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Regulation of immune responses and tolerance: the microRNA perspective

Chang-Zheng Chen^{1,2}, Steven Schaffert^{1,2}, Rita Fragoso^{1,2}, and Christina Loh^{1,2}

¹Department of Microbiology and Immunology, Stanford University School of Medicine, Stanford, CA, USA.

²Department of Microbiology and Immunology, Baxter Laboratory in Stem Cell Biology, Stanford University School of Medicine, Stanford, CA, USA.

Summary

Much has been learned about the molecular and cellular components critical for the control of immune responses and tolerance. It remains a challenge, however, to control the immune response and tolerance at the system level without causing significant toxicity to normal tissues. Recent studies suggest that microRNA (miRNA) genes, an abundant class of non-coding RNA genes that produce characteristic approximately 22 nucleotides small RNAs, play important roles in immune cells. In this article, we discuss emerging knowledge regarding the functions of miRNA genes in the immune system. We delve into the roles of miRNAs in regulating signaling strength and threshold, homeostasis, and the dynamics of the immune response and tolerance during normal and pathogenic immunological conditions. We also present observations based on analyses of miR-181 family genes that indicate the potential functions of primary and/ or precursor miRNAs in target recognition and explore the impact of these findings on target identification. Finally, we illustrate that despite the subtle effects of miRNAs on gene expression, miRNAs have the potential to influence the outcomes of normal and pathogenic immune responses by controlling the quantitative and dynamic aspects of immune responses. Tuning miRNA functions in immune cells, through gain- and loss-of-function approaches in mice, may reveal novel approach to restore immune equilibrium from pathogenic conditions, such as autoimmune disease and leukemia, without significant toxicity.

Keywords

microRNA; miR-181; leukemia; hematopoiesis; autoimmunity; T-cell receptors

Introduction

The immune system mediates dynamic interactions between animals and their environment. Throughout an animal's lifetime, the immune system plays critical roles in maintaining homeostasis and defending against invading pathogens. To maintain homeostasis, the immune system must provide stability and withstand challenges. Simultaneously, to defend against invading pathogens, the immune system must possess the ability to mount rapid and specific responses and to develop and maintain memories of invaders. Moreover, the immune system must have built-in mechanisms that allow it to distinguish self from foreign

antigens. Dysregulation of the immune system results in various pathological disorders including defects in host–pathogen interactions, autoimmune disease, and cancer. How the immune system encompasses these interesting properties – some seemingly contradictory – and how to restore a dysfunctional immune system to the healthy state have captivated the imaginations of immunologists for many decades.

Much has been learned about the molecular, cellular, and organismal components of the immune system and the functions of these components in immune responses and tolerance. At the molecular level, the focus of most studies has been on protein-coding genes. The discovery of microRNA (miRNA) genes revealed an unexpected layer of genetic programs that regulate the immune response at the post-transcriptional levels (1, 2). The non-coding RNAs produced from miRNA genes have widespread effects on gene expression by influencing target mRNA stability and/or translational efficiency. Emerging evidence indicates that miRNA play important roles in diverse biological processes (reviewed in 3, 4), and a number of recent reviews have summarized the growing body of knowledge regarding miRNA functions in immune cells (5–7). In this review, we examine the impact of miRNAs on immune response and tolerance at the molecular, cellular, and system levels with a focus on genetic analyses in mice.

Diverse functions of animal miRNAs

The discovery of the first miRNA gene, *lin-4*, and its regulatory control of *lin-14* mRNA at the translational level defined the essential characteristics of a previously unsuspected form of RNA regulators and mode of gene regulation (8, 9). The importance of miRNA genes only became apparent following the identification of another *lin-4*-like non-coding RNA gene, *let-7*, and the discovery of RNA interference (10, 11). *let-7* controls the timing of *C. elegans* cell lineage development during the later larval to adult transition through suppression of the expression of *lin-41* target mRNAs. Unlike *lin-4*, the mature *let-7* miRNA is conserved across a wide range of animal phyla. These discoveries prompted systematic cloning of *lin-4*- and *let-7*-like approximately 22-nt small RNAs in animals and resulted in the identification of thousands of mature miRNAs (12–14). miRNA genes encode long primary RNA transcripts (pri-miRNAs) that are sequentially processed by nuclear and cytoplasmic ribonuclease III enzymes first into stem-loop pre-miRNAs (pre-miRNAs) and then into mature miRNAs. Cellular proteins, including Drosha and Dicer, are important for the biogenesis of these small RNAs (15–18). miRNA genes control diverse biological processes in vertebrates such as hematopoietic lineage differentiation (19), oncogene activities (20, 21), heart development (22, 23), and immune responses (24–26).

Intriguing functions of miRNA genes have been revealed through gain-of-function and loss-of-function genetic manipulations in animals. One of the paramount observations is that loss of a specific miRNA gene often results in subtle phenotypes. In fact, deletion of most miRNA genes individually does not affect the viability or the development of *C. elegans* (27). Among the characterized miRNA-null mouse strains, only a few result in lethality with varied penetrance. Deletion of the *miR-17-92* cluster results in smaller embryos and immediate postnatal death of all animals (28, 29). Loss of *mir-126* causes defects in angiogenesis and approximately 50% embryonic lethality (29); however, deletion of many other miRNA genes in mice cause little or no observable changes in animal growth or behavior.

Many reasons may contribute to the subtle phenotypes of miRNA deletion in animals. Some miRNAs may function in a relatively small set of cells or even in a single cell type. For example, *Isy-6* and *miR-273* miRNAs reciprocally control the left and right asymmetry of chemosensory receptor expression in two morphologically bilateral taste receptor neurons in

worms (30, 31). The consequences of deleting this type of miRNA gene are difficult to detect without prior knowledge of gene targets and sensitive functional readout. Furthermore, some miRNA genes produce identical or nearly identical mature miRNAs, and these miRNA gene products may have redundant functions that can only be revealed when combinatorial deletions are generated. For example, deletion of multiple *let-7* family miRNA genes in worms is necessary to produce observable developmental defects (32). Yet, not all members of miRNA gene families are functionally redundant. The *mir-181* family genes, *mir-181a-1/b-1*, *mir-181a-2/b-2*, and *mir-181c/d*, are not functionally equivalent in normal thymocyte development or in tumor transformation induced by the Notch oncogene (33, 34). Finally, the phenotypes of deleting individual miRNA genes may be detected only under stressed and/or perturbed conditions (35). Indeed, many *C. elegans* mutations in individual miRNA genes have detectable phenotypes under genetically sensitized backgrounds (36). Similarly, when certain miRNA-knockout mouse strains are subjected to cardiovascular insults, the roles of miRNAs in normal and pathological hearts are apparent (37). These studies provide conceptual insights that may be useful for understanding miRNA functions in the immune system.

