Dicer-Dependent MicroRNAs Control Maturation, Function, and Maintenance of Langerhans Cells In Vivo

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Dendritic cells (DCs) are central for the induction of T cell immunity and tolerance. Fundamental for DCs to control the immune system is their differentiation from precursors into various DC subsets with distinct functions and locations in lymphoid organs and tissues. In contrast to the differentiation of epidermal Langerhans cells (LCs) and their seeding into the epidermis, LC maturation, turnover, and MHC class II Ag presentation capacities are strictly dependent on the presence of Dicer, which generates mature microRNAs (miRNAs). Absence of miRNAs caused a strongly disturbed steady-state homeostasis of LCs by increasing their turnover and apoptosis rate, leading to progressive ablation of LCs with age. The failure to maintain LCs populating the epidermis was accompanied by a proapoptotic gene expression signature. Dicer-deficient LCs showed largely increased cell sizes and reduced expression levels of the C-type lectin receptor Langerin, resulting in the lack of Birbeck granules. In addition, LCs failed to properly upregulate MHC class II, CD40, and CD86 surface molecules upon stimulation, which are critical hallmarks of functional DC maturation. This resulted in inefficient induction of CD4 T cell proliferation, whereas Dicer-deficient LCs could properly stimulate CD8 T cells. Taken together, Dicer-dependent generation of miRNAs affects homeostasis and function of epidermal LCs. The Journal of Immunology, 2010, 185: 400–409.

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Dendritic cells (DCs) are specialized for uptake, processing, and presentation of Ag and, as a consequence, do control tolerance and immunity (1). DCs develop from hematopoietic precursors into a heterogeneous population of subsets, which are distinct in their locations, phenotypes, and functions (2–4). In the absence of inflammation, two major subsets of DCs can be distinguished, conventional (cDCs) and plasmacytoid DCs (pDCs). In addition to these blood-derived cDCs and pDCs, tissue-derived migratory DCs can be found in lymph nodes. In skin-draining lymph nodes (sLNs), this group of migratory DCs mainly consists of Langerin-expressing epidermal Langerhans cells (LCs) as well as Langerin+ and Langerin−dermal DCs that migrated from the skin (5–7).

The complexity of different DC subtypes and their relative functions was elucidated only recently. Their central role in the maintenance of immune homeostasis was demonstrated, for example, by constitutive ablation of DCs in vivo, which resulted in spontaneous fatal autoimmunity (8). In addition, induction of T cell immunity also depends on DCs (9). A division of labor can be observed for certain tissue-derived DCs, such as LCs, which may induce potent T cell immunity either directly (10) or indirectly by transporting Ag from skin to lymph nodes for transfer to CD8α+ resident DCs. The latter then cross-present the transferred Ag from skin-derived viral infections to CD8 T cells (11). In contrast, CD8−DCs preferentially prime CD4 T cells via MHC class II presentation (12). Moreover, DCs mediate peripheral T cell tolerance by deleting self-reactive CD8 T cells, which otherwise could induce autoimmune reactions (13). Despite this striking complexity of different phenotypes and functions, DCs seem to differentiate from common precursors by largely unknown mechanisms. Cytokines, particularly Il3, and transcription factors have been shown to contribute to this process (4, 14, 15).

A novel control mechanism of immune homeostasis has become evident with the discovery of microRNAs (miRNAs), which are noncoding RNA molecules of an average of 21–22 nt in length (16). miRNAs bind to the 3′ untranslated region of mRNAs, resulting in inhibition of translation or degradation of mRNA, thereby providing mechanisms for posttranscriptional regulation of gene expression. A key enzyme in the miRNA biogenesis pathway is the microRNA-processing enzyme Dicer, which generates 22-nt mature miRNAs. Therefore, the deficiency for the gene encoding Dicer, dicer1, abolishes generation of miRNAs and their regulatory functions.

It has been shown that a large number of specific miRNAs are involved in immunological processes, with major roles for miR-150 and miR-155 in lymphocyte differentiation and function (17, 18). Furthermore, several miRNAs have been identified that are involved in human monocyte differentiation to DCs in vitro (19–21). Dicer has also been specifically deleted in several immunological cell lineages, such as T cells (22–24), regulatory T cells (25–27), or B cells (28), with drastic consequences for development and functions of the relevant cells. In contrast, nothing is known about the role of Dicer in DCs in vivo. In this report, we analyzed development and function of Dicer-deficient DCs. Upon
conditional deletion of dicer1 in CD11c+ DCs, we could not detect an effect of Dicer deficiency on short-lived resident DC subtypes in spleen and lymph nodes. However, the dense LC network in the epidermis could not be maintained in the absence of Dicer. In addition, LCs displayed a defect in maturation and Ag presentation. These data identify Dicer-generated miRNAs as key regulators of LC homeostasis and function.

