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REVIEW

microRNA dynamic expression regulates invariant NKT cells

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Abstract

Invariant natural killer T cells (iNKT) are a prevalent population of innate-like T cells in mice, but quite rare in humans that are critical for regulation of the innate and adaptive immune responses during antimicrobial immunity, tumor rejection, and inflammatory diseases. Multiple transcription factors and signaling molecules that contribute to iNKT cell selection and functional differentiation have been identified. However, the full molecular network responsible for regulating and maintaining iNKT populations remains unclear. MicroRNAs (miRNAs) are an abundant class of evolutionarily conserved, small, non-coding RNAs that regulate gene expression post-transcriptionally. Previous reports uncovered the important roles of miRNAs in iNKT cell development and function using Dicer mutant mice. In this review, we discuss the emerging roles of individual miRNAs in iNKT cells reported by our group and other groups, including miR-150, miR-155, miR-181, let-7, miR-17 ~ 92 cluster, and miR-183-96-182 cluster. It is likely that iNKT cell development, differentiation, homeostasis, and functions are orchestrated through a multilayered network comprising interactions among master transcription factors, signaling molecules, and dynamically expressed miRNAs. We provide a comprehensive view of the molecular mechanisms underlying iNKT cell differentiation and function controlled by dynamically expressed miRNAs.

Keywords iNKT cells · microRNAs · miR-155 · miR-150 · miR-183-96-182 · miR-181 · miR-17~92 · let-7

Introduction

MicroRNAs (miRNAs) are 18- to 23-nucleotide, single-stranded RNAs that modulate the stability and translation of mRNAs at the post-transcriptional level [1]. To date, more than 2500 mature human miRNAs have been identified [2]. Over the past decade, miRNAs have been increasingly recognized as a discrete layer of gene regulation controlling the cellular development and physiological workings of the immune system, and miRNAs are the best-characterized class of noncoding RNAs in regard to genetic expression and cellular function [3]. miRNAs are encoded in the genome as miRNA genes and are transcribed by RNA polymerase II, which generates a primary miRNA (pri-miRNA) molecule [4]. This primary transcript is then cleaved in the nucleus by the conserved endogenous double-strand RNaseIII-type endonuclease Drosha, resulting in a precursor miRNA (pre-miRNA) molecule. The pre-miRNA translocates to the cytoplasm with assistance from Exportin 5, where it is processed by the enzyme Dicer, which cleaves it into a double-stranded miRNA duplex. The resulting 18- to 23-nucleotide long single-stranded mature miRNA is then loaded onto the RNA-induced silencing complex (RISC) [5], which interacts with its target mRNAs via complementary binding, causing either post-transcriptional degradation or translational repression [6].

Invariant natural killer T (iNKT) cells are a subset of regulatory T cells that co-express a rearranged T cell receptor α chain (Vα14-Jα18 in mice; Vα24-Jα18 in humans) in combination with a limited set of Vβ chains (Vβ8.2, 7 or Vβ2 in mice; Vβ11 in humans) [7]. iNKT cells branch out of the conventional T cell lineage at the double-positive (DP) thymocyte stage and are selected by homotypic DP thymocytes expressing CD1d,

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a nonclassical MHC-I–like molecule [8, 9]. After positive selection, signals initiated through TCR (T cell receptor) and SLAM (signaling lymphocytic activation molecule) family receptors allow iNKT cells to progress to the earliest detectable subset, stage 0, where cell surface molecules CD24 and CD69 are expressed [10]. Subsequently, thymus iNKT cells proliferate and proceed through three sequential maturation stages that are phenotypically distinguished by the variable expression of CD24, CD44, and NK1.1 as follows: stage 1 ($CD24^{low}CD44^{low}NK1.1^{-}$), stage 2 ($CD24^{low}CD44^{hi}NK1.1^{-}$), and stage 3 ($CD24^{low}CD44^{hi}NK1.1^{+}$) [11, 12]. Upon antigenic stimulation, iNKT cells rapidly and robustly produce a broad range of cytokines, which allows them to modulate the functions of a variety of immune cells and regulate both innate and adaptive immune responses [13, 14].

Recently, studies on iNKT cell development focus on the lineage differentiation model in addition to the linear maturation model [15]. Three major functionally distinct iNKT cell subsets originate in the thymus—iNKT1, iNKT2, and iNKT17—with subset delineation based on transcription factor expression and cytokine profiles. Each subset can be further characterized by the expression of distinct transcription factors that correlate with a cytokine secretion profile upon activation. iNKT1 cells express T-bet, predominantly secrete IFN-γ, and are mainly equivalent to stage 3 iNKT cells; iNKT17 cells have intermediate levels of PLZF, are $ROR\gamma t^{+}$ and secrete IL-17 and IL-23 [16, 17]. iNKT2 cells are not readily defined, and typically, iNKT2 express high levels of GATA3, PLZF, and IL17RB, and secrete IL-4 and IL-13 [15, 18, 19]; and more precisely, recent high dimensional single-cell RNA sequencing studies indicated the transcriptional heterogeneity and developmental path of iNKT cells development [20–22]. These new discoveries in iNKT subset differentiation, along with the recent global transcriptional analysis of iNKT cell development and functional subsets, have revealed that iNKT cell development and differentiation in the thymus are controlled by a complex signaling network, which ensures proper induction of genes essential for iNKT cell differentiation and maturation. All of these iNKT cell subsets differentiation and population expansion mechanisms depend on tunable responses that are sensitive to minor perturbations in protein expression cascades. In particular, antigen receptor signaling can be quantitatively manipulated through minor changes in expression that limit regulators in the downstream signaling pathways, such as the PI3K and NF-κB. This type of manipulation can be carried out by multiple epigenetic mechanisms, including miRNA regulation. In this review, we provide an overview of the current literatures to outline the roles of miRNAs at various stages of iNKT cell development, differentiation, and activation.

