**Title: A miRNA181a/NFAT5 axis links impaired T cell tolerance induction with autoimmune Type 1 diabetes**

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**Overline:** Autoimmunity

**One Sentence Summary: A miRNA181a/NFAT5 signaling axis promotes immune activation and interferes with the induction of FOXP3+ regulatory T cells in islet autoimmunity.**

**Abstract**: Molecular checkpoints that trigger the onset of islet autoimmunity or progression to human type 1 diabetes (T1D) are incompletely understood. Here, using T cells from children at an early stage of islet autoimmunity without clinical T1D we find that a microRNA (miRNA)181a-mediated increase in signal strength of stimulation and costimulation links Nuclear factor of activated T cells 5 (NFAT5) with impaired tolerance induction and autoimmune activation. We show that enhancing miRNA181a activity increases NFAT5 expression while inhibiting FOXP3+ regulatory T cell (Treg) induction *in vitro*. In contrast, blocking the binding of miRNA181a to *Nfat5* can enhance Treg induction. Accordingly, Treg induction is improved using T cells from NFAT5 knockout (ko) animals whereas altering miRNA181a activity does not affect Treg induction in NFAT5ko T cells. Moreover, high costimulatory signals result in Phosphoinositide-3-kinase (PI3K)-mediated NFAT5 which interferes with FoxP3+Treg induction. Blocking miRNA181a or NFAT5 increases Treg induction in murine and humanized models and reduces murine islet autoimmunity *in vivo*. These findings suggest targeting miRNA181a and/or NFAT5 signaling for the development of innovative personalized medicines to limit islet autoimmunity.

**[Main Text: ]**

**Introduction**

Clinical Type 1 diabetes (T1D) is presumed to develop from autoimmune destruction of the pancreatic insulin-producing β cells ([*1*](#_ENREF_1)) resulting in hyperglycemia. The incidence of T1D is rising dramatically, especially in young children with the appearance of multiple islet autoantibodies marking the onset of islet autoimmunity ([*2*](#_ENREF_2)). Thereafter, the time from this asymptomatic phase of islet autoimmunity to progression to metabolic T1D is highly plastic, ranging from several months to more than two decades ([*3*](#_ENREF_3)). A rapid progression to clinical T1D is indicative of multiple layers of tolerance defects and aberrant immune activation. However, despite recent insights from identifying a divergent autoantigen-responsive CD4+T cell population in infants prior to developing islet autoimmunity ([*4*](#_ENREF_4)) molecular underpinnings involved in triggering the onset of islet autoimmunity remain incompletely understood.

Peripheral T cell tolerance is mainly executed by regulatory T cells (Tregs). The X chromosome-encoded forkhead domain containing transcription factor FOXP3 is a lineage-specifying factor responsible for the differentiation and function of CD25+CD4+Tregs ([*5*](#_ENREF_5)*,* [*6*](#_ENREF_6)). Binding of a strong-agonistic antigen to the T cell receptor (TCR) on naïve CD4+T cells under subimmunogenic conditions results in efficient FOXP3+Treg induction ([*7-10*](#_ENREF_7)). High doses of TCR ligands and strong costimulatory signals activate the PI3 kinase (PI3K)/Akt/mTOR pathway thereby interfering with Treg induction ([*11*](#_ENREF_11)). Therefore, control of PI3K signaling by phosphatase and tensin homolog (PTEN) is essential for Treg function and lineage stability.

*Ex* *vivo* frequencies of human leucocyte-antigen (HLA)-DQ8-restricted insulin-specific Tregs were critically reduced during islet autoimmunity onset or in children with a fast progression to clinical T1D ([*12*](#_ENREF_12)) accompanied by an increase in insulin-specific T follicular helper (TFH) precursor cells ([*13*](#_ENREF_13)). In contrast, high frequencies of insulin-specific Tregs were associated with profound delays in progressing to symptomatic T1D ([*12*](#_ENREF_12)). However, a mechanistic understanding of relevant promoters of T cell activation involved in triggering islet autoimmunity is still lacking.

Based on their ability to regulate cellular states including T cell activation, we focused on microRNAs (miRNAs) ([*14*](#_ENREF_14)). miRNA-mediated gene regulation comprises a variety of mechanisms including the canonical function of target gene inhibition, relief of miRNA-mediated repression or miRNAs that in dependence of cellular state and function can contribute to a potential activation of targeting sites ([*15-17*](#_ENREF_15)).

The Nuclear factor of activated T cells 5 (NFAT5) represents a functionally and structurally unique member of this transcription factor family ([*18*](#_ENREF_18)*,* [*19*](#_ENREF_19)). Besides its role in regulating transcription in response to hyperosmolar stimuli, NFAT5 exerts important functions following other stimuli including TCR-dependent mechanisms ([*20-22*](#_ENREF_20)), whereas PI3K can contribute to NFAT5 activation ([*23*](#_ENREF_23)).

Here, we provide evidence for a profound impairment of Treg induction during islet autoimmunity onset. We demonstrate that a miRNA181a-mediated enhanced signal strength of TCR stimulation and costimulation links increased NFAT5 expression with impaired Treg induction. A miRNA181a antagomir or a pharmacological NFAT5 inhibitor improves Treg induction and reduces murine islet autoimmunity *in vivo*.

**Results**

***Impaired Treg induction during human islet autoimmunity onset***

We studied Treg induction in vitro using naïve CD4+T cells from individual children with different durations of islet autoimmunity without clinical T1D (overview in **Fig.S1**). Given the critical role of insulin epitopes as target autoantigens, we focused on insulin-specific Treg induction using highly-pure naïve CD4+T cells and premature withdrawal of TCR stimulation after 18 hours without TGF([*11*](#_ENREF_11)*,* [*12*](#_ENREF_12)). We therefore employed previously established protocols using HLA-DQ8 insulin-specific monomers coated onto streptavidin pre-coated plates ([*12*](#_ENREF_12)).

