

# miR-150 regulates the development of NK and iNKT cells

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**Natural killer (NK) and invariant NK T (iNKT) cells are critical in host defense against pathogens and for the initiation of adaptive immune responses. miRNAs play important roles in NK and iNKT cell development, maturation, and function, but the roles of specific miRNAs are unclear. We show that modulation of miR-150 expression levels has a differential effect on NK and iNKT cell development. Mice with a targeted deletion of miR-150 have an impaired, cell lineage-intrinsic defect in their ability to generate mature NK cells. Conversely, a gain-of-function miR-150 transgene promotes the development of NK cells, which display a more mature phenotype and are more responsive to activation. In contrast, overexpression of miR-150 results in a substantial reduction of iNKT cells in the thymus and in the peripheral lymphoid organs. The transcription factor c-Myb has been shown to be a direct target of miR-150. Our finding of increased NK cell and decreased iNKT cell frequencies in *Myb* heterozygous bone marrow chimeras suggests that miR-150 differentially controls the development of NK and iNKT cell lineages by targeting c-Myb.**

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Abbreviations used: iNK cell, immature NK cell; iNKT cell, invariant NK T cell; MFI, mean fluorescence intensity; miRNA, microRNA; NKP, NK cell precursor; qRT-PCR, quantitative RT-PCR.

The innate immune system provides early host defense against viruses, bacteria, and tumor cells. Two of its cell lineages, NK and invariant NK T (iNKT) cells, do not require prior sensitization for effector function and play critical roles in both initially fighting infection and subsequently activating the adaptive immune response.

NK cells develop in the BM and share a common lymphoid progenitor with T and B cells (Kondo et al., 1997). The committed NK cell precursors (NKPs) express the shared IL-2 and IL-15 receptor common  $\beta$  subunit (CD122; Rosmaraki et al., 2001). NK cell development is critically dependent on IL-15, as mice lacking IL-15 or any component of its receptor or signaling have a block in NK cell differentiation in the BM (DiSanto et al., 1995; Suzuki et al., 1997; Kennedy et al., 2000; Gilmour et al., 2001; Vosshenrich et al., 2005). As NK cells mature, they sequentially acquire the cell surface receptors NK1.1 (in C57BL/6 mice), CD94-NKG2A, Ly49 receptors, and the  $\alpha 2$ -integrin CD49b (DX5; Hesslein and Lanier, 2011). Terminal maturation is accompanied by loss of CD27 expression and up-regulation of CD11b expression, together with optimal cytolytic

function and IFN- $\gamma$  production. NK cell specificity is fine-tuned by stochastic expression of activating and inhibitory receptors of the Ly49 family. Some activating Ly49 members can detect specific virus-encoded products (e.g., Ly49H recognizes the mouse cytomegalovirus m157 glycoprotein; Arase et al., 2002; Smith et al., 2002). Individual inhibitory Ly49 members recognize distinct MHC class I allotypes (e.g., Ly49C recognizes H2<sup>b</sup> in C57BL/6 mice) and are important in self-recognition, whereas other inhibitory Ly49 genes produce receptors that do not recognize the autologous MHC class I (e.g., Ly49A in C57BL/6 mice; Orr and Lanier, 2011).

NKT cells share properties with both NK cells and T cells in that they express receptors of the NK cell lineage, including NK1.1 and members of the Ly49 family, along with a TCR. The majority of NKT cells express an invariant TCR $\alpha$  (V $\alpha$ 14J $\alpha$ 18 in mice) that pairs with a

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limited repertoire of TCR $\beta$  chains (V $\beta$ 8, V $\beta$ 7, or V $\beta$ 2 in mice; Godfrey et al., 2010). These NKT cells, also referred to as iNKT cells, can be identified using CD1d tetramers loaded with the glycosphingolipid antigen  $\alpha$ -galactosylceramide (Matsuda et al., 2000). iNKT cells develop from CD4<sup>+</sup>CD8<sup>+</sup> double-positive V $\alpha$ 14J $\alpha$ 18 TCR<sup>+</sup> thymic precursors (Godfrey et al., 2010). After positive selection, iNKT-cell precursors down-regulate their expression of CD24 (HSA), and transition through several maturation stages accompanied by the expression of NK1.1, CD44, and CD122. After CD122 up-regulation, iNKT cells proliferate in response to IL-15 and migrate from the thymus to the periphery, where they are most abundant in the spleen, liver, and BM.

Although much has been learned about the transcriptional regulation of NK and iNKT lineage development (Godfrey et al., 2010; Hesslein and Lanier, 2011), less is known about the posttranscriptional mechanisms that regulate NK and iNKT cells. microRNAs (miRNAs) are noncoding RNAs, expressed from endogenous genes, which act on protein-encoding mRNAs, targeting them for translational repression or degradation (Bartel, 2004). Many miRNAs are expressed in a stage- and cell-specific fashion in the hematopoietic system (Kuchen et al., 2010), and emerging evidence suggests that they regulate lymphocyte differentiation and function (Muljo et al., 2005; Cobb et al., 2006; Koralov et al., 2008; X. Zhou et al., 2008; Liston et al., 2008; Zhou et al., 2009; Fedeli et al., 2009). Deletion of the miRNA-processing enzymes Dicer or Dgcr8 leads to defects in NK cell activation, survival, and function during mouse cytomegalovirus infection (Bezman et al., 2010). In addition, loss of miRNAs in the BM or thymus leads to impaired iNKT cell development and function (Fedeli et al., 2009; Zhou et al., 2009; Seo et al., 2010). These results, and the description of a subset of miRNAs differentially expressed in NK and iNKT versus other hematopoietic cells (Fedeli et al., 2009; Bezman et al., 2010; Kuchen et al., 2010), raise a question as to how individual miRNAs present in high amounts in these cell types contribute to specific aspects of their biology.

miR-150 is expressed in both mature NK and iNKT cells (Fedeli et al., 2009; Bezman et al., 2010; Fehniger et al., 2010) and, as in B and T lymphocytes, miR-150 is up-regulated during cellular maturation. Constitutive expression of miR-150 blocks B cell development at the transition from the pro-B to pre-B cell developmental stage (Zhou et al., 2007). miR-150-deficient mice have an accumulation of B-1 B cells in the spleen and peritoneal cavity (Xiao et al., 2007). In nonlymphoid lineages, where it drives megakaryocyte differentiation at the expense of erythropoiesis, miR-150 regulates the cell fate of megakaryocyte-erythroid progenitors (Lu et al., 2008). The transcription factor c-Myb has been shown to be a direct and functional target of miR-150 (Xiao et al., 2007; Lu et al., 2008). In this study, we show that miR-150 differentially affects the development of NK and iNKT cell lineages. These results show a critical developmental role for miR-150 in the innate immune system and suggest that there are distinct molecular pathways during the maturation stages of NK and iNKT cells.

