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Dicer-Dependent MicroRNA Pathway Controls Invariant NKT Cell Development

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Invariant NK T (iNKT) cells are a separate lineage of T lymphocytes with innate effector functions. They express an invariant TCR specific for lipids presented by CD1d and their development and effector differentiation rely on a unique gene expression program. We asked whether this program includes microRNAs, small noncoding RNAs that regulate gene expression posttranscriptionally and play a key role in the control of cellular differentiation programs. To this aim, we investigated iNKT cell development in mice in which Dicer, the RNase III enzyme that generates functional microRNAs, is deleted in cortical thymocytes. We find that Dicer deletion results in a substantial reduction of iNKT cells in thymus and their disappearance from the periphery, unlike mainstream T cells. Without Dicer, iNKT cells do not complete their innate effector differentiation and display a defective homeostasis due to increased cell death. Differentiation and homeostasis of iNKT cells require Dicer in a cell-autonomous fashion. Furthermore, we identify a miRNA profile specific for iNKT cells, which exhibits features of activated/effector T lymphocytes, consistent with the idea that iNKT cells undergo agonist thymic selection. Together, these results define a critical role of the Dicer-dependent miRNA pathway in the physiology of iNKT cells. The Journal of Immunology, 2009, 183: 2506–2512.

Invariant natural killer T cells are a separate lineage of innate-like T lymphocytes that in mice express a TCR made of the invariant Vα14–Jα18 rearrangement paired with Vβ8.2, −7, or −2 (1). Invariant NK T (iNKT) cells recognize both self and exogenous lipids presented by the MHC class I-like molecule CD1d and play a role in infection, autoimmunity, allergy, and cancer (1). iNKT cell developmental pathway is distinct from that of mainstream T lymphocytes (1, 2). CD4CD8 double positive (DP) precursors that have randomly rearranged the canonical invariant TCR are positively selected into the iNKT cell lineage upon recognition of endogenous agonist lipids, presented by CD1d on DP thymocytes (1, 2). Postselection iNKT cells undergo an ordered phenotypic maturation characterized by four stages: the first, detectable immediately after positive selection, is the HSAhighCD69highCD44 highNK1.1 + stage 0, which becomes HSA lowCD44 lowNK1.1 + stage 1, followed by CD44 high NK1.1 + stage 2, and finally by the mature CD44highNK1.1 + stage 3, which occurs both in the thymus and periphery (3–5). Maturation is accompanied by a massive cellular expansion between stage 1 and 2 (4). Furthermore, developing iNKT cells acquire also effector cytokine expression in the thymus independently of foreign Ag encounter (4, 5), resulting in the rapid display of effector functions in thymic iNKT cell emigrants.

The iNKT cell development is selectively controlled by the SLAM/Fyn/SAP/PKC-θ signal transduction pathway, the NFκB, T-bet, PLZF, and Egr2 transcription factors, and the cytokine IL-15 (1, 2, 6–8). Deletion of one of these molecules impairs iNKT cell ontogeny, whereas it has little or no effects on the development of mainstream T lymphocytes.

Collectively, this evidence argues for a unique genetic program controlling iNKT cell development.

Small microRNAs (miRNAs) are short (~22 nt) noncoding RNAs that control gene expression at the posttranscriptional level by binding to specific mRNA sequences, regulating the expression of target proteins and mRNAs. Their role in the development and function of immune cells is now well established. Here, we tested whether miRNAs play a role in the development of iNKT cells.

We found that Dicer deletion results in a substantial reduction of iNKT cells in thymus and their disappearance from the periphery, unlike mainstream T cells. Without Dicer, iNKT cells do not complete their innate effector differentiation and display a defective homeostasis due to increased cell death. Differentiation and homeostasis of iNKT cells require Dicer in a cell-autonomous fashion. Furthermore, we identified a miRNA profile specific for iNKT cells, which exhibits features of activated/effector T lymphocytes, consistent with the idea that iNKT cells undergo agonist thymic selection. Together, these results define a critical role of the Dicer-dependent miRNA pathway in the physiology of iNKT cells.
Foxp3-driven deletion of Dicer selectively in Treg cells impaired their peripheral homeostasis (16) and suppressor functions (15, 16), supporting the critical requirement for Dicer-controlled miRNA pathway in this T cell lineage.

