Investigations on long-term effects of the N-methyl-D-aspartate receptor antagonist dextromethorphan on islet inflammation and beta cell survival in type 1 diabetes

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1. Abstract

Albeit achievements in identifying people at risk to develop type 1 diabetes mellitus in recent years, none of the current treatment options is able to sustainably prevent type 1 diabetes progression indicating the urgent need for novel agents. On the basis of previous studies of our lab, which revealed anti-diabetic effects of the widely-used over-the-counter cough suppressant dextromethorphan (DXM) in mice and humans with type 2 diabetes, we aimed to investigate whether DXM could also be a potential novel candidate for the preventive treatment of type 1 diabetes.

By performing *in vitro* experiments with isolated mouse and human islets, we could confirm the previously reported islet cell protective characteristics of DXM. Precisely, we demonstrated that by treating islets with dextrorphan (DXO), which is the active metabolite of DXM, pancreatic islets are protected against streptozotocin-induced cell death, a commonly used beta cell-specific toxin.

Furthermore, in order to evaluate the anti-diabetic effects of DXM *in vivo*, we conducted a long-term study with a well-known type 1 diabetes model, the non-obese diabetic (NOD) mouse. Long-term treatment of these mice with DXM via the drinking water reduced blood glucose levels, delayed diabetes onset, and reduced diabetes incidence. Additionally, DXM-treated NOD mice displayed increased residual beta and alpha cell mass, as well as islets numbers. To be specific, at 30 weeks of age, islet numbers were five times higher in NOD mice which had been treated with DXM compared to control NOD mice. These effects were mediated by reduced apoptosis in pancreatic islets, as assessed by the cell death marker cleaved caspase-3, whereas changes in the rate of islet cell proliferation were not observed.

In addition, DXM treatment reduced the amount of infiltrating immune cells into pancreatic islets. Particularly, the numbers of $CD8^+$ and $CD4^+$ T lymphocytes in the immune cell infiltrate were reduced, but not $FoxP3^+$ regulatory T cells. An improvement in inflammation of pancreatic islets was also confirmed by determining the expression of chemokines in pancreatic islets treated with DXO. Several chemokines involved in the attraction of different types of immune cells were expressed at lower levels under standard culture conditions, as well as in an inflammatory environment in the presence of DXO.

As a conclusion, the study proved that DXM exerts anti-diabetic effects in type 1 diabetes and should therefore be further investigated as a potential preventive treatment. Considering recently suggested novel approaches for effective disease prevention using combinatory treatments and the fact that DXM improves beta cell function and survival, DXM might be particularly useful in combination with an additional immune-modulating agent.

2. Zusammenfassung

Obwohl es in den letzten Jahren gelungen ist, Personen zu identifizieren, die ein erhöhtes Risiko besitzen an Typ 1 Diabetes mellitus zu erkranken, gibt es bis heute keine Behandlungsmöglichkeit, welche in der Lage ist das Fortschreiten des Typ 1 Diabetes langfristig zu verhindern. Daher besteht dringender Bedarf neue Wirkstoffe zur Prävention von Typ 1 Diabetes zu entwickeln. Frühere Studien unseres Labors konnten zeigen, dass der weit verbreitete rezeptfreie Hustenstiller Dextromethorphan (DXM) bei Mäusen und Menschen, die an Typ 2 Diabetes erkrankt sind, anti-diabetische Effekte besitzt. Daher wollten wir untersuchen, ob DXM auch ein potenzieller neuer Kandidat für die präventive Behandlung von Typ 1 Diabetes sein könnte.

Durch *in vitro* Experimente mit isolierten murinen und menschlichen Inseln konnten wir die zuvor berichteten schützenden Eigenschaften von DXM auf die Langerhans-Inseln weiter bestätigen. Wir konnten zeigen, dass die Behandlung der Langerhans-Inseln mit Dextrorphan (DXO), welches der aktive Metabolit von DXM ist, die Inselzellen vor Streptozotocin-induziertem Zelltod schützt. Bei Streptozotocin handelt es sich um ein häufig verwendetes Betazell-spezifisches Toxin.

Um die antidiabetische Wirkung von DXM *in vivo* zu untersuchen, haben wir eine Langzeitstudie mit einem bekannten Typ 1 Diabetes Modell, der NOD Maus (engl. non-obese diabetic), durchgeführt. Die Langzeitbehandlung dieser Mäuse mit DXM über das Trinkwasser senkte den Blutzuckerspiegel, verzögerte die Manifestation des Diabetes und reduzierte die Diabetesinzidenz. Darüber hinaus wiesen die mit DXM behandelten NOD-Mäuse eine erhöhte Menge an verbliebenen Beta- und Alphazellen, sowie eine erhöhte Anzahl an Langerhans-Inseln auf. Im Alter von 30 Wochen war die Anzahl der Langerhans-Inseln bei NOD-Mäusen, die mit DXM behandelt worden waren, fünfmal höher als bei NOD-Kontrollmäusen. Diese Effekte wurden durch eine verringerte Apoptose in den Zellen der Langerhans-Inseln vermittelt, während eine Veränderung der Proliferationsrate in den Inselzellen nicht beobachtet wurde.

Darüber hinaus reduzierte die Behandlung mit DXM die Anzahl der Immunzellen, die in die Langerhans-Inseln infiltrieren. Insbesondere die Anzahl der $CD8^+$ und $CD4^+$ T-Lymphozyten war in den insulitischen Läsionen reduziert, nicht jedoch die Anzahl der $FoxP3^+$ regulatorischen T-Zellen. Ebenfalls wurde eine Linderung der Entzündung in den Langerhans-Inseln durch die Bestimmung der Expression von Chemokinen nach DXO-Behandlung festgestellt. Mehrere Chemokine, die an der Anziehung verschiedener Arten von Immunzellen beteiligt sind, wurden sowohl unter Standard-Kulturbedingungen als auch in einer entzündlichen Umgebung in Gegenwart von DXO in geringerem Maße exprimiert.

Zusammenfassend beweist die Studie, dass DXM beim Typ 1 Diabetes antidiabetische Wirkungen entfaltet und daher als potenzielle präventive Behandlung weiter untersucht

werden sollte. In Anbetracht der kürzlich vorgeschlagenen neuen Ansätze für eine wirksame Krankheitsprävention mittels Kombination mehrerer Wirkstoffe und da DXM insbesondere die Funktion und das Überleben der Betazellen verbessert, könnte DXM zusammen mit einem zusätzlichen immunmodulierenden Wirkstoff besonders nützlich sein.

3. Introduction

3.1. Islets of Langerhans – blood glucose regulating mini-organs of the pancreas

Responsible for controlling glucose homeostasis in the human body and therefore of great interest in the field of diabetes mellitus (DM) research is the pancreas with its endocrine islets of Langerhans. The pancreas is a glandular organ located in the upper abdomen. It consists of 5 anatomical parts, namely head, uncinate process (in the ventral lobe of the head), neck, body, and tail (see Figure 1 a) [1]. The head lies in direct vicinity of the duodenum, whereas the tail reaches to the spleen (see Figure 1 a) [2].

Regarding its function, the pancreas is separated into an exocrine and an endocrine compartment, both parts participating in main functions of the body such as regulation of glucose metabolism and food digestion. The exocrine part, which is responsible for the secretion of digestive enzymes, makes up about 95% of pancreatic mass [3]. It consists of acinar cells arranged in acini which secrete digestive enzymes. These secreted proteins are then transported to the duodenum via the ductal epithelium (see Figure 1 b) [1]. The endocrine part, although only covering 1 to 2% of pancreas mass, consists of up to 3.2 million islets of Langerhans [1, 4]. The diameter of islets of Langerhans can vary between 50 and 250 µm, but is on average between 100 and 150 µm [3].

Islets of Langerhans contain several endocrine cell types, each of them producing and secreting a specific hormone (see Figure 1 c). The majority of endocrine cells are the so-called beta cells. The main function of beta cells is to synthesize and secret the blood glucose lowering hormone insulin. In humans, beta cells display 40-60% of endocrine cells in pancreatic islets [1]. The second most common endocrine cell type of pancreatic islets are called alpha cells. These cells are responsible for the synthesis of the blood glucose increasing hormone glucagon, thereby counteracting insulin function. Other cell types found in islets of Langerhans are delta cells secreting somatostatin, PP cells synthesizing pancreatic polypeptide (PP), and epsilon cells which are responsible for ghrelin production.

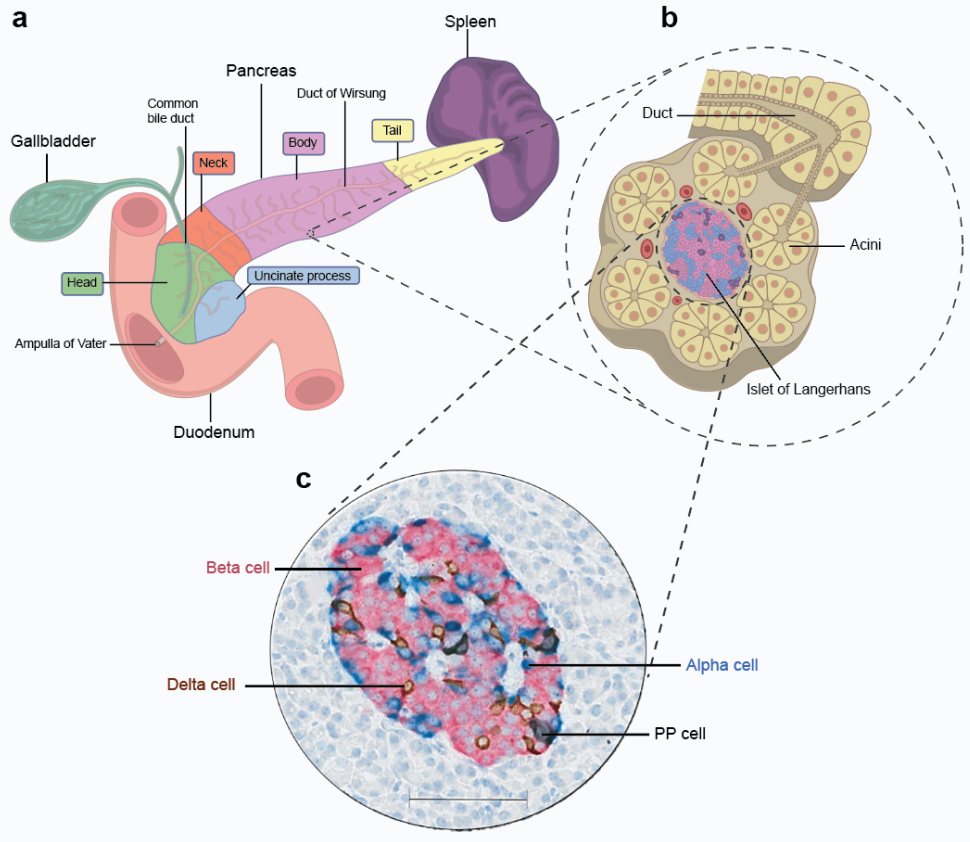

Figure 1: Macroscopic and microscopic architecture of the pancreas. (a) The pancreas anatomically consists of 5 different regions: head, uncinate process, neck, body, and tail. It is located between duodenum and spleen and (b) comprises two parts, the exocrine and the endocrine part. The exocrine compartment consists of ducts and acinar cells arranged in acini, together responsible for the secretion and transport of digestive enzymes to the duodenum. The main duct (also known as duct of Wirsung) joins the common bile duct (coming from the gallbladder) in the head of the pancreas and forms the ampulla of Vater connecting the ducts with the duodenum. The endocrine part consists of islets of Langerhans which are shown in (c). Each islet of Langerhans contains different endocrine cell types, namely alpha, beta, delta, and PP cells. The likewise associated endocrine epsilon cells are not shown in this illustration. scale bar: 100 µm. (Illustration reprinted from Atkinson, M.A., et al., *Organisation of the human pancreas in health and in diabetes.* Diabetologia, 2020. 63(10): p. 1966-1973. [1].)

