Dicer-Dependent MicroRNA Pathway Controls Invariant NKT Cell Development

Maya Fedeli, Anna Napolitano, Molly Pui Man Wong, Antoine Marciais, Claudia de Lalla, Francesco Colucci, Matthias Merkenschlager, Paolo Dellabona and Giulia Casorati

*J Immunol* 2009; 183:2506-2512; Prepublished online 22 July 2009; doi: 10.4049/jimmunol.0901361

http://www.jimmunol.org/content/183/4/2506

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/07/23/jimmunol.0901361.DC1

References

This article cites 33 articles, 14 of which you can access for free at:

http://www.jimmunol.org/content/183/4/2506.full#ref-list-1

Subscription

Information about subscribing to *The Journal of Immunology* is online at:

http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:

http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:

http://jimmunol.org/alerts
Dicer-Dependent MicroRNA Pathway Controls Invariant NKT Cell Development

Maya Fedeli,* Anna Napolitano,* Molly Pui Man Wong,‡ Antoine Marcais,‡ Claudia de Lalla,* Francesco Colucci, ‡ Matthias Merkenschlager,‡ Paolo Dellabona,2* and Giulia Casorati2*

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–Ja18 rearrangement paired with Vβ8.2, −7, or −2 (1). Invariant NK T (iNKT)3 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).

I
nvariant T lymphocytes (1, 2). Postselection iNKT cells undergo an ordered phenotypic maturation characterized by four stages: the first, detectable immediately after positive selection, is the HSAhighCD69 stage 0, which becomes HSA−CD44highNK1.1 stage 1, followed by CD44highNK1.1− 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 target protein-encoding mRNAs, inducing their translational repression or degradation, depending on the degree of complementarity (9). Functional mature miRNAs are generated in the cytoplasm by a complex containing the RNase III enzyme Dicer and TRBP (HIV 1–trans-activating response RNA-binding protein) (9).

miRNAs regulate basic functions such as proliferation, apoptosis, lineage commitment, or differentiation of cells that constitute different tissues and organs, including the immune system (9, 10). Conditional deletion of Dicer provided insight into the role of miRNAs in controlling the development of B and T lymphocytes (11–16). Dicer deletion early in T cell development (DN stage) caused a 10-fold decrease in DP and single-positive (SP) thymocyte numbers, though the CD4/CD8 lineage choice was unaffected (13). Dicer deletion at later stage of T cell development (DP stage) did not alter number and composition of mainstream thymocytes, though it resulted in a substantial reduction in thymic CD4+CD25+/Foxp3+ natural regulatory T (Treg) cells (14, 17). In the peripheral compartment, these mice showed a modest reduction in CD4+ and CD8+ T cells, Th1 polarization, and increased apoptosis in CD4+ T cells (12).
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 microRNA 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**

Dicerlox/lox, cd4DicerΔ, and lckDicerΔ transgenic mice were described (13, 14). DicerΔ mice were crossed with both hCD2Cre and R26r-EYFP transgenic mice (18) to delete one (cd2Dicerlox/Δ) or both (cd2DicerΔ/Δ) Dicelox 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-TCRα/β, CD4-PacificOrange, CD8α-PE, CD44-PerCP, HSA-FITC, CD1d-PE, streptavidin-allophycocyanin, SLAMF3-bio (BD Biosciences), SLAMF1-APC, SLAMF5-bio (BioLegend), SLAMF6-bio (eBioscience). Enrichment of HSAΔ mature thymocytes by anti-HSA mAb and rabbit complement was performed as described (19). CD1d-IgG Dimix (BD Biosciences) 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 × 10⁶ thymocytes were stained for 1 h on ice with 0.5 μg Dicerlox/ΔD1d 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 instruction and stained with anti-TCRβ-allophycocyanin and anti-HSA-FITC mAbs. As control, empty DimerX-C1d1 did not stain thymocytes above the background level. Cells were analyzed on FACSCounto or LSRII and sorted on MoFlo (Beckman Coulter), always excluding dead cells by the use of the DNA specific dye DAPI (Santa Cruz Biotechnology) and doublets by comparing side scatter width to forward scatter area.