In the light of these considerations, we asked whether Dicer-dependent miRNAs play any role in controlling the unique iNKT cell developmental program. To this aim, we investigated the iNKT cell development in a set of mice in which Dicer has been deleted from cortical thymocytes.

Materials and Methods

**Mice**

Dicer<sup>lox/lox</sup>, cd4Dicer<sup>−/−</sup>, and cd2Dicer<sup>−/−</sup> transgenic mice were described (13, 14). Dicer<sup>−/−</sup> were crossed with both hCD2Cre and R26R-EYFP transgenic mice (18) to delete one (cd2Dicer<sup>lox/lox</sup>) or both (cd2Dicer<sup>−/−</sup>) Dicer<sup>−/−</sup> alleles expressing EYFP upon deletion of a floxed transcriptional stop cassette inserted into the rosa26 locus. All mice were housed in a pathogen-free environment. Procedures involving animals were approved by the Institutional Animal Care and Use Committee at San Raffaele Scientific Institute or performed according to the Animals (Scientific Procedures) Act.

**Flow cytometry and cell sorting**

Cells from thymus, spleen, and liver were purified, stained, and sorted as described (19) using the following mAbs: anti-TCR<sub>α/β</sub>-allophycocyanin, CD3-PECy5, anti-NK1.1-PerCP, HSA-FITC, CD4-PerCP or allophycocyanin, CD8α-PE, CD44-allophycocyanin, CD19-FITC, CD1d-PE, streptavidin-allophycocyanin, SLAMF3-bio (BD Bioscience), SLAMF1-APC, SLAMF5-bio (Biolegend), SLAMF6-bio (eBiology). Enrichment of HSA<sup>+</sup> mature thymocytes by anti-HSA mAb and rabbit complement was performed as described (19). CD1d-IgG Dimetix (BD Bioscience) or mCD1d tetramers (Proimmune) were loaded with αGalCer (Alexis) as described (19). BrdU and 7-aminoactinomycin D stainings were performed using the BrdU Flow Kit (BD Biosciences). To investigate the iNKT cell stage 0 of development, 2 × 10<sup>5</sup> thymocytes were stained for 1 h on ice with 0.5 μM DimerX-CD1d followed by 0.5 μg rat anti-mouse IgG1 PE (clone A85-1, BD). DimerX-positive cells were enriched with anti-PE immunomagnetic beads (Miltenyi Biotec) following the manufacturer’s instructions. Expression values were normalized to snor420 and reconfirmed using snor202.

**Quantitative real-time RT-PCR**

To quantify miRNA expression, gene-specific reverse transcription was performed using TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems). RT-PCR was performed using TaqMan MicroRNA Assay Mix containing PCR primers and TaqMan probes (Applied Biosystems) according to manufacturer instructions. Expression values were normalized to 0.05 was considered statistically significant.

**Results**

**Absence of Dicer in cortical thymocytes results in a substantial iNKT cell reduction**

To investigate the role for Dicer-controlled miRNAs in the regulation of the iNKT cell developmental program, we first determined the frequency and number of these cells in the thymus and peripheral organs of mice carrying a conditional mutation of dicer-1 gene (Dicer<sup>lox/lox</sup>), activated by Cre recombinase under the control of the cd4 enhancer/promoter/silencer (CD4Cre) (14). CD4Cre causes 90% deletion of Dicer in DP thymocytes that, however, still express abundant mature miRNAs (12, 14). Dicer<sup>lox/lox</sup> deletion induced by CD4Cre is essentially complete in SP thymocytes and mature miRNAs are reduced ~10-fold in naive T cells (12, 14). As shown in Fig. 1, Dicer deletion in Dicer<sup>lox/lox</sup> mice induced by CD4Cre (cd4Dicer<sup>−/−</sup> mice) indeed resulted in >10-fold reduction in thymic iNKT cells compared with control thymi from Dicer<sup>lox/lox</sup> littermate mice. iNKT cell depletion in cd4Dicer<sup>−/−</sup> mice was even more profound in the peripheral compartment. iNKT cells could be hardly detected by CD1d-tetramer staining in the liver and spleen of cd4Dicer<sup>−/−</sup> mice (Fig. 1), approaching background values obtained in CD1d<sup>−/−</sup> mice that completely lack iNKT cells. In lymph nodes, iNKT cells from cd4Dicer<sup>−/−</sup> mice were also markedly reduced compared with Dicer<sup>lox/lox</sup> littermates, although this difference did not reach statistical significance (Fig. 1).

