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MicroRNA miR-150 Is Involved in Vα14 Invariant NKT Cell Development and Function

Quanhui Zheng,*† Li Zhou,*†‡§ and Qing-Sheng Mi*†‡§

CD1d-restricted Vα14 invariant NKT (iNKT) cells play an important role in the regulation of diverse immune responses. MicroRNA-mediated RNA interference is emerging as a crucial regulatory mechanism in the control of iNKT cell differentiation and function. Yet, roles of specific microRNAs in the development and function of iNKT cells remain to be further addressed. In this study, we identified the gradually increased expression of microRNA-150 (miR-150) during the maturation of iNKT cells in thymus. Using miR-150 knockout (KO) mice, we found that miR-150 deletion resulted in an interruption of iNKT cell final maturation in both thymus and periphery. Upon activation, iNKT cells from miR-150KO mice showed significantly increased IFN-γ production compared with wild-type iNKT cells. Bone marrow-transferring experiments demonstrated the cell-intrinsic characteristics of iNKT cell maturation and functional defects in mice lacking miR-150. Furthermore, miR-150 target c-Myb was significantly upregulated in miR-150KO iNKT cells, which potentially contribute to iNKT cell defects in miR-150KO mice. Our data define a specific role of miR-150 in the development and function of iNKT cells. The Journal of Immunology, 2012, 188: 2118–2126.

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Abbreviations used in this article: DN, double-negative; DP, double-positive; αGalCer, α-galactosylceramide; iNKT, invariant NKT; KO, knockout; miR-150, microRNA-150; miRNA, microRNA; WT, wild-type.

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mice. We found that miR-150 deletion impaired iNKT cell final maturation in both thymus and peripheral lymphoid organs, and that iNKT cells from miR-150KO mice showed increased cytokine production capacity. Furthermore, bone marrow transfer experiments indicated that the maturation and functional changes of iNKT cells in miR-150KO mice are intrinsic to iNKT cells. Together, our results define a specific role of miR-150 in the development and function of iNKT cells.

Materials and Methods

Mice

Conventional miR-150KO mice in C57BL/6 background and WT C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and bred in our animal facility. In this study, 6- to 10-wk-old age and sex-matched miR-150KO and WT mice were used. Handling of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee.

Genotyping

MiR-150KO mice were genotyped using the following PCR primer pairs: 5'-CAAGGACAGGAAGCCTCATGCA-3' and 5'-CCGTGACCGCTGG-AAGACATTTC-3'. The miR-150 deletion allele produced a 262-bp PCR product, whereas the wild-type allele resulted in a 866-bp product.

Flow cytometry

Single-cell suspensions were washed twice with staining buffer (PBS, 2% FCS, HEPES, penicillin and streptomycin, pyruvate, nonessential amino acids, FCS, HEPES, penicillin and streptomycin, pyruvate, nonessential amino acids) and incubated with Fc block (clone 2.4G2). Cells were stained with PBS57-CD1d tetramers, as described (31). The following conjugated mAbs were used: NK1.1 (PK136), TCR-β (H57-597), CD44 (IM7), CD122 (SH4), CD69 (H12F5), B220 (RA3-6B2), Ly49C (5E6), Ly49G2 (4D11), CD1d (1B1), CD5 (53-6.7), CD4 (RM4-5), CD25 (PC61, Foxp3 (FJK-16s), IL-4 (11B11), and IFN-γ (XM1.2). All mAbs were from BD Biosciences or eBioscience. Data were analyzed using CELLQuest Pro (BD Biosciences) or FlowJo software. Apoptosis assays were carried out by staining with annexin V (BD Biosciences), according to the manufacturer’s instructions. For iNKT cell proliferation in vivo, as revealed by staining with annexin V (BD Biosciences), according to the manufacturer’s instructions. The expression of miR-150 was examined using TaqMan real-time PCR. As shown in Fig. 1B, miR-150 expression increased gradually during the maturation of iNKT cells and reached the highest level at the final maturation stage 3 (CD44+NK1.1+), consistent with a previous report that miR-150 is highly expressed in conventional thymic and splenic T cells (Supplemental Fig. 1A). This is consistent with previous studies (31). iNKT cells from spleen have a relatively higher miR-150 expression level compared to thymus iNKT cells, and miR-150 is also highly expressed in conventional thymic and splenic T cells (Supplemental Fig. 1A). This is consistent with a previous report that miR-150 is highly expressed in mature and resting lymphocytes (29). Nevertheless, in contrast with the report showing downregulated miR-150 in B cells after activation (29), in vivo stimulation of iNKT cells with α-GalCer for 3 d resulted in a significantly increased miR-150 expression in spleen iNKT cells (Fig. 1C). Thus, the stage-specific expression pattern of miR-150 during iNKT cell development and activation suggests its potential involvement in iNKT cell development and function.

