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microRNA regulation of T lymphocyte immunity: modulation of molecular networks responsible for T cell activation, differentiation and development

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Abstract

MicroRNAs (miRNA) are a class of small non-coding RNAs that constitute an essential and evolutionarily conserved mechanism for post-transcriptional gene regulation. Multiple miRNAs have been described to play key roles in T lymphocyte development, differentiation and function. In this review we highlight the current literature regarding the differential expression of miRNAs in various models of mouse and human T cell biology and emphasize mechanistic understandings of miRNA regulation of thymocyte development, T cell activation, and differentiation into effector and memory subsets. We describe the participation of miRNAs in complex regulatory circuits shaping T cell proteomes in a context-dependent manner. It is striking that some miRNAs regulate multiple processes, while others only appear in limited functional contexts. It is also evident that the expression and function of specific miRNAs can differ between mouse and human systems. Ultimately, it is not always correct to simplify the complex events of T cell biology into a model driven by only one or two master regulator miRNAs. In reality, T cell activation and differentiation involves the expression of multiple miRNAs with many mRNA targets and thus, the true extent of miRNA regulation of T cell biology is likely far more vast than currently appreciated.

Keywords

microRNA; T cell; T cell activation; T cell function; T cell development; microRNA regulation of molecular networks

INTRODUCTION

Basics of miRNA Gene Structure and Biogenesis

One of the surprises of the “post-genomic era” is the discovery that the majority of the human genome is transcribed into RNA, some protein-coding but mostly non-coding.^{1–6} microRNAs (miRNAs) are a class of small non-coding RNA, discovered twenty years ago in *Caenorhabditis elegans*^{7, 8} and since shown to be broadly evolutionarily conserved^{9–12} and implicated in many developmental, physiological and pathogenic events.^{13–15} In

animals, miRNAs are dynamically expressed as a function of embryonic as well as hematopoietic stem cell development and are necessary for these processes.^{16–18} Animal miRNAs regulate gene expression at the level of translation by using imperfect target complementarity with cognate mRNA in the 3' untranslated regions (UTRs), resulting in translational repression and/or degradation of the target transcript.¹⁹ In balance, it appears that in mammalian cells miRNAs exert their effects more predominantly through increased degradation of target mRNA transcripts.²⁰

The genomic organization of miRNA-coding genes is multi-tiered. Intergenic miRNAs are found in genomic regions outside of other known open reading frames and are transcribed from their own promoters, normally located within 2kb upstream of the miRNA.²¹ miRNAs can be mono- or polycistronic; in the latter case, multiple miRNAs are transcribed from the same promoter and often referred to as a cluster.²² Intragenic miRNAs are located within introns of protein-coding or long non-coding RNA genes. These miRNAs are usually transcribed together with their host genes and can be excised from the pre-mRNA transcript before or after the splicing event.^{23, 24} However, even some of these intragenic miRNA genes have their own promoters that are separate from the parent gene's promoter.^{25, 26} The length of the primary miRNA gene transcript (pri-miRNA) can vary from 0.5 to 7kb.²¹ In some cases, the entire sequence of the mature miRNA (18–24nt) is encoded by multiple genes at different loci in the genome, suggesting evolution via gene duplication, transposon/retrovirus integration or Alu-mediated recombination.^{27–29} Genes that encode the same mature miRNA are designated by a dashed number (e.g. miR-181a-1 and miR-181a-2).

miRNA genes are similar to protein coding genes in that the majority of miRNAs are transcribed by RNA Polymerase II, pri-miRNA primary transcripts are capped and polyadenylated³⁰ and miRNA promoters are regulated by the same epigenetic marks as those of protein coding genes.²⁶ In the nucleus, pri-miRNA transcripts are processed by a type III ribonuclease called Drosha and a non-catalytic protein DGCR8 into 60–80nt stem-loop structures called precursor miRNAs (pre-miRNAs) (Figure 1). In effect, this is a form of RNA splicing and growing evidence including our own unpublished work suggests that a number of other classic nuclear splicing proteins, such as SFRS1, are involved in this process though their role is presently unknown.³¹ Moreover, canonical splicing can substitute Drosha-mediated processing of some intragenic miRNAs.³² Pre-miRNAs are transported to the cytoplasm by Exportin-5, a member of the karyopherin family of energy-dependent transporters, where a second type III ribonuclease, Dicer, cleaves the pre-miRNA into 18–24bp duplexes, called mature miRNAs. One of the strands of the mature miRNA duplex is loaded into the Argonaute (Ago) protein that is part of the RNA-Induced Silencing Complex (RISC). Other key proteins currently known to comprise the miRNA-RISC in animals are GW182, TRBP and PABP.^{33–35}

Basics of miRNA Function

The RISC is guided by the miRNA, incorporated into the Ago protein component, to bind partially complementary sequences, primarily located in the 3' untranslated regions (3'UTRs) of mRNA transcripts, resulting in translational repression or degradation of target mRNA transcripts. This suppressive impact of miRNA-guided RISC is due to blocking

initiation of translation or inducing mRNA degradation by cellular nucleases and decapping enzymes found primarily in sub-cellular structures called processing bodies (or P-bodies) (reviewed in ¹⁹). Early studies suggested that animal miRNAs primarily function by interfering with translation,^{8, 36} but recently it was demonstrated that the majority of miRNA effects on the mammalian cell proteome can be explained by miRNA-guided mRNA degradation.²⁰ Further evidence obtained from experiments in *Drosophila melanogaster* suggests that the two mechanisms may also be linked and that mRNA deadenylation induced by the miRNA-guided RISC results in inhibition of an early stage of mRNA translation, which is followed by the decay of the mRNA.³⁷ miRNAs have also been shown to physically bind to sequences within the protein coding region of the mRNA.³⁸ Though the impact of such binding is uncertain, this event is probably transient due to displacement of the RISC complex by polyribosomes.

In animals, miRNAs canonically recognize mRNA molecules that have site complementarity in their 3'UTRs to the 6–8nt long sequences located in the 5' regions of miRNAs called “seed” sequences. Different miRNAs can have identical seed sequences and in this case they belong to the same miRNA family, because they are thought to recognize the same mRNA target transcripts. Thus, miR-29a and miR-29b have identical 5' seed sequences but otherwise their sequences differ within the downstream portion of the mature miRNA molecule. Emergence of miRNA families is likely due to mutations within orthologous genes constrained by secondary structure and targeting specificities.²⁹ Downstream sequences in the 3' part of the miRNA can, however, supplement or compensate imperfect seed sequence/mRNA interactions, possibly explaining differences in target preferences between some miRNA family members.³⁹

Because the region of complementarity between the miRNA and the mRNA is short, computational algorithms designed to predict miRNA/mRNA interactions (e.g. Targetscan, PicTar, miRanda) generate thousands of potential target sites for any given miRNA. For example, if we assumed that the sequence of 3'UTRs were random, then for the approximately 21Mb of total human 3' UTR sequence there is a 1 in 4,096 chance of finding any given 6nt seed sequence resulting in over 5,000 predictions. But we know that 3'UTR sequences are not random but rather have been evolved under selective pressures linked to organismal survival. Accordingly, these algorithms have gotten progressively better by weighting predictions according to evolutionary conservation. We also know that 3'UTRs are highly structured and many predicted miRNA binding sites are actually not accessible when the transcript is folded and this constraint has now also been integrated into miRNA target prediction tools.

However, the number of predictions remains high and whether all the predicted targets for a given miRNA are biologically real still has to be experimentally determined. Thus, it is important to remember that these tools are powerful but still far short of perfectly predictive. In experimental terms, one cannot establish a profile of differentially expressed miRNAs, plug these into any of the current tools to predict possible targets and then simply conclude that these targets are really engaged in the cell or tissue profiled. It is equally important to recognize that an interaction that is real in one cell type or under one set of conditions is not necessarily generalizable to another cell type or condition. These differences in miRNA

functions between cell types and conditions are due to phenomena such as, differential target gene expression, 3'UTR splicing,^{40, 41} alternative poly-adenylation sites resulting in 3' UTR truncations⁴² and/or regulation of miRNA binding to mRNA by the presence of other RNA binding proteins.^{43–45} On the other hand, miRNAs can regulate multiple genes at once, similar to transcription factors.^{46–48} More importantly, this level of multiple gene regulation is not just random because there are examples where a single miRNA has been shown to regulate multiple genes in the same signaling or regulatory network to achieve or reinforce the desired phenotype.^{49, 50} Conversely, most genes contain binding sites for multiple miRNAs, which may or may not all be functional at any given moment in time.^{46, 47, 51} The major point here is that mapping miRNA binding sites to target transcripts and then correlating that information to predict the impact of a single miRNA that is changing in a given experiment is not simple. Consideration of these sources of variation and miRNA targeting complexity in experimental designs is critical for proper conclusions about reductionist miRNA paradigms.

Basics of miRNA in T lymphocyte immunity

While it is evident that the miRNA pathway constitutes a critical part of post-transcriptional regulation in all cells, we need to start our review of miRNA regulation of T lymphocyte biology with an overview of the observational studies and studies of global miRNA disruptions that demonstrate the importance of miRNAs specifically in T cells.

In studies of thymic T cell development miRNA expression profiles at each developmental stage are unique, with some miRNAs undergoing expression changes up to 3 orders of magnitude during maturation.⁵² Accordingly, ablation of all mature miRNAs at early stages of thymocyte development via Dicer or Drosha knockouts results in a developmental block and a consequent reduction of the peripheral mature alpha-beta T and invariant natural killer T (iNKT) cell pool.^{53–56} Interestingly, development of gamma-delta T cells is not impaired by miRNA disruption and in contrast, there is a substantial increase of gamma-delta T cells in the double negative thymic compartment of mice conditionally lacking Dicer in early thymocytes.⁵⁵

miRNAs regulate functions of mature T cells as well. One interesting observation is that CD4 T cells shorten mRNA 3'UTRs upon activation by antigen.⁴² This study showed that truncation of the UTR at alternative polyadenylation sites resulted in the loss of specific miRNA binding sites and this protected the target transcripts from miRNA-mediated degradation. These results provide evidence indicating that miRNAs play an important role in T activation and also demonstrate a mechanism for regulating miRNA impacts. Dicer deletion in mature CD8 T cells results in enhanced cell activation, but a defect in survival and migration to peripheral tissues.⁵⁷ CD4 T cells with miRNAs that are globally disrupted by knocking out different miRNA processing molecules exhibit decreased proliferation and produce aberrant Th1 cytokines.^{54, 58, 59} Regulatory T cells that lack Drosha or Dicer lose suppressor function and fail to prevent autoimmunity in mice.^{59–61} Taken as a whole, it appears that one critical role for miRNAs in mature T cells is to help maintain functional phenotypes of distinct T cell lineages. It is interesting to consider how this effect is mediated

differently by miRNAs in CD8 effectors, CD4 effectors and CD4 regulatory T cells and additional information is provided in the sections below.