Specific and dynamic miRNA expression in T and iNKT cells

Unique spatial and temporal expression patterns in distinct hematopoietic cell lineages suggest that miRNAs may play multiple roles in immune cell development, differentiation, and function, which have been explored over the past decade [23–25]. The dynamic, cell-specific regulation of miRNA expression during sequential stages of T cell development has been well documented [26, 27]. Even though T cells at early DP developmental stages have relatively high miRNA expression levels, individual miRNAs show dynamic changes in distinct thymic T cell subpopulations, including at the DP, double-negative (DN), and single-positive (SP) stages [26, 28]. In addition to defining the miRNA genome (miRNome) of mouse lymphocyte subsets, a recent study combined high-throughput microRNA-seq, mRNA-seq, and CHIP-seq to gain insights into the epigenetic regulation of lymphocyte miRNA genes [29]. This study offers a complete view of the mouse immune system miRNome while pointing out that miRNA abundance results from the interplay of epigenetic, transcriptional, and post-transcriptional mechanisms. To generate a comprehensive view of the human lymphocytic miRNome, Rossi et al. isolated 17 human T and B lymphocyte subsets from peripheral blood mononuclear cells and sorted them by flow cytometry, analyzing the miRNA expression profiles by TaqMan low-density arrays (TLDAs) [30]. This study defined human lymphocyte miRNA expression signatures and identified 29 miRNAs that showed subset-specific expression patterns. To determine the miRNA profiles expressed by thymic iNKT cells relative to mature thymic conventional T cells, miRNAs from mouse HSA^{low} thymic iNKT and T cells were profiled with locked nucleic acid (LNA)-based miRNA microarrays [31]. Among 70 miRNAs detected, the study revealed 17 miRNAs that were differentially expressed in thymic iNKT cells compared to thymic conventional T cells. Real-time PCR confirmed that the thymic iNKT cells had higher expression of miR-21 and lower expression of let-7g, miR-106a, miR-34b-3p, miR-17, miR-30c, miR-106b, miR-16, miR-467a, miR-467b, miR-690, miR-15b, miR-669f, and miR-150 than T cells. These miRNA signatures exhibited features of activated/effector iNKT cells.

Dynamic changes of miRNA expression during the sequential stages of T cell development have been well documented [26]. Coinciding with this phenomenon, studies from other groups, including ours, have shown dynamic changes in levels of individual miRNAs during distinct iNKT cell developmental stages. While miR-142 [32] and miR-96 [33] are equally expressed at developmental stages 1 to 3, the following molecules are expressed at

higher levels in stage 1 and lower levels in stages 2 and 3: miR223 [34], miR-155 [35, 36], miR-150 [37, 38], miR-182, miR-183 [33], miR-181a and b [39], miR-30c and e [32]. Also, miR-25 and miR-92 are selectively down-regulated at stage 3 [32], whereas miR-21a, miR-23b, and miR-30a and b are upregulated at stage 3 [32]. Finally, miR-17, miR-18a and b, miR-19a and b, miR-20a and b, miR-93, miR-106a and b, and miR-363 are selectively up-regulated at stage 2 [32]. The specific and dynamic miRNA expression changes in iNKT cells strongly suggest a potential role for miRNAs in maintaining and balancing signaling networks during iNKT cell development, maturation, and activation.

Global miRNA regulation in iNKT cells

As knowledge of multiple defined pathways has contributed to the identification of the miRNA pool, initial studies determining the importance of miRNAs in the murine immune system utilized a broad disruption of all miRNAs in specific cell types by deleting key proteins, such as Dicer, required for miRNA biogenesis. With the deletion of Dicer, miRNA gene expression profiles display remarkable changes within immune cells, including iNKT and conventional T cells [31, 40]. Consistent with the distinct miRNA expression patterns, the overall critical regulatory roles of miRNAs in hematopoiesis, including during the development and maturation of iNKT cells, have been revealed through the use of Dicer mutation animal models [31, 40–42]. To investigate the role of miRNAs in iNKT cell regulation, our group generated a hematopoietic progenitor-specific Dicer deletion mouse model (Tie2-cre;Dicer$^{flox/flox}$), which uncovered the critical relevance of miRNAs in iNKT cell development and function [40]. Our results indicate that Dicer deletion leads to a severe abrogation of iNKT cells in both the thymus and in peripheral organs. Strikingly, Dicer-deficient iNKT cells were not able to properly mature and showed dramatic blockade in the transitions from DP to DN or CD4 + SP and from NK1.1- immature stage to NK1.1 + mature stage [40]. Moreover, Dicer-deficient iNKT cells show remarkably reduced activation and cytokine secretion upon both TCR-dependent and TCR-independent stimulation. Soon after our report, Fedeli et al. independently identified the critical role of miRNAs in iNKT cell development using CD4-Cre- and Lck-Cre-mediated thymus-specific Dicer knockout (KO) mouse models [31]. Their results indicate that defective iNKT cell homeostasis in thymus-specific Dicer deletion mice is presumably caused by a mitotic defect and increased iNKT cell death [31]. In agreement with these results, our studies using the similar thymus-specific Dicer deletion mouse model further confirm the profound developmental and functional defects in Dicer deletion iNKT cells [43]. In addition to iNKT cells, Lck mediated Dicer deletion showed that in a profound loss of conventional immature and mature T cells [44]. Furthermore, T cell-specific Dicer deletion leads to impaired conventional T cell development and aberrant T helper cell differentiation and reduced T regulatory cell (T_{reg}) development [45, 46]. These studies demonstrated the critical roles of Dicer-dependent miRNAs in the signaling pathways and networks controlling iNKT cell development and function. Meanwhile, they also emphasize the necessity of identifying the specific miRNAs and related molecular mechanisms contributing to iNKT cell maturation, homeostasis, and function.

