Review

MOLECULAR METABOLISM

The role of T cell miRNAs for regulatory T cell induction in islet autoimmunity

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ABSTRACT

Background: microRNAs (miRNAs) have emerged as critical contributors to immune regulation and homeostasis, and their dysregulation is involved in the aberrant differentiation and function of T cell subsets. In type 1 diabetes (T1D), the clinically overt disease is preceded by a presymptomatic phase which is marked by the presence of islet autoantibodies while the individual is still normoglycemic. Recent analyses revealed impaired regulatory T (Treg) cell induction from naive $CD4^+$ T cells during this early phase of autoimmunity.
Scope of the review: In this review article, we aim to discuss important recent insights into miRNA regulation of immune homeostasis and activation. Specifically, we highlight the role of miRNAs as biomarkers in autoimmunity and T1D as well as the contribution of specific miRNAs and their downstream pathways to the onset and progression of islet immunity. Furthermore, we focus on critical next steps required to establish miRNAs as biomarkers to predict disease onset and progression and as novel targets of future prevention and treatment strategies to control autoimmunity.
Major conclusions: Several recent studies have provided considerable insight into the miRNA regulation of immune homeostasis and how dysregulated miRNAs contribute to onset and progression of islet autoimmunity. Specifically, high levels of individual miRNAs such as miR92a and miR181a are involved in impaired Treg induction during the onset of islet autoimmunity, thereby contributing to disease pathogenesis. The recent advancements in the field suggest miRNAs as potential biomarkers for islet autoimmunity and their direct targeting, especially in a T cell-specific manner, could contribute to the reestablishment of immune homeostasis and ultimately interfere with the onset of islet autoimmunity.

Keywords Immune regulation; Islet autoimmunity; Type 1 diabetes; Regulatory T cell; miRNA; Biomarker

1. INTRODUCTION

The immune system detects and eliminates harmful pathogens and discriminates them from the organism's own tissues. The balance between immunity and tolerance is a complex and tightly regulated process which requires precise control of lymphocyte development and function. The dysregulation of these control mechanisms critically contributes to the development and activation of autoreactive lymphocytes which can lead to autoimmunity.
There are more than 80 diseases with an autoimmune etiology, and their high prevalence early in life and rising incidence makes them a significant burden for the healthcare system. Autoimmune diseases can be classified into systemic or organ-specific diseases, such as T1D which is the most common metabolic disease in young children [1]. In T1D, impaired immune tolerance mechanisms result in a breakdown of self-tolerance to the insulin-producing islet beta cells, their destruction by autoreactive T cells and consequently impaired blood glucose control [2].

2. ONSET AND PROGRESSION OF ISLET AUTOIMMUNITY

Longitudinal studies of individuals at risk for developing T1D show that the disease progresses through distinct identifiable stages prior to the onset of clinical symptoms [3,4]. The presymptomatic phase of T1D is termed "islet autoimmunity" and it is characterized by the appearance of autoantibodies against islet autoantigens. Islet autoantigens include insulin [5], glutamic acid decarboxylase (GAD) [6], insulinoma-antigen 2 (IA2) [7,8] and zinc transporter 8 (ZnT8) [9], and autoantibodies can be present in the blood years or even decades before the onset of hyperglycemia [4,10]. Despite ongoing research efforts, the molecular mechanisms underlying the heterogeneity, the onset of islet autoimmunity and especially the progression to symptomatic diabetes remain poorly understood.
Given that most of the beta cell mass is already destroyed at the onset of the symptomatic disease, the presymptomatic phase has become the focus of research approaches aiming at the mechanistic dissection of the molecular underpinnings triggering islet autoimmunity. The specific roles of different T cell subsets during the presymptomatic stage of T1D and their contribution to immune activation and autoimmunity have recently been highlighted [11–14]. The analysis of longitudinal samples collected starting shortly after birth from children at risk for T1D revealed an altered autoantigen response in $CD4^+$ T cells even prior to autoantibody seroconversion [14]. Regulatory T cell (Treg) induction from naive $CD4^+$ T cells was shown to be impaired in children with recent onset of islet autoimmunity, and a slow

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progression from islet autoimmunity to clinical T1D in children was associated with high frequencies of insulin-specific Tregs [12]. These findings suggest a crucial role for Tregs in delaying or possibly preventing the progression from islet autoimmunity to clinical T1D.

