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Targeting RORγt/DHODH signaling fosters Treg-based immune modulation to control autoimmune Type 1 Diabetes

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Abstract

Incidences of Type 1 Diabetes, an organ-specific autoimmune disease, are on the rise throughout the globalized world. It oftentimes manifests very early in life and patients rely on life-long insulin treatment which all in all adds up to a major health and mental burden for the patients and their families. Furthermore, due to life-long treatment and various long-term complications it has a tremendous impact on healthcare and society. The disease is characterized by the autoimmune-mediated destruction of the pancreatic beta cells, which results in the disruption of their main function, namely insulin production in response to glucose. This eventually leads to a complete loss of blood glucose control making the patients dependent on exogenous insulin. The autoimmune reaction results from increased and aberrant immune activation and defective tolerance mechanisms and is mainly mediated by T cells. Peripheral immune tolerance is executed by regulatory T (Treg) cells which suppress self-reactive T cells and their impaired generation, function and stability contribute to the loss of immune tolerance and autoimmune activation.

Therapies that target T cells or inflammatory pathways are under investigation for potential treatment options. Targeting TNF-α or CD3 for instance resulted in better beta cell function compared to the control group. Furthermore, treatment of at-risk individuals with Teplizumab, an anti-CD3 antibody, was shown to delay the onset of overt T1D and was approved by the FDA in November 2022. As the first approved disease modifying immunotherapy, this lays the ground work for future immunotherapies. To avoid general immunosuppression, more specific and targeted approaches will be pivotal in the future.

Therefore, in this thesis, I studied the new potential drug candidates IMU-935 and -838, which were developed by Immunic AG. IMU-838 is an inhibitor of dihydroorotate dehydrogenase (DHODH), an enzyme which catalyzes the de novo pyrimidine synthesis and is essential for activated lymphocytes. IMU-935 is a potent RORγt inverse agonist, while it can also inhibit, although to a lower extent, DHODH. Importantly, DHODH inhibition specifically blocks activated lymphocytes while avoiding general immunosuppression. The strong immune activating conditions during islet autoimmunity and T1D hinder de novo Treg induction. We hypothesized, that reducing immune activation by targeting RORγt and/or DHODH could open a broader window of opportunity to induce Tregs.

Using in vitro experiments I could show that IMU-935 and -838 both improved murine Treg induction. The impact was more pronounced in conditions that hinder Treg induction including immune-activating conditions or using T cells from NOD mice with ongoing insulin autoantibody positive autoimmunity. Importantly, the drug candidates fostered an anti-inflammatory phenotype and their presence resulted in the upregulation of RORγt. Using loss-of-function models IL6Rα signaling was identified as one contributing mechanism by which the drug candidates can improve Foxp3 and RORγt expression. Furthermore, I identified DHODH inhibition as the main contributor on the mode of action of the drug candidates in our in vitro studies. Importantly, in vivo experiments using a preclinical mouse model of T1D showed a reduction in T1D incidences upon treatment with IMU-

838. This was accompanied by reduced immune activation and infiltration in the pancreas. Furthermore, preliminary data on increased Treg frequencies with a higher expression of functional Treg markers suggest positive influence on immune tolerance as well.

In summary, I showed that targeting DHODH results in reduced immune activation during islet autoimmunity and T1D which opens a broader window of opportunity to induce and stabilize Tregs. The improved interplay between immune activation and tolerance restored the balance of the immune system. My work gives important insights into the immune tolerogenic properties of the potential drug candidates IMU-838 and -935 and their potential use for T1D. The encouraging results let us to hypothesis that the drug candidates present an opportunity for prospective clinical trials in the future.

Zusammenfassung

Type 1 Diabetes (T1D) ist eine organspezifische Autoimmunerkrankung, deren Inzidenz auf der gesamten Welt stetig steigt. Sie tritt häufig schon im frühen Kindesalter auf und Betroffene sind auf Insulin Injektionen angewiesen, was insgesamt eine große gesundheitliche und psychische Belastung für die Patienten und ihre Familien darstellt. Darüber hinaus hat T1D aufgrund der lebenslangen Behandlung und verschiedener Langzeitkomplikationen große soziale und volkswirtschaftliche Auswirkungen. T1D ist durch die autoimmunvermittelte Zerstörung der Beta Zellen der Bauchspeicheldrüse gekennzeichnet, was zu einer Störung ihrer Hauptfunktion führt, nämlich der Insulinproduktion als Reaktion auf Glukose. Dies führt schließlich zum vollständigen Verlust der Blutzuckerkontrolle, weshalb die Patienten von körperfremder Insulin Zufuhr abhängig sind. Die Autoimmunreaktion resultiert aus einer überschießenden Immunaktivierung und defekten Immuntoleranzmechanismen und wird hauptsächlich durch T-Zellen vermittelt. Die periphere Immuntoleranz wird durch regulatorische T-Zellen (Tregs) vermittelt, die selbstreaktive T-Zellen unterdrücken und deren gestörte Bildung, Funktion und Stabilität zum Verlust der Immuntoleranz und zur Autoimmunreaktion beitragen.

Verschiedene Therapien, die T-Zellen oder Entzündungswege unterdrücken, werden als mögliche Behandlungsoptionen für T1D untersucht. Hierbei haben zum Beispiel Studien zur Inhibition von TNF-α oder CD3 in kürzlich diagnostizierten T1D Patienten eine verbesserte Beta Zell-Funktion im Vergleich zu der Kontroll-Gruppe gezeigt. Darüber hinaus hat die Behandlung von Risikopersonen mit Teplizumab, einem anti-CD3 Antikörper, den Ausbruch von symptomatischen T1D verzögert, was zu der Zulassung durch die FDA als Präventionstherapie in November 2022 führte. Dies ist die erste zugelassene krankheits-modifizierende Immuntherapie und legt somit den Grundstein für künftige Immuntherapien. Um eine generelle Immunsuppression zu vermeiden, werde insbesondere spezifischere und gezieltere Ansätze von entscheidender Bedeutung sein.

Daher wurden in dieser Arbeit die neuen Wirkstoffe IMU-935 und -838, welche von Immunic AG entwickelt wurden, untersucht. IMU-838 ist ein Inhibitor der Dihydroorotat-Dehydrogenase (DHODH). Das Enzym katalysiert einen wichtigen Schritt in der *de novo* Pyrimidin-Synthese, welcher für aktivierte Lymphozyten unerlässlich ist. MU-935 ist ein potenter RORγt inverser Agonist, der auch, wenn auch in geringerem Maße, DHODH hemmt. Wichtig hierbei ist, dass der DHODH-Inhibitor spezifisch aktivierte Lymphozyten hemmt und damit eine generelle Immunsuppression verhindert. Die starken immunaktivierenden Bedingungen, die während der Inselautoimmunität und T1D herrschen, behindern die Induktion von Tregs. Ich untersuchte, ob die reduzierte Immunaktivierung, die durch die Inhibition von RORγt und/oder DHODH erreicht wird, bessere Bedingungen für die Treg-Induktion bieten kann.

In *in vitro* Experimenten konnte ich zeigen, dass IMU-935 und -838 die Treg-Induktion verbessern. Die Wirkung war ausgeprägter in Bedingungen, die die Treg-Induktion erschweren. Diese beinhalteten experimentelle Konditionen, die zu erhöhter Immunaktivierung führen oder die Verwendung von T Zellen aus NOD Mäuse mit laufender IAA^+ Autoimmunreaktion. Zudem förderten die beiden Wirkstoffe einen entzündungshemmenden Phänotyp und die Hochregulation von RORγt in $CD4^+$ T-Zellen und Tregs. Mit Hilfe von Funktionsverlustmodellen, konnte der IL6Rα-Signalweg als ein Mechanismus identifiziert werden, der zu der verbesserten Expression von Foxp3 und RORyt in Anwesenheit der Wirkstoffe während der *in vitro* Treg-Induktion beiträgt. Darüber hinaus konnte ich die DHODH-Hemmung als Hauptfaktor für die Wirkungsweise der Wirkstoffe in unseren *in vitro* Studien identifizieren. In einem präklinischen Mausmodel für T1D führte die Behandlung mit IMU-838 außerdem zu einer Verringerung der T1D Inzidenzen. Dies ging mit einer verringerten Immunaktivierung und Infiltration der Bauchspeicheldrüse einher. Darüber hinaus bestätigten vorläufige Daten eine erhöhte Treg-Frequenz mit einer gesteigerten Expression von funktionellen Treg-Markern einen positiven Einfluss auf die Immuntoleranz.

Zusammenfassend konnte ich zeigen, dass die Hemmung der Immunaktivierung während der Inselautoimmunität oder T1D durch die DHODH Inhibition zu besseren Bedingungen für die Induktion von Tregs führt. Das verbesserte Zusammenspiel von Immunaktivierung und –toleranz führt zu einem wiederhergestellten Gleichgewicht des Immunsystems. Meine Arbeit gibt wichtige Einblicke in die Eigenschaften der Wirkstoffe Immuntoleranz zu stärken und deren Möglichkeit für Therapie Optionen für T1D. Die hier dargelegten Ergebnisse lassen somit die Hypothese zu, dass IMU-838 und -935 gute Kandidaten für mögliche zukünftige klinische Studien für T1D sind.

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Akt	Protein kinase B
APC	Antigen presenting cell
BSA	Bovine serum albumin
CD	Cluster of differentiation
CNS	Conserved non-coding sequence
CpG	5'-C-phosphate-G-3'
CTLA4	Cytotoxic T lymphocyte antigen 4
DC	Dendritic cell
CY	Cyclophosphamide
DHODH	Dihydroorotate dehydrogenase
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
Foxp3	Forkhead box protein 3
FCS	Fetal calf serum
GAD	Glutamic acid decarboxylase
Gp130	Glycoprotein 130
GWAS	Genome-wide association studies
HEPES	Hydroxyethylpiperazin-ethansulfonacid
HLA	Human leukocyte antigen
HPAP	Human Pancreas Analysis Program
IA2	Insulinoma antigen 2
IAA	Insulin autoantibodies
IFN-γ	Interferon γ
IL	Interleukin
IL2R	Interleukin 2 receptor
IL6Rα	Interleukin 6 receptor α
IPEX	Immunedysregulation polyendocrinopathy enteropathy X-linked syndrome
i.v.	Intravenous
KO	Knock-out
MACS	Magnetic activated cell separation
mesLN	Mesenteric lymph nodes
MHC	Major histocompatibility complex
MS	Multiple Sclerosis
mTOR	Mammalian target of rapamycin
NFAT	Nuclear factor of activated T cells
NOD	Non-obese diabetic
Nrp1	Neuropilin 1
nPOD	Network for Pancreatic Organ Donors
NSG	NOD Scid IL2Rg knockout
PBMC	Peripheral blood mononuclear cell

PMA	Phorbol 12-myristate 13-acetate
PBS	Dulbecco's phosphate-buffered saline
PI3K	Phophatidylinositol-3-kinase
POInT	Primary Oral Insulin Trial
PTEN	Phosphatase and tensin homolog
pLN	Pancreatic lymph node
qPCR	quantitative real time polymerase chain reaction
RA	Rheumatoid arthritis
Rorγt	RAR-related orphan receptor gamma
RPMI	Roswell Park Memorial Institute medium
Scid	Severe combined immunodeficiency
Stat	Signal transducers and activators of transcription
STZ	Streptozotocin
T1D	Type 1 Diabetes
TCR	T cell receptor
T_H	T helper
TGF-β	Transforming growth factor beta
TKO	T cell-specific knock-out
TNF-α	Tumor necrosis factor
Treg	Regulatory T cell
TSDR	Treg-specific demethylated region
wt	Wildtype
ZnT8	Zinc transporter 8

1. Introduction

1.1 Immune Tolerance and Autoimmunity

Our body faces constant threats from infectious invaders. The immune system recognizes the pathogens and eliminates them by starting a well-orchestrated immune attack. Immune tolerance mechanisms ensure on the one hand that an immune attack stays controlled and on the other hand that immune attacks are only directed against foreign pathogens. The discrimination between self and non-self is executed by two distinct mechanisms: central and peripheral tolerance (reviewed in [1]).

Central tolerance takes place during T cell development in the thymus. Lymphocytes recognizing self-antigens presented by antigen-presenting cells with high affinity are either removed by apoptotic cell death or inactivated (anergy) [2, 3]. However, some autoreactive T cells can escape the thymus and reach the periphery. Here, tolerance is maintained by a specialized T cell subset, so-called regulatory T cells (Tregs), which are able to suppress autoreactive T cells [4, 5].

The balance between immune activation and tolerance is critical for a healthy immune system and is tightly controlled. Failure of those mechanisms can lead to the escape of autoreactive T cells that eventually can cause autoimmune diseases. The autoimmune attack can be either systemically including systemic lupus erythematosus or organ specific such as observed in multiple sclerosis (MS) or Type 1 Diabetes (T1D) [6, 7]. The latter is the most common autoimmune disease in young children and recent studies suggest a doubling of cases in Europe in the next 20 years [8].

1.2 Type 1 Diabetes

In T1D, the loss of immune tolerance and aberrant immune activation results in impaired self-tolerance to the insulin-producing beta cells which consequently lead to their destruction in the pancreas [9]. In a healthy state the pancreatic beta cells sense glucose in the blood stream and in a tightly regulated and complex network they release insulin to promote storage of glucose in other cells and tissues. Destruction of the beta cells and the decreased or absent insulin production in T1D results in a defective blood glucose control which renders patients dependent on insulin replacement therapies (reviewed in [10]). This traditional model in which islet autoimmunity as the primary source of beta cell destruction results sooner or later in impaired glucose homeostasis was extended in the last years. Several studies show that beta cells themselves also contribute to the pathogenesis of T1D. For instance, beta cells exhibit first activation of the unfolded protein response which secondary leads to ER stress in later stages of islet autoimmunity but before the overt disease contributing to beta cell dysfunction [11, 12]. In addition, the progression to T1D is accompanied by a shift to a senescent phenotype in a subset of

beta cells which was shown to contribute to the disease development [13]. The beta cell fragility is among other reasons also attributed to a genetic predisposition in T1D [14, 15]. A recent study by Warncke et al. investigated alterations in blood glucose before and after the onset of islet autoimmunity by making use of the study population of POInt, in which 1050 infants between the ages of four to seven months with elevated genetic risk are enrolled [16]. They observed that postprandial blood glucose levels slightly rise be-fore the detection of autoantibodies and that post-and pre-prandial levels further increase afterwards [16]. This highlights that changes or insults of the pancreatic beta cells occur earlier than previously thought and that beta cells themselves together with the autoim-mune reaction promote T1D progression.

1.2.1 Aberrant activation of immune cells in T1D

The autoimmune destruction of the pancreas is initiated by an immune cell infiltration in the pancreatic islets, a process called insulitis [17, 18]. Infiltrating immune cells include $CD4^+$ and $CD8^+$ T cells, B cells, macrophages and dendritic cells (DC) with $CD8^+$ T cells being the most abundant cell type [19-21]. In mouse models of T1D, such as the non-obese diabetic (NOD) mice, insulitis is even more prominent and starts early in life at around 3-4 weeks of age [22]. Treatment of NOD mice with anti-T cell agents, such as anti-thymocyte serum, anti-Thy 1,2 [23] or anti-CD3 [24] suppressed or delayed the occurrence of overt T1D highlighting that T cells play a major role in disease development and progression. Islet-specific $CD8^+$ T cells are found in insulitic lesions and peripheral blood from T1D patients as well as in peripheral blood, pancreatic lymph nodes (pLN) and islet infiltrates of NOD mice [25-27]. Here, they acquire a cytotoxic effector memory phenotype with expression of granzyme B and IFN-γ [28]. Noteworthy, this was specifically observed independently of beta cells in inflamed islets and not in the pLN [29]. $CD8^+$ T cells are thought to be the main mediators for direct beta cell destruction [30, 31]. They can do so by cytotoxic degranulation in a contact-dependent manner through perforin or granzyme molecules [32, 33]. In addition, beta cell death can be mediated by the released proinflammatory cytokines, such as IFN-γ or TNF-α, which are toxic to beta cells [34, 35]. Hyperexpression of HLA class I molecules in pancreatic islets might explain the prevalence of $CD8^+$ T cells in the pancreas [36, 37]. Likewise, aberrant expression of HLA class II was observed, suggesting that beta cells might directly be involved in the presentation of autoantigens to infiltrating $CD4^+$ T cells [38]. Animal experiments showed that $CD4^+$ and $CD8^+$ T cells are required to induce T1D after transfer of splenic cells to immunodeficient recipients indicating the destruction of beta cells depends on both T cell subsets [39]. Indeed, islet-specific $CD4^+$ T cells were identified and further characterized. Several studies observed autoreactive $CD4^+$ T cells producing the pro-inflammatory cytokine IFN-γ in peripheral blood of T1D patients [40, 41]. Increased levels of IFN-γ were also observed in sera, pLNs and pancreata of diabetic NOD mice [42]. The same study also correlated increased T_H17 activity with disease onset of NOD mice [42]. Furthermore, upregulated or islet-specific T_H17 immune responses were found in human studies

[43-45]. In addition to their own effector function, $CD4^+$ T cells are able to activate M1 macrophages which promote inflammation [46]. They are also known to be activated in response to IFN-γ and TNF-α, adding to a vicious circle of increasing immune activation in the pancreas thereby perpetuating beta cell destruction [35].

1.2.2 Pathology of human T1D

T1D development is associated with a genetic component as well as environmental factors, including dietary factors, early viral infections and the microbiome [47-49]. The strong genetic contribution was indicated by an increased risk of first degree relatives of T1D patients [50, 51]. Specifically, the human leucocyte antigen (HLA) class II haplotypes HLA-DR4 and -DQ8 have been identified as the strongest risk factors. Moreover, other non-HLA T1D risk loci, including those for insulin, cytotoxic T cell-associated antigen-4 (CTLA-4) and IL2RA were identified using genome-wide association studies (GWAS) [52-54].

Longitudinal studies of individuals at risk for developing T1D have shown that, prior to the onset of clinical symptoms, the disease progresses through distinct stages which resulted in the well-accepted staging classification system proposed by Insel *et. al.* [55]. The appearance of two or more autoantibody against islet autoantigens marks the onset of the presymptomatic stage of T1D, also known as islet autoimmunity [55]. Islet autoantigens include insulin [56], glutamic acid decarboxylase (GAD)[57], insulinom-antigen 2 (IA2)[58, 59] and zinc transporter 8 (ZnT8) [60]. Over time, beta cell mass declines, resulting in dysglycemia which is referred to as stage 2 [55]. In stage 3, clinical symptoms manifest [55]. It was shown that autoantibodies can be detected in the blood years or even decades before the onset of hyperglycemia which highlights the heterogeneity of T1D [61]. However, the presence of two or more autoantibodies coincides with a lifelong risk for developing T1D of almost 100% [62]. Hence, autoantibodies can serve as biomarkers and prediction tool. A lot of effort is put into establishing screening protocols and platforms to identify people at risk for developing T1D. Ziegler and colleagues, for example, initiated a study in Bavaria in which children, regardless of genetic risk factors, are tested for the presence of islet autoantibodies as part of pediatric well-baby visits [63, 64]. Together with many other screening programs across the world, this helps researches to learn more about early stages of islet autoimmunity and the progression to clinical disease. For instance, the German DiMelli cohort and a classification and regression tree analysis was used to ask the question whether the highly heterogeneous disease can be divided into subtypes, endotypes or theratypes [65]. This analysis observed several subgroups of young patients that got newly diagnosed with T1D and are below 20 years of age, which can be relevant for potential therapy options [65]. Furthermore, a recent study which combined several cohorts, provided new insights into a more precise prediction of T1D development which is dependent on islet autoantibodies at seroconversion and HLA genotype [66]. This highlights also the potential of large and diverse data sets which could be used for machine learning algorithms and could provide more

precise approaches for the prediction and prevention of T1D. Screening platforms and better predication tools will further help patients and their families to get counseling which can prevent life-threatening complications such as ketoacidosis [67]. In addition, longitudinal samples from those cohorts could help identify early impairments in immune tolerance.

1.3 Regulatory T cells mediate immune tolerance

Regulatory T cells are key players for the establishment and maintenance of immune tolerance and their failure contributes to development and progression of autoimmunity [68]. Regulatory cells, which are now known as Tregs, were first characterized in 1995. Sakaguchi et al. observed that a subpopulation of $CD4^+$ T cells, that expressed the high-affinity α-chain of the IL-2 receptor CD25, were able to prevent autoimmunity [69]. Later, the transcription factor Foxp3 was identified as the master regulator for Treg differentiation [5, 70-72]. The human autoimmune disease Immunedysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) and the scurfy mouse with a lethal autoimmune phenotype both harbor a mutation in the Foxp3 gene, highlighting the critical role of Foxp3 for the development and maintenance of Tregs as well as their role in immune regulation [73, 74].

Tregs can exert their immune-regulatory functions through various mechanisms, including inhibitory cytokines, cytolysis, metabolic disruption and targeting of DC. The release of inhibitory cytokines, such as IL-10 and TGF-β, suppress the function of effector T cells [75, 76]. In addition, Tregs can exhibit cytotoxic activity by secreting perforin and granzyme A/B [77, 78] or deprive their surroundings of IL-2 causing metabolic disruption of effector T cells [79]. Most importantly, Tregs can target DCs via direct cell-cell contact modulating their maturation or function through expression of the surface markers Lag3 or CTLA4, respectively [80-83]. Recently, a novel suppressive pathway, in which strong interaction of antigen-specific Tregs with DCs resulted in the depletion of the peptide-MHC class II complex, was described [84]. Since not all suppressive mechanisms rely on direct cell-cell contact but on the secretion or consumption of cytokines and other soluble mediators, they can also manipulate cells in their close proximity, in a process called bystander suppression [85].

1.3.1 Generation of Tregs

Two origins for Tregs have been described. They can either develop in the thymus during T cell maturation [86, 87] or differentiate in the periphery [87-89]. Several studies have shown that Treg induction in the periphery is most efficient when a strong agonistic ligand is applied under subimmunogenic conditions [89-91]. Subimmunogenic doses avoid strong immune activation and result in minor cellular proliferation [90, 92-94]. This coincides with only limited activation of the PI3K/Akt/mTOR (phophatidylinositol-3-kinase/protein kinase B/mammalian target of rapamycin) pathway, while higher doses or

strong costimulatory signals result in high activation of the PI3K/Akt/mTOR pathway and T cell activation, consequently hindering Treg induction [89-91]. Thus, negative modulators of that pathway, such as the mTOR inhibitors rapamycin or everolimus, enhance Treg induction [90, 92-94]. In addition, phosphatase and tensin homolog (PTEN) counteracts PI3K activity and is crucial for Treg induction and function [92, 95]. Importantly, our lab identified that *in vitro* Treg induction is impaired during islet autoimmunity and T1D which is likewise observed using T cell of children with recent onset of islet autoimmunity or of NOD with ongoing islet autoimmunity [92, 96].

Antigen-specific Tregs play a major role in the development and progression of T1D. Serr *et al.* showed that insulin-specific Tregs are severely reduced in children with recent onset of T1D while enhanced insulin-specific Tregs were observed in children with long-term autoimmunity without progression to the overt disease [97]. This highlights the importance of studying the induction of autoantigen-specific Tregs for delaying T1D progression. The essential role of insulin as an autoantigen was shown in various studies in the NOD mouse model. Elimination of insulin prevented the disease whereas tolerance to overexpressed preproinsulin 2 reduce the onset and severity of T1D [98, 99]. Furthermore, islet infiltrates harbor predominantly insulin-specific T cells, specifically recognizing the B:9-23 peptide region of the B chain [100, 101]. Human studies also confirmed that B:9-23 insulin is a major target of the immune system in T1D [102].

In NOD mice the immune response is focused on the IA^{g7} conferring the high risk for the development of T1D [103, 104]. A polymorphism in the IA^{g7} modifies the binding affinity to the insulin B:9-23 epitope resulting in a poor binding and an inefficient presentation of the peptide [105, 106]. This negatively influences both tolerance mechanisms increasing the risk for the development of an autoimmune disease. First of all, the insufficient presentation of the epitopes for thymic deletion allows the escape of autoreactive T cells [106, 107]. Secondly, a weak agonist results in inefficient Treg induction in the periphery thereby limiting peripheral tolerance [106, 107]. To overcome that obstacle, effort was put into finding insulin variant with increased binding affinity and consequently improved antigen presentation [106, 108]. Based on this, Carolin Daniel and colleagues showed that a strong agonistic ligand supplied under subimmunogenic conditions to young NOD mice was able to improve the induction of antigen-specific Tregs *in vivo* [91].

Standard protocols to induce Tregs *in vitro* involve the stimulation of naïve T cells via the TCR in presence of TGF-β. However, these induced Tregs rapidly lost Foxp3 expression in restimulation experiments in absence of TGF-β and consequently also their suppressive function [109, 110]. Thus, despite of high levels of induced Tregs in presence of TGF-β, they do not resemble the stable, long-lasting phenotype of their *in vivo* counterparts and are distinct from *in vivo* induced Tregs. However, maintenance of a stable and functional Treg phenotype is crucial for a potential therapeutic approaches. With the goal to induce Tregs *in vitro* that resemble *in vivo* induced Tregs the group of Matthias Merkenschlager established a TGF-β independent Treg induction protocol by limited activity of the PI3K/Akt/mTOR pathway. This can be achieved either by pharmacological

inhibition of the pathway or by limiting the TCR stimulation by premature withdrawal of the stimulus resembling subimmunogenic TCR stimulation [93]. Consequently, the resulting Tregs are more stable and long-lasting and resemble *in vivo* induced Tregs [93].

1.3.2 Regulatory elements for Treg generation and maintenance

In order to exert their immune regulatory functions Tregs rely on a stable and persistent Foxp3 expression. A stable Foxp3 expression and therefore a stable Treg phenotype is associated with a Treg-specific hypomethylation pattern [109-111]. Several regulatory elements in the Foxp3 locus including the promoter and three conserved non-coding sequences (CNS1-3) have been identified and can be differentially methylated, thus controlling Foxp3 expression [109-111]. In addition to a hypomethylated state the Foxp3 promoter shows a stronger association with acetylated histones in Tregs compared to conventional T cells, indicating a better chromatin accessibility in Tregs [112, 113]. TCR signaling triggers a signaling cascades which leads to the recruitment of several transcription factors to the promoter, including NFAT, AP1 and FOXO proteins, resulting in the activation of the Foxp3 promoter [112, 114]. CNS1 was shown to be dispensable for thymic Treg differentiation as well as for the maintenance of Foxp3 [111]. But Zheng *et al.* observed that it is critical for the peripheral induction of Foxp3 in naïve T cells in response to TGF-β [111]. CNS3 is required for the initiation of Foxp3 expression since it promotes an epigenetically poised state through the accumulation of permissive histone modifications at the Foxp3 promoter [111, 115]. The methylation status of CNS2 is linked to the stability of Tregs [109, 110, 116].

1.3.3 Treg stability

CNS2, also known as the Treg-specific demethylated region (TSDR), is completely demethylated in Tregs, while it is fully methylated in conventional T cells. It is critical for sustained Foxp3 expression and thus is most important for the Treg phenotype and its stability [109, 110, 116]. *In vitro* induced Tregs in presence of TGF-β have a methylated TSDR which explains their aforementioned unstable Foxp3 expression [109, 110]. In contrast, *in vivo* induced Tregs under subimmunogenic conditions have a demethylated TSDR and can be maintained for long periods of time, thus resembling a more stable phenotype of induced Tregs [97]. In accordance, forced demethylation of *in vitro* induced Tregs by 5-azadeoxycytidine, an inhibitor of CpG methylation, resulted in a stable Foxp3 expression [110]. Moreover, in the absence of the enzyme mediating methylation, DNA methyltransferases 1 (DMNT1), efficient induction of Foxp3 upon TCR stimulation of con-ventional T cells was observed [117]. *Vice versa*, Tet proteins are main mediators for removing DNA methylation and establishing a hypomethylated state and accordingly, *Tet2/Tet3*-defecient mice show an increased TSDR methylation and impaired Foxp3 stability [118]. Scherm et al. observed that by inhibiting miR142-3p, which targets Tet2, Treg differentiation and stability can be improved [96]. In the same study, they linked insulin autoantibody positive autoimmunity in NOD mice and recent onset of

T1D in human with higher expression of miR142-3p, reduced Tet2 protein abundance and therefore higher CNS2 methylation suggesting impaired Treg stability in islet autoimmunity and T1D [96].

Apart from T1D, Treg instability was also observed in other autoimmune diseases, such as MS or rheumatoid arthritis (RA) [119, 120]. Treg instability under proinflammatory conditions as observed in autoimmune diseases was suggested to lead to the loss of Foxp3 expression and the subsequent conversion into pathogenic exTregs [68, 121]. Despite extensive research and the development of fate mapping models, exTreg development remains controversial and two different models have been proposed. First, under proinflammatory conditions, remethylation of the Foxp3 CNS2 region results in an unstable Foxp3 expression and reprogramming into T_H cells [120, 121]. A second model suggests, that Tregs are a stable lineage and ex-Tregs arise from a minor population of uncommitted Tregs [122, 123]. The latter was based on studies conducted largely under homeostatic conditions in the steady state *in vitro* or under setting of acute lymphopenia *in vivo* [122, 123]. The group of Xuyu Zhou developed a model in which they could specifically trace thymic-derived Tregs (tTregs) [124]. While only around 1 % of tTregs lost Foxp3 expression under homeostatic conditions, the activation and sequential specialization of Tregs resulted in higher conversion into ex-Tregs [124]. Overall, tTregs likely constitute a stable phenotype under physiological conditions. However, when exposed to proinflammatory triggers or overstimulation a fraction of Tregs may become ex-Tregs.

1.3.4 Treg plasticity

In addition to Treg instability, their plasticity became a focus of research in the last years. Treg plasticity is defined as de-differentiation into more specialized Treg subsets which is accompanied by a change in migratory and functional capabilities. They still express Foxp3 and retain suppressive function but acquire T_H cell-like characteristics including transcription factors and chemokine receptors and are therefore referred to as T_H-like Tregs. This adaptation and functional specialization may allow them travel to the site of inflammation and enables them to better restrain different types of $CD4^+$ T cell immune responses (reviewed in [68]).

T-bet is the master transcription factor for IFN-γ-producing T_H1 cells [125]. Koch *et al.* observed that a subset of Tregs express T-bet which is critical for Treg homeostasis and function during T_H1-inflammatory responses [126, 127]. Similarly, expression of interferon regulatory factor 4 (IRF4) or Gata3, both transcription factors involved in the T_H2 cell differentiation, in Tregs is required for the Treg-mediated control of T_H2-inflammatory responses [128-133]. Finally, Tregs can express RORγt (retinoic acid receptor-related orphan receptor-γt) or STAT3 (signal transducer and activator of transcription 3) to control T_H17-dependent immune responses [134-139]. The latter is most prominently observed in the intestine and represents a stable subset with suppressive function [139]. $ROR\gamma t^+$ Tregs are mostly negative for Neuropilin 1 (Nrp1) and Helios and are induced by

microbial stimuli, which all together suggests that they are peripherally induced Tregs (pTregs) [137-139].

Several studies indicated that IL-6 is important for RORγt-expressing $CD4^+$ T and Treg cells. *In vitro* T_H17 cells can be induced by TCR stimulation in presence of TGF-β, IL-6 and IL-23 [140, 141]. The importance of IL-6 for the development in vivo was demonstrated in various studies. Ohnmacht ***et al***. observed that mice with a global knockout (KO) of IL-6 have reduced frequencies of RORγt$^+$ Tregs in the colon [137]. Furthermore, it was suggested that RORγt expression in thymic-derived Treg cells is dependent on IL-6-mediated Stat3 signaling [142] and recent study reported that enteric neurons are an important source of IL-6 for the induction of RORγt$^+$ Tregs [143]. TGF-β has overlapping functions in T_H17 vs. Tregs development since its downstream signaling can upregulate both, Foxp3 and RORγt [144, 145]. Therefore, they engage in an antagonistic competition such that different stimuli including IL-6 or the TGF-β concentration can shift the balance to one side or the other [144, 145]. On a mechanistic basis, it was shown that Foxp3 can bind RORγt resulting in transcriptional inactivation [146].

IL-6 can signal via different mechanisms which are all based on a complex formation of the IL6Rα (IL6 Receptor α), glycoprotein 130 (gp130) and IL-6. In the classical mechanism membrane-bound IL6Rα binds IL-6. In the trans-signaling soluble IL6Rα which can be cleaved from the membrane-bound receptor upon TCR activation can bind IL-6 [147, 148]. Both complexes can engage with membrane bound gp130 to initiate the signaling cascade [147, 148]. In a third mechanism called trans-presentation, membrane-bound IL6R on DCs can first bind IL-6 in trans and then form a complex with gp130 expressed on T cells [149]. While IL-6R is only expressed by a few cell types like hepatocytes, neutrophils, monocytes and $CD4^+$ T cells, gp130 is present on all cell types [149]. Therefore, the last two mechanisms allow cells that do not express the IL6Rα to be responsive to IL-6. Trans-signaling was shown to be important for pro-inflammatory actions of IL-6 and is required for the generation of pathogenic T_H17 cells *in vivo* [150].

As explained above, Tregs display functional and phenotypical heterogeneity matched to the type of immune response to be restrained. To further deepen the concept of Treg heterogeneity, Tregs were identified in various non-lymphoid tissues.

1.3.5 Tissue Tregs

The presence of so-called tissue Tregs has been reported for several non-lymphoid tissues, including adipose tissue [151], skin [152], lung [153], muscle [154] and small intestine/colon [137-139]. Here, tissue Tregs adapt to the new environment and acquire a tissue-specific phenotype which is characterized by the unique expression of tissue-specific surface markers, transcription factors and TCR repertoires (reviewed in [155]). In addition to their classical function of maintaining immune tolerance and restraining inflammation, they exert non-canonical functions such as maintaining tissue homeostasis, integrity and function [151, 154].

Tregs reside also in the pancreas and are mainly described in context of an inflamed pancreas such as in T1D in which they show a distinct transcriptional profile from other tissue Tregs and lymphoid Tregs [156]. In NOD mice, islet antigen-specific Tregs are enriched in the inflamed islets which are able to slow down islet destruction [157]. Compared to spleen and pLNs, the Tregs residing in the islets show signs of antigen stimulation [157]. One could however speculate that the presence of Tregs in the pancreas is only helpful to a limited extent due to the aforementioned Treg impairments in T1D. Evidence for the high importance of pancreatic Tregs for the development of T1D were obtained from a temporally controlled Treg ablation experiment. Deletion of Tregs in pre-diabetic BDC2.5 NOD mice with ongoing insulitis resulted in a rapid and uncontrolled activation of effector $CD4^+$ T cells residing in the autoimmune lesions but not in the pLN [158, 159]. The mice in which Treg were ablated became diabetic within 3-5 days, which highlights an ongoing need for Tregs to restrain the autoimmune attack [158, 159]. *Vice versa*, treatment of pre-diabetic NOD mice with short-term low-dose IL-2 resulted in an increase of Tregs specifically in the pancreas [160]. A similar treatment strategy starting at the time of T1D onset was able to reverse T1D [160]. In line with that, our group could show that by reversing miR142-3p/Tet2-mediated Treg instability Treg numbers in the pancreas were increased which was accompanied by an improved insulitis score [96].

Translating the finding for pancreas residing cells obtained from mice experiments into the human setting remains challenging. Consortia such as the network for Pancreatic Organ Donors (nPOD) and the Human Pancreas Analysis Program (HPAP) which collect and study these rare tissue samples in detail will be pivotal for addressing the remaining questions [161, 162]. In addition, the recent advantages of next-generation sequencing, especially at the single cell level, provide new technologies to obtain large amount of data [161, 162]. However, results obtained from peripheral blood show that presence of islet-specific Tregs is associated with disease protection and that insulin-specific Tregs are severely reduced in children with newly diagnosed T1D while they are enhanced in non-diabetic children with long-term autoimmunity [97, 163]. In addition, various deficits in numbers, suppressive capacity and stability have been reported for circulating Tregs in T1D (reviewed in [164]). Overall, these results show that in mice and human, Tregs residing in the pancreas are main mediators for the development of overt T1D. Thus, therapeutic strategies that are strengthening Tregs may provide a promising perspective to improve immune tolerance with the overarching goal to reverse autoimmunity in T1D. In combination with inhibitors of immune activation that could be even more beneficial.

1.4 Immunotherapeutic interventions in T1D

1.4.1 Current immunotherapeutic intervention studies

The pathogenesis and staging of T1D implies a window of opportunity for treatment options. In stage 1, the autoimmune process is initiated and is marked by the appearance of islet autoantibodies. With time beta cell mass declines which results in dysglycemia in stage 2 and finally leads the overt disease (stage 3) which requires insulin supplementation [55]. When beta cell function declines, the autoimmune process also subsides which may result in a lower effect of immune therapies at later stages of the disease. The staging implies also different preventive or treatment approaches. The primary intervention aims at delaying or stopping the initiation of autoimmunity while secondary preventions have the goal to prevent the clinical disease by stopping or slowing down the beta cell destruction [165]. Studies have shown that shortly after diagnosis most patients have remaining endogenous insulin production which reduces further with time [166-168].Thus, the purpose of tertiary intervention is the preservation of residual beta cell function with the aim to reduce complications [165].