Composition and architecture of islets of Langerhans were shown to differ with size, age, and location within the organ [1, 5], as well as between species [2, 6, 7]. Precisely, rodent islets were reported to have a higher proportion of beta cells, but less alpha cells compared to human islets [2, 6, 7]. The endocrine cells in islets of mice are arranged in a mantle-core architecture where beta cells are located in the center of the islets and other endocrine cells in the periphery [6]. In humans, this pattern is only found in smaller islets with a diameter below 100 µm, whereas bigger human islets are reported to have a more complex and heterogenous architecture [2, 5, 8].

Pancreatic islets not only consist of the before mentioned endocrine cells, other cell types such as epithelial cells and nerve cells are essential for proper regulation of blood glucose levels as well [9]. In fact, islets of Langerhans are highly vascularized, as seen by a five-fold higher density of capillaries and a five-to-ten-fold increased blood flow compared to the exocrine

compartment [2, 10]. Besides this quite pronounced vascularization, islets of Langerhans are likewise excessively innervated [9, 11]. The nerve density in islets was stated to be six times higher than in the exocrine compartment [11]. This complex network of different cell types enables islets of Langerhans to sense and immediately react to changing blood glucose concentrations with the secretion of the respective hormone to control glucose homeostasis in a narrow range.

3.2. Regulation of blood glucose levels

Glucose is the most important energy supplier in the human body and must be available in sufficient quantities at all times. Still, blood glucose concentrations need to be controlled strictly. Neither blood glucose levels that are too high (hyperglycemia), nor ones that are too low (hypoglycemia) are healthy and derailments in both directions can entail severe consequences. In healthy individuals, blood glucose levels are therefore tightly regulated by insulin and glucagon to lie between 4 mM during a fasting period and 6 mM after meal intake [12]. Insulin thereby lowers blood glucose levels as it induces the uptake of glucose out of the bloodstream into muscle cells and adipose tissues, as well as by inhibiting hepatic gluconeogenesis, whereas glucagon counteracts by elevating glucose concentrations in the blood [12].

The optimized interaction between glucose as most important insulin secretagogue and insulin secretion is called stimulus-secretion coupling and describes a signaling cascade in beta cells coupling metabolization of glucose with exocytosis of insulin vesicles via electrical activity and intracellular Ca^{2+} concentrations $[Ca^{2+}]_i$ (see Figure 2). As the strong vascularization of islets enables a fast exchange of glucose between blood and beta cells, glucose is taken up into beta cells via the glucose transporter 1 (GLUT-1) in humans and GLUT-2 in mice as the first step [13, 14]. Once in the beta cells, the ingested glucose can be metabolized, triggering the mechanisms that stimulate insulin secretion. The phosphorylation of glucose by the glucokinase as the initial step of glucose metabolism is considered the rate-limiting step in this signaling cascade [15]. The generated glucose-6-phosphate is then further metabolized via the tricarboxylic acid cycle (TCA), subsequently leading to the generation of adenosine triphosphate (ATP) and an increased ATP/ADP ratio [16].

Under non-stimulatory glucose concentrations below 3 mM, beta cells are in a hyperpolarized state with a membrane potential of approximately -70 mV [17]. This is mostly maintained by K^+ outward currents via K_{ATP} channels [18]. Increased ATP/ADP ratios due to enhanced glucose metabolism now block the K_{ATP} channel preventing K^+-efflux and thereby depolarizing the cell membrane [16]. This depolarization induces the opening of voltage-gated calcium channels (VGCC) so that $[Ca^{2+}]_i$ rises due to Ca^{2+}-influx. This elevated $[Ca^{2+}]_i$ finally triggers exocytosis of insulin vesicles and the release of insulin into the bloodstream [16, 17]. As an additional

consequence of elevated $[Ca^{2+}]_i$, large-conductance Ca^{2+}-activated K^+ channels (BK channels and Kv2.1 channels) open. These are crucial for repolarization of the plasma membrane besides the rapid inactivation of channels responsible for the depolarizing inward currents (Ca^{2+} dependent inactivation of Ca^{2+} channels and voltage-dependent inactivation of Na^+ channels) [14, 17]. As a consequence, membrane potentials, as well as $[Ca^{2+}]_i$ are described to follow an oscillatory pattern and stimulate a pulsatile insulin secretion [19-21].

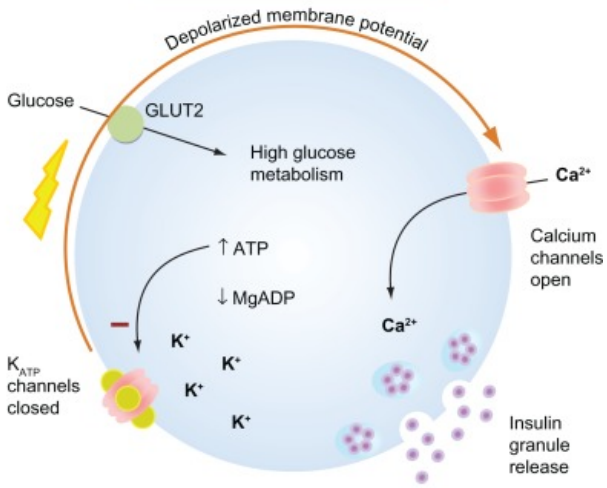

Figure 2: Mechanism of insulin secretion. Glucose is taken up into mouse pancreatic beta cells via the glucose transporter GLUT2. Inside the cell, glucose is metabolized leading to the generation of ATP and simultaneous consumption of MgADP. The consequential increased ATP/ADP ratio closes K_{ATP} channels and thus prevents K^+-efflux leading to the depolarization of the plasma membrane. Upon depolarization, voltage-gated calcium-channels open, enabling Ca^{2+} influx. Increased intracellular Ca^{2+} is the final trigger for exocytosis of insulin vesicles and the secretion of insulin. (Illustration adapted from Lang, V. and P.E. Light, *The molecular mechanisms and pharmacotherapy of ATP-sensitive potassium channel gene mutations underlying neonatal diabetes.* Pharmgenomics Pers Med, 2010. 3: p. 145-61. [22] according to the Creative Commons Attribution – Non-Commercial (unported v3.0) License.)

Furthermore, insulin is secreted in a bi-phasic pattern. It is believed that the stimuli of elevated $[Ca^{2+}]_i$ induces insulin secretion from a readily releasable pool (RRP) of insulin vesicles located close to the plasma membrane accounting for the first phase of insulin secretion [16, 18]. The evolving initial peak of insulin secretion rapidly declines within 3 to 10 minutes and is followed by a second phase of insulin release at a slower, but prolonged rate [23]. The latter is responsible for most of the released insulin and dependent upon the recruitment of secretory granules from intracellular storages [24]. However, in addition to glucose, a second set of stimuli besides glucose, including several amino acids or specific fatty acids, as well as incretins like glucagon-like peptide-1 (GLP-1) are able to amplify insulin secretion. Therefore, this pathway is known as the amplifying pathway [12, 14, 23]. Yet, these fuels have little stimulatory effects on their own and are dependent on membrane depolarization and $[Ca^{2+}]_i$ elevation induced by the stimulus-secretion coupling [16, 25].

Thus, it can be concluded that glucose is not the only, but the most important trigger for insulin secretion. By this perfectly tuned stimulus-secretion coupling, blood glucose levels in healthy

individuals are regulated in a narrow range. However, this system is susceptible to disturbances, so that proper feedback regulation of insulin secretion fails and blood glucose levels may exceed the optimal range. If this happens permanently, it is a serious pathological condition, called diabetes mellitus (DM).

3.3. Diabetes mellitus (DM) – a global health issue

Increased blood glucose levels resulting from either insufficient insulin secretion of beta cells or deficient insulin action in target tissues like skeletal muscles, adipose tissue or the liver are the main characteristics of one of the biggest health problems of the 21st century which is called DM [26]. In 2019, 463 million people worldwide (age 20-79 years) were reported to suffer from DM and numbers still tend to increase [27]. It is estimated that in 2045 this number will rise to over 700 million people concerned which would be 1 out of 10 of the world's population [27].

Typical symptoms of DM are polyuria, polydipsia, polyphagia, and weight loss. Sometimes blurred vision, impaired growth, and susceptibility to certain infections can also occur [26]. Severity of symptoms is dependent on disease progression. If the disease is not diagnosed and blood glucose levels remain uncontrolled, this can lead to serious conditions like stupor or coma. In the worst case even death due to ketoacidosis or more rarely from nonketotic hyperosmolar syndrome can occur [28]. In fact, it is estimated that over 4 million people worldwide between 20 and 79 years of age died because of diabetes or at least their deaths were related to diabetes [27].

Besides these acute conditions, increased blood glucose levels can also induce several long-term complications affecting different organs such as eyes, kidneys, nerves, and especially the cardiovascular system including heart and blood vessels with severe consequences [27].

