Tie2cre-induced inactivation of the miRNA-processing enzyme Dicer disrupts invariant NKT cell development

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MicroRNAs (miRNAs) are a class of evolutionarily conserved small noncoding RNAs that are increasingly being recognized as important regulators of gene expression. The ribonuclease III enzyme Dicer is essential for the processing of miRNAs. CD1d-restricted invariant natural killer T (*i*NKT) cells are potent regulators of diverse immune responses. The role of Dicer-generated miRNAs in the development and function of immune regulatory *i*NKT cells is unknown. Here, we generated a mouse strain with a tissue-specific disruption of Dicer, and showed that lack of miRNAs after the deletion of Dicer by Tie2-Cre (expressed in hematopoietic cells and endothelial cells) interrupted the development and maturation of *i*NKT cells in the thymus and significantly decreased the number of *i*NKT cells in different immune organs. Thymic and peripheral *i*NKT cell compartments were changed in miRNA-deficient mice, with a significantly increased frequency of $CD4^+CD8^+$ *i*NKT cells in the thymus and a significantly decreased frequency of $CD4^+$ *i*NKT cells in the spleen. MiRNA-deficient *i*NKT cells display profound defects in α-GalCer-induced activation and cytokine production. Bone marrow (BM) from miRNA-deficient mice poorly reconstituted *i*NKT cells compared to BM from WT mice. Also, using a thymic iNKT cell transfer model, we found that *i*NKT cell homeostasis was impaired in miRNA-deficient recipient mice. Our data indicate that miRNAs expressed in hematopoietic cells and endothelial cells are potent regulators of *i*NKT cell development, function, and homeostasis.

T cell | thymus | galactosylceramide | bone marrow

Although only a small number of genetic transcripts (2–3%) code for proteins in higher organisms, a large fraction of the genome is transcribed. MicroRNAs (miRNAs) are a class of 21–25 nt single-stranded non-coding small RNAs that are transcribed from DNA but are not translated into protein. miRNAs are increasingly being recognized as important regulators of gene expression through the inhibition of effective mRNA translation via imperfect base pairing with the 3′-untranslated region (3′ UTR) of target mRNAs in animals. Primary miRNAs are transcribed from DNA segments and are then cleaved by Drosha (a nuclear enzyme) to form a premiRNA that is actively transported out of the nucleus. In the cytoplasm, the ribonuclease III enzyme Dicer cuts the hairpin loop to form the mature miRNA, which is incorporated into the RNA-induced silencing complex (RISC) to impede mRNA translation into protein (1–3). Dicer is required for the processing of mature and functional miRNAs. Therefore, the deletion of Dicer provides a genetic test for the relevance of miRNAs in mammalian development. Emerging evidence suggests that miRNA-mediated gene regulation represents a fundamental layer of genetic programming at the posttranscriptional level and has diverse functional roles in animals, including development, differentiation, and homeostasis (4–10).

Defining the role of Dicer-generated miRNAs in mammalian development is complicated by the embryonic lethality of constitutive Dicer knockouts in mice (10). The overall importance of miRNAs during hematopoiesis has been investigated by specific disruption of steps in miRNA biogenesis (4, 6, 7, 11–13). Using Cre-loxP tissue-specific Dicer deletion, Cobb et al. (11) and Muljo et al. (7) each reported that deletion of Dicer in early T cell development results in reduced thymocyte number and increased thymocyte susceptibility to cell death. Dicer-deficient helper T cells preferentially expressed IFN-γ, the hallmark effector cytokine of the Th1 lineage. Using a similar mouse model, Cobb et al. (4) further reported that deletion of Dicer in the thymus interrupted $CD4^+CD25^+Foxp3^+$ regulatory T (Treg) cell development as well as reduced in vitro $CD4^+CD25^+Foxp3^+$ Treg cell induction by TGF-β. In addition, Dicer-deficient mice spontaneously developed colitis and other inflammatory diseases, indicating a role for Dicer in regulation of the immune system. More recently, 2 groups reported that depletion of Dicer within the $CD4^+CD25^+Foxp3^+$ Treg cell lineage resulted in fatal systemic autoimmune diseases, and that Dicer-deficient $CD4^+CD25^+Foxp3^+$ Treg cells lose suppression activity in vivo (6, 13). Thus, the role of Dicer in immune regulation has clearly demonstrated that miRNAs are important regulators of the development and regulatory function of $CD4^+CD25^+Foxp3^+$ Treg cells.

Natural killer T (NKT) cells comprise another major subset of regulatory T cells in mice and humans. NKT cells possess the properties of both T cells and NK cells as they co-express a rearranged T cell receptor (TCR) and several NK cell receptors, including NK1.1. Most NKT cells are restricted to the non-classical MHC-I like molecule CD1d and preferentially use an invariant TCR consisting predominantly of the Vα14-Jα18/Vβ8 pair in mice (14–16). These invariant NKT (*i*NKT) cells are almost uniformly reactive to the synthetic glycolipid ligand α-galactosylceramide (α-GalCer) presented by CD1d, and they can be identified using α-GalCer-loaded CD1d tetramers. *i*NKT cells are potent regulators of diverse immune responses, including the onset of cancer, infection, and autoimmune diseases (14–19). The role of miRNAs in *i*NKT cell development is currently unknown. Here, we tested the role of miRNAs in *i*NKT cell development by generating a mouse strain with Tie2 Cre mediated tissue-specific disruption of Dicer (Tie2 is expressed in hematopoietic progenitors and endothelial cells). We found that deficiency of miRNAs during hematopoiesis resulted in significantly fewer *i*NKT cells in the thymus, spleen, and liver, and interrupted the development and maturation of *i*NKT cells in the thymus. miRNA-deficient peripheral *i*NKT cells display profound defects in α-GalCer-induced activation and cytokine production. Bone marrow (BM) from miRNA-deficient mice poorly reconstituted *i*NKT cells compared to BM from wild-type (WT) mice. Further more, using a thymic *i*NKT cell transfer model,