Individual miRNAs regulate iNKT cell development, differentiation, and activation

Even though genetic studies have clearly demonstrated the central role of miRNAs and miRNA-dependent pathways in various aspects of iNKT cell biology, identification of the roles of individual miRNAs and their related molecular mechanisms is essential for comprehensively understanding the signaling pathways and molecular networks in iNKT cell developmental regulation. The principle that miRNAs target multiple mRNAs to form regulatory networks is evident in multiple cell types, diseases, and animal models [47, 48]. The positive or negative regulation by individual miRNAs is often achieved through the modulation of key signaling pathways, and multiple miRNAs can coordinately regulate shared signaling pathways to induce integrated broader changes in related signaling networks [47, 49, 50]. Through years of effort, multiple laboratories have independently identified several individual miRNAs critically involved in iNKT cell development, differentiation, activation, and function, which will be briefly reviewed here (Fig. 1; Table 1).

miR-150

miR-150 has been reported to be particularly expressed in mature B and T cells, but not in their progenitors [51]. The ectopic expression of miR-150 in hematopoietic progenitor cells leads to a substantially reduced number of mature B cells, especially B1 cells, by blocking B cell development during the transition from the pro-B to pre-B cell stage [52]. The importance of miR-150 in B cell development was further demonstrated through gain- and loss-of-function mouse models, but no apparent T cell developmental defects were identified except a slightly delayed T cell development observed in miR-150 transgenic mice early in life [53]. Furthermore, *c-Myb*, a predicted target of miR-150, was found to play a crucial role in modulating B cell development [53]. Bezman NA et al. and our group have found that, in addition to expression in conventional B and

