Declaration

Prohlašuji, že jsem závěrečnou práci zpracoval/a samostatně a že jsem uvedl/a všechny použité informační zdroje a literaturu. Tato práce ani její podstatná část nebyla před-ložena k získání jiného nebo stejného akademického titulu.

V Praze, 03.08.2024 Anna Zelenska

I hereby declare that my thesis represents my own original research work. Wherever the contribution of others is involved, every effort is made to indicate this clearly including reference to the literature. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma.

Prague, 03.08.2024 Anna Zelenska

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"Success is not final, failure is not fatal:
it is the courage to continue that counts."

— *Winston S. Churchill*

Abstract

This thesis explores the effects of low-carbohydrate (LC) diet on development of T1D, insulitis and alterations of immune parameters using spontaneous diabetes model, specific pathogen-free (SPF) non-obese diabetic (NOD) mice, when introduced from the age of 4 weeks or from *in utero*. The study includes glycaemia screening for evaluation of T1D incidence in NOD mice, insulitis scoring, cell staining and flow cytometry analysis focused mainly on FoxP3+Tregs, CD4+IL-10+, Tr1, potentially immunoregulatory γδ T cells, and CD8+ memory/effector T cells in mucosal and non-mucosal lymphoid organs.

We did not observe any significant effect of LC diet on diabetes incidence when it was introduced to NOD females from the age of 4 weeks. In line with these data, we found no substantial differences in cell subsets percentages in mucosal (mLNs, pLNs, PPs) and non-mucosal (spleen, iLNs) lymphoid organs in 12-week-old NOD mice fed with LC diet from the age of 4 weeks. This finding was reflected by no substantial differences in insulitis scoring in pancreata of NOD mice fed LC and standard diet from 4 weeks of age.

When we investigated the effects of LC diet introduced to NOD females from *in utero*, we obtained an unexpected and interesting finding of statistically significantly accelerated diabetes incidence in NOD mice fed with LC diet versus standard diet, which was confirmed by three independent experiments. This was reflected by changes in immunoregulatory T cell subsets in LC diet group, with a statistically significantly decreased CD4+IL-10+ T cells (spleen, mLNs), Foxp3+ Tregs (spleen, iLNs), Tr1 cells in the spleen; the percentage of γδ T cells was also decreased in the spleen. CD8+ central memory T cells were generally increased on the LC diet introduced from in utero (spleen, pLNs and iLNs). Reciprocal decrease was found in CD8+ effector T cells, possibly reflecting an accelerated migration of effector T cells to the pancreas in mice fed with LC diet from in utero compared with mice kept on a standard diet. This was consistent with the insulitis scoring in panreata of NOD mice fed with LC and standard diet from *in utero*.

Thus, this study brings new findings about the influence of LC diet on immunoregulatory and effector T cell subsets and acceleration of T1D when it is introduced to NOD mice from in utero. A very rare acceleration of the development of T1D in NOD mice by an environmental factor – the LC diet, may shed more light on the pathogenesis of the disease. Further multidisciplinary experiments are needed to elucidate the complex effects of LC diet on developing immune system in NOD mice.

Key words: T1D, low-carbohydrate diet, NOD mice, Tregs, Tr1 cells, γδ T cells, effector T cells; memory T cells.

Abstrakt

Tato práce se zabývá vlivem nízkosacharidové (LC) diety na rozvoj diabetu 1. typu (T1D), inzulitidy a změny imunitních parametrů s využitím modelu spontánního diabetu, neobézních diabetických myší (NOD) bez specifických patogenů (SPF). Dieta byla podávána buď od věku 4 týdnů, nebo ještě in utero. Studie zahrnuje screening glykémie pro hodnocení incidence T1D u NOD myší, hodnocení insulitidy, barvení buněk a analýzu průtokovou cytometrií, zaměřenou zejména na FoxP3+Tregs, CD4+IL-10+, Tr1, potenciálně imunoregulační γδ T buňky a CD8+ paměťové/efektorové T buňky ve slizničních a neslizničních lymfoidních orgánech.

Nepozorovali jsme žádný významný vliv LC diety na incidenci diabetu, pokud byla podávána NOD samicím od věku 4 týdnů. V souladu s těmito údaji jsme také nezjistili žádné podstatné rozdíly v procentuálním zastoupení buněčných subpopulací ve slizničních (mLN, pLN, PP) a neslizničních (slezina, iLN) lymfoidních orgánech u 12týdenních NOD myší krmených LC dietou od věku 4 týdnů. Tento nález byl potvrzen také absencí podstatných rozdílů rozvoje insulitidy v pankreatech NOD myší krmených LC a standardní dietou od věku 4 týdnů.

Při zkoumání účinků LC diety podávané NOD samicím prenatálně, in utero, jsme získali nečekané a zajímavé výsledky: statisticky významně rychlejší rozvoj diabetu u NOD myší krmených LC dietou ve srovnání se standardní dietou, což bylo potvrzeno třemi nezávislými experimenty. Tento jev se projevil změnami v imunoregulačních subpopulacích T buněk ve skupině s LC dietou, přičemž došlo ke statisticky významným poklesu CD4+IL-10+ T buněk (slezina, mLNs), Foxp3+ Tregs (slezina, iLNs), a Tr1 buněk ve slezině. Ve slezině se také snížil podíl γδ T buněk. CD8+ centrální paměťové T buňky byly obecně zvýšeny na LC dietě zavedené od doby in utero (slezina, pLNs a iLNs). Reciproční pokles byl zjištěn u CD8+ efektorových T buněk, což může odrážet zrychlenou migraci efektorových T buněk do pankreatu u myší krmených LC dietou od doby in utero ve srovnání s myšmi chovanými na standardní dietě. Tyto data jsou v souladu s výsledky hodnocení inzulitidy v pankreatech NOD myší krmených LC a standardní dietou od in utero.

Tato studie tedy přináší nové poznatky o vlivu LC diety na imunoregulační a efektorové subpopulace T buněk a akceleraci T1D při jejím podání NOD myším od doby in utero. Velmi

vzácná akcelerace rozvoje T1D u NOD myší vlivem environmentálního faktoru – LC diety, může vrhnout více světla na patogenezi tohoto onemocnění. Další multidisciplinární experimenty jsou potřebné k objasnění komplexních účinků LC diety na vyvíjející se imunitní systém u NOD myší.

Klíčová slova: T1D, nízkosacharidová dieta, NOD myši, Tregs, Tr1 buňky, γδ T buňky, efektorové T buňky; paměťové T buňky.

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List of Abbreviations

APCs - antigen-presenting cells

ASF - Altered Schaedler Flora

DCs - dendritic cells

GFD - gluten-free diet

iLNs - inguinal lymph nodes

LC - low-carbohydrate diet

mLNs- mesentheric lymph nodes

NK - natural killer cells

NOD - non-obese diabetic mice

pLNs - pancreatic lymph nodes

PPs - Peyer's patches

SPF - specific pathogen-free (SPF)

STD - standard diet

T1D - type I diabetes

TCR - T cell receptor

Tr1 - type 1 regulatory T cells

Tregs - regulatory T cells

2. Literature overview

2.1. T1D: introduction

Type 1 diabetes (T1D) is a chronic autoimmune disease in which pancreatic islet β-cells are destroyed by immune cells, ultimately leading to a clinically apparent diabetes with a chronic dependence on exogenous insulin. In 2022, the number of individuals living with T1D was estimated to be around 8.75 million people, with 530,000 new cases diagnosed at all ages. 201,000 of these new cases were in children and young people less than 20 years of age [link].

T1D is a multifactorial disease that involves multiple gene variants that have been associated with the disease along with multiple environmental factors. Genome-wide association studies (GWAS) identified over 60 genes predisposing to T1D susceptibility, with functions both in pancreatic beta cells and innate and adaptive immune systems. The strongest association have been established for HLA-DR3-DQ2 or HLA-DR4-DQ8 haplotypes, PTPN22, CD14, CTLA 4, STAT 4, IL2, SUMO4, IL2RA and other genes [Zajec et al., 2022; Chiou et al., 2021].

However, different lines of evidence indicate the role of environmental factors in triggering or accelerating T1D pathogenesis. First, a progressive increase in T1D incidence including increase in childhood onset diabetes has been reported in many countries over the past decades, with the overall annual increase has been estimated around 3%. According to the ranking of T1D incidence rate in the 0-14 year age group, the Nordic countries of Finland, Sweden and Norway are in the top five of countries worldwide [Patterson et al., 2019]. Second, according to different studies, disease concordance in monozygotic twins constitutes only 30-50% [Triolo et al., 2018]. Third, an increase in T1D incidence has been observed among immigrants from regions with low T1D incidence to regions with high disease incidence. Fourth, a wide gradient in disease incidence in neighbouring countries/regions where two populations have the same frequency of the high-risk HLA-DQ genotypes but had different hygienic conditions [Haupt-Jorgensen et al., 2018]. Finally, a high heterogeneity of the disease is observed between diabetic patients. Thus it is commonly hypothesized that environmental factors trigger a T-cell-mediated immune response in genetically susceptible individuals [Houeiss et al., 2022].

It is also important to add that in 2009, the overall annual increase in T1D cases in European countries was 5.4% in the youngest age group (0-4 years) [Patterson et al., 2009], which may speak in favour of the hygiene hypothesis, combined with the influence of early life events, such as caesarian section, breastfeeding and time of implementation of dietary components such as

gluten and cow's milk [Ogrotis et al., 2023]. According to the hygiene hypothesis, decreasing childhood infections due to a better hygiene conditions impair the development of the immune system by limiting the competition between self and non-self, which impairs normal development of Tregs and T-cell regulation and thus increases the risk of autoimmune diseases [Houeiss et al., 2022].

To date, current evidence allows to suggest the significant contribution of several environmental factors in T1D development: 1) viral infections; 2) alterations in the gut microbiome; 3) dietary factors; 4) early life events (cesarian section, breastfeeding, etc.) [Ogrotis et al., 2023].

Immune infiltrates of the Langerhans islets observed in T1D are very heterogeneous even if the patients are at a similar disease stage. Different studies investigating immune cell composition reported that the majority of the infiltrates are composed of CD8+ T cells followed by macrophages, CD4+ T cells and B cells [Willcox, 2009; Michels, 2017; Gianani, 2010]. More detailed analysis revealed that CD4+ and CD8+ T cells from pancreatic islets of T1D patients recognize a broad range of native islet autoantigens as well as post-translationally modified islet peptides, indicating the crucial role of these cells in immunopathogenesis of T1D and their potential as a target for immunotherapy in T1D [Babon, 2016; Michels, 2017].

T1D progresses in 3 stages: 1) stage 1 (prediabetes), which is presymptomatic and defined by the presence of islet autoantibodies indicating β-cell autoimmunity, while glucose levels do not exceed a normal range; 2) stage 2 is also presymptomatic, but characterized by the presence of islet autoantibodies and hyperglycaemia (fasting plasma glucose of ≥ 5.6 mmol/L or ≥ 6.2 mmol/L); 3) stage 3 is clinically overt diabetes [Insel et al., 2015]. Although anti-islet autoantibodies do not play a significant role in β-cell destruction since T1D is a primarily T-cell-mediated disease, they are used as a biological marker for T1D; for instance, anti-insulin (IAA) autoantibodies are detected in up to 100% of young children (< 5 years of age) before the disease onset and seem to be an early marker of β-cell destruction. A high titers of IAA autoantibodies at a younger age has been associated with a more aggressive disease course. Among other known ant-islet autoantibodies there are anti-glutamic acid decarboxylase (anti-GAD), anti-islet antigen 2 (anti-IA2), and anti-zinc transporter-8 (ZnT8). In GAD65- and IA-2-positive first-degree relatives of T1D patients, presence of these two autoantibodies correlated with 100% T1D incidence by 11 years of follow-up [Pietropaolo et al., 2012].

Islet-specific antigens recognized by diabetogenic T cells partially overlap with those for B cells and include native proteins and epitopes of proinsulin, GAD65, IA-2, islet-specific glucose-6-

phosphatase catalytic subunit-related protein (IGRP), ZnT8, chromogranin, islet amyloid polypeptide (IAPP), etc. [Coppieters et al., 2012; Pugliese, 2017]

Immune mechanisms involved in the autoimmune destruction of pancreatic β-cells are mostly studied in non-obese diabetic (NOD) mice spontaneously developing autoimmune diabetes. Despite the great value of this model in T1D research, human T1D is a much more heterogeneous disease due to the multiple environmental exposures and very different disease processes in patients.

2.2. NOD mouse model

Non-obese diabetic (NOD) mouse is the most commonly used model for T1D. Unlike many other models of autoimmune diseases, NOD mouse is characterized by spontaneous development of autoimmune diabetes that has many similarities with human T1D including genetic susceptibility and natural development of diabetogenic T cells driving the disease progression. NOD mice spontaneously develop autoimmune diabetes starting at the age around 10 weeks in females, and disease incidence increases over time until the age of around 25 weeks. NOD female mice are much more predisposed to T1D development compared with males, with diabetes incidence in females and males constituting approximately 80% and 30%, respectively. The immune cell infiltrates (insulitis) of pancreas of NOD mice consist of macrophages, neutrophils, DCs on early stages of insulitis, then (at the age of 12-14 weeks) CD8+ T cells and CD4+ T cells become predominant, while macrophages, NK cells and B cells are present in lower numbers. These cells are similarly found in the human islet infiltrates, however, in case of NOD mice, there is a massive accumulation of inflammatory cells, and pancreatic beta-cells destruction is much more aggressive compared with humans [*Pearson et al., 2016; Bruggeman et al., 2023; Magnuson AM et al., 2015; Catrina et al., 2021; Aldrich VR et al., 2020; *In't Veld, 2014].

NOD mouse has multiple genes/loci that predispose to the development of autoimmune diabetes similar to those found in human, identified by genome-wide associated studies, including MHC gene variants (in particular, MHC-II molecule *I-Ag7)* and different insulin-dependent diabetes (*Idd*) loci. It is known that FoxP3+ Tregs require IL-2, which is encoded by *Idd3* loci associated with T1D susceptibility and expressed at reduced levels in NOD mice. This leads to an imbalance between Tregs and islet-reactive T cells, activation of the latter, and specific destruction of pancreatic beta-cells [*Mullen, 2017; *Pearson et al., 2016]. NOD mice have been

reported to have a generalized decreased Treg numbers with increased proportion of effector/effector memory T cells (CD44+CD62L-) compared with BALB/c mice; moreover, naïve CD4+CD25- T cells from NOD mice showed reduced functionality in vitro [Godoy et al., 2020]. All these traits make NOD mice prone to spontaneous and multifactorial development of autoimmune diabetes.

Autoreactive T cells in NOD mice have been reported to recognize diabetes-specific autoantigens similar to those observed in diabetic patients, such as Insulin, GAD, IA-2, IA-2β, IGRP, ZnT8, chromogranin A [*Pearson et al., 2016]. It has been demonstrated that early (at 3 weeks-old age) removal of pancreatic lymph nodes, but not the spleen, in NOD mice protected mice against insulin autoantibodies, insulitis, and diabetes development almost completely, but had no effect when performed at 10 weeks-old age when NOD mice were very close to diabetes onset. This experiment suggests that the activation of islet-reactive T cells takes place first in the pancreatic lymph nodes [Gagnerault et al., 2002].

Environmental conditions also play a very important role in T1D development in NOD mice, and it is well known that NOD mice in different institutions and laboratories have different diabetes incidence as well as the speed of the disease development. There are many environmental factors that can alter T1D susceptibility in NOD mice, including exposure to dietary factors such as wheat and gluten, exposure to the microorganisms/infections, and changes in the gut microbiota. T1D incidence is the highest when NOD mice are maintained in conditions maximally close to germ-free, and sharply declines when mice are exposed to infections or their environments are dirty/contaminated. It has been demonstrated that infections of NOD mice with mouse hepatitis virus (MHV), mouse norovirus 4 (MNV4), Salmonella typhimurium, and Schistosoma mansoni abrogated T1D development. For these reasons, NOD mice are generally maintained in specific pathogen-free (SPF) facilities that are carefully screened for the presence of mouse pathogens and correspond to FELASA requirements [Chen et al., 2020; Pearson, Tai, et al., 2019].

It has been found that germ-free (GF) and SPF NOD mice have very similar T1D incidence, but insulitis in GF mice was reported to be more accelerated and intense. Germ-free NOD mice showed increased levels of Th17 and Th1 cells in the mesenteric and pancreatic lymph nodes and increased levels of IL-17 mRNA in the colon, while FoxP3+ Tregs and Foxp3 mRNA were reduced compared with SPF NOD mice. These data indicate that lack of intestinal microbiota in GF mice promotes an imbalance between Th17, Th1 and Treg differentiation in the intestine, with reduction in Tregs and accelerated insulitis [Alam et al., 2010].

2.3. Viruses in T1D and generation of neoepitopes

A growing evidence obtained from clinical epidemiological and experimental studies supports a role of viruses in T1D development, particularly human enterovirus and rotavirus infections, as potential triggers for the development of T1D in individuals genetically predisposed to autoimmunity.The first evidence came from epidemiological studies, which reported increased T1D incidence following enterovirus epidemics. Enteroviral RNA has been detected in the blood of newly diagnosed T1D patients, and a positive association between enteroviral infection and T1D, particularly for the coxsackievirus B4 (CVB4), has been reported in different studies using PCR-based detection methods. The presence of antibodies against coxsackievirus B1 (CVB1) was also associated with T1D [Houeiss et al., 2022; Op de Beeck, Eizirik, 2016].

Furthermore, the Diabetes Virus Detection study (DiViD) has demonstared that enteroviral VP1 protein was detected in the islets of all newly diagnosed T1D patients included into the study, but only in two of nine non-diabetic controls. Hyperexpression of class I HLA molecules was found in the islets of all patients [Krogvold et al., 2015], and islet cell hyperexpression of HLA I antigens was found to be a defining feature in type 1 diabetes [Richardson et al., 2016]. Another sudy has found that upregulation of IFN-stimulated genes in pancreatic tissue samples taken from T1D patients correlated with the insulitis [Ferreira et al., 2014]. Finally, a recent meta-analysis of 25 studies with 4,854 participants has shown a clinically significant association between enteroviral infection and T1D, which was consistent with a previous large meta-analysis [Yang et al., 2021; Yeung et al., 2011].

Based on current evidence, one of the hypotheses suggests that viral infection/s could induce an immune response with a local production of cytokines, including type I interferons, and subsequent upregulation of HLA-I on pancreatic beta-cells. At the same time, viruses could induce a translational arrest in beta-cells, hampering insulin production. Also, other forms of environmental stress, such as chemicals, dietary components may promote ER or oxidative stress [*Rodriguez-Calvo et al., 2021]. The effects of these environmental triggers may result in the generation of a first wave of non-conventional proteins by pancreatic beta-cells. Impaired clearance of such stressed or dying beta-cells expressing modified proteins will cause activation of APCs and presentation of neoepitopes to CD4+ T-cells, which in turn can trigger a wide range of immune responses, including activation of B cells with subsequent production of islet-specific autoantibodies, and activation of autoreactive effector T cells that can directly kill beta-cells presenting modified islet peptides (neoepitopes). This first cascade of immune activation may promote further beta-cell stress or death, resulting in an autoreactive loop, with further modification of beta-cell proteins, epitope spreading, amplification of immune response, and

disease exacerbation. This model could provide evidence for a role of neoepitopes both in initiation and exacerbation of the disease [*Rodriguez-Calvo et al., 2021].

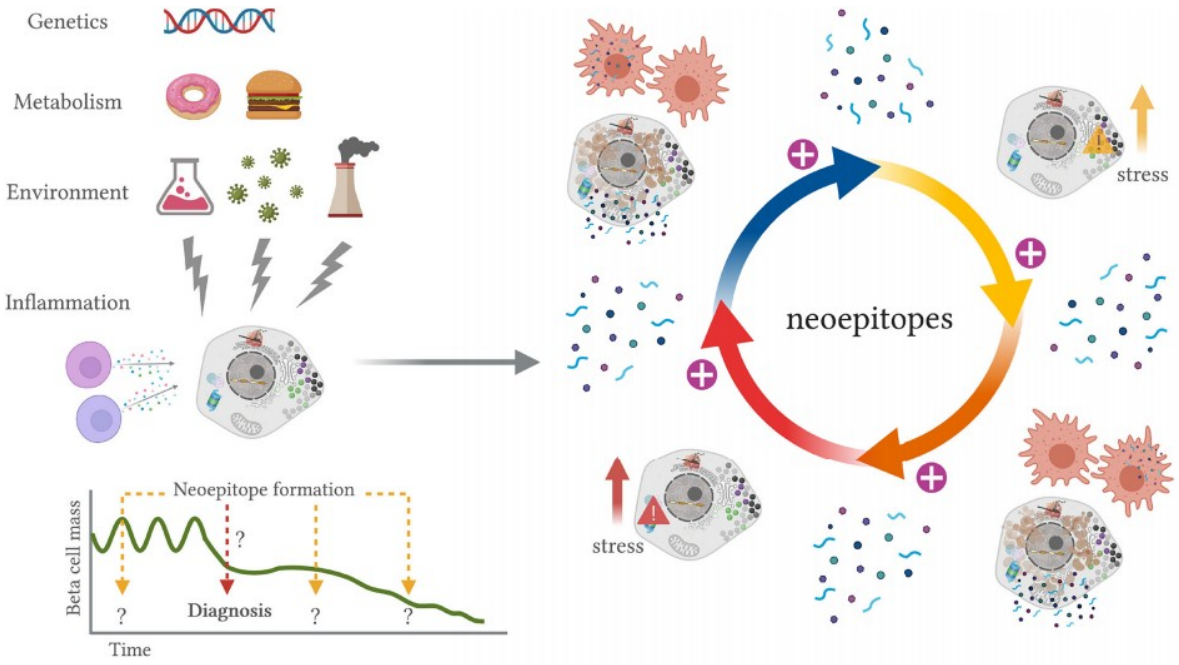

Fig. 1. Schematic representation of generation of neoepitopes and how they could lead to the targeted killing of pancreatic β-cells in T1D [Rodriguez-Calvo et al. *Front Immunol.* 2021 Apr 19;12:667989].

2.4. Gut microbiome in T1D

Data from recent years indicate that alterations in the gut microbiota can be an important factor in T1D pathogenesis as well as in other autoimmune diseases. Different studies including recent ones using high-throughput sequencing observed marked differences between the intestinal microbiome profile of T1D patients compared with healthy subjects, suggesting a link between gut microbiota alterations and development of T1D. One of the most profound changes observed in different studies is a significant decrease in the *Firmicutes/Bacteroides* (F/B) ratio in T1D patients [Murri et al., 2013; Giongo et al., 2011; Leiva-Gea et al., 2018; Demirci et al., 2019]. As for the *Bacteroides* abundance, one study has revealed that in children with genetic risk of autoimmune diabetes, changes in the intestinal microbiome become evident approximately 8

months before the seroconversion, with a significant increase in *Bacteroides dorei* and *Bacteroides vulgatus* compared to controls [Davis-Richardson et al., 2014].

Another common feature in T1D patients microbiome is a reduced gut microbiome diversity [Giongo et al., 2011; Leiva-Gea et al., 2018; de Goffau et al., 2013; Kostic et al., 2015]. One study has found a 25% decline in microbiome diversity in seroconverters who progressed into T1D during the study period compared to seroconverters without clinically apparent diabetes. Reduced microbial diversity was paralleled with changes in the microbiome associated with a proinflammatory environment [Kostic et al., 2015].

It is known that short-chain fatty acids (SCFA) such as acetate (C2), propionate (C3), and butyrate (C4) are generated by the gut microbiota as a result of enzymatic processing of complex carbohydrates/dietary fibers and play an important role in maintaining intestinal homeostasis on many levels. SCFA strengthen epithelial barrier function by stimulating epithelial growth and innate responses to damage as well as microbial invasion. It has been shown that SCFA have a strong ability to modulate the function of intestinal macrophages and DCs, rendering them into anti-inflammatory, tolerogenic phenotype and making them more efficient in inducing regulatory T cells, and many studies have reported butyrate-induced induction of FoxP3+Tregs [Kim CH 2018; Smith et al., 2013]. One study has shown that SCFA can directly promote T cell differentiation into both effector and regulatory T cells producing IL-17, FN-γ, and/or IL-10 depending on cytokine milieu and presence of infection, thus promoting either immune response or immune tolerance depending on immunological context [Park et al., 2015].

It has been shown that some commensal microbiota species, for instance, some *Clostridia* species (including clusters IV and XIVa) which are involved in production of SCFA, can induce CD4+FoxP3+Tregs [Atarashi et al., 2010; Atarashi et al., 2013; Furusawa et al., 2013]. Other gut microbiota species can stimulate proinflammatory T cells, for instance, segmented filamentous bacteria can promote the differentiation of Th17 and Th1 cells [Ivanov et al., 2009]

Several studies using metagenomic analysis revealed a significant reduction in lactate- and butyrate-producing bacteria, such as *Clostridium* clusters IV and XIVa and mucin-degrading bacteria, such as *Akkermansia* and *Prevotella* in T1D patients compared with unaffected children [de Goffau 2013; Brown et al., 2011; Leiva-Gea et al., 2018]. A decrease in *Bifidobacterium* in the gut microbiome of T1D children has also been reported by several studies [Murri et al., 2011; Soyucen et al., 2014; Leiva-Gea et al., 2018]. A consortium of lactate- and butyrate-producing

bacteria in healthy gut can induce mucin synthesis sufficient for maintaining integrity of the mucosal intestinal barrier, while lack of these bacteria observed in diabetic patients, as well as the presence of non-butyrate producing lactate-utilizing bacteria prevent optimal mucin synthesis and lead to increased gut permeability [Brown et al., 2011].

