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Original Paper

Role of Dicer Enzyme in the Regulation of Store Operated Calcium Entry (SOCE) in CD4$^+$ T Cells

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Key Words
CD4$^+$ T cells • Dicer • SOCE and miRNAs

Abstract

Background/Aims: Activation of T cell receptors (TCRs) in CD4$^+$ T cells leads to a cascade of signalling reactions including increase of intracellular calcium (Ca^{2+}) levels with subsequent Ca^{2+} dependent stimulation of gene expression, proliferation, cell motility and cytokine release. The increase of cytosolic Ca^{2+} results from intracellular Ca^{2+} release with subsequent activation of store-operated Ca^{2+} entry (SOCE). Previous studies suggested miRNAs are required for the development and functions of CD4$^+$ T cells. An enzyme called Dicer is required during the process of manufacturing mature miRNAs from the precursor miRNAs. In this study, we explored whether loss of Dicer in CD4$^+$ T cells affects SOCE and thus Ca^{2+} dependent regulation of cellular functions. ***Methods:*** We tested the expression of Orai1 by q-RT-PCR and flow cytometry. Further, we measured SOCE by an inverted phase-contrast microscope with the Incident-light fluorescence illumination system using Fura-2. Intracellular Ca^{2+} was also measured by flow cytometry using Ca^{2+} sensitive dye Fluo-4. ***Results:*** We found that in Dicer deficient ($Dicer^{\Delta/\Delta}$) mice Orai1 was downregulated at mRNA and protein level in CD4$^+$ T cells. Further, SOCE was significantly smaller in $Dicer^{\Delta/\Delta}$ CD4$^+$ T cells than in CD4$^+$ T cells isolated from wild-type ($Dicer^{fl/fl}$) mice. ***Conclusion:*** Our data suggest that miRNAs are required for adequate Ca^{2+} entry into CD4$^+$ T cells and thus triggering of Ca^{2+} sensitive immune functions.

Introduction

After antigen-specific T cell activation, an increase of intracellular Ca^{2+} levels is required to induce gene expression, proliferation, cell motility and cytokine expression [1-3]. In resting T cells, Ca^{2+} is stored in the endoplasmic reticulum (ER) where it is sensed by stromal

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cell-interaction molecules (STIM) 1 and 2. In T cells, signalling through the T cell receptor results in the production of inositol trisphosphate (IP_3), which binds to IP_3 receptors on the ER and results in the release of Ca^{2+} into the cytosol. The depletion of ER Ca^{2+} results in Ca^{2+} influx across the plasma membrane by store-operated Ca^{2+} entry (SOCE) [2, 4-7]. It entails activation of the calcium release-activated calcium (CRAC) channel protein 1 (encoded by Orai1 gene) through the binding of the ER Ca^{2+} sensors STIM1 and 2. Ca^{2+} influx through Orai1 in T cells depends on a negative membrane potential that provides the electrical driving force for Ca^{2+} entry [2, 6, 8, 9]. The importance of ion channel function in T cells comes largely from genetic studies in mice through knockout or siRNA mediated knock down of specific ion channel genes [2, 6]. Mice genetically defective for STIM1/2 or Orai1 have impaired T cell development, which is not surprising considering the important role these molecules play in Ca^{2+} signalling [5, 8-10]. The regulation of the function of Orai1 channels could affect the signalling in T cells and play an important role in $CD4^+$ T cells development and function [1-3].

MicroRNAs (miRNAs) are transcribed in the genome as a part of Pol II transcribed messages and function as post-transcriptional gene regulators [11]. Biogenesis of miRNAs involves the RNase Dicer [11, 12]. In the cell nucleus, primary miRNAs are transcribed and processed into precursor miRNAs by the enzyme complex containing the RNase Drosha. These precursor miRNAs then move out from the nucleus to cytoplasm by experotin-5, where these miRNAs are processed into mature miRNAs by Dicer. Thus in the absence of Dicer, miRNAs are not produced. Generally, miRNAs bind to the 3' untranslated region (3'UTR) of mRNAs and inhibit translation and induce message degradation [13-21]. Bioinformatics studies have predicted that almost one third of the genome is targeted by miRNAs [13, 21]. Therefore, miRNAs play decisive roles in the regulation of gene expression. Dicer is involved in the regulation of diverse biological processes including development as well as organogenesis and Dicer contributes to the development of several pathologies including cancer, infection susceptibility as well as autoimmunity [22].

