Original Paper

Enhanced Reactive Oxygen Species Production, Acidic Cytosolic pH and Upregulated Na⁺/H⁺ Exchanger (NHE) in Dicer Deficient CD4⁺ T Cells

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Key Words

miRNAs • CD4⁺ T cells • Dicer • pH and NHE1 • miR-15b • miR-200b

Abstract

Background: MicroRNAs (miRNAs) negatively regulate gene expression at a post-transcriptional level. Dicer, a cytoplasmic RNase III enzyme, is required for the maturation of miRNAs from precursor miRNAs. Dicer, therefore, is a critical enzyme involved in the biogenesis and processing of miRNAs. Several biological processes are controlled by miRNAs, including the regulation of T cell development and function. T cells generate reactive oxygen species (ROS) with parallel H⁺ extrusion accomplished by the Na⁺/H⁺-exchanger 1 (NHE1). The present study explored whether ROS production, as well as NHE1 expression and function are sensitive to the lack of Dicer (miRNAs deficient) and could be modified by individual miRNAs. **Methods:** CD4⁺ T cells were isolated from CD4 specific Dicer deficient (Dicer^{Δ/Δ}) mice and the respective control mice (Dicer^{fl/fl}). Transcript and protein levels were quantified with RT-PCR and Western blotting, respectively. For determination of intracellular pH (pHi) cells were incubated with the pH sensitive dye bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) and Na⁺/H⁺ exchanger (NHE) activity was calculated from re-alkalinization after an ammonium pulse. Changes in cell volume were measured using the forward scatter in flow cytometry, and ROS production utilizing 2',7'-dichlorofluorescein diacetate (DCFDA) fluorescence. Transfection of miRNA-control and mimics in T cells was performed using DharmaFECT3 reagent. **Results:** ROS production, cytosolic H⁺ concentration, NHE1 transcript and protein levels, NHE activity, and cell volume were all significantly higher in CD4⁺ T cells from Dicer^{Δ/Δ} mice than in CD4⁺ T cells from Dicer^{fl/fl} mice. Furthermore, individual miR-200b and miR-15b modify pHi and NHE activity in Dicer^{fl/fl} and Dicer^{Δ/Δ} CD4⁺ T cells, respectively. **Conclusions:** Lack of Dicer leads to

oxidative stress, cytosolic acidification, upregulated NHE1 expression and activity as well as swelling of CD4⁺ T cells, functions all reversed by miR-15b or miR-200b.

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Introduction

Micro-ribonucleic acids (microRNAs; miRNAs) are small, highly conserved non-coding RNA molecules involved in the regulation of gene expression. miRNAs, small double stranded RNA molecules approximately 22 nucleotides in length, negatively regulate gene expression at the post-transcriptional level [1]. miRNAs are evolutionarily conserved and are found from *Caenorhabditis elegans* to most eukaryotes, including humans [2-5]. It is thought that miRNAs account for 1-5% of the human genome and regulate at least 30% of protein-coding genes [3, 6]. Thus far, it is evident that miRNAs play a crucial role in the regulation of gene expression, controlling diverse cellular and metabolic pathways thus, influencing diverse physiological processes, such as embryogenesis and in pathologies, such as infection, cancer and autoimmune disorders [7].

Mature miRNAs are generated *via* a two-step cleavage of primary miRNA (pri-miRNA), which incorporates into the effector complex RNA-induced silencing complex (RISC) [8, 9]. The miRNA functions as a guide by base-pairing with target homologous sequences on the respective mRNA to negatively regulate its expression [5, 10]. miRNAs bind to target messenger RNA (mRNA) sequences to prevent protein translation by one of two distinct mechanisms. The level of complementarity between the guide and mRNA target determines which silencing mechanism will be employed; cleavage of target mRNA with subsequent degradation or translation inhibition [5, 8, 11-15].

miRNAs are encoded within the genome and are transcribed as pol II transcript messages by enzyme complexes containing the RNases, Drosha and Dicer [1, 11, 12]. Dicer mutation in mice or in mouse embryonic stem cells results in developmental failure [16]. Recent evidence suggests that certain miRNAs exhibit tissue- or cell type-specific expression and may play a role in mammalian biology [17]. Employment of the loxP-Cre technology has enabled investigators to study the immune cell type-specific function of Dicer [18-21]. Conditional mutation or deletion of Dicer or Drosha specifically in CD4⁺ T cells is used as a tool to elucidate the effect of individual miRNAs in order to understand the physiological functions of immune cells [11, 18, 19, 22, 23]. Many studies have described that miRNAs are important regulators of T cell development and function [11, 12, 24]. These studies have incorporated the T cell specific deletion of Dicer and Drosha in addition to the analysis of specific miRNAs [18, 19, 23].