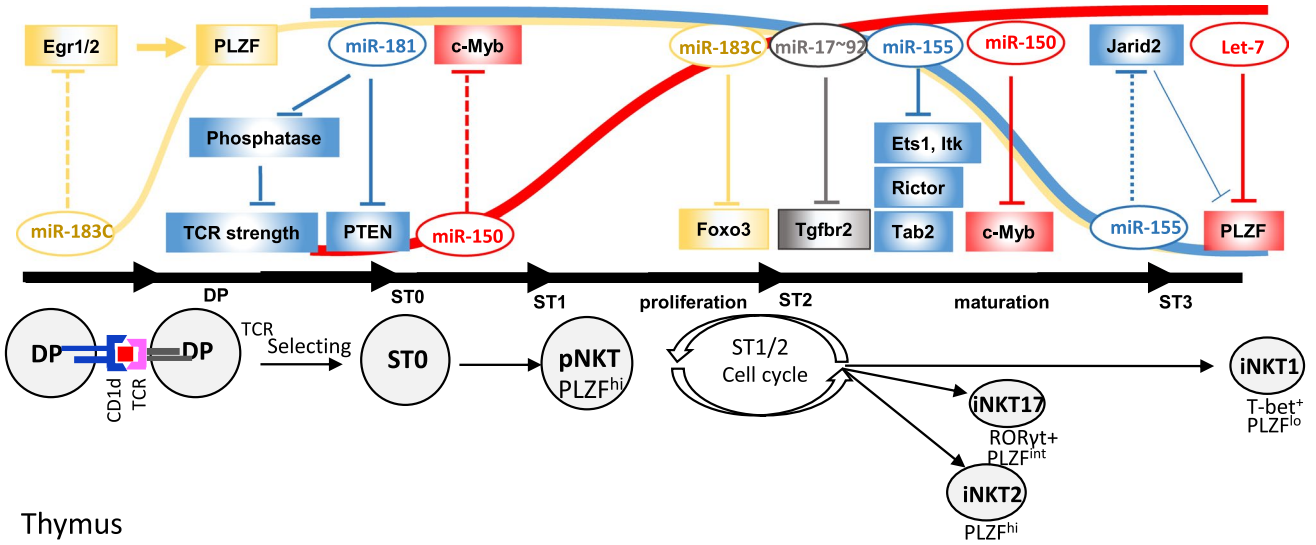

Fig. 1 microRNA dynamic expression mediates iNKT cell development. iNKT cells are selected from DP thymocytes and proceed through 4 sequential stages (stage 0–3) that are phenotypically distinguished by the expression of CD24, CD44, and NK1.1. During development, iNKT cells differentiate into iNKT1, iNKT2, and iNKT17 cells based on the activity of the transcription factors T-bet, PLZF, and RORγt. Dynamically expressed miRNAs have been identified that regulate iNKT cell development. The effects of these miRNAs depend on their timing of expression throughout iNKT cell development. miR-181ab, miR-183-96-182 cluster (miR-183C), and miR-155 control the early development stages of iNKT cell, whereas miR-17~92, miR-150, and let-7 direct the final steps of iNKT cell maturation via targeting multiple genes that critical for iNKT cell development. During iNKT cells development, miRNAs showing in red (miR-150 and let-7) are gradually increased; miRNAs showing in blue (miR-181 and miR-155) are gradually down regulated; miRNA in yellow (miR-183C) is repressed in DP, increased in ST0, and then gradually down regulated during iNKT cells development; miRNA in grey (miR-17~92) is highly expressed in ST2. *DP* double positive, *ST* stage, *pNKT* iNKT precursor

T cells, miR-150 is expressed in iNKT cells and is gradually upregulated during iNKT cell maturation in the thymus [37, 38]. Using conventional miR-150 KO mice, we found that miR-150 deletion significantly reduces iNKT cell frequency and cell number, and partially blocks iNKT cell maturation from stage 2 to stage 3 in both thymus and peripheral organs. Moreover, unlike Dicer-deficient iNKT cells, miR-150–deficient iNKT cells display increased IFN-γ production after stimulation with α-GalCer in vivo, but not after phorbol myristate acetate/ionomycin treatment, indicating that miR-150 may negatively regulate iNKT activation mainly through targeting proximal components of TCR signaling pathway [37]. In contrast to the maturation and functional defects in miR-150-deficient iNKT cells, the miR-150-deficient conventional T cells, Tregs, and γδ T cells from thymus and peripheral organs show no apparent phenotypic changes, suggesting that iNKT cell lineage-specific regulation is mediated by miR-150. Notably, Lanier's group reported that overexpression of miR-150 also dramatically reduces the iNKT cell number and partially blocks iNKT cell development at the transition from stage 0 to stage 1, resulting in an increased proportion of $CD24^{hi}$ $NK1.1^{-}$ iNKT cells compared to wild type [38]. These results indicate the essential role of dynamic miR-150 regulation in the development of iNKT cells [38, 54, 55]. Deficiency in the potential miR-150 target *c-Myb* in the thymus results in a complete absence of iNKT cells. *c-Myb* plays a central role in priming DP thymocytes to enter the iNKT cell lineage by simultaneously regulating CD1d expression, the half-life of DP cells, and the expression of SLAMF1, SLAMF6, and SAP [56]. c-Myb is also highly expressed in immature iNKT cells and downregulated during iNKT cell maturation, which is the opposite of miR-150 dynamic expression changes during iNKT cell maturation. Furthermore, in miR-150 KO mice, c-Myb expression is significantly upregulated in both immature (stages 1 and 2) and mature (stage 3) iNKT cells [37], and partial ablation of *c-Myb* phenocopies impaired iNKT cell development in $miR\text{-}150^{Tg}$ mice[38]. These data suggest that a dynamically and tightly regulated expression of miR-150 is required for optimal iNKT cell development by controlling the levels of *c-Myb* expression.

miR-181

The miR-181 family is composed of six mature miRNAs encoded in three independent miRNA clusters: miR-181a1, miR-181a2, miR-181b1, miR-181b2, miR-181c, and miR-181d [57]. One of these clusters, miR-181a1b1, has been shown to be particularly important in conventional T cell selection and activation. Inhibition of miR-181 in thymocytes impairs positive and negative T cell selection and decreases TCR sensitivity, while overexpression of

Table 1 miRNAs and their potential targets involved in iNKT cell development

miRNA	Expression		Potential targets	Function	Signaling pathway	References
	Up-reg	Down-reg				
miR-155	Stages 1/2	Stage 3	*Jarid2*	A negative regulator of *Zbtb16* at later stage		[35]
			Rictor	Control iNKT17 differentiation and *i*NKT survival	mTORC2 signaling	
			Tab2	TGF-b activated kinase 1-binding protein 2	TGF-β & NF-κB signaling	
			Est1	Critical for cell maturation		[36]
			Itk	Inducible T cell kinase, downstream of TCR		
miR-183C	Stages 1/2	DP stage, stage 3	*Egr1/2*	A positive regulator of *Zbtb16* at early stage		[33]
			Foxo1	Control iNKT17 effector function	NF-κB signaling	
			Foxo3	Control NF-kB signaling in T cells	NF-κB signaling	
miR-181	DP, Stage 1	Stage 3	*Pten*	Governs iNKT cell homeostasis	PI3K-PTEN signaling	[60]
			Ptptn11	Phosphatases	Negatively regulate TCR signaling	[39]
			Ptptn22			
			Dusp6			
			Aplp2	Modulates phosphoinositide-mediated calcium flux		[32]
			Atp1b1	Catalyzes the hydrolysis of ATP coupled with the exchange of Na + and K + ions		
			Klf6	A transcriptional activator		
			Man2a1	Catalyzes the first step in the biosynthesis of complex N-glycans		
			Samhd1	A modulator of the innate immune response		
			Txndc5	Functions as antioxidant		
miR-150	Stage 3	Stages 1/2	*c-Myb*	Control T cell proliferation and differentiation		[37, 38]
let-7	Stages 1/2	Stage 3	*Zbtb16*	A master of iNKT cell lineages		[94]
miR-133b	–	–	*Th-pok*	Control iNKT17 differentiation		[108]

