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MicroRNA-183 and microRNA-96 are associated with autoimmune responses by regulating T cell activation

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ABSTRACT

MircoRNAs (miRs) are small molecules that regulate gene expression at the posttranscriptional level. They have been proposed to be involved in the regulation of several immune responses including autoimmunity. Here, we identified miR-183 and miR-96 to be highly expressed in $CD4^+$ T cells from peripheral blood of Graves' orbitopathy (GO) patients as well as in human and murine T cells upon activation *in vitro*. By using Luciferase-based binding assays, we identified EGR-1 as target for miR-183 and miR-96. Overexpression of miR-183 and miR-96 in murine $CD4^+$ T cells by retroviral gene transfer resulted in decreased EGR-1 and PTEN expression, elevated Akt phosphorylation and enhanced proliferation. In contrast, treatment of murine $CD4^+$ T cells with specific antagomiRs increased EGR-1 and PTEN expression and interfered with the proliferative activity upon stimulation *in vitro*. Strikingly, adoptive transfer of miR-183 and miR-96 overexpressing antigen-specific T cells into INS-HA/Rag2KO mice accelerated the development of autoimmune diabetes, whereas transfer of antagomiR-treated cells delayed the disease onset. These results indicate that miR-183 and miR-96 have the ability to regulate the strength of T cell activation and thereby the development and severity of T cell-dependent autoimmune diseases.

1. Introduction

MicroRNAs (miRs) are small endogenous non-coding RNAs that regulate gene expression at the posttranscriptional level. They bind to their respective target sequences located usually within the 3′ untranslated region (UTR) of their target mRNAs resulting in mRNA degradation, destabilization or blocking of translation [1,2]. Similarly to transcription factors, miRs are supposed to be integral players of the gene expression network [3]; thereby playing a pivotal role in a wide range of cellular and developmental processes such as differentiation, proliferation and apoptosis [4]. The importance of miRs for T cell biology was demonstrated in mice deficient for the miR-processing enzyme Dicer. These mice exhibit an impaired T cell development and function [5,6]. Strikingly, deficiency of Dicer in regulatory T cells resulted in lethal autoimmune inflammatory disease [7,8]. In this regard, the identification of specific T cell miRs contributing to several pathological disorders is of great interest as potential mediators of pathology and hence putative therapeutic targets.

Changes in miR expression patterns have been described in numerous immunological disorders including autoimmune diseases such as diabetes, rheumatoid arthritis, systemic lupus erythematosus (SLE) and Graves' disease (GD) [9–15]. Type 1 diabetes (T1D) and GD, including Graves' orbitopathy (GO) or thyroid eye disease, are organ-specific autoimmune disorders affecting the endocrinological system and caused i.a. by uncontrolled T cell responses. Several specific miRs have already been identified to be associated with T1D and GD [16–19]. Among others miR-96 was shown to be up-regulated in serum samples derived from GD patients and was correlated with higher severity of disease [19]. MiR-96 is clustered with miR-182 and miR-183 and likely be generated from the same transcript [20]. Well in line, Qin and colleagues demonstrated elevated miR-183 expression in thyroid tissues from GD patients in comparison to controls [17]. However, the expression pattern of miR-183 and miR-96 in $CD4^+$ T cells from peripheral blood of GO patients and its impact on T cell function during acute autoimmunity still remain elusive.

Here, we demonstrate enhanced expression of miR-183 and miR-96 in both $CD4^+$ T cells from GO patients and activated T cells from human and mice. Functional analysis of T cells overexpressing miR-183

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and miR-96 or treated with respective antagomiRs revealed that both miRs are involved in T cell proliferation by targeting the transcription factor early growth response protein 1 (EGR-1) which in turn down-regulates the phosphatase and tensin homolog (PTEN) resulting in increased protein kinase B (Akt) phosphorylation. Strikingly, over-expression of miR-183 and miR-96 in antigen-specific T cells accelerated the development of autoimmune diabetes whereas transfer of T cells with blocked miR expression delayed the disease onset.

2. Material and methods

2.1. Patients

Blood samples derived from patients suffering from Graves' orbitopathy (GO) and age- and sex-matched healthy donors. GO activity and severity was classified according to the actual guidelines [21]. All patients gave their written informed consent. The study was approved by the medical ethical committee of the University Hospital Essen, Germany.

2.2. Mice

TCR-HA/Thy.1.1 transgenic mice express a αβTCR specific for the peptide 110–120 from influenza hemagglutinin (HA) presented by I-Ed [22] and were backcrossed to Thy1.1 mice. INS-HA/Rag2KO transgenic mice express the HA protein under control of the insulin promoter [23] and are deficient for Rag2 expression. BALB/c mice were obtained from Envigo (Rossdorf, Germany). Mice were housed and bred in the animal facility at the University Hospital Essen under specific pathogen-free conditions. All animal experiments were carried out in accordance with the guidelines of the German Animal Protection Law and the state authority for nature, environment and customer protection, North Rhine-Westphalia, Germany. The protocol was approved by the state authority for nature, environment and customer protection, North Rhine-Westphalia, Germany.

2.3. Cell isolation and stimulation

Murine $CD8^+$ and $CD4^+CD25^-$ T cells were isolated from erythrocyte depleted splenocytes by using the $CD8^+$ or $CD4^+$ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany) according to the manufacturer's recommendation and by addition of biotinylated anti-CD25 antibody (BD Biosciences, Heidelberg, Germany). Peripheral blood mononuclear cells (PBMCs) were isolated from blood of healthy controls or patients suffering from GO by density gradient centrifugation using Bicoll (Biochrom, Berlin, Germany). Afterwards human $CD4^+$ and $CD8^+$ T cells were purified from PBMCs by using the $CD4^+$ or $CD8^+$ T cell isolation kit (Miltenyi Biotec, Bergisch-Gladbach, Germany). For retroviral transduction and miRNA-183 and miRNA-96 expression analysis cells were activated with 0.75 μg/ml anti-CD3 plate-bound and 1 μg/ml anti-CD28 soluble.

2.4. Retroviral vectors and transduction

A 671 bp fragment encoding the mature miR-183 and miR-96 sequences was amplified from genomic DNA isolated from BALB/c mice using specific primers including *Bgl*II and *Xho*I restriction sites (5′- AGA TCT TGC CGG GGG AGG TGA ACG T – 3′ and 5′- CTC GAG CTG CCC ACT TGG GAG TAG GTG AGG – 3′), cloned into the TOPO2.1 vector (Invitrogen, Darmstadt, Germany), sequenced and inserted into a murine stem cell virus-based (MCSV) retroviral vector encoding eGFP under control of an internal ribosomal entry site (RV-eGFP). Alternatively, the 671bp fragment encoding mature miR-183 with six point mutations within the seed sequence (Fig. 2B) together with the mature miR-96 was synthesized by Eurofins Genomics (Ebersberg, Germany) and inserted into RV-eGFP. These retroviral vector constructs (RV-miR-183/96 and RV-miR-183mut/96) or the empty control vector (RV-eGFP) was used to stably transfect the ectopic packaging cell line GPE86+ [24]. Concentrated and filtrated retrovirus-containing cell culture supernatants supplemented with 20 mM Hepes and 8 μg/ml Polybrene were used to transduce stimulated $CD4^+CD25^-$ T cells by centrifugation at 500 × g for 2 h. Subsequently 50 U/ml IL-2 was added and cells were cultivated at 37 °C.