In fact, diabetic retinopathy occurs in one third of diabetic patients and is the major cause of blindness in adults [27, 29]. In the kidney, high blood glucose levels can lead to renal failure in the long-run, which is stated to account for over 10% of deaths in people with diabetes [30]. Furthermore, it is estimated that every 30 seconds a lower limb is amputated as a consequence of diabetes and its subsequent peripheral neuropathy [27, 31]. However, the main cause of morbidity and mortality of people with DM remains cardiovascular conditions due to increased blood glucose levels. The relative risk of cardiovascular diseases, such as ischemic heart diseases or other diseases of the circulatory system, is approximately doubled in diabetic patients, finally resulting in 30 to 50% of all diabetes-associated deaths [27, 30, 32]. Therefore, it is essential to diagnose diabetes as early as possible to prevent long-term complications by tight control of blood glucose levels with lifestyle interventions or drugs.

DM can be diagnosed on the basis of various parameters according to the following criteria defined by the World Health Organization (WHO) [33]. The most important parameters are based on the measurement of blood glucose levels. DM is diagnosed if either random plasma

glucose levels exceed 200 mg/dL or fasting plasma glucose levels are above 126 mg/dL. Another indicator useful for the diagnosis of DM is the HbA1c value. This value is a measure of glycated hemoglobin levels and thus correlates with blood glucose levels over the past two to three months. HbA1c values above 6.5% also meet the criteria established for diabetes diagnosis. Furthermore, disease state can be determined using a 75 g oral glucose tolerance test (oGTT). If plasma glucose concentrations are still above 200 mg/dL after two hours, the diagnosis of DM will be made [33]. Preliminary stages indicated by impaired fasting glucose values or glucose tolerance are applied to define people at risk to develop diabetes and its complications [27].

In general, patients of DM are united by increased circulatory blood glucose levels, but underlying pathogenesis can be heterogenous. Therefore, DM is further classified as described by the WHO [33]. There are two main forms of DM which will be described in the following. Type 1 DM (T1DM) accounts for 5 to 10% of diabetes cases and Type 2 DM (T2DM) is diagnosed in around 90% of cases [26, 27]. Other forms of diabetes include gestational diabetes, diabetes due to pancreas diseases or monogenetic forms, but these are less common [33].

3.3.1. Type 1 Diabetes mellitus (T1DM) – an autoimmune disease

T1DM is an autoimmune disease in which pancreatic beta cells are progressively destroyed by the body's own immune system so that the remaining islets of Langerhans are not able to secrete enough insulin to control blood glucose levels. In 2019, over 1 million children and adolescents under the age of 19 years were reported by the International Diabetes Federation (IDF) to have T1DM, with numbers rising [27]. Precisely, the EURODIAB study reported an annual increase of 3.4% in the incidence of T1DM from 1989 to 2013 in children under 14 years of age [34]. Although T1DM is often diagnosed in childhood, up to 50% of cases occur later during adulthood [35].

T1DM pathogenesis has a strong genetic background. Till this day, more than 60 different loci were identified by genome wide association studies (GWAS) to be linked to T1DM [36]. Most of these loci are related to the immune system or main functions of beta cells like insulin secretion. Especially mutations in the human leukocyte antigen (HLA) are associated with the risk of autoimmunity against beta cells [36, 37]. The two HLA class 2 haplotypes HLA-DR3-DQ2 and HLA-DR4-DQ8 were identified as primary risk factors to develop T1DM [37]. Due to this hereditary component, the relative risk of T1DM is determined to be 15-fold increased when a first-degree relative is already diagnosed with T1DM [36]. But genetics does not appear to be the only explanation for the development of T1DM, as 85% of children diagnosed with diabetes have no known case in the family [36]. Therefore, it is suggested that besides genetics there are additional environmental triggers, which originally induce autoimmunity against beta

cells. Several factors such as viruses (e.g. enteroviruses), diet, and gut microbiota are discussed to be implicated in disease onset. However, the correlation is not yet comprehensively understood [38].

The main characteristic to distinguish between T1DM and other forms of diabetes is the presence of autoantibodies against beta cell specific antigens such as glutamate decarboxylase 65 (GAD65), insulin-associated antigen 2 (IA-2), zinc transporter 8 (ZnT8) or insulin itself. They are found in the blood of over 90% of individuals at diabetes onset [35]. In most cases they can be detected before the disease manifests, sometimes even years before, which makes them useful biomarkers to identify individuals who will likely progress to T1DM. Children with multiple autoantibodies will be more likely to progress to T1DM within the next 15 years compared to children with only one single islet autoantibody found [39]. Thus, a consensus statement of the American Diabetes Association (ADA), Juvenile Diabetes Research Foundation (JDRF), and Endocrine Society introduced a new staging of T1DM [40]. Due to the correlation between islet autoantibodies and the development of T1DM, the presence of two or more islet antibodies without further symptoms was already defined as stage 1 of T1DM. If blood glucose levels increase, the disease is classified as stage 2. Stage 3 describes a disease state in which additionally typical symptoms, such as polyuria, polydipsia or weight loss, occur [36, 40]. Often the disease is not diagnosed until stage 2 or 3 is reached, when the disease has already led to the destruction of over 70% of the beta cells [41, 42]. However, nowadays the percentage of lost beta cells upon diagnosis is questioned. There are studies showing that the remaining beta cell mass depends on age at diagnosis. Children are reported to have left lower islet numbers, whereas older patients still have 30 to 60% remaining beta cell mass when the disease manifests [43-45]. A small amount of beta cells seems to be able to survive the autoimmune attack anyway, since in 75% of patients with long-standing T1DM low levels of endogenous insulin are still measurable years after diagnosis [36].

Because the majority of beta cells is lost, endogenous insulin is not sufficient to fulfill the body's need to control blood glucose levels in T1DM patients. Thus, patients require lifelong insulin substitutions. Thanks to the discovery of insulin by Banting and Best one century ago, the options to treat T1DM with insulin are well-established, at least in developed countries, and patients can live long and healthy lives. Nonetheless, the WHO reports that T1DM still reduces the life expectancy by over ten years in high income countries [33, 46]. Interestingly, in 80% of children there is a so-called honeymoon phase, when insulin treatment is initiated [47]. This means that insulin substitution leads to a partial regain of beta cell function so that less exogenous insulin is needed temporarily for weeks or even years proving that beta cells are able to recover [43]. However, this phase is not permanent and the progressive beta cell decline continues [47].

Against this background, further options for the treatment of T1DM besides insulin are needed. Taking in the fact that there are studies showing that screening for autoantibodies early in life is feasible [39, 48, 49], it seems possible to prevent diabetes manifestation in people at risk, if drugs were available which could stop the autoimmune-mediated beta cell destruction. For the development of such agents, further knowledge on the complex underlying mechanisms around disease onset is necessary.

3.3.2. Insulitis in T1DM causes beta cell destruction

A hallmark of T1DM is the infiltration of immune cells into islets of Langerhans which is called insulitis. It was defined as the presence of ≥ 15 $CD45^+$ cells per islet in three or more islets [50]. In the human pancreas, peri-insulitis is the most described form of insulitis, which means that immune cells are only found in the periphery of islets [51, 52]. Still, the number of infiltrated islets in humans varies with age of diagnosis and disease duration [51, 53]. Only 10 to 30% of human islets are reported to show signs of insulitis at all, whereas in the widely used non-obese diabetic (NOD) mouse model the infiltration is much stronger [51, 54, 55].

Among the infiltrating immune cells, cytotoxic $CD8^+$ T cells are the most frequent ones, accompanied by $CD4^+$ T helper cells and also B cells [38, 44, 54]. The latter are found more frequently in advanced stages of disease or in young children where the disease manifests early before 7 years of age [56]. Other cell types which are found in insulitic lesions are antigen presenting cells (APC) and cells of the innate immune system like natural killer (NK) cells and neutrophils [42, 57]. Notably, in the T1DM NOD mouse model, APCs like dendritic cells and macrophages are described to be among the first infiltrating immune cells followed by T cells suggesting that APCs are involved in early disease onset and attributing them an essential function in the onset of T1DM [42, 57-60].

Actually, it is widely accepted that a breakdown in self-tolerance allows the escape of several autoantigen-specific immune cells from thymic selection which cause beta cell death and consequently induce the release of beta cell self-antigens [38, 61]. These antigens are then taken up by islet-resident APCs (dendritic cells, macrophages, B cells) which upon activation secret cytokines causing an inflammatory milieu in pancreatic islets and travel to the pancreatic lymph nodes where they present beta cell autoantigens to T cells (see Figure 3 A) [62, 63]. Thus activated islet-specific $CD4^+$ and $CD8^+$ T cells in turn circulate back and infiltrate the islets to attack beta cells, induce further beta cell death, and simultaneously worsen inflammation (see Figure 3 B) [35, 62, 63]. The ongoing damage of beta cells provokes release of further self-antigens which leads to epitope spreading exacerbating immune cell infiltration as well as the progressive beta cell destruction [57].

Major contributor to the destruction of beta cells are cytotoxic $CD8^+$ T cells [35, 58, 64] which can kill beta cells via the secretion of perforins and granzymes, or inflammatory cytokines like

tumor necrosis factor α (TNFα) and interferon γ (IFNγ) as well as via the Fas/FasL pathway [58, 65, 66]. Cytokines are not only secreted by cytotoxic $CD8^+$ T cells, but also by $CD4^+$ cells and other immune cell subsets like macrophages and B cells in order to orchestrate immune cell interactions (see Figure 3 C) [67]. The released cytokines in islets for example further activate cytotoxic $CD8^+$ T cells and attract even more immune cells to infiltrate islets, but also stimulate B cells to produce and secrete beta cell-specific autoantibodies which can be used as biomarkers for the diagnosis of T1DM (see Figure 3 E) [35, 58].

