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MicroRNA-21 silencing prolongs islet allograft survival by inhibiting Th17 cells

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ABSTRACT

It has been reported that microRNA-21 (miR-21) augments Th17 responses and contributes to the pathogenesis of autoimmune diseases. Gene knockout or siRNA-induced knockdown of miR-21 in mice resulted in impaired Th17 differentiation and strong resistance to experimental autoimmune encephalomyelitis (EAE). Recently, we validated the miR-21 target IL-10 mRNA and showed that it exerts a pro-inflammatory role by inhibiting IL-10-expressing regulatory B cell (B10) differentiation. The administration of miR-21 antisense oligonucleotides (antagomiR-21) in vivo potently suppressed the severity of EAE, and the suppressive activity was mediated by an increased number of B10 cells. However, the contribution of the miR-21 pathways involved in transplant rejection remains obscure. In this study, we examined the impact of systemic administration of miR-21 inhibitor on allografts in a pancreatic islet transplantation model. We showed that specific miR-21 silencing in vivo significantly prolonged allograft survival (median survival time (MST) 21 days with antagomiR-21 vs 13 days for the control, $p < 0.05$), and the change was associated with a decrease in Th17 cells (~3-fold) and an increase in B10 cells (~2.4-fold). Moreover, we found that miR-21-silenced B cells mediate this protective role through pro-inflammatory Th17 responses in an IL-10-dependent fashion. Thus, we have revealed a novel mechanistic pathway that modulates Th17 development and alloimmunity. Targeting miR-21 may represent a valuable therapeutic intervention strategy for preventing transplant rejection.

1. Introduction

Pancreatic islet transplantation is considered one of the most promising therapeutic approaches for type 1 diabetes mellitus (T1DM). However, alloreactive effector Tcell response and inflammation contribute to transplant rejection and remain a major obstacle in achieving long-term graft survival [1]. Considering the failure of islet transplantation and the recurrence of diabetes, many studies are still focused on increasing islet survival rates.

IL-17-expressing $CD4^+$ T cells (Th17 cells) are involved in allograft rejection and autoimmune diseases. Th17 cells have been shown to accumulate in draining lymph nodes (LNs) and infiltrate rejected grafts in both mice and humans [2]. Cellular immune response may exert a critical role in transplant rejection. In particular, Th17 cells play a central role in orchestrating a broad range of pro-inflammatory activities in graft rejection process such as the acute rejection of lung transplants, chronic kidney rejection and graft-versus-host disease (GVHD). Additionally, activated Tregs can be converted into Th17 cells [3,4]. Blockade of IL-17 led to a marked prolongation in allograft survival, suggesting that Th17 cells contribute to allograft rejection [2]. Neutralization of IL-17A activity has been shown to reduce transplant rejection; however, Th17 cells are not indispensable to the process, and other effector T lymphocytes (Th1, Th2, etc.) are also associated with graft rejection [5]. Nonetheless, the redundant effector mechanisms associated with allograft rejection have made no allowance for conclusive evidence of a vital role for the Th17 response in this setting.

MicroRNAs (miRNAs, miRs) are a class of small (~22-nucleotide) noncoding RNAs that regulate gene expression through translational repression and mRNA degradation, leading to a decrease in protein expression encoded by their cognate mRNAs [6]. MiRNAs are involved in various physiological processes such as the development of hematopoietic and immune cells and immune homeostasis and response. Abnormal miRNA expression has been implicated in a wide range of human diseases, including tumorigenesis and autoimmune disorders [7]. Among these miRNAs, miR-21 plays a crucial role in the development of autoimmune diseases [8,9]. Specifically, elevated expression of miR-21 in T cells was observed in association with autoimmune diseases such as systemic lupus erythematosus (SLE), multiple sclerosis (MS) and psoriasis. Moreover, miR-21-deficient mice selectively influence differentiation of Th17 cells but not Th1, Th2 or Treg cells and

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resulted in strong resistance to dextran sulfate sodium (DSS)-induced colitis [10] and experimental autoimmune encephalomyelitis (EAE) [11]. Blockade of miR-21 strikingly ameliorated the EAE symptoms and affected Th17 differentiation by directly targeting smad7 [11]. We have previously demonstrated that IL-10 is a miR-21 target and elucidated that miR-21 negatively controls the development of IL-10-expressing regulatory B cells (B10) [12]. However, the contribution of the miR-21 pathway to the development of allograft rejection remains unclear.

The observed ameliorating effects of miR-21 silencing on EAE and multiple autoimmune disease models illustrate a pro-inflammatory role for miR-21. At the same time, the transcriptome profile of miR-21 also reported increased miR-21 expression in MS patients and other human autoimmune diseases. Employment of miR-21 silencing may be an effective therapeutic approach for the treatment of autoimmune and other inflammatory diseases. We hypothesized that miR-21 knockdown could reduce allogeneic immune response and prolong allograft survival.

miR-21 regulates cell survival, inflammation and immune response given the observed ameliorating effects in EAE models and other human autoimmune diseases [9,12,13]. Compared to other mediators, miR-21 induction may dynamically impact the balance between pro-inflammatory and immunosuppressive states. To test the potential role of miR-21 in the allograft rejection process, we established an STZ-induced diabetic mouse model for islet transplantation whereby antagomiR-21 was administered to examine whether miR-21 silencing may prolong islet allograft survival in mice. We also measured lymphocyte infiltration, inflammatory cytokines and the immune response.