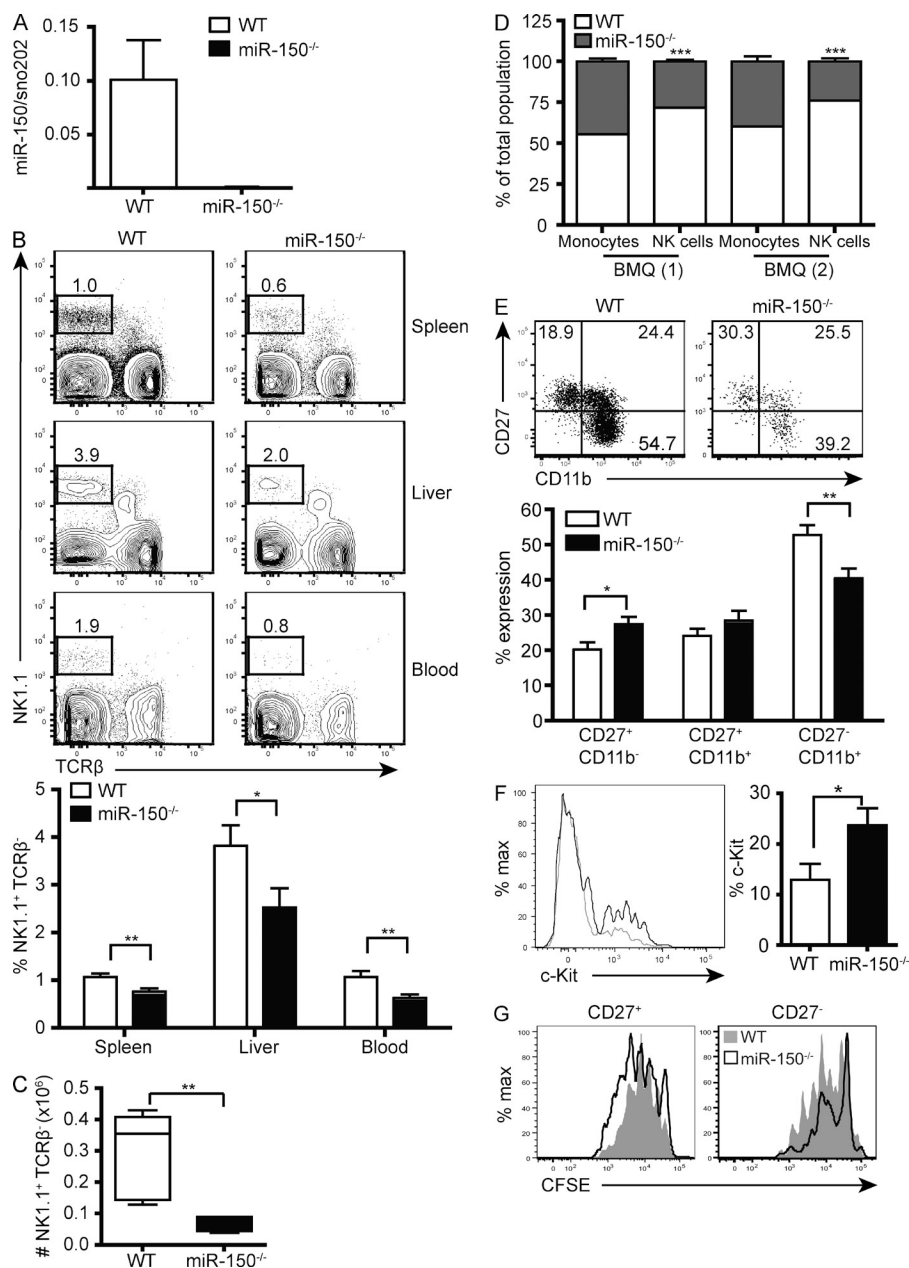
## RESULTS

### Mice lacking miR-150 have cell-intrinsic defects in the development and maturation of NK cells

To define the role for miR-150 in NK cells, we generated mixed BM chimeras by reconstituting lethally irradiated mice with BM from WT (CD45.1<sup>+</sup>) and miR-150<sup>-/-</sup> (CD45.2<sup>+</sup>) mice (Xiao et al., 2007). Analysis of the resulting chimeric animals (WT:miR-150<sup>-/-</sup>) allowed us to study the cell-intrinsic effects of miR-150 deficiency on NK cells. We confirmed the absence of miR-150 in CD45.2<sup>+</sup> NK1.1<sup>+</sup> TCR $\beta$ <sup>-</sup> NK cells (Fig. 1 A). Flow cytometric analyses of WT and miR-150<sup>-/-</sup> compartments showed a significant reduction in the percentage of miR-150<sup>-/-</sup> NK cells in the spleen (WT, 1.1  $\pm$  0.1% vs. miR-150<sup>-/-</sup>, 0.8  $\pm$  0.1%), liver (WT, 3.6  $\pm$  0.5% vs. miR-150<sup>-/-</sup>, 2.5  $\pm$  0.4%), and blood (WT, 1.1  $\pm$  0.1% vs. miR-150<sup>-/-</sup>, 0.6  $\pm$  0.1%; Fig. 1 B). Concurrently, the absolute numbers of miR-150<sup>-/-</sup> NK cells were also reduced compared with that of WT NK cells in the spleen of WT:miR-150<sup>-/-</sup> chimeras (WT, 0.30  $\times$  10<sup>6</sup>  $\pm$  0.05  $\times$  10<sup>6</sup> vs. miR-150<sup>-/-</sup>, 0.10  $\times$  10<sup>6</sup>  $\pm$  0.01  $\times$  10<sup>6</sup>; Fig. 1 C). The frequency of miR-150<sup>-/-</sup> monocytes was similar to that of WT monocytes in peripheral blood (WT, 7.3  $\pm$  0.2% vs. miR-150<sup>-/-</sup>, 7.4  $\pm$  0.4%; P = 0.8; n = 10), and was even elevated in spleen of WT:miR-150<sup>-/-</sup> chimeras (WT, 2.9  $\pm$  0.3% vs. miR-150<sup>-/-</sup>, 5.0  $\pm$  0.6%; P = 0.008; n = 11). Furthermore, we observed no significant differences between WT and miR-150<sup>-/-</sup> donor cells in the monocyte compartment, whereas miR-150<sup>-/-</sup> NK cells were underrepresented (Fig. 1 D). Together, these results suggest that the reduced frequency of miR-150<sup>-/-</sup> NK cells is not caused by a competitive disadvantage of miR-150<sup>-/-</sup> hematopoietic stem cells to reconstitute the host.

The maturation status of peripheral NK cells can be classified based on their expression of CD27 and CD11b, following the pathway of CD27<sup>+</sup> CD11b<sup>-</sup>  $\rightarrow$  CD27<sup>+</sup> CD11b<sup>+</sup>  $\rightarrow$  CD27<sup>-</sup> CD11b<sup>+</sup> (Chiossone et al., 2009). A significant accumulation of more immature CD27<sup>+</sup> CD11b<sup>-</sup> and a corresponding reduction of mature CD27<sup>-</sup> CD11b<sup>+</sup> subsets was observed among miR-150<sup>-/-</sup> NK cells in chimeric mice (Fig. 1 E). Similarly, miR-150<sup>-/-</sup> NK cells showed increased expression of c-Kit, a receptor that is expressed on developing BM and immature splenic NK cells but is down-regulated upon maturation (Fig. 1 F; Di Santo, 2006; Chiossone et al., 2009).

To determine whether the reduction of miR-150<sup>-/-</sup> NK cells was the result of increased cell death, increased expansion of immature NK cells, or both, we stained freshly isolated cells with Annexin V and a viability dye to assess apoptosis. There was no significant increase in apoptotic miR-150<sup>-/-</sup> compared with WT NK cells (unpublished data). The rate of NK cell turnover at steady state (measured by BrdU incorporation during a 3-d labeling period) was also not different between WT and miR-150<sup>-/-</sup> splenic NK cells (unpublished data). However, when WT and miR-150<sup>-/-</sup> NK cells were co-transferred into a lymphopenic environment (*Rag2*<sup>-/-</sup>  $\times$  *Il2rg*<sup>-/-</sup> mice) for 3 d, immature CD27<sup>+</sup> miR-150<sup>-/-</sup>

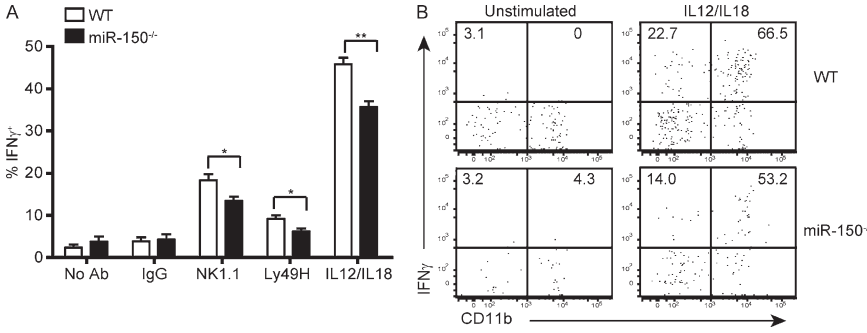


**Figure 1. Loss of miR-150 results in reduction and impaired maturation of NK cells.** Mixed BM chimeras were generated by injecting WT and miR-150<sup>-/-</sup> BM cells into irradiated hosts, which were analyzed 8–14 wk later. All stains contained  $\alpha$ CD45.1 and  $\alpha$ CD45.2 to distinguish between WT (CD45.1<sup>+</sup>) and miR-150<sup>-/-</sup> (CD45.2<sup>+</sup>) populations. Three independent sets of WT:miR-150<sup>-/-</sup> chimeras were made. (A) RNA was isolated from CD45.1<sup>+</sup> NK1.1<sup>+</sup> TCRβ<sup>-</sup> and CD45.2<sup>+</sup> NK1.1<sup>+</sup> TCRβ<sup>-</sup> cells sorted from five chimeras. miR-150 level was quantified by qRT-PCR. Sno202 was used as normalization control. Data are presented as mean  $\pm$  SEM ( $n = 3$ ). (B and C) Cells were isolated from the indicated tissues and stained for NK1.1 and TCRβ. (B) The frequencies of NK cells are shown as a percentage of WT and miR-150<sup>-/-</sup> lymphocytes. Bar graphs represent mean percentages  $\pm$  SEM of data ( $n = 11$ ). (C) Absolute numbers of WT and miR-150<sup>-/-</sup> NK cells  $\pm$  SEM ( $n = 6$ ). (D) Contribution of WT and miR-150<sup>-/-</sup> cells to monocyte (CD11b<sup>+</sup> NK1.1<sup>-</sup>) and NK cell (NK1.1<sup>+</sup> TCRβ<sup>-</sup>) compartments in peripheral blood of WT:miR-150<sup>-/-</sup> chimeras. Data are from two independent chimeras experiments (BMQ [1] and BMQ [2]) with nine mice per experiment. (E and F) NK cell maturation was determined by staining splenocytes with NK1.1, TCRβ, CD11b, CD27, and c-Kit antibodies. Gated NK1.1<sup>+</sup> TCRβ<sup>-</sup> cells are shown. Bar graph represents mean percentage  $\pm$  SEM of data ( $n = 11$  [E] and  $n = 8$  [F]). (G) Equal numbers (10<sup>6</sup>) of WT (CD45.1<sup>+</sup>) and miR-150<sup>-/-</sup> (CD45.2<sup>+</sup>) NK cells were labeled with CFSE and adoptively transferred into *Rag2*<sup>-/-</sup> *Il2rg*<sup>-/-</sup> mice. At 3 d after transfer, splenocytes were isolated and stained for NK1.1, TCRβ, CD45.2, CD45.1, and CD27. Histograms show CFSE dilution by CD27<sup>+</sup> (left) and CD27<sup>-</sup> (right) WT and miR-150<sup>-/-</sup> NK cells. (B, C, E, and F) \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (D) \*\*\*,  $P < 0.001$  (paired Student's *t* test). Data shown are from two (A, D, and G) and at least five (B, C, E, and F) experiments.

NK cells proliferated more than CD27<sup>+</sup> WT NK cells, whereas mature CD27<sup>-</sup> miR-150<sup>-/-</sup> NK cells proliferated less than their WT counterparts (Fig. 1 G). Thus, the immature phenotype of peripheral miR-150<sup>-/-</sup> NK cells might be because miR-150<sup>-/-</sup> NK cells spend more time at the immature stage, and CD27<sup>-</sup> miR-150<sup>-/-</sup> NK cells have reduced proliferative capacity compared with WT NK cells.

To determine whether the function of NK cells is affected by miR-150 deficiency, freshly isolated splenic NK cells were stimulated either by cross-linking cell surface receptors, including NK1.1 and Ly49H, or by a combination of IL-12 and IL-18 cytokines. Whereas control NK cells produced high amounts of IFN- $\gamma$  in response to stimulation, a significantly smaller proportion of miR-150<sup>-/-</sup> NK cells

made IFN- $\gamma$  (~30% decrease in the frequency of miR-150<sup>-/-</sup> IFN- $\gamma$ <sup>+</sup> NK cells; Fig. 2 A). The amounts of IFN- $\gamma$  produced by individual control and miR-150<sup>-/-</sup> NK cells were similar (unpublished data). The reduction in IFN- $\gamma$ -producing cells might be caused by the reduction in the CD27<sup>-</sup> CD11b<sup>+</sup> subset within the NK cell population, because this subset plays a dominant role in cytokine production (Chiossoni et al., 2009). We co-stained NK cells with anti-CD11b antibody and measured intracellular IFN- $\gamma$  levels. Both CD11b<sup>+</sup> and CD11b<sup>-</sup> subsets of miR-150<sup>-/-</sup> NK cells still produced less IFN- $\gamma$  compared with that in WT NK cells (Fig. 2 B). Collectively, these experiments provide strong evidence for a cell-lineage intrinsic role for miR-150 in the generation and maturation of NK cells.