Potential outcomes of miRNA regulation in the immune system

The diverse functions and complexity of the immune system also makes it difficult to detect and make sense of subtle and quantitative phenotypes. One might wonder whether characterizing miRNA functions will provide fundamental insights into the control of the immune response and tolerance beyond discovering new activities. To facilitate discussion of miRNA functions in this review, we have categorized the immune system into three basic layers, the molecular, cellular, and system layers, and have postulated various outcomes of genetic manipulation of molecular components of the immune system (Fig. 1). The molecular layer, which consists of RNA, proteins, and other genetic elements, provides molecular signals for lineage differentiation and cellular responses. The cellular layer, which consists of immune cell types, provides the cellular basis of immune responses and homeostasis. The system layer integrates the components of molecular and cellular layers and executes the homeostasis and defense functions of the immune system. Regulation of immune responses and tolerance can be dissected through genetic manipulation of the components in the molecular layer. Genetic manipulation may result in varied effects: switching (on/off), quantitative, or no effect. Moreover, these manipulations may have varied penetrance into the cellular and/or system layers (Fig. 1).

Genetic manipulation of molecular components, such as protein-coding and miRNA genes, may reveal the functions of these components in the immune system. Manipulation of switching molecules with on/off effects may result in fully or partially penetrant effects in the cellular and the system layers (Fig. 1A). Significant efforts in immunological research have been dedicated to identifying molecules that constitute the structural components of the immune system. Manipulation of these molecules often results in fully penetrant on/off effects in all three layers (Fig. 1A). For example, deletion of either the B-cell or T-cell receptor (BCR and TCR, respectively) disrupts a key immune function (antigen recognition) and results in the loss of B or T cells and their function in all three layers. As disruption of structural components often results in unambiguous phenotypes, such analyses have contributed a great deal to our current understanding of the structural components of the immune system. Molecules with partially penetrant phenotypes beyond the molecular layer are likely to play redundant and/or less essential roles. Of note, although identification of the structural components laid the foundation for our understanding of the framework of the immune system, therapeutic manipulation of these targets remains challenging and causes severe toxicity as a result of structural damage to the immune system.

The immune system also consists of regulatory components that control the dynamic aspects of its functions. Emerging evidence suggest that miRNAs likely play important roles in controlling the strength and spatiotemporal dynamics of the immune responses (26, 34, 38). Manipulation of molecules with this type of tuning effect may also have fully or partially penetrant effects in the cellular and system layers (Fig. 1B). Dissecting molecular components with quantitative effects may illustrate yet-to-be appreciated dynamic aspects of immune regulation and their contribution to immune function at the system level. It will be interesting to determine whether immune functions at the system level can be turned on or off by modulating molecular components with quantitative effects. This type of molecules may be good target as genetic or therapeutic manipulation of these molecules may alter the outcome of immune responses without causing major structural defects in the immune system.

Control of immune signaling networks by miRNAs

Immune signals provide the basic instructions for immune responses. miRNA-mediated gene regulation, situated at the stage immediately preceding protein synthesis, plays a critical role in determining the level of protein expression by targeting mRNAs for degradation or translational repression (Fig. 2). Unlike more upstream gene regulatory mechanisms, such as chromatin remodeling and transcriptional regulation, miRNA-mediated regulation allows rapid control and fine-tuning of protein levels. The highly degenerate nature of miRNA and target gene interactions enables a single miRNA to control a network of genes (39). Importantly, the relationships between a miRNA and its cognate targets were established through evolutionary selection processes (40). Therefore, connecting miRNA targets to functions in signaling provides a unique opportunity to decipher the evolutionarily selected molecular networks in immune cells (Fig. 2). It is important to emphasize that information that can be used to identify the targets of a miRNA is encoded in the nucleotide sequence of the miRNA gene and is translated into activity through sequence-specific interactions with targets (Fig. 2). It is conceivable that this information may be readily decoded in the future.

Modes of miRNA-controlled signaling

miRNAs may control immune signaling through a few basic modes (Fig. 3). In the linear model, miRNAs exert their activities through the control of a single mRNA target. In the divergent model, a single miRNA controls the expression of multiple targets and each regulatory interaction is associated with distinct biological outcomes. In the network model, coordinated regulation of multiple targets is required to accomplish a specific biological outcome. Finally, in the convergent model, many targets are coordinately controlled by cognate miRNAs in a linear fashion, and all regulatory events contribute additively to a specific biological phenotype.

Each of these models is supported by abundant experimental observations. In support of the linear model of target regulation, Xiao *et al.* (41) showed that miR-150 controls B-lymphocyte development by precisely regulating the concentration of c-Myb within a narrow range. A number of miRNAs control biological activities through the divergent model. For example, miR-155 miRNA determines the level of SOCS1 (suppressor of cytokine signaling 1) and proliferation of regulatory T (Treg) cells (42), and it also controls the quantitative and temporal expression of AID (activation-induced cytidine deaminase) to influence class-switch recombination, affinity maturation, and the degree of *Myc-Igh* translocation in B cells (43, 44). An example of the network regulation model is provided by miR-181a, which tunes the strength and threshold of TCR signaling by coordinated regulation of multiple phosphatases, including SHP-2, PTPN-22, DUSP5, and DUSP6, at discrete steps of the signaling cascade (26). Similarly, miR-146a dampens

lipopolysaccharide (LPS) signaling through simultaneous targeting of TRAF6 [tumor necrosis factor receptor (TNFR)-associated factor 6] and IRAK1 [interleukin-1 (IL-1) receptor-associated kinase], thus controlling production of TNF- α (45). Finally, multiple miRNAs can control a particular function through regulation of distinct targets. miR-146a and miR-155, both unregulated by Foxp3, function through the convergent model to control the development and function of Treg cells by targeting STAT1 (signal transducer and activator of transcription 1) and SOCS1, respectively (42, 46).

One can interrogate the model by which a particular miRNA controls immune signals by analyzing the effects of target knockdown with specific short-hairpin RNAs (shRNAs) and target restoration by expressing miRNA-insensitive targets (47). If a miRNA acts through the linear model (Fig. 3A), knocking down the target mRNA should mimic miRNA function. However, if expression of miRNA-insensitive targets is sufficient to block the miRNA function and knocking down a single target gene by shRNAs is not sufficient to mimic the miRNA function, then the miRNA likely acts according to the network model (Fig. 3C). The divergent model can only be established by expressing miRNA-insensitive targets and miRNA loss-of-function analyses (Fig. 3B), through genetic deletion or miRNA silencing agents, such as sponges and antisense oligonucleotides (also known as antagomirs) (48, 49). In the convergent model (Fig. 3D), the phenotype is the sum of multi-target regulation; thus, knocking down a single target with shRNA or restoring the expression of an individual target genes will only result in a partial phenotype.

miRNA-controlled immune signaling networks

In immune cells, miRNAs may act as feedbacks to either potentiate or dampen signals. Specifically, miRNAs may be turned on or off at the transcriptional level upon ligand stimulation to influence the expression of signaling components either positively or negatively (Fig. 4A, B). For example, LPS stimulation of innate immune cells turns on the expression of miR-146a, which dampens the expression TRAF6 and IRAK1, which are positive regulators of the Tolllike receptor 4 (TLR4) signaling pathway; thus, the miRNA serves to reduce LPS-controlled TLR4 signals (45, 50). In contrast, Foxp3 expression upregulates miR-155 in Treg cells, which dampens the expression of SOCS1, a negative regulator of cytokine receptor signal pathway, and potentiates IL-2 signals (42). These results illustrate that miRNAs can either reduce or enhance immune signals by modulating the expression of the positive or negative components of immune signaling pathways.

