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Effect of gluten-free diet on potentially regulatory immune mechanisms in human type 1 diabetes

Efekt bezlepkové diety na potenciálně regulační imunitní mechanismy u lidského diabetu 1. typu

Diploma thesis

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Prohlášení

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Podpis

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Abstract

Type 1 diabetes (T1D) is an autoimmune disease, whose incidence is rising every year, and its prevention or a cure does not exist. T1D is influenced by multiple genetic factors but environmental factors represent the major contributor to the recent almost epidemic increase of T1D incidence worldwide, primarily in developed countries. Amongst these factors belong for example enteroviral infections, microbiota dysbiosis or gluten-free diet (GFD). GFD has been proven to have a protective effect in NOD mice, which is a spontaneous model of T1D, and a beneficial effect on glycemic control in humans, when administered after T1D onset. This diploma thesis examined changes of regulatory and potentially regulatory T-cells and their cytokines in peripheral blood mononuclear cells (PBMC) of T1D children, who underwent 12-month intervention trial of GFD. Secondly, the thesis assessed if the influence of GFD on immune regulatory functions can be transferred by colonization of germ-free NOD mice with gut microbiota of these children. We have found that intervention with GFD increases percentage of Tr1 cells and IL-10 producing $CD4^+$ T-cells in PBMC of T1D children. Furthermore, the beneficial effect on immune regulation can be at least partially transferred to NOD mice by the colonization with human microbiota because this also increased the percentage of regulatory, potentially regulatory T-cells and IL-10 producing $CD4^+$ T-cells. Thus, this diploma thesis brings novel findings about the influence of GFD on immune regulatory functions in the context of human T1D and indicates a possibility that this influence may be, at least from a part, mediated by a diet shaping of microbiota composition.

Key words: type 1 diabetes, T-cells, regulatory cells, gluten-free diet, human, microbiome transfer, NOD mouse, gnotobiotic

Abstrakt

Diabetes 1. typu (T1D, type 1 diabetes) je autoimunitní onemocnění, jehož incidence vzrůstá každý rok, a prevence nebo léčba této nemoci neexistuje. T1D je ovlivňován mnoha genetickými faktory, nicméně environmentální faktory představují hlavní důvod pro současný prudký, až téměř epidemický, celosvětový nárůst T1D, především pak ve vyspělých zemích. Mezi tyto faktory patří například enterovirové infekce, dysbióza mikrobiomu nebo bezlepková dieta (GFD, gluten-free diet). Bezlepková dieta má protektivní efekt u NOD myší, které jsou spontánním modelem T1D, a prospěšný vliv na kontrolu glykémie u lidí, pokud je dodržována po diagnóze T1D. Tato diplomová práce zkoumala změny regulačních a potenciálně regulačních buněk a jejich cytokinů v mononukleárních buňkách periferní krve (PBMC, peripheral blood mononuclear cells) u dětí s T1D, které se zúčastnily 12 měsíční intervenční studie ohledně GFD. Dále se práce zabývala zkoumáním, zda lze vliv GFD na regulační funkce imunitního systému přenést do bezmikrobního NOD myšího modelu pomocí jejich kolonizace mikrobiomem od T1D dětí. Zjistili jsme, že GFD zvýšila procenta Tr1 buněk a IL-10 produkujících $CD4^+$ T-buněk v periferní krvi dětí s T1D. Dále se zdá, že prospěšný vliv GFD může být alespoň částečně přenesen do NOD myší pomocí mikrobiomu, protože myši, které byly kolonizované mikrobiomem od dětí dodržující GFD, měly vyšší zastoupení regulačních a potenciálně regulačních buněk a IL-10 produkujících $CD4^+$ T-buněk. Tudíž tato diplomová práce přináší nové poznatky ohledně vlivu GFD na imunitní regulační funkce v kontextu lidského T1D a nastiňuje možnost, že tento vliv může být alespoň z části zprostředkován změnami ve složení mikrobiomu.

Klíčová slova: diabetes 1. typu, T buňky, regulační buňky, gluten-free dieta, lidský, přenos mikrobiomu, NOD myš, gnotobiotický

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List of abbreviations

ALN	axillary lymph nodes
APCs	antigen presenting cells
DCs	dendritic cells
GADA	glutamic acid decarboxylase 65 autoantibody
GFD	gluten-free diet
IA-2A	insulinoma-associated antigen-2 autoantibody
IEL	intraepithelial lymphocytes
ILN	inguinal lymph nodes
INS	insulin autoantibody
MLN	mesenteric lymph nodes
NK	natural killer
NOD	nonobese diabetic mouse
PBMC	peripheral blood mononuclear cells
PLN	pancreatic lymph nodes
PMA	phorbol myristate acetate
PP	Peyer's patches
STD	standard diet
T1D	type 1 diabetes
TCR	T-cell receptor
Tr1	type 1 regulatory T-cells
Tregs	regulatory T cells
ZnT8A	zinc transporter 8 autoantibody

1 Introduction

Type 1 diabetes is an autoimmune disease, of which annual incidence is rising by 3 % every year. Pancreatic islets are rendered incapable of sufficient production of insulin by the autoimmune reaction and even with compensation by external insulin injections the disease is associated with many health complications, which can manifest later in life [1]. At present, no effective cure or prevention of T1D exists.

The pathogenesis is very heterogeneous, conditioned by multiple genetic factors but to a large extent also by environmental ones. Amongst suggested environmental factors supporting the development of T1D belong for example enteroviral infections, dysbiosis of gut microbiota, gluten or cow's milk. On the other hand, it seems that some agents such as vitamin D or polyunsaturated fatty acids as well as bacteria may have beneficial effects [2].

Environmental factors play a substantial role in the recent epidemic increase of T1D in developed countries. T1D was a rare but well diagnosed disease in several countries during the first half of the last century but its incidence is on a steady rise in developed countries (well documented in the last 20-30 years) [3]. Both epidemiological surveys in humans and controlled animal studies in spontaneous models of T1D (NOD mice and BB rats) implicate environmental factors as modulators of the diseases onset and progression [4]. Among them diets and microbiome represent the major ones. Dietary factors, more specifically proteins, have been documented to modify diabetes incidence in the spontaneous animal models and wheat flour proteins were identified as important components within the non-purified animal diets to keep a high (>60%) standard diabetes incidence in clean SPF animal facilities [5]–[7]. Gluten-free diets were repeatedly shown to highly prevent diabetes in NOD mice if introduced early in life [8]–[11]. The first human prospective trial with at risk first-degree relatives kept on gluten-free diet has been carried out in Italy by Pastore et al. It demonstrated that gluten-free diet improved insulin secretion in subjects at risk of T1D [12].

It has been proposed that the development of type 1 diabetes could be associated with gut microbiota dysbiosis but also with disruption of the intestinal barrier [13], [14] and both of these phenomena can be influenced by gluten [15], [16]. Gluten can also promote the immune system towards more inflammatory milieu and on the contrary, gluten-free diet returns this milieu back to balance. *In vitro* experiments show that stimulation of T-cells with gliadin, which is a component of gluten, causes their proliferation and production of proinflammatory cytokines [17] whereas gluten-free diet fed prenatally to mice lowers production of proinflammatory cytokines and increases percentage of regulatory T cells [18].

The balance of inflammatory and regulatory part of the immune system is shifted towards proinflammation during autoimmune disease. Numbers or at least function of T regulatory cells are reduced during T1D, thus some therapies try to shift the balance back to normal by promoting these T regulatory cells. Amongst such T-cells belong well-established regulatory populations such as FoxP3 Tregs [19] or type 1 regulatory cells [20] but also some other populations like γδ T-cells [21], $CD45RB^{low}$ T-cells [22] and $CD62L^{+}$ T-cells [23] seem to have potentially regulatory properties in the context of T1D. Another cellular populations such as naïve, central memory or effector memory $CD4^{+}$ T-cells seem to be influenced during T1D [24].

When gluten-free diet is introduced early in life to NOD mice it not only greatly protects against T1D but also improves the immune milieu [25]. Up to this date there is only a sporadic information with respect to the influence of gluten-free diet on T1D in humans [26] and it is not possible or very difficult to carry on similar early intervention studies with gluten-free diets in humans. Recently it has been documented by our collaborators at the Motol Univ. Hospital, that GFD does have an influence on improved glycemic control when administered shortly after T1D diagnosis [27].

So far no human study has addressed the impact of gluten-free diet on immune parameters, thus this is the first aim of this diploma thesis. Specifically, to examine the changes in populations of regulatory and potentially regulatory T cells and their cytokines in peripheral blood mononuclear cells of newly diagnosed T1D children, who underwent a 12-month prospective intervention trial of gluten-free diet.

The second and the third aim of this thesis were carried out on the spontaneous NOD mouse model of T1D. They also address changes of immune parameters (Tregs, IL-10 cytokine) but in ex-germ-free NOD mice, which were previously colonized with microbiota from the T1D children, who had been on gluten-free or standard diets (and with different pace of residual β-cell loss). These aims were designed to assess how diet-related changes in their microbiota may influence diabetes preventive capacity and immune characteristics in the NOD mouse model of spontaneous T1D.

2 Current state of knowledge

2.1 Type 1 diabetes

Type 1 diabetes is a disease considered an autoimmune due to involved immune reactions directed towards autoantigens of β-cells in pancreas. It leads to insufficient production of insulin by β-cells and subsequently to total dependence on external insulin injections [2]. Despite the compensation of insulin demands there is a whole list of health complications, which can manifest later in life, and according to the International Diabetes Federation the overall annual incidence of T1D is rising approximately by 3 % worldwide every year [1].

Even though T1D has been extensively researched in the past fifty years many aspects of its pathogenesis remain unclear, nor it is known how to prevent or cure this disease. Biopsy of pancreas is very risky and therefore it is done very rarely, so the main difficulty in researching this condition in humans is a lack of samples and thus it is hard to examine what is going on at the cellular and molecular level in pancreas [28]. The situation is slowly resolving thanks to projects such as the Network for Pancreatic Organ Donors with Diabetes (web 1) or Human Pancreas Analysis Program (web 2), which collect high-quality pancreatic samples from organ donors and forms biobanks so the number of available and examined samples is rising. But it seems that T1D is very complex and heterogeneous disease and it is important to differentiate between individual groups of patients [2].

Type 1 diabetes is associated with a presence of autoantibodies in blood even though they do not seem to possess pathogenetic effects in the disease. Nevertheless they are very useful diagnostic and disease-progression tools. In humans the four most common autoantibodies are targeted against: insulin (IAA), glutamic acid decarboxylase 65 (GADA), insulinoma-associated antigen-2 (IA-2A) and zinc transporter 8 (ZnT8A). A seroconversion, which is an appearance of at least one autoantibody, occurs before clinical manifestation of T1D [2]. The seroconversion becomes evident by appearance of multiple autoantibodies at once or there is initially only one autoantibody and others develop in time or there is a persistent presence of only one autoantibody [29]. The first autoantibody to develop is usually IAA or less often GADA and it has a connection with HLA class II genetic background [30]. The highest frequency of appearance is around 1-2 years with IAA and around 4-5 years with GADA [29][31]. The risk of progressing to T1D is rising with rising number of individual types of autoantibodies present in blood and with age. With 2 or more types of autoantibodies the risk of developing T1D at any point in life is very high. But there are differences in the probability of progression to T1D depending on the combination of 2 autoantibodies, the

most prone being a combination of IAA plus IA-2A. Regarding the single autoantibody seroconversion then the progression to T1D is faster with emergence of IA-2A than IAA or GADA [32].

The disease can be divided to several stages according to the presence of autoantibodies, glucose intolerance and clinical symptoms of T1D. Stage 1 is characterized with the presence of 2 or more autoantibodies, normal blood glucose and symptoms are not present. In stage 2 there is an emergence of glucose intolerance and in stage 3 the patient develops clinical symptoms of type 1 diabetes [33]. Some literature even distinguish 4th stage and that is a long duration of T1D with 3rd stage as a recent-onset of the disease as it is depicted in Figure 1 [34].

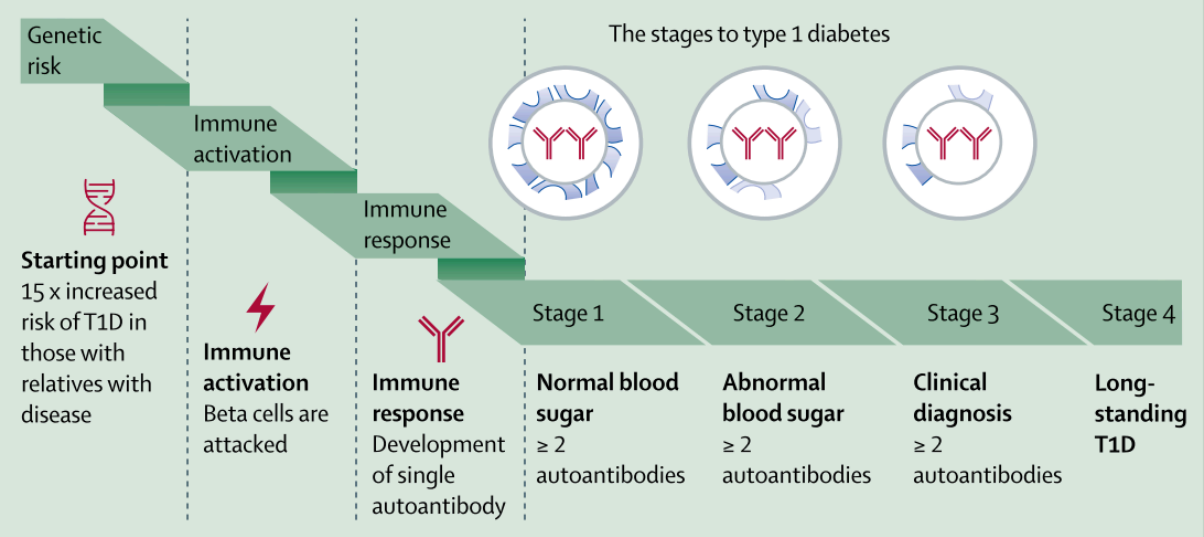


Figure 1: Staging of type 1 diabetes and some events leading to activation of autoimmunity

Genetic risk for T1D increases the probability of subsequent activation of immune response against β-cells and emergence of single autoantibody. Characterization of stages of T1D. Stage 1: characterized by emergence of at least 2 autoantibodies in blood. Stage 2: there is an appearance of glucose intolerance. Stage 3: occurrence of symptoms associated with type 1 diabetes that results in a clinical diagnosis. Stage 4: lifelong duration of T1D [34].

Important differences amongst individual groups of patients are seen also in insulitis, which is an infiltration of immune cells into pancreas and subsequent localization in the surroundings of pancreatic islets. These islets contain β-cells and other specialized endocrine cell types. In humans the infiltrated immune cells are localized more on the outside periphery of islets (peri-insulitis) and only some localize directly inside of them as it is seen in Figure 2 [35]. The number of insulitic islets is higher in the recent-onset versus the long-

duration of T1D [36] and it seems that it is also more extensive in children versus adults. Regarding the proportion of individual infiltrating cell types, the most frequent are $CD8^+$ T-cells followed by macrophages, $CD4^+$ T-cells and B-cells [37]. $CD8^+$ T-cells and $CD4^+$ T-cells are recruited simultaneously to the islets during the recent-onset of T1D and then their numbers slowly decline with progression of the disease [38]. But proportions of composition of the infiltrating immune cell types changes in time with reduction of β-cells in islets and the overall amount of immune cells decreases [37].

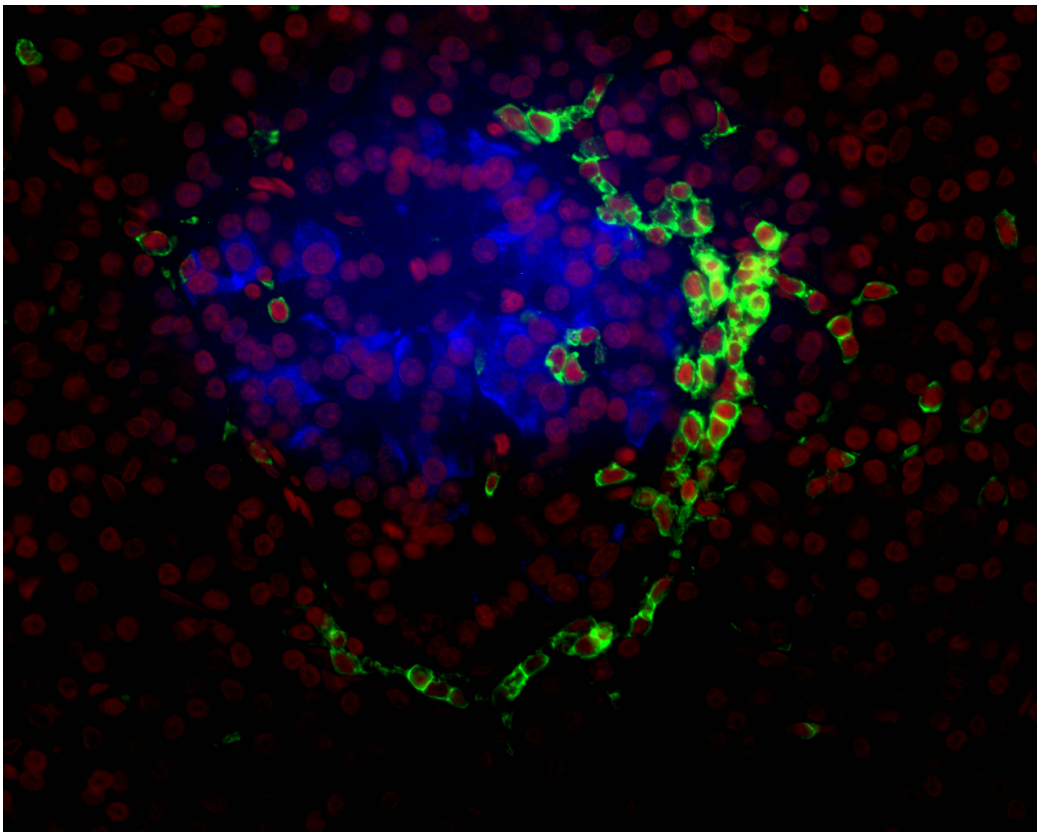


Figure 2: Insulitis in a pancreas of human patient with type 1 diabetes

As it can be seen in this figure human insulitis is more in the form of peri-insulitis, where immune cells (green immunofluorescent labeling of CD45) surround the pancreatic islet (blue labeling of insulin in β-cells) and only a few infiltrate into the islet, than a complete infiltration [35].

It is possible to differentiate between two types of insulitis at recent-onset of T1D according to the quantity of infiltrated B-cells or more precisely according to the ratio of B-cells to $CD4^+$ T-cells. If the ratio is higher than 1 then it is associated with younger age of T1D onset (≤ 6 years) and the age group has very low number of residual insulin-containing islets.

Conversely, if the ratio is lower than 1 then the onset of disease is at older age (≥ 13 years) and the number of residual insulin-containing islets is much higher [39].

It seems that β-cells themselves are undergoing through some phenotypic changes before the onset of T1D because they are loosing their signature phenotype and it precedes β-cell loss [38]. On the other hand they acquire some specific features like upregulation of HLA class I expression [40] or upregulation of PD-L1 in insulin-containing islets of T1D patients but the overexpression of these molecules is not increased in insulin-deficient islets and healthy subjects [41].

Overall β-cell amount in pancreas is lower in recent-onset of T1D than in healthy subjects and is declining even further with the progression to long-duration [38]. Plus the overall weight of pancreas is much lower in T1D subjects than in healthy ones [36]. However the rate of loss of β-cells is not uniform and can be distinguished to two categories (slow or rapid β-cell loss). One of the proposed reasons could be the different types of insulitis based on the quantity of infiltrated B-cells [39]. Sometimes the patients can be described as slow, with milder autoimmune reaction, or rapid progressors, with more aggressive reaction, of T1D based on their rate of β-cell loss [42]. Usually the immune response is more aggressive in children and they are loosing β-cells faster than adults with the recent-onset of T1D. Nevertheless there can be some β-cells preserved even after long-duration of T1D and it looks like their function is suppressed [43].

Susceptibility to type 1 diabetes is associated with genetic factors among other things. The most studied relevant genes belong to HLA class II region but there are also important genes from HLA class I region, some genes mediating processing of antigens and some even outside of the HLA region. Altogether more then 60 genes are specified as susceptible to T1D [2]. The strongest association is with HLA DR3-DQ2 and HLA DR4-DQ8 haplotypes and the predisposition is [44] even higher if the patient is heterozygotic in these haplotypes. But on the other hand, some give strong protection against T1D [45]. HLA class II alleles also influence the type of autoantibody, which is detected as the first during seroconversion. IAA occurs as the first in genotypes HLA DR3-DQ2/DR4-DQ8 and HLA DR4-DQ8/DR4-DQ8 and in contrast GADA is the first in patients homozygous for HLA DR3-DQ2 [29]. With the highly susceptible haplotype comes faster progression to seroconversion but the HLA class II alleles have no effect on the rate of progression to T1D after 2 autoantibodies are present [46]. Some HLA class I alleles speed up the progression to disease after seroconversion but the effect is prominent only when individual HLA I allele is associated with specific HLA II allele. Amongst the genes located outside of HLA regions are ones such as insulin, IL2RA or

CTLA4 [2]. The main differences for insulin genes are located outside of the coding region and play role in effectiveness of gene expression. Thus some forms of insulin genes are more probable to aid in developing T1D due to impaired expression in thymus and establishing central tolerance [47].