Table 1 (continued)

miRNA	Expression		Potential targets	Function	Signaling pathway	References
	Up-reg	Down-reg				
miR-30e,-c	Stage 1	Stages 2/3	*Timp2*	Inhibitor of the matrix metalloproteinases		[32]
miR-30a,-b	Stage 3	Stages 1/2	*Timp2*	Inhibitor of the matrix metalloproteinases		
miR-23	Stage 3	Stages 1/2	*Sgk1*	Regulate cellular progress such as cell survival		
miR-223	Stages 1/2	Stage 3	–	–		
miR-21	Stage 3	Stages 1/2	–	–		
miR-17 ~ 92	Stage 2	Stages 1/3	*Sgk1*	Regulate cellular progress such as cell survival		
			Timp2	Inhibitor of the matrix metalloproteinases		
			Tgfbr2	Binds TGF-beta, regulate the transcription of genes related to cell proliferation, cell cycle arrest	TGF-β signaling	
miR-106a ~ 363	Stage 2	Stages 1/3	*Tgfbr2*			
miR-106b ~ 25	Stage 2	Stages 1/3	*Tgfbr2*			
miR-495	–		*Sgk1*	Regulate cellular progress such as cell survival		
miR-495	–		*Timp2*	Inhibitor of the matrix metalloproteinases		
miR-142	Equally expressed at stage 1–3		*Sgk1*	Regulate cellular progress such as cell survival		

Down-reg downregulated, *Up-reg* upregulated, *DP* double positive, *iNKT* invariant natural killer T cell

miR-181a in mature T cells increases their TCR sensitivity to peptide antigens [58]. In addition, miR-181 reduces the TCR engagement threshold for cellular responses through targeting multiple phosphatases, including PTPN22, SHP-2, DUSP5, and DUSP6 [58]. In innate immunity, miR-181 promotes human NK cell development from $CD34^+$ hematopoietic progenitor cells and IFN-γ production in primary $CD56^+$ NK cells, partially through the suppression of the Notch signaling inhibitor NLK [59].

In addition to having an effect on conventional T cells, miR-181 has been shown to play an essential role in iNKT cell development, as was shown in two parallel studies [39, 60]. Deletion of the miR-181a1b1 cluster was shown to block iNKT cell development during the metabolically demanding transition from stage 0 to 1 and to almost completely abrogate early iNKT cell development, resulting in dramatically reduced iNKT cell numbers in the thymus and in peripheral organs [39, 60]. These findings suggest that iNKT cell development is essentially dependent on miR-181. Furthermore, miR-181a1b1 deletion results in reduced iNKT cell activation upon TCR stimulation, an altered TCR repertoire, and reduced peripheral iNKT cell function [39]. Completely eliminating the miR-181 target PTEN (phosphatase tensin homolog) restores iNKT cell numbers in miR-181a1b1 deficient mice [60]. This indicates a critical role for miR-181 in regulating phosphoinositide 3-kinase (PI3K) signaling downstream of TCR activation and suggests that miR-181 may possibly control mTOR (mammalian target rapamycin) signaling and regulation of global metabolic fitness. Interestingly, iNKT cell development appears to be particularly sensitive to alterations in metabolic fitness, especially during development from stage 0 to stage 1, which likely reflects multiple unique features in iNKT cell ontogenesis [61, 62]. Moreover, increasing the concentration of TCR ligands or elevating levels of a Vα14Jα18 TCRα chain [63] could rescue defective iNKT cell development in miR-181a1b1 KO mice, which indicates the important role of miR-181 in setting a TCR signaling threshold for agonist selection through high-affinity TCR ligands [39]. The TCR signaling threshold alteration seen during iNKT cell development in miR-181a1b1 KO mice maybe, at least partially, related to the modest upregulation of miR-181 target genes such as *Ptpn22*, *Shp-2*, and *Dusp6*, which negatively regulate TCR signaling [39]. Therefore, the paucity of iNKT cells

in miR-181a1b1 deficient mice most likely results from a coordinated interplay of players that regulate TCR and PI3K signaling, which has been well-documented in the control of iNKT cell selection, population expansion, and homeostasis.

