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Defining the let-7 microRNA-mediated molecular mechanisms regulating T cell differentiation

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DEFINING THE LET-7 MICRORNA-MEDIATED MOLECULAR MECHANISMS REGULATING T CELL DIFFERENTIATION

A Dissertation Presented

by

CONSTANCE C. ANGELOU

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**DEFINING THE LET-7 MICRORNA-MEDIATED MOLECULAR
MECHANISMS REGULATING T CELL DIFFERENTIATION**

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DEDICATION

For my parents, who, despite not having a single clue of what I wanted to do, let me do it anyways. If that is not true love, I don't know what is.

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ABSTRACT

DEFINING THE LET-7 MICRORNA-MEDIATED MOLECULAR MECHANISMS REGULATING T CELL DIFFERENTIATION

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$CD4^+$ and $CD8^+$ T cells are lymphocytes of the adaptive immune system that play essential roles in immunity. Both T cell subsets recognize their cognate antigen through the T cell receptor (TCR), which induces the proliferation and differentiation of these antigen-specific cells into effector T cells. $CD4^+$ T cells have the potential to differentiate into one of multiple lineages of helper T (Th) cells and participate indirectly in antigen clearance by orchestrating the function of other cells. $CD8^+$ T cells differentiate into cytotoxic T lymphocytes (CTL), which directly contributes to the resolution of an infection by killing cancerous or virally-infected cells. Upon antigen clearance, most effector T cells die, but some survive and generate long-lived memory T cells that will respond faster and more efficiently to subsequent encounters with the same antigen. When antigen fails to be cleared, such as in chronic infections and cancer, effector T cells are diverted into a hyporesponsive state, exhaustion, characterized by the upregulation of co-inhibitory receptors that transmits inhibitory signals resulting in the loss of effector function and memory potential. Moreover, when T cell differentiation is dysregulated, T cell responses become aberrant, causing autoimmune diseases. Therefore, understanding

the molecular mechanisms controlling T cell responses is important to develop innovative treatments that can enhance T cell activity during infections and cancer, and dampen the generation of disease-causing T cells in autoimmunity. We have uncovered a novel post-transcriptional mechanism regulating T cell differentiation. Particularly, we showed that the let-7 family of miRNAs is highly expressed in naive T cells, but gets dramatically downregulated upon antigen encounter, proportionally to both the strength and duration of TCR stimulation. Specifically, let-7 downregulation was required for the differentiation of pathogenic Th17 cells in experimental autoimmune encephalomyelitis (EAE), a mouse model of the autoimmune disease multiple sclerosis (MS). In CD8$^+$ T cells, although let-7 inhibits CTL differentiation *in vitro*, let-7 was demonstrated both *in silico* and *in vivo* to promote memory CD8$^+$ T cell formation, while repressing the differentiation of terminal effectors, which are susceptible to exhaustion. Thus, let-7 constitutes a promising tool for the therapeutic manipulation of T cell responses.

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CHAPTER 1

INTRODUCTION

1.1 The immune system

The immune system comprises numerous types of bone marrow-derived (hematopoietic) cells that have evolved to recognize and react to foreign substances, known as antigens (Janeway, 1992; Medzhitov, 2007). The immune system's reaction, or immunity, constitutes the body's defense against diseases caused by infectious pathogens, such as viruses, bacteria, protozoa, fungi, and parasites, as well as cancer (Janeway et al., 2017). The immune system can be divided into two distinct, but intertwined, components – the innate and adaptive immune systems **(Figure 1.1)** (Vivier & Malissen, 2005). The innate immune system is a series of broad and immediate defense mechanisms that serve as a first physical and chemical line of defense against pathogens that gets established within minutes after the onset of infection. Innate defense mechanisms consist of physical barriers or epithelia (e.g. skin, oral mucosa, respiratory epithelium, intestine), as well as circulating antimicrobial proteins, and innate immune cells. Innate immune cells include granulocytes, macrophages, dendritic cells, innate lymphoid cells, and natural killer cells, which recognize a wide variety of pathogen- or damage-associated molecules (Akira et al., 2006; Bird et al., 2018). The adaptive immune system is unique to vertebrates, and encompasses B and T cells, which express surface receptors that specifically recognize individual immune determinants, or epitopes, on antigens (Burnet, 1959; Pancer & Cooper, 2007). Adaptive immunity gets activated by antigen-presenting cells, such as dendritic cells, when pathogens resist to innate immune responses, and participates in pathogen elimination by using processes that specifically target antigen-expressing

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pathogens (Busso, 2008; Iwasaki & Medzhitov, 2015). B cells develop in the bone marrow, and contribute to immune responses through the production of antibodies (humoral immunity), while T cells mature in the thymus and secrete effector molecules and cytokines which contribute either directly or indirectly to pathogen clearance (cellular immunity) (Waksman et al., 1962; Ryser & Vassalli, 1974; Spits, 2002; Cooper, 2015). Unlike the innate immune system, adaptive immune responses are slower, as adaptive immune cells need to differentiate and acquire their function after responding to presented antigen. However, the duration of these responses lasts longer and results, after the resolution of an infection, in the formation of long-lived memory cells. These memory cells respond faster, with higher affinity, and more efficiently upon subsequent exposures to the same antigen **(Figure 1.2)** (Hammarlund et al., 2003).

1.2 T lymphocyte development in the thymus

T lymphocyte progenitors are generated following Notch signaling in common lymphoid progenitors in the bone marrow and then migrate to the thymus where they become thymocytes and develop into mature T cells **(Figure 1.3)** (Kondo et al., 1997; Pui et al., 1999; Spits, 2002). The thymus is also the site where thymocytes acquire their lineage identity, characterized by the mutually exclusive surface expression of the co-receptors CD4 and CD8, which will determine whether the generated mature T cells will have the potential to differentiate into helper CD4$^+$ T (Th) cells or CD8$^+$ cytotoxic T lymphocytes (CTLs) (Singer et al., 2008). At the beginning of thymic education, thymocytes are double-negatives (DN), as they do not express any of these surface co-receptors, and undergo irreversible random genetic recombinations of T cell receptor (TCR) gene segments, thereby generating a broad diversity (repertoire) of different

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antigen-specific TCR from only a limited pool of gene segments (Davis & Bjorkman, 1988; Engel & Hedrick, 1988). Each thymocyte then expresses a unique TCR that is transmitted to its progeny, or clone. After productive TCR rearrangement, thymocytes upregulate both co-receptors, thereby becoming double-positives (DP), and are subjected to repertoire (positive) selection (Nikolić-Žugić, 1991; Mombaerts et al., 1992; Starr et al., 2003). During this process, thymocytes interact with thymic epithelial cells, which express self-peptide antigens presented on major histocompatibility complex (MHC) molecules (Scott et al., 1989; Anderson et al., 1996). Thymocytes that fail to recognize self-MHC die "by neglect", while cells that are able to do so are positively selected and thus receive survival signals, thus establishing an MHC-restricted T cell repertoire (Klein et al., 2014). Positively selected thymocytes subsequently go through the process of negative selection, during which thymocytes that have too strong of an affinity for self-peptides receive apoptotic signals, resulting in the deletion of these potentially harmful autoreactive T cells (Ashton-Rickardt et al., 1994). During the same process, DP thymocytes also transition to an intermediate stage during which CD8 gets downregulated, and CD4 versus CD8 lineage commitment occurs following mutually exclusive signaling events (Brugnera et al., 2000). Persistence of positively selecting TCR signals induces the transient expression of the transcription factor ThPOK and results in the generation of single-positive CD4^{+} T cells (He et al., 2005; Luckey et al., 2014). In contrast, cessation of TCR signaling following positive selection allows thymocytes to respond to the cytokine IL-7, the signaling of which drives the upregulation of the transcription factor Runx3, leading to ThPOK repression, co-receptor reversal, and

commitment of intermediate thymocytes to the CD8^{+} T cell lineage (Sato et al., 2005; McCaughtry et al., 2012).

The goal of T cell development in the thymus is thus to produce a functional pool of mature MHC-restricted and self-tolerant mature T cells with distinct differentiation potentials. After thymic education, mature T cells exit the thymus and migrate to secondary lymphoid organs, such as the spleen and lymph nodes (McCaughtry et al., 2007).

1.3 T cell differentiation

1.3.1 Activation of naive T cells

Mature T lymphocytes that migrate to the secondary lymphoid organs after developing in the thymus are naive and appear small and round, with a low cytoplasm-to-nucleus ratio (Donnadieu et al., 1994). Naive T cells are antigen-inexperienced and exist in an inactive, or quiescent, state defined by rare mitotic divisions, minor transcriptional activity, and absence of any effector function (Hamilton & Jameson, 2012). Because of their low activity level, naive T cells have limited metabolic needs, which they fulfill by using oxidative phosphorylation as the predominant metabolic pathway (Chapman et al., 2020). Although devoid of any effector function, naive T cells constitute a sustained homeostatic pool of potential effector cells that will only acquire their function upon antigen recognition, thereby avoiding any spontaneous activity that could become detrimental to the host and would result in the depletion of the naive T cell reservoir (Tzachanis et al., 2004).

Antigen presentation to antigen-specific naive T cells initiates effector T cell differentiation with T cell activation, a process achieved by the combination of three distinct, but synergistic signals **(Figure 1.4)** (Smith-Garvin et al., 2009). The first signal directly derives from the recognition of the cognate antigen presented on MHC molecules (Cone, 1981). MHC class I is recognized by $CD8^+$ T cells and is expressed by all nucleated cell types, while $CD4^+$ T cells recognize MHC class II, which is expressed only on antigen-presenting cells (APCs), such as dendritic cells, macrophages, and B cells, as well as by thymic epithelial cells (Rudolph et al., 2006). Antigens recognized by $CD4^+$ T cells consist of exogenous peptides that get processed in acidified endocytic vesicles (endocytic pathway) into fragments of up to 18 residues-long, whereas $CD8^+$ T cells recognize short, 8-10 residues-long endogenous peptides generated in the cytosol (Stern & Wiley, 1994). The interaction between the TCR and peptide-antigen:MHC complex is stabilized by co-receptor binding to constant domains on MHC molecules (Gao et al., 2002). This interaction further induces conformation changes in the intracellular portion of the co-receptor, which is connected to the kinase Lck, and brings Lck close to the immunoreceptor tyrosine-based activation motifs (ITAMs) of the CD3 complex, which constitutes the signaling component of the TCR (Samelson et al., 1986; Artyomov et al., 2010). Lck phosphorylates the CD3 ITAMs, which trigger TCR signaling, a highly complex series of signal transduction pathways that initiate T cell activation (Glaichenhaus et al., 1991). These pathways promote the nuclear translocation and subsequent activity of transcription factors that are already expressed in naive T cells, but remain in the cytosol in an inactive state until they receive activating signals to translocate into the nucleus and transcribe genes required for the initiation of T cell differentiation

(Figure 1.5). For instance, calcium influx, triggered upon TCR stimulation, activates calmodulin, which interacts with and thereby activates the phosphatase calcineurin (Jain et al., 1993). In turn, calcineurin dephosphorylates the transcription factor NFAT, which is initially sequestered in the cytoplasm, thereby enabling NFAT nuclear translocation and transcriptional activity. Similarly, the MAPK pathway leads to c-Jun phosphorylation, which interacts with phosphorylated c-Fos in the nucleus to form the active transcriptional complex AP-1 (Maciàn et al., 2001). Notably, NFAT and AP-1, by binding to common gene promoters, enhance the strength of TCR activation (Maciàn, 2005). On the other hand, the transcription factor NF-κB is kept in the cytoplasm when bound to the inhibitor IκB. TCR signaling events induce the phosphorylation and subsequent degradation of IκB, which enables the translocation of NF-κB to the nucleus, where it drives the transcription of target genes (Schulze-Luehrmann & Ghosh, 2006). Enabling the transcriptional activity of these three transcription factors thus initiates the expression of genes required for effector T cell differentiation in a timely manner, which is critical to achieve efficient T cell responses.

The second signal results from interactions between co-stimulatory ligands and receptors, such as the CD28 receptor on the T cell and molecules of the B7 family on APCs, and leads to the amplification of the TCR signal, and in the generation of mitogenic, metabolic, and survival signals (Boise et al., 1995; Pagès et al., 1996, Vella et al., 1998; Frauwirth et al., 2002). In fact, absence of co-stimulation during T cell activation elicits a dysfunctional state known as anergy (Harding et al., 1992).

The third signal is mediated by cytokines, which are secreted by multiple immune and non-immune cell types, including APCs, and differ in nature according to the type of

pathogen detected by the host (Curtsinger et al., 1999). For instance, CD28-dependent co-stimulation of activated T cells leads to the expression of IL-2 and the IL-2 receptor high-affinity chain, IL-2Rα, also known as CD25. Binding of IL-2 to its high-affinity receptor is critical during early T cell activation as it promotes T cell survival, growth, and clonal expansion (Pape et al., 1997, Refaeli et al., 1998). Cytokine signaling also plays a decisive role in the acquisition of effector T cell function, which will be discussed later.

1.3.2 Metabolic reprogramming and proliferation

In response to these three stimulatory signals, activated T cells exit quiescence and undergo dramatic transcriptional, morphological, and metabolic transitions, as they prepare to rapidly proliferate in order to produce a large progeny of antigen-specific effector T cells that will mount an efficient response against the antigen (Butz & Bevan, 1998; Frauwirth et al., 2002). The intensified rates of RNA and protein synthesis result in the accumulation of newly produced molecules in the cytoplasm of activated T cells, which leads to cell growth characterized by a greater cytoplasm-to-nucleus ratio (Donnadieu et al., 1994). The exit of activated T cells from quiescence is also marked by their entry into the cell cycle, which promotes the rapid expansion of antigen-specific T cell clones (Butz & Bevan, 1998). This increased cellular activity requires greater amounts of energy and biomacromolecules, which activated T cells satisfy by switching their metabolism from oxidative phosphorylation to glycolysis. In addition to providing ATP more rapidly, glycolysis also results in the production of a large variety of metabolites necessary for the cellular processes needed for T cell proliferation and

functional differentiation, including DNA replication and biomacromolecules synthesis (Chapman et al. 2020).

The initiation of the metabolic switch and cell cycle entry requires the expression of the transcription factor Myc, which is rapidly induced upon T cell activation by both TCR and co-stimulatory signals, and enhanced by IL-2 signaling at later stages of T cell activation (Reed et al., 1985; Wang et al., 2011). On the one hand, Myc drives the expression of glucose transporters and glycolytic enzymes, which contributes to increased glucose uptake and ATP production by glycolysis. On the other hand, Myc also directly induces the expression of cyclins, cyclin-dependent kinases, and transcription factor of the E2F family, while repressing cell cycle inhibitors, thereby promoting cell cycle progression from the G1 to S phase (Grandori & Eisenman, 1997). However, because the continuous expression of Myc is detrimental to T cell survival, Myc is only expressed in a transient manner, but its activity is preserved by upregulation of the transcription factor AP-4, a direct Myc target gene which maintains the metabolically active and proliferative state of differentiating T cells after Myc downregulation (Butz & Bevan, 1998; Chang et al., 2000; Hoffman & Liebermann, 2008; Jung et al., 2008; Chou et al., 2014).

1.3.3 The acquisition of effector T cell function

1.3.3.1 CD4^{+} helper T cells

As mentioned earlier, cytokine signaling plays an important role in the acquisition of effector T cell function. In the case of CD4^{+} T cells, which have the potential to differentiate into one of several helper T (Th) cell lineages, cytokines present during CD4^{+} T cell activation play a central role in inducing specific Th differentiation programs

(Zhu et al., 2010). Each Th cell subset is tailored to respond effectively to distinct types of pathogens, as they each express a signature transcription factor which controls the expression of specific cytokines **(Figure 1.6)** (Zhu & Paul, 2008). The cytokines IL-12 and IFNγ drive the Th1 differentiation program, which is characterized by the expression of the transcription factor T-bet and secretion the cytokine IFNγ. Th1 cells contribute to the clearance of intracellular pathogens and tumor cells by promoting the phagocytic activity and antigen-presenting function of macrophages, as well as the function of CD8$^+$ cytotoxic T lymphocytes (CTLs) (Hsieh et al., 1993; Szabo et al., 2000; Lighvani et al., 2001). Th2 differentiation is induced by IL-4 signaling, which induces the expression of the transcription factor GATA-3 and secretion of the cytokines IL-4, IL-5, and IL-13. Th2 cells activate eosinophils, basophils, and mast cells, and elicit mucus secretion in goblet cells and smooth muscle contraction, all of which work towards the elimination of extracellular pathogens (Swain et al., 1990; Zheng et al., 1997, Kishikawa et al., 2001; Min et al., 2004). The Th17 lineage is promoted by IL-6 in combination with TGF-β, which drives RORγt expression, secretion of IL-17, IL-21, and IL-22, and helps in the clearance of mucosal bacteria and fungi by activating neutrophils and inducing antimicrobial peptide secretion in epithelial cells (Aggarwal et al., 2003; Mangan et al., 2006; Zhou et al., 2007; Yang et al., 2008). Follicualr T (T$_{FH}$) cells are generated upon exposure to IL-6 alone, express the transcription factor Bcl-6, and can secrete different cytokines, depending on the type of immune response, including IL-21, IFNγ, IL-4, IL-5, and TGF-β (Yu et al., 2009). The role of T$_{FH}$ cells is to specify the production of high-affinity antigen-specific antibodies of the appropriate isotype by B cells, phenomena known as somatic hypermutation and immunoglobulin isotype switching, which are

dependent on the interaction between the co-stimulatory molecules CD40 and CD40L (Kawabe et al., 1994; Xu et al., 1994). Thus, through cytokine secretion and cell-cell interactions, Th cells play a critical role in the establishment, coordination, and potentiation of adaptive immune responses by enabling or enhancing the function of immune and non-immune cells, as well as by attracting appropriate immune cells to the site of infection, hence their "helper" appellation. A distinct type of effector CD4$^+$ T cells, regulatory T (Treg) cells, are induced upon exposure to TGF-β and IL-2, express FOXP3, and secrete the immunosuppressive cytokines IL-10 and TGF-β to regulate the magnitude and intensity of immune responses and prevent detrimental consequences on the host (Sakaguchi et al., 1995; Chen et al., 2003; Fontenot et al., 2003; DiPaolo et al., 2007; Kim et al., 2007). The regulation of Th cell responses is essential, as spontaneous or sustained Th cell activity can result in the development of pathologies, such as hypersensitivities and autoimmune disorders (Ohashi, 2002). However, despite undergoing negative selection in the thymus and being kept in check by Tregs, some autoreactive T cell clones are able to escape these regulatory mechanisms and drive autoimmune diseases upon recognition of self-peptide antigens (Yui et al., 1990; Kariv et al., 1993).

1.3.3.2 CD8$^+$ cytotoxic T lymphocytes

Following antigen encounter, CD8$^+$ T cells differentiate into cytotoxic T lymphocytes (CTLs) in the presence of the cytokine IL-2 (Andrus et al., 1984). As the name suggests, CTLs are able to kill targets cells, specifically those infected by intracellular pathogens, such as viruses, or that are cancerous, thus directly contributing to infection clearance **(Figure 1.7)** (Sawamura et al., 1989; Dharakul et al., 1990; Harty et al., 1992; Rodrigues et al., 2003). Differentiation of naive CD8$^+$ T cells into CTLs

requires a complex regulatory interplay between the transcription factors Notch, Runx3, T-bet, Eomesodermin (Eomes), Blimp-1, Id2, and Zeb2 (Pearce et al., 2003; Sullivan et al., 2003; Cannarile et al., 2006; Cho et al., 2009; Cruz-Guilloty et al., 2009; Kallies et al., 2009; Backer et al., 2014; Dominguez et al., 2015). This differentiation program induces the expression by CTLs of the effector molecules Perforin, Granzyme A, and Granzyme B, as well as the death receptor ligand FasL, and the cytokines IFNγ and TNFα (Ruby & Ramshaw, 1991; Suda & Nagata, 1994; Janas et al., 2005, Brehm et al., 2005). Effector molecules are contained within lysosome-derived structures, called secretory granules, and mediate direct cytolysis of target cells upon release, which is triggered upon antigen recognition by CTLs on target cells through TCR:MHC I interactions, a process known as degranulation (Peters et al., 1991). Degranulation involves the reorientation of the Golgi apparatus towards the target cell, where perforin forms a pore in the target cell membrane to enable the entry of the proteases Granzymes A and B (Masson & Tschopp, 1985). Granzyme A is a tryptase that can initiate caspase-independent cell death by cleaving NDUFS3, a protein from complex I of the mitochondrial electron chain, resulting in the disruption of the NADH oxidation process and reactive oxygen species production (Martinvalet et al., 2008). Other identified substrates of Granzyme A are histones and proteins involved in DNA repair pathways, which sensitizes target cells to DNA damage and degradation upon Granzyme A-mediated cleavage (Lieberman, 2010). Granzyme B is a serine-protease that induces apoptosis by cleaving inactive initiator pro-caspases-8 and -10, as well as inactive executioner pro-caspases-3 and -7 into their active forms, which contributes to cell disassembly (Adrain et al., 2005). In human but not in mouse, Granzyme B is also able to induce the intrinsic pathway of apoptosis by cleaving

the pro-apoptotic protein BID, leading to cytochrome c release (Sutton et al., 2000, Casciola-Rosen et al., 2007). Similarly to granule-mediated cytolysis, expression of FasL on CTLs can trigger apoptosis of the target cell through the extrinsic apoptotic pathway by binding to its cognate receptor Fas on the target cell (Rouvier et al., 1993; Suda & Nagata, 1994). In contrast to effector molecules, cytokines secreted by CTLs do not contribute directly to target cell killing. Rather, IFNγ promotes CTL proliferation, motility, and cytotoxicity by stimulating perforin expression (Whitmire et al., 2005; Bhat al., 2017), potentiates Th1 cell differentiation and function, and enhances antigen presentation and Fas expression on target cells (Portnoy et al., 1989; Früh & Yang, 1999; Müllbacher et al., 2002). CTLs are able to produce both soluble and membrane-bound forms of TNFα. Soluble TNFα activates and recruits immune cells to the site of infection, and enhances T cell survival and CTL cytotoxicity (Bancroft et al., 1989; McKenzie et al., 2006; Calzascia et al., 2007; Allie et al., 2013). On the other hand, membrane-bound TNFα has been suggested to participate in "slow lysis" of target cells upon interaction with its receptor TNFR1 (Ratner & Clark, 1993).

1.4 T cell motility, homing and migration to tissues

Efficient immune responses require T cells to adopt appropriate trafficking patterns throughout their lifetime. T cell motility arises from the combination of three distinct processes: autonomous cell locomotion, physical guidance, and response to chemical signals (Krummel et al., 2016). Autonomous cell locomotion depends on the cell-intrinsic motility machinery, including the actin cytoskeleton and motor proteins, while physical guidance is controlled by interactions with the extracellular matrix and surrounding cells, and the response to chemical signals relies on the expression of

chemokine receptors on the T cell surface **(Figure 1.8)**. Depending on their stage of differentiation, T cells express distinct chemokine receptors that sense specific chemokines. The signaling events resulting from these interactions lead to the rearrangement of the actin cytoskeleton, which enables the migration of T cells towards peripheral tissues, where these cells participate in processes ranging from development in the thymus, homing, antigen encounter, and ongoing immune responses at inflamed sites (Griffith et al., 2014). For instance, mature naive T cells traveling in the bloodstream express CCR7, the receptor that recognizes and guides these cells towards gradients of the chemokines CCL19 and CCl21, which are secreted by stromal cells of the secondary lymphoid organs, such as the spleen and lymph nodes, where antigen presentation occurs (Gunn et al., 1998; Förster et al., 1999; Luther et al., 2000). T cells constantly interact with endothelial cells through repeated bind-release events between adhesion molecules, such as L-selectin (CD62L), and their ligands, a process termed "rolling" (Kishimoto et al., 1990). T cells that respond to CCL19/CCL21 upregulate surface adhesion molecules that will bind more strongly to the endothelium, and undergo rearrangement of their actin cytoskeleton that promotes the transmigration of these cells from the bloodstream into the secondary lymphoid organs through specialized blood vessels, high endothelial venules (HEV) (Stein et al., 2000). Engagement of the TCR with peptide antigen:MHC results in the transient upregulation of CD69, as well as the reciprocal downregulation of S1PR1 on activated T cells, which results in their retention in secondary lymphoid organs during activation and differentiation (Matloubian et al., 2004; Shiow et al., 2006). TCR engagement is also accompanied by the reciprocal downregulation of CD62L and upregulation of the adhesion molecule CD44 (DeGrendele et al., 1996). After

differentiation, effector T cells downregulate CD69 expression and restore S1PR1 expression, which facilitates their exit from the secondary lymphoid organs and re-entry into the circulation through diapedesis by following gradients of the S1P1R ligand S1P (Pappu et al., 2007; Bankovich et al., 2010). Many cell types at sites of ongoing immune responses secrete chemokines that will also generate gradients, and result in the recruitment of the appropriate types of effector T cells, which will be specifically responsive to these signals through the expression of the corresponding cognate chemokine receptors (Kagnoff & Eckmann, 1997; Griffith et al., 2014). Once in the inflamed tissue, effector T cells keep following chemokine gradients, and come to an arrest upon antigen re-encounter.

1.5 Memory T cell formation

Upon entry into inflamed sites, large numbers of antigen-specific T cells, peaking at about a week after antigen challenge, accomplish their effector function and participate in antigen clearance (De Boer et al., 2003). After resolution of an infection, these cells are no longer needed, and may even be detrimental to the host, due to the tissue toxicity of the effector molecules and cytokines they secrete (Hutcheson et al., 2008). Accordingly, antigen clearance is followed by the withdrawal of co-stimulatory signals and cytokines, including the survival factor IL-2, which will cause approximately 90% of expanded effector T cell clones to undergo apoptosis mediated by the pro-apoptotic factor Bim, an event known as contraction (Badovinac et al., 2002; Pellegrini et al., 2003, McKinstry et al., 2010). Because they express high levels of the chemokine receptor IL-7α (CD127), Bcl-2, and other anti-apoptotic proteins, the remaining 10% are able to survive and differentiate into long-lived memory T cells. These cells preserve the memory

of the initial antigen exposure and respond more rapidly and efficiently to subsequent challenges with the same antigen **(Figure 1.9)** (Akbar et al., 1993; Mueller et al., 1996, Grayson et al., 2000; Wojciechowski et al., 2006; Whitmire et al., 2008). Memory CD4^{+} T cell differentiation remains poorly understood, but shares many similarities with the mechanisms of CD8^{+} T cell memory formation, which have been more extensively studied (Seder & Ahmed, 2003). In contrast to effector T cells, which are highly active and glycolytic, memory T cells exist in a quiescent state characterized by the preferential utilization of oxidative phosphorylation and fatty acid oxidation, but retain an intermittent self-renewal capacity, and can survive in the absence of interaction with their cognate antigen (Seder & Ahmed, 2003; Pearce et al., 2009; van der Windt et al., 2012).

Several models have proposed distinct explanations for the determination of this terminal-effector (contraction-sensitive)-versus-memory (contraction-resistant) T cell fate dichotomy (Kaech & Cui, 2012). In the asymmetric cell fate model, a single T cell precursor gives rise to both a terminal effector and a memory T cell precursor at the time of the first division during antigen presentation, which depends on APC-proximal versus -distal T cell identity, respectively (Chang et al., 2007). The underlying uneven acquisition of factors following cell division programs the daughter cells to adopt distinct differentiation programs (Arsenio et al., 2014). For instance, the proximal daughter cell obtains more of T-bet, as well as the high-affinity alpha chain of the IL-2 receptor (IL-2Rα or CD25), the signaling of which negatively regulates IL-7Rα (CD127), and thus contributes to inducing terminal effector differentiation (Xue et al., 2002; Kalia et al., 2010; Chang et al., 2011). In contrast, the signal-strength model suggests that the strength of signals 1, 2, and 3 initially received by a T cell during antigen presentation determines

T cell fate. That is, a weak signal strength generates memory-like T cells, while strong signals induce terminal effector-like T cells (Stemberger et al., 2007; Gerlach et al., 2010). In the decreasing-potential model, the cumulative amounts of signals 1, 2, and 3 received throughout the effector T cell stage will similarly shape T cell fate (Kaech & Ahmed, 2001; Harbertson et al., 2002; Williams et al., 2008; Plumlee et al., 2013). Finally, the T memory stem cell model proposes the existence of a subset of memory T cells with stem-like properties that can maintain the memory T cell pool, while being able to give rise to effector T cells (Gattinoni et al., 2011; Graef et al., 2014).

T cells that will become terminal effectors or memory cells can already be distinguished during the primary immune response by specific markers. Memory precursor effector cells (MPECs) highly rely on IL-7 signaling for their maintenance, and accordingly express high levels of its cognate receptor IL-7Rα (CD127), whereas short-lived effector cells (SLECs) express high levels of the terminal effector marker KLRG1 (Kaech et al., 2003; Kondrack et al., 2003; Seddon et al. 2003, al; Lenz et al., 2004; Joshi et al., 2007). Memory T cell differentiation also requires the expression of transcription factors, including Eomes, FOXO1, and Id3, as well as IL-15 and Wnt signaling, which induces the expression of the transcription factors TCF-1 and LEF1 (Zhang et al., 1998; Becker et al., 2002; Gattinoni et al., 2009; Zhou et al., 2010; Yang et al., 2011; Zhou & Xue, 2012; Hess Michelini et al., 2013). However, the memory T cell pool is heterogeneous, and as such exhibit distinct differentiation capacities, proliferative potentials, effector function, and homing characteristics (Buchholz et al,. 2013). T memory stem cells (T_{SCM}) are multipotent and can give rise to all types of memory T cells **(Figure 1.10)**, and accordingly exhibit a high proliferative and self-renewal ability, but

do not display any effector function, and reside in the secondary lymphoid organs (Gattinoni et al., 2011; Lugli et al., 2013). On the contrary, tissue-resident memory T cells (T_{RM}) display the lowest differentiation capacity, but proliferate rapidly and exhibit strong effector function upon recall, and accordingly occupy non-lymphoid organs, such as the lung, liver, skin, and adipose tissue (Beura et al., 2019). In between T_{SCM} and T_{RM} are central-memory (T_{CM}) and effector-memory T (T_{EM}) cells, T_{CM} being less differentiated than T_{EM}, which can be distinguished by their level of CD62L expression (Sallusto et al., 1999; Ahmadzadeh et al., 2001; Pepper & Jenkins, 2011). T_{CM} exhibit high CD62L expression, and accordingly reside in the secondary lymphoid organs. These cells also have a higher proliferative potential and can give rise to T_{RM} that populate the skin (Osborn et al., 2019), but display weaker effector function upon antigen re-challenge (Wherry et al., 2003). T_{EM} express low levels of CD62L, and are found in the circulation (Masopust et al., 2001). This memory T cell subset has a lower proliferation ability than T_{CM}, but elicits a more potent effector response upon antigen re-encounter (Sallusto et al., 2004; Olson et al., 2013; Roberts et al., 2014). Despite their apparent more differentiated phenotype, T_{RM} and T_{EM} have the capacity to give rise to T_{CM} that populate the secondary lymphoid organs (Wherry et al., 2003; Bromley et al., 2013; Beura et al., 2018).

1.6 T cell exhaustion

In distinction from acute infections during which antigen is effectively cleared by functional effector T cells and memory T cells are formed following contraction, antigen persists during chronic infections and cancer, resulting in an unresponsive state known as exhaustion which has been more extensively studied in CD8^{+} T cells than in CD4^{+} T cells (Moskophidis et al., 1993; Crawford et al.; 2014). The exhausted state is characterized by

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compromised effector T cell proliferation, cytotoxic function, and pro-inflammatory cytokine production, as well as by the loss of memory T cell potential (Zajac et al., 1998; Fuller et al., 2003; Wherry et al., 2004; Angelosanto et al., 2012). T cell exhaustion is established progressively through continuous TCR signaling provoked by recurring contacts with the lingering antigen, during which T cells upregulate co-inhibitory receptors, such as PD-1, TIM-3, LAG-3, CD160, 2B4, and CTLA-4 **(Figure 1.11)** (Day et al., 2006; Grosso et al., 2007; Wherry, 2007; Cai et al., 2008; Jones et al., 2008; Bucks et al., 2009; Razziorrouh et al., 2010). During acute infections, these surface molecules get expressed transiently on activated T cells following antigen encounter, and promote self-tolerance by negatively regulating T cell activation and proliferation when bound to their ligand, hence their designation as "immune checkpoints" (Brunet et al., 1987; Triebel et al., 1990; Maïza et al., 1993; Valiante & Trinchieri, 1993; Agata et al., 1996; Monney et al., 2002; Probst et al., 2005). In contrast, during chronic inflammation, exhausted T cells continuously express high levels of these receptors, which can inhibit T cell responses by transmitting negative signals or inducing cell death upon ligand binding (Blackburn et al., 2009). Cognate ligands for these co-inhibitory receptors are upregulated in response to immune signals, such as cytokines, on APCs, as well as on non-hematopoietic cells, including endothelial cells, and are also abundantly expressed in immunosuppressive environments typical of chronic infections and cancer (Mazanet & Hughes, 2002; Liang et al., 2003; Rodig et al., 2003; Mühlbauer et al., 2006). For instance, PD-1 suppresses effector T cell function after interaction with PD-L1 or PD-L2, TIM-3 elicits calcium-dependent cell death when bound to Galectin-9, and CD160 inhibits T cell activation upon binding herpesvirus entry mediator (HVEM) (Freeman et

al., 2000; Latchman et al., 2001; Iwai et al., 2002; Zhu et al., 2005; Mühlbauer et al., 2006; Cai et al., 2008; Mengshol et al.; 2010; Malissen et al., 2019). Other co-inhibitory receptors function by competing for co-stimulatory ligand binding (Blackburn et al., 2009). Specifically, LAG-3 competes with its homolog CD4 for binding to MHC-II, 2B4 competes with CD2 for interacting with CD48, and CTLA-4 competes with CD28 for binding to the B7-family molecules (Baixeras et al., 1992; Brown et al., 1998; Fallarino et al, 1998). Suppression of T cell function following co-inhibitory receptor signaling occurs in a sequential fashion, starting with compromised IL-2 production, proliferation, motility, and cytotoxicity, followed by the loss of the ability to produce TNFα and IFNγ, and eventually concluding with the death of the exhausted T cells (Wherry et al., 2003; Zinselmeyer et al., 2013).

The establishment of the exhausted state in T cells is induced by a dysregulated terminal effector differentiation program derived from persistent TCR stimulation that results in aberrant expression of multiple transcription factors, including BATF, Blimp-1, Eomes, Id2, IRF4, NFAT, TOX, as well as transcription factors from the NR4A family, which in turn control the expression of some co-inhibitory receptors (Cannarile et al., 2006; Wherry, 2007; Oestreich et al., 2008; Shin et al., 2009; Quigley et al., 2010; Buggert et al., 2014; Martinez et al., 2015; Man et al., 2017; Li et al., 2019; Seo et al., 2019). In recent years, the possibility of reverting exhausted T cells back to a functional state has been demonstrated by inhibiting the interaction between co-inhibitory receptors and their ligands using blocking antibodies, thereby preventing co-inhibitory receptor signaling (Leach et al., 1996; Barber et al., 2006; Sakuishi et al,. 2010; Wolchok et al., 2013). This strategy, termed "checkpoint blockade immunotherapy" (CBI), has since

been developed for human therapeutic purposes, and many "checkpoint inhibitors", mainly targeting PD-1, PD-L1, and CTLA-4, have already been approved for the treatment of various types of cancer (Hargadon et al., 2018). However, most patients are resistant to CBI, and some of them experience detrimental effects due to the tissue toxicity of these treatments, especially when used in combination (Topalian et al., 2012; Wolchok et al., 2013; Topalian et al., 2014; Larkin et al., 2015). Studies investigating the possibility of using CBI to treat chronic infectious diseases, such as HIV, hepatitis B, tuberculosis, and malaria, are ongoing (Wykes & Lewin, 2018).

Aside from co-inhibitory receptors, anti-inflammatory cytokine signaling also participates in the induction of T cell exhaustion, as these are abundant during chronic infections and in the tumor microenvironment (Landskron et al., 2014; Beltra & Decaluwe, 2016). Two potent immunosuppressive cytokines are IL-10 and TGFβ, which are secreted by Tregs, but also other T cells, including exhausted T cells and APCs (Tinoco et al., 2009; Ng & Oldstone, 2012; Parish et al., 2014). These cytokines contribute to the suppression of effector T cell function both directly, by restraining T cell proliferation, cytokine production, and cytotoxicity, as well as indirectly, by preventing the upregulation of MHC molecules on APCs, and by suppressing the response of other innate immune cells that normally support effector T cell function, notably macrophages (Geiser et al., 1993; Lee et al., 1997; Corinti et al., 2001; Thomas & Massagué, 2005; Wilson & Brooks, 2011; Stephen et al., 2014; Ip et al., 2017). Similarly to CBI, blocking IL-10 signaling during chronic viral infection using neutralizing antibodies has been shown to restore effector T cell function and result in viral clearance (Brooks et al., 2006; Ejrnaes et al., 2006). However, TGFβ blockade alone

fails to ameliorate effector T cell function in chronic disease, and needs to be combined with CBI to enhance T cell-mediated anti-tumor responses (Garidou et al., 2012; Mariathasan et al., 2018; Tauriello et al., 2018).