Although the biological activity of a particular miRNA can sometimes be attributed to the repression of a single target gene, accumulating evidence suggests that many miRNAs exert their biological functions by controlling multiple targets. In fact, the degenerate nature of miRNA target recognition enables multi-target regulation that may be considered as an important feature of miRNA-based control (39). For example, miR-181a controls the expression of multiple phosphatases that are negative signaling molecules in the TCR signaling pathway (26) (Fig. 4C). These phosphatase mRNA targets share little sequence homology, and their protein products of these mRNAs act at distinct stages of the TCR signaling pathway. Moreover, shRNAs that can knockdown the phosphatase more effectively than the miRNA cannot recapitulate the function of miR-181a in controlling TCR signaling (39). These observations highlight the unique power of a single miRNA to regulate gene networks, illustrating that the evolutionarily selected molecular networks that are critical for immune signaling can be deciphered by connecting miRNA function to their functional targets (Fig. 2). Although it is possible to approach the same goal with global gene expression analysis and *de novo* network constructions using bioinformatics, characterization of miRNA targets in immune cells may have unique advantage in revealing evolutionarily selected gene networks.

miRNA control of immune signaling strength and threshold

miRNAs have the potential to modulate the strength and threshold of immune signals through quantitative control of target expression (Fig. 4D). An increase of about threefold in miR-181a levels in effector T cells, from approximately 25 copies/cell to approximately 70 copies/cell, caused a decrease in the levels of target phosphatases by 30–80% (26). These somewhat subtle changes in target expression are sufficient to reduce the threshold and potentiate the strength of TCR signaling. This result suggests that TCR signaling threshold is determined by two opposing forces in T cells: phosphorylation and dephosphorylation of receptor-associated tyrosine kinase Lck and serine/threonine kinase Erk. Indeed, an increase in miR-181a expression, which dampens the expression of phosphatases, elevates the basal phosphorylation of Lck and Erk kinases and reduces the activation threshold. In contrast, a decrease in miR-181a expression has the opposite effects on signal threshold. A change in signaling strength may be a direct outcome of altering TCR signaling threshold, which allows the same ligand stimulation to deliver a stronger signal as the result of reduction in threshold (Fig. 4D). Thus, miRNAs can have both quantitative and binary effects on the outcome of TCR stimulation.

miRNAs appear to control T-cell sensitivity to antigens during development. For example, miR-181a expression during T-cell development and maturation in the thymus or the periphery is dynamically regulated and positively correlated with developmental changes in T-cell sensitivity (26). Inhibition of miR-181a function in CD4 and CD8 double positive (DP) thymocytes, which results in upregulation of phosphatases and reduces TCR signal strength and T-cell sensitivity to antigens, shifts selection toward a TCR repertoire with higher affinity against self-antigens (38). Although it had been known for decades that T-cell sensitivity to antigens was controlled by intrinsic regulators during T-cell development and maturation (51), the nature of the regulator remained elusive until the discovery of miR-181a (26). Moreover, *mir-181a-1/b-1* controls the strength and threshold of Notch oncogenes in tumor transformation by dampening the targets in both the Notch and pre-TCR signaling pathways (34). These examples illustrate that deciphering miRNA function and target networks in immune cells may fill important gaps in our knowledge about the control of immune signaling in normal immune cells and cancer cells.

Spatiotemporal control of immune signaling by miRNAs

Dynamic regulation of miRNA expression may play an important role in the temporal and spatial regulation of immune signals (Fig. 5A). Most immune signaling studies have been carried out in cell culture, focusing on triggering events, and immediate signaling events. Immune signaling *in vivo* is likely to be subjected to temporal and spatial controls. Differential miRNA expression may modulate the level of positive or negative signals and thus provide spatio-temporal control of immune signaling. As an example, *miR-181a* provides spatiotemporal control of Notch, pre-TCR, and TCR signaling during T-cell maturation in the thymus (34) (Fig. 5B). The presence of high levels of miR-181a in early thymocytes may suppress the expression of Notch-regulated ankyrin repeat protein (Nrarp), a negative regulator of Notch signaling, and potentiate Notch signaling in double negative (DN) stage 1 and 2 cells. At the DN3 stage, miR-181a may potentiate both Notch and pre-TCR signals to provide essential synergy between these two signaling pathways that share no protein-based signaling molecules (26, 34). At this stage in development, miR-181a dampens the expression of negative signaling molecules in the pre-TCR pathway, the same set of phosphatases it controls in the effector T cells, and the negative feedback loop in the Notch pathway. After β -selection, miR-181a potentiates TCR signaling in DN4, DP, CD4, or CD8 single positive (SP) cells. Upregulation of miR-181a expression from DN1 to DN3 may provide strong suppression of Nrarp expression and potentiate Notch signaling, whereas stepwise downregulation of miR-181a expression after β -selection may release

Nrarp from miR-181a suppression and dampen Notch signaling. In another example, miR-155 expression is dynamically regulated in B cells during a 48 h time frame after the activation of BCR signaling (24), suggesting that miR-155 may provide temporal control of events after ligand-triggered activation. The effects of this regulation and its biological implications *in vivo* remain unclear. Nevertheless, miRNA regulation can provide exquisite spatiotemporal control of immune signaling, and characterizing these mechanisms may reveal yet-to-be appreciated aspects of signaling control.

miRNA control of immune responses and tolerance at the system level

Abundant examples illustrate miRNA functions in diverse molecular and cellular processes that are integral to innate and adaptive immune responses. Indeed, some recent studies illustrate that inactivation of miRNA genes in mice may have profound effects on immune response and tolerance at the system level. One can envision that systematic characterization of miRNA functions will establish the scope and effects of miRNAs in immune response and tolerance.

