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Regulation of T cell responses by microRNAs

By

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Doctor of Philosophy

Graduate Division of Biological and Biomedical Sciences

Biochemistry, Cell, and Developmental Biology

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B.S., Peking University, 2007

Advisor: Rafi Ahmed, Ph.D.

An abstract of

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Abstract

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Understanding the mechanisms underlying the generation of effector functions and immune memory by T cells is essential to the development of more effective vaccination strategies. Various molecular pathways involved in effector and memory T cell development have been elucidated over the last few decades. However, how translational regulation mechanisms are involved in this process is less clear. MicroRNAs play a major role in translational regulation. My dissertation research focuses on how microRNAs govern the differentiation of T cells during the immune response to viral infection. In the first study, I demonstrate that the miR-17-92 cluster is highly expressed in proliferating effector CD8 T cells. miR-17-92 enhances mTOR signaling and promotes CD8 T cell clonal expansion through enhancing proliferation. However, excessive levels of miR-17-92 drive effector CD8 T cells into terminal differentiation and result in compromised memory CD8 T cell development. In my second study, I investigate how miR-17-92 regulates the antiviral immune response mediated by CD4 T cells. Similar to what is seen in CD8 T cells, miR-17-92 is necessary for the clonal expansion of CD4 T cells after viral infection. The generation of IFN-γ-producing CD4 T cells is especially sensitive to the level of miR-17-92 expression. In addition, miR-17-92 over-expression preferentially expands the Th1 response, which indicates that different subsets of effector CD4 T cells are differentially regulated by miR-17-92. Interestingly, virus-specific CD4 T cells that over-express miR-17-92 are not terminally differentiated and can generate a normal memory compartment. Thus, these studies provide insight into how microRNAs are involved in the differentiation of virus-specific T cells and highlight the importance of translational regulation in the immune response to viral infection.

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Chapter 1: Introduction

Part I: Immunity and the immune system

Immunity is the capacity of an organism to defend against diseases caused by infection, or other harmful biological invasions. There are two types of immunity: innate immunity and adaptive immunity. Innate immunity is present in most organisms. It acts as a first line of defense and immediately responds to infection. This type of immunity is acquired during development and can protect the host against a broad range of pathogens in a non-specific manner. However, an innate immune response does not result in long-lasting protection to the host. Adaptive immunity, which is unique to vertebrates, is acquired during the lifetime of an organism. The adaptive immune response is specific to a particular pathogen and often results in the protective immunity against re-encounters with the same pathogen, which is known as immune memory. Lymphocytes make up the backbone of the adaptive immune system. B lymphocytes (B cells) and T lymphocytes (T cells) are two main sub-populations of lymphocytes, each with unique functions. Although they have evolved differently, the innate and adaptive immune systems have extensive crosstalk during the immune response.

Part II: CD8 T cell responses during viral infection

Lymphocytic choriomeningitis virus (LCMV) infection as a model system to study the T cell antiviral immune response

LCMV is a rodent borne virus and a member of the *Arenaviridae* family and was first isolated by Charles Armstrong in 1933. The genome of LCMV is composed of two single-stranded RNAs. The L strand is an ambisense RNA encoding the polymerase and the zinc binding protein (1). The S strand is also an ambisense RNA encoding the nucleocapsid protein (NP) and the glycoprotein GP-C (2, 3). GP-C is cleaved to produce GP-1 and GP-2, which form spikes that mediate the interaction between the virion and its cellular receptor α-dystroglycan (3, 4). The house mice, *Mus musculus*, are natural hosts of LCMV. Infected mice can transmit virus through urine, nasal secretion, milk, or bites (5). Infected females can vertically transmit the virus to their offspring, which will become carriers of the virus (6). The immune response to LCMV varies depending on the LCMV strain and the route of infection. Peritoneal inoculation of adult mice with the LCMV Armstrong strain induces a vigorous CD8 T cell response, which results in the clearance of the virus within one week. In contrast, intravenous inoculation of the LCMV clone 13 strain induces a suppressed CD8 T cell response and leads to protracted viremia (7, 8). The LCMV clone 13 differs from LCMV Armstrong by three amino-acid mutations: K1076Q in the polymerase, and F260L and N176D in GP-1 (9). None of the mutations cause a change in T cell epitopes (10). However, F260L enhances the binding affinity of the virion to α-dystroglycan, which is preferentially expressed by dendritic cells (DCs), and thus leads to viral persistence by altering DC function (9). The different immune responses induced by the two strains of LCMV allow for the study of memory T cell development during acute infections and T cell exhaustion during chronic infections.

CD8 T cells are essential to immunity against viral infection

Viruses and intracellular bacteria can replicate inside host cells. Although antibodies can effectively eliminate pathogens outside the cells and/or block their entry, they are no longer effective after these pathogens gain access to the host cells. An alternative way to control these pathogens is to destroy infected cells and stop the intracellular pathogens from propagating. This task is carried out by cytotoxic lymphocytes, the majority of which are CD8 T cells, also referred to as cytotoxic T lymphocytes (CTLs). CD8 T cells express the co-receptor glycoprotein CD8 and T cell receptors (TCRs) that recognize their cognate peptide epitopes bound to major histocompatibility complex class I molecules (MHC-I). CD8 T cell responses to acute viral infections can be divided into four phases. In the initial phase, naïve CD8 T cells are primed by antigen-presenting cells (APCs), which present their cognate antigens in peripheral lymphoid organs. Activated CD8 T cells acquire effector functions and start to proliferate extensively, a process known as clonal expansion. During clonal expansion, effector CD8 T cells undergo more than 15 consecutive cell divisions and expand from several thousand antigen-specific naïve T cells into millions of effector cells (11, 12). Effector CD8 T cells migrate to infected tissues and control the infection by directly killing infected cells and secreting cytokines. After the infection is cleared, the contraction phase begins. The majority of effector CD8 T cells undergo apoptosis during this phase, which lasts several weeks (13). During this period, a subset of effectors destined to survive the contraction phase starts to differentiate into memory CD8 T cells. Memory CD8 T cells can survive for years after infection during memory maintenance phase, when they slowly and continuously divide

to renew the memory pool. Upon re-infection by the same virus, memory CD8 T cells mount a recall response that is faster and stronger than the primary response.

Priming of CD8 T cells

Naïve T cells are primed in secondary lymphoid organs, such as lymph nodes. Migration of naïve T cells to secondary lymphoid organs takes multiple steps. Naïve T cells enter lymph nodes through high endothelial venules (HEVs). Naïve T cells express L-selectin (CD62L), which recognizes peripheral node addressin (PNAD) expressed on the surface of HEVs (14). Upon binding of PNAD, naïve T cells start rolling. HEV cells present CCL21 and CCL19 to rolling T cells, which express CCR7, the receptor for these chemokines (14). Signaling through CCR7, a G-protein coupled receptor, activates Gαi signaling and leads to the interaction between leukocyte function-associated antigen 1 (LFA1) on the T cell and intercellular adhesion molecule 1 (ICAM1) on the endothelial cell. This interaction arrests the rolling lymphocytes onto endothelial cells. T cells then transverse the HEV and enter the T cell zone.

DCs are necessary for the priming of CD8 T cells (15). In infected tissues, DCs that are exposed to inflammatory stimuli undergo maturation, down-regulate receptors for inflammatory chemokines, and up-regulate chemokine receptors for the migration to secondary lymphoid organs (16). Naïve T cells migrate quickly in random directions to screen T cell zones for APCs presenting their cognate antigens (17). A DC can make contact with ~500 T cells in an hour and more than 10 T cells simultaneously (18). The

interaction between T cells and DCs can be divided to three phases: 1) During the first 8 hrs, T cells make short contacts with DCs, slow down their motion, and up-regulate activation markers; 2) During the next 12 hrs, T cells form stable interactions with DCs and produce cytokines such as interleukin-2 (IL-2) and interferon-γ (IFN-γ); 3) After 24 hrs, T cells return to quick movement, form transient contacts with DCs, and start to proliferate (19).

Effector CD8 T cell differentiation and clonal expansion

Effector CD8 T cells are equipped with cytotoxic granules, which are derived from lysosomes. Cytotoxic granules store perforin, granzymes, and granulysin. Perforin can form pores on the plasma membrane of target cells, so that granzymes can get access into the cytoplasm and trigger apoptosis via activating the caspase cascade and mitochondrial apoptotic pathways (20). When effector CD8 T cells recognize their cognate epitope presented by MHC-I on infected cells, signals through the TCR trigger the calcium-dependent release of cytotoxic granules. Effector cells orient their Golgi complexes and microtubule-organizing centers, and form cytotoxic immunological synapses with their target cells (21). The contents of cytotoxic granules are only released via the secretory domain of the immunological synapse into the space between the CTL and its target cell, so that bystander killing is minimized and the efficiency of killing the target cell is enhanced. Effector CD8 T cells also secrete cytokines such as IFN-γ, TNF-α, and IL-2. IFN-γ directly inhibits viral replication and enhances antigen presentation by MHC-I. TNF-α induces inflammation, activates macrophages, and triggers apoptosis of infected

cells and tumor cells. IL-2 is a growth factor for T cells and was recently shown to promote the expression of perforin and granzymes by CTLs (22).

Clonal expansion of effector CD8 T cells can last for one week or longer. During this period, these cells can complete one cell cycle within 6 hrs (23). An interesting question is whether the maintenance of effector differentiation and cell division requires the drive of continuous antigen signaling through the TCR. It has been shown that CD8 T cells can start proliferating after 2 hrs of antigen stimulation (24). However, longer priming is necessary for subsequent antigen-independent expansion and the development of effector functions (25). After this initial programming, CD8 T cells can undergo at least seven cell divisions without further antigen stimulation and can develop into memory cells (26). The initial dose of pathogens only affects the number of naïve CD8 T cells recruited to the immune response but not the extent of cell division nor effector/memory programming (26). More recently, experiments in which antigen presentation was halted by curtailing infection by antibiotics or depleting DCs confirmed that antigen is no longer required for clonal expansion or effector/memory differentiation after the initial window of programming (27, 28). Another question is how the affinity of the TCR for its cognate antigen affects effector differentiation and clonal expansion. Using mouse T cells that express a transgenic TCR specific for a peptide from ovalbumin (OVA) and a collection of WT and mutated OVA peptides with different affinities to the transgenic TCR, a group showed that weak TCR-antigen interactions can still trigger effector and memory cell differentiation but lead to reduced clonal expansion (29). Weak TCR affinity also leads to the early egress of CD8 T cells from lymphoid organs (29).

Early during the immune response, T cells are temporarily trapped in lymphoid organs to achieve maximal activation. This is mediated by inhibiting T cell egress, a process dependent on sphingosine 1-phosphate receptor 1 (S1P1) signaling (30, 31). Further studies have demonstrated that CD69 acts downstream of type I interferons and inhibits the S1P1 signal, leading to the temporary lymphopenia seen in the first few days of infection (32, 33). After effector differentiation, CD8 T cells begin to down-regulate the expression of secondary lymphoid organ homing molecules such as CD62L and CCR7, as a result of the activation of the PI3K-mTOR axis (34). During vaccinia virus infection, effector CD8 T cells up-regulate tissue-homing molecules and exit lymphoid organs at ~60 hrs post-infection (35). The inflammatory chemokine receptor CXCR3 is also expressed on effector CD8 T cells, allowing for their migration into infection sites (36, 37). During migration and inside infected tissues, effector CD8 T cells continue to proliferate, driven by cognate antigen presented by MHC-I on infected cells (38). Moreover, antigen presentation by DCs also occurs in infected tissues, which can further modify the effector differentiation programming. Such presentation is likely to be mediated by monocyte-derived DCs recruited to the site of infection (39).

After their differentiation from naïve cells into effectors, CD8 T cells undergo rapid proliferation, produce a large amount of cytotoxic proteins and cytokines and migrate to inflamed tissues. It is crucial to understand how the physiology of these cells changes to accommodate such a dramatic shift in cellular function and tissue environment. Upon differentiation into effector cells, CD8 T cells increase their uptake of glucose and amino

acids by up-regulating the expression of the corresponding transporters (40-42). Naïve and memory T cells depend on mitochondrial dependent pathways, such as glucose oxidation and fatty acid oxidation, to provide energy (41, 43, 44). However, effector T cells switch to aerobic glycolysis and glutaminolysis in order to meet the increased metabolic demands associate with the assembly of macromolecules (45). The PI3K-Akt-mTOR cascade, downstream of the co-stimulatory receptor CD28, and the ERK signaling cascade, downstream of TCR, are essential for the shift to glycolysis and for enhanced glucose and amino acid uptake (40, 46, 47). mTOR serves as a hub linking growth factor signaling, metabolism, and cell growth, and is a critical regulator of effector and memory CD8 T cell differentiation (44, 48, 49).

Effector CD8 T cell heterogeneity and memory CD8 T cell differentiation

Several models of memory CD8 T cell development have been proposed: 1) All effector cells have the same potential to develop into memory cells; 2) The potential of effectors to form memory cells decreases as the signals driving terminal differentiation, such as TCR stimulation, increases/prolongs; 3) The commitments to effector and memory lineages are mutually exclusive and predetermined; 4) All effector cells possess effector functions but can be divided into populations with different potentials of forming memory cells (50). Adoptive transfers of single antigen-specific CD8 T cell or molecular barcode-labeled CD8 T cells have shown that an antigen-specific naïve CD8 T cell can contribute to both effector and memory pools (51, 52). Experiments using transgenic mouse strains that irreversibly mark effector T cells through the expression of reporter

genes also have demonstrated that memory T cells are derived from effector cells (53-55). Therefore, the precursors of memory T cells have effector functions before they differentiate into memory cells. After antigen clearance, the surface markers and transcriptomes of effector cells gradually change and eventually become indistinguishable from those of memory cells (50). Moreover, central memory cells also gradually dominate over time, as indicated by the conversion of the $CD62L^{low}CCR7^{low}IL\text{-}2^{-}$ population into the $CD62L^{high}CCR7^{high}IL\text{-}2^{+}$ population (56). However, this does not exclude the possibility that memory precursor cells have different characteristics compared to other effectors that are eliminated during the contraction phase. Indeed, evidence supporting the heterogeneity of effector CD8 T cells is accumulating. Two subsets of effector CD8 T cells were observed in various infection models: $CD127(IL\text{-}7R\alpha)^{high}$killer cell lectin-like receptor g1 $(KLRG1)^{low}$ memory precursor effector cells (MPECs) and $CD127^{low}KLRG1^{high}$ short-lived effector cells (SLECs), or terminally differentiated effector cells (57-61) (Figure 1-1). Both MPECs and SLECs express cytotoxic proteins and IFN-γ. However, MPECs demonstrate a much higher potential to survive the contraction phase and differentiate into self-renewing memory T cells (50). MPECs also express higher levels of CD62L and the co-stimulatory receptor CD27 than SLECs (50, 59, 62). Besides these differences in surface markers, the gene expression profile of MPECs is also different from that of SLECs (57, 59). Moreover, the lineage commitments of MPECs and SLECs are stable, and there is little conversion between the two populations (57). Great effort has been spent to determine when the fate of an effector cell is determined. One study revealed that a biphasic expression of CD25 occurs by day 3.5 p.i. and that the $CD25^{low}$ cells have higher potential to form memory cells than

their $CD25^{high}$ counterparts (63). Another study claimed that the cell fate determination occurs as early as the first division of activated CD8 T cells (64).

It is important to understand the extracellular signals that govern the lineage commitment of different effector subsets and memory cell development. Blunting infection or increasing clonal competition can induce effector CD8 T cells to up-regulate CD127 and CD62L and leads to an accelerated transition to memory T cells (57, 65, 66). While the amount of antigenic stimulation through the TCR does not seem to affect the balance between MPECs and SLECs, the duration of antigenic stimulation appears to be inversely correlated with the potential to form memory cells. This is consistent with the observation that effector CD8 T cells gradually lose their ability to form memory cells during chronic infection (56, 57, 59). Co-stimulatory signals are also important regulators of memory T cell development. Co-stimulatory receptors including CD27, CD28, OX-40, and 4-1BB have been shown to regulate memory cell differentiation and survival (67-70). Not surprisingly, IL-7 is necessary for the survival of CD8 T cells during the transition to memory cells (71). IL-2, which belongs to the same cytokine family as IL-7, is a growth factor produced predominately by activated CD4 T cells, and to a lesser extent by activated CD8 T cells. Strong or prolonged IL-2 signaling leads to increased terminal differentiation of effector cells and decreased memory cell formation (22, 63, 72). T cells lacking CD25 (high affinity IL-2 receptor α chain) form fewer $KLRG1^{high}CD127^{low}$ effector cells. Inflammatory cytokines, such as IL-12 and IFN-γ, promote terminal differentiation of effector CD8 T cells and compromise their longevity (57, 73). Moreover, cytokines may specifically act on one subset of effector cells. For example,

TGF-β induces apoptosis of SLECs, while IL-15 promotes their survival during the contraction phase (74).

It is also important to understand the intracellular signaling pathways that control memory differentiation. As discussed above, during the transition from naïve to effector cells, CD8 T cells switch from oxidative phosphorylation to aerobic glycolysis. After viral clearance, in order to return to the quiescent state and deal with the withdrawal of nutrients and growth factors, CD8 T cells need to return to the oxidation of glucose and fatty acids during the transition from effector to memory cells (62). Therefore, suppression of mTOR signals by drugs, such as rapamycin or metformin, or protein inhibitors, such as AMPK, promotes memory T cell development (44, 49). The PI3K-AKT axis is a key pathway downstream of IL-2. Strong PI3K or AKT signaling promotes terminal differentiation of effector CD8 T cells and impairs their homing to secondary lymphoid organs by down-regulating corresponding homing molecules (34, 75, 76). IL-2 signaling also induces expression of the transcriptional repressor Blimp-1 (77). SLECs express higher levels of Blimp-1 than MPECs, and knocking out Blimp-1 promotes MPEC and central memory CD8 T cell differentiation (78, 79). Blimp-1 and another transcriptional repressor, Bcl6, antagonize each other (80). Bcl6 has been shown by several studies to promote the generation of memory CD8 T cells (81, 82). IL-12 also enhances the terminal differentiation of effector cells by stimulating mTOR activity as well as by increasing the expression of T-bet and suppressing Eomes expression (48, 57). T-bet and Eomes both belong to the T-box family of transcription factors. T-bet enhances terminal differentiation of effectors, while Eomes promotes memory cell development

(48, 57, 83). The Wnt signaling pathway is critical for various developmental processes and is conserved from invertebrates to vertebrates (84). One study showed that the Wnt-β-catenin signaling pathway suppresses effector T cell differentiation and promotes the generation of memory CD8 T cells (85). Another transcription factor downstream of the Wnt pathway, Tcf7, has also been shown to promote memory differentiation and persistence (86). The E-box transcription factor E2A and its antagonists, Id2 and Id3, play critical roles in B/T lymphocyte fate commitment and various steps of lymphocyte development (87, 88). Id2 deficient cells undergo normal clonal expansion but fail to persist due to up-regulation of pro-apoptotic factors (89). Knocking out Id2 appears to compromise the generation of short-lived effector cells while promoting the differentiation of $CD62L^{high}$ memory cells (89, 90). Id3, a target of the transcriptional repressor Blimp-1, is highly expressed in memory precursor cell (90, 91). Id3 over-expression and E2A deficiency favor memory CD8 T cell development (91).

Memory CD8 T cells

Memory CD8 T cells are antigen-experienced cells generated after infection, vaccination, or tumor challenge. Memory CD8 T cells have two cardinal features: 1) They have the capacity to self-renew, so that they can maintain a stable memory pool that conveys long-term immune protection; 2) They can mount a rapid recall response upon re-encountering their cognate antigens.

Memory CD8 T cells can survive for decades in humans and for a lifetime in mice. The memory CD8 T cell pool is maintained by matching the rate of homeostatic proliferation to the rate of cell death. Cell transfer experiments and statistical analyses determined that the length of the intermitotic interval of LCMV-specific memory T cells is ~50 days, and is roughly the same across all epitopes (92). Such proliferation occurs stochastically in a small fraction of memory cells (92). The homeostatic proliferation of memory CD8 T cells does not depend on cognate antigen but instead on IL-15 (93). DCs and macrophages produce and present IL-15 to memory CD8 T cells (94). Interestingly, IL-15 is not secreted by their producers but trans-presented together with the IL-15 receptor α chain to memory CD8 T cells (94). Central memory CD8 T cells are thought to reside largely in their niches in the bone marrow (95).

During a second exposure to antigen, memory CD8 T cells mount a rapid and strong recall response. This is partially a result of the increased numbers of antigen-specific T cells generated by the previous immune response. The number of memory CD8 T cells can be several thousand times higher than that of naïve cells specific for the same antigen (96). A second factor that enhances the recall response is the change in mRNA profile and chromatin structure that occurs when a naïve T cells differentiates into a memory cell (97). These changes not only increase the baseline expression levels of critical genes, such as IFN-γ and granzyme B, but also poise the cells to rapidly re-express genes important to effector functions and reactivate critical signaling pathways upon antigen recognition (96, 98). Thirdly, the TCRs of memory cells are believed to have a lower activation threshold than those of naïve cells, due to functional avidity maturation (99).

Fourthly, memory cells acquire features of innate immunity, and can respond to cytokines such as IL-12 and IL-18 by producing IFN-γ independent of TCR signaling during infection (100). Moreover, unlike naïve cells, some subsets of memory cells can enter and reside in non-lymphoid tissues and previously infected sites, so that they can mount an immediate response upon re-infection (101). A recent study reported that, because of their expression of CXCR3, memory CD8 T cells enter the outermost regions of lymph nodes, where pathogens are enriched, more efficiently than naïve CD8 T cells (102).

Memory CD8 T cell heterogeneity

Like effector cells, memory CD8 T cells are also heterogeneous. There are two major memory CD8 T cell populations: central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells). T_{CM} cells express CCR7 and CD62L and home to secondary lymphoid organs, while T_{EM} cells do not express CCR7 or CD62L and preferentially home to peripheral tissues (103, 104). T_{CM} cells are mostly found in lymph nodes, spleen, and blood, and are much less frequent in non-lymphoid tissues (50, 104). While seldom present in lymph nodes, T_{EM} cells can be found in various tissues as well as spleen and blood (50, 104). T_{EM} cells retain considerable cytotoxicity and reside at portals of pathogen invasion, so that they can respond immediately upon re-infection. T_{CM} cells produce IL-2 and have higher proliferation potential than T_{EM} cells. The relative contribution to immune protection by the two populations is difficult to determine and also depends on the infection model (105-108). The lineage relationship between T_{CM} cells and T_{EM} cells and whether the two populations are inter-convertible are under

continuous debate. While some studies suggest that the two lineages are not inter-convertible, others argue that T_{EM} cells are in a transitional state between effector T cells and T_{CM} cells after antigen clearance (56, 66, 109-113). There are also studies showing that T_{CM} cells can up-regulate effector molecules and turn into T_{EM} cells after entering peripheral tissues (114, 115). It is possible that the lineage relationship between T_{CM} cells and T_{EM} cells depends on the type of antigen inoculated. It is also worth pointing out that these two populations may not be homogenous themselves, especially considering that T_{CM} cells and T_{EM} cells are distinguished by only a few surface markers in most of the studies described above. Moreover, the expression of CCR7 or CD62L may not fully reflect the migratory behavior of memory T cells.

Recently, another memory CD8 T cell population, tissue-resident memory T cells (T_{RM} cells), was described (105). T_{RM} cells do not leave their resident tissues and can maintain a stable pool with less homeostatic proliferation (105). Moreover, most T_{RM} cells are not derived from their circulating counterparts (116-118). T_{RM} cells can be found in the nervous system, gut, and epidermis of the skin (116-121). T_{RM} cells often express CD103 and CD69 (122-124). CD103 can bind to E-cadherin expressed by epithelial cells, which may help to retain T_{RM} cells in their resident tissues (105). CD69 inhibits S1P1 function and may inhibit the egress of T_{RM} cells (33). A recent study of brain-resident memory CD8 T cells showed that T_{RM} cells have a distinct gene expression signature compared to circulating memory T cells (125). Skin T_{RM} cells have been shown to offer better immune protection than their circulating counterparts in several immunization models (122, 126,

127). Finally, T_{RM} cells can interact with DCs and CD4 T cells simultaneously, and initiate the recall response in extra-lymphoid tissues (119).

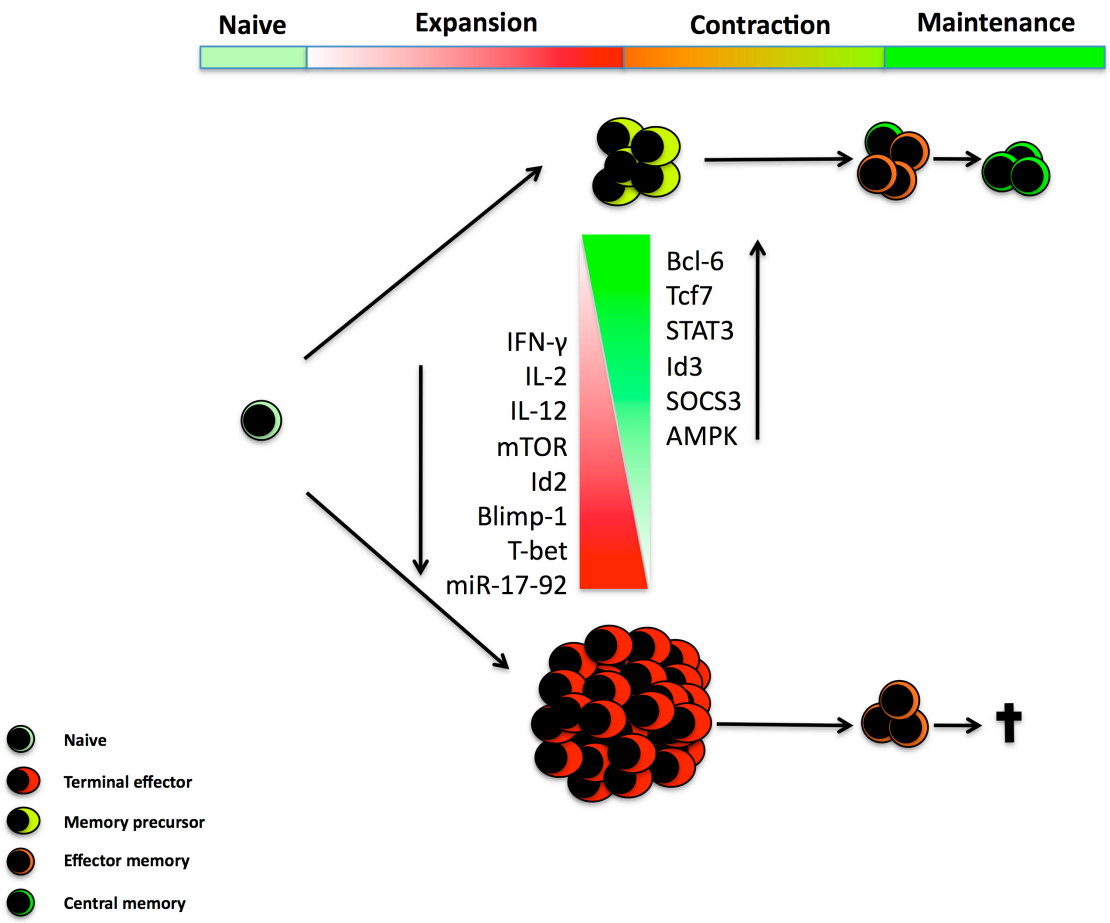

Figure 1- 1. CD8 T cell differentiation during viral infection.