During islet autoimmunity onset insulin-specific Treg induction was significantly (*p*<0.001) impaired compared to children without islet autoimmunity (**Fig.1A**). Insulin-specific Tregs were first identified as CD4+CD3+CD127loCD25hiT cells (example in **Fig.S2A upper row:** e.g. 0.302% of all CD4+CD3+T cells were identified as CD127loCD25hi upon stimulation with HLA-DQ8 insulin-specific monomers). Within this CD127loCD25hi population percentages of CD25hiFOXP3hiTregs were assessed (**Fig.S2A upper row**: e.g. 55.6% CD25hiFOXP3hi of 0.302% of CD127lowCD25hi cells). No FOXP3hiTregs were identified upon stimulation with HLA-DQ8 control monomers (example in **Fig.S2A lower row**). Functionality of induced Tregs was demonstrated previously ([*12*](#_ENREF_12)) and was confirmed in Treg suppression assays (**Fig.S2B**). Consistent with the reduced insulin-specific Treg induction in children with onset of autoimmunity, frequencies of FOXP3intCD4+T cells were significantly (*p*<0.05) increased thereby pointing to activated T cells (no autoimmunity vs. recent onset of autoimmunity: 16.9±8.4 vs. 67.2±11.3 CD25hiFOXP3int cells as a % of CD127loCD25hiCD4+T cells, *p*<0.05, **Fig.1B, Fig.S2A**).

Upon stimulation with HLA-DQ8 insulin-specific tetramer-based artificial antigen presenting cells (aAPCs, **Fig.S3**) naïve CD4+T cells from children with islet autoimmunity onset proliferated more vigorously (0.11±0.01 CFSEdimCD25+ cells as a % of CD4+T cells) compared to autoantibody negative children (0.01±0.003, *p*<0.001) and non-diabetic children with longterm autoimmunity (0.04±0.01, *p*<0.05, **Fig.1C**). These findings suggest a higher sensitivity to antigenic stimulation during islet autoimmunity onset and are in line with studies showing best Treg induction in T cells that proliferated the least *(*[*7*](#_ENREF_7)*,* [*8*](#_ENREF_8)*,* [*10*](#_ENREF_10)*,* [*24*](#_ENREF_24)*)*. The reduced Treg induction potential during islet autoimmunity onset was confirmed in non-autoantigen-specific assays with a hemagglutinin A peptide (HA307–319 epitope) and in polyclonal assays (**Fig.1D+E**).

***Reduced Treg induction by miRNA181a-mediated increase in signal strength of stimulation***

To mechanistically dissect the impaired Treg induction, we determined miRNA expression profiles by next generation sequencing (NGS) using CD4+T cells from children with or without islet autoimmunity (**Fig.2A; Fig.S4A**). Given the reduced Treg induction accompanied by increased T cell proliferation, we focused on miRNAs regulating signal strength of antigenic stimulation. Specifically, we identified enhanced miRNA181a abundance in ex vivo stimulated CD4+T cells from children with ongoing islet autoimmunity (**Fig.2A+B**). Importantly, murine studies had highlighted that miRNA181a regulates TCR signaling strength during T cell development in the thymus ([*25*](#_ENREF_25)).

Validation experiments in CD4+T cells from individual children with onset of autoimmunity showed increased miRNA181a abundance (**Fig.2C)**. By contrast, miRNA181a expression in CD4+T cells from non-diabetic children with longterm autoimmunity was as low as that in CD4+T cells from children without ongoing islet autoimmunity (**Fig.2C**).

We used NGS to determine miRNA181a-mediated regulation on mRNA expression in CD4+T cells focusing on target genes regulating signal strength of antigenic stimulation. Consistent with enhanced miRNA181a expression, CD4+T cells from autoantibody positive children had diminished expression of target genes that negatively impact T cell activation, such as *Pten*, Transducer Of ERBB2, 1 (*Tob1)* and cytotoxic T-lymphocyte-associated Protein 4 (*Ctla4)* (**Fig.2D**).

Additionally, predicted miRNA181a target genes which promote immune activation (**Fig.2D**), were upregulated accompanied by enhanced CD28 expression suggesting increased costimulatory signaling and enhanced signal strength during autoimmunity. Accordingly, expression of NFAT transcription factor family members was enhanced (**Fig.2D+E, Fig.S4F**); with *Nfat5* being most prominently increased (**Fig.2D**).

Validation experiments using CD4+T cells from individual children confirmed increased *Nfat5* expression during islet autoimmunity onset (**Fig.2E**) accompanied by reduced abundance of negative regulators of T cell activation, as *Pten, Tob1* and *Ctla4* (**Fig.S4C-E**). In accordance, a miRNA181a-mediated suppression of *Pten* promotes PI3K signaling which in turn can contribute to NFAT5 activation ([*23*](#_ENREF_23)).

***Promotion of NFAT5 expression by enhancing miRNA181a activity***

Next, we studiedwhether modulating miRNA181a activity impacts Treg induction during autoimmunity. MiRNA181a mimics (**Fig.S5**) were delivered to CD4+T cells using established chitosan-coated poly(lactic-co-glycolic acid) (PLGA) nanoparticles ([*26-28*](#_ENREF_26)). A miRNA181a mimic significantly lowered Treg induction (*p*<0.01 human; *p*<0.05 murine) while enhancing *Nfat5* abundance (*p*<0.05) and cellular proliferation (*p*<0.001) (**Fig.3A-G**). In contrast, blocking miRNA181a significantly reduced *Nfat5* abundance (*p*<0.05) (**Fig.3H**). Furthermore, stimulated emission depletion (STED) microscopy showed that a miRNA181a mimic distinctly enhanced nuclear NFAT5 expression (**Fig.3I**). Higher costimulation interfered with FoxP3+Treg induction, increased *Nfat5* and reduced Forkhead box protein O1 (*Foxo1*) expression (**Fig.S6A+B**).

Blocking miRNA181a significantly enhanced human Treg induction (without islet autoimmunity *p*<0.001; longterm autoimmunity *p*<0.01; with T1D *p*<0.01) (**Fig.4A**). The *FOXP3* Treg-specific demethylated (TSDR) region shows the most distinct differences concerning the methylation status of the *FOXP3* locus: it is completely demethylated in Tregs and fully methylated in conventional T cells and in Tregs induced by continuous TCR stimulation *in vitro (*[*29*](#_ENREF_29)*)*. We recently had demonstrated that subimmunogenic stimulation of human naïve CD4+T cells *in vitro* resulted in higher Treg induction efficacy and improved Treg stability as seen from restimulation cultures using sort-purified induced Tregs ([*12*](#_ENREF_12)). Here, using Treg induction with naïve CD4+T cells and early withdrawal of TCR stimulation, after 14 hours some T cells had already upregulated high FOXP3 expression and at that early time point presented with a reduced *FOXP3* TSDR methylation (**Fig.S7**). A miRNA181a antagomir significantly lowered the *FOXP3* TSDR methylation in such early FOXP3hiCD4+T cells (*p*<0.001) (**Fig.S7**).