These initial observational studies revealed that the miRNA pathway is necessary for both T cell development and function. However, advances in profiling methods allowed identification of specific miRNAs associated with cell types of interest and after many different kinds of experimental manipulations (e.g. activation, cytokine treatment, differentiation). Hence, the direction of the field in the last 5 years has involved increasingly sophisticated and informative experimental designs including unbiased profiling using large qPCR panels, microarrays and deep miRNA sequencing (miRNAseq) to identify hundreds of different miRNAs. There are also miRNA knockouts, targeted *in vitro* perturbations and conditional knockouts of individual miRNAs to elucidate a much more detailed and dynamic picture for the roles that individual miRNAs play in T lymphocyte immunity. The objective of this review is to describe the emerging view that T cell-associated miRNAs engage in regulation of key molecular networks underlying T lymphocyte activation by antigen, acquisition of effector functions, formation of memory and thymic development. We also note that dysregulation of these miRNAs leads to development of T cell related cancers and autoimmune disorders. The key miRNAs discussed in this review and the processes in which they are implicated are listed in Table 1.

I. Proliferation and activation by antigen

Within the classic paradigm of T cell activation, encounter of antigen and co-stimulatory ligands presented by professional antigen presenting cells (APCs) triggers a signaling cascade downstream of the T cell receptor (TCR) in a resting T lymphocyte, whereby phosphorylation changes in numerous immune molecules leads to expression of activation-induced genes. However, T cell activation is also a complex metabolic process. Activated T cells grow in size due to increased protein synthesis, derive their energy from oxidative glycolysis and glutaminolysis instead of fatty acid oxidation and activate cell cycle molecules in order to undergo a proliferative burst.⁶² Thus, to fully understand the impact of miRNAs on regulation of T cell activation, it is important to consider that critical targets of activation-induced miRNAs are not limited to immune molecules but also include molecules critical for cell proliferation, cell cycle, metabolism and cell death. Figure 2 depicts the currently published data on miRNA regulation of specific molecules in T cell activation and will be reviewed here. Table 2 summarizes these miRNAs and their functions. However, it is important to note that we recently published a miRNA profiling of human T cell activation, revealing significant differential expression of 71 miRNAs,⁶³ suggesting that the current mechanistic understanding of miRNA regulation of T cell activation is not fully complete

A. Co-stimulation

Co-stimulation is necessary for functional activation of T cells by antigen, as evidenced by the fact that TCR signaling in the absence of sufficient co-stimulation leads to T cell anergy.⁶⁴ ICOS is one co-stimulatory molecule expressed on the surface of T cells early after antigen recognition and when engaged by the ICOS ligand on an APC, provides a survival signal to the activated cell by increasing activation of phosphatidylinositol 3-kinase. Roquin is a ubiquitin ligase that destabilizes ICOS mRNA by binding to the carboxy-

terminal region of its 3'UTR. The same region contains a predicted binding site for miR-101 and disruption of this site within the ICOS 3'UTR of human T cells reverses its repression by Roquin, suggesting that Roquin-mediated repression of ICOS requires miR-101.⁶⁵ In mouse embryonic fibroblasts, Roquin recruits mRNA to P-bodies and stress granules, subcellular structures where RNA decapping enzymes and nucleases are located, by interacting with P-body proteins.⁶⁶ However, this mRNA recruitment occurs in a miRNA-independent manner in fibroblasts, because Dicer depletion in these cells does not reduce the ability of Roquin to destabilize ICOS mRNA. The question is whether this is a discrepancy or simply reflects the fact that the rules of miRNA involvement are determined in each cell and can be very different. In fact, in experimental terms we suggest caution in taking studies of T cell-specific phenomena, like ICOS signaling, and studying them in an unrelated genetically modified cell line because it is a more tractable experimental model. It is also possible that Roquin and miR-101 are independent mechanisms of ICOS regulation and that both miR-101 and Roquin recognize the same binding site within the ICOS 3'UTR. In agreement with this, mutation in Roquin that prevents it from binding to the ICOS 3'UTR is accompanied by elevated miR-101 expression, suggesting a compensatory mechanism. In summary, miR-101 appears to negatively regulate ICOS expression in CD4 T cells and this has possible implications in systemic lupus erythematosus (SLE), where ICOS overexpression has been shown to contribute to the disease.⁶⁵

miRNAs can also favor co-stimulation. CTLA4 is an inhibitory T cell co-receptor, which due to its high affinity for B7, competes with the CD28-B7 interaction and blocks co-stimulatory signaling provided by CD28 crosslinking.⁶⁷ miR-155, encoded by an exon of a long non-coding RNA gene called *BIC*, is strongly up-regulated upon TCR stimulation.^{68–70} It targets and down-regulates expression of CTLA4, conferring increased proliferation to T cells upon TCR stimulation by promoting CD28 signaling.⁷¹

B. Phosphatidylinositol 3-kinase signaling

The miR-17~92 cluster is strongly up-regulated during T cell activation^{72, 73} and regulates T cell survival at multiple stages (Figure 2), but its regulation of phosphatidylinositol 3-kinase (PI3K) signaling has been especially well studied, due to the involvement of this regulatory interaction in both T cell activation and oncogenesis. In humans, the miR-17~92 gene cluster is transcribed as a single transcript and processed into seven mature miRNAs (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b-1 and miR-92a-1). An interesting feature of the miR-17~92 cluster is that there are two homologous miRNA gene clusters that share some of the same mature miRNAs as well as several similar miRNA sequences – miR-106a~363 (miR-106a, miR-18b, miR-20b, miR-19b-2, miR-92a-2 and miR-363) and miR-106b~25 (miR-106b, miR-93 and miR-25). The 14 mature miRNAs encoded by these three polycistronic genes, each at different loci in the human genome, fall within 5 miRNA families based on sharing identical seed sequences. Thus, cells can use any one or a combination of these genes to regulate targets like PI3K in complex networks. Moreover, all three gene clusters are highly conserved in vertebrates,⁷⁴ further underlining their functional significance.

The first evidence for miR-17~92 function in T cells came from gain of function studies in thymocytes.⁷⁵ Mice, made transgenic for the human miR-17~92 cluster at the double negative stage 1 (DN1) of thymocyte development, had an expanded peripheral lymphocyte compartment, with the largest effect on the CD4 T cells (i.e. expanded 10-fold). Transgenic mice also developed systemic autoimmunity, characterized by lymphocyte infiltration into tissues and high titers of self-reactive antibodies. Accordingly, the cluster is up-regulated in splenic T cells of three different murine models of SLE.⁷⁶ The correlation between miR-17~92 and systemic autoimmunity is present in humans as well, where CD4 T cells from patients with relapsing-remitting multiple sclerosis have elevated levels of and more variability in miR-17~92 expression than cells from healthy controls.⁷⁷

Mechanistically, miR-17~92 promotes CD4 T cell proliferation upon TCR stimulation and inhibits activation-induced cell death (AICD).⁷² As a result, miR-17~92 transgenic cells proliferate more rapidly in response to TCR crosslinking and interestingly, do not require CD28 co-stimulation, the signal that normally activates PI3K.⁷⁵ This effect is due to direct targeting by a member of the miR-17~92 cluster, miR-19b, of phosphatase and tensin homolog (PTEN), a negative regulator of PI3K signaling.⁷² As a result, miR-17~92 overcomes the requirement for additional CD28-mediated PI3K activation, leading to enhanced proliferation and cell survival without co-stimulation. Unsurprisingly, miR-17~92 displays a proto-oncogenic potential. Indeed, expression of the genomic locus of this miRNA cluster, c13orf25, had long been known to be amplified in cancers of hematopoietic origins.^{78–80} miR-17~92 was also shown to accelerate development of B cell lymphoma induced by c-myc in mice.⁸¹ miR-17~92 and the homologous miR-106~363 cluster are overexpressed in human T cell leukemias, such as T cell acute lymphoblastic leukaemia.^{82–85} Interestingly, in a rare but aggressive primary cutaneous CD4 T cell lymphoma, Sezary Syndrome, miR-17~92 is down-regulated and the ectopic overexpression of the first miRNA in the cluster, miR-17-5p, decreases cell proliferation in contrast to all other reports where such overexpression increased CD4 proliferation.⁸⁶ These results suggest that the mechanism of this form of T cell cancer is due to the targeting of a different set of genes by miR-17~92, though this paradoxical effect has never been further studied to our knowledge.

TCR/CD28 signaling also induces expression of miR-214 in mouse primary T cells.⁸⁷ Like the miR-17~92 cluster, miR-214 targets PTEN and promotes PI3K signaling and cell proliferation. In this case, we know that miR-214 up-regulation is co-stimulation dependent. This was shown by *in vivo* inhibition of CD28 signaling through blocking its ligand, CD80 (B7), with CTLA4-Ig, which prevented the up-regulation of miR-214. Removing the miR-214 inhibition of PTEN resulted in expression levels of PTEN similar to its levels in unstimulated cells. In summary, the miR-17~92 cluster and miR-214 are induced during T cell activation and work in tandem to reduce expression of PTEN, thus promoting PI3K signaling and cell proliferation. miR-17~92 is a proto-oncogene dysregulated in a number of human B and T cell cancers. While there are no reports of miR-214 in hematopoietic lineage cancers there are multiple reports linking it to the regulation of solid tumors including pancreatic and ovarian cancer.^{88, 89}

Interestingly, we did not detect any expression of miR-214 in our miRNA profiling of Jurkat T cells, a human leukemic T cell line, or during CD3/CD28 activation of primary human total, naïve or memory CD4 T cells done by both qPCR and deep sequencing (⁶³ and unpublished data). In contrast, we readily confirmed the up-regulation of all the members of the miR-17~92 cluster with activation of primary T cells. Our results underline the potential dangers of assuming that results in one model of miRNA regulation will be translatable to another, whether in different cells or different species. Thus, while miR-214 might indeed regulate PTEN in murine T cells and human solid cancers, it may not be an important miRNA in activation of human T cells.

C. IL-2 expression

T cell activation, mediated in part through PI3K, culminates in production of the cytokine, IL-2. miR-181d, a member of the miR-181 family, directly targets IL-2 mRNA in human CD4 T cells, leading to reduction of IL-2 production and inhibition of cell activation, as evidenced by reduced CD69, CD25 and CD40L surface expression.⁹⁰ Accordingly, miR-181d is down-regulated upon activation of Jurkat and peripheral human CD4 T cells.

Besides targeting IL-2 mRNA directly, two other miRNAs (miR-184 and miR-31) have been shown to regulate NFAT, a transcription factor that mediates IL-2 transcription. This is another example of how different miRNAs can target and regulate several molecules in a single pathway. It has been known for a while that CD4 T cells derived from umbilical cord blood have lower NFAT expression and express less inflammatory cytokines upon activation than adult cells.^{91, 92} This difference in the immune capacity of cord blood lymphocytes is clinically relevant for patients receiving hematopoietic stem cell transplants from stem cells purified from cord blood, where less well-matched donors can be used due to the decreased risk of developing T cell-mediated graft vs. host disease post transplant. Mechanistically, miR-184 reduces the translation of NFAT mRNA at the ribosomal level in these cells, resulting in reduced levels of the NFAT protein, but in this case the data indicate that miR-184 does not effect the mRNA stability.⁹³ miR-184 is over 50-fold down-regulated in adult CD4 T cells relative to cord blood cells this repression is induced at the level of miRNA gene transcription. While the CpG sequences of the miR-184 promoter are equally methylated in cord blood and adult CD4 T cells, miR-184 transcription is regulated by differences in di- or tri-methylation of histone 3–lysine 9, a repressive histone mark.⁹⁴ It is worth noting here that one of the significant gaps in our current knowledge of miRNA gene regulation is the “epigenetic code” by which individual miRNA genes are regulated by changes in CpG methylation and various histone modifications.

