Canonical microRNAs in thymic epithelial cells promote central tolerance

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Medullary thymic epithelial cells (mTECs) facilitate the deletion of developing self-reactive T cells by displaying a diverse repertoire of tissue-specific antigens, a process which largely depends on the expression of the autoimmune regulator (Aire) gene. Mature microRNAs (miRNAs) that regulate gene expression post-transcriptionally are generated in a multistep process. The microprocessor complex, including DGCR8, cleaves canonical miRNAs, but alternative DGCR8-independent miRNA biogenesis pathways exist as well. In order to study the role of canonical miRNAs in thymic epithelial cells (TECs), we ablated Dgcr8 using a FoxN1-Cre transgene. We report that DGCR8-deficient TECs are unable to maintain proper thymic architecture and exhibit a dramatic loss of thymic cellularity. Importantly, DGCR8-deficient TECs develop a severe loss of Aire⁺ mTECs. Using a novel immunization approach to amplify and detect self-reactive T cells within a polyclonal TCR repertoire, we demonstrate a link between the loss of Aire expression in DGCR8-deficient TECs and the breakdown of negative selection in the thymus. Thus, DGCR8 and canonical miRNAs are important in TECs for supporting central tolerance.

Keywords: Aire · Central tolerance · DGCR8 · MicroRNAs · Thymic epithelial cells

Introduction

Thymic epithelial cells (TECs) support T-cell development in two distinct stages. Cortical thymic epithelial cells (cTECs) facilitate the positive selection of thymocytes that have undergone TCR rearrangements capable of recognizing self-MHC [1]. Positively selected thymocytes undergo negative selection by medullary thymic epithelial cells (mTECs) to eliminate self-reactive T cells [2]. To prevent autoimmunity, mTECs display a diverse repertoire of tissue-specific antigens (TSAs) whose expression is otherwise restricted to peripheral tissues [3–5]. Developing thymocytes bearing a TCR recognizing the TSAs undergo apoptosis to purge the developing T-cell pool of self-reactive T cells [6–8]. TSA expression in mTECs is largely dependent on autoimmune regulator (Aire), which is expressed in a subset of mature mTECs expressing high levels of MHC II and the costimulatory molecule CD80 [5, 9, 10]. Both patients and mice with mutations in Aire develop multiorgan autoimmunity which underscores the importance of TSA expression for the elimination of self-reactive T cells in maintaining tolerance [3, 11, 12].

MicroRNAs (miRNAs) are ~22 nucleotide-long noncoding RNAs that mediate sequence-dependent post-transcriptional gene repression [13, 14]. The primary miRNA transcripts of canonical miRNAs are processed by a complex formed by DROSHA and DGCR8 to generate ~60–80 nucleotide hairpin precursor miRNAs. After export to the cytoplasm, these hairpins are further processed by the RNase III enzyme Dicer to produce mature miRNAs. However, Dicer does not exclusively process miRNA precursors
but rather includes a variety of small RNAs such as endogenous siRNAs, endogenous shRNAs, mirtrons, and Alu RNAs [15–17]. By ablating key genes required for miRNA biogenesis, we and others have previously demonstrated the importance of miRNAs in various lymphocyte populations [18–22]. Similarly, Dicer is important for TEC biology [23–25]. However, since Dicer is not restricted to processing miRNAs it remains unclear whether TEC development and function are truly dependent on the canonical miRNA pathway [15–17].

To further define the role of canonical miRNAs in TECs, we generated mice with TEC-specific deletion of Dgcr8, a component of the miRNA-specific microprocessor complex [16, 26]. Here, we find that DGCR8 is critical for maintaining the proper expression of Aire and the overall architecture of the thymic medulla. Furthermore, we demonstrate a breakdown in thymic negative selection in these animals by detecting pathogenic autoreactive T-cell clones in the periphery that are normally deleted in the thymus. Thus, proper thymic architecture and central tolerance depend on canonical miRNAs expressed in TECs.