Given the major role of T cells in the disease progression, therapies targeting T cells are highly investigated, most promisingly anti-CD3e monoclonal antibodies, such as Teplizumab. First indications for a potential treatment option were obtained more than 25 years ago. Chatenoud *et al*. showed that treatment of NOD mice with anti-CD3 at first signs of hyperglycemia reversed disease development [24]. This led to various clinical trials which failed to mirror results of the murine study. Despite lacking the reversal of T1D, these trials showed improved stimulated C-peptide responses which is a measure for endogenous insulin production [169-171]. Furthermore, high-risk non-diabetic individuals treated with Teplizumab showed delayed a progression to clinical T1D [172]. A follow-up study observed better beta cell function and metabolic improvement with Teplizumab treatment even before diagnosis of T1D [173]. This was accompanied by an increased frequency of $TIGIT^{+}KLRG1^{+}$ memory $CD8^{+}$T cells and a reduced secretion of IFN-γ and TNF-α indicating T cell exhaustion [173]. In addition, anti-inflammatory and cytokine therapies are under investigation for treatment of new-onset T1D patients which were diagnosed within 100 days before the trials. While blocking the inflammatory pathways signaling through the IL1Rα or IL6Rα did not show any clinical effects, targeting TNF-α using the antibody Golimumab resulted in a higher endogenous insulin production and a lower need to use exogenous insulin [174-176].

To prolong the time before diagnosis of the overt disease means an expanded time without the burden of constant insulin injections for the patients and a beneficial impact on the risk for long-term complications [165]. Positive results of the Teplizumab-prevention trial which resulted its FDA approval for the secondary prevention of T1D in at-risk individuals this year is a milestone for the T1D community and the first immunotherapy approved for T1D [177]. This together with the help of expanding screening programs for

islet autoantibodies and genetic risk and the establishment of more precise risk scores, paved the way for additional secondary prevention studies with other immune-intervening agents which are planned or already ongoing [178].

The balance of immune activation and immune tolerance is key to a healthy immune system, thus improving regulatory mechanisms is an alternative to achieve an equilibrium between these two sites. Expansion of Tregs by low-dose IL-2 was shown to be safe, however clinical trials for their effect on C-peptide preservation are still ongoing [179, 180]. The group of Jeffery Bluestone showed that adoptive transfer of antigen-specific Tregs could reverse T1D in NOD mice [181]. Moving forward, they tested expanded polyclonal Tregs in patients with recent onset of T1D [182]. Despite being a safe approach, they observed a rapid decline in the percentage of infused Tregs [182]. Combining Treg transfer with low-dose IL-2 resulted in an expansion of infused Tregs, but also of cytotoxic T cells [182]. The increase in cytotoxic T cells, NK cells and eosinophils was also observed in other studies, highlighting the need for mutant IL-2 therapeutics avoiding aforementioned activation of other cell types (reviewed in [183]). Although Treg transfer is now known to be safe in the clinic, the efficacy will be largely dependent on the antigen-specific disease-relevant Tregs. Their low frequency in autoimmune diseases require the manipulation or expansion of Tregs before transfer. Various approaches, such as introducing a synthetic TCR to generate antigen-specific Tregs or the forced expression or deletion of genes that promote Treg induction, function, stability could help to overcome that obstacle [184].

An alternative approach was used by the Pre-POINT study group. In a pilot clinical trial, islet autoantigen negative, but genetically at-risk children at the age of two to seven years were given daily oral insulin, with the rational that exposing the oral mucosa to insulin may induce immune tolerance [185]. Indeed, they observed an increased T cell response to insulin with features of immune regulation [185]. These findings opened up the opportunity for the currently ongoing primary intervention trial called Primary Oral Insulin Trial (POInT) in which 1050 infants at the age of four to seven months with an elevated risk for T1D have been enrolled and are given daily oral insulin [186]. This antigen-specific vaccination strategy could prevent early stages of islet autoimmunity, namely the first appearance of islet autoantibodies.

1.4.2 Targeting DHODH as a potential treatment strategy

Induction of antigen-specific tolerance is an ultimate goal in preventing and treating T1D. However, aforementioned Treg impairments during islet autoimmunity and T1D including impaired Treg stability and induction will pose as an obstacle [92, 96]. Thus, this will probably result in the need for combinatorial treatment strategies with immune modulators to control overshooting immune activation and thereby providing a better window of opportunity for tolerance-inducing agents to induce and stabilize Tregs. Commonly used

immune suppressants such as FK506, rapamycin or mycophenolate mofetil have shown negative side effects on pancreatic islets and in order to reduce side-effects general immune-suppression should be avoided [187]. This highlights the need for new targets and approaches for alternative treatment options.

Among others, DHODH (Dihydroorotate dehydrogenase) inhibitors became a research interest. DHODH catalyzes the fourth step in the pyrimidine *de novo* biosynthesis and is localized in the mitochondria [188-191]. In a DHODH independent pathway pyrimidines can also be synthesized by a salvage pathway in which pyrimidine nucleotides are recycled [188-191]. Pyrimidines serve as precursors for RNA and DNA and are therefore critical mediators for the cellular metabolism [188-191]. Lymphocytes harbor only a few mitochondria suggesting that activity of DHODH is the rate-limiting step of *de novo* biosynthesis for lymphocytes [191]. In contrast to resting lymphocytes, proliferating and activated lymphocytes have a higher pyrimidine demand and expand their pool by 8-fold [192]. Thus, they rely on *de novo* biosynthesis of pyrimidines while resting lymphocytes can cover their demand with the salvage pathway [192]. This suggests that inhibition of DHODH and therefore the pyrimidine *de novo* biosynthesis can be highly efficient in suppressing activation of lymphocytes and their effector function without affecting basic homeostatic functions, which makes it a promising candidate for selectively interfering with overshooting immune responses [193].

Most commonly used up to date are leflunomide and its active metabolite teriflunomide. They were shown to inhibit T cell proliferation *in vitro* [194, 195]. Furthermore, many studies reported altered T cell function in response to DHODH inhibition. Leflunomide was shown to impair generation of IFN-γ producing T_H1 cells and to reduce TNF-α and IL-1β production by human synovial cells [196]. Reduced IFN-γ production was also observed upon antigen-specific stimulation of myelin basic protein (MBP)-specific T cells which are able to induce experimental autoimmune encephalomyelitis (EAE) when adoptively transferred in rats in a model of MS [197]. *In vivo* models for MS or RA showed reduced or absent disease severity upon treatment with leflunomide or teriflunomide [197-199]. The following clinical trials showed better results with respect to relapse rates, imaging outcomes and accumulation of disability in RRMS and delayed radiographic progression and clinical outcomes in RA [200-202]. This led to the approval of leflunomide for the treatment of RA in 1998, while teriflunomide was approved for relapsing-remitting multiple sclerosis (RRMS) in 2012 [203, 204]. However, more adverse events attributed to general anti-proliferative effects, such as neutropenia, alopecia, and diarrhea, were observed and follow-up studies identified a high discontinuation rate [193, 205-207]. Experiments with uridine supplementation in addition to leflunomide or teriflunomide which circumvents the DHODH inhibition showed a restoration of immune cell proliferation, while immune cell functions, such as migration and cytokine production stayed unchanged [197]. These off-target effects were attributed to inhibition of various protein kinases, such as Jak 1 and 3,[208] or epidermal growth factor (EGF) receptor [209].

Therefore, second generation DHODH inhibitors were studied, including vidofludimus or its calcium salt vidofludimus calcium, which was previously developed by 4SC AG and further modified by Immunic AG and is now termed IMU-838 [210]. Both depend on the same active substance (vidofludimus) for their mechanism of action and the structure is distinct from the structure of leflunomide and teriflunomide [193, 211]. Consequently, vidofludimus shows no inhibition of protein kinases and is thus highly selective for DHODH resulting in reduced production of proinflammatory cytokines of activated lymphocytes and eventually leads to their apoptosis [193, 212]. Furthermore, in various *in vivo* models of colitis and EAE administration of vidofludimus resulted in improved disease outcomes. Fitzpatrick *et. al.* observed improved disease outcomes in chronic dextran sodium sulfate (DSS)-induced colitis in mice as well colitis induced by TNBS (2,4,6-tritrobenzene sulfonic acid) upon treatment of vidofludimus with better histology score, reduced body weight loss and reduced expression IL-17 [212, 213]. In an EAE rat model, administration of vidofludimus resulted in a decreased disease score similar to what was observed with Leflunomide [193]. Furthermore, in contrast to teriflunomide, which has a prolonged plasma half-life of 18-19 days, IMU-838 has a half-life of about 30h resulting in only little accumulation after daily dosing [193, 214]. An additional benefit of vidofludimus is its antiviral effect which is explained by the finding that virus-infected cells are metabolically active and are dependent on pyrimidine *de novo* biosynthesis [215, 216]. These advantages resulted in a good safety profile of vidofludimus and IMU-838 as assessed in the COMPONENT study with patients with RA, which showed little to no difference between the treatment groups [211, 217]. In addition, the recently evaluated phase 2 clinical trial (EMPhASIS) for RRMS showed a favorable safety profile of IMU-838 as well as reductions in new magnetic resonance imaging lesions [211, 217]. These findings justified a longer and larger phase 3 clinical trial (ENSURE-1, NCT05134441) which is now open for recruitment [211, 217].

In addition to inhibition of activated and proliferating lymphocytes, DHODH inhibition was shown to delay beta cell destruction and improve glucose metabolism and metabolic balance in T2D which was accompanied by an increase of growth/differentiation factor 15 (GDF15) [218]. GDF15 levels were reduced in islets from NOD mice with insulitis as well as in islets from donors with T1D [219]. In addition administration of GDF15 to prediabetic NOD mice resulted in a decrease in insulitis as well as reduced incidences of diabetes [219]. Overall this highlight the good potential of DHODH inhibitors for the treatment of T1D.

1.4.3 RORγt as a target for new immunotherapeutics

Immunic devised another DHODH inhibitor called IMU-935 which inhibits DHODH to a lesser extent than IMU-838 but in addition functions as a RORγt inverse agonist [220, 221]. They showed that IMU-935 is able to inhibit differentiation of murine and human T_H17 cells as well as their expression of IL-17 [220, 221]. In contrast to a full knockout of RORγt or other small pharmacological inhibitors of RORC, it does not induce thymocyte

apoptosis nor does it lead to an increased risk for thymic lymphoma [220, 222]. IMU-935 further showed activity in mouse models for colitis and psoriasis suggesting a potential for the treatment of autoimmune diseases [220, 221, 223]. For instance, in an imiquimod induced psoriasis model treatment of IMU-935 reduced IL-17F expression in the skin and the histological pathology scores in all skin layers [223]. Recently, Immunic reported first results from their phase 1 clinical trial of IMU-935 in healthy human subjects, including part A with single ascending doses and part B with multiple ascending doses. Daily dosing of IMU-935 in healthy humans over a 2 week period was observed to be safe and well tolerated, with no serious adverse events, or dose-dependent adverse events [223, 224]. Therefore, part C has been initiated in which moderate-to-severe psoriasis patients are going to be randomized to a 28-day treatment period with IMU-935 or placebo [223, 224].

There are several lines of evidence that suggest the involvement of T_H17 cells in the pathogenesis of T1D. Inhibition of T_H17 cells by neutralizing anti-IL-17 antibodies delayed the progression to diabetes with reduced insulitis and increased frequency of Tregs residing in the pLNs [225]. Accordingly, diabetes onset is delayed in IL-17 single deficient NOD mice [226]. Conversely, several studies reported a protective role of T_H17 cells and IL-17 [227, 228]. In addition, plasticity of T_H17 cells and their ability to reprogram into IFN-γ-producing T_H1-like cells complicates the investigation and results in opposing findings [42, 229-231]. Noteworthy, T1D patients showed increased circulating IL-17 producing cells as well as islet antigen-specific T cells [43, 232]. In addition, Ferraro *et. al.* observed a higher frequency of T_H17 cells in pancreatic lymph nodes of T1D subjects compared to non-diabetic donors [44]. Thus, clinical data support that IL-17 production and T_H17 cells may be pathogenic, opening the field for therapeutic T_H17 cell-targeting strategies for the treatment of T1D. In addition, due to the antagonistic competition between Foxp3 and RORγt described before, targeting RORγt could shift the balance towards immune tolerance.

The group of Thomas Burris reported a selective RORγ-specific synthetic ligand, which inhibited T_H17 cell development and function while increasing Treg induction [233]. Furthermore, they investigated the role of a selective RORα/γ inverse agonist in T1D [234]. The inverse agonist prevented the development of autoimmune diabetes in NOD mice with reduced immune infiltration in the islets [234]. This was accompanied by an inhibition of the release of proinflammatory cytokines, such as IL-17 of IFN-γ, while increasing the frequency of Tregs in the spleen [234]. Overall, the studies outlined above suggest that targeting RORγt as well as DHODH could be a novel approaches to reduce aberrant immune activation and open a window of opportunity to strengthen immune tolerance in autoimmune T1D.

2. Objective

T1D is the most common autoimmune disease in childhood and adolescence and can start early in life which can be a major health, mental and financial burden for the patients and their families [235]. Despite extensive research, patients rely on life-long insulin treatment. T1D results from overshooting immune activation and a loss of immune tolerance and therefore many drugs directed against the immune system are currently tested in clinical trials. A milestone for the treatment of T1D was reached in November 2022 with the FDA approval of Teplizumab for the prevention of T1D. The first approved immunotherapy in T1D opens up new opportunities for innovative treatment strategies. As a general immunosuppressant Teplizumab can also cause various side effects. This highlights the need for more specific or targeted approaches in the future.

Therefore, new potential drug candidates are a constant research focus. In my thesis, I aimed to investigate the drug candidates IMU-935 and IMU-838 which target RORγt and/or DHODH, respectively. Especially, DHODH inhibition selectively blocks activated lymphocytes thereby leaving homeostatic functions unaffected. Both are already in clinical trials for other autoimmune diseases such as MS or Psoriasis. However, their effect on Tregs and on the progression of T1D has yet to be explored. Thus, the ultimate goal of my work was to be able draw conclusion for their effectiveness in treating T1D and to pave the way for a clinical trial.

The first objective of this thesis was to assess the impact of the drug candidates on immune tolerance. Therefore, their effect on Treg induction and on the Treg phenotype was investigated in several *in vitro* experiments. They importantly also included experiments that mimic increased immune activation as observed during islet autoimmunity and T1D. This was reported to impair Treg induction. We hypothesize that reducing immune activation by targeting DHODH or RORγt could open a broader window of opportunity to induce Tregs. Furthermore, in order to specifically test their effect during islet autoimmunity I employed the mouse model of choice for T1D, the NOD mouse.

Secondly, having established an impact of the drug candidates on *in vitro* Treg induction I sought to investigate the responsible mechanism. Therefore, I used a T cell or Treg-specific loss-of-function mouse model and *in vitro* experiments, to investigate the influence of signaling pathways, specifically the IL6Rα signaling in T cells and Tregs.

In a third objective, I tested our hypothesis of the effectiveness of one of the drug candidates on the development of T1D *in vivo*. A preclinical mouse model in which diabetes was induced by adoptive transfer was used. It is characterized by a strong and aggressive phenotype and a fast progression to T1D. This gave insights into the immune activation and tolerance after treatment with the drug candidate and the impact on the progression to T1D.

3. Material and Methods

3.1 Material

Table 1. Buffers and medium

Buffer/Medium	Components
Hank's Balanced Salt Solution with supplements (HBSS+)	500 mL HBSS 5% (v/v) FCS 10 mM HEPES
MACS-PBS	500 mL PBS 0.5% (v/v) BSA 2 mM Ethylenediaminetetraacetic acid (EDTA)
RPMI 1640 with supplements (RPMI+)	500 mL RPMI 10% (v/v) FCS 1x Penicillin / Streptomycin 1x Sodium pyruvate 1x Non-essential amino acids 50 µM 2-Mercaptoethanol
Coating Buffer	0.1 M sodium bicarbonate buffer, pH 8.2
5x Tail Lysis Buffer	100 mM Tris 5 mM EDTA 0.2% (w/v) SDS 200 mM NaCl pH 8.0

Table 2. Chemicals and reagents

Chemical/reagent	Source	Identifier
Beta-Mercaptoethanol	BioConcept	5-69F00-E

Bovine serum albumin (BSA)	Sigma Aldrich	A7906
Collagenase D	Roche	11088882001
DNA Ladder 100bp	New England Biolabs	N3231 L
DreamTaq Green DNA-Polymerase (5 U/µl)	Thermo Fisher Scientific	EP0712
Dulbecco's phosphate buffered saline (DPBS)	Fisher Scientific	14190169
Ethylenediaminetetraacetic acid (EDTA)	Lonza	51234
Fc-block	Bioledgend	101320
Fetal calf serum (FCS)	Biowest	S1810-500
Fixable Viability Dye eFluor450	Thermo Fisher Scientific	65-0863-18
GolgiPlug™ (Brefeldin A)	BD Bioscience	555029
Hank's balanced salt solution	Sigma Aldrich	H6648
Heparin	AppliChem	APA3004.0005
Hydroxyethyl-piperazineethanesulfonic acid solution (HEPES, 1M)	VWR	15630056
Nunc-Immuno™ maxi Sorp 96-well plate	Thermo Fisher Scientific	439454
Ionomycin	Cayman Chemicals	10004974-1
Midori Green	Biozym	617004
Non-essential amino acids (100x)	Biochrom AG	K 0293
Penicillin/Streptomycin (Pen/Strep)	Sigma Aldrich	P4333
Phorbol 12-Myristate 13-Acetate (PMA)	abcam	ab120297
Proteinase K	Carl Roth	7528.5
RPMI 1640 + Glutamax	Life technologies	61870-010
Recombinant human IFN-γ	PeproTech	300-02
recombinant human IL-2	PeproTech	200-02
recombinant human IL-6	PeproTech	200-06

recombinant human IL-1β	PeproTech	200-01B
Recombinant human insulin	Sigma-Aldrich	I9278
Sodium Pyruvate Solution	Sigma Aldrich	S8636
Streptavidin Pacific Blue	Invitrogen	S11222
Streptavidin Microbeads	Miltenyi	130-048-101
Sytox Red	Thermo Fischer Scientific	S34859
Sytox Blue	Thermo Fischer Scientific	S34857

Table 3. Commercial assays

Assay	**Source**	**Identifier**
TMB Substrate Reagent Set	BD Bioscience	555214
Foxp3/Transcription factor staining buffer	Thermo Fischer Scientific	00-5523-00

Table 4. Technical equipment

Device	**Manufacturer**
Accu-Check® Aviva Glucometer	Roche
BD FACS Aria III	BD Bioscience
BD LSR Fortessa	BD Bioscience
CO_2 incubator BBD6220	Thermo Scientific
Cooling System HAAKE SC100	Thermo Scientific
Epoch	BioTek
Heraeus Multifuge 3 S-R	Thermo Scientific
Heraeus Multifuge X3R	Thermo Scientific
MACS Multistand, QuadroMACS	Miltenyi Biotec
Mars Safety Class Cabinet II	ScanLaf
Microscope, Primo star	Zeiss

peqStar 2X thermal cycler	Peqlab
Rotator	VWR
Thermal Cycler T100	BioRad
Vortex Mixture, Lab Dancer	VWR

Table 5. Antibodies

Antibody (anti-mouse)	Fluorophore	Source	Identifier
Fc-Block (2.4G2)	-	BioLegend	422302
CD11b (clone M1/70)	PB	BioLegend	101224
CD11c (clone N418)	BV421	BioLegend	117330
CD126 (clone REA620)	PE	Milteney	130-109-566
CD14 (clone rmC5-3)	V450	BD Bioscience	560639
CD25 (clone PC61)	PercpCy5.5 BV785	BioLegend	102051, 102030
CD28 (clone 37.51)	Purified	BD Bioscience	553294
CD3 (clone 145-2C11)	BV711, PE-Texas Red Purified	BioLegend BD Bioscience	100349, 100348 553057
CD4 (clone GK1.5, RM4-5)	Alexa Fluor 700 Biotin	BioLegend	100536, 100404
CD44 (clone IM7)	PE APC	BioLegend	103011, 103008
CD45R/B220 (clone RA3-6B2)	PB	BioLegend	103227
CD62L (clone MEL-14)	BV510 APC	BioLegend	104441 104412
CD8a (clone 53-6.7)	PB	BioLegend	00725
F4/80 (clone CI:A3-1)	PB	BioLegend	122611

Foxp3 (clone FJK-16s)	FITC PE	Thermo Fisher Scientific	11-5773-82 12-5773-82
IFN-γ (clone XMG1.2)	Alexa Fluor 647	BioLegend	505810
IL-10 (clone JES5-16E3)	PE	Biolegend	505007
IL-17A (clone TC11-18H10.1)	BV605	Biolegend	506927
Ki67 (clone 16A8)	BV605 APC	Biolegend	652413 652406
RORγt (clone AFKJS-9)	PE	Thermo Fisher Scientific	12-6988-82
TCRVβ4 (clone KT4)	FITC	BD Pharmingen	553365
Streptavidin	PB	Invitrogen	S11222

Table 6. mouse strains

Mouse strain	**Source/Description**
NOD/ShiLtJ	Polygenic model for autoimmune T1D, Jackson Laboratory, #001976
Foxp3 GFP Balb/c (C.Cg-*Foxp3*^{tm2Tch}/J)	Co-expression of eGFP and Foxp3, Jackson Laboratory, #006769
CD90.1 Balb/c (CBy.PL(B6)-*Thy1*^{a}/ScrJ)	Strain carries a T cell-specific Thy1.1 allele, Jackson Laboratory, #005443
NOD SCID (NOD.Cg- *Prkdc*^{scid}/J)	Homozygous for SCID mutation resulting in immunodeficiency Jackson Laboratory, #001303
NOD BDC2.5 (NOD.Cg-Tg (TcraBDC2.5,TcrbBDC2.5)1Doi/DoiJ)	NOD mice with a transgenic TCR which is specific for a Chromogranin A related epitope [236] Jackson Laboratory, #004460
IL6Rα TKO (C57Bl/6 CD4^{Cre} IL6Rα$^{fl/fl}$) and floxed IL6Rα$^{fl/fl}$ littermate controls	T cell-specific deletion of the *Il6rα* gene by CD4-Cre mediated excision of IL6Rα floxed exons, CD4^{Cre} mouse line purchased from Jackson Laboratory, #017336, IL6Rα$^{fl/fl}$ line

	provided by J. Brüning and first described here [237]
IL6Rα TregKO (Foxp3$^{eGFP/Cre}$ IL6Rα$^{fl/fl}$) and floxed IL6Rα$^{fl/fl}$ litter-mate controls	Treg cell-specific deletion of the *Il6rα* gene by Foxp3-Cre mediated excision of IL6Rα floxed exons, Foxp3$^{eGFP/Cre}$ mouse line Jackson Laboratory, # 023161
Foxp3-RFP Rorgt-GFP reporter, C57BL/6	Kindly provided by C. Ohnmacht and described previously [137]

Table 7. Primer for Genotyping

	Fdw sequence (5´-3´)		**Rev sequence (5´-3´)**
TCRVβ4-transgen	CATGTTTCCCTGCACATC		CCAGATCCAAAGATGAGTTGC
CD4 Cre	TGTGGCTGATGAT CCGAATA		GCTTGCATGATC TCCGGTAT
internal control for CD4 Cre	TTCCATCCAGTT GCCTTCTTGG		TTCTCATTTCCA CGATTTCCCAG
Foxp3 Cre	CAGTTTCAGTCC CCATCCTC		CGGGTCAGAA GAATGGTGT
internal control for Foxp3 Cre	CAAATGTTGCTTG TCTGGTG		GTCAGTCGA GTGCACAGTTT
IL6Rα flox	5GK12: CCGCGGGCGA TCGCCTAGG	5IL6Ex3: CCAGAGGAGCC CAAGCTCTC	3IL6A: TAGGGCCCAG TTCCTTTAT

3.2 Mice

Mice were bred and maintained on a 12 hours/12 hours light dark cycle at 25°C with ad libitum access to water and a chow diet under specific pathogen free conditions at the

animal facility of the Helmholtz Center Munich, Germany, according to the Institutional Animal Committee Guidelines. NOD/ShiLtJ mice were stratified according to their IAA status. For experiments using NOD or NOD BDC2.5 mice blood sugar was measured using Accu-Check Aviva® glucometer and blood from NOD mice was collected in tubes provided with 50 µL Heparin.

Ear punches collected during weaning were used to determine the genotype of the NOD BDC2.5, IL6Rα TKO and Treg KO mice by PCR as described in section 3.4. During each experiment the genotype was confirmed by FACS, either by staining of TCRVβ4 or IL6Rα by the respective fluorochrome-labeled antibodies, or by the Foxp3-GFP expression in Cre^{+} $Foxp3^{eGFP/Cre}$ mice.

Ethical approval for all mouse experimentations has been received by the District Government of Upper Bavaria, Munich, Germany (approval # ROB-55.2-2532.Vet_02-18-173, # ROB-55.2-2532.Vet_02-17-130 and # ROB-55.2-2532.Vet_02-17-63).

3.3 Murine insulin autoantibody (IAA) assay

In order to obtain the insulin autoantibody levels of NOD mice, a mouse high specificity/sensitivity competitive IAA assay in an ELISA format using sera from NOD mice was performed as described earlier [238]. To obtain the serum blood was collected in heparin and centrifuge for 8min for 3000g at RT (room temperature). Serum was collected and stored at -80°C until use. For the ELISA, high binding flat bottom 96-well plates were coated with 10 µg/mL insulin overnight at 4°C. Unspecific-blocking was performed with PBS containing 2% BSA for 2 hours. All steps were carried out at room temperature. Pre-incubated NOD serum with or without insulin competition was added and incubated for 2 hours. After wash steps biotinylated anti-mouse IgG1 was added for 30 min, fol-lowed by washing and 15 min incubation with by horseradish peroxidase-labeled strep-tavidin. After additional washing steps TMB substrate was added. All samples were measured in duplicates with and without competition using human insulin.

Serum from wildtype (wt) C57Bl/6 or BALB/c mice was used as negative control. The cutoff for IAA+ was set to 0.01.

3.4 Genotyping

Genotyping of the NOD BDC2.5, IL6Rα TKO and TregKO mice was performed by PCR. Ear punches from respective mice were digested using 1x Tail lysis buffer and 20mg/mL Proteinase K overnight at 55°C. The genotyping PCR was carried out using Dream Taq Green DNA Polymerase and the primer listed in **Table 7. Primer for Genotyping** ac-cording to the manufacturer's instructions. For analysis the PCR was run on a 2% Agarose gel with Midori Green.

3.5 T cell isolation

3.5.1 Preparation of T cells from murine lymphoid organs

Lymph nodes (LN) and spleen were collected and stored in HBSS+ (Hank's Balanced Salt Solution with supplements) on ice until use. Single cell suspensions were obtained by grinding LNs and spleen through a 70µM cell strainer. Cells were centrifuged for 5min at 400xg and 4°C, resuspended in HBSS+ and stored on ice for further use.

3.5.2 Preparation of T cells from pancreas

Pancreas was homogenized at 2500rpm for 30s using 1.4mm ceramic beads in MACS PBS. The suspension and remaining tissue was passed and grinded through a 40µm cell strainer. Cells were centrifuged at 300g for 10min to remove debris. Cells were re-suspended in 30% percoll prepared with RPMI and overlaid on 80% percoll prepared with HBSS. After centrifugation at 500xg for 30 min without brake and acceleration the interphase was collected in HBSS+, washed and stored in HBSS+ before use.

3.6 Flow cytometry and FACS

Isolated cells were incubated with Fc-Block to prevent unspecific signals for 10min on ice and stained with fluorochrome-labeled antibodies specific for surface markers for 30min on ice. Afterwards, cells were washed with HBSS+. Dead cells were excluded by using viability stains using Sytox Blue/Red Live Dead Stain for unfixed cells or fixable viability dye eFluor450 for fixed cells. For *ex vivo* stainings cells were fixed using the Foxp3 Staining Buffer Set for 30min to 1h one ice followed by intracellular staining using 1x Perm buffer for 30min to 1h. After 2 washing steps with Perm buffer and one with HBSS+ cells were filtered through a 40µm nylon mesh and acquired. For sorting live cells, cells are filtered after surface staining using a 40µm nylon mesh and sorted for purity with the FACS AriaIII (Beckton Dickinson) using the FACS DIVA software (v6.1.3-8.0.1). Doublets were excluded based on forward and side scatter gating. Dead cells were excluded based on live/dead staining.

For cytokine staining, cells were stimulated in presence of 0.5µg/mL PMA and 0.5µg/mL Ionomycin in RMPI+ with 1.5mM CaCl2 for 2-4 h. The protein transport inhibitor brefeldin A (Golgi Plug, BD) was added (1:1000) after 1-2 h. Samples were processed as described earlier using the Foxp3 Staining Buffer Kit.

Used antibodies are listed in **Table 5. Antibodies**.

For large murine sorts, T cells were pre-enriched using CD4-Biotin antibodies, Streptavidin beads and MACS (magnetic activated cell sorting) following manufacturer’s protocol (Miltenyi) on LS columns.

Flow cytometry data were acquired at the BD FACS Aria III or BD LSR Fortessa and analyzed using FlowJo (v7.6.1-v10.8).

3.7 Murine *in vitro* Treg induction assay

For *in vitro* Treg induction assays, 10 000 murine live naïve $CD4^+$ $CD25^-$ $CD44^{low}$ T cells or activated $CD4^+$ $CD25^-$ $CD44^{high}$ $Foxp3^-$ T cells were sort purified with the BD FACS Aria III into tubes provided with RPMI+ (Gating strategy in **Figure 1**). Cells were resuspended in RPMI+ and 100U/mL recombinant IL-2 and seeded in a 96-well plate precoated with 5µL/mL anti-CD3 and 5µL/mL anti-CD28 in coating buffer. Three different culturing conditions were used. First, for limited TCR stimulation cells were transferred into uncoated plates after 18h and cultured for additional 36h. Secondly, cells were continuously cultured for 54h (continuous TCR stimulation). Thirdly, for Treg induction in presence of pro-inflammatory cytokines, cells were cultured in presence of 10ng/mL IL-6, IFN-y and IL-1β. Cells were transferred into uncoated plates after 18h and cultured for additional 36h. IMU-935 and -838 were provided by Immunic Therapeutics. IMU-935 and -838 were dissolved in DMSO to a concentration of 50mM and 100.4mM, respectively. IMU-935 and IMU-838 were added at the indicated concentration at the start of the culture. Teriflunomide was dissolved in DMSO to a concentration of 15mM. IMU-935 was first diluted 1:10 with DMSO and further dilutions were done using RPMI+ with a final concentration of 1µM. IMU-838 and Teriflunomide were diluted with RPMI+ to 50.2µM or 7.5µM, respectively. For experiments with uridine, uridine was dissolved to 200mM in water and further diluted with RPMI+.

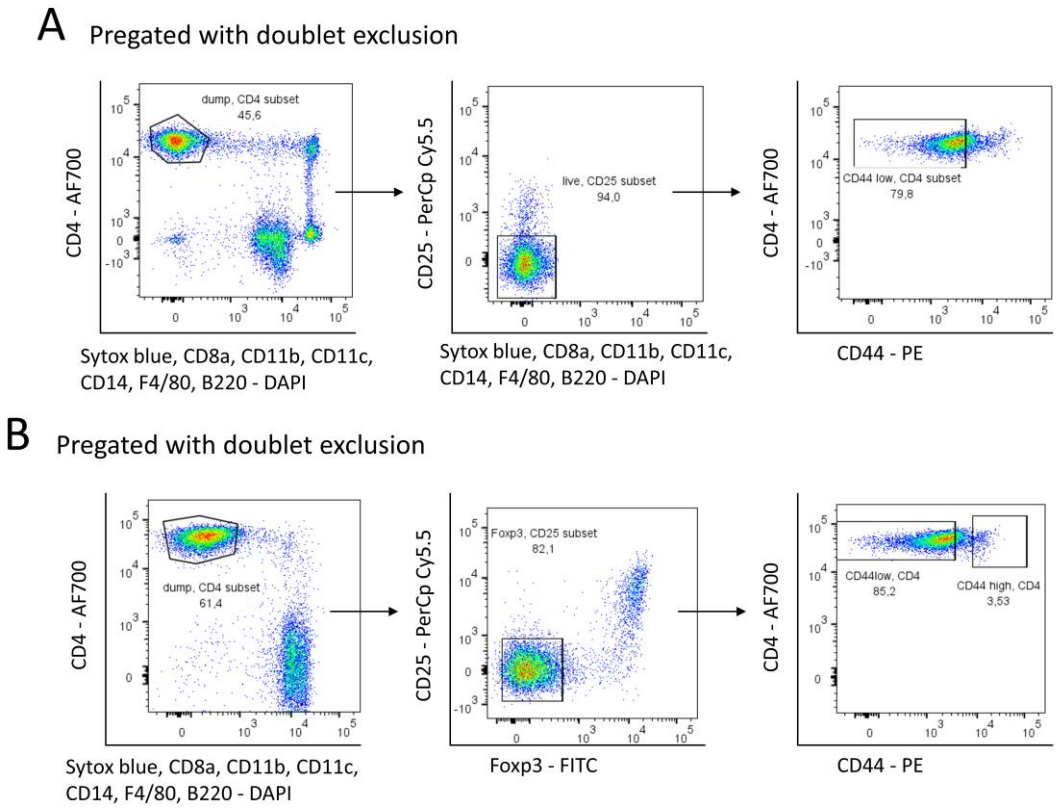

Figure 1: Gating strategy for FACS sorting of T cells used in Treg induction assays. (A) Live naïve $CD4^+$ $CD25^-$ $CD44^{low}$ T cells from CD90.1 BALB/c or NOD mice. (B) Naïve $CD4^+$ $CD25^-$ $CD44^{low}$ or activated $CD4^+$ $CD25^-$ $CD44^{high}$ $Foxp3^-$ T cells from Foxp3 BALB/c reporter mice.

3.8 Diabetes induction by adoptive transfer

To induce diabetes, diabetogenic T cells from TCR transgenic BDC2.5 NOD mice were transferred into NOD SCID mice as described earlier [236]. In brief, $CD4^+$ $CD62L^-$ $CD25^-$ $TCRV\beta4^+$ T cells from pre-diabetic BDC2.5 NOD mice were FACS-purified using MACS enrichment for CD4 and 1.4 Mio sorted cells per mouse were transferred i.v. into NOD SCID mice. Gating strategy and purity control, which was performed by fixation and in-tracellular staining for Foxp3 using an aliquot of the sorted cells, is depicted in **Figure 28**.

Mice were either treated daily with 150mg/kg IMU-838 which was dissolved in PEG-400 or PEG-400 alone as vehicle control by oral gavage (4mL/kg) starting the day before the transfer. Blood sugar was measured every other day from day 5 onwards from the tail vein with the Accu-Check Aviva® glucometer. Mice with two consecutive measurements above 250mg/dL were considered diabetic.

3.9 Statistics

All data were analyzed using GraphPad Prism (v7-9) and represented as box-and-whiskers plots. For normally distributed data, unpaired Student's t-test or a Student's t-test for paired values was used to compare means of two independent groups or comparing values from the same sample which was treated under different conditions, respectively. For multiple comparison, one-way ANOVA with Tukey's or Dunnett's multiple comparison test was used. Diabetes incidences were plotted in a survival curve and significance was tested using a Mantel-Cox test. For all tests, a two-tailed P value < 0.05 was considered as significant. Statistical significance is shown as *= $P < 0.05$; **= $P < 0.01$; ***= $P < 0.001$, non-significant (ns).

4. Results

4.1 Presence of IMU-935 increases *in vitro* Treg induction

Balancing immune activation vs. immune tolerance is the major goal when interfering with autoimmune processes. This could be achieved by directly stabilizing and improving immune tolerance or by inhibiting aberrant immune activation which in turn could open a window of opportunity to improve Treg induction. Therefore, the effect of IMU-935, a RORγt inverse agonist and, although to a lesser extent, a DHODH inhibitor, on Treg induction was assessed first.

4.1.1 Improved *in vitro* Treg induction from naïve T cells of non-autoimmune prone BALB/c mice in response to IMU-935

In order to investigate the impact of the drug candidate on *in vitro* Treg induction, Treg induction assays using limited TCR stimulation were performed.

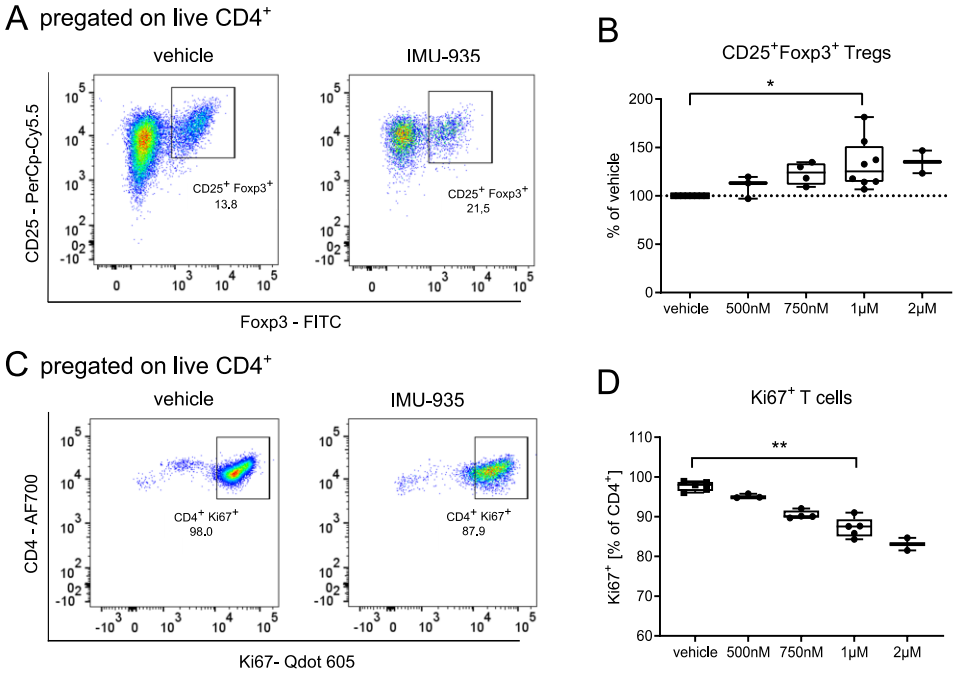

Figure 2: Increased Treg induction and reduced proliferation *in vitro* in presence of IMU-935. Treg induction assays using limited TCR stimulation of naïve CD4+ T cells isolated from LNs of wt BALB/c mice in presence of increasing concentrations of IMU-935 or the corresponding vehicle control. (A) Representative FACS staining and (B) corresponding summary plot of CD25+Foxp3+ Tregs. (C) Representative FACS staining and (D) corresponding summary plot of Ki67+CD4+ T cells. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing the mean of 2-3 technical replicates. *P > 0.05, **P < 0.01, One-way ANOVA with Dunnett's multiple comparisons test.

