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MicroRNAs Control the Maintenance of Thymic Epithelia and Their Competence for T Lineage Commitment and Thymocyte Selection

Saulius Zuklys,*1 Carlos E. Mayer,*1 Saule Zhanybekova,* Heather E. Stefanski,†‡* Gretel Nusspaumer,* Jason Gill,* Thomas Barthollt,* Stephane Chapraz,§ Takeshi Nitta,¶ James Dooley,‖ Ruben Nogales-Cadenas,§ Yousuke Takahama,§ Daniela Finke,§ Adrian Liston,‖ Bruce R. Blazar,†‡ Alberto Pascual-Montano,§ and Georg A. Holländer****

Thymic epithelial cells provide unique cues for the lifelong selection and differentiation of a repertoire of functionally diverse T cells. Rendered microRNA (miRNA) deficient, these stromal cells in the mouse lose their capacity to instruct the commitment of hematopoietic precursors to a T cell fate, to effect thymocyte positive selection, and to achieve promiscuous gene expression required for central tolerance induction. Over time, the miroenvironment created by miRNA-deficient thymic epithelia assumes the cellular composition and structure of peripheral lymphoid tissue, where thymopoiesis fails to be supported. These findings emphasize a global role for miRNA in the maintenance and function of the thymic epithelial cell scaffold and establish a novel mechanism how these cells control peripheral tissue Ag expression to prompt central immunological tolerance. *The Journal of Immunology, 2012, 189: 3894–3904.

The thymus provides a unique stromal microenvironment that instructs the differentiation of blood-borne precursors to functionally mature T lymphocytes proficient in effecting an immune response against microbial pathogens while unable to elicit an autoimmune reaction (1). The major structural components of the thymus are thymic epithelial cells (TEC) that can further be classified as cortical (cTEC) or medullary TEC (mTEC) subpopulations based on distinct structural, antigenic, and functional features (2, 3). The molecular programs that control TEC growth, differentiation, and maintenance are, however, only incompletely characterized.

The most immature T cell precursors differentiate within the thymic cortex, where they acquire the expression of both CD4 and CD8 (double-positive [DP] stage of development) and eventually express the complete αβ TCR (4). As their Ag specificity is randomly generated, DP thymocytes are subjected to a selection process aimed at testing their suitability for a given individual. Known as positive selection, thymocytes with a TCR that recognizes self-peptide/MHC complexes on cTEC with sufficient affinity will continue their intrathymic maturation and migrate to the medulla. There, thymocytes are exposed to a negative selection. This process purges TCR-bearing cells with an affinity for self-peptide/MHC complexes above a critical threshold and thus prevents self-Ag recognition by T cells of extra thymic tissue, which may elicit autoimmunity.

MicroRNA (miRNA) represent an essential class of small (19–25 nt), noncoding RNAs indispensable for biological processes including cell fate determination, self-renewal, differentiation, proliferation, apoptosis, and cellular homeostasis (5). Primary miRNA transcripts are processed by nuclear RNAse III enzyme Drosha and its cofactor DGCR8 to intermediate miRNAs, which are exported to the cytoplasm. There, second RNAse III enzyme, designated Dicer, catalyzes the formation of miRNA duplexes. These short sequences are integrated into the RNA-induced silencing complexes and control protein synthesis by interacting with target miRNA either repressing translation or mediating RNA cleavage and degradation (6). A single miRNA species may regulate the expression of hundreds of proteins, though the repression is usually mild and frequently the result of both downregulation of miRNA levels and inhibition of translation (7–9). Cell- and tissuespecific miRNA expression patterns have been identified, suggesting unique biological roles for specific miRNA. However, the precise functions for almost all of the at least 1055 mouse miRNA...
remain to be experimentally verified (10). To judge the global role of miRNA for the development, function, and homeostasis of TEC, we generated mice with a TEC targeted Dicer deficiency.