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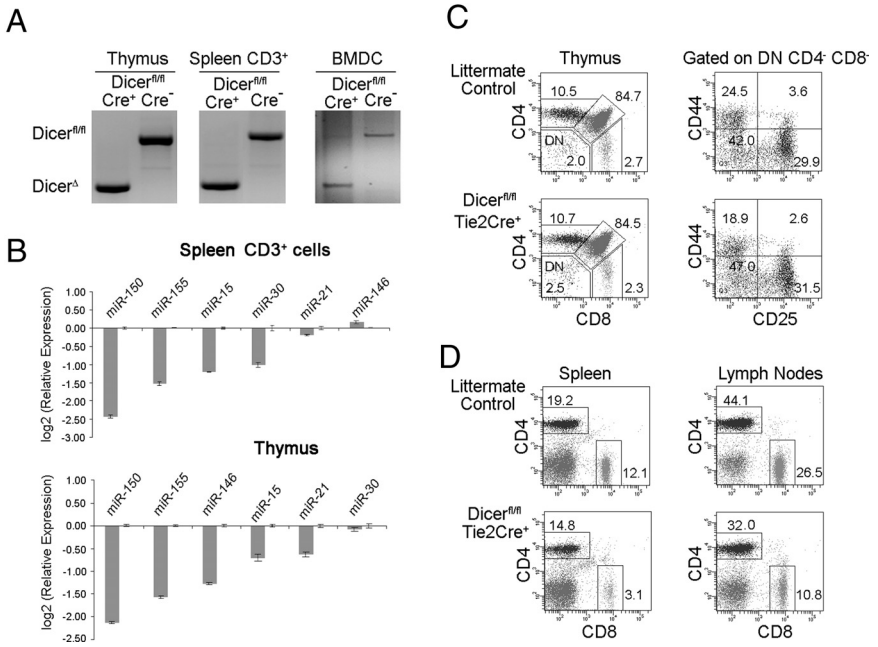

Fig. 1. Ablation of dicer expression in hematopoietic stem cells. (*A*) PCR typing of genomic DNA isolated from thymocytes, CD3$^+$ T cells, and bone marrow-derived dendritic cells (BMDC) of *Dicer*$^{fl/fl}$*Tie2cre*$^+$ and *Dicer*$^{fl/fl}$*Tie2cre*$^-$ mice. The deletion Dicer allele produced a 471-bp PCR product whereas the WT allele resulted in a 1,300-bp product. (*B*) Micro RNA gene expressions in thymus and spleen CD3$^+$ cells by Taqman real-time PCR. Results were the average measured in duplicate and normalized to a control gene (snoRNU 202). Error bars are SD. The expression of miRNAs in thymus and splenic T cells was significantly reduced in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice compared to *Dicer*$^{fl/fl}$*Tie2cre*$^-$ mice. (*C*) Flow cytometric analysis of thymocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^+$ and *Dicer*$^{fl/fl}$*Tie2cre*$^-$ littermates. Contour plots depict CD4 (*y* axis) versus CD8 (*x* axis) staining profiles in thymus cells (*Left*), CD44 (*y* axis) versus CD25 (*x* axis) staining profile in gated CD4$^-$CD8$^-$ DN thymus cells (*Right*). (*D*) Flow cytometric analyses of spleen (*Right*) and lymph nodes (*Left*) from *Dicer*$^{fl/fl}$*Tie2cre*$^+$ and *Dicer*$^{fl/fl}$*Tie2cre*$^-$ littermates. Percentages of cells in each quadrant are indicated. Data are representative of 3 independent experiments (2–3 mice per group).

we found that WT *i*NKT cell homeostasis was impaired in miRNA-deficient recipient mice. Our data indicate that miRNAs expressed in hematopoietic cells and endothelial cells are potent regulators of *i*NKT cell development, function, and homeostasis.