3. TREGS: MEDIATORS OF IMMUNE TOLERANCE

Tregs are key players for the maintenance of peripheral immune tolerance by inhibiting their autoreactive counterparts in various ways, and defects in Treg induction and function are important contributors to autoimmune disorders like T1D [15–17]. Tregs are characterized by the expression of CD4, the high-affinity α chain of the interleukin 2 receptor (CD25), and the transcription factor Foxp3, which is a master regulator of Tregs and required for their development and function [18,19]. The crucial role of Foxp3 in Treg differentiation and its impact on immune regulation is highlighted by the deleterious consequences of mutations in the *FOXP3* gene, leading to autoimmune phenotypes in both mice (scurfy mice) and humans (IPEX – immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [20,21].

3.1. Requirements for efficient Treg induction

In addition to their differentiation in the thymus, Tregs can also originate from naive T cells in the periphery upon antigen exposure. The efficient *in vivo* induction of stable Tregs requires the binding of a strong-agonistic antigen to the TCR under subimmunogenic conditions [15,22–24]. These subimmunogenically induced Tregs are stable, even upon antigen exposure and stimulation. By contrast, higher doses of TCR ligands and strong co-stimulatory signals fail to induce stable Tregs by activating the PI3K/Akt/mTOR pathway, resulting in T cell activation. The costimulatory molecule CD28 activates PI3K [25] which phosphorylates Akt, resulting in the activation of mTOR. mTOR induces the phosphorylation of Foxo proteins Foxo1 and Foxo3a, resulting in their export from the nucleus which interferes with Foxp3 induction and expression. Therefore, factors inhibiting the PI3K/Akt/mTOR pathway such as PTEN are crucial for Treg induction and function [26–28]. Treg induction from naive $CD4^+$ T cells *in vitro* is most commonly achieved by TCR stimulation in the presence of TGFβ. Even though the resulting Tregs are functional, they do not resemble the stable phenotype and long-lasting suppressive function of their *in vivo* counterparts. This decreased stability is reflected by a completely methylated conserved non-coding sequence within the Foxp3 gene (Treg-specific demethylated region (TSDR)). In contrast, the TSDR is unmethylated in Tregs induced *in vivo*, and thus maintains stable Foxp3 expression [29,30]. TGFβ independent *in vitro* Treg induction can be achieved by limiting the activity of the PI3K/Akt/mTOR pathway, by direct inhibition of this pathway or by limited TCR stimulation [28]. Proper immune function, including the regulation of lymphocyte development in the thymus and the prevention of autoimmune reactions in the periphery, requires the precise control of signaling pathways. Both effector cell differentiation and activation as well as Treg homeostasis and function are critical for the balance of the immune system and their regulation depends on tunable responses to minor changes in their environment. Specifically, TCR signaling can be regulated precisely by the expression of regulators of downstream signaling pathways including PI3K and NF-κB [31]. One potential mechanism for the fine tuning of these pathways is miRNAs.

4. MIRNAS: REGULATORS OF THE IMMUNE SYSTEM

Small non-coding RNAs (ncRNAs), including miRNAs, have emerged as key players in the regulation of various biological processes, including immune function and homeostasis. Small ncRNAs are defined by their length of 20–30 nucleotides and their Argonaute (AGO) family protein-mediated mode of action. There are three distinct families of regulatory small ncRNAs: miRNA, siRNA (small interfering RNA) and piRNA (PIWI-interacting RNA), of which miRNAs are the most abundant class in most tissues. miRNAs are transcribed from the genome, alone or in polycistronic clusters. They are ~22 nucleotides long, single-stranded and control gene expression by complementary binding of their target mRNA, recruiting AGO family proteins and inducing translational repression, mRNA deadenylation and mRNA degradation.