1.7 Regulation of T cell differentiation by microRNAs

1.7.1 Biogenesis and function of microRNAs

MicroRNA (miRNA)-mediated RNA interference is one of the most well-studied post-transcriptional mechanisms that potently regulates gene expression (Elbashir et al., 2001; Kim et al., 2008). MiRNAs are short, 20-22 nucleotide-long, non-coding RNAs that bind target mRNAs in a sequence-specific manner to inhibit translation by inducing target mRNA degradation or ribosome stalling (Lee et al., 1993; Lee & Ambros, 2001; Bartel et al., 2009). Several hundreds of miRNAs have been identified in the mammalian genome, and many of these are evolutionarily conserved (Lagos-Quintana et al., 2001; Lewis et al., 2003). Single miRNAs have many target mRNAs, and individual mRNAs can be directly regulated by multiple miRNAs (Lim et al., 2005; Friedman et al., 2009). MiRNAs are involved in the control of a broad range of cellular functions, including cell differentiation, cell cycle progression and apoptosis, which makes these an essential component of the cellular machinery, as they confer robustness to a wide range of biological processes, including development and immunity (Abbott et al., 2005; Farh et al., 2005; Stark et al., 2005). It has been estimated that miRNAs control the expression of approximately one third of the total human gene pool, including genes involved in immune cell differentiation (Lewis et al., 2005). However, dysregulation of miRNA-mediated RNA interference has been shown to result in pathologies, such as

developmental defects, cancer, obesity, and autoimmune diseases (Zhao et al., 2007; Wang et al., 2008; Frost & Olson, 2011; Xu et al., 2013).

MiRNAs can be encoded by genes located either in intergenic regions of the genome, where they rely on their own promoter for transcription, or within gene introns, in which case the transcription of these miRNAs is controlled by the promoter of the neighboring gene (Rodriguez et al., 2004; Baskerville & Bartel, 2005). Because in this situation both the adjoined gene and the miRNA are transcribed at the same time, mRNA splicing and processing is needed to separate the miRNA from the gene mRNA (Kim & Kim, 2007; Melamed et al., 2013; Ramalingam et al., 2014). In addition, miRNA genes can include either a single miRNA (monocistronic) or a cluster of multiple miRNAs (polycistronic), which all share the same promoter (Altuvia et al., 2005; Ozsolak et al., 2008).

MiRNA gene transcription, mediated by RNA polymerases II and III, produces long primary miRNA (pri-miRNA) transcripts, consisting of a succession of hairpin structures **(Figure 1.12)** (Lee et al., 2004; Borchert et al., 2006). Pri-miRNAs are then cleaved by the ribonuclease (RNase) III enzyme Drosha of the microprocessor complex in the nucleus into precursor miRNAs (pre-miRNAs), which are composed of 60-70 nucleotide-long single hairpin structures (Lee et al., 2003). Pre-miRNAs are next exported from the nucleus to the cytoplasm by Exporting 5, where they undergo cleavage of their hairpin loop, mediated by the RNase III enzyme Dicer, which generates mature miRNA duplexes (Knight & Bass, 2001; Yi et al., 2003). Only one strand of this double-stranded mature miRNA, the guide strand, will bind to the RNAse III enzyme Argonaute (AGO) and other proteins to generate the RNA-induced silencing complex (RISC), while

the other strand, the passenger strand, will be degraded (Williams & Rubin, 2002; Kloosterman et al., 2004; Rand et al., 2004; Leuschner et al., 2006; Ameres et al., 2007; MacRae et al., 2008).

MiRNAs recognize their target mRNAs through their seed region, located at their 5' end at positions 2-8, and miRNAs with conserved seed regions are grouped into families which share common mRNA targets (Lewis et al., 2005; Bartel, 2018). RISC antagonizes protein translation by mediating the degradation of target mRNAs when the loaded miRNA seed region is perfectly complementary to the mRNA, or by ribosome stalling in cases of partial mRNA-miRNA seed region complementarity (Vella et al., 2004; Bagga et al., 2005; Baek et al., 2008). MiRNA seed regions most commonly bind sequences canonically located within the 3' untranslated region (UTR) of mRNAs, but some miRNAs binding sites can be found in non-canonical sites outside the 3' UTR, such as within the mRNA 5' UTR or coding sequence (CDS) (Lai, 2002; Lytle et al., 2007; Majoros & Ohler, 2007; Schnall-Levin et al., 2010). Interestingly, recent studies have described a role for sequences outside the seed region in miRNA-mediated RNA interference (Grimson et al., 2007; Brancati & Großhans, 2018).

1.7.2 Role of microRNAs in CD4^{+} T cell differentiation

The crucial regulatory functions of miRNAs in many aspects of T cell development, homeostasis, and differentiation, have been extensively studied (Baumjohann & Ansel, 2013; Wells et al., 2020). Pioneer studies using ectopic overexpression of miRNAs demonstrated that miRNAs were able to direct hematopoietic lineage differentiation (Chen et al., 2004). Moreover, T cell-specific deletion of factors involved in miRNA biogenesis, such as Dicer and Drosha, led to diminished survival and

proliferation of activated T cells (Cobb et al., 2005), but also resulted in enhanced Th1 cell differentiation and inflammatory disease with reduced T_{reg} presence, which suggested that miRNAs can control the quiescent state of naive T cells (Muljo et al., 2005; Cobb et al., 2006; Chong et al., 2008). Subsequent studies identified regulatory roles for individual miRNAs at distinct steps of CD4^{+} T cell differentiation (Baumjohann & Ansel, 2013). For instance, miR-155 and miR-181a enhance CD4^{+} T cell activation and clonal selection (Banerjee et al., 2010; Palin et al., 2013), while miR-146a inhibits it (Yang et al., 2012). Moreover, the miR-17-92 miRNA cluster promotes CD4^{+} T cell proliferation and survival, ultimately resulting in lymphoproliferative disease and autoimmunity (Xiao et al., 2008). Furthermore, Th1 cell differentiation is enhanced by the miR-17-92 cluster and miR-155, but negatively regulated by miR-29, miR-125b, and miR-146a (O'Connell et al., 2010; Jiang et al., 2011; Rossi et al., 2011; Steiner et al., 2011; Yang et al., 2012). MiR-21 facilitates the differentiation of Th2 cells, while miR-27, miR-126, and miR128 prevents it (Mattes et al., 2009; Guerau-de-Arellano et al., 2011; Sawant et al., 2013). Th17 cell differentiation is increased by miR-155, miR-301a, and miR-326, but suppressed by miR-10a (Du et al., 2009; O'Connell et al., 2010; Mycko et al., 2012; Takahashi et al., 2012). The differentiation of T_{FH} cells is positively regulated by the miR-17-92 cluster, whereas miR-10a prevents it (Baumjohann et al., 2013; Takahashi et al., 2012). Finally, T_{reg} cell differentiation is promoted by miR-10a, miR-146a and miR-155, but repressed by the miR-17-92 cluster (Lu et al., 2009; Lu et al., 2010; Jiang et al., 2011; Jeker et al., 2012; Takahashi et al., 2012).

1.7.3 Role of microRNAs in CD8^{+} cytotoxic T lymphocyte differentiation

The importance of miRNA-mediated regulation of CD8^{+} T cell responses was demonstrated through CD8-specific deletion of Dicer, which led to enhanced activation and proliferation, but also diminished survival, effector function, and migratory potential (Zhang & Bevan, 2010). A later study found that Dicer deletion at the naive stage, but not at the activated stage, potentiated CD8^{+} T cell responses through upregulation of Granzyme B, IFNγ, as well as Eomes and its direct transcriptional target Perforin, due to the absence of mature miR-139 and miR-342 (Trifari et al., 2013). Other miRNAs, such as miR-15a/16 and miR-150, were also identified as critical drivers and suppressors of CTL differentiation, respectively (Smith et al., 2015; Yang et al., 2017). Interestingly, some miRNAs that play a role in the regulation of Th1 cell differentiation also control CTL differentiation, such as the miR-17-92 cluster, miR-29, and miR-155, which inhibit the T-bet/IFNγ signaling axis that both CTL and Th1 differentiation programs rely on (Ma et al., 2011; Wu et al., 2012; Gracias et al., 2013).

Although studies profiled the expression of a wide array of miRNAs at different stages of CD8^{+} T cell differentiation, the molecular mechanisms underlying the miRNA-mediated regulation of the CD8^{+} T cell memory versus terminal-effector fate specification remain largely unknown (Wu et al., 2007). Some miRNAs, such as miR-143, miR-155, and the miR-200 family, have been shown to promote memory CD8^{+} T cell formation (Tsai et al., 2013; Guan et al., 2018; Zhang et al., 2018). In contrast, other miRNAs, including miR-15/16, the miR-17-92 cluster, miR-23a, miR-31, and miR-150, have been found to skew the CD8^{+} T cell fate towards terminal effectors and exhausted cells (Wu et al., 2012; Khan et al., 2013; Lin et al., 2014; Ban et al., 2017; Chen et al.,

2017; Moffett et al., 2017; Gagnon et al, 2019). Despite the fact that all these studies significantly contributed to the understanding of the miRNA-mediated control of T cell differentiation and fate determination, whether specific miRNAs consistently play regulatory roles throughout T cell differentiation remains an outstanding question in the field.

1.8 The lethal-7 (let-7) family of microRNAs

1.8.1 Phylogeny and genomic organization of let-7 microRNAs

Lethal-7 (let-7) miRNAs were discovered in the nematode *Caenorhabditis elegans* and were initially described as critical regulators of larval development, in which let-7 deficiency results in premature death (Meneely & Herman, 1984; Lee et al., 1993; Reinhart et al., 2000; Slack et al., 2000). The let-7 family of miRNAs was later found to be highly conserved across bilaterian animals, in which more let-7 isoforms, or members, are found in comparison to *C. elegans*, due to multiple let-7 miRNA gene duplications (Pasquinelli et al., 2000; Hertel et al., 2012). In the mouse, 14 paralog let-7 genes produce 8 mature let-7 members, while in humans 12 miRNA genes give rise to 10 mature let-7 members (Lagos-Quintana et al., 2002). Paralog let-7 miRNA genes are located on distinct chromosomes and different members are identified by a unique letter (e.g. let-7a, let-7b). In addition, identical mature let-7 miRNAs can be generated from distinct let-7 genes, which is indicated by a unique number after the let-7 member name (e.g. let-7a-1, let-7a-2) (Ambros et al., 2003; Roush & Slack, 2008).

1.8.2 Regulation of let-7 microRNA expression

Let-7 miRNAs are expressed in numerous different cell types and control many biological processes, such as cell survival, metabolism, and differentiation (Büssing et al., 2008; Zhu et al., 2011). Particularly, let-7 miRNAs were found to silence the mRNAs of cell cycle progression regulator genes such as Cdc25a and Cdk6, as well as genes involved in carcinogenesis, including HMGA2, Myc, and Ras, demonstrating their robust tumor-suppressing function (Mayr et al, 2007; Büssing et al., 2008). The tight and complex regulation of let-7 miRNAs is well illustrated by the differences in its expression levels in distinct cell types (Reinhardt et al., 2000, Johnson et al., 2003). For instance, in embryonic stem cells, mature let-7 miRNAs cannot be detected, but both pri- and pre-let-7 forms are present, which indicates that let-7 expression is regulated at the post-transcriptional level, at different steps of let-7 biogenesis (Suh et al,. 2004; Thomson et al., 2006). Well-studied factors that inhibit mature let-7 miRNA expression are the fetal proteins Lin28 and Lin28B, which bind to a specific and conserved sequence in the stem-loop region of pri- and pre-let7, thereby preventing Drosha- and Dicer-mediated processing (Piskounova et al., 2008; Viswanathan et al., 2008). Moreover, binding of Lin28 to pre-let-7 leads to the recruitment of the terminal uridylyl transferases (TUTases) TUT4 and Zcchc11, which polyuridylate pre-let-7, thereby marking it for degradation by the exoribonuclease Dis3l2 (Heo et al., 2008; Hagan et al., 2009; Heo et al., 2009; Piskounova et al,. 2011; Chang et al., 2013). Lin28-mediated let-7 miRNA repression therefore constitutes a potent regulatory mechanism during early embryonic development, and has also been shown to play an important role in establishing neonatal immunity (Wang et al., 2016).

1.8.3 Let-7 microRNAs in immunity

In contrast to embryonic stem cells, let-7 miRNAs are highly expressed in lymphocytes (Kuchen et al., 2010). As such, many lymphocyte subsets have been shown to be dependent on let-7 miRNAs for their development, homeostasis, and differentiation. For instance, the upregulation of let-7 miRNAs during natural killer T (NKT) cell development in the thymus is necessary for the differentiation of IFNγ-producing effectors (Pobezinsky et al., 2015). In the periphery, let-7 miRNA expression is required for the survival and maintenance of the quiescent phenotype in naive T cells (Wells et al., 2017; Pobezinskaya et al., 2019). During lymphocyte activation, let-7 miRNAs have been shown to inhibit the metabolic reprogramming of both B cells and $CD8^+$ T cells by negatively regulating the expression of the transcription factor Myc and the glycolytic enzyme hexokinase 2 (Wells et al., 2017; Jiang et al., 2018). Moreover, let-7 miRNAs have been shown to play a regulatory role in $CD8^+$ cytotoxic T lymphocyte (CTL) function by directly targeting the transcription factor Eomes, as well as in $CD4^+$ helper T cell responses during HIV infection and airway inflammation by regulating the expression of the cytokines IL-10 and IL-13, respectively (Polikepahad et al., 2010; Kumar et al., 2011; Swaminathan et al., 2012; Wells et al., 2017). One study also proposed a regulatory role for let-7f expression in Th17 cell function in women with severe asthma (Newcomb et al., 2015). In addition, the exosome-mediated transfer of let-7 miRNAs to various immune cells has been proposed as a suppressive mechanism used by regulatory T (Treg) cells, and has also been reported to inhibit the generation and function of Treg cells (Okoye et al., 2014; Kimura et al., 2018). Although many studies have described regulatory roles for let-7 in T cells, the molecular mechanisms underlying

the control of T cell differentiation and the determination of memory versus terminal effector/exhausted T cell fate remains unclear.

1.9 Rationale, central hypothesis, specific aims, and significance

We have previously shown that all let-7 family members are abundantly expressed in naive $CD8^+$ T cells, but get dramatically downregulated upon antigen recognition, which enables CTL differentiation and function (Wells et al., 2017). Based on these findings, the central hypothesis of this dissertation is that let-7 acts as a central regulator of effector T cell differentiation and fate specification. This main hypothesis will be investigated through two specific aims. The first aim will be to determine the regulatory role of let-7 miRNAs during $CD4^+$ T helper cell differentiation. The second aim will elucidate the signaling pathways controlled by let-7 miRNAs in memory versus terminal-effector/exhausted $CD8^+$ T cell fate specification. Completion of the proposed aims will identify let-7 miRNAs as key regulators of T cell responses. Determining the let-7-mediated regulatory mechanisms governing the differentiation of $CD4^+$ T helper cells and specifying the differentiation of $CD8^+$ T cells towards the memory or terminal effector/exhausted fate will significantly advance our understanding of the post-transcriptional mechanisms controlling the function and fate of T cells. Moreover, this research has the potential to establish let-7 miRNAs as a promising novel therapeutic target or cargo that can adjust T cell responses in the contexts of infectious diseases, cancer, and autoimmunity.
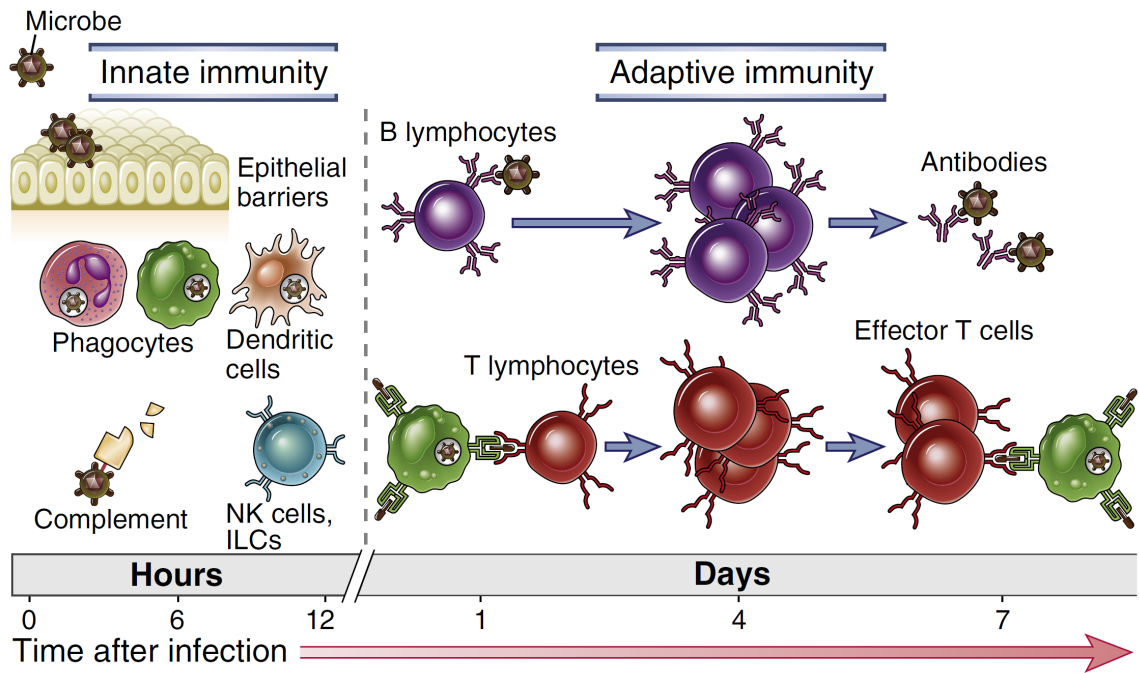
Figure 1.1: Innate and adaptive immune cells and response kinetics. Innate processes of the immune system constitute the first defenses against pathogens, while adaptive mechanisms of immunity, which are mediated by activated lymphocytes, are slower to appear. ILC, innate lymphoid cell; NK, natural killer. Adapted from Cellular and Molecular Immunology, 8th edition, Figure 1-1.
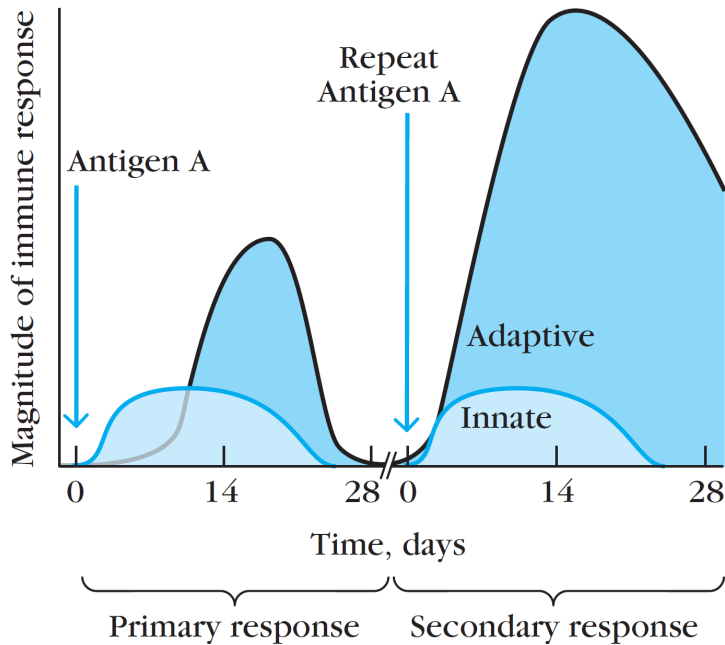
Figure 1.2: The innate and adaptive immune systems show distinct kinetics and amplitudes in primary and secondary response to antigen. The primary adaptive immune response to antigen is low in both duration and amplitude, while subsequent exposure to the same antigen results in secondary responses. These secondary responses recruit memory cells and are thus faster to appear, stronger in amplitude, and more specific to the antigen than was the primary response (dark blue). In contrast, innate immune responses, which do not form immunological memory, remain unchanged every time the antigen is encountered (light blue). Adapted from Cellular and Molecular Immunology, 8th edition, Figure 1-8.
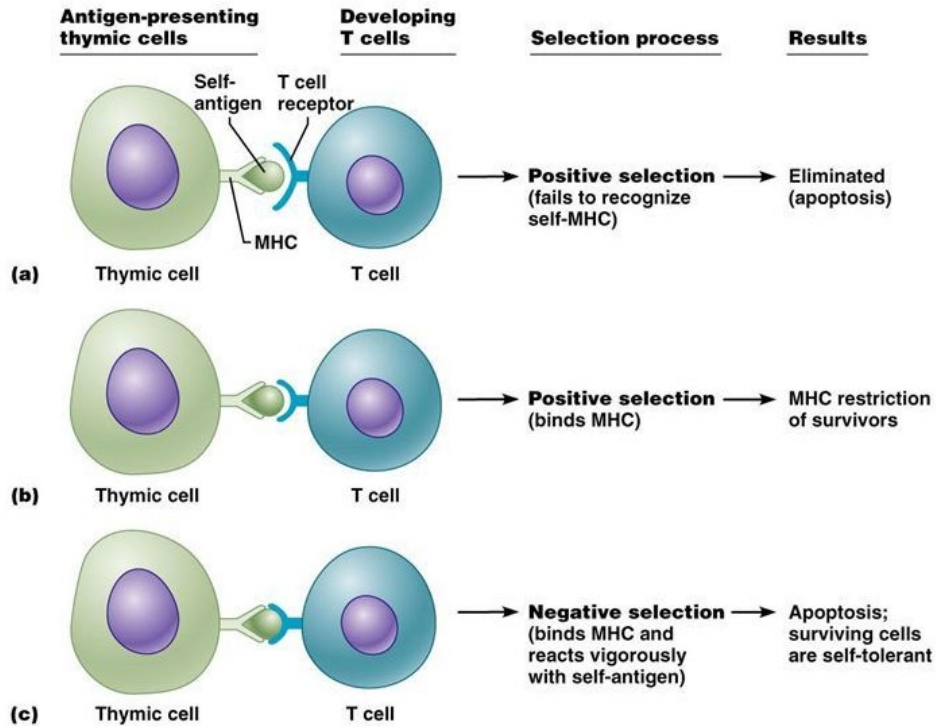
Figure 1.3: T cell selection in the thymus. T cell progenitors migrate from the barrow to the thymus, where they become thymocytes and complete their development by rearranging their T cell receptor genes and undergoing repertoire selection. (a & b) During the process of positive selection, immature T cells that are able to bind self-MHC receive survival signals, while those which fail to recognize self-MHC are eliminated through the induction of apoptosis. (c) Next, the process of negative selection selects self-tolerant thymocytes by providing survival signals, whereas those which react too strongly with self-antigen are deleted from the repertoire by apoptosis.
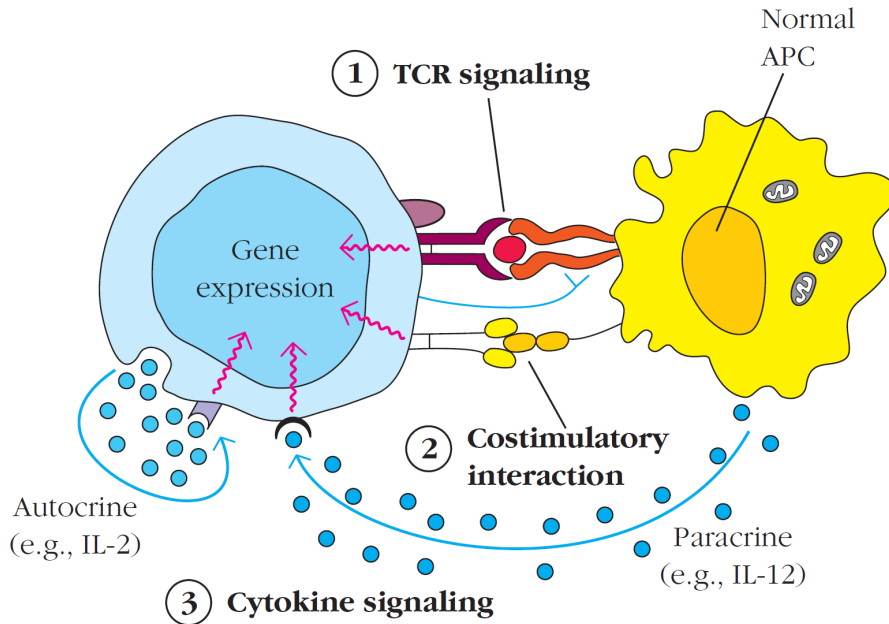
Figure 1.4: Efficient activation of naive T cells by antigen-presenting cells require three types of signals. Upon antigen recognition in the context of peptide:self-MHC complex by the T cell receptor (TCR) on the surface of an antigen-presenting cell (APC), the CD3 complex of the TCR and the co-receptor (CD4 in this example) send signal 1 to the activated T cell (arrow 1). Signal 2 is transmitted by ligation of co-stimulatory receptors, expressed on T cells (CD28 in this example), to their ligand expressed by the APC (molecules of the B7 family in this example) and provide survival, metabolic, and mitogenic signals (arrow 2). Signal 3 is mediated by cytokine signaling, which is important for directing the differentiation of T cells, particularly in the case of CD4^{+} T cells which can differentiate into multiple distinct lineages, depending on the nature of signal 3. Adapted from Owen Kuby Immunology, 7th edition, Figure 11-3.
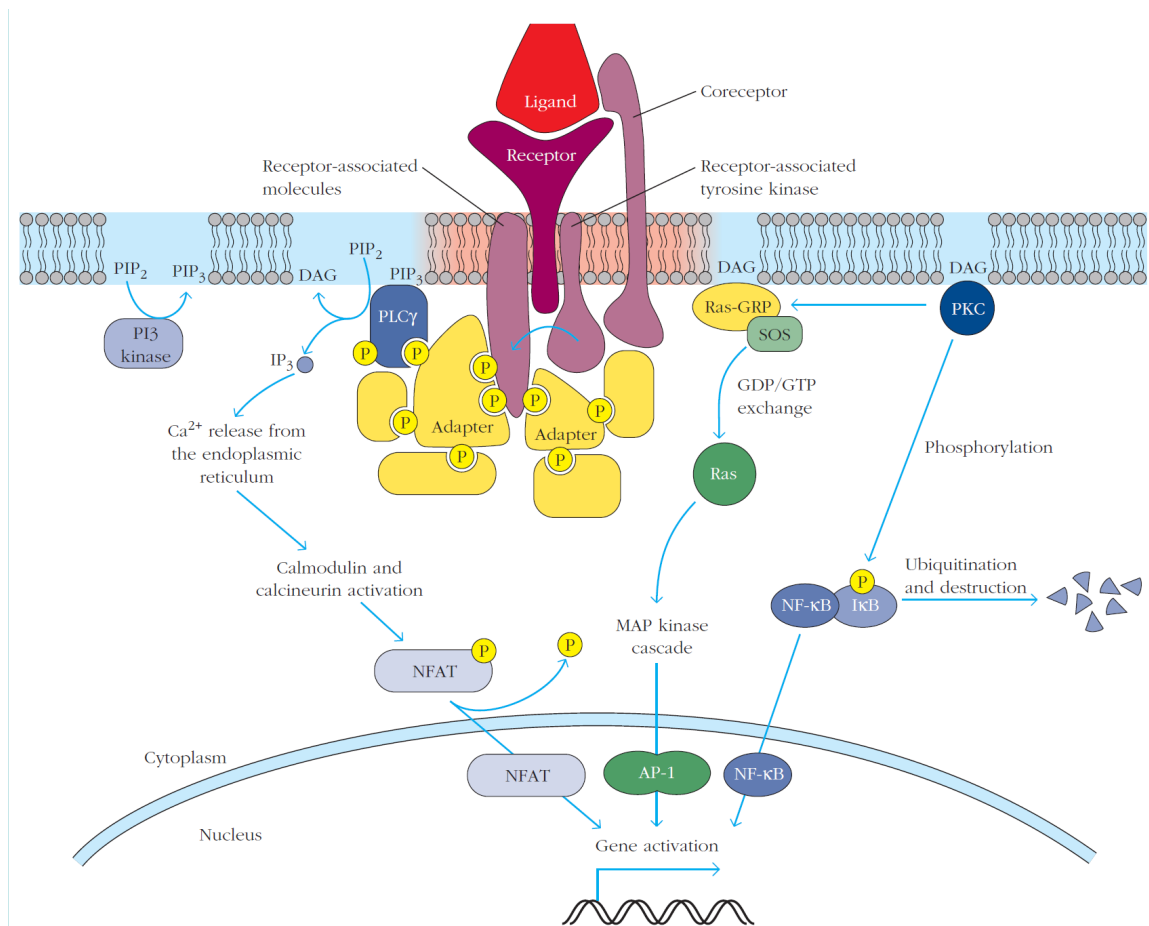
Figure 1.5: Engagement of the T cell receptor results in the activation of transcription factors that initiate the differentiation process. Model representing signaling events occurring upon T cell receptor (TCR) engagement. Recognition of cognate antigen by TCR, which is associated to the CD3 complex, elicits a cascade of phosphorylation events eventually leading to the activation and nuclear translocation of the transcription factors NFκB, NFAT, and AP-1, the latter being a complex comprising c-Fos and c-Jun. Adapted from Cellular and Molecular Immunology, 8th edition, Overview Figure 3-5.
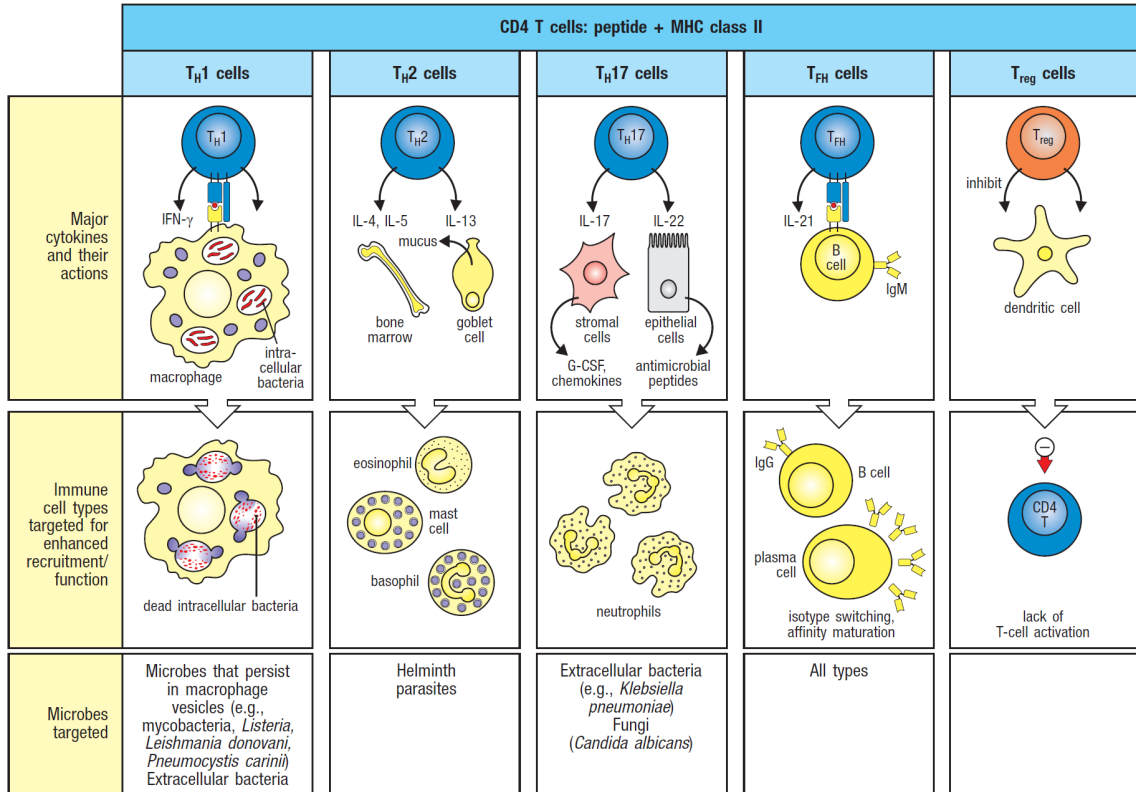
Figure 1.6: CD4⁺ T cells can differentiate into multiple subsets of helper T cells. Each helper T cell lineage is induced by distinct cytokines and specialize in enhancing the function of or recruiting other cells that will directly participate in the elimination of different types of pathogens. Adapted from Janeway's Immunobiology, 9th edition, Figure 9.30.
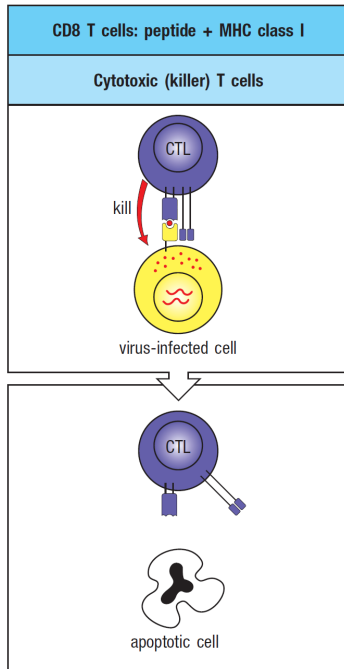
Figure 1.7: CD8+ T cells differentiate into cytotoxic T lymphocytes that can directly kill target cells. Differentiated cytotoxic CD8+ T lymphocytes (CTLs) specialize in lysing cancerous cells and cells infected with intracellular pathogens upon recognition of pathogen fragments presented on MHC class I molecules on the surface of target cells. Adapted from Janeway's Immunobiology, 9th edition, Figure 9.22.
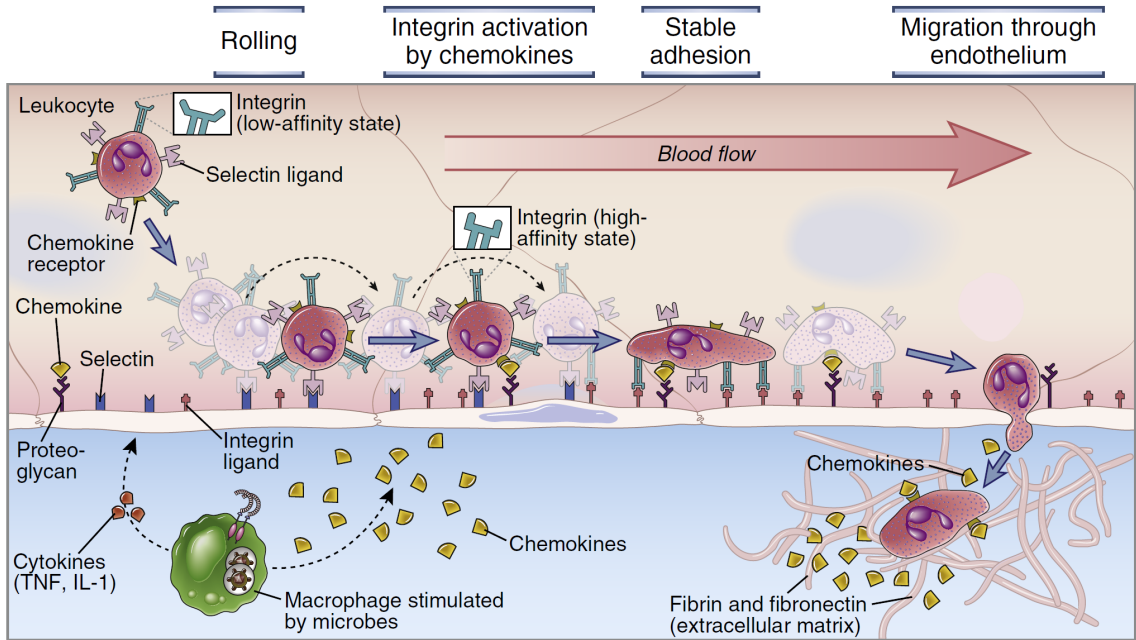
Figure 1.8: Effector T cells reach sites of ongoing immune responses by following physical and chemical cues that induce rearrangements in their actin cytoskeleton.
Effector T cells express surface receptors for cell-adhesion molecules such as selectins and integrins, as well as chemokine receptors. Interactions with cells expressing ligands for selectins and integrins, as well as chemokine gradients, direct the migration of these cells to inflames tissues. Adapted from Cellular and Molecular Immunology, 8th edition, Figure 3-3.
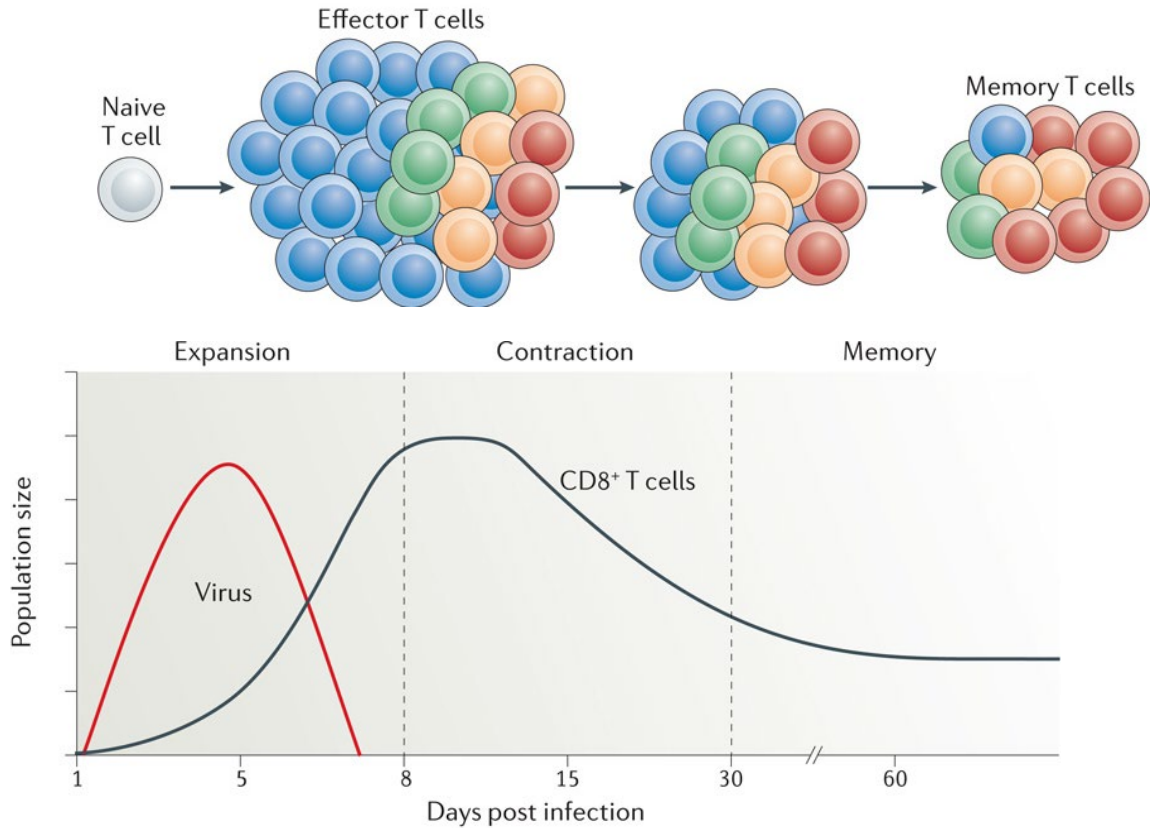
Figure 1.9: Phases of a T cell response in the context of acute infections. Antigen-specific T cells undergo massive clonal expansion upon activation, but most effector T cells undergo apoptosis upon antigen clearance during the contraction phase, while only a small T cell population remains and form long-lasting immunological memory. Adapted from Kaech & Cui, 2012.
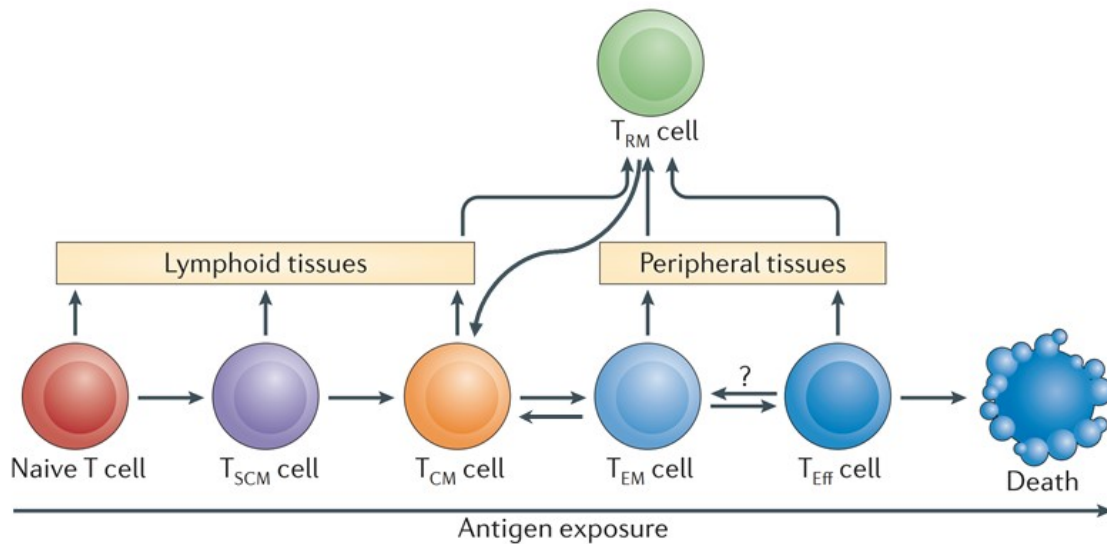
Figure 1.10 Heterogeneity of the memory T cell pool. Multiple memory T cell subsets can differentiate from naïve T cells and exhibit distinct differentiation potential and preferential anatomic location. T memory stem cells (T_{SCM}) are multipotent, reside in the secondary lymphoid organs, and have the greatest proliferative, self-renewal and differentiation capacity. On the other hand, tissue-resident memory T (T_{RM}) cells show the lowest differentiation ability and populate peripheral tissues. In between T_{SCM} and T_{RM} are central (T_{CM}) and effector (T_{EM}) memory T cells, which can be distinguished by differential expression of CD62L. T_{CM} express high levels of CD62L and thus reside in the secondary lymphoid organs, while T_{EM} exhibit low expression of CD62L and are found in the circulation. Despite their apparent more differentiated phenotype, T_{RM} and T_{EM} can give rise to T_{CM} that migrate to the lymphoid organs. Effector T cells (T_{Eff}) cells represent terminally-differentiated cells, which eventually undergo cell death. is one outcome of increased antigen exposure and proliferation. Adapted from Farber et al., 2014.
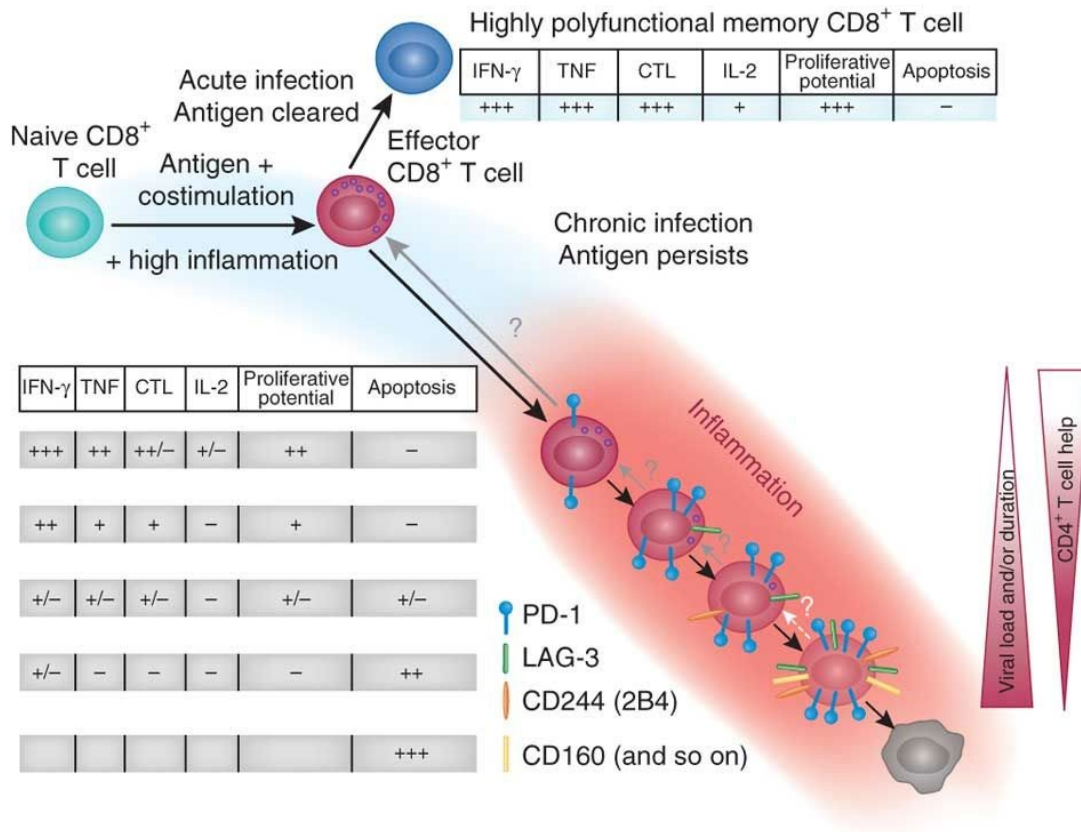
Figure 1.11: Chronic inflammation induces T cell exhaustion. In contrast to acute infection, during which antigen gets cleared efficiently, antigen lingers during persistent viral infection and in the tumor microenvironment, which recurrently signals to the T cell receptor, resulting in the upregulation of co-inhibitory receptors, such as PD-1, LAG-3, 2B4, and CD160. In turn, ligation of these receptors to their cognate ligands present in these immunosuppressive environments transmits negative signals to the T cell, leading to the loss of effector function and memory potential. Adapted from Wherry, 2007.
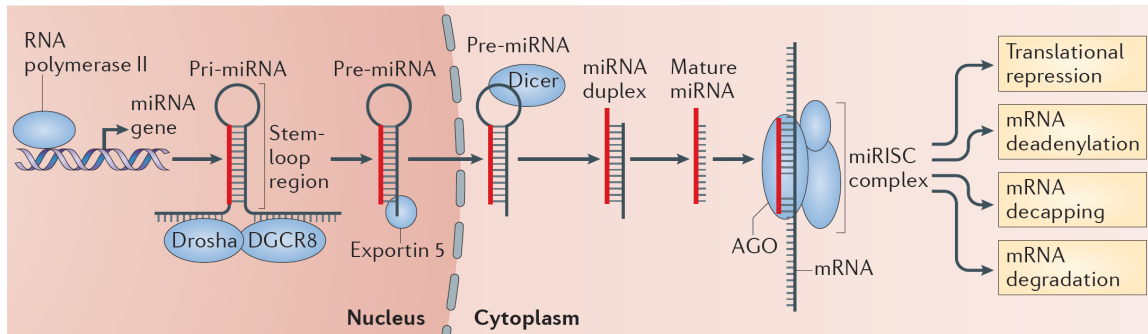
Figure 1.12: MicroRNA biogenesis and function. MicroRNAs (miRNAs) are encoded as genes in the genome and are transcribed by RNA polymerase II into long primary miRNAs (pri-miRNAs), which undergo a first cleavage by the endonuclease Drosha in the nucleus, resulting in the generation of precursor miRNAs (pre-miRNAs.) These pre-miRNAs then get exported by Exportin 5 into the cytoplasm , where the endonuclease Dicer will cleave the loop region to produce mature miRNA duplexes. Only the guide strand will be incorporated into the RNA-induced silencing complex (RISC) and participate in the silencing of messenger RNA (mRNA) targets, while the passenger strand will undergo degradation. Adapted from Baumjohann & Ansel, 2013.