Materials and Methods

Mice

Mice were kept under specific pathogen-free conditions and used according to federal and institutional regulations. For developmental staging, the day of the vaginal plug was designated as embryonic day (E) 0.5. C57BL/6 mice were obtained in-house from the departmental breeding facility, whereas nude (nu/nu) mice were obtained from a commercial vendor (Janvier SAS, Chassal, France). Mice were kept and handled according to Cantonal and federal regulations and permissions.

Histology and immunofluorescence

Frozen thymus tissue sections (8 μm) were fixed in gradient ethanol solutions and stained with Mayer’s hematoxylin (Re®actifs RAL) and eosin (J.T. Baker). For immunohistochemistry, aceton-fixed sections (8 μm) were stained using Abs specific for cytokeratin (CK) 5 (Covance), CK8 (Progen), CK14 (Covance), MTS10 (a gift from R. Boyd, Melbourne, Australia), ERTR7 (provided by W. van Ewijk, Utrecht, The Netherlands), Psnb1 (MBL), Lyvel (ReliaTech), CR1 (8C12; BD Biosciences), autoimmune regulator (Aire) (5H12; provided by S. Hamish, Adelaide, Australia), PNAad (MECA-79; BD Biosciences), and reactivity to lectin UEA-1 (Reactolab). Alexa Fluor-conjugated anti-IgG Abs (Invitrogen) were used as secondary reagents. Images were acquired using a Zeiss LSM510 (Carl Zeiss).

Flow cytometry, cell sorting, and intracytoplasmic staining

Hematopoietic cells from thymus and spleen were stained with Abs against CD3 (KT-3), CD4 (GK1.5; BioLegend), CD8 (53–67; BioLegend), CD19 (MEL-14; BD Biosciences), CD69 (H1.2F3; BD Biosciences), CD93 (aa8.1, eBioscience) and IgM (R33.24.12). For intracellular staining, cells were marked for cell-surface molecules, fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained using Abs specific for epithelial cell adhesion molecule (EpCAM) (G8.8, DSHB; University of Iowa), CD45 (30F11; eBioscience), MHC class I (MHC I; BioLegend), MHC class II (MHC II; Af6-120.1; BioLegend), Ly51 (6C3; BioLegend), Dil-I (gift from Robson MacDonald, University of Lausanne, Lausanne, Switzerland), and UEA-1 (Reactolab). For intracellular staining, TECs were marked for cell-surface molecules, fixed, permeabilized (Cytofix/Cytoperm; BD Biosciences), and stained with BrdU or Aire-specific Abs. Flow cytometric analysis and cell sorting were performed (FACSaria) using FACSDiva (BD Biosciences) and FlowJo software (Tree Star).

miRNA detection and gene expression profiling

Total RNA was isolated from sorted cells with the miRNeasy Mini Kit (Qiagen). The QuantiMir RT Kit (System Biosciences) was used to analyze the expression of specific miRNA. cDNA was assessed by quantitative real-time PCR with SYBR Green (SensiMix; Bioline). Primer sequences are available upon request. gapdh was used as an internal control for miRNA and mRNA analysis, respectively. The mRNA expression profiles in TEC isolated from 2-wk-old Foxn1Cre::Dicerfl/fl and Dicerfl/fl mice were generated using the GeneChip Gene 1.0 ST Array System (Affymetrix). The array data were submitted to the ArrayExpress repository under accession number E-MEXP-3303 (http://www.ebi.ac.uk/arrayexpress/experiments/E-MEXP-3303).

Bioinformatic and statistical analysis

Differential expression analysis for miRNA was carried out using Partek application software (http://www.partek.com) and visualized with Integromics Biomarker Discovery for Tibco Spotfire (http://www.integromics.com). The gene expression data were normalized using Affymetrix robust multi-array analysis (Affymetrix), and differential expression was obtained by ANOVA (two-way ANOVA). The p value correction for multiple hypotheses was performed with Benjamin-Hochberg’s false discovery rate (11). Functional analyses of biological processes were determined by GeneCodis determining the significantly enriched gene ontology terms in the list of gene targets (12, 13).

