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Glucose determines unstable FoxP3 expression and favours the development of poorly functional Treg cells in recent-onset Type 1 Diabetes children

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2. **Bruzzaniti S**, Piemonte E, Mozzillo E, Bruzzese D, Lepore MT, Carbone F, de Candia P, Strollo R, Marigliano M, Maffeis C, Bifulco M, Ludvigssson J, Franzese A, Matarese G, Galgani M.High levels of blood circulating immune-checkpoint molecules in new-onset Type 1 Diabetes children associate with the risk of developing a subsequent additional autoimmune disease. *Diabetologia* (2022).

3. De Vito F, Musella A, Fresegna D, Rizzo FR, Gentile A, Bassi MS, Gilio L, Buttari F, Procaccini C, Colamatteo A, Bullitta S, Guadalupi L, Caioli S, Vanni V, Balletta S, Sanna K, Bruno A, Dolcetti E, Furlan R, Finardi A, Licursi V, Drulovic J, Pekmezovic T, Fusco C, **Bruzzaniti S**, Hornstein E, Uccelli A, Salvetti M, Matarese G, Centonze D, Mandolesi G. MiR-142-3p regulates synaptopathy-driven disease progression in multiple sclerosis. *Neuropathol. Appl. Neurobiol.* (2021).

4. Proto MC, Fiore D, Piscopo C, Pagano C, Galgani M, **Bruzzaniti S**, Laezza C, Gazzerro P, Bifulco M. Lipid homeostasis and mevalonate pathway in COVID-19: Basic concepts and potential therapeutic targets. *Prog Lipid Res.* (2021).

5. Palma C, La Rocca C, Gigantino V, Aquino G, Piccaro G, Di Silvestre D, Brambilla F, Rossi R, Bonacina F, Lepore MT, Audano M, Mitro N, Botti G, **Bruzzaniti S**, Fusco C, Procaccini C, De Rosa V, Galgani M, Alviggi C, Puca A, Grassi F, Rezzonico-Jost T, Norata GD, Mauri P, Netea MG, de Candia P, Matarese G. Caloric Restriction Promotes Immunometabolic Reprogramming Leading to Protection from Tuberculosis. *Cell Metab.* (2021).

6. Perna F, **Bruzzaniti S**, Piemonte E, Maddaloni V, Atripaldi L, Sale S, Sanduzzi A, Nicastro C, Pepe N, Bifulco M, Matarese G, Galgani M, Atripaldi L. Serum levels of SARS-CoV-2 nucleocapsid antigen associate with inflammatory status and disease severity in COVID-19 patients. *Clin Immunol.* (2021).

7. Galgani M, **Bruzzaniti S**, La Rocca C, Micillo T, de Candia P, Bifulco M, Matarese G. Immunometabolism of regulatory T cells in cancer. *Mol Aspects Med.* (2020).

8. **Bruzzaniti S**, Cirillo E, Prencipe R, Giardino G, Lepore MT, Garziano F, Perna F, Procaccini C, Mascolo L, Pagano C, Fattorusso V, Mozzillo E, Bifulco M, Matarese G, Franzese A, Pignata C, Galgani M. $CD4^+$ T Cell Defects in a Mulibrey Patient With Specific TRIM37 Mutations. *Front Immunol.* (2020).

9. Galgani M, **Bruzzaniti S**, Matarese G. Immunometabolism and autoimmunity. *Curr Opin Immunol.* (2020).

10. Garavelli S, **Bruzzaniti S**, Tagliabue E, Di Silvestre D, Prattichizzo F, Mozzillo E, Fattorusso V, La Sala L, Ceriello A, Puca AA, Mauri P, Strollo R, Marigliano M, Maffeis C, Petrelli A, Bosi E, Franzese A, Galgani M, Matarese G, de Candia P. Plasma circulating miR-23~27~24 clusters correlate with the immunometabolic derangement and predict C-peptide loss in children with type 1 diabetes. *Diabetologia* (2020).

11. Barisciano G, Colangelo T, Rosato V, Muccillo L, Taddei ML, Ippolito L, Chiarugi P, Galgani M, **Bruzzaniti S**, Matarese G, Fassan M, Agostini M, Bergamo F, Pucciarelli S, Carbone A, Mazzoccoli G, Colantuoni V, Bianchi F, Sabatino L. miR-27a is a master regulator of metabolic reprogramming and chemoresistance in colorectal cancer. *Br J Cancer.* (2020).

12. Terrazzano G*, **Bruzzaniti S***, Rubino V*, Santopaolo M, Palatucci AT, Giovazzino A, La Rocca C, de Candia P, Puca A, Perna F, Procaccini C, De Rosa V, Porcellini C, De Simone S, Fattorusso V, Porcellini A, Mozzillo E, Troncone R, Franzese A, Ludvigsson J, Matarese G, Ruggiero G, Galgani M. Type 1 diabetes progression is associated with loss of $CD3^+CD56^+$ regulatory T cells that control CD8+ T-cell effector functions. *Nat Metab.* (2020). *These authors equally contributed to the paper.

13. Colamatteo A, Carbone F, **Bruzzaniti S**, Galgani M, Fusco C, Maniscalco GT, Di Rella F, de Candia P, De Rosa V. Molecular Mechanisms Controlling Foxp3 Expression in Health and Autoimmunity: From Epigenetic to Post-translational Regulation. *Front Immunol.* (2020).

14. Garavelli S*, **Bruzzaniti S***, Tagliabue E, Prattichizzo F, Di Silvestre D, Perna F, La Sala L, Ceriello A, Mozzillo E, Fattorusso V, Mauri P, Puca AA, Franzese A, Matarese G, Galgani M, de Candia P. Blood Co-Circulating Extracellular microRNAs and Immune Cell Subsets Associate with Type 1 Diabetes Severity. Int *J Mol Sci.* (2020). *These authors equally contributed to the paper.

15. Colamatteo A, Micillo T, **Bruzzaniti S**, Fusco C, Garavelli S, De Rosa V, Galgani M, Spagnuolo MI, Di Rella F, Puca AA, de Candia P, Matarese G. Metabolism and Autoimmune Responses: The microRNA Connection. *Front Immunol.* (2019).

ABSTRACT

Type 1 diabetes (T1D) is an autoimmune disorder caused by the destruction of insulin-producing β-cells in the pancreas by autoreactive T cells. The disease starts years before the clinical manifestation with a progressive loss of pancreatic functions, leading to unbalanced blood glucose levels, due to low insulin production. Damage of the β-cells is largely ascribed to unrestrained activation of effector T cells associated with defective suppressive activity of regulatory T (Treg) cells expressing the forkhead box P3 (FoxP3) transcription factor. This cell subset plays a key role in the maintenance of immune tolerance and it is now established that a reduced frequency and function of Treg cells are one of the main causes of uncontrolled autoreactive responses. While contrasting results are present about their frequency in literature, it is now clear that in autoimmune diabetes Treg cells are defective in their suppressive capability. However, the molecular mechanisms at the basis of their impaired functions are not completely elucidated.

We believed that a dysregulated glucose homeostasis in T1D individuals may affect the capacity of the immune system to generate an appropriate regulatory response, thus accelerating the loss of immunological self-tolerance.

For this reason, this thesis has been designed to ascertain whether glucose levels and their oscillations during T1D progression can impact a proper Treg cell generation and function, by affecting FoxP3 expression.

Here, by analyzing a large cohort of T1D children at diagnosis we provided evidence indicating an increased frequency of poor functional Treg cells which is directly associated with reduced β-cell mass and poor glycaemic control. Of note, the frequency of peripheral Treg cells decreased one year later the disease onset

reflecting the differences in glucose control (measured by Continuous Glucose Monitoring). To the best of our knowledge, this thesis is the first to show that the frequency of Treg cells, measured by the expression of the transcription factor FoxP3, correlates with glycaemic levels in T1D. Further, we noticed that Tconv cells from T1D children at onset had higher glycolytic capacity than those derived from healthy children, and this event was associated with an increased ability to generate induced (i) FoxP3 Treg cells. Mechanistically, in these conditions, the enolase-1 enzyme, engaged in the glycolytic pathway, was unable to bind the regulator region of *FOXP3* and repress its transcription. However, iTreg cells from T1D individuals had a reduced capability to suppress the proliferation of effector $CD4^+$ T cells due to the transient expression of FoxP3. Strikingly, TGF-β and IL-2 supplementation, upon iTreg cell generation, stabilized FoxP3 expression and restored Treg cell suppressive capability in T1D children at onset.

Collectively, these data suggest that dysregulated glucose levels represent an accelerating factor of autoimmune reaction by favouring the generation of poorly functional Treg cells, due to unstable FoxP3 expression. Results of this thesis could open the way to identify drug interventions aiming at stabilizing FoxP3 and restoring their suppressive functions during T1D development and progression.

1. BACKGROUND

1.1 Natural history of type 1 diabetes

Type 1 Diabetes (T1D) is an autoimmune disease occurring mainly in childhood, characterized by high blood glucose levels due to insulin deficiency as the consequence of pancreatic β-cell loss (1-4). In the majority of subjects (70–90%), the loss of β-cells is the consequence of T1D-related autoimmune responses; while, in a smaller subset of individuals with genetic susceptibility is identified by the absence of self-immune responses or autoantibodies, and the cause of β-cell destruction is not completely understood (idiopathic T1D or type 1b diabetes mellitus) (5).

Over the last few years, it has been observed that T1D incidence is increasing worldwide and it is estimated that nearly 100,000 children will be affected by this disease each year (**Figure 1**) (6).

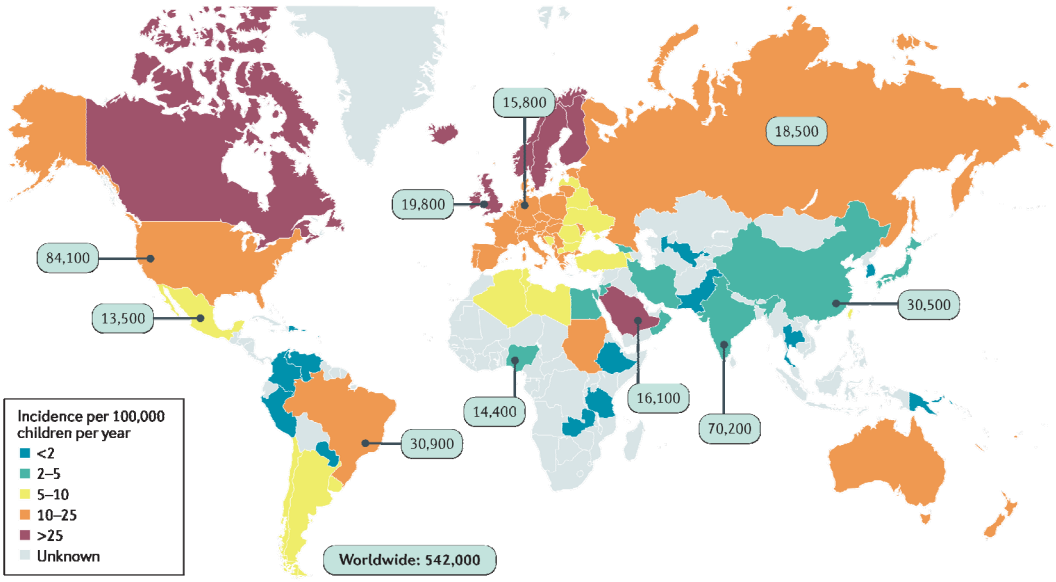


Figure 1. The incidence and prevalence of T1D in children. The estimated number of new cases of T1D in children (<15 years) per 100,000 individuals in 2015. The prevalence of T1D in the 10 most-affected countries is noted. Data from the International Diabetes Federation (http://www.diabetesatlas.org/across-the-globe.html). *Katsarou et al. Nat Rev Dis Primers, 2017.*

It is interesting to note that the rate of incidence varies is different worldwide; indeed, is highest in Nordic regions (Sweden, Finland, Denmark, and Norway), followed by European countries (Italy, France, Spain and Germany) United Kingdom, North America and Australia (**Figure 1**) (6). On the other hand, T1D incidence in Asian countries, such as China, Korea and Japan is very rare and the reasons for this remain to be fully investigated but may be related to genetic susceptibility, environmental and lifestyle factors (**Figure 1**) (7).

Thanks to scientific progress, the ability to understand the natural history of T1D has improved dramatically, particularly through the combined use of genetic, autoantibody, and metabolic markers of the disease (8). Among the models developed to define the natural history of T1D, in the mid-1980s, Eisenbarth proposed a model integrating each of these three features (3). This model indicates that genetically susceptible individuals are exposed to a putative environmental trigger, which induces β-cell autoimmunity. This process, characterized by the appearance of islet-reactive autoantibodies, leads to the development of activated autoreactive T cells able to destroy insulin-producing cells, resulting in a progressive and predictable loss of this hormone (3). It has been proposed that T1D individuals are asymptomatic until >80%–90% of the β-cells have been destroyed, suggesting a marked chronological gap between the onset of autoimmunity and the clinical manifestation of the disease (3). Although, this model has been considered for a long time a road map for investigators helping them to understand the natural history of this disease, recently, some aspects of this model have been modified (9). In comparison with the classical model, experimental and epidemiological data suggest that pancreatic β-cells may persist in some individuals with T1D for a more extended period (10). Indeed, it has been reported that 40%–50% β-cell viability

may be present at the onset of hyperglycemia, with a low exogenous insulin requirement (11).

In this scenario, an improved understanding of the natural events of asymptomatic phases (pre-diabetic stages) is critical for directing future studies aimed at the prevention of T1D development. Indeed, continued identification of genes controlling disease susceptibility, the unveiling of cellular/molecular mechanisms at the basis of loss of immune regulation, and identification of environmental determinants influencing the disease are needed to impact efforts toward the goal of disease prevention.

1.2 Pathogenesis of type 1 diabetes: the involvement of genetics, environment and cellular immunity

Decades of studies have shown that, in genetically susceptible individuals, the disease is a continuum that progresses sequentially through distinct identifiable stages before the onset of symptoms (12; 13). The pathogenesis of T1D can be divided into three different stages depending on the presence of islet-autoantibodies, hyperglycemia and hyperglycemia-associated symptoms (such as polyuria and thirst) (**Figure 2**). The first stage is asymptomatic, but the immune system has already begun attacking the insulin-producing β-cells, as testified by the presence of two or more islet autoantibodies [i.e. islet antigens insulin (IAA), glutamate decarboxylase (GADA), and the protein tyrosine phosphatase-like protein IA-2 (IA-2A), zinc transporter 8 (Znt8)] (**Figure 2**) (14). The second stage is still asymptomatic however, the presence of autoantibodies is associated with

dysglycemia due to the increasing loss of β-cells (**Figure 2**) (14). In the third stage, there is significant destruction of pancreatic β-cells, and individuals develop T1D symptoms, including hyperglycemia and metabolic imbalance (**Figure 2**).

Although B lymphocytes and autoantibodies are associated with the progression of T1D, it is well known that T cells ultimately exert the killing of insulin-producing β-cells (15).

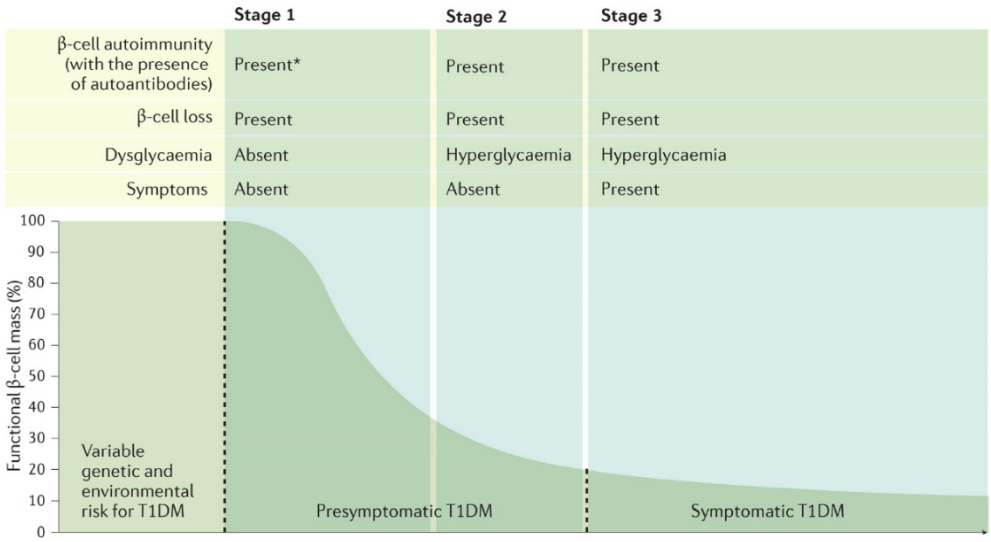


Figure 2. Type 1 Diabetes staging. Type 1 Diabetes can be subdivided into three stages: stage 1 is characterized by the presence of autoantibodies and the absence of dysglycaemia; stage 2 is characterized by the presence of both autoantibodies and dysglycaemia; symptoms only appear at stage 3, which corresponds to symptomatic T1D. *β□cell-directed autoimmunity, marked by the presence of autoantibodies targeting β□cell autoantigens, is usually present months to years before the onset of β□cell loss. T1DM, type 1 diabetes mellitus. *Katsarou et al. Nat Rev Dis Primers, 2017.*

Despite the T1D stages are predictable and well-defined, the sequel of immune pathogenic events responsible for the loss of the immunological self-tolerance during the different phases of disease is not fully elucidated.

As reported above, a clear dissection of contributing factors may help to better define the pathogenesis of this autoimmune condition.

1.2.1 Genetics

Pioneer familial aggregation and twin studies supported the importance of genetic risk factors in T1D (16), as individuals in the United States having a first-degree relative with T1D have about 1 in 20 risks of developing autoimmune diabetes, comparing to 1 in 300 risks in the general population (17). In particular, monozygotic twins have historically been considered to have a disease concordance rate of 30%–50%, with dizygotic twins having a concordance of 6%–10%. Of note, another interesting observation is that differences in disease risk are also dependent on which parent has diabetes: children of T1D mothers have about 2% risk of developing T1D, while children of T1D fathers have approximatively 7% risk (17) of diseases. Later studies found that nearly 50 genetic loci are associated with susceptibility to T1D (18-20); however, no single gene is itself either necessary or sufficient to predict the development of autoimmune diabetes.

In this scenario, the Human Leukocyte Antigen (HLA) was the first T1D susceptibility genetic locus, providing insight into the contribution to the overall genetic susceptibility. Among the classes of HLA genes, class II genes had the strongest association with T1D (17). As class II HLA genes encode for molecules that participate in antigen presentation, impacts of MHC allelic variability on T1D risk may be explained by differences in the presentation of β-cell antigens, by favouring the selection of anti-self-reactive immune cells (21). Regarding the HLA genes, HLA-DR3-DQ2 and HLA-DR4-DQ8, on chromosome 6, are the most haplotypes linked to T1D susceptibility (22-24). It is important to note that the risk of developing β-cell-targeted autoimmunity on the extended HLA-DR-DQ haplotype is complicated by many HLA-DRB1 alleles in humans. Specifically, on

the HLA-DQ8 haplotype, HLA-DRB1*04:01 and HLA-DRB1*04:05 are associated with greater susceptibility to T1D than HLA-DRB1*04:04, whereas HLA-DRB1*04:03 is protective (25-27). These latter are often associated with insulin autoantibodies (55) however, the extended haplotype HLA-DRB1*03:01-DQ2 (HLA-DQA1*05:01-DQB1*02:01) was associated with the presence of GAD65 autoantibody (28; 29). The afore mentioned genetic risk factors are common in general populations in western countries, although had a low penetrance (30; 31), which might explain why many people do not develop islet-targeted autoimmunity or T1D despite having these specific risk factors.

Genome-wide association studies (GWAS) allowed us to identify more than 50 non-HLA genetic loci that contribute to T1D risk (32; 33). Most of these genetic factors are associated with the general immune response, whereas only a limited number of them are associated with the development of β-cell-targeting autoantibodies (34). For example, non-receptor protein tyrosine phosphatase type (PTPN)-22, a molecule involved in T and B cell responsiveness and *INS* (which encodes insulin) influences the development of stage 1 in T1D (34; 35). Future investigations will be needed to reveal the extent to which these non-HLA genes contribute to disease pathogenesis in stage 2 and stage 3 of T1D. An additional non-HLA gene associated with T1D is *CTLA-4* (cytotoxic T lymphocyte associated-4) which encodes for a molecule that plays an important role in the regulation of T-cell activation and overall immune responsiveness (18; 36). Another specific gene associated with autoimmune diabetes is the interleukin-2 receptor alpha chain (*IL-2RA/CD25*) gene region (on 10p15) (37; 38), each thought to influence immune responsiveness (19). *IL-2RA/CD25* association were motivated by results from the NOD mouse model showing a strong genetic effect for T1D mapped to the

chromosome 3 region encompassing the *IL-2* gene (*Idd3*) (39; 40). Indeed, mice possessing T1D susceptibility alleles at *Idd3* have reduced IL-2 levels in comparison to mice with C57BL/6 (B6)-derived resistance alleles (41).

HLA-II and *INS* polymorphisms are also suggested to influence processes that are involved in the proper thymic immune selection, which determines an inadequate deletion of β-cell autoreactive T cells or defective generation of specific Treg cell populations (42; 43). Indeed, some *INS* polymorphisms protect against T1D development, by increasing insulin expression in thymic cells that present self-antigens to newly forming T cells (43).

All these findings indicated that immune cells expressed many genes that confer T1D susceptibility, confirming that T1D development is generally influenced by the magnitude and control of the response to immune stimuli, such as those encountered during childhood.

1.2.2 Environment

Environmental factors have been recognized as critical determinants in the pathogenesis of T1D. An increase in the number of patients with a low level of HLA-defined genetic risk also supports the suggestion of increased environmental pressure (**Figure 3**) (44-48).