Materials and Methods

Mice

To generate mice deficient in mature miRNAs in DCs, previously described Dicer−/− mice (29), were backcrossed to C57BL/6 background (CD11c-Cre-Dicerfl/fl mice) (29). To analyze recombination of dicer1 alleles in sorted CD11c+ splenocytes, genomic DNA was extracted using a commercial kit according to the manufacturer’s instructions (Qiagen, Hilden, Germany) and genotyping PCR was performed as described (29). To quantify mRNA expression levels, RNA was isolated from sorted red fluorescent protein (RFP+) spleen DCs using a RNeasy Mini Kit (Qiagen) or from isolated LCs using a RNAneuMicro-Kit (Ambion, Darmstadt, Germany). cDNA was generated with SuperScript III First-Strand Synthesis System using random primers (Invitrogen, Darmstadt, Germany). Quantitative PCR reactions were run in a CFX36 PCR machine (Bio-Rad, Munich, Germany) using the LightCycler TaqMan Master Kit (Roche) and gene-specific primers (Applied Biosystems, Darmstadt, Germany) according to the manufacturer’s instructions. Expression levels were normalized to ubiquitin c, and relative expression was calculated using the ΔΔCt method. For quantitative real-time RT-PCR of miRNAs, total RNA including the low m.w. fraction was isolated from RFP+ spleen DCs or LCs using a mirNeasy Kit (Qiagen) or a RNAneuMicro-Kit (Ambion) modified for recovery of small RNAs according to manufacturer’s instructions. Gene-specific reverse transcription was performed for each miRNA using the TaqMan MicroRNA Reverse Transcription Kit and gene-specific primers (Applied Biosystems) following the manufacturer’s protocol. Quantitative PCR (qPCR) reactions were run in a CFX36 PCR machine (Bio-Rad) using a LightCycler TaqMan Master Kit (Roche) according to the manufacturer’s instructions. Expression levels were normalized to RNU19, and relative expression was calculated using the ΔΔCt method.

Flow cytometry and cell purification

The mAbs used were FITC-, PE-, allophycocyanin-, PECy5.5-, Alexa Fluor 647-, or PerCP-conjugated anti-I-Ab, H-2Kb, H-2Db, F4/80, CD11c, PDCD1-1, Langerin, CD24, CD40, CD45, CD68, CCR7, and epithelial cell adhesion molecule (EpCAM) (eBioscience, Frankfurt, Germany) and BD Biosciences, Heidelberg, Germany). H-2Kb/HSV-glycoprotein B (B14.189-505) tetrantens were purchased from ProdiMune (Oxford, U.K.). Single-cell suspensions of lymphoid organs were obtained by Liberase Cl (0.42 mg/ml Rote) and DNaseI (0.2 mg/ml Rote) treatment. Fc block (anti-FcγRI/II Ab) was included in every staining. To separate epidermal and dermal sheets, mouse ears were split into dorsal and ventral parts and incubated in 0.5 M ammonium thiocyanate (Sigma-Aldrich) for 20 min at 37°C to allow for separation of epidermal sheets from the dermis. After fixation in acetone for 5 min at room temperature, sheets were blocked in PBS containing 0.25% BSA and 10% mouse serum and stained with biotin-conjugated anti-I-A/I-E, followed by Alexa Fluor 555-conjugated streptavidin (Invitrogen). Sheets were then mounted in Fluoromount (Southern Biotechnology Associates, Birmingham, AL) and analyzed on an BX41TF-5 microscope equipped with a F-View II Digital camera and CELL-BND-F software (Olympus, Hamburg, Germany).

Electron microscopy

Ear tips were fixed in a solution of 3.5% glutaraldehyde or 2.5% glutaraldehyde and 2% formaldehyde, freshly prepared from paraformaldehyde, in 0.1 M sodium cacodylate-HCl buffer (pH 7.3). The tissue specimens were cut in small blocks of 2–1 mm in length and, after thorough washing in the buffer, postfixed in 2% OsO4 in 0.1 M sodium cacodylate-HCl buffer, dehydrated in ethanol, and embedded in Araldite (Ted Pella, Redding, CA). Thin sections were stained with lead citrate and uranyl acetate and examined in a CM10 electron microscope (Philips, Eindhoven, The Netherlands) at 80 kV. The occurrence of Birbeck granules, LCs were investigated in three blocks from each animal of the two groups. A total of 100 LCs in the Dicer−/− mice and of 60 LCs in Dicer+/+ mice were examined.

Contact hypersensitivity response

To induce ear swelling, mice were sensitized with either 25 μl 0.3% 2,4-dinitrofluorobenzene (DNFB) or 50 μl 0.5% Oxaizolene (both Sigma-Aldrich) in acetone/oil (4:1) on the shaved abdomen and on day 5 challenged with 5 μl 0.15% DNFB or 10 μl 0.25% Oxazolone in acetone/oil (4:1), respectively, on both sides of one ear. Swelling was measured by comparing ear thickness before and 24 h after challenge using a micrometer (Mitutoyo, Eisenach, Germany).

Migration assay

Mouse ears were mechanically split into ventral and dorsal halves, and the dorsal halves were floated dermal side down on complete RPMI media containing 0.1 μg/ml CCL21 (R&D Systems, Wiesbaden-Nordenstadt, Germany). Ear halves were cultured for 3 d at 37°C, and after 24, 48, and 72 h, migratory DCs were collected and incubated in the culture media. Collected cells were washed and stored at 4°C until analysis on day 3 via flow cytometry.

