Dissertation

Autophagy Induction as a Therapeutic Intervention in Type 1 Diabetes

submitted by

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Statutory Declaration

“I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Guidelines of the Medical University of Graz on Good Scientific Practice “.

Ceren Karacay

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Disclosures

Part of this thesis was published in the following original paper:

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The Effect of Spermidine on Autoimmunity and Beta Cell Function in NOD mice

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„Hayatta en hakiki mürşit, ilimdir”

„The most genuine guide in life is science.”

Mustafa Kemal Atatürk

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Abbreviations

ANOVA	analysis of variance
BW	body weight
cDCs	conventional dendritic cells
Cd1	cluster of differentiation 1
CTRL	control
DCs	dendritic cells
DNA	deoxyribonucleic acid
ER	endoplasmic reticulum
H&E	hematoxylin-eosin
HFD	high fat diet
IFN-ɣ	interferon gamma
IL-1β	interleukin 1 beta
IL-2	interleukin 2
IL-5	interleukin 5
IL-6	interleukin 6
IL-10	interleukin 10
LC-MS	liquid chromatography- mass spectrometry
LPS	lipopolysaccharide
µM	micromolar
mM	millimolar
min	minutes
mTORC1	mammalian target of rapamycin complex 1
NK	natural killer cells
NKT	natural killer T cells

NOD	non-obese diabetic
pDCs	plasmacytoid dendritic cells
pLN	pancreatic lymph nodes
RNA	ribonucleic acid
ROS	reactive oxygen species
RT	room temperature
SPD	spermidine
STZ	streptozotocin
T1D	type 1 diabetes mellitus
T2D	type 2 diabetes mellitus
TNF-α	tumor necrosis factor alpha
Tregs	regulatory T cells
wt/vol	weight per volume

Zusammenfassung

Die physiologische Autophagie ist für die Erhaltung gesunder, funktionsfähiger Beta-Zellen von entscheidender Bedeutung, und ein Mangel an Autophagie führt bei Mäusen zu einer Verschlimmerung von Diabetes. Die Induktion der Autophagie hat in Modellen für Typ-2-Diabetes eine schützende Wirkung auf Beta-Zellen gezeigt. Spermidin ist ein natürliches Polyamin und ein Autophagie-Induktor. Es hat sich gezeigt, dass das Polyamin Spermidin die Lebensspanne vieler Organismen verlängert und die kognitiven und kardialen Funktionen alternder Mäuse verbessert. Es wurde auch berichtet, dass Spermidin Entzündungen reduziert und Immunzellpopulationen wie T-Zellen, B-Zellen, Makrophagen und dendritische Zellen moduliert. Die Wirkung von Spermidin auf die Entwicklung von Typ-1-Diabetes wurde bisher noch nicht erforscht. Ziel dieser Arbeit war es, die Rolle der Autophagie-Induktion durch tägliche orale Spermidin-Behandlung in der Pathogenese des Typ-1-Diabetes bei NOD-Mäusen zu untersuchen.

Wir untersuchten drei Spermidin-Konzentrationen (1 mM, 3 mM und 10 mM) in zwei Studien mit männlichen NOD-Mäusen. Diese Studien dienten dazu, die Wirkung von Spermidin auf den Polyamin-Stoffwechsel und auf Vitalparameter der Mäuse wie Körpergewicht, Futter- und Wasserverbrauch zu erheben. Im nächsten Schritt untersuchten wir in zwei Präventionsstudien die Wirkung von 3 mM und 10 mM Spermidin auf die Entwicklung von Typ-1-Diabetes. Wir analysierten die gesamte Population der Immunzellen, den Grad der Insulitis, den Pool der Insulingranula und die Autophagie im Pankreas bei weiblichen NOD-Mäusen mit Spermidinbehandlung.

Die Supplementierung von 3 mM und 10 mM Spermidin im Trinkwasser wurde bei männlichen und weiblichen NOD-Mäusen gut toleriert, was sich in einem ähnlichen Körpergewicht und einer ähnlichen Wasser- und Nahrungsaufnahme zwischen der Kontrollgruppe und der Spermidin-Gruppe zeigte. 10 mM Spermidin beeinflusste den Polyamin-Stoffwechsel in mehreren relevanten Organen von männlichen NOD-Mäusen. In der Präventionsstudie bewirkte 3 mM Spermidin im Trinkwasser keine signifikante Veränderung der Diabetesinzidenz bei weiblichen NOD-Mäusen. Die Veränderungen bei CD8+ T-Zellen, Tregs und NKT-Zellen waren statistisch nicht unterschiedlich. 3 mM Spermidin veränderte den Grad der Insulitis der

Langerhans'schenInseln und die Plasmakonzentration der proinflammatorischen Zytokine bei nichtdiabetischen Überlebenden und diabetischen Mäusen nicht. Im Gegensatz zur ursprünglichen Hypothese erhöhte 10 mM Spermidin im Trinkwasser in der Präventionsstudie das Auftreten von Diabetes bei NOD-Mäusen, parallel mit einer Erhöhung der pro-inflammatorischen CD8+ T-Zellen in den pLN von diabetischen Mäusen. Darüber hinaus wirkte sich 10 mM Spermidin auf Tregs sowohl bei nicht-diabetischen Überlebenden als auch bei diabetischen Mäusen sowie auf dendritische Zellpopulationen und NK-Zellen bei nicht-diabetischen Überlebenden aus. 10 mM Spermidin reduzierte signifikant den IL-6-Plasmazytokinspiegel bei nicht-diabetischen Überlebenden, hatte aber keinen Einfluss auf andere gemessene Plasmazytokine (IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5 und IL-10) sowohl bei nicht-diabetischen Überlebenden als auch bei diabetischen Mäusen. 10 mM Spermidin hatte keinen Effekt auf die Insulitis und die thymischen T-Zell-Populationen sowohl bei nicht-diabetischen Überlebenden als auch bei diabetischen Mäusen. Darüber hinaus erhöhte 10 mM Spermidin die Autophagie-Werte in der gesamten Bauchspeicheldrüse von Mäusen mit spät einsetzendem Diabetes. Weiters hatte 10 mM Spermidin keinen Effekt auf den Pool der Insulingranula und auf die Autophagie-abhängige Lyse der Insulingranula.

Insgesamt deuten diese Ergebnisse darauf hin, dass eine Behandlung mit 10 mM Spermidin das Auftreten von Diabetes und die Zahl der proinflammatorischen T-Zellen in NOD-Mäusen erhöht, möglicherweise über die Induktion von Autophagie.

Abstract

Physiological autophagy is crucial for maintenance of healthy functional beta cells and autophagy deficiency results in exacerbated diabetes in mice. Induction of autophagy has shown protective effects on beta cells in type 2 diabetes models. Spermidine is a natural polyamine and an autophagy inducer. The polyamine spermidine was shown to prolong lifespan of many organisms and to enhance cognitive and cardiac functions in aging mice. Spermidine was also reported to reduce inflammation and to modulate immune cell populations such as T cells, B cells, macrophages, and dendritic cells. The effect of spermidine on progression of type 1 diabetes has yet to be explored. The aim of this thesis was to study the role of autophagy induction by daily oral spermidine treatment in type 1 diabetes pathogenesis in NOD mice.

We investigated three spermidine concentrations (1 mM, 3 mM, and 10 mM) in two studies employing male NOD mice. These studies were designed to observe the effect of spermidine on polyamine metabolism and on vital parameters of mice such as body weight, food, and water consumption. Later we investigated the effect of 3 mM and 10 mM spermidine on diabetes incidence, immune cell populations, islet insulitis, insulin granule pool, and autophagy in female NOD mice in two prevention studies.

Supplementation of 3 mM and 10 mM spermidine in drinking water was tolerable in male and female NOD mice, indicated by similar body weight (BW) and water and food consumption between the control and spermidine groups. 10 mM spermidine affected polyamine metabolism in several relevant organs of male NOD mice. In the prevention study, 3 mM spermidine in drinking water did not significantly change diabetes incidence in female NOD mice. The changes in CD8+ T cells, Tregs and NKT cells were inconclusive. 3 mM spermidine did not alter islet insulitis and plasma cytokine levels in nondiabetic survivors and diabetic mice. In the prevention study, 10 mM spermidine in drinking water increased the diabetes incidence in NOD mice and elevated pro-inflammatory CD8+ T cells in pancreatic lymph nodes (pLN) of diabetic mice. Additionally, 10 mM spermidine affected Tregs both in nondiabetic survivors and diabetic mice and dendritic cell populations and NK cells in nondiabetic survivors. 10 mM spermidine significantly reduced IL-6 plasma cytokine levels in nondiabetic survivors but it did not affect other measured plasma cytokines (IFN-ɣ, IL-1β, TNF-α,

IL-2, IL-5, and IL-10) both in nondiabetic survivors and diabetic mice. 10 mM spermidine did not change insulitis and the thymic T cell populations both in nondiabetic survivors and diabetic mice. Additionally, 10 mM spermidine increased the autophagy levels in total pancreas in late-onset diabetic mice. Last but not least, 10 mM spermidine did not change the insulin granule pool and autophagy-dependent insulin granule digestion.

Altogether these results suggest that 10 mM spermidine treatment increased diabetes incidence and increased pro-inflammatory T cells in NOD mice, possibly via autophagy induction.

1. INTRODUCTION

Diabetes is characterized by high blood glucose levels due to either complete loss of insulin hormone production or insulin resistance. While the loss of insulin production leads to type 1 diabetes mellitus (T1D), insulin resistance is associated with type 2 diabetes mellitus (T2D). To put the results of the dissertation into a broader perspective, the introduction will elaborate on both T1D and T2D.

1.1. Type 1 Diabetes

T1D is an autoimmune disease characterized by pancreatic beta cell loss. The clinical onset of T1D is usually recognized by high blood glucose levels due to decreased insulin levels [1]. Individuals with non-fasting blood glucose levels higher than 200 mg/dl or fasting glucose levels higher than 126 mg/dl are diagnosed with T1D [2]. Observation of clinical symptoms such as polyuria (excess urination), polydipsia (excess thirst), and weight loss also assist the diagnosis of T1D [2].

Observation studies have shown that the incidence of T1D has been increasing by about 3-4% annually [3]. These studies have illustrated that both genetic and environmental factors play a role in the development of T1D. Hence, it is defined as a multifactorial disease [4]. Studies with identical twins, siblings, or parents of individuals with T1D showed that diabetes risk becomes higher with an increasingly common genetic background [4]. While identical twins were shown to be diagnosed with diabetes with 30-70% concordance, siblings or children of individuals with T1D were diagnosed with diabetes with 6-7% and 1-9%, respectively [4]. However, environmental factors such as geography, vitamin D sufficiency, gut-microbiome diversity, obesity, socioeconomic status and early exposure to enteroviruses have been also shown to be critical in the pathophysiology of T1D [4,5].

The modern staging of type 1 diabetes in humans was described for the first time more than three decades ago in a model by Eisenbarth [6]. In genetically predisposed individuals, immune activation leads to beta cell stress. This is followed by T-cell immune response and development of T1D-related autoantibodies such as against insulin (INS), glutamic acid decarboxylase isoform 65 (GAD65), islet antigen 2 (IA-2), and zinc transporter (ZnT8) [7]. Detection of two or more autoantibodies with normal

blood glucose levels characterizes stage 1 of the disease [7]. Stage 2 is defined by two or more autoantibodies and elevated but still normal blood glucose levels due to the beginning of the loss of beta cell function. At this stage, hyperglycemic episodes can occur [7]. Stage 3 corresponds to the clinical diagnosis of type 1 diabetes as described before. At this stage, individuals show clinical symptoms such as polyuria, polydipsia, weight loss, fatigue, and diabetic ketoacidosis [7]. Figure 1 illustrates the stages in T1D progression.

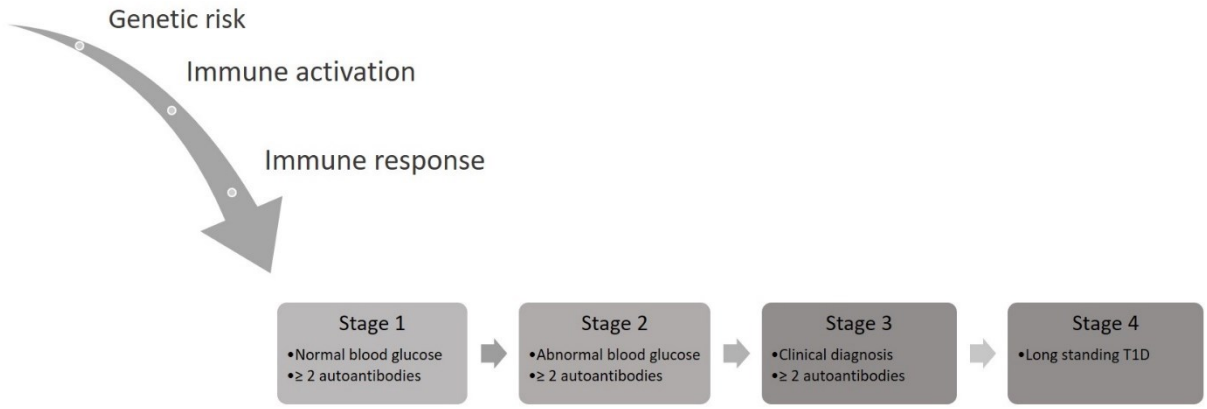

Figure 1: The stages in type 1 diabetes progression.

1.1.1. Non-Obese Diabetic Mice in Type 1 Diabetes Research

Since the discovery of pancreatic islets by Paul Langerhans in 1869, pancreatic islets have been accepted as the hubs for insulin-producing cells in the pancreas. As the access to human pancreatic islet biospecimens is complex and resource intense, animal models play an important role in diabetes research. The non-obese diabetic (NOD) mouse model is the best-characterized animal model, which facilitated T1D research for over 30 years [8]. T1D in NOD mice and T1D in humans show both similarities and differences. Similar to human T1D, diabetes onset in NOD mice is diagnosed by polyuria, glycosuria, and non-fasting glucose levels over 200 mg/dl [9]. Additionally, NOD mice share common characteristics with human T1D, such as genetic susceptibility carried on MHC complexes, infiltration of islets by T cells, and antigen-presenting cells [9]. Several autoantibodies, such as against Insulin, GAD, IA-2, IA-2β, ZnT8, IGRP can be found both in NOD mice and in humans with T1D [9]. Other autoantibodies against IAPP, HSP60, and Carboxypeptidase-H can be found in

human disease but not in NOD mice. In contrary to human T1D, NOD mice have more severe insulitis and milder ketoacidosis [9].

In addition to easy access to pancreatic tissue, the NOD mouse model also allows more complex interventions, such as the generation of transgenic mouse models and genetic manipulations. Specifically, NOD mice helped researchers to understand the pathogenesis of T1D by revealing the involvement of T cells, B cells, and antigen-presenting cells in the disease process [10–14]. NOD mice also allowed researchers to investigate important factors of T1D, such as environmental factors, e.g. the hygiene theory [15,16], infections by enteroviruses [17,18], and modulation of the innate immune system [19], all of them are related to the progression of T1D. It is important to note that above mentioned determinants of T1D are currently being investigated in the clinical study program named TEDDY (The Environmental Determinants of Diabetes in the Young) [20,21]. Nevertheless, NOD mice still serve as a crucial model enabling the examination of each environmental factor separately, which is not feasible in human disease.

1.1.2. Prevention and Intervention Studies in Type 1 Diabetes Research

The NOD mouse model is also widely used to test novel therapies aiming for prevention or reversal of diabetes. In prevention studies, treatment starts before the clinical onset of diabetes, and the success of the intervention is evaluated by the absence or delay of diabetes manifestation in the treated group. Prevention studies need to start early to allow the tested intervention to have an effect on the pathophysiologic process. Currently, it is standard to start the prevention studies at 4 weeks of age (after weaning) or shortly later and carried out until diabetes occurs or until 35 weeks of age.

In intervention studies, treatment starts when mice are diagnosed with diabetes. Depending on the treatment strategy, mice can be treated for a few days to a few weeks while non-fasting blood glucose levels are continuously monitored. It is generally agreed that interventions lasting longer than 4 weeks also should add insulin therapy, either insulin pellets or regular insulin injections, to maintain animal welfare [22]. Furthermore, in animals not treated with insulin for a longer time, hyperglycemia

above 600 mg/dl and/or ketoacidosis can occur; therefore, they are considered substantial confounders of the read-out of interest.

Corresponding to the concept of prevention or intervention in NOD mice, the same strategy is used in human T1D; either prevention or intervention studies are currently designed and executed. As in animals, the purpose of prevention studies in humans is to arrest and delay the loss of beta cell function before the clinical onset of T1D. Depending on the time of the intervention, prevention studies are typically classified into two groups as primary and secondary [23]. Primary prevention targets individuals with a completely normal glucose metabolism but specific risk factors that have been associated with T1D. Those risk factors are related to nutrition, such as early exposure to cow milk protein or early exposure to gluten or other dietary factors. In primary prevention, those risk factors are modified, e.g. the use of highly hydrolyzed cow milk to reduce the suspected exposure. [23]. E.g., one of the biggest recent trials in prevention investigated the exposure to complex cow milk proteins in 2159 infants. Those children had a high genetic risk for T1D confirmed by HLA screening and first-degree relationship to individuals with T1D [24]. In this clinical study, participants were randomized to either casein hydrolysate or a cow´s milk formula supplemented with 20% of the casein hydrolysate [24]. In contrast to earlier association studies, this randomized controlled trial concluded that hydrolyzed formula did not reduce the risk of T1D in high-risk children [24]. This trial as well as others using different interventions are very valuable for understanding the plausible triggers of T1D.

Secondary prevention studies target individuals in stage 1 and stage 2, defined as the presence of two islet autoantibodies with or without abnormal glucose tolerance [23]. Several different strategies such as nicotinamide, oral insulin, parenteral insulin, nasal insulin, teplizumab (anti-CD3 monoclonal antibody), and alum-formulated glutamate decarboxylase have been tested in secondary prevention studies [23]. Of all these interventions, only teplizumab led to promising results. A recent placebo control clinical trial investigated teplizumab in 76 nondiabetic participants in stage 2 (relatives to patients with T1D and with two or more autoantibodies and dysglycemia) [25]. Teplizumab or placebo was given for 14 days, and patients were followed for diabetes incidence. The onset of diabetes was recorded, and beta cell function was tested at 6-month intervals with C-peptide measurements during oral glucose tolerance tests

[25,26]. Teplizumab was able to delay the progression to clinical type 1 diabetes in stage 2 individuals for up to 923 days, and it was able to increase C-peptide responses in OGTT [25,26]. For the first time, with this study, progression to T1D could be delayed. These promising results are currently repeated in a second study.

Intervention (sometimes also named tertiary prevention) employs individuals recently diagnosed with T1D, in other words, individuals at stage 3 of the disease [27]. The key objective of these studies is to halt disease progression and to preserve remaining beta cell mass and function at the time of diabetes diagnosis [27]. Many therapies have been proposed and tested to delay beta cell loss, such as abatacept (CTLA4/Fc fusion protein) [28,29], alefacept (LFA-3/Fc fusion protein) [30], teplizumab (anti-CD3) [31,32], etanercept (anti-TNF-a) [33], rituximab (anti-CD20) [34,35], antithymocyte globulin (ATG) [36,37], the combination of ATG and pegylated granulocyte colony-stimulating factor GCSF [38], verapamil [39] and ex-vivo expanded Tregs [40]. In most of these studies, there was a shift for improved beta cell function, but no sustainable preservation of beta cell function was achieved. To overcome the efficacy of monotherapy, a combination of different approaches is currently under investigation. Recent clinical trials have investigated interventions with ATG and/or GCSF in participants with clinically overt diabetes. In this study, 89 participants with new-onset diabetes (duration < 100 days) were randomized to either placebo, ATG, or ATG and GCSF group [36]. At 1-year analysis, C-peptide levels of ATG treated group were higher compared to the placebo group [36]. However, C-peptide levels of the ATG+GCSF group were not significantly different from the placebo group [36]. At 2-year analysis, C-peptide levels of both ATG monotherapy were significantly higher than the placebo group and ATG+GCSF group [37]. Another recent clinical trial has investigated the effect of the combination of a monoclonal anti-IL-21 antibody and the GLP-1 receptor agonist liraglutide in participants with recently-diagnosed T1D [41]. In this study, 308 participants with new-onset diabetes (duration < 20 weeks) were randomized to either placebo, liraglutide, IL-21, or combination group and received the treatment for 54 weeks [41]. This study showed that at week 54, combination treatment led to the preservation of endogenous insulin secretion significantly better than placebo, which was illustrated by a higher C-peptide secretion in the mixed meal tolerance test [41].

The striking finding relates to the fact that almost all immune interventions tested in humans so far only showed a transient effect on the progressive loss of beta cell function. This leads to a new concept that not only the immune system has an effect but also the beta cell might play a substantial role. The beta cell concept is described in chapter 1.2.

The autoimmune destruction of beta cells in T1D was initially explained by a concept based on a defective immune-system leading to an autoimmune response. In T1D susceptible individuals, T cell selection fails to distinguish and discard autoimmune T cells due to lack or low expression of beta cell specific antigens [42]. After the autoimmune response is initiated via the capture of beta cell specific antigens by islet resident immune cells, naïve autoimmune T cells recognize these antigens and become activated in pancreatic lymph nodes [42]. Activated T cells migrate to the pancreas and start a cascade of immune cell reactions by secreting pro-inflammatory cytokines [42]. Many studies have demonstrated a role for T cells in T1D. Some examples can be listed as the presence of autoreactive CD4+ T cells and CD8+ T cells in insulitis, recurrent islet autoimmunity in pancreas transplantations, development of T1D after co-stimulation blockade therapy in cancer patients, functional abnormalities in regulatory T cells in T1D [43]. On the other hand, there is evidence demonstrating that T cells might not be the driving force in T1D. For example, islet specific autoimmune T cells were found not only in T1D patients but also in healthy subjects, which means autoimmune T cells are part of the healthy T cell repertoire [44–46]. Additionally, multiple studies have shown that immunotherapies do not provide a long lasting effect on the preservation of beta cells [28,43,47,48].

Via a defect of T cell selection in the thymus or by secondary autoimmune phenomenon, beta cells are the target for killing. To better understand the role of beta cells and immune cells in T1D pathogenesis and treatment, further chapters will outline the role of each immune cell type and beta cell stress and death.

Table 1: Comprehensive summary of key prevention and intervention studies.

Intervention	**Stage**	**Primary outcome results**	**Reference**
Hydrolyzed cow milk	Stage 0	Diabetes was not delayed or prevented	[24]
Nicotinamide	Stage 1	Diabetes was not delayed or prevented	[49]
Oral insulin	Stage 1	Diabetes was not delayed or prevented	[50]
Nasal insulin	Stage 1	Diabetes was not delayed or prevented	[51]
Alum-formulated glutamate decarboxylase	Stage 1 and 2	Diabetes was not delayed or prevented	[52]
Parenteral insulin	Stage 2	Diabetes was not delayed or prevented	[53]
Teplizumab	Stage 2	Delay of diagnosis	[25]
Abatacept (CTLA4/Fc fusion protein)	Stage 3	Improved preservation of beta cell function up to 24 months	[28,29]
Alefacept (LFA-3/Fc protein fusion)	Stage 3	Improved preservation of beta cell function up to 12 months	[30]
Teplizumab (anti-CD3)	Stage 3	Improved preservation of beta cell function up to 24 months	[31,32]
Etanercept (anti-TNF-a)	Stage 3	Improved preservation of beta cell function up to 6 months	[33]
Rituximab (anti-CD20)	Stage 3	Improved preservation of beta cell function up to 8 months	[34,35]
Antithymocyte globulin (ATG)	Stage 3	Improved preservation of beta cell function up to 24 months	[36,37]
ATG + GCSF (pegylated colony- granulocyte stimulating	Stage 3	Improved preservation of beta cell function up to 6 months	[38]
Verapamil	Stage 3	Improved preservation of beta cell function up to 12 months	[39]
Ex-vivo expanded Tregs	Stage 3	No effect	[40]
Liraglutide+ IL21	Stage 3	Improved preservation of beta cell function up to 54 weeks	[41]

1.1.3. T Cell Selection in The Thymus

Autoreactive T cells are accepted as an indicator of impaired selection activity within the thymus. Typically, immature T cells go through positive and negative selection prior to leaving the thymus as mature T cells to join the peripheral immune pool. Hence, positive selection is the first checkpoint for immature T cells, where they are tested for their recognition of self-antigens on cortical thymic epithelial cells. Immature T cells which bind antigens with high enough signals survive, and those immature T cells which fail to bind to the antigens undergo apoptosis. Immature T cells which survived positive selection migrate from the cortex to the medulla and undergo negative selection. Through negative selection, immature T cells encounter self-antigens loaded on MHC complexes presented by medullary thymic epithelial cells. Immature T cells which optimally bind antigens survive, while those that bind with a very high affinity undergo apoptosis. Following positive and negative selection, mature T cells migrate to the periphery.

Despite these two steps of selection in the thymus, self-antigen specific T cells might escape the thymus. Three possible mechanisms describe this phenomenon. One possible mechanism was described as the lack of presentation of T cell antigen in thymus due to alternative splicing of the respective gene [54]. An alternative mechanism proposed by Liu *et al*. described a low avidity recognition of self-antigen by T cells which may allow T cells to escape thymic selection [55]. Another mechanism proposed the degradation of the self-antigens in the thymus by the thymus proteases [56].

Independent of the escape of T cells, in fact, self-antigen recognizing T cells contribute to T1D development. Based on the studies in both human and mice up to now, several types of native autoantigens have been found to be recognized by CD4+ T cells and CD8+ T cells [57]. Both CD4+ T cells and CD8+ T cells recognize common self-antigens, which are insulin (INS), glutamic acid decarboxylase isoform 65 (GAD65), islet antigen 2 (IA-2), zinc transporter (ZnT8), islet amyloid polypeptide (IAPP) and islet-specific glucose-6-phosphatase catalytic subunit related protein (IGRP) [57]. Additionally, CD4+ T cells were shown to recognize glucose regulatory protein 78 (GRP 78), heat shock protein 60 (HSP60), and heat shock 70 (HSP70) [57]. Next to the conventional self-antigens, T cells can recognize modified proteins generated by

post-translational modifications [58]. These modifications and resulting neoepitopes can be categorized as following: hybrid proteins (hybrid insulin peptides, crosslinked proinsulin and secretory granule peptides), oxidation (oxidized insulin), crosslinking (transglutaminase crosslinked ChgA derived peptide, WE14), deamidation (conversion of glutamine to glutamic acid in GAD65, IA-2 and proinsulin), citrullination (citrullinated GPR78, IAPP and GAD65), spliced peptides (IAPP fusion peptides, IA-2 fusion peptides), alternative splicing (IGRP and secretogranin 5) and defective ribosomal products (aberrant translation products of insulin) [58] .

1.1.4. T Cells

T cells are a class of lymphocytes that can be divided into two main groups based on the incorporated receptor as CD4+ T cells and CD8+ T cells. Both of these T cell types play a major role in the pathogenesis of T1D, as shown by multiple studies since the development of the NOD mouse model. Early studies focusing on the role of CD4+ T cells in T1D showed that depletion of CD4+ T cells prevented and reversed diabetes onset in NOD mice [59,60]. Furthermore, studies reported that the transfer of diabetic NOD CD4+ T cells alone was able to cause diabetes [61,62]. Similarly, activated CD4+ T cells in the pancreas were able to trigger pancreatic beta cell death by secreting pro-inflammatory cytokines such as IFN-ɣ and TNF-α [63] as well as by modulating CD8+ T cells, macrophages, B cells and dendritic cells (DCs). Although CD4+ T cells were shown to be the first T cell type to infiltrate into islets [64], CD8+ T cells were also found within the inflamed islets as early as 6 weeks in NOD mice [65]. Particularly CD8+ T cells can recognize major histocompatibility complex I (MHC class I), consisting of beta 2 microglobulin, which has been targeted for the treatment of diabetes in several studies with NOD mice. These studies investigating beta-2-microglobulin deficient NOD mice demonstrated how reduced MHC class I molecules correlated with reduced frequency of CD8+ T cells and protection from diabetes [10,11,66,67].

Extensive research on CD4+ T cells and CD8+ T cells enabled the development of antibody therapies targeting both classes of T cells, which have been proposed among the most promising therapies for the treatment of T1D. One promising study showed that anti-CD3 monoclonal antibody therapy led to the depletion of T cells which

improved insulin production for up to 2 years in T1D patients [47,68]. Investigation of the anti-CD3 therapy in NOD revealed that anti-CD3 therapy reversed diabetes by inducing apoptosis of effector T cells, impairing the function of effector T cells, and promoting Treg differentiation [69–71]. Another promising therapy tested the combination of anti-thymocyte globulin (ATG), which transiently depleted T cells and induced apoptosis, and granulocyte colony-stimulating factor (G-CSF), which induced mobilization of Tregs and tolerogenic DCs [72]. This combination therapy was able to reverse diabetes in NOD mice [72]. In human T1D, monotherapy of ATG was more efficient than combination therapy, and ATG alone could improve beta cell function for up to 2 years in recent-onset T1D patients [37,73]. Currently, there is an ongoing phase II study investigating the efficacy of ATG monotherapy in new-onset T1D patients [74] In addition to successful therapies with depleting anti-CD3 antibodies, non-depleting antibody therapies with anti-CD4 and/or anti-CD8 demonstrated to reverse diabetes in NOD mice by reducing TCR signaling and by suppressing pro-inflammatory cytokines from T cells [75–79].

Regulatory T cells (Tregs) are a subset of CD4+ T cells, and they were initially identified as CD25-expressing CD4+ T cells, which suppress autoimmunity [80–82]. Later, the FoxP3+ transcription factor was identified as the major key factor driving the phenotype and function of Tregs [83–85]. The connection between FoxP3+ T cells and autoimmunity was exhibited by two key studies in the field. In the first human study, it was observed that loss of function mutations in the FoxP3 gene led to several autoimmune disorders, including T1D in individuals affected with the disorder immune dysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) [86]. Similar to IPEX syndrome, the scurfy mouse lacking a functional FoxP3 gene was shown to be affected by autoimmunity and lymphoproliferative disease [87]. In line with these two studies, direct evidence regarding the role of Tregs in T1D was shown by a study employing Treg deficient NOD mice in which lack of Tregs led to accelerated diabetes [88].

Numerous studies presented that the treatments targeting the amplified function of Tregs led to the prevention or reversal of T1D in NOD mice. IL-2 injections aiming to increase the number of Tregs were partially successful in reversing diabetes in NOD mice [89]. Contrary to the attempts focusing on the mere increase of the number of

Tregs, the utilization of adoptive transfer of CD25+ Tregs showed that CD25+ Tregs, which were transferred from healthy mice, can reverse already manifested diabetes in NOD mice [88]. Similarly, the transfer of *in vitro* expanded CD25+ Tregs could successfully reverse diabetes in NOD mice [90]. Based on adoptive transfer studies in mice, several clinical trials investigated the possibility of this treatment model in individuals with T1D. Recently it has been confirmed that infusion of autologous *ex vivo* expanded CD25+ Tregs could serve as a safe and feasible treatment for T1D [91,92]. Infusion of autologous and exogenously expanded Tregs, as well as low dose IL-2, led to an increased endogenous Treg pool. However, the impact on diabetes progression should be demonstrated [40].

A subset of Tregs express also cytotoxic T-lymphocyte associated protein 4 (CTLA4) in addition to CD25 and FoxP3, therefore, they are described as CTLA4+ Tregs. The contribution of CTLA4 to peripheral regulation was elegantly illustrated by Tivol *et al*. through experiments with CTLA4-deficient mice [93]. The CTLA4 deficient mice were shown to develop lymphoproliferative disease and die by 3-4 weeks of age [93]. The crucial role of CTLA4 in Tregs was also reported in the suppression of pancreatic islet inflammation [94]. The role of CTLA4 was also investigated in the co-stimulation modulation concept. Blocking co-stimulation by CTLA4 antibody before the onset of diabetes in NOD mice could prevent the development of diabetes [95]. Furthermore, in human T1D, treatment with abatacept, a soluble CTLA4, led to higher C-peptide levels for up to 3 years [28,29].

1.1.5. B Cells

B cells are another class of lymphocytes, and they play a crucial role in the pathogenesis of T1D. Unlike T cells, B cells mature in the bone marrow and recognize antigens through the B cell receptor. Recently the presence of B cells in insulitis has been shown to correlate with earlier diabetes onset, faster beta cell destruction, and more aggressive disease phenotype [96,97]. The communication between B cells and T cells in T1D pathogenesis was demonstrated by two studies reporting that B cell specific deletion of both MHC-I and MHC-II complexes prevented diabetes in NOD mice [98,99]. Additionally, B cell deletion in NOD mice protected mice from diabetes development [100]. Similarly, B cell depletion by monoclonal antibodies led to

prevention and reversal of diabetes in NOD mice [101–104]. Clinical research has also shown that B cell targeting antibodies delayed the reduction of C-peptide levels and reduced the need for insulin therapy in diabetic patients at 1 year and 2 years evaluation [34,35].

1.1.6. Natural Killer Cells

Natural killer (NK) cells are lymphocytes that are capable of lysing foreign cells. NK cells were detected in insulitis lesions of NOD mice as early as 1985 [105]. Compared to other mouse strains, NK cells in NOD mice were shown to have impaired cell cytotoxicity [106,107]. A defective IL-15 pathway has been implicated in the impaired NK cell function in NOD mice [108]. In NOD mice, pancreatic NK cells were shown to have the higher proliferative capacity than NK cells in lymph nodes and spleen [109]. In a diabetic state, expression of stimulatory receptors on NK cells was reported to be elevated both in diabetic NOD mice [110] and in human T1D [111].

NKp46 (CD335) is an activating receptor on NK cells, and it can recognize both mouse and human ligands presented by pancreatic beta cells. A study investigating the role of NKp46 in NOD mice and STZ-induced diabetic mice found that beta cells led to the activation of NKp46 signaling resulting in the degranulation of NK cells and death of beta cells [112]. A recent study analyzing nPOD (network for pancreatic organ donors) pancreas samples showed that autoantibody-positive patients have higher NKp46 ligands in their islets compared to the islets from nondiabetic donors [113]. The presence of NKp46 ligands seemed to decrease when the donors were fully diabetic [113]. Depletion of NKp46 in NOD mice at early (5-6 weeks old) or late (11-12 weeks old) age by fusion proteins induced the production of antibodies against NKp46, which resulted in the prevention of the development of diabetes [112]. Furthermore, NKp46 specific antibody, inducing impairment of NK cell function, halted diabetes in NOD mice and STZ-induced diabetic mice [114]. Recently a monoclonal antibody against human NKp46 has been developed, and it has been proposed as an immunotherapeutic drug to treat NKp46-dependent diseases, including T1D [115]. In contrast, some studies demonstrated that NK cell induction was beneficial preventing diabetes. Complete Freund´s adjuvant (CFA) consisting of heat-killed *Mycobacterium tuberculosis* prevented diabetes in NOD mice by inducing the production of IFN-ɣ from NK cells

[116]. NK cells were also shown to be crucial in islet allografts as their presence was required to kill dendritic cells (DCs) in perforin dependent manner [117]. Interestingly transgenic NOD mice lacking NK cells showed that NK cells were not required for diabetes development [106]. Further mice and clinical studies will be necessary to clarify the role of NK cells in T1D pathogenesis.

1.1.7. Natural Killer T Cells

Natural killer T (NKT) cells are a subset of T cells carrying NK cell markers. In contrary to T cells, NKT cells recognize lipids presented by Cd1 molecules. NKT cells are classified into three groups according to their antigen specificity and expression of the T cell receptor: invariant natural killer (iNKT), type II NKT and NKT-like cells [118]. Since iNKT represents the majority of NKT cells and most of the studies investigating the role of NKT cells in T1D have focused on iNKT cells, the literature review of NKT cells in this chapter will refer to iNKT cells.

NKT cells have regulatory functions in T1D. In NOD mice, deficiency of Cd1 molecules was shown to accelerate diabetes [119,120]. Additionally, the studies increasing the frequency of NKT cells in NOD mice showed that elevated frequency of NKT cells prevented diabetes in NOD mice [121,122]. Many studies also reported that NKT cell stimulation by exogenous treatments such as α-galactosylceramide prevented diabetes in NOD mice [123,124]. Initial theory for a such protective effect of NKT cells was explained by NKT cells-induced Th2 cell response towards islet autoantigens [123–125]. Later, NKT cell-inhibited T cell differentiation was proposed as an another mechanism leading to NKT cell-induced protection [126,127]. The impact on the T cell differentiation was described by the recruitment of tolerogenic dendritic cells such as plasmacytoid DCs (pDCs) into pancreatic lymph nodes (pLN) [127,128]. Recent studies proposed an association between Tregs and NKT cells, which suggested that viral infection in NOD mice prevented diabetes by inducing Tregs via NKT cell-pDCs interaction [129]. Similarly, NKT cell stimulation by α-galactosylceramide treatment was shown to induce Tregs and prevent diabetes in NOD mice [130,131].

Clinical studies investigating the role of NKT cells in T1D patients revealed contradictory results. While some studies showed that the frequency and function of NKT cells were reduced in diabetic patients [132,133], others reported no difference in

the frequency and function of NKT cells in diabetic patients [134–136]. The contradictory results might arise from the heterogeneity of NKT cell subsets in humans, the low number of patients, and different detection methods for NKT cells.

1.1.8. Dendritic Cells

Dendritic cells (DCs) are leukocytes and antigen-presenting cells, which process antigens to present to T cells. They play a role at the interface of innate and adaptive immune systems. DCs are classified as plasmacytoid DCs (pDCs) and conventional DCs (cDCs). cDCs are further categorized according to their CD8 expression as CD11b+ cDCs and CD4+ CD11b+ cDCs and CD8+ cDCs.

The phenotype and functions of DCs are largely unknown in the NOD mice during the development of T1D. One recent study showed that cDCs were present in the pancreatic islets of NOD mice as of 4 weeks of age whereas pDCs were not observed in the pancreatic islets of NOD mice until 10 weeks of age [137]. In addition, the presence of peri-islet dendritic cells correlated with lymphocyte infiltration to the pancreatic islets of NOD mice [137]. Initial characterization studies on DCs in NOD mice showed that DCs present defects during cell development mainly in spleen and other lymphoid organs [138–140]. These deficiencies in DC development were suggested to lead to a reduced CD8+ cDCs population [141,142] which was described to have a regulatory function [143,144]. Similarly, increased CD8+ cDCs correlated with an increased number of Tregs after treatment of NOD mice with a growth factor, Flt3 ligand, which eventually protected mice from diabetes [145,146]. However, administration of Flt3 treatment at a later stage in mice such as 11 weeks of age led to exacerbated diabetes [147].