2. Materials and methods

2.1. Islet transplantation

Pancreatic islets from wild-type (WT) BALB/c mice were perfused and digested with collagenase V (Sigma-Aldrich) and separated by discontinuous Ficoll gradient centrifugation. Islets were then hand-picked under a stereomicroscope and implanted beneath the left renal capsule of sex-matched C57BL/6 mice recipients with streptozotocin (STZ, 50 mg/kg in 10 mmol/l of citrate buffer, pH 4.5, i.p.)-induced diabetes (400 islets per recipient), as previously described [14–16]. Blood glucose levels and the graft survival time after transplantation were monitored. All recipients had glycemia < 150 mg/dl within 2 d after the transplant was identified as engrafted. Blood glucose > 250 mg/dl after engraftment was considered as rejection. Moreover, we analyzed the residual islets and graft-infiltrating immune cells. In some experiments, mice were immunized with alloantigen by an i.p. injection of 2×10^7 mitomycin C-treated allogeneic BALB/c splenocytes.

2.2. Histology

For the histopathological studies, the area of the kidney bearing the grafted islets was dissected, fixed in 4% paraformaldehyde and processed for paraffin embedding. The sections (6–10 μm thick), which were mounted on poly-L-lysine glass slides, were stained with hematoxylin and eosin (H&E). To visualize the islets transplanted beneath the kidney capsule, IHC staining for insulin was performed with a monoclonal antibody (Abcam).

2.3. RNA extraction and qPCR (quantitative PCR) analysis

Total RNA was extracted with the RNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. cDNA synthesis was performed using SuperScript™ III Reverse Transcriptase (Invitrogen). For analysis of miR-21-5p expression, miRNA-specific RT (reverse transcription) primers for miR-21a-5p and the internal control U6 are listed in Table 1. Real-time PCR was performed using the SYBR Green mix (Arraystar) on a ViiA 7 Real-Time PCR System (Applied Biosystems). The PCR program consisted of a 10 min hot-start at 95 °C followed by 40 cycles of 10 s at 95 °C and 1 min at 60 °C. After completion, a melting curve was completed to ensure the specificity of the RT-PCR. The relative expression was normalized to U6 and calculated by the comparative $2^{-\Delta\Delta CT}$ method. The reaction was run in triplicate and the results are represented as the mean ± SD from three independent experiments.

2.4. AntagomiR-21 synthesis and treatment

The miRNA inhibitor (antagomiR-21) was synthesized by GenePharma. The terminal nucleotides of the antagomiR-21 are modified by with cholesteryl at the 3′-end, *O*-methyl at the 2′-ribose position and a PS linkage. The oligonucleotide sequences of antagomiR-21 and the negative control sequence are 5′-UCAACAUCAGUCUGAUAAG CUA-3′ and 5′-CAGUACUUUUGUGUAGUACAA-3′, respectively. To silence miR-21 in vivo, we used the intravenous systemic delivery of liposome-encapsulated antagomiR-21 to transplant-recipient mice. To generate miR-21 knockdown mice, antagomiR-21 and the negative controls were administered by vein injection to the transplant-recipient mice as previously described [11].

2.5. Flow cytometry

Splenocytes from antagomiR-21-treated or untreated mice recipients of islet allografts were harvested 10 days after transplantation. Subsequently, the splenocytes were stained with fluorochrome-conjugated anti-CD4, -CD44, or -CD62L to detect the effector/memory $CD4^+$ T cells. Data were acquired through FACSCalibur flow cytometry and analyzed using the CellQuest Pro software.

2.6. In vitro coculture assays

B cells (1×10^6/ml) from antagomiR-21- or control-treated recipient B6 mice of Balb/C islets (day 14) were collected from splenocytes through MACS enrichment. Then, 10 μg/ml agonistic anti-CD40 mAb and 10 μg/ml LPS were added for B cell stimulation. MACS-purified $CD4^+$ T cells (2×10^5/ml) from alloimmunized WT B6 mice were labeled with CFSE and cultured alone or with stimulated B cells with anti-CD3 and soluble anti-CD28 bound on the plate as described previously [12,17]. After the cells were harvested, the CFSE dilution was assessed as a measure of proliferation [12,18].

2.7. Cytokine measurement

For the detection of intracellular IL-17, T cells were cultured for 4 h with PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and brefeldin A (5 μg/ml). For the detection of IL-10, B cells were cultured for 5 h with LPS (10 μg/ml), PMA (50 ng/ml; Sigma-Aldrich), ionomycin (500 ng/ml; Sigma-Aldrich), and monensin (2 μM), as described previously [19]. Cultured cells were collected and IL-17 and IL-10 cytokine concentrations were measured using intracellular staining kits (BD Biosciences) according to the manufacturer's recommendations.

For the detection of cytokines by ELISA assays, cell cultures were prepared as described above and the supernatants were collected at the indicated times and stored at −20 °C. The supernatant concentrations of IL-17 and IL-10 were determined using cytokine ELISA kits (eBioscience). The results were analyzed spectrophotometrically at 450 nm using an ELISA microplate reader.

2.8. Statistics

Statistical analyses used the log-rank (Mantel-Cox) test to analyze allograft survival. Other experiments were statistical analyzed by 2-tailed Student's *t*-test. *p*-Values < 0.05 (*) were considered to be

Table 1
Sequences of RT-PCR primers.

Reverse transcription primer		
U6 RT primer	5′-CGCTTCACGAATTTGCGTGTCAT-3′	
miR-21 RT primer	5′-GTCGTATCCAGTGCGTGTCGTGGAGTCGGCAATTGCACTGGATACGACTCAACA-3′	
qRT-PCR		
	Sense primer	Anti-sense primer
U6 primers	5′-GCTTCGGCAGCACATATACTAAAAT-3′	5′-CGCTTCACGAATTTGCGTGTCAT-3′
miR-21a-5p primers	GSP: 5′-GGGGGGTAGCTTATCAGACTG-3′	5′-CAGTGCGTGTCGTGGAGT-3′

significant.

2.9. Ethic statement

All animals were maintained under clean conditions in the animal facility at Jiangsu University School of Medicine. Housing and experimental protocols for this study were approved by the Ethics Committee of Jiangsu University (Zhenjiang, China).