CHAPTER 2

DIFFERENTIATION OF PATHOGENIC TH17 CELLS IS NEGATIVELY REGULATED BY LET-7 MICRORNAS IN A MOUSE MODEL OF MULTIPLE SCLEROSIS

2.1 Introduction

CD4^{+} helper T cells play essential roles in the function of the immune system by orchestrating immune responses against a broad range of pathogens, supporting the function of CD8^{+} T cells, helping B cells produce antibodies, activating macrophages and granulocytes, eliciting memory responses, as well as by regulating the magnitude and persistence of immune responses (Zhu & Paul, 2008). However, the dysregulation of CD4^{+} T cell responses causes aberrant effector CD4^{+} T cell activity, including overproduction of pro-inflammatory cytokines, eventually resulting in the manifestation of autoimmune disorders, such as multiple sclerosis (MS) and related diseases (Wucherpfennig, 2001; Goodnow, 2007). MS is a chronic inflammatory disease of the central nervous system (CNS) that affects approximately 2.5 million people worldwide, with a strong predominance in women (Compston & Coles, 2008). The animal model of neuroinflammation, experimental autoimmune encephalomyelitis (EAE), recapitulates the pathological and clinical symptoms of MS and has been extensively used to study this disorder (Rangachari & Kuchroo, 2013). In both MS and EAE, autoreactive CD4^{+} type-17 helper T (Th17) cells that are generated by exposure to IL-23 and IL-1β (Langrish et al., 2005; Sutton et al., 2006, Komuczki et al., 2019) migrate to the CNS and cross the blood-brain barrier by following gradients of chemokines secreted by CNS-resident innate lymphoid cells (Kwong et al., 2017). The transcription factor Bhlhe40, which is

induced in encephalitogenic Th17 by IL-1β signaling, positively regulates the secretion of granulocyte-macrophage colony-stimulating factor (GM-CSF) (Martínez-Llordella et al., 2013; Lin et al., 2014; Lin et al., 2016). GM-CSF is a proinflammatory cytokine essential for disease induction as it promotes the activation, differentiation, and recruitment of monocytes and dendritic cells to the CNS, as well as the mobilization of the local microglia (McQualter et al., 2001; Ponomarev et al., 2007; Codarri et al., 2011; El-Behi et al., 2011; Spath et al., 2017; Komuczki et al., 2019). In turn, GM-CSF-stimulated glial and dendritic cells secrete IL-6 and IL-23, thereby reinforcing the differentiation and maintenance of pathogenic Th17 cells (Sonderegger et al., 2008). Inflammatory myeloid cells produce reactive oxygen species and cytokines that cause neuron demyelination and axonal damage, which leads to the disruption of neuronal signaling, eventually resulting in disabling physical symptoms, including progressive loss of motor function, which reflect the extent of neurodegeneration (Compston & Coles, 2008).

About a third of the total risk factors for MS development can be attributed to genetic variation, and genome-wide association studies have identified more than 100 different genetic variants associated with MS and related autoimmune disorders (Lock et al., 2002; Duerr et al., 2006; Stuart et al., 2010; International Multiple Sclerosis Genetics Consortium et al., 2013). Many of these susceptibility factors consist of variants of genes which are involved in Th17 pathways and contain single nucleotide polymorphisms within the untranslated regions (UTRs) of their mRNA. Given that UTR sequences are targeted by factors controlling mRNA translation and stability (Barrett et al., 2012), the post-transcriptional dysregulation of these genes, particularly by miRNAs, may be

responsible for the aberrant cytokine responsiveness and effector function observed in autoreactive Th17 cells. In fact, altered miRNA expression has been shown in encephalitogenic Th17 cells from active MS lesions, and specific upregulated miRNAs, such as miR-155 and miR-326, were demonstrated to promote the pathogenic Th17 phenotype (Du et al., 2009; Junker et al., 2009; O'Connell et al., 2010; Ma et al., 2014).

Although regulatory roles have been described for let-7 miRNAs in the control of the differentiation of cytotoxic CD8$^+$ T cells and various subsets of effector CD4$^+$ T cells (Polikepahad et al., 2010; Kumar et al., 2011; Swaminathan et al., 2012; Okoye et al., 2014; Wells et al., 2017; Kimura et al., 2018), the precise contribution of let-7 miRNAs in autoreactive Th17 cell differentiation and MS pathogenesis has been controversial (Ma et al., 2014). Disease-promoting roles have been proposed for individual let-7 miRNA family members (Junker et al., 2009; Guan et al., 2013; Kimura et al., 2018), but other reports have suggested that let-7 miRNA expression may confer protection against MS (Cox et al., 2010; Martinelli-Boneschi et al., 2012). Therefore, the objective of this project was to investigate the role of let-7 miRNAs in the regulation of pathogenic Th17 cell generation and identify the underlying molecular mechanisms.

We demonstrated that the differentiation of pathogenic Th17 cells in EAE requires the downregulation of let-7 miRNAs in naive CD4$^+$ T cells upon antigen stimulation. Specifically, we found that high let-7 miRNA expression in activated CD4$^+$ T cells prevents EAE development by inhibiting the clonal expansion, IL23R/IL-1R1-dependent acquisition of pathogenic function, and CCR2/CCR5-dependent chemokine-mediated migration of Th17 cells to the CNS, while depletion of let-7 miRNAs enhances Th17 cell pathogenicity and aggravates EAE. Therefore, let-7 miRNA delivery to pathogenic Th17

cells may be a promising therapeutic strategy for the treatment of autoimmune diseases such as MS.

2.2 Results

2.2.1 Let-7 expression in CD4^{+} T cells inhibits EAE development

The role of let-7 miRNAs in the differentiation of pathogenic CD4^{+} T cells remains unclear (Junker et al., 2009; Cox et al., 2010; Martinelli-Boneschi et al., 2012; Guan et al., 2013; Ma et al., 2014; Kimura et al., 2018). Previously, we have shown that let-7 miRNA expression in CD8^{+} T cells prevents the differentiation of CTLs and must be downregulated during antigen stimulation (Wells et al., 2017). Here, we found that, similarly to CD8^{+} T cells, naive CD4^{+} T cells expressed high levels of let-7, which were rapidly downregulated upon activation, proportionally to both the duration **(Figure 2.1A)** and strength of TCR stimulation **(Figure 2.1B)**. Based on these observations, we hypothesized that let-7 miRNAs inhibit the differentiation of CD4^{+} T cells and may therefore compromise the generation of pathogenic Th17 cells, thereby suppressing the development of autoimmune disorders.

To test our hypothesis *in vivo*, we acquired mice with a doxycycline-inducible let-7g transgene (Let-7Tg) in order to maintain high let-7g expression in activated CD4^{+} T cells (Zhu et al., 2011). We used EAE susceptibility as a readout of CD4^{+} T cell differentiation in doxycycline- or vehicle-treated Let-7Tg and WT control mice immunized with the peptide antigen myelin oligodendrocyte glycoprotein, residues 35-55 (MOG$_{35-55}$), in complete Freund's adjuvant (CFA). Only doxycycline-treated Let-7Tg mice developed a significantly milder disease in comparison to control mice **(Figure**

2.2A). Strikingly, the number of mononuclear cells **(Figure 2.2B)** and CD4$^+$ T lymphocytes **(Figure 2.2C)** that infiltrated the CNS was strongly diminished in these mice. Overall, the frequencies and numbers of cytokine-producing CD4$^+$ T cells were greatly reduced in the CNS of doxycycline-treated Let-7Tg mice **(Figure 2.2D)**. In addition, *in vitro* MOG$_{35-55}$-restimulated splenocytes from the same mice secreted less IL-17, IFNγ, and GM-CSF in comparison to that of control mice **(Figure 2.2E)**. We obtained similar results using WT and Let-7Tg mice on a 2D2 RAG2-deficient (2D2Rag2KO) background, in which all CD4$^+$ T cells express the 2D2 transgenic T cell receptor that recognizes the MOG$_{35-55}$ peptide (Bettelli et al., 2003) **(Figure 2.3)**. To assess whether the absence of let-7 miRNAs in CD4$^+$ T cells leads to aggravated EAE, we used Lin28 transgenic mice (Lin28Tg) with T-cell specific ectopic overexpression of the fetal protein Lin28B, which blocks let-7 miRNA biogenesis (Heo et al., 2008; Piskounova et al., 2008; Newman et al., 2008; Heo et al., 2009; Piskounova et al., 2011; Pobezinsky et al., 2015). 2D2Rag2KO Lin28Tg mice developed stronger symptoms of EAE, during which the phenotype of cytokine-producing pathogenic CD4$^+$ T cells was enhanced, even though the extent of CD4$^+$ T cell infiltration into the CNS was unchanged in comparison to controls **(Figure 2.3)**, suggesting that let-7 miRNAs inhibit EAE development.

To determine whether the let-7 miRNA-mediated protection against EAE is CD4$^+$ T cell-intrinsic, we adoptively transferred naive 2D2Rag2KO CD4$^+$ T cells from Let-7Tg, Lin28Tg and control mice, into Rag2KO recipient mice that were subsequently immunized with MOG$_{35-55}$ in CFA. At day 9 post-EAE induction, disease outcome **(Figure 2.4A)**, CNS infiltration **(Figure 2.4B)**, as well as frequencies and numbers of

cytokine-producing donor 2D2Rag2KO CD4$^+$ T cells that had differentiated into pathogenic CD4$^+$ T cells **(Figures 2.4C and 2.4D)** recapitulated the results from our previous EAE experiments **(Figures 2.2 and 2.3)**. These results demonstrate that let-7 abolishes the development of EAE in a CD4$^+$ T cell-intrinsic manner.

Although Let-7Tg CD4$^+$ T cells were largely absent in the CNS of EAE-induced mice, they were found in the spleen, albeit at lower numbers than control cells **(Figure 2.4E)**. To explain this phenotype, we proposed four potential mechanisms: 1) poor cell survival, 2) reduced proliferation, 3) compromised differentiation, or 4) impaired trafficking.

2.2.2 Let-7 promotes survival but restricts the proliferation of activated CD4$^+$ T lymphocytes

To examine whether let-7 miRNAs negatively regulate the survival of activated CD4$^+$ T cells during EAE, we measured the survival rate of Let-7Tg, Lin28Tg and WT control CD4$^+$ T cells activated *in vitro* for 3 days. Interestingly, Let-7Tg cells survived better than their WT counterparts, while the recovery of Lin28Tg lymphocytes was significantly lower **(Figure 2.5A)**. In fact, these results are in agreement with our recently published findings in naive CD4$^+$ and CD8$^+$ T cells (Pobezinskaya et al., 2019), suggesting that let-7 expression also supports the survival of activated CD4$^+$ T cells. Thus, the reduced numbers of CNS-infiltrated Let-7Tg CD4$^+$ T cells recovered during EAE cannot be explained by increased cell death.

Based on our data in CD8$^+$ T cells (Wells et al., 2017), we tested whether let-7 miRNAs suppress CD4$^+$ T cell proliferation as well. To address this question, Let-7Tg, Lin28Tg and WT control naive CD4$^+$ T cells were labeled with CellTrace Violet (CTV)

and activated *in vitro*. We observed that Let-7Tg CD4$^+$ T cells proliferated less than activated control T cells, while Lin28Tg CD4$^+$ T lymphocytes proliferated more **(Figure 2.5B)**, even though this effect was less pronounced than in CD8$^+$ T cells. These findings suggest that let-7 restricts CD4$^+$ T cell proliferation, which, in turn, can contribute to the diminished numbers of CNS-infiltrated Let-7Tg CD4$^+$ T cells observed in EAE.

Given that Let-7Tg CD4$^+$ T cells exhibit a lower proliferative potential, we investigated whether let-7 inhibits the expression of genes that regulate cell cycle progression and the metabolic switch in antigen-stimulated CD4$^+$ T cells. Similarly to the results observed in CD8$^+$ T cells, activated Let-7Tg CD4$^+$ T cells expressed low levels of cyclin D2 (*Ccnd2*), cyclin-dependent kinase 6 (*Cdk6*), phosphatase Cdc25a (*Cdc25a*), and ubiquitin-conjugating enzyme Cdc34 (*Cdc34*), as well as the transcription factor *Myc* and several Myc target genes involved in glycolysis and protein synthesis (*Tfap4*, *Glut3*, *Hk2*, *Ldha*, *Qars*, *Yars*), while in Lin28Tg cells those genes were derepressed (Galaktionov et al., 1996; Johnson et al., 2007; Legesse-Miller et al., 2009; Wells et al., 2017) **(Figures 2.5C and 2.5D)**. These results indicate that let-7 miRNAs may restrain CD4$^+$ T cell proliferation by suppressing the metabolic switch and cell cycle progression. These data also suggest that Lin28Tg CD4$^+$ T cells are able to elicit aggravated EAE because of their proliferative and metabolic advantage over WT CD4$^+$ T cells, despite a survival defect.

2.2.3 Let-7 negatively regulates the differentiation of pathogenic Th17 cells

Alternatively, the low frequency of effector CD4$^+$ T cells in the CNS of Let-7Tg mice during EAE could be due to a defect in autoreactive CD4$^+$ T cell differentiation. Therefore, we tested the ability of let-7 miRNAs to influence the differentiation of

pathogenic Th17 cells. Naive 2D2Rag2KO CD4$^+$ T cells with different levels of let-7 expression were polarized *in vitro* towards the TGFβ-independent pathogenic Th17 lineage in the presence of the cytokines IL-1β, IL-6, and IL-23 (Chung et al., 2009; Ghoreschi et al., 2010). We confirmed that, while the expression of let-7 miRNAs was downregulated over time in 2D2Rag2KO WT cells **(Figure 2.6A)**, the expression of the let-7g transgene remained high in 2D2Rag2KO Let-7Tg cells before and after differentiation **(Figure 2.6B)**, and Lin28 expression could only be detected at substantial levels in 2D2Rag2KO Lin28Tg cells **(Figure 2.6C)**. Notably, very low frequencies of Let-7Tg pathogenic Th17 cells expressed IL-17 and GM-CSF as compared to WT controls, whereas Lin28Tg cells had increased percentages of cells that expressed these cytokines **(Figure 2.7A)**. This trend was also observed in the frequencies of IL-17$^+$GM-CSF$^+$ double positive cells, suggested to be the most pathogenic Th17 cells in EAE (Ponomarev et al., 2007; Sonderegger et al., 2008; Codarri et al., 2011; El-Behi et al., 2011; Spath et al., 2017). Furthermore, the extent of *Il17a* and *Csf2* mRNA expression correlated with the percentage range of IL-17A- and GM-CSF-producing cells **(Figure 2.7B)**. These results suggest that let-7 expression in CD4$^+$ T lymphocytes prevents the differentiation of pathogenic Th17 cells.

We observed that the expression of the cytokine receptors *Il1r1* and *Il23r*, both previously described as essential for the differentiation of pathogenic Th17 cells in EAE (Langrish et al., 2005; Matsuki et al., 2006; McGeachy et al., 2009; El-Behi et al., 2011; Komuczki et al., 2019), was profoundly inhibited in Let-7Tg and derepressed in Lin28Tg pathogenic Th17-polarized cells. Analysis of *Il1r1* and *Il23r* mRNA sequences revealed multiple potential let-7 binding motifs which can be directly targeted by let-7 **(Figure**

2.7C). In fact, such regulation was previously proposed for the mRNA of *IL23R* in human memory CD4^{+} T cells (Li et al., 2011). Interestingly, although miRNA binding sites are most commonly found within the 3' UTRs of mRNAs, some let-7 binding sites were located within the coding sequence of mouse and human *Il1r1* and *Il23r* mRNAs. On the other hand, the expression of the transcription factor *Bhlhe40*, shown to be induced by IL-1R signaling (Lin et al., 2016) and essential for the pathogenicity of Th17 cells in EAE (Martínez-Llordella et al., 2013; Lin et al., 2014), was only slightly reduced in Let-7Tg pathogenic Th17-polarized cells, but drastically enhanced in Lin28Tg lymphocytes **(Figure 2.7B)**. Let-7 miRNAs did not repress the expression of other genes controlling the Th17 cell fate, such as *Il6ra*, *Il6st*, *Irf4* and *Rorc*, and had little impact on *Stat3* expression **(Figure 2.7D)**, even though *Il6ra*, *Stat3*, and *Rorc* are predicted direct let-7 targets (Agarwal et a;., 2015). Thus, these results suggest that let-7 miRNAs negatively regulate the acquisition of pathogenic Th17 phenotype and may act through directly targeting *Il1r1* and *Il23r* transcripts.

Of note, in agreement with previously published reports (Polikepahad et al., 2010; Kumar et al., 2011; Swaminathan et al., 2012), we confirmed that let-7 expression also blocks the differentiation of Th0, Th1, and Th2 cells. Surprisingly, let-7 did not suppress the differentiation of non-pathogenic Th17 cells generated in the presence of IL-6 and TGF-β **(Figures 2.8A and 2.8B)** (McGeachy et al., 2007). Moreover, there was no obvious effect of let-7 on the generation of iTregs in polyclonal polarization cultures, but in monoclonal 2D2Rag2KO cells iTreg differentiation was quite substantially inhibited by let-7 **(Figures 2.8A and 2.8B)**. The expression level of *Ifng*, *Il4*, and *Csf2* mRNAs was consistent with the range of frequencies of IFNγ[+], IL-4^{+} and GM-CSF^{+} cells in Th0,

Th1, and Th2 cultures **(Figures 2.9)**. The expression of genes encoding lineage-specific cytokine receptors, such as *Il12rb2* (Th1) and *Il4ra* (Th2), as well as lineage-specific transcription factors, including *Tbx21* (Th1) and *Gata3* (Th2), and the transcription factor *Bhlhe40*, which has been shown to promote a proinflammatory phenotype in Th1 cells (Yu et al., 2018), was also repressed in Let-7Tg Th0, Th1, and Th2 cells, but increased in Lin28Tg cells. Thus, these results suggest a broader suppressive role for let-7 miRNAs in the regulation of effector CD4$^+$ T cell differentiation.

2.2.4 Let-7 prevents the chemokine-mediated migration of pathogenic Th17 cells

In addition to reduced proliferation potential and compromised differentiation, the lower number of effector Let-7Tg CD4$^+$ T cells in the CNS may be due to impaired cell trafficking. Antigen-stimulated T cells upregulate chemokine receptors to sense, migrate, and home to the location of inflammatory sites by following gradients of chemokines (Griffith et al., 2014). Two chemokine receptors, CCR2 and CCR5, have been shown to be critical for the migration of pathogenic T cells to the CNS and subsequently for EAE development (Fife et al., 2000; Szczuciński & Losy, 2007; Kara et al., 2015; Gu et al., 2016). To determine whether let-7 regulates CCR2 and CCR5 expression, we measured *Ccr2* and *Ccr5* mRNA levels in *in vitro*-generated 2D2Rag2KO pathogenic Th17 cells from WT, Let-7Tg and Lin28Tg mice. Surprisingly, the expression of both *Ccr2* and *Ccr5* was very low in Let-7Tg cells, while in Lin28Tg cells it was enhanced **(Figures 2.10A)**. Interestingly, we found potential let-7 binding sites within the mRNA of both *Ccr2* and *Ccr5* **(Figures 2.10B)**. To test whether these binding sites were functional, we transfected NIH3T3 fibroblasts, which have high endogenous expression of let-7 miRNAs, with dual luciferase vectors containing the wild-type sequence of these binding motifs. An ability

for let-7 to bind to both sites in the *Ccr2* mRNA, and one site in *Ccr5* mRNA was demonstrated by a significant reduction in luciferase activity **(Figures 2.10C)**. Mutation of these binding sites restored luciferase activity, confirming direct let-7 targeting. To test whether the let-7-mediated suppression of CCR2 and CCR5 expression is sufficient to prevent chemokine-mediated migration of Th17 cells towards their specific ligands, CCL2 and CCL4, we subjected *in vitro*-generated 2D2Rag2KO WT, Let-7Tg and Lin28Tg pathogenic Th17 cells to chemokine-mediated migration assays. Indeed, 2D2Rag2KO Let-7Tg cells exhibited compromised trafficking in response to both chemokines alone and in combination, while Lin28Tg cells migrated more efficiently than WT cells **(Figures 2.10D)**. Even though changes in cell motility can contribute to the difference in trafficking of Th17 cells, neither speed nor other intrinsic motility variables (track length, track straightness, and cell displacement) were negatively affected by let-7 expression **(Figures 2.11)**. To test whether let-7 miRNAs prevent the migration of pathogenic Th17 cells by inhibiting CCR2 and CCR5 expression, we overexpressed *Ccr2* or *Ccr5* in *in vitro*-generated 2D2Rag2KO WT and 2D2Rag2KO Let-7Tg pathogenic Th17 cells **(Figures 2.10E)** and tested the migratory potential of these cells using chemokine-mediated migration assays. Surprisingly, only overexpression of *Ccr5*, but not *Ccr2*, partially rescued the chemotaxis of 2D2Rag2KO Let-7Tg pathogenic Th17 cells towards CCL4, and enhanced the migration of 2D2Rag2KO WT pathogenic Th17 cells **(Figures 2.10F)**. These results strongly suggest that let-7 miRNAs restrict the CCR5-mediated migration of pathogenic Th17 cells by directly binding to *Ccr5* mRNA and inhibiting CCR5 expression, and additional let-7-mediated regulatory mechanisms are involved in the CCR2-mediated migration of these cells.

Altogether, our data show that let-7 miRNAs control the pathogenicity of Th17 cell in EAE by restricting their clonal expansion, inhibiting IL-1R1/IL-23R-dependent differentiation and preventing CCR2/CCR5-mediated migration to the CNS. As such, we propose that let-7 miRNAs may constitute a promising therapeutic target for the treatment of autoimmune diseases such as MS.

2.3 Discussion

In the present study, we have identified let-7 miRNAs as critical negative regulators of pathogenic Th17 cell differentiation and EAE development. Specifically, we found that, similarly to CD8$^+$ T cells (Wells et al., 2017), the expression of let-7 miRNAs in naive CD4$^+$ T cells is downregulated upon activation, which is essential for the clonal expansion, acquisition of pathogenic Th17 phenotype, and migration to the CNS. We demonstrate that high let-7 miRNA expression in activated CD4$^+$ T cells confers almost complete protection against EAE by preventing CD4$^+$ T cell pathogenicity and infiltration in the CNS, while in the absence of let-7 miRNAs the development of EAE is exacerbated.

The role of miRNAs in the regulation of T cell differentiation and function has been extensively studied (Baumjohann & Ansel, 2013). However, despite the growing number of reports describing miRNA dysregulation in MS patients (Ma et al., 2014), their contribution to MS pathogenesis remains largely unknown. Although let-7 is one of the most highly expressed miRNA families in CD4$^+$ T cells (Kuchen et al., 2010) and it has been shown to play regulatory roles in helper T cell responses (Polikepahad et al., 2010; Kumar et al., 2011; Swaminathan et al., 2012; Okoye et al., 2014; Kimura et al., 2018), published reports have yielded conflicting conclusions regarding the contribution of let-

7 miRNAs to Th17 cell pathogenicity during MS and EAE, and have remained unresolved (Ma et al., 2014). For example, Junker et al., 2009 found an upregulation of let-7c in MS lesions, while Kimura et al., 2018 described an increase in exosomal let-7i in MS patients and proposed a disease-promoting role for let-7i. On the other hand, Cox et al., 2010 showed a downregulation of let-7d, f, i, and, together with Martinelli-Boneschi et al., 2012, let-7g, in peripheral blood samples of MS patients, whereas Guan et al., 2013 reported a decrease in let-7g and let-7i in pathogenic CD4+ T cells in EAE, but at the same time let-7b, c, d, f and especially let-7e, were upregulated. Moreover, overexpression of let-7e in CD4+ T cells led to aggravated EAE, while knockdown of this miRNA attenuated the disease. Our findings are in discordance with Guan et al., 2013, since we show that EAE is aggravated upon adoptive transfer of Lin28Tg naive CD4+ T cells, in which all let-7 members are suppressed, into Rag2KO recipients. These disparities could be due to the use of different mouse models and treatments, as the cited study employed CD44 KO CD4+ T cells and lentivirus-mediated overexpression or silencing of let-7e expression, while we used transgenic mice with specific modulation of let-7 miRNA expression.

Our most striking finding is that let-7 miRNAs keep pathogenic CD4+ T cells from infiltrating the CNS. This effect was not due to a detrimental impact of let-7 miRNAs on the survival of activated CD4+ T cells, as maintenance of high let-7 miRNA expression improved the survival rate of activated CD4+ T cells, while let-7 deficiency caused an increase in cell death, which is consistent with our recently published observations in both naive CD4+ and CD8+ T cells that let-7 miRNAs promote

homeostatic survival through IL-7-independent stabilization of Bcl2 expression (Pobezinskaya et al., 2019).

In comparison to CD8$^+$ T cells (Wells et al., 2017), we observed only an incremental contribution of let-7 to the proliferation of CD4$^+$ T cells, despite the fact that we found let-7-mediated suppression of *Myc* and Myc target genes involved in glycolysis and protein synthesis. Furthermore, we demonstrate that let-7 miRNAs inhibit the expression of *Cdc25* and *Cdc34*, both of which are involved in the positive regulation of cell cycle progression and are also documented direct Myc and let-7 miRNA target genes (Gakationov et al., 1996; Johnson et al., 2007; Legesse-Miller et al., 2009). Thus, our results indicate that let-7 miRNAs may regulate cell cycle progression in CD4$^+$ T cells both directly by inhibiting *Cdc25a* and *Cdc34*, as well as indirectly through *Myc*.

It is well-known that regulatory T cells play an indispensable role in preventing autoimmunity (Wing & Sakaguchi, 2010). Based on our EAE experiments, it was reasonable to hypothesize that let-7 miRNA expression may enhance the development or function of Tregs while let-7 deficiency may compromise it. Surprisingly, our data show the opposite results, in which let-7 miRNAs inhibited the differentiation of monoclonal (2D2Rag2KO) iTregs *in vitro*. Furthermore, we noticed that, even though Lin28Tg CD4$^+$ T cells have an enhanced potential to differentiate into pathogenic Th17 cells, Lin28Tg mice are healthy and do not show any sign of autoimmunity, suggesting unaltered Treg function in the absence of let-7 miRNAs. Based on these observations we can conclude that let-7 expression does not enhance Treg differentiation or function. Therefore, in order to understand the observed phenotype, we focused our research on the role of let-7 miRNAs in the differentiation and function of pathogenic Th17 cells.