Even though genetic factors are a strong predisposition for development of type 1 diabetes they explain emergence of the disease only partially. Quickly rising incidence of T1D, seasonal occurrence, the same rate of incidence in offsprings of immigrants as the rate in the country, in which they were born, irrespective of the rate in the country of origin of their ancestors [2] or incomplete occurrence of the disease in monozygotic twins are all examples advocating for a big contribution of environmental factors to incidence of T1D [34]. Over the years of research many environmental factors have been proposed and it seems that they have the biggest influence especially in the early years of life. Among the associated factors belong viral infections, microbiota dysbiosis or dietary and nutritional factors such as vitamin D intake, breastfeeding, cow's milk protein or gluten [48].

2.2 NOD mouse – a spontaneous animal model of T1D

Nonobese diabetic (NOD) mouse is an animal model that spontaneously develops type 1 diabetes. When maintained under specific-pathogen-free conditions, the incidence in female NOD mice is around 85 % whereas it is only 50 % in males by 30 weeks of age. Therefore usually only female NOD mice are used in T1D studies [49]. The model was established in 1980 through inbreeding and it brought valuable insights to pathogenesis of T1D ever since. Nevertheless there are some similarities and differences between T1D in humans and in NOD mice, as it is with every animal model, even spontaneous, and this has to be considered when translating animal results to human trials [50].

The general pathology of type 1 diabetes in NOD mice seems to be very similar to that in humans. NOD mice also develop autoantibodies against islet antigens, have an insulitis in pancreas before the onset of T1D, as well as loose β-cell mass and show a genetic susceptibility to the disease mainly by MHC class II locus [51].

But there are also some differences such as it seems that in NOD mice the main initiating autoantibody is IAA [51]. Compared to humans the insulitis in NOD mice is quite extensive and aggressive and only from the begging in the peri-insulitic manner, which then changes to a profound intra-islet infiltration by immune cells [52]. At the beginning there are more cells of myeloid origin than of lymphoid one and with time the situation turns around with predominant lymphocytes in the infiltrate and the most abundant cell types being $CD4^+$ T-cells and B-cells over $CD8^+$ T-cells, which is the opposite to the situation in humans [53]. The elemental similarities and differences are summarized in Table 1.

	Human	NOD
Age at onset:	>6 months up to late adolescence	>10 weeks
Ketoacidosis:	Severe	Mild
Lymphopenia:	None	None
Insulitis:	DCs, Macrophages, B cells, NK cells, CD4 & CD8 T cells	DCs, Macrophages, B cells, NK cells, CD4 & CD8 T cells
Autoantigens:	Insulin, GAD, IA-2, IA-2β, ZnT8, IGRP, IAPP, HSP60, Carboxypeptidase H	Insulin, GAD, IA-2, IA-2β, ZnT8, IGRP, Chromogranin A
TCR repertoire bias:	None	None
Genetic susceptibility:	MHC most important, >40 non-MHC genes	MHC most important, >40 non-MHC genes
Gender effect:	Males and females equally affected before puberty, small male preponderance after puberty	Females predominantly affected

Table 1: Summarization of differences and similarities of human and NOD mouse T1D

(Taken and modified [54]*)*

In order to dissect the complexity and interplay of various environmental factors (e.g. diet, microbiome, metabolome) gnotobiotic NOD mice represent an irreplaceable tool. Gnotobiotic (environmentally–defined, including germ-free) animals can be used for e.g. studying the microbiome dependency of dietary effects or to elucidate effect of a single bacterium on disease development, immune parameters and many more [55]. Furthermore, they could be colonized with human microbiota to study its effects and characteristics in disease development [56]. So far gnotobiotic NOD mice have brought valuable information for example about gut microbiota influence on immunity and through it on development of T1D [57] or about viral influence on development of T1D, composition of microbiota and mutual cross-talk between these two factors [58]. In addition, study by Yurkovetskiy et al. nicely documented that gender difference in diabetes incidence of NOD mice is, at least partly, due to influences of gut microbiota [59].

The germ-free NOD females (when fed non-purified standard diets e.g. NIH-06 or Altromin Standard 1434) displayed a remarkably high diabetes incidence of 100 % and an accelerated onset of the disease compared to typically around 80% or less diabetes incidence in high quality SPF facilities [60]. There is a very limited number of studies using germ-free NOD mice, some of them reporting the same 100% diabetes incidence [61] or 100% incidence of T1D in both genders in the commercial Taconic germ-free production colony (Figure 3).

Germ-free NOD mice have altered balance between inflammatory Th1, Th17 and regulatory Foxp3 Tregs in intestine, MLN and PLN. The balance is shifted towards Th1 and Th17 populations whereas FoxP3 Tregs are represented in smaller percentage. Therefore, microbiota, or at least beneficial microbiota, contribute to generation of regulatory T-cells and through that to control of the autoimmune reaction [62].

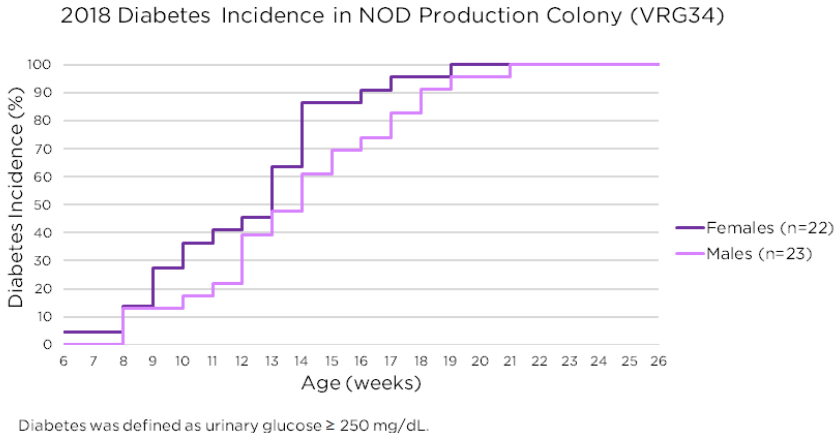


Figure 3: Diabetes incidence in males and females of germ-free NOD mice (Taconic colony)

The development and 100% incidence of T1D in germ-free NOD mice, both in males and females (web 3)

2.3 Pancreatic lymph nodes as an induction site of the autoimmune reaction

The first proposal that pancreatic lymph nodes (PLN) are important for development of T1D in NOD mice came from two studies in the year 1992. First, it was noted that PLN from NOD mice are enlarged at 8 weeks but not at 4 weeks of age. Moreover, dendritic cells (DCs) from PLN of 8 weeks old mice form clusters with T-cells, which show proliferative properties, but the clustering is not seen at 4 weeks of age, thus the profound autoimmune reaction in NOD mice starts after 4 weeks of age [63]. The second study showed, that DCs from PLN of NOD mice older than 8 weeks have protective effect on developing T1D when they are given to 4 weeks old NOD mice in a footpad injection and these mice subsequently have lower insulitis [64].

Subsequently, it was confirmed that PLN are required for the full development of the autoreactive response because removal of these lymph nodes at 3 weeks of age greatly decreases insulitis and the following development of T1D. Even the production of insulin autoantibody is inhibited. But when the removal is done later at 4 weeks of age the protection has much lesser extent and at 10 weeks of age has no effect whatsoever. Therefore, the induction of autoimmune reaction starts around 4 weeks and is fully developed at 10 weeks. Nevertheless, the protection by removal of PLN at 3 weeks is not absolute [65] and it was also proposed that at very young age immune cells from mesenteric lymph nodes (MLN) have higher diabetogenic potential during adoptive transfer of diabetes than cells from PLN. When immune cells from MLN of NOD mice are transferred to NOD-SCID mice at 3 weeks of age the diabetogenic potential is very weak but is higher than a transfer from PLN. At 6 weeks of age the situation reverses and PLN have much higher potential to induce T1D in the adoptive transfer. So it seems that MLN also have a role in T1D development, especially at early stages of diabetes development in NOD mice [66]. Cells from both PLN as well as MLN express mucosal homing marker α4β7 integrin and $CD8^+$ T cells can be activated in gut associated lymphatic tissues and thereafter migrate to pancreatic islets [66].

During the pathogenesis of type 1 diabetes pancreatic lymph nodes are a site of activation of autoreactive T-cells. The general activated phenotype is characterized by higher expression of IL-2 receptor and CD45R [67]. This activation and subsequent proliferation in PLN precedes the infiltration of pancreatic islets. Furthermore, the activation occurs only after certain developmental age of mice, when pancreatic autoantigens are presented in PLN,

because autoreactive T-cells are not activated during adoptive transfer to 10 days old mice but they are activated after transfer to adult ones [68]. In case of autoreactive $CD4^+$ T-cells there is an activated $CD44^{hi}CD62L^{low}$ phenotype in PLN from the age of 3 weeks onward. Once autoreactive T-cells are primed they do not need PLN to infiltrate pancreatic islets and induce type 1 diabetes [65]. Even though autoreactive $CD8^+$ T-cells are primed and proliferate in PLN, they undergo further proliferation and extensive activation to their full cytotoxic potential in pancreatic islets. This activation is independent of β-cells presenting autoantigens or on their production of cytokines but it seems to depend on activation signal and stimulation by CD40-expressing cells [69].

Even B-cells are involved in full activation of autoreactive T-cells and development of T1D because NOD mice without them fail to develop diabetes and insulitis in pancreas is minimal compared to wild-type NOD mice. Moreover, there is an impairment in activation and subsequent proliferation of autoreactive $CD4^+$ T cells in PLN of B-cell deficient NOD mice [70].

Additionally, there is an imbalance in regulation of the immune response such as it is tilted towards proinflammatory response and the regulatory branch of the immune system is attenuated. NOD mice are characterized by lower expression of peripheral tissue antigens in PLN of 12 weeks old NOD mice and the same seems to be true in PLN of humans with T1D. Hence, the induction of peripheral tolerance does not have to be so efficient [71]. Even though numbers of T regulatory cells (Tregs) in PLN are comparable between T1D patients and healthy subjects, they have reduced regulatory potential in T1D patients. In addition the immune response is shifted towards Th17 due to higher percentage of IL-17 producing Th17 cells in PLN of T1D patients compared to healthy controls. Thus, the ratio of Tregs to Th17 is lower in PLN of T1D patients and the immune response is shifted to a more proinflammatory setting [19].

2.4 Regulatory and potentially regulatory T-cells

The importance to re-establish a balance between effector and regulatory arms of immune response is often emphasized as a key goal in various autoimmune diseases. Several studies reported defects of well-established regulatory populations of immune cells (FoxP3 Tregs, type 1 regulatory cells) as well as in populations of T-cells, which show some regulatory properties in the context of type 1 diabetes or their proportions are altered in T1D (γδ T-cells, $CD4^+$ $CD45RB^{low}$, $CD4^+CD62L^+$, naïve/central memory/effector memory $CD4^+$ T-cells) and thus could play a part in maintaining the homeostasis.

2.4.1 FoxP3 Tregs

FoxP3 Tregs are a well-established population of T regulatory cells. They are naturally generated in thymus or can be induced in periphery and their suppressive properties are mediated in a contact dependent manner or by production of cytokines. They can directly influence dendritic cells to become tolerogenic, cause cytolysis via secreting granzyme B and perforin or produce suppressive cytokines like TGF-β, IL-10 or IL-35. FoxP3 Tregs can be characterized like $CD4^+$ $CD25^+$ $FoxP3^+$ or only by surface markers $CD4^+$ $CD25^+$ $CD127^-$ [72].

In NOD mice the proportion of FoxP3 Tregs is decreased prior to diabetes onset [73] and their depletion leads to more severe insulitis, faster destruction of β-cells and establishment of T1D [74]. Interestingly, also peripherally induced Tregs, which are largely generated in the gut, contribute to maintaining homeostasis of immune tolerance in T1D. They can be found in PLN and pancreas of NOD mice and their depletion increases T1D incidence too [75].

Protection by FoxP3 Tregs can be experimentally enhanced by administration of cytokines such as IL-2 or IL-33, which then results in reduction of insulitis and incidence of T1D and conversely in increase of percentage of Tregs in PLN, pancreas and spleen, respectively [76][77]. Administration of IL-2 increases expression of molecules, which have a role in FoxP3 Treg mediated suppression (CD25, FoxP3, CTLA-4, ICOS and GITR), and on the other hand it reduces production of IFN-γ in pancreas [76]. In another experimental designs of induced protection against T1D occurs an increase of FoxP3 Tregs which show suppression towards T effector cells and reduce T1D incidence in adoptive co-transfer [78][79].

There are controversial findings regarding the frequency of FoxP3 Tregs in blood of T1D patients. Some studies report reduction [80][81][82][83][84], some even slight increase in the

percentage of total FoxP3 Tregs [85] whereas others found no difference of total FoxP3 Tregs [86][19][87][88][89][90][91] or proinsulin specific FoxP3 Tregs [88] when compared to healthy subjects. There are no conclusive results when considering factors like age of patients and duration of the disease in relation to the frequency of FoxP3 Tregs.

Nevertheless, the studies, which report no change in frequency of FoxP3 Tregs in blood, also report reduction of Tregs in PLN [19], increase of activated Tregs [89], bigger proportion of proinsulin specific memory Tregs in blood [88] and reduced expression of FoxP3 transcription factor [90].

Taken together he findings about frequency of FoxP3 Tregs are inconclusive but it seems that their suppressive function is reduced in blood of T1D patients [89][90] even though one study found that the function of Tregs residing in blood is without a difference but it is reduced in Tregs residing in PLN [19]. Tregs from T1D patients also have an impaired signaling by IL-2. The expression of FOXP3 in Tregs after IL-2 *in vitro* activation is reduced and this reduction is due to a defect in signaling pathway of IL-2R, namely to reduced phosphorylation of STAT5, which correlates with higher expression of PTPN2 [92]. Plus, they have a lower expression of CD25, which is an alpha subunit of receptor for IL-2 [80].

It seems that better function of FoxP3 Tregs is associated with milder display of the disease due to the fact that patients with residual insulin capacity have higher frequency of activated Tregs [89], higher concentration of IL-35 in serum and have higher percentage of IL-35 producing FoxP3 Tregs [91]. Furthermore, children with uncontrolled T1D have lower frequency of total Tregs [84].

2.4.2 Tr1 T-cells

Type 1 regulatory (Tr1) T-cells are a recently established and important type of peripheral immune regulating cells. They produce high amounts of IL-10 and TGF-β, low amount of IL-2 and INF-γ and do not produce IL-4. Tr1 cells suppress T effector cells or they can mediate their suppressive properties by influencing antigen presenting cells (APCs) [72]. Tr1 cells are characterized by a combination of surface markers LAG-3 and CD49b in humans and mice or they can be also labeled as $CD4^+$ $FoxP3^-$ $IL\text{-}10^+$ T-cells even though it is not so accurate and bit outdated [93]. Nevertheless, it has come to notion recently that expression of LAG-3 and CD49b is not solely specific for Tr1 T-cells and there can be double positivity of these markers also in IL-10 producing FoxP3+ CD4+ and CD8+ T-cells of mice and men after in

vitro and also in vivo induction [94]. Tr1 cells can transiently express FoxP3 upon their activation and in smaller amounts than CD25+ FoxP3+ Tregs [95].

In vitro development of Tr1 cells is more efficient from memory $CD4^+$ T-cells than from all $CD4^+$ T-cells and only the Tr1 memory $CD4^+$ T-cells have protective effect against T1D in adoptive co-transfer [96]. They produce high amount of IL-10 after *in vitro* stimulation with insulin autoantigen but not with GAD65 [79] and, furthermore, they suppress proliferation of diabetogenic T effector cells, which express receptor for IL-10 [96], in a contact independent manner mainly by secretion of IL-10 [97].

Tr1 T-cells are generated in NOD mouse models with induced protection against T1D and they have essential protective effect in adoptive co-transfers [79][96][78][97]. Moreover, autoantigen specific Tr1 T-cells migrate to peripheral lymph nodes and block migration of diabetogenic T-cells into these lymph nodes [97] and more importantly into PLN [78]. Tr1 T-cells can be induced in the small intestine and adoptive co-transfer with only gut Tr1 T-cells also delays the onset of the disease and reduces number of INF-γ producing cells in pancreas. Moreover, intestinal Tr1 cells express chemokine receptors, which enables them to migrate to periphery and home to sites of inflammation [96].

Up to this day there are not many studies about human Tr1 T-cells in context of T1D but one study found that patients with type 1 diabetes have lower percentage of autoantigen specific Tr1 T-cells in blood [20].

2.4.3 γδ T-cells

Prior to proceeding to a human trial, it was reported that repeated inhalation of aerosol insulin significantly reduced incidence of T1D in NOD mice and this effect was transferrable by γδ $CD8^+$ T-cells into the NOD-SCID model of adoptive co-transfer (splenocytes from pre-diabetic mice) [98]. Moreover, NOD mice are deficient in intraepithelial lymphocytes (IEL), which are mainly γδ T-cells, and transfer of these CD8αα TCRγδ IELs have also protective effect on development of T1D. In addition, CD8αα TCRγδ IELs are essential for induction of mucosal $CD4^+$ $CD25^+$ regulatory T cells. They abrogated accelerated diabetes in the neonatally thymectomized NOD model and are required in oral tolerance induction [99]. NOD mice, which already have developed T1D, have lower percentage of γδ T-cells in blood than non-diabetic NOD mice [100]. Moreover, diabetes prevention by intranasal administration of gliadin in NOD mice is associated with increased proportion of γδ T cells, preferentially within the mucosal lymphoid (PLN, MLN, NALT) compartment [101].

A reduced percentage of γδ T-cells can be seen also in T1D patients that have a lower γδ T-cell proportion in peripheral blood at the time of diagnosis and it decreases even more during the continuation of the disease. The lower count of γδ T-cells is mainly due to reduced numbers of $CD8^+$ γδ T-cells. There is also a decrease in activated $CD25^+$ and $CD56^+$ γδ T-cell subpopulations during T1D [21].

Interestingly, IL-17 producing γδ T-cells isolated from 8 weeks old NOD mice were ported to suppress the development of T1D after adoptive transfer and it seems that the protective effect is mediated by production of TGF-β [100]. On the other hand, it was reported that IL-17 producing γδ T-cells, even though they do not transfer the disease by themselves, do potentiate αβ T-cells to induce the development of T1D [102] and higher numbers of γδ T cells was documented in NOD compared to BALB/c mice and their proportion increases in the periphery at onset of diabetes [103], thus, they are quite controversial regarding the potential regulatory function.

2.4.4 $CD45RB^{low}$ T-cells

Another potentially regulatory T-cells in context of T1D are $CD45RB^{low}$ T-cells but it seems it is important to differentiate between age of the mice and individual subset of $CD4^+$ $CD45RB^{low}$ T-cells since the cells loose their regulatory potential with increasing age of the mice and only some subsets mediate the regulation.

$CD45RB^{low}$ T-cells have inhibitory potential on proliferation of effector T-cells [104]. $CD4^+$ $CD45RB^{low}$ T-cells from non-diabetic NOD mice have protective effect in adoptive co-transfer of diabetes to NOD-SCID mice, whereas $CD4^+$ $CD45RB^{low}$ T-cells from diabetic NOD mice do not have this protective effect [105]. The difference in protective abilities corresponds with diverse production of IFN-γ and IL-4 by individual subsets. $CD4^+$ $CD45RB^{low}$ T-cells from non-diabetic NOD mice have much lower IFN-γ to IL-4 ratio, which could have at least partially explain their protective role [105]. The inhibition of TNF-α has protective effect on development of T1D and it influences percentages of $CD45RB^{low}$ T-cells because they are highly represented among $CD4^+$ splenocytes after the inhibition. However, this protection is true only with cells from 6 weeks old NOD mice but not older [106]. The increase of $CD4^+$ $CD45RB^{low}$ T-cells is confirmed in another model of induced protection against T1D in NOD mice plus specifically there is increase in $CD38^+$ subset of $CD4^+$ $CD45RB^{low}$ T-cells [107].

So it seems that the regulatory properties are characteristic only to some subset of CD45RB^{low} T-cells. Indeed, CD4$^+$ CD38$^+$ CD45RB^{low} T-cells inhibit proliferation of stimulated T-cells and need to be activated via T-cell receptor (TCR) to be able to mediate this inhibition. This regulation is due to a contact depended manner and do not depend on production of IL-10 and TGF-β cytokines. The expression of CD38 divides CD4$^+$ CD45RB^{low} T-cells to two equally represented populations in spleen and MLN of BALB/c mice whereas the CD4$^+$ CD38$^+$ CD45RB^{low} T-cell population is present in higher percentage in Peyer's patches [108].

CD4$^+$FoxP3$^-$CD62L$^-$CD44$^+$CD45RB^{low} T-cells produce IL-10 by themselves *in vitro* and they also induce IL-10 production by FoxP3 Tregs but on the other hand production of TGF-β is reduced in co-culture. The regulating effect is mediated in contact independent manner by production of IL-4 by CD45RB^{low} T-cells and is more heightened by production of IL-2. Moreover, the regulating effect of this population is also seen *in vivo* [22].

2.4.5 CD62L$^+$ T-cells

Another cell subset with potentially regulatory properties in the context of T1D are CD4$^+$ CD62L$^+$ T-cells. Their percentage is higher in NOD mice models with induced protection against the disease and the increase is seen in spleen and lymph nodes such as PLN [109][110][23]. CD4$^+$ CD62L$^+$ T-cells from pre-diabetic NOD mice but not from already diabetic ones inhibit development of T1D in adoptive co-transfer [111][112][104].

These cells are increased also in an model with orally induced protection and adoptive co-transfer of these cells mediate protection against T1D and like so transferred cells migrate to PLN and MLN [113]. They infiltrate the surrounding of pancreatic islets and protect them from intra-islet infiltration by diabetogenic T-cells [112].

But in *in vitro* studies CD4$^+$ CD62L$^+$ T-cells do not inhibit proliferation of effector T-cells [104] and their protective effect is not dependent on production of IL-4 or IL-10 and they produce low amount of IL-2 and IFN-γ [112].