Defective iNKT cell development in miR-150KO mice

To assess the role of miR-150 in iNKT cell development, conventional miR-150KO mice were evaluated and compared with age- and sex-matched WT B6 mice. TaqMan real-time PCR analysis confirmed the substantial deletion of miR-150 in the thymus and spleen of miR-150KO mice compared with that of WT control mice (Supplemental Fig. 1B). To detect the possible in-
volvement of miR-150 in conventional T cell development, we compared the ratio and number of conventional T cell subsets in the thymus, including CD4+ and CD8+ single-positive, CD4+CD8+ DP, and CD4−CD8− DN T cells between miR-150 KO and WT mice. No substantial difference was identified (Supplemental Fig. 2A, 2B). Early T cell precursors, CD4−CD8− DN T cells, progress through the CD44+CD25− (DN1) and the CD44+CD25+ (DN2) stages to the CD44−CD25− (DN4) stage. To further dissect the early T cell development profile in miR-150KO mice, we gated DN thymocytes and analyzed their CD44 and CD25 expression profiles (Supplemental Fig. 2C). The miR-150KO mice showed a DN thymocyte developmental profile comparable to that of WT control mice, indicating that miR-150 deficiency did not affect conventional T cell development. In addition, we also found no significant changes of CD4+Foxp3+ regulatory T cell and γδ T cell ratios in the thymus of miR-150KO mice compared with that of WT control mice (Supplemental Fig. 2D, 2E). Interestingly, we found significant reduction of the percentage ($p = 0.001$) and cell number ($p = 0.04$) of thymus iNKT cells stained by anti–TCR-b and PBS57-CD1d tetramers in the miR-150KO mice compared with that of WT mice.

FIGURE 1. Expression of miR-150 is upregulated during the development and activation of iNKT cells. (A) Different developmental stages (CD44−NK1.1−, CD44+NK1.1−, and CD44+NK1.1+) of thymic iNKT cells were sorted from C57BL/6 mice. (B) TaqMan real-time PCR analysis of miR-150 transcript in CD44−NK1.1−, CD44+NK1.1−, and CD44+NK1.1+ iNKT cells from the thymuses of C57BL/6 mice. Results were the mean of triplicate and normalized to a control gene (snoRNU 202). Error bars are SD. *$p < 0.05$, **$p < 0.01$. Results are representatives of three independent experiments. (C) C57BL/6 mice were stimulated in vivo with α-GalCer for 3 d, spleen iNKT cells were sorted, and RNA was extracted. MiR-150 expression was detected by TaqMan real-time PCR. Results were the mean of triplicate and normalized to a control gene (snoRNU 202). Error bars are SD. Results are representatives of three independent experiments.

FIGURE 2. Defective iNKT cell development in miR-150KO mice. (A) Representative dot plots of thymus iNKT cells stained by anti–TCR-β Ab and CD1d tetramer from miR-150KO and WT mice. (B and C) The frequency (B) and number (C) of TCR-β+CD1d-tetramer+ iNKT cells in the thymus of miR-150KO and WT mice. Each point represents one individual mouse, and the mean values are indicated by the middle horizontal lines from three to five independent experiments (3–5 mice/experiment). (D) The representative staining of iNKT cells (gated on B220-negative cells) in lymph nodes, spleen, bone marrow, and liver from miR-150KO and WT mice. (E and F) The frequency (E) and number (F) of iNKT cells in the lymph nodes, spleen, bone marrow, and liver of miR-150KO and WT mice. Each point represents one individual mouse, and the mean values are indicated by middle horizontal lines from three to five independent experiments (3–5 mice/experiment). Statistical analysis was performed with Prism 5.0 (GraphPad Software). Differences were considered statistically significant when values of $p < 0.05$. 
with that in WT mice (Fig. 2A–C). The iNKT cells from spleen, lymph nodes, bone marrow, and liver were further analyzed. As shown in Fig. 2D–F, the frequencies and number of iNKT cells in the spleen, bone marrow, and liver from miR-150KO mice were comparable with that from WT control mice. However, significantly increased frequency \( (p = 0.028) \) and number \( (p = 0.035) \) of iNKT cells were observed in the lymph nodes from miR-150KO mice compared with that from WT control mice. Thus, deficiency of miR-150 causes an impairment of development in thymus iNKT cells, but not in conventional T cells, CD4+Foxp3+ regulatory T cells, and γδ T cells.