By contrast, total thymocyte numbers, and both frequency and number of mainstream DP and SP thymocytes, were normal in cd4Dicer<sup>−/−</sup> thymi, while peripheral CD<sup>4+</sup> and CD8<sup>+</sup> T cells were moderately reduced (2-fold and 4-fold, respectively) in cd4Dicer<sup>−/−</sup> mice (supplemental Fig. 1) compared with Dicer<sup>lox/lox</sup> control mice, and consistent with published data (12, 14).

Thus, Dicer deletion in DP thymocytes resulted in a dramatic reduction of iNKT but not of T cells, in both thymic and peripheral compartments.

We also investigated the effects of Dicer<sup>lox/lox</sup> deletion on iNKT cell development by Cre recombinase driven by hCD2 promoter (cd2Dicer<sup>−/−</sup>) or proximal plKc (lkDicer<sup>−/−</sup>) promoters, which delete at the DN3 stage (18, 21) significantly earlier in T cell development than CD4Cre, resulting in the depletion of Dicer and of mature miRNAs already at the DP stage (13) (and supplemental Fig. 2A). Dicer<sup>lox/lox</sup> deletion by pCD2cre resulted in a 14-fold reduction in the total number thymocytes compared with heterozygous deleted control mice (8.4 ± 6 × 10<sup>5</sup> in cd2Dicer<sup>−/−</sup> vs 120 ± 45.1 × 10<sup>5</sup> in cd2Dicer<sup>−/−</sup> mice), which essentially depended on a dramatic drop in the number of DP (>100-fold), CD4 SP (>20-fold) and, to a lesser extent, CD8 SP (6-fold) thymocyte (supplemental Fig. 2B), while DN thymocytes were even slightly increased in cd2Dicer<sup>−/−</sup> compared with cd2Dicer<sup>−/−</sup> thymi (6.3 ± 5 × 10<sup>5</sup> and 2.6 ± 1.4 × 10<sup>5</sup>, respectively). In the peripheral

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1 The online version of this article contains supplemental material.

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**Mixed radiation bone marrow chimeras**

Mixed radiation bone marrow chimeras were described as (20).

**MicroRNA array profiling**

Total RNA was extracted from total thymocytes, and from sorted mature thymic iNKT cells or T cells. Three independent samples for each cell type were obtained from 18 pooled thymi of 4-wk-old C57BL/6 mice. Its quality was verified by an Agilent 2100 Bioanalyzer profile. Ninety nanograms of total RNA from samples (iNKT or T cells) and reference (total thymocytes) was verified by an Agilent 2100 Bioanalyzer profile. Ninety nanograms of total RNA from samples (iNKT or T cells) and reference (total thymocytes) was verified by an Agilent 2100 Bioanalyzer profile. Ninety nanograms of total RNA from samples (iNKT or T cells) and reference (total thymocytes) was verified by an Agilent 2100 Bioanalyzer profile.
FIGURE 1. Dicer deletion in cortical thymocytes results in a substantial iNKT cell reduction. Quantification of iNKT cells from thymus and periphery of 8-wk-old cd4Dicerlox/lox mice. A, Cells were stained with αGalCer-loaded CD1d tetramers (CD1d tet), TCRβ, and HSA (thymus) or CD19 (periphery) specific mAbs. Shown are the percentages of iNKT cells in thymus (HSA-αGalCer-CD1d TCRβ+) and periphery (CD19 CD1d TCRβ+). B, Frequency and number of iNKT cells in thymus and periphery of cd4Dicerlox/lox mice and Dicerlox/lox controls. Data are representative of five pairs of mice. (t test: *, p ≤ 0.05; **, p ≤ 0.01).