miR-155

miR-155 is processed by Dicer from BIC (B cell integration cluster), a non-coding transcript highly expressed in B cells, T cells, and in monocytes/macrophages. In Treg cells, miR-155 is directly regulated by FoxP3 and facilitates Treg homeostasis by targeting SOCS1 (suppressors of cytokine signaling1), leading to increased sensitivity of the IL-2 receptor to IL-2 [64, 65]. Beyond the role of miR-155 in Treg homeostasis, Baltimore and colleagues have noted the upregulation of miR-155 as a consistent feature of the mammalian inflammatory response [66]. Both B and T lymphocytes display a similar induction of miR-155 in response to activating stimuli [67, 68], and other studies using miR-155 gene targeting mouse models have further demonstrated a broad role of miR-155 in the immune system, including for B cell memory formation, Th1 and Th17 cell-dependent tissue inflammation, Th2 cell differentiation, and $CD8^+$ T cell effector function, which have been comprehensively reviewed previously [3, 49, 69–75]. High expression of miR-155 is implicated in human B cell malignancies, which aligns with the B cell proliferative disorders observed in miR-155 transgenic mice [76, 77]. Using a thymus-specific (Lck-cre) miR-155 over-expression mouse model, Burocchi and colleagues found the crucial role of tightly controlled miR-155 expression in the development of iNKT cells [36]. Overexpression of miR-155 results in a substantial block of iNKT cell maturation at the stage 2 to stage 3 transition in both thymus and in peripheral organs, which leads toward an overall reduction of peripheral iNKT cells, including in the liver, spleen, and bone marrow. These differentiation and maturation iNKT cell defects induced by miR-155 overexpression were shown to be cell autonomous. Furthermore, the transcription factors *Ets1* and *Itk* have been identified as targets of miR-155 during iNKT cell differentiation [36]. Both *Itk* and *Ets1* are also linked to TCR signaling [78] and may be targeted by miR-155 in different cell types [79, 80]. Both *Ets1* KO [81, 82] and *Itk* KO [83] mice display a defect in iNKT maturation at stage 2 to stage 3 transition, which phenocopies Lck-miR-155 transgenic mice. The expression of *Ets1* and *Itk* is consistently downmodulated in miR-155 transgenic iNKT cells, except in stage 1, which could be due to a shorter isoform of the 3′-UTR during stage 1 development. Moreover, at both transcriptional and protein levels, *Ets1* and *Itk* are dynamically upregulated in wild type mice during thymic iNKT cell maturation, which is reciprocally correlated to the dynamic downregulation of miR-155.

Of note, our recent study using a knock-in (KI), thymic cell-specific miR-155 overexpression mouse model showed an additional mechanism underlying miR-155 regulation of iNKT cells [35]. RNA-seq analysis of sorted stage1/2 $NK1.1^-$ and stage 3 $NK1.1^+$ cells showed that potential miR-155 targets, including *Jarid2*, *Rictor*, and *Tab2*, were significantly repressed in miR-155 overexpressing iNKT cells. *Jarid2* is a member of the JmjC domain-containing protein family, a novel component of polycomb repressive complex 2. A previous study has confirmed that *Jarid2* is a negative regulator of *Zbtb16* (encoded by PLZF), which is mediated by H3K9me3 demethylation at a later stage of iNKT cell development [84]. Of note, the iNKT phenotype in a T cell-specific *Jarid2* deletion closely resembles what was observed in miR-155 KI mice, as judged by the disturbed differentiation of iNKT1/2 cells. Rictor is an obligatory component of mTORC2 [85], and the defective iNKT phenotype in *Rictor* KO mice is reminiscent of miR-155 KI iNKT cells, particularly in regard to diminished iNKT17 lineage differentiation [86, 87]. Furthermore, miR-155 overexpressing iNKT cells also show downregulation of the NF-κB signaling pathway, which may possibly occur through direct targeting of *Tab2*, an upstream activation kinase complex component of NF-κB [88]. Overall, the dynamic expression of miR-155 and its regulation in thymic iNKT cells is essential for the development, functional lineage differentiation, and homeostasis of iNKT cells [35, 36]. It should be mentioned that unlike dramatic phenotype change of iNKT cell with miR-155 overexpression, loss of miR-155 showed a minor role in the development of iNKT cells, and no effect on peripheral iNKT cells [89]. It is possible that loss of miR-155 is compensated for by other miRNAs or other proteins, in which would account for normal iNKT cell development in miR-155 deficient mice.

let-7

The let-7 (lethal-7) family encompasses the most highly abundant miRNAs in the human genome and consists of 12 to 14 individual members encoded on different chromosomes in mammals [90]. Let-7 miRNA family members are highly conserved across the majority of bilateral species, including mammals [91]. And the potential target genes of let-7 are involved in a variety of cellular functions, such as cell-cycle progression, metabolism, and oncogenesis [92, 93]. To investigate the regulatory roles of let-7 miRNAs during T cell differentiation, Singer's group created a mouse model with reduced thymus-specific let-7 miRNA expression. Their strategy took advantage of the fetal protein LIN28, which prevents let-7 precursor molecules from being processed into the functionally mature miRNAs [94]. They found that in LIN28 transgenic mice, where the T cell-specific human CD2 promoter/enhancer was used to drive

