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Understanding mechanisms of immune regulations in islet autoimmunity

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Abstract

Our immune system is tightly regulated by a balance between immunity and immunological tolerance. As part of peripheral tolerance mechanisms, regulatory T cells (Tregs) play an essential role as cellular mediators and consequently, their dysregulation has been implicated in the development of autoimmune diseases. In autoimmune Type 1 Diabetes (T1D), loss of tolerance to insulin-producing beta cells concomitant with an aberrant activation of autoreactive T cells destroying these cells lead to the initiation of islet autoimmunity. Therefore, therapeutic strategies aiming at restoring this balance by the induction of Tregs are intensively studied. Multiple impairments in Treg function, stability and induction during islet autoimmunity have been linked to T1D pathogenesis. Additionally, several studies could show that high T cell activation, as during islet autoimmunity, impairs an efficient Tregs induction. Therefore, to overcome limitations of strong T cell activation novel targetable pathways during early stages need to be identified which can serve as efficient Treg inducers.

In recent years, microRNAs (miRNAs) have gained much of attention as poten-tial therapeutic agents since they are able to regulate complex cellular states in different immune cells, among others Tregs. Due to their complex regulatory network, it is essential to decipher potential upstream regulators. In this regard, it has been shown that *all-trans* retinoic acid (atRA) strongly induces the expression of miR-99a-5p in Tregs. Notably, one major target of this miRNA is mTOR, a well-known negative regulator of Treg cell differentiation. Interestingly, studies from our group could show decreased expression of miR-99a-5p in naïve T cells of patients with established T1D. Moreover, Treg induction has been demonstrated to be significantly impaired in children with recent onset of islet autoimmunity. Therefore, for the first part of this thesis I sought to dissect a potential link between the atRA-mediated increase in miR-99as-5p expression in inducing Tregs during islet autoimmunity.

Following up on the finding that miR-99a-5p shows very low expression in naïve T cells from individuals with T1D, I first studied the expression patterns of this miRNA in T cells from individuals with different stages of islet autoimmunity. Interestingly, I could see an increase in expression in naïve T cells of non-diabetic individuals with long-term islet

autoimmunity as well as patients with short-term duration of T1D. Likewise in the murine setting of T1D there was an increase in its expression which declined again in the course of islet autoimmunity. These first data suggested that miR-99a-5p might play important roles in ongoing tolerance mechanisms at early stages of disease and hence, it might offer an interesting target for immunotherapies. To get more insights in its regulatory network, I performed several *in vitro* Treg induction assays with stimulation of its upstream regulator atRA. Importantly, I could show that atRA is able to significantly increase Treg induction capacity in T cells from autoimmune-prone NOD mice compared to control Balb/c in spite of challenging conditions. I could show that the atRA-mediated effect was at least in parts dependent on miR-99a-5p expression, as evidenced by *in vitro* miRNA modulation assays. *In vivo*, atRA treatment led to a significant reduction in infiltrating lymphocytes and a strong enhancement in Tregs with a superior anti-inflammatory and suppressive phenotype in NOD mice, thereby further supporting its well-known role as inducer of immune tolerance. Moreover, I could demonstrate that there were first hints of a positive correlation between Treg frequencies and the expression of miR-99a-5p in T cells of the target tissue of atRA-treated NOD mice, suggesting that atRA acts via miR-99a-5p to promote tolerogenic responses during ongoing islet autoimmunity.

To sum up, I could provide evidence for a link between atRA-mediated miR-99a-5p upregulation and enhanced Treg induction during islet autoimmunity. Targeting this new signaling pathway opens a new opportunity to develop therapeutic strategies with the vision to restore the balance of immunity and immune tolerance in T1D.

The translation of findings derived from the NOD mouse model to the human settings still hinders the development of efficient and safe therapeutic approaches. Furthermore, the vast heterogeneity in disease progression from the onset of islet autoimmunity to the development of clinical T1D, calls for a better understanding of the plasticity in immune activation and tolerance in the different disease stages. For this purpose, immunodeficient mice engrafted with a human immune system provide an excellent tool to study human immune responses *in vivo*.

Abstract

Thus, I aimed to apply two different approaches with the goal to model islet autoimmunity in immunodeficient mice. For the first approach I received patient-specific peripheral blood mononuclear cells (PBMCs). Here, I could show some first insights in the different immune responses in humanized mice reconstituted with donors at different stages of islet autoimmunity. As a second approach, we sought to optimize conditions for the re-programming of patient-specific PBMCs into induced-pluripotent stem cells (iPSCs) and their further differentiation into hematopoietic stem cells (HSCs) with the goal to generate a 'truly' humanized mouse mode. I could demonstrate that we were able to successfully generate patient-specific iPSCs which were further differentiated into HSCs. This mouse model will provide an excellent tool to analyze the dialogue between the human immune system and disease-specific target tissues for long-term studies. Furthermore, these mice can be used as drug testing platform or to develop novel immunotherapeutics.

Zusammenfassung

Unser Immunsystem wird durch ein Gleichgewicht zwischen Immunität und immunologischer Toleranz streng reguliert. In peripheren Toleranzmechanismen spielen regulatorische T-Zellen (Tregs) eine wesentliche Rolle als zelluläre Mediatoren, und folglich wurde ihre Fehlregulation mit der Entwicklung von Autoimmunerkrankungen in Verbindung gebracht. Bei autoimmunem Typ-1-Diabetes (T1D) führt der Verlust der Toleranz gegenüber insulinproduzierenden Betazellen zu einer fehlgeleiteten Aktivierung von autoreaktiven T-Zellen, die diese Zellen zerstören, und demnach zur Initiierung der Inselautoimmunität führt. Daher werden intensiv neue, therapeutische Strategien untersucht, die darauf abzielen, dieses Gleichgewicht durch die Induktion von Tregs wiederherzustellen. Mehrere Beeinträchtigungen der Funktion, Stabilität und Induktion der Tregs während der Inselautoimmunität wurden mit der Entstehung von T1D in Verbindung gebracht. Darüber hinaus konnten mehrere Studien zeigen, dass eine hohe T-Zell-Aktivierung, wie sie während der Inselautoimmunität auftritt, die effiziente Induktion von Tregs beeinträchtigt. Um die Einschränkungen wegen einer starken T-Zell-Aktivierung zu überwinden, müssen daher in frühen Stadien neue zielgerichtete Signalwege identifiziert werden, die als effiziente Induktoren von Tregs dienen können.

In den letzten Jahren haben microRNAs (miRNAs) als potenzielle Immunregulatoren viel Aufmerksamkeit erlangt, da sie in der Lage sind, komplexe zelluläre Zustände in verschiedenen Immunzellen, unter anderem Tregs, zu regulieren. Aufgrund ihres komplexen Regulierungsnetzwerks ist es unerlässlich, potenzielle vorangeschaltene Regulierungen zu entschlüsseln. Diesbezüglich wurde gezeigt, dass *all-trans* Retinsäure (atRA) die Expression von miR-99a-5p in Tregs stark induziert. Angriffspunkt dieser miRNA ist insbesondere mTOR, ein bekannter negativer Regulator der Treg-Zelldifferenzierung. Studien aus unserer Gruppe konnten in diesem Zusammenhang eine verminderte Expression von miR-99a-5p in naiven T-Zellen von Patienten mit etabliertem T1D zeigen. Weiters wurde gezeigt, dass die Treg-Induktion bei Kindern mit kürzlich aufgetretener Inselautoimmunität signifikant beeinträchtigt ist. Daher habe ich im ersten Teil dieser Arbeit versucht, einen möglichen Zusammenhang zwischen der

atRA-vermittelten Zunahme der Expression von miR-99as-5 bei der Induktion von Tregs während der Inselautoimmunität herzustellen.

Darauf aufbauend, dass miR-99a-5p eine sehr geringe Expression in naiven T-Zellen von Personen mit T1D zeigt, untersuchte ich zunächst die Expressionsmuster dieser miRNA in verschiedenen Stadien der Insel-Autoimmunität. Hier konnte ich eine Zunahme der Expression in naiven T-Zellen von nicht-diabetischen Personen mit langfristiger Inselautoimmunität sowie von Patienten mit kurzzeitiger T1D-Dauer feststellen. Auch im murinen Setting von T1D kam es zu einer Zunahme seiner Expression, die im Verlauf der Inselautoimmunität aber wieder abnahm.

Diese Daten deuten darauf hin, dass miR-99a-5p eine wichtige Rolle bei laufenden Toleranzmechanismen in frühen Krankheitsstadien spielen könnte und daher ein interessantes Ziel für Immuntherapien darstellen könnte. Um mehr Einblicke in das regulatorisches Netzwerk zu erhalten, führte ich mehrere In-vitro-Treg-Induktionsexperimente mit Stimulation seines vorangeschaltene Regulators atRA durch. Ich konnte zeigen, dass atRA in der Lage ist, die Treg-Induktionskapazität in autoimmunanfälligen NOD-Mäusen im Vergleich zu Kontrollmäusen trotz schwieriger Bedingungen signifikant zu erhöhen. Ich konnte weiters zeigen, dass die atRA-vermittelte Wirkung zumindest teilweise von der miR-99a-5p-Expression abhängig war, wie durch in-vitro-miRNA-Modulationsassays belegt wurde. *In vivo* führte die atRA-Behandlung zu einer signifikanten Verringerung der infiltrierenden Lymphozyten und einer starken Erhöhung der Tregs mit einem markanten entzündungshemmenden und suppressiven Phänotyp bei NOD-Mäusen. Dadurch wurde die Rolle von atRA als Induktor der Immuntoleranz weiter unterstützt. Darüber hinaus konnte ich zeigen, dass es erste Hinweise auf eine positive Korrelation zwischen Treg-Frequenzen und der Expression von miR-99a-5p in T-Zellen des Zielgewebes von atRA-behandelter NOD-Mäuse gibt. Dies deutet darauf hin, dass atRA über miR-99a-5p zur Förderung tolerogener Reaktionen während einer andauernden Inselautoimmunität wirkt.

Zusammenfassend konnte ich einen Zusammenhang zwischen atRA-vermittelter Hochregulierung von miR-99a-5p und verstärkter Treg-Induktion während Inselautoimmunität zeigen. Die Entdeckung dieses neuen Signalwegs ermöglicht es

neue therapeutische Strategien mit dem Ziel, das Gleichgewicht von Immunität und Immuntoleranz bei T1D wiederherzustellen, zu entwickeln.

Die Übertragung von Erkenntnissen aus dem NOD-Mausmodell auf den Menschen erschwert noch immer die Entwicklung effizienter und sicherer Therapieansätze. Darüber hinaus erfordert die große Heterogenität des Krankheitsverlaufs vom Beginn der Inselautoimmunität bis zur Entwicklung des klinischen T1D ein besseres Verständnis der Plastizität der Immunaktivierung und Toleranz in den verschiedenen Krankheitsstadien. Zu diesem Zweck bieten immundefiziente Mäuse, denen ein menschliches Immunsystem eingepflanzt wurde, eine hervorragende Möglichkeit, um menschliche Immunantworten *in vivo* zu untersuchen.

Hierfür war es mein Ziel, zwei verschiedene Ansätze anzuwenden, um die Insel-Autoimmunität in immundefizienten Mäusen zu modellieren. Für den ersten Ansatz erhielt ich patientenspezifische periphere mononukleäre Blutzellen (PBMCs). Hier konnte ich einige erste Einblicke in die unterschiedlichen Immunantworten in humanisierten Mäusen zeigen, die mit Spendern in verschiedenen Stadien der Inselautoimmunität rekonstituiert wurden. Als zweiten Ansatz versuchten wir, die Bedingungen für die Umprogrammierung patientenspezifischer PBMCs in induzierte pluripotente Stammzellen (iPSCs) und deren weitere Differenzierung in hämatopoetische Stammzellen (HSCs) zu optimieren, mit dem Ziel, ein krankeitsrelevantes humanisiertes Mausmodell zu generieren. Ich konnte zeigen, dass wir erfolgreich patientenspezifische iPSCs generieren konnten, die weiter in HSCs differenziert wurden. Dieses Mausmodell wird aufbauend auf weiteren Optimierungen eine hervorragende Methode darstellen, um den Dialog zwischen dem menschlichen Immunsystem und krankheitsspezifischen Zielgeweben für Langzeitstudien zu analysieren. Darüber hinaus können diese Mäuse als Plattform für Arzneimitteltests oder zur Entwicklung neuartiger Immuntherapeutika verwendet werden.

List of abbreviations

3’ UTR	3’ untranslated region
AP-1	Activating protein–1
atRA	all-*trans* retinoic acid
APCs	Antigen presenting cells
API	Autoimmune protocol
ATG	Antithymocyte globulin
BCR	B cell receptor
CAR T cells	Chimaeric antigen receptor T cells
CAAR T cells	Chimaeric autoantigen receptor T cells
CMML	Chronic myelomonocytic leukemia
CNS2	Conserved non-coding sequence 2
CRS	Cytokine Release Syndrome
CTLA-4	Cytotoxic T-lymphocyte-associated protein 4
DCs	Dendritic cells
EAE	Experimental autoimmune encephalomyelitis
FDA	Food and Drug Administration
Foxp3	Forkhead box protein 3
GWAS	Genome-wide association studies
GAD	Glutamic acid decarboxylase
GLP-1	Glucagon-like peptide-1
GvHD	Graft-vs.-Host-Disease
HCV	Hepatitis C virus
HE	Hemogenic endothelium
HSCs	Hematopoietic stem cells
HSPCs	Hematopoietic stem/progenitor cells
HLA	Human leukocyte
iPSC	Induced pluripotent stem cells

List of abbreviations

iTregs	Induced Tregs
IA2	Inulinoma-antigen 2
IAA	Insulin autoantibody
IBD	Inflammatory bowel disease
IL-2	Interleukin 2
i.p.	intraperitoneal
IPEX	Immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome
lncRNAs	long-non-coding RNAs
mRNA	Messenger RNA
MS	Multiple Sclerosis
MG	Myasthenia gravis
miRNAs	MicroRNAs
NK cells	Natural killer cells
NFAT5	Nuclear factor of activated T cells 5
NSG	NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tmWjl}/Sz
NOD	Non-obese diabetic
PD-1	Programmed-cell-death protein 1
PBMCs	Peripheral blood mononuclear cells
pre-miRNAs	Precursor miRNAs
pri-miRNAs	Primary miRNAs
Tregs	Regulatory T cells
SCID	Severe combined immunodeficiency
SLE	Systemic lupus erythematosus
STAT5	Signal transducer 5
TCR	T cell receptor
TGF-β	Transforming growth factor β
T1D	Type 1 Diabetes
TNF-α	Tumor necrosis factor α
ZnT8	Zinc transporter 8

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1. Introduction

1.1. Immune system

The immune system comprises a complex network of organs, cells and proteins with the task to defend our body against infections while sparing our own tissues. Thus, the immune response is the reaction we make against potential pathogens. Here, we have two major arms of defense mechanisms: adaptive immune response and innate immunity. The latter is immediately available and combats most of the pathogens that reach our body without any specificity. The adaptive immune response, however, develops over lifetime in course of adaption to infections, and thereby creating an immunological memory.

Since lymphocytes are generated constantly during lifetime, it is inevitable that some of them are potentially dangerous and recognize an individual's own self-antigen. A critical part of the immune response is therefore to distinguish between "self and non-self", which is exerted by two distinct mechanisms: recessive and dominant immunological tolerance.

1.1.1. Immunological tolerance

Recessive tolerance is the first arm of T cell education during their development in the thymus, which is important to eliminate autoreactive T cell receptors (TCRs). Lymphocytes that bind strongly to self-antigens during the process of somatic rearrangement [1, 2] are deleted by clonal deletion [3]. However, some of these autoreactive T cells manage to escape the negative selection process in the thymus and migrate to the periphery. Here, the second arm of immunological tolerance – dominant tolerance – assists in taming these autoreactive T cells. One important cellular mediator of dominant tolerance are $CD4^+CD25^+$ regulatory T cells (Tregs) [4].

1.1.2. Regulatory T cells as mediators of peripheral tolerance

Tregs are able to suppress the effector function, activation and proliferation of several immune cells including T cells, B cells, natural killer (NK) cells as well as antigen presenting cells (APCs) [5, 6]. They exert their suppressive function via different ways: direct killing of effector T cells through lytic mechanisms [7, 8], secretion of inhibitory cytokines (e.g. IL-10) [9] and/or transforming growth factor β (TGF-β) [10, 11], or

metabolic deprivation [12]. Importantly, Tregs either develop in the thymus [13, 14] or are induced in the periphery from naïve $CD4^+$ T cells [15-17]. Characteristic for Tregs are the expression of the high-affinity α chain of the interleukin 2 (IL-2) receptor (CD25) [4] and their master transcription factor Forkhead box protein 3 (Foxp3) [18-21], and in the human setting the absence of CD127 [22].

Their vital role in peripheral tolerance is emphasized by the fact that mutations in the FOXP3 gene lead to a fatal autoimmune phenotype, which occurs both in mice (scurfy mice) and humans (IPEX – immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome) [20, 23].

1.2. Autoimmunity

In an autoimmune setting, the immune system is directed against cells from our own body, initiated by the aberrant activation of autoreactive T cells concomitant with a breakdown of immunological tolerance. Autoimmunity is often characterized by different stages of initiation and propagation and some of them even include stages of remission [24]. There are two major forms of autoimmune diseases: systemic and organ-specific. Type 1 Diabetes (T1D) is one of the most frequent forms of autoimmune diseases that are directed against only one specific organ. T1D afflicts approximately 5% of all diabetes cases, with an overall prevalence of 200 million individuals worldwide and the numbers are increasing yearly (https://diabetesatlas.org/). This disease predominantly occurs in the youth (80%).

In autoimmune T1D the aberrant immune response is directed against the insulin-producing beta cells in the pancreatic islets. Autoreactive T cells infiltrate the pancreas and initiate the onset of islet autoimmunity. Thereafter the progressive destruction of beta cells leads to overt T1D with its symptoms of glucose intolerance and the necessity for patients suffering to administer exogenous insulin throughout their lifetime.

1.2.1. Stages of autoimmune T1D disease progression

During the past years, it has become clear that T1D can be classified into different disease stages ranging from a pre-symptomatic stage (stage 1) to the clinical, symptomatic disease (stage 3) (Fig. 1).

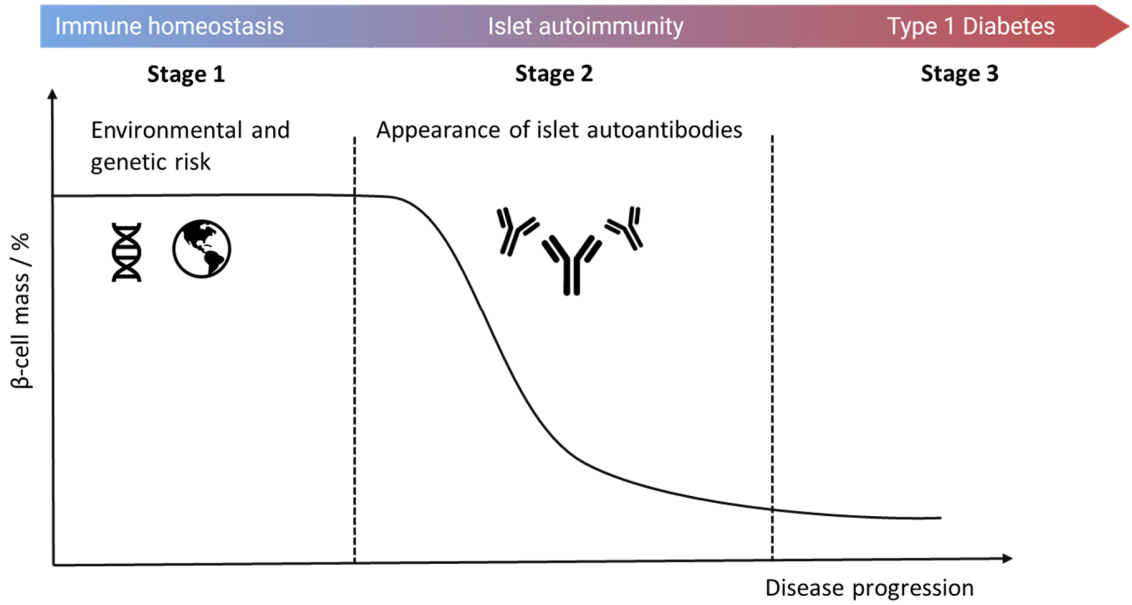


Figure 1: Stages of T1D. The graph shows beta cell mass in % to indicate relevant disease stages. Adapted from Katsarou *et al.* [25].

It is now well established that T1D develops in individuals with a genetic predisposition (stage 1). Specifically, genome-wide association studies (GWAS) mapped a strong association to human leukocyte (HLA) class II molecules, with HLA-DR/DQ haplotypes being the most robust predictor of risk development [26-28]. Additionally, environmental stimuli such as diet or virus infections trigger the development of islet autoimmunity.

Islet autoimmunity (stage 2), refers to the pre-symptomatic phase of the disease and is characterized by the presence of one or more islet autoantibodies [29] marking the onset of the autoimmune process. So far, four islet autoantigens have been identified: insulin [30], glutamic acid decarboxylase (GAD) [31], inulinoma-antigen 2 (IA2) [32, 33], and zinc transporter 8 (ZnT8) [34]. Importantly, islet autoimmunity occurs already very early in life, with a peak incidence of 2 to 3 years of age. Due to this early onset of autoantibody seroconversion, primary prevention studies in children with yet no signs of autoimmunity have to be conducted with extreme caution.

Throughout stage 2 the gradual destruction of pancreatic beta cells leads to dysglycemia, eventually leading to stage 3 with the onset of clinical, symptomatic T1D.

Despite these advances in T1D disease characterization, the underlying mechanisms leading to the development of islet autoimmunity remain still poorly understood. Moreover, the rate of progression from the onset of islet autoimmunity to the clinical disease varies widely among all individuals. At autoantibody-positivity, there are children that progress to the symptomatic disease only within some months or a few years, whereas others remain diabetes-free for more than a decade, despite ongoing islet autoimmunity, as evidenced by the presence of multiple islet autoantibodies [35, 36].

Given this vast heterogeneity in disease progression, a concept of disease endotypes has been developed and further investigated by several groups, allowing to tailor therapeutic interventions to a given disease stage [37, 38]. In 2022, a group could identify several parameters being substantially different among groups stratified based on their autoantibody status, including among others genetics, inflammatory markers, 25-OH-Vitamin D_3 and insulin autoimmunity [37]. Interestingly, especially in young individuals with diabetes they could find increased inflammatory signaling pathways which could be used as target for therapeutic interventions. In line with this finding, in previous work our group could show an increase in the frequency of insulin-specific Tregs in children harboring multiple autoantibodies for more than 10 years without progression to the clinical disease, thereby demonstrating that high frequencies of disease-relevant Tregs associate with a slower disease progression. This highlights the potential of antigen-specific Treg induction for immunotherapies of autoimmune diseases [39].

1.2.2. Immunotherapies for T1D

Despite major advances in insulin administration and glucose monitoring, many patients with T1D do not meet the recommended blood glucose levels. Fluctuating waves of hyper- and hypoglycemia have a great impact on the patient's life quality and life expectancy. Even though our understanding of disease pathogenesis has greatly improved over the past years, there are still no effective immunotherapies for the treatment or prevention of the disease. Although, various nonspecific immunotherapies for the treatment of T1D have been investigated in clinical trials, potential side effects and limited efficacy have are major hurdles [40-42].

A major breakthrough was the approval by the US FDA in 2022 of an anti-CD3 antibody as first immunomodulating drug for the treatment of T1D [43]. Dating back to 1992, a group identified a non-activating (i.e. prevention of binding to the Fc receptor) form of anti-CD3 monoclonal antibody (teplizumab) as potential target for T-cell mediated immunotherapy. The treatment of mice with this modified anti-CD3 monoclonal antibody prevented or reverted disease onset [44]. In a randomized, controlled, phase 1-2 trial of a humanized anti-CD3 molecule, they tested the efficacy of this therapy in patients with new-onset T1D. They could find improvements in metabolic control during the first years of disease in most of their patients, possibly due to its direct effects on $CD8^+$ T cells [45].

Given these promising results, the group investigated the effect of teplizumab in individuals with high-risk of developing T1D. Accordingly, they could show that teplizumab was able to delay disease onset in their high-risk study participants [43]. Based on the approval by the US FDA the drug will be applied for adults or pediatric individuals that are in stage 2 T1D in order to delay the onset of stage 3 T1D. Even though anti-CD3 treatment has not led to major side effects in the clinical trials, the treatment with general immunosuppressive agents still harbors the risk of serious side effects. This is especially important when aimed at primary or secondary intervention, early in the disease process, which is, in the majority of cases, very early in life. Especially for early prevention efforts the restoration of immune tolerance by fostering of Tregs is of great potential since it is more targeted and reduces the risk of severe side effects.

1.3. Fostering Tregs during ongoing islet autoimmunity

Given their aforementioned essential role as mediators of peripheral tolerance, modulating Treg activity as therapeutic intervention for autoimmune diseases has also gained interest in the past. Especially their antigen-specific induction is of great interest due to its safe and specific action avoiding general immune suppression. However, we are just starting to understand the requirements for efficient *de novo* Treg induction, especially in settings of autoimmunity.

1.3.1. Challenges for antigen-specific Treg induction in T1D

Tregs can be induced *in vivo* and *in vitro* by stimulation through the TCR in combination with TGF-β treatment or by limiting the activation through subimmunogenic (i.e. non-activating) TCR stimulation. Importantly, Tregs induced *in vitro* with TGF-β display and unstable phenotype, as they lose Foxp3 expression after expansion and *in vivo* injection. *In vivo*, the most efficient conversion of naïve T cells into induced (iTreg) cells can be achieved through the systemic, subimmunogenic (i.e. non-activating) delivery of strong-agonistic TCR ligands [16, 46-51].

The therapeutic potential of their *in vivo* induction has been demonstrated in a study in 2011, where subimmunogenic delivery of strong-agonistic insulin mimetopes efficiently converted naïve T cells into Tregs in the non-obese diabetic (NOD) mouse model, resulting in protection from T1D development [52]. However, efficient Treg induction was only achieved in young NOD mice with rather moderate insulin autoantibody levels since increased autoantibody titers and hence, high immune activation was accompanied by higher frequencies of already activated insulin-specific T cells.

In this regard, we and others have shown that in states of increased immune activation (as during ongoing islet autoimmunity) enhanced signaling through the PI3K/AKT/mTOR pathway limits Tregs induction [39, 52-55]. Accordingly, children with recently developed islet autoimmunity or with overt T1D and thus, high T cell activation display markedly reduced Treg induction capacity. Of note, impaired Treg induction was not only seen in an antigen-specific manner, but was also observed for hemagglutinin-specific and polyclonal Treg induction. Additionally, we can also see these early impairments of Treg induction in T cells of young NOD mice, suggesting that defects at initial stages of islet autoimmunity might contribute to disease progression (unpublished data).

Apart from impairments in Treg induction, Tregs have been shown to become unstable during inflammation. Notably, proper Treg function requires a stable and long-term suppressor phenotype. This is partly ensured by the complete demethylation of a regulatory DNA region within the *Foxp3* gene which is called conserved non-coding sequence 2 (CNS2) [56, 57]. Our group could show elevated DNA methylation of the CNS2 region in NOD mice with islet autoimmunity and children with clinical T1D,

indicating an unstable Treg phenotype [58]. Importantly, increased methylation of the CNS2 region and hence, Treg instability was also already observed in infant NOD mice right after weaning.

Moreover, these unstable Tregs display some degree of plastic differentiation under certain inflammatory conditions as during islet autoimmunity [reviewed in [59]]. Accordingly, some Treg subtypes have been identified in T1D, which secrete pro-inflammatory cytokines characteristic for effector T cells [60-62]. In line with this, we observe plasticity in Tregs already at very early stages of islet autoimmunity in NOD mice (unpublished data).

Overall, these previous findings suggest that during ongoing islet autoimmunity, Tregs are greatly impaired in their function, stability, and induction capacity. Importantly, these defects are particularly observed at early stages of disease, highlighting the importance of considering preventive strategies for the administration of antigen-specific Treg therapies. However, for the development of efficient antigen-specific Treg induction therapies, these impairments need to be overcome and it is thus required to get a better understanding of the underlying mechanisms of defective Treg induction.