It has been shown that when the integrity of the intestinal barrier is impaired, intestinal toxins, food antigens, gut microbiome products, and infection factors may leak to intestinal mucosal components, and finally to the pancreatic lymph nodes to induce or exacerbate T1D. In such cases, pancreatic-draining lymph node T cells, including diabetogenic CD8+ T cells, can be activated, proliferate, invade pancreatic islets and promote insulitis. It has been reported that children with T1D have significantly higher intestinal permeability compared to controls, and certain bacteria, such as *Dialister invisus*, *Gemella sanguinis*, and *Bifidobacterium longum*, were associated with compromised gut integrity in T1D [*Zheng, Li, Zhou 2018].

Interestingly, some studies also reported that the Firmicutes:Bacteroidetes ratio and the number of *Bifidobacterium* and *Lactobacillus* correlated negatively and significantly with the plasma glucose levels in T1D children; at the same time, the quantity of *Clostridium* correlated positively and significantly with glucose levels in plasma of diabetic children [Murri 2013].

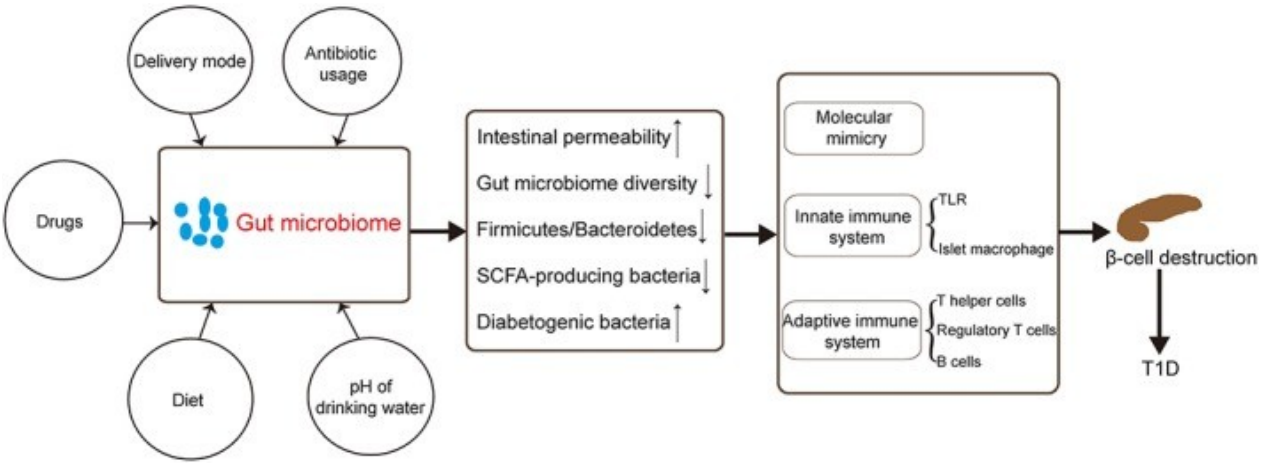

Fig. 2. The role of the gut microbiome in T1D [Zheng P, Li Z, Zhou Z. *Diabetes Metab Res Rev.* 2018 Oct;34(7):e3043]

2.5. Dietary factors in T1D

Epidemiological and animal studies have shown that dietary factors contribute to the development of diabetes. Diet is one of the main modulators of the gut microbiome in newborns, and combination of these two factors is suggested to play an essential role in a proper maturation of the immune system in infants. Diet serves not only as a source of nutrients, but also as a main route of entry for antigens to the newborn organism; at the same time, early microbiota colonizing the newborn's gut provide the stimuli that guide the differentiation of immune cells and organs and define the balance between regulatory and inflammatory responses. At this stage, the infant's immune system also learns to distinguish self and non-self through different environmental exposures [Mejía-León, Barca 2015].

Accumulating evidence suggests that various dietary factors can regulate Tregs development directly or indirectly through changes in the gut microbiota and their metabolites [Tan J, Ni D et al., 2022]. Therefore, dietary components implemented in the early life or even during pregnancy can affect the risk of development of T1D, especially in genetically predisposed individuals. Distinguishing between fetal, newborn, and childhood exposures to certain dietary factors is very important in T1D research since the immunological effects can differ depending on the developmental stage.

According to the American Diabetes Association, normal diet should be composed in a way to obtain 15-20% of total energy intake from protein, 20-35% of energy from dietary fat, and 45-60% of energy from carbohydrates [Talmadge et al., 2018]. It has been suggested that a high-fat Western diet can play a role in the increase of T1D observed in westernized societies. In cell and animal models, high-fat diet have been shown to induce beta-cell endoplasmic reticulum (ER) stress, mitochondrial damage, oxidative stress, insulin resistance, dysfunctional insulin production and beta-cell death. Moreover, independent experiments have demonstrated that stimulation of beta-cell stress pathways may result in an enhanced production of neo-antigens and activation of diabetogenic T cells [Clark et al., 2021].

Among dietary components, gluten exposure is known to be one of the strongest factors in development of T1D. It was shown a long time ago that gluten-free diet introduced to NOD mice from *in utero* and postnatally lowered diabetes incidence from 64% to 15% [Funda et al., 1999], which was confirmed by subsequent studies [Marietta et al., 2013; Funda et al., 2008; Hansen et al., 2006]. A more recent study has shown that feeding NOD mice with a gluten-free diet (GFD) only during pregnancy turned out to be sufficient to prevent T1D development in offspring, with T1D incidence reduction from 62.5% in gluten-consuming mice to 8.3% in the gluten-free

group. This *in utero* exposure to GFD was also associated with reduction in insulitis and intestinal expression of RORγt (Th17) in offspring mice [Antvorskov et al., 2016]. Interestingly, a recent study of Hansen et al. has shown that GFD can reduce T1D in NOD mice across multiple generations in a microbiota-independent manner. In that study GFD introduced to NOD females until the weaning of their offsprings significantly delayed T1D in both parent generation and offspring (F1) of untreated NOD mice and in mice treated with a strong cocktail of antibiotics killing most of the existing microbiota [Hansen et al., 2022].

Furthermore, epidemiological studies have suggested that increased consumption of high glycaemic index carbohydrates and sugars may trigger or exacerbate the progression of T1D. A recent study has shown that sustained high glucose drinking significantly exacerbated islet inflammation and accelerated the T1D onset in NOD mice. High glucose intake promoted excessive endoplasmic reticulum stress and upregulated expression of autoantigens in islet beta-cells. In addition, high glucose treatment significantly increased the abililty of small extracellular vesicles to promote DCs maturation and stimulate proinflammatory response This study provides evidence for a negative effect of high glucose intake on the pathogenesis of T1D in individuals susceptible to the disease. Therefore, restriction of sugar intake may be an effective strategy for prevernting T1D in children or adults genetically predisposed to T1D [Li, Wang, Meng et al., 2022].

2.5.1. Low-carbohydrate diet in T1D

Before the discovery of insulin in 1921, strict low-carbohydrate diets (≤ 10 g/day) were the only available option to treat T1D patients. Since dietary carbohydrates significantly increase blood glucose levels, patients with T1D are recommended to count the carbohydrate intake to control glycaemia and adjust insulin dosage for meals [Bolla M. et al., 2019]. A recent meta-analysis of Lampousi et al. came up with a conclusion that there are positive associations between T1D and childhood intake of sugar, carbohydrates, and sugar-sweetened beverages, with a strongest association for carbohydrates [Lampousi et al., 2021].

There is quite a large heterogeneity in definitions of low carbohydrate diet between different studies and clinicians. According to the American Diabetes Association (ADA) which is widely used by many experimental studies, low-carbohydrate (LC) diets are defined as less than 130 g/day or 26% total energy intake from carbohydrates [Feinman R. et al, 2015].

Several studies have reported that after of period of adherence to LC diet, adult T1D patients had significantly lower levels of glycated haemoglobin (HbA1c) and daily insulin use [Turton J., 2023; Krebs J et al., 2016; Nielsen J. et al., 2012]. Due to the potential benefits of the LC diet in reducing insulin doses and better glycaemic control, it can be a feasible approach for people with T1D. Thus, there is a need in larger, long-term non-randomised controlled trials to confirm these findings and implement LC diets into clinical practice.

Despite different studies reporting a reduction in HbA1c levels in T1D patents adhering to LC diet, there is still a question regarding long-term tolerability of LC diet, and clinical audit avaluating the long-term adherence to LC diet in diabetic patients showed that after two years about 50% of the people ceased the diet. Furthermore, it has been reported that carbohydrate restriction in growing children led to anthropometrical deficits, metabolic profile associated with higher cardiovascular risk and fatigue [Bolla et al., 2019].

Despite a high popularity of LC and ketodiet in recent years, there is still a need in studies investigating possible effects of long-term LC diet on immune system, especially for understanding its potential risks in growing children with genetical predisposition to T1D and children born from diabetic mothers adhering to LC diet during pregnancy. Our study is trying to shed the light on this question using NOD mice exposed to LC diet at different developmental stages.

2.6. Regulatory and potentially immunoregulatory cell subsets in T1D

2.6.1. CD4+FoxP3+ Tregs

CD4+CD25+FoxP3+ Tregs are known to play a vital role in preventing development of autoimmune diseases including T1D. The majority of Tregs is generated in thymus (tTregs), but some Tregs can be induced on the periphery (pTregs) from FoxP3-CD4+ T cell precursors [Schuster et al., 2018]. Mechanisms exploited by Tregs to suppress autoreactive effector T cells

include depriving pathogenic T cells from IL-2 due to the high levels of CD25 (the IL-2 receptor α chain) expressed on Tregs; secretion of TGF-β, IL-10, IL-35, adenosine, as well as expression of molecules such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4), lymphocyte-activation gene 3 (LAG3), and granzyme B [*Hull, Peakman, Tree, 2017].

CD4+Foxp3+Tregs have been known to play an essential role in controlling the progression of T1D. Experimental depletion of CD4+FoxP3+ Tregs in NOD mice is known to promote activation of proinflammatory T cells and NK cells in the pancreas, resulting in very fast T1D onset with a more aggressive beta-cell destruction. In contrast, adoptive transfer or therapeutic boosting of Tregs with IL-2 has been shown to have protective effects [Feurer et al., 2009; D'Alise AM et al., 2008; Tarbell et al., 2007].

The studies on the number of Tregs in T1D patients gave very controversial results with some reporting an increase in Tregs frequency at T1D onset [Viisanen et al., 2019; Ferreira et al., 2017; Marwaha et al., 2010; Sanda, Roep, Herrath, 2008], some reporting decrease [Ghonaim et al., 2017; Ryba-Stanisła- wowska et al., 2014; Szypowska et al., 2012], while some did not find significant differences in Tregs frequencies compared with controls [Hamari et al, 2016; McClymont et al., 2011; Brusko et al., 2007; Putnam et al., 2005]. However, mounting evidence suggests that it is not the number of Tregs, but their fitness and functionality that are altered in individuals with T1D [Lindley et al., 2005; Brusko et al., 2005; Brusko et al., 2007; Haseda et al., 2013]. This conclusion is consistent with the genetic variants associated with T1D susceptibility, e.g. IL2RA, IL2, CTLA4, IL10, and PTPN2, which may profoundly affect Tregs function [Bakay et al, 2013].

Independent studies confirmed that Tregs from the blood of T1D patients have much lower suppressive effect on proliferation of autologous effector T cells compared with Tregs from healthy HLA- and age-matched individuals [Lindley et al., 2005; Brusko et al., 2005; Lawson et al., 2008]. Moreover, it has been shown that recent-onset T1D patients have much higher levels of apoptosis of CD4+CD25+high Tregs compared with healthy controls [Glisic-Milosavljevic et al., 2005; Glisic-Milosavljevic et al., 2007]. Furthermore, in co-cultures studies FoxP3+ Tregs from T1D patients were shown to secrete proinflammatory cytokines, such as IL-17 and IFN-γ, while in healthy individuals they predominantly secreted IL-10 [Marwaha et al., 2010; McClymont et al., 2011].

Evidence obtained from the studies on NOD mice indicate that Tregs dysfunction in T1D is mainly restricted to the pancreas and pancreatic lymph nodes. It has been shown that insufficient

IL-2 amounts in the pancreas led to an increased apoptosis of Tregs in this compartment, thus promoting break of self-tolerance and development of T1D in NOD mice [Tang et al., 2008]. Another study demonstrated that T1D onset was associated with an increase in Tregs in pancreatic lymph nodes of NOD mice, while Treg numbers in the pancreas were decreased due to the apoptosis. Low-dose IL-2 injections from the point of T1D onset reversed established disease due to an increase in Tregs numbers, expression of FoxP3, CD25, CTLA-4, ICOS, GITR and restoration of their functional activity specifically in pancreas [Grinberg-Bleyer et al., 2010]. Due to the high levels of CD25 (IL2R) expression by Tregs, low dose IL-2 can preferentially target Tregs with few side effects, which is exploited in human clinical trials [Pham et al., 2016; Todd et al, 2016] .

2.6.2. CD4+FoxP3-Tregs, Tr1 cells

It has been demonstrated that peripherally induced Tregs (pTregs) arise from the mature CD4+FoxP3- T cells after enocuntering with tolerogenic stimuli, including TGF-β. A recent study on NOD mice showed that deletion of the FoxP3 enhancer CNS1, which is essential for the development of peripheral but not thymic Tregs, increased diabetes incidence in NOD mice, suggesting a role of peripheral Tregs in development of T1D [Schuster et al., 2018]. The highest pTregs frequency is found in the gut, reflecting the ability of commensal gut microbiome to induce pTregs development; it is also known that pTregs play an important role in the gut immune regulation [Schuster et al., 2018].

An original study of Arif et al. has identified a CD4+ T cell subset secreting IL-10 in response to islet-specific antigens; these T cells were enriched in people carrying T1D tisk HLA haplotype who were diabetes-free, and were reduced in T1D patients [Arif et al., 2004]. Another study has shown that CD4+IL-10+ T cells observed in T1D patients were associated with a reduced percentage of proinflammatory islet-specific T cells, fewer autoantibodies, and less aggressive pancreatic autoimmunity [Arif et al., 2014]. It has also been found that higher mean numbers of CD4+FoxP3-IL10+ T cells at T1D diagnosis were associated with a better glycaemic control in children after diagnosis [Sanda et al., 2008].

Type 1 regulatory T cells known as Tr1 cells was found as a subtype of Tregs also induced on a periphery and not expressing high levels of FoxP3, but characterized by CD49b and lymphocyte-activation gene 3 (LAG3) expression, both in humans and in mice. This cell subset has potent immunoregulatory effects, suppressing other T cell subsets via high levels of production of IL-10

and TGF-β, as well as contact-dependent mechanisms mediated by PD-1 or CTLA-4, and by disrupting metabolic state of effector T cells via production of CD39 and CD73. Moreover, Tr1 cells can also modulate APCs function via variety of mechanisms, including inhibitory surface receptors, release of perforin B and granzyme B, and secretion of soluble factors. Independent experimental models showed an essential role of Tr1 cells in maintaining tolerance to both self-antigens and commensal gut microbiota [Roncarolo et al, 2014].

The study of Yu et al. showed that alteration of gut microbiota in NOD mice through cohousing with dysbiotic inflammasome-deficient mice or through administration of anti-CD3 promoted a significant increase in Tr1 cells producing IL-10, which resulted in 2-fold decrease in T1D incidence compared with control NOD mice. These data suggest an important role of intestinal Tr1 cells in the control of effector T cells and development of autoimmune diabetes [Yu H et al., 2017].

2.6.3. γδ T cells

Alterations in γδ T cells frequency and functional properties have been reported for different autoimmune diseases including multiple sclerosis, celiac disease, systemic lupus erythematous (SLE), and T1D [Kucharska & Noczyńska, 2016]. Results of the studies investigating the role of γδ T cells in T1D development have been heterogeneous and sometimes contradictory, overall suggesting the immunoregulatory role in T1D and that different γδ T cell subsets can play different roles in the disease.

γδ T cells specific for modified insulin peptide B:9-23 were reported in NOD mice [Zhang et al., 2010; Kemal Aydintug M. et al, 2014]. It has been shown that aerosolized intranasal insulin induced intraintestinal regulatory CD8+ γδ T cells in NOD mice, and these CD8+ γδ T cells were required for induction of CD4+CD25+Tregs by oral insulin and for diabetes prevention in adoptive co-transfer model. When gut intraepithelial CD8+ γδ T cells from normal NOD mice were transferred to NOD mice that underwent neonatal thymectomy, they significantly delayed T1D onset, and this effect was prevented when mice were co-injected with TCR γδ-depleting mAb, GL3 clone [Locke et al., 2006].

On a systemic level, one human study observed that the percentage of CD8+ γδ T cells in peripheral blood in patients with new-onset T1D and after 12-months follow-up was

significantly lower than in controls and was consistent with a previous study reporting the association between γδ T cells percentage and T1D onset [Zubkiewicz-Kucharska A. & Noczyńska A., 2016].

In NOD mice, the number of γδ T cells in the spleen has been shown to drop with the onset of T1D [O'Brien R.L. et al., 2022; Han G. et al., 2010]. Vγ1+ γδ T cells were found to infiltrate the pancreas of NOD mice with insulitis [Aydintug M.K. et al., 2014]. Another study reported that pancreatic islets of 12-weeks old prediabetic NOD mice were infiltrated with γδ TCR+ cells, including both the CD27-CD44^{hi} secreting IL-17 and CD27+CD44^{lo} secreting IFN-γ. An adoptive transfer of diabetogenic αβ T cells alone led to 50% of T1D incidence in NOD/SCID mice compared with 100% T1D incidence when αβ T cells were transferred together with γδ T cells, suggesting that CD27-CD44^{hi} γδ T cells producing IL-17 cooperated with αβ T cells to induce T1D. In that study, the effects of this pathogenic γδ T cell subset was blocked in vivo by IL-17-neutralizing mAb, leading to a significant drop in T1D incidence [Markle et al., 2013].

However, a study of Han et al. has shown that in vivo neutralization of IL-17 did not protect NOD/SCID mice from T1D when they underwent the transfer of diabetogenic splenocytes from NOD mice. In fact, an adoptive co-transfer experiment showed that γδ T cells markedly reduced insulitis and prevented T1D incidence in NOD/SCID mice injected with diabetic splenocytes. In contrast to the aforementioned study, in a study of Han et al. the γδ T cell subset which was dominant in NOD mice, IL-17-producing γδ T cells, did not exacerbate T1D in adoptive transfer model, but rather provided a regulatory effect, protecting recipient mice from the disease through upregulated production of TGF-β [Han et al., 2010].

The immunoregulatory role of IL-17 producing γδ T cells has been confirmed by a recent study which used Vγ-gene-targeted NOD mice to discriminate the functional roles of different γδ T cell subsets. The authors have found that NOD Vγ4+ γδ T cells inhibited the development of diabetes in NOD mice due to their ability to produce IL-17 and/or promote CD4+ Tregs in pancreatic lymph nodes. In contrast, Vγ1+ γδ T cells, in particular those producing IFNγ, were found to promote T1D development in NOD mice [O'Brien R.L. et al., 2022].

Taken together, these heterogeneous data may indicate that γδ T cells can play a dualistic role in diabetes development, with some subsets providing immunoregulatory role by inducing CD4+ Tregs and secreting TGF-β, while others having potential to contribute to T1D pathogenesis.

2.7. Naive, effector and memory T cells in T1D

CD8+ T cells are known to play a crucial role in destruction of pancreatic ß-cells and are one of the predominant cell types infiltrating pancreatic islets both in human T1D [Babon et al., 2016] and in NOD mice [Bruggeman et al., 2023], correlating with disease progression. An essential observation that was made quite a long time ago is that islet-reactive CD8+ and CD8+ T cells are also found in the peripheral blood of healthy controls, but in case of nondiabetic subjects they are kept under robust immune control. Later studies revealed that autoreactive T cells in healthy subjects predominantly have a naïve phenotype, whereas islet-specific T cells in T1D patients are memory T cells [Ehlers and Rigby, 2015].

The study of Chee et al. has shown that islet-specific CD8+ T cells (namely, $IGRP_{206–214}$-specific CD8+ T cells) in NOD mice increased with age in lymphoid tissues, and their numbers significantly correlated with the severity of insulitis. The majority of IGRP-specific CD8+ T cells detected in lymphoid tissues of older (12-15 weeks-old) NOD mice were antigen-experienced T cells (Tcm) ($CD44^{hi}CD62L^{lo}$) compared with younger (6-8 weeks old) mice having mostly naive T cells (Tn) ($CD44^{lo}CD62L^{hi}$). Furthermore, autoreactive $CD8^+$ T cells in the peripheral lymphoid tissues of older NOD mice expressed markers of effector memory T cells (Tem) ($IL\text{-}7Ra^{hi}KLRG1^{hi}$) compared with $CD4^+$ and $CD8^+$ T cells not specific for IGRP. Importantly, transfer experiment with labeled naive IGRP-specific CD8+ T cells has shown that IGRP-specific CD8+ T cells acquire their effector-memory phenotype in islets, not in the pancreatic lymph nodes. Finally, in this study islet-infiltrating T cells emigrated from the islets that were transplanted to syngeneic immunodeficient NOD.$RAG^{-/-}$ mice, were found in peripheral lymphoid tissues, invaded the recipient's pancreas and cause diabetes [Chee et al., 2014].

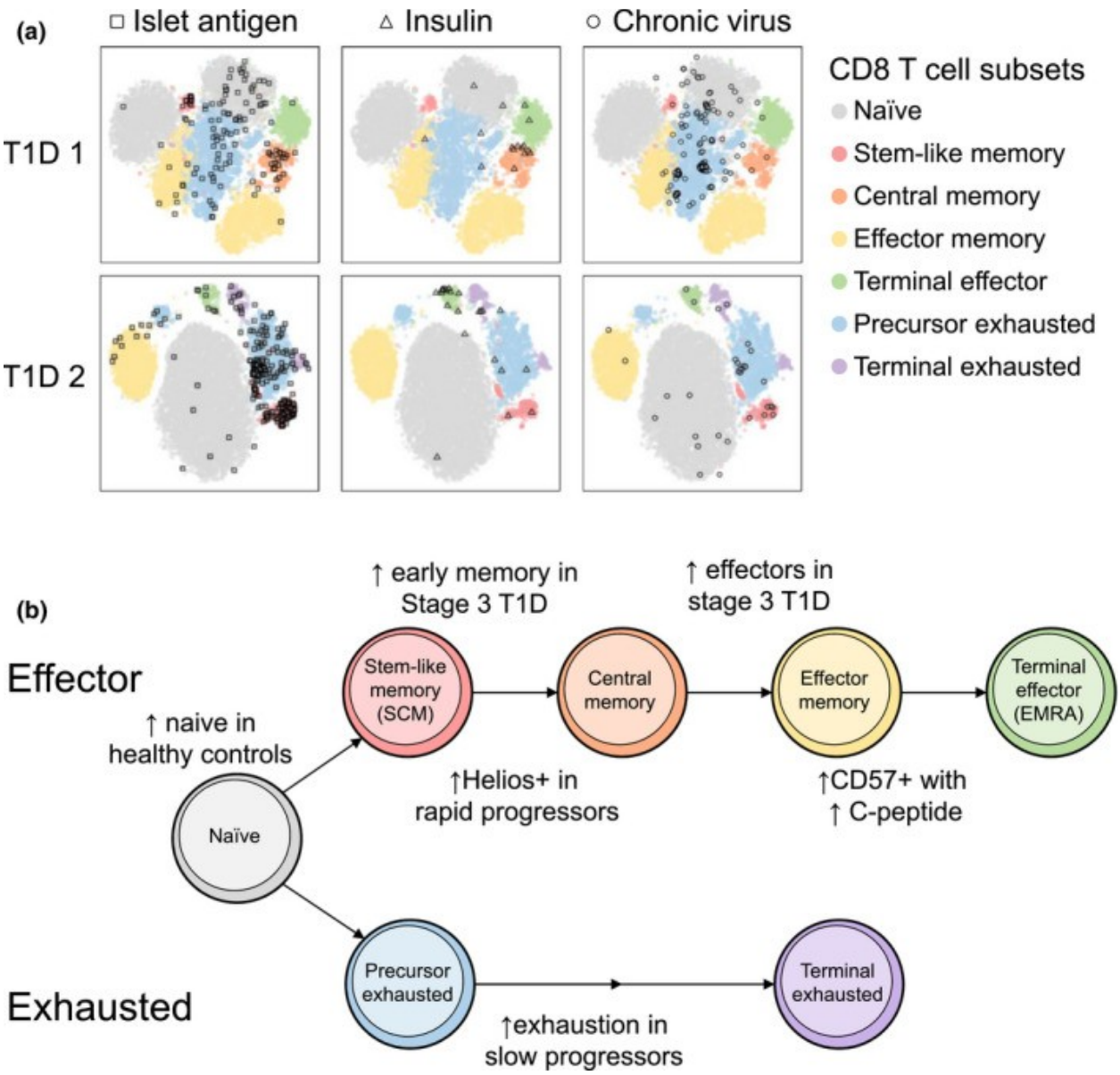

Figure 3. Islet antigen-specific CD8 T cells are phenotypically heterogeneous and differ by disease stage and rate of progression in type 1 diabetes (T1D). [Wiedeman AE, Speake C, Long SA. Immunol Cell Biol. 2021 May;99(5):475-485.]

A study of Wiedeman et al. provides a scheme with trends in T cell phenotypes distinguished at different T1D stages (Fig.3) [Wiedeman et al.,. 2021]. Early memory subsets including stem-like memory (Tscm), transitional memory and effector cells tend to be increased at stage 3 of T1D; terminal effector T cells (T_{EMRA}) characterised by CD57 expression and exhaustion markers have also been found. Importantly, prevalence of a Helios+ early memory phenotype is associated with more rapid progression of disease after onset, while an increase in terminal

CD57+ effector memory or exhausted phenotype is associated with beta-cell health and preservation of C-peptide or slow T1D progression [Wiedeman et al.,. 2021].