The miRNA database MirBase contains more than 2,500 human miRNA sequences [32] although the actual number is considered to be up to ten times higher [33]. Most miRNAs target a multitude of genes [34] and more than 60% of human protein-coding genes contain conserved miRNA binding sites in addition to various non-conserved sites [35,36], illustrating the complexity of miRNA-induced gene regulation. Consequently, the dysregulation of miRNA expression is associated with various diseases, including autoimmunity, cancer [37] and neurological diseases [38]. In line with their role in tissue homeostasis and function miRNAs exhibit tissue-specific expression patterns, which are regulated on the level of transcription [35,39]. The biogenesis of miRNAs is a multistep process, involving the transcription into primary miRNA (pri-miRNA) transcripts and processing into pre-miRNAs and finally into mature miRNAs. Pri-miRNAs are mainly transcribed by RNA polymerase II [40], and the primary transcript can contain a single miRNA or a cluster of multiple miRNAs [41]. The pri-miRNA is processed by Drosha [42] and DGCR8 [43,44] and the resulting pre-miRNA by Dicer [45] to produce the mature miRNA duplex. One strand of the mature miRNA is loaded into AGO to form the miRNA-induced silencing complex (miRISC) and guide the complex to its target mRNAs, while the other strand is discarded [46].

The miRNA seed sequence, spanning from nucleotide position 2 to 7, facilitates target recognition via complementary binding to miRNA binding sites which are slightly enriched for the 3' UTRs (untranslated region) but can occur anywhere in the target mRNA. While the seed sequence is pivotal for target recognition the miRNA to mRNA interaction is also supported by nucleotides 8 and 13–16 of the miRNA [35]. miRNAs which share a highly similar seed sequence are grouped into families or clusters and their target genes overlap substantially [34].

4.1. Circulating miRNAs as biomarkers in islet autoimmunity and T1D

Given the importance of miRNAs for the proper function of the immune system, a number of studies have investigated the impact of circulating miRNAs on disease development and their potential role as biomarkers to predict disease progression [47–49]. miRNA profiles from whole blood and serum of newly diagnosed T1D patients and healthy controls revealed differentially expressed miRNAs involved in the function of lymphocytes and beta cells. The abundance of miR21a and miR93, which are involved in NF-κB signaling and negatively regulate apoptotic and inflammatory genes, was significantly reduced in peripheral blood mononuclear cells (PBMCs) of T1D patients [49]. In contrast, the high expression of miR25 was associated with residual beta cell function and glycemic control in individuals with recent onset of T1D [48].

A meta-analysis of studies investigating profiles of circulating miRNAs in T1D patients revealed eleven miRNAs (miR21-5p, miR24-3p, miR100-5p, miR146a-5p, miR148a-3p, miR150-5p, miR181a-5p, miR210-5p, miR342-3p, miR375 and miR1275), which were involved in immune regulation, cell proliferation and insulin processing, and

suggested them as potential biomarkers for T1D [50]. In PBMCs of patients with newly diagnosed T1D miR146a was significantly downregulated and the decreased miRNA expression was significantly associated with high GAD autoantibody titers in the serum [51] while an upregulation of miR326 correlated with the presence of autoantibodies against GAD and IA2 [47].

So far, few studies have investigated miRNA levels in individuals with islet autoimmunity before the onset of clinical T1D. The comparison of serum miRNAs in high-risk individuals positive for multiple islet autoantibodies, age-matched healthy children and recent-onset T1D patients revealed similar miRNA levels in the high-risk group and the healthy controls and no specific miRNA profile was identified for the high-risk group. In addition, high-risk individuals progressing to clinical disease could not be distinguished from non-progressors based on serum miRNA expression. However, serum miRNAs from high-risk individuals correlated with glycemic status and ongoing islet autoimmunity, since several miRNAs were associated to glucose homeostasis and autoantibody titers [52]. The analysis of serum miRNA levels of a large cohort of 150 autoantibody-positive and 150 autoantibody-negative family-matched siblings revealed several miRNAs reflecting islet autoimmunity and progression to T1D with miR21-3p, miR29a-3p and miR424-5p showing the most robust associations [53]. Nevertheless, at present it seems doubtful that circulating miRNAs hold much power in T1D risk assessment.