We show that let-7 miRNAs prevent the *in vitro* differentiation of naive CD4^{+} T cells towards the pathogenic Th17 lineage, as reflected by the reduced frequencies of IL-17A^{+}, GM-CSF^{+}, and IL-17A^{+}GM-CSF^{+} cells, and the downregulation of the cytokine genes *Il17a* and *Csf2* (encoding GM-CSF), as well as the cytokine receptor genes *Il1r1*, and *Il23r*. Both IL-1R1 and IL-23R signaling play critical roles in Th17 cell differentiation, as mice deficient in either cytokine receptor or their respective ligand are completely resistant to EAE development (Cua et al., 2003; Matsuki et al., 2006; Sutton et al., 2006; McGeachy et al., 2009). It was later found that IL-23R signaling, as well as IL-1R-mediated expression of the transcription factor Bhlhe40, induces the expression of the cytokine GM-CSF, which stimulates peripheral inflammatory macrophages and promotes their migration to the CNS, where they are responsible for demyelination and neuroaxonal damage (El-Behi et al., 2011; Lin et al., 2016; Spath et al., 2017; Komuczki et al., 2019). Both Bhlhe40 and GM-CSF have been shown to be indispensable for EAE induction, since deficiency in either factor confers protection against EAE (McQualter et al., 2001; Ponomarev et al., 2007; Codarri et al., 2011; Martínez-Llordella et al., 2013; Lin et al., 2014), and elevated GM-CSF levels have been correlated with the active phase of MS (Carrieri et al., 1998). In addition, two earlier reports proposed a role for specific members of the let-7 family in the regulation of *Il23r* expression. Specifically, potential let-7f binding sites were identified in *IL23R* mRNA in human memory CD4^{+} T cells (Li et al., 2011), and loss of let-7e- and let-7f-mediated regulation of a human *IL23R* gene variant was shown to be associated with inflammatory bowel disease, due to a polymorphism in the 3' UTR of *IL-23R* mRNA sequence (Zwiers et al., 2012). In our study, we found additional let-7 binding sites within the mRNA sequence of *Il23r* and

described the regulatory role of let-7 miRNAs in the context of pathogenic Th17 cell differentiation. Furthermore, we identified potential let-7 binding sites within the mRNA sequence of *Il1r1*, which has never been suggested to be a direct let-7 miRNA target. Therefore, we propose a novel let-7 miRNA-mediated regulatory mechanism in which let-7 miRNAs prevent both IL-1R1 and IL-23R expression in CD4^{+} T cells by directly targeting their respective transcripts. Consistent with knock-out studies, CD4^{+} T cells that do not express these cytokine receptors are unable to receive the necessary signals for the induction of Bhlhe40 and GM-CSF, thereby aborting the differentiation of encephalitogenic Th17 cells and EAE development.

We also tested whether the let-7 miRNA-mediated restriction of CNS infiltration by pathogenic CD4^{+} T cells in EAE was due to the inhibition of cell migration to the CNS. Even though there was no difference in intrinsic motility of *in vitro*-generated pathogenic Th17 cells, we demonstrated, using transwell assays, that let-7 repressed the chemokine-mediated migration of these cells in response to the chemokines CCL2 and CCL4 by suppressing the expression of their cognate receptors CCR2 and CCR5. Although chemokine receptors are normally upregulated in differentiating T cells, enabling them to migrate and home to the location of ongoing immune responses, CCL2 and CCL4 have been detected at high levels in the cerebrospinal fluid, brain tissue, and active lesions of patients with MS, and elevated expression of both chemokine receptors on pathogenic CD4^{+} T cells has been correlated with the active phase of MS (Sørensen & Sellebjerg, 2011; Teleshova et al., 2002; Szczuciński & Losy, 2007). In fact, the role of CCR2 and CCR5 in pathogenic CD4^{+} T cell trafficking to the CNS in MS has been confirmed in EAE using receptor-deficient mice (Fife et al., 2000; Gu et al., 2016).

Despite the fact that the observed dysregulation of these receptors could be indirectly resulting from the let-7-mediated inhibition of pathogenic Th17 differentiation, we found that the mRNA sequences of both chemokine receptors contain potential let-7 miRNA binding sites. We showed, using luciferase reporter assays, that both sites identified in *Ccr2* mRNA, but only one site in *Ccr5* mRNA, are functional let-7-binding sites. Furthermore, we showed, in transwell assays using *Ccr2-* and *Ccr5-*overexpressing cells, that let-7 can inhibit the CCR5-, but not the CCR2-mediated migration of pathogenic Th17 cells. Therefore, we propose that let-7 miRNAs, in addition to possibly targeting *Il1r1* and *Il23r* transcripts, also inhibit CCR5-mediated chemotaxis by directly targeting *Ccr5* mRNA in pathogenic CD4$^+$ T cells. The repression of CCR2-mediated chemotaxis appears to be controlled by unknown let-7-mediated regulatory mechanisms, as it is not rescued upon CCR2 overexpression. In accordance with chemokine receptor deficiency studies in EAE, we can conclude that the inability to express the adequate levels of CCR5 suppresses the responsiveness of pathogenic Th17 cells to their respective ligands, thereby preventing their migration to the CNS and EAE induction.

Our study strongly highlights a potential therapeutic application for let-7 miRNAs in the treatment of autoimmune diseases such as MS. Besides, we show that let-7 expression did not interfere with the differentiation of non-pathogenic Th17 cells, which are generated with IL-6 and TGF-β (McGeachy et al., 2007). This was consistent with our findings that let-7 expression does not inhibit the expression of genes expressed in both Th17 subsets, such as the transcription factors *Rorc* and *Irf4*, as well as the IL-6 receptor components *Il6ra* and *Il6st*. In contrast to their pathogenic counterparts, these cells are not able to induce EAE, likely due to their inability to secrete GM-CSF in the

absence of IL-1β and IL-23 signaling. Instead, non-pathogenic Th17 cells secrete the anti-inflammatory cytokine IL-10, which antagonizes autoimmune responses (Kühn et al., 1993). Therefore, it would be interesting to test whether let-7 affects the normal function of these cells, the preservation of which would be important in the context of the proposed therapeutic strategy. Furthermore, because the phenotypic plasticity of the Th17 subset is well known, assessing whether the let-7-mediated inhibition of pathogenic Th17 differentiation can contribute to the conversion of these cells towards the non-pathogenic Th17 lineage in the presence of the adequate signals would also have significant implications for this therapy (McGeachy et al., 2007; McGeachy et al., 2009).

Altogether, our data demonstrate that let-7 miRNAs have a protective effect in EAE rather than a role in promoting disease pathogenesis. Therefore, delivering let-7 miRNAs to pathogenic Th17 cells may constitute a promising therapeutic strategy for the treatment of MS and related autoimmune diseases.
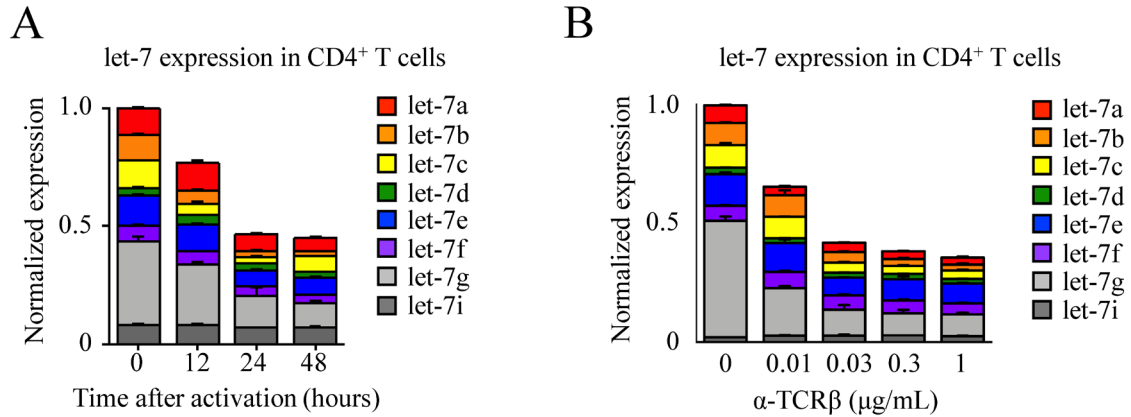
Figure 2.1 Let-7 miRNAs are highly expressed in naive CD4$^+$ T cells, but get downregulated upon activation, proportionally to the duration and strength of TCR stimulation. (A) Quantitative RT-PCR analysis of individual let-7 miRNA expression in naive CD4$^+$ T cells activated with plate-bound α-CD3 mAbs (5 µg/mL) and α-CD28 mAbs (5 µg/mL) for increasing time periods as indicated, presented relative to results obtained for the small nuclear RNA U6 (control) and normalized to the unstimulated (0 hours) control. **(B)** Quantitative RT-PCR analysis of individual let-7 miRNA expression in naive CD4$^+$ T cells activated for 24 hours with plate-bound α-TCR mAbs (as indicated) and α-CD28 mAbs (5 µg/mL), presented relative to results obtained for the small nuclear RNA U6 (control) and normalized to the unstimulated (0 hours) control. Data are from one experiment representative of two independent experiments (A, B; mean ± S.E.M. of technical triplicates).

A EAE

Mean clinical score vs Days post-induction

- WT no dox
- WT + dox
- Let-7Tg no dox
- Let-7Tg + dox

B Mononuclear cells (CNS)

Cell number (x10⁶)

Dox: − + − +
WT Let-7Tg

C CD4⁺ T cells (CNS)

Cell number

Dox: − + − +
WT Let-7Tg

- WT no dox
- WT + dox
- Let-7Tg no dox
- Let-7Tg + dox

D Gated on CD4⁺ T cells (day 9 post-EAE induction)

Isotype control | WT no dox | WT + dox | Let-7Tg no dox | Let-7Tg + dox

IL-17A vs IFNγ

| 0 | 0.4 | 27.7 | 15.1 | 49.7 | 13.2 | 10.4 | 9.9 | 29.5 | 4.6 |
| 0 | 0 | 20.8 | | 7.4 | | 36.6 | | 15.9 | |

IL-17A⁺ | IFNγ⁺ | IL-17A⁺IFNγ⁺

IL-17A vs GM-CSF

| 0 | 0.4 | 32.1 | 15.1 | 44.8 | 18.0 | 13.2 | 7.1 | 22.7 | 11.4 |
| 0 | 0 | 8.6 | | 5.1 | | 18.3 | | 4.6 | |

IL-17A⁺ | GM-CSF⁺ | IL-17A⁺GM-CSF⁺

IFNγ vs GM-CSF

| 0 | 0.4 | 17.6 | 16.2 | 7.9 | 11.5 | 21.4 | 21.9 | 13.6 | 6.8 |
| 0 | 0 | 4.9 | | 13.4 | | 5.5 | | 9.1 | |

IFNγ⁺ | GM-CSF⁺ | IFNγ⁺GM-CSF⁺

- WT no dox
- WT + dox
- Let-7Tg no dox
- Let-7Tg + dox

E ELISA on MOG-restimulated splenocytes from EAE-induced mice

IL-17 | IFNγ | GM-CSF

Concentration (ng/mL) vs MOG (μg/mL)

- WT
- Let-7Tg

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Figure 2.2 Downregulation of let-7 miRNAs upon activation is required for CD4$^+$ T cell pathogenicity in EAE. (A) Mean clinical scores in vehicle- (no dox) treated wild-type (WT) (n=3) and Let-7Tg (n=4) mice or doxycycline- (+ dox) treated WT (n=7) and Let-7Tg (n=7) mice immunized with MOG$_{35-55}$ in complete Freund's adjuvant (CFA) and pertussis toxin (60 ng). **(B)** Number of total mononuclear cells at the peak of the disease (day 9-15 post-immunization) in the CNS of vehicle- (no dox) or doxycycline- (+ dox) treated WT versus Let-7Tg mice. **(C)** Number of CNS-infiltrated CD4$^+$ T cells at the peak of the disease (day 9-15 post-immunization) in vehicle- (no dox) or doxycycline- (+ dox) treated WT versus Let-7Tg mice as analyzed by flow cytometry. **(D)** Intracellular staining of CD4$^+$ T cells from the CNS of vehicle- (no dox) or doxycycline- (+ dox) treated WT versus Let-7Tg mice (left). Numbers indicate the frequencies of cytokine-positive cells within the indicated gates. Quantification of the numbers of cytokine-positive cells as assessed by flow cytometry for each staining strategy (right). **(E)** ELISA analysis of IL-17, IFNγ and GM-CSF concentration in the supernatants of splenocytes from vehicle- (no dox) or doxycycline- (+ dox) treated WT versus Let-7Tg mice harvested at the peak of disease (day 9-15 post-immunization) and restimulated for 5 days in vitro with 20 µg/mL MOG$_{35-55}$. $*$ $p < 0.05$, $**$ $p < 0.01$; $***$ $p < 0.001$, $****$ $p < 0.0001$ (A, B, C, D, E), employing two-way ANOVA (A) or compared with WT using two-tailed Student's t test (B, C, D, E). Data are from two combined independent experiments (A, B, C; mean ± S.E.M. of each population from all mice), from one experiment representative of two independent experiments (D; mean ± S.E.M. of each population from all mice), or from one experiment (C; mean ± S.E.M. of technical triplicates of each population from all mice).

A EAE

B Mononuclear cells (CNS)

C CD4$^+$ T cells (CNS)

- 2D2Rag2KO WT
- 2D2Rag2KO Let-7Tg
- 2D2Rag2KO Lin28Tg

D Gated on CD4$^+$ T cells (day 9 post-EAE induction)

Isotype control | 2D2Rag2KO WT | 2D2Rag2KO Let-7Tg | 2D2Rag2KO Lin28Tg

63
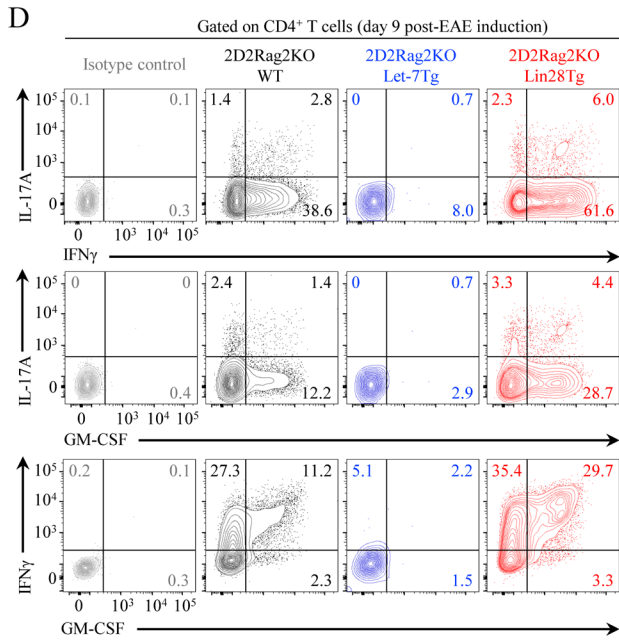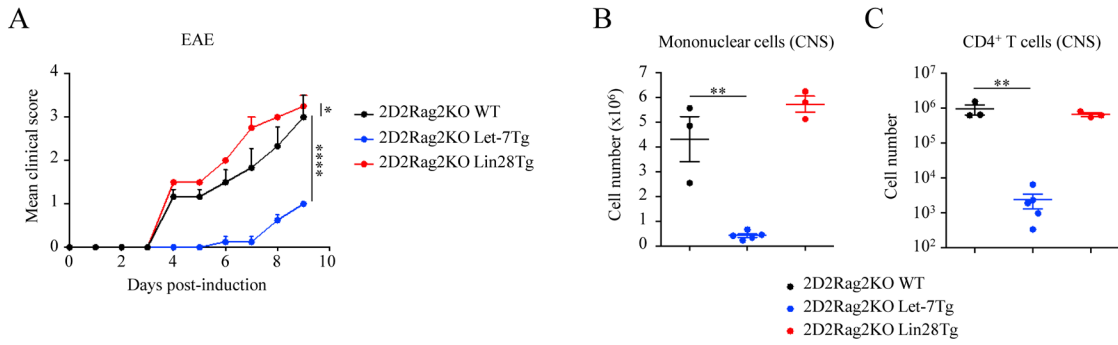
Figure 2.3 Let-7 miRNAs control the development of active EAE by negatively regulating the pathogenicity of monoclonal CD4$^+$ T cells. (A) Mean clinical scores in 2D2Rag2KO WT (n=4), 2D2Rag2KO Let-7Tg (n=5) and 2D2Rag2KO Lin28Tg (n=3) mice immunized with MOG$_{35-55}$ in complete Freund's adjuvant (CFA) and pertussis toxin (60 ng). **(B)** Number of total mononuclear cells at the peak of the disease (day 9 post-immunization) in the CNS of 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice. **(C)** Number of CNS-infiltrated CD4+ T cells at the peak of the disease (day 9-15 post-immunization) in 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice as analyzed by flow cytometry. **(D)** Intracellular staining of CD4$^+$ T cells from the CNS of 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice (left). Numbers indicate the frequencies of cytokine-positive cells within the indicated gates. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ (A, B, C, D), compared with WT employing two-way ANOVA (A) or using two-tailed Student's t test (B, C, D). Data are from two combined independent experiments (A, B, C; mean ± S.E.M. of each population from all mice) or one experiment representative of two independent experiments (D).

A EAE

B Mononuclear cells (CNS)

C CD4$^+$ T cells (CNS)

● 2D2Rag2KO WT → Rag2KO
● 2D2Rag2KO Let-7Tg → Rag2KO
● 2D2Rag2KO Lin28Tg → Rag2KO

D Gated on donor 2D2Rag2KO CD4$^+$ T cells (day 9 post-EAE induction)

Isotype control | WT | Let-7Tg | Lin28Tg

E Spleen cellularity   CD4$^+$ T cells (Spleen)

● 2D2Rag2KO WT → Rag2KO
● 2D2Rag2KO Let-7Tg → Rag2KO
● 2D2Rag2KO Lin28Tg → Rag2KO

65
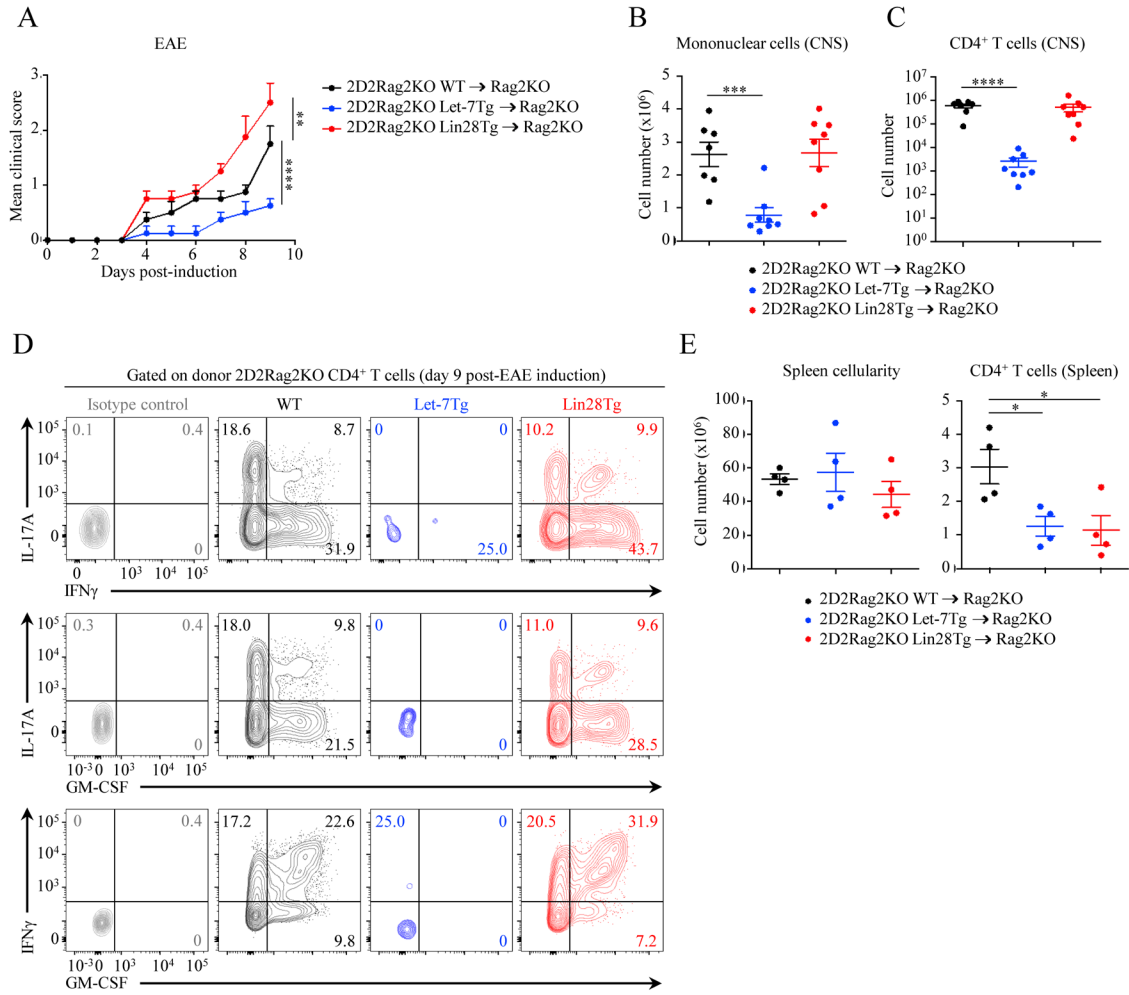
Figure 2.4 Let-7 miRNAs negatively regulate CD4$^+$ T cell pathogenicity in a cell-intrinsic manner in EAE. (A) Mean clinical scores in Rag2KO recipient mice that received 2D2Rag2KO WT (n=7), 2D2Rag2KO Let-7Tg (n=7) or 2D2Rag2KO Lin28Tg (n=8) naive CD4$^+$ T cells (2-2.5 x 10^6 cells/recipient) and that were subsequently immunized with MOG$_{35-55}$ in complete Freund's adjuvant (CFA) and pertussis toxin (60 ng). **(B)** Number of total mononuclear cells at the peak of the disease (day 9 post-immunization) in the CNS of Rag2KO recipients that received 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg, and 2D2Rag2KO Lin28Tg cells. **(C)** Number of CNS-infiltrated 2D2Rag2KO CD4+ T cells at the peak of the disease (day 9 post-immunization) in Rag2KO recipients transferred with 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg, and 2D2Rag2KO Lin28Tg cells as analyzed by flow cytometry. **(D)** Intracellular staining of donor CD4$^+$ T cells from the CNS of Rag2KO recipients that received 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg, and 2D2Rag2KO Lin28Tg cells (left). Numbers indicate the frequencies of cytokine-positive cells within the indicated gates. **(E)** Quantification of total cell numbers and CD4$^+$ T cell numbers in the spleens of Rag2KO recipient mice that received 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg, and 2D2Rag2KO Lin28Tg cells. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ (A, B, C, D, E), compared with WT employing two-way ANOVA (A) or using two-tailed Student's t test (B, C, D, E). Data are from two combined independent experiments (A, B, C; mean ± S.E.M. of each population from all mice), from one experiment representative of two independent experiments (D), or from one experiment (E; mean ± S.E.M. of each population from all mice).
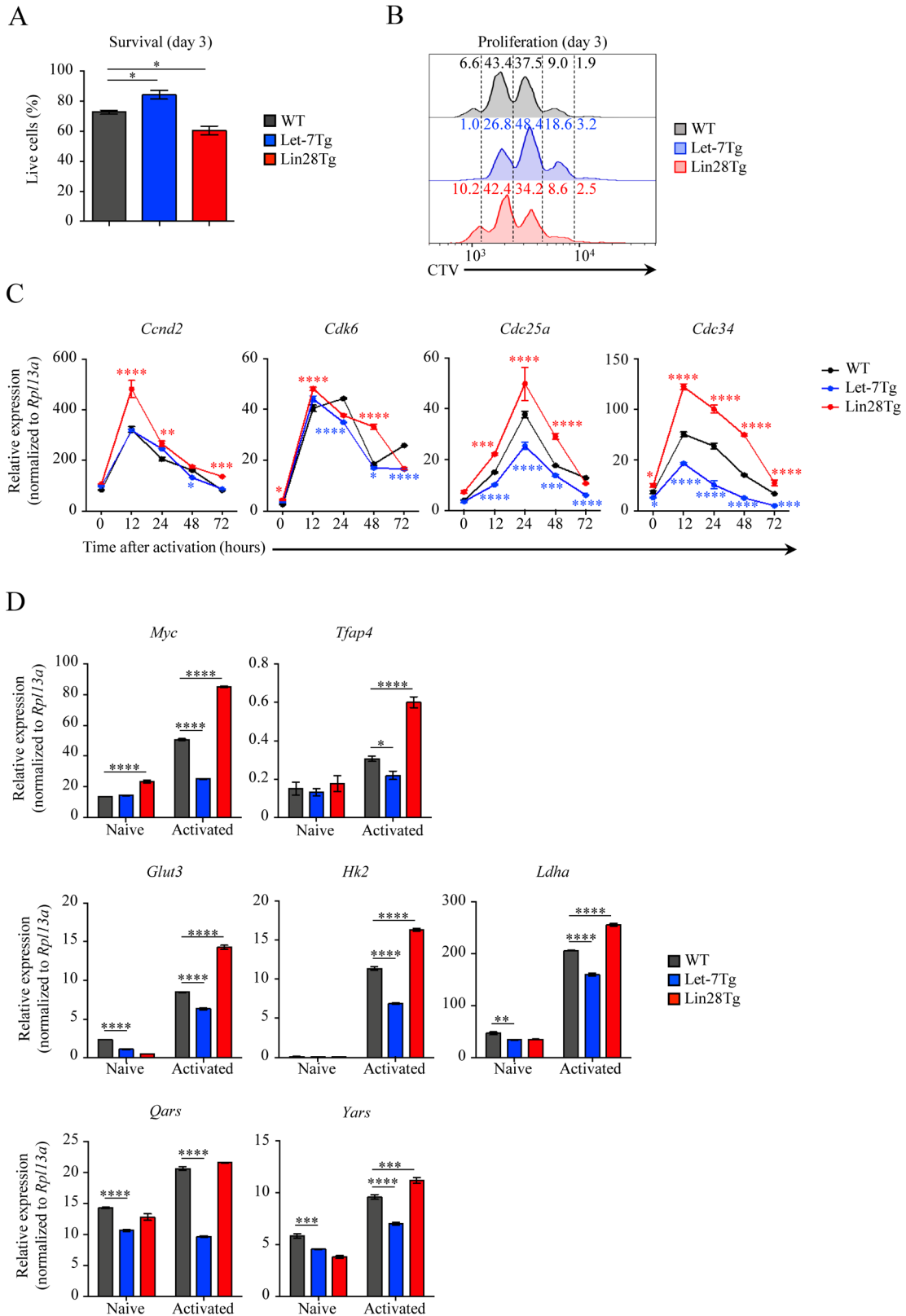
Figure 2.5 Let-7 miRNAs control the proliferation of CD4$^+$ T cells by negatively regulating metabolic reprogramming and cell cycle progression. (A) Survival rate of WT, Let-7Tg and Lin28Tg CD4$^+$ T cells activated *in vitro* for 3 days with α-CD3 and α-CD28 mAbs (5 μg/mL each) as analyzed by trypan blue exclusion. **(B)** Proliferation of Cell-Trace Violet-labeled WT, Let-7Tg and Lin28Tg CD4$^+$ T cells activated *in vitro* for 3 days α-CD3 and α-CD28 mAbs (5 μg/mL each) as analyzed by flow cytometry. Numbers indicate the cell frequencies within the indicated gates for each genotype. **(C)** Quantitative RT-PCR analysis of the cell cycle regulators, cyclin D2 (*Ccnd2*), cyclin-dependent kinase 6 (*Cdk6*), cell division cycle 25a phosphatase (*Cdc25a*), and ubiquitin-conjugating enzyme E2 Cdc34 (*Cdc34*) in naive CD4$^+$ T cells activated with plate-bound α-CD3 and α-CD28 mAbs (5 μg/mL each) for increasing time periods as indicated, presented relative to results obtained for the ribosomal protein Rpl13a (control). **(D)** Quantitative RT-PCR analysis of the transcription factors Myc (*Myc*) and AP-4 (*Tfap4*), as well as Myc direct target genes involved in glycolysis and protein synthesis, glucose transporter 3 (*Glut3*), hexokinase 2 (*Hk2*), lactate dehydrogenase A (*Ldha*), glutamyl-tRNA synthetase (*Qars*) and tyrosyl-tRNA synthetase (*Yars*) in naive CD4$^+$ T cells activated with plate-bound α-CD3 mAbs and α-CD28 mAbs (5 μg/mL each) for 48 hours, presented relative to results obtained for the ribosomal protein Rpl13a (control). * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ (A, C, D), compared with WT using two-tailed Student's t test. Data are from one experiment representative of two independent 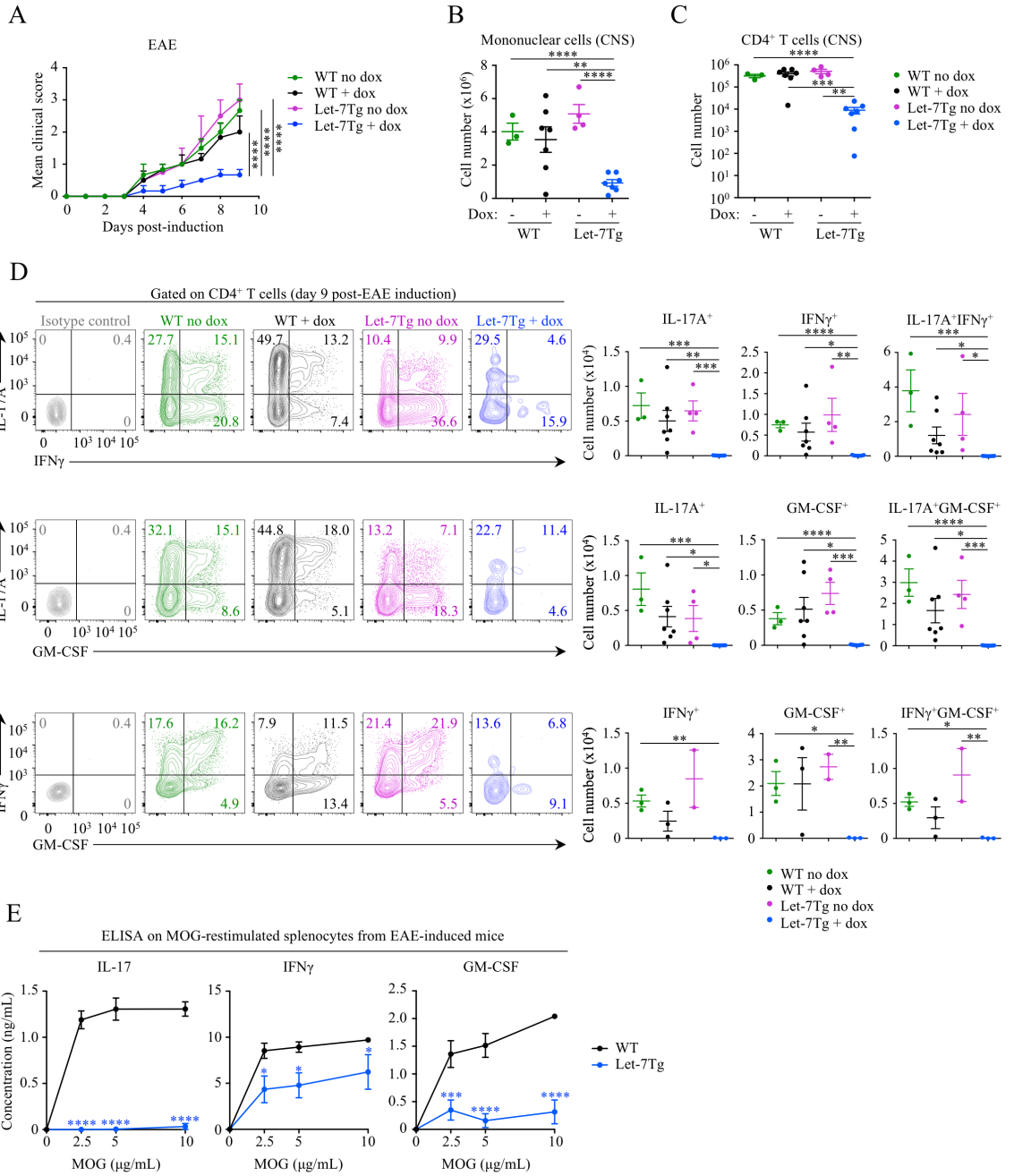experiments (A, C, D; mean ± S.E.M. of technical triplicates of each population from all mice) or from two independent experiments (B).
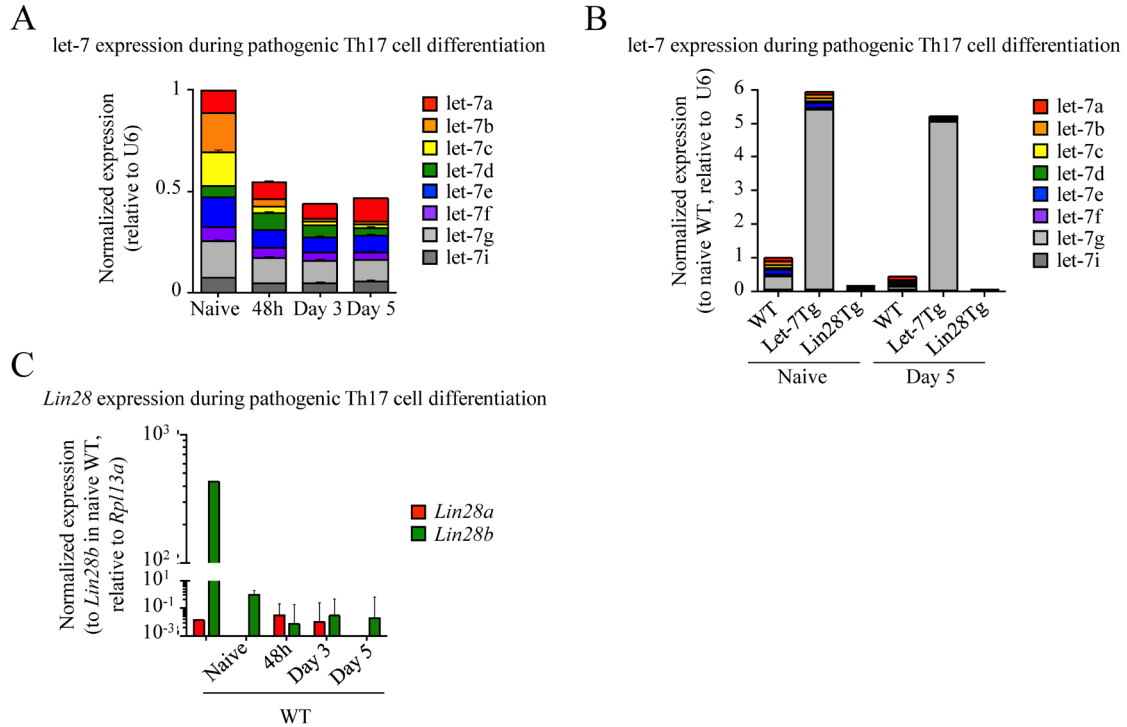
Figure 2.6 Let-7 miRNAs also get downregulated over time during pathogenic Th17 differentiation. (A) Quantitative RT-PCR analysis of individual let-7 miRNA expression in naive 2D2Rag2KO WT CD4^{+} T cells and during *in-vitro*-generation of 2D2RagKO WT pathogenic Th17 cells at the indicated time points, presented relative to results obtained for the small nuclear RNA U6 (control), and normalized to results obtained for naive 2D2Rag2KO WT CD4^{+} T cells. **(B)** Quantitative RT-PCR analysis of individual let-7 miRNA expression in naive CD4^{+} T cells and day-5 *in-vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg, and 2D2Rag2KO Lin28Tg mice, presented relative to results obtained for the small nuclear RNA U6 (control), and normalized to results obtained for naive 2D2Rag2KO WT CD4^{+} T cells. **(C)** Quantitative RT-PCR analysis of *Lin28a* and *Lin28b* in naive 2D2Rag2KO WT and 2D2Rag2KO Lin28Tg CD4^{+} T cells, as well as during *in-vitro*-generation of 2D2RagKO WT pathogenic Th17 cells at the indicated time points presented relative to results obtained for the small nuclear RNA U6 (control), and normalized to results obtained for naive 2D2Rag2KO WT CD4^{+} T cells. . * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$, compared with WT using two-tailed Student's t test. Data are from one experiment representative of at least two experiments (A, B, C; mean ± S.E.M. of technical replicates).

