resident memory cells, the liver of vaccinated mice which received adoptive transfer of let-7Tg P14 T cells were analyzed, as this is a large reservoir for T_{RM} cells. However, no let-7Tg P14 T cells were found (Figure 4.2D). It is also possible that although the mice from which let-7Tg P14 cells are isolated are restricted to MHC H-2D^b, there may be differences in minor antigens that lead to allogeneic

responses against these cells upon transfer into host mice. To address this possibility, let-7Tg P14 cells were transferred into Perforin deficient mice (*Prf1*^{-/-}), such that T cells and natural killer cells cannot kill the MHC-mismatched cells. Yet, there was no improvement of recovery of let-7Tg cells in *Prf1*^{-/-} mice (Figure 4.3E). These data suggested the let-7 mediated block on proliferation may in part be responsible for the failure to identify let-7Tg CD8 T cells during adoptive transfer experiments; however, it is not possible to rule out the possibility that differences in genetic background may also be a contributing factor, or that naïve let-7Tg CD8 T cells home to a unique peripheral site. There are several possibilities to address the latter two concerns.

First, to completely rule out the possibility that let-7Tg cells are rejected from the host based on minor antigens, let-7Tg P14 T cells could be transferred into agouti mice (C57BL/6J-*A*^{w-J}/J) which should share any minor antigens, as let-7Tg mice were originally generated on the agouti background, thus eliminating rejection of the let-7Tg cells. However, to simply completely eliminate any differences in genetic background and still use the pure C57BL/6J genetic background on which all the other mouse models are based, DNA encoding the let-7 miRNAs can be cloned into a GFP-expressing retroviral vector that is transfected into WT P14 T cells which can be successfully transferred into host mice without any complications. In this way, both GFP positive (let-7 overexpressing) and negative (“wild type”) cells can be analyzed for their ability to differentiate into the memory fate. Further, in this model it may be possible that only GFP negative cells will be identified in secondary lymphoid organs, which would provide more insight into whether let-7 miRNAs direct the localization of memory cells to a unique niche. If this were the case, it would be prudent to check other tissues for the presence of let-7 transduced cells.

While the liver and gut are important niche for T_{RM} cells, the skin, lung, white adipose tissue, and bone marrow also serve as reservoirs for T_{RM} cells. Checking the presence of GFP positive cells in these other organs may help not only in locating let-7-overexpressing cells, but also may further illuminate the role of let-7 miRNAs in CD8 T cell differentiation. In fact, it was recently demonstrated that T_{CM} cells (which are the memory population let-7Tg cells most resemble phenotypically) can seed the T_{RM} population in the skin (Osborn et al., 2019). Thus, it will be important to analyze this organ for let-7Tg cells regardless of the results from the experiments described above.

It will also be interesting to fit our data demonstrating that let-7 miRNAs promote memory CD8 T cell differentiation into the proposed models of this process. The observation that let-7 miRNA expression during the first 48 hours of activation is sufficient to generate memory CD8 T cells, would be consistent with the asymmetric division model. It would be interesting to sort the proximal and distal daughter cells generated as a result of T cell activation by antigen presenting cells, and assess let-7 expression in these two populations. We would hypothesize that let-7 expression would be higher in the distal daughter cell which received weaker TCR stimulation and will differentiate into a memory cell, as compared to the proximal daughter cell which would have lower let-7 expression due to stronger TCR stimulation. As a follow-up to these experiments, further dissection of the function of let-7 miRNAs within this 48-hour window will be enlightening. In fact, preliminary data in the lab suggests that let-7 makes critical ‘decisions’ to determine the fate of CD8 T cells as early as 12 hours after activation.

4.2.4 Expansion of let-7 miRNA regulation of CD4 T cell differentiation and function

This study has focused primarily on CD8 T cells, but the let-7 miRNAs are also very abundantly expressed in naïve CD4 T cells, and have been shown by others in the lab to be downregulated by TCR signaling. Thus, the role of let-7 miRNAs during CD4 T cell differentiation should be examined. This is especially important because CD4 T cells are the primary mediators of autoimmune diseases. It will be interesting to test *in vivo* whether maintaining let-7 miRNAs or depleting let-7 miRNAs ameliorates autoimmune disease progression. Based on the *in vitro* data presented in this dissertation, one would hypothesize that expression of let-7 miRNAs would inhibit autoreactive cells from attacking healthy host cells, while depletion of let-7 miRNAs would expedite disease progression. This can easily be tested by using a mouse model of multiple sclerosis called experimental autoimmune encephalomyelitis in WT, let-7Tg, or Lin28Tg mice. In fact, this is an active area of investigation in the lab. CD4 T cells also have the unique ability to differentiate into distinct subsets of T helper cells based on the invading pathogen and cytokine milieu associated with the infection. It will be interesting to test whether modulation of let-7 miRNAs is important to direct CD4 T cells into the different T helper subsets. Further, based on the cytokine milieu present during CD4 T cell differentiation, some of these helper subsets can also become pathogenic, contributing to autoimmune disease progression (Lee et al., 2012). Using the distinct cytokine conditions used to differentiate pathogenic CD4 T helper cells on WT, let-7Tg, and Lin28Tg CD4 T cells will help determine whether let-7 miRNAs negatively regulate the pathogenic differentiation of CD4 T cells, and will also help to understand the role of let-7 miRNAs in CD4 T cell-mediated autoimmune diseases.