A considerable number of studies analyzed miRNA expression in whole blood or serum of patients to reveal differentially expressed miRNAs which may function as potential disease biomarkers and give insight into the mechanisms underlying disease pathogenesis and progression. Theses samples are readily available, but there are also considerable limitations: especially in organ-specific disease profiles from whole blood or serum might not reflect the situation in the affected organ. In addition, changes in miRNA profiles of these highly diverse, mixed populations of hematopoietic cells may be due to changes in the abundance or miRNA expression of specific cell subsets, rather than global changes in miRNA expression. Therefore, miRNA expression and function in cell types directly involved in autoimmune pathogenesis, such as Tregs, are of great interest and can provide considerable insight into the underlying mechanisms. Furthermore, miRNA expression profiles of specific immune cell subsets, in whole blood or ideally in the organ-specific context, are essential to validate circulating miRNAs as potential biomarkers and gain further insight into their contribution to onset and progression of autoimmunity.

4.2. miRNA regulation of Treg differentiation and function

The essential role of miRNAs for Treg development, homeostasis and function has been highlighted by the Treg-specific ablation of Dicer or Drosha in mouse models. Although Treg development in the thymus was unaffected, the lineage-specific miRNA deficiency resulted in disturbed Treg homeostasis, reduced suppressive function and fatal systemic autoimmune disease [54–57]. Further studies identified specific miRNAs and their contribution to these defects, including effects on thymic Treg development, Treg induction, Foxp3 expression, Treg stability and suppressive function.

miR155 is highly expressed in Tregs and directly regulated by Foxp3. Murine miR155 deficiency resulted in a reduced abundance of thymic and splenic Tregs as a consequence of impaired Treg development and homeostasis [58,59]. In miR155-deficient Tregs the expression of Foxp3 is reduced and instable while *in vitro* Treg induction is unaffected. miR155 targets suppressor of cytokine signaling 1 (SOCS1), a negative regulator of STAT5 signaling which determines the responsiveness to IL2, a critical regulator of Treg homeostasis [60]. However, miR155-deficient Tregs can prevent autoimmune diseases in mice, indicating that miR155 does not directly affect Treg suppressive function [59].

The *in vitro* Treg induction in presence of TGFβ is also dependent on proper regulation by miRNAs. Using naive $CD4^+$ T cells from both T cell-specific Dicer and Drosha deficient mice resulted in a significantly reduced expression of Foxp3 in induced Tregs compared to wildtype mice [54,57]. An extensive miRNA screen identified multiple miRNAs with both positive and negative regulatory effects on *in vitro* Treg induction [61]. Several miRNAs form networks to cooperatively regulate Treg induction, for example miR150 reduced the abundance of mTOR only in presence of miR99a and there was a similar cooperation between miR15a-16 and 15b-16. The PI3K/Akt/mTOR pathway is also a target of miR126. Specifically, miR126 reduces the expression p85β, a regulatory subunit of PI3K, limiting the activity of the PI3K/Akt/mTOR pathway and favoring Treg induction. The inhibition of miR126 enhanced the activity of the PI3K/Akt/mTOR pathway, diminishing Foxp3 expression and Treg induction [62]. In line with its role for thymic Treg generation miR155 is also supports proper Treg induction *in vitro* by targeting SOCS1 [63].