Ag presentation assay

Epidermal sheets were prepared as described and incubated with 0.25 mg/ml OVA protein (Sigma-Aldrich) overnight. Migrated cells and sheets were washed and incubated further in fresh medium. At day 2 of culture,
migrated cells were collected, and light-density LCs were enriched using OptiPrep (Axis-Shift, Oslo, Norway) in a density gradient centrifugation. Equal numbers of LCs were seeded into the wells of a round-bottom 96-well plate and cocultured with 2.5 \times 10^4 CFSE-labeled OT-I or OT-II T cells in complete RPMI medium containing GM-CSF. T cell proliferation was analyzed by flow cytometry after 60–65 h of culture.

**BrdU and TUNEL labeling**

Mice were injected with 1 mg BrdU i.p. and kept on 0.8 mg/ml BrdU-supplemented water for 7 d. Epidermal cell suspensions were stained for surface markers and BrdU incorporation using the BrdU Flow Kit (BD Biosciences) following the manufacturer’s protocol. For apoptosis detection by TUNEL assay (In Situ Cell Death Detection Kit; Roche), epidermal cell suspensions were stained for surface markers. Cells were fixed, permeabilized, and enzymatically labeled with fluorescein–dUTP according to the manufacturer’s instructions. The percentage of LCs that incorporated BrdU or dUTP, respectively, was determined by flow cytometry.

**Statistical analysis**

Data were analyzed using the unpaired, two-tailed Student t test. A p value <0.05 was considered to be significant. For analysis of fluorescence intensities, the median fluorescence intensity was calculated.

**Results**

**Characterization of Dicer-deficient DCs**

To study the role of Dicer and miRNAs in DCs, we crossed mice containing loxP-flanked dicer1 alleles (floxed; Dicer0/0) (29) with CD11c-Cre transgenic mice expressing Cre in CD11c+ DCs (30). The resulting CD11c-Cre-Dicer0/0 (DicerΔ/Δ) mice did not exhibit any obvious abnormalities, were viable and fertile, and were born at the expected Mendelian ratio (data not shown). We first determined whether the dicer1 alleles were effectively excised from the DC genome and analyzed expression of Dicer transcripts and protein from sorted CD11c+ spleen DCs. PCR on genomic DNA from FACS-sorted CD11c+ splenocytes showed that the loxP-flanked alleles (fl/fl) were efficiently recombined in Cre-positive cells (ΔΔ) (Fig. 1A). This deletion led to disappearance of Dicer mRNA (Fig. 1B). qPCR analysis of the deletion of Dicer mRNA indicated 99.9% efficiency of deletion. Consequently, also Dicer protein was present only at minimal amounts, as detected by Western blot (Fig. 1C). However, qPCR quantification of some mature miRNAs, which are known to be expressed in DCs (33; data not shown), revealed only modestly reduced mRNA levels in Dicer-deficient mice.
DCs (Fig. 1D). For example, miR-223 and miR-320 were ~2-fold reduced in DCs from Dicer\(^{Δ\lambda}\) mice, whereas we could not detect reduced expression of miR-10a. Consequently, the frequencies and total cell counts of splenic cDCs (CD11c\(^{hi}\)MHC II\(^{+}\)) and pDCs (CD11c\(^{lo}\)PCDA-1\(^{+}\)) were not significantly different between both mouse strains (Fig. 1E), and these cells showed normal surface expression of typical surface markers (data not shown). Further characterization of cDC subsets showed that also the frequencies of CD8a\(^{+}\) and CD8a\(^{-}\) DCs were not significantly altered (data not shown). The numbers of other leukocyte cell populations from spleens, such as T and B cells, NK cells, macrophages, thymocytes, and immature as well as mature neutrophils, were also similar between Dicer\(^{Δ\lambda}\) and CD11c-Cre control (Dicer\(^{wt/wt}\)) mice (Supplemental Fig. 1). To test if this apparent normality of DC phenotype and differentiation was eventually accompanied by functional defects, we infected mice i.v. with HSV. However, Dicer\(^{Δ\lambda}\) mice (Supplemental Fig. 1). To test if this apparent normality of DC phenotype and differentiation was eventually accompanied by functional defects, we infected mice i.v. with HSV. However, Dicer\(^{Δ\lambda}\) mice (Supplemental Fig. 1). To test if this apparent normality of DC phenotype and differentiation was eventually accompanied by functional defects, we infected mice i.v. with HSV. However, Dicer\(^{Δ\lambda}\) mice did not exhibit impaired immunity to systemic viral infection. At day 0, Dicer\(^{wt/wt}\) (\(n = 5\)) and Dicer\(^{Δ\lambda}\) (\(n = 5\)) mice were infected systemically with HSV-1 strain KOS (i.v. 1\(^{×}\)10\(^7\) PFU), Control mice (\(n = 5\)) received PBS i.v. A. The frequency of HSV-gB–specific CD8 T cells in peripheral blood at day 10 postinfection was determined by staining with gB\(^{498–505–}\)MHC tetramer complexes. Peripheral blood cells were acquired by flow cytometry and gated on CD8\(^{+}\) lymphocytes. FACS plots are representative for a sample from each group. Numbers denote frequency of H-2K\(^{b}\)gB\(^{498–505–}\) MHC tetramer complexes-positive cells as percentage of CD8\(^{+}\) T cells \(±\) SEM. B. On day 25 postinfection, mice were subjected to an in vivo cytotoxic T cell assay. A mixture of HSV-gB–SSIEFARL peptide-loaded target cells (CFSE\(^{hi}\)) and control cells (CFSE\(^{lo}\)) was injected i.v., and the specific lysis was analyzed by flow cytometry 24 h later. A representative plot from each group is shown. Numbers denote frequency of cells in each gate as a percentage of the total CFSE-labeled population. C. On days 6 and 23, sera were analyzed for HSV-specific total IgG and IgG2a Abs using ELISA. Error bars denote SEM.