One of the most used markers of pancreatic beta-cell function, i.e. secretion of endogenous insulin, is a C-peptide, which is generated during the cleavage of insulin from proinsulin, thus corresponding to insulin with a ratio 1:1. One study examined a group of T1D who are characterized as slow progressors, known to have C-peptide values much better than most of T1D patients long after diagnosis. Using peptide-tetramer technology, the authors have shown that slow progressors had minimal islet autoantigen-specific CD8+ T cell responses, lower than new-onset and long-standing T1D patients, and comparable to healthy controls. Furthermore, while higher frequencies of CD4+ T cells with a central memory phenotype (Tcm) were detected in newly diagnosed T1D patients compared with healthy donors, low progressors had lower frequencies of these cells compared with controls [Hanna et al., 2020].

Another study was tracking circulating β cell-reactive CD8+ T cell subsets and measuring C-peptide in newly diagnosed T1D patients longitudinally for 2 years. The authors have found that islet-autoreactive CD8+Tem expressing the terminal differentiation marker CD57 and displaying enhanced cytotoxic potential, significantly and positively correlated with C-peptide levels, whereas decline in these cells was associated with C-peptide loss. It is interesting that increase in C-peptide observed in several children at 6 months from T1D diagnosis (<12 years old at diagnosis) was associated with β cell-specific CD8+CD57+ Tem, likely reflecting so-called honeymoon period, during which endogenous β cell-function recovers and there is lower dependence on exogenous insulin injections. The authors suggest that temporal functional recovery of β cells in honeymoon period promotes the appearance of β cell-specific CD8+CD57+ effector memory T cells in the peripheral blood, followed by increased β-cell killing by these cells, which correlates with the decline in this cell subset in the blood and reflects its function in T1D pathogenesis [Yeo, Woodwyk et al., 2018].

A recent study of Gearty et al. has identified a subpopulation of β-cell-specific stem-like autoimmune progenitor CD8+TCF1+CD44+ T cells in the pancreatic lymph nodes (pLNs) of NOD mice, which self-renewed and gave rise to islet-specific autoimmune mediators in the pLNs. These autoimmune mediators have been found to migrate to the pancreas, where they differentiated and destroyed β-cells. Adoptive transfer experiments has shown that just 20 IGRP-specific CD8+TCF1+ T cells from diabetic pLNs promoted T1D in recipient NOD/SCID mice, while as many as 100,000 autoreactive CD8+TCF1- T cells from the pancreas failed to promote T1D. Blocking the migration of CD8+TCF1+ T cells from pLNs prevented the induction of T1D

in NOD mice, which suggests that TCF1- effector T cells in the pancreas cannot persist without resupply from pLN autoimmune progenitors. This finding holds a great potential for developing immunotherapeutic strategies targeting the stem-like autoimmune progenitor pool in T1D [Gearty et al., 2022].

A recent outstanding study of Vignali et al. has revealed that CD8+ Tscm specific for beta-cell antigens insulin, GAD65, and IGRP are present in the blood of T1D patients. The authors have found that high expression of GLUT1 (necessary for IL-7 uptake) observed in diabetic patients was a hallmark of circulating Tscm, and selective targeting of glucose uptake via GLUT1 inhibitor resulted in blockade of Tscm generation and expansion. This finding suggests that islet-reactive Tscm can be selectively targeted in T1D and paves the way for novel therapeutic strategies in T1D research [Vignali et al., 2018].

3. Thesis Aims

The general aim of this thesis was to study the possible effects of open-formula low-carbohydrate (LC) diet on development of T1D compared with open-formula standard (STD) diet in SPF NOD mice when introduced from the age of 4 weeks and from *in utero*. The study included:

1) T1D incidence studies;

2) isolation of cells from mucosal and systemic lymphoid organs of NOD mice, cell staining and flow cytometry analysis for evaluation of immunological parameters;

3) insulitis scoring.

4. Materials and Methodology

4.1. Materials

4.1.1 Laboratory disposables

Test tubes 5 ml: Becton Dickinson, USA

Gloves: Nitrile nonsterile, 9018 – S, 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

4.1.2. Solutions, buffers, antibodies

Complete medium: RPMI-1640 with L-glutamine (Lonza, USA), fetal bovine serum (FBS)(10%, Gibco-Life Technologies, USA)

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

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

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

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

Compensation beads: UltraComp eBeads™, Invitrogen, USA

Antibodies: see Table 1.

Extracellular staining							
Antigen	**CD3**	**CD4**	**CD8**	**CD44**	**CD62L**	**γδ**	**CD45RB**

Fluorochrome	FITC	PerCP Cy5.5	Alexa Fluor 700	APC	APC eFluor 780	PE-Cyanine 7	PE
Manufacturer	eBioscience	Invitrogen	Invitrogen	eBioscience	eBioscience	eBioscience	eBioscience
Clone	0KT3	RM4-5	53-6-7	IM7	MEL-14	eBioGL3	C363.16A
Extracellular staining							
Antigen	**CD3** (both panels)	**CD4** (panel 2)	**CD49b**	**LAG3**			
Fluorochrome	PerCP Cy5.5	Alexa Fluor 700	FITC-A	APC eFluor 780			
Manufacturer	eBioscience	BD Pharmingen	Invitrogen	Invitrogen			
Clone	145-2C11	RM4-5	DX5	C9B7W			

Intracellular staining						
Antigen	**FoxP3**	**IL-10**	**IFN-γ**			
Fluorochrome	PE-Cyanine 7	PE-A	APC-A			
Manufacturer	eBioscience	eBioscience	BD Pharmingen			
Clone	FJK-16S	JES3-9D7	XMG1.2			

Table 1. The list of fluorochrome-conjugated antibodies used in the experiments.

4.1.3 Laboratory equipment

Flow cytometer: BD LSR II, BD Biosciences, USA

Flowbox: Biocyt 150, Esi Flufrance, France

Centrifuge 1: Rotanta 460R, Hettich, Germany

Centrifuge 2: IEC CL31R Multispeed centrifuge, Thermo Electron, USA

Microcentrifuge: Micro-Centrifuge II, LabTech, Korea

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

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

Bürker chamber: Assistent, Germany

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

Manual repetitive pipette: HandyStep, BRAND, Germany

Combitip: Eppendorf, Germany

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

Surgical instruments: scalpel, tweezers, scissors

4.2. Methods

Gnotobiotic female SPF NOD mice with Altered Schaedler Flora (ASF) from Jackson Laboratories were studied since they exhibit much higher diabetes incidence compared with male mice. Mice were kept in SPF conditions according to FELASA and fed with open-formula Altromin diets, Altromin 1324 standard diet or carbohydrate-deficient diet Altromin C1009 (herein referred as LC diet). The contents of the diets are outlined on Fig. 4 and Fig.5.

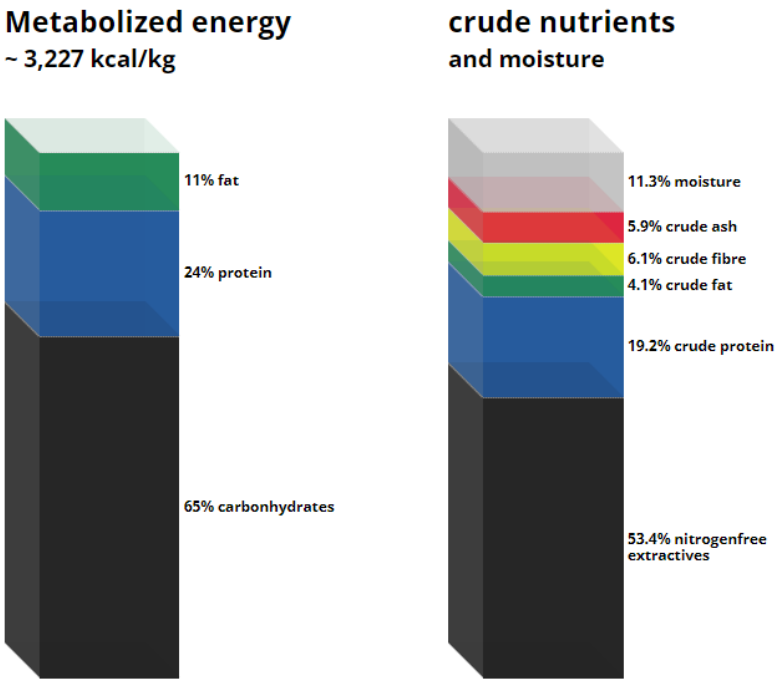

Fig. 4. Altromin 1324 standard diet with contents by total energy intake and proportion of crude nutrients.
(https://altromin.com/products/standarddiets/mice/1320)

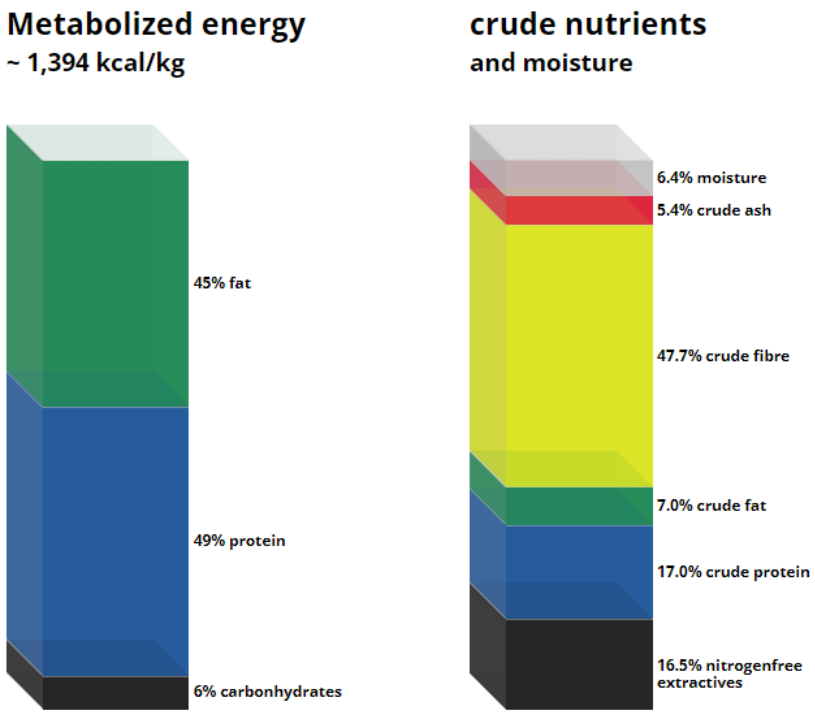

Fig. 5. Carbohydrate-deficient Altromin C1009 diet (LC) with contents by total energy intake and proportion of crude nutrients. (https://altromin.com/products/specialdiets/deficientdiets/C1009)

Experimental design for the study of the LC introduced from the age of 4 weeks and from *in utero* are shown and described of Fig. 6 and Fig. 7, respectively.

4.2.1. T1D incidence

Blood glucose levels were measured in mice every week in the morning via the tail vein cuts, and cut-off value for T1D onset in NOD mice was considered as 12.0 mMol/L. The observation period was from the age of 100 days (14 weeks) to the age of 300 days (42 weeks).

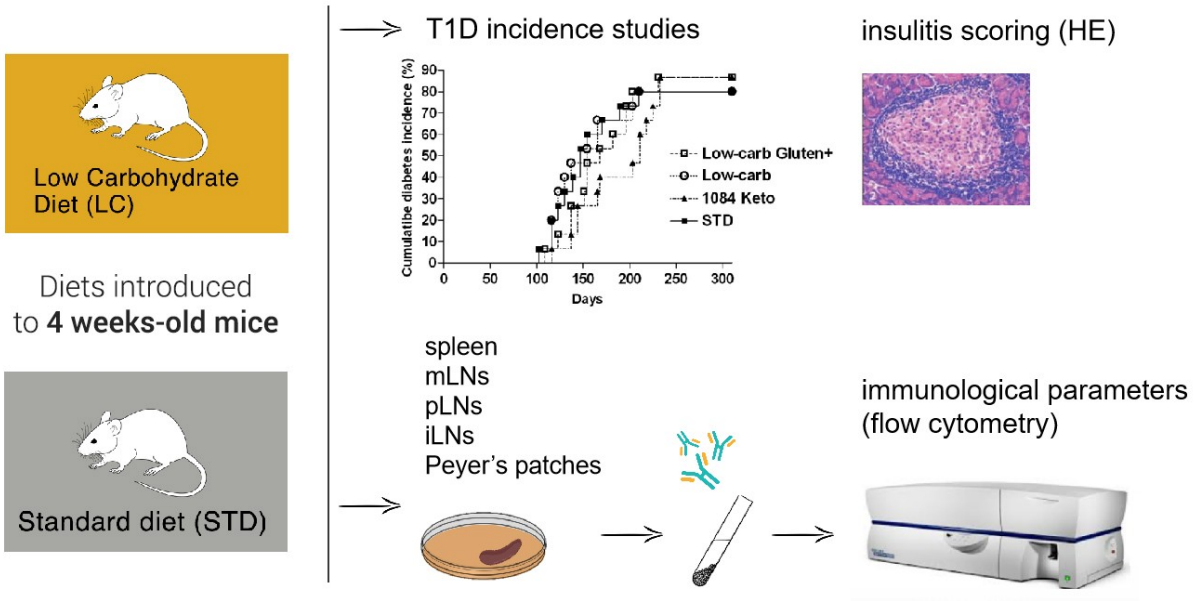

Fig. 6. **LC introduced from the age of 4 weeks, experimental design.** Low-carbohydrate (LC) and standard (STD) Altromin diets were introduced to SPF NOD female mice from the age of 4 weeks until the age of 12 weeks, when they were sacrificed for cytometry analysis and insulitis scoring. T1D incidence in mice was monitored every week from the age of 100 days (14 weeks) to the age of 300 days (42 weeks).

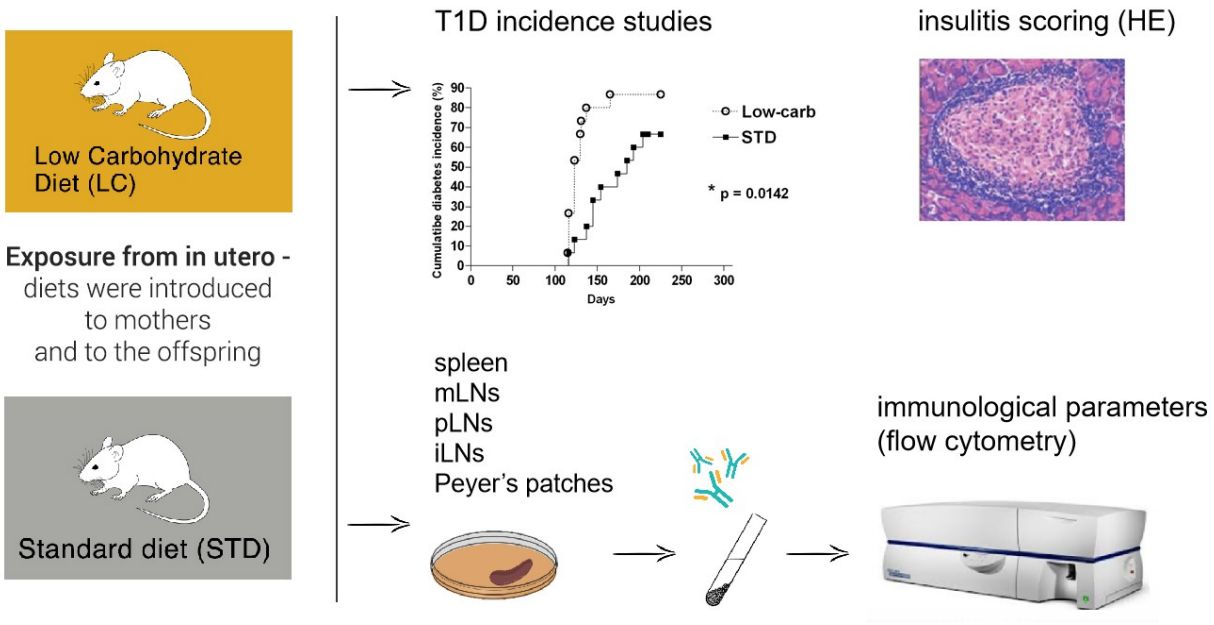

Fig. 7. LC introduced from *in utero*, experimental design. LC and standard (STD) Altromin diets were introduced to SPF NOD female mice from *in utero*, i.e. NOD females were kept on one of these diets from the mating through pregnancy and weaning period, and their offspring were kept on the same diet from the birth until the age of 12 weeks, when they were sacrificed for cytometry analysis and insulitis scoring. T1D incidence in offspring kept on LC and STD was monitored every week from the age of 100 days (14 weeks) to the age of 300 days (42 weeks).

4.2.2. Isolation of mouse organs

To evaluate the effects of LC diet on immunological parameters, in both branches of experiments (exposute to LC diet from the age of 4 weeks or from *in utero*) NOD mice were sacrificed at the age of 12 weeks and dissected for mucosal lymphoid organs - mesentheric lymph nodes (mLNs), pancreatic lymph nodes (pLNs), Peyer's patches (PPs), and systemic lymhoid organs - spleen, inguinal lymph nodes (iLNs).

The organs were placed into a Petri dish with 3 ml of complete medium. In the experiment with LC diet exposure from the age of 4 weeks, small lymhoid organs (PPs, pLNs, and iLNs) were pooled. Then the organs were mechanistically but gently triturated by rubbing them with a tweezer against a rough part of a microscope slide, slide was washed with 400 µl of FACS medium by a pipette and the sample was filtered through a filter into a test tube. Spleen spleens

were further processed to remove erythrocytes. First, the tubes were centrifuged (1200 RPM, 20 °C, 5 minutes), then the supernatant was poured off. The pellet was resuspended, with 1 ml of lysing buffer added, followed by samples incubation for 4 minutes to lyse erythrocytes. To stop the lysis, 30 ml of complete medium were added to the samples, followed by a next round of centrifugation (1200 RPM, 20 °C, 5 minutes), and the supernatant was discarded. The pellet was resuspended, and 4 ml of complete medium was added, followed by cell counting. Absolute number of live cells per sample was counted using 20 µl of the sample, trypan blue stain, Bürker chamber and light microscope. All samples were further processed for flow cytometry staining, which is described below.

4.2.3. Samples processing for flow cytometry

The samples were divided to test tubes according to the required number of cells per tube. 500 µl of FACS solution were added, samples were centrifuged (1300 RPM, 4 °C, 4 minutes), and supernatant was discarded. Next, the pellet was resuspended, and samples were stained with Fixable Viability Dye and fluorochrome-conjugated antibodies according to the used cytometry panels. FACS solution was added to the samples to reach a final volume of 100 µl, followed by 25 minutes incubation of the samples on ice and in dark. After that, unconjugated fluorochrome-conjugated antibodies were washed out from the samples by adding 1 ml of FACS, vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes), and then discarding the supernatant. The pellet was resuspended, fixed, and permeabilized by adding 250 µl of fixation and permeabilization solution (BD Cytofix/CytopermTM), vortexed for at least five seconds, and then the samples were incubated for 20 minutes on ice in the dark. The solution was washed out by adding 1 ml of Perm/Wash solution (diluted from BD Perm/Wash™ Buffer 10x solution), vortexing, centrifuging (1300 RPM, 4 °C, 4 minutes), and discarding the supernatant.

Then antibodies against intracellular markers were added to the pellet, Perm/Wash solution was added to the samples to a final volume of 100 µl, and samples were incubated on ice and in dark for 25 minutes. After the incubation, 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 discarding the supernatant. Finally, FACS solution was added to each sample to reach a final volume of 120 µl, and thereby samples were ready for measurement.

All samples were measured with BD LSR II flow cytometer, and obtained data were analyzed using FlowJo v.10 software.

4.2.4. Insulitis scoring

Insulitis was scored from haematoxylin and eosin (H&E) stained sections of pancreas and graded as follows:
1 – intact islet, 2 – periinsulitis, 3 – insulitis with < 50% of islet infiltration, 4 – insulitis with > 50% infiltration. Minimum of 25 islets per mouse and 6 mice per group were evaluated. Data are expressed as % of a given score.

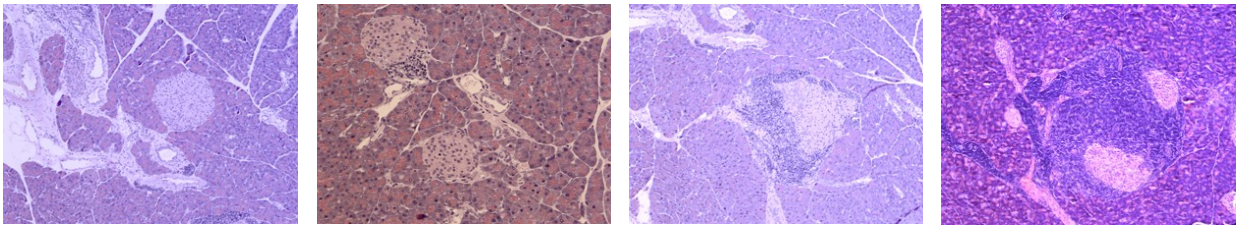

Fig. 8. Insulitis scoring. A) intact islet; B) periinsulitis; C) insulitis with < 50% of islet infiltration; D) insulitis with > 50% infiltration.

4.3. Statistical analysis

Statistical analysis and graphs were done in GraphPad Prism 8.

The log-rank test was used for analysis of T1D incidence in NOD mice. For the cytometry experiments and insulitis scoring, P-values were calculated with the two-tailed unpaired t-test. A P-value of $\leq 0{\cdot}05$ was considered to be statistically significant.

5. Results

5.1. Effects of low-carbohydrate diet introduced to NOD mice from the age of 4 weeks

5.1.1. T1D incidence in NOD mice

When low-carbohydrate diets were introduced to NOD females from the age of 4 weeks, no significant differences were observed in T1D incidence compared with standard Altromin diet. Despite I joined just the end of this experiment, I was allowed to demonstrate these data since they were important for tailoring experiments aimed at investigating the effects of LC diet introduced to NOD mice from *in utero*.

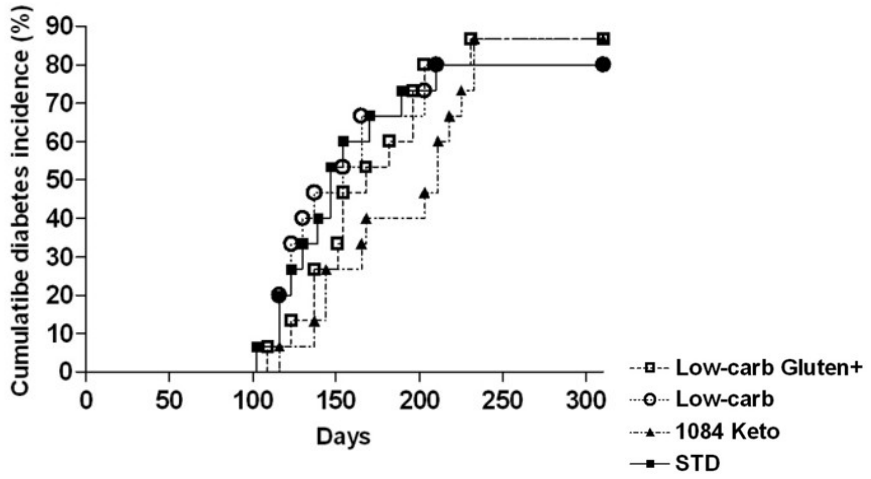

Fig. 9. Cumulative T1D incidence in NOD females exposed to low carbohydrate diets from the age of 4 weeks. 3 low-carbohydrate diets from Altromin were implemented: low-carbohydrate enriched with gluten (Low-carb Gluten+), Low-carb (C1009), and 1084 Keto, compared with standard Altromin diet (1324).

5.1.2. Immunological parameters of different T cell subsets in NOD mice exposed to LC diet from the age of 4 weeks

First, we were evaluating the percentages of immunoregulatory CD4+IL10+, CD4+FoxP3+ Tregs, FoxP3+ IL10+ Tregs in mice exposed to LC diet from the age of 4 weeks. Gating strategy for the above mentioned cell subsets is shown on Fig. 10.

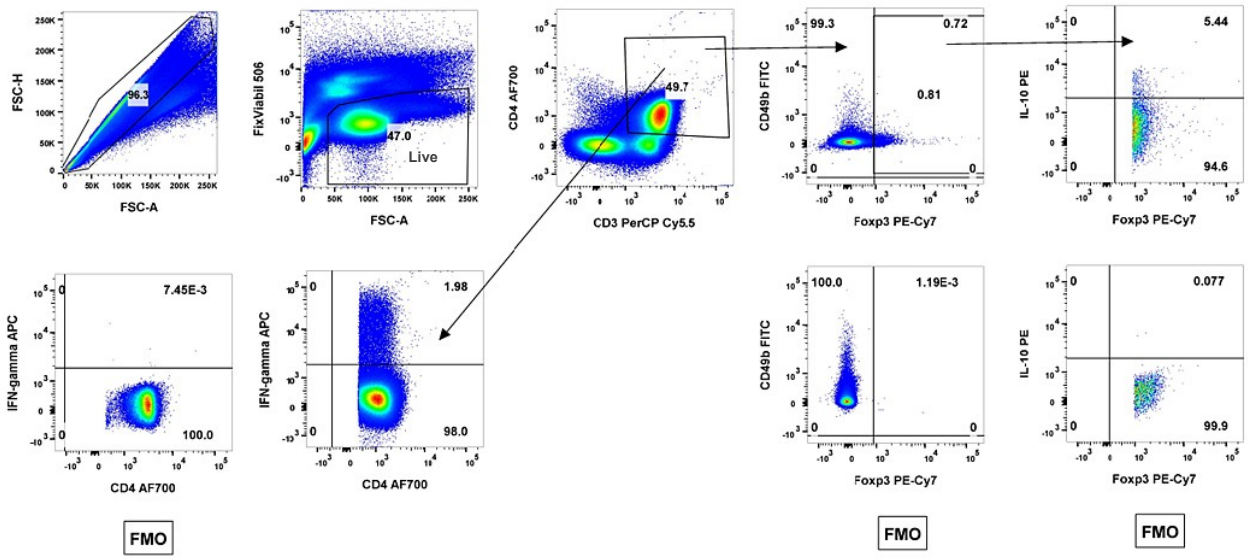

Fig. 10. Gating strategy for CD4+FoxP3+ Tregs, FoxP3+ IL10+ Tregs, CD4+IFNγ+ T cells. The first steps of the gating were excluding doublets, gating leukocytes (FSC-A/SSC-A (not shown), selecting live cells (Fix Viability 506-), followed by CD3+ T cells and CD4+ T cells. Then CD4+ were gated for subsequent analysis of CD4+IFNγ+ T cells (bottom left), FoxP3+ Tregs and FoxP3+IL10+ (first row, right). FMO controls are shown for IFNγ_APC, FoxP3_PE-Cy7, and IL-10_PE. The gating was done with FlowJo v.10 software.