While miR-184 inhibits NFAT, miR-31 positively regulates NFAT expression upon activation of T cells. Transfection of miR-31 mimics increased IL-2 mRNA and protein levels in total human T cells and increased IL-2 promoter activity in Jurkat cells upon PMA/ ionomycin stimulation, while miR-31 silencing had the opposite effect.⁹⁵ The mechanism appears to involve RhoA, a small GTP binding protein that negatively regulates the nuclear localization of NFAT. miR-31 inhibits RhoA expression in T cells. In proof, RhoA knockdown enhances NFAT traffic to the nucleus and has the same effect on IL-2 promoter activity and IL-2 expression as miR-31 overexpression.

D. Cell Cycle

miRNAs also directly regulate cell cycle progression in T cells (Figure 2). Cyclin T1 protein levels increase as a function of macrophage and T cell activation without a corresponding change in the mRNA, indicative of a post-transcriptional regulatory mechanism.^{96, 97} In human T cells miR-27b directly targets the Cyclin T1 3'UTR in resting CD4 T cells and is down-regulated with activation, thus promoting T cell proliferation.⁹⁸ In mice, miR-182 targets the Foxo1 transcriptional regulator and promotes activated CD4 T cell expansion.⁹⁹ Foxo1 suppresses proliferation in resting CD4 T cells by up-regulating the expression of a Cdk inhibitor, p27Kip1.¹⁰⁰ While initially Foxo1 is inactivated by phosphorylation immediately upon murine T cell activation, at the late stage of activation (after approximately 66 hours) Foxo1 is downregulated by miR-182. Correspondingly, IL-2 induces miR-182 after 48 hours and this is true for Th1, Th2, Th17 and naïve CD4 T cell activation. It then follows that inhibition of miR-182 in mouse CD4 cells limits their expansion *in vitro* and *in vivo*.

An example of an indirect regulation of the cell cycle is the roles of miR-19a and miR-19b of the miR-17~92 cluster. These miRNAs inhibit a de-ubiquitinating enzyme, CYLD, which normally blocks NFκB nuclear translocation, thus helping to induce NFκB-mediated expression of cyclin and growth factor genes.⁸⁴ Overexpression of these two members of the cluster might explain sustained NFκB activation and cell proliferation in T cell lymphoblastic leukemia as well as their resistance to apoptosis.

Finally, the miR-17~92 cluster has also been shown to regulate a cell cycle inhibitor, E2F1, in T cell lymphoblastic leukemia cells. Transcription of pri-miR-17~92 in leukemic T cells is induced by dysregulated expression of two members of the homeobox-containing NKX transcription factor family, NKX2-5 or NKX3-1.¹⁰¹ In turn, the NKX transcription factors are driven by the expression of the transcription factor, TAL-1, that is up-regulated in more than 40% of T cell leukemias¹⁰² and is also a critical factor in hematopoietic stem cell differentiation.¹⁰³ This is a good example of how miRNA regulation of a critical cellular pathway like cell cycle can involve multiple levels of transcriptional signals, miRNA and protein regulators.

E. Apoptosis

miRNAs negatively regulate both extrinsic and intrinsic apoptotic pathways in T cells. Let-7 is a highly conserved miRNA family that plays a critical role stem cell differentiation.¹⁰⁴ In mature human CD4 T cells, miR-98 (a let-7 family member) plays a different role and negatively regulates Fas (CD95), a TNF receptor family member that triggers the extrinsic apoptotic pathway when associated with the Fas ligand.¹⁰⁵ During AICD miR-98 expression is reduced leading to increased Fas expression.

On the other hand, the miR-17~92 cluster regulates the intrinsic apoptotic pathway. Overexpression of miR-17~92 leads to down-regulation of Bim, a pro-apoptotic molecule. This inhibition is likely one mechanism for the enhanced survival of miR-17~92 transgenic cells.⁷⁵ In a murine T cell lymphoma line, WEHI7.2, miR-19~72 was shown to directly target Bim.¹⁰⁶ However, there is some ambiguity about whether miR-17~92 targets Bim

mRNA in normal CD4 T cells. While luciferase reporter experiments in murine NIH3T3 fibroblasts suggested that Bim is directly targeted by miR-17~92,⁷⁵ loss of function studies in murine CD4 T cells did not reveal any increase in Bim protein expression.⁷² Earlier we pointed to examples of paradoxical results between studies in murine and human T cells. In this case, the discrepancy is between two different types of murine cells. Though currently the field emphasizes luciferase reporter assays as the final validation of a given miRNA/mRNA interaction, we would express some caution with this assumption. If a reporter construct is designed to contain the 3'UTR sequence under investigation and is co-expressed with the miRNA of interest in a tractable cell line with no lineage connection to the cell of interest, it is probably not surprising, on a simple biochemical basis, that the result will be inhibition of the reporter gene expression (e.g. luciferase). However, the intracellular milieu of the cell under study, in this case CD4 T cells, may influence the results due to factors such as the activation-induced truncation of 3'UTRs, alternative splicing, regulation by other miRNAs and binding of other proteins to the 3'UTR that may inhibit or regulate the impact of miRNA/RISC binding.

F. Global miRNA changes with T cell activation

While we have discussed how the expression of individual miRNAs is altered (up- or down-regulated) during specific states of T cell activation, a recent paper reported that activated mouse CD4 T cells also have a global reduction in their repertoire of mature miRNAs.¹⁰⁷ This depletion occurs at a post-transcriptional level and likely results from activation-dependent proteasomal degradation of Ago proteins, which in addition to guiding miRNAs to their targets also stabilize and increase the half-life of mature miRNAs. Following 48 hours of CD3/CD28 co-stimulation the majority of miRNAs were down-regulated to less than 25% of their expression levels in the naïve cells. These results have an important implication for analyzing miRNA expression changes that occur in all model systems in which the miRNA processing machinery is differentially regulated or which have vastly different miRNA expression profiles.

Statistical methods that rely on standardization of control and experimental population means (e.g. quantile, LOESS, global geometric mean) or medians (e.g. Mann-Whitney test) assume that the majority of miRNAs in the two populations do not change. As a result, a change in the expression of the majority of miRNAs, such as depletion of miRNAs with T cell activation, could complicate the assumptions about absolute changes in the expression of individual miRNAs. In the context of this new report on changing Ago levels with activation, one possibility is that certain miRNAs are more prone to post-transcriptional regulation by Ago than others. These would be selectively down-regulated when Ago levels fall and the remaining miRNAs would be up-regulated relative to the population mean even though their absolute levels have not changed. Alternatively, specific miRNAs can be transcriptionally up-regulated with activation resulting in absolute changes in the levels of the mature miRNA, though the measured change may also be affected by how sensitive these miRNAs are to Ago-dependent degradation.

Proper normalization is critical for successful identification of biologically relevant miRNA changes. Unfortunately, there is currently a lack of consensus about the best method to

normalize genome-wide miRNA expression data and the choice in part depends on the platform used (RT-qPCR, microarray or deep sequencing). One method that captures global changes in miRNA levels is normalization of miRNA expression data to an empirically derived list of small RNAs that do not change under the conditions of interest. An alternative method for deep sequencing experiments is to use a set of spike-in controls of known sequences and concentrations that would not normally be detected in the sample under study.¹⁰⁸ For a detailed overview of currently available methods for genome-wide miRNA data normalization and their limitations see Meyer *et al.* 2010.¹⁰⁹

II. Effector Functions

A. Th1/Th2 lineage specification

Peripheral T cells from mice in which Dicer is knocked out in thymocytes preferentially express IFN γ , suggesting that the net effect of miRNAs in activated T cells is to inhibit Th1 polarization.⁵⁴ However, several miRNAs promote Th1 polarization and production of Th1 cytokines, albeit indirectly (Figure 3 and Table 3). Mature CD4 T cells from mice made transgenic for the human miR-17~92 cluster at the double negative (DN) stage of thymic development produce more IFN γ , while miR-17~92 deficient CD4 T cells are less able to produce IFN γ in response to Th1 polarizing conditions than normal cells.^{72,75} In addition, miR-17~92 is overexpressed in murine Th1 relative to Th2 cells and is sufficient to induce Th1 differentiation upon activation.¹¹⁰ In fact, Th2 polarizing environments induce down-regulation of miR-17~92, with T cells from tumor bearing mice having less miR-17~92, consistent with a mechanism where the tumor down-regulates a tumor specific Th1 response. Most recently, it was shown that the induction of IFN γ production is regulated indirectly by just one of the miRNAs in the miR-17~92 cluster, miR-19b, but that expression of either miR-19b or miR-17 can exacerbate an inflammatory Th1 response *in vivo*.⁷² Though many targets of the miR-17~92 cluster responsible for cell survival and proliferation are known, the targets of miR-19b and miR-17 that regulate IFN γ and Th1 polarization are presently undefined. The effect of miR-17~92 may be to prevent differentiation of other Th subsets, thus removing competing signaling networks and allowing the Th1 lineage specification program to proceed.

Another miRNA that indirectly promotes Th1 polarization is miR-155. miR-155 deficient mice exhibit skewed CD4 T cell polarization toward Th2^{70, 111} and produce higher levels of the Th2 cytokines IL-4, IL-5, and IL-10 *in vivo*.^{70, 112} An orthogonal decrease in IFN γ -producing (Th1) cells is only observed under non-polarizing *in vitro* activation conditions,⁹⁶ suggesting that miR-155 promotes Th1 only in the absence of Th2 polarizing cytokines, which may override the pro-Th1 effect of miR-155. Specifically, miR-155 attenuates Th2 by targeting c-Maf, a transactivator of the IL-4 promoter.¹¹² It is less clear how miR-155 promotes Th1 polarization. It may do so simply by inhibiting Th2, via c-Maf, since the two differentiation programs are antagonistic to each other.^{113–115} One interesting way in which miR-155 expression is regulated in T cells is by a cytoplasmic ribonuclease MCPIP1, which acts antagonistically to Dicer and broadly degrades pre-miRNAs. In immune cells, MCPIP1 controls inflammatory cytokine production by macrophages and T cells.¹¹⁶ MCPIP1 knockdown has been shown to induce an increase in miR-155 expression in Jurkat cells,¹¹⁷

accompanied by a decrease in c-Maf expression and IL-4 induction by PMA/ionomycin, implicating MCPIP1 in the Th1/Th2 fate decision.