**FIGURE 2.** CD11c-Cre-Dicer\(^{fl/ff}\) mice do not exhibit impaired immunity to systemic viral infection. At day 0, Dicer\(^{wt/wt}\) (\(n = 5\)) and Dicer\(^{Δ\lambda}\) (\(n = 5\)) mice were infected systemically with HSV-1 strain KOS (i.v. 1\(^{×}\)10\(^7\) PFU). Control mice (\(n = 5\)) received PBS i.v. A. The frequency of HSV-gB–specific CD8 T cells in peripheral blood at day 10 postinfection was determined by staining with gB\(^{498–505–}\)MHC tetramer complexes. Peripheral blood cells were acquired by flow cytometry and gated on CD8\(^{+}\) lymphocytes. FACS plots are representative for a sample from each group. Numbers denote frequency of H-2K\(^{b}\)gB\(^{498–505–}\) MHC tetramer complexes-positive cells as percentage of CD8\(^{+}\) T cells \(±\) SEM. B. On day 25 postinfection, mice were subjected to an in vivo cytotoxic T cell assay. A mixture of HSV-gB–SSIEFARL peptide-loaded target cells (CFSE\(^{hi}\)) and control cells (CFSE\(^{lo}\)) was injected i.v., and the specific lysis was analyzed by flow cytometry 24 h later. A representative plot from each group is shown. Numbers denote frequency of cells in each gate as a percentage of the total CFSE-labeled population. C. On days 6 and 23, sera were analyzed for HSV-specific total IgG and IgG2a Abs using ELISA. Error bars denote SEM.

by in vivo cytotoxic assays (Fig. 2B). In addition, Dicer\(^{Δ\lambda}\) mice generated identical HSV-specific Ab responses as compared with wild-type control animals (Fig. 2C). Because DCs are necessary for the induction of antiviral T cell and B cell responses, these findings suggest that DCs from Dicer\(^{Δ\lambda}\) mice are not functionally defective.

**CD11c-mediated Dicer ablation results in reduction of specific DC subsets**

To further assess the phenotype of DCs in the absence of Dicer, we performed flow cytometric analysis of DC subsets in lymph nodes. We could not detect differences in cDCs of mesenteric lymph nodes (Fig. 3A). The cDC population in sLNs can be further subdivided into blood-derived, resident DCs (CD11c\(^{hi}\)MHC II\(^{+}\)) and migratory DCs (CD11c\(^{lo}\)MHC II\(^{hi}\)) consisting of epidermal LCs and dermal DCs immigrating from skin. This migratory DC subgroup from skin was significantly decreased in Dicer\(^{Δ\lambda}\) mice (Fig. 3A). To differentiate the various DC populations from skin, we performed further characterization by flow cytometry. Using mAbs specific for MHC class II, Langerin, and EpCAM, we differentiated between LCs and Langerin\(^{−}\)/Langerin\(^{+}\) dermal DCs. As shown in Fig. 3B, we found a 12-fold reduction of MHC II\(^{+}\)Langerin\(^{−}\) LCs in the epidermis of CD11c-specific Dicer\(^{Δ\lambda}\) animals (\(p = 0.0032\)). Consequently, also the transmigrating DCs found in underlying dermal regions were nearly undetectable (Fig. 3B, dermis). In contrast, the other two main dermal DC subpopulations, the Langerin\(^{+}\) and Langerin\(^{−}\) dermal DCs, which are both EpCAM\(^{−}\), were present at normal frequencies (Fig. 3B).

To ensure that the few LCs found in the epidermis of Dicer\(^{Δ\lambda}\) mice were actually expressing Cre and would not be cells escaping the loxp rearrangement due to lack of Cre expression, we analyzed the LCs from epidermal sheets of CD11c-Cre-tdRFP-Dicer\(^{fl/ff}\) mice. Despite significantly lower numbers of DCs in the epidermis of CD11c-Cre-tdRFP-Dicer\(^{fl/ff}\) mice, these DCs occupied a larger area and had longer dendrites (Fig. 4A). The morphology of the remaining Dicer\(^{Δ\lambda}\) DCs was similar to that of their wild-type counterparts (Fig. 4A). This selective loss of LCs from the skin was also confirmed by histological analysis of epidermal sheets (Fig. 4A). In 8- to 9-wk-old mice, Dicer\(^{Δ\lambda}\) LCs were distributed sparsely but equally in the dermal layers of the epidermis. This selective loss of LCs from the skin was also confirmed by histological analysis of epidermal sheets (Fig. 4A). In 8- to 9-wk-old mice, Dicer\(^{Δ\lambda}\) LCs were distributed sparsely but equally in the dermal layers of the epidermis. This selective loss of LCs from the skin was also confirmed by histological analysis of epidermal sheets (Fig. 4A). In 8- to 9-wk-old mice, Dicer\(^{Δ\lambda}\) LCs were distributed sparsely but equally in the dermal layers of the epidermis. This selective loss of LCs from the skin was also confirmed by histological analysis of epidermal sheets (Fig. 4A). In 8- to 9-wk-old mice, Dicer\(^{Δ\lambda}\) LCs were distributed sparsely but equally in the dermal layers of the epidermis. This selective loss of LCs from the skin was also confirmed by histological analysis of epidermal sheets (Fig. 4A). In 8- to 9-wk-old mice, Dicer\(^{Δ\lambda}\) LCs were distributed sparsely but equally in the dermal layers of the epidermis.
a consequence of decreased LC density. In contrast, no differences in other leukocyte populations of the skin, such as dendritic epidermal T cells (CD45+TCRγδ+), could be detected (data not shown).