3. Results

3.1. Silencing of miR-21 leads to prolonged islet allograft survival in mice

Since miR-21 blockade is a very effective therapeutic approach for suppressing EAE and weakening the inflammatory response [11,12], we hypothesized that miR-21 silencing may suppress the undesired immune responses that mediate allograft rejection. To verify this, we examined the effect of systemic administration of a miR-21 inhibitor on allograft outcome in a pancreatic islet transplantation model. AntagomiRs are chemically synthesized analogs with a 2′-*O*-methyl modification, phosphorothioate linkages, and 3′-cholesterol to elevate cellular uptake. In the present study, we used antagomiR-21 to silence miR-21 in mice transplanted with allogeneic pancreatic islet cells. We initially investigated whether antagomiR-21 treatment could prolong islet allograft survival. BALB/c islet allografts were transplanted under the kidney (renal) capsule of chemically (streptozotocin, STZ)-induced diabetic C57BL/6 (B6) recipients. Islet graft survival in the recipient mice was assessed. As shown in Fig. 1A, islet allograft rejection occurred with a median survival time (MST) of 13 days in mice that received the negative controls. In contrast, systemic delivery of antagomiR-21 by four intravenous (i.v.) injections of 30 μg/mouse markedly prolonged islet allograft survival (MST, 21 days; $p < 0.05$), with approximately 20% of mice achieving long-term graft survival (> 100 days). These data suggest that antagomiR-21 treatment enhanced survival after allograft transplantation.

To identify the mechanism through which miR-21 silencing prolonged islet allograft survival, the grafts were dissociated 10 days after transplantation and processed for histological analysis. Immunohistochemical staining with anti-insulin antibody in formalin-fixed paraffin-embedded grafts tissue sections showed more residual islets and an insulin-positive mass (Fig. 1B) in the grafts in miR-21-silenced mice compared with control recipients. Histochemical analysis (hematoxylin-eosin (HE) staining) revealed that fewer immune cells infiltrated the islet allografts in the miR-21-silenced recipients than in the control-treated recipient mice at 10 days after transplantation (Fig. 1C). Thus, miR-21 silencing prevented leukocyte infiltration and increased insulin secretion by islet allografts. These results clearly indicate that antagomiR-21-treated recipients have prolonged allograft survival compared with control-treated recipients. Moreover, increased insulin secretion and decreased immune cell infiltration were observed in the antagomiR-21-treated recipients.

3.2. In vivo silencing of miR-21 significantly inhibits Th17 responses

Murugaiyan et al. [20] demonstrated that miR-21 expression is increased in Th17 cells and that miR-21-deficient mice are defective in Th17 differentiation and are resistant to the development of myelin oligodendrocyte glycoprotein (MOG)-induced EAE. Moreover, they uncovered that miR-21 augments Th17 differentiation by specifically regulating SMAD-7 through TGF-β signaling negative feedback. Based on these previous findings and our own results [11,12], we aimed to investigate whether miR-21 silencing inhibits allogeneic Th17 differentiation and prolongs allograft survival in a transplantation model.

Splenocytes from antagomiR-21- and control-treated mice recipients of islet allografts were harvested 10 days after transplantation. First, we examined the percentages and absolute numbers of overall splenic $CD4^+$ T cells in antagomiR-21- and negative control-treated mice recipients. As shown in Fig. 2A, the percentage of $CD4^+$ T cells (21.7 ± 1.5%) in antagomiR-21-treated mice vs. control mice (20.3 ± 1.2%) showed no differences between the two groups ($p > 0.05$, Fig. 2A, left). Moreover, no statistically significant differences were found in the absolute number of splenic $CD4^+$T cells between the two groups ($p > 0.05$, Fig. 2A, right). Subsequently, the frequency of effector/memory $CD4^+$ T ($CD4^+$ $CD44^{high}$ $CD62^{low}$) cells in the splenocytes of both recipient groups was analyzed. As presented in Fig. 2B, the number of $CD4^+$ effector/memory T cells in the splenocytes collected from recipients treated with antagomiR-21 (21.3 ± 1.6%) is similar to the control group (20.3 ± 1.1%, $p > 0.05$). Moreover, intracellular flow cytometry was performed to quantify the frequency of IL-17-producing $CD4^+$ T (Th17) cells in the spleen and draining lymph nodes. However, we observed that both the spleen and draining lymph nodes from antagomiR-21-treated recipient mice exhibited significantly lower numbers of Th17 cells compared with control-treated mice (~3-fold decreased, Fig. 2C, $p < 0.01$, spleen; ~4-fold decreased, Fig. 2D, $p < 0.01$, lymph nodes). Furthermore, no differences were observed in the frequency of $Foxp3^+$ Tregs in the splenocytes from antagomiR-21-treated recipients compared with the control mice ($p > 0.05$, Fig. 2E). Thus, antagomiR-21 treatment inhibits recipient alloimmune responses by inhibiting Th17 responses.

To evaluate the transfection efficiency, we examined miR-21 expression levels in $CD4^+$ T cells after in vivo transfection with antagomiR-21 for 24 h. The endogenous $CD4^+$ T cells displayed a significant (~70%) decrease in miR-21 expression (Fig. 2F). Thus, the systemic delivery of antagomiR-21 by intravenous injection of 30 μg/mouse efficiently inhibited endogenous miR-21 expression.