BrdU analysis

Mice were injected i.p. with 1 mg BrdU in PBS, and TECs were analyzed 4 h later by flow cytometry (see above).

Bone marrow chimera mice

Sublethally irradiated newborn Foxn1Cre::Dicerfl/fl and Dicerfl/fl mice were injected i.p. with OT1 transgenic fetal liver cells (2 × 10⁶) from E13.5–14.5 embryos. Successfully grafted animals (with an average chimerism of at least 30% for OT1) were analyzed 4 wk later.

T cell depletion

Two-week-old mice were injected i.p. three times every 3 d with 200 μg anti-CD4 (GK1.5), 100 μg anti-CD8 (53–67), and 50 μg anti-Thy1.2 (T24), a dose that efficiently depletes peripheral T cells.

Fetal thymus transplants

Thymic lobes were collected from E15.5 embryos and both lobes from a single embryo were placed under the kidney capsule of the recipient nu/nu mice. Four weeks later, thymic lobes were removed and analyzed.

Statistical analysis

Statistical analysis was performed using Student t test (unpaired, two-tailed). Probability values were classified into four categories: p > 0.05 (NS), *0.05 ≥ p > 0.01, **0.01 ≥ p > 0.001, and ***p ≤ 0.0001.

Results

Thymus cellularity and T lymphopoietic activity are decreased in the absence of Dicer expression in TEC

To study the integrated and global role of miRNA in TEC development and function, we generated mice that lack dicer1 expression in thymic epithelia. For this purpose, mice with a conditional dicer1 allele [Dicerfl/fl (14)] were crossed to transgenic animals expressing the Cre recombinase in all TECs (15). Because Cre expression in heterozygote Foxn1-Cre transgenic mice is nontoxic to TECs (Supplemental Fig. 1) (16), Dicerfl/fl mice negative for the expression of Cre were used as controls and compared with heterozygous Cre-transgenic Dicerfl/fl mice (designated Foxn1-Cre::Dicerfl/fl). Thymus cellularity was unaffected in fetal and early postnatal Foxn1-Cre::Dicerfl/fl mice despite a complete depletion of the dicer1 locus and the absence of distinct miRNA species in TEC at E16.5 of gestation (Fig. 1A, 1B, 1D). A significant reduction in thymus size and absolute cell number was first observed in 3-wk-old Foxn1-Cre::Dicerfl/fl mice (Fig. 1A, 1C). In contrast, changes in intrathymic T cell differentiation were already apparent in Foxn1-Cre::Dicerfl/fl mice as early as the first week of life (Fig. 1E, 1F). Although the frequency of double-negative (DN) cells was increased, CD4+ CD8+ single-positive (SP) thymocytes with a mature phenotype (CD24loCD3hi) were consistently increased though all subsequent maturation stages were diminished (Fig. 1E, 1F). By 30 wk of age, DP thymocytes were almost completely absent in Foxn1-Cre::Dicerfl/fl mice. The competence of Dicer-deficient TEC to support subsequent maturation to SP, naive T cells.

Commitment to the T cell lineage requires Dicer expression in cTEC

We next investigated the nature of the CD4+ CD8+ thymocytes as these cells were already increased in Foxn1-Cre::Dicerfl/fl mice as early as 1 wk of age and represented more than half of all the thymocytes in 30-wk-old mutant animals (Fig. 1E). The screening
of these cells for the expression of non-T cell lineage markers identified a relative and absolute increase of CD19+ cells (Fig. 2A). Although a substantial proportion of these cells expressed high levels of CD93 similar to the pattern observed for B cells in the bone marrow of both wild-type and mutant mice (Fig. 2A), they did not express IgM. We therefore conclude that these were immature thymic B cells that developed in situ, possibly due to changes in the microenvironment. Indeed, the thymic medulla progressively displayed increased numbers of Lyve-1+ lymphatic vessels, PNAd+ high endothelial venules, and CR-1+ follicular dendritic cells (Fig. 2B) that in aggregate resulted in older animals in a histological structure reminiscent of secondary lymphoid tissue.