Results and discussion

Thymic architecture and TEC composition depend on miRNAs

To study the role of canonical miRNAs in TEC function we first analyzed Dgcr8 expression in mTECs and cTECs from C57BL/6J WT mice and found no significant differences in expression (data not shown). We then utilized FoxN1-Cre knock-in mice, which express Cre recombinase in all TECs without disrupting FoxN1 function, to conditionally inactivate Dgcr8 in TECs (Dgcr8 ΔTEC) [26, 27]. We used qPCR analysis to verify that the deletion of Dgcr8 in Dgcr8 ΔTEC mice was comparable between mTECs and cTECs (data not shown). At 2 weeks of age Dgcr8 ΔTEC mice exhibited evidence of disrupted thymic architecture with a loss of the distinct keratin-8 (K8) and keratin-5 (K5) staining patterns as compared with the characteristic separation between cortex and medulla in littermate control mice (Fig. 1A). The TECs in Dgcr8 ΔTEC mice appeared to be thinned out and many expressed both keratin markers (K5+/K8+) (Fig. 1A). Although Dgcr8 ΔTEC mice showed increased frequencies and absolute numbers of cTECs at 2 weeks of age, cTEC numbers were comparable to those of littermate controls by 6 weeks. In contrast, mTEC cellularity was reduced by nearly 80% in 2-week-old Dgcr8 ΔTEC mice and progressed to a 95% loss by 6 weeks (Fig. 1B). Within the mTEC compartment in 2-week-old mice, the relative frequency of Aire+ cells was reduced while the immature mTEC (MHC IIlow Aire−) and the more mature mTEC (MHC IIhi Aire−) cell subsets were relatively enriched (Fig. 1C) [9, 10]. In contrast, absolute cell numbers were reduced across all mTEC subsets at both 2-week and 6-week time points (Fig. 1D). However, the loss was most prominent in the Aire+ cells. Thus, proliferating immature mTEC precursors could be partially compensating for the loss of the most mature mTECs. Supporting this notion, increased frequencies of the relatively enriched mTEC hi and mTEC hi cell subsets expressed the proliferation marker Ki67 (Fig. 1D). By 6 weeks of age, both mTEC hi and Aire+ cells were relatively depleted in Dgcr8 ΔTEC mice while the mTEC lo subset was enriched. Similar to the 2-week time point, a larger proportion of mTEC hi cells expressed the proliferation marker Ki67 (Fig. 1D). Thus, increased proliferation rates of mTEC precursor cells partially compensated for the loss of the more differentiated mTECs.

To investigate whether the loss of Aire+ mTEC resulted from the TEC-intrinsic loss of Dgcr8 expression in mTEC or was an indirect consequence of disturbed TEC-thymocyte cross-talk we analyzed neonatal mice. While overall thymocyte cellularity was comparable between Dgcr8 ΔTEC and control mice 2 days postnatally, Dgcr8 ΔTEC mice exhibited a significant loss of both mTEC and cTEC cellularity (Supporting Information Fig. 2 A–E). The mTEC loss was specific to the mature mTEC hi and Aire+ subsets, which is indicative of an initial TEC-intrinsic maturation defect in the thymi of Dgcr8 ΔTEC mice. Additional impaired TEC-thymocyte cross-talk may occur at later time points.

Together, these findings demonstrate that DGCR8-dependent canonical miRNAs are essential for TEC cellularity and mTEC maturation, particularly the accumulation and maintenance of Aire-expressing mTECs. This suggests that the histologically apparent mTEC voids in 6-week-old mice represent a true absence of mTECs. In addition, the altered relative TEC composition suggests a superimposed differentiation defect in which the mature mTEC hi and Aire+ mTEC subsets are diminished while the immature mTEC lo cells accumulate and exhibit increased proliferation. These findings are consistent with the increased presence of K5+/K8+ cells in Dgcr8 ΔTEC thymic sections suggesting that the loss of the most differentiated mTEC may trigger a proliferative response in immature TECs to compensate for the overall loss of TEC cellularity.