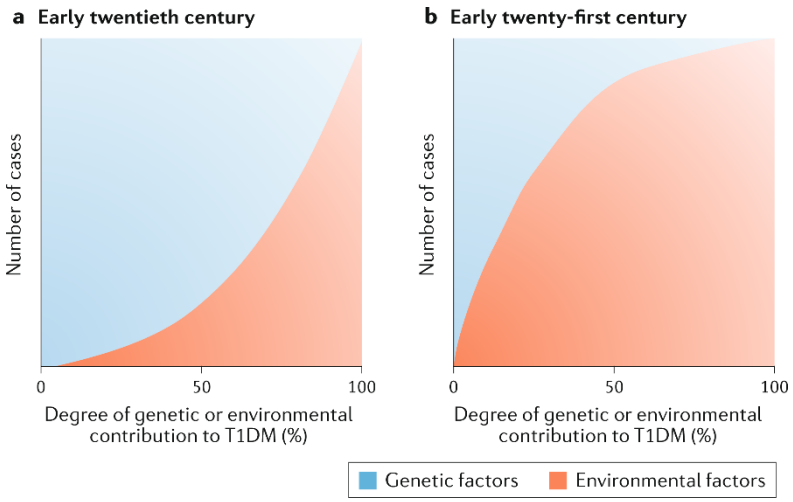


Figure 3. Estimated proportion of genetic (blue) and environmental (red) components in children with T1D.
The frequency distributions of diagnosed cases in the (part a) early twentieth century and (part b) early twenty-first century are shown. The relative number of cases with a strong genetic component has decreased according to multiple studies. T1DM, type 1 diabetes mellitus. *Ilonen et al. Nat Rev Endocrinol, 2019.*

Indeed, viral infections (49-51), the timing of the first introduction of food (52), gestational infections (53-55) and vitamin D deficiency (56) have been proposed as candidate etiological factors.

Microbial infections, particularly those associated with the gut, have been identified as prominent risk factors for T1D development (57). Among these, the relationship between β-cell autoimmunity and enteroviral infections has long been reported (58). Specifically, an increased frequency of anti-enteroviral antibodies and viral RNA in the pancreas and blood have been observed in T1D individuals (59; 60). However, the presence of antibodies against enteroviruses in those with T1D, even if true, does not prove a causal relationship. For example, subjects with autoimmunity may also be more prone to enteroviral infection, may have a stronger humoral response to infection due to their specific HLA genotype, or may be in a hyper-immune state. Indeed, different hypothetic models have been proposed to

explain the relationship between virus infections and the development of T1D. The so-called “hygiene hypothesis” attributes the rising incidence of autoimmune diseases, including T1D, to a reduced or altered stimulation of the immune system by environmental factors (61-63). Conversely, the “fertile field hypothesis” proposes that viral infections in pancreas tissues provide a fertile field, where autoreactive T cells can react with self-molecules due to bystander activation and molecular mimicry (64).

Also, environmental factors modulate gut microbiota and potentially contribute to T1D. In this regard, the “old friends hypothesis,” which is based on the role of normal gastrointestinal microbes, indicates food exposure as a possible regulator of the immune system through the alteration of gut permeability and microbiota (65). In this context, recent experimental evidence from T1D human cohorts and mouse models of the disease support the protective effects of gut microbial metabolites, such as the short-chain fatty acids (SCFAs), which limit the frequency of auto-reactive T lymphocytes (66). Other evidence further supports the protective role of gut microbiota in controlling the progression of autoimmune diabetes. For example, faecal microbiota transplantation (FMT) halts the progression of T1D, as several microbiota-derived plasma metabolites and bacterial strains preserved residual beta cell function (67).

The contribution of various nutritional factors to the risk of T1D has been intensively studied. The increase in overweight and obesity might be of importance, especially in children with T1D who have lower-risk HLA genotypes (68; 69). Numerous dietary components have been linked to the development of anti-islet autoantibodies since it has been reported that the late introduction of gluten, fruit, and cow's milk may reduce the risk of T1D (70). The most predominant example

of this association is the effect of breastfeeding and/or early exposure to cow's milk on the incidence of autoimmune diabetes (71). In support of this association, a meta-analysis revealed a weak but statistically significant correlation between T1D and both a shortened period of breastfeeding and cow's milk exposure (72). Furthermore, a large and well-organized cohort study (i.e., the Trial to Reduce IDDM in the Genetically at Risk, TRIGR) showed that supplementing breast milk with highly hydrolyzed milk formula decreased the incidence of diabetes-associated autoantibodies (71). Furthermore, several milk factors, ranging from casein to bovine insulin, as well as bovine serum albumin (BSA), have been reported in T1D development (73-76). Of note, cross-reactivity between insulin and bovine α-casein has been found, probably due to a potential molecular mimicry (77). However, no significant association between early exposure to cow's milk and β-cell damage in young siblings and offspring of T1D subjects have been reported (78; 79). Also, the ingestion of plant-derived nutrients has been associated with the development of T1D. Indeed, two large studies (i.e., the Diabetes Autoimmunity Study in the Young [DAISY] and the German study of offspring of T1D parents [BABY-DIAB]) reported that T1D risk is associated with the timing of exposure to cereal and gluten (80; 81). Furthermore, the "accelerator" and "overload" hypotheses suggest that childhood obesity increases insulin demand, determining the overloading of islet cells and β-cell damage (81-83). Despite their interesting findings, more investigations are needed to determine whether early dietary exposures affect autoimmune diabetes risk (84).

It has been reported that perinatal risk factors such as maternal-child blood group incompatibility, pre-eclampsia, neonatal respiratory distress, neonatal

infections, caesarian section, birth weight, gestational age, birth order and maternal age are associated with T1D development (85).

As previously discussed, the highest incidence of T1D worldwide occurs in northern Europe, leading some to suggest that low serum concentrations of vitamin D may be implicated in T1D development (86). In this regard, published evidence suggested that recent-onset T1D individuals had lower serum concentrations of Vitamin D compared to healthy individuals (87-89). In this context, the "North-South Gradient Hypothesis" would be consistent with the notion that the amount of UV-B exposure, which influences the synthesis of vitamin D, could modulate this metabolite and consequently the hyper-activated immune responses (90). Moreover, polymorphisms in the genes involved in vitamin D metabolism have recently been associated with T1D pathogenesis (91). However, not all reports have found an association between T1D development and Vitamin D; indeed, Bierschen et al. suggested that in the general population, most individuals are either vitamin D deficient or insufficient (92). Thus, a clear cause-effect relationship between vitamin D and autoimmune diabetes in not completely confirmed.

Finally, a very recent theory was put forward to provide a mathematical model for calculating T1D risk. The "threshold hypothesis" considered the contributions of genetics and environment as a quantifiable function of invariables subject to calculation (93). This model hypothesized that a single person has a T1D combinatorial-based risk based on the sum of single genetic and environmental odds ratios (93). However, proper testing of this hypothesis requires prospective analysis of large cohorts with high variability in their genetic and environmental T1D risks.

1.2.3 Cellular immunity and antibody response

As largely documented, dysregulation of both arms of immune responses, such as innate and adaptive immunity, is strongly implicated in the pathogenesis of autoimmune diabetes. A complex interaction between pancreatic β-cells and immune cells leads to the progressive loss of self-tolerance and T1D development (**Figure 4**).

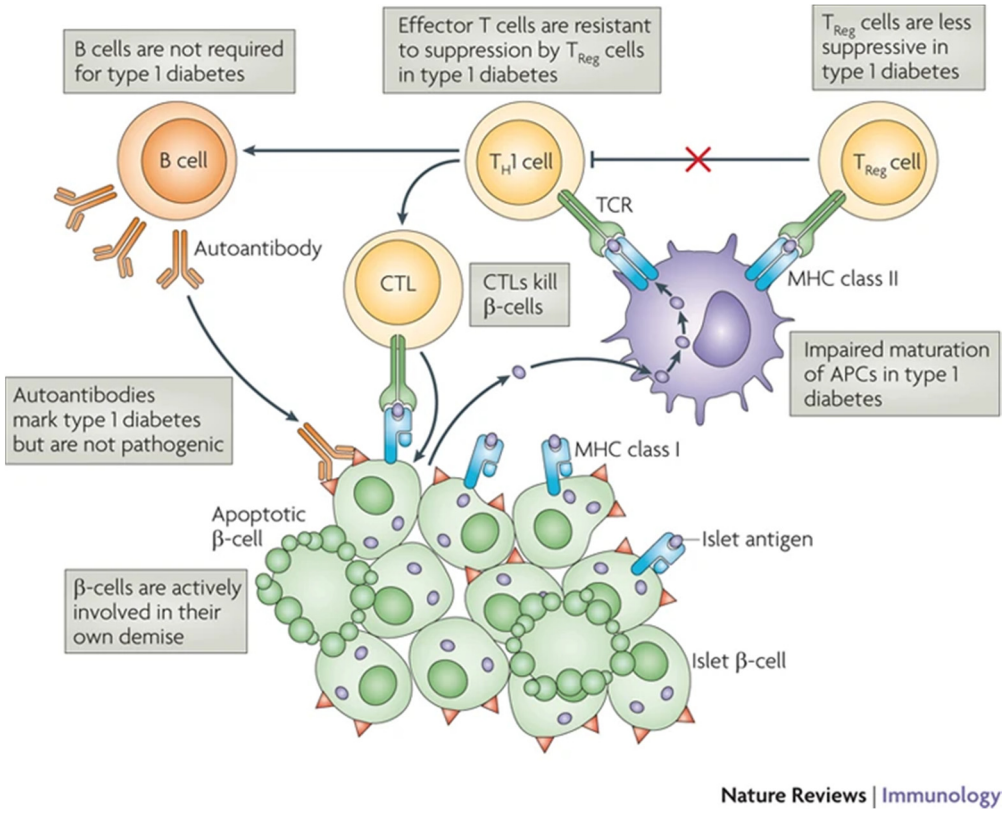


Figure 4. The main immune components involved in the multi-step process that leads to β-cell destruction in T1D.
β-cell proteins are phagocytosed by professional antigen-presenting cells (APCs), such as dendritic cells. APCs process these proteins to peptide fragments that are presented by MHC class II molecules to pro-inflammatory T helper 1 (Th1) cells, which in turn activate a cascade of immune responses, including activation of B cells (which produce islet antigen-specific autoantibodies) and islet antigen-specific cytotoxic T lymphocytes (CTLs) that can directly lyse β-cells presenting islet peptides by MHC class I molecules on their surface. Alternatively, APCs can present islet antigenic peptides to Treg cells that suppress the pro-inflammatory cascade, preventing β-cells from being destroyed. Several immunological features (boxes) distinguish patients with T1D from healthy individuals, collectively predisposing them to disease. *Roep & Peakman Nat Rev Immunol, 2010.*

Over the last decades, different theories have been proposed to explain the activation of β-cell autoimmunity, among these: molecular mimicry, defective MHC antigens expression on antigen-presenting cells (APCs), trafficking of dendritic cells from β-cells to pancreatic lymph nodes and loss of immunological self-tolerance (94).

It has been reported that the appearance of the first islet-targeting autoantibody is linked to the self-antigen presentation by APCs, and the subsequent $CD4^+$ and $CD8^+$ T cell effector responses (14). However, the real triggering event for T1D development is completely unknown. In this scenario, the appearance of an autoimmune process in the pancreas of a T1D individual is only identified by the presence of autoantibodies to islet and/or β-cell antigens, which can often be detected long before the disease becomes clinically evident (95). The five most prevalent and best-characterized autoantibodies in T1D include: islet cell autoantibodies (ICAs), autoantibodies to glutamic acid decarboxylase (GADAs), insulin autoantibodies (IAAs), and autoantibodies to transmembrane tyrosine phosphatase (IA2As), as well as those against the ZnT8 molecule (ZnT8As) (96; 97). Concerning autoantibody diagnostic and predictive role, T1D-associated autoantibodies are typically present in 70%–80% of recent onset T1D individuals (98). Of note, 0.5% of the general population and about 3%–4% of relatives of T1D individuals are autoantibody-positive (99). In this regarding, it is noteworthy that several factors influence autoantibody values, including age, gender, race and ethnicity of the population studied and, the quality of the autoantibody assay (98; 100). Considering islets autoantibodies as surrogates of T1D progression, the absolute number of autoantibodies and autoantibody titer is considered both independent predictors of T1D risk (101). More in detail, when present at high titers in younger subjects with HLA susceptibility, autoantibodies allow for a more

accurate prediction of T1D risk (8). Furthermore, the risk for T1D increases significantly when more autoantibodies are present in combination (102). For example, the risk of T1D in 5 years was 20%–25% for individuals with one autoantibody, 50%–60% for those with two autoantibodies, about 70% for those with three autoantibodies, and 80% for those with four anti-islets antibodies (102). The use of autoantibodies for predicting T1D has also been supported by several large natural history trials, including The Environmental Determinants of Diabetes in the Young (TEDDY) and the NIH TrialNet efforts, as well as in general population-based efforts (71; 103; 104). Although autoantibodies were important for T1D diagnosis, few studies have reported a pathogenic role for B lymphocytes in T1D in human. A central role of B cells in autoimmune diabetes is well documented in the NOD mouse model of the disease. Genetic or antibody deletion of B cells completely arrests the disease development at a pre-insulitis stage in mice (105). In addition, depletion of mouse B cells using anti-CD20 mAb proved effective in reversing hyperglycemia at onset (106; 107). This preclinical data proposed a rationale for developing B cell-directed therapy for T1D patients with a rapid course of the disease and unresponsive to the clinical trials that targeted T cells. However, when applied in human setting several limitations of B-cell targeted therapy should be taken into account.

Antigen-specific interactions between B and T lymphocytes can lead to the expansion of autoreactive T cell clone targeting β-cells, and it is now clear that β-cell damage is mediated by the combined actions of $CD4^+$ and $CD8^+$ T cells with specificity for islet autoantigens (108). It has been proposed that β-cell-targeted autoimmunity started a long time before disease manifestation as indicated by the presence of $CD4^+$ and $CD8^+$ T cells, dendritic cells, macrophages and B cells into

islets of Langerhans T1D subjects at T1D onset (2; 109). Indeed, both $CD4^+$ and $CD8^+$ T lymphocytes specific for β-cell antigens are detectable in both pre-diabetic and T1D individuals (110; 111). The inflammatory lesion within islets of those with T1D is typically characterized by a progressive decrease of insulin-producing β-cells along with infiltration in the pancreas of T, B-lymphocytes, and macrophages (112). Pancreatic histology, either through postmortem examination or via biopsy, is considered the "real" picture to define the immune-cell pathogenesis of T1D. However, as pancreatic biopsies have not been considered ethical and tissue autopsy from cadaveric subjects recently diagnosed with T1D is rare, major programs have recently been established to obtain these tissues for research (8). Among such, examples are the Belgium T1D registry, PEVNET (Finland), and the Network for Pancreatic Organ donors with Diabetes (nPOD) (113-115). Recent experimental evidence indicates that pathogenic T cells recognize post-translationally modified peptides from β-cells, which suggests that the loss of tolerance to β-cell autoantigens might result from changes to proteins that occur in response to stress within the β-cell (116-120). Recently it has been shown that individuals with T1D (121) or children at risk of diabetes (122) have autoantibodies to oxidative post-translationally modified insulin (oxPTM-INS). Also, the oxPTM-INS peptides are able to generate a T cell response in T1D children, supportig the concept that oxidative stress, and neoantigenic epitopes of insulin, may be involved in the immunopathogenesis of autoimmune diabetes (123).

1.3 Altered immune tolerance involved in the progression of type 1 diabetes: the impact of Treg cells

Several intricate regulatory mechanisms are taken in place to maintain immune homeostasis and prevent autoimmune conditions. In healthy subjects, pathogenic T cells are held in check by immunological tolerance processes which start in the thymus with "central" mechanisms that remove autoreactive T cells by an affinity cut-off of the self-antigens expressed by APCs (124). However, when self-reactive T lymphocytes escape the "negative selection" in the primary lymphoid organ, additional cell-mediated mechanisms operate in the periphery to warrant tissue homeostasis. In this regard, a specialized subpopulation of T cells, known as regulatory (Treg) cells, is considered a key player in maintaining immunological unresponsiveness to self-antigens and in suppressing exaggerated immune responses deleterious to the host. In particular, Treg cells ensure immune homeostasis through their defining ability to suppress the activation and function of other immune cell populations, including $CD4^+$, $CD8^+$ T lymphocytes and APCs. The expression of the transcription factor forkhead box protein P3 (FoxP3) is a well-recognized master gene of Treg cells, and FoxP3 is centrally involved in the establishment and maintenance of Treg cell phenotype and functions (125; 126). Regulatory mechanisms used by Treg cells include the production of inhibitory cytokines, cytolysis of autoreactive immune cells, metabolic disruption and modulation of dendritic cell maturation or function (125).

In the last two decades, there has been an explosion in research investigating the role of Treg cells and their relevance in the development and progression of autoimmune conditions. Specifically, a plethora of studies have analyzed the

frequency of Treg cells in T1D subjects with contrasting results; some of them reported no alterations in Treg cell frequency (127-133), and others revealed either increased (134-136) or decreased frequency in T1D (137). However, most of them analyzed the frequency of Treg cells only by the expression of CD25 molecules on $CD4^+$ lymphocytes, and only few studies on large populations utilized FoxP3 as a marker to identify Treg cells.

Recently, it has also become clear that $FoxP3^+$ Treg cells are a heterogeneous mixture of immune lymphocytes sharing a common phenotype with different states of maturation, differentiation and activation, which use different methods of suppression (138; 139). It is therefore possible that a shift in the balance or alteration in the frequency of a subtype of Treg cells might be present in T1D. For example, Okubo *et al.* recently demonstrated that the frequency of activated $FoxP3^+$ Treg cell subsets was reduced in T1D when compared with healthy subjects (132). Alterations in Treg cell population in T1D could be related to decrease stability of FoxP3 expression (140; 141) and an increase in the frequency of Treg cell subset producing pro-inflammatory cytokines, such as IFN-γ and IL-17 (131; 134).

In this scenario, a general consensus is that Treg cell suppressive capability is defective in T1D individuals, as it has been amply documented that they were less able to control the proliferation of autologous T effector (Teff) cells in comparison to healthy controls (131; 134; 138; 141). In 2005, Lindley and colleagues showed, for the first time, that Treg cells from T1D individuals were less able to control the proliferation of autologous Teff than those from HLA- and age-matched healthy individuals (127), a finding later confirmed by many other researcher laboratories (142-146). Furthermore, it has been shown that Treg cells from T1D individuals

predominantly released pro-inflammatory cytokines, whereas those from healthy subjects produced the anti-inflammatory cytokine IL-10 (147).

It has also been observed that Treg cell functions are influenced by many of the T1D susceptibility loci (e.g., *IL2RA*, *IL2*, *PTPN2*, *CTLA4* and *IL10*), contributing to disease pathogenesis (148). In this context, Pesenacker and colleagues recently examined the expression of a panel of FoxP3-specific transcripts in Treg cells from T1D recent-onset individuals and well-matched healthy subjects (149). They identified a panel of six genes [i.e., *FOXP3*, *TNFRSF1B* (*CD120b*) and *LRRC32* (*GARP*)] directly linked to Treg cell function and stability which were differentially expressed in Treg cells from T1D individuals and healthy subjects (149). Similarly, other studies have identified differences in Treg cell gene expression profiles according to T1D development or not (150). Recently, Yang and colleagues linked many of these associations, demonstrating that T1D individuals with IL-2 sensitivity in $CD4^+$ T-cell subsets, had Treg cells that were less able to maintain FoxP3 expression under limiting concentrations of IL-2, which also displayed reduced suppressive functions (151).

To better understand how immune tolerance is lost in T1D, a key issue to address is whether the altered Treg cell suppressive function is a consequence of the disease or whether is involved in disease initiation. Studies examining Treg cell function in autoantibody-positive individuals suggested that Treg cell defects are present before clinical disease, supporting a causative role for Treg cell suppressive dysfunction in T1D (144; 152).

1.4 Regulatory T cells: the control of FOXP3 gene

In the late 1990s, the investigation on suppressor cells, redefined as "regulatory" T cells, gained new light thanks to the findings of Sakaguchi and colleagues who showed that a subset of $CD4^+$ T lymphocytes, expressing the surface expression of the IL-2 receptor α-chain (CD25), contributes to maintaining immunological self-tolerance in a mouse model of autoimmune disease (153). The identification by the same group, of the transcription factor FoxP3, as the master regulator for lineage stability and suppressive function (154), has then accelerated Treg cell biology research in both physiological and pathological settings (155; 156). It has been shown that FoxP3 controls Treg cell differentiation and stability, and its expression is required for the maintenance of the Treg cell transcriptional program and suppressive capability (155). Treg cell suppressive activity is exerted *via* cell-to-cell contact on different immune cell subsets and is mediated by several proteins expressed by Treg cells, such as the cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), programmed cell death (PD)-1, Lymphocyte Activation Gene 3 (LAG-3) and T cell immunoglobulin and ITIM domain (TIGIT) (157-160). Among the other cell-surface molecules involved in the suppressive activity of Treg cells, CD39 and CD73, two ectoenzymes highly expressed on these cells, have also great importance, as they act by facilitating the elaboration of adenosine, which acts as a paracrine immune modulatory factor (161; 162). Another well-described mechanism of Treg cell action is based on the release of specific anti-inflammatory cytokines, mainly represented by IL-10 and TGF-β (163-165); more recently, an increasing interest is also been conveyed in the analysis of Treg cell-derived

extracellular vesicles (EVs) which are able to target and inhibit effector T (Teff) cell function in both human and mice settings (166-169).

1.4.1 Regulation of the FOXP3 gene

The human *FOXP3* gene is located in the p-arm of the X chromosome, includes 11 exons and displays a high degree of conservation with the mouse genes, especially at the exon-intron interfaces (170; 171). The control of FoxP3 expression depends on several transcription factors and regulatory elements within the *FOXP3* gene locus (**Figure 5**).