3.3. In vivo silencing of miR-21 significantly promotes IL-10-expressing regulatory B (B10) cells

An immunosuppressive effect of B10 cells has been proven in several autoimmune models in mice. Depletion of B10 contributes to exacerbation of systemic autoimmunity in various mouse models. IL-10-expressing regulatory B (B10) cells have been observed to exert an immunosuppressive effect in autoimmune diseases and organ

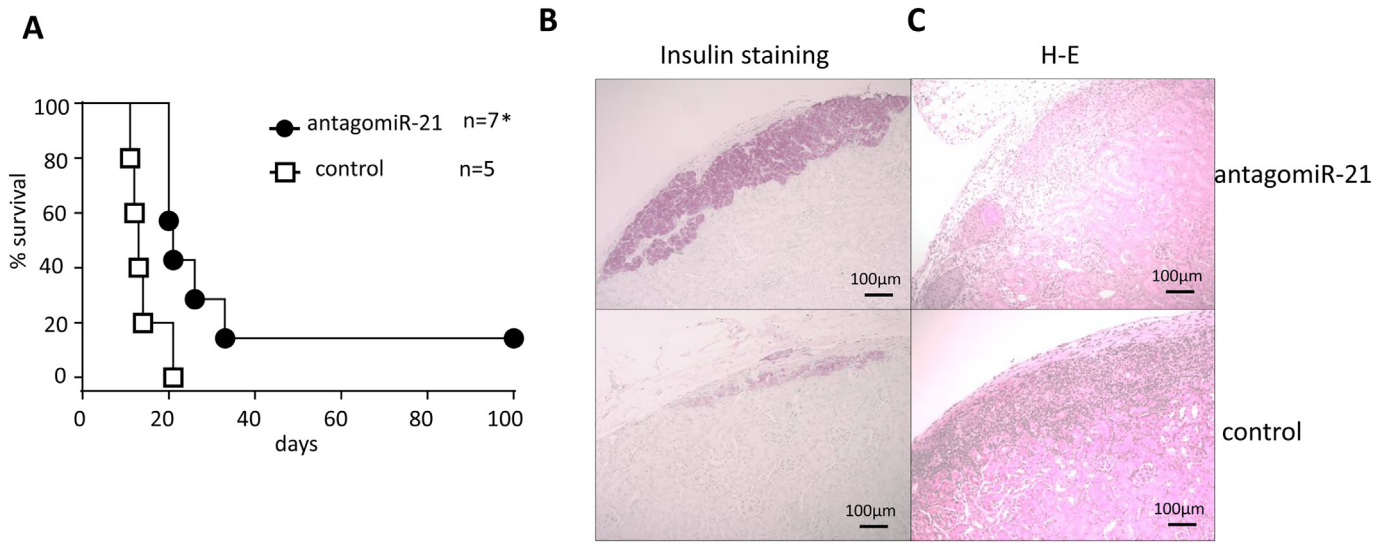

Fig. 1. AntagomiR-21 treatment inhibits alloimmune inflammation and mediates the prolongation of islet allograft survival in mice. STZ-induced chemically diabetic B6 mice recipients of BALB/c islet allografts were treated with antagomiR-21 or negative control as described in the Materials and methods section. (A) Kaplan-Meir plots for graft survival. (MST 21 d vs. 13 d). Graft survival was compared using the log-rank test (*$p < 0.05$). (B) Representative images of the residual islet β cells in the renal capsules at 10 days post-transplantation. Grafts were harvested and 5-μm thick sections were subsequently stained for insulin with anti-insulin antibody and visualized with peroxidase and diaminobenzidine (in red). (C) The infiltration of inflammatory cells in islet grafts. Representative images of H&E staining of immune cells in paraffin-embedded allografts at 10 d post-transplantation (100× magnification). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

transplantation [19,21]. Our previous study showed a miR-21-mediated regulatory mechanism for the differentiation of B10 cells [12]. AntagomiR-mediated knockdown of miR-21 significantly upregulated IL-10 levels in B cells. Moreover, miR-21 knockdown in vivo markedly ameliorated the severity of EAE by promoting B10 cells [12]. However, whether miR-21 is involved in B10 cell differentiation in the transplant model remains unclear.

We first examined the percentages and absolute numbers of overall $CD19^+$ B cells in the recipients' spleens. There were no statistically significant differences between the antagomiR-21-treated mice and control mice in terms of the percentages and absolute numbers of splenic $CD19^+$ B cells ($p > 0.05$) (Fig. 3A). Next, we sought to determine the impact of antagomiR-21 on IL-10 expression in B cells. IL-10 was detected in stimulated splenocytes from transplanted mice treated with antagomiR-21 or the negative control. We observed ~2.5-fold higher levels of IL-10 in antagomiR-21-treated transplanted mice compared with control-treated mice (Fig. 3B). Further work will be required to determine whether antagomiR-21-induced B10 cells can directly modulate alloimmune responses and suppress the development of pro-inflammatory Th17 cells.