When LC diet was introduced to SPF NOD female mice from the age of 4 weeks, no significant changes were observed in percentages of immunoregulatory CD4+ T cell subsets, such as CD4+IL-10+, CD4+FoxP3+, CD4+FoxP3+IL-10+ Tregs.

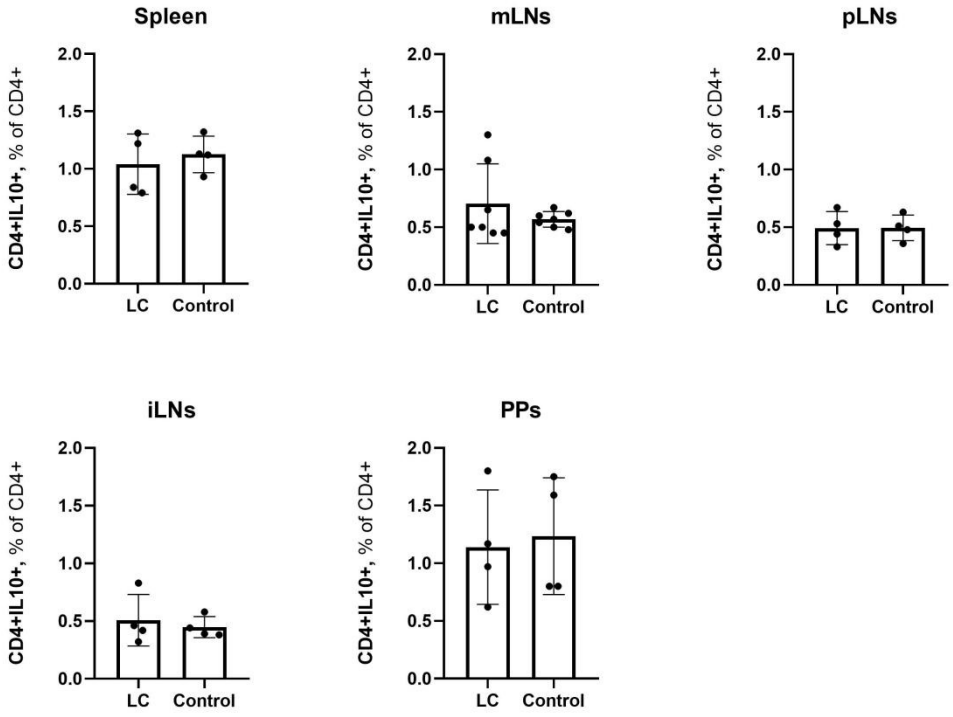

Fig. 11. Percentage of CD4+IL10+ T cells (of total CD4+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*$p<0.05$).

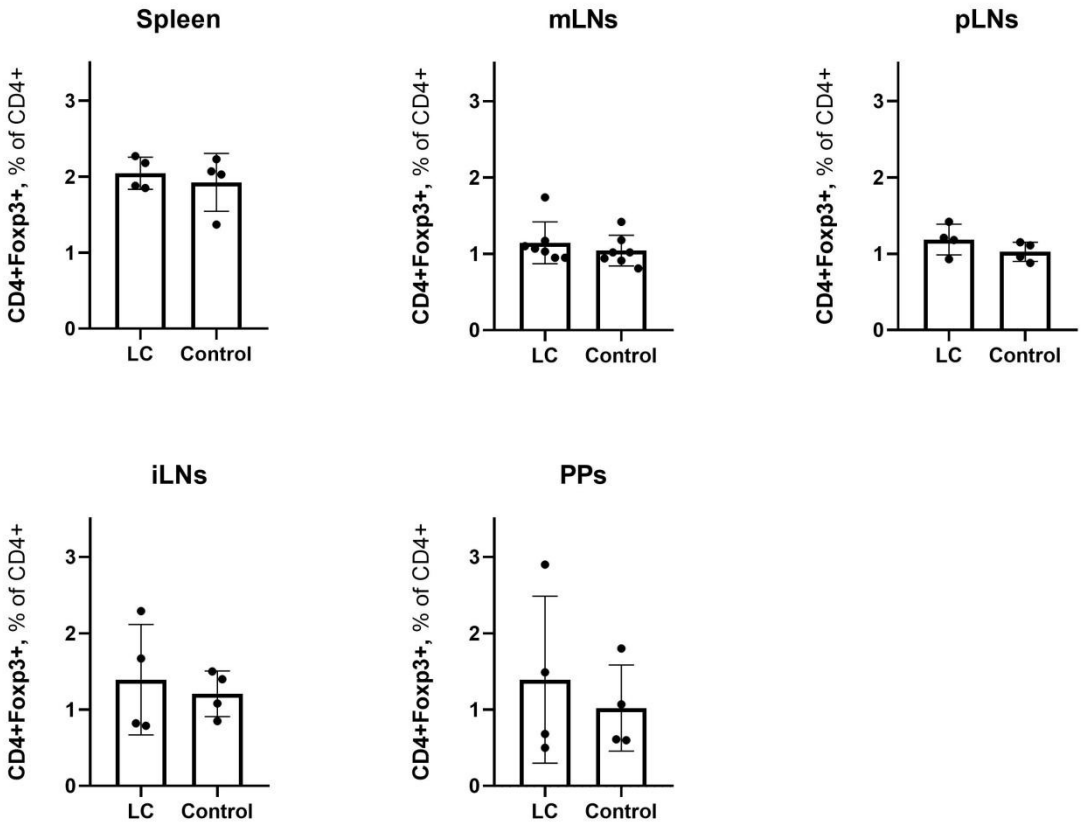

Fig.12. Percentage of CD4+FoxP3+ Tregs (of total CD4+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*$p<0.05$).

Analysis of cytotoxic CD4+ and CD8+ T cells secreting IFN-γ also did not show any statistically significant changes.

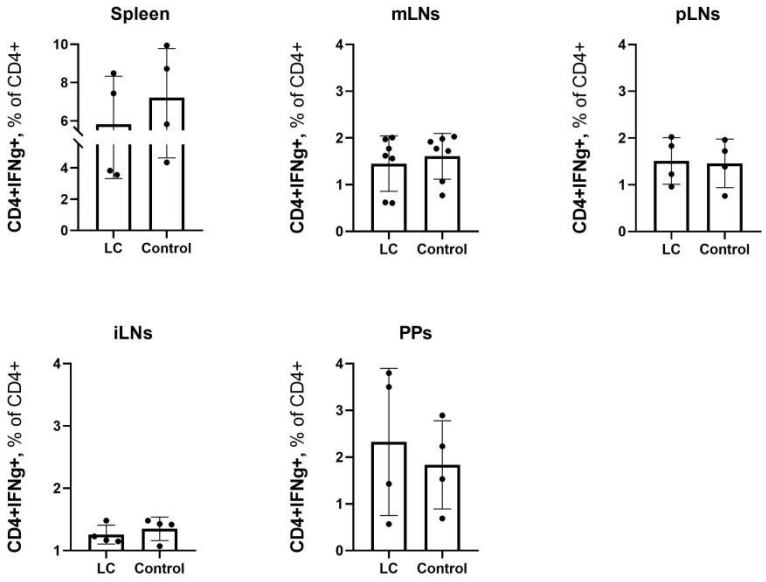

Fig. 13. Percentage of CD4+IFNγ+ T cells (of total CD4+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

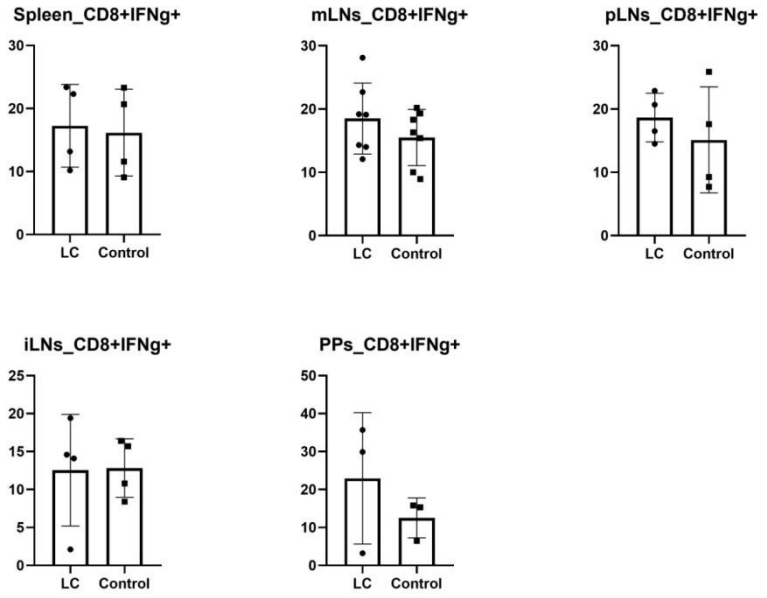

Fig. 14. Percentage of CD8+IFNγ+ T cells (of total CD8+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

Assessing the percentages of potentially immunoregulatory γδ T cells in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks

Since several studies have shown that γδ T cells can provide immunoregulatory effects in T1D, this cell subset was of special interest in this study. The gating strategy is shown below (Fig. 15).

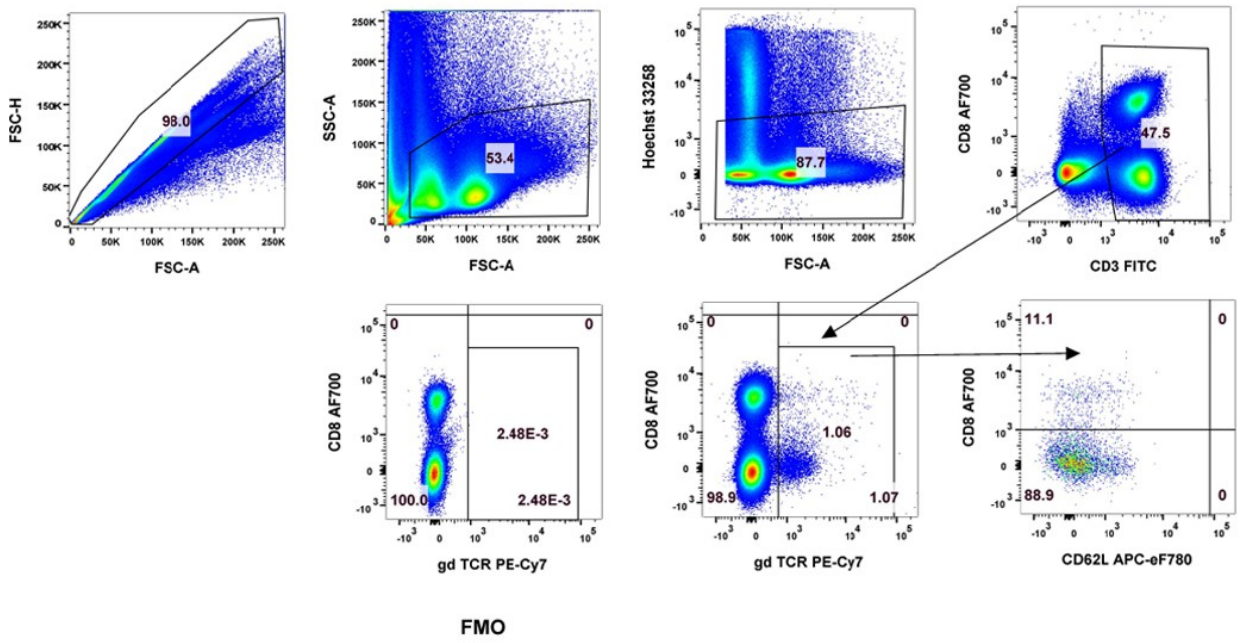

Fig. 15. Gating strategy for γδ T cells and CD8+γδ T cells. Gates were set for the singlets, leukocytes, live cells, CD3+ T cells, followed by CD3+ γδ T cells and CD8+ γδ T cells. The FMO control for γδ TCR_PE-Cy7 is shown in a second row.

Again, when LC diet was introduced to SPF NOD female mice from the age of 4 weeks, no significant changes were observed neither in percentages of total γδ T cells, nor in CD8+γδ T cells compared with the mice kept on standard Altromin diet.

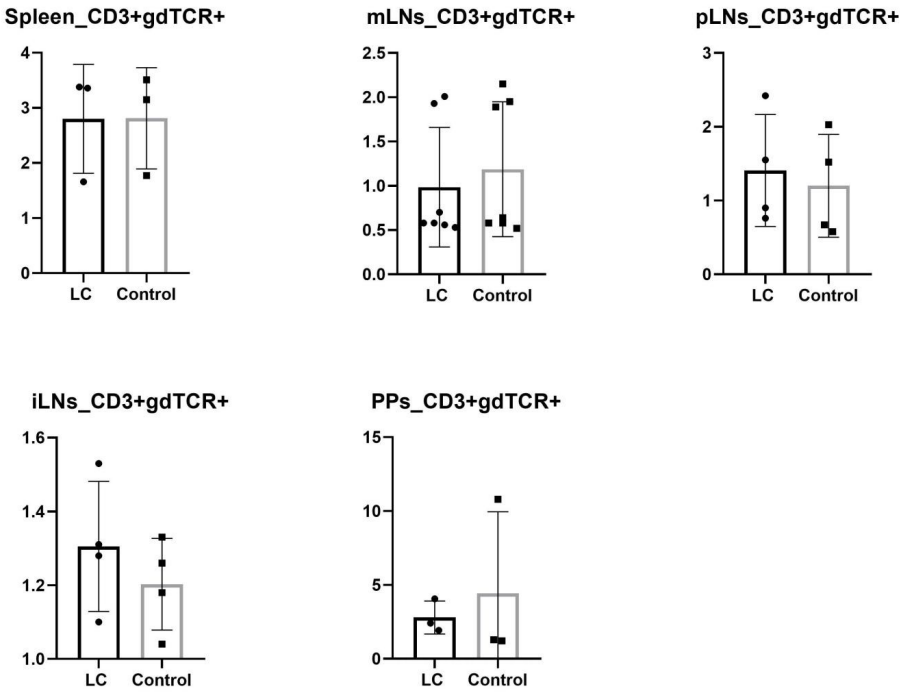

Fig. 16. Percentage of γδ T cells (of total CD3+ T cells) in 12-weeks old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

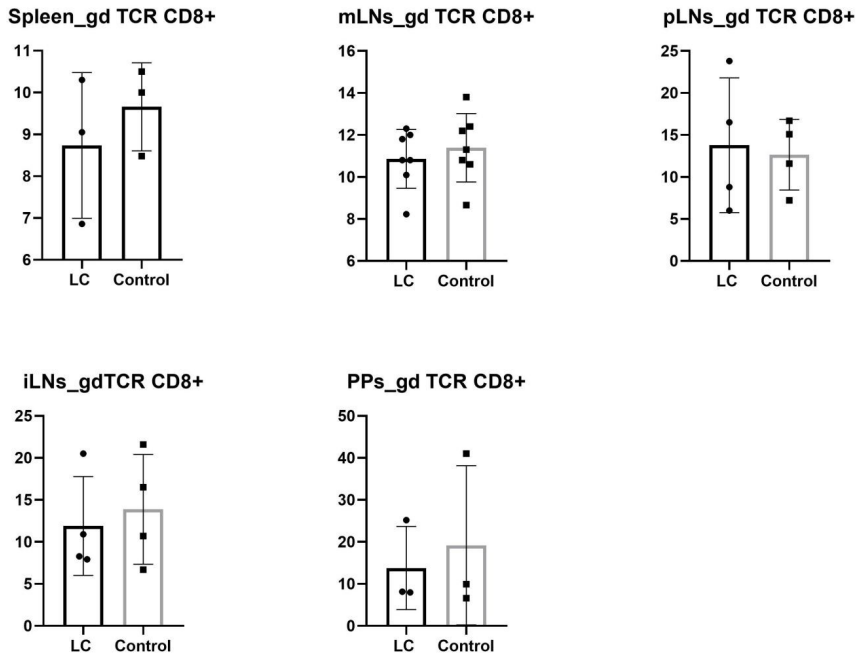

Fig. 17. Percentage of CD8+ γδ T cells (of total CD3+γδ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

Assessing the percentages of migratory T cell subsets in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks

On the next step, we were evaluating the percentages of migratory CD8+ T cell subsets - naive T cells (Tn, CD8+CD44-CD62L+), T central memory (Tcm, CD44+CD62L+), T effector/ effector memory (Teff/em, CD44+CD62L-), and double negative CD44-CD62L- subsets.

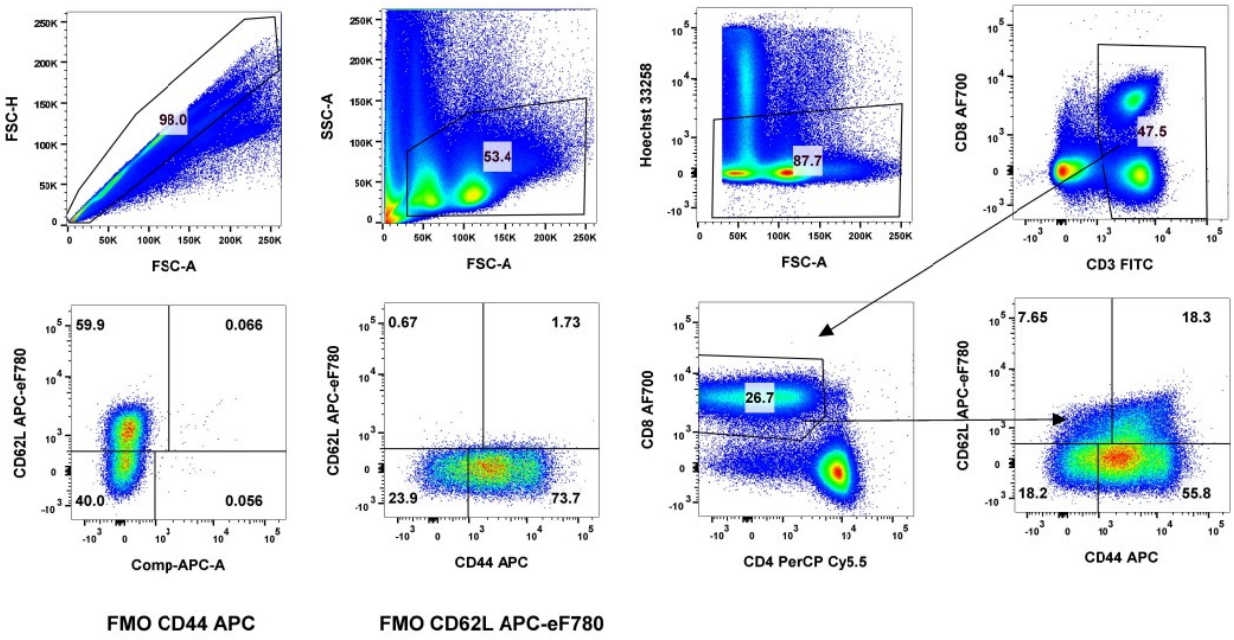

Fig. 18. Gating strategy for migratory CD8+ T cells subsets. Gates were set for the singlets, leukocytes, live total CD3+ T cells, followed by the gating of CD8+ T cells and subsequent discrimination into naive T cells (Tn, CD44-CD62L+), T central memory (Tcm, CD44+CD62L+), T effector memory (Tem, CD44+CD62L-) and double negative CD44-CD62L- subsets. FMO controls are shown for CD62L_APC-H7 and CD44_APC-A.

Considering these T cell subsets, we again did not find any statistically significant changes in NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled. The statistical analysis was done with unpaired t-test (*$p<0.05$).

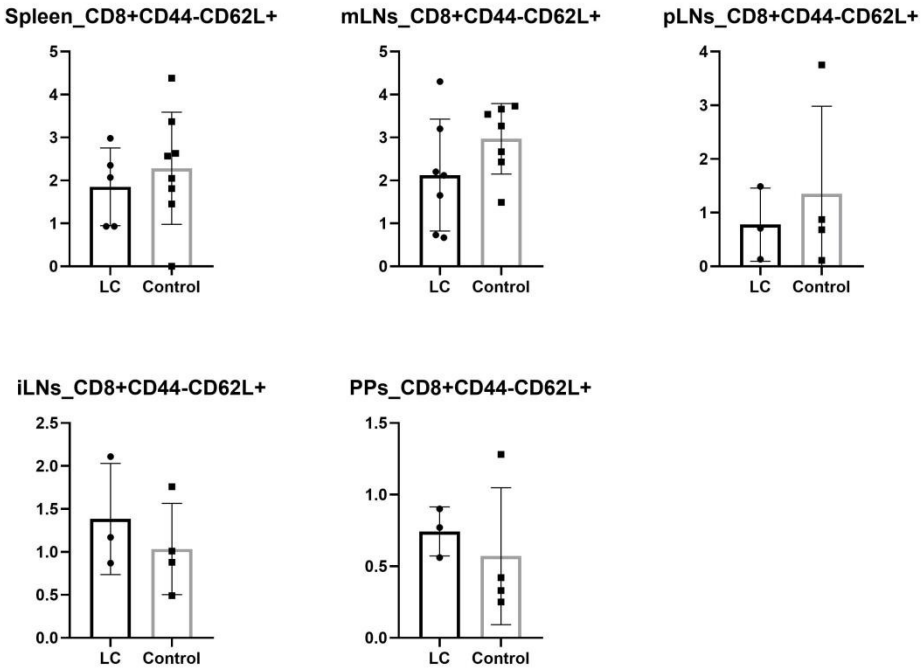

Fig. 19. Percentage of Tn, CD8+CD44-CD62L+ T cells (of total CD8+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

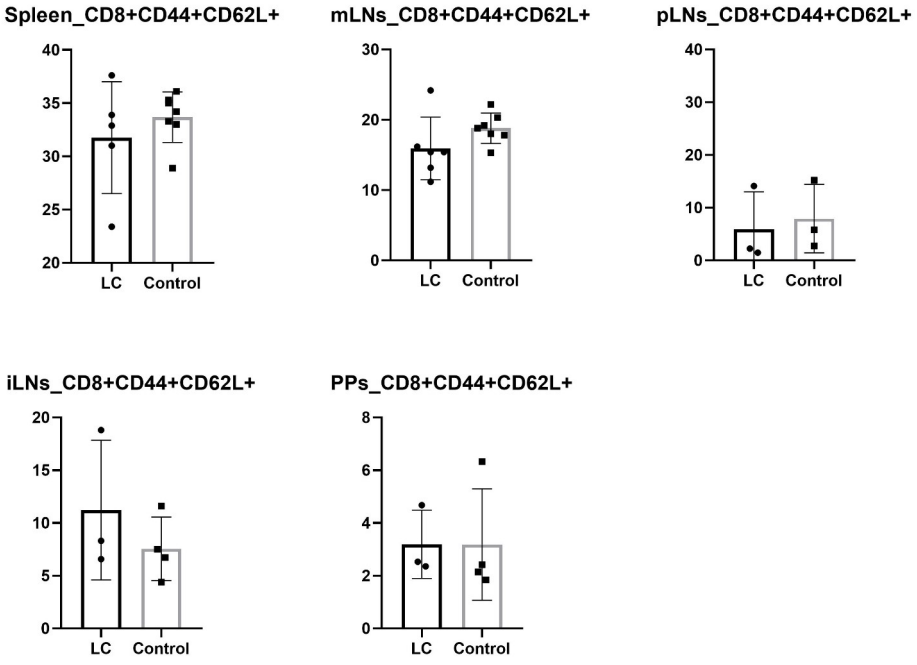

Fig. 19. Percentage of Tcm, CD8+CD44+CD62L+ T cells (of total CD8+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*p<0.05).