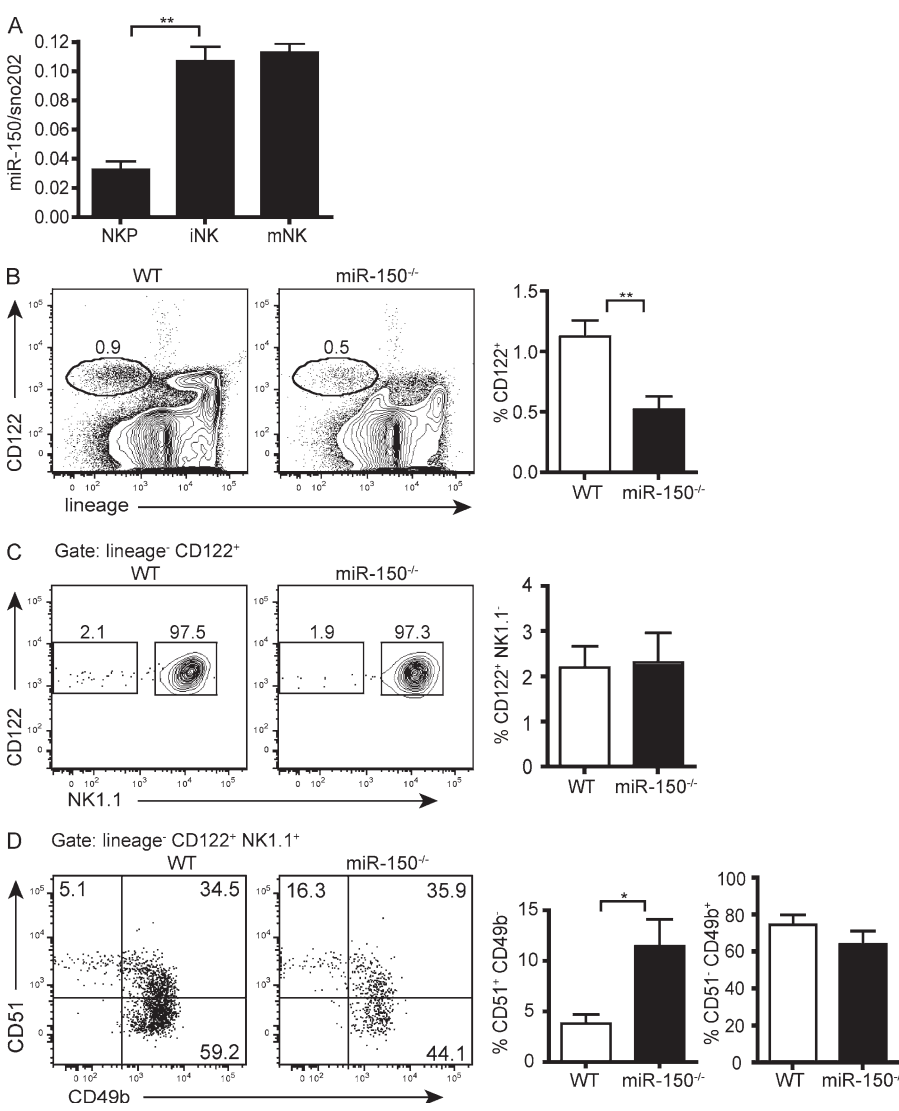


**Figure 2. Defective IFN- $\gamma$  production in miR-150<sup>-/-</sup> NK cells.** NK cells from WT:miR-150<sup>-/-</sup> chimeras were stimulated by either immobilized mAbs (control IgG,  $\alpha$ NK1.1, and  $\alpha$ Ly49H), by incubation with IL-12 and IL-18, or left untreated. Cells were stained for NKp46, TCR $\beta$ , CD45.2, and CD45.1 (A) or NKp46, TCR $\beta$ , CD45.2, CD45.1, and CD11b (B), and intracellular IFN- $\gamma$  was determined. (A) Bars represent mean percentages  $\pm$  SEM of data ( $n = 6-9$ ). Data shown are from five experiments. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . (B) Numbers indicate the percentage of CD11b<sup>+</sup> or CD11b<sup>-</sup> NK cells staining positive for IFN- $\gamma$ . Data are representative of two experiments.

**miR-150 promotes NK cell development in the BM**

Because miR-150 is expressed early in NK cell development, and is up-regulated during maturation (Fig. 3 A), we tested whether the absence of this miRNA influenced the development of NK cells in the BM. NK cells can be identified by the expression of CD122 and the absence of cell surface markers associated with mature hematopoietic lineages,

such as CD4, CD8, CD3, CD11c, Ter119, Gr-1, and CD19 (Di Santo, 2006). A significant reduction of miR-150<sup>-/-</sup> NK cells was observed in the BM of WT:miR-150<sup>-/-</sup> chimeras (WT, 1.1  $\pm$  0.1% vs. miR-150<sup>-/-</sup>, 0.5  $\pm$  0.1%;  $P = 0.003$ ;  $n = 9$ ; Fig. 3 B). A scheme for the differentiation stages of developing BM NK cells has been refined based on cell surface markers (Di Santo, 2006). NKPs are defined as CD122<sup>+</sup> NK1.1<sup>-</sup>, as well as being lineage marker negative, and are limited to cells differentiating into NK cells (Rosmaraki et al., 2001). Acquisition of NK1.1 marks the beginning of the immature NK cell (iNK) stage



**Figure 3. miR-150 promotes development of NK cells in the BM.** (A) NKP (lineage [CD4, CD8, CD3, GR1, CD19, and Ter119]-negative, CD122<sup>+</sup> NK1.1<sup>-</sup>), iNK (lineage<sup>-</sup> CD122<sup>+</sup> NK1.1<sup>+</sup> CD51<sup>+</sup>), and mature NK (mNK; lineage<sup>-</sup> CD122<sup>+</sup> NK1.1<sup>+</sup> CD51<sup>-</sup> CD49b<sup>+</sup>) cells were sorted from the BM of WT mice ( $n = 3$ ). miR-150 expression was measured using qRT-PCR. Sno202 was used as a normalization control. Mean relative miR-150 level  $\pm$  SEM is shown. (B-D) BM developmental progression was determined by staining BM cells isolated from WT:miR-150<sup>-/-</sup> chimeras with CD122, CD4, CD8, CD3, GR1, CD19, Ter119, CD51, CD49b, CD45.1, and CD45.2 antibodies. (B and C) The number of CD122<sup>+</sup> (lineage<sup>-</sup> CD122<sup>+</sup>; B) and NKP (lineage<sup>-</sup> CD122<sup>+</sup> NK1.1<sup>-</sup>; C) cells is shown as a percentage of either WT (CD45.1<sup>+</sup>) or miR-150<sup>-/-</sup> (CD45.2<sup>+</sup>) BM cells. Bar graphs represent mean percentages  $\pm$  SEM of data.  $n = 9$  (B);  $n = 5$  (C). (D) Analysis of CD51 and CD49b expression. Percentages of BM lineage<sup>-</sup> CD122<sup>+</sup> NK1.1<sup>+</sup> cells positive for each marker among WT (CD45.1<sup>+</sup>) and miR-150<sup>-/-</sup> (CD45.2<sup>+</sup>) cells are shown. Bar graph represents mean percentages  $\pm$  SEM of data ( $n = 5$ ). \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ . Data are from at least three experiments.

(Rosmaraki et al., 2001; Kim et al., 2002). Expression of CD51 is up-regulated at this stage, and then down-regulated upon further maturation into mature NK cells when CD49b is expressed. Whereas the frequency of NKP cell subsets among CD122<sup>+</sup> miR-150<sup>-/-</sup> NK cells was not affected (Fig. 3 C), a significant accumulation of CD51<sup>+</sup>CD49b<sup>-</sup> iNK cells was observed among CD122<sup>+</sup> NK1.1<sup>+</sup> miR-150<sup>-/-</sup> NK cells (Fig. 3 D). Together with the corresponding increase in miR-150 expression at the iNK cell stage (Fig. 3 A), these results suggest that the reduction of miR-150<sup>-/-</sup> NK cells occurs during the transition from immature to mature NK cells rather than earlier.

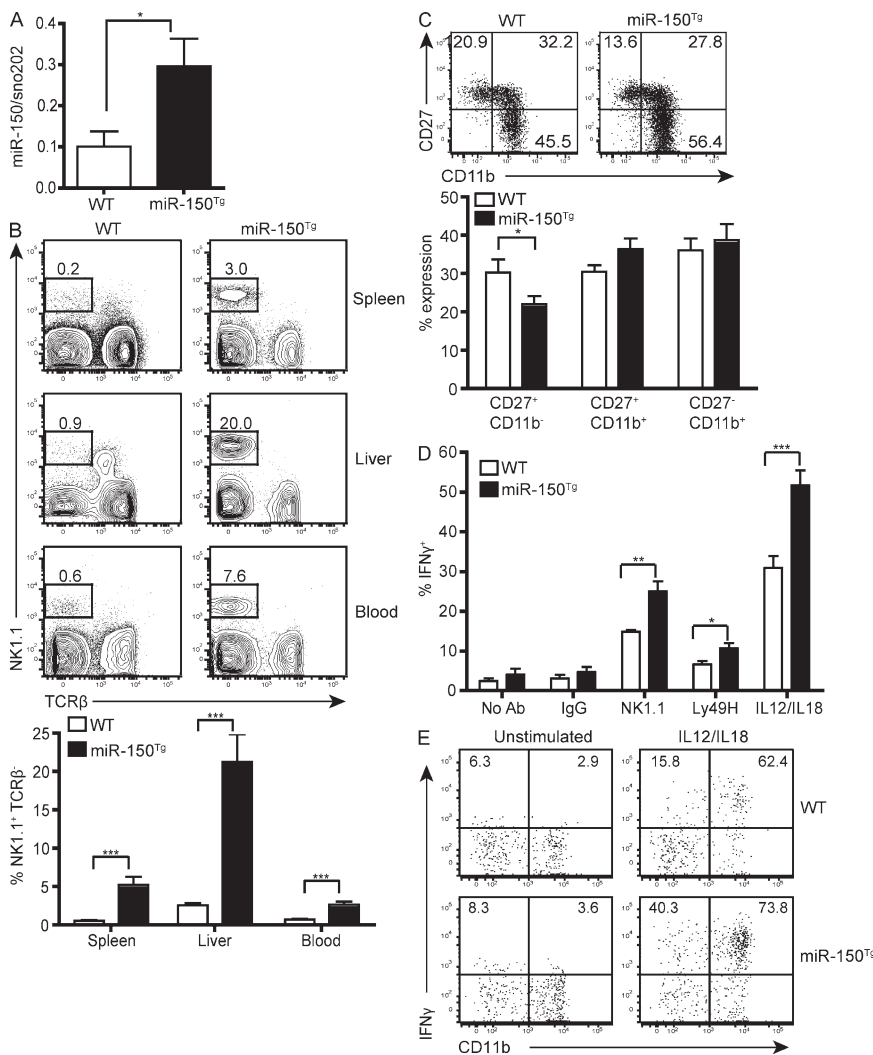
**Mature hyperfunctional NK cells accumulate in miR-150<sup>Tg</sup> mice**

Having shown that WT:miR-150<sup>-/-</sup> chimeras display an impaired development of miR-150<sup>-/-</sup> NK cells, we hypothesized that increased miR-150 expression in BM could result in the accumulation of NK cells. To test this hypothesis, we used transgenic mice with moderate ectopic expression of miR-150 under the control of the CAG promoter in the

context of *Rosa26* locus (Xiao et al., 2007). Analogous to our studies with miR-150<sup>-/-</sup> mice, we performed BM reconstitution where lethally irradiated hosts were reconstituted with an equal mix of miR-150<sup>Tg</sup> and congenic WT BM cells. miR-150 expression level in sorted splenic miR-150<sup>Tg</sup> NK cells was 3.0 ± 0.7× higher than the endogenous level in splenic WT NK cells (Fig. 4 A).

We observed a significantly greater frequency of miR-150<sup>Tg</sup> NK cells in the spleen (WT, 0.5 ± 0.1% vs. miR-150<sup>Tg</sup>, 5.2 ± 1.1%), liver (WT, 2.6 ± 0.3% vs. miR-150<sup>Tg</sup>, 21.3 ± 3.6%), and blood (WT, 0.7 ± 0.1% vs. miR-150<sup>Tg</sup>, 2.7 ± 0.4%) of WT:miR-150<sup>Tg</sup> chimeric mice (Fig. 4 B). The absolute numbers of splenic miR-150<sup>Tg</sup> NK cells were also increased compared with that of WT NK cells (WT, 0.10 × 10<sup>6</sup> ± 0.04 × 10<sup>6</sup> vs. miR-150<sup>Tg</sup>, 0.30 × 10<sup>6</sup> ± 0.03 × 10<sup>6</sup>; P = 0.003; n = 6; Fig. 4 C). Among miR-150<sup>Tg</sup> NK cells, there was a significant reduction in the CD27<sup>+</sup> CD11b<sup>-</sup> subset in the spleen of WT:miR-150<sup>Tg</sup> chimeras (Fig. 4 C). This decrease in CD27<sup>+</sup>CD11b<sup>-</sup> miR-150<sup>Tg</sup> NK cells was correlated with a concomitant mild increase in CD27<sup>+</sup> CD11b<sup>+</sup> and CD27<sup>-</sup> CD11b<sup>+</sup>

NK populations. Next, we tested the ability of miR-150<sup>Tg</sup> NK cells to produce IFN-γ upon activation with various stimuli. NK cells isolated from WT:miR-150<sup>Tg</sup> chimeras were stimulated by immobilized antibodies to several activating receptors or by a combination of