IL-10 is an anti-inflammatory cytokine secreted by regulatory T cells, monocytes and Th2 cells and that inhibits Th1 proliferation. Human let-7c targets IL-10 mRNA and suppresses its expression, promoting the Th1 response.¹¹⁸ Let-7 is down-regulated in CD4 T cells of chronically infected HIV patients, leading to higher IL-10 expression and contributing to suppression of an anti-viral Th1 response.¹¹⁹ Let-7i also promotes the Th1 response in rats by aiding in maturation of dendritic cells (DC) through translational inhibition of suppressor of cytokine signaling-1 (SOCS1) and preventing DC-promoted expansion of regulatory T cells.¹²⁰

While the miR-17~92 cluster, miR-155 and let-7 promote the Th1 response, miR-29 inhibits it by directly targeting key Th1 transcriptional regulators and effector molecules. T-bet transcription factor initiates the Th1 lineage commitment in naïve activated CD4 cells by inducing transcription of IFN γ .¹²¹ Eomes is another member of the T-box containing transcription factor family, which can induce IFN γ in CD4 cells.¹²² miR-29a/b targets both Eomes and T-bet, repressing IFN γ production in wildtype cells. DGCR8-deficient cells produce an excess of IFN γ similar to Dicer knockouts and expression of miR-29 restores normal IFN γ production.⁵⁸

miR-29 can also target IFN γ directly. *In vivo* evidence for this came from infection models with intracellular pathogens like *Listeria monocytogenes* and *Mycobacterium bovis* Calmette-Guerin. Infected IFN γ -producing natural killer, CD4 and CD8 cells had significantly reduced expression of miR-29, which was shown to directly target IFN γ mRNA.¹²³ While miR-29 is reduced in IFN γ producing cells *in vivo*, IFN γ treatment of activated CD4 T cells *in vitro* can induce miR-29 expression levels through Stat1-dependent transcription of the miR-29 gene.¹²⁴ It has been known for some time that IFN γ initiates a positive feedback loop to reinforce the Th1 phenotype, but this evidence suggests that miR-29 mediates an IFN γ -induced negative feedback loop, which may play a role in autoimmunity (Figure 3). Indeed, memory CD4 cells from multiple sclerosis patients have high IFN γ , T-bet and miR-29 expression levels relative to healthy control individuals, suggesting that miR-29 is up-regulated in response to chronic inflammation with high levels of IFN γ to achieve some kind of balance. Furthermore, miR-29 is down-regulated upon reactivation of memory cells from multiple sclerosis patients, potentially contributing to the disease pathogenesis by allowing increased IFN γ expression that favors Th1 polarization.

An example of a miRNA impact on Th1 polarization by regulation of non-T cells is miR-21 mediated suppression of the Th1 polarizing cytokine IL-12A in DCs and macrophages.^{125, 126} DCs from miR-21 knockout mice produce more IL-12A and these mice have increased levels of IFN γ and decreased levels of IL-4. Allergen-specific memory CD4 cells from these mice restimulated *in vitro* also produce more IFN γ .

GATA3 is a transcription factor that induces Th2 cell differentiation and cytokine production and is regulated by several miRNAs with opposing functions. First, miR-126 promotes Th2 polarization in mice by targeting POU2F3, an activator of the transcription

factor PU.1, which in turn inhibits the DNA binding activity of GATA3.¹²⁷ Inhibition of miR-126 *in vivo* results in a reduced Th2 response to allergy-promoting dust mite antigens. Second, Bmi1 is a ring finger protein that promotes differentiation of murine Th2 cells by stabilizing the GATA3 protein.¹²⁸ In human patients with multiple sclerosis, miR-27b and miR-128 are up-regulated in naïve CD4 and miR-340 is up-regulated in memory CD4.¹²⁹ By seed sequences, all three miRNAs directly target Bmi1, resulting in increased GATA3 ubiquitination and a decrease in Th2 polarization. miR-340 also directly targets the Th2 effector cytokine IL-4. Together, the up-regulation of these miRNAs in multiple sclerosis patients presumably promotes the pro-inflammatory state by deviating activated T cells to a Th1 response.

B. Th17 and inflammation

Th17 cells secrete IL-17, IL-21 and IL-22 to induce inflammatory responses at mucosal sites and are implicated in a broad range of autoimmune diseases (Reviewed in ¹³⁰). miRNAs currently known to regulate Th17 differentiation and function are listed in Table 4. IL-23 helps maintain the Th17 phenotype in mice and is sufficient to induce *de novo* Th17 differentiation in humans.¹³¹ Human CD4 memory cells were found to express higher levels of the IL-23 receptor (IL-23R) and secrete more IL-17 than naïve cells.¹³² Also, in a heterogeneous population of human CD4 memory cells, let-7f is significantly under-expressed relative to naïve cells and was found to directly target the IL-23R mRNA (Figure 4). Specific attenuation of IL-23R expression by higher levels of let-7f in naïve as compared to memory cells helps explain the higher levels of IL-23R and IL-17 in memory cells. Together these findings suggest that memory cells may be the primary drivers of inflammation in autoimmune conditions such as multiple sclerosis and rheumatoid arthritis. We have also demonstrated the significant activation and proliferative expansion of CD4 memory cells in kidney transplant patients during the early allo-immune response.¹³³

While let-7f inhibits Th17 polarization, 3 other miRNAs enhance differentiation of Th17 cells. *In vitro*, naïve miR-155 deficient murine CD4 cells exhibit impaired Th17 differentiation¹³⁴ and accordingly, CD4 T cells from mice with experimental autoimmune encephalomyelitis (EAE) have elevated levels of miR-155.¹³⁵ *In vivo*, EAE mice that lack miR-155 have less inflammation, fewer Th17 cells in lymphoid tissues and the brain and are resistant to EAE induction.^{134, 135} miR-155 knockout mice are also resistant to collagen-induced arthritis (a model for rheumatoid arthritis) and colitis in response to *Helicobacter pylori* infection.^{136, 137} Besides contributing to EAE in a T cell intrinsic manner, miR-155 also stimulates production of Th17 biasing cytokines by activated DCs (IL-6, IL-23 and IL-12).¹³⁴

miR-326 is up-regulated in the CD4 T cells of relapsing multiple sclerosis patients and levels of miR-326 and IL-17 mRNA are positively correlated in these patients and in mice with acute EAE.¹³⁸ In these studies *in vivo* inhibition of miR-326 with a miRNA “sponge” reduced severity of EAE in mice and *in vitro* inhibition of miR-326 blocked Th17 differentiation. Finally, Ets-1 was identified as a negative transcriptional regulator of Th17 polarization and is directly targeted and suppressed by miR-326. miR-301 also promotes Th17 differentiation from activated CD4 T cells. miR-301 targets PIAS3, an E3 SUMO

ligase that inhibits STAT3 transcription factor, critical for IL-6 and IL-23-induced Th17 development.¹³⁹ miR-301 is over-expressed in Th17 cells relative to other helper subsets and its inhibition results in decreased IL-17 production.

Interestingly, the IL-17A gene is co-expressed in humans and mice with a miRNA cluster comprised of miR-133b and miR-206.¹⁴⁰ In mice and humans this miRNA cluster is located 40kb and 140kb upstream of the genes encoding IL17a/f, respectively. However, no function has yet been assigned to these miRNA in Th17 cells. Together these findings suggest that multiple miRNAs act to primarily enhance Th17 polarization and function, while a single known miRNA, let-7f, inhibits cell responsiveness to a Th17 polarizing cytokine through the regulation of the receptor, IL-23R.

C. Immunomodulation

1. Treg Development and Lineage Stability—Dicer knockout experiments demonstrated that miRNAs are necessary for thymic development as well as the peripheral function of natural regulatory T cells (nTregs; CD4⁺Foxp3⁺). Depletion of miRNAs at the DN/Double Positive (DP) transition in the thymus resulted in a substantial reduction of nTreg differentiation and a defect in the production of Foxp3 in CD4⁺CD25⁻ cells exposed to TGFβ during the production of induced Tregs (iTregs).¹⁴¹ A conditional Dicer knockout in Foxp3⁺ cells resulted in normal nTreg development and emigration from the thymus, but these nTregs lost their suppressive ability over time leading to systemic autoimmunity.⁶⁰ Similarly, Tregs in the gut can transform into T follicular helper cells, which results in the loss of Treg-mediated immunomodulation and promotes B cell responses.¹⁴² Several lines of evidence demonstrate that this transformation is mediated by Bcl-6 through inhibition of the transcription factors, T-bet and RORγt, assisted by the co-repressor protein Ncor2.^{143–145} In turn, both Bcl-6 and Ncor2 are regulated by miR-10a.¹⁴⁵ Together all these findings indicate that miRNAs enhance the development and lineage stability of Tregs (Table 5).

miR-155 is an important positive regulator of nTreg development and miR-155 gene transcription is driven by Foxp3.^{146–148} Accordingly, in mice, miR-155 is up-regulated in mature Tregs (CD4⁺CD25⁺Foxp3⁺) relative to conventional T cells (Tconv; CD4⁺CD25⁻FOXP3⁻) as well as in FOXP3⁺ DP and single positive (SP) thymocytes.^{141, 146, 149} miR-155 knockout mice have reduced Treg numbers in both thymus and periphery and miR-155 deficient Tregs have a reduced proliferative potential and impaired IL-2 signaling (i.e. STAT5 phosphorylation) under limiting IL-2 conditions.^{146, 149} Exogenous IL-2 is a critical factor for Treg development and survival, because Tregs do not produce IL-2.^{150–152} In this context, miR-155 promotes Treg survival and proliferation in the thymus and periphery by enhancing their sensitivity to IL-2.¹⁴⁹ As shown in Figure 5, miR-155 achieves this by targeting SOCS1, an inhibitor of IL-2 signaling, thus increasing levels of activated STAT5 and enhancing IL-2 signaling.

It has been shown that a different miRNA, let-7i, regulates SOCS1 expression in DCs.¹²⁰ However, the let-7 family is expressed at relatively low levels in Tregs relative to Tconv¹⁴¹, so it is unlikely that let-7 specifically regulates the survival of Tregs. The point is that a given gene can be targeted by multiple miRNAs but the expression of individual miRNAs

can vary as a function of different cell types and differentiation stages. It is tempting to speculate that genes have evolved multiple miRNA binding sites to accommodate regulation by different cell-specific miRNAs; miR-155 regulates SOCS1 in Tregs and let7i regulates it in DCs. Indeed, we have found that the number of miRNA binding sites in a 3'UTR positively correlates with the number of cell types in which the gene is expressed (unpublished data). This is in line with the fact that miRNA regulation is easy to evolve by simple RNA base changes, allowing rapid specification of cellular functions.

SOCS1 also suppresses IFN γ receptor signaling by negatively regulating the Stat1 transcription factor (Figure 5). Therefore, while SOCS1-deficient Tregs proliferate better due to enhanced IL-2 signaling, they have an activated phenotype and impaired suppressor function due to increased expression of IFN γ inducible genes.¹⁵³ To compensate this, Stat1 is down-regulated by a different Treg-associated miRNA, miR-146a. Mice with miR-146a deficient Tregs succumb to IFN γ -induced autoimmunity due to a decreased suppressive ability of their Tregs. Thus, both miR-155 and miR-146a are required for the development and maintenance of Tregs, demonstrating again how combinations of miRNAs can be evolved to regulate multiple gene targets within the same signaling pathway to achieve the necessary cellular phenotype.