After infection, naïve CD8 T cells are programmed into effector CD8 T cells and undergo clonal expansion. Strong inflammatory signals from cytokines, such as IFN-γ, IL-2, and IL-12, miR-17-92 over-expression (this study), and strong mTOR signaling promote the generation of terminal effectors. In addition, transcription factors, such as Id2, Blimp-1, and T-bet, program terminal differentiation. On the other hand, Bcl-6, Tcf7, STAT3, Id3, SOCS3, and AMPK drive memory precursor differentiation. Most memory precursor cells survive the contraction phase and gradually transition from effector memory to central memory cells. Terminal effectors undergo a stronger contraction and only generate effector memory cells.

Part III: Follicular helper T cells

B cell immune response

Subcapsular sinus macrophages in lymph nodes can capture soluble antigens and present them to follicular B cells (128). B cells can take up non-cognate antigens presented by macrophages in a non-antigen-specific manner through complement receptors and pass them to follicular dendritic cells (FDCs) (128). FDCs can prime B cells that recognize the antigens presented by them (128). Effective recognition of cognate antigens triggers the formation of immunological synapses and calcium signaling downstream of B cell receptors (BCRs) (129). In a T-cell-dependent B cell response, activated B cells up-regulate CCR7 and MHC-II and enhance their responsiveness to CD40 signaling (130). Activated B cells express CCR7 and EBI2, which guide activated B cells to the T:B cell zone border, where they meet cognate helper T cells (131-133).

Antibody class switching is a process that changes the isotype of an antibody, e.g. IgM to IgG. During class switching, germline transcription occurs in two selected switch regions, which recruits activation-induced cytidine deaminase (AID) that deaminates cytosines in switch regions (134). Deaminated loci recruit the DNA repair machinery that breaks the DNA in the switch regions and joins the two free ends through non-homologous end joining recombination. This irreversible process deletes the DNA sequence between the two switch regions and brings the exon encoding the constant region of the desired isotype to the end of exons encoding the variable region. AID is only expressed in

activated B cells. The initiation of class switching is believed to occur after the initial contact between activated B cells and their cognate helper T cells (135). Helper T cells not only signal through CD40L-CD40 interaction, but also secrete cytokines that determine the isotype that their cognate B cells switch to (129).

After the initial interaction between activated B cells and helper T cells, some B cells differentiate into plasma cells (129). The plasma cell fate is stabilized by the transcriptional repressor Blimp-1 (80). Other activated B cells maintain CXCR5 expression, down regulate EBI2, and migrate to follicle centers to start the germinal center (GC) reactions (136). Germinal center B cell (GC B cell) fate is stabilized by the transcriptional repressor Bcl-6 (80). The GC is compartmentalized into the light zone and the dark zone (137). The light zone is organized by a network formed by FDCs, and is positioned towards the antigen-rich region of secondary lymphoid organs: the marginal zone in spleen, or the subcapsular sinus in lymph nodes (138). GC B cells that undergo clonal expansion and somatic hypermutation in GC are called centroblasts, which form the dark zone of GCs (129). Somatic hypermutation is initiated by cytosine deamination in the hotspots of the variable region by AID (139). Cytosine deamination is followed by error-prone mismatch repair and base excision repair, which introduces point mutations in the variable region (139). The mutations introduced in the variable region may change the affinity or specificity of antibodies/BCRs. Therefore, somatic hypermuation is crucial for the affinity maturation of antibodies. Centroblasts exit the cell cycle, migrate to the light zone, and re-express surface BCRs, which turn them into centrocytes. Centrocytes in the light zone compete for antigens captured by FDCs. Only the centrocytes that

express high affinity BCRs can capture antigen, present it to cognate CD4 T cells, and receive T cell help. These centrocytes may then either migrate back to the dark zone to initiate a new round of proliferation or exit the GCs to become memory B cells or long-lived plasma cells. Centrocytes with ineffective or auto-reactive BCRs are deleted. These mechanisms allow GCs to continuously select high-affinity clones and export them as memory B cells and long-lived plasma cells (129). The migration of GC B cells between the dark zone and the light zone is governed by chemokines. The dark zone contains a higher amount of the CXCR4 ligand, CXCL12 (SDF-1), while the CXCR5 ligand, CXCL13, is more highly expressed in the light zone (140). Correspondingly, centroblasts express higher CXCR4, which positions them in the dark zone.

Follicular helper T cells

Follicular helper T cells (T_{FH} cells) are a subset of effector CD4 T cells that are found in B cell follicles. T_{FH} cells are indispensible for the GC reaction (141). T_{FH} cells were first identified in human tonsils as a subset of effector CD4 T cells that express CXCR5, ICOS, and CD40L and support antibody production by B cells (142-144) (Figure 1-2). CXCR5 is required to guide T_{FH} cells to B cell follicles, and CXCR5 deficiency in CD4 T cells impairs the GC response (143, 145, 146). Signals from T_{FH} cells are required for the initiation of GCs, and they promote the proliferation, survival, and affinity maturation of GC B cells. During the initiation of a GC reaction, T_{FH} cells stimulate Bcl-6 expression by B cells, and thus induce the commitment of B cells to the GC fate (141). Prior to GC formation, the up-regulation of Bcl-6 is also required for the maintenance of long-term

contacts between T_{FH} and B cells in the interfollicular zone (147, 148). During the GC reaction, antigen presentation to T_{FH} cells by GC B cells rather than BCR signaling seems to be the rate-limiting factor in the selection of GC B cells with high affinity BCRs (149). GC B cells that present MHC-peptide complexes to cognate T_{FH} cells receive survival and proliferation signals through proteins expressed by T_{FH} cells such as CD40L, IL-21, IL-4, and PD-1 (141). CD40L signals through CD40 expressed on B cells, promotes survival of GC B cells, and prevents GC B cells from differentiating to plasma cells (150-152). The absence of CD40 signaling results in termination of the GC reaction (153, 154). SAP is a signaling adaptor of SLAM family receptors. SAP is critical for the formation of sustained T_{FH} cell /GC B cell conjugates, and is required for SLAM dependent IL-4 secretion by T_{FH} cells (155-157). SAP deficiency results in the loss of the GC reaction and defective B cell memory (157). IL-4 secreted by GC T_{FH} cells prevents apoptosis of GC B cells by inducing Bcl-XL expression and enhancing glucose uptake in these cells (155, 158, 159). IL-21, another cytokine produced by T_{FH} cells, supports GC B cell proliferation through induction of Bcl-6 (160, 161). In addition, cytokines secreted by T_{FH} cells also guide the class switching of B cells. For example: IL-4 promotes switching to IgG1 and IgE, whereas IFN-γ favors the switching to IgG2a (162). T_{FH} cells express high levels of the inhibitory receptor PD-1, while GC B cells express its ligands, PD-L1 and PD-L2. Deficiency in PD-1 or its ligands leads to defective GC formation and enhanced apoptosis of GC B cells (163).

There has been debate over whether T_{FH} cells are a distinct lineage separate from Th1, Th2, Th17, and regulatory T (Treg) cells. Early observations that SAP deficiency in CD4

T cells blocks T cell help in the GC without affecting LCMV-specific Th1 responses suggested that T_{FH} cells may be an independently regulated population (157). The discovery that Bcl-6 functions as a master regulator of T_{FH} cells established T_{FH} cells as a distinct CD4 T cell lineage (164-166). Bcl-6 and Blimp-1 are mutually antagonistic transcription factors (167). Expression of Bcl-6 or Blimp-1 determines whether an activated CD4 T cell becomes a T_{FH} or non-T_{FH} effector (164, 167, 168). Interestingly, T-bet, the master regulator of Th1 differentiation, was shown to physically associate with Bcl-6 and recruit Bcl-6 to its binding loci, which may contribute to determining whether an effector CD4 T cell becomes a T_{FH} or Th1 cell (169). Although regulated by a distinct differentiation program, T_{FH} cells can produce some cytokines that were once considered as hallmarks of other effector subsets, such as IFN-γ, IL-4, and IL-17 (162, 170). T_{FH} cells secrete less IFN-γ than Th1 cells, probably as a result of lower T-bet expression (164). IL-4 was long considered a Th2 cytokine and a mediator of B cell help provided by CD4 T cells (171, 172). However, it is now known that GC T_{FH} cells also produce IL-4 (155, 162). Moreover, blocking the Th2 response shows little effect on the GC reaction (141). Now, it is clear that during helminth infections, IL-4-producing CD4 T cells in lymph nodes are mostly T_{FH} cells, while peripheral IL-4^+ CD4 T cells are Th2 cells (162). While Th2 cells produce both IL-4 and IL-13, T_{FH} cells produce only IL-4 (173).

T_{FH} cell differentiation is a multi-step process. Although T_{FH} cells are often associated with B cell responses, early T_{FH} cell development is independent of B cells (174, 175). The induction of CXCR5 and Bcl-6 expression in T_{FH} cells occurs as early as day 2 post-infection prior to their movement into the T:B cell zone border (147, 174). The

instruction of early T_{FH} cell development is instead provided by DCs (174, 175). DCs may signal through ICOSL-ICOS interaction or IL-6 to promote T_{FH} cell differentiation (174, 176). There are two populations of effector CD4 T cells generated early after LCMV infection: Bcl-6^{+}CXCR5^{+}CD25^{-}Blimp-1^{-} T_{FH} cells and Bcl-6^{-}CXCR5^{-} CD25^{+}Blimp-1^{+} non-T_{FH} cells (174). Differences in the strength of IL-2 signaling and subsequently differences in STAT5 activity have been shown to contribute to the divergence of T_{FH} cells and non-T_{FH} cells (177). After being primed by DCs, T_{FH} cells up-regulate Bcl-6 and CXCR5, down-regulate CCR7, and migrate to T:B cell zone border (146, 174, 178, 179). Migration of T_{FH} cells to the T:B cell zone border is independent of B cells (148). After T_{FH} cells are localized to the T:B cell zone border, their development becomes dependent on the interaction with B cells. T_{FH} cells and cognate B cells form long-lived interactions at the border and then move to the follicle interior to initiate GC reactions (148). Two populations of T_{FH} cells can be observed during GC reactions: T_{FH} cells and GC T_{FH} cells (141). GC T_{FH} cells are found in GCs, while other T_{FH} cells are localized in B cell follicles (146, 155). Although both populations are CXCR5^{+}PD-1^{+}ICOS^{+}Bcl-6^{+}, the expression of CXCR5 and Bcl-6 is higher in GC T_{FH} cells (146, 155). The transcriptional signatures of T_{FH} cells and GC T_{FH} cells are similar, but there are still genes that are differentially regulated between the two populations (155). For example, GC T_{FH} cells express higher levels of SAP, which is required for GC T_{FH} development (155). Moreover, partial loss of Bcl-6 function more severely impairs the ability of GC T_{FH} cells to enter GCs than follicles, indicating that the dosage of Bcl-6 may contribute to the divergence of T_{FH} cells and GC T_{FH} cells (147). In addition, c-Maf induces expression of IL-4 and IL-21 by GC T_{FH} cells, and deficiency of c-Maf impairs T_{FH} cell

differentiation (141, 180, 181). After antigen clearance and the collapse of the GC, Bcl-6 expression in antigen-specific CD4 T cells is lost and GC T_{FH} cells also disappear (182). Interestingly, a $CXCR5^{+}CCR7^{+}CD62L^{+}$ population can persist independent of GCs and is therefore called follicular helper-like central memory cells (182). During recall responses, follicular helper-like central memory cells produce higher frequencies of T_{FH} cells and GC T_{FH} cells than their counterparts, Th1 memory cells (182, 183). It remains to be determined how the cell fate of follicular helper-like central memory cells is maintained in the absence of Bcl-6 expression and GCs. Interestingly, the T-bet and Bcl-6 genes contain active histone marks in both T_{FH} cells and non-T_{FH} cells, indicating that both populations may be poised to differentiate into either lineage (184). Other factors, such as DNA methylation or additional transcription factors, may play a role in maintaining T_{FH} cell fates after the primary GC reaction.

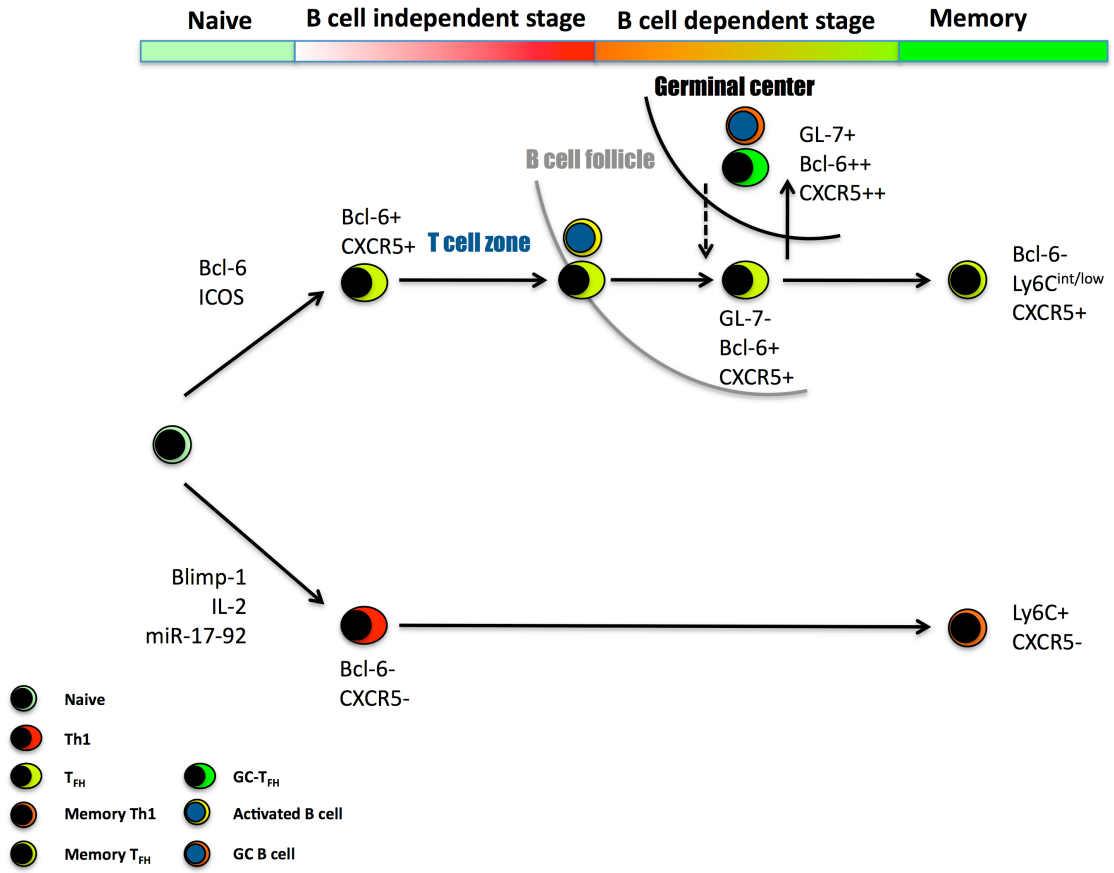

Figure 1- 2. CD4 T cell differentiation during viral infection.

After LCMV infection, naïve CD4 T cells are programmed into either T_{FH} (Bcl-6^{+}CXCR5^{+}) or Th1 (Bcl-6^{-}CXCR5^{-}) cells. ICOS signaling and Bcl-6 are essential for T_{FH} differentiation, while Blimp-1 and IL-2 signaling favor Th1 differentiation. In addition, our current study has shown that miR-17-92 preferentially expands Th1 cells. T_{FH} and Th1 cells diverge early after infection independent of B cells. T_{FH} cells later migrate to the T:B cell zone border to interact with cognate B cells. T_{FH} cells and activated B cells then migrate back to the B cell follicle to trigger the germinal center (GC) reaction. At this stage, a GC reaction is indispensible for T_{FH} cells. T_{FH} cells can further differentiate

into GC T_{FH} cells. After the primary immune response, memory T_{FH} and Th1 cells are generated from the corresponding effectors.

Part IV: microRNA

In 1993, Victor Ambros and colleagues found that small RNAs encoded by the *C. elegans* gene *lin-4* contain sequences complementary to regions in the 3' untranslated region (3'UTR) of the lin-14 mRNA (185). This pioneering work and numerous follow-up studies identified microRNAs (miRNAs) as a family of ~22-nucleotide (nt) small single-stranded RNAs that play a critical role in the post-transcriptional regulation of gene expression in animals and plants (186, 187). miRNAs silence genes by recognizing the complementary sequences in the 3'UTR of target mRNAs and recruit a silencing protein complex, which blocks protein expression and promotes mRNA degradation (188). There are ~800 miRNAs identified in humans. Each miRNA can target hundreds of mRNAs, and the 3'UTR of one mRNA may contain binding sites for multiple miRNAs (189).

miRNAs are transcribed by RNA polymerase II. The primary transcript containing a miRNA is called a pri-miRNA. Some pri-miRNAs contain a single miRNA, while the others such as miR-17-92 are grouped into clusters and transcribed together (188). miRNAs fall into three groups based on their location within a gene: 1) miRNAs in the exons of non-coding transcripts, 2) miRNAs in the introns of non-coding transcripts, and 3) miRNAs in the introns of protein-coding transcripts (190). The stem-loop structure containing the miRNA on the primary transcript is recognized and cleaved by the Microprocessor complex, which is comprised of the nuclear RNase III Drosha and co-factor DGCR8 (191-195). The stem-loop generated by this process is called a pre-

miRNA. Pre-miRNAs are then exported to the cytosol through the nuclear pore complexes, which is mediated by exportin-5 (196, 197). Upon arriving in the cytosol, pre-miRNAs are processed by the cytoplasmic RNase III Dicer into ~22-nt double-stranded RNAs (198-202). Typically, one strand of the duplex remains, while the other strand is degraded. The remaining strand is incorporated into the miRNA-containing RNA-induced silencing complex (miRISC), which contains the Argonaute protein (190). miRISC is recruited to the 3'UTR of target mRNAs recognized by the miRNA. Seed regions of 6-8 nt long at the 5' end of miRNAs appear to be the major determinant of target recognition by miRNAs (203). A miRISC inhibits gene expression by reducing protein translation and/or promoting uncapping and deadenylation of target mRNAs (204, 205).

microRNAs in the immune system

Recent studies have demonstrated that miRNAs play important roles in the development of immune cells and in immune responses. The importance of miRNAs in immune cell development was first demonstrated by studies in which enzymes critical to miRNA biogenesis, such as *Dicer,* were conditionally knocked out in T or B cells. Inactivation of *Dicer* in double negative thymocytes causes a drastic reduction in thymocyte numbers as a result of increased apoptosis (206). When *Dicer* is removed in double positive thymocytes, CD8 T cell development in the periphery is blocked (207). Although the defect in the development of *Dicer* deficient CD4 T cells is milder, the effector differentiation of these cells is aberrant. *Dicer* deficiency also compromises the

homeostasis and function of regulatory T cells (Tregs), which results in lethal inflammation (208). Experiments in Chapter 2 and a similar study from another group have demonstrated that loss of *Dicer* in mature CD8 T cells results in a defective CD8 T cell immune response and a failure to control pathogens (209, 210). Knocking out *Dicer* in B cells blocks B cell development partially due to increased apoptosis (211). These pioneering studies have demonstrated that miRNAs are indispensible for the development and function of the immune system.

Following the discovery that miRNA deficiency affects almost every compartment of the immune system, studies have focused on the identification of specific miRNAs involved in the differentiation and/or function of various immune cells. For example, the aberrant Th1 differentiation in Dicer deficient mice is mostly caused by the loss of miR-29, which suppresses the expression of T-bet, the master regulator of Th1 differentiation (212). miR-181a, which is highly expressed in thymocytes, augments TCR signaling by repressing the expression of multiple phosphatases, and thus influences T cell selection in the thymus (213). Besides T cell development, miR-181a also participates in NKT cell development. miR-181a deficient mice fail to develop NKT cells due to impaired PI3K signaling (214). miR-182, whose expression is induced by IL-2 following T cell activation, reduces the expression of the anti-proliferative protein FOXO1, and enhances the clonal expansion of effector CD4 T cells (215). miR-155 was first noted as one of several miRNAs induced by TLR signaling (216). As a positive regulator of TLR signaling, miR-155 activates AKT and IFN response genes by suppressing the expression of SOCS1 and SHIP1 (217, 218). Over-expression of miR-155 causes unchecked

proliferation of the myeloid compartment, which resembles chronic inflammation and some hematopoietic malignancies (216). It was later found that miR-155 is also up-regulated in activated B and T cells (203). Gain- and loss-of-function approaches have demonstrated that miR-155 is indispensible for GC reactions, class switching, and antibody secretion (219, 220). miR-155 also promotes CD8 T cell immune responses by suppressing the anti-proliferative effect of type I interferon signaling (221). In addition, miR-155 enhances Treg cell proliferation stimulated by IL-2 through suppressing SOCS1, which inhibits IL-2 induced STAT5 activity (222). Another miRNA highly expressed by Treg cells, miR-146a, targets STAT1 and is indispensable for the suppressor function of Treg cells (223). In addition to its role in Treg cells, miR-146a also functions as a suppressor of effector T cell responses by acting as a negative feedback signal for NF-κB activity and repressing the expression of TRAF6 and IRAK1 (224). Like miR-155, miR-146a is also induced by TLR signaling (225). However, unlike miR-155, miR-146a dampens the innate immune response by suppressing NF-κB activity (226). Similar to miR-146a, miR-21 is up-regulated by TLR signaling, and suppresses NF-κB activity by targeting PDCD4, a pro-inflammatory protein (227). Thus, miR-21 promotes IL-10 production and represses IL-6 production of cells activated by LPS, resulting in the inhibition of the inflammatory response. miR-21 is also markedly induced after T cell activation and suppresses apoptosis of activated T cells (228). Besides miR-146a, miR-10a is also expressed in Treg cells. It is induced by TGF-β and retinoic acid and constrains Treg cells from developing into other helper cell lineages by targeting Bcl-6 (229). Various autoimmune diseases show unique miRNA signatures. For example, the expression of miR-326 positively correlates with the disease severity of multiple sclerosis.

The mechanism seems to involve the ability of miR-326 to promote the generation of pathogenic Th17 cells by targeting Ets-1, which suppresses Th17 differentiation (230).

The miR-17-92 cluster

miR-17-92 is a cluster of six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a) localized in an intron of the host gene known as *C13orf25* (231, 232). The sequence encoding this cluster is highly conserved across all vertebrates, while the exonic sequences of the host gene do not seem to be conserved. Therefore, it is speculated that the sole function of the host gene is to carry this miRNA cluster. The six mature miRNAs in the miR-17-92 cluster can be categorized into four families based on the sequences of their seed regions: the miR-17 family (miR-17 and miR-20), the miR-18 family (miR-18a), the miR-19 family (miR-19a and miR-19b), and the miR-92 family (miR-92a) (231). The six miRNAs encoded in the miR-17-92 cluster are transcribed together in a primary transcript and function independently after being processed into mature miRNAs. The cluster structure raises the possibility that different target transcripts may interact with a combination of several members in the cluster with different affinities. This would further increase the complexity of miRNA:mRNA interaction, and expand the dynamic range of miR-17-92-mediated silencing effects (233).

There are two paralogs of miR-17-92 in mammals as a result of genomic duplications: miR-106a-363 and miR-106b-25, each containing a cluster of miRNAs homologous to a subset of members of the miR-17-92 cluster. This implies that miRNAs from different

clusters may have overlapping sets of target transcripts and that the existence of three homologous miRNA clusters may provide functional redundancy (234). Both miR-17-92 and miR-106b-25 are widely expressed across different cell types, while the expression of miR-106a-363 in the tissues tested so far is extremely low (234). Therefore, it is not surprising that *miR-106a-363* knockout mice do not have any developmental defect. However, knocking out *miR-106b-25* alone or in combination with *miR-106a-363* does not cause an overt defect (234). Only *miR-17-92* deficiency results in defective embryonic development, manifested by smaller embryo sizes compared to wildtype controls (234). *miR-17-92* knockout mice die shortly after birth due to defects in their lungs and hearts. Embryos deficient in both *miR-17-92* and *miR-106b-25* show more severe defects than *miR-17-92* deficient embryos and die at midgestation.

The miR-17-92 signaling network

The miR-17-92 cluster first attracted research interest as a potential oncogenic microRNA cluster. 13q31.3, the human genomic locus where miR-17-92 is located, is amplified in different hematopoietic malignancies (232). The minimal amplicon contains the region encoding miR-17-92. A correlation between tumorigenesis and miR-17-92 is further solidified by studies showing that miRNAs in this cluster are over-expressed in various tumors (235, 236). Furthermore, large-scale screenings for oncogenes through ectopic gene activation mediated by retroviral insertion have shown that the locus containing miR-17-92 is frequently inserted in several types of retrovirally-induced leukemias (237, 238). A causal relationship between miR-17-92 and oncogenesis is

provided by a study from the Hannon group (239). This group used a mouse model in which *c-Myc* expression is driven by the immunoglobulin heavy chain enhancer to study B cell lymphomagenesis. They have demonstrated that over-expression of the miR-17-92 cluster in this model accelerates tumor development, reduces apoptosis of tumor cells, and increases lymphoma invasion. Following this study, the oncogenic activity of miR-17-92 has been confirmed in various other cancer models (240-242).