The loss of LCs was increasing with age, because young DicerΔ/Δ mice at the age of 10 d still contained normal numbers of LCs (Fig. 4B,C). This was not due to lack of Cre expression in the animals, because 10-d-old CD11c-Cre-tdRFP-Dicerfl/fl mice had already uniformly RFP+ LCs in the skin (data not shown). In contrast, with increasing age, their LC numbers decreased drastically, and at 6 mo of age hardly any LCs could be found (Fig. 4B, DicerΔ/Δ, 6 mo). The extremely few LCs in old mice were mostly clustered in “patches” (Fig. 4B, DicerΔ/Δ, 6 mo). In the epidermis of 18-mo-old animals, only extremely few LCs could be found, among them some LCs with giant extremities (Fig. 4B, bottom panel).

To further analyze LCs, we performed electron microscopy studies. LCs were distinguished from keratinocytes in thin sections by the presence of Birbeck granules and the absence of thick bundles of intermediate keratin filaments and desmosomes (Fig. 4D). Although the frequency of LCs was not studied quantitatively with this method, these cells were clearly less frequently present in the epidermis of DicerΔ/Δ mice as compared with control Dicer wt/wt animals, confirming our analysis by flow cytometry (Fig. 3B) and histology (Fig. 4A, 4B). Whereas Birbeck granules were present in 87% of randomly sectioned LCs in the Dicer wt/wt mice, they were observed in only ~7% of the LCs examined in the DicerΔ/Δ mice. Taken together, ablation of Dicer led to a progressive loss of LCs in the epidermis with time, with some remaining cells that showed altered morphology, lower expression levels of Langerin (Fig. 3E), and lack of Birbeck granules (Fig. 4D).

Dicer-deficient Langerhans cells have a proapoptotic gene expression signature

Given the pivotal function of Dicer in miRNA biogenesis, we expected that absence of Dicer in LCs would result in profound

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**FIGURE 3.** CD11c-mediated deletion of Dicer results in the absence of specific DC populations in peripheral lymphoid organs and in the skin. A, FACS analysis of DC populations in mesenteric lymph nodes and sLNs obtained from control Dicer wt/wt and DicerΔ/Δ mice. Percentages of cells that fall into each gate are indicated (mean ± SEM; n = 5–6). All of the populations depicted were gated on live cells; NK cells and macrophages were gated out by staining with CD49b and F4/80, respectively. Combined data from two independent experiments with similar results are shown (p < 0.05). B, FACS analysis of single-cell suspensions from epidermis and dermis obtained from Dicer wt/wt and DicerΔ/Δ animals. Epidermal and dermal cell populations shown are gated on CD45+ cells or, for analysis of dermal cell populations based on Langerin and EpCAM, on CD45+MHC II+ cells. Numbers on plots indicate cells in each gate as a percentage of CD45+ cells (mean ± SEM; n = 4). C, FACS analysis of CD45+-gated epidermal cells derived from Dicer wt/wt and DicerΔ/Δ animals. Percentages of RFP+ LCs are indicated (mean ± SEM; n = 3). D, qPCR analysis of Dicer mRNA and miR-223 miRNA levels in FACS-sorted LCs pooled from 3 Dicer wt/wt or 14 DicerΔ/Δ animals. Values indicated represent relative expression levels in LCs from Dicer wt/wt and DicerΔ/Δ animals. Expression was normalized to ubiquitin c (Dicer) or RNU19 (miR-223). E, CD45+MHC II+Langerin+ LCs from Dicer wt/wt and DicerΔ/Δ animals were assessed for their size by forward scatter measurement (top panel) and their Langerin (middle panel) and TGFβRII (bottom panel) expression levels by FACS staining. Representative histogram overlays of Dicer wt/wt (gray) and DicerΔ/Δ (open) LC measurements are shown. Graphs indicate mean ± SEM (n = 3). Data are representative of at least three independent experiments. **p < 0.01; ***p < 0.001.
changes in gene expression profiles. To determine whether individual genes or gene sets were differentially expressed between Dicer-deficient and wild-type LCs, we purified LCs from epidermal layers of the skin and analyzed gene expression using microarrays. Due to the extremely low numbers of LCs in Dicer-deficient animals, we had to include amplification steps before analysis. However, we could not detect individual genes that were differentially expressed with statistical significance (data not shown). Gene set enrichment analysis using Gene Ontology (GO) biological process terms identifies overrepresentation of genes belonging to a given functional GO term in an ordered list of genes. Differences in biological processes between DicerΔ/Δ LCs and wild-type LCs can be revealed this way, and we therefore analyzed the differentially expressed gene list for enrichment of genes belonging to biological process GO terms. The amount of overrepresentation is assessed with a statistical score. Two enriched GO terms, “induction of apoptosis by intracellular signals” (GO.ID: 0008629; p = 0.0145) and “regulation of caspase activity” (GO.ID: 0043281; p = 0.0111) contain proapoptotic genes. Also the proapoptotic gene bcl2l11 (encoding for Bim), belonging to the enriched GO category “postembryonic organ development” (GO.ID: 0048569; p = 0.0064), is differentially regulated in DicerΔ/Δ LCs. Verification of differential gene expression by qPCR on mRNA samples from LCs proved to be very difficult due to the extremely low numbers of DicerΔ/Δ LCs in the skin. Nevertheless, pooling the few DicerΔ/Δ LCs from the skin of dozens of mice allowed the isolation of sufficient amounts of mRNA for the verification of a few genes (Fig. 5A). This analysis showed that the expression of dicer1 in the purified DicerΔ/Δ LCs was reduced to >99% as compared with Dicer-sufficient LCs and served as a quality control (Fig. 5A). Furthermore, genes predicted by the GO term analysis, such as myc and bcl2l11 (bim), were indeed expressed differentially in DicerΔ/Δ LCs (Fig. 5A). The absence of Dicer reduced the expression of Myc transcripts 17-fold in LCs, whereas the expression levels of Bim increased 5.3-fold (Fig. 5A). Altogether, these data suggest that Dicer-deficient LCs might be engaged in active apoptosis.