Moreover, it will be interesting to test the role of let-7 miRNAs in the development of CD4 T cell memory, which is less understood than CD8 T cell memory. As such, the importance of let-7 miRNAs in generating memory CD8 T cells presented here may provide important insight into the generation of memory CD4 T cells. First, WT, let-7Tg, and Lin28Tg mice can be crossed onto a SMARTA TCR background, in which SMARTA is a T cell receptor that recognizes the gp66-81 epitope from LCMV. This way, these cells can be transferred into mice which are subsequently infected with *Listeria* that express the target antigen (LM-66), in order to determine the extent to which let-7 expression directs memory CD4 T cell differentiation. However, it is entirely possible that these cells will also be rejected in the host. If this is the case, the previously generated retroviruses which encode let-7 miRNA can be used to overexpress let-7 in CD4 T cells to test the role of let-7 in memory CD4 T cell differentiation. Moreover, the let-7 miRNAs are expressed throughout the immune system. Determining the function of let-7 miRNAs in other immune cell populations, and whether modulating let-7 expression in one immune cell may determine the effects of let-7 in another, will surely be illuminating.

4.2.5 Identify the mechanism regulating let-7 miRNA expression

As an important outcome of this research is the identification of the let-7 miRNAs as a therapeutic tool, it is extremely important to determine the molecular mechanism that controls the expression of these miRNAs in response to TCR signaling, as this might identify a means by which to artificially regulate let-7. Along these lines, it would be very interesting to perform bisulfite sequencing and ATACseq in memory, terminal effector, and exhausted CD8 T cells in genomic locations where let-7 miRNAs are found. One might

expect that as cells progress further into the terminal effector program, as let-7 expression decreases, there would be increased deposition of repressive marks, and closed chromatin in these areas. To perform these experiments, WT P14 CD8 T cells will be adoptively transferred into congenically marked host mice which are subsequently infected with either LCMV Armstrong to establish an acute infection, or LCMV clone 13 to establish a chronic infection. In this way, naïve, terminal effector cells, memory precursor cells, memory cells and exhausted cells can all be identified and sorted based on well-established phenotypic markers. Once these populations of CD8 T cells have been separated, they can be sent for bisulfite sequencing and ATAC-seq to determine the epigenetic landscapes of let-7 miRNA loci during the different stages of CD8 T cell differentiation, providing novel information regarding the regulation of let-7 miRNA expression.

Moreover, the molecular mechanism mediated by TCR signaling to downregulate let-7 miRNA expression in activated CD8 T cells remains to be determined. First, whether let-7 miRNAs are downregulated by transcriptional or post-transcriptional mechanisms should be determined. To assess this, the relative levels of let-7 primary miRNAs and precursor miRNAs will be assessed. If the let-7 pri-miRNAs are dramatically downregulated upon TCR signaling, this would indicate strong transcriptional regulation, whereas downregulation of let-7 pre-miRNAs would be indicative of post-transcriptional regulation. This would be determined by northern blot, as probes for pri-miRNA and pre-miRNA qPCR are limited. In the case of transcriptional regulation, some factor may bind to promoter or enhancer regions of let-7 genes to inhibit their expression. Analysis of chromatin accessibility around the promoters of let-7 genes and characterization of the binding sites in these regions of open chromatin will help to identify factors that bind to

promoters of let-7 miRNAs to inhibit their expression. Once several candidate factors have been identified, ChIP-seq can be used to determine if they do in fact bind to the promoters of let-7 genes. ChIP-seq data from activated versus naïve CD8 T cells could be compared. Proteins bound in activated, but not naïve CD8 T cells may negatively regulate let-7 miRNA expression. In the case of post-transcriptional regulation, it is possible that the let-7 miRNAs themselves are inhibited by some factor in a manner analogous to Lin28. To address the possibility of post-transcriptional regulation, immature let-7 miRNAs will be pulled down, such that any proteins bound to the immature let-7 miRNA are also pulled down. The proteins will be dissociated from the let-7 miRNA and sent for mass spectrometry to determine the identity of the bound proteins.

Once these proteins have been identified, their function in regulating let-7 expression can be tested by overexpressing the protein via retroviral vector in NIH 3T3 cells, which have high endogenous let-7 expression, and assessing if let-7 expression is downregulated. Upon confirmation of a role in inhibiting let-7 expression, whether these proteins regulate let-7 expression in CD8 T cells can be tested by both gain and loss of function approaches. A potential loss of function approach would first be to generate shRNAs that prevent the expression of this protein. It would be hypothesized that by inhibiting expression of this protein, let-7 miRNAs will remain highly expressed upon TCR stimulation. Accordingly, determining the phenotype of CTLs generated in the absence of this protein will prove interesting. If inhibiting this protein maintained let-7 expression, one would hypothesize that these CD8 T cells would differentiate into the memory phenotype. If a phenotype is observed using shRNAs, generation of a conditional knockout mouse, in which the protein is deleted only in T cells, may be warranted for future studies.

In these studies, conditional knockout mice could be bred to a P14 TCR background, such that CD8 T cells from these mice could be transferred into host mice subsequently infected with LM-33 to study antigen specific responses. The differentiation of terminal effector, memory precursor, and memory populations would be assessed. Further, CTLs could be generated *in vitro*, and the extent to which they eliminate tumors *in vivo* could be determined. Contrastingly, gain of function approaches could also be taken. In this situation, the previously generated retroviral vectors would be used to overexpress the protein in CD8 T cells. First, let-7 expression would be analyzed, expecting that it would be reduced in the presence of this protein. Further, analyzing the phenotype of these cells *in vitro* would demonstrate functional significance of the expression of this protein. It would be hypothesized that overexpressing this protein would drive cells into a terminal effector/ exhausted state due to downregulation of let-7. If a phenotype were observed, transgenic mice which constitutively overexpress this protein in T cells under the control of the human CD2 promoter (analogous to Lin28Tg mice) could be generated. These mice would also be crossed to a P14 background to study the ability of cells overexpressing this protein to generate memory cells in the LM-33 model and to eliminate tumors in the B16^{gp33} tumor model. These studies would identify novel candidates for manipulating let-7 miRNAs therapeutically.