E2F transcription factors are essential for the expression of the S phase specific genes and the progression from G1 to S phase (243). Studies have shown that E2F1 and E2F3 can directly bind to the promoter of miR-17-92 and transactivate this gene (244, 245) (Figure 1-3). This implies that the expression of miR-17-92 may have a periodic nature as cells progress through the cell cycle. p53 has also been shown to repress miR-17-92 expression (246), further suggesting that miR-17-92 is integrated into the regulatory network of cell cycle progression. In addition, oncogenes such as c-Myc and N-Myc directly transactivate miR-17-92 (243, 247). It is now known that there are additional regulatory mechanisms besides transcriptional regulation that control miR-17-92 expression. For example, VEGF selectively increases the expression of miR-17, miR-18 and miR-20 in this cluster (248). Moreover, RNA-binding protein hnRNP A1 has been shown to be required for the generation of miR-18a but not other members of miR-17-92 (249). These findings indicate that miRNAs within the miR-17-92 cluster can be differentially expressed due to post-transcriptional mechanisms and that miR-17-92 may function differently in different cell types and under different physiological conditions.

miR-17-92 represses the expression of several tumor suppressor genes. Multiple miRNAs in the miR-17-92 cluster target the transcript of the pro-apoptotic gene *Bim*, which may contribute to the repression of c-Myc induced apoptosis by miR-17-92 (234, 239). The repression of Bim by miR-17-92 may also play a role in TGF-β resistance in cancer (250). In addition to supporting the survival of cancer cells, miR-17-92 also increases their proliferation. miR-17 and miR-20a promote cell cycle progression by reducing cellular levels of CDKN1A (p21), an important inhibitor of the G1-S transition (250, 251). Through this mechanism, miR-17-92 helps cells bypass the cell cycle arrest caused by DNA damage or TGF-β signaling. Another prominent target of miR-17-92 is PTEN (252). Multiple members of this cluster recognize the 3'UTR of *Pten*, and inhibit PTEN-mediated apoptosis of cancer cells (242, 252). miR-17-92 also enhances the angiogenic effect of c-Myc through targeting TSP-1 and CTGF (241).

miR-17-92 in the immune system

Ablation of *Dicer* causes a marked reduction in the number of pre-B cells in mice, as a result of increased apoptosis (211). A further analysis has shown that transcripts with a 3'UTR containing the binding sites for miRNAs of the miR-17-92 cluster are up-regulated in *Dicer* deficient pro-B cells, which indicates that the loss of miR-17-92 may cause the phenotype seen in *Dicer* knockout cells. Indeed, the development of pro-B cells to pre-B cells and more mature B cells is defective in *miR-17-92* deficient mice due to the marked increase in apoptosis of pro-B cells (234). This is caused by the derepression of Bim as a result of *miR-17-92* deficiency. Another group engineered a transgenic mouse

strain that over-expresses miR-17-92 in T and B cells (253). They found that these transgenic mice died prematurely due to lymphoproliferative autoimmune disease. Lymphocytes in these mice show a predominately activated phenotype and display markedly increased proliferation and decreased activation-induced cell death. This phenotype can be partially explained by the dysregulation of Bim and PTEN: mice that are heterozygous for both *Bim* and *Pten* demonstrate a phenotype similar to that observed in transgenic mice that over-express miR-17-92 (253). In this dissertation, I have shown that miR-17-92 represses PTEN and PD-1 expression in T cells and promotes terminal differentiation of effector CD8 T cells and the Th1 response during LCMV infection.

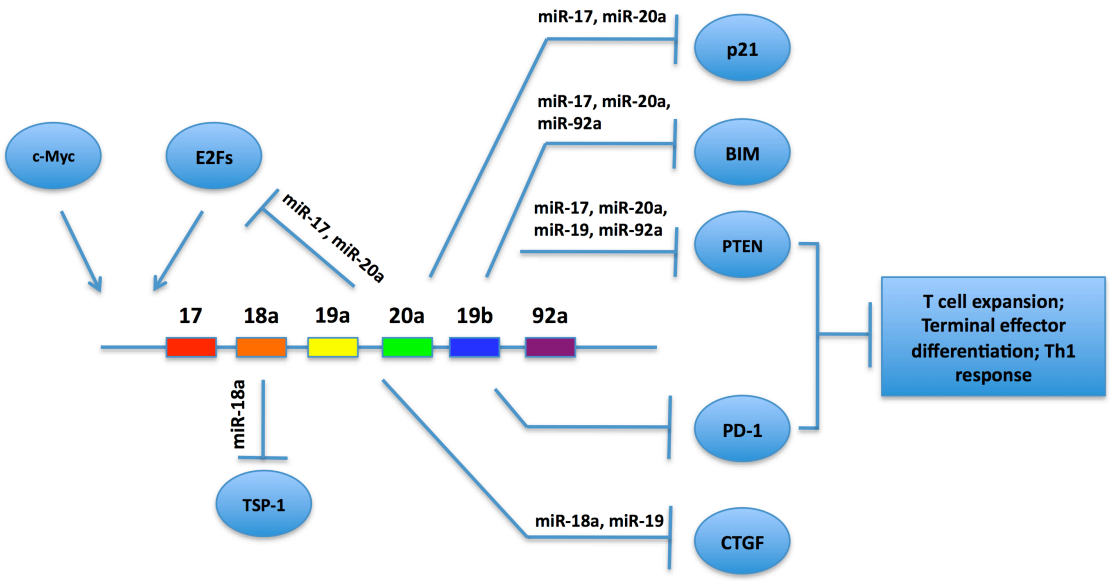

Figure 1- 3. The miR-17-92 pathway.

The miR-17-92 cluster encodes 6 mature miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a). Transcription of the cluster is initiated by c-Myc and by E2F family transcription factors. Members of the miR-17-92 cluster promote proliferation by repressing p21 and PTEN, and enhance cell survival by reducing BIM expression. miR-17-92 also represses E2F expression as a negative feedback mechanism. Moreover, miR-17-92 enhances angiogenesis by targeting TSP-1 and CTGF. Our current study has shown that in LCMV-specific effector CD8 T cells, miR-17-92 represses PTEN and PD1 levels and drives their terminal differentiation. miR-17-92 also enhances the Th1 response during viral infection.

Part V: Significance

T cells play an important role in protecting the host against various infections and cancers. Vaccines whose protection is mediated by memory T cells have been successfully developed (254). Understanding the mechanisms of memory T cell development may help in the development of future T cell-based vaccines or in improving the efficacy of current vaccines by leading to strategies for optimizing the quality of memory T cells. Moreover, understanding the differentiation status of antigen-specific T cells in patients with chronic infections or cancer is necessary for the design of strategies that can elicit effective T cell immune responses by therapeutic vaccination. Immunotherapies that employ the adoptive transfer of *in vitro* activated T cells or genetically engineered T cells have shown promising results in cancer treatment (255, 256). Chronic infection and cancer usually induce an immunosuppressive microenvironment that results in T cell dysfunction. Several immunotherapies that aim to improve T cell effector functions by blocking immunosuppressive signals through antibody treatment have proved to be successful in treating cancers and chronic infections (257-259). A better understanding of the positive and negative signals that regulate the effector functions of T cells will help to develop more effective immunotherapies for treatment of cancer and infectious diseases. Moreover, knowledge of the signals required for the differentiation of certain effector T cell lineages may help us to harness the immune system to confer better protection against pathogens. For example, a stronger T_{FH} response may be beneficial for HIV vaccines or immunotherapies (260).

In the last decades, extensive effort has been spent to study the transcriptomes of different lineages of effector and memory T cells and understand how transcription factors regulate their differentiation. Less attention has been paid to the post-transcriptional mechanisms involved in T cell differentiation, especially in the context of antiviral immune responses. miRNAs are major players of post-transcriptional regulation. As discussed in the previous sections of this chapter, miRNAs have been shown to regulate the differentiation of various immune cells. However, how the differentiation of virus-specific effector and memory T cells is regulated by miRNAs still demands more comprehensive studies. In chapter 2 and 3, I will describe how the miR-17-92 cluster regulates the virus-specific CD4- and CD8-T cell immune responses. Knowledge obtained from these studies will not only help us understand the basic molecular mechanisms of antiviral immune responses but also have significant clinical implications. For example, miRNA mimics that ectopically introduce miRNA activity and miRNA antagonists that repress miRNA activity have potential clinical applications (261, 262).

Chapter 2: Temporal expression of microRNA cluster miR-17-92 regulates effector and memory CD8$^+$ T-cell differentiation

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Author contributions: Tuoqi Wu and Rafi Ahmed designed research; Tuoqi Wu and Andreas Wieland performed research; Tuoqi Wu, Koichi Araki, and Carl W. Davis contributed new reagents/analytic tools; Tuoqi Wu, Lilin Ye, and Rafi Ahmed analyzed data; and Tuoqi Wu, J. Scott Hale, and Rafi Ahmed wrote the paper.

Abstract

MicroRNAs are important regulators of various developmental and physiological processes. However, their roles in the $CD8^+$ T-cell response are not well understood. Using an acute viral infection model, we show that microRNAs of the miR-17-92 cluster are strongly induced after T-cell activation, down-regulated after clonal expansion, and further silenced during memory development. miR-17-92 promotes cell-cycle progression of effector $CD8^+$ T cells, and its expression is critical to the rapid expansion of these cells. However, excessive miR-17-92 expression enhances mammalian target of rapamycin (mTOR) signaling and strongly skews the differentiation toward short-lived terminal effector cells. Failure to down-regulate miR-17-92 leads to a gradual loss of memory cells and defective central memory cell development. Therefore, our results reveal a temporal expression pattern of miR-17-92 by antigen-specific $CD8^+$ T cells during viral infection, the precise control of which is critical to the effector expansion and memory differentiation of $CD8^+$ T cells.

Introduction

$CD8^+$ T cells play a pivotal role in the control of numerous intracellular infections and malignancies. Upon antigen encounter, a program triggers the few antigen-specific naïve precursor cells to undergo extensive proliferation and differentiate into effector cells, which are able to produce cytokines and cytolytic proteins (13, 50, 263). In an acute

infection, antigen clearance is followed by a contraction phase during which the majority of effector $CD8^+$ T cells undergo apoptosis. However, a small fraction of effector cells manage to survive through this phase and gradually differentiate into memory cells, which are capable of long-term self-renewal and rapid response to antigen reencounter (50, 263).

It is now well established in several different infection models that effector $CD8^+$ T cells comprise a heterogeneous population consisting of at least two subsets: (*i*) CD127(IL-7Rα)^{high} killer cell lectin-like receptor G1 (KLRG1)^{low} memory precursor cells, which are more likely to survive the contraction phase and differentiate into memory cells and (*ii*) CD127^{low}KLRG1^{high} terminal effectors, which are short-lived, more terminally differentiated, and lack the capacity for antigen-independent homeostatic proliferation (57-59). Additional features such as high expression of CD27 as well as rapid reexpression of CD62L can also be used to distinguish memory precursors from terminal effectors (50, 59). Memory cells are also considered to be heterogeneous, consisting of central memory T cells (T_{CM} cells) and effector memory T cells (T_{EM} cells) (103). Central memory T cells, which express higher levels of lymph node homing receptors (e.g., CD62L and CCR7) and have better homeostatic turnover, gradually dominate in the lymphoid organs, whereas effector memory T cells preferentially reside in the peripheral organs (264). High expression of CD27, secretion of IL-2 upon restimulation, and greater proliferation potential upon antigen reencounter are also hallmarks of central memory T cells.

T-cell differentiation is regulated by an orchestration of T-cell receptor (TCR), costimulatory, and cytokine signals and is further stabilized by lineage-specific transcription factors in response to these signals (13, 50, 265). It was recently shown that microRNA (miRNA) is also a major regulator of the T-cell immune response (203). miRNAs are small noncoding RNAs consisting of ~22 nt that bind to the 3′ UTR of the target mRNA and suppress the expression of the encoded protein by blocking translation as well as promoting degradation of the transcript (188). Experiments using mice deficient in enzymes critical to miRNA biogenesis have demonstrated an indispensable role of miRNAs in T-cell development (207). A recent study has shown that Dicer, an enzyme involved in miRNA synthesis, is indispensable for $CD8^+$ T-cell responses (210). However, less is known about the specific miRNAs regulating effector and memory $CD8^+$ T-cell differentiation in the context of viral infection.

In this study, we profiled the miRNA expression of naïve, effector, and memory $CD8^+$ T cells by using the mouse model of lymphocytic choriomeningitis virus (LCMV) infection and demonstrated that multiple miRNAs in the miR-17-92 cluster and its paralogs are highly expressed in proliferating effector cells. We then showed that miR-17-92 is critical to maintain a proliferative and terminally differentiated effector state and that down-regulation of the cluster after viral clearance is necessary for $CD8^+$ T cells to transit into the quiescent memory phenotype.

Results

miRNAs Are Crucial for CD8$^+$ Effector T-Cell Expansion During Acute Viral Infection. We used Dicer conditional knockout mice to examine the role of miRNAs in regulating the effector CD8$^+$ T-cell response to an acute viral infection. To avoid defective thymic T-cell development caused by Dicer deficiency, we crossed mice bearing floxed *Dicer* alleles (*Dicer* loxP/loxP) to a transgenic strain expressing Cre recombinase driven by a truncated human granzyme B promoter (GzB-cre) (54), which is only active among mature T cells activated by TCR signal. In this study, *Dicer* loxP/loxP;GzB-cre$^+$ (*Dicer*$^{-/-}$) mice were compared with their littermate controls (*Dicer* loxP/loxP;GzB-cre$^-$ or *Dicer* loxP/wt;GzB-cre$^+$). Although *Dicer*$^{-/-}$ mice had normal T-cell compartments before infection (Figure 2-S1*A*), they mounted a severely dampened CD8$^+$ T-cell response compared with littermate controls (Figure 2-S1*B*) on day 8 postinfection (p.i.) with LCMV Armstrong strain (Arm). The overall numbers of LCMV-specific CD8$^+$ T cells for the two main epitopes, $D^bGP_{33-41}$and $D^bNP_{396–404}$, were ~20-fold lower in the spleens of *Dicer*$^{-/-}$ mice than in those of the littermate controls, as determined by both tetramer staining (Figure 2-S1*C*) and intracellular IFN-γ staining (Figure 2-S1*D*). Accordingly, the frequency and total number of CD44^{high} effector CD8$^+$ T cells were also significantly reduced in the knockout mice (Figure 2-S1 *E* and *F*). Moreover, the defective expansion of *Dicer*$^{-/-}$ effector CD8$^+$ T cells was accompanied with impaired viral clearance (Figure 2-S1*G*). Therefore, our results in the LCMV infection model are consistent with the previous observation that Dicer is essential for CD8$^+$ T-cell responses during *Listeria monocytogenes* and vesicular stomatitis virus

infections (210).

miR-17-92 Cluster and Its Paralogs Are Up-Regulated in Expanding Effector CD8$^+$ T Cells. We next sought to identify the miRNAs whose loss of function could account for the defective CD8$^+$ T-cell expansion observed in *Dicer*$^{-/-}$ mice by profiling miRNA expression in LCMV-specific CD8$^+$ T cells at different stages of the immune response. We used the P14 TCR transgenic system (TCR specific to $D^bGP_{33-41}$of LCMV) (5) and sorted naïve, day 5 effector, day 8 effector, and memory (day >60) P14 cells. Effector CD8$^+$ T cells are rapidly proliferating on day 5 p.i., and their number reaches the peak on day 8 p.i., when proliferation largely stops (Figure 2-S2*A*). Therefore, miRNAs more highly expressed in day 5 effectors than in the other three populations are more likely to play a role in clonal expansion. Unsupervised hierarchical analysis successfully segregated the four groups representing four different stages of LCMV-specific CD8$^+$ T-cell differentiation (Figure 2-S2*B*). A one-way ANOVA analysis identified 160 miRNAs that were differentially regulated among the four populations (Dataset S1). miRNAs that were upregulated in day 5 effectors by more than twofold ($P < 0.05$) relative to naïve cells are shown in Figure 2-S2*C*. Remarkably, multiple miRNAs in the miR-17-92 cluster and its paralogs, namely miR-106a-363 and miR-106b-25, were up-regulated on day 5 p.i. but down-regulated during the differentiation from effector to memory T cells. We identified 10 miRNAs up-regulated (fold change ≥ 2; $P < 0.05$) in day 5 effectors relative to all three other populations (Figure 2-1*A*). Strikingly, six of those miRNAs belong to the miR-17-92 or miR-106a-363 cluster, suggesting a potential role of these miRNAs in

the expansion phase (Figure 2-1*B*). The expression kinetics of individual members in the miR-17-92 cluster were confirmed by quantitative RT-PCR (QRT-PCR) (Figure 2-1*C*).

miR-17-92 Deficiency Impairs Effector CD8$^+$ T-Cell Proliferation. The observation that expression of miRNAs in the miR-17-92 cluster positively correlates with the proliferation of effector CD8$^+$ T cells prompted us to speculate that miR-17-92 may promote CD8$^+$ T-cell expansion during the immune response. We bred *miR-17-92* loxP/loxP mutants (234) to GzB-cre transgenic mice to generate *miR-17-92* loxP/loxP;GzB-cre (*miR-17-92*$^{-/-}$) mice. Although a previous study showed that conventional miR-17-92 knockout mice had normal T-cell development (234), we still confirmed in our system that the *miR-17-92*$^{-/-}$ mice showed no obvious defect in the T-cell compartment (Figure 2-S3*A*). We infected *miR-17-92*$^{-/-}$ mice and their littermate controls (*miR-17-92* loxP/loxP or *miR-17-92* loxP/loxP;GzB-cre) with LCMV Arm. The frequency and number of effector CD8$^+$ T cells were examined on day 8 p.i. As predicted, both D^{b}GP$_{33\text{-}41}$and D^{b}NP$_{396–404}$ tetramer$^+$ CD8$^+$ T-cell frequencies in the spleens of *miR-17-92*$^{-/-}$ mice were lower than those of the littermate controls (Figure 2-S3*B*). The total numbers of D^{b}GP$_{33–41}$- or D^{b}NP$_{396–404}$-specific cells, determined by either tetramer staining or intracellular IFN-γ staining after peptide stimulation, were three- to fourfold lower in *miR-17-92*$^{-/-}$ mice than in the littermate controls (Figure 2-2 *A* and *B*). Also, fewer activated CD44^{high} CD8$^+$ T cells were found in the knockout mice (Figure 2-S3 *C* and *D*). *miR-17-92*$^{-/-}$ mice had slightly higher frequencies of CD127^{high}KLRG1^{low} (memory precursor) effector CD8$^+$ T cells, although not statistically significant, than their littermate controls on day 8 p.i. (Figure 2-S3 *E* and *F*). The trend toward higher

frequencies of $CD127^{high}KLRG1^{low}$ LCMV-specific $CD8^+$ T cells was also observed in the knockout mice on day 91 p.i. (Figure 2-S3 *G* and *H*). The diminished expansion of effector $CD8^+$ T cells observed in the *miR-17-92*$^{-/-}$ mice is likely to be caused by impaired proliferation. After stimulated for 48 h with anti-CD3 and anti-CD28 antibodies in vitro, $CD8^+$ T cells from *miR-17-92* loxP/loxP;CD4-cre (CD4-cre *miR-17-92*$^{-/-}$) mice proliferated less than those from their littermate controls (*miR-17-92* loxP/loxP or *miR-17-92* loxP/loxP;CD4-cre) (Figure 2-2*C*) despite similar expression of TCR, CD3ε, and CD28 (Figure 2-S3*I*). Our results indicate that miR-17-92 is necessary for optimal proliferation of $CD8^+$ effector T cells.

Overexpression of miR-17-92 Promotes Effector $CD8^+$ T-Cell Expansion. To test whether miR-17-92 has an effect on cell-cycle progression of effector $CD8^+$ T cells, we overexpressed miR-17-92 in P14 $CD8^+$ T cells. P14 cells were infected by retrovirus packaged with MSCV-IRES-Thy1.1 (MIT) vector with or without a miR-17-92 insert. After infection, all P14 cells, both transduced ($Thy1.1^+$) and nontransduced ($Thy1.1^-$), were transferred into C57BL/6 recipients, which were subsequently infected with LCMV (Figure 2-3*A*). This procedure allows us to determine the effect of a vector by directly comparing transduced to nontransduced cells within the same mouse. Thus, any environmental factors can be ruled out, and better sensitivity is achieved. BrdU was i.p. injected into mice on day 6 or 7 after LCMV infection; 6 h later, mice were killed, and cells were checked for BrdU incorporation. Although the transduction with MIT empty vector showed little effect on the $BrdU^+$ frequency, the transduction with miR-17-92-MIT

clearly increased BrdU incorporation in the P14 cells (Figure 2-3*B*). Therefore, increasing the expression of miR-17-92 promotes cell-cycle progression of effector $CD8^+$ T cells. To test whether miR-17-92 overexpression enhances clonal expansion, we transduced purified P14 $CD8^+$ cells with MSCV-Puro-IRES-GFP (MSCV-PIG) vector with or without a miR-17-92 insert, cultured the cells with IL-2 for 2–3 d, sorted for GFP^+-transduced cells, and adoptively transferred the GFP^+ T cells to C57BL/6 recipients (Figure 2-S4 *B* and *C*). The chimeras were subsequently infected with LCMV and killed on day 5 p.i. As shown in Figure 2-S4 *D* and *E*, the P14 cells transduced with the miR-17-92 overexpression vector accumulated approximately threefold more on day 5 p.i. than the P14 cells transduced with the empty MSCV-PIG.

Overexpression of miR-17-92 Skews Effector $CD8^+$ T Cells to CD127 (IL-7Rα)^{low}KLRG1^{high} Terminal Effectors. As described above, the expression of miRNAs in the miR-17-92 cluster peaks when the $CD8^+$ T cells are rapidly proliferating, decreases by day 8 p.i. when the proliferation nearly stops, and further decreases during memory development (Figure 2-1 *B* and *C*). Therefore, we reasoned that maintaining the high expression of miR-17-92 by overexpression might affect the differentiation of LCMV-specific effector and memory $CD8^+$ T cells. We first examined the impact of miR-17-92 overexpression on effector $CD8^+$ T-cell differentiation by comparing the day 8 P14 cells transduced with miR-17-92-MIT to the nontransduced P14 cells in the same mice as well as the empty MIT-transduced P14 cells. The nontransduced P14 cells in both the MIT and miR-17-92-MIT groups, as well as the empty MIT-transduced P14 cells, showed similar expression patterns of CD127 and KLRG1 (Figure 2-4*A* and Figure 2-S5 *A* and

B). In striking contrast, the P14 cells transduced with miR-17-92-MIT were almost exclusively $CD127^{low}KLRG1^{high}$, a pattern associated with short-lived terminal effector cells (57, 59). Moreover, miR-17-92-MIT–transduced P14 cells also expressed lower levels of CD62L (L-selectin), CD27, and Bcl2, but higher levels of granzyme B, all consistent with a more terminally differentiated effector phenotype (Figure 2-4*B* and Figure 2-S5 *C–E*). Interestingly, miR-17-92-MIT–transduced P14 cells also showed heightened expression of 2B4 (Figure 2-4*C* and Figure 2-S5*F*), which is highly expressed in exhausted $CD8^+$ T cells (266). In addition, consistent with a previous report that miR-19 directly targets TNF-α mRNA (267), the TNF-α production after restimulation by $GP_{33-41}$was lower with miR-17-92 overexpression (Figure 2-S5*G*).

We examined the mRNA profiles of miR-17-92-MIT–transduced and nontransduced P14 cells by microarray analysis. Student's *t* test identified 350 probes down-regulated by more than 1.5-fold ($P < 0.05$) and 546 probes up-regulated by more than 1.5-fold ($P < 0.05$) in the transduced P14 cells relative to the nontransduced P14 cells (Dataset S2). Consistent with our FACS data, Il7r, Sell (CD62L), and Bcl2 were lower and Cd244 (2B4) was higher at the transcript level in the cells transduced with miR-17-92-MIT (Figure 2-4*D*). Notably, Tcf7, a transcription factor essential for central memory T-cell development (86), as well as Traf1 and serpina3g, which facilitate memory $CD8^+$ T-cell survival by suppressing Bim or cathepsin B, respectively (268, 269), were also down-regulated when miR-17-92 was overexpressed. To determine whether the overall gene expression pattern of miR-17-92–overexpressing cells resembles that of terminal effector cells, we compared our data with published microarray data of terminal effector cells and

memory precursors (57). Geneset enrichment analysis (GSEA) showed that the gene signature of $CD127^{low}$ effectors was overrepresented in the miR-17-92- MIT–transduced P14 cells whereas the gene signature of $CD127^{high}$ effectors was underrepresented in these cells (Figure 2-4*E* and Figure 2-S5*H*).

miR-17-92 Enhances Mammalian Target of Rapamycin (mTOR) Signaling in Effector $CD8^+$ T Cells. Our previous work demonstrated that reducing mTOR signaling in effector $CD8^+$ T cells favors their differentiation into memory precursors and increases the generation of central memory T cells (49). Interestingly, our microarray data showed that the transcripts of multiple negative regulators of the PI3K–Akt–mTOR axis, namely phosphatase and tensin homolog (Pten), programmed cell death 1 (Pdcd1; PD1), B- and T-lymphocyte associated (Btla), and Fc fragment of IgG, low affinity IIb, receptor (Fcgr2b), were significantly lower in the miR-17-92-MIT–transduced P14 cells (Figure 2-5*A*). The results of Western blots and FACS confirmed that the protein levels of PTEN, PD1, and BTLA were lowered by miR-17-92 overexpression on day 4.5 p.i. (Figure 2-5 *B* and *C*), when mTOR signaling is high. Notably, the 3′ UTR of Pten mRNA contains target sites for five of the six miRNAs in the cluster (miR-17, miR-19a, miR-19b, miR-20a, and miR-92a), suggesting that miR-17-92 can directly suppress PTEN expression by interacting with its mRNA (253, 270). To determine mTOR pathway activity in the P14 cells, we stained for the phosphorylated ribosome protein S6 (Ser235/236) on day 4.5 p.i. As shown in Figure 2-5*D*, miR-17-92 overexpression increased the phosphorylation of S6, indicating heightened mTOR signaling. Therefore, miR-17-92 relieves the suppression on the PI3K–Akt–mTOR axis and enhances mTOR activity. Strengthened

mTOR signaling by miR-17-92 overexpression may explain the absence of memory precursor cells and indicates a potential defect in memory differentiation.