But the situation is inverse in humans because T1D patients have upregulated expression of SELL (CD62L) in peripheral blood mononuclear cells [114] and higher percentage of CD4$^+$ CD62L$^+$ T-cells [115]. The increase in expression of SELL and percentage of CD4$^+$ CD62L$^+$ T-cells is even more prominent in T1D patients with some associated complications than without them [114][115].

2.4.6 Naïve and memory T-cells

T-cells can be distinguished into different populations based on their experience with antigen and functional differences. Aside from effector T-cells we can describe naïve and memory population, which can be further divided to several subsets. Naïve T-cells emerge from thymus and circulate the body until they die or encounter their specific antigen. Upon activation they become effector T-cells, which combat the infection and after fulfilling their distinctive functions undergo apoptosis or minority differentiate into memory T-cells [116].

In the context of T1D naïve $CD4^+CD45RA^+$ T-cells are represented in lower percentages in peripheral blood than in healthy age matched controls. Conversely, all memory $CD4^+CD45R0^+$ T-cells, which are also increased in PLN [19] and can be found infiltrating pancreatic islets [117], are represented in higher percentages [118]–[120] and it is also true for subsets of memory cells namely for central memory ($CD4^+CD45R0^+CCR7^+$) and effector memory ($CD4^+CD45R0^+CCR7^-$) T-cells, which produce higher amount of IFN-γ [118]. There is a positive correlation between the percentage of memory T-cells at the time of diagnosis and the length of subsequent partial remission [121]. Similar findings were also in another study where was a positive association between the percentage of central memory T-cells and the decline of β–cell function [122].

Memory T-cells from T1D and autoantibody positive children proliferate after *in vitro* stimulation with proinsulin, insulin and GAD65 whereas memory T-cells from controls do not [123], [124]. The higher rate of proliferation was also confirmed by shorter telomere length in these responsive memory T-cells [124]. The higher proliferation of all memory but not naïve T-cells was confirmed also *in vivo* in T1D patients [125] but not in only autoantibody positive ones [126].

Furthermore, human trials for a treatment drug, which slows down the progress of T1D after its onset, show that there is a decrease of central memory T-cells and conversely, an increase of naïve T-cells after this treatment [122], [127]. Effector memory T-cells are also deceased but only after prolonged period of treatment [127].

2.5 Environmental factors

As it was previously mentioned, the pathogenesis of type 1 diabetes is greatly influenced by environmental factors. The findings advocating for environmental influence include seasonal peaks in diagnosis, incomplete occurrence in monozygotic twins or increase in incidence in offsprings of immigrants and more. Thus, it is clear that only genetic predisposition is not sufficient for mounting the autoimmune reaction and type 1 diabetes is a very heterogeneous disease to whose induction contribute many factors [2].

Type 1 diabetes is diagnosed mostly in autumn and winter and this seasonal occurrence hints on association with some seasonal infections. Indeed, an infection by enteroviruses has been confirmed to be a predisposition for subsequent islet autoimmunity. The winter months are typical for a changes in lifestyle, e.g. exercise, diet, sun exposure and others, which all contribute to more proinflammatory immune milieu. But it is not sufficient to initiate the autoimmunity by itself and other factors, especially genetic ones, need to weight in [128].

Another entity that could add to initiation of T1D are neoepitopes. These epitopes are generated through post-translational modifications and therefore immune system cannot be schooled not to react towards them. Neoepitopes can be generated by various inductors, amongst which belongs e.g. viral infection or stress. Moreover, unlike in case of classical autoantigens, T-cells specific for these new epitopes are increasingly present in type 1 diabetics [129].

Microbiota and diet belong amongst T1D promoting environmental factors, which can be easily influenced. Dysbiosis of gut microbiota is present in T1D and it has been proposed to accelerate the disease by products of their metabolism. Even though the causality is still unclear it is thought that microbiota can support the development of T1D especially by disrupting intestinal barrier. Diet can influence T1D indirectly through changing microbiota composition or directly through affecting mechanisms leading to higher T1D incidence. Such dietary factor is for example gluten but on the other hand some factors have possible preventive influence and they include breastfeeding, vitamin D and polyunsaturated fatty acids [130].

A summarizing infographic about the influence of environmental factors on development of T1D can be seen in Figure 4.

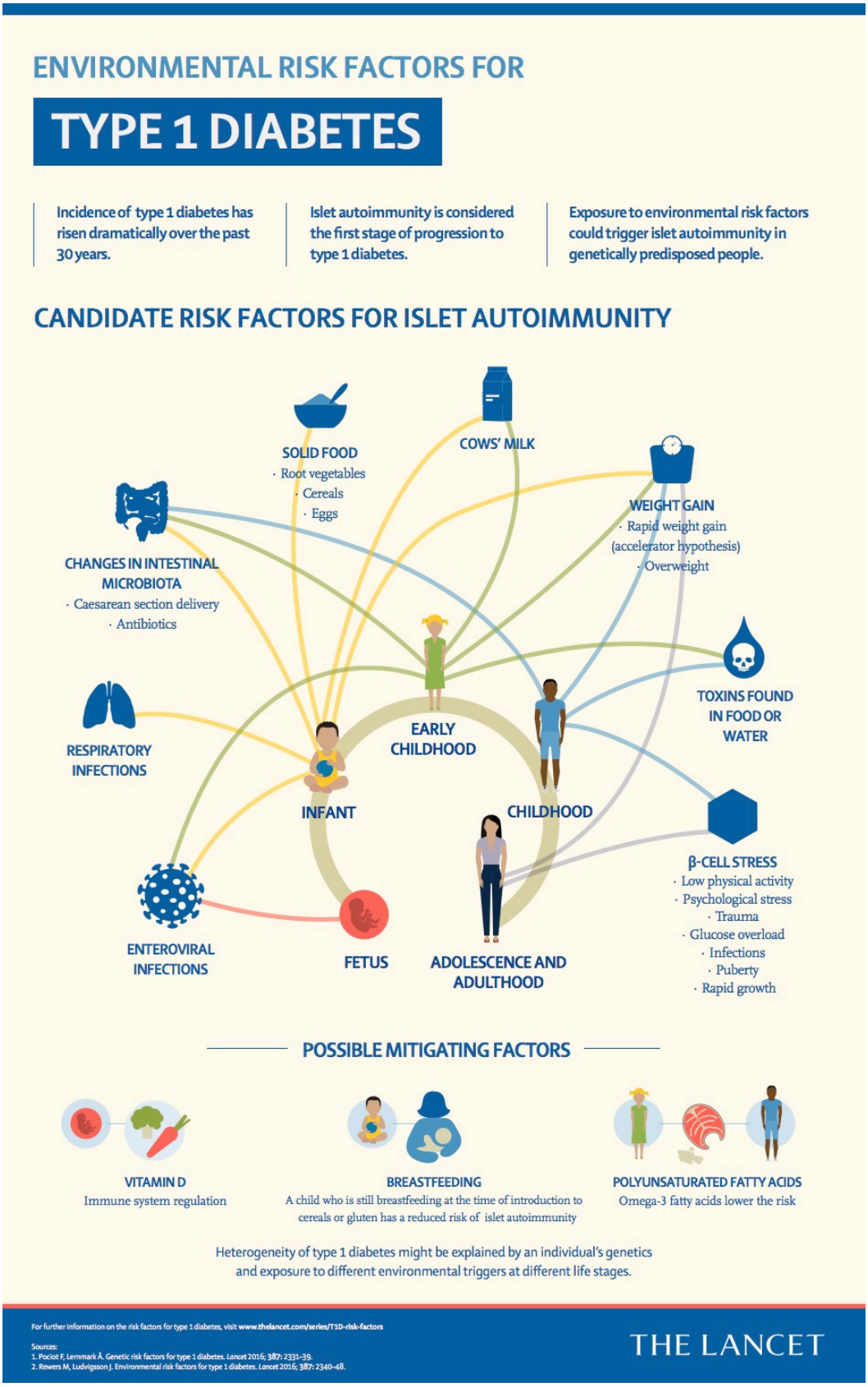


Figure 4: The infographic about possible environmental agents that could influence the development of type 1 diabetes (web 4)

2.5.1 Gut permeability in type 1 diabetes and its influencing factors

Gut permeability is higher in patients with type 1 diabetes but it is not clear if the higher permeability precedes T1D and thus is a cause for its development, or not. Integrity of intestinal barrier is influenced by many factors to which, interestingly, belongs also gluten and gut microbiota. There is a proposed necessary coincidence of damaged intestinal barrier and dysbiosis of gut microbiota for NOD mice to subsequently develop type 1 diabetes [14]. So it seems that gut permeability influenced by gluten intake directly or indirectly through affecting microbiota dysbiosis can be at least partially responsible for development of T1D.

Intestinal barrier is mediated mainly by tight junctions between individual enterocytes and layer of mucus, which covers intestinal epithelium [13]. Type 1 diabetes susceptible NOD mice have higher permeability of intestinal barrier than control groups of mice and this discrepancy begins around 10 - 12 weeks of age, which is prior to onset of T1D. There is a lower expression of some of the tight junctions composing proteins in the intestine, namely tight junction protein 1 and claudin-1, and also microscopically detectable deterioration of the mucus layer, which is also demonstrated by lower expression of some of the mucus constituting mucins [14]. Tight junctions disassembly is directed by zonulin, which can thereby influence gut permeability and levels of zonulin in serum can by used as a marker of said permeability [13]. Intestinal permeability represented by higher zonulin expression is increased in NOD mice that progress to T1D as opposed to non-progressors and a control group of mice [131]. Blocking zonulin pathway in animals with high intestinal permeability lowers permeability and subsequent development of T1D [13]. The higher gut permeability is found also in humans. T1D patients have elevated markers of intestinal barrier damage in serum [132] and the higher permeability is seen also in seroconverted children, who later progress to T1D as opposed to non-progressors [133], and in children with full blown T1D [134]. But the causality of intestinal permeability in the pathogenesis of type 1 diabetes is still not clear [135].

Gut permeability can be influenced by gliadin, which is a component of gluten. Gliadin affects signaling pathway that releases zonulin from enterocytes and thus increases gut permeability [15]. On the other hand, the expression of genes encoding proteins, which promote barrier function, namely occludin, tight junction protein 1 and claudin-15, is higher in offsprings of NOD mice fed gluten-free diet. This particular effect of GFD is probably mediated through altered microbiota that is transferred from the mothers to their pubs [25]. Gliadin peptides can get through intestinal barrier and are found in pancreas of NOD mice but also control groups of mice BALB/c and C57BL/c after oral administration [136] and

they can probably directly stimulate β-cells in pancreas because *in vitro* stimulation of β-cells with enzymatically digested gliadin and mainly gliadin 33-mer peptide results in higher secretion of insulin. Furthermore, NOD mice intravenously given digested gliadin gain weight most likely due to higher insulin secretion [137].

Gluten-free diet changes representation of individual groups of intestinal microbiota in NOD mice [138] and thus indirectly influences intestinal permeability and zonulin release because microbiota itself directs integrity of the barrier [13]. NOD mice maintained on GFD for 3 generation have increased abundance of *Akkermansia*, whereas diets containing gluten increase *Bifidobacterium*, *Barnesiella* and *Tannerella* [139]. Different composition of microbiota is further confirmed by another study, where NOD mice on GFD and their offsprings have distinct abundance of bacteria compared to controls on standard diet. GFD elevates abundance of *Proteobacteria*, *TM7*, *Verrucomicrobia*, namely *Akkermansia*, and some genera of *Firmicutes* and on the other hand decreases abundance of *Bacteroidetes*, *Cyanobacteria* and other genera of *Firmicutes* [25]. The effect of GFD is seen also in humans, where gluten-free diet eaten by healthy humans for 1 month influences the composition of microbiota and metabolic pathways in the gut, especially increases butyrate metabolism [16].

Patients with T1D have different representation of some individual species of bacteria in comparison with healthy subjects. Even though the findings are somewhat controversial some aspects seem to be common. T1D children have lower overall microbial diversity and seroconverted children, who later progress to T1D, have lower alpha diversity than non-progressors [133]. Next, T1D children have lower *Firmicutes*/*Bacteroides* ratio, less bacteria producing butyrate, namely *Clostridium* clusters IV and XIVa, and less abundant *Prevotella* and *Akkermasia*, also a lower proportion of *Bifidobacterium* [135]. Seroconverted children, who subsequently progress to T1D, have lower abundance of phylum *Verrucomicrobia* to which *Akkermansia muciniphila* belongs [133]. Intervention studies, which manipulated intestinal microbiota, executed on NOD mice suggest a causal relation between microbiota dysbiosis and subsequent development of T1D. For example, colonization of NOD mice with *Akkermansia muciniphila* is associated with reduced T1D incidence and higher production of mucus [140]. Human studies addressing the causality in this phenomenon are lacking but there are a few studies hinting on the possibility [135].

Some microbiota can influence the barrier function of intestinal epithelium mainly by producing short chain fatty acids (SCFA) and also by influencing production of zonulin. Bacteria producing SCFA butyrate have positive effect on intestinal barrier and on the other hand bacteria, such as *Bacteroides*, producing other SCFAs have negative effect.

Bifidobacterium and *Lactobacillus* species are associated with barrier protection [135]. Pathways of bacterial fermentation, namely some individual steps in pathways producing SCFA such as butyrate, acetate and propionate are decreased in T1D children [141].

2.5.2 Influence of gluten-free diet and gluten on the immune system

In general, gluten-free diet shifts the immune milieu towards a less inflammatory one. When given to BALB/c mice, it reduces production of IL-2, IL-4, IL-17 and IFN-γ, has no effect on production of IL-10 but increases production of TGF-β by T-cells in mucosa associated organs namely MLN, PLN and PP [142].

NOD mice, which spontaneously develop type 1 diabetes, have higher intestinal permeability, increased expression of several inflammatory cytokines (namely TNF-α, IL-17 and IL-23) and higher percentage of Th17 cells and on the other hand lower percentage of FoxP3 Tregs in the intestine. Islet autoreactive T-cells acquire activated phenotype in mice with high permeability of gut barrier and start to produce more IFN-γ and IL-17. These activated Th17 cells migrate to PLN and pancreatic islets, where they can induce T1D [14]. Gluten-free diet with its anti-inflammatory properties protects against T1D development, as mentioned earlier, and reduces percentage of these IL-17 producing T-cells in pancreatic lymph nodes [18], moreover lowers production of inflammatory cytokines IFN-γ and TNF-α in the intestine but has no effect on production of anti-inflammatory ones IL-10 and TGF-β [143]. Regarding other sites, GFD also reduces production of IFN-γ by $CD4^+$ T-cells and IL-22 by γδ T-cells [144] and has no effect on production of IL-10 in spleen [145]. So the anti-inflammatory properties of GFD reside in shifting the immune milieu towards a less inflammatory one mainly by reducing the production of inflammatory cytokines and thereby increasing the balance in favor of anti-inflammation.

Regarding the influence of gluten-free diet on abundance of individual populations of immune cells, then in BALB/c mice it increases percentage of $CD4^+$ $CD62L^+$ T-cells in PP and PLN, γδ T-cells in spleen, ILN, MLN, PLN and PP but decreases percentage of γδ T-cells expressing CD8 marker in PLN. Next, it increases memory $CD45RB^{low}$ T-cells and on the other hand decreases naïve $CD45RB^{high}$ T-cells and there is no difference in percentage of FoxP3 Tregs [18]. Although it was reported that percentage of FoxP3 Tregs is higher after GFD in PLN [25] and MLN in NOD mice [139]. GFD also lowers the percentage of $CD8^+$ T-cells especially in pancreatic lymph nodes [146].

Gluten-free diet influences the innate part of immune system too, such as dendritic cells (DCs) and natural killer (NK) cells. GFD increases percentage of $CD11c^+$ DCs and among these $CD11c^+$ cells increases the CD103 positive fraction but on the other hand lowers the CD11b positive fraction of cells. GFD also reduces surface molecules on DCs in PLN such as MHCII, CD40 and CCR7, which are considered as activation markers, so it seems the phenotype of dendritic cells is more tolerogenic due to influence of gluten-free diet [147].

Regarding the population of natural killer cells, GFD lowers expression of activating receptor NKG2D on these cells and also lowers expression of CD71, which is a marker of proliferation. Furthermore, GFD reduces overall expression of NKG2D [146] and NKp46 [148] in intestine and pancreatic islets and also reduces expression of NKG2D ligands in the intestine, by which NK cells are less likely to be activated [146]. On the other hand, *in vitro* stimulation of NK cells with gliadin peptides potentiates their production of IFN-γ and overall cytotoxicity towards β-cells in co-culture. And it increases expression of a ligand of activating receptor NKp46 on these β-cells, so stimulation with gliadin potentiates activation of NK cells towards β-cells [148].

Interestingly, *intra nasal* administration of gliadin peptides to NOD mice induces their protection against development of T1D and reduces severity of insulitis. This administration of gliadin leads to higher percentage of FoxP3 Tregs and γδ T-cells in mucosal lymph nodes MLN and especially in PLN but not in other lymphoid organs. Moreover, it influences the production of cytokines towards anti-inflammatory IL-10 and suppresses production of IFN-γ [101]. One case-control study looked at type 1 diabetes incidence in bakers and agriculture workers, who come to a contact with gluten-containing cereals, and found that these occupations are associated with lower incidence of T1D. This phenomenon could be due to inhaling of gliadin, which was found intra nasally in these groups of workers [149].

There are no human studies, which would inspect the influence of gluten-free diet on individual parts of immune system, but there are some, which look into the effect of *in vitro* gliadin stimulation on immune cells. One study focused on stimulation of immune cells in biopsy specimens of small intestine from T1D children. It found that challenge with gliadin peptides increases number of activated $CD25^+$ T-cells and expression of CD80 in lamina propria and number of intraepithelial T-cells [150]. Other studies investigated the immune response to gliadin on samples of PBMCs. T-cells from approximately half of the patients proliferate after stimulation with wheat peptides (Figure 6), gliadin and the greatest response is after stimulation with α-gliadin 33-mer peptide. Most of these proliferating cells are $CD4^+$ T-cells and $CD8^+$ T-cells react only weakly. This proliferation is dependent on

HLA-DR and almost all of the responding patients have HLA-DR4 allele. Moreover, these proliferating T-cells produce high amount of IFN-γ, TNF, IL-6 and IL-17 [17]. On the other hand, after stimulation with another peptide from α-gliadin, specifically 13-mer peptide, only $CD8^+$ T-cells do proliferate [151]. Nevertheless, the proliferation of $CD4^+$ T-cells after gliadin stimulation is controversial because another study found that the proliferation of T-cells from seroconverted children, with 2 or more autoantibodies, and from recent-onset of T1D children is by contrast reduced after this challenge [44].

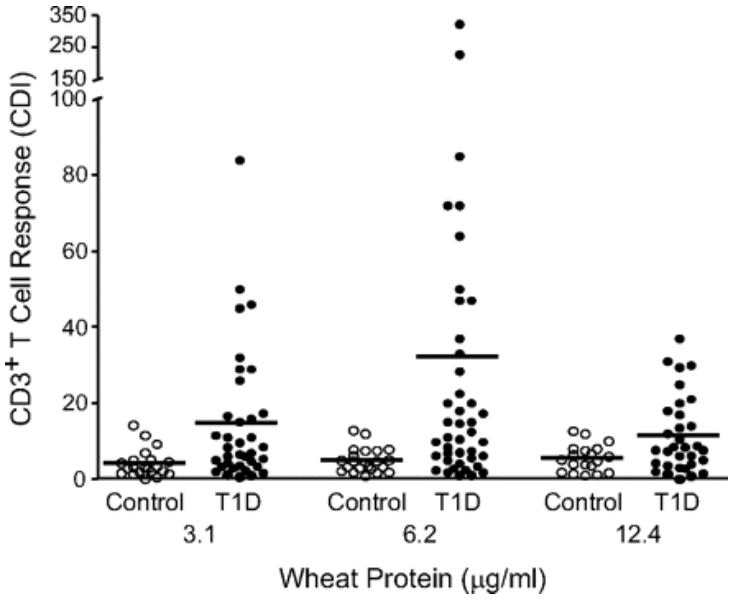


Figure 6: Proliferation of T-cells in control T1D groups after stimulation with wheat peptides

Proliferation of T-cells after stimulation of peripheral blood mononuclear cells from control subjects or type 1 diabetic patients by different concentrations of wheat peptides [17]

2.5.3 Gluten-free diet

First studies have reported that dietary protein alters diabetes incidence in animal models and hypoallergenic diets based on hydrolyzed casein were first shown to prevent T1D in BB rats and NOD mice [5]–[7].

Non-purified, open formula gluten-free diet (GFD) prepared by modification of protein sources (grain versus meat protein) as a possible preventing remedy for type 1 diabetes was firstly described in the year 1999 on the NOD mouse model. The study claims that NOD

mice maintained on GFD *in utero* and postnatally have substantially lower incidence of T1D [8]. This effect has been confirmed several times in another studies [10][9][139]. But the protective effect of GFD is also seen when the diet is administered to mice only from 3 weeks of age at weaning [10][138] or only to mothers during pregnancy and the protectiveness is transferred to their offsprings [25][152]. Nevertheless, also a diet which is very high in gluten content has the same effect on protection against T1D as the gluten-free one, when mothers and their offsprings of NOD mice were maintained on GFD or gluten enriched diet [9][153]. Gluten-free diet also reduces titers of autoantibodies against insulin [10] and insulitis [152][9] in 13 weeks old but not in 4 weeks old NOD mice, which were given GFD only *in utero* [144].