In conclusion, our data demonstrate a novel role for the let-7 miRNAs in determining CD8 T cell fate, and has identified them as a unique therapeutic target. The further characterization of these miRNAs in CD8 T cells should prove fascinating and productive for manipulating immune responses to improve human health.

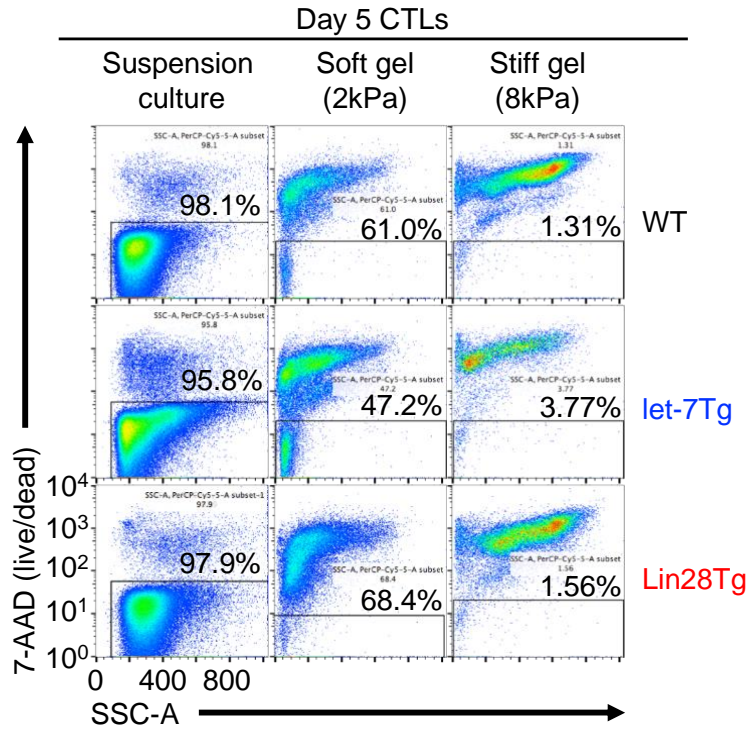


Figure 4.1 Encapsulation of T cells in hydrogels with different stiffnesses

On day 3 after activation, 1×10^6 cells were encapsulated into functionalized hydrogels with either a 2kPa stiffness, representative of healthy tissues, an 8kPa stiffness, representative of a cancerous tissue, or cells were kept in suspension as usually cultured. Two days later, cells were analyzed by flow cytometry. Numbers indicate the frequency of live cells after isolation from the gels. Data are from one experiment, representative of two experiments.