This approach resembles subimmunogenic TCR stimulation which has been shown to be most beneficial for *in vivo* Treg induction [89-91].

First, I performed titration experiments with increasing concentrations of IMU-935 and the respective vehicle control using naïve CD4$^+$ T cells isolated from LNs of non-autoimmune prone BALB/c mice. The highest increase of induced Tregs in response to IMU-935 was observed at a concentration of 1µM (**Figure 2 A, B**). This was accompanied by a reduced proliferation of T cells as assessed by Ki67 staining (**Figure 2 C, D**) which is in line with previous findings showing optimal Treg induction conditions in settings of limited cellular proliferation [89]. Thus, the following experiments were performed using a concentration of 1µM.

4.1.2 IMU-935 enhanced *in vitro* Treg induction during challenging conditions or islet autoimmunity

Next, the potential of IMU-935 to enhance Treg induction under conditions that mimic strong immune activation as observed during islet autoimmunity was assessed. Therefore, I established Treg induction assays under these so-called challenging conditions. Specifically, Tregs were induced either from activated T cells or from naïve CD4$^+$ T cells using limited TCR stimulation in presence of the pro-inflammatory cytokines IL-6, IFN-γ and IL-1β or using continuous TCR stimulation. In all three conditions, Treg induction was dramatically hindered and ranged from 0-5%. However, the addition of IMU-935 resulted in a significant improvement in Treg induction potential (**Figure 3 A-C**).

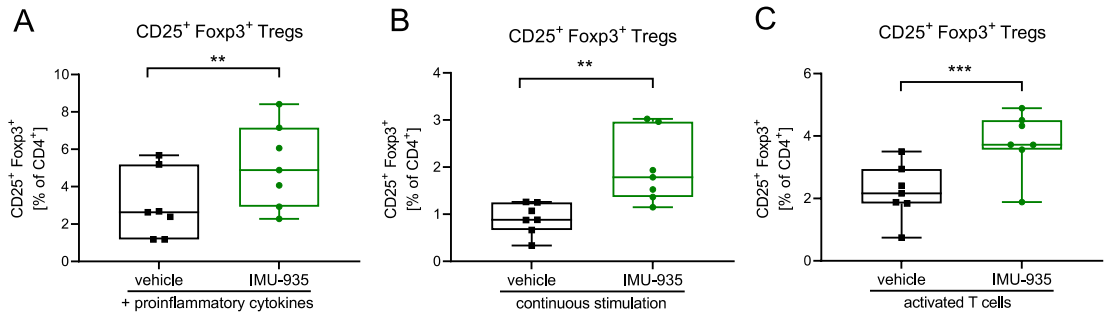

Figure 3: Addition of IMU-935 enhanced Treg induction in an immune-activating environment. *In vitro* Treg induction under conditions mimicking strong immune activation in presence of IMU-935 or the vehicle control using naïve CD4$^+$ T cells isolated from LNs of Foxp3 BALB/c reporter mice (A) in presence of 10ng/mL IL-6, IFN-γ and IL-1β and limited TCR stimulation or (B) using continuous TCR stimulation. (C) Tregs were induced from activated T cells FACS-sorted as CD4$^+$CD25$^-$CD44^{high}Foxp3$^-$ from LNs of Foxp3 BALB/c reporter mice using limited TCR stimulation. Experiments were performed in three technical replicates per mouse. Data are represented as box-and-whiskers plots with min and max values, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, Student's t-test.

In previous work, our group observed a profound impairment in Treg induction during murine and human islet autoimmunity [92, 96]. Therefore, I assessed the impact of IMU-935 on Treg induction capacity using naïve CD4$^+$ T cells from NOD mice at different stages of islet autoimmunity. Specifically, addition of IMU-935 significantly improved Treg induction using naïve CD4$^+$ T cells from BALB/c mice as well as from NOD mice without and with ongoing islet autoimmunity, as assessed by the presence of insulin au-toantibodies. In line with our previous findings, *in vitro* Treg induction of naïve CD4$^+$ T cells from IAA$^+$ NOD mice was impaired compared to non-autoimmune prone BALB/c mice (**Figure 4 A**). Of note, the effect of IMU-935 on Treg induction potential was greater using T cells from IAA$^+$ NOD mice compared to non-autoimmune prone BALB/c mice. This raised the frequency of induced Tregs from NOD mice to levels similar to what was observed for BALB/c mice (**Figure 4 A, B**).

Furthermore, Treg induction assays were performed using CD4$^+$ T cells from NOD mice younger than 30 days. Mice with an early onset of islet autoantibodies could face a more

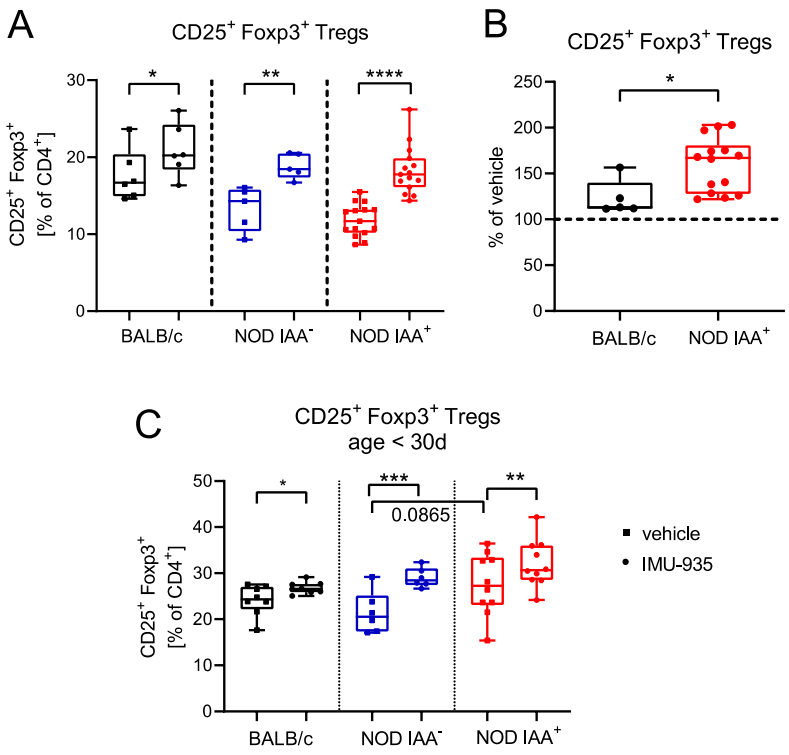

Figure 4: Improved Treg induction in presence of IMU-935 during ongoing islet autoimmunity. *In vitro* Treg induction assays in presence or absence of IMU-935 using limited TCR stimulation of naïve CD4$^+$ T cells isolated from pLN of BALB/c or NOD mice with different stages of islet autoimmunity (A) at the age of 80-100d, (B) Comparison of BALB/c or IAA$^+$ NOD mice represented as % of vehicle control from (A) and (C) at the age of below 30 days. Experiments were performed in three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student's t-test.

aggressive form of islet autoimmunity whereas those with no IAAs over a long period of time could be more protected. Supplementation of cultures with IMU-935 was able to increase frequencies of *in vitro* induced Tregs using naïve CD4$^+$ T cells from NOD mice with our without ongoing islet autoimmunity, whereas there was only a minor impact using naïve CD4$^+$ T cells from BALB/c mice. Interestingly, naïve CD4$^+$ T cells isolated from young NOD mice with an early development of insulin autoantibody show increased frequencies of induced Tregs (**Figure 4 C**).

Next, I assessed the effect of IMU-935 on *in vitro* Treg induction from naïve CD4$^+$ T cells isolated from NOD mice with ongoing islet autoimmunity under the aforementioned challenging conditions. Although, inducing Tregs is maximally hindered in these conditions, the presence of IMU-935 increased Treg induction (**Figure 5 A, B**).

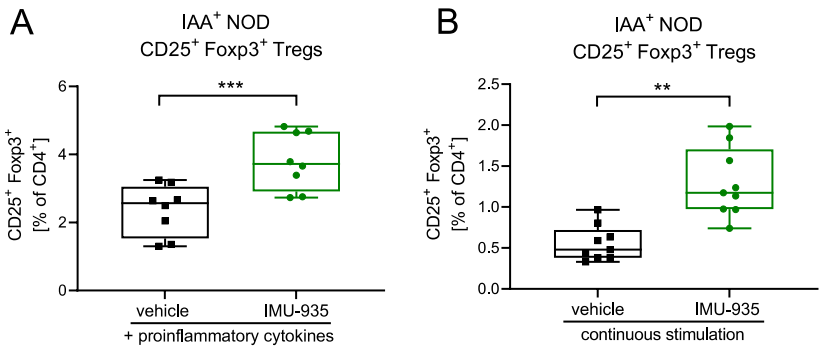

Figure 5: Increased Treg induction from IAA$^+$ NOD mice under challenging conditions. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from IAA$^+$ NOD mice under challenging conditions in presence of IMU-935 or the respective vehicle control (A) using limited TCR stimulation in presence of proinflammatory cytokines IL-6, IFN-γ and IL-1β or (B) by culturing under continuous TCR stimulation. Experiments were performed in three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, Student's t-test.

Overall, these data suggest that IMU-935 most efficiently enhances *in vitro* Treg induction under challenging conditions and islet autoimmunity. This supports our hypothesis, that under conditions of strong immune activation, that are known to be suboptimal for Treg induction, the inhibition of immune activation by IMU-935 can broaden the window of opportunity to induce Tregs.

4.2 RORγt expression is upregulated in T cells and Tregs in *in vitro* Treg induction upon addition of IMU-395

Tregs and T_H17 cells were observed to be reciprocally regulated during differentiation. In addition, upon Treg skewing conditions with TGF-β both RORγt upregulation in T cells as well as induced Tregs was observed [144, 239]. In addition, T_H17-like Tregs

express both master transcription factors, Foxp3 and RORγt [136-138]. This relationship together with the inverse agonistic properties of IMU-395 towards RORγt prompted me to exam-ine the expression of RORγt expression after Treg induction.

First, RORγt expression was evaluated in *in vitro* Treg induction using limited TCR stimulation of naïve CD4$^+$ T cells isolated from BALB/c and NOD mice with islet autoimmunity. For both, I observed an upregulation of RORγt in presence of IMU-935 in induced Tregs, as well as in CD4$^+$ T cells that did not express Foxp3 after Treg induction (**Figure 6 A-C**). In addition, I validated the RORγt expression after *in vitro* Treg induction using

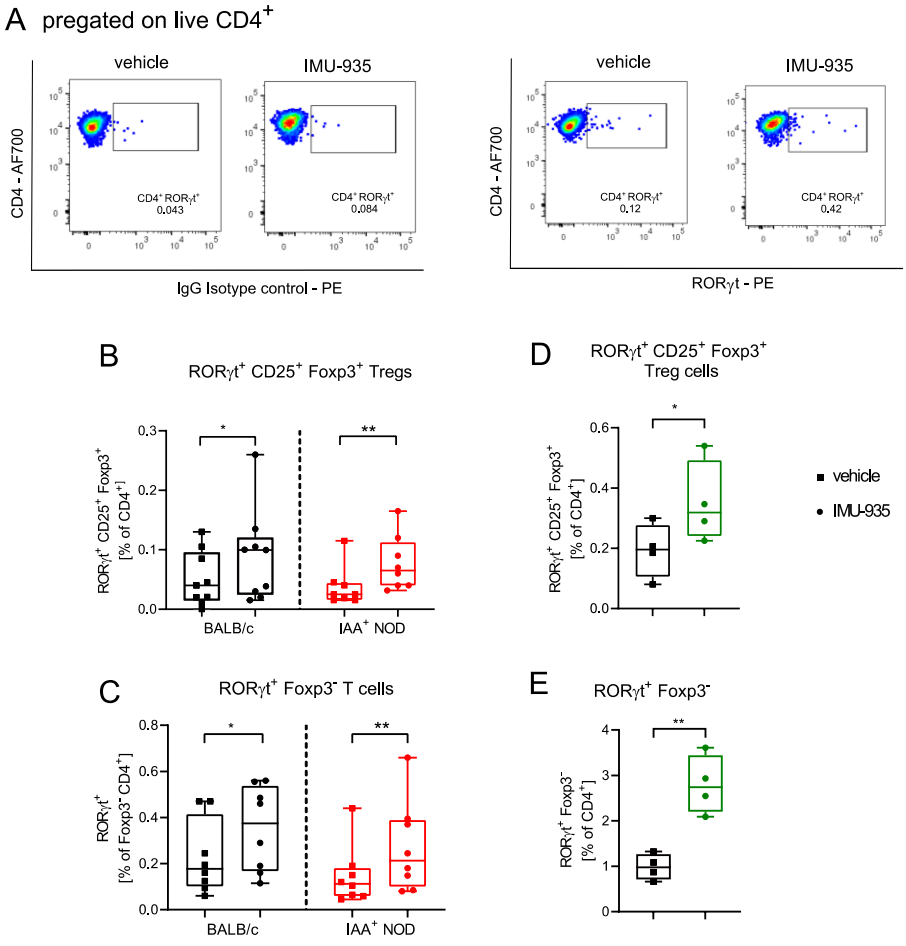

Figure 6: Increased RORγt expression in CD4$^+$ T cells and Tregs after *in vitro* Treg induction in presence of IMU-935. *In vitro* Treg induction in presence of IMU-935 or the respective vehicle control using limited TCR stimulation of naïve CD4$^+$ T cells isolated from LNs of BALB/c or IAA$^+$ NOD mice. (A) Staining examples for RORγt and the respective isotype control staining. Evaluation of RORγt expression in (B) Tregs or (C) Foxp3$^-$ T cells. *In vitro* Treg induction in presences of IMU-935 or the respective vehicle control using limited TCR stimulation of naïve T cells isolated from LNs of RORγt-GFP Foxp3-RFP double reporter mice. Evaluation of RORγt expression in (D) Tregs or (E) Foxp3$^-$ T cells. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, Student's t-test.

RORγt-GFP Foxp3-RFP double reporter mice, in which RORγt expression was assessed without intracellular staining. Likewise, addition of IMU-935 increased the frequency of RORγt$^+$ Tregs and RORγt$^+$ Foxp3$^-$ CD4$^+$ T cells (**Figure 6 D, E**).

Furthermore, I assessed RORγt expression in *in vitro* Treg induction in settings of strong immune activation. Similar to results obtained using optimal Treg inducing conditions, higher frequencies of RORγt$^+$ Treg and RORγt$^+$ Foxp3$^-$ CD4$^+$ T cells were observed in *in vitro* Treg induction in the presence of pro-inflammatory cytokines (**Figure 7 A, B**). For continuous stimulation experiments, RORγt$^+$ upregulation was quantified using its mean fluorescence intensity (MFI), which was normalized to the isotype control. RORγt$^+$ expression was likewise increased in Tregs and Foxp3$^-$ CD4$^+$ T cells in presence of IMU-935 (**Figure 7 C, D**). Noteworthy the upregulation of RORγt was higher in settings of continuous stimulation.

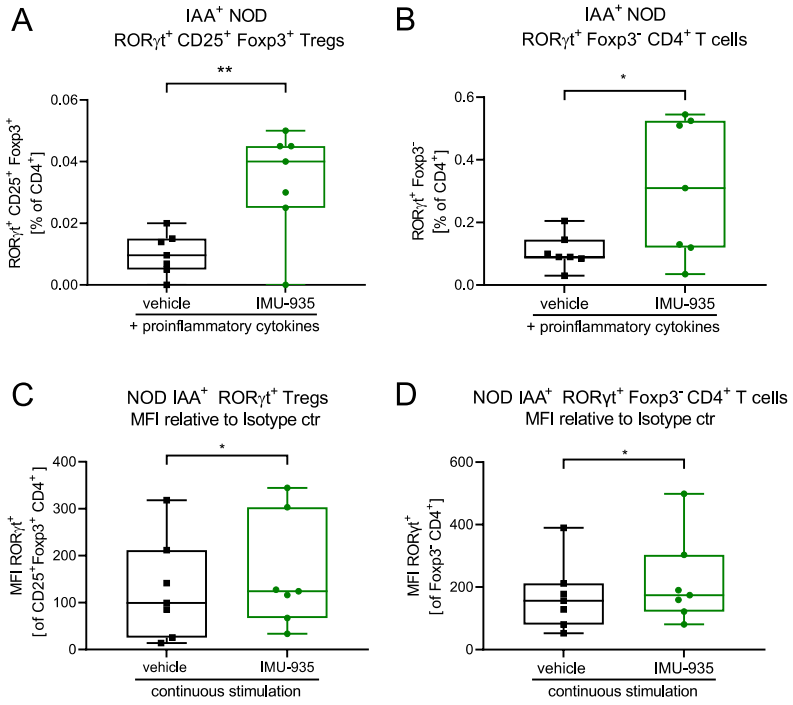

Figure 7: Upregulated RORγt expression in presence of IMU-935 in NOD Treg induction *in vitro* under challenging conditions. RORγt expression in *in vitro* Treg induction using naïve CD4$^+$ T cells isolated from IAA$^+$ NOD mice under the indicated challenging conditions in presence of IMU-935 or the respective vehicle control using limited TCR stimulation. Frequencies of RORγt$^+$ (A) CD25$^+$ Foxp3$^+$ Tregs or (B) Foxp3$^-$ CD4$^+$ T cells. Mean fluorescence intensity (MFI) of RORγt in (C) CD25$^+$ Foxp3$^+$ Tregs or (D) Foxp3$^-$ CD4$^+$ T cells, which was normalized to the isotype control. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, Student's t-test.

These experiments all together show that RORγt expression was increased in response to IMU-935 in *in vitro* Treg induction independent of the degree of activation the cells face during the induction.

4.3 Induced Tregs in presence of IMU-935 have an anti-inflammatory phenotype

RORγt$^+$ expression in Foxp3$^-$ CD4$^+$T cells is associated with the secretion of IL-17 and therefore effector function of T_H17 cells [134]. On the basis of studies showing the involvement of T_H17 cells in the pathogenesis of T1D, increased IL-17 production would be an unfavorable side effect. Therefore, I examined the production of IL-17A as well as the anti-inflammatory cytokine IL-10.

I did not observe any impact of IMU-935 on the production of IL-17A in Treg induction assays using naïve CD4$^+$ T cells from NOD mice with ongoing islet autoimmunity (**Figure 8 A**). In addition, IL-10 production was increased in *in vitro* induced Tregs as well as in Foxp3$^-$ CD4$^+$ T cells (**Figure 8 B, C**), which indicates anti-inflammatory properties.

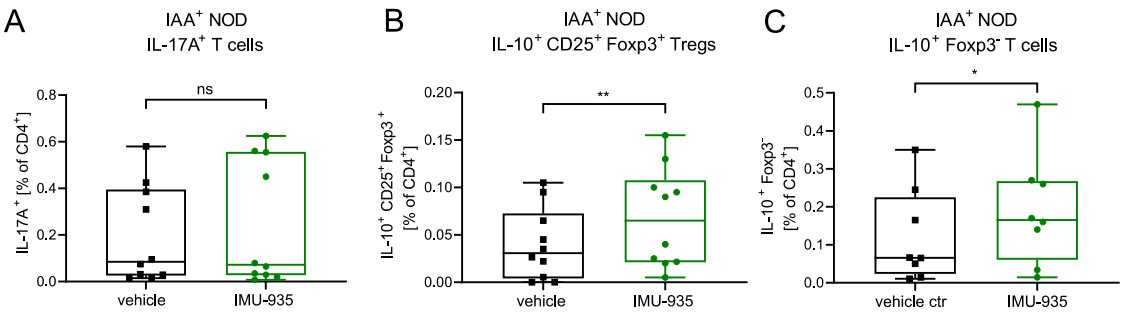

Figure 8: Anti-inflammatory phenotype of induced Tregs in presence of IMU-935. Cytokine production after *in vitro* Treg induction using limited TCR stimulation using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice. (A) IL17 production in CD4$^+$ T cells. IL10 expression (A) in CD25$^+$ Foxp3$^+$ Tregs or (B) in Foxp3$^-$ CD4$^+$ T cells. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, Student's t-test.

Similarly, addition of IMU-935 did not result in an increase in IL17A$^+$ CD4$^+$ T cells in Treg induction assays with supplemented pro-inflammatory cytokines, while IL-10 production was increased in Tregs as well as in Foxp3$^-$ CD4$^+$ T cells (**Figure 9 A-C**). Furthermore, *in vitro* Treg induction using continuous TCR stimulation in presence of IMU-935 tended to result in a decrease in IL17A$^+$ CD4$^+$ T cells compared to the vehicle control (**Figure 9 D**). In addition, IL-10 production was slightly increased in Tregs and significantly increased in Foxp3$^-$ CD4$^+$ T cells **(Figure 9 E, F**). Overall, these results suggest that Tregs

induced *in vitro* in presence of IMU-935 have an anti-inflammatory phenotype. Especially, the higher production of IL-10 in CD4$^+$ T cells that did not convert to Tregs point towards a high anti-inflammatory potential of IMU-935.

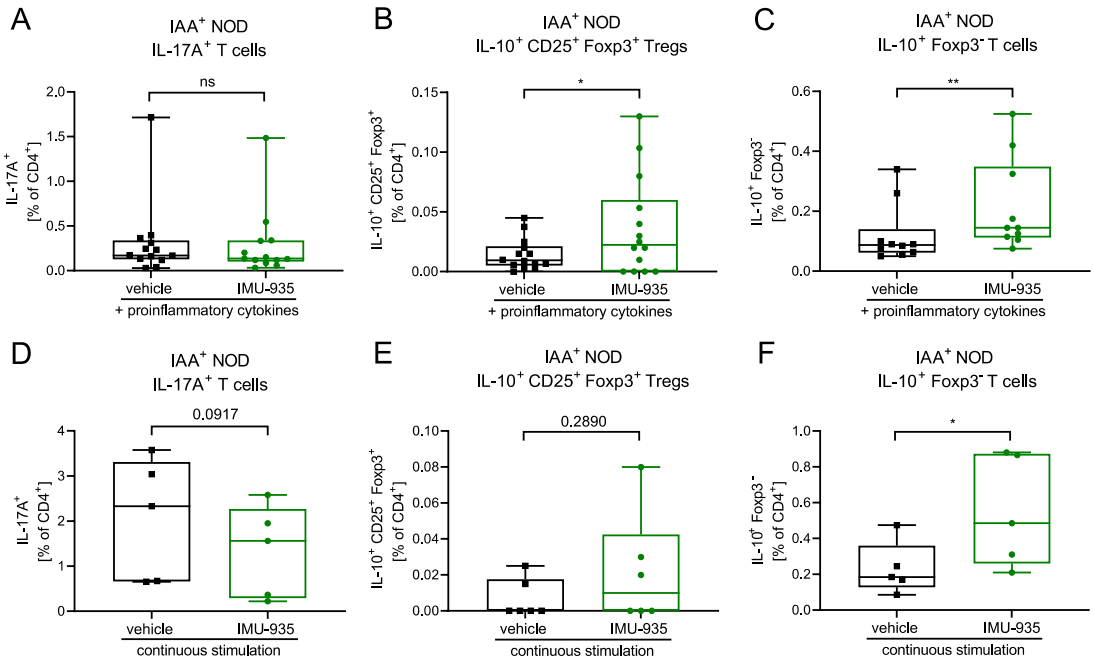

Figure 9: Increased IL-10 production in NOD Treg induction *in vitro* under immune-activating conditions upon addition of IMU-935. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice under challenging conditions in presence of IMU-935. (A) IL-17A secretion in CD4$^+$ T cells and (B) IL-10 production in Tregs and (C) Foxp3$^-$ CD4$^+$ T cells in assays with additional pro-inflammatory cytokines. (D) IL-17A secretion in CD4$^+$ T cells and (E) IL-10 production in Tregs and (F) Foxp3$^-$ CD4$^+$ T cells in assays using continuous TCR stimulation. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, Student's t-test.

4.4 IMU-838 is most capable at promoting Treg induction in settings of strong immune activation

The results so far showed an increase in *in vitro* induced Tregs with a higher production of anti-inflammatory IL-10 in presence of IMU-935, which is a RORγt inverse agonist and to a lesser extend also a DHODH inhibitor. This raised the question whether one of the two components of the drug candidate is responsible for the effect on Tregs or whether it is an interplay between them.

In order to address this issue I performed experiments with IMU-838 which only inhibits DHODH. While only a tendency to increased *in vitro* Treg induction capacity using naïve $CD4^+$ T cells from non-autoimmune prone BALB/c mice in presence of IMU-838 was observed, it was significantly enhanced when using naïve $CD4^+$ T cells from NOD mice with ongoing islet autoimmunity (**Figure 10 A**). This resulted in a trend towards a greater impact of IMU-838 on Treg induction potential using T cells from NOD mice compared to BALB/c mice (**Figure 10 B**). Similar to IMU-935, addition of IMU-838 reduced the $CD4^+$ T cell proliferation as assessed by Ki67 staining (**Figure 10 C**).

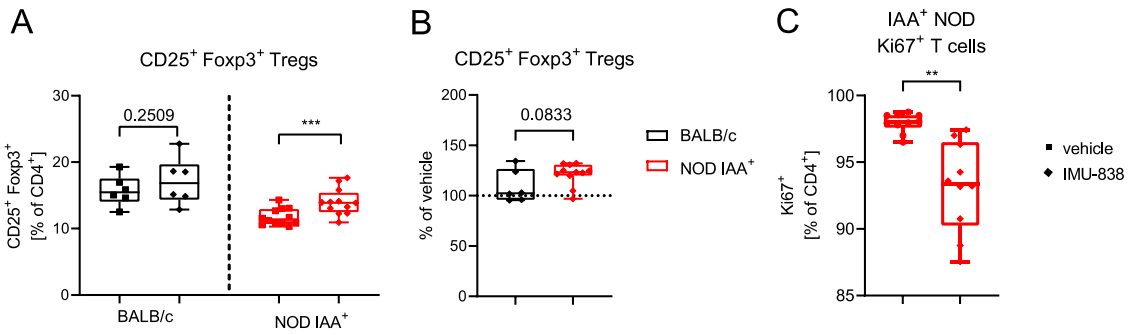

Figure 10: IMU-838 improves NOD Treg *in vitro*. *In vitro* Treg induction assays in presence or absence of IMU-838 using limited TCR stimulation of naïve $CD4^+$ T cells isolated from LNs of BALB/c or NOD mice with ongoing islet autoimmunity. Summary plots for frequencies of (A, B) induced Tregs and (C) proliferating $CD4^+$ T cell (Ki67). Experiments were performed in two to three technical replicates per subject. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, Student's t-test.

Next, I examined the influence of IMU-838 on *in vitro* Treg induction under challenging conditions that mimic autoimmune inflammation as outlined above. IMU-838 was able to improve Treg induction potential in all three conditions that hinder Treg induction (**Figure 11 A-C**). Noteworthy, the impact of IMU-838 on Treg induction using continuous TCR stimulation was significantly greater compared to IMU-935. Similar tendencies were observed in Treg induction experiments in presence of pro-inflammatory cytokines or when using activated $CD4^+$ T cells (**Figure 11 D-F**). The greater impact of IMU-838 in settings of stronger immune activation was likely due to the higher dependency of activated $CD4^+$

T cells on *de novo* pyrimidine synthesis and thus being a better target for DHODH inhibition.

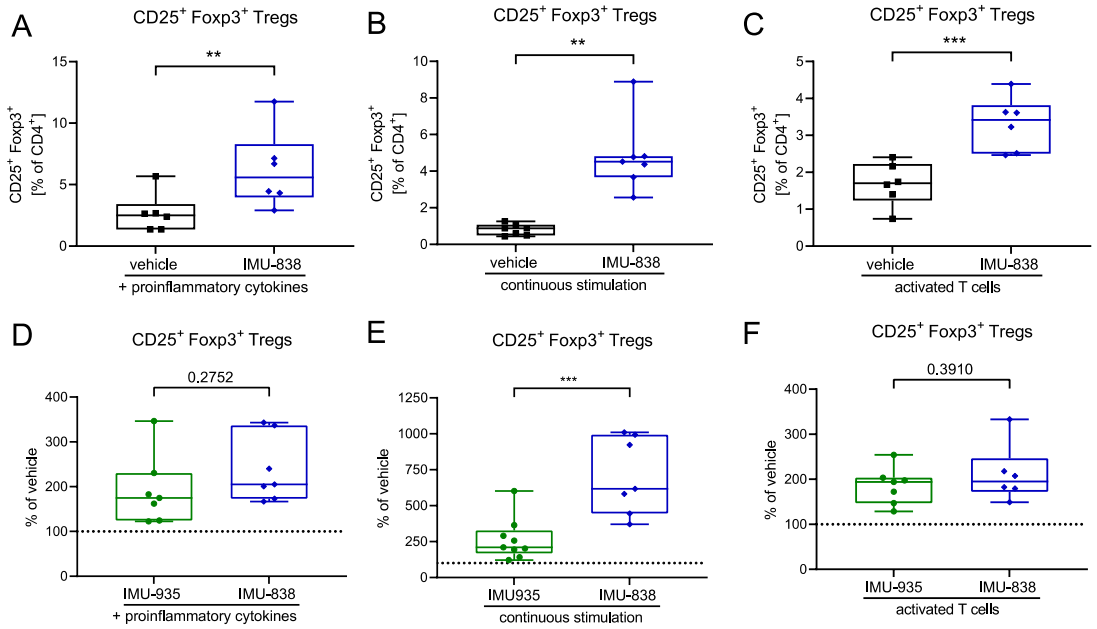

Figure 11: Addition of IMU-838 enhanced Treg induction in a pro-inflammatory environment. *In vitro* Treg induction under challenging conditions in presence of IMU-838 or the vehicle control from naïve CD4$^+$ T cells isolated from LNs from Foxp3 BALB/c reporter mice (A) in presence of 10ng/mL IL-6, IFN-γ and IL-1β and limited TCR stimulation or (B) using continuous TCR stimulation. (C) Induced Treg from activated CD4$^+$ T cells sorted as CD4$^+$CD25$^-$CD44^{high}Foxp3$^-$ LNs from Foxp3 BALB/c reporter mice using limited TCR stimulation. (D-F) Comparison between IMU-935 and -838 as % of vehicle in the indicated experimental setting. Experiments were performed in three technical replicates per mouse. Data are represented as box-and-whiskers plots with min and max values, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, Student's t-test.

In addition, I assessed the effect of IMU-838 on *in vitro* Treg induction from naïve CD4$^+$ T cells isolated from NOD mice with ongoing islet autoimmunity under the aforementioned strong activating conditions. Here, the conditions to induce Tregs *in vitro* are maximally unfavorable. Nonetheless, IMU838 improved *in vitro* Treg induction potential also in these challenging conditions (**Figure 12 A, B**). Overall, these experiments show that IMU-838 is capable of improving *in vitro* Treg induction under conditions of strong immune activation. Furthermore, these results suggests that IMU-838 might do so more efficiently than IMU-935 under conditions of continuous TCR stimulation. In addition, preliminary data suggest also a stronger impact of IMU-838 on Treg induction in presence of pro-inflammatory cytokines or when using activated CD4$^+$ T cells.

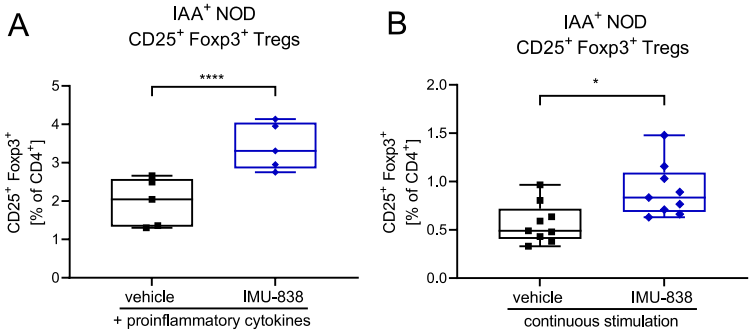

Figure 12: IMU-838 improves NOD Treg induction under immune-activating conditions *in vitro.* *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from IAA$^+$ NOD mice under immune-activating conditions in presence of IMU-838 or the respective vehicle control (A) using limited TCR stimulation in presence of pro-inflammatory cytokines IL-6, IFN-γ and IL-1β or (B) by culturing under continuous TCR stimulation. Experiments were performed in three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, ****P < 0.0001, Student's t-test.

4.5 Presence of IMU-838 increases RORγt expression in CD4$^+$ T cells and Tregs and induces an anti-inflammatory phenotype of Tregs *in vitro*

Next, I analyzed RORγt expression after *in vitro* Treg induction in order to assess whether DHODH inhibition alone was also able to increase RORγt expression in CD4$^+$ T cells and Tregs. Frequencies of RORγt$^+$ Tregs as well as RORγt$^+$ Foxp3$^-$ CD4$^+$ T cells was elevated in presence of IMU-838 in *in vitro* Treg induction using limited TCR stimulation and CD4$^+$ T cells from either non-autoimmune prone BALB/c or IAA$^+$ NOD mice. Noteworthy, IMU-838 had only a minimal effect on *in vitro* induced Tregs using CD4$^+$ T cells from non-autoimmune prone BALB/c mice, however RORγt expression was still increased (**Figure 13 A, B**).The increased RORγt expression in Tregs and Foxp3$^-$ CD4$^+$ T cells in *in vitro* Treg induction was confirmed using CD4$^+$ T cells from RORγt-GFP Foxp3-RFP double reporter mice (**Figure 13 C, D**). In addition, in *in vitro* Treg induction in presence of pro-inflammatory cytokines IMU-838 likewise increased RORγt$^+$ Tregs and RORγt$^+$ Foxp3$^-$ CD4$^+$ T cells (**Figure 14 A, B**). A slight enhancement in RORγt expression in Tregs and Foxp3$^-$ CD4$^+$ T cells was observed in response to IMU-838 as assessed by the MFI which was normalized to the isotype control. (**Figure 14 C, D**)

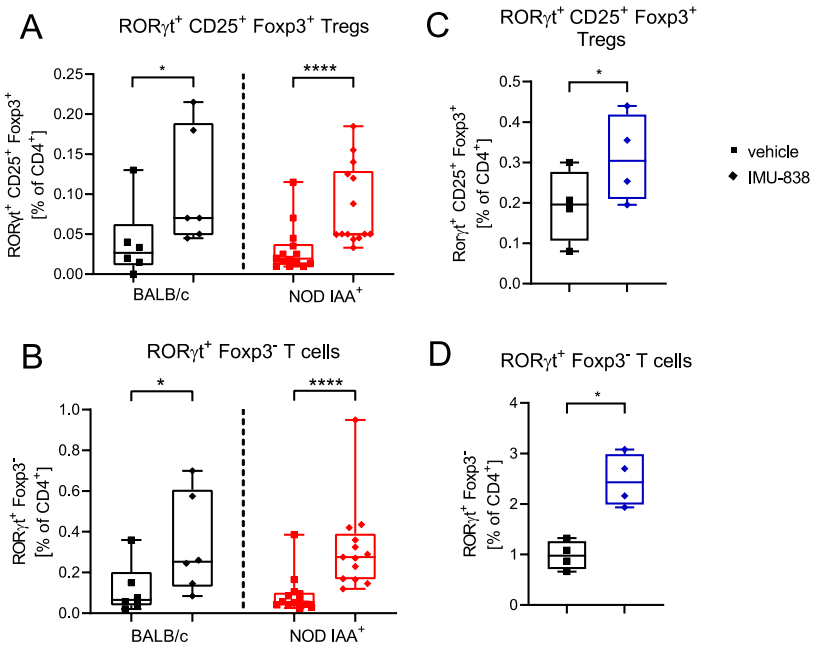

Figure 13: Increased RORγt expression in CD4$^+$ T cells and Tregs after *in vitro* Treg induction in presence of IMU-838. *In vitro* Treg induction in presence of IMU-838 or the respective vehicle control using limited TCR stimulation of naïve CD4$^+$ T cells isolated from LNs of BALB/c or IAA$^+$ NOD mice. Evaluation of RORγt expression on (A) Tregs or (B) Foxp3$^-$ CD4$^+$ T cells. *In vitro* Treg induction in presence of IMU-838 or the respective vehicle control using limited TCR stimulation of naïve CD4$^+$ T cells isolated from LNs of RORγt-GFP Foxp3-RFP double reporter mice. Evaluation of RORγt expression on (C) Tregs or (D) Foxp3$^-$ CD4$^+$ T cells. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, ****P < 0.0001, Student's t-test.