2.5. AntagomiR treatment

The protocol for antagomiR transfection of T cells was adapted from Haftmann and colleagues [25]. Briefly, $CD4^+CD25^-$ T cells were washed with ice cold PBS, resuspended in 0.25 vol of the final culture volume serum-free medium and incubated for 90 min with 1 μM antagomiRs against miR-183, mir-96 or miRIDIAN microRNA Hairpin Inhibitor Negative Control #1 as scrambled control (Dharmacon, Lafayette, CO, USA). Transfected cells were stimulated with 0.75 μg/ml anti-CD3 plate-bound and 1 μg/ml anti-CD28 soluble in serum-containing culture medium.

2.6. Proliferation

Four days after retroviral transduction $eGFP^+$ RV-miR-183/96, RV-miR-183mut/96 and RV-eGFP transduced T cells were sorted by using the ARIA II cell sorter (BD Biosciences, Heidelberg, Germany). AntagomiR-treated cells were used three days after initial activation. 1×10^5 cells were stained with the cell proliferation dye eFlour670 (eBioscience, Frankfurt, Germany) according to the manufacturer's recommendations and stimulated with 1 μg/ml anti-CD3 in the presence of 4×10^5 $CD4^+$ T cell-depleted irradiated splenocytes for 72 h. Proliferation was assessed as loss of the proliferation dye by flow cytometry (LSR II, BD Bioscience, Heidelberg, Germany).

2.7. Flow cytometry

Flow cytometric expression analysis of surface proteins was performed by using anti-CD4, anti-CD25, and anti-CD90.1 (Thy1.1) antibodies (all BD Biosciences, Heidelberg, Germany). Antibodies were used as Allophycocyanin (APC), AlexaFlour647, Brillant Violet (BV421), Fluorescinisocyanate (FITC), Pacific Blue (PB), Phycoerythrin (PE) or Phycoerythrin Cyanine 7 (PE-Cy7) conjugates. The anti-6.5 (anti-TCR-HA) monoclonal antibody was purified from hybridoma supernatant and labeled with AlexaFluor647. Intracellular Akt and Akt-P staining of sorted and activated (1 μg/ml anti-CD3; 30 min at 37 °C) $eGFP^+$ retroviral transduced T cells was performed by using the Cytoperm/Cytofix kit (BD Biosciences, Heidelberg, Germany) according to the manufacturer's recommendations and anti-Akt, anti-Akt-P-S473 and anti-Akt-P-T308 antibodies (all Cell Signaling Technology, Leiden, Netherlands). For EGR-1 expression analysis cells were resuspended in 2% formaldehyde, incubated for 1 h at 4 °C, washed with permeabilization buffer (eBioscience, Frankfurt, Germany), stained with unconjugated anti-EGR-1 antibody prior to incubation with BV421-labeled anti-rabbit IgG (eBioscience, London, UK). Intracellular staining of PTEN was performed by using the Cell signaling buffer set A and APC-conjugated anti-PTEN antibody (Miltenyi Biotech, Bergisch-Gladbach, Germany) according to the manufacturer's recommendation. For AnnexinV staining the Annexin-V-PE Detection kit (BD Bioscience, Heidelberg, Germany) was used according to the manufacturer's recommendation. Flow cytometric analyses were done on a LSRII with Diva or FlowJo Software (BD Biosciences, Heidelberg, Germany; Flow Jo LLC, Ashland, OR).

2.8. Luciferase assay

GPE86 + cells were stably transfected with the miR-183/-miR96, miR-183mut/96 encoding retroviral vector or the control vector (RV-

eGFP). Thereafter, cells were transfected with a plasmid encoding Renilla-luciferase and the target sequence of miR-183 (pLightSwitch_3UTR183) or miR-96 (pLightSwitch_3UTR96) in addition to a plasmid encoding Firefly-luciferase for normalization (all Switch Gear Genomics, La Hulpe, Belgium). For analyzing EGR-1 3′UTR as miRNA target the Luc-Pair Luciferase Assay (Gene Copoeia, BioCat, Heidelberg, Germany) was used. MiR-183/96, miR-183mut/96 or control vector transduced GPE86 + cells were transfected with the EGR-1 mouse miTarget expression vector (pEZX-MT01-EGR-1UTR-fLUC) or control miTarget expression vector (both Gene Copoeia, BioCat, Heidelberg, Germany). At day 1 post transfection cells were lysed and luciferase activity was analyzed in the presence of Firefly- and Renilla-luciferase substrates by using Orion II (Berthold Detection Systems, Pforzheim, Germany). The ratio of luminescence from the Firefly-luciferase to the Renilla-luciferase was calculated as the luciferase activity.

2.9. Quantitative RT-PCR

Total RNA was isolated by using the RNeasy Mini kit followed by using the RNeasy MinElute Cleanup kit (Qiagen, Hilden, Germany) to obtain the miRNA containing fraction according to the manufacturer's recommendations. MiR was reverse transcribed by using the miScript Reverse Transcription kit (Qiagen, Hilden, Germany) and miR expression analysis was performed with the miScript SYBR Green PCR kit and the miScript Primer Assays (Qiagen, Hilden, Germany) with RNU6B as housekeeping control according to the manufacturer's recommendations.

2.10. Adoptive transfer

$CD4^+CD25^-$ T cells isolated from TCR-HA/Thy1.1 mice were transduced with either RV-miR-183/96, RV-miR-183mut/96 or RV-eGFP retroviral vectors as control. Two days after transduction $CD4^+6.5^+eGFP^+$ cells were separated by cell sorting. A total of 1×10^5 sorted cells were adoptively transferred i.v. to INS-HA/Rag2KO mice. Alternatively, sorted $CD4^+CD25^-6.5^+$ T cells from TCR-HA/Thy1.1 mice were transfected with 1 μM fluorescein-labeled antagomiR-183/antagomiR-96 or antagomiR-scrambled and stimulated with 0.75 μg/ml anti-CD3 plate-bound and 1 μg/ml anti-CD28 soluble for 72 h. Cells equivalent to 1×10^5 $fluorescein^+$ cells were adoptively transferred i.v. to INS-HA/Rag2KO mice. Blood glucose concentrations were monitored using the StatStripXpress-i (Nova Biochemical, Runcorn, Cheshire, UK). Mice were considered diabetic when glycaemia was > 200 mg/dl.

2.11. Statistical analysis

Statistical analyses were performed with One-Way ANOVA or Mann-Whitney test with significance set as levels of * = $p < 0.05$, ** = $p < 0.01$, and *** = $p < 0.001$. All analyses were calculated with Graph Pad Prism software (Graph Pad software, La Jolla, CA).

3. Results

3.1. Elevated miR-183 and miR-96 expression in $CD4^+$ T cells from peripheral blood of patients suffering from Graves' orbitopathy and in activated T cells from healthy controls

First, we investigated the expression pattern of miR-183 and miR-96 in $CD4^+$ T cells from GO patients and age- and sex-matched healthy donors by quantitative RT-PCR. As shown in Fig. 1, both miR-183 (Fig. 1A) and miR-96 (Fig. 1B) are significantly upregulated in T cells from diseased patients compared to healthy controls. It is well established that autoimmunity is associated with uncontrolled activation of T cells. Therefore, we next asked whether *in vitro* activated T cells also show elevated miR-183 and miR-96 expression. We isolated $CD4^+$ as well as $CD8^+$ T cells from healthy donors, left them either untreated or stimulated with anti-CD3/anti-CD28 and performed quantitative RT-PCR analysis with specific primers to examine the miR-183 and miR-96 expression profile. We detected significantly enhanced miR-183 and miR-96 expression in $CD4^+$ T cells (Fig. 1C and D) as well as in $CD8^+$ T cells (Fig. 1E and F) upon activation for four days in comparison to unstimulated cells.