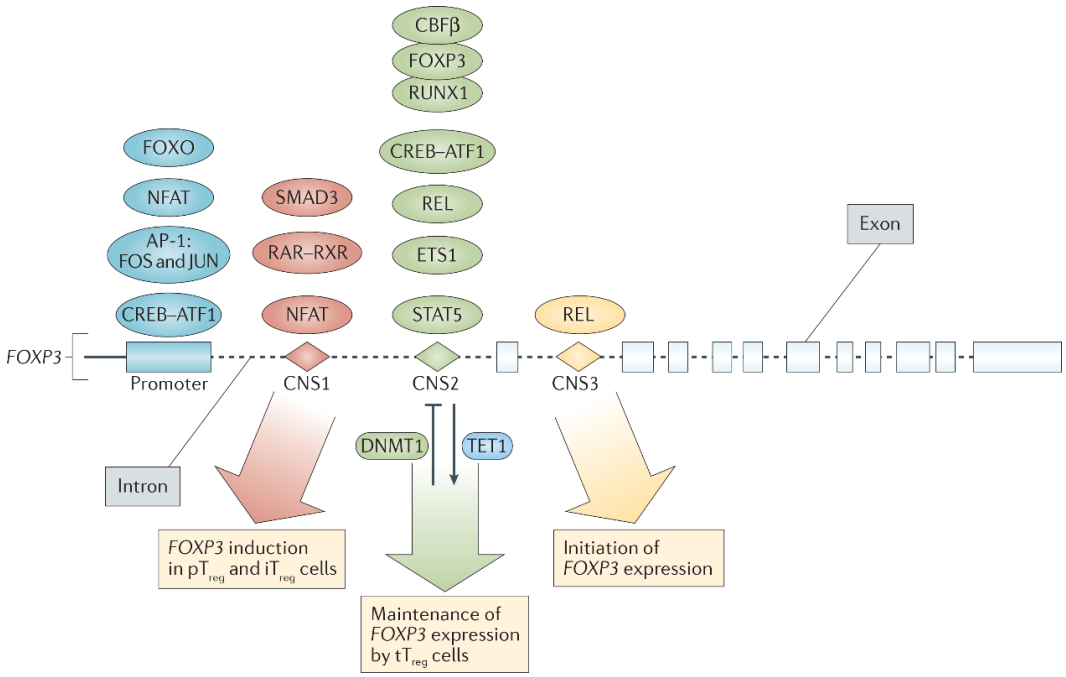


Figure 5. The control of FoxP3 expression by transcription factors and regulatory elements within the FOXP3 gene locus.
The figure depicts the characterized coding and non-coding elements of the gene that encodes forkhead box protein P3 (*FOXP3*) along with the transcription factors that are reported to activate the transcription of the gene and their sites of interaction. Transcription factors that bind to the promoter, conserved non-coding sequence 1 (CNS1), CNS2 and CNS3 regions of FOXP3 are shown in blue, red, green and yellow, respectively. Also depicted are the CNS2-targeting methylating enzyme DNA methyltransferase 1 (DNMT1) and the demethylating enzyme ten-eleven translocation 1 (TET1), which influence the inactive and active transcriptional status of that region, respectively. ATF1, activating transcription factor 1; CBFβ, core-binding factor subunit-β; CREB, cAMP-responsive element-binding protein; FOXO, forkhead box protein O; iTreg, in vitro-induced regulatory T; NFAT, nuclear factor of activated T cells; pTreg, peripherally derived regulatory T; RAR, retinoic acid receptor; RUNX1, Runt-related transcription factor 1; RXR, retinoid X receptor; STAT5, signal transducer and activator of transcription 5; tTreg, thymus-derived regulatory T cells. *Lu et al. Nat Rev Immunol , 2017.*

In response to TCR engagement and co-stimulation signals, the *FOXP3* promoter is bound and activated by transcription factors such as NFAT and AP-1 (172). In addition, the forkhead box O (FOXO) proteins FOXO1 and FOXO3 have been reported to bind to the *FOXP3* promoter as well as to other regulatory elements (173); cAMP-responsive element-binding protein (CREB)–activating transcription factor 1 (ATF1) complexes also drive the activation of the *FOXP3* promoter (174). Studies aim at characterizing the promoter of this transcription factor, showed that its transcription relied on the contribution of conserved enhancer regions (172). Indeed, both the initiation and maintenance of *FOXP3* transcription are dependent on key conserved non-coding sequences (CNSs), which serve as binding sites for several transcription factors (**Figure 5**).

CNS3 is found between exon 1 and exon 2 of *FOXP3*, promoting the accumulation of permissive histone modifications at the *FOXP3* promoter, leaving it in an epigenetically controlled -state in both established FoxP3^{+} cells and FoxP3^{-} Treg cell precursors (175). Thus, CNS3 has a crucial role as an epigenetic switch that controls *FOXP3* transcription in Treg cells in response to binding by the transcription factor REL, which is a crucial regulator of this cell subset development in the thymus (176; 177). However, although it is indispensable for initiating *FOXP3* expression, CNS3 does not seem to be necessary for its maintenance.

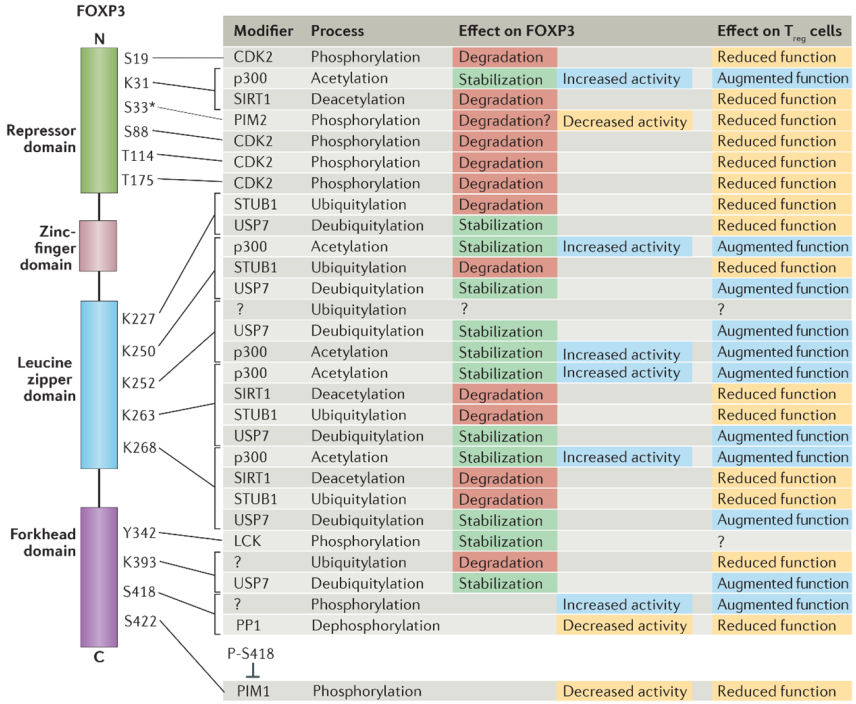
On the other hand, CNS2 (also known as Treg cell-specific demethylated region) is important for maintaining the expression of FoxP3 in thymus-derived (t)Treg cells once they reach the periphery (125). The CNS2 region is located within the first intron of *FOXP3*, and CpG elements within CNS2 become extensively hypo-methylated during Treg cell development (178). Demethylated CpG motifs in

CNS2 and other regulatory elements in the *FOXP3* gene enable the binding of several transcription factors, including REL, CREB–ATF1, RUNX1–core-binding factor subunit-β (CBFβ), ETS1, signal transducer and activator of transcription 5 (STAT5), and FoxP3 itself (172; 175). It has been established that the maintenance of this epigenetic state allows the stable expression of FoxP3 by tTreg cells under different conditions, including tissue inflammation (125). Moreover, *in vitro*-induced Treg (iTreg) cells, which are less stable than tTreg cells in terms of their *FOXP3* expression and suppressive activity, have a methylated or partially demethylated CNS2 region (179; 180). By contrast, peripherally derived (pTreg) cells with an epigenetic profile that resembles that of tTreg cells, are thought to be functionally stable (179). Hypo-methylation state of CNS2 is warranted by methyl-CpG-binding domain protein 2 (MBD2) which binds CNS2 and in turn recruits the ten-eleven translocation (TET) demethylases (125). Loss of function of MBD2 in mice results in decreased numbers of Treg cells, with a reduced suppressive capability (179). Conversely, DNA methyltransferase 1 (DNMT1) is known as the main enzyme that promotes methylation events at the CNS2 region (125). DNMT1 and similar factors may counteract the epigenetic programming that is responsible for tTreg cell activity (125). Indeed, IL-6, a pro-inflammatory molecule, triggers the DNMT1-dependent methylation of CNS2 motifs in Treg cells, whereas exposure to IL-2 induces TET enzymes which determines hypomethylation at the CNS2 region (181; 182). Also, exposure of Treg cells to IL-6 reduces the acetylation of histone H3 at the upstream promoter as well as negatively affects the *FOXP3* transcription (183; 184). On the other side, transforming growth factor-β (TGF-β) signalling operates the epigenetic stabilization of *FOXP3* by the suppression of DNMT1 expression (185).

The CNS1 enhancer is pivotal for the induction of extrathymic *FOXP3* expression in T cells and it is indispensable for activating *FOXP3* expression in response to TGFβ-induced SMAD signalling (186). Of note, the binding of activated SMAD3 to CNS1 is a key event in the induction of FoxP3 during the differentiation of pTreg cells but not that of tTreg cells (175). Accordingly, CNS1-deficient mice have a defective pTreg cell compartment and consequently loss the maintenance of immune homeostasis at barrier sites (187).

1.4.2 Post-translational modifications of FoxP3

Compelling evidence revealed that the expression and regulatory activity of FoxP3 are also controlled at the protein level. The pathways that lead to distinct



Modifier	Process	Effect on FOXP3		Effect on T_{reg} cells
CDK2	Phosphorylation	Degradation		Reduced function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
PIM2	Phosphorylation	Degradation?	Decreased activity	Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
CDK2	Phosphorylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Reduced function
p300	Acetylation	Stabilization	Increased activity	Augmented function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
?	Ubiquitylation	?		?
USP7	Deubiquitylation	Stabilization		Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
p300	Acetylation	Stabilization	Increased activity	Augmented function
SIRT1	Deacetylation	Degradation		Reduced function
STUB1	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
LCK	Phosphorylation	Stabilization		?
?	Ubiquitylation	Degradation		Reduced function
USP7	Deubiquitylation	Stabilization		Augmented function
?	Phosphorylation		Increased activity	Augmented function
PP1	Dephosphorylation		Decreased activity	Reduced function
PIM1	Phosphorylation		Decreased activity	Reduced function

*Functional observations for PIM2 were made in mice

Figure 6. Post-translational modifications of FoxP3 and their impact on Treg cell function. Depicted on the left is a schematic representation of the mature forkhead box protein P3 (FOXP3) molecule showing its functional domains and reported post-translational modification sites. The table on the right summarizes the types of modification that can occur in each region of FOXP3, the effects of these modifications on FOXP3 protein stability and function, and the subsequent impact on the suppressive function of regulatory T (Treg) cells. CDK2, cyclin-dependent kinase 2; PP1, protein phosphatase 1; SIRT1, sirtuin 1; USP7, ubiquitin-specific peptidase 7. *Lu et al. Nat Rev Immunol , 2017.*

post-translational modifications of FoxP3 — including acetylation, phosphorylation and ubiquitination — have been recently shown to be important in this newly appreciated layer of control over Treg cell function (**Figure 6**) (125).

1.4.2.1 Acetylation of FoxP3. The enzymes lysine acetyltransferases (KATs) catalyse the attachment of acetyl groups at specific lysine residues of the FoxP3 protein (125). Acetylated FoxP3 molecules are more stable than those that are not acetylated, as they avoid ubiquitination at targeted lysine residues and thus resist proteasomal degradation (125). Acetylation acts by improving the ability of FoxP3 to bind to chromatin and carry out its functions as a transcriptional regulator (188; 189). In this context, it has been shown that FoxP3 interacts with two specific KATs, such as TIP60 and p300, which promote its acetylation at residues K63, K263 and K268 (188-191). Of note, the inhibition or deletion of p300 reduced the levels of both acetylated and total FoxP3 in Treg cells, and negatively influenced the fitness and function of these immune cell subsets (190; 192). Genetic ablation of *TIP60* also markedly decreased FoxP3 expression levels, leading to Treg cell dysfunction and the development of autoimmune disorders (190; 192). Indeed, TIP60-deficient Treg cells showed reduced expression of FoxP3 and CTLA4 molecules, as well as exaggerated production of the pro-inflammatory cytokines IL-6 and IL-17 (192). IL-6-induced STAT3 activity can downregulate the levels of the FoxP3 protein by inhibiting its acetylation (189; 193), while TGF-β augments the FoxP3 stability and/or functional activity by driving its acetylation (189).

Lysine deacetylases (KDACs) and Histone deacetylases (HDACs) remove acetyl groups and negatively affect FoxP3 expression and Treg cell function; while their inhibitors are well known to augment FoxP3 expression in Treg cells and improve

regulatory capability (188; 191; 194; 195). Specifically, Sirtuin 1 (SIRT1) is a class III KDAC, and its expression has been observed to be inversely proportional to the expression of Foxp3 during iTreg cell differentiation (196). Interestingly, SIRT1 is differently regulated by TCR activation depending on the T cell subset considered. Indeed, while TCR stimulation in non-Treg cells induced SIRT1 levels, on the contrary in Treg cells TCR engagement reduced SIRT1 expression (196). Moreover, forced SIRT1 expression in Treg cells not only leads to the deacetylation of FoxP3 but also triggers the rapid polyubiquitylation of the transcription factor and its degradation by the proteasome (196). Conversely, Treg cell-specific *SIRT1* ablation determined an increase of FoxP3 expression and Treg cell suppressive functions by upregulating the expression of Treg cell-associated genes (for example, *Ctla4*) (197). Also, chemical inhibition of this enzyme similarly increases FoxP3 levels and the *in vivo* suppressive capability of iTreg cells (198). Recently, it has been observed that the serine/threonine kinase and Hippo pathway participant mammalian sterile 20-like kinase 1 (MST1) antagonize the FoxP3-deacetylating activity of SIRT1 (199). In particular, MST1 interacts with FoxP3 and promotes its activity as a transcriptional suppressor by increasing its acetylation; Treg cells isolated from MST1-deficient mice have reduced levels of FoxP3 protein owing to its degradation (199).

Finally, commensal microorganisms in the gut are involved in the induction of pTreg cells by the acetylation of FoxP3 (200; 201). For example, $CD4^+$ T cell exposure to short-chain fatty acids (SCFAs), such as butyrate, produced by certain species of gut bacteria, increased FoxP3 induction by promoting histone modifications (200). This ability of SCFAs to support Treg cell induction seems to

involve HDAC inhibition and results in the reduced acetylation of the FoxP3 protein and histones at the *FOXP3* locus (201).

1.4.2.2 Phosphorylation of FoxP3. It is now well-established that FoxP3 regulation also depends on phosphorylation processes. The C terminus region of FoxP3 can be modified by an unknown kinase at S418 which improves the ability of FoxP3 to bind to DNA and drives Treg cell-associated gene expression(125). By contrast, Foxp3 protein phosphorylation at different sites inhibits its ability to promote Treg cell suppressive capability; further, cyclin-dependent kinase 2 (CDK2), which is activated by TCR signalling, is capable of phosphorylating four CDK motifs within the N-terminal domain of FoxP3 (202). CDK-mediated phosphorylation might negatively influence FoxP3 expression, as CDK2-deficient Treg cells are more suppressive compared to wild-type cells (203). Indeed, specific phosphorylation by CDK-2 of S19 was observed *in vitro*, and mutation of this and other residues (S88, T114 and T175) in the repressor domain was associated with an increased FoxP3 half-life and increased repression of target genes (125). In agreement, the ectopic expression in Tconv cells of a mutant FoxP3 construct insensitive to S19 phosphorylation determined a high suppressive function *in vitro* and *in vivo* (202).

Among the protein kinase that controls FoxP3, it has been shown that the kinase PIM1 interacts with FoxP3 and exerts its phosphorylation (204). Indeed, Li *et al*. found that PIM1, highly expressed by human Treg cells, interacts with the C-terminal domain of FoxP3 and targets S422 residue (204). The PIM1-induced phosphorylation of S422 affects FoxP3 activity and hampers the expression of Treg cell-associated genes (204). Although TCR signalling limits the induction of

PIM1 expression, IL-6 can upregulate the expression of the kinase. Of note, inhibitory modification at S422 prevents phosphorylation at S418 (204), improving Treg cell function (205).

A related kinase known as PIM2 also exerts inhibitory phosphorylation of FoxP3 in Treg cells, as it targets multiple N-terminal sites (i.e., S33 and S41), thus interfering with the expression of Treg cell-associated genes; indeed, inhibition of PIM2 in mouse Treg cells increases their suppressive capabilities (206). Interestingly, it has also been reported that PIM2 expression is FoxP3-dependent and involved in the expansion of Treg cells (207). It is possible to hypothesize that the upregulation of this kinase impairs the suppressive function of Treg cells in order to favour proliferative response.

Taken together, these findings suggest that phosphorylation event may modulate FoxP3 stability, thus representing an additional mechanism through which is possible to control Treg cell fitness.

1.4.2.3 Ubiquitination of FoxP3. Ubiquitination at lysine residue 48 (K48 polyubiquitylation) is critical for facilitating the proteasomal degradation of several proteins (208). Scientific evidence has shown that the FoxP3 protein can be modified by this process which has major implications for Treg cell functions (209). For example, it has been demonstrated that in differentiating $CD4^+$ T cells, hypoxia-inducible factor 1-α (HIF-α) was found to physically interact with FoxP3 and trigger K48 polyubiquitylation; accordingly, knocking down components of the HIF1-α stabilized the FoxP3 protein pool (210).

The ubiquitin-dependent degradation processes control FoxP3 stability also in established Treg cells under inflammatory response. In this context, it is interesting

to note that the cellular levels of the FoxP3 protein are subject to constant turnover depending on K48-type polyubiquitylation-induced proteasomal degradation (211; 212). Also, the half-life of FoxP3 is reduced upon exposure to a range of inflammatory conditions *in vitro*, including lipopolysaccharide, which leads to the degradation of Foxp3 through K48-linked polyubiquitination by the E3 ubiquitin ligase Stub1 (211). Mechanistically, the chaperone molecule heat shock 70 kDa protein (HSP70) recruits the stress-activated U-box domain type E3 ubiquitin ligase Stub1, which in turn mediates the degradation of FoxP3 and other important Treg cell transcription factors, such as RUNX2, HIF1-α (211; 213; 214). This downregulation results from K48-type polyubiquitylation at residues K227, K250, K263 and K268 in human FoxP3 by Stub1, with subsequent proteasome activity (211). Ectopic Stub1-mediated FoxP3 loss hampers Treg cell suppressive functions while knocking down *STUB1* stabilizes FoxP3 expression and increases their regulatory capability (211).

1.4.3 Instability of Treg cell lineage and generation of ex-Treg cells

As stated before FoxP3 is indispensable for the development and functions of Treg cells. However, increasing evidence indicates that under specific micro-environmental conditions (such as IL-2 depletion, strong TCR engagement with autoantigens and pro-inflammatory cytokines), Treg cells can lose FoxP3 expression and differentiate in so-called 'ex-Treg' cells *in vivo* (**Figure 7**) (215-217).

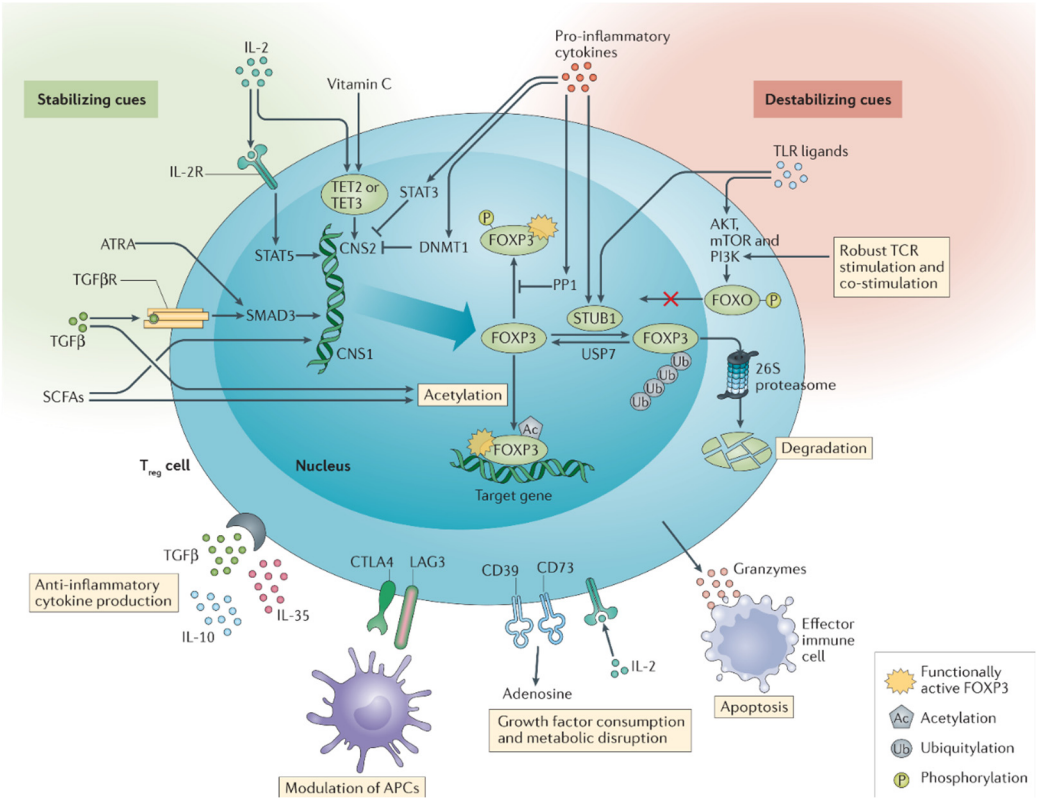


Figure 7. Environmental cues modulate the transcription, stability and function of FoxP3.
The induction and maintenance of forkhead box protein P3 (*FOXP3*) transcription can be positively influenced by cytokines such as transforming growth factor-β (TGF-β) and interleukin-2 (IL-2), and by other factors in the tissue microenvironment, such as retinoic acid, vitamin C and short-chain fatty acids (SCFAs). By contrast, FOXP3 is negatively regulated by pro-inflammatory cytokines, such as tumour necrosis factor (TNF) and IL-6, and by other factors, such as Toll-like receptor (TLR) activation or robust T cell receptor (TCR) and co-stimulatory molecule signalling. These factors can alter the post-translational modifications that are made to the mature FOXP3 protein to either stabilize or deplete the cellular pools of FOXP3 and modulate its functional capacity. By affecting FOXP3 expression and function, these factors influence the many functions of regulatory T (Treg) cells, such as their production of anti-inflammatory cytokines, their modulation of antigen-presenting cell (APC) function, their consumption of growth factors and their ability to induce apoptosis in effector immune cells. ATRA, all-trans-retinoic acid; CNS, conserved non-coding sequence; CTLA4, cytotoxic T lymphocyte antigen 4; DNMT1, DNA methyltransferase 1; FOXO, forkhead box protein O; IL-2R, IL-2 receptor; LAG3, lymphocyte activation gene 3 protein; mTOR, mechanistic target of rapamycin; PI3K, phosphoinositide 3-kinase; PP1, protein phosphatase 1; STAT, signal transducer and activator of transcription; TET, ten-eleven translocation; TGFβR, TGFβ receptor; USP7, ubiquitin-specific peptidase 7. *Lu et al. Nat Rev Immunol, 2017.*

The TCR repertoire of ex-Treg cells substantially overlaps with that of both Treg and Tconv cells, suggesting that ex-Treg cells are a heterogeneous cell subset derived in part from established Treg cells that have lost FoxP3 expression and in part from Tconv cells that expressed unstable FoxP3 (215; 218). FoxP3 expression in Treg cells is maintained intrinsically by a positive feedback loop exerted by

several transcription factors, including the transcription complex Cbfβ-Runx1-FoxP3 which binds the CNS2 region (175; 219-222); indeed, Treg cells lacking either CNS2 or Cbfβ, or Runx1 have an unstable FoxP3 expression (175; 220). A recent study revealed that several STAT proteins, such as STAT5, STAT6 and STAT3 bind to the CNS2 *FOXP3* locus (223). While STAT5 (downstream of the IL2 pathway) stabilized FoxP3 expression, STAT6 and STAT3 activated by pro-inflammatory cytokines may effectively compete with STAT5 for the binding and may bring DNA methyltransferases silencing *FOXP3* transcription (223). Also, in the absence of CNS2, Treg cells are unable to maintain heritable FoxP3 expression levels either under IL-2-limiting conditions (223). A relevant study shows that the CNS2-mediated feedback loop is critical for the maintenance of Treg cell lineage stability. Indeed, Li *et al.* revealed that NFAT-mediated looping between CNS2 and the FoxP3 promoter is critical for the expression of FoxP3 in activated Treg cells (224). Recent evidence reports that PTEN is essential for maintaining Treg cell stability by limiting phosphoinositide 3-kinase (PI3K) signalling, which also supports the notion that TCR overstimulation destabilizes Treg cells (225; 226). In addition, other signalling pathways are involved in the control of FoxP3 stability; it has been established that both CDK2 and PIM1 mediated the down-regulation of DNA binding activity of human *FoxP3*, thus inducing Treg cell instability (202; 204). Upon loss of *FoxP3*, Treg cells showed an impaired suppressive capability and acquired an effector phenotype characterized by the production of IFN-γ and IL-17. The stability of Treg cells is pivotal to ensure immune homeostasis especially in local tissues, such as pancreas islets and loss of FoxP3 alters the immune balance and unleashes a local pathogenic T-cell response (227). Also, the Th cell-like

effector functions of 'ex-Treg' cells directly contribute to the further implement pathogenic immune responses in local tissues (227).