Gluten-free diet in humans has not been tested in similar settings as in NOD mice as it would have to be introduced prenatally to representative sample of whole population, however some benefits can be seen in individual phases of the disease progression. The amount of gluten intake by mothers during pregnancy has no effect on development of type 1 diabetes in their children according to one study [154] but according to another study the increasing amount of gluten in a diet of mothers during pregnancy is positively associated with increasing risk of T1D in their children with two times higher risk in children with mothers on the highest gluten intake [155].

It seems that gluten intake in genetically predisposed children, who have not become seroconverted yet, has some influence on subsequent emergence of islet autoantibodies and then T1D but the results are in some cases ambiguous. However, it is clear that introduction of gluten before 3 months of age to these children is associated with higher risk of developing islet autoimmunity and subsequent T1D [156] and the highest rate of increased risk is in children with HLA genotype DR3/DR4-DQ8 [157]. Another study confirmed that very early introduction of gluten to the diet of infants (before 4 months of age) is associated with worse prognosis about development of T1D [158]. Moreover, it seems that not only the time of gluten introduction but also the amount of gluten intake influences islet autoimmunity and T1D because high amount of gluten in the diet is positively associated with higher risk of seroconversion [159] and then with higher incidence of T1D [154]. Nevertheless, another study claims there is no relation between the amount of gluten and development of autoantibodies and T1D [158].

At the first stage of the disease, which is emergence of islet autoantibodies, gluten-free diet has no effect on titers of individual autoantibodies when given during intervention trials for

6 months [160] or 12 months [161] and does not change probability of subsequent development of T1D [162], but it does improve insulin secretion [160].

The insulin secretion improves even when the diet is given to children with recent onset of T1D. It was firstly mentioned in a case report about 6 year old boy, who had been given GFD, and then entered a long remission phase without any insulin therapy for at least 20 months after diagnosis [163]. Then followed a pilot study about children with T1D, who had been on GFD for 12 months. This study found lower HbA1c and IDAA1c in these patients and thus more children were in partial remission [26]. And finally, children kept on GFD for 12 months after diagnosis with T1D had better residual β-cell capacity (measured by C-peptide AUC), lower HbA1c, lower insulin dose, higher incidence of partial remission (IDAA1c) and even three children could stop altogether with insulin substitution for a few months. These findings are represented in Figure 5 [27].

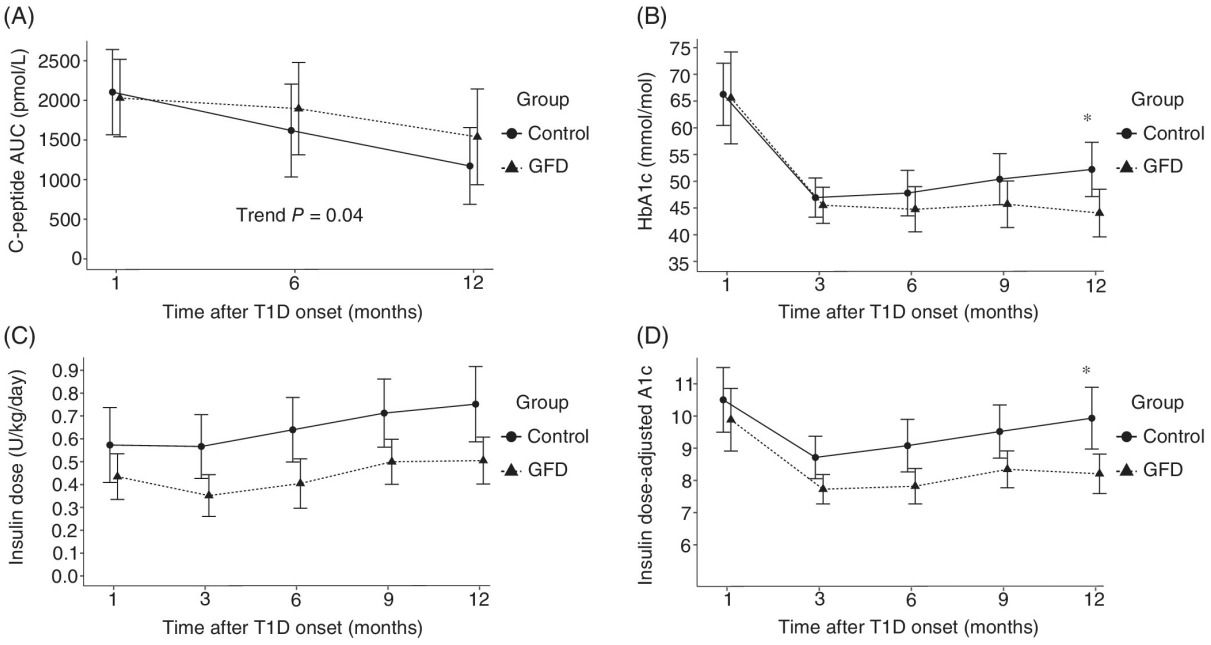


Figure 5: The progress of C-peptide AUC, HbA1c, insulin dose and adjusted insulin dose values during 12-month intervention trial by gluten-free diet in T1D children

*Graphs depict the progress in control (full line) and GFD (dashed line) groups in values: (A) C-peptide area under the curve, (B) HbA1c, (C) insulin dose and (D) insulin dose adjusted A1c. * P < 0,05* [27]

3 Aims

This diploma thesis is a part of a larger project, which was done in collaboration with Department of Pediatrics, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital, Laboratory of Molecular Genetics at University Hospital Prague-Motol and Laboratory of Gnotobiology at the Institute of Microbiology in Nový Hrádek.

Specific aims related directly to this thesis were to study the influence of gluten-free diet on composition of immune system and thus to:

1. Examine numbers of regulatory and potentially regulatory T-cells and their cytokine signature in peripheral blood mononuclear cells of children with type 1 diabetes, who participated in the 12-month prospective intervention trial with GFD
2. Assess the same immunological features as in aim 1 but in mucosal and systemic lymphoid organs of NOD mice, which were colonized with microbiota from T1D children adhered to GFD or STD for 12 months
3. Measure the percentage of IL-10 producing $CD4^+$ T helper cells from these NOD mice after *in vitro* restimulation

4 Materials and methodology

4.1 Materials

4.1.1 Laboratory disposables

Test tubes 5 ml: Becton Dickinson, USA

Gloves: Nitril non sterile, 9018 – M, Vulkan Medical, a.s., Czech Republic

Petri dishes 60 mm: GAMA GROUP a.s, Czech Republic

Serological pipettes 10 ml: Jet Biofil, China

Pipette tips 1000 µl: Greiner bio-one, Austria

Pipette tips 200 µl: Greiner bio-one, Austria

Pipette tips 20 µl: Greiner bio-one, Austria

Tubes with Cell-Stainer caps 5 ml: Falcon, Mexico

Microcetrifuge tubes: Snap Cap Low Retention Microcentrifuge Tubes, Thermo Scientific, USA

Centrifuge tubes 50 ml: Jet Biofil, China

Microscope slides: Thermo Scientific, USA

Cover glass: Hounisen Laboratorieudstyr, Denmark

4.1.2 Solutions, buffers, antibodies

Protein Transport Inhibitor: BD Cytofix/Cytoperm™ Plus, GolgiStop™,BD Biosciences, USA

Viability Dye: Fixable Viability Dye, eFluor 780, eBiosciences, USA

Compensation beads: UltraComp eBeads™, Invitrogen, USA

Trypan Blue solution 0,4 %: Sigma-Aldrich, USA

Red Blood Cell Lysing Buffer Hybri-Max™: Sigma Life Science, USA

Fixation and Permeabilization Solution: BD Cytofix/Cytoperm™ Plus, BD Biosciences, USA

Phorbol myristate acetate (PMA): Sigma-Aldrich, USA

Ionomycin: Sigma-Aldrich, USA

Complete medium: RPMI-1640 with L-glutamine (Lonza, USA), fetal bovine serum (10%, Gibco-Life Technologies, USA), penicillin-streptomycin (0,5%, Sigma-Aldrich, USA), sodium pyruvate (1%, Sigma-Aldrich, USA), β-mercaptoethanol (0,00035%, Serva Electrophoresis GmbH, Germany), MEM Non-essential Amino Acid Solution (1%, Sigma-Aldrich, USA)

FACS solution: PBS with added 0,1% sodium azide, 0,02% EDTA, 2% FBS

FACS + monensin solution: FACS solution + protein transport inhibitor (0,66 µl/ml, BD GolgiStop, BD Biosciences, USA)

Perm/Wash solution: distilled water + BD Cytofix/Cytoperm™ Plus, Perm/Wash™ Buffer (10%, BD Biosciences, USA)

Antibodies: see Table 2

HUMAN SAMPLES

Surface markers								
Antigen	CD3	CD4	CD49b	LAG3	CD3	CD45R0	CD62L	CD45RA
Fluorochrome	FITC	eFluor 450	APC	PE-Cyanine 7	APC	BV510	PE-Cyanine7	FITC
Manufacturer	eBioscience	Invitrogen	Invitrogen	Invitrogen	Invitrogen	BD Biosciences	eBioscience	eBioscience
Clone	0KT3	RPA-T4	P1H5	3DS223H	UCHT1	UCHL1	DREG-56	HI100

Intracelular markers			
Antigen	IL-17	IFN-γ	IL-10
Fluorochrome	PE	PE	PE
Manufacturer	Invitrogen	Invitrogen	eBioscience
Clone	eBio64DEC1	4S.B3	JES3-9D7

MOUSE SAMPLES

Surface markers								
Antigen	CD4	CD3	CD25	γδ	CD45RB	CD4	CD62L	CD3
Fluorochrome	PB	PerCP Cy5.5	APC	PE	PE	PE-Cy7	APC eFluor 780	FITC
Manufacturer	Invitrogen	eBioscience	eBioscience	eBioscience	eBioscience	eBioscience	eBioscience	Invitrogen
Clone	RM4-5	145-2C11	PC61.5	eBioGL3	C363.16A	GK1.5	MEL-14	145-2C11

Intracelular markers		
Antigen	FoxP3	IL-10
Fluorochrome	PE	PE
Manufacturer	eBioscience	eBioscience
Clone	FJK-16s	eBioscience

Table 2: The list of used fluorochrome-conjugated antibodies

4.1.3 Laboratory equipment

Light microscope: Motic® BA310 Professional Light Microscope, Motic, USA

Bürker chamber: Assistent, Germany

Surgical instruments: scalpel, tweezers, scissors

Pipettes (0,5–10, 5-50, 20-200, 200-1000 µl): Finnpipette, Labsystems, Hungary

Pipette: Pipetman, Gilson, USA

Manual repetitive pipette: HandyStep, BRAND, Germany

Combitip: Eppendorf, Germany

Pipette Controller: FastPette V-2, Labnet International, USA

CO2 inkubator: MCO-170AICUV-PE, Panasonic, Schoeller, Czech Republic

Flowbox: Biocyt 150, Esi Flufrance, France

Centrifuge: Rotanta 460R, Hettich, Germany

Centrifuge: IEC CL31R Multispeed centrifuge, Thermo Electron, USA

Microcentrifuge: Micro-Centrifuge II, LabTech, Korea

Vortex: MS2 Minishaker, IKA Works, INC., USA

Flow cytometer: BD LSR II, BD Biosciences, USA

4.2 Methods

As it was already mentioned this thesis was a part of a larger project, which was done in collaboration with other laboratories.

The Department of Pediatrics, 2nd Faculty of Medicine, Charles University in Prague and Motol University Hospital recruited children with recent onset of type 1 diabetes into this study and divided them to a control group, which adhered to standard diet (n = 13), and an intervention group, which adhered to gluten-free diet (n = 12) for 12 months. The adherence to gluten-free diet was controlled by food questionnaires and also by a presence of gluten immunogenic peptides in stool samples. These children were monitored for residual β-cell capacity and control of T1D, and blood samples were collected. Further, peripheral blood mononuclear cells were isolated from these blood samples on Ficoll gradient and stored at -150 °C. The frozen samples were further processed and analyzed by our laboratory as it is described below. The experimental design of this part of the study is depicted in Figure 7.

After 12 months of chosen diets five children were picked and samples of stool were collected. These samples were then processed and frozen in glycerol at -80 °C. At the Laboratory of Gnotobiology at the Institute of Microbiology in Nový Hrádek specimens were inoculated with 1.7×10^8 to 1.9×10^{10} bacteria (assayed by quantitative pan-bacteria RT-PCR) by administration on skin, enema and oral gavage into 5 colonies of 3 - 4 weeks old germ-free female NOD mice (originally obtained from Taconic USA, NY, Albany), which were kept in gnotobiotic isolators and given sterile water and standard diet. In addition, also one germ-free colony was established as a control group. All of the colonies were observed for a period of 250 days to asses the development of T1D by measuring blood glucose. Nevertheless, some of these mice were sacrificed at 12 - 13 weeks of age to examine insulitis and changes in T-cells, which was done by our laboratory as it is described below. The experimental design of this part of the study can be seen in Figure 8.

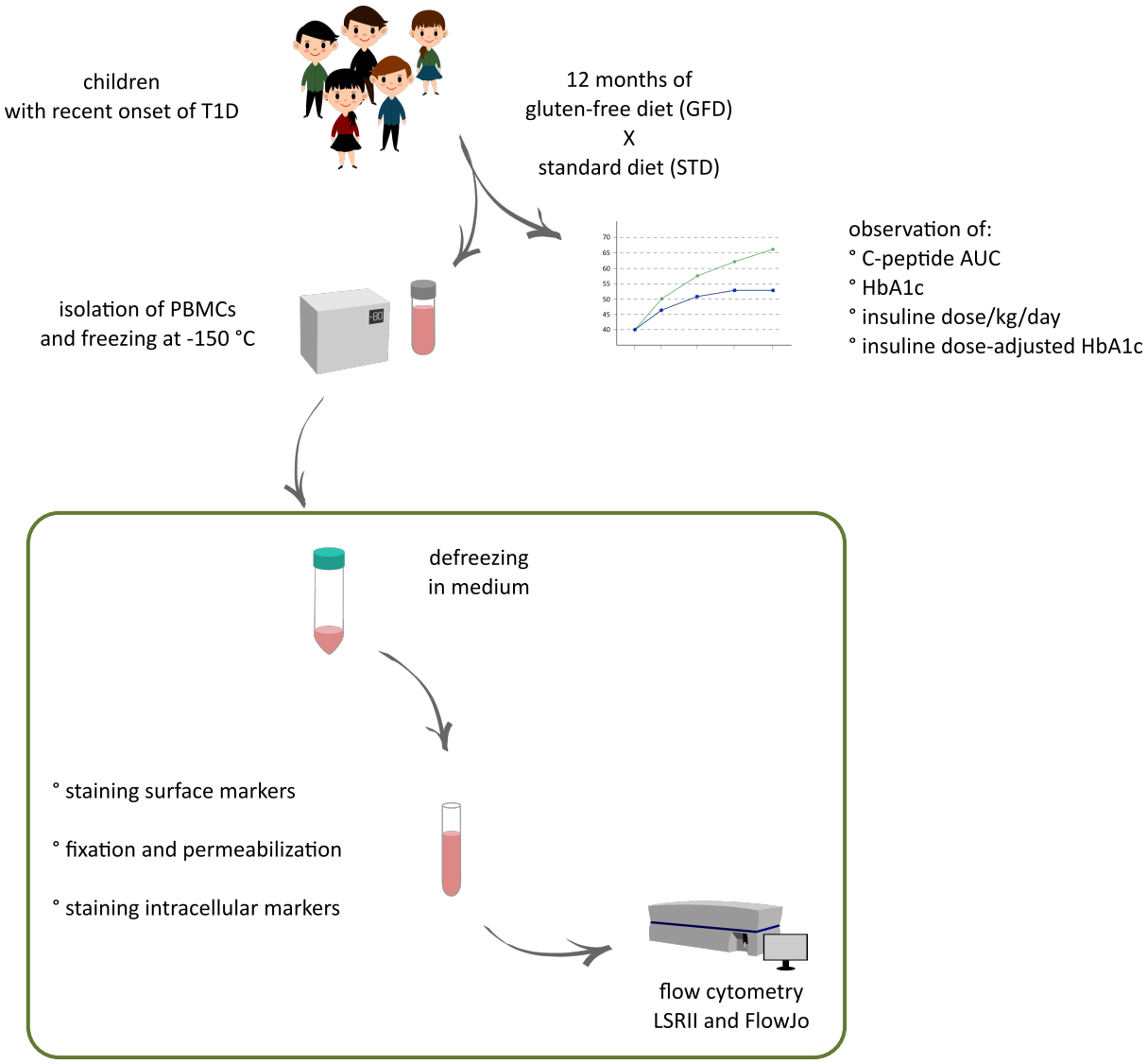


Figure 7: The experimental design of the first part of this project

Children with recent onset of T1D were recruited into the study and administered to standard or gluten-free diet for duration of 12 months. They were monitored for C-peptide AUC, HbA1c, insulin dose/kg/day and insulin dose-adjusted HbA1c. Also samples of PBMCs were collected and frozen at -150 °C. In the green box is a depiction of the part of the work that was done directly at our laboratory: samples were defrosted in medium and prepared for fluorescence staining, stained for surface markers, fixated and permeabilized, stained for intracellular markers and then measured by flow cytometer BD LSR II and analyzed by FlowJo software.

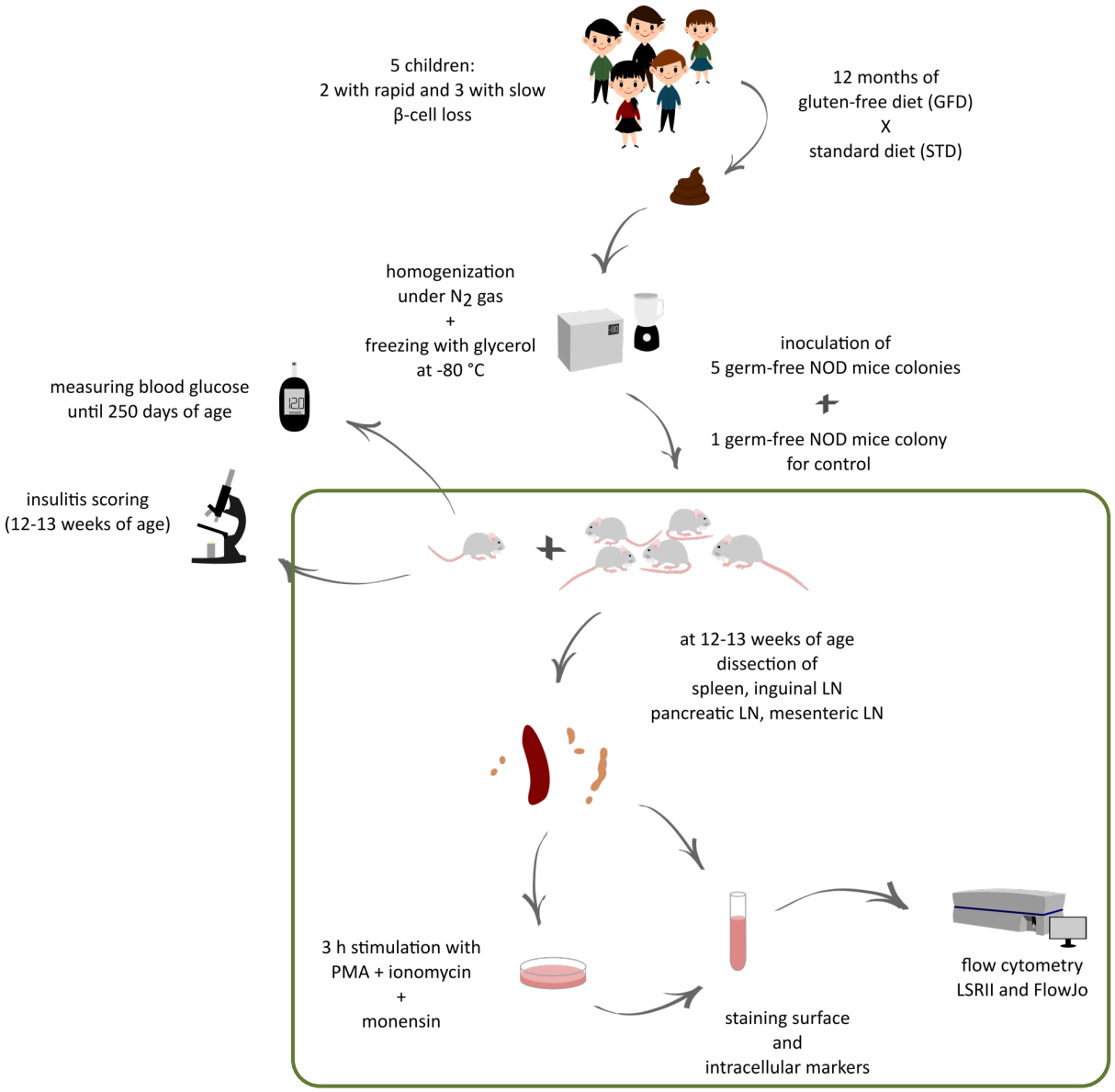


Figure 8: The experimental design of the second part of this project

After 12 months of administered diet five children were picked and samples of stool were collected. These samples were processed and frozen at -80 °C and then inoculated to 5 colonies of female NOD mice. Also 1 germ-free colony was established as a control group. Blood glucose was then measured up to 250 days for observing of T1D incidence. Some mice were sacrificed at 12-13 weeks for scoring of insulitis. In the green box is a part of the work that was done directly by our laboratory: sacrificed mice were dissected for spleen, ILN, PLN and MLN and these organs were prepared for fluorescence staining. Part of the samples was stimulated with phorbol myristate acetate (PMA) and ionomycin in the presence of monensin to examine production of cytokines. All of the samples were stained for surface and then after fixation and permeabilization for intracellular markers and at last measured by flow cytometer BD LSR II and analyzed by FlowJo software.