The accepted interpretation of the Dicer knockout experiments is that miRNAs in general promote Treg differentiation. However, two members of the miR-17~92 cluster negatively regulate multiple signaling components involved in the differentiation of iTregs. One member of the cluster, miR-19b, targets PTEN, discussed above in the context of enhancing co-stimulation-induced proliferation of CD4 T cells and the regulation of PI3K signaling. Another member, miR-17, targets TGF β receptor II (TGF β RII) and cAMP responsive element binding protein 1 (CREB1).⁷² In CD4 T cells, PTEN-mediated inhibition of the PI3K/AKT signaling arm inhibits the activity of mammalian target of rapamycin (mTOR) kinase and mTOR inhibition favors differentiation of iTregs both *in vitro* and *in vivo*.^{154, 155} This is clinically relevant to the use of the immunosuppressive drug, rapamycin, that inhibits mTOR and enhances iTreg development post-transplantation as we showed recently in liver transplant patients.¹⁵⁶ TGF β and CREB1 also promote iTreg differentiation.^{157, 158} Thus, miRNA-mediated inhibition of these three proteins has a counter-regulatory effect on iTreg induction. Importantly, perturbation of miR-17~92 in CD4 T cells does not result in a defect in nTreg development *in vivo*, suggesting that this miRNA cluster plays a role in production of iTregs but not nTregs.⁷²

2. Immunomodulation by Tregs—The function of miRNAs in mediating the suppressor effects of Tregs can operate at two levels, either regulating the Tregs themselves or modifying the response of target cells to Tregs (Table 5). One example of the latter mechanism is that miR-155 is up-regulated with the activation of CD4 Tconv¹⁴¹ and miR-155 up-regulation decreases the sensitivity of these cells to Treg-mediated suppression.⁶⁹ Tregs are thought to suppress T cells in part by competing with effector cells for IL-2.^{159–162} Thus, it is possible that the function of activation-induced miR-155 in conventional CD4 T cells is to make them more sensitive to IL-2 via the impact on SOCS1 as discussed above, and hence more resistant to Treg-mediated suppression during the early stages of an immune response.

The opposite happens in human intestinal lamina propria T cells where miR-155 enhances the inhibitory effects of Treg-secreted TGF β on T cell activation.¹⁶³ In this case, effector T cells respond to TGF β by reducing IL-2 and IFN γ expression and increasing miR-155 expression 9-fold. In turn, miR-155 targets IL-2 inducible kinase Itk, inhibiting TCR signaling, IL-2 transcription and cell proliferation.

miRNAs may also directly regulate the function of Tregs. For example, miR-155 regulates CD62L expression. High CD62L (L-selectin) expression is associated with increased Treg suppressor function in mice with EAE and this effect is correlated with increased levels of ICOS, CTLA-4 and TGF β .¹⁶⁴ A similar finding of high levels of CD62L correlating with a population of highly suppressive Tregs was demonstrated in a mouse model of autoimmune gastritis/colitis.¹⁶⁵ Moreover, Tregs from an SLE-prone mouse model were shown to lack expression of CD62L and Tregs from normal mice transfected with miR-155 mimics showed decreased CD62L expression.¹⁶⁶

A striking example of how miRNAs directly regulate Treg function is the control of cAMP levels by miR-142-3p. Tregs can suppress activation of T cells and APCs by transferring cAMP to effector T cells via gap junctions¹⁶⁷ and the increase in intracellular levels of cAMP reduces pro-inflammatory cytokine production.¹⁶⁸ Mechanistically, a family of adenylyl cyclases converts ATP to cAMP, which binds to protein kinase A. The catalytic subunits of this kinase are released to translocate to the nucleus and phosphorylate NFAT, leading to its export from the nucleus and inhibiting transcription of multiple T cell-activating cytokines.^{169, 170} Adenylyl cyclase 9 is a target of miR-142-3p and this miRNA is down-regulated by FOXP3 in Tregs. The result of decreased miR-142-3p in Tregs is high cAMP levels and that facilitates the suppression of target T cells by the transfer of cAMP.¹⁷¹

3. Human Tregs—Up to this point, we have only reviewed the literature established for the roles of miRNAs in murine models of Treg differentiation and function. However, there are some important and unresolved differences between murine and human Tregs (reviewed in ¹⁷²). First, expression of FOXP3 in human Tregs appears to be regulated differently. Unlike mouse cells, naïve human CD4 T cells transiently express FOXP3 upon activation.¹⁷³ Second, several studies have shown that the activation of naïve human CD4 T cells in the presence of exogenous TGF β results in high and stable FOXP3 expression.^{174, 175} However, despite the fact that this treatment produces functional murine Tregs, these TGF β -induced human Tregs lack regulatory functions *in vivo*.^{176, 177} Thus, the stable induction of FOXP3 expression is not sufficient to confer a regulatory phenotype upon human CD4 Tconv cells. Third, human nTregs express a different splice variant of FOXP3 than mouse nTregs.¹⁷⁸ Finally, the human nTreg population appears to be comprised of several subsets. Resting nTregs (CD25^{int}CD45RA⁺FOXP3^{lo}) expand into activated nTregs (CD25^{hi}CD45RA⁻FOXP3^{hi}) upon TCR stimulation by self-antigen, though both are functional. Activated nTregs eventually down-regulate FOXP3, lose suppressive activity and produce proinflammatory cytokines such as IL-17 (CD25^{int}CD45RA⁻FOXP3^{lo}).¹⁷⁹

Some of the differences between human and mouse Tregs may be due to differences in regulation by miRNAs. Indeed, the miRNA expression profile of human nTregs differs from

that of the mouse.¹⁸⁰ miRNA profiling of human nTregs (CD4⁺CD25⁺) isolated from umbilical cord blood identified 10 differentially expressed miRNAs: miR-491, -21, -425-5p, -586, -181c, -340, -26b, -374 were up-regulated and miR-31 and miR-125a were down-regulated. Of these, only miR-21 was found to be up-regulated in murine nTregs (CD4⁺CD25⁺GITR⁺) isolated from lymph nodes.¹⁴¹ Importantly, none of the miRNAs with currently defined functions in murine Tregs are preferentially expressed in human nTregs from cord blood. Yet, these human Treg-associated miRNAs appear to be functional in human cells. miR-31, which is down-regulated in human CD4⁺CD25⁺ cord blood cells, targets FOXP3, while miR-21, which is up-regulated, indirectly increases FOXP3 expression.¹⁸⁰

However, it is important to point out that results obtained by miRNA profiling of different populations of human Tregs have not been consistent either. For example, the profiling of miRNAs in CD4⁺CD25⁺CD127^{lo} cells from adult human peripheral blood identified an nTreg signature that does not overlap with the one from the CD4⁺CD25⁺ cord blood nTregs discussed above.¹⁸⁰ Instead, miR-9, -18a, -24, -27b, -126, -133a, -134, -145, -181b, -181d, -210, -224, -335 were down-regulated and miR-95 and miR-509 were up-regulated.¹⁸¹ These miRNAs also appear to be functional, with miR-24 and miR-210 targeting FOXP3 and miR-145 targeting CTLA4. These discrepancies may be due to differences in the Treg populations being studied as well as differences in the detection technologies being used. Thus, it is important to be cautious regarding any claims of absolute Treg-specific miRNA signatures. Nonetheless, it is striking that given all the mechanistic detail involving miRNA functions in murine Tregs described in the preceding sections, these have not been found to date in similar surveys of human Tregs. Given the assumption that mouse immunology is a good model for human immunology, the mechanistic significance of these very large differences in miRNA expression will be important to resolve.

4. CD8 Suppressor Cells—After fading from focus for a few decades, CD8 suppressor T cells (Ts) are again emerging as alternative modulators of effector T cell responses.¹⁸² Ts have a limited TCR repertoire and preferentially suppress activated T cells by mechanisms that may involve both cytokine and contact-dependent inhibition.¹³⁹ Ts can be induced by exposure to the extracellular domains of the Immunoglobulin-like transcript 3 (ILT3) in the form of an Fc fusion protein.¹⁸³ ILT3 is normally expressed on activated monocytes, macrophages and dendritic cells, has an intracellular inhibitory ITIM domain and appears to function as a down-regulatory circuit for APC-mediated activation of CD4 T cells.^{184, 185} During alloantigen-stimulated induction of Ts with soluble ILT3Fc there is down-regulation of miR-155, miR-21 and miR-30b, accompanied by an upregulation of SOCS1, DUSP10 and Bcl-6.¹⁸⁶ Mechanistically, ILT3Fc inhibits the transcription of miR-155 and miR-21 by interfering with the localization of Fos to the nucleus and formation of the AP-1 transcription factor complex that drives expression of these two miRNAs. Inhibition of all 3 miRNAs in peripheral blood CD8 cells can induce a Ts phenotype and these cells suppress proliferation of CD3/CD28-activated CD4 Tconv cells.

III. T cell Memory

Recent evidence in a mouse model links the miR-17~92 cluster to the conversion of CD8 T cells from effector to memory phenotypes.⁷³ miR-17~92 is induced in CD8 T cells during the expansion phase following a viral infection, but is down-regulated during the contraction phase and down-regulated even further during memory CD8 T cell formation. The proposed mechanism involves down-regulation of the mTOR inhibitory phosphatase, PTEN, by miR-17~92 (Figure 6). The expression of miR-17~92 positively correlates with the activation status of the mTOR complex, the kinase activity of which induces cell cycle progression and inhibits memory formation¹⁸⁷. Thus, the down-regulation of the miR-17~92 cluster during the later stages of the immune response to a murine viral infection results in the loss of CD8 effector T cells and favors memory T cell development. These results beg the question of how these mechanisms might regulate CD4 T cell activation and the evolution of effector and memory phenotypes.

miR-146a was originally discovered as part of a negative feedback loop regulating the activation of Toll-like receptor signaling in cells of myeloid origin. Upon treatment with lipopolysaccharide, NF κ B drives transcription of the miR-146a gene and in turn, the mature miRNA directly targets two key drivers of NF κ B and AP-1 activation, TRAF6 and IRAK1.¹⁸⁸ Unsurprisingly, miR-146a was later found to also regulate NF κ B activation down-stream of the TCR.¹⁸⁹ miR-146a knock-out mice present with autoimmune disease accompanied by expansion of monocytes and macrophages with a strong deviation of T cells to effector cells.¹⁹⁰ These results are consistent with the role of miR-146a in inhibiting NF κ B activation and reinforcing the Treg lineage stability (see Figure 5).

miR-146a is also up-regulated in human memory CD4 and CD8 T cells relative to naïve cells.¹⁹¹ The expression of miR-146a begins to increase in naïve CD4 T cells after 8 days of activation, accompanied by the appearance of CD45RO expression (i.e. memory), suggesting that acquisition of miR-146a expression is a function of memory development. Our own data in human cells supports this observation in showing a low level of miR-146a in naïve CD4 and a high level of expression in memory CD4 T cells (unpublished data). Expression of the miR-146a gene in T cells is induced by TCR signaling and is dependent on NF κ B (similar to myeloid cells) and Ets-1 binding sites in its promoter. miR-146a appears to play a dual role in memory T cells. First, it impairs IL-2 production by interfering with AP-1 transcriptional activity, though the exact mechanism is unknown. Second, miR-146a protects cells from Fas-mediated apoptosis, or AICD, by targeting the Fas adaptor molecule, FADD. FADD levels decrease 14 days after TCR stimulation, at the same time as miR-146a expression increases and CD45RO⁺/CD45RA⁻ cells emerge. Thus, changes in miR-146a expression correlate with the transition of effector cells (high IL-2, high AICD) to resting memory cells (low IL-2, low AICD).

The let-7 miRNA family may play a role in the regulation of central T cell memory though the currently published results are incomplete. In one study, up-regulation of let-7b indirectly increased the expression of CD62L in mouse memory CD4 and CD8 T cells and human peripheral blood lymphocytes.¹⁹² CD62L is an adhesion molecule that mediates central memory T cell homing to secondary lymphoid organs. In contrast, another study

demonstrated that when mouse effector and central memory CD8 T cells were generated *in vitro* by antigen stimulation in the presence of IL-2 or IL-15, respectively, let-7a/b/c/d/g were specifically downregulated in central memory cells.¹⁹³ Unfortunately, the more recent publication did not reference or explain the earlier work and the different results could reflect the different models involved. The miRNAs known to be involved in regulation of T cell memory are listed in Table 6.