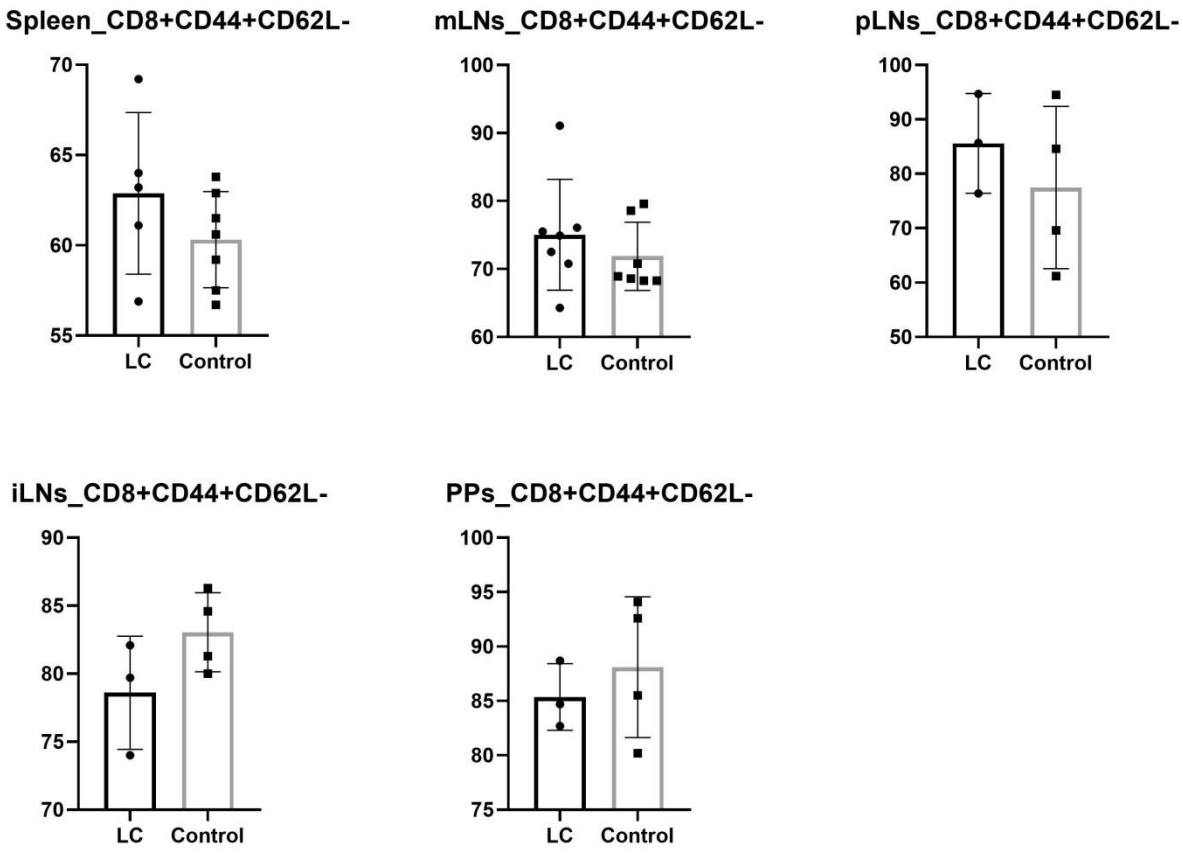

Fig. 20. Percentage of Teff/em, CD8+CD44+CD62L- T cells (of total CD8+ T cells) in 12-weeks-old NOD mice exposed to LC diet from the age of 4 weeks compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from pLNs, iLNs, and PPs were pooled (n=7). The statistical analysis was done with unpaired t-test (*$p<0.05$).

5.1.3. Insulitis scoring in NOD mice exposed to LC diet from the age of 4 weeks

Consistently with T1D incidence and cytometry data, no statistically significant differences were observed in NOD mice exposed to LC diet from the age of 4 weeks compared with standard Altromin diet.

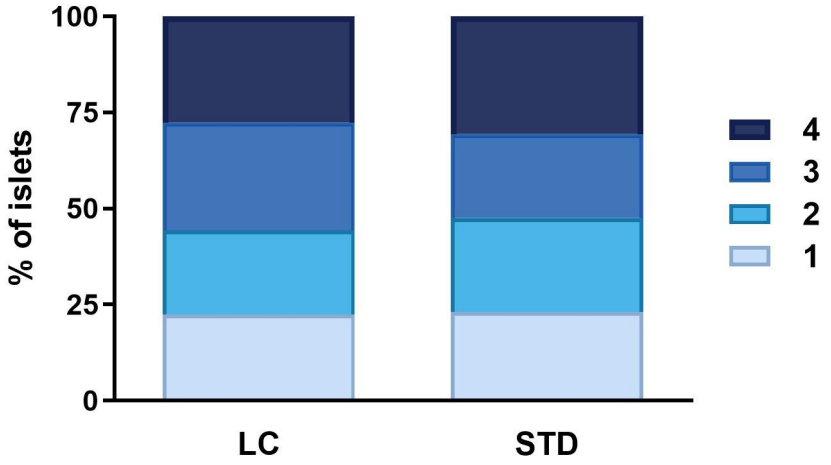

Fig. 21. Insulitis scoring in NOD mice exposed to LC diet from the age of 4 weeks compared with the standard Altromin diet (6 mice per group).

5.2. Effects of low-carbohydrate diet introduced to NOD mice from *in utero*

5.2.1. T1D incidence in NOD mice exposed to LC diet from *in utero*

When LC diet was introduced to SPF NOD mice from *in utero*, surprisingly, there was a highly significant acceleration in cumulative T1D incidence compared with the group kept on a standard diet (p=0.0141).

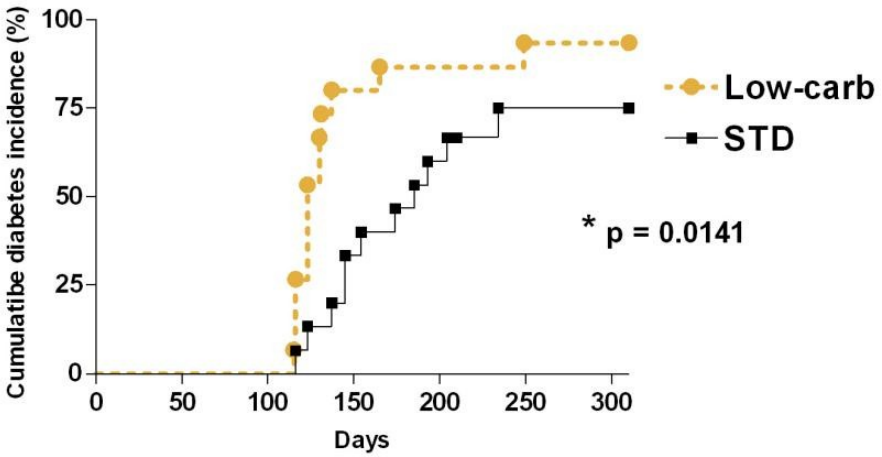

Fig.22. Effect of the LC diet introduced to pregnant females on diabetes incidence in F1 NOD female mice (up to the age of 310 days, n=15). Experiment 1 of 3.

So far, there have been very few environmental exposures which had been shown to accelerate T1D onset in NOD mice. This finding was so unusual, that it has been decided to repeat this experiment. The second T1D incidence experiment on NOD mice with LC diet exposure from *in utero* confirmed the previous results, again with high statistic significance (p=0.0329). The ongoing third experiment confirmed these results (data are being processed), which makes this finding highly reliable.

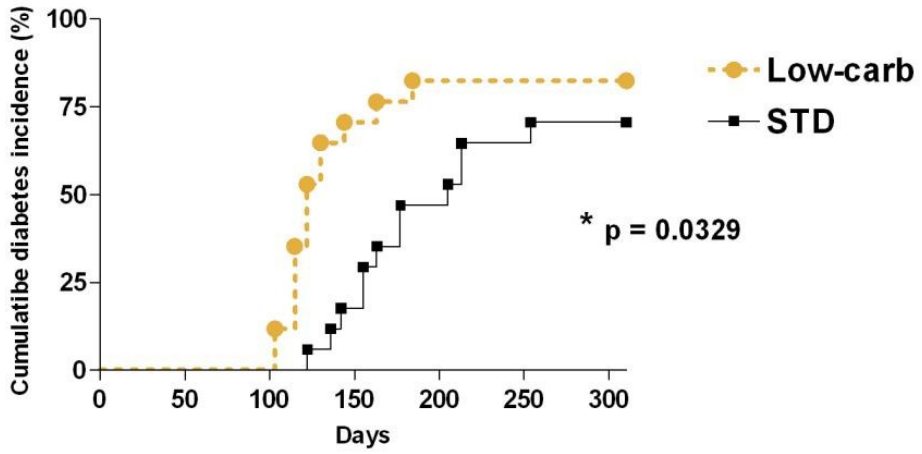

Fig. 23. Effect of the low-carbohydrate diet introduced to pregnant females on diabetes incidence in F1 NOD female mice (up to the age of 310 days, n=17). Experiment 2 of 3.

The next step was to evaluate the immunological effects of LC diet in NOD mice that reached the age of 12 weeks.

5.2.2. Evaluating the percentages of T cell subsets in 12-weeks-old NOD mice exposed to LC diet from *in utero*

When LC diet was introduced from *in utero*, there were statistically significant changes in different immunoregulatory subsets. Among CD4+IL-10+ T cells, a significant decrease was observed in LC group compared with the standard diet group (p=0.0018), while in mLNs the percentage of CD4+ T cells secreting IL-10 was higher than in a control group.

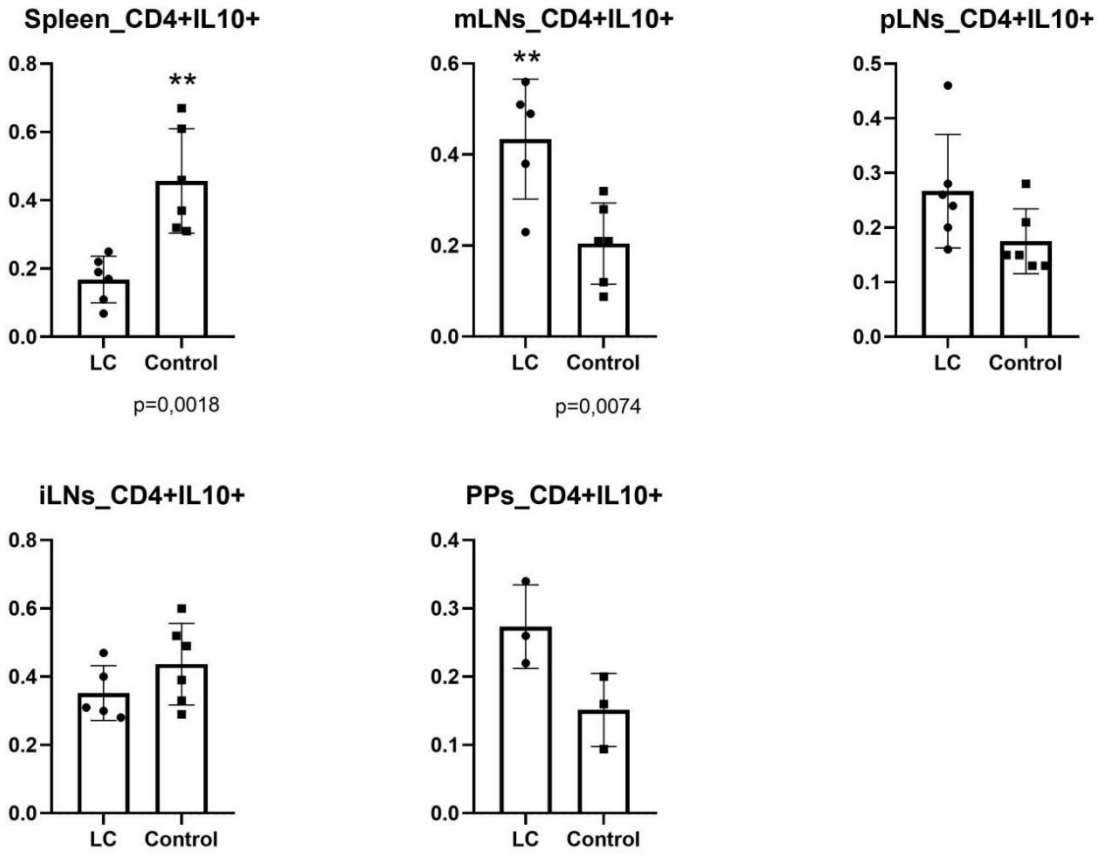

Fig. 24. Percentage of CD4+IL10+ T cells (of total CD4+ T cells) in 12-weeks old NOD mice exposed to LC diet from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*p<0.05).

The percentage of CD4+FoxP3+ Tregs was significantly reduced in spleen in NOD mice exposed to LC diet from *in utero*, in parallel with the reduction of CD4+IL10+ T cells. Moreover, Tregs were also decreased in iLNs, together with the data on spleen, indicating a systemic reduction of FoxP3+Tregs under exposure to LC diet in NOD mice at the stage close to T1D onset.

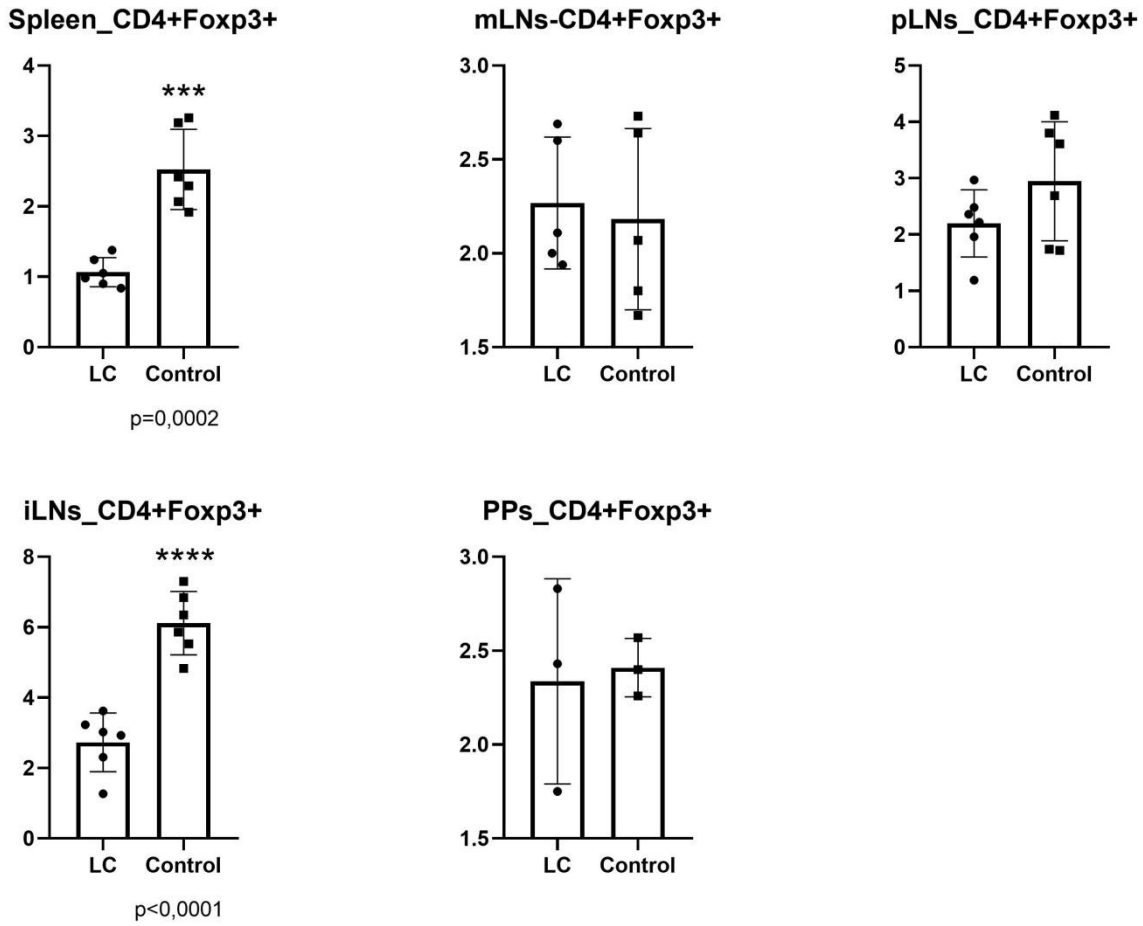

Fig. 25. Percentage of CD4+FoxP3+ T cells (of total CD4+ T cells) in 12-weeks old NOD mice exposed to LC diet from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*$p<0.05$).

Next, when CD4+FoxP3+IL10+ were studied, surprisingly, there were no differences observed in spleen from LC and standard diet groups, while in LC group in mLNs and in iLNs, the Tregs percentages were increased compared with control.

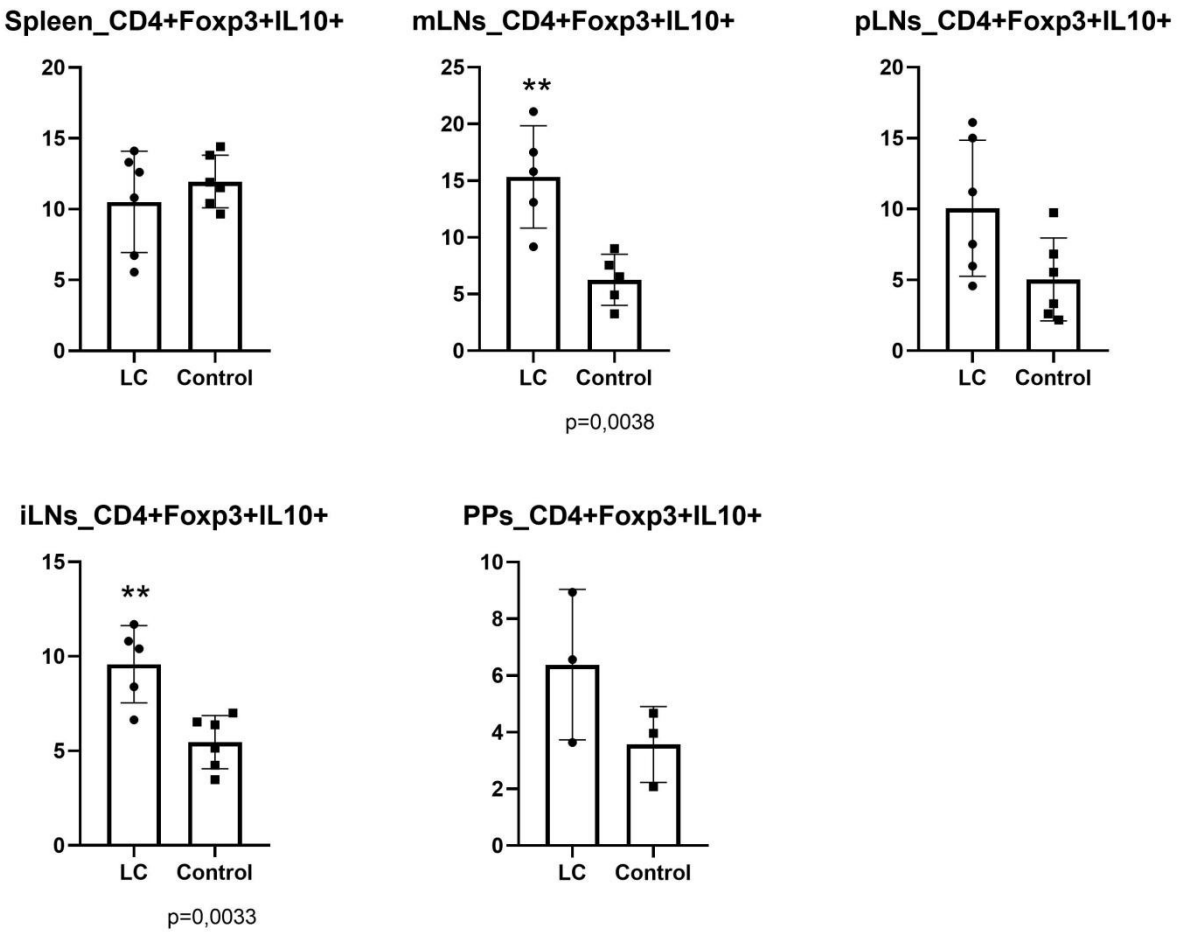

Fig. 26. Percentage of FoxP3+IL10+ T cells (of CD4+FoxP3+ T cells) in 12-weeks old NOD mice exposed to LC diet from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*$p<0.05$).

The next evaluated T cell subset was a subset of Tr1 cells, characterized by CD4+CD49b+ LAG3+ phenotype. Due to the fact that Tr1 cells are highly metabolically active and able to produce high amounts of IL-10 and TGF-β, they are also rapidly dying, which was taken into account when gating live/dead cells as shown below (Fig. 27).

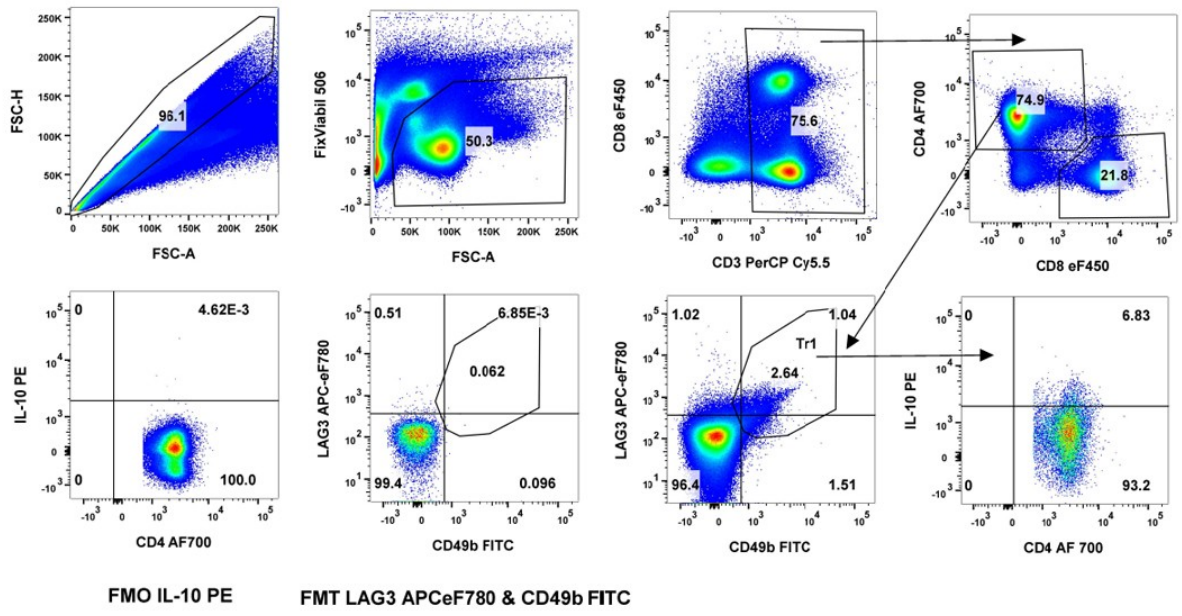

Fig. 27. Gating strategy for CD49b+LAG3+ Tr1 and CD49b+LAG3+IL10+ Tr1 cells. The first steps of the gating were the same as for the previous subsets, i.e. separating singlets, selection of live cells (Fix Viability 506), gating lymphocytes (FSC-A/SSC-A (not shown), CD3+ T cells, and CD4+ T cells. Then CD4+ T cells were gated for Tr1 subset (CD49b+LAG3+) and CD49b+LAG3+IL10+. FMO controls are shown for CD49b_FITC + LAG3+APC-eF7870 and for IL10_PE.

The percentages of Tr1 cells were significantly lower in mice exposed to LC from in utero only in spleen, which was consistent with a decrease in CD4+IL-10+ T cells in spleen in this group. In other lymphoid organs no statistically significant difference was observed.

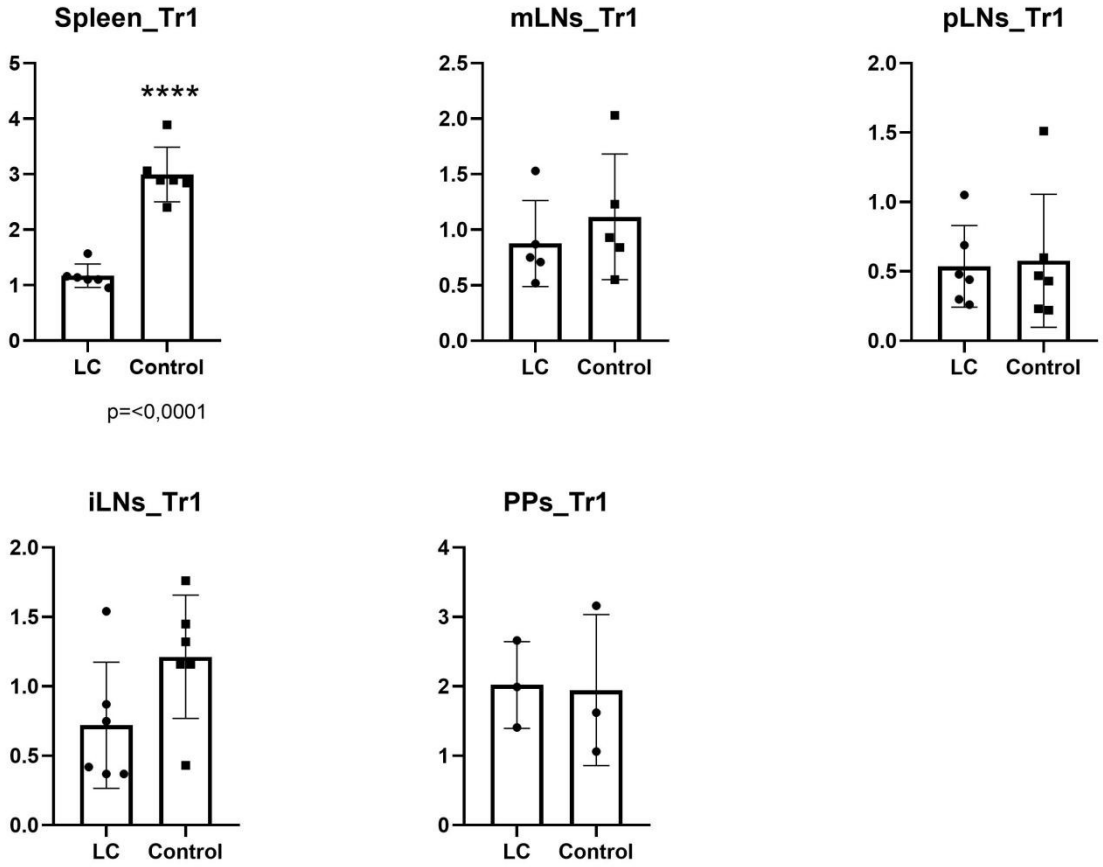

Fig. 28. Percentage of Tr1 T cells (from total CD4+ T cells) in 12-weeks-old NOD mice exposed to LC diet from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*$p<0.05$).

When the percentage of Tr1 IL10+ was assessed, no statistically significant differences were found between the studied groups.