Since CD8 expression in DCs determines the regulatory function of CD8+ cDCs, lack of CD8 expression in CD11b+ cDCs was shown to be pathological in T1D. Depletion of CD11b+ cDCs in NOD mice protected mice from diabetes by loss of CD4+ T cell activation and insulitis due to reduced pro-inflammatory T cell infiltration to pancreatic islets [148]. Furthermore, the addition of CD11b+ cDCs exacerbated the diabetes and mediated the activation of T cells. Depletion of pDCs during ongoing insulitis accelerated the infiltration of immune cells to islets and the development of diabetes [148]. This study suggests that CD11b+ cDCs were required for the initial priming of

DCs and pDCs was required to oppose immune filtration in pancreatic islets by possibly inducing Tregs [148].

Cell therapy with tolerogenic DCs is one of the emerging treatments for T1D. Tolerogenic DCs can be expanded ex-vivo using GM-CSF and IL-4 or IL-10. Several research groups showed that expanded tolerogenic DCs prevented [149–151] or reversed [152,153] diabetes in NOD mice. In human T1D, clinical studies reported the safety and feasibility of intradermal injection of tolerogenic DCs [154,155]. Ongoing clinical trials will shed light on the therapeutic potential of tolerogenic DCs in near future.

1.1.9. Macrophages

Macrophages are activated monocytes, which are the first line of defense along with the neutrophils. They are capable of phagocyting pathogens and considered part of the innate immune system. The first study describing the role of macrophages in T1D showed that blocking macrophage adhesion promoting receptors prevented insulitis in NOD mice [156]. Following studies found that the recruitment of macrophages to the pancreatic islets is mediated through CCR8 and CCL1 secretion by CD4 T cells [157] and CCL2 which was secreted by pancreatic beta cells [158]. Macrophages were suggested to play role in destruction of pancreatic beta cells by producing TNF-α and IL1-β [159,160]. Specifically high levels of TNF-α and IL-1β were produced by NOD macrophages when they faced necrotic and apoptotic cells [161]. In comparison to macrophages isolated from non-obese diabetes-resistant (NOR) mice, macrophages isolated from NOD mice secreted reduced TNF-α and IL-1 and elevated IL-12 [162]. Elevated IL-12 secretion in NOD mice is one of the proposed pathways which macrophages employ to mediate the differentiation of diabetogenic cytotoxic CD8+ T cells [163].

There are not many studies investigating the potential therapeutic role of macrophages in the treatment of T1D. One recent study has shown that the adoptive transfer of M2 macrophages into prediabetic NOD mice was successful in the prevention of diabetes [164].

1.1.10. Neutrophils

Neutrophils are polymorphonuclear leukocytes, which are the first line of defense against pathogens, and they are characterized as part of the innate immune system [165]. While earlier studies showed an increased frequency of neutrophils in human T1D compared to healthy individuals [166], recent studies reported a reduced proportion of neutrophils in human T1D compared to healthy individuals [167,168]. Dysregulated functions of neutrophils were also suggested to contribute to T1D pathogenesis. In human T1D and T1D mouse models, both adhesion [169] and microbicidal activities [170–172] of neutrophils were shown to be downregulated while apoptosis of neutrophils [173,174] was shown to be upregulated. However, there is no consensus regarding the regulation of other functions of neutrophils which are used in host-defense mechanisms such as oxidative burst activity [171,175] and phagocytosis [170–172] in the diabetic state.

In NOD mice it was shown that CXCR2-expressing neutrophils were trafficked to the pancreatic islets by beta cells and by macrophages producing CXCR2 ligands such as CXCL1 and CXCL2 [176]. Additionally, pharmacological inhibition of CXCR2 increased the survival of pancreatic islet transplantation both in mice and human subjects [177]. A recent study also showed that pharmacological inhibition of CXCR2 reversed established diabetes in NOD mice and STZ-induced diabetes mouse models [178].

1.2. Beta Cell in Type 1 Diabetes

Pancreatic islets or islets of Langerhans were defined as endocrine cells in pancreas tissue by Paul Langerhans in 1869. They constitute 1-2% of all pancreatic cells. Pancreatic islets consist of 5 types of endocrine cells: beta, alpha, delta, epsilon, and PP (gamma). Beta cells constitute 50-70% of the pancreatic islets and produce insulin, the blood glucose-lowering hormone. Alpha cells constitute 20-40% of the pancreatic islets and produce glucagon. Glucagon is a direct antagonist of insulin but exhibits also other metabolic functions in lipid and amino acid metabolism. Delta, epsilon, and PP cells constitute ≤ 10% of the pancreatic islet and they produce somatostatin, ghrelin, and pancreatic polypeptide respectively [179]. It is worth noting that the architecture of pancreatic islets is different in rodent and human islets. In rodent islets, beta cells mainly localize at the center of the islets, and alpha, epsilon, and PP (gamma) cells

locate at the rim of the islets. On the contrary, in human islets, beta and alpha cells are dispersed throughout the islet [180].

One hallmark of the T1D is the loss of beta cell function. This can result from reduced beta cell mass and/or reduced beta cell function. T1D is considered to be an autoimmune disease and which most likely to be initiated in the pancreas. Surveillant antigen presenting cells (APCs) recognize and process beta cell antigens and eventually transfer them to pancreatic lymph nodes. In the second phase, beta cell antigens presented by APCs are recognized by autoantigen-specific CD4+ T cells in pancreatic lymph nodes and these activated CD4+ T cells lead to the activation of CD8+ T cells which eventually circulate to pancreatic islets. Meanwhile, in pLN B cells are also stimulated by activated T cells to generate autoantibodies against beta cell antigens. These autoantibodies are then secreted into circulation and they can be used as biomarkers which also help to define the stages of T1D. In the third phase, autoantigen specific CD8+ T cells interact with beta cell carrying self-antigens on their MHC molecules and they directly lyse beta cells via perforin-granzyme pathway. In the meantime, other immune cell types such as NK cells, macrophages and neutrophils contribute to beta cell death by secreting pro-inflammatory cytokines and reactive oxygen species. To suppress pro-inflammation, normally Tregs should intervene, however, in T1D defects in Tregs contribute to the exacerbation of the beta cell death [4].

In pancreatic beta cells, cytokine-induced cell death is one of the most investigated mechanisms *in vitro*. Cytokine-induced cell death is triggered by pro-inflammatory cytokines, such as TNF-α, interleukin 1β (IL-1β), and interferon gamma (IFN-ɣ), which are secreted by islet-infiltrating immune cells in the pancreatic islets. Upon secretion, these cytokines bind to their respective receptors on the beta cell membrane and they activate downstream transcription factors JNK, NF-κB, and STAT-1 which enable transcription of INOS and consequently lead to cell death [181–183]. Thus, cytokine-induced beta cell death can be a suitable model to study beta cell death *in vitro*.

Beta cell death can occur via apoptosis or regulated necrosis [184]. Apoptosis is an energy-dependent programmed cell death defined morphologically by the condensed nucleus, fragmented DNA, and apoptotic bodies [185]. Mechanisms of apoptosis are

discussed in the next chapter. Necrosis follows an energy-independent mode of cell death [186]. It is defined morphologically by increased cell volume, swelling of the organelles, formation of cytoplasmic vacuoles, distended endoplasmic reticulum, condensed, swollen or ruptured mitochondria, swollen and ruptured lysosomes, disaggregation and detachment of ribosomes, and ruptured plasma membrane [187]. Ruptured plasma membrane facilitates the release of cytoplasmic content which eventually recruits inflammatory cells [187].

1.2.1. Extrinsic Apoptosis

The extrinsic pathway is defined by the activation of a transmembrane receptor by its ligand. Secreted TNF-α and Fas ligand (FasL), bind to their respective receptor molecules which are located on the cell membrane [184]. Upon ligand binding, cytoplasmic adapter proteins bind to the death receptor which activates pro-caspase 8 to Caspase 8. Caspase-8 triggers the executioner caspases such as caspase 3, caspase 6, and caspase 7 which eventually lead to apoptosis in the cell [184]. In contrast to necrosis, in apoptosis cytoplasmic content is not released to the surrounding tissue, instead apoptotic cells are phagocytosed immediately [187]. Because apoptosis is energy dependent, ongoing apoptosis can be converted to necrosis due to the lack of caspases and intracellular ATP.

1.2.2. Intrinsic Apoptosis

1.2.2.1. Endoplasmic Reticulum Stress, Unfolded Protein Response and Endoplasmic Reticulum Stress-Induced Autophagy

In the process of developing T1D an early sign of beta cell dysfunction is related to ER stress. Islet inflammation and exposure to cytokines secreted by infiltrating immune cells lead to ER stress through the JNK-MAP signaling pathway [188,189]. Reactive oxygen species (ROS) produced by extracellular and intracellular sources initiate the release of Ca^{+2} from ER lumen to cytosol and lead to ER stress [190,191]. Elevated blood glucose levels also contribute to ER stress via glucose sensing pathways, insulin production, and secretion [192]. Another mechanism, infection with coxsackie virus, which is associated with T1D, can also disrupt ER membrane leading to the release of Ca^{+2} from ER into the cytosol [193,194]. Untreated and continuous ER stress leads to

the accumulation of unfolded/misfolded proteins, which in turn activate unfolded protein response (UPR). UPR aims to improve ER stress by initiating translational attenuation and digestion of unfolded/misfolded protein and activating expression of molecular chaperons which facilitate protein folding [195]. Accumulation of unfolded/misfolded protein in ER is recognized by three mechanisms which are located on the ER membrane: RNA-dependent protein kinase like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring ER-to-nucleus signal kinase 1 (IRE1) [196]. Upon activation, PERK phosphorylates eukaryotic translation initiation factor 2α (eIF2α) leading to translational attenuation and induces activating transcription factor 4 (ATF4) which translocate into the nucleus and increases the expression of C/EBP homologous protein (CHOP) [196]. As a response to ER stress, activating transcription factor 6 (ATF6) is cleaved in the Golgi apparatus to become fully active and is translocated to the nucleus to induce the expression of X-box binding protein 1 (XBP1) [196]. Activation of IRE1 leads to the splicing of XBP1 creating an active transcription factor (sXBP1) [196]. As a result of UPR activation, expression of XBP1, sXBP1, and ATF4 lead to transcription activation of UPR-targeted genes participating in several mechanisms such as autophagy, apoptosis, protein secretion, ER-associated degradation (ERAD), folding, and lipid synthesis [196].

Unresolved ER stress may lead to apoptosis. Several reports showed that ER-stress induced autophagy pathway eliminates the misfolded or unfolded proteins and dysfunctional parts of ER [197–199]. Treatment of neuroblastoma cells with ER stressors notably induced autophagosomes as evidenced with GFP labeled LC3 structures and ER-induced autophagy-promoted cell survival [197]. Another study reported that ER-stress stimulated autophagy by activating the assembly of pre-autophagosome structure in yeast cells [198]. During unfolded protein response, it was shown that ER expansion was counterbalanced by induction of autophagosome formation [199].

ER stress-induced autophagy does not always induce cell survival in every cell model. The effect of autophagy depends on the status of the cells. Although treatment of murine embryonic fibroblasts, colon cancer cells, and prostate cancer cells with ER stressors led to autophagy induction, increased autophagy contributed to cell death [200]. ER stress-induced autophagy has been also observed in a mouse model of

transient middle cerebral artery occlusion in which autophagy worsened neuronal apoptosis [201].

ER stress-induced autophagy has been also illustrated in pancreatic beta cells. A recent study reported ER-stress induced autophagy as a feedback mechanism to protect the pancreatic beta cells from apoptosis [202]. Additionally, it was shown that blocking autophagy leads to increased ER-stress while blocking ER-stress leads to inhibited autophagy [202]. Another study also reported that primary rat islets underwent delayed apoptosis compared to the immortalized beta cell line after treatment with ER-stress inducing agents such as thapsigargin and etoposide [203]. Although autophagy was induced in both models by increased ER-stress, rapid induction of the apoptotic pathway in the beta cell line did not allow the autophagy pathway to contribute to cell survival [203]. In islets, however, delayed apoptosis enabled autophagy to improve cell survival [203].

1.2.2.2. Mitochondrial Stress

Intrinsic signals such as hypoxia, ROS, nutrient withdrawal, and DNA damage can also lead to apoptosis via the mitochondrial pathway [204]. These intrinsic stress factors activate the pro-apoptotic BCL-2 proteins (Bim, Bid, Bad, Bax, and Bak) which are normally present in an inactive form on the outer membrane of the mitochondrial membrane [204]. Upon activation, pro-apoptotic BCL-2 proteins are translocated to mitochondria from the outer membrane [204]. Activated pro-apoptotic BCL-2 proteins can either bind anti-apoptotic BCL-2 proteins (BCL-2 and BCL-XL) or they can form pores in mitochondria which leads to the release of cytochrome-c into cytosol [204]. Eventually, the accumulation of cytochrome-c in the cytosol triggers caspase-3 and the cascade of apoptosis [204].

1.2.3. Perforin and Granzyme Pathway

Another mechanism leading to beta cell death is the perforin/granzyme pathway executed by cytolytic lymphocytes (CTLs) which are CD8+ T cells and NK cells. Perforins and granzymes are proteins which are found in the secretory granules of CTLs [205]. Upon recognition of target cells, granules containing perforins and granzymes are released and perforins form transmembrane pores on the membrane

of target cells allowing granzymes to enter the target cells [205]. Granzymes are serine proteases and they induce apoptosis through different mechanisms based on their type. While Granzyme B and A are well described, many granzyme types such as H, K, and M in humans and C, D, E, F, G, K, L, M. and N in mice are not well described up to now [206]. Granzyme B induces apoptosis by cleaving proteins at their aspartate residues which activates caspase-dependent apoptosis and activates the mitochondrial pathway by cleavage of Bid [186]. Granzyme A induces apoptosis via a caspase-independent pathway by cleaving SET complex (an endonuclease Ape1, an endonuclease NM23-H1, and a 5'-3' exonuclease Trex1) which leads to DNA cleavage [186].

Many studies have investigated the effect of perforin/granzyme deficiency on diabetes development in NOD mice. After the generation of perforin-deficient NOD mice [207], two independent studies showed that perforin-deficient NOD mice have reduced diabetes compared to control mice [208,209]. Studies investigating the development of diabetes in NOD mice lacking Granzyme B showed that deletion of Granzyme B did not affect diabetes incidence, suggesting that lack of Granzyme B was compensated by other granzyme types or other beta cell death-inducing mechanisms [210,211].

As mentioned before immune therapies could not always provide preservation or enhancement of beta cell function in human T1D. Interestingly, verapamil, a calcium channel blocker, was able to improve beta cell function without altering the immune cell profile. Thus, an alternative hypothesis suggests that beta cells contribute to their own demise. According to this hypothesis, the early steps in the disease process are related to beta cell stress due to several triggers. These triggers lead to antigen presentation in dendritic cells which is later recognized by immune cells [43]. This concept is also recognized as “beta cell suicide theory”. One beta cell stress might be the nature of the islet [43]. It has been shown that cytokine-induced generation of neoantigens which are created by alternative splicing [212,213], post-translational modifications [214–216] and peptide fusion [217] can more robustly activate immune response. Another stress trigger might be reduced beta cell mass or reduced beta cell function which leads to increased stress on beta cells to maintain glycemic control. Eventually increased metabolic stress leads to ER stress and beta cell death. Last but not least, beta cells are also vulnerable to viral infection since coxsackievirus and

adenovirus receptors are found in insulin granules of beta cells [218]. Viral infection might also lead to ER stress [193,219].

1.3. Autophagy

Autophagy is an evolutionarily conserved pathway mainly responsible for the degradation and recycle of cytosolic material. In the autophagy pathway, double-membrane vesicles which are also called autophagosomes, are formed around the cytoplasmic material to be delivered to lysosomes.

Autophagy can be initially categorized into nonselective and selective autophagy. Nonselective or bulk autophagy is generally observed upon starvation by the presence of double membrane phagophores and cargo containing autophagosomes. Several ATG proteins regulate the autophagy pathway from the initiation of phagophores to the delivery of cargo to the lysosomes [220]. One of the vital proteins in the autophagy pathway is Atg8-PE (phosphatidylethanolamine) which is recruited to the phagophore assembly and regulates the elongation of the phagophore [221]. Atg8-PE is also known as LC3-II which is the lipidated version of LC3-I in mammals. Lipidation of LC3-II takes place during the initiation of the autophagy pathway. Thus, an increased ratio of LC3-II to LC3-I shows the turnover of the autophagosome formation and this ratio is widely accepted as an autophagy marker [222]. Another protein in the autophagy pathway is Vps30/Atg6 which is recognized as Beclin1 in mammals. Beclin1 is also recruited to phagophore assembly [223] and it could be found on the autophagosome membrane. Consequently, increased Beclin1 protein levels suggest increased autophagy function [222]. Other nonselective autophagy types include microautophagy and chaperone-mediated autophagy which directly deliver the cargo to lysosomes without the need for phagophores [220].

Similar to bulk autophagy, selective autophagy requires the same core ATG proteins and additionally some selective autophagy receptors such as SQSTM1/p62, Nbr1, Optineurin, and Ndp52 which can bind selectively to individual proteins [220]. p62/SQSTM1 is an important receptor protein playing a role in the autophagy pathway. It selectively binds other proteins that are targeted for degradation via autophagy pathway. Along with the targeted proteins, p62 is similarly degraded through the autophagy pathway. Usually, a reduction in p62 levels within cells suggests elevated

autophagy. Alternatively, the abundance of p62 in a cell is accepted as a marker for defective autophagy [224].

In beta cells, selective autophagy affects mitochondria, insulin/proinsulin granules, lipids, protein aggregates, ER, and peroxisomes [220]. Crinophagy is a process where lysosomes merge with insulin/pro-insulin granules and lead to the degradation of these granules. Recently another autophagy-dependent degradation mechanism has been described where double-membrane forms around insulin/pro-insulin granules and later on these double-membrane vesicles merge with lysosomes for degradation of the granules [225]. These double-membrane vesicles were initially identified in an autophagy-induced T2D model, where Beclin-1 was constitutively expressed [225]. Different than crinophagy, these vesicles had double-membranes, thus they were proposed as a new type of selective autophagy mechanism and they were named as vesicophagy [225]. Supporting this vesicophagy concept, previously it was shown that rapamycin-induction led to an increased number of autophagic vesicles containing insulin granules [226]. In summary, autophagy can affect cell homeostasis by modulating several aspects of beta cell function.

1.3.1. Spermidine

Spermidine is a natural polyamine associated with crucial cellular functions and cell homeostasis. In general polyamines bind and stabilize DNA and RNA, show antioxidant activities, regulate enzymatic reactions and they are involved in regulation of translation [227,228]. Therefore, polyamines play a role in cell growth, cell proliferation, and regeneration. Recently, spermidine has been shown to act as a novel autophagy inducer [229]. Spermidine was reported to induce autophagy by inhibiting acetyltransferase EP300 and by the hypusination of transcription factor eIF5a depending on the cell type [230–232]. Spermidine-induced autophagy was proposed to prolong the life span of many experimental models such as yeast, flies, worms, mammalian cells, and mice [233,234]. Spermidine-induced autophagy was also reported to promote cardioprotection and to improve cognitive function in aging mice [233,235]. Besides the role of spermidine in aging, spermidine has been suggested to have an anti-inflammatory function in a both autophagy-dependent and autophagy-independent manner. Multiple studies revealed that spermidine treatment

reduced the production of pro-inflammatory cytokines [236–238] or reduced inflammation by modulation of macrophages in various inflammation models [239,240]. Additionally, spermidine-induced autophagy was demonstrated to modulate Tregs and inhibit tumorigenesis [241]. In addition to the effects of spermidine on inflammation, autophagy induction by spermidine was also suggested to induce stem cell function in aging muscle stem cells [242] and in epithelial stem cells [243] and to reprogram somatic cells into induced pluripotent stem cells [244].

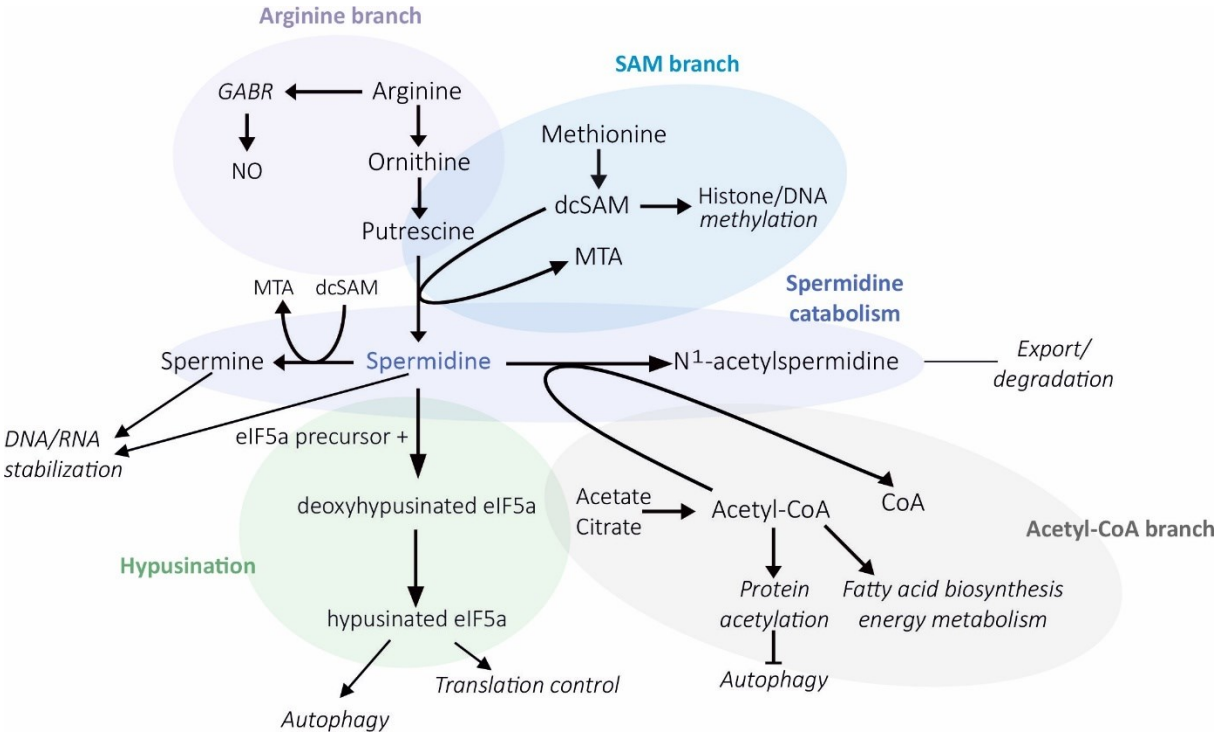

Figure 2: Overview of the diverse functions of spermidine in cellular processes. dcSAM: decarboxylated S-adenosylmethionine, GABR: global arginine bioavailability ratio, MTA: 5′-methylthioadenosine.

1.3.2. The Effect of Autophagy on Beta Cell Health

Basal autophagy has been suggested to be crucial for the maintenance of islet cell survival and function. This phenomenon was established with two studies employing loss of autophagy function: Specific deletion of pancreatic autophagy related 7 (Atg7) led to decreased beta cell mass, reduced serum insulin level, and reduced insulin production [245,246]. Additionally, high-fat diet (HFD) and high-glucose (HG) treated mice had reduced beta cell mass when autophagy was inhibited by Atg7 shRNA [247]. Furthermore, beta cell specific autophagy deficiency in obese mice revealed that autophagy deficiency might accelerate the progression from obesity to T2D [202].

Similarly, the hyperreactivity of the mammalian targets of rapamycin complex 1, a negative regulator of autophagy, led to reduced autophagy and beta cell failure [248].

Autophagy deficiency has been linked with diabetes susceptibility in genome-wide association studies [249,250] and with loss of function studies [245,246] in mouse models. However, there are currently conflicting results regarding autophagy levels in diabetes state. Ebato *et al*. showed that T2D mouse models such as HFD-treated mice and db/db mice had higher autophagy levels in their pancreatic beta cells [245]. Another research group supported that finding in HFD and HFD + HG treated mice exhibiting higher autophagy levels [247]. A transgenic beta cell line generated from Akita mice, which is another T2D model, was also reported to have higher autophagy levels in comparison to the beta cell line generated from wild-type mice [251]. In opposite to the above findings, Liu *et al*. found that the islets from HFD-treated mice had less autophagy compared to the islets from control mice [252]. In line with that, another study presented that streptozotocin (STZ) induced diabetic mice and HFD-treated mice also had lower autophagy levels in their pancreas [253]. In agreement with the results from mouse models, a clinical study reported that pancreatic islets from individuals with T2D had less autophagy compared to pancreatic islets from individuals with no diabetes [254]. Similarly, a recent study has shown that islets isolated from individuals with T2D have a higher amount of p62 in comparison to nondiabetic individuals indicating inhibited autophagy [255]. Further studies are needed in T2D models to clarify the effect of diabetes on autophagy levels. Furthermore, the role of autophagy in T1D has not been examined extensively except an *in vitro* study investigating the effect of pro-inflammatory cytokines on autophagy levels. This study has illustrated that treatment of the MIN6 beta cells and primary rat islets with pro-inflammatory cytokines led to autophagy inhibition [256].

Targeting autophagy as a therapeutic intervention has been widely investigated in T2D models such as HFD and/or HG-treated mice and in insulin-deficient models such as STZ-induced and alloxan-induced diabetes. Induction of autophagy by rapamycin has been shown to affect diabetes and beta cell function both positively and negatively. Rapamycin is an inhibitory substrate of the mammalian target of rapamycin (mTOR) and an immunosuppressant which has been mainly used to prevent rejection in kidney transplants due to its inhibitory function on activation of T cells and B cells [257].

Bugliani *et al* presented that ER stress-induced apoptosis in isolated human islets by palmitate and brefeldin was reduced by rapamycin-induced autophagy [255]. Rapamycin treatment restored reduced insulin granules triggered by palmitate and brefeldin [255]. The analysis of isolated human islets from individuals with T2D showed that rapamycin treatment decreased the expression of ER-stress markers [255]. Similarly, rapamycin-induced autophagy ameliorated ER-stress-induced diabetes model by improving glucose tolerance, increasing plasma insulin levels and pancreatic insulin content, and by preventing beta cell apoptosis [251].

There are also numerous studies showing the negative impact of rapamycin-induced autophagy induction on diabetes or pancreatic beta cells. *In vitro* experiments with pancreatic beta cell lines and *ex vivo* pancreatic islet studies demonstrated that rapamycin treatment increased apoptosis, reduced viability, and glucose stimulated insulin secretion (GSIS) [258]. *In vivo* studies also demonstrated that chronic rapamycin treatment led to either impaired or unaffected insulin tolerance depending on the gender, the concentration of rapamycin, and the route of treatment [259–262] and furthermore chronic rapamycin treatment increased mortality in db/db mice, a model for T2D [263]. Initially, rapamycin was suggested to affect insulin homeostasis by diminishing beta cell mass and reducing plasma insulin levels and insulin content [264,265]. Later, it was proposed that the inactivation of mTORC1 signaling could impair insulin secretion due to autophagy induction and elevated autophagic bodies containing insulin granules [226]. A recent study confirmed this assumption by reporting that those autophagic bodies were indeed loaded with insulin granules [225]. Yamamoto *et al* suggested that those structures differed from crinophagic bodies, another insulin degradation pathway, stemming from the fusion of insulin granules with lysosomes, as they possess double membranes [225]. Furthermore, it was shown that constitutive autophagy due to overexpression of an autophagy gene Beclin1 led to decreased insulin secretion due to the digestion of granules by autophagy [225,266]. In line with those findings, it was shown that HFD treated VAMP7-deficient mice showed reduced insulin secretion and glucose intolerance [267]. It was hence suggested that pancreatic beta cell-specific deletion of VAMP7, a snare protein playing role in vesicle trafficking, led to defective autophagosome formation and accumulation of dysfunctional mitochondria [267]. In conclusion, while basal autophagy is required

for autophagy-dependent insulin granule digestion, excessive and chronic autophagy induction might impair insulin homeostasis. Future studies employing different diabetes mouse models and autophagy-inducing agents will be necessary to clarify the role of autophagy in insulin granule homeostasis.

Similar to rapamycin, spermidine treatment in HFD-treated mice reduced weight gain, improved insulin sensitivity, and counteracted diabetes which was achieved by spermidine-induced elevated autophagy levels [268]. Additionally, intermittent fasting was shown to prevent beta cell loss in HFD-treated mice in an autophagy-dependent manner [252]. Another interesting finding relates to GLP1 agonist liraglutide, which is widely used to treat T2D. Liraglutide was reported to alleviate diabetes in HFD-treated mice and STZ-induced diabetic mice by increasing beta cell proliferation and autophagic levels in fasted islets [253]. Liraglutide treatment was also described to be protective in pancreatic beta cells in an alloxan-induced diabetes mouse model [269]. Another study also showed that liraglutide protect against high glucose induced apoptosis in pancreatic beta cells in an autophagy-dependent manner [270].

Although the relationship between autophagy induction and beta cell health was studied in T2D models, there are only indirect reports investigating the role of autophagy-inducing agents in beta cell health in T1D models. Those reports did not study whether the effect was mediated in an autophagy-dependent manner. Additionally, studies in T1D models using autophagy-inducing agents revealed conflicting findings. For example rapamycin, an autophagy inducer [258], failed to prevent diabetes development in some studies [271,272], while others reported that rapamycin treatment prevented diabetes [273–275] in NOD mice. Differences in those studies probably arise from the variations in treatment duration and frequency, application route, dosage, and diagnosis of diabetes onset. Vitamin D, another autophagy inducer [276], was shown to prevent diabetes in NOD mice by decreasing IFN-ɣ+ CD8+ cells and inducing regulatory T cells (Tregs) [277]. On the contrary, liraglutide, which showed a protective effect in beta cells in T2D and insulin-deficient models, was shown to be not sufficient to prevent diabetes in NOD mice [278]. Although above mentioned studies did not investigate the role of autophagy induction, several studies showed that modulation of apoptosis in pancreatic beta cell lines might

be mediated through autophagy pathway after rapamycin, Vitamin D, and liraglutide treatment [253,258,275,279].

1.3.3. The Role of Autophagy in Immune System

Besides being one of the most important players in cell homeostasis, autophagy also plays a significant role in the immune system. In this chapter, I will briefly outline the role of autophagy in each immune cell population which is relevant for T1D pathogenesis.

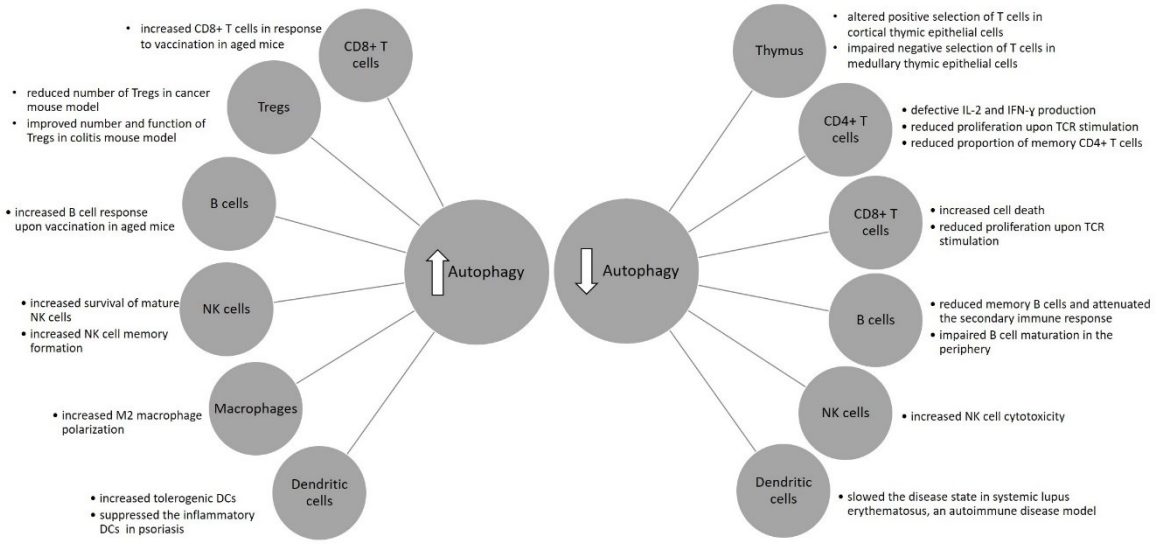

Figure 3: The role of autophagy in immune system.

1.3.3.1. The Role of Autophagy in T Cell Selection in The Thymus

As it is widely recognized, dysregulated positive and negative selection lead to increased autoimmune T cells in the periphery. Although, there is no study investigating the leak of autoimmune T cells to periphery in humans, dysregulated thymic T cell selection was proposed to contribute to the development of diabetes in NOD mice [280–282]. Autophagy in thymic epithelial cells was shown to play a significant role in the generation of the MHC-II peptide pool which regulated thymocyte development [283,284]. The role of autophagy in producing MHC-II peptides has been also previously shown by many groups in different cell models [285–288]. One study found that autophagy impairment in cortical thymic epithelial cells led to an altered

selection of MHC-II peptides thereby altering the positive selection of T cells [284]. Additionally, the same study showed that autophagy deficiency in medullary thymic epithelial cells impaired the negative selection of T cells by defective presentation of tissue restricted antigens [284].

1.3.3.2. T Cells

Many studies have proposed that autophagy is critical for the homeostasis of T cells [289–292]. Autophagy-deficient CD8+ T cells showed increased cell death and reduced proliferation upon TCR stimulation [292]. Interestingly, energy metabolism during CD4+ T cell activation was also regulated by autophagy. Autophagy-deficient CD4+ T cells showed defective IL-2 and IFN-ɣ production and reduced proliferation upon TCR stimulation [292,293]. It was observed that autophagosomes contained mitochondria and other organelles in activated T cells in comparison to resting T cells [293]. Autophagy was also suggested to regulate memory T cells. Specifically, it is essential for the memory formation of CD8+ T cells [231,294]. Similarly, autophagy impairment led to reduced proportion of memory CD4+ T cells, but it did not affect the survival of naive CD4+ T cells [295]. Autophagy seems to regulate long-term memory of antibody production in T cells in a study where autophagy-deficient T cells were transferred to a naïve host [295]. Similarly, autophagy induction by spermidine led to increased CD8+ T cells in response to vaccination in aged mice [231].

Up to now the role of autophagy in Tregs has been investigated in various but limited number of experimental models. The impact of autophagy induction on Tregs has been shown in an *ex vivo* differentiation study where spermidine treatment led to an elevated number of Tregs in an autophagy-dependent manner although spermidine did not change the suppression capacity of Tregs [232]. On the contrary, it was also proposed that spermidine treatment could prevent the development of T cell-induced transfer of colitis in mice [232]. In fact, in a cancer study, spermidine treatment led to a reduction of Tregs in an autophagy-dependent manner in order to reduce the tumor size [241]. Overall, the role of autophagy in Tregs remains to be elucidated.

1.3.3.3. B Cells

Numerous studies explored the role of autophagy in B cells. These studies mainly showed that autophagy could be dispensable in the development of B cells based on the stage of development but not in the memory function of B cells. Early B cell development was not affected by the deletion of Atg5 [296]. However, maturation of B cells in the periphery was impaired by autophagy deficiency generated by deletion of Atg5 or Beclin1 genes [297,298]. While maintenance of mature B cells in the periphery was not affected by autophagy deficiency [299], innate-like B cells, (B1a memory B cells) were shown to be susceptible to autophagy deficiency [300]. B cell memory formation was suggested to occur normally in the absence of autophagy [301,302]; however, autophagy deficiency led to reduced memory B cells in time and attenuated the secondary immune response [302]. The clinical relevance of the impact of autophagy in B cells was shown in systemic lupus erythematosus, a B cell-driven autoimmune disease. Multiple studies have proposed that autophagy deficiency led to reduced autoantibodies and diminished disease phenotype [296,303,304]. Similar to the spermidine-induced memory T cell increase in aged mice, autophagy induction by spermidine was shown to increase B cell response upon vaccination in aged mice [305].

1.3.3.4. Natural Killer Cells

The association of NK cells and autophagy has been investigated only in a limited number of studies. These studies suggested that autophagy is essential for the survival and memory function of NK cells. A recent study showed that the deletion of Atg5 can lead to impaired survival and development of innate lymphocytes [306]. Similarly, the deletion of mitophagy related gene, Clec16a, has been shown to increase NK cell cytotoxicity [307]. In addition to basal autophagy necessity, autophagy induction also increased the survival of mature NK cells [306]. Additionally, autophagy-inducing agents metformin and rapamycin were shown to increase NK cell memory formation via the regulation of mitophagy [308].

1.3.3.5. Dendritic Cells

Various studies have shown that autophagy plays a significant role in the regulation of dendritic cells. It is extensively accepted that DCs both reduce and exacerbate inflammation in autoimmune diseases. Autophagy induction by combination treatment of Flt3 receptor-ligand and rapamycin stimulated tolerogenic DCs, which promoted the production of Tregs as a protective mechanism in allograft survival [309]. Moreover, a recent study suggested that Tregs are controlled by DCs, which means that autophagy deficiency in DCs regulates the stability and function of Tregs, triggering autoimmune diseases [310]. In inflammatory bowel disease, an autoimmune disease, autophagy was required for DCs to activate Tregs, which suppressed the mucosal inflammation [311]. Similarly, in psoriasis, also an autoimmune disease, spermidine was reported to suppress the inflammatory DCs through an enhanced FOXO pathway, possibly by regulating autophagy [312]. Conversely, loss of autophagy in DCs in systemic lupus erythematosus, an autoimmune disease model, slowed the disease state [313]. Downregulation of autophagy by Tregs inhibited immunogenic DC maturation, leading to reduced autoantigen presentation, decreased T cell priming, and alleviated autoimmune response [314]. Additionally, Atg5 was shown to be required for antigen presentation on MHC-II complexes of DCs [315]. DC autophagy was shown to be responsible for the presentation of citrullinated antigens, which are important players in autoimmune diseases including T1D [182,214]. Autophagosomes were reported to transport peptidylarginine deiminases (PAD) to antigen containing-endolysosomes, following which PADs generate citrullinated peptides, which were presented on DCs to CD4+ T cells [316]. Further studies are required to clarify the role of autophagy in DCs.