Because commitment to a T cell fate is dependent on Dll4 expression by cTEC (17), we next tested whether Foxn1-Cre::Dicerfl/fl mice lacked the correct expression of Dll4+. The relative frequency and absolute cell number of Dll4+ cTEC was halved in 3-wk-old Foxn1-Cre::Dicerfl/fl mice when compared with controls (Fig. 2C). The relative frequency of early thymic progenitors (defined as Lin$^-$CD25$^-$CD24$^-$CD44$^{hi-}$Kit$^+$ cells) among thymocytes was in parallel decreased by 2-fold when compared with age-matched controls (0.02 ± 0.011 versus 0.04 ± 0.017; p < 0.05, n = 4). Thus, Dicer-deficient TECs created an altered microenvironment reduced in the molecular cues critical for the attraction of early thymic progenitors and their commitment to the T cell lineage.

Dicer-deficient TEC fail to maintain a regular thymic microenvironment

To further investigate the consequences, a TEC-restricted loss of Dicer-dependent miRNA expression, control, and mutant thymus tissue sections were analyzed for the composition and organization of their stroma. In 2-d- and 3-wk-old mice of both groups, histological analyses demonstrated well-demarcated medullary islands surrounded by a cell-dense cortex (Fig. 3A, Supplemental Fig. 2). In 3-wk-old mutant mice, the cortical TEC network was, however, less dense and, contrary to the wild-type thymus, mainly composed of cytokeratin (CK)5$^+$CK8$^+$ cTEC (MTS10$^+$). The thymic cortex of mutant mice had also changed in as much as its epithelia expressed lower amounts of Psmb11 (a.k.a. β5),
a cTEC-specific component of the thymoproteasome pivotal for the differentiation of MHC I-restricted CD8 SP thymocytes (18) (Fig. 3A, 3B). mTEC (CK5$^+$MTS10$^+$) were reduced in number and mostly located at the cortico-medullary junction. In parallel, ERTR7$^+$ fibroblasts had accumulated in the presumed medulla, where the few remaining mTEC were usually less reactive with UEA-1, though Aire$^+$ epithelia and dendritic cells could still be detected (Fig. 3A and G. Nusspaumer and G.A. Hollander, unpublished observations).

The small thymus remnant detected in 30-wk-old Foxn1-Cre::Dicerfl/fl mice had lost its typical cortico-medullary organization, and the few remaining TEC were now arranged in several solitary islands devoid of mTEC contributions (Fig. 3B). Though almost all of the epithelia displayed a cortical phenotype (CK8$^+$MTS10$^+$UEA-1$^+$), the expression of Psmb11 could not be detected anymore. The loss of Dicer expression differentially affected TEC cellularity of Foxn1-Cre::Dicerfl/fl mice. In 2- and 3-wk-old mutant mice, the relative frequency and the absolute cell numbers of mTEC were decreased (Fig. 3C). In contrast, both absolute and relative cTEC numbers of cTEC were increased (Fig. 3C), a change that correlated with a higher proliferation rate (Fig. 3D). Thus, a lack of Dicer expression in TEC progressively precludes the normal differentiation, patterning, maintenance, and function of these cells.