***Mechanisms of impaired Treg induction in murine islet autoimmunity***

To mechanistically dissect Treg induction during islet autoimmunity we next studied non-obese diabetic (NOD) mice which share many similarities with human T1D ([*30-32*](#_ENREF_30)). Non-diabetic NOD mice with recent onset of insulin autoantibodies (IAAs) likewise showed significantly impaired Treg induction (*p*<0.001) (**Fig.4B**) accompanied by significantly enhanced miRNA181a (*p*<0.01) and *Nfat5* (*p*<0.05) abundance ex vivo (**Fig.4C+D)**.Moreover,the percentage of cells that are NFAT5 positive (and the MFI of the entire population) was increased in CD4+T cells from IAA+NOD mice (**Fig.S8A+B**).

In ex vivo Tregs of IAA+NOD mice miRNA181a expression was reduced whereas *Nfat5* and *Pten* expression was unaltered when compared to IAA-NOD mice (**Fig.S8C-E**). Moreover, CD4+T cells from IAA+NOD mice had higher miRNA181a expression upon TCR stimulation and ex vivo PTEN protein expression was reduced (**Fig.S8F-H**) thereby supporting PI3K signaling and NFAT5 activation.

Next, to dissect a potential involvement of hypertonicity-related signaling in mediating *Nfat5* enhancement, we analyzed A-Kinase Anchoring Protein 13 (*Akap13)* and Serum/Glucocorticoid Regulated Kinase 1 (*Sgk1*) expression in CD4+T cells from IAA+NOD mice. In contrast to *Nfat5*, *Akap13* and *Sgk1* mRNA were unaltered during IAA+autoimmunity (**Fig.S9A+B**). Likewise, AKAP13hiT cells were unchanged (**Fig.S9C+D**). Furthermore, Treg induction was unaltered with T cells from AKAP13 haploinsufficient (+/-) animals ([*33*](#_ENREF_33)) compared to wildtype (WT) T cells or with T cells from IAA+NOD mice with an *Akap13* small interfering RNA (siRNA) compared to control siRNA (**Fig.S9E-H**). Again, blocking miRNA181a distinctly enhanced Treg induction using naïve CD4+T cells from IAA+NOD mice (*p*<0.001) (**Fig.4E**).

***Treg induction potential in CD4+T cells from NFAT5ko mice and PTEN Tg mice***

Using loss of function experiments naïve CD4+T cells from NFAT5ko mice ([*34*](#_ENREF_34)) showed a significantly increased Treg induction potential (CD4+CD25+FoxP3+Tregs [% of CD4+T cells]: 17.1±2.8 vs. 30.3±1.2, *p*<0.01, **Fig.5A+B**). Accordingly, *Pten* and *Foxo1* mRNA abundance were significantly upregulated in activated CD4+T cells from NFAT5ko mice (*p*<0.05) (**Fig.S10A+B**). An improved Treg induction was also seen when modifying TCR signal strength or costimulation (**Fig.S10C-E**).

Blocking miRNA181a increased induced Tregs only with naïve CD4+T cells from WT animals (WT: + miRNA181a antagomir: 19.8±6.9% increase in induced CD4+CD25+FoxP3+Tregs compared to control mice, *p*<0.05, **Fig.5C**) and not with naïve CD4+T cells from NFAT5ko animals (**Fig.5C**). Accordingly, Treg induction was unaltered with naïve CD4+T cells from NFAT5ko mice and a miRNA181a mimic (**Fig.5C**). Modulating miRNA181a activity likewise did not impact Treg induction with naïve CD4+T cells from NFAT5ko mice and low dose anti-CD3 stimulation (**Fig.S11A-D**). PI3K-inhibition improved while a PTEN-inhibitor decreased Treg induction in T cells from both WT and NFAT5ko (**Fig.5D+E**).

Next, to study the association between costimulation, PTEN and NFAT5 signaling with Treg induction we used naïve CD4+T cells from PTEN transgenic (Tg) animals ([*35*](#_ENREF_35)) which harbor constitutively increased PTEN expression and reduced PI3K activation. High PTEN expression significantly enhanced Treg induction compared to WT T cells (*p*<0.01) (**Fig.5F+G**). The increased Treg induction from PTEN Tg animals was accompanied by reduced *Nfat5* expression, enhanced *Foxo1* expression in CD4+T cells ex vivo (**Fig.S12A+B**) and lower *Nfat5* expression after TCR stimulation (**Fig.5H**). A miRNA181a mimic reduced whereas a miRNA181a antagomir enhanced Treg induction and inhibited NFAT5 expression using naïve CD4+T cells from PTEN Tg animals also with low dose TCR stimulation (**Fig.S12C+D, Fig.S13A+B**).

***Enhancement of Treg induction by a specific NFAT5 inhibitor***

Therapeutic agents that can specifically inhibit NFAT5 have been lacking till now. A recently identified compound was shown to block the pro-inflammatory NFAT5 activity by inhibiting *Nfat5* transcriptional activation without affecting NFAT1-4, nuclear factor (NF)-κB, p38 mitogen-activated protein (MAP) kinase and cAMP response element-binding protein (CREB) activity, excluding potential off-target effects ([*36*](#_ENREF_36)). NFAT5 inhibition improved murine and human Treg induction (**Fig.6A+C; B+D**) in vitro in WT and PTEN Tg mice as well as in a humanized mouse model in vivo (**Fig.6E+F; Fig.S14A-F**). Humanized mice are immunodeficient mice that after reconstitution with human hematopoietic cells or tissues do develop a human immune system with a highly diverse TCR repertoire. These mice permit the assessment of human T cell responses in vivo. Here, we made use of the murine MHCII deficient, HLA-DQ8 transgenic NOD.Cg-*Prkdcscid Il2rg*tm1Wjl (NSG) mouse model ([*12*](#_ENREF_12)).

***Amelioration of murine islet autoimmunity by a miRNA181a antagomir or NFAT5 inhibitor in vivo***

Next, we tested the impact of blocking miRNA181a to modulate islet autoimmunity in IAA+NOD mice in the absence symptomatic T1D in vivo. The miRNA181a antagomir [10 mg/kg *i.p*.] every other day for 14 days enhanced FoxP3+Tregs in the peripheral blood (**Fig.7A-C**). The fact that the Treg enhancement was significant in peripheral blood (*p*<0.01) but not in lymph nodes suggests that a further miRNA181a antagomir dose titration might be needed and/or a daily application.