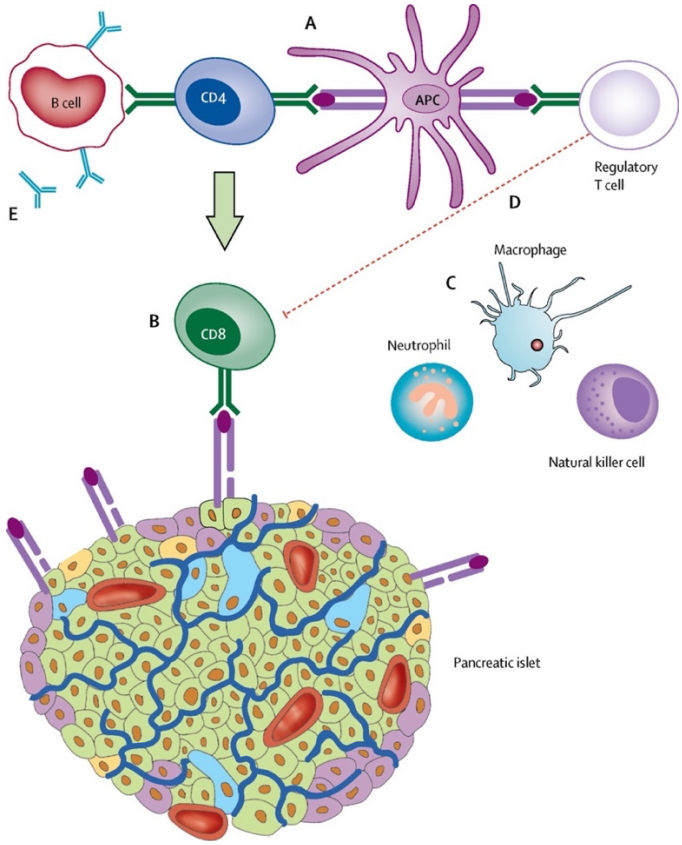

Figure 3: Immune cell interactions in the pathogenesis of T1DM. (A) It is believed that the initiation of autoimmunity starts with the presentation of beta cell-specific antigens by antigen-presenting cells (APC) to $CD4^+$ T cells in pancreatic lymph nodes. (B) Activated $CD4^+$ T cells in turn activate $CD8^+$ T cells which traffic to pancreatic islets mediating beta cell death and inducing the presentation of beta cell-specific antigens on the major histocompatibility complex (MHC) class 1 molecules on islets. (C) The emerging inflammatory environment is further aggravated by various infiltrating innate immune cells such as neutrophils, macrophages, and NK cells and their release of proinflammatory cytokines. (D) Deficient regulatory T cells (Treg) are not able to efficiently suppress the function of autoreactive $CD8^+$ T cells which enhances the autoimmune-mediated destruction. (E) In the pancreatic lymph node, activated T cells stimulate B cells to generate beta cell-specific autoantibodies which can be detected in the blood of T1DM patients. (Illustration reprinted from DiMeglio, L.A., C. Evans-Molina, and R.A. Oram, *Type 1 diabetes.* Lancet, 2018. 391(10138): p. 2449-2462. [35].)

Once autoimmune attacks have started, beta cells themselves are involved in the deterioration of their own destruction [68, 69]. In the inflammatory environment of secreted cytokines, beta cells upregulate the expression of major histocompatibility complex (MHC) 1 molecules so that they more efficiently present self-antigens to the immune system which facilitates increasing

numbers of immune cells to infiltrate into the islets [42, 44, 68, 70]. In addition, when stimulated with inflammatory cytokines, islets of Langerhans upregulate the expression and secretion of chemokines like C-X-C motif chemokine ligand 10 (CXCL10) and CC-chemokine ligand 2 (CCL2) among others. This additionally attracts further immune cells [62, 71-74]. Consequently, T1DM is not exclusively described as a disease of the immune system anymore, contribution of beta cells to the pathogenesis is postulated to be crucial as well [68, 69].

Although knowledge about the ongoing process of autoimmunity has increased greatly in recent years, it is still not known what causes the breakdown in immune regulation in the first place. It is described that decreased regulatory immune function might be involved. Regulatory T cells (Treg), whose original function is to prevent autoimmune diseases by maintaining self-tolerance, show deficits in immunosuppressive functions in patients with T1DM, although numbers remain unchanged (see Figure 3 D) [75, 76].

Another theory trying to explain the special vulnerability of beta cells to autoimmune-mediated destruction involves excessive endoplasmic reticulum (ER) stress. Beta cells in healthy individuals are already burdened with the need to produce a high amount of insulin, which must be folded in the ER, thus placing stress on the beta cells. Upon inflammation beta cells then get additionally stressed which leads to protein misfolding and finally results in more ER stress [64, 77]. This may be responsible for the generation of neoepitopes by mechanisms like generation of post-translationally modified and hybrid antigens, the use of different open reading frames or RNA splicing variants. Neoepitopes are not known to the immune system and therefore might provoke the initial autoimmune reaction responsible for beta cell destruction [64, 69, 77, 78]. However, we are just beginning to understand these processes and their contribution to T1DM are not fully elucidated yet. Further research needs to confirm the actual event provoking onset of autoimmunity, eventually revealing new targets and options to prevent T1DM onset at an early disease state.

3.3.3. Type 2 Diabetes mellitus (T2DM) and its treatment options

T2DM is characterized by peripheral insulin resistance accompanied by progressive beta cell dysfunction which results in chronically elevated blood glucose levels. It accounts for the vast majority of over 90% of all diabetes cases, but is often not diagnosed for years because dramatic symptoms as observed in T1DM are rare [27, 33]. Still, elevated blood glucose levels tremendously increase the risk of long-term complications. As a consequence, the disease should be diagnosed and treated as early as possible [27, 79, 80]. In contrast to T1DM, T2DM occurs mainly in adulthood, but numbers of concerned children and adolescents are increasing too [27, 81, 82]. This is most likely due to general society changes towards sedentary lifestyles represented by physical inactivity and unhealthy diets finally resulting in obesity [27, 83]. These

factors, especially obesity, contribute to the development of T2DM besides age, smoking, and prior gestational diabetes [27, 33, 83, 84].

Ectopic fat accumulation in general leads to insulin resistance, especially in peripheral organs of insulin action, such as liver, skeletal muscle, and adipose tissue [85, 86]. Insulin resistance means that higher amounts of insulin are needed in target organs to provoke downstream responses comparable to healthy individuals [27, 87]. Nevertheless, since not all obese people become diabetic, beta cells are usually able to cope with this increased demand by secreting higher amounts of insulin [88-91]. But in some individuals, beta cell dysfunction evolves parallel so that beta cells are not able to secrete enough insulin to compensate the insulin resistance which results in T2DM manifestation [86, 92-95]. It is widely accepted that beta cells get exhausted due to the increased demand of insulin resistant effector tissues which contributes to their dysfunction [27, 85, 90]. In fact, it is reported that beta cells are lost in T2DM patients, but the degree of beta cell mass decline varies between studies from 20 to 50% [95-97]. Additionally, it is still under debate, whether this loss of beta cell mass is only due to increased beta cell apoptosis or if other mechanisms such as dedifferentiation contribute to the loss of function too [97-100].

Based on the conclusion that obesity is clearly associated with insulin resistance, it is not surprising that treatment of T2DM always begins with lifestyle interventions including a healthy diet and regular physical activity [27]. Precisely, two studies, the Finnish Diabetes Prevention Study and the US Diabetes Prevention Program, showed that managing obesity through lifestyle changes prevents the manifestation of T2DM in individuals at risk by over 50% compared to placebo treatment [101, 102]. Moreover, in individuals, that are already diabetic, remission can be achieved by losing body weight as shown by the Diabetes Remission Clinical Trial (DiRECT) [103, 104]. In many cases, however, weight reduction is not achieved sustainably or to the necessary extent, so that pharmacological treatment becomes unavoidable and one of the numerous antidiabetic drugs is prescribed. Different classes of antidiabetic drugs are on the market which aim to restore blood glucose control by different mechanisms. They either increase insulin secretion (sulfonylurea), slow down and prolong glucose digestion (a-glucosidase inhibitors), improve insulin sensitivity (thiazolidinediones), reduce hepatic glucose output (metformin), induce glucosuria (sodium-glucose linked transporter 2 (SGLT2) inhibitors) or stimulate GLP-1 induced insulin secretion (GLP-1 analogues, DPP-4 inhibitors). If lifestyle changes alone do not suffice to lower blood glucose concentrations, metformin is recommended as first-line therapy [93]. In most patients with T2DM a combinatory treatment with several oral antidiabetics becomes necessary at some point to control blood glucose levels efficiently. The majority of patients finally ends up with exogenous insulin substitution as last option for proper glycemic control, although insulin is not

necessarily required for the treatment of T2DM, which is in contrast to T1DM. Yet, this gradual adaptation of medications shows the progressive nature of the disease [27, 85, 105].
Regarding the fact that different studies proved that the most effective treatment for T2DM involves excess weight loss, it seems admissible to conclude that changing our current lifestyle to a healthier one is the best disease prevention we can do to relieve the burden of this global health problem.

3.4. Current and future options to treat T1DM

As outlined before, T1DM is a complex disease based on genetics, the immune system, and as an additional factor the environment. This complex interplay reveals many possible targets to treat this disease, although until today, exogenous insulin administration remains the only option to properly control blood glucose levels. Other drugs can only be used as additives and so far, preventive strategies have been only partially successful in clinical use [106].
Exogenous insulin administration has improved largely since its discovery 100 years ago. New insulin modifications with varying pharmacokinetics due to altered absorption or clearance like fast-acting insulin lispro or long-acting insulin glargine improve quality of life for patients and make it easier to approach physiological insulin kinetics [61, 107, 108]. However, the risk of hypoglycemia induced by exogenous insulin remains, so that still only the minority of 20% of patients reaches their target HbA1c levels as reported by a US national registry study [109].
The only approved additional treatment for T1DM by the U.S. Food and Drug Administration (FDA) besides insulin is pramlintide which is an amylin analog. Thus, physiological secreted together with insulin, amylin is known to decrease glucagon secretion and retard nutrient absorption resulting in prolonged satiety. Pramlintide was shown to moderately reduce HbA1c values and lower the amount of needed insulin [110-112]. However, it is not widely used as adjunct prandial therapy since it causes nausea and can induce postprandial hypoglycemia [61, 106].
Moreover, two SGLT2 inhibitors (sotagliflozin and dapagliflozin), which are commonly used for the treatment of T2DM, were recently approved by the European Medicine Agency (EMA) to treat T1DM patients with a body mass index (BMI) $\geq$ 27 kg/m^2. Both SGLT2 inhibitors have the ability to induce weight loss and were shown to reduce HbA1c levels by 0.3 to 0.5% [113]. These drugs inhibit SGLT2 in the proximal tubule of the kidney, thereby preventing reabsorption of glucose and simultaneously inducing glucosuria. The FDA denied drug approval due to severe adverse events like diabetic ketoacidosis associated with sotagliflozin treatment [61, 106].
Progress for the treatment of T1DM not only concerns the development of new drugs, but technical innovations are at least as important. For instance, continuous subcutaneous insulin infusion (CSII) and continuous glucose monitoring (CGM) devices facilitate everyday life of

patients by making frequent injections or glucose measurements avoidable. In 2016, the FDA approved the first hybrid closed-loop system which is a combination of both [114]. These systems are able to automatically regulate blood glucose levels during day and night. However, manual administration of insulin as bolus before meals is necessary to control prandial glucose levels. Since the first approval, various systems have been launched on the market with increasing numbers of utilizers. Benefits of closed-loop systems are increased time in target glucose range, less hypoglycemic events, and a reduced HbA1c value of 0.3% [115-117]. Further improvements of a prospective artificial pancreas are currently under development [114].