3.2. Upregulation of miR-183 and miR-96 expression in activated murine T cells

To evaluate whether miR-183 and miR-96 are also upregulated in murine T cells upon activation, we isolated $CD4^+CD25^-$ and $CD8^+$ T cells from splenocytes of naïve BALB/c mice, left the cells unstimulated or stimulated them with anti-CD3/anti-CD28 for indicated time points and analyzed the miR-183 and miR-96 expression profile. As depicted in Fig. 2, $CD4^+CD25^-$ T cells (Fig. 2A and B) as well as $CD8^+$ T cells (Fig. 2C and D) significantly upregulated miR-183 and miR-96 expression upon stimulation *in vitro*, comparable to the results we obtained from expression analysis of human T cells (Fig. 1C–F).

3.3. Overexpression of biological active miR-183 and miR-96 in $CD4^+CD25^-$ T cells by retroviral gene transfer

To gain further insights into the role of miR-183 and miR-96, in particular during T cell activation, we overexpressed the pri-miR-183 and pri-miR-96 by retroviral gene transfer. For this purpose we cloned a 671bp genomic DNA fragment containing about 300 bp upstream and downstream of the miR-183 seed sequence into a retroviral vector that additionally encodes for eGFP as marker molecule under control of an internal ribosomal entry side (RV-miR-183/96, Fig. 3A). This construct also encodes for the miR-96 seed sequence which is located 171 bp downstream of the miR-183 seed sequence. To define effects derived from either miR-183 or miR-96 overexpression, we cloned an additional retroviral vector containing again the 671 bp fragment, but with six point mutations within the miR-183 seed sequence (RV-miR-183mut/96, Fig. 3B). Transduction of $CD4^+CD25^-$ T cells with RV-miR-183/-96 resulted in a threefold higher miR-183 (Fig. 3C) and miR-96 (Fig. 3D) expression than transduction with the control vector RV-eGFP. In contrast, RV-miR-183mut/-96 transduced $CD4^+$ T cells did not exhibit upregulation of miR-183 expression as expected, but showed a sixfold increase in miR-96 expression as compared to controls (Fig. 3C and D). To verify that miR-183 and miR-96 are correctly processed from our constructs, we analyzed their biological activity. For this purpose, we transfected GPE86 + cells with plasmids encoding for luciferase either fused to the target 3′UTR of miR-183 or of miR-96 together with the retroviral vectors RV-miR183/96, RV-miR-183mut/96 or RV-eGFP as control and measured the luciferase activity. As depicted in Fig. 3E, miR-183/96 but not miR-183mut/96 overexpression significantly interfered with luciferase activity in cells co-transfected with the miR-183 target sequence. In contrast, we detected a significantly reduced luciferase activity in both miR-183/96 as well as miR-183mut/96 transfected cells (Fig. 3F). These results demonstrate that our constructs are suitable to express and properly process either miR-183 and miR-96 or miR-96 alone.

3.4. Targeting of EGR-1 by miR-183 and miR-96 results in modulated PTEN expression, Akt phosphorylation and proliferation

The transcription factor EGR-1 has been described as a target for miR-183, at least in tumor cells [26]. To analyze whether this is also true in murine T cells, we transduced $CD4^+CD25^-$ T cells with retroviral vectors encoding for miR-183/96, miR-183mut/96 or eGFP as control and analyzed the EGR-1 expression by flow cytometry. Interestingly, both miR-183 and also miR-96 overexpression interfered with

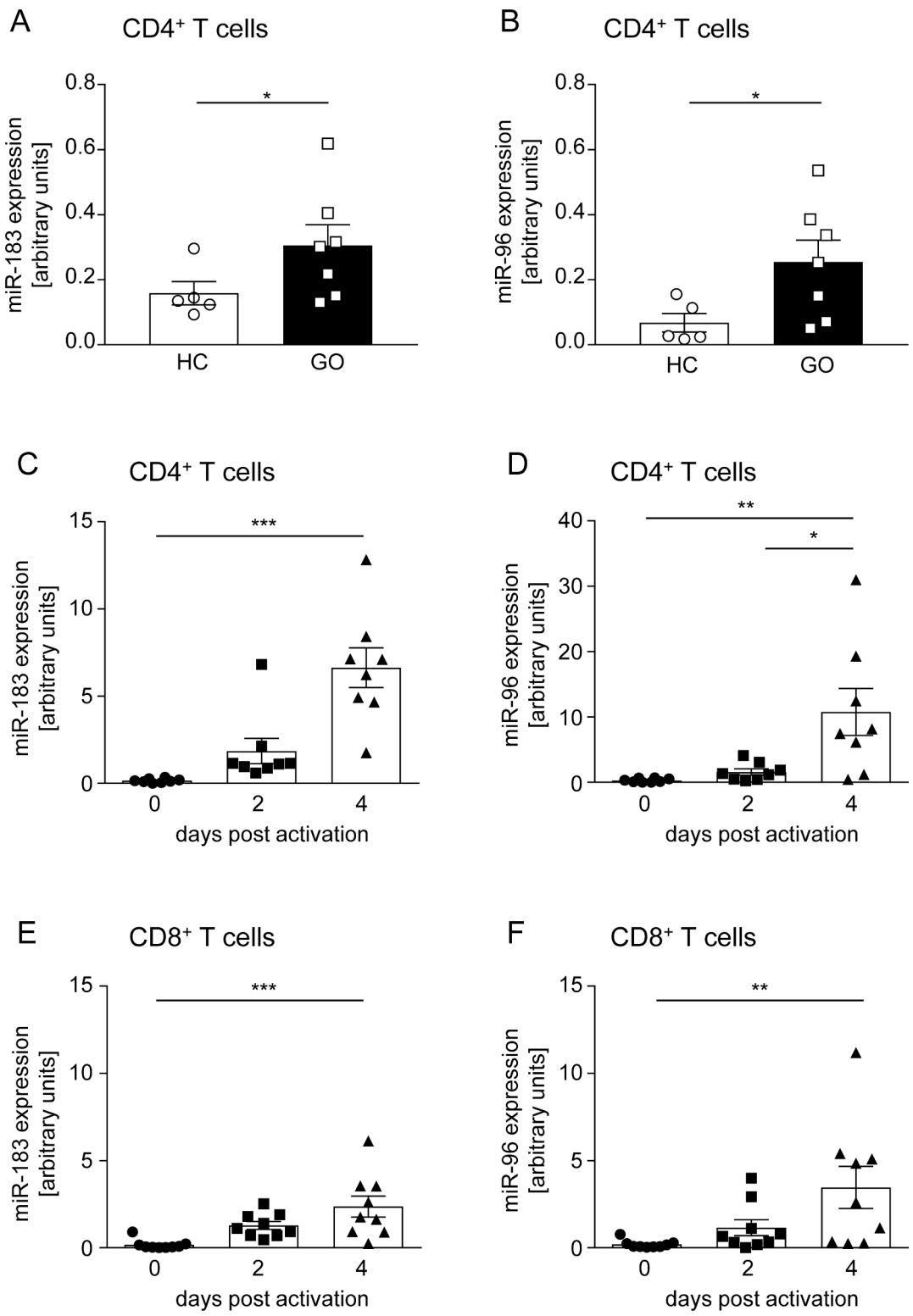

Fig. 1. T cells from GO patients and *in vitro* stimulated T cells from healthy controls exhibit enhanced miR-183 and miR-96 expression. (A, B) Untreated $CD4^+$ T cells from peripheral blood of GO patients (n = 7) and healthy controls (n = 5) as well as *in vitro* stimulated (C, D) $CD4^+$ T cells and (E, F) $CD8^+$ T cells from healthy donors (n = 8–9) were analyzed for (A, C, E) miR-183 and (B, D, F) miR-96 expression by qRT-PCR. Mann-Whitney test (A, B) or One-Way ANOVA with Dunett's multiple comparisons test was used for statistical analysis. *p > 0.05, **p > 0.01, ***p < 0.001.