Blocking miRNA181a significantly enhanced ex vivo PTEN expression in CD4+T cells from lymph nodes of IAA+NOD mice (*p*<0.05) (**Fig.S15A+B**), while significantly reducing CD28 expression (*p*<0.001) (**Fig.7D+E**) and lowering IAA levels (*p*<0.01) (**Fig. S15C)**. The amelioration of autoimmune activation was verified by histopathological and immunofluorescence analyses of pancreatic sections, which showed a reduction in T cell infiltration (**Fig.7F-G, Fig.S15D**).

Applying a NFAT5 inhibitor to IAA+NOD mice at 3 mg/kg *i.p.* every day for 14 days significantly enhanced FoxP3+Tregs in lymph nodes (*p*<0.001) (**Fig.7H+I**) s and reduced pancreatic immune infiltration (**Fig.7J+K; Fig.S15F)**. Additionally, NFAT5 inhibition distinctly reduced NFAT5 expression in pancreas-infiltrating T cells from IAA+NOD mice (**Fig.8A+B; Fig.S15G).** Blocking NFAT5 did not cause any changes in metabolic parameters (e.g. body mass and blood glucose, **Fig.8C+D**).

**Discussion**

A better understanding of the molecular underpinnings impacting immune tolerance defects is pivotal to develop personalized medicines that limit autoimmune progression. Highly variable progression from islet autoimmunity to clinical T1D ([*3*](#_ENREF_3)) underscores plasticity in regulating immune activation vs. tolerance. Therefore, T cells from non-diabetic children with ongoing islet autoimmunity offer a valuable resource for studying signaling pathways involved in this plasticity.

An important weakness of this study is that no longitudinal samples from individual children have been available to assess and integrate Treg induction potential from naïve CD4+T cells in accordance with the duration of islet autoimmunity over time. Therefore and given the differences in the median age within respective disease groups, within the present human data set a confounding role of age per se in influencing Treg induction potential from naïve CD4+T cells cannot be excluded.

Here, we provide evidence for a broad Treg induction impairment during islet autoimmunity onset. Consistent with the reduced Treg induction potential, proliferation and frequencies of FOXP3intCD4+T cells were enhanced, indicative of T cell activation, thereby limiting possibilities of subimmunogenic stimulation necessary for efficient FOXP3+Treg induction ([*7*](#_ENREF_7)*,* [*11*](#_ENREF_11)*,* [*24*](#_ENREF_24)).

By contrast, with naïve CD4+T cells from non-diabetic children with longterm autoimmunity, the Treg induction potential was restored to frequency observed in children without autoimmunity thereby suggesting that children with longterm autoimmunity might have regulated autoimmune activation or might at least be in a transient state of immune tolerance. Accordingly, children with longterm autoimmunity phenotypes show an accumulation of protective genotypes in T1D susceptibility genes ([*37*](#_ENREF_37)) most notably *IL-2, IL2-Rα, INS VNTR* and *IL-10*. In line, such children with longterm autoimmunity harbored TFH precursor cell frequencies as low as autoantibody-negative children ([*13*](#_ENREF_13)) accompanied by an increase in insulin-specific Tregs ([*12*](#_ENREF_12)).

As one possible means to explain this Treg induction impairment, we identified enhanced miRNA181a abundance in CD4+T cells during islet autoimmunity onset. Murine miRNA181a regulates signal strength of stimulation by modulating the sensitivity to antigenic stimulation and signaling thresholds in CD4+T cells during thymic T cell development ([*25*](#_ENREF_25)). These properties of miRNA181a suggest that an increased sensitivity to antigenic stimulation boosts T cell activation thereby promoting islet autoimmunity and antagonizing Treg induction. Accordingly, a miRNA181a mimic distinctly impeded while a miRNA181a antagomir enhanced human and murine Treg induction. The miRNA181a-mediated enhancement of signal strength of stimulation also involves suppression of negative regulators of T cell activation such as the phosphatase PTEN. PTEN functions as a key signaling intermediate controlling the activation of PI3K. Consequently, miRNA181a-mediated PTEN inhibition promotes PI3K signaling which in turn can contribute to NFAT5 activation ([*23*](#_ENREF_23)). Blockade of PTEN signaling by miRNA181a might therefore function as one possible mechanism of indirect regulation to boost NFAT5 expression. Accordingly, in CD4+T cells from children with ongoing islet autoimmunity we find enhanced miRNA181a abundance together with a decrease in *Pten* and an increase in *Nfat5* expression. Moreover, upon miRNA181a blockade in IAA+NOD mice we identify increased PTEN expression in CD4+T cells accompanied by reduced NFAT5 expression .

Furthermore, the observed NFAT5 induction by miRNA181a might also result from additional indirect control of repression pathways and/or potentially involve relief of miRNA181a-mediated repression of NFAT5 ([*17*](#_ENREF_17)*,* [*38*](#_ENREF_38)). Additionally, the increase in signal strength of stimulation induced by miRNA181a includes an enhancement of costimulatory signals such as CD28 ([*25*](#_ENREF_25)). Specifically, increased miRNA181a levels promoted CD28 expression while reducing CTLA4 surface expression . CD28 signaling triggers PI3K signaling ([*11*](#_ENREF_11)) which can contribute to NFAT5 activation ([*23*](#_ENREF_23)) thereby offering a potential additional explanation how miRNA181a might indirectly contribute to NFAT5 upregulation. Accordingly, we observe enhanced CD28 mRNA abundance in CD4+T cells from children with ongoing islet autoimmunity. Moreover, miRNA181a blockade *in vivo* significantly reduced CD28 expression in CD4+T cells while reducing murine islet autoimmunity.

In the absence of NFAT5, Treg induction was enhanced, T cells harbored increased *Pten* and *Foxo1* expression, both critically involved in favoring Treg induction. Specifically, Foxo1 can directly regulate Treg differentiation ([*39*](#_ENREF_39)). In contrast, high PI3K/Akt signaling activity inactivates Foxo1 proteins by excluding them from the nucleus ([*39*](#_ENREF_39)).

These results suggest that a low activity of a miRNA181a-NFAT5 signaling axis can link improved FOXP3 inducibility with limited PI3K/Akt/mTOR activation. Accordingly, PTEN-deficient T cells presented with a constitutive activation of the PI3K/Akt/mTOR signaling pathway accompanied by impaired FoxP3+Treg induction which was restored by PI3K inhibition.

In sum, here we show that a miRNA181a/NFAT5 axis critically contributes to an impairment of Treg induction during islet autoimmunity. These findings suggest that targeting miRNA181a and/or NFAT5 signaling could contribute to the development of translational strategies aimed at inhibiting the signaling intermediates of T cell activation to limit T1D islet autoimmunity while improving Treg induction.