Comparative depletion of iNKT cells and mainstream T cells were observed in thymus and periphery of lckDicerlox/lox mice (data not shown).

Together, these results showed that, unlike mainstream T cells that require Dicer during the early developmental stage (DN) but not after their lineage commitment (DP stage), iNKT cells continue to require Dicer-dependent miRNAs also after this stage, suggesting a critical role for miRNAs in controlling the iNKT cell differentiation program.

Dicer deletion in cortical thymocytes impairs iNKT cell differentiation program

We next investigated whether Dicer-dependent miRNAs played any role in the phenotypic maturation followed by iNKT cells in thymus. We concentrated on cd4Dicerlox/lox mice because they had detectable iNKT cells in the thymus. As shown in Fig. 2A, the differentiation program of thymic iNKT cells in 4-wk-old cd4Dicerlox/lox mice was arrested at the stage 2. In Dicerlox/lox littermate controls, iNKT cells maturation progressed to stage 3, although the percentage of NK1.1 expressing cells was low because of the young age. The defective maturation of iNKT cells in cd4Dicerlox/lox mice did not improve with age (8-wk-old mice) (Fig. 2B), when the majority of thymic iNKT cells from Dicerlox/lox littermate were NK1.1+. Interestingly, the great majority of thymic iNKT cells from cd4Dicerlox/lox mice expressed CD4 (Fig. 2C), unlike iNKT cells from Dicerlox/lox thymi, which developed into both CD4+ and CD4− subsets, a bifurcation that is suggested to occur between stage 1 and 2 (22). Thus, without Dicer-controlled miRNAs, iNKT cells may not reach the maturation stage at which the CD4− subset emerges. Alternatively, but not mutually exclusive, CD4 shut off and the emergence of the CD4− iNKT cell subset could be directly controlled by miRNAs.

Dicer deletion in DP thymocytes by CD4Cre, however, did not affect the very early stages of iNKT cell development (stage 0), immediately following positive selection (Fig. 2D), in line with the normal miRNA content displayed by DP thymocytes in this model (12, 14). Comparable quantities of canonical invariant Vα14-Jα18 transcripts were also detected by quantitative RT-PCR (qRT-PCR) in sorted DP thymocytes from cd4Dicerlox/lox mice and Dicerlox/lox littermate controls, supporting the flow cytometry data on stage 0 iNKT cells.

Collectively, these findings strongly suggested that the iNKT cell differentiation program is critically controlled by Dicer-dependent miRNAs.

Altered homeostasis of the iNKT cells in the absence of Dicer

The reduced number of thymic iNKT cells caused by the lack of Dicer could result either from an impaired expansion of immature iNKT cells or from an increased cell death, or both. As determined by BrdU incorporation in vivo, depletion of Dicer in cd4Dicerlox/lox mice did not modify the proliferation of thymic iNKT cell precursors compared with control mice (Fig. 3A). Coupling BrdU incorporation in vivo with in vitro staining for the DNA content revealed that the frequency of immature iNKT cells at the G1/M phase was significantly higher in cd4Dicerlox/lox thymi than Dicerlox/lox control thymi (8 and 1%, respectively, Fig. 3B), suggesting a possible mitotic defect in iNKT cells that lack Dicer.

In contrast to the rate of cell division, cell death in thymic iNKT cells from cd4Dicerlox/lox mice exceeded by 10-fold that of iNKT cells from Dicerlox/lox controls (Fig. 3C). Cell death in Dicer-deficient iNKT cells occurred mainly at stage 2 (CD44hiNK1.1−), which is normally characterized by marked proliferation (4), suggesting a possible link between cell division and death of iNKT cells in the absence of miRNAs.
Unlike iNKT cells, both cell division and death of mainstream T cells were comparable in \textit{cd4Dicer}\textsuperscript{lox/lox} and \textit{Dicer\textsuperscript{lox/lox}} littermates, indicating that the homeostasis of thymic T cells was not affected by the deletion of Dicer at the DP stage (data not shown).