Targets of microRNA include the redox-sensitive nuclear factor erythroid 2-related factor 2 (Nrf2) transcription factor, a key master regulator of cell survival by induction of phase II and antioxidant defence enzymes reducing oxidative stress and by controlling redox signalling [25]. microRNAs are able to buffer the regulation of these events by directly interacting with the Nrf2 pathway and the generation of reactive oxygen species (ROS) [25]. In endothelial cells, many biological functions such as generation of ROS and redox reactions are regulated by miRNAs and thus, sensitive to Dicer [25, 26]. Production ROS requires H⁺ extrusion by the sodium hydrogen exchanger 1 (Slc9a1; NHE1) which maintains the intracellular cytoplasmic pH in immune cells such as dendritic cells and T cells [27-29]. NHE1 activity is further involved in cell volume regulation, cell survival, cell proliferation and cell migration [30]. Previous studies have suggested that miR-200 family members are involved in the regulation of Na⁺ ion balance and cell size using zebrafish and drosophila models [31-34]. We have previously shown that miR-15b/16 further regulates phosphorylation of Akt, which is required for the accurate maintenance of Na⁺/H⁺ exchanger activity [27, 35]. However, whether Dicer or individual miRNAs (miR-200b and miR-15b) are involved in the regulation of ROS production and NHE1 expression and activity in CD4⁺ T cells remained elusive.

In this study, we found that Dicer^{fl/fl} CD4⁺ T cells compared with Dicer^{Δ/Δ} CD4⁺ T cells have significantly higher ROS production. Furthermore, Dicer^{Δ/Δ} CD4⁺ T cells have significantly lower cytoplasmic intracellular pH (pHi) and significantly higher Na⁺/H⁺ exchanger 1 (Nhe1) mRNA expression and activity, as well as significantly larger cell volume compared with control CD4⁺ T cells. Transfection of miR-200b further increased ROS production, reduced resting pHi and increased Na⁺/H⁺ exchanger activity in Dicer^{fl/fl} CD4⁺ T cells. In contrast miR-15b downregulated the ROS production as well as Na⁺/H⁺ exchanger activity in Dicer^{Δ/Δ} CD4⁺ T cells. Thus, miRNAs are powerful regulators of Nhe1 in CD4⁺ T cells. miRNA sensitive functions are disclosed by genetic knockout of Dicer.

Materials and Methods

Mice

Dicer^{fl/fl} mice (mixed C57BL/6/129 background) were bred with CD4^{Cre} mice to produce CD4 specific Dicer^{Δ/Δ} mice as described earlier [18, 19] and kept under specific pathogen free (SPF) conditions. C57BL/6 (WT) mice were also used for some experiments. Mice used for the experiments were between 8-16 weeks old. Experiments were performed in age and sex matched male and female mice. All experiments were performed according to the EU Animals Scientific Procedures Act and the German law for the welfare of animals. All procedures for the experiments were approved by the authorities of the state of Baden-Württemberg.

CD4⁺ T cell isolation

CD4⁺ naïve T cells were isolated from Dicer^{fl/fl} and Dicer^{Δ/Δ} mice using the MagniSort® Mouse naïve T cell Enrichment kit, using a biotinylated antibody cocktail and streptavidin-coated magnetic bead method as described by the manufacturer (eBioscience, Frankfurt, Germany). Firstly, unwanted cells were targeted for removal with antibody complexes that recognized non CD4⁺ T cells and secondly, unwanted cells bound biotinylated antibody complexes were reacted with streptavidin-coated magnetic beads. The labelled cells (biotinylated antibody-streptavidin magnetic beads mix) were separated using a magnet and the (non-bound) desired cells were present in the suspension.

From each mouse, the spleen and all lymph nodes were isolated and macerated through 70 μm filter in cell buffer (PBS supplemented with 3% FBS and 10 mM EDTA) per well of a 6 well plate. The cell mixture was transferred to 15 ml tubes and centrifuged at 600 x g for 5 minutes at 4°C, to yield a concentration of 1x10⁷ cells/ml.

A volume of 100 μl/1x10⁸ cells of CD4 Naïve T cell Enrichment Antibody-Cocktail was added and vortexed, and then incubated for 10 minutes at room temperature. The antibody cocktail contained the following antibodies; *Anti-Mouse CD8 Biotin*, *Anti-Mouse CD11b Biotin*, *Anti-Mouse CD19 Biotin*, *Anti-Mouse CD24 Biotin*, *Anti-Mouse CD44 Biotin*, *Anti-Mouse CD45R (B220) Biotin*, *Anti-Mouse CD49b (Integrin alpha 2) Biotin*, *Anti-Mouse Ly-6G (Gr-1) Biotin*, *Anti-Mouse γδ TCR Biotin*, *Anti-Mouse TER-119 Biotin*.