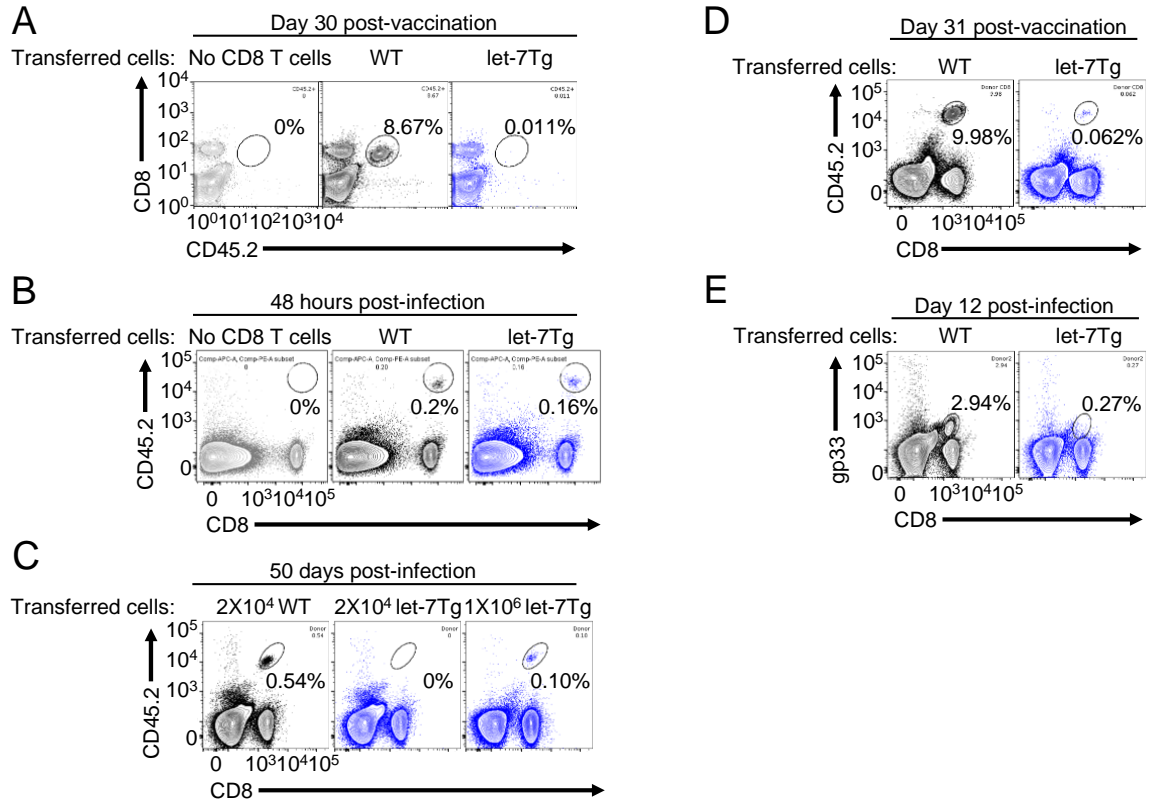


Figure 4.2 Identification of let-7Tg cells in host mice

(a) 2X10⁴ naïve CD45.2 P14 or CD45.2 P14 let-7Tg CD8 T cells were transferred into CD45.1 host mice, and one day later vaccinated with gp33, poly(I:C), and anti-CD40 monoclonal antibodies. The spleens of host mice were analyzed for donor cells 30 days after vaccination. (b) 1X10⁶ naïve CD45.2 P14 or CD45.2 P14 let-7Tg CD8 T cells were transferred into CD45.1 host mice, and one day later infected with LM-33. The spleens of host mice were analyzed for host cells 48 hours after infection. (c) 2X10⁴ naïve CD45.2 P14, or either 2X10⁴ or 1X10⁶ naïve CD45.2 P14 let-7Tg CD8 T cells were transferred into CD45.1 host mice, and one day later infected with LM-33. The spleens of host mice were analyzed for host cells 48 hours after infection. (d) 2X10⁴ naïve CD45.2 P14 or CD45.2 P14 let-7Tg CD8 T cells were transferred into CD45.1 host mice, and one day later vaccinated with gp33, poly(I:C), and anti-CD40 monoclonal antibodies. The livers of host mice were analyzed for donor cells 30 days after vaccination. (e) 2X10⁴ naïve CD45.2 P14 or CD45.2 P14 let-7Tg CD8 T cells were transferred into Prf1^{-/-} host mice, and one day later infected with LM-33. The spleens of host mice were analyzed for donor cells 12 days after infection, using a gp33 tetramer. Data are from one experiment, representative of one experiment.

CHAPTER 5

MATERIALS AND METHODS

5.1 Animals

C57BL/6J (CD45.2⁺ wild type, stock no. 000664), B6.SJL- *Ptprca^aPepcb^b*/ BoyJ (CD45.1⁺ wild type, stock no. 002014), B6(Cg)- *Rag2^{Tm1.1Cgn}*/J (*Rag2^{-/-}*, stock no. 008449), B6 Tg(CD4-cre)1Cwi/BfluJ (*CD4-Cre*, stock no. 017336), and B6.129S1 (Cg)- *Eomes^{Tm1.1Bflu}*/J (*Eomes^{fl/fl}*, stock no. 017293) were obtained from the Jackson Laboratory. B6.Cg- *Col1a1^{Tm3(tetO-Mirlet7g/Mir21)Gqda}*/J (let-7g, stock no. 023912) and B6.Cg- *Gt(ROSA)26^{Sor^{Tm1(rtTA*M2)Jae}}*/J (M2rtTA, stock no. 006965) were also obtained from the Jackson Laboratory and subsequently crossed to generate let-7Tg mice. Mice with the Lin28B transgene (Lin28Tg) driven under the control of the human CD2 promoter (Pobezinsky et al., 2015), and B6 Tg(TcrLCMV)327Sdz/JDvs/J (P14) mice were a generous gift from Alfred Singer (NCI, NIH). P14⁺ mice, and wild type C57Bl/6J mice on a *Rag2^{-/-}* background were crossed to generate wild type P14 mice. let-7Tg mice, and P14⁺ mice were crossed on a *Rag2^{-/-}* background to generate P14⁺ doxycycline- inducible let-7 transgenic mice. Lin28B Tg mice were crossed with P14⁺ mice on a *Rag2^{-/-}* background to generate P14⁺ Lin28Tg mice. let-7Tg, Lin28B Tg, P14⁺ mice were crossed on a *Rag2^{-/-}* background to generate 4Tg mice. *CD4-Cre* and *Eomes^{fl/fl}* mice were crossed to generate mice with a T cell- specific conditional knockout of Eomes. *CD4-Cre*, *Eomes^{fl/fl}* Lin28B Tg, P14⁺ mice were crossed to generate Lin28Tg mice with a T cell- specific deletion of Eomes. R26^{STOP-Lin28-GFP} mice were generated in collaboration with Alfred Singer, NCI and Georg Hollander (University of Oxford). Gzmb^{Cre+}R26^{STOP-Lin28-GFP} were generated by

crossing R26^{STOP-Lin28-GFP} mice with FVB-Tg(GZMB-cre)1 Jcb/J (GzmbCre) mice obtained from Jackson Labs (stock number: 003734). iCreR26^{STOP-Lin28-GFP} mice were generated by crossing R26^{STOP-Lin28-GFP} mice with Tg(tetO-cre)1Jaw/J (iCre) mice obtained from Jackson Labs (006224). Littermates or age and sex-matched mice were used as controls. All breedings were maintained at the University of Massachusetts, Amherst. This study was performed in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All of the animals were handled according to approved institutional animal care and use committee (IACUC) protocols (#2014-0045, 2014-0065, 2015-0035) of the University of Massachusetts.

5.2 Doxycycline-mediated induction of let-7 transgene expression

Experimental mice including control animals (unless specifically stated otherwise) were fed with 2 mg/mL doxycycline in drinking water supplemented with 10 mg/mL sucrose for four days prior to the initiation of experimental procedures to ensure maximal induction of let-7g expression. Doxycycline drinking water was replaced every other day. *In vitro*, lymphocytes were cultured with 2 µg/mL doxycycline in CTL culture media (see cell sorting and *in vitro* culture below).