**Materials and Methods**

**Study Design**

This study was designed to investigate Treg induction in T cells from children at an early state of islet autoimmunity without symptomatic T1D. Therefore, peripheral blood from children who are first degree relatives of T1D patients that consented to the Munich Bioresource project was analyzed. Treg induction during different phases of islet autoimmunity was assessed. T cell-specific miRNAs and miRNA-regulated pathways involved in Treg induction were studied using NGS and qPCR analyses. Treg induction and autoimmune modulation upon miRNA181a or NFAT5 blockade was analyzed in NOD mice and in humanized NSG mice. Specifically, we focused on miRNA181a, due to its implication in mediating signal strength of antigenic stimulation and on the role of *Nfat5* in interfering with Treg induction. We performed loss and gain of function studies using NFAT5ko animals and PTEN overexpressing mice to assess the role of a miRNA181a NFAT5 axis in Treg induction. NOD mice were monitored for weight changes, blood glucose levels and clinical signs throughout the experiment. The investigators were not blinded and no animals were excluded due to illness. Primary data are located in table S1.

**Human subjects and blood samples.**

Blood samples were collected from children or adults who are first degree relatives of T1D patients and who consented to the *Munich Bioresource project (approval number #5049/11, Technische Universität München, Munich, Germany)*. All subjects have been already enrolled into longitudinal studies with prospective follow-up from birth ([*40-42*](#_ENREF_40)) with the documented age of islet autoantibody seroconversion (initiation of islet autoimmunity). Venous blood was collected using sodium heparin tubes and blood volumes collected were based on EU guidelines with a maximal blood volume of 2.4 ml per kg of body weight. Subjects have been stratified based on the presence or absence of multiple islet autoantibodies (= with or without pre-T1D) and based on the duration of islet autoantibody positivity. Details on included subjects can be found in the supplementary material. Peripheral blood mononuclear cells (PBMC) were isolated by density centrifugation over Ficoll-Paque PLUS (GE Healthcare). Human Dendritic cells (DCs) were purified to >90% purity from autologous PBMC samples using the Blood DC Isolation Kit II human (Miltenyi Biotec) according to the manufacturer’s instructions. Human CD4+T cells were isolated at a purity of >90% from fresh PBMCs via positive MACS enrichment, using CD4 microbeads (Miltenyi).

**Mice.**

Female NOD/ShiLtJ mice were obtained from Jackson and stratified according to their IAA status (IAA+NOD mice: median age 118 days, IQR 74-134 days; IAA-NOD mice: median age 99 days, IQR 51-120 days). Analyses of murine IAAs was done from serum as described previously using a mouse high specificity/sensitivity competitive IAA assay in an ELISA format ([*43*](#_ENREF_43)) or a Protein A/G radiobinding assay ([*42*](#_ENREF_42)). For more details see supplementary information. NFAT5ko mice were provided by Christoph Küper. PTEN Tg mice were provided by Manuel Serrano. AKAP13 (Brx) haploinsufficient mice were received from James Segars. NOD.Cg-*Prkdcscid H2-Ab1tm1Gru* *Il2rg*tm1Wjl Tg(HLA-DQA1,HLA-DQB1)1Dv//Sz (NSG HLA-DQ8) mice were developed by Leonard Shultz at Jackson. Ethical approval for all mouse experimentations has been received by the District Government of Upper Bavaria, Munich, Germany. No animals were excluded due to illness or outlier results; therefore, no exclusion determination was required. Further information on knockout and transgenic mouse lines can be found in the supplementary material.

CD4+T cells were isolated at >90% purity by positive MACS enrichment using CD4 Biotin (BD Biosciences) and Streptavidin microbeads (Miltenyi).

**Treg induction using subimmunogenic TCR stimulation *in vitro*.**

***Polyclonal TCR stimulation.***

Naïve CD4+T cells were defined as CD3+CD4+CD45RA+CD45RO-CD127+CD25-, (human) or as CD4+CD25-CD44- (murine) and sorted with the BD FACS AriaIII at a purity of >95% 10,000 (murine assays) or 100,000 (human assays) naïve CD4+T cells per well were cultured for 18 hours in 96-well plates pre-coated with 5 µg/ml (unless indicated otherwise) anti-CD3 and anti-CD28 antibodies. Subimmunogenic TCR stimulation was achieved by pipetting cells into uncoated wells, after 18 hours, where they were cultured for additional 36 hours.

***Insulin-specific TCR stimulation.***

Human naïve CD4+T cells were sorted as described above and cultured at 500,000 per well in 96-well plates, pre-coated with streptavidin and 5 µg/ml HLA-DQ8 monomers in complex with either insulin-mimetopes or control peptides (for information on peptides see supplementary information). 50 ng/ml anti-CD28 was added to the medium. Subimmunogenic TCR stimulation was achieved by changing the medium and pipetting the cells into uncoated wells after 12 hours. Analysis was performed after an additional 36 hours. Insulin-specificity of T cell responses has been verified previously using HLA-DQ8 restricted insulin-specific tetramers and control tetramers fused to irrelevant peptides ([*12*](#_ENREF_12)), sort-purification of tetramer+T cells and insulin-specific restimulation ([*12*](#_ENREF_12)).

***Dendritic cell (DC)-dependent Treg induction.***

500,000 human naïve CD4+T cells per well were co-cultured with autologous CFSE-labeled DCs at a ratio of 100:1 in the presence of a human Hemaggluttinin A epitope. After 12 hours antigen-presenting cells were removed and CD4+T cells were sorted as CFSE- followed by a culture for additional 36 hours in new wells without further peptide stimulation. Information on peptides can be found in the supplementary materials.

**CFSE-T cell proliferation assays.**

CD4+CD25-T cells were labeled with CFSE (0.25 µM) and propagated with artificial antigen presenting cells (aAPCs) for 5 days. Information on aAPCs is in the supplementary material. After 5 days, cells were analyzed by FACS. Responsiveness was measured by the presence and quantity of CD4+CD25+CFSEdimT cells.

**Isolation and processing of miRNAs** **and mRNAs**.

For the isolation of miRNAs and mRNAs the miRNeasy Micro Kit (Qiagen) was used according to the manufacturer’s instructions. RNA concentration and purity were determined by nanodrop (Epoch, Biotech) or RNA Nano Chip and Agilent 2100 Bioanalyzer (Agilent Technologies). For cDNA synthesis the Universal cDNA Synthesis Kit II (Exiqon, for miRNAs) or the iScript cDNA Synthesis Kit (Biorad, for mRNAs) were used according to the instructions. qPCR was performed using the ExiLENT SYBR Green PCR Master Mix (Exiqon, for miRNAs) or the SsoFast Evagreen Supermix (Biorad, for mRNAs) and a CFX96 real time system (Biorad). Information on primers can be found in the supplementary material.