Down-Regulation of miR-17-92 Is Necessary for Optimal Memory CD8$^+$ T-Cell Development. Normally, during the CD8$^+$ T-cell response to acute LCMV infection, the proportions of CD127^{high} and CD62L^{high} cells gradually increase after day 8 during the contraction and memory development. Meanwhile, the expression of miR-17-92 decreases to a level similar to that in naïve T cells (Figure 2-1 *B* and *C*). However, when ectopically overexpressing miR-17-92, the reexpression of CD127 and CD62L in the P14 cells was strongly delayed, suggesting a defective memory differentiation program (Figure 2-6 *A* and *B*). As a result, although the frequency of the empty MIT-transduced cells within the donor P14 cell pool was largely unchanged over time, the miR-17-92-MIT–transduced P14 cells were outcompeted by the nontransduced cells in the same mice (Figure 2-6*C*). The remaining miR-17-92-MIT–transduced cells displayed a phenotype closer to what seen in effector T cells or effector memory T cells: The majority of the cells were CD127^{low}KLRG1^{high} with limited expression of CD62L and high levels of granzyme B (Figure 2-6 *D–F* and Figure 2-S6 *A–E*). The impaired central memory T-cell development was further supported by the observation that the miR-17-92-MIT–transduced memory P14 cells expressed lower CD27 and Bcl2 and produced less IL-2 upon restimulation than the control P14 cells did (Figure 2-6 *G–I* and Figure 2-S6 *F–H*).

Discussion

In this study, we determined the miRNA profiles of LCMV specific $CD8^+$ T cells during and after an acute viral infection and demonstrated that a group of miRNAs, predominantly members of the miR-17-92 cluster or its paralogs, are more expressed in rapidly proliferating effectors than in naïve, memory, or nonproliferating effector cells. The miR-17-92 cluster encodes precursors for six miRNAs (miR-17, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92a) and has two paralogs (miR-106b-25 and miR-106a-363) generated by ancient genomic duplication (231). miR-17-92 is frequently involved in genomic translocation and amplification and is overexpressed in various hematopoietic malignancies and solid tumors (231). Studies on $CD4^+$ T cells showed that overexpressing the miR-17-92 cluster overrides the need for costimulatory signals (253) and that several miRNAs in the cluster can enhance proliferation and inhibit activation-induced cell death of T-helper cells after in vitro antigen stimulation (212, 253, 271). Accordingly, using the in vivo LCMV acute infection model, we showed that knocking out miR-17-92 with GzB-cre reduced the number of LCMV-specific $CD8^+$ T cells and thus demonstrated that the loss of miR-17-92 at least partially accounts for the phenotype observed in Dicer knockout mice. In addition to the loss-of-function experiments, we found that overexpressing miR-17-92 promotes the expansion of effector cells. Altogether, our data reveal a proproliferative role of miR-17-92 in effector $CD8^+$ T cells. Although the effector $CD8^+$ T-cell response to LCMV infection is largely independent of $CD4^+$ T-cell help (272), it is worth pointing out that GzB-cre can also induce recombination in effector $CD4^+$ T cells that express granzyme B and potentially delete

miR-17-92 in this subset of $CD4^+$ T cells (14). Whether this process has any effect on the differentiation of memory $CD8^+$ T cells needs to be determined in future studies.

Our data demonstrated that miR-17-92 expression is downregulated when clonal expansion approaches the end and further reduced to levels seen in naïve cells during the contraction phase. Given the prosurvival role of miR-17-92 in malignancies as well as primary lymphocytes in the autoimmune model (252, 253), one might have predicted that maintaining high levels of miR-17-92 would make effector $CD8^+$ T cells less vulnerable to apoptosis and favor the accumulation of memory cells. In sharp contrast, instead of surviving better, the P14 cells ectopically expressing miR-17-92 contracted more than the control P14 cells did. Memory development was also impaired, manifested by the loss of markers usually associated with memory or central memory cells. Phenotypic analysis of miR-17-92–overexpressing effector cells provides a logical link between the enhanced clonal expansion and increased contraction of these cells. Our data demonstrated that the enhanced cell-cycle progression driven by miR-17-92 overexpression is accompanied by a strong tendency toward terminal effector differentiation. In fact, the miR-17-92–overexpressing P14 cells on day 8 p.i. were almost exclusively $CD127^{low}KLRG1^{high}$. The expression pattern of other markers such as CD62L and CD27 as well as the global transcription signature assessed by GSEA further support the idea that these cells resemble short-lived terminal effectors. However, knocking out miR-17-92 only slightly increased the frequencies of $CD127^{high}KLRG1^{low}$ LCMV-specific $CD8^+$ T cells at effector and memory time points. One likely explanation of the lesser impact on $CD8^+$ T-cell differentiation caused by loss of function than gain of function of miR-17-92 is that the

miR-106a-363 and miR-106b-25 may compensate for the loss of miR-17-92, given that the two share extensive targets with miR-17-92. In addition, the reduced $CD8^+$ T-cell response in *miR-17-92*$^{-/-}$ mice may result in delayed antigen clearance or prolonged proinflammatory cytokine stimulation, which may impair the generation of memory precursor cells.

Interestingly, previous studies in our laboratory showed that extending antigen stimulation leads to more proliferation toward the tail end of clonal expansion, drives effectors toward terminal differentiation, and impedes the conversion from effector memory cells to central memory cells (59), which closely resembles our observation in miR-17-92–overexpressing $CD8^+$ T cells. Therefore, miR-17-92 may be an intracellular signaling component that promotes proliferation and effector differentiation in response to antigen stimulation. In support of this hypothesis, NF-κB, a transcription factor downstream of TCR, was shown to bind to the human miR-17-92 promoter (273). Additional transcription factors such as STAT3 and E2Fs are also involved in the transcriptional regulation of miR-17-92 in human cell lines (244, 274), indicating that cytokine signals and proliferation itself may also regulate miR-17-92 expression.

In conclusion, we showed that miRNA expression patterns undergo dramatic changes in the course of a $CD8^+$ T-cell response, and we identified the high expression of miR-17-92 cluster and its paralogs as a miRNA signature of proliferating effectors. We then dissected the role of miR-17-92 in clonal expansion and effector/memory differentiation.

Our results may provide useful insights for the development of vaccines and therapies that target to enhance $CD8^+$ T-cell effector function or maximize memory cell formation by modulating miR-17-92.

Materials and Methods

Standard procedures and methods such as mouse handling, plaque assay, in vitro T-cell activation, lymphocyte isolation, flow cytometry, retroviral transduction, BrdU labeling, RNA isolation, microarray analysis, QRT-PCR, and statistical analysis are described in SI Materials and Methods.

Acknowledgements

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Supporting Information

SI Materials and Methods

Mice, Infection, and Plaque Assays. Six- to eight-week-old C57BL/6 mice were purchased from The Jackson Laboratory. Thy1.1$^+$ or Thy1.1$^-$ CD45.1$^+$ P14 TCR transgenic mice were on the C57BL/6 background and maintained in the laboratory as described previously (58). *Dicer* flox/flox and *miR-17-92* flox/flox mutant mice were purchased from The Jackson Laboratory (234, 275). GzB-cre transgenic mice were a kind gift from Joshy Jacob (Emory Vaccine Center). CD4-cre transgenic mice were purchased from Taconic (276). For acute infection, mice were i.p. injected with 2×10^5 pfu of LCMV Arm. Plaque assays were used to determine viral titers (264). All animal experiments were approved by the Institutional Animal Care and Use Committee of Emory University.

In Vitro T-Cell Stimulation. Splenic T cells were purified by MACS magnetic beads (Miltenyi Biotec), labeled with carboxyfluorescein succinimidyl ester (CFSE), plated at 10^5 cells per well in the 96-well plate, and stimulated with plate-bound anti-CD3 and soluble anti-CD28 (BD Biosciences).

Lymphocyte Isolation and Flow Cytometry. Splenocytes and peripheral blood mononuclear cells (PBMC) were isolated as described previously (104). MHC class I tetramer and intracellular cytokine staining were performed as previously described

(264). Live cells were determined by LIVE/DEAD Fixable Near-IR Dead Cell Stain Kit (Invitrogen). For phospho-S6 staining, splenocytes were fixed with BD Lyse/Fix Buffer (BD Biosciences) immediately after isolation, permeabilized with BD Phosflow Perm/Wash Buffer I (BD Biosciences), and stained with anti–phospho-S6 antibody (Cell Signaling Technology). Samples were analyzed on a BD FACSCanto II flow cytometer (BD Biosciences).

Retroviral Transduction. The MSCV-Puro-IRES-GFP (MSCVPIG) empty vector and MSCV-PIG with miR-17-92 (252) were gifts from Andrea Ventura (Memorial Sloan-Kettering Cancer Center, New York, NY). For transduction with MSCV-PIG, Thy1.1$^+$ P14 mice were i.v. injected with 2 × 10^6 pfu of lymphocytic choriomeningitis virus (LCMV) Armstrong strain (Arm). CD8$^+$ T cells were purified from P14 splenocytes at 24 h postinfection (p.i.), and infected by retrovirus packaged with either MSCV-PIG or MSCV-PIG inserted with miR-17-92. The cells were then cultured with IL-2 for 2–3 d and sorted for GFP$^+$ T cells. Then, 2 × 10^4 GFP$^+$ T cells were transferred to each C57BL/6 recipient. The MSCV-IRES-Thy1.1 (MIT) plasmid (17442; Addgene) was kindly provided by Anjana Rao (La Jolla Institute for Allergy and Immunology, La Jolla, CA) (277). A genomic fragment containing miR-17-92 was cloned as previously described (278) and inserted into the MIT vector. Transduction with the MIT vector was performed by first infecting CD45.1$^+$ Thy1.1$^-$ P14 mice i.v. with 2 × 10^6 pfu of LCMV Arm. Splenocytes were collected 24 h later, infected with retrovirus packaged with MIT or miR-17-92-MIT vector, and immediately transferred into C57BL/6 recipients at 10^5 P14

$CD8^+$ T cells per mouse.

In Vivo BrdU Incorporation. Mice were injected with 1 mg of BrdU solution i.p. on day 6 p.i. or day 7 p.i. and killed 4 h (as in Figure 2-S2*A*) or 6 h (as in Figure 2-3B) later. The BrdU staining was performed with BrdU Flow Kit (BD Biosciences).

RNA Isolation, Microarray Analysis, and Quantitative RT-PCR (QRT-PCR). For the microRNA (miRNA) microarray, each mouse was transferred with 10^4 P14 $CD8^+$ T cells and infected i.p. with LCMV Arm. Day 5 p.i., day 8 p.i., or memory (day 60–70 p.i.) P14 $CD8^+$ T cells were sorted on the basis of the expression of Thy1.1 congenic marker. Naïve P14 $CD8^+$ T cells were directly sorted from naïve P14 mice. Total RNA including the small RNA fraction was isolated with miRNeasy kit (Qiagen), then labeled and hybridized to Mouse miRNA Microarray (Agilent Technologies). The data analysis was performed with Gene-Spring GX 11.5 (Agilent Technologies) according to the manufacturer's manual. The raw data were normalized by 90thpercentile shift, baseline-transformed, and filtered on the basis of flag. For the mRNA microarray, retroviral transduction with the miR-17-92-MIT vector was performed as described above. Splenocytes were collected on day 8 p.i. Transduced ($CD45.1^+$ $CD8^+$ $Thy1.1^+$) and nontransduced ($CD45.1^+$ $CD8^+$ $Thy1.1^-$) P14 cells were sorted on the basis of the surface markers. RNA was isolated with RNeasy kit (Qiagen), labeled, and hybridized to mouse 430.2 microarray (Affymetrix). Data analysis was performed with GenePattern (Broad Institute). The raw data were normalized by the robust multichip averaging method.

Gene-set enrichment analysis (GSEA) was performed as previously described (279). The microarray data of $CD127^{high}$ memory precursors and $CD127^{low}$ terminal effectors were obtained from a previous study (57), the Gene Expression Omnibus (GEO) accession no. of which is GSE8678. The genes up-regulated by greater than or equal to twofold ($P < 0.05$) in the $CD127^{high}$ or $CD127^{low}$ effectors relative to $CD127^{low}$ or $CD127^{high}$ effectors were selected into the gene set representing the gene signature of the $CD127^{high}$ or $CD127^{low}$ effectors. miRNA QRT-PCR was performed with TaqMan MicroRNA Reverse Transcription Kit, TaqMan MicroRNA Assays, and TaqMan Universal PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions.

Statistical Analysis. All data analysis was performed with Prism 5.

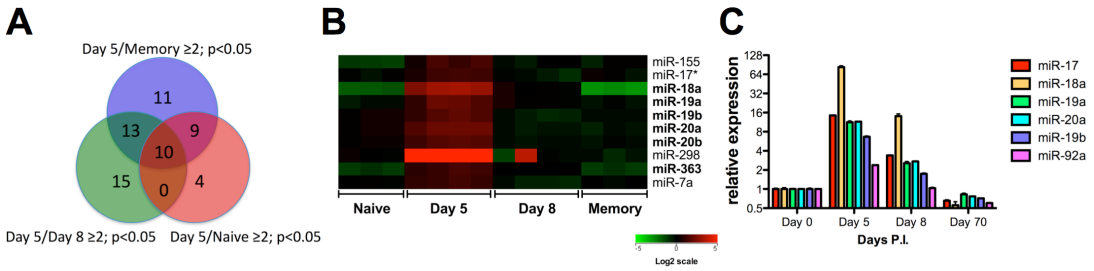

Figure 2- 1. miRNAs in the miR-17-92 cluster and its paralogs are up-regulated in the proliferating effector CD8⁺ T cells.

(*A*) Venn diagram of genes up-regulated by more than twofold ($P < 0.05$) in the day 5 effector P14 CD8⁺ T cells relative to the naïve (red), day 8 effector (green), or memory P14 (blue) cells. (*B*) Heat map of the expression of miRNAs that were up-regulated more than twofold ($P < 0.05$) in the day 5 effector P14 CD8⁺ T cells relative to naïve, day 8 effector, and memory P14 cells. miRNAs belonging to the miR-17-92 or miR-106a-363 cluster are in bold. (*C*) QRT-PCR analysis of the expression of individual members in the miR-17-92 cluster in naïve, day 5 p.i., day 8 p.i., and memory P14 cells. Bars represent the fold changes relative to naive. Sno-142 was used as the loading control.

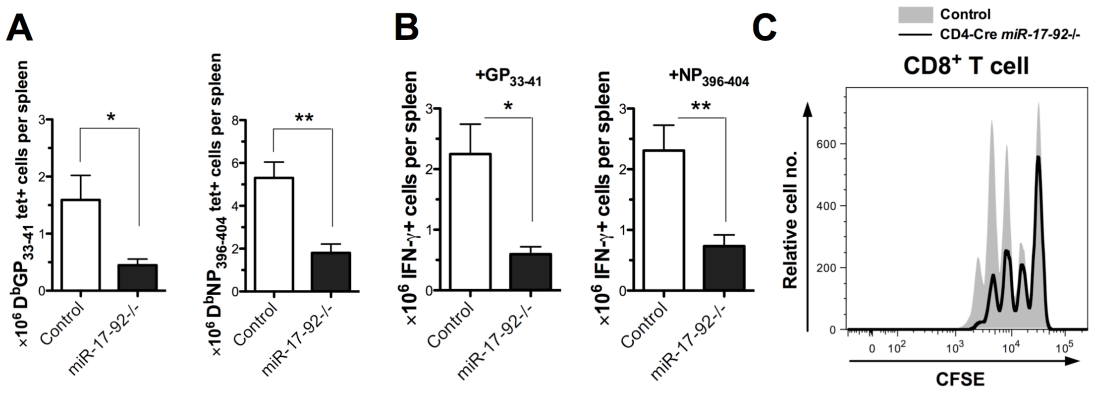

Figure 2- 2. miR-17-92 deficiency reduces effector CD8$^+$ T-cell response by inhibiting proliferation.

miR-17-92$^{-/-}$ mice and littermate controls were infected with LCMV Arm and killed on day 8 p.i. (*A* and *B*) Numbers of tetramer$^+$ cells per spleen (*A*) and numbers of IFN-γ^+ cells per spleen after 5-h stimulation with $GP_{33–41}$ or $NP_{396–404}$ (*B*) were determined. Results are representative of at least three independent experiments with at least five mice per group. Student's *t* test was used. $^*P < 0.05$, $^{**}P < 0.01$. (*C*) Carboxyfluorescein succinimidyl ester (CFSE) dilution of purified CD4-cre *miR-17-92*$^{-/-}$ and WT CD8$^+$ T cells after culture with plate-bound anti-CD3 and soluble anti-CD28 for 48 h. Results are representative of at least two experiments with $n \geq 6$.

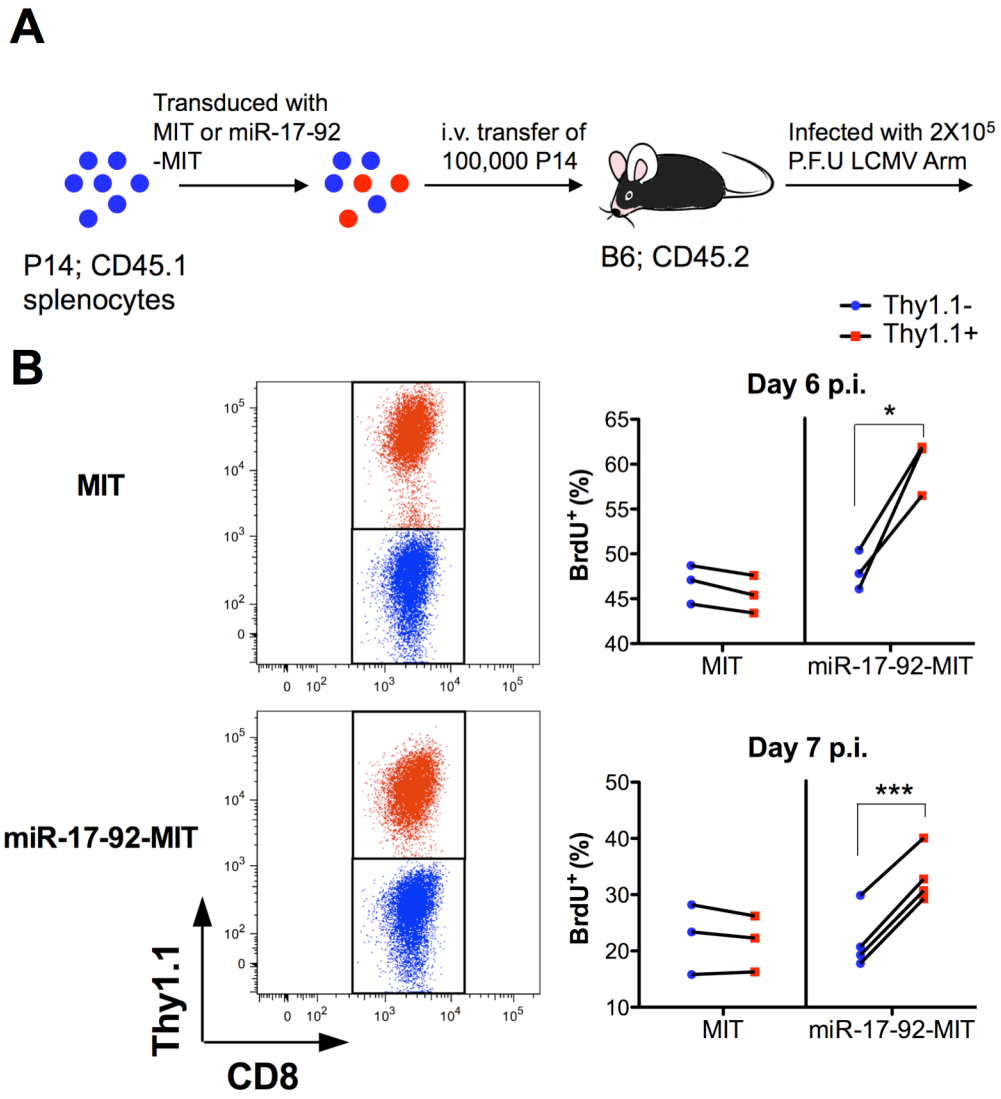

Figure 2- 3. Overexpression of miR-17-92 promotes cell-cycle progression of effector CD8$^+$ T cells.

(*A*) Splenocytes from Thy1.1$^-$ P14 mice were transduced with MIT or miR-17-92-MIT and transferred to C57BL/6 mice, which were subsequently infected with LCMV Arm and pulse-labeled with BrdU on day 6 or 7 p.i. (*B Left*) Gating of transduced (Thy1.1$^+$; red) and nontransduced (Thy1.1$^-$; blue) P14 cells. (*Right*) Frequencies of BrdU$^+$ cells

within transduced and nontransduced P14 T cells in the spleens of each group (MIT or miR-17-92-MIT) on days 6 and 7 p.i. Each line represents data from one individual mouse. Results are representative of at least two experiments with $n \geq 3$. Paired Student's *t* test was used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

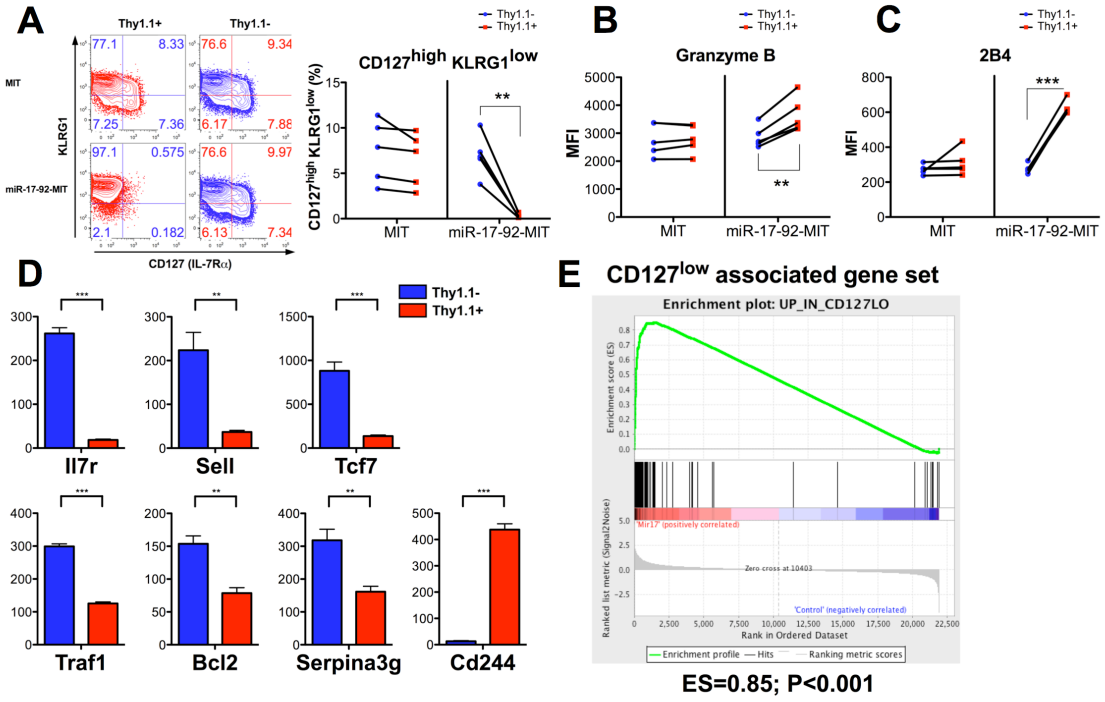

Figure 2- 4. Overexpressing miR-17-92 compromises the differentiation of memory precursor effector CD8⁺ T cells.

Chimeras transferred with MIT- or miR-17-92-MIT–transduced P14 cells were generated and infected as described in Figure 2-3. Phenotypic analysis of transduced (Thy1.1^{+}) or nontransduced (Thy1.1^{-}) P14 cells in the spleens on day 8 p.i. was performed. (*A*) Representative plots and statistics of CD127 and KLRG1 expression on transduced (red) and nontransduced (blue) P14 CD8^{+} T cells from each group (MIT or miR-17-92-MIT). Paired Student's *t* test was performed. Each line represents the frequencies of CD127^{high}KLRG1^{low} cells in the transduced and nontransduced P14 from one individual mouse. (*B* and *C*) The same statistical analysis was performed on the expression of granzyme B, and 2B4. Experiments were repeated at least three times with $n \geq 3$. (*D*) Relative gene expression values of Il7r (CD127), Sell (CD62L), Tcf7, Traf1, Bcl2,

serpina3g, and Cd244 (2B4) in the miR-17-92-MIT–transduced (Thy1.1^{+}; red) or nontransduced (Thy1.1^{-}; blue) P14 cells. Student's *t* test was used. (*E*) Gene signature of CD127^{low} effector CD8^{+} T cells is overrepresented in miR-17-92-MIT–transduced P14 on day 8 p.i., as determined by GSEA. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

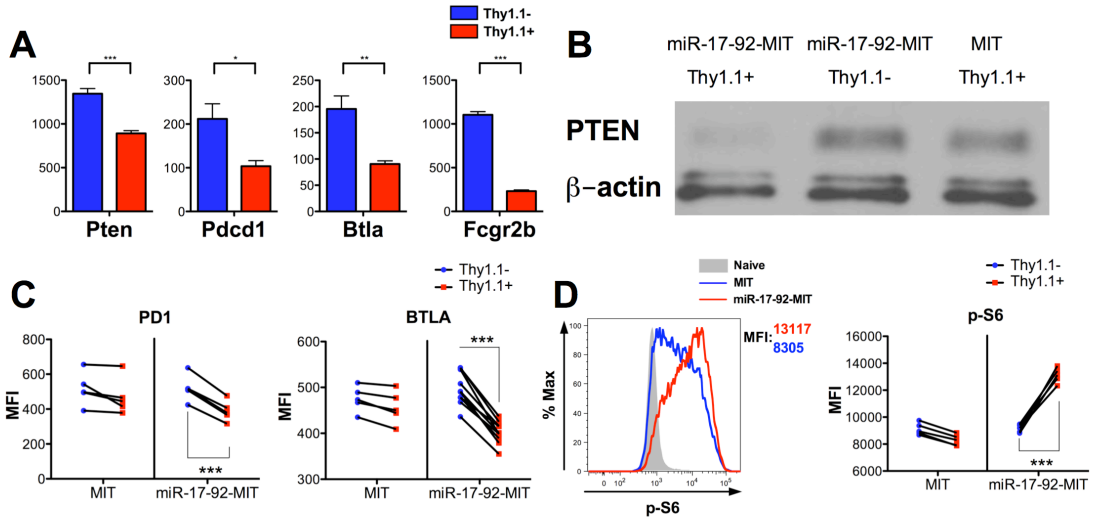

Figure 2- 5. miR-17-92 enhances mTOR signaling by suppressing multiple negative regulators up-stream of mTOR.