A

Monoclonal (2D2Rag2KO) *in vitro*-directed pathogenic Th17 polarizations

B

Il17a *Csf2* *Il1r1* *Il23r* *Bhlhe40*

Relative expression (normalized to *Rpl13a*)

- 2D2Rag2KO WT
- 2D2Rag2KO Let-7Tg
- 2D2Rag2KO Lin28Tg

C

Mouse *Il1r1* mRNA
Human *IL1R1* mRNA

Mouse *Il23r* mRNA
Human *IL23R* mRNA

5' UTR
CDS
3' UTR
let-7 binding site

D

Il6ra *Il6st* *Stat3* *Irf4* *Rorc*

Relative expression (normalized to *Rpl13a*)

- 2D2Rag2KO WT
- 2D2Rag2KO Let-7Tg
- 2D2Rag2KO Lin28Tg

Figure 2.7 Let-7 miRNAs specifically inhibit the acquisition of the pathogenic Th17 phenotype. (A) Intracellular staining of CD4$^+$ T cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice polarized *in vitro* towards the pathogenic Th17 lineage with IL-6, IL-1β, and IL-23. Numbers indicate the frequencies of cytokine-positive cells within the indicated gates. **(B)** Quantitative RT-PCR analysis of the cytokines IL-17A (*Il17a*) and GM-CSF (*Csf2*), the cytokine receptors IL-1R1 (*Il1r1*) and IL-23R (*Il23r*), and the transcription factor Bhlhe40 (*Bhlhe40*) in *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice from (A), presented relative to results obtained for the ribosomal protein Rpl13a (control). **(C)** Diagram positioning *in silico*-identified let-7 binding sites (black vertical lines) within the mouse and human mRNA sequences of the cytokine receptors IL1-R1 (*Il1r1* and *IL1R1*, respectively) and IL-23R (*Il23r* and *IL-23R*, respectively). **(D)** Quantitative RT-PCR analysis of the IL-6 cytokine receptor components IL-6Rα (*Il6ra*) and IL-6ST (*Il6st*), and the transcription factors STAT3 (*Stat3*), IRF4 (*Irf4*), and RORγt (*Rorc*) in *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice.* $p < 0.05$, *** $p<0.001$, **** $p < 0.0001$ (B, D), compared with WT using two-tailed Student's t test. Data are from one experiment representative of two independent experiments (A) or from two independent experiments (B, D; mean ± S.E.M. of technical triplicates of each population from all mice).
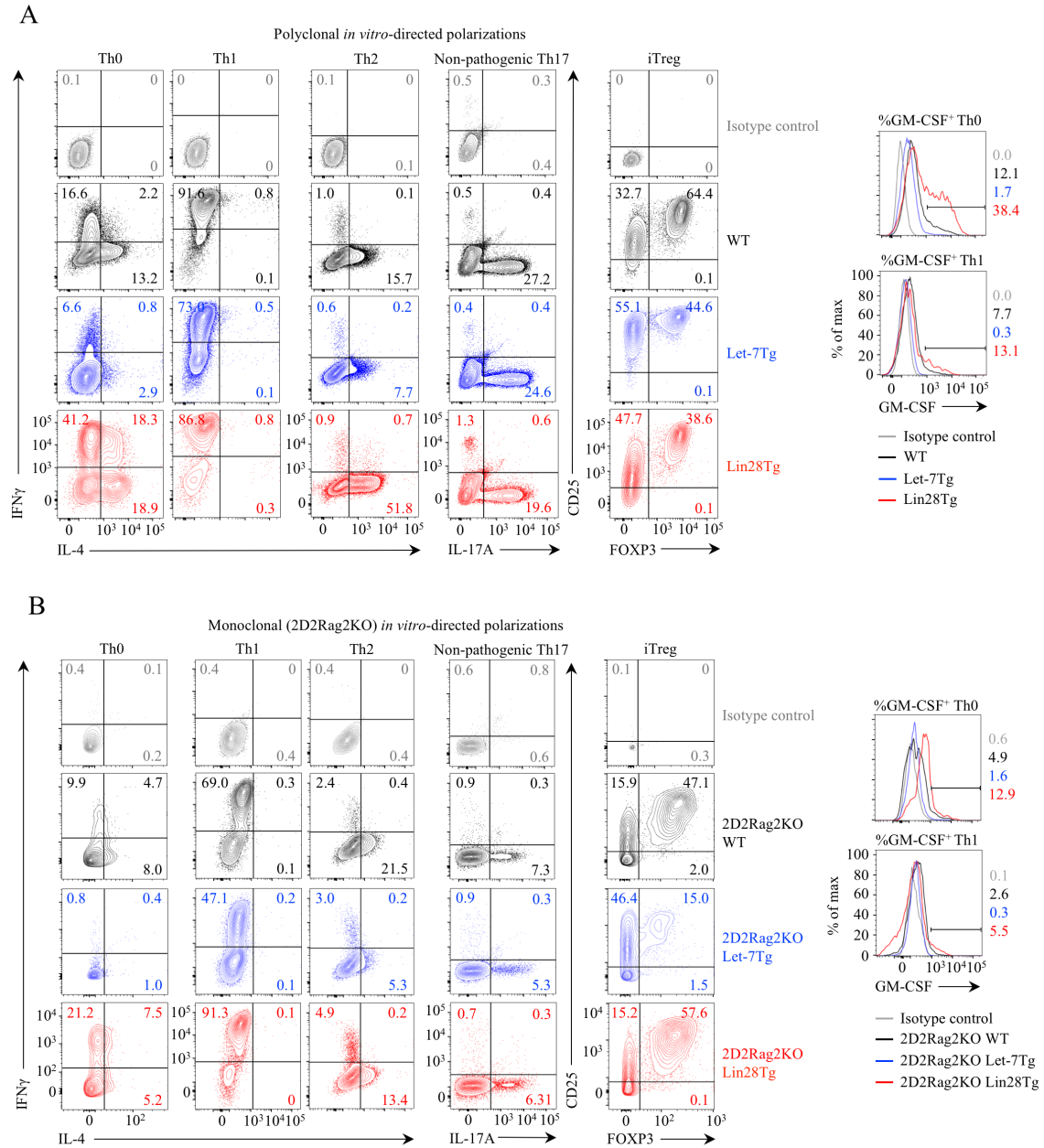
Figure 2.8 Let-7 miRNAs inhibit the differentiation of several helper T cell subsets generated from both polyclonal and monoclonal naive CD4+ T cells *in vitro*. Intracellular staining of CD4+ T cells from polyclonal **(A)** or monoclonal 2D2Rag2KO **(B)** WT, Let-7Tg and Lin28Tg mice polarized *in vitro* towards the Th0, Th1, Th2, non-pathogenic Th17 and iTreg lineages. Numbers indicate the frequencies of cytokine-positive cells within the indicated gates. Data are from one experiment representative of seven (A) or six (B) independent experiments.
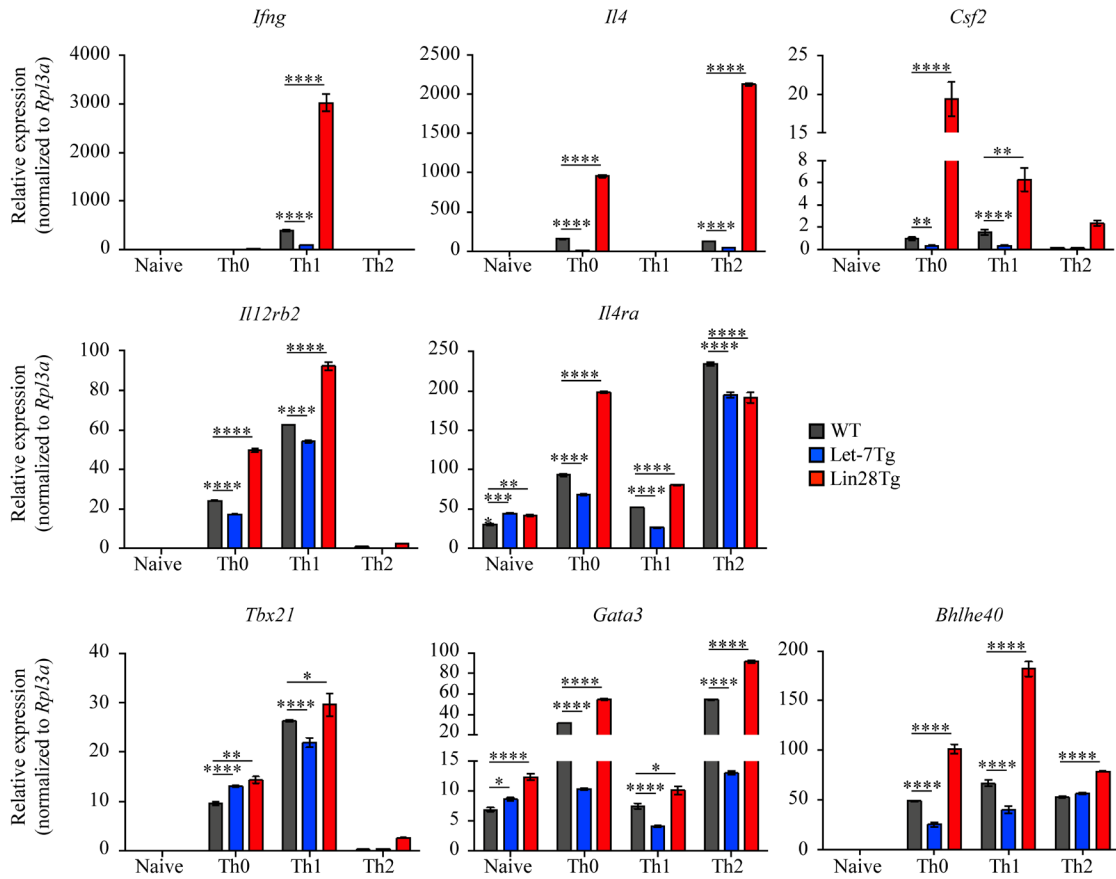
Figure 2.9 Let-7 miRNAs negatively regulate the expression of genes controlling the differentiation of Th0, Th1 and Th2 cells generated *in vitro*. Quantitative RT-PCR analysis of the cytokines IFNγ (*Ifng*), GM-CSF (*Csf2*), and IL-4 (*Il4*), the cytokine receptors IL12Rβ2 (*Il12rb2*) and IL4Rα (*Il4ra*), as well as the transcription factors T-bet (*Tbx21*), Bhlhe40 (*Bhlhe40*) and GATA3 (*Gata3*) in naive CD4⁺ T cells and *in vitro*-generated Th0, Th1 and Th2 cells from WT, Let-7Tg and Lin28Tg mice, presented relative to results obtained for the ribosomal protein Rpl13a (control). Data are from one experiment representative of two independent experiments (mean ± S.E.M. of technical triplicates of each population from all mice).

A

Ccr2

Ccr5

- 2D2Rag2KO WT
- 2D2Rag2KO Let-7Tg
- 2D2Rag2KO Lin28Tg

B

Mouse *Ccr2* mRNA

Human *CCR2* mRNA

Mouse *Ccr5* mRNA

Human *CCR5* mRNA

- 5' UTR
- CDS
- 3' UTR
- Non-conserved let-7 binding site
- Conserved let-7 binding site

C

Luciferase reporter assay (NIH/3T3)

D

Pathogenic Th17 cell migration assay (3h)

- 2D2Rag2KO WT
- 2D2Rag2KO Let-7Tg
- 2D2Rag2KO Lin28Tg

E

Ccr2 and *Ccr5* overexpression in *in vitro*-generated pathogenic Th17 cells

Ccr2

- 2D2Rag2KO WT – Empty vector
- 2D2Rag2KO WT – *Ccr2* overexpression
- 2D2Rag2KO WT – *Ccr2* overexpression (no reverse transcriptase)
- 2D2Rag2KO Let-7Tg – Empty vector
- 2D2Rag2KO Let-7Tg – *Ccr2* overexpression
- 2D2Rag2KO Let-7Tg – *Ccr2* overexpression (no reverse transcriptase)

Ccr5

- 2D2Rag2KO WT – Empty vector
- 2D2Rag2KO WT – *Ccr5* overexpression
- 2D2Rag2KO WT – *Ccr5* overexpression (no reverse transcriptase)
- 2D2Rag2KO Let-7Tg – Empty vector
- 2D2Rag2KO Let-7Tg – *Ccr5* overexpression
- 2D2Rag2KO Let-7Tg – *Ccr5* overexpression (no reverse transcriptase)

F

Pathogenic Th17 cell migration assay (3h)
with *Ccr2* or *Ccr5* overexpression

- 2D2Rag2KO WT – Empty vector
- 2D2Rag2KO WT – *Ccr2* overexpression
- 2D2Rag2KO WT – *Ccr5* overexpression
- 2D2Rag2KO Let-7Tg – Empty vector
- 2D2Rag2KO Let-7Tg – *Ccr2* overexpression
- 2D2Rag2KO Let-7Tg – *Ccr5* overexpression

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Figure 2.10 Let-7 miRNAs prevent the chemokine-dependent migration of *in vitro*-generated pathogenic Th17 cells by suppressing the expression of the chemokine receptors CCR2 and CCR5. (A) Quantitative RT-PCR analysis of the chemokine receptors CCR2 (*Ccr2*) and CCR5 (*Ccr5*) in *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice, presented relative to results obtained for the ribosomal protein Rpl13a (control). **(B)** Diagram positioning *in silico*-identified conserved (red vertical lines) and non-conserved (black vertical lines) let-7 binding sites within the mouse and human mRNA sequences of the chemokine receptors CCR2 (*Ccr2* and *CCR2*, respectively) and CCR5 (*Ccr5* and *CCR5*, respectively). **(C)** Luciferase reporter assay of let-7 targeting *in-silico*-identified let-7-binding sites in mouse *Ccr2* or *Ccr5* mRNA, in NIH/3T3 cells transfected with a luciferase reporter vector containing either the wild-type or mutated variants of these binding sites, or either the wild-type or a mutated variant of the antisense seed sequence of let-7g (controls). Results are presented as relative luminescence units (RLU), calculated by normalization of Firefly luciferase activity to Renilla luciferase activity (control). **(D)** Transwell migration assay of *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice from (A) in response to the chemokines CCL2 (50 ng/mL) and CCL4 (50 ng/mL) alone or in combination (50 ng/mL or 10 ng/mL each). Results are presented as percentage of cell migration in media only control, defined as 100%. **(E)** Quantitative RT-PCR analysis of the chemokine receptors CCR2 (*Ccr2*) and CCR5 (*Ccr5*) in *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT and 2D2Rag2KO Let-7Tg mice, transduced with empty vector (solid bars), *Ccr2*-overexpression vector (horizontally-striped bars), and *Ccr5*-overexpression vector (diagonally-striped bars), presented relative to results obtained for the ribosomal protein Rpl13a (control). **(F)** Transwell migration assay of *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT and 2D2Rag2KO Let-7Tg mice, transduced with empty vector (solid bars), *Ccr2*-overexpression vector (horizontally-striped bars), and *Ccr5*-overexpression vector (diagonally-striped bars) in response to the chemokines CCL2 (50 ng/mL) and CCL4 (50 ng/mL) alone. Results are presented as percentage of cell migration in media only control, defined as 100%. * $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$ (A, C, D, F), compared with WT using two-tailed Student's t test. Data are from one experiment representative two independent experiments (A, C, D, F; mean ± S.E.M. of technical triplicates).
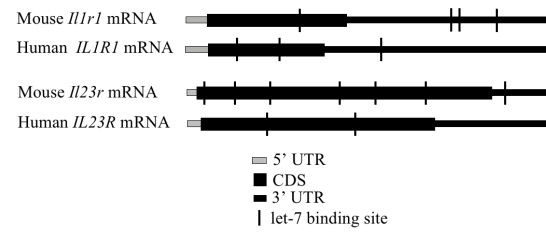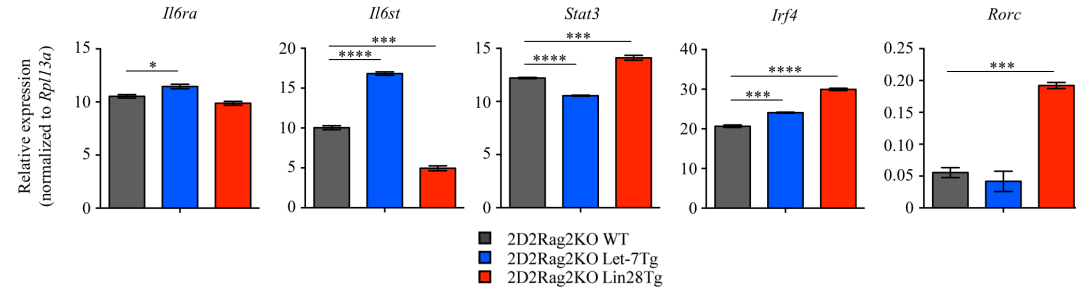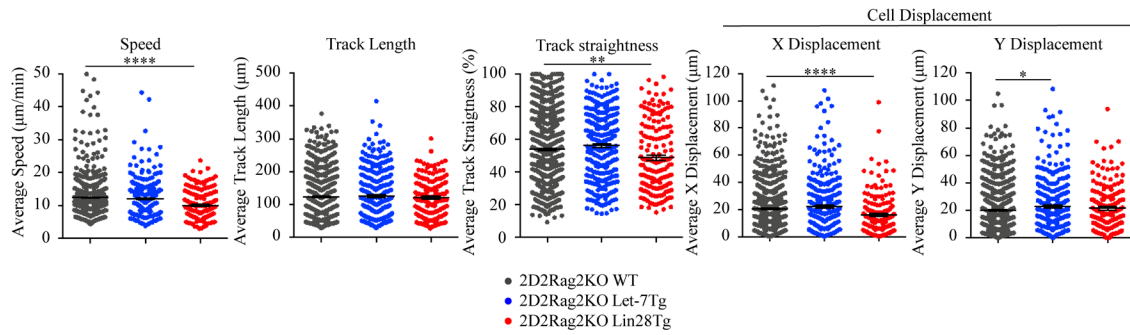
Figure 2.11 Let-7 miRNAs do not control the intrinsic motility of *in vitro*-generated pathogenic Th17 cells. Speed, track length, track straightness, and displacement of migrating CFSE-labeled *in vitro*-generated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice embedded in collagen matrices. * p < 0.05, ** p < 0.01, **** p < 0.0001, compared with WT using two-tailed Student's t test. Data are from one experiment (mean ± S.E.M. of at least 175 analyzed cells of each population from all mice).

CHAPTER 3

COMPUTATIONAL ANALYSES PREDICT THE REGULATORY ROLE OF LET-7 MICRORNAS IN SHAPING THE FATE OF CD8$^+$ T CELLS TOWARDS MEMORY FORMATION WHILE SUPPRESSING TERMINAL EFFECTOR DIFFERENTIATION/EXHAUSTION

3.1 Introduction

Cytotoxic CD8$^+$ T lymphocytes (CTLs) are critical players of the immune response, as they are able to directly contribute to antigen clearance by recognizing and killing cells infected by intracellular pathogens, as wells as tumor cells (Sawamura et al., 1989; Dharakul et al., 1990; Harty et al., 1992; Rodrigues et al., 2003). Upon antigen encounter, antigen-specific CD8$^+$ T cells proliferate and generate a large pool of CTLs that migrate to the inflamed sites and execute their effector function to participate in the resolution of the infection (Butz & Bevan, 1998). After antigen elimination, the majority of CTLs undergo apoptosis during a contraction phase, as they are terminally differentiated, but a small proportion survives and generates long-lived memory CD8$^+$ T cells that will respond faster and more effectively to subsequent challenges with the same antigen (Grayson et al, 2000; Badovinac et al., 2002). This terminal-effector versus memory fate specification depends on the strength of signals 1, 2, and 3, received by CD8$^+$ T cells upon activation, with stronger signals leading to a decrease in memory potential (Stemberger et al., 2007; Gerlach et al., 2010; Angelosanto et al., 2012). These signals also result in the upregulation of co-inhibitory receptors or "immune checkpoints", such as PD-1, TIM-3, LAG-3, 2B4, CD160, and CTLA-4 (Wherry, 2007). During acute

infections, these receptors are only expressed in a transient manner, and contribute to the adjustment of the strength and duration of the CTL response to prevent toxicity towards host tissues (Agata et al., 1996; Monney et al., 2002; Probst et al., 2005). However, when antigen fails to be efficiently cleared, such as during chronic infections and cancer, the persistence of TCR signals on $CD8^+$ T cells results in a more abundant, permanent, and joint expression of these receptors (Blackburn et al., 2009). Following the interaction of these receptors with their specific ligands that are upregulated in immunosuppressive environments, such as the tumor microenvironment, CTLs are diverted towards a dysfunctional state known as exhaustion. Exhausted CTLs are unable to clear the infection and to differentiate into long-lasting memory cells (Moskophidis et al., 1993; Zajac et al., 1998; Wherry et al., 2004).

Although memory $CD8^+$ T cell formation is compromised during chronic inflammation, memory $CD8^+$ T cells introduced under these pathological conditions have the capacity to elicit highly protective responses (Shoukry et al., 2003; Malik et al., 2017). These efficient responses may be due to the high survival capacity of memory cells, coupled to low immune checkpoint expression that may confer resistance to exhaustion (Shoukry et al., 2003; Wirth et al., 2010). In addition, the generation of memory $CD8^+$ T cells by vaccination possess outstanding potential for preventing infectious diseases (Lauvau et al., 2001; Akondy et al., 2017). However, the molecular mechanisms promoting memory formation while inhibiting terminal effector differentiation and restraining exhaustion are still incompletely understood.

RNA interference mediated by miRNAs is a potent and well-conserved post-transcriptional mechanism that regulates gene expression in a sequence-specific manner

(Elbashir et al., 2001; Kim et al., 2008). Many individual miRNAs have been shown to play a role in the control of terminal-effector versus memory CD8$^+$ T cell fate determination. For instance, miR-143, miR-155, and miRNAs of the miR-200 family promote memory CD8$^+$ T cell differentiation (Tsai et al., 2013; Guan et al., 2018; Zhang et al., 2018), while miR-15/16, the miR-17-92 cluster, miR-23a, miR-31, and miR-150, direct terminal-effector specification (Wu et al., 2012; Khan et al., 2013; Lin et al., 2014; Ban et al., 2017; Chen et al., 2017; Moffett et al., 2017; Gagnon et al, 2019).

We previously showed that the let-7 miRNA family is highly expressed in naive CD8$^+$ T cells, thereby keeping these cells in a quiescent state and promoting their survival (Wells et al., 2017; Pobezinskaya et al., 2019). Moreover, we demonstrated that let-7 gets substantially downregulated upon antigen encounter, as these miRNAs negatively regulate CTL differentiation (Wells et al., 2017). Surprisingly, follow-up studies in our lab have found that, even though cytotoxic function is diminished upon let-7 overexpression in *in vitro*-generated CTLs, these cells confer superb anti-cancer protection upon adoptive transfer into tumor-bearing mice. Conversely, although *in vitro*-differentiated let-7-deficient CTLs exhibit enhanced cytotoxic function, these cells failed to control the same tumors, leading to poor recipient survival. Because the CTL response observed *in vitro* is not reflective of the *in-vivo* outcome, the purpose of the research described here was to elucidate this paradox.

RNA sequencing (RNA-Seq) is a well-established quantitative method that measures the expression of all genes in a tissue sample at a given time using next-generation sequencing of the transcriptome (Wang et al., 2009). In addition, bioinformatics has emerged as a powerful tool to understand this type of big biological data by executing *in*

silico analyses that use mathematical and statistical methods embedded in specific analysis pipelines (Van den Berge et al., 2019). Thus, we sought to apply RNA-Seq and bioinformatics analyses in order to explain this *in-vitro* versus *in-vivo* discrepancy in the let-7-mediated regulation of CTL function in an unbiased manner.

We found that the modulation of let-7 expression in CD8$^+$ T cells results in transcriptional changes that shape the terminal-effector versus memory fate. Specifically, we showed that maintaining high let-7 expression during the early stages of CTL differentiation led to the upregulation of a memory-like gene signature, whereas absence of let-7 during the differentiation of CTLs led to the upregulation of a gene signature associated with terminally-differentiated effectors and exhaustion. This was consistent with the identification of multiple early T cell activation-associated pathways inhibited by let-7, including ERK1/2 and Notch. Finally, we identified multiple direct let-7 target genes that were negatively regulated throughout CD8$^+$ T cell differentiation, among which we found that *Hk2* and *Mycn* functionally contribute to the control of terminal-effector versus memory fate. Altogether, this study has solved the paradoxical outcomes in the let-7-regulated CTL function *in-vitro* versus *in-vivo* using an unbiased approach that uncovered a novel regulatory role for let-7 in promoting the generation of memory CD8$^+$ T cells differentiation while preventing terminal-effector differentiation. Thus, let-7 miRNAs exhibit an utmost encouraging therapeutic potential as an innovative treatment against chronic infections and cancer.

3.2 Results

3.2.1 Modulation of let-7 expression in CTLs results in transcriptional changes

To understand the discrepancy between the *in-vitro* and *in-vivo* performance of CTLs expressing different levels of let-7 **(Figure 3.1)**, we isolated naive CD8$^+$ T cells from P14Rag2KO WT, Let-7Tg, and Lin28Tg mice, where P14 is a transgenic TCR which recognizes the gp$_{33-41}$ epitope from the lymphocytic choriomeningitis virus (LCMV) glycoprotein (Pircher et al., 1987). We differentiated these cells into CTLs *in vitro* in the presence of the cytokine IL-2, and we performed RNA-Seq on these CTLs at day 5 of culture to analyze their transcriptome.

To assess whether let-7 regulates the transcriptome of CTLs, we used Principal Component Analysis (PCA) to reduce the complexity of all 35,276 transcripts examined to only ranked 9 variables, or principal components (PCs), which reflect the overall variance between all samples analyzed in a small range of dimensions (Abdi & Williams, 2010). We chose to project our experimental samples along PC1 and PC2, which together accounted for most of the total variance (74% and 13%, respectively). Notably, PCA analysis revealed that WT, Let-7Tg, and Lin28Tg CTLs exhibited transcriptionally distinct gene signatures, as all samples from the same genotype were clustered together, but separated from those with distinct genotypes **(Figure 3.2A)**. This genetic distance was statistically significant between all groups, as represented by the non-overlapping 95% confidence ellipses from each cluster. These results show that let-7 expression alters the transcriptome of CTLs.

To examine the extent to which let-7 modulates the CTL transcriptome, we performed differential gene expression analysis on Let-7Tg-versus-WT, and Lin28Tg-

versus-WT CTLs. The output data showed that, in comparison to WT CTLs, 216 genes were downregulated and 286 genes were upregulated in Let-7Tg CTLs, while in Lin28Tg CTLs 448 genes were downregulated and 428 genes were upregulated, as compared to WT CTLs **(Figures 3.2B)**. Moreover, we found that several genes involved in memory T cell differentiation and terminal-effector differentiation/exhaustion were differentially expressed in a reversed manner in Let-7Tg and Lin28Tg CTLs, when compared to WT CTLs. For instance, memory markers, such as *Ccr7*, *Id3*, *Il7ra*, *Lef1*, *Sell* (CD62L) and *Tcf7* (TCF-1), were upregulated in Let-7Tg CTLs, but downregulated in Lin28Tg CTLs, whereas terminal-effector/exhaustion markers, including *Cd244a* (2B4), *Entpd1* (CD39), *Eomes*, *Havcr2* (TIM-3), *Id2*, and *Pdcd1* (PD-1), were upregulated in Lin28Tg CTLs, but downregulated in Let-7Tg CTLs. These findings suggest that let-7 globally manipulates the transcriptional signature of CTLs.

To identify and compare the expression of all genes that were either positively or negatively regulated by let-7 in CTLs, we used the normalized gene expression reads from WT, Let-7Tg, and Lin28Tg CTL replicate samples to generate a heatmap plot of all the genes that were differentially expressed at least by 30% ($|\log_2(\text{fold change}|= 0.5$) in a statistically significant manner in Let-7Tg and Lin28Tg CTLs, in comparison to WT CTLs. Heatmaps are bi-dimensional matrices that represent, across comparable samples, the differences in the expression level of genes, which are ranked according to the extent of differential expression (Eisen et al., 1998). Under these criteria, we were able to categorize 177 of these genes into 4 distinct clusters **(Figure 3.2C)**. Cluster I was the largest and comprised all genes that were negatively regulated by let-7 (n=98), such as *Cd160*, *Gzmb*, *Havcr2*, and the direct let-7 target *Hk2*, and were thus downregulated in

Let-7Tg CTLs, but upregulated in Lin28Tg CTLs, as compared to WT CTLs. Conversely, cluster II contained all genes that were positively regulated by let-7 (n=70), including *Il7ra*, thereby upregulated in Let-7Tg CTLs, but downregulated in Lin28Tg CTLs, in comparison to WT CTLs. Cluster III and cluster IV were very small and consisted of genes downregulated (n=2) or upregulated (n=7) in both Let-7Tg and Lin28Tg CTLs, when compared to WT CTLs, respectively. These results demonstrate that let-7 has both substantial negative and positive impacts on the CTL transcriptome.

To determine which biological processes the genes significantly downregulated in Let-7Tg CTLs and upregulated in Lin28Tg CTLs (cluster I), as well as the genes significantly upregulated in Let-7Tg CTLs and downregulated in Lin28Tg CTLs (cluster II) belonged to, we performed functional annotation of these genes using the Database for Annotation, Visualization and Integrated Discovery (DAVID) (Huang et al., 2009). This analysis determined that cluster I was enriched for functionally-related gene groups involved in the defense response to viruses and the response to biotic stimulus, whereas in cluster II, genes implicated in the immune response and the apoptotic signaling pathway were over-represented **(Figure 3.2D)**. These data indicate that let-7 expression in CTLs regulates biological processes associated with the immune response and cell survival, which is consistent with our previous reports on the regulatory role of let-7 in CTL differentiation (Wells et al., 2017), as well as the role of let-7 in promoting T cell survival (Pobezinskaya et al., 2019).

3.2.2 Let-7 expression promotes a memory-like gene signature in CTLs, while also repressing genes associated with terminal effector differentiation and exhaustion

Because several memory markers were upregulated in Let-7Tg CTLs and downregulated in Lin28Tg CTLs, as compared to WT CTLs, and multiple terminal-effector/exhaustion markers followed the opposite expression pattern in the same cells, we hypothesized that let-7 may regulate memory versus terminal-effector CD8$^+$ T cell fate determination.

To support our hypothesis that let-7 promotes memory formation in CD8$^+$ T cells, we employed gene set enrichment analysis (GSEA), which determines whether a defined set of genes is differentially expressed in a statistically significant manner between two samples (Subramanian et al., 2005). To do so, we ranked the differential gene expression data from Let-7Tg-versus-WT, and Lin28Tg-versus-WT CTLs by increasing statistical significance using the adjusted p-value. We next ran GSEA by subjecting our ranked gene lists to a memory-associated gene signature (n=56 genes), as well as a terminal-effector/exhaustion-associated gene set (n=62 genes), both of which were generated by gathering well-defined markers from the literature (Yang et al., 2011; Im et al., 2016; Schietinger et al., 2016; Yu et al., 2017; Snell et al., 2018; Miller et al., 2019). In fact, the memory gene signature was significantly enriched in Let-7Tg CTLs (NES=1.458; p-value<0.001), whereas in Lin28Tg CTLs this gene set was significantly depleted (NES=-1.518; p-value<0.001) **(Figure 3.3A)**. In contrast, the terminal-effector/exhaustion gene signature was over-represented in Lin28Tg CTLs (NES=1.431; p-value=0.118), but depleted in Let-7Tg CTLs (NES=-1.153; p-value=0.213), although the statistical

significance threshold was not reached. These findings suggest that let-7 may play a role in the specification of memory versus terminal-effector/exhausted CD8^{+} T cell fate.

To define and compare the expression of the genes contained in the gene sets used in GSEA, we used the normalized gene expression reads from WT, Let-7Tg, and Lin28Tg CTL replicate samples and we grouped all genes from both signatures examined into functional categories. We then generated heatmaps of each gene category, namely transcription factors, effector molecules and receptors, chemokines and chemokine receptors, co-stimulatory molecules and receptors, and inhibitory receptors **(Figure 3.3B)**. This analysis revealed that Let-7Tg CTLs upregulated the transcription factors *Foxo1*, *Id3*, *Lef1*, and *Tcf7*, which drive the memory fate of CD8^{+} T cells (Zhou et al., 2010; Gattinoni et al., 2011; Yang et al., 2011; Zhou & Xue, 2012; Hess Michelini et al., 2013), but also *Tbx21* (T-bet) and *Zeb2*, which promote cytotoxic activity (Dominguez et al., 2015). Moreover, the secondary lymphoid organ homing receptor *Ccr7*, as well as the cell-adhesion molecule *Sell* (CD62L), which direct the migration of central memory cells to the lymph nodes, also showed increased expression in Let-7Tg CTLs (Kishimoto et al, 1990; Förster et al., 1999; Sallusto et al., 1999). In addition, Let-7Tg CTLs exhibited high expression of the cytokine receptors *Il7ra* (CD127), emblematic of memory precursor effector cells (MPECs), as well as *Il6ra*, which is important for memory CD8^{+} T cell survival (Brown et al., 2019). Interestingly, Let-7Tg CTLs also upregulated most co-stimulatory molecules and receptors assessed, including *Cd28*, *Icos*, and *Ox40*, the engagement of which is particularly important for optimal recall responses of memory CD8^{+} T cells (Borowski et al., 2007). Most strikingly, Let-7Tg CTLs expressed high levels of T memory stem cell markers, including the transcription factor *Sox4*, the anti-

apoptotic protein *Bcl2*, the secondary lymphoid organ homing receptor *S1pr1*, as well as the Wnt signaling target *Axin2* **(Figure 3.3C)** (Gattinoni et al., 2009; Gattinoni et al., 2011). Thus, these data indicate that let-7 directs the fate of CD8^{+} T cells towards memory formation.

On the other hand, Lin28Tg CTLs showed increased expression of the transcription factors *Eomes*, *Id2*, *Ikzf2* (Helios), and *Prdm1* (Blimp-1), which are highly expressed in terminally-differentiated effectors and exhausted CD8^{+} T cells (Wherry, 2007; Shin et al., 2009). Besides, *Eomes* and *Ikzf2* are direct let-7 targets (Agarwal et al., 2015; Wells et al., 2017). Moreover, most effector molecules and cytokines, including *GzmA*, *GzmB*, *Prf1*, *Ifng*, *Tnf*, and *Fasl*, were upregulated in Lin28Tg CTLs. In addition, these cells also expressed high levels of the immunosuppressive cytokine *Il10*, which, in addition to being directly targeted by let-7, is also abundantly expressed in exhausted cells CD8^{+} T cells (Wilson et al., 2011). Furthermore, numerous pro-inflammatory chemokines, including *Ccl3*, *Ccl4*, *Ccl5*, as well as the chemokine receptors *Cx3cr1* and *Cxcr1*, were highly expressed in Lin28Tg CTLs. In contrast, these cells showed enhanced expression of only a few co-stimulatory molecules and receptors, namely *Tnfrsf9* (4-1BB), its ligand *Tnfsf9* (4-1BBL), and *Tnfrsf8* (CD30). Most remarkably, Lin28Tg CTLs showed upregulation of numerous co-inhibitory receptors, including *Pdcd1* (PD-1), *Havcr2* (TIM-3), *Cd160*, and *Cd244a* (2B4), as well as the short-lived effector cell (SLEC) marker *Klrg1*. Therefore, these results suggest that let-7 deficiency leads to terminal-effector differentiation, which renders CD8^{+} T cells susceptible to exhaustion upon engagement with their cognate ligands, which can be found in immunosuppressive conditions such as the tumor microenvironment.

To test whether let-7 expression influences the memory potential of differentiating CD8$^+$ T cells, we injected $2x10^4$ P14Rag2KO WT or P14Rag2KO Lin28Tg naive CD8$^+$ T cells into congenic CD45.1$^+$ recipient mice which were subsequently infected with $6x10^6$ colony-forming units (cfu) of a strain of the bacterium *Listeria monocytogenes*, a well-established model for the study of memory T cell responses (Khan & Badovinac, 2015), that expresses the LCMV gp$_{33-41}$ peptide antigen (Lm-gp33). Strikingly, the frequencies of donor CD8$^+$ T cells in the spleen of mice that received Lin28Tg cells were dramatically diminished, and the proportions of effector-memory (CD44^{hi}CD62L^{lo}) to central-memory cells (CD44^{hi}CD62L^{hi}) were increased, in comparison to mice that received WT cells **(Figure 3.3D)**. These results demonstrate that the formation of memory CD8$^+$ T cells is compromised in the absence of let-7 miRNAs, and that in these conditions the cells that survive contraction preferentially generate effector-memory cells. Overall, our results strongly indicate that let-7 plays a regulatory role in the fate determination of CD8$^+$ T cells into memory and terminally-differentiated cells.

3.2.3 Let-7 expression primes CD8$^+$ T cells towards the memory fate early upon antigen encounter by negatively regulating T-cell receptor signaling pathways

The asymmetric-cell-fate model and the signal-strength model propose that the memory CD8$^+$ T cell fate is specified following antigen encounter (Kaech & Cui, 2012). To determine whether let-7 can direct the fate of CD8$^+$ T cells towards memory formation early during activation, we performed RNA-Seq on P14Rag2KO WT, Let-7Tg, and Lin28Tg naive CD8$^+$ T cells, as well as CD8$^+$ T cells activated *in vitro* for 12 hours.

To examine whether let-7 expression has an impact on the gene signatures of CD8$^+$ T cells at early stages of differentiation, PCA analysis was run on normalized expression reads of naive and 12h-activated CD8$^+$ T cells. Again, the output data demonstrated that the transcriptomes of WT, Let-7Tg, and Lin28Tg CD8$^+$ T cells clustered separately both at the naive and 12h-activated stage, as shown by the shift of samples from different genotypes along PC1 (accounting for 61% and 57% of the total variance in naive and 12h-activated, respectively) and PC2 (accounting for 21% and 22% of the total variance in naive and 12h-activated, respectively), as well as by the grouping of all samples from the same genotype into non-overlapping clusters **(Figure 3.4A and 3.4B)**. These results demonstrate that let-7 expression induces transcriptional changes early during CD8$^+$ T cell differentiation.

To assess the magnitude of let-7 influence on the transcriptome of naive and 12h-activated CD8$^+$ T cells, we analyzed the differential gene expression between WT and Let-7Tg or Lin28Tg CD8$^+$ T cells at these two time points. In naive CD8$^+$ T cells, 220 genes were downregulated and 131 genes were upregulated in Let-7Tg cells, whereas in Lin28Tg cells 1,687 genes were downregulated, and 2,718 genes were upregulated **(Figure 3.4C)**. The same analysis in 12h-activated CD8$^+$ T cells revealed that 357 genes were downregulated and 241 genes were upregulated in Let-7Tg cells, while in Lin28Tg cells 580 genes were downregulated and 1,177 genes were upregulated. These findings demonstrate that let-7 expression already has a substantial impact on the transcriptome of CD8$^+$ T cells at early stages of differentiation **(Figure 3.4D)**.