IV. Thymic Development

miR-150 is a critical regulator of lymphocyte development and its expression is up-regulated during the course of T and B cell development.¹⁹⁴ When expressed prematurely, miR-150 blocks B cell development, blocking the transition from pro-B to the pre-B cell stage. The effect of miR-150 over-expression on T cell development is less severe, but nonetheless, in the thymus it blocks the DN3 to DN4 transition, resulting in fewer CD4 and CD8 cells in the periphery.¹⁹⁵ Expression of miR-150 inversely correlates with expression of the transcriptional regulator c-Myb, which is gradually down-regulated as a function of T cell maturation and is implicated in thymocyte survival at multiple stages of development.¹⁹⁶ It was shown that miR-150 directly targets c-Myb mRNA and by titrating c-Myb expression it promotes the completion of the lymphocyte maturation cycle (Figure 7). miR-150 regulation of c-Myb also regulates thymic development of iNKT cells. As in conventional T cells, miR-150 expression increases as a function of iNKT maturation and iNKT development is inhibited by premature miR-150 expression in the thymus.¹⁹⁷ miR-150 deletion, on the other hand, results in functional defects in mature iNKT cells, which exhibit increased IFN γ production.¹⁹⁸ In human thymocytes miR-150 also targets NOTCH3, a cell surface receptor that plays an important role in T cell development by regulating pre-TCR and NF κ B signaling, and disruption of which leads to leukemogenesis.¹⁹⁹

miR-181 is also highly expressed in the thymus. The first clue that miR-181 may regulate lymphocyte development came from studies in which bone marrow cells transduced with a miR-181 expressing lentivirus were transferred into host mice and gave rise to an increased peripheral B-lymphoid (CD19+) population and a substantially reduced Thy1.2+ T-lymphoid population, especially affecting the CD8+ T cells.²⁰⁰ Myeloid-derived cells were largely unaffected by miR-181 overexpression. At this point, however, it was unclear whether the decrease in the peripheral T cell pool was due to an increased commitment to the B-lymphoid lineage or due to an intrinsic defect in thymocyte development. The first evidence for the mechanistic function of miR-181 in the thymus came from the observation that expression of this miRNA family is enriched in DP thymocytes and that two of its predicted targets, CD69 and TCR have known roles in positive selection and are overexpressed in DP thymocytes of Dicer knockout mice.²⁰¹ Luciferase reporter assays in HeLa cells confirmed targeting of both of these genes by miR-181, but reconstitution of miR-181 into Dicer deficient DP thymocytes only reduced expression of CD69, possibly due to the long half-life of a fully assembled TCR. CD69 promotes egress of T cells from the thymus,²⁰² so a miR-181-mediated reduction of CD69 expression is expected to result in retention of thymocytes and a diminished peripheral T cell pool. However, there is no evidence for accumulation of miR-181 transgenic cells in the thymus.

The complete picture of the role miR-181 plays in thymic development emerged with the discovery that a member of the miR-181 family, miR-181a, regulates TCR signaling thresholds. These thresholds are particularly important at the DP stage in thymic development during positive and negative selection. Thus, miR-181 inhibition in fetal thymic organ culture with antagomirs results in a 50% inhibition of negative selection and a defect in positive selection, accompanied by a 70% decrease in the CD4 SP thymocyte pool. Accordingly, mRNA and protein levels of miR-181a targeted proteins are increased in DP thymocytes treated with antagomirs. This mechanism of miR-181 function in DP thymocytes involves the targeting of multiple phosphatases that negatively regulate signaling downstream of the TCR: PTPN22, which dephosphorylates and inactivates Lck and Zap70; DUSP5 and DUSP6, which dephosphorylate and inactivate Erk; and SHP-2, which mediates CTLA-4 signaling.²⁰³ Inhibition of these negative regulators by miR-181a results in more TCR signaling for any given MHC peptide/TCR interaction as measured by calcium release. This enhanced TCR signaling increases T cell sensitivity to peptide antigens effectively lowering the TCR signaling threshold.

miR-181a function during positive selection is required for the induction of central tolerance. DP thymocytes signal stronger through their TCRs than mature cells with the same receptor specificity in order to successfully induce clonal deletion of cells with moderate receptor affinities. Thus, by increasing TCR signaling sensitivity, miR-181a prevents the survival of cells that react even moderately to positively selecting self-antigens.²⁰⁴ Accordingly, while miR-181a depletion in DP thymocytes results in drastically reduced numbers of mature T cells, CD4 T cells that do emerge from the thymus exhibit high CD69 expression and release TNF α , IFN γ and IL-17 in response to self-antigen recognition.

Interestingly, the reduction of TCR signaling thresholds in the mouse thymus appears to be unique to miR-181a because other family members such as miR-181c fail to promote DP cell development.²⁰⁵ Interestingly, miR-181a and 181c have the same seed sequence and differ by only a single nucleotide in the sequence of the mature miRNA. However, there are multiple sequence differences in the loop region of the pre-miRNA (see Figure 1). It was shown that the levels and functionality of miR-181a is determined by the pre-miRNA loop sequence, possibly due to differences in processing by Dicer and/or the efficiency of loading the mature miRNA into the RISC complex.²⁰⁵ In agreement with this, our own studies of the human CD4 T cell line, Jurkat, and activation of naïve CD4 T cells, consistently demonstrate significantly higher expression levels of miR-181a compared to miR-181c (unpublished data).

miR-181a regulation of the SHP-2 phosphatase may also play a role in the recognition of antigen by mature peripheral T cells. miR-181a is gradually down-regulated in T cells of patients chronically infected with *Mycobacterium leprae*.²⁰⁶ The decrease in the miRNA is accompanied by increased expression of SHP-2, suggesting that miR-181a regulation of TCR sensitivity may contribute to T cell hyporesponsiveness.

Wnt signaling is important in thymocyte development during pre-TCR and TCR-dependent stages as well as for differentiation of activated naïve T cells into Th effector cells.^{207, 208}

Unfortunately, we could not find any published data on miRNA regulation of Wnt signaling in the thymus. However, there is some data from other cell types that suggests such miRNA-mediated signaling could also be important in the thymus. Canonical Wnt signaling induces miR-29a transcription in osteoblasts, which in turn promotes Wnt signaling by targeting the negative Wnt regulators Dkk1, Kremen2, and secreted frizzled related protein 2 (sFRP2).²⁰⁹ miR-34, on the other hand, is induced by p53 and suppresses canonical Wnt signaling by directly targeting WNT1, WNT3 and β -catenin 3'UTRs in epithelial and breast cancer cells.²¹⁰ All the miRNAs discussed in this section are listed in Table 7.

Conclusions

The focus of the miRNA field in the last decade has been the identification of miRNA gene targets in cells and biological processes of interest. Though many studies start with profiling of miRNA expression the resulting publications tend to describe the role of only one or two specific miRNAs. However, what is increasingly apparent when these studies are reviewed in aggregate is the fact that in any model of changing biology multiple miRNAs engage in complex regulatory networks with many targets to shape cell-specific proteomes. In the present review of miRNAs in T lymphocytes we have identified and discussed 34 well-studied miRNAs with defined roles in activation, effector function, memory and development and reviewed how these miRNAs can act synergistically or antagonistically. A key point to make in this context is that there is a natural tension between trying to explain the regulation of a complex network during a complex biological state by the function of a single miRNA and the necessity to focus an individual research project's limited resources on a very defined part of this natural complexity. However, the reader should be reminded that this common tendency for reductionism in the literature should not obscure the reality that the real molecular mechanisms of events as complex as T cell differentiation, activation and function are rarely the consequence of only one or two miRNAs.

The complexity of these miRNA-mediated events is revealed in this review at several levels. First, a single miRNA can have cell-intrinsic targets and effects at different stages of development or differentiation. Let-7 promotes Th17 polarization by targeting IL-23R and regulates memory cell homing by targeting CD62L. miR-155 inhibits SOCS1 to promote survival in Tregs, c-Maf to inhibit Th2 polarization and CTLA to promote activation in CD4 T cells. miR-17~92 cluster inhibits Treg differentiation and emergence of CD8 memory cells, while in activated CD4 T cells it inhibits apoptosis and promotes proliferation. Second, the same miRNA can regulate a key developmental or signaling pathway at multiple levels. For example, miR-150 regulates thymocyte development at the DN stage by targeting c-myb and NOTCH3; members of the miR-17~92 gene inhibit Treg differentiation at the level of mTOR, TGF β and TCR signaling; and miR-340 inhibits Th2 polarization by inhibiting IL-4 and Bmi1. Finally, key molecules in T lymphocytes can be targeted by multiple different miRNAs, such as Bmi1 by miR-128, miR-27b and miR-340; CD62L by let-7 and miR-155; CTLA4 by miR-145 and miR-155; and PTEN by miR-19b and miR-214. These regulatory signals can be delivered in parallel or in series depending on the state of a cell's activation or the arc of an immune response developing in time. Thus, the evidence supports the view that miRNAs target both effector molecules and other regulators to help adjust the proteome of the T cell to suit its precise temporal needs.

The fact that miRNAs reported so far regulate key molecules in T cells, begs the question whether all miRNAs are “master-regulatory”. The term “master regulator” often has more than one definition. It is sometimes understood to be a molecule with several upstream inputs and downstream targets, a node in a molecular network (e.g. PI3K), while it can also refer to a regulator that functions as a binary switch (e.g. T-bet for Th1 differentiation). Master-regulatory miRNAs can also be those that regulate master-regulatory protein-coding genes. Though miRNAs usually fine-tune, rather than completely shut off gene expression, small changes in transcription factor levels can have fate-determining effects on the cell, and small changes induced by miRNAs can do the same. For example, incremental changes in miR-150 expression in pre-B cells and thymocytes result in gradual changes in c-Myb expression, which is essential for proper B cell and T cell development.¹⁹⁵ Most miRNAs and their T cell targets described in this review can be considered master regulators by at least one of the above definitions. However, in the latest version of miRBase (v.19), there are annotations for 2042 mature human and 1281 mature mouse miRNAs. By our own work using quantitative PCR there are 71 miRNAs differentially expressed with human T cell activation, only a dozen of which have clearly defined molecular targets in this process and 57 have not been implicated in T lymphocyte function at all.⁶³ Thus, it is unclear whether all or most miRNAs have master regulatory functions, similar to the ones reported in the literature and discussed in this review or if the miRNAs with assigned biological functions have been the easiest to study precisely because they are master-regulatory. If only a small number of miRNAs are truly master-regulatory, it would suggest that many of the other miRNAs expressed in T cells are either redundant, not functional due to ongoing evolution or contribute incrementally to the silencing of any particular gene. In support of the latter possibility, it was reported that miRNA regulation is additive and that genes targeted by multiple miRNAs exhibit larger changes.^{46, 63} This, of course, could be because the more miRNAs are predicted to target a gene, the higher is the probability that at least one of them actually binds to its target site under the conditions being investigated.