These results show that RORγt expression was increased upon *in vitro* Treg induction assays in presence of IMU-838 again raising the question about a potential pro-inflammatory phenotype. Therefore, I assessed the cytokine production by intracellular staining of IL-17A and IL-10 after Treg induction under settings of strong immune activation since IMU-838 is most potent under these conditions. There was no effect on IL-17A production, but I observed increased IL-10 production in Tregs as well as Foxp3$^-$ CD4$^+$ T cells in presence of IMU-838 in Treg induction assays in presence of pro-inflammatory cytokines (**Figure 15 A-C**). Similar to IMU-935, there was a trend towards lower expression of IL-17A in Treg induction assays performed using continuous TCR stimulation which coincided with an increased IL-10 production in Tregs as well as Foxp3$^-$ CD4$^+$ T cells in presence of IMU-838 (**Figure 15 D-E**).

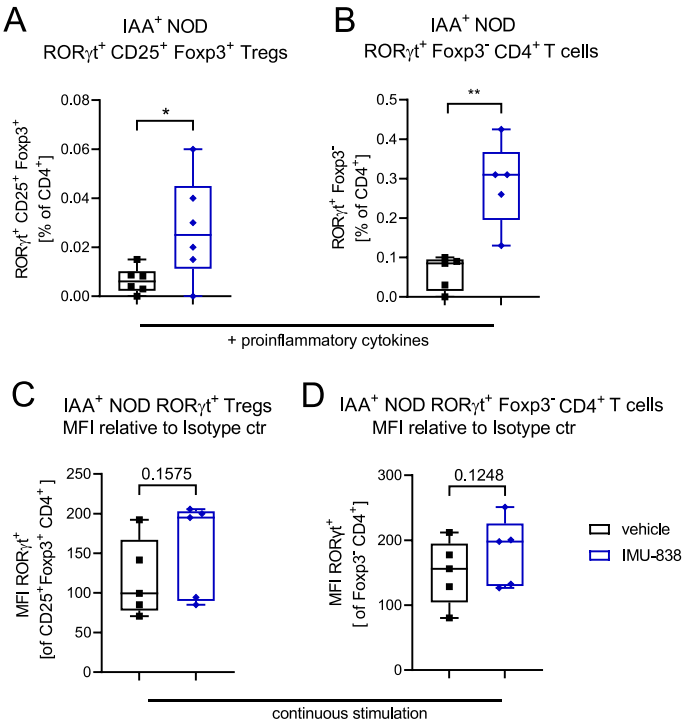

Figure 14: IMU-838 enhances RORγt expression upon *in vitro* NOD Treg induction under immune-activating conditions. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from IAA$^+$ NOD mice under the indicated immune-activating conditions in presence of IMU-838 or the respective vehicle control. RORγt expression (A, C) in Tregs and (B, D) in Foxp3$^-$ CD4$^+$ T cells. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, Student's t-test.

Overall, these data suggest that induced Tregs in presence of IMU-838, despite the up-regulation of RORγt, have an enhanced anti-inflammatory phenotype. Furthermore, the increased IL-10 production also in Foxp3$^-$ CD4$^+$ T cells underlines the anti-inflammatory potential. Importantly, all in all these data are similar to results obtained with IMU-935.

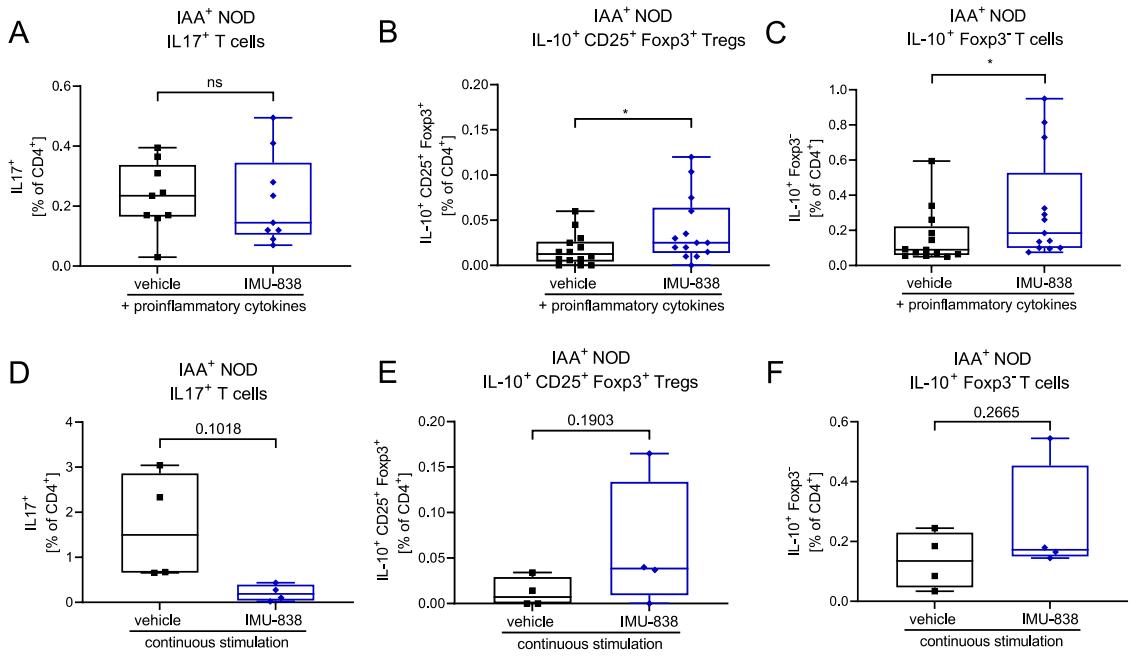

Figure 15: Increased IL-10 production in presence of IMU-838 in NOD Treg induction under immune-activating conditions *in vitro*. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice under immune-activating conditions in presence of IMU-838. (A) IL-17A production in CD4$^+$ T cells and (B) IL-10 production in Tregs and (C) Foxp3$^-$ CD4$^+$ T cells in assays with additional pro-inflammatory cytokines. (D) IL-17A production in CD4$^+$ T cells and (B) IL-10 production in Tregs and (C) Foxp3$^-$ CD4$^+$ T cells in assays using continuous TCR stimulation. Experiments were performed in two technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, Student's t-test.

4.6 Effects of IMU-838 and -935 are dependent on DHODH inhibition

Noteworthy, data obtained from *in vitro* Treg induction assays in presence of either IMU-935 or IMU-838 resembled each other in terms of upregulation of Foxp3, RORγt and IL-10. Both drug candidates share the property of DHODH inhibition, but only IMU-935 is additionally a RORγt inverse agonist. This raised the question, whether RORγt inverse agonistic properties are necessary for the impact of IMU-935 on Treg induction and RORγt upregulation in T cells and Tregs. Therefore, I performed Treg induction assays with IMU-935 in presence of increasing concentrations of uridine. Addition of uridine circumvents DHODH inhibition by supplementing an exogenous source for the pyrimidine synthesis, turning IMU-935 into a RORγt inverse agonist.

At a high concentration of uridine and in presence of IMU-935 the frequencies of induced Tregs decreased compared to IMU-935 alone, eliminating the positive effect on Treg induction of IMU-935 (**Figure 16 A**). In accordance with restored pyrimidine biosynthesis upon exogenous uridine supplementation and therefore fully functional proliferative capacity, Ki67 expression was restored with addition of uridine (**Figure 16 B**). This highlights the reciprocal relationship between cellular proliferation and optimal Treg induction. In addition, the increase in RORγt$^+$ Tregs in response to IMU-935 is abrogated at high concentrations of uridine (**Figure 16 C**).

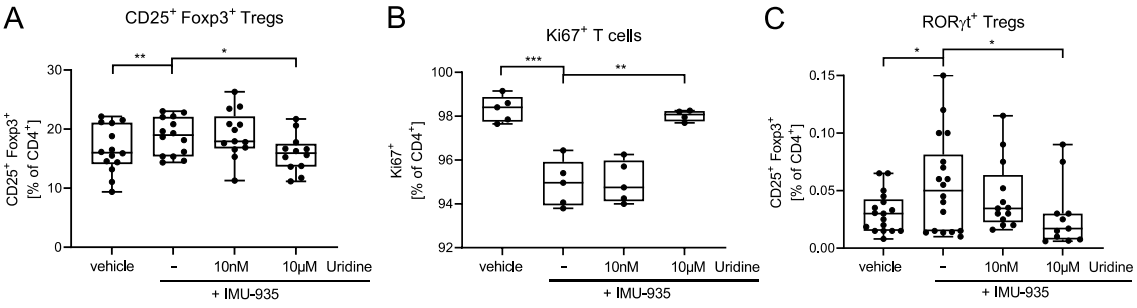

Figure 16: Uridine supplementation eliminates the effect of IMU-935 on NOD Treg induction. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice in presence of IMU-935 with increasing concentrations of uridine using limited TCR stimulation. Frequencies of (A) CD25$^+$ Foxp3$^+$ Tregs (B) Ki67$^+$ CD4$^+$ T cells (C) RORγt$^+$ Tregs. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, ***P < 0.001, One-way ANOVA with Tukey's multiple comparisons test.

In line with these results, uridine supplementation in Treg induction assays in presence of pro-inflammatory cytokines reduced the impact of IMU-935 on Treg induction potential and RORγt expression in CD4$^+$ T cells and Tregs (**Figure 17 A-C**).

The eliminated effect of IMU-935 in presence of excess uridine suggests that DHODH inhibition is the major driver in enhancing *in vitro* Treg induction as well as RORγt expression. These data support the concept that the RORγt inverse agonist contributes only little to the effect on Treg frequencies as well as RORγt expression in T cells and Tregs in *in vitro* Treg induction.

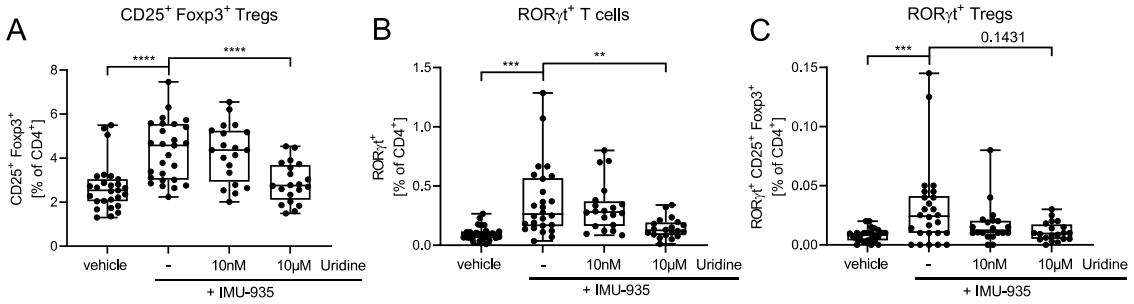

Figure 17: Uridine supplementation eliminates the effect of IMU-935 in NOD Treg induction under immune-activating conditions. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice in presence of IMU-935 with increasing concentrations of uridine using limited TCR stimulation and of pro-inflammatory cytokines. Frequencies of (A) CD25$^+$ Foxp3$^+$ Tregs (B) RORγt$^+$ CD4$^+$ T cells (C) RORγt$^+$ Tregs. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, ****P < 0.0001, One-way ANOVA with Tukey's multiple comparisons test.

As proof of concept, I also tested the impact of excess of uridine on *in vitro* Treg induction under challenging condition in presence of IMU-838. Similarly, the increase in induced Tregs as well as RORγt$^+$ CD4$^+$ T cells is diminished at higher concentrations (**Figure 18 A, B**). Furthermore, although not yet significant, there is a trend towards impaired potential of IMU-838 to increase RORγt$^+$ Tregs upon restored pyrimidine biosynthesis (**Figure 18 C**). Together, these data suggest that DHODH inhibition enhances *in vitro* Treg induction and RORγt$^+$ T cells and Tregs which can be eliminated by restored pyrimidine biosynthesis.

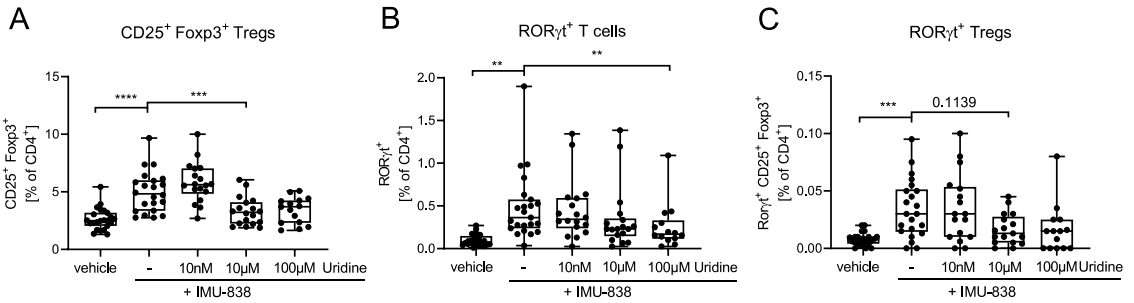

Figure 18: Uridine supplementation eliminates effect of IMU-838 in NOD Treg induction under immune-activating conditions. *In vitro* Treg induction using naïve CD4$^+$ T cells isolated from LNs of IAA$^+$ NOD mice in presence of IMU-838 with increasing concentrations of uridine using limited TCR stimulation and pro-inflammatory cytokines. Frequencies of (A) CD25$^+$ Foxp3$^+$ Tregs (B) RORγt$^+$ CD4$^+$ T cells (C) RORγt$^+$ Tregs. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, ***P < 0.001, ****P < 0.0001, One-way ANOVA. ANOVA with Tukey's multiple comparisons test.

4.7 RORγt upregulation in CD4⁺ T cells and Tregs *in vitro* is a common phenomenon of DHODH inhibitors

My results indicate that in *in vitro* Treg induction DHODH inhibition results in an increased RORγt expression in Tregs as well as in Foxp3⁻ CD4⁺ T cells. This raised the question whether the impact on RORγt expression is a specific characteristic of the next-generation DHODH inhibitor, IMU-838. Therefore, I compared IMU-838 and Teriflunomide (Tef), a commonly used DHODH inhibitor, in *in vitro* Treg induction assays under immune-activating conditions using CD4⁺ T cells from IAA⁺ NOD mice. Similar to IMU-838, Teriflunomide was able to improve Treg induction in presence of pro-inflammatory cytokines *in vitro* (**Figure 19 A**). Furthermore, IMU-838 potential to increase Treg induction capacity was significantly greater compared to Teriflunomide. In addition, presence of Teriflunomide likewise enhanced RORγt expression in Tregs and Foxp3⁻ CD4⁺ T cells, but with a tendency towards a lower potential than IMU-838 (**Figure 19 B, C**). Overall, these results suggests that IMU-838 is more potent in improving Treg induction *in vitro* and in increasing RORγt expression in Tregs and CD4⁺ T cells compared to Tef.

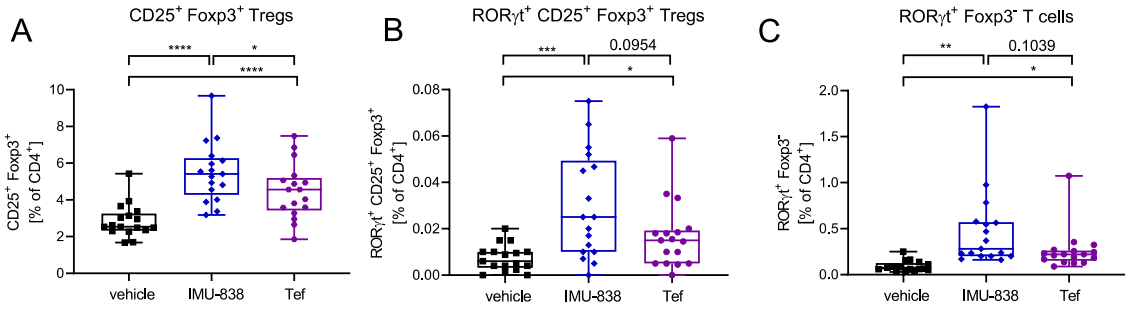

Figure 19: Teriflunomide increases Treg frequencies and RORγt expression in *in vitro* Treg induction under immune-activating conditions. *In vitro* Treg induction using naïve CD4⁺ T cells isolated from IAA⁺ NOD mice and limited TCR stimulation in presence of pro-inflammatory cytokines IL-6, IFN-γ and IL-1β and IMU-838 (50µM), Teriflunomide (7.5µM) or the vehicle control. Summary plots for (A) induced Tregs, (B) RORγt⁺ Tregs and (C) RORγt⁺ Foxp3⁻ CD4⁺ T cells. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, One-way ANOVA with Tukey's multiple comparisons test.

4.8 IL6Rα signaling influences RORγt expression in T cells and Tregs *in vitro* in presence of IMU-935 and IMU-838

Since increased RORγt expression in *in vitro* Treg induction was observed in presence of IMU-935 and -838 in optimal and suboptimal conditions to induce Tregs, I next sought to identify the mechanism involved in increased RORγt expression in response to the drug candidates. Many studies report that IL-6 signaling is important for RORγt expression in $CD4^+$ T cells and in Tregs. First of all, IL-6 is used for optimal T_H17 induction *in vitro* [140, 141]. Secondly, mice with a global IL-6 KO have lower frequencies of T_H17-like Tregs in the colon [137]. Therefore, I assessed the impact of IL-6 signaling on RORγt expression in *in vitro* Treg induction in presence of IMU-935 and -838.

I made use of mice harboring a T cell specific knockout (TKO) of the IL6Rα which was generated by crossing $CD4^{Cre}$ and $IL6R\alpha^{fl/fl}$ mice [237, 240, 241]. While the classical signaling pathway is abrogated in these cells, they can still be responsive to IL-6 via trans-signaling or trans-presentation [149, 237, 240, 241]. Naïve $CD4^+$ T cells from $IL6R\alpha^{TKO}$ of floxed control mice were isolated from LNs and stimulated under subimmunogenic con-ditions to induce Tregs. The Treg induction potential using $CD4^+$ T cells with an IL6Rα KO was higher compared to $CD4^+$ T cells from floxed control mice. Presence of IMU-935 increased in vitro Treg induction using either IL6Rα deficient of sufficient T cells (**Figure 20 A**). However, the improvement was significantly lower using IL6Rα deficient $CD4^+$ T cells compared to control $CD4^+$ T cells (**Figure 20 B**). Furthermore, RORγt expression in $CD4^+$ T cells or Tregs was enhanced or showed a trend towards it upon addition of IMU-935. Importantly, the IMU-935-mediated increase in $ROR\gamma t^+$ $CD4^+$ T cells and Tregs relative to the vehicle control was reduced in absence of the IL6Rα (**Figure 20 C-F**). These results indicate that the IL6Rα and therefore IL-6 signaling in $CD4^+$ T cells is im-portant for the upregulation of RORγt as well as the improved Treg induction in response to IMU-935 in *in vitro* Treg induction assays under subimmunogenic conditions.

In addition, the influence of IL6Rα signaling for the IMU-935-mediated upregulation of Treg induction and RORγt expression in a setting of strong immune activation was ana-lyzed. Here, I focused on *in vitro* Treg induction using continuous TCR stimulation, since the IL6Rα deficient $CD4^+$ T cells cannot properly react to IL-6. *In vitro* Treg induction using continuous TCR stimulation was improved in presence of IMU-935, independently of a functional IL6Rα signaling (**Figure 21 A**). But, the improvement of Treg induction potential in presence of IMU-935 relative to the vehicle was slightly better in absence of the IL6Rα (**Figure 21 B**). Furthermore, IMU-935 increased RORγt expression in $CD4^+$ T cells but it did so to a similar extend when comparing IL6Rα sufficient and deficient $CD4^+$ T cells (**Figure 21 C**). It likewise increased RORγt expression in Tregs and preliminary studies suggest a trend towards higher RORγt expression in Tregs in the absence of the IL6Rα and presence of IMU-935 as represented as fold change over the vehicle control in **Figure 21 D**.

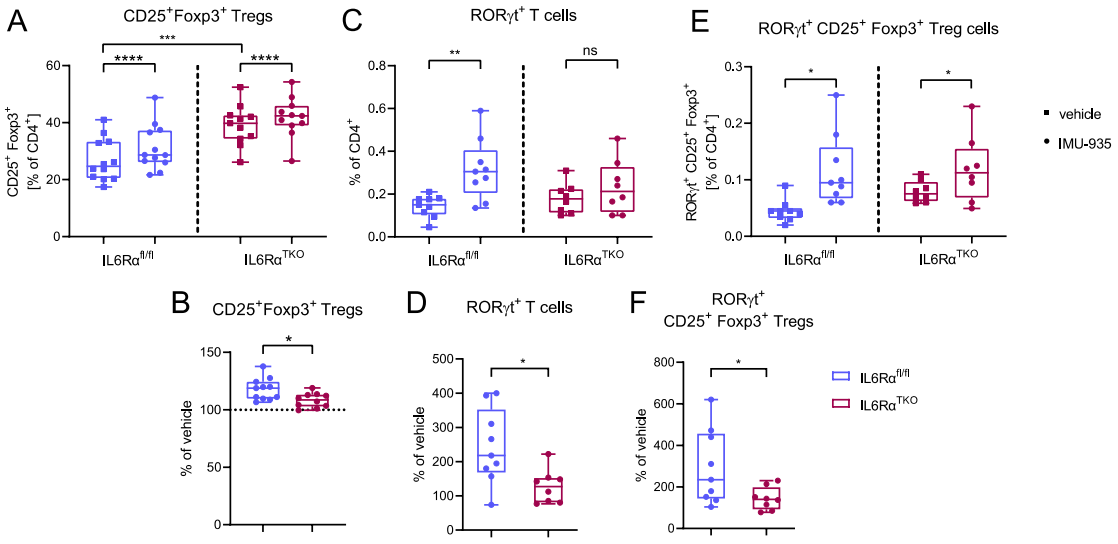

Figure 20: Reduced potential of IMU-935 to induce Tregs and increase RORγt expression in CD4⁺ T cells and Tregs in absence of the IL6Rα. *In vitro* Treg induction using naïve CD4⁺ T cells isolated from LNs of IL6Rα^{TKO} or floxed control mice in presence of IMU-935. (A) Induced CD25⁺Foxp3⁺ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt expression in CD4⁺ T cells and (D) normalized to vehicle control and represented as % of vehicle control. (E) RORγt expression in Treg and (F) normalized to vehicle control and represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001, Student's t-test.

Furthermore, loss of the classical IL-6 signaling did not completely resulted in the loss of RORγt expression in in vitro Treg induction which might indicates that other signaling mechanism are also involved in RORγt expression.

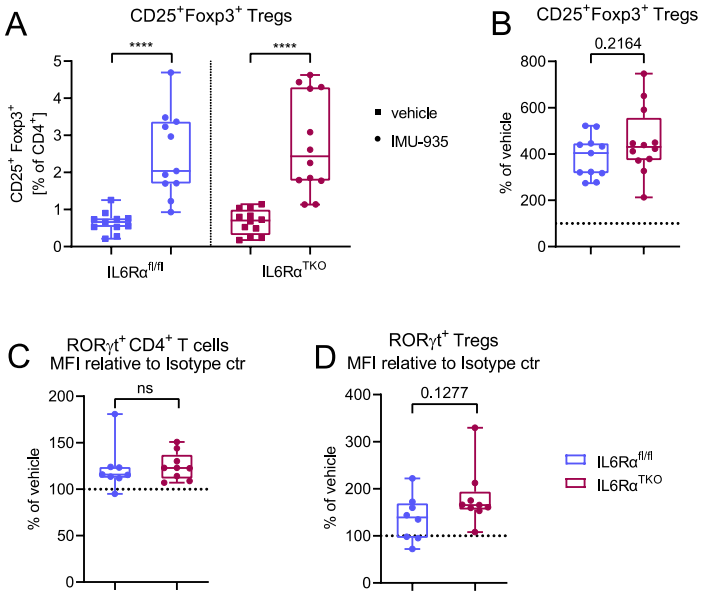

Figure 21: IMU-935 increased Treg induction potential and RORγt expression under challenging conditions independent of IL6Rα signaling. *In vitro* Treg induction using continuous TCR stimulation and naïve CD4$^+$ T cells isolated from LNs of IL6Rα^{TKO} or floxed control mice in presence of IMU-935. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt MFI of CD4$^+$ T cells and (D) RORγt MFI of Tregs, normalized to isotype control and vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. ****P < 0.0001, Student's t-test.

In addition, I also examined IMU-838-mediated increase in *in vitro* Treg induction and RORγt expression in absence of the IL6Rα. As observed before, in settings of limited TCR stimulation IMU-838 slightly increases Treg induction capacity from floxed control T cells or IL6Rα deficient CD4$^+$ T cells, respectively (**Figure 22 A, B**). RORγt expression in IL6Rα deficient and sufficient CD4$^+$ T cells and Tregs cells was increased in presence of IMU-838 compared to the vehicle control, which is shown by values above 100% in normalized expression to the vehicle control in **Figure 22 C, D**. Furthermore, a tendency towards a loss of IMU-838-mediated RORγt-increasing potential in the absence of IL6Rα was observed in these preliminary studies. This is in line with results of IMU-935 leading to the hypothesis that IL6Rα signaling is important for the increased Treg induction capacity and RORγt expression in response to the drug candidates using limited Treg induction assays. This, however, will have to be confirmed in additional studies.

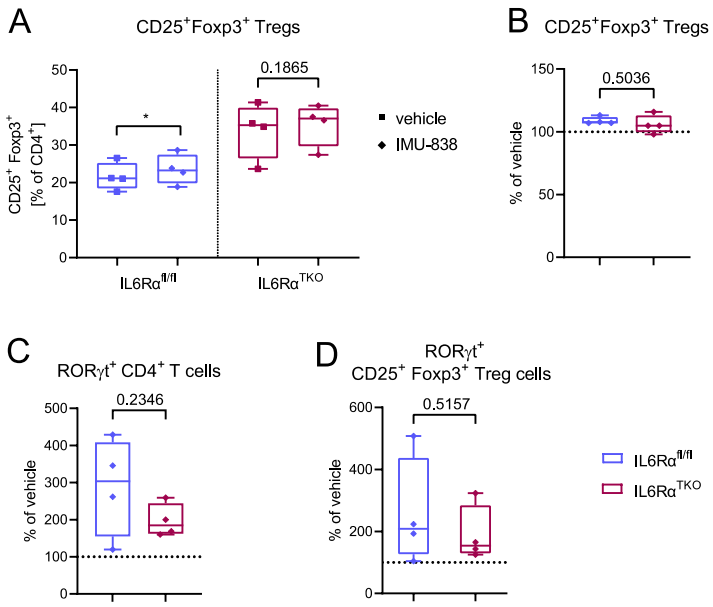

Figure 22: Tendency towards reduced potential of IMU-838 to induce Tregs and increase RORγt expression in CD4⁺ T cells and Tregs in absence of the IL6Rα in *in vitro* Treg induction. *In vitro* Treg induction using limited TCR stimulation and naïve CD4⁺ T cells isolated from LNs of IL6Rα^{TKO} or floxed control mice in presence of IMU-838. (A) Induced CD25⁺Foxp3⁺ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt expression in CD4⁺ T cells and (D) RORγt⁺ Tregs, frequencies normalized to vehicle control, represented as % of vehicle control. Experiments were per-formed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, Student's t-test.

Furthermore, in Treg induction assays using continuous TCR stimulation the absence of the IL6Rα tended to improve the IMU-838-mediated increase of Treg induction potential compared to the floxed control, which is in line with results using IMU-935 (**Figure 23 A, B**). RORγt expression in Tregs but not in CD4⁺ T cells was increased in presence of IMU-838 without an impact of the absence of IL6Rα (**Figure 23 C, D**), which stands in contrast to results observed with IMU-935.

These findings might suggest that different signaling mechanisms may be important for the increase in RORγt expression in T cells and Tregs in response to IMU-935 and -838 in Treg induction assays using continuous TCR stimulation. However, further studies and models will need to be validated to gain a full picture of these initial results which will be important to dissect the different signaling mechanisms involved in the IMU-mediated increase in RORγt expression.

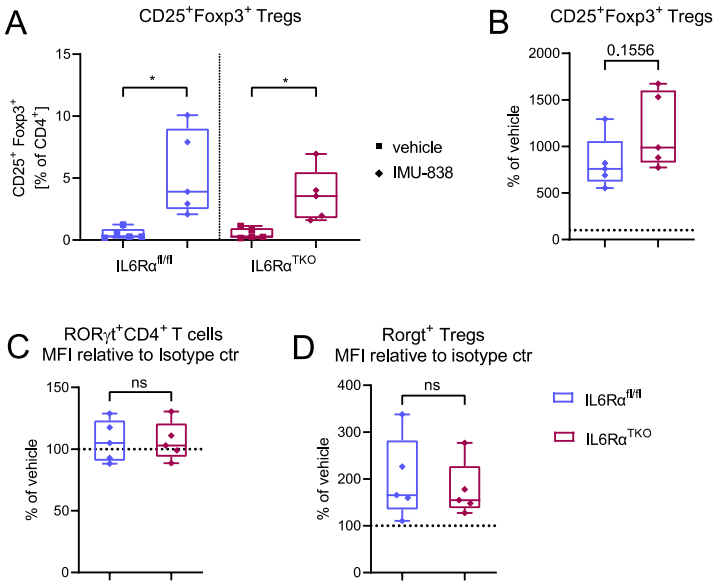

Figure 23: IMU-838 increases the Treg induction potential and RORγt expression in Tregs under immune-activating conditions independent of IL6Rα signaling. *In vitro* Treg induction using continuous TCR stimulation and naïve CD4$^+$ T cells isolated from LNs of IL6Rα^{TKO} or floxed control mice in presence of IMU-838. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt MFI of CD4$^+$ T cells and (D) RORγt MFI of Tregs, normalized to isotype control and vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, Student's t-test.

In the data shown above, IL6Rα signaling is absent from the beginning of Treg induction based on the fact that IL6Ra is lacking in all T cells. Two different scenarios could therefore mediate the impact of the drug candidates on Treg induction and RORγt expression in the absence of the IL6Rα compared to functional IL-6 signaling. On the one hand, IL6Rα signaling could be important in early phases of Treg induction and the initial upregulation of Foxp3 and RORγt in CD4$^+$ T cells. On the other hand, it might be also important at later stages when Foxp3 expression is already initiated, e.g. mediating the reciprocal relationship between Foxp3 and RORγt. In order to elucidate that, I performed Treg induction assays using Foxp3 IL6Rα knockout (TregKO) mice. Here, knockout of IL6Rα is only initiated once Foxp3 is expressed. Therefore, in the early phase of Treg induction IL6Rα is still functional.

At first, the impact of IMU-935 on Treg induction using limited TCR stimulation and IL6Rα^{TregKO} mice was investigated. Presence of IMU-935 improved Treg induction independently of absent IL6Rα signaling in Tregs (**Figure 24 A**). The fold increase normalized to the vehicle control did not change indicating that the TregKO of the IL6Rα does

not have an impact on the effect of IMU-395 on Treg induction (**Figure 24 B**). Furthermore, an increase in RORγt$^+$ T cells and RORγt$^+$ CD4$^+$ Tregs was observed in response to IMU-935 as indicated by values above 100% when normalizing the expression to the vehicle control (**Figure 24 C, D**). Preliminary data suggest a trend towards a better improvement in the IMU-935-mediated increase of RORγt$^+$ CD4$^+$ T cells using CD4$^+$ T cells from IL6Rα^{TregKO} mice. In addition, the effect of IMU-935 on RORγt$^+$ Tregs tended to be greater when the IL6Rα signaling was absent in Tregs (**Figure 24 C, D**).

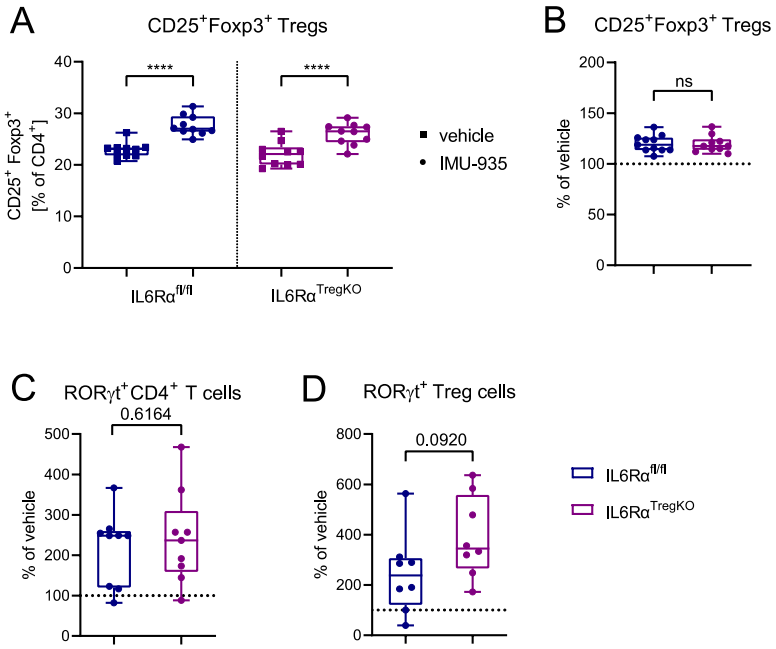

Figure 24: Foxp3-specific deletion of IL6Rα does not impact the IMU-935-mediated increase in Treg induction potential. *In vitro* Treg induction using limited TCR stimulation and CD4$^+$ naïve T cells isolated from LNs of IL6Rα^{TregKO} or floxed control mice in presence of IMU-935. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt expression in CD4$^+$ T cells and (D) RORγt$^+$ Tregs, normalized to vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. ****P < 0.0001, Student's t-test.

Next, I performed Treg induction assays under immune-activating conditions using continuous TCR stimulation. IMU-935 improved Treg induction to a greater extend using CD4$^+$ T cells from IL6Rα^{TregKO} mice compared to floxed controls (**Figure 25 A, B**). Furthermore, RORγt expression was increased in presence of IMU-935 with a trend towards lower potential of IMU-935 to increase RORγt expression in CD4$^+$ T cells in the absence of IL6Rα signaling in Tregs, while no differences were observed on the Treg level (**Figure 25 C, D**).

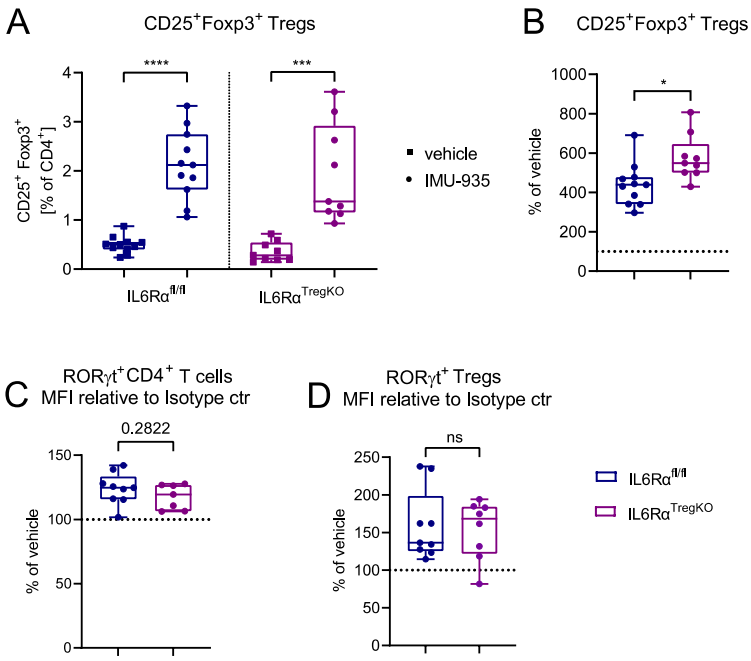

Figure 25: Presence of IMU-935 improved Treg induction using continuous TCR stimulation in Foxp3-specific absence of the IL6Rα. *In vitro* Treg induction using continuous TCR stimulation and naïve CD4$^+$ T cells isolated from LNs of IL6Rα^{TregKO} or floxed control mice in presence of IMU-935. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt MFI of CD4$^+$ T cells and (D) RORγt MFI of Tregs, normalized to isotype control and vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. *P < 0.05, ***P < 0.001, ****P < 0.0001, Student's t-test.

In addition, I investigated the impact on IMU-838 on Treg induction using limited TCR stimulation and IL6Rα^{TregKO} mice. IMU-838 increased Treg induction using both floxed control and IL6Rα^{TregKO} mice, without any differences in efficiency (**Figure 26 A, B**). This is in line observed with results using IMU-935. In addition, RORγt expression in CD4$^+$ T cells and Tregs was increased in response to IMU-838, here represented as frequency normalized to the vehicle control. In contrast to data with IMU-935, no differences were observed between sufficient and deficient IL6Rα signaling in Tregs using IMU-838 **(Figure 26 C, D**).