1.3.3.6. Macrophages

The role of autophagy in the macrophage population is not clear despite a few reports proposing a possible role in regulating polarization from M1 to M2. These reports employed spermidine, implying that the mode of action could be autophagy; however, no direct evidence was reported. Spermidine has been shown to inhibit T cell activation by promoting M2 polarization mediated by a reduction in NF-KB dependent pro-inflammatory cytokine production in several inflammatory disease models

[238,317,318]. Additionally, in experimental autoimmune encephalomyelitis, which is a mouse model for the autoimmune disease multiple sclerosis, spermidine also favored M2 macrophage polarization [239,240]. However, these studies fail to show the association of M2 polarization with elevated autophagy levels.

1.4. Hypothesis and Aims

1.4.1. Hypothesis

Autophagy plays significant roles in both beta cell health and the immune system. It was first associated with T1D in genome-wide studies [249,250]. Later, loss of function studies showed that basal autophagy is required for functional beta cells [245,246]. Additionally, autophagy deficiency leads to exacerbated diabetes in mice. Therefore, we hypothesized that autophagy induction could protect non-obese diabetic (NOD) mice from T1D. To examine the effect of autophagy induction in T1D, we chose spermidine as the autophagy-inducing agent. Spermidine is a natural polyamine, and it has been shown to be protective in myocardial and neural function in aging mice. Based on current evidence, it is not clear how autophagy can be protective in T1D. Since previous studies have not investigated the direct effect of autophagy induction on the beta cells and a wide range of immune cells in NOD mice, this thesis examined for the first time the impact of spermidine and spermidine-induced autophagy in NOD mice.

Taken together, our hypothesis was that spermidine might protect NOD mice from T1D via autophagy induction.

1.4.2. Aims

The aim of this thesis was to investigate the impact of spermidine and spermidine-induced autophagy on T1D pathogenesis in NOD mice.

Specific aims:

1. Investigate the effect of spermidine treatment on diabetes incidence
2. Examine the effect of spermidine on polyamine and autophagy levels
3. Elucidate the impact of spermidine on immune cell populations and islet insulitis
4. Analyze the influence of spermidine on insulin granule pool in pancreatic islets

2. MATERIAL and METHODS

2.1. Mice Experiments

NOD/ShiLtJ strain was purchased from Charles River and inbred under specific pathogen-free conditions in the animal facility of the Medical University of Graz. Approval for conducting all mice experiments was granted by the Federal Ministry Republic of Austria Education, Science, and Research (TVA number: BMWFW-66.010/0142-WF/V/3b/2017). Mice were maintained in individually ventilated cages under pathogen-free conditions during our studies. In each study, mice were assigned to the experimental group randomly and by matching the parental background. Mice received food and water ad libitum. In each study, water consumption was checked and recorded twice a week, and body weight (BW) and food consumption were checked and recorded once a week.

All mice in four studies were sacrificed at the animal facility of the Medical University of Graz. Mice were anesthetized using ketamine/xylazine solution (0.1 ml/ 10 g BW) with a 25-gauge needle. Ketamine/Rompun solution was prepared by mixing 1 ml of Ketasol (AniMedica, Germany), 0.8 ml of Rompun (Bayer, Germany), and 8.2 ml of distilled water (Fresenius Kabi, Austria). Once the mice were sedated, cervical dislocation was performed. Organs and blood were collected and fixed in liquid nitrogen, and tissues were also fixed in formalin.

Spermidine (Sigma-Aldrich, USA) was reconstituted in 1 M sterile water and stored in aliquots at -80°C for a maximum of 3 months. Water bottles were prepared freshly containing respective spermidine concentrations and changed on Mondays and Thursdays. Mice studies were summarized in table 2.

Table 2: Summary of mice studies. Control (ctrl) and spermidine (SPD).

Study	Treatment Groups	Number of Mice	Treatment Start (weeks of age)	Treatment End	Gender
Study-I	CTRL 1 mM SPD 3 mM SPD 10 mM SPD	2 3 3 2	4 weeks	8 weeks	Male
Study-II	CTRL 3 mM SPD	14 14	4 weeks	At diabetes onset or 35 weeks	Female
Study-III	CTRL 10 mM SPD	10 12	4 weeks	8 weeks	Male
Study-IV	CTRL 10 mM SPD	30 30	4 weeks	At diabetes onset or 35 weeks	Female

2.1.1. Studies in Male NOD Mice

Female mice become diabetic at a rate of 50-85%, while male mice become diabetic at a rate of 10-30%. Due to this gender bias, female NOD mice are employed in prevention studies. To make use of male mice, we set up the pilot studies with male mice to define the suitable spermidine concentrations for prevention studies.

Although different concentrations of spermidine treatment have been tested in different strains of mice in previous studies, so far spermidine has not been tested in NOD mice before. Therefore, we designed a study to define the tolerability of spermidine (SPD) in NOD mice and to determine the spermidine dose for the prevention studies of this thesis. Based on the literature, we initially decided to test 1 mM, 3 mM, and 10 mM of spermidine (Table 2). After the conclusion of the prevention study (study-II) in female NOD mice, we increased the spermidine concentration to 10 mM. In these studies, male NOD mice were given untreated or above-mentioned spermidine concentrations treated drinking water ad libitum for 28 days starting from the age of 4 weeks until the age of 8 weeks (Table 2). The effect of SPD on body weight (BW), water and food consumption, and polyamine levels were measured in relevant tissues and blood.

2.1.2. Studies in Female NOD Mice

Based on the literature and the dose-response study in male mice, we initially tested the effect of 3 mM SPD on diabetes in female NOD mice, and in the following prevention study, 10 mM SPD was tested in female NOD mice (Table 2). In these studies, the effect of spermidine was investigated in diabetes incidence, pancreatic

insulitis, immune cells, and autophagy levels. The treatment started when the mice were 4 weeks old and continued until diabetes onset or the mice reached 35 weeks of age. Control mice were given untreated regular drinking water, and the spermidine group was given the above-mentioned SPD concentration via drinking water. Mice were sacrificed as diabetic when diagnosed with two consecutive blood glucose (BG) measurements equal to or above 200 mg/dl. Diabetic mice were sacrificed on the same day of the second BG measurements. Mice were diagnosed as nondiabetic when they were 35 weeks old, and their blood glucose level was below 200 mg/dl. In study-IV, two control mice were excluded from the data analysis since one mouse was found dead in its cage, and the other mouse was severely sick.

2.2. Blood Collection

For flow cytometric analysis, blood was obtained by cardiac puncture using a 23-gauge needle and a syringe flushed with 150 U/ml lithium heparin (Li-He) (Sigma-Aldrich, USA). Blood was collected in Li-He-coated tubes (Sarstedt, Germany). For other blood analyses, heart was punctured a second time by 23-gauge needle and syringe flushed with EDTA (0.5 M pH 8 EDTA, Sigma Aldrich, USA). The obtained blood was stored in Eppendorf tubes containing EDTA or in K3-EDTA coated tubes (Sarstedt, Germany). Plasma was obtained by centrifugation of whole blood at 2500 g for 20 min at RT. Collected whole blood and plasma were kept on ice until storage at -80°C.

2.3. Liquid Chromatography-Tandem Mass Spectrometry

Tissues collected from study-I and study-III were analyzed for the polyamines by liquid chromatography-tandem mass spectrometry.

The method for polyamine measurements performed in this thesis was previously published by Magnes *et al.* [319]. Further details regarding the method may be found in that article. For this thesis, polyamine extraction was performed in the laboratory of Tobias Eisenberg at the Institute of Molecular Biosciences, University of Graz and polyamine measurements were conducted in the laboratory of Bioanalysis and Metabolomics at Joanneum Research supervised by Dr. Christoph Magnes.

Polyamines isolated from the thymus, spleen, pancreas, pancreatic lymph nodes (pLN), heart, plasma, and whole blood were measured by liquid chromatography-

tandem mass spectrometry. Measured polyamines included spermidine, spermine, ornithine, and putrescine. Mice were sacrificed, and organs were snap-frozen in liquid nitrogen and stored at -80°C until analysis. Blood and plasma were collected freshly and stored at -80°C as described before. Since each tissue contains different amounts of polyamines, a dilution factor (D) for each tissue was considered. Hence measured polyamines levels fall within a calibration range. Dilution was only performed for putrescine, spermidine, and spermine measurements, and it is calculated for 30 mg tissue or 25 µl whole blood samples. 15-30 mg of tissue was pulverized by mortar and pestle on dry ice. Then 25 µl * D internal standards and D* 125 µl trichloroacetic acid were added to pulverized tissue or 20 µl whole blood sample. The samples were incubated on ice for 1 h and centrifuged for 10 min at 10000 g at 4°C. 150 µl of supernatant was mixed with 800 µl double distilled water, 125 µl sodium carbonate buffer (1 M, pH 9), and 25 µl isobutyl chloroformate, then incubated for 15 min at 35°C. The solution was centrifuged for 1 min at 10000 g, and the resulting supernatant was measured in the Ultimate 3000 HPLC system (Thermo Fisher Scientific, USA) coupled to a triple-quadrupole mass spectrometer, a Quantum TSQ Ultra AM controlled by Xcalibur Software 4.0 (both Thermo Fisher Scientific, USA).

2.4. Histology

Pancreata from sacrificed mice were fixed in formalin for at least 24h and processed in Tissue-Tek VIP (Sakura, Japan) (Table 3). Later samples were embedded in paraffin blocks using Tissue-Tek TEC at 56°C (ATS-200856, Sakura, Japan). Tissue sections (2-2.5 µm) were cut and selected 50 µm apart for hematoxylin and eosin (H&E) staining to avoid overlapping islets. Deparaffinization and H&E staining were performed according to Table 4. Each stained section was analyzed, and images were taken with an Aperio ScanScope digital slide scanner (Leica Biosystems, Germany) at 40-fold magnification. Islets were counted and classified in a blinded fashion by two independent observers. The results are the average of these two datasets. Immune cell filtration in islets was classified as published before [320]: grade 0: no infiltration, grade 1: peri-insulitis to 10% insulitis, grade 2: 10-50% insulitis, grade 3: more than 50% insulitis, grade 4: 75-100% insulitis.

Table 3: Tissue processing

Solution	Temperature	Duration
Formalin 6%	40°C	1 h
Formalin 6%	40°C	1 h
Ethanol 70%	40°C	1 h
Ethanol 80%	40°C	1 h
Ethanol 96%	40°C	1 h
Ethanol 96%	40°C	1 h
Ethanol 100%	40°C	1 h
Ethanol 100%	40°C	1 h
Tissue Clear	40°C	1 h
Tissue Clear	40°C	1 h
Paraffin	53°C	1 h
Paraffin	53°C	1 h
Paraffin	53°C	2 h
Paraffin	53°C	2 h

Table 4: Deparaffinization and H&E Staining

Solution	Duration
Xylene	2 min
Xylene	2 min
Ethanol 100%	2 min
Ethanol 100%	2 min
Ethanol 90%	2 min
Ethanol 90%	2 min
Ethanol 70%	2 min
Ethanol 70%	2 min
Ethanol 50%	2 min
Ethanol 50%	2 min
Distilled Water	2 min
Distilled Water	2 min
Hematoxylin (Gatt-Koller, Austria)	5 min
Warm tap water	1 min
Eosin Stain (Gatt-Koller, Austria)	4 min
Distilled Water	1 min
Distilled Water	1 min

2.5. Flow Cytometry

Flow cytometry experiments were conducted at CBmed under the supervision of Dr. Barbara Prietl with the help of CBmed GmbH-Center for Biomarker Research in Medicine Immunology core facility and Dr. Clemens Harer.

When mice were sacrificed, blood was obtained by cardiac puncture as described before, and spleen, pancreatic lymph nodes (pLN), and thymus were collected immediately, and stored briefly in cold RPMI buffer (pH 7.4, Thermo Fisher Scientific, USA) including 5% FBS (Thermo Fisher Scientific, USA), and 1% penicilin/ streptomycin (Thermo Fisher Scientific, USA) during the transfer of the samples. Samples were transferred on ice from animal facility of the Medical University of Graz to CBMed. The tissues were processed immediately to obtain single cell suspensions. Briefly tissues were crushed through 100 µm cell strainer (BD Biosciences, USA), and centrifuged at 400 g for 5 min. After supernatants were discarded, pellets of blood, spleen and thymus were treated with ACK lysis buffer (Lonza, Switzerland) for 5 min at RT to remove red blood cells. Meanwhile, pLN were resuspended in 1 mL PBS. After centrifugation of blood, spleen, and thymus at 400 g for 5 min, pellets were resuspended in 1 ml PBS. Single cell suspensions from blood, spleen, pLN, and thymus were blocked with Fc block (Thermo Fischer, USA), stained with the surface antigens, and fixable viability dye (Thermo Fischer, USA) according to Table 5 and Table 6. For intracellular staining which was required in panel A and D, cells were fixed, and permeabilized with transcription factor buffer set (BD Biosciences, USA), and stained with FoxP3 and CTLA4 antibodies. Appropriate isotype controls and FMO (fluorescence minus one) tubes were included for an appropriate identification of positive signals. Cells were analyzed using a BD LSR Fortessa SORP device and Diva software (v8.0.1; BD Biosciences, USA). One mouse data was excluded to avoid confounders due to the high blood glucose level at sacrifice which was over 600 mg/dl.

Antibodies were purchased from Miltenyi Biotech (Germany); CD4 (GK1.5), CD25 (REA568), CD44 (IM7.8.1), CD62L (REA828), FoxP3 (REA788), CD19 (REA749), CD317 (REA818), CD11c (REA754), Ly6C (REA796), F4/80 (REA126), CD335 (REA815), CD45R/ B220 (RA3-6B2), from Thermo Fisher Scientific (USA); CD3 (145-2C11), CD8a (53-6.7), CD11b (M1/70), Ly6G (RB6-8C5), from BD Biosciences (USA); CD45 (30-F11), CD25 (PC61), CTLA4 (UC10-4F10-11), and from Biolegend (USA); CD301 (LOM-14).

All immune cell populations were always gated for singularity (FSC-H vs FSC-A) and viability. T cell subpopulations were quantified in blood, spleen, and pLN by panel A using following antibodies: CD45 (30-F11), CD4 (GK1.5), CD8a (53-6.7), CD3 (145-

2C11), CD44 (IM7.8.1), CD62L (REA828), CD25 (REA568), CD25 (PC61), FoxP3 (REA788), CTLA4 (UC10-4F10-11), CD196/ CCR6 (REA277). T cell populations were gated accordingly: CD4+ T cells (CD45+, CD3+, CD4+), CD8+ T cells (CD45+, CD3+, CD8+), naïve CD4+ T cells (CD45+, CD3+, CD4+, CD44(-), CD62L+), effector memory CD4+ T cells (CD45+, CD3+, CD4+, CD44+, CD62L(-)), central memory CD4+ T cells (CD45+, CD3+, CD4+, CD44+, CD62L+), naïve CD8+ T cells (CD45+, CD3+, CD8+, CD44(-), CD62L+), effector memory CD8+ T cells (CD45+, CD3+, CD8+, CD44+, CD62L(-)), central memory CD8+ T cells (CD45+, CD3+, CD8+, CD44+, CD62L+), FoxP3 T cells (CD45+, CD3+, CD4+, FoxP3+), regulatory T cells (CD45+, CD3+, CD4+, FoxP3+, CD25+), and activated regulatory T cells (CD45+, CD3+, CD4+, FoxP3+, CD25+, CTLA4+). Dendritic cells and monocytes were quantified in blood, spleen, and pLN by panel B using following antibodies: CD4 (GK1.5), CD317 (REA818), CD11c (REA754), Ly6C (REA796), CD8a (53-6.7), CD11b (M1/70), Ly6G (RB6-8C5), CD45 (30-F11), F4/80 (REA126) and CD301 (LOM-14). Dendritic cells were gated accordingly: CD8+ conventional DCs (CD45 +, CD11c+, CD11b (-), CD8+), CD11b+ CD4+ conventional DCs (CD45 +, CD11c+, CD11b+, CD4+), CD11b conventional DCs (CD45 +, CD11c+, CD11b+, CD8(-)), plasmacytoid DCs (CD45+, CD317+, CD11c low, Ly6C high). Monocytes were gated as (CD45+, CD11c (-), CD11b+, Ly6C+, Ly6G (-), CD317(-), and macrophages were gated as (CD45+, F4/80+, CD11b+). M2 macrophages were gated as (CD45+, F4/80+, CD11b+, CD301+), and neutrophils were gated as (CD45+, Ly6G high, CD11b high).

B cells, NK cells, and NKT cells were quantified in blood and spleen by panel C by using following antibodies: CD19 (REA749), CD335 (REA815), CD45R/ B220 (RA3-6B2), CD3 (145-2C11) and CD45 (30-F11). These cell populations were gated as following: B cells (CD45+, CD3(-), CD19+, B220+), NK cells (CD45+, CD3(-), CD335+), and NKT cells (CD45+, CD3+, CD335+).

Maturation of T cells, Tregs, and positive selection [321] in thymus were analyzed by panel D using following antibodies: CD4 (GK1.5), CD8a (53-6.7), FoxP3 (REA788), CD25 (REA568), TCR beta (REA318), CD24 (REA743), CD5 (REA421), CD69 (REA937). All immune cell populations were always gated for singularity (FSC-H vs FSC-A) and viability. Cells were gated accordingly: CD4+ thymocytes (CD4+ CD8(-)), CD8+ thymocytes (CD4+ CD8+), double positive thymocytes (CD4+ CD8+), double

negative thymocytes (CD4(-) CD8(-)), mature CD4+ Thymocytes (TCRβ+ CD24(-) CD4+), mature CD8+ thymocytes (TCRβ+ CD24(-) CD8+), FoxP3 T cells (CD4+, FoxP3+), and Tregs (CD4+, FoxP3+, CD25+).

Table 5: Panel designs of antibodies used in flow cytometry experiments.

	Panel ID			
Fluorochrome	Panel A	Panel B	Panel C	Panel D
V450	CD4	CD4	CD19	CD4
Brilliant Violent 510	FVS eflour 506	FVS eflour 506	FVS eflour 506	FVS eflour 506
Brilliant Violet 711	CD8a	CD8a		CD8a
FITC	CD44	Ly6C	CD335 (Nkp46)	CD69
PerCP-Cy5.5	CD3	CD11b* or	CD3	TCR-β
PE	FoxP3	CD11b*		FoxP3
PE-CF594	CD62L	F4/80		
PE-Cy5.5		Ly6G		
PE-Cy7	CD25	CD11c		CD24* or CD25
APC	CD196 /CCR6	CD317	CD45R/ B220	CD5
APC-R700	CTLA4			
APC-Cy7	CD45	CD45	CD45	CD24*

Table 6: Tissue analysis in flow cytometry experiments.

	Panel A	Panel B	Panel C	Panel D
Blood	√	√	√	
Spleen	√	√	√	
pLN	√	√		
Thymus				√

2.6. Measurement of Cytokines in Plasma by Mesoscale Discovery

Plasma samples were collected as described before. Samples were measured for their plasma cytokine levels by V-plex pro-inflammatory panel 1 (K15048D, MSD, USA). This kit measures IFN-ɣ, IL-1β, IL-6, IL-5, IL-10, TNF-α and IL-2. Samples were processed according to the manufacturer´s guidelines. Samples were measured with Spectrostar Omega (BMG Lab Tech, Germany).

2.7. Immunoblot Analysis

For immunoblot analysis of tissues, frozen tissues were pulverized by mortar and pestle on dry ice. Tissue powders were lysed with lysis buffer (150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, and 20 mM Tris pH. 7.5) in the presence of HaIt™ Protease and Phosphatase Inhibitor Cocktail (Thermo Fisher Scientific, USA).

Lysates were immediately sonicated at Amplitude 40 for 5 sec twice, and they were incubated on a shaker in a cold room (+4°C) for 45 min. Later, lysates were centrifuged at 8000 g for 20 min at 4°C, and supernatants were transferred to new Eppendorf tubes. Total protein concentration was determined by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA). Unless otherwise stated materials for immunoblot analysis were purchased from Bio-rad Laboratories (USA). An equal amount of tissue lysates ware heated at 95°C for 5 min and loaded on pre-casted gels, and transferred to polyvinylidene difluoride membranes by Trans-Blot Turbo transfer system. The blots were probed for LC3 (1:1000, # 2775, CST, USA), p62 (1:1000, # 5114, CST, USA), Beclin1 (1:1000, # 3738, CST, USA) primary antibodies, and goat anti-rabbit HRP-linked secondary antibody (1:5000, # 7074, CST, UK) and developed with ECL. Then blots were stained with Coomassie brilliant blue for normalization purposes. The bands were detected by the Biorad Chemidoc Touch imaging system and analyzed by Image Lab software.

2.8. Electron Microscopy

Sample processing for electron microscopy and imaging was performed in Core Facility Ultrastructure Analysis under the supervision of Dr. Dagmar Kolb-Lenz.

Pancreata were fixed in 2.5% (wt/vol) glutaraldehyde and 2% (wt/vol) paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) for 2 h. Post-fixation was performed in 1% (wt/vol) osmium tetroxide for 2 h at RT. After tissues were dehydrated in graded series of ethanol, they were infiltrated with ethanol and pure TAAB epoxy resin and then placed in TAAB epoxy resin for 8 h. Later tissues were transferred into embedding moulds, and polymerized at 60°C for 48 h. Ultrathin sections (70 nm) were cut with a UC 7 Ultramicrotome (Leica Microsystems, Vienna, Austria) and sections were stained with lead citrate for 5 min and platin blue for 15 min. Photomontages (Serial EM) were taken using a Tecnai G2 20 transmission electron microscope (FEI, Eindhoven, Netherlands) with a Gatan UltraScan 1000 charge-coupled device (CCD) camera (temperature −20°C; acquisition software Digital Micrograph; Gatan, Munich, Germany). The acceleration voltage was 120 kV. Image calculations (granules and cluster counting) were done with Image J Software [322].

2.9. Statistical Analysis

Data are presented as mean ± SD. Data points were tested for normality. In normally distributed data, two groups were compared by unpaired two-tailed Student´s t-test and statistical analysis for more than two groups was performed by one-way ANOVA with Sidak´s post-hoc test. In non-normally distributed data, two groups were compared by the Mann-Whitney U test, and statistical analysis for more than two groups was performed by the Kruskal-Wallis test with Dunn´s post-hoc test. To analyze the effect of two factors on data points, two-way ANOVA was used. In diabetes incidence analysis, two groups were statistically evaluated by the Mantel-Cox log-rank test. Graphs and statistics were prepared in GraphPad Prism version 8 (GraphPad Software, USA). Results were considered significant for $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$, $p^{****} < 0.0001$.

3. RESULTS

An overview of mice studies is given in Table 2.

3.1. Study-I: Dose-response in Male NOD Mice

Previous studies have shown that 3 mM SPD can show its effect on polyamine levels and autophagy levels when given to mice via drinking water. Thus, we designed a dose-response study with 1 mM, 3 mM, and 10 mM SPD in male NOD mice to study the effect of spermidine on BW development, water and food consumption, and polyamine levels. In this study, male mice were given untreated or the above-mentioned concentrations of SPD via drinking water for 28 days.

3.1.1. Study-I: Vital Parameters

After 4 weeks of treatment, initial BW, BW gain, water, and food consumption were analyzed. In general, mice exhibited consistent well-being and did not show any symptoms of sickness. Mice with similar BW were paired and housed together since the beginning of the study (Fig. 4A) and BW gain remained similar between the groups during the study (Fig. 4B). Food and water consumption were comparable between the treatment groups (Fig. 4C and D).

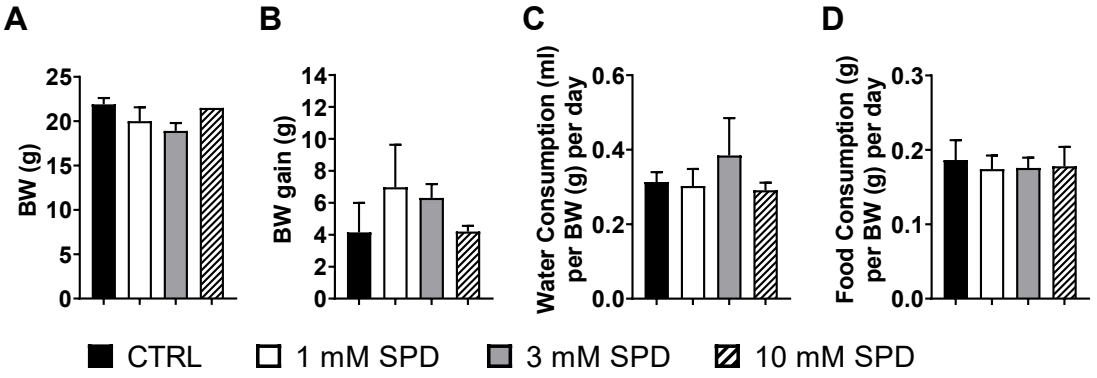

Figure 4: Vital parameters in Study-I. (A) Body weight (BW) at the beginning of the , (B) BW gain, (C) water consumption, and (D) food consumption during the study. Spermidine concentrations were used as following: ctrl (n=2), 1 mM SPD (n=3), 3 mM SPD (n=3) and 10 mM SPD (n=3). Each treatment group was compared to the ctrl group. The one-way ANOVA with Sidak´s post-hoc test or the Kruskal-Wallis with Dunn´s post-hoc test was performed as statistical analysis.

3.1.2. Study-I: Polyamine Analysis

To determine the effect of spermidine treatment on the endogenous polyamine pool, we analyzed spermidine, putrescine, and ornithine by liquid chromatography-tandem mass spectrometry to obtain a comprehensive understanding of polyamine levels. This approach was essential to facilitate the accurate interpretation of the results regarding the effects of spermidine administration in various tissues. Higher spermidine concentrations led to significantly increased endogenous spermidine levels in the thymus (Fig. 5A) and significantly reduced putrescine levels in the thymus and heart (Fig. 5B). Spermidine treatment did not affect the spermidine and putrescine levels in any other tissue (Fig. 5A and B). Spermidine treatment did not change the ornithine levels in any of the analyzed tissue (Fig. 5C).

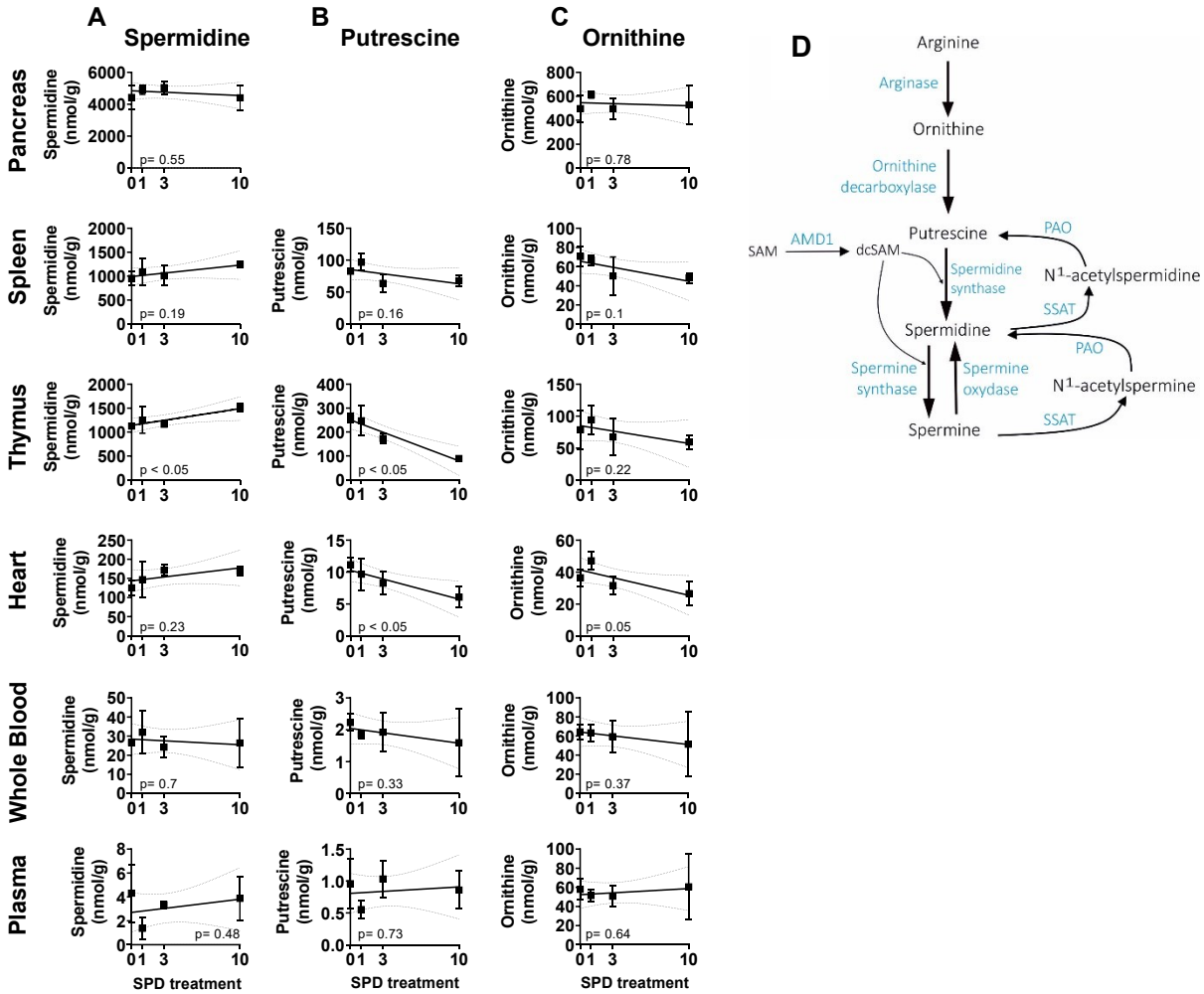

Figure 5: Polyamine analysis in Study-I. After 4 weeks of spermidine treatment, pancreas, spleen, thymus, heart, whole blood, and plasma samples were analyzed in SPE- LC/MS/MS for (A) spermidine,

(B) putrescine, and (C) ornithine. (D) Illustration of polyamine pathway. AMD1: S-adenosylmethionine decarboxylase, dcSAM: decarboxylated S-adenosylmethionine, PAO: polyamine oxidase, SAM: S-adenosylmethionine, SSAT: spermidine/spermine-N1-acetyltransferase. Spermidine concentrations were used as following: CTRL group (n=2), 1 mM SPD (n=3), 3 mM SPD (n=3) and 10 mM SPD (n=3). Data is shown as nmol/ g and mean ± SD. Linear regression analysis was performed as statistical analysis. p-values were shown.

3.2. Study-II: Control vs 3 mM SPD Treatment in Female NOD Mice

Interpretation of spermidine and putrescine measurements in Study-I showed that 3 mM SPD led to turnover of polyamines in relevant tissues. Our findings are also consistent with existing literature which shows that supplementation of 3 mM SPD in drinking water leads to elevated endogenous spermidine concentrations and stimulated autophagy in heart and brain [233,235]. Therefore, 3 mM SPD was chosen to investigate the effect of spermidine on diabetes incidence in female NOD mice. In this study, female mice were given untreated or 3 mM SPD treated water from the age of 4 weeks until diabetes onset or the age of 35 weeks. The effects of spermidine on BW, water and food consumption, diabetes incidence, immune cell profile, islet insulitis, and plasma cytokine levels were examined.

3.2.1. Study-II: Vital Parameters

There was no difference in the BW of mice between treatment groups at the start and throughout the study (Fig. 6A and D). We did not observe any significant difference in BW gain when mice were sacrificed at diabetes onset or at the age of week 35 (Fig. 6B and C). Each treatment group consumed similar amounts of water and food at the indicated time points (Fig. 6E and F).

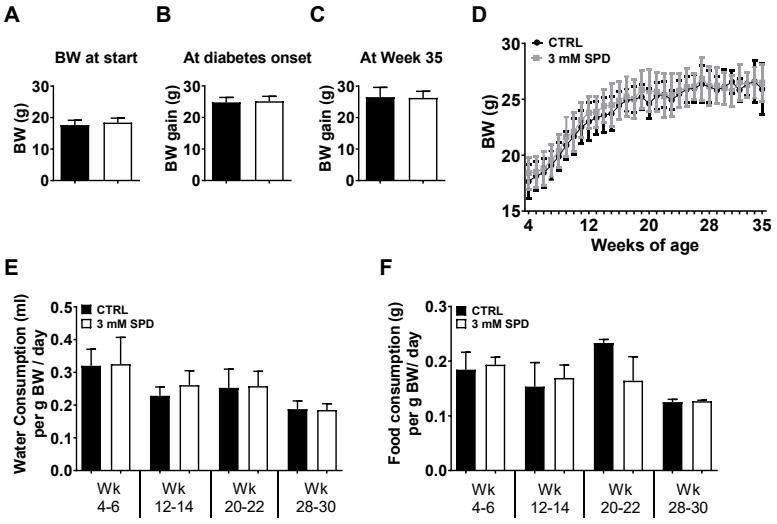

Figure 6: Vital parameters in Study-II. (A) BW at the beginning of the study (CTRL n=14 and SPD n=14), (B) BW gain at diabetes onset (CTRL n=12 and SPD n=10), and (C) at week 35 when the mice sacrificed as nondiabetic (CTRL n=4 and SPD n=2), (D) BW monitoring during the study (week 4-35), (E) water consumption during the study, the average was calculated from 9-10 independent measurements from 2 cages (wk. 4-14) and 4-9 independent measurements from 1-2 cages (wk. 20-30) and (F) food consumption during the study, the average was calculated from 2-6 independent measurements from 1-2 cages. Mice with blood glucose levels over 200 mg/dl in two consecutive measurements were determined as diabetic. (A-C and E-F) To analyze the difference between CTRL and SPD groups, the Mann-Whitney U test or the unpaired Student´s t-test was performed as statistical analysis and (D) to analyze BW graph, the two-way ANOVA with Sidak´s post -hoc test was performed.

3.2.2. Study-II: Diabetes Incidence

As shown in the study design (Fig. 7A), female mice were given untreated or 3 mM SPD-treated water ad libitum. We analyzed the non-fasting blood glucose of the mice twice a week from 10 weeks of age until diabetes onset or 35 weeks of age. Mice started to become diabetic at 11 weeks of age. Diabetes incidence was calculated at 35 weeks of age. We found that control and 3 mM SPD mice had similar diabetes incidence at week 35 (Fig. 7B). We also analyzed diabetes incidence based on the age of diabetes onset: early-onset and late-onset. Sub-analysis of diabetes incidence between 21 weeks of age and 35 weeks of age showed that there was no difference between the ctrl mice and 3 mM SPD mice (Fig. 7C).

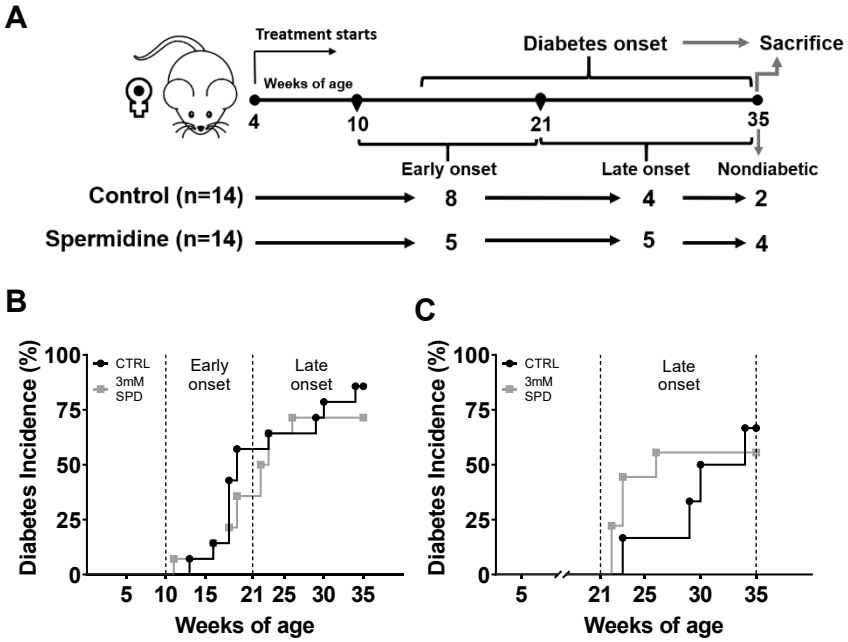

Figure 7: Diabetes incidence in Study-II. (A) Study design, (B) diabetes incidence from week 10 to week 35, and (C) from week 21 to week 35. Mice with blood glucose levels over 200 mg/dl in two consecutive measurements were determined as diabetic. The mantel-Cox log-rank test was performed as statistical analysis.

3.2.3. Study-II: Pancreatic Islet Insulitis

We stained two nondiabetic CTRL, three diabetic CTRL, four nondiabetic SPD, and three diabetic SPD mice with H&E staining. Islet insulitis was assessed according to the grading classification presented in Fig. 8A. Each analyzed mouse had a minimum of 20 islets. Spermidine treatment did not change the insulitis levels in pancreatic islets of nondiabetic 3 mM SPD mice compared to nondiabetic CTRL mice (Fig. 8B and D). Similarly, spermidine treatment did not alter the insulitis levels in pancreatic islets of diabetic 3 mM SPD mice compared to diabetic CTRL mice (Fig. 8C and E).