T cell specific deletion of Dicer results in impaired T cell development and severely reduced numbers of regulatory T cells [23, 24]. Dicer deficient mice develop inflammatory bowel disease by age of 6 months [23, 25, 26], which is a similar phenotype as seen in Orai1 and STIM1/2 deficient mice [2, 8, 27]. Dicer impacts on the development of autoimmunity by controlling the immune functions of regulatory T cells and conventional T cells. Therefore, we explored whether Dicer may influence Ca^{2+} signalling in $CD4^+$ T cells.

In this study we found that Dicer deficient ($Dicer^{\Delta/\Delta}$) $CD4^+$ T cells have reduced expression of Orai1 at mRNA and protein levels. Further studies suggested that Dicer deficient $CD4^+$ T cells have less Ca^{2+} influx after activation with anti-CD3 and anti-CD28 compared with control ($Dicer^{fl/fl}$) $CD4^+$ T cells. Thus, our data suggest that miRNAs are required for proper influx of Ca^{2+} after activation of T cells.

Material and Methods

Mice

$Dicer^{fl/fl}$ mice (mixed C57BL/6/129 background) were bred with $CD4^{Cre}$ mice to generate CD4 specific $Dicer^{\Delta/\Delta}$ mice described earlier [23, 24] and kept in specific pathogen free conditions. Mice used for the experiments were in between 8-16 weeks of age. All the experiments were performed according to the EU Animals Scientific Procedures Act and the German law for the welfare of animals. All the procedures for the experiments were approved by the authorities of the state of Baden-Württemberg.

$CD4^+$ T cell isolation and culture

$CD4^+$ naïve T cells were isolated from $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ mice using the MagniSort® Mouse naïve T cell Enrichment kit as described by the manufacturer (eBioscience, Frankfurt, Germany). Purified T cells were cultured in plate-bound anti-CD3: and anti-CD28 (1:2 dilution; 1μg/ml anti-CD3 and 2μg/ml anti-CD28) for 2-3 days and then subjected to the measurement of intracellular Ca^{2+} by flow cytometry (Fluo-4) and fluorescence microscopy (Fura-2/AM).

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Calcium measurements

Fluorescence measurements were performed using an inverted phase-contrast microscope with the Incident-light fluorescence illumination system (Axiovert 100, Zeiss, Germany). Cells were excited alternatively at $\lambda = 340$ or 380 nm and the light deflected by a dichroic mirror into either the objective (Fluar 40×/1.30 oil, Zeiss, Germany) or a camera (Proxitronic, Germany). Emitted fluorescence intensity was recorded at $\lambda = 505$ nm and data were acquired by using specialized computer software (Metafluor, Universal Imaging, USA) [28].

Activated cells (anti-CD3/anti-CD28) for 3 days from both mice strains were loaded with Fura-2-AM (2 µM, Molecular Probes, Germany) for 30 min at 37°C in a CO_2 incubator. To measure SOCE, changes in intracellular Ca^{2+} $[Ca^{2+}]i$ were monitored on depletion of the intracellular Ca^{2+} stores. In brief, $[Ca^{2+}]i$ was measured using Ca^{2+} containing standard HEPES buffer [125mM/L NaCl, 5mM KCl, 1.2 mM $MgSO_4*7H_2O$, 32.2 mM HEPES, 2mM $Na_2HPO_4*2H_2O$, 5mM Glucose, 1mM $CaCl_2*2H_2O$; pH=7.4] for 2 minutes and then changed to Ca^{2+} free HEPES buffer [125mM NaCl, 5mM KCl, 1.2 mM $MgSO_4*7H_2O$, 32.2 mM HEPES, 2mM $Na_2HPO_4*2H_2O$, 5mM Glucose, 0.5 mM EGTA; pH=7.4] for 3 minutes. In the absence of Ca^{2+}, the intracellular Ca^{2+} stores were depleted by inhibition of the vesicular Ca^{2+} pump by thapsigargin (1 µM, Sigma, Germany) and $[Ca^{2+}]i$ activity was measured for another 5 minutes. Furthermore, Ca^{2+} containing HEPES buffer was added for 5 minutes, which allowed assessing SOCE.