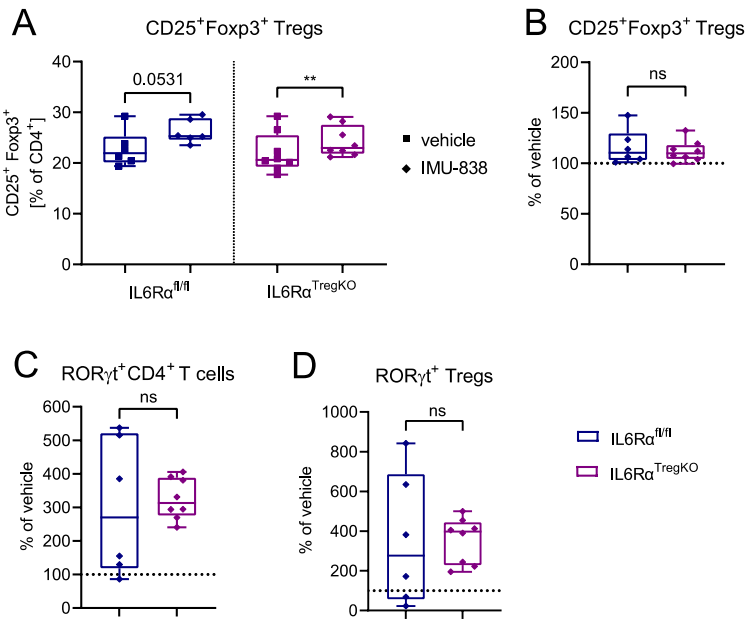

Figure 26: Foxp3-specific absence of IL6Rα does not impact the IMU-838-mediated increase in Treg induction potential. *In vitro* Treg induction using limited TCR stimulation and naïve CD4$^+$ T cells isolated from LNs of IL6Rα^{TregKO} or floxed control mice in presence of IMU-838. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt expression in CD4$^+$ T cells and (D) RORγt$^+$ Tregs, normalized to vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, Student's t-test.

The impact of the Treg-specific absence of the IL6Rα on IMU-838-mediated effects on Treg induction potential was also assessed using continuous TCR stimulation. IMU-838 improved Treg induction potential better in absence of IL6Rα signaling in Tregs (**Figure 27 A, B**). *Vice versa*, the impact on RORγt expression was reduced using CD4$^+$ T cells from IL6Rα^{TregKO} mice compared to floxed controls (**Figure 27 C, D**). This findings are consistent with effects observed using IMU-935.

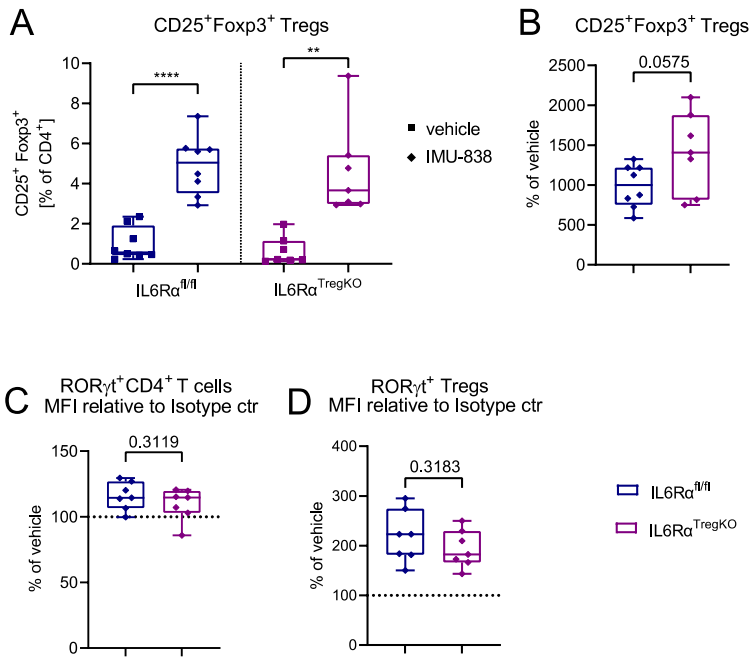

Figure 27: Presence of IMU-838 improved Treg induction using continuous TCR stimulation in Foxp3-specific absence of the IL6Rα. *In vitro Treg* induction using continuous TCR stimulation and naïve CD4$^+$ T cells isolated from LNs of IL6Rα^{TregKO} or floxed control mice in presence of IMU-838. (A) Induced CD25$^+$Foxp3$^+$ T cells and (B) normalized to vehicle control and represented as % of vehicle control. (C) RORγt MFI of CD4$^+$ T cells and (D) RORγt MFI of Tregs, normalized to isotype control and vehicle control, represented as % of vehicle control. Experiments were performed in two to three technical replicates per mouse. Data are represented as box-and-whiskers plots, each data point representing a biological replicate. **P < 0.01, ****P < 0.0001, Student's t-test.

Overall, these findings indicate an involvement of IL6Rα signaling on the effects on Treg induction and RORγt expression mediated by IMU-935 and -838. While IL6Rα signaling in CD4$^+$ T cells seems to be more important for the impact of IMU-935 and -838 on Treg induction using limited TCR stimulation, specific abrogation of IL6Rα signaling in Tregs has a greater effect on Treg induction using continuous TCR stimulation in presence of the drug candidates. This suggests that different signaling pathways might be important for the impact of the drug candidates on Treg induction capacity and RORγt expression in CD4$^+$ T cells and Tregs dependent on the strength of immune activation during *in vitro* Treg induction. Additional validation experiments will be required to allow for precise conclusions based on statistically significant differences.

4.9 IMU-838 reduces disease incidences in a model of accelerated T1D

Having established that the drug candidates improve immune tolerance *in vitro*, I next tested them in vivo for their ability to reduce immune activation and progression to T1D. As described before, IMU-838 has an already advanced clinical profile. Furthermore, my *in vitro* results suggest that DHODH inhibition is the main contributor to the effects on Treg induction. Therefore, this drug candidate was tested first. I reasoned that IMU-838, as a DHODH inhibitor has maximum potential to interfere with aberrant immune activation and disease progression in settings of an aggressive disease phenotype. Thus, IMU-838 was applied in a model of accelerated T1D induced by adoptive transfer. To this end, naïve $CD62L^+CD25^+TCRV\beta4^+CD4^+$ T cells were isolated from LNs and spleen of BDC2.5 NOD mice, which harbor a transgenic TCR for a Chromogranin A related epitope, an autoantigen in T1D [242]. These diabetogenic $CD4^+$ T cells were used to re-constitute immunodeficient NOD SCID mice which rapidly become diabetic after T cell transfer. The drug candidate or a vehicle control was applied to the mice via oral gavage with a concentration of 150 mg/kg BW (**Figure 28 A, B**).

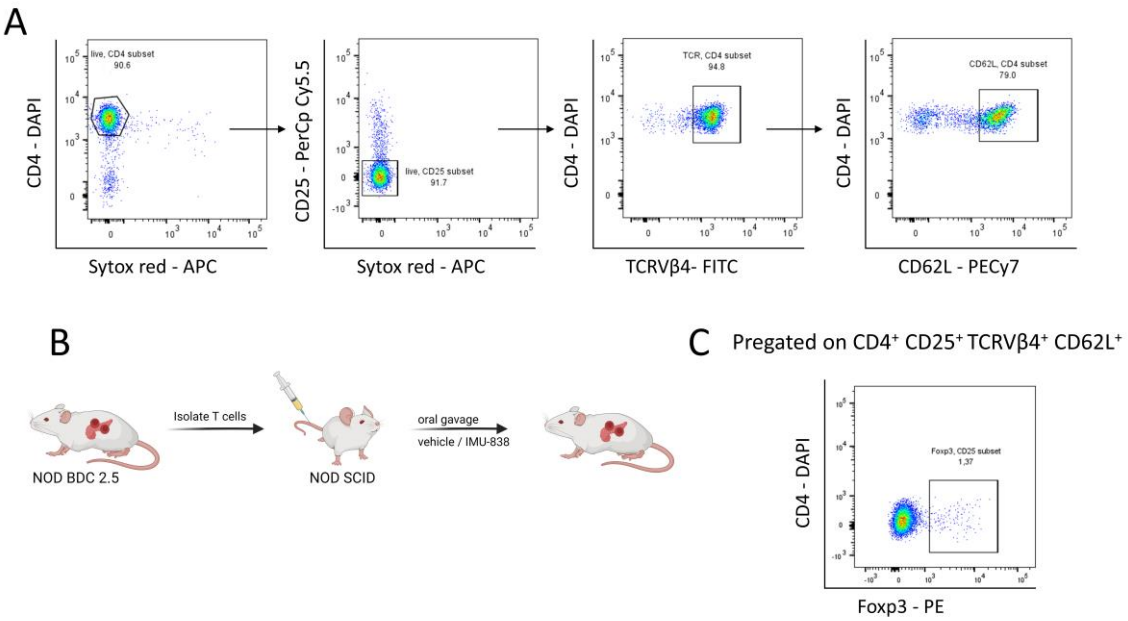

Figure 28: Experimental design of the adoptive transfer model of accelerated T1D. (A) Sorting strategy for diabetogenic $CD62L^+$ $CD25^+$ $TCRV\beta4^+$ $CD4^+$ T cells, pregated on lymphocyte scatter and doublet exclusion. (B) Schematic representation of experimental design. (C) Representative purity control staining for Tregs on sorted cells used for T cell transfer.

Diabetes development was defined as a blood sugar of above 250 mg/dL on two consecutive days. Control mice developed diabetes after 10-13 days, while the IMU-838 treated mice were normoglycemic until the end of the observation period (**Figure 29 A**).

This was accompanied by significantly reduced activation of T cells in the pLN and spleen, analyzed as $CD44^+CD62L^-$ $CD4^+$ T cells (**Figure 29 B**). Furthermore, Ki67 expression in $CD4^+$ T cells was lower in pLN and spleen of IMU-838 treated mice indicating lower $CD4^+$ T cell proliferation (**Figure 29 C**). There was no difference in activation and proliferation of $CD4^+$ T cells in the pancreas between vehicle and IMU-838 treated mice (**Figure 29 B, C**). However, the number of $CD4^+$ T cells infiltrating the pancreas was drastically reduced in IMU-838 treated mice (**Figure 29 D**), which likely contributes to lower beta cell destruction and therefore maintained glucose homeostasis and absence of diabetes development.

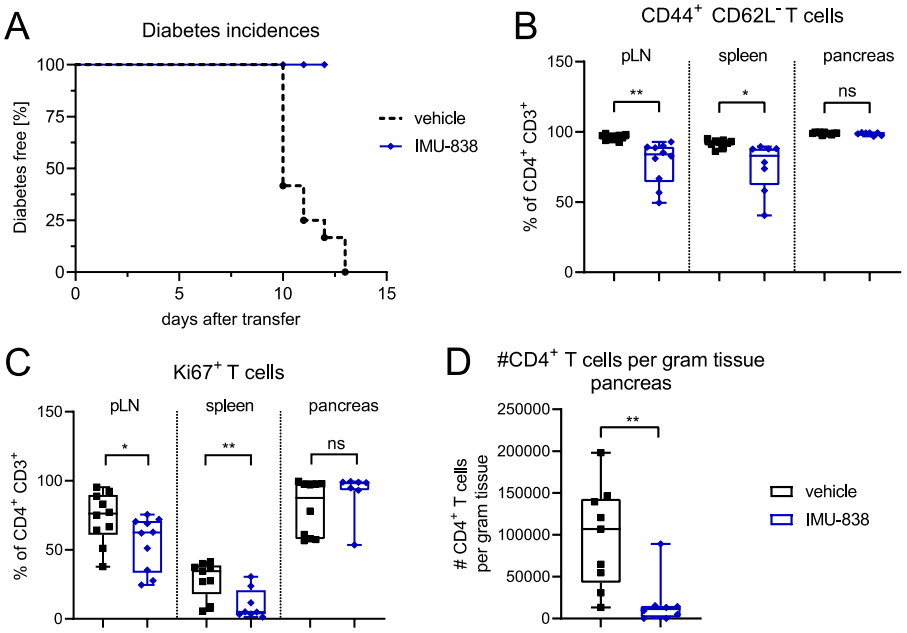

Figure 29: Treatment with IMU-838 reduces disease incidences in a model of accelerated T1D. NOD SCID mice are reconstituted with diabetogenic $CD4^+$ T cells and treated with vehicle or IMU-838 as shown in **Figure 28** and monitored for hyperglycemia. Mice were analyzed between 10-12d after transfer when the vehicle controls of the cohort got diabetic**.** (A) Diabetes incidence of vehicle or IMU-838 treated mice. (B) Frequencies of activated $CD44^+CD62^-$ $CD4^+$ T cells and (C) $Ki67^+$ $CD4^+$ T cells in pLN, spleen and pancreas. (D) Number of $CD4^+$ T cells in the pancreas represented per gram tissue. Data are represented as box-and-whiskers plots, each data point representing a biological replicate from four independent experiments. *P < 0.05, **P < 0.01, Student's t-test.

Furthermore, the frequency of Tregs in pLN and spleen slightly increased in IMU-838 treated mice which has to be confirmed by additional studies (**Figure 30 A**). This coincided with a trend towards higher expression of CTLA-4, TIGIT, FR4 and PD1 on Treg which are markers for functional Tregs in one preliminary experiment [243-246]. Since NOD SCID mice do not themselves have immune cells, the observed Treg population is either induced from the transferred T cells or expanded from contaminating Tregs in the

transferred T cell pool. In addition, CD4⁺ T cells in the spleen tend to have a more exhausted phenotype as assessed by CD4⁺ T cells expressing high levels of PD1 (**Figure 30 C**). In accordance with reduced T cell activation and increased immune tolerance CD4⁺ T cells in pLN and spleen secret lower levels of the pro-inflammatory cytokine IFN-γ (**Figure 30 D**).

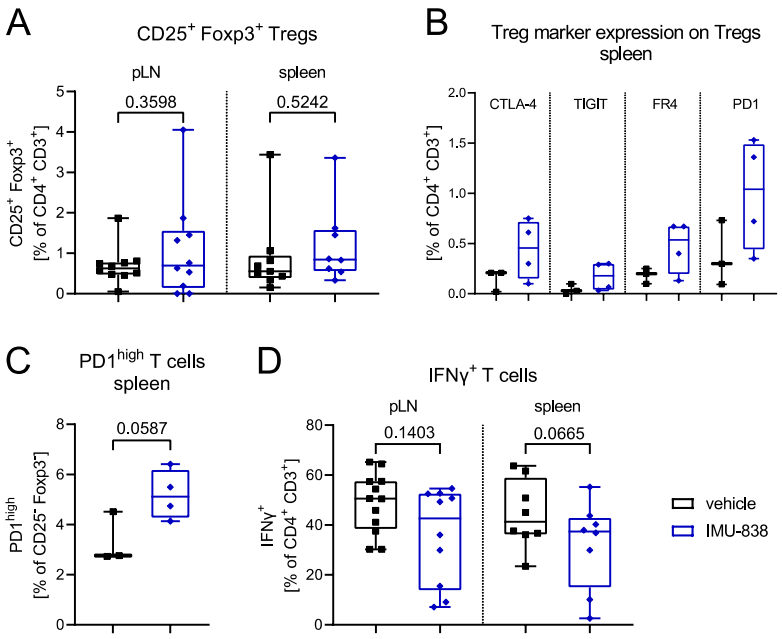

Figure 30: Trend to improved tolerance in a mouse model of accelerated T1D upon treatment with IMU-838. NOD SCID mice were reconstituted with diabetogenic T cells and treated with vehicle or IMU-838 as shown in **Figure 28**. (A) Frequencies of CD25⁺ Foxp3⁺ Tregs in pLN and spleen. (B) Expression of CTLA4, TIGIT, FR4 and PD1 on Tregs, shown here as frequencies of CD4⁺CD3⁺ T cells. (C) Frequencies of splenic CD25⁻ Foxp3⁻ T cells with high expression of PD1. (D) Secretion of IFN-γ from CD4⁺ T cells in pLN and spleen. Data are represented as box-and-whiskers plots, each data point representing a biological replicate from four independent experiments, except B and C which are from one experiment. Student's t-test.

Collectively, these data show reduced T1D incidences in a model of accelerated T1D induced by adoptive transfer after treatment with IMU-838. This coincides with reduced CD4⁺ T cell activation and proliferation which opens the window for better Treg induction or expansion, which is in line results showing improved Treg induction *in vitro* under conditions of strong immune activation in presence of IMU-838. This interplay between immune activation and tolerance might lead to the reduced secretion of the pro-inflammatory cytokine IFN-γ and the reduced CD4⁺ T cell infiltration in the pancreas which helps to reduce beta cell destruction to maintain normal blood glucose control.

5. Discussion

T1D is the most common autoimmune disease in children and incidences throughout the world are on the rise [8]. Being oftentimes diagnosed very early in life, T1D confers a major burden on the patients and their families for their rest of their lives. They have to follow a daily routine of blood glucose monitoring and insulin-replacement therapy in order to maintain glucose homeostasis. In addition to the impact on the health of the body itself, it is also accompanied by constant challenges on the mental health, starting with the constant need to monitor blood glucose levels and ending with the fear of unrecognized hyper- or hypoglycemia or possible long-term complications later in life [247-249]. Furthermore, T1D causes a great financial burden for the individuals and the health care system, which is oftentimes disproportionally higher than for T2D [235].

Extensive research over the last years deepened our knowledge in the development and manifestation of the disease. Genetic as well as environmental factors contribute to the risk for developing T1D [50, 51]. Several HLA haplotypes as well as other risk loci have been identified by GWAS studies and islet autoantibodies, which mark the onset islet autoimmunity, can serve as biomarkers [52-54, 61]. Longitudinal studies resulted in the well-established staging of T1D, starting with the presymptomatic appearance of two or more islet autoantibodies. This highlights that T1D starts already years to decades earlier before the onset of clinical symptoms [55]. T1D and the underlying autoimmune destruction of the pancreatic beta cells is assumed to be the result of a breakdown of immune tolerance mechanism and aberrant immune activation [9]. Here, several impairments in immune tolerance mechanism were identified [92, 96]. Despite ongoing research efforts, patients rely on life-long insulin treatment. Many clinical studies treated established T1D with immunotherapeutics, including the targeting of T cells and inflammatory pathways (summarized in section 1.4). However, the ultimate goal of reversing T1D and eliminating the need for exogenous insulin injections could so far not been reached. Encouragingly though, endogenous insulin production was improved which resulted in lower demand for exogenous insulin and the first immunotherapy for the prevention of T1D was approved in November 2022 [177]. This will open the door for new immunotherapies for the treatment of T1D and specific and targeted approaches to avoid general immune suppression are an important new research focus.

Among others the drug candidates, IMU-935 and IMU-838, which I investigated here, became a research focus. IMU-935 is a RORγt inverse agonist and to a lesser extend a DHODH inhibitor, whereas IMU-838 is only a DHODH inhibitor. The rational to investigate the drug candidates was found in the literature. Firstly, DHODH as the key enzyme of the pyrimidine *de novo* biosynthesis is metabolically required for activated lymphocytes [191-193]. Inhibition results in the suppression of exclusively activated lymphocytes, which represents an elegant way to treat overshooting immune activation in autoimmune diseases. Indeed, DHODH inhibition was observed to improve colitis and

EAE in several mouse models [193, 212, 250]. Furthermore, this was accompanied by reduced expression of pro-inflammatory cytokines and T cell proliferation [193, 250]. In T2D, DHODH inhibition had beneficial effects on beta cell survival and glucose metabolism, which was attributed to increased levels of the cytokine GDF15 [218]. The link to T1D was established by Nakayasu *et al.* who showed that GDF15 levels were decreased in islets from NOD mice with insulitis or T1D donors. Administration of recombinant GDF15 to NOD mice decreased incidences of diabetes [219].

Secondly, treating NOD mice with a RORα/γ inverse agonist or anti-IL-17 antibodies delayed the progression to T1D which was accompanied by reduced insulitis, increased Treg frequencies and inhibition of the proinflammatory cytokines IL-17 and IFN-γ [225, 234]. The inhibition of T_H17 cells as well as the production of the proinflammatory cytokines IL-17 and IFN-y was also observed in *in vitro* and *in vivo* studies using IMU-935 [220, 221]. Furthermore, clinical data support the hypothesis that T_H17 cells and their IL-17 production might be pathogenic [43, 44, 232]. Overall, these data support the concept of targeting DHODH and/or RORγt as a new approach to treat T1D. Importantly, targeting these pathways could directly or indirectly improve Tregs and therefore strengthen immune tolerance mechanisms. On the one hand, the antagonistic competition between RORγt and Foxp3 suggest that targeting RORγt could shift the balance to Foxp3 expression and therefore improve immune tolerance [144, 145]. On the other hand, reducing immune activation could also open a broader window of opportunity to increase Treg induction. In line with these hypothesis, I observed an improved *in vitro* Treg induction using limited Treg induction and T cells from non-autoimmune prone BALB/c mice in presence of IMU-935 and -838. This was accompanied by a reduced T cell proliferation, which was also represented by an overall reduced number of recovered T cells (data not shown). The reciprocal relationship is in line with published and well established results that show that limited cellular proliferation coincides with lower activation of the PI3K/Akt/mTOR pathway, which in turn is beneficial for Treg induction [90, 92, 93].

We and others already presented various deficits in immune tolerance during islet autoimmunity and at the same time there are several lines of evidence suggesting increased immune activation. First of all, miRNA181a which regulates TCR signaling strength is increased in children with recent onset autoimmunity [92]. This correlated with an increased expression of genes that promote immune activation, including PI3K and NFAT5, while genes associated with reducing $CD4^+$ T cell activation are downregulated. Accordingly, naïve $CD4^+$ T cells from children with recent onset of islet autoimmunity proliferated more compared to islet autoantibody negative children and they harbored increased frequencies of $FOXP3^{int}CD4^+$ T cells all of which point towards increased T cell activation [92, 251, 252]. In line with that, *in vitro* Treg induction was shown to be reduced during human islet autoimmunity onset as well as in T cells from NOD mice with ongoing insulin autoantibody positivity [92, 96]. At first, I aimed at mimicking the environment of strong immune activation that is observed during islet autoimmunity and T1D *in vitro*. Therefore, I established Treg induction assays under challenging conditions.

Specifically, Tregs were induced either from activated CD4$^+$ T cells or from naïve CD4$^+$ T cells using limited TCR stimulation in presence of the pro-inflammatory cytokines IL-6, IFN-γ and IL- 1β or using continuous TCR stimulation. These experimental conditions are known to be suboptimal for Treg induction and mimic increased immune activation [92, 97, 253]. As expected, *in vitro* Treg induction was drastically decreased and ranged from 0-5%. However, the pres-ence of IMU-935 or -838 was able to improve Treg induction potential. This indicates that the drug candidates are potent Treg inducers in conditions that are unfavorable for Treg induction.

Following up on that, I performed *in vitro* Treg induction using naïve CD4$^+$ T cells from NOD mice with or without ongoing autoimmunity under subimmunogenic conditions. Here, I was able to confirm impaired Treg induction *in vitro* using T cells from IAA$^+$ NOD mice compared to non-autoimmune prone BALB/c mice. In accordance with results using immune-activating conditions, presence of IMU-935 or -838 improved Treg induction from CD4$^+$ T cells of autoimmune-prone NOD mice. In contrast to immune-activating conditions, the naïve CD4$^+$ T cells are already intrinsically more activated [92, 97]. This highlights that the compounds are not only able to reduce immune activation the cells experience by the experimental conditions, but also the intrinsic aberrant activation that result from islet autoimmunity. Importantly, the drug candidates are more efficient in improving Treg induction capacity using CD4$^+$ T cells from IAA$^+$ NOD mice compared to BALB/c mice. Since T cells isolated from an autoimmune environment are more activated, they provide a better target for immunosuppression. Furthermore, in conditions that are maximally unfavorable for Treg induction, such as inducing Tregs from T cells of IAA$^+$ NOD mice in an immune-activating environment, IMU-935 or -838 were able to increase *in vitro* Treg induction. Here, there is only a very narrow window for Treg induction. This indicates that the drug candidates are able to open a broader window of opportunity to induce Tregs by potentially reducing immune activation.

Importantly, using CD4$^+$ T cell from very young mice with an age below 30d IMU-935 was able to improve Treg induction capacity from NOD but to a lesser extend from BALB/c mice. In autoimmune-prone NOD mice autoantibodies can be detected as early as 3 weeks highlighting that the autoimmune reaction starts very early in life [254]. Of note, this is also accompanied by an increased TSDR methylation of young IAA$^+$ NOD mice compared to age-matched BALB/c mice indicating reduced Treg stability and autoimmune reaction already at an early age [96]. This might explain the higher impact of IMU-935 on Treg induction potential using young NOD mice in contrast to non-autoimmune BALB/c mice. Importantly, Treg induction was increased using young IAA$^+$ NOD mice compared to IAA$^-$ NOD mice. This could hint towards a mechanism to compensate the already impaired Treg stability in young IAA$^+$ NOD mice [96].

Overall, the Treg induction capacity was increased in CD4$^+$ T cells from young mice compared to mice at the age of 80-1100d (BALB/c mice age<30d: 24.43% vs age >80d: 17.67%, direct comparison not shown). This is in line with several studies that observed similar trends in *in vivo* and *in vitro* Treg induction [255, 256]. Many studies report age-

associated defects and higher activation in the T cell compartment with age which could hinder Treg induction [257-259]. First of all, miR21 which is normally upregulated upon $CD4^+$ T cell activation is drastically increased in elderly individuals [260]. In agreement with its proposed target being PTEN, upon activation of naïve $CD4^+$ T cells, those from old individuals expressed lower amounts of PTEN but showed an increased phosphorylation of AKT or mTOR. Increased miR21 expression in naïve $CD4^+$ T cells of older individuals further resulted in the induction of T cells with a proinflammatory effector phenotype after stimulation [260]. In addition, others studies also reported higher activation of the PI3K/Akt/mTOR pathway with aging [261]. Noteworthy, studies in the literature were performed either with elderly individuals that faced numerous infections throughout their life or with mice aged more than one year and compared results to teenagers or young adult mice. Here, mice were either studied shortly after weaning with an age below 30d or with an age between 80-100d. Neither of the reported study compared their results to mice shortly after weaning. The group of Gérard Eberl observed a phenomenon in the mouse intestinal immune system that they termed “weaning reaction” that was specific during a certain time window between 10d and 3-4 weeks after birth [262]. The microbiota expands which results in an immune reaction shaped by the generation of microbiota-induced Tregs [262]. Thus, the higher capacity for Treg induction of T cells isolated from very young mice shortly after weaning could also reflect the “weaning reaction”. Additionally, the impact of IMU-935 was slightly higher using T cells from older BALB/c mice compared to young ones (% of vehicle: age<30d: 112% vs age >80d: 123.3%, direct comparison not shown). This can be explained by the higher activation of T cells in older mice which therefore represent a better target for IMU-935.

Next, based on the reported reciprocal relationship between Foxp3 and RORγt I assessed RORγt expression after Treg induction. Furthermore, both transcription factors were reported to get upregulated in Treg induction assays with TGF-β which raises the question whether a RORγt inverse agonist might be able to shift the balance towards more Foxp3 expression [144]. Surprisingly, I observed an increase in $ROR\gamma t^+$ Tregs as well as $Foxp3^-$ $CD4^+$ T cells after *in vitro* Treg induction in presence of IMU-935 which was unexpected due to the reciprocal relationship. Increased RORγt expression did not result in an enhanced secretion of the proinflammatory cytokine IL-17A. In contrast, the production of IL-10 was improved in Tregs and $Foxp3^-$ $CD4^+$ T cells in presence of IMU-935. The latter particularly highlights the anti-inflammatory potential of the drug candi-date. Importantly, the anti-inflammatory phenotype is maintained also when inducing Tregs in an immune-activating environment. The importance of IL-10 secretion was demonstrated by the rapid development of T1D in BDC2.5 NOD mice or following cyclophosphamide (CY)-treatment in the absence of IL-10 [263, 264].
Vice versa, systemic administration of IL-10 prevented diabetes at high concentrations with reduced insulitis and an increase in $CD25^+$ $CD4^+$ regulatory T cells [265]. IL-10 is not only important for its direct suppression of effector T cells but it also license Tregs to facilitates their suppressive function [75]. E.g. IL10R signaling was reported to induce IL-10 expression in Tregs and its deficiency in Tregs results in

in failed suppression of T_H17 responses [266]. Furthermore, IL-10 engagement with its receptor on Tregs is important for a maintained Foxp3 expression in inflammatory conditions and it was also shown to enhance *in vitro* human Treg induction [267, 268]. Thus, increased IL-10 production could not only have a direct suppressive function but could also enhance Tregs in a feed-back loop. In addition, a non-pathogenic T_H17 cell subset which expressed high levels of IL-10 was described [269, 270]. Thus, the drug candidates also might induce non-pathogenic T_H17 in addition to Treg cells that could act in concert to suppress autoimmunity.

IMU-935 inhibits RORγ and DHODH at an IC_{50} of 20nM or 240nM in stimulated human PBMCs, respectively [271]. This results in a synergistic inhibition of the proinflammatory cytokines IL-17 and IFN-γ with an IC_{50} of 3-5nM in human T cells and an inhibition of murine T_H17 differentiation at an IC_{50} of 135nM [271]. Thus, this raised the question which of the components is most responsible for the observed effects. Results using IMU-838, a potent DHODH inhibitor, mirrored results using IMU-935 in terms of improved Treg induction which was accompanied by increased frequencies of RORγt$^+$ Tregs and RORγt$^+$Foxp3$^-$ CD4$^+$ T cells and enhanced IL-10 production in Tregs and Foxp3$^-$ CD4$^+$ T cells. Additional evidence of an only minor contribution of the RORγt inverse agonist in IMU-935 came from uridine supplementation experiments. Here, uridine supports T cells with an exogenous source for the pyrimidine synthesis thereby circumventing DHODH inhibition. High concentrations of uridine abrogated the impact of IMU-935 on induced Tregs as well as RORγt expression in CD4$^+$ T cells and Tregs, which was accompanied by a restored proliferation. The increase in proliferation is in line with the reduced Treg induction capacity as described before. In settings of higher immune activation, which e.g. is achieved by the addition of proinflammatory cytokines, one could think of two scenarios that could point towards a higher impact of either RORγt or DHODH inhibition in immune activating conditions. First of all, in immune activation conditions, especially in presence of IL-6, CD4$^+$ T cell might be more prone to convert into T_H17 cells than into Tregs, which could point towards a higher effect of the RORγt inverse agonist. Second of all, under conditions of strong immune activation, T cells become activated and more DHODH-dependent which could make them more susceptible to DHODH inhibition. However, results obtained with exogenous uridine supplementation were not dependent on the degree of immune activation the naïve CD4$^+$ T cells experience during differentiation. Thus, this points towards a dependence on DHODH inhibition using IMU-935 also in conditions of high immune-activating conditions. Overall, this highlights that in *in vitro* Treg induction DHODH inhibition by IMU-838 or IMU-935 is responsible for the observed effects. An improved Treg induction in response to DHODH inhibition was also observed in other studies. A771726, the active metabolite of leflunomide, enhances induction of Tregs from naive CD4$^+$ T cells *in vitro* which was attributed to the inhibition of Akt by A771726 [272]. Another DHODH inhibitor, M62, also suppressed the PI3K/Akt/mTor pathway which confirms a positive role of DHODH inhibition in reducing immune activation and thereby opening a broader window of opportunity for Treg induction [273].

Using Teriflunomide (Tef), a commonly used DHODH inhibitor, I observed similar to IMU-838 a positive impact on Treg induction capacity which was accompanied by an upregulation of RORγt expression in Foxp3$^+$ and Foxp3$^-$ CD4$^+$ T cells. This highlights that the observed effects are a common phenomenon of DHODH inhibitors. Importantly, the impact on Treg induction and RORγt expression was greater in presence of IMU-838 compared to Tef. This might indicate a superior effect of the next-generation DHODH inhibitor IMU-838. Noteworthy, both are used here above their reported IC_{50}, which excludes any difference based on their ability to suppress DHODH. Especially, Tef which has an IC_{50} of 156nM for murine DHODH is here used at 7.5µM [193]. The concentration of Tef is furthermore below the reported concentration needed for its off-target effect on protein kinases, making off-target effects less possible [274]. The induced RORγt$^+$ Tregs in presence of the DHODH inhibitors might be superior in restraining T_H17 cell-mediated autoimmune diseases [139]. In line with that, teriflunomide and IMU-838 show improved disease outcomes in colitis and EAE, which are both T_H17 cell-driven [193, 212].

Surprisingly, as outlined above our findings suggest that DHODH inhibition and not RORγt inverse agonism is responsible for the upregulation of RORγt in response to the drug candidates in *in vitro* Treg induction. This was at first counterintuitive because it was shown in an *in vivo* transfer model that CD4$^+$ T cells that upregulated low levels of RORγt proliferated more compared to RORγt$^-$ CD4$^+$ T cells establishing a positive association between T cell proliferation and RORγt expression [275]. In addition, it was long believed that Foxp3 inhibits the expression of RORγt [141]. However, the upregulation of RORγt can be a compensatory mechanism due to the blocked secretion of proinflammatory cytokines or the increased expression of Foxp3. A possible feedback mechanism of increased RORγt expression due to repressed IL-17 expression was indicated in a study using another T_H17 cell-targeting compound. It inhibited IL-17 production in human T_H17 differentiation which was accompanied by an increased expression of RORC2 [276]. In line with the latter hypothesis, Zeng et al. observed that CD4$^+$ T cells that were *in* vitro differentiated into T_H17 cells and infected with a Foxp3-expressing retrovirus expressed higher levels of RORγt compared to cells infected with a control virus [277]. Thus, forced Foxp3 expression resulted in higher RORγt expression, which might suggest a compensatory upregulation of RORγt. Furthermore, after culturing Tregs under T_H17 conditions they co-expressed significant amounts of RORγt [277]. This suggests, that the reciprocal relationship between Foxp3 and RORγt is not as strict as previously thought. Accordingly years later, the existence of Foxp3$^+$ RORγt$^+$ Tregs was confirmed which represent a stable and functioning Treg subset [139].

Worth mentioning, for optimal Treg inducing capacity I used a concentration of IMU-935 of 1µM which is considerable higher than the reported IC_{50} for murine T_H17 induction [271]. In addition, the IC_{50} for DHODH inhibition is higher than the one for RORγt inhibition. Thus, it could be that at the high concentration used for *in vitro* Treg induction DHODH inhibition outweighs RORγt inverse agonism. Treg titration experiments observed a higher Treg induction capacity starting from 500nM which incr-

eases with increasing concentration. At lower concentrations the RORγt inverse agonist may play a contributing role.

Furthermore, I overall observed a higher RORγt expression in Treg induction experiments using continuous TCR stimulation in which frequencies of induced Tregs were reduced to around 1%. This is in line with results from the literature. While continuous TCR stimulation and therefore high activation of the T cells is unfavorable for the generation of Tregs, it is optimal for T_H17 differentiation and therefore IL-17 production [89-91, 140]. Consequently, Treg induction protocols rely on limited TCR stimulation for efficient induction of functional Tregs and common T_H17 induction experiments use continuous TCR stimulation with the respective cytokine cocktails [92, 278]. This is also in line with higher IL-17 production that was observed after Treg induction using continuous TCR stimulation which is repressed in the presence of IMU-935 and -838. Although T_H17 induction pathways seem to be more involved in Treg induction using continuous TCR stimulation, my results suggest, that still DHODH inhibition is the major contributor for the effect of the two drug candidates, due to the fact that results for IMU-935 and IMU-838 resemble each other. However, in order to draw a definite conclusion experiments with uridine supplementation need to be performed.

Many studies show the importance of IL6Rα signaling for T_H17 cells while it is suppressing Tregs. As outlined in section 1.3.4 IL-6 is a pleiotropic cytokine which can signal via three different signaling pathways, the classical and trans-signaling or trans-presentation [147, 149, 150]. *In vitro* and *in vivo* studies observed that IL-6 is important for the efficient generation of T_H17 cells [141]. For instance, IL-6-deficient mice are resistant to EAE induced by myelin oligodendrocyte glycoprotein (MOG) which was associated with defects in T_H17 cell generation and elevated Treg frequencies [279, 280]. Heink at al. observed increased Treg frequencies in in the draining lymph node of IL6Rα^{TKO} mice that were immunized with MOG(35-55) compared to floxed controls, highlighting the importance of the classical IL-6 signaling for the IL-6 mediated suppression of Tregs [149]. Their results further show that IL-6 trans-presentation is required for the generation of pathogenic T_H17 cells *in vivo* [149]. Tocilizumab, a monoclonal antibody against the soluble and membrane-bound IL6R is used for the treatment of e.g. rheumatoid arthritis [281]. Several studies observed that treatment with tocilizumab increased the frequency of Tregs which resulted in an increased ratio of Tregs vs. Th17 cells [282, 283]. In order to assess the impact of IL6Rα signaling on Treg induction and RORγt expression in response to IMU-935 and -838 I employed various mouse models. First of all, T cells of mice with a T cell specific deletion of the IL6Rα which were generated by crossing CD4^{Cre} with IL6Rα $^{fl/fl}$ mice, cannot respond to the classical signaling but are still responsive via trans-signaling or -presentation. Secondly, by using Foxp3^{Cre} mice classical signaling is specifically abrogated in Tregs [237, 240, 241].