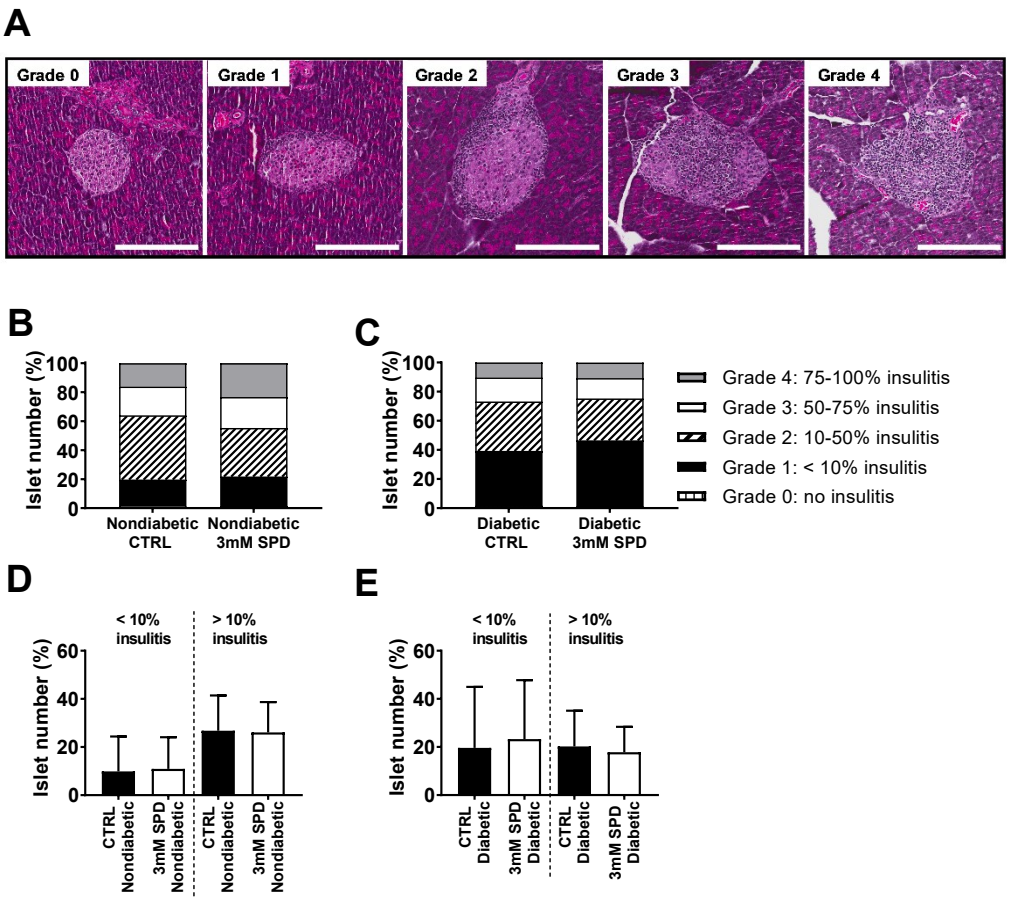

Figure 8: Analysis of islet insulitis by H&E staining. A) Insulitis grading map: grade 0 (no insulitis), grade 1 (peri insulitis to 10% insulitis), grade 2 (10- 50% insulitis), grade 3 (50- 75% insulitis), grade 4 (75- 100% insulitis), mice were analyzed according to (B and C) insulitis grading, and (D and E) insulitis levels < 10% or > 10%, 2 datapoints per mouse in each group. Mice were sacrificed as diabetic at diabetes onset (week 11-35 of age) or as nondiabetic at week 35 of age. Nondiabetic CTRL mice (n=2), nondiabetic 3 mM SPD mice (n=4), diabetic CTRL mice (n=3), and diabetic 3 mM SPD mice (n=3). Scale bar: 200 µm. The two-way ANOVA with Sidak´s post-hoc test and the Mann-Whitney U test or the unpaired Student´s t-test were used as statistical analysis.

3.2.4. Study-II: Immunophenotyping of Blood, Spleen, pLN and Thymus

We examined the proportions of total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells, FoxP3+ CD25+ CD4+ Tregs, total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, central memory CD8+ T cells in the blood, spleen, and pLN and B cells, NK cells and NKT cells in the blood and spleen, CD11b+ CD4+ cDCs, CD8+ cDCs, CD11b+ cDCs, pDCs, monocytes, macrophages, and neutrophil granulocytes in the spleen. In the

thymus, we examined the proportions of CD4+, CD8+, CD4+CD8+, CD4(-) CD8(-), FoxP3+ CD4+, mature CD4+, and mature CD8+ thymocytes. Diabetic and nondiabetic mice were analyzed separately to observe the direct impact of spermidine treatment independent of diabetes status.

We examined the proportions of total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells, FoxP3+ CD25+ CD4+ Tregs, total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells in the spleen of nondiabetic mice. Blood and pLN could not be examined due to a low number of samples. We found that spermidine treatment did not change any CD4+ and CD8+ T cell subsets in the spleen of nondiabetic mice (Fig. 9).

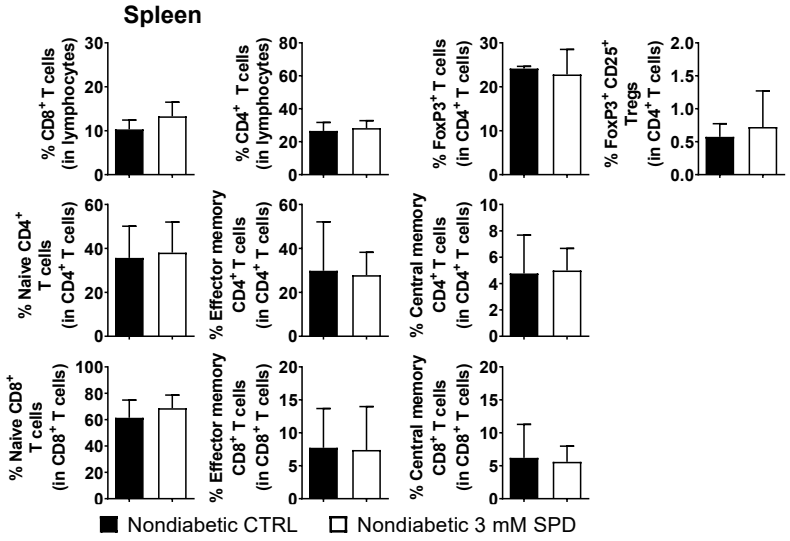

Figure 9: Analysis of CD4+ T cell subsets and CD8+ T cell subsets in the spleen of nondiabetic mice. Total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells and FoxP3+ CD25+ Tregs and total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells were examined in the spleen of nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=2) and open bar shows nondiabetic 3 mM SPD mice (n=4). Data is shown as mean ± SD. The Mann-Whitney U test was used as statistical analysis.

We examined the proportions of total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells, FoxP3+ CD25+ CD4+ Tregs, total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells in the blood, spleen, and pLN of diabetic mice. Spermidine treatment reduced only the proportion of CD4+ T cells (1.2-fold, $p < 0.05$) in the spleen of diabetic mice (Fig. 10B), while it did not alter any other CD4+ and CD8+ T cell subsets in the blood, spleen, and pLN of diabetic mice (Fig. 10 and 11).

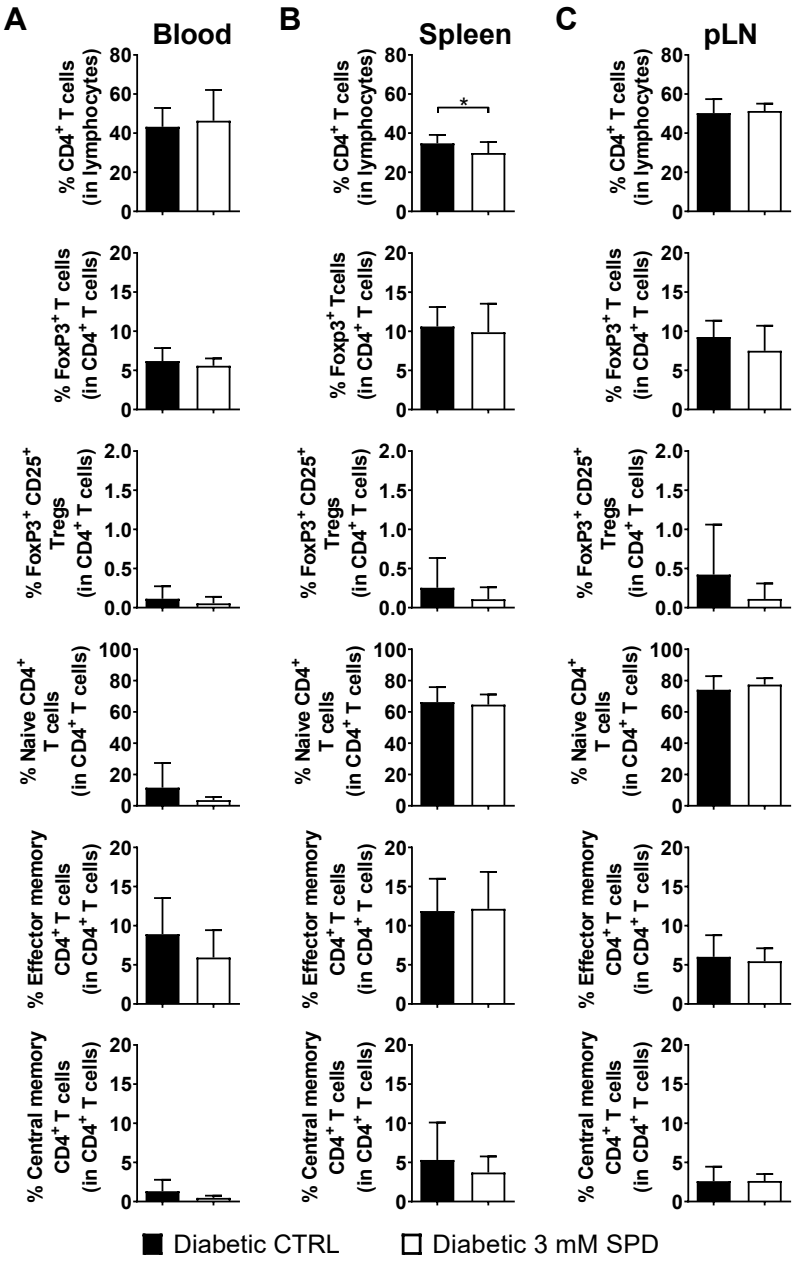

Figure 10: Analysis of CD4+ T cell subsets in blood, spleen, and pLN of diabetic mice. Total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells, and FoxP3+ CD25+ Tregs were examined in (A) blood, (B) spleen, and (C) pLN of diabetic mice. Black bar shows diabetic CTRL mice (n=8-11) and open bar shows diabetic 3 mM SPD mice (n=4-9). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

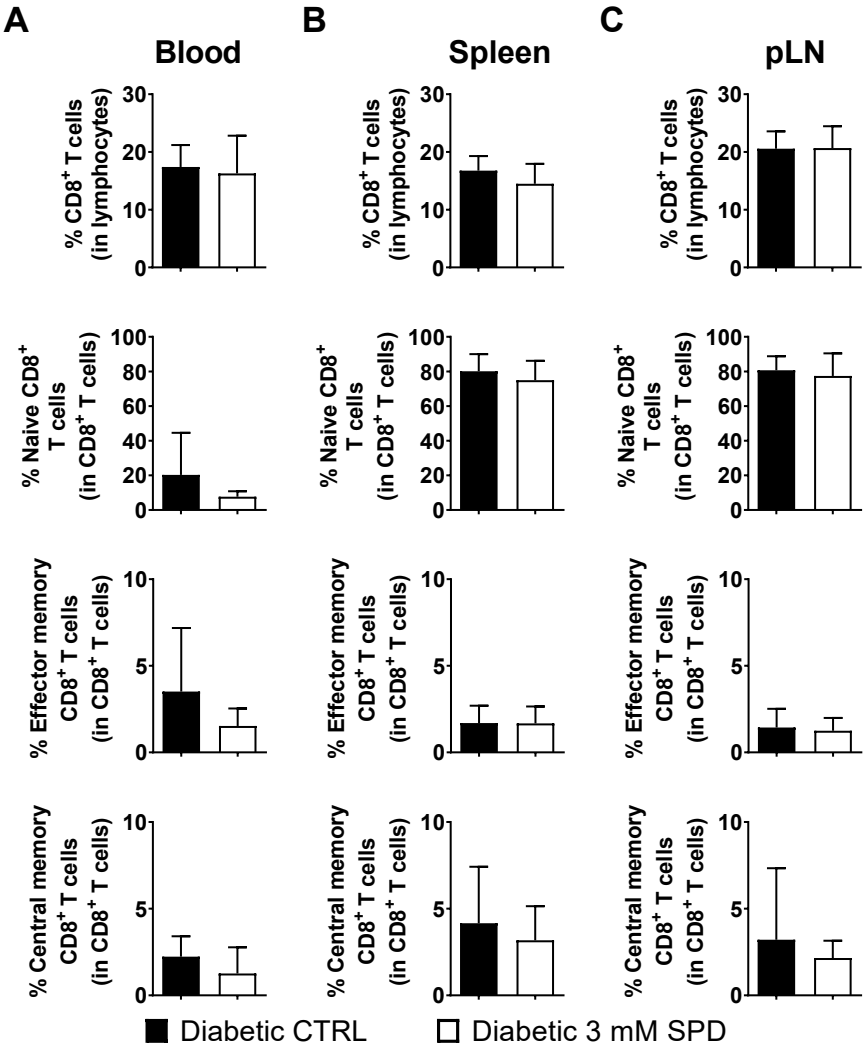

Figure 11: Analysis of CD8+ T cell subsets in blood, spleen and pLN of diabetic mice. Total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells were examined in (A) blood, (B) spleen, and (C) pLN of diabetic mice. Black bar shows diabetic CTRL mice (n=8-11) and open bar shows diabetic 3 mM SPD mice (n=4-9). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used.

Since spermidine was shown to modulate macrophages in previous studies, we examined monocytes, neutrophil granulocytes, and plasmacytoid dendritic cells in the spleen of NOD mice. Spermidine treatment did not alter the proportion of monocytes, neutrophil granulocytes, and pDCs in the spleen of nondiabetic mice (Fig. 12).

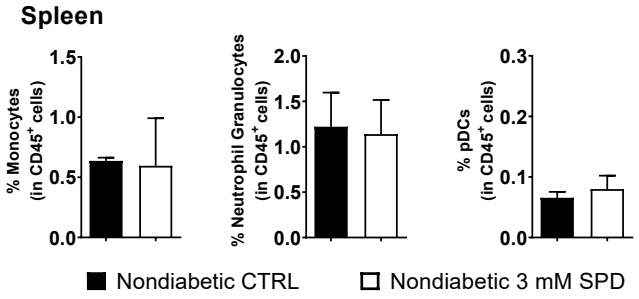

Figure 12: Analysis of monocytes, neutrophil granulocytes and pDCs in the spleen of nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=2) and open bar shows nondiabetic 3mM SPD mice (n=4). Data is shown as mean ± SD. The Mann-Whitney U test was used as statistical analysis.

After analysis of antigen-presenting cells in nondiabetic mice, we explored the dendritic cell populations, monocytes, macrophages, and neutrophil granulocytes in the spleen of diabetic mice. 3 mM spermidine treatment reduced the proportion of CD8+ cDCs (1.5-fold, $p < 0.05$) (Fig. 13) compared to control mice while it did not change the proportion of CD11b+ CD4+ cDCs, CD11b+ cDCs, and CD8+ cDCs compared to control mice (Fig. 13). Similar to the findings in nondiabetic mice, in the spleen of diabetic mice, monocytes, macrophages, and neutrophil granulocytes were similar in control and spermidine treated mice (Fig. 13).

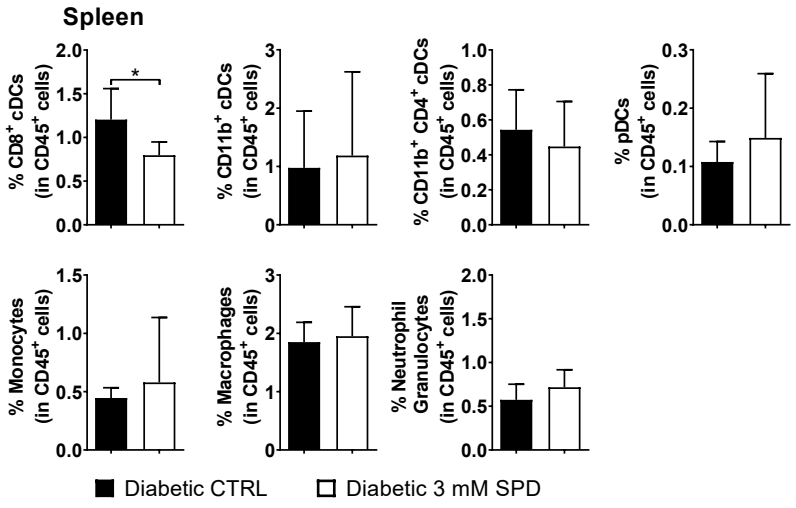

Figure 13: Analysis of CD8+ cDCs, CD11b+ cDCs, CD11b+ CD4+ cDCs, pDCs, monocytes, macrophages, and neutrophil granulocytes in the spleen of diabetic mice. Black bar shows diabetic CTRL mice (n=6-9) and open bar shows diabetic 3 mM SPD mice (n=4-7). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

Next, we explored B cells, natural killer cells, and natural killer T cells in the blood and spleen of nondiabetic mice. Spermidine treatment did not change the proportion of B cells, NK cells, and NKT cells in the blood and spleen of nondiabetic mice (Fig. 14).

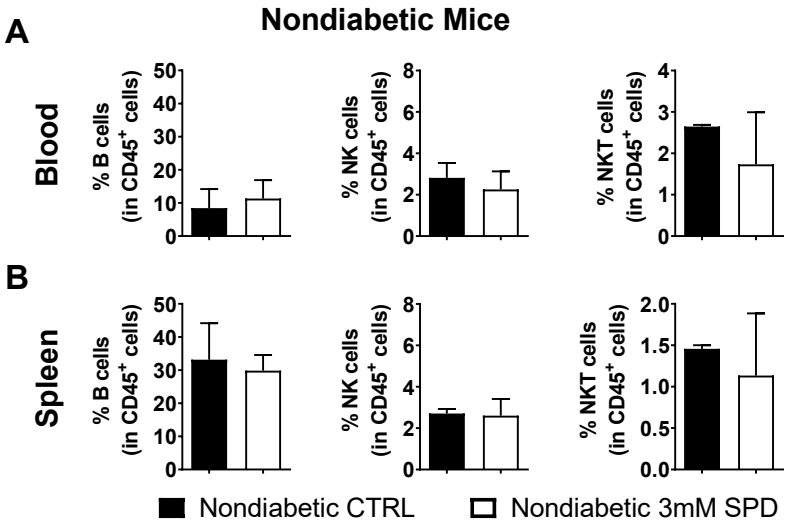

Figure 14: Analysis of B cells, natural killer (NK) cells, and natural killer T (NKT) cells in nondiabetic mice. (A) Blood and (B) spleen. Black bar shows nondiabetic CTRL mice (n=2) and open bar shows nondiabetic 3 mM SPD mice (n=4). Data is shown as mean ± SD. The Mann-Whitney U test was used as statistical analysis

After analyzing nondiabetic mice, we investigated the effect of spermidine on the same immune cell populations in diabetic mice. In the blood of diabetic mice, spermidine treatment elevated the proportion of NKT cells (5.2-fold, $p < 0.05$), while it did not change the proportion of B cells and NK cells (Fig. 15A). In the spleen of diabetic mice, spermidine treatment did not alter the proportion of B cells, NK cells, and NKT cells (Fig. 15B).

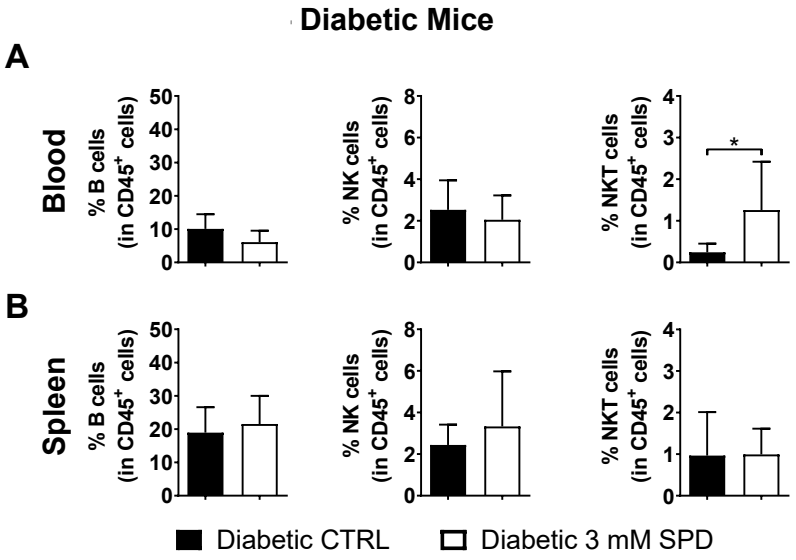

Figure 15: Analysis of B cells, NK cells, and NKT cells in diabetic mice. (A) Blood and (B) spleen. Black bar shows diabetic CTRL mice (n= 11) and open bar shows diabetic 3 mM SPD mice (n=7-8). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

To investigate the hypothesis that NOD mice might be affected by disrupted positive and negative selection processes, we studied thymocytes in control and spermidine-treated nondiabetic and diabetic mice. We showed that spermidine treatment did not change the proportions of CD4+ and CD8+ thymocytes in the thymus of nondiabetic and diabetic mice (Fig. 16 and 17).

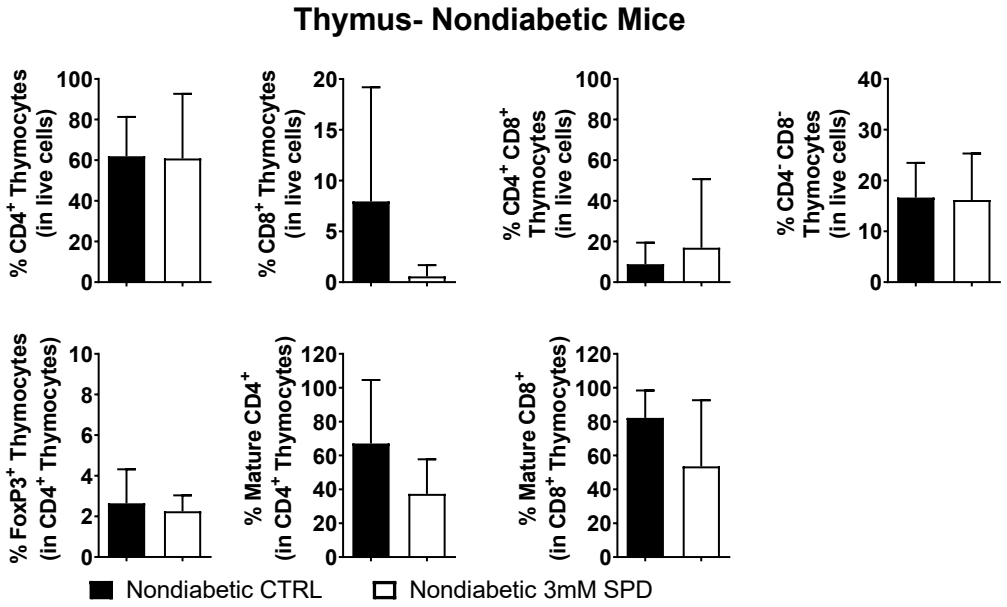

Figure 16: Analysis of thymocytes in the thymus of nondiabetic mice. CD4+, CD8+, CD4+ CD8+, CD4(-) CD8(-), mature CD4+, mature CD8+, and FoxP3+ thymocytes were analyzed. Black bar shows nondiabetic CTRL mice (n=2) and open bar shows nondiabetic 3 mM SPD mice (n=4). Data is shown as mean ± SD. The Mann-Whitney U test was used as statistical analysis.

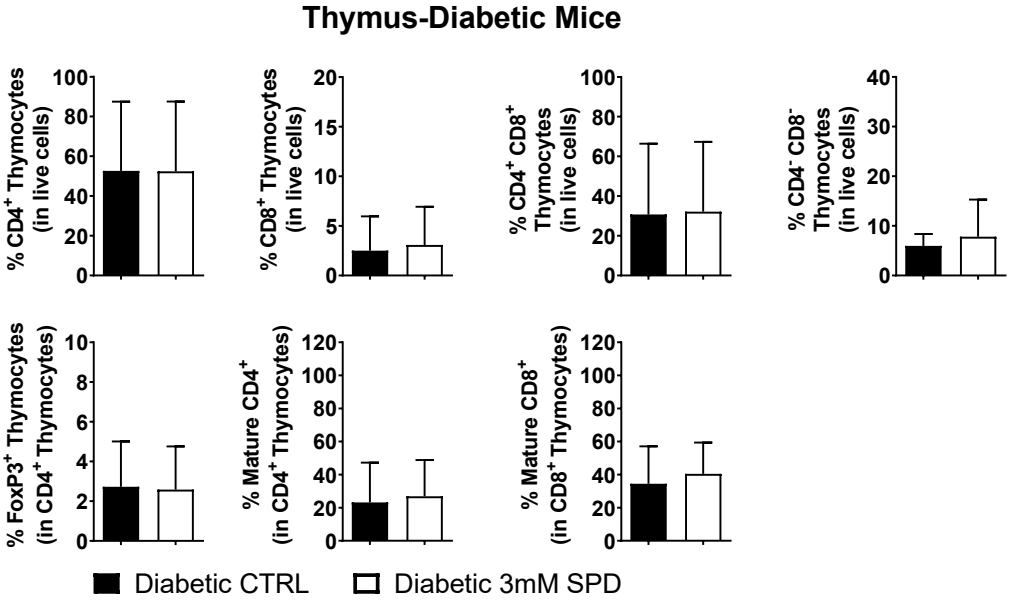

Figure 17: Analysis of thymocytes in the thymus of diabetic mice. CD4+, CD8+, CD4+CD8+, CD4(-) CD8(-), FoxP3+, mature CD4+, and mature CD8+ thymocytes were analyzed. Black bar shows diabetic CTRL mice (n=11) and open bar shows diabetic 3 mM SPD mice (n=9). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

After exploring the effect of 3 mM spermidine on different immune cell populations in nondiabetic and diabetic mice, we examined the plasma cytokine levels (IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10) by mesoscale discovery plates. We observed that spermidine did not change the plasma cytokine levels in diabetic and nondiabetic mice (Fig. 18 and 19).

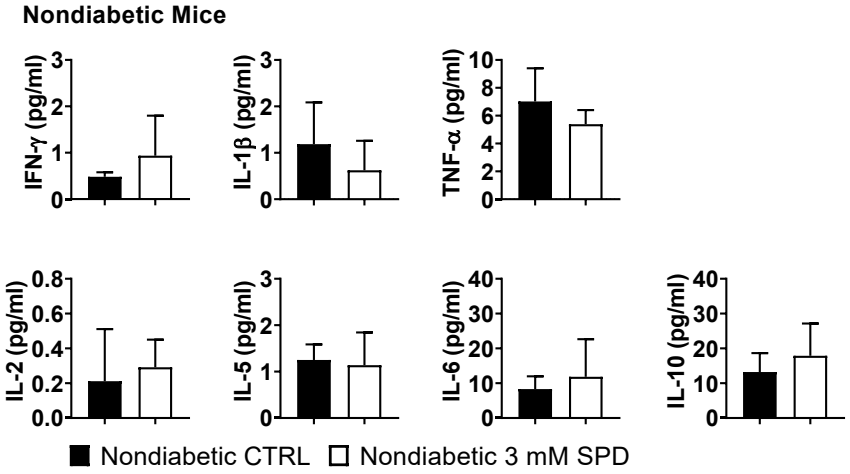

Figure 18: Analysis IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10 in the plasma of nondiabetic mice. Black bar shows nondiabetic control (n=2) and open bar shows nondiabetic 3 mM SPD (n=4). Data is shown as pg/ml and mean ± SD. The Mann-Whitney U test was used as statistical analysis.

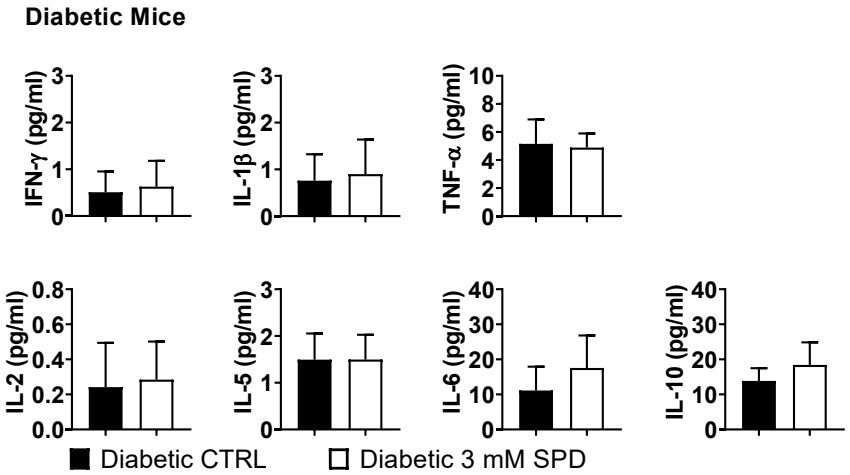

Figure 19: Analysis of IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10 in the plasma of diabetic mice. Black bar shows diabetic CTRL mice (n=7) and open bar shows diabetic 3 mM SPD mice (n=7). Data is shown as pg/ml and mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

3.3. Study-III: Control vs 10 mM SPD Treatment in Male NOD Mice

3 mM SPD did not show any effect on diabetes incidence in female NOD mice. One possibility was that the 3 M spermidine concentration was insufficient to protect beta cells. To address this, we considered using higher spermidine concentrations. Before initiating the long-term prevention study in female mice, we took the step to evaluate male NOD mice by administering 10 mM spermidine in their drinking water to ensure that there were no side effects. In this study, mice were given untreated or 10 mM SPD-treated water from the age of 4 weeks until the age of 8 weeks. The effect of 10 mM SPD on BW, water and food consumption and polyamine levels were analyzed.

3.3.1. Study-III: Vital Parameters

CTRL and 10 mM SPD mice had similar BW at the beginning of the study (Fig. 20A). During the study BW gain was similar between CTRL and SPD mice (Fig. 20B and C). Water and food consumption were similar between CTRL and SPD mice at indicated time points (Fig. 20D and E).

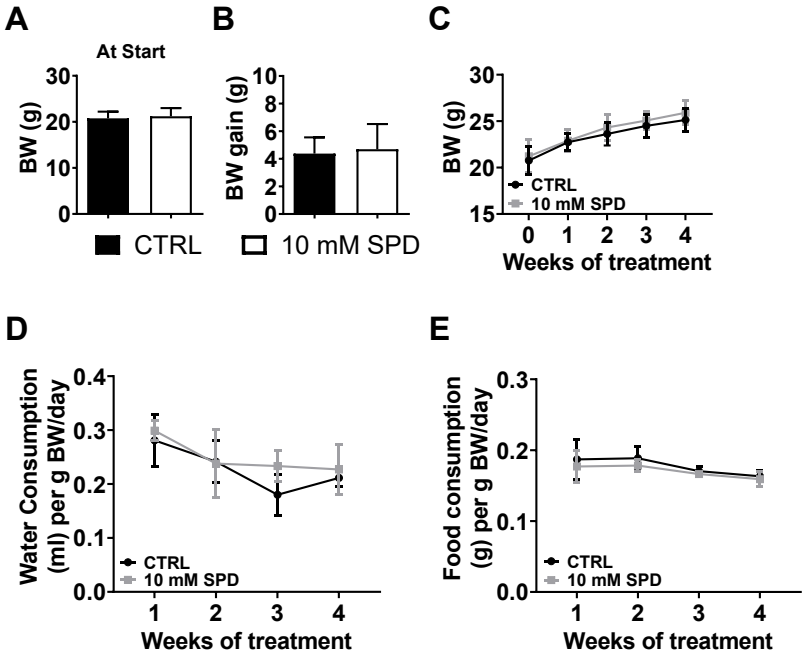

Figure 20: Vital parameters in Study-III. (A) BW at the beginning of the study, (B) BW gain during the study, (C) BW monitoring during the study, (D) water consumption during the study, and (E) food consumption during the study. CTRL (n=10) and 10 mM SPD (n=12). Each data point in water and food consumption graphs represents one measurement of respective cage including 2 or 3 mice. The two-way ANOVA with Sidak´s post-hoc test or the unpaired Student´s t-test was performed as statistical analysis.

3.3.2. Study-III: Polyamine Analysis

Spermidine treatment significantly increased the endogenous spermidine levels in the spleen, plasma, and heart (Fig. 21A). There was no significant accumulation of spermidine in the thymus, pLN, pancreas and whole blood (Fig. 21A). Spermidine treatment significantly reduced the putrescine levels only in the thymus, spleen, pLN, and heart (Fig.21B). Spermidine treatment did not alter the spermine and ornithine levels in any of the analyzed tissues (Fig. 21C and D).

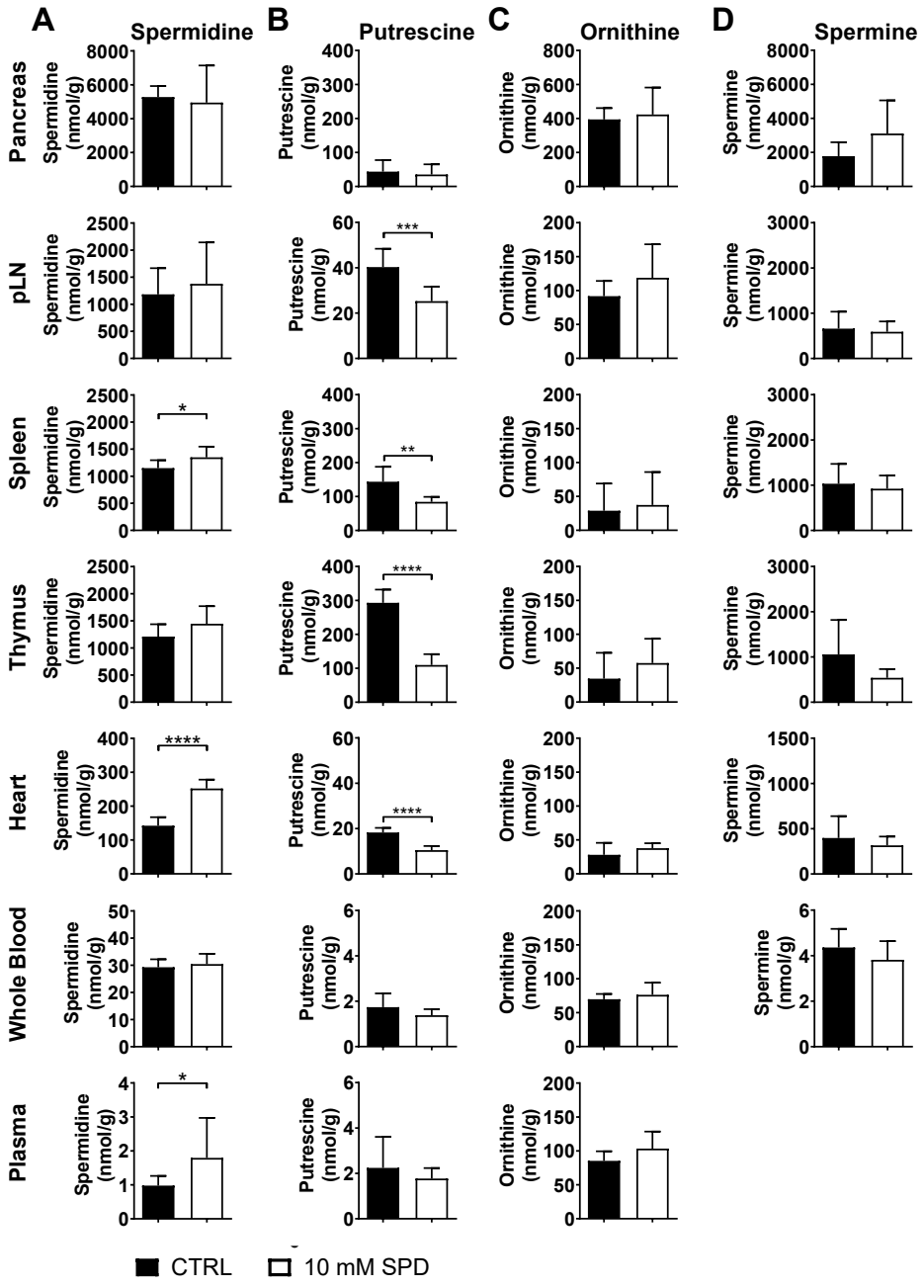

Figure 21: Tissue polyamine levels in Study-III. After 4 weeks of spermidine treatment pancreas, pLN, spleen, thymus, heart, whole blood, and plasma samples were analyzed in SPE- LC/MS/MS for (A) spermidine, (B) putrescine, (C) ornithine, and (D) spermine levels. Black bar shows CTRL mice (n=8) and white bar shows SPD mice 10 mM SPD (n=10). Data is shown as nmol/ g and mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05, p** < 0.01, p*** < 0.001, p**** < 0.0001.

3.4. Study-IV: Control vs 10 mM SPD Treatment in Female NOD Mice

After determining the robust effect of 10 mM SPD on polyamine levels and absence of toxicity of 10 mM SPD, 10 mM SPD was tested for its ability to prevent diabetes development in female NOD mice. Mice were given untreated or 10 mM SPD-treated water from the age of 4 weeks until diabetes onset or the age of 35 weeks. The effect of 10 mM spermidine on BW, water and food consumption, diabetes incidence, immune cell profile, islet insulitis, plasma cytokine levels, pancreatic autophagy levels, and insulin granules was examined.

3.4.1. Study-IV: Vital Parameters

The body weight of mice in the control and treatment groups were similar at the start of the study (Fig. 22A). We did not observe any difference in BW gain of the mice sacrificed at diabetes onset or 35 weeks of age (Fig 22B and C). Spermidine did not alter water and food consumption during the study (Fig. 22E and F). Kidney and heart weight were similar in control and spermidine-treated mice both at diabetes onset and at week 35 (Fig. 22G-J).