q-RT-PCR

Total mRNA was isolated from *Dicer*$^{fl/fl}$ and *Dicer*$^{\Delta/\Delta}$ mice using the mRNAeasy isolation kit (QIAGEN, Germany) as described by the manufacturer. 1µg mRNA was converted into cDNA using the cDNA synthesis kit (Invitrogen, Germany). Briefly, in 10 µl reactions, 10 ng cDNA, 2X SYBR green mastermix (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 [29]. Universal cycling conditions were used (95° C for 10 minutes, 95° C for 15 seconds and 60° C for 1 minute for 40 cycles followed by melting curve analysis) for q-RT-PCR [30]. The following primers were used:

Orai1-F 5’- CCTGGCGCAAGCTCTACTTA-3’
Orai1-R 5’- CATCGCTACCATGGCGAAGC-3’,
GAPDH-F 5’-TCTGACCACAGTGAGGAATGTCCAC-3’
GAPDH-R 5’-TTGATGGCAACAATCTCCAC-3’

Fluo-4 Calcium measurement by flow cytometry

Activated $CD4^+$ T cells from *Dicer*$^{fl/fl}$ and *Dicer*$^{\Delta/\Delta}$ mice were washed once with PBS and then incubated in Ringer solution with calcium containing Fluo4 and Fluo3 (Invitrogen, Germany) for 30 minutes in a 37 °C incubator as described earlier [31]. After incubation, cells were washed 3 times with Ringer solution. 200 µl of Ringer solution was added, and intracellular calcium levels were measured by flow cytometry analysing 20,000 cells. Data were analysed by FlowJo (Treestar, USA)

Statistics

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

Results

Orai1 expression by q-RT-PCR and flow cytometry

Previous studies suggested that various ion channels such as Ca^{2+} release activated Ca^{2+} (CRAC) channels, K^+ channels, TRPM4 channels, TRPM7 channels and chloride channels contribute to the pathophysiology of asthma, allergy and inflammatory bowel disease [2, 6, 10]. According to published microarray data from *Dicer*$^{\Delta/\Delta}$ mice potassium channel K^+ two pore domain channel subfamily K (KCNK) 1, KCNK6 and Mg^{2+} permeable channel (TRPM7) are upregulated in regulatory T cells compared to *Dicer*$^{fl/fl}$ regulatory T cells [32]. Thus, these data suggested that ion channels could be dysregulated in miRNAs deficient immune T cells.

Given the important role of both Ca^{2+} signalling and miRNAs in key physiological processes, the role of miRNAs in the regulation of calcium channels in T cell differentiation and effective function was addressed. Channels accomplishing Ca^{2+} entry into lymphocytes include CRAC channels composed of the pore-forming units Orai1, 2 and 3 and their regulators STIM1 and 2, which can be activated by emptying of the intracellular Ca^{2+} stores [2, 33]. SOCE following activation of T cells is mediated by the Orai1 channel protein [2]. Therefore, we measured Orai1 expression at mRNA transcript and protein levels. We observed significantly lower mRNA expression in $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells than in $Dicer^{fl/fl}$ $CD4^+$ T cells (Fig. 1A). Similarly, measurement of Orai1 expression by flow cytometry revealed that Orai1 protein abundance was significantly lower in $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells than in $Dicer^{fl/fl}$ $CD4^+$ T cells (Fig. 1B).