In line with the aforementioned beneficial effect of absent IL6Rα signaling on Treg I observed an increased potential of IL6Rα-deficient CD4$^+$ T cells to convert into Tregs when stimulated *in vitro* under subimmunogenic conditions. This phenomena is specific to the

deletion of the IL6Rα in T cells because the abrogation of the signaling specifically in Tregs did not impact *in vitro* Treg induction. Naïve $CD4^+$ T cells express high levels of the IL6Rα, which makes them sensitive to even low levels of IL-6 [284]. Therefore, naïve T cells could sense IL-6 *in vivo* before isolation, which would negatively impact their Treg induction. In contrast, $CD4^+$ T cells without the expression of IL6Rα do not sense IL-6 via the classical signaling making them more prone to convert into Tregs. Accordingly, a study with human naïve $CD4^+$ T cells showed that pre-exposing them to pathophysiological levels of IL-6 prior to TCR activation enhanced their proliferative capacity and activation [285]. This would hinder Treg induction. Certainly, pathophysiological levels are higher than observed in healthy unchallenged mice. *In vitro* Treg induction is, however, sensitive to already minor perturbations. Thus, whether naïve $CD4^+$ T cells did sense small amounts or never sensed any IL-6 could impact Treg induction potential. Naïve cells might potentially secrete small amounts of IL-6 once activated via the TCR during Treg induction since activation of splenoyctes by anti-CD3 and CD28 did result in the secretion of small amounts of IL-6 [286]. Activation of $CD4^+$ T cells furthermore induces shedding of the membrane-bound IL6R producing a soluble receptor [287]. And indeed especially the trans-signaling via the soluble IL6Rα was shown to negatively impact Treg induction [288]. In line with the hypothesis of sensing of IL-6 by naïve $CD4^+$ T cells which may make them more intrinsically activated or proliferative and thereby being a better target for suppressive agents, the impact of IMU-935 and -838 on Treg induction was reduced using $CD4^+$ T cells from $IL6R\alpha^{TKO}$ mice. Furthermore, there was no difference in Treg induction potential as well as no differential impact of IMU-935 or -838 on Treg induction using $CD4^+$ T cells from $IL6R\alpha^{TregKO}$ mice compared to floxed controls. This supports a concept in which naïve $CD4^+$ T cells from $IL6R\alpha^{TregKO}$ mice are similar to floxed controls before the upregulation of Foxp3 and thus were able to encounter IL-6 *in vivo* or *in vitro* before upregulating Foxp3. This findings supports a mechanism in which presence of the IL6Rα in the early phase of Treg induction is responsible for the IL-6 mediated suppression of Treg generation. In accordance with IL-6 signaling being important for T_H17-like Tregs and T cells, the IMU-mediated increase in RORγt$^+$ T cells and Tregs was reduced using IL6Rα-deficient $CD4^+$ T cells in limited Treg induction assays [289]. Worth mentioning, abrogation of the classical IL6Rα signaling did not impact RORγt expression in limited Treg induction without the addition of the drug candidates. This indicates, that similar to reports from other studies, RORγt expression is not exclusively dependent on IL-6 signaling [280]. The findings presented here suggest that it might become more important in settings of reduced immune activation, as experienced during the presence of IMU-935 and -838, to compensate for the lack of activation.

In contrast to the TKO of IL6Rα, using the TregKO preliminary findings suggest increased frequencies of RORγt$^+$ Tregs in limited Treg induction experiments in presence of IMU-935. This might indicate that the absence of the classical signaling specifically on Tregs might help to mediate the increase of RORγt$^+$ Tregs in response to IMU-935. This stands in contrast to various studies that reported the importance of IL-6 for RORγt$^+$ expression

[137, 275]. One possible explanation might be a compensatory upregulation of RORγt in response to the sudden deletion of classical IL-6 signaling in combination with immunosuppression. IL6Rα^{TregKO} naïve CD4$^+$ T cells express the IL6Rα until the upregulation of Foxp3. Thus, while not being responsive to the classical signaling anymore, IL6Rα-deficient Tregs from IL6Rα^{TregKO} mice are still responsive to IL-6 via trans-signaling because naïve CD4$^+$ T cells upon activation were able to produce soluble IL6R by shedding of the membrane bound receptor [287]. This is an important difference between the T cell and Treg knockout. Studies with the sgp130Fc protein which blocks trans-signaling without affecting the classical signaling suggest that IL-6 trans-signaling is pro-inflammatory (reviewed in [290]). Importantly, diminished trans-signaling blocked *in vitro* T_H17 induction and is therefore important for RORγt expression [149]. Trans-signaling might be overrepresented in IL6Rα-deficient Tregs due to the fact that it is the only possible signaling pathway while control Tregs can employ additionally the classical signaling. This could facilitate an increased expression of RORγt. Similar to the TKO, there is no difference between RORγt expression after limited Treg induction between IL6Rα^{TregKO} or flox control T cells. These preliminary results highlights again that RORγt expression is not exclusively dependent on IL-6 signaling but it might become more important in settings of reduced immune activation. Thus, trans-signaling might facilitate the increase in RORγt expression in response to IMU-935 using T cells from IL6Rα^{TregKO} mice in settings of reduced immune activation, which has to be validated in further experiments. Noteworthy, similar results were not observed using IMU-838, but here the variability in RORγt expression is big thus making conclusions difficult. Overall, this suggests that deletion of the IL6Rα on CD4$^+$ T cells during the early phase of Treg induction is important for efficient upregulation of RORγt in response to the drug candidates while the specific deletion in Tregs tends to increase RORγt expression. These preliminary findings suggest a differential effects of IL6Rα signaling during different phases of Treg induction.

The results observed using limited TCR stimulation were different to preliminary experiments from Treg induction assays under immune-activating conditions using continuous TCR stimulation suggesting that different mechanism are involved in the increase of Tregs and RORγt$^+$ CD4$^+$ T cells and Tregs in response to IMU-935 and -838. In contrast to limited TCR stimulation, upon continuous TCR stimulation, preliminary findings using a T cell-specific deletion of IL6Rα suggest a trend towards increased Treg induction potential as well as RORγt expression in Tregs in presence of IMU-935 and -838 compared to control T cells. During continuous TCR stimulation T cells get more activated which likely increases on the one hand side secretion of IL-6 but also the shedding of the membrane-bound receptor which results in higher responsiveness of the control T cells towards trans-signaling driving them towards a pro-inflammatory state. Both IMU-935 and -838 were able to increase Treg induction from the control T cells but to a lesser extend compared to T cells that cannot respond to IL-6 signaling. Thus, the degree of activation the IL-6 responsive T cell face during T cell induction might be too great for restoring a

less-activated state in those T cells by the drug candidates compared to IL6Rα-deficient T cells. In addition, IMU-mediated RORγt upregulation is not affected on the T cell-level but an increased frequency of RORγt$^+$ Tregs was observed using IL6Rα-deficient CD4$^+$ T cells in presence of IMU-935. This might suggest that continuous TCR stimulation might be able to compensate for the absent IL6Rα signaling, in contrast to subimmunogenic TCR stimulation. This is also line with the hypothesis of a compensatory upregulation of RORγt in response to increased Foxp3 expression.

Similarly to the T cell-specific knockout, the IMU-mediated increase in Treg induction using continuous TCR stimulation further tended to increase when IL6Rα was absent on Tregs compared to IL6Rα-sufficient Tregs. This however, coincided with a trend towards reduced frequencies of RORγt$^+$ CD4$^+$ T cells and Tregs in presence of the drug candidates. These preliminary findings indicate that using continuous TCR stimulation the absence of IL6Rα on Treg is important for the IMU-mediated increase of RORγt expression. Overall, my results show an involvement of IL6Rα signaling on the impact of IMU-935 and -838 on Treg induction and RORγt expression in CD4$^+$ T cells and Tregs. Prelimi-nary results suggest that the effect is different depending on the degree of TCR stimula-tion indicating that different signaling pathways might be involved. It further depends on the timing of the absence of the membrane-bound IL6Rα. This highlights that the impact of IL6Rα signaling on Treg induction in presence of IMU-935 and -838 is a complex interplay between TCR stimulation and timing of IL6Rα signaling. Noteworthy, many of the findings using T of Treg-specific deletion of the IL6Rα are preliminary and need to be validated for final conclusions.

In the end, after getting mechanistic insights into the impact of the drug candidates on Treg induction and RORγt upregulation in CD4$^+$ T cells and Tregs, IMU-838 was administered to mice in a T1D mouse model induced by adoptive transfer. This represents a model for an aggressive phenotype of the disease, due to the strong immune activation and fast development of T1D. IMU-838 administration delayed the progression to T1D while the control mice developed diabetes within 10-13 days. As reported for other disease models, treatment of mice with IMU-838 resulted in a decrease in CD4$^+$ T cell activation, their pro-inflammatory cytokine release and proliferation in secondary lymphoid organs [193, 212, 213, 291]. When T cells are transferred into immunodeficient hosts, they get activated and proliferate heavily to fill the empty niche. They therefore represent a good target for the DHODH inhibitor IMU-838. The transferred CD4$^+$ T cells have an islet-specific TCR which is specific for chromogranin A, a peptide in pancreatic beta cells. They therefore readily infiltrate and destroy the pancreas. While no impact on T cell activation and proliferation in the pancreas was observed, the number of CD4$^+$ T cells that infiltrated the pancreas was drastically reduced. This likely results in a lower degree of destruction of the pancreas, higher remaining insulin production and slower development of T1D. The CD4$^+$ T cells in the periphery might be hindered to infiltrate the pancreas by reduced expression of chemokine receptors on the CD4$^+$ T cells or reduced release of chemokines from the beta cells which attracts the CD4$^+$ T cells. In human and mice it

was shown that the CXCL10/CXCR3 axis is important for the generation of T1D. Serum concentrations of CXCL10 are increased in patients at high risk for developing T1D or in patients with newly diagnosed T1D [292, 293]. Importantly, elevated levels of CXCL10 were increased directly in the pancreas of diabetic patients which also coincided with increased frequencies of infiltrating lymphocytes expressing the corresponding chemokine receptor CXCR3 [294, 295]. These studies identified that CXCL10 is produced directly by beta cells and that CXCR3-expressing cells mainly consist of T cells. In animal models of T1D, this axis was also confirmed [296-298]. Consistently, blocking CXCL10 or CXCR3 prevents or delays insulitis and T1D in various mouse models [297, 299, 300]. Conversely, mice with an overexpression of CXCL10 in islets show spontaneous infiltration and present with accelerated virus-induced T1D which was likely caused by an enhanced migration and retention of antigen-specific lymphocytes in the pancreas [301]. Importantly, IFN-γ^+ T_H1 cells almost exclusively express CXCR3 and the expression was shown to be important for the migration to the site of T_H1-driven inflammation [300, 302-304]. I also observed reduced frequencies of $CD4^+$ T cells expressing IFN-γ in secondary lymphoid organs, which might hint towards a reduced expression of CXCR3. Accordingly, no decreased IFN-γ production was observed in the pancreas which could suggest that fully functional T_H1 cells, with expression of CXCR3 and IFN-γ are able to enter the pancreas. Since these cells are already suppressed in the periphery only a small number of cells can infiltrate the pancreas and facilitate the destruction. In order to establish a possible relationship with CXCR3, this marker will be included in further experiments. Interestingly, not only T_H1 cells but also Tregs can express CXCR3 which is important for their trafficking into the pancreas [300]. These cells also co-express Tbet and are important to control T1D [156].

In accordance with my *in vitro* data I observed a preliminary trend towards higher Treg frequencies in secondary lymphoid organs *in vivo* upon treatment with IMU-838. These Tregs are either induced from transferred $CD4^+$ T cells or expanded from contaminating Tregs, which represent a minor fraction of around 1%. They, however, do not express CD25. The T cells originated from the islet autoimmune environment of NOD BDC2.5 mice and stability of Tregs from NOD mice with ongoing islet autoimmunity was show to be impaired [96]. Their conversion into pathogenic ex-Tregs when exposed to proinflammatory triggers or overstimulation is a constant debate. Especially, a minor population of $CD25^-$ Tregs was reported to convert into ex-Tregs. In a model of collagen-induced arthritis transferred $CD25^{low}Foxp3^+$ Tregs lost Foxp3 expression more easily than their counterparts with high CD25 expression [119]. Thus, the isolated fraction of $CD25^-$ $Foxp3^+$ T cells could represent an unstable subpopulation of Tregs which can easily lose Foxp3 expression and convert into pathogenic ex-Tregs in a lymphopenic environment. Treatment of IMU-838 might directly stabilize Foxp3 expression in these transferred Tregs while in control mice they lose Foxp3 expression. In addition, the reduced immune activation and proliferation in the treated mice most likely provide a better environment for stabilization and/or induction of Foxp3 expression. This is also in agreement with the

here presented *in vitro* data. In addition, preliminary data suggest an increased frequency of Tregs expressing functional markers such as CTLA-4, TIGIT, FR4 and PD1 which are known to correlate with suppressive function and a higher expression of PD1 in non-Treg cells points towards higher exhaustion [243-246]. Here, the impact might be a direct effect of the DHODH inhibition. DHODH inhibition in activated T cells stresses the cells which in turn might get exhausted and therefore show a lower immune-pathological profile. All of the above mentioned points, including reduced activation and proliferation of T cell with higher percentages of them being exhausted as well as a higher frequency of more functional Tregs likely act in concert to reduce pancreas infiltration and destruction and incidences of T1D.

The increase of Tregs was already observed in other disease models that were treated with DHODH inhibitors. In a collagen-induced arthritis (CIA) model, mice treated with leflunomide showed decreased incidences of CIA which coincided with reduced frequency of IL17-producing $CD4^+$ T cells and a slight increase of $Foxp3^+$ Tregs in the spleen compared to the untreated control group [305]. Similar results were also observed in a CIA model in Wistar rats [306]. In addition, A771726, the active metabolite of leflunomide, also shifted the balance of T_H17/Treg cells towards the tolerance site *in vivo* [272]. In the human setting inhibition of *de novo* pyrimidine synthesis by PALA which blocks the first three reactions in the pyrimidine synthesis, resulted in an increase of $Foxp3^{high}$ T cells during T cell activation [307]. Thus, increased Treg frequencies *in vivo* in presence of IMU-838 are also in agreement with the literature, but the preliminary results on the impact on Tregs *in vivo* have to be validated in additional experiments.

So far, I concentrated on the impact of IMU-838 treatment on $CD4^+$ T cells and pancreas infiltration. However, DHODH inhibition can also have a direct impact on the beta cells which could contribute to better disease management. A recent study observed that DHODH inhibitors promote the expression and secretion of GDF15 *in vitro* and *in vivo* after treatment of db/db mice, which are a model of Type 2 diabetes and obesity [218]. This resulted in improved glucose control and prevented beta cell mass loss. Interestingly, not only the GDF15 serum levels were increased but they also observed increased GDF15 expression directly in the pancreatic islets upon treatment [218]. GDF15 is a stress-inducible cytokine and a distant member of the TGF-β family [308]. In a healthy state GDF15 expression is low, but observable in various tissues including kidney, colon, liver and pancreas and is secreted by different cell types such as cardiomyocytes, adipocytes or macrophages [308]. The group of T. Metz directly linked GDF15 to reduced apoptosis of islets and beta cells, thereby promoting survival [219]. Pro-inflammatory cytokines, including IFN-γ or IL-1β, are toxic to beta cells and mediate their destruction. They observed that islets stressed in such a pro-inflammatory environment either in cell culture or by an autoimmune setting as observed in NOD mice or T1D patients expressed lower amounts of GDF15 [219]. This was accompanied by an increased activity of caspase 3 which can be reversed by supplementation of GDF15. Treatment of pre-diabetic NOD mice with recombinant GDF15 reduced insulitis and diabetes incidences

[219]. Furthermore, overexpression of GDF15 decreased apoptosis of pancreatic islets in a STZ-induced T1D model [309]. Importantly, in patients with advanced hepatocellular carcinoma GDF15 levels positively correlated with increased frequencies of Tregs in the tumor [310]. Ablation of GDF15 resulted in slowed tumor growth, prolonged survival of the mice which was accompanied by a decrease in Treg frequencies [310]. This led the authors to hypothesize that GDF15 plays an important role for the generation or regulation of Tregs. Indeed, in *in vitro* Treg induction assays GDF15 induced high frequencies of Tregs similar to TGF-β which also correlated with reduced proliferation of naïve T cells and increased expression of key suppressive molecules such as CTLA-4 and TIGIT [310]. Noteworthy, in allergic airway inflammation it was shown that lung regulatory Tregs are a major source of GDF15 which could represent a positive feedback loop [311]. Thus, IMU-838 might also induce expression of GDF15 to increase beta bell survival and strengthen immune tolerance.

In order to test this hypothesis GFD15 expression in the pancreas will be determined with pancreatic immunofluorescence staining in future experiments. As an additional model to probe an influence of a DHODH inhibition/GDF15 axis we propose a model of streptozotocin (STZ)-accelerated T1D in NOD mice. STZ is a beta cell toxin thus inducing diabetes through directly decreasing beta cell mass [312]. Multiple injections of low-dose STZ accelerates autoimmune-mediated T1D in young, pre-diabetic NOD mice which was accompanied by increased insulitis [313]. In a pilot experiment (data not shown) I observed that injecting young NOD mice with low-dose STZ for 5 consecutive days resulted in the development of diabetes after 8-12 days after the last injection. We are planning to give NOD mice low-dose STZ and treat them with IMU-838 throughout the experiment. Before the development of overt diabetes, a potential protective role of IMU-838 and GDF15 to reduce the STZ-induced beta cell toxicity and beta cell death will be analyzed.

Additionally, the impact of IMU-838 can also be assessed in a model of accelerated T1D induced by CY [314]. Here, the development of T1D is mediated by the immunomodulatory properties of CY, which is attributed to a reduction of Tregs [315]. Therefore, while the STZ-induced T1D model aims to decipher a potential mechanism of IMU-838 to support beta cell survival the CY-induced T1D model probes IMU-838 for the potential to enhance immune tolerance. Surely, the final mechanism by which IMU-838 might be able to decrease incidences of T1D is not based on one or the other mechanism but is most likely an interplay between all of them. However, the mechanism by which diabetes development is accelerated in the different models might give priority to one of the properties of IMU-838 to achieve reduce immune activation, pancreas infiltration and diabetes progression.

In vivo studies so far were performed only using IMU-838 which is mainly attributed to its advances clinical profile. However, in all mentioned animal models mice are going to be treated with IMU-935 as well. Results suggesting its potential to reduce proinflammatory responses *in vitro* as well as *in vivo* and to induce immune tolerance mechanism make us confident towards a positive outcome. As mentioned before the IC_{50} of DHODH

inhibition of IMU-935 is ten-times lower than the one for RORγt inhibition [271]. Whether a potential DHODH inhibition/GDF15 axis will positively influence beta cell survival in response to IMU-935 treatment will need to be analyzed in the respective models. Most likely in *in vivo* models RORγt inhibition will play a bigger role in the effect of IMU-935 than observed in our *in vitro* results. Observations with other RORγt inverse agonists point towards a positive impact on immune tolerance and T1D development. The group of Megan Levings investigated the role of a RORC2 inverse agonist (BMS-336) on T_H17 and T_H17-like Treg responses [276]. While BMS-336 inhibited human T_H17 differentiation their data indicated improved stability of T_H17-like Tregs in presence of BMS-366 in inflammatory conditions [276]. They could show that T_H17-like Tregs that are characterized in human peripheral blood as $CD4^+CD25^{hi}CD127^{lo}CXCR3^-CCR4^+CCR6^+$ lose Foxp3 expression when cultured under pro-inflammatory conditions and Tregs present with an increased TSDR methylation. Presence of BMS-366 reduced loss of Foxp3 expression [276]. Likewise, a RORγ-specific synthetic ligand, increased Treg induction and a selective RORα/γ inverse agonist prevented the development of T1D in NOD mice [233, 234].

IMU-935 and -838 are already tested in clinical trials for the treatment of psoriasis and MS, respectively. IMU-935 just started its phase 1 part C clinical trial because part A and B showed promising safely results [224]. IMU-838 has already a more advanced clinical profile. A good safety profile and a reduction in new magnetic resonance imaging lesions resulted in a large phase 3 clinical trial for treatment of MS (ENSURE-1, NCT05134441) [217]. The approval of the first immunotherapy for T1D by the FDA in November 2022 is a milestone for the treatment of T1D [177]. Teplizumab was shown to delay the progression of overt T1D in at-risk patients by around 3 years [172, 173]. This lays the foundation for the development of new therapies for T1D which will for sure be needed to prevent or even cure T1D. Foremost, general immune suppression as observed for Teplizumab has many side effects including lymphopenia and rash [172]. These side effects might lead to the treatment refusal of some patients. In addition, the delayed progression will be beneficial for reducing long-term complications related to T1D, but a continued delay might need more infusions of Teplizumab or a combinatorial treatment with other drugs. In contrast, IMU-838 is more selective in its immune suppression. It exclusively inhibits activated lymphocytes and does not impact their basic homeostatic functions. This resulted in a good safety and tolerability profile without the most prominent side effect of Teplizumab, lymphopenia [172, 217]. While an increased risk for infects which is associated with lymphopenia and other immunosuppressants was not observed in clinical trials with Teplizumab, IMU-838 itself has antiviral properties [316]. This not only contributes to the good safety and tolerability profile but it also implies IMU-838 as a good candidate for a prospective preventive trial. Viral infections early in life have been implicated in the T1D [49]. To that end a vaccine specific for coxsackie virus has been discussed for a preventive option [317]. Indeed, a study correlated the introduction of the rotavirus vaccination in the US with a decrease in T1D incidences [318]. Thus, preventing viral

infections by IMU-838, together with reduced immune activation and a potential beneficial impact on beta cell survival might help to slow down the progression to the overt disease. The latter might be particularly important because recent reports show that beta cells themselves also play a role in the disease pathogenesis, including their fragility and senescence [13, 14]. Noteworthy, the aforementioned combinatorial treatment strategy are discussed to involve first an immune-suppressing drug while a secondary therapy could improve Tregs and immune tolerance [319]. We show here *in vitro* and in preliminary *in vivo* results that IMU-838 might be able to shape an environment which is beneficial for Treg induction and stability. Thus, IMU-838 together with another drug which supports or induces Tregs might be a beneficial combinatorial treatment or prevention strategy.

All in all, the data presented in this thesis show that IMU-935 and -838 both improve *in vitro* Treg induction with increased expression of RORγt in $CD4^+$ T cells and Tregs and an anti-inflammatory phenotype especially in conditions of strong immune activation. This indicated that by targeting DHODH and/or RORγt immune activation during autoimmune T1D can be reduced which results in a broader window of opportunity to induce and stabilize Tregs. Mechanistically, I could show that *in vitro* the observed effects are attributed to DHODH inhibition. Furthermore, IL6Rα signaling influenced the impact on Treg induction and RORγt expression in response to the drug candidates. Treatment of a T1D mouse models which is characterized by strong immune activation and a fast disease progressing with IMU-838 reduced T1D incidences which was accompanied by reduced immune activation and a trend towards improved immune tolerance. Overall, this suggests that IMU-838 and -935 are potentially good candidates for new treatment options for T1D and represent an opportunity for prospective clinical trials in the future.

References

1. Daniel C, Nolting J, von Boehmer H. Mechanisms of self-nonself discrimination and possible clinical relevance. Immunotherapy. 2009;1(4):631-44.

2. Kappler JW, Roehm N, Marrack P. T cell tolerance by clonal elimination in the thymus. Cell. 1987;49(2):273-80.

3. Ramsdell F, Lantz T, Fowlkes BJ. A nondeletional mechanism of thymic self tolerance. Science. 1989;246(4933):1038-41.

4. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155:1151-64.

5. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol. 2003;4(4):330-6.

6. Wang L, Wang FS, Gershwin ME. Human autoimmune diseases: a comprehensive update. J Intern Med. 2015;278(4):369-95.

7. Mbongue J, Nicholas D, Firek A, Langridge W. The role of dendritic cells in tissue-specific autoimmunity. J Immunol Res. 2014;2014:857143.

8. Patterson CC, Harjutsalo V, Rosenbauer J, Neu A, Cinek O, Skrivarhaug T, Rami-Merhar B, Soltesz G, Svensson J, Parslow RC, Castell C, Schoenle EJ, Bingley PJ, Dahlquist G, Jarosz-Chobot PK, Marciulionyte D, Roche EF, Rothe U, Bratina N, Ionescu-Tirgoviste C, Weets I, Kocova M, Cherubini V, Rojnic Putarek N, deBeaufort CE, Samardzic M, Green A. Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989-2013: a multicentre prospective registration study. Diabetologia. 2019;62(3):408-17.

9. Martins CP, Piganelli JD. Targeting T cell Metabolism to Combat Autoimmunity: Implications for the Future of Type 1 Diabetes Therapeutics. Immunometabolism. 2020;2(2):e200010.

10. Aronoff SL, Berkowitz K, Shreiner B, Want L. Glucose Metabolism and Regulation: Beyond Insulin and Glucagon. Diabetes Spectrum. 2004;17(3):183-90.

11. Tersey SA, Nishiki Y, Templin AT, Cabrera SM, Stull ND, Colvin SC, Evans-Molina C, Rickus JL, Maier B, Mirmira RG. Islet beta-cell endoplasmic reticulum stress precedes the onset of type 1 diabetes in the nonobese diabetic mouse model. Diabetes. 2012;61(4):818-27.

12. Marhfour I, Lopez XM, Lefkaditis D, Salmon I, Allagnat F, Richardson SJ, Morgan NG, Eizirik DL. Expression of endoplasmic reticulum stress markers in the islets of patients with type 1 diabetes. Diabetologia. 2012;55(9):2417-20.

13. Thompson PJ, Shah A, Ntranos V, Van Gool F, Atkinson M, Bhushan A. Targeted Elimination of Senescent Beta Cells Prevents Type 1 Diabetes. Cell Metab. 2019;29(5):1045-60 e10.

14. Dooley J, Tian L, Schonefeldt S, Delghingaro-Augusto V, Garcia-Perez JE, Pasciuto E, Di Marino D, Carr EJ, Oskolkov N, Lyssenko V, Franckaert D, Lagou V, Overbergh L, Vandenbussche J, Allemeersch J, Chabot-Roy G, Dahlstrom JE, Laybutt DR, Petrovsky N, Socha L, Gevaert K, Jetten AM, Lambrechts D, Linterman MA, Goodnow CC, Nolan CJ, Lesage S, Schlenner SM, Liston A. Genetic predisposition for beta cell fragility underlies type 1 and type 2 diabetes. Nat Genet. 2016;48(5):519-27.

15. Mallone R, Eizirik DL. Presumption of innocence for beta cells: why are they vulnerable autoimmune targets in type 1 diabetes? Diabetologia. 2020;63(10):1999-2006.

16. Warncke K, Weiss A, Achenbach P, von dem Berge T, Berner R, Casteels K, Groele L, Hatzikotoulas K, Hommel A, Kordonouri O, Elding Larsson H, Lundgren M, Marcus BA, Snape MD, Szypowska A, Todd JA, Bonifacio E, Ziegler AG, Gppad, Groups POS. Elevations in blood glucose before and after the appearance of islet autoantibodies in children. J Clin Invest. 2022;132(20).

17. Gepts W. Pathologic Anatomy of the Pancreas in Juvenile Diabetes Mellitus. Diabetes. 1965;14(10):619-33.

18. In't Veld P, Lievens D, De Grijse J, Ling Z, Van der Auwera B, Pipeleers-Marichal M, Gorus F, Pipeleers D. Screening for insulitis in adult autoantibody-positive organ donors. Diabetes. 2007;56(9):2400-4.

19. Turley S, Poirot L, Hattori M, Benoist C, Mathis D. Physiological beta cell death triggers priming of self-reactive T cells by dendritic cells in a type-1 diabetes model. J Exp Med. 2003;198(10):1527-37.

20. Hanninen A, Jalkanen S, Salmi M, Toikkanen S, Nikolakaros G, Simell O. Macrophages, T cell receptor usage, and endothelial cell activation in the pancreas at the onset of insulin-dependent diabetes mellitus. J Clin Invest. 1992;90(5):1901-10.

21. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol. 2009;155(2):173-81.

22. Magnuson AM, Thurber GM, Kohler RH, Weissleder R, Mathis D, Benoist C. Population dynamics of islet-infiltrating cells in autoimmune diabetes. Proc Natl Acad Sci U S A. 2015;112(5):1511-6.

23. Harada M, Makino S. Suppression of overt diabetes in NOD mice by anti-thymocyte serum or anti-Thy 1, 2 antibody. Jikken Dobutsu. 1986;35(4):501-4.

24. Chatenoud L, Thervet E, Primo J, Bach JF. Anti-CD3 antibody induces long-term remission of overt autoimmunity in nonobese diabetic mice. Proc Natl Acad Sci U S A. 1994;91:123-7.

25. Coppieters KT, Dotta F, Amirian N, Campbell PD, Kay TW, Atkinson MA, Roep BO, von Herrath MG. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med. 2012;209(1):51-60.

26. Wong CP, Stevens R, Long B, Li L, Wang Y, Wallet MA, Goudy KS, Frelinger JA, Tisch R. Identical beta cell-specific CD8(+) T cell clonotypes typically reside in both peripheral blood lymphocyte and pancreatic islets. J Immunol. 2007;178(3):1388-95.

27. Ogura H, Preston-Hurlburt P, Perdigoto AL, Amodio M, Krishnaswamy S, Clark P, Yu H, Egli D, Fouts A, Steck AK, Herold KC. Identification and Analysis of Islet Antigen-Specific CD8(+) T Cells with T Cell Libraries. J Immunol. 2018;201(6):1662-70.

28. Chee J, Ko HJ, Skowera A, Jhala G, Catterall T, Graham KL, Sutherland RM, Thomas HE, Lew AM, Peakman M, Kay TW, Krishnamurthy B. Effector-memory T cells develop in islets and report islet pathology in type 1 diabetes. J Immunol. 2014;192(2):572-80.

29. Graham KL, Krishnamurthy B, Fynch S, Mollah ZU, Slattery R, Santamaria P, Kay TW, Thomas HE. Autoreactive cytotoxic T lymphocytes acquire higher expression of cytotoxic effector markers in the islets of NOD mice after priming in pancreatic lymph nodes. Am J Pathol. 2011;178(6):2716-25.

30. Bottazzo GF, Dean BM, McNally JM, Mackay EH, Swift PGF, Gamble DR. In Situ Characterization of Autoimmune Phenomena and Expression of HLA Molecules in the Pancreas in Diabetic Insulitis. N Engl J Med. 1985;313(6):353-60.

31. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M. Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. J Clin Investig. 1993;92(5):2313-22.

32. Knight RR, Kronenberg D, Zhao M, Huang GC, Eichmann M, Bulek A, Wooldridge L, Cole DK, Sewell AK, Peakman M, Skowera A. Human beta-cell killing by autoreactive preproinsulin-specific CD8 T cells is predominantly granule-mediated with the potency dependent upon T-cell receptor avidity. Diabetes. 2013;62(1):205-13.

33. Trivedi P, Graham KL, Krishnamurthy B, Fynch S, Slattery RM, Kay TW, Thomas HE. Perforin facilitates beta cell killing and regulates autoreactive CD8+ T-cell responses to antigen in mouse models of type 1 diabetes. Immunol Cell Biol. 2016;94(4):334-41.

34. Suk K, Kim S, Kim Y-H, Kim K-A, Chang I, Yagita H, Shong M, Lee M-S. IFN-γ/ TNF-α Synergism as the Final Effector in Autoimmune Diabetes: A Key Role for STAT1/IFN Regulatory Factor-1 Pathway in Pancreatic β Cell Death. J Immunol. 2001;166(7):4481-9.

35. Calderon B, Suri A, Pan XO, Mills JC, Unanue ER. IFN-γ-Dependent Regulatory Circuits in Immune Inflammation Highlighted in Diabetes. J Immunol. 2008;181(10):6964-74.

36. Foulis AK, Farquharson MA, Hardman R. Aberrant expression of Class II major histocompatibility complex molecules by B cells and hyperexpression of Class I major histocompatibility complex molecules by insulin containing islets in Type 1 (insulin-dependent) diabetes mellitus. Diabetologia. 1987;30(5):333-43.

37. Richardson SJ, Rodriguez-Calvo T, Gerling IC, Mathews CE, Kaddis JS, Russell MA, Zeissler M, Leete P, Krogvold L, Dahl-Jorgensen K, von Herrath M, Pugliese A, Atkinson MA, Morgan NG. Islet cell hyperexpression of HLA class I antigens: a defining feature in type 1 diabetes. Diabetologia. 2016;59(11):2448-58.

38. Russell MA, Redick SD, Blodgett DM, Richardson SJ, Leete P, Krogvold L, Dahl-Jorgensen K, Bottino R, Brissova M, Spaeth JM, Babon JAB, Haliyur R, Powers AC, Yang C, Kent SC, Derr AG, Kucukural A, Garber MG, Morgan NG, Harlan DM. HLA

Class II Antigen Processing and Presentation Pathway Components Demonstrated by Transcriptome and Protein Analyses of Islet beta-Cells From Donors With Type 1 Diabetes. Diabetes. 2019;68(5):988-1001.

39. Phillips JM, Parish NM, Raine T, Bland C, Sawyer Y, De La Peña H, Cooke A. Type 1 diabetes development requires both CD4+ and CD8+ T cells and can be reversed by non-depleting antibodies targeting both T cell populations. Rev Diabet Stud. 2009;6(2):97-103.

40. Durinovic-Bello I, Schlosser M, Riedl M, Maisel N, Rosinger S, Kalbacher H, Deeg M, Ziegler M, Elliott J, Roep BO, Karges W, Boehm BO. Pro- and anti-inflammatory cytokine production by autoimmune T cells against preproinsulin in HLA-DRB1*04, DQ8 Type 1 diabetes. Diabetologia. 2004;47(3):439-50.

41. Arif S, Tree TI, Astill TP, Tremble JM, Bishop AJ, Dayan CM, Roep BO, Peakman M. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. J Clin Invest. 2004;113(3):451-63.

42. Li CR, Mueller EE, Bradley LM. Islet antigen-specific Th17 cells can induce TNF-alpha-dependent autoimmune diabetes. J Immunol. 2014;192(4):1425-32.

43. Arif S, Moore F, Marks K, Bouckenooghe T, Dayan CM, Planas R, Vives-Pi M, Powrie J, Tree T, Marchetti P, Huang GC, Gurzov EN, Pujol-Borrell R, Eizirik DL, Peakman M. Peripheral and islet interleukin-17 pathway activation characterizes human autoimmune diabetes and promotes cytokine-mediated beta-cell death. Diabetes. 2011;60(8):2112-9.

44. Ferraro A, Socci C, Stabilini A, Valle A, Monti P, Piemonti L, Nano R, Olek S, Maffi P, Scavini M, Secchi A, Staudacher C, Bonifacio E, Battaglia M. Expansion of Th17 cells and functional defects in T regulatory cells are key features of the pancreatic lymph nodes in patients with type 1 diabetes. Diabetes. 2011;60(11):2903-13.

45. Reinert-Hartwall L, Honkanen J, Salo HM, Nieminen JK, Luopajarvi K, Harkonen T, Veijola R, Simell O, Ilonen J, Peet A, Tillmann V, Knip M, Vaarala O, Group DS, Group DS. Th1/Th17 plasticity is a marker of advanced beta cell autoimmunity and impaired glucose tolerance in humans. J Immunol. 2015;194(1):68-75.

46. Calderon B, Suri A, Unanue ER. In CD4+ T-cell-induced diabetes, macrophages are the final effector cells that mediate islet beta-cell killing: studies from an acute model. Am J Pathol. 2006;169(6):2137-47.

47. Serrano-Rìos M, Goday A, Martìnez Larrad T. Migrant populations and the incidence of Type 1 diabetes mellitus: an overview of the literature with a focus on the Spanish-heritage countries in Latin America. Diabetes/Metabolism Research and Reviews. 1999;15(2):113-32.

48. Akerblom HK, Knip M. Putative environmental factors in Type 1 diabetes. Diabetes Metab Rev. 1998;14(1):31-67.

49. Hyoty H. Viruses in type 1 diabetes. Pediatr Diabetes. 2016;17 Suppl 22:56-64.

50. Bonifacio E, Hummel M, Walter M, Schmid S, Ziegler A-G. IDDM1 and Multiple Family History of Type 1 Diabetes Combine to Identify Neonates at High Risk for Type 1 Diabetes. Diabetes Care. 2004;27(11):2695-700.

51. Winkler C, Jolink M, Knopff A, Kwarteng NA, Achenbach P, Bonifacio E, Ziegler AG. Age, HLA, and Sex Define a Marked Risk of Organ-Specific Autoimmunity in First-Degree Relatives of Patients With Type 1 Diabetes. Diabetes Care. 2019;42(9):1684-91.

52. Bell GI, Horita S, Karam JH. A Polymorphic Locus Near the Human Insulin Gene Is Associated with Insulin-dependent Diabetes Melliitus. Diabetes. 1984;33(2):176-83.

53. Nisticò L, Buzzetti R, Pritchard LE, Van der Auwera B, Giovannini C, Bosi E, Martinez Larrad MT, Serrano Rios M, Chow CC, Cockram CS, Jacobs K, Mijovic C, Bain SC, Barnett AH, Vandewalle CL, Schuit F, Gorus FK, Belgian Diabetes R, Tosi R, Pozzilli P, Todd JA. The CTLA-4 Gene Region of Chromosome 2q33 Is Linked to, and Associated with, Type 1 Diabetes. Human Molecular Genetics. 1996;5(7):1075-80.

54. Lowe CE, Cooper JD, Brusko T, Walker NM, Smyth DJ, Bailey R, Bourget K, Plagnol V, Field S, Atkinson M, Clayton DG, Wicker LS, Todd JA. Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes. Nat Genet. 2007;39(9):1074-82.

55. Insel RA, Dunne JL, Atkinson MA, Chiang JL, Dabelea D, Gottlieb PA, Greenbaum CJ, Herold KC, Krischer JP, Lernmark A, Ratner RE, Rewers MJ, Schatz DA, Skyler JS, Sosenko JM, Ziegler AG. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care. 2015;38(10):1964-74.

56. Palmer JP, Asplin CM, Clemons P, Lyen K, Tatpati O, Raghu PK, Paquette TL. Insulin Antibodies in Insulin-Dependent Diabetics Before Insulin Treatment. Science. 1983;222(4630):1337-9.

57. Baekkeskov S, Aanstoot H-J, Christgai S, Reetz A, Solimena M, Cascalho M, Folli F, Richter-Olesen H, Camilli P-D. Identification of the 64K autoantigen in insulin-dependent diabetes as the GABA-synthesizing enzyme glutamic acid decarboxylase. Nature. 1990;347(6289):151-6.