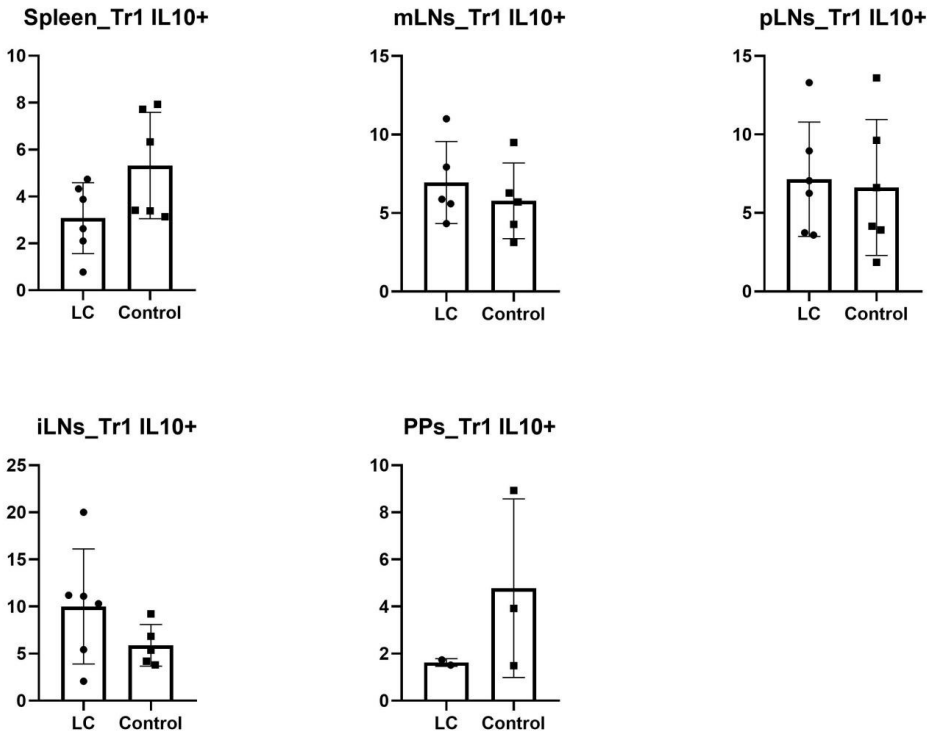

Fig. 29. Percentage of Tr1 IL10+ T cells (of Tr1 cells) in 12-weeks-old NOD mice exposed to LC diet from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*$p<0.05$).

The next cell subset analyzed was potentially immunoregulatory γδ T cells (Fig. 30). The percentages of γδ T cells in 12-weeks old mice exposed to LC diet since in utero were significantly lower in spleen compared with the standard diet group, while in mLNs it was the opposite. No statistically significant differences were observed in other lymphoid organs.

Furthermore, in mice exposed to LC diet from *in utero*, the percentage of CD8+ γδ T cells turned out to be significantly increased in spleen compared to the standard diet group (Fig. 31). At the same time, CD8+ γδ T cells were significantly reduced in PPs in LC diet compared with control.

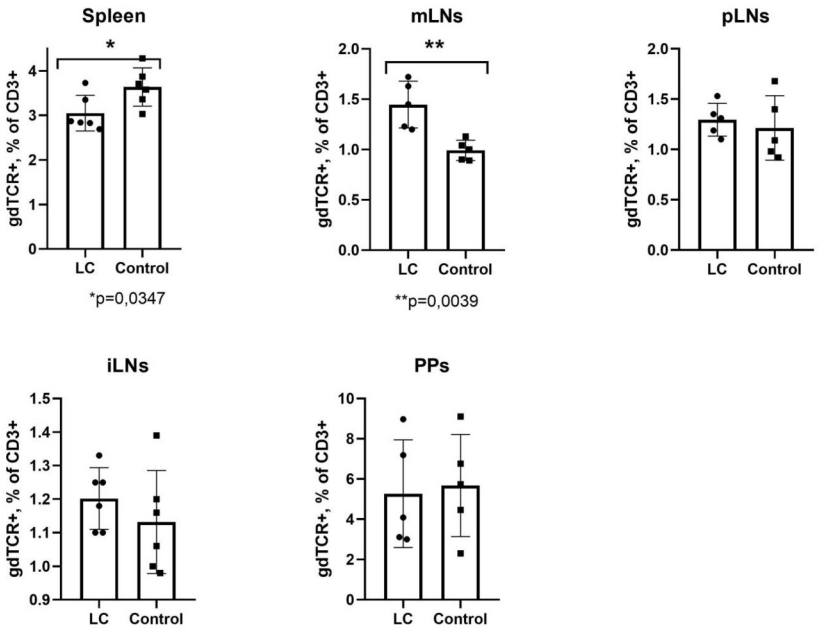

Fig. 30. Percentage of γδ T cells (of CD3+ T cells) in 12-weeks-old NOD mice exposed to LC from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*p<0.05).

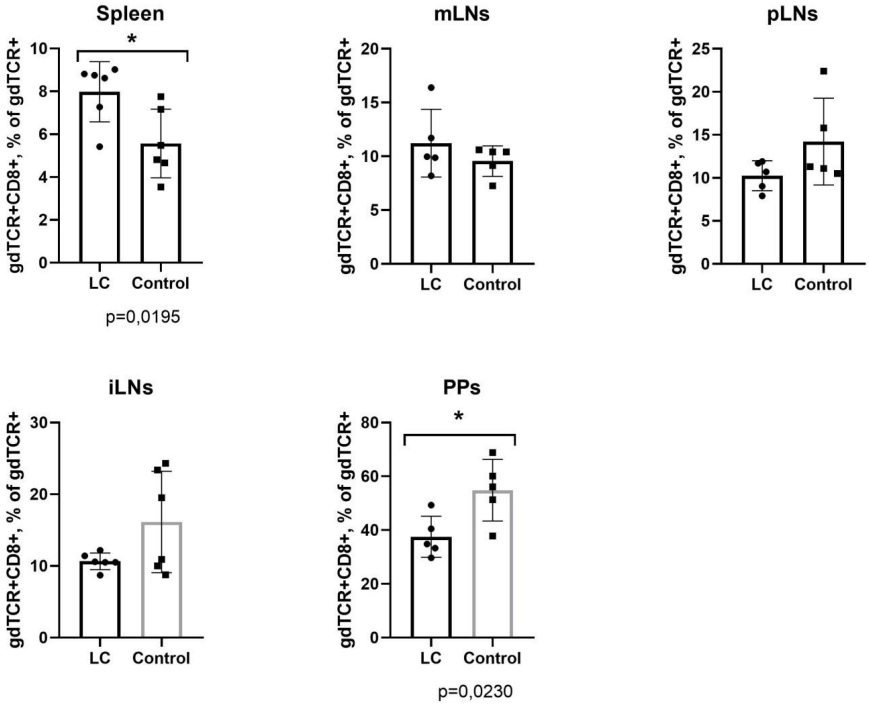

Fig. 31. Percentage of CD8+ γδ T cells (of total γδ T cells) in 12-weeks-old NOD mice exposed to LC from in utero compared with a standard Altromin diet (control) in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. The statistical analysis was done with unpaired t-test (*p<0.05).

Evaluating migratory CD8+ T cell subsets (Tn, Tcm, Teff/em)

Analysis of CD8+ naive T cells (Tn) characterized by the CD8+CD44-CD62L+ phenotype has shown a significant increase of this cell subset in pLNs and iLNs of LC-treated group.

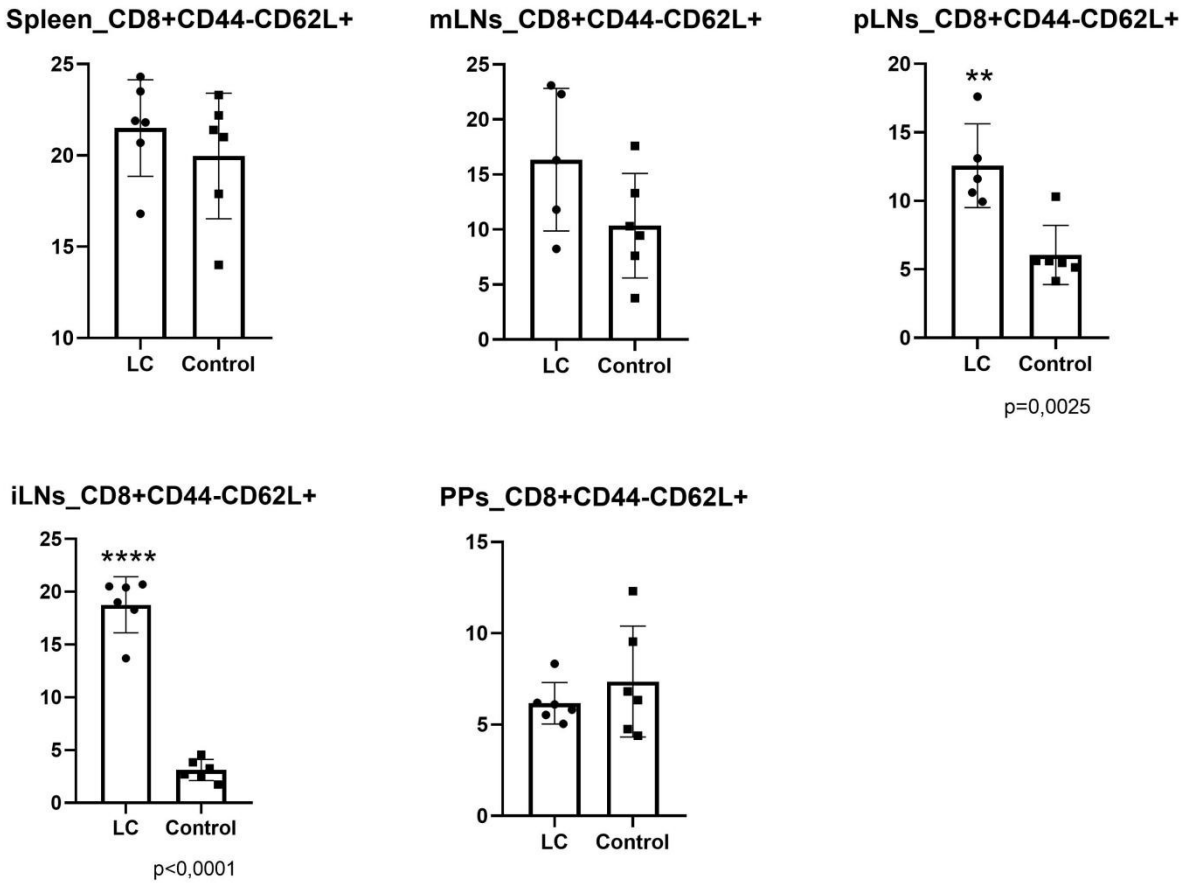

Fig. 32. Percentage of CD8+CD44-CD62L+ T cells (Tn, of total CD8+T cells) in 12-weeks-old NOD mice exposed to LC and standard Altromin (control) diets from in utero in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. . The statistical analysis was done with unpaired t-test (*p<0.05).

As for the CD8+ central memory T cells (Tcm), a decrease in this cell subset was observed in spleen, mLNs, pLNs, and iLNs of LC-treated group.

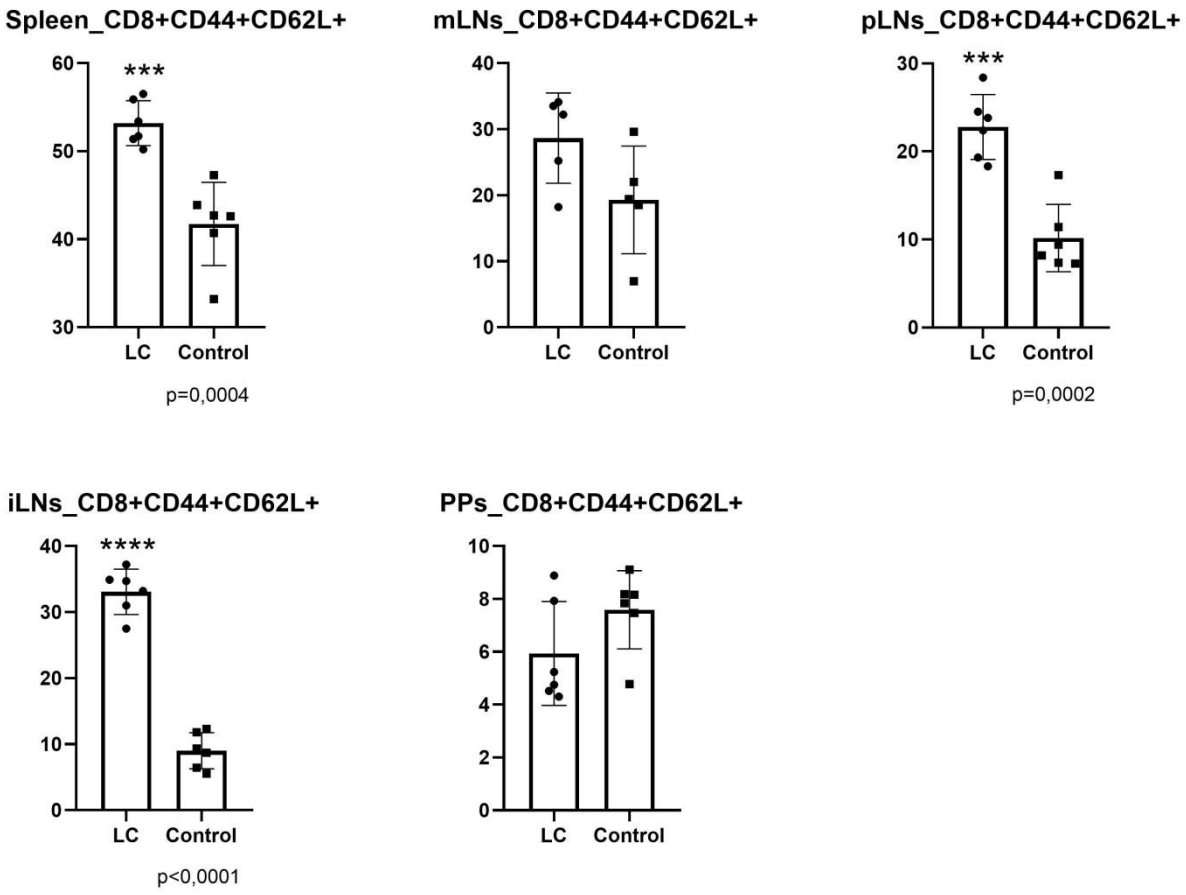

Fig. 33. Percentage of CD8+CD44+CD62L+ T cells (Tcm, of total CD8+T cells) in 12-weeks-old NOD mice exposed to LC and standard Altromin (control) diets from in utero in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. . The statistical analysis was done with unpaired t-test (*$p<0.05$).

Finally, analysis of CD8+ effector/effector memory (Teff/em, CD8+CD44+CD62L-) cells has shown a decrease in these cells in spleen, mLNs, pLNs, and iLNs in a group exposed to LC from *in utero*, also indicating a localization of Teff/em in the pancreas of NOD mice.

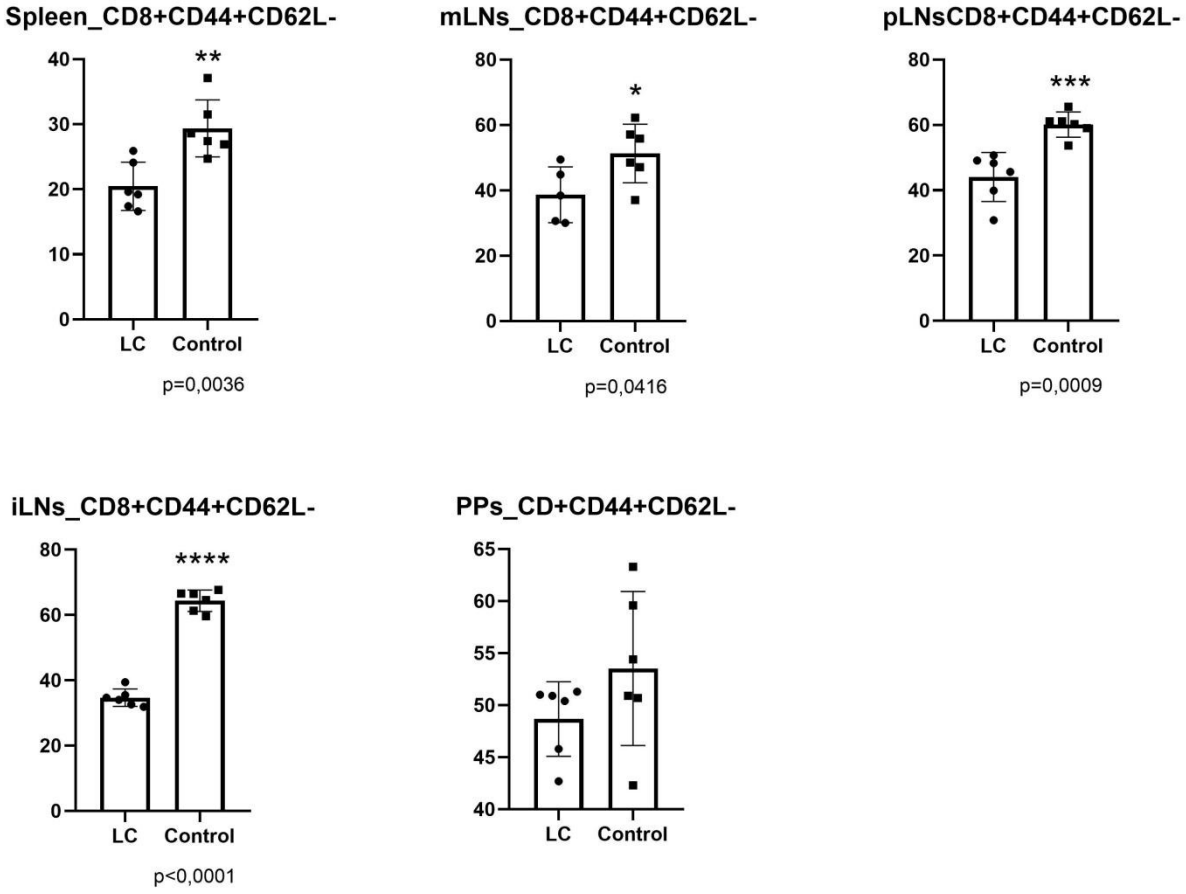

Fig. 34. Percentage of CD8+CD44+CD62L- T cells (Teff/em, of total CD8+T cells) in 12-weeks-old NOD mice exposed to LC and standard Altromin (control) diets from in utero in spleen, mLNs, pLNs, iLNs, and PPs. Samples from PPs were pooled. . The statistical analysis was done with unpaired t-test (*$p<0.05$).

5.2.3. Insulitis scoring in NOD mice exposed to LC diet from *in utero*

Insulitis scoring of pancreas of NOD mice has shown a statistically significant increase in stage 4 with highly infiltrated islets in NOD mice exposed to LC diet from *in utero* compared with a control group. These data were consistent with T1D incidence data and immunological changes we observed in the LC group.

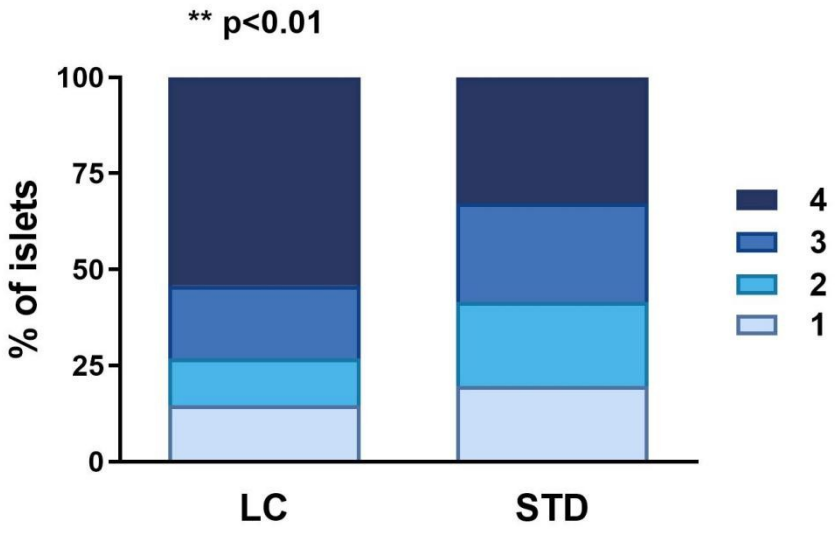

Fig. 35. Insulitis scoring in NOD mice exposed to LC diet from *in utero* compared with the standard Altromin diet (6 mice per group).

5. Discussion

Accumulating evidence suggests that various dietary factors can regulate Tregs development directly or indirectly, e.g. through changes in the gut microbiota and their metabolites. To date, two dietary factors have been known to induce thymic Tregs (tTregs): 1) short-chain fatty acids (SCFA), which is a byproduct of fermentation of complex carbohydrates (found in dietary fibre and resistance starch) by the gut microbiome, and 2) dietary cholesterol, derived from animal products and prevalent in the Western diet [Tan, Taitz et al., 2022]. As for peripheral Tregs (pTregs), they can arise from naive CD4+ T cells dependently or independently of peripheral APCs, and their differentiation is dependent on environmental stimuli, such as TGF-β, microbiome-derived metabolites and dietary factors. Under physiological conditions, the majority of Tregs in the gut are pTregs, with pTregs playing an essential role in maintaining the gut homeostasis [Cheru, Hafler, Sumida 2023].

It has been shown by multiple studies that alterations in the gut microbiome composition and diversity can delay and/or suppress the natural development of T1D in NOD mice [Simon et al., 2020; Livanos et al., 2016;]. Studies on germ-free NOD mice have shown that lack of microbial signals from the gut promoted Tregs dysfunction and a rapid development of autoimmune diabetes [Ostman et al., 2006].

In our experiments, we have used F1 and F2 generations of SPF NOD mice (NOD/ShiLtJ strain from the Jackson Lab., Bar Harbour, USA). The breeding nucleus imported from the Jackson Laboratories is from positively-defined SPF conditions in which the mice are colonized with only 8 bacterial species (altered Shaedler flora, ASF). Unlike many years ago, when frequent germ-free rederivations of breeding nucleus of NOD mice was required to maintain high diabetes incidence in the commercial breeding colonines, today`s NOD/ShiLtJ mice from the Jackson Lab. are phenotypically very close to the germ-free NOD mice (diabetes incidence, insulitis) [Lyte et al., 2019]. Due to such a restricted microbiome composition and short term exposure (F1 or F2 generations) to negatively-defined SPF conditions (according to the FELASA guidelines) [Guillen, 2012] in our animal facility, mice used in our experiments are already displaying very rapid development and high incidence of diabetes, most likely due to a lack of stimulation of the immune system with danger stimuli (in line with the hygiene hypothesis). Thus, in addition to a polygenic predisposition, these mice are strongly prone to the development of autoimmune diabetes [Pfefferle et al., 2021].

Complex carbohydrates such as dietary fibre and resistant starch are known to be a major source of energy for commensal bacteria, being fermented to SCFA, which in turn have prebiotic properties by stimulating the growth of beneficial bacteria that can utilize these substrates [Tan et al., 2021]. In our study, the low carbohydrate diet was composed in a way that 45% of energy came from fat, 49% from protein, and only 6% from carbohydrates. Analyzing cytometry data, we observed a decrease in CD4+FoxP3+, CD4+IL-10+, and Tr1 cells in the spleen of NOD mice kept on LC diet from in utero.

Despite very limited gut microbiota composition of commercial SPF mice, the study of Smith et al. has shown that even in SPF mice, altered Schaedler microbiota produce large amounts of SCFAs in vivo and in vitro [Smith et al., 2013]. The study of Bihan et al. using an isotope-based strategy for absolute quantification of SCFAs in complex biological samples (SQUAD) analyzed SCFAs in the caecal contents of germ-free versus conventionally raised SPF mice. This study has found that acetate was the most abundant SCFA in both types of mice and in SPF mice it was present at 200-fold higher concentration compared to the germ-free animals [Bihan et al., 2022].

Our study did not include metabolomic and microbiome analyses, but since bacteria are known having mainly preventive effects on diabetes incidence in NOD mice (reviewed in [Zhou et al., 2020]) , and the NOD/ShiLtJ mice from the Jackson Lab. are phenotypically very close to the germ-free NOD mice, we hypothetize, that it is more likely that the effect of LC diet since in utero is gut microbiota-independent. Although there is only a limited window for testing increased/accelerated diabetes incidence compared to germ-free NOD mice, such experiment should be carried out using long-term germ-free NOD mouse colony to give a clear-cut answer. The data we obtained still allows to raise several hypothesis: a hypothesis that LC diet introduced to NOD mice early from in utero induced via direct metabolic influences changes in early Treg development and maturation, thus resulting in accelerated development of autoimmune diabetes. The metabolomics of direct effects of diets without presence of gut microbiota has not yet been adequately developed [Castro-Alves et al., 2022], at least in the field of T1D. It is also possible to hypothetize, that low levels of dietary carbohydrates in LC diet could still reduce SCFA production by the limited gut microbiota in our NOD mice, and thus influence Tregs development at least partly via gut microbiota. This would however require a careful comparison with the germ-free NOD mice. A recent study of Jacob et al. has shown that butyrate administration to NOD mice from diabetes onset (15-25 weeks of age) and at 4 weeks of age reduced the progression of hyperglycaemia in diabetic mice and delayed diabetes onset in the early-intervention group with a significant reduction in insulitis. The authors observed

butyrate-induced increase in Tregs in mLNs, colon, PPs; consistently, Tregs depletion abrogated the effects promoted by butyrate administration [Jacob N. et al., 2020]. This study demonstrates the importance of SCFA in maintaining Tregs function and protection from diabetes in NOD mice.