To determine which genes were significantly controlled by let-7 early during CD8$^+$ T cell differentiation, heatmap clusters representing the same 4 trends as in our

CTL bioinformatics studies were generated using all genes exhibiting at least a 30% change in expression ($|\log_2(\text{fold change})| = 0.5$) in Let-7Tg and Lin28Tg CD8$^+$ T cells, as compared to WT cells, at the naive and 12h-activated stage. In naive cells, 148 of these genes could be incorporated in these 4 clusters (**Figure 3.4E)**, whereas in 12h-activated cells, 224 of these genes could be included **(Figure 3.4F)**. Cluster I, which contained genes repressed by let-7, was again the largest for both time points assessed, especially in 12h-activated cells. In this cluster, there were 63 genes from the naive stage, including the let-7 target genes *Cdc34*, *Efhd2*, *Gng5*, *Hk2*, and *Nme4*, as well as the proliferating cell nuclear antigen *Pcna*. At the 12h-activated time point, cluster I comprised 157 genes, including additional targets of let-7, such as *Arid3a*, *Arid3b*, and *Ero1l*, but also numerous genes involved in cell cycle regulation, including *Ccna2*, *Cdc25a*, and *Mki67*, consistent with the inhibitory role of let-7 in CD8$^+$ T cell proliferation (Wells et al., 2017). Moreover, this cluster contained T cell activation-associated genes, such as *Jund*, and *Notch2*, as well as the effector molecule *Ifng,* the activation marker *Cd44*, and the anti-apoptotic factor *Bcl2l1* (Bcl-xL) (Boise et al., 1993). Cluster II, which contained genes positively regulated by let-7, was once again the second largest cluster at both stages tested, and comprised 41 genes from the naive time point, including the co-stimulatory receptor *Cd28*, which was consistent with the same cluster in CTLs **(Figure 3.2C)**. At the 12h-activated time point, cluster II contained 47 genes, and comprised the transcription factor *Ets1*, which drives the expression of *Il7ra* (CD127) (Grenningloh et al., 2011), as well as *Il6st*, a component of the IL-6 receptor complex, the signaling of which promotes memory CD8$^+$ T cell survival (Nish et al., 2014; Harker et al., 2015). Cluster III and IV, which respectively incorporated genes either upregulated or downregulated in both Let-

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7Tg and Lin28Tg CTLs when compared to WT CTLs, were once more the least represented at both stages assessed. In naive cells, cluster III comprised 24 genes and cluster IV contained 20 genes while in 12h-activated cells cluster III incorporated 6 genes and cluster IV contained 14 genes. These results reveal that let-7 has a mostly negative impact on the transcriptome of CD8$^+$ T cells that gets established very early during T cell activation.

To identify the biological processes enriched in cluster I in both naive and 12h-activated CD8$^+$ T cells, we ran the DAVID functional gene annotation algorithm. To our surprise, the output data revealed that, in naive CD8$^+$ T cells, many functionally-related gene groups from this cluster belonged to processes associated with T cell activation. These let-7-regulated pathways included signal transduction, protein phosphorylation, gene expression regulation, cell cycle progression, apoptosis, the immune response, as well as signaling pathways downstream of TCR signaling, such as Notch, JNK, MAPK, and ERK1/2 **(Figure 3.5A)**. Consistently, in 12h-activated CD8$^+$ T cells, there was an over-representation of let-7-inhibited processes that resulted from these pathways, such as the regulation of gene expression, protein phosphorylation, and cell cycle progression **(Figure 3.5B)**, which is in agreement with our previously published findings that let-7 represses CD8$^+$ T cell proliferation (Wells et al., 2017). These data indicate that let-7 regulates CD8$^+$ T cell differentiation at early stages of CD8$^+$ T cell activation.

To validate our predictions, we first examined the extent of ERK1/2 phosphorylation (pERK1/2) in 5-minute *in vitro*-activated P14Rag2KO WT, Let-7Tg, and Lin28Tg CD8$^+$ T cells. Outstandingly, the levels of pERK1/2 were diminished in Let-7Tg cells, while in Lin28Tg cells the amount of pERK1/2 was increased, which suggests

that let-7 regulates the strength of TCR signaling in CD8^{+} T cells **(Figure 3.5C)**. These findings confirm that let-7 controls the differentiation of CD8^{+} T cells during the very first minutes following activation by inhibiting early signaling pathways, including the ERK1/2 pathway.

We next sought to determine whether let-7 controls the fate of CD8^{+} T cells through the negative regulation of the Notch signaling pathway, which has already been reported to promote short-lived effector cell (SLEC) over memory precursor effector cell (MPEC) generation (Backer et al., 2014). Notch is a transmembrane receptor which, upon ligand binding on its extracellular portion, undergoes cleavage of its intracellular portion, Notch intracellular domain (NICD) (Artavanis-Tsakonas et al., 1995). NICD is the active form of Notch which can participate both in the transcriptional control of gene expression in the nucleus (canonical Notch signaling) and in signaling pathways in the cytoplasm (non-canonical Notch signaling) (Nam et al., 2003; Kwon et al., 2011; Minter & Osborne, 2012; Dongre et al., 2014; Shin et al., 2014). We overexpressed NICD, containing either a nuclear export signal (NES) or a nuclear localization signal (NLS), in P14Rag2KO CD8^{+} T cells by transduction using the pMRX-IRES-GFP retroviral vector that contains a GFP reporter (Saitoh et al., 2002), and assessed their phenotype *in vivo*. We injected $2x10^5$ of these cells into congenic CD45.1^{+} recipient mice which were subsequently infected with Lm-gp33. Surprisingly, both NICD-NES and NICD-NLS overexpression in CD8^{+} T cells had a dramatic effect on memory formation in the spleen of these mice 30 days post-infection (dpi) **(Figure 3.5D)**. Specifically, the frequencies of NICD-NES and NICD-NLS-overexpressing CD8^{+} T cells were substantially reduced in comparison with cells that overexpressed the empty vector, as represented by GFP expression, which

demonstrates that these cells lost their memory potential. Moreover, the percentages of effector-memory cells were increased, while central-memory cell proportions were diminished in NICD-NES- and NICD-NLS-overexpressing CD8$^+$ T cells that persisted at this time point. This was consistent with the diminished frequencies and enhanced effector-memory to central-memory cell identity ratio of donor P14Rag2KO Lin28Tg CD8$^+$ T cells present in the spleen of Lm-gp33-challenged CD45.1$^+$ mice at the same time point **(Figure 3.3D)**. These results suggest that let-7 antagonizes the Notch signaling pathway that promotes terminal-effector CD8$^+$ T cell differentiation over memory formation both by participating in signaling pathways and driving gene transcription.

Since we showed that let-7 negatively regulates early T cell activation signaling pathways, and that we previously demonstrated that let-7 downregulation depends on the strength of TCR signaling upon CD8$^+$ T cell activation (Wells et al., 2017), we next sought to examine whether the generation of SLECs and MPECs depended on strong and weak TCR signals, in accordance with the signal-strength and asymmetric-cell-fate models. To this end, we took advantage of Nur77-GFP mice, which express a GFP reporter under the control of the *Nr41a* (Nur77) promoter, the expression level of which directly correlates with the strength of TCR signaling received by T cells upon activation (Moran et al., 2011). We crossed these mice to P14Rag2KO mice to generate P14Rag2KO Nur77-GFP mice, and we injected 2×10^4 naive CD8$^+$ T cells from these mice into CD45.1$^+$ congenic recipient mice that were subsequently challenged with Lm-gp33. Remarkably, we observed that, at day 9 dpi, SLECs (KLRG1^{hi}CD127^{lo}) expressed higher levels of Nur77 than MPECs (KLRG1^{lo}CD127^{hi}), as reflected by GFP MFI **(Figure 3.5E)**. Of note, intermediate cells (KLRG1^{hi}CD127^{hi}) shared similar Nur77

expression to MPECs. These findings directly validate the signal-strength model of CD8^{+} T cell fate determination. Moreover, our data suggest that let-7 promotes the memory CD8^{+} T cell fate by suppressing the strength of TCR signaling.

These analyses strongly indicate that let-7 negatively regulates CD8^{+} T cell differentiation early on by diminishing the strength of TCR signaling, thereby inhibiting early downstream activation pathways, including ERK1/2 and Notch. These results coincide with the signal-strength and asymmetric-cell-fate models of CD8^{+} T cell fate specification, and thus provide a mechanism for the let-7-mediated regulation of memory versus terminal-effector CD8^{+} T cell fate determination.

3.2.4 Let-7 consistently regulates target genes throughout CD8^{+} T cell differentiation

To deepen our understanding of the let-7-regulated molecular mechanisms controlling the fate of CD8^{+} T cells, we compared all the let-7 target genes contained in cluster I at all stages of CD8^{+} T cell differentiation assessed (naive, 12h-activated, and CTLs). Remarkably, this analysis revealed that multiple genes were negatively regulated by let-7 throughout CD8^{+} T cell differentiation **(Figure 3.6A)**. For instance, the ubiquitin-conjugating enzyme *Cdc34*, which is involved in the regulation of the cell cycle, as well as in the NF-κB and Wnt signaling pathways, was negatively regulated by let-7 in both naive and 12h-activated CD8^{+} T cells (Semplici et al., 2002 ; Legesse-Miller et al., 2010; Wu et al., 2010). Moreover, the expression of the transcription factor *Mycn*, which promotes hematopoietic stem cell proliferation, differentiation, survival, was inhibited by let-7 at both the 12h-activated and CTLs stage of CD8^{+} T cell differentiation (Laurenti et al., 2008). Most interestingly, let-7 consistently repressed several target genes at all time

points tested, namely *Efhd2*, *Gng5*, and *Hk2*. *Efhd2*, or EF Hand Domain Family Member D2, is a calcium-binding adapter protein which has been shown to promote CD8$^+$ T cell cytotoxicity and contribute to PD-1 inhibitory activity (Peled et al., 2018). *Gng5* is a G protein-coupled receptor gamma subunit that participates in chemokine receptor signaling, which regulates the migration of T cells (Griffith et al., 2014). *Hk2* is the glycolytic enzyme hexokinase 2, which catalyzes the rate-limiting step of glucose phosphorylation into glucose-6-phosphate, and is highly upregulated in T cells upon activation as well as in terminal effectors, but gets downregulated in memory CD8$^+$ T cells (Tan et al., 2017; Sukumar et al., 2013). These results provide candidate let-7 target genes that may play a role in the regulation of CD8$^+$ T cell fate determination.

To test whether the candidate let-7 target genes identified above contribute to the regulation of terminal-effector versus memory CD8$^+$ T cell fate specification, we overexpressed the open reading frame (ORF) of *Cdc34*, *Efhd2*, *Gng5*, *Hk2*, and *Mycn* in CD8$^+$ T cells by transduction using the pMRX-IRES-GFP retroviral vector. Overexpression of *Cdc34* in *in vitro*-generated Let-7Tg CTLs resulted in a slight increase in PD-1 expression, and, unexpectedly, in enhanced frequencies of central memory-like (CD44^{hi}CD62L^{hi}) cells over effector memory-like (CD44^{hi}CD62L^{lo}) cells **(Figure 3.6B)**. However, this effect was also observed in cells that were not transduced (GFP$^-$), making these results inconclusive. To test the biological relevance of the remaining candidate genes in the fate of CD8$^+$ T cells, P14Rag2KO CD8$^+$ T cells overexpressing the ORF of *Efhd2*, *Gng5*, *Hk2*, or *Mycn* were injected into CD45.1$^+$ congenic host mice that were subsequently infected with Lm-gp33. At 30 dpi, only overexpression of *Hk2* and *Mycn* had substantial effects on the formation of memory CD8$^+$ T cells in the spleen of these

mice **(Figure 3.6C and 3.6D)**. In fact, the percentages of *Hk2*- and *Mycn*-overexpressing CD8$^+$ T cells were significantly diminished when compared to cells that overexpressed the empty vector. Moreover, the frequencies of effector-memory cells were increased, while central-memory cell proportions were diminished in *Hk2*- or *Mycn*- overexpressing cells that persisted at this time point. These findings are consistent with the phenotype observed upon Notch NICD overexpression **(Figure 3.5D)**, and are in agreement with previous reports describing Notch as a positive regulator of glycolysis, and *Mycn* as a positive regulator of Notch (Landor et al., 2011; Tong et al., 2019). Thus, these data demonstrate that *Hk2* and *Mycn* are let-7 target genes that play a role in establishing the terminal-effector differentiation program and repressing memory formation in CD8$^+$ T cells.

Taken together, our results demonstrate that let-7 miRNA expression specifies the fate of CD8$^+$ T cells following antigen encounter towards memory formation while preventing terminal-effector differentiation, and thus the susceptibility to exhaustion, thereby solving the *in-vitro* versus *in-vivo* paradox of let-7-regulated CTL function. Specifically, absence of let-7 leads to the upregulation of co-inhibitory receptors that induce exhaustion upon engagement with their cognate ligands, which are abundantly expressed in immunosuppressive environments encountered during chronic infections and cancer. In fact, we show that let-7 alters the CD8$^+$ T cell transcriptome, reduces TCR signaling strength, and inhibits downstream signaling pathways during early stages of activation, including ERK1/2 and Notch. Finally, we identified multiple let-7 target genes that were continuously dysregulated during CD8$^+$ T cell differentiation, and among these we found that *Hk2* and *Mycn* drive the terminal-effector CD8$^+$ T cell fate, while inhibiting

the generation of memory cells. Consequently, our discovery that let-7 skews CD8$^+$ T cell fate towards memory cell generation may lead to the development of novel therapeutic strategies contributing to substantial advances in the treatment of cancer and chronic infections.

3.3 Discussion

Our study uncovered a novel post-transcriptional mechanism regulating the fate of CD8$^+$ T cells towards memory and terminally-differentiated effector cell formation, using RNA-Seq and bioinformatics analyses as an unbiased approach. Particularly, we identified let-7 miRNAs as central regulators of this dichotomy, promoting the generation of memory CD8$^+$ T cells while restraining the terminal effector program early during CD8$^+$ T cell differentiation, thereby preventing exhaustion in chronic inflammatory conditions. This discovery elucidates the paradoxical CTL performance *in-vitro* and *in-vivo*, as let-7 deficiency resulted in high expression of co-inhibitory receptors that can induce exhaustion upon interaction with their cognate ligands, which are upregulated in immunosuppressive conditions, such as the tumor microenvironment. Most importantly, our findings directly establishes let-7 has a promising innovative therapeutic means to treat chronic pathologies such as cancer.

Analysis of the CTL transcriptome showed that let-7 expression significantly altered the phenotype of these cells, in which many genes were negatively regulated by let-7. Consistently with the *in-vitro* phenotype of CTLs, Let-7Tg CTLs upregulated genes involved in the immune response and the regulation of apoptosis, whereas processes such as the defense response to viruses and the response to biotic stimuli were over-represented

in let-7 deficient CTLs. Moreover, let-7 overexpression contributed to the enrichment of a memory-associated gene signature, whereas in the absence of let-7 a gene set characteristic of terminally-differentiated CD8$^+$ T cells was over-represented. Specifically, we found that Let-7Tg CTLs expressed high levels of the well-defined memory markers *Ccr7*, *Sell* (CD62L), *Il7ra*, *Bcl2*, *Foxo1*, *Id3*, *Tcf7*, and *Lef1*, as well as T stem cell memory markers, including *Axin2*, *S1pr1* and *Sox4*, but also transcription factors involved in CTL differentiation, such as *Tbx21* (T-bet) and *Zeb2* (Gattinoni et al., 2009; Gattinoni et al., 2011; Dominguez et al., 2015). Additionally, these cells expressed high levels of co-stimulatory receptors, such as *Cd28*, *Icos*, and *Ox40*, the signaling of which contributes to efficient memory responses (Borowski et al., 2007). Based on these results, we could infer that, although they exhibit reduced cytotoxicity *in vitro*, Let-7Tg CTLs maintain a central/stem cell-like memory potential and are thus able to elicit the robust, long-lasting anti-tumor immunity observed *in vivo*. This is in accordance with our previously published findings that let-7 preserves the quiescence of naive T cells, as memory cells are characterized by a return to the quiescent state (Kalia et al., 2015; Wells et al., 2017). Our predictions are also consistent with another publication in which we demonstrated that let-7 expression is required for the survival of naive T cells, as Let-7Tg CTLs had increased expression of the anti-apoptotic genes *Bcl2* and *Bcl11b* (Bcl-xL) (Pobezinskaya et al., 2019). Thus, let-7 may contribute to the resistance of memory precursors to the contraction phase and prolonged survival of memory cells through enhanced *Bcl2* expression (Akbar et al., 1993; Grayson et al., 2000; Pobezinskaya et al., 2019). In contrast, Lin28Tg CTLs preferentially expressed the transcription factors *Eomes*, *Id2*, *Ikzf2* (Helios), and *Prdm1* (Blimp-1), the co-inhibitory receptors *Pdcd1* (PD-

1), *Havcr2* (TIM-3), *Cd160*, and *Cd244a* (2B4), effector molecules and cytokines *GzmA*, *GzmB*, *Prf1*, *Ifng*, *Tnf*, and *Fasl*, as well as the immunosuppressive cytokine *Il10*, all of which are highly expressed in terminally-differentiated and exhausted CD8$^+$ T cells (Wherry, 2007; Blackburn et al., 2009; Shin et al., 2009). These cells also had increased expression of the co-stimulatory receptor *Tnfrsf9* (4-1BB), which has been described as an exhaustion marker in CD8$^+$ T cells (Williams et al., 2017). Several of these genes were also direct targets of let-7, namely *Eomes*, which we previously reported, as well as *Ikzf2* and *Il10* (Swaminathan et al., 2012; Agarwal et al., 2015; Wells et al., 2017). Our data indicate that, despite their superior *in-vitro* cytotoxic activity, Lin28Tg CTLs fail to control tumor growth *in vivo* due to the high expression of these co-inhibitory receptors, which likely interacted with their cognate ligands in the tumor microenvironment, inducing exhaustion in these cells. We further show experimentally that Lin28Tg CD8$^+$ T cell have a poor memory potential, as only few of these cells persisted after Lm-gp33 clearance, which are skewed towards the effector-memory phenotype. Thus, these results suggest that absence of let-7 leads to the terminal differentiation of CD8$^+$ T cells, which renders these cells prone to exhaustion.

In fact, we show that this let-7-mediated-regulation of CD8$^+$ T cell fate determination occurs soon after antigen encounter, which is consistent with previous work in our lab that recapitulated the phenotype of Let-7Tg CTLs by inducing the let-7g transgene only during the first 48h following CD8$^+$ T cell activation *in vitro* **(Figure 3.7)**. Specifically, transcriptome analysis of naive CD8$^+$ T cells predicted that numerous signaling pathways downstream of TCR signaling were negatively regulated by let-7, including ERK1/2 and Notch, which we experimentally validated. Interestingly, both the

cytoplasmic (NICD-NES) and nuclear (NICD-NLS) active forms of Notch inhibited memory CD8^{+} T cell formation and skewed the persisting memory cells towards effector-memory cell formation at the expense of central-memory cell generation, thereby showing that both canonical and non-canonical Notch signaling contribute to the regulation of CD8^{+} T cell fate. These results indicate that let-7 expression diminishes the strength of TCR signaling in these cells, thereby specifying the memory CD8^{+} T cell fate minutes following antigen encounter. In fact, we show, using P14Rag2KO donor CD8^{+} T cells that express the Nur77-GFP reporter of TCR signal strength, that SLECs exhibit a stronger TCR signaling history than MPECs. These data are consistent with previous work in our lab demonstrating that let-7 inhibits TCR signal strength in *in vitro*-activated P14Rag2KO WT, Let-7Tg, and Lin28Tg CD8^{+} T cells expressing Nur77-GFP **(Figure 3.8)**. Our data are in accordance with the signal-strength and asymmetric-cell-fate models of CD8^{+} T cell fate determination, both of which propose that the strength of TCR signaling received upon T cell activation inversely correlates with the extent of memory potential (Kaech & Cui, 2012). Our predictions are also consistent with a previously published report in which we showed that the magnitude of let-7 downregulation upon CD8^{+} T cell activation depends on the strength and duration of TCR signaling, where high let-7 expression negatively regulates CTL differentiation (Wells et al., 2017). Moreover, examination of the CD8^{+} T cell transcriptome at both the naive and 12h-activated stage revealed that let-7 inhibited cell cycle progression, which we had already shown before (Wells et al., 2017). Thus, reduced proliferation, in addition to the enhanced survival promoted by let-7 in CD8^{+} T cells, may also contribute to the maintenance of the quiescent state in these cells, thereby preserving the memory cell pool.

Even though our study provides strong evidence that let-7 miRNAs promote the fate of CD8$^+$ T cells towards memory CD8$^+$ T cells while restraining terminal-effector differentiation and exhaustion, the relative expression of let-7 at these different stages of CD8$^+$ T cell differentiation *in vivo* remains to be examined. It would be reasonable to hypothesize that, because weak TCR signaling induces the retention of let-7 expression, these cells would adopt the memory fate, while CD8$^+$ T cells receiving strong TCR signals downregulate let-7 to a greater extent will become terminally-differentiated, and thus more susceptible to exhaustion. Along these lines, recurrent TCR stimulation during chronic inflammation due to persisting antigen may gradually cause the loss of let-7 expression in CD8$^+$ T cells, which would progressively direct these cells towards the exhausted state. In fact, we confirmed, using CD8$^+$ T cells expressing the Nur77-GFP reporter of TCR signal strength, that SLECs do receive stronger TCR signals than MPECs, which is in agreement with our hypothesis. In addition, it would be interesting to test the memory versus terminal-effector differentiation potential of CD8$^+$ T cells activated *in vitro* with different strengths of TCR stimulation at the phenotypic and functional levels.

We also identified multiple direct target genes of let-7 that were consistently dysregulated throughout CD8$^+$ T cell differentiation, which provided candidates to study the let-7-mediated mechanisms that inhibit terminal effector differentiation and exhaustion. The ubiquitin-conjugating enzyme *Cdc34* was common to the naive and 12h-activated time points, the oncogenic transcription factor *Mycn* was found at both the 12h-activated and CTL stages, and the calcium-binding adapter protein *Efhd2*, the G-protein gamma subunit *Gng5*, as well as the rate-limiting glycolytic enzyme *Hk2* were negatively

regulated by let-7 at all stages of CD8$^+$ T cell differentiation examined. Among these, the rate-limiting glycolytic enzyme *Hk2* and the oncogenic transcription factor *Mycn* were confirmed to prevent memory CD8$^+$ T cell formation and enhance the effector- to central-memory cell ratios using the Lm infection model. Since *Hk2* and *Mycn* overexpression recapitulated the phenotype observed upon overexpression of Notch, and given that Notch signaling induces glycolysis and is regulated by *Mycn*, additional overexpression studies in Let-7Tg CD8$^+$ T cells or knockdown in Lin28Tg cells would determine whether these factors have redundant or distinct roles in CD8$^+$ T cell fate regulation (Landor et al., 2011; Tong et al., 2019). Although they were not investigated in this study, we do not rule out the possibility that genes which are regulated by let-7 at a single stage of CD8$^+$ T cell differentiation may play a role in the regulation of terminal effector differentiation and memory formation. In fact, previous work in our lab has identified such a role for the transcription factor *Arid3a*, the expression of which is inhibited by let-7 at the 12h-activated stage, as well as the transcription factors *Eomes* and *Hmga1*, which are both negatively regulated by let-7 at the CTL stage **(Figure 3.9)**. Thus, together with the early T cell activation signaling pathways we had identified above, assessing the function of these genes will advance our knowledge of the molecular mechanisms through which let-7 controls the fate of CD8$^+$ T cells.

Although the present study offers significant insights into the let-7-mediated mechanisms regulating terminal effector versus memory CD8$^+$ T cell differentiation, it only examined the role of let-7 miRNAs in the fate of CD8$^+$ T cells at the transcriptional level, and thus provides an incomplete perspective in this regard. Therefore, validating whether let-7 shapes the proteome of differentiating CD8$^+$ T cells consistently with our

transcriptomic study will be important. Moreover, because the memory and terminal-effector fates are also regulated at the epigenetic level, whether let-7 performs its regulatory function by modeling the chromatin state of differentiating CD8$^+$ T cells remains to be examined (Araki et al., 2009; Crompton et al., 2016; Kakaradov et al., 2017; Henning et al., 2018). Furthermore, since it is well understood that terminally-differentiated and memory T cells rely on distinct metabolic processes, investigating whether let-7 plays a role in modulating these metabolic pathways will be interesting, especially since we already showed that the expression of glycolytic genes, including *Glut1*, *Glut3*, *Hk2*, *Pfk1*, *Pkm*, and *Tpi*, were repressed by let-7 (Wells et al., 2017). Moreover, previous work in our lab showed that, when compared to WT CTLs, Let-7Tg CTLs exhibit a lower glycolytic rate, while in Lin28Tg CTLs glycolysis is increased **(Figure 3.10)**, which is in agreement with studies describing that memory CD8$^+$ T cells preferentially use fatty acid metabolism, whereas glycolysis is used to a higher extent in terminally-differentiated cells (Araki et al., 2009; Pearce et al., 2009; Michalek & Rathmell, 2010; Sukumar et al., 2013; Pollizzi et al., 2016). This is also consistent with our results showing that *Hk2*-overexpressing CD8$^+$ T cells fail to form memory cells.

Our data emphasize a strong potential for the translation of the let-7-mediated control of memory CD8$^+$ T cell formation into novel therapies for the treatment of chronic pathologies such as cancer and persistent infections. Such innovative medical solutions could consist of let-7 miRNA delivery to CD8$^+$ T cells during the *ex vivo* T cell expansion phase of CAR-T cell production for adoptive T cell therapy, during which these cells would be directed towards the memory fate, and thus would not be as prone to exhaustion (Ho et al., 2003; Rosenberg et al., 2011). Besides, in the case of anti-cancer therapy,

delivering let-7 miRNAs directly to tumor cells could also be performed, since let-7 is a well-known tumor suppressor, and thus may have at least an additive effect on the outcome of tumor control. Moreover, the combination of these let-7-based therapies with checkpoint blockade immunotherapy may elicit a superior anti-tumor response. Furthermore, our study may substantially contribute to advances in vaccine development, since effective vaccination relies on the generation of memory cells (Lauvau et al., 2001; Akondy et al., 2017). Finally, although it remains to be tested experimentally, inhibiting let-7 expression in CD8^{+} T cells may constitute a promising healthcare solution to treat CD8^{+} T cell-driven autoimmune disorders, such as type-1 diabetes, by inducing exhaustion in these self-reactive cells (McKinney et al., 2015).

Altogether, our study elucidated the paradoxical outcome of let-7 expression on the *in-vitro* versus *in-vivo* activity of CTLs by predicting that let-7 expression regulates CD8^{+} T cell differentiation towards the generation of memory cells, while inhibiting the differentiation of terminal effectors. Additionally, our data establish let-7 miRNA as novel therapeutic tool for the manipulation of CD8^{+} T cell function in the contexts of cancer, persistent infections, and autoimmune diseases.

A Cytotoxicity assay of *in vitro*-generated P14Rag2KO CTLs (4h)

Data by Alexandria Wells, Ph.D.

B

Data by Elena Pobezinskaya, Ph.D., and Alexandria Wells, Ph.D.
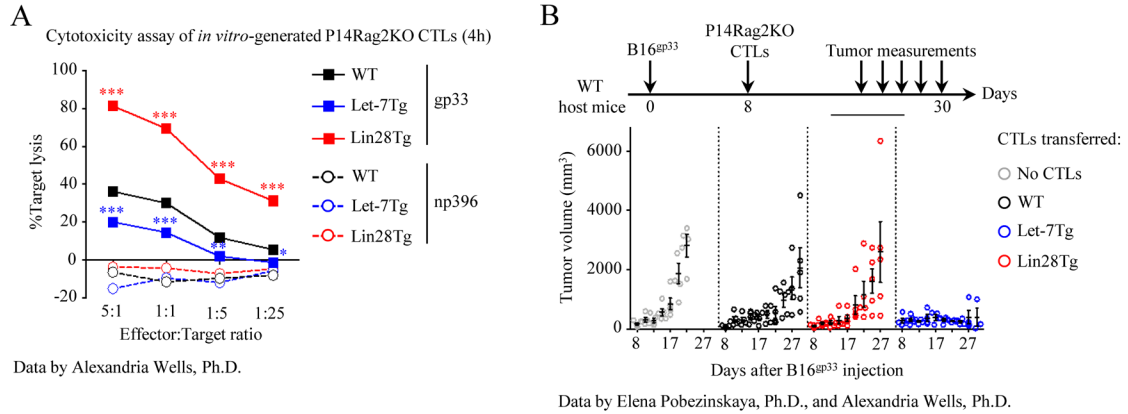
Figure 3.1 Let-7 expression in CTLs results in paradoxical functional outcomes *in vitro* and *in vivo*. (A) Cytotoxicity assay of *in vitro*-differentiated CTLs from P14Rag2KO WT, P14Rag2KO Let-7Tg, and P14Rag2KO Lin28Tg mice co-cultured for 4h at the indicated ratios with splenocytes pulsed with either LCMV gp$_{33-41}$ peptide (cognate P14 epitope) or LCMV np396 peptide (non-specific epitope). **(B)** Tumor growth curves in mice inoculated subcutaneously with 0.25×10^6 B16^{gp33} tumor cells. Tumor-bearing mice received adoptive transfer of 1.5×10^6 CTLs generated *in vitro* from either P14Rag2KO WT (n=5), P14Rag2KO Let-7Tg (n=5), P14Rag2KO Lin28Tg (n=5) mice. Some mice did not receive CTLs (n=5). * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$, compared with WT using two-tailed Student's t-test. Data are from one experiment representative of three experiments (A; mean and S.E.M of technical triplicates), or from one experiment representative of two experiments (B; mean and S.E.M. of 5 biological replicates).

A — PCA on P14Rag2KO CTLs

B — Differential gene expression analysis on P14Rag2KO CTLs

Lin28Tg vs WT · Let-7Tg vs WT

● Upregulated   ● Downregulated

C — Differentially-expressed genes with |log2FoldChange| ≥ 0.5
P14Rag2KO CTLs

D — DAVID GO Biological process enrichment analysis on genes
from Cluster I and Cluster II in P14Rag2KO CTLs
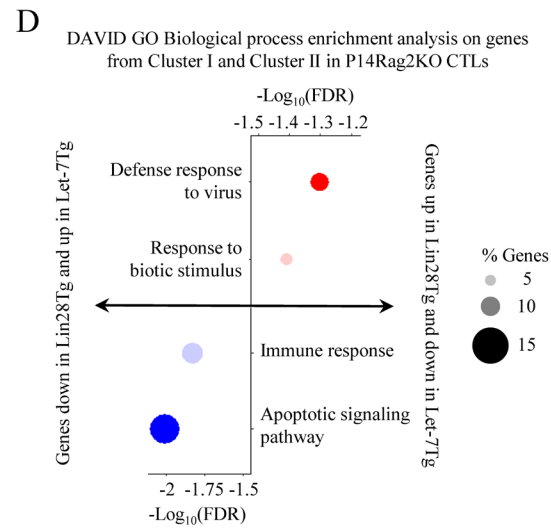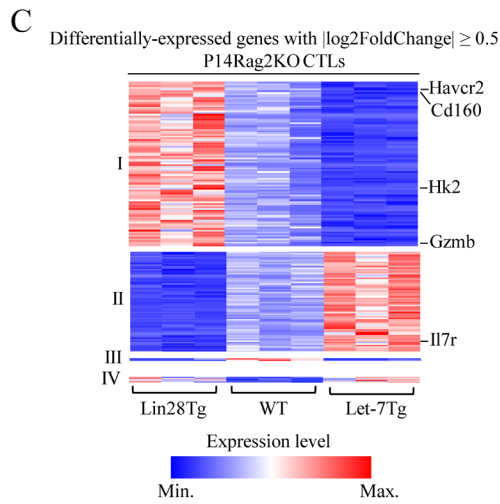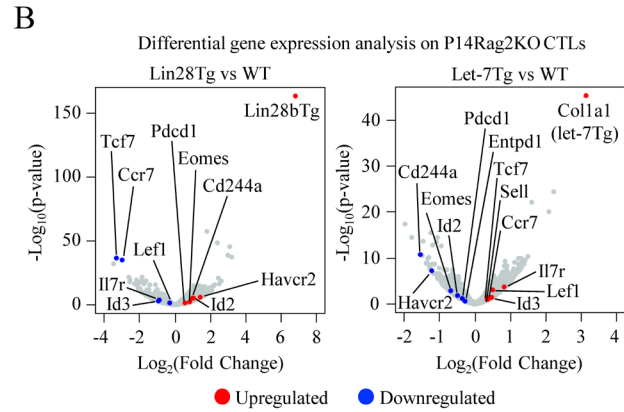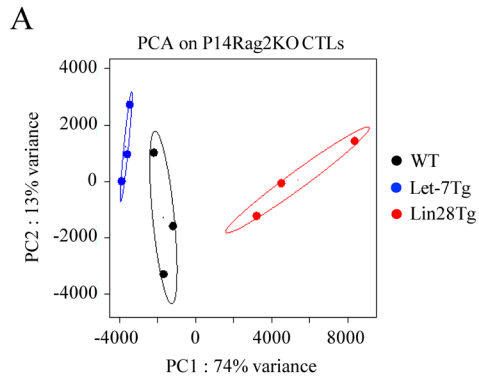
Figure 3.2 Let-7 miRNAs significantly alter the CTL transcriptome. (A) Principal component analysis (PCA) on normalized expression read counts from the RNA-Seq data obtained from *in vitro*-generated CTLs from P14Rag2KO WT, P14Rag2KO Let-7, and P14Rag2KO Lin28Tg mice, separated by PC1 (y axis) and PC2 (x axis), which explain 74% and 13% of the total variance between samples, respectively. All replicate samples from each genotype were clustered by applying 95% confidence ellipses (p <0.05). **(B)** Volcano plots displaying differential gene expression in *in vitro*-differentiated P14Rag2KO Lin28Tg (left panel) and P14Rag2KO Let-7Tg (right panel) CTLs, as compared with P14Rag2KO WT CTLs, by the mean expression value of $-\log_{10}$(p-value) (y axis) and the $\log_2$ value of the expression fold change (Log2(Fold Change), x axis). Each differentially-expressed gene is represented by a gray solid circle, and examples of upregulated (red solid circles) and downregulated (blue solid circles) associated with CTL differentiation are indicated in each plot. **(C)** Heatmap clusters of normalized expression read counts from the RNA-Seq data showing all genes differentially expressed by $|\log_2$(Fold Change)$| \geq 0.5$ in a statistically-significant manner (p<0.05) in *in vitro*-generated CTLs from P14Rag2KO WT (center), P14Rag2KO Let-7 (right), and P14Rag2KO Lin28Tg (left) mice. Downregulated genes are indicated in blue and upregulated genes are represented in red. Cluster I shows genes downregulated in Let-7Tg CTLs and upregulated in Lin28Tg CTLs, cluster II indicates genes upregulated in Let-7Tg CTLs and downregulated in Lin28Tg CTLs, cluster III represents genes downregulated in both Let-7Tg and Lin28Tg CTLs, and cluster IV shows genes upregulated in both Let-7Tg and Lin28Tg CTLs, in comparison with WT CTLs. Examples of differentially-expressed genes important for CTL differentiation are indicated. **(D)** Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of gene ontology (GO) biological processes significantly enriched (p <0.05) in cluster I (red bubbles) and cluster II (blue bubbles) from (C), based on the over-representation of functionally-related gene groups. Data is presented relative to the $-\log_{10}$ of the false discovery rate ($-\log_{10}$(FDR), x axis). The size of the bubbles indicates the percentage of genes from each cluster contributing to the enriched biological process.

A

GSEA on P14Rag2KO CTLs

Enrichment plot: Memory-associated genes

NES = 1.458
p < 0.001

NES = -1.518
p < 0.001

Let-7Tg ← → WT

Lin28Tg ← → WT

Enrichment plot: Terminal effector-associated genes

NES = -1.153
p = 0.213

NES = 1.431
p = 0.118

Let-7Tg ← → WT

Lin28Tg ← → WT

B

Heatmap clustering of the genes contained in the P14Rag2KO CTL GSEA signatures

Transcription factors

Prdm1
Eomes
Id2
Tcf7
Lef1
Id3
Foxo1

Lin28Tg   WT   Let-7Tg

Effector molecules and receptors

GzmB
GzmA
Tnf
TRAIL
Fasl
Il10
Ifng
Prf1
Il6ra
Il7r

Lin28Tg   WT   Let-7Tg

Chemokines and chemokine receptors

Ccl5
Ccl4
Csf1
Ccl3
Xcl1
Cx3cr1
Cxcr1
Cxcl11
Ccr7
Cxcr6
Cxcl10

Lin28Tg   WT   Let-7Tg

Costimulatory molecules and receptors

Tnfrsf9 (4-1BB)
Tnfrsf8 (CD30)
Tnfsf9 (4-1BBL)
Klrg1
Cd27
Cd28
Sell (CD62L)
Icos
Tnfrsf4 (OX40)
Tnfsf8 (CD30L)
Tnfsf14 (LIGHT)
Tnfsf4 (OX40L)

Lin28Tg   WT   Let-7Tg

Inhibitory receptors

Entpd1 (CD39)
Havcr2 (TIM-3)
Cd160
Pdcd1 (PD-1)
Cd244a (2B4)
Pdcd1lg2 (PD-L2)
Mt1
Lag3
Ctla4
Mt2
Tigit
Mt3
Cd101

Lin28Tg   WT   Let-7Tg

Expression level

Min.   Max.