3.4. MiR-21 silencing inhibits the Th17 response by promoting B10 cells

Whereas Murugaiyan et al. [20] revealed that miR-21 augments the Th17 response by directly targeting SMAD-7, a negative regulator of the TGF-β pathway, we found that miR-21 directly targets IL-10 mRNA and inhibits IL-10 expression in B cells [12]. IL-10-expressing regulatory B cells (B10) have been ascribed important anti-inflammatory functions in autoimmune diseases and transplant tolerance in both humans and mice [22]. In the light of this evidence, we theorized that miR-21 promotes Th17 differentiation by inhibiting B10 cells. To check this speculation, we set up a coculture model system to examine the impact of miR-21-silenced B cells on Th17 responses [17]. First, purified splenic $CD19^+$ B cells from antagomiR-21-treated alloimmunized mice were incubated in vitro with agonistic anti-CD40 mAb (48 h) and LPS (last 5 h of culture). Then, these cells were added to cultures in which purified splenic $CD4^+$ cells from alloimmunized WT B6 mice were labeled with CFSE (carboxyfluorescein succinimidyl ester) and stimulated with plate-bound anti-CD3 and soluble anti-CD28 together with recombinant mouse IL-2. On day 3 of culture, the CFSE dilution was assessed as a marker for T-cell proliferation by flow cytometry. Similar numbers (~80%) of $CD4^+$ T cells in both groups were identified as dividing cells based on the CFSE intensity (Fig. 4A). Thus, $CD4^+$ T cells cultured with miR-21-silenced or control-treated B cells proliferated equally as fast in response to anti-CD3/CD28 stimulation, and antagomiR-21-treated B cells did not regulate T-cell proliferation in these in vitro assays. However, $CD4^+$ T cells cultured with miR-21-silenced B cells had dramatically reduced IL-17 production when compared to $CD4^+$ T cells that were cultured with control B cells (Fig. 4B). To further confirm this effect, we collected the cell culture supernatants and assayed them for released IL-17 and IL-10 using as enzyme-linked immunosorbent assay (ELISA). Markedly decreased IL-17 expression and increased IL-10 expression were observed in the supernatants of $CD4^+$ T cells cultured with miR-21-silenced B cells. These changes relied upon B cell IL-10 production because anti-IL-10 neutralizing antibodies could effectively reverse the IL-17 expression (Fig. 4B). To strengthen the conclusions, the coculture experiments were repeated with sorting-purified Breg ($CD5^+CD1d^{hi}$) cells. $CD4^+$ T cells cultured with miR-21-silenced Breg ($CD5^+CD1d^{hi}$) cells had a dramatically reduced frequency of $IL\text{-}17^+$ cells compared to $CD4^+$ T cells that were cultured with control Breg cells, and these changes relied upon B cell IL-10 production (Supplementary Fig. 1). This result also suggests that miR-21-silenced Bregs exhibit enhanced suppressive capacity.

Therefore, miR-21-silenced B cells regulate effector Th17 cell responses but do not constrain T-cell proliferation, and their suppressive function is dependent on IL-10.

4. Discussion

Type 1 diabetes mellitus (T1DM) is an autoimmune disease characterized by the body's inability to produce insulin due to the destruction of pancreatic β cells. Whereas multiple therapeutic strategies for T1DM have been developed, islet transplantation could be of great benefit to patients with serious and unstable type 1 diabetes. Islet transplantation may produce physiological insulin in patients, reducing the risk of hypoglycemia and end-organ damage and improving the