**Defective iNKT cell maturation in miR-150KO mice**

Accumulated studies have indicated that iNKT cells undergo a series of maturation stages in the thymus immediately after positive selection, based on their CD44 and NK1.1 expressions. Given that iNKT cells exhibit a stage-specific miR-150 expression pattern, the CD44/NK1.1 profile of CD1d-tetramer+ thymocytes was analyzed to test the potential role of miR-150 in iNKT cell maturation. We found that the frequency of mature NK1.1+CD44+ iNKT cells decreased significantly \( (p = 0.004) \), whereas the semimature NK1.1−CD44+ stage 2 iNKT cells increased significantly \( (p = 0.019) \), but the early immature NK1.1−CD44− iNKT cells remain unchanged in miR-150KO mice compared with WT mice (Fig. 3A, 3B). Furthermore, the number of mature NK1.1+CD44+ iNKT cells decreased significantly \( (p = 0.006) \) in miR-150KO mice compared with that of WT mice, whereas the number of NK1.1−CD44− and NK1.1−CD44+ iNKT cells was comparable between miR-150KO and WT control mice (Fig. 3C).

The development and maturation of iNKT cells are also accompanied by the upregulation of several other cell surface receptors, such as CD122, CD69, and Ly-49 in addition to NK1.1, and the final steps of iNKT cell development also need the transcription factor T-bet and cytokine signaling initiated by IL-15 (8, 33, 34). To further dissect the developmental impairment and the potential molecular mechanisms involved in iNKT cell development in miR-150KO mice, the expression levels of these molecules in iNKT cells were evaluated and compared between miR-150KO and WT control mice. As shown in Fig. 3D and 3E, the frequency of CD122-positive iNKT cells was significantly decreased in miR-150KO mice compared with WT control mice \( (p = 0.005) \), consistent with the defective iNKT cell maturation based on NK1.1 expression.
expression. Interestingly, the expressions of CD69, Ly49C, and T-bet in thymus iNKT cells were comparable between miR-150KO and WT control mice. Thus, miR-150KO mice have a partial defect in iNKT cell maturation at the late stage, suggesting that miR-150 regulates thymus iNKT cell development and maturation.

Immature iNKT cells also leave the thymus and are matured in the peripheral immune organs. Using NK1.1 as a peripheral iNKT cell maturation marker, peripheral iNKT cell maturation status was further evaluated in miR-150KO mice. As shown in Fig. 3F and 3G, iNKT cell maturation was slightly, but significantly interrupted in spleen and lymph nodes in miR-150KO mice (p = 0.021 and 0.046, respectively). Together, our data suggest that miR-150 also regulates peripheral iNKT cell maturation.

**MiR-150 contributes to thymus iNKT cell survival during iNKT cell development**

CD1d expressed in CD4+CD8+ DP thymocyte controls iNKT selection and development, and mice lacking CD1d have severe defects in iNKT development (35). To ascertain that the impaired iNKT cell development was not due to defective CD1d expression, thymocytes from miR-150KO and WT control mice were compared for CD1d expressions. As shown in Fig. 4A, CD1d expressions were found to be almost equivalent between miR-150KO and WT mice, ruling out the possibility of CD1d deficiency as a cause of the thymic iNKT developmental defects in miR-150KO mice.