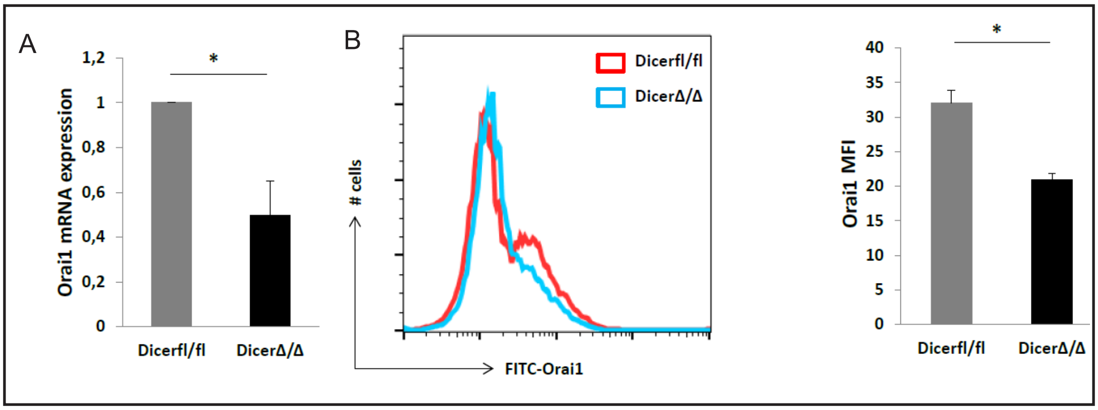

Fig. 1. $Dicer^{\Delta/\Delta}$ mice express reduced Orai1 at transcript and protein levels in $CD4^+$ T cells. A. mRNA was isolated from $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells and equal amount of mRNA was converted into cDNA. q-RT-PCR was performed for quantification of Orai1 transcript levels. B. Protein expression of Orai1 in $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells by flow cytometry. Left hand side shows the representative FACS histogram of Orai1 expression and right hand side shows arithmetic means ± SEM of n=3 independent experiments. * indicates statistical significance difference ($p<0.05$).

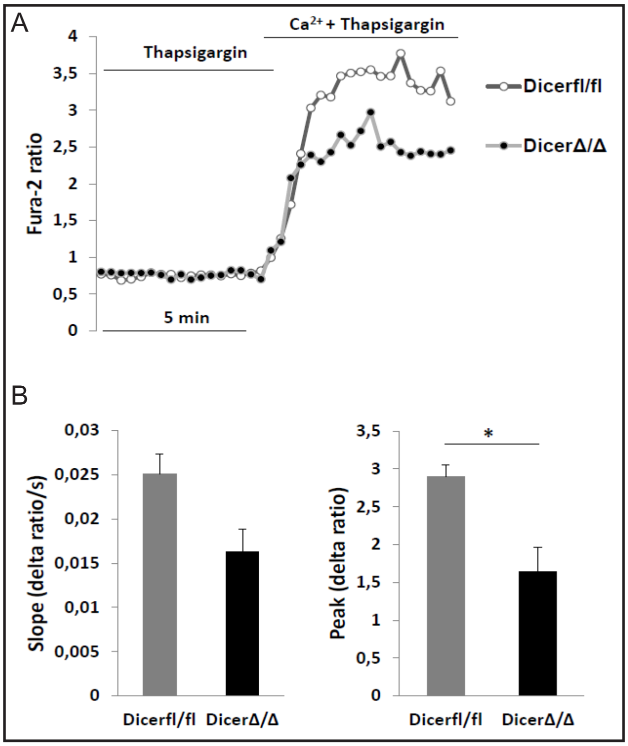

Fig. 2. $Dicer^{\Delta/\Delta}$ mice have decreased calcium influx (SOCE) in ex vivo isolated $CD4^+$ T cells . $CD4^+$ T cells were isolated from $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ mice spleen and lymph nodes and Ca^{2+} entry into $CD4^+$ T cells measured using Fura-2 fluorescence under an inverted phase-contrast microscope with the incident-light fluorescence illumination system. A. The average of 100-120 cells was used for measuring the Ca^{2+} entry. Representative tracings showing the 340/380 nm fluorescence ratio in Fura-2/AM loaded T cells from $Dicer^{\Delta/\Delta}$ and $Dicer^{fl/fl}$ mice upon removal of extracellular Ca^{2+} followed by exposure to thapsigargin (1µM) and Ca^{2+} readmission. B. Arithmetic means ± SEM of the slope (left) and peak (right) of the fluorescence ratio change between $Dicer^{\Delta/\Delta}$ and $Dicer^{fl/fl}$ $CD4^+$ T cells (n=3; independent experiments). * indicates statistical significance difference ($p<0.05$).