1.4. Small non-coding microRNAs as regulators of immune function

In the past years, the important roles of small non-coding microRNAs (miRNAs) in regulating several essential biological processes have emerged.

miRNAs belong to a family of small RNAs of about 22 nucleotides in length that are able to regulate gene expression by the inhibition of a target messenger RNA (mRNA). The majority of these miRNAs is transcribed from DNA sequences into primary miRNAs (pri-miRNAs). They are then further processed into precursor miRNAs (pre-miRNAs) and finally mature miRNAs. Mostly, these mature miRNAs bind to the 3' untranslated region (3' UTR) of their target mRNAs to trigger their degradation or translational silencing. Due to imperfect matching of the 3' UTR to the sequence of their target miRNA, one miRNA can have multiple targets.

Thus, it has become clear that they are able to regulate complex cellular states of T cells, including their development, differentiation and function, which has made them a desirable target for the development of novel immunomodulating therapeutics.

Accordingly, dysregulation of miRNA expression contributes to the development of several diseases, including autoimmunity where they contribute to aberrant immune activation and Treg impairments. In this regard, our group could provide evidence for the role of aberrantly expressed miRNAs in T cells of individuals with islet autoimmunity mediating impaired Treg induction.

For instance, in one study our group could show a link between miR-181a-mediated increased activation thresholds of T cells and consequently, impaired Treg induction capacity in T cells of non-diabetic individuals with recent onset of islet autoimmunity. Accordingly, blocking of this miRNA or its downstream target led to improved Treg induction *in vitro* and decreased islet autoimmunity *in vivo* in mouse models [55].

Importantly, another study demonstrated that aberrant expression of miR-142-3p in activated T cells from individuals with islet autoimmunity led not only to impairments in Treg induction but also to dysregulated DNA methylation at the *Foxp3* locus, hence destabalizing Tregs [58]. A targeting approach for this miRNA with a specific inhibitor led to improved Treg induction and stability *in vitro* and reduced murine islet autoimmunity *in vivo*.

The relevance of targeting miRNAs as therapy is further evidenced in several ongoing clinical studies that investigate the potential therapeutic effects of miRNA-based therapies [reviewed in: [63]]. For instance, one clinical trial has been conducted testing the effects of miravirsen, a target for miR-122, and its effects on levels of hepatitis C virus (HCV) RNA in patients with chronic hepatitis C [64].

However, due to the ubiquitous expression of miRNAs, cell-type specific targeting remains one of the major challenges in miRNA therapeutics. Understanding upstream regulation of miRNAs that might contribute to disease-related dysregulated pathways therefore opens up new opportunities for the development of specific targeting interventions.

1.4.1. External stimuli as upstream regulators of miRNA expression

Likewise, environmental stimuli such as diet are also considered as key drivers for the pathogenesis of T1D [65].

In this regard, Vitamins have gained much of attention since they can act as mediators of immune responses. Consequently, impaired Vitamin metabolism is linked to the development of several diseases. Specifically, the strong anti-inflammatory properties of Vitamin A on immunity have been long acknowledged [66] and the role of Vitamin A in regulating several biological processes such as embryonic development, visual cycle and immunity is well known [67-69]. Accordingly, synthetic analogues to retinol, called retinoids, are in use for treatment of dermatological disorders [68].

Of interest, several studies were able to show that individuals with T1D had lower circulating levels of Vitamin A compared to non-diabetic individuals, suggesting that Vitamin A with its potential anti-inflammatory properties might be important in T1D progression [70-72].

In this regard, one group investigated the role of Vitamin A or in its bioactive form, all-*trans* retinoic acid (atRA), in T1D pathogenesis in NOD mice. Interestingly, the group found that atRA administration not only delayed the onset of T1D but also protected beta cell mass destruction [73]. Another study showed that atRA treatment in mice was able to expand the number of Tregs, which led to enhanced suppression of interferon (IFN)-gamma-producing $CD4^+$ and $CD8^+$ effector T cells, thereby preventing immune infiltration in the islets and destruction of beta cells [74].

Of interest, atRA was also linked to the regulation of miRNA expression in Tregs. Specifically, one study found that treatment with atRA in combination with TGF-β strongly enhanced the expression of miR-99a-5p in Tregs *in vitro* [75]. The important functions of this miRNA have been revealed in an miRNA overexpression screen in naive murine $CD4^+$ T cells during Treg cell differentiation. Here, they found that exposure of naïve T cells to atRA significantly enhanced miR-99a-5p expression, resulting in effective repression of mTOR, a well-known negative regulator of Treg cell differentiation.

Taken together, these studies show how external stimuli likewise contribute to miRNA regulation and consequently, how changes in miRNA expression levels are able to shape immune responses and impact disease pathogenesis. This newly discovered regulatory network of miRNA modulation would offer promising targets for the development of novel strategies with the vision to reestablish Treg induction *in vitro* and *in vivo*. However, the mode of action of the atRA/miR-99a-5p pathway during islet autoimmunity remains elusive.

1.4.2. The role of atRA/miR-99a-5p axis in Treg induction during islet autoimmunity

Importantly, in previous work we showed that miR-99a-5p expression was very low in naïve T cells of individuals with ongoing islet autoimmunity compared to individuals without islet autoimmunity, thereby suggesting a potential role of this miRNA in contributing to dysregulated Treg induction at early disease stages [55].

Of interest, there is increasing body of evidence showing that atRA promotes and stabilizes TGF-β-induced Tregs under steady state conditions as well as in inflammatory environments [76-78]. However, the role of the Vitamin A/atRA/miR-99a-5p signaling pathway in Treg induction during islet autoimmunity is still elusive and is therefore an interesting novel target. Deciphering the potential beneficial effects of atRA on miR-99a-5p expression in order to enhance Treg induction would provide critical insights in its regulatory network and how this pathway is fine-tuned in an inflammatory milieu. Importantly, it would help to improve future therapeutic targeting of dysregulated pathways during islet autoimmunity with the aim to foster Treg function.

Despite all these important insights of molecular pathways and cellular mediators involved in disease pathogenesis, the implementation of their targeting to the clinics has had only little success so far. Even though the use of animal models to study human biology has advanced our understanding in the complex mechanisms of human diseases, its translatability is very limited. Many clinical studies with promising results in the murine model failed to meet their secondary endpoints in clinical trials. Therefore, there is an urgent need to improve model systems for efficient translation from bench to bedside.

1.5. Humanized mice as preclinical models for T1D

Most of our knowledge of the pathogenesis and progression of T1D derives from animal models. The most commonly used mouse model for the study of T1D is the NOD, which has been first used in 1980 [79]. At a young age, immune cell infiltration can be detected in these NOD mice and eventually, they spontaneously develop diabetes [80, 81]. Similar to the human disease, islet autoantibodies can be measured prior to disease onset [82]. Another important similarity is that the murine MHC class II molecule, I-A^{g7} (*H2-Aa*^{g7}) shares homology with the human T1D susceptibility HLA-DQB1 locus [83].

Given these outstanding similarities to the human disease, the NOD mouse model has been used for pre-clinical testing of several drugs. Some of these agents were able to delay or even prevent diabetes in mice, for example Abatacept [84], Rapamycin and IL-2 [85] or oral administration of insulin [86]. Unfortunately, their translation to the human setting has failed or has had only minor effects so far.

Despite the aforementioned similarities between human and murine T1D, there are some T1D-specific differences in the nature of T1D pathogenesis, which potentially impede the clinical translation. For instance, insulin appears to be the only autoantigen required for the initiation of disease onset [82], whereas in human T1D multiple autoantigens have been described [87, 88]. Additionally, there are some considerable differences in the mouse and human immune system, among others leukocyte subset composition, different cytokines and cytokine receptors, and antigen-presenting function of endothelial cells. Accordingly, one has to consider that immune responses in mice might be different in humans.

Therefore, to safely and effectively translate novel therapeutics to the human setting, pre-clinical models that reflect the human disease in the most related way are required. In this regard, human immunesystem engrafted mice are emerging as an important tool.

In 1988, a group could show for the first time that mice with severe combined immunodeficiency (*Prkcd*^{scid} or SCID) can efficiently develop a human immune system after injection with either human hematopoietic stem cells (HSCs) or human peripheral blood mononuclear cells (PBMCs) [89]. These first humanized models were immunodeficient due to the *scid* mutation, which leads to defective TCR and B cell

receptor (BCR) rearrangement, resulting in a loss of functional T and B cells [90], while the innate immune system is intact.

After this groundbreaking finding, immense research was conducted in order to improve the engraftment of human immune cells including the use of different immunodeficient mouse lines. For instance, the NOD mouse background offered improvements in engraftment efficacy due to a polymorphism in the gene Sirpα. Sirpα encodes the signal regulatory protein alpha (Sirpα), which leads to an interaction with CD47 on hematopoietic cells resulting in a protection of their phagocytosis by macrophages [91].

Another milestone was the generation of mouse strains bearing two mutations on the NOD background, the aforementioned *scid* mutation and an interleukin-2 receptor γ-chain (*Il2rg*^{null}) mutation. The latter one is an important receptor for cytokine signaling of innate immune cells [92, 93]. As a consequence, such NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tmWjl}/Sz (NSG) mouse strains displayed severely disrupted murine cytokine signaling as well as disabled development of innate immune cells, which led to a significant enhancement of engraftment efficacy.

Another hurdle for efficient reconstitution of humanized mice is human T cell xeno-reactivity, leading to the development of Graft-versus-host disease (GVHD). In this regard, the deletion of murine MHC class I and class II molecules was a major improvement [94, 95]. Our group successfully engrafted murine MHC class II deficient NSG mice bearing a human HLA DQ8 transgene with HSCs for the identification of HLA-DQ8-restricted insulin-specific CD4$^+$ T cells. Importantly, by using this model we were able to induce insulin-specific Tregs upon subimmunogenic vaccination with strong agonistic insulin mimetopes [39], opening a window of opportunity for therapeutic interventions.

Taken together, all these improvements in engraftment efficacy to recapitulate the human immune system in humanized mice have enabled the generation of a valuable pre-clinical model for immunotherapies.

1.5.1. Establishment of Humanized mice

The two most common approaches for the reconstitution of humanized mice are either injection with human PBMCs or HSCs. Engraftment with PBMCs is the most direct approach and offers short-time analysis of human immune responses. Unfortunately, due to the potential development of xenogeneic GVHD, these mice cannot be used for long-term studies. Alternatively, human HSCs from umbilical cord blood, bone marrow, fetal liver or adult mobilized HSCs can be used. Since T and B cells mature from human stem cells by negative selection during the differentiation process, these cells are tolerant of the mouse host, thereby enabling long-term studies.

However, due to low frequencies of circulating HSCs in peripheral blood of adult donors, efficient reconstitution is hindered. Additionally, non-mobilized peripheral blood HSCs are already fully mature and thus, impede engraftment efficacy.

The use of patient-specific induced pluripotent stem cells (iPSC) for differentiation into HSC could be a means for the efficient engraftment of humanized mice and their longevity and long-term stability. Researchers have put immense efforts to enhance *in vivo* engraftment and multi-lineage potential of these iPSC-specific HSC [96, 97]. For example, the therapeutic benefit of this approach is highlighted by a group who used patient-derived iPSC for successful engraftment of immunodeficient mice to study chronic myelomoncytic leukemia and used their humanized mouse model to develop a drug-testing system [98].

There is a huge plasticity in immune activation vs. aberrations in immune tolerance as evidenced by the variable degree of disease progression from onset of islet autoimmunity to the development of clinical T1D. Despite our knowledge in the broad impairments in human immune tolerance during islet autoimmunity, underlying mechanisms guiding these impairments in Treg cell function, induction and stability remain elusive and *in vivo* models addressing these questions are missing. Thus, the improvements in humanized mouse models will enable a functional *in vivo* evaluation of human immune cells for the purpose to study the different stages of islet autoimmunity and/or T1D and to dissect the plasticity of immune activation and impairments in immune tolerance in a human setting *in vivo.*

2. Objectives

Tregs are critical cellular mediators of peripheral tolerance and thus, antigen-specific Treg induction is a long-awaited goal for immunotherapies of autoimmune diseases, such as T1D. The onset of islet autoimmunity is accompanied by reduced frequencies of insulin-specific Tregs concomitant with high immune activation, resulting in impaired Treg induction and stability *in vitro*. However, the underlying molecular mechanisms leading to these impairments remain poorly understood.

Small non-coding miRNAs are important mediators of immune homeostasis. Accordingly, previous studies from our group could show how dysregulated miRNA expression in activated T cells during islet autoimmunity can lead to aberrant immune activation and impairments in Treg induction.

Since high immune activation at the onset of islet autoimmunity interferes with efficient Treg induction, strategies aiming at boosting Tregs at earlier stages, i.e. at a naïve T cell level, are required in order to enable a window of opportunity for Treg therapies.

Thus, for the first objective, I aimed at dissecting underlying mechanisms of Treg induction in the context of a specific miRNA during islet autoimmunity, which shows different regulations already on the naïve T cell level. Specifically, it was the goal to unravel a link between an upstream regulator and the subsequent induction of this miRNA in fostering Treg cells in an autoimmune setting.

One major challenge in the development of new therapeutics for T1D is the heterogeneity of the disease. Specifically, the progression from the onset of islet autoimmunity to overt T1D shows a huge heterogeneity among individuals. This heterogeneity has been characterized as fast and slow progressor phenotypes [35] and led to the concept of different disease endotypes [37, 38]. However, cellular and molecular mechanisms that underlie these differences are still unknown and studies investigating the plasticity in immune activation vs. impaired immune tolerance mechanisms during human islet autoimmunity in an *in vivo* setting are still missing.

As a second objective, I aimed at filling this knowledge gap by modelling islet autoimmunity in humanized mice. As first approach, I used PBMCs from donors at various disease stages for the reconstitution of NSG-HLA-DQ8 mice to dissect differences in T cell responses in the target tissue.

Objectives

For the second approach, we generated patient-specific iPSC-derived HSCs for the reconstitution of humanized mice in collaboration with the iPSC Core Facility at the Helmholtz Center Munich. Successful reconstitution will permit the long-term study of the human immune system and will contribute to a better understanding of how the plasticity in human autoimmune activation vs. tolerance can be modelled in humanized mice *in vivo.*

3. Materials and Methods

3.1. Materials

Table 1 Chemicals and reagents

Chemical/reagent	Source	Identifier
Foxp3 Staining Buffer Set	eBioscience	Cat #00-5523-00
BD Cytofix/Cytoperm™ Fixation/Permeabilization Solution Kit	BD Bioscience	Cat # BDB554714
Fixable Viability Dye eFluor450	eBioscience	Cat #65-0863-18
Sytox Red	Thermo Fisher Scientific	Cat #S34859
Sytox Blue	Thermo Fisher Scientific	Cat #S34857
Streptavidin Mircobeads	Miltenyi	Cat #130-048-101
Human $CD4^+$ Microbeads	Miltenyi	Cat #130-045-101
Ficoll-Paque PLUS	GE Healthcare	Cat #17-1440-03
Recombinant human IL-2	ReproTech	Cat #200-02
Recombinant human IL-6	PeproTech	Cat #200-06
Recombinant human IL-1β	PeproTech	Cat #200-01B
Recombinant human IFN-γ	PeproTech	Cat #300-02
Recombinant human insulin	Sigma-Aldrich	Cat #I9278
Retinoic acid	Sigma-Aldrich	Cat # R2625
Percoll™	Sigma-Aldrich	Cat #P1644
Streptavidin Pacific Blue	Invitrogen	Cat #S11222
Beta-Mercaptoethanol	BioConept	Cat #5-69F00-E
Bovine serum albumin (BSA)	Sigma-Aldrich	Cat #A7906

Collagenase D	Roche	Cat #11088882001
Dulbecco's phosphate buffered saline (DPBS)	Thermo Fisher Scientific	Cat #14190169
Ethylenediaminetetraacetic acid (EDTA)	Lonza	Cat #51234
Fetal calf serum (FCS)	Biowest	Cat #S1810-500
GolgiPlug™ (Brefeldin A)	BD Bioscience	Cat #555029
Hank's balanced salt solution	Sigma Aldrich	Cat #H6648
Heparin	AppliChem	Cat #APA3004.0005
Human serum	PAN Biotech	Cat #P40-3001
Hydroxyethyl-piperazineethanesulfonic acid solution (HEPES, 1M)	VWR	Cat #15630056
Nunc-Immuno™ maxi Sorp 96-well plate	Thermo Fisher Scientific	Cat #439454
Ionomycin	Cayman Chemicals	Cat #10004974-1
Non-essential amino acids (100x)	Biochrom AG	Cat #K 0293
Penicillin/Streptomycin (Pen/Strep)	Sigma Aldrich	Cat #P4333
Phorbol 12-Myristate 13-Acetate (PMA)	abcam	Cat #ab120297
Sodium Pyruvate Solution	Sigma Aldrich	Cat #S8636
Streptavidin Pacific Blue	Invitrogen	Cat #S11222
One Step Vitamine A und E im Serum/Plasma - UHPLC	Chromosystems	Cat # 34900/UHPLC

Table 2 Cell culture media and supplements

Medium	Manufacturer	Components
X-Vivo 15	Lonza	50 ml X-Vivo 15 10% Human serum 1x Penicillin Streptomycin 1x Sodium pyruvate 500µl 1x Non-essential aminoacids 1x GlutaMAX
RPMI 1640	Thermo Fisher Scientific	500 ml RPMI 10% (v/v) FCS 1x Sodium pyruvate 1x Non-essential aminoacids 1x Penicillin Streptomycin 50 µM Mercaptoethanol

Table 3 Buffers

Buffer	Manufacturer	Components
Hank's Balanced Salt Solution (HBSS)	Sigma-Aldrich	500ml HBSS 5% (v/v) FCS 10 mM HEPES
MACS PBS	Thermo Fisher Scientific	500 ml PBS 0.5% (v/v) BSA 2 mM Ethylenediaminetetraacetic acid (EDTA)
Coating Buffer		0.1 M sodium bicarbonate buffer, pH 8.2

Table 4 Commercial assays

Assay	Source	Identifier
miRNeasy Micro Kit	Qiagen	Cat #217084
ssoAdvanced Universal Probes Supermix	Bio-Rad	Cat #1725281
miRCURY LNA RT Kit	Qiagen	Cat #339340
miRCURY LNA SYBR Green PCR Kit (600)	Qiagen	Cat #339346
Quick-DNA™ Miniprep Kit	Zymo Research	Cat #D3025
EasySep™ Cell Separation	StemCell™ Technologies	Cat #17952
TMB Substrate Reagent Set	BD Bioscience	Cat #555214

Table 5 Techniqual equipment

Device	**Model**	**Manufacturer**
Cell sorter	FACS Aria III	BD Biosciences
Accu-Check® Glucometer	Aviva	Roche
Centrifuge	Heraeus Multifuge X3R	Thermo Scientific
Microcentrifuge		
CO_2 Incubator	BBD 6220	Thermo Scientific
MACS cell seperator	MACS Multistand, QuadroMACS	Miltenyi Biotec
Sterile workbench	Scanlaf Mars Class 2	LaboGene
Photometer	NanoPhotometer® N120	Implen
Microplate spectrophotometer	Epoch	BioTek
Real-time PCR system	CFX96 Touch	Bio-Rad
BD Vacutainer®	Sodium Heparin	BD Bioscience
Cooling System	HAAKE SC100	Cat #368480
Heraeus Multifuge 3 S-R		BioTek
Heraeus Multifuge X3R		Thermo Scientific
Microscope, Primo star		Thermo Scientific
peqStar 2X thermal cycler		Zeiss
Vortex Mixture, Lab Dancer		Peqlab
Rotator		VWR
Thermal Cycler T100		VWR
Bio-Rad CFX Manager 3.1		BioRad
Ultimate 3000 (U3000-ECD)		

Table 6 Experimental models

Experimental model	Description/Source	Identifier
NOD/ShitLtJ	Polygenic model for autoimmune T1D Jackson Laboratory	Cat #001976
CD90.1 Balb/c (CBy.PL(B6)-*Thy1*^{a}/ScrJ)	Strain carries a T cell-specific Thy1.1 allele Jackson Laboratory	Cat #005443
HLA-DQ8 transgenic NOD-Cg-Prkdcscid Il2rgtm1Wjl	Strain lacks murine MHC class II and expresses a mutant human leukocyte antigen (HLA-DQ8) on the mmunodeficient NSG background Jackson Laboratory	Cat #026561

Table 7 Antibodies

Antibody	Source	Identifier
anti-human CD3 A700	Biolegend	Cat. No: #300324; clone: HIT3a
anti-human CD3 PerCP-Cy5.5	Biolegend	Cat. No: #300328; clone: HIT3a
anti-human CD4 V500	BD Biosciences	Cat. No: # 560768; clone: RPA-T4
anti-human CD25 APC	BD Biosciences	Cat. No: #340907; clone: 2A3
anti-human CD45 A700	Biolegend	Cat. No: #304024; clone: HI30
anti-human CD45 APC-Cy7	BD Biosciences	Cat. No: #560274; clone: 2D1
anti-human CD45RA FITC	Biolegend	Cat. No: #304106; clone: HI100
anti-human CD45RO APC-Cy7	BD Biosciences	Cat. No: #561137; clone: UCHL1
anti-human CD127 PE-Cy7	Biolegend	Cat. No: #351320; clone: A019D5
anti-human CD8a PB	Biolegend	Cat. No: #301023; clone: RPA-T8
anti-human CD11b PB	Biolegend	Cat. No: #301334; clone: ICRF44
anti-human CD14 PB	Biolegend	Cat. No: #325616; clone: HCD14
anti-human CD19 PB	eBioscience	Cat. No: # 48019842; clone: SJ25C1
anti-human FOXP3 PE	eBioscience	Cat. No: # 12477742; clone: 236A/E7

anti-human FOXP3 FITC	eBioscience	Cat. No: #11477641; clone: PCH101
anti-human Ki67 Qdot	Biolegend	Cat. No: # 350522; clone: Ki-67
anti-human IL-10 FITC	eBioscience	Cat. No: #53-7108; clone: JES3-9D7
anti-human IL-17A	Biolegend	Cat. No: #512326; clone: BL168
anti-human IFNγ PerCP-Cy5.5	Biolegend	Cat. No: #502525; clone: 4S.B3
anti-human Tbet QDot	Biolegend	Cat. No: # 644817; clone: 4B10
Fc block (Human TruStain FcX)	Biolegend	Cat. No: #422302
anti-mouse CD3 BV711	Biolegend	Cat. No: #100349; clone: 145-2C11
anti- mouse CD4 A700	Biolegend	Cat. No: #100536; clone: RM4-5
anti- mouse CD4 Biotin	Biolegend	Cat. No: #100404; clone: GK1.5
anti-mouse CD25 PerCP-Cy5.5	Biolegend	Cat. No: #102030; clone: PC61
anti-mouse CD44 PE	Biolegend	Cat. No: #103008; clone: IM7
anti-mouse CD62L PE-Cy7	Biolegend	Cat. No: #104418; clone: MEL-14
anti-mouse FOXP3 PE	Biolegend	Cat. No: # #126403; clone: MF-14
anti-mouse FOXP3 FITC	eBioscience	Cat. No: # #11-5773-82; clone: FJK-16s

anti-mouse Ki67 APC	Biolegend	Cat. No: #652406; clone: 16A8
anti-mouse Ki67 QDot	Biolegend	Cat. No: #652413; clone: 16A8
anti-mouse HELIOS PE-Dazzle	Biolegend	Cat. No: #313512; clone: C398.4A
anti-mouse IL-10 PE	eBioscience	Cat. No: #25480182; clone: BM8
anti-mouse IL-17A QDot	Biolegend	Cat. No: #506927; clone: TC11-18H10.1
anti-mouse IFNγ APC	BD Biosciences	Cat. No: #560078; clone: L50-823
Fc block (CD16/CD32)	BD Biosciences	Cat. No: #553142; clone: 2.4G2

Table 8 Tetramers

Antibody	**Sequence**
DQB1-03:02	HLVEELYLVCGEEG
DQB1-03:02	HLVEELYLVCGGEG
DQB1-03:02	PVSKMRMATPLLMQA

Table 9 Primer sequences qPCR

Primer/Probe	Sequence	Source
rs3104413_fwL_4	GAGCTGAGCACTGAGTAG	Sigma-Aldrich
rs3104413_revL_4	GCAGTTGAGAAGTGAGAG	Sigma-Aldrich
FAM-Probe rs3104413_LPC	[6FAM]CAGCCT[+G]CT[+C]TC[+C]TA[+T]TGG[BHQ1]	Sigma-Aldrich
HEX-Probe rs3104413_LPG	[HEX]CAGCCT[+G]CT[+G]TC[+C]TA[+T]TGG[BHQ1]	Sigma-Aldrich
rs2854275_fwL_6	CCAGAACCAAGCCTTAAC	Sigma-Aldrich
rs2854275_revL_6	GCATCATCCTAGTGTCTAAC	Sigma-Aldrich
FAM-Probe rs2854275 G	[6FAM]TCCACA[+T]TT[+C]AC[+A]AG[+A]AGA[BHQ1]	Sigma-Aldrich
HEX-Probe rs2854275 T	[HEX]TCCACA[+T]TT[+A]AC[+A]AG[+A]AGA[BHQ1]	Sigma-Aldrich
rs9273363_fw_2	GAGGGAGAAAGGAAGATG	Sigma-Aldrich
rs9273363_rev_2	GAAGCTGGTCTACATCTC	Sigma-Aldrich
FAM-Probe rs9273363_LPA	[6FAM]CATGGC[+C]TT[+A]CA[+T]AA[+C]CTC[BHQ1]	Sigma-Aldrich
FAM-Probe rs9273363_LPC	[HEX]CATGGC[+C]TT[+C]CA[+T]AA[+C]CTC[BHQ1]	Sigma-Aldrich

Table 10 Sequences miRNA inhibitors and mimics

Inhibitor/mimic	Sequence	Source
miR99a-5p inhibitor hs/mm	AACCCGUAGAUCCGAUCUUGUG	Qiagen miRCURY LNA™ miRNA inhibitor
miR99a-5p mimic hs/mm	AACCCGUAGAUCCGAUCUUGUG	Qiagen miRCURY LNA™ miRNA mimic
control inhibitor hs/mm	TAACACGTCTATACGCCCA	Qiagen miRCURY LNA™ miRNA inhibitor
control mimic hs/mm	UCACCGGGUGUAAAUCAGCUUG	Qiagen miRCURY LNA™ miRNA mimic

Table 11 Softwares

Software	Source	Identifier
FlowJo™ software (10.8.1)	TreeStar Inc., OR	https://www.flowjo.com/solutions/flowjo/downloads/
BD FACSDiva™	BD Biosciences	https://www.bdbiosciences.com/en-be/products/software/instrument-software/bd-facsdiva-software
Prism (version 9.4.1)	GraphPad	https://www.graphpad.com/scientific-software/prism/
Chromeleon (7.2.9)	ThermoFisher Scientific	

3.2. Methods

3.2.1. Human subjects

Blood samples were obtained from participants of the BABYDIAB or DiMELLI study at the Institute of Diabetes Research, Helmholtz Center Munich or from patients of Munich Municipal Hospital with the relevant ethical regulation and all participants consented to the "B11 – Mechanismen der Immunaktivierung vs. Toleranz in Autoimmunem Typ 1 Diabetes" project (approval number 2019-510_4-S-SR approval committee: Technische Universität München, Munich, Germany). Venous blood was collected using sodium heparin tubes. The obtained volume was in accordance with EU guidelines, where a maximal blood volume of 2.4 ml per kg of body weight is recommended. Subjects were stratisfied based on the absence or presence of multiple islet autoantibodies and duration of T1D: healthy donors (no risk genes for T1D, no autoimmunity), autoantibody negative (no autoimmunity but at-risk haplotypes for T1D based on the presence of HLA-DR4/DQ8), autoantibody positive (presence of multiple autoantibodies), recent onset of T1D (newly diagnosed children with T1D), short-term T1D (disease onset <5 years) and long-term T1D (disease onset >5 years).