58. Lan MS, Lu JIA, Goto Y, Notkins AL. Molecular Cloning and Identification of a Receptor-Type Protein Tyrosine Phosphatase, IA-2, from Human Insulinoma. DNA and Cell Biology. 1994;13(5):505-14.

59. Rabin DU, Pleasic SM, Shapiro JA, Yoo-Warren H, Oles J, Hicks JM, Goldstein DE, Rae PM. Islet cell antigen 512 is a diabetes-specific islet autoantigen related to protein tyrosine phosphatases. The Journal of Immunology. 1994;152(6):3183-8.

60. Wenzlau JM, Juhl K, Yu L, Moua O, Sarkar SA, Gottlieb P, Rewers M, Eisenbarth GS, Jensen J, Davidson HW, Hutton JC. The cation efflux transporter ZnT8 (Slc30A8) is a major autoantigen in human type 1 diabetes. Proceedings of the National Academy of Sciences. 2007;104(43):17040-5.

61. Achenbach P, Hummel M, Thumer L, Boerschmann H, Hofelmann D, Ziegler AG. Characteristics of rapid vs slow progression to type 1 diabetes in multiple islet autoantibody-positive children. Diabetologia. 2013;56(7):1615-22.

62. Ziegler AG, Rewers M, Simell O, Simell T, Lempainen J, Steck A, Winkler C, Ilonen J, Veijola R, Knip M, Bonifacio E, Eisenbarth GS. Seroconversion to Multiple Islet Autoantibodies and Risk of Progression to Diabetes in Children. JAMA. 2013;309(23):2473-9.

63. Ziegler AG, Kick K, Bonifacio E, Haupt F, Hippich M, Dunstheimer D, Lang M, Laub O, Warncke K, Lange K, Assfalg R, Jolink M, Winkler C, Achenbach P, Fr1da Study G. Yield of a Public Health Screening of Children for Islet Autoantibodies in Bavaria, Germany. JAMA. 2020;323(4):339-51.

64. Raab J, Haupt F, Scholz M, Matzke C, Warncke K, Lange K, Assfalg R, Weininger K, Wittich S, Lobner S, Beyerlein A, Nennstiel-Ratzel U, Lang M, Laub O, Dunstheimer D, Bonifacio E, Achenbach P, Winkler C, Ziegler AG, Fr1da Study G. Capillary blood islet autoantibody screening for identifying pre-type 1 diabetes in the general population: design and initial results of the Fr1da study. BMJ Open. 2016;6(5):e011144.

65. Achenbach P, Hippich M, Zapardiel-Gonzalo J, Karges B, Holl RW, Petrera A, Bonifacio E, Ziegler AG, DiMelli Study g, group DPVS. A classification and regression tree analysis identifies subgroups of childhood type 1 diabetes. EBioMedicine. 2022;82:104118.

66. Anand V, Li Y, Liu B, Ghalwash M, Koski E, Ng K, Dunne JL, Jonsson J, Winkler C, Knip M, Toppari J, Ilonen J, Killian MB, Frohnert BI, Lundgren M, Ziegler AG, Hagopian W, Veijola R, Rewers M, Group TDS. Islet Autoimmunity and HLA Markers of Presymptomatic and Clinical Type 1 Diabetes: Joint Analyses of Prospective Cohort Studies in Finland, Germany, Sweden, and the U.S. Diabetes Care. 2021.

67. Elding Larsson H, Vehik K, Bell R, Dabelea D, Dolan L, Pihoker C, Knip M, Veijola R, Lindblad B, Samuelsson U, Holl R, Haller MJ, Group TS, Group SS, Swediabkids Study G, Group DPVS, Finnish Diabetes Registry Study G. Reduced prevalence of diabetic ketoacidosis at diagnosis of type 1 diabetes in young children participating in longitudinal follow-up. Diabetes Care. 2011;34(11):2347-52.

68. Sakaguchi S, Vignali DAA, Rudensky AY, Niec RE, Waldmann H. The plasticity and stability of regulatory T cells. Nat Rev Immunol. 2013;13(6):461-7.

69. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol. 1995;155(3):1151-64.

70. Hori S, Nomura T, Sakaguchi S. Control of Regulatory T Cell Development by the Transcription Factor Foxp3. Science. 2003;299(5609):1057-61.

71. Khattri R, Kasprowicz D, Cox T, Mortrud M, Appleby MW, Brunkow ME, Ziegler SF, Ramsdell F. The amount of scurfin protein determines peripheral T cell number and responsiveness. J Immunol. 2001;167(11):6312-20.

72. Khattri R, Cox T, Yasayko SA, Ramsdell F. An essential role for Scurfin in CD4+CD25+ T regulatory cells. Nat Immunol. 2003;4(4):337-42.

73. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. Nat Genet. 2001;27(1):20-1.

74. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko S-A, Wilkinson JE, Galas D, Ziegler SF, Ramsdell F. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. Nat Genet. 2001;27(1):68-73.

75. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. An Essential Role for Interleukin 10 in the Function of Regulatory T Cells That Inhibit Intestinal Inflammation. J Exp Med. 1999;190(7):995-1004.

76. Fahlén L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, Powrie F. T cells that cannot respond to TGF-β escape control by CD4+CD25+ regulatory T cells. J Exp Med. 2005;201(5):737-46.

77. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. Immunity. 2004;21(4):589-601.

78. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. J Immunol. 2005;174(4):1783-6.

79. Pandiyan P, Zheng L, Ishihara S, Reed J, Lenardo MJ. CD4+CD25+Foxp3+ regulatory T cells induce cytokine deprivation-mediated apoptosis of effector CD4+ T cells. Nat Immunol. 2007;8(12):1353-62.

80. Liang B, Workman C, Lee J, Chew C, Dale BM, Colonna L, Flores M, Li N, Schweighoffer E, Greenberg S, Tybulewicz V, Vignali D, Clynes R. Regulatory T cells inhibit dendritic cells by lymphocyte activation gene-3 engagement of MHC class II. J Immunol. 2008;180(9):5916-26.

81. Misra N, Bayry J, Lacroix-Desmazes S, Kazatchkine MD, Kaveri SV. Cutting Edge: Human CD4+CD25+ T Cells Restrain the Maturation and Antigen-Presenting Function of Dendritic Cells. J Immunol. 2004;172(8):4676-80.

82. Tadokoro CE, Shakhar G, Shen S, Ding Y, Lino AC, Maraver A, Lafaille JJ, Dustin ML. Regulatory T cells inhibit stable contacts between CD4+ T cells and dendritic cells in vivo. J Exp Med. 2006;203(3):505-11.

83. Oderup C, Cederbom L, Makowska A, Cilio CM, Ivars F. Cytotoxic T lymphocyte antigen-4-dependent down-modulation of costimulatory molecules on dendritic cells in CD4+ CD25+ regulatory T-cell-mediated suppression. Immunology. 2006;118(2):240-9.

84. Akkaya B, Oya Y, Akkaya M, Al Souz J, Holstein AH, Kamenyeva O, Kabat J, Matsumura R, Dorward DW, Glass DD, Shevach EM. Regulatory T cells mediate specific suppression by depleting peptide-MHC class II from dendritic cells. Nat Immunol. 2019;20(2):218-31.

85. Miller A, Lider O, Weiner HL. Antigen-driven bystander suppression after oral administration of antigens. J Exp Med. 1991;174(4):791-8.

86. Itoh M, Takahashi T, Sakaguchi N, Kuniyasu Y, Shimizu J, Otsuka F, Sakaguchi S. Thymus and Autoimmunity: Production of CD25+CD4+ Naturally Anergic and Suppressive T Cells as a Key Function of the Thymus in Maintaining Immunologic Self-Tolerance. J Immunol. 1999;162(9):5317-26.

87. Apostolou I, Sarukhan A, Klein L, von Boehmer H. Origin of regulatory T cells with known specificity for antigen. Nat Immunol. 2002;3(8):756-63.

88. Apostolou I, von Boehmer H. In Vivo Instruction of Suppressor Commitment in Naive T Cells. J Exp Med. 2004;199(10):1401-8.

89. Kretschmer K, Apostolou I, Hawiger D, Khazaie K, Nussenzweig MC, von Boehmer H. Inducing and expanding regulatory T cell populations by foreign antigen. Nature Immunology. 2005;6(12):1219-27.

90. Daniel C, Wennhold K, Kim HJ, von Boehmer H. Enhancement of antigen-specific Treg vaccination in vivo. Proc Natl Acad Sci U S A. 2010;107(37):16246-51.

91. Daniel C, Weigmann B, Bronson R, von Boehmer H. Prevention of type 1 diabetes in mice by tolerogenic vaccination with a strong agonist insulin mimetope. J Exp Med. 2011;208(7):1501-10.

92. Serr I, Scherm MG, Zahm AM, Schug J, Flynn VK, Hippich M, Kälin S, Becker M, Achenbach P, Nikolaev A, Gerlach K, Liebsch N, Loretz B, Lehr C-M, Kirchner B, Spornraft M, Haase B, Segars J, Küper C, Palmisano R, Waisman A, Willis RA, Kim W-U, Weigmann B, Kaestner KH, Ziegler A-G, Daniel C. A miRNA181aNFAT5 axis links impaired T cell tolerance induction with autoimmune type 1 diabete. Sci Transl Med. 2018;10(422).

93. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, Shokat KM, Fisher AG, Merkenschlager M. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc Natl Acad Sci U S A. 2008;105(22):7797-802.

94. Haxhinasto S, Mathis D, Benoist C. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J Exp Med. 2008;205(3):565-74.

95. Huynh A, DuPage M, Priyadharshini B, Sage PT, Quiros J, Borges CM, Townamchai N, Gerriets VA, Rathmell JC, Sharpe AH, Bluestone JA, Turka LA. Control of PI(3) kinase in Treg cells maintains homeostasis and lineage stability. Nat Immunol. 2015;16(2):188-96.

96. Scherm MG, Serr I, Zahm AM, Schug J, Bellusci S, Manfredini R, Salb VK, Gerlach K, Weigmann B, Ziegler AG, Kaestner KH, Daniel C. miRNA142-3p targets Tet2 and impairs Treg differentiation and stability in models of type 1 diabetes. Nat Commun. 2019;10(1):5697.

97. Serr I, Furst RW, Achenbach P, Scherm MG, Gokmen F, Haupt F, Sedlmeier EM, Knopff A, Shultz L, Willis RA, Ziegler AG, Daniel C. Type 1 diabetes vaccine candidates promote human Foxp3(+)Treg induction in humanized mice. Nat Commun. 2016;7:10991.

98. Nakayama M, Abiru N, Moriyama H, Babaya N, Liu E, Miao D, Yu L, Wegmann DR, Hutton JC, Elliott JF, Eisenbarth GS. Prime role for an insulin epitope in the development of type 1 diabetes in NOD mice. Nature. 2005;435(7039):220-3.

99. Jaeckel E, Lipes MA, von Boehmer H. Recessive tolerance to preproinsulin 2 reduces but does not abolish type 1 diabetes. Nat Immunol. 2004;5(10):1028-35.

100. Wegmann DR, Norbury-Glaseru M, Danielf D. Insulin-specific T cells are a predominant component of islet infiltrates in pre-diabetic NOD mice. European Journal of Immunology. 1994;24(8):1853-7.

101. Daniel D, Gill RG, Schloot N, Wegmann D. Epitope specificity, cytokine production profile and diabetogenic activity of insulin-specific T cell clones isolated from NOD mice. European Journal of Immunology. 1995;25(4):1056-62.

102. Alleva DG, Crowe PD, Jin L, Kwok WW, Ling N, Gottschalk M, Conlon PJ, Gottlieb PA, Putnam AL, Gaur A. A disease-associated cellular immune response in type 1 diabetics to an immunodominant epitope of insulin. J Clin Invest. 2001;107(2):173-80.

103. Hattori M, Buse JB, Jackson RA, Glimcher L, Dorf ME, Minami M, Makino S, Moriwaki K, Kuzuya H, Imura H, Strauss WM, Seidman JG, Eisenbarth GS. The NOD Mouse: Recessive Diabetogenic Gene in the Major Histocompatibility Complex. Science. 1986;231(4739):733-5.

104. Prochazka M, Leiter EH, Serreze DV, Coleman DL. Three Recessive Loci Required for Insulin-Dependent Diabetes in Nonobese Diabetic Mice. Science. 1987;237(4812):286-9.

105. Acha-Orbea H, McDevitt HO. The first external domain of the nonobese diabetic mouse class II I-A beta chain is unique. Proceedings of the National Academy of Sciences. 1987;84(8):2435-9.

106. Stadinski BD, Zhang L, Crawford F, Marrack P, Eisenbarth GS, Kappler JW. Diabetogenic T cells recognize insulin bound to IAg7 in an unexpected, weakly binding register. Proceedings of the National Academy of Sciences. 2010;107(24):10978-83.

107. Wucherpfennig KW, Sethi D. T cell receptor recognition of self and foreign antigens in the induction of autoimmunity. Semin Immunol. 2011;23(2):84-91.

108. Crawford F, Stadinski B, Jin N, Michels A, Nakayama M, Pratt P, Marrack P, Eisenbarth G, Kappler JW. Specificity and detection of insulin-reactive CD4+ T cells in type 1 diabetes in the nonobese diabetic (NOD) mouse. Proc Natl Acad Sci U S A. 2011;108(40):16729-34.

109. Floess S, Freyer J, Siewert C, Baron U, Olek S, Polansky J, Schlawe K, Chang H-D, Bopp T, Schmitt E, Klein-Hessling S, Serfling E, Hamann A, Huehn J. Epigenetic Control of the foxp3 Locus in Regulatory T Cells. PLOS Biology. 2007;5(2):e38.

110. Polansky JK, Kretschmer K, Freyer J, Floess S, Garbe A, Baron U, Olek S, Hamann A, von Boehmer H, Huehn J. DNA methylation controls Foxp3 gene expression. Eur J Immunol. 2008;38(6):1654-63.

111. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature. 2010;463(7282):808-12.

112. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol. 2006;176(6):3593-602.

113. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med. 2007;204(7):1543-51.

114. Ouyang W, Beckett O, Ma Q, Paik JH, DePinho RA, Li MO. Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells. Nat Immunol. 2010;11(7):618-27.

115. Feng Y, van der Veeken J, Shugay M, Putintseva EV, Osmanbeyoglu HU, Dikiy S, Hoyos BE, Moltedo B, Hemmers S, Treuting P, Leslie CS, Chudakov DM, Rudensky

AY. A mechanism for expansion of regulatory T-cell repertoire and its role in self-tolerance. Nature. 2015;528(7580):132-6.

116. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, Turbachova I, Hamann A, Olek S, Huehn J. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. Eur J Immunol. 2007;37(9):2378-89.

117. Josefowicz SZ, Wilson CB, Rudensky AY. Cutting edge: TCR stimulation is sufficient for induction of Foxp3 expression in the absence of DNA methyltransferase 1. J Immunol. 2009;182(11):6648-52.

118. Yue X, Trifari S, Aijo T, Tsagaratou A, Pastor WA, Zepeda-Martinez JA, Lio CW, Li X, Huang Y, Vijayanand P, Lahdesmaki H, Rao A. Control of Foxp3 stability through modulation of TET activity. J Exp Med. 2016;213(3):377-97.

119. Komatsu N, Okamoto K, Sawa S, Nakashima T, Oh-hora M, Kodama T, Tanaka S, Bluestone JA, Takayanagi H. Pathogenic conversion of Foxp3+ T cells into TH17 cells in autoimmune arthritis. Nat Med. 2014;20(1):62-8.

120. Bailey-Bucktrout SL, Martinez-Llordella M, Zhou X, Anthony B, Rosenthal W, Luche H, Fehling HJ, Bluestone JA. Self-antigen-driven activation induces instability of regulatory T cells during an inflammatory autoimmune response. Immunity. 2013;39(5):949-62.

121. Zhou X, Bailey-Bucktrout SL, Jeker LT, Penaranda C, Martinez-Llordella M, Ashby M, Nakayama M, Rosenthal W, Bluestone JA. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. Nat Immunol. 2009;10(9):1000-7.

122. Miyao T, Floess S, Setoguchi R, Luche H, Fehling HJ, Waldmann H, Huehn J, Hori S. Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells. Immunity. 2012;36(2):262-75.

123. Komatsu N, Mariotti-Ferrandiz ME, Wang Y, Malissen B, Waldmann H, Hori S. Heterogeneity of natural Foxp3 + T cells: A committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity. Proc Natl Acad Sci U S A. 2009; 106(6):1903-8.

124. Zhang Z, Zhang W, Guo J, Gu Q, Zhu X, Zhou X. Activation and Functional Specialization of Regulatory T Cells Lead to the Generation of Foxp3 Instability. J Immunol. 2017;198(7):2612-25.

125. Szabo SJ, Kim ST, Costa GL, Zhang X, Fathman CG, Glimcher LH. A novel transcription factor, T-bet, directs Th1 lineage commitment. Cell. 2000;100(6):655-69.

126. Koch MA, Tucker-Heard G, Perdue NR, Killebrew JR, Urdahl KB, Campbell DJ. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. Nat Immunol. 2009;10(6):595-602.

127. Levine AG, Mendoza A, Hemmers S, Moltedo B, Niec RE, Schizas M, Hoyos BE, Putintseva EV, Chaudhry A, Dikiy S, Fujisawa S, Chudakov DM, Treuting PM, Rudensky AY. Stability and function of regulatory T cells expressing the transcription factor T-bet. Nature. 2017;546(7658):421-5.

128. Lohoff M, Mittrücker H-W, Prechtl S, Bischof S, Sommer F, Kock S, Ferrick DA, Duncan GS, Gessner A, Mak TW. Dysregulated T helper cell differentiation in the absence of interferon regulatory factor 4. Proceedings of the National Academy of Sciences. 2002;99(18):11808-12.

129. Zhu J, Min B, Hu-Li J, Watson CJ, Grinberg A, Wang Q, Killeen N, Urban JF, Guo L, Paul WE. Conditional deletion of Gata3 shows its essential function in TH1-TH2 responses. Nat Immunol. 2004;5(11):1157-65.

130. Cretney E, Xin A, Shi W, Minnich M, Masson F, Miasari M, Belz GT, Smyth GK, Busslinger M, Nutt SL, Kallies A. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. Nat Immunol. 2011;12(4):304-11.

131. Zheng Y, Chaudhry A, Kas A, deRoos P, Kim JM, Chu TT, Corcoran L, Treuting P, Klein U, Rudensky AY. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses. Nature. 2009;458(7236):351-6.

132. Wohlfert EA, Grainger JR, Bouladoux N, Konkel JE, Oldenhove G, Ribeiro CH, Hall JA, Yagi R, Naik S, Bhairavabhotla R, Paul WE, Bosselut R, Wei G, Zhao K, Oukka M, Zhu J, Belkaid Y. GATA3 controls Foxp3(+) regulatory T cell fate during inflammation in mice. J Clin Invest. 2011;121(11):4503-15.

133. Wang Y, Su MA, Wan YY. An essential role of the transcription factor GATA-3 for the function of regulatory T cells. Immunity. 2011;35(3):337-48.

134. Ivanov, II, McKenzie BS, Zhou L, Tadokoro CE, Lepelley A, Lafaille JJ, Cua DJ, Littman DR. The orphan nuclear receptor RORgammat directs the differentiation program of proinflammatory IL-17+ T helper cells. Cell. 2006;126(6):1121-33.

135. Yang XO, Panopoulos AD, Nurieva R, Chang SH, Wang D, Watowich SS, Dong C. STAT3 Regulates Cytokine-mediated Generation of Inflammatory Helper T Cells*. Journal of Biological Chemistry. 2007;282(13):9358-63.

136. Chaudhry A, Rudra D, Treuting P, Samstein RM, Liang Y, Kas A, Rudensky AY. CD4+ Regulatory T Cells Control TH17 Responses in a Stat3-Dependent Manner. Science. 2009;326(5955):986-91.

137. Ohnmacht C, Park J-H, Cording S, Wing JB, Atarashi K, Obata Y, Gaboriau-Routhiau V, Marques R, Dulauroy S, Fedoseeva M, Busslinger M, Cerf-Bensussan N, Boneca IG, Voehringer D, Hase K, Honda K, Sakaguchi S, Eberl G. The microbiota regulates type 2 immunity through RORγt^{+} T cells. Science (New York, NY). 2015;349(6251):989-93.

138. Sefik E, Geva-Zatorsky N, Oh S, Konnikova L, Zemmour D, McGuire AM, Burzyn D, Ortiz-Lopez A, Lobera M, Yang J, Ghosh S, Earl A, Snapper SB, Jupp R, Kasper D, Mathis D, Benoist C. Individual intestinal symbionts induce a distinct population of RORγ^{+} regulatory T cells. 2015;349(6251):993-7.

139. Yang BH, Hagemann S, Mamareli P, Lauer U, Hoffmann U, Beckstette M, Fohse L, Prinz I, Pezoldt J, Suerbaum S, Sparwasser T, Hamann A, Floess S, Huehn J, Lochner M. Foxp3(+) T cells expressing RORgammat represent a stable regulatory T-cell effector lineage with enhanced suppressive capacity during intestinal inflammation. Mucosal Immunol. 2016;9(2):444-57.

140. Mangan PR, Harrington LE, O'Quinn DB, Helms WS, Bullard DC, Elson CO, Hatton RD, Wahl SM, Schoeb TR, Weaver CT. Transforming growth factor-beta induces development of the T(H)17 lineage. Nature. 2006;441(7090):231-4.

141. Bettelli E, Carrier Y, Gao W, Korn T, Strom TB, Oukka M, Weiner HL, Kuchroo VK. Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. Nature. 2006;441(7090):235-8.

142. Kim BS, Lu H, Ichiyama K, Chen X, Zhang YB, Mistry NA, Tanaka K, Lee YH, Nurieva R, Zhang L, Yang X, Chung Y, Jin W, Chang SH, Dong C. Generation of RORgammat(+) Antigen-Specific T Regulatory 17 Cells from Foxp3(+) Precursors in Autoimmunity. Cell Rep. 2017;21(1):195-207.

143. Yan Y, Ramanan D, Rozenberg M, McGovern K, Rastelli D, Vijaykumar B, Yaghi O, Voisin T, Mosaheb M, Chiu I, Itzkovitz S, Rao M, Mathis D, Benoist C. Interleukin-6 produced by enteric neurons regulates the number and phenotype of microbe-responsive regulatory T cells in the gut. Immunity. 2021;54(3):499-513 e5.

144. Yang XO, Nurieva R, Martinez GJ, Kang HS, Chung Y, Pappu BP, Shah B, Chang SH, Schluns KS, Watowich SS, Feng XH, Jetten AM, Dong C. Molecular antagonism and plasticity of regulatory and inflammatory T cell programs. Immunity. 2008;29(1):44-56.

145. Zhou L, Lopes JE, Chong MM, Ivanov, II, Min R, Victora GD, Shen Y, Du J, Rubtsov YP, Rudensky AY, Ziegler SF, Littman DR. TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function. Nature. 2008;453(7192):236-40.

146. Ichiyama K, Yoshida H, Wakabayashi Y, Chinen T, Saeki K, Nakaya M, Takaesu G, Hori S, Yoshimura A, Kobayashi T. Foxp3 inhibits RORgammat-mediated IL-17A mRNA transcription through direct interaction with RORgammat. J Biol Chem. 2008;283(25):17003-8.

147. Taga T, Hibi M, Hirata Y, Yamasaki K, Yasukawa K, Matsuda T, Hirano T, Kishimoto T. Interleukin-6 triggers the association of its receptor with a possible signal transducer, gp130. Cell. 1989;58(3):573-81.

148. Tamura T, Udagawa N, Takahashi N, Miyaura C, Tanaka S, Yamada Y, Koishihara Y, Ohsugi Y, Kumaki K, Taga T, et al. Soluble interleukin-6 receptor triggers osteoclast formation by interleukin 6. Proc Natl Acad Sci U S A. 1993;90(24):11924-8.

149. Heink S, Yogev N, Garbers C, Herwerth M, Aly L, Gasperi C, Husterer V, Croxford AL, Moller-Hackbarth K, Bartsch HS, Sotlar K, Krebs S, Regen T, Blum H, Hemmer B, Misgeld T, Wunderlich TF, Hidalgo J, Oukka M, Rose-John S, Schmidt-Supprian M, Waisman A, Korn T. Trans-presentation of IL-6 by dendritic cells is required for the priming of pathogenic TH17 cells. Nat Immunol. 2017;18(1):74-85.

150. Rose-John S. IL-6 Trans-Signaling via the Soluble IL-6 Receptor: Importance for the Pro-Inflammatory Activities of IL-6. International Journal of Biological Sciences. 2012;8(9):1237-47.

151. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, Shoelson S, Mathis D. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 2009;15(8):930-9.

152. Sather BD, Treuting P, Perdue N, Miazgowicz M, Fontenot JD, Rudensky AY, Campbell DJ. Altering the distribution of Foxp3(+) regulatory T cells results in tissue-specific inflammatory disease. J Exp Med. 2007;204(6):1335-47.

153. D'Alessio FR, Tsushima K, Aggarwal NR, West EE, Willett MH, Britos MF, Pipeling MR, Brower RG, Tuder RM, McDyer JF, King LS. CD4+CD25+Foxp3+ Tregs resolve experimental lung injury in mice and are present in humans with acute lung injury. J Clin Invest. 2009;119(10):2898-913.

154. Burzyn D, Kuswanto W, Kolodin D, Shadrach JL, Cerletti M, Jang Y, Sefik E, Tan TG, Wagers AJ, Benoist C, Mathis D. A special population of regulatory T cells potentiates muscle repair. Cell. 2013;155(6):1282-95.

155. Panduro M, Benoist C, Mathis D. Tissue Tregs. Annu Rev Immunol. 2016;34:609-33.

156. Tan TG, Mathis D, Benoist C. Singular role for T-BET+CXCR3+ regulatory T cells in protection from autoimmune diabetes. Proc Natl Acad Sci U S A. 2016;113(49):14103-8.

157. Spence A, Purtha W, Tam J, Dong S, Kim Y, Ju CH, Sterling T, Nakayama M, Robinson WH, Bluestone JA, Anderson MS, Tang Q. Revealing the specificity of regulatory T cells in murine autoimmune diabetes. Proc Natl Acad Sci U S A. 2018;115(20):5265-70.

158. Feuerer M, Shen Y, Littman DR, Benoist C, Mathis D. How punctual ablation of regulatory T cells unleashes an autoimmune lesion within the pancreatic islets. Immunity. 2009;31(4):654-64.

159. Chen Z, Herman AE, Matos M, Mathis D, Benoist C. Where CD4+CD25+ T reg cells impinge on autoimmune diabetes. J Exp Med. 2005;202(10):1387-97.

160. Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, Cagnard N, Carpentier W, Tang Q, Bluestone J, Chatenoud L, Klatzmann D, Salomon BL, Piaggio E. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. J Exp Med. 2010;207(9):1871-8.

161. Kaestner KH, Powers AC, Naji A, Consortium H, Atkinson MA. NIH Initiative to Improve Understanding of the Pancreas, Islet, and Autoimmunity in Type 1 Diabetes: The Human Pancreas Analysis Program (HPAP). Diabetes. 2019;68(7):1394-402.

162. Campbell-Thompson M, Wasserfall C, Kaddis J, Albanese-O'Neill A, Staeva T, Nierras C, Moraski J, Rowe P, Gianani R, Eisenbarth G, Crawford J, Schatz D, Pugliese A, Atkinson M. Network for Pancreatic Organ Donors with Diabetes (nPOD): developing a tissue biobank for type 1 diabetes. Diabetes Metab Res Rev. 2012;28(7):608-17.

163. Wen X, Yang J, James E, Chow IT, Reijonen H, Kwok WW. Increased islet antigen–specific regulatory and effector CD4+ T cells in healthy individuals with the type 1 diabetes–protective haplotype. Science Immunology. 2020;5(44):eaax8767.

164. Bettini M, Bettini ML. Function, Failure, and the Future Potential of Tregs in Type 1 Diabetes. Diabetes. 2021;70(6):1211-9.

165. Bollyky J, Sanda S, Greenbaum CJ. Type 1 diabetes mellitus: primary, secondary, and tertiary prevention. Mt Sinai J Med. 2008;75(4):385-97.

166. Madsbad S, Faber OK, Binder C, McNair P, Christiansen C, Transbøl I. Prevalence of Residual Beta-cell Function in Insulin-Dependent Diabetics in Relation to Age at Onset and Duration of Diabetes. Diabetes. 1978;27(Supplement_1):262-4.

167. O'Meara NM, Sturis J, Herold Kc, Ostrega DM, Polonsky KS. Alterations in the Patterns of Insulin Secretion Before and After Diagnosis of IDDM. Diabetes Care. 1995;18(4):568-71.

168. Grajwer LA, Pildes RS, Horwitz DL, Rubenstein AH. Control of juvenile diabetes mellitus and its relationship to endogenous insulin secretion as measured by C-peptide immunoreactivity. The Journal of Pediatrics. 1977;90(1):42-8.

169. Hagopian W, Ferry RJ, Jr., Sherry N, Carlin D, Bonvini E, Johnson S, Stein KE, Koenig S, Daifotis AG, Herold KC, Ludvigsson J, Protege Trial I. Teplizumab preserves C-peptide in recent-onset type 1 diabetes: two-year results from the randomized, placebo-controlled Protege trial. Diabetes. 2013;62(11):3901-8.

170. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, Gitelman SE, Harlan DM, Xu D, Zivin RA, Bluestone JA. Anti-CD3 Monoclonal Antibody in New-Onset Type 1 Diabetes Mellitus. N Engl J Med. 2002;346(22):1692-8.

171. Keymeulen B, Vandemeulebroucke E, Ziegler AG, Mathieu C, Kaufman L, Hale G, Gorus F, Goldman M, Walter M, Candon S, Schandene L, Crenier L, De Block C, Seigneurin J-M, De Pauw P, Pierard D, Weets I, Rebello P, Bird P, Berrie E, Frewin M, Waldmann H, Bach J-F, Pipeleers D, Chatenoud L. Insulin Needs after CD3-Antibody Therapy in New-Onset Type 1 Diabetes. N Engl J Med. 2005;352(25):2598-608.

172. Herold KC, Bundy BN, Long SA, Bluestone JA, DiMeglio LA, Dufort MJ, Gitelman SE, Gottlieb PA, Krischer JP, Linsley PS, Marks JB, Moore W, Moran A, Rodriguez H, Russell WE, Schatz D, Skyler JS, Tsalikian E, Wherrett DK, Ziegler AG, Greenbaum CJ, Type 1 Diabetes TrialNet Study G. An Anti-CD3 Antibody, Teplizumab, in Relatives at Risk for Type 1 Diabetes. N Engl J Med. 2019;381(7):603-13.

173. Sims EK, Bundy BN, Stier K, Serti E, Lim N, Long SA, Geyer SM, Moran A, Greenbaum CJ, Evans-Molina C, Herold KC, null n, DiMeglio LA, Gitelman SE, Gottlieb PA, Marks JB, Moore W, Rodriguez H, Russell WE, Schatz D, Tsalikian E, Wherrett DK, Ziegler A-G. Teplizumab improves and stabilizes beta cell function in antibody-positive high-risk individuals. Sci Transl Med. 2021;13(583):eabc8980.

174. Cabrera SM, Wang X, Chen YG, Jia S, Kaldunski ML, Greenbaum CJ, Type 1 Diabetes TrialNet Canakinumab Study G, Mandrup-Poulsen T, Group AS, Hessner MJ. Interleukin-1 antagonism moderates the inflammatory state associated with Type 1 diabetes during clinical trials conducted at disease onset. Eur J Immunol. 2016;46(4):1030-46.

175. Greenbaum CJ, Serti E, Lambert K, Weiner LJ, Kanaparthi S, Lord S, Gitelman SE, Wilson DM, Gaglia JL, Griffin KJ, Russell WE, Raskin P, Moran A, Willi SM, Tsalikian E, DiMeglio LA, Herold KC, Moore WV, Goland R, Harris M, Craig ME, Schatz DA, Baidal DA, Rodriguez H, Utzschneider KM, Nel HJ, Soppe CL, Boyle KD, Cerosaletti K, Keyes-Elstein L, Long SA, Thomas R, McNamara JG, Buckner JH, Sanda S, Team IAES. IL-6 receptor blockade does not slow beta cell loss in new-onset type 1 diabetes. JCI Insight. 2021;6(21).

176. Quattrin T, Haller MJ, Steck AK, Felner EI, Li Y, Xia Y, Leu JH, Zoka R, Hedrick JA, Rigby MR, Vercruysse F. Golimumab and Beta-Cell Function in Youth with New-Onset Type 1 Diabetes. N Engl J Med. 2020;383(21):2007-17.

177. FDA. FDA Approves First Drug That Can Delay Onset of Type 1 Diabetes. [Internet] 2022 Nov 12 [cited 2022 Dez 02]. Available from: https://www.fda.gov/news-events/press-announcements/fda-approves-first-drug-can-delay-onset-type-1-diabetes.

178. Dayan CM, Besser REJ, Oram RA, Hagopian W, Vatish M, Bendor-Samuel O, Snape MD, Todd JA. Preventing type 1 diabetes in childhood. Science. 2021;373(6554):506-10.

179. Hartemann A, Bensimon G, Payan CA, Jacqueminet S, Bourron O, Nicolas N, Fonfrede M, Rosenzwajg M, Bernard C, Klatzmann D. Low-dose interleukin 2 in patients with type 1 diabetes: a phase 1/2 randomised, double-blind, placebo-controlled trial. The Lancet Diabetes & Endocrinology. 2013;1(4):295-305.

180. Rosenzwajg M, Salet R, Lorenzon R, Tchitchek N, Roux A, Bernard C, Carel JC, Storey C, Polak M, Beltrand J, Amouyal C, Hartemann A, Corbeau P, Vicaut E, Bibal C, Bougneres P, Tran TA, Klatzmann D. Low-dose IL-2 in children with recently diagnosed type 1 diabetes: a Phase I/II randomised, double-blind, placebo-controlled, dose-finding study. Diabetologia. 2020;63(9):1808-21.

181. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. J Exp Med. 2004;199(11):1455-65.

182. Dong S, Hiam-Galvez KJ, Mowery CT, Herold KC, Gitelman SE, Esensten JH, Liu W, Lares AP, Leinbach AS, Lee M, Nguyen V, Tamaki SJ, Tamaki W, Tamaki CM, Mehdizadeh M, Putnam AL, Spitzer MH, Ye CJ, Tang Q, Bluestone JA. The effect of low-dose IL-2 and Treg adoptive cell therapy in patients with type 1 diabetes. JCI Insight. 2021;6(18).

183. Overwijk WW, Tagliaferri MA, Zalevsky J. Engineering IL-2 to Give New Life to T Cell Immunotherapy. Annu Rev Med. 2021;72:281-311.

184. Raffin C, Vo LT, Bluestone JA. Treg cell-based therapies: challenges and perspectives. Nat Rev Immunol. 2020;20(3):158-72.

185. Bonifacio E, Ziegler A-G, Klingensmith G, Schober E, Bingley PJ, Rottenkolber M, Theil A, Eugster A, Puff R, Peplow C, Buettner F, Lange K, Hasford J, Achenbach P, for the Pre PSG. Effects of High-Dose Oral Insulin on Immune Responses in Children at High Risk for Type 1 Diabetes: The Pre-POINT Randomized Clinical Trial. JAMA. 2015;313(15):1541-9.

186. Ziegler AG, Achenbach P, Berner R, Casteels K, Danne T, Gundert M, Hasford J, Hoffmann VS, Kordonouri O, Lange K, Elding Larsson H, Lundgren M, Snape MD, Szypowska A, Todd JA, Bonifacio E, group GS. Oral insulin therapy for primary prevention of type 1 diabetes in infants with high genetic risk: the GPPAD-POInT (global platform for the prevention of autoimmune diabetes primary oral insulin trial) study protocol. BMJ Open. 2019;9(6):e028578.

187. Johnson JD, Ao Z, Ao P, Li H, Dai L-J, He Z, Tee M, Potter KJ, Klimek AM, Meloche RM, Thompson DM, Verchere CB, Warnock GL. Different Effects of FK506,

Rapamycin, and Mycophenolate Mofetil on Glucose-Stimulated Insulin Release and Apoptosis in Human Islets. Cell Transplantation. 2009;18(8):833-45.

188. Forman HJ, Kennedy J. Mammalian dihydroorotate dehydrogenase: Physical and catalytic properties of the primary enzyme. Archives of Biochemistry and Biophysics. 1978;191(1):23-31.

189. Löffler M, Jöckel J, Schuster G. Dihydroorotat-ubiquinone oxidoreductase links mitochondria in the biosynthesis of pyrimidine nucleotides. Molecular and Cellular Biochemistry. 1997;174(1):125-9.

190. Evans DR, Guy HI. Mammalian Pyrimidine Biosynthesis: Fresh Insights into an Ancient Pathway*. J Biol Chem. 2004;279(32):33035-8.

191. Peters GJ, Veerkamp JH. Purine and pyrimidine metabolism in peripheral blood lymphocytes. International Journal of Biochemistry. 1983;15(2):115-23.

192. Fairbanks LD, Bofill M, Ruckemann K, Simmonds HA. Importance of ribonucleotide availability to proliferating T-lymphocytes from healthy humans. Disproportionate expansion of pyrimidine pools and contrasting effects of de novo synthesis inhibitors. J Biol Chem. 1995;270(50):29682-9.