**Figure 4. Mature hyperfunctional NK cells accumulate in miR-150<sup>Tg</sup> mice.** Mixed BM chimeras were generated by injecting WT and miR-150<sup>Tg</sup> BM cells into irradiated hosts, which were analyzed 8–14 wk later. All stains contained αCD45.1 and αCD45.2, as in Fig. 1. Two independent sets of WT:miR-150<sup>Tg</sup> chimeras were made. (A) RNA was isolated from CD45.1<sup>+</sup> NK1.1<sup>+</sup> TCRβ<sup>-</sup> and CD45.2<sup>+</sup> NK1.1<sup>+</sup> TCRβ<sup>-</sup> cells sorted from five chimeras. miR-150 level was quantified by qRT-PCR. Sno202 was used as normalization control. Data are presented as mean ± SEM (n = 5). (B) Cells from the indicated tissues were stained for NK1.1 and TCRβ. The numbers are shown as percentage of WT and miR-150<sup>Tg</sup> lymphocytes. Bar graph represent mean percentages ± SEM of data (n = 13). (C) NK cell maturation was determined by staining splenocytes with NK1.1, TCRβ, CD11b, and CD27 antibodies. Gated NK1.1<sup>+</sup> TCRβ<sup>-</sup> cells are shown. Bar graph represents mean percentage ± SEM of data (n = 11). (D) NK cells were stimulated as described in Fig. 2. Cells were stained for Nkp46 and TCRβ, and intracellular IFN-γ was determined. Bars represent mean percentages ± SEM of data (n = 6–9). (E) Numbers indicate the percentage of CD11b<sup>+</sup> or CD11b<sup>-</sup> NK cells staining positive for IFN-γ. Data are from two (A and E) and at least five (B–D) experiments. \*, P < 0.05; \*\*, P < 0.01; \*\*\*, P < 0.001.

IL-12 and IL-18. After either type of stimulation, a larger fraction of miR-150<sup>Tg</sup> NK cells produced IFN- $\gamma$  (~40% increase in miR-150<sup>Tg</sup> as compared with WT NK cells; Fig. 4 D). Controlling for an increased number of NK cells expressing CD11b, a greater proportion of miR-150<sup>Tg</sup> NK cells generated IFN- $\gamma$  compared with WT NK cells (Fig. 4 E). The amounts of IFN- $\gamma$  produced by individual miR-150<sup>Tg</sup> NK cells, however, were similar to those made by control NK cells (unpublished data).

Because we observed the accumulation of mature miR-150<sup>Tg</sup> NK cells in WT:miR-150<sup>Tg</sup> chimeras, we tested whether this could be caused by faster expansion and/or lower susceptibility to apoptosis of peripheral miR-150<sup>Tg</sup> NK cells or alterations in the development of BM miR-150<sup>Tg</sup> NK cells. We did not find a significant difference in either basal turnover rate or Annexin V staining of splenic miR-150<sup>Tg</sup> NK cells in comparison to WT NK cells (unpublished data). However, when adoptively transferred into *Rag2*<sup>-/-</sup> *x Il2rg*<sup>-/-</sup> mice, mature CD27<sup>-</sup> miR-150<sup>Tg</sup> NK cells underwent more rounds of proliferation compared with CD27<sup>-</sup> subset of WT NK cells (Fig. 5 A). Furthermore, there was a significant increase in the percentage of lineage<sup>-</sup> CD122<sup>+</sup> miR-150<sup>Tg</sup> NK cells in the BM (WT, 1.0  $\pm$  0.2% vs. miR-150<sup>Tg</sup>, 7.8  $\pm$  2.7%; *P* = 0.04; *n* = 9) of WT:miR-150<sup>Tg</sup> chimeras (Fig. 5 B). Among miR-150<sup>Tg</sup> NK cells in the BM, there was also a significant reduction in iNK cell fraction (Fig. 5 C). Thus, a larger proportion of mature miR-150<sup>Tg</sup> NK cells in the periphery of WT:miR-150<sup>Tg</sup> chimeras is likely caused by both increased maturation of miR-150<sup>Tg</sup> NK cells in the BM and expansion of mature miR-150<sup>Tg</sup> NK cells in the spleen.

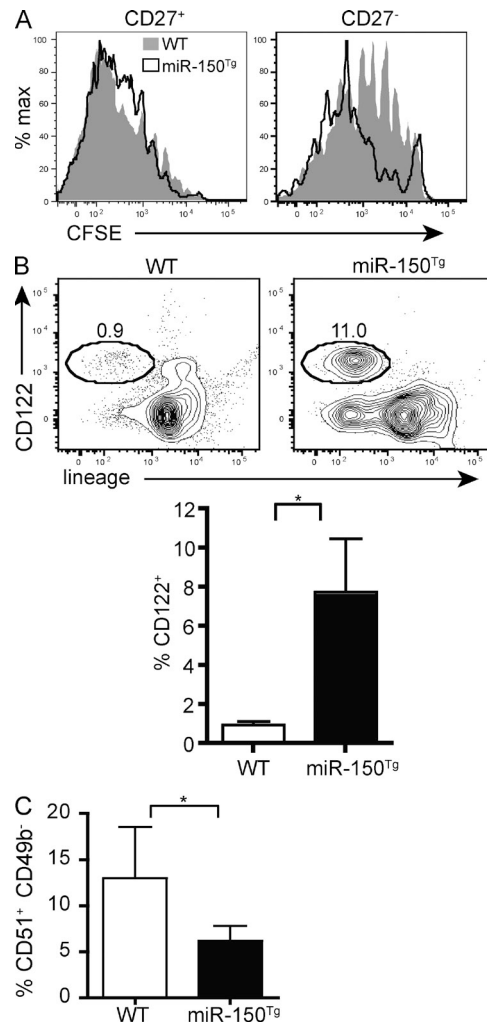
In contrast to the NK cell compartment, the frequency of mature T cells was decreased in the peripheral lymphoid organs in WT:miR-150<sup>Tg</sup> chimeras (unpublished data). Analysis of thymocytes by flow cytometry revealed a partial block in T cell development, at the double negative-3 (DN3) to the double negative-4 (DN4) transition. These data are in agreement with the phenotype observed in miR-150<sup>Tg</sup> mice (Xiao et al., 2007).

**Partial loss of c-Myb recapitulates the phenotype of miR-150<sup>Tg</sup> mice and leads to enhanced NK cell development**

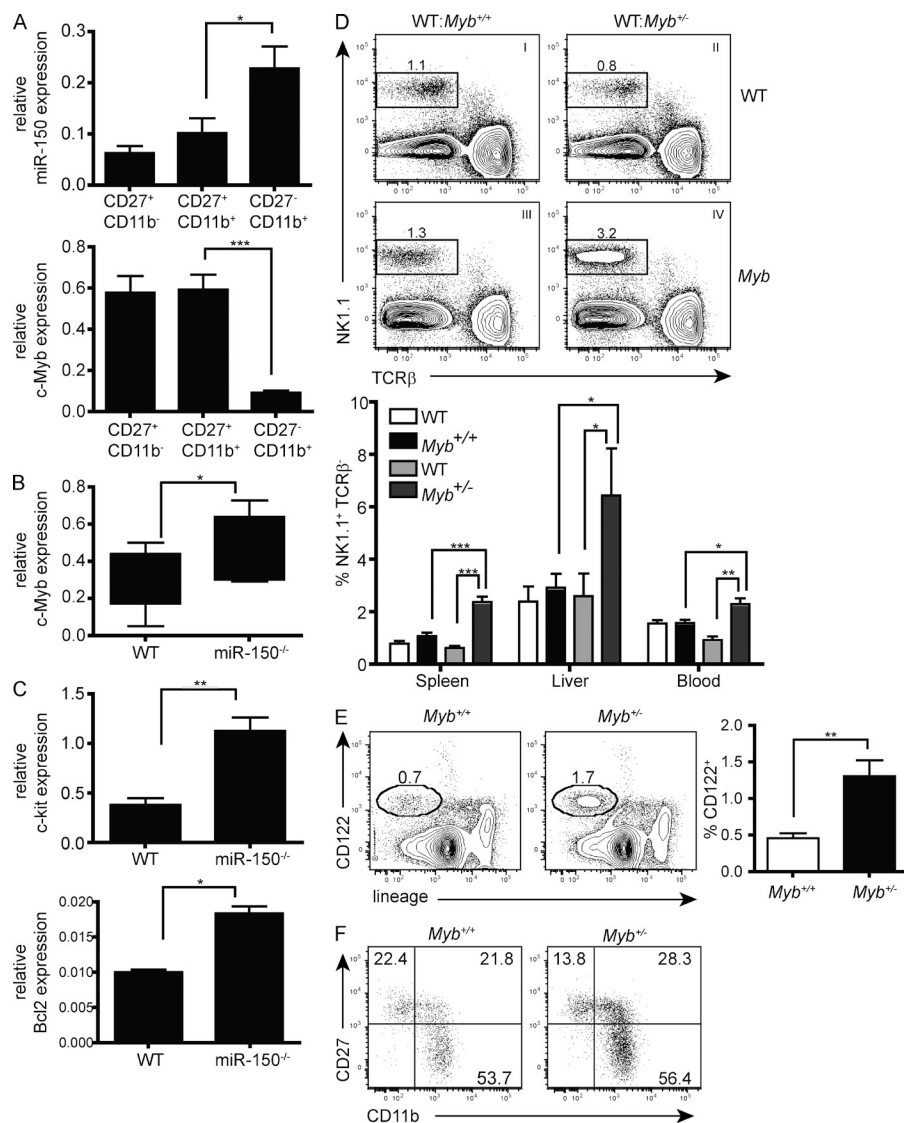
We explored the mechanism underlying miR-150-dependent NK cell development. Several studies in cell lines, as well as primary cells, have shown that a transcription factor c-Myb is a target of miR-150 (Xiao et al., 2007; Lin et al., 2008; Lu et al., 2008). We tested whether there is an inverse relationship between miR-150 and c-Myb expression in NK cells. CD27<sup>+</sup>CD11b<sup>-</sup>, CD27<sup>+</sup>CD11b<sup>+</sup>, and CD27<sup>-</sup>CD11b<sup>+</sup> subsets of NK cells were purified from spleens of WT mice and analyzed for their expression of miR-150 and *c-Myb* mRNA. miR-150 expression level correlates with the progression of NK cell maturation, with the highest being in the most mature CD27<sup>-</sup> CD11b<sup>+</sup> subset of NK cells (Fig. 6 A). The expression of *c-Myb* mRNA shows a reverse trend with the lowest being in CD27<sup>-</sup> CD11b<sup>+</sup> subset of NK cells, consistent with a previous study showing c-Myb as a transcription factor

differentially expressed by CD27<sup>+</sup> CD11b<sup>-</sup> and CD27<sup>-</sup> CD11b<sup>+</sup> subsets of NK cells (Chiossone et al., 2009).