Results and Discussion

Ablation of Dicer Expression in Hematopoietic Stem Cells. To investigate the role of miRNAs in immunological function, we generated a hematopoietic specific Dicer mutation mouse by mating loxP-flanked Dicer gene mutation *Dicer*$^{fl/fl}$ mice (9) with *Tie2*-Cre transgenic mice (20). *Tie2* kinase is specifically expressed by hematopoietic progenitors and endothelial cells. The *Tie2* gene promoter driving Cre expression was used to delete the genes in bone marrow and endothelial cells (20). We obtained mice homozygous for *Dicer*$^{fl/fl}$ with *Tie2*-Cre expression, which are conditional Dicer knockout (KO) mice. These mice are designated as *Dicer*$^{fl/fl}$*tie2cre*$^+$ (Dicer KO) mice. Littermate *Dicer*$^{fl/fl}$*tie2cre*$^-$ mice were used as wild-type (WT) controls. There was substantial deletion of Dicer in hematopoietic cells, including thymocytes, CD3$^+$ splenocytes, and bone marrow-derived dendritic cells in these mice (Fig. 1*A*). Taqman real-time PCR further indicated that miRNA gene expression was significantly reduced in the thymus and spleen CD3$^+$ T cells, including miR-150, mir-155, and miR-15 (Fig. 1*B*). *Dicer*$^{fl/fl}$*tie2cre*$^+$ mice were viable and fertile. We then examined the development of B cells, macrophages, and dendritic cells in the bone marrow. *Dicer*$^{fl/fl}$*tie2cre*$^+$ mice showed no significant defects in the number and frequencies of macrophages, different stages of B cells, and bone marrow-derived dendritic cells compared to WT littermate controls. Analyses of T cell development in the thymus showed that the total number of thymocytes in *Dicer*$^{fl/fl}$*tie2cre*$^+$ mice was comparable to that in *Dicer*$^{fl/fl}$*Tie2cre*$^-$ WT littermate controls. Flow cytometry analyses demonstrated that the percentages of the thymic subsets, CD4 and CD8 single-positive (SP) T cells, CD4$^+$CD8$^+$ double-positive (DP) and CD4$^-$CD8$^-$ double-negative (DN) T cells, were also normal compared to littermate controls (Fig. 1*C Left*). 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$^+$ (DN3) stage. To further dissect the early T cell development profile in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice, we gated DN thymocytes and analyzed their CD44 and CD25 expression profiles. As shown in Fig. 1*C Right*, the *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mouse showed a DN thymocyte developmental profile comparable to the WT littermate control. Previous studies indicated that expression of Dicer in the thymus is required for the optimal maturation and homeostasis of peripheral T cells, particularly CD8$^+$ T cells (4, 7, 11). Therefore, we next analyzed peripheral lymphocytes. As shown in Fig. 1*D*, lymphocytes in spleen and lymph nodes showed a marked reduction of CD8$^+$ T cells (4- and 2-fold reduction, respectively) with a smaller reduction in CD4$^+$ cells in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice. Thus, our data lend further support to previous studies (4, 7, 11), as deletion of Dicer in hematopoietic progenitors did not significantly affect thymoctye development but did influence peripheral CD8 T cell homeostasis. In addition, we found that *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice had significantly reduced CD4$^+$CD25$^+$Foxp3$^+$ Treg cells in the thymus, spleen and lymph nodes compared to WT mice. This observation supports recent findings that miRNAs are required for CD4$^+$CD25$^+$Foxp3$^+$ Treg cell development (4, 6, 13). Taken together, the immune phenotypes in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice are very similar to *Dicer*$^{fl/fl}$*CD4cre*$^+$ or *Dicer*$^{fl/fl}$*Lckcre*$^+$ mice (4, 7, 11).

Number of *i*NKT Cells Is Reduced in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ Mice. To assess the role of miRNAs in *i*NKT cell development, we first examined the frequency and number of *i*NKT cells in the different hematopoietic organs of *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice and WT littermate controls by flow cytometry analysis. The percentages of *i*NKT cells stained by anti-TCRβ and α-GalCer-loaded CD1d tetramer in the liver, spleen, and thymus from *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice were severely decreased compared with littermate controls, with an almost complete absence in the liver (Fig. 2*A*). We further confirmed that the observed defect is due neither to Cre expression in hematopoeitic progenitor cells nor to Dicer-loxp insertion (Fig. S1). Consistent with the decreased proportions of *i*NKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice, the absolute numbers of *i*NKT cells in the liver, spleen, and thymus were drastically reduced in *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice compared to WT littermate controls (Fig. 2*B*). Thus, *Dicer*$^{fl/fl}$*Tie2cre*$^+$ mice lack CD1d-restricted *i*NKT cells.

*i*NKT cells are known to proliferate and produce IFN-γ and IL-4 upon engagement of their invariant TCR with CD1d-presented α-GalCer (17, 21). Stimulation of splenocytes and thymocytes from WT littermate control mice with α-GalCer resulted in robust cell proliferation. In contrast, no significant cell proliferation was

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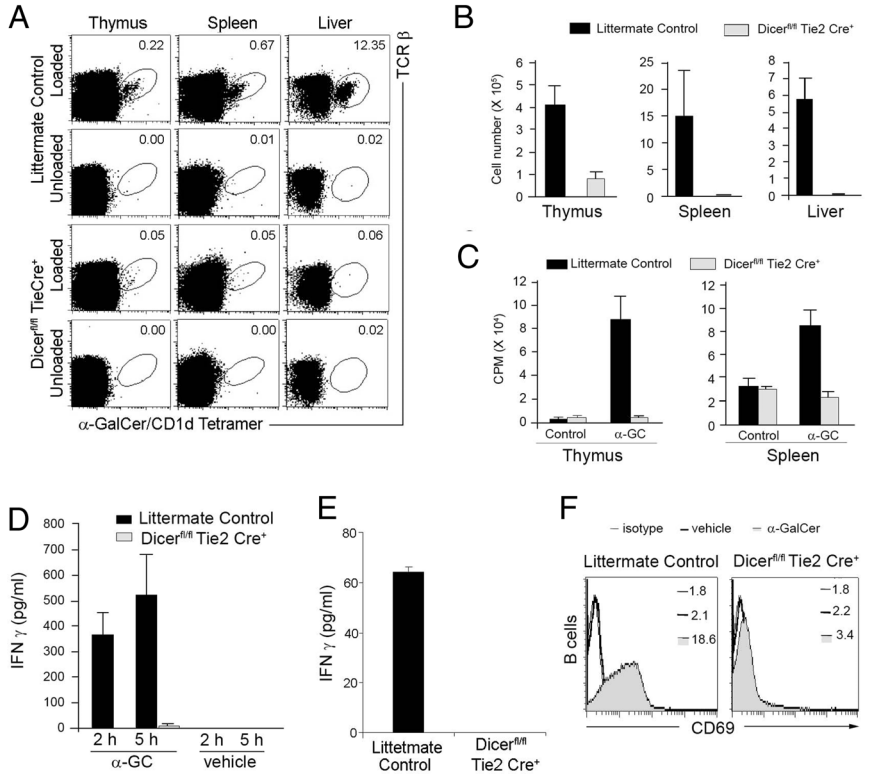