There are several explanations for why one miRNA might have a master regulatory phenotype, while an equally highly expressed or differentially expressed miRNA might appear to only have a marginal effect besides the obvious explanation of targeting genes with different functional significance. If the target transcript of a specific miRNA had a very brief period of transcriptional activation and a short half-life, it might be much more sensitive to miRNA-mediated degradation. Its regulation might then have a much greater relative impact on a cell's function, causing this miRNA to be viewed as master-regulatory. Another possibility is that miRNA-mediated binding of the RISC complex to the mRNA may be enhanced by the presence of other RNA binding proteins with recognition sequences located outside the immediate miRNA binding site. The assembly of very large, multi-protein complexes on RNA as part of regulating splicing and alternative splicing is well established.^{211, 212} However, almost nothing is known about additional proteins assembled after RISC binding to target mRNA transcripts that might regulate the subsequent events mediated by miRNAs, such as accelerated mRNA degradation.

Another explanation for a limited number of miRNAs with assigned master-regulatory functions is that miRNAs are usually selected from a screening study for a mechanistic follow-up based on conservation of the target site across species. While this is a good metric

for minimizing false positive predictions, using it as a filter may result in overlooking functional and important species-specific interactions. There are differences in miRNA expression profiles between species (e.g. in mouse and human Tregs, as described in this review), suggesting that miRNAs play different roles in regulation of mouse and human immunity and may thus help explain some of the functional differences between the cells of the two species (e.g. induction of suppressive function by FOXP3). Indeed, the short recognition site (the seed sequence) required for miRNA-mediated regulation can be readily evolved as a system because there are few structural constraints to the newly acquired mutation. It is therefore widely viewed that co-evolution of miRNAs and their targets are in part responsible for increasing organismal complexity in animals.²¹³ The fascinating question remains how and why a specific immune cell type like a Th17 or a Treg has responded to evolutionary pressures to evolve a specific group of regulatory miRNAs and then fix these mutations in the germ line.

Future Directions

There are new emerging mechanisms by which miRNAs may regulate gene expression that are beyond the scope of the current review but worth mentioning in brief. Besides silencing gene expression post-transcriptionally, mammalian miRNAs have also been shown to repress gene transcription in *trans* by recognizing antisense non-coding RNAs that overlap gene promoters and inducing repressive histone modifications.²¹⁴ Some miRNAs can also regulate transcription in *cis*. miR-320 is encoded in an anti-sense direction within the POLR3D promoter and its transcription results in recruitment of Ago1 and the polycomb component, EZH2, leading to H3K27 tri-methylation, which induces transcriptional silencing of the POLR3D gene.²¹⁵ miRNAs may also play a role in inter-cellular communication. There is a report that T cells can transfer miRNAs to APCs via exosomes at the immune synapse.²¹⁶ The transfer is unidirectional and antigen-dependent. Not all miRNAs can be packaged into exosomes, but the mechanism responsible for this selectivity is unknown. Other studies have demonstrated that miRNAs are highly resistant to RNase activity in the plasma and thus, are found free and in readily measured levels in plasma with unknown biological significance.²¹⁷ There are also multiple recent reports of non-canonical target recognition by miRNAs, including incomplete seed sequence matches, which suggest a broader scope of miRNA regulation.^{218, 219} Further exploration of these newly emerging miRNA functions and non-canonical target recognitions, together with investigation of species-specific and non-master-regulatory roles of miRNAs will provide a more complete picture of the full extent to which these short noncoding RNAs shape not only T cell immunity, but the protein landscapes of all cells.

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Abbreviations

3' UTR	3' untranslated region
Ago	argonaute
AICD	activation-induced cell death
APC	antigen presenting cell
DCs	dendritic cells
DN	double negative thymocyte
DP	double positive thymocyte
EAE	experimental autoimmune encephalomyelitis
ILT3	immunoglobulin-like transcript 3
iNKT	invariant natural killer T cell
iTreg	induced regulatory CD4 T cell
miRNA	microRNA
mTOR	mammalian target of rapamycin
nTreg	natural regulatory CD4 T cell
P-body	processing body
PI3K	phosphatidylinositol 3-kinase
pre-miRNA	precursor miRNA
pri-miRNA	primary miRNA
PTEN	phosphatase and tensin homolog
RISC	RNA-induced silencing complex
SLE	systemic lupus erythematosus
SOCS1	suppressor of cytokine signaling-1
SP	single positive thymocyte
Tconv	conventional CD4 T cell
TCR	T cell receptor
Treg	regulatory CD4 T cell
Ts	CD8 suppressor T cell

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FIGURE 1. A brief overview of miRNA biogenesis and function

miRNA genes are transcribed primarily by RNA Pol II and sometimes RNA Pol III, into 5' G-capped, polyadenylated, hairpin-containing primary miRNA transcripts (pri-miRNAs). A type III ribonuclease Drosha, assisted by a non-catalytic protein DGCR8 required for pri-miRNA recognition, cleaves the pri-miRNA, leaving ~80nt stem-loops called pre-miRNAs. pre-miRNAs are transported from the nucleus through the nuclear pore complex by Exportin 5 in a RanGTP-dependent manner. In the cytoplasm, another type III ribonuclease, Dicer, cleaves the hairpin into a ~22nt duplex, leaving mono-phosphate overhangs at the 5' end of

each strand. One of the strands of this duplex is loaded into the RNA-induced silencing complex (RISC) and a member of the Argonaute (Ago) protein family catalyzes miRNA binding to its target site within the mRNA 3' untranslated region. The RISC complex inhibits mRNA translation by interfering with ribosome assembly and/or recruits the miRNA-bound mRNA to cytoplasmic structures called processing bodies (P-bodies), where the mRNA is deadenylated, decapped and subsequently degraded. It has been demonstrated, however, that the macroscopic integrity of the P-body is not necessary for miRNA-mediated mRNA decay and that the individual protein components of the P-bodies are sufficient.²²⁰ RNA Pol II - RNA polymerase II; DGCR8 - DiGeorge syndrome critical region gene 8; Ago - argonaute; RISC - RNA-induced silencing complex; GW182 - glycine-tryptophan protein of 182 kDa; ORF - open reading frame; pri-miRNA - primary miRNA; pre-miRNA - precursor miRNA.

FIGURE 2. Multiple life and death signaling pathways involved in T cell activation are regulated by miRNAs

miRNAs modulate the proliferative response of T cells to activation by antigen at multiple levels. First, miR-101, possibly in collaboration with a ubiquitin ligase Roquin, inhibits the expression of an essential co-stimulatory molecule ICOS, while miR-155 inhibits the expression of an inhibitory co-receptor CTLA4. Second, the miR-17~92 cluster (specifically miR-19b) and miR-214 promote PI3K signaling by targeting the inhibitory phosphatase PTEN. Third, expression of IL-2 is regulated by 3 different miRNAs in human T cells: miR-181d targets IL-2 mRNA directly, miR-184 targets the IL-2 transcriptional activator, NFAT, thus indirectly reducing IL-2 transcription, and miR-31 promotes IL-2 transcription by targeting an inhibitor of NFAT nuclear localization, RhoA. Fourth, miRNAs regulate cell cycle progression in T cells. miR-27b inhibits the cell cycle in human resting T cells by directly inhibiting expression of Cyclin T1 and miR-182 promotes proliferation of activated murine T cells by targeting the inhibitor of cell cycle Foxo1. NFκB activation leads to transcription of cytokines as well as cyclins and growth factors. The miR-17~92 cluster (specifically miR-19a or miR-19b) promotes NFκB-mediated gene expression by targeting CYLD, an inhibitor of NFκB nuclear translocation. miR-17~92 also directly inhibits expression of a cell cycle inhibitor, E2F1, in leukemic cells. Finally, miRNAs inhibit

apoptosis in T cells. A let-7 family member, miR-98, targets extrinsic apoptosis-inducing receptor Fas and miR-17~92 likely directly targets an intrinsic apoptosis inducer Bim. Of note, miR-17~92 has several targets involved in regulation of cell proliferation - PTEN, E2F1, CYLD and Bim. Solid boxes indicate miRNAs that promote T cell activation; dashed boxes indicate miRNAs that inhibit T cell activation. TCR - T cell receptor; ICOS - inducible T-cell co-stimulator; CTLA4 - cytotoxic T-lymphocyte-associated protein 4; PI3K - phosphoinositide-3-kinase; PTEN - phosphatase and tensin homolog; mTOR – mammalian target of rapamycin; IL-2 - interleukin 2; NFAT - nuclear factor of activated T-cells; RhoA - ras homolog family member A; NFκB - nuclear factor of kappa light polypeptide gene enhancer in B-cells; CYLD – cylindromatosis; E2F1 - E2F transcription factor 1; Fas - TNF receptor superfamily, member 6; Bim - bcl-2 interacting mediator of cell death.

FIGURE 3. miRNAs regulate the balance between Th1 and Th2 lineage commitment
 miR-19b, miR-17, miR-155 and let-7c indirectly promote differentiation of Th1 effector cells. miR-19b and miR-17 are members of the miR-17~92 cluster and promote IFN γ production and Th1 differentiation, but the targeted genes responsible for this regulation are unclear. miR-155 inhibits differentiation of Th2 cells by targeting c-Maf, an IL-4 gene trans-activator, thus indirectly promoting Th1 polarization. IL-10, a Th1 suppressing cytokine produced by Th2 and regulatory T cells is inhibited by let-7c, and thus let-7c also indirectly promotes differentiation of Th1 effector cells. miR-29a and miR-29b inhibit Th1 polarization and Th1 effector cytokine production by targeting T-bet, Eomes and IFN γ . miR-21 inhibits production of the Th1 polarizing cytokine IL-12A by dendritic cells and macrophages. Several miRNAs influence Th2 differentiation by regulating the activity of GATA3. miR-126 promotes Th2 differentiation by targeting POU2F3, an activator of a GATA3 inhibitor PU.1, while miR-27b, miR-128 and miR-240 have been suggested to inhibit Th2 polarization by directly targeting an enhancer of GATA3 stability Bmi1. miR-34 also directly targets the Th2 effector cytokine IL-4. Solid boxes indicate miRNAs that promote Th or Th2 differentiation or function; dashed boxes indicate miRNAs that inhibit Th1 or Th2 differentiation or function. miR-155 promotes Th1, but inhibits Th2 differentiation so is marked by both types of boxes. Dashed arrows indicate indirect

induction. IFN γ - interferon gamma; IL-10 - interleukin 10; T-bet - T-box expressed in T cells; Eomes - eomesodermin; IL-12A - interleukin 12A; GATA3 - GATA binding protein 3; POU2F3 - POU class 2 homeobox 3; PU.1 - hematopoietic transcription factor PU.1; Bmi1 -BMI1 polycomb ring finger oncogene; IL-4 - interleukin 4.

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FIGURE 4. miRNAs regulate Th17 effector cell differentiation and IL-17 production

Let-7f inhibits expression of IL23R and is down-regulated in human memory CD4 T cells, poising these cells for higher IL-17 production. miR-155 promotes Th17 differentiation via an undefined mechanism. miR-301a and miR-326 also promote IL-17 production and Th17 differentiation in mice by targeting STAT3 and ROR γ t inhibitors PIAS3 and Ets-1, respectively. Solid boxes indicate miRNAs that promote Th17 differentiation or function; dashed boxes indicate miRNAs that inhibit Th17 differentiation or function. Dashed arrow indicates indirect induction. IL23R - interleukin 23 receptor; STAT3 - signal transducer and activator of transcription 3; ROR γ t - RAR-related orphan receptor t; PIAS3 - protein inhibitor of activated STAT, 3; Ets-1 - v-ets erythroblastosis virus E26 oncogene homolog 1.