Do changes in miR-150 expression inversely correlate with c-Myb expression? Because *c-Myb* mRNA levels were already low in freshly isolated splenic NK cells, we asked whether miR-150 deficiency leads to c-Myb up-regulation. We found an increased level of *c-Myb* mRNA in miR-150<sup>-/-</sup> NK cells compared with WT cells (Fig. 6 B). Transcription levels of the c-Myb targets *c-kit* and *Bcl2* (Greig et al., 2008) were also increased in sorted miR-150<sup>-/-</sup>



**Figure 5. Increased maturation and expansion of miR-150<sup>Tg</sup> cells in BM and spleen of WT:miR-150<sup>Tg</sup> chimeras.** (A) 10<sup>7</sup> splenocytes from WT:miR-150<sup>Tg</sup> chimeras were labeled with CFSE and adoptively transferred into *Rag2*<sup>-/-</sup> *x Il2rg*<sup>-/-</sup> mice. At 5 d after transfer, splenocytes were isolated and stained for NK1.1, TCR $\beta$ , CD45.2, CD45.1, and CD27. Histograms show CFSE dilution by CD27<sup>+</sup> (left) and CD27<sup>-</sup> (right) subsets among WT (CD45.1<sup>+</sup>) and miR-150<sup>Tg</sup> (CD45.2<sup>+</sup>) NK cells. (B and C) The number of CD122<sup>+</sup> (lineage<sup>-</sup> CD122<sup>+</sup>; B) and iNK (lineage<sup>-</sup> CD122<sup>+</sup> NK1.1<sup>+</sup> CD51<sup>+</sup> CD49b<sup>-</sup>; C) cells is shown as a percentage of either WT or miR-150<sup>Tg</sup> BM cells. Bar graphs represent mean percentages  $\pm$  SEM of data. *n* = 9 (B); *n* = 5 (C). \*, *P* < 0.05. Data are from two (A) and at least five (B and C) experiments.



**Figure 6. Increased NK cell frequency in *Myb*<sup>+/-</sup> mice.** (A) miR-150 and c-Myb expression in sorted NK cell subsets ( $n = 6$  for each subset) defined by CD27/CD11b expression. (B and C) Levels of c-Myb, c-kit, and Bcl2 mRNAs in miR-150<sup>-/-</sup> NK cells. The amount of c-Myb, c-kit, and Bcl2 transcripts in sorted WT and miR-150<sup>-/-</sup> NK cells were determined by qPCR with normalization to the amount of HPRT in each sample. Data are presented as mean  $\pm$  SEM ( $n = 2-6$ ). (D-F) Two sets of mixed BM chimeras were generated by injecting either WT (CD45.1<sup>+</sup>) and *Myb*<sup>+/-</sup> (CD45.2<sup>+</sup>) or WT (CD45.1<sup>+</sup>) and *Myb*<sup>-/-</sup> (CD45.2<sup>+</sup>) BM cells into irradiated hosts. All stains contained  $\alpha$ CD45.1 and  $\alpha$ CD45.2 to distinguish between WT (CD45.1<sup>+</sup>) and *Myb* (CD45.2<sup>+</sup>) populations. (D) Frequency of *Myb*<sup>+/-</sup> NK cells. Splenocytes from WT:*Myb*<sup>+/-</sup> and WT:*Myb*<sup>-/-</sup> chimeras were stained for NK1.1 and TCR $\beta$ . Gated WT (I and II), *Myb*<sup>+/-</sup> (III), and *Myb*<sup>-/-</sup> (IV) cells are shown. Bottom, bar graph represents mean percentage  $\pm$  SEM of data from WT:*Myb*<sup>+/-</sup> ( $n = 6$ ) and WT:*Myb*<sup>-/-</sup> ( $n = 7$ ) mice. (E) The number of CD122<sup>+</sup> (lineage<sup>-</sup> CD122<sup>+</sup>) cells is shown as a percentage of either *Myb*<sup>+/-</sup> or *Myb*<sup>-/-</sup> BM cells. Bar graphs represent mean percentages  $\pm$  SEM of data ( $n = 5$ ). (F) NK cell maturation was determined by staining splenocytes with NK1.1, TCR $\beta$ , CD11b, CD27, CD45.2, and CD45.1 antibodies. Gated CD45.2<sup>+</sup> NK1.1<sup>+</sup> TCR $\beta$ <sup>-</sup> cells are shown. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ . Data are from two (A-C) and at least three (D-F) experiments.

NK cells (Fig. 6 C), suggesting that the c-Myb protein level is likely to be altered in miR-150<sup>-/-</sup> NK cells as well.

To ascertain whether the enhanced NK cell development induced by miR-150 overexpression might be the consequence of c-Myb repression by miR-150, we studied mice with a partial loss of *Myb*. We generated two sets of chimeric mice by reconstituting irradiated hosts with an equal mix of BM from WT (CD45.1<sup>+</sup>) and experimental (*Myb*<sup>+/-</sup> [2 doses of c-Myb gene] or *Myb*<sup>-/-</sup> [c-Myb heterozygous knockout]; CD45.2<sup>+</sup>) mice. The proportion of NK cells was significantly increased in the CD45.2<sup>+</sup> compartment of WT:*Myb*<sup>+/-</sup> mice, compared with that in the WT:*Myb*<sup>+/-</sup> chimeras (Fig. 6 D, III vs. IV in the flow cytometry plots). The frequency of NK cells among *Myb*<sup>+/-</sup> lymphocytes was also significantly higher than the frequency of NK cells among WT lymphocytes in WT:*Myb*<sup>+/-</sup> mice (Fig. 6 D, II vs. IV in the flow cytometry plots), thus suggesting a cell intrinsic mechanism for c-Myb regulation of NK cell generation. Furthermore, there was a

significant increase in the percentage of lineage<sup>-</sup> CD122<sup>+</sup> *Myb*<sup>+/-</sup> NK cells compared with that of *Myb*<sup>+/-</sup> cells (*Myb*<sup>+/-</sup>,  $0.5 \pm 0.1\%$  vs. *Myb*<sup>+/-</sup>,  $1.3 \pm 0.2\%$ ;  $P = 0.005$ ;  $n = 5$ ; Fig. 6 E) in the BM of WT:*Myb*<sup>+/-</sup> and WT:*Myb*<sup>+/-</sup> mice. Splenic *Myb*<sup>+/-</sup> NK cells expressed higher amounts of CD11b compared with their *Myb*<sup>+/-</sup> counterparts (Fig. 6 F), suggesting that *Myb*<sup>+/-</sup> NK cells resembled more mature NK cells. Together, these results indicate the NK cell developmental phenotype observed in WT:*Myb*<sup>+/-</sup> chimeras resembles that in WT:miR-150<sup>Tg</sup> chimeras, suggesting that the latter could be explained, at least partially, by the control of c-Myb expression by miR-150. This is reminiscent of the observation that *Myb*<sup>+/-</sup> mice have fewer mature B-1 B cells and block at the transition from pro-B to pre-B cells, which is similar to the phenotype of miR-150<sup>Tg</sup> mice (Xiao et al., 2007).

#### Acquisition of the Ly49A NK cell receptor is dependent on the dosage of miR-150 and c-Myb

NK cells express a repertoire of activating and inhibitory receptors, including members of the Ly49 gene family and CD94 that complexes with NKG2A, NKG2C, or NKG2E. CD27<sup>+</sup> CD11b<sup>-</sup> and CD27<sup>-</sup> CD11b<sup>+</sup> subsets of NK cells

express a distinct NK cell repertoire (Hayakawa and Smyth, 2006). In particular, CD27<sup>+</sup> CD11b<sup>-</sup> NK cells display higher expression of NKG2A, whereas CD27<sup>-</sup> CD11b<sup>+</sup> NK cells contain a greater proportion of cells that express inhibitory Ly49 receptors (C and I isoforms), which recognize self-MHC class I molecule, H-2<sup>b</sup> (C57BL/6 mice), and the activating Ly49 receptor Ly49D. Consistent with the higher frequency of immature CD27<sup>+</sup> CD11b<sup>-</sup> NK cell subset among miR-150<sup>-/-</sup> NK cells, expression of NKG2A was higher and expression of Ly49C/I was lower in miR-150<sup>-/-</sup> NK cells (Table I). Although we did not observe a corresponding change in these receptor expressions in miR-150<sup>Tg</sup> NK cells, they did show an impaired expression of Ly49D. Expression of neither the activating Ly49D or Ly49H, nor the inhibitory NKG2A, Ly49C/I, and Ly49G2 receptors, was affected in *Myb*<sup>+/-</sup> NK cells.

The striking exception was Ly49A whose expression was exquisitely sensitive to the dosage of miR-150 and *c-Myb*. Ly49A is an inhibitory receptor that recognizes MHC class I molecules H2D<sup>d</sup> and H2D<sup>k</sup> (Orr and Lanier, 2011). The size of Ly49A subset was significantly higher in splenic miR-150<sup>-/-</sup> NK cells (WT, 14.5 ± 0.8% vs. miR-150<sup>-/-</sup>, 17.5 ± 0.8%; P = 0.03; n = 14), and lower in splenic miR-150<sup>Tg</sup> NK cells (WT, 13.2 ± 0.8% vs. miR-150<sup>Tg</sup>, 4.0 ± 0.3%; P < 0.0001; n = 14; Fig. 7 A). Furthermore, Ly49A expression was lower in splenic *Myb*<sup>+/-</sup> NK cells compared with either internal WT control NK cells (WT, 17.1 ± 0.3% vs. *Myb*<sup>+/-</sup>, 7.7 ± 0.3%; P < 0.0001; n = 3) or *Myb*<sup>+/+</sup> NK cells (*Myb*<sup>+/+</sup>, 15.6 ± 1.2% vs. *Myb*<sup>+/-</sup>, 7.7 ± 0.3%; P = 0.004; n = 3; Fig. 7 B). To determine at what point in NK cell development miR-150 mutant NK cells first show altered Ly49A expression, we measured Ly49A frequencies among NK cell populations in the BM of WT:miR-150<sup>Tg</sup> mice. NK1.1<sup>+</sup> CD51<sup>+</sup> iNKT cells begin to acquire Ly49 receptors as they up-regulate CD49b (Fig. 7 C). miR-150<sup>Tg</sup> NK cells showed a marked reduction in Ly49A frequency at both intermediate (CD51<sup>+</sup> CD49b<sup>+</sup>) and mature (CD51<sup>-</sup> CD49b<sup>+</sup>) stages. This deficit in Ly49A

receptor acquisition is specific to Ly49A, as we observed similar expression of Ly49G2 receptor in miR-150<sup>Tg</sup> and WT NK cells. These data suggest that the percentage of NK cells expressing the *Klra1* gene in C57BL/6 mice encoding Ly49A is determined at an early point in NK cell development.

The presence of occasional Ly49A-bearing NK cells among miR-150<sup>Tg</sup> and *Myb*<sup>+/-</sup> NK cells suggests that normal Ly49A expression levels are not dependent on miR-150 or *c-Myb* dosage. This is evident from the mean fluorescence intensity (MFI) of Ly49A<sup>+</sup> NK cells, which is identical among the different miR-150 mutant (Fig. 7 D), and between WT and *Myb*<sup>+/-</sup> (Fig. 7 E) NK cells. Therefore, miR-150 and *c-Myb* are necessary for the acquisition of Ly49A, but they are dispensable for Ly49A cell surface expression once its expression is initiated.

Ly49A is also expressed by a small fraction of memory CD8<sup>+</sup> T cells (Coles et al., 2000). We found that Ly49A expression was present in higher fraction of miR-150<sup>-/-</sup> T cells compared with WT T cells (WT, 0.40 ± 0.04% vs. miR-150<sup>-/-</sup>, 0.80 ± 0.07%; P = 0.0001; n = 9); accordingly, Ly49A expression was displayed in lower proportion of miR-150<sup>Tg</sup> T cells compared with control T cells (WT, 0.30 ± 0.02% vs. miR-150<sup>Tg</sup>, 0.08 ± 0.02%; P < 0.0001 [n = 9]). Together, these data indicate that the dosage of miR-150 and *c-Myb* influences, in a cell-autonomous manner, the percentage of cells expressing *Klra1*. Because potential *c-Myb* binding sites exist in the upstream region of *Klra1* (Tanamachi et al., 2004), *c-Myb* might represent the transcription factor that determines *Klra1* gene variegation.