All these innovations only help to tightly control blood glucose levels, but cannot cure T1DM. The only known treatments leading to insulin independence are beta cell replacement strategies with either allogenic whole organ transplants or islet transplantations. Of note, diabetes remission is not achieved in all transplanted patients and even if achieved, it only lasts for 5 years in half of the cases [107, 118]. Furthermore, both methods necessarily require immunosuppression so that the grafted organs or islets are not rejected by the immune systems which is a great burden for affected patients and entails a lot of other complications. Therefore, the criteria to get a transplantation are quite strict. Transplantations are only indicated in patients with additional renal transplantation, in patients with recurrent ketoacidosis or in patients with severe hypoglycemia albeit intensive glycemic management [61, 118, 119]. The low amount of available donor material is a further challenge and makes this procedure only applicable to a low number of patients. Hence, other sources of beta cells are under investigation, including usage of porcine islets for transplantation, increasing the proliferation of existing beta cells, the trans-differentiation of other pancreatic cell types like alpha or duct cells, and the differentiation of pluripotent stem cells into beta-like cells [107, 118, 119]. Despite great progress over the past years, this field is still in its infancy and needs further studies until one of these methods can be clinically used to cure T1DM. Moreover, the problem with autoimmune-mediated destruction of the new beta cells remains. If transplanted beta cells cannot be protected by some sort of encapsulation, the immune system or the new beta cells themselves need to be modified to circumvent autoimmune attack [107, 118, 119].

None of the drugs or methods used nowadays stops or prevents disease progression, they rather focus on establishing glycemic control. However, screening for biomarkers like autoimmune antibodies and genotypes makes it possible to detect people at risk in an early disease state. These individuals would benefit from preventive strategies before clinical manifestation takes place. Thus, preventive strategies as described in detail in the following chapter are an important field in T1DM research.

3.4.1. Prevention strategies for T1DM

Strategies to prevent T1DM can be divided according to their different goals. Primary prevention includes strategies to prevent autoimmune-mediated beta cell destruction which is most useful in T1DM patients with stage 1. Secondary prevention aims to avoid blood sugar fluctuations by stimulating remaining beta cells to adequately secrete insulin and additionally preserve residual beta cell function, whereas tertiary prevention tends to prevent associated long-term complications [120, 121]. Taking in the various possible targets involved in T1DM disease initiation and progression, preventive strategies become manifold. They comprise strategies to prohibit autoimmune attacks by modifying the immune system, strategies to avoid environmental triggers associated with disease onset, strategies to increase self-tolerance against beta cell antigens, and strategies to overall improve inflammatory status in islets. Due to the large number of conducted trials, this chapter will concentrate on phase 2 primary and secondary prevention studies.

Targeting environmental factors seems very difficult since they are not truly identified yet, nor comprehensively understood. Hints on predictive triggers and therefore possible targets come from observational studies like the Environmental Determinants of Diabetes in the Young (TEDDY), Diabetes Autoimmunity Study in the Young (DAISY), and the Type 1 Diabetes Prediction and Prevention (DIPP) whose aim is to identify environmental triggers associated with disease onset in children with high-risk profiles [122-125]. Since primary prevention is very appealing, several studies were conducted to investigate whether modifying exposure to the suggested environmental triggers can prevent beta cell autoimmunity in children at risk. For example, avoidance of certain nutrients like gluten [126], bovine insulin [127] or cow's milk [128, 129] early in life of children with increased risk of developing T1DM were investigated [121, 130]. However, only in the Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes (FINDIA) pilot study, which investigated the avoidance of bovine insulin, beneficial effects on the development of autoantibodies were observed at the age of 3 years, whereas the other studies were unsuccessful in preventing autoimmunity [126-130].

A different preventive strategy, which was shown to be effective in other autoimmune diseases, tries to increase self-tolerance against beta cell-specific autoantigens by getting used to them. Therefore, the application of insulin in several forms (orally [131-133], nasal [134, 135], subcutaneous [136]), as well as using GAD65 as antigen [137], were tested [121]. Yet, study results were so far disappointing albeit achievement of some study goals on immune response or on the basis of subgroup analysis [61, 121, 133].

Most promising preventive strategies currently under investigation focus on modulating the immune system since former studies using the immunosuppressant cyclosporin for the treatment of T1DM achieved diabetes remission [138, 139]. But this therapy is accompanied by severe side-effects such as nephrotoxicity, which is why using cyclosporin is not an

acceptable option. However, this proof-of-concept study paved the way for current prevention studies for T1DM more specifically targeting certain aspects of the immune system [61].

Since T cells are key mediators of beta cell destruction in T1DM, many studies target these cells for disease prevention. Currently, most promising results were obtained using the anti-CD3 antibody teplizumab. A phase 2 trial on primary T1DM prevention showed that 14 days of intravenous teplizumab application delayed progression to T1DM manifestation in high-risk individuals for a median of 2 years [140]. Conclusively, teplizumab is currently investigated in a phase 3 trial (PROTECT, NCT03875729). The mechanism beyond this prevention is not completely understood, but it is believed that teplizumab increases exhaustion of autoreactive $CD8^+$ T cells and anergy of $CD4^+$ T cells [140-143].

Inhibition of T cell activation was exploited in a secondary prevention trial using abatacept (CTLA4-Ig fusion protein). Abatacept has already proven its effectiveness in rheumatoid arthritis, another autoimmune disease to whose treatment it is already approved for [144]. It inhibits CD80 and CD86 on APC so that they cannot bind the costimulatory molecule CD28 on T cells and thereby prohibits T cell activation, proliferation, and survival [144]. In the TrialNet study with patients, who were recently diagnosed with T1DM, treatment with abatacept for 2 years delayed decline in beta cell function by more than 9 months compared to placebo. The latter was measured by C-peptide levels in a mixed meal tolerance test (MMTT). Furthermore, abatacept decreased HbA1c levels [144]. This effect even persisted 1 year after the trial ended [145]. Due to this promising results abatacept is currently tested in a phase 2 clinical primary prevention trial with T1DM patients of stage 1 (NCT01773707) [145].

Another tested molecule which aims to restore T cell balance is alefacept (LFA3-Ig fusion protein). Alefacept binds CD2 which is predominantly expressed on effector T cells (Teff) (including memory Teff), but not on Tregs and therefore alefacept was shown to induce a rise in the Treg/Teff ratio for $CD4^+$ and $CD8^+$ T cells [146]. The T1DAL study investigated effects of alefacept in recently diagnosed T1DM patients. Still 15 months after the treatment ended, increased C-peptide levels in MMTT, decreased insulin requirements and 50% reduced hypoglycemic events were found in treated patients suggesting increased remaining beta cell function [146].

Another strategy for T1DM prevention is based on the maintenance of self-tolerance by improving Treg function. For example, one group tried to increase the number of Tregs by interleukin 2 (IL-2) administration [147-149]. Another group used *ex vivo* expanded Tregs [150-153]. Both treatments were shown to be safe in initial studies and are currently under further investigation to test their effect on beta cell function and survival [147-153]. Relative preservation of Tregs was also found in a phase 2 trial in patients with established T1DM treated with anti-thymocyte globulin (ATG) in combination with granulocyte-colony stimulating factor (G-CSF). This effect was accompanied by increased insulin secretion at 12 months

follow-up and tendentially lower HbA1c values at 6 months follow-up [154]. This study outcome is in so far interesting as that monotherapy with each one of these drugs was not effective [154], indicating that combining different drugs might be more successful in the prevention of T1DM than using single ones.

Since T cells are not the sole immune cell type involved in T1DM progression, modulating other immune subsets like B cells is also of interest [155]. Indeed, a four-dose course of treatment with the anti-CD20 antibody rituximab delayed the fall in C-peptide for around 8 months, reduced HbA1c levels, and required insulin doses 1 year after treatment, but could not maintain this effect after 2 years [156, 157].

Besides the before mentioned strategies, current studies do not exclusively focus on immune cells themselves, but also on improving the inflammatory environment. Cytokines under investigation include the central proinflammatory cytokine TNFα, interleukin 6 (IL-6), whose inhibition with tocilizumab was successful in other autoimmune diseases [158], anti-interleukin 21 (IL-21) antibody together with the GLP-1 analog liraglutide [159], and inhibition of interleukin 1 (IL-1) signaling with canakinumab or anakinra [160]. Most promising results were achieved by inhibiting TNFα signaling [106]. For instance, the TNFα inhibitor etanercept was shown to reduce HbA1c values in a phase 2 clinical study in newly diagnosed children, accompanied by a decreased need for exogenous insulin and increased remaining insulin secretory capacity after 24 weeks of treatment [161]. Furthermore, these results were recently confirmed in the T1GER trial using golimumab, a TNFα-antibody, which is already approved for treating different autoimmune diseases [162].

The Ver-A-T1D study tries to prevent T1DM progression from a completely different angle than the before mentioned studies which concentrate on modulating the immune system. In this lately enrolled trial, the anti-hypertensive calcium channel blocker verapamil shall be used in order to improve beta cell function and survival (NCT04545151). A phase 2 study on safety and efficacy of verapamil in adults recently diagnosed with T1DM has already been conducted with promising results. It revealed increased C-peptide area under the curve (AUC) in MMTT at 3 and 12 months accompanied by lower increases in insulin requirements after add-on therapy with verapamil. This suggests that verapamil might be a new approach useful in T1DM secondary and maybe even primary prevention [163].

Regarding the numerous conducted studies targeting different aspects of T1D pathogenesis, it becomes evident that successful prevention might require a combination of different drugs to target several aspects of the immune system, as well as simultaneously improve beta cell function and survival. A lot of effort has been put into the development of immune-modulating agents so far as outlined in this chapter. Hopefully, some of them will prove efficacy in phase 3 trials and become an approved prevention strategy for T1D. However, on the side of beta

cells, new drugs are still lacking to allow combinatory treatments for the immune system and the beta cells likewise.

3.5. Dextromethorphan as potential new agent for the treatment of T1DM

New antidiabetic drugs need to fulfill several requirements. Firstly, novel anti-diabetic drugs should be safe and well tolerated, as drugs currently on the market do not induce severe adverse events. Secondly, they need to improve glycemic control by increasing insulin secretion without inducing life-threatening hypoglycemic events. Thirdly, especially in the context of T1DM, new drugs need to preserve beta cell mass sustainably and prevent their immune-mediated destruction. Last, but not least important, novel therapeutics should also be considered in order to improve diabetic long-term complications as they are causative for most of diabetes-associated deaths. Interestingly, a lot of preclinical and clinical studies suggest that all these aspects might apply to the dextrorotary morphinan derivate dextromethorphan (DXM).