Subsequently, the cells were washed once with cell buffer at 600xg for 5 minutes at 4°C and the supernatant discarded. The cell pellet was re-suspended in 1ml of cell buffer and 100 μl of MagniSort® Negative Selection Beads added per 1x10⁸ cells and the cells incubated at room temperature for 5 minutes. The cells were then placed on a magnet for 5 minutes and the supernatant (containing CD4⁺ T cells) collected whilst the tubes were on a magnetic stand. This method yielded approximately 10-15x10⁶ naïve CD4⁺ T cells (90% pure based on CD4⁺CD62L^{high} staining).

Transfection of Dicer^{Δ/Δ} and Dicer^{fl/fl} CD4⁺ T cells with miR-15b and miR-200b mimics

Naïve T cells (1x10⁶ cells in a 24 well plate) were isolated and activated with plate-bound anti-CD3 and anti-CD28 antibodies [35]. Naïve T cells were washed 3x with PBS to remove any residual antibiotics from the cells and 1x10⁶ cells per well cultured in the presence of 500 μl of R-10 medium without any antibiotics in 24 well plates, which were coated with anti-CD3:anti-CD28 (1:2 ratio; 1μg/ml anti-CD3 and 2 g/ml anti-CD28 antibodies).

After culturing the cells (same day), 2 μl of 50 μM stock concentration of non-targeting miRNA-control mimic, mimic miR-15b, inhibitor miR-15b and 200b-mimic (Exiqon, Denmark) were added to 8 μl

of antibiotic-free and serum-free RPMI1640 medium and incubated for 5 minutes in tube 1 respectively. In tube 2, 0.5 μ l of DharmaFECT 3 was added to 9.5 μ l of antibiotic-free and serum-free RPMI1640 medium and the content of tube 1 was added to tube 2, then incubated for an additional 20 minutes. After 20 minutes of incubation, the reaction mixture from tube 2 was added to corresponding wells to control and miR-mimics wells and the cells cultured for 48 hours. After a 48 hour incubation, cells were used for pHi, NHE activity and ROS measurements.

pH_i and NHE1 measurement

To measure the pHi and Na⁺/H⁺ exchanger activity, 300 μ l T cells (0.3×10^6) were activated for two days in presence of anti-CD3 and anti-CD28. Cells were fixed on a coverslip (coated with Poly-L-lysine from Sigma) then placed in a chamber [37]. For digital imaging of cytosolic pH (pHi), the cells were incubated in a HEPES-buffered Ringer solution containing 10 μ M bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF)-AM (Invitrogen, Leiden, The Netherlands) for 15 min at 37°C. After loading the dye, the chamber was flushed for 5 min with Ringer solution to remove any de-esterified dye. The perfusion chamber was mounted on the stage of an inverted microscope (Zeiss Axiovert 135), which was used in the epifluorescence mode with a 40 x oil immersion objective (Zeiss Neoplan, Germany). BCECF was successively excited at λ 490/10 nm and λ 440/10 nm, and the resultant fluorescent signal was monitored at λ 535/10 nm using an intensified charge-coupled device camera (Proxitronic, Germany) and specialized computer software (Metafluor, USA). Approximately 10-20 cells were outlined and monitored during the course of the measurements. The results from each cell were averaged and taken for final analysis. Intensity ratio (490/440) data were converted into pHi values using the high-K⁺/nigericin calibration technique. To this end, the cells were perfused at the end of each experiment for 5 minutes with standard high-K⁺/nigericin (10 μ g/ml) solution (pH 7.0). The intensity ratio data thus obtained were converted into pH values using the r_{max} , r_{min} , pK_a values previously generated from calibration experiments to generate a standard nonlinear curve (pH range 5 to 8.5).

For acid loading, cells were transiently exposed to a solution containing 20 mM NH₄Cl leading to initial alkalization of cytosolic pH (pHi) due to entry of NH₃ and binding of H⁺ to form NH₄⁺. The acidification of cytosolic pH upon removal of ammonia allowed calculating of the mean intrinsic buffering power (β) of the cells. Assuming that NH₄⁺ and NH₃ are in equilibrium between cytosolic and extracellular fluid and that ammonia leaves the cells as NH₃:

$$\beta = \Delta[NH_4^+]_i / \Delta pHi$$

where ΔpHi is the decrease of cytosolic pH (pHi) following ammonia removal and $\Delta[NH_4^+]_i$ is the decrease of cytosolic NH₄⁺ concentration, which is identical to the concentration of [NH₄⁺]_i immediately before the removal of ammonia. The pK for NH₄⁺/NH₃ is 8.9 and at an extracellular pH (pH_o) of 7.4 the NH₄⁺ concentration in extracellular fluid ([NH₄⁺]_o) is 19.37 [20/(1+10^{pH_o-pK})]. The intracellular NH₄⁺ concentration ([NH₄⁺]_i) was calculated from:

$$[NH_4^+]_i = 19.37 \cdot 10^{pH_o - pHi}$$

The calculation of the buffer capacity required that NH₄⁺ exits completely. After the initial decline, pHi indeed showed little further change in the absence of Na⁺, indicating that there was no relevant further exit of NH₄⁺.