**The development of iNKT cells is impaired in miR-150<sup>Tg</sup> and *Myb*<sup>+/-</sup> mice**

Previous studies have shown that global miRNA loss as a result of Dicer deficiency results in severe defects in iNKT cell development (Fedeli et al., 2009; Zhou et al., 2009; Seo et al., 2010). iNKT cells developing in the thymus initially acquire Vα14-Jα18 TCR rearrangement, and then down-regulate CD24, and finally up-regulate CD44 and NK1.1 as the last maturation step (Godfrey et al., 2010). Populations of thymic iNKT cells, identified using α-galactosylceramide-loaded CD1d tetramer (CD1d-tet; Matsuda et al., 2000), were purified and analyzed for their expression of miR-150 (Fig. 8 A). Because miR-150 is up-regulated during iNKT cell maturation in the thymus, we hypothesized that miR-150 could also play a role in the development of this lineage. We isolated thymocytes from WT:miR-150<sup>-/-</sup> and WT:miR-150<sup>Tg</sup> chimeras and stained for iNKT cells. Although the frequency of thymic miR-150<sup>-/-</sup> iNKT cells was mildly decreased (1.5 ± 0.2-fold compared with that of WT cells), the proportion of thymic miR-150<sup>Tg</sup> iNKT cells was significantly reduced (4.6 ± 0.8-fold compared with WT cells; Fig. 8 B). Analysis of CD1d-tet<sup>+</sup> thymocytes showed that the majority of WT cells down-regulate CD24 and up-regulate NK1.1 (Fig. 8 C). In contrast, the proportion of CD24<sup>hi</sup> NK1.1<sup>-</sup> cells was significantly higher among miR-150<sup>Tg</sup> iNKT cells as compared with WT cells, whereas

**Table I.** Receptor repertoire of NK cells from miR-150<sup>-/-</sup>, miR-150<sup>Tg</sup>, *Myb*<sup>+/+</sup>, and *Myb*<sup>+/-</sup> mixed BM chimeras

Receptor	miR-150 <sup>-/-</sup>		miR-150 <sup>Tg</sup>	
	WT	WT	WT	WT
Ly49H	48.5 ± 4.4	58.5 ± 1.5 <sup>a</sup>	51.0 ± 2.7	54.6 ± 1.2
	53.3 ± 3.9	47.3 ± 2.6	46.9 ± 0.6	51.7 ± 0.6
Ly49D	43.5 ± 1.8	36.3 ± 2.0 <sup>a</sup>	41.9 ± 3.3	48.1 ± 2.7
	47.6 ± 1.7	44.0 ± 1.6	46.6 ± 4.1	49.0 ± 0.8
NKG2A	55.3 ± 2.3 <sup>b</sup>	55.3 ± 2.9	52.0 ± 1.9	47.6 ± 0.9
	43.4 ± 1.3	50.7 ± 2.0	43.9 ± 1.5	44.1 ± 0.6
Ly49C/I	26.2 ± 1.2 <sup>b</sup>	41.3 ± 2.1	33.6	43.3
	38.9 ± 1.6	37.4 ± 2.6	33.7	40.9
Ly49G2	48.4 ± 1.6	38.4 ± 1.6	36.8 ± 10.1	37.3 ± 0.6
	46.4 ± 0.9	40.8 ± 1.9	44.7 ± 1.4	43.7 ± 0.3

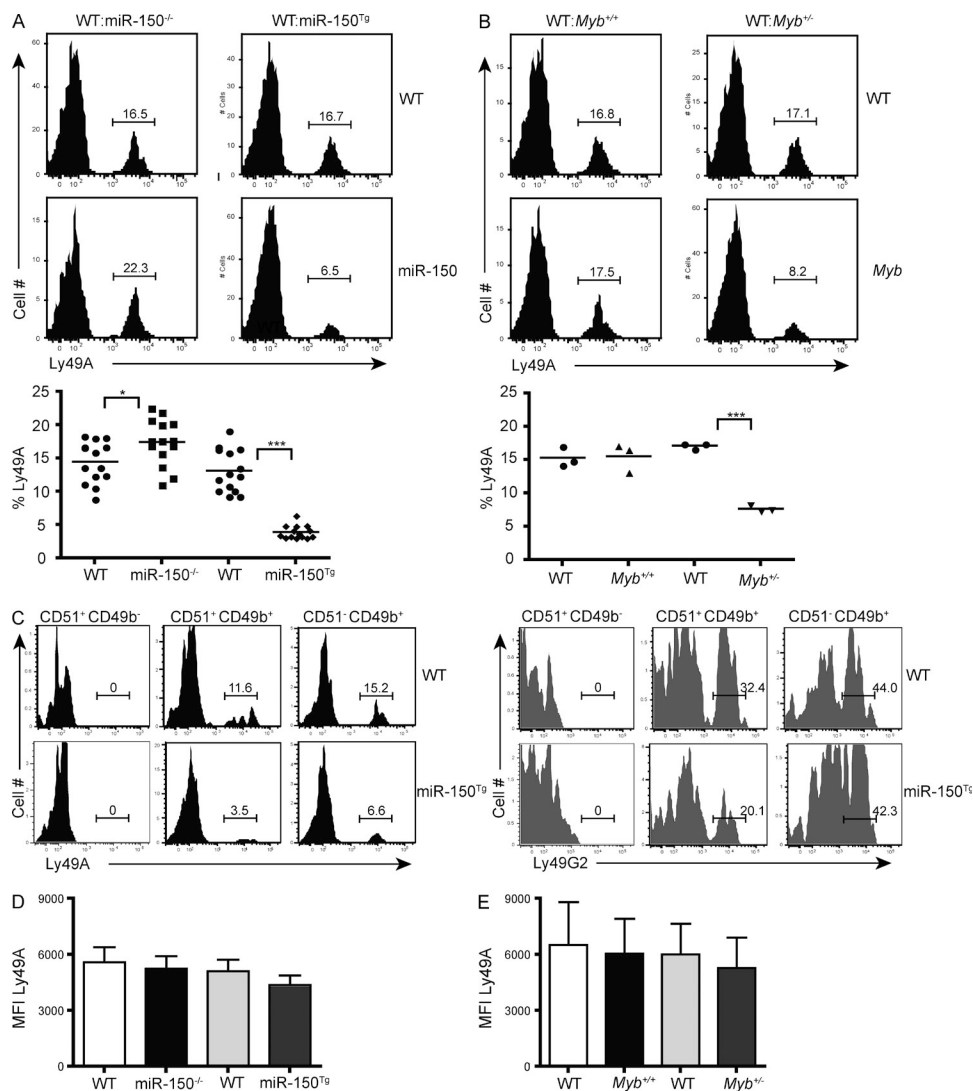
<sup>a</sup>Significant P < 0.01 compared with WT.

<sup>b</sup>Significant P < 0.001 compared with WT.



the proportion of mature stage CD24<sup>lo</sup> NK1.1<sup>+</sup> miR-150<sup>Tg</sup> iNKT cells was reduced. Analysis of miR-150<sup>-/-</sup> iNKT developmental intermediates revealed a mild reduction in the fully mature CD24<sup>lo</sup> NK1.1<sup>+</sup> iNKT cells; however, the data were not statistically significant. As CD24 is normally down-regulated during positive selection, these results suggest that miR-150 is important during the positive selection of iNKT cells. The terminal maturation stages during iNKT cell development involve up-regulation of Ly49 receptors (Gapin et al., 2001) and CD122 (Matsuda et al., 2002)

in addition to NK1.1 (Benlagha et al., 2002). miR-150<sup>Tg</sup> iNKT cells had lower proportion of Ly49G2-expressing cells compared with that among WT iNKT cells (WT 49.2%  $\pm$  2.5% vs. miR-150<sup>Tg</sup> 36.6%  $\pm$  3.7%;  $P = 0.05$  [ $n = 3$ ]), although they had normal levels of CD122 (unpublished data). The observed decrease of miR-150<sup>Tg</sup> iNKT cells was also found in spleen (3.7  $\pm$  0.6-fold compared with WT cells; Fig. 8 D). Together these data suggest that optimal dose of miR-150 is necessary for the development and maturation of iNKT cells.



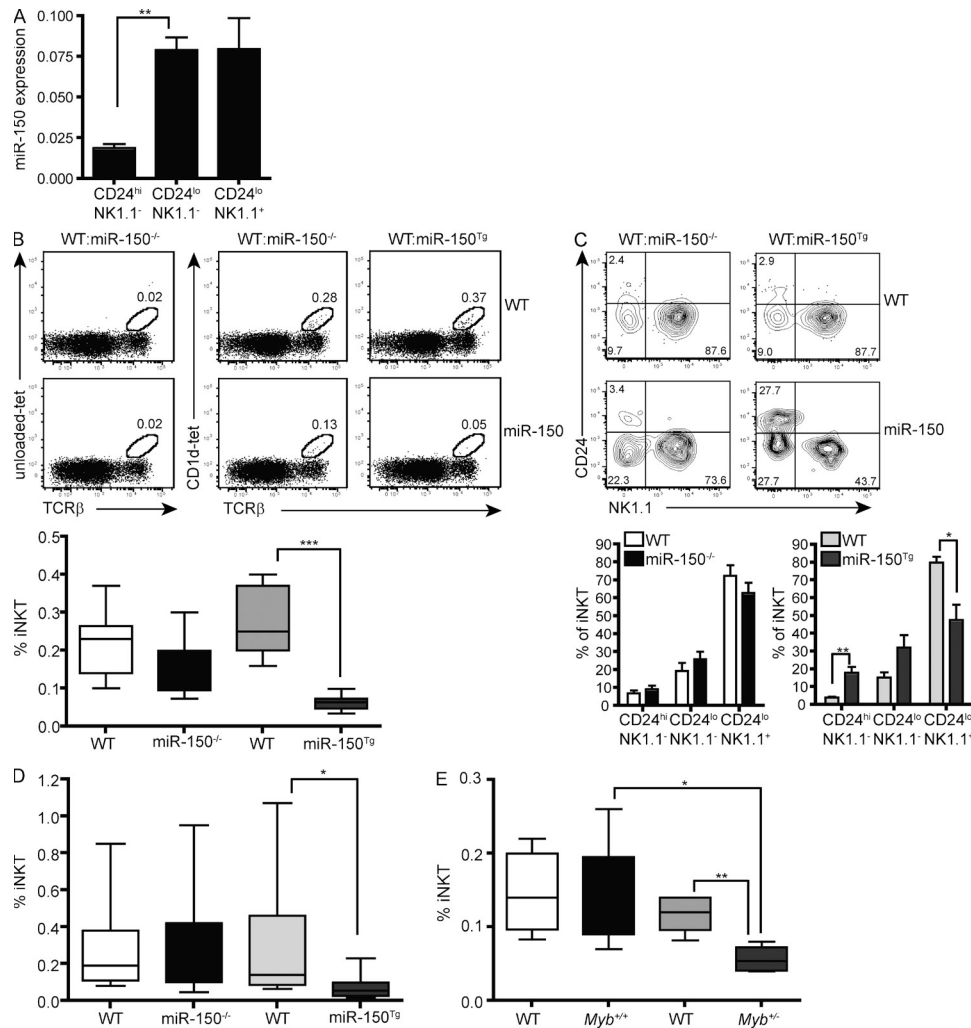
**Figure 7. Acquisition of the Ly49A NK cell receptor is dependent on the dosage of miR-150 and c-Myb.** (A) Splenocytes were isolated from the mixed WT:miR-150<sup>-/-</sup> and WT:miR-150<sup>Tg</sup> chimeras described in Figs. 1 and 4, respectively, and stained with NK1.1, TCR $\beta$ , CD45.1, CD45.2, and Ly49A antibodies. Histograms show percentages of Ly49A<sup>+</sup> cells in WT (CD45.1<sup>+</sup>) and miR-150 mutant (CD45.2<sup>+</sup>) NK cells. Cumulative data are shown in a scatter plot. Each symbol represents an individual mouse ( $n = 14$ ). Horizontal lines represent mean values. (B) Splenocytes were isolated from the mixed WT:Myb<sup>+/-</sup> and WT:Myb<sup>-/-</sup> chimeras described in Fig. 6, and stained as described in A. Histograms show percentage of Ly49A<sup>+</sup> cells in WT (CD45.1<sup>+</sup>) and c-Myb mutant (CD45.2<sup>+</sup>) NK cells. Each symbol in the scatter plot represents an individual mouse ( $n = 3$ ). Horizontal lines represent mean values. (C) Percentage of Ly49A<sup>+</sup> (left) and Ly49G2<sup>+</sup> (right) cells among WT and miR-150<sup>Tg</sup> NK cell subsets at various stages of maturation in the BM. (D and E) The mean fluorescence intensity (MFI) values of Ly49A staining in miR-150<sup>-/-</sup>, miR-150<sup>Tg</sup> and WT Ly49A<sup>+</sup> NK cells (D) or between WT, Myb<sup>+/-</sup>, and Myb<sup>-/-</sup> Ly49A<sup>+</sup> NK cells (E). Data are shown as mean MFI  $\pm$  SEM.  $n = 11$  (D);  $n = 3$  (E). \*,  $P < 0.05$ ; \*\*\*,  $P < 0.001$ . Data are from nine (A and D), three (B and E) and two (C) experiments.