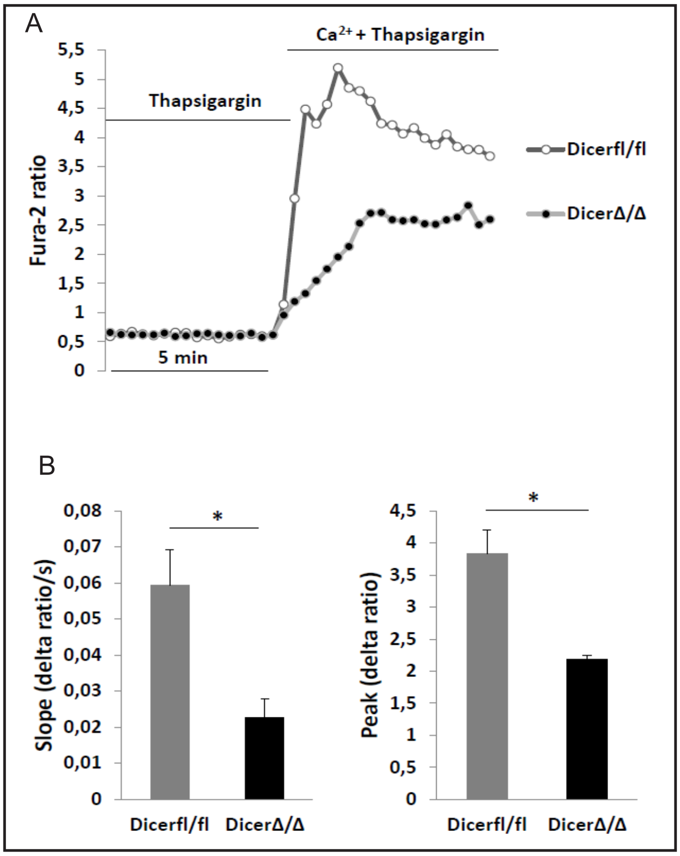

Fig. 3. SOCE is decreased in activated CD4$^+$ T cells of Dicer$^{\Delta/\Delta}$ mice. CD4$^+$ T cells were isolated from Dicer$^{fl/fl}$ and Dicer$^{\Delta/\Delta}$ mice spleen and lymph nodes and activated in the presence of anti-CD3 and anti-CD28 (plate bound) and Ca^{2+} entry into CD4$^+$ T cells measured using Fura-2 fluorescence. A. Representative tracings showing the 340/380 nm fluorescence ratio in Fura-2/AM loaded activated CD4$^+$ T cells from Dicer$^{\Delta/\Delta}$ and Dicer$^{fl/fl}$ mice upon removal of extracellular Ca^{2+} followed by exposure to thapsigargin (1µM) and Ca^{2+} readmission. B. Arithmetic means ± SEM of the slope (left) and peak (right) of the fluorescence ratio change between Dicer$^{\Delta/\Delta}$ and Dicer$^{fl/fl}$ CD4$^+$ T cells (n=3; independent experiments). * indicates statistical significance difference (p<0.05).

Ca^{2+} entry in ex vivo isolated Dicer$^{fl/fl}$ and Dicer$^{\Delta/\Delta}$ CD4$^+$ T cells

We explored whether SOCE into *ex vivo* CD4$^+$ T cells is modified by Dicer deficiency [3, 34]. Thus, we measured the intracellular Ca^{2+} activity ([Ca^{2+}]i) and SOCE from *Dicer*$^{fl/fl}$and *Dicer*$^{\Delta/\Delta}$ CD4$^+$ T cells using the Fura-2 AM dye. *Ex vivo* isolated CD4$^+$ T cells from both *Dicer*$^{fl/fl}$ and *Dicer*$^{\Delta/\Delta}$ mice were loaded with Fura-2 for 30 minutes in standard HEPES buffer containing Ca^{2+} and washed once with standard HEPES buffer containing Ca^{2+} and then with Ca^{2+} free HEPES buffer. Ca^{2+} stores were then depleted by addition of sarco-/endoplasmic reticulum Ca^{2+} ATPase (SERCA) inhibitor thapsigargin (1µM) in the nominal absence of extracellular Ca^{2+}. The intracellular Ca^{2+} ([Ca^{2+}]i) manoeuver was similar in between *Dicer*$^{fl/fl}$ and *Dicer*$^{\Delta/\Delta}$ CD4$^+$ T cells (Fig. 2A, B). Addition of extracellular Ca^{2+} (1µM) in the continued presence of thapsigargin triggered SOCE. Peak of SOCE was significantly lower whereas slope of SOCE was not significantly different between *Dicer*$^{\Delta/\Delta}$ and *Dicer*$^{fl/fl}$ CD4$^+$ T cells (Fig. 2A, B).