(*A*) Relative expression values of Pten, Pdcd1 (PD1), Btla, and Fcgr2b in miR-17-92-MIT–transduced and nontransduced P14 cells on day 8 p.i., as determined by microarray. (*B* and *C*) Protein levels of PTEN (*B*) as well as PD1 and BTLA (*C*) on day 4.5 p.i. were measured by Western blotting or FACS, respectively. β-Actin was used as loading control for Western blots. (*D*) Representative histogram of S6 phosphorylation in MIT- or miR-17-92-MIT–transduced P14 cells on day 4.5 p.i. (*Left*) and statistical analysis of the effect of MIT or miR-17-92-MIT transduction on S6 phosphorylation (*Right*). The results shown, except the microarray data, were representative of at least two independent experiments with $n \geq 4$. Paired *t* test (*C* and *D*) and unpaired *t* test (*A*) were used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

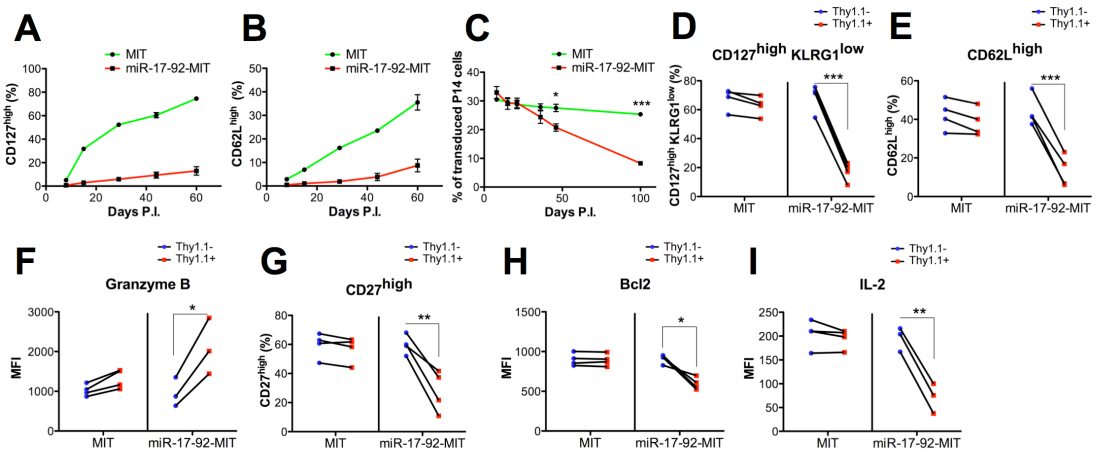

Figure 2- 6. Overexpression of miR-17-92 impairs the development of LCMV-specific memory CD8⁺ T cells.

(*A–C*) Frequencies of CD127^{high} (*A*) and CD62L^{high} (*B*) cells within transduced P14 cells as well as the portion of transduced cell (Thy1.1^{+}) within donor P14 cells in the peripheral blood mononuclear cells (PBMC) of each group (MIT or miR-17-92-MIT) (*C*) were tracked longitudinally starting from day 8 p.i. (*D–I*) Phenotypic analysis of transduced (Thy1.1^{+}) or nontransduced (Thy1.1^{-}) P14 cells in the spleens on day 66 p.i. was performed. Experiments were repeated at least three times with $n \geq 3$. Unpaired *t* test (*A–C*) and paired *t* test (*D–I*) were used. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

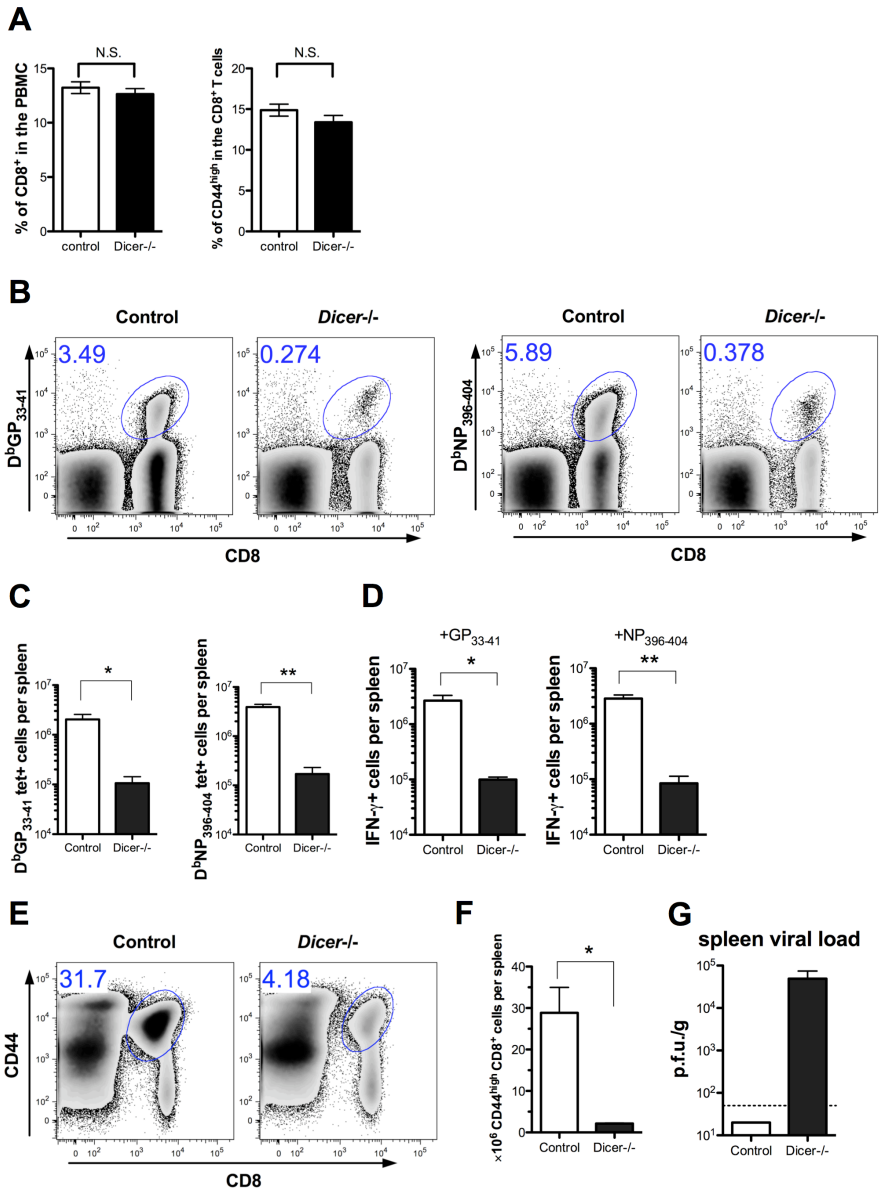

Figure 2-S 1. Dicer deficiency impairs effector CD8⁺ T-cell response.

(*A*) Percentages of CD8⁺ T cells and CD44^{high} cells within the CD8⁺ T-cell population in the PBMC of naïve *Dicer*$^{-/-}$ mice or littermate controls. *Dicer*$^{-/-}$ mice and littermate controls were infected with LCMV Arm and killed on day 8 p.i. (*B*) Representative plots of D^{b}GP$_{33–41}$ tetramer⁺ and D^{b}NP$_{396–404}$ tetramer⁺ CD8⁺ T cells in the spleens of *Dicer*$^{-/-}$ mice or littermate controls on day 8 p.i. (percentages of tetramer⁺ cells indicated; gated

on live splenocytes). (*C*) Numbers of tetramer$^+$ cells per spleen. (*D*) Numbers of IFN-γ^+ cells per spleen after 5-h stimulation with $GP_{33–41}$ or $NP_{396–404}$. (*E*) Representative plots of $CD44^{high}$ $CD8^+$ T cells in the spleens (percentages of $CD44^{high}$ $CD8^+$ T cells indicated; gated on live splenocytes). (*F*) Number of $CD44^{high}$ $CD8^+$ T cells per spleen. (*G*) Splenic viral load. Results are representative of at least three independent experiments with at least three mice per group. Student's *t* test was used. $*P < 0.05$; $**P < 0.01$; N.S., $P > 0.05$.

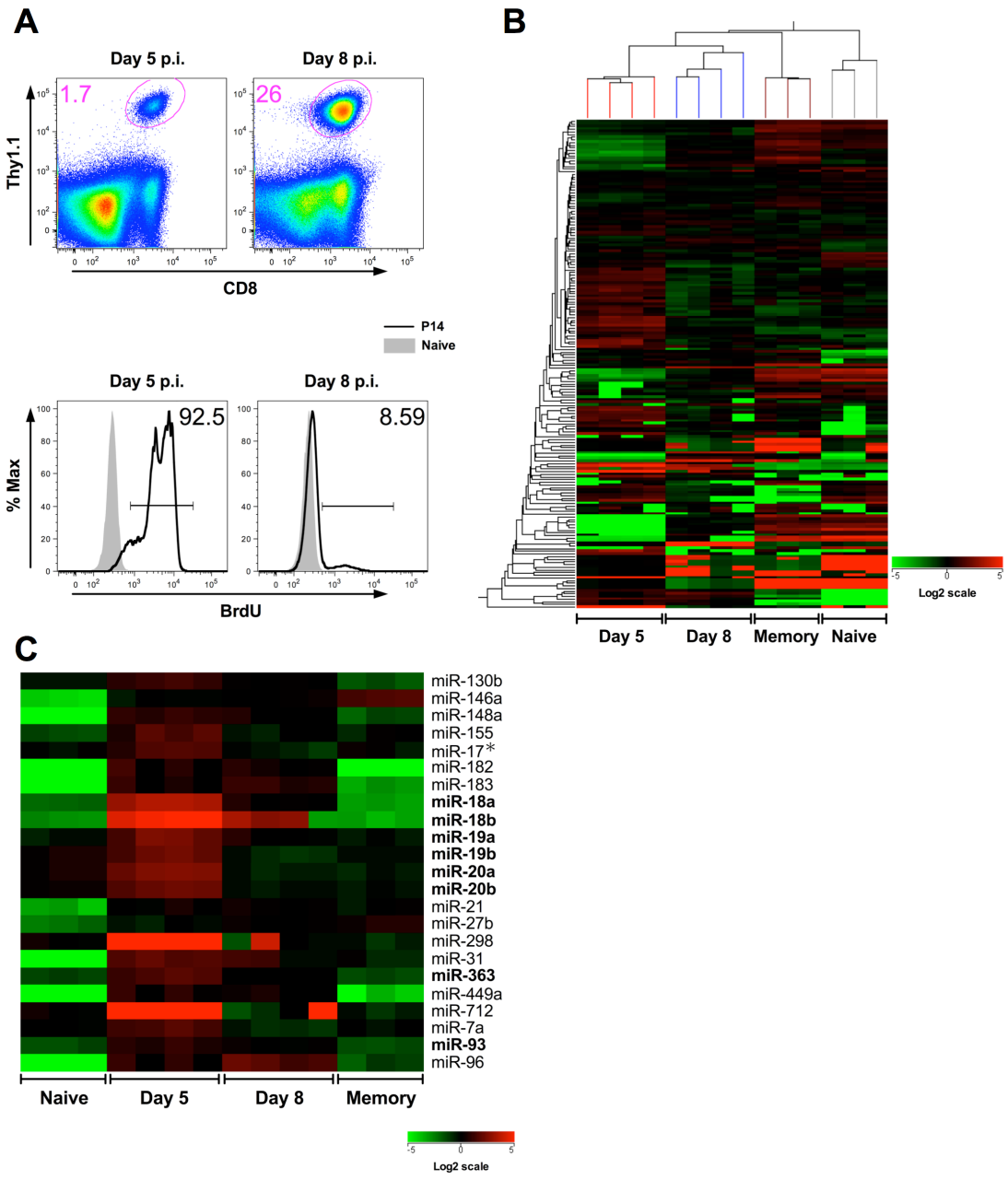

Figure 2-S 2. miRNAs up-regulated in day 5 effectors relative to naïve P14 CD8$^+$ T cells.

(*A*) Mice were transferred with 10^4 P14 CD8$^+$ T cells and infected with LCMV Arm. On day 5 or day 8 p.i., the mice were injected with 1 mg of BrdU i.p. and killed 4 h later. For

each time point, the frequency of P14 $CD8^+$ T cells ($Thy1.1^+$) is shown in *Upper* (percentage of P14 cells indicated; gated on live splenocytes) and histograms of BrdU staining in P14 cells (line) and $CD44^{low}$ naïve $CD8^+$ T cells (shaded) are shown in *Lower* (percentages of $BrdU^+$ P14 cells indicated). (*B*) Unsupervised hierarchical clustering of naïve, day 5 p.i., day 8 p.i., and memory P14 $CD8^+$ T cells with the miRNAs present in all of the samples from at least one of the four populations. Colors represent the fold change in expression compared with the mean of all samples for each probe. (*C*) Heat map of the expression at all time points of the miRNAs that were up-regulated more than twofold ($P < 0.05$) in day 5 effector P14 $CD8^+$ T cells relative to naïve P14 cells. Those miRNAs belonging to the miR-17-92, miR-106a-363, or miR-106b-25 clusters are in bold.

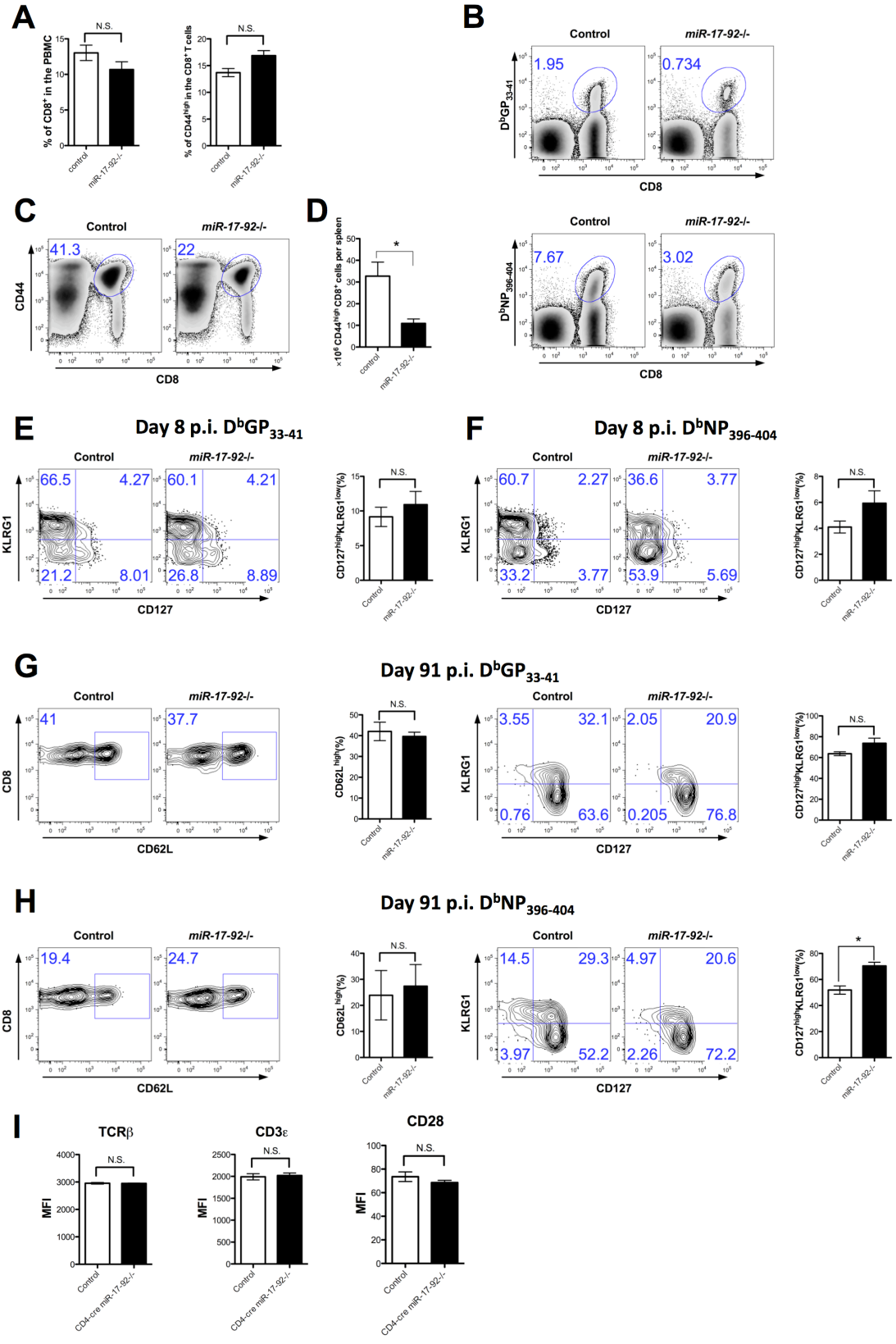
A
N.S.
% of CD8+ in the PBMC
control
miR-17-92-/-
N.S.
% of CD44high in the CD8+ T cells
control
miR-17-92-/-
B
Control
miR-17-92-/-
1.95
0.734
DbGP33-41
CD8
C
Control
miR-17-92-/-
41.3
22
CD44
CD8
D
*
×10⁶ CD44high CD8+ cells per spleen
control
miR-17-92-/-
Control
miR-17-92-/-
7.67
3.02
DbNP396-404
CD8
E
Day 8 p.i. DbGP33-41
Control
miR-17-92-/-
66.5
4.27
60.1
4.21
21.2
8.01
26.8
8.89
KLRG1
CD127
N.S.
CD127highKLRG1low(%)
Control
miR-17-92-/-
F
Day 8 p.i. DbNP396-404
Control
miR-17-92-/-
60.7
2.27
36.6
3.77
33.2
3.77
53.9
5.69
KLRG1
CD127
N.S.
CD127highKLRG1low(%)
Control
miR-17-92-/-
G
Day 91 p.i. DbGP33-41
Control
miR-17-92-/-
41
37.7
CD8
CD62L
N.S.
CD62Lhigh(%)
Control
miR-17-92-/-
Control
miR-17-92-/-
3.55
32.1
2.05
20.9
0.76
63.6
0.205
76.8
KLRG1
CD127
N.S.
CD127highKLRG1low(%)
Control
miR-17-92-/-
H
Day 91 p.i. DbNP396-404
Control
miR-17-92-/-
19.4
24.7
CD8
CD62L
N.S.
CD62Lhigh(%)
Control
miR-17-92-/-
Control
miR-17-92-/-
14.5
29.3
4.97
20.6
3.97
52.2
2.26
72.2
KLRG1
CD127
*
CD127highKLRG1low(%)
Control
miR-17-92-/-
I
TCRβ
N.S.
MFI
Control
CD4-cre miR-17-92-/-
CD3ε
N.S.
MFI
Control
CD4-cre miR-17-92-/-
CD28
N.S.
MFI
Control
CD4-cre miR-17-92-/-

Figure 2-S 3. Characterization of the LCMV-specific CD8$^+$ T-cell response in *miR-17-92*$^{-/-}$ mice.

(*A*) Frequencies of CD8$^+$ T cells and CD44^{high} cells within the CD8$^+$ T-cell population in the PBMC of naïve *miR-17-92*$^{-/-}$ mice or littermate controls. (*B*) Frequencies of $D^bGP_{33–41}$ tetramer$^+$ and $D^bNP_{396–404}$ tetramer$^+$ CD8$^+$ T cells in the spleens of *miR-17-92*$^{-/-}$ mice or littermate controls on day 8 p.i. (gated on live splenocytes). (*C*) Representative plots of CD44^{high} CD8$^+$ T cells in the spleens (percentages of CD44^{high} CD8$^+$ T cells indicated; gated on live splenocytes). (*D*) Number of CD44^{high} CD8$^+$ cells per spleen. (*E* and *F*) Frequencies of CD127^{high} killer cell lectin-like receptor G1 (KLRG1)^{low} cells in $D^bGP_{33–41}$ tetramer$^+$ CD8$^+$ T cells (*E*) and $D^bNP_{396–404}$ tetramer$^+$ CD8$^+$ T cells (*F*) in *miR-17-92*$^{-/-}$ mice or littermate controls on day 8 p.i. (*G* and *H*) Frequencies of CD62L^{high} or CD127^{high}KLRG1^{low} cells in $D^bGP_{33–41}$ tetramer$^+$ CD8$^+$ T cells (*G*) and $D^bNP_{396–404}$ tetramer$^+$ CD8$^+$ T cells (H) in *miR-17-92*$^{-/-}$ mice or littermate controls on day 91 p.i. (*I*) Expression of T-cell receptor β (TCRβ), CD3ε, and CD28 on naïve CD8$^+$ T cells from CD4-cre *miR-17-92*$^{-/-}$ or control mice. Student's *t* test was used. $*P < 0.05$; N.S., $P > 0.05$.

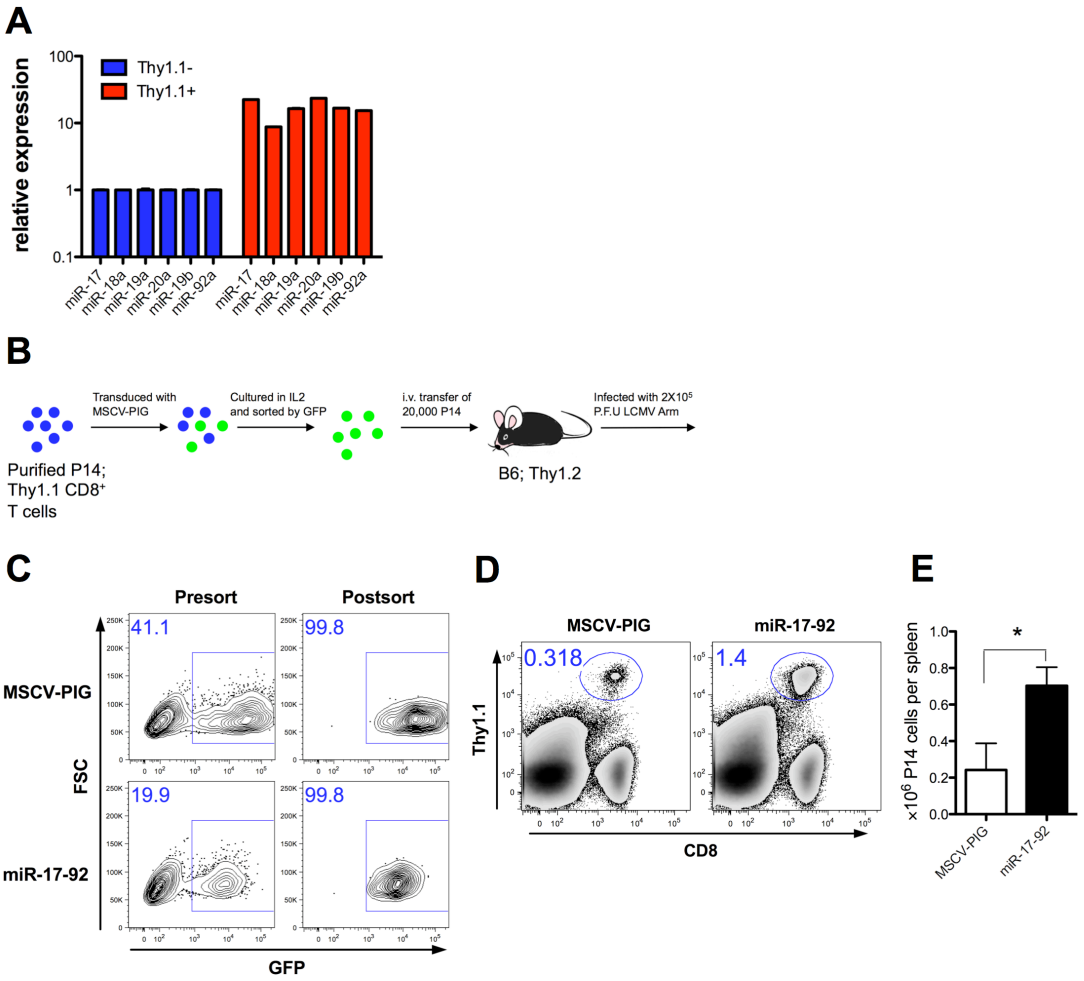

Figure 2-S 4. Overexpression of miR-17-92 increases the accumulation of effector CD8⁺ T cells.