Fig. 2. The number of *i*NKT cells is reduced in *Dicer*$^{fl/fl}$*Tie2re*$^{+}$ mice. *i*NKT cells were analyzed by flow cytometory in the thymus, spleen, and liver of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and WT littermates (*Dicer*$^{fl/fl}$*Tie2cre*$^{-}$) (2 to 3 mice per group, 4–8 weeks old). (*A*) Cells isolated from the indicated tissues of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice were co-stained with α-GalCer-loaded CD1d tetramers and a TCRβ-specific mAb. The numbers of *i*NKT cells are shown as a percentage of gated B220^{-} lymphocytes from spleen and liver, and of total lymphocytes from thymus. (*B*) Absolute numbers of *i*NKT cells. Using the total cell count obtained from each organ and the proportion of *i*NKT cells (as shown in *A*), the absolute numbers of *i*NKT lymphocytes were determined. Data (mean ± SD) are representative of 4 separate experiments in which 5 to 8 mice of each strain were analyzed. (*C*) Defective proliferation of thymic and splenic *i*NKT cells. Thymocytes and splenocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice were cultured with 100 ng/mL α-GalCer for 48 h. Proliferation of cells was assessed by [^{3}H]thymidine incorporation. Spontaneous proliferation in the absence of α-GalCer was similar with both splenocytes and thymocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice. Data are mean ± SD, representative of 3 independent experiments. (*D*) Impaired α-GalCer-dependent *i*NKT cell responses in vivo in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice were injected with 2 μg of α-GalCer or vehicle. Serum was collected at 2 and 5 h for detection of IFN-γ by ELISA. (*E*) Defective cytokine production of thymocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. Thymocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice were cultured with 100 ng/mL α-GalCer. The culture supernatant was collected at 24 h for detection of IFN-γ by ELISA. (*F*) The expression of CD69 by splenic B cells was analyzed 5 h after α-GalCer injection. CD69 mean fluorescence intensity (MFI) is indicated.

observed with *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ thymocytes and splenoytes (Fig. 2*C*). Consistent with the proliferation results, robust production of IFN-γ was induced after α-GalCer stimulation in vivo and in vitro from WT littermate control mice, but not from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice (Fig. 2 *D* and *E*). Up-regulation of CD69 has been demonstrated in B cells, a consequence of the *i*NKT-induced cytokine storm after α-GalCer stimulation (22). However, we did not detect significant up-regulation of CD69 in B cells from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice compared to WT littermate control mice (Fig. 2*F*). Taken together, these results further indicate that the compartment of *i*NKT cells was functionally impaired in the absence of miRNAs in hematopoietic stem cells.

The *i*NKT Cell Development and Maturation Are Impaired in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ Mice. During *i*NKT cell development in the thymus, CD1d-restricted *i*NKT cell precursors primarily acquire the V14α-Jα18/Vβ8 TCR that allows their subsequent selection by the CD1d-presented self-glycolipid expressed by CD4^{+}CD8^{+} thymocytes (23). To ascertain that the defect of *i*NKT cells was not due to defective CD1d expression, thymocytes and splenocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice and littermate controls were compared for CD1d expression and were found to be almost equivalent (Fig. 3*A*). Thus, we conclude that loss of CD1d expression does not account for the defect of *i*NKT cells observed in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. After positive selection, *i*NKT precursors progress through CD44^{lo} and CD44^{hi} stages and lastly acquire NK1.1 expression during their final maturation (23–25). Therefore, the CD44/NK-1.1 profiles of tetramer^{+} thymocytes were analyzed to determine if *i*NKT cell development and maturation were defective in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. The frequencies of mature CD44^{hi}NK-1.1^{+} *i*NKT cells were significantly reduced by >3-fold in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice, while the percentage of immature CD44^{lo}NK-1.1^{-} *i*NKT cells was increased by 2-fold (Fig. 3*B*). Differences between *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice and littermate controls were even larger when the absolute numbers of the different subpopulations were compared (Fig. 3*C*). A previous study indicated that the most immature iNKT cells found in the thymus express heat stable antigen (HAS)^{high}, while mature iNKT cells are NK1.1^{+}Tet^{+}HSA^{low} (26). As shown in Fig. S2, the majority of thymic iNKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice were indeed immature Tet^{+}HSA^{high}, and few mature NK1.1^{+}Tet^{+}HSA^{low} iNKT cells were detected in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. Collectively, these results identify a severe impairment in *i*NKT cell development and maturation at the CD44^{low} to CD44^{hi} and NK1.1^{-} to NK1.1^{+} checkpoints in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice.

