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

Harmjan Kuipers, Frauke M. Schnorfeil, Hans-Jörg Fehling, Helmut Bartels and Thomas Brocker

*J Immunol* 2010; 185:400-409; Prepublished online 7 June 2010;
doi: 10.4049/jimmunol.0903912
http://www.jimmunol.org/content/185/1/400

Supplementary Material
http://www.jimmunol.org/content/suppl/2010/06/04/jimmunol.0903912.DC1

References
This article cites 57 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/185/1/400.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

Permissions
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts

*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc.,
1451 Rockville Pike, Suite 650, Rockville, MD 20852
Copyright © 2010 by The American Association of Immunologists, Inc. All rights reserved.
Print ISSN: 0022-1767 Online ISSN: 1550-6606.
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 complexity of different DC subsets 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, CD8α- 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 into various phenotypes.

Cytokines, particularly IL-1, 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 biogenesis pathway.

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 DicerΔDicer mice (29) on a mixed C57BL/6 background were crossed with CD11c-Cre mice (30) (CD11c-Cre-DicerΔDicer). For tracking Cre expression, CD11c-Cre-DicerΔDicer mice were bred to ROSA26-tdRFP mice (CD11c-Cre-tdRFP-DicerΔDicerΔDicer) (31). OT-I and OT-II mice (expressing a transgenic TCR specific for OVA257–264/MHC II-κ or OVA253–336/MHC II-κ, respectively) were originally obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred and maintained in a conventional facility at the Institute of Immunology (Munich, Germany) and used according to protocols approved by the local animal ethics committee.

Gene expression analysis

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 RNAqueous-Micro 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 CFX96 PCR machine (Bio-Rad, Munich, Germany) using the LightCycler TaqMan Master Kit (Roche, Mannheim, Germany) 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 miRNaseq-Micro 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 CFX96 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-, PE-Cy5.5-, Alexa Fluor 647-, or PerCP-conjugated anti-mouse I-Ab, H-2Kb, F4/80, CD11c, PDCA-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 (gB) 498–505 peptide (SSIEFARL) for 2 h at 37˚C and subsequently labeled with 1.67 μM CFSE (CFSElow population). A total of 5 × 10^6 cells of each CFSE-labeled cell cohort were mixed and injected i.v. Twenty-four hours later, mice were sacrificed and spleens were analyzed by flow cytometry.

ELISA

For the detection of HSV-specific Abs in the serum, ELISA was performed as described (32), using peroxidase-labeled second-step Abs specific for mouse total IgG or IgG2a.

Immunofluorescence

Mouse ears were mechanically split into dorsal and ventral halves 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-G (Southern Biotechnology Associates, Birmingham, AL) and analyzed on a 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 and 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 1–2 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. To determine 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 DicerΔDicer mice and of 60 LCs in DicerΔDicer mice were examined.

Contact hypersensitivity response

To induce ear swelling, mice were sensitized with either 25 μl 0.2% 3,4-dihydroxy-2-nitrobenzene (DNFB) or 50 μl 0.5% Oxazone (both Sigma-Aldrich) in acetone/olive oil (4:1) on the shaved abdomen and on day 5 challenged with 5 μl 0.15% DNFb or 10 μl 0.25% Oxazone in acetone/olive 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 0.5% trypsin and 5 μM EDTA (5). Epidermal sheets were subsequently incubated for 2.5 h in collagenase 1 (4.1 mg/ml Worthington, Lakewood, NJ), whereas dermal sheets were further processed using an enzyme mixture as described by Bursch et al. (6). Intracellular staining against Langerin was performed with the BD Cytofix/Cytoperm reagents (BD Biosciences) according to the manufacturer’s protocol. To analyze LC maturation, epidermal sheets were incubated for 48 h at 37˚C on complete RPMI media and emigrated cells were analyzed by flow cytometry. To obtain OT-I and OT-II T cells, cell suspensions of spleens and lymph nodes of TCR transgenic mice were prepared. OT-I and OT-II T cells were purified using a CD8+ or CD4+ T cell Isolation Kit (Miltenyi Biotec, Bergisch Gladbach, Germany), respectively, following the manufacturer’s protocol. For all of the experiments, data were acquired on FACSCanto2 or sorted on FACSARia flow cytometers (BD Biosciences) to 88–98% purity after gating out dead cells. Flow cytometric data were analyzed using FlowJo software (Tree Star, Ashland, OR).