To further dissect the mechanisms involved in thymus iNKT cell homeostasis, proliferation and apoptosis of thymus iNKT cells were evaluated in miR-150KO mice. To examine the former possibility, we injected miR-150KO and WT mice with BrdU and determined the rate of iNKT cell proliferation in the thymus. As shown in Fig. 4B and 4C, thymus iNKT cells from miR-150KO mice incorporated BrdU at a comparable rate compared with WT control mice. Thus, the disparity between the comparable proliferation observed and the overall decreased numbers of iNKT cells in the thymus suggested that increased cell death might be occurring in iNKT cells from miR-150KO mice. Therefore, fresh thymocytes from WT and miR-150KO mice were stained with the apoptotic marker annexin V to assess levels of cell apoptosis. As shown in Fig. 4D and 4E, miR-150KO mice showed a higher frequency of annexin V-positive iNKT cells than that of WT control mice (p = 0.031). Collectively, these data suggest that miR-150 regulates thymus iNKT cell survival, and may not be required for iNKT cell selection and proliferation.

**Increased α-GalCer-mediated iNKT cell IFN-γ production in miR-150KO mice**

The prompt production of large amounts of cytokines in response to TCR signaling is a unique feature of iNKT cells, which is closely related to their potent immune regulatory functions (36). To investigate the role of miR-150 in iNKT cell function, we injected mice with α-GalCer and evaluated the production of cytokines by iNKT cells with intracellular cytokine staining. As shown in Fig. 5A and 5B, 2 h after in vivo α-GalCer treatment, more spleen iNKT cells from miR-150KO mice produced IL-4 and IFN-γ compared with that from WT control mice, especially for IFN-γ-producing NKT cells (p = 0.0004), suggesting the increased iNKT cell responsiveness to α-GalCer stimulation in the miR-150KO mice. However, CD69 expression in iNKT cells from miR-150 KO and WT mice was comparable. Interestingly, when spleen iNKT cells were stimulated in vitro with PMA and ionomycin, which bypass proximal TCR-mediated signaling events, comparable numbers of both IFN-γ- and IL-4–producing iNKT cells were detected in miR-150KO and WT control mice (Fig. 5C, 5D). These results suggest that miR-150 may regulate iNKT cell cytokine production, possibly through targeting the upstream TCR-mediated signaling molecular pathways.

**MiR-150 deficiency affects iNKT cell maturation and function via a cell-autonomous mechanism**

Accumulated studies suggest that iNKT cell development and function are regulated by both the bone marrow and micro-environments. To determine whether the altered iNKT cell development and cytokine secretion in miR-150KO mice are cell intrinsic or related to the defects in their environment, we used a mixed bone marrow transfer model, in which both WT and miR-150KO iNKT precursors develop in the same WT microenvironment. B6.SJL (CD45.1+) mice were lethally irradiated and reconstituted with a 1:1 mixture of bone marrow cells from the age- and sex-matched B6.SJL and miR-150KO mice (CD45.2+). iNKT cell development, maturation, and cytokine production capacity were analyzed after 8 wk postreconstitution. As shown in Fig. 6A, the bone marrow from miR-150KO mice reconstituted iNKT cells comparably to that from bone marrow of B6.SJL mice. However, significantly decreased mature CD44+NK1.1+ and increased immature CD44+NK1.1+ iNKT cell frequency (p = 0.011) appeared in miR-150KO bone marrow-derived iNKT cells compared with that from B6.SJL mice (Fig. 6B, 6C). In addition, miR-150KO bone marrow-derived iNKT cells showed significantly reduced CD122 expression compared with that from B6.SJL mice (p = 0.024) (Fig. 6D, 6E). Furthermore, as shown in Fig. 6F, more IFN-γ– and IL-4–producing iNKT cells were observed in miR-150KO bone marrow-derived iNKT cells compared with that from B6.SJL bone marrow after in vivo α-GalCer stimulation. Thus, the iNKT cell phenotypes in the bone marrow chimeras, including interrupted iNKT cell final stage maturation, reduced CD122 expression, and elevated α-GalCer–induced cytokine production, are consistent with the iNKT phenotypes identi-
fied in miR-150 KO mice. These observations therefore indicate the cell-intrinsic nature of the maturation and functional changes of iNKT cells in mice lacking miR-150.