Thymic and peripheral *i*NKT cell compartments are composed of well-defined subsets, and the majority are CD4^{+} and CD4^{-}CD8^{-} DN *i*NKT cells. The existence of very rare α-Galcer CD1d tetramer-positive DP thymocytes is controversial (26, 27). We next examined these subsets on gated α-GalCer CD1d tetramer-positive *i*NKT cells in the thymus and spleen. Surprisingly, in the thymus (Fig. 3 *D* and *E*), a dramatic increase in frequency of CD4^{+}CD8^{+} *i*NKT cells (26.64 ± 6.67%, $n = 6$) was seen in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice compared to littermate controls (4.18 ± 3.2%, $n = 9$), $P = 0.00016$, while a significantly decreased frequency of CD4^{-}CD8^{-} *i*NKT cells (21.16 ± 3.5% vs. 34.95 ± 8.49%, $P = 0.0011$) was observed. An increase of CD4^{+}CD8^{+} *i*NKT cells in the thymus is further suggestive of an early block in *i*NKT cell development (26). As shown in Fig. S2, most immature Tet^{+}HSA^{high} *i*NKT cells were CD4^{+}CD8^{+} and the CD4^{-}CD8^{-} *i*NKT cells were significantly reduced in Tet^{+}HSA^{low} iNKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice, suggesting a defect in iNKT cell development from CD4^{+}CD8^{+} to CD4^{+} iNKT cells and from CD4^{+} to CD4^{-}CD8^{-} iNKT cells. A higher frequency of CD4^{+}CD8^{+} *i*NKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice was also seen in the spleen (Fig. 3*F*, $P = 0.0001$). Strikingly, there was more than a 2-fold reduction of splenic CD4^{+} *i*NKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice compared to control mice ($P = 1.9E^{-10}$). Together, these results suggest that the development of *i*NKT cell subsets in the thymus and their main-

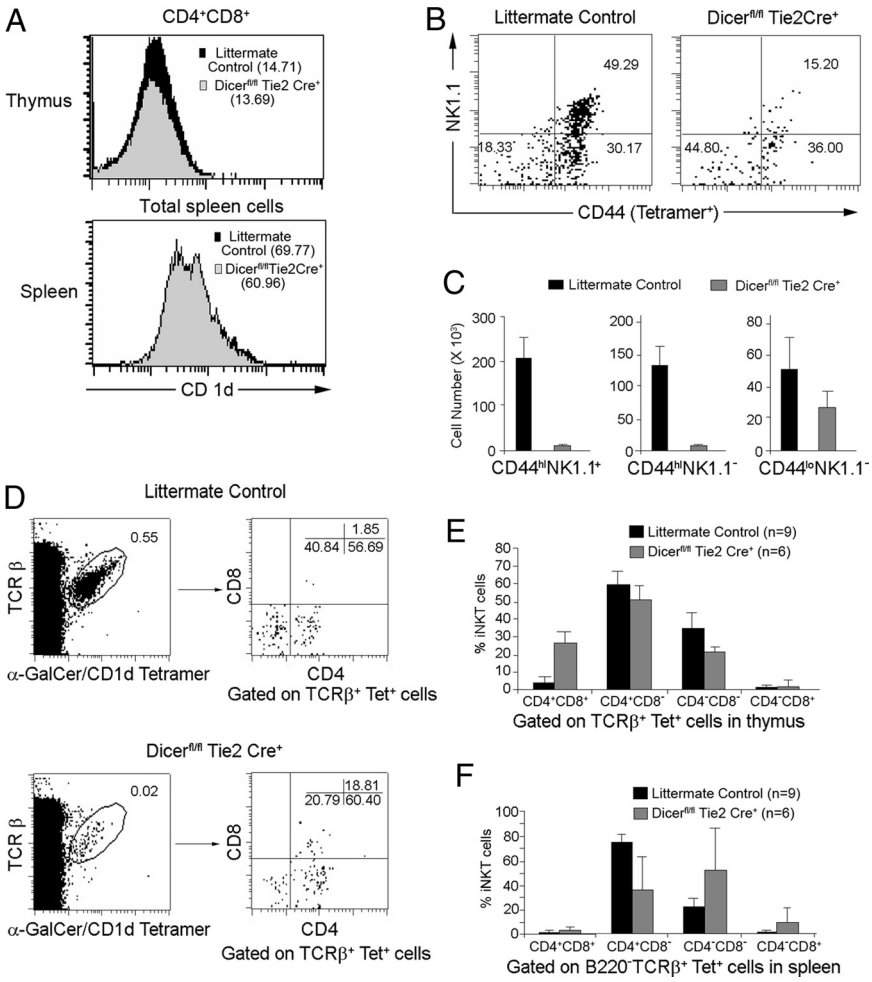

Fig. 3. Abnormal development of iNKT cells in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. (*A*) CD1d expression on CD4CD8 DP thymocytes and total splenocytes from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermates were compared. CD1d MFI is shown in parentheses. Data are representative of 3 independent experiments. (*B*) CD44 and NK1.1 expression in thymic *i*NKT cells. Events shown are gated on TCRβ and CD1d tetramer double-positive cells. (*C*) Absolute number of *i*NKT cell subpopulations (mean ± SD) based on their CD44 and NK1.1 expression patterns. (*D–F*) The *i*NKT cell subset distribution in thymus and spleen are abnormal in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. TCRβ and CD1d tetramer double-positive cells were analyzed in the thymus (*D* and *E*) and spleen (*F*) of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermates and the relative frequencies of subsets defined by CD4 and CD8 expression were assessed (mean ± SD).

tenance in the periphery are affected by the absence of miRNAs in the hematopoietic progenitors.