Collectively, these results suggested that the profound depletion of iNKT cells observed in the absence of Dicer resulted from an altered homeostasis of this lymphocyte subset due to increased cell death.

To determine the mechanisms by which Dicer deletion impaired iNKT cell development, we first investigated whether DP thymocytes from \textit{cd4Dicer}\textsuperscript{lox/lox} mice exhibited defective CD1d expression, CD1d-dependent lipid Ag presentation or expression of the SLAMF molecules shown to play a role in iNKT cell development (23). DP thymocytes from \textit{cd4Dicer}\textsuperscript{lox/lox} and \textit{Dicer\textsuperscript{lox/lox}} littermate control mice expressed similar levels of CD1d and presented with comparable efficiency the exogenous glycolipid HSA\textsubscript{2501}GalCer to an iNKT cell hybridoma (supplemental Fig. 3). Furthermore, SLAMF1, F3, F5, and F6 molecules were also expressed at comparable levels in DN, DP, CD4 SP, and CD8 SP thymocyte subsets from \textit{cd4Dicer}\textsuperscript{lox/lox} and \textit{Dicer\textsuperscript{lox/lox}} control mice (supplemental Fig. 3). These results ruled out that the lack of Dicer-dependent miRNAs was affecting key molecular interactions necessary for iNKT cell development.

To determine whether the iNKT cell developmental defect caused by the lack of Dicer was cell autonomous, we verified whether the development of Dicer-deficient iNKT cells could be rescued by Dicer-sufficient thymocytes in mixed BM chimeras. Lethally irradiated \textit{cd4Dicer}\textsuperscript{lox/lox} mice were reconstituted with an equal mixture of BM cells derived from CD45.1 wild type (wt) and CD45.1\textsuperscript{B6} mice (supplemental Fig. 3). These results ruled out that the lack of Dicer-dependent miRNAs was affecting key molecular interactions necessary for iNKT cell development.
mice and CD45.2 cd4Dicer<sup>Δ/Δ</sup> mice. As shown in Fig. 4, the majority of iNKT cells present in the thymi of the BM chimeras were mature NK1.1<sup>+</sup> cells derived from the CD45.1 wt BM. Only a minority of iNKT cells were derived from the CD45.2 cd4Dicer<sup>Δ/Δ</sup> BM cells and displayed an immature NK1.1<sup>+</sup> phenotype. Unlike iNKT cells, T cells developed normally from both wt and cd4Dicer<sup>Δ/Δ</sup> mice (Fig. 4).

Hence, the impaired iNKT cell development caused by the deletion of Dicer could not be rescued by wt thymocytes, arguing strongly for an iNKT cell-autonomous defect.

**iNKT cells display a distinct miRNA profile**

Given the selective role played by Dicer-dependent miRNAs in the control of iNKT cell development, we sought to determine the miRNA profile expressed by iNKT cells in comparison with mature thymic T cells. The miRNAs obtained from sorted HSA<sub>low</sub>-enriched iNKT and T cells, in triplicate samples each originating from a pool of six C57BL/6 mice, were profiled with LNA (locked nucleotide acid)-based miRNA microarray. The analysis identified 70 miRNAs expressed in thymic iNKT and T cells, 17 of which were differentially expressed between the two cell subsets at a statistically significant level (Fig. 5A). Quantitative RT-PCR confirmed that miR-21 was overexpressed, while 13 miRNAs were underexpressed in iNKT cells compared with T cells (Fig. 5B). The overexpression of miR-290, miR-483* and miR-720 in iNKT cells was not confirmed by qRT-PCR.
miRNA clustering (top left) and sample clustering trees (top) are shown. A, Fold change expression of miRNAs in iNKT cells compared with mature thymic T cells, validated by qRT-PCR.

Thus, this analysis revealed an iNKT lineage-specific miRNA profile, substantially different from that displayed by mature thymocytes, in line with the selective role of Dicer-controlled miRNAs in the control of the iNKT lineage-specific genetic program.