3.2.1.1. iPSCs Generation

Ficoll-separated PBMCs (GE Healthcare) were seeded at a density of 5×10^5 cells/mL. Sendai virus reprogramming was performed in line with the manufacturer's instructions (CytoTune iPS 2.0 Sendai Reprogramming Kit, Thermo Fisher Scientific). The culture was monitored daily for emergence of iPSC colonies. First colonies were single-picked and further cultivated. After 7-10 passages, iPSC clones were tested for presence of the virus. Clones that were Sendai virus-free, were further passaged for 1-2 times and tested for pluripotency performed by the iPSC Core Facility at the Helmholtz Center Munich.

3.2.1.2. Hematopoietic Stem Cell Differentiation

iPSC clones were differentiated into hematopoietic progenitor cells expressing CD34, CD45, and CD43 using the STEMdiff™ Hematopoietic Kit according to the manufacturer's instructions (StemCell™ Technologies). Differentiation efficacy was evaluated by flow cytometry based on HSC marker expression ($CD34^+$, $CD38^-$, $CD45^+$, $CD45RA^-$).

3.2.1.3. HLA-Genotyping

For the determination of the HLA genotypes of human donors, DNA from whole blood was extracted using the Quick-gDNA™ MiniPrep Kit (Zymo Research). For the detection of high-risk HLA-DR and HLA-DQ types, three single nucleotide polymorphism (SNPs) were used based on the scheme published by Nguyen *et al*. [99]. Briefly, 15ng/µl of DNA were used for the RT-qPCR reaction. Primers and probes for the specific SNPs were used for the analysis. Samples with known HLA haplotypes were used as standards: DR4DQ8/DR4DQ8, DR3/DR4DQ8, DRX/DRX, DR3/DRX, DR3/DR3, DR4DQ7/X, DR4DQ7/DR4DQ7, DR4DQ8/X. For analysis, Bio-Rad CFX Manager 3.1 was used.

3.2.2. Mouse experiments

CBy.PL(B6)-Thy1^{a}/ScrJ (CD90.1 BALB/c) and NOD/ShiLtJ mice were obtained from the Jackson Laboratory. NOD/ShiLtJ mice were stratified according to their IAA status into IAA-negative and IAA-positive groups. Humanized mice, NOD.Cg-Prkdc^{scid} H2-Ab1^{tm1Gru}Il2rg^{tm1Wjl} Tg(HLA-DQA1,HLA-DQB1) 1Dv//Sz mice lacking mouse MHC class II and transgenically expressing human HLA-DQ8 were obtained from and developed by Dr. Leonard D. Shultz. The DQ8 transgene was backcrossed for ten generations on the NSG strain background, and these NSG-DQ8 mice were further intercrossed with NSG mice, which lack the mouse MHC class II (NOD.Cg-*Prkdc^{scid} H2-Ab1^{tm1Gru} Il2rg^{tm1Wjl}*). Mice were held under specific pathogen-free conditions on 12-h/12-h light dark cycle at 25 °C. All mice were supplied with ad libitum access to water and a standard diet if not indicated otherwise at the animal facility of Helmholtz Zentrum München, Munich, Germany according to guidelines established by the Institutional Animal Committees. These also included all required ethical regulations for all performed animal experiments and research. Ethical approval for all mouse experiments has been received by the District Government of Upper Bavaria, Munich, Germany (approval # ROB-55.2-2532.Vet_02-17-130).

3.2.2.1. Murine insulin autoantibody assay

Levels of insulin autoantibodies were measured at below 30, 40 and 70 days of age from serum of NOD mice. For that, a mouse high specificity competitive insulin autoantibody assay with an enzyme-linked immunosorbent assay (ELISA) was applied. 96-well plates

were coated overnight with human recombinant insulin (100 U/ml; Humulin, Lilly) at 4°C. The next day, unspecific blocking was performed with PBS containing 2% BSA for 2 h at room temperature. NOD sera were preincubated for 1 h (1:10 dilution) with or without insulin competition and then added to the place for 2 h at room temperature on a shaking platform. After washing. Biotinylated antimouse IgG1 (Abcam) was incubated in a 1:10,000 dilution in PBS/BSA for 30 min at room temperature on a shaking platform. After 4 washing steps, horseradish peroxidase-labeled streptavidin was added for another 15 min. The plate was washed 5 times and then a TMB substrate solution was added to the wells (OptEIA reagent set; BD). All measurements were performed in duplication with and without human insulin competition.

3.2.2.2. Vitamin A excess and deficient diet

All diets had the same composition of nutrients with only a difference in the Vitamin A supplemention. Vitamin A diets were purchased from ssniff Spezialdiäten GmbH. NOD mice were subjected to either a diet with normal Vitamin A supplemention (15,000 IU/kg), deficient in Vitamin A (0 IU/kg) or an excess Vitamin A supplemention (350,000 IU/kg).

3.2.2.3. In vivo all-trans retinoic acid application

All-trans retinoic acid (Sigma-Aldrich R2625) was reconstituted in pure DMSO at a concentration of 200 mM. The stock solution (60 µg/µl) was diluted in 200 µl with corn oil to a final concentration of 0.5 mg atRA per mouse.

atRA was injected i.p. into NOD mice with mixed IAA status at a concentration of 0.5 mg/mouse every other day for 2 weeks. Prior injection, total $CD4^+$ T cells were sorted from PBMCs and used for RNA extraction. Serum was taken for Vitamin A measurement. For the analysis, Treg frequencies, stability and cytokine production were analyzed from pLNs and pancreas. Total $CD4^+$ T cells were sorted for RNA extraction.

3.2.2.4. Reconstitution of NSG mice with human PBMCs

HLA-DQ8 transgenic NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl} (NSG) mice lacking the murine MHC class II molecules were intravenously engrafted with 5-10 x 10^6 PBMCs from an HLA-DQ8-positive donor in 50 µl volume per mouse. Human donors and mice were sex-matched.

3.2.3. Cell isolation, staining and sorting

Venous blood was collected with Heparin vials (BD Vacutainer®, BD 368480). In order to isolate PBMCs, blood was centrifuged using Ficoll-Plaque PLUS (GE Healthcare). $CD4^+$ T cells were enriched using the EasySep™ Human $CD4^+$ T Cell Isolation Kit (StemCell™ Technologies) according to the manufacturer's protocol.

Murine lymph nodes and spleen from mice were passed through 70 µm cell strainers. Spleen was lysed with ACK lysis buffer for 10min at room temperature.

Whole pancreas was homogenized in Precellys tube with 1.4 mm ceramic beads and filled with 1 ml MACS PBS for 30 seconds at 2500 RPM. The homogenized tissue was collected and passed through a 40 µm cell strainer. The sample was centrifuged at 300 x g for 10 min and the supernatant was discarded. Cells were resuspended in 5 ml of 30% Percoll in RPMI medium without supplements and overlaid on 2 ml of 80% Percoll in Hank's Balanced Salt Solution. After centrifugation of 30 min at 500 x g at 1/1 acceleration/brake and room temperature, the interphase was collected and added to a Falcon prefilled with Hank's Balanced Salt Solution.

Murine PBMCs were separated from whole blood by Ficoll density gradient centrifugation (GE Healthcare).

To avoid unspecific binding, all isolated cells were preincubated with Fc-block (Biolegend) for 10 min. Subsequently, the cells were stained with fluorochrome-labeled antibodies for 30 min on ice and light-protected. After surface staining, fixation of the cells was performed followed by permabilization with either the Foxp3 Staining Buffer Kit (eBioscience) or CytoPerm/Cytofix Kit (BD) for the detection of intracellular proteins or cytokines, respectively. After optimizing compensation and gating strategies, cells were acquired on the BD FACS Aria III cell sorting system using FACS Diva software. Doublets were excluded based on SSC-A vs. SSC-W plots. In order to acquire live cell populations, cells were gated on the basis of cell side and forward scatter, and the exclusion of cells positive for Sytox Blue (Life Technologies) or Fixable Viability Dye eFluor450 (ebioscience). The FlowJo software version 10.8.1 (TreeStar Inc., OR) was used for the analysis.

3.2.3.1. Insulin-specific HLA-DQ8-restricted tetramer staining

Two fluorescent-labelled insulin-specific HLA-DQ8-tetramers were developed by NIH Tetramer facility based on insulin-B-chain-10-23-mimetopes: 14E-21G-22E (ins.mim.1) and 14E-21E-22E (ins.mim.2) as in [39]. PBMCs were isolated from whole blood of humanized mice and incubated with "insulin-specific HLA-DQ8-tetramers" for 1 hour at 37°C in humidified 5% CO_2 with moderate vortexing every 20 min. Subsequently, cells were stained for surface markers and exclusion of dead cells (Sytox Blue) for another 20 min on ice. To exclude unspecific binding of insulin-specific T cells, a fluorescent-labelled negative control tetramer using an irrelevant peptide (PVSKMRMATPLLMQA) was used as in [39]. Live $CD3^+CD4^+$tetramer$^+$ T cells were analysed.

3.2.3.2. In vitro studies with primary T cells

Human naïve $CD4^+$ T cells were cultivated at 37°C in a humidified CO_2 incubator in cell culture-treated-96-well U bottom plates (Bio-Greiner) as in [58]. For the culturing medium, X-Vivo15 Medium (Lonza) was supplemented with 2mM glutamine, 100 U/ml human recombinant IL-2 (ReproTech), 10 ng IL-6 (ReproTech), 50 U/ml penicillin, 50 mg/ml streptomycin (Sigma Aldrich), and 5% heat-inactivated human AB serum (Invitrogen).

Murine naïve $CD4^+$ T cells were cultured at 37°C in a humidified CO_2 incubator in cell culture-treated-96-well U bottom plates (Bio-Greiner) as in [58]. RPMI media (Gibco by life technologies) was prepared with the following supplementations: 10% FCS, 1 mM sodium pyruvate (Sigma Aldrich), 50 mM b-mercaptoethanol (Amimed), 1x nonessential amino acids (Merck Millipore), 100 U/ml human recombinant IL-2 (ReproTech), 100 U/ml penicillin, and 100 mg/ml streptomycin (Sigma Aldrich) Human in vitro Treg induction by limited TCR stimulation and atRA treatment

Human naïve $CD4^+$ T cells ($CD3^+$, $CD4^+$, $CD45RA^+$, $CD45RO^-$, $CD127^{hi}$, $CD25^{lo}$) were sorted using the BD FACS Aria III cell sorting system for purity. The sorted cells were cultured (50,000 cells / well) in a 96-well plate pre-coated with 5 µg/ml anti-CD3 and 15 µg/ml anti-CD28 antibody and IL-2 (100 U/ml) and IL-6 (10 ng/ml) (Fig. 2A). 1 µM atRA (5 mM) or vehicle were added directly to the cells. After 18 h of incubation, limited TCR stimulation was performed by transferring the cells into uncoated wells, followed by

additional 36 h of incubation. Efficiency of Treg induction was measured by flow cytometry based on percentage of Foxp3 expression in $CD25^{+}CD127^{-}CD4^{+}$ T cells (Fig. 2B).

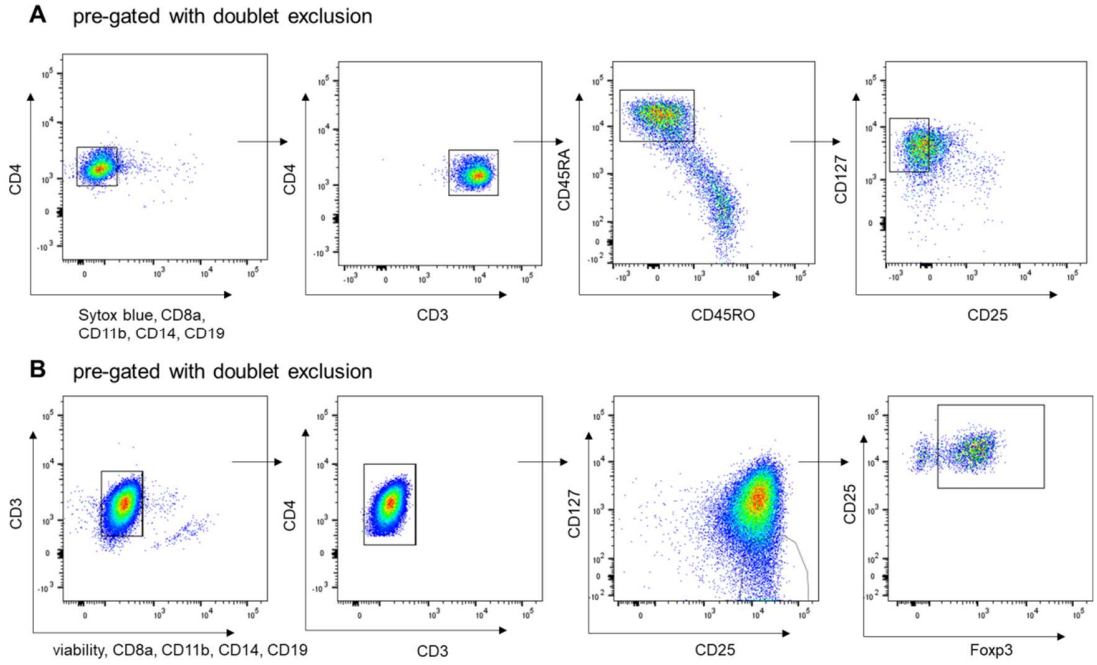


Figure 2: Gating strategy for FACS sorting of human T cells and Treg induction assays. (A) Live naïve $CD3^{+}CD4^{+}CD45RA^{+}CD45RO^{-}CD127^{hi}CD25^{lo}$ from human PBMCs. (B) Representative FACS staining for identification of human $CD127^{lo}CD25^{hi}CD25^{hi}Foxp3^{+}$ T cells after Treg induction.

3.2.3.3. Murine in vitro Treg induction by continuous TCR stimulation and atRA treatment

Murine naïve $CD4^{+}$ T cells ($CD3^{+}$, $CD4^{+}$, $CD62L^{hi}$, $CD44^{lo}$) were sorted with the BD FACS Aria III cell sorting system for purity. Sorted cells were cultured (10,000 cells / well) in a 96-well plate pre-coated with 5 µg/ml anti-CD3 and 15 µg/ml anti-CD28 antibody and IL2 (100 U/ml) for 56 h (Fig. 2A). 1 µM atRA (5 mM) or vehicle were added directly to the cells. Efficiency of Treg induction was assessed by flow cytometry based on percentage of Foxp3 expression in $CD25^{+}CD4^{+}$ T cells (Fig. 3B).

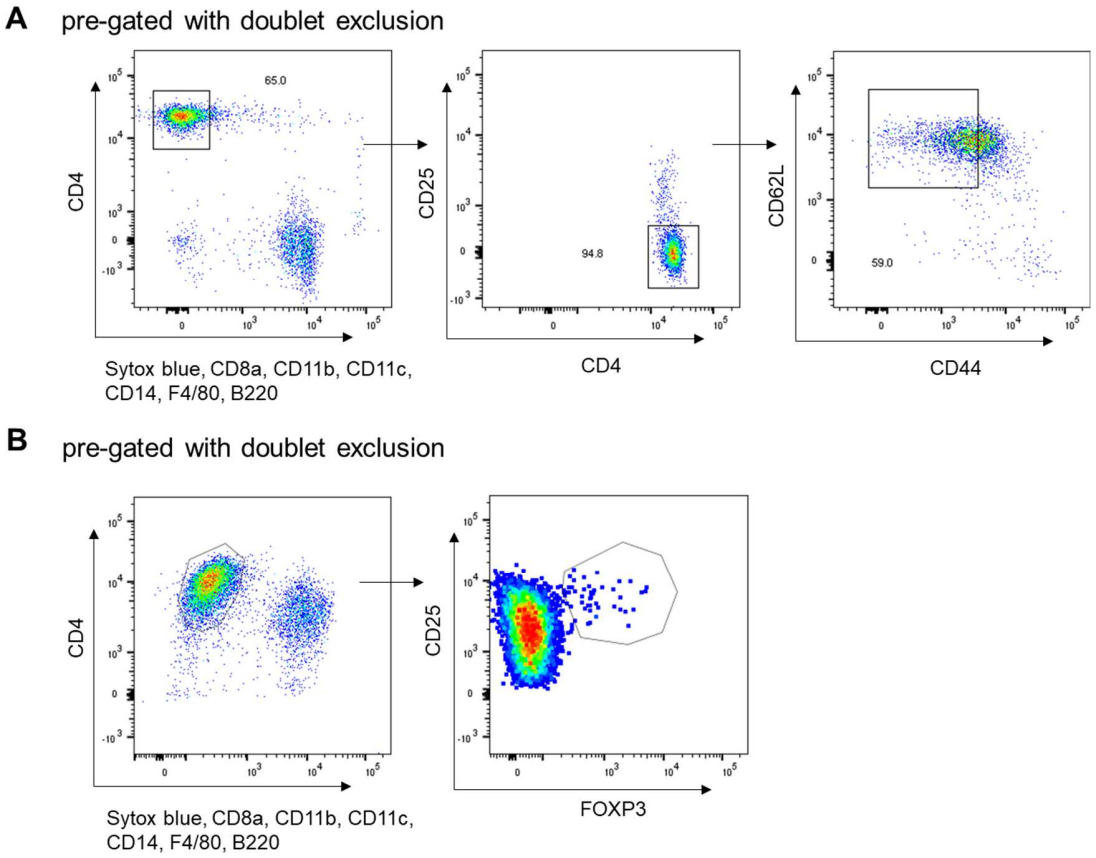


Figure 3: Gating strategy for FACS sorting of murine T cells and Treg induction assays. (A) Live naïve $CD4^{+}CD25^{-}$ $CD62L^{hi}CD44^{lo}$ from CD90.1 Balb/c or NOD mice. (B) Representative FACS staining for identification of murine $CD4^{+}CD25^{hi}Foxp3^{+}$ T cells after Treg induction.

3.2.4. Nanoparticles

Chitosan-coated PLGA nanoparticles for *in vitro* miRNA mimic application were kindly provided by Prof Dr Claus-Michael Lehr at the Helmholtz Institute for Pharmaceutical Research in Saarland, Germany.

3.2.4.1. Application of miR99a-5p mimic

To load chitosan-coated PLGA nanoparticles with the mimic (miRCURY LNA miRNA mimic, Qiagen), both components were mixed at a weight ratio of nanoparticles:mimic of 50:1 and mixed on a shaking device at 300 rpm at room temperature for 30 minutes as in [58]. The complexed nanoparticle/mimic was added to the wells at a final concentration of 40 pmol per well. As a control, nanoparticles loaded with a miRNA

mimic control (Qiagen) were added to the cultures. For mimic/control sequences see Table 10.

3.2.5. Application of miR99a-5p inhibitor

Invitrogen Lipofectamine RNAiMAX Transfection Reagent (ThermoFisher) was used to form a complex with a miR-99a-5p inhibitor (miRCURY LNA miRNA inhibitor, Qiagen) according to the manufacturer's instructions. The miRNA-Lipid complex was added to the naïve T cells for Treg induction assay at a final concentration of 10 pmol per well. As a control, a miRNA inhibitor control (Qiagen) was added to the wells. For inhibitor/control sequences see Table 10.

3.2.6. Isolation and analysis of miRNAs

MiRNAs was extracted using the miRNeasy Micro Kit (Qiagen). RNA concentration was measured using Epoch (BioTek). cDNA libraries were performed using the miRCURY LNA RT Kit (Qiagen). For the detection of the miR-99a-5p, 60 ng of RNA were used instead of the recommended 5 ng due to low expression of this miRNA. qPCR was performed using the miRCURY LNA SYBR Green PCR Kit (Qiagen) in combination with miRCURY LNA primers for miR-99a-5p. miRCURY LNA primers for the housekeeper 5s rRNA (Exiqon) were used for normalization,. For primer sequences see Table 9. The reaction was performed on a CFX96 real time system (Biorad).

3.2.7. Vitamin A concentration

Vitamin A concentration in serum of human and murine samples were measured with the One Step Vitamins A and E in Serum/Plasma kit according to the manufacturer's instructions (Chromosystems, #34900/UHPLC) using the Ultimate 3000 (U3000-ECD) HPLC system. Experiments were performed by Fabien Riols at the Metabolomics & Proteomics Core (MPC), Helmholtz Center Munich. For data acquisition and quantitation the Chromeleon software version 7.2.9 (Thermo Fisher Scientific) was used.

3.2.8. Statistical analysis

Data is presented as mean and standard error of the mean (s.e.m) or in percentage (%). For all data that shows normal distribution, Student's t test for unpaired values was applied to compare "means between independent groups". For multiple comparisons,

the one-way ANOVA was applied. All tests that showed a two-tailed P value of < 0.05 were considered to be significant. Statistical significance is shown as *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$, or not significant (NS) $P > 0.05$. Analyses were performed using the program GraphPad Prism version 9.4.1 (La Jolla, CA).

4. Results

4.1. The role of the atRA/miR-99a-5p axis during islet autoimmunity

In previous work, our group could show a direct link between the aberrant activation of specific miRNAs in activated T cells of human and mice with islet autoimmunity and impairments in Treg induction and stability. In order to dissect underlying mechanisms that affect Treg impairments already on a naïve T cell level, a miRNA – miR-99a-5p - has been identified which showed very low expression in individuals with T1D. Of note, *all-trans* retinoic acid (atRA), the active metabolite of Vitamin A, has been described by other groups as upstream regulator of this miRNA in Tregs [75], thereby favoring Treg cell differentiation. In the first section, I will focus on a role of this upstream regulator atRA on miR-99a-5p in fostering Tregs during islet autoimmunity in a human and murine setting using several *in vitro* and *in vivo* approaches.

4.1.1. miR-99a-5p expression in naïve T cells correlates with Vitamin A concentrations during human islet autoimmunity

Specifically, in order to dissect mechanisms that are already impaired at the naïve T cell level, I looked for miRNAs that were differentially expressed in naïve T cells isolated from PBMCs from children with or without islet autoimmunity using data from a previous NGS screen. Here, the expression of miR-99a-5p showed lower expression in naïve T cells from individuals with established T1D compared to those individuals without islet autoimmunity [55]. To study differences of miRNA expression at various disease stages, I received donor blood from either healthy donors (HD; no risk for T1D), non-diabetic autoantibody-negative donors (at-risk for T1D but no presence of islet autoantibodies), non-diabetic autoantibody-positive donors (presence of multiple autoantibodies), clinical T1D (different disease durations).

When I investigated the expression levels of this miRNAs in sorted naïve T cells ($CD3^+CD4^+CD45RA^+CD45RO^-CD127^+CD25^-$) from PBMCs of individuals at different stages of islet autoimmunity, I could see trends towards lower expression of this miRNA in individuals with long-term clinical T1D (Fig. 4A). However, severe heterogeneity among the individuals together with limited *n* numbers makes a definitive conclusion difficult. Importantly, the individuals from this group were not matched for time of onset

of the clinical disease after autoantibody seroconversion, which could potentially affect the expression levels of this miRNA.

Critically, autoantibody positive individuals without progression to the clinical disease showed a trend towards higher expression of miR-99a-5p (Fig. 4A), which is in line with previous data showing that those individuals that harbored multiple autoantibodies without the progression to the clinical disease had the highest frequencies of insulin-specific Tregs [39].

In contrast, donors that developed the clinical disease not longer than 5 years ago showed the highest expression of miR-99a-5p (Fig. 4A, $P < 0.05$). It would be interesting to follow up on these patients and to include more donors at this stage of disease duration to see whether the expression level of this miRNA in naïve T cells will decline again in the course of established disease.

To see whether there are differences in the expression profile of miR-99a-5p on already fully differentiated Tregs in healthy donors versus patients with T1D, I sorted $CD3^+CD4^+CD127^-CD25^+$ Tregs and analyzed the expression levels of miR-99a-5p. There were trends towards decreased expression of this miRNA in donors with established T1D compared to healthy donors, however, the data was heterogeneous among those individuals with T1D (Fig. 4B).

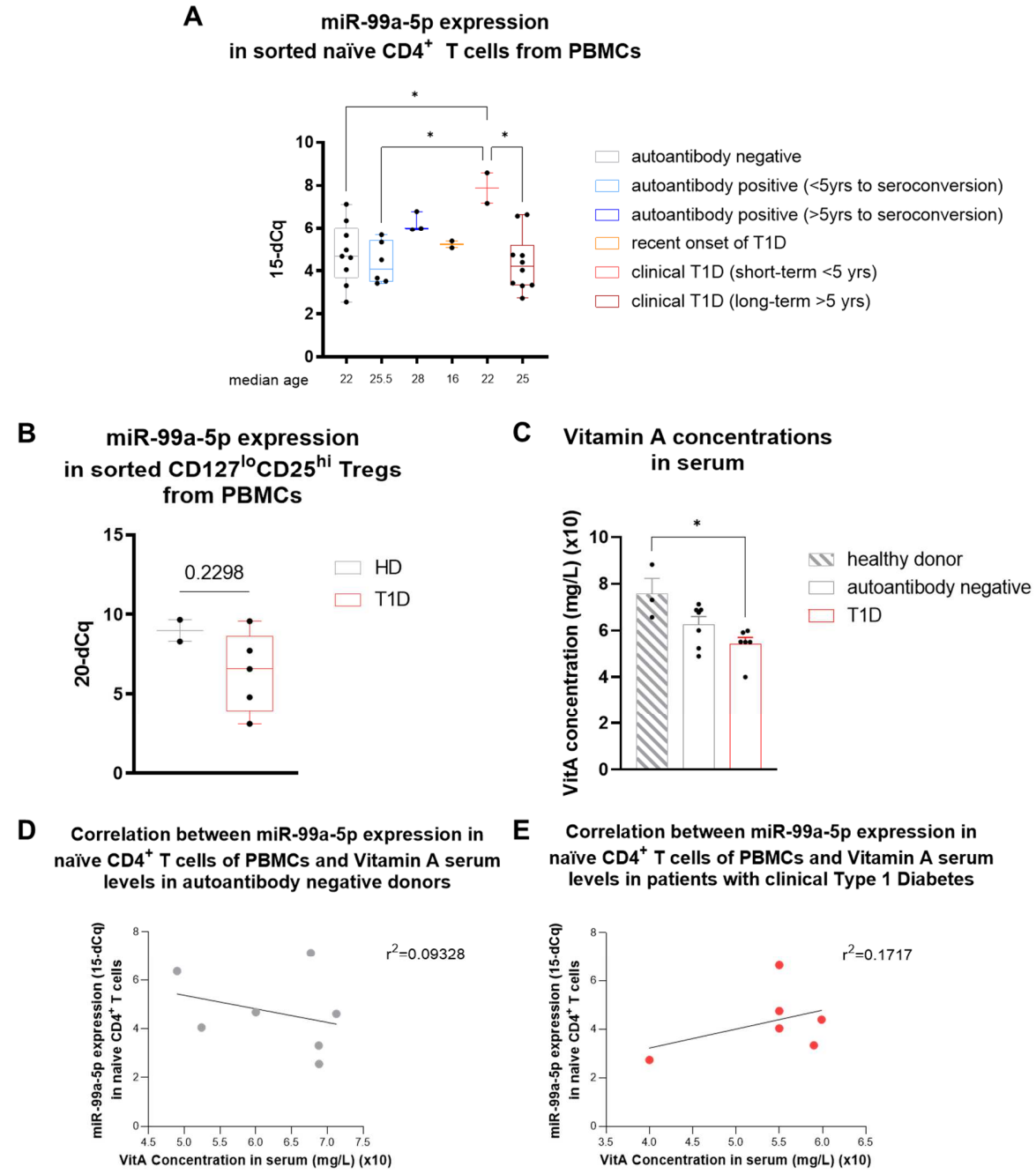


Figure 4: Correlation between miR-99a-5p expression in naïve T cells and Vitamin A concentrations in serum of individuals at different disease stages. (A) Expression of miR-99a-5p in *ex vivo* naïve CD4+ T cells of PBMCs from individuals with different disease stages as assessed by RT-qPCR. (B) Expression of miR-99a-5p in *ex vivo* CD127^{lo}CD25^{hi} Tregs of PBMCs from healthy donors or patients with T1D as assessed by RT-qPCR. (C) Vitamin A concentrations in serum of individuals with indicated health status. Data represent the mean ± SEM. (D-E) Correlation of miR-99a-5p abundance in

naïve CD4+ T cells of PBMCs with Vitamin A levels in serum of indicated donors. (E) HD=healthy donor; PBMCs=peripheral blood mononuclear cells; yrs=years; T1D=Type 1 Diabetes. Ordinary one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

Since atRA, as the major metabolite of Vitamin A, has been shown to strongly upregulate miR-99a-5p in Tregs [75], I wanted to reveal a potential link between the expression levels of miR-99a-5p in naïve T cells and Vitamin A levels in donors at different disease durations. First, I compared the concentrations of Vitamin A in serum between individuals without any risk (healthy donor) for developing T1D, individuals harboring at-risk HLA genotypes but without presence of islet autoantibodies (autoantibody negative), and patients with established T1D to see whether there are differences in Vitamin A levels with respect to the disease stage. Of note, I found significantly reduced levels of Vitamin A in the sera of patients with clinical T1D compared to healthy donors (Fig. 4C, P < 0.05).