Western blotting

Approximately 0.5 × 10^6 CD11c+ sorted splenocytes were lysed in buffer containing 1% Nonidet P-40 and protease inhibitors and loaded onto a 4–15% gradient gel (Bio-Rad). To detect Dicer protein on Western blots, Dicer 1416 (a kind gift from the D. Livingston laboratory, Dana Farber Cancer Institute, Boston, MA) was used. To control for cell loading, blots were subsequently stained with anti-β-actin Ab (Sigma-Aldrich, Munich, Germany). Bound primary Abs were detected with appropriate peroxidase-conjugated secondary Abs (Dako, Hamburg, Germany and Amersham Biosciences, Freiburg, Germany), and signal intensities were quantified with ImageJ software (National Institutes of Health, Bethesda, MD).

In vivo cytotoxic T cell assay

Erythrocyte-depleted C57BL/6 splenocytes were pulsed with 2 μg/ml HSV-gB498–505 peptide (SSIEFARL) for 2 h at 37˚C and subsequently labeled with 1.67 μM CFSE (CFSEhigh population). Unpulsed control cells were labeled with 0.07 μM CFSE (CFSElow population). A total of 5 × 10^6 cells of each CFSE-labeled cell cohort were mixed and injected i.v. Twenty-four hours later, mice were sacrificed and spleens were analyzed by flow cytometry.
migrated cells were collected, and light-density LCs were enriched using OptiPrep (Axis-Shield, 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 × 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 dicer alleles (floxed; Dicer<sup>fl/fl</sup>) (29) with CD11c-Cre transgenic mice expressing Cre in CD11c<sup>+</sup> DCs (30). The resulting CD11c-Cre-Dicer<sup>fl/fl</sup> (Dicer<sup>Δ/Δ</sup>) 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 dicer<sup>1</sup> alleles were effectively excised from the DC genome and analyzed expression of Dicer transcripts and protein from sorted CD11c<sup>+</sup> spleen DCs. PCR on genomic DNA from FACS-sorted CD11c<sup>+</sup> splenocytes showed that the loxP-flanked alleles (fl/fl) were efficiently recombined in Cre-positive cells (Δ/Δ) (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 miRNA levels in Dicer-deficient DCs.
DCs (Fig. 1D). For example, miR-223 and miR-320 were ~2-fold reduced in DCs from DicerΔΔ mice, whereas we could not detect reduced expression of miR-10a. Consequently, the frequencies and total cell counts of splenic cDCs (CD11c<sup>hi</sup>MHC II<sup>+</sup>) and pDCs (CD8<sup>+</sup>PDCA-1<sup>+</sup>) 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 CD8<sup>α<sup>+</sup></sub> and CD8<sup>α<sup>−</sup></sub> 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 DicerΔΔ and CD11c-Cre control (Dicer<sup>wt/wt</sup>) mice (Supplemental Fig. 1). To test if this apparent normality of DC phenotype and differentiation was actually accompanied by functional defects, we infected mice i.v. with HSV. However, DicerΔΔ mice mounted normal HSV-gB–specific cytotoxic T cell responses as detected with the respective MHC multimers (Fig. 2A). These CD8<sup>+</sup> HSV-gB–specific CTLs were functionally indistinguishable from those found in wild-type mice as revealed by in vivo cytotoxic assays (Fig. 2B). In addition, DicerΔΔ 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 DicerΔΔ 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 DC population in sLNs can be further subdivided into blood-derived, resident DCs (CD11c<sup>hi</sup>MHC II<sup>+</sup>) and migratory DCs (CD11c<sup>lo</sup>MHC II<sup>high</sup>) consisting of epidermal LCs and dermal DCs immigrating from skin. This migratory DC subgroup from skin was significantly decreased in DicerΔΔ 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<sup>+</sup> dermal DCs. As shown in Fig. 3B, we found a 12-fold reduction of MHC II<sup>+</sup>Langerin<sup>+</sup> LCs in the epidermis of CD11c-specific DicerΔΔ animals (p = 0.0032). Consequently, also the transmigrating LCs found in underlying dermal regions were nearly undetectable (Fig. 3B, dermis). In contrast, the other two main dermal DC subpopulations, the Langerin<sup>+</sup> and Langerin<sup>−</sup> dermal DCs, which are both EpCAM<sup>+</sup>, were present at normal frequencies (Fig. 3B).