miRNA control of immune homeostasis

Targeted deletion of some miRNA genes can affect the homeostasis of the immune system. Loss of miR-155, miR-146a, and miR-223 all result in mild inflammatory responses in aged mice (typically more than 1 year old) (24, 25, 52, 53). *miR-155*-null mice display lung and enteric inflammation (25). *miR-223*-null mice spontaneously develop inflammatory lung pathology and exhibit exaggerated tissue destruction after endotoxin challenge (52). Loss of miR-146a in mice causes incomplete penetrant of chronic systemic inflammation in aged mice (53). These results demonstrate that miRNAs are integral components of the homeostatic maintenance machinery. The mechanisms through which each of these miRNAs contributes to immune homeostasis differ, because they regulate different pathways in distinct cell types. miR-223 regulates the development of granulocytes and their sensitivity to endotoxin. miR-146a dampens NF- κ B controlled inflammatory responses in macrophages, dendritic cells, and T cells. Notably, miR-155 controls antibody generation and contributes to diverse signaling pathways in B, T, and myeloid cells. The degree of contribution to immune homeostasis also varies. For example, loss of miR-146a results in the development of tumors in secondary lymphoid organs in approximately 25% of aged mice (53), whereas loss of either miR-223 or miR-155 does not have such effects (24, 25, 52). In humans, reduced miR-181a expression in naive T cells from aged individuals may contribute to their defective responses to antigen stimulation and vaccination (54). Effects of miRNAs on immune homeostasis are usually subtle and gradual and manifest more profoundly in older than younger animals. Nevertheless, these results demonstrate that the quantitative effects of these miRNAs on immune signaling may result in phenotypes that penetrate into the system layer [Fig. 1 (3)].

System control of immune responses and tolerance by miRNAs

Inactivation of miRNA genes can have either positive or negative influence on the overall strength of immune responses. Loss of miR-155 causes defects in the development of adaptive immune responses (25). *miR-155*-null mice are incapable of mounting a protective immune response against the virulent enteric pathogen *Salmonella typhimurium* after immunization with the live, attenuated form of the bacteria. These mice also have defects in B cell development and antibody generation, antigen-presentation by dendritic cells, and T cell activation, polarization, and cytokine production (24, 43, 44). Not surprisingly, miR-155-null mice are also resistant to the induction of autoimmune diseases, including experimental autoimmune encephalomyelitis (EAE) and arthritis, presumably due to defects in Th17 cell differentiation and IL-17 production (55, 56). Thus, although miR-155 deletion

has subtle effects on various cellular processes, these quantitative effects can result in switching outcomes on immune responses in the system layer [Fig. 1B (2)].

In contrast, loss of miR-146a in mice causes exaggerated innate and adaptive immune responses (53, 57). Macrophages from *miR-146a*-null mice are hypersensitive to endotoxin stimulation, and T cells are hyperactive in chronic and acute inflammatory responses to antigen stimulation. Consistent with these phenotypes, *miR-146a*-null mice are hypersensitive to endotoxin stimulation and succumb to autoimmunity. It has been postulated that miR-146a may be an intrinsic molecular component of the endotoxin-induced tolerance pathway; priming human THP-1 cells, an undifferentiated promonocytic cell line, with low doses of LPS induces the expression of miR-146a and represses the expression of TRAF6 and IRAK1, which in turn dampens the responses of these cells to subsequent higher doses of LPS (50). It is conceivable that miR-146a-null cells, both innate and adaptive immune cells, are unable to be primed by low-level stimulation. It will be of great interest to investigate whether endotoxin-induced or self-antigen-induced tolerance is defective in *miR-146a*-null mice in vivo. Collectively, these results demonstrate that the quantitative effects of miR-146a deletion on innate and adaptive immune signals result in fully penetrated outcomes at the system layer (Fig. 1B).

Loss of a miRNA gene may also influence the outcome of immune responses by reshaping the dynamics of immune responses at the system level. miR-181a regulates T-cell sensitivity to antigens, and inhibition of miR-181a function in DP cells decreases their sensitivity and enables the selection of TCRs with higher affinity for self-peptides (38). The fact that dynamic regulation of miR-181a corresponds to changes in sensitivity in developmental T-cell populations suggests that miR-181a contributes to central tolerance by setting positive and negative selection thresholds. Thus, one would expect that deletion of *mir-181a-1/b-1* might result in the selection of T cells with high affinity for self-antigens and that *mir-181a-1/b-1*-null mice should be prone to development of autoimmune diseases. Interestingly, however, *mir-181a-1/b-1*-null mice are normal and have no sign of autoimmunity at 1 year of age (34). Moreover, these mice are more resistant to EAE induction and have a delayed disease onset (Schaffert *et al.*, unpublished observations). Thus, the effects of miR-181a on antigen sensitivity at the molecular and cellular level does not significantly affect immune equilibrium at the system layer under unperturbed conditions, but may have profound effects on the outcomes of autoimmune perturbations (Fig. 1A).

These paradoxical observations raise many interesting questions. If *mir-181a-1/b-1* deletion in mice does result in T cells with higher affinity for self-antigens, then why are *mir-181a-1/b-1*-null mice more resistant to autoimmune disease induction than wildtype mice? Why don't these mutant mice develop spontaneous autoimmunity? It may be that the detrimental shift in central tolerance as a result of loss of *mir-181a-1/b-1* is compensated by changes in peripheral responses controlled by this miRNA gene. Other questions also remain unanswered. Why is such an antigen-sensitivity rheostat needed in the DP thymocytes? What are the physiological signals that might control this T-cell sensitivity rheostat? What are the physiological implications of such tuning? Is this antigen-sensitivity rheostat be exploited by pathogens? Finally, is this rheostat be utilized in other immune cell types such as B cells, dendritic cells, or myeloid cell types? The answers to these questions may provide further insights into the control of immune responses and tolerance.

Since characterized miRNA-null mice generally have an intact immune system but react differently to various perturbations, one may consider that the immune system in these mouse strains is in an 'alternative immune equilibrium' that is reactive but differs in response to perturbations from the wildtype system. In contrast, genetic manipulation of

essential immune molecules (e.g. BCR, TCR, and FoxP3) and clinically used immune suppressive agents generally cause massive structural damage to the immune system. Thus, it is possible to influence immune response and tolerance at the system level without causing major structural damage as illustrated by analyses of mice with genetic deletion of certain miRNAs.