The above-mentioned miRNA screen also revealed miRNAs with a negative effect on *in vitro* Treg induction [61]. miR17 and miR19, members of the miR17∼92 cluster, function as negative regulators of Treg induction while being dispensable for thymic Treg development [64]. miR17 directly targets the TGFβ-receptor II and the cAMP-responsive element binding protein 1 (CREB1), both involved in proper Treg induction. The TGFβ signaling pathway is also a target of the miR23-miR27-miR24 cluster, and consequently over-expression of this cluster impairs Treg induction [65].

miR10a is a Treg-specific miRNA which is induced following retinoic acid exposure and high levels of miR10a correlate with low autoimmune diseases susceptibility in mouse models [66,67]. miR10a targets several effector T cell genes including *Bcl6* and *Ncor2*, thereby stabilizing the Treg-specific gene expression signature. The function of miR10a seems to overlap with other miRNAs since miR10a deficiency does not result in impaired Treg function or autoimmunity.

4.3. Role of T cell specific miRNAs in autoimmune activation

The studies described above have provided considerable insight into the contribution of specific miRNAs to immune and Treg function. However, the precise mechanisms of how they affect signaling pathways in Tregs, leading to islet autoimmunity remain to be elucidated. As described earlier, impairments in Treg function seem to be a pivotal contributor to insufficient tolerance induction and the onset of islet autoimmunity. Recently, our lab has reported a direct link between the upregulation of two specific miRNA in T cells and impairments in Treg induction from naive $CD4^+$ T cells during the onset of islet autoimmunity.

4.3.1. miR181a

Serr et al. provide evidence for impaired Treg induction capacity in naive $CD4^+$ T cells from children with recent onset of islet autoimmunity compared to children without islet autoantibodies or with long term islet autoimmunity without developing clinical T1D. The reduced Treg induction was accompanied by enhanced frequencies and proliferation of $Foxp3^{int}CD4^+$ T cells, pointing towards increased T cell activation, which interferes with subimmunogenic stimulation and efficient Treg induction. Increased T cell activation could be linked to differential miRNA expression during onset of islet autoimmunity, specifically to enhanced levels of miR181a which modulates the