To assess whether miRNAs are specifically required by TEC for MHC expression and their differentiation from immature (MHClo) to mature (MHChi) mTEC, we next analyzed TEC for their MHC expression and mTEC for the detection of Aire. Both cTEC and mTEC from Foxn1-Cre::Dicerfl/fl and Dicerfl/fl mice expressed comparable amounts of MHC I and II molecules (Fig. 3E). However, the relative frequency of MHC IIloAire$^2$mTEC was reduced and that of MHC IIhiAire$^2$mTEC was increased in Foxn1-Cre::Dicerfl/fl mice. Thus, miRNAs appear to be required for the regular maintenance of MHC IIlo mTEC but dispensable for their differentiation into MHC IIhi Aire$^+$ mTEC, which express an array of peripheral tissue Ags (PTA) and are critical for negative selection of maturing thymocytes (see below).
FIGURE 3. Dicer-deficient TEC fail to maintain a regular thymic microenvironment. H&E staining (top panels) and immunofluorescence analysis of thymic tissue sections from 3-wk-old (A) and 30-wk-old (B) Dicer<sup>fl/fl</sup> or Foxn1-Cre::Dicer<sup>fl/fl</sup> mice. For immunohistology, Abs specific for ERTR7 were used to identify fibroblasts, CK8 and Psmbl1 for the detection of cTEC, and MTS10, CK5, and Aire as well as reactivity with UEA-1 for the identification of mTEC. Original magnification ×20. Bottom panels display close-ups in the upper right corner. Data are representative of at least three separate experiments with at least two mice each. (C) Flow cytometric analysis of TEC (CD45<sup>-</sup> EpCAM<sup>+</sup> MHC II<sup>+</sup>) subpopulations isolated from 2- and 3-wk-old mice. The relative frequency (left panels) and absolute cell numbers (right panel) of cTEC (UEA<sup>-1</sup>-Ly5<sup>1+</sup>) and mTEC (UEA<sup>-1</sup>-Ly5<sup>1+</sup>) in Dicer<sup>fl/fl</sup> (black bars) and Foxn1-Cre::Dicer<sup>fl/fl</sup> (white bars) mice are shown. Data indicate the mean ± SD of at least three mice per group. Data are representative of three independent experiments. (D) BrdU incorporation in cTEC and mTEC isolated from 2-wk-old Dicer<sup>fl/fl</sup> and Foxn1-Cre::Dicer<sup>fl/fl</sup> mice pulsed for 4 h. The percentage of TEC incorporating the label (mean ± SD, three mice per group) is shown. Data are representative of at least two separate experiments. (E) MHC cell-surface expression on Dicer-deficient TEC. cTEC and mTEC from Dicer<sup>fl/fl</sup> (solid lines) and Foxn1-Cre::Dicer<sup>fl/fl</sup> (Figure legend continues)
Dicer-deficient cTEC fail to impose efficient positive selection

Given the changes in cTEC, we next investigated in Foxn1-Cre::Dicerfl/fl and control mice the sequential changes in thymocyte CD3 and CD69 cell-surface expressions as phenotypic markers of positive selection (19). Under physiological conditions, positive selection sets off a transient upregulation of CD69 among CD3int DP thymocytes. In turn, these cells sequentially adopt a CD3hiCD69+ and eventually a CD3hiCD69− cell-surface phenotype. The relative frequencies of these distinct thymocyte populations were undisturbed in 1-wk-old Foxn1-Cre::Dicerfl/fl mice, implying thymocyte positive selection to be normal (Fig. 4A). In contrast, 2-wk-old and older mutant mice displayed a progressively compromised positive selection, as the frequencies of their CD3int CD69+ and CD3hiCD69+ DP thymocytes were gradually reduced (Fig. 4A) despite an increased frequency of cTEC (Fig. 3C). To detail early postselection steps in DP thymocyte differentiation, we also analyzed changes in CD4 and CD8 expression on either CD3intCD69+ or CD3hiCD69+ DP cells (Fig. 4B). Although the downregulation of CD8 on CD3intCD69+ cells occurred normally in 1- and 2-wk-old Foxn1-Cre::Dicerfl/fl mice, this was impaired in 3-wk-old mutants (Fig. 4B, left panels). Focusing on younger Foxn1-Cre::Dicerfl/fl mice with a seemingly undisturbed cortical epithelial microenvironment, we also noticed a gradual defect in the progression of CD3hiCD69hi DP thymocytes (via the intermediate CD3hiCD69med stage) to cells with a mature SP CD8 phenotype (Fig. 4B, right panels). Thus, positive selection of DP thymocytes is significantly impaired in Foxn1-Cre::Dicerfl/fl mice as early as the second week of life and is preceded by a defect in DP maturational progression toward the CD8 SP lineage.