To calculate the $\Delta pH/min$ during re-alkalinization, a manual linear fit was placed over a narrow pH range with time which could be applied to all measured cells.

The solutions were composed of (in mM): standard Hepes: 115 NaCl, 5 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 NaH₂PO₄, 10 glucose, 32.2 Hepes; sodium free Hepes: 132.8 NMDG Cl, 3 KCl, 1 CaCl₂, 1.2 MgSO₄, 2 KH₂PO₄, 32.2 Hepes, 10 mannitol, 10 glucose (for sodium free ammonium chloride 10 mM NMDG and mannitol were replaced with 20 mM NH₄Cl); high K⁺ for calibration 105 KCl, 1 CaCl₂, 1.2 MgSO₄, 32.2 Hepes, 10 mannitol, 5 μ M nigericin. The pH of the solutions was titrated to 7.4 or 7.0 with HCl/NaOH, HCl/NMDG and HCl/KOH, respectively, at 37°C [29, 38-41]

Semi-quantitative-RT-PCR for NHE1

Total mRNA were isolated from 2 day activated CD4⁺ T cells using the miRNAeasy isolation kit (QIAGEN, Germany) as described by the manufacturer. 1.0 μ g mRNA was converted into cDNA using the Superscript III cDNA synthesis kit (Invitrogen, Germany). Briefly, in 10 μ l reactions, 10 ng cDNA, 2x Taq polymerase Master-mix (Peqlab, Germany) and 250 nM primers were used for RT-PCR reactions [42]. Nhe1 and Gapdh primers were used in reactions [37]. PCR was run using standard PCR conditions (95°C for 3 minutes, 95°C for 15

seconds, 60°C for 30 seconds and 72°C for 30 second for 40 cycles followed by final extension at 72°C for 10 minutes. All primers were obtained from Sigma.

q-RT-PCR

Total mRNAs (1 µg) was converted into cDNA using cDNA synthesis kit (Invitrogen, Germany). Briefly, in 10 µl reactions, 10 ng cDNA, KAPA SYBR® FAST q-PCR kit Master Mix (2x) Bio-Rad iCycler™ (Peqlab, Erlangen, Germany) and 250 nM primers were used for q-RT-PCR reactions. q-RT-PCR run and data analysis was performed as described previously [43] for *Nhe1* and *Gapdh* [37] using universal cycling conditions (95° C for 10 minutes, 95° C for 15 seconds and 60° C for 1 minute for 40 cycles followed by melting curve analysis) [43].

Confocal Imaging

Activated T cells from *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} mice were fixed using -20 °C cooled methanol on poly-L-Lysine coated glass slides. These fixed cells were washed with PBS immediately and blocked with 10%FCS, 0.1% saponin in PBS buffer solution. Further, the cells were washed again with PBS and incubated with anti-Rabbit primary polyclonal NHE1- antibody (1:500; #PRS4377, Sigma). After a 1 hour incubation with primary antibody, the cells were washed with 10% FCS, 0.1% saponin in PBS buffer solution. At the end of the incubation, the cells were washed in 0.1% saponin 3x for 3 minutes each and then 1x in water. Finally, coverslips were mounted onto slides with 2 µl vector shield containing 1 µl DRAQ5, a nuclear stain (eBioscience, Germany) and subjected to confocal laser-scanning microscopy (LSM 5 Exciter, Carl Zeiss, Germany) with a C-Apochromat 63/1.3 NA DIC water immersion objective.

Flow cytometry for cell volume and ROS production

To quantify cell volume and ROS production from both *Dicer*^{Δ/Δ} and *Dicer*^{fl/fl} CD4⁺ T cells, 2×10⁵ cells were co-incubated with 10 µM 2',7'-dichlorofluorescein diacetate (DCFDA) (Sigma) for 30 min in the dark at room temperature. Cells were then washed 2x with 200 µl PBS and then re-suspended in 200 µl of fresh PBS. ROS in T cells were detected by flow cytometry using the FACS Calibur (BD Biosciences, Heidelberg, Germany). DCFDA fluorescence intensity was measured in FL-1 channel. Geometric mean of the FL-1 signal intensity was used to show the amount of ROS production. Cell volume was calculated by using the geometric mean of forward scatter. All flow data were analysed with FlowJo® software (FLOWJO, LLC, Ashland, Oregon, USA).

Statistics

Data are provided as arithmetic means ± SEM, n represents the number of independent experiments. All data were tested for significance using Student's *t*-test. Data were analysed by Excel 2010 or GraphPad Prism Software, USA. *p* value ≤0.05 was considered statistically significant.