In a study of Clark et al. using high-fat diet with 44.6% calories coming from fat, 40.7% from carbohydrates, and 14.7% from protein, exposure of NOD mice from the age of 4 weeks up to 40 weeks to this high-fat diet prevented development of autoimmune diabetes and protected pancreatic beta-cells from destruction. These protective effects turned out to be mediated by alterations in gut microbiome, increase in Tregs proportion, and decreased insulitis [Clark et al., 2021]. These data suggest the important role not only of fats, but also of carbohydrates in maintaing gut microbiome and immune homeostasis. Moreover, the mentioned study found that in NOD mice fed with a high-fat diet, CD4+FoxP3+ Tregs were significantly increased in the spleen but not in pancreatic lymph nodes, and when the authors depleted Tregs in 25-week-old nondiabetic NOD mice on high-fat diet, there was a 6-fold increase in diabetes incidence compared with age-matched untreated NOD mice on the same high-fat diet (60% compared with 10.5%, respectively). In our study, CD4+FoxP3+Tregs were significantly decreased in the spleen of mice fed with LC diet since in utero compared with the mice kept on standard chow diet, suggesting that LC diet introduced since in utero influnces/targets early Tregs development and maturation, that is necessary for prevention of full penetrance of diabetes incidence in NOD mice..

The study of Schuster et al. using helios and neuropilin-1 (Nrp1) to distinguish tTregs from pTregs has shown that CD4+Foxp3+ Tregs in the spleen and mLNs are predominantly of thymic origin (tTregs), while gut-associated lymphocytes contented a high frequency of pTregs (25-30% of all colonic CD4+pTregs) [Schuster et al., 2018]. Our data documenting a decrease in CD4+FoxP3+Tregs and peripherally-induced CD4+IL-10+ and Tr1 cells in the spleen of mice exposed to LC since in utero are in line with the literature data on the essential role of early life dietary components on development of peripheral Tregs and Tr1 cells [Tan, Taitz, et al., 2022]. Thus, we suggest that prenatal exposure to low carbohydrate diet since in utero affects both thymic-derived and peripheral Tregs.

These data are consistent with the curves of cumulative T1D incidence (and insulitis scoring) , which were significantly higher in NOD mice exposed to LC diet since in utero compared with the standard diet group. It would be interesting to expand our data with experiments including adoptive transfer of diabetes to NOD-SCID recipients, to test the diabetogenicity of leukocyte

subsets from LC fed NOD mic as well as to investigate changes within the pancreas infiltrate, and how LC diet effects the gut microbiome composition in SPF NOD mice.

Dietary manipulations should affect mainly the mucosal immune compartment (mLNs) , however, our data did not show a prominent changes in the draining pLNs that is also a mucosal lymphoid organ, and the most critical LN for induction of effector but also regulatory immune responses, and thus most important lymphoid organ affecting development of diabetes in NOD mice [Jaakkola et al., 2003; Gagnerault et al., 2002; Clare-Salzler et al., 1992].

The fact that the LC diet has no influance on diabetes incidence if applied at age of 4 weeks corresponds well with similar findings in case of the highly diabetes-preventive gluten-free diet in NOD mice, that is also effective only if introduced prenately and has no effect on diabetes incidence from the age of 4 weeks or later [Hansen et al., 2014].

Interestingly, the percentages of γδ T cells in a group exposed to LC from in utero followed the same pattern as CD4+FoxP3+, CD4+IL-10, and Tr1 in spleen, showing significantly lower percentage compared with the control. This pattern corresponds to the opposite change found with the highly preventive gluten-free diet in T1D, if introduced prenatally. In addition it is in line with the data on immunoregulatory role of γδ T cells in T1D and their role in mechanism of induction of mucosal, oral tolerance [Harrison et al., 1996; Locke et al., 2006].

Finally, considering the effects of LC diet from in utero on effector and memory T cell subsets, we observed that CD8+ Tcm (CD44+CD62L+) cells were generally increased on the LC diet introduced from in utero, with highly significant increases in spleen, pLNs and iLNs. Reciprocal decrease was found in effector (CD44+CD62L-) CD8+ T cells, possibly indicating a localization of Teff/em in the pancreas of NOD mice corresponding to our findings of a faster and higher cumulative T1D incidence as well as insulitis scoring in the LC diet fed group of NOD mice. It has been recently shown that a very few effector T cells propagating from pancreatic lymph nodes may effectively lead to the development of T1D in NOD mice [Gearty SV et al., 2022]

Our data present a surprising finding - an accelerated development of T1D in NOD mice if exposed to the LC diet prenatally, and this topic, indeed, requires further investigations (e.g. adoptive transfer models, germ-free conditions, studies on pancreas-infiltrating lymphocytes, sc-RNAseq etc.). Because LC diet is one of a few, rare manipulations that leads to the disease acceleration, our data also call for caution with the use of LC diet in humans.

6. Summary

Despite the advances in immunological research and a many clinical trials in more than the last two decades, exogenous insulin therapy still remains a standard of care in T1D. Apart from the insulin therapy, LC diet can be considered as a feasible approach for a better glycaemia control in diabetic patients. However, the studies on possible long-term effects of LC diet on immune parameters, especially during early life stages and immune system formation, are still missing. This thesis explores the effects of LC diet on development of T1D, insulitis and alterations of immune parameters using SPF NOD mice, when introduced from the age of 4 weeks and from in utero.

We did not observe any significant effect of LC diet on diabetes incidence when it was introduced to NOD females from the age of 4 weeks. In line with these data, we found no substantial differences in cell subsets percentages in mucosal (mLNs, pLNs, PPs) and non-mucosal (spleen, iLNs) lymphoid organs in 12-week-old NOD mice fed with LC diet from the age of 4 weeks. This finding was reflected by no substantial differences in insulitis scoring in pancreata of NOD mice fed LC and standard diet from 4 weeks of age.

When investigating the effects of LC diet introduced to NOD females since??? in utero, we obtained an unexpected and interesting finding of statistically significantly accelerated diabetes incidence in NOD mice fed with LC diet versus the standard diet, which was confirmed by three independent experiments. This effect was reflected by changes in immunoregulatory T cell subsets in the LC diet group, with a statistically significantly decreased CD4+IL-10+ T cells , Foxp3+ Tregs , Tr1 cells, and γδ T cells preferentially in the spleen, but also in some other lymphoid organs (e.g. mLNs). CD8+ Tcm were generally increased on the LC diet introduced since in utero, while CD8+ effector T cells were decreased, possibly reflecting an accelerated migration of effector T cells to the pancreas of mice fed with the LC diet compared to the NOD mice fed a standard diet. These data are in line with the insulitis scoring in panreata of NOD mice fed with the LC and standard diet since in utero.

Thus, this study brings new findings about the influence of LC diet on immuno-regulatory and effector T cell subsets and an acceleration of T1D if introduced early enough, i.e. when it is introduced to NOD mice prenatally, since in utero. A very rare acceleration of the development of T1D in NOD mice by an environmental factor – the LC diet, may shed more light on the pathogenesis of the disease. Further multidisciplinary experiments are needed to elucidate the complex effects of LC diet on developing immune system in NOD mice.

7. References

* - reviews

1. https://diabetesatlas.org/idfawp/resource-files/2022/12/IDF-T1D-Index-Report.pdf

2.*Zajec A, Trebušak Podkrajšek K, Tesovnik T, Šket R, Čugalj Kern B, Jenko Bizjan B, Šmigoc Schweiger D, Battelino T, Kovač J. Pathogenesis of Type 1 Diabetes: Established Facts and New Insights. Genes. 2022; 13(4):706. https://doi.org/10.3390/genes13040706

3. Chiou, J., Geusz, R.J., Okino, ML. et al. Interpreting type 1 diabetes risk with genetics and single-cell epigenomics. Nature 594, 398–402 (2021). https://doi.org/10.1038/s41586-021-03552-w

4. Patterson CC, Harjutsalo V, Rosenbauer J, Neu A, Cinek O, Skrivarhaug T. Trends and cyclical variation in the incidence of childhood type 1 diabetes in 26 European centres in the 25 year period 1989–2013: a multicentre prospective registration study. Diabetologia. 2019;62(3):408–417.

5. Triolo TM, Fouts A, Pyle L, Yu L, Gottlieb PA, Steck AK; Type 1 Diabetes TrialNet Study Group. Identical and Nonidentical Twins: Risk and Factors Involved in Development of Islet Autoimmunity and Type 1 Diabetes. Diabetes Care. 2019 Feb;42(2):192-199. doi: 10.2337/dc18-0288.

6. *Haupt-Jorgensen M, Holm LJ, Josefsen K, Buschard K. Possible Prevention of Diabetes with a Gluten-Free Diet. Nutrients. 2018 Nov 13;10(11):1746. doi: 10.3390/nu10111746. PMID: 30428550; PMCID: PMC6266002.

7. *Houeiss P, Luce S, Boitard C. Environmental Triggering of Type 1 Diabetes Autoimmunity. Front Endocrinol (Lausanne). 2022 Jul 22;13:933965. doi: 10.3389/fendo.2022.933965. PMID: 35937815; PMCID: PMC9353023.

8. Patterson CC, Dahlquist GG, Gyurus E, Green A, Soltesz G. Incidence trends for childhood type 1 diabetes in Europe during 1989–2003 and predicted new cases 2005–20: a multicentre prospective registration study. Lancet. 2009;373:2027–2033.

9. *Ogrotis I, Koufakis T, Kotsa K. Changes in the Global Epidemiology of Type 1 Diabetes in an Evolving Landscape of Environmental Factors: Causes, Challenges, and Opportunities. Medicina. 2023; 59(4):668. https://doi.org/10.3390/medicina59040668

10. Willcox A, Richardson SJ, Bone AJ, Foulis AK, Morgan NG. Analysis of islet inflammation in human type 1 diabetes. Clin Exp Immunol. 2009 Feb;155(2):173-81. doi: 10.1111/j.1365-2249.2008.03860.x.

11. Michels AW, Landry LG, McDaniel KA, et al. Islet-Derived CD4 T Cells Targeting Proinsulin in Human Autoimmune Diabetes. Diabetes. 2017 Mar;66(3):722-734. doi: 10.2337/db16-1025. Epub 2016 Dec 5. PMID: 27920090; PMCID: PMC5319719..

12. Gianani R, Campbell-Thompson M, Sarkar SA, et al. Dimorphic histopathology of long-standing childhood-onset diabetes. Diabetol 2010; 53: 690-698. doi: 10.1007/s00125-009-1642-y.

13. Babon JA, DeNicola ME, Blodgett DM, et al. Analysis of self-antigen specificity of islet-infiltrating T cells from human donors with type 1 diabetes. Nat Med. 2016 Dec;22(12):1482-1487. doi: 10.1038/nm.4203.

14. Insel RA, Dunne JL, Atkinson MA, et al. Staging presymptomatic type 1 diabetes: a scientific statement of JDRF, the Endocrine Society, and the American Diabetes Association. Diabetes Care. 2015 Oct;38(10):1964-74. doi: 10.2337/dc15-1419.

15. Pietropaolo M, Towns R, Eisenbarth GS. Humoral Autoimmunity in Type 1 Diabetes: Prediction, Significance, and Detection of Distinct Disease Subtypes. Cold Spring Harb Perspect Med (2012) 2:10. doi: 10.1101/cshperspect.a012831

16. Coppieters KT, et al. Demonstration of islet-autoreactive CD8 T cells in insulitic lesions from recent onset and long-term type 1 diabetes patients. J Exp Med. 2012;209(1):51–60.

17. Pugliese A. Autoreactive T cells in type 1 diabetes. J Clin Invest. 2017 Aug 1;127(8):2881-2891. doi: 10.1172/JCI94549.

18. *Pearson JA, Wong FS, Wen L. The importance of the Non Obese Diabetic (NOD) mouse model in autoimmune diabetes. J Autoimmun. 2016 Jan;66:76-88. doi: 10.1016/j.jaut.2015.08.019.

19. Bruggeman Y, Martens PJ, Sassi G, Viaene M, Wasserfall CH, Mathieu C, Gysemans C. Footprint of pancreas infiltrating and circulating immune cells throughout type 1 diabetes development. Front Endocrinol (Lausanne). 2023 Nov 10;14:1275316. doi: 10.3389/fendo.2023.1275316.

20. Magnuson AM, Thurber GM, Kohler RH, Weissleder R, Mathis D, Benoist C. Population dynamics of islet-infiltrating cells in autoimmune diabetes. Proc Natl Acad Sci U S A. 2015 Feb 3;112(5):1511-6. doi: 10.1073/pnas.1423769112.

21. Catrina AM, Popa MA, Văcaru AM, Fenyo IM. Inflammatory status of the pancreas in NOD mice that do not develop overt diabetes. Rom J Morphol Embryol. 2021 Jan-Mar;62(1):109-115. doi: 10.47162/RJME.62.1.10. PMID: 34609413; PMCID: PMC8597392.

22. Aldrich VR, Hernandez-Rovira BB, Chandwani A, Abdulreda MH. NOD Mice-Good Model for T1D but Not Without Limitations. Cell Transplant. 2020 Jan-Dec;29:963689720939127. doi: 10.1177/0963689720939127.

23. *In't Veld P. Insulitis in human type 1 diabetes: a comparison between patients and animal models. Semin Immunopathol. 2014 Sep;36(5):569-79. doi: 10.1007/s00281-014-0438-4.

24. *Mullen Y. Development of the Nonobese Diabetic Mouse and Contribution of Animal Models for Understanding Type 1 Diabetes. Pancreas. 2017 Apr;46(4):455-466. doi: 10.1097/MPA.0000000000000828.

25. Godoy GJ, Paira DA, Olivera C, Breser ML, Sanchez LR, Motrich RD, Rivero VE. Differences in T regulatory cells between mouse strains frequently used in immunological research: Treg cell quantities and subpopulations in NOD, B6 and BALB/c mice. Immunol Lett. 2020 Jul;223:17-25. doi: 10.1016/j.imlet.2020.04.006.

26. Gagnerault MC, Luan JJ, Lotton C, Lepault F. Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. J Exp Med. 2002 Aug 5;196(3):369-77. doi: 10.1084/jem.20011353. PMID: 12163565; PMCID: PMC2193939.

27. *Chen D, Thayer TC, Wen L, Wong FS. Mouse Models of Autoimmune Diabetes: The Nonobese Diabetic (NOD) Mouse. Methods Mol Biol. 2020;2128:87-92. doi: 10.1007/978-1-0716-0385-7_6. PMID: 32180187; PMCID: PMC8253669.

28. Pearson JA, Tai N, Ekanayake-Alper DK, et al. Norovirus Changes Susceptibility to Type 1 Diabetes by Altering Intestinal Microbiota and Immune Cell Functions. Front Immunol. 2019 Nov 12;10:2654. doi: 10.3389/fimmu.2019.02654.

29. Alam C, Bittoun E, Bhagwat D, et al. Effects of a germ-free environment on gut immune regulation and diabetes progression in non-obese diabetic (NOD) mice. Diabetologia. 2011 Jun;54(6):1398-406. doi: 10.1007/s00125-011-2097-5.

30. *Op de Beeck A, Eizirik DL. Viral infections in type 1 diabetes mellitus--why the β cells? Nat Rev Endocrinol. 2016 May;12(5):263-273. doi: 10.1038/nrendo.2016.30.

31. Krogvold L., Edwin B., Buanes T., Frisk G., Skog O., Anagandula M., Korsgren O., Undlien D., Eike M.C., Richardson S.J., et al. Detection of a low-grade enteroviral infection in the islets of langerhans of living patients newly diagnosed with type 1 diabetes. Diabetes. 2015;64:1682–1687. doi: 10.2337/db14-1370.

32. Richardson SJ, Rodriguez-Calvo T, Gerling IC, et al. Islet cell hyperexpression of HLA class I antigens: a defining feature in type 1 diabetes. Diabetologia. 2016 Nov;59(11):2448-2458. doi: 10.1007/s00125-016-4067-4.

33. Ferreira RC, Guo H, Coulson RM, Smyth DJ, Pekalski ML, Burren OS, et al. A Type I Interferon Transcriptional Signature Precedes Autoimmunity in Children Genetically at Risk for Type 1 Diabetes. Diabetes (2014) 63:2538–50. doi: 10.2337/db13-1777

34. *Yang S, Zhao B, Zhang Z, Dai X, Zhang Y, Cui L. Association between enterovirus infection and clinical type 1 diabetes mellitus: systematic review and meta-analysis of observational studies. Epidemiol Infect. 2021 Dec 10;150:e23. doi: 10.1017/S0950268821002442. PMID: 35144715; PMCID: PMC8851353.

35. *Yeung WC, Rawlinson WD, Craig ME. Enterovirus infection and type 1 diabetes mellitus: systematic review and meta-analysis of observational molecular studies. BMJ. 2011 Feb 3;342:d35. doi: 10.1136/bmj.d35. PMID: 21292721; PMCID: PMC3033438.

36. *Rodriguez-Calvo T, Johnson JD, Overbergh L, Dunne JL. Neoepitopes in Type 1 Diabetes: Etiological Insights, Biomarkers and Therapeutic Targets. Front Immunol. 2021 Apr 19;12:667989. doi: 10.3389/fimmu.2021.667989. PMID: 33953728; PMCID: PMC8089389.

37. Murri M, Leiva I, Gomez-Zumaquero JM, Tinahones FJ, Cardona F, Soriguer F, et al. Gut microbiota in children with type 1 diabetes differs from that in healthy children: a case-control study. BMC Med. (2013) 11:46. doi: 10.1186/1741-7015-11-46

38. Giongo A, Gano KA, Crabb DB, Mukherjee N, Novelo LL, Casella G, et al. Toward defining the autoimmune microbiome for type 1 diabetes. ISME J. (2011) 5:82–91. doi: 10.1038/ismej.2010.92

39. Leiva-Gea I, Sanchez-Alcoholado L, Martin-Tejedor B, Castellano-Castillo D, Moreno-Indias I, Urda-Cardona A, et al. Gut microbiota differs in composition and functionality between children with type 1 diabetes and MODY2 and healthy control subjects: a case-control study. Diabetes Care. (2018) 41:2385–95. doi: 10.2337/dc18-0253

40. Demirci M, Bahar Tokman H, Taner Z, Keskin FE, Cagatay P, Ozturk Bakar Y, et al. Bacteroidetes and Firmicutes levels in gut microbiota and effects of hosts TLR2/TLR4 gene expression levels in adult type 1 diabetes patients in Istanbul, Turkey. J Diabetes Complications. (2019) 34:107449. doi: 10.1016/j.jdiacomp.2019.107449

Brown CT, Davis-Richardson AG, Giongo A, Gano KA, Crabb DB, Mukherjee N, et al. Gut microbiome metagenomics analysis suggests a functional model for the development of autoimmunity for type 1 diabetes. PLoS ONE. (2011) 6:e25792. doi: 10.1371/journal.pone.0025792

Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, et al. Induction of Colonic Regulatory T Cells by Indigenous Clostridium Species. Science. 2010

Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, et al. Treg induction by a rationally selected mixture of Clostridia strains from the human microbiota. Nature. 2013;500:232–236.

Furusawa Y, Obata Y, Fukuda S, Endo TA, Nakato G, Takahashi D, Nakanishi Y, Uetake C, Kato K, Kato T, et al. Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 2013;504:446–450

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013 Aug 2;341(6145):569-73. doi: 10.1126/science.1241165. Epub 2013 Jul 4. PMID: 23828891; PMCID: PMC3807819.

Ivanov, Atarashi K, Manel N, Brodie EL, Shima T, Karaoz U, Wei D, Goldfarb KC, Santee CA, Lynch SV, et al. Induction of intestinal Th17 cells by segmented filamentous bacteria. Cell. 2009;139:485–498.

*Dedrick S, Sundaresh B, Huang Q, Brady C, Yoo T, Cronin C, Rudnicki C, Flood M, Momeni B, Ludvigsson J, Altindis E. The Role of Gut Microbiota and Environmental Factors in Type 1 Diabetes Pathogenesis. Front Endocrinol (Lausanne). 2020 Feb 26;11:78. doi: 10.3389/fendo.2020.00078. PMID: 32174888; PMCID: PMC7057241.

Zheng P, Li Z, Zhou Z. Gut microbiome in type 1 diabetes: A comprehensive review. Diabetes Metab Res Rev. 2018 Oct;34(7):e3043. doi: 10.1002/dmrr.3043. Epub 2018 Jul 17. PMID: 29929213; PMCID: PMC6220847.

Cerf-Bensussan N., Gaboriau-Routhiau V. The immune system and the gut microbiota: Friends or foes? Nat. Rev. Immunol. 2010;10:735–744. doi: 10.1038/nri2850.

Traversi D, Rabbone I, Ignaccolo MG, Carletto G, Racca I, Vallini C, et al. Gut microbiota diversity and T1DM onset: preliminary data of a case-control study. Human Microb J. (2017) 5–6:11–3. doi: 10.1016/j.humic.2017.11.002

de Goffau MC, Luopajarvi K, Knip M, Ilonen J, Ruohtula T, Harkonen T, et al. Fecal microbiota composition differs between children with beta-cell autoimmunity and those without. Diabetes. (2013) 62:1238–44. doi: 10.2337/db12-0526

Kostic AD, Gevers D, Siljander H, Vatanen T, Hyotylainen T, Hamalainen AM, et al. The dynamics of the human infant gut microbiome in development and in progression toward type 1 diabetes. Cell Host Microbe. (2015) 17:260–73. doi: 10.1016/j.chom.2015.01.001

Park J, Kim M, Kang SG, Jannasch AH, Cooper B, Patterson J, Kim CH. Short-chain fatty acids induce both effector and regulatory T cells by suppression of histone deacetylases and regulation of the mTOR-S6K pathway. Mucosal Immunol. 2015 Jan;8(1):80-93. doi: 10.1038/mi.2014.44. Epub 2014 Jun 11. PMID: 24917457; PMCID: PMC4263689.

Soyucen E, Gulcan A, Aktuglu-Zeybek AC, Onal H, Kiykim E, Aydin A. Differences in the gut microbiota of healthy children and those with type 1 diabetes. Pediatr Int. (2014) 56:336–43. doi: 10.1111/ped.12243

Verduci E, Mameli C, Amatruda M, Petitti A, Vizzuso S, El Assadi F, Zuccotti G, Alabduljabbar S, Terranegra A. Early Nutrition and Risk of Type 1 Diabetes: The Role of Gut Microbiota. Front Nutr. 2020 Dec 23;7:612377. doi: 10.3389/fnut.2020.612377. PMID: 33425976; PMCID: PMC7785819.

Davis-Richardson et al., 2014

Zheng P, Li Z, Zhou Z. Gut microbiome in type 1 diabetes: A comprehensive review. Diabetes Metab Res Rev. 2018 Oct;34(7):e3043.

Brugman S., Perdijk O., van Neerven R.J.J., Savelkoul H.J. Mucosal immune development in early life: Setting the stage. Arch. Immunol. Ther. Exp. 2015;63:251–268. doi: 10.1007/s00005-015-0329-y.

Longman R.S., Yang Y., Diehl G.E., Kim S.V., Littman D.R. Microbiota: Host interactions in mucosal homeostasis and systemic autoimmunity. Cold Spring Harbor Symp. Quant. Biol. 2013;78:193–201. doi: 10.1101/sqb.2013.78.020081.

Kim CH. Microbiota or short-chain fatty acids: which regulates diabetes? Cell Mol Immunol. 2018 Feb;15(2):88-91. doi: 10.1038/cmi.2017.57. Epub 2017 Jul 17. PMID: 28713163; PMCID: PMC5811682.

Harjutsalo V, Sjöberg L, Tuomilehto J. Time trends in the incidence of type 1 diabetes in Finnish children: a cohort study. Lancet. 2008;371:1777–1782

Nigi L, Maccora C, Dotta F, Sebastiani G. From immunohistological to anatomical alterations of human pancreas in type 1 diabetes: New concepts on the stage. Diabetes Metab Res Rev. 2020 May;36(4):e3264. doi: 10.1002/dmrr.3264. Epub 2019 Dec 30. PMID: 31850667.

James EA, Joglekar AV, Linnemann AK, Russ HA, Kent SC. The beta cell-immune cell interface in type 1 diabetes (T1D). Mol Metab. 2023 Dec;78:101809. doi: 10.1016/j.molmet.2023.101809. Epub 2023 Sep 20. PMID: 37734713; PMCID: PMC10622886.

Colli ML, Szymczak F, Eizirik DL. Molecular Footprints of the Immune Assault on Pancreatic Beta Cells in Type 1 Diabetes. Front Endocrinol (Lausanne) (2020) 11:568446. doi: 10.3389/fendo.2020.568446

Funda DP, Kaas A, Bock T, Tlaskalová-Hogenová H, Buschard K. Gluten-free diet prevents diabetes in NOD mice. Diabetes Metab Res Rev. 1999 Sep-Oct;15(5):323-7.

Marietta E.V., Gomez A.M., Yeoman C., Tilahun A.Y., Clark C.R., Luckey D.H., Murray J.A., White B.A., Kudva Y.C., Rajagopalan G. Low incidence of spontaneous type 1 diabetes in non-obese diabetic mice raised on gluten-free diets is associated with changes in the intestinal microbiome. *PLoS ONE.* 2013;8:e78687. doi: 10.1371/journal.pone.0078687.

Funda DP, Kaas A, Tlaskalová-Hogenová H, Buschard K. Gluten-free but also gluten-enriched (gluten+) diet prevent diabetes in NOD mice; the gluten enigma in type 1 diabetes. Diabetes Metab Res Rev. 2008 Jan-Feb;24(1):59-63. doi: 10.1002/dmrr.748. PMID: 17607660.

Hansen A.K., Ling F., Kaas A., Funda D.P., Farlov H., Buschard K. Diabetes preventive gluten-free diet decreases the number of caecal bacteria in non-obese diabetic mice. *Diabetes Metab. Res. Rev.* 2006;22:220–225. doi: 10.1002/dmrr.609

Antvorskov J.C., Josefsen K., Haupt-Jorgensen M., Fundova P., Funda D.P., Buschard K. Gluten-Free Diet Only during Pregnancy Efficiently Prevents Diabetes in NOD Mouse Offspring. J. Diabetes Res. 2016;2016:3047574. doi: 10.1155/2016/3047574.

Haupt-Jorgensen M, Holm LJ, Josefsen K, Buschard K. Possible Prevention of Diabetes with a Gluten-Free Diet. Nutrients. 2018 Nov 13;10(11):1746. doi: 10.3390/nu10111746. PMID: 30428550; PMCID: PMC6266002.