miRNA control of autoimmunity

miRNAs may play important roles in the pathogenesis of autoimmune diseases. Both gain and loss of miRNA function seem to have strong effects on the tolerance and response of the immune system. For example, loss of miR-146a causes spontaneous autoimmunity in aged mice (53), and transgenic miR-17–92 expression drives lymphoproliferative disease and systemic autoimmunity due to augmented T and B-cell activation, proliferation, and survival (58). Moreover, aberrant miRNA expression often correlates with onset and prognosis of autoimmune diseases, suggesting that miRNA dysregulation may directly contribute to the inflammatory responses and autoimmune disease in human and mice. miR-155 overexpression in CD4⁺ T cells inhibits natural Treg-mediated suppression (59), and increased miR-155 levels have been observed in T cells of lupus-prone B6.Sle123 congenic mice and in peripheral blood mononuclear cells of rheumatoid arthritis patients (60, 61). Interestingly, active lesions in multiple sclerosis patients are characterized by strong upregulation of miR-155 expression (62). These results suggest that miR-155 overexpression in T cells may contribute the development of lupus, rheumatoid arthritis, and EAE. As *miR-155*-null mice are compromised in mounting adaptive immune responses (25) and are resistant to EAE induction (55, 56), it would be interesting to test whether antagomirs against miR-155 reduce symptoms of those affected by rheumatoid arthritis and lupus. Further supporting the roles of miRNAs in autoimmune diseases, mutations and polymorphisms observed in patients affect miRNA expression or miRNA regulation. A mutation in the miR-101 binding site enhances ICOS expression on T cells and mediates spontaneous systemic autoimmunity (63). Luo and colleagues (64) identified a genetic variant in the promoter region of miR-146a that may contribute to reduced binding by the Ets-1 transcription factor and decreased miR-146a expression in patients with lupus. Finally, miRNAs may contribute to autoimmune responses by modulating tissue responses to inflammatory signals. As an example, IL-17-mediated inflammatory responses are in part a result of the suppression of miR-23b expression in radio-resistant cells at the site of autoimmune inflammation. Downregulation of miR-23b by IL-17 in these cells causes the up regulation of miR-23b targets, including TAB 2, TAB 3, and IKK- α , and promotes pro-inflammatory cytokine expression (65). Collectively, this evidence strongly supports the role of miRNAs in the pathogenesis of autoimmune diseases.

miRNA control of hematopoietic lineage differentiation

Diverse cell types constitute the vertebrate immune system. Remarkably, hundreds (if not thousands) of immune cell types are generated and sustained by a small number of hematopoietic stem cells that occur at a frequency of approximately one in 100 000 murine bone marrow cells (66). To establish the homeostasis of immune cell milieu, the types, quantities, timing, and rates of cell production are tightly regulated. Moreover, the development of these cells must be responsive to regulatory signals for the homeostatic and defense functions. Cells that are exhausted must be replenished, cells needed for action must be propagated, and cells that are in excess must be eliminated. Lineage commitment, one of the best-characterized processes of hematopoietic lineage differentiation, plays critical roles in determining the cell types made (67). Many transcription factors, receptors, and secreted factors responsible for lineage commitment have been identified. For example, Notch signaling is essential for the commitment of early thymic progenitors into T-cell lineages in the thymus (68), and the transcription factor ThPOK is essential for the commitment of

double-positive cells into CD4 SP lineages (69). However, the mechanisms underlying the dynamic regulation of immune cell equilibrium are still poorly understood.

Effects of miRNA deletion on lineage commitment

A growing body of evidence indicates that miRNAs regulate hematopoietic/lymphoid differentiation, but deletion of individual miRNA genes often has minor effects on the development of the immune system. Only subtle changes in various immune cell populations are observed in the *miR-155*, *miR-150*, *miR-146a*, *miR-451*, *miR-21*, or individual *miR-181* knockout mouse strains. Homozygous *miR-155*-null mice have reduced numbers of germinal center B cells in gut-associated lymphoid tissue, including Peyer's patches and mesenteric lymph nodes, compared with wildtype mice (24). Loss of *miR-150* results in quantitative changes in numbers of splenic and peritoneal B1 and/or B2 cells but does not affect T-cell, follicular B-cell, or marginal zone B-cell development in mice (41). Germline deletion of the *mir-181a-1/b-1* alleles results in quantitative reductions of early thymic progenitors, CD4 and CD8 DN3, CD4 and CD8 DP cells, germinal center B cells, and peripheral blood B cells, and increases in CD4 SP cells and marginal zone B cells (34). In contrast, germline deletion of *mir-181cd* causes more than a twofold increase in the percent of CD8 SP T cells in the thymus, but does not cause notable changes in other T or B-lymphocyte populations (34). Deletion of *miR-451* causes slight reduction in hematocrit and no apparent changes in white blood cells (70–72). Mice in which *mir-181a-2/b-2*, *miR-21*, or *miR-146a* are deleted do not have significant changes in hematopoietic or lymphoid differentiation (34, 53, 73).

Effects of miRNA deletion on differentiation are more apparent *in vitro* than *in vivo*. For example, the effects of gain or loss of *mir-181a-1/b-1* expression on DP, B cell, and early thymocyte development is more robust when analyzed using *in vitro* assays than when evaluated *in vivo* (34). Germline deletion of *mir-181a-1/b1* only results in subtle decreases in early thymocyte populations *in vivo* (34). Immune cell development *in vivo* is likely to be subjected to extensive and redundant levels of regulation. This means that changes in the steady-state levels of immune cell types in the miRNA-null mice may have been compensated for through homeostatic mechanisms. By inference, these miRNAs likely play quantitative roles rather than deterministic roles in hematopoietic lineage differentiation, and the quantitative effects of miRNA deletion do not cause fully penetrant phenotypes at the system layer (Fig. 1B).

Effects of miRNA overexpression on lineage differentiation

Overexpression of miRNA genes can have drastic effects on immune cell development, sometimes leading to the development of cancer. Ectopic expression of miR-181a in hematopoietic stem/progenitor cells promotes peripheral B-cell differentiation in a mouse bone marrow transplantation assay (19), whereas deletion of *mir-181a-1/b-1* only results in modest effects on peripheral B-cell differentiation (34). More strikingly, miR-155 and miR-21 are tumorigenic when individually expressed in targeted cell populations. Transgenic expression of miR-155 at the pro-B cell stage under the control of a V_H promoter-Ig heavy chain E_μ enhancer initially causes a preleukemic pre-B-cell proliferation in the spleen and bone marrow and later results in B-cell malignancy (74). Inducible transgenic expression of miR-21 in hematopoietic cells leads to B-cell lymphoma; interestingly, tumors regress completely soon after miR-21 is inactivated (75). Intriguingly, pan-tissue transgenic expression of miR-21 using the CAG promoter does not induce tumor development in mice (73). These results demonstrate that tumors can become addicted to miRNAs. Clearly, miRNA overexpression, despite the quantitative effects on lineage differentiation, often results in fully penetrant phenotypes at the system layer (Fig. 1B).