**Mixed radiation bone marrow chimeras**

Mixed radiation bone marrow chimeras were prepared as described (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/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) were labeled with H3 and H3 fluorescent label, respectively, using the miRCURY LNA Array power labeling kit (Exiqon). The H3-labeled samples and a H3-labeled reference RNA sample were mixed pair-wise and hybridized to the miRCURY LNA array version 11.0 (Exiqon), which contains capture probes targeting all miRNAs for human, mouse, or rat registered in the miRBASE version 12.0 at the Sanger Institute. The hybridization was performed according to the miRCURY LNA array manual using a Tecan HS4800 hybridization station (Tecan). After hybridization, the microarray slides were scanned and stored in an ozone-free environment (ozone level below 2.0 ppb) to prevent potential bleaching of the fluorescent dyes. Scanning was performed with the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and the image analysis was conducted using the ImaGene 8.0 software (BioDiscovery). The quantified signals were background corrected (normexp with offset value 10) and normalized using a global Lowess (Locally Weighted Scatterplot Smoothing) regression algorithm.


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 snor240 and reconfirmed using snor202.

**Statistical analysis**

The results were analyzed by a two-tailed Student t test. A value of p < 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 (Dicerlox/lox), 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). Dicerlox/lox 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 Dicerlox/lox mice induced by CD4Cre (cd4DicerΔ) mice indeed resulted in >10-fold reduction in thymic iNKT cells compared with control thymi from Dicerlox/lox littermate mice. iNKT cell depletion in cd4DicerΔ 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 cd4DicerΔ mice (Fig. 1), approaching background values obtained in CD1d−/− mice that completely lack iNKT cells. In lymph nodes, iNKT cells from cd4DicerΔ mice were also markedly reduced compared with Dicerlox/lox 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 cd4DicerΔ thymin, while peripheral CD4+ and CD8+ T cells were moderately reduced (2-fold and 4-fold, respectively) in cd4DicerΔ mice (supplemental Fig. 1) compared with Dicerlox/lox 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 Dicerlox/lox deletion on iNKT cell development by Cre recombinase driven by hCD2 promoter (cd2DicerΔ) or proximal pLck (lckDicerΔ) 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). Dicerlox/lox deletion by pCD2cre resulted in a 14-fold reduction in the total number miRNAs compared with heterozygous deleted control mice (8.4 ± 6 × 10^6 in cd2DicerΔ vs 120 ± 45.1 × 10^6 in cd2DicerΔ 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 cd2DicerΔ compared with cd2DicerΔ thymin (6.3 ± 5 × 10^6 and 2.6 ± 1.4 × 10^6, respectively).

The online version of this article contains supplemental material.
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 cd4Dicer\textsuperscript{lox/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 G\textsubscript{2}/M phase was significantly higher in cd4Dicer\textsuperscript{lox/lox} thymi than Dicer\textsuperscript{lox/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 cd4Dicer\textsuperscript{lox/lox} mice exceeded by 10-fold that of iNKT cells from Dicer\textsuperscript{lox/lox} controls (Fig. 3C). Cell death in Dicer-deficient iNKT cells occurred mainly at stage 2 (CD44\textsuperscript{+}NK1.1\textsuperscript{+}), 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.

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 cd4Dicer\textsuperscript{lox/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 cd4Dicer\textsuperscript{lox/lox} mice was arrested at the stage 2. In Dicer\textsuperscript{lox/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 cd4Dicer\textsuperscript{lox/lox} mice did not improve with age (8-wk-old mice) (Fig. 2B), when the majority of thymic iNKT cells from Dicer\textsuperscript{lox/lox} littermate were NK1.1\textsuperscript{+}.

Interestingly, the great majority of thymic iNKT cells from cd4Dicer\textsuperscript{lox/lox} mice expressed CD4 (Fig. 2C), unlike iNKT cells from Dicer\textsuperscript{lox/lox} thymi, which developed into both CD4\textsuperscript{+} and CD4\textsuperscript{−} 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\textsuperscript{−} subset emerges. Alternatively, but not mutually exclusive, CD4 shut off and the emergence of the CD4\textsuperscript{−} 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 differentiation. DP thymocytes in this model (12, 14). Comparable quantities of canonical invariant V\textalpha\textsubscript{1}4-J\textalpha\textsubscript{18} transcripts were also detected by quantitative RT-PCR (qRT-PCR) in sorted DP thymocytes from cd4Dicer\textsuperscript{lox/lox} mice and Dicer\textsuperscript{lox/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.

miRNAs CONTROL iNKT CELLS

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 cd4Dicer\textsuperscript{lox/lox} mice. A. Cells were stained with αGalCer-loaded CD1d tetramers (CD1d tet), TCR\textgamma, and HSA (thymus) or CD19 (periphery) specific mAbs. Shown are the percentages of iNKT cells in thymus (HSA\textsuperscript{−}/CD1d\textsuperscript{+}TCR\textgamma\textsuperscript{+}) and periphery (CD19\textsuperscript{−}/CD1d\textsuperscript{+}TCR\textgamma\textsuperscript{+}). B. Frequency and number of iNKT cells in thymus and periphery of cd4Dicer\textsuperscript{lox/lox} and Dicer\textsuperscript{lox/lox} controls. Data are representative of five pairs of mice. (t test: *, p ≤ 0.05; **, p ≤ 0.01).