Hansen CHF, Larsen CS, Zachariassen LF, Mentzel CMJ, Laigaard A, Krych L, Nielsen DS, Gobbi A, Haupt-Jorgensen M, Buschard K, Hansen AK. Gluten-free diet reduces autoimmune diabetes mellitus in mice across multiple generations in a microbiota-independent manner. J Autoimmun. 2022 Feb;127:102795. doi: 10.1016/j.jaut.2022.102795. Epub 2022 Jan 31. PMID: 35101708.

Li X, Wang L, Meng G, Chen X, Yang S, Zhang M, Zheng Z, Zhou J, Lan Z, Wu Y, Wang L. Sustained high glucose intake accelerates type 1 diabetes in NOD mice. Front Endocrinol (Lausanne). 2022 Dec 5;13:1037822. doi: 10.3389/fendo.2022.1037822. PMID: 36545340; PMCID: PMC9760976.

*Bolla AM, Caretto A, Laurenzi A, Scavini M, Piemonti L. Low-Carb and Ketogenic Diets in Type 1 and Type 2 Diabetes. Nutrients. 2019 Apr 26;11(5):962. doi: 10.3390/nu11050962. PMID: 31035514; PMCID: PMC6566854.

*Lampousi AM, Carlsson S, Löfvenborg JE. Dietary factors and risk of islet autoimmunity and type 1 diabetes: a systematic review and meta-analysis. EBioMedicine. 2021 Oct;72:103633. doi: 10.1016/j.ebiom.2021.103633. Epub 2021 Oct 14. PMID: 34656932; PMCID: PMC8523874.

*Feinman R.D., Pogozelski W.K., Astrup A., Bernstein R.K., Fine E.J., Westman E.C., Accurso A., Frassetto L., Gower B.A., McFarlane S.I., et al. Dietary carbohydrate restriction as the first approach in diabetes management: Critical review and evidence base. Nutrition. 2015;31:1–13. doi: 10.1016/j.nut.2014.06.011.

Turton JL, Brinkworth GD, Parker HM, Lim D, Lee K, et al. (2023) Effects of a low-carbohydrate diet in adults with type 1 diabetes management: A single arm non-randomised clinical trial. PLOS ONE 18(7): e0288440. https://doi.org/10.1371/journal.pone.0288440

Krebs JD, Parry Strong A, Cresswell P, Reynolds AN, Hanna A, Haeusler S. A randomised trial of the feasibility of a low carbohydrate diet vs standard carbohydrate counting in adults with type 1 diabetes taking body weight into account. Asia Pacific Journal of Clinical Nutrition. 2016;25(1):78–84. Pmid:26965765

Nielsen JV, Gando C, Joensson E, Paulsson C. Low carbohydrate diet in type 1 diabetes, long-term improvement and adherence: A clinical audit. Diabetology and Metabolic Syndrome. 2012;4 (1) (23).

*Bolla AM, Caretto A, Laurenzi A, Scavini M, Piemonti L. Low-Carb and Ketogenic Diets in Type 1 and Type 2 Diabetes. Nutrients. 2019 Apr 26;11(5):962. doi: 10.3390/nu11050962. PMID: 31035514; PMCID: PMC6566854.

*Turton JL, Raab R, Rooney KB (2018) Low-carbohydrate diets for type 1 diabetes mellitus: A systematic review. PLoS ONE 13(3): e0194987. https://doi.org/10.1371/journal.pone.0194987.

Schuster C, Jonas F, Zhao F, Kissler S. Peripherally induced regulatory T cells contribute to the control of autoimmune diabetes in the NOD mouse model. Eur J Immunol 2018;48:1211–1216

*Hull, Peakman, Tree, 2017.

Feurer et al., 2009;

D'Alise AM et al., 2008.

Tarbell KV, Petit L, Zuo X, Toy P, Luo X, Mqadmi A, Yang H, Suthanthiran M, Mojsov S, Steinman RM. Dendritic cell-expanded, islet-specific CD4+CD25+CD62L+ regulatory T cells restore normoglycemia in diabetic NOD mice. J Exp Med. 2007;204:191–201.

Viisanen T, Gazali AM, Ihantola EL, Ekman I, Näntö-Salonen K, Veijola R, Toppari J, Knip M, Ilonen J, Kinnunen T. FOXP3+ Regulatory T Cell Compartment Is Altered in Children With Newly Diagnosed Type 1 Diabetes but Not in Autoantibody-Positive at-Risk Children. Front Immunol. 2019 Jan 22;10:19. doi: 10.3389/fimmu.2019.00019.

Ferreira RC, Simons HZ, Thompson WS, Rainbow DB, Yang X, Cutler AJ, et al. Cells with Treg-specific FOXP3 demethylation but low CD25 are prevalent in autoimmunity. J Autoimmun. (2017) 84:75–86. doi: 10.1016/j.jaut.2017.07.009

Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q, et al. Cutting edge: increased IL-17-secreting T cells in children with new-onset type 1 diabetes. J Immunol. (2010) 185:3814–8. doi: 10.4049/jimmunol.1001860

Sanda S, Roep BO, von Herrath M. Islet antigen specific IL-10+ immune responses but not CD4+CD25+FoxP3+ cells at diagnosis predict glycemic control in type 1 diabetes. Clin Immunol. 2008 May;127(2):138-43. doi: 10.1016/j.clim.2007.12.003. Epub 2008 Mar 4. PMID: 18304876.

Ryba-Stanisławowska M, Rybarczyk-Kapturska K, Myśliwiec M, Myśliwska J. Elevated levels of serum IL-12 and IL-18 are associated with lower frequencies of CD4(+)CD25 (high)FOXP3 (+) regulatory t cells in young patients with type 1 diabetes. Inflammation. 2014 Oct;37(5):1513-20. doi: 10.1007/s10753-014-9878-1.

Ghonaim MM, El-Edel RH, Kamal Eldein SM, Abo El Fotoh WMM, Salman SS. T-Regulatory Cell Subsets in Children with Type 1 Diabetes Mellitus: Relation to Control of the Disease. Endocr Metab Immune Disord Drug Targets. 2017;17(3):238-245. doi: 10.2174/1871530317666170818115116.

Szypowska A, Stelmaszczyk-Emmel A, Demkow U, Luczyński W. Low frequency of regulatory T cells in the peripheral blood of children with type 1 diabetes diagnosed under the age of five. Arch Immunol Ther Exp (Warsz). 2012 Aug;60(4):307-13. doi: 10.1007/s00005-012-0177-y.

Brusko T, Wasserfall C, McGrail K, Schatz R, Viener HL, Schatz D, et al. No alterations in the frequency of FOXP3+ regulatory T-cells in type 1 diabetes. Diabetes (2007) 56:604–12. doi: 10.2337/db06-1248

Putnam AL, Vendrame F, Dotta F, Gottlieb PA. CD4+CD25high regulatory T cells in human autoimmune diabetes. J Autoimmun. (2005) 24:55–62. doi: 10.1016/j.jaut.2004.11.004

McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, et al. Plasticity of human regulatory T cells in healthy subjects and patients with type 1 diabetes. J Immunol. (2011) 186:3918–26. doi: 10.4049/jimmunol.1003099

Hamari S, Kirveskoski T, Glumoff V, Kulmala P, Simell O, Knip M, et al. Analyses of regulatory CD4+ CD25+ FOXP3+ T cells and observations from peripheral T cell subpopulation markers during the development of type 1 diabetes in children. Scand J Immunol. (2016) 83:279–87. doi: 10.1111/sji.12418

Viisanen T, Gazali AM, Ihantola EL, Ekman I, Näntö-Salonen K, Veijola R, Toppari J, Knip M, Ilonen J, Kinnunen T. FOXP3+ Regulatory T Cell Compartment Is Altered in Children With Newly Diagnosed Type 1 Diabetes but Not in Autoantibody-Positive at-Risk Children. Front Immunol. 2019 Jan 22;10:19. doi: 10.3389/fimmu.2019.00019.

Brusko TM, Wasserfall CH, Clare-Salzler MJ, Schatz DA, Atkinson MA. Functional defects and the influence of age on the frequency of CD4+ CD25+ T-cells in type 1 diabetes. Diabetes. 2005 May;54(5):1407-14. doi: 10.2337/diabetes.54.5.1407.

Lindley S, Dayan CM, Bishop A, Roep BO, Peakman M, Tree TI. Defective suppressor function in CD4(+)CD25(+) T-cells from patients with type 1 diabetes. Diabetes. 2005 Jan;54(1):92-9. doi: 10.2337/diabetes.54.1.92.

Lawson JM, Tremble J, Dayan C, Beyan H, Leslie RD, Peakman M, Tree TI. Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes. Clin Exp Immunol. 2008 Dec;154(3):353-9. doi: 10.1111/j.1365-2249.2008.03810.x.

Glisic-Milosavljevic S, Wang T, Koppen M, Kramer J, Ehlenbach S, Waukau J, Jailwala P, Jana S, Alemzadeh R, Ghosh S. Dynamic changes in CD4+ CD25+(high) T cell apoptosis after the diagnosis of type 1 diabetes. Clin Exp Immunol. 2007 Oct;150(1):75-82. doi: 10.1111/j.1365-2249.2007.03475.x.

Glisic-Milosavljevic S, Waukau J, Jailwala P, Jana S, Khoo HJ, Albertz H, Woodliff J, Koppen M, Alemzadeh R, Hagopian W, Ghosh S. At-risk and recent-onset type 1 diabetic subjects have increased apoptosis in the CD4+CD25+ T-cell fraction. PLoS One. 2007 Jan 3;2(1):e146. doi: 10.1371/journal.pone.0000146.

Marwaha AK, Crome SQ, Panagiotopoulos C, Berg KB, Qin H, Ouyang Q, Xu L, Priatel JJ, Levings MK, Tan R. Cutting edge: Increased IL-17-secreting T cells in children with new-onset type 1 diabetes. J Immunol. 2010 Oct 1;185(7):3814-8. doi: 10.4049/jimmunol.1001860.

McClymont SA, Putnam AL, Lee MR, Esensten JH, Liu W, Hulme MA, Hoffmüller U, Baron U, Olek S, Bluestone JA, Brusko TM. Plasticity of human regulatory T cells in healthy subjects

and patients with type 1 diabetes. J Immunol. 2011 Apr 1;186(7):3918-26. doi: 10.4049/jimmunol.1003099. Epub 2011 Mar 2. PMID: 21368230; PMCID: PMC3091943.

Grinberg-Bleyer Y, Baeyens A, You S, Elhage R, Fourcade G, Gregoire S, Cagnard N, Carpentier W, Tang Q, Bluestone J, Chatenoud L, Klatzmann D, Salomon BL, Piaggio E. IL-2 reverses established type 1 diabetes in NOD mice by a local effect on pancreatic regulatory T cells. J Exp Med. 2010 Aug 30;207(9):1871-8. doi: 10.1084/jem.20100209.

Pham MN, von Herrath MG, Vela JL. Antigen-Specific Regulatory T Cells and Low Dose of IL-2 in Treatment of Type 1 Diabetes. Front Immunol. 2016 Jan 11;6:651. doi: 10.3389/fimmu.2015.00651.

Todd JA, Evangelou M, Cutler AJ, Pekalski ML, Walker NM, et al. Regulatory T Cell Responses in Participants with Type 1 Diabetes after a Single Dose of Interleukin-2: A Non-Randomised, Open Label, Adaptive Dose-Finding Trial. PLoS Med. 2016 Oct 11;13(10):e1002139. doi: 10.1371/journal.pmed.1002139.

*Roncarolo MG, Gregori S, Bacchetta R, Battaglia M. Tr1 cells and the counter-regulation of immunity: natural mechanisms and therapeutic applications. *Curr Top Microbiol Immunol.* 2014;380:39–68.

Schuster C, Jonas F, Zhao F, Kissler S. Peripherally induced regulatory T cells contribute to the control of autoimmune diabetes in the NOD mouse model. Eur J Immunol. 2018 Jul;48(7):1211-1216. doi: 10.1002/eji.201847498. Epub 2018 Apr 25. PMID: 29604048; PMCID: PMC6033626.

Arif S, Tree TI, Astill TP, et al. Autoreactive T cell responses show proinflammatory polarization in diabetes but a regulatory phenotype in health. J Clin Invest. 2004;113:451–463. doi: 10.1172/JCI19585.

Arif S, Leete P, Nguyen V, Marks K, Nor NM, Estorninho M, Kronenberg-Versteeg D, Peakman M., et al. Blood and islet phenotypes indicate immunological heterogeneity in type 1 diabetes. Diabetes. 2014 Nov;63(11):3835-45. doi: 10.2337/db14-0365. Epub 2014 Jun 17. Erratum in: Diabetes. 2015 Sep;64(9):3334. doi: 10.2337/db15-er09a.

Sanda S, Roep BO, von Herrath M. Islet antigen specific IL-10+ immune responses but not CD4+CD25+FoxP3+ cells at diagnosis predict glycemic control in type 1 diabetes. Clin Immunol. 2008;127:138–143. doi: 10.1016/j.clim.2007.12.003.

Mitchell AM, Michels AW. Self-Antigens Targeted by Regulatory T Cells in Type 1 Diabetes. *International Journal of Molecular Sciences*. 2022; 23(6):3155. https://doi.org/10.3390/ijms23063155

Freeborn RA, Strubbe S, Roncarolo MG. Type 1 regulatory T cell-mediated tolerance in health and disease. Front Immunol. 2022 Oct 28;13:1032575. doi: 10.3389/fimmu.2022.1032575. PMID: 36389662; PMCID: PMC9650496.

Yu H, Gagliani N, Ishigame H, Huber S, Zhu S, Esplugues E, Herold KC, Wen L, Flavell RA. Intestinal type 1 regulatory T cells migrate to periphery to suppress diabetogenic T cells and prevent diabetes development. Proc Natl Acad Sci U S A. 2017 Sep 26;114(39):10443-10448. doi: 10.1073/pnas.1705599114. Epub 2017 Sep 11. PMID: 28894001; PMCID: PMC5625908.

Hanna SJ, Powell WE, Long AE, et al. Slow progressors to type 1 diabetes lose islet autoantibodies over time, have few islet antigen-specific CD8+ T cells and exhibit a distinct CD95hiB cell phenotype. Diabetologia 2020; 63: 1174–1185.

Yeo L, Woodwyk A, Sood S, et al. Autoreactive T effector memory differentiation mirrors β cell function in type 1 diabetes. J Clin Invest 2018; 128: 3460–3474.

Yeo L, Woodwyk A, Sood S, Lorenc A, Eichmann M, Pujol-Autonell I, Melchiotti R, Skowera A, Fidanis E, Dolton GM, Tungatt K, Sewell AK, Heck S, Saxena A, Beam CA, Peakman M. Autoreactive T effector memory differentiation mirrors β cell function in type 1 diabetes. J Clin Invest. 2018 Aug 1;128(8):3460-3474. doi: 10.1172/JCI120555. Epub 2018 Jul 16. PMID: 29851415; PMCID: PMC6063477.

Wiedeman AE, Muir VS, Rosasco MG, et al. Autoreactive CD8+ T cell exhaustion distinguishes subjects with slow type 1 diabetes progression. J Clin Invest 2020; 130: 480–490.

Wiedeman AE, Muir VS, Rosasco MG, DeBerg HA, Presnell S, Haas B, Dufort MJ, Speake C, Greenbaum CJ, Serti E, Nepom GT, Blahnik G, Kus AM, James EA, Linsley PS, Long SA. Autoreactive CD8+ T cell exhaustion distinguishes subjects with slow type 1 diabetes progression. J Clin Invest. 2020 Jan 2;130(1):480-490. doi: 10.1172/JCI126595. PMID: 31815738; PMCID: PMC6934185.

Wiedeman AE, Speake C, Long SA. The many faces of islet antigen-specific CD8 T cells: clues to clinical outcome in type 1 diabetes. Immunol Cell Biol. 2021 May;99(5):475-485. doi: 10.1111/imcb.12437. Epub 2021 Feb 21. PMID: 33483981; PMCID: PMC8248166.

Gearty SV, Dündar F, Zumbo P, Espinosa-Carrasco G, Shakiba M, Sanchez-Rivera FJ, Socci ND, Trivedi P, Lowe SW, Lauer P, Mohibullah N, Viale A, DiLorenzo TP, Betel D, Schietinger

A. An autoimmune stem-like CD8 T cell population drives type 1 diabetes. Nature. 2022 Feb;602(7895):156-161. doi: 10.1038/s41586-021-04248-x. Epub 2021 Nov 30. PMID: 34847567; PMCID: PMC9315050.

Vignali D, Cantarelli E, Bordignon C, Canu A, Citro A, Annoni A, Piemonti L, Monti P. Detection and Characterization of CD8+ Autoreactive Memory Stem T Cells in Patients With Type 1 Diabetes. Diabetes. 2018 May;67(5):936-945. doi: 10.2337/db17-1390. Epub 2018 Mar 5. PMID: 29506985.

Teniente-Serra A, Pizarro E, Quirant-Sánchez B, Fernández MA, Vives-Pi M, Martinez-Caceres EM. Identifying Changes in Peripheral Lymphocyte Subpopulations in Adult Onset Type 1 Diabetes. Front Immunol. 2021 Dec 6;12:784110. doi: 10.3389/fimmu.2021.784110. PMID: 34938295; PMCID: PMC8685245.

Jameson SC, Masopust D. Understanding Subset Diversity in T Cell Memory. Immunity. 2018 Feb 20;48(2):214-226. doi: 10.1016/j.immuni.2018.02.010. PMID: 29466754; PMCID: PMC5863745.

Ehlers, M.R., Rigby, M.R. Targeting Memory T Cells in Type 1 Diabetes. Curr Diab Rep 15, 84 (2015). https://doi.org/10.1007/s11892-015-0659-5

Jonathan Chee, Hyun-Ja Ko, Ania Skowera, Gaurang Jhala, Tara Catterall, Kate L. Graham, Robyn M. Sutherland, Helen E. Thomas, Andrew M. Lew, Mark Peakman, Thomas W. H. Kay, Balasubramanian Krishnamurthy; Effector-Memory T Cells Develop in Islets and Report Islet Pathology in Type 1 Diabetes. J Immunol 15 January 2014; 192 (2): 572–580. https://doi.org/10.4049/jimmunol.1302100

Okada M, Zhang V, Loaiza Naranjo JD, Tillett BJ, Wong FS, Steptoe RJ, Bergot AS, Hamilton-Williams EE. Islet-specific CD8+ T cells gain effector function in the gut lymphoid tissues via bystander activation not molecular mimicry. Immunol Cell Biol. 2023 Jan;101(1):36-48. doi: 10.1111/imcb.12593. Epub 2022 Nov 1. PMID: 36214093; PMCID: PMC10092732.

*Tan J, Taitz J, Sun SM, Langford L, Ni D, Macia L. Your Regulatory T Cells Are What You Eat: How Diet and Gut Microbiota Affect Regulatory T Cell Development. Front Nutr. 2022 Apr 20;9:878382. doi: 10.3389/fnut.2022.878382. PMID: 35529463; PMCID: PMC9067578.

*Cheru N, Hafler DA, Sumida TS. Regulatory T cells in peripheral tissue tolerance and diseases. Front Immunol. 2023 May 1;14:1154575. doi: 10.3389/fimmu.2023.1154575. PMID: 37197653; PMCID: PMC10183596.

Ostman S, Rask C, Wold AE, Hultkrantz S, Telemo E. Impaired regulatory T cell function in germ-free mice. Eur J Immunol. 2006 Sep;36(9):2336-46. doi: 10.1002/eji.200535244.

Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly-Y M, Glickman JN, Garrett WS. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. Science. 2013 Aug 2;341(6145):569-73. doi: 10.1126/science.1241165. Epub 2013 Jul 4. PMID: 23828891; PMCID: PMC3807819.

Wymore Brand M, Wannemuehler MJ, Phillips GJ, Proctor A, Overstreet AM, Jergens AE, Orcutt RP, Fox JG. The Altered Schaedler Flora: Continued Applications of a Defined Murine Microbial Community. ILAR J. 2015;56(2):169-78. doi: 10.1093/ilar/ilv012. PMID: 26323627; PMCID: PMC4554250.

Livanos AE, Greiner TU, Vangay P, Pathmasiri W, Stewart D, McRitchie S, Li H, Chung J, Sohn J, Kim S, Gao Z, Barber C, Kim J, Ng S, Rogers AB, Sumner S, Zhang XS, Cadwell K, Knights D, Alekseyenko A, Bäckhed F, Blaser MJ. Antibiotic-mediated gut microbiome perturbation accelerates development of type 1 diabetes in mice. Nat Microbiol. 2016 Aug 22;1(11):16140. doi: 10.1038/nmicrobiol.2016.140.

Simon MC, Reinbeck AL, Wessel C, Heindirk J, Jelenik T, Kaul K, Arreguin-Cano J, Strom A, Blaut M, Bäckhed F, Burkart V, Roden M. Distinct alterations of gut morphology and microbiota characterize accelerated diabetes onset in nonobese diabetic mice. J Biol Chem. 2020 Jan 24;295(4):969-980. doi: 10.1074/jbc.RA119.010816. Epub 2019 Dec 10. PMID: 31822562; PMCID: PMC6983849.

Alam C, Bittoun E, Bhagwat D, Valkonen S, Saari A, Jaakkola U, Eerola E, Huovinen P, Hänninen A. Effects of a germ-free environment on gut immune regulation and diabetes progression in non-obese diabetic (NOD) mice. Diabetologia. 2011 Jun;54(6):1398-406. doi: 10.1007/s00125-011-2097-5. Epub 2011 Mar 5. PMID: 21380595.

Bihan DG, Rydzak T, Wyss M, Pittman K, McCoy KD, Lewis IA. Method for absolute quantification of short chain fatty acids via reverse phase chromatography mass spectrometry. PLoS One. 2022 Apr 20;17(4):e0267093. doi: 10.1371/journal.pone.0267093. PMID: 35443015; PMCID: PMC9020710.

Jacob, N., Jaiswal, S., Maheshwari, D. et al. Butyrate induced Tregs are capable of migration from the GALT to the pancreas to restore immunological tolerance during type-1 diabetes. Sci Rep 10, 19120 (2020). https://doi.org/10.1038/s41598-020-76109-y

Lyte JM, Proctor A, Phillips GJ, Lyte M, Wannemuehler M. Altered Schaedler flora mice: A defined microbiota animal model to study the microbiota-gut-brain axis. Behav Brain Res. 2019 Jan 1;356:221-226. doi: 10.1016/j.bbr.2018.08.022. Epub 2018 Aug 25. PMID: 30153465.

Guillen J. FELASA guidelines and recommendations. J Am Assoc Lab Anim Sci. 2012 May;51(3):311-21. PMID: 22776188; PMCID: PMC3358979.

Pfefferle PI, Keber CU, Cohen RM, Garn H. The Hygiene Hypothesis - Learning From but Not Living in the Past. Front Immunol. 2021 Mar 16;12:635935. doi: 10.3389/fimmu.2021.635935. PMID: 33796103; PMCID: PMC8007786.

Zhou H, Sun L, Zhang S, Zhao X, Gang X, Wang G. Evaluating the Causal Role of Gut Microbiota in Type 1 Diabetes and Its Possible Pathogenic Mechanisms. Front Endocrinol (Lausanne). 2020 Mar 24;11:125. doi: 10.3389/fendo.2020.00125. PMID: 32265832; PMCID: PMC7105744.

Castro-Alves V, Orešič M, Hyötyläinen T. Lipidomics in nutrition research. Curr Opin Clin Nutr Metab Care. 2022 Sep 1;25(5):311-318. doi: 10.1097/MCO.0000000000000852. Epub 2022 Jul 5. PMID: 35788540.

Jaakkola I, Jalkanen S, Hänninen A. Diabetogenic T cells are primed both in pancreatic and gut-associated lymph nodes in NOD mice. Eur J Immunol (2003) 33:3255-64. doi:10.1002/eji.200324405

Gagnerault MC, Luan JJ, Lotton C, Lepault F. Pancreatic lymph nodes are required for priming of beta cell reactive T cells in NOD mice. J Exp Med (2002) 196:369-77. doi:10.1084/jem.20011353

Clare-Salzler MJ, Brooks J, Chai A, van Herle K, Anderson C. Prevention of diabetes in nonobese diabetic mice by dendritic cell transfer. J Clin Invest (1992) 90:741-8. doi:10.1172/JCI115946

Hansen CH, Krych L, Buschard K, Metzdorff SB, Nellemann C, Hansen LH, Nielsen DS, Frøkiær H, Skov S, Hansen AK. A maternal gluten-free diet reduces inflammation and diabetes incidence in the offspring of NOD mice. Diabetes. 2014 Aug;63(8):2821-32. doi: 10.2337/db13-1612. Epub 2014 Apr 2. PMID: 24696449.

Harrison LC, Dempsey-Collier M, Kramer DR, Takahashi K (1996) Aerosol insulin induces regulatory CD8 gamma delta T cells that prevent murine insulin-dependent diabetes. J Exp Med 184: 2167–2174

Locke NR, Stankovic S, Funda DP, Harrison LC. TCR gamma delta intraepithelial lymphocytes are required for self-tolerance. J Immunol. 2006 Jun 1;176(11):6553-9. doi: 10.4049/jimmunol.176.11.6553. PMID: 16709812.

Gearty SV, Dündar F, Zumbo P, Espinosa-Carrasco G, Shakiba M, Sanchez-Rivera FJ, Socci ND, Trivedi P, Lowe SW, Lauer P, Mohibullah N, Viale A, DiLorenzo TP, Betel D, Schietinger A. An autoimmune stem-like CD8 T cell population drives type 1 diabetes. Nature. 2022 Feb;602(7895):156-161. doi: 10.1038/s41586-021-04248-x. Epub 2021 Nov 30. PMID: 34847567; PMCID: PMC9315050.