miRNA control of rates and timing of lineage differentiation

The results mentioned above suggest that miRNAs can affect hematopoietic lineage differentiation without disrupting the commitment processes. What are the processes controlled by miRNAs during the hematopoietic/lymphoid lineage differentiation? Regulation of the commitment processes, which determines the type of cell made, is likely to be digital and not sufficient to provide robust control of the homeostasis of the immune cell milieu. Other types of graded and quantitative regulations must also play important roles in controlling immune cell development. During hematopoietic lineage differentiation, there is a rapid expansion of committed progenitors. This process is critical for generating large numbers of differentiated cells from a limited number of stem/progenitor cells and is utilized repeatedly during hematopoietic/lymphoid differentiation processes, from hematopoietic stem cells to T, B, and myeloid cells. Therefore, another parameter that can be controlled during the expansion phase is the rate of expansion (Fig. 6A). In this model, both commitment and rate of expansion can affect the ratio of daughter 1(D1) and daughter 2 (D2) cells, the progenies of the corresponding progenitor. The commitment process will influence the ratio in a digital manner (1, 2, ..., n-fold), whereas the control of the rate of expansion can have a graded and non-digital influence. Without the control of differentiation rates and other dynamic factors, immune cell differentiation would respond to extrinsic and intrinsic stimuli with large swings in cell numbers. Moreover, timing, duration, and location of differentiation may also be regulated (Fig. 6A). We postulate that miRNAs may play a critical role in controlling these kinetic factors of lineage differentiation. As the stability and robustness of the immune cell milieu is the foundation of immune homeostasis and defense, significant redundancy must be built into the regulation of these dynamic parameters. This model may explain why miRNA overexpression, which renders miRNA expression insensitive to homeostatic signals, may eventually drive immune equilibrium out of balance.

Distinct miRNA contributions to leukemogenesis

Many studies have illustrated the importance of miRNAs in leukemogenesis (reviewed in 3, 76). miRNAs were shown to function as oncogene or tumor suppressors, and miRNA expression profiles can be used to classify leukemia types and predict prognosis (77–79). Of note, most known protein leukemogenic factors have essential roles in hematopoietic lineage differentiation, whereas miRNAs often have quantitative roles in hematopoietic lineage differentiation as discussed elsewhere in this article. Therefore, interfering with miRNA function may inhibit cancer cell growth without severe side effects.

The critical roles of oncogenic drivers in normal development and leukemia

Many transcription factors, receptors, kinases, and chromatin-remodeling factors play critical roles in controlling normal hematopoietic lineage differentiation (80–82). Genetic disruption of these factors in mice often results in aberrant lineage development and genes encoding these proteins are often mutated or rearranged and function as oncogenes in human leukemia. For example, *Notch1* mutations are found in over 50% human T-cell acute lymphoblastic leukemia (T-ALL) patients (83). Notch1 mutant proteins can transform hematopoietic stem and progenitor cells and induce T-ALL in mice (84). These mutated or recombined lineage-specification factors often act as drivers to induce tumor transformation. During this process, loss of various homeostatic control mechanisms marks the transitions from a normal cell to a tumor cell (Fig. 6B).

Although these genes have provided the basis for targeted treatment of human leukemia, most protein oncogenes are difficult therapeutic targets. These factors (or their fusion partners) often have essential roles in normal hematopoietic lineage differentiation or tissue development. Thus, effective inhibition these proteins may disrupt normal hematopoiesis

and result in significant toxicity to normal tissues (Fig. 6C). For example, in clinical trials involving Notch-induced T-ALL patients, a γ secretase inhibitor that effectively inhibits Notch function by blocking the processing and release of intracellular Notch (ICN) caused severe gastrointestinal toxicity and had limited anti-leukemic activity (85).

Essential roles of miRNAs in leukemogenesis

Oncogenes and tumor suppressors can have profound effects on remodeling miRNA profiles in tumor cells (21, 86, 87). The altered miRNA profiles in tumor cells, revealed by miRNA profiling analyses, indicate that some miRNAs are dysregulated to facilitate the expression of oncogenic programs. Here, we use the studies on Notch-induced T-ALL to illustrate the critical functions of miRNAs in leukemogenesis (34, 88, 89). Expression of the Notch oncogene in hematopoietic stem/progenitor inhibits the expression of miR-451 and miR-709 and causes the upregulation of their common target Myc, which is essential for Notch oncogenic function (88). Interestingly, Notch expression also causes upregulation of many miRNAs, including miR-9b, miR-20a, miR-26a, miR-92, and miR-223, that potentiate Notch-induced T-ALL by suppressing the expression of IKAROS (also known as IKZF1), PTEN, BIM, PHF6, NF1, and FBXW7 (89). Both upregulated and downregulated miRNAs may contribute to T-ALL induced by the Notch oncogenes. Retroviral delivery of miR-451 into leukemia cells inhibits Notch-induced TALL upon transplantation into mice (88). It is possible that knockdown of individual miRNAs or a combination of the Notch upregulated miRNAs (miR-9b, miR-20a, miR-26a, miR-92, and miR-223) will inhibit T-ALL development.

In addition to the above examples, miRNAs that are directly regulated by Notch oncogene may play a critical role in Notch-induced T-ALL, a recent study has illustrated that miRNA programs important for normal development may be utilized by oncogenes for tumor transformation (34). It is known that Notch-induced T-ALL transformation blocks T-cell development at the immature DP thymocyte stage and permits extrathymic DP cell development in humans and mice (90), indicating that Notch1 oncogenes utilize the genetic programs that operate in normal DP thymocytes for transformation. Interestingly, miR-181a is highly expressed in normal DP cells and potentiates both Notch and pre-TCR signaling pathways during normal DP cell development (Fig. 5B). As Notch and pre-TCR signaling pathways act synergistically to promote TALL (91–93) and miR-181a expression and function in normal DP cells coincides with the developmental arrest of T-ALL cells at this stage, it is likely that miR-181a may contribute to T-ALL. Indeed, targeted deletion of *mir-181a1-1/b-1* in mice and antisense inhibition of miR-181a in T-ALL cells demonstrated that miR-181a is essential for leukemia development and maintenance. Importantly, the deletion of *mir-181a1-1/b-1* effectively inhibits NOTCH-induced T-ALL with only subtle effects on normal development (34). As *mir-181a1-1/b-1* is essential for oncogene activity in tumor development but is not critical for normal development, this miRNA may be targeted to treat T-ALL without causing significant toxicity (Fig. 6C).

Emerging evidence illustrated that miRNAs may be therapeutic targets for treating leukemias (3, 34, 75). However, it remains a challenge to deliver therapeutic molecules to replace miRNAs or to inhibit miRNA function in tumor cells. Among various strategies for delivering miRNAs or miRNA sponges into tumor cells, the delivery system based on adeno-associated virus has showed promise (94). The challenge ahead involves the development of effective antisense reagents that can be delivered to tumor cells and silence miRNAs that support tumor development. Of note, the toxicity and effectiveness of miRNA overexpression as a cancer therapy may need to be rigorously tested as a few studies have shown that miRNA overexpression in hematopoietic cells causes leukemia and lymphoproliferative disorders (74, 75).