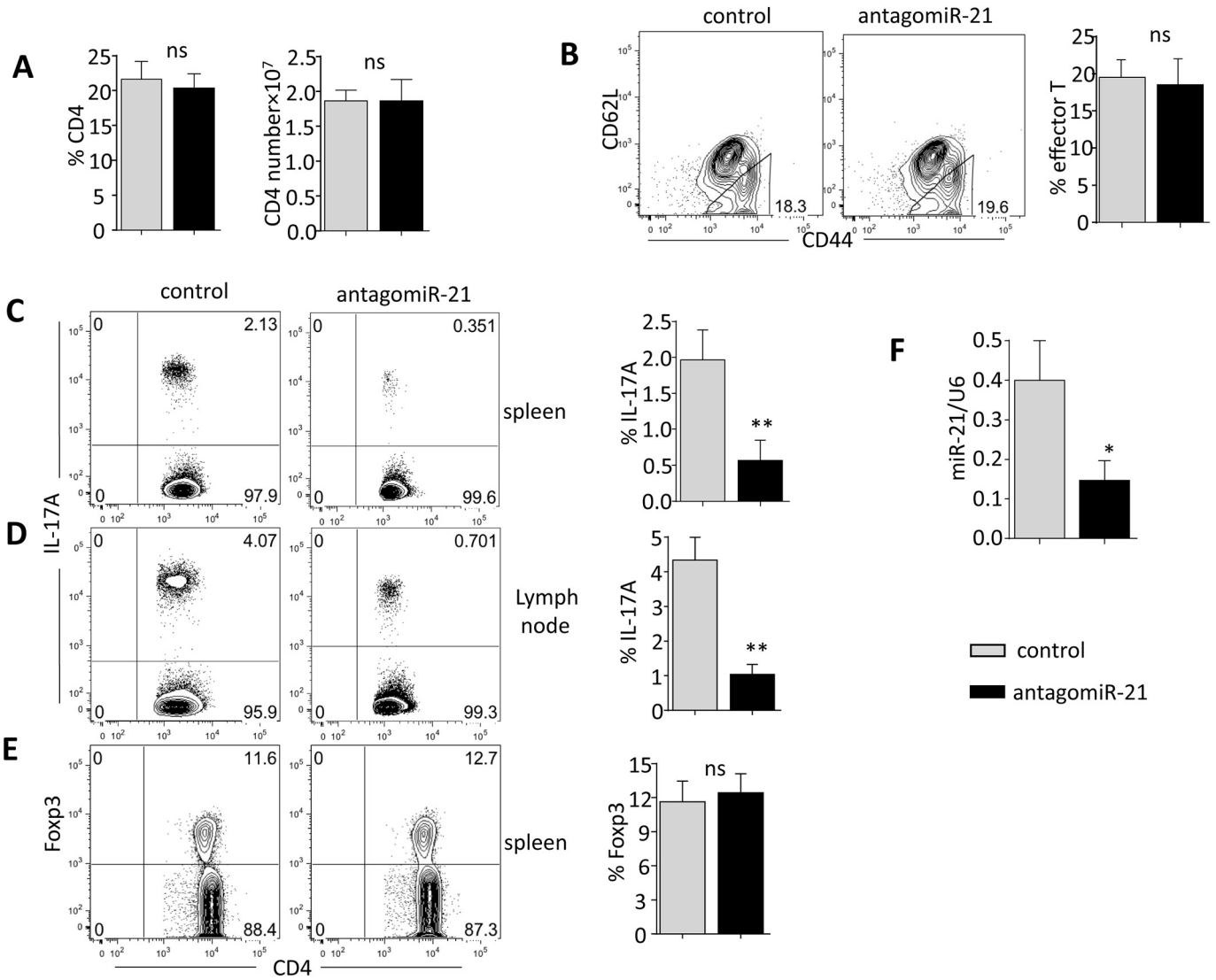

Fig. 2. Analysis of effector T cells, Tregs and Th17 cells. Splenocytes and lymph node cells from antagomiR-21- and control-treated mice recipients of islet allografts were harvested 10 days after transplantation. (A, B, and C) Splenocytes were surface-stained for CD4, CD62L and CD44. (A) The frequency (*left*) and absolute number (*right*) of $CD4^+$ T cells (mean ± SD) for each indicated group. (B). Gated $CD4^+$ T cells were analyzed for CD62L and CD44 expression for each indicated group. *Left*, Representative flow cytometry plots show the % of effector $CD4^+$ T cells ($CD62L^{low}$, $CD44^{high}$). *Right*, The frequency (mean ± SD) of effector $CD4^+$ T cells. (C and D) IL-17 expression by gated $CD4^+$ T cells was determined by intracellular staining using flow cytometry after stimulation of fresh splenocytes (C) or lymph node cells (D) with PMA/ionomycin/Brefidine A. (E) The frequency (means ± SD) of Tregs ($Foxp3^+$ $CD4^+$T cells) was determined by flow cytometry. (F) miR-21 expression was analyzed by qRT-PCR using U6 snRNA as an endogenous control. The numbers in the dot plots indicate the percentage of each gated population. The bar graphs represent the means ± SD of 4–6 mice per group (ns indicates no significance, $*p < 0.05$, and $**p < 0.01$).

quality of life. Additionally, islet transplantation is helpful for decreasing several complications induced by T1DM [23]. Thus, islet transplantation is becoming an exciting and attractive approach to treat T1DM. However, allograft rejection seriously affects graft survival, and the overall long-term survival of islets is still unsatisfactory. The infiltration of T lymphocytes is a main event in transplant recipients. Many immunosuppressive drugs have been clinically tested for their potential to reduce the risk of transplant rejection over the past few decades. However, these medications may cause serious side effects, including infection, nephrotoxicity and cancer. Thus, safer agents need to be developed to improve allograft survival.

Th17 cells can promote the rejection of cardiac allografts [24,25]. Blockade of IL-17 promoted the obvious prolongation of allograft survival, proposing that Th17 cells contributed to allograft rejection [2]. While Th17 cells have the ability to mediate rejection, other studies have also indicated that IFN-γ production by Th1 cells also promotes allograft rejection, suggesting their effects are redundant [2,3]. Additional evidence showed that Th17 cells contribute to the rejection process but are not fully responsible for the rejection of allografts derived from a corneal transplant model [26]. Mice lacking IL-17 experienced delayed graft rejection compared to wild-type mice, though overall graft survival remained unaffected. The contribution of Th17 cells in clinical transplantation needs to be determined in clinical trials. Additionally, the implication of Th17 cells in allograft rejection in the presence of the full orchestra of T helper cells remains elusive [5].

Over the last decade, research in immunology has increasingly intersected with the field of miRNAs. Previous studies on miR-21 have been majorly focused on its role in autoimmune diseases and tumorigenesis; in this study, we examined the function of miR-21 in detail in the context of alloimmune responses. MiR-21 knockdown mice showed prolonged graft survival associated with increased B10 and decreased Th17 cells. Thus, we provide definitive evidence that the main function of miR-21 is to promote Th17 cells and regulate B10 cells in transplantation models. Efficient targeting of miR-21 induced the