Upregulation of c-Myb expression in the thymus iNKT cells of miR-150KO mice

A top predicted target of miR-150 is c-Myb, a transcription factor controlling lymphocyte differentiation and proliferation. Combining loss- and gain-of-function gene-targeting approaches for miR-150 with conditional and partial ablation of c-Myb, Xiao et al. (30) have recently confirmed that miR-150 indeed controls c-Myb expression in vivo, through which B cell development and differentiation are regulated. The c-Myb is highly expressed in lymphocyte progenitors and downregulated upon maturation, and ectopic expression of c-Myb blocked the differentiation of hematopoietic cells. Thus, the appropriate levels of c-Myb are strictly defined at distinct differentiation steps of each hematopoietic cell lineage (30, 37, 38). To investigate the potential involvement of c-Myb in miR-150-mediated iNKT cell regulation, we first evaluated c-Myb expression in thymus immature (stages 1 and 2) and mature (stage 3) iNKT cells by a TaqMan real-time RT-PCR. As shown in Fig. 7, consistent with the observation in other lymphocytes, c-Myb is also highly expressed in immature iNKT cells and downregulated in mature iNKT cells. Thus, during iNKT cell development in thymus, the dynamic expression pattern of c-Myb is in mirror contrast with that of miR-150 (Figs. 1A, 7), which may be required for iNKT cell maturation. Furthermore, c-Myb expression was significantly upregulated in both immature (stages 1 and 2) and mature (stage 3) iNKT cells from miR-150KO mice compared with that in WT mice (Fig. 7). Thus, these data indicate that c-Myb may serve as one of the potential targets in miR-150-mediated late-stage iNKT cell development defect in the thymus.

Discussion

iNKT cells are a separate lineage of T lymphocyte that goes through a distinct developmental pathway controlled by a unique gene expression pattern (10). In this study, we demonstrate an important role for miR-150 in directing the maturation and function of iNKT cells. Absence of miR-150 results in an impairment of iNKT cell maturation in both thymus and peripheral lymphoid organs and thymus iNKT cell homeostasis, but upregulates IFN-γ cytokine production capacity. The maturation and functional changes of iNKT cells in miR-150KO mice are iNKT cell intrinsic. As miR-150 deletion resulted in upregulated c-Myb expression in both immature and mature thymus iNKT cells, c-Myb may be a major target through which miR-150 regulates NKT cell development, maturation, and function. During the submission, Lanier and colleagues (32) reported that miR-150 regulates the development of NK and NKT cells. In line with their findings, we also found the reduced frequency of NK cells in thymus and spleen from miR-150KO mice (data not shown), indicating that appropriate miR-150 expression is necessary for both NK and iNKT cell development. Furthermore, using a mixed bone marrow-transferring chimera model, they also found that the frequency of thymic miR-150KO iNKT cells from WT: miR150KO chimera was mildly decreased (1.5 ± 0.2-fold compared with WT iNKT cells), and that loss of miR-150 in
InNKT cells revealed a mild reduction in the population of thymic mature NK1.1⁺ iNKT cells. However, the reductions they detected were not statistically significant. One possible reason is that in their transferring experiments, four to five mice per group may not have enough power to detect a difference for this mild reduction, compared with the almost complete blocking of early iNKT cell development in the mice with miR-150 overexpression. Compared with iNKT cells in bone marrow- or thymus-specific total miRNA deletion mouse models (26–28), miR-150 deletion alone is unlikely accountable for the profound interruption of iNKT cell development, maturation, and function identified in those mice. This may suggest the potential involvement of multiple miRNAs or groups of miRNAs in iNKT cell developmental and functional regulation, which is also supported by our recent miRNA expression profiles of iNKT cells (Q.H. Zheng, L. Zhou, and Q.S. Mi, unpublished observations).

INKT cells differentiate from DP conventional T cells and develop in thymus, with the final maturation step (NK1.1 expression) occurring in both the thymus and the periphery (1). Numerous transcription factors, cytokines, and signaling molecules have been identified as unique requirements for iNKT cell maturation once commitment to the NKT lineage has been confirmed. In particular, transcription factor T-bet is essential for final iNKT cell maturation (10, 34). However, a difference in T-bet expression was not identified in the iNKT cells of miR-150KO mice compared with that of WT control mice, indicating that defective iNKT cell maturation in miR-150KO mice is not T-bet dependent. NF-κB, IL-15, IL-15Rb (CD122), and IL-2Rb also play an essential role in the maturation and overall population size of iNKT cells (39–41). CD122 is upregulated between stages 2 and 3 of thymus iNKT development. Whether the immature iNKT cell phenotype in miR-150KO mice is induced by a failure to upregulate CD122 or a developmental blockage needs to be further identified. CD122 is involved in inducing expression of anti-apoptotic proteins Bcl-2 and Bcl-xL and is important for NKT cell homeostasis (42). We have found that thymus iNKT cells from miR-150KO mice showed increased apoptosis compared with that from WT controls, which could result in a decreased iNKT cell ratio and number in miR-150KO mice.