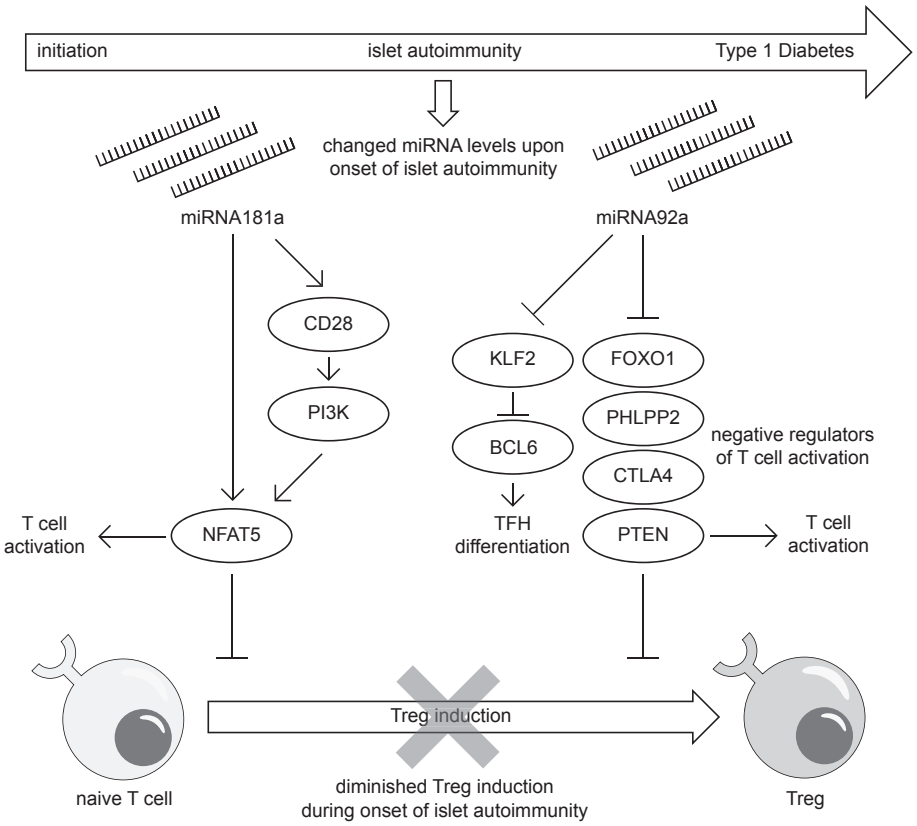

Figure 1: Role of T cell specific miRNAs in autoimmune activation. High levels of miR181a and miR92a contribute to enhanced T cell activation and impairments in Treg induction from naive CD4$^+$ T cells during the onset of islet autoimmunity. PI3K: phosphatidylinositol-3-kinase, NFAT5: nuclear factor of activated T cells 5, KLF2: krueppel-like factor 2, BCL6: B-cell lymphoma 6, FOXO1: forkhead box protein O1, PHLPP2: PH domain and leucine rich repeat protein phosphatase 2, CTLA4: cytotoxic T-lymphocyte associated protein 4, PTEN: phosphatase and tensin homolog.

sensitivity to antigenic stimulation and alters signaling thresholds in CD4$^+$ T cells [68]. Consequently, the inhibition of miR181a enhanced murine and human Treg induction, whereas a miR181a mimic had the opposite effect. High levels of miR181a were accompanied by an increased abundance of nuclear factor of activated T cells 5 (NFAT5) which plays an important role in T cell activation [69]. Furthermore, the negative regulator of T cell activation, PTEN, was identified as an additional target of miR181a and its suppression promoted the activation of PI3K signaling, culminating in the activation of NFAT5. Consequently, elevated miR181a levels in CD4$^+$ T cells from children with recent onset of islet autoimmunity are accompanied by a decrease in PTEN and an increase in NFAT5 expression, leading to T cell activation and impaired Treg induction. Moreover, high levels of miR181a promoted the expression of the costimulatory molecule CD28 in CD4$^+$ T cells from children with ongoing islet autoimmunity which further contributed to activation of the PI3K pathway [28], NFAT5 upregulation and T cell activation.

Additional studies in NOD mice with recent development of islet autoantibodies (IAA) revealed impaired Treg induction capacity and high levels of miR181a and NFAT5 levels, as well as decreased levels of PTEN. The *in vivo* blockade of miR181a in IAA$^+$ NOD mice resulted in significantly reduced CD28 and NFAT5 expression, enhanced PTEN levels and reduced islet autoimmunity. Using an NFAT5 inhibitor or NFAT5 null mice enhanced Treg induction, and CD4$^+$ T cells showed increased levels of PTEN and also Foxo1, which is directly involved in the positive regulation of Treg differentiation [27].

These studies show that the dysregulation of an individual miRNA and the downstream signaling pathway resulting in NFAT5 upregulation critically contribute to an impairment of Treg induction during islet autoimmunity (Figure 1).

4.3.2. miR92a

In a second study, the investigation of the effects of miR92a on T follicular helper (TFH) cell regulation in islet autoimmunity revealed important insights into the regulation of Treg induction by this miRNA [11]. TFH cells are a subset of CD4$^+$ T cells which are an essential part of humoral immunity by providing support to B cells to produce high-affinity antibodies [70]. The precursors of TFH cells circulate in the blood and their function of inducing antibody responses also suggests a critical contribution to the development of autoimmune diseases [71,72]. miR92a belongs to the miR17∼92 cluster which is involved in the onset of autoimmunity and antibody production in mice [73]. This provided the rationale for a miRNA screen to investigate T cell-specific miRNAs in children with ongoing islet autoimmunity compared to healthy controls. miR92a was significantly upregulated in CD4$^+$ T cells from children with ongoing islet autoimmunity, and the confirmation using qPCR showed that this increase was restricted to children with recent onset of islet autoimmunity whereas children with long-term autoimmunity showed miR92a levels comparable to healthy controls. Furthermore, the abundance of miR92a correlated with the frequency of TFH cell precursors in the peripheral blood. Inhibition of miR92a function resulted in decreased TFH cell induction *in vitro*, whereas a