In this regard, it has been proposed a model to understand the pathogenesis of autoimmune diseases on the basis of the presence of ex-Treg cells (**Figure 8**) (227). First, exposure of self-antigens and pro-inflammatory cytokines in damaged tissues continually activates effector Th cell-mediated immune responses, which in turn attack the self-tissues, resulting in more self-antigen exposure and production of pro-inflammatory cytokines *in situ* (227). Next, self-antigens and pro-inflammatory cytokines in damaged tissues promote the instability of Treg cells; unstable Treg cells further sustain the autoimmune attack, by favouring the activation and expansion of autoreactive effector T cells which in turn promote the generation of ex-Treg cells by their cytokine secretions (e.g., IL-4 and IL-6) (227).

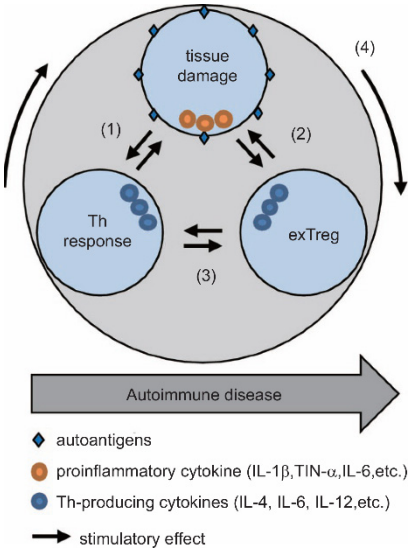


Figure 8. The multilayer feed-forward loop model of autoimmune diseases.
(1) Exposed autoantigens and pro-inflammatory cytokines in damaged tissues activate effector Th cells, which in turn exacerbate self-tissue damage. (2) Exposed self-antigens and pro-inflammatory cytokines in damaged tissues promote the generation of exTreg cells, which are pathogenic for the self-tissues. (3) exTreg cells favor the activation and expansion of autoreactive effector Th cells, which may further induce the generation and effector Th-like functions of exTreg cells through Th-producing cytokines. Together, these feed-forward loops constitute a higher-level feed-forward loop (4) that drives the development of autoimmune diseases forward. Th, T helper cells; Treg, regulatory T cells. *Guo & Zhou Cell Mol Immunol, 2015.*

2. AIM OF THE STUDY

The main objective of this PhD thesis was to ascertain at cellular and molecular levels whether altered glucose levels and their oscillations during T1D progression impact a proper Treg cell generation and function, affecting FoxP3 expression and stability. Our working hypothesis is that the absence of appropriate glucose homeostasis, typically observed in T1D individuals, may affect the capability of the immune system to generate an effective regulatory response, thus accelerating the loss of immunological self-tolerance. Although glucose is an energetic fuel essential for Treg cells, it is possible to hypothesize that high amounts and/or defective control of its levels may lead to impaired Treg cell functions, thus associating with the development of pathological inflammatory/autoimmune conditions. In agreement, high glucose intake has been proposed as one of the main environmental risk factors responsible for the increasing incidence of autoimmune disorders (228-232). As a relevant example, the study cohort Diabetes Autoimmunity Study in the Young (DAISY) showed that a higher glycemic index and increased intake of total sugars were associated with a major risk of T1D progression (233; 234). The observed association between increased total sugar consumption and more rapid T1D progression in children with islet autoantibodies may be driven by direct or indirect biological mechanisms not yet discovered.

Therefore, to test our hypothesis several objectives were explored:

- Frequency of peripheral Treg cells and their suppressive capability in T1D children at onset and one year later, in correlation with β-cell function and glucose levels.
- Impact of blood glucose levels on *in vitro* FoxP3 induction and its stability in iTreg cells from T1D subjects.

Results of this PhD thesis could open the way to understanding whether altered glucose metabolism is able to affect the molecular mechanisms regulating FoxP3 protein expression, thus explaining the defective functional capability of Treg cells observed in T1D. Targeting manipulation of glucose concentration during T1D progression phase may contribute to developing novel immune-therapeutic strategies with a view to controlling the loss of immunological self-tolerance.

3. RESEARCH DESIGN AND METHODS

3.1 Study population

Newly diagnosed T1D children (n = 100) were recruited after glycaemic stabilization (10 days from clinical onset) by treatment with exogenous insulin (glucose values between 3.5-10 mmol/l or 80-180 mg/dl) at the Department of Translational Medical Sciences, Paediatric section, University of Naples 'Federico II' and were followed-up for 1 year. Diagnosis of T1D was defined according to the Global International Diabetes Federation/International Society for Paediatric and Adolescent Diabetes Guidelines for Diabetes in Childhood and Adolescence and included symptoms of diabetes in addition to casual plasma glucose concentration ≥11.1 mmol/L (200 mg/dl), or fasting plasma glucose ≥7.0 mmol/l (≥126 mg/dl), or 2 hours post-load glucose ≥11.1 mmol/l (≥200 mg/dl) during an oral glucose tolerance test, and glycated haemoglobin (HbA1c) ≥6.5 and all of them were positive for at least two anti-islet autoantibodies.

Glucose control was assessed by HbA1c and CGM by FreeStyle Libre (at 1-year follow-up), defined according to the international consensus on time in range. CGM measurement included: the percentage of readings within the target glucose range of 70-180 mg/dl (TIR), time below (TBR) and time above the target glucose range (TAR).

In parallel, healthy control children with similar sex and age distribution (n = 100) were recruited in the same Paediatric department, upon selection for the following criteria: fasting blood glucose <5.5 mmol/l (<100 mg/dl); negative personal and familial history of autoimmune disorders; and negativity for islet autoantibodies at the 99th percentile.

The Institutional Review Board of the Ethics Committee of the University of Naples "Federico II" approved the study. All adult human subjects, or parents of participating children, provided written informed consent.

3.2 Laboratory testing

Blood samples from T1D and healthy children were withdrawn at 8.00 a.m. into heparinized BD Vacutainers and processed within the following 4 hours. Serum or plasma was obtained after centrifugation and kept at –80°C until use. Fasting C-peptide levels were measured in duplicate serum samples, at the same time for all samples, using a commercial ELISA kit (Merck Millipore Corporation). Glucose levels were measured using the enzymatic hexokinase method and HbA1c by high-performance liquid chromatography (HLC-723 G7 TOSOH, Bioscience). Islet autoantibodies (GADA, IA-2A, IAA, ZnT8) were measured by a commercial ELISA kit (Pantec). The remaining part of the blood samples was processed and after Ficoll-Hypaque (GE-Healthcare) gradient centrifugation, PBMCs were obtained.

3.3 Multiparametric flow-cytometry analyses

Multiparametric flow cytometry was used for the evaluation of Treg cells from PBMCs: APC-H7 anti-human CD4 (BD Pharmingen, clone RPA-T4), FITC anti-human CD45RA (Miltenyi Biotec, clone REA562), BB700 anti-human CCR7 (BD Horizon, clone 3D12), PECy7 anti-human CD25 (BD Pharmingen, clone M-A251), BV421 anti-human PD-1 (BD Horizon, clone EH12-1), PE anti-human

FoxP3-all (BD Pharmingen, clone 259D/C7), APC anti-human CD152/CTLA-4 (BD Pharmingen, clone BN13), BV421 anti-human GITR (BD Horizon, clone V27-580). Staining for intracellular factors was performed by using the fixation and permeabilization FoxP3 buffer kit (BD Pharmingen), according to the manufacturer's instructions. Samples were acquired by using two lasers equipped FACSCanto II (BD Bioscience); at least $3x10^4$ events in the lymphocyte gate. For the evaluation of positive events, fluorescence minus one (FMO) controls were used for setting the gate; non-viable cells were detected by 7-AAD viability staining (BD Pharmingen). Cytofluorimetric analyses were performed by using FlowJo Software (FlowJo, LLC).

3.4 Induction of Treg cells in vitro, isolation and re-stimulation assay

For the generation of iTreg cells, $CD4^+CD25^-$ T cells (Tconv cells) were isolated from PBMCs by negative selection with a human $CD4^+CD25^+$ T cell kit (Miltenyi) (cell purity >98%). Cells were cultured (2×10^6 cells/ml) with RPMI-1640 medium supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and 5% autologous plasma and were stimulated for 12 or 36 hours with anti-CD3/CD28 microbeads (Invitrogen) at a density of 0.1 beads per cell. To isolate iTreg cells, stimulated Tconv cells were stained with the following mAbs–PE–Cy7 - anti-human CD25 and APC-H7 anti-human CD4; then cells were sorted by flow cytometry on the basis of CD25 surface expression (purity > 98%) with a BD FACSJazz (BD, Biosciences). For the re-stimulation assay isolated iTreg cells

were re-stimulated with a low dose of anti-CD3/CD28 microbeads (0.1 beads per cell) for 24, 48 and 96 hours, respectively, with or without supplementation of TG-Fβ (20 ng/mL) and IL-2 (100 UI/mL).

3.5 Suppression assay

For the assessment of Treg cell suppressive capability, autologous $CD4^+CD25^-$ T cells were labelled with the fluorescent dye Celltrace Violet (Thermo Fischer Scientific). Then labelled $CD4^+CD25^-$ T cells were stimulated for 72 hours with anti-CD3/CD28 microbeads (0.2 beads per cell) alone or with different numbers of peripheral Treg or iTreg cells (ratios from 1:1 to 8:1, labelled $CD4^+CD25^-$ T cells/Treg cells) in round-bottomed 96-well plates (all from Becton-Dickinson). All tests were performed in the presence of RPMI-1640 medium supplemented with 100 UI/ml penicillin, 100 μg/ml streptomycin (Life Technologies) and with heat-inactivated 5% autologous plasma, with or without supplementation of TGF-β (20 ng/mL) and IL-2 (100 UI/mL), as reported. Celltrace Violet dilution analysis was performed by gating on labelled $CD4^+CD25^-$ T cells using BD FACSCanto II and analysed by FlowJo software.

3.6 Seahorse analyses

Metabolic profile was evaluated in Tconv cells stimulated for 12 and 36 hours in the presence of anti-CD3/CD28 microbeads (0.1 beads/cells) to generate iTreg cells. Extracellular acidification rates (ECAR) were performed by an XFe-96 Analyzer

(Seahorse Bioscience). Specifically, cells were plated in XFe-96 plates (Seahorse Bioscience) at the concentration of $4x10^5$ cells/well and cultured with RPMI-1640 medium supplemented with 5% autologous plasma. ECAR was measured in XF media in basal condition and in response to 10 mM glucose, 5 μM oligomycin and 100 mM of 2-deoxyglucose (2-DG) (all from Sigma Aldrich). Experiments with the Seahorse were done with the following assay conditions: 3 minutes mixture; 3 minutes wait; 3 minutes measurement.

3.7 Western Blot analyses

To perform Western blotting assay, freshly isolated or TCR-stimulated Tconv and iTreg cells were purified (90–95% purity) from PBMCs by magnetic cell separation with $CD4^+CD25^-$ T Cell Kit (from Miltenyi) or by cell-sorting on the basis of CD25 surface expression, as reported above. Subcellular fractionations were performed with a nuclear-cytosol fractionation kit (NE-PER Nuclear and Cytoplasmic Extraction Reagents; Thermo-Fisher). Cells were lysed on ice in RIPA buffer (Sigma-Aldrich) plus SIGMAFAST Protease Inhibitor (Sigma-Aldrich) and Sigma Phosphatase Inhibitor (Sigma-Aldrich) for 20 min. Protein concentration was calculated by BCA protein assay kit (Pierce, Thermo Fisher Scientific) and 15 μg of proteins were separated by SDS-PAGE under reducing conditions, as previously described (235). The membrane was incubated overnight at 4°C with anti-FoxP3-All (PCH101) (eBioscience); anti-aldolase, anti-hexokinase, and anti-enolase-1 (all from Santa Cruz Biotechnology). Then, the filter was washed three times in phosphate-buffered saline 0.5% Tween 20 (PBST) and incubated with a peroxidase-conjugated secondary antibody (GE Healthcare) for 1 hour. After washing with

PBST, peroxidase activity was detected with ECL system (Roche) or Femto (Pierce, Thermo Fisher Scientific). We scanned four films with different timing exposures, and all signals were quantified normalizing to β-actin (clone C4, Santa Cruz Biotechnology), α-tubulin (for cytoplasmic extracts) or PARP (for nuclear extracts). Densitometric analysis was performed using ImageJ Software (NIH).

3.8 Statistical analyses

Statistical analyses of data were carried out by GraphPad Prism 7 software (GraphPad, California, USA). Comparisons were performed by Mann-Whitney U-test, Student's t-test, one-way ANOVA and two-way ANOVA-corrected for multiple comparisons using the Bonferroni test and Wilcoxon matched pairs test as indicated. Correlation analyses were performed by Pearson's correlation. For all analyses, a two-tailed test was used, with $P<0.05$ values denoting statistical significance.

4. RESULTS

4.1 Recent-onset T1D children had high levels of peripheral Treg cells with impaired suppressive function.

To explore the expression levels of FoxP3 in T1D, we analysed peripheral Treg cells from T1D at onset (10 days after disease manifestation) in comparison to age-matched healthy children. Flow cytometry analysis revealed a higher frequency of $CD4^+FoxP3^+$ Treg cells in T1D children at onset (n=100) compared with matched healthy donors (n=100) (**Figure 9**).

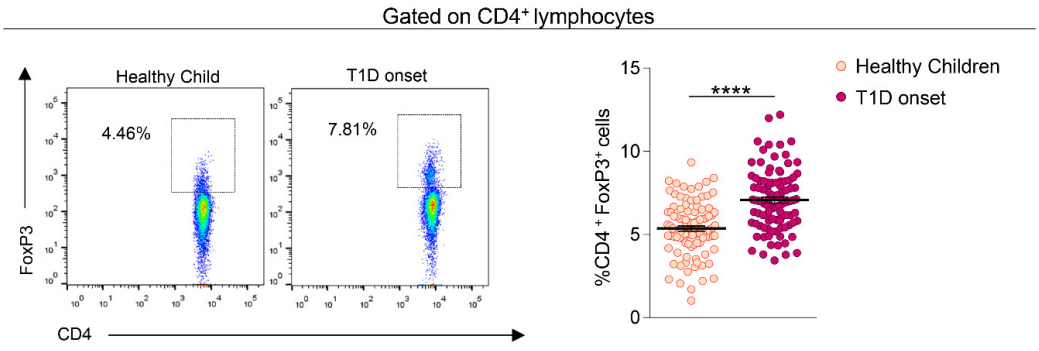


Figure 9. High frequency of peripheral Treg cells in T1D children at disease onset.
Left, representative flow cytometry plots show the percentage of $CD4^+FoxP3^+$ cells in PBMCs from healthy and recent-onset T1D children. Numbers in plots indicate the percentage of positive cells. Right, cumulative data of $CD4^+FoxP3^+$ cells in PBMCs from healthy and recent-onset T1D children. Data are expressed as mean ± SEM. Each symbol represents an individual healthy or T1D child, as indicated. ****P <0.0001 by two-tailed Student's t-test.

To assess whether high expression of $CD4^+FoxP3^+$ cells in T1D children is associated with increased Treg cell regulatory properties, we tested *in vitro* their ability to suppress the proliferation of TCR-activated autologous $CD4^+CD25^-$ Tconv cells. Surprisingly, we found that freshly isolated peripheral Treg cells from T1D children at onset were less able to suppress Tconv cell proliferation *in vitro* (**Figure 10**).

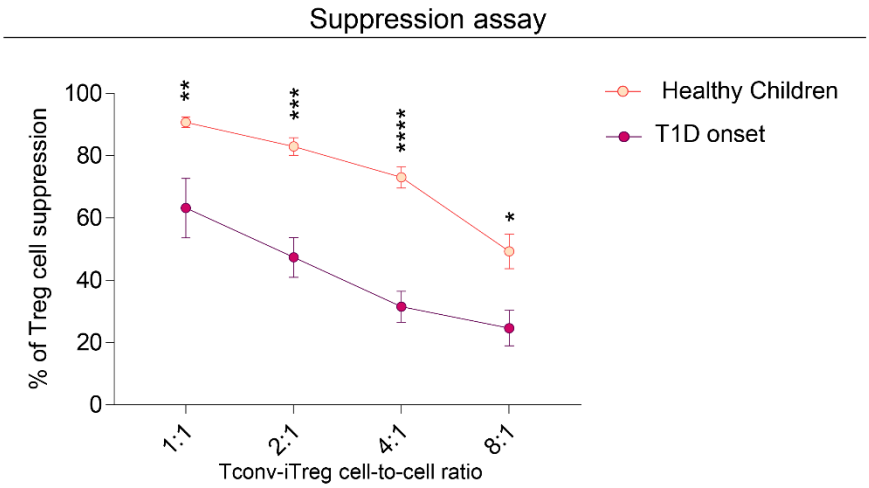


Figure 10. Impaired regulatory capability of peripheral Treg cells in recent-onset T1D children.
Percent of suppression exerted by peripheral Treg cells isolated from healthy and T1D children on proliferation of CellTrace Violet-labeled Tconv cells stimulated for 72 hours in vitro with anti-CD3/CD28 beads (at indicate cell-to-cell ratio). Data are expressed as mean ± SEM. **P*<0.05; ****P*<0.001; *****P*<0.0001 by two-way ANOVA corrected for Bonferroni's multiple comparisons test.

Next, through multiparametric flow cytometry assay, we investigated the expression levels of the main Treg cell-regulatory markers associated with suppressive functions. Flow cytometry analysis displayed that peripheral Treg cells from T1D children had reduced expression of CTLA4 and PD-1 molecules compared with those of healthy individuals (**Figure 11**).

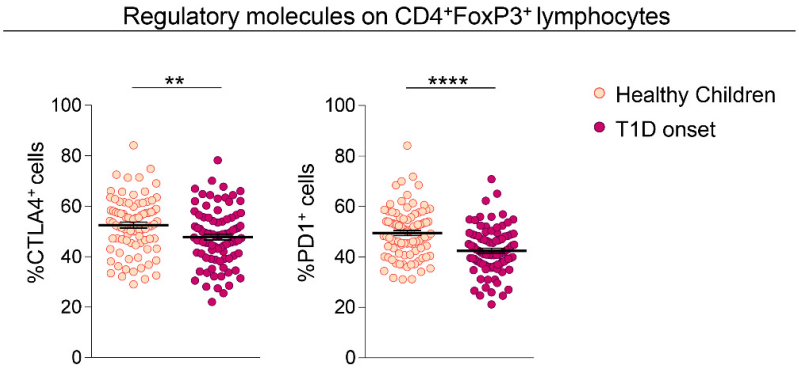


Figure 11. Reduced expression of regulatory molecules on peripheral Treg cells from recent-onset T1D children.
Cumulative data of the expression of Treg cell-specific markers (CTLA-4, PD-1) in peripheral $CD4^+FoxP3^+$ cells in PBMCs from healthy and recent-onset T1D children, as indicated. Data are expressed as mean ± SEM. Each symbol represents an individual healthy or T1D child, as indicated. ***P* <0.01; *****P* <0.0001 by two-tailed Student's *t*-test.

Taken together these results indicated that although the high frequency of Treg cells in T1D subjects at onset, the expression levels of FoxP3 were uncoupled to their regulatory ability.

4.2 Pancreatic β-cell function and blood glucose levels associated with frequency of peripheral Treg cells in recent-onset T1D children.

Since we found that recent-onset T1D children had a higher frequency of peripheral Treg cells compared with healthy children, here we evaluated whether Treg cell expression of FoxP3 associated with β-cell function and glucose levels, measured as circulating c-peptide levels, glycated haemoglobin (HbA1c) and insulin dose required. We showed a significantly negative correlation between the frequency of $CD4^{+}FoxP3^{+}$ cells and C-peptide levels (**Figure 12A**); on the contrary, a positive correlation among peripheral Treg cells, HbA1c and insulin dose was observed (**Figure 12B-C**).

In all, these results suggest that the higher frequency of peripheral Treg cells observed in T1D children depended on the altered blood glucose levels presented before the onset, since their Treg cell rate reflected the low β-cell functionality and the high Hba1c levels.