To test this hypothesis, we performed in vivo BrdU labeling and TUNEL assays to determine turnover and cell death of LCs. During the 7-d period of BrdU labeling, ~13% of wild-type LCs did incorporate BrdU (Fig. 5B). This rate of BrdU incorporation indicates the total turnover time for wild-type LCs to be ~53.8 d and confirms previous publications that estimated 53–78 d as turnover time of LCs in absence of inflammation (reviewed in Ref. 34). In marked contrast, 36% of Dicer-deficient LCs incorporated BrdU (Fig. 5B), indicating a nearly three times faster turnover of only 19.4 d. TUNEL assay revealed an 7-fold increased rate of apoptosis in DicerΔ/Δ LCs as compared with that in Dicer-sufficient LCs (Fig. 5B). Therefore, ablation of Dicer leads to modulation of gene expression involved in apoptosis of LCs. This enhanced the turnover and increased the apoptosis rate, resulting in progressive loss of DicerΔ/Δ LCs. From these results, we conclude that miRNAs...
regulate key cellular events, such as proliferation and survival of LCs in the steady state.

**Dicer-deficient Langerhans cells are migratory but display differential capacities to activate CD8+ versus CD4+ T cells**

To analyze if DicerΔΔ LCs displayed functional deficiencies before they undergo apoptosis, we next assessed the LC migratory capacity, a hallmark of LC biology. To this end, ear explants were cultured in medium containing the chemokine CCL21, and we analyzed by FACS cells migrating out of the explants. As shown in Fig. 6A, numbers of migratory epidermal LCs from DicerΔΔ mice are strongly reduced compared with those from controls. This reduction highly correlated with the reduced number of LCs in the skin of DicerΔΔ mice in steady-state conditions (Fig. 3B), implying that the Dicer-deficient LCs can be mobilized comparable to wild-type LCs upon a chemotactic stimulus. Culture of epidermal sheets is known to induce maturation of LCs, and a concomitant modulation of typical maturation markers on the surface of LCs can be observed (35). Consequently, wild-type LCs upregulated surface expression of MHC class I and class II molecules, CCR7, CD40, and CD86 upon exit of the epimids (Fig. 6B). In marked contrast, Dicer-deficient LCs were unable to upregulate these molecules to the same extent (Fig. 6B), except for MHC class I and CCR7, which were modulated normally (Fig. 6B). Uregulation of CCR7, the chemokine receptor for CCL21, allowed DicerΔΔ LCs to migrate out of the epidermal layers of the skin (Fig. 6A). Functional consequences of this maturation deficiency were observed in Ag presentation assays with T cells. When LCs were incubated with OVA protein overnight and cocultured with OVA-specific CD8+ OT-I or CD4+ OT-II T cells, Dicer wt/wt LCs efficiently induced proliferation of OT-II cells, as measured by dilution of the CFSE dye (Fig. 6C). In contrast, DicerΔΔ LCs could induce only a 2- to 3-fold lower percentage of divided T cells in these assays (Fig. 6C). Although Ag presentation via MHC class II was severely inhibited in absence of Dicer, we could not detect differences in priming of CD8+ OT-I T cells. These findings indicate that the uptake of exogenous protein Ag and its consecutive cross-presentation via MHC class I was not affected by Dicer deficiency (Fig. 6C) and are consistent with the intact upregulation of MHC class I by DicerΔΔ LCs (Fig. 6B). Taken together, these data show that Dicer regulates the phenotypical and functional maturation of LCs and that absence of Dicer renders LCs defective to activate MHC class II-restricted CD4+ T cells.