Potential roles of primary and precursor miRNAs in target recognition

To decipher the molecular network controlled by miRNAs in immune cells, it is imperative to identify the functionally relevant target mRNAs. Target identification through both computational prediction and experimental approaches remains challenging. The target prediction programs based on the ‘seed-recognition’ principle, that is, perfect base pairing between the nucleotides at positions 2 through 7 of mature miRNAs and their targets, have high false positive rates (approximately 40% or more) and undetermined false negative rates (39, 95). Importantly, many biologically relevant targets do not require perfect seed pairing for recognition by miRNAs, and recognition of certain targets is actually mediated by the nucleotides in the center of mature miRNA sequences (96). Experimental approaches based on the enrichment of cross-linked target and mature miRNA RISC (RNA-induced silencing complexes), such as HIT-CLIP and Par-CLIP, also have limitations (97, 98). Notably, only about 10% of mature miRNAs in HeLa cells are in the RISC (99). Further investigation is necessary to determine the fraction of mature miRNAs in RISC for other cell types. Such analyses are necessary to establish the inclusiveness and validity of using mature miRNA-RISC pull-down to identify miRNA targets.

The puzzle of target-recognizing miRNA species

The constraints in target identification may be in part caused by our incomplete knowledge of the target-recognizing miRNA species (33, 100, 101). In the original article describing the discovery of *lin-4* miRNAs, the authors pointed out that both precursor and mature *lin-4* miRNAs (referred to as *lin-4L* and *lin-4S*, respectively) could function in gene repression as both contain sequence complementary to the target mRNA (8). The primary *lin-4* miRNA, which was not detected in the original study, also has the potential to be a functional species following the same logic. Genetic deletion could not be used to attribute the phenotype to one of the RNA species as such operation results in loss of both precursor and mature species. The authors did favor the hypothesis that the mature *lin-4* miRNA plays the major role because mature *lin-4S* is significantly more abundant than *lin-4L* and the secondary structure of *lin-4L* may sequester the bases required for interactions with the target mRNA rendering it inactive. However, as the major RNA species made from a miRNA gene, the pri-, pre-, and mature miRNAs, each contain a region of identical sequence (that of the mature miRNA), all have the potential to interact with target mRNAs. It is intrinsically difficult to distinguish the contributions of miRNA species in target recognition and repression by either biochemical or genetic approaches.

Although the miRNA field has largely accepted that mature miRNAs are the sole functional RNA species produced from all miRNA genes, this general assumption actually has never been tested for miRNA genes. Importantly, examples of antisense regulatory RNAs from the RNA World demonstrate that structured RNAs are common target-recognizing species (102, 103). These examples illustrate that the regulatory information encoded in secondary and tertiary structures of these RNAs is interpreted and translated into gene regulatory activities through the interaction between structured regulatory RNAs and their cognate targets (102). The possibility of pri-/pre-miRNAs as target-recognizing RNA species should be considered and is required for elucidating the principles of target-recognition and mechanisms of repression. Intuitively, whether pri-/pre-miRNAs or mature miRNAs are the target-recognizing species would determine the nucleotide information that should be used for target prediction and the baits that should be used for target pull-down. Similarly, the answer to the puzzle of target-recognizing miRNA species is critical for properly interpreting results on the regulation of miRNA gene activities. Within the framework of canonical mature miRNA model, proteins that control the activity of pri-/pre-miRNAs in target recognition and repression can only be explained through the control of mature miRNA activities, leading to misconceptions and confusion.

Lessons from the miR-181 family miRNAs

Our efforts to understand the biological functions of *mir-181* family genes in immune cells provide support for the potential functions of pri-/pre-miRNAs in target recognition (33, 34). The members of the *mir-181* family of genes produce four nearly identical mature miRNAs (miR-181a, miR-181b, miR-181c, and miR-181d) from three polycistronic miRNA genes: *mir-181a-1/b-1*, *mir-181a-2/b-2*, and *mir-181c/d*. Based on small RNA-seq analyses, the miR-181 miRNAs are co-expressed by many T-cell types, albeit at varied levels (104). This observation that members of the same miRNA family are often co-expressed in the same cells and tissues is not unique to the *mir-181* family miRNAs. For example, the *let-7* family miRNAs, which consist of eight essentially identical mature let-7 miRNAs, are co-expressed in many immune cell types (104). These observations are puzzling and suggest that the expanded members of miRNA family may have quantitatively and qualitatively distinct biological activities, despite that they may also contribute to diverse expression patterns in tissues and cell types of the miRNA family members.

We tested this hypothesis using quantitative biological readouts for the *mir-181* family genes in T cells. It is important that these assays depend on the control of multiple targets by the miRNA gene products in its native milieu. In contrast, report assays measure the repression of a single target by a miRNA in an arbitrary cell culture. We demonstrated that *mir-181a-1* and *mir-181c*, which produce mature miRNAs with only a single nucleotide difference in the center of mature miRNAs (Fig. 7A, B), have distinct functions in CD4 and CD8 DP T-cell development *in vitro* and *in vivo* (33, 34). Furthermore, we showed that *mir-181a-1*, but not *mir-181c*, can promote DP T-cell development when ectopically expressed in thymic progenitor cells (Fig. 7C). Importantly, the distinct activities of *mir-181a-1* and *mir-181c* are largely determined by their unique pri/pre-miRNA loop nucleotides not by the single nucleotide difference in their mature miRNA sequences (Fig. 7C). Moreover, the effect of *mir-181a-1* gene products on DP cell development is quantitatively influenced by nucleotides in the pri/pre-miRNA loop region; both the strength and the functional specificity of *mir-181a-1* depends on pri-/pre-miRNA loop sequences (33). This phenomenon is not unique to the *mir-181a-1* and *mir-181c* during early T-cell development. Through loss-of-function analyses, we demonstrated that deletion of *mir-181a-1/b-1*, but not *mir-181-c/d* or *mir-181a-2b-2*, selectively inhibits tumor transformation induced by Notch oncogenes (34). Finally, we found that all three *mir-181* alleles are expressed in embryonic stem cells (ESCs); however, deletion of *mir-181a-2b-2*, but not *mir-181a-1/b-1* and *mir-181-c/d*, affects the rate of ESC self-renewal (Arnold *et al.*, unpublished observations). We also observed that loop nucleotides play critical roles in controlling target repression by *let-7* miRNA genes independent of mature miRNA biogenesis (100, 101).