LIN28 expression, the frequency of SP CD8 + memory-like populations in the thymus were increased, possibly due to an increased number of IL-4 producing cells. In addition to this phenotype, LIN28 transgenic mice showed dramatically increased iNKT2 and iNKT17 but decreased iNKT1 frequencies, indicating a requirement for let-7 miRNAs in iNKT thymocyte terminal differentiation. Although the overall number of thymic iNKT cells in LIN28 transgenic mice remains comparable to wild type, higher expression of let-7 miRNAs quantitatively inhibits the generation of iNKT thymocytes in a cell-intrinsic manner [94, 95]. The transcription factor PLZF (promyelocytic leukemia zinc finger) belongs to the BTB-zinc finger family and is the signature transcription factor expressed in innate-like T cells, including iNKT and γδT cells [96]. In iNKT cells, PLZF expression is induced by *Egr2* after iNKT cell positive selection, and *Egr2* is one of the earliest transcription factors induced by TCR signaling [97]. A deficiency in PLZF leads to a dramatic interruption of iNKT cell development. The few iNKT cells in PLZF deficient mice are phenotypically immature with defective cytokine secretion [95, 98] In line with the iNKT and T cell phenotypes in let-7 and PLZF mutant mice, let-7 miRNAs have been shown to target *Zbtb16* mRNA (which encodes PLZF) to post-transcriptionally inhibit PLZF protein expression. As the expression of let-7 miRNAs is enhanced as iNKT cells mature, PLZF expression is decreased. The dynamic expression changes of PLZF and let-7 miRNAs in opposite directions emphasizes the key role of let-7 in regulating PLZF expression during iNKT cell differentiation and maturation [94]. Furthermore, let-7 miRNAs are dynamically upregulated during iNKT cell development and maturation in the thymic medulla through exogenous stimuli, including IL-15, vitamin D, and retinoic acid. One might speculate that the elevated and prolonged TCR-induced expression of *Egr2* in iNKT cell precursors activates PLZF transcription, which may promote iNKT cell development and acquisition of effector phenotypes [97]. As iNKT cell differentiation progresses, exogenous stimuli may induce the upregulation of let-7, which concomitantly downregulates PLZF, thus supporting a potential model where increased let-7 expression in iNKT cells at stage 2 results in differentiation and maturation toward iNKT1 cells, rather than iNKT2 and iNKT17 cells [94]. These results suggest that a temporal, specific, and dynamic expression pattern of miRNAs regulates iNKT cell effector differentiation.

miR-183-96-182

The miR-183-96-182 cluster (miR-183C) is a family of miRNAs with evolutionarily conserved sequence homology and genomic organization and comprises three molecules: miR-183, miR-96, and miR-182 [99]. Our recent study has shown that miR-183C regulates iNKT cell development and effector function through targeting multiple transcription factors [33]. Of these miRNAs, miR-183 and miR-182 are severely repressed in the DP stage, dramatically upregulated in stage 1, and then gradually downregulated in stages 2 and 3. However, miR-96 is evenly repressed throughout iNKT cell development. The number of iNKT cells in miR-183C KO mice is reduced twofold in the thymus, and tissue-resident iNKT cells show a modified distribution, with a reduced level of iNKT1 cells, increased level of iNKT2 cells, and unchanged iNKT17 cells. The developmental defect of iNKT cells in the absence of miR-183C is cell-intrinsic. This study also showed that miR-183C regulates iNKT cell development via targeting *Foxo3*, *Foxo1*, *Egr1*, and *Egr2*, which may be required for iNKT development and function. *Egr1* and *Egr2* are the earliest transcription factors induced by TCR signaling and play a critical role in the positive selection of iNKT cells [97, 100]. In the DP stage, repressed miR-183C may promote upregulation of *Egr1* and *Egr2* expression, consequently supporting iNKT cell selection [33]. While upregulated, *Foxo3* could interrupt the NF-κB signaling pathway by binding NF-κB RelA in the cytosol and preventing RelA nuclear translocation [101], and miR-183C might target *Foxo3*. NF-κB is thought to be critical for iNKT cell maturation [102]. In fact, the iNKT phenotype observed in mice that have deletions in various NF-κB components closely resembles the iNKT phenotype of miR-183C–deficient mice. Finally, the study observed that IL-17 production in iNKT cells from the thymus and from peripheral organs of miR-183C KO mice is reduced, even though the differentiation of RORγt-expressing iNKT17 cells is unchanged. A recent study from Chen's lab demonstrated that miR-183C enhances IL-17 production and pathogenic function in Th17 cells through targeting *Foxo1*, which inhibits RORγt-induced IL-1R1 expression and subsequent IL-17 production, without affecting RORγt expression [103]. Consistently, this study found that *Foxo1* expression in iNKT cells is significantly upregulated in the absence of miR-183C and showed that iNKT cells with a *Foxo1* deletion have dramatically increased IL-17 production. These data indicate that miR-183C is involved in maintaining iNKT17 effector function through targeting *Foxo1*. Taken together, miR-183C plays a subtle but indispensable role in iNKT cell development, differentiation, and effector function via targeting multiple genes.

miR-17 ~ 92 family clusters

The miRNAs discussed up until now (miR-150, miR-155, miR-181, miR183C, and let-7) have been shown to play independent roles in iNKT cell development. However, deleting them results in defects that only partially recapitulate those found in iNKT cells with a Dicer deletion. Recently, a study by Fedeli et al. demonstrated that miR-17 ~ 92 family clusters

play a role in controlling iNKT cell development [32]. The miR-17 ~ 92 family clusters (miR-17 ~ 92, miR-106a ~ 363, and miR-106b ~ 25) are some of the best-characterized polycistronic miRNAs. They encode 15 individual miRNAs and are grouped into 4 "seed" families (miR-17, miR-18, miR-19, and miR-92) [104]. Many oncogenic transcription factors have been reported that regulate the expression of miR-17 ~ 92 cluster, including MYC, MYCN, and E2F family, e.g. E2F1, E2F2, and E2F3, which are critical for cell cycle procession [105–107], therefore, cycling cells are likely to have elevated levels of miR-17 ~ 92. That could explain that miR-17 ~ 92 was highly increased in stage 2 of iNKT cells, where iNKT cells undergo quick expansion. Mice lacking all 3 miR-17 ~ 92 family clusters (triple knockout, TrKO) had markedly reduced iNKT cell frequency and absolute number. iNKT cells that can be isolated from TrKO mice display a severely immature phenotype, and the mice have an increased level of iNKT2 cells and reduced iNKT1 and iNKT17 cells, which is similar to what is seen in Dicer KO mice. iNKT cell maturation defect we observed in TrKO phenocopy the maturation defect we observed in overall miRNAs deletion mice (Dicer KO). Of note, iNKT cells with a deletion in the miR-17 ~ 92 family show increased TGF-βRII expression but relatively normal TGF-βRI expression. Furthermore, in developing iNKT cells, TGF-βRII and its downstream molecules pSMAD2/3 show an expression pattern that is inverse to miR-17 ~ 92 family clusters. Consistently, TGF-βRII expression and signaling are augmented in Dicer KO iNKT cells [32]. Therefore, Fedeli et al. suggest that miR-17 ~ 92 family clusters may directly target and fine-tune TGF-β signaling at stage 2, controlling iNKT cell terminal maturation. Finally, genetic ablation of TGF-β signaling in the absence of miRNAs rescues iNKT cell maturation and differentiation [32]. Overall, miR-17 ~ 92 could be the prominent miRNA that direct the final steps of iNKT cell maturation by targeting on TCF-βII.