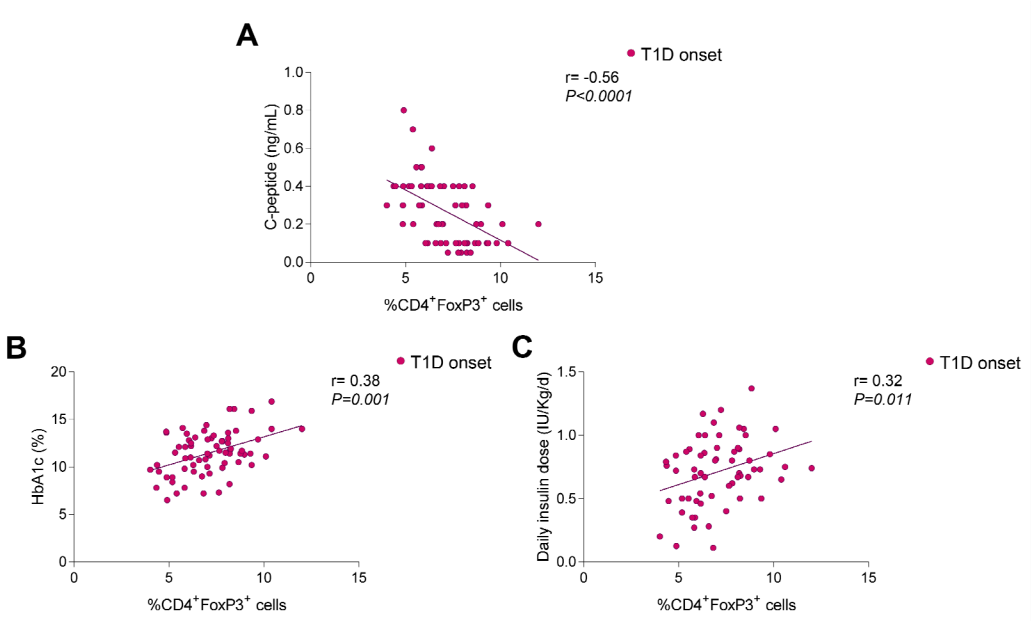


Figure 12. Frequency of circulating Treg cells correlated with β-cell function and blood glucose levels in T1D children at disease onset.
Statistical correlation between $CD4^+FoxP3^+$ cells and (**A**) c-peptide levels, as a measure of β-cell function, (**B**) HbA1c, as a measure of altered blood glucose levels and (**C**) daily insulin dose in T1D children at disease onset. $P<0.05$ denoting statistical significance by Spearman's correlation.

4.3 In vivo glycemic normalization associated with nTreg cell frequency in T1D children one-year later the disease onset

To test whether *in vivo* glucose normalization upon insulin treatment restored Treg cell frequency, we followed-up n=41 T1D individuals from T1D diagnosis to one year later (T1D-FU); continuous glucose monitoring (CGM), including time in range (TIR) and above range (TAR), were collected. Flow cytometry analysis revealed that Treg cell frequency (measured as FoxP3 expression on $CD4^+$ T cells) was reduced in T1D individuals one year later the disease onset (**Figure 13A**).

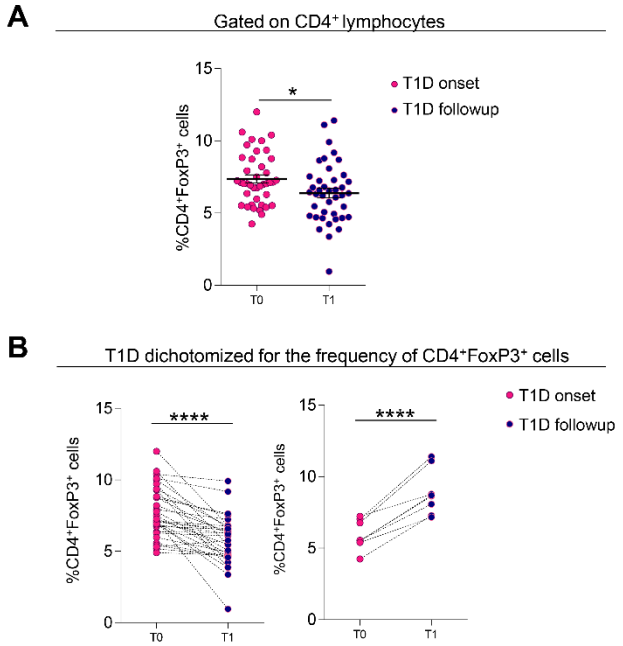


Figure 13. Frequency of peripheral Treg cells in T1D children one year after diagnosis.
(**A**)Cumulative data of $CD4^+FoxP3^+$ cells in PBMCs from T1D children at disease onset (T0) and one year later (T1). (**B**) Cumulative data shows the frequency of $CD4^+FoxP3^+$ cells in PBMCs from T1D who had decreased (left) or increased number of Treg cells at disease onset (T0) and one year later (T1), respectively. Data are expressed as mean ± SEM. Each symbol represents an individual healthy or T1D child, as indicated. **P*<0.05, *****P* <0.0001 by paired Wilcoxon test.

Strikingly, despite a general decrease of Treg cells in T1D overtime (**Figure 13B, left panel**), we noticed that a few T1D-FU children had an increased percentage of

Treg cells one year later (**Figure 13B, right panel**). Also, we observed that the FoxP3 differences relied on glycaemic control, as T1D individuals with a TIR≥70% had low Treg cell frequency compared to T1D subjects with poor glucose control (TAR≥25%) that expressed higher Treg cells (**Figure 14**).

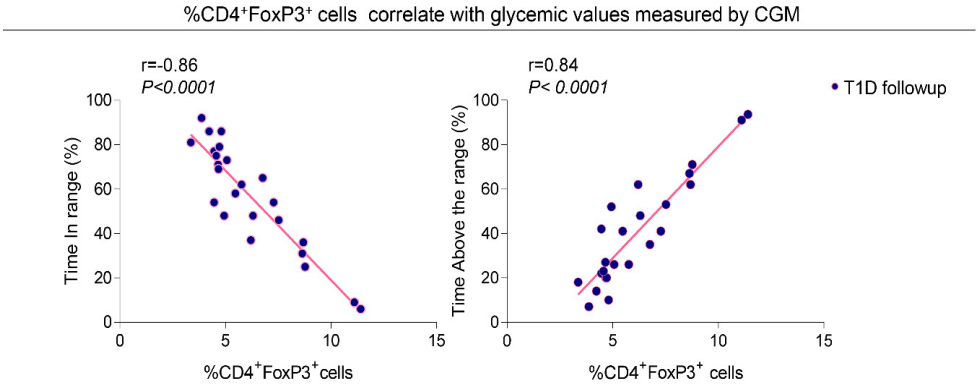


Figure 14. Correlation between peripheral Treg cells and glycaemic values in T1D-FU children. Statistical correlation between $CD4^+FoxP3^+$ cells and either percentage of time in range (left) or above the range (right) of blood glucose levels, measured by CGM in T1D-FU children. $P<0.05$ denoting statistical significance by Spearman's correlation.

All together these data indicate that dysregulated glucose levels *in vivo* impact FoxP3 expression during T1D progression.

4.4 Increased glycolytic metabolism during iTreg cell generation in recent-onset T1D children associated with the enolase-1 cytoplasmic localization.

Since our published data revealed that glycolysis contributes to the induction of FoxP3 during Treg cell generation (236), herein we tested the hypothesis that high amount and/or defective control of glucose levels during T1D progression may affect Tconv cells metabolic responses, thus impairing a proper differentiation in iTreg cells. To this aim, *ex-vivo* extracellular acidification rate (ECAR) was measured as a method for the detection of glycolytic flux in 12 hours and 36 hours TCR-stimulated Tconv cells during iTreg cell generation. We found that TCR-stimulated Tconv cells from T1D at onset showed a high glycolytic rate in comparison with healthy children (**Figure 15, upper panels**).

More in detail, analysis of glycolytic parameters revealed increased glycolysis and glycolytic capacity of Tconv cells during iTreg cell generation in T1D children at onset (**Figure 15, lower panels**).

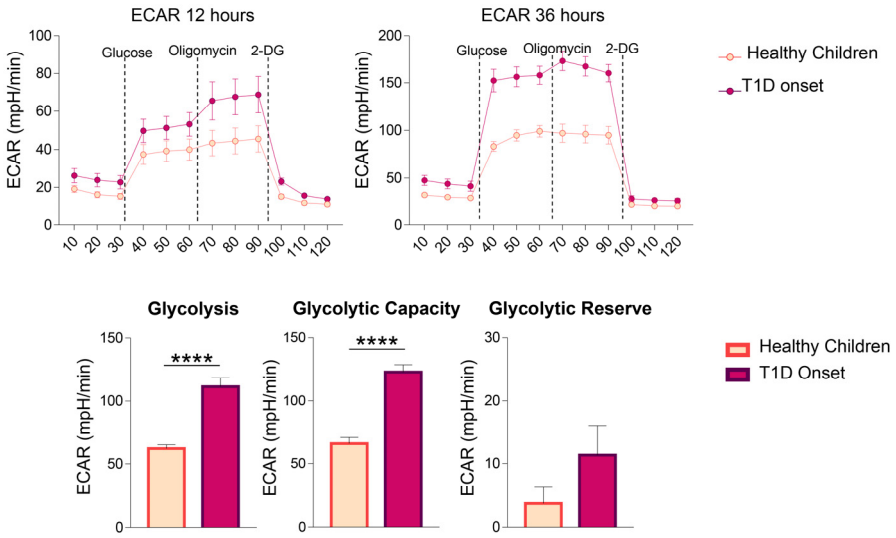


Figure 15. Increased glucose metabolism in Tconv cells from T1D children at disease onset during iTreg cell generation.
Upper panel, ExtraCellular Acidification Rate (ECAR) profile, as an indicator of glycolysis, of isolated Tconv cells from healthy and recent-onset T1D children stimulated for 12 hours (left) or 36 hours (right) with anti-CD3/CD28 beads. Injection of glucose, the ATP-synthase inhibitors oligomycin and 2-deoxy-D-glucose (2DG), as indicated. Lower panel, parameters of the glycolytic pathway of TCR-stimulated Tconv cells upon 36 hours from healthy and recent-onset T1D children. Parameters of the glycolytic pathway were calculated from the ECAR profile. Data are expressed as mean ± SEM. ****P<0.0001 by two-tailed Mann-Whitney test.

To corroborate this finding, we analysed the levels of the main glycolytic enzymes in iTreg cells by Western blot. An increased expression of intracellular levels of hexokinase, aldolase, and enolase-1 in iTreg cells was observed in T1D children at onset compared with healthy children (**Figure 16**).

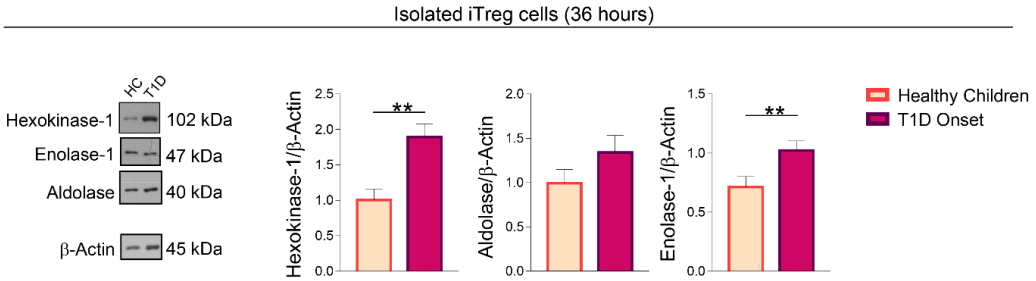


Figure 16. High expression of the glycolytic enzymes in iTreg cells from T1D children at disease onset. Left panel shows representative immunoblot for glycolytic enzymes and actin proteins in isolated iTreg cells from healthy and recent-onset T1D children. Right panel shows the relative densitometric quantitation of glycolytic enzymes normalized on actin, in the aforementioned experimental conditions. Data are shown as mean ± SEM. $^{**}P<0.01$ by two-tailed Mann-Whitney test.

Since data from our laboratory revealed that the enzyme enolase-1 is responsible for *FOXP3* transcription in iTreg cells when engaged in glucose metabolism, we evaluated its cytoplasmic and nuclear localization in freshly isolated Tconv cells and upon TCR stimulation in T1D and healthy children. We observed that enolase-1 was more located in the cytoplasm of freshly isolated Tconv cells from T1D children at onset compared with those of healthy individuals (**Figure 17**). Furthermore, upon early TCR-engagement (15, 30 minutes), enolase-1 remained in the cytoplasm of Tconv cells from T1D children, supporting glucose metabolism; upon 36 hours its levels decreased in TCR-stimulated Tconv cells from T1D individuals and enolase-1 protein localization increased in the nucleus compartment (**Figure 17**).

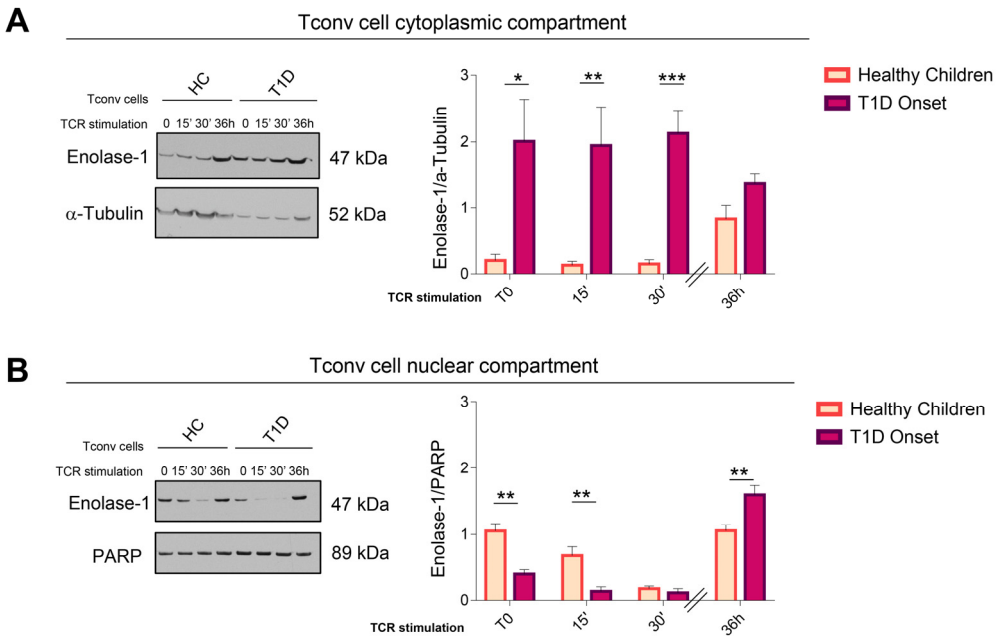


Figure 17. Increased localization of enolase-1 in the cytoplasm of Tconv cells from T1D children at disease onset.

(**A**) Left panel shows representative immunoblot for the enzyme enolase-1 and tubulin proteins in the cytoplasmic compartment of freshly-isolated Tconv cells and upon 15 minutes, 30 minutes and 36 hours of TCR-stimulation from healthy and recent-onset T1D child. Right panel shows the relative densitometric quantitation for the enzyme enolase-1 normalized on tubulin, in the aforementioned experimental conditions. Data are shown as mean ± SEM. **P*<0.05, ***P*<0.01, ****P*<0.01 by two-way ANOVA corrected for Bonferroni's multiple comparisons test. (**B**) Left panel shows representative immunoblot for the enzyme enolase-1 and PARP proteins in the nuclear compartment of freshly-isolated Tconv cells and upon 15 minutes, 30 minutes and 36 hours of TCR-stimulation from healthy and recent-onset T1D children. Right panel showing the relative densitometric quantitation for the enzyme enolase-1 normalized on PARP, in the aforementioned experimental conditions. Data are shown as mean ± SEM. ***P*<0.01 by two-way ANOVA corrected for Bonferroni's multiple comparisons test.

All together these data indicate that Tconv cells from T1D subjects were more prone to utilize glucose for the glycolytic pathway during iTreg cell generation, also recruiting enolase-1 in the cytoplasm.

4.5. Recent onset T1D children had a high frequency of poorly suppressive iTreg cells which was associated with unstable FoxP3 expression.

To ascertain whether high glucose metabolism and cytoplasmic localization of enolase-1 in Tconv cells observed in T1D children are able to influence their capacity to differentiate in iTreg cells, we analysed CD25 and FoxP3 induction levels in TCR-stimulated Tconv cells from children at T1D diagnosis. Flow cytometry experiments showed that FoxP3 expression levels were higher in iTreg cells from T1D (n=16) in comparison with matched healthy children (n=16), while no differences were observed for CD25 induction in the two studied groups (**Figure 18**).

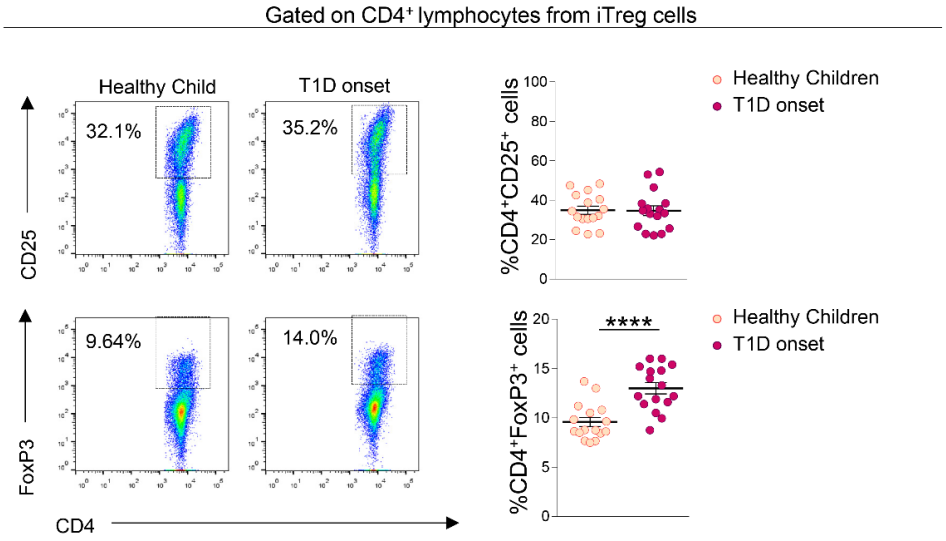


Figure 18. High frequency of iTreg cells in T1D children at disease onset.
Upper panels, representative flow cytometry plots and cumulative data show the expression of CD25 molecules in iTreg cells from healthy and recent at onset T1D children. Lower panels, representative flow cytometry plots and cumulative data show the expression of FoxP3 in iTreg cells from healthy and recent-onset T1D children. Numbers in plots indicate the percentage of positive cells. Data are expressed as mean ± SEM. Each symbol represents an individual healthy or T1D child, as indicated. ****P <0.0001 by two-tailed Mann-Whitney test.

To verify whether the observed high FoxP3 expression levels were associated with iTreg cell suppressive capability in T1D condition, we isolated iTreg cells (on the basis of CD25 expression) and tested their functional ability to control the proliferation of autologous TCR-activated $CD4^{+}CD25^{-}$ Tconv cells. Surprisingly, we noticed that iTreg cells from newly-diagnosed T1D subjects had reduced capability to control the proliferation of TCR-activated T cells, despite the highest FoxP3 levels (**Figure 19**).

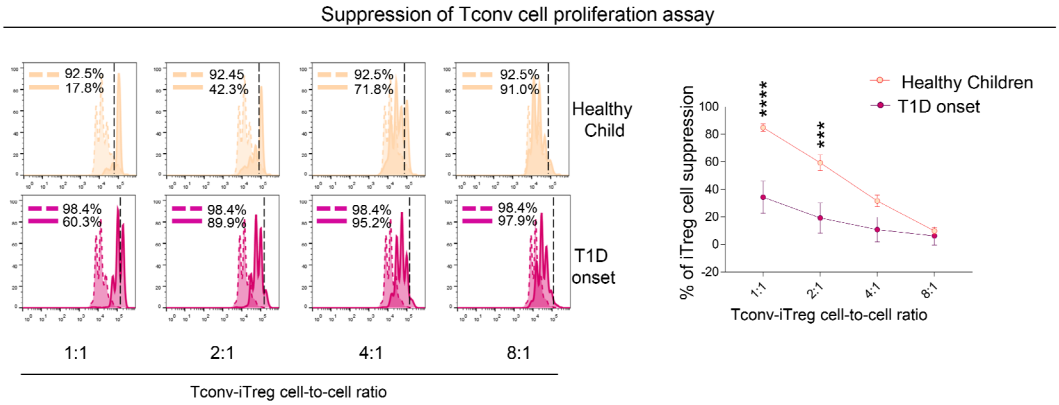


Figure 19. Impaired regulatory capability of iTreg cells in recent-onset T1D children.
Left, flow cytometry histograms showing proliferation of CellTrace-labeled autologous Tconv cells stimulated for 72 hours in vitro with anti-CD3/CD28 beads and cultured alone (dashed curves) or in the presence of various numbers of isolated iTreg cells (from 1:1 to 8:1 cell-to cell ratio), from healthy and recent-onset T1D children. Numbers in plots indicate the percentage of CellTrace Violet (CTV) dilution in Tconv cells cultured alone (upper) or in the presence of iTreg cells at different disease stages, as reported. Right, the percentage of suppression exerted by isolated iTreg cells isolated from healthy and T1D children on the proliferation of CTV Tconv cells stimulated for 72 hours in vitro with anti-CD3/CD28 beads (at indicate cell-to-cell ratio). Data are expressed as mean ± SEM. *P<0.05; ***P<0.01; ***P<0.001; ****P<0.0001 by two-way ANOVA corrected for Bonferroni's multiple comparison test.

Since stable expression of FoxP3 was pivotal for Treg cell lineage and regulatory capability, we investigated whether its altered stability is responsible for reduced iTreg cell suppressive functions observed in recent-onset T1D children. To this aim, we performed a Western Blot analysis revealing that, TCR-stimulated iTreg cells from T1D children progressively reduced FoxP3 expression levels at 24 and 48 hours upon iTreg cell generation from Tconv cells, compared with those of healthy

control (**Figure 20A**). Furthermore, cytofluorimetric experiments showed that upon 96-hour induction in Tconv cells the FoxP3 protein was completely lost in iTreg cells from T1D children at onset, likely determining the formation of poorly suppressive *ex*-Treg cells (**Figure 20B**).

In all these findings indicate high glucose levels in T1D lead to unstable FoxP3 expression in Treg cells.