EGR-1 expression in T cells (Fig. 4A). To confirm that the EGR-1 mRNA is indeed a target for both miRs, we transfected RV-miR183/96, RV-miR183mut/96 or RV-eGFP control cells with a plasmid encoding for luciferase fused to the 3′UTR of EGR-1. The luciferase activity was significantly reduced in miR-183/96 as well as miR-183mut/96 overexpressing cells (Fig. 4B), confirming that both miRs efficiently target EGR-1. EGR-1 has been supposed to directly activate PTEN [27,28], and thereby affecting the Akt signaling pathway [29]. Flow cytometric expression analysis revealed that downregulation of EGR-1 in miR-183/96 and in miR-183mut/96 transduced murine $CD4^+CD25^-$

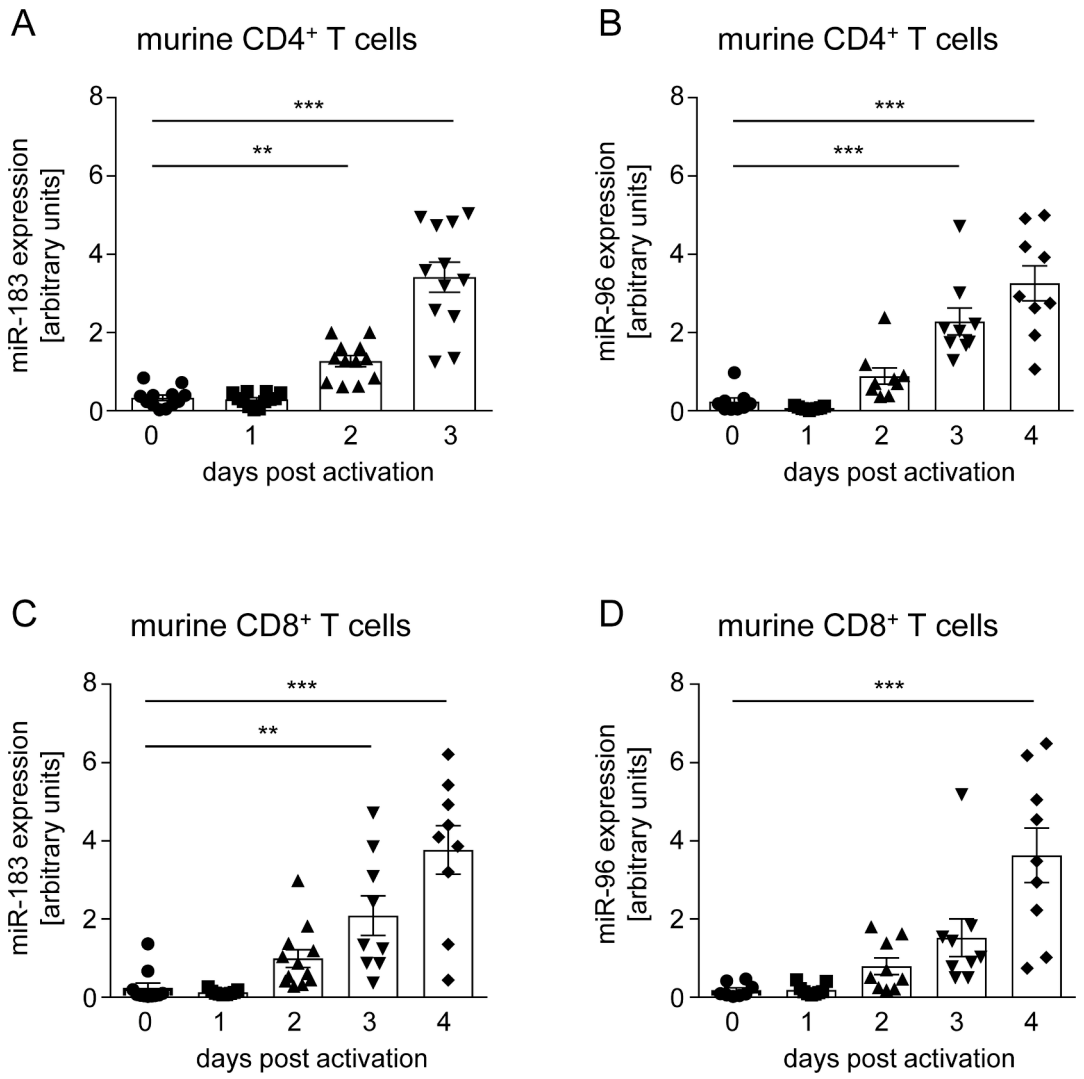

Fig. 2. Elevated miR-183 and miR-96 expression in activated murine CD4$^+$CD25$^-$ and CD8$^+$ T cells. (A, B) CD4$^+$CD25$^-$ and CD8$^+$ (C, D) T cells were isolated from BALB/c mice left un-stimulated or activated for indicated time points with anti-CD3 and anti-CD28. miRs were isolated, reverse transcribed and analyzed for (A, C) miR-183 expression, (B, D) miR-96 expression and RNU6B as housekeeping control by qRT-PCR. Results from three independent experiments with n = 9–11 were summarized as mean ± SEM. One-Way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis. **p < 0.01, ***p < 0.001.

T cells was accompanied by significantly reduced PTEN expression and elevated Akt phosphorylation as compared to control vector transduced T cells (Fig. 4C and D). Based on these results, we wondered whether the proliferative activity of CD4$^+$CD25$^-$ T cells is also modulated by overexpression of either miR. Therefore, we transduced primary murine CD4$^+$CD25$^-$ T cells with miR-183/96, miR-183mut/96 or control retroviral vectors, isolated eGFP$^+$ cells by cell sorting, stained them with an eFlour670-labeled proliferation dye and re-stimulated the cells for three days with anti-CD3 in the presence of irradiated splenocytes. As shown in Fig. 4E, overexpression of miR183 as well as miR-96 resulted in significantly enhanced proliferative activity (Fig. 4E). These findings indicate that miR-183 and miR-96 regulate molecules involved in the activation process of CD4$^+$ T cells *in vitro*.