C

T stem cell memory (Tscm) markers

P14Rag2KO CTLs

Ly6a
Kit
Bcl2
Lef1
Bcl11b
S1pr1
Axin2
Slamf1
Sox4

Lin28Tg   WT   Let-7Tg

Expression level

Min.   Max.

D

Day 30 post-Lm-gp33 infection

Gated on live singlets

WT   0.37

Lin28Tg   0.04

Gated on CD45.2⁺ donor CD8⁺ T cells

7.1   92.7

29.5   68.9

Donor CD8⁺ T cells

CD44hiCD62Llo donor CD8⁺ T cells

CD44hiCD62Lhi donor CD8⁺ T cells

● P14Rag2KO WT
● P14Rag2KO Lin28Tg

Figure 3.3 Let-7 miRNA expression promotes a memory gene signature in CTLs, while let-7 deficiency is characterized by am enrichment in genes associated with terminal effectors. (A) Gene set enrichment analysis (GSEA) using the adjusted p-value-ranked normalized expression read counts from the RNA-Seq data obtained from *in vitro*-generated P14Rag2KO Let-7 and P14Rag2KO Lin28Tg CTLs scored against gene sets containing memory-associated genes (n=56; left panel) and terminal effector-associated genes (n=62; right panel), and compared to the transcriptome of P14Rag2KO WT CTLs. Gene signatures were created by gathering well-characterized genes from the literature (see Materials and methods). Normalized enrichment score (NES), as well as adjusted p-values, are shown for each analysis. Positive NES values indicate gene signature enrichment, while negative NES values show gene set under-representation. **(B)** Heatmap clusters of normalized expression read counts from the RNA-Seq data showing differential expression by P14Rag2KO WT (center), Let-7Tg (right), and Lin28Tg (left) CTLs of the genes used for GSEA in (A) that were classified into heatmap categories. Downregulated genes are indicated in blue and upregulated genes are represented in red. Examples of differentially-expressed genes important for memory and terminal effector differentiation are indicated. **(C)** Heatmap showing differential expression of T stem cell memory (Tscm) markers in P14Rag2KO WT (center), Let-7Tg (right), and Lin28Tg (left) CTLs. Downregulated genes are indicated in blue and upregulated genes are represented in red. **(D)** Staining of CD45.2$^+$ donor P14Rag2KO WT (n=3) and P14Rag2KO Lin28Tg (n=3) CD8$^+$ T cells in the spleen of CD45.1$^+$ host mice for CD44 and CD62L at day 30 post-infection with Lm-gp33. Numbers represent the frequencies of each population on the indicated gates (left). Quantification of the frequencies of total, effector memory (CD44^{hi}CD62L^{lo}), and central memory (CD44^{hi}CD62L^{hi}) CD45.2$^+$ donor P14Rag2KO CD8$^+$ T cells for each genotype as assessed by flow cytometry (right) * p <0.05, ** p < 0.01, compared with P14Rag2KO WT CD8$^+$ T cells using two-tailed Student's t-test (D). Data are from one experiment representative of two experiments (D, mean ± S.E.M. of each population from all mice).

A PCA on naive P14RagKO CD8⁺ T cells

B PCA on 12h-activated P14RagKO CD8⁺ T cells

C Differential gene expression analysis on P14Rag2KO naïve CD8⁺ T cells

Lin28Tg vs WT

Let-7Tg vs WT

D Differential gene expression analysis on P14Rag2KO 12h-activated CD8⁺ T cells

Lin28Tg vs WT

Let-7Tg vs WT

E Differentially-expressed genes with |log2FoldChange| ≥ 0.5 Naive P14RagKO CD8⁺ T cells

F Differentially-expressed genes with |log2FoldChange| ≥ 0.5 12h-activated P14Rag2KO CD8⁺ T cells

Figure 3.4 Let-miRNAs substantially modify the transcriptome of naive and 12h-activated CD8$^+$ T cells. (A and B) Principal component analysis (PCA) on normalized expression read counts from the RNA-Seq data obtained from P14Rag2KO WT, P14Rag2KO Let-7, and P14Rag2KO Lin28Tg naive **(A)** or 12h-activated **(B)** CD8$^+$ T cells, separated by PC1 (y axis) and PC2 (x axis), which explain 61% and 21% (naive) or 57% and 22% (12h-activated) of the total variance between samples, respectively. All replicate samples from each genotype were clustered by applying 95% confidence ellipses (p<0.05). **(C and D)** Volcano plots displaying differential gene expression in naive **(C)** and 12h-activated **(D)** P14Rag2KO Lin28Tg (left panel) and P14Rag2KO Let-7Tg (right panel) CD8$^+$ T cells, as compared with P14Rag2KO WT CD8$^+$ T cells, by the mean expression value of -log$_{10}$(p-value) (y axis) and the log$_2$ value of the expression fold change (Log2(Fold Change), x axis). Each differentially-expressed gene is represented by a gray solid circle, and significantly upregulated and downregulated genes are indicated in red and blue, respectively. **(E and F)** Heatmap clusters of normalized expression read counts from the RNA-Seq data showing all genes differentially expressed by |log$_2$(Fold Change)| ≥ 0.5 in a statistically-significant manner (p<0.05) in naive **(E)** and 12h-activated **(F)** P14Rag2KO WT (center), P14Rag2KO Let-7 (right), and P14Rag2KO Lin28Tg (left) mice. Downregulated genes are indicated in blue and upregulated genes are represented in red. Cluster I shows genes downregulated in Let-7Tg CD8$^+$ T cells and upregulated in Lin28Tg cells, cluster II indicates genes upregulated in Let-7Tg CD8$^+$ T cells and downregulated in Lin28Tg cells, cluster III represents genes downregulated in both Let-7Tg and Lin28Tg CD8$^+$ T cells, and cluster IV shows genes upregulated in both Let-7Tg and Lin28Tg cells, in comparison with WT CD8$^+$ T cells. Examples of differentially-expressed genes important for CD8$^+$ T cell differentiation are indicated.

A DAVID GO Biological process enrichment analysis on genes from Cluster I in naive P14Rag2KO CD8$^+$ T cells

% Genes: 1, 3, 6, 12

Apoptotic process
Positive regulation of Notch signaling pathway
Cell differentiation
Positive regulation of gene expression
Signal transduction
Activation of MAPK activity
Positive regulation of JUN kinase activity
Positive regulation of ERK1 and ERK2 cascade
Cell adhesion
Protein phosphorylation
Chemotaxis
Inflammatory response
Immune system process
Cell cycle

-Log10(FDR)

B DAVID GO Biological process enrichment analysis on genes from Cluster I in 12h-activated P14Rag2KO CD8$^+$ T cells

% Genes: 10, 20, 30, 40

Negative regulation of transcription from RNA polymerase II promoter
Protein phosphorylation
Microtubule-based movement
Cell cycle

-Log10(FDR)

C pERK1/2 expression in P14Rag2KO activated CD8$^+$ T cells (5 minutes)

Normalized MFI (relative to WT)

WT
Let-7Tg
Lin28Tg

E Nurr77-GFP expression in P14Rag2KO Nurr77-GFP CD8$^+$ T cells (Day 9 post-Lm-gp33 infection)

MFI (GFP)

SLECs
MPECs
Intermediates

D Day 30 post-Lm-gp33 infection

Gated on CD45.2$^+$ donor CD8$^+$ T cells

Gated on GFP$^+$ CD45.2$^+$ donor CD8$^+$ T cells

Empty vector — 17.9 — 20.0 / 80.0
NES — 3.1 — 40.7 / 58.1
NLS — 2.0 — 54.2 / 45.8

CD8 / GFP
CD44 / CD62L

Donor CD8$^+$ T cells
Cell frequency

CD44^{hi}CD62L^{lo} donor CD8$^+$ T cells
Cell frequency

CD44^{hi}CD62L^{hi} donor CD8$^+$ T cells
Cell frequency

● Empty vector
● NES
● NLS

Figure 3.5 Let-7 miRNAs controls the fate of differentiating CD8$^+$ T cells by suppressing the strength of TCR signaling, thereby inhibiting early signaling pathways, including ERK1/2 and Notch. Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of gene ontology (GO) biological processes significantly enriched (p <0.05) in cluster I of naive **(A)** and 12h-activated **(B)** CD8$^+$ T cells, based on the over-representation of functionally-related gene groups. Data is presented relative to the -$\log_{10}$ of the false discovery rate (-Log_{10}(FDR), x axis). The size of the bubbles indicates the percentage of genes from each cluster contributing to the enriched biological process. **(C)** Normalized MFI of phosphor-ERK1/2 (pERK1/2) in P14Rag2KO WT, P14Rag2KO Let-7Tg, and P14Rag2KO Lin28Tg CD8$^+$ T cells activated *in vitro* for 5 minutes with plate-bound anti-CD3e (1 µg/mL) and anti-CD28 mAbs (5 µg/mL). Data is presented relative to results obtained for 5-minute activated P14Rag2KO WT CD8$^+$ T cells. **(D)** Staining of CD45.2$^+$ donor P14Rag2KO WT CD8$^+$ T cells transduced with GFP reporter-containing empty retroviral vector (n=3), or retrovirus expressing Notch intracellular domain (NICD) fused to either a nuclear export signal (NES, n=3) or a nuclear localization signal (NLS, n=3) in the spleen of CD45.1$^+$ host mice for CD44 and CD62L at day 30 post-infection with Lm-gp33. Numbers represent the frequencies of each population on the indicated gates (left). Quantification of the frequencies of total, effector memory (CD44^{hi}CD62L^{lo}), and central memory (CD44^{hi}CD62L^{hi}) CD45.2$^+$ donor P14Rag2KO CD8$^+$ T cells for each condition as assessed by flow cytometry (right). **(E)** MFI of GFP of P14Rag2KO Nur77-GFP donor CD8$^+$ SLECs (KLGR1^{hi}CD62L^{lo}) and MPECs (KLRG1^{lo}CD127^{hi}) in the blood of CD45.1$^+$ host mice at day 9 post-infection with Lm-gp33. Data is presented relative to MFI obtained for control P14Rag2KO WT CD8$^+$ SLECs and MPECs. * p < 0.05; ** p <0.01, *** p < 0.001, compared with WT using two-tailed Student's t-test. Data are from one experiment representative of two experiments (C; mean and S.E.M. of technical triplicates), or from one experiment (D, E; mean and S.E.M. of each cell population).
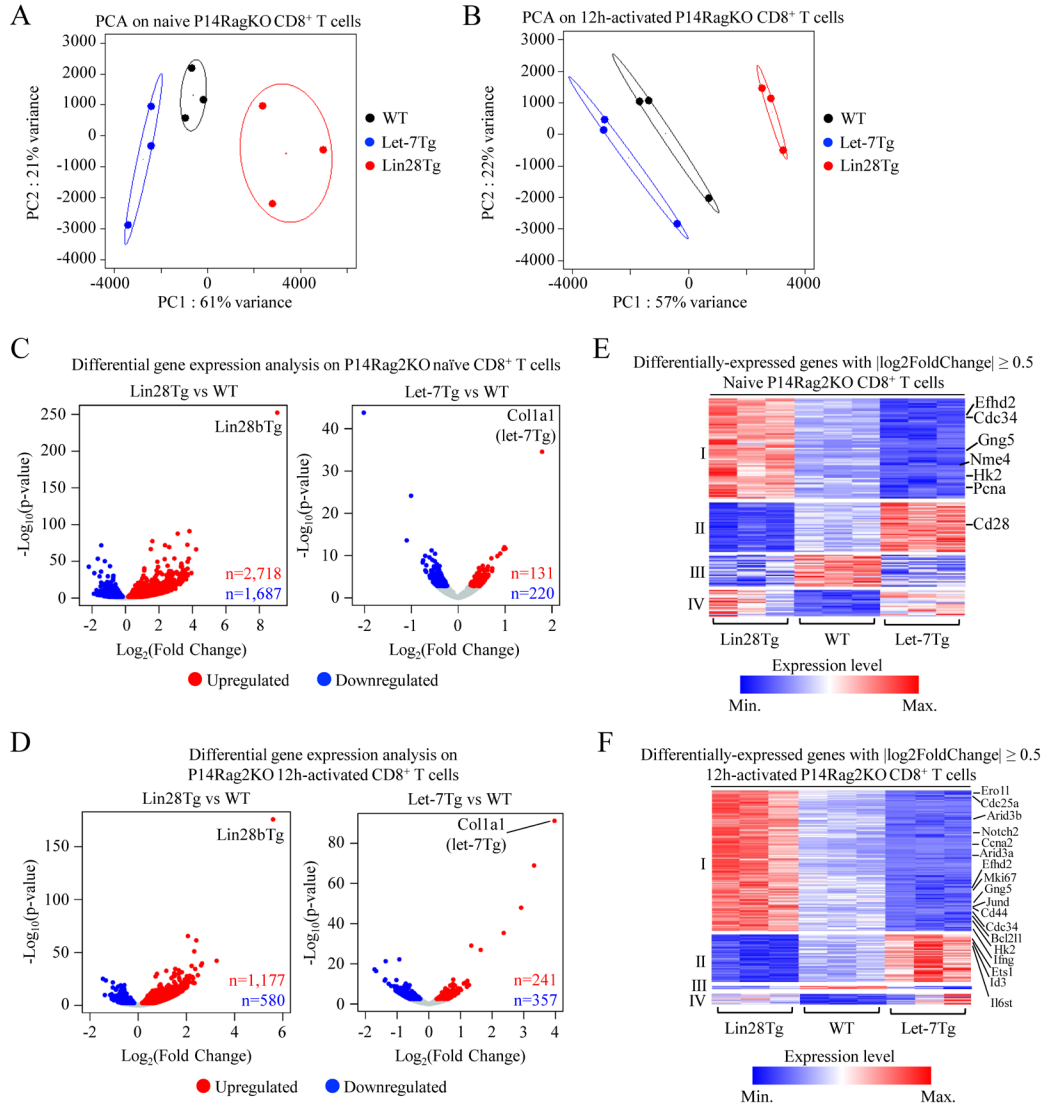
A

Distribution of let-7 target genes differentially expressed in Cluster I at all time points tested

CTLs

9

0   3   5

Naive   8   3   20   12h-activated

B

In vitro-generated P14Rag2KO Let-7Tg CTLs

Gated on CD8+ T cells | Gated on GFP^{hi} CD8+ T cells | Gated on GFP^{hi} CD8+ T cells

Empty vector

23.7

30.7   68.1

Cdc34

20.5

6.2   93.4

5.02
137
209

— Isotype control
— Empty vector
— Cdc34

PD-1

C

Day 30 post-Lm-gp33 infection

Gated on CD45.2+ donor CD8+ T cells | Gated on GFP+ CD45.2+ donor CD8+ T cells

Empty vector

18.6

29.7   70.3

Hk2

6.0

47.9   52.1

Donor CD8+ T cells

**

CD44^{hi}CD62L^{lo} donor CD8+ T cells

*

CD44^{hi}CD62L^{hi} donor CD8+ T cells

*

● Empty vector
● Hk2

D

Day 30 post-Lm-gp33 infection

Gated on CD45.2+ donor CD8+ T cells | Gated on GFP+ CD45.2+ donor CD8+ T cells

Empty vector

36.3

23.7   75.8

Mycn

8.7

9.1   72.7

113

Figure 3.6 Let-7 miRNAs consistently represses the expression of multiple target genes throughout CD8$^+$ T cell differentiation, and restrain terminal effector differentiation through inhibiting *Hk2* and *Mycn* expression. (A) Diagram representing the numbers of let-7 direct targets that are differentially expressed in cluster I a statistically significant manner at all stages of CD8$^+$ T cell differentiation examined. **(B)** Staining of P14Rag2KO Let-7Tg CD8$^+$ T cells transduced with GFP reporter-containing empty retroviral vector or retrovirus expressing Cdc34 and differentiated *in vitro* into CTLs for CD44 and CD62L. Numbers represent the frequencies of each population on the indicated gates (left). Histogram of PD-1 expression in GFP^{hi} cells from each condition, as assessed by flow cytometry. Numbers represent the MFI of PD-1 for each condition (right). **(C)** Staining of CD45.2$^+$ donor P14Rag2KO WT CD8$^+$ T cells transduced with GFP reporter-containing empty retroviral vector (n=2), or retrovirus expressing Hk2 (n=3) in the spleen of CD45.1$^+$ host mice for CD44 and CD62L at day 30 post-infection with Lm-gp33. Numbers represent the frequencies of each population on the indicated gates (left). Quantification of the frequencies of total, effector memory (CD44^{hi}CD62L^{lo}), and central memory (CD44^{hi}CD62L^{hi}) CD45.2$^+$ donor P14Rag2KO CD8$^+$ T cells for each condition as assessed by flow cytometry (right). **(D)** Staining of CD45.2$^+$ donor P14Rag2KO WT CD8$^+$ T cells transduced with GFP reporter-containing empty retroviral vector (n=1), or retrovirus expressing Mycn (n=3) in the spleen of CD45.1$^+$ host mice for CD44 and CD62L at day 30 post-infection with Lm-gp33. Numbers represent the frequencies of each population on the indicated gates. * p < 0.05; ** p <0.01, compared with WT using two-tailed Student's t-test. Data are from one experiment representative of two experiments (C; mean and S.E.M. of each cell population), or from one experiment (B, D).
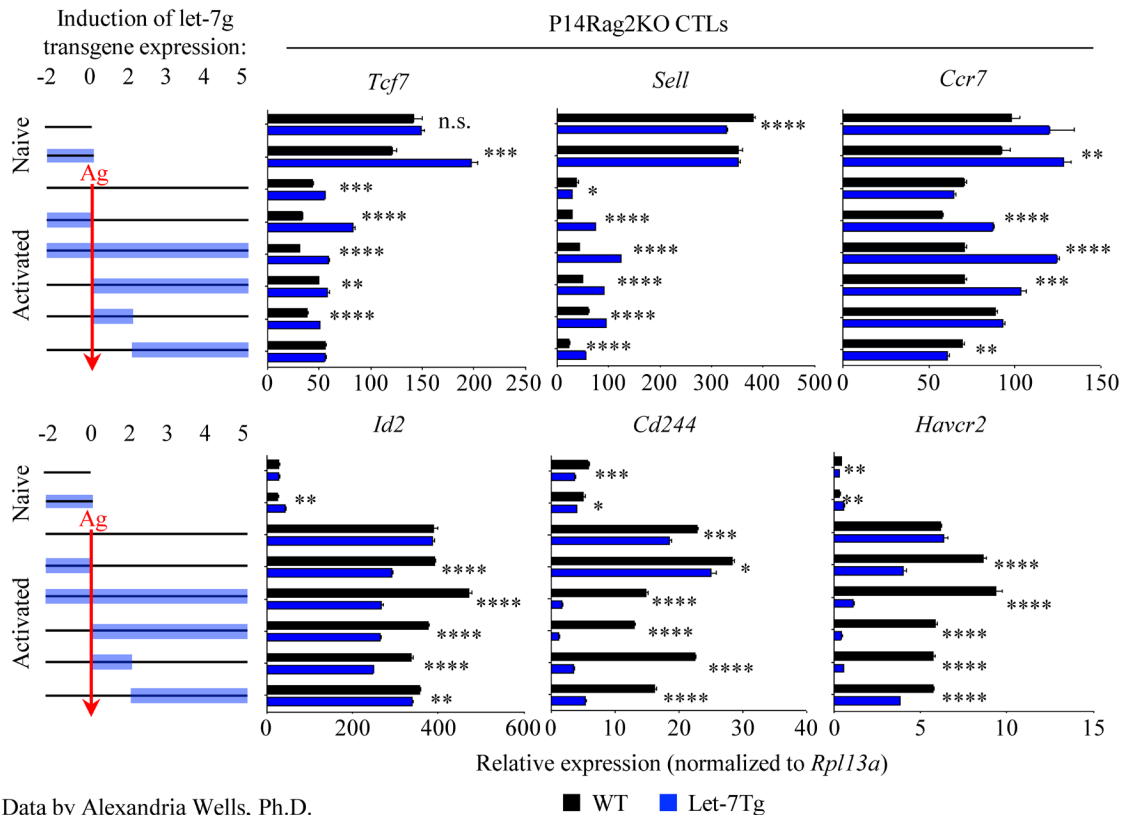
Figure 3.7 Maintenance of let-7 expression during the first 48h of CTL differentiation is sufficient to recapitulate the memory phenotype of Let-7Tg CTLs *in vitro*. Quantitative RT-PCR analysis of mRNA expression in: *Tcf7* (TCF-1), *Sell* (CD62L), *Ccr7*, *Id2, Havcr2* (TIM-3), and *Cd244* (2B4) in *in vitro*-differentiated P14Rag2KO CTLs WT and Let-7Tg CTLs which received doxycycline either prior to stimulation or during the culture, as indicated (left, blue boxes), presented relative to the expression of the ribosomal protein *Rpl13a*. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.001$ compared with WT cells from matched culture conditions using two-tailed Student's t-test. Data are from one experiment representative of two experiments (mean ± S.E.M. of technical triplicates).

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Nurr77-GFP expression in P14Rag2KO Nurr77-GFP CD8$^+$ T cells (12h)
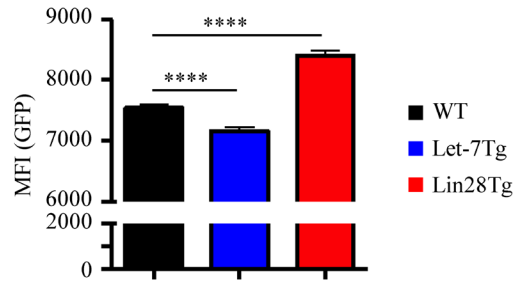
Data by Elena Pobezinskaya, Ph.D.

Figure 3.8 Let-7 miRNAs negatively regulate the strength of TCR signaling in CD8$^+$ T cells. MFI of GFP in P14Rag2KO Nur77-GFP WT, P14Rag2KO Nur77-GFP Let-7Tg, and P14Rag2KO Nur77-GFP Lin28Tg CD8$^+$ T cells activated *in vitro* for 12h with plate-bound anti-CD3e and anti-CD28 mAbs (5 µg/mL each). **** $p < 0.0001$, compared with WT using two-tailed Student's t-test. Data are from one experiment representative of two experiments (mean and S.E.M. of technical triplicates).
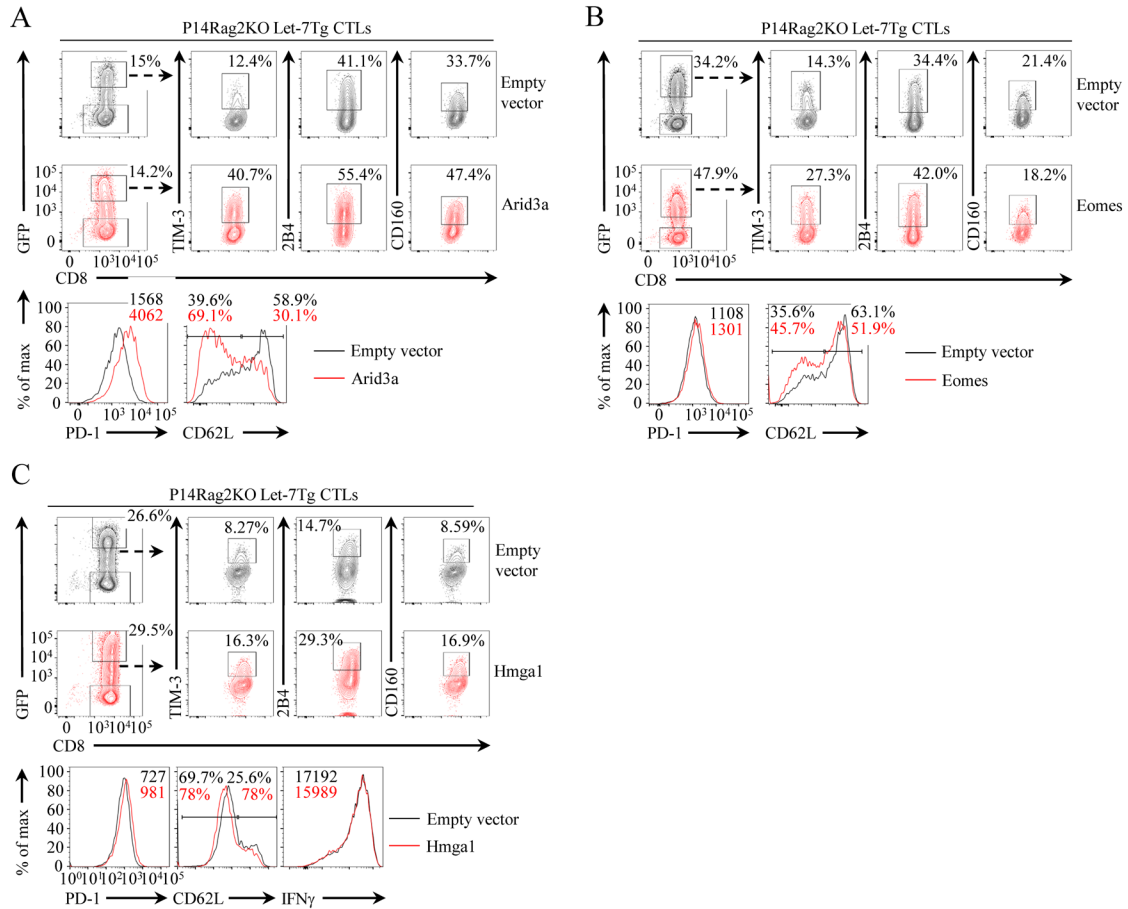
Data (A, B, C) by Alexandria Wells, Ph.D.

Figure 3.9 Let-7 miRNAs inhibit terminal effector differentiation through a complex transcriptional mechanism involving *Arid3a*, *Eomes*, and *Hmga1*. Staining of P14Rag2KO Let-7Tg CD8$^+$ T cells transduced with GFP reporter-containing empty retroviral vector or retrovirus expressing Arid3a **(A)**, Eomes **(B)**, or Hmga1 **(C)** and differentiated *in vitro* into CTLs for TIM-3, 2B4, CD160, PD-1, and CD62L and intracellular staining of IFNγ on P14Rag2KO Let-7Tg CD8 T cells transduced with either GFP-expressing empty vector or Hmga1. Numbers represent either the frequencies of positive cells (GFP, TIM-3, 2B4, CD160, CD62L), negative cells (CD62L), or mean fluorescence intensities of protein expression (MFI, PD-1, IFNγ) of each population on the indicated gates.

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Glycolytic rate assay of *in vitro*-generated P14Rag2KO CTLs
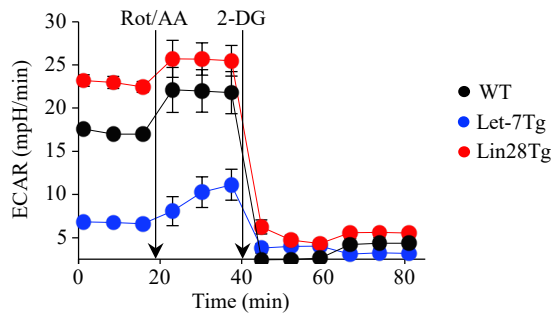
Data by Elena Pobezinskaya, Ph.D., and Alexandria Wells, Ph.D.

Figure 3.10 Let-7 miRNAs regulate glycolysis in CTLs. Glycolytic rate assay of *in vitro*-generated P14Rag2KO WT, P14Rag2KO Let-7Tg, and P14Rag2KO Lin28Tg CTLs. Arrows indicate the times of rotenone/antimycin A (Rot/AA, inhibitor of mitochondrial activity) and 2-deoxyglucose (2-DG, inhibitor of glycolysis) treatments. Data from one experiment representative of two experiments (mean ± S.E.M. of technical triplicates.

CHAPTER 4

CONCLUSIONS AND FUTURE DIRECTIONS

4.1 Conclusions

Overall, the data presented in this thesis show that let-7 miRNAs are global regulators of T cell differentiation. We first investigated the role of let-7 in CD4$^+$ T cell differentiation in the context of autoimmunity, and demonstrated that, as observed in CD8$^+$ T cells, the expression level of let-7 miRNAs is high in naive CD4$^+$ T cells, but gets downregulated upon activation, proportionally to both the strength and duration of TCR signaling. Moreover, we show that this reduction in let-7 expression is required for the differentiation of pathogenic Th17 cells in EAE, the mouse model of the autoimmune disease MS. Specifically, maintenance of high levels of let-7 during the differentiation of these cells resulted in almost complete protection from disease, while absence of let-7 in pathogenic Th17 cells led to aggravated EAE. In fact, we demonstrated that let-7 negatively regulated the proliferation, IL-1β/IL-23-dependent acquisition of function, and CCR2/CCR5-dependent migration of these cells to the CNS. This first study thus established a protective role for let-7 miRNAs in EAE, which has promising therapeutic implications for the treatment of MS and related autoimmune diseases.

We further predicted a regulatory role of let-7 miRNAs in the fate determination of CD8$^+$ T cells towards memory cell formation and terminal-effector differentiation using RNA-Seq and bioinformatics analyses as an unbiased approach, which we validated through *in-vitro* and *in-vivo* experiments. Specifically, we elucidated the paradoxical outcomes of let-7-mediated regulation of CTL function *in-vitro* versus *in-vivo*, during which we had observed that let-7-deficient CTLs that exhibited superior cytolytic

function *in vitro* failed to control tumor growth *in vivo*. Conversely, overexpression of let-7 in CTLs *in vitro* resulted in diminished cytotoxic activity, whereas the same cells elicited outstanding tumor control *in vivo*. Transcriptomic analyses of these cells uncovered that let-7 promoted memory CD8$^+$ T cell differentiation, while inhibiting the differentiation of terminally-differentiated effectors. Conversely, absence of let-7 resulted in the activation of the terminal-effector differentiation program, characterized, among others, by the upregulation of co-inhibitory receptors. These receptors may render let-7-deficient CD8$^+$ T cells susceptible to exhaustion upon engagement with their cognate ligands, which are upregulated in immunosuppressive conditions, such as the tumor microenvironment. In fact, we showed that the influence of let-7 miRNAs on CD8$^+$ T cell fate gets established early upon activation, as let-7 dampened the strength of TCR signaling, which resulted in the inhibition of signaling pathways associated with T cell activation, including ERK1/2 and Notch. Moreover, we identified direct let-7 target genes that were dysregulated throughout CD8$^+$ T cell differentiation, among which we validated the rate-limiting glycolytic enzyme *Hk2* and the transcription factor *Mycn* as drivers of the terminally-differentiated state *in vivo*. Thus, we have identified specific genes that are functionally repressed by let-7 to prevent terminal effector differentiation and maintain the memory potential of CD8$^+$ T cells.

4.2 Future directions

Even though the discoveries described in this dissertation significantly advance our knowledge of the let-7-mediated mechanisms regulating T cell differentiation, they also raise many other questions that remain to be investigated, such as: (1) uncovering the molecular mechanisms governing let-7 expression in T cells (2) determining the

common let-7-mediated regulatory mechanisms of T cell differentiation, (3) assessing the possible role of let-7 in the control of Treg activity, (4) deepening our understanding of the role of let-7 in the induction of memory T cells, and (5) identifying the function of *Hk2* and *Mycn* in the differentiation of terminal effector CD8$^+$ T cells.

4.2.1 Determine the molecular mechanisms controlling let-7 expression in T cells

An outstanding question remaining from our studies on the role of let-7 miRNAs in T cell differentiation is to identify the molecular mechanisms regulating the expression of let-7 in T cells, especially given the therapeutic potential of modulating let-7 expression in these cells to control T cell responses. We previously demonstrated that let-7 miRNAs are abundantly expressed in both naive CD4$^+$ and CD8$^+$ T cells, but get dramatically downregulated over time upon T cell activation (Wells et al., 2017; Angelou et al., 2020). Moreover, we showed that the extent of let-7 downregulation following antigen encounter depends on the strength and duration of TCR signaling, both in CD4$^+$ and CD8$^+$ T cells. Because mature miRNAs are encoded in genes and generated following a sequence of transcriptional and post-transcriptional steps during biogenesis, it would be interesting to examine whether the TCR-mediated regulatory mechanisms of let-7 expression are of epigenetic, transcriptional and/or post-transcriptional nature.

To test whether let-7 is regulated at the epigenetic level, the chromatin state at the distinct let-7 miRNA loci can first be analyzed in naive and activated T cells using methods such as ATAQ-Seq and bisulfite sequencing. ATAQ-Seq, or assay for transposase-accessible chromatin with high-throughput sequencing, enables the analysis of global epigenetic profiles through the construction of a sequencing library using the hyperactive transposase Tn5, which integrates the Tn5 transposon modified with flanking

sequencing adapters, at accessible chromatin regions of the genome (Buenrostro et al., 2013), which can be identified by sequencing. Bisulfite sequencing consists of treating DNA with bisulfite before sequencing to uncover methylation profiles, particularly of cytosine residues at the carbon-5 position of CpG dinucleotides, which are found at high frequencies at gene promoters, and where methylation represses transcriptional activity (Frommer et al., 1992). The predicted outcomes of such experiments would be that the genomic loci of let-7 miRNAs in naive T cells consist of open chromatin, and would therefore be enriched following ATAQ-Seq, but become less accessible to the transcriptional machinery in activated T cells, in which these loci would not be detected, or at very low levels, by ATAQ-Seq. Conversely, CpG dinucleotides at let-7 miRNA promoters in naive T cells would not be marked by methylation, as they are transcriptionally active, while in activated T cells CpG methylation at these sites would be increased, as let-7 miRNA transcription is repressed.

To identify the potential transcriptional mechanisms regulating let-7 miRNA expression in T cells, proteomics of isolated chromatin segments (PICh), can be run on naive and activated T cells. This technique employs DNA probes that hybridize to genomic loci of interest and pull these down together with associated proteins, which can be subsequently identified using mass spectrometry (Déjardin & Kingston, 2009). Proteins bound to let-7 loci in activated, but not naive T cells, may be responsible for the negative regulation of let-7 expression following antigen encounter. These candidates can first be validated by overexpression, where the open-reading frame (ORF) of their respective genes is cloned into a retroviral vector containing a GFP reporter that gets transduced in NIH/3T3 fibroblasts, in which let-7 miRNAs are abundantly expressed.

Candidates that induce a reduction in let-7 expression in this system can then be tested functionally by transducing activated T cells with specific shRNAs targeting these genes. Recovery of high let-7 expression upon silencing of these candidates in activated T cells will confirm their role in the TCR-mediated control of let-7 expression.

Previous work in our lab showed that, while mature let-7 miRNAs, including let-7b, let-7c, and let-7g, are depleted in activated T cells, their respective pre-let-7 miRNAs accumulate, suggesting that a post-transcriptional mechanism controls let-7 expression. Subsequent mass spectrometry experiments using pre-let-7b pull-down have identified numerous candidate proteins that bind to pre-let-7b during T cell activation. We have already tested the potential role of some candidates in the regulation of let-7 expression by overexpression in NIH/3T3 fibroblasts, using the gain-of-function strategy described above. Although none of the candidates assessed so far were found to control let-7 expression in this system, more candidates remain to be examined. Once candidates are validated in NIH/3T3 fibroblasts, their potential role in the regulation of let-7 expression in T cells can be assessed by silencing the expression of these genes using shRNAs, as mentioned before. Maintenance of high let-7 miRNA expression upon silencing of these genes will confirm their role in controlling let-7 expression in T cells.

To further understand how let-7 is regulated at different stages of T cell differentiation, the experimental approaches proposed above can also be expanded to SLECs, MPECs, memory, and exhausted T cells. To this end, donor $CD8^+$ T cells adoptively transferred into congenic recipient mice challenged with Lm or cancer can be electronically sorted at these differentiation stages. In addition to offering important insights into the molecular mechanisms regulating the expression of let-7 miRNAs in T

cells, these findings will provide a therapeutic strategy to modulate let-7 expression to enhance T cell responses against infections and cancer, and suppress the activity of autoreactive T cells.

4.2.2 Identify the common let-7-mediated mechanisms regulating T cell differentiation

We show, in accordance with previous publications (Polikepahad et al., 2010; Kumar et al., 2011; Swaminathan et al., 2012), that the let-7-mediated suppression of effector CD4$^+$ T cell differentiation is not limited to the pathogenic Th17 cell subset and extends to the Th0, Th1, and Th2 lineages. Together with our published data on the suppressive function of let-7 in the differentiation of effector CD8$^+$ T cells (Wells et al., 2017), our findings indicate that let-7 plays a global regulatory role in T cell differentiation, and suggest that common let-7-mediated regulatory mechanisms are involved in the control of both CD4$^+$ and CD8$^+$ T cell differentiation. Because let-7, initially highly expressed in naive T cells, is downregulated in both subsets within the first 48 hours following TCR stimulation, and since both CD4$^+$ and CD8$^+$ T cells undergo shared early activation signaling pathways downstream of TCR signaling, a major point to address will be to determine the common genes within these pathways that are regulated by let-7 during T cell differentiation. A first step in answering this question would be to perform RNA-Seq on monoclonal WT, Let-7Tg, and Lin28Tg CD4$^+$ and CD8$^+$ T cells at the naive and early-activated stage, followed by bioinformatics analyses. Such analyses that will be relevant in identifying the shared signaling pathways regulated by let-7 in both CD4$^+$ and CD8$^+$ T cells are contained within pathway analysis softwares, which interact with pathways collections and protein interaction networks databases. The

algorithm of such softwares will calculate the number of genes from the RNA-Seq data that are differentially expressed in the pathway collection databases between WT versus Let-7Tg, and WT versus Lin28Tg activated CD4$^+$ and CD8$^+$ T cells, and generate a list of predicted let-7-regulated pathways ranked according to the statistical significance of the overlap between the list of differentially expressed genes and the genes contained in a given pathway. The pathways that are most probably regulated by let-7 will be the ones that are shared between the WT-versus-Let-7Tg and WT-versus-Lin28Tg hits in both the CD4$^+$ and CD8$^+$ T cell samples.