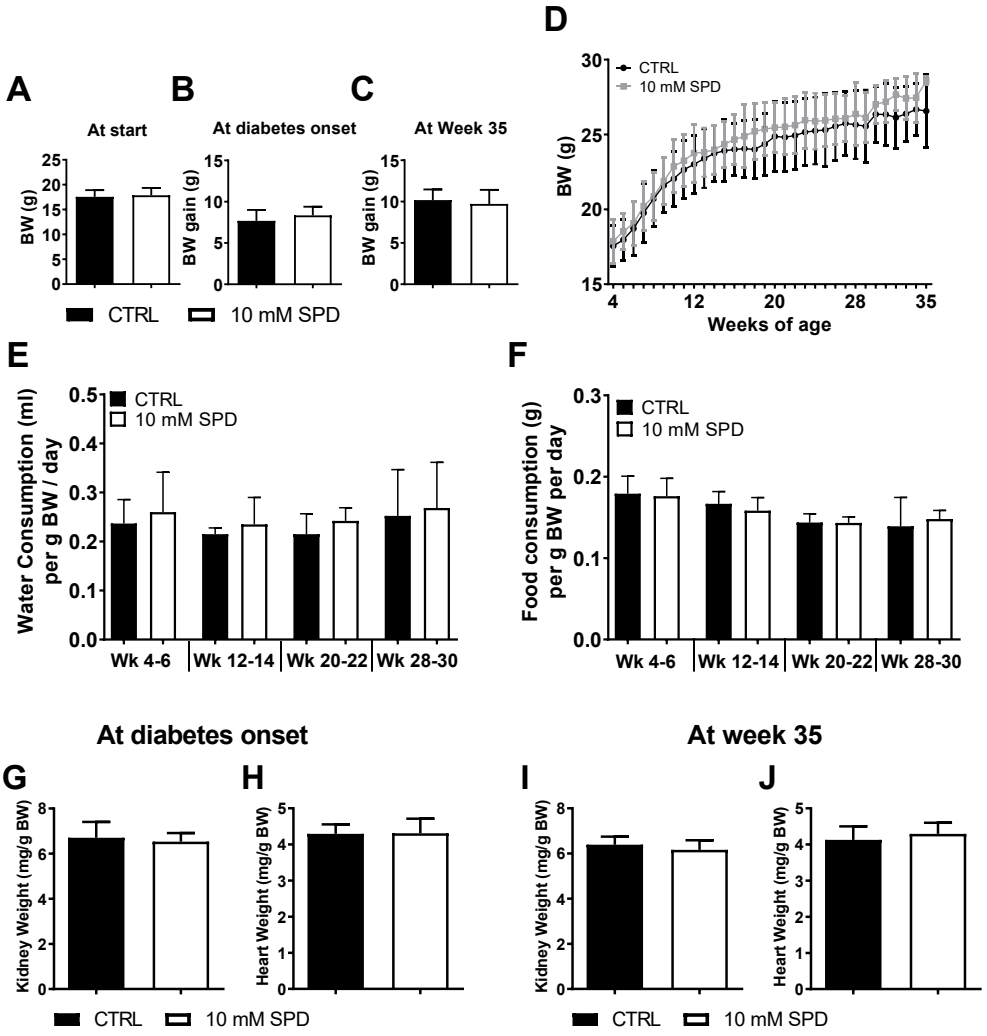

Figure 22: Vital parameters in Study-IV. (A) BW at the start of the study, (B) BW gain at diabetes onset and (C) at week 35, (D) BW monitoring during the study (week 4-35), (E) water consumption during the study, each bar represents 4-6 independent measurements from two cages, (F) food consumption during the study, each bar represents 2-3 independent measurements from two cages, (G and I) kidney weight at diabetes onset and at week 35 respectively, and (H and J) heart weight at diabetes onset and at week 35 respectively. Mice with BG levels over 200 mg/dl in two consecutive measurements were determined as diabetic. (A-C and E-J) The Mann-Whitney U test or the unpaired Student´s t-test and (D) two-way ANOVA with Sidak´s post-hoc test were performed as statistical analyses.

3.4.2. Study-IV: Diabetes Incidence

The study design and the number of mice are depicted in Fig. 23A. We observed that spermidine treatment did not lead to a significant change in diabetes incidence until 21 weeks of age (Fig. 23B). Sub-analysis of age at diabetes onset showed that spermidine treatment led to higher diabetes incidence in female NOD mice from 21 weeks of age (Fig. 23C).

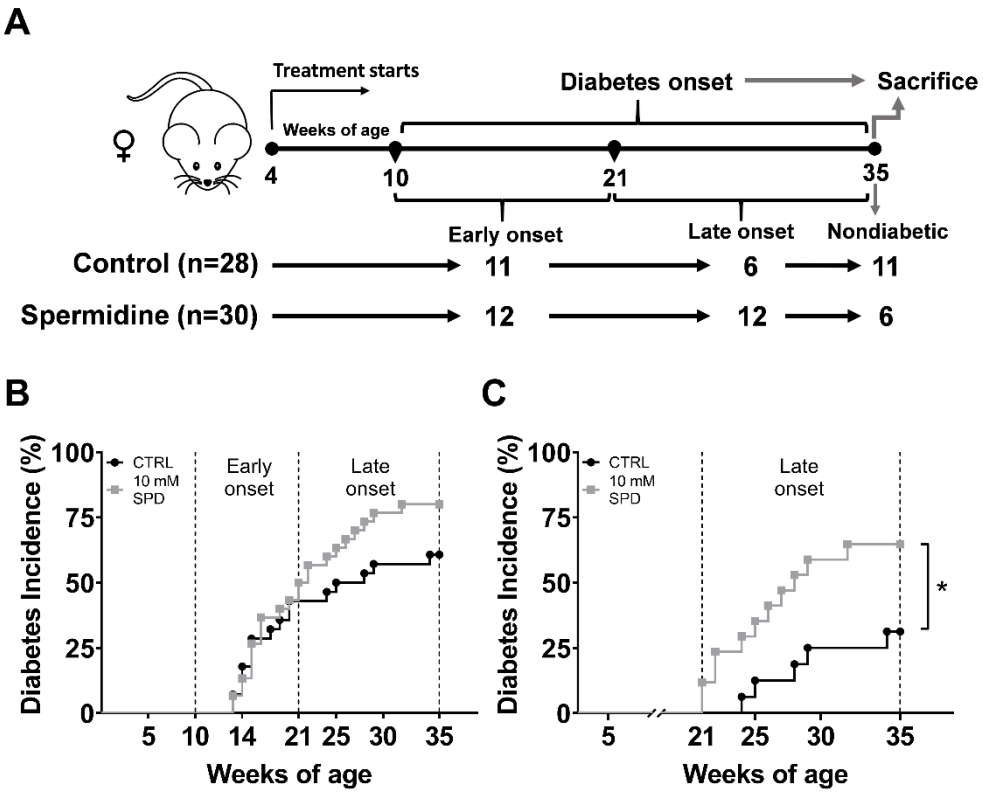

Figure 23: Diabetes incidence in Study-IV. (A) Study design, (B) diabetes incidence from week 10 to week 35, and (C) from week 21 to week 35. Mice with blood glucose levels over 200 mg/dl in two consecutive measurements were determined as diabetic. The Mantel-Cox log-rank test was performed as statistical analysis. $p^* < 0.05$.

3.4.3. Study-IV: Pancreatic Islet Insulitis

Spermidine treatment did not change the insulitis levels in pancreatic islets of nondiabetic 10 mM SPD-treated mice compared to nondiabetic CTRL mice (Fig. 24B and D). Similarly, spermidine treatment did not alter the insulitis levels in the pancreatic islets of diabetic 10 mM SPD-treated mice compared to diabetic CTRL mice (Fig. 24C and E). Insulitis was assessed according to the grading classification presented in Fig. 24A

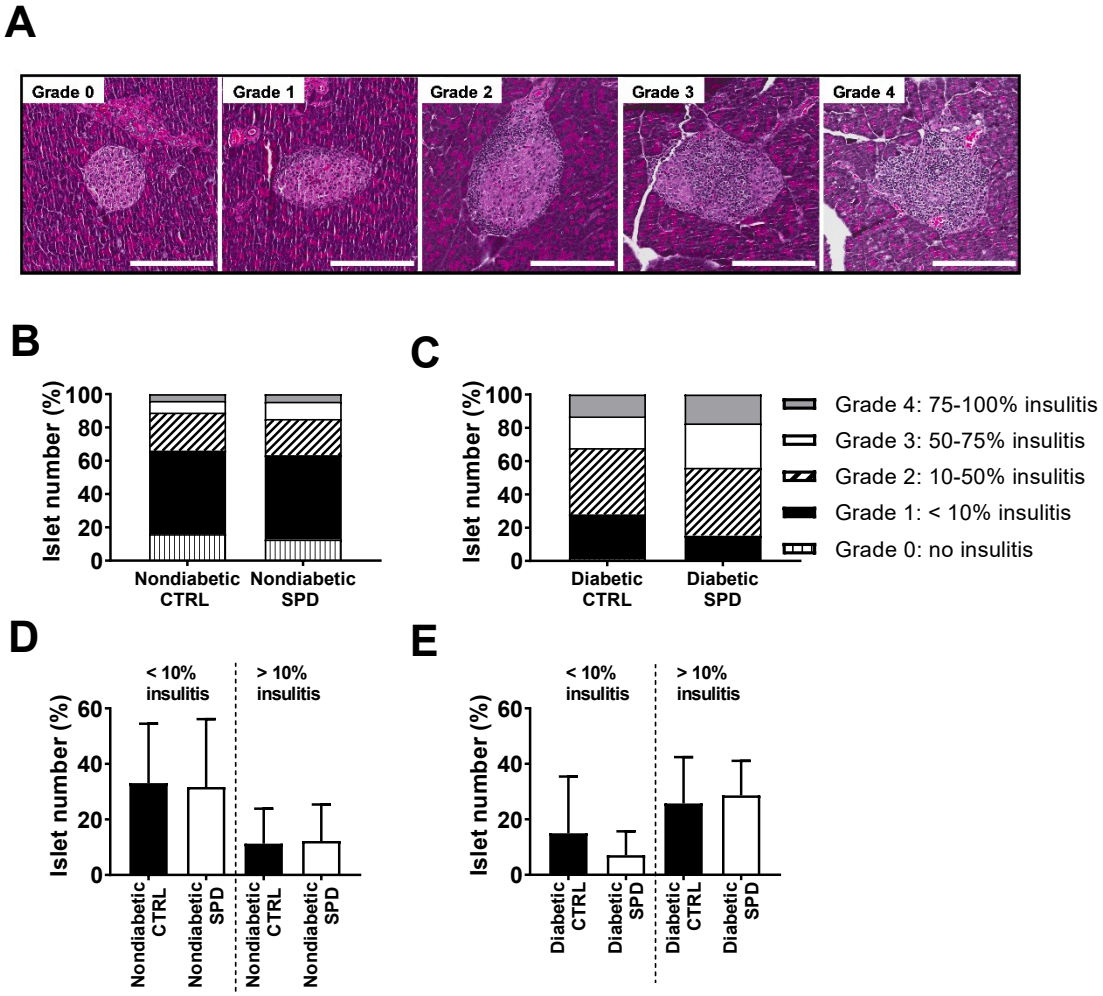

Figure 24: Analysis of islet insulitis by H&E staining. (A) Insulitis grading: grade 0 (no insulitis), grade 1 (peri insulitis to 10% insulitis), grade 2 (10- 50% insulitis), grade 3 (50- 75% insulitis), grade 4 (75- 100% insulitis), mice were analyzed according to (B and C) insulitis grading, and (D and E) insulitis levels < 10% or > 10%. Mice were sacrificed as diabetic at diabetes onset (week 14-35) or as nondiabetic at week 35 of age. Diabetic CTRL mice (n=4), diabetic 10 mM SPD mice (n=4), nondiabetic CTRL mice (n=4), and nondiabetic 10mM SPD mice (n=4). (B and C) The two-way ANOVA with Sidak´s post-hoc test and (D and E) the Mann-Whitney U test or the unpaired Student´s t-test were used as statistical analyses.

3.4.4. Study-IV: Immunophenotyping of Blood, Spleen, pLN and Thymus

We analyzed immune cell types in blood, spleen, pLN, and thymus from diabetic control, diabetic spermidine, nondiabetic control, and nondiabetic spermidine groups. We examined total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, central memory CD4+ T cells, FoxP3+ T cells, FoxP3+ CD25+ Tregs, FoxP3+ CD25+ CTLA4+ Tregs, total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, central memory CD8+ T cells, CD8+ cDCs, CD11b+ cDCs, CD11b+ CD4+ cDCs, and pDCs in blood, spleen, pLN and B cells, NK cells, and NKT cells, monocytes, macrophages, neutrophil granulocytes in blood and spleen. In thymus, we examined CD4+, CD8+, CD4+ CD8+, CD4(-) CD8(-), FoxP3+ CD4+, FoxP3+ CD25+ CD4+, mature CD4+ and mature CD8+ thymocytes.

Nondiabetic mice were sacrificed at week 35 of age with non-fasting blood glucose levels lower than 200 mg/dl. Diabetic mice were sacrificed between the age of 14 and the age of 35 weeks age following diagnosis with two consecutive measurements of non-fasting blood glucose levels higher than 200 mg/dl. Each immune cell population was compared between CTRL and 10 mM SPD-treated mice. Furthermore, each immune cell population was investigated in nondiabetic and diabetic groups as well as the early-onset and late-onset in diabetic mice separately to observe the direct impact of spermidine on each immune cell population. The gating strategy of each population was described in the methods chapter and was illustrated in the appendix (Fig. 55-57).

Nondiabetic control and nondiabetic spermidine mice sacrificed at the age of 35 weeks were examined for CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, and central memory CD4+ T cells. Spermidine treatment reduced total CD4+ T cells (1.5-fold, $p < 0.05$) and naïve CD4+ T cells (1.9-fold, $p=0.05$) in the blood of nondiabetic mice (Fig. 25A). There was no difference between CTRL and SPD groups in all analyzed cell populations in the spleen and pLN (Fig. 25B and C).

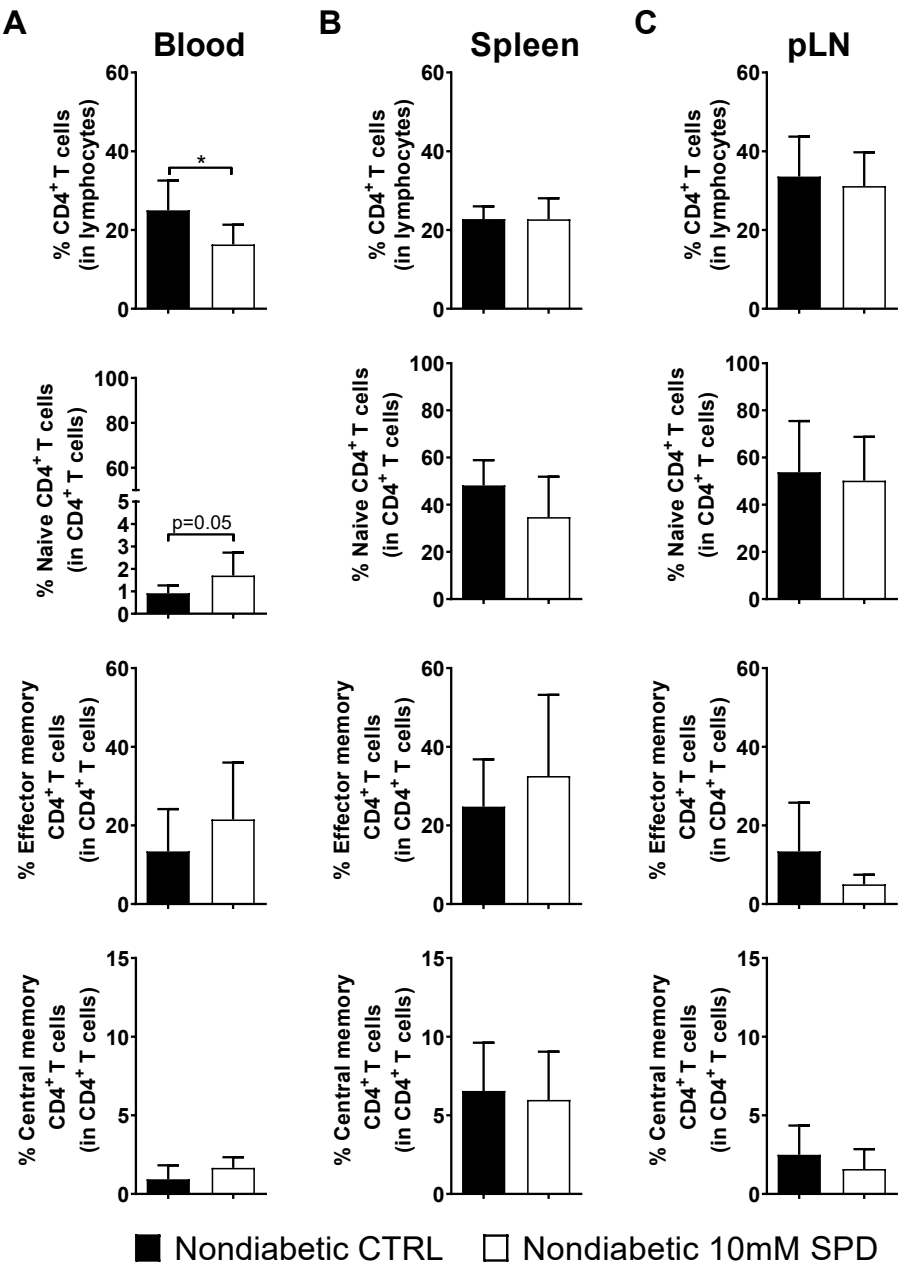

Figure 25: Analysis of CD4+ T cell subsets in nondiabetic mice. Total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, and central memory CD4+ T cells in (A) blood, (B) spleen, and (C) pLN were examined in nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=10-11) and open bar shows nondiabetic 10 mM SPD mice (n=5-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used. p* < 0.05.

Next, we examined FoxP3+ T cells and Tregs. Spermidine treatment increased the proportions of FoxP3+ T cells (1.5-fold, $p < 0.05$) in the blood of nondiabetic mice and FoxP3+ CD25+ Tregs (3.7-fold, $p < 0.05$) in the spleen of nondiabetic mice (Fig. 26A and B). There was no difference between CTRL and SPD groups in Treg populations in the pLN (Fig. 26C).

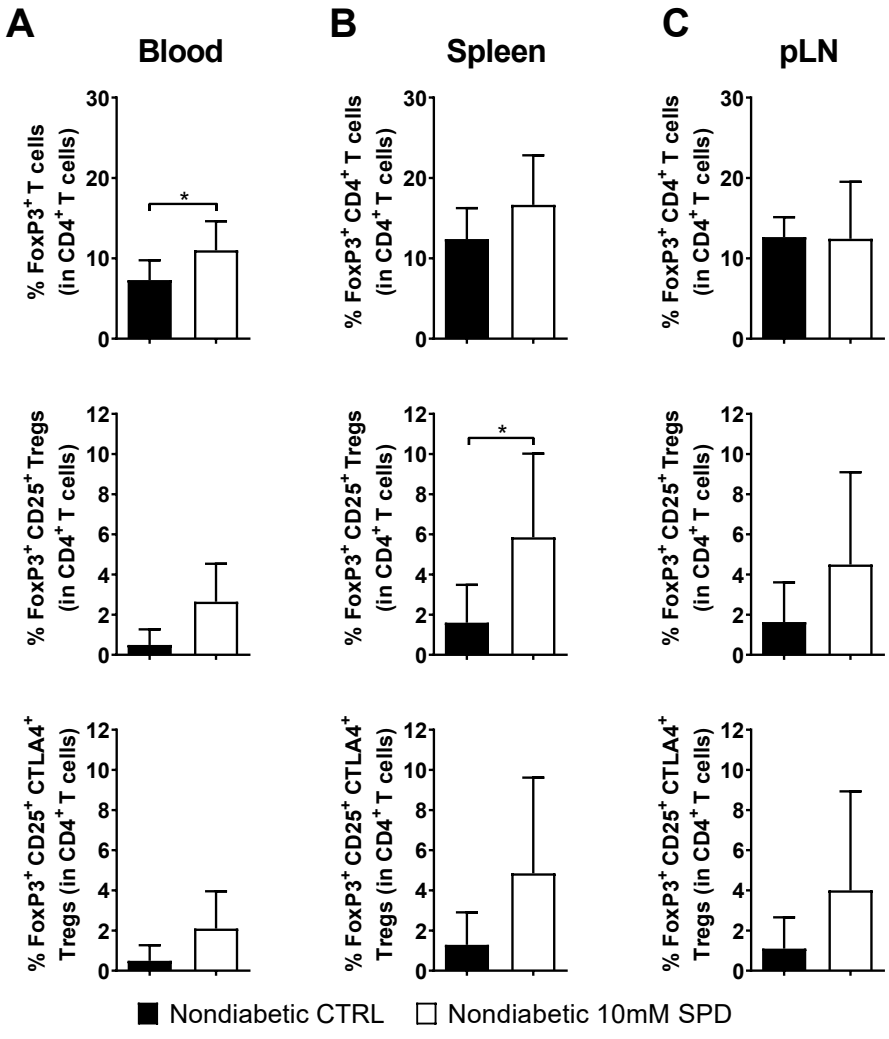

Figure 26: Analysis of FoxP3+ T cell subsets in nondiabetic mice. FoxP3+ T cells, FoxP3+ CD25+ Tregs, and FoxP3+ CD25+ CTLA4+ Tregs in (A) blood, (B) spleen, and (C) pLN were examined in nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=10-11) and open bar shows nondiabetic 10 mM SPD mice (n=5-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used. $p^* < 0.05$.

We explored CD4+ T cell subsets in the blood, spleen, and pLN of diabetic mice. In the blood of diabetic SPD mice, spermidine treatment increased the proportion of naïve CD4+ T cells (6.5-fold, $p < 0.05$), while it did not change any other CD4+ T cell subsets (Fig. 27A). Spermidine treatment did not alter any CD4+ T cell subsets in the spleen of diabetic mice (Fig. 27B). In the pLN of diabetic SPD mice, spermidine increased the proportion of total CD4+ T cells (1.1-fold, $p < 0.05$), while it did not change the proportion of other CD4+ T cell subsets (Fig. 27C).

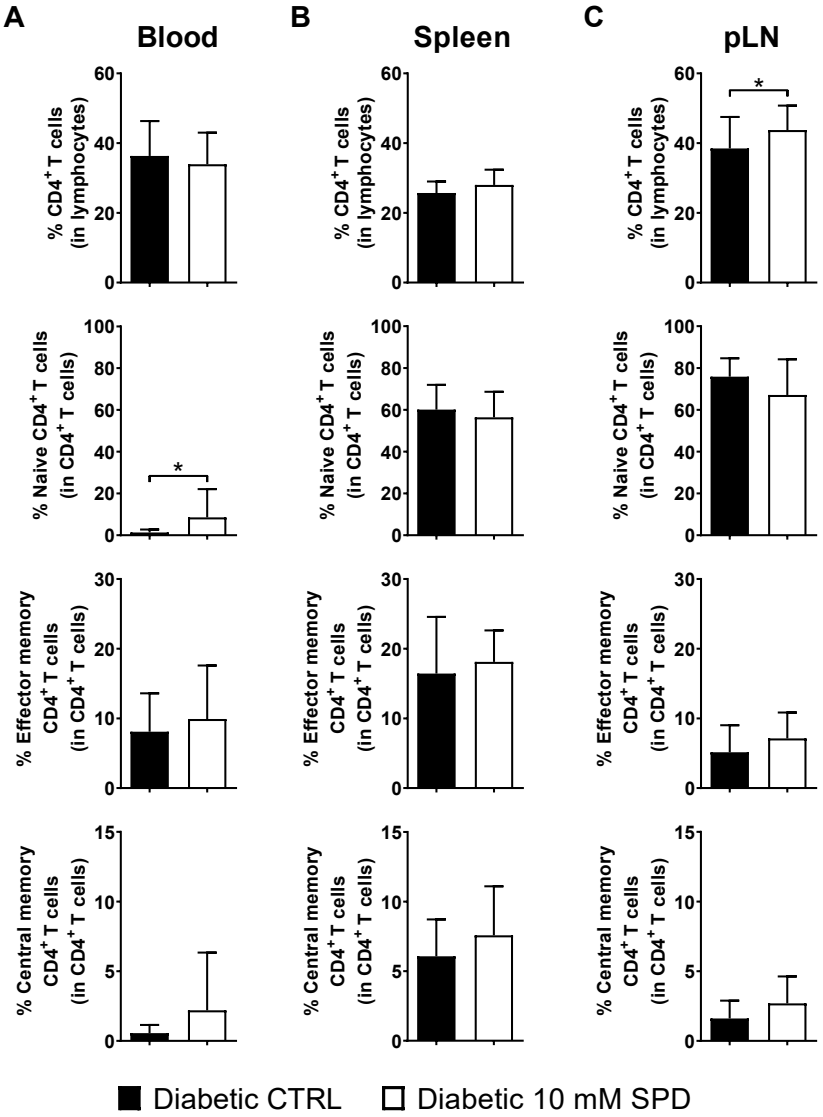

Figure 27: Analysis of CD4+ T cell subsets in diabetic mice. Total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, and central memory CD4+ T cells were examined in (A) blood, (B) spleen, and (C) pLN. Black bar shows diabetic CTRL mice (n=14-15) and open bar shows diabetic 10 mM SPD mice (n=22-24). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. $p^* < 0.05$.

In contrast to the findings in nondiabetic mice, in diabetic mice, spermidine treatment did not change the proportion of FoxP3+ T cells, total Tregs and activated CTLA4+ Tregs in blood, spleen, and pLN (Fig. 28).

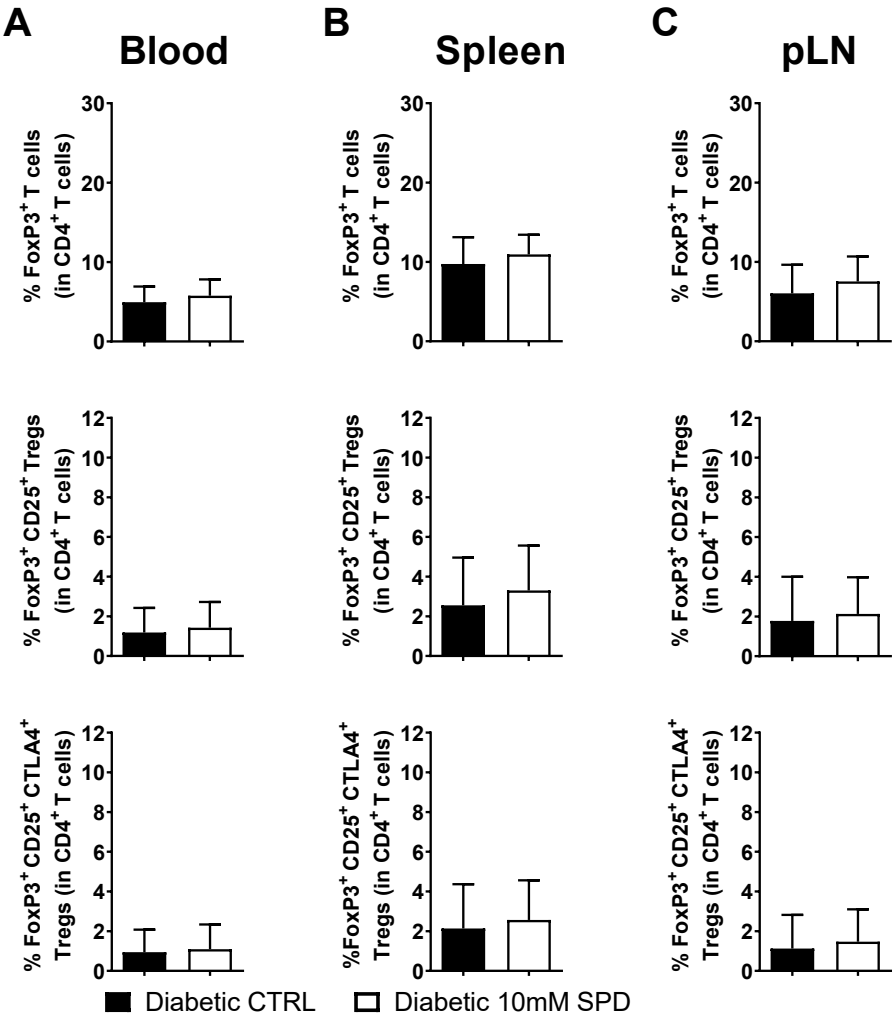

Figure 28: Analysis of FoxP3+ T cell subsets in diabetic mice. FoxP3+ T cells, FoxP3+ CD25+ Tregs, and FoxP3+ CD25+ CTLA4+ Tregs were examined in (A) blood, (B) spleen, and (C) pLN. Black bar shows diabetic CTRL mice (n=14-15) and open bar shows diabetic 10 mM SPD mice (n=22-24). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

Based on the effect of spermidine on diabetes incidence, mice were grouped as early-onset and late-onset diabetic mice to investigate further the effect of spermidine on immune cell composition. In the blood of early-onset SPD mice, spermidine treatment decreased the proportion of total CD4+ T cells (1.2-fold, $p < 0.05$) (Fig. 29A). In the spleen, early and late-onset mice had a similar proportion of CD4+ T cell subsets (Fig. 29B). In the pLN of early-onset SPD mice spermidine treatment increased the proportion of total CD4+ T cells (1.2-fold, $p < 0.05$) (Fig. 29C). Remaining CD4+ T cell populations were similar between CTRL and SPD groups in early-onset or late-onset (Fig. 29).

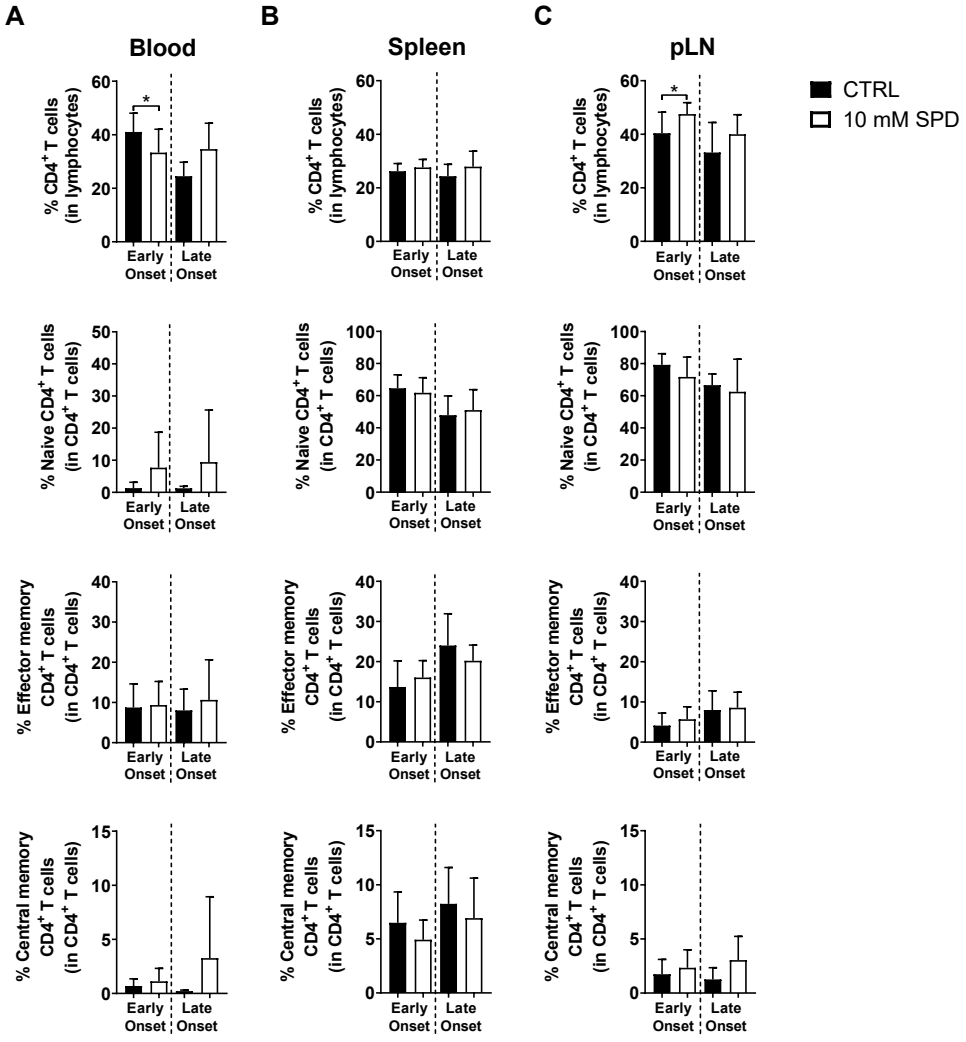

Figure 29: Analysis of CD4+ T cell subsets in early-onset and late-onset diabetic mice. Total CD4+ T cells, naïve CD4+ T cells, effector memory CD4+ T cells, and central memory CD4+ T cells were examined in (A) blood, (B) spleen, and (C) pLN. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=10-11) and open bar 10 mM SPD (n=11-12), late-onset; black bar control (n=4) and open bar 10mM SPD (n=11-12). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. $p^* < 0.05$.

Next, we explored the effect of spermidine on FoxP3+ T cells and Tregs based on the age of onset. In the pLN of early-onset SPD mice, spermidine treatment increased the proportion of FoxP3+ Tregs (1.7-fold, $p < 0.05$), FoxP3+ CD25+ Tregs (3.2-fold, $p < 0.05$) and FoxP3+ CD25+ CTLA4+ Tregs (3.8-fold, $p < 0.05$) (Fig. 30C). In the pLN of late-onset SPD mice, spermidine treatment reduced the proportion of FoxP3+ CD25+ Tregs (2.6-fold, $p < 0.05$) and FoxP3+ CD25+ CTLA4+ Tregs (3-fold, $p < 0.05$) (Fig. 30C). Spermidine treatment did not change the Treg populations in the blood and spleen of early-onset and late-onset mice (Fig. 30).

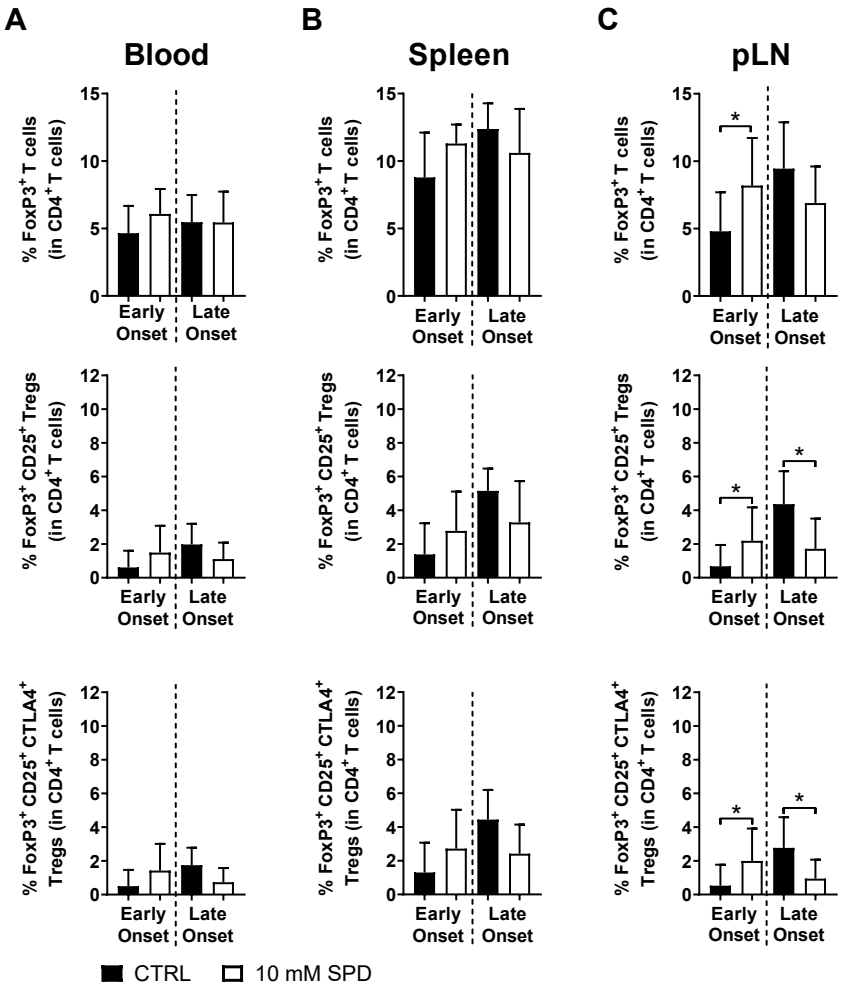

Figure 30: Analysis of FoxP3+ T cell subsets in early-onset and late-onset diabetic mice. FoxP3+ T cells, FoxP3+ CD25+ Tregs, and FoxP3+ CD25+ CTLA4+ Tregs were examined in (A) blood, (B) spleen, and (C) pLN. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=10-11) and open bar 10 mM SPD (n=11-12), late-onset; black bar CTRL (n=4) and open bar 10 mM SPD (n=11-12). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

Next, we studied CD8+ T cell subsets in the blood, spleen, and pLN of nondiabetic mice. Specifically, we analyzed the proportions of total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells. We did not observe any change in any of the analyzed CD8+ T cells subsets in nondiabetic mice (Fig. 31).

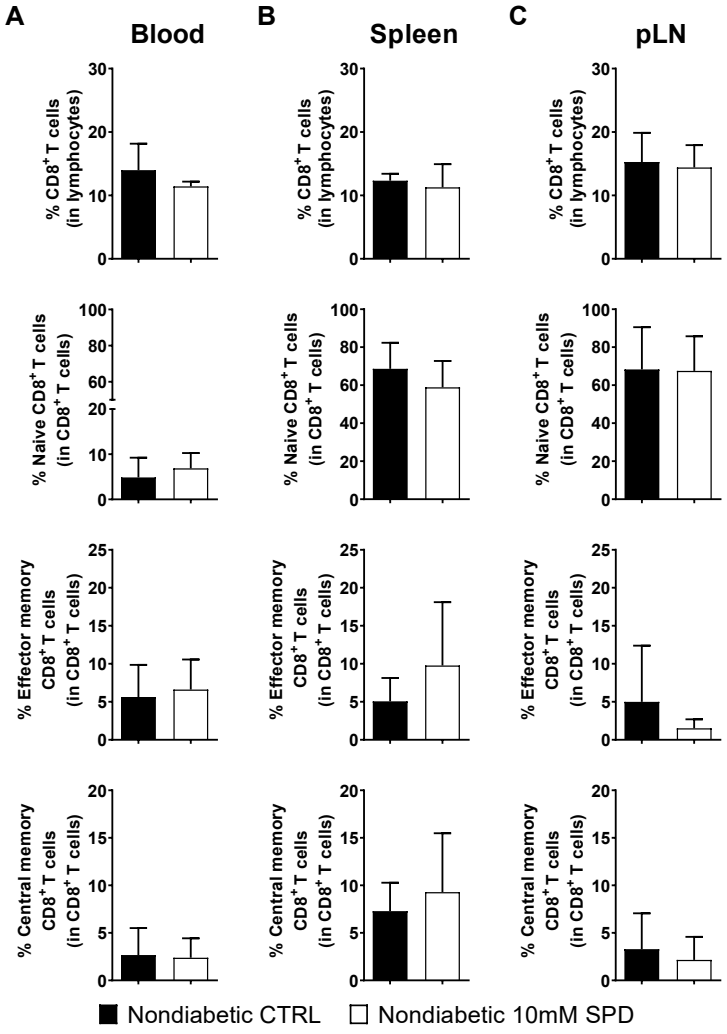

Figure 31: Analysis of CD8+ T cell subsets in nondiabetic mice. Total CD8+ T cells, naïve CD8+ T cells, effector memory CD4+ T cells, and central memory CD8+ T cells in (A) blood, (B) spleen, and (C) pLN were examined in nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=9-11) and open bar shows nondiabetic 10 mM SPD mice (n=5-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used.

Although there was no effect of spermidine in CD8+ T cell populations in nondiabetic mice, in the blood of diabetic SPD mice, spermidine treatment increased the proportion of naïve CD8+ T cells (2.5-fold, $p < 0.05$) (Fig. 32A). In the pLN of diabetic SPD mice, spermidine treatment increased the proportion of effector memory CD8+ T cells (1.8-fold, $p < 0.01$) and central memory CD8+ T cells (1.9-fold, $p < 0.05$) (Fig. 32C). Spermidine treatment did not change the proportion of other CD8+ T cells subsets in blood, spleen, and pLN of diabetic mice (Fig. 32).