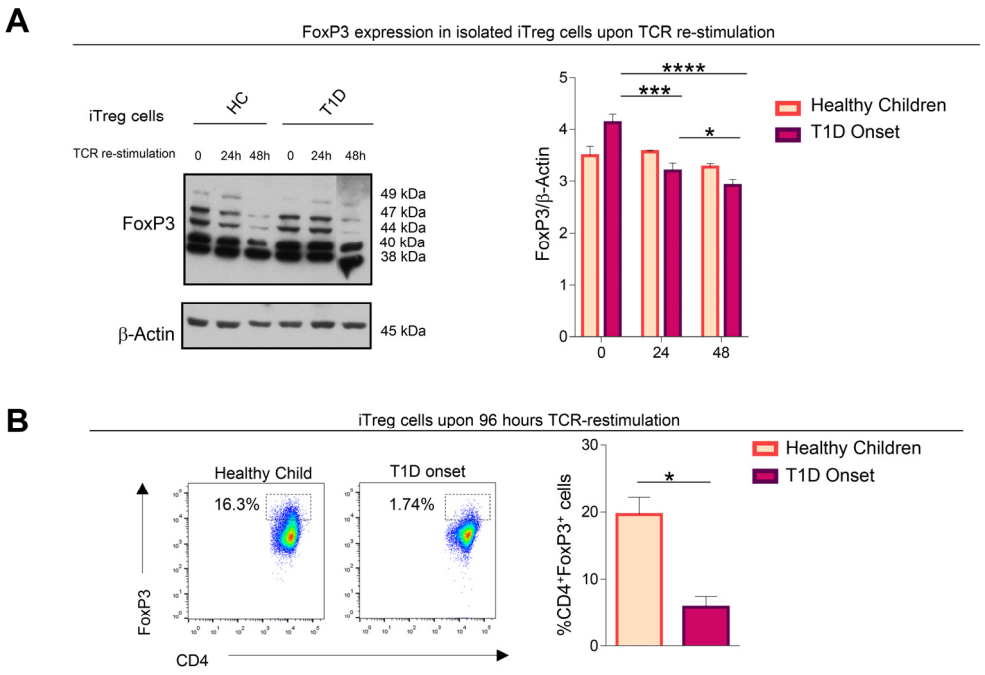


Figure 20. Progressive decrease of FoxP3 expression levels in iTreg cells from T1D children at disease onset.
(**A**) Left panel shows representative immunoblot for FoxP3 and actin proteins in freshly isolated iTreg cells (0) and upon 24, 48 hours of TCR re-stimulation from healthy and recent-onset T1D children. Right panel shows the relative densitometric quantitation of FoxP3 normalized on actin, in the aforementioned experimental conditions. Data are shown as mean ± SEM. **P*<0.05, ****P*<0.001, *****P*<0.0001 by two-way ANOVA corrected for Bonferroni's multiple comparison test. (**B**) Representative flow cytometry plots and cumulative data show the FoxP3 expression in isolated iTreg cells upon 96 hours of TCR re-stimulation from healthy and recent-onset T1D children. Numbers in plots indicate the percentage of positive cells. Data are expressed as mean ± SEM. **P* <0.05 by two-tailed Mann-Whitney test.

4.6 Stabilization of FoxP3 expression in iTreg cells from T1D children at the onset by a high dose of TGF-β and IL-2 treatment

It has been reported that TGF-β and IL-2 had synergistic effects needed for maintaining Treg cell phenotype as well as FoxP3 expression (237). Thus, to determine whether unstable FoxP3 expression observed in iTreg cells from T1D children at onset could be recovered by *in vitro* supplementation of these cytokines, we cultured isolated iTreg cells in presence of TCR stimulation and the addition of TGF-β and IL-2 for 96 hours. Flow-cytometry analyses revealed that cytokines supplementation in iTreg cells led to an increased and stable FoxP3 expression at levels similar to those observed in healthy children (**Figure 21A**). Furthermore, we found that cytokine-stabilization of induced FoxP3 in iTreg cells from T1D children at onset was associated with a 10-fold increased suppressive ability (**Figure 21B**). As expected, iTreg cells from healthy children did not improve their functionality when treated with TGF-β and IL-2 (**Figure 21B**). Our data revealed that TGF-β and IL-2 stabilized defective levels of induced FoxP3 in iTreg cells from T1D individuals, thus suggesting that cytokine supplementation represents a possible therapeutic strategy to restore the functionality of Treg cells in autoimmune diabetes.

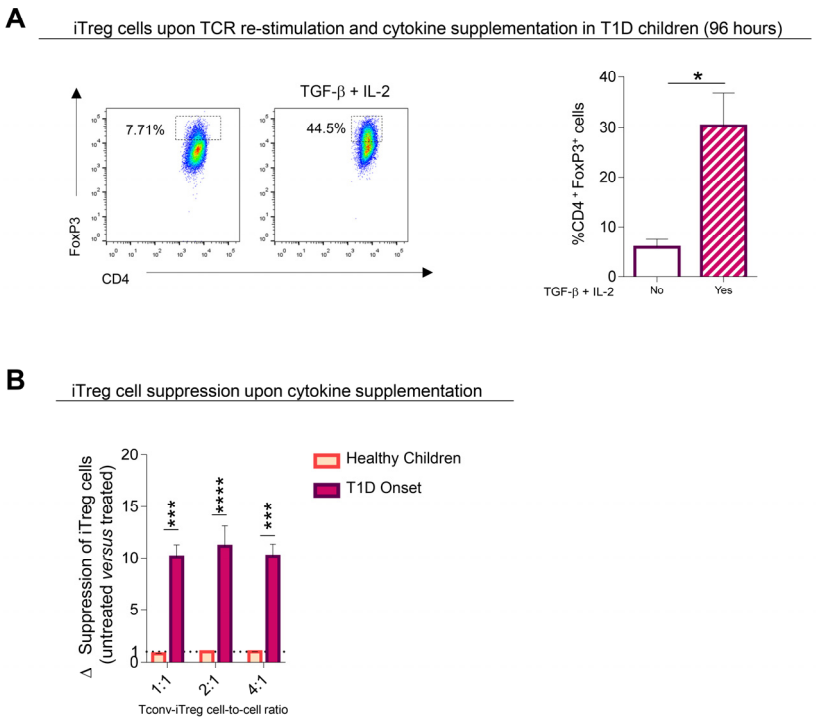


Figure 21. TGF-β and IL-2 addition maintain stable FoxP3 expression and restore iTreg cell suppression in reent-onset T1D children.
(**A**)Representative flow cytometry plots and cumulative data show the FoxP3 expression in isolated iTreg cells untreated or with TGF-β and IL-2 addition upon 96 hours of TCR re-stimulation, from T1D children at disease onset. Numbers in plots indicate the percentage of positive cells. Data are expressed as mean ± SEM. **P* <0.05 by two-tailed Mann-Whitney test. (**B**) TGF-β and IL-2 addition improved iTreg cell suppressive function specifically in T1D individuals. Δsuppression was calculated as follows: the percentage of untreated-iTreg cell suppression – the percentage of treated-iTreg cell suppression. Data are expressed as mean ± SEM. ****P* <0.001, *****P* <0.0001 by two-way ANOVA corrected for Bonferroni's multiple comparison test

5. DISCUSSION

This PhD project assessed the effects of dysregulated glucose levels on FoxP3 expression, Treg cell frequency and their functionality in children with T1D at the onset. Our data revealed that blood glucose levels, in T1D children, influence FoxP3 expression Treg cell stability and their suppressive functions.

The role of Treg cells in the maintenance of immune tolerance was fully documented, and their functional defects and reduced number are considered pivotal for the development and progression of autoimmune diseases (156). Over the past years, several studies have analysed the frequency and functional activity of Treg cells in T1D reporting contrasting results. Some studies described no alterations in Treg cell frequency (127-133), while others revealed either increased (134-136) or decreased frequency in T1D (137). On the other hand, mounting evidence from mouse models, as well as from human disease, strongly indicated defects in Treg cell-mediated suppression in autoimmune diabetes.

In this study, by analyzing a large cohort of T1D children at diagnosis we provided evidence of an increased Treg cell frequency, which is associated with reduced β-cell mass and poor glycaemic control, leading us to hypothesize that glucose levels *per se* are able to affect Treg cell fitness. Published evidence from our laboratory showed that glycolysis is involved in FoxP3 induction (236), here we confirmed these results in an *in vivo* disease model, finding that blood glucose levels affected *FOXP3* gene expression and its stability in Treg cells from T1D children. Glucose is the critical energy source in the human body and it is considered the major fuel source used by immune cells for their biological functions (238; 239). However, increased glucose load has been associated with a high incidence of various

autoimmune and inflammatory syndromes (230; 240-242). Evidence from the study cohort DAISY showed that higher glycemic index and increased intake of total sugars were associated with a major risk of T1D progression (233; 234). Of note, a very recent study revealed increased blood glucose concentrations in children who developed islet autoantibodies (243); this finding indicates that metabolic shifts are present much earlier in the disease process than previously considered and that they may precede the appearance of autoimmune reactions.

Although most of the studies revealed that high glucose intake can exacerbate the development of autoimmune diseases, by sustaining hyper-activation of Th17 cells (231), our working hypothesis is that chronic dysregulation in glucose fluctuation, during T1D progression, influence also Treg cell-mediated immune tolerance.

It has been shown that glycolysis is required for the generation of iTreg cells through the engagement of the glycolytic enzyme enolase-1, which is unable to repress *FOXP3* transcription through its binding to *FOXP3* regulatory regions (236). Herein we showed that in a high glucose microenvironment, typical of T1D condition, enolase-1 is mostly located into the cytoplasmic compartment, as a consequence of its engagement into the glycolytic pathway, supporting the FoxP3 expression and higher frequency of iTreg cells in T1D children at the onset. However, our data revealed that in T1D individuals, FoxP3 expression was transient, thus generating poorly functional iTreg cells. It has been established that transcription of the *FOXP3* in Treg cells is intrinsically maintained by a series of molecular determinants which operate a positive feedback loop favouring *FOXP3* regulatory regions in a permissive status (244). In agreement with these data, we revealed that iTreg cells from T1D children, when sustained with a high amount of TGF-β and IL-2, restored

their suppressive capability as a consequence of FoxP3 protein expression stabilization.

Our data have identified a previously unknown molecular mechanism that links glucose metabolism to the regulation of *FOXP3*, whose transcription is necessary for the induction and function of Treg cells. These findings unveiled an apparent paradox in T1D: while abnormal glucose levels lead to a high frequency of Treg cells by forcing FoxP3 induction, on the other hand, FoxP3 levels are unstable determining its rapid loss that is associated with the generation of poorly functional Treg cells. In this context, alteration in glycolytic metabolism at asymptomatic early stages of disease represents a novel contributing factor which may "accelerate" T1D progression by impairing the regulatory T cell compartment.

Given the crucial role of Treg cells in immune balance, the instability of Treg cells, especially in local tissues, such as pancreas islets, disturbs the immune balance and unleashes a local pathogenic T-cell response. Targeting such poorly functional Treg cells, to stabilize their FoxP3 expression could prevent and or/delay T1D clinical onset. Also, the development of therapies aiming at controlling possible glucose oscillation in the asymptomatic stages of T1D could help to maintain a stable amount of FoxP3 protein, thus generating a fully functional Treg cell population.

6. CONCLUSIONS

T1D development is due to a complex interplay among β-cells, the immune system, and the environmental factors in genetically susceptible individuals. Although the initiating mechanism(s) behind T1D development and progression are under intensive investigation, defects in Treg cell-mediated suppression have been largely shown. The discovery of FoxP3, as an essential transcription factor, for the differentiation and suppressive capability of Treg cells, has provided new insights into the role of this cell subset in the pathogenesis of immune-mediated diseases, in particular T1D. By using an *in vivo* human setting, this PhD project explored the different aspects of the impact of altered glucose levels on Treg cell biology in children with autoimmune diabetes.

Herein, we found that the frequency of the Treg cell population was increased in recent-onset T1D children and associated with impaired suppressive ability. Also, we showed that altered glucose metabolism influenced FoxP3 stability as well as the functional activity of Treg cells. Finally, our data revealed that exogenous administration of IL-2 and TGF-β after Treg cell generation stabilized FoxP3 protein and restored in T1D children their Treg cell suppressive capability.

Overall, results from this PhD research pointed out that dysregulated glucose levels may represent one of the pathogenic determinants affecting Treg cell fitness with subsequent loss of the immunological self-tolerance and β-cell failure in T1D. This finding reveals a previously unrecognized adverse effect of imbalanced glucose concentration in T1D development, which may open the way to novel immunometabolic strategies to prevent T1D in high-risk individuals.

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"Somewhere,

something incredible is waiting to be known"

Carl Sagan

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REFERENCES

1. Group SS. SEARCH for Diabetes in Youth: a multicenter study of the prevalence, incidence and classification of diabetes mellitus in youth. Control Clin Trials 2004;25:458-471

2. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes 1965;14:619-633

3. Eisenbarth GS. Type I diabetes mellitus. A chronic autoimmune disease. N Engl J Med 1986;314:1360-1368

4. Atkinson MA, Eisenbarth GS, Michels AW. Type 1 diabetes. Lancet 2014;383:69-82

5. American Diabetes A. 2. Classification and Diagnosis of Diabetes. Diabetes Care 2017;40:S11-S24

6. Diaz-Valencia PA, Bougneres P, Valleron AJ. Global epidemiology of type 1 diabetes in young adults and adults: a systematic review. BMC Public Health 2015;15:255

7. Katsarou A, Gudbjornsdottir S, Rawshani A, Dabelea D, Bonifacio E, Anderson BJ, Jacobsen LM, Schatz DA, Lernmark A. Type 1 diabetes mellitus. Nature reviews Disease primers 2017;3:17016

8. Atkinson MA. The pathogenesis and natural history of type 1 diabetes. Cold Spring Harb Perspect Med 2012;2

9. Atkinson MA, Eisenbarth GS. Type 1 diabetes: new perspectives on disease pathogenesis and treatment. Lancet 2001;358:221-229

10. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia 2005;48:2221-2228

11. Akirav E, Kushner JA, Herold KC. Beta-cell mass and type 1 diabetes: going, going, gone? Diabetes 2008;57:2883-2888

12. Couper JJ, Haller MJ, Greenbaum CJ, Ziegler AG, Wherrett DK, Knip M, Craig ME. ISPAD Clinical Practice Consensus Guidelines 2018: Stages of type 1 diabetes in children and adolescents. Pediatr Diabetes 2018;19 Suppl 27:20-27

13. 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;38:1964-1974

14. 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;309:2473-2479

15. Roep BO. The role of T-cells in the pathogenesis of Type 1 diabetes: from cause to cure. Diabetologia 2003;46:305-321

16. Tattersall RB. Mild familial diabetes with dominant inheritance. Q J Med 1974;43:339-357

17. Redondo MJ, Yu L, Hawa M, Mackenzie T, Pyke DA, Eisenbarth GS, Leslie RD. Heterogeneity of type I diabetes: analysis of monozygotic twins in Great Britain and the United States. Diabetologia 2001;44:354-362

18. Cooper JD, Smyth DJ, Smiles AM, Plagnol V, Walker NM, Allen JE, Downes K, Barrett JC, Healy BC, Mychaleckyj JC, Warram JH, Todd JA. Meta-analysis of genome-wide association study data identifies additional type 1 diabetes risk loci. Nat Genet 2008;40:1399-1401

19. Concannon P, Rich SS, Nepom GT. Genetics of type 1A diabetes. N Engl J Med 2009;360:1646-1654

20. Pociot F, Lernmark A. Genetic risk factors for type 1 diabetes. Lancet 2016;387:2331-2339

21. Mallone R, Kochik SA, Reijonen H, Carson B, Ziegler SF, Kwok WW, Nepom GT. Functional avidity directs T-cell fate in autoreactive CD4+ T cells. Blood 2005;106:2798-2805

22. Nerup J. Platz R Andersen OO et al.(1974) HL-A antigens and diabetes mellitus. Lancet II:864-866

23. Singal D, Blajchman M. Histocompatibility (HL-A) antigens, lymphocytotoxic antibodies and tissue antibodies in patients with diabetes mellitus. Diabetes 1973;22:429-432

24. Cudworth A, Woodrow J. Evidence for HL-A-linked genes in" juvenile" diabetes mellitus. Br Med J 1975;3:133-135

25. Erlich HA, Valdes AM, McDevitt SL, Simen BB, Blake LA, McGowan KR, Todd JA, Rich SS, Noble JA, Consortium TDG. Next generation sequencing reveals the association of DRB3* 02: 02 with type 1 diabetes. Diabetes 2013;62:2618-2622

26. Caillat-Zucman S, Garchon H-J, Timsit J, Assan R, Boitard C, Djilali-Saiah I, Bougneres P, Bach J. Age-dependent HLA genetic heterogeneity of type 1 insulin-dependent diabetes mellitus. The Journal of clinical investigation 1992;90:2242-2250

27. Cucca F, Lampis R, Frau F, Macis D, Angius E, Masile P, Chessa M, Frongia P, Silvetti M, Cao A. The distribution of DR4 haplotypes in Sardinia suggests a primary association of type I diabetes with DRB1 and DQB1 loci. Hum Immunol 1995;43:301-308

28. Zhao LP, Alshiekh S, Zhao M, Carlsson A, Elding Larsson H, Forsander G, Ivarsson SA, Ludvigsson J, Kockum I, Marcus C. Next-generation sequencing reveals that HLA-DRB3,-DRB4, and-DRB5 may be associated with islet autoantibodies and risk for childhood type 1 diabetes. Diabetes 2016;65:710-718

29. Graham J, Hagopian WA, Kockum I, Li LS, Sanjeevi CB, Lowe RM, Schaefer JB, Zarghami M, Day HL, Landin-Olsson M. Genetic effects on age-dependent onset and islet cell autoantibody markers in type 1 diabetes. Diabetes 2002;51:1346-1355

30. Dahlquist G, Blom L, Holmgren G, Hägglöf B, Larsson Y, Sterky G, Wall S. The epidemiology of diabetes in Swedish children 0–14 years—a six-year prospective study. Diabetologia 1985;28:802-808

31. Parkkola A, Härkönen T, Ryhänen SJ, Ilonen J, Knip M, Register FPD. Extended family history of type 1 diabetes and phenotype and genotype of newly diagnosed children. Diabetes Care 2013;36:348-354

32. Cooper J, Howson J, Smyth D, Walker N, Stevens H, Yang J, She J-X, Eisenbarth G, Rewers M, Todd J. Confirmation of novel type 1 diabetes risk loci in families. Diabetologia 2012;55:996-1000

33. Pociot F, Lernmark Å. Genetic risk factors for type 1 diabetes. The Lancet 2016;387:2331-2339

34. Torn C, Hadley D, Lee H-S, Hagopian W, Lernmark A, Simell O, Rewers M, Ziegler A, Schatz D, Akolkar B. Role of type 1 diabetes-associated SNPs on risk of autoantibody positivity in the TEDDY study. Diabetes 2015;64:1818-1829

35. Bell GI, Pictet R, Rutter WJ. Analysis of the regions flanking the human insulin gene and sequence of an Alu family member. Nucleic Acids Res 1980;8:4091-4110

36. Nistico L, Buzzetti R, Pritchard LE, Van der Auwera B, Giovannini C, Bosi E, Larrad MT, Rios MS, Chow CC, Cockram CS, Jacobs K, Mijovic C, Bain SC, Barnett AH, Vandewalle CL, Schuit F, Gorus FK, Tosi R, Pozzilli P, Todd JA. The CTLA-4 gene region of chromosome 2q33 is linked to, and associated with, type 1 diabetes. Belgian Diabetes Registry. Hum Mol Genet 1996;5:1075-1080

37. 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;39:1074-1082

38. Vella A, Cooper JD, Lowe CE, Walker N, Nutland S, Widmer B, Jones R, Ring SM, McArdle W, Pembrey ME, Strachan DP, Dunger DB, Twells RC, Clayton DG, Todd JA. Localization of a type 1 diabetes locus in the IL2RA/CD25 region by use of tag single-nucleotide polymorphisms. Am J Hum Genet 2005;76:773-779

39. Lyons PA, Armitage N, Argentina F, Denny P, Hill NJ, Lord CJ, Wilusz MB, Peterson LB, Wicker LS, Todd JA. Congenic mapping of the type 1 diabetes locus, Idd3, to a 780-kb region of mouse chromosome 3: identification of a candidate segment of ancestral DNA by haplotype mapping. Genome Res 2000;10:446-453

40. Podolin PL, Wilusz MB, Cubbon RM, Pajvani U, Lord CJ, Todd JA, Peterson LB, Wicker LS, Lyons PA. Differential glycosylation of interleukin 2, the molecular basis for the NOD Idd3 type 1 diabetes gene? Cytokine 2000;12:477-482

41. Wicker LS, Clark J, Fraser HI, Garner VE, Gonzalez-Munoz A, Healy B, Howlett S, Hunter K, Rainbow D, Rosa RL, Smink LJ, Todd JA, Peterson LB. Type 1 diabetes genes and pathways shared by humans and NOD mice. J Autoimmun 2005;25 Suppl:29-33

42. Polychronakos C, Li Q. Understanding type 1 diabetes through genetics: advances and prospects. Nature Reviews Genetics 2011;12:781-792

43. Pugliese A, Zeller M, Fernandez A, Zalcberg LJ, Bartlett RJ, Ricordi C, Pietropaolo M, Eisenbarth GS, Bennett ST, Patel DD. The insulin gene is transcribed in the human thymus and transcription levels correlate with allelic variation at the INS VNTR-IDDM2 susceptibility locus for type 1 diabetes. Nat Genet 1997;15:293-297

44. Hermann R, Knip M, Veijola R, Simell O, Laine AP, Akerblom HK, Groop PH, Forsblom C, Pettersson-Fernholm K, Ilonen J, FinnDiane Study G. Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure? Diabetologia 2003;46:420-425

45. Gillespie KM, Bain SC, Barnett AH, Bingley PJ, Christie MR, Gill GV, Gale EA. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 2004;364:1699-1700

46. Vehik K, Hamman RF, Lezotte D, Norris JM, Klingensmith GJ, Rewers M, Dabelea D. Trends in high-risk HLA susceptibility genes among Colorado youth with type 1 diabetes. Diabetes Care 2008;31:1392-1396

47. Fourlanos S, Varney MD, Tait BD, Morahan G, Honeyman MC, Colman PG, Harrison LC. The rising incidence of type 1 diabetes is accounted for by cases with lower-risk human leukocyte antigen genotypes. Diabetes Care 2008;31:1546-1549

48. Ilonen J, Lempainen J, Veijola R. The heterogeneous pathogenesis of type 1 diabetes mellitus. Nat Rev Endocrinol 2019;15:635-650

49. Beyerlein A, Donnachie E, Jergens S, Ziegler AG. Infections in Early Life and Development of Type 1 Diabetes. JAMA 2016;315:1899-1901

50. Ashton MP, Eugster A, Walther D, Daehling N, Riethausen S, Kuehn D, Klingel K, Beyerlein A, Zillmer S, Ziegler AG, Bonifacio E. Incomplete immune response to coxsackie B viruses associates with early autoimmunity against insulin. Sci Rep 2016;6:32899