5.3 *In vivo* BrdU labeling

Mice were injected i.p. with 1 mg BrdU in PBS, and subsequently fed with 0.8 mg/mL BrdU in drinking water supplemented with 2% sucrose for four days. BrdU water was kept in the dark to eliminate light-sensitivity effects of BrdU and was replaced daily.

Incorporation of BrdU in CD8 T cells from the spleen and lymph nodes were analyzed by flow cytometry (see flow cytometry analysis below).

5.4 Flow cytometry analysis

Flow cytometry data were acquired on a BD Fortessa or a MilliPore Amnis ImageStream. For restimulation *in vitro*, 2×10^6 cells were stimulated with phorbol myristate acetate (PMA) and Ionomycin for 4 h at 37°C and Monensin A for 2 h at 37°C. Cells were first stained for surface proteins then fixed, permeablized, and stained for intracellular proteins according to the manufacturer's instructions (BD Pharmingen, eBio).

For LCMV studies, 2×10^6 cells were stimulated for 4 hr at 37°C with 2 µg/ mL of GP₃₃₋₄₁ or NP₃₉₆₋₄₀₄ peptides, and 1 µl/ mL GolgiPlug (BD Pharmingen). Cells were first stained for surface proteins then fixed, permeablized, and stained for intracellular proteins according to the manufacturer's instructions (BD Pharmingen). Flow cytometry data were acquired on a BD LSR II. All flow cytometry data was analyzed with FlowJo software (TreeStar; RRID:SCR_008520). MilliPore Amnis ImageStream data was analyzed with IDEAS software (EMD Millipore).

The monoclonal antibodies used in these studies can be found in Table 5.1.

5.5 Western blot analysis

Cells were collected and lysed in M2 lysis buffer (20mM Tris, pH7.0, 0.5% NP40, 250mM NaCl, 3mM EDTA, 3mM EGTA, 2mM DTT, 05mM PMSF, 20mM β-glycerol phosphate, 1mM sodium vanadate, 1µg/mL Leupeptin), then resolved by SDS-PAGE. Blots were probed with anti-Perforin (CB5.4, Abcam; RRID:AB_302236) and anti-actin (AC40,

Sigma; RRID:AB_2686923), and visualized using enhanced chemiluminescence (ThermoScientific) with horse-radish peroxidase conjugated anti-rat IgG (712-035-150, Jackson ImmunoResearch; RRID:AB_2340638) or anti-mouse IgG (401215, Calbiochem; RRID_AB:2686924).

5.6 T cell isolation, sorting, and *in vitro* culture

Lymph nodes were harvested and gently tweezed to remove lymphocytes. CD8 lymph node T cells were enriched for via antibody-mediated depletion of B cells using anti-mouse IgG magnetic beads (BioMag, Qiagen). CD4 T cells were removed via anti-rat IgG magnetic beads (BioMag, Qiagen) following incubation with anti-mouse CD4 antibodies conjugated with rat IgG (GK1.5). Lymphocytes were electronically sorted for the further purification of naïve CD8 T cells ($CD44^{lo} CD25^{lo} CD8^{+} CD4^{-}$) (Figure 3—figure supplement 1A).

Cells were stimulated either with irradiated splenocytes loaded with anti-CD3 mAbs (10 $\mu\text{g}/\text{mL}$), or plate-bound anti-TCR β mAbs (10 $\mu\text{g}/\text{mL}$) and anti-CD28 mAbs (5 $\mu\text{g}/\text{mL}$), then differentiated for five days in RPMI supplemented with 10% fetal bovine serum, 1% HEPES, 1% sodium pyruvate, 1% penicillin/ streptomycin, 1% L-glutamine, 1% non-essential amino acids, 0.3% β -mercaptoethanol, 100 U/mL IL-2, 100 mg/mL gentamicin, and 2 $\mu\text{g}/\text{mL}$ doxycycline when necessary. When appropriate 100 U/mL IL-15 was used.

5.7 Eomes site-directed mutagenesis

Site-directed mutagenesis of the let-7 binding site in the Eomes ORF was performed using the Agilent QuikChange II Site-Directed Mutagenesis kit according to the manufacturer's protocol.

5.8 Retroviral transduction of candidate genes

Retrovirus expressing Eomes, Eomes^{MUTANT}, Nupr1, Helios, Hmga1, or Arid3a cDNA with a GFP reporter were produced from the transfection of PlatE cells using Lipofectamine plus (Invitrogen). For retroviral transduction, naïve lymphocytes were stimulated with irradiated splenocytes in the presence of anti-CD3 mAbs (10 µg/mL) for 24 hours, then spin-fected (2000 RPM, 90 minutes, 30°C) with virus and polybrene (4 µg/mL). Retrovirally transduced cells were obtained by sorting on the GFP⁺ population.

Retrovirus expressing the gp33 minigene with a GFP reporter was produced from the transfection of PlatE cells using Lipofectamine plus (Invitrogen). For retroviral transduction, B16 and EL-4 cells were spin-fected (2000 RPM, 90 minutes, 30°C) with virus and polybrene (4 µg/mL). Retrovirally transduced cells were obtained by sorting on the GFP⁺ population. Antibodies used for flow cytometry are listed in Table 5.1.

5.9 Prediction of miRNA targets

Eomes was independently identified in an unbiased search of all ORFs in the mouse and humans genomes, for matches to an extended 9 bp let-7 seed, “TACTACCTC“. This search utilized a hashing algorithm as described in ⁸¹ and identified 119 genes in the mouse genome, and 159 genes in the human genome that have one or more matches to the 9 bp let-7 seed in their ORF sequences. Interestingly, humans have three splice variants of Eomes, one of which lacks the exon containing the match to let-7, thus opening the

possibility that Eomes may escape let-7 repression in some cells by alternative splicing of the target sequence. This may require further investigation.