Upon identification of these predicted shared let-7-regulated pathways, and following validation of these *in silico* predictions by quantifying the transcript and protein expression of the direct let-7 target genes contained in the identified pathways in WT, Let-7Tg, and Lin28Tg activated CD4$^+$ and CD8$^+$ T cells, whether these factors promote T cell differentiation will be tested using both gain-of-function and loss-of-function approaches. Gain-of-function can be achieved through transduction of early-activated T cells with a retroviral overexpression vector containing the open reading frame of these candidate genes, as well as a reporter such as GFP, upon which cells overexpressing these factors can be identified through GFP expression. Similarly, loss-of-function can be accomplished by designing shRNAs capable of inhibiting the expression of the candidate genes. Implementing these approaches in both *in vitro* and *in vivo* experimental strategies, using the outcome on effector T cell differentiaton as a readout, will enable us to directly test the hypothesis that let-7 miRNAs negatively regulate both CD4$^+$ and CD8$^+$ T cell differentiation by mechanistically targeting our candidate genes.

In vitro, when compared with the same cells overexpressing the empty vector, recovery of effector T cell phenotype upon overexpression of these genes in Let-7Tg CD4$^+$ and CD8$^+$ T cells, as well as diminished effector T cell phenotype in WT and Lin28Tg CD4$^+$ and CD8$^+$ T cells expressing shRNAs targeting these factors, will confirm that the candidate genes are regulated by let-7, and functionally relevant in both CD4$^+$ and CD8$^+$ T cell differentiation. *In vivo* models of infection, autoimmunity, and cancer, can also be used to determine whether these candidate genes control T cell differentiation, thereby establishing their biological significance. To investigate the potential regulatory role of these genes in T cell differentiation during acute infection, transduced OT-I Rag2KO CD8$^+$ T cells, which specifically recognize the OVA$_{257-264}$ epitope from the chicken ovalbumin (OVA), or OT-II Rag2KO CD4$^+$ cells, which specifically recognize the OVA$_{323-339}$ epitope, can be transferred into congenically-marked recipient mice which are subsequently challenged with an OVA-expressing Lm strain (Lm-OVA). In comparison with T cells expressing the empty vector, shRNA-expressing donor WT and Lin28Tg T cells will become more MPEC-like, resulting in enhanced memory T cell formation.

To investigate the function of the candidate genes in T cell differentiation during the anti-cancer immune response, transduced OT-I Rag2KO CD8$^+$ T cells or OT-II Rag2KO CD4$^+$ cells can be adoptively transferred into recipient mice challenged with subcutaneous OVA-expressing B16 melanoma tumors (B16-OVA). In the case of CD8$^+$ T cells, it would be predicted that, when compared to the same cells expressing the empty vector, Let-7Tg T cells overexpressing the candidate genes would divert towards the terminal-effector state and thus would not be able to control tumor growth, while shRNA-

expressing donor WT and Lin28Tg T cells would control the tumors more efficaciously, as they would adopt a memory-like phenotype. For CD4$^+$ T cells, in comparison to empty vector-expressing cells, donor Let-7Tg T cells overexpressing the candidate genes would become better helpers and contribute to effective tumor control, while donor WT and Lin28Tg T cells that express shRNAs would be detrimental to the anti-tumor response, as their helper capacity would be compromised.

To assess whether the predicted let-7 target genes regulate the differentiation of both T cell subsets in the context of autoimmunity, T cell transfers in EAE and the RIP-mOVA model of diabetes, in which membrane-OVA is expressed under the control of the rat insulin promoter, can be respectively used as CD4$^+$ and CD8$^+$ T cell-driven autoimmunity *in vivo* experimental methods. 2D2Rag2KO donor CD4$^+$ T cells can be used in EAE, while OT-IRag2KO donor CD8$^+$ T cells can be employed in the diabetes model. In comparison with T cells expressing the empty vector, Let-7Tg T cells overexpressing the candidate genes are expected to exhibit enhanced pathogenicity, while the autoreactive phenotype of shRNA-expressing donor WT and Lin28Tg T cells would be diminished.

If some candidate genes, in addition to meet our predictions following the above-mentioned gain-of-function and loss-of function *in vitro* and *in vivo* studies, are not well characterized in regards to their role in T cell differentiation, it would be interesting to generate conditional overexpression and knock-out mouse models, in which these factors are specifically overexpressed or deleted in T cells, respectively. Further crossing of these mice with TCR-transgenic mice on a Rag2KO background would provide powerful tools

for the study of the function of these genes in T cell differentiation *in vitro* and *in vivo* using the disease models mentioned before.

4.2.3 Investigating the potential role of let-7 in Treg function

Although we observed substantial suppression of monoclonal 2D2Rag2KO iTreg differentiation *in vitro* in Chapter 2, we showed that mice which received 2D2Rag2KO Let-7Tg CD4$^+$ T cells upon EAE induction did not develop autoimmunity, due to the let-7-mediated inhibition of pathogenic effector CD4$^+$ T cells. This global let-7-mediated inhibition of effector CD4$^+$ T cell differentiation may have masked a potential role of let-7 in the regulation of Treg activity, which will be important to determine given that Tregs are critical regulators of immune responses and are very different from other effector CD4$^+$ T cell lineages. To study the potential role of let-7 in the regulation of Treg activity, we could acquire FOXP3-GFP mice, in which only Tregs express the GFP reporter that is constitutively expressed under the control of the Treg-specific FOXP3 promoter, and can thus be electronically sorted by gating on GFP$^+$ cells. Breeding FOXP3-GFP mice to Let-7Tg and Lin28Tg mice will enable the direct investigation of the potential regulatory role of let-7 miRNAs in the immunosuppressive function of Tregs both *in vitro* and *in vivo*.

To test whether let-7 suppresses Treg activity, an *in-vitro* suppression assay can be performed using FACS-sorted FOXP3-GFP (GFP$^+$) WT, Let-7Tg, and Lin28Tg Tregs co-cultured with CTV-labeled electronically-sorted GFP$^-$ naive WT CD4$^+$ T cells activated *in vitro* with anti-CD3e mAbs and CD4 T cell-depleted irradiated splenocytes. In this assay, the extent of GFP$^-$ CD4$^+$ T cell proliferation, represented by CTV dilution, is inversely correlated with the potential of Treg immunosuppressive function. The

predicted outcome of this assay would be, in comparison to WT Tregs, a diminished suppressive capacity in Let-7Tg Tregs, whereas in Lin28Tg Tregs the immunosuppressive potential would be increased.

To confirm the biological significance of let-7 in the negative regulation of Treg-mediated immunosuppression *in vivo*, the T cell transfer model of colitis can be employed, in which FACS-sorted FOXP3-GFP WT, Let-7Tg, and Lin28Tg Tregs are co-transferred with electronically-sorted GFP$^-$ CD45RB^{hi} naive WT CD4$^+$ T cells, which are pathogenic and induce the disease, into Rag2KO recipient mice. In this model, weight loss and colon inflammation are inversely correlated with Treg immunosuppressive capacity and disease control. When compared to WT Tregs, Let-7Tg Tregs would be expected to show reduced suppressive activity, while Lin28Tg Tregs would exhibit enhanced disease control.

To investigate the let-7-mediated molecular mechanisms regulating Treg function, FACS-sorted FOXP3-GFP WT, Let-7Tg, and Lin28Tg Tregs can be subjected to RNA-Seq, and the transcriptome of these cells can be subsequently analyzed by bioinformatics analyses. The potential mechanistic role in Treg function of genes that are negatively regulated by let-7 in Tregs and that are also predicted direct let-7 targets can be tested by gain-of-function and loss-of-function approaches described before, using *in-vitro* suppression assays and colitis.

If the hypothesis that let-7 inhibits Treg-mediated immunosuppression is validated through the experiments proposed above, let-7 miRNAs may be established as a novel therapeutic target that could be artificially deleted in Tregs from patients suffering from autoimmune disorders such as inflammatory bowel disease, transplant rejection, and

graft-versus-host disease to enhance Treg-mediated immunosuppression of autoimmunity. On the other hand, let-7 miRNAs could be specifically delivered to Tregs that populate the the tumor microenvironment of cancer patients to inhibit their immunosuppressive activity towards the anti-tumor response.

4.2.4 *In-vivo* assessment of the memory-promoting role of let-7 in T cells

Although we demonstrated in Chapter 3 that the memory potential of let-7-deficient CD8$^+$ T cells is compromised *in vivo* by the adoptive transfer of P14Rag2KO Lin28Tg naive CD8$^+$ T cells in congenic host mice subsequently challenged with Lm-gp33, we did not show that CD8$^+$ memory T cell formation is enhanced when high let-7 expression is maintained by adoptive transfer of Let-7Tg CD8$^+$ T cells in this system. Despite the fact that this experiment is conceptually feasible, previous work in our lab revealed that adoptively transferred Let-7Tg cells could not be retrieved from the spleen of Lm-gp33-challenged host mice at 30 dpi. Because we previously showed that let-7 inhibits CD8$^+$ T cell clonal expansion (Wells et al., 2017), this phenotype could be explained by impaired proliferation. It was also described that central-memory T cells, the memory subset which Let-7Tg CTLs shares the most phenotypic attributes with, can give rise to tissue-resident memory T cells that populate non-lymphoid organs, including the skin (Osborn et al., 2019). Thus, it will be interesting to test whether the preferential location of memory Let-7Tg CD8$^+$ T cells is in peripheral tissues, which would provide an explanation for their absence in lymphoid organs. Moreover, minor differences in the genetic background of Let-7Tg and host mice exist and could therefore cause allogeneic rejection of donor Let-7Tg cells. Therefore, whether complete genetic background matching of Let-7Tg mice with host mice through backcrossing can rescue this technical

difficulty would be interesting to assess. A direct solution to this issue that would avoid the lengthy process of backcrossing would be to clone let-7g into a retroviral vector containing a GFP reporter, and use this method to overexpress let-7 to the same extent as in Let-7Tg CD8$^+$ T cells in donor WT CD8$^+$ T cells of the same genetic background as host mice, which prevents any risk of allogeneic rejection. Using this approach, the role of let-7 in the formation of memory CD8$^+$ T cells *in vivo* can be assessed using the Lm-gp33 model of infection and compared in the same host mice between let-7g-overexpressing (GFP$^+$) and WT (GFP$^-$) donor P14Rag2KO CD8$^+$ T cells. Potential differences in the preferential anatomical location between these populations can also be examined by comparing the ratios of GFP$^+$ versus GFP$^-$ frequencies in lymphoid and non-lymphoid organs.

The same approach can be used to investigate the potential role of let7 miRNAs in the control of memory CD4$^+$ T cell generation, which was not addressed in this dissertation, with the hypothesis that, similarly to CD8$^+$ T cells, let-7 directs the fate of CD4$^+$ T cells towards memory formation. In fact, we have generated a strain of Lm that expresses the gp$_{66-81}$ epitope of LCMV (Lm-gp66), which is specifically recognized by TCR-transgenic SMARTA CD4$^+$ T cells. Thus, crossing SMARTA mice to WT and Lin28Tg mice on a Rag2KO background will enable the study of the potential regulatory role of let-7 miRNA in memory CD4$^+$ T cell generation *in vivo* by adoptive transfer of transduced SMARTARag2KO WT CD4$^+$ T cells into congenically-marked host mice subsequently challenged with Lm-gp66. To investigate the effect of let-7 deficiency on memory CD4$^+$ T cell formation, donor naive SMARTARag2KO Lin28Tg CD4$^+$ T cells can be adoptively transferred into congenic recipient mice subsequently infected with

Lm-gp66 . In comparison with WT (GFP$^-$) CD4$^+$ T cells, let-7-overexpressing (GFP$^+$) CD4$^+$ T cells would be predicted to show enhanced memory cell formation, while absence of let-7 would be expected, as in CD8$^+$ T cells, to result in compromised generation of memory CD4$^+$ T cells.

4.2.5 Determine the function of *Hk2* and *Mycn* in terminal effector CD8$^+$ T cell differentiation

In Chapter 3, we identified *Hk2* and *Mycn* as let-7 direct targets that functionally promote the terminal effector fate in CD8$^+$ T cells, using retroviral transduction to overexpress these genes as a gain-of-function approach. To test whether absence of these genes results in the specification of CD8$^+$ T cells towards the memory fate, conditional genetic knock-out mouse models in which these genes would be specifically deleted in CD8$^+$ T cells could be generated. In fact, Hk2$^{fl/fl}$ and Mycn$^{fl/fl}$ mice were already produced by other groups (Knoepfler et al., 2002; Patra et al., 2013). Thus, breeding these mice to mice that express the Cre recombinase under the control of a T cell-specific promoter, such as CD4 or CD2, will result in the deletion of *Hk2* and *Mycn* only in T cells. These mouse model will enable the study of the role of these genes in terminal effector CD8$^+$ T cell differentiation using *in-vitro* CTL differentiation and the *in vivo* model of Lm infection used throughout Chapter 3. It would be expected that, consistently with our overexpression studies, the generation of memory T cells would be enhanced in CD8$^+$ T cells deficient in either gene.

Altogether, this dissertation establishes a central role for let-7 miRNAs in the regulation of T cell differentiation, and highlights the very promising medical potential

of using let-7 miRNA delivery as a novel therapeutic mean to modulate T cell-mediated

responses for the treatment of pathological conditions, such as autoimmunity and cancer.

CHAPTER 5

MATERIALS AND METHODS

5.1 Mice

C57BL/6J (WT CD45.2$^+$, stock no. 000664), B6.SJL- *Ptprc^{a}Pepc^b*/ BoyJ (WT CD45.1$^+$, stock no. 002014), B6(Cg)-Rag2$^{tm1.1Cgn}$/J (Rag2KO, stock no. 008449), B6.Cg-Col1a1$^{tm3(tetO-Mirlet7g/Mir21)Gqda}$/J (let-7g, stock no. 023912), B6.Cg- Gt(ROSA)26 Sor$^{tm1(rtTA*M2)Jae}$/J (M2rtTA, stock no. 006965), and C57BL/6-Tg(Nr4a1-EGFP/cre)820Khog/J (Nur77-GFP, stock no. 016617) mice were acquired from the Jackson Laboratory. let-7g and M2rtTA mice were bred to generate Let-7Tg mice. Mice with a human CD2 promoter-driven Lin28B transgene (Lin28Tg) (Pobezinsky et al., 2015), as well as B6 Tg(TcrLCMV)327Sdz/JDvs/J (P14) mice were generously provided by Alfred Singer (NCI, NIH) and C57BL/6-Tg(Tcra2D2,Tcrb2D2)1Kuch/J (2D2) mice were a kind gift from Barbara Osborne (UMass Amherst, VASCI). 2D2 mice were crossed on a Rag2KO background to produce 2D2Rag2KO WT mice. Let-7Tg and 2D2 mice were bred on a Rag2KO background to generate 2D2Rag2KO Let-7Tg mice. Lin28Tg and 2D2 mice were crossed on a Rag2KO background to produce 2D2Rag2KO Lin28Tg mice. P14 mice were crossed on a Rag2KO background to produce P14Rag2KO WT mice. Let-7Tg and P14 mice were bred on a Rag2KO background to generate P14Rag2KO Let-7Tg mice. Lin28Tg and P14 mice were crossed on a Rag2KO background to produce P14Rag2KO Lin28Tg mice. Control mice used were either littermates or age and sex-matched mice. All breedings were maintained at the University of Massachusetts, Amherst. All experiments were executed according to the recommendations in the Guide for the Care and Use of Laboratory Animals of the

National Institutes of Health. All mice were handled in accordance with reviewed and approved institutional animal care and use committee (IACUC) protocols (#2017-0041, #2017-0053) of the University of Massachusetts.

5.2 Doxycycline treatment for the induction of let-7 transgene expression

All experimental mice (including controls) were fed with 2 mg/mL doxycycline hyclate (Sigma) and 10 mg/mL sucrose in drinking water that was replaced once over the course of 4 days before the start of experiments to induce maximal let-7g expression. For EAE experiments, doxycycline treatment was maintained throughout disease course analysis, during which doxycycline-containing water was replaced every other day. For *in-vitro* lymphocyte cultures, lymphocyte culture media (see cell sorting and *in-vitro* culture below) was complemented with 2 µg/mL doxycycline hyclate.

5.3 Cell sorting and *in-vitro* culture

Lymph nodes were collected and gently dissociated using sharp-ended forceps to release lymphocytes. Naive CD4$^+$CD44^{lo}CD25$^-$CD8$^-$ T cells were either purified using electronic sorting after removal of B cells from whole-lymphocyte suspensions using α-mouse IgG-coated magnetic beads (BioMAg, Qiagen) or directly isolated from whole-lymphocyte suspensions using the EasySep^{TM} Mouse Naive CD4+ T Cell Isolation kit (Stem Cell Technologies) according to the manufacturer's instructions. Cells were cultured in RPMI media supplemented with 10% fetal bovine serum, 1% penicillin/streptavidin, 1% L-glutamine, 1% non-essential amino-acids, 1% sodium pyruvate, 1% HEPES and 0.3% β-mercaptoethanol. Culture media was supplemented with 2 µg/mL doxycycline, and 100

μg/mL gentamicin when necessary. Unless otherwise indicated, cells were activated with plate-bound α-CD3 (clone 2C11, 1 or 5 µg/mL) and α-CD28 (clone 37.51, 5 µg/mL).

5.4 Induction of EAE and disease analysis

EAE was induced by subcutaneous immunization with the MOG_{35-55} peptide in complete Freund's adjuvant (Hooke Laboratories EK-2110) according to the manufacturer's instructions. Intraperitoneal injection of 60 ng pertussis toxin (Hooke Laboratories BT-0105) was performed 2-4 hours and 26-28 hours post-immunization. For adoptive-transfer experiments, intravenous injection of $2-2.5x10^6$ WT, Let-7Tg or Lin28Tg 2D2Rag2KO naive $CD4^+$ T cells was performed 12 hours prior to immunization with MOG_{35-55}. EAE symptoms were scored according to standard criteria: 0, asymptomatic; 1, limp tail; 2, hindlimb weakness; 3, hindlimb paralysis; 4, complete hindlimb and partial frontlimb paralysis; 5, moribund or death.

5.5 Isolation of CNS-infiltrating cells

Experimental mice were sacrificed at the peak of EAE and perfused through the left cardiac ventricle with PBS containing 1% fetal bovine serum. Brain and spinal cord tissues were dissociated and digested with 1 mg/mL DNaseI (Roche) and 2.5 mg/mL collagenase D (Roche) for 30 minutes at 37°C using a gentleMACS dissociator (Miltenyi), filtered through 100-µm mesh strainers, and centrifuged through a Percoll density gradient (37% and 70%). Mononuclear cells in the interphase were collected, washed twice with PBS and resuspended in lymphocyte culture media prior to *in-vitro* restimulation.

5.6 Enzyme-linked immunosorbent assay (ELISA)

Spleens from experimental mice were harvested at the peak of EAE and splenocytes were released by gentle organ dissociation using sharp-ended forceps. After erythrocyte lysis, duplicates of 2×10^7 splenocytes from each mouse were restimulated in lymphocyte culture media supplemented with either 2.5, 5 or 10 µg/mL MOG$_{35-55}$ (Hooke Laboratories DS-0111) in the presence of 2 µg/mL doxycycline hyclate. Cytokine concentrations were measured in supernatants collected from restimulated cells after 5 days in culture. Concentrations of secreted IL-17, GM-CSF and IFNγ were measured using matching capture and biotinylated detection mAbs (BD Pharmingen) in a sandwich ELISA. HRP-conjugated streptavidin and HRP substrate from the TMB ELISA kit (Pierce) were applied for the quantification of HRP activity at 450 nm using a Synergy™ 2 Multi-Mode Microplate Reader (Biotek).

5.7 CTV and CFSE labeling

Naive CD4$^+$ T cells were labeled at 1×10^6 cells/mL in PBS containing 2.5 µM CTV or 1 µM CFSE, both obtained from Invitrogen, for 15 minutes at 37°C. The labeling reaction was stopped by washing the cells with lymphocyte culture media prior to use in experiments.

5.8 In-vitro proliferation assay

CTV-labeled WT, Let-7Tg and Lin28Tg cells were activated with plate-bound α-CD3e (clone 2C11, 5 µg/mL) and α-CD28 (clone 37.51, 5 µg/mL). Cells were cultured for 3 days prior to CTV dilution profile analysis by flow cytometry.

5.9 *In-vitro* differentiation of CD4$^+$ T helper (Th) cells

Naive CD4$^+$ T cells (1x10^6) were activated with soluble α-CD3 (clone 2C11, 2 µg/mL) in the presence of irradiated WT splenocytes (5x10^6) and cultured for 5 days in lymphocyte culture media. In some experiments, whole-splenocyte suspensions were depleted of CD4$^+$ and CD8$^+$ T cells using α-mouse CD4 (clone L3T4) and α-mouse CD8 (clone Ly-2) microbeads (Miltenyi) followed by magnetic-activated cell sorting. For pathogenic Th17 differentiation, culture media was further supplemented with 20 ng/mL IL-6 (Miltneyi), 10 ng/mL IL-1β (Miltenyi), 10 ng/mL IL-23 (R&D Systems), 10 µg α-IFNγ mAbs (clone XMG1.2, BioXCell) and 10 µg/mL α-IL-4 mAbs (clone 11B11, BioXCell). For Th0 differentiation, culture media was further supplemented with 200 U/mL IL-2 (Peprotech). For Th1 differentiation, culture media was further supplemented with 200 U/mL IL-2, 10 ng/mL IL-12 (Peprotech) and 10 µg/mL α-IL-4 mAbs (clone 11B11, BioXCell). For Th2 differentiation, culture media was further supplemented with 200 U/mL IL-2, 10 ng/mL IL-4 (Peprotech) and 10 µg/mL α-IFNγ mAbs (clone XMG1.2, BioXCell). For non-pathogenic Th17 cell differentiation, culture media was further supplemented with 20 ng/mL IL-6 (Miltenyi), 2 ng/mL TGF-β (Miltenyi), 10 µg/mL α-IFNγ mAbs (clone XMG1.2, BioXCell) and 10 µg/mL α-IL-4 mAbs(clone 11B11, BioXCell). For iTreg differentiation, naive CD4$^+$ T cells were stimulated with 10 µg/mL soluble α-CD3 (clone 2C11, BD Pharmingen) and culture media was further supplemented with 100 U/mL IL-2 (Peprotech) and 5 ng/mL TGF-β (Miltenyi).

5.10 In-vitro differentiation of cytotoxic CD8$^+$ T lymphocytes (CTLs)

Naive CD8$^+$ T cells were stimulated either with irradiated splenocytes loaded with anti-CD3e mAbs (10 µg/mL), or plate-bound anti-CD3e mAbs (10 µg/mL) and anti-CD28

mAbs (5 µg/mL), then differentiated for 5 days in lymphocyte culture media supplemented with 100 U/mL IL-2 (Peprotech). Culture media was further supplemented with 2µg/mL doxycycline when necessary.

5.11 Overexpression and retroviral transduction of candidate genes

The open reading frame (ORF) of *Ccr2*, *Ccr5*, *Cdc34*, *Hk2*, *Mycn*, *NICD-NES*, and *NICD-NLS* were cloned into the pMRX-IRES-GFP plasmid, which contain a green fluorescent protein (GFP) reporter (Saitoh et al., 2002). Empty pMRX-IRES-GFP plasmids were used as controls. Retrovirus supernatants were produced by transfecting Platinum-E (Plate-E) retroviral packaging cells (Morita et al., 2000) using Transporter 5 transfection reagent (Polysciences). Retrovirus supernatants were concentrated 10x in lymphocyte culture media with PEG-it™ virus concentration reagent (System Biosciences) prior to cell transduction. T cells were retrovirally transduced 24h after activation with 10x-concentrated retrovirus supernatants by spin-infection (660 xg for 90 minutes at 37°C) in the presence of polybrene (4 µg/mL). Transduction media was replaced with lymphocyte culture media appropriately supplemented 4h after spin-infection. Analysis of transduced cells was performed by gating on the GFP$^+$ cell population.

5.12 Flow cytometry

For analysis of surface markers, live cells were treated with α-CD16/32 Fc block (2.4G2, BD Pharmingen, RRID:AB_394657) prior to staining with antibodies against surface markers for 30 minutes at 4°C. For intracellular cytokine staining, cell suspensions were restimulated *in vitro* for a total of 4 hours with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 µM Ionomycin (Sigma) with addition of 2 µM monensin

(eBioscience) in the last 2 hours of restimulation to inhibit secretion. After surface marker staining, cells were stained with the Live/Dead fixable Aqua Dead Cell Stain Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Before intracellular staining, cells were fixed and permeabilized for 30 minutes at 4°C using the Cytofix/Cytoperm solution kit (BD Biosciences) for cytokine staining or the Foxp3/ Transcription factor staining buffer set (eBioscience) for transcription factor staining according to the manufacturer's instructions. Samples were acquired on a BD LSR Fortessa flow cytometer (BD Biosciences) and data analysis was performed using FlowJo software (TreeStar).

5.13 Antibodies

The following monoclonal antibodies were used for flow cytometry: 2B4 (m2B4, Biolegend), CD4 (RM4-5, Biolegend), CD8a (53-6.7, eBioscience; 5H10, Invitrogen), CD25 (PC61, Biolegend), CD45.2 (104, BD Pharmingen), CD44 (IM7, BD Pharmingen), CD62L (MEL-14, Biolegend), CD127 (A7R34, Biolegend), CD160 (CNX46-3, eBioscience), FOXP3 (FJK-16S, eBioscience), GFP (FM264G, Biolegend), GM-CSF (MP1-22E9, Biolegend), IFNγ (XMG1.2, Biolegend), IL-4 (11B11, BD Pharmingen), IL-17A (17B7, eBioscience), KLRG1 (2F1, BD Pharmingen), PD-1 (29F.A12, Biolegend), PE-Streptavidin (Biolegend), APC-Streptavidin (Biolegend), TIM-3 (RMT3-23, Biolegend).

5.14 RNA isolation and quantitative RT-PCR

RNA was isolated using the QIAGEN miRNeasy (QIAGEN) or the Total RNA Purification kit (Norgen Biotek) according to the manufacturer's instructions. Genomic

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DNA was eliminated using the DNA-free DNA removal kit (Invitrogen). cDNA of mRNA-encoded genes was synthesized using the SuperScript III Reverse Transcriptase kit (Invitrogen) or the SensiFast^{TM} cDNA synthesis kit (Bioline). cDNA of miRNAs was synthesized using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). SYBR Green or TaqMan quantitative RT-PCR were executed using the SensiFast^{TM} SYBR Lo-Rox kit (Bioline) or the SensiFast ^{TM} Probe Lo-Rox kit (Bioline), respectively. The list of specific SYBR Green amplification primers (Integrated DNA Technologies), TaqMan gene (Integrated DNA Technologies or Thermo Fisher Scientific) and TaqMan microRNA assays (Thermo Fisher Scientific) used can be found in **Table 5.1**. Quantitative RT-PCR data was acquired using a QuantStudio 6 Flex Real-Time PCR system and analyzed using QuantStudio Real-Time PCR software (Applied Biosystems).

5.15 *In-silico* prediction of let-7 binding sites

Let-7 binding sites were identified by searching for complete or partial continuous matches to the extended let-7 seed sequence "TACTACCTCA" in the complete mRNA sequences of the indicated mouse and human genes, and are available in **Table 5.2**. A 6 bp-long perfect match was considered as minimum requirement for a potential binding site. Conservation was assessed according to the retention of the binding site position within corresponding mouse and human mRNA sequences upon optimal GLOBAL pairwise alignment using BioEdit software (Tom Hall, Ibis Therapeutics).

5.16 Luciferase reporter assays

NIH/3T3 cells (ATCC) were transfected with the pmirGLO vector (Promega) containing either the wild-type *in-silico* predicted let-7-binding sites within *Ccr2* and *Ccr5* mouse mRNA, or mutated variants of these binding sites, or either the wild-type or a mutated variant of the antisense seed sequence of let-7g, using Lipofectamine and Plus Reagent (Invitrogen). Firefly luciferase activity was measured 48h post-transfection and was normalized to Renilla luciferase activity, using the Dual-Luciferase Assay Reporter kit (Promega), on a POLARstar Omega 96-well plate reader (BMG Labtech).

5.17 Motility in collagen matrices

In vitro-differentiated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice were harvested at day 5, labeled with CFSE, and resuspended in RPMI/10% FBS. PureCol EZ Gel (Advanced BioMatrix) was added to cells in RPMI/10% FBS to obtain a final collagen gel concentration of 1.6 mg/mL with a final cell concentration of 1.25×10^6 cells/mL. Collagen gels were allowed to fully polymerize for 1 hour at 37°C prior to imaging the cells for 20 minutes at 10-second intervals with a modified inverted epi-fluorescence microscope (Axio Observer.Z1, Carl Zeiss). Data was analyzed using Imaris software (Bitplane).

5.18 Transwell assay

In vitro-differentiated pathogenic Th17 cells from 2D2Rag2KO WT, 2D2Rag2KO Let-7Tg and 2D2Rag2KO Lin28Tg mice were harvested at day 5, washed in RPMI/10% FBS and resuspended at 5×10^6 cells/mL in RPMI/10% FBS. Chemotaxis towards 600 µL control media, Ccl2 and Ccl4 alone (50 ng/mL) or in combination (50 ng/mL or 10 ng/mL

each) in the lower chamber of a 24-well plate was assessed by incubating 100 µL cell suspension in the upper chamber of 24-well 6.5 mm transwell inserts with a 5-µm pore polycarbonate membrane (Corning) at 37°C for 3 hours. Percent chemotaxis was measured by manually counting the number of cells present in the lower chamber and normalized to cell counts obtained in control media for each condition.

5.19 *Listeria monocytogenes-gp33* (Lm-gp33) infection and adoptive CD8^{+} T cell transfer

$2x10^4$ CD45.2^{+} P14Rag2KO donor cells, or $2x10^5$ transduced CD45.2^{+} P14Rag2KO donor cells from the indicated mice were transferred intravenously (i.v.) into CD45.1[+] congenic hosts. Mice were challenged i.v. with $6x10^6$ colony-forming units (cfu) Lm-gp33 grown to log phase in TSB with 50 µg/mL streptomycin the next day, or 1h later, respectively.

5.20 RNA Sequencing (RNA-Seq) and bioinformatics analyses

$20x10^6$ *in vitro*-generated CTLs were sent out for RNA-Seq. Unless specified, the R scripting language was used for bioinformatics analyses.

5.20.1 Reads mapping to the reference genome

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

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junctions based on the gene model annotation file and thus achieve a better mapping result than other nonsplice mapping tools.

5.20.2 Quantification of gene expression level

HTSeq v0.6.1 was used to count the read numbers mapped of each gene. Reads Per Kilobase of exon model per Million mapped reads (RPKM), which considers the effect of sequencing depth and gene length for the reads count at the same time, was calculated for each gene based on the length of the gene and reads count mapped to this gene.

5.20.3 Differential gene expression analysis

Differential expression analysis between two conditions/groups (three biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting p-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate (FDR). Genes with an adjusted p-value <0.05 found by DESeq2 were assigned as differentially expressed.

5.20.4 Plot generation for data visualization

Principal component analysis (PCA) was performed with normalized expression read counts using the "PCAtools", "car", "RColorBrewer", and "ggplots2" R packages. Volcano plots were generated with differential gene expression analysis output data using the "ggplots2" R package. Heatmaps were generated with normalized expression read

counts using the "ggplots2" and "RColorBrewer" R packages. Bubble plots were generated using the "ggplots2" R package.

5.20.5 Database for Annotation, Visualization and Integrated Discovery (DAVID) analysis of gene ontology (GO) biological processes

Lists of genes of interest were subjected to the gene ontology (GO) Functional Annotation Tool of the Database for Annotation, Visualization and Integrated Discovery (DAVID) database, which finds functionally-related groups of genes that belong to biological processes that are enriched in the submitted gene list (Huang et al., 2009). GO terms with p-value < 0.05 were considered significantly enriched.

5.20.6 Gene set enrichment analysis

The differential gene expression data output was ranked according to the adjusted p-value. Gene signatures were generated by compiling well-defined memory and terminal-effector/exhaustion markers from the literature (Yang et al., 2011; Im et al., 2016; Schietinger et al., 2016; Yu et al., 2017; Snell et al., 2018; Miller et al., 2019). The GSEA software (Broad Institute) was used for data analysis (Mootha et al., 2003; Subramanian et al., 2005).

5.20.7 Venn diagram

Genes contained in Cluster I at all time points tested were analyzed for predicted let-7 miRNA target genes using the TargetScan database (Agarwal et al., 2015). A Venn diagram representing the let-7 target genes dysregulated at all stages of CD8$^+$ T cell differentiation tested was generated using the "ggplots2" R package.

5.21 Statistics

Data statistical analysis was performed with Prism 7 (GraphPad software) or RStudio software (RStudio Team). P-values were determined using a two-tailed Student's t test or a two-way ANOVA, as indicated on the figure legends. A p value < 0.05 was considered significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

Table 5.1: Primers and probes used for quantitative RT-PCR

SYBR primers		
Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Bhlhe40	ACGGAGACCTGTCAGGGATG	GGCAGTTTGTAAGTTTCCTTGC
Ccnd2	GAGTGGGAACTGGTAGTGTTG	CGCACAGAGCGATGAAGGT
Ccr2	ATCCACGGCATACTATCAACATC	CAAGGCTCACCATCATCGTAG
Ccr5	TTTTCAAGGGTCAGTTCCGAC	GGAAGACCATCATGTTACCCAC
Cdc25a	ACAGCAGTCTACAGAGAATGGG	GATGAGGTGAAAGGTGTCTTGG
Cdc34	CCCCAACACCTACTATGAGGG	ACATCTTGGTGAACCGGA
Cdk6	GGCGTACCCACAGAAACCATA	AGGTAAGGGCCATCTGAAAACT
Csf2	TGGAAGCATGTAGAGGCCATCA	GCGCCCTTGAGTTTGGTGAAAT
Gata3	CGAGATGGTACCGGGCACTA	GACAGTTCGCGCAGGATGT
Glut3	ATGGGGACAACGAAGGTGAC	GTCTCAGGTGCATTGATGACTC
Hk2	TGATCGCCTGCTTATTCACGG	AACCGCCTAGAAATCTCCAGA
Il1r1	GTGCTACTGGGGCTCATTTGT	GGAGTAAGAGGACACTTGCGAAT
Il4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT
Il4ra	TCTGCATCCCGTTGTTTTGC	GCACCTGTGCATCCTGAATG
Il6ra	CCTGAGACTCAAGCAGAAATGG	AGAAGGAAGGTCGGCTTCAGT
Il6st	CCGTGTGGTTACATCTACCCT	CGTGGTTCTGTTGATGACAGTG
Il12rb2	AGAGAATGCTCATTGGCACTTC	AACTGGGATAATGTGAACAGCC
Il17a	TTTAACTCCCTTGGCGCAAAA	CTTTCCCTCCGCATTGACAC
Il23r	TTCAGATGGGCATGAATGTTTCT	CCAAATCCGAGCTGTTGTTCTAT
Irf4	TCCGACAGTGGTTGATCGAC	CCTCACGATTGTAGTCCTGCTT
Myc	AGTGCTGCATGAGGAGACAC	GGTTTGCCTCTTCTCCACAG
Qars	CCTTGGCCTTAGCGAGAACAA	TGTCGATGGTAGAACCCAGAATC
Rorc	GACCCACACCTCACAAATTGA	AGTAGGCCACATTACACTGCT
Rpl13a	CGAGGCATGCTGCCCCACAA	AGCAGGGACCACCATCCGCT
Stat3	AGCTGGACACACGCTACCT	AGGAATCGGCTATATTGCTGGT
Tfap4	GGAGAAGCTAGAGCGGGAAC	TTTTGCCGGGATGTAGAGAC
TaqMan probes		
Gene	Assay ID	Source
Ifng	Mm.PT.58.30096391	Integrated DNA Technologies
Rpl13a	Mm.PT.58.43547045.g	Integrated DNA Technologies
Ldha	Mm01612132_g1	Thermo Fisher Scientific
TaqMan microRNA assays		
microRNA	Assay ID	Source
hsa-let-7a	00377	Thermo Fisher Scientific
hsa-let-7b	00378	Thermo Fisher Scientific
hsa-let-7c	00379	Thermo Fisher Scientific
hsa-let-7d	02283	Thermo Fisher Scientific
hsa-let-7e	02406	Thermo Fisher Scientific
hsa-let-7f	00382	Thermo Fisher Scientific
hsa-let-7g	02282	Thermo Fisher Scientific
hsa-let-7i	02221	Thermo Fisher Scientific
U6	01973	Thermo Fisher Scientific

Table 5.2: Let-7-binding sites identified in mouse and human Ccr2 and Ccr5 mRNA sequences

Species	Receptor	Accession number	Binding site	Sequence	Positon
Mouse	*Ccr2*	NM_009915.2	1	TACCTCA	153-159
			2	ACCTCA	2025-2030
	Ccr5	NM_009917.5	1	CTACCT	311-316
			2	ACTACC	356-361
Human	*CCR2*	NM_001123396.2	1	ACCTCA	211-216
			2	CTACCTC	360-366
			3	ACCTCA	1679-1684
	CCR5	NM_000579.3	1	CTACCT	558-563
			2	ACTACCTC	1274-1281

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