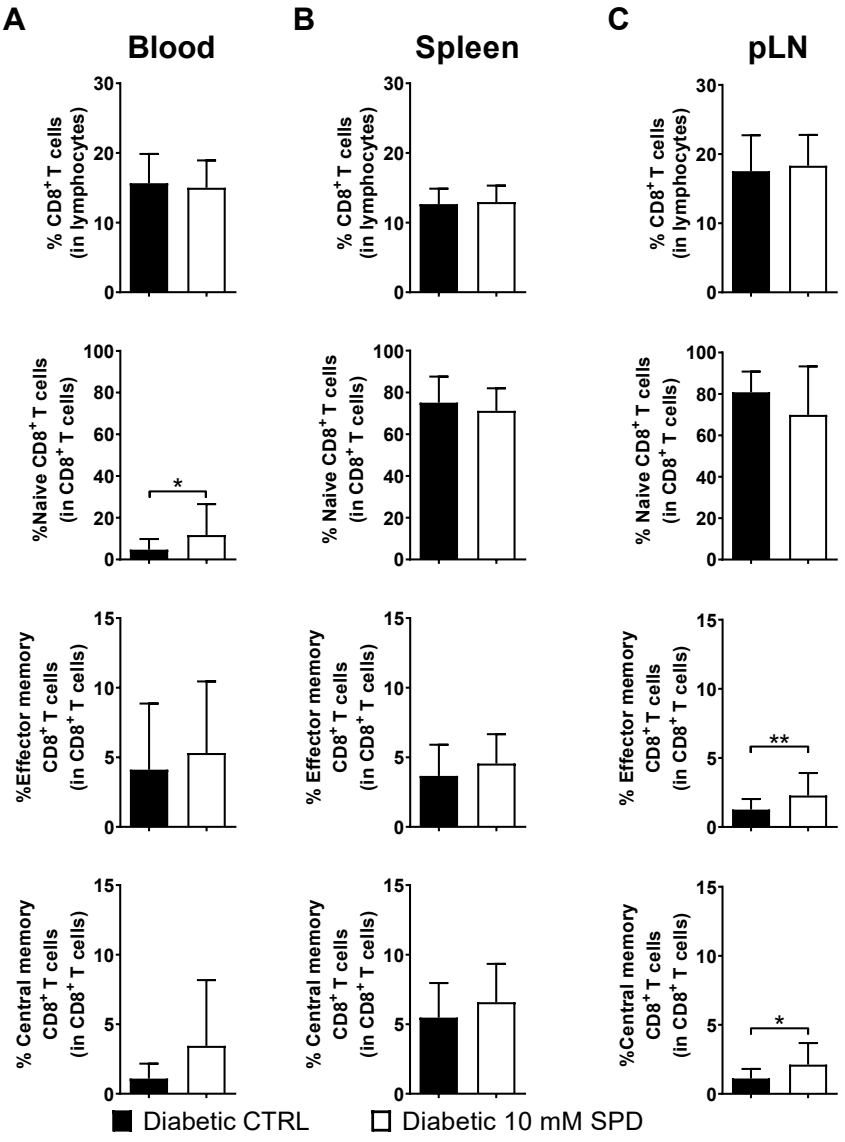

Figure 32: Analysis of CD8+ T cell subsets in diabetic mice. Total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells were examined in (A) blood, (B) spleen and (C) pLN of diabetic mice. Black bar shows diabetic CTRL mice (n=14-15) and open bar shows diabetic 10 mM SPD mice (n=22-24). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used. $p^* < 0.05$, $p^{**} < 0.01$.

CD8+T cell subsets were further analyzed according to the age of diabetes onset. In the blood of early-onset SPD mice, spermidine decreased the proportion CD8+ T cells (1.3-fold, $p < 0.0.5$) (Fig. 33A). In contrast, in the blood of late-onset SPD mice, spermidine increased the proportion of total CD8+ T cells (1.5-fold, $p < 0.0.5$) (Fig. 33A). In the spleen, early and late-onset mice had similar proportion of CD4+ T cell subsets and CD8+ T cells subsets (Fig. 33B). In the pLN of early-onset SPD mice, spermidine reduced the proportion of naïve CD8+ T cells (1.2-fold, $p < 0.05$) (Fig. 33C).

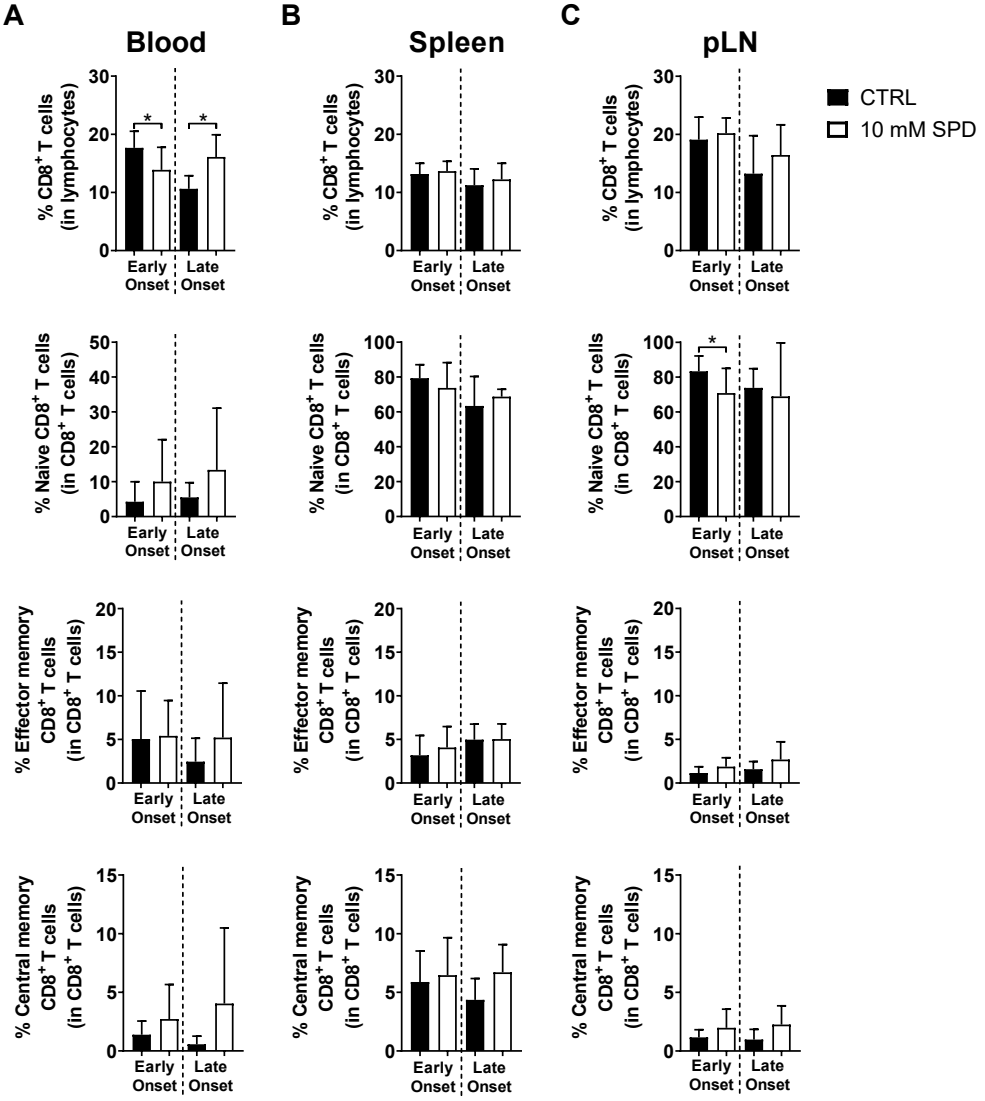

Figure 33: Analysis of CD8+ T cell subsets in early-onset and late-onset diabetic mice. Total CD8+ T cells, naïve CD8+ T cells, effector memory CD8+ T cells, and central memory CD8+ T cells were examined in (A) blood, (B) spleen, and (C) pLN. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=4-6) and open bar 10 mM SPD (n=6-7), late-onset; black bar CTRL (n=5-8) and open bar 10mM SPD (n=7-8). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. $p^* < 0.05$.

Another important immune cell population in T1D, which are dendritic cell populations, was analyzed in nondiabetic mice. Spermidine treatment significantly elevated the proportion of CD8+ cDCs (6-fold, $p < 0.001$) and pDCs (2.2-fold, $p < 0.05$) in blood of nondiabetic mice (Fig. 34A). Spermidine treatment increased the proportion of CD11b+ CD4+ cDCs (2.2-fold, $p < 0.05$) and reduced the proportion of pDCs (1.4-fold, $p < 0.05$) in the spleen of nondiabetic mice (Fig. 34B). We did not observe any alteration in the other DCs populations in nondiabetic mice (Fig. 34).

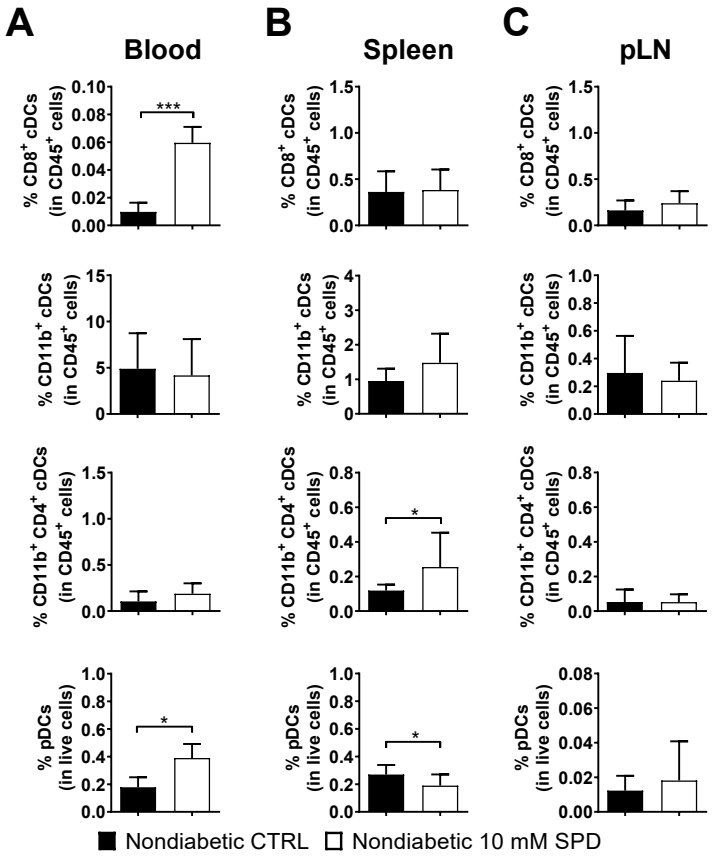

Figure 34: Analysis of dendritic cell subsets in nondiabetic mice. CD8+ cDCs, CD11b+ cDCs, CD11b+ CD4+ cDCs, and pDCs were examined in (A) blood, (B) spleen, and (C) pLN of nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=4-11) and open bar shows nondiabetic 10 mM SPD mice (n=3-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. $p^* < 0.05$, $p^{***} < 0.001$.

When dendritic cells were analyzed in diabetic mice, in contrast to our observation in nondiabetic mice, we observed that spermidine treatment did not change the proportion of dendritic cell populations in the blood, spleen and pLN of diabetic mice (Fig. 35).

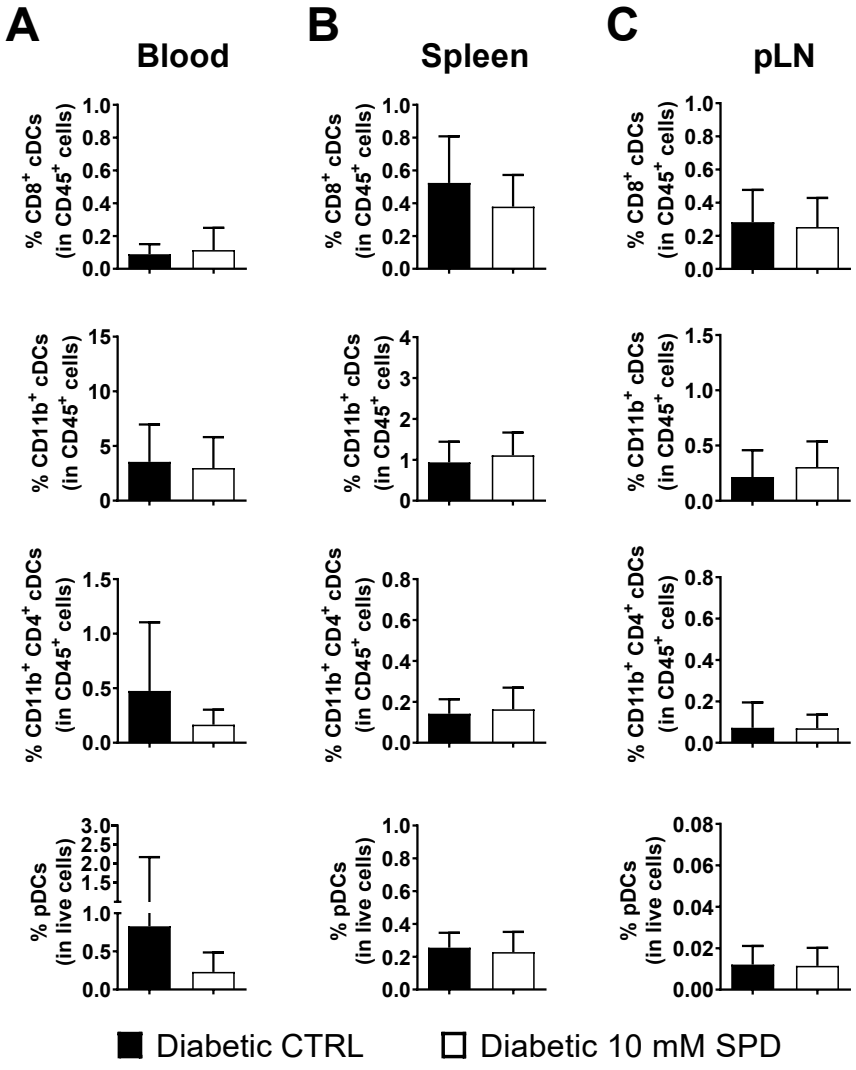

Figure 35: Analysis of dendritic cell subsets in diabetic mice. CD8+ cDCs, CD11b+ cDCs, CD11b+ CD4+ cDCs, and pDCs were examined in (A) blood, (B) spleen, and (C) pLN of diabetic mice. Black bar shows diabetic CTRL mice (n=4-15) and open bar shows diabetic 10 mM SPD mice (n=10-18). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

Analysis of dendritic cell populations according to the age of diabetes onset revealed that in early-onset mice, spermidine treatment increased only the proportion of CD11b+ cDCs in pLN (Fig. 36C). Otherwise, early-onset and late-onset mice had similar proportion of dendritic cell populations in the spleen and pLN (Fig. 36).

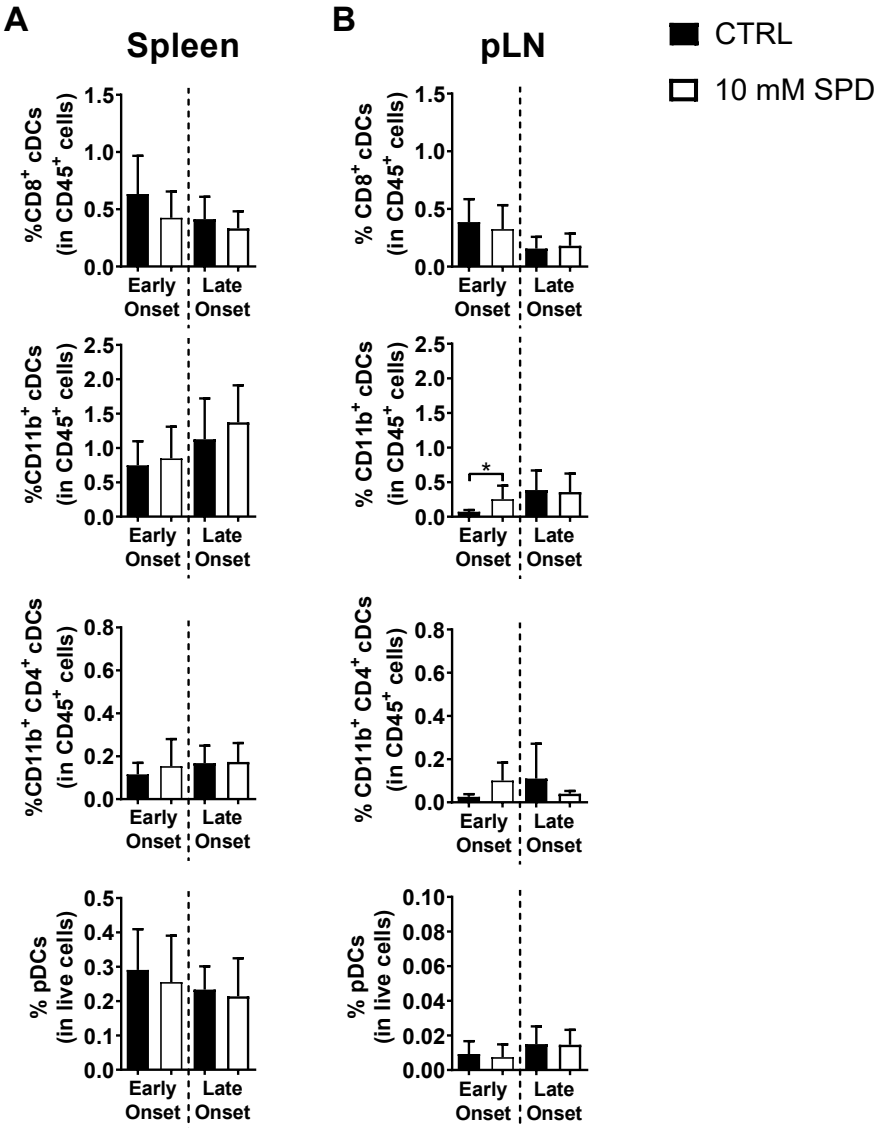

Figure 36: Analysis of dendritic cell subsets in early-onset and late-onset diabetic mice. CD8+ cDCs, CD11b+ cDCs, CD11b+ CD4+ cDCs, and pDCs were analyzed in (A) spleen and (B) pLN of diabetic mice. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=4-6) and open bar 10 mM SPD (n=6-7), late-onset; black bar CTRL (n=5-8) and open bar 10 mM SPD (n=7-8). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

Furthermore, we explored the effect of spermidine on myeloid immune cells in nondiabetic mice. Spermidine treatment did not alter the proportion of monocytes, macrophages, M2 macrophages, and neutrophil granulocytes in the blood and spleen of nondiabetic mice (Fig. 37).

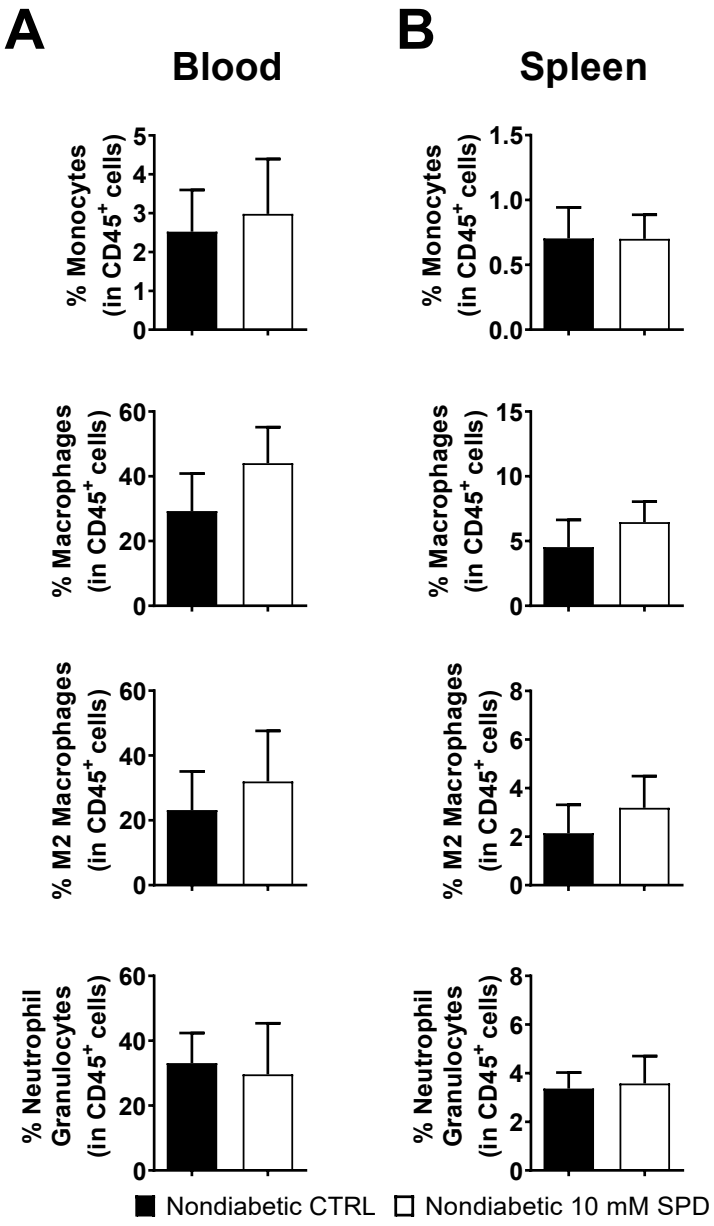

Figure 37: Analysis of monocytes, macrophages, M2 macrophages, and neutrophil granulocytes in nondiabetic mice. (A) Blood and (B) spleen. Black bar shows nondiabetic CTRL mice (n=4-11) and open bar shows nondiabetic 10 mM SPD mice (n=3-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

Similar to our results in nondiabetic mice, spermidine treatment did not change the proportion of monocytes, macrophages, M2 macrophages, and neutrophil granulocytes in the blood, spleen, and pLN of diabetic mice (Fig. 38).

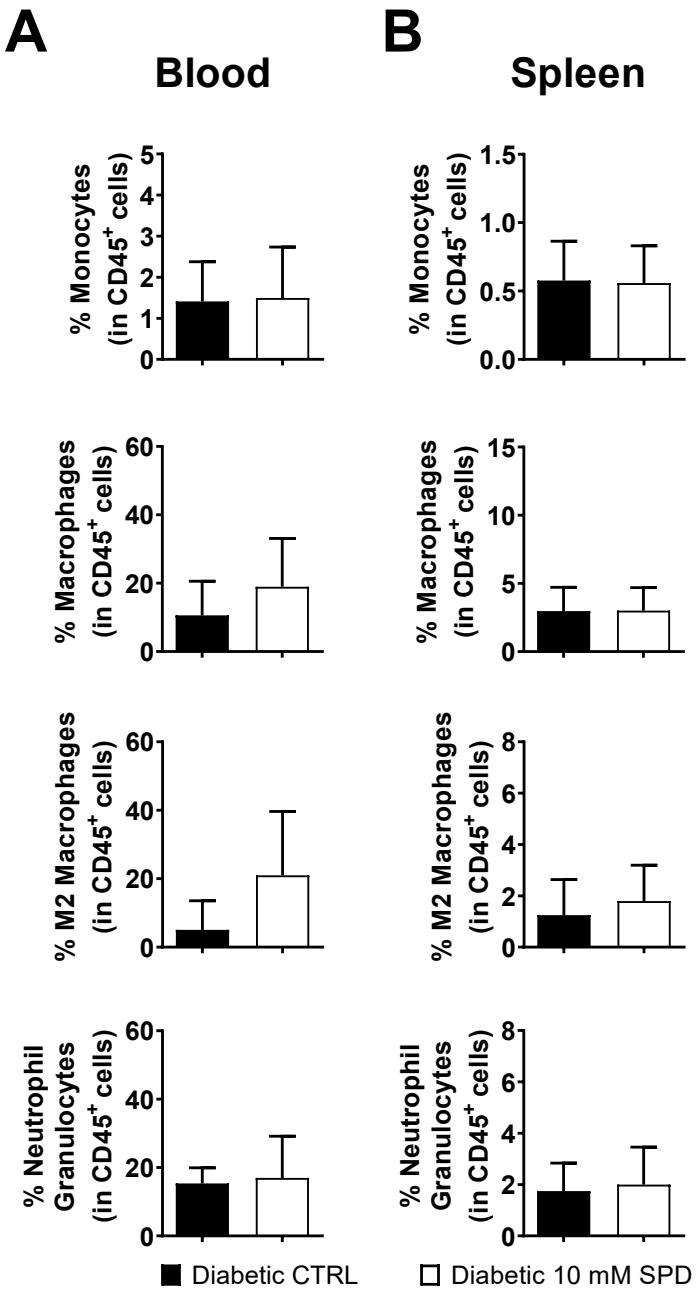

Figure 38: Analysis of monocytes, macrophages, M2 macrophages and neutrophil granulocytes in diabetic mice. (A) Blood and (B) spleen. Black bar shows diabetic CTRL mice (n=3-12) and open bar shows diabetic 10 mM SPD mice (n=3-16). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

Finally, we analyzed the proportions of myeloid cells in the spleen based on the age of diabetes onset. The proportions of monocytes, macrophages, M2 macrophages, and neutrophil granulocytes were similar between CTRL and SPD mice in early-onset and late-onset mice (Fig. 39).

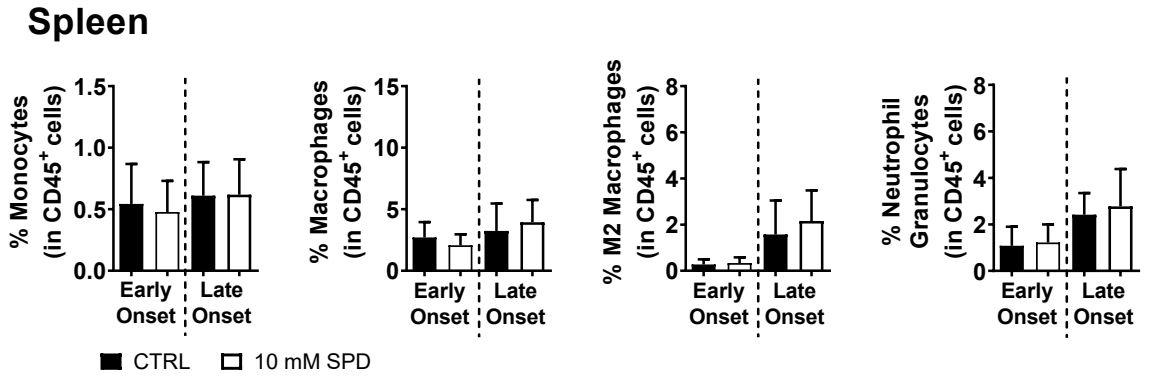

Figure 39: Analysis of monocytes, macrophages, M2 macrophages, and neutrophil granulocytes in the spleen of early-onset and late-onset diabetic mice. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=2-6) and open bar 10 mM SPD (n=2-8), late-onset; black bar CTRL (n=6) and open bar 10 mM SPD (n=8). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

We further explored the effect of spermidine on B cells, natural killer (NK) cells, and natural killer T (NKT) cells. Spermidine treatment elevated the proportion of NK cells (1.9-fold, $p < 0.05$) in the blood of nondiabetic mice (Fig. 40A). We did not detect any other change in B cells, NK cells, and NKT cells by spermidine treatment in nondiabetic mice (Fig. 40).

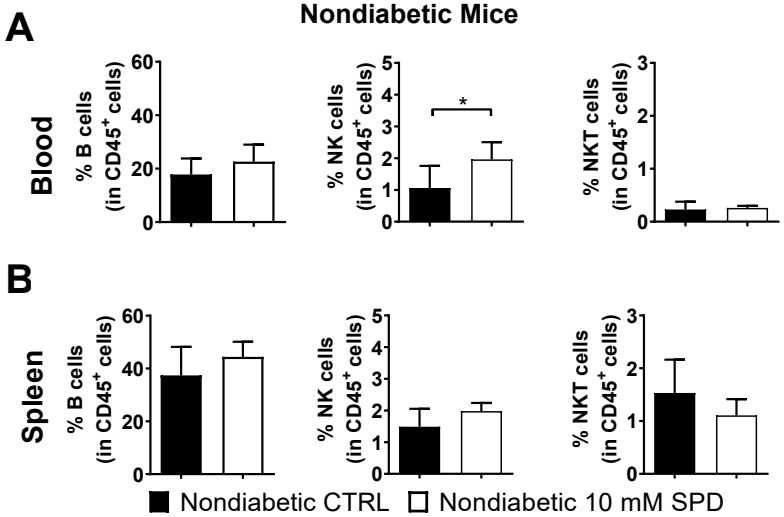

Figure 40: Analysis of B cells, natural killer (NK) cells, and natural killer T cells (NKT) cells in nondiabetic mice. (A) Blood and (B) spleen. Black bar: nondiabetic CTRL mice (n=10) and open bar: nondiabetic 10 mM SPD mice (n=4-6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

Similar to nondiabetic mice, spermidine treatment did not alter the proportion of B cells, natural killer (NK) cells, and natural killer T (NKT) cells in blood and spleen of diabetic mice (Fig. 41).

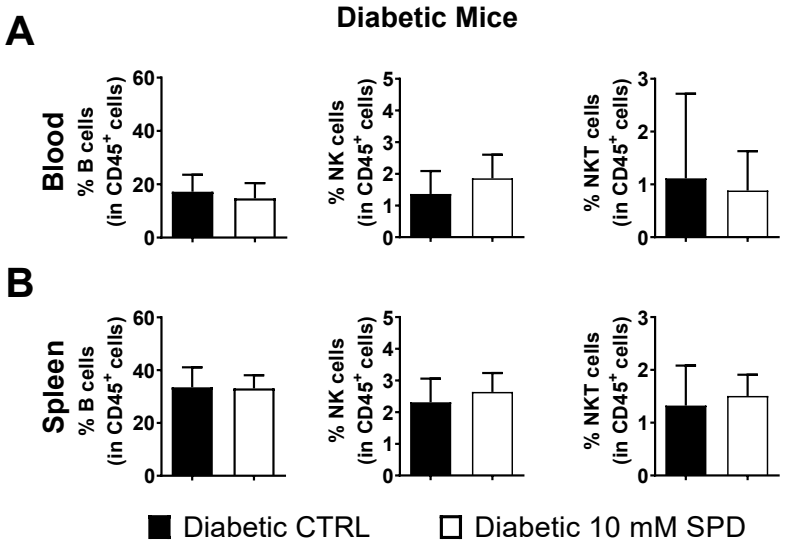

Figure 41: Analysis of B cells, NK cells, and NKT cells in diabetic mice. (A) Blood and (B) spleen. Black bar shows diabetic CTRL mice (n=8-16) and open bar shows diabetic 10 mM SPD mice (n=21-24). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired student t-test was used as statistical analysis.

Lastly, we examined B cells, natural killer (NK) cells, and natural killer T (NKT) cells based on the age of diabetes onset. We detected that the proportion of B cells, NK cells, and NKT cells were similar between CTRL and SPD mice in early-onset and late-onset mice in blood and spleen (Fig. 42).

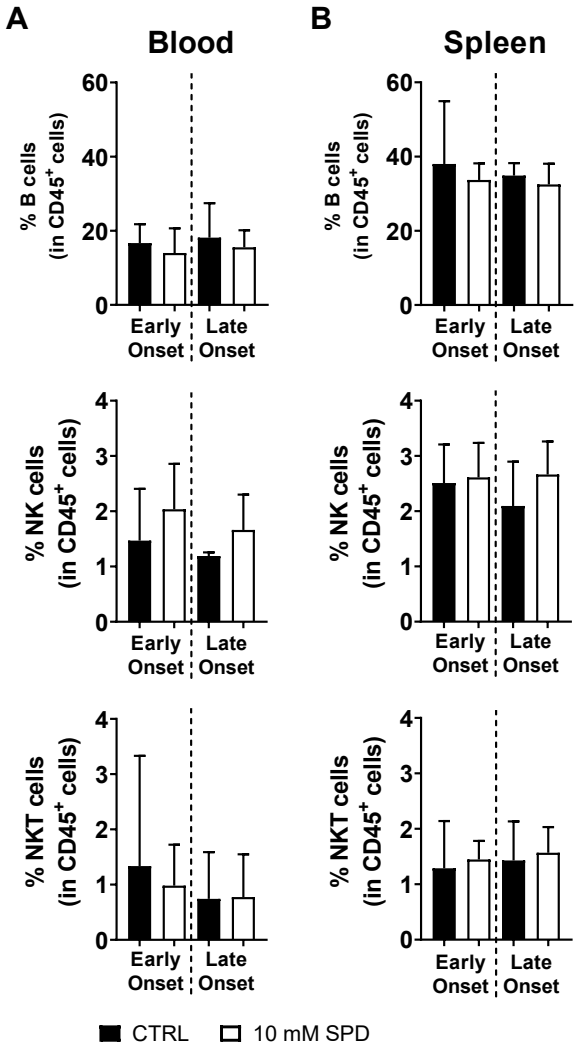

Figure 42: Analysis of B cells, NK cells, and NKT cells in early-onset and late-onset diabetic mice. (A) Blood and (B) spleen. Mice are grouped as early-onset diabetic (< 21 weeks of age) and late-onset diabetic (≥ 21 weeks of age). Early-onset; black bar CTRL (n=5-10) and open bar 10 mM SPD (n=11-12), late-onset; black bar CTRL (n=3-6) and open bar 10 mM SPD (n=10-12). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

To examine the hypothesis of disrupted thymic selection in NOD mice, we examined CD4+, CD8+, CD4+CD8+, CD4 (-) CD8(-), FoxP3+, FoxP3+ CD25+, mature CD4+, and mature CD8+ thymocytes. We found that spermidine treatment did not affect the proportion of CD4+ and CD8+ thymocyte subsets in nondiabetic mice (Fig. 43).

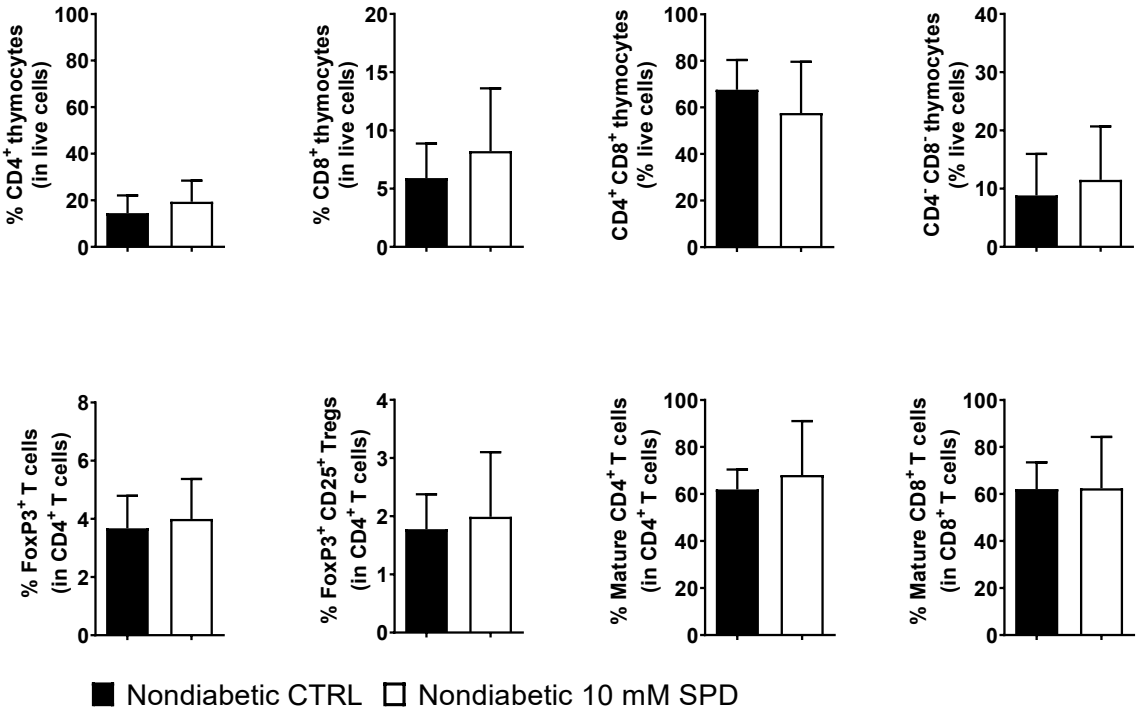

Figure 43: Analysis of thymocytes in the thymus of nondiabetic mice. CD4+, CD8+, CD4+CD8+, CD4(-) CD8(-), FoxP3+, FoxP3+ CD25+, mature CD4+, and mature CD8+ thymocytes were analyzed. Black bar shows nondiabetic CTRL mice (n=11) and open bar shows nondiabetic 10mM SPD mice (n= 6). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

Comparable to our results in nondiabetic mice, in the thymus of diabetic mice, spermidine treatment did not change the proportion of CD4+ and CD8+ thymocytes subsets, and the thymocyte populations such as TCR lo CD69(-), TCR int CD69+, TCR hi CD69+, TCR hi CD69(-) populations which have been shown to play a role in positive selection (Fig. 44 and 45).