*Myb* deficiency in the thymus results in a complete absence of iNKT cells (Hu et al., 2010). If miR-150 regulates iNKT cell development by regulating *c-Myb*, partial loss of *c-Myb* would be predicted to have the same effect on the development of this lineage as the overexpression of miR-150. To test this hypothesis, we stained for iNKT cells in the spleen of WT:*Myb*<sup>+/+</sup> and WT:*Myb*<sup>+/-</sup> chimeras. The frequency of *Myb*<sup>+/-</sup> iNKT cells was significantly reduced (2.8 ± 1-fold compared with WT cells; Fig. 8 E). We also examined the iNKT cell population in the thymus of WT:*Myb*<sup>+/+</sup> and WT:*Myb*<sup>+/-</sup> chimeras and found fewer *Myb*<sup>+/-</sup> iNKT cells that exhibited

more immature (CD24<sup>hi</sup> NK1.1<sup>-</sup> and CD24<sup>hi</sup> NK1.1<sup>-</sup>) phenotype (unpublished data). These data suggest that the iNKT cell developmental phenotype observed in miR-150<sup>Tg</sup> chimeras is at least partially caused by the alteration in *c-Myb* levels.

DISCUSSION

Although miR-150 was initially appreciated as a key miRNA in B cell development (Zhou et al., 2007), it is becoming increasingly apparent that it also functions in other cell types of the hematopoietic system. miR-150 modulates lineage fate in megakaryocyte progenitors, driving differentiation



**Figure 8. Partial ablation of *c-Myb* phenocopies impaired iNKT cell development in miR-150<sup>Tg</sup> mice.** (A) Up-regulation of miR-150 during iNKT cell maturation in the thymus. Developmental intermediates (CD24<sup>hi</sup> NK1.1<sup>-</sup>, CD24<sup>lo</sup> NK1.1<sup>-</sup>, and CD24<sup>lo</sup> NK1.1<sup>+</sup>) of thymic PBS57-loaded CD1d tetramer (CD1d-tet)<sup>+</sup> TCRβ<sup>+</sup> iNKT cells were sorted from WT mice. miR-150 expression was measured using qRT-PCR. Sno202 was used as a normalization control. Mean relative miR-150 level ± SEM is shown (*n* = 3). (B) Percentage of miR-150<sup>Tg</sup> CD1d-tet<sup>+</sup> TCRβ<sup>+</sup> cells in thymus of WT:miR-150<sup>Tg</sup> chimeras. A representative dot-plot analysis of CD1d tetramer and TCRβ staining is shown. Bottom, box plots represent mean percentages ± SEM of data from WT:miR-150<sup>-/-</sup> (*n* = 5) and WT:miR-150<sup>Tg</sup> (*n* = 7) chimeras. (C) Frequency of CD24<sup>hi</sup> NK1.1<sup>-</sup>, CD24<sup>lo</sup> NK1.1<sup>-</sup>, and mature CD24<sup>hi</sup> NK1.1<sup>+</sup> iNKT cells among CD1d-tet<sup>+</sup> NK1.1<sup>+</sup> TCRβ<sup>+</sup> cells from thymus of WT:miR-150<sup>-/-</sup> and WT:miR-150<sup>Tg</sup> chimeric mice. Bar graphs depict the mean ± SEM for the proportion of developmental intermediates within miR-150<sup>-/-</sup> (left) and miR-150<sup>Tg</sup> (right) CD1d-tet<sup>+</sup> iNKT cells. *n* = 4 for each group. (D and E) Percentage of splenic miR-150<sup>Tg</sup> and *Myb*<sup>+/-</sup> CD1d-tet<sup>+</sup> iNKT cells. Bar graphs depict mean ± SEM for the proportion of CD1d-tet<sup>+</sup> TCRβ<sup>+</sup> iNKT cells in the spleens of WT:miR-150<sup>-/-</sup> (*n* = 11), WT:miR-150<sup>Tg</sup> (*n* = 11), WT/*Myb*<sup>+/+</sup> (*n* = 6), and WT/*Myb*<sup>+/-</sup> (*n* = 7) mice. \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001. Data are from two (A and C) and at least three (B, D, and E) experiments.

of megakaryocytes at the expense of erythroid cells (Lu et al., 2008). Overexpression of miR-150 in hematopoietic stem cells leads to a mild increase in granulocytes (Zhou et al., 2007). In this study, we have demonstrated an important role for miR-150 in directing that development and maturation of the NK and the CD1d-restricted NKT cell lineages as well. In the absence of miR-150, peripheral numbers of NK cells are decreased; the cells appear to be halted at a stage before final maturation, and their ability to produce IFN- $\gamma$  after activation is diminished. That miR-150 promotes NK cell development and maturation was corroborated in experiments in which miR-150 was ectopically expressed in BM. This led to an accumulation of more mature hyperfunctional NK cells. Furthermore, proper dosage of miR-150 is also necessary for the development and maturation of iNKT cells. Importantly, we find that miR-150 acts in a cell-intrinsic manner in both lineages. Because NK cells and iNKT cells come from different committed precursors and develop in different locations (Di Santo, 2006; Godfrey et al., 2010), miR-150 most likely acts independently in both cell types after lineage commitment. Given that miR-150 expression is low in the progenitor populations of each of these lineages compared with mature NK and iNKT cells, it is unlikely that it would regulate the formation of these committed precursors.

miR-150 effects in NK and iNKT cells are at least partially mediated by its target gene *Myb*. Transcript levels of *c-Myb* and its target genes *c-kit* and *Bcl2* are increased in miR-150<sup>-/-</sup> NK cells. Partial ablation of *c-Myb* phenocopies increased NK cell frequency and impaired iNKT cell development observed in miR-150<sup>Tg</sup> chimeric mice. It is possible that other miR-150 target genes may also contribute to the NK and iNKT cell developmental phenotypes observed in miR-150<sup>-/-</sup> and miR-150<sup>Tg</sup> chimeric mice. Bioinformatics analyses predict hundreds of target genes for each miRNA (Rajewsky, 2006). Only a few of these, however, may represent targets with a significant biological function or be relevant in a particular cellular context. *Pim-1*, a predicted target of miR-150 (Xiao et al., 2007), is known to stimulate *c-Myb* activity (Levenson et al., 1998), indicating that miR-150 may suppress multiple genes in the *c-Myb* pathway. The expression pattern of *Pim-1* mimics *c-Myb*, and is inversely correlated with that of miR-150 during NK cell activation (Heng and Painter, 2008), suggesting that it might be functionally relevant for the regulation of NK cells. Recently, miR-150 was shown to target the transcription factor *Egr2* (Wu et al., 2010). *Egr2* is an essential regulator of iNKT cell development and is thought to control iNKT cell proliferation and apoptosis (Lazarevic et al., 2009). Interestingly, although expressed in developing NK cells (unpublished data), *Egr2* is not necessary for NK cell development (Lazarevic et al., 2009), suggesting that miR-150-mediated regulation of *Egr2* levels might have functional consequences for iNKT but not NK cells. Furthermore, another miR-150 target, the purinergic P2X7 receptor (L. Zhou et al., 2008), has been implicated in regulating iNKT cell activation (Kawamura et al., 2006). Targeted mutagenesis of the miR-150-binding

sites in the *Myb* 3'UTR or a genetic rescue of miR-150<sup>-/-</sup> phenotype by the partial loss of *c-Myb* will ultimately be required to determine to what extent contributions of target genes other than *c-Myb* might explain the phenotypes of NK and iNKT cell compartments.

Several transcription factors that regulate the development of NK cells have been described (Hesslein and Lanier, 2011). Members of the Ets family of transcription factors control several aspects of hematopoiesis in general, and NK cell development in particular. PU.1 deficiency results in the generation of reduced numbers of NKP and mature NK cells (Colucci et al., 2001), whereas deficiencies in *Ets1* and MEF result in severe defects in the development of NK and iNKT cells (Barton et al., 1998; Walunas et al., 2000). T-bet, a member of the T-box family of transcription factors, controls various aspects of NK cells (Hesslein and Lanier, 2011). Mice lacking T-bet have a profound, cell-intrinsic defect in their ability to generate mature NK and iNKT cells (Townsend et al., 2004). Both cell lineages fail to complete maturation, leading to decreased numbers in the periphery. *Bcl11b* is a zinc finger protein that plays an essential role in specifying T cell fate by both up-regulating T cell-specific genes and suppressing NK cell genes (Li et al., 2010). When *Bcl11b* was deleted, T cells acquired expression of NK cell lineage molecules including CD122, T-bet, NKp46, and NKG2A receptors, perforin, and IFN- $\gamma$  after in vitro culture. Perhaps the accumulation of miR-150<sup>Tg</sup> NK cells is a result of reprogrammed NK-like cells with altered *Bcl11b* expression? This scenario is unlikely as the mouse *Bcl11b* gene does not contain the miR-150 seed sequence in its 3' or 5' UTR or its coding sequence (Dweep et al., 2011), and there is no evidence suggesting that *Bcl11b* mRNA levels are altered in miR-150 mutant NK cells (unpublished data). *c-Myb* has recently been shown to be required for the development and function of iNKT cells (Hu et al., 2010). Further investigation of a role for *c-Myb* in NK cell development requires loss of *c-Myb* specifically in NK cell lineage. With the development of a NK cell-specific Cre mice (Eckelhart et al., 2011), it will now be feasible to test the hypothesis that *c-Myb* is a negative regulator of NK cell development.