FIGURE 5. The development and lineage stability of CD4 regulatory T cells is regulated by miRNAs

miR-10a favors the iTreg lineage stability in the gut by suppressing a Tfh master-regulatory transcription factor Bcl-6 and the associated co-repressor Ncor2. miR-155 favors Treg thymic development and peripheral survival by directly inhibiting the expression of SOCS1, an inhibitor of STAT5 and IL-2 signaling. miR-146a compensates for miR-155 induced activation of STAT1 and the downstream IFN γ response by targeting STAT1. On the other hand, the miR-17~92 cluster attenuates the expression of three positive regulators of iTreg induction: miR-19b of the miR-17~92 cluster targets PTEN, and miR-17 targets TGF β RII and CREB1, resulting in inhibition of iTreg development. Solid boxes indicate miRNAs that enhance Treg development or function; dashed boxes indicate miRNAs that inhibit it. iTreg - induced regulatory T cell; Tfh - follicular helper T cell; Bcl-6 - B-cell lymphoma 6 protein; Ncor2 - nuclear receptor co-repressor 2; SOCS1 - suppressor of cytokine signaling 1; STAT5 - signal transducer and activator of transcription 5; STAT1 - signal transducer and activator of transcription 1; PTEN - phosphatase and tensin homolog; TGF β RII - transforming growth factor, beta receptor II; CREB1 - cAMP responsive element binding protein 1; mTOR - mammalian target of rapamycin.

FIGURE 6. Several miRNAs regulate T cell memory formation

The miR-17~92 cluster inhibits the effector to memory CD8 T cell transition by activating mTOR and is down-regulated in memory cells. Conversely, miR-146a may promote CD4 and CD8 memory cell formation and is more highly expressed in memory cells. miR-146a inhibits IL-2 transcription by negatively regulating AP-1 activity and also directly targets FADD, a Fas adaptor molecule, thus preferentially protecting memory cells from AICD. Let-7b may indirectly promote CD62L (L-selectin) expression in memory central T cells, allowing homing to secondary lymphoid organs and formation of the central memory niche. Solid boxes indicate miRNAs that promote memory cell formation and/or maintenance; dashed boxes indicate miRNAs that inhibit memory cell formation and/or maintenance. mTOR - mammalian target of rapamycin; IL-2 - interleukin 2; AP-1 - activator protein 1;

FADD - Fas (TNFRSF6)-associated via death domain; AICD - activation-induced cell death.

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FIGURE 7. miR-150 and miR-181a regulate thymocyte development at multiple stages
 miR-150 regulates thymic development of T cells and invariant natural killer T cells at multiple stages. It impairs progression through the double negative (DN) 3 stage and survival of CD4 single positive (SP) thymocytes in mice by targeting c-Myb, a critical regulator of the DN3 to DN4 transition and SP cell survival. In human thymocytes, miR-150 also inhibits NOTCH3, thus attenuating pre-TCR signaling required for the survival of double positive (DP) thymocytes during the DN to DP transition. miR-181a increases TCR sensitivity in DP thymocytes by directly inhibiting PTPN22, DUSP5/6 and SHP-2, phosphatases that inhibit distinct signaling molecules downstream of the TCR. This is critical for survival of DP thymocytes undergoing positive selection and deletion of DP clones with moderate TCR affinities to self-antigens. miR-181a may also directly target CD69, thus attenuating egress of mature thymocytes from the thymus. Solid boxes indicate miRNAs that positively regulate thymocyte survival; dashed boxes indicate miRNAs that negatively regulate thymocyte survival. miR-181a promotes survival of DP thymocytes, but also mediates removal of cells with potentially self-reactive TCRs so is marked by both types of boxes. c-Myb - v-myb myeloblastosis viral oncogene homolog; PTPN22 - protein tyrosine phosphatase, non-receptor type 22; DUSP5/6 - dual specificity phosphatase 5/6; SHP-2 - protein tyrosine phosphatase, non-receptor type 11; CD69 - early T-cell activation antigen.

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TABLE 1

T cell-associated miRNAs and their functions *

	Process						
miRNA	Activation ^	Th1/2	Th17	Treg	Ts	Memory	Development
let-7							
miR-10							
miR-101							
miR-126							
miR-128							
miR-142							
miR-145							
miR-146							
miR-150							
miR-155							
miR-17~92							
miR-181							
miR-182							
miR-184							
miR-21							
miR-210							
miR-214							
miR-24							
miR-27							
miR-29							
miR-30							
miR-301							
miR-31							
miR-326							
miR-34							

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TABLE 2

miRNAs that regulate T cell proliferation upon activation by antigen.

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
miR-98	let-7	let-7f-2/miR-98	Fas	Inhibits cell extrinsic apoptosis	Human	105
miR-101	miR-101	miR-101-1/3671 miR-101-2	ICOS	Inhibits co-stimulation	Human	66 65
miR-155	miR-155	miR-155	CTLA4	Promotes CD28 signaling	Human	71
miR-17~92	miR-17~92	miR-17~92	Bim	Inhibits cell intrinsic apoptosis	Mouse	75 72 106
miR-17~92	miR-17~92	miR-17~92	E2F1	Promotes cell cycle	Human	101
miR-181d	miR-181	miR-181c/181d	IL-2	Inhibits IL-2 production	Human	90
miR-182	miR-182	miR-183/96/182	Foxo1	Promotes long-term expansion of activated CD4 T cells	Mouse	99
miR-184	miR-184	miR-184	NFAT	Inhibits IL-2 production	Human	93 94
miR-19a/b	miR-19	miR-17~92 miR-106~363	CYLD	Promotes NFκB nuclear localization	Human	84
miR-19a/b	miR-19	miR-17~92 miR-106~363	PTEN	Promotes PI3K signaling	Mouse	75 72
miR-214	miR-214	miR-199a-2/214	PTEN	Promotes PI3K signaling	Mice	87
miR-27b	miR-27b	miR-23b/27b/3074/24-1	CyclinT1	Promotes cell cycle progression	Human	98
miR-31	miR-31	miR-31	RhoA	Promotes IL-2 production	Human	95

TABLE 3

miRNAs that regulate Th1/Th2 polarization.

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
let-7c	let-7	miR-99a/let-7c	IL-10	Inhibits an anti-inflammatory cytokine	Human	118
let-7i	let-7	let-7i	SOCs1	Inhibits DC-mediated Treg expansion	Rat	120
miR-126	miR-126	miR-126	POU2F3	Promotes Th2 polarization	Mouse	127
miR-128	miR-128	miR-128-1 miR-128-2	BMI1	Inhibits Th2 polarization	Human	129
miR-155	miR-155	miR-155	c-maf	Inhibits Th2 polarization	Mouse	112
miR-17	miR-17	miR-17~92	n/a *	Induces Th1 polarization	Mouse	72
miR-19b	miR-19	miR-17~92 miR-106~363	n/a *	Induces IFN γ production and Th1 polarization	Mouse	72 75 110
miR-21	miR-21	miR-21	IL-12A	Inhibits Th1 polarization	Mouse	125 126
miR-27b	miR-27b	miR-23b/27b/3074/24-1	BMI1	Inhibits Th2 polarization	Human	129
miR-29a/b	miR-29	miR-29b-1/miR-29a miR-29b-2/miR-29c	T-bet Eomes IFN γ	Inhibits Th1 polarization	Mouse	58 123 124
miR-340	miR-340	miR-340	BMI1	Inhibits Th2 polarization	Human	129
miR-340	miR-340	miR-340	IL-4	Inhibits Th2 polarization	Human	129

* miRNA target gene is unidentified

TABLE 4

miRNAs that regulate Th17 differentiation.

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
let-7f	let-7	let-7a-1/let-7f-1/let-7d let-7f-2/miR-98	IL23R	Inhibits Th17 polarization	Human	132
miR-301	miR-130	miR-301a miR-301b/miR-130b	PIAS3	Promotes Th17 polarization	Mouse	139
miR-155	miR-155	miR-155	n/a*	Promotes Th17 polarization	Mouse	134 135 136 137
miR-326	miR-326	miR-326	Ets-1	Promotes Th17 polarization	Mouse	138

* miRNA target gene is unidentified

TABLE 5

miRNAs that regulate CD4 Treg development, lineage stability and function

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
miR-10a	miR-10	miR-10a	Bcl6 Ncor2	Prevents conversion of Tregs into T _H in the gut	Mouse	145
miR-142-3p	miR-142	miR-142	AC9	Inhibits cAMP/PKA signaling in conventional T cells	Mouse	171
miR-145	miR-145	miR-143/145	CTLA4	Reduces CTLA expression on Tregs	Human	181
miR-146a	miR-146	miR-146	Stat1	Inhibits IFN γ signaling in Tregs	Mouse	153
miR-155	miR-155	miR-155	CD62L	Reduces Treg suppressor function	Mouse	166
miR-155	miR-155	miR-155	Itk	Mediates TGF β signaling in intestinal lamina propria T cells	Human	163
miR-155	miR-155	miR-155	SOCS1	Promotes Treg survival and thymic development	Mouse	146 149
miR-17	miR-17	miR-17~92	CREB1	Inhibits formation of iTregs	Mouse	72
miR-17	miR-17	miR-17~92	TGF β RII	Inhibits formation of iTregs	Mouse	72
miR-19b	miR-19	miR-17~92 miR-106~363	PTEN	Inhibits formation of iTregs	Mouse	72
miR-210	miR-210	miR-210	Foxp3	Reduces Foxp3 expression	Human	181
miR-24	miR-24	miR-23b/27b/3074/24-1 miR-23a/27a/24-2	Foxp3	Reduces Foxp3 expression	Human	181
miR-31	miR-31	miR-31	Foxp3	Reduces Foxp3 expression	Human	180

TABLE 6

miRNAs that regulate T cell memory.

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
let-7b	let-7	let-7a-3/miR-4763/let-7b	CD62L	Prevents effector memory cell homing to lymph nodes	Human Mouse	192
miR-146a	miR-146	miR-146	FADD	Protects memory cells from AICD	Human	191
miR-17~92	miR-17~92	miR-17~92	PTEN	Inhibits memory formation by activating mTOR in CD8 T cells	Mouse	73

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TABLE 7

miRNAs that regulate thymic development of T cells.

miRNA	miRNA family	miRNA Gene	Target gene	Function	Species	Ref.
miR-150	miR-150	miR-150/5121	c-myc	Blocks DN3 to DN4 transition	Mouse	195
miR-150	miR-150	miR-150	NOTCH3	Regulates pre-TCR and NFkB signaling	Human	199
miR-181a	miR-181	miR-181a-1/181b-1 miR-181a-2/181b-2	PTPN22 Dusp5/6 SHP-2	Increases TCR sensitivity, regulates positive selection	Mouse	203 204
miR-29a	miR-29	miR-29b-1/29a	Dkk Kremen2 sFRP2	Promotes canonical Wnt signaling in osteoblasts	Human	209
miR-34	miR-34	miR-34a miR-34b/34c	Wnt1 Wnt3 β-catenin	Suppresses canonical Wnt signaling in epithelial and breast cancer cells	Human	210