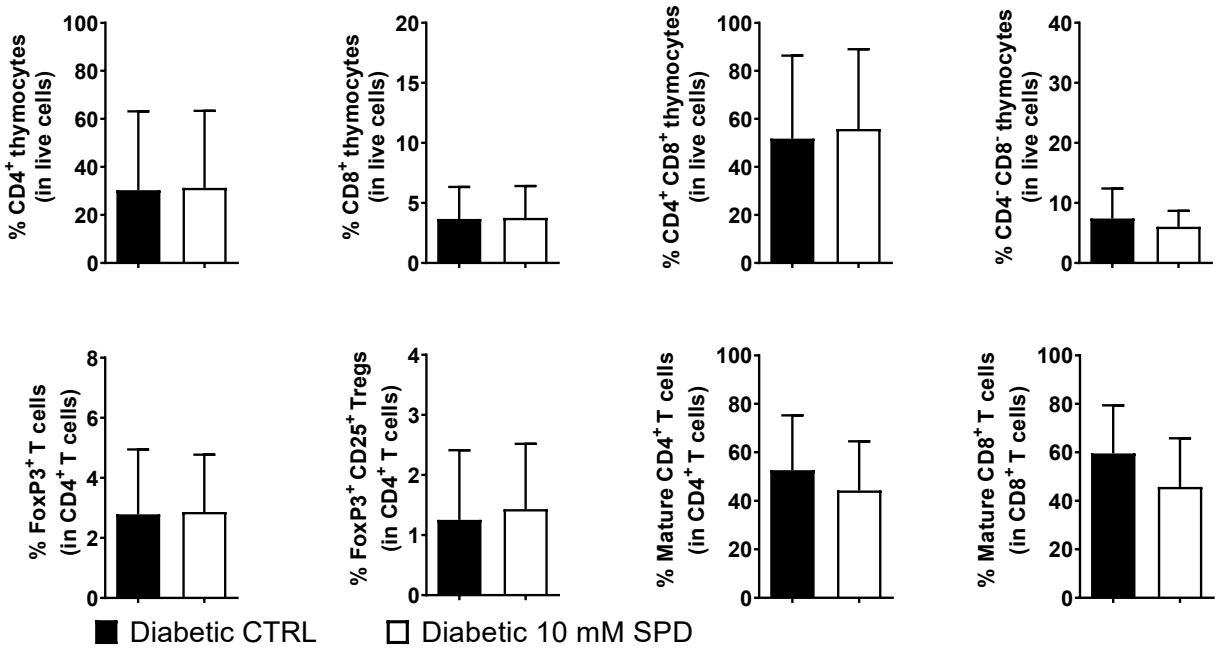

Figure 44: Analysis of thymocytes in the thymus of diabetic mice. CD4+ thymocytes, CD8+ thymocytes, CD4+CD8+ thymocytes, CD4(-)CD8(-) thymocytes, FoxP3+, FoxP3+ CD25+ thymocytes, and mature CD4+ and mature CD8+ thymocytes were analyzed. Black bar shows diabetic CTRL mice (n=11-17) and open bar shows diabetic 10 mM SPD mice (n=13-20). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

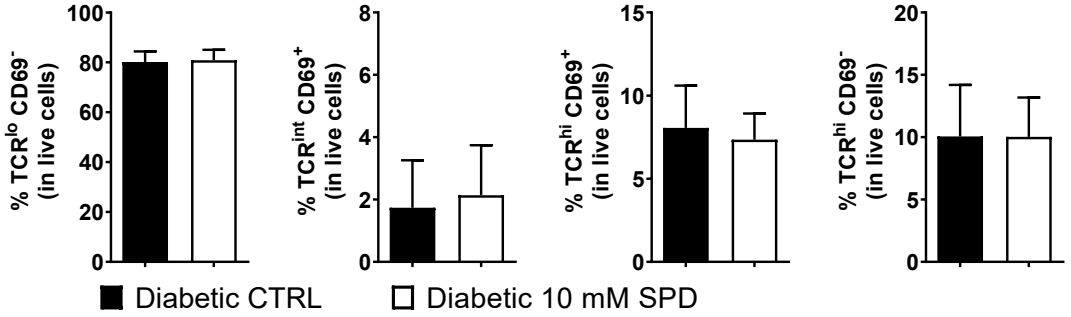

Figure 45: Analysis of positive selection in the thymus of diabetic mice. TCR lo CD69(-), TCR int CD69+, TCR hi CD69+, and TCR hi CD69(-) populations were analyzed. Black bar shows diabetic CTRL mice (n=13) and open bar shows diabetic 10 mM SPD mice (n=13). Data is shown as mean ± SD. The unpaired Student´s t-test was used as statistical analysis.

Next, we examined the plasma cytokine levels (IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10) in nondiabetic and diabetic mice. Spermidine treatment reduced IL-6 levels in spermidine-treated nondiabetic mice (Fig. 46). Other cytokine levels were not altered by spermidine in nondiabetic mice (Fig. 46). Spermidine did not modulate the cytokine levels in diabetic mice (Fig. 47).

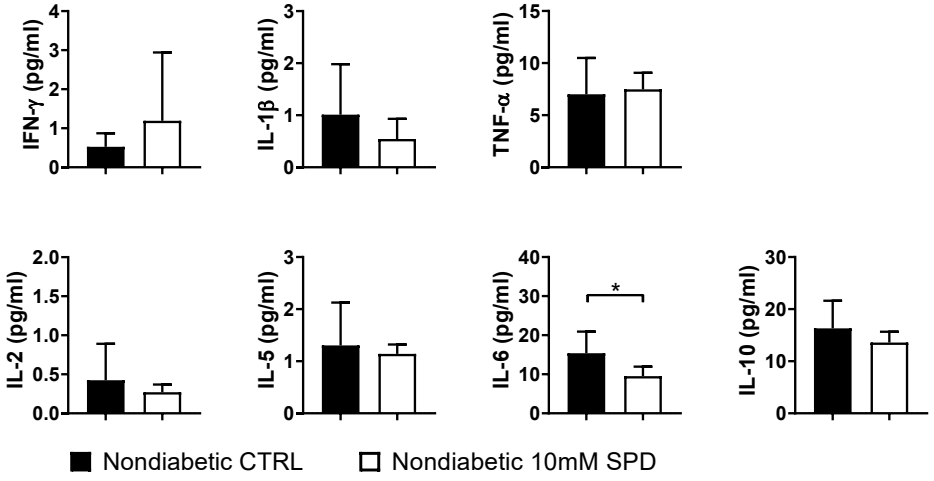

Figure 46: Analysis of IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10 in the plasma of nondiabetic mice. Black bar shows nondiabetic CTRL mice (n=9-10) and open bar shows nondiabetic 10 mM SPD mice (n=6). Data is shown as pg/ml and mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

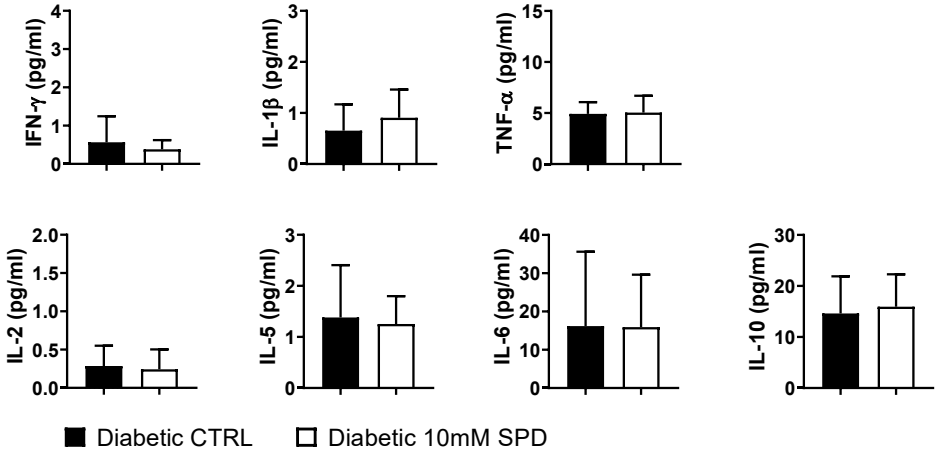

Figure 47: Analysis of IFN-ɣ, IL-1β, TNF-α, IL-2, IL-5, IL-6, and IL-10 in the plasma of diabetic mice. Black bar shows diabetic control (n=16) and open bar shows diabetic 10 mM SPD (n=22). Data is shown as pg/ml and mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis.

3.4.5. Study-IV: Analysis of Autophagy Markers in Pancreas and Thymus

We investigated the autophagy markers in nondiabetic and diabetic mice to observe the effect of diabetes on autophagy. Additionally, we checked autophagy markers in control and spermidine-treated mice to observe the effect of spermidine on autophagy. The whole pancreas and whole thymus were analyzed by immunoblotting. Mice are grouped as early-onset (< 21 weeks of age), late-onset (≥ 21 weeks of age), and nondiabetic (35 weeks of age). LC3-I, LC3-II, Beclin1, and p62 were used as autophagy markers.

We found that diabetes onset alone did not change the autophagy markers in the pancreas. We found that LC3-II to LC3-I ratio was elevated in diabetic mice in comparison to nondiabetic mice independent of the treatment group (Fig. 48). Beclin1 and p62 levels were similar between nondiabetic and diabetic mice independent of the treatment (Fig. 48).

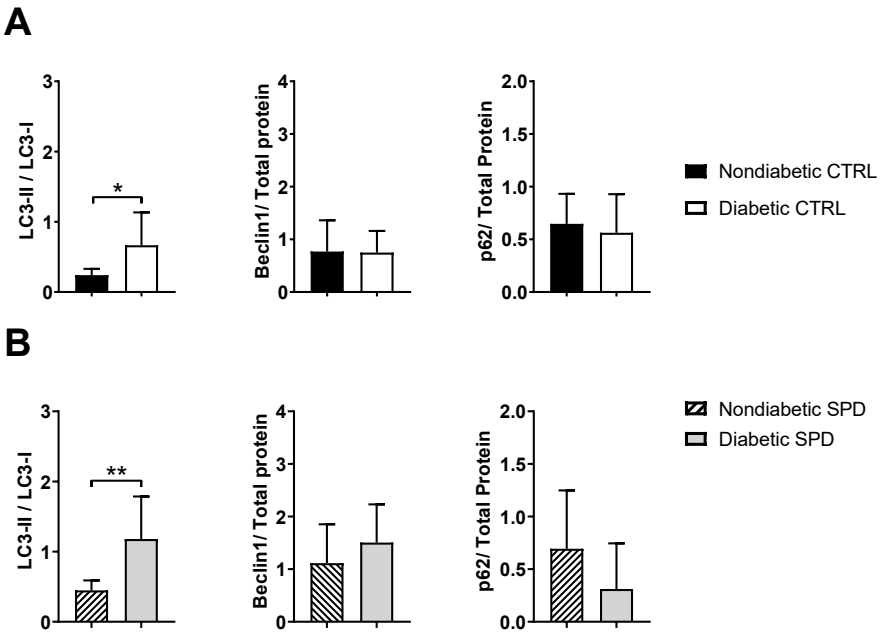

Figure 48: Analysis of autophagy markers in the pancreas of diabetic and nondiabetic mice. Mice are grouped as diabetic (BG > 200 mg/dl, 14-35 weeks of age) and nondiabetic (BG < 200 mg/dl, 35 weeks of age). Samples were probed for LC3, Beclin1, p62 and stained with coomassie blue for total protein by immunobloting in (A) nondiabetic CTRL, and diabetic CTRL mice and in (B) nondiabetic 10 mM SPD, and diabetic 10 mM SPD mice. n= 6-8 for nondiabetic mice and n=17-19 for diabetic mice. Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05, p** < 0.01.

Next, we checked whether spermidine treatment altered the pancreatic autophagy markers in nondiabetic mice. We found that spermidine treatment only increased LC3-II to LC3-I ratio in nondiabetic mice, while spermidine did not change Beclin1 and p62 levels compared to control mice (Fig. 49). Representative immunoblot images are shown in Fig. 49B.

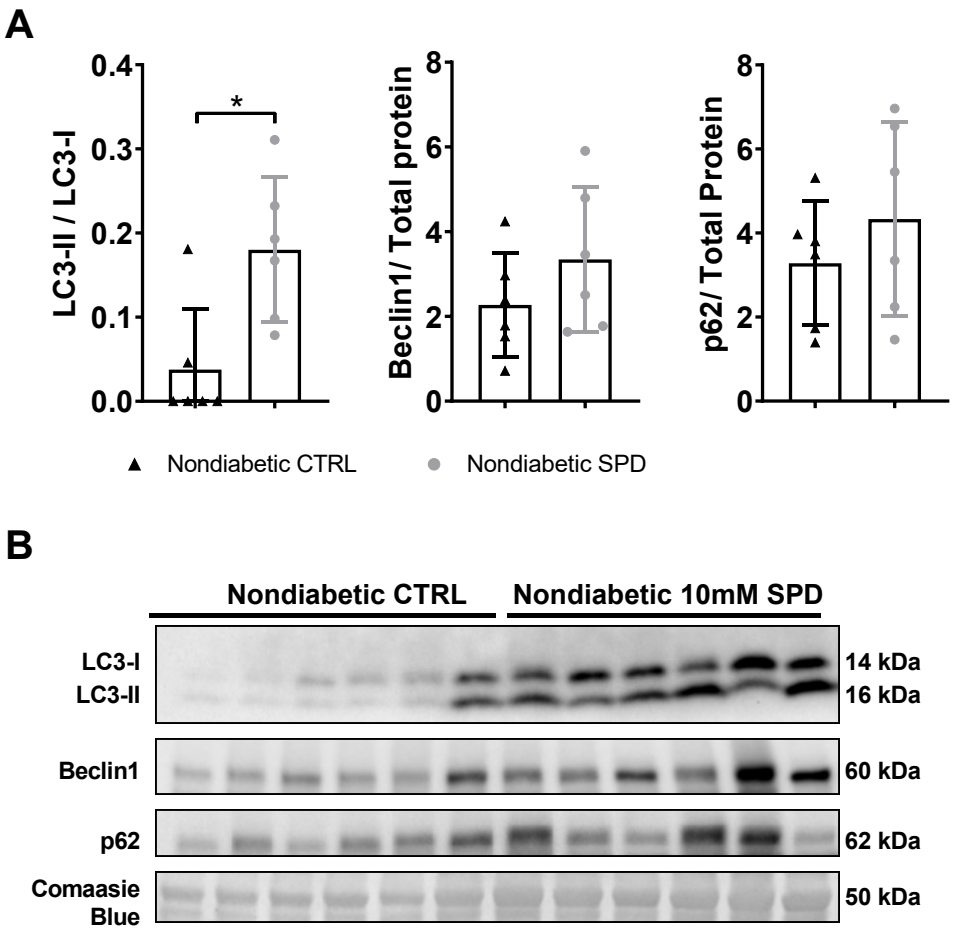

Figure 49: Analysis of autophagy markers in the pancreas of nondiabetic mice after life-long 10 mM spermidine treatment . Nondiabetic mice (BG < 200mg/dl, 35 weeks of age). Samples were probed for (A) LC3, Beclin1, p62 and stained with coomassie blue for total protein as shown in the (B) image from immunoblots. n= 6 for each group. Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis. p* < 0.05.

We then tested the effect of spermidine treatment on pancreatic autophagy markers in diabetic mice. We found that spermidine treatment increased the LC3-II to LC3-I ratio and Beclin1 levels, while it did not change p62 levels (Fig. 50A-C). When mice were analyzed according to the age of diabetes onset, we showed that spermidine treatment specifically increased the autophagy markers by elevating the Beclin1 levels and by reducing the p62 levels in late-onset SPD mice (Fig. 50E and F). Spermidine treatment increased the LC3-II to LC3-I ratio only in early-onset SPD mice (Fig. 50D). Representative images are shown in Fig. 50G.

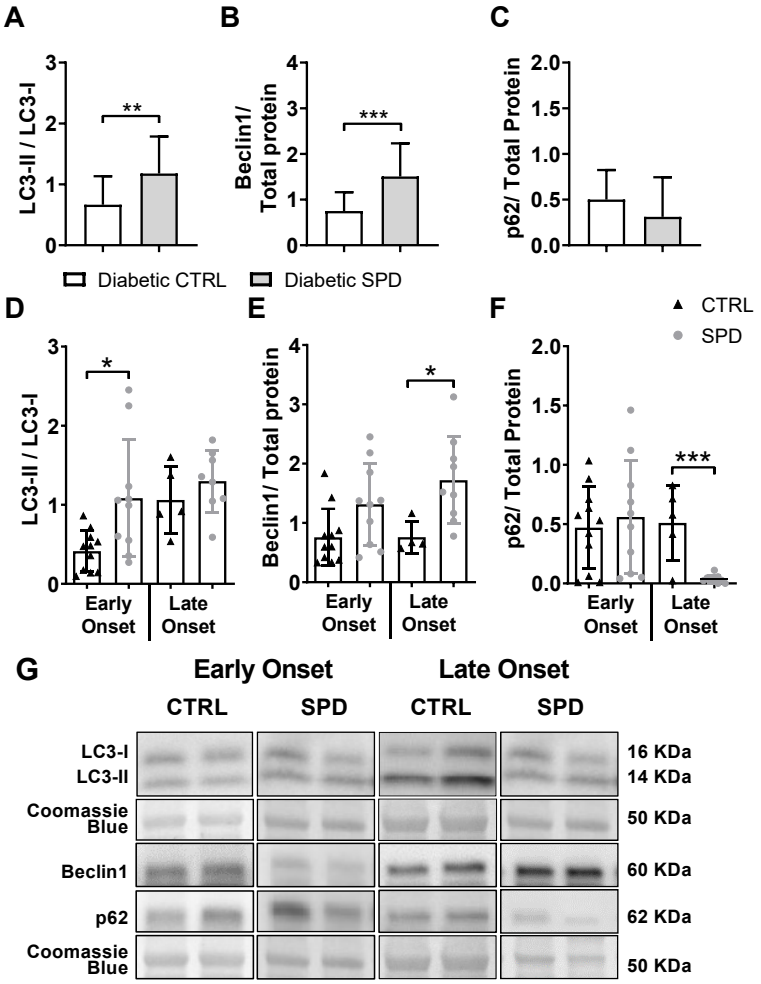

Figure 50: Analysis of autophagy markers in the pancreas of diabetic mice. Mice are grouped as early-onset diabetic (< 21 weeks of age), late-onset diabetic (≥ 21 weeks of age) and nondiabetic (35 weeks of age). Samples were probed for LC3, Beclin1, p62 and stained with coomassie blue for total protein in western blot. (A- C) diabetic CTRL (n=16-17) vs diabetic SPD (n=18-19), (D-F) early-onset CTRL (n=11) vs early-onset SPD (n=10) and late-onset CTRL (n=4-5) vs late-onset SPD (n=8-9). (G) Representative immunoblot images. Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used as statistical analysis to compare CTRL and SPD groups. $p^* < 0.05$, $p^{**} < 0.01$, $p^{***} < 0.001$.

Similar to the pancreas, in the thymus, we also observed that diabetes onset did not change autophagy markers (Fig. 51A and B). In the thymus, spermidine treatment did not increase the autophagy markers shown by similar levels of the LC3-II to LC3-I ratio, Beclin1, and p62 levels in nondiabetic mice (Fig. 51C-E). Representative immunoblot images are shown in Fig 51F and G.

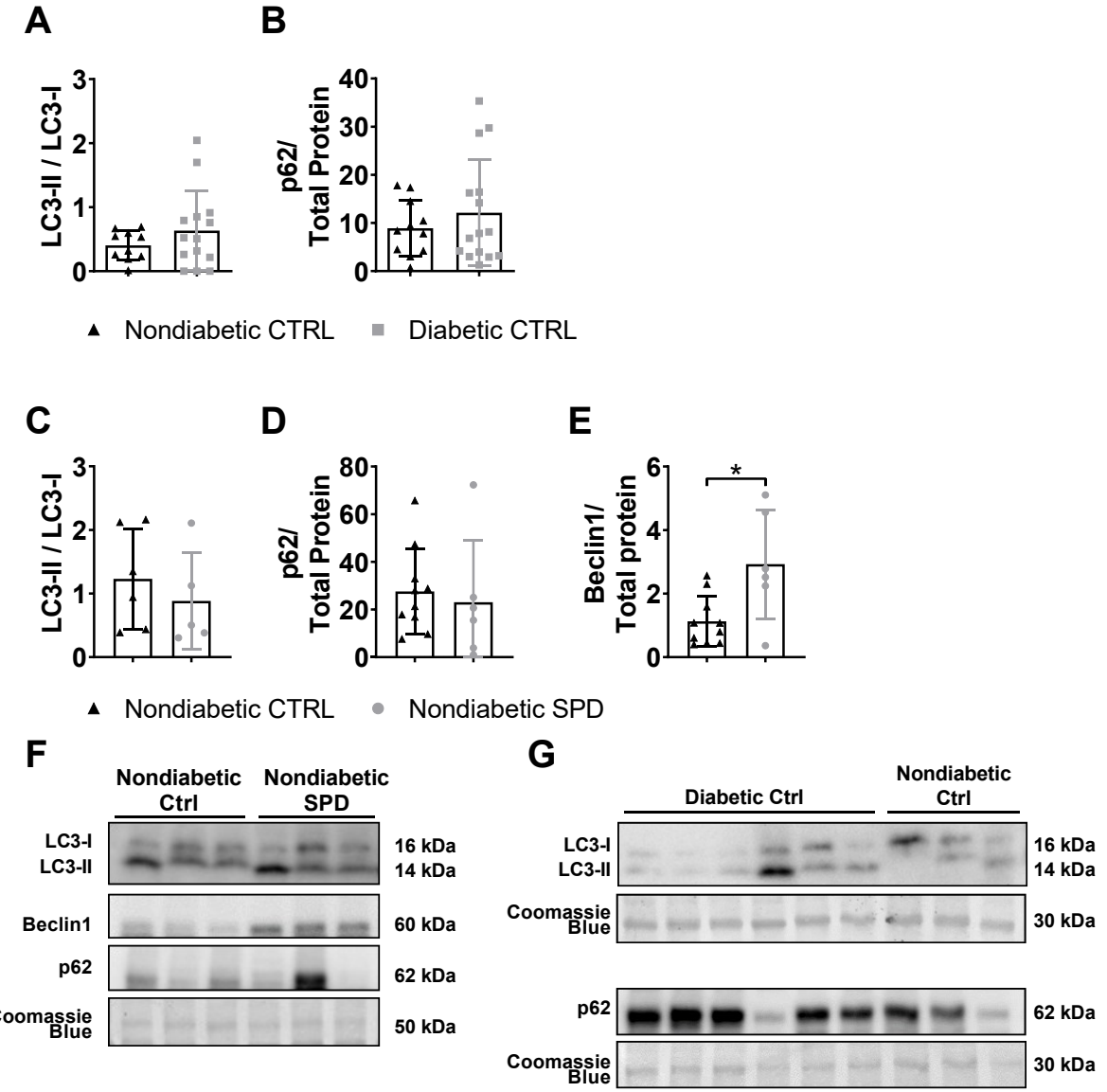

Figure 51: Analysis of autophagy markers in thymus. Samples were probed for LC3, Beclin1, p62. and stained with coomassie blue for total protein in western blot. Mice are grouped as diabetic (BG > 200 mg/dl, 14-35 weeks of age) and nondiabetic (BG < 200mg/dl, 35 weeks of age). (A-B) nondiabetic CTRL (n=10-11) vs diabetic CTRL (n=14-15), (C-E) nondiabetic CTRL (n=6-10) vs diabetic CTRL (n=5-6). (F-G) Representative immunoblot images. Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test was used for statistical analysis. p* < 0.05.

3.4.6. Study-IV: Analysis of Insulin Granule Homeostasis

To get an insight into crinophagy and vesicophagy, we examined insulin granules and autophagy pathway structures in beta cells of pancreatic islets in nondiabetic control and nondiabetic 10 mM SPD mice by electron microscopy at week 35. Representative images are shown in Fig. 52. For the insulin granule analysis, we counted mature, immature, and rod-like granules. We found that lifelong spermidine treatment did not alter the composition of insulin granules in nondiabetic SPD mice compared to nondiabetic CTRL mice (Fig. 53A, 54A, and B). Interestingly, we observed that there were many structures consisting of a fusion of immature and mature or a fusion between mature granules. We found that spermidine treatment did not alter the composition of fusion granules in nondiabetic SPD mice compared to nondiabetic CTRL mice (Fig. 54C). We counted autophagy pathway structures such as phagophores, autophagosomes, and autolysosomes. Although there was a tendency for an increase in phagophores and autophagosomes, spermidine treatment did not significantly affect the number of autophagy pathway structures in nondiabetic SPD mice compared to nondiabetic CTRL mice (Fig. 53C and 54D). We analyzed insulin degradation pathways, which are vesicophagy and crinophagy. Vesicophagy is described as an autophagy-dependent insulin degradation pathway in which insulin granules are digested after the formation of a double membrane around the vesicle. Crinophagy is also autophagy-dependent, but different than vesicophagy crinophagic bodies have a single membrane. We observed that spermidine treatment did not change vesicophagy and crinophagy in nondiabetic SPD mice compared to nondiabetic CTRL mice (Fig. 53D and E).

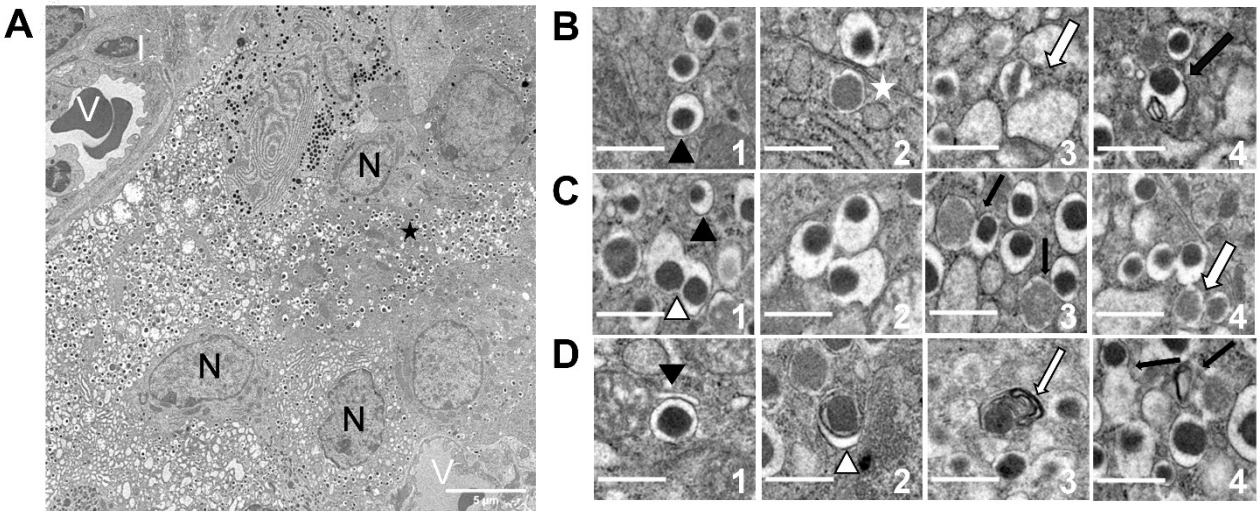

Figure 52: Analysis of insulin granules by electron microscopy. (A) A stiched micrograph including 25 single micrographs (V: vessel, N: nucleus, I: immune cell), scale bar: 5 µm, (B) type of insulin granules (B1) black arrowhead indicates mature granules with a condensed core containing insulin, (B2) white asterisk marks immature transforming granules with a white halo, (B3) white arrow indicates rod-like insulin granules – crystallized structure surrounded by a bright halo, (B4) black arrow shows mature granules with an acentric dark core and membrane inclusions (membrane stacks) inside the bright halo area, (C) type of granule fusions, (C1) black arrowhead indicates mature granules, white arrowhead indicates fusion of mature granules-bright surrounding halo of both granules is fused, (C2) 3 mature granules show a fusion of their surrounding membranes, (C3) black arrows indicate fusion of transforming immature granules with mature granules, membrane of both types of granules is open, (C4) white arrow indicates cluster of immature granules – fusion of the white surrounding halo, (D) autophagy pathway structures, (D1) black arrowhead indicates a phagophore (dilated membrane) enwrapping a mature granule with a dark core, (D2) white arrowhead marks autophagosome (double bilayer) surrounding a mature granule, (D3) autolysosome: white arrow indicates enclosed digested granule without a halo; membrane stacks within the autolysosome, (D4) crinophagy, black arrows indicate a fusion of mature granules with electron-bright lysosomes. (B-D) Scale bar: 0.5 µm.

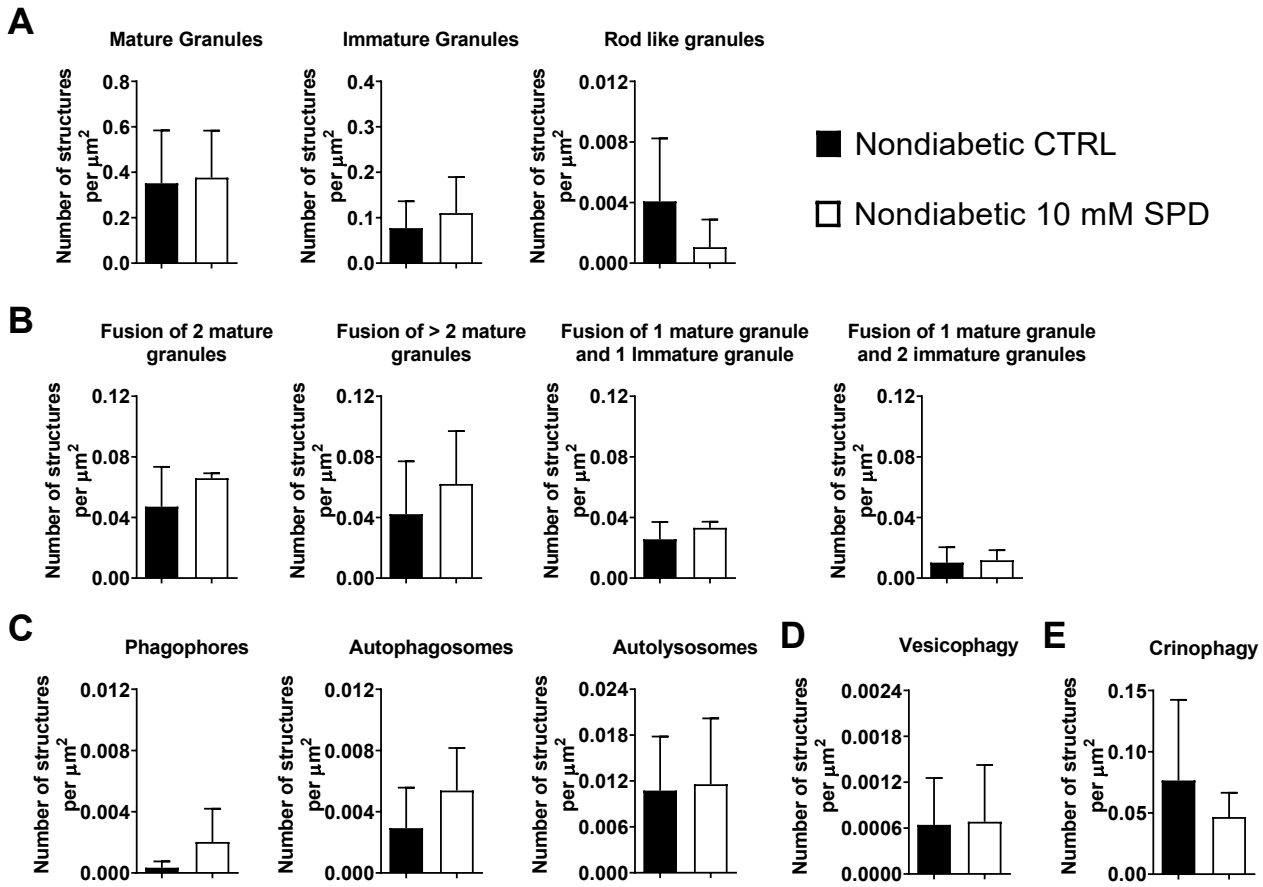

Figure 53: Quantitative analysis of insulin granule pool and autophagy pathway structures. (A) Number of types insulin granules, (B) number of types of insulin granule fusions, (C) number of autophagy pathway structures, (D) number of vesicophagy (insulin granules enwrapped in autophagosomes and autolysosomes), and (E) number of crinophagy (insulin granules enwrapped in lysosomes) were shown as per µm^2. Mice with blood glucose levels lower than 200 mg/dl in two consecutive measurements were determined as nondiabetic at 35 weeks of age. Each bar represents the average of 3 islets from one mouse; min. area of 332 µm^2 and max. area of 2272 µm^2 from each islet was analyzed. Black bar shows nondiabetic CTRL mice (n=3) and open bar shows nondiabetic 10 mM SPD mice (n=3). Data is shown as mean ± SD. The Mann-Whitney U test or the unpaired Student´s t-test were used as statistical analysis.

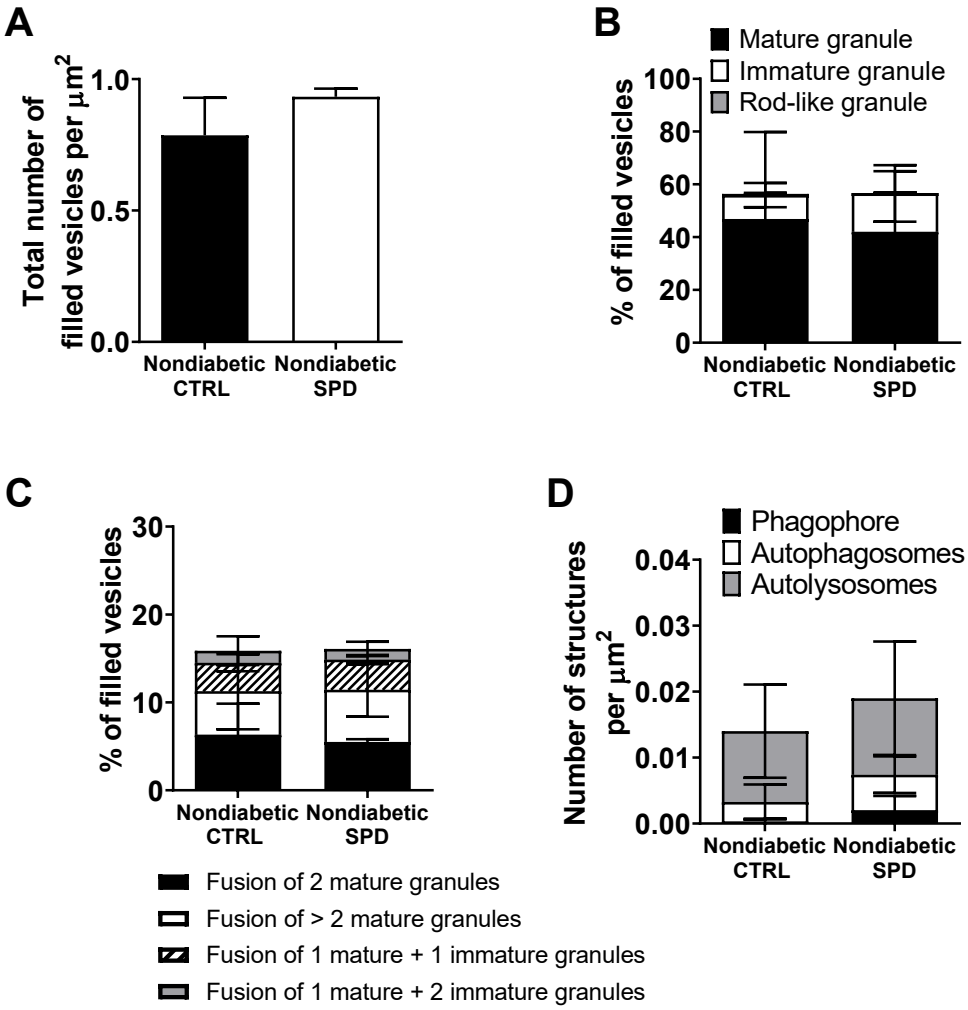

Figure 54: Analysis of the insulin granule pool. (A) Total number of filled vesicles per µm^2, (B) insulin granule types normalized to filled vesicles, (C) type of insulin granule fusions normalized to filled vesicles, and (D) number of autophagy pathway structures per µm^2. Each bar represents the average of 3 islets from one mouse; min. area of 332 µm^2 and max. area of 2272 µm^2 from each islet was analyzed. Black bar shows nondiabetic CTRL mice (n=3) and open bar shows nondiabetic 10 mM SPD mice (n=3). Data is shown as mean ± SD. The unpaired Student´s t-test or the two-way ANOVA with Sidak´s post-hoc test was used as statistical analysis.

4. Discussion

Autophagy was initially suggested to be linked to T1D via genome-wide association studies [249,250]. Following knock-out studies showed that autophagy is indispensable for the maintenance of functional beta cells [245,246]. Furthermore, subtypes of selective autophagy such as crinophagy, and vesicophagy play an important role in maintaining beta cell function [220,225]. Spermidine is a naturally occurring polyamine and an autophagy-inducing agent. It was shown to promote cardioprotection and improve cognitive function in aging mice via autophagy [233,235]. Additionally, spermidine was shown to affect immune cell populations such as Tregs, CD8+ T cells, and B cells [231,232,241,305]. To the best of our knowledge, no study investigated the direct impact of autophagy induction by spermidine on T1D in NOD mice. Therefore, this thesis aimed to get a deeper insight into the effect of autophagy induction on beta cell health and immune cell populations in NOD mice.

The first step was to establish the pharmacokinetics of oral spermidine in different tissues of NOD mice. In previous studies, spermidine supplementation in different strains of mice showed that 3 mM spermidine in drinking water is able to achieve increased endogenous spermidine concentrations and induce autophagy in heart and brain tissue [233,235]. Also, even higher enrichment with spermidine (up to 30 mM in drinking water), was also generally well tolerated [239]. So far, spermidine was not tested in NOD mice; thus, we set up a dose-response study in male NOD mice. Male NOD mice have lower diabetes incidence compared to their female counterparts [15]. Therefore, the use of female NOD mice is the international standard for prevention and intervention studies. Thus, we employed male NOD mice for short-term studies to assess the pharmacokinetics of spermidine. In other mice strains, no sex differences in spermidine metabolism have been described. Thus, the use of male NOD mice enabled us to avoid possible strain-specific differences. For study-I, we chose 1 mM as the lower concentration, 3 mM as the middle concentration, and 10 mM as the higher spermidine concentration for 4 weeks of treatment. The main aim was to assess the tolerability of spermidine supplementation in NOD mice and to assess dose-response relationship of polyamine levels in different tissues. We noticed that mice tolerated all spermidine concentrations well based on their food and water

consumption and body weight development which were similar to control mice. With this study, we also observed that treatment with higher spermidine concentration was associated with increased tissue concentrations of spermidine. In contrast to increased concentrations of spermidine in other tissues, no changes in spermidine tissue concentration were observed in the pancreas. It is well-known that compared to other tissues, spermidine concentrations in the pancreas are relatively high [323]; therefore, spermidine treatment might not lead to further elevation of physiologic higher baseline spermidine levels.

Another read-out of the changes in the polyamine pool is the reduced levels of putrescine. Putrescine is generated from ornithine by ornithine decarboxylase, which is then converted to spermidine by spermidine synthase. In our study, as expected, we observed that spermidine treatment led to reduced tissue concentrations of putrescine. This is potentially caused by negative feedback inhibition of increased exogenous spermidine [324]. According to this mechanism, ornithine levels are expected to be higher in spermidine-treated mice. However, we could not observe a higher ornithine level, possibly due to insufficient sample size to detect the difference.