Next, I analyzed the serum of collected samples for Vitamin A concentration and correlated it with the expression levels of the miRNA in naïve T cells from PBMCs. There was no correlation of the expression of miR-99a-5p in naïve T cells and Vitamin A in autoantibody negative individuals (Fig. 4D), however, we can see a trend towards a positive correlation between the expression of miR-99a-5p in naïve T cells and Vitamin A concentrations in individuals with established T1D (Fig. 4E).

These findings show that miR-99a-5p might play an important role at early stages of islet autoimmunity, at a stage where also higher frequencies of Tregs are present [39], indicative for transient tolerance mechanisms. The expression levels decline during the course of islet autoimmunity, which could be correlated with Vitamin A concentrations in the serum of patients with T1D.

4.1.2. atRA improves Treg induction *in vitro* under pro-inflammatory conditions using T cells from individuals with long-term T1D

In vitro, efficient conversion of naïve T cells into Tregs is achieved under limited TCR stimulation, whereas continuous TCR stimulation (with or without the presence pro-inflammatory cytokines) hinders efficient Treg induction. To test whether atRA treatment can improve Treg induction under such challenging conditions, I tested several settings

of Treg induction: continuous TCR stimulation (continuous) limited TCR stimulation (limited) or limited TCR stimulation and addition of a pro-inflammatory cytokine (limited + IL-6) in the presence of atRA (Fig. 5A-B). Here, I observed the strongest effect of atRA-mediated Treg induction in settings of limited TCR stimulation in the presence of the pro-inflammatory cytokine IL-6 when I analyzed the fold change of atRA treatment over vehicle in T cells of a patient with T1D compared to a healthy donor.

Accordingly, atRA has been shown to improve Treg induction capacity even in the presence of a pro-inflammatory milieu. To investigate the impact of atRA on Treg induction capacity, I stimulated naïve $CD4^+$ T cells using limited TCR stimulation in the presence of the pro-inflammatory cytokine IL-6 to mimic an inflammatory milieu and treated them with atRA. Due to the small sample size, I also compared the frequencies of Tregs between vehicle and atRA-treated naïve T cells from healthy donors and patients with established T1D. I could see an increase of Treg induction in naive T cells from healthy donors (Fig. 5C, $P < 0.05$), and an even stronger response in naïve T cells from patients with T1D *in vitro* (Fig. 5D, $P < 0.01$). When I directly compared Treg induction capacity upon atRA treatment between these two groups, I could see a trend towards increased capability to induce Tregs in T cells from individuals with established T1D (Fig. 5E).

Taken together, these data emphasize that atRA plays an important role in inducing Tregs during challenging conditions as during ongoing islet autoimmunity in the human setting.

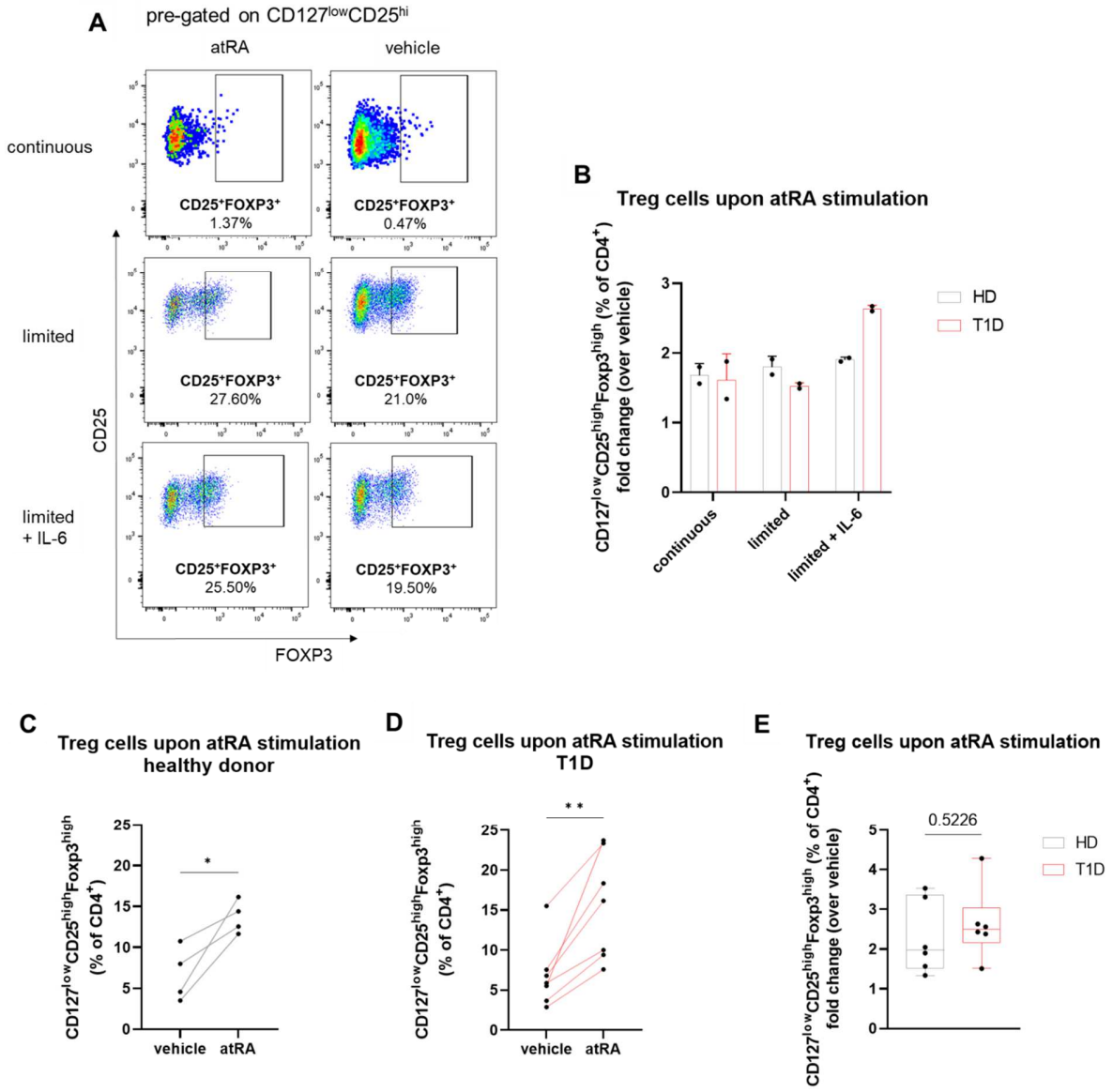


Figure 5: Increased *in vitro* Treg induction upon atRA treatment. (A) Representative FACS stainings of *in vitro* Treg induction upon 1µM atRA treatment using different stimulation conditions and (B) corresponding quantification of $CD127^{lo}CD25^{hi}Foxp3^{high}$ Tregs as fold change atRA over vehicle; continuous=continuous TCR stimulation; limited=limited TCR stimulation; limited+IL-6=limited TCR stimulation in presence of 10ng/mL IL-6. Summary bar plots, each data point represents technical replicate. (C-D) *In vitro* Treg induction using limited TCR stimulation in the presence of 1µM atRA and 10ng/mL IL-6 using naïve $CD4^+$ T cells of PBMCs from indicated donors. (E) *In vitro* Treg induction depicted as fold change of conditions with atRA stimulation normalized to vehicle (fold change over vehicle) using limited TCR stimulation in the presence of 10ng/mL IL-6 using naïve $CD4^+$ T cells of PBMCs

from healthy donors (HD) or patients with established T1D. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

4.1.3. Increased expression of miR-99-5p in early stages of murine islet autoimmunity

In order to get more insights in underlying mechanisms of the atRA-mediated impact on Treg induction during islet autoimmunity, I tested whether this miRNA has also a biological relevance in NOD mice. Therefore, I assessed miR-99a-5p expression levels in naïve T cells from axillary lymph nodes (axLN) of non-autoimmune-prone Balb/c mice versus autoimmune-prone NOD mice. In T cells from young NOD mice (<30 days of age), I saw an increase in the expression levels of miR-99a-5p in naïve T cells compared to Balb/c mice which reached significance in insulin autoantibody positive (IAA^+) NODs (Fig. 6A, P < 0.01). This could be indicative for ongoing counter-regulatory mechanisms, because during the progression of islet autoimmunity, these expression levels significantly decreased again in T cells from old IAA^+ NOD mice (> 70 days of age) (Fig. 6A, P < 0.001). I also looked at the expression levels in naïve T cells from disease-relevant pancreatic lymph nodes (pLN), where I could observe similar trends (Fig 6B).

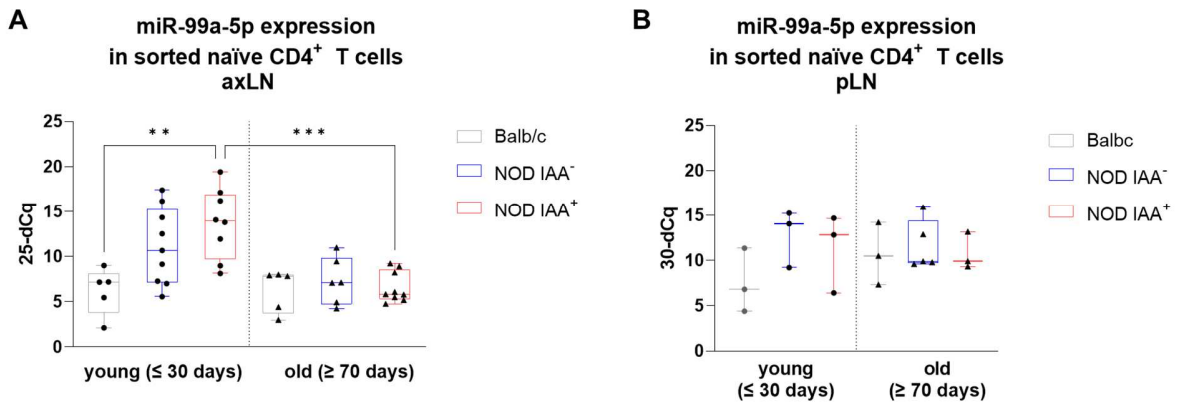


Figure 6: miR-99a-5p abundance in naïve $CD4^+$ T cells from different LNs. (A-B) Expression of miR-99a-5p in naïve $CD4^+$ T cells isolated from axLNs (A) or pLNs (B) in Balb/c control mice and NOD mice without the presence of insulin autoantibodies (IAA^-) and with insulin autoantibodies (IAA^+) at different ages as assessed by RT-qPCR. axLN=auxiliary lymph nodes; pLN=pancreatic lymph nodes. Ordinary one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001.

4.1.4. High levels of induced Tregs correlate with enhanced miR-99a-5p expression upon atRA treatment

Similar as to the human setting, I tested whether atRA treatment can improve Treg induction under challenging conditions. To do so, I either used continuous TCR stimulation in the presence of atRA (continuous), limited TCR stimulation with atRA (limited) or limited TCR stimulation where I added atRA after 18 hours (limited + atRA after 18hrs) (Fig. 7A, B). The effect of additional cytokine stimulation upon atRA treatment during Treg induction was tested in later settings. Since there was the most pronounced effect when using continuous stimulation upon atRA treatment, I performed all further experiments with this condition, if not indicated otherwise.

I next analyzed the expression levels of miR-99a-5p after Treg induction upon atRA stimulation. It has been shown that atRA is able to strongly induce the expression of this miRNA in Tregs in the presence of TGF-β. In order to achieve stable Tregs *in vitro*, we induce Tregs independently of TGF-β. To assess the expression levels of miR-99a-5p upon atRA stimulation after Treg induction, I extracted RNA from whole T cells of the assay. As displayed in fold change, there are slight increases in the expression levels of miR-99a-5p upon atRA treatment after Treg induction (Fig. 7C). However, these culturing conditions might not be optimal for the assessment of miR-99a-5p expression, since only a few Tregs in frequencies are induced compared to limited TCR stimulation. Interestingly, when I correlated the expression levels of miR-99a-5p upon atRA treatment after Treg induction with induced Treg frequencies in T cells of NOD mice with ongoing islet autoimmunity, there was a positive trend between these two factors (Fig. 7D).

Overall, these data show that similar to the human setting, miR-99a-5p showed highest expression at early stages of islet autoimmunity in T cells of autoimmune-prone NOD mice, which also declined during the course of islet autoimmunity.

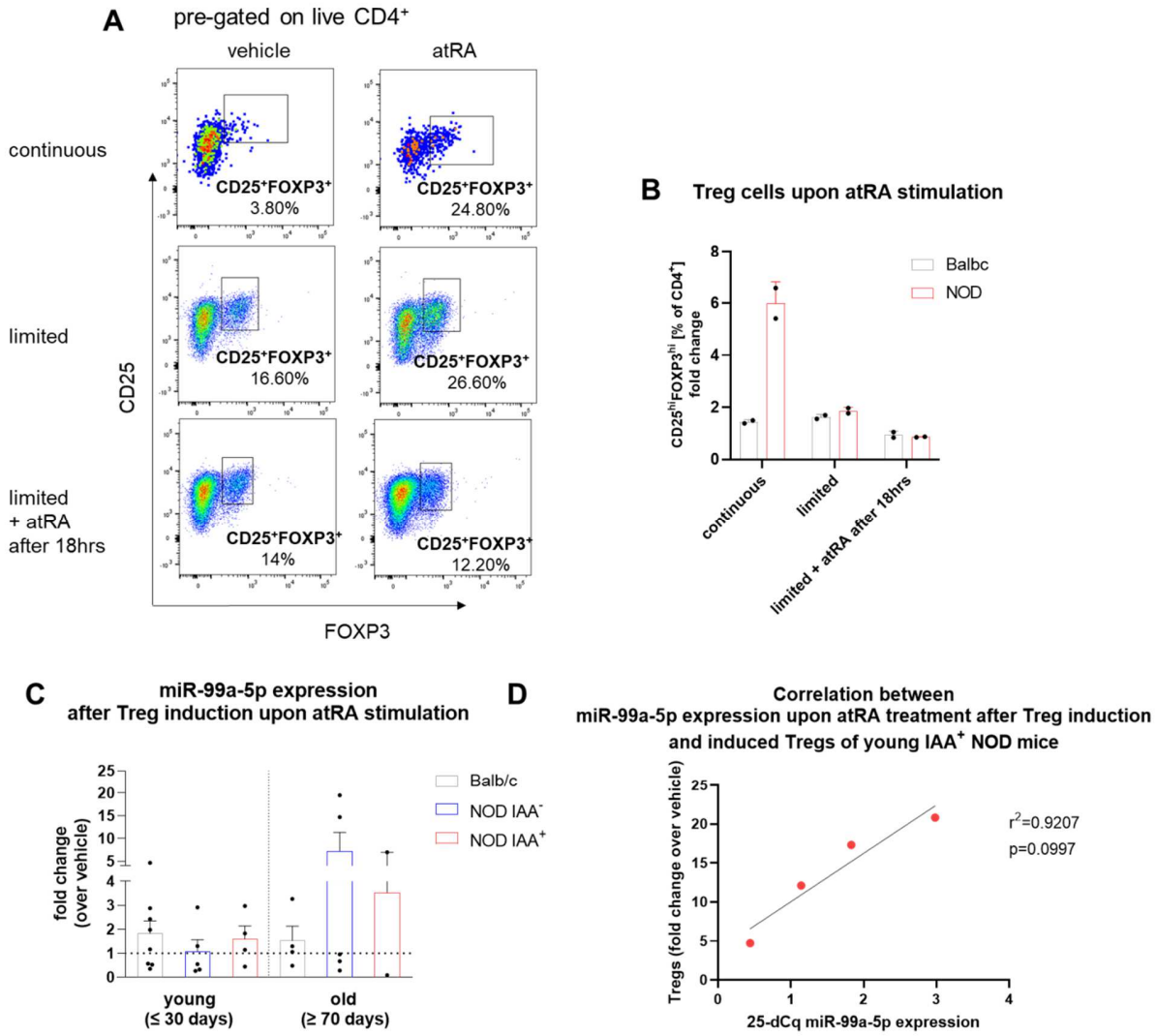


Figure 7: Correlation of miR-99a-5p abundance in induced Tregs and Treg frequencies. (A) Representative FACS stainings of *in vitro* Treg induction upon 1µM atRA treatment using different stimulation conditions and (B) corresponding quantification of CD25^{hi}FOXP3^{+} Tregs as fold change atRA over vehicle using naïve CD4^{+} T cells of axLN from control Balb/c mice and NOD mice; continuous=continuous TCR stimulation; limited=limited TCR stimulation; limited+atRA after 18hrs=limited TCR stimulation and atRA treatment after 18hrs of culture. Summary bar plots, each data point represents technical replicate. (C) miR-99a-5p expression as assessed by RT-qPCR after Treg induction upon atRA stimulation depicted as fold change over vehicle. Data represent the mean ± SEM. (D) Correlation of miR-99a-5p expression after Treg induction with frequencies of induced Tregs in young NOD IAA^{+} mice.

4.1.5. Strong TCR stimulation impairs *in vitro* Treg induction in T cells from NOD mice

It was previously shown that Treg induction using limited TCR stimulation was reduced in T cells from autoimmune-prone NOD mice. I tested if there are also impairments in Treg induction for challenging conditions. Indeed, Treg induction was significantly impaired in T cells from autoimmune-prone NOD mice, irrespectively of their insulin autoantibody (IAA) status when compared to a non-autoimmune-prone control Balb/c mouse using strong activating conditions (Fig. 8A, $P < 0.0001$). In very challenging conditions upon stimulation with a pro-inflammatory cytokine mix, only a few Tregs were induced, and they showed a significant reduction in older NOD mice compared to Balb/c mice (Fig. 8B, $P < 0.05$). This data demonstrate that Treg induction is greatly impaired during islet autoimmunity.

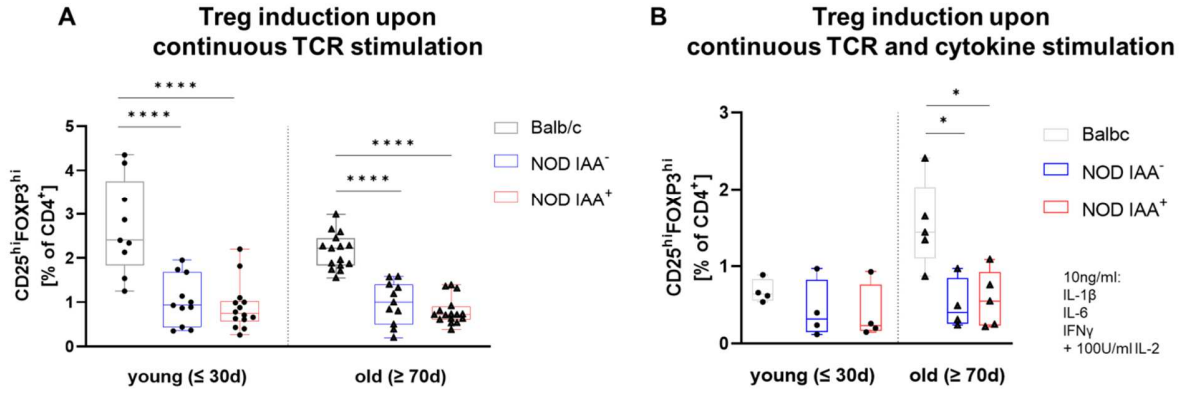


Figure 8: Impairments in Treg induction in autoimmune-prone NOD mice. (A) Frequencies of $CD25^{hi}FOXP3^{+}$ Tregs after continuous *in vitro* Treg induction of naïve $CD4^{+}$ T cells isolated from axLN of control Balb/c mice or NOD mice with different IAA status and ages (B) or using a mix of pro-inflammatory cytokines in indicated concentrations. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001, **** P < 0.0001.

4.1.6. atRA fosters Treg induction *in vitro* during inflammation

Treg induction is significantly increased in T cells from both young and old NOD mice upon atRA stimulation under challenging conditions (Fig. 9A, $P < 0.01$ and $P < 0.001$). To further challenge Treg conversion and to mirror a strong inflammatory setting, pro-inflammatory cytokines were added to the cells and Treg induction capacity was assessed with our without atRA treatment. There is a significant increase in Treg induction using naïve T cells from autoimmune-prone NOD mice upon atRA treatment

as compared to a non-autoimmune Balb/c mouse (Fig. 9B, P < 0.05). This is indicative of a strong anti-inflammatory role of atRA during inflammation by fostering Treg induction.

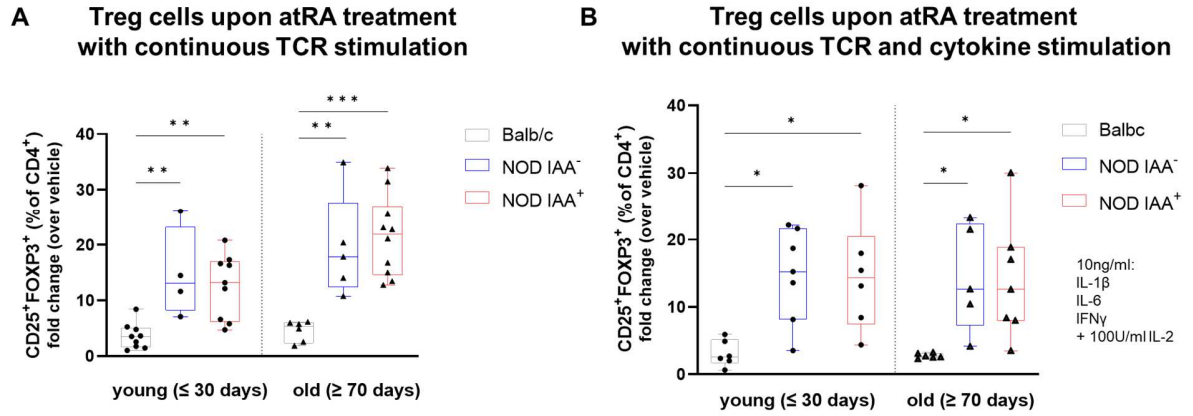


Figure 9: Enhancement of Treg induction upon atRA treatment. (A) Frequencies of CD25^{hi}FOXP3^{+} Tregs after continuous *in vitro* Treg induction of naïve CD4^{+} T cells isolated from axLN of control Balb/c mice or NOD mice with different IAA status and ages upon 1µM atRA treatment (B) or also adding a mix of pro-inflammatory cytokines in indicated concentrations. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

4.1.7. Inhibition of miR-99a-5p reduces atRA effect on *in vitro* Treg induction

To test whether the effect of atRA on Treg induction capacity is mediated through miR-99a-5p, I first stimulated naïve CD4^{+} T cells with atRA and added a LNA™ miR-99a-5p inhibitor complexed in Lipofectamine RNAiMAX Transfection Reagent after 18 hours. The inhibition of miR-99a-5p was confirmed by qPCR, where a decrease in the expression of miR-99a-5p could be observed upon inhibition of miR-99a-5p as compared to a control inhibitor (Fig. 10A). Next, I investigated the effect of miR-99a-5p inhibition on *in vitro* Treg induction upon atRA treatment. Therefore, in a first step, I normalized Treg frequencies from atRA-treated groups to vehicle-treated groups and, in a second step, compared the miR-99a-5p inhibitor treatment with the control inhibitor treatment (shown are changes compared to the control treatment "control inhibitor + atRA"). Importantly, inhibition of miR-99a-5p reduced the positive effect of atRA on Treg induction in young and old NOD (Fig. 10B, C), which was significant when using T cells from NOD mice with ongoing islet autoimmunity (Fig. 10B, P < 0.01; Fig. 10C, P < 0.05).

These data suggest that atRA acts as upstream regulator of miR-99a-5p to induce Treg cell differentiation.

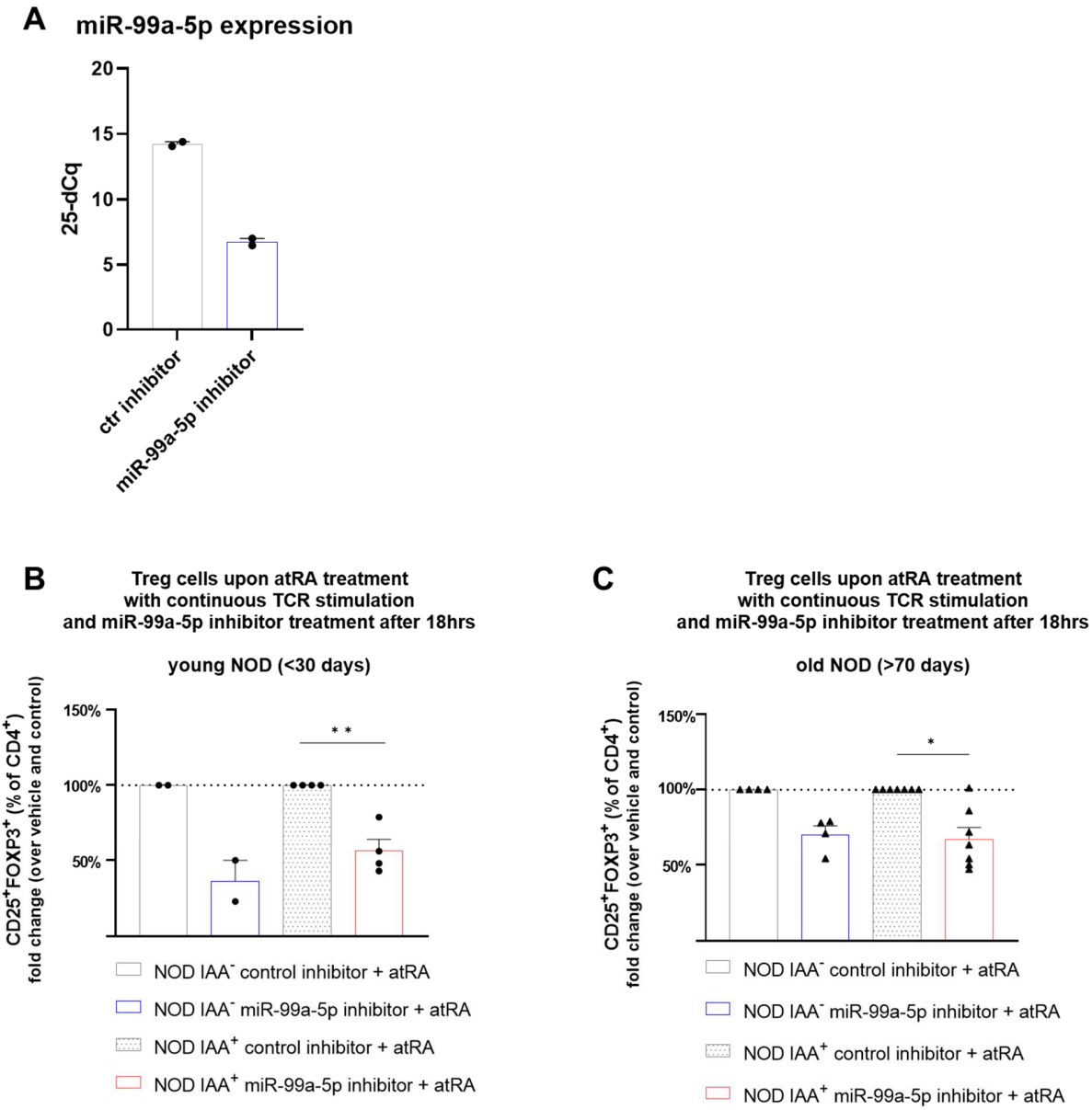


Figure 10: Inhibition of *in vitro* Treg induction upon miR-99a-5p inhibition in the presence of atRA. (A) Validation of miR-99a-5p expression upon treatment with a miR-99a-5p inhibitor compared to a control (ctr) inhibitor for 24 hours as assessed by RT-qPCR. Summary bar plots, each data point represents technical replicate. (B,C) Frequencies of $CD25^{hi}FOXP3^{+}$ Tregs after continuous *in vitro* Treg induction of naïve $CD4^{+}$ T cells isolated from axLN of young NOD mice (left panel) and old NOD mice (right panel) with different IAA status upon treatment with 1µM atRA and addition of a miR-99a-5p inhibitor after 18hrs of culture. Shown is the fold change in % with 100% depicting the control inhibitor treatment in presence of atRA, normalized to vehicle; groups treated with miR-99a-5p inhibitor are depicted as atRA treatment

normalized to vehicle treatment and subsequent normalization to control inhibitor treatment. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

4.1.8. Enhancing miR-99a-5p expression *in vitro* slightly improves Treg induction

Since atRA treatment is at least partially mediated by miR-99a-5p, this miRNA should be able to improve Treg induction independently of atRA. To test this, I applied a LNA™ miR-99a-5p mimic complexed with chitosan PLGA-coated nanoparticles using strong activating conditions. Uptake of the miR mimic by T cells was confirmed by qPCR (Fig. 11A). The miR-99a-5p mimic treatment resulted in a slight increase in the frequency of *in vitro* induced Tregs in all groups, with a more profound effect in older mice. Treg frequencies upon miR-99a-5p mimic treatment were analyzed as percentage relative to the control mimic (Fig. 11B,C). As seen in Fig. 6A,B, young NOD mice had already higher endogenous levels of miR-99a-5p compared to older NODs. Therefore, mimicking this miRNA *in vitro* might not have a strong impact in the setting of young mice.