Spleen and lymph node iNKT cells from miR-150KO mice showed a decrease in NK1.1 expression akin to the defective maturation of thymus iNKT. Consistent with previous studies showing that immature CD44⁺NK1.1⁻ iNKT cells preferentially accumulate in the lymph nodes (43, 44), lymph nodes of miR-150KO mice have increased iNKT cell frequency compared with that in WT control mice. As thymus and peripheral microenvironments differentially mediated development and maturation of iNKT cells, these results suggest that miR-150 is involved in both thymus and peripheral iNKT cell maturation. Given that iNKT cell maturation defects appear to be cell intrinsic, as demonstrated by our bone marrow chimera experiment (Fig. 6), the
differential iNKT cell maturation phenotype in individual peripheral lymphoid organs may come from different migration capacities of iNKT cells toward individual peripheral lymphoid organs.

iNKT cells rapidly secrete cytokines upon TCR stimulation, thus modulating acquired immune responses. In this study, we show that in addition to a role in iNKT cell maturation, miR-150 indeed negatively regulates iNKT cell production of IFN-γ and IL-4, for a lesser extent, IL-4 upon TCR stimulation. It is well known that immature CD44+NK1.1+ iNKT cells produce more IL-4, whereas mature CD44+NK1.1+ iNKT cells produce more IFN-γ (8). Considering that the lower ratio of mature CD44+NK1.1+ iNKT cells present in the miR-150KO mice, the increased IFN-γ production of iNKT cells in miR-150KO mice cannot be explained by the defective iNKT cell maturation. Other mechanisms must be involved in miR-150 regulating iNKT cell IFN-γ production. Several transcription factors known to regulate cytokine gene transcription, such as T-bet, GATA-3, and NF-κB, have been implicated in iNKT cells (34, 39, 44, 45). In addition, epigenetic regulation of IL-4 and IFN-γ expression is reported to occur during iNKT cell development (46). It was known that Notch signaling regulates IL-4 expression, whereas GM-CSF signaling controls IL-4 and IFN-γ secretion without disturbing the transcription and translation of these two cytokines (47, 48). Previous studies showed that c-Myb mRNA is induced upon B and T cell activation, whereas miR-150 expression was reduced upon B cell activation, suggesting the involvement of miR-150 and its target, c-Myb, in T and B cell activation and function (29, 49, 50). In contrast with these results identified in T and B cells, however, iNKT cells stimulated with α-GalCer for 3 d showed increased miR-150 expression (Fig. 1C), suggesting the potentially discrete mechanisms that exist in miR-150–mediated activation and functional regulation of iNKT cells versus T and B cells. The increased iNKT cytokine production upon α-GalCer stimulation instead of PMA/ionomycin stimulation in miR-150KO mice suggests that miR-150 controls IFN-γ production through targeting proximal components of TCR signaling pathway. The detailed mechanisms of miR-150–mediated iNKT cell activation and cytokine production remain to be defined.

The transcription factor c-Myb is expressed in hematopoietic stem cells and progenitors of all hematopoietic lineages and is required for differentiation along individual hematopoietic cell lineages. In the thymus, DN and DP thymocyte subsets have the highest expression of c-Myb, and its expression decreases after positive selection (51, 52). Conditional deletion of c-Myb at various stages of T cell development has suggested that c-Myb influences the DN to DP transition, the survival of DP thymocytes, and the differentiation of CD4+ thymocytes (51). Alberola-Ila and colleagues (21) recently identified the central role of c-Myb in priming DP thymocytes to enter the iNKT lineage by simultaneously regulating CD1d expression, the t1/2 of DP cells, and expression of SLAMF1, SLAMF6, and SAP. MiR-150 is specifically expressed in mature lymphocytes, but not their progenitors, which is opposite to the pattern of c-Myb expression. Combining loss- and gain-of-function gene-targeting approaches for miR-150, studies from Rajewsky and colleagues (30) showed miR-150 controls B cell differentiation through targeting c-Myb. Consistent with upregulated c-Myb expression in early iNKT cells from miR-150KO mice (Fig. 7), we do not expect the abnormal iNKT cell selection and early development in miR-150KO mice. Most interestingly, Lanier and colleagues (32) recently reported that overexpression of miR-150 dramatically reduced iNKT cell number and blocked iNKT cell development in the early stage, which is related to downregulated expression of c-Myb, further indicating the importance of downregulation of miR-150 and upregulation of c-Myb for the early development of iNKT cells.