Results

Dicer deficiency enhances ROS production in CD4⁺ T cells

Activation of T cells leads to stimulation of ROS production. To examine whether deficiency of *Dicer* protein affects ROS production, after 2 days of activation, CD4⁺ T cells were incubated with 2',7'-dichlorofluorescein diacetate (DCFDA) dye for 1 hour and ROS production quantified using flow cytometry. DCFDA fluorescence was significantly higher in *Dicer*^{Δ/Δ} (*Dicer* deficient) than in *Dicer*^{fl/fl} CD4⁺ T cells, reflecting enhanced ROS production in *Dicer* deficient cells (Fig. 1A-B).

*Nhe1 expression is higher in *Dicer*^{Δ/Δ} than in *Dicer*^{fl/fl} activated CD4⁺ T cells*

ROS production requires up-regulation of H⁺ extrusion by the Na⁺/H⁺ exchanger *Nhe1*. To investigate whether deficiency of *Dicer* protein affects *Nhe1* transcript levels, we first quantified the mRNA abundance by semi-quantitative RT-PCR. We found that the *Nhe1* mRNA levels were higher in *Dicer*^{Δ/Δ} CD4⁺ T cells than in *Dicer*^{fl/fl} CD4⁺ T cells (Fig. 2A). Quantification of *Nhe1* mRNA by qRT-PCR yielded similar results, i.e. significantly higher

Fig. 1. ROS production in *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells. A. FACS histograms displaying ROS-dependent DCFDA fluorescence in CD4⁺ T cells from *Dicer*^{Δ/Δ} (red) and *Dicer*^{fl/fl} (blue) following 2 days of activation in the presence of anti-CD3 and anti-CD28. B. Arithmetic means $\pm$ SEM (n = 4 independent experiments) of the ROS Geometric Mean Fluorescence (MFI) intensity in *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells. * (p < 0.05) indicates statistically significant difference.

Fig. 2. NHE1 expression in *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells. A. RT-PCR with equal amount of cDNA from *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells activated in the presence of anti-CD3 and anti-CD28 for 2 days. B. Arithmetic means $\pm$ SEM (n = 5) of NHE1 mRNA levels in *Dicer*^{fl/fl} (blue) and *Dicer*^{Δ/Δ} (red) CD4⁺ T cells activated in the presence of anti-CD3 and anti-CD28 for 2 days. * (p < 0.05) indicates statistically significant difference between *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells. C. *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells stained with NHE1 antibody and characterised by confocal microscopy. NHE1 expression (green) and nuclear staining (DRAQ5) is shown in the upper and lower panels for *Dicer*^{fl/fl} and *Dicer*^{Δ/Δ} CD4⁺ T cells.

expression of *Nhe1* mRNA in *Dicer*^{Δ/Δ} CD4⁺ T cells than in *Dicer*^{fl/fl} CD4⁺ T cells (Fig. 2B). Immunofluorescence was employed to quantify the surface or cytoplasmic abundance of Nhe1 protein. As a result, Nhe1 protein on the cell surface and in the cytoplasm were both significantly more abundant in *Dicer*^{Δ/Δ} CD4⁺ T cells than in *Dicer*^{fl/fl} CD4⁺ T cells (Fig. 2C). Our mRNA and protein expression data suggest that in the absence of Dicer protein, Nhe1 is upregulated in CD4⁺ T cells.

*Na⁺/H⁺ exchanger activity is higher in *Dicer*^{Δ/Δ} than in *Dicer*^{fl/fl} CD4⁺ T cells*

After validation of mRNA and protein expression of Nhe1 in CD4⁺ T cells from *Dicer* deficient mice, we quantified cytosolic (pHi) and functional Na⁺/H⁺ exchanger activity in activated CD4⁺ T cells from *Dicer*^{Δ/Δ} and *Dicer*^{fl/fl} mice. Both *Dicer*^{Δ/Δ} and *Dicer*^{fl/fl} CD4⁺ T cells were loaded with the pH sensitive dye BCECF for 15 minutes and the resting intracellular pHi measured. As a result, resting pHi was significantly lower in *Dicer*^{Δ/Δ} than in *Dicer*^{fl/fl} CD4⁺ T cells (Fig. 3A-B).

Further, Na⁺/H⁺ exchanger activity was measured using the ammonium pulse technique. As shown in the Fig. 3A, after the addition of NH₄Cl to the extracellular bath, there was a sharp cytosolic alkalization due to NH₃ entry into the cells followed by binding to cytosolic H⁺ to form NH₄⁺. Subsequently NH₄Cl was removed from the extracellular bath, leading to a sharp cytosolic acidification due to NH₃ exit and cellular H⁺ retention (Fig. 3). Neither in