3.5. Adoptive transfer of antigen-specific miR-183 and miR-96 transduced CD4$^+$ T cells accelerate the development of autoimmune diabetes in vivo

We next asked whether miR-183/96 overexpression not only modulates T cell activation *in vitro*, but has also an impact on the development of autoimmunity *in vivo*. To our knowledge transgenic mice with T cell-specific miR-183/96 overexpression or deficiency are not available yet. Thus, detailed analysis on the impact of miR-183 and miR-96 upregulation in T cells, as observed in EO patients (Fig. 1A and B), are difficult in EO mouse models which are based on immunization of immunocompetent mice [30,31]. Therefore, we used the well-established T cell transfer INS-HA/Rag2KO mouse model of acute autoimmune diabetes allowing for T cell-specific modulation of miR expression. Adoptive transfer of hemagglutinin (HA)-specific CD4$^+$ T cells (6.5$^+$) isolated from TCR-HA transgenic mice elicits autoimmune diabetes in INS-HA/Rag2KO mice, expressing the HA model antigen under control of the rat-insulin promotor specifically in the beta cells of the pancreas [32]. We transduced CD4$^+$CD25$^-$ T cells isolated from Thy1.1$^+$TCR-HA mice with miR-183/96, miR-183mut/96 or eGFP encoding retroviral vectors. Two days after transduction CD4$^+$6.5$^+$eGFP$^+$ T cells were separated by cell sorting and adoptively transferred to INS-HA/Rag2KO mice. Blood glucose levels were measured continuously as sign for diabetes development. Mice that received miR-183/96 or miR-183mut/96 overexpressing T cells developed diabetes at day 8 post transfer, whereas mice adoptively transferred with control cells (RV-eGFP) had blood glucose concentration below 200 mg/dl at least until day 10 post transfer (Fig. 5A). Blood glucose levels of INS-HA/Rag2KO mice were significantly increased at day 10 after transfer of RV-miR-183/96 or RV-miR-183mut/96 transduced T cells (Fig. 5B), accompanied by significantly elevated frequencies of

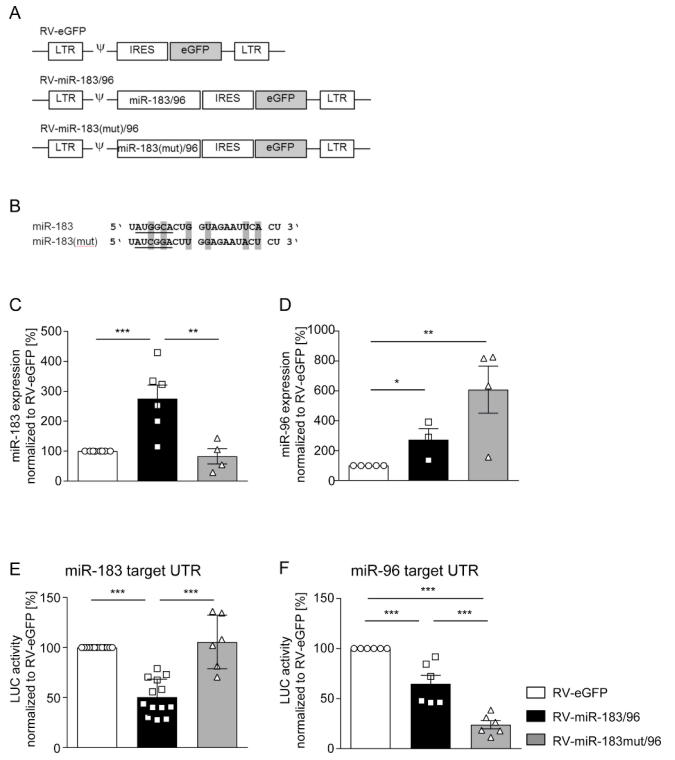

Fig. 3. Overexpression of biological active miRs in CD4$^+$ T cells by retroviral gene transfer. (A) Schematic drawing of retroviral vector constructs RV-eGFP, RV-miR-183/96 and RV-miR-183mut/96 containing long terminal repeats (LTR), the packaging signal ψ, an internal ribosomal entry side (IRES) and enhanced GFP (eGFP). RV-miR-183/-96 contains a genomic DNA fragment compromising the mature miR-183 and miR-96 sequence with additional upstream and downstream sequences. (B) The miR-183 seed sequence was point-mutated at six positions in RV-miR-183mut/-96. (C) miR-183 and (D) miR-96 expression of sorted CD4$^+$eGFP$^+$ cells transduced with RV-miR-183/96, RV-miR-183mut/96 and control vector (RV-eGFP) was analyzed by qRT-PCR. (E) Luciferase activity was determined in lysed RV-miR-183/96, RV-miR-183mut/96 and control vector (RV-eGFP) transduced GPE86 + cells 24 h after transfection with a plasmid encoding for luciferase fused to a 3′UTR containing the miR-183 target sequence or (F) miR-96 target sequence, respectively. Data from three to six independent experiments are summarized as mean ± SEM. One-way ANOVA followed by Bonferroni's post-hoc comparisons test was used for statistical analysis. *p < 0.05, **p < 0.01, ***p < 0.001.

CD4$^+$Thy1.1$^+$ T cells (Fig. 5C). From these results we conclude that overexpression of miR-183 and miR-96 contributes to the expansion of antigen-specific CD4$^+$ T cells, which in turn promotes the development of autoimmune diabetes *in vivo*.

3.6. MiR-183 and miR-96 antagomiR treatment results in elevated EGR-1 and PTEN expression and interferes with proliferation of CD4$^+$ T cells

To further dissect the impact of miR-183 and miR-96 on T cell function, we treated CD4$^+$CD25$^-$ T cells with 1 μM of specific antagomiRs prior to stimulation with anti-CD3 for 72 h. As expected, transfection of CD4$^+$CD25$^-$ T cells with a specific antagomiR against miR-183 and against miR-183 and miR-96 resulted in a significant reduction of miR-183 (Fig. 6A), whereas treatment with a specific antagomiR against miR-96 alone or in combination with an antagomiR against miR-183 downregulated miR-96 (Fig. 6B). Thus, antagomiR transfection is a suitable method to downregulate specific miRs. Our overexpression experiments revealed that EGR-1 and PTEN expression are regulated by miR-183 and miR-96 (Fig. 4B and C). Therefore, we treated freshly isolated CD4$^+$CD25$^-$ T cells with an antagomiR against miR-183, miR-96 or both, or left cells untreated as negative control and