5.10 Luciferase assay

NIH 3T3 cells (ATCC) were transfected with the pmirGLO vector (Promega) containing either the intact let-7 binding motif from Eomes, or a mutated version of this binding motif, or either intact antisense or mutated antisense seed regions of let-7b, let-7g, or let-7i using Lipofectamine and Plus reagent (Invitrogen). Luciferase activity was measured 48 hours later on a POLARstar Omega 96-well plate reader (BMG Labtech), using the Dual-Luciferase Reporter Assay System (Promega).

5.11 IL-2 withdrawal assay

P14⁺ CD8 T cells were stimulated with anti-TCR β mAbs (10 μ g/mL) and anti-CD28 mAbs (5 μ g/mL) plate-bound antibodies, differentiated into CTLs for 5 days in the presence of IL-2, and 2 μ g/mL doxycycline. On day 5, CTLs were washed twice with PBS to remove all cytokines from the cells. 5X10⁴ CTLs were plated into cytokine-free media for up to 24 hours. Survival was assessed by flow cytometry using propidium iodide.

5.12 CellTrace Violet proliferation assay

Electronically sorted naïve CD8 T cells were labeled with CellTrace Violet (Invitrogen) for 15 minutes at 37°C. Cells were stimulated using plate-bound anti-TCR β mAbs (10 μ g/mL) and anti-CD28 mAbs (5 μ g/mL), cultured for 72 hours, and analyzed by flow cytometry.

5.13 CellTrace Violet cytotoxicity assay

P14⁺ CD8 T cells were stimulated with anti-TCR β mAbs (10 μ g/mL) and anti-CD28 mAbs (5 μ g/mL) plate-bound antibodies, differentiated into CTLs for 5 days in the presence of IL-2, gentamicin, and 2 μ g/mL doxycycline when appropriate. On day 5, live splenocytes were warmed for 10 minutes at 37°C, then labeled with CellTrace Violet (Invitrogen) at two different concentrations (CTV^{high} or CTV^{low}) for 15 minutes at 37°C. CTV^{low} splenocytes were then loaded with either LCMV gp33-41 peptide (1 μ M, GenScript) or LCMV np396-404 peptide (1 μ M, GenScript) for 1 hour at 37°C, and are referred to below as “experimental splenocytes”. CTV^{high} splenocytes remained peptide-free, and were used as a reference control, referred to below as “control splenocytes”. Equal amounts of both experimental and control splenocytes were co-cultured with CTLs at different ratios for four to five hours. Cytotoxicity was assessed by flow cytometry using propidium iodide. Measured as the percent target lysis of live experimental splenocytes loaded with either target (gp33-41) or control (np396-404) peptide from the lymphocytic choriomeningitis virus. The following formula was used to calculate the percent target lysis:

$$\left(1 - \left(\frac{A}{B} \times \frac{C}{D}\right)\right) \times 100$$

A- frequency of live experimental splenocytes co-cultured with CTLs; B- frequency of live control splenocytes co-cultured with CTLs; C- frequency of live control splenocytes incubated in the absence of CTLs; D- frequency of live experimental splenocytes incubated in the absence of CTLs.

5.14 Tumor cytotoxicity assay

P14⁺ CD8 T cells were stimulated with anti-TCR β mAbs (10 μ g/mL) and anti-CD28 mAbs (5 μ g/mL) plate-bound antibodies, differentiated into CTLs for 5 days in the presence of IL-2, and 2 μ g/mL doxycycline. On day 5, titrated amounts of CTLs were added to 1X10⁶ B16^{gp33} adherent tumor cells plated one day prior. Cells were co-cultured for 12-16 hours. Cytotoxicity was assessed by propidium iodide in GFP⁺ tumor cells by flow cytometry.

5.15 Lymphocytic choriomeningitis virus infection and T cell adoptive transfer

10X10³ CD45.2⁺ P14⁺ donor cells from the indicated mice were transferred i.v. into CD45.1⁺ congenic hosts. Mice were inoculated with 5X10⁴ p.f.u. of LCMV Armstrong i.p.. Spleens were harvested and processed 7 days post- infection.

5.16 *L. monocytogenes*^{gp33} bacterial infection and T cell adoptive transfer

2X10⁴ CD45.2⁺ P14⁺ donor cells from the indicated mice were transferred i.v. into CD45.1⁺ congenic hosts. Mice were inoculated with 6X10⁶ c.f.u. LM-33 grown to log phase in TSB with 50 μ g/mL streptomycin.

5.17 Isolation of RNA and quantitative PCR

RNA was isolated according to the manufacturer's instructions (QIAGEN miRNeasy), and genomic DNA removed using the DNA-free DNA removal kit (Ambion). mRNA-encoding cDNA was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen), while miRNA- encoding cDNA was synthesized using the Taqman MicroRNA Reverse Transcription kit (Applied Biosystems). SYBR Green quantitative PCR was performed using the Biorad SensiFAST SYBR Lo-Rox kit and Taqman

quantitative PCR was performed using the Bioline SensiFAST Lo-Rox kit. Both SYBR Green and Taqman amplification primers (Integrated DNA Technologies, or Applied Biosystems) are listed in Tables 5.2 and 5.3.