Micro RNAs Expressed in Bone Marrow and Endothelial Cells Regulate *i*NKT Cell Development and Homeostasis. Accumulated studies suggest that *i*NKT cell development and function are regulated by both the bone marrow and microenvironments. Although the findings from the *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice are suggestive of a cell-intrinsic (hematopoietic cell) defect in *i*NKT development, these do not exclude the possible role of subtle yet important defects in the microenvironment of the *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mouse, since the *Tie2* gene promoter drives Cre expression in both bone marrow and endothelial cells. Recent studies indicate that the maintenance and regulation of endogenous miRNA levels via Dicer mediated processing is also critical for endothelial cell gene expression and function (28, 29). The reduced number of *i*NKT cells in the *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mouse may reflect either a defect in *i*NKT cell precursors, positive selection and maturation of *i*NKT cells, and/or a block in *i*NKT cell homeostasis in the peripheral tissues, which is regulated by both the bone marrow and microenvironments. Initially, we used a bone marrow transfer model to further investigate if a *Tie2*-mediated *Dicer* deficiency influences *i*NKT cell development via a stem cell-intrinsic mechanism. Lethally irradiated normal C57/B6 mice were reconstituted with donor *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mouse or *Dicer*$^{fl/fl}$*Tie2cre*$^{-}$ littermate bone marrow. As shown in Fig. 4*A*, the bone marrow from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice poorly reconstituted *i*NKT cells in the thymus, spleen, and liver compared to bone marrow from littermate controls, suggesting a cell-intrinsic defect in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. Interestingly, slightly recovered *i*NKT cells were detected in mice that received *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ BM compared to unmanipulated *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice, particularly in the liver, indicating the miRNA expression in endothelial cells may also regulate *i*NKT cell trafficking and/or homeostasis. Recent studies indicate that lymphocyte trafficking to lymphoid organs or non-lymphoid tissues is governed in large part by the interactions of selectively expressed adhesion receptors and chemokine receptors on the surface of diverse lymphocytes with ligands on the endothelium, including CXCR6/CXCL16 interaction for *i*NKT trafficking (30). To further test an extrinsic defect on *i*NKT cell trafficking in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice, a thymic *i*NKT cell transfer experiment was performed in which CD8-depleted thymus cells (*i*NKT cells enriched up to 10-fold) from B6.CD45.1 congenic mice were transferred to irradiated *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice and WT littermate (CD45.2 receipts). The CD45.1^{+} lymphocytes from the spleen and liver were analyzed for their frequency of *i*NKT cells at day 5 after transfer. As shown in Fig. 4*B*, the *i*NKT cells were moderately reduced in the liver but not in the spleen from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ recipient mice compared to their WT littermates. Interestingly, CD4^{+} *i*NKT cells were reduced in spleen from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ recipient mice (Fig. S3), which may provide a possible explanation why CD4^{+} *i*NKT cell frequency was largely decreased in the spleen of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice (Fig. 3*F*), while a relatively normal population of CD4^{+} *i*NKT cells is still present in the thymus (Fig. 3*E*). These data suggest that a lack of miRNAs in endothelial cells may affect *i*NKT cell homeostasis. Therefore, the defective *i*NKT cells observed in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice may result from both intrinsic (hematopoietic cells) and extrinsic (endothelia cells) defects.

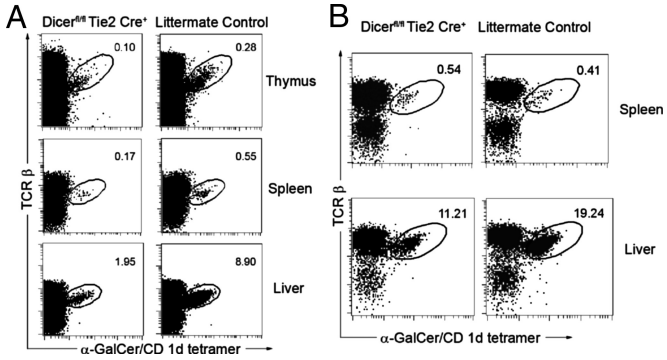

Fig. 4. The defect of *i*NKT cells in Dicer deletion mice are both cell intrinsic and extrinsic. (*A*) B6 lethally irradiated hosts (4 to 5 mice per group) were reconstituted with *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ or littermate control bone marrow cells. Eight weeks later, host thymus, spleen and liver were examined by staining with α-GalCer/CD1d tetramers and anti-TCRβ antibody. (*B*) CD8-depleted thymocytes (up to 4% of recovered thymocytes were Tetramer^{+} *i*NKT cells) from CD45.1-congenic mice were transferred to irradiated 6 week-old *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ or littermate control mice (2 to 3 mice per group). The CD45.1^{+} lymphocytes from the spleen and liver were analyzed for *i*NKT cells at day 5 after transferring. The percentages of *i*NKT cells are shown. Data are representative of 2–3 experiments.