Because Dicer-deficient mice express an impaired thymoproteasome (Fig. 3A), we next investigated the development of MHC I-restricted thymocytes expressing a T cell Ag receptor known to depend on Psmb11 expression for its efficient positive selection (20). For this purpose, sublethally irradiated neonatal Foxn1-Cre::Dicerfl/fl and Dicerfl/fl mice were engrafted with day 14 fetal liver (20). For this purpose, sublethally irradiated neonatal Foxn1-Cre::Dicerfl/fl and Dicerfl/fl mice were engrafted with day 14 fetal liver (20). To this end, 2-wk-old and older mutant mice displayed a concomitant reduction in OT-1 TCR-transgenic thymocytes was severely compromised in chimeric Foxn1-Cre::Dicerfl/fl mice, and this resulted in a significant reduction of cells with an immediate postselection phenotype (i.e., CD4hiCD8int and CD4hiCD8hi) (Fig. 4C). The partial block in positive selection resulted in parallel in an increase in preselection DP thymocytes and a concomitant reduction in OT-1 transgenic T cells committed to the CD8 lineage (Fig. 4C, left and middle panel). Moreover, chimeric Foxn1-Cre::Dicerfl/fl mice displayed a reduced frequency of mature transgenic SP CD8 thymocytes expressing high surface Vα2 concentrations (Fig. 4C, right panel). Taken together, the absence of Dicer expression in TEC resulted in reduced positive thymocyte selection and an altered Ag receptor repertoire.

To test whether the thymus phenotype in Foxn1-Cre::Dicerfl/fl mice was affected by Foxn1-Cre mediated recombination in keratinocytes and consequent systemic influences, we transplanted embryonic thymic tissue from both Dicerfl/fl and Foxn1-Cre::Dicerfl/fl mice under the kidney capsule of nu/nu recipients. Analysis of the graft tissue 4 wk after transplantation revealed a phenotype identical to that observed in age-matched Foxn1-Cre::Dicerfl/fl mice (Supplemental Fig. 3A–C). This finding is in keeping with the observation that Foxn1-Cre::Dicerfl/fl mice have a normal skin (Supplemental Fig. 3D).

Gene expression analysis in Dicer-deficient TEC uncover miRNA-sensitive cellular processes

Gene expression profiles were established for both cortical and medullary TEC subpopulations to identify transcripts that are significantly up- or downregulated as a consequence of Dicer deficiency (Fig. 5A, 5B). For this purpose, Foxn1-Cre::Dicerfl/fl and Dicerfl/fl mice were investigated at 2 wk of age because the former animals still had a relatively intact cTEC cellularity but already displayed functional deficiencies. Gene ontology analysis of significantly upregulated and downregulated transcripts (corrected \(p < 0.05\)) predicted multiple cellular processes to be affected in cTEC, mTEC, and both types of TEC (Fig. 5C, 5D). Specifically, transcription, cell signaling, differentiation, adhesion, apoptosis, and the organization of extracellular matrix were predicted to be altered as a consequence of Dicer deficiency in TEC.

Dicer deficiency in TEC alters peripheral T cell phenotype

The lack of Dicer expression in TEC led to significant phenotypic changes among peripheral T cells. Both naive CD4+ and CD8+ T cells (i.e., CD62L+CD44hi) were reduced in Foxn1-Cre::Dicerfl/fl mice at 8 wk of age, whereas the relative frequency of memory T cells was 2- to 3-fold more abundant, a finding likely caused by moderate T lymphopenia (Fig. 6A, 6B). Moreover, Foxn1-Cre::Dicerfl/fl mice displayed a proportional increase in CD8+ effector (CD44+CD62Llow) and central memory (CD44hiCD62Lhi) T cells. In keeping with the degree of lymphopenia, the relative frequency of Foxp3+CD4+ regulatory T cells was only modestly increased, though their cellularity was not significantly different from that of Dicerfl/fl mice (Fig. 6C).