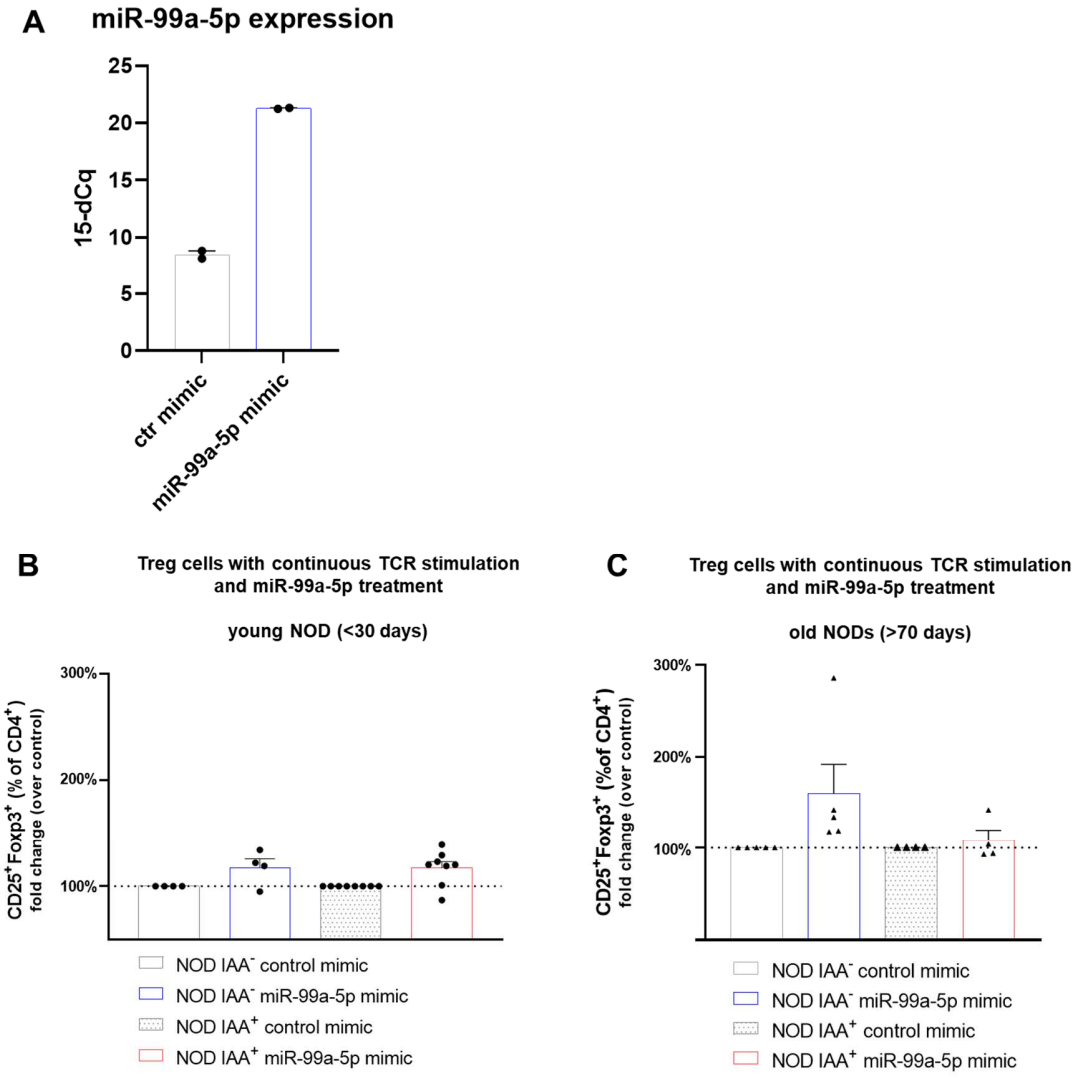


Figure 11: Slight increase of *in vitro* Treg induction upon miR-99a-5p. (A) Validation of miR-99a-5p expression upon treatment with a miR-99a-5p mimic compared to a control (ctr) mimic as assessed by RT-qPCR. Summary bar plots, each data point repre- sents technical replicate. (B,C) Frequencies of $CD25^{hi}FOXP3^{+}$ Tregs after continuous *in vitro* Treg induction of naïve $CD4^{+}$ T cells isolated from axLN of young NOD mice (left panel) and old NOD mice (right panel) with different IAA status and ages upon treatment with a miR-99a-5p mimic. Shown is the fold change in % of the control mimic.

4.1.9. miR-99a-5p expression declines after long-term exposure of Vitamin A diet

Since we see a significant reduction in Vitamin A concentrations in serum of patients with T1D compared to healthy donors (Fig. 4C) and decreased expression of miR-99a-5p in naïve T cells of patients with long-term T1D (Fig. 4A), I wanted to evaluate the

expression patterns of miR-99a-5p in naïve T cells of PBMCs in a Vitamin A excess diet time-course during the course of islet autoimmunity in NOD mice.

NOD mice were subjected to different diet forms right after weaning (21 days of age): Vitamin A **normal** diet (15,000 IU/kg Vitamin A), Vitamin A **deficient** diet (0 Vitamin A [<120 IU/kg]), and Vitamin A **excess** diet (350,000 IU/kg Vitamin), and blood was taken at time points 0, after 2 weeks, 4 weeks and 8 weeks to sort for $CD4^+$ T cells to assess miR-99a-5p expression. I could see a peak of miR-99a-5p expression in naïve $CD4^+$ T cells isolated from PBMCs after 2 weeks, which declined again (Fig. 12). Therefore, for further experiments we subjected mice to a short-term exposure of 2 weeks to Vitamin A excess diet.

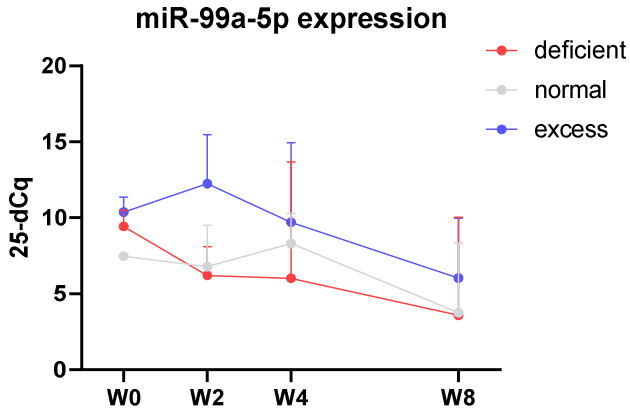


Figure 12: miR-99a-5p abundance in naïve T cells of mice fed with different Vitamin A diets. miR-99a-5p expression in naïve $CD4^+$ T cells from PBMCs of mice fed with different Vitamin A diets (deficient=0 IE/IU Vitamin A; normal=15,000 IE/IU Vitamin A; excess=350,000 IE/IU) after indicated time points as assessed by RT-qPCR. W=week. n=9 mice per diet group.

4.1.10. Increased Vitamin A concentrations correlate with higher miR-99a-5p abundance in NOD mice

Since in T cells from young NOD mice, we see already significantly higher endogenous miR-99a-5p levels as compared to older NODs (Fig. 6A), I subjected old NOD mice (> 77 days of age) to either a Vitamin A excess diet (350,000 IE/IU) or Vitamin A deficient diet (0 IE/IU) for 2 weeks.

When I looked at the expression levels of miR-99a-5p in total $CD4^+$ T cells in the disease-specific target draining lymph node pLN and tissue pancreas, I did not see any difference

between the diet groups (Fig. 13A). However, due to small *n* numbers definitive conclusions cannot be drawn. Vitamin A concentrations significantly increased in NOD mice fed with the Vitamin A excess diet, as expected and were significantly higher than the Vitamin A concentrations in serum of mice fed with a Vitamin A deficient diet (Fig. 13B, $P < 0.0001$). There was no correlation between miR-99a-5p expression in total CD4⁺ T cells sorted from PBMCs and Vitamin A concentrations in serum of NOD mice fed the Vitamin A deficient diet. However, there was rather a trend towards a positive correlation between miR-99a-5p expression in total CD4⁺ T cells of PBMCs and Vitamin A concentrations of serum when looking at NOD mice fed with the Vitamin A excess diet (Fig. 13D) compared to the NODs fed with the Vitamin A deficient diet (Fig. 13C).

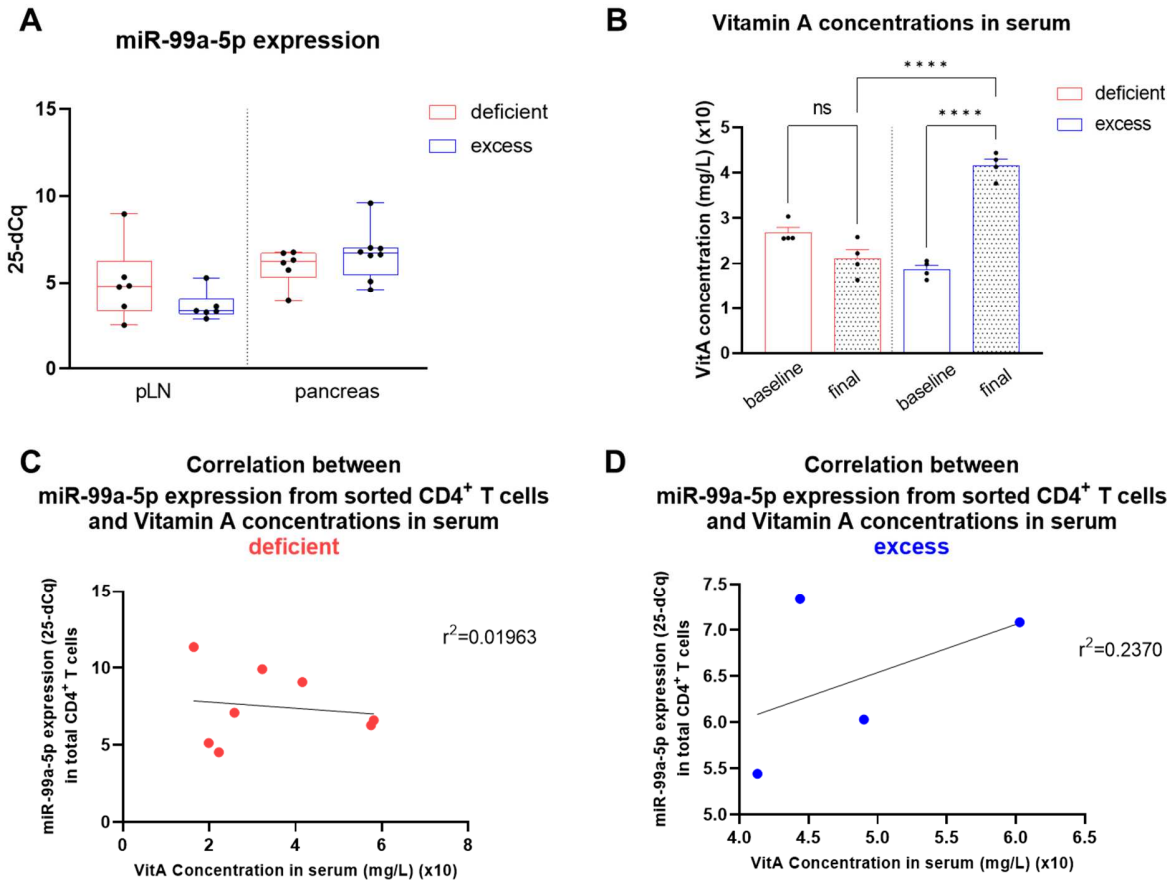


Figure 13: Correlation between miR-99a-5p expression in naïve CD4⁺ T cells and Vitamin A concentrations in serum of mice fed a Vitamin A excess diet. (A) miR-99a-5p expression as assessed by RT-qPCR in NOD mice fed either a Vitamin A deficient diet (=deficient) or Vitamin A excess diet (=excess) in CD4⁺ T cells of pLN and pancreas. (B) Vitamin A concentrations in serum of NOD mice fed

either a Vitamin A deficient diet or Vitamin A excess diet at start of the experiment (=baseline) and at the end (=final). pLN=pancreatic lymph nodes. (C-D) Correlation of miR-99a-5p expression in $CD4^+$ T cells of PBMCs and Vitamin A concentrations in serum from NOD mice fed either a Vitamin A deficient diet (C) or Vitamin A excess diet (D). Ordinary one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

4.1.11. Short-term treatment with a Vitamin A excess diet leads to an enhanced anti-inflammatory phenotype

To see the effect of the Vitamin A excess diet on different immune subsets, I also analyzed *ex vivo* disease-relevant tissues and draining lymph nodes. When I looked at pLN, I could see some trends towards more frequencies of Tregs (Fig. 14A), but no changes in regard to Helios expression (Fig. 14B). Moreover, there was a slight increase in IL-10-producing T cells in the target tissues (Fig. 14C), and a significant increase in the frequency of IL-10-producing Tregs in pLN (Fig. 14D, $P < 0.05$).

Overall, I could not see any differences in T cells or Tregs producing pro-inflammatory cytokine between the diet groups (Fig. 15A-D).

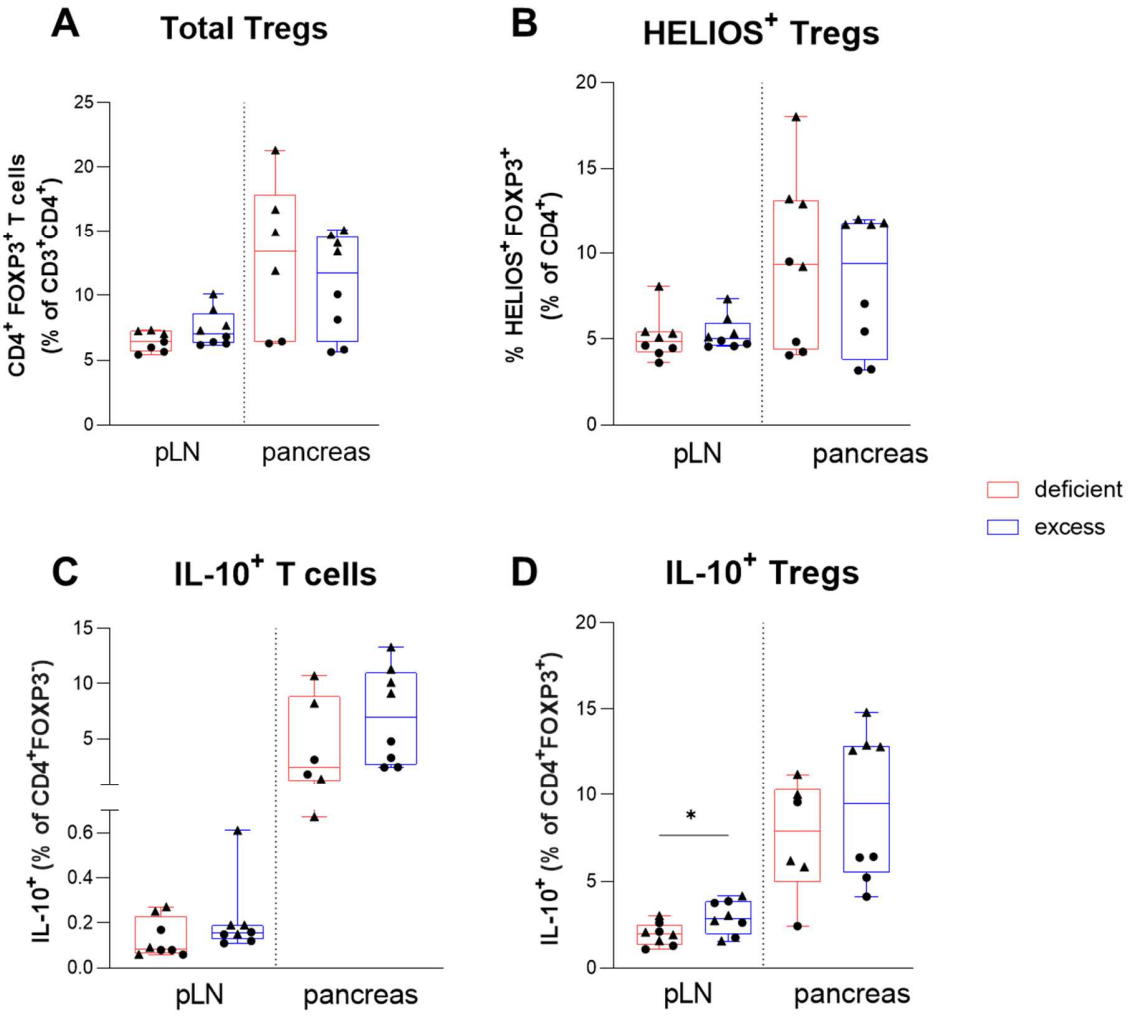


Figure 14: Anti-inflammatory signatures in Tregs in disease-relevant tissue of NOD mice fed a Vitamin A excess diet. (A) Frequencies of $CD4^+FOXP3^+$ Tregs in pLN and pancreas of NOD mice fed either a Vitamin A deficient (=deficient) or Vitamin A excess (excess) diet. (B) Frequencies of $HELIOS^+FOXP3^+$ of $CD4^+$ in pLN and pancreas of NOD mice fed a Vitamin A- deficient of -excess diet. (C) Frequencies of IL-10^+ T cells and IL-10^+ Tregs (D) in pLN and pancreas of NOD mice fed a Vitamin A-deficient of -excess diet. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

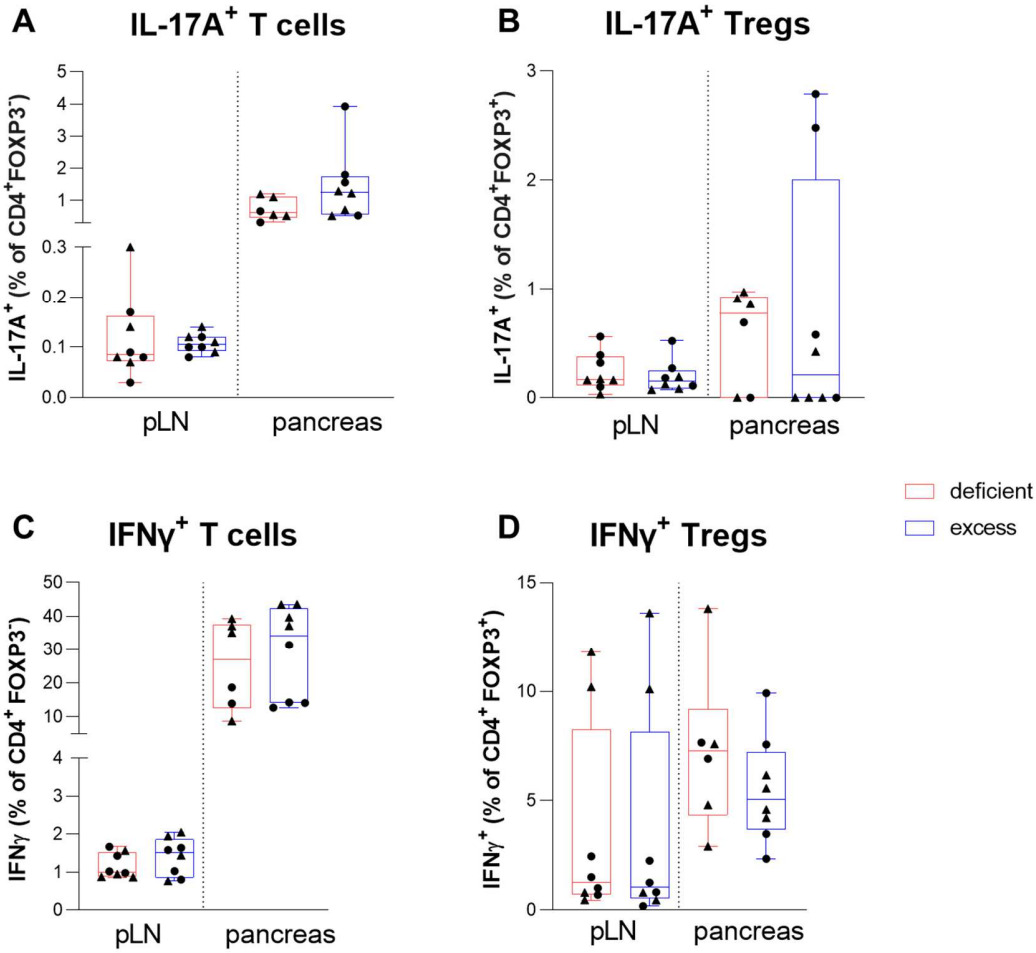


Figure 15: Pro-inflammatory signatures of T cells in disease-relevant tissue in NOD mice fed a Vitamin A excess diet. (A) Frequencies of IL-17⁺ T cells and IL-17⁺ Tregs (B) in pLN and pancreas of NOD mice fed either a Vitamin A deficient (=deficient) or Vitamin A excess (excess) diet. (C) Frequencies of IFNγ⁺ T cells and IFNγ⁺ Tregs (D) in pLN and pancreas of NOD mice fed a Vitamin A- deficient of -excess diet.

4.1.12. Trends towards a positive correlation between miR-99a-5p expression in T cells and Treg frequencies in atRA-treated NOD mice

Since not all of the Vitamin A provided in the diet of these mice will be metabolized to atRA, I could not exclude the possibility that the time frame was not sufficient enough to achieve an effect on miR-99a-5p-mediated Treg upregulation. Therefore, as an alternative approach and in order to further confirm the role as upstream regulator of atRA on miR-99a-5p and their impact on improved Treg induction during islet

autoimmunity *in vivo*, I subjected old NOD mice (> 77 days of age) to atRA treatment for 2 weeks with intraperitoneal (i.p.) injections every other day.

In order to see if atRA treatment *in vivo* also leads to an increase in miR-99a-5p expression, I sorted total $CD4^+$ T cells from different tissues of atRA- or vehicle-treated mice. I could not observe any differences in miR-99a-5p expression in $CD4^+$ T cells between the treatment groups (Fig. 16A), which could potentially be due to the mixed populations present in total $CD4^+$ T cells, also including the pathogenic T cells. To get a better idea of miR-99a-5p expression in Tregs, I correlated the expression of miR-99a-5p from total $CD4^+$ T cells with Treg frequencies (Fig. 16B-E). While there were trends towards a negative correlation which did not reach significance yet between these two factors in vehicle-treated mice (Fig. 16B. 16D), there were trends towards a positive correlation between miR-99a-5p expression in total $CD4^+$ T cells and Treg frequencies in atRA-treated mice, especially in their pancreata (Fig. 16C, 16E). In spite of low *n* numbers, this data give first hints that most of the Tregs were induced via atRA-mediated upregulation of miR-99a-5p.

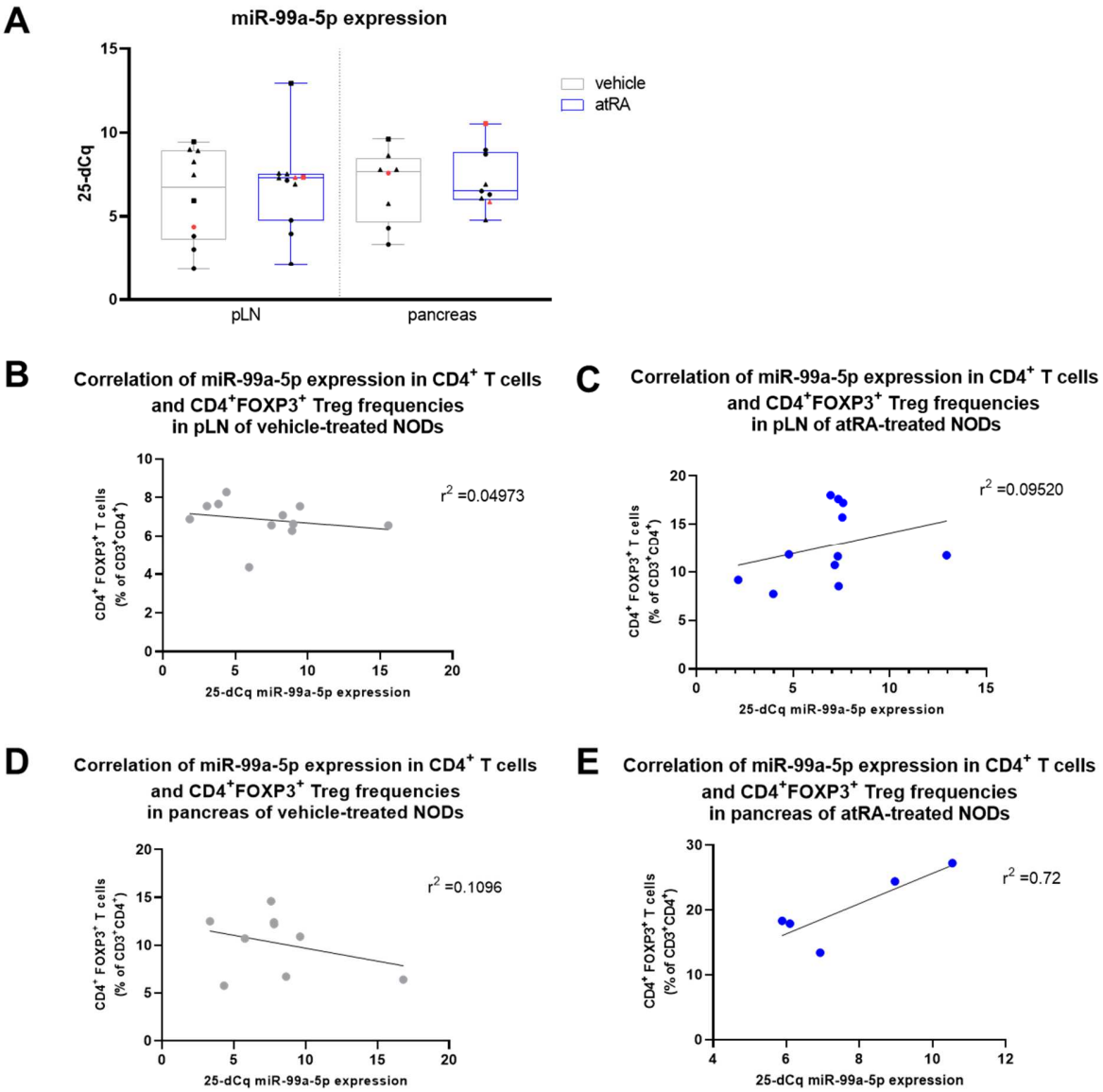


Figure 16: Correlation of miR-99a-5p expression in T cells and Treg frequencies upon atRA treatment. (A) miR-99a-5p expression of CD4+ T cells isolated from pLN and pancreas of mice treated either with vehicle or atRA. Red dots indicate mice with a blood glucose level above 250mg/dL at the end of analysis. (B-E) Correlation of miR-99a-5p expression in CD4+ T cells of pLN or pancreas and Vitamin A concentrations in serum from NOD treated either with vehicle or atRA.