Impaired *i*NKT Cell Function in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ Mice. One unique feature of *i*NKT cells is the prompt production of large amounts of cytokines in response to TCR signaling. The lack of proliferation and cytokine response to α-GalCer in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice could be explained by the markedly reduced numbers of *i*NKT cells. In addition, *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ *i*NKT cells may have an intrinsic defect precluding them from activation and producing cytokines. To test the latter possibility, we again injected mice *i.v.* with α-GalCer and examined the production of cytokines by *i*NKT cells with intracellular cytokine staining. As shown in Fig. 5*A*, the number of splenic *i*NKT cells that express intracellular IL-4 and IFN-γ were increased 2- to 3-fold in WT littermate controls after in vivo α-GalCer stimulation (40 min), while splenic *i*NKT cells from *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice had almost no response to α-GalCer stimulation. The decreased cytokine production response to α-GalCer stimulation in Dicer-deficient mice could result from either a defect in cytokine synthesis or TCR signaling, thereby affecting the activation of *i*NKT cells. We therefore further investigated whether Dicer-deficient *i*NKT cells are activated normally upon *i.v.* α-GalCer stimulation. As shown in Fig. 5*A Bottom*, α-GalCer stimulation resulted in up-regulation of CD69 expression, a downstream marker of TCR signaling, in *i*NKT cells from WT mice. In contrast, α-GalCer stimulation did not significantly up-regulate CD69 expression in Dicer-deficient *i*NKT cells. Those observations strongly argue that miRNA deficiency leads to a defect in TCR signaling. To further localize the block in TCR-mediated signal transduction targeted by miRNAs, we stimulated *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ *i*NKT cells in vitro with phorbol myristate acetate (PMA) and ionomycin, which bypass proximal TCR-mediated signaling events and activates cells most likely at a stage proximal to protein kinase C and calcium flux (31). Three hours after stimulation with PMA and ionomycin, >60% of splenic *i*NKT cells from WT control mice stained positive for IL-4 and IFN-γ, respectively, while only 26.9% and 18.2% of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ *i*NKT cells stained positive for IL-4 and IFN-γ, respectively (Fig. 5*B*). Taken together, miRNAs seem to be critical for *i*NKT cell activation and cytokine production.

In summary, depletion of miRNAs by eliminating Dicer at the bone marrow stem cell stage significantly reduces the frequencies of *i*NKT cells, but seems to be dispensable for early thymocyte development and CD4/CD8 lineage commitment. Dicer-generated miRNAs in hematopoietic cells are required for *i*NKT cell development, maturation, and function. Defects in the number and function of *i*NKT cells may also contribute to autoimmunity in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. In addition, miRNAs expression in endothelial cells regulates iNKT cell homeostasis in the liver. The identification of specific miRNAs differentially required for the proper development, function, and homeostasis of *i*NKT cells and the downstream genes targeted by miRNAs may further unravel the immunological and molecular mechanisms underlying *i*NKT cell development, and may also facilitate the development of new intervention strategies for cancer and autoimmune diseases related to *i*NKT cell function.

Materials and Methods

Mice. Mice carrying a conditional floxed allele of *Dicer* (*Dicer*^{flox}) (9) were backcrossed onto the C57BL/6 background for 5 generations and then mated to C57BL/6 mice carrying the Tie2 Cre allele (obtained from The Jackson Laboratory) (20) to generate *Dicer*$^{flox/flox}$ *Tie-2*$^{cre/+}$ conditional knockout mice, designated as *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$. All WT mice (*Dicer*$^{fl/fl}$*Tie2cre*$^{-}$), unless indicated otherwise, are littermate controls of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ mice. Experiments were conducted at 4–8 weeks of age, unless otherwise indicated. Mice were housed in a specific pathogen-free barrier unit. Handling of mice and experimental procedures were in accordance with requirements of the Institutional Animal Care and Use Committee.

Genotyping. Offspring were genotyped using the following PCR primer pairs: for Cre, 5′-TGATGAGGTTCGCAAGAACC-3′ and 5′-CCATGAGTGAACGAACCTGG-3′ (product size: 420 bp); and for *Dicer*, 5′-CCTGACAGTGACGGTCCAAAG-3′ (DicerF1) and 5′-CATGACTCTTCAACTCAAACT-3′ (product sizes: 420 bp from the *Dicer*^{flox} allele and 351 bp from the wild-type *Dicer* allele). The deletion allele was genotyped using primers DicerF1 and DicerDel (5′-CCTGAGCAAGGCAAGTCATTC-3′). The deletion allele produced a 471-bp PCR product whereas the WT allele resulted in a 1,300-bp product.