4.2.1 Defrosting of human PBMCs samples

PBMCs samples, which had been frozen at -150 C°, were defrosted in water bath at 37 °C. Each sample was then slowly, drop by drop, transferred by pipette to 10 ml of complete medium, which was also preheated to 37 °C. Samples were vortexed and centrifuged (1200 RPM, 20 °C, 10 minutes). Then the supernatant was poured off and the pellet resuspended in 2 ml of complete medium. Absolute number of viable cells per sample was counted by usage of 20 µl of the sample, trypan blue stain, Bürker chamber and light microscope. After that all samples were further processed for flow cytometry staining, which is described below.

4.2.2 Isolation of mouse organs

Mice were sacrificed at the age of 12-13 weeks and dissected for desired organs. Spleen and inguinal, mesenteric and pancreatic lymph nodes were taken and placed into a petri dish with 3 ml of complete medium. The organs were then mechanistically but gently triturated by rubbing them with a tweezer against a rough part of a microscope slide. The microscope slide was washed with 400 µl of FACS medium by a pipette and the sample was filtered through a filter into a test tube. Samples of spleens were further processed to remove erythrocytes. Firstly, the tubes were centrifuged (1200 RPM, 20 °C, 5 minutes) and then the supernatant was poured off. The pellet was resuspended, 1 ml of lysing buffer was added and samples were incubated for 4 minutes to lysate erythrocytes. Adding 30 ml of complete medium stopped the lysis and samples were again centrifuged (1200 RPM, 20 °C, 5 minutes) and supernatant poured off. The pellet was resuspended and 4 ml of complete medium was added. Absolute number of viable cells per sample was counted by usage of 20 µl of the sample, trypan blue stain, Bürker chamber and light microscope. After that all samples were further processed for flow cytometry staining, which is described below.

4.2.3 Stimulation by PMA and ionomycin

Prior to flow cytometry staining some samples were stimulated *in vitro* to measure production of cytokines. Cell suspension with 1-2 milions of cells were taken from each sample and placed into different tube. Next, previously prepared solution of PMA (final concentration 25 ng/ml) and ionomycin (final concentration 1 µg/ml) was added to each sample and incubated for 30 minutes at 37 °C and 5% CO_2. After this incubation period Protein Transport Inhibitor containing monensin was added to final concentration of 0,66 µl/ml and all samples were incubated for 3 hours at 37 °C and 5% CO_2. After the stimulation had been completed, samples were processed for flow cytometry staining.

4.2.4 Preparation of samples for flow cytometry

The samples were divided to test tubes according to the desired number of cells per tube. 0,5 ml of FACS + monensin solution was added and samples were centrifuged (1300 RPM, 4 °C, 4 minutes) and the supernatant was poured out. Pellet was resuspended and samples were stained with Fixable Viability Dye and by adding fluorochrome conjugated antibodies towards surface markers according to used panels. The samples were filled with FACS + monensin solution to a final volume of 100 µl and incubated for 25 minutes on ice and in dark. After the incubation period fluorochromes were wash out from samples by adding of 1 ml of FACS + monensin solution, vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes) and pouring out the supernatant. The pellet was resuspended and then fixated and permeabilized by 250 µl of fixation and permeabilization solution (BD Cytofix/Cytoperm™), vortexed for at least 5 seconds and incubated on ice and in dark for 20 minutes. The solution was washed out by adding 1 ml of Perm/Wash solution (prepared from BD Perm/Wash™ Buffer 10x solution), vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes) and pouring out the supernatant. Then antibodies against intracellular markers were added to the pellet, samples were filled with Perm/Wash solution to a final volume of 100 µl and incubated on ice and in dark for 25 minutes. After the incubation period fluorochromes were washed out for the last time by adding 1 ml of Perm/Wash solution, vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes) and pouring out the supernatant. Finally, FACS solution was added to a final volume of 120 µl per sample and thereby samples were ready for measurement.

Measurement of all samples was done by BD LSR II flow cytometer and obtained data analyzed by FlowJo software.

4.2.5 Statistical analysis

Statistical analyzes were done by means of GraphPad Prism 5 (GraphPad Software, San Diego, California, USA), which was used also for subsequent generation of graphs. For the human samples Unpaired t-tests were used to analyze the difference between STD and GFD groups. Differences of p-value < 0,05 were considered statistically significant. * $P < 0,05$. Mouse samples were analyzed by One-way ANOVA with follow-up by Tukey's Multiple Comparisons Test to compare the differences between each pair of multiple obtained datasets. p-values < 0,05 were considered statistically significant. * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$.

5 Results

The incidence of T1D and insulitis in NOD mice colonized with microbiota from T1D patients was not done by our laboratory but it was carried out as a part of the project by the Laboratory of Gnotobiology at the Institute of Microbiology in Nový Hrádek, where the mice were held in gnotobiologic isolators. Hence, the figures about incidence of T1D (Figure 9) and insulitis (Figure 10) are shown here only for illustration and further discussion of diabetes-protective effect of microbiota that were transferred from T1D children on GFD or STD diets (and with a "slow" or a "fast" β-cell loss) to germ-free NOD mice. Immune parameters were assessed in ex-germ-free NOD mice from three isolators: Slow3 and Rapid2 (children on STD) and Slow2 (child on GFD).

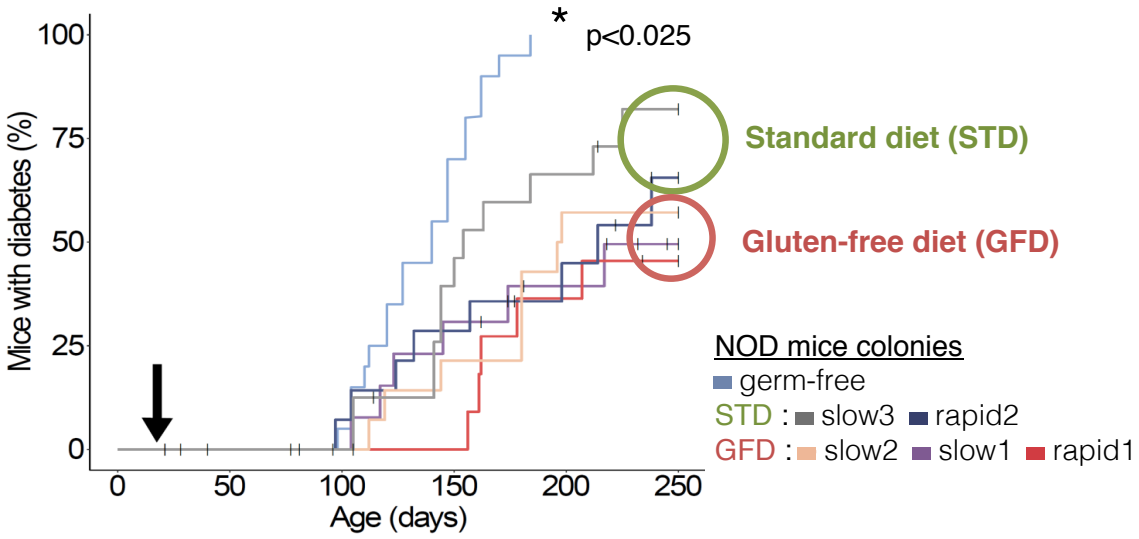


Figure 9: Cumulative incidence of T1D in NOD mice colonized with microbiota from T1D children

Colonization of germ-free NOD mice with microbiota from T1D patients significantly reduced the cumulative incidence of T1D, which is in line with previous data. However, the effect was more prominent with colonization from T1D children, who had been on GFD for 12 months. The difference is not statistically significant due to the compensation for multiple comparisons of diabetes incidences in this graph. On the other hand the slow or rapid pace of loss of residual β-cell capacity does not indicate such tendency. NOD, non-obese diabetes; T1D, type 1 diabetes; GFD, gluten-free diet; STD, standard diet

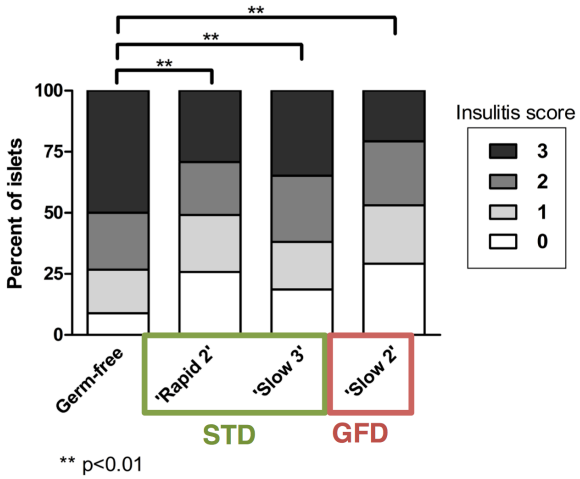


Figure 10: Insulitis score of NOD mice colonized with microbiota from T1D children

The colonization significantly reduced insulitis compared to a colony of germ-free NOD mice. NOD, non-obese diabetes; T1D, type 1 diabetes; GFD, gluten-free diet; STD, standard diet

5.1 Assessing the percentage of regulatory T cells and cytokines in PBMCs of T1D children

Samples of peripheral blood mononuclear cells were isolated from blood of T1D children, who participated in the 12-month prospective intervention trial, and frozen at -150 °C. They were defrosted at our laboratory and stained for surface and, after fixation and permeabilization, for intracellular markers and measured by flow cytometry in order to determine the effects of GFD on chosen immune parameters.

The first population, which was studied, were type 1 regulatory T-cells. They were characterized as $CD4^+$ $CD49b^+$ $LAG3^+$ T-cells as it can be seen in the gating strategy below (Figure 11). An example of dot plots of Tr1 cells (Figure 12A) and IL-10 producing Tr1 cells of STD compared to GFD group is shown. As it can be seen, the majority of Tr1 cells produced IL-10 (Figure 12B). The percentages of Tr1 cells were significantly higher ($P < 0,05$) in PBMCs of T1D children who had been on GFD compared to STD (Figure 12C).

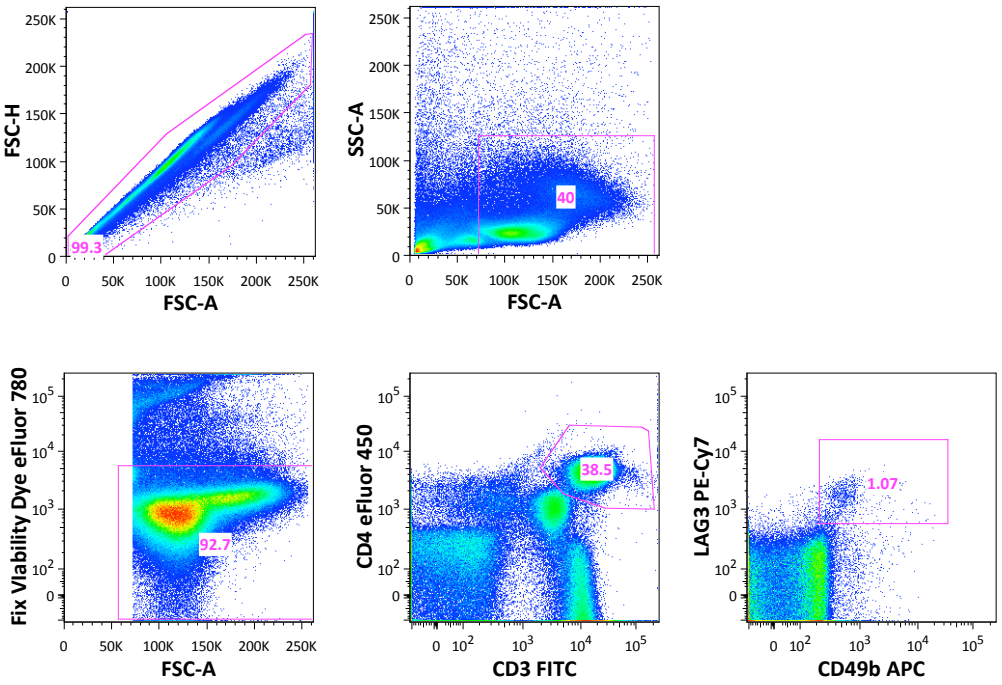


Figure 11: The gating strategy of Tr1 cells ($CD4^+$ $CD49b^+$ $LAG3^+$ T-cells)

The first gate was set only on single cells to exclude doublets, then lymphocytes and monocytes were gated and the third gate was set only on viable cells to exclude dead cells. Next were gated $CD3^+CD4^+$ cells and the last gate was set on $CD49b^+LAG3^+$ cells out of the $CD3^+CD4^+$ gate. The gating was done in FlowJo software.

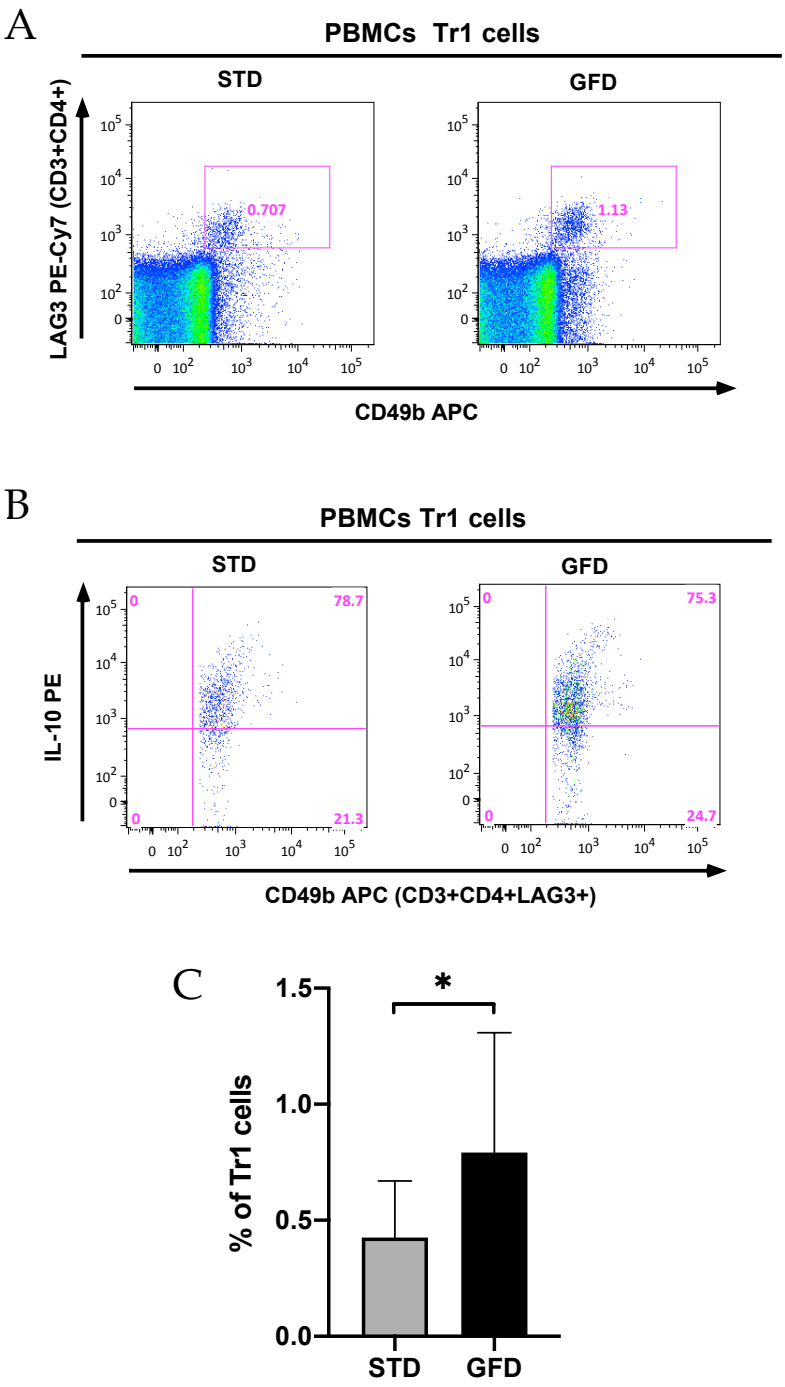
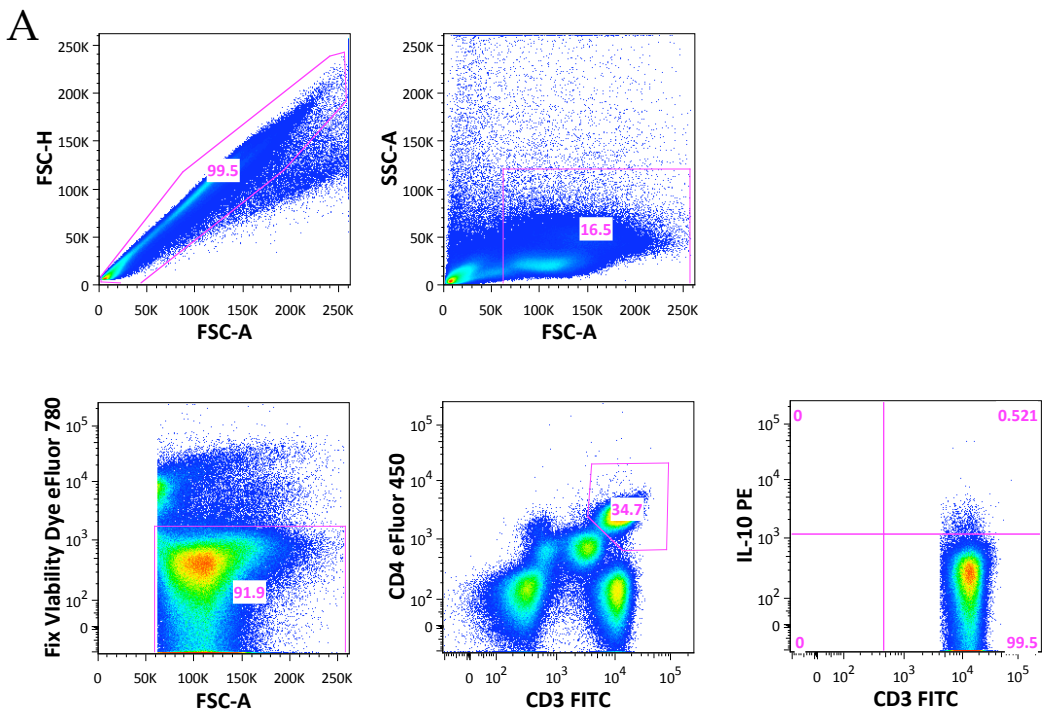


Figure 12: Example dot plots of Tr1 cells and IL-10 producing Tr1 cells on STD vs. GFD and the percentages of Tr1 cells among all T-cells in PBMCs of T1D children

*(A) An example of dot plots of Tr1 cells in STD and GFD groups of T1D children. (B) An example of dot plots of IL-10 producing Tr1 cells in STD and GFD groups of T1D children. Majority of these cells produce IL-10. (C) Tr1 cells ($CD4^+$ $CD49b^+$ $LAG3^+$ T-cells) were significantly increased in PBMCs of T1D children who had previously been on GFD compared to STD. The statistical analysis was done with Unpaired t-test, * $P < 0,05$. Tr1 cells, type 1 regulatory cells; T1D, type 1 diabetes; PBMCs, peripheral blood mononuclear cells; GFD, gluten-free diet; STD, standard diet*

Next, cytokines IL-10, IFN-γ and IL-17 were assessed on CD4⁺ T-cells. Due to the fact that Tr1 cells are very active cells, producing high amounts of cytokines, thus rapidly dying even without polyclonal stimulation, the human samples in this study were not re-stimulated *in vitro* for the assessment of cytokines. The gating strategies of all three examined cytokines can be seen below (Figure 13) and an example of dot plots of IL-10, IFN-γ and IL-17 producing CD4⁺ T-cells in T1D children on STD compared to GFD is enclosed (Figure 14). At last the statistically processed percentages of CD4⁺ T-cells producing IL-10, IFN-γ and IL-17 in PBMCs of T1D patients on two different diets are depicted in the graphs below. Anti-inflammatory IL-10 producing CD4⁺ T-cells were significantly increased in GFD group ($P < 0,05$) (Figure 15A). However, GFD had no significant effect on the percentages of CD4⁺ T-cells producing proinflammatory cytokines IFN-γ (Figure 15B) and IL-17 (Figure 15C). Although a trend towards lower proportion of these cells can be observed.