Ca^{2+} entry in activated Dicer$^{fl/fl}$ and Dicer$^{\Delta/\Delta}$ CD4$^+$ T cells

The increase of [Ca^{2+}]i plays a decisive role during the initial phase of T cell activation, particularly for the production of cytokines, cell proliferation and cell death [2, 28, 34-37]. Therefore, we also measured the SOCE from activated CD4$^+$ T cells. CD4$^+$ T cells from both *Dicer*$^{fl/fl}$ and *Dicer*$^{\Delta/\Delta}$ mice were activated for 2 days in the presence of plate-bound anti-CD3 and anti-CD28, after which Ca^{2+} entry was measured. Cells were loaded with Fura-2 for 30 minutes in standard HEPES buffer and washed once with standard HEPES buffer and further with Ca^{2+} free HEPES buffer as described in Fig.2. First, depletion of Ca^{2+} stores by the SERCA inhibitor thapsigargin (1µM) in the nominal absence of extracellular Ca^{2+} was performed. Then, [Ca^{2+}]i measurements were performed prior to and following addition of extracellular Ca^{2+} (1µM) in the continued presence of thapsigargin resulting in SOCE. Slope and peak of SOCE were significantly lower in *Dicer*$^{\Delta/\Delta}$ than in *Dicer*$^{fl/fl}$ CD4$^+$ T cells (Fig. 3A, B). In addition to this, when we compared the Ca^{2+} entry in between unactivated and activated T cells from

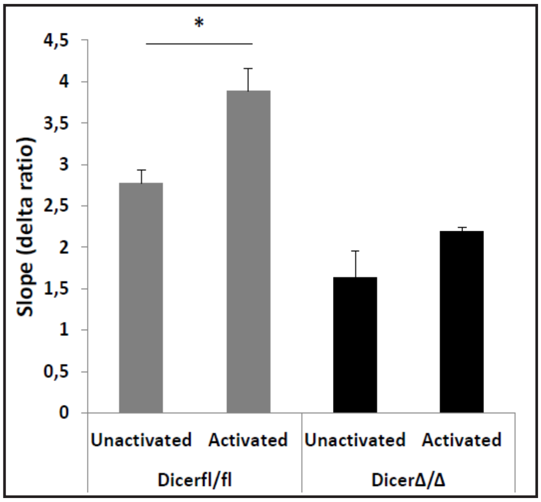

Fig. 4. Intracellular Ca^{2+} is increased after activation of T cells in $CD4^+$ T cells of $Dicer^{\Delta/\Delta}$ mice. Measurement of intracellular Ca^{2+} measurement by Fluo-2/AM dye fluorescence in unactivated and activated $Dicer^{\Delta/\Delta}$ and $Dicer^{fl/fl}$ $CD4^+$ T cells. Activated $Dicer^{fl/fl}CD4^+$ T cells have significantly higher levels of SOCE compared with unactivated cells, whereas no difference was observed in $Dicer^{\Delta/\Delta}$ T cells. * indicates statistical significance difference ($p<0.05$).

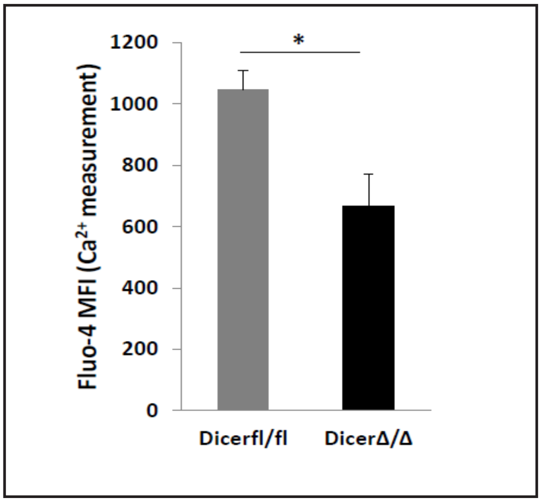

Fig. 5. Intracellular Ca^{2+} is decreased in anti-CD3 and anti-CD28 activated $CD4^+$ T cells of $Dicer^{\Delta/\Delta}$ mice. Measurement of intracellular Ca^{2+} by Fluo-4/AM dye fluorescence in activated $Dicer^{\Delta/\Delta}$ and $Dicer^{fl/fl}$ T cells. $Dicer^{\Delta/\Delta}$ T cells have significantly less intracellular Ca^{2+} in comparison with $Dicer^{fl/fl}$ T cells. * indicates statistical significance difference ($p<0.05$).