Fig. 3. Cytosolic pH and NHE1 activity in $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ CD4⁺ T cells. A. Original tracings (490/440 nm intensity ratio) showing alterations of cytosolic pH (pH_i) following an ammonium pulse in CD4⁺ T cells isolated after 2 days of activation from $Dicer^{fl/fl}$ (left-hand side) and $Dicer^{\Delta/\Delta}$ (right-hand side) mice. CD4⁺ T cells were loaded with H⁺ by adding 20 mM NH_4Cl and Na^+ was removed (replaced by NMDG) in a first step, NH_4Cl was removed in a second step, Na^+ was added again in a third step and nigericin (pH_o 7.0) was applied in a fourth step to calibrate each individual experiment. B. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of cytosolic pH (pH_i) prior to the ammonium pulse. ****($p < 0.0001$) indicates statistically significant difference between $Dicer^{fl/fl}$ (blue) and $Dicer^{\Delta/\Delta}$ (red) CD4⁺ T cells. C. Na^+ -dependent recovery (Na^+/H^+ exchanger activity) of cytosolic pH ($\Delta pH/min$) following an ammonium pulse from 2 days activated $Dicer^{fl/fl}$ (blue) and $Dicer^{\Delta/\Delta}$ (red) CD4⁺ T cells. ** indicates statistically significant difference ($p < 0.01$) between $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ CD4⁺ T cells.

Fig. 4. $Dicer^{\Delta/\Delta}$ CD4⁺ T cell increases cell volume. A. FACS histograms displaying forward scatter of CD4⁺ T cells from $Dicer^{\Delta/\Delta}$ mice ($Dicer^{\Delta/\Delta}$, red) and their wild type littermates ($Dicer^{fl/fl}$, blue) following 2 days of activation in the presence of anti-CD3 and anti-CD28. B. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of forward scatter (FSC) of $Dicer^{fl/fl}$ (blue) and $Dicer^{\Delta/\Delta}$ (red) CD4⁺ T cells. *($p < 0.05$) indicates statistically significant difference.

$Dicer^{\Delta/\Delta}$ CD4⁺ T cells nor in $Dicer^{fl/fl}$ CD4⁺ T cells acidification was followed by significant re-alkalinization as long as Na^+ was absent. Therefore, CD4⁺ T cells did not express appreciable Na^+ -independent H^+ extrusion irrespective of absence or presence of Dicer. Addition of Na^+ was, however, followed by cytosolic re-alkalinization, pointing to Na^+/H^+ exchanger activity in both, $Dicer^{\Delta/\Delta}$ and $Dicer^{fl/fl}$ CD4⁺ T cells. As illustrated in Fig. 3A-C, after 2 days of CD4⁺ T cell activation, the pH_i recovery following addition of Na^+ was significantly more rapid in $Dicer^{\Delta/\Delta}$ CD4⁺ T cells than in $Dicer^{fl/fl}$ CD4⁺ T cells, pointing to enhanced Na^+/H^+ exchanger activity in $Dicer^{\Delta/\Delta}$ CD4⁺ T cells (Fig. 3A,C). Thus, Dicer deficiency enhanced Na^+ dependent re-alkalinization reflecting higher Na^+/H^+ exchanger activity.

Dicer deficiency increases cell volume in CD4⁺ T cells

Activation of Nhe1 was expected to increase cell volume. We thus tested whether Dicer protein modifies cell volume. According to forward scatter in flow cytometry, cell volume was significantly larger in $Dicer^{\Delta/\Delta}$ than in $Dicer^{fl/fl}$ CD4⁺ T cells (Fig. 4A-B).

Effect of miR-200b on pH_i and Na^+/H^+ exchanger activity in $Dicer^{fl/fl}$ CD4⁺ T cells

As shown in embryonic epidermis of developing zebrafish, miRNAs participate in the salt balance homeostasis through targeting *Nherf1* [33, 34, 44]. *Nherf1* was originally shown to regulate the activity of Nhe3 (an isoform of Nhe1), but also to control trafficking of further ion transporters and of receptors [44]. Disruption of *miR-200* miRNAs disrupts the response of zebrafish embryos to osmotic stress, and compromises ion channel function, acid-base homeostasis as well as body size regulation [31-34]. These studies suggest that the miR-200

Fig. 5. miR-200b overexpression increases the ROS and cell volume but does not affect the Na^+/H^+ exchanger activity in WT CD4^+ T cells. A. ROS production from WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b (green) transfected CD4^+ T cells. $^*(p<0.05)$ indicates statistically significant difference. B. Cell volume from WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b (green) transfected CD4^+ T cells. $^*(p<0.05)$ indicates statistically significant difference. C. Original tracings (490/440 nm intensity ratio) showing alterations of cytosolic pH (pH_i) following an ammonium pulse in activated CD4^+ T cells transfected with miRNA mimics (48 hours) from WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b (green) mice. D. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of cytosolic pH (pH_i) prior to the ammonium pulse between WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b CD4^+ T cells (green). **** indicates statistically significant difference ($p<0.0001$) between WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b (green) transfected CD4^+ T cells. E. Na^+/H^+ exchanger activity of cytosolic pH ($\Delta\text{pH}/\text{min}$) following an ammonium pulse in activated CD4^+ T cells transfected with miRNA mimic (48 hours) from WT+miRNA-mimic-Control (grey) and WT+miRNA-mimic-miR-200b (green) mice.