In summary, we showed that 3 mM spermidine is well tolerated in NOD mice, and we achieved higher spermidine concentrations in some of the relevant tissues. But we have to acknowledge here that, similar to other studies, we were not able to measure higher spermidine concentrations in the pancreas, most likely due to higher turnover. By combining readouts of spermidine and putrescine, we concluded that, in concordance with the literature, we were achieving a higher turnover of polyamines in relevant tissues. Thus, based on our study-I, we decided to use 3 mM spermidine in drinking water to test the hypothesis that T1D can be prevented in NOD mice.

In study-II, we examined the effect of 3 mM spermidine on diabetes development in female NOD mice. In this study, mice either received untreated drinking water or drinking water enriched with 3 mM spermidine ad libitum from the age of 4 weeks until diabetes onset or the age of 35 weeks. Unchanged water and food consumption, normal development of body weight, and overall well-being of the mice demonstrated the tolerance to long-term treatment with 3 mM enriched drinking water in NOD mice. Other than expected, spermidine treatment did not change diabetes incidence in this

study in our female NOD mice. At the end of the study, two control and four spermidine mice remained nondiabetic at week 35 and were analyzed as nondiabetic survivors. Next, we checked whether 3 mM spermidine treatment was able to reduce insulitis in pancreatic islets. In line with the lack of impact on diabetes incidence, we also did not observe any difference in insulitis levels between control and spermidine-treated groups in nondiabetic survivors and diabetic mice. Previous studies have shown that spermidine might affect several immune cell populations, such as CD4+ T cells, Tregs, CD8+ T cells, B cells, and macrophages in different mouse models. All these cell types might be relevant for T1D pathogenesis in NOD mice. Thus, we decided to examine different immune cell populations, from T cells to B cells and macrophages to dendritic cells in blood, spleen, and pLN.

Interestingly, spermidine treatment did not affect any immune cell population in nondiabetic survivors. When analyzing the same populations in diabetic mice, we found that the proportion of total CD4+ T cells and Tregs were reduced by spermidine treatment in the spleen of diabetic mice. Additionally, the proportion of total CD8+ T cells in the spleen of diabetic mice was also reduced by spermidine treatment. Tregs are known to have an anti-inflammatory role in T1D [88], and CD8+ T cells are known to have a pro-inflammatory role in T1D [10,11,65–67]. Based on these observations, it is hard to conclude whether 3 mM spermidine is pro-inflammatory or anti-inflammatory in NOD mice. Furthermore, we observed that spermidine led to an increased proportion of NKT cells in the blood of diabetic mice. NKT cells have been shown to contribute to immune tolerance in T1D [121,122]. However, our results are inconclusive regarding NKT cells. In addition to immune cell analysis, measurement of plasma cytokine levels displayed that 3 mM spermidine treatment did not significantly affect any cytokine level in NOD mice.

In summary, in our study with 3 mM spermidine, we observed a trend towards less diabetes incidence by spermidine treatment; however, the difference between control and spermidine groups was not significant. The reasons for our findings could be explained by the too-small sample size or by spermidine concentrations that were inadequate to protect beta cells. Therefore, we considered treating with higher spermidine concentrations. Previous studies investigated several mouse models

treated with higher than 3 mM spermidine in drinking water e.g. 5 mM, 6 mM, 30 mM, and 100 mM [235,237,239,325–327]. None of these studies reported side effects of high doses of spermidine. Thus, we decided to use 10 mM spermidine in drinking water in our next studies. As the next step, we decided to increase the sample size of our pharmacokinetic investigations, and we restudied male NOD mice with 10 mM spermidine added to drinking water.

In study-III, we had 10 control mice and 12 spermidine-treated mice. The study design was similar to study-I. As shown in study-I, we observed that 10 mM spermidine was well tolerated, and it did not change water and food consumption and body weight development. Although study-I showed that there is a correlation between increasing spermidine concentration supplementation and increasing endogenous spermidine concentration in some tissues, increased sample size led to a better understanding of the effect of 10 mM spermidine on polyamine levels: Similar to study-I, we observed that 10 mM spermidine led to increased spermidine levels in the spleen, thymus and heart and reduced the putrescine levels in the respective organs. As mentioned before, in relation to spermidine biosynthesis from ornithine, we observed a trend for higher tissue concentrations of ornithine in all tissues, as expected. In this study, we were able to measure the polyamine levels in the pLN as well. Similar to other immune-cell-relevant tissues, we observed that 10 mM spermidine led to increased spermidine and reduced putrescine levels in the pLN. Concurrent with study-I, we did not observe a change of tissue concentration of spermidine or putrescine in the pancreas. It is worth noting that we only measured the polyamine tissue concentrations of the total pancreas, not in small endocrine tissue. Overall, our observations in study-III were in line with the results of study-I.

Based on unchanged body weight, and food/water consumption in male mice in study-III with 10 mM spermidine, we decided to conduct study-IV with 10 mM spermidine in female NOD mice as a prevention study. In a recent toxicity study, food supplemented with 60 mg/kg spermidine (~3 mM) in wild-type mice was able to modulate polyamine levels with similar body weight development, water and food consumption compared to untreated mice but with a minor increase in kidney weight compared to untreated mice [328]. Thus, to exclude a toxic side effect of high

spermidine supplementation, we also measured the weight of the kidney and heart in our experiments with 10 mM spermidine. We did not observe any difference between the control and 10 mM spermidine groups in kidney and heart weight, as shown in Fig. 22.

In study-IV, similar to study-II, we used spermidine-enriched drinking water. Here, the mice were given either untreated or 10 mM spermidine-containing drinking water after weaning from the age of 4 weeks until diabetes onset or until the age of 35 weeks. Our aim was to check the effect of 10 mM spermidine on the progression to diabetes as the primary outcome. Diabetes diagnosis was performed as in study-II. Contrary to the 3 mM spermidine treatment, here we observed an increased diabetes incidence in 10 mM spermidine-treated female mice after 21 weeks of age. Based on this observation, we decided to divide our NOD mice into “early-onset” (< 21 weeks of age) and “late-onset” (≥ 21 weeks of age), and we performed statistical analysis with these groups. The increased diabetes incidence observed with 10 mM spermidine contradicts our initial hypothesis and our findings with 3 mM spermidine. In the following section, we highlight several possible mechanisms.

The first mechanism which might explain the higher diabetes incidence could be the role of spermidine in eIf5a hypusination. Hypusination is a posttranslational modification where spermidine is used as a substrate. In hypusination, first deoxyhypusine synthase transfers 4-aminobutyl moiety of spermidine to an ε-amino group of a specific lysine residue in the eIF5A precursor protein, which generates deoxyhypusine eIF5A [329]. Then, deoxyhypusine hydroxylase converts deoxyhypusine eIF5A to hypusine eIF5A [329]. As spermidine is used as a substrate for eIF5A hypusination, exogenously administered spermidine might have led to increased eIF5A hypusination in our study, as described before [305]. Previous studies have shown that eIF5A hypusination is associated with T1D. In line with our findings with 10 mM spermidine, previously, it was shown that depletion of polyamines leads to reduced levels of eIF5A hypusination and that leads to delayed diabetes onset in NOD mice [330]. Additionally, in pancreas samples from diabetic NOD mice and from people with T1D, higher levels of hypusinated eIF5A were detected in comparison to controls [331], suggesting that increased levels of hypusinated eIF5a are associated with T1D.

Similarly, inhibition of eIF5A led to an increased number of Tregs in the pancreas, pLN and spleen and delayed diabetes onset in humanized type 1 diabetic mice [332]. In summary, others have shown that inhibition of eIF5A hypusination is protective against T1D. Thus, higher diabetes incidence in 10 mM spermidine study might be explained by spermidine-induced elevated eIF5A hypusination. Unfortunately, we did not measure hypusination of eIF5A in our study, however, we cannot exclude the effect of 10 mM spermidine on eIF5A hypusination.

The second mechanism which might explain the higher diabetes incidence could be the dose of spermidine. A high dose of spermidine might have a direct cytotoxic effect on beta cells. Nevertheless, this is unlikely as others reported no direct cytotoxicity of spermidine, and our histology data do not indicate direct toxic effects. Additionally, a high dose of spermidine could put further stress on beta cells, leading to a more pronounced number of immune cell infiltration into the islets. Unfortunately, we did not study beta cell stress directly; therefore, we cannot rule a direct toxic effect out completely. However, we have assessed the immune cell infiltration in the islets of Langerhans by H&E staining. Interestingly, analysis of insulitis level indicated a trend for higher grade inflammation in 10 mM spermidine-treated mice. These differences did not achieve statistical significance, this could be explained by the small sample size or by a lack of sensitivity of the H&E staining for insulitis assessment. As H&E staining does not allow full characterization of immune cells, we were not able to detect any change in the composition of the immune cell infiltrate in our experiments.

The third mechanism which might have led to an increased diabetes incidence could be the increased number of particular immune cells, as described in our flow cytometry experiments. We performed an in-depth analysis of immune cells in the blood, spleen, and pLN to further investigate the effect of a high dose of spermidine. We found an increased proportion of effector memory and central memory CD8+ T cells in the pLN of spermidine-treated diabetic NOD mice. In nondiabetic survivors, there was a trend for reduction in these cell types in the pLN of spermidine-treated mice. This difference in effector and central memory CD8+ T cells between diabetic and nondiabetic survivors indicate that spermidine might have led to higher diabetes incidence by increasing the proportion of these pro-inflammatory CD8+ T cell types [333]. The effect

of spermidine on CD8+ T cells might be mediated via autophagy induction. Autophagy is suggested to affect CD8+ T cells by removing accumulated damaged organelles such as mitochondria and/or by inducing the switch from glycolysis to mitochondrial respiration [334,335]. A previous study reported that spermidine treatment rejuvenates the effector memory CD8+ T cells and central memory CD8+ T cells in elderly mice after vaccination in an autophagy-dependent manner [231]. Furthermore, metformin and rapamycin have been shown to lead to an increased number of CD8+ T cells, probably via autophagy induction [334]. Another study with rapamycin and IL-2 showed that the combination treatment increased IFN-ɣ+ CD8+ T cells in NOD mice with the consequence that diabetes could not be reversed [336]. Additionally, autophagy deficiency in CD8+ T cells was shown to lead to compromised memory formation [231,294]. Taking the above-reported effects of spermidine into our results, it is probable that spermidine treatment led to increased diabetes incidence by increasing the number or function of CD8+ T cells.

We also analyzed several subsets of CD4+ T cells, such as effector memory CD4+ T cells and central memory CD4+ T cells. We observed a trend towards an increased proportion of effector memory and central memory of CD4+ T cells in pLN of diabetic mice. On the contrary, in nondiabetic survivors, there was a trend for reduction in these cell types in the pLN of spermidine-treated mice. This difference in effector and central memory CD4+ T cells between diabetic and nondiabetic survivors indicate again that spermidine might have led to higher diabetes incidence by increasing the proportion of these pro-inflammatory CD4+ T cell types [337]. On the other hand, in spleen and blood of both nondiabetic survivors and diabetic mice, 10 mM spermidine treatment led to a small increase in effector memory and central memory CD4+ T cells without statistical significance.

The increased proportions of effector memory and central memory T cells were accompanied by reduced proportions of naïve CD4+ and CD8+ T cells in the spleen and pLN of both nondiabetic survivors and diabetic mice. However, in peripheral blood analysis of nondiabetic survivors and diabetic mice, we observed that spermidine significantly elevated proportions of naïve CD4+ T cells and naïve CD8+ T cells. These

distinct differences were not seen in all organs investigated here but support the concept that a high dose of spermidine overall shifts towards inflammation.

In addition to the effect on naïve, effector, and central memory CD4+ T cells, another significant effect of spermidine was observed in classical types of Tregs (CD4+ FoxP3+ CD25+). There was a visible trend towards elevation in FoxP3+ CD4+ T cells, CD4+ Tregs, and CTLA4+ CD4+ Tregs in the blood, spleen, and pLN of spermidine-treated diabetic mice and spermidine-treated nondiabetic survivors compared to their controls. In contrary to the pro-inflammatory role of CD8+ T cells, typical Tregs (CD4+ FoxP3+ CD25+) contribute to tolerance in T1D by suppressing inflammatory T cells [89,94]. It is important to highlight that the increased suppressive capacity of Tregs can result from an increased number of Tregs or a change in the suppressive function. Thus, an increased number of Tregs can be recognized as an indicator of tolerance, and it has been related to diabetes prevention. Interestingly, the effect of spermidine was diametrical when Treg populations were compared between early and late-onset mice. Spermidine treatment led to an increased proportion of Tregs in early-onset, which did not change diabetes incidence. Similar to our findings, a recent *ex vivo* study also showed that spermidine treatment increased the proportion of FoxP3+ CD4+ T cells but did not increase the suppression capacity of isolated FoxP3+ CD4+ T cells [232]. Additionally, the same authors showed that spermidine-treated mice were protected from colitis in a transfer model, and it was suggested that the protection was achieved by an increased number of FoxP3+ CD4+ T cells [232]. The discrepancy in their study might be explained by different subtypes of Tregs with different suppressive functions. In our study, we found an increased number of Tregs with no change in diabetes incidence, which can be explained by either insufficient suppression activity of the Tregs or by the elevated memory CD8+ T cells which are considered to heighten an inflammatory process. Future studies should clarify the impact of spermidine on the suppression capacity of Tregs in disease models, including the NOD mice.

Interestingly, while 10 mM spermidine treatment increased the proportion of Tregs in early-onset mice, spermidine treatment reduced the proportion of Tregs in late-onset mice. The increased diabetes incidence in NOD mice in late-onset group, therefore could be explained by the reduced number of Tregs and/or by the elevation of pro-

inflammatory CD8+ T cells, Similar to our findings, other studies also reported that a reduced proportion of Tregs increases diabetes incidence in NOD mice [338,339]. Interestingly, in a cancer model, spermidine can reduce tumor growth by generating a differential effect on CD8+ T cells and Tregs, respectively. This spermidine effect is only seen in the presence of CD8+ T cells and is explained by the autophagy-dependent reduction of Tregs [241]. At the current stage, we cannot explain the differences in Tregs between early and late-onset groups. One potential explanation for the differential impact of spermidine on T cells could lie in the heterogeneity of the disease, as also observed in human T1D. [340]. Accordingly, genetic predisposition in early-onset mice might have led to a more pronounced insulitis and an increased diabetes incidence. Furthermore, the long duration of spermidine treatment in late-onset mice might have unknown effects on beta cell function and/or on the immune system. Therefore, it might be worth testing the effect of short-term spermidine treatment. Further studies investigating various duration of spermidine treatment in different age groups of pre-diabetic and diabetic mice might characterize potential underlying mechanisms.

To assess the impact of spermidine on other immune cells, we also checked NKT cells, NK cells, B cells, myeloid cells, and DCs. We observed that 10 mM spermidine treatment did not change the proportion of NKT cells in nondiabetic survivors and diabetic mice. To our knowledge, there is also no literature regarding the connection between autophagy and NKT cells. Based on our data, we can only speculate that spermidine does not have any effect on NKT cells in NOD mice.

Spermidine treatment led to an increased proportion of NK cells. The change was significant in the blood of nondiabetic survivors, and it was a trend in other tissues in both diabetic and nondiabetic survivors. The role of NK cells in T1D pathogenesis is not clear. While most of the studies showed that NK cells are pro-inflammatory [112,114,115], a few studies showed that NK cells could be protective in NOD mice [116,117]. Others also showed that NK cells are not essential for the development of T1D [106]. The literature is also limited regarding the connection between NK cells and autophagy. Previous studies showed that autophagy induction leads to survival and memory function of NK cells [306,308]. In our study, we also observed that spermidine

treatment led to an increased proportion of NK cells, possibly via autophagy induction. Based on our findings, we can speculate that the increased NK cell proportion might contribute to the increased diabetes incidence in spermidine-treated mice.

In B cells, there was a trend towards a reduced proportion of B cells in diabetic mice, whereas there was a trend towards an increased proportion of B cells in nondiabetic survivors. Spermidine-induced-increased proportion of B cells in nondiabetic survivors is in line with the limited knowledge from the literature on B cells. The connection of autophagy and B cells was reported by a recent study where B cells were elevated upon spermidine treatment in aged mice after vaccination leading to better vaccination response [305].

We analyzed also myeloid immune cells such as monocytes, macrophages, and neutrophils. The proportion of neutrophils and monocytes was similar between control and spermidine-treated mice, both in nondiabetic survivors and diabetic mice. It is worth noting that a recent report showed a potential effect of ketamine/xylazine injection on neutrophil population [341]. In this study, pre-treatment of ketamine/xylazine before induction of pancreatitis reduced the proportion of neutrophil population in this rodent model of acute pancreatitis [341]. While we did not assess the neutrophil population in the pancreas, the measured neutrophil population in the blood falls within the expected range in mice [342]. Furthermore, we, and the authors in above-mentioned paper, used ketamine/xylazine immediately before sacrificing the mice. Thus, we believe that the short time exposure of a few minutes is unlikely to impact our results, however, we cannot exclude an effect on neutrophil function. Macrophages and M2 macrophages were elevated in spermidine-treated groups both in nondiabetic survivors and diabetic mice without reaching statistical significance. Our finding correlates with the previous studies, as described in the earlier chapters, which have shown that spermidine treatment elevates the proportion of M2 macrophages in several inflammatory disease models [238,317,318] and in a mouse model for the autoimmune disease multiple sclerosis [239,240]. Our findings confirm that spermidine can modulate the macrophage population. Nevertheless, we could not observe any positive impact of the increased proportion of M2 macrophages on diabetes incidence.

In literature, spermidine was shown to modulate dendritic cells in mouse models. A recent study has shown that spermidine can suppress inflammatory DCs in psoriasis in an autoimmune disease mouse model [312]. We also showed that spermidine treatment changes the proportion of DCs in NOD mice. In nondiabetic survivors, we observed an increased proportion of pDCs and CD8+ cDCs in blood and a reduced proportion of pDCs and an increased proportion of CD11b+ cDCs in spleen. Based on the literature, we know that pDCs and CD8+ cDCs have a regulatory function, while CD11b+ cDCs have a cytotoxic role in T1D [141,145,146,148]. We did not analyze DC population in the pancreatic islets; however, our data illustrated that 10 mM spermidine treatment might have increased the regulatory DCs in the blood and the inflammatory DCs in the spleen. Further studies examining the direct impact of spermidine on subsets of DCs might help to uncover the inflammatory and regulatory role of DCs in T1D. On the other hand, regulatory DCs were shown to regulate the suppressive function of Tregs by indoleamine 2,3- dioxygenase (IDO) production [343]. Thus, the effect of spermidine on the axis of Tregs and pDCs can be further elucidated to understand the effect of spermidine on immune tolerance in T1D.

In addition to the analysis of immune cell populations, we also measured several cytokines (IFN-ɣ, IL-1β, IL-6, IL-5, IL-10, TNF-α, and IL-2) in diabetic NOD mice and nondiabetic survivors in study-IV. All of them did not show any difference, except IL-6 which was significantly reduced in 10 mM spermidine-treated nondiabetic survivors. The significance of this finding is unclear since the role of IL-6 in T1D pathogenesis is currently being challenged [344].

Next, we examined the effect of spermidine treatment in the thymus by analyzing different subsets of thymocytes and positive selection by TCR-β and CD69 [321]. It was proposed that thymic selection in NOD mice was disrupted [280–282], and autophagy induction was suggested to improve thymic selection [283,284]. Thus, we hypothesized that spermidine treatment might improve thymic selection and lead to reduced autoimmune T cells in an autophagy-dependent way. In our study, we found that 10 mM spermidine treatment did not change the proportion of thymic T cell subsets and did not alter the positive selection process in the thymus of diabetic NOD mice and nondiabetic survivors. Although we could show a trend for an elevation of spermidine

levels in the thymus by spermidine treatment, we could not observe increased autophagy levels in the thymus of spermidine-treated nondiabetic survivors. Of note, autophagy levels in the thymus of spermidine-treated diabetic mice were not measured. Additionally, our autophagy estimates should be cautiously interpreted as we did not assess autophagy flux using lysosome inhibitors (e.g., leupeptin) [345].

The fourth mechanism which might have led to increased diabetes incidence could be that induction of the autophagy pathway might not lead to protective effects in T1D. As described in chapter 1.3.2, other pharmacological substances which induce autophagy pathways different than spermidine have shown conflicting results in diabetes prevention in NOD mice. For example, rapamycin was not able to prevent diabetes in some NOD studies [271,272], while it was shown to prevent diabetes in other NOD studies [273–275]. These conflicting results might be due to differences in treatment duration and frequency, application route, dosage, and diagnosis of diabetes onset. We were able to test two spermidine doses in two separate NOD studies, in which spermidine could not demonstrate a protective effect.

To understand the relationship between basal autophagy and diabetes in NOD mice, we analyzed the protein markers of autophagy in the pancreas and thymus in diabetic mice and nondiabetic survivors in the absence of spermidine treatment. As described in detail before, it is well known that basal autophagy is required for normal beta bell function; however, the link between stressed beta cells in diabetes and autophagy is not clear yet. Especially, there are not many studies in T1D models investigating the pathophysiological link, except one *in vitro* study in which pro-inflammatory cytokines led to reduced autophagy levels [256]. Based on the literature in T1D and T2D models, we hypothesized that autophagy levels might be lower in diabetic NOD mice compared to nondiabetic survivors. Unexpectedly, we could not observe a significant difference in all autophagy markers between diabetic mice and nondiabetic survivors. We could only demonstrate that the pancreatic LC3-II/LC3-I ratio was higher in diabetic mice compared to nondiabetic survivors, which might hint towards either activated autophagy or blocked autophagy. Only the detection of a change in at least two autophagy markers can lead to a meaningful conclusion about autophagy levels. Since we could not observe any difference in other autophagy markers (p62 and Beclin1),

our findings are inconclusive regarding the relationship between basal autophagy levels and diabetes pathophysiology in NOD mice. In summary, further studies should investigate the autophagy levels in the pancreas and especially in the isolated islets of different age groups of diabetic and nondiabetic NOD mice.

Next, we analyzed the effect of pharmacological spermidine treatment on beta cell autophagy. Based on the literature, we hypothesized that spermidine should protect beta cells from apoptosis via an autophagy-dependent pathway. Since we observed a significant difference in diabetes incidence in study-IV with 10 mM spermidine, we analyzed the pancreas and thymus. In our 10 mM spermidine study, correlating with higher diabetes incidence at late-onset, spermidine treatment had an age-dependent impact on autophagy markers of the pancreas in diabetic mice. We showed that spermidine increased the autophagy levels in late-onset NOD mice, shown by increased Beclin1 levels and reduced p62 levels. It is worth mentioning that our autophagy estimates should be carefully interpreted since we did not evaluate autophagy flux using lysosome inhibitors (e.g. leupeptin) [345]. We did not directly measure beta cell apoptosis in NOD mice; however, we suggest that increased diabetes incidence by spermidine treatment might be caused by autophagy-induced beta cell apoptosis as it has been shown by other studies in which increased autophagy led to increased apoptosis [346,347]. Beta cell apoptosis was reported both in vitro and in vivo to be driven by rapamycin-induced autophagy [258,348]. It was also reported that combination treatment of rapamycin and IL-2 momentarily reduced C-peptide, indicating reduced beta cell function, in T1D patients [349]. In summary, spermidine treatment might have increased apoptosis levels via an autophagy-dependent pathway.

It is worth mentioning that recently published studies have raised the question of potential effects of ketamine/xylazine injections on autophagy and apoptosis [341,350]. In one study ketamine/xylazine was used before induction of pancreatitis and 6 h before scarification [341]. The authors have shown that pre-treatment with ketamine/xylazine reduced apoptosis and autophagy levels in pancreas [341]. The other study tested long-term exposure of ketamine/xylazine in rats (daily injections for 3 months) [350]. This study showed that daily injections of ketamine/xylazine led to

increased autophagy and apoptosis levels in bladder tissue [350]. In our studies, we used ketamine/xylazine for only a few minutes immediately before the sacrifice of the mice. Additionally, in our mice we observed that the elevated autophagy levels were only detected under specific conditions (e.g. spermidine treatment). Therefore, we conclude that ketamine/xylazine is unlikely to have any significant effect on our autophagy read-out, however, future studies should investigate this topic.

Spermidine-induced autophagy might have modulated insulin granules as well. A recent study described that continuous and long-term autophagy induction might lead to insulin granule digestion via lysosome-vesicophagy axis [225]. Similarly, it was also shown that the inactivation of mTORC1 signaling diminished insulin secretion via autophagy induction and increased the number of autophagic bodies containing insulin granules [226]. Thus, we analyzed insulin granules in pancreatic beta cells by electron microscopy. We analyzed only nondiabetic survivors, as diabetic mice were not expected to have many remaining pancreatic islets. Interestingly, we observed that spermidine treatment did not change the insulin granule pool in pancreatic islets of nondiabetic NOD mice. Additionally, we did not observe a change in crinophagic and vesicophagic vesicles in spermidine-treated nondiabetic mice. Technical restrictions, such as the determination of autophagy flux, might be a reason for not detecting increased crinophagy and vesicophagy. However, we believe that if there was a substantial change in insulin degradation pathways (crinophagy and vesicophagy), this change would have been reflected in the number of insulin granules. Our data is limited to the investigation of solely nondiabetic survivors, which restricts the comparison with the published study examining pre-diabetic mice [225].

Alternatively, spermidine can impact insulin granules through the polyamine pathway. Previous studies imply that increased spermidine levels may improve insulin homeostasis. Earlier, it was shown that the depletion of polyamines reduced insulin content through the inhibition of spermidine synthase. This led to diminished endogenous spermidine levels, which resulted in reduced Ca^{+2} levels and subsequently diminished insulin content [351]. Supplementation of spermidine was able to reverse the Ca^{+2} levels and insulin content [351]. Similarly, an *in vivo* study

demonstrated that reduced spermine and spermidine levels as a result of overexpression of N-acetyltransferase triggered reduced insulin secretion shown by impaired glucose-induced insulin tolerance [352]. Based on the literature, spermidine, as a polyamine, is required for insulin granule homeostasis and supplementation of spermidine might improve this homeostasis. However, in our experiments, we did not observe a difference in the insulin granule pool between the control and spermidine groups.

Conclusion

In conclusion, this thesis provides novel information regarding the effect of spermidine on the pathogenesis of T1D in NOD mice. Previous studies showed that autophagy is vital for the maintenance of functional beta cells under physiological conditions. Autophagy induction by several pharmacological interventions, including spermidine improved T2D in mouse models. Additionally, spermidine treatment was reported to reduce inflammatory milieu in autoimmune models. In this thesis, we demonstrate that long-term supplementation with 3 mm and 10 mM spermidine is tolerable in NOD mice. We report that low-dose 3 mM spermidine treatment did not affect diabetes incidence, and the minor changes in the immune cell pool were inconclusive. Surprisingly high dose 10 mM spermidine treatment increased diabetes incidence in NOD mice. Possible explanations for increased diabetes incidence in 10 mM spermidine treated mice are increased eIF5A hypusination, high dose spermidine relevant cytotoxicity, increased pro-inflammatory T cells, and autophagy-induced apoptosis in beta cells. Our findings suggest that autophagy induction via 3 mM and 10 mM spermidine does not merely result in protection from T1D in NOD mice. Despite basal autophagy being indispensable for beta cell health and immune cells, autophagy induction via pharmacological intervention or genetic manipulation should be carefully assessed in different autoimmune disease models to avoid exacerbation of inflammation.

5. References

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6. Appendix

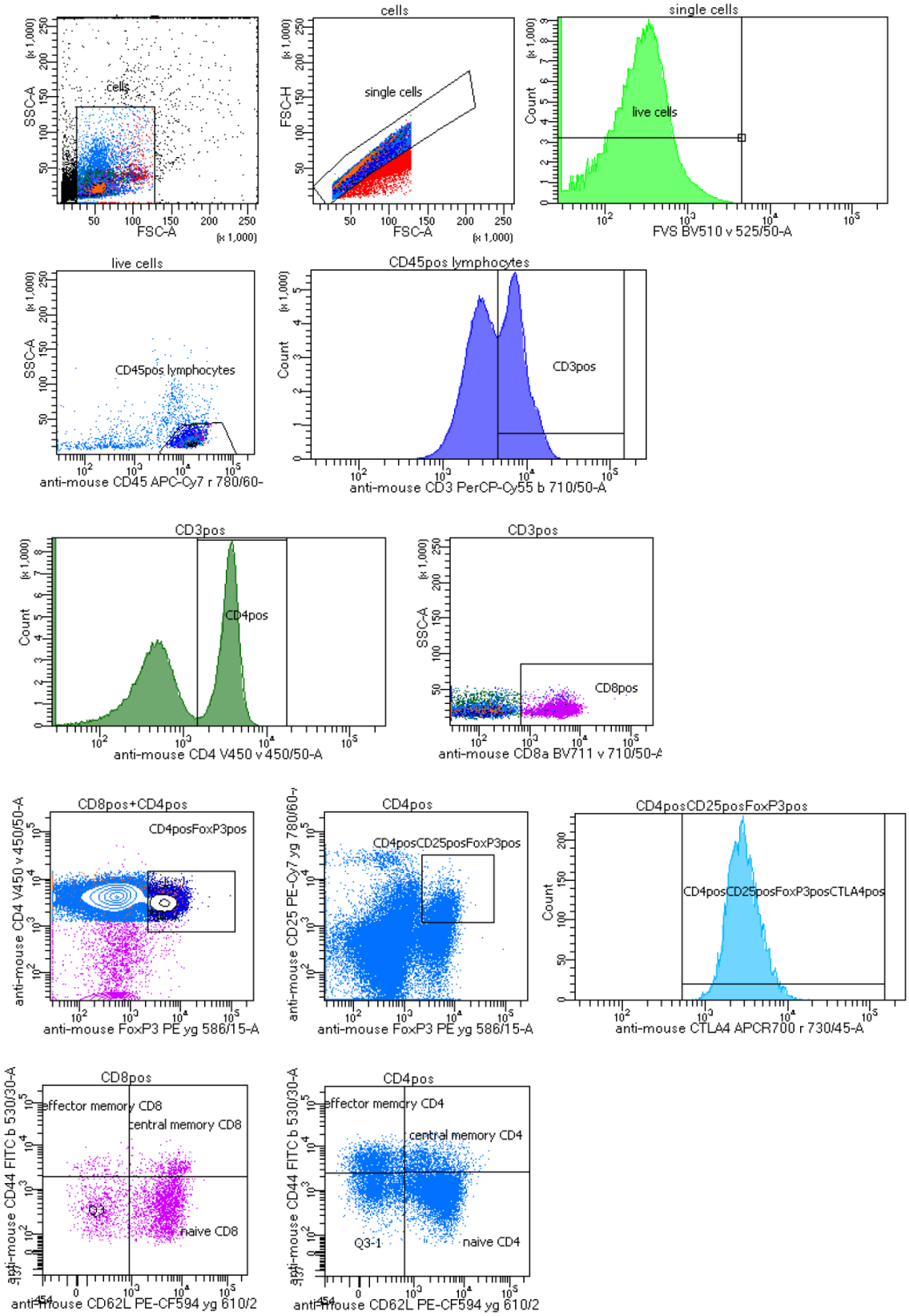
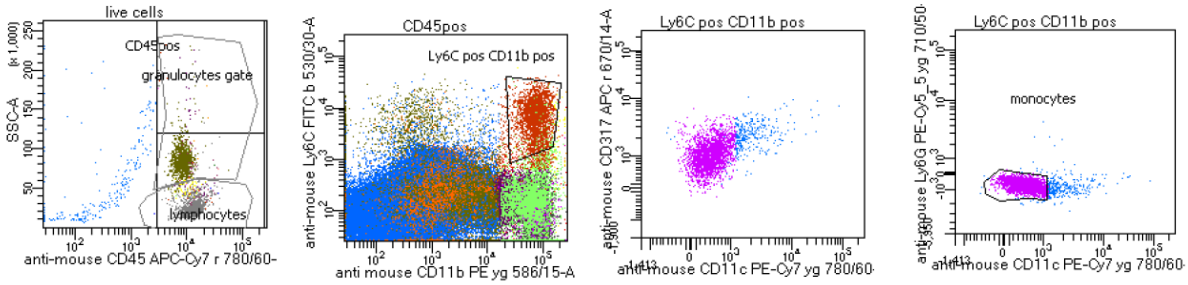


Figure 55: Dot plots of panel A.

CD45+ cells

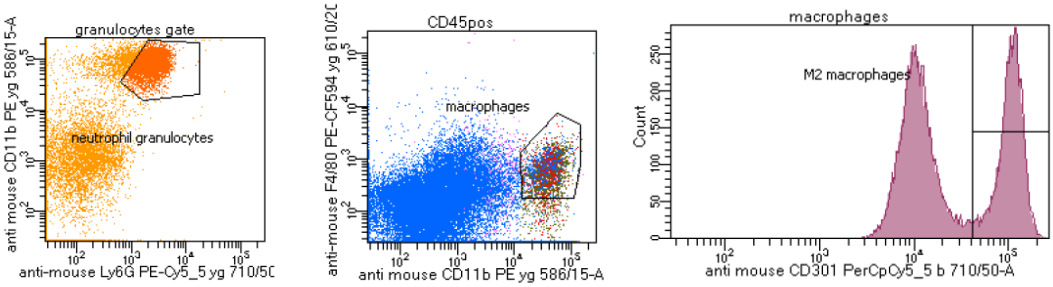
Monocytes
CD11b+ Ly6C+ CD11c (-) CD317 (-) Ly6G (-)



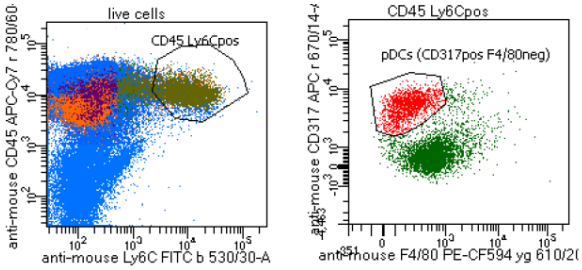
Neutrophil granulocytes
CD11b+ Ly6G+

Macrophages
CD11b+ F4/80+

M2 Macrophages
CD11b+ F4/80+ CD301+



Plasmacytoid dendritic cells (pDCs)
Ly6Chi CD317+ F4/80(-)



CD8+ cDCs: CD45+ CD11c+ CD11b (-) CD8+ CD4(-)
CD11b+ cDCs: CD45+ CD11c+ CD11b+ CD8 (-)
CD11b+ CD4+ cDCs: CD45+ CD11c+ CD11b+ CD4+ CD8(-)

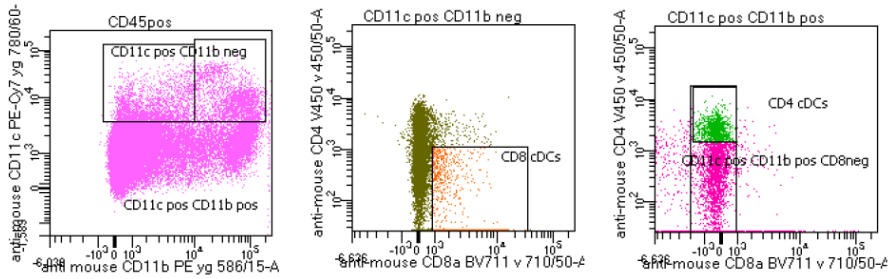
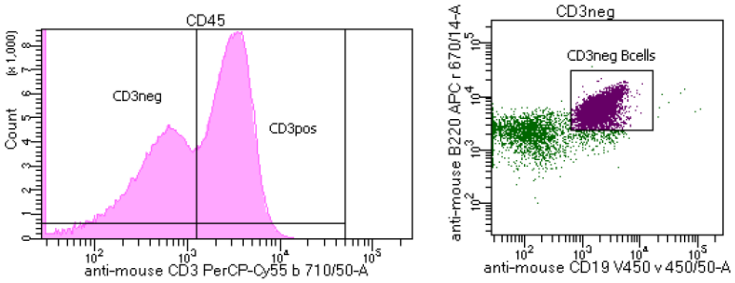


Figure 56: Dot plots of panel B.

B cells
CD19+ B220+ CD3(-)



NK cells
CD335+ CD3(-)

NKT cells
CD335+ CD3+

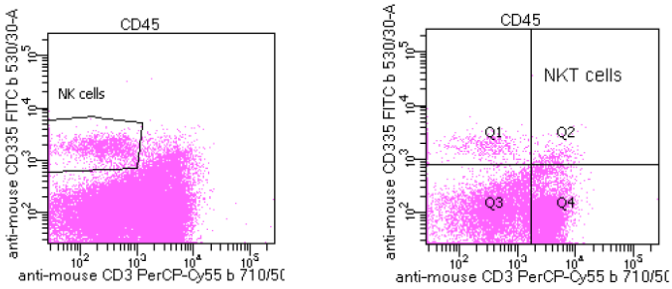
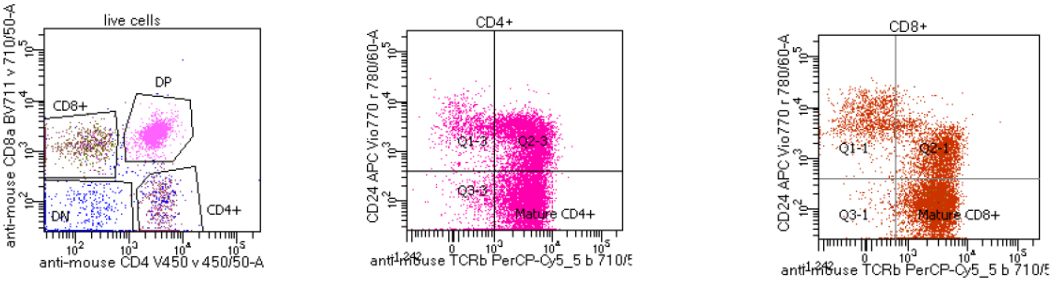


Figure 56: Dot plots of panel C

CD4+ thymocytes
CD8+ thymocytes
CD4+ CD8+ thymocytes
CD4(-) CD8(-) thymocytes

Mature CD4+
Thymocytes
TCRβ+ CD24(-) CD4+

Mature CD8+
Thymocytes
TCRβ+ CD24(-) CD8+



FoxP3+ Tregs

FoxP3+ CD25+ Tregs

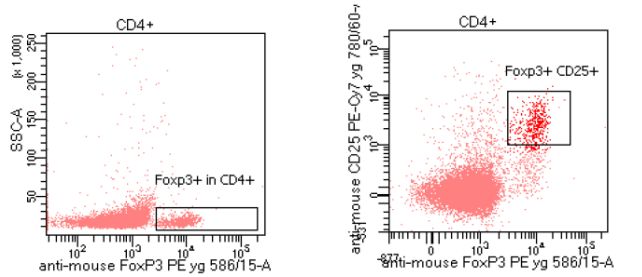


Figure 57: Dot plots of panel D