DXM is widely used as over-the-counter cough suppressant since its approval by the FDA in 1958. Thus, a lot of data demonstrates its good safety profile, so that it is even routinely used in pediatric patients older than 4 years [164]. DXM can be administered orally and is available in liquids, as tablets, lozenges, and capsule formulations which is advantageous for new drug formulations regarding patient compliance [165]. Because DXM is orally applied, it undergoes extensive hepatic first-pass metabolism and is rapidly metabolized to its active metabolite dextrorphan (DXO) via the CYP2D6 enzyme [164]. However, DXM and DXO both show pharmacological effects. Nonetheless, a new formulation approved in 2013 by the FDA for the treatment of pseudobulbar affects included the CYP2D6 inhibitor quinidine together with DXM to increase bioavailability of the active agent DXM [166]. This combination facilitated to test the impact of DXM on several other conditions such as neuropathic pain, nonketotic hyperglycinemia, and different neurological and psychiatric disorders with promising outcomes [164, 166-169]. DXM was shown to exert its pharmacological effects by interacting with several sites of action. It antagonizes N-methyl-D-aspartate receptors (NMDARs), it is an agonist at Sigma-1 receptors, and it inhibits serotonin and noradrenaline transporters, as well as nicotinic and acetylcholine receptors [164, 166]. Albeit sharing structural similarities with opioids, DXM shows no relevant interaction with these receptors [164, 166].

Previously, our group could show that DXM exerts anti-diabetic effects including improvement of beta cell function and survival. In fact, DXO increased glucose-stimulated insulin secretion of mouse and human islets *in vitro*, without affecting basal insulin secretion under low glucose concentrations [170]. These results were confirmed by Suwandhi et al. observing similar effects of DXO on insulin secretion [171]. However, a third group reported increased insulin secretion, not only glucose-stimulated, but also under basal conditions [172]. Yet, they used a

10-times higher concentration of DXM (100µM). The insulin secretagogue effects of DXO only under glucose-stimulated conditions were corroborated by *in vivo* experiments of our group. Mice, which have either been intraperitoneally (i.p.) injected or treated orally with DXO or DXM respectively, displayed better glycemic control during a glucose tolerance test (GTT) with no signs of hypoglycemia [170]. These effects were mediated by increased insulin secretion as elevated circulatory plasma insulin levels in DXO-treated mice were found [170]. Moreover, positive effects of DXM on glycemic control could even be translated to humans in a single-dose proof-of concept study. In this study, probands receiving a high dose of DXM (270 mg) displayed a decreased AUC during the first 2h of an oGTT, which was accompanied by elevated maximal insulin concentrations compared to the same patient receiving a placebo [170]. Strikingly, fasting blood glucose levels were again not influenced by DXM treatment further supporting the idea that DXM does not induce severe hypoglycemia [170].

Based on these very promising effects, DXM was also tested in the context of T2DM. DXM was used as add-on therapy to sitagliptin in a randomized, placebo-controlled, double-blinded, multiple crossover, single-dose clinical trial in T2DM patients. A dose of 60 mg DXM revealed the strongest effects and lowered maximal blood glucose concentrations in an oGTT and enhanced early insulin response compared to sitagliptin alone or placebo treatment [173]. Additionally, preclinical long-term studies with the T2DM mouse model *db/db* were conducted. Mice were treated with a high dose of DXM (3 g/L) in their drinking water and compared to *db/db* mice receiving only a low dose (1 g/L, control group). Notably, in this model of T2DM, long-term DXM application was able to improve glucose tolerance and lower fasting blood glucose levels. Furthermore, histochemical analysis of pancreatic sections of these mice revealed an insulin and glucagon area twice as large in *db/db* mice treated with 3 g/L DXM compared to control mice. The same mice also displayed higher islet numbers compared to the control group. This was likely due to less islet cell apoptosis as determined by reduced expression of the apoptosis marker cleaved caspase-3 (CC3), whereas proliferation remained unchanged [170]. What is even more interesting regarding the pathogenesis of T1DM is that Marquard et al. were able to show that DXO protects human islets against cytokine-induced cell death *in vitro* (IFNγ, interleukin 1β (IL-1β), TNFα) [170]. Together these results strongly suggest that DXM and DXO are able to improve islet cell survival in an inflammatory environment which could be beneficial for treating T1DM. In line with that and as proof that the NMDAR might actually be a very interesting new target for antidiabetic drugs, another NMDAR antagonist called memantine was likewise able to improve beta cell function and survival in a different T2DM mouse model using the beta cell-specific toxin streptozotocin (STZ) in combination with high-fat diet [174]. These effects were partially mediated by improving inflammatory status of islets as shown by reduced pancreatic expression of TNFα and IL-1β [174].

Anti-inflammatory properties are not exclusively assigned to memantine, but were also attributed to DXM. For instance, DXM lowered the expression of proinflammatory cytokines and chemokines, in particular TNFα, in different cells of the immune system *in vitro* including macrophages and dendritic cells [175-178]. Additionally, it improved inflammation *in vivo* in rodent models of several pathological conditions such as traumatic brain injury [179], Parkinson's disease [180], different models of sepsis [181, 182], and hepatotoxicity [178, 183]. Furthermore, Chen et al. could proof that these anti-inflammatory properties of DXM have a functional impact in autoimmune diseases by investigating effects of DXM in rheumatoid arthritis. DXM improved severity of arthritis accompanied by reduced serum levels for TNFα, IL-6, and interleukin 17A (IL-17A) in mice, as well as human patients [184]. Besides, they could show that *in vitro* treatment of dendritic cells with DXM suppressed the activation of collagen-reactive $CD4^+$ T cells as observed by less proliferation and production of proinflammatory cytokines [184]. This indicates that DXM might also exert anti-inflammatory and immune-modulatory effects in T1DM.

These described anti-inflammatory characteristics of DXM might also be relevant in regard to micro- and macrovascular long-term complications as they often involve inflammatory mechanisms. Notably, DXM showed beneficial effects concerning atherosclerosis and neointima formation, as well as blood pressure and vascular protection in rodents and humans, which are likely partially mediated by reducing oxidative stress [176, 185, 186]. Moreover, 6 months of DXM application improved endothelial function and inflammatory state of male heavy smokers [187]. Even just a single dose of DXM was sufficient to increase flow-mediated dilation (FMD), which is a measure of endothelial function, in 4 probands including a smoker, a diabetic, and an obese one [188]. Besides its effects on the cardiovascular system, DXM and NMDAR antagonists in general were also suggested for the treatment of other diabetic complications like nephropathy [189], retinopathy [190], and neuropathy [188, 191]. In fact, DXM with quinidine was shown to improve diabetic neuropathic pain by relieving leg pain intensity in a phase 3 clinical trial [191]. These studies suggest that the manifold effects of DXM might, as another advantage of this drug, contribute to a better outcome regarding micro- and macrovascular diabetic long-term complications.

Summing up, DXM markedly improves beta cell function and survival in models of T2DM, exerts anti-inflammatory effects in the context of several diseases, and might even ameliorate the outcome of diabetic long-term complications. In conclusion, it seems very reasonable to believe, that DXM might also be a good candidate for the prevention and treatment of T1DM.

3.6. Aims of this study

Current prevention strategies for T1DM focus mainly on modulating the autoreactive immune response, whereas agents targeting beta cells are rather neglected. Still, such agents could

play a crucial role in successful T1DM prevention as they would allow combinatory treatments with immune-modulating agents, which might be necessary to achieve sustainable disease prevention. Therefore, the purpose of this study was to investigate whether DXM is able to improve beta cell function and survival, thereby preventing the progressive decline of beta cell mass in the natural course of T1DM.

Indeed, previous data obtained from *in vitro* experiments with cytokines and *in vivo* experiments using the T2DM *db/db* mouse model suggest that DXM is able to convey islet cell protection which would be a great characteristic regarding T1DM prevention. In order to further confirm these beta cell protective properties of DXO, we performed, as a first step, *in vitro* experiments with isolated mouse and human islets. Since STZ is a beta cell toxin which is commonly used to model T1DM, we investigated whether DXO is able to prevent STZ-induced beta cell death, besides the already shown protection against cytokines.

On the basis of promising *in vitro* data, the next crucial step in the development of a new anti-diabetic drugs includes to test the efficacy of a potential drug candidate *in vivo* using a model which mimics the pathogenesis of the human disease. Hence, we investigated effects of DXM long-term treatment in the NOD mouse, which is widely used as a model for human T1DM. This preclinical study enabled us to explore *in vivo* effects of DXM on T1DM manifestation and disease progression by monitoring blood glucose levels and analyzing islet numbers and sizes of remaining pancreatic islets in an environment of autoimmune-mediated beta cell destruction. Furthermore, we aimed to investigate underlying mechanisms leading to the observed anti-diabetic effects of DXM, especially regarding the number of remaining islets. Thus, we analyzed pancreatic sections for apoptosis and proliferation markers. As the immune system is a major contributor to T1DM pathogenesis, in particular to beta cell death, we additionally assessed whether DXM long-term administration affects the grade of insulitis in NOD mice with the goal of further elucidating possible mechanisms involved in mediating the observed anti-diabetic effects of DXM. Finally, in order to provide information on possible causes leading to less immune cell infiltration in DXM-treated NOD mice, *in vitro* experiments with isolated pancreatic islets were performed with the aim to determine possible effects of DXO on chemoattraction.

In conclusion, the conducted preclinical studies should answer the question whether DXM could be a potential novel candidate for the preventive treatment of T1DM. Hence, this study aimed to investigate whether DXM improves beta cell function and survival, as well as elucidate possible mechanisms involved in the anti-diabetic effects of DXM in the context of T1DM.

4. Material and methods

Parts of the following chapter containing detailed descriptions of applied methods will be used for publication of herein presented data in a scientific journal (see chapter 7).

4.1. Mouse experiments

4.1.1. Mouse models

Different mouse models were used to investigate effects of DXM in the context of T1DM. For *in vitro* studies with isolated islets from wild-type mice, male C57BL/6J mice older than 9 weeks purchased from Janvier were used. To conduct a preclinical study investigating the effects of DXM in a T1DM mouse model, female non-obese diabetic mice (NOD/ShiLtJ, Stock No: 001976) were purchased from The Jackson Laboratory. These mice will be referred to as NOD mice in the following. All mice were housed in rooms with a controlled temperature of 22°C, a humidity of 55%, and a 12:12 hour light/dark cycle in which lights were switched on at 6 a.m. The mice had access to standard laboratory chow and drinking water *ad libitum*. The local Animal Ethics Committee of the Landesamt für Natur, Umwelt und Verbraucherschutz Nordrhein-Westfalen (LANUV North Rhine-Westphalia) approved all animal experiments and they were all performed at the Heinrich Heine University Düsseldorf according to the German Animal Protection Laws.