**miRNA/mRNA expression profiling.**

We employed NGS for expression profiling in a test set of each four pooled samples of naïve vs. activated CD4+T cells purified from children with or without ongoing islet autoimmunity. cDNA libraries for miRNA and mRNA sequencing were transcribed from total RNA using the NEBnext Multiplex Small RNA Library Prep Set and NEBnext mRNA Library Prep Master Mix (New England Biolabs), mRNA library preparation was conducted with TruSeq RNA Sample Preparation Kit v2 (Illumina), according to the manufacturer’s instruction. NGS was performed on a HiSeq2000 (Illumina) with 50bp single end reads for small RNA and mRNA using Illumina reagents and following the manufacturer’s instruction. Information on data processing and statistical analysis can be found in the supplementary materials.

**Application of miR181a antagomir or mimic to human and murine CD4+T cells.**

Chitosan-coated PLGA nanoparticles (for information on nanoparticle preparation, characterization and testing, please refer to the supplementary materials) were loaded with a miR181a-5p inhibitor (miRCURY LNA microRNA inhibitor, Exiqon) or a miR181a-5p mimic (miRCURY LNA microRNA mimic, Exiqon) at a ratio of nanoparticles:inhibitor/mimic of 50:1 and incubated at room temperature for 30 minutes with gentle agitation (sequences can be found in the supplementary materials). The loaded nanoparticles were added to the wells of a polyclonal Treg induction assay at a final concentration of: miR181a inhibitor: 0.95 ng/µl per 100,000 cells for human cells and 0.19 ng/µl per 10,000 cells for murine cells, miR181a-5p mimic: 2.1 ng/µl per 100,000 cells for human assays and 0.21 ng/µl per 10,000 cells for murine assays. As a control a negative control mimic or inhibitor with no known targets were used at the same concentration.

**Application of PI3K-, PTEN- and NFAT5- inhibitors**

The PTEN inhibitor SF1670 (Abcam Biochemicals) was added at a concentration of 0.5 µM directly with the start of the stimulation of naïve CD4+T cells, the PI3K inhibitor LY294002 (SYNkinase, final concentration 10 µM) was added after 18h of TCR stimulation. A small molecule inhibitor of NFAT5, developed by Wan-Uk Kim ([*36*](#_ENREF_36)), was added simultaneously to the TCR stimulation at a concentration of 0.01 µM or 0.01 nM in case of low dose anti-CD3 stimulation.

***In vivo* NFAT5 inhibition and miRNA181a antagomir application**

For assessment of *in vivo* NFAT5 inhibition, IAA+NOD mice or human immune-system-engrafted NSG HLA-DQ8 mice ([*12*](#_ENREF_12)), were injected i.p. with 3 mg/kg NFAT5 inhibitor once daily for 14 days (NOD mice) or 4 days (NSG mice). A miRNA181a antagomir (Inhibitor Probe mmu-miR-181a-5p, Exiqon) was injected i.p. into IAA+NOD mice at 10mg/kg every other day for 14 days. Control mice were injected with sodium chloride. On day 15 or day 5 Treg frequencies were analyzed in peripheral blood and pancreatic lymph nodes (NOD mice) or in peripheral blood and spleen (NSG HLA-DQ8 mice). Pancreata of NOD mice were embedded for cryosections and analysis of pancreas pathology.

**Histopathology of NOD pancreata.**

Pancreata were embedded with TissueTek O.C.T. Compound, frozen on dry ice and serial sections were stained with hematoxylin and eosin. Insulitis scoring was performed as previously described ([*44*](#_ENREF_44)*,* [*45*](#_ENREF_45)). The following scores were assigned: 0: intact islets / no lesions; 1: periislet infiltrates; 2: <25% islet destruction; 3: >25% islet destruction; 4: complete islet destruction. Investigators were blinded for group allocations.

**Immunofluorsecence staining of NOD pancreata.**

Immunofluorescence staining was done using rabbit–anti-mouse insulin antibodies (Cell Signaling) and donkey–anti-rabbit Alexa647 antibodies (Dianova). For CD4 staining, rat–anti-mouse (Becton, Dickinson and Company) antibodies were used, followed by goat–anti-rat AlexaFluor488 (Dianova). For NFAT5 staining a rabbit-anti-mNFAT5 (ThermoScientific) was used. For FoxP3 staining, cells were incubated with rat–anti-mouse antibodies (eBioscience) and goat–anti-rabbit (Becton, Dickinson and Company) combined with TSA Cyanine3 amplification (PerkinElmer). Nuclei were counterstained with HOECHST 33342 dye (Invitrogen). Negative control slides were incubated with secondary antibodies. Cells were analyzed by confocal microscopy (Olympus).

**Statistics.**

Results are presented as mean and standard error of the mean (s.e.m) or as percentages, where appropriate or as box and whisker plots with minimum to maximum values for data distribution. For normally distributed data, Student’s t test for unpaired values was used to compare means between independent groups and the Student’s t test for paired values was used to compare values for the same sample or subject tested under different conditions. The nonparametric Wilcoxon signed-rank test was applied when data did not show Gaussian distribution. Group size estimations were based upon a power calculation to minimally yield an 80% chance to detect a significant difference in the respective parameter of *p*<0.05 between the relevant groups. For all tests, a two-tailed *p* value of <0.05 was 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 programs GraphPad Prism 6 and the Statistical Package for the Social Sciences (SPSS 19.0; SPSS Inc.).

**Supplementary Materials**

**Supplementary material and methods**

**Fig. S1.** Categories of human islet autoimmunity in children at risk of developing Type 1 diabetes.

**Fig. S2.** Identification of insulin-specific Tregs after Treg induction assays *in vitro*.

**Fig. S3.** Insulin-specific CD4+T cell proliferation in accordance with the duration of islet autoimmunity.

**Fig. S4.** miR181a targeted signaling pathways in CD4+T cells.

**Fig. S5.** Nanoparticle-mediated miRNA uptake in CD4+T cells.

**Fig. S6.** *Pten* and *Foxo1* expression upon TCR stimulation and increasing doses of costimulation.

**Fig. S7.** DNA-methylation analysis of the human *FOXP3* Treg-specific demethylated region (TSDR) in *in vitro* induced Tregs.