miRNAs are required for the maintenance of thymocyte cellularity

The profoundly altered thymic architecture and TEC cellularity suggested that thymocyte development could be affected by TEC-specific miRNA-deficiency. Thymi from 6- to 8-week old Dgcr8 ΔTEC mice showed a significant reduction of over 60% in thymic

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cellularity (Fig. 2A). In contrast, the relative frequencies of CD4−CD8− double negative, CD4+CD8+ double positive, CD4+ single positive, CD8+ single positive thymocytes, and CD4+FoxP3+ Treg cells were not affected in Dgcr8ATEC mice. As a consequence, absolute numbers of all thymocyte developmental stages were proportionally reduced. These results suggest that although Dgcr8ATEC mice have a severely disrupted thymic architecture and significant reduction in TECs, the remaining TECs are sufficient to support T-cell development. This finding is reminiscent of Smad4-deficient TEC that lead to substantial thymic hypoplasia but intact relative thymocyte development [29]. Thus, the thymus appears to have a remarkable ability to maintain thymocyte development despite severely impaired TEC numbers and composition. Next, we analyzed whether the reduction of
Thymic T-cell numbers resulted in peripheral T-cell lymphopenia. In contrast to the thymic cellularity, total splenic cellularity was not different between Dgcr8\(^{\Delta TEC}\) and control mice, and CD4\(^{+}\) and CD8\(^{+}\) T-cell numbers were only modestly reduced (Fig. 2B). Thus, homeostatic proliferation in the periphery most likely compensated for the reduced thymic cellularity. However, despite the relatively normal thymocyte development and the presence of substantial numbers of T cells in lymph nodes and spleen, we could not exclude that the thymocytes developing in a Dgcr8\(^{\Delta TEC}\) microenvironment were functionally impaired or had a skewed TCR repertoire due to defective thymocyte selection. Indeed, mice with Dicer-deficient TECs develop collagen-induced arthritis with increased incidence but decreased severity suggesting both an altered T-cell repertoire and possibly impaired T-cell function [23]. Thus, Dicer-deficient TEC are not able to support numerically and functionally normal thymocyte development.

miRNA deficiency in TECs causes a breakdown in central tolerance

Given the complex consequences on T cells developing in Dicer-deficient TEC [23] and the prominent and progressive loss of Aire\(^{+}\) mTECs we sought to determine whether Dgcr8\(^{\Delta TEC}\) mice had a defect in central tolerance. Dgcr8\(^{\Delta TEC}\) mice did not develop spontaneous autoimmunity as evidenced by immune infiltrates in various organs or the presence of autoantibodies when compared with littermate controls, even when aged out beyond 45 weeks (data not shown). These findings are consistent with previous work which found that Aire expression during the perinatal period is sufficient to induce central tolerance [30]. In addition, similar results have been reported for mice with Dicer-deficient TECs [25]. In these studies, depletion of T cells at 2 weeks of age to allow the seeding of potentially autoreactive T cells developing in a Dicer-deficient TEC microenvironment led to multiorgan autoimmune disease after 30 weeks [25]. Thus, the presence of some Aire\(^{+}\) TECs during the perinatal period, peripheral Aire expression, and other peripheral tolerance mechanisms likely cooperated to prevent the development of spontaneous autoimmunity in Dgcr8\(^{\Delta TEC}\) mice [5, 31].