Discussion

CD1d-dependent iNKT cells are a separate lineage of T lymphocyte that undergoes a distinct developmental pathway controlled by a unique gene expression program. In this study, we show that this program includes microRNAs, small noncoding RNAs that regulate gene expression posttranscriptionally and play a key role in the control of cellular differentiation programs. We provide a set of compelling evidences suggesting that the differentiation and homeostasis of iNKT cells require Dicer, the RNase III enzyme that generates functional miRNAs, in a cell-autonomous fashion. Remarkably, the development of mainstream T cells is largely unaffected by Dicer deletion, underscoring the uniqueness of the iNKT cell program.

Furthermore, we have identified an iNKT cell-specific miRNA profile, different from that of mature mainstream thymocytes, which exhibits features of activated/effector T cells.

These findings reveal the critical role of the Dicer-dependent miRNA pathway in the physiology of iNKT cells. Furthermore, they provide new targets to investigate the molecular pathways controlling iNKT cell development in future work.

Several studies have shown that conditional Dicer ablation is associated with an increased cell death in the targeted cells (24, 25), suggesting that a Dicer-dependent miRNA pathway plays an important role in controlling cell survival and tissue homeostasis (26). Dicer deletion in B cells resulted in the block of B cell development that was partially due to increased apoptosis at the pre-B stage, linked to an aberrantly high expression of the pro-apoptotic gene Bim (11). Without Dicer, cells seem particularly vulnerable to cell death during cell division, possibly because of mitotic defects due to centromer dysfunctions and premature sister chromatid separation (24). The extensive cell division occurring in thymocytes between the DN and DP stages has also been implicated in the marked apoptosis of DP thymocytes observed in Ick-DicerΔ/Δ mice, in which Dicer is deleted at the DN stage (13). Furthermore, Muljo et al. (12) showed that mature peripheral T cells from cd4DicerΔ/Δ undergo increased cell death compared with controls upon TCR-dependent activation, which induces a potent proliferative stimulus.

Interestingly, the miRNA profile found in thymic iNKT cells shares several similarities with that described for effector T cells (14, 27), obtained upon in vitro activation of peripheral naive CD8+ or CD4+ T cells. Indeed, miR-15b, miR-16, miR-30c, miR-150, and Let-7 family were down-regulated in thymic iNKT cells as well as in activated CD8+ and CD4+ T cells; while, among the five miRNAs that are up-regulated in iNKT cells, miR-21 was found also preferentially expressed in the peripheral T cells activated in vitro (14, 27). miR-21 was shown to inhibit apoptosis in tumor cells (28) and to down-regulate the miRNA encoding the proapoptotic PTEN (phosphatase and tensin homolog deleted on chromosome TEN) molecule (29). The fact that Dicer deletion from DP thymocytes results in a selective cell death in immature iNKT cells could be therefore compatible with the lack of miRNA-21 expression in these cells. Nevertheless, it is likely that also other miRNAs take part in the coordinate regulation of the molecular pathways involved in iNKT cell development.

Cobb et al. (14) have also shown that Treg cells express a characteristic set of miRNAs that is distinct from that of naive CD4+ T cells, and more similar to that acquired by effector CD4+ T cells upon activation in vitro. Accordingly, iNKT and Treg cells seem to share part of their miRNA profile: miR-21 is preferentially expressed in iNKT and Treg cells compared with naive T cells, while, among the miR-30c, miR-106a, miR-106b, miR-150, and Let-7 family are down-regulated in both iNKT and Treg cells. The “effector-like” miRNA profiles exhibited by iNKT cells and Tregs would be consistent with the evidence suggesting that both iNKT and Treg cells undergo an agonist selection process in the thymus, resulting in the early activation and acquisition of the effector/memory phenotype (1, 30, 31). The miRNAs shared by iNKT, Treg, and activated T cells might be implicated in the regulation of common molecular pathways(s) that lead to the acquisition of the effector phenotype.