5.18 Generation of gp33-expressing tumor cell lines

Retrovirus expressing the gp33 minigene with a GFP reporter was produced from the transfection of PlatE cells using Lipofectamine plus (Invitrogen). For retroviral transduction, B16 and EL-4 cells were spin-fected (2000 RPM, 90 minutes, 30°C) with virus and polybrene (4 µg/mL). Retrovirally transduced cells were obtained by sorting on the GFP⁺ population. To select for tumor cells which grow in syngeneic hosts, 0.5X10⁶ sorted gp33-expressing cells were injected into C57BL/6J mice. Solid tumors were homogenized, and single cells were isolated and cultured *in vitro*. Mouse-passaged cells were sorted on the GFP⁺ again before considering the tumor cell line established.

5.19 P815 and MC57 tumor transplantation

For P815 (ATCC; RRID:CVCL_2124) survival studies, 30X10⁶ tumor cells were injected i.p. into host mice. For P815 tumor burden studies, 20X10⁶ tumor cells were injected i.p. into host mice. Seven days post-injection, tumor burden was assessed by washing the peritoneum with cold PBS and counting the collected cells. For studies involving let-7Tg mice, all mice were fed with doxycycline for the duration of the study. For studies involving adoptive CD8 T cell transfer, 10X10⁶ naïve CD8 T cells were injected i.v. into P14⁺Rag2^{-/-} mice, concurrently with i.p. tumor injection. The P815 cell line was authenticated by analyzing H-2K^d expression via flow cytometry.

For MC57 tumor growth studies 1×10^6 cells were injected s.c. into host mice. Tumors were measured by caliper every other day. Once tumor sizes reached 3000 mm^3 , mice were euthanized.

5.20 B16^{gp33} and EL-4^{gp33} tumor transplantation and CTL adoptive transfer

For solid tumor studies, 0.25×10^6 B16^{gp33} or EL-4^{gp33} were injected s.c. into host mice that had been sub-lethally irradiated (300 rad) 24 hours prior. Seven days later, 1.5×10^6 P14 CTLs were injected i.v. into host mice. Tumors were measured by caliper every other day. For survival studies, once tumor sizes reached 3000 mm^3 , mice were euthanized and considered to not have survived the study. For metastatic tumor studies, 0.2×10^6 B16^{gp33} were injected i.v. into host mice. Two days later, 1.5×10^6 P14 CTLs were injected i.v. into host mice. Fourteen day after adoptive transfer of CTLs, tumor burden was assessed by lung perfusion and counting metastatic nodes. For studies involving let-7Tg CTL transfer, all mice were fed with doxycycline for the duration of the study. For studies involving anti-PD-L1 antibody treatment, mice were injected i.p. with $200 \mu\text{g}$ anti-PD-L1 antibodies (BioXCell, clone: 10F.9G2). Treatments were scheduled according to the study design.

5.21 Seahorse glycolytic rate assay

CTLs were plated in XF media and the ECAR was measured using the Seahorse XF glycolytic rate assay kit (Agilent; Catalog #:103344-100), on an XFe96.

5.22 RNA-seq analysis

5.22.1 Data Analysis

Downstream analysis was performed using a combination of programs including Bowtie2, Tophat2, HTseq, Cufflink and our wrapped scripts. Alignments were parsed using Tophat program and differential expressions were determined through DESeq2/DEGseq. GO and KEGG enrichment were implemented by the Goseq R package and KOBAS. Gene fusion and difference of alternative splicing event were detected by MISO and TopHatfusion software.

5.22.2 Reads mapping to the reference genome

Reference genome and gene model annotation files were downloaded from genome website browser (NCBI/UCSC/Ensembl) directly. Indexes of the reference genome were built using Bowtie v2.0.6 and paired-end clean reads were aligned to the reference genome using TopHat v2.0.9. Bowtie uses a BWT(Burrows-Wheeler Transformer) algorithm for mapping reads to the genome and Tophat can generate a database of splice junctions based on the gene model annotation file and thus achieve a better mapping result than other non-splice mapping tools.

5.22.3 Quantification of gene expression level

HTSeq v0.6.1 was used to count the read numbers mapped of each gene. And then RPKM of each gene was calculated based on the length of the gene and reads count mapped to this gene. RPKM, Reads Per Kilobase of exon model per Million mapped reads, considers the effect of sequencing depth and gene length for the reads count at the same time, and is currently the most commonly used method for estimating gene expression levels (Mortazavi et al., 2008).

5.22.4 Differential expression analysis

(For DESeq2 with biological replicates) Differential expression analysis between two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate(FDR). Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed. (For DESeq without biological replicates) Prior to differential gene expression analysis, for each sequenced library, the read counts were adjusted by edgeR program package through one scaling normalized factor. Differential expression analysis of two conditions was performed using the DESeq R package (1.12.0). The P values were adjusted using the Benjamini & Hochberg method. Corrected P-value of 0.005 and $\log_2(\text{Fold change})$ of 1 were set as the threshold for significantly differential expression.

5.22.5 Correlations

To allow for log adjustment, genes with 0 RPKM are assigned a value of 0.001. Correlation were determined using the cor.test function in R with options set alternative = "greater" and method = "Spearman".

5.22.6 Clustering

To identify the correlation between difference, we clustered different samples using expression level RPKM to see the correlation using hierarchical clustering distance method with the function of heatmap, SOM(Self-organization mapping) and kmeans using silhouette coefficient to adapt the optimal classification with default parameter in R.

5.23 Statistical analysis

P-values were determined using a two-tailed Student's t-test, or one-tailed Student's t-test where indicated.