(*A*) QRT-PCR analysis of the individual miRNAs of miR-17-92 in miR-17-92-MIT–transduced (red) and nontransduced (blue) P14 cells on day 8 p.i. The data are shown as the fold change in expression relative to the nontransduced cells. Sno-142 was used as the loading control. (*B–E*) Thy1.1⁺ P14 CD8⁺ T cells were purified, transduced by MSCV-PIG with or without miR-17-92 insert, expanded in culture supplemented with IL-2, and sorted on the basis of GFP expression. Each C57BL/6 mouse was transferred with 20,000

GFP$^+$ cells and infected with LCMV Arm. Mice were killed on day 5 p.i. (*B*) Experimental procedure of the transduction. (*C*) Frequencies of GFP$^+$ cells in the P14 cells before and after sorting. (*D*) Frequencies of empty vector (MSCV-PIG) or overexpression vector (miR-17-92)–transduced Thy1.1$^+$ donor P14 cells in the spleens (gated on live splenocytes). (*E*) Numbers of Thy1.1$^+$ P14 cells per spleen in each group. Results are representative of at least two experiments with more than four mice per group. Student's *t* test was used. $*P < 0.05$.

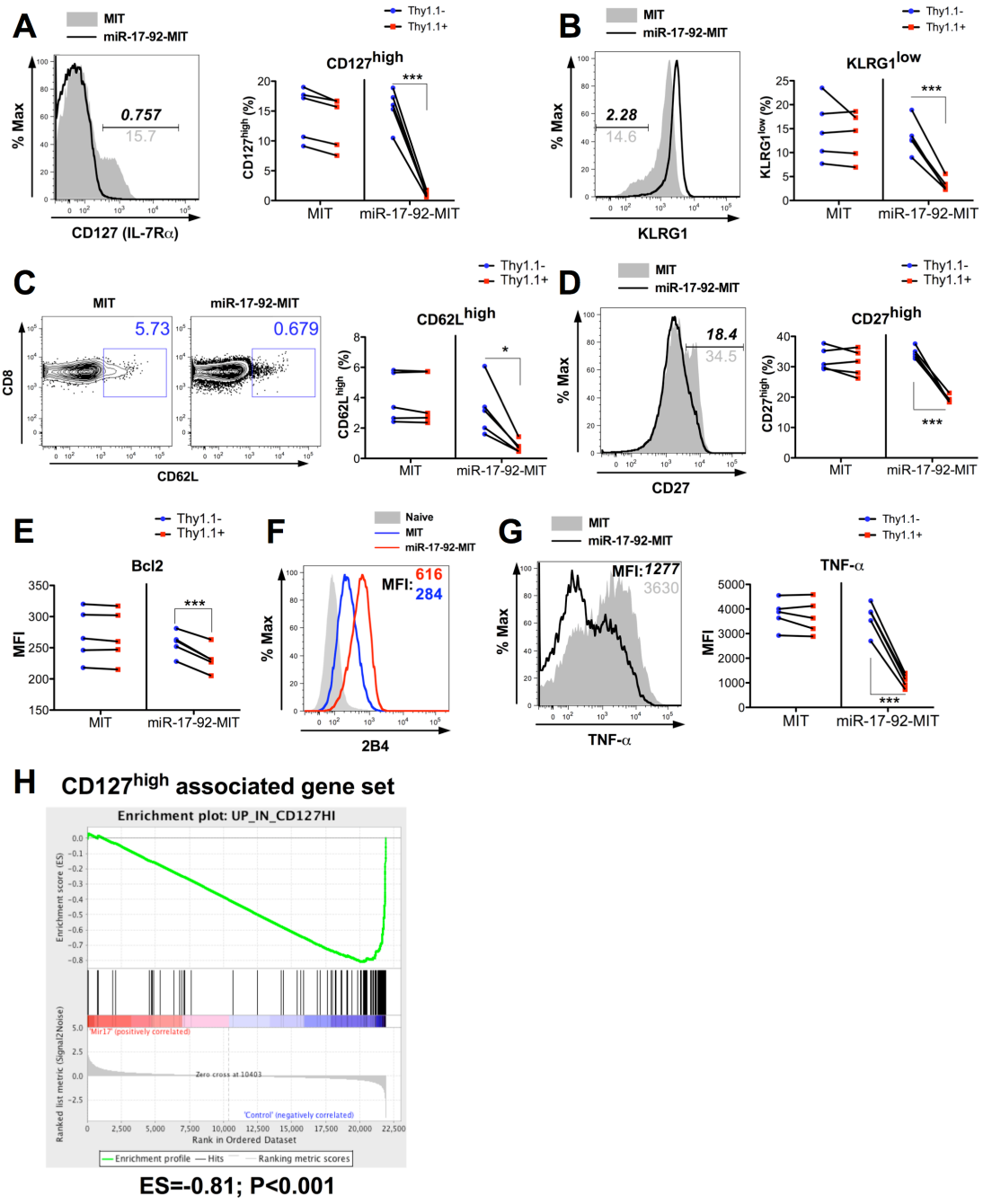

Figure 2-S 5. Changes in phenotypic markers on day 8 p.i. induced by miR-17-92 overexpression.

(*A* and *B*) (*Left*) Representative histograms of CD127 or KLRG1 expression on P14 cells transduced with MIT (shaded) or miR-17-92-MIT (line). Percentages of CD127^{high} or

KLRG1^{low} cells in P14 cells transduced with MIT (gray) or miR-17-92-MIT (italic) are indicated. (*Right*) Statistical analysis of CD127^{high} or KLRG1^{low} frequencies in the transduced (red) and nontransduced (blue) P14 cells. (*C*) Representative plots of CD62L expression on the MIT- or miR-17-92-MIT–transduced P14 cells (percentages of CD62L^{high} cells in MIT- or miR-17-92-MIT–transduced P14 cells are indicated). Statistical analysis of CD62L^{high} frequencies in the transduced (red) and nontransduced (blue) P14 cells. (*D*) Representative histograms of CD27 expression on MIT- or miR-17-92-MIT–transduced P14 cells (percentages of CD27^{high} cells in MIT- or miR-17-92-MIT–transduced P14 cells are indicated). Statistical analysis of CD27^{high} frequencies in the transduced (red) and nontransduced (blue) P14 cells. (*E*) Statistical analysis of Bcl2 expression in the transduced (red) and nontransduced (blue) P14 cells. (*F*) Representative histograms of 2B4 expression in naïve CD8$^+$ T cells (gray) or P14 cells transduced with MIT (blue) or miR-17-92-MIT (red). Mean fluorescence intensity (MFI) is indicated. (*G*) Representative histograms of TNF-α production in MIT- or miR-17-92-MIT–transduced P14 cells after 5-h restimulation with $GP_{33–41}$ (MFI indicated) and statistical analysis of the TNF-α MFI of the transduced cells and nontransduced P14 cells of each group are shown. Experiments were repeated at least three times with $n \geq 3$. Paired *t* test was used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (H) Gene signature of CD127^{high} effectors is underrepresented in miR-17-92-MIT–transduced P14 on day 8 p.i., as determined by GSEA.

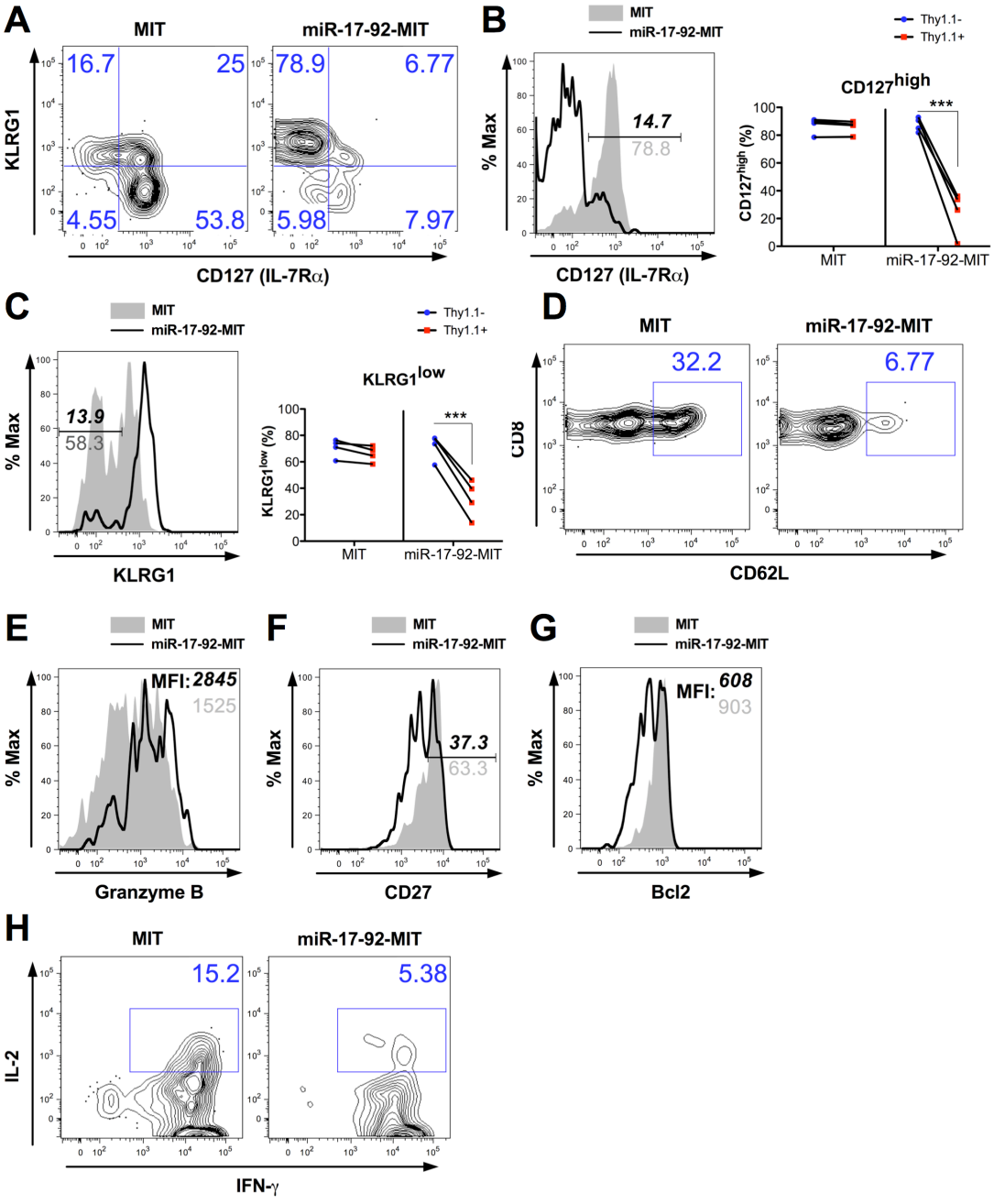

Figure 2-S 6. Changes in phenotypic markers on day 66 p.i. induced by miR-17-92 overexpression.

(*A*) Representative plots of CD127 and KLRG1 expression on MIT or miR-17-92-MIT–

transduced P14 cells. (*B* and *C*) (*Left*) Representative histograms of CD127 or KLRG1 expression on P14 cells transduced with MIT (shaded) or miR-17-92-MIT (line). Percentages of $CD127^{high}$ or $KLRG1^{low}$ cells in P14 cells transduced with MIT (gray) or miR-17-92-MIT (italic) are indicated. (*Right*) Statistical analysis of $CD127^{high}$ or $KLRG1^{low}$ frequencies in the transduced (red) and nontransduced (blue) P14 cells. (*D*) Representative plots of CD62L expression on MIT or miR-17-92-MIT–transduced P14 cells (the frequencies of $CD62L^{high}$ cells are indicated). (*E–G*) Representative histograms of granzyme B, CD27, or Bcl2 expression on MIT- or miR-17-92-MIT–transduced P14 cells. (*H*) Representative plots of IL-2 and IFN-γ production in MIT- or miR-17-92-MIT–transduced P14 cells after 5-h restimulation with $GP_{33–41}$ (frequencies of IFN-γ^+IL-2^+ cells are indicated). Experiments were repeated at least three times with $n \geq 3$. Paired *t* test was used. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Dataset S1. The list of miRNAs in the Agilent miRNA microarray that were identified as differentially expressed within the four time points (naïve, day 5 p.i., day 8 p.i., and memory) by one-way ANOVA

http://www.pnas.org/content/suppl/2012/06/03/1207327109.DCSupplemental/sd01.xls

Dataset S2. The list of genes in the Affymetrix mouse 430 microarray that were differentially expressed in the miR-17-92-MIT–transduced vs. nontransduced P14 cells by ≥1.5-fold ($P < 0.05$) identified by Student's *t* test

http://www.pnas.org/content/suppl/2012/06/03/1207327109.DCSupplemental/sd02.xls

Chapter 3: miR-17-92 regulates CD4 T cell differentiation during antiviral immune responses

Abstract

The miR-17-92 cluster plays an important role in virus-specific effector and memory CD8 T cell differentiation. However, how this cluster regulates CD4 T cell differentiation during viral infection is less clear. In this study, we show that effective antiviral CD4 T cell response requires miR-17-92. The lack of miR-17-92 compromises CD4 T cell clonal expansion and their ability to provide help to B cells. Moreover, miR-17-92 is particularly important to the development of virus-specific IFN-γ^+ CD4 T cells. miR-17-92 over-expression enhances the CD4 T cell response and the generation of IFN-γ^+ CD4 T cells. In addition, miR-17-92 elevates mTOR signaling in effector CD4 T cells and preferentially expands Th1 cells. Unlike our previous observation in CD8 T cells, miR-17-92 over-expressing CD4 T cells did not show an overt defect in memory cell development. In summary, our results demonstrate miR-17-92 as a critical regulator of CD4 T cell antiviral immune responses.

Introduction

Upon infection, antigen-specific naïve CD4 T cells recognize cognate antigens presented by professional antigen presenting cells (APCs), and differentiate into effector CD4 T cells. Effector CD4 T cells instruct the immune system to combat pathogens efficiently by providing other immune cells with signals via cytokines and direct engagement of cell surface molecules. CD4 T cells can differentiate into distinct effector subsets, such as T helper 1 (Th1), Th2, Th17, and induced regulatory T cells under different priming

conditions. Each subset is controlled by a unique master regulator transcription factor, and secretes a distinct group of cytokines, which are often the hallmark of this subset. For example, Th1 cells, whose differentiation is controlled by T-bet, secrete the signature cytokine IFN-γ, which boosts the activity of CD8 effectors and macrophages, and facilitates isotype switching of immunoglobulin to IgG2a (280, 281). A fraction of effector CD4 T cells survive and differentiate into memory cells after antigen clearance, while the others undergo apoptosis (53, 282). Memory CD4 T cells maintain a stable memory pool through long-term self-renewal, and can mount a strong recall response upon re-exposure to their cognate antigen. Interestingly, accumulating evidence supports the idea that different subsets of effector helper cells may give rise to distinct memory CD4 T cells that retain the characteristics of their progenitors (283-286).

A major role of CD4 T cells is to provide help to B cells (141). B cell helper activity is provided by a subset of effector CD4 T cells, known as follicular helper T cells (T_{FH} cells), which express the B cell follicle homing chemokine receptor CXCR5 and high levels of ICOS and PD-1 (141-144). The germinal center (GC) reaction is critical to affinity maturation, class switching, and generation of long-term humoral immunity. T_{FH} cells are indispensible for the initiation and maintenance of GCs (141). Only GC B cells that are able to present antigen to their cognate T_{FH} cells can receive survival and proliferation signals conveyed by proteins expressed by T_{FH} cells, such as CD40L, IL-21, and IL-4 (141). In addition, cytokines expressed by T_{FH} cells also influence the class switching of activated B cells. Bcl-6 is the master regulator of T_{FH} differentiation, while Blimp-1 enforces non-T_{FH} differentiation (164-166). Induction of Bcl-6 and repression of

Blimp-1 occur as early as day 3 post-infection in T_{FH} cells, which precedes GC formation and depends on dendritic cells rather than B cells (147, 174). Signaling induced by ICOS-ICOSL interaction is indispensible for Bcl-6 expression (174). On the other hand, IL-2 signals through STAT5 to induce Blimp-1 expression, and thus promotes the polarization to Th1 instead of T_{FH} during viral infection (177).

While transcriptional regulation of CD4 T cell differentiation is well documented, how the CD4 T cell response is regulated at the post-transcriptional level is less clear. MicroRNAs (miRNAs) are a family of small non-coding RNAs of ~22 nucleotides, which play a major role in post-transcriptional regulation of gene expression (186). Binding of the complementary sequences at the 3' UTR of target transcripts by seed regions of miRNAs appears to be critical for miRNA-mediated silencing of target mRNAs (203). Such silencing may involve blockade of mRNA translation and/or degradation of target mRNAs (204, 205). Early studies on *Dicer* knockout mice have suggested that miRNAs are required for B and T cell development (206, 207, 211). More recently, miR-29, miR-126, miR-326, and miR-146a have been shown to regulate Th1, Th2, Th17, and Treg differentiation respectively (212, 223, 230, 287). Our group recently demonstrated that the oncogenic miRNA miR-17-92 cluster regulates CD8 T cell differentiation (209). miR-17-92 was also shown to promote CD4 proliferation and Th1 polarization upon *in vitro* stimulation or tumor challenge (271). However, the role of this cluster in CD4 T cell response during viral infection is still unclear.

In this study, we have demonstrated that miR-17-92 is indispensible for CD4 T cell clonal expansion during viral infection and that miR-17-92 promotes Th1 differentiation and IFN-γ production of virus-specific effector CD4 T cells.

Materials and methods

Mice and infection

SMARTA mice with transgenic TCR specific to the GP_{66-77} epitope of lymphocytic choriomeningitis virus (LCMV) were bred in the lab and were congenically marked as $CD45.1^+$ (288). C57BL/6 ($CD45.1^-$) mice purchased from Jackson Laboratory were used as recipients. Mice with miR-17-92 null conditional allele and mice with miR-17-92 transgene conditional allele were also purchased from Jackson Laboratory and were bred to a CD4-cre transgenic strain (Taconic) (234, 253, 276). For acute infection, mice were injected intraperitoneally (i.p.) with 2×10^5 PFU of LCMV Armstrong. Animal experiments were performed under Emory University IACUC protocols.

In vitro T cell stimulation

Splenic CD4 T cells were purified by magnetic beads (Miltenyi Biotec), and labeled with carboxyfluorescein succinimidyl ester (CFSE). Labeled cells were plated at a density of 10^5 cells per well in 96-well plates. Cells were stimulated for 48 hours with plate-bound anti-CD3 and soluble anti-CD28 antibodies (BD Biosciences).

Flow cytometry

To label GP_{66-77}-specific CD4 T cells, splenocytes were incubated with 2ug/mL IA^bGP66-77 tetramer in RPMI medium supplemented with 10% FBS at 37 ºC for 2 hours. IA^bGP66-77 tetramer was generated at NIH Tetramer Core Facility (Emory University). CXCR5 staining was performed using a three-step staining protocol as previously described (164). Phosphorylated-S6 was stained by with anti-phospho-S6 antibodies (Cell Signaling Technology) as described in our previous study (209). Cell sorting was performed on FACSAria™ II (BD Biosciences). Flow cytometry data were acquired on FACSCanto™ II (BD Biosciences) and analyzed using FlowJo (Threestar, Inc.).

ELISA and ELISPOT assays

LCMV-specific antibody titers were measured by ELISA. Briefly, lysate of BHK-21 cells infected with LCMV-clone 13 was sonicated and coated to 96-well polysorp plates 48 hours before assay (NUNC, Rochester, NY). Diluted sera were incubated and later detected by HRP-conjugated goat anti-mouse immunoglobulin (SouthernBiotech). O-Phenylenediamine dihydrochloride (OPD) was used as substrate in the reaction. Absorbance was read at 490 nm.

LCMV-specific IgG secreting cells were detected via ELISPOT. Sonicated BHK-21 cells infected by LCMV-clone 13 were coated on plates 48 hours before assay. Splenocytes were cultured on plates for 8 hours and removed. LCMV-specific IgG secreted by antibody secreting cells (ASC) was first detected by biotinylated goat-anti-mouse IgG (Caltag Laboratories) followed by HRP-conjugated avidin-D (Vector Laboratories inc.).

Retroviral transduction

MSCV-PGK-GFP vector was inserted with miR-17-92 as previously described (209). SMARTA CD4 T cells simulated by GP61-80 peptides for 18 hours were purified, and incubated with packaged retrovirus with MSCV construct with or without miR-17-92 insert. Cells were cultured in complete medium supplemented with 10ng/mL IL-2 for 2-3 days, and sorted for the GFP^+ subset. 2×10^4 GFP^+ SMARTA T cells were adoptively transferred into each C57BL/6 recipient.

Statistical analysis

Data were analyzed on Prism 5. Student's t-test was performed. *$p<0.05$, ** $p<0.01$, ***$p<0.001$.

Results

miR-17-92 knockout reduces CD4 T cell response and alters effector CD4 differentiation

To determine whether miR-17-92 regulates the CD4 T cell response, we bred *miR-17-92* loxP/loxP to CD4-cre, which deletes loxP flanked genes efficiently in T cells (276). Knockout mice (*miR-17-92* loxP/loxP; CD4-cre) and their littermate controls (*miR-17-92* loxP/loxP) were infected with the Armstrong strain of lymphocytic choriomeningitis virus (LCMV Arm). Splenocytes from both groups were analyzed on day 8 post-infection (p.i.). Numbers of LCMV-specific CD4 T cells in knockout mice were strikingly reduced

compared to those in littermate controls, as manifested by a ~10 fold reduction in CD4 T cells bound to $IA^bGP66\text{-}77$ tetramer and a ~16 fold reduction in CD4 T cells producing IFN-γ in response to $GP_{61\text{-}80}$ peptides (Figure 3-1*A* and *B*). miR-17-92 seems to be critical for the differentiation of IFN-γ-producing effector CD4 T cells, as the ratios between IFN-γ^+ cells and tetramer$^+$ cells for the same MHCII-restricted epitope were lower in the knockout mice than in the controls (Figure 3-1*C*). In addition, LCMV-specific CD4 effector cells from knockout mice produce less IFN-γ and TNF-α on a per cell basis than their counterparts from controls (Figure 3-1*D* and *E*). Therefore, mir-17-92 deficiency leads to defective CD4 T cell clonal expansion as well as impaired differentiation into IFN-γ-producing cells. The diminished clonal expansion seen in miR-17-92 deficient CD4 T cells is likely a result of compromised proliferative capacity, as knockout cells showed less CFSE dilution than their wild-type counterparts after stimulation with anti-CD3 and anti-CD28 antibodies for 48 hrs (Figure 3-1*F*).

An important role of effector CD4 T cells during viral infection is to provide necessary signals to help B cells. Therefore, we speculated that the B cell response to LCMV might be compromised in T cell-specific miR-17-92 deficient mice as a result of a dampened CD4 T cell response. Indeed, LCMV-specific IgG titer was significantly reduced in the knockout mice on day 8 p.i. as a result of less LCMV-specific IgG secreting plasma cells (Figure 3-2*A* and *B*). Moreover, knockout mice had ~ 6 times less germinal center B cells than controls (Figure 3-2*C*).

Follicular helper T cell (T_{FH} cells), which can be distinguished by their expression of CXCR5, are the subset of effector CD4 T cells critical to the B cell response (141). To determine whether the defective B cell response is caused by altered T_{FH} cell differentiation, we analyzed CXCR5 expression on LCMV-specific CD4 T cells. As shown in Figure 3-3*A*, neither the frequency of $CXCR5^+$ cells nor the protein expression level of CXCR5 seemed to decrease after deletion of miR-17-92, indicating that miR-17-92 deficiency does not selectively impact the T_{FH} subset. This may indicate that the reduced B cell response could be caused merely by a general reduction in the LCMV-specific CD4 T cell response. PD-1 expression was significantly higher in the *miR-17-92-/-* CD4 T cells, which may suggest persistence of antigen (Figure 3-3*A*). Ly6C, T-bet, granzyme B expression has been shown to be associated with a Th1 phenotypes (289). We found a severe reduction in the frequency of $Ly6C^{high}$ cells and a moderate reduction in T-bet expression in the miR-17-92 knockout cells, which may suggest impaired Th1 differentiation in these cells (Figure 3-3*B* and *C*). However, knockout cells expressed granzyme B at a level comparable to wild-type cells (Figure 3-3*D*). A previous study identify Bcl-6 as a target repressed by T-bet (290). Accordingly, we observed that a higher expression of Bcl-6 coincided with lower T-bet expression in miR-17-92 deficient effector CD4 cells (Figure 3-3*E*). In summary, while loss of miR-17-92 does not preferentially compromise the T_{FH} cells, it alters effector CD4 T cell differentiation during viral infection.

Memory CD4 differentiation is influenced by miR-17-92 deficiency

To investigate how miR-17-92 knockout impacts CD4 memory differentiation, we sacrificed knockout and control mice on day 108 p.i.. There were ~4 fold less IA^bGP66-77 tetramer$^+$ CD4 T cells and ~7 fold less IFN-γ^+ CD4 T cells after GP_{61-80} peptide stimulation in the knockout mice than in their littermate controls (Figure 3-4*A* and *B*). Similar to our observation on day 8 p.i., the ratios between IFN-γ^+ cells and tetramer$^+$ cells for the same epitope were lower in the knockout mice than in the control mice, which confirms that loss of miR-17-92 seems to preferentially undermine the generation of IFN-γ^+ cells (Figure 3-4*C*). Moreover, production of IFN-γ was much reduced in miR-17-92 knockout memory CD4 T cells, suggesting that miR-17-92 is important for the functionality of virus-specific CD4 T cells (Figure 3-4*D*). Our recent study has shown that LCMV-specific memory CD4 T cells can be segregated into three populations: $Ly6C^{high}CXCR5^-$ memory Th1 cells, $Ly6C^+CXCR5^+$ memory T_{FH} cells and $Ly6C^-CXCR5^+$ memory T_{FH} cells (291). miR-17-92 deficient CD4 memory cells did not seem to form a distinct $Ly6C^{high}CXCR5^-$ or $Ly6C^+CXCR5^+$ population (Figure 3-5*A*). This may be explained by a potential defect in the generation of the $Ly6C^+$ population, because Ly6C expression was significantly reduced while CXCR5 expression was mostly undisturbed in miR-17-92 deficient memory CD4 T cells (Figure 3-5*B* and *C*). While the B cell response was compromised at the effector time point caused by defective CD4 T cell response, serum LCMV-specific IgG titers in knockout mice eventually reached similar levels as control mice at the memory phase (Figure 3-5*D*).

miR-17-92 over-expression promotes the generation of IFN-γ^+ and Th1 effector CD4 T cells

Our data demonstrate that miR-17-92 deficiency greatly compromises CD4 clonal expansion and seems to preferentially affect the generation of IFN-γ^+ effector CD4 T cells. Therefore, we sought to determine whether increasing miR-17-92 expression can enhance CD4 response and generate more IFN-γ^+ effectors. We utilized a previously described conditional over-expression strain that carries a CAG promoter and the human miR-17-92 cluster whose expression is blocked by a loxP-flanked STOP cassette inserted between the promoter and the cluster (253). The transgenic strain was bred to CD4-cre so that miR-17-92 is over-expressed only in T cells. Cre^+ mice that contain two copies of the miR-17-92 transgene (TG) are designated as TG/TG, and their cre^- littermates were used as controls.