**Fig. S8.** NFAT5 and PTEN protein expression in CD4+T cells of NOD mice.

**Fig. S9.** Hypertonicity independent NFAT5 induction in CD4+T cells of IAA+NOD mice.

**Fig. S10.** *Pten* and *Foxo1* expression in CD4+T cells from NFAT5ko mice.

**Fig. S11.** Treg frequencies after *in vitro* induction using CD4+ T cells from NFAT5ko animals.

**Fig. S12.** *Nfat5* and *Foxo1* expression in CD4+T cells of PTEN Tg mice.

**Fig. S13.** Effect of a miRNA181a antagomir or mimic on Treg induction from PTEN Tg mice with decreasing anti-CD28 stimulation.

**Fig. S14.** Effect of NFAT5 inhibition in CD4+T cells from humanized NSG mice or from PTEN Tg mice *in vivo*.

**Fig. S15.** Reduction of immune activation by blocking miRNA181a or NFAT5 in NOD mice.

**Table S1.** Primary source data.

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**Competing interests:** None.

**Figures**

**Fig.1**. **Analyses on immune activation vs. tolerance induction during islet autoimmunity onset. (A)** Frequencies of induced insulin-specific CD127loCD25hiFOXP3hiCD4+Tregs after stimulation with insulin-specific HLA-DQ8–monomers and 100 U/ml IL2 *in vitro* using T cells from children with different durations of islet autoimmunity (no autoimmunity [autoantibody negative]: n=5; recent onset of autoimmunity [<5 years autoantibody positivity]: n=7; longterm autoimmunity [>10 years autoantibody positivity]: n=4). **(B)** Activated T cell (CD127loCD25hiFOXP3intCD4+T cells) frequencies in assays from **(A)**. **(C)** Proliferative responses of CD4+T cells from children with different durations of islet autoimmunity to HLA-DQ8–restricted insulin-specific artificial antigen-presenting cellsshown aspercentages of CD25+CD45RO+CFSEdimCD4+T cells (no autoimmunity: n=14; recent onset of autoimmunity: n=6; persistent autoimmunity: n=6; longterm autoimmunity: n=14). **(D)** Non-autoantigen-specific Treg induction *in vitro* with the hemaggluttinin A peptide (n=5 per group). **(E)** CD127loCD25hiFOXP3hiCD4+Treg frequencies after polyclonal Treg induction (n=5 per group). Data are presented as the mean ± s.e.m. with individual valuesfor data distribution. Student´s t-test. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Fig.2.** **miRNA181a targets NFAT5 in human CD4+T cells. (A)** MiRNA expression profiles in *ex vivo* CD4+T cells with an activated phenotype from children with or without autoantibodies by NGS of pooled samples (n=4 per group). Shown is a set of most abundant miRNAs relevant for T cell activation and or Treg induction. **(B)** MiRNA181a reads by NGS as in **(A)** (n=4 per group). **(C)** MiRNA181a abundance in *ex vivo* CD4+T cells from children with different durations of autoimmunity (no autoimmunity: n=9; recent onset of autoimmunity: n=10; longterm autoimmunity: n=5) by RT-qPCR. **(D)** Abundance of signaling intermediates involved in T cell activation in *ex vivo* CD4+T cells from autoantibody negative or positive children, by NGS from pooled samples, (n=4 per group). Open bars: predicted as direct targets of miRNA181a, hatched bars: not predicted as direct targets of miRNA181a. (**E**) Human *Nfat5* mRNA abundance in *ex vivo* CD4+T cells from individual children with or without islet autoimmunity by RT-qPCR (no autoimmunity: n= 6, recent onset of autoimmunity: n= 7, longterm autoimmunity: n= 6). Data are presented as mean ± s.e.m. **(B)** or as box and whisker plots with min to max values for data distribution **(C+E)**. Student´s t-test.\* *p*<0.05.

**Fig.3.** **Human** **Treg induction using subimmunogenic TCR stimulation with a miRNA181a antagomir or mimic. (A)** Frequencies of induced CD127loCD25hiFOXP3hiCD4+Tregs after polyclonal TCR stimulation of naïve CD4+T cells from healthy individuals for 18h with 100 U/ml IL2 with a miRNA181a mimic (n=5 independent experiments). **(B)** Human *Nfat5* mRNA abundance by RT-qPCR upon TCR stimulation of CD4+T cells from healthy individuals for 48 hours with a control mimic or a miRNA181a mimic (n=8 per group). **(C)** Representative confocal microscopy images of human CD4+T cells from healthy individuals stimulated for 54 hours with anti-CD3/anti-CD28 with a control mimic or miRNA181a mimic and 100 U/ml IL2 stained for CD4, NFAT5 and DAPI (n= 5 individuals/samples for either control mimic or miRNA181a mimic with 5 images per individual/sample, scale bar: 25 m). (**D**) Human NFAT5 abundance in samples from **(C)** (n=5 per group). **(E)** Representative FACS plots indicating induced CD4+CD25+FoxP3+Tregs after TCR stimulation and 100 U/ml IL2 from Balbc mice with a control or miRNA181a mimic. (**F**) Frequencies of CD25hiFoxP3hiCD4+Tregs as in **(E)** (n=5 independent experiments). **(G)** Ki67 expression in induced Tregs as in **(E)** (n=5 per group). **(H)** *Nfat5* mRNA abundance in Balbc CD4+T cells after TCR stimulation for 54 hours with a miRNA181a or control antagomir and 100 U/ml IL2 (n=5 per group). **(I)** Representative STED microscopy images of Balbc CD4+T cells stimulated with anti-CD3/anti-CD28 and either a control mimic or miRNA181a mimic and 100 U/ml IL2 for 54 hours, stained for CD4, NFAT5 and DAPI (n=4 mice/samples for control mimic (10 images per sample) and n=5 mice/samples for miRNA181a mimic (12 images per sample). Scale bar: 25 m. Negative control slides were incubated with secondary antibodies (goat-anti-rabbitSTAR635P (Abberior)) only. Data are presented as box and whisker plots with min to max values for data distribution **(A, B, F, G)** or as mean ± s.e.m. **(D+H)**. Student´s t-test. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Fig.4.** **Murine** **Treg induction in the presence of a miRNA181a antagomir or mimic. (A)** Effects of a miRNA181a antagomir on Treg induction in cells from children without islet autoimmunity (n=7 for control and n=8 for miRNA181a antagomir) with islet autoimmunity and no clinical disease (n=5 per group), or with established T1D (n=5 per group). **(B)** Frequencies of induced CD25hiFoxP3hiCD4+Tregs from *in vitro* polyclonal Treg induction assays with naïve T cells from NOD mice with or without IAA+autoimmunity (n=14 per group, IAA+NOD: median age 118 days, IQR 74-134 days; IAA-NOD: median age 99 days, IQR 51-120 days). **(C)** MiRNA181a expression in *ex vivo* CD4+T cells with a naïve or activated phenotype from NOD mice with or without IAA+autoimmunity (n=5 per group). **(D)** *Nfat5* mRNA abundance in *ex vivo* CD4+T cells from NOD mice with (n=8) or without IAA+autoimmunity (n=9). **(E)** Frequencies of CD25hiFoxP3hiTregs induced with subimmunogenic TCR stimulation for 18h from naïve CD4+T cells from NOD mice with or without IAA+autoimmunity and a control or miRNA181a antagomir (n=6 per group). Data are presented as box and whisker plots with min to max values for data distribution. Student´s t-test. \**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Fig.5.** **Treg induction potential in CD4+T cells from NFAT5ko mice and PTEN Tg mice. (A)** Representative FACS plots indicating CD4+CD25+FoxP3+Tregs upon Treg induction with subimmunogenic TCR stimulation and 100 U/ml IL2 from WT or NFAT5ko mice **(B)** Frequencies of CD4+CD25+FoxP3+Tregs upon Treg induction as in **(A)** (n=5 experiments). **(C)** % change in Treg induction with subimmunogenic TCR stimulation and 100 U/ml IL2 from WT or NFAT5ko mice with a miRNA181a antagomir or mimic. % change refers to the difference in Treg frequency obtained with either a control mimic or antagomir respectively (n=4 experiments). **(D+E)** Frequencies of CD4+CD25+FoxP3+Tregs upon Treg induction with low-dose TCR stimulation (0.01µg/ml anti-CD3) and 100 U/ml IL2 in combination with a PI3K- **(D)** or a PTEN inhibitor **(E)** (n=4). **(F)** Representative FACS plots indicating CD4+CD25+FoxP3+Tregs upon Treg induction with subimmunogenic TCR stimulation and 100 U/ml IL2 from WT or PTEN Tg mice. **(G)** Frequencies of CD4+CD25+FoxP3+Tregs as in **(F)** (n=7 per group). **(H)** *Nfat5* mRNA expression in CD4+T cells after TCR stimulation for 54h from WT or PTEN Tg mice (n=6 per group).Data are presented as box and whisker plots with min to max values for data distribution **(B, G, H)** or asmean ± s.e.m. **(C-E**). Student´s t-test.\**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001.