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

52. Knip M, Virtanen SM, Akerblom HK. Infant feeding and the risk of type 1 diabetes. Am J Clin Nutr 2010;91:1506S-1513S

53. Lynch KF, Lernmark B, Merlo J, Cilio CM, Ivarsson SA, Lernmark A, Diabetes Prediction in Skane Study G. Cord blood islet autoantibodies and seasonal association with the type 1 diabetes high-risk genotype. J Perinatol 2008;28:211-217

54. Resic Lindehammer S, Honkanen H, Nix WA, Oikarinen M, Lynch KF, Jonsson I, Marsal K, Oberste S, Hyoty H, Lernmark A. Seroconversion to islet autoantibodies after enterovirus infection in early pregnancy. Viral Immunol 2012;25:254-261

55. Viskari HR, Roivainen M, Reunanen A, Pitkaniemi J, Sadeharju K, Koskela P, Hovi T, Leinikki P, Vilja P, Tuomilehto J, Hyoty H. Maternal first-trimester enterovirus infection and future risk of type 1 diabetes in the exposed fetus. Diabetes 2002;51:2568-2571

56. Mathieu C, Gysemans C, Giulietti A, Bouillon R. Vitamin D and diabetes. Diabetologia 2005;48:1247-1257

57. Morse ZJ, Horwitz MS. Virus Infection Is an Instigator of Intestinal Dysbiosis Leading to Type 1 Diabetes. Front Immunol 2021;12:751337

58. Jaidane H, Sauter P, Sane F, Goffard A, Gharbi J, Hober D. Enteroviruses and type 1 diabetes: towards a better understanding of the relationship. Rev Med Virol 2010;20:265-280

59. Hober D, Sane F. Enteroviral pathogenesis of type 1 diabetes. Discov Med 2010;10:151-160

60. Oikarinen S, Martiskainen M, Tauriainen S, Huhtala H, Ilonen J, Veijola R, Simell O, Knip M, Hyoty H. Enterovirus RNA in blood is linked to the development of type 1 diabetes. Diabetes 2011;60:276-279

61. Nerup J, Mandrup-Poulsen T, Helqvist S, Andersen HU, Pociot F, Reimers JI, Cuartero BG, Karlsen AE, Bjerre U, Lorenzen T. On the pathogenesis of IDDM. Diabetologia 1994;37 Suppl 2:S82-89

62. Kukreja A, Maclaren NK. NKT cells and type-1 diabetes and the "hygiene hypothesis" to explain the rising incidence rates. Diabetes Technol Ther 2002;4:323-333

63. Cooke A. Review series on helminths, immune modulation and the hygiene hypothesis: how might infection modulate the onset of type 1 diabetes? Immunology 2009;126:12-17

64. von Herrath MG, Fujinami RS, Whitton JL. Microorganisms and autoimmunity: making the barren field fertile? Nature reviews Microbiology 2003;1:151-157

65. Vaarala O, Atkinson MA, Neu J. The "perfect storm" for type 1 diabetes: the complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Diabetes 2008;57:2555-2562

66. Marino E, Richards JL, McLeod KH, Stanley D, Yap YA, Knight J, McKenzie C, Kranich J, Oliveira AC, Rossello FJ, Krishnamurthy B, Nefzger CM, Macia L, Thorburn A, Baxter AG, Morahan G, Wong LH, Polo JM, Moore RJ, Lockett TJ, Clarke JM, Topping DL, Harrison LC, Mackay CR. Gut microbial metabolites limit the frequency of autoimmune T cells and protect against type 1 diabetes. Nat Immunol 2017;18:552-562

67. de Groot P, Nikolic T, Pellegrini S, Sordi V, Imangaliyev S, Rampanelli E, Hanssen N, Attaye I, Bakker G, Duinkerken G, Joosten A, Prodan A, Levin E, Levels H, Potter van Loon B, van Bon A, Brouwer C, van Dam S, Simsek S, van Raalte D, Stam F, Gerdes V, Hoogma R, Diekman M, Gerding M, Rustemeijer C, de Bakker B, Hoekstra J, Zwinderman A, Bergman J, Holleman F, Piemonti L, De Vos W, Roep B, Nieuwdorp M. Faecal microbiota transplantation halts progression of human new-onset type 1 diabetes in a randomised controlled trial. Gut 2021;70:92-105

68. Corbin KD, Driscoll KA, Pratley RE, Smith SR, Maahs DM, Mayer-Davis EJ, Advancing Care for Type D, Obesity N. Obesity in Type 1 Diabetes: Pathophysiology, Clinical Impact, and Mechanisms. Endocr Rev 2018;39:629-663

69. Carlsson A, Kockum I, Lindblad B, Engleson L, Nilsson A, Forsander G, Karlsson AK, Kernell A, Ludvigsson J, Marcus C, Zachrisson I, Ivarsson SA, Lernmark A, Swedish Better Diabetes Diagnosis Study G. Low risk HLA-DQ and increased body mass index in newly diagnosed type 1 diabetes children in the Better Diabetes Diagnosis study in Sweden. Int J Obes (Lond) 2012;36:718-724

70. Lampousi AM, Carlsson S, Lofvenborg JE. Dietary factors and risk of islet autoimmunity and type 1 diabetes: a systematic review and meta-analysis. EBioMedicine 2021;72:103633

71. Knip M, Virtanen SM, Becker D, Dupre J, Krischer JP, Akerblom HK, Group TS. Early feeding and risk of type 1 diabetes: experiences from the Trial to Reduce Insulin-dependent diabetes mellitus in the Genetically at Risk (TRIGR). Am J Clin Nutr 2011;94:1814S-1820S

72. Gerstein HC. Cow's milk exposure and type I diabetes mellitus. A critical overview of the clinical literature. Diabetes Care 1994;17:13-19

73. Borch-Johnsen K, Joner G, Mandrup-Poulsen T, Christy M, Zachau-Christiansen B, Kastrup K, Nerup J. Relation between breast-feeding and incidence rates of insulin-dependent diabetes mellitus. A hypothesis. Lancet 1984;2:1083-1086

74. Karjalainen J, Martin JM, Knip M, Ilonen J, Robinson BH, Savilahti E, Akerblom HK, Dosch HM. A bovine albumin peptide as a possible trigger of insulin-dependent diabetes mellitus. N Engl J Med 1992;327:302-307

75. Vaarala O. Is type 1 diabetes a disease of the gut immune system triggered by cow's milk insulin? Adv Exp Med Biol 2005;569:151-156

76. Luopajarvi K, Savilahti E, Virtanen SM, Ilonen J, Knip M, Akerblom HK, Vaarala O. Enhanced levels of cow's milk antibodies in infancy in children who develop type 1 diabetes later in childhood. Pediatr Diabetes 2008;9:434-441

77. Adler K, Mueller DB, Achenbach P, Krause S, Heninger AK, Ziegler AG, Bonifacio E. Insulin autoantibodies with high affinity to the bovine milk protein alpha casein. Clin Exp Immunol 2011;164:42-49

78. Atkinson MA, Bowman MA, Kao KJ, Campbell L, Dush PJ, Shah SC, Simell O, Maclaren NK. Lack of immune responsiveness to bovine serum albumin in insulin-dependent diabetes. N Engl J Med 1993;329:1853-1858

79. Norris JM, Beaty B, Klingensmith G, Yu L, Hoffman M, Chase HP, Erlich HA, Hamman RF, Eisenbarth GS, Rewers M. Lack of association between early exposure to cow's milk protein and beta-cell autoimmunity. Diabetes Autoimmunity Study in the Young (DAISY). JAMA 1996;276:609-614

80. Norris JM, Barriga K, Klingensmith G, Hoffman M, Eisenbarth GS, Erlich HA, Rewers M. Timing of initial cereal exposure in infancy and risk of islet autoimmunity. JAMA 2003;290:1713-1720

81. Ziegler AG, Schmid S, Huber D, Hummel M, Bonifacio E. Early infant feeding and risk of developing type 1 diabetes-associated autoantibodies. JAMA 2003;290:1721-1728

82. Dahlquist G. Can we slow the rising incidence of childhood-onset autoimmune diabetes? The overload hypothesis. Diabetologia 2006;49:20-24

83. Fourlanos S, Harrison LC, Colman PG. The accelerator hypothesis and increasing incidence of type 1 diabetes. Curr Opin Endocrinol Diabetes Obes 2008;15:321-325

84. Atkinson M, Gale EA. Infant diets and type 1 diabetes: too early, too late, or just too complicated? JAMA 2003;290:1771-1772

85. Dahlquist GG, Patterson C, Soltesz G. Perinatal risk factors for childhood type 1 diabetes in Europe. The EURODIAB Substudy 2 Study Group. Diabetes Care 1999;22:1698-1702

86. Todd JA. Etiology of type 1 diabetes. Immunity 2010;32:457-467

87. Pozzilli P, Manfrini S, Crino A, Picardi A, Leomanni C, Cherubini V, Valente L, Khazrai M, Visalli N, group I. Low levels of 25-hydroxyvitamin D3 and 1,25-dihydroxyvitamin D3 in patients with newly diagnosed type 1 diabetes. Horm Metab Res 2005;37:680-683

88. Littorin B, Blom P, Scholin A, Arnqvist HJ, Blohme G, Bolinder J, Ekbom-Schnell A, Eriksson JW, Gudbjornsdottir S, Nystrom L, Ostman J, Sundkvist G. Lower levels of plasma 25-hydroxyvitamin D among young adults at diagnosis of autoimmune type 1 diabetes compared with control subjects: results from the nationwide Diabetes Incidence Study in Sweden (DISS). Diabetologia 2006;49:2847-2852

89. Svoren BM, Volkening LK, Wood JR, Laffel LM. Significant vitamin D deficiency in youth with type 1 diabetes mellitus. J Pediatr 2009;154:132-134

90. Karvonen M, Viik-Kajander M, Moltchanova E, Libman I, LaPorte R, Tuomilehto J. Incidence of childhood type 1 diabetes worldwide. Diabetes Mondiale (DiaMond) Project Group. Diabetes Care 2000;23:1516-1526

91. Bailey R, Cooper JD, Zeitels L, Smyth DJ, Yang JH, Walker NM, Hypponen E, Dunger DB, Ramos-Lopez E, Badenhoop K, Nejentsev S, Todd JA. Association of the vitamin D metabolism gene CYP27B1 with type 1 diabetes. Diabetes 2007;56:2616-2621

92. Bierschenk L, Alexander J, Wasserfall C, Haller M, Schatz D, Atkinson M. Vitamin D levels in subjects with and without type 1 diabetes residing in a solar rich environment. Diabetes Care 2009;32:1977-1979

93. Wasserfall C, Nead K, Mathews C, Atkinson MA. The threshold hypothesis: solving the equation of nurture vs nature in type 1 diabetes. Diabetologia 2011;54:2232-2236

94. Bluestone JA, Herold K, Eisenbarth G. Genetics, pathogenesis and clinical interventions in type 1 diabetes. Nature 2010;464:1293-1300

95. Ziegler AG, Nepom GT. Prediction and pathogenesis in type 1 diabetes. Immunity 2010;32:468-478

96. Taplin CE, Barker JM. Autoantibodies in type 1 diabetes. Autoimmunity 2008;41:11-18

97. Zhang L, Nakayama M, Eisenbarth GS. Insulin as an autoantigen in NOD/human diabetes. Curr Opin Immunol 2008;20:111-118

98. Bingley PJ. Clinical applications of diabetes antibody testing. J Clin Endocrinol Metab 2010;95:25-33

99. Knip M, Korhonen S, Kulmala P, Veijola R, Reunanen A, Raitakari OT, Viikari J, Akerblom HK. Prediction of type 1 diabetes in the general population. Diabetes Care 2010;33:1206-1212

100. Tsirogianni A, Pipi E, Soufleros K. Specificity of islet cell autoantibodies and coexistence with other organ specific autoantibodies in type 1 diabetes mellitus. Autoimmunity reviews 2009;8:687-691

101. Skyler JS. Prediction and prevention of type 1 diabetes: progress, problems, and prospects. Clin Pharmacol Ther 2007;81:768-771

102. Winter WE, Schatz DA. Autoimmune markers in diabetes. Clin Chem 2011;57:168-175

103. Jacobsen LM, Larsson HE, Tamura RN, Vehik K, Clasen J, Sosenko J, Hagopian WA, She JX, Steck AK, Rewers M, Simell O, Toppari J, Veijola R, Ziegler AG, Krischer JP, Akolkar B, Haller MJ, Group TS. Predicting progression to type 1 diabetes from ages 3 to 6 in islet autoantibody positive TEDDY children. Pediatr Diabetes 2019;20:263-270

104. Orban T, Sosenko JM, Cuthbertson D, Krischer JP, Skyler JS, Jackson R, Yu L, Palmer JP, Schatz D, Eisenbarth G, Diabetes Prevention Trial-Type 1 Study G. Pancreatic islet autoantibodies as predictors of type 1 diabetes in the Diabetes Prevention Trial-Type 1. Diabetes Care 2009;32:2269-2274

105. Serreze DV, Fleming SA, Chapman HD, Richard SD, Leiter EH, Tisch RM. B lymphocytes are critical antigen-presenting cells for the initiation of T cell-mediated autoimmune diabetes in nonobese diabetic mice. J Immunol 1998;161:3912-3918

106. Hu CY, Rodriguez-Pinto D, Du W, Ahuja A, Henegariu O, Wong FS, Shlomchik MJ, Wen L. Treatment with CD20-specific antibody prevents and reverses autoimmune diabetes in mice. J Clin Invest 2007;117:3857-3867

107. Xiu Y, Wong CP, Bouaziz JD, Hamaguchi Y, Wang Y, Pop SM, Tisch RM, Tedder TF. B lymphocyte depletion by CD20 monoclonal antibody prevents diabetes in nonobese diabetic mice despite isotype-specific differences in Fc gamma R effector functions. J Immunol 2008;180:2863-2875

108. Wong FS. How does B-cell tolerance contribute to the protective effects of diabetes following induced mixed chimerism in autoimmune diabetes? Diabetes 2014;63:1855-1857

109. Krogvold L, Wiberg A, Edwin B, Buanes T, Jahnsen FL, Hanssen KF, Larsson E, Korsgren O, Skog O, Dahl-Jorgensen K. Insulitis and characterisation of infiltrating T cells in surgical pancreatic tail resections from patients at onset of type 1 diabetes. Diabetologia 2016;59:492-501

110. Roep BO, Peakman M. Antigen targets of type 1 diabetes autoimmunity. Cold Spring Harb Perspect Med 2012;2:a007781

111. Oling V, Reijonen H, Simell O, Knip M, Ilonen J. Autoantigen-specific memory CD4+ T cells are prevalent early in progression to Type 1 diabetes. Cell Immunol 2012;273:133-139

112. Foulis AK. Pancreatic pathology in type 1 diabetes in human. Novartis Found Symp 2008;292:2-13; discussion 13-18, 122-129, 202-123

113. 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;56:2400-2404

114. Oikarinen M, Tauriainen S, Honkanen T, Vuori K, Karhunen P, Vasama-Nolvi C, Oikarinen S, Verbeke C, Blair GE, Rantala I, Ilonen J, Simell O, Knip M, Hyoty H. Analysis of pancreas tissue in a child positive for islet cell antibodies. Diabetologia 2008;51:1796-1802

115. Atkinson MA, Gianani R. The pancreas in human type 1 diabetes: providing new answers to age-old questions. Curr Opin Endocrinol Diabetes Obes 2009;16:279-285

116. van Lummel M, Duinkerken G, van Veelen PA, de Ru A, Cordfunke R, Zaldumbide A, Gomez-Tourino I, Arif S, Peakman M, Drijfhout JW, Roep BO. Posttranslational modification of HLA-DQ binding islet autoantigens in type 1 diabetes. Diabetes 2014;63:237-247

117. Delong T, Wiles TA, Baker RL, Bradley B, Barbour G, Reisdorph R, Armstrong M, Powell RL, Reisdorph N, Kumar N, Elso CM, DeNicola M, Bottino R, Powers AC, Harlan DM, Kent SC, Mannering SI, Haskins K. Pathogenic CD4 T cells in type 1 diabetes recognize epitopes formed by peptide fusion. Science 2016;351:711-714

118. McLaughlin RJ, Spindler MP, van Lummel M, Roep BO. Where, How, and When: Positioning Posttranslational Modification Within Type 1 Diabetes Pathogenesis. Current diabetes reports 2016;16:63

119. Yang J, Chow IT, Sosinowski T, Torres-Chinn N, Greenbaum CJ, James EA, Kappler JW, Davidson HW, Kwok WW. Autoreactive T cells specific for insulin B:11-23 recognize a low-affinity peptide register in human subjects with autoimmune diabetes. Proc Natl Acad Sci U S A 2014;111:14840-14845

120. Yang J, James EA, Sanda S, Greenbaum C, Kwok WW. CD4+ T cells recognize diverse epitopes within GAD65: implications for repertoire development and diabetes monitoring. Immunology 2013;138:269-279

121. Strollo R, Vinci C, Arshad MH, Perrett D, Tiberti C, Chiarelli F, Napoli N, Pozzilli P, Nissim A. Antibodies to post-translationally modified insulin in type 1 diabetes. Diabetologia 2015;58:2851-2860

122. Strollo R, Vinci C, Napoli N, Pozzilli P, Ludvigsson J, Nissim A. Antibodies to post-translationally modified insulin as a novel biomarker for prediction of type 1 diabetes in children. Diabetologia 2017;60:1467-1474

123. Strollo R, Vinci C, Man YKS, Bruzzaniti S, Piemonte E, Alhamar G, Briganti SI, Malandrucco I, Tramontana F, Fanali C, Garnett J, Buccafusca R, Guyer P, Mamula M, James EA, Pozzilli P, Ludvigsson J, Winyard PG, Galgani M, Nissim A. Autoantibody

and T cell responses to oxidative post-translationally modified insulin neoantigenic peptides in type 1 diabetes. Diabetologia 2022;

124. Kurd N, Robey EA. T-cell selection in the thymus: a spatial and temporal perspective. Immunol Rev 2016;271:114-126

125. Lu L, Barbi J, Pan F. The regulation of immune tolerance by FOXP3. Nat Rev Immunol 2017;17:703-717

126. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010;10:490-500

127. Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4+ CD25+ T-cells from patients with type 1 diabetes. Diabetes 2005;54:92-99

128. Putnam AL, Vendrame F, Dotta F, Gottlieb PA. CD4+ CD25high regulatory T cells in human autoimmune diabetes. J Autoimmun 2005;24:55-62

129. Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, Haller M, Rockell J, Gottlieb P, Clare-Salzler M. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes 2007;56:604-612

130. Ferraro A, Socci C, Stabilini A, Valle A, Monti P, Piemonti L, Nano R, Olek S, Maffi P, Scavini 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;60:2903-2913

131. McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, Hoffmüller U, Baron U, Olek S, Bluestone JA. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. The Journal of Immunology 2011;186:3918-3926

132. Okubo Y, Torrey H, Butterworth J, Zheng H, Faustman DL. Treg activation defect in type 1 diabetes: correction with TNFR2 agonism. Clinical & translational immunology 2016;5:e56

133. Hamari S, Kirveskoski T, Glumoff V, Kulmala P, Simell O, Knip M, Veijola R. Analyses of regulatory CD 4+ CD 25+ FOXP 3+ T cells and observations from peripheral T cell subpopulation markers during the development of type 1 diabetes in children. Scand J Immunol 2016;83:279-287

134. Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q, Xu L, Priatel JJ, Levings MK, Tan R. Cutting edge: increased Il-17–secreting T cells in children with new-onset type 1 diabetes. The Journal of Immunology 2010;185:3814-3818

135. Ferreira RC, Simons HZ, Thompson WS, Rainbow DB, Yang X, Cutler AJ, Oliveira J, Dopico XC, Smyth DJ, Savinykh N. Cells with Treg-specific FOXP3 demethylation but low CD25 are prevalent in autoimmunity. J Autoimmun 2017;84:75-86

136. Viisanen T, Gazali AM, Ihantola EL, Ekman I, Nanto-Salonen K, Veijola R, Toppari J, Knip M, Ilonen J, Kinnunen T. FOXP3+ Regulatory T Cell Compartment Is Altered in Children With Newly Diagnosed Type 1 Diabetes but Not in Autoantibody-Positive at-Risk Children. Front Immunol 2019;10:19

137. Kukreja A, Cost G, Marker J, Zhang C, Sun Z, Lin-Su K, Ten S, Sanz M, Exley M, Wilson B. Multiple immuno-regulatory defects in type-1 diabetes. The Journal of clinical investigation 2002;109:131-140

138. Miyara M, Yoshioka Y, Kitoh A, Shima T, Wing K, Niwa A, Parizot C, Taflin C, Heike T, Valeyre D. Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity 2009;30:899-911

139. Mason GM, Lowe K, Melchiotti R, Ellis R, de Rinaldis E, Peakman M, Heck S, Lombardi G, Tree TI. Phenotypic complexity of the human regulatory T cell compartment revealed by mass cytometry. The Journal of Immunology 2015;195:2030-2037

140. Long SA, Cerosaletti K, Bollyky PL, Tatum M, Shilling H, Zhang S, Zhang Z-Y, Pihoker C, Sanda S, Greenbaum C. Defects in IL-2R signaling contribute to diminished maintenance of FOXP3 expression in CD4+ CD25+ regulatory T-cells of type 1 diabetic subjects. Diabetes 2010;59:407-415

141. Garg G, Tyler JR, Yang JH, Cutler AJ, Downes K, Pekalski M, Bell GL, Nutland S, Peakman M, Todd JA. Type 1 diabetes-associated IL2RA variation lowers IL-2 signaling and contributes to diminished CD4+ CD25+ regulatory T cell function. The Journal of Immunology 2012;188:4644-4653

142. Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes 2005;54:1407-1414

143. Glisic-Milosavljevic S, Wang T, Koppen M, Kramer J, Ehlenbach S, Waukau J, Jailwala P, Jana S, Alemzadeh R, Ghosh S. Dynamic changes in CD4+ CD25+(high) T cell apoptosis after the diagnosis of type 1 diabetes. Clin Exp Immunol 2007;150:75-82

144. Glisic-Milosavljevic S, Waukau J, Jailwala P, Jana S, Khoo HJ, Albertz H, Woodliff J, Koppen M, Alemzadeh R, Hagopian W, Ghosh S. At-risk and recent-onset type 1 diabetic subjects have increased apoptosis in the CD4+CD25+ T-cell fraction. PLoS One 2007;2:e146

145. Lawson JM, Tremble J, Dayan C, Beyan H, Leslie RD, Peakman M, Tree TI. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin Exp Immunol 2008;154:353-359

146. Schneider A, Rieck M, Sanda S, Pihoker C, Greenbaum C, Buckner JH. The effector T cells of diabetic subjects are resistant to regulation via CD4+ FOXP3+ regulatory T cells. J Immunol 2008;181:7350-7355