The quantitative variation of miRNAs that are differentially expressed between iNKT cells and T cells should result in the modulation of expression levels of their target miRNAs. As suggested by the data showing a dose-dependent regulation of c-Myb expression by graded concentration of miR-150 (10), this would have a major impact on the regulation of protein synthesis between the two T cell subsets, resulting in a lineage-specific expression level of particular proteins in iNKT and in T cells. A finely regulated
level of expression would be critical for proteins that function over a narrow range of concentration, such as PTEN and Bim (10), which control lymphocyte proliferation and survival and are targeted by miR-21 and miR-17, differentially expressed in iNKT and T cells.

Nevertheless, we cannot discount the possibility that miRNAs that are equally expressed by both iNKT and T cells may also play a lineage-specific role in the development of the two subsets, because the target miRNAs may code for proteins involved in pathways critical for the program of one but not the other lineage, as suggested for other biological processes (10).

Harnessing miRNA function (32) and identification of the transcripts that are targets of iNKT cell-expressed miRNAs might provide further insights into the molecular pathways that specifically control the development of this subset, and also shed light on molecular cues common to Treg and effector T cells.

In conclusion, our results suggest that differentiation and homoeostasis of iNKT cells depend critically on Dicer-dependent miRNAs, consistent with the idea that miRNAs control the canalization of cell differentiation by buffering stress-related variations in the expression of genes specifically involved in the programs (33).

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Disclosures
The authors have no financial conflict of interest.

References
Figure S1. Dicer deletion in DP thymocytes does not result in major loss of mainstream T compared to iNKT cells. Comparison of total lymphocytes, total T cells (CD3^+), CD4^+ T (CD3^+CD4^+), CD8^+ T (CD3^+CD8^+) and iNKT (CD3^+CD1d tet^+) T cells from thymus and spleen of cd4Dicer^{Δ/Δ} mice and Dicer^{lox/lox} littermate controls. The number of each cell subset in cd4Dicer^{Δ/Δ} mice is expressed as a % relative to Dicer^{lox/lox} control mice, represented by the dashed line. Data represent mean values from ten 8 wks old mice.

Figure S2. NKT cell development is severely perturbed in cd2Dicer^{Δ/Δ} mice.
A. Genotype of cd2Dicer mice. PCR of genomic DNA from the tail of a cd2Dicer^{wt} (one 390bp Dicer^fl allele and one 259bp wild type allele) and a cd2Dicer^{Δ/Δ} (two Dicer^fl alleles) mouse respectively. Efficient deletion of Dicer exon 20-21 transcript by hCD2Cre in DP thymocytes, detected by RT-PCR of Dicer exons 19-22 of cDNA samples from the indicated populations. B. Total thymocytes and tymphocytes subset numbers in cd2Dicer^{Δ/Δ} and cd2Dicer^{wt} mice. C. Peripheral T cell numbers in cd2Dicer^{Δ/Δ} and cd2Dicer^{wt} mice. D. iNKT cells (TCRβ^+CD1d tet^+) present in HSA^{lo} thymocytes and in CD19^- splenic and hepatic lymphocytes. Percentage and numbers of invariant NKT cells are significantly reduced (* p≤0.05; ** p≤0.005). B and D: data from 8-9 wk old cd2Dicer^{Δ/Δ} (n= 9) and cd2Dicer^{wt} controls (n=6).
Figure S3. CD1d expression, antigen presentation functions and SLAM family expression is normal in DP thymocytes from cd4DicerΔ/Δ mice.

A. Comparative CD1d expression on DP thymocytes from cd4DicerΔ/Δ, Dicerlox/lox littermate controls, C57BL/6 and CD1d+/− mice. B. Sorted DP thymocytes from either cd4DicerΔ/Δ or Dicerlox/lox littermate controls were cultured with the iNKT cell hybridoma DN3A4-1.2 in the presence or absence of 100ng αGalCer or anti-CD1d blocking mAb or control rat IgG. After 18h, culture supernatant was collected and analyzed for IL-2 content by standard ELISA. Data are expressed as the mean± S.D. calculated from triplicate wells. One experiment representative of two is shown. C. Total thymocytes from cd4DicerΔ/Δ or Dicerlox/lox littermate controls were stained with anti-CD4 and anti-CD8 mAbs together with one of the mAb specific for the indicated SLAM family member. Shown is the expression of each SLAM member in the gated thymocytes subsets.