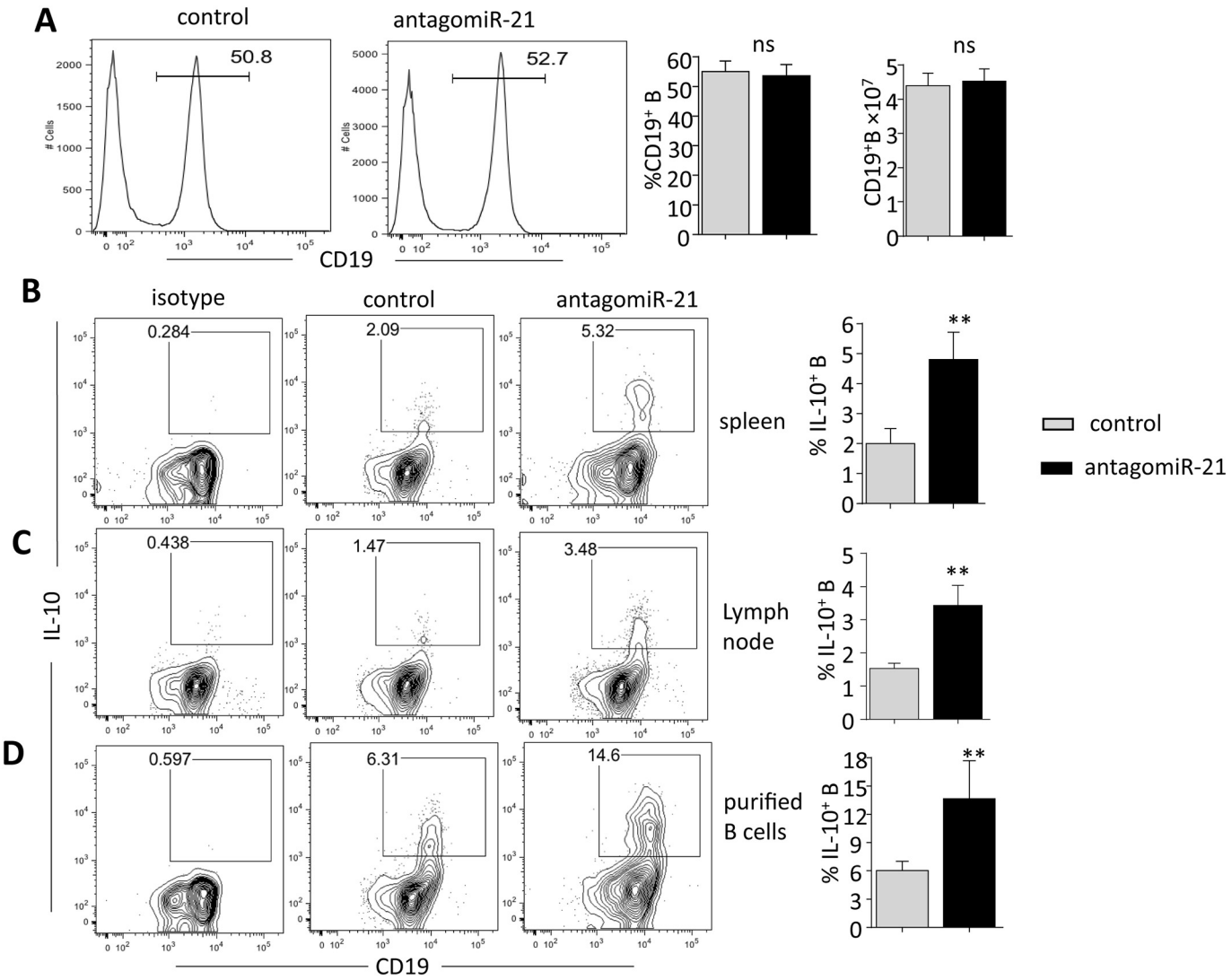

Fig. 3. Silencing of miR-21 significantly promotes IL-10-producing B cells. Splenocytes and lymph node cells from antagomiR-21- and negative control-treated mice transplanted with islet allografts were harvested 10 days after transplantation. (A) Representative histograms (*left*) and frequency (mean ± SD) (*middle*) showing the percent of $CD19^+$ B cells in the spleen. (*Right*) Absolute number of $CD19^+$ B cells per spleen compiled for each indicated group. (B and C) IL-10 expression by gated $CD19^+$ B cells was determined by intracellular staining using flow cytometry after stimulation of fresh splenocytes (B) or lymph node cells (C) with PMA/ionomycin/monensin/LPS. *Left*, Representative flow cytometry plots showing the % of $IL\text{-}10^+CD19^+$ B cells. *Right*, Frequency (mean ± SD) of $IL\text{-}10^+CD19^+$ B cells. (D) $CD19^+$ B cells were purified by MACS from alloimmunized B6 mice and cultured for 24 h under LPS/PMA/ionomycin/monensin conditions. IL-10 levels were measured by intracellular staining and flow cytometry analysis. The numbers represent the frequencies of $CD19^+$ B cells. N = 4–5 for each indicated group. $^{**}p < 0.01$ and "ns" indicates no significance.

differentiation of B10 cells, which in turn alleviated transplant rejection by inhibiting pro-inflammatory Th17 cells. To the best of our knowledge, this is the first study demonstrating the role of miR-21 in regulating B10 versus Th17 responses in vivo. Our finding that this critical step in immunity is regulated by a specific microRNA represents a new paradigm with broad and deep implications.

MiRNAs are highly pleiotropic, with a given miRNA having the potential to target different genes, meaning that they can potentially affect multiple biological processes. To address this issue, researchers usually focus on the functions or pathways in which they converge. Thus, a miRNA can target different molecules in converging pathways to have a large effect upon the cell. Additionally, different microRNAs can converge on the same messenger RNA target [7,27,28]. In the matter of miR-21, several targets have been verified, and most of them are tumor suppressors; notable targets include PDCD4, FasL, and PTEN [9,18,29–31]. We have, for the first time, established that miR-21 is a signal that negatively regulates $IL\text{-}10^+$ Breg differentiation. It is also known that microRNA target prediction tools have limitations with regard to specificity; additionally, a specific microRNA has the ability to target different mRNAs involved in many biological processes. This is illustrated in part by the recent report that miR-21 knockout mice show no changes in cardiac remodeling, even though this outcome was expected based on predictive algorithms and antagomiR studies [32]. Whereas silencing miR-21 efficiently increased $IL10^+$ Bregs and inhibited Th17 responses, only slightly prolonged graft survival was observed in antagomiR-21-treated allograft recipients; the discrepancy between the regulatory cytokine profile and graft survival could be mechanistically explained by the pleiotropic effects of miR-21.

Many lines of evidence indicate that miR-21 plays a crucial role in regulating apoptosis by targeting the tumor suppressors PDCD4 and PTEN [33]. The miR-21-PDCD4 axis regulates cell apoptosis through the Bax family in T1DM [34]. MiR-21 has also been shown to be closely associated with autoimmune diseases. The ability of miR-21 to inhibit $IL\text{-}10^+$ Bregs and augment Th17 responses may be an additional mechanism for their action against transplant tolerance. In line with the hypothesis that miRNA function is determined by the specific cellular