4.1.13. Decreased lymphocyte infiltration upon atRA treatment in NOD mice

Other groups could provide evidence for a delay in disease onset when diabetic splenocytes from NOD were transferred into NOD/*scid* mice and those mice were subsequently treated with atRA compared to mice that received the vehicle control [74]. In line with these findings, I observed a significant reduction in infiltrating CD4$^+$ T cells into the pancreas in atRA-treated mice after two weeks of treatment with atRA (Fig. 17A, $P < 0.001$). When I normalized infiltrating lymphocytes to pancreas weight, I could see a tendency to less infiltrating immune cells in atRA-treated mice (Fig. 17B). This data demonstrates that atRA is able to shape a less inflammatory milieu in spite of ongoing autoimmunity.

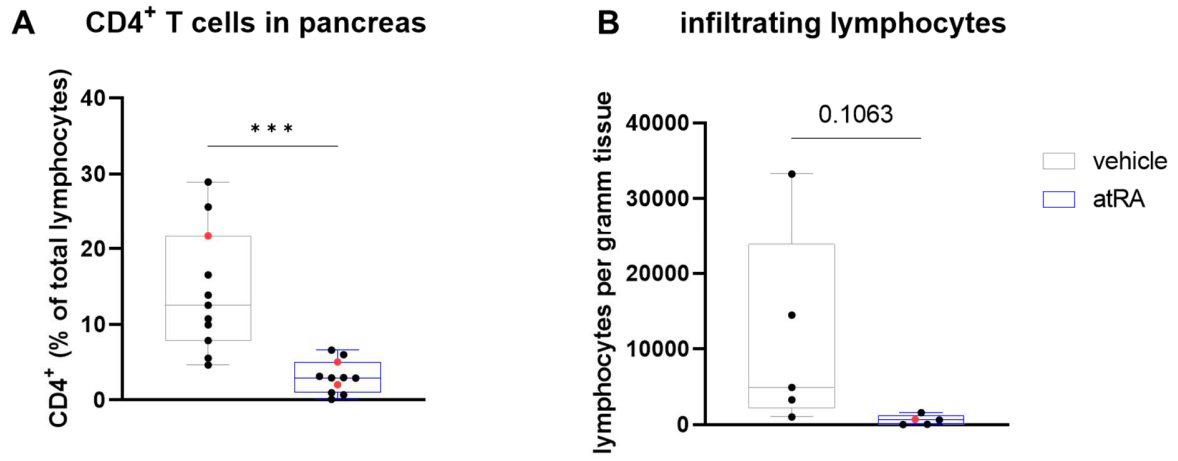


Figure 17: Infiltrating lymphocytes in disease-relevant tissue of NOD mice upon atRA treatment. (A) CD4$^+$ T cell frequencies in pancreas of NOD mice treated either with vehicle or atRA. (B) Infiltrating lymphocytes per gramm pancreas weight in NOD mice treated either with vehicle or atRA. Red dots indicate mice with a blood glucose level above 250mg/dL at the end of analysis. Student's t-test, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

4.1.14. Fully differentiated IFNγ$^+$ T cells remain in target tissues of atRA-treated NOD mice

There was a significant increase in the frequencies of IFNγ$^+$ T cells in atRA-treated mice in both target tissues (Fig. 18B, $P < 0.01$, $P < 0.001$). However, total numbers of IFNγ$^+$ T cells (Fig. 18A) as well as frequencies of IFNγ-producing Tregs (Fig. 18C) did not differ between the treatment groups. The increase in IFNγ$^+$ T cells could result from already

fully differentiated pathogenic T cells in the progressed inflammatory milieu since we used old NOD mice. Other pro-inflammatory T cells, such as IL-17A-producing T cells, did not show any differences between vehicle- or atRA-treated NODs (Fig. 18D). Moreover, there were almost no detectable IL-17A-producing Tregs in both treatment groups (Fig. 18E).

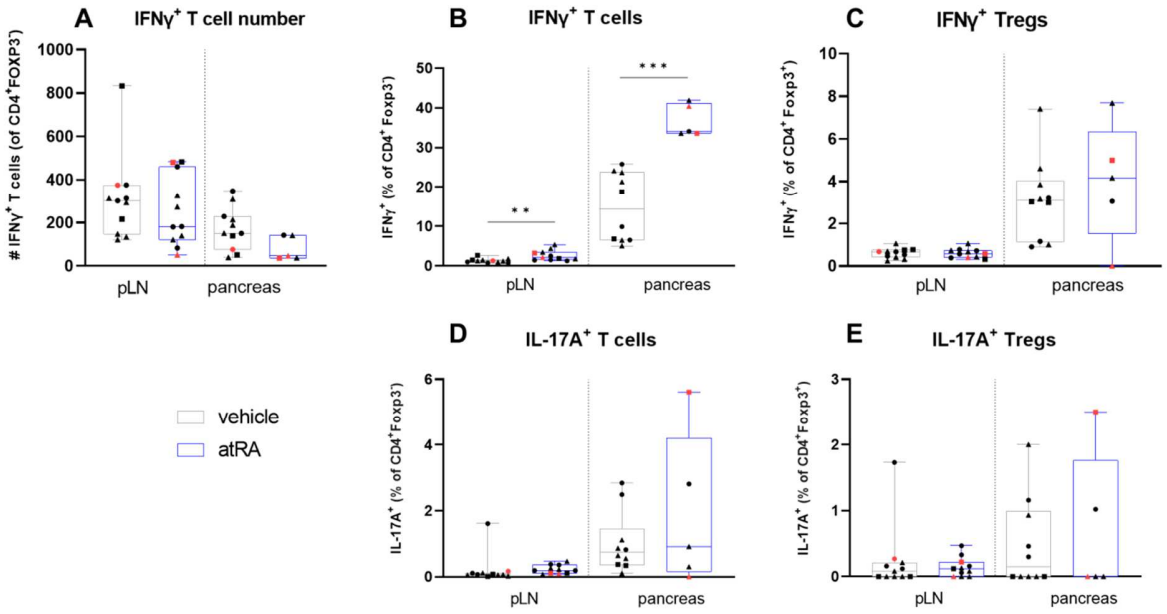


Figure 18: Pathogenic IFNγ⁺ T cells in target tissues of atRA-treated NOD mice. (A) Total number of IFNγ⁺ T cells, (B) Frequencies of IFNγ⁺ T cells and (C) IFNγ⁺ Tregs in pLN and pancreas of NOD mice treated either with vehicle or atRA. (D) Frequencies of IL-17A⁺ T cells and (E) IL-17A⁺ Tregs in pLN and pancreas of NOD treated either with vehicle or atRA. Red dots indicate mice with a blood glucose level above 250mg/dL at the end of analysis. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

4.1.15. In vivo atRA application leads to increased Treg frequencies with a stable suppressive phenotype in NOD mice *ex vivo*

In line with findings by other groups that atRA treatment leads to increased Treg frequencies, I could also see a significant increase of Treg frequencies in pLN and pancreas of atRA-treated mice (Fig. 19A, P < 0.0001, P <0.001) without any differences in total Treg numbers (Fig. 19B). Moreover, there was a significant increase in Helios⁺ Tregs in the target tissues of atRA-treated mice, thereby further indicating superior suppressive function of these Tregs upon atRA treatment (Fig. 19C, P < 0.001, P < 0.01).

This data highlight the fact that Tregs with a stable suppressor phenotype are strongly induced upon atRA treatment.

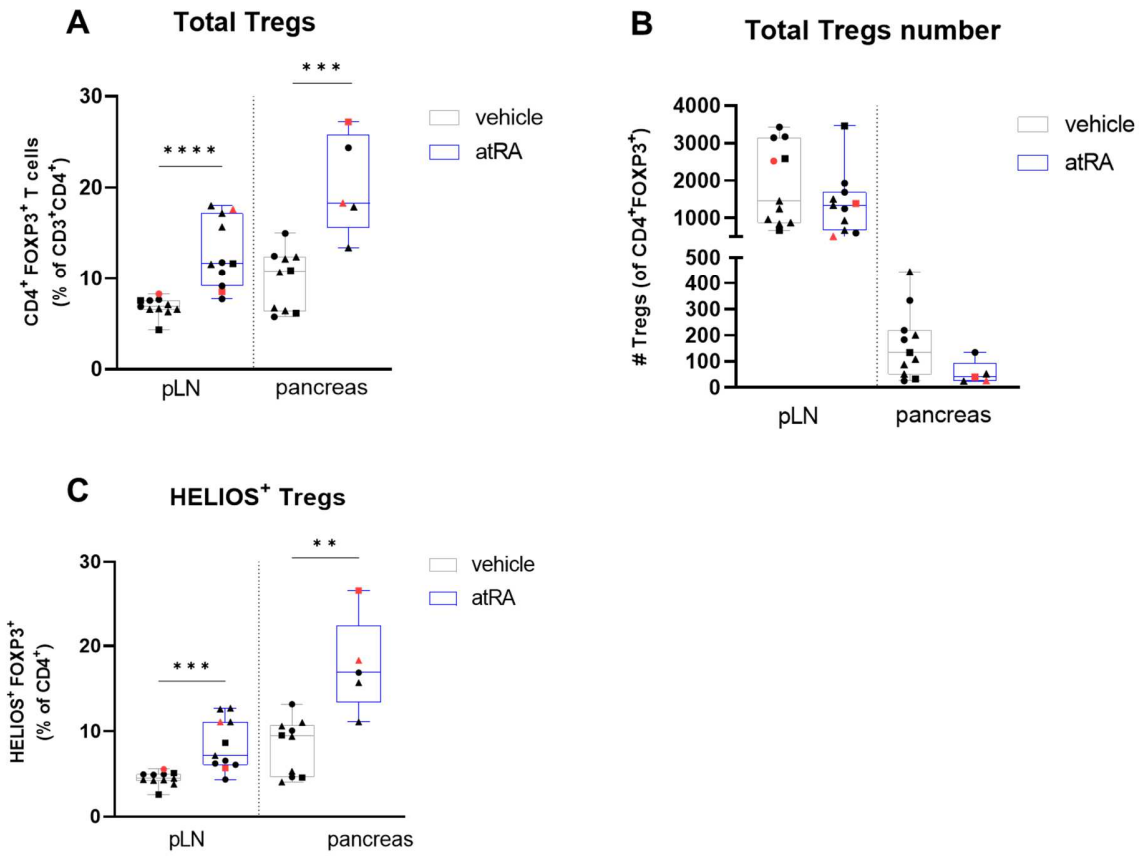


Figure 19: Strong immunoregulatory responses upon atRA treatment in NOD mice. (A) Frequencies of CD4⁺FOXP3⁺ Tregs and (B) total number of CD4⁺FOXP3⁺ Tregs in pLN and pancreas of NOD treated either with vehicle or atRA. (C) Frequencies of HELIOS⁺FOXP3⁺ of CD4⁺ Tregs in pLN and pancreas of NOD treated either with vehicle or atRA. Red dots indicate mice with a blood glucose level above 250mg/dL at the end of analysis. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

4.1.16. Increased production of anti-inflammatory cytokines upon atRA treatment

Cytokines signatures reveal important insights into function and behavior of T cells. Expression of IL-10 has strong anti-inflammatory properties and almost all cells of the innate and adaptive immune system are able to express this cytokine [100-108].

Results

Treatment of atRA in old NOD mice led to a significant increase in IL-10-producing T cells in pLN and pancreas (Fig. 20A, $P < 0.05$) and in pLN of atRA-treated mice, there was also a significant increase in IL-10^+ Tregs (Fig. 20C, $P < 0.01$). Total numbers of IL-10^+ T cells and Tregs were significantly reduced in pancreata of atRA-treated mice (Fig. 20B, D, $P < 0.05$), which could result from the overall reduced infiltrating T cells in atRA-treated mice (Fig. 17A, B).

Overall, in the first section I was able to show that the expression of miR-99a-5p could play an important role in counter-regulatory mechanisms in early stages of human and murine islet autoimmunity. Moreover, treatment with atRA led to pronounced enhancements of Treg induction capacity and a trend towards a positive correlation between miR-99a-5p expression and Treg frequencies after Treg induction *in vitro*. In the *in vivo* setting, I provided evidence for a significant increase in Treg frequencies with superior suppressive functions as well as first trends that miR-99a-5p expression correlates with Treg frequencies in the target tissue of NOD mice treated with atRA.

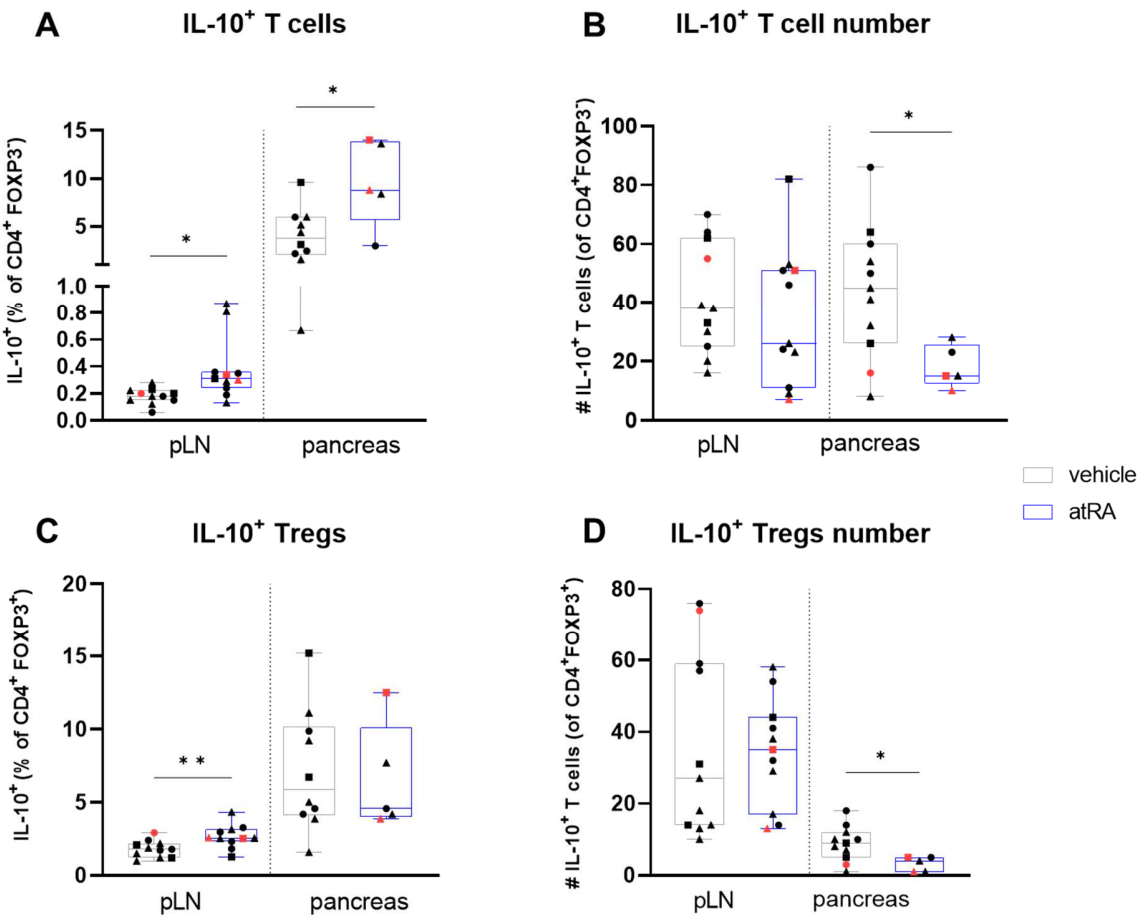


Figure 20: Increased anti-inflammatory signatures upon atRA treatment in NOD mice. (A) Frequencies of IL-10$^+$ T cells and total number of IL-10$^+$ T cells (B) in pLN and pancreas of NOD treated either with vehicle or atRA. (C) Frequencies of IL-10$^+$ Tregs and total number of IL-10$^+$ Tregs in pLN and pancreas of NOD treated either with vehicle or atRA. Red dots indicate mice with a blood glucose level above 250mg/dL at the end of analysis. Student's t-test, *P < 0.05, **P < 0.01, ***P < 0.001.

4.2. Modelling of islet autoimmunity in humanized mice

Underlying mechanisms that lead to impairments in immune tolerance and aberrant immune activations in human islet autoimmunity are still poorly defined. Most studies dealing with these questions are conducted in animal models of T1D, such as the NOD mouse model. Even though there are similarities between human and murine T1D, there are profound differences in T1D pathogenesis. Furthermore, studies from humans are restricted either to PBMCs, which might not reflect the actual situation of the disease, or they are analyzed from pancreatic sections of deceased donors. Therefore, for the second section I used humanized mouse models reconstituted with human immune cells from donors at different stages of islet autoimmunity to study immune responses in a living animal in the target tissue during ongoing islet autoimmunity.

In the second section of the presented work, I received donor blood from individuals with different islet autoimmunity stages and disease durations. For the reconstitution of humanized mice, I used either PBMCs or PBMCs reprogrammed into iPSCs and further differentiated into HSCs.

4.2.1. Reconstitution of humanized mice with PBMCs

For the first approach, I used PBMCs for the reconstitution of humanized mice as outlined in Fig. 21A. Engraftment efficacy was evaluated by frequencies of infiltrating human $CD45^+$ T cells into the tested tissues (Fig. 21B).

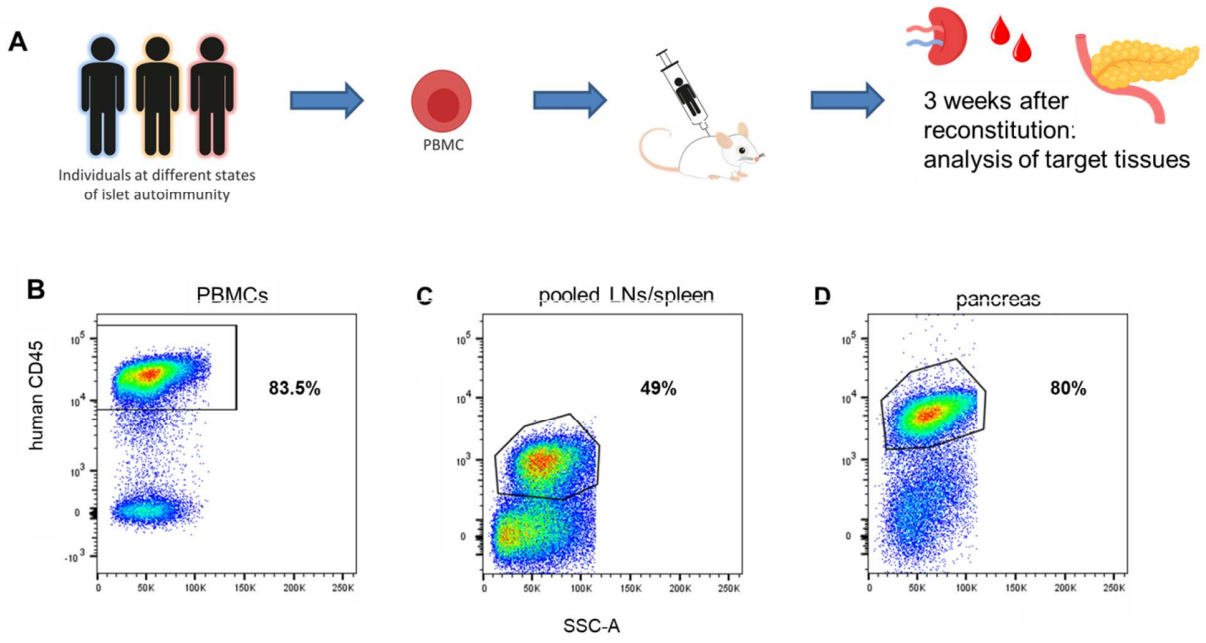


Figure 21: Reconstitution of humanized mice with PBMCs of donors at different disease stages. (A) Schematic representation of experimental design. (B-D) Represenative FACS stainings for engraftment efficacy of human CD45⁺ T cells in indicated tissues. PBMCs=peripheral blood mononuclear cells. LNs=lymph nodes.

4.2.2. Increased Treg expansion in target tissues after reconstitution with PBMCs from autoantibody positive individuals

Individuals that are autoantibody positive show fluctuating waves of islet autoantibodies in their blood without progression to the clinical disease, indicative for ongoing tolerance mechanisms. Accordingly, studies from our group could provide evidence for an increase in the frequencies of insulin-specific Tregs in individuals with long-term islet autoimmunity but without progression to the clinical disease, further highlighting that these children might be in a state of immune tolerance at least for a period of time [39]. To see whether I can mirror these immune tolerance responses in the humanized mouse model, in preliminary studies I analyzed insulin-specific CD4⁺ T cell frequencies in PBMCs of humanized mice. In a pilot study, I observed trends towards higher frequencies of HLA-DQ8 insulin-specific tetramers in PBMCs of humanized mice reconstituted with donor blood from donors at autoantibody-positivity (=autoantibody positive) (Fig. 22B). Of note, in PBMCs of humanized mice reconstituted from the same

donors also showed a tendency towards higher frequencies of Tregs in lymphoid structures after the reconstitution (Fig. 23A). Overall, frequencies of Tregs in the pancreas were very low (Fig. 23B).

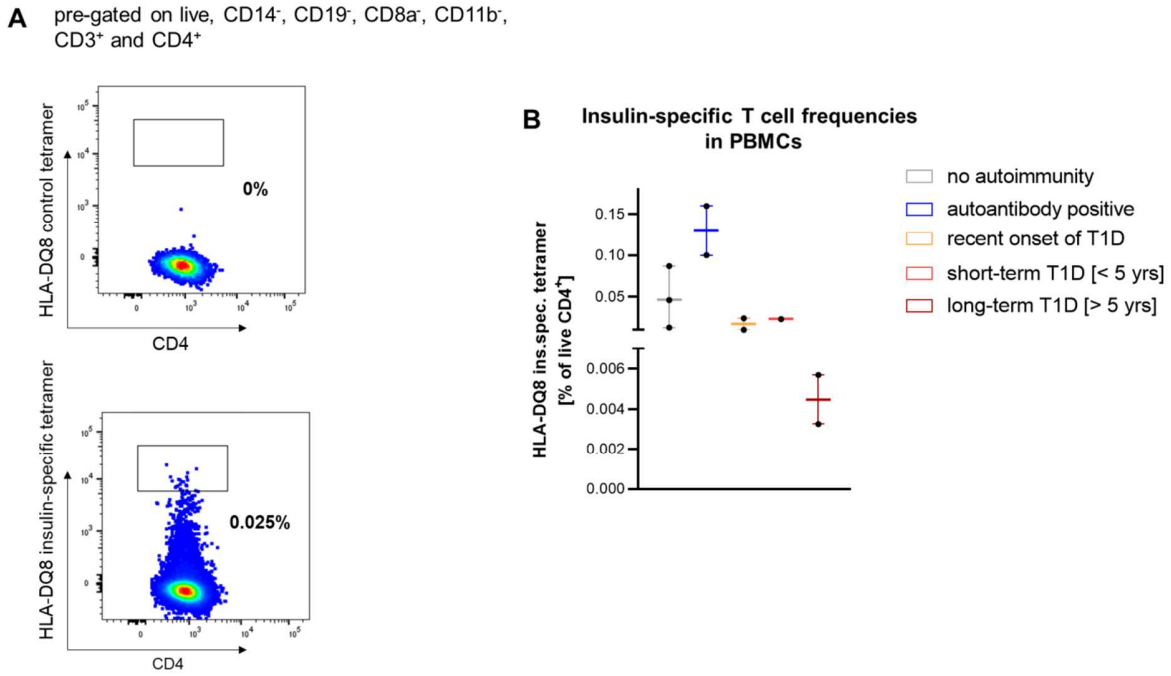


Figure 22: Frequencies of insulin-specific tetramers in humanized mice. (A) Representative FACS stainings for *ex vivo* identification of HLA-DQ8-restricted insulin-specific CD4+ T cells. For the HLA-DQ8 control staining a combination of two control tetramers fused to irrelevant peptides were used (upper panel). (B) Quantification of HLA-DQ8-restricted insulin-specific CD4+ T cells in PBMCs of humanized mice reconstituted with donors at different stages of islet autoimmunity.

Likewise, individuals at-risk due to the presence of HLA-DR4/HLA-DQ8 haplotypes but without autoimmunity (=no autoimmunity) showed also trends towards higher frequencies of these tetramers compared to donors with T1D (Fig. 22B). Patients with established T1D have to inject exogenous insulin for treatment, insulin-specific TCRs get downregulated in the course of the disease. Accordingly, in circulating PBMCs of humanized mice engrafted with donor blood from patients with long-term T1D, i.e. disease duration longer than 5 years, there was a tendancy towards a reduction in the frequency of HLA-DQ8 insulin-specific tetramers (Fig. 22B).

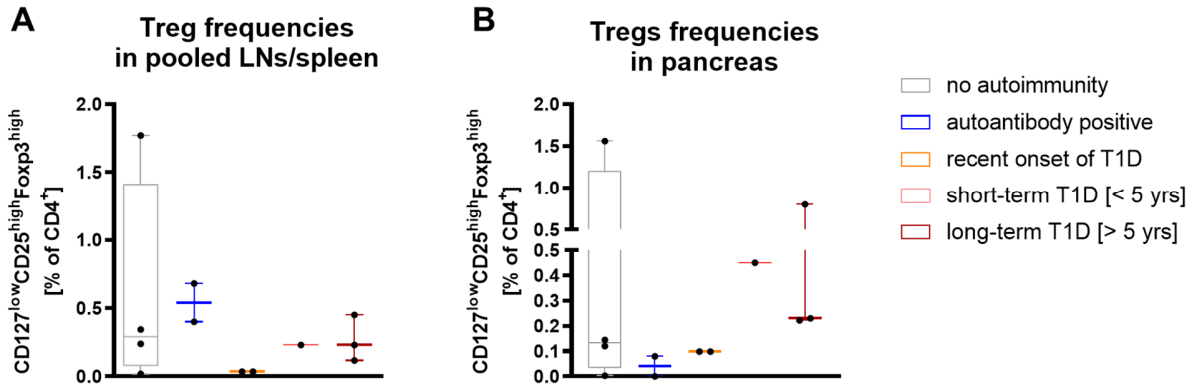


Figure 23: Frequencies of human Tregs in LN/spleen of humanized mice. (A) Frequency of $CD127^{lo}CD25^{high}Foxp3^{high}$ of $CD4^+$ T cells in pooled LNs and spleen (A) and pancreas (B) of humanized mice reconstituted with PBMCs from donors at different stages of islet autoimmunity.

4.2.3. Cytokine profiling of T cells in target tissues

Due to the slight increase in Tregs and insulin-specific T cells, I wanted to further phenotypically characterize the different T cell responses in the target tissues and therefore, I looked at the cytokine profiles. In preliminary experiments, I could see trends towards higher frequencies of IL-10-producing T cells in LNs (Fig. 24A) and a significant increase in IL-10-producing T cells in the pancreas of humanized mice engrafted with PBMCs from an autoantibody negative donor (Fig. 24B, $P < 0.05$). Contrarily, in the pancreata of humanized mice reconstituted with PBMCs from the donors with recently manifested T1D, there was a tendancy towards higher frequency of immune cells producing pro-inflammatory cytokines (Fig. 24C-D).

Overall, these data show efficient engraftment efficacy of PBMCs in humanized mice. Even though patient numbers were very limited, these initial preliminary results highlight that this short-term model can be used to gain better insights in the immune responses in target tissues between different stages of islet autoimmunity. Moreover, this model can be further expanded to characterize cytokine signatures of Tregs in the different stages of islet autoimmunity.