A common method to test for cutaneous immune responses is the topical application of hapten Ag to the skin to induce contact hypersensitivity (CHS). It is thought that LCs play a central role in this skin-mediated immunity, although recent findings with a different LC ablation model generated controversial data (reviewed in Ref. 36). To test the function of DicerΔΔ LCs in vivo, we sensitized and challenged the mice with DNFB. The DicerΔΔ mice used for these tests were older than 9 wk and had already considerably lower numbers of LCs in their epimids as compared with those in wild-type mice (Fig. 4C). Nevertheless, the measured ear swelling was similar in both groups (Fig. 6D). This might in part be due to the fact that DNFB can also be transported to the lymph nodes by dermal DCs, and therefore this test seems not to address specifically the function of LCs, as previously suggested (37). We therefore performed CHS with the hapten Oxazolone at a low dose previously described to selectively target LCs (38). However, also in this setting, no difference between DicerΔΔ mice and Dicer wt/wt mice could be observed (Fig. 6D). Taken together, our data suggest that Dicer controls LC maturation and Ag presentation via MHC class II, but this deficiency does not directly cause deficient skin immunity as tested with CHS.

**Discussion**

In this study, we have shown that loss of Dicer leads to progressive ablation of LCs in the epimids of the skin. As indicated by the almost complete absence of miR-223, the CD11c promoter-driven expression of Cre recombinase resulted in efficient disruption of dicer1 in these cells. Dicer-deficient LCs showed lack of Birbeck granules, normally a typical attribute of LCs, furthermore, a disturbed expression of surface molecules and reduced Ag presentation capacities to CD4+ T cells, increased turnover, reduced halflives, and increased rates of apoptosis.

A complex molecular network of signal transducers, transcription factors, and miRNAs is emerging controlling proliferation and apoptosis (39, 40). One of the best-studied miRNA families in this regard is the miR-17-92 cluster. Two studies identified Bim as a target for miRNAs of the miR-17-92 cluster and showed elevated levels of Bim protein in its absence (28, 41). Dicer-deficient LCs in our study expressed nearly 6-fold elevated mRNA levels of the
proapoptotic Bim, indicating a potential involvement of this apoptosis pathway also in the disappearance of Dicer\textsuperscript{Δ/Δ} LCs. One possibility for the lack of effect on spleen DC populations after dicer\textsuperscript{1} deletion could be the appearance of DCs that escaped deletion of the dicer\textsuperscript{1} alleles. However, in very young mice also, Dicer\textsuperscript{Δ/Δ} LCs were present in the epidermis in normal numbers but decreased to almost complete absence with increasing age of the mice. This is in contrast to a previous report describing skin-repopulating LCs that escaped Cre-mediated deletion of TGF\textbeta\textsuperscript{1} and TGF\textbeta\textbeta\textsuperscript{II} alleles (42), but such a phenomenon certainly depends on the type of promoter driving the Cre transgene.

However, although >90% of LCs do already express Cre and are RFP\textsuperscript{+} at the age of 10 d, the initial seeding of the epidermis with LCs in Dicer\textsuperscript{Δ/Δ} mice is normal. We therefore assume that at this time point Dicer has been inactivated recently, but effects are not yet visible. Similarly, >90% of DCs in spleen and lymph nodes were RFP\textsuperscript{+} in CD11c-Cre-tdRFP-Dicer\textsuperscript{fl/fl} mice (data not shown), and Dicer mRNA was undetectable in these cells, but they developed and functioned normally. The presence of substantial amounts of miRNAs at nearly undetectable levels of Dicer protein in spleen DCs could have several possible reasons. First, miRNAs can eventually be generated in the absence of Dicer. However, this seems unlikely because other enzymes could not replace Dicer functionally in ubiquitously Dicer-deficient mice, which are not viable (43). Also, Dicer is necessary to generate mature miRNAs in T cells, and its function was not redundant (24). Second, the highly reduced
amounts of Dicer protein in spleen DCs are sufficient to generate mature miRNAs. Although it seems unlikely, we cannot generally rule out this possibility. Third, the half-life of miRNAs is too long and does not decrease beyond critical levels during the life span of Dicer<sup>Δ/Δ</sup> spleen DCs. A delay between the disappearance of Dicer protein and mature miRNAs might be considered as a reason for an absent phenotype in spleen and lymph node DCs as well as normal initial LC development. A recent study also described such a delay after Cre-mediated Dicer deletion early in embryogenesis. Careful examination of residual miRNA expression in affected tissues suggests a 3–10 d delay between elimination of Dicer and depletion of specific miRNAs (44). The mechanisms influencing miRNA stability are not yet fully understood, although multiple factors including cis- and trans-acting modifications and proteins regulating their half-lives have been discovered (45). Generally, miRNAs are considered to be stable molecules, with half-lives ranging from hours to days. Certain miRNAs, such as miRNA-208, have a half-life of >12 d (46). In contrast, BrdU studies determined the half-life of DCs in mesenteric lymph nodes and spleen to be 1.5–3 d and of DCs in cutaneous lymph nodes as 7–9 d (47, 48). Tissue-derived lymph node DCs had a half-life of 5–7 d as determined in parabiosis studies (49). It is therefore possible that the half-life of at least some mature miRNAs might be longer as compared with the half-life of spleen DCs. Therefore, Dicer<sup>Δ/Δ</sup> DCs of spleen and lymph nodes may contain functional miRNAs despite efficient deletion of Dicer. We detected a <50% decrease for some selected miRNAs, which could indicate that only a part of the spleen DC miRNA content might be due to de novo synthesis, whereas a large part could be residual molecules.