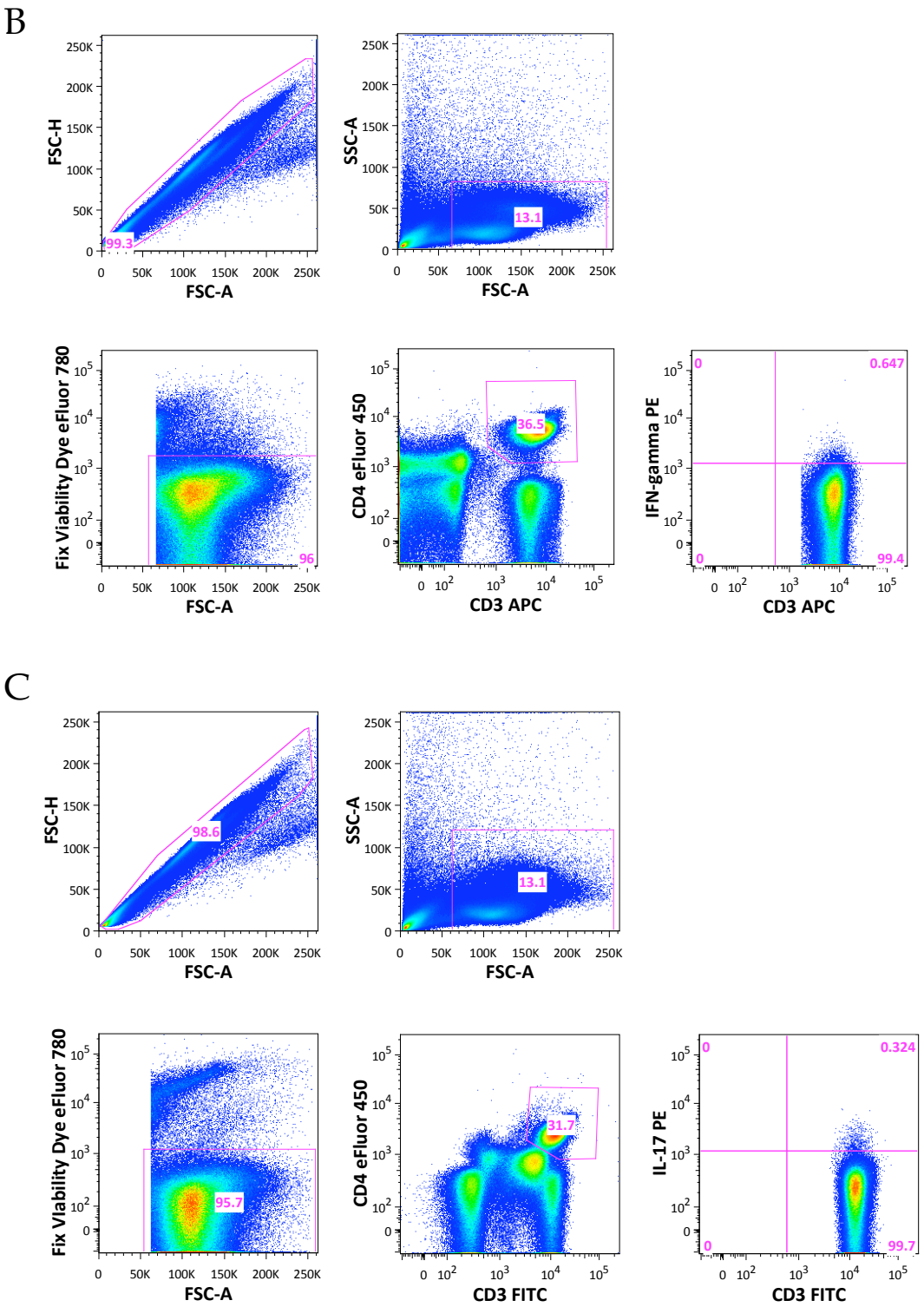


Figure 13: Gating strategies of IL-10, IFN-γ and IL-17 producing CD4⁺ T-cells

Gating strategies of all three cytokines were done similarly at the beginning. The first gate was set to exclude doublets, then lymphocytes and monocytes were gated, next only viable cells were included in the analysis and then the gate was set at CD3⁺CD4⁺ cells. As the last step, cells from the CD3⁺CD4⁺ gate were plotted according to the (A) IL-10, (B) IFN-γ and (C) IL-17 staining. The gating was done in FlowJo software.

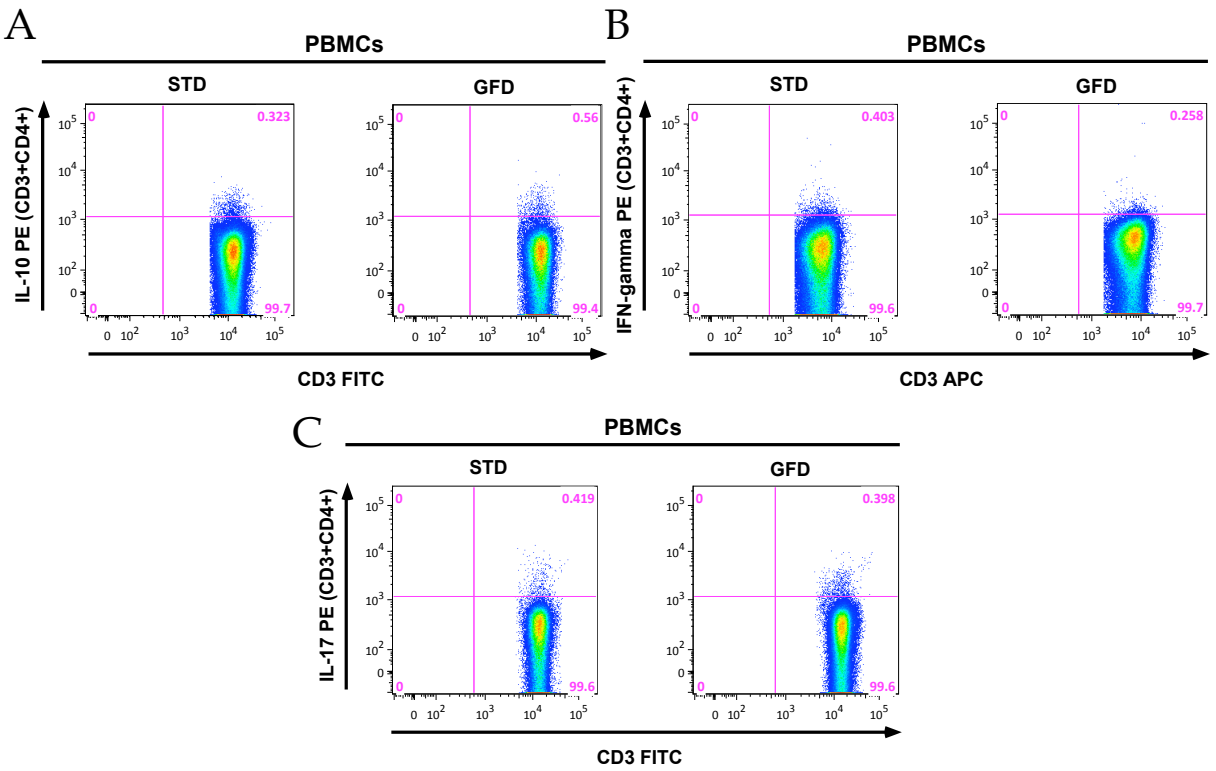


Figure 14: Examples of dot plots of (A) IL-10, (B) IFN-γ and (C) IL-17 producing CD4⁺ T-cells in PBMCs of T1D children on STD compared to GFD

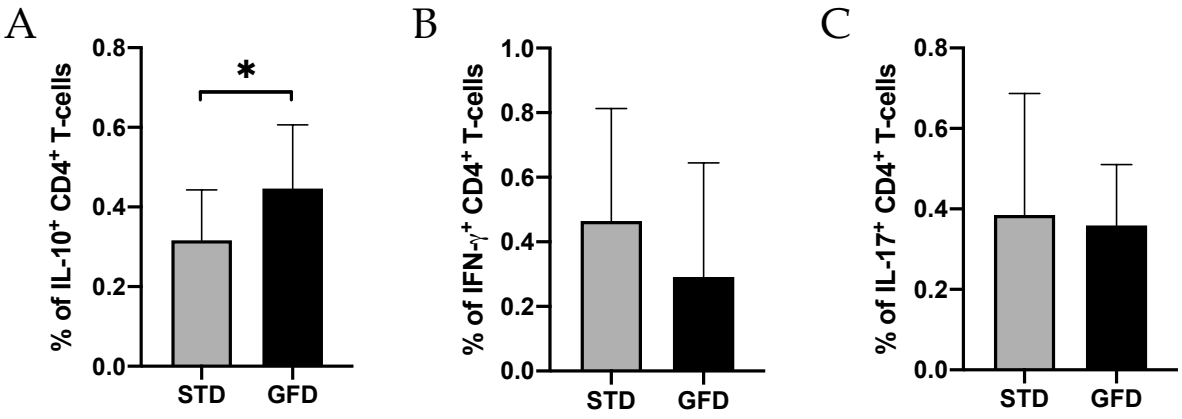


Figure 15: The percentages of CD4⁺ T-cells producing IL-10, IFN-γ and IL-17 cytokines in PBMCs of T1D children

*(A) A significantly higher proportion of CD4⁺ T-cells producing anti-inflammatory IL-10 was observed in GFD group. The percentages of (B) IFN-γ and (C) IL-17 producing CD4⁺ T-cells were not significantly influenced by GFD, though a trend towards a lower proportion of these proinflammatory cytokines (IL-17 and IFN-γ) could be seen. The statistical analysis was done with Unpaired t-test, * P < 0,05. T1D, type 1 diabetes; PBMCs, peripheral blood mononuclear cells; GFD, gluten-free diet; STD, standard diet*

Amongst another populations, which could have been influenced by GFD, belong naïve, central memory and effector memory CD4+ T-cells. Naïve CD4+ T-cells were characterized by surface markers as $CD3^{+}CD4^{+}CD45RA^{+}CD45R0^{-}CD62L^{+}$, central memory CD4+ T-cells were characterized as $CD3^{+}CD4^{+}CD45RA^{-}CD45R0^{+}CD62L^{+}$ and the last population of effector memory CD4+ T-cells as $CD3^{+}CD4^{+}CD45RA^{-}CD45R0^{+}CD62L^{-}$. The gating strategies with FL-1 controls are enclosed (Figure 16) and an example of dot plots of PBMCs from T1D children on STD compared to GFD too (Figure 17). The percentages of naïve, central memory and effector memory CD4+ T-cells were not significantly influenced by gluten-free diet (Figure 18).

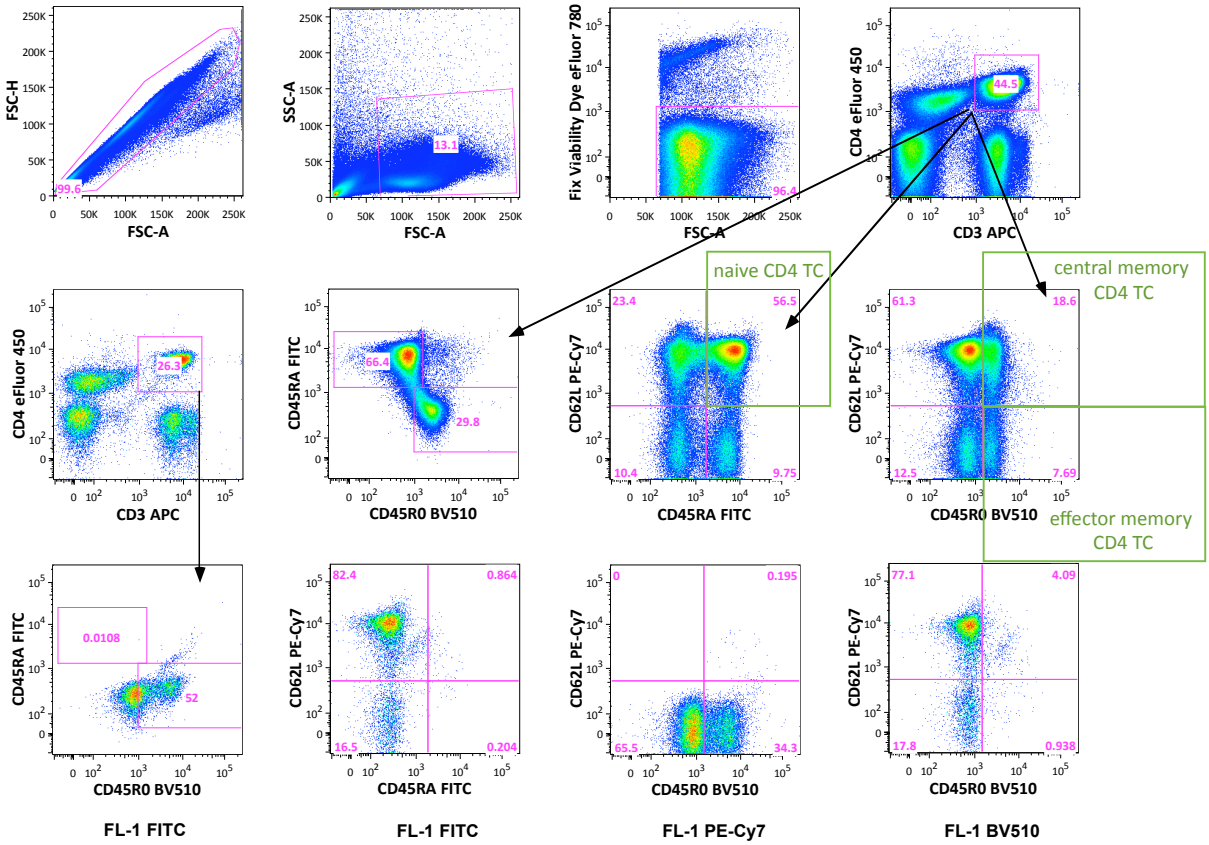


Figure 16: The gating strategy of naïve, central memory and effector memory CD4+ T-cells

The cells were again gated to exclude doublets and then the gate was set at lymphocytes and monocytes, followed by gating of viable cells and then of CD3+CD4+ cells. FL-1 controls were used for further settings of gates. Out of the CD3+CD4+ gate cells were gated for CD45RA and CD45R0 markers. And finally naïve CD4+ T-cells were gated as CD45RA+CD62L+, central memory CD4+ T-cells as CD45R0+CD62L+ and effector memory CD4+ T-cells as CD45R0+CD62L-. The gating was done in FlowJo software.

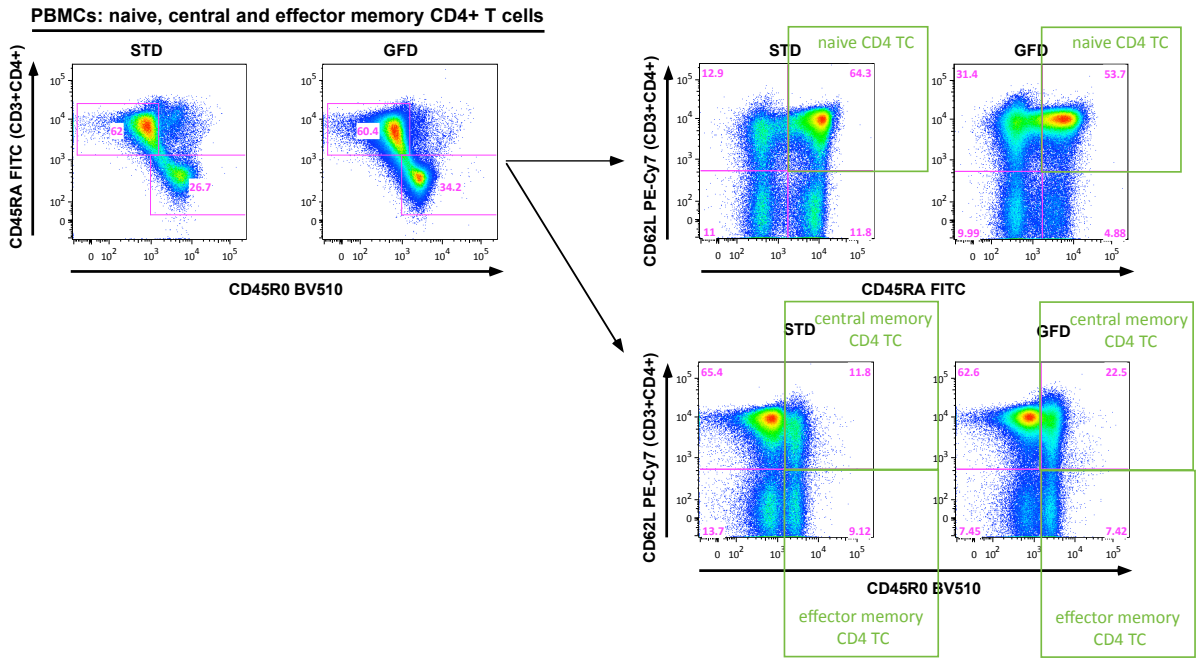


Figure 17: Examples of dot plots of naïve, central memory and effector memory CD4⁺ T-cells in PBMCs of T1D children on STD compared to GFD

On the left is a comparison of CD45RA/CD45R0 dot plots of STD and GFD samples. These gates were then used for further characterization of naïve CD4⁺ T-cells that can be seen on the right. They were plotted for expression of CD62L. Below is a comparison of dot plots of central and effector memory CD4⁺ T-cells that were further characterized by expression of CD62L

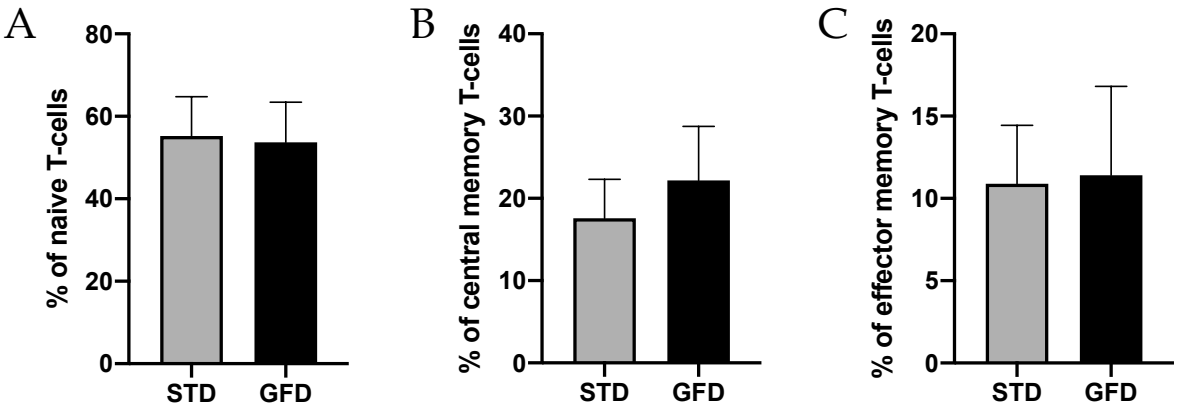


Figure 18: The percentages of naïve, central memory and effector memory CD4⁺ T-cells among all T-cells in PBMCs of T1D children

The percentages of (A) ($CD3^+CD4^+CD45RA^+CD45R0^-CD62L^+$) naïve, (B) ($CD3^+CD4^+CD45RA^-CD45R0^+CD62L^+$) central memory and (C) ($CD3^+CD4^+CD45RA^-CD45R0^+CD62L^-$) effector memory $CD4^+$ T-cells in PBMCs of T1D children were not influenced by GFD. The statistical analyzes were done with Unpaired t-test. T1D, type 1 diabetes; PBMCs, peripheral blood mononuclear cells; GFD, gluten-free diet; STD, standard diet

5.2 Assessing the percentage of regulatory and potentially regulatory T cells in NOD mice colonized with human microbiota from T1D children on gluten-free or standard diet

To see if the beneficial effect of GFD could be transferred from humans by microbiota, NOD mice were colonized at 3 – 4 weeks of age by microbiota from 5 chosen children with T1D after 12 months of standard or gluten-free diet. These 5 colonies with 1 germ-free colony were held in gnotobiotic isolators. Mice were sacrificed at 12 – 13 weeks of age and dissected for spleen, ILN, MLN and PLN. Organs were then processed and stained for flow cytometry and measured for differences in regulatory and potentially regulatory T-cells.

Regulatory population of FoxP3 Tregs was characterized as $CD4^+FoxP3^+CD25^+$ T-cells. Gating (Figure 19A) and illustration of dot plots taken into statistics is shown (Figure 19B, 19C). The colonization of NOD mice by microbiota from T1D children on GFD increased FoxP3 Tregs in systemic lymphoid organs, spleen ($P < 0,05$) and ILN ($P < 0,01$), but had no effect on percentages of these cells in mucosal lymphoid organs (MLN, PLN) (Figure 20).