TG/TG mice and their littermate controls were infected with LCMV Arm and analyzed on day 10 p.i.. TG/TG contained more IA^bGP66-77 tetramer labeled CD4 T cells in the spleens than controls (Figure 3-6*A*). TG/TG mice also had ~3 fold higher numbers of IFN-γ^+ CD4 cells after GP_{61-80} re-stimulation than controls (Figure 3-6*B* and *C*). Interestingly, the ratios between IFN-γ^+ and $tetramer^+$ CD4 T cells specific for the same epitope in TG/TG mice were ~1.5, while the ratios in control mice were ~1 (Figure 3-6*D*). This indicates that there is a population of LCMV-specific CD4 T cells that cannot be detected by tetramer but can produce IFN-γ after re-stimulation with cognate antigen. TG/TG LCMV-specific CD4 effectors seemed to produce slightly more IFN-γ on a per cell basis than their counterparts in control mice (Figure 3-6*E*), while the production of

TNF-α and IL-2 were lower in TG/TG effectors compared to control cells (Figure 3-6*F* and *G*).

Next, we went on to investigate whether the balance between Th1 and T_{FH} cell differentiation is affected by miR-17-92 over-expression. Interestingly, the frequencies of T_{FH} cells within LCMV-specific effector CD4 T cells were significantly lower in TG/TG mice than those in control mice (Figure 3-7*A*). However, the total numbers of LCMV-specific T_{FH} cells in TG/TG mice were no less than those in controls, which rules out a potential defect in T_{FH} cell development (Figure 3-7*B*). The decrease in T_{FH} cell frequency seemed to be solely caused by an enhanced Th1 response (Figure 3-7*B*). Accordingly, the numbers of germinal center B cells were comparable between the two groups (Figure 3-7*C*). Similar to our observation in effector CD8 T cells (209), miR-17-92 over-expression reduced PD-1 expression on effector CD4 T cells (Figure 3-7*D*). Interestingly, we found a striking increase in surface Ly6C levels in miR-17-92 over-expressing effector CD4 T cells (Figure 3-7*E*), which is consistent with our observation that loss of miR-17-92 causes the loss of $Ly6C^+$ effector CD4 T cell subset. Moreover, TG/TG effector CD4 T cells expressed lower levels of Bcl-6 than control cells (Figure 3-7*F*). Therefore, miR-17-92 over-expression in virus-specific effector CD4 T cells promotes the generation of Th1 cells.

miR-17-92 over-expression affects CD4 memory differentiation

In our previous studies, we showed that miR-17-92 over-expression impairs memory CD8 T cell differentiation (209). Therefore, we sought to determine whether miR-17-92 over-expression has the same effect on memory CD4 T cell differentiation. TG/TG and control mice were sacrificed on day 115 p.i., and numbers of LCMV-specific memory CD4 T cells were determined by tetramer staining and IFN-γ staining after cognate peptide re-stimulation. Interestingly, there were similar or even slightly more LCMV-specific memory CD4 T cells in TG/TG mice as compared to the controls, indicating that miR-17-92 over-expression does not compromise the generation of memory CD4 T cells (Figure 3-8*A* and *B*). However, the MFI of tetramer was lower in LCMV-specific TG/TG memory CD4 T cells than that in the control cells, which may suggest that higher miR-17-92 expression selects memory CD4 T cells with lower avidity (Figure 3-8*C*). Consistent with our observation in the effector phase, the ratios between IFN-γ^+ cells and tetramer$^+$ cells for the same LCMV epitope in TG/TG mice were significantly higher than the ratios in control mice (Figure 3-8*D*). TG/TG memory CD4 T cells produced less TNF-α upon re-stimulation, while their production of IFN-γ was comparable to or even slightly higher than that of controls (Figure 3-8*E* and *F*). While expressing a similar level of CXCR5 as control cells, TG/TG cells had significantly higher Ly6C expression than control cells (Figure 3-9*A*-*C*). Accordingly, there were more Ly6C$^+$CXCR5$^+$ memory T_{FH} cells in TG/TG mice.

miR-17-92 enhances mTOR signaling in effector CD4 T cells

As documented in our previous study, miR-17-92 potentiates mTOR activity in effector CD8 T cells (209). To test whether miR-17-92 has the same effect on effector CD4 T cells, we used the MSCV-PGK-GFP retroviral system to over-express this cluster in SMARTA transgenic CD4 T cells that are specific for the GP_{61-80} epitope of LCMV (288), and used phosphorylation of ribosomal protein S6 at Ser235/236 as a readout of mTOR activity. As shown in Figure 3-10, SMARTA cells over-expressing miR-17-92 had more phosphorylated ribosome protein S6 than SMARTA cells transduced with empty vector, indicating that miR-17-92 indeed enhances mTOR signaling in effector CD4 T cells.

Discussion

In this study, we used both loss-of-function and gain-of-function approaches to demonstrate the critical role of miR-17-92 in regulating the CD4 T cell response to viral infection. We showed that miR-17-92 is necessary for CD4 T cell proliferation and is indispensible for CD4 T cell clonal expansion during acute LCMV infection. miR-17-92 seemed to be particularly important for IFN-γ expression and the generation of the IFN-γ^+ subset of virus-specific CD4 T cells. Moreover, miR-17-92 also regulates effector and memory CD4 T cell differentiation. Specifically, miR-17-92 favors the generation of Th1 effector cells and $Ly6C^+$ memory CD4 T cells. However, unlike our previous observation in CD8 T cells, miR-17-92 over-expression did not compromise memory CD4 T cell development.

miR-17-92 is frequently over-expressed in tumor cells, and possesses oncogenic activity (231). Over-expression of this cluster in T and B cells results in the development of lymphoproliferative disease and autoimmunity in mice (253). Further studies showed that miR-17-92 drives CD4 T cell proliferation *in vitro* and CD8 T cell proliferation in response to viral infection (209, 212, 271). However, the role of this cluster in virus-specific CD4 T cell response was still unclear. This study demonstrates that miR-17-92 is indispensible for the antiviral immune response mediated by CD4 T cells. While knocking out miR-17-92 in T cells resulted in diminished CD4 expansion, over-expression of the cluster increased the magnitude of the CD4 T cell response. The humoral immune response to viral infection depends on T cell help. As the effector CD4 response was compromised when knocking out miR-17-92 specifically in T cells, the germinal center reaction and virus-specific IgG production at the effector phase were also reduced. However, the virus-specific IgG levels were indistinguishable between knockout mice and controls at the memory phase, indicating that the remaining effector CD4 T cells in the knockout were sufficient to support the efficient generation of long-lived plasma cells.

Our previous study showed that miR-17-92 reduces PTEN protein levels and enhances PI3K/mTOR signaling in virus-specific effector CD8 T cells (209). Consistently, phosphorylation of ribosomal protein S6, an indicator of mTOR activity, was significantly elevated upon miR-17-92 over-expression in effector CD4 T cells. Upon activation, T cells switch from catabolism to anabolism, a process tightly regulated by mTOR, in order to meet the robust metabolic demands resulting from clonal expansion

and effector functions (292). It is possible that miR-17-92 drives the CD4 T cell response by maintaining a metabolic signature associated with effector T cells through promoting mTOR signaling strength.

Besides supporting the B cell response, virus-specific effector CD4 T cells also produce cytokines, such as IFN-γ, to modulate the antiviral immune response. Interestingly, there was a greater decrease in IFN-γ-producing CD4 T cells than tetramer bound CD4 T cells after knocking out miR-17-92. Correspondingly, miR-17-92 over-expression preferentially promoted the generation of IFN-γ^+ CD4 effectors. These results suggest that differentiation of IFN-γ-producing CD4 effectors is more sensitive to the level of miR-17-92. This may be explained by the fact that IFN-γ production by T cells is enhanced by PI3K signaling (293), which can be elevated by miR-17-92 over-expression. Although control mice for both loss-of-function and gain-of-function experiments showed a ratio between IFN-γ^+ and tetramer$^+$ CD4 T cells of ~1, it should be noticed that not all antigen-specific CD4 T cells produce IFN-γ after stimulation with cognate antigen, while not all IFN-γ-producing T cells have enough TCR avidity to be labeled with tetramer (294). The lower than 1 ratio between IFN-γ^+ and tetramer$^+$ CD4 T cells seen in *miR-17-92-/-* CD4 T cells may indicate that CD4 T cells deficient for miR-17-92 need higher TCR signaling to pass the threshold required for cytokine production. On the other hand, the fact that there were more IFN-γ^+ than tetramer$^+$ CD4 T cells in TG/TG CD4 T cells may suggest that more CD4 T cells with low avidity to their antigen were enabled to produce IFN-γ by miR-17-92 over-expression.

miR-17-92 not only regulates virus-specific CD4 T cell expansion but also modulates their differentiation. Two primary effector CD4 subsets are generated after viral infection: T_{FH} and Th1 cells. The two can be effectively distinguished by their expression of CXCR5, a chemokine receptor necessary for migration to B cell follicles, along with other markers such as SLAM, PD-1, ICOS, and Ly6C (164, 291). The transcriptional repressor Bcl-6 is essential for T_{FH} differentiation (164). Interestingly, one study showed that Bcl-6 suppresses the expression of a wide range of microRNAs including miR-17-92, and that miR-17-92 over-expression reduces CXCR5 expression in mouse B cells (166). The authors therefore concluded that miR-17-92 inhibits T_{FH} differentiation by suppressing CXCR5 expression. Although we did find a consistent decrease in the frequency of $CXCR5^+$ virus-specific CD4 T cells when over-expressing miR-17-92, this decrease was the result of an increase in Th1 response, which accounted for most of the increase in total virus-specific effector CD4 T cells after miR-17-92 over-expression. The numbers of T_{FH} cells in miR-17-92 TG/TG mice were no less than those in control mice. Therefore, we conclude that although essential for T cell expansion, miR-17-92 does not disrupt T_{FH} differentiation.

$Ly6C^+$ CD4 T cells express markers associated with Th1 cells and were shown to be associated with terminal differentiation of effector CD4 T cells (289). Interestingly, we found a loss of the $Ly6C^+$ subset when knocking out miR-17-92 and an increase in this subset when over-expressing miR-17-92. Moreover, our previous study on the role of miR-17-92 in CD8 T cell differentiation clearly demonstrated that miR-17-92 promotes terminal differentiation of virus-specific CD8 T cells. It is possible that miR-17-92 also

drives virus-specific CD4 T cells into terminal differentiation. However, unlike CD8 T cells, CD4 T cells over-expressing miR-17-92 generated a memory pool similar to, if not larger than, the wildtype memory CD4 population, which indicates that miR-17-92 over-expressing CD4 T cells are not more terminally differentiated than their wildtype counterparts. The changes in the frequencies of the $Ly6C^+$ subset in miR-17-92 knockout and over-expression mice may be explained by corresponding changes in virus-specific CD4 T cell differentiation. Moreover, the $Ly6C^+$ subset contains not just Th1 cells, and $Ly6C^-$ CD4 T cells are not necessarily T_{FH} cells (291). More comprehensive studies in the future regarding the nature of $Ly6C^+$ and $Ly6C^-$ effector and memory CD4 T cells are needed.

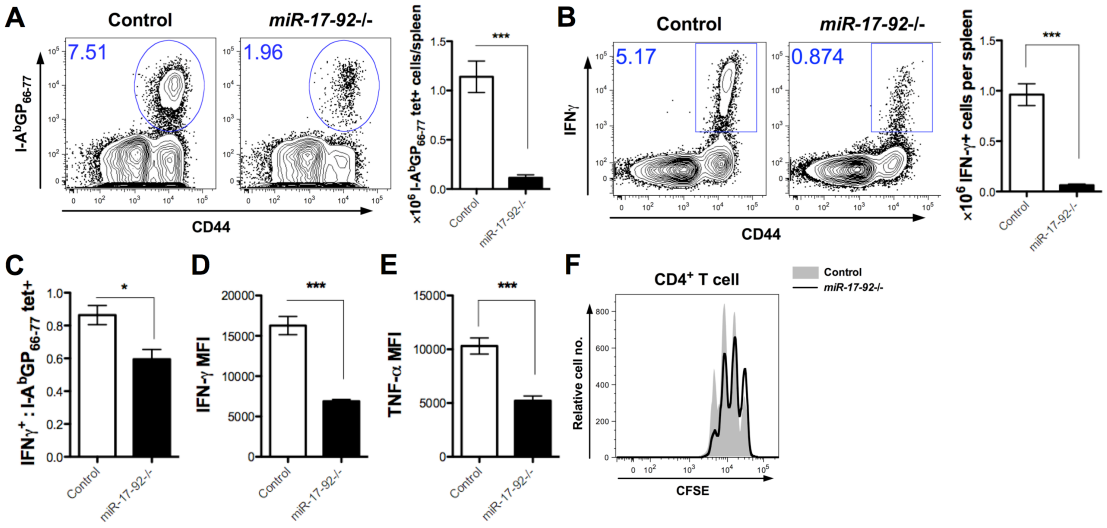

Figure 3- 1. miR-17-92 deficiency compromises CD4 T cell immune response.

(*A*) Representative plots (percentages of tetramer$^+$ cells indicated; gated on CD4 T cells) and numbers of IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleens of *miR-17-92-/-* mice and littermate controls on day 8 p.i.. (*B*) Representative plots (percentages of IFN-γ$^+$ cells indicated; gated on CD4 T cells) and numbers of IFN-γ$^+$ CD4 T cells in the spleen after 5-h stimulation with GP_{61-80} peptide. (*C*) The ratio between numbers of IFN-γ$^+$ CD4 T cells in response to GP_{61-80} peptide stimulation and IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleen. (*D*) and (*E*) Expression of IFN-γ and TNF-α as measured by mean fluorescence intensity (MFI) in IFN-γ$^+$ CD4 T cells after GP_{61-80} peptide stimulation. (*F*) Naïve CD4 T cells were purified, labeled with carboxyfluorescein succinimidyl ester (CFSE), and stimulated with plate bound anti-CD3 and soluble anti-CD28 for 48 hours. The extent of cell divisions was measured by CFSE dilution.

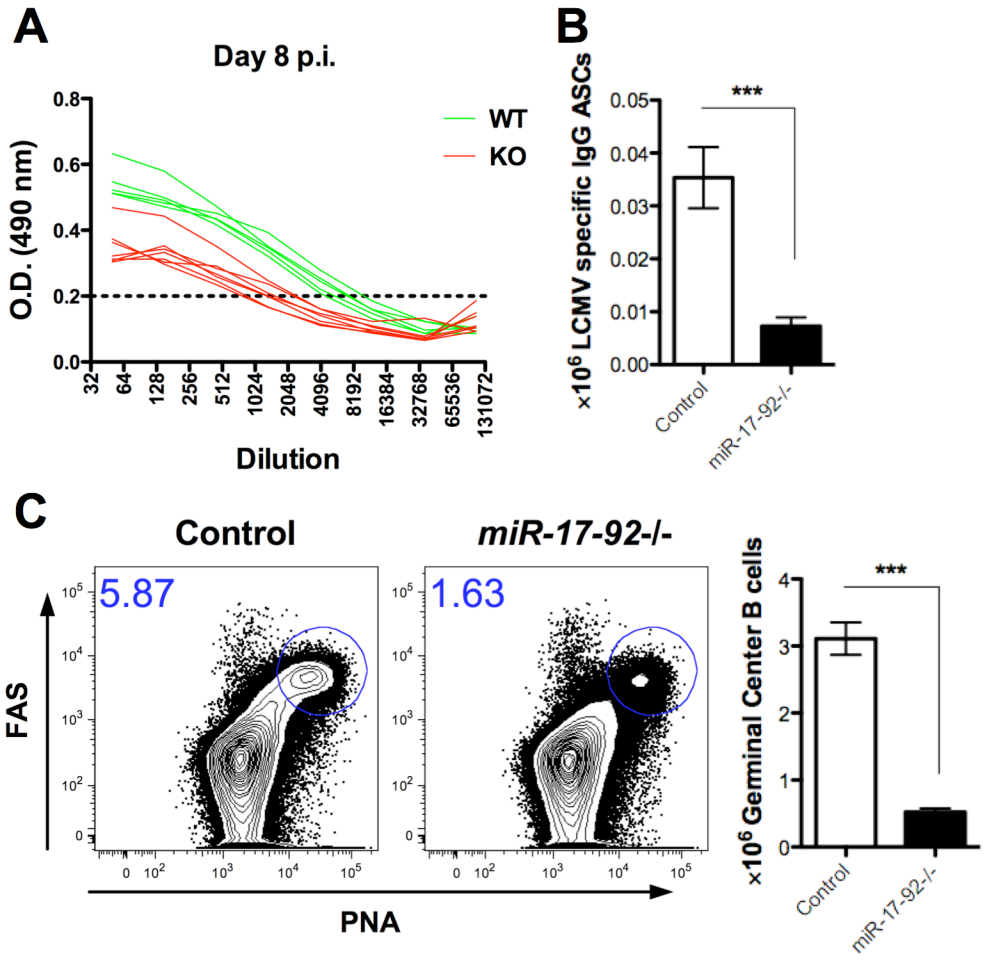

Figure 3- 2. Defective B cell response in miR-17-92-/- mice.

(*A*) LCMV-specific IgG titers on day 8 p.i. in the sera of *miR-17-92*-/- mice and littermate controls were determined by ELISA. (*B*) Numbers of LCMV-specific IgG antibody secreting cells (ASCs) in the spleen. (*C*) Representative plots (percentages of germinal center B cells indicated; gated on total B cells) and number of germinal center B cells in the spleens.

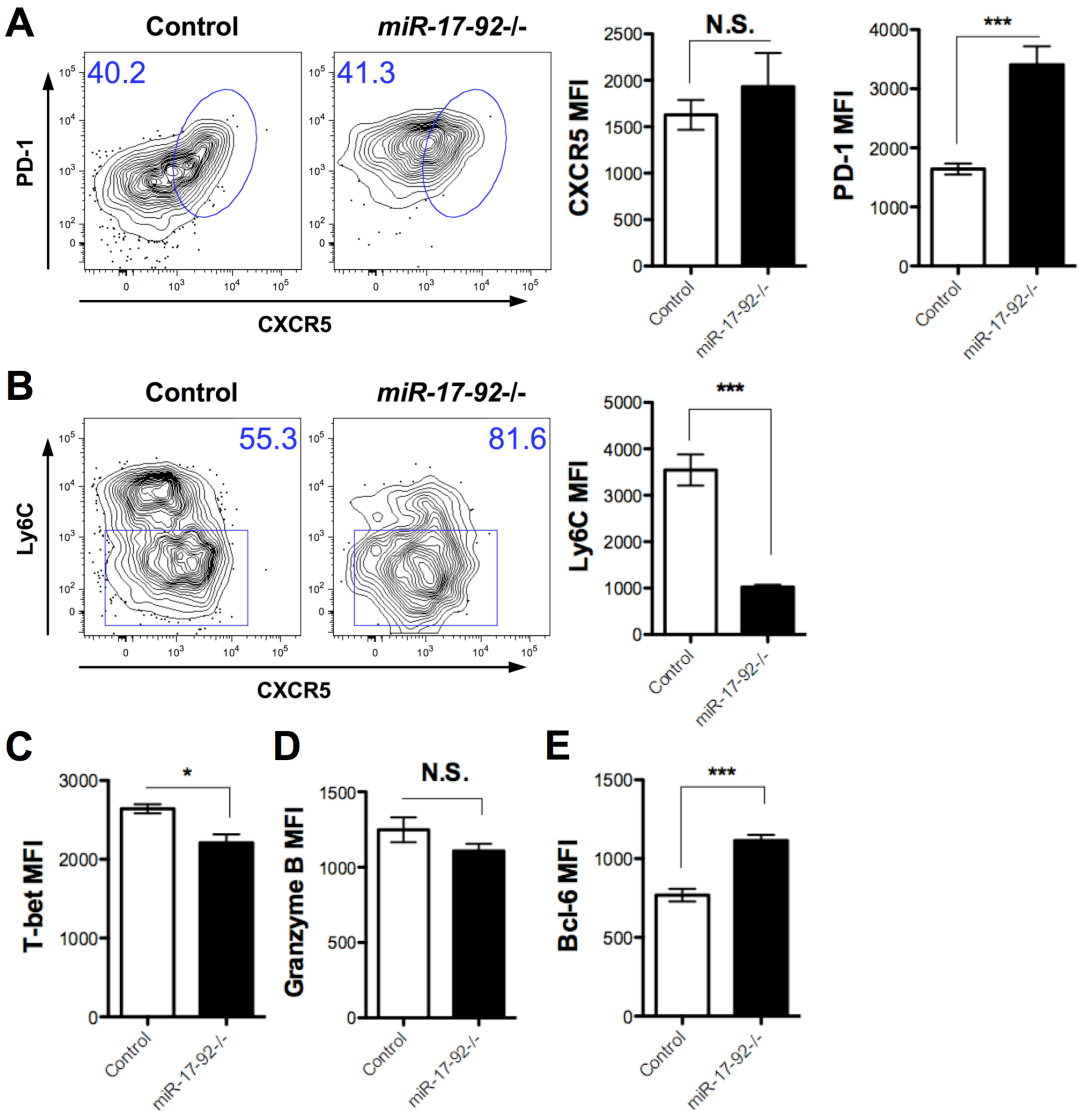

Figure 3- 3. Effector CD4 T cell differentiation is altered by miR-17-92 deficiency.

(*A*) Representative plots of T_{FH} staining (percentages of $CXCR5^+$ cells indicated; gated on $tetramer^+$ CD4 T cells) and expressions of CXCR5 and PD-1 on $tetramer^+$ CD4 T cells. (*B*) Representative plots of Ly6C staining (percentages of $Ly6C^-$ cells indicated; gated on $tetramer^+$ CD4 T cells) and expression of Ly6C on $tetramer^+$ CD4 T cells. (*C-E*) Expressions of T-bet, granzyme B, and Bcl-6 in $tetramer^+$ CD4 T cells.

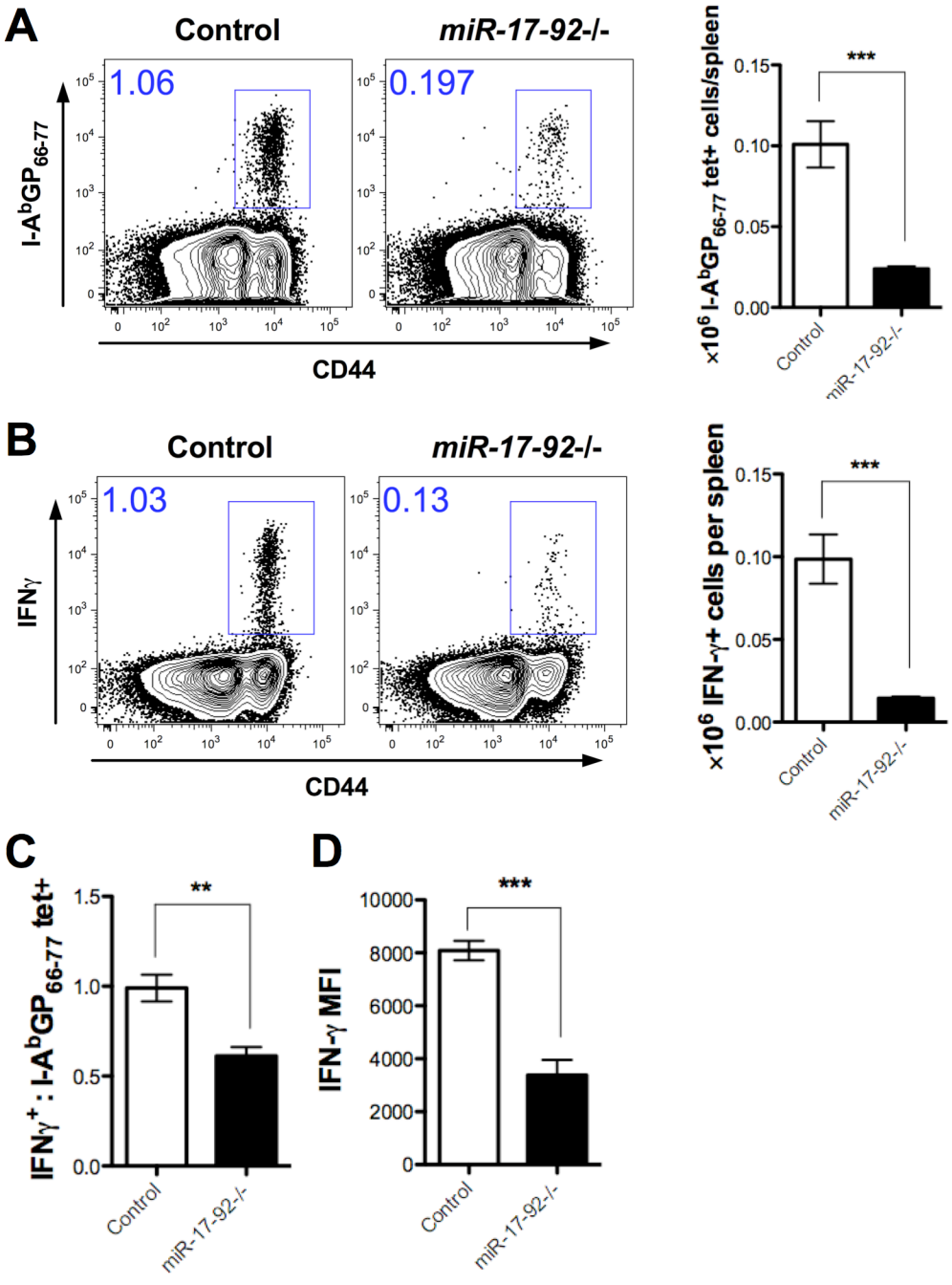

Figure 3- 4. Less memory CD4 T cells are generated in *miR-17-92-/-* mice after infection.