T cells selected in a thymus with Dicer-deficient TEC elicit autoimmunity

We next examined whether T cells selected by Foxn1-Cre::Dicerfl/fl mice were prone to elicit autoimmunity because their selection was altered by thymic microenvironmental changes, possibly resulting in a T cell repertoire with different functional properties. Although Foxn1-Cre::Dicerfl/fl mice were followed and regularly analyzed for as long as 45 wk, spontaneous lymphocytic organ infiltrates were not observed at a frequency different to that of control mice (S. Zuklys and G.A. Holländer, unpublished observations). As peripheral tolerance may have been maintained in Foxn1-Cre::Dicerfl/fl mice by T cells that had early on developed in a yet largely normal though already miRNA-deficient thymic microenvironment (22), mutant and wild-type mice were efficiently T cell depleted at the age of 2 wk using a mixture of anti-CD4, -CD8, and -Thy1.2 Abs. In the course of autologous reconstitution, newly formed T cells were now selected in a thymic microenvironment, which, in the case of Foxn1-Cre::Dicerfl/fl mice, had adopted an altered architecture and had lost its capacity for normal TCR selection. Multiorgan infiltration developed in Foxn1-Cre::Dicerfl/fl but not control mice over the course of 30 wk, variably affecting eyes, pancreas, salivary glands, and liver...
The inflammatory cell infiltrations of the eyes altered the retinal architecture in its entire thickness, leading to a partial destruction of the photoreceptor outer segment and the nuclear layer. As thymic expression of self-Ags is critical for the maintenance of self-tolerance, transcripts for selective single self-Ags were quantified in purified mTEC of 2-wk-old Foxn1-Cre::Dicerfl/fl and Dicerfl/fl mice (Fig. 7B, 7C). This time point had specifically been chosen because the relative frequency of MHC IIhiAire+ mTEC was comparable for both mouse strains (Fig. 3F). A significant decrease in both Aire-dependent and -independent...
scripts was detected with retinal, salivary gland (salivary protein 1), hepatic (C-reactive protein), and pancreatic (Insulin-2; glutamic acid decarboxylase 1) PTA invariably reduced in Dicer-deficient mTEC (Fig. 7B–D). These changes correlated with the pattern of tissue infiltrations linking defects in PTA expression to organ-specific autoimmune pathologies.

FIGURE 5. Analysis of gene expression changes in Dicer-deficient TEC and its impact on cell signaling. Volcano plot analysis of mRNA expression changes in cTEC (A) and mTEC (B) from 2-wk-old Foxn1-Cre::Dicer<sup>fl/fl</sup> mice compared with Dicer<sup>fl/fl</sup> mice. Positive values on the x-axis indicate an upregulation of transcripts in mutant TEC presented as fold changes, whereas negative values specify downregulated transcripts shown as fold changes. The y-axis represents a log scale revealing the corrected p value for a two-way ANOVA of the differences between samples. The data represent two separate biological replicates with at least 10 mice per group. The bar graphs show gene ontology processes significantly affected (corrected p < 0.05) in cTEC (C) and mTEC (D), respectively.

FIGURE 6. Dicer deficiency in TECs results in altered T cell numbers. (A) Relative frequencies and absolute cell numbers (mean ± SD) of splenic CD4 and CD8 T cells in 8-wk-old Dicer<sup>fl/fl</sup> (black bars) and Foxn1-Cre::Dicer<sup>fl/fl</sup> mice (white bars). Data are representative of three separate experiments with at least three mice per group. (B) Left panels. Representative analysis of CD44 and CD62L expression among splenic T cells from 8-wk-old Dicer<sup>fl/fl</sup> and Foxn1-Cre::Dicer<sup>fl/fl</sup> mice. The numbers show the relative frequency of a given subpopulation as identified by the drawn gate. Right panels, Absolute numbers (mean ± SD) of T cells from 8-wk-old Dicer<sup>fl/fl</sup> (black bars) and Foxn1-Cre::Dicer<sup>fl/fl</sup> mice (white bars) with naive and memory phenotypes. Data are representative of three separate experiments with at least three mice per group. (C) Relative frequency (upper graph) and absolute cell numbers (lower graph) of splenic CD4<sup>+</sup>Foxp3<sup>+</sup> T cells in 8-wk-old Dicer<sup>fl/fl</sup> (black bars) and Foxn1-Cre::Dicer<sup>fl/fl</sup> mice (white bars). Data are representative of two separate experiments with at least three mice per group. *p < 0.05.
Discussion