193. Muehler A, Peelen E, Kohlhof H, Groppel M, Vitt D. Vidofludimus calcium, a next generation DHODH inhibitor for the Treatment of relapsing-remitting multiple sclerosis. Mult Scler Relat Disord. 2020;43:102129.

194. Li L, Liu J, Delohery T, Zhang D, Arendt C, Jones C. The effects of teriflunomide on lymphocyte subpopulations in human peripheral blood mononuclear cells in vitro. Journal of Neuroimmunology. 2013;265(1):82-90.

195. Cherwinski HM, Cohn RG, Cheung P, Webster DJ, Xu YZ, Caulfield JP, Young JM, Nakano G, Ransom JT. The immunosuppressant leflunomide inhibits lymphocyte proliferation by inhibiting pyrimidine biosynthesis. Journal of Pharmacology and Experimental Therapeutics. 1995;275(2):1043-9.

196. Dimitrova P, Skapenko A, Herrmann ML, Schleyerbach R, Kalden JR, Schulze-Koops H. Restriction of de novo pyrimidine biosynthesis inhibits Th1 cell activation and promotes Th2 cell differentiation. J Immunol. 2002;169(6):3392-9.

197. Korn T, Magnus T, Toyka K, Jung S. Modulation of effector cell functions in experimental autoimmune encephalomyelitis by leflunomide--mechanisms independent of pyrimidine depletion. J Leukoc Biol. 2004;76(5):950-60.

198. Bartlett RR, Schleyerbach R. Immunopharmacological profile of a novel isoxazol derivative, HWA 486, with potential antirheumatic activity — I. Disease modifying action on adjuvant arthritis of the rat. International Journal of Immunopharmacology. 1985;7(1):7-18.

199. Merrill JE, Hanak S, Pu SF, Liang J, Dang C, Iglesias-Bregna D, Harvey B, Zhu B, McMonagle-Strucko K. Teriflunomide reduces behavioral, electrophysiological, and histopathological deficits in the Dark Agouti rat model of experimental autoimmune encephalomyelitis. J Neurol. 2009;256(1):89-103.

200. O'Connor P, Wolinsky JS, Confavreux C, Comi G, Kappos L, Olsson TP, Benzerdjeb H, Truffinet P, Wang L, Miller A, Freedman MS. Randomized Trial of Oral

Teriflunomide for Relapsing Multiple Sclerosis. New England Journal of Medicine. 2011;365(14):1293-303.

201. Cohen S, Cannon GW, Schiff M, Weaver A, Fox R, Olsen N, Furst D, Sharp J, Moreland L, Caldwell J, Kaine J, Strand V. Two-year, blinded, randomized, controlled trial of treatment of active rheumatoid arthritis with leflunomide compared with methotrexate. Arthritis & Rheumatism. 2001;44(9):1984-92.

202. Confavreux C, O'Connor P, Comi G, Freedman MS, Miller AE, Olsson TP, Wolinsky JS, Bagulho T, Delhay J-L, Dukovic D, Truffinet P, Kappos L. Oral teriflunomide for patients with relapsing multiple sclerosis (TOWER): a randomised, double-blind, placebo-controlled, phase 3 trial. The Lancet Neurology. 2014;13(3):247-56.

203. Leflunomide approved for rheumatoid arthritis; other drugs nearing approval. American Journal of Health-System Pharmacy. 1998;55(21):2225-6.

204. Dunn B. Cross-Discipline Team Leader Review: Treatment of patients with relapsing forms of multiple slerosis. Center For Drug Evaluation And Research; 2012.

205. Duquette P, Yeung M, Mouallif S, Nakhaipour HR, Haddad P, Schecter R. A retrospective claims analysis: Compliance and discontinuation rates among Canadian patients with multiple sclerosis treated with disease-modifying therapies. PLoS One. 2019;14(1):e0210417.

206. Johnson KM, Zhou H, Lin F, Ko JJ, Herrera V. Real-World Adherence and Persistence to Oral Disease-Modifying Therapies in Multiple Sclerosis Patients Over 1 Year. Journal of Managed Care & Specialty Pharmacy. 2017;23(8):844-52.

207. Jones PB, White DH. Reappraisal of the clinical use of leflunomide in rheumatoid arthritis and psoriatic arthritis. Open Access Rheumatol. 2010;2:53-71.

208. Siemasko K, Chong AS-F, Jäck H-M, Gong H, Williams JW, Finnegan A. Inhibition of JAK3 and STAT6 Tyrosine Phosphorylation by the Immunosuppressive Drug Leflunomide Leads to a Block in IgG1 Production. The Journal of Immunology. 1998;160(4):1581-8.

209. Bartlett RR, Dimitrijevic M, Mattar T, Zielinski T, Germann T, Rüde E, Thoenes GH, Küchle CC, Schorlemmer HU, Bremer E, et al. Leflunomide (HWA 486), a novel immunomodulating compound for the treatment of autoimmune disorders and reactions leading to transplantation rejection. Agents Actions. 1991;32(1-2):10-21.

210. Immunic Inc. Form 8-K [Internet]. 2019 Jul 16 [cited 2022 Dez 02].Available from: https://www.sec.gov/Archives/edgar/data/1280776/000119380519000646/e618557_8k-immunic.htm.

211. Muehler A, Kohlhof H, Groeppel M, Vitt D. The Selective Oral Immunomodulator Vidofludimus in Patients with Active Rheumatoid Arthritis: Safety Results from the COMPONENT Study. Drugs R D. 2019;19(4):351-66.

212. Fitzpatrick LR, Deml L, Hofmann C, Small JS, Groeppel M, Hamm S, Lemstra S, Leban J, Ammendola A. 4SC-101, a novel immunosuppressive drug, inhibits IL-17 and attenuates colitis in two murine models of inflammatory bowel disease. Inflamm Bowel Dis. 2010;16(10):1763-77.

213. Fitzpatrick LR, Small JS, Doblhofer R, Ammendola A. Vidofludimus inhibits colonic interleukin-17 and improves hapten-induced colitis in rats by a unique dual mode of action. J Pharmacol Exp Ther. 2012;342(3):850-60.

214. Cada DJ, Demaris K, Levien TL, Baker DE. Teriflunomide. Hosp Pharm. 2013;48(3):231-40.

215. Marschall M, Niemann I, Kosulin K, Bootz A, Wagner S, Dobner T, Herz T, Kramer B, Leban J, Vitt D, Stamminger T, Hutterer C, Strobl S. Assessment of drug candidates for broad-spectrum antiviral therapy targeting cellular pyrimidine biosynthesis. Antiviral Research. 2013;100(3):640-8.

216. Bonavia A, Franti M, Pusateri Keaney E, Kuhen K, Seepersaud M, Radetich B, Shao J, Honda A, Dewhurst J, Balabanis K, Monroe J, Wolff K, Osborne C, Lanieri L, Hoffmaster K, Amin J, Markovits J, Broome M, Skuba E, Cornella-Taracido I, Joberty G, Bouwmeester T, Hamann L, Tallarico JA, Tommasi R, Compton T, Bushell SM. Identification of broad-spectrum antiviral compounds and assessment of the druggability of their target for efficacy against respiratory syncytial virus (RSV). Proceedings of the National Academy of Sciences. 2011;108(17):6739-44.

217. Fox RJ, Wiendl H, Wolf C, De Stefano N, Sellner J, Gryb V, Rejdak K, Bozhinov PS, Tomakh N, Skrypchenko I, Muehler AR. A double-blind, randomized, placebo-controlled phase 2 trial evaluating the selective dihydroorotate dehydrogenase inhibitor vidofludimus calcium in relapsing-remitting multiple sclerosis. Ann Clin Transl Neurol. 2022;9(7):977-87.

218. Zhang J, Teran G, Popa M, Madapura H, Ladds M, Lianoudaki D, Grunler J, Arsenian-Henriksson M, McCormack E, Rottenberg ME, Catrina SB, Lain S, Darekar S. DHODH inhibition modulates glucose metabolism and circulating GDF15, and improves metabolic balance. iScience. 2021;24(5):102494.

219. Nakayasu ES, Syed F, Tersey SA, Gritsenko MA, Mitchell HD, Chan CY, Dirice E, Turatsinze JV, Cui Y, Kulkarni RN, Eizirik DL, Qian WJ, Webb-Robertson BM, Evans-Molina C, Mirmira RG, Metz TO. Comprehensive Proteomics Analysis of Stressed Human Islets Identifies GDF15 as a Target for Type 1 Diabetes Intervention. Cell Metab. 2020;31(2):363-74 e6.

220. Peelen E, Wu H, Muehler A, Vitt D, Z S, H K. IMU-935, a potent RORgt inverse agonist, effectively inhibits T helper 17 cells but maintains normal thymocyte development. [Internet] 16th World Immune Regulation Meeting Davos, Switzerland 2022. Available from https://imux.com/publications/.

221. H. Kohlhof BH, M. Schenk, M. Gröppel, A. Mühler, Z. He, Z. Sun, S. Tasler, D. Vitt. IMU-935: Orally available small molecule inhibitor of IL-17 with unique molecular profile for the treatment of autoimmune diseases. [Internet] 2nd Conference on Molecular Mechanisms of Inflammation; Trondheim, Norway 2019. [cited 2022 Dez 02] Available from https://imux.com/publications/.

222. Guntermann C, Piaia A, Hamel ML, Theil D, Rubic-Schneider T, Del Rio-Espinola A, Dong L, Billich A, Kaupmann K, Dawson J, Hoegenauer K, Orain D, Hintermann S, Stringer R, Patel DD, Doelemeyer A, Deurinck M, Schumann J. Retinoic-acid-orphan-receptor-C inhibition suppresses Th17 cells and induces thymic aberrations. JCI Insight. 2017;2(5):e91127.

223. Peelen E. MU-935, a Potent RORgt Inverse Agonist, Effectively Inhibits T Helper 17 Cells but Maintains Normal Thymocyte Development. [Internet] 3rd B & T Cell-Mediated Autoimmune Disease Drug Development Summit; Boston 2022. [cited 2022 Dez 02] Available from https://imux.com/publications/.

224. Immunic, Inc. Positive Results from the Single and Multiple Ascending Dose Parts of its Phase 1 Clinical Trial of IMU-935, a Potentially Best-in-Class Oral IL-17 Inhibitor [Internet]. 2021 Dez 14 [cited 2022 Dez 02]. Available from https://ir.imux.com/2021-12-14-Immunic,-Inc-Publishes-Positive-Results-from-the-Single-and-Multiple-Ascending-Dose-Parts-of-its-Phase-1-Clinical-Trial-of-IMU-935,-a-Potentially-Best-in-Class-Oral-IL-17-Inhibitor.

225. Emamaullee JA, Davis J, Merani S, Toso C, Elliott JF, Thiesen A, Shapiro AM. Inhibition of Th17 cells regulates autoimmune diabetes in NOD mice. Diabetes. 2009;58(6):1302-11.

226. Kuriya G, Uchida T, Akazawa S, Kobayashi M, Nakamura K, Satoh T, Horie I, Kawasaki E, Yamasaki H, Yu L, Iwakura Y, Sasaki H, Nagayama Y, Kawakami A, Abiru N. Double deficiency in IL-17 and IFN-gamma signalling significantly suppresses the development of diabetes in the NOD mouse. Diabetologia. 2013;56(8):1773-80.

227. Kriegel MA, Sefik E, Hill JA, Wu HJ, Benoist C, Mathis D. Naturally transmitted segmented filamentous bacteria segregate with diabetes protection in nonobese diabetic mice. Proc Natl Acad Sci U S A. 2011;108(28):11548-53.

228. Joseph J, Bittner S, Kaiser FM, Wiendl H, Kissler S. IL-17 silencing does not protect nonobese diabetic mice from autoimmune diabetes. J Immunol. 2012;188(1):216-21.

229. Bending D, De la Pena H, Veldhoen M, Phillips JM, Uyttenhove C, Stockinger B, Cooke A. Highly purified Th17 cells from BDC2.5NOD mice convert into Th1-like cells in NOD/SCID recipient mice. J Clin Invest. 2009;119(3):565-72.

230. Martin-Orozco N, Chung Y, Chang SH, Wang YH, Dong C. Th17 cells promote pancreatic inflammation but only induce diabetes efficiently in lymphopenic hosts after conversion into Th1 cells. Eur J Immunol. 2009;39(1):216-24.

231. Liu SM, Lee DH, Sullivan JM, Chung D, Jager A, Shum BO, Sarvetnick NE, Anderson AC, Kuchroo VK. Differential IL-21 signaling in APCs leads to disparate Th17 differentiation in diabetes-susceptible NOD and diabetes-resistant NOD.Idd3 mice. J Clin Invest. 2011;121(11):4303-10.

232. Honkanen J, Nieminen JK, Gao R, Luopajarvi K, Salo HM, Ilonen J, Knip M, Otonkoski T, Vaarala O. IL-17 immunity in human type 1 diabetes. J Immunol. 2010;185(3):1959-67.

233. Solt LA, Kumar N, He Y, Kamenecka TM, Griffin PR, Burris TP. Identification of a selective RORgamma ligand that suppresses T(H)17 cells and stimulates T regulatory cells. ACS Chem Biol. 2012;7(9):1515-9.

234. Solt LA, Banerjee S, Campbell S, Kamenecka TM, Burris TP. ROR inverse agonist suppresses insulitis and prevents hyperglycemia in a mouse model of type 1 diabetes. Endocrinology. 2015;156(3):869-81.

235. Tao B, Pietropaolo M, Atkinson M, Schatz D, Taylor D. Estimating the cost of type 1 diabetes in the U.S.: a propensity score matching method. PLoS One. 2010;5(7):e11501.

236. Berry G, Waldner H. Accelerated Type 1 Diabetes Induction in Mice by Adoptive Transfer of Diabetogenic CD4+ T Cells. JoVE. 2013(75):e50389.

237. Wunderlich FT, Strohle P, Konner AC, Gruber S, Tovar S, Bronneke HS, Juntti-Berggren L, Li LS, van Rooijen N, Libert C, Berggren PO, Bruning JC. Interleukin-6 signaling in liver-parenchymal cells suppresses hepatic inflammation and improves systemic insulin action. Cell Metab. 2010;12(3):237-49.

238. Babaya N, Liu E, Miao D, Li M, Yu L, Eisenbarth GS. Murine high specificity/sensitivity competitive europium insulin autoantibody assay. Diabetes Technol Ther. 2009;11(4):227-33.

239. Lochner M, Peduto L, Cherrier M, Sawa S, Langa F, Varona R, Riethmacher D, Si-Tahar M, Di Santo JP, Eberl G. In vivo equilibrium of proinflammatory IL-17+ and regulatory IL-10+ Foxp3+ RORgamma t+ T cells. J Exp Med. 2008;205(6):1381-93.

240. Lakso M, Sauer B, Mosinger B, Jr., Lee EJ, Manning RW, Yu SH, Mulder KL, Westphal H. Targeted oncogene activation by site-specific recombination in transgenic mice. Proc Natl Acad Sci U S A. 1992;89(14):6232-6.

241. Sawada S, Scarborough JD, Killeen N, Littman DR. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell. 1994;77(6):917-29.

242. Stadinski BD, Delong T, Reisdorph N, Reisdorph R, Powell RL, Armstrong M, Piganelli JD, Barbour G, Bradley B, Crawford F, Marrack P, Mahata SK, Kappler JW, Haskins K. Chromogranin A is an autoantigen in type 1 diabetes. Nat Immunol. 2010;11(3):225-31.

243. Read S, Malmström V, Powrie F. Cytotoxic T Lymphocyte–Associated Antigen 4 Plays an Essential Role in the Function of Cd25+Cd4+ Regulatory Cells That Control Intestinal Inflammation. J Exp Med. 2000;192(2):295-302.

244. Yamaguchi T, Hirota K, Nagahama K, Ohkawa K, Takahashi T, Nomura T, Sakaguchi S. Control of immune responses by antigen-specific regulatory T cells expressing the folate receptor. Immunity. 2007;27(1):145-59.

245. Bedke T, Pretsch L, Karakhanova S, Enk AH, Mahnke K. Endothelial cells augment the suppressive function of CD4+ CD25+ Foxp3+ regulatory T cells: involvement of programmed death-1 and IL-10. J Immunol. 2010;184(10):5562-70.

246. Joller N, Lozano E, Burkett PR, Patel B, Xiao S, Zhu C, Xia J, Tan TG, Sefik E, Yajnik V, Sharpe AH, Quintana FJ, Mathis D, Benoist C, Hafler DA, Kuchroo VK. Treg cells expressing the coinhibitory molecule TIGIT selectively inhibit proinflammatory Th1 and Th17 cell responses. Immunity. 2014;40(4):569-81.

247. Roy T, Lloyd CE. Epidemiology of depression and diabetes: a systematic review. J Affect Disord. 2012;142 Suppl:S8-21.

248. Smith KJ, Béland M, Clyde M, Gariépy G, Pagé V, Badawi G, Rabasa-Lhoret R, Schmitz N. Association of diabetes with anxiety: A systematic review and meta-analysis. Journal of Psychosomatic Research. 2013;74(2):89-99.

249. Diabetes Canada Clinical Practice Guidelines Expert C, Robinson DJ, Coons M, Haensel H, Vallis M, Yale JF. Diabetes and Mental Health. Can J Diabetes. 2018;42 Suppl 1:S130-S41.

250. H. Kohlhof MG, A. Mühler, D. Vitt. Preclinical Investigations of IMU-838, an Orally Available Small Molecule Inhibitor of Dihydroorotate Dehydrogenase for the Treatment of Inflammatory Bowel Disease. United European Gastroenterology Week; Barcelona, Spain 2019. Available from: https://imux.com/wpcontent/uploads/2021/07/2019022_UEGW2019_P1021_Kohlhof_Immunic.pdf.

251. Serr I, Furst RW, Ott VB, Scherm MG, Nikolaev A, Gokmen F, Kalin S, Zillmer S, Bunk M, Weigmann B, Kunschke N, Loretz B, Lehr CM, Kirchner B, Haase B, Pfaffl M, Waisman A, Willis RA, Ziegler AG, Daniel C. miRNA92a targets KLF2 and the phosphatase PTEN signaling to promote human T follicular helper precursors in T1D islet autoimmunity. Proc Natl Acad Sci U S A. 2016;113(43):E6659-E68.

252. Wang J, Ioan-Facsinay A, van der Voort EI, Huizinga TW, Toes RE. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. Eur J Immunol. 2007;37(1):129-38.

253. Korn T, Mitsdoerffer M, Croxford AL, Awasthi A, Dardalhon VA, Galileos G, Vollmar P, Stritesky GL, Kaplan MH, Waisman A, Kuchroo VK, Oukka M. IL-6 controls Th17 immunity in vivo by inhibiting the conversion of conventional T cells into Foxp3+ regulatory T cells. Proceedings of the National Academy of Sciences. 2008;105(47):18460-5.

254. Anderson MS, Bluestone JA. The NOD mouse: a model of immune dysregulation. Annu Rev Immunol. 2005;23:447-85.

255. Chougnet CA, Tripathi P, Lages CS, Raynor J, Sholl A, Fink P, Plas DR, Hildeman DA. A major role for Bim in regulatory T cell homeostasis. J Immunol. 2011;186(1):156-63.

256. Takano T, Kotaki R, Park J, Yoshida T, Wakatsuki Y, Tanokura M, Miyakawa T, Takahashi K, Nakajima-Adachi H, Hachimura S. Age-Dependent Decrease in the Induction of Regulatory T Cells Is Associated With Decreased Expression of RALDH2 in Mesenteric Lymph Node Dendritic Cells. Frontiers in Immunology. 2020;11.

257. Tsukamoto H, Clise-Dwyer K, Huston GE, Duso DK, Buck AL, Johnson LL, Haynes L, Swain SL. Age-associated increase in lifespan of naïve CD4 T cells contributes to T-cell homeostasis but facilitates development of functional defects. Proceedings of the National Academy of Sciences. 2009;106(43):18333-8.

258. Martinez-Jimenez CP, Eling N, Chen H-C, Vallejos CA, Kolodziejczyk AA, Connor F, Stojic L, Rayner TF, Stubbington MJT, Teichmann SA, de la Roche M, Marioni JC, Odom DT. Aging increases cell-to-cell transcriptional variability upon immune stimulation. Science. 2017;355(6332):1433-6.

259. Haynes L, Eaton SM, Burns EM, Rincon M, Swain SL. Inflammatory cytokines overcome age-related defects in CD4 T cell responses in vivo. J Immunol. 2004;172(9):5194-9.

260. Kim C, Hu B, Jadhav RR, Jin J, Zhang H, Cavanagh MM, Akondy RS, Ahmed R, Weyand CM, Goronzy JJ. Activation of miR-21-Regulated Pathways in Immune Aging Selects against Signatures Characteristic of Memory T Cells. Cell Rep. 2018;25(8):2148-62 e5.

261. Quinn KM, Palchaudhuri R, Palmer CS, La Gruta NL. The clock is ticking: the impact of ageing on T cell metabolism. Clin Transl Immunology. 2019;8(11):e01091.

262. Al Nabhani Z, Dulauroy S, Marques R, Cousu C, Al Bounny S, Dejardin F, Sparwasser T, Berard M, Cerf-Bensussan N, Eberl G. A Weaning Reaction to Microbiota Is Required for Resistance to Immunopathologies in the Adult. Immunity. 2019;50(5):1276-88 e5.

263. Huang J, Tan Q, Tai N, Pearson JA, Li Y, Chao C, Zhang L, Peng J, Xing Y, Zhang L, Hu Y, Zhou Z, Wong FS, Wen L. IL-10 Deficiency Accelerates Type 1 Diabetes Development via Modulation of Innate and Adaptive Immune Cells and Gut Microbiota in BDC2.5 NOD Mice. Front Immunol. 2021;12:702955.

264. Balasa B, Van Gunst K, Jung N, Katz JD, Sarvetnick N. IL-10 Deficiency Does Not Inhibit Insulitis and Accelerates Cyclophosphamide-Induced Diabetes in the Nonobese Diabetic Mouse. Cellular Immunology. 2000;202(2):97-102.

265. Goudy KS, Burkhardt BR, Wasserfall C, Song S, Campbell-Thompson ML, Brusko T, Powers MA, Clare-Salzler MJ, Sobel ES, Ellis TM, Flotte TR, Atkinson MA. Systemic overexpression of IL-10 induces CD4+CD25+ cell populations in vivo and ameliorates type 1 diabetes in nonobese diabetic mice in a dose-dependent fashion. J Immunol. 2003;171(5):2270-8.

266. Chaudhry A, Samstein RM, Treuting P, Liang Y, Pils MC, Heinrich JM, Jack RS, Wunderlich FT, Bruning JC, Muller W, Rudensky AY. Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity. 2011;34(4):566-78.

267. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, Kronenberg M. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. Nat Immunol. 2009;10(11):1178-84.

268. Hsu P, Santner-Nanan B, Hu M, Skarratt K, Lee CH, Stormon M, Wong M, Fuller SJ, Nanan R. IL-10 Potentiates Differentiation of Human Induced Regulatory T Cells via STAT3 and Foxo1. J Immunol. 2015;195(8):3665-74.

269. McGeachy MJ, Bak-Jensen KS, Chen Y, Tato CM, Blumenschein W, McClanahan T, Cua DJ. TGF-beta and IL-6 drive the production of IL-17 and IL-10 by T cells and restrain T(H)-17 cell-mediated pathology. Nat Immunol. 2007;8(12):1390-7.

270. Zielinski CE, Mele F, Aschenbrenner D, Jarrossay D, Ronchi F, Gattorno M, Monticelli S, Lanzavecchia A, Sallusto F. Pathogen-induced human TH17 cells produce IFN-gamma or IL-10 and are regulated by IL-1beta. Nature. 2012;484(7395):514-8.

271. Immunic Inc. Virtual R&D Day: Update on the Preclinical and Clinical Development of IMU-935 [Internet] 2021 Jul 21 [cited 2022 Dez 02]. Available from: https://imux.com/publications/virtual-rd-day-update-on-the-preclinical-and-clinicaldevelopment-of-imu-935/.

272. Qiao G, Yang L, Li Z, Williams JW, Zhang J. A77 1726, the active metabolite of leflunomide, attenuates lupus nephritis by promoting the development of regulatory T cells and inhibiting IL-17-producing double negative T cells. Clin Immunol. 2015;157(2):166-74.

273. Yang X, Li C, Gou K, Liu X, Zhou Y, Zou J, Chen Q, Luo Y, Zhao Y. A novel and potent dihydroorotate dehydrogenase inhibitor suppresses the proliferation of colorectal cancer by inducing mitochondrial dysfunction and DNA damage. MedComm – Oncology. 2022;1(1).

274. Elder RT, Xu X, Williams JW, Gong H, Finnegan A, Chong AS. The immunosuppressive metabolite of leflunomide, A77 1726, affects murine T cells through two biochemical mechanisms. The Journal of Immunology. 1997;159(1):22-7.

275. Li B, Jones LL, Geiger TL. IL-6 Promotes T Cell Proliferation and Expansion under Inflammatory Conditions in Association with Low-Level RORgammat Expression. J Immunol. 2018;201(10):2934-46.

276. Boardman DA, Garcia RV, Ivison SM, Bressler B, Dhar TM, Zhao Q, Levings MK. Pharmacological inhibition of RORC2 enhances human Th17-Treg stability and function. Eur J Immunol. 2020.

277. Zeng WP, Chang C, Lai JJ. Immune suppressive activity and lack of T helper differentiation are differentially regulated in natural regulatory T cells. J Immunol. 2009;183(6):3583-90.

278. Gomez-Rodriguez J, Sahu N, Handon R, Davidson TS, Anderson SM, Kirby MR, August A, Schwartzberg PL. Differential expression of interleukin-17A and -17F is coupled to T cell receptor signaling via inducible T cell kinase. Immunity. 2009;31(4):587-97.

279. Samoilova EB, Horton JL, Hilliard B, Liu TS, Chen Y. IL-6-deficient mice are resistant to experimental autoimmune encephalomyelitis: roles of IL-6 in the activation and differentiation of autoreactive T cells. J Immunol. 1998;161(12):6480-6.

280. Korn T, Bettelli E, Gao W, Awasthi A, Jager A, Strom TB, Oukka M, Kuchroo VK. IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells. Nature. 2007;448(7152):484-7.

281. Sheppard M, Laskou F, Stapleton PP, Hadavi S, Dasgupta B. Tocilizumab (Actemra). Hum Vaccin Immunother. 2017;13(9):1972-88.

282. Chandran S, Leung J, Hu C, Laszik ZG, Tang Q, Vincenti FG. Interleukin-6 blockade with tocilizumab increases Tregs and reduces T effector cytokines in renal graft inflammation: A randomized controlled trial. Am J Transplant. 2021;21(7):2543-54.

283. Pesce B, Soto L, Sabugo F, Wurmann P, Cuchacovich M, Lopez MN, Sotelo PH, Molina MC, Aguillon JC, Catalan D. Effect of interleukin-6 receptor blockade on the balance between regulatory T cells and T helper type 17 cells in rheumatoid arthritis patients. Clin Exp Immunol. 2013;171(3):237-42.

284. Jones GW, McLoughlin RM, Hammond VJ, Parker CR, Williams JD, Malhotra R, Scheller J, Williams AS, Rose-John S, Topley N, Jones SA. Loss of CD4+ T cell IL-6R expression during inflammation underlines a role for IL-6 trans signaling in the local maintenance of Th17 cells. J Immunol. 2010;184(4):2130-9.

285. Ridgley LA, Anderson AE, Maney NJ, Naamane N, Skelton AJ, Lawson CA, Emery P, Isaacs JD, Carmody RJ, Pratt AG. IL-6 Mediated Transcriptional Programming of Naive CD4+ T Cells in Early Rheumatoid Arthritis Drives Dysregulated Effector Function. Front Immunol. 2019;10:1535.

286. Wu W, Dietze KK, Gibbert K, Lang KS, Trilling M, Yan H, Wu J, Yang D, Lu M, Roggendorf M, Dittmer U, Liu J. TLR ligand induced IL-6 counter-regulates the anti-viral CD8(+) T cell response during an acute retrovirus infection. Sci Rep. 2015;5:10501.

287. Briso EM, Dienz O, Rincon M. Cutting edge: soluble IL-6R is produced by IL-6R ectodomain shedding in activated CD4 T cells. J Immunol. 2008;180(11):7102-6.

288. Dominitzki S, Fantini MC, Neufert C, Nikolaev A, Galle PR, Scheller J, Monteleone G, Rose-John S, Neurath MF, Becker C. Cutting edge: trans-signaling via the soluble IL-6R abrogates the induction of FoxP3 in naive CD4+CD25 T cells. J Immunol. 2007;179(4):2041-5.

289. Hagenstein J, Melderis S, Nosko A, Warkotsch MT, Richter JV, Ramcke T, Herrnstadt GR, Scheller J, Yan I, Mittrucker HW, Kluger MA, Steinmetz OM. A Novel Role for IL-6 Receptor Classic Signaling: Induction of RORgammat(+)Foxp3(+) Tregs with Enhanced Suppressive Capacity. J Am Soc Nephrol. 2019;30(8):1439-53.

290. Rose-John S. IL-6 trans-signaling via the soluble IL-6 receptor: importance for the pro-inflammatory activities of IL-6. Int J Biol Sci. 2012;8(9):1237-47.

291. Fitzpatrick L, Small J, Ammendola A. Inhibition of IL17 Release by the Novel Anti-Inflammatory Drug Vidofludimus Involves Attenuation of STAT3 and NF-kappa B Signaling Pathways in Murine Splenocytes and Hapten-Induced Colitis. Gastroenterology. 2011;140.

292. Nicoletti F, Conget I, Di Mauro M, Di Marco R, Mazzarino MC, Bendtzen K, Messina A, Gomis R. Serum concentrations of the interferon-gamma-inducible chemokine IP-10/CXCL10 are augmented in both newly diagnosed Type I diabetes mellitus patients and subjects at risk of developing the disease. Diabetologia. 2002;45(8):1107-10.

293. Antonelli A, Fallahi P, Ferrari SM, Pupilli C, D'Annunzio G, Lorini R, Vanelli M, Ferrannini E. Serum Th1 (CXCL10) and Th2 (CCL2) chemokine levels in children with newly diagnosed Type 1 diabetes: a longitudinal study. Diabetic Medicine. 2008;25(11):1349-53.

294. Roep BO, Kleijwegt FS, Van Halteren AGS, Bonato V, Boggi U, Vendrame F, Marchetti P, Dotta F. Islet inflammation and CXCL10 in recent-onset type 1 diabetes. Clinical and Experimental Immunology. 2010;159(3):338-43.

295. Uno S, Imagawa A, Saisho K, Okita K, Iwahashi H, Hanafusa T, Shimomura I. Expression of chemokines, CXC chemokine ligand 10 (CXCL10) and CXCR3 in the inflamed islets of patients with recent-onset autoimmune type 1 diabetes. Endocrine Journal. 2010;57(11):991-6.

296. Bender C, Christen S, Scholich K, Bayer M, Pfeilschifter JM, Hintermann E, Christen U. Islet-Expressed CXCL10 Promotes Autoimmune Destruction of Islet Isografts in Mice With Type 1 Diabetes. Diabetes. 2017;66(1):113-26.

297. Frigerio S, Junt T, Lu B, Gerard C, Zumsteg U, Holländer GA, Piali L. β cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. Nature Medicine. 2002;8(12):1414-20.

298. Bradley LM, Asensio VC, Schioetz L-K, Harbertson J, Krahl T, Patstone G, Woolf N, Campbell IL, Sarvetnick N. Islet-Specific Th1, But Not Th2, Cells Secrete Multiple Chemokines and Promote Rapid Induction of Autoimmune Diabetes. The Journal of Immunology. 1999;162(5):2511-20.

299. Lasch S, Muller P, Bayer M, Pfeilschifter JM, Luster AD, Hintermann E, Christen U. Anti-CD3/Anti-CXCL10 Antibody Combination Therapy Induces a Persistent Remission of Type 1 Diabetes in Two Mouse Models. Diabetes. 2015;64(12):4198-211.

300. Yamada Y, Okubo Y, Shimada A, Oikawa Y, Yamada S, Narumi S, Matsushima K, Itoh H. Acceleration of diabetes development in CXC chemokine receptor 3 (CXCR3)-deficient NOD mice. Diabetologia. 2012;55(8):2238-45.

301. Rhode A, Pauza ME, Barral AM, Rodrigo E, Oldstone MB, von Herrath MG, Christen U. Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. J Immunol. 2005;175(6):3516-24.

302. Groom JR, Luster AD. CXCR3 in T cell function. Exp Cell Res. 2011;317(5):620-31.

303. Campanella GS, Medoff BD, Manice LA, Colvin RA, Luster AD. Development of a novel chemokine-mediated in vivo T cell recruitment assay. J Immunol Methods. 2008;331(1-2):127-39.

304. Xie JH, Nomura N, Lu M, Chen SL, Koch GE, Weng Y, Rosa R, Di Salvo J, Mudgett J, Peterson LB, Wicker LS, DeMartino JA. Antibody-mediated blockade of the CXCR3 chemokine receptor results in diminished recruitment of T helper 1 cells into sites of inflammation. J Leukoc Biol. 2003;73(6):771-80.

305. Zhu M, Xu Q, Li XL, He Q, Wang WF. Modulating effects of leflunomide on the balance of Th17/Treg cells in collageninduced arthritis DBA/1 mice. Cent Eur J Immunol. 2014;39(2):152-8.

306. Wang T-Y, Li J, Li C-Y, Jin Y, Lü X-W, Wang X-H, Zhou Q. Leflunomide induces immunosuppression in collagen-induced arthritis rats by upregulating CD4+CD25+ regulatory T cells. Canadian Journal of Physiology and Pharmacology. 2010;88(1):45-53.

307. Metzler B, Gfeller P, Guinet E. Restricting Glutamine or Glutamine-Dependent Purine and Pyrimidine Syntheses Promotes Human T Cells with High FOXP3 Expression and Regulatory Properties. J Immunol. 2016;196(9):3618-30.

308. Wischhusen J, Melero I, Fridman WH. Growth/Differentiation Factor-15 (GDF-15): From Biomarker to Novel Targetable Immune Checkpoint. Front Immunol. 2020;11:951.

309. Wang Y, Chen J, Sang T, Chen C, Peng H, Lin X, Zhao Q, Chen S, Eling T, Wang X. NAG-1/GDF15 protects against streptozotocin-induced type 1 diabetes by inhibiting apoptosis, preserving beta-cell function, and suppressing inflammation in pancreatic islets. Molecular and Cellular Endocrinology. 2022;549:111643.

310. Wang Z, He L, Li W, Xu C, Zhang J, Wang D, Dou K, Zhuang R, Jin B, Zhang W, Hao Q, Zhang K, Zhang W, Wang S, Gao Y, Gu J, Shang L, Tan Z, Su H, Zhang Y, Zhang C, Li M. GDF15 induces immunosuppression via CD48 on regulatory T cells in hepatocellular carcinoma. J Immunother Cancer. 2021;9(9).

311. Harb H, Stephen-Victor E, Crestani E, Benamar M, Massoud A, Cui Y, Charbonnier LM, Arbag S, Baris S, Cunnigham A, Leyva-Castillo JM, Geha RS, Mousavi AJ, Guennewig B, Schmitz-Abe K, Sioutas C, Phipatanakul W, Chatila TA. A regulatory T cell Notch4-GDF15 axis licenses tissue inflammation in asthma. Nat Immunol. 2020;21(11):1359-70.

312. Rossini AA, Like AA, Dulin WE, Cahill GF, Jr. Pancreatic Beta Cell Toxicity by Streptozotocin Anomers. Diabetes. 1977;26(12):1120-4.

313. Reddy S, Chang M, Robinson E. Young NOD Mice Show Increased Diabetes Sensitivity to Low Doses of Streptozotocin. Annals of the New York Academy of Sciences. 2006;1079(1):109-13.

314. Yasunami R, Bach J-F. Anti-suppressor effect of cyclophosphamide on the development of spontaneous diabetes in nod mice. European Journal of Immunology. 1988;18(3):481-4.

315. Brode S, Raine T, Zaccone P, Cooke A. Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells. J Immunol. 2006;177(10):6603-12.

316. Hahn F, Wangen C, Hage S, Peter AS, Dobler G, Hurst B, Julander J, Fuchs J, Ruzsics Z, Uberla K, Jack HM, Ptak R, Muehler A, Groppel M, Vitt D, Peelen E, Kohlhof H, Marschall M. IMU-838, a Developmental DHODH Inhibitor in Phase II for Autoimmune Disease, Shows Anti-SARS-CoV-2 and Broad-Spectrum Antiviral Efficacy In Vitro. Viruses. 2020;12(12).

317. Stone VM, Hankaniemi MM, Laitinen OH, Sioofy-Khojine AB, Lin A, Diaz Lozano IM, Mazur MA, Marjomäki V, Loré K, Hyöty H, Hytönen VP, Flodström-Tullberg M. A hexavalent Coxsackievirus B vaccine is highly immunogenic and has a strong protective capacity in mice and nonhuman primates. Sci Adv. 2020;6(19):eaaz2433-eaaz.

318. Rogers MAM, Basu T, Kim C. Lower Incidence Rate of Type 1 Diabetes after Receipt of the Rotavirus Vaccine in the United States, 2001-2017. Sci Rep. 2019;9(1):7727.

319. Cully M. T cell-regulating therapies for autoimmune diseases take FDA rejection in stride. Nat Rev Drug Discov. 2021;20(9):655-7.

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Eidesstattliche Versicherung

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Ich erkläre hiermit an Eides statt, dass ich die vorliegende Dissertation mit dem Thema

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