**Fig.6. A NFAT5 inhibitor increases Treg induction. (A+B)** Representative FACS plots indicating CD4+CD25+FoxP3+Tregs upon murine **(A)** or human **(B)** Treg induction with subimmunogenic TCR stimulation and 100 U/ml IL2 using naïve CD4+T cells with or without NFAT5 inhibitor [0.01 M]. **(C+D)** Frequencies of CD4+CD25+FoxP3+Tregs upon Treg induction as in **(A+B)** (n=5 experiments). **(E)** Representative FACS plots indicating *ex vivo* CD4+CD25+FOXP3hiTregs in peripheral blood of humanized NSG mice after treatment with either saline or a specific NFAT5 inhibitor [3 mg/kg, i.p.] for 4 days. **(F)** Frequencies of CD4+CD25+FOXP3hiTregs as in **(E)** (n=5 per group). Data are presented as the mean ± s.e.m. **(C, D, F)**. Student´s t-test. \* *p*<0.05, \*\* *p*<0.01, \*\*\* *p*<0.001.

**Fig.7. NFAT5 or miR181a inhibition *in vivo* improves islet autoimmunity. (A)** Representative FACS plots indicating *ex vivo* CD4+CD25+FoxP3+Tregs from lymph nodes of IAA+NOD mice treated with a miRNA181a or control antagomir for 14 days with 10mg/kg i.p. every other day. **(B)** Summary graphs for CD4+CD25+FoxP3hiTregs as in **(A)** (n=8 per group). **(C)** Frequencies of *ex vivo*CD4+CD25+FoxP3hiTregs from peripheral blood of IAA+NOD mice treated as in **(A)** (n=5 per group). **(D)** Representative histogram of CD28 staining in *ex vivo* CD4+T cells from IAA+NOD mice treated with a control antagomir (red) or miRNA181a antagomir (blue). **(E)** Summary graph for CD28+T cells as in **(D**) (n=4 per group). **(F)** Representative hematoxylin and eosin-stained pancreas cryosections from IAA+NOD mice treated as in **(A)** (n=5 mice per group, 3 sections/mouse, scale bar: 100 m). **(G)** Grading of insulitis from mice as in **(F)** (n=5 per group). **(H)** Representative FACS plots indicating *ex vivo* CD4+CD25+FoxP3+Tregs from lymph nodes of IAA+NOD mice treated with a NFAT5 inhibitor or vehicle control for 14 days with 3mg/kg i.p. every day. **(I)** Summary graphs for CD4+CD25+FoxP3hiTregs as in **(H)** (n=7 per group). **(J)** Representative hematoxylin and eosin-stained pancreas cryosections from IAA+NOD mice treated as in **(H)** (n=5 mice per group, 3 sections/mouse, scale bar: 100 m). **(K)** Grading of insulitis from mice as in **(J**) (n=5 per group). Data are presented as box and whisker plots with min to max values for data distribution. Student´s t-test. \*\* *p*<0.01, \*\*\**p*<0.001.

**Fig.8. NFAT5 inhibition *in vivo* decreases NFAT expressing T cells in the pancreas in the absence of metabolic side effects. (A)** Representative confocal microscopy images of pancreatic cryosections of NOD mice given a vehicle control (left) or NFAT5 inhibitor (right) at 3mg/kg i.p. every day for 14 days. Staining for CD3 (green), NFAT5 (red) and DAPI (blue). **(B)** Frequencies of CD3+NFAT5+T cells in pancreata from NOD mice treated as in **(A)** (n=4 mice per group/2 sections per mouse, scale bar: 50 m).  **(C)** Blood glucose levels in NOD mice treated as in **(A)**. **(D)** Body mass development in NOD mice treated as in **(A)**. **(C+D)** n=4 per group for NFAT5 inhibitor and n=5 per group for vehicle control. Data are presented as box and whisker plots with min to max values for data distribution **(B+C)** or as mean ± s.e.m. **(D)**. Student´s t-test. \*\* *p*<0.01.