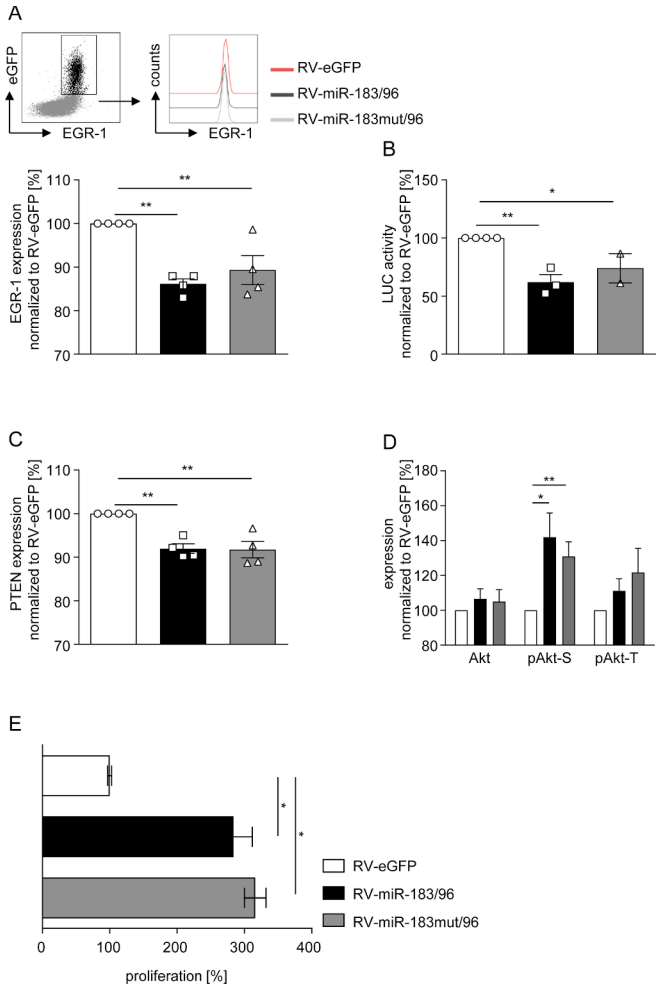

Fig. 4. MiR-183 and miR-96 target EGR-1 resulting in decreased EGR-1 and PTEN expression, elevated Akt phosphorylation and proliferation. (A) Murine CD4$^+$CD25$^-$ T cells were transduced with RV-miR-183/96, RV-miR-183mut/96 or control vector and CD4$^+$eGFP$^+$ cells were analyzed for EGR-1 expression by flow cytometry. (B) GPE86 + cells were co-transfected with RV-eGFP, RV-miR-183/96 or RV-miR-183mut/96 and a plasmid encoding for luciferase fused to the 3′UTR of EGR-1. Luciferase activity was determined 24 h post transfection. (C) PTEN and (D) Akt expression and phosphorylation of CD4$^+$eGFP$^+$ gated RV-miR-183/96, RV-miR-183mut/96 or control vector transduced CD4$^+$CD25$^-$ T cells were determined by flow cytometry two days after transduction. (E) CD4$^+$CD25$^-$ T cells were transduced with RV-miR-183/96, RV-miR-183mut/96 or RV-eGFP retroviral vectors. After 4 days, eGFP$^+$ cell were isolated by cell sorting, labeled with the eFlour670 proliferation dye and stimulated with anti-CD3 in the presence of CD4$^+$ T cell-depleted irradiated splenocytes for 72 h. Proliferation was assessed as loss of eFluor670 dye by flow cytometry. Proliferation of RV-miR-183/96 and RV-miR-183mut/96 transduced cells was normalized to control vector transduced T cells (set as 100%). Results from two to eight independent experiments are summarized as mean ± SEM. One-Way ANOVA with Bonferroni's post-hoc comparisons test was used for statistical analysis. *p < 0.05, **p > 0.01, ***p < 0.001.

analyzed the expression of EGR-1 and PTEN. As shown in Fig. 6, downregulation of miR-183 and miR-96 resulted in an increased protein expression of EGR-1 (Fig. 6C) and also PTEN (Fig. 6D). In particular, cells treated with both miR-183 and miR-96 antagomiRs exhibited significant higher EGR-1 and PTEN expression than controls (Fig. 6C and D). Additionally, we detected significantly impaired proliferative activity of cells transfected with either antagomiR-183 or antagomiR-96 alone or in combination upon re-stimulation *in vitro* (Fig. 6E).

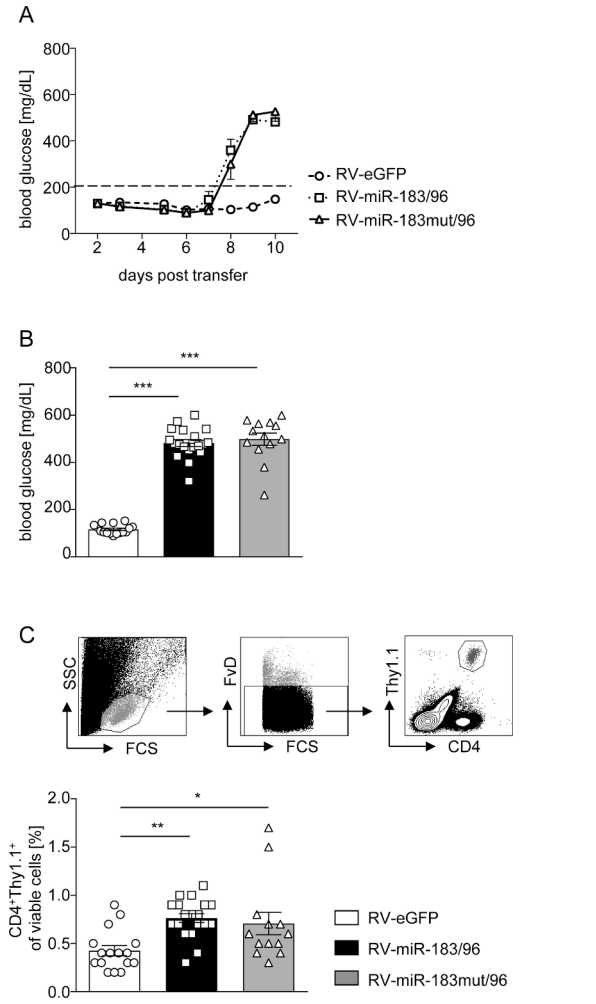

Fig. 5. Overexpression of miR-183 and miR-96 in adoptively transferred CD4$^+$CD25$^-$ T cells accelerates the development of autoimmune diabetes *in vivo*. Sorted eGFP$^+$6.5$^+$ RV-miR-183/96, RV-miR-183mut/96 and control vector transduced CD4$^+$CD25$^-$Thy1.1$^+$ T cells were adoptively transferred to INS-HA/Rag2KO mice. (A) The development of diabetes was monitored by blood glucose level at indicated time points. Mice with blood glucose > 200 mg/dl were considered as diabetic. (B) Endpoint blood glucose levels at day 10 post cell transfer. (C) The frequency of CD4$^+$Thy1.1$^+$ T cells within the spleen was determined by flow cytometry. Data from five independent experiments with n = 13–19 mice are summarized as mean ± SEM. One-Way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis. *p < 0.05, **p > 0.01, ***p < 0.001.

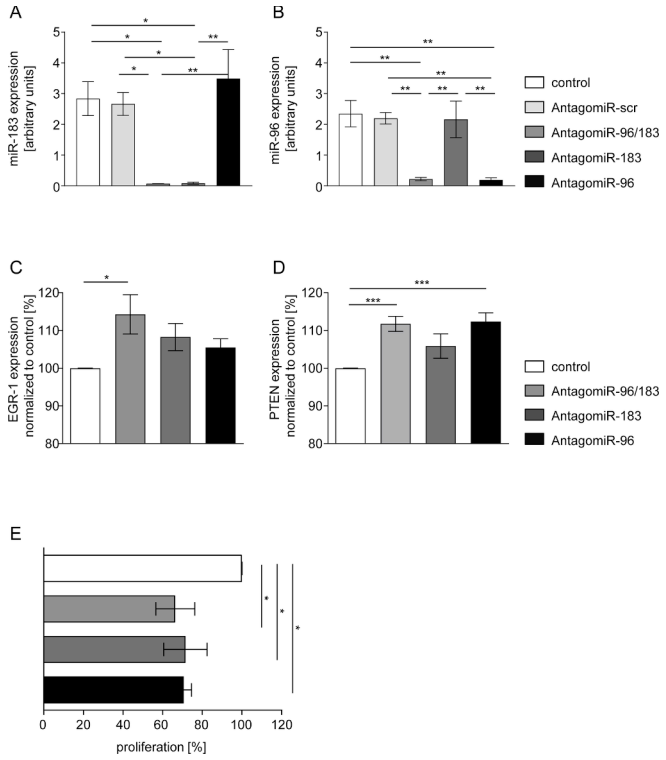

Fig. 6. Downregulation of miR-183 and miR-96 in murine CD4$^+$CD25$^-$ T cells increases EGR-1 and PTEN expression and interferes with their proliferative activity. CD4$^+$CD25$^-$ T cells were transfected with antagomir-183, antagomiR-96, both, antagomir-183 and antagomiR-96, antagomiR-scr control or without antagomiR (control) prior to stimulation with anti-CD3 and anti-CD28 for 72 h (A) miR-183 and (B) miR-96 expression were analyzed by qRT-PCR. (C) EGR-1 and (D) PTEN expression was determined in antagomiR-treated cells by flow cytometry. (E) AntagomiR-treated cells were labeled with the eFluor670 cell proliferation dye and stimulated with anti-CD3 in the presence of CD4$^+$ T cell-depleted irradiated splenocytes for 72 h. Proliferation was assessed as loss of eFluor670 dye by flow cytometry and normalized to control (set as 100%). Data from two to six independent experiments are summarized as mean ± SEM. One-Way ANOVA with Dunnett's multiple comparisons test or Bonferroni's post-hoc comparisons test was used for statistical analysis. *p < 0.05, **p > 0.01, ***p < 0.001.