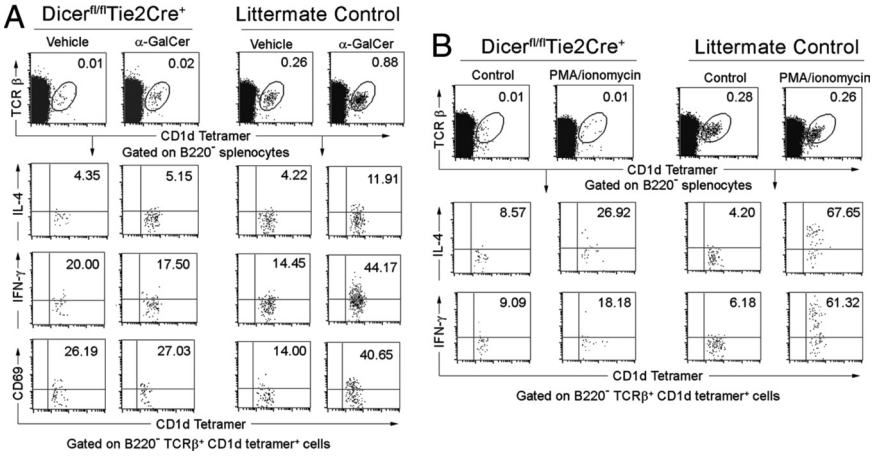

Fig. 5. Impaired *i*NKT cell function upon in vivo α-GalCer treatment and in vitro PMA/ionomycin stimulation. (*A*) IL-4 and IFN-γ production by splenic *i*NKT cells was analyzed after α-GalCer or vehicle injection by intracellular cytokine staining. Events shown are gated on B220-negative, TCRβ-positive, CD1d tetramer-positive events. α-GalCer or vehicle-stimulated *i*NKT cells were also analyzed for the expression of the activation marker CD69. (*B*) Whole splenocytes of *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ and littermate control mice were treated with or without PMA and ionomycin for 3 h in vitro. The production of IL-4 and INF-γ by splenic *i*NKT cells were analyzed with intracellular cytokine staining. Data are representative of 3 experiments.

Flow Cytometry. Single-cell suspensions were washed twice with staining buffer (PBS, 2% FCS) and incubated with Fc Block (clone 2.4G2). Cells were stained with α-GalCer/CD1d tetramers as described (17). The following conjugated monoclonal antibodies (mAbs) were used: NK1.1 (PK136), TCRβ (H57–597), CD44 (IM7), CD1d (1B1), FoxP3 (FJK-16s), CD69 (H1.2F3), CD8 (53–6.7), CD4 (RM4–5), CD25 (PC61), IL-4 (11B11), and IFN-γ (XMG1.2). All mAbs were from BD Biosciences or eBioscience. Data were analyzed using CELLQuest Pro software (BD Biosciences).

Cell Proliferation Assay. Spleen and thymus cell suspensions were incubated in complete medium supplemented with or without 100 ng/mL of α-GalCer (Alexis). After 30 h in culture, [^{3}H]thymidine was added and the culture continued for an additional 18 h. Cells were then harvested and counted in a microbetaplate counter (Wallac).

In Vivo αGalCer-Induced Activation Assays. Two micrograms of α-GalCer or vehicle in 100 μL of PBS were injected into the tail vein. For serum cytokine determination, mice were euthanized and bled at 2 and 5 h. The blood was allowed to clot, and the serum was separated from the clot by centrifugation. IL-4 and IFN-γ were detected by sandwich ELISA using capture and biotinylated antibodies (Abs) from R & D Systems. For secondary stimulation of B cells, splenocytes were collected 5 h after α-GalCer injection and stained with anti-CD69 and anti-B220. For intracellular cytokine staining, splenocytes were collected at 40 min after injection and cultured in T cell medium (RPMI 1640 with 10% FCS, Hepes, penicillin and streptomycin, pyruvate, nonessential amino acids, L-glutamine, and 2-ME). Monensin was added to a final concentration of 3 μM, and the cells were incubated for an additional hour. Cells were extracellularly stained with anti-TCRβ and CD1d-tetramer. After washing and fixing with 2% paraformaldehyde, cells were permeabilized with 0.1% saponin and stained with anti-IFN-γ and anti-IL4 Abs and analyzed by flow cytometry.

Bone Marrow and *i*NKT Cell Transfer Experiments. To generate bone marrow chimeras, 6–8 week-old female recipient mice (C57/B6) were lethally irradiated initially with 900 rads. Donor bone marrow was harvested from 4 to 6 week-old *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ or littermate control mice by flushing with a syringe containing sterile tissue culture medium. After erythrocyte lysis, mature T cells were depleted by biotin-conjugated anti-mouse CD3 (BD Biosciences) mAbs and anti-biotin magnetic beads (Miltenyi Biotec) using an AutoMACS sorter (Miltenyi Biotec.). Depletion of >90% mature T cells was confirmed by flow cytometry. Donor bone marrow cells after T cell depletion (5×10^6 cells per mouse, in a volume of 100 μL) were then injected into irradiated recipients by the intraocular vein in the retro-orbital plexus. The chimeras were analyzed 8 weeks after reconstitution for *i*NKT cell development. For *i*NKT cell transfer, CD8 depleted thymocytes (3–4% of recovered thymocytes were Tetramer^{+} *i*NKT cells) from CD45.1-congenic mice were transferred to irradiated 6 week-old *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ or littermate control mice. The CD45.1^{+} lymphocytes from the thymus, spleen, and liver were analyzed for *i*NKT cells at day 5 after transfer.

Real-Time RT-PCR for miRNA Expression. Total thymic cells and splenic CD3^{+} T cell RNA from 4 to 6 week-old *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ or littermate control mice, respectively, were harvested using the Ambion mirVana miRNA isolation kit (Ambion) according to the manufacturer's instructions. The expression levels of miR-150, miR-155, miR-15, miR-30, miR-21, and miR-146 were examined using the Applied Biosystems TaqMan MicroRNA Assay kit (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions. The kit uses gene-specific stem-loop reverse transcription primers and TaqMan probes to detect mature miRNA transcripts. PCR amplification was carried on the Applied Biosystems 7900 Real-Time PCR system. The assay was run in duplicate for each case to allow for assessment of technical variability. snoRNU202 was used as endogenous control. Relative quantitation using the $\Delta\Delta$ct method in *Dicer*$^{fl/fl}$*Tie2cre*$^{+}$ versus littermate control mice was carried out and fold changes were calculated for each gene.

Statistics. Data were analyzed by the 2-tailed Student's *t* test. The differences were considered significant with a $P < 0.05$.

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