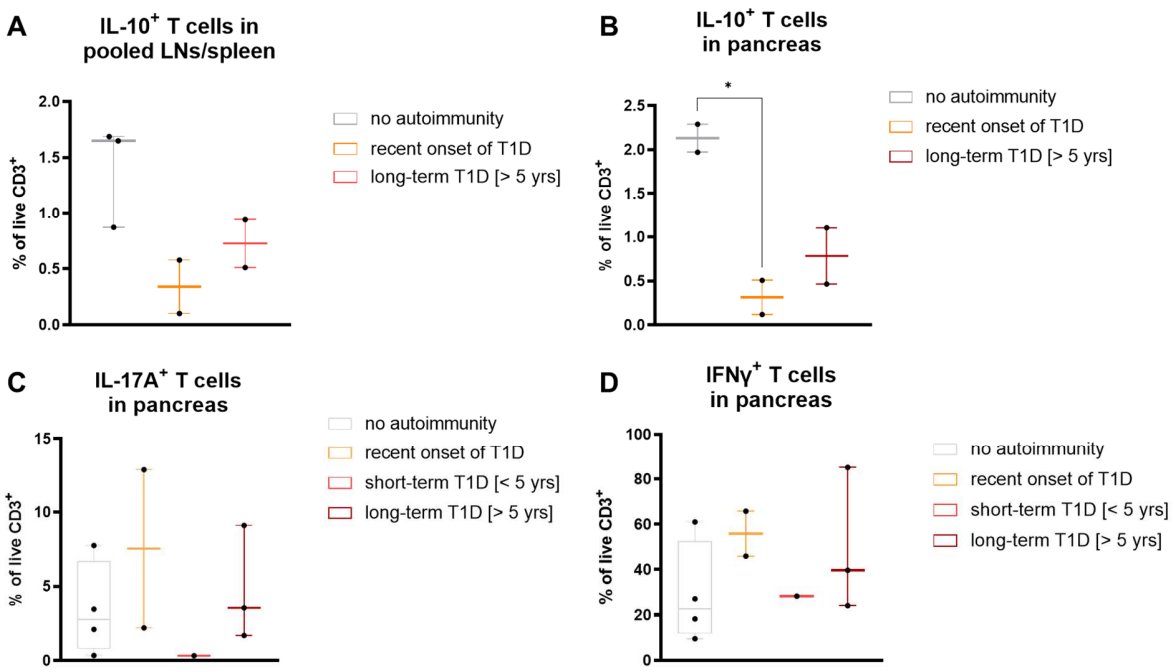


Figure 24: T cell cytokine signature in target tissues of humanized mice. (A) Frequency of IL-10$^+$ T cells in pooled LN and spleen (A) and pancreas (B) or IL-17A$^+$ T cells (C) and IFNγ$^+$ T cells (D) in the pancreata of humanized mice reconstituted with PBMCs from donors at different stages of islet autoimmunity. Ordinary one-way ANOVA, *P < 0.05, **P < 0.01, ***P < 0.001.

4.2.4. Generation of donor-specific iPSC-derived HSCs for reconstitution of humanized mice for long-term studies

Even though the PBMC model allows for a better understanding of human immune responses in a living animal, it has some critical drawbacks. Most importantly, the potential development of Graft-versus-Host disease (GvHD) after three to four weeks, which limits the use of this model to short-term studies. Reconstitution with patient-derived HSCs can be used to overcome this limitation. Of note, efficient reconstitution of humanized mice with human HSCs has been previously shown by our group [39]. Importantly, successful engraftment with HSCs allows for maturation of human T and B cells during differentiation and thus, these cells are tolerant of the host. However, frequencies of circulating HSCs are very low in PBMCs. Therefore, as an alternative approach, we wanted to reprogram patient-derived PBMCs into iPSCs and further differentiate them into HSCs.

Results

This work was performed in collaboration with scientists from the iPSC Core Facility at the Helmholtz Center Munich, led by Ejona Rusha (Head of the Core Facility), Dr. Anna Pertek and Polyxeni Nteli.

For most reprogramming methods, fibroblasts are used as main source due to well established and efficient reprogramming methods. For the use of PBMCs, however, we had to first test different protocols to achieve efficient reprogramming. Moreover, it was important to reach similar reprogramming efficiencies for iPSCs derived from PBMCs of healthy donors as well as of donors with T1D. Initial reprogramming attempts of PBMCs using Episomal vectors did not achieve promising results. As a next step, we tried Sendai virus reprogramming kit, which uses three reprogramming vectors for the delivery of genetic factors that are essential for reprogramming of somatic cells into iPSCs. The kit offers high success rate for blood reprogramming and a rapid clearance of virus, thereby allowing for applications for translational research. Moreover, this reprogramming method is performed under feeder-free conditions, thus enhancing cell viability of PBMCs.

After applying the Sendai virus reprogramming kit as outlined in Fig. 25A, we were able to single-pick and further passage the first emerging iPSC colonies from both a HD and T1D donor (Fig. 25B, C).

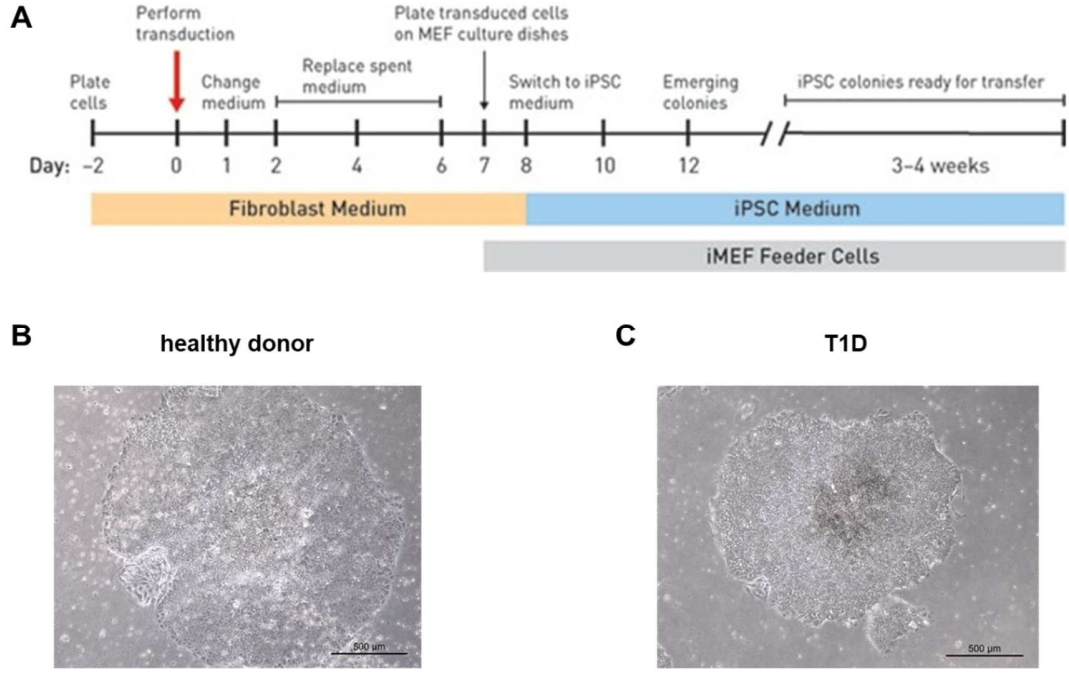


Figure 25: Reprogramming of patient-specific PBMCs into iPSCs. (A) Schematic representation of experimental design. (B-C) Representative pictures of first emerging iPSC clones from PBMCs of a healthy donor (B) and a donor with established T1D (C).

Successful sendai virus clearance was performed by the iPSC Core Facility (Fig. 26A, B) and the sendai virus-free clones (Fig. 26C) were then characterized for pluripotency, based on endoderm differentiation(Fig. 27 A) and the expression of pluripotency markers OCT4, SOX2, SOX17, CXRC4 and FOXA2 (Fig. 27B,C).

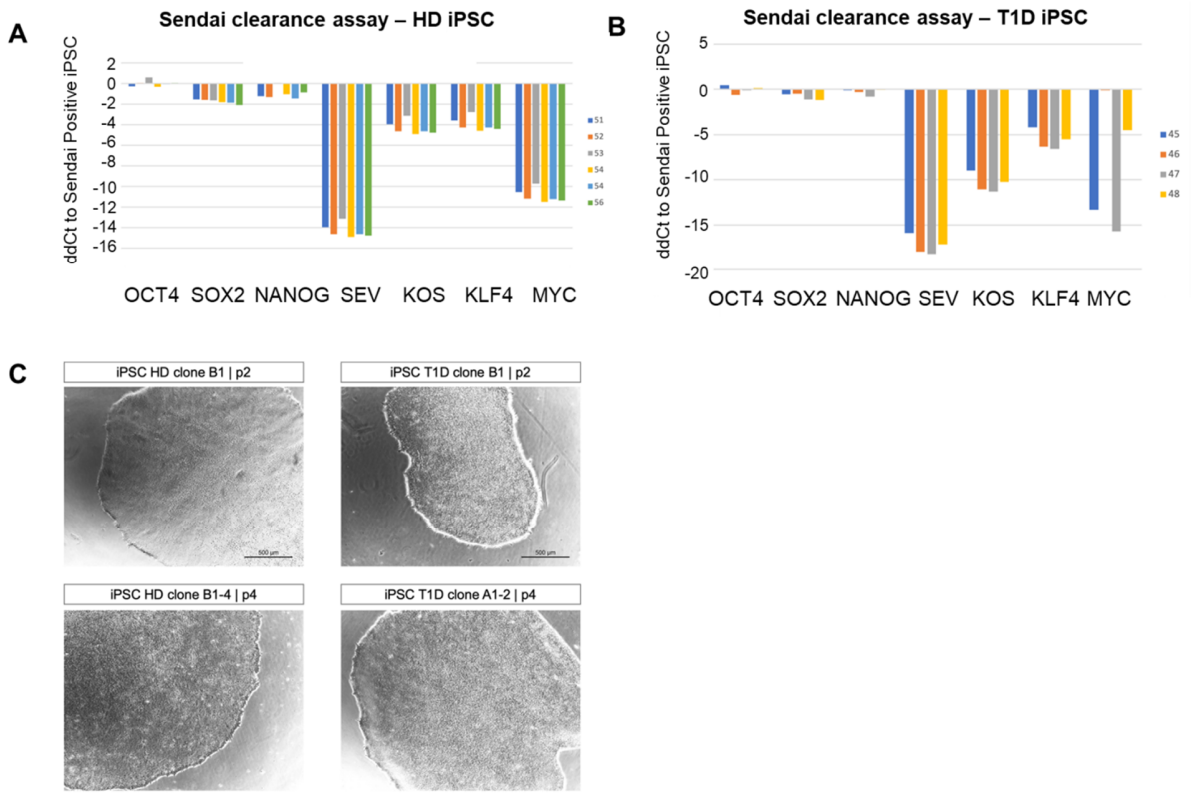
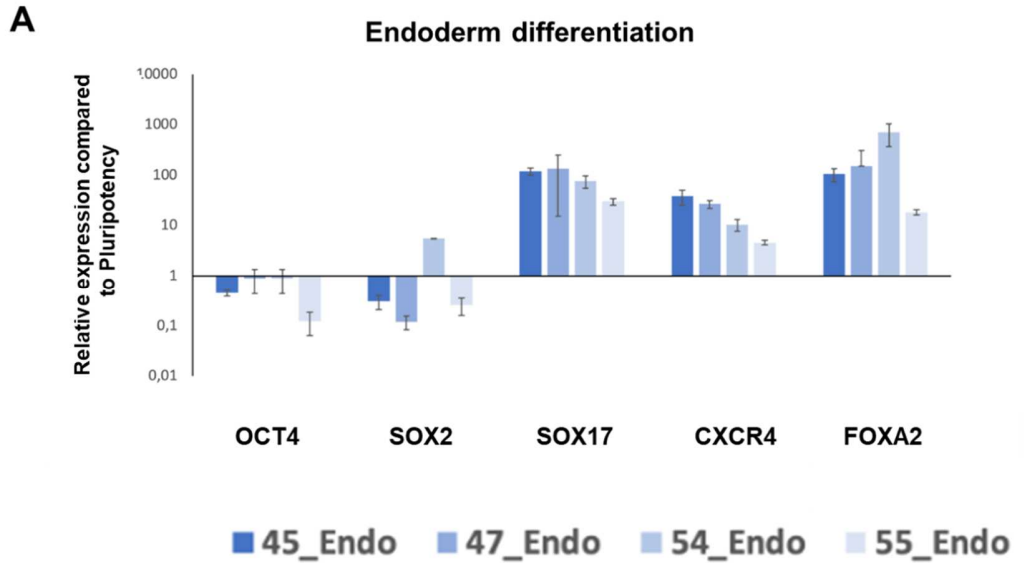
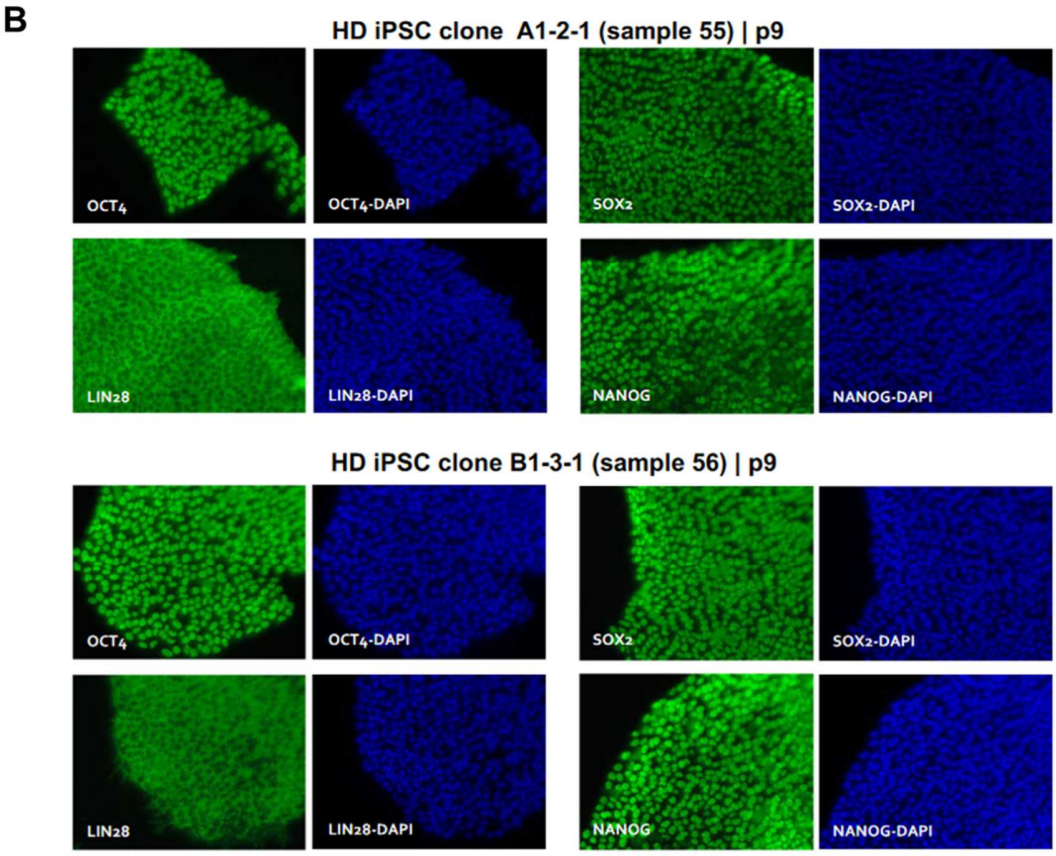


Figure 26: Clearance of Sendai virus. (A-B) Sendai clearance assay as assessed by RT-qPCR, performed by Dr. Anna Pertek, iPSC Core Facility. (C) Representative pictures of iPSC clones derived from a healthy donor (upper and lower left picture) and a donor with T1D (upper and lower right picture).

In parallel, we optimized conditions for the HSC differentiation from control PBMC-derived iPSCs reprogrammed with Episomal vectors. Fig. 28A shows the protocol timeline and in Fig. 28B there are representative pictures from each differentiation steps. Subsequently, the cells were tested for expression of the HSC markers $CD34^{+}CD38^{-}CD45^{+}CD45RA^{-}$ at Flow Cytometry after the 12-day protocol, where we achieved around 20% of hematopoietic pluripotent stem cell differentiation efficiency (Fig. 28C).


A
Endoderm differentiation
Relative expression compared to Pluripotency
10000
1000
100
10
1
0,1
0,01
OCT4
SOX2
SOX17
CXCR4
FOXA2
45_Endo
47_Endo
54_Endo
55_Endo


B
HD iPSC clone A1-2-1 (sample 55) | p9
OCT4
OCT4-DAPI
SOX2
SOX2-DAPI
LIN28
LIN28-DAPI
NANOG
NANOG-DAPI
HD iPSC clone B1-3-1 (sample 56) | p9
OCT4
OCT4-DAPI
SOX2
SOX2-DAPI
LIN28
LIN28-DAPI
NANOG
NANOG-DAPI

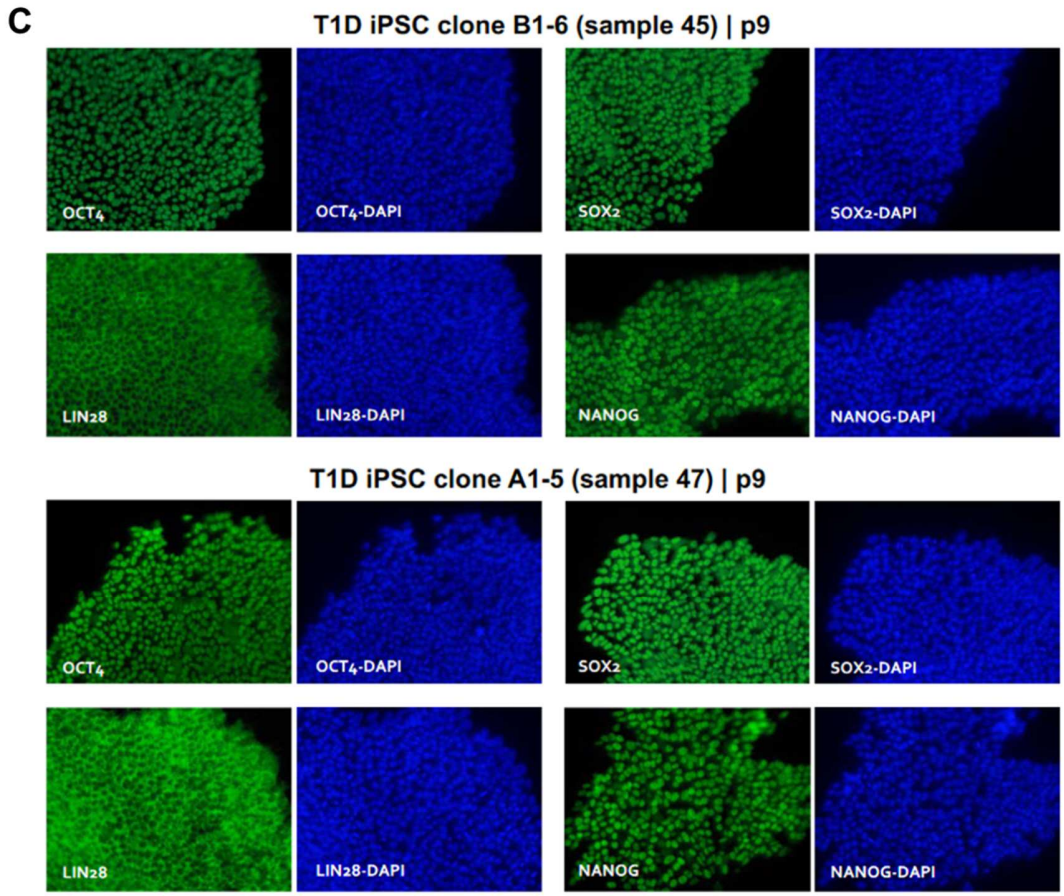


Figure 27: Characterization of iPSC clones after HSC differentiation. (A) Endoderm differentiation for pluripotency testing of the iPSC lines as assessed by RT-qPCR. Sample 54/55=T1D iPSC line; sample 45/47=HD iPSC line. (B-C) Immunocytochemistry for Oct4, Sox2, Lin28 and Nanog of the HD iPSC clone (left picture) and the T1D iPSC clone (right picture). HD=healthy donor. p=passage.

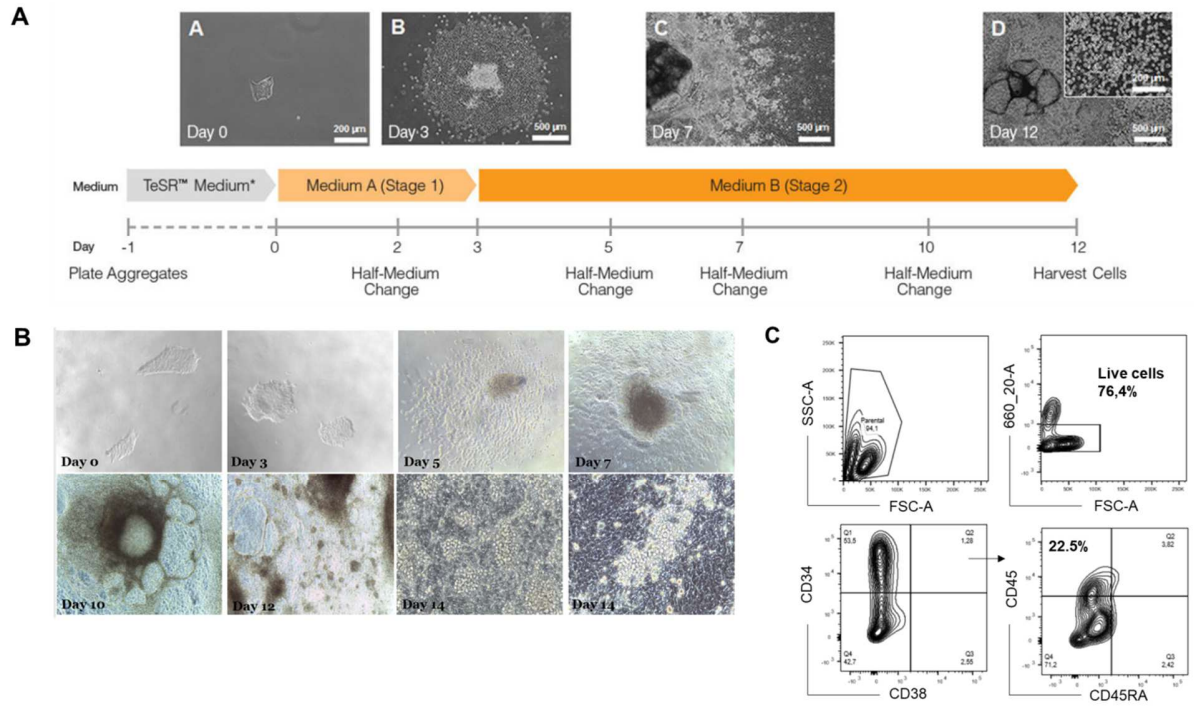


Figure 28: HSC differentiation of iPSC clones. (A) Schematic representation of experimental design (STEMdiff™ Hematopoietic Kit). (B) Representative pictures of the protocol diagram for hematopoietic progenitor cell differentiation. (C) Analysis of $CD34^{+}CD38^{-}CD45^{+}CD45RA^{-}$ hematopoietic progenitor cells by FACS for assessing differentiation efficacy.

These first steps show that we are able to efficiently reprogram patient-derived PBMCs into iPSC, which can be further used for the differentiation into HSC and their engraftment into immunedeficient mice. This mouse model would enable the long-term study of a patient-specific immune system *in vivo* and can be further used for test the safety and efficacy for novel therapeutic approaches.

5. Discussion

5.1. Dissecting the immunoregulatory role of Vitamin A/atRA in islet autoimmunity

T1D develops in individuals with a genetic predisposition and external trigger initiate the onset of islet autoimmunity [109, 110]. There are currently no other therapies for the treatment of established T1D than to administer exogenous insulin injections on a daily routine based on their glucose measurements. However, fluctuating levels of blood glucose are an enormous burden for the patient's life quality and life expectancy. Moreover, the number of incidences for T1D is rising dramatically, an incident for which genetic susceptibility alone cannot account for. As one means, environmental stimuli such as diet have been considered as key drivers for the pathogenesis of T1D [65].

The balance of immunity and immune tolerance is a tightly regulated process, and its dysregulation can contribute to the development of autoimmune diseases. Tregs are critical cellular mediators of peripheral tolerance and hence, their dysregulation has been implicated in the development of autoimmune diseases. In autoimmune T1D loss of self-tolerance to insulin-producing pancreatic beta cells due to the destruction by autoreactive T cells results from an imbalance of immune tolerance and immunity. In this regard, studies from our group could demonstrate broad impairments in immune tolerance concomitant with an aberrant high immune activation [39, 55, 58, 111].

Therefore, the goal of this thesis was to contribute to a better understanding of underlying mechanisms that lead to these impairments in immune homeostasis in islet autoimmunity and to identify new targetable candidates for the development of novel therapeutic approaches. Since Vitamin A has important regulatory functions as anti-inflammatory vitamin in enhancing immune functions and controlling Treg maintenance, the focus was first to dissect the role of Vitamin A/atRA in regulating immune responses in islet autoimmunity.

Several studies investigated the impact of vitamins on T1D pathogenesis, with the main focus on Vitamin D [112-114]. Strikingly, however, low concentrations of Vitamin A have also been observed in the serum of patients with T1D [70-72, 115], but studies in humans remain few in number. Based on these observations, in preliminary studies, I also found

significant lower concentrations of Vitamin A in serum of patients with established T1D compared to healthy donors, but the sample size is limited and larger cohorts are required to identify a possible correlation between Vitamin A serum levels and T1D progression.

It is well known that Vitamin A exerts essential functions in regulating several processes such as embryonic development, visual cycle and immunity [116, 117]. Moreover, the strong anti-inflammatory properties of Vitamin A have been acknowledged already in the 1980s and 1990s [118, 119], and owing to these properties, several derivatives of atRA are in clinical use, for instance for the treatment of acne [120]. However, how Vitamin A or its active metabolite atRA mediate immunoregulatory mechanisms is not completely understood.

Importantly, dietary Vitamin A supplementation has been shown to be able to induce immune tolerance [74, 121] and to inhibit the production of pro-inflammatory cytokines [122]. In the setting of murine T1D, one study conducted in NOD mice could demonstrate that upon dietary Vitamin A supplementation immune cell infiltration could be reduced and consequently, diabetes onset was delayed [121]. To get more insights into differences of T cell subsets upon Vitamin A supplementation, I subjected NOD mice to an excess Vitamin A diet for two weeks. During this short-term exposure of dietary Vitamin A, I could observe a tendency towards increased frequencies of total Tregs in disease-specific draining pancreatic lymph nodes (pLN) and IL-10-producing T cells in both pLN and the target tissue pancreas in NOD mice fed a Vitamin A excess diet. Moreover, I saw a significant higher frequency of IL-10-producing Tregs in pLN, while there were no differences in the production of pro-inflammatory cytokines between the two different diets, which is in accordance with the observation from the aforementioned studies.

Similar to Vitamin A, atRA exerts strong anti-inflammatory properties. In an inflamed milieu, atRA has been shown to stabilize TGF-β-induced Tregs while inhibiting the differentiation of pro-inflammatory Th17 cells [76, 123, 124]. Accordingly, a study investigated the impact of atRA treatment on cytokine release in DBA/1J mice with collagen-induced arthritis [125]. Interestingly, after a 35-week period of atRA treatment,

the group could demonstrate a decrease in arthritis score concomitant with reduced infiltration of macrophages to the inflamed sites. Moreover, the production of pro-inflammatory cytokines was significantly reduced upon atRA treatment.