Short, noncoding RNAs typically modulate the regulation of organ development via subtle though efficient parallel targeting of multiple components within a regulatory network (10, 23). Because the molecular targets of most miRNA remain experimentally unverified, their role in tissue differentiation and function has largely been interrogated via global interference with their biogenesis (5). In this study, the dependence of TEC differentiation and function on the biological properties of miRNA has been investigated in mice in which Dicer is ablated after formation of the thymus primordium but before the complete patterning of its microenvironment. Our results reveal that miRNA are essential for the maintenance of a regularly composed and correctly functioning thymic microenvironment.

The TEC-targeted, embryonic loss of Dicer results in a sequential emergence of structural and functional alterations, with defects apparent only after the first week of life. The asynchronous appearance of a complex phenotype is likely the result of a substantial variation in the decay of individual miRNA following dicer1 gene ablation (24) and may also reflect a variable susceptibility of separate TEC subpopulations for the loss of specific miRNA.

The hematopoietic cells settling into the thymus require signals from DI4 expressed by cTEC for their commitment to a T cell fate (17). In the absence of Dicer, fewer cTEC express DI4, which suggests DI4 as a likely indirect target of miRNA. Diminished expression of DI4 on cTEC correlates with the observed reduction of early thymic progenitors and a robust B cell differentiation in situ. Because the Notch ligand density is important in vitro for the commitment of hematopoietic precursors to a T cell fate (25), quantitative signaling differences suffice to explain the in situ B cell development in younger Foxn1-Cre::Dicerfl/fl mice. These findings provide a molecular mechanism (i.e., reduced Dll4 expression by cTEC) for the increase in intrathymic B cell development (this report and Ref. 26).

In older mutant animals, the microenvironment is unable to attract uncommitted hematopoietic precursors and consequently neither immature B nor T cells can be detected. Rather, the cellular composition and organization of the microenvironment demonstrates a structure reminiscent of secondary lymphoid tissues where lymphocyte homing is directed through PNAd+ venules (27), and new lymphatic vessels are established (in the absence of overt inflammation), possibly sprouting from pre-existing endothelial cells (28). The increase and persistence of the two vascular
Thymocyte negative selection is likely impaired in Foxn1-Cre::Dicerfl/fl mice because their mTEC display a significant deficiency in promiscuous gene expression. The molecular signature of this defect implies a common yet undefined mechanism that controls some but not all known PTA transcripts. Though the differentiation of Aire+ mTEC was apparently independent of miRNAs, the expression of a significant number of Aire-dependent PTAs was, however, affected by the lack of Dicer expression. It is thus conceivable that miRNAs may act either in concert or downstream of Aire to regulate the expression of a subset of Aire-dependent PTAs. In parallel, our data likewise reveal that miRNA also control the expression of a number of Aire-independent PTA, possibly via a common pathway. As a corollary, central tolerance induction is defective, and autoimmunity ensues. Because PTA expression confined to the perinatal period suffices to induce long-lasting tolerance (22), functionally relevant defects in negative selection become apparent only once the initially chosen T cell pool is replaced by cells selected in the distorted thymic microenvironment of ≥2-wk-old Foxn1-Cre::Dicerfl/fl mice. The pattern of organ-specific autoimmunity observed in these mice correlates with the altered expression profile of tissue-specific self-Ags. It is therefore conceivable that some forms of autoimmunity may also occur as a consequence of known or not yet described miRNA polymorphisms that either affect the biogenesis of miRNA or influence their ability for target repression.

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