To ensure that the few LCs found in the epidermis of DicerΔΔ 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<sup>fl/fl</sup> mice. Despite significantly lower numbers of LCs in the epidermis of CD11c-Cre-tdRFP-DicerΔΔ mice as compared with those found in CD11c-Cre-tdRFP-Dicer<sup>wt/wt</sup> littermates, the percentages of RFP<sup>+</sup> LCs were nearly identical (Fig. 3C) and therefore indicative for Cre expression. As a consequence, only background levels of Dicer mRNA could be detected by qPCR in sorted LCs from DicerΔΔ mice (Fig. 3D). This indicated that the few LCs found in DicerΔΔ mice actually deleted dicer<sup>1</sup> with a deletion efficacy of 99.1%. Also, the analysis of miRNA-223 indicated a deletion efficacy of 93.2% for this miRNA in LCs (Fig. 3D). The Dicer-deficient remaining LCs showed ~30% enlarged cell sizes as indicated by an increased forward scatter signal during flow cytometry (Fig. 3E, p = 0.0002). Steady-state surface expression levels of other typical LC markers, such as MHC class II and CD24 (Fig. 3C), and CD11c, CD40, CD86, and CCR7 (see below, Fig. 6B) were unaltered as compared with those of Dicer<sup>wt/wt</sup> mice. However, Dicer-deficient LCs expressed only half the amount of Langerin as compared with that of their wild-type counterparts (Fig. 3E, p = 0.0057) as well as substantially decreased levels of TGF-β<sub>RII</sub> (Fig. 3E).

Dicer-deficient Langerhans cells do not develop Birbeck granules and are lost with age

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<sup>ΔΔ</sup> LCs were distributed sparsely but equally in the epidermis. Quantification of LCs revealed ~12-fold reduction of DicerΔΔ LCS (Fig. 4C), similarly to our observations by flow cytometry (Fig. 3B). The morphology of the remaining DicerΔΔ LCS was different, because they occupied a larger area and had longer dendrites (Fig. 4A). It remains to be seen whether this change of cell shape is directly correlated to the loss of dicer1 expression or

---

**FIGURE 2.** CD11c-Cre-Dicer<sup>fl/fl</sup> mice do not exhibit impaired immunity to systemic viral infection. At day 0, Dicer<sup>wt/wt</sup> (n = 5) and Dicer<sup>ΔΔ</sup> (n = 5) mice were infected systemically with HSV-1 strain KOS (i.v. 1 × 10<sup>7</sup> PFU). Control mice (n = 5) received PBS i.v. A, The frequency of HSV-gB–specific CD8<sup>T</sup> cells in peripheral blood at day 10 postinfection was determined by staining with gB<sub>498–505</sub>-MHC tetramer complexes. Peripheral blood cells were acquired by flow cytometry and gated on CD8<sup>+</sup> lymphocytes. FACS plots are representative for a sample from each group. Numbers denote frequency of H-2K<sub>B</sub>/gB<sub>498–505</sub>-positive cells as percentage of CD8<sup>+</sup> 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<sup>hi</sup>) and control cells (CFSE<sup>lo</sup>) 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.
a consequence of decreased LC density. In contrast, no differences in other leukocyte populations of the skin, such as dendritic epidermal T cells (CD45+ TCRgd+), could be detected (data not shown).

The loss of LCs was increasing with age, because young Dicer Δ/Δ mice at the age of 10 d still contained normal numbers of LCs (Fig. 4B,4C). This was not due to lack of Cre expression in the animals, because 10-d-old CD11c-Cre-tdRFP-Dicer fl/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 Dicerwt/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 Dicerwt/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...
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:0008629; p = 0.0145) and “regulation of caspase activity” (GO:0043281; p = 0.0111) contain proapoptotic genes. Also the proapoptotic gene bc12111 (encoding for Bim), belonging to the enriched GO category “postembryonic organ development” (GO: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 bc12111 (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