family may participate in the regulation of NHE3 and NHERF1 (slc9a3r2) protein [33, 34, 45]. NHERF1 negatively regulates NHE3 [44, 45]. We thus tested whether miR-200b could also regulate Na^+/H^+ exchanger activity and ROS production in CD4^+ T cells [33]. Indeed, overexpression of miR-200b enhanced the ROS production as well as cell volume significantly (Fig. 5A-B). Transfection of miR-200b further significantly decreased pH_i (Fig. 5C-D). miR-200b tended to increase Na^+/H^+ exchanger activity, an effect, however, not reaching statistical significance (Fig. 5C and E).

Over-expression of miR-15b decreases Na^+/H^+ exchanger activity in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells

According to previous studies Akt is involved in the regulation of Nhe1 as Akt1 deficient mice cannot upregulate Na^+/H^+ exchanger activity [29]. Moreover, Akt is also involved in the regulation of cell size [46]. Our recent findings also suggest that miR-15b/16 can regulate Akt phosphorylation by regulating mTOR/Rictor expression, thus leading to reduced functional activity [47]. At least in theory miR-15b or 16 could regulate Na^+/H^+ exchanger activity. Thus, we overexpressed miR-15b using miR-15b mimic in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells and measured the pH_i and Na^+/H^+ exchanger activity. Overexpression of mimic-miR-15b in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells did not change significantly cytoplasmic pH_i (Fig. 6A-B). However, overexpression of mimic-miR-15b in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells significantly reduced Na^+/H^+ exchanger activity (Fig. 6A-C).

Fig. 6. miR-15b overexpression inhibits the Na^+/H^+ exchanger activity in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells. A. Original tracings (490/440 nm intensity ratio) showing alterations of cytosolic pH (pH_i) following an ammonium pulse in activated CD4^+ T cells transfected with miRNA mimics (48 hours) from $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-Control (grey) and $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-miR-15b (purple) mice. B. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of cytosolic pH (pH_i) prior to the ammonium pulse between $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-Control (grey) and $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-miR-15b (purple) CD4^+ T cells. C. Na^+ -dependent recovery (Na^+/H^+ exchanger activity) of cytosolic pH ($\Delta\text{pH}/\text{min}$) following an ammonium pulse in activated CD4^+ T cells

transfected with miRNA mimics (48 hours) from $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-Control (grey) and $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-miR-15b (purple) mice. * indicates statistically significant difference ($p < 0.05$) between $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-Control and $\text{Dicer}^{\Delta/\Delta}$ +miRNA-mimic-miR-15b transfected CD4^+ T cells.

Fig. 7. ROS production in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells after miR-15b mimic expression and cell volume regulation by miR-15b inhibitor and mimic in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells. A. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) displaying ROS-dependent DCFDA fluorescence in CD4^+ T cells from $\text{Dicer}^{\Delta/\Delta}$ mimic control (grey) and $\text{Dicer}^{\Delta/\Delta}$ mimic miR-15b (purple) following 2 days of activation in the presence of anti-CD3 and anti-CD28. * ($p < 0.05$) indicates statistically significant difference. B. Arithmetic means $\pm$ SEM ($n = 4$ independent experiments) of the FSC Geometric Mean Fluorescence (MFI) intensity in $\text{Dicer}^{\Delta/\Delta}$ mimic control (grey), $\text{Dicer}^{\Delta/\Delta}$ inhibitor miR-15b (blue) and $\text{Dicer}^{\Delta/\Delta}$ mimic miR-15b (purple) CD4^+ T cells. * ($p < 0.05$) and ** ($p < 0.01$) indicates statistically significant difference.

miR-15b mimic reduces ROS production and cell volume in CD4^+ T cells

Overexpression of miR-15b mimic in $\text{Dicer}^{\Delta/\Delta}$ CD4^+ T cells decreased ROS production (Fig. 7A). Furthermore, inhibition of miR-15b slightly, but significantly, increased, whereas miR-15b mimic significantly decreased cell volume (Fig. 7B).

Discussion

The present observations reveal an important role of Dicer protein in the regulation of ROS production, cytosolic pH (pH_i), Na^+/H^+ exchanger activity and cell volume in CD4^+ T cells. Lack of miRNAs enhanced ROS formation, acidified pH_i , enhanced Nhe1 expression and activity, as well as increased cell volume.