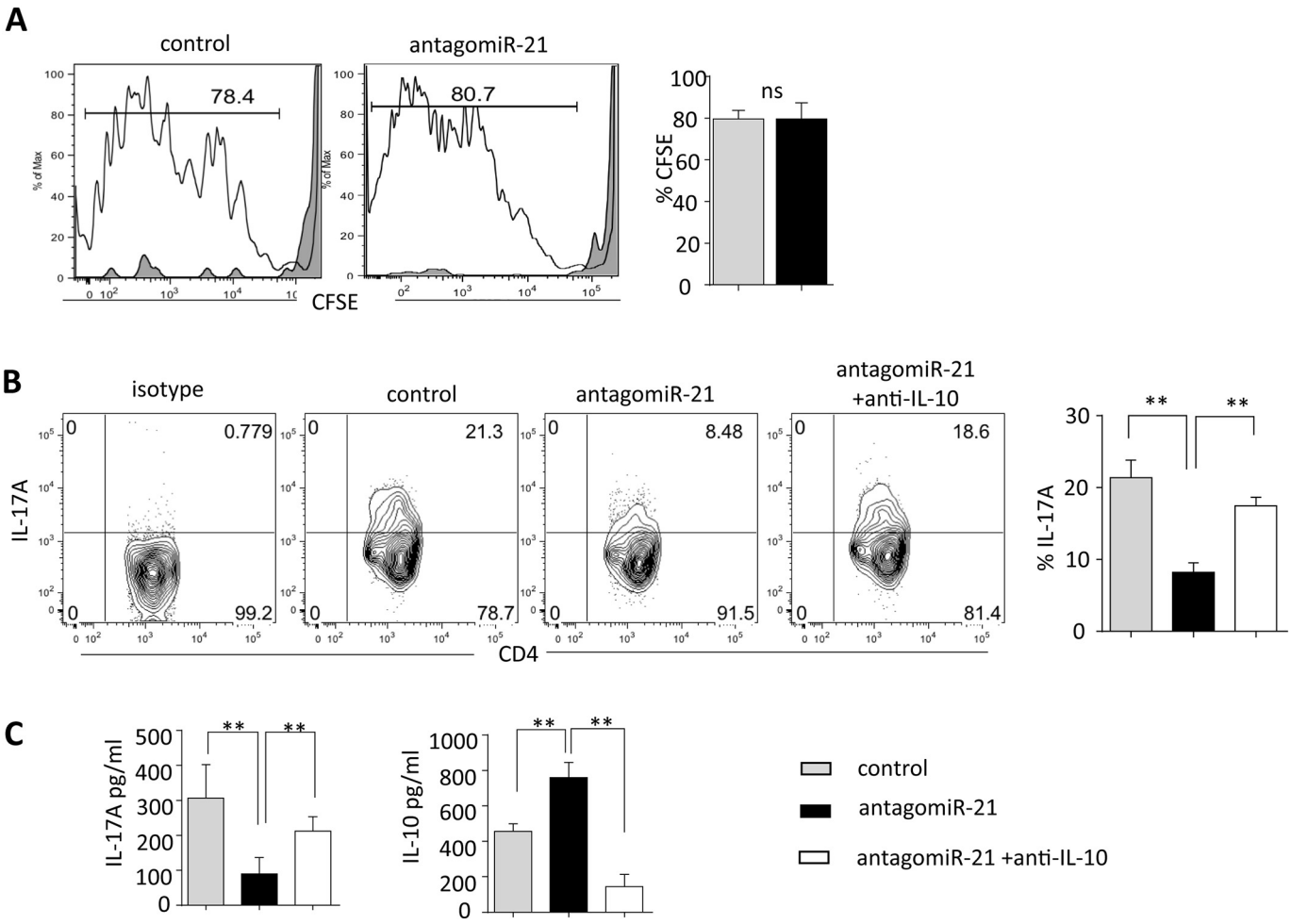

Fig. 4. Silencing of miR-21 in B cells alters the Th17 response. MACS-purified splenic CD19$^+$ B cells from control- or antagomiR-21-treated alloimmunized mice (day 10) were stimulated with agonistic CD40 mAb for 48 h, and LPS was added during the final 5 h. Purified CD4$^+$ T cells from alloimmunized WT B6 mice were labeled with CFSE and cultured with CD40/LPS-stimulated B cells in the presence of plate-bound anti-CD3 and soluble anti-CD28 for 72 h. (A) The cultured cells were stained for CD4 and analyzed for CFSE dilution by flow cytometry. Representative frequencies of dividing CFSE-labeled cells are shown. Bar graphs indicate the mean frequencies of the dividing CFSE-labeled CD4$^+$ T cells (mean ± SD) from 5 to 6 mice. ns indicates no significance. (B) PMA, ionomycin, and Brefeldin A were added during the final 4 h of culture. IL-17 expression by CD4$^+$ T cells was determined by intracellular staining and flow cytometry analysis. Numbers indicate the percentages of IL17$^+$CD4$^+$T cells within the indicated gates among all CD4$^+$ T cells in the sample. Bar graphs indicate the mean (± SD) percentages of IL-17-expressing CD4$^+$ T cells from 5 to 6 mice. $^{**}p < 0.01$ and ns indicates no significance. (C) Cells were cultured similar to as described for A and B except that Brefeldin A was not added. Then, the culture supernatants were collected and used for the estimation of IL-17 and IL-10 secretion by ELISA. 3 wells/sample. Bar graphs indicate the mean (± SD). IL-17 or IL-10 levels are representative of 5–6 mice per group. $^{**}p < 0.01$.

context, we highlight a novel role for miR-21, which is a repressor of signaling through this pathway [35]. Our findings are consistent with the observation that miR-21 augments adaptive immune response. Moreover, our findings do not rule out the possibility that miR-21 may modulate other pathways.

Collectively, our results have identified miR-21 as a potent regulator of IL-10$^+$ Bregs and Th17 responses in a model of alloimmune inflammation. Even though multiple regulatory steps are involved in alloimmune responses, IL-10$^+$ Breg and Th17 decisions appear to rely on miR-21. The present study has provided evidence that the imbalance between Bregs and Th17 cells in the development of transplant rejection is attributed to miR-21 overexpression. Thus, treatment with antagomiR-21 and the latter studies on its effect on the dynamic equilibrium of Breg and Th17 signaling will pave the road for potential clinical studies on islet transplantation. Overall, our findings identified a new mechanism for miR-21 in shifting a polarized immune-inflammatory response.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.intimp.2018.11.022.

Conflicts of interest

The authors have no financial conflicts of interest.

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