$Dicer^{\Delta/\Delta}$ and *$Dicer^{fl/fl}$* $CD4^+$ T cells. We found that *$Dicer^{fl/fl}$* $CD4^+$ T cells significantly upregulated the SOCE in activated T cells compared with unactivated T cells whereas no significant difference was observed in *$Dicer^{\Delta/\Delta}$* $CD4^+$ T cells (Fig. 4)

Ca^{2+} measurement by Fluo-4 in activated $Dicer^{fl/fl}$ and $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells

To test the effect of Dicer deletion on Ca^{2+} signalling by a second, independent method, we employed flow cytometry and measured $[Ca^{2+}]i$ by Fluo-4 dye. Similar to the experiments with fluorescence microscopy, *$Dicer^{\Delta/\Delta}$* $CD4^+$ T cells had significantly lower $[Ca^{2+}]i$ than *$Dicer^{fl/fl}$* $CD4^+$ T cells (Fig. 5).

Discussion

The present study revealed a role of miRNAs in Ca^{2+} homeostasis as a positive regulator of SOCE into T cells. SOCE was lower in the *$Dicer^{\Delta/\Delta}$* $CD4^+$ T cells compared with *$Dicer^{fl/fl}$* $CD4^+$ T cells. Orai1 expression was lower in *$Dicer^{\Delta/\Delta}$* $CD4^+$ T cells compared with *$Dicer^{fl/fl}$* $CD4^+$ T cells. Therefore, we speculate that miRNAs are required to suppress the expression of a repressor of Orai1.

Dicer is essential for maturation of miRNAs and is involved in the pathophysiology of various diseases such as cancer, infection and autoimmunity [23, 24]. In this study, we found that activation of T cells with anti-CD3 and anti-CD28 leads to enhanced Ca^{2+} entry. This was significantly reduced in $Dicer^{\Delta/\Delta}$ $CD4^+$ T cells even though most miRNAs show decreased expression in activated T cells due to the degradation of Argonaute proteins, which are the key component of the effector complex that binds the miRNA and mediate the effects on gene regulation [12, 38]. Therefore, there are presumably still sufficient levels of key compensatory miRNAs to regulate Ca^{2+} homeostasis in activated *$Dicer^{fl/fl}$* $CD4^+$ T cells.

Stimulation of T cells with anti-CD3 and anti-CD28 initiates a cascade of signalling events resulting in activation of various downstream pathways such as PI3K/mTOR, JAK/Stat etc [39]. The influence of miRNAs on SOCE and thus activation of T lymphocytes may play a role in enhancing T lymphocyte activation and function. Limiting the extent and duration of TCR signalling ensures a tightly constrained response, protecting cells from harmful effects of chronic activation [28]. MiRNAs play an additional role in T cell activation through their regulation of mTOR, which is important for regulating the strength of TCR signalling leading to activation or anergy [40]. In contrast to *Dicer*$^{fl/fl}$ $CD4^+$ T cells, *Dicer*$^{\Delta/\Delta}$ $CD4^+$ T cells fail to adequately discriminate between activating and anergy-inducing stimuli as the TCR signal can elicit full activation with effective functions or state of anergy [40]. We speculate that Ca^{2+} signalling could also play a decisive role in this process because in the absence of miRNAs there was no significant increase in SOCE upon activation in between activated and unactivated $CD4^+$ T cells from *Dicer*$^{\Delta/\Delta}$ mice. Further studies are required to find out which key miRNAs are involved in Ca^{2+} homeostasis.

Conclusion

The present observations uncovered a novel role of miRNAs in the regulation of Ca^{2+} entry into $CD4^+$ T cells. Further studies are required to define the specific miRNAs and target genes necessary for the regulation of Ca^{2+} entry into T cells. In any case, our results suggest that miRNAs are essential for the maintenance of Ca^{2+} homeostasis. Our study could have an important implication in infection, autoimmunity and cancer progression.

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Disclosure Statement

The authors of this manuscript state that they do not have any financial conflict of interests and nothing to disclose.

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