147. Hull CM, Peakman M, Tree TIM. Regulatory T cell dysfunction in type 1 diabetes: what's broken and how can we fix it? Diabetologia 2017;60:1839-1850

148. Todd JA, Walker NM, Cooper JD, Smyth DJ, Downes K, Plagnol V, Bailey R, Nejentsev S, Field SF, Payne F, Lowe CE, Szeszko JS, Hafler JP, Zeitels L, Yang JH, Vella A, Nutland S, Stevens HE, Schuilenburg H, Coleman G, Maisuria M, Meadows W, Smink LJ, Healy B, Burren OS, Lam AA, Ovington NR, Allen J, Adlem E, Leung HT, Wallace C, Howson JM, Guja C, Ionescu-Tirgoviste C, Genetics of Type 1 Diabetes in F, Simmonds MJ, Heward JM, Gough SC, Wellcome Trust Case Control C, Dunger DB, Wicker LS, Clayton DG. Robust associations of four new chromosome regions from genome-wide analyses of type 1 diabetes. Nat Genet 2007;39:857-864

149. Pesenacker AM, Wang AY, Singh A, Gillies J, Kim Y, Piccirillo CA, Nguyen D, Haining WN, Tebbutt SJ, Panagiotopoulos C, Levings MK. A Regulatory T-Cell Gene Signature Is a Specific and Sensitive Biomarker to Identify Children With New-Onset Type 1 Diabetes. Diabetes 2016;65:1031-1039

150. Ferraro A, D'Alise AM, Raj T, Asinovski N, Phillips R, Ergun A, Replogle JM, Bernier A, Laffel L, Stranger BE, De Jager PL, Mathis D, Benoist C. Interindividual variation in human T regulatory cells. Proc Natl Acad Sci U S A 2014;111:E1111-1120

151. Yang JH, Cutler AJ, Ferreira RC, Reading JL, Cooper NJ, Wallace C, Clarke P, Smyth DJ, Boyce CS, Gao GJ, Todd JA, Wicker LS, Tree TI. Natural Variation in Interleukin-2 Sensitivity Influences Regulatory T-Cell Frequency and Function in Individuals With Long-standing Type 1 Diabetes. Diabetes 2015;64:3891-3902

152. Jana S, Campbell H, Woodliff J, Waukau J, Jailwala P, Ghorai J, Ghosh S, Glisic S. The type of responder T-cell has a significant impact in a human in vitro suppression assay. PLoS One 2010;5:e15154

153. 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;155:1151-1164

154. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. Science 2003;299:1057-1061

155. Fontenot JD, Gavin MA, Rudensky AY. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol 2003;4:330-336

156. Sakaguchi S, Mikami N, Wing JB, Tanaka A, Ichiyama K, Ohkura N. Regulatory T Cells and Human Disease. Annu Rev Immunol 2020;38:541-566

157. Huang CT, Workman CJ, Flies D, Pan X, Marson AL, Zhou G, Hipkiss EL, Ravi S, Kowalski J, Levitsky HI, Powell JD, Pardoll DM, Drake CG, Vignali DA. Role of LAG-3 in regulatory T cells. Immunity 2004;21:503-513

158. Kumar S, Malik S, Singh UP, Ponnazhagan S, Scissum-Gunn K, Manne U, Mishra MK. PD-1 expression on Foxp3⁺ Treg cells modulates CD8⁺ T cell function in prostatic tumor microenvironment. The Journal of Immunology 2017;198:155.111

159. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. CTLA-4 control over Foxp3+ regulatory T cell function. Science 2008;322:271-275

160. Yu X, Harden K, Gonzalez LC, Francesco M, Chiang E, Irving B, Tom I, Ivelja S, Refino CJ, Clark H, Eaton D, Grogan JL. The surface protein TIGIT suppresses T cell activation by promoting the generation of mature immunoregulatory dendritic cells. Nat Immunol 2009;10:48-57

161. Borsellino G, Kleinewietfeld M, Di Mitri D, Sternjak A, Diamantini A, Giometto R, Hopner S, Centonze D, Bernardi G, Dell'Acqua ML, Rossini PM, Battistini L, Rotzschke O, Falk K. Expression of ectonucleotidase CD39 by Foxp3+ Treg cells: hydrolysis of extracellular ATP and immune suppression. Blood 2007;110:1225-1232

162. Kobie JJ, Shah PR, Yang L, Rebhahn JA, Fowell DJ, Mosmann TR. T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine. J Immunol 2006;177:6780-6786

163. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. J Immunol 2001;166:3008-3018

164. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. Nature 2007;450:566-569

165. Li B, Samanta A, Song X, Furuuchi K, Iacono KT, Kennedy S, Katsumata M, Saouaf SJ, Greene MI. FOXP3 ensembles in T-cell regulation. Immunol Rev 2006;212:99-113

166. Okoye IS, Coomes SM, Pelly VS, Czieso S, Papayannopoulos V, Tolmachova T, Seabra MC, Wilson MS. MicroRNA-Containing T-Regulatory-Cell-Derived Exosomes Suppress Pathogenic T Helper 1 Cells. Immunity 2014;41:503

167. Torri A, Carpi D, Bulgheroni E, Crosti MC, Moro M, Gruarin P, Rossi RL, Rossetti G, Di Vizio D, Hoxha M, Bollati V, Gagliani C, Tacchetti C, Paroni M, Geginat J, Corti L, Venegoni L, Berti E, Pagani M, Matarese G, Abrignani S, de Candia P. Extracellular MicroRNA Signature of Human Helper T Cell Subsets in Health and Autoimmunity. J Biol Chem 2017;292:2903-2915

168. Tung SL, Boardman DA, Sen M, Letizia M, Peng Q, Cianci N, Dioni L, Carlin LM, Lechler R, Bollati V, Lombardi G, Smyth LA. Regulatory T cell-derived extracellular vesicles modify dendritic cell function. Sci Rep 2018;8:6065

169. Tung SL, Fanelli G, Matthews RI, Bazoer J, Letizia M, Vizcay-Barrena G, Faruqu FN, Philippeos C, Hannen R, Al-Jamal KT, Lombardi G, Smyth LA. Regulatory T Cell Extracellular Vesicles Modify T-Effector Cell Cytokine Production and Protect Against Human Skin Allograft Damage. Frontiers in cell and developmental biology 2020;8:317

170. Brunkow ME, Jeffery EW, Hjerrild KA, Paeper B, Clark LB, Yasayko SA, 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;27:68-73

171. 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;27:20-21

172. Josefowicz SZ, Lu LF, Rudensky AY. Regulatory T cells: mechanisms of differentiation and function. Annu Rev Immunol 2012;30:531-564

173. 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;11:618-627

174. Kim HP, Leonard WJ. CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med 2007;204:1543-1551

175. 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;463:808-812

176. Ruan Q, Kameswaran V, Tone Y, Li L, Liou HC, Greene MI, Tone M, Chen YH. Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. Immunity 2009;31:932-940

177. 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;528:132-136

178. Toker A, Engelbert D, Garg G, Polansky JK, Floess S, Miyao T, Baron U, Duber S, Geffers R, Giehr P, Schallenberg S, Kretschmer K, Olek S, Walter J, Weiss S, Hori S, Hamann A, Huehn J. Active demethylation of the Foxp3 locus leads to the generation of stable regulatory T cells within the thymus. J Immunol 2013;190:3180-3188

179. Ohkura N, Hamaguchi M, Morikawa H, Sugimura K, Tanaka A, Ito Y, Osaki M, Tanaka Y, Yamashita R, Nakano N, Huehn J, Fehling HJ, Sparwasser T, Nakai K, Sakaguchi S. T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development. Immunity 2012;37:785-799

180. 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;38:1654-1663

181. Nair VS, Song MH, Ko M, Oh KI. DNA Demethylation of the Foxp3 Enhancer Is Maintained through Modulation of Ten-Eleven-Translocation and DNA Methyltransferases. Mol Cells 2016;39:888-897

182. 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;213:377-397

183. Lu L, Ma J, Li Z, Lan Q, Chen M, Liu Y, Xia Z, Wang J, Han Y, Shi W, Quesniaux V, Ryffel B, Brand D, Li B, Liu Z, Zheng SG. All-trans retinoic acid promotes TGF-beta-induced Tregs via histone modification but not DNA demethylation on Foxp3 gene locus. PLoS One 2011;6:e24590

184. Lal G, Zhang N, van der Touw W, Ding Y, Ju W, Bottinger EP, Reid SP, Levy DE, Bromberg JS. Epigenetic regulation of Foxp3 expression in regulatory T cells by DNA methylation. J Immunol 2009;182:259-273

185. Li C, Ebert PJ, Li QJ. T cell receptor (TCR) and transforming growth factor beta (TGF-beta) signaling converge on DNA (cytosine-5)-methyltransferase to control forkhead box protein 3 (foxp3) locus methylation and inducible regulatory T cell differentiation. J Biol Chem 2013;288:19127-19139

186. Schlenner SM, Weigmann B, Ruan Q, Chen Y, von Boehmer H. Smad3 binding to the foxp3 enhancer is dispensable for the development of regulatory T cells with the exception of the gut. J Exp Med 2012;209:1529-1535

187. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT, Rudensky AY. Extrathymically generated regulatory T cells control mucosal TH2 inflammation. Nature 2012;482:395-399

188. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, van Beekum O, Brenkman AB, Hijnen DJ, Mutis T, Kalkhoven E, Prakken BJ, Coffer PJ. Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood 2010;115:965-974

189. Samanta A, Li B, Song X, Bembas K, Zhang G, Katsumata M, Saouaf SJ, Wang Q, Hancock WW, Shen Y, Greene MI. TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. Proc Natl Acad Sci U S A 2008;105:14023-14027

190. Xiao Y, Nagai Y, Deng G, Ohtani T, Zhu Z, Zhou Z, Zhang H, Ji MQ, Lough JW, Samanta A, Hancock WW, Greene MI. Dynamic interactions between TIP60 and p300 regulate FOXP3 function through a structural switch defined by a single lysine on TIP60. Cell reports 2014;7:1471-1480

191. Li B, Samanta A, Song X, Iacono KT, Bembas K, Tao R, Basu S, Riley JL, Hancock WW, Shen Y, Saouaf SJ, Greene MI. FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci U S A 2007;104:4571-4576

192. Wang L, Kumar S, Dahiya S, Wang F, Wu J, Newick K, Han R, Samanta A, Beier UH, Akimova T, Bhatti TR, Nicholson B, Kodrasov MP, Agarwal S, Sterner DE, Gu W, Weinstock J, Butt TR, Albelda SM, Hancock WW. Ubiquitin-specific Protease-7 Inhibition Impairs Tip60-dependent Foxp3+ T-regulatory Cell Function and Promotes Antitumor Immunity. EBioMedicine 2016;13:99-112

193. Gao Z, Gao Y, Li Z, Chen Z, Lu D, Tsun A, Li B. Synergy between IL-6 and TGF-beta signaling promotes FOXP3 degradation. Int J Clin Exp Pathol 2012;5:626-633

194. Tao R, de Zoeten EF, Ozkaynak E, Chen C, Wang L, Porrett PM, Li B, Turka LA, Olson EN, Greene MI, Wells AD, Hancock WW. Deacetylase inhibition promotes the generation and function of regulatory T cells. Nat Med 2007;13:1299-1307

195. Wang L, de Zoeten EF, Greene MI, Hancock WW. Immunomodulatory effects of deacetylase inhibitors: therapeutic targeting of FOXP3+ regulatory T cells. Nature reviews Drug discovery 2009;8:969-981

196. van Loosdregt J, Brunen D, Fleskens V, Pals CE, Lam EW, Coffer PJ. Rapid temporal control of Foxp3 protein degradation by sirtuin-1. PLoS One 2011;6:e19047

197. Beier UH, Wang L, Bhatti TR, Liu Y, Han R, Ge G, Hancock WW. Sirtuin-1 targeting promotes Foxp3+ T-regulatory cell function and prolongs allograft survival. Mol Cell Biol 2011;31:1022-1029

198. Kwon HS, Lim HW, Wu J, Schnolzer M, Verdin E, Ott M. Three novel acetylation sites in the Foxp3 transcription factor regulate the suppressive activity of regulatory T cells. J Immunol 2012;188:2712-2721

199. Li J, Du X, Shi H, Deng K, Chi H, Tao W. Mammalian Sterile 20-like Kinase 1 (Mst1) Enhances the Stability of Forkhead Box P3 (Foxp3) and the Function of

Regulatory T Cells by Modulating Foxp3 Acetylation. J Biol Chem 2015;290:30762-30770

200. Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, Takahashi M, Fukuda NN, Murakami S, Miyauchi E, Hino S, Atarashi K, Onawa S, Fujimura Y, Lockett T, Clarke JM, Topping DL, Tomita M, Hori S, Ohara O, Morita T, Koseki H, Kikuchi J, Honda K, Hase K, Ohno H. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature 2013;504:446-450

201. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature 2013;504:451-455

202. Morawski PA, Mehra P, Chen C, Bhatti T, Wells AD. Foxp3 protein stability is regulated by cyclin-dependent kinase 2. J Biol Chem 2013;288:24494-24502

203. Chunder N, Wang L, Chen C, Hancock WW, Wells AD. Cyclin-dependent kinase 2 controls peripheral immune tolerance. J Immunol 2012;189:5659-5666

204. Li Z, Lin F, Zhuo C, Deng G, Chen Z, Yin S, Gao Z, Piccioni M, Tsun A, Cai S, Zheng SG, Zhang Y, Li B. PIM1 kinase phosphorylates the human transcription factor FOXP3 at serine 422 to negatively regulate its activity under inflammation. J Biol Chem 2014;289:26872-26881

205. Nie H, Zheng Y, Li R, Guo TB, He D, Fang L, Liu X, Xiao L, Chen X, Wan B, Chin YE, Zhang JZ. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. Nat Med 2013;19:322-328

206. Deng G, Nagai Y, Xiao Y, Li Z, Dai S, Ohtani T, Banham A, Li B, Wu SL, Hancock W, Samanta A, Zhang H, Greene MI. Pim-2 Kinase Influences Regulatory T Cell Function and Stability by Mediating Foxp3 Protein N-terminal Phosphorylation. J Biol Chem 2015;290:20211-20220

207. Basu S, Golovina T, Mikheeva T, June CH, Riley JL. Cutting edge: Foxp3-mediated induction of pim 2 allows human T regulatory cells to preferentially expand in rapamycin. J Immunol 2008;180:5794-5798

208. Metzger MB, Hristova VA, Weissman AM. HECT and RING finger families of E3 ubiquitin ligases at a glance. J Cell Sci 2012;125:531-537

209. Ben-Neriah Y. Regulatory functions of ubiquitination in the immune system. Nat Immunol 2002;3:20-26

210. Dang EV, Barbi J, Yang HY, Jinasena D, Yu H, Zheng Y, Bordman Z, Fu J, Kim Y, Yen HR, Luo W, Zeller K, Shimoda L, Topalian SL, Semenza GL, Dang CV, Pardoll DM, Pan F. Control of T(H)17/T(reg) balance by hypoxia-inducible factor 1. Cell 2011;146:772-784

211. Chen Z, Barbi J, Bu S, Yang HY, Li Z, Gao Y, Jinasena D, Fu J, Lin F, Chen C, Zhang J, Yu N, Li X, Shan Z, Nie J, Gao Z, Tian H, Li Y, Yao Z, Zheng Y, Park BV, Pan Z, Zhang J, Dang E, Li Z, Wang H, Luo W, Li L, Semenza GL, Zheng SG, Loser K, Tsun A, Greene MI, Pardoll DM, Pan F, Li B. The ubiquitin ligase Stub1 negatively modulates

regulatory T cell suppressive activity by promoting degradation of the transcription factor Foxp3. Immunity 2013;39:272-285

212. van Loosdregt J, Fleskens V, Fu J, Brenkman AB, Bekker CP, Pals CE, Meerding J, Berkers CR, Barbi J, Grone A, Sijts AJ, Maurice MM, Kalkhoven E, Prakken BJ, Ovaa H, Pan F, Zaiss DM, Coffer PJ. Stabilization of the transcription factor Foxp3 by the deubiquitinase USP7 increases Treg-cell-suppressive capacity. Immunity 2013;39:259-271

213. Li X, Huang M, Zheng H, Wang Y, Ren F, Shang Y, Zhai Y, Irwin DM, Shi Y, Chen D, Chang Z. CHIP promotes Runx2 degradation and negatively regulates osteoblast differentiation. J Cell Biol 2008;181:959-972

214. Luo W, Zhong J, Chang R, Hu H, Pandey A, Semenza GL. Hsp70 and CHIP selectively mediate ubiquitination and degradation of hypoxia-inducible factor (HIF)-1alpha but Not HIF-2alpha. J Biol Chem 2010;285:3651-3663

215. 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;10:1000-1007

216. 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;29:44-56

217. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. J Exp Med 2005;201:723-735

218. 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;36:262-275

219. Williams LM, Rudensky AY. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. Nat Immunol 2007;8:277-284

220. Rudra D, Egawa T, Chong MM, Treuting P, Littman DR, Rudensky AY. Runx-CBFbeta complexes control expression of the transcription factor Foxp3 in regulatory T cells. Nat Immunol 2009;10:1170-1177

221. Birzele F, Fauti T, Stahl H, Lenter MC, Simon E, Knebel D, Weith A, Hildebrandt T, Mennerich D. Next-generation insights into regulatory T cells: expression profiling and FoxP3 occupancy in Human. Nucleic Acids Res 2011;39:7946-7960

222. Ono M, Yaguchi H, Ohkura N, Kitabayashi I, Nagamura Y, Nomura T, Miyachi Y, Tsukada T, Sakaguchi S. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. Nature 2007;446:685-689

223. Feng Y, Arvey A, Chinen T, van der Veeken J, Gasteiger G, Rudensky AY. Control of the inheritance of regulatory T cell identity by a cis element in the Foxp3 locus. Cell 2014;158:749-763

224. Li X, Liang Y, LeBlanc M, Benner C, Zheng Y. Function of a Foxp3 cis-element in protecting regulatory T cell identity. Cell 2014;158:734-748

225. Shrestha S, Yang K, Guy C, Vogel P, Neale G, Chi H. Treg cells require the phosphatase PTEN to restrain TH1 and TFH cell responses. Nat Immunol 2015;16:178-187

226. 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;16:188-196

227. Guo J, Zhou X. Regulatory T cells turn pathogenic. Cell Mol Immunol 2015;12:525-532

228. De Rosa V, Galgani M, Santopaolo M, Colamatteo A, Laccetti R, Matarese G. Nutritional control of immunity: Balancing the metabolic requirements with an appropriate immune function. Semin Immunol 2015;27:300-309

229. Lerner A, Matthias T. Changes in intestinal tight junction permeability associated with industrial food additives explain the rising incidence of autoimmune disease. Autoimmunity reviews 2015;14:479-489

230. Manzel A, Muller DN, Hafler DA, Erdman SE, Linker RA, Kleinewietfeld M. Role of "Western diet" in inflammatory autoimmune diseases. Current allergy and asthma reports 2014;14:404

231. Zhang D, Jin W, Wu R, Li J, Park SA, Tu E, Zanvit P, Xu J, Liu O, Cain A, Chen W. High Glucose Intake Exacerbates Autoimmunity through Reactive-Oxygen-Species-Mediated TGF-beta Cytokine Activation. Immunity 2019;51:671-681 e675

232. Hu Y, Costenbader KH, Gao X, Al-Daabil M, Sparks JA, Solomon DH, Hu FB, Karlson EW, Lu B. Sugar-sweetened soda consumption and risk of developing rheumatoid arthritis in women. Am J Clin Nutr 2014;100:959-967

233. Lamb MM, Yin X, Barriga K, Hoffman MR, Baron AE, Eisenbarth GS, Rewers M, Norris JM. Dietary glycemic index, development of islet autoimmunity, and subsequent progression to type 1 diabetes in young children. J Clin Endocrinol Metab 2008;93:3936-3942

234. Lamb MM, Frederiksen B, Seifert JA, Kroehl M, Rewers M, Norris JM. Sugar intake is associated with progression from islet autoimmunity to type 1 diabetes: the Diabetes Autoimmunity Study in the Young. Diabetologia 2015;58:2027-2034

235. De Rosa V, Procaccini C, Cali G, Pirozzi G, Fontana S, Zappacosta S, La Cava A, Matarese G. A key role of leptin in the control of regulatory T cell proliferation. Immunity 2007;26:241-255

236. De Rosa V, Galgani M, Porcellini A, Colamatteo A, Santopaolo M, Zuchegna C, Romano A, De Simone S, Procaccini C, La Rocca C, Carrieri PB, Maniscalco GT, Salvetti M, Buscarinu MC, Franzese A, Mozzillo E, La Cava A, Matarese G. Glycolysis controls the induction of human regulatory T cells by modulating the expression of FOXP3 exon 2 splicing variants. Nat Immunol 2015;16:1174-1184

237. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA. IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol 2007;178:2018-2027

238. Qi X, Tester RF. Fructose, galactose and glucose - In health and disease. Clinical nutrition ESPEN 2019;33:18-28

239. Palmer CS, Ostrowski M, Balderson B, Christian N, Crowe SM. Glucose metabolism regulates T cell activation, differentiation, and functions. Front Immunol 2015;6:1

240. Hu R, Xia CQ, Butfiloski E, Clare-Salzler M. Effect of high glucose on cytokine production by human peripheral blood immune cells and type I interferon signaling in monocytes: Implications for the role of hyperglycemia in the diabetes inflammatory process and host defense against infection. Clin Immunol 2018;195:139-148

241. Qi Q, Chu AY, Kang JH, Jensen MK, Curhan GC, Pasquale LR, Ridker PM, Hunter DJ, Willett WC, Rimm EB, Chasman DI, Hu FB, Qi L. Sugar-sweetened beverages and genetic risk of obesity. N Engl J Med 2012;367:1387-1396

242. Yu Y, Yang W, Yu T, Zhao X, Zhou Z, Yu Y, Xiong L, Yang H, Bilotta AJ, Yao S, Golovko G, Plasencia A, Quintana FJ, Zhou L, Li Y, Cong Y. Glucose promotes regulatory T cell differentiation to maintain intestinal homeostasis. iScience 2022;25:105004

243. 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

244. Colamatteo A, Carbone F, Bruzzaniti S, Galgani M, Fusco C, Maniscalco GT, Di Rella F, de Candia P, De Rosa V. Molecular Mechanisms Controlling Foxp3 Expression in Health and Autoimmunity: From Epigenetic to Post-translational Regulation. Front Immunol 2019;10:3136