The factors that control Ly49 gene expression are beginning to be defined. We show here that miR-150 and *c-Myb* are required for the acquisition of the Ly49A receptor. Several scenarios are possible to explain the reduced frequency of Ly49A<sup>+</sup> NK cells in miR-150 transgenic and *c-Myb* heterozygous mice. First, miR-150 and *c-Myb* may be involved in the formation of Ly49 receptor repertoire at the level of its adaptation to the MHC class I environment. This is unlikely for Ly49A because Ly49A is not known to recognize the MHC class I H2<sup>b</sup> allele in C57BL/6 mice (Orr and Lanier, 2011), and our studies were conducted in mixed BM chimeras where both WT and mutant cells develop in the same environment. Alternatively, the reduced frequency of Ly49A<sup>+</sup> NK cells might result from differences within the CD27<sup>+</sup> CD11b<sup>-</sup> and CD27<sup>+</sup> CD11b<sup>-</sup> cell subsets between miR-150<sup>Tg</sup> and *Myb*<sup>+/-</sup> NK cells. To this end, we co-stained NK cells from different

chimeric mice with anti-CD11b antibody and measured Ly49A expression. Both CD27<sup>+</sup> CD11b<sup>-</sup> and CD27<sup>-</sup> CD11b<sup>+</sup> subsets among miR-150<sup>Tg</sup> NK cells showed a lower proportion of Ly49A<sup>+</sup> NK cells compared with WT cells; similar findings were observed in *Myb*<sup>+/-</sup> chimeras (unpublished data). In a third possible scenario, a lack of transcription factors necessary for Ly49A expression could explain the reduction of Ly49A subset in miR-150<sup>Tg</sup> and *Myb*<sup>+/-</sup> NK cells. Gata-3 has been shown to be a direct target of c-Myb (Maurice et al., 2007), and Gata-3 deficiency leads to a reduction of the Ly49A subset of NK cells (Samson et al., 2003). Trans-acting factor transcription factor-1 has been shown to bind the *Klra1* promoter and regulate the Ly49A-bearing NK cell subset in a dosage-dependent manner (Held et al., 1999). Putative binding sites for the transcription factors Runx1 and Atf2 have also been suggested to control Ly49A expression (Kubo et al., 1999; Kunz and Held, 2001), but at least in the case of Atf2, the Ly49 receptor repertoire analysis does not seem to indicate a requirement for this transcription factor for Ly49A expression (Kim et al., 2006). Lastly, it is possible that c-Myb itself binds to and regulates the *Klra1*. A c-Myb-binding sequence is found in the upstream region of *Klra1* (Tanamachi et al., 2004), and we find that the size of the Ly49A-expressing NK cell subset is dependent on the dosage of c-Myb. Future studies in c-Myb-deficient NK cells will definitively prove whether c-Myb is necessary for the acquisition of Ly49A.

We find that miR-150 differentially affects the development of NK and iNKT cell lineages. The literature supports the notion that miR-150 plays different roles depending on cell context. Forced expression of miR-150 promoted proliferation of gastric cancer cells (Wu et al., 2010), whereas it increased the rate of apoptosis and reduced proliferation of NK/T lymphoma cells (Watanabe et al., 2011). miR-150<sup>Tg</sup> mice have a reduced number of splenic B cells, and miR-150<sup>Tg</sup> B cells from the BM showed increased sensitivity to apoptosis when cultured in vitro (Xiao et al., 2007). In contrast, there was an accumulation of NK cells in miR-150<sup>Tg</sup> chimeric mice and no difference was seen in the rate of apoptosis between WT and miR-150<sup>Tg</sup> NK cells. The differences in susceptibility to apoptosis can be partially explained by the higher frequency of mature NK cells and greater percentage of immature B cells (Xiao et al., 2007), but the maturation status alone is probably insufficient to explain the entire phenotype of miR-150<sup>Tg</sup> NK cells. B cells depend on positive signaling through the B cell receptor to promote survival and terminal differentiation (Meffre et al., 2001). B-1 B cells, which require strong BCR signaling to develop (Hayakawa et al., 1999), are significantly reduced in miR-150<sup>Tg</sup> mice (Xiao et al., 2007). The opposite is true for NK cells, which rely on negative signaling through the inhibitory receptors to reach a mature, functionally competent state (Fernandez et al., 2005; Kim et al., 2005; Anfossi et al., 2006). Future studies will examine how miR-150 effects the survival of iNKT cells. Whereas c-Myb is necessary for the survival of double-positive thymocytes (Bender et al., 2004), ectopic expression of miR-150 did not significantly effect T cell

development in the thymus (Xiao et al., 2007; Zhou et al., 2007). Another unexpected finding was the relatively unchanged number of miR-150<sup>-/-</sup> iNKT cells. Perhaps developing iNKT cells are exquisitely sensitive to the dose of miR-150 and its regulated genes; too much miR-150 inhibits iNKT cell development, whereas lack of miR-150 also leads to a mild reduction. It is also possible that miR-150 might have both a negative and a positive role at different stages of iNKT cell development, depending on the expression of the target genes it regulates. This context-specific property may perhaps explain why miR-150 expression varies between different lineages and different developmental stages within the hematopoietic system (Kuchen et al., 2010). In conclusion, this is the first study showing the requirement of a specific miRNA for the development and maturation of NK and iNKT cells.

## MATERIALS AND METHODS

**Mice.** M. Ansel (University of California, San Francisco, San Francisco, CA) provided miR-150<sup>-/-</sup> mice. K. Rajewsky (Harvard Medical School, Boston, MA) provided miR-150<sup>Tg</sup> BM cells (Xiao et al., 2007). T.P. Bender (University of Virginia Health System, Charlottesville, VA) provided *Myb*<sup>+/+</sup> and *Myb*<sup>+/-</sup> BM cells (Bender et al., 2004). To generate mixed BM chimeras, 6–8-wk-old CD45.1-congenic C57BL/6 mice (National Cancer Institute, Frederick, MD) were lethally irradiated with 1,000 rads, and then reconstituted with a 1:1 mix of CD45.1 and either miR-150<sup>-/-</sup>, miR-150<sup>Tg</sup>, *Myb*<sup>+/+</sup>, or *Myb*<sup>+/-</sup> BM cells. Mixed BM cells were then retroorbitally injected into the host mice. Mice were analyzed 8–14 wk after reconstitution. CD45.1<sup>+</sup> C57BL/6 mice were purchased from the National Cancer Institute. *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> C57BL/6 mice were purchased from Taconic. Experiments were done according to the University of California, San Francisco Institutional Animal Care and Use Committee guidelines.

**Flow cytometry.** Fc receptors on cells isolated from peripheral blood, spleen, liver, BM, and thymus were blocked with anti-CD16 + CD32 mAb (clone 2.4G2) at 10 µg/ml before surface staining. Cells were stained with antibodies against NK1.1, TCRβ, CD45.1, CD45.2, CD27, CD11b, NKp46, CD122, CD49b, CD51, CD4, CD8, CD11c, CD19, GR1, Ter119, Ly49H, Ly49D, CD94, NKG2A, Ly49C/I, Ly49G2, Ly49A, CD24, and CD117 (from BD, eBioscience, or BioLegend). Intracellular IFN-γ staining was performed according to the manufacturer's instructions (BD). iNKT cells were identified with a Pacific blue-conjugated mouse CD1d tetramer either unloaded or loaded with PBS57. For apoptosis analysis, freshly isolated splenocytes were first stained with antibodies against NK1.1, TCRβ, CD45.1, CD45.2, and Live/Dead fixable near-IR stain (Invitrogen), washed, and then stained with PE-conjugated Annexin V (BD), according to the manufacturer's protocol. For BrdU labeling, mice were injected i.p. with 200 µg of BrdU (Sigma-Aldrich) in PBS every 24 h for 3 consecutive days, and then sacrificed. For the detection of incorporated BrdU, cells were first stained for surface antigens, and then fixed, permeabilized, treated with DNase I, and stained with APC-conjugated anti-BrdU mAb (BD). Flow cytometry was performed on an LSRII, and data were analyzed with FlowJo software (Tree Star).

**CFSE labeling and adoptive transfer.** Enriched (60–80% NK1.1<sup>+</sup> TCRβ<sup>-</sup>) cells from WT and miR-150<sup>-/-</sup> mice (mixed at a 1:1 ratio) or splenocytes from WT:miR-150<sup>Tg</sup> chimeras were labeled for 8 min with 10 µM CFSE in accordance with the manufacturer's instructions (Invitrogen), and transferred into *Rag2*<sup>-/-</sup> × *Il2rg*<sup>-/-</sup> mice. 3 (Fig. 1) or 5 d (Fig. 5) later, NK cells were analyzed by flow cytometry for CFSE dilution and expression of CD27.

**Ex vivo stimulation assay.** Splenocytes were stimulated in Immulon microtiter plates (Thermo Fisher Scientific) coated with 10  $\mu$ g anti-NK1.1, anti-Ly49H, or control IgG in PBS for 4 h at 37°C in the presence of Golgi-Stop (BD), followed by staining for intracellular IFN- $\gamma$  (BD). For cytokine stimulation, splenocytes were incubated with 20 ng/ml IL-12 (R&D Systems) and 10 ng/ml IL-18 (R&D Systems).

**Cell sorting.** NK cells from spleen were enriched with antibodies against CD5, CD4, CD8, Ter119, Gr-1, CD19 (UCSF Antibody Core), and anti-rat IgG-coated magnetic beads (Miltenyi Biotec). NK cells were stained with antibodies against NK1.1, TCR $\beta$ , CD45.1, CD45.2, NK1.1, TCR $\beta$ , CD11b, and CD27 and were sorted using a FACSAria (BD). NK populations from the BM were sorted using antibodies against CD4, CD8, CD19, GR-1, CD11c, Ter119, CD122, NK1.1, CD51, and CD49b. Thymic iNKT cells were sorted with PBS57-loaded CD1d tetramer and antibodies against NK1.1, TCR $\beta$ , and CD24. The purity of the recovered populations was typically >98%.

**Quantitative RT-PCR (qRT-PCR).** Total RNA was extracted from sorted cells with RNAqueous-Micro kit (Invitrogen). miR-150 expression was examined by using the TaqMan MicroRNA Assay kit (Applied Biosystems) with gene-specific stem-loop PCR primers and TaqMan probe (IDT). SnoRNA202 was used as endogenous control. cDNA was generated by using a SuperScript III First-Strand kit (Invitrogen) and real-time PCR was performed on an Applied Biosystems 7500. PCR of cDNA was conducted using ABI TaqMan kits (c-Myb and HPRT) or SYBR Green PCR Master Mix (Invitrogen). The primer sequences for SYBR green PCR reactions were as follows: c-kit, forward, 5'-TCATCGAGTGTGATGGGAAA-3'; reverse, 5'-GGTGACTTGTTCAGGCACA-3'; Bcl2, forward, 5'-TACCGTCGTGACTTCGCAGAG-3'; reverse, 5'-GGCAGGCTGAGCAGGTCTT-3'; HPRT, forward, 5'-CACAGGACTAGAACACCTGC-3'; reverse, 5'-GCTGGTGAAAAGGACCTCT-3'. Data were analyzed by the CT method (Livak and Schmittgen, 2001).

**Statistical analysis.** All data shown are the mean  $\pm$  SEM. Comparisons between samples were performed by using a two-tailed unpaired Student's *t* test, unless otherwise stated. Statistics were calculated using Prism software (GraphPad Software, Inc.). *P* values were denoted in the following manner: \*, *P* < 0.05; \*\*, *P* < 0.01; \*\*\*, *P* < 0.001.

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