4.1.2. Long-term treatment of NOD mice

Female NOD mice housed under specific pathogen free (SPF) – conditions were used in a preclinical study to investigate effects of DXM on T1DM onset. From the age of 4 weeks on, half of the NOD mice were treated with 3 g/L DXM (Sigma Aldrich) in their drinking water (DXM group), whereas the other half of mice received no DXM (control group). To determine the influence of DXM on the incidence of diabetes, this incidence cohort including a total of 24 mice with 12 mice per treatment group, was monitored up to an age of 30 weeks. A second prediabetic cohort consisting of 12 mice in total was only monitored up to an age of 10 weeks until none of the mice had become diabetic yet to get insights into mechanisms starting before diabetes onset. Random blood glucose levels of all NOD mice were measured weekly at the tail vein using GlucoSmart® Swing2 glucometer (MSP Bodman GmbH) and their corresponding test strips (MSP Bodman GmbH). A mouse with blood glucose levels exceeding 250 mg/dL on two consecutive days was considered diabetic and sacrificed at this point of the study. At the end of the study, tissues were collected for histochemical analysis.

4.2. *In vitro* methods

4.2.1. Isolation of mouse pancreatic islets

Mouse pancreatic islets were isolated from wild-type mice for *in vitro* experiments according to the protocol published by Yesil et al. with few minor changes [192]. Enzymatic digestion of pancreatic tissue with Liberase TL Research Grade (Roche) at 37°C was stopped after 16 minutes with Dulbecco's Modified Eagle's Medium + GlutaMAX (1 mg/mL glucose) (DMEM, Gibco by Life Technologies) supplemented with 15% heat-inactivated fetal bovine serum (FBS, Gibco by Life Technologies). After washing and filtering steps, islets were separated from exocrine tissues by gradient centrifugation at 1,200 rpm for 25 minutes and collected from the interphase between Lymphoprep™ (Stemcell Technologies) and DMEM. Afterwards, islets were washed twice with islet medium consisting of Connaught Medical Research Laboratories medium 1066 (CMRL, Gibco by Life Technologies) supplemented with 15% heat-inactivated FBS, 100 U/mL penicillin (Gibco by Life Technologies), 100 µg/mL streptomycin (Gibco by Life Technologies), 50 µM β-mercaptoethanol (Gibco by Life Technologies), 0.15% $NaHCO_3$ (Gibco by Life Technologies), and 10 mM glucose (Sigma Aldrich). All further assays with isolated islets were performed after overnight (o/n) culture in islet medium in a humidified atmosphere at 37°C and 5% CO_2 for recovery.

4.2.2. Human pancreatic islets

Human islets were ordered from the NIDDK-funded Integrated Islet Distribution Program (IIDP) at City of Hope in Duarte, California, United States, NIH Grant # 2UC4DK098085 and the JDFR award 31-2008-416: ECIT islet for Basic Research Program at San Raffaele Scientific Institute, Milan, Italy. The respective ethics committees (ethics committee of the Medical Faculty, Heinrich Heine University Düsseldorf, study number 3921; ethics committee of the Instituto Scientifico Ospedale San Raffaele; ethics committee of the IIDP centers) approved all experiment protocols. Only islet isolations displaying a purity >80% and a viability > 80% were accepted for purchase. After arrival, human islets were washed up to three times and cultured in islet medium as described in 4.2.1. Before further usage in functional assays, human islets were cultured o/n in a humidified atmosphere at 37°C and 5% CO_2.

4.2.3. *In vitro* treatment of mouse and human islets for cell death assay

The beta cell toxin STZ (Sigma Aldrich) was used to investigate whether DXO protects murine and human islets *in vitro* against STZ-induced cell death. For this, islets were either cultured in the presence or absence of DXO before STZ was added to induce cell death. Mouse islets were cultured in islet medium containing 1 µM DXO (Sigma Aldrich) for 24 h, before 2 mM STZ, solved in 0.1 M citrate buffer, pH 4.5, was added for another 24 h. Human islets were pretreated with 10 µM DXO in islet medium for 24 h, before 50 mM STZ was added for

additional 24 h. During all incubation steps mouse as well as human islets were cultured at 37°C and 5% CO_2.

4.2.4. *In vitro* treatment of mouse islets for analysis of chemokine expression

For the analysis of mRNA expression of different chemokines under the influence of DXO in islets isolated from C57BL/6J mice, islets were incubated in the presence or absence of 1 µM DXO for 24 h at 37°C and 5% CO_2. Furthermore, to analyze mRNA expression in an inflammatory environment, islets were treated with a cytokine mixture consisting of 1,000 U/mL recombinant mouse TNFα (R&D Systems), 1,000 U/mL recombinant mouse IFNγ (Thermo Fisher Scientific), and 50 U/mL recombinant mouse IL-1β (R&D Systems) for 24 h in the presence or absence of 1 µM DXO. These islets were either pretreated with 1 µM DXO for 1 h or left untreated before the cytokine mixture was added.

4.3. Molecular biological methods

4.3.1. Isolation of RNA

RNA was isolated using the RNeasy Mini Kit (50, Qiagen). After treatment, islets were centrifuged for 2 min, at 3,300 rpm and 4°C. Supernatants were stored at -80°C until usage for analysis of secreted proteins. Remaining cell pellets were suspended in RLT buffer and homogenized for 10 min at 4°C using a cell disruptor (Scientific Industries, Inc). Samples were either processed directly or stored at -80°C until RNA was isolated. From here on, RNA was isolated according to the instructions of the manufacturer (Qiagen). An additional DNA digestion step was included in the RNA isolation process using the RNase-free DNase Kit (Qiagen). RNA yield and quality was measured after isolation with BioMate™ 3 (Thermo Fisher Scientific).

4.3.2. Quantitative real-time polymerase chain reaction (qPCR)

To measure gene expression levels, isolated RNA was transcribed into complementary DNA (cDNA) using SuperScript II Reverse Transcriptase (Invitrogen by Thermo Fisher Scientific) according to manufacturer's protocol. cDNA was diluted 1/5 with dH_2O and stored at -20°C until further usage. Quantitative real-time polymerase chain reaction (qPCR) was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies). Reaction volumes per sample were used as described in the following:

5 µL Brilliant III SYBR Green QPCR Master Mix

2 µL H_2O

1 µL Primer Mix (0.3 µM of reverse and forward primer)

2 µl cDNA

Primers were purchased from Eurogentec and utilized primer sequences can be found in Table 1.

Table 1: List of primers used for qPCR containing gene name, host species and primer sequence.

Gene	Species	Primer Sequence
Ccl2	Mouse	Forward: 5'- TTA AAA ACC TGG ATC GGA ACC AA
		Reverse. 5'- GCA TTA GCT TCA GAT TTA CGG GT
Cxcl1	Mouse	Forward: 5'- ACT GCA CCC AAA CCG AAG TC
		Reverse: 5'- TGG GGA CAC CTT TTA GCA TCT T
Cxcl2	Mouse	Forward: 5'- CCA ACC ACC AGG CTA CAG G
		Reverse: 5'- GCG TCA CAC TCA AGC TCT G
Cxcl16	Mouse	Forward: 5'- CCT TGT CTC TTG CGT TCT TCC
		Reverse: 5'- TCC AAA GTA CCC TGC GGT ATC
Cx3cl1	Mouse	Forward: 5'- ACG AAA TGC GAA ATC ATG TGC
		Reverse: 5'- CTG TGT CGT CTC CAG GAC AA
Gapdh	Mouse	Forward: 5'- TGA GGC CGG TGC TGA GTA TGT CG
		Reverse: 5'- CCA CAG TCT TCT GGG TGG CAG TG
Hprt	Mouse	Forward: 5'-GCT GGT GAA AAG GAC CTC T
		Reverse: 5'-CAC AGG ACT AGA ACA CCT GC
Rplp0	Mouse	Forward: 5' - GAT GCC CAG GGA AGA CAG
		Reverse: 5' - ACA ATG AAG CAT TTT GGA TAA TCA

Quantitative real-time PCR was performed using QuantStudio 1 with QuantStudio™ Design & Analysis Software (Thermo Fisher Scientific) with the following program settings (Table 2):

Table 2: Settings for qPCR using QuantStudio1.

Program	Settings	
Preincubation	95°C for 120s	1 cycle
Amplification	95°C for 5s 60°C for 20s	40 cycles
Melting	95°C for 1s 60°C for 20s 95°C for 1s	1 cycle

Samples were run in triplicates and relative mRNA expression was analyzed according to the comparative ΔCt method as previously described using three different housekeeping genes for normalization (*Gapdh*, *Hprt*, *Rplp0)* [193, 194].

4.3.3. Cytokine measurements in supernatants

Supernatants of islets were used to determine concentrations of secreted chemokines using an enzyme-linked immunosorbent assay (ELISA). For the quantification of CCL2, Mouse CCL2 Quantikine ELISA (MJE00b, R&D Systems) was performed according to manufacturer's instruction and measured on GloMax® Discover Microplate Reader (Promega).

4.4. Staining methods and image analysis

4.4.1. Live cell imaging

To detect dead and viable cells of murine and human islets the LIVE-DEAD Viability-Cytotoxicity Kit (Thermo Fisher Scientific) was used. Islets were stained for 1 h in the dark at 37°C and 5% CO_2 with 4 µM ethidium homodimer-1 (dead cells), 2 µM calcein (viable cells) and Hoechst (1:1000, Thermo Fisher Scientific, cell nuclei) in Krebs Ringer HEPES (KRH) buffer containing 15 mM HEPES (Gibco by Life Technologies), 5 mM KCl (Chemsolute, Th.Geyer), 120 mM NaCl (Carl Roth), 2 mM $CaCl_2$ (Sigma-Aldrich), 10 µM glycine (Sigma-Aldrich), and 24 mM $NaHCO_3$ (Sigma-Aldrich) supplemented with 0.1% bovine serum albumin (BSA, Sigma Aldrich) and 10 mM glucose (Sigma Aldrich).

Images were acquired using a Zeiss laser scanning microscope (LSM) 710 coupled to an Axio Observer.Z1 microscope (Carl Zeiss Microscopy GmbH) equipped with a Plan-Apochromat 20x/0.8 objective. Whole islets were imaged using maximum intensity projections of Z-stacks obtained under 20x magnification. To quantify the amount of dead cells per islet, the image analysis software Fiji (Image J) was used [195]. Briefly, area of ethidium homodimer-1-positive cells (dead cells), determined by global Otsu thresholding, was normalized to islet area.