FIGURE 4. Progressive loss and altered morphology of LCs in the absence of Dicer. A and B, Immunofluorescence analysis using Alexa Fluor 555-labelled MHC II-mAb of epidermal sheets from (A, original magnification ×40) 9-wk-old or (B, original magnification ×20) 10-d-, 6-, or 18-mo-old Dicerwt/wt and DicerΔ/Δ animals are shown. The skin of 6-mo-old and older mice barely contained LCs. The rare areas containing LCs were chosen for display (10 d and 6 mo, scale bar, 100 μm; 18 mo, scale bar, 50 μm). C, Quantification of LCs in epidermal sheets at the indicated age. The graph shows the mean of at least five fields counted from at least two mice per genotype (***p < 0.0001). D, Electron microscopy images of the epidermis from Dicerwt/wt and DicerΔ/Δ animals show LCs surrounded by keratinocytes with bundles of keratin filaments (asterisks). The arrows point at three Birbeck granules, two of which are enlarged in the insets (original magnification ×6500; insets, ×39000). Note the absence of Birbeck granules in the LCs of the DicerΔ/Δ mouse. Scale bars, 1 μm; inset scale bar, 0.1 μm.
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 DicerΔ/Δ 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 DicerΔ/Δ mice are strongly reduced compared with those from controls. This reduction highly correlated with the reduced number of LCs in the skin of DicerΔ/Δ mice in steady-state conditions (Fig. 3Δ), 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 epidermis (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). Upregulation of CCR7, the chemokine receptor for CCL21, allowed DicerΔ/Δ 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, DicerΔ/Δ LCs efficiently induced proliferation of OT-II cells, as measured by dilution of the CFSE dye (Fig. 6C). In contrast, DicerΔ/Δ 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 DicerΔ/Δ 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 DicerΔ/Δ LCs in vivo, we sensitized and challenged mice with DNFB. The DicerΔ/Δ mice used for these tests were older than 9 wk and had already considerably lower numbers of LCs in their epidermis 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 DicerΔ/Δ mice and DicerΔ/ΔΔ 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 epidermis 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 half-lives, 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 DicerΔΔ LCs. One possibility for the lack of effect on spleen DC populations after dicer1 deletion could be the appearance of DCs that escaped deletion of the dicer1 alleles. However, in very young mice also, DicerΔΔ 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 TGFb1 and TGFbRII 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+ at the age of 10 d, the initial seeding of the epidermis with LCs in DicerΔΔ 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+ in CD11c-Cre-tdRFP-Dicerfl/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

FIGURE 6. Dicer deficiency compromises maturation and function of LCs. A, DCs migrating out of ear explants from Dicerwt/wt and DicerΔΔ animals in the presence of CCL21 were analyzed by FACS. Percentages of migrated Langerin+CD103+ LCs are displayed (mean ± SEM, n = 6; combined results from two independent experiments are shown). B, Epidermal sheets from Dicerwt/wt and DicerΔΔ animals were digested to release LCs immediately (day 0) or cultured for two more days (day 2). Expression of surface markers on CD45+Langerin+ LCs was assessed using FACS. Shown are histogram overlays of Dicerwt/wt (gray) and DicerΔΔ (open) LC staining intensities. Results are representative of two experiments (n = 2–6). C, Epidermal skin explants were cultured overnight in medium containing 0.25 mg/ml OVA protein and after washing further cultured until day 2. Emigrated LCs were enriched, and equal numbers of LCs were cocultured with CFSE-labeled OT-I or OT-II T cells. T cell proliferation was assessed by flow cytometry after 60–65 h. One representative experiment out of two independent experiments with a similar outcome is shown (OT-II, n = 6; OT-I, n = 3–4). Dashed line, control without LCs. The graph shows the percentage of T cells having proliferated with graded doses of LCs. D, Dicerwt/wt and DicerΔΔ animals were sensitized with hapten (0.3% DNFB or 0.5% Oxazolone) on their backs. Five days later, ear swelling was elicited by challenging the mice with the same hapten on both sides of one ear. After 24 h, ear swelling was measured as the difference between ear thickness prior to and after challenge. Points represent individual mice (n = 6–7). N.D., not detectable.
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 DicerΔΔ 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, DicerΔΔ 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 DicerΔΔ LCs, we could identify differential regulation of myc, 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 myc in LCs, and it is hardly conceivable that such a loss of expression may lead to the observed increased cell size of DicerΔΔ 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- and down-regulation of myc expression are linked to apoptosis, depending on the experimental system used (53, 54). Thus, the decreased levels of Myc mRNA in DicerΔΔ 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 DicerΔΔ LCs, the observed lower expression levels could be sufficient to cause survival disadvantages.

We found that CHS is not altered in DicerΔΔ 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 DicerΔΔ 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 DicerΔΔ 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 monocye-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 DicerΔΔ LCs are likely to be involved in the maturation process. Thus, the reduced Ag presentation capacity observed in DicerΔΔ 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 DicerΔΔ 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.

Acknowledgments

We thank G. Hannon and B. Reizis for providing the DicerΔΔ mice and the CD11c-Cre mice, respectively, and D. Livingston for the anti-Dicer Ab. We thank C. Ried and U. Fazekas for expert technical assistance and A. Bol, W. Mertl, S. Heinzl, and M. Gridic for excellent animal care.

Disclosures

The authors have no financial conflicts of interest.

References