Overall, these studies clearly demonstrate that miRNAs direct important functions during the regulation of iNKT cell development, maturation, and homeostasis. Positive and negative regulation by individual miRNAs was often achieved through the modulation of key signaling pathways. Multiple miRNAs may coordinately regulate shared signaling pathways to induce integrated broad changes in related signaling networks, such as the TCR signaling pathway, while individual miRNAs might target multiple molecules to induce cumulative effects during multiple stages of iNKT cell development and activation.

Conclusions

Tremendous progress has been made in identifying the transcription factors and signal transduction components involved in iNKT cell development and function. However, the mechanisms controlling the expression of these factors and coordinating their activities in promoting iNKT cell positive selection, lineage specification, acquisition of functional activity, and homeostasis remain mysterious. As the functions of miRNAs in regulating iNKT cell development and function become evident, the potential roles of miRNAs as key intermediates in the molecular networks of iNKT cell regulation become clear. The ability of individual miRNAs to target molecules in crucial signaling pathways related to iNKT cell differentiation and maturation emphasizes the critical role of miRNAs in iNKT cell modulation. Until now, individual miRNAs, including miR-150, miR-155, miR-181, let-7, miR-17 ~ 92 cluster, and miR-183C have been reported that exhibit dynamic expression pattern during iNKT cells development, and play critical roles by targeting key signature markers for iNKT cell development and function (Fig. 1). Beyond those, miR-133b was also considered as a regulator for iNKT17 cell differentiation by targeting Th-POK in NOD mice [108]. However, miRNAs dynamic regulation does not always support iNKT cell functional relevance, for example, despite prominent and highly dynamic expression in thymocytes and iNKT cells, miR-21 [109] and miR-223 [34] expression may not be required for iNKT cell development and function. Since multiple miRNAs regulate cellular events and may act redundantly [110], it is possible that the unaffected iNKT cell development in miR-21 KO and miR-233 KO could be due to redundant function on cellular events regulated by multiple miRNAs. It has been well known that TCR signaling plays a critical role in subset differentiation [111–113], weakened TCR signaling led to abrogation in iNKT2 and to a lesser extent iNKT17 cell development, while not reducing iNKT1 cell development [111, 114]. Among the miRNAs reviewed above, both miR-155 and miR-17 ~ 92 cluster family have been reported could be induced upon TCR activation [115]. And miR-181, miR-183C, and miR-155 have been considered are implicated in TCR signaling strength in some degrees [33, 35, 39, 63]. Since Mucosal-Associated Invariant T (MAIT) cells share at least some developmental features with iNKT cells, both iNKT cell and MAIT cell are selected on DP thymocytes and depend on PLZF. However, thymic MAIT cells can be divided into MAIT1 and MAIT17 cells in the mature stage [116]. In fact, investigations of MAIT cells unveiled that miRNAs are MAIT cells required for MAIT cells development as well [117]. The role of individual miRNAs, such as miR-181 and miR-155, in MAIT cell development have been reported. Similar to iNKT cells, miR-181 could regulate

MAIT cells via regulating TCR signal, deletion of miR-181 mice displayed an almost complete block in early MAIT cells development, resulting in dramatically reduced MAIT cell number [118]. Strikingly, like iNKT cells, overexpression of miR-155 reduces MAIT cell frequency and number, and blocks MAIT17 differentiation. Both iNKT17 and MAIT17 cells decreased in miR-155 overexpression mice indicated that miR-155 targets on signature transcription factors, such as *Rictor,* in innate T cells development [119]. However, MAIT1 cells in miR-155 overexpression MAIT cells are increased, the discrepancy of miRNAs in regulating iNKT cells and MAIT cells could due to the differentiation programs between iNKT cells and MAIT cells are different. Future investigations that identify new miRNAs, elucidate miRNA targets, and reveal the molecular mechanisms of miRNA regulation will not only provide new perspectives on the key molecular mechanisms of iNKT cell regulation but will also offer opportunities to identify novel genes and pathways involved in the iNKT cell regulatory network. Important challenges remain to identify the related molecular mechanisms among various miRNAs and to understand how they control the integrated context of iNKT cell differentiation, homeostasis, and function. These studies will ultimately form the basis for future utilization of iNKT cells in disease diagnosis and therapies.

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Declarations

Conflict of interest The authors declare that they have no competing interests.

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