In the setting of islet autoimmunity, one study reported that upon atRA treatment, immune tolerance could be induced by promoting Treg cell differentiation in NOD/scid mice that were adoptively transferred with splenocytes from newly-diagnosed NOD mice [74]. Specifically, the authors showed that atRA treatment blocked IFNγ production by $CD4^+$ as well as $CD8^+$ effector T cells, thereby resulting in an expansion of Tregs. Another study tested the effect of a combinatorial treatment of atRA and exendin-4, a glucagon-like peptide-1 (GLP-1) receptor agonist which has been shown to increase beta cell mass [126], on T1D development. Interestingly, the authors could show that both treatments given individually were able to preserve beta cell mass. While treatment with atRA and atRA in combination with exendin-4 could delay the development of T1D prior disease onset, after the onset of overt T1D, treating diabetic mice with atRA and/or exendin-4 did not lead to reversal of symptoms [73]. This observation highlights the importance of finding the window of opportunity for future therapeutic intervention studies.

Based on these findings, in the data presented here, I see a strong reduction in infiltrating T cells into the target tissue of NOD mice with ongoing islet autoimmunity treated with atRA in a 2-week treatment period. In order to assess the influence of atRA on Treg functionality during islet autoimmunity, I phenotypically characterized the induced Tregs *in vivo*. In addition to a significant increase in Treg frequencies upon exposure of a short-term atRA treatment, these Tregs present a superior suppressive phenotype based on Helios expression. Moreover, I could observe a significant increase in T cells as well as Tregs producing the anti-inflammatory cytokine IL-10. This is in accordance with previous work showing that atRA is able to modulate cytokine production by increased IL-10 availability, while pro-inflammatory cytokines were limited [127], further supporting its essential role in promoting immune tolerance.

A large body of evidence showed that atRA is able to induce Tregs in the periphery [123, 128-133]. However, the exact mechanisms by which atRA induces Foxp3 induction are

still controversial. In the presence of TGF-β atRA treatment is known to induce Tregs via nuclear receptor RARα [131, 134]. Binding of atRA to RARα leads to recruitment of histone acetyl transferases, thereby allowing for an open conformation of the chromosome and hence, transcription of genes, which drives the differentiation of naïve T cells into Tregs [129]. Initial studies posed the hypothesis that atRA mediates the conversion into Tregs either by inhibiting IL-6 signaling [135] or by blocking the transcriptional activity of activating protein–1 (AP-1)[136]. Importantly, AP-1 is induced upon co-stimulation of T cells and plays an important role as regulator of the IL-2 gene by forming a DNA binding complex with nuclear factor of activated T cells (NFAT). Interestingly, Foxp3 has also been shown to cooperate with NFAT in order to regulate Treg function [137, 138], and AP-1 is able to disrupt their formation [137]. Accordingly, involvement of atRA leads to inhibition of AP-1 and hence, enhances Treg conversion[136]. Intriguingly, one study proposed a different mode of action of a so-called “counterconversion” to induce Tregs by which atRA acts as an indirect negative regulator of memory and effector $CD4^+$ T cells. Specifically, they state that atRA indirectly enhances Tregs by lifting signaling of inhibitory cytokines for TGF-β-induced Foxp3 expression, which are produced by effector $CD4^+$ T cells [139]. This finding was controversial to other studies showing a direct effect of atRA in enhancing Treg induction [140, 141]. Shortly after, it has been proposed that atRA acts directly on the conversion of naïve T cells into Tregs [123], which could result from an indirect suppression of secreted cytokines by naïve T cells [142].

To shed light on these controversial findings, a study in 2009 investigated the molecular mechanisms of atRA-mediated Treg induction in more detail. They concluded that the proposed indirect mechanism only partially explains atRA-mediated Treg conversion. They could further provide evidence that atRA was able to convert Tregs even in absence of inhibitory cytokines, which was mediated by RARα in a Smad3-independent mechanism [139].

In conclusion, the exact mechanisms of atRA-mediated Treg induction and inhibition of Th17 cell differentiation still warrant further studies especially in the context of islet autoimmunity and T1D.

5.2. Understanding atRA-mediated Treg induction during islet autoimmunity

I performed murine and human *in vitro* Treg induction experiments to dissect the role of Vitamin A/atRA-mediated induction of Tregs during islet autoimmunity. To achieve a stable phenotype of these induced Tregs, the *in vitro* induction assays were performed in the absence of TGF-β. Moreover, with the goal to achieve the best atRA-mediated effects on Treg induction, I used challenging culturing conditions, i.e. continuous TCR stimulation.

Interestingly, *in vitro* conversion from naïve T cells into Tregs in the presence of atRA achieved stronger Treg induction capacity using T cells from patients with established T1D as compared to healthy donors, probably due to the progressed inflammatory milieu. This is in accordance with previous studies demonstrating that atRA stabilizes human Tregs in spite of ongoing inflammation *in vitro* and *in vivo* [76].

Remarkably, in the murine setting of T1D, I could observe an in average 20-fold increase in *in vitro* Treg induction upon atRA treatment in T cells of autoimmune-prone NOD mice, whereas there was only a minor increase in T cells of non-autoimmune-prone Balb/c mice. Conversely, *in vitro* Treg induction without atRA led to great impairments in the capacity to induce Tregs in T cells of NOD mice compared to Balb/c mice. It is therefore striking to see that atRA treatment without TGF-β was able to strongly induce Treg induction in spite of these unfavorable conditions for Treg cell differentiation. This finding highly supports the fact that atRA is able to foster Treg induction during ongoing inflammation. This is in line with observations from other groups showing that atRA is able to stabilize murine Tregs despite the presence of the pro-inflammatory cytokine IL-6 [124].

Given its strong anti-inflammatory properties and its function to stabilize Tregs during inflammation, therapies targeting atRA-mediated pathways might be beneficial also in the setting of autoimmune diseases. However, despite these important insights in atRA-mediated Treg cell differentiation, the molecular underpinnings regulating these Tregs are not completely understood. Broad impairments in Treg induction and high immune

activation have made it difficult to develop efficient immunotherapies. Moreover, we are just beginning to understand the requirements for efficient *de novo* Treg induction during ongoing islet autoimmunity.

5.3. Impairments of Treg induction during islet autoimmunity

In previous work, we and others have demonstrated that the best *de novo* Treg conversion from naïve T cells *in vivo* is achieved by subimmunogenic delivery of strong-agonistic TCR ligands, since high doses of antigens activate the PI3K/AKT/mTOR pathway, which negatively regulates Treg induction [39, 52-54, 58]. In the setting of human islet autoimmunity, however, it has been shown that naïve T cells from children with new onset of islet autoimmunity react with a strong proliferation upon antigen stimulation. Accordingly, the resulting increased activation of T cells during islet autoimmunity hinders efficient Treg conversion [55]. Importantly, these impairments were also seen at a polyclonal level, suggesting that there are broad impairments in Treg induction [55].

Moreover, Tregs were shown to have reduced stability in human and murine T1D, as evidenced by increased methylation of the CNS2 region [58]. This instability is accompanied by the presence of Treg subtypes in T1D expressing pro-inflammatory cytokines typical for effector T cells, which is referred to as Treg plasticity [60-62]. Importantly, in the murine setting of T1D, all these Treg defects were observed in very young NOD mice, indicating that Treg impairments occur already at early disease stages and might be causative instead of a mere consequence of the autoimmune process (unpublished data). It is thus required to gain more mechanistic insights of molecular underpinnings guiding impairments in Treg function, stability and induction during islet autoimmunity.

5.4. Identification of molecular mechanisms guiding Treg impairments during islet autoimmunity

As one means, small non-coding miRNAs have attracted a lot of interest in the past years, as they are involved in fine-tuning complex signaling pathways and cellular states, such as T cell activation. Moreover, they are able to regulate gene expression of several

immune cells, such as Tregs. Of note, several miRNAs are also being recognized as disease-relevant and levels of circulating miRNAs in peripheral blood can serve as biomarkers for several diseases, including T1D [143-145]. Recent studies have also been able to link dysregulation of specific miRNAs to the development of islet autoimmunity and its progression to the clinical disease [146-148].
In this regard, in previous work we performed a miRNA sequencing of $CD4^+$ T cells from PBMCs of individuals with various disease durations of islet autoimmunity in order to identify miRNAs being differentially expressed on T cells of individuals with or without islet autoimmunity. Importantly, our group could show a direct link between aberrant expression of miRNAs on activated T cells and impairments in Treg induction and stability [55, 58, 111]. Of note, blocking of these miRNAs or their downstream targets markedly improved Treg induction *in vitro* and also led to a decrease in islet infiltration in mouse models *in vivo*, highlighting the potential of applying miRNA-targeting approaches for the treatment of islet autoimmunity.

Notably, the fact that T cells show a reduced activation threshold in the setting of islet autoimmunity highlights that changes on the naive T cell level contribute to aberrant immune activation and impaired tolerance in islet autoimmunity. Thus, I investigated the role of miR-99a-5p that has been shown to be differentially regulated on naïve T cells of individuals with islet autoimmunity compared to individuals without islet autoimmunity as evidenced by NGS data [55].

Importantly, differentially regulated expression of miR-99a-5p has been brought into context of other diseases as well. For instance, miR-99a-5p is well known for its role as tumor suppressor in several cancers, including oral squamous cell carcinoma (OSCC) [149], breast cancer [150], and hepatocellular carcinoma [151] and its downregulation has been associated with worse survival. Importantly, one study found reduced levels of miR-99a-5p in T cells in an animal model for experimental autoimmune encephalomyelitis (EAE) [152]. However, its specific role in islet autoimmunity remains poorly understood.

Therefore, I wanted to identify the function of this miRNA in naïve T cells during islet autoimmunity. Specifically, I started to look at the expression levels of miR-99a-5p in

naïve T cells from individuals at different stages of islet autoimmunity. Accordingly, I saw low expression of miR-99a-5p in naïve T cells of patients with long-term T1D. Contrarily, those individuals harboring multiple autoantibodies for several years without the progression to the clinical disease showed higher expression of miR-99a-5p in naïve T cells compared to individuals at-risk or long-standing T1D. Interestingly, those donors that suffer from T1D for a short period showed a significant higher expression of miR-99a-5p in naïve T cells. Patients with this short-term duration of T1D often experience the so-called “honeymoon phase”, which is a remission phase in T1D and has been first described in 1940 [153]. The underlying mechanisms of this phenomenon still remain uncertain. Several studies addressed this question and found an increase in several immune subsets, among others Tregs in the early stages of the honeymoon phase [154-156]. Importantly, the duration of this remission phase could also be positively correlated with increased frequencies of Tregs, $CD25^{+}CD127^{hi}$ T cells, $CD45RO^{+}$ memory T cells, neutrophils as well as the production of the anti-inflammatory cytokine IL-10 [155]. Moreover, changes in circulating levels of miRNAs in the course of disease duration have been described by other groups. Of note, one study found that the expression of miR-99a-5p increased in the first year of established T1D and subsequently decreased again [157] which is in accordance with our observations. However, low *n* numbers for donors at the different stages of islet autoimmunity make unambiguous conclusions yet difficult.

Likewise, in the murine model of T1D we see an early and late onset of islet autoimmunity. Accordingly, there was an increase in the expression of miR-99a-5p in naïve T cells from autoimmune-prone NOD mice at early stages, which could also result from counter-regulatory mechanisms during early inflammation. During the course of islet autoimmunity, however, the levels of this miRNA declined again in older NOD mice with ongoing islet autoimmunity.

The observation that miR-99a-5p downregulation has been associated with disease exacerbation in different conditions [149-152] was in accordance with our findings of a decline in miR-99a-5p expression levels during the course of islet autoimmunity. Based on these data, the observed differences in expression levels during the course of

disease could hint to an important immunoregulatory function of miR-99a-5p in T cells at early stages of islet autoimmunity. This hypothesis is further supported by the observation that those individuals with a so-called slow progressor phenotype, i.e. non-diabetic individuals with long-term islet autoimmunity had also higher frequencies of insulin-specific Tregs [39].

In this regard, miR-99a-5p has been identified as one of the top candidates in a miRNA screen for relevant regulators of efficient Treg induction *in vitro* [75]. Specifically, they found that miR-99a-5p and miR-150 cooperatively repress mTOR, a negative regulator of Treg induction. Thus, this finding prompted us to further dissect its role in regulating Treg induction in an autoimmune setting.

5.5. atRA as upstream regulator of miR-99a-5p

Since the authors of the aforementioned study observe low endogenous expression levels of this miRNA, they tested different Treg-polarizing conditions in presence of TGF-β to enhance its expression and to identify potential upstream regulatory pathways [75]. Importantly, *in vitro* Treg polarizing conditions often include treatment with TGF-β due to a strong increase in Tregs. However, it has been shown that these induced Tregs lose their Foxp3 expression and as a consequence, are less stable compared to their *in vivo* counterparts [158, 159].

Strikingly, this study found that atRA together with TGF-β was able to strongly induce the expression of miR-99a-5p in Tregs *in vitro*, thereby acting as upstream regulator of miR-99a-5p. Since I see a strong upregulation of Tregs upon atRA stimulation in a TGF-β-independent manner, I wanted to further decipher the mechanisms by which atRA mediates miR-99a-5p expression in fostering Tregs during islet autoimmunity.

5.5.1. Dissecting the role of atRA-mediated miR-99a-5p expression in inducing Tregs during islet autoimmunity

Based on the finding of a tendency towards a positive correlation between miR-99a-5p expression in T cells and corresponding induced Tregs in young NOD mice with ongoing

islet autoimmunity, I wanted to further support the hypothesis that atRA acts upstream of miR-99a-5p in inducing Tregs during islet autoimmunity. To do so, I pre-treated naïve T cells with atRA and a subsequent miR-99a-5p inhibition markedly decreased Treg induction capacity in T cells from autoimmune-prone NOD mice. Limited TCR stimulation, i.e. TCR withdrawal after 18 hours, has been shown to elude activation of the PI3K/Akt/mTOR pathway and a thereby favors Treg cell differentiation *in vitro*. Hence, as one possible explanation, pre-treatment with atRA in the first 18 hours of strong stimulation initially enhanced Treg conversion. Due to continuous TCR stimulation in our setting, subsequent blocking of miR-99a-5p-mediated mTOR repression might lead to the observed decease in Tregs due to activation of the PI3K/Akt/mTOR signaling pathway in spite of the presence of atRA. In light of these observations, studies from our group could demonstrate Foxp3 CNS demethylation is significantly increased after 12 hours compared to other time points during *in vitro* Treg induction, thereby opening a window of opportunity to stabilize Tregs in this time frame [58].

In vivo, I could see a trend towards a positive correlation between miR-99a-5p expression in T cells and Treg frequencies in the pancreas of NOD mice treated with atRA, whereas in the control group it appeared to have a negative correlation. This data could point towards an immunoregulatory function of atRA in controlling miR-99a-5p-mediated increase of Tregs in spite of ongoing inflammation in the target tissue. However, the sample size was small and therefore, further studies are required to support this hypothesis.

In order to get first hints of a link between Vitamin A concentration and miR-99a-5p expression in T cells in the human setting of T1D, I correlated Vitamin A concentrations in sera of patients with different durations of islet autoimmunity and the expression of miR-99a-5p in naïve T cells from PBMCs. Whereas individuals at autoantibody-negativity did not show any correlation between these two factors, there was a trend towards a positive correlation in patients with T1D. However, low number of recruited donors hinder a conclusive judgement of these results.

In conclusion, I could provide insights into the complex regulatory network of the atRA-mediated increase in miR-99a-5p expression in T cells for improved Treg induction during islet autoimmunity. Mechanistically, the increased expression of miR-99a-5p in T cells could result from an inhibition of mTOR signaling, thereby enhancing Treg cell differentiation. The overall low expression of miR-99a-5p in T cells of patients with established T1D and the concomitant lower frequencies of Tregs in peripheral blood support the notion that this miRNA might play an important role in the early stages of T1D.

Antigen-specific Treg induction is a long-awaited goal for the treatment of autoimmune diseases. Since efficient Treg induction is impaired by strong activation of T cells during onset of islet autoimmunity, it is important to find novel targets for immunotherapies that boost Treg induction at early stages. Initial findings provide first evidence that miR-99a-5p modulation can have a positive impact on immunoregulatory mechanisms in the early phases of islet autoimmunity and thus, might be an interesting candidate for novel targeting approaches. However, since most miRNAs exert different functions depending on the cell type, for therapeutic interventions it would be required to develop cell type-specific targeting strategies. Therefore, the finding that the effect of miR-99a-5p could be enhanced via atRA would offer an alternative approach by targeting its upstream regulator. Accordingly, in a recent clinical trial, atRA was used to expand *ex vivo* Tregs for the treatment of Crohn's disease (ClinicalTrials.gov Identifier: NCT03185000).

The results obtained from this work reveal novel targetable candidates for the development of intervention strategies aiming at restoring the imbalance of immune tolerance and immunity in islet autoimmunity. Even though our understanding in T1D pathogenesis has greatly improved over the past years, the vast disease heterogeneity has limited the translation to immunotherapies so far.

5.6. Current perspectives for immunotherapies of T1D

Immense research over the past 30 years has led to groundbreaking discoveries for novel immunotherapy approaches. Yet, none of them has made it to the clinics so far, except for the historical moment in 2022, with the FDA approval of the first disease-

modifying therapy with anti-CD3 teplizumab-mzwv (Tzield, Provention Bio). Dating back to 1992, the authors could show that treatment with a modified monoclonal antibody against CD3 that prevented binding to the Fc receptor led to a prevention of disease onset in NOD mice [44]. In their first clinical trial, they could see an improvement of residual insulin response in patients with newly-diagnosed T1D [45]. Strikingly, for the next clinical trial, they could observe a delay in disease onset by 2 years after administration of teplizumab over a 2-week course to at-risk patients with stage 2 disease [43]. Hereof, treatment with teplizumab has been approved as first immunomodulatory drug for patients at risk for developing T1D (ClinicalTrials.gov Identifier: NCT01030861). Importantly, anti-CD3 monoclonal antibodies have been shown to modify $CD8^+$ T cell responses, leading to a partial exhaustion of this cell type and consequently, preserved beta cell mass [160, 161].

Despite the major achievement of the first-ever FDA-approved immunomodulatory drug for T1D, the onset of the clinical disease is inevitable in those individuals at high risk of developing T1D. Prolongation of the asymptomatic stage is desirable since over time patients suffering from T1D might develop late complications including neuropathy, nephropathy or eye and foot damage. Moreover, treatment with general immunosuppressive agents can still cause serious side effects.

Since Tregs themselves are powerful, naturally occurring cells with immunosuppressive functions, they are an attractive candidate for immunomodulatory therapies for autoimmune diseases. In this regard, several studies aim at restoring immune tolerance by targeting molecules that are crucial for Treg induction. For instance, the immunosuppressive agent rapamycin acts via the inhibition of the serine/threonine kinase mTOR, thereby promoting TCR-induced T cell anergy and expansion of Tregs *in vitro* and *in vivo* [162, 163]. Blockade of mTOR leads to the preferential expression of signal transducer 5 (STAT5) which is crucial for Treg development [164]. Combinatorial treatment of rapamycin and IL-2 led to prevention of T1D development in NODs [85]. Based on that, a clinical trial tested the combined effects of rapamycin and IL-2 in inducing Tregs in patients with T1D. Even though they observed an increase in Tregs in the first months, most of the clinical parameters worsened in the course of the

intervention study [165]. These findings highlight the challenges in the translation of findings obtained in the murine setting for the treatment of human T1D and in addition underscore the complexity of human T1D.

Antigen-specific Treg therapy is intensively investigated as a treatment opportunity since it is a targeted approach and thereby, limits the risk of side effects of general immunosuppression. Importantly, studies from our group could demonstrate that individuals harboring multiple autoantibodies without progression to the clinical disease had the highest frequencies of insulin-specific Tregs [39]. This highlights the rationale of using antigen-specific Treg induction as primary preventive approach with the goal to delay disease onset.

Even though first results from clinical studies using antigen-specific Treg therapy are promising [166], the long-term phenotype of these Tregs is not studied in detail yet, including the possibility that Tregs might differentiate into T cell subsets expressing pro-inflammatory cytokines, thereby causing safety concerns.
In order to advance the development of effective treatment strategies, it is important to improve the translatability from animal models to humans. In the setting of T1D, the NOD mouse model offers a unique tool for testing novel therapeutic approaches. However, the differences in disease pathogenesis and progression between man and mouse as well as immune responses within the organisms are by far not comparable which greatly hinders the implementation of new therapies [167, 168]. Thus, there is an urgent need for improvements of preclinical models.

5.7. Modelling of islet autoimmunity in humanized mice

For this purpose, severely immunodeficient mice engrafted with a human immune system provide an excellent system for *in vivo* immunology research, as summarized in section 1.5. This mouse model can be used for different applications, ranging from studying human immune reactions in an *in vivo* model [169-171] up to the evaluation of T cells responses upon treatment with immunotherapies [172-174]. In this regard, our group successfully established such humanized mouse models reconstituted with HSCs to study requirements for *in vivo* Treg induction in a human immune system [39]. First

studies using T1D patient-derived PBMCs for the reconstitution of immunodeficient mice date back to 1993, where the authors could demonstrate a production of islet-antigen-specific autoantibodies upon reconstitution [175]. Immense research over the past years by other groups using several *in vitro* and *in vivo* personalized models has provided invaluable critical insights in T1D pathogenesis [summarized in [176]]. Given the vast heterogeneity in T1D disease progression, the aim of the second part of this thesis was to get a better understanding of the plasticity in immune tolerance and immunity in islet autoimmunity. For this purpose, I wanted to model different stages of islet autoimmunity in humanized mice.

First, to see whether I can mirror the differences in insulin-specific $CD4^+$ T cell frequencies in PBMCs from donors at different stages of islet autoimmunity [39], I analyzed HLA-DQ8-restricted insulin mimetope-specific $CD4^+$ T cells in PBMCs of mice reconstituted with $HLA\text{-}DQ8^+$ donors with different disease durations. In accordance with the data obtained from *ex vivo* human donor stainings [39], there are first indications of higher frequencies of insulin mimetope-specific $CD4^+$ T cells in PBMCs of humanized mice reconstituted with PBMCs of autoantibody negative as well as autoantibody positive children. It is well known that upon exogenous insulin administration as it is the case in patients with clinical T1D, its cognate TCR gets downregulated. Accordingly, there are reduced frequencies of insulin mimetope-specific $CD4^+$ T cells in PBMCs of mice reconstituted with PBMCs from donors with long-term T1D.

Thus, in a pilot study, I could successfully mimic human antigen-specific T cell responses in PBMCs of humanized mice reconstituted with PBMCs from donors at various stages of islet autoimmunity. Similarly, another group successfully generated a humanized mouse model reconstituted with HSCs from a patient with IPEX syndrome that developed fatal autoimmunity [177]. Therefore, the establishment of such mouse models recapitulating human autoimmunity will help us to obtain critical insights in differences of immune responses that can facilitate the development of future treatments.

Additionally, this mouse model enables the analysis of T cell phenotypes in the disease-relevant draining lymph nodes and target tissue. Accordingly, cytokine profiling revealed a more predominant pro-inflammatory phenotype in the pancreas of mice reconstituted

with PBMCs from donors with T1D of various disease durations. This finding is supported by a recent study that had the goal to classify T1D into different endotypes. Here, they found a predominant pro-inflammatory signature in young individuals with T1D [38]. In this regard, it has also been demonstrated that the production of the pro-inflammatory cytokine IFNγ produced by autoreactive T cells appears in patients just prior the onset or at onset of the clinical disease [178].

In contrast, mice reconstituted with PBMCs from donors at an autoantibody-negative status but harboring at-risk haplotypes had higher frequencies of IL-10-producing T cells in the pooled LNs and of pancreata. Conversely, the production of this anti-inflammatory cytokine was severely reduced in both LNs and pancreas of humanized mice engrafted with PBMCs from donors at various durations of T1D. Importantly, IL-10 has strong anti-inflammatory functions and its loss has been linked to T1D disease acceleration in mouse models [179]. Noteworthy, the same study as described before could observe an IFNγ response to peptides in patients with T1D which was accompanied by an increased secretion of IL-10. They hypothesize that enhanced IL-10 production during the immune assault is a result of ongoing efforts to promote immune tolerance [178]. Since cytokines are important immune mediators, they are inevitable to provide crucial information about immune responses. Therefore, cytokine profiling in humanized mice which mimic the different stages of islet autoimmunity enables valuable insights in immune reactions that can be used for manipulation to develop novel intervention strategies.

In this regard, human cytokine profiling in humanized mice upon reconstitution has been applied also by other groups. In one study, humanized mice were reconstituted with human $CD34^+$ fetal liver hematopoietic stem/progenitor cells (HSPCs) to test the immunotoxicity of drugs for the treatment of cancer. Here, the authors could recapitulate the clinical scenario of a “cytokine storm”, i.e. the release of high levels of cytokines, in these mice, which will help in evaluating the safety of immunotoxic biologics [180]. Another study assessed human cytokine profiles in a humanized mouse model for colon cancer after immune checkpoint blockade therapy. Owing to the functioning T cells in this *in vivo* model, they could evaluate cytokine signatures upon treatment with PD-1 immunotherapy to better understand underlying mechanisms leading to the potential

development of hyperprogressive disease during immune checkpoint inhibitor therapy [181]. These studies further support the wide range of possible applications for these humanized mouse models and their important impact on studying immune reactions in response to treatments.

In conclusion, first results obtained from my studies revealed important insights in human-specific immune responses during islet autoimmunity upon reconstitution with PBMCs in humanized mice in spite of the small sample size used in this study. This mouse model facilitates our understanding of underlying mechanisms of human pathophysiology and further allows us to phenotypically characterize T cells in the different stages of islet autoimmunity. Moreover, it helps us to understand which pathways are involved in the immune reactions and this knowledge can be further used to target specific signaling molecules for therapeutic intentions. However, the potential of developing GvHD limits the use of this mouse model to short-term analyses [182].

Therefore, as an alternative approach, in a first step we reprogrammed patient-specific PBMCs into iPSCs. iPSCs are widely applied in disease-modeling settings with the aim to study human diseases or for drug safety tests [183]. In this regard, one group established an *in vitro* platform to study the dialogue between T1D patient-specific iPSC-derived beta cells and autologous PBMC-derived immune cells [184]. For *in vivo* application, another study used patient-specific iPSCs for a humanized mouse model of chronic myelomonocytic leukemia (CMML) in order to study requirements for drug candidates. Owing to their personalized model, they were able to identify drug candidates for the treatment of CML [185]. These studies highlight the invaluable substantial fields of application of the iPSC technology and its enormous potential to aid in the development of personalized medicine.

With the goal to enable long-term analysis of human immune responses *in vivo*, we further differentiated the fully characterized iPSCs into HSCs, which were used for the reconstitution of humanized mice.

Even though the *de novo* generation of functional HSCs after differentiation of iPSCs remains still challenging, recent advances in generating HSCs with multi-lineage potential for the reconstitution of humanized mice have shown promising results. For

instance, one group established a protocol of a morphogen-directed differentiation of iPSCs into hemogenic endothelium (HE). Subsequent introduction of seven HSC-specific transcription factors enabled the conversion into HSPCs with robust engraftability in immunodeficient mice [96]. However, the intermediate stage of HE added another layer of complexity and hence, impeded the efficacy of this differentiation method. Thereafter, another group published their results of using only a single transcription factor MLL-AF4 for the re-specification of iPSCs into HSPCs. Their MLL-AF4-induced iHSPCs, however, were shown to be more prone to leukemic transformation, thereby limiting the translatability of their results to the clinical setting [186]. Nonetheless, numerous studies are being conducted with the goal to improve the generation of HSCs from iPSCs and to enable their application for disease modeling and drug testing platforms, among others [reviewed in [187]].

Overall, by optimizing conditions for efficient reprogramming of patient-derived PBMCs and their further differentiation into HSCs we were able to generate first humanized mice to study immune responses during islet autoimmunity *in vivo*. In future, this mouse model offers many opportunities to study interactions between the immune system and the disease-specific target tissue and will thereby help to enable studies addressing mechanisms of aberrant immune activation vs. impairments in Treg cells in human islet autoimmunity *in vivo*. Moreover, these mice can be used as an efficient tool for the development of novel immunotherapeutics and to tailor a patient's specific outcome to a treatment with the aim to advance the field of personalized medicine.

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Affidavit

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I hereby declare, that the submitted thesis entitled

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