In contrast, constitutive expression of c-Myb has been reported to block erythrocyte maturation (53, 54). A more recent study using a dose- and timing-controlled gene expression system showed overexpression of c-Myb; however, it prevented the terminal differentiation of erythrocytes and megakaryocytes (37), indicating that appropriate levels of c-Myb protein are strictly defined at distinct development and differentiation steps of each hematopoietic cell lineage. In agreement with this notion, our study showed that miR-150 deletion, resulting in upregulated c-Myb expression level, mediated defective iNKT cell final maturation and changed function. This suggests the target role of c-Myb for miR-150 in iNKT cells and the necessity of miR-150–mediated c-Myb downregulation in the terminal maturation of iNKT cells. Therefore, the expression of the correct level at the appropriate time during differentiation seems to be important for c-Myb to regulate development and maturation of iNKT cells, in which miR-150 may be a critical player. However, we cannot exclude that other miR-150 potential target genes may also contribute to the iNKT cell phenotypes observed in miR-150 KO mice. For examples, miR-150 was demonstrated to target the transcription factor early growth response–2 and the purinergic P2X7 receptor, which has been suggested in controlling iNKT cell proliferation, apoptosis, and activation (55–58). Therefore, more credible evidence is needed to further confirm the target role of c-Myb in miR-150–mediated iNKT cell regulation.

In conclusion, we demonstrated a role for miR-150 in regulating the maturation and function of iNKT cells. Absence of miR-150 results in a moderate interruption of iNKT cell maturation in both thymus and peripheral lymphoid organs and thymus iNKT cell homeostasis, and upregulates their IFN-γ production capacity. MiR-150–mediated iNKT maturation and function are cell intrinsic. Given the opposite expression levels of miR-150 and c-Myb in iNKT cells during their development and the critical role of miR-150 and c-Myb in iNKT cell development and maturation, it is likely that the dynamic expression of miR-150 is required for normal NKT cell development through controlling the proper levels of c-Myb expression.

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Disclosures
The authors have no financial conflicts of interest.

References


Supplemental figure 1. Substantial deletion of miR-150 expression in miR-150KO mice. (A) Real-time PCR detection of miR-150 expression in iNKT and conventional T cells in the thymus and spleen of C57BL/6 mice. Results were the average measured in triplicate and normalized to a control gene (snoRNU 202). Error bars are SD. Results are representative of three independent experiments (B) Real-time PCR detection of miR-150 expression in the total thymocytes and spleen T cells of WT control and miR-150KO mice. Results were the average measured in duplicate and normalized to a control gene (snoRNU 202). Results are representative of two independent experiments.
Supplemental figure 2. Normal developments of conventional T cells, Treg cells and γδ T cells in miR-150 knock mice (A) Flow cytometry analysis of thymocyte profile from the WT control and miR-150KO mice (left), and percentage of each cell subset are shown (right). (B) Total cell number of thymocytes from WT and miR-150KO mice. (C) Dot plots depict CD44 (y axis) versus CD25 (x axis) staining in gated CD4−CD8− DN thymocytes (left), and percentage of CD44+CD25−(DN1), CD44+CD25+(DN2), CD44−CD25+(DN3) and CD44−CD25−(DN4) stage of thymocytes from WT control and miR-150KO mice are shown (right). Results are representative of 3-5 independent experiments (3-5 mice/experiment). (D) Flow cytometry analysis of thymus CD4+foxp3+T reg cells from WT control and miR-150KO mice. Results are representative of 3 independent experiments (3-5 mice/experiment). (E) Flow cytometry analysis of thymus γδ t cells from WT control and miR-150KO mice, Data are representative of 3 experiments (3-5 mice/experiments).