miR92a mimic increased TFH cell formation. Increased miR92a activity resulted in the downregulation of PTEN, PHLPP2, FOXO1 and CTLA4 which are negative regulators of T cell activation and known targets of miR92a. The reduced abundance of PTEN resulted in the activation of the PI3K/Akt/mTOR pathway which interferes with Treg induction. Consequently, *in vitro* Treg induction was significantly impaired in the presence of a miR92a mimic, and high miR92a levels in children with recent onset of islet autoimmunity are accompanied by reduced frequencies of insulin-specific Treg. Conversely, PI3K signaling promotes TFH cell induction, since the effect of a miR92a mimic is reduced in the presence of a PI3K inhibitor and increased when PTEN signaling is inhibited. KLF2 was shown to interfere with TFH cell differentiation by induction of S1pr1 expression and inhibition of the TFH master regulator BCL6 via upregulation of BLIMP1 [74]. The study revealed KLF2 as a novel target of miR92a, thereby offering an additional mechanism of miR92a-mediated TFH cell differentiation.

In conclusion, this work provided evidence that high levels of miR92a during onset of islet autoimmunity mediate the induction of TFH precursors as well as impaired Treg induction from naive $CD4^+$ T cells, two mechanisms that are likely involved in the onset and progression of islet autoimmunity (Figure 1).

5. TARGETING MIRNAS TO REESTABLISH TREG HOMEOSTASIS IN ISLET AUTOIMMUNITY

Multiple studies show the importance of miRNAs for immune homeostasis and highlight how miRNA dysregulation can contribute to various diseases including autoimmunity. miRNAs can be easily targeted using highly specific inhibitors, while mimics can be used to increase their activity, making miRNAs promising novel potential drug targets. miRNA-targeting agents have been successfully used *in vitro*, in mouse models of autoimmune diseases, and in humanized mice [11,13], resulting in downregulation of the respective miRNA targets, improvements in Treg function and reduced islet autoimmunity. Of note, a miRNA inhibitor to treat HCV infection has been successfully tested in a clinical trial [75]. Unfortunately, the treatment of an organ-specific autoimmune diseases such as T1D will required the delivery of miRNA-targeting drugs to the desired cell type, which remains a major challenge [76]. However, the use of nanoparticles and encapsulation of oligonucleotides significantly improved their uptake by lymphocytes as intended [77]. The specific targeting of T cells or even T cell subsets can be facilitated using combinations of antibodies and nanoparticles or single chain fusion antibodies which already have been used successfully for T cell specific delivery in humanized mice [78]. Despite these considerable advancements, therapeutic T cell specific targeting of miRNAs remains challenging and will require the development of additional targeted strategies. In particular, the optimization of both specificity and efficiency is crucial and will facilitate the translation of mechanistic insights into therapeutic strategies to delay or even prevent the onset of autoimmunity.

6. CONCLUSIONS

To gain insight into the presymptomatic phase of T1D and to identify the mechanisms underlying the onset of islet autoimmunity and the progression to T1D remain critical steps towards prevention and/or treatment of T1D and other autoimmune diseases. Several studies have shown that miRNAs play crucial roles in Treg induction and function and immune tolerance. Importantly, even the targeting of individual miRNAs resulted in relevant changes in gene expression and effects on downstream signaling pathways as well as improvements of islet autoimmunity in mouse models. Therefore, the targeting of individual miRNAs or a combination of several miRNAs with improved, highly specific and efficient delivery techniques, could reestablish immune homeostasis and ultimately interfere with the onset of islet autoimmunity. At present, miRNAs as potential biomarkers to predict disease onset and progression require further validation, including strategies to confirm that circulating miRNAs reflect the relevant alterations in individual T cell subsets in the periphery or the affected organ. The development and validation of future treatment approaches will require optimized humanized mouse models to mimic the mechanisms underlying human autoimmune diseases in a preclinical model. These models are valuable tools to underpin findings based on both murine and human studies and to considerably advance the field towards novel translational treatment strategies based on specific miRNA targeting.

CONFLICT OF INTEREST

None.

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