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 cd4Dicer\textsuperscript{lox/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 cd4Dicer\textsuperscript{lox/lox} mice was arrested at the stage 2. In Dicer\textsuperscript{lox/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 cd4Dicer\textsuperscript{lox/lox} mice did not improve with age (8-wk-old mice) (Fig. 2B), when the majority of thymic iNKT cells from Dicer\textsuperscript{lox/lox} littermate were NK1.1\textsuperscript{+}.

Interestingly, the great majority of thymic iNKT cells from cd4Dicer\textsuperscript{lox/lox} mice expressed CD4 (Fig. 2C), unlike iNKT cells from Dicer\textsuperscript{lox/lox} thymi, which developed into both CD4\textsuperscript{+} and CD4\textsuperscript{−} 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\textsuperscript{−} subset emerges. Alternatively, but not mutually exclusive, CD4 shut off and the emergence of the CD4\textsuperscript{−} 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 V\textalpha\textsubscript{1}4-J\textalpha\textsubscript{18} transcripts were also detected by quantitative RT-PCR (qRT-PCR) in sorted DP thymocytes from cd4Dicer\textsuperscript{lox/lox} mice and Dicer\textsuperscript{lox/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.
Unlike iNKT cells, both cell division and death of mainstream T cells were comparable in \textit{cd4Dicer^{lox/lox}} mice. Total thymocytes were stained with CD1d tet or CD1d dimerX, HSA, CD3, NK1.1, and either CD4 or CD44 specific mAbs. Phenotype, frequency and number of iNKT cells (HSA^{+}/CD3^{+}CD1d tet^{+}) from 4-wk-old \textit{cd4Dicer^{V/D}} mice and controls. Histograms data are expressed as the mean ± SD. Three to four mice per group were independently analyzed in each experiment. CD4 and NK1.1 expression on iNKT cells from 4-wk-old \textit{cd4Dicer^{V/D}} mice and controls. D. Phenotype of iNKT cells enriched from total thymocytes of 2-wk-old mice by CD1d dimerX staining and immunomagnetic sorting. Thymocytes pooled from five mice per group were analyzed. One experiment representative of two is shown.

**The iNKT cell developmental defect caused by Dicer deficiency is cell-autonomous**

To determine the mechanisms by which Dicer deletion impaired iNKT cell development, we first investigated whether DP thymocytes from \textit{cd4Dicer^{V/D}} 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^{V/D}} and \textit{Dicer^{lox/lox}} littermate control mice expressed similar levels of CD1d and presented with comparable efficiency the exogenous glycolipid αGalCer 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^{V/D}} and \textit{Dicer^{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^{V/D}} mice were reconstituted with an equal mixture of BM cells derived from CD45.1 wild type (wt)
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 HSAlow-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 (left) and sample clustering trees (top) are shown. A, Heat map: overexpression (red), underexpression (blue), and identical expression (white) of miRNA in iNKT or T cells relative to the reference. The miRNA expression in iNKT and T cells was normalized against a reference consisting of miRNAs from total thymocytes. Shown are the miRNAs expressed at statistically different levels between iNKT and T cells ($p \leq 0.05$). B, 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 evidence 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 proapoptotic 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 lck-Dicer<sup>−/−</sup> mice, in which Dicer is deleted at the DN stage (13). Furthermore, Muljo et al. (12) showed that mature peripheral T cells from cd4Dicer<sup>−/−</sup> 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<sup>+</sup> or CD4<sup>+</sup> 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<sup>+</sup> and CD4<sup>+</sup> 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 mRNA 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<sup>+</sup> T cells, and more similar to that acquired by effector CD4<sup>+</sup> 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 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 pathway(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).

Acknowledgments

We thank Drs. Massimiliano Pagani, Riccardo Rossi, Graziza Rossetti (Milano), Aurore Saudemont, Klaus Okkenhaug, and Elena Vigorito (Babraham), as well as the Babraham Small Animal Facility, for help and discussions.

Disclosures

The authors have no financial conflict of interest.

References