In other cell types such as endothelial cells, adipocytes, trophoblasts, miRNAs participate in the production of ROS, which is thus sensitive to Dicer enzyme expression or levels in various cell types [25, 26]. The ROS formation is paralleled by intracellular H^+

generation [28] and could thus well contribute to or even account for the observed lower cytosolic pH (pHi) in Dicer^{Δ/Δ} CD4⁺ T cells than in Dicer^{fl/fl} CD4⁺ T cells. Cytosolic acidification in turn up-regulates NHE1 expression and activity [43]. The up-regulation of NHE1 activity could thus have been due to cytosolic acidification. Along those lines, expression of NHE1 mRNA increases in human lymphocytes during metabolic acidosis [44, 45]. Cytosolic pH in turn modifies ROS production, i.e. NHE1 activity and ROS production mutually activate each other [27, 46-48]. Dicer is involved in the miRNAs maturation process, and in the absence of miRNAs or lack of Dicer protein, ROS, resting pHi, Na⁺/H⁺ exchanger activity and cell volume, respectively, could be altered due to lack of either an individual or several miRNAs. Earlier studies in Zebrafish ionocytes revealed that the miR-200a/b family is involved in the regulation of cell volume and pHi by regulating NHERF1 at the post-transcriptional level [33, 34]. We also found that resting pHi was decreased and Na⁺/H⁺ exchanger activity tended to be higher after overexpression of miR-200b in CD4⁺ T cells. Additionally, we have shown that LeftyA is involved in the downregulation of Na⁺/H⁺ exchanger activity, reduces the resting pHi, lactate production and cell volume in Ishikawa cells [48, 49]. Interestingly, LeftyA simultaneously augments the miR-200a expression in Ishikawa cells (endometrial cancer cells) and leads to reduced migration of cells by regulating focal adhesion kinase and E-cadherin expression [50]. miR-200b could be similarly involved in maintenance of resting pHi and cell volume regulation in CD4⁺ T cells.

The present study does not rule out the participation of further mechanisms. For instance, phosphorylation of Akt on both Ser473 and Thr308 after TCR activation with anti-CD3 and anti-CD28 antibodies is stronger in Dicer^{Δ/Δ} CD4⁺ T cells than in Dicer^{fl/fl} CD4⁺ T cells [35]. Akt activation could in turn lead to increase in NHE1 expression [27, 36]. In Dicer^{Δ/Δ} CD4⁺ T cells, the enhanced Nhe1 expression could further result from acidic pHi. According to a recent study, inhibition of miR-15b increases the ROS production, decreases the mitochondrial membrane potential, and modulates mRNA levels of nuclear encoded mitochondrial genes (CytC, TFAM, NRF1) as well as components of the senescence-associated secretory phenotype (IL-6, IL-8, VEGF, MMP1, IL-1-α, IL-1β, and IFN-γ) via Sirtuin4 (SIRT4) [51]. In Dicer^{Δ/Δ} CD4⁺ T cells, phosphorylation of Akt on both Ser473 and Thr308 after TCR activation is augmented [35]. Thus, Akt activation could lead to increase in Nhe1 expression [27, 36]. Furthermore, miR-15b/16 regulates phosphorylation of Akt by downregulating mTOR/Rictor [35]. Thus, in principle, ROS production could be regulated by miR-15b/16 in T cells. Indeed, our data suggest that miR-15b overexpression in Dicer^{Δ/Δ} CD4⁺ T cells suppresses Na⁺/H⁺ exchanger activity.

Control of oxidative stress and cell size are important mechanisms that control gene integrity and tissue homeostasis. Deranged regulation of cell and organ size may be the first step leading to tumorigenesis [52]. Akt regulates T cell size in the thymus and miRNAs are also under the control of Akt [53, 54], supporting that the Akt pathway and miRNA deregulation participate in the regulation of cell size and subsequently tissue homeostasis. Furthermore, miR-200b is also involved in controlling the size of *Drosophila* [31]. Indeed, our data also suggest that miR-200b or miR-15b is involved in the regulation of cell size in CD4⁺ T cells.

Na⁺/H⁺ exchanger Nhe1 contributes to regulatory cell volume increase, which is in part accomplished by parallel activity of Na⁺/H⁺ and Cl⁻/HCO₃⁻ exchanger [55, 56]. Increase of cell volume is a precondition for cell proliferation and stimulation of cell proliferation is paralleled by a shift of Na⁺/H⁺ exchanger cell volume regulatory set point to larger volumes [57-59]. Cell proliferation is further supported by intracellular alkalinization [60]. Based on our data we are tempted to speculate that the acidic cytosolic pH could be responsible for the reduced proliferation and increased apoptosis of Dicer^{Δ/Δ} CD4⁺ T cells compared with Dicer^{fl/fl} CD4⁺ T cells [20].

In summary, lack of the Dicer enzyme enhances oxidative stress, leads to cytosolic acidification, up-regulates Na⁺/H⁺ exchanger activity and increases cell volume. These

observations disclose an impact of Dicer dependent miRNA formation on oxidative stress, cytosolic pH and cell volume regulation. Specifically, miR-200b and miR-15b could belong to the miRNAs regulating this important physiological function.

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The authors of this manuscript state that they do not have any financial conflict of interests to disclose.

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