These findings demonstrate that miRNA genes that produce identical or nearly identical mature miRNAs have distinct biological activities that are controlled by their pri/ pre-miRNA loop sequences. The interpretation of these findings depends on whether or not one assumes that mature miRNAs are the sole functional species of miRNA genes. According to the canonical model, members of the same miRNA family genes should have the same activity when co-expressed. However, we found that the distinct activities of these miRNA genes producing identical or nearly identical mature miRNAs are not controlled through differential mature miRNA biogenesis, as shown by extensive loop mutagenesis and domain-swapping analyses (33). These results indicate that pri-miRNAs and/or pre-miRNAs may have direct roles in target recognition and repression and loop nucleotides control their activities. This model enables direct translation of regulatory information encoded in the structural elements of pri/pre-miRNAs into regulatory function through RNA:RNA interaction. While proteins can be evolved to regulate the process of target and pri/pre-miRNAs interactions, it is unlikely that evolution would discard this simple and elegant

approach to interpret the regulatory information in the structured RNAs through RNA:RNA interaction.

Further supporting this model, we demonstrated that primary *cel-let-7* represses the expression of a target reporter with lin-41 3' untranslated region (UTR) in the absence of functional pre- and mature let-7 (100). Moreover, we demonstrated that *cel-let-7* pri-miRNAs form complexes with target mRNAs containing the lin-41 3' UTR *in vitro* and *in vivo* and showed that the loop nucleotides control the activities of pri-miRNA in target repression by influencing complex formation between pri-*cel-let-7* and lin-41 3' UTR. We went on to show that human *let-7a-3* pri-miRNA also forms complexes with target mRNAs. Thus, pri-miRNAs can recognize and repress target expression in the absence and presence of functional mature miRNAs (100, 101). Collectively, our studies on the function of *miR-181* and *let-7* family genes illustrate that the regulatory information encoded in the structure and sequence elements of pre- and pri-miRNAs can be directly translated into function in target recognition and repression through interactions between two structured RNA molecules.

Perspectives and concluding remarks

In this article, we examined the diverse functions of miRNAs in immune cells and the impacts of miRNAs on immune responses and tolerance at the molecular, cellular, and system levels. miRNA expression may be turned on or off at the transcriptional level upon ligand stimulation and may have either positive or negative effects on the expression of signaling components that impact immune cell development or function. Interestingly, despite the quantitative nature of miRNA-mediated regulation, it is possible to dampen autoimmune responses and leukemia by genetically manipulating miRNA expression in mice. These examples suggest that miRNAs may be targeted to treat autoimmune diseases and leukemias without causing significant toxicity. Moreover, the multi-target regulation potential of miRNAs presents opportunities to dissect immune signaling networks by decoding the target-recognition information encoded in the miRNA genes. However, to improve our ability in miRNA target identification, we first need to figure out the contribution of primary and precursor miRNAs in target-recognition and repression. The answer to this puzzle may have significant implications for the understanding of the principles underlying miRNA target recognition and for the development of better methods for computational and experimental target identification. Such efforts will ultimately help us to decipher the evolutionarily selected immune signaling networks controlled by miRNAs.

We have only begun to appreciate the impact of miRNAs on immune response and tolerance at the system level. The activities and mechanisms of action of these regulators are significantly different from the essential protein molecules that immunologists have characterized in the past decades. We postulate that miRNAs contribute to the temporal, spatial, and dosage aspects of immune regulation. These components may have important roles in controlling the dynamic aspects of immune responses that are important for maintaining tissue homeostasis and defending the host against foreign pathogens. The phenotypes that result from genetic manipulation of these regulatory components are often subtle and/or pleiotropic. To date, there are no well-established techniques to quantify the stability or buffering potential of the immune system. It is also challenging to measure the strength and the spatiotemporal dynamics of immune responses *in vivo*. However, manipulation of miRNA genes in mice, which generally results in subtle changes to the immune system, presents a unique opportunity to study how tuning the strength and the spatiotemporal dynamics of immune signals may affect immune tolerance and functions at the system layer. It is evident that researchers must develop a new conceptual framework and experimental techniques for elucidating the functions of miRNAs and other quantitative

regulators of the immune response and tolerance. Controlling these molecules could be the key to manipulating immune tolerance and restoring a healthy immune equilibrium from pathogenic conditions without significant toxicity.

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Fig. 1. Representative scenarios of phenotype penetrance caused by genetic manipulation of (A) switching (on/off) molecules and (B) tuning molecules on the immune system at the molecular, cellular, and system layers.

Fig. 2. Control of evolutionarily selected target networks by miRNA species at the post-transcriptional levels

The regulatory information for target recognition and degree of repression is encoded in the nucleotide sequence of miRNA genes. Understanding how such regulatory information is translated into activity is essential in decoding the target networks controlled by miRNA gene products. It is important to note that the major RNA species made from a miRNA gene – primary, precursor, and mature miRNA – all contain the same mature miRNA sequence and, in principle, can bind mRNA targets. The optimal method for computational and experimental target identification will differ depending on which miRNA species are the target-recognizing species.

Fig. 3. Basic models of miRNA target recognition and the corresponding functional consequences (A) Linear, (B) divergent, (C) network, and (D) convergent model of target control by miRNA species.

Fig. 4. Models of miRNA-controlled signaling networks and the potential impact of miRNA regulation on the strength and threshold of signaling

(A) miRNAs as negative feedback to dampen the expression of multiple positive signaling molecules. (B) miRNAs as a positive feedback to dampen a single negative signaling molecule. (C) miRNAs as a negative tuner of immune signaling by controlling the expression of multiple negative signaling molecules. (D) Effects of miRNA control on the strength and threshold of immune signaling. A1, A2, and An represent positive signaling molecules in the pathway (i.e. the positive kinases). B1, B2, and Bn represent negative signaling molecules in the pathway (i.e. the phosphatases that inactive positive kinases).

Fig. 5. miRNA control of immune signaling

(A) Multi-dimensional control of immune signaling by miRNAs. The strength and spatiotemporal of immune signaling may be controlled by differential miRNA expression. (B) miRNAs as modular signaling regulators. An miRNA can control the same or different signaling pathways in different cell populations. Schematic of thymic T-cell development and signaling pathways controlled by miR-181a these cell populations. Cell types and corresponding signal pathways controlled by miR-181a are matched by color codes.

Fig. 6. miRNA control of normal lineage differentiation and leukeomogenesis

(A) Schematic depicting the parameters that are important for hematopoietic/lymphoid lineage differentiation. (B) Tumor transformation by driver mutations in oncogenes and tumor suppressors and effects on the loss of homeostasis maintenance mechanisms. (C) Targeted inhibition of driver oncogenes and miRNAs has distinct effects on normal and leukemogenic lineage development.

Fig. 7. Pri/pre-miRNAs are potential target-recognizing RNA species of a miRNA gene
 (A) Schematics of *mir-181a-1* and *mir-181c*. (B) Mature miR-181a and miR-181c. (C) Pri-/pre-miRNA loop nucleotides control the distinct activities of *mir-181a-1* and *mir-181c* in promoting early T cell development. Effects of domain-swapping mutations on the activities of *mir-181a-1* and *mir-181c* in promoting early T-cell development. See Liu *et al.* (33) for complete data and in depth analyses.