3.7. Downregulation of miR-183 and miR-96 in antigen-specific CD4$^+$ T cells delays the development of autoimmune diabetes in vivo

Next, we aimed to analyze whether downregulation of miR-183 and miR-96 in CD4$^+$CD25$^-$ T cells interferes with the ability to induce autoimmune diabetes *in vivo*. For this purpose, we isolated CD4$^+$CD25$^-$ T cells from TCR-HA/Thy1.1 mice, transfected them with fluorescein-labeled antagomiRs against miR-183 and miR-96, with fluorescein-labeled scrambled antagomiR or left the cells untreated as controls. After stimulation for three days, we sorted fluorescein$^+$CD4$^+$6.5$^+$ HA-specific T cells and adoptively transferred them into INS-HA/Rag2KO mice. Whereas control animals developed diabetes at day 9–10 after transfer, mice that received cells with downregulated miR-183 and miR-96 remained healthy at least until day 14 (Fig. 7A). Accordingly, blood glucose levels (Fig. 7B) and frequencies of CD4$^+$Thy1.1$^+$ cells (Fig. 7C) were significantly reduced in mice adoptively transferred with T cells transfected with antagomiRs against miR-183 and miR-96 than of mice that received control cells at day 14 post transfer. Staining with AnnexinV showed no differences between adoptively transferred T cells transfected with antagomiRs against miR-183 and miR-96, scrambled antagomiRs or controls (Fig. 7D). Hence, we exclude that increased apoptosis of antagomiR-treated HA-specific T cells as reason for reduced percentages of CD4$^+$Thy1.1$^+$ cells. In summary, these results indicate that miR-183 and miR-96 expression modulates T cell activation and has thereby an impact on the development of T cell-dependent autoimmune disease *in vivo*.

4. Discussion

MiRs are involved in the regulation of a broad variety of cellular processes and are important for the normal function of the immune system, including immune tolerance mechanisms and autoimmunity [33–35]. Aberrant miR expression patterns were detected in patients or mice suffering from several autoimmune disorders such as T1D, SLE and GD. Among others, miR-96 and miR-183 were described to be dysregulated in serum, different tissues and cell types from diseased patients. Qin and colleagues detected elevated expression of miR-183 in thyroid tissue from GD patients [17] and miR-96 was described to be upregulated in serum samples of patients suffering from the

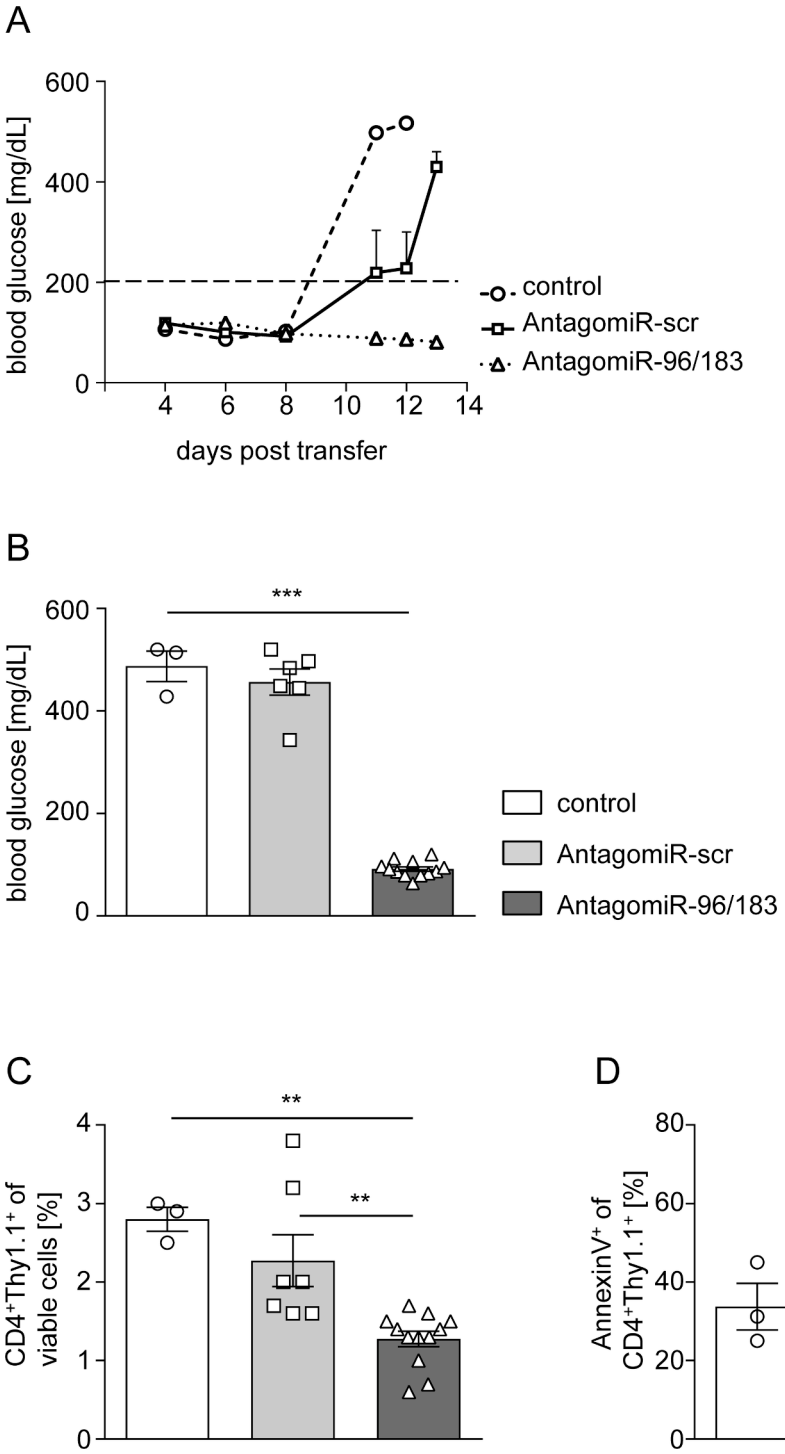

Fig. 7. Delayed development of autoimmune diabetes upon adoptive transfer of $CD4^+CD25^-$ T cells transfected with miR-183 and miR-96 antagomiRs. Sorted HA-specific $CD4^+CD25^-$ T cells form TCR-HA/Thy1.1 mice were transfected with fluorescein-labeled antagomir-183 and antagomiR-96, antagomiR-scr control or without antagomiR (control). After 72 h 1×10^5 fluorescein$^+$ cells were adoptively transferred to INS-HA/Rag2KO mice. (A) The development of diabetes was monitored by blood glucose level at indicated time points. Mice with blood glucose > 200 mg/dl were considered as diabetic. (B) Endpoint blood glucose levels. (C) The frequency of $CD4^+Thy1.1^+$ T cells and $AnnexinV^+$ $CD4^+Thy1.1^+$ T cells within the spleen was determined by flow cytometry. Data from three independent experiments with n = 3–12 mice are summarized as mean ± SEM. One-Way ANOVA with Dunnett's multiple comparisons test was used for statistical analysis. **p > 0.01, ***p < 0.001.

autoimmune thyroid diseases Hashimoto thyroiditis and GD [19]. Interestingly, splenic T cells from three different lupus mouse models also expressed elevated levels of miR-183 and miR-96 [36], suggesting that both miRs play a crucial role in T cell-mediated autoimmunity.