We hypothesized that although Aire expression is partially maintained in young Dgcr8\(^{\Delta TEC}\) mice, self-reactive T cells could have escaped thymic deletion due to the disturbance of thymic architecture and the progressive loss of Aire\(^{+}\) mTECs, but be kept in check by peripheral tolerance mechanisms. We aimed at testing this hypothesis in the polyclonal T-cell repertoire employing a novel approach to expand and detect Aire-dependent autoreactive T cells. In previous work, we determined that IRBP-specific
T cells are normally deleted efficiently in the thymus of Aire-sufficient hosts and that such cells escape deletion in Aire-deficient thymi and provoke autoimmune uveitis [6]. Utilizing a previously described tetramer enrichment protocol, we developed methods to detect T cells with this specificity in the polyclonal repertoire of Aire-deficient hosts [8, 32]. Thus, we hypothesized that escaped self-reactive IRBP-specific CD4+ T cells could be detected in Dgcr8ΔTEC mice given the loss of proper Aire expression in these mice. To expand T cells for detection, we immunized Dgcr8ΔTEC and control mice with a MHC II binding IRBP peptide epitope (P2) and 10 days later pooled lymph nodes and spleen to enumerate CD4+ P2-I-AΔ1-reactive T cells. Consistent with the loss of Aire+ mTECs, immunized Dgcr8ΔTEC mice showed a significant expansion of P2-specific CD4+ T cells when compared with littermate controls (Fig. 3A and Supporting Information 1B). Importantly, this expansion was also associated with a breakdown in immune tolerance with the generation of IRBP-specific autoantibodies and autoimmune uveitis in immunized Dgcr8ΔTEC mice when compared with control mice (Fig. 3B-C).

Concluding remarks

In summary, we show here that Dgcr8 expression in TECs is critical for the maintenance of proper corticomedullary thymic architecture and that canonical miRNAs are unequivocally required to support both TEC and thymocyte cellularity. miRNAs are critical for TEC differentiation and composition and for the development and maintenance of Aire+ mTECs. Using a novel immunization approach to expand and detect autoreactive T cells in a polyclonal TCR repertoire, we demonstrate that TECs rely on miRNAs to prevent a breakdown in central tolerance. Furthermore, we show that immunization with self-antigen followed by tetramer-mediated detection of expanded self-reactive T-cell clones can be used as an effective and rapid tool to screen for central tolerance defects in animal models. Thus, such an approach may be useful to screen for hidden central tolerance defects in large scale mutagenesis projects.

Materials and methods

Mice

FoxN1-Cre knock-in mice were kindly provided by N. Manley [27]. Floxed Dgcr8 mice were kindly provided by R. Blelloch [26]. IRBP+/− mice were described previously [6]. Throughout this study, Dgcr8ΔTEC represents B6.FoxN1-Cre+ Dgcr8Δ/Δ mice and littermate controls are both B6.FoxN1-Cre+ Dgcr8Δ/Δ mice and all B6.FoxN1-Cre− mice. Mice were housed and bred under specific-pathogen free conditions at the University of California, San Francisco (UCSF) Animal Barrier Facility. Animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at UCSF.

Histology and immunofluorescence

Thymi were harvested and embedded in Tissue-Tek Optimal Cutting Temperature media. Eight micrometer frozen thymic sections were fixed in 100% acetone and blocked in 10% goat serum.

Figure 3. miRNA deficiency in TECs causes a breakdown in central tolerance. (A) Mice were immunized with P2 peptide and then harvested 10 days later by flow cytometry following a tetramer pulldown assay. Plots are pregated on DAPI+, NK1.1−, CD11b−, CD11c+, F4/80−, B220−, CD3− events. Absolute numbers of P2-specific cells are inset within the flow cytometry plots. Tetramer data are pooled from four to five samples in three independent experiments. IRBP−/− mice were included as a positive control for immunization and tetramer pulldown. (B) The IRBP-specific immune response was assessed by an IRBP autoantibody assay in mice immunized with P2 peptide and harvested 21 days later. (C) Eyes harvested from mice in (B) were H&E stained and scored for infiltrates. Scale bars = 200 μm. Data in (B) and (C) is shown as mean ± SEM of 7–8 samples pooled from two independent experiments. * denotes p ≤ 0.05, and ** denotes p ≤ 0.01, Mann–Whitney test.
before incubation with primary antibodies. Primary antibodies were purchased from either Abcam (keratin-5, keratin-8) or eBio-
sience (Aire) and all secondary antibodies were purchased from
Invitrogen. Immunofluorescence slides were visualized using a
Zeiss Apotome widefield microscope. For eye disease scoring, eyes
were processed by formalin fixation and H&E staining as previ-
ously described [6, 8]. Sections were blindly scored for severity of
infiltration and tissue destruction. H&E slides were imaged using a
Zeiss Axioimager brightfield microscope.