LCs gain cell size, and it is possible that this is due to the decrease of LC population density, leaving more “space” or “niches” available for each individual remaining LC. In Dicer<sup>Δ/Δ</sup> LCs, we could identify differential regulation of mVy, which, when overexpressed, increases cell size during all stages of B cell differentiation (50). However, the loss of Dicer instead caused a strongly decreased expression of mVy in LCs, and it is hardly conceivable that such a loss of expression may lead to the observed increased size of Dicer<sup>Δ/Δ</sup> LCs. Myc is also an important member of the cellular proliferation regulatory network and is part of a well-studied feedback loop involving the miR-17-92 cluster miRNAs and the critical cell cycle regulator E2F (39). Recently, other miRNAs have also been discovered that target Myc (51, 52). Both up- as well as down-regulation of mVy expression are linked to apoptosis, depending on the experimental system used (53, 54). Thus, the decreased levels of Myc mRNA in Dicer<sup>Δ/Δ</sup> LCs might also contribute to apoptosis. Also, the observed loss of TGFβRII expression might contribute to LC disappearance, because TGFβRII-deficient LCs are lost from epidermis (42). Although TGFβRII expression did not disappear completely in Dicer<sup>Δ/Δ</sup> LCs, the observed lower expression levels could be sufficient to cause survival disadvantages.

We found that CHS is not altered in Dicer<sup>Δ/Δ</sup> mice. Currently, the role of LCs in skin immunity is controversially discussed. With gene-targeted mice where LCs can be ablated by the use of diphtheria toxin, it was found that CHS responses were either reduced in one model (37) or completely unaffected (55) by LC ablation in a second model. With a constitutive LC ablation model, it was even found that CHS was increased, suggesting a regulatory function for LCs (56). Therefore, it is still unclear which roles LCs play in skin immunity (34). We used Dicer<sup>Δ/Δ</sup> mice that were older than 9 wk in our CHS assays. We could show that at this age the absence of Dicer had caused already a severe loss of LCs, with nearly no LCs left in the skin. Therefore, our results indicate that either the few remaining Dicer<sup>Δ/Δ</sup> LCs were sufficient to elicit hypersensitivity or, as suggested in other models (55), that LCs play no obvious role in CHS.

Our finding that Dicer-deficient LCs fail to upregulate maturation markers is in line with other reports showing implications for miRNAs at certain developmental stages (17). MiR-155, which is upregulated in human monocyte-derived DCs in response to LPS (19, 21), has been shown to play a role in DC maturation. However, miR-155–deficient DCs do not fail to upregulate maturation markers (18); therefore, other miRNAs lacking in Dicer<sup>Δ/Δ</sup> LCs are likely to be involved in the maturation process. Thus, the reduced Ag presentation capacity observed in Dicer<sup>Δ/Δ</sup> LCs might be a consequence of miRNA-dependent regulation of DC activation. The fact that only MHC class II-mediated presentation is inhibited, but not MHC class I cross-presentation, indicates that uptake of protein Ag might not be affected by loss of Dicer. Currently, we do not know which genes are targets of miRNA regulation in this context nor if the deficiency to upregulate MHC class II can be overcome by further signals, such as anti-CD40 or microbial stimuli. This issue is subject to further studies.

We also attempted to identify whether specific miRNAs contributed to the observed phenotype in Dicer-deficient LCs via correlation of Dicer-dependent gene expression changes with occurrence of specific miRNA seed motifs in the 3′ untranslated region of affected genes, as has been reported for Dicer-deficient B cells (28). However, using the recently developed Sylamer software (57), we did not identify a specific miRNA responsible for the gene expression changes between wild-type and Dicer<sup>Δ/Δ</sup> LCs. The fact that reduced levels of many miRNAs affect simultaneously the expression of many genes, compounded by secondary effects of genes influencing each other’s expression, might account for the failure to identify specific miRNAs. Specific deletion of individual miRNAs or miRNA clusters in LCs is required to gain more insight into the role of individual miRNAs in these processes.

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Disclosures
The authors have no financial conflicts of interest.

References


ONLINE SUPPLEMENTAL MATERIAL:

Supplemental Figure 1:

Figure S1. CD11c-Cre-mediated deletion of Dicer has no effect on non-DC leukocyte populations. FACS analysis of B cells, T cells, NK cells, macrophages and neutrophils in the spleen (A) and T cells in the thymus (B). Percentages of cells that
fall into each gate (mean ± SEM, n=6) are indicated. All populations depicted were gated on live cells; for analysis of neutrophils F4/80⁻ cells were gated.