In the present study, we identified miR-183 and miR-96 to be highly expressed in $CD4^+$ T cells from GO patients and in *in vitro* activated human and murine T cells. Our gain- and loss-of-function experiments revealed that miR-183 and miR-96 expression in $CD4^+$ T cells modulate their proliferative activity *in vitro* and their ability to induce autoimmune responses in an antigen-specific diabetes model *in vivo*. Increased miR-183 and miR-96 expression was observed in retinas during the progress of diabetic retinopathy in rats [37]. For human

patients suffering from autoimmune diabetes elevated expression of miR-183 was detected in serum samples of children with recent-onset T1D [38]. Only a limited number of miRNA expression analyses of specific peripheral $CD4^+$ T cell subsets such as regulatory T cells or memory T cells from T1D patients was performed [39] with no significant differential expression of miR-183 or miR-96 [40,41]. We also determined miR-183 and miR-96 expression levels in $CD4^+$ T cells from eight adult long-standing T1D patients in comparison to $CD4^+$ T cells from age-matched healthy controls and detected no differences in the relative expression of both miRs (data not shown). Interestingly, specific miRs in sera from children with recent-onset diabetes were upregulated only in the early phase of disease and showed expression levels comparable to controls in patients with diabetes longer than six months [38]. Thus, further longitudinal analysis of miR-183 and miR-96 expression in $CD4^+$ T cells from at-risk and new-onset patients are necessary to further dissect the impact of both miRs on the development and progression of autoimmune diabetes.

Elevated expression of miR-96 and miR-183 was also described in several tumors and tumor cell lines including breast cancer, bladder cancer and prostate cancer as well as in stem/progenitor cells and correlated with increased proliferative activity [42–46]. Downregulation of miR-183 in human stem/progenitor cells resulted in impaired proliferation [46], whereas overexpression of miR-96 in a prostate cancer cell line triggered their cell growth activity [45]. With regard to T cells, members of the miR-183 cluster were identified to be highly expressed in *in vitro* differentiated T helper cell subsets. Activation of naïve T cells under specific polarizing conditions for Th1, Th2 and Th17 cells promoted elevated miR-182, miR-183 and miR-96 expression [47,48]. It was further demonstrated that the miR-183 cluster increases the pathogenic cytokine production from Th17 cells during their development and exacerbates experimental autoimmune encephalomyelitis *in vivo* [48]. Moreover, specific inhibition of miR-182 in activated T helper lymphocytes interfered with clonal expansion, and overexpression of miR-182 resulted in increased proliferation [47]. Hence, these studies and our data clearly indicate that the miR-183 cluster is involved in the regulation of T cell function.

By using a luciferase-based reporter assay we identified EGR-1 as target for miR-183 and miR-96. Expression of EGR-1 has been described to be directly regulated by miR-183 in multiple tumor types [26] and human stem/progenitor cells [46]. Well in line with these findings, we detected significantly reduced EGR-1 expression in miR-183 overexpressing $CD4^+$ T cells and elevated EGR-1 expression in miR-183 antagomiR-treated cells, indicating that miR-183 interferes with EGR-1 expression not only in tumor and stem cells but also in T cells. Interestingly, by using a dual luciferase approach, we identified EGR-1 also as target for miR-96. In accordance, our gain- and loss-of-function experiments of miR-96 resulted in modulated EGR-1 protein expression.

EGR-1 was shown to transactivate the negative regulator PTEN by binding to a consensus EGR-1 binding motif in the PTEN promoter [27]. Well in line, we observed EGR-1 and PTEN downregulation in miR-183 and miR-96 overexpressing T cells, whereas antagomiR-treated cells exhibited elevated EGR-1 expression correlating with increased PTEN protein levels. PTEN is a protein with lipid phosphatase function that directly opposes PI3K signaling and thereby negatively regulates the Akt signaling pathway [49]. Indeed, enforced miR-183 and miR-96 expression resulted in enhanced Akt phosphorylation in $CD4^+$ T cells. Hence, we propose that miR-183 and miR-96 contribute to $CD4^+$ T cells activation by modulating EGR-1 expression that is linked to the PTEN/Akt signaling axis. Importantly, loss of PTEN in mature T cells has been described to promote hyperproliferation and the development of severe multiorgan autoimmune disorders [50], linking this pathway to T cell-mediated autoimmunity. Adoptive transfer of miR-183 and miR-96 overexpressing antigen-specific T cells resulted in accelerated development of autoimmune diabetes, whereas transfer of specific antagomiR-treated cells prolonged the disease onset. In these mice, we detected increased percentages of miR-183 and miR-96 transduced T cells upon adoptive transfer in contrast to reduced frequencies of HA-specific T cells transfected with miR-183 and miR-96 specific antagomiRs. Based on our *in vitro* studies, we conclude that both miR-183 and miR-96 foster the proliferative activity of $CD4^+$ T cells resulting in their expansion *in vivo* and thereby promoting the development of autoimmune diseases. However, one might also speculate that modulation of miR expression resulted in changes in T cell apoptosis which in turn led to differences in the percentage of antigen-specific T cells in diseased mice, since an anti-apoptotic function of miR-183 and miR-96 has been described in several tumor cells [51]. However, we did not observe increased apoptosis of antagomiR transfected T cells upon adoptive transfer to INS-HA/Rag2KO mice.

It is well known that miRs generally have multiple targets and thereby, miR-96 and miR-183 probably also targets other mRNAs in addition to EGR-1. The transcription factor forkhead box O 1 (Foxo1) has been described to be regulated by members of the miR-183 cluster [47,48]. Within T cells, Foxo1 is required for the development of induced regulatory T cells [52], for the maintenance of memory T cells [53] and it inhibits the differentiation of Th17 cells. Moreover, negative regulation of Foxo1 expression by the miR-183 cluster promotes Th17 cell pathology [48]. Interestingly, binding of Foxo1 to a region 5 kb upstream of the PTEN coding sequence was demonstrated in $CD4^+$ T cells and knockdown of Foxo1 resulted in decreased PTEN protein levels [54]. Since we have not analyzed regulation of Foxo1 in our experiments, we could not exclude that miR-183 and miR-96 might also bind to Foxo1 in addition to EGR-1 in activated $CD4^+$ T cells, thereby contributing to the observed regulation of PTEN expression and Akt phosphorylation. However, our data clearly indicate that miR-183 and miR-96 expression regulates T cell activation *in vitro* and *in vivo* and might therefore represent a suitable target for the treatment of T cell-mediated autoimmune diseases.

Conflict of interest

The authors have no conflict of interest to disclose.

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