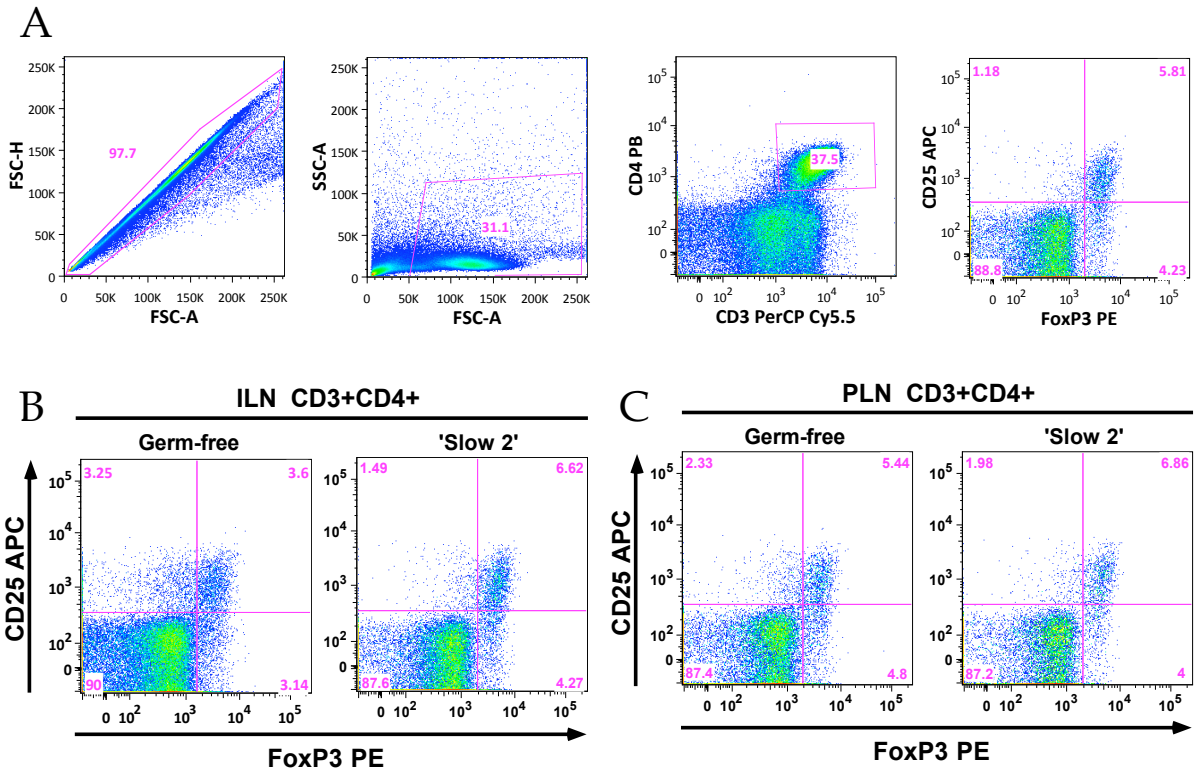


Figure 19: Gating of FoxP3 Tregs and their examples of dot plots from ILN and PLN of NOD mice after colonization with microbiota from T1D children

(A) The gating strategy of FoxP3 Tregs. Gates were set for single cells, lymphocytes and monocytes, $CD3^+CD4^+$ cells and at last for $CD25^+FoxP3^+$ cells. (B) ILN and (C) PLN examples of dot plots from NOD mice colonized with microbiota from T1D children. The gating was done in FlowJo software. 'Slow2' = GFD

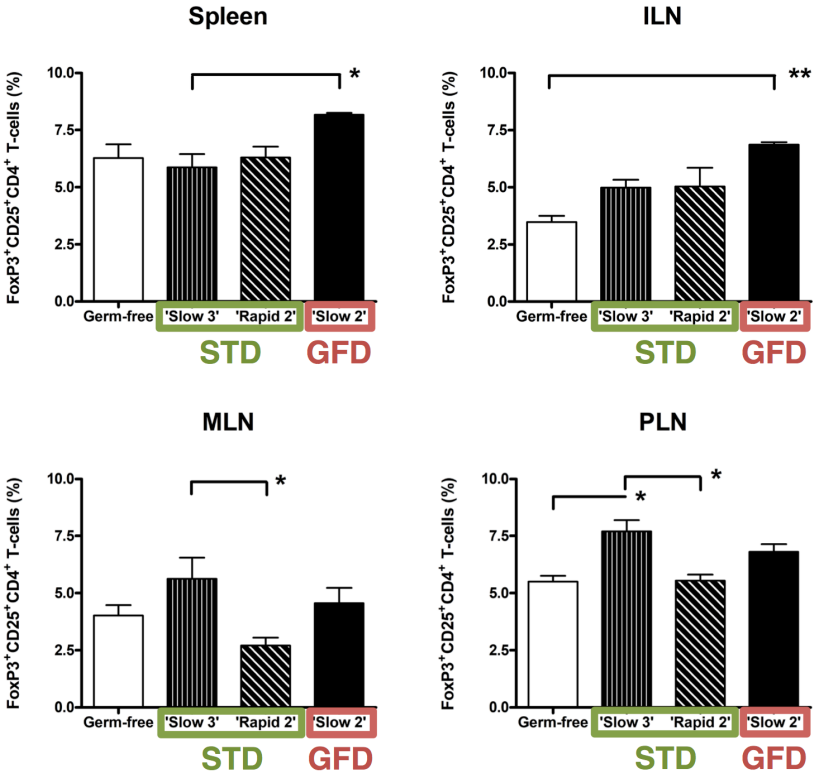


Figure 20: The depiction of FoxP3 Tregs in organs of colonized NOD mice

*Graphs depicting changes of percentages of FoxP3 Tregs in individual organs from colonized NOD mice. Significant increase of FoxP3 Tregs in organs of mice colonized with microbiota from GFD T1D patient is seen in spleen and ILN but not in mucosal lymphoid organs (MLN, PLN). The statistical analysis was done with One-way ANOVA and followed by Tukey's Multiple Comparisons Test, * P < 0,05; ** P < 0,01. T1D, type 1 diabetes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes; PLN, pancreatic lymph nodes; GFD, gluten-free diet; STD, standard diet*

Another population of T-cells that was studied were γδ T-cells because it was shown they could have regulatory properties towards development of T1D in NOD mice. The gating strategy and an example of dot plots from ILN and PLN used for further processing in statistical analysis is shown (Figure 21). There is a depiction of percentual changes of γδ T-cells in individual organs of NOD mice colonized with microbiota from T1D children on STD or GFD. Colonization with microbiota from GFD T1D patient increased the percentage of γδ T-cells in spleen ($P < 0,05$), MLN ($P < 0,05$) and especially in PLN ($P < 0,01$) but had no effect on γδ T-cells in ILN (Figure 22).

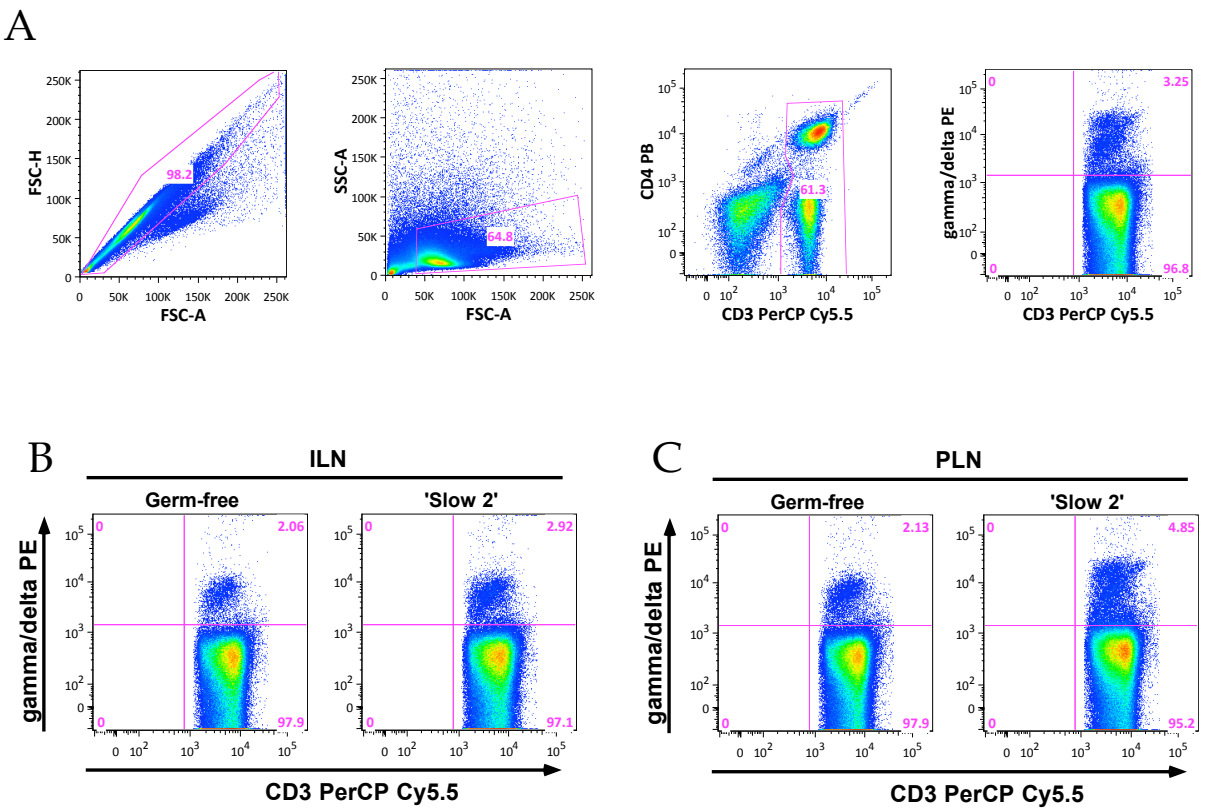


Figure 21: The gating strategy and examples of dot plots of γδ T-cells in NOD mice colonized with microbiota from T1D children

(A) The gating strategy of γδ T-cells. Gates were set for single cells, lymphocytes and monocytes, CD3⁺ and at last for γδ T-cells. (B) ILN and (C) PLN examples of dot plots from NOD mice colonized with microbiota from T1D children. The gating was done in FlowJo software. 'Slow2' = GFD

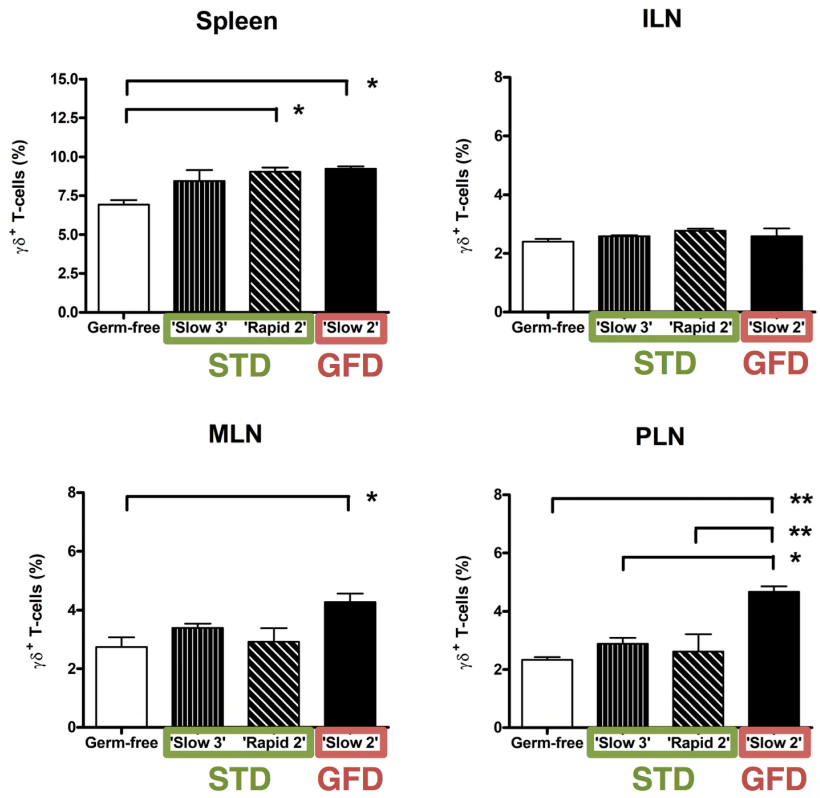


Figure 22: The depiction of γδ T-cells in organs of colonized NOD mice

*The influence of colonization with microbiota from T1D children on percentages of γδ T-cells in individual organs of NOD mice is depicted in these graphs. The colonization with microbiota from GFD T1D patient significantly increased the percentage of γδ T-cells in spleen, MLN and especially in PLN but had no effect on percentage of γδ T-cells in ILN. The statistical analysis was done with One-way ANOVA and followed by Tukey's Multiple Comparisons Test, * P < 0,05; ** P < 0,01. T1D, type 1 diabetes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes; PLN, pancreatic lymph nodes; GFD, gluten-free diet; STD, standard diet*

Another cell population with potentially regulatory properties in the context of T1D are CD4+ CD45RB^{low} T-cells. There is a depiction of these cells after colonization with microbiota from T1D children. Again, the gating strategy (Figure 23A) and an example of dot plots from ILN and PLN, which were used for further statistics, are shown (Figure 23B, 23C). The colonization from GFD T1D patient but not from patients on STD raised the percentage of CD4+ CD45RB^{low} T-cells only in PLN ($P < 0,05$) but not in other studied organs (Figure 24).

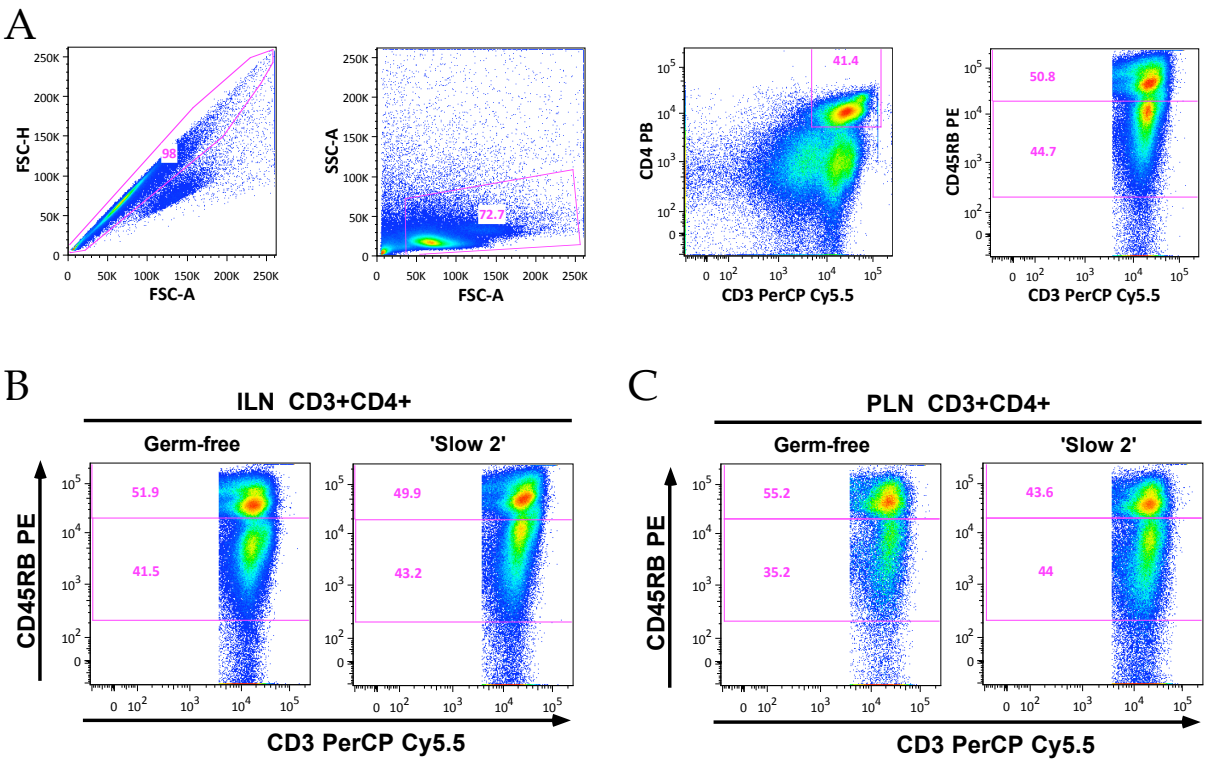


Figure 23: The gating strategy and examples of dot plots of CD45RB^{low} T-cells in NOD mice colonized with microbiota from T1D children

(A) The gating strategy of CD45RB^{low} T-cells. Gates were set for single cells, lymphocytes and monocytes, CD3+CD4+ and at last for CD45RB^{low} T-cells. (B) ILN and (C) PLN examples of dot plots from NOD mice colonized with microbiota from T1D children. The gating was done in FlowJo software. 'Slow2' = GFD

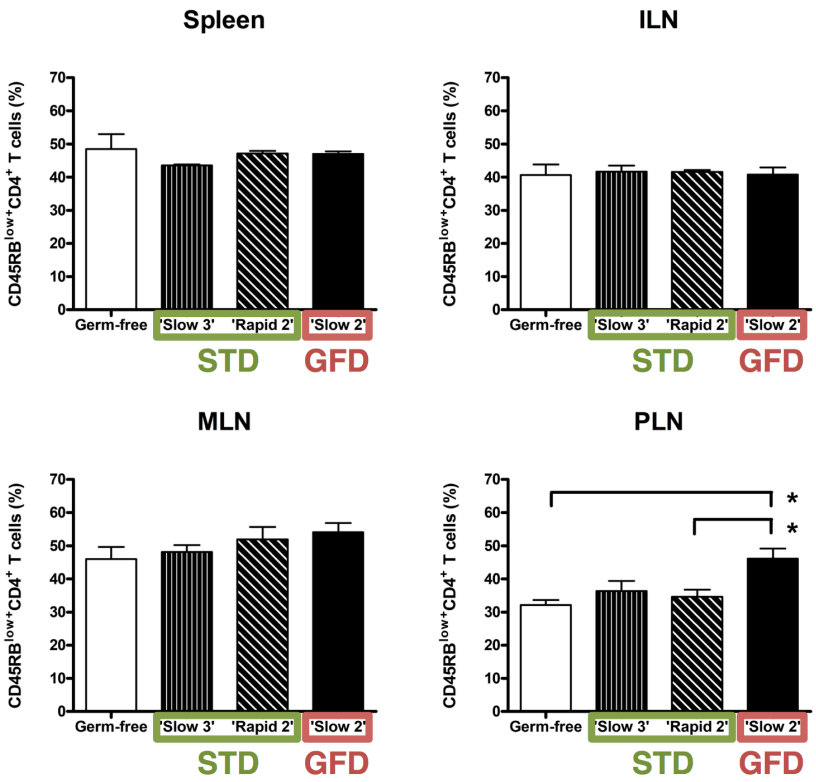


Figure 24: The depiction of CD4⁺CD45RB^{low} T-cells in organs of colonized mice

*The percentage of CD4⁺CD45RB^{low} T-cells was influenced by the colonization from T1D patients only in PLN but not in other studied organs. The percentage was significantly increased after colonization from GFD T1D patient but not from T1D patients on STD. The statistical analysis was done with One-way ANOVA and followed by Tukey's Multiple Comparisons Test, * P < 0,05. T1D, type 1 diabetes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes; PLN, pancreatic lymph nodes; GFD, gluten-free diet; STD, standard diet*

The last studied population of T-cells in colonized NOD mice were CD4⁺CD62L⁺ T-cells. Gating (Figure 25A) and an example of dot plots from PLN are enclosed (Figure 25B). The colonization with microbiota from GFD or STD T1D patients had no effect at all on this cellular population in any studied organ (Figure 26).

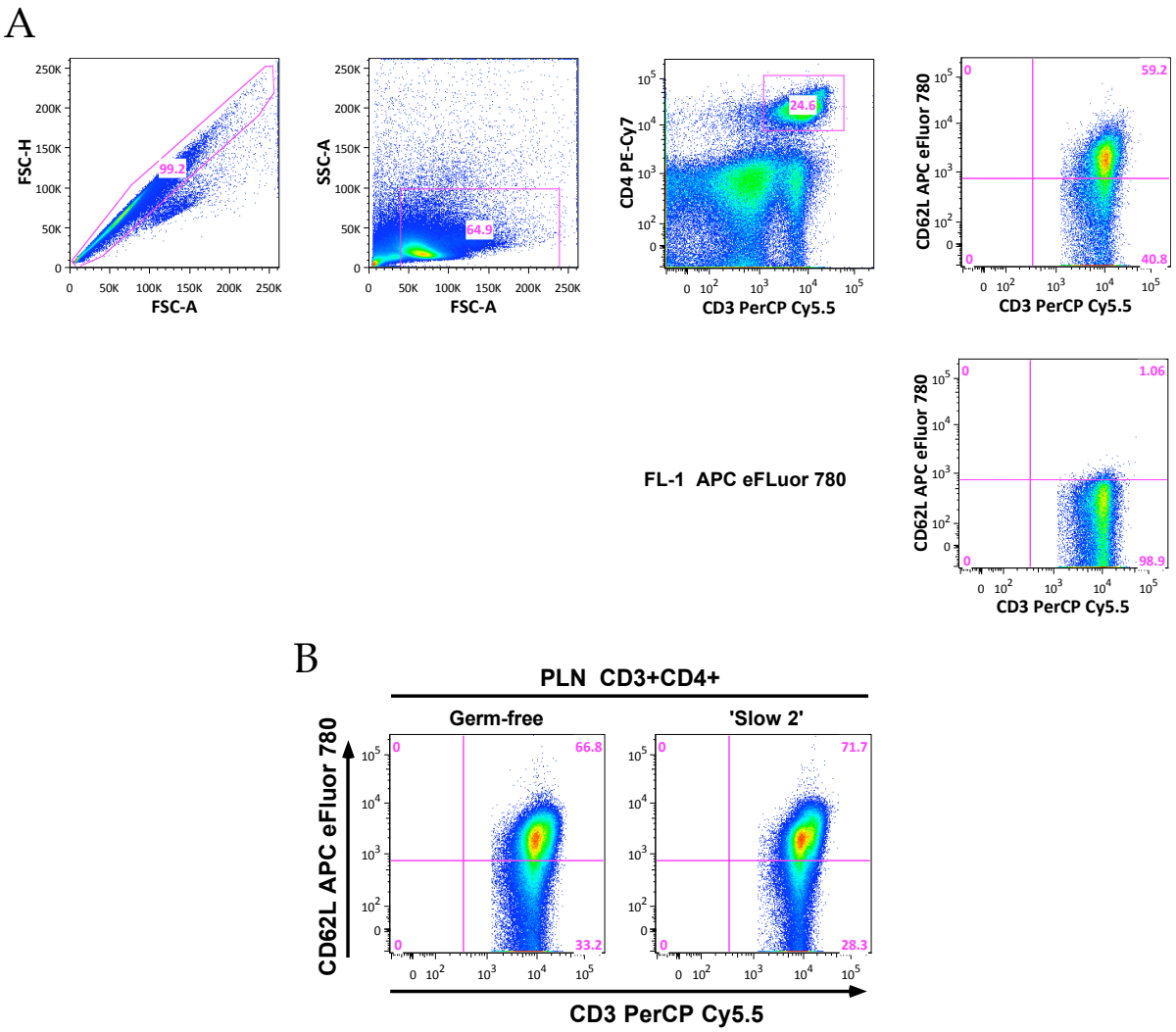


Figure 25: The gating strategy and example of dot plots of CD62L⁺ T-cells in NOD mice colonized with microbiota from T1D children

(A) Gating strategy of CD62L⁺ T-cells. Gates were set for single cells, lymphocytes and monocytes, CD3⁺CD4⁺ and at last for CD62L⁺ T-cells. FL-1 control for CD62L is shown below the gating strategy. (B) Example of dot plots from PLN of NOD mice colonized with microbiota from T1D children. The gating was done in FlowJo software. 'Slow2' = GFD

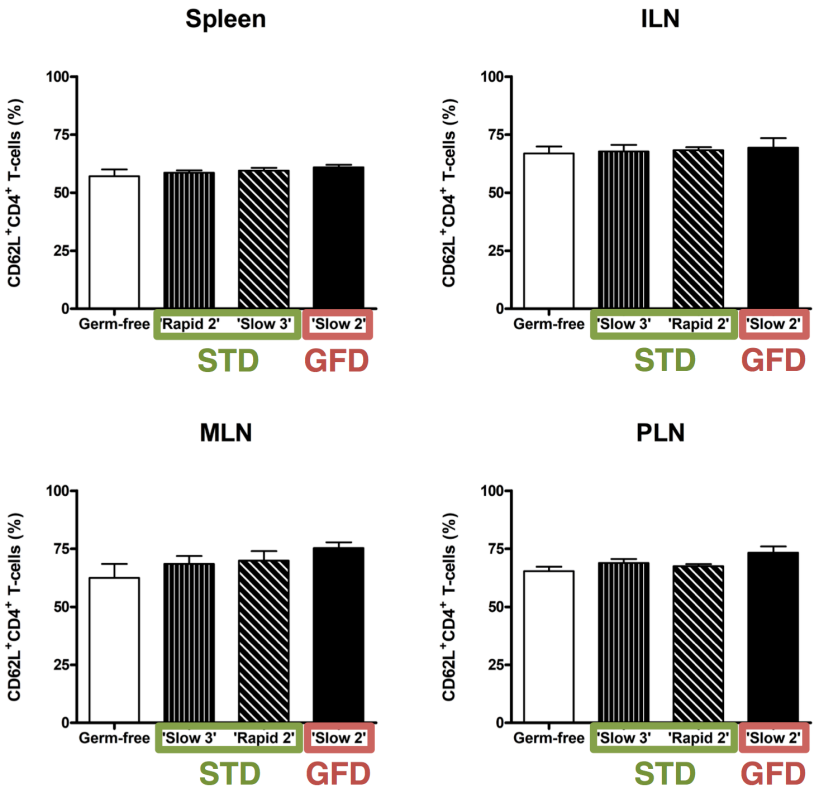


Figure 26: The depiction of CD4⁺CD62L⁺ T-cells in organs of colonized mice

Colonization with microbiota from T1D children on GFD or STD did not change percentages of CD4⁺CD62L⁺ T-cells in any studied organ (spleen, ILN, MLN, PLN). The statistical analysis was done with One-way ANOVA and followed by Tukey's Multiple Comparisons Test. T1D, type 1 diabetes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes; PLN, pancreatic lymph nodes; GFD, gluten-free diet; STD, standard diet

5.3 Measuring the proportion of IL-10 producing CD4+ T helper cells from colonized NOD mice

A part of samples of mice organs was polyclonally stimulated prior to fluorescence staining. The stimulation was done for 3 hours with PMA and ionomycin in the presence of Protein Transport Inhibitor containing monensin to stop the transport from the Golgi apparatus. After the stimulation period samples were stained for flow cytometry and measured for differences in percentages of IL-10 producing CD4+ T-cells.