Table 5.1: Antibodies used for flow cytometry

Target protein	Clone	Company
CD8a	53-6.7	eBioscience
	5H10	Invitrogen
CD8b	YTS156.7.7	BioLegend
CD4	RM4-5	BioLegend
BrdU	3D4	BioLegend
CD44	IM7	BD Pharmingen
CD25	PC61	BioLegend
	PC61.5	eBioscience
CD122	TM-b1	BioLegend
Granzyme A	3G8.5	BioLegend
Granzyme B	GB11	BioLegend
IFN-g	XMG1.2	eBioScience
Eomes	Dan11mag	eBioScience
Ki67	SolA15	eBioScience
Tbet	O4-46	BD Pharmingen
PE-Streptavidin		BioLegend
APC-Streptavidin		BioLegend
CD45.1	A20	BD Pharmingen
CD45.2	104	BD Pharmingen
KLRG1	2F1	BD Pharmingen
TNF-a	MP6-XT22	BD Pharmingen
CD62L	MEL-14	BioLegend
CD127	A7R34	BioLegend
TIM-3	RMT3-23	BioLegend
PD-1	29F.A12	BioLegend
CD160	CNX46-3	eBioscience
2B4	m2B4	BioLegend
IL-10	JES5-16E3	eBioscience
GFP	FM264G	BioLegend

Table 5.2: Taqman primers used for qRT-PCR

IDT Taq Assay	
Eomes	Mm.PT.58. 32833544
Tbx21	Mm.PT.58. 5261453
Gzma	Mm.PT.58. 43238753
Gmzb	Mm.PT.58. 42155916
Ifng	Mm.PT.58. 30096391
Prfl	Mm.PT.58. 41904164
Rpl13a	Mm.PT.58. 43547045.g
AB Taqman Assay	
Hes1	Mm01342805_m1
Hey1	Mm00468865_m1
Heyl	Mm00516558_m1
Dtx1	Mm00492297_m1
Ldha	Mm01612132_g1
Yars	Mm00460301_m1
let-7a	377
let-7b	378
let-7c	379
let-7d	2283
let-7e	2406
let-7f	382
let-7g	2282
let-7i	2221
U6	1973

Table 5.3: Primers used for qRT-PCR

SYBR primers	Forward	Reverse
Cdc25a	ACAGCAGTCTACAGAGAATGGG	GATGAGGTGAAAGGTGTCTTGG
Ccnd2	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT
Ccnf	GTAGGCGATAGGTCATACGGA	ACAATGGATCACTACCCCGTG
Cdk6	GGCGTACCCACAGAAACCATA	AGGTAAGGGCCATCTGAAAAC
Glut1	CAGTTCGGCTATAACTGTTG	GCCCCGACAGAGAAGATG
Glut3	ATGGGGACAACGAAGGTGAC	GTCTCAGGTGCATTGATGACTC
Gpd2	GAAGGGGACTATTCTTGTGGGT	GGATGTCAAATTCGGGTGTGT
Pfk1	GGAGGGGAGAACATCAAGCC	CGGCCTTCCCTCGTAGTGA
Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
Tpi	CCAGGAAGTCTCTCGTTGGGG	CAAAGTCGATGTAAGCGGTGG
Pkm	GCCGCCTGGACATTGACTC	CCATGAGAGAAATTCAGCCGAG
Myc	AGTGCTGCATGAGGAGACAC	GGTTTGCCTCTTCTCCACAG
Tfap4	GGAGAAGCTAGAGCGGGAAC	TTTTGCCGGGATGTAGAGAC
Zbtb32	CCCACTCCAGGATCTTTTCCC	TGACTCACACAGGTTGCCAG
Prdm1	GACGGGGTACTTCTGTTC	GGCATTCTTGGGAACTGTGT
Runx3d	GCGACATGCCTTCCAACAGC	CTTAGCGCGCCGCTGTTCTCGC
Id3	CTGTGGAACGTAGCCTGG	GTGGTTCATGTGCTCCAAGAG
Tcf7	NA	NA
Ccr7	NA	NA
Sell	TACATTGCCAAAAGCCCTTAT	CATCGTTCCATTTCCCAGAGTC
Bcl2	CACCCCTGGCATCTTCTCCTTC	CATCTCCCTGTTGACGCTCTCC
Id2	ATGAAAGCCTTCAGTCCGGTG	AGCAGACTCATCGGGTCTGT
Ikzf2	CCGTACCTGGTCATCACAGAG	CAGTCTCGAAGCTCGATGGC
Havcr2	TCAGGTCTTACCCTCAACTGTG	GGCATTCTTACCAACCTCAAACA
Pdcd1	ACCCTGGTCATTCACTGGG	CATTGCTCCCTCTGACACTG
Cd160	GGGGCTAATACTTCTTGGTGC	CTTTTCAGTGATGCCATCTGTCT
Cd244	AGCCCTGGACTAATGGGACTT	GCTGGCGTCAATCTGGTCT
Il10	GCTCTTACTGACTGGCATGAG	CGCAGCTCTAGGAGCATGTG
Wnt10b	GAAGGGTAGTGGTGAGCAAGA	GGTTACAGCCACCCCATTC
Cd127	GGATGGAGACCTAGAAGATG	GAGTTAGGCATTTCACTCGT
Arid3a	GCTTGGGACATCCGTCCTC	CAAATGCCTATCTCCCTCAGC
Nupr1	CCCTTCCCAGCAACCTCTAAA	TCTTGGTCCGACCTTTCCGA
Hmga1	GGTCGGGAGTCAGAAAGAGC	ATTCTTGCTTCCCTTTGGTGC
Tnfrsf9	CCTTGCAGGTCTTACCTTGT	GTTGCTTGAATATGTGGGGGA
Tnfrsf4	ATGTCATCCGTGTGAGACTGG	CCACTTCGATGGTTGCACTGT
RPL13a	CGAGGCATGCTGCCCCACAA	AGCAGGGACCACCATCCGCT

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