(*A*) Representative plots (percentages of tetramer$^+$ cells indicated; gated on CD4 T cells) and numbers of IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleens of knockout mice and

littermate controls on day 108 p.i.. (*B*) Representative plots (percentages of IFN-γ^+ cells indicated; gated on CD4 T cells) and numbers of IFN-γ^+ CD4 T cells in the spleen after $GP_{61\text{-}80}$ peptide stimulation. (*C*) The ratio between numbers of IFN-γ-producing CD4 T cells and IA^bGP66-77 tetramer$^+$ CD4 T cells in the spleen. (*D*) MFI of IFN-γ in IFN-γ^+ CD4 T cells.

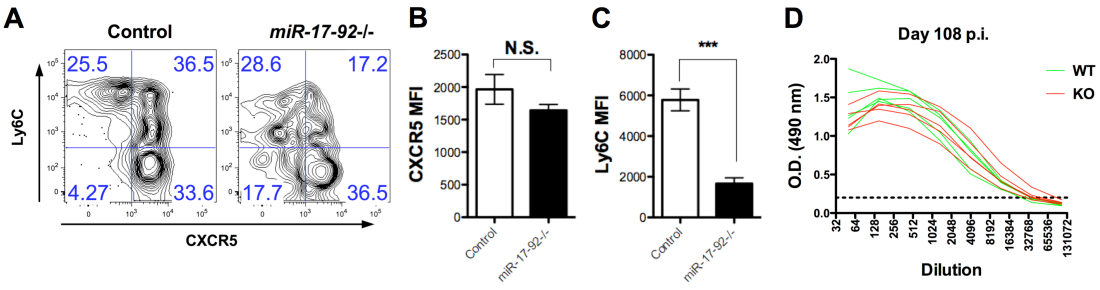

Figure 3- 5. Differentiation of memory CD4 T cell subsets in miR-17-92-/- mice.

(*A*) Representative plots of memory CD4 subsets (percentage of each subset indicated; gated on tetramer$^+$ CD4 T cells). (*B*) and (*C*) Expressions of CXCR5 and Ly6C on tetramer$^+$ CD4 T cells. (*D*) LCMV-specific IgG titers in the sera on day 108 p.i. as determined by ELISA.

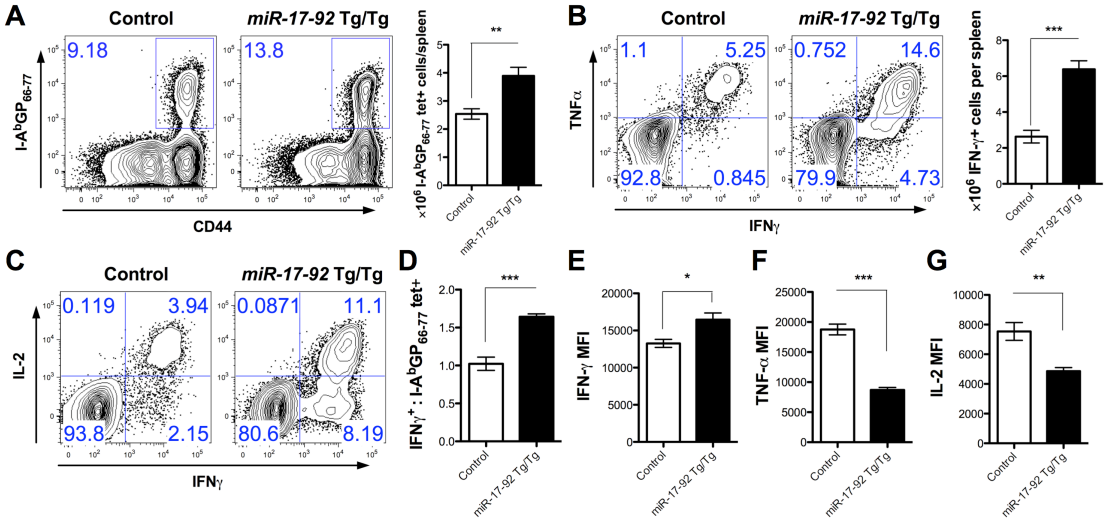

Figure 3- 6. miR-17-92 over-expression enhances CD4 T cell response to viral infection.

(*A*) Representative plots (percentages of tetramer$^+$ cells indicated; gated on CD4 T cells) and numbers of IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleens of TG/TG mice and littermate controls on day 10 p.i.. (*B*) Representative plots (gated on CD4 T cells) of IFN-γ and TNF-α staining and numbers of IFN-γ$^+$ CD4 T cells in the spleen after GP$_{61-80}$ peptide stimulation. (*C*) Representative plots (gated on CD4 T cells) of IFN-γ and IL-2 staining after peptide stimulation. (*D*) The ratio between IFN-γ$^+$ and IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleen. (*E-G*) MFIs of IFN-γ, TNF-α, and IL-2 in IFN-γ$^+$ CD4 T cells.

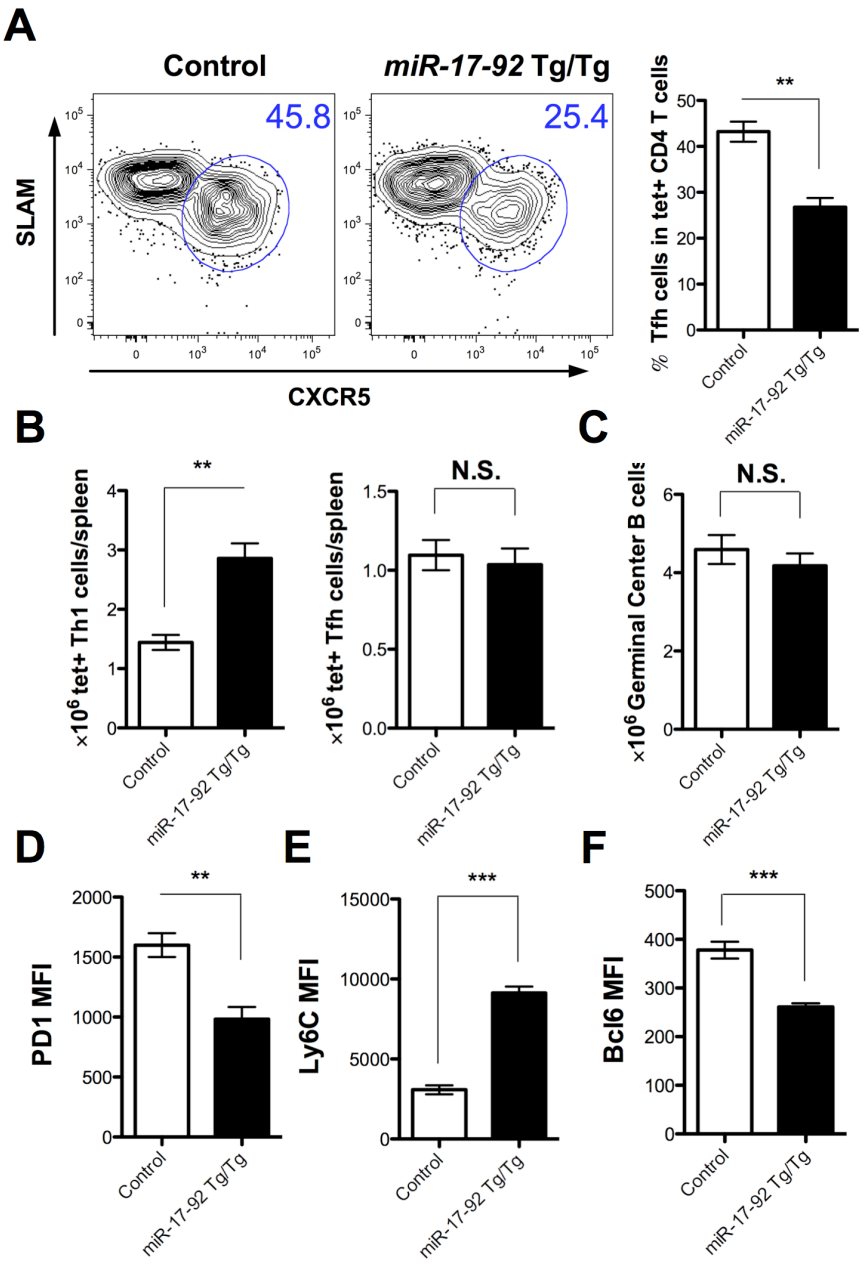

Figure 3- 7. miR-17-92 favors the generation of Th1 effector cells.

(*A*) Representative plots of T_{FH} staining (percentages of T_{FH} cells indicated; gated on tetramer$^+$ CD4 T cells) and frequencies of T_{FH} cells within tetramer$^+$ CD4 T cells. (*B*) Numbers of tetramer$^+$ Th1 and T_{FH} CD4 T cells. (*C*) Numbers of germinal center B cells. (D-E) Expression of PD-1, Ly6C, and Bcl-6 as measured by MFIs.

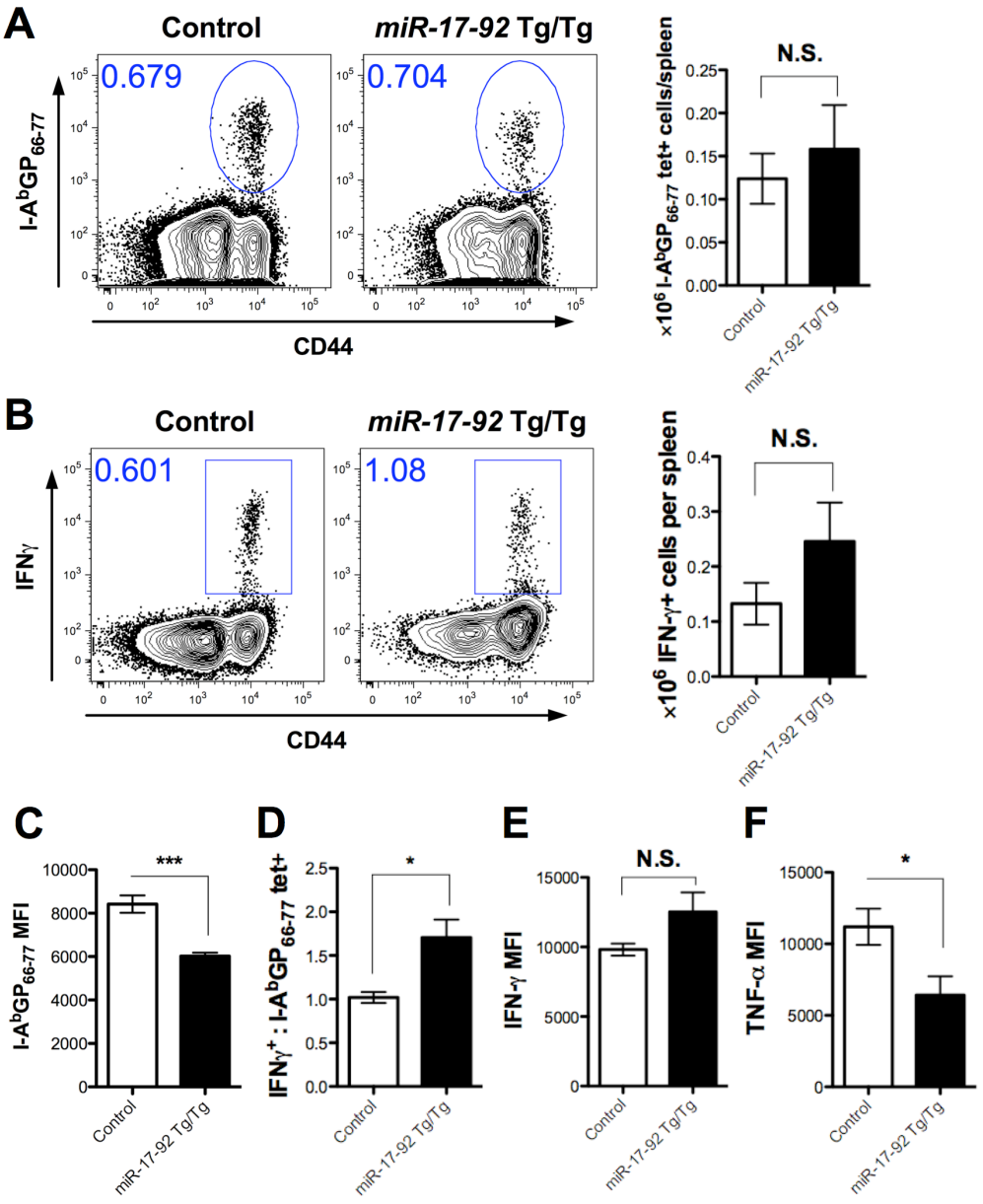

Figure 3- 8. miR-17-92 over-expression does not compromise memory CD4 T cell formation.

(*A*) Representative plots (gated on CD4 T cells) and numbers of IA^{b}GP66-77 tetramer$^+$ CD4 T cells in the spleens of knockout mice and littermate controls on day 115 p.i.. (*B*) Representative plots (gated on CD4 T cells) and numbers of IFN-γ$^+$ CD4 T cells in the

spleen after peptide stimulation. (*C*) Tetramer fluorescence intensity of tetramer$^+$ CD4 T cells. (*D*) Ratio between IFN-γ^+ and IA^{b}GP66-77 tetramer$^+$ CD4 T cells. (*E*) and (*F*) MFIs of IFN-γ and TNF-α in IFN-γ^+ CD4 T cells.

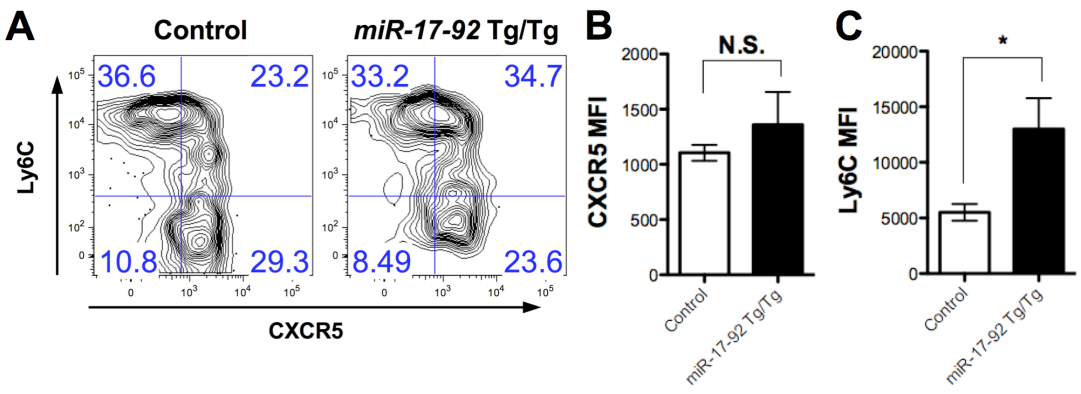

Figure 3- 9. miR-17-92 influences memory CD4 T cell differentiation.

(*A*) Representative plots of memory CD4 subsets (gated on tetramer$^+$ CD4 T cells). (*B*) and (*C*) Surface expression of CXCR5 and Ly6C.

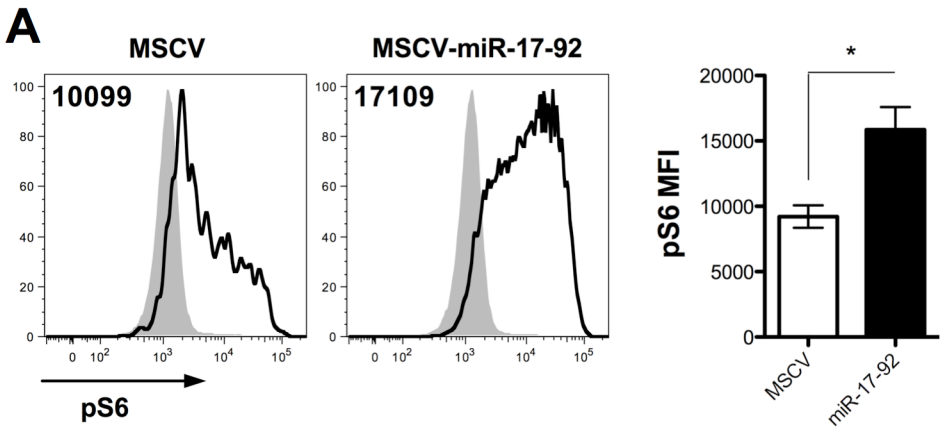

Figure 3- 10. miR-17-92 enhances mTOR signaling.

(*A*) Representative histogram of phosphorylated S6 (pS6) staining (numbers indicate the MFIs). Solid lines represent SMARTA cells in each group (MSCV: empty vector transduced; MSCV-miR-17-92: miR-17-92 over-expression construct transduced), and shades represent naïve cells in the same samples. Statistics of pS6 staining MFIs are shown on the right.

Chapter 4: Conclusion and Future Directions

Non-coding RNAs (ncRNAs) are RNAs that do not encode proteins. ncRNAs have been known since the discovery of transport RNAs (tRNAs) and ribosomal RNAs (rRNAs). However, it was not until recently that ncRNAs were shown to regulate gene expression. Since Victor Ambros identified the first miRNA, lin-4, in *Caenorhabditis elegans* in 1993, the regulatory role of ncRNAs has attracted great interest (185). Based on the size of ncRNAs, they can be categorized into two groups: long ncRNAs and small ncRNAs. Long ncRNAs regulate various cellular processes such as chromatin modification, transcriptional regulation, inhibition of miRNA function, and imprinting (295). Small ncRNAs include miRNAs, piwi-interacting RNAs (piRNAs), small-interfering RNAs (siRNAs), small nucleolar RNAs (snoRNAs), etc. (296). The importance of miRNAs in the immune system was first demonstrated by experiments using mice deficient in enzymes critical to miRNA biogenesis (206, 211). Since then, research has focused on understanding the roles of specific miRNAs in the differentiation and function of different immune cell lineages (219, 223). However, the miRNA signatures of T cells responding to viral infections and the specific miRNAs that regulate this process are less clear. In this dissertation, I seek to address the role of miRNAs in the T-cell antiviral immune response.

In Chapter 2, I have demonstrated how miRNAs regulate the CD8 T cell immune response using LCMV acute infection in mice as a model system. First, *Dicer*, a gene encoding an enzyme essential to miRNA biogenesis, was knocked out using Granzyme

B-cre. The effector CD8 T cell response was severely compromised in *Dicer* conditional knockout mice due to a cell-autonomous defect (54). In order to identify specific miRNAs that are critical to the effector CD8 T cell response, we profiled miRNA expression in naïve, day 5 and day 8 effectors, as well as memory CD8 T cells. Members of the miR-17-92 cluster and its paralogs were among the most up-regulated miRNAs in proliferating effector CD8 T cells. Interestingly, miR-155, which was later shown by other groups as an important regulator of the CD8 T cell response (221), was also found to be induced after naïve CD8 T cells differentiate into effectors. To examine the role of miR-17-92 in the CD8 T cell response, we conditionally knocked out miR-17-92 in effector CD8 T cells and found that the number of LCMV-specific effector CD8 T cells was reduced by ~3 fold. To test whether the two paralogs of miR-17-92 (miR-106a-363 and miR-106b-25) act redundantly, future experiments with triple knockouts of all three clusters seem warranted. In addition to a reduced effector response, knockout mice seemed to generate a slightly higher proportion of memory precursor effector CD8 T cells, at least among those cells specific for the $D^bNP_{396-404}$ epitope. In addition, we used *in vitro* and *in vivo* experiments to prove that miR-17-92 drives the clonal expansion of effector CD8 T cells by enhancing T cell proliferation. The importance of miR-17-92 for T cell clonal expansion is also supported by the findings from other groups (212, 271).

It has long been speculated that effector and memory CD8 T cell differentiation is influenced by the magnitude of the primary response. To test whether excessive amounts of miR-17-92 have an impact on CD8 T cell differentiation in addition to clonal expansion, we over-expressed miR-17-92 in LCMV-specific T cells and found that

almost all miR-17-92 over-expressing effector CD8 T cells underwent terminal differentiation. This conclusion was furthered supported by Gene Set Enrichment Analysis, which revealed that the transcriptome of miR-17-92 over-expressing effectors resembled that of terminally differentiated effector cells. Moreover, miR-17-92 over-expressing cells could not generate a stable memory population and failed to develop into central memory cells, which also suggests that excessive miR-17-92 skews virus-specific CD8 T cells towards terminal differentiation. miR-17-92 was the first miRNA cluster shown to function as an oncogene (239). Multiple members of this cluster repress tumor suppressors such as PTEN, BIM, and p21, and promote oncogenesis (233). We did observe a drastic increase in mTOR signaling, which correlated with a decrease in PTEN expression, when over-expressing miR-17-92 in effector CD8 T cells, although down-regulation of BIM was not observed. However, virus-specific CD8 T cells over-expressing miR-17-92 did not possess unchecked proliferative and survival capacity as tumor cells, but instead had compromised fitness when transitioning into quiescent memory cells. This suggests that additional checkpoints must exist in effector CD8 T cells to prevent malignancy. For example, the mice used in the experiments demonstrating that miR-17-92 over-expression facilitates the survival of malignant T cells or B cells had additional mutations in oncogenes, such as *Myc* and *Notch* (242, 252). In addition, over-expressing miR-17-92 in immature lymphocytes may allow self-reactive lymphocytes to pass thymic selection and to be constitutively stimulated by their cognate antigens when they develop into mature lymphocytes, which may facilitate oncogenesis (253). Besides the fact that additional factors are required for miR-17-92 to transform lymphocytes, it is also worth noting that while aerobic glycolysis is prominent among

cancer cells, this metabolism pattern is only found in effector T cells. Metabolic changes such as returning to the oxidation of glucose and fatty acids are required for effector CD8 T cells to survive the contraction phase and develop into memory T cells (45). The increased contraction observed in miR-17-92 over-expresssing CD8 T cells may be explained by the failure of this metabolic transition due to excessive mTOR activity. Therefore, future studies to investigate the mechanisms that prevent rapidly proliferating effector T cells from undergoing oncogenesis seem warranted. In addition to PTEN, we also found that SOCS3, a suppressor of cytokine signaling, was down-regulated by miR-17-92. SOCS3 expression is induced by STAT3 in response to IL10 or IL21 signaling and is critical to CD8 T cell memory development (82). It will be interesting to further elucidate additional downstream targets of miR-17-92 in effector CD8 T cells.

In Chapter 3, the importance of miR-17-92 in the CD4 T cell antiviral immune response was examined. Similar to CD8 T cells, CD4 T cells expanded significantly less following infection in the absence of miR-17-92. The B cell response was also reduced as a result of inadequate CD4 T cell help. Interestingly, loss of miR-17-92 seemed to have a stronger impact on IFN-γ^+ CD4 T cells, as the ratio between IFN-γ^+ and tetramer$^+$ CD4 T cells was lower in knockout mice than controls. Consistently, miR-17-92 over-expression enhanced the CD4 T cell response, and preferentially expanded IFN-γ^+ CD4 T cells. Moreover, the ratio between CXCR5$^+$ T_{FH} and CXCR5$^-$ Th1 cells decreased in miR-17-92 over-expressing effector CD4 T cells, as a result of a disproportionate increase in the Th1 response rather than a compromised T_{FH} cell response. Interestingly, memory CD4 T cell population appeared to be stable in mice that over-express miR-17-92 in T cells. Since

differences in the generation of CD4 and CD8 memory T cells have long been documented (297), it would be interesting to further dissect the molecular mechanisms that cause such differences. In addition, we observed that the Ly6C$^+$ LCMV-specific CD4 T cell population was reduced when knocking out miR-17-92 and increased when over-expressing miR-17-92. It would require future studies to illustrate the nature of Ly6C$^+$ effector and memory CD4 T cells and how they are related to antiviral immune responses.

Extensive effort has been made to elucidate the transcriptional mechanisms that regulate the differentiation of different CD4 T cell lineages (171). Transcription factors have been identified as master regulators of different CD4 T cell subsets (e.g. Foxp3 for Treg cells). However, whether master regulator miRNAs exist for each CD4 T cell lineage remains elusive. miRNA flow cytometry or miRNA reporter transgenic mice may serve as useful tools for immunologists to further dissect how translational regulation participates in CD4 T cell lineage commitment (298). Since many miRNAs have been shown to be involved in cell proliferation (299), it would be interesting to examine how the expression of a particular miRNA involved in cell proliferation changes at different phases of the cell cycle. Another challenge in the field is to identify the downstream targets of miRNAs. One miRNA can have hundreds of targets predicted by algorithms (296). These targets need to be verified by experiments such as luciferase reporter assays performed on cell lines (300). Whether a miRNA can suppress the expression of a certain gene depends not only on the existence of the binding site in the 3'UTR of the target transcript, but also on the context of that particular 3'UTR (i.e. the existence of other regulatory elements

including binding sites for other miRNAs). It is obvious that primary immune cells have different expression patterns of translational regulators than cell lines. A miRNA may silence a transcript in a certain subset of immune cells but not other immune cells or cell lines. Therefore, methods such as High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation (HITS-CLIP) performed on primary cells of interest may be more valuable for the identification of direct interactions between miRNAs and target transcripts (301). HITS-CLIP can also identify other machineries that may cooperate with miRNAs in regulating the translation of target transcripts.

In summary, this dissertation has demonstrated that miRNAs are important regulators of the antiviral immune response mediated by T cells. The differentiation of virus-specific effector and memory T cells can be modulated by changing the levels of specific miRNAs, such as miR-17-92. The expression of miR-17-92 is tightly regulated during an antiviral immune response to achieve effective control of viral infection and minimize the chance of autoimmunity or even malignancy. The knowledge obtained from this study and future work on the miR-17-92 signaling network in T cells will not only help us to understand the molecular mechanisms of effector and memory T cell development but also shed light on how the balance between oncogenes and tumor suppressors in somatic cells is shifted during oncogenesis. In addition, this study has also demonstrated the potential to develop more effective vaccination and therapeutic strategies by harnessing miRNA activities.

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