Flow cytometry

Thymic stromal cells were isolated as previously described [33].
Briefly, thymi were minced with razor blades and digested with
DNase I and Liberase TM (Roche) before gradient centrifuga-
tion with Percoll PLUS (GE Healthcare). Enriched stromal cells
were blocked with the Fc-receptor blocking antibody 2.4 G2 and
stained with the indicated surface marker antibodies (BioLegend).
For intracellular staining with anti-Aire-A647 (eBiosciences) and
anti-Ki67-PE (BD Biosciences), cells were stained with the Foxp3
Staining Buffer Set (eBiosciences). For staining of lymphocytes, all
surface marker antibodies were obtained from BioLegend except
anti-Foxp3-APC, which was obtained from eBiosciences. Flow
cytometry was performed using a LSR II flow cytometer (BD Bio-
cytometry), and raw data were analyzed using FACS Diva (BD Bio-
sciences) and Flow Jo (Tree Star).

Immunization

As described previously, 7- to 8-week old mice were immunized
subcutaneously with 100 μg of P2 peptide emulsified in 100 μL of
CFA [8]. For induction of autoimmune uveitis, mice were given an
i.p. injection of 400 ng pertussis toxin at the time of immunization.
Mice were harvested 10 days following immunization and lymphocytes were pooled
previously described protocols [8, 32]. Briefly, mice were harvested 10 days following immunization for tetramer
and 21 days following immunization for uveitis analysis.

Tetramer analysis

P2-I-A^b tetramer (Interphotoreceptor retinoid binding protein 3,
 amino acids 294–306) was generated by the NIH Tetramer Core
Facility, and tetramer staining was performed according to pre-
viously described protocols [8, 32]. Briefly, mice were harvested 10 days following immunization and lymphocytes were pooled
from lymph nodes and spleen. Cells were stained with tetramer for
1 hour at room temperature and enriched for tetramer^+ cells using
anti-APC microbeads and MACS columns (Miltenyi Biotec). Pos-
itively selected cells were stained with antibodies for flow cytom-
etry, and counting beads (Invitrogen) were used to enumerate
tetramer^+ cells.

Generation of ^35S-radiolabeled IRBP and autoantibody
assay

The autoantibody assay was described previously [7]. Briefly, full-
length cDNA for mouse IRBP (Thermo Scientific, #MMM1013) was used for in vitro transcription and translation and labeling with
^35S-methionine using the TNT system kit (Promega). The
^35S-IRBP was immunoprecipitated with serum samples in 96-well
PVDF filtration plates (Millipore). Serum samples were analyzed
in triplicate with 20 000 cpm of ^35S-IRBP per well. Radioactivity
of immunoprecipitated material was evaluated with a liquid scintil-
tillation counter (1450 MicroBeta Trilux, Perkin Elmer). Serum samples from Aire^+/+ and Aire^−/− mice were used as negative
and positive standards, respectively (data not shown). The IRBP
autoantibody index for each serum sample was found by the fol-
lowing calculation: (cpm in unknown sample−cpm in negative
standard) = (cpm in positive standard−cpm in the negative stan-
dard) × 100).

Statistical analysis

Statistical analysis was performed using Prism 6.0 (Graph-
pad). Mann–Whitney testing was performed for tetramer anal-
ysis, autoantibody indices, and histological analyses. Student’s
^t^ test was performed for TEC and lymphocyte analyses. ^p^ denotes
p ≤ 0.05, ** denotes p ≤ 0.01, and *** denotes p ≤ 0.001.

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Abbreviations: Aire: autoimmune regulator · miRNA: MicroRNA · TEC: thymic epithelial cell · cTEC: cortical thymic epithelial cell · mTEC: medullary thymic epithelial cell · TSA: tissue-specific antigen

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