An example of dot plots from spleens of colonized NOD mice is shown (Figure 27). The colonization with microbiota from GFD T1D patient raised the percentage of IL-10 producing CD4+ T-cells significantly only in systemic lymph nodes, namely spleen ($P < 0,05$) and ILN ($P < 0,05$), whereas it had no effect on the production in mucosal lymph nodes (MLN and PLN). Colonization from STD T1D patients raised the production only in spleen ($P < 0,001$). No colonization influenced the production in MLN and PLN (Figure 28).

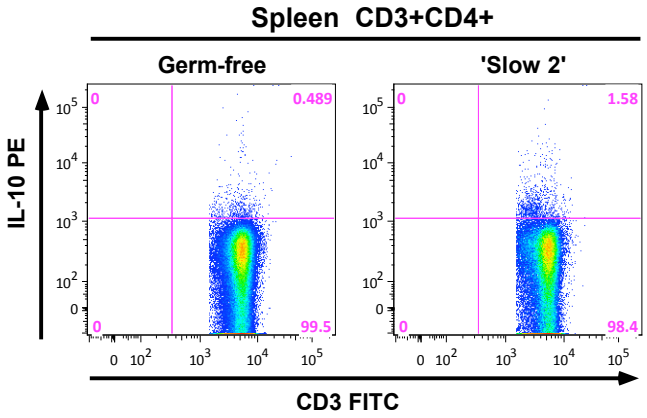


Figure 27: An example of dot plots of IL-10 producing CD4+ T-cells in spleens of colonized NOD mice

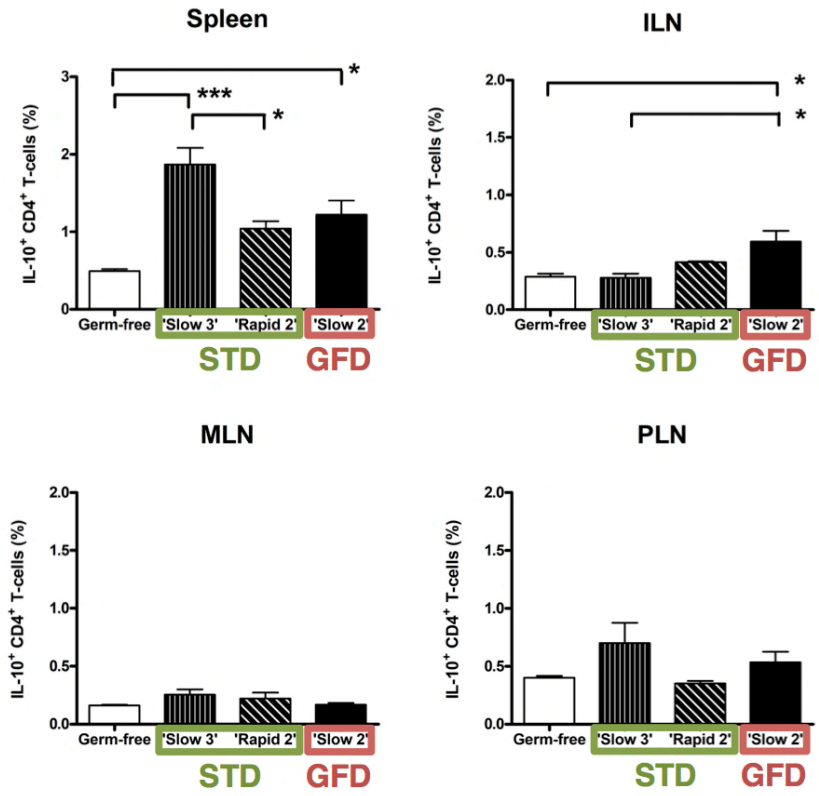


Figure 28: The depiction of IL-10 producing CD4⁺ T-cells in organs of colonized NOD mice

*The colonization with microbiota from GFD T1D patient increased IL-10 producing CD4⁺ T-cells in spleen and ILN and colonization from STD patients only in spleen. No colonization had effect on the production in MLN and PLN. The statistical analysis was done with One-way ANOVA and followed by Tukey's Multiple Comparisons Test, * P < 0,05; ** P < 0,01; *** P < 0,001. T1D, type 1 diabetes; ILN, inguinal lymph nodes; MLN, mesenteric lymph nodes; PLN, pancreatic lymph nodes; GFD, gluten-free diet; STD, standard diet*

6 Discussion

Gluten-free diet is one of the strongest known environmental interventions in decreasing diabetes incidence in the spontaneous NOD mouse model [8]–[10]. Several mechanism may be involved, even simultaneously – it could be due to a direct effect and/or metabolite driven effect on gut mucosal immunity [17], [60], the diet may influence the immune system through changes in composition of gut microbiota [25], [60], or altered permeability of the intestinal barrier [15]. Ongoing gnotobiotic experiments indicate that at least part of the diabetes preventive effect of the Altromin GFD is microflora-independent.

Though the mechanism is not fully understood, gluten-free diet repeatedly showed to have a very substantial protective effect against development of type 1 diabetes in animal models. This applies not only to the non-purified, open formula Altromin GFD used in this study but also to the hypoallergenic, hydrolyzed casein-based diets, that are by their nature also wheat flour/gluten-free and were previously tested in NOD mice and BB rats [5]–[7]. Parallel studies in humans are difficult to design since the diets have to be introduced early in the life, preferentially before birth. However, human case reports [163] and trials, which have been carried out from T1D onset [26], [27], point out on significantly better control of the disease and prolonged partial remission. There is only a limited number of studies addressing the effect of GFD on some immune parameters and cytokine signatures in BALB/c mice [18], [142] and NOD mice [152], [164]. To my knowledge, no study has examined the influence of GFD on human immune parameters in the context of T1D so far. Thus, this is the first aim of this diploma thesis, whereas the next aims focused on changes of immune parameters in NOD mice after colonization with microbiota from T1D children, who had been on standard or gluten-free diet.

6.1 Assessing the percentage of regulatory T cells and cytokines in PBMCs of T1D children

It is widely accepted that pathogenesis of type 1 diabetes is mainly driven by Th1 and Th17 polarization of immune responses [165] and that the regulatory part of immunity is suppressed either due to reduced frequency or function of regulatory cells [19]. In animal models gluten-free diets decreases the incidence of T1D in NOD mouse [8]–[10] and modulates the immune response towards a less inflammatory one [18], [142]. T1D children participating in this study had better metabolic control of T1D after GFD [27], thus the first aim of this thesis was to examine if GFD had an effect on chosen immune parameters.

Firstly, we looked at Tr1 cells ($CD3^+CD4^+CD49b^+LAG3^+$) which were only recently better defined but are a well-established population of regulatory cells that is reduced in human T1D and the reduction is seen also in islet autoantigen specific ones [20]. Tr1 cells or only intestinal Tr1 cells inhibit T1D development in NOD mice after adoptive co-transfer [96]. Thus, this thesis examined if percentage of Tr1 cells could be increased by GFD intervention trial. In line with the previously published data on Tr1 in T1D, adherence to gluten-free diet significantly increased the percentage of Tr1 cells in PBMCs of T1D children (Figure 12C). In addition, majority of these cells produced IL-10 (Figure 15A), which is in concordance with published findings [166]. To our knowledge, no studies determined an influence of GFD on Tr1 cells in the context of T1D so far. Nevertheless, NOD model has shown that these cells could be induced in small intestine by different gut microbiota and they do express chemokine receptors, which enable them to migrate to mucosal lymph nodes and also pancreas. This was confirmed in further experiments carried out by Yuu et al. [96]. Moreover, autoantigen specific Tr1 T-cells were shown to migrate specifically into PLN [78], where they can suppress diabetogenic T-cells [97], most likely by high levels of IL-10 secretion, which they produce after stimulation with islet autoantigen in *vitro* [79]. The suppression of diabetogenic T-cells could be done through receptor for IL-10 that is expressed on Th1 [96] and Th17 cells [167]. Therefore, Tr1 cells represent important peripheral Tregs that could be induced in the gut and contribute to protection against T1D by migrating to PLN and pancreas and suppressing diabetogenic Th1 and Th17 cells by production of IL-10 *in situ*.

Findings are advocating for the importance of IL-10 cytokine in T1D. There is a positive correlation between higher numbers of these cells at the time of T1D diagnosis and improved glucose control (HbA1c) 3 months later [168]. Besides, as it was already mentioned, IL-10 suppress diabetogenic IFN-γ producing $CD4^+$ T-cells [96] and also IL-17 producing ones because they do express receptor for IL-10 and its ligation leads to inhibition of proliferation of these cells [167]. Furthermore, aside from reducing the incidence of T1D, adoptive co-transfer of Tr1 cells, which are substantial producers of IL-10 [166], decreases number of INF-γ producing cells in pancreas [96].

In order to assess first natural occurrence of cytokine producing cells and also due to the fact that Tr1 cells detected from the same sample are rapidly dying, perhaps due to their high metabolic activity, these experiments were carried out on unstimulated cells. We have found that GFD significantly increased percentage of IL-10 producing $CD4^+$ T-cells in PBMCs of T1D children (Figure 15A). However, in BALB/c mice GFD only unsignificantly increases

IL-10 producing cells in spleen, which is a site that may best reflect possible effects in blood [142]. Nevertheless, GFD does influence the composition of $CD4^+$ T-cells to a lower portion of IL-17 and IFN-γ producing ones in spleen, ILN, MLN, PP and PLN in this animal model [142]. A similar study in NOD mice, shows that production of IFN-γ by $CD4^+$ T-cells was reduced but production of IL-17 and IL-10 were not significantly influenced in the spleen [144]. This thesis showed that the percentages of IFN-γ and IL-17 producing $CD4^+$ T-cells were not significantly changed by the GFD intervention, though they showed tendency towards lower portions in PBMCs of T1D children who had been on GFD (Figure 15B, 15C).

Thus, it seems that GFD alters ratio of regulatory and inflammatory parts of the immune response but unlike in mouse models it is done more by increasing the regulatory arms than by decreasing the inflammatory ones.

Another cell populations, which had been studied, were naïve and memory $CD4^+$ T-cells. The percentages of these populations are altered in T1D. Namely, that is fewer naïve $CD4^+$ T-cells and more central and effector memory $CD4^+$ T-cells are present [118]. An intervention trial with a treatment drug that results in better T1D control returns proportions of naïve and memory $CD4^+$ T-cells back, that is, it increases portion of naïve $CD4^+$ T-cells and conversely decreases them in central memory and effector memory $CD4^+$ T-cells [127]. Therefore, this thesis investigated if intervention with gluten-free diet would have the same effect. However, there were no differences in percentages of naïve, central memory and effector memory $CD4^+$ T-cells between T1D children on STD and GFD (Figure 18). This could have been due to usage of one different marker because this experimental design used CD62L compared to CCR7, which is usually used according to literature, nonetheless, these two markers are reported to be quite interchangeable [116]. At present gluten-free diet had no effect on portions of these cell subsets.

To conclude the human part of this thesis, gluten-free diet has a beneficial effect on the course of T1D when administered after diagnosis and this phenomenon can be due to its influence on higher percentage of Tr1 cells and overall IL-10 producing $CD4^+$ T-cells (as documented in PBMCs). Indeed it would be more desirable to explore these changes in pancreas and PLNs but that is left to be addressed by animal studies. These results also do not exclude that other cellular populations can contribute to the milder course of T1D progression by GFD.

6.2 Assessing the percentage of regulatory and potentially regulatory T cells in NOD mice colonized with human microbiota from T1D children on gluten-free or standard diet

GFD was shown to have protective effect against T1D in NOD mice as well as to have some beneficial properties when administered to T1D children. The data from the Laboratory of Gnotobiology at the Institute of Microbiology in Nový Hrádek not surprisingly show that T1D incidence (Figure 9) and insulitis (Figure 10) of colonized NOD mice are statistically significantly reduced after the microbiota transfer. Interestingly, microbiota from children on GFD have a tendency towards more protective effect (not statistically significant due to compensations for multiple comparisons). This thesis examined if the effect of GFD on immune parameters in T1D children could be conveyed by transfer of their gut microbiota into NOD mice.

First population that was examined after the transfer of microbiota were FoxP3 Tregs. These regulatory cells are decreased in NOD mice before the onset of T1D [73] and their experimental depletion leads to faster manifestation of T1D [74]. Conversely, numbers of FoxP3 Tregs are enhanced in models with induced protection against T1D and they can be found in spleen, PLN and pancreas [77]. These cells can be generated also in the intestine and then migrate to PLN and pancreas, where they contribute to maintaining immune regulation [75]. Therefore, this thesis examined if colonization of NOD mice with gut microbiota from T1D children increased percentages of FoxP3 Tregs in studied organs and if this increase would have been higher in mice colonized with GFD T1D microbiota. The increase of percentage of FoxP3 Tregs after colonization with GFD microbiota had been seen in spleen and ILN but not in MLN and PLN, which are associated with mucosal tissues (Figure 20). This finding was opposite to our expectation because GFD was previously reported to increase these cells in MLN [139] and PLN [25]. On the other hand, there are also data reporting no changes induced by GFD in proportion of FoxP3 Tregs [18].

Next we investigated some populations that had been reported to have potentially regulatory properties in the context of T1D. One such population of cells are γδ T-cells. Diabetic NOD mice have lower percentage of these cells then non-diabetic ones (Han et al. 2010) and adoptive co-transfer of γδ T-cells protects against development of T1D [98]. Furthermore, NOD mice are deficient in intraepithelial lymphocytes, which are mainly γδ T-cells, and transfer of these cells also have protective effect on development of T1D [99]. This thesis showed that colonization of NOD mice with GFD T1D microbiota raised

percentage of these cells in spleen, MLN and especially in PLN (Figure 22), which is an important site for induction of both effector [66] but conversely also of the regulatory T-cell response against β-cells [64]. This finding was in concordance with preceding studies, which reported that GFD also increases percentages of γδ T-cells in spleen, ILN, MLN, PP and PLN [18] and that intranasal administration of gliadin, which protects against T1D, also increases these cells in MLN and PLN [101].

$CD4^+$ $CD45RB^{low}$ T-cells are another population that could have been influenced by the transfer of microbiota into NOD mice because one study shows that BALB/c mice after GFD had higher percentage of these cells in all studied organs but the difference was significant only in PP and spleen [18]. In accord with this data, the colonization of NOD mice with microbiota from GFD T1D children raised the percentage of $CD4^+$ $CD45RB^{low}$ T-cells but only in PLN and not in any other studied organ (Figure 24). These cells demonstrate ability to inhibit proliferation of effector T-cells [104] but more importantly they protect against development of T1D in adoptive co-transfer [105]. And they are more represented in animal models of induced protection against T1D [106], [107].

The last examined cellular population that seems to have some regulatory potential in the context of T1D are $CD4^+$ $CD62L^+$ T-cells. This population has protective effect in adoptive co-transfer but only when taken from pre-diabetic NOD mice [111]. In models with induced protection against T1D, $CD62L^+$ T-cells are represented in higher numbers and the increase is seen in spleen and also PLN [23]. They migrate to MLN and PLN after the adoptive co-transfer [113] and also infiltrate pancreas, where they protect islets from intra-islet infiltration by diabetogenic T-cells [112]. In our experiments colonization with gut microbiota from T1D children did not alter the percentage of these cells in any studied organ (Figure 26). This is perhaps not surprising, as it was reported that, although, GFD increases percentage of these cells, it is increased significantly only in PP of BALB/c mice [18].

6.3 Measuring the proportion of IL-10 producing $CD4^+$ T helper cells from colonized NOD mice

As the last aim presented in this thesis, we determined percentages of IL-10 producing $CD4^+$ T-cells. As it was mentioned earlier IL-10 is a regulatory cytokine with a protective function also in T1D, most likely by mechanism of suppression of Th1 and Th17 cells at the site of autoimmune reaction. These results document percentage of IL-10 producing $CD4^+$ T-cells in NOD mice colonized with microbiota from T1D children on gluten-free and standard diet.

The percentages increased after the colonization with microbiota from GFD T1D patient only in systemic lymphoid organs such as spleen and ILN but the colonization had no effect on percentages of IL-10 producing $CD4^+$ T-cells in MLN and PLN, which are associated with mucosal tissues (Figure 28). This observation is in accord with animal GFD studies, in which GFD had little effect on IL-10 production or proportions of IL-10 positive $CD4^+$ T-cells [142], [143], [145]. The anti-inflammatory properties of GFD regarding its influence on production of cytokines in mice lie more in reducing the proinflammatory part of the immune response and thus shifting the balance towards a less inflammatory milieu than particularly increasing the anti-inflammatory properties [142].

7 Conclusions

Unlike many other immune-intervention approaches in the current T1D research (mucosal administration of autoantigens, long term regulatory but in the end immunosuppressive interventions e.g. based on anti-CD3 mAbs, or autoantigen specific cell therapies such as Tregs and tolerogenic dendritic cells), GFD is completely safe, inexpensive and without a danger of promoting autoimmunity. After decades of negative outcomes of T1D human trials, GFD diet even led to positive effects in children with recent onset of T1D.

This thesis focused on determination of immune changes related to gluten-free diet, which was proven to have protective effect in NOD mice but also some beneficial properties in newly diagnosed children with T1D. Specifically, the main aim was to assess regulatory populations of T-cells and production of IL-10 in peripheral blood mononuclear cells of type 1 diabetic children after GFD. The other aims of this thesis were to determine if colonization of gnotobiotic germ-free NOD mice with gut microbiota from T1D children, who had been on standard or gluten-free diet for 12 months, was sufficient for transfer of the beneficial aspects also at the level of immune characteristics.

We have found out that gluten-free diet significantly increased percentage of regulatory Tr1 cells and overall percentage of IL-10 producing $CD4^+$ T-cells in PBMCs of T1D children. This could at least partially explain the beneficial effect of GFD on metabolic control of T1D after its onset in these children.

Furthermore, the colonization of NOD mice with microbiota from T1D children showed that the profitable influence of GFD on immune characteristics can be transferred by microbiota that had been shaped by GFD in humans. Indeed, mice colonized with GFD T1D microbiota had higher percentages of regulatory and some uncommon regulatory T-cells, which had been proven to have some regulatory function in the context of T1D by previous studies. Thus, it seems that the beneficial influence of GFD on T1D may be at least partially propagated via its influence on microbiota composition.

This thesis brings some novel information about influence of gluten-free diet on immune regulatory cells in children with type 1 diabetes and suggests that it could be the reason behind their better metabolic control of T1D. Moreover, it claims that the beneficial effect of GFD on immune regulation can be transferred by human gut microbiota to NOD mice, thus contribute a small piece of information to the growing and increasingly popular puzzle of how diet components and composition of gut microbiota are important in health and disease.

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