4.4.2. Immunohistochemical analysis

For immunohistochemical analysis, collected pancreata were fixed in 4% paraformaldehyde (PFA, Chemsolute, Th. Geyer) at 4°C o/n under rotation. Afterwards, tissues were cryopreserved in 8% sucrose (Sigma Aldrich) in PBS for around 4 h and then in 30% sucrose o/n. Both steps were performed at 4°C under rotation. Finally, tissues were embedded in embedding molds (Peel-A-Way®, Polyscience Inc.) with Tissue Tek OCT embedding media (Thermo Fisher Scientific) and stored at -80°C. With a cryostat microtome HM 560 (Thermo Fisher Scientific) 12 µm cryo-sections were made and stored until staining at -20°C. Each Superfrost® plus microscope slide (Thermo Scientific) contained four pancreatic sections per

mouse representing different pancreatic regions, meaning that there was a minimal cutting distance of around 1 mm between two sections on one slide. For immunohistochemical staining, slides were washed three times at room temperature (RT) on a rocking platform with PBS^{++} (5.4 mM KCl, 0.27 M NaCl, 3 mM KH_2PO_4, 1.3 mM Na_2HPO_4, 5 mM $MgCl_2$, 9 mM $CaCl_2$ in H_2O) supplemented with 0.2% Triton-X-100 (AppliChem) before they were blocked in PBS^{++} with 0.2% Triton-X-100 containing 10% normal donkey serum (Jackson Immuno Research) at RT in a humidified atmosphere. Afterwards, slides were incubated o/n at 4°C with primary antibodies (see Table 3) diluted in blocking solution as indicated in Table 3 using a humidified chamber. On the next day, slides were washed five times with PBS^{++} supplemented with 0.2% Triton-X-100 for 5 min at RT on a rocking platform, before slides were incubated for 45 min at RT in a humidified chamber with secondary antibodies (see Table 3) as well as DAPI (Sigma Aldrich, diluted 1:1000 in blocking solution) to counterstain cell nuclei. Finally, slides were washed once more in PBS^{++} with 0.2% Triton-X-100 (3 times for 5 min) at RT, until they were mounted with Fluoroshield™ (Sigma Aldrich). Prior to imaging, stained slides were stored at 4°C.

Table 3: List of used antibodies for immunohistochemical staining and their applied dilution in blocking solution.

Primary Antibodies	Dilution	Secondary Antibodies	Dilution
Guinea-pig anti-mouse insulin (Dako A0564)	1/300	Donkey anti-guinea pig AF488-conjugated (Jackson Immuno Research)	1/200
Rabbit anti-mouse CD4 (abcam ab183685)	1/50	Donkey anti-guinea pig Cy3-conjugated (Jackson Immuno Research)	1/200
Rabbit anti-mouse cleaved caspase-3 (Cell Signaling Technology 9664)	1/75	Donkey anti-rabbit AF488-conjugated (Invitrogen)	1/200
Rabbit anti-mouse glucagon (Santa Cruz sc-13091)	1/100	Donkey anti-rabbit Cy3-conjugated (Jackson Immuno Research)	1/200
Rabbit anti-mouse Ki67 (Merck AB9260)	1/100	Donkey anti-rabbit Cy5-conjugated (Jackson Immuno Research)	1/200
Rat anti-mouse CD45 (R&D Systems MAB114)	1/50	Donkey anti-rat AF488-conjugated (Invitrogen)	1/200
Rat anti-mouse CD8a (Invitrogen 14-0081-82)	1/50	Donkey anti-rat Cy3-conjugated (Jackson Immuno Research)	1/200
Rat anti-mouse FoxP3 (Invitrogen 14-5773-82)	1/100		

4.4.3. Imaging and image analysis

For quantification of insulin- and glucagon-positive area, as well as determination of islet numbers and sizes, images were acquired blinded using a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam MRm and 10x/0.45 as well as 20x/0.8 Plan Apochromat objective lenses (Carl Zeiss Microscopy GmbH). Overview images of pancreatic sections were obtained via tile scans using the 10x objective. For a more detailed view of the islets, each islet consisting of more than five insulin-positive cells was additionally imaged using the 20x objective lens. Per mouse, four sections of different depths of the pancreas were imaged. For all other immunohistochemical analyses, the images of all islets per mouse containing more than five insulin-positive cells were obtained using the Zeiss LSM 710 with a 40x/1.4oil DIC M27 Plan Apochromat objective lens (Carl Zeiss Microscopy GmbH).

Acquired Images were analyzed using FIJI [195]. Insulin- and glucagon-positive areas were determined by Otsu thresholding and as well as counted islet numbers normalized to total pancreatic nuclei area obtained by Li or Otsu thresholding of overview images of pancreatic sections. The sum of obtained insulin- and glucagon-positive areas was calculated as a measure for the islet size. CD4- and CD8-positive area were determined by RenyiEntropy thresholding and also normalized to total pancreatic nuclei area determined by Otsu thresholding of images of pancreatic sections. To determine CC3-positive area of the islets, the positive area was measured by RenyiEntropy thresholding in the islet. Islet area used for normalization was defined by Li thresholding of insulin area. Numbers of Ki67-and FoxP3-positive cell nuclei were obtained by manual counting and normalized to islet area defined by Li thresholding of the insulin area.

4.4.4. Insulitis scoring

To measure insulitis, pancreatic sections were stained for $CD45^+$ cells. Images were acquired blinded using a Zeiss Axio Observer.Z1 microscope equipped with an AxioCam MRm and a 20x/0.8 Plan Apochromat objective lens (Carl Zeiss Microscopy GmbH). The CD45-positive area was measured by RenyiEntropy or Triangle thresholding and normalized to the islet area including the surrounding immune cells. The corresponding insulitis score of the analyzed islet was then defined by the calculated ratio. Score 0 means that < 2% of islet area is infiltrated by immune cells. Score 1 stands for present peri-insulitis with less than 10% of islet area infiltrated, but immune cells still surround the islet and are not invading them. Score 2 means that between 10 and 50% of the islet area is infiltrated. Score 3 includes islets where between 50 and 90% of the islet area is infiltrated, whereas score 4 comprises islets where over 90% of the islet area is infiltrated. To get the mean insulitis score per mouse the average is calculated.

4.5. Statistical analysis

To calculate statistical significance the software Prism 9 (GraphPad Inc.) or Excel 2019 (Microsoft Corporation) were used. The performed statistical test was dependent on the number of groups which had to be compared within one experiment. For the comparison of two groups, either paired two-tailed Student's t-test or unpaired Student's t-test with Welch correction was performed. The performed test is indicated in the figure legends. To analyze more than two groups, one-way ANOVA followed by Tukey's multiple comparisons test was performed. The statistical evaluation of diabetes incidence curves was done utilizing Mantel-Cox log-rank test. Statistical significance was defined by P values < 0.05. Exact P values are given in the figure and its corresponding figure legend. Data are presented as single values

with mean ± standard error of the mean (SEM) except for paired data, diabetes incidence curve, and insulitis score.

4.6. Personal contributions

L. Wörmeyer, funded by the DFG (Deutsche Forschungsgemeinschaft, grant no. 434472323), performed most of the experiments presented in this thesis. L. Wörmeyer was co-supervised by E. Lammert and A. Welters.

J. Marquard, A. Welters, and S. Otter started the research on NMDAR and DXM/DXO in the context of diabetes. A. Welters initiated the project related to type 1 diabetes, performed basic experiments to this work, and handed the project over to L. Wörmeyer.

L. Wörmeyer co-supervised A. Hamacher during her bachelor thesis (2019) in which she established a semi-automatic image quantification tool (FIJI) and performed immunohistochemical analysis of the diabetic NOD mice cohort as stated in figure legends.

L. Wörmeyer co-supervised L. Lucks during her master thesis (2021). L. Lucks investigated effects of DXM and DXO on the cytokine response, proliferation, and apoptosis of T lymphocytes, after isolation and culture of mouse T lymphocytes were established by L. Wörmeyer.

Y. Koh created parts of Figure 6.

5. Results

Previous studies of our lab revealed that the NMDAR antagonist DXM and its active metabolite DXO, which are widely known as ingredients of over-the-counter anti-cough medications, have anti-diabetic effects in mice and humans [170, 173]. In the T2DM *db/db* mouse model and a registered phase 2a proof-of-concept study, DXM treatment led to better glycemic control and higher insulin levels [170]. In addition, and of even greater importance with regard to T1DM, DXM and DXO have been demonstrated to provide beta cell protection in the context of diabetes *in vitro* and *in vivo*. DXM-treated *db/db* mice displayed not only more remaining insulin-positive cells, but also more pancreatic islets due to less beta cell apoptosis [170]. This finding was corroborated by *in vitro* experiments showing that DXO protects islet cells against cytokine-induced cell death in mouse and even human islets [170]. However, it is not known so far whether the observed protective effects could be of benefit in the treatment of T1DM, in which the beta cell destruction is more dramatic than in T2DM. Thus, the following study aimed to elucidate whether DXM might be a new potential candidate for the adjunct treatment of T1DM.

5.1. Protective effects of DXO on pancreatic islets in T1DM

As cytokines are known to play a major role in the destruction of beta cells in T1DM [58, 66] and since our lab already proved that DXO is able to protect islet cells against cytokine-induced cell death [170], it seemed likely that DXM/DXO will beneficially affect the pathogenesis of T1DM. In fact, it seemed reasonable to believe that these effects will be mediated, at least to some extent, by protecting beta cells against the progressive destruction observed during T1DM progression.

5.1.1. DXO protects pancreatic islets against STZ-induced cell death *in vitro*

To further verify that DXO has islet cell protective effects in a model of T1DM, *in vitro* experiments with isolated islets and the beta cell toxin STZ were conducted. STZ is a beta cell-specific cell death stimulus which is commonly used to model T1DM in rodents *in vivo* [196]. Hence, as a first step, the ability of DXO to convey protection against this insult *in vitro* was investigated.

For this purpose, isolated mouse pancreatic islets were pretreated with 1 µM DXO for 24 h before the beta cell toxin STZ was added at a concentration of 2 mM. After another incubation period of 24 h in the presence of 2 mM STZ with or without 1 µM DXO, islets were stained to visualize both dead as well as living cells (Figure 4 A-C). Quantifying the amount of dead islet cells per islet area revealed that STZ treatment significantly increased cell death in islets compared to control-treated islets (Figure 4 D, $p = 0.0004$). Notably, mouse islets which were

pretreated with 1 µM DXO for 24 h, were protected against STZ-induced cell death by DXO. Consequently, these islets showed fewer dead cells compared to STZ-treated islets which had not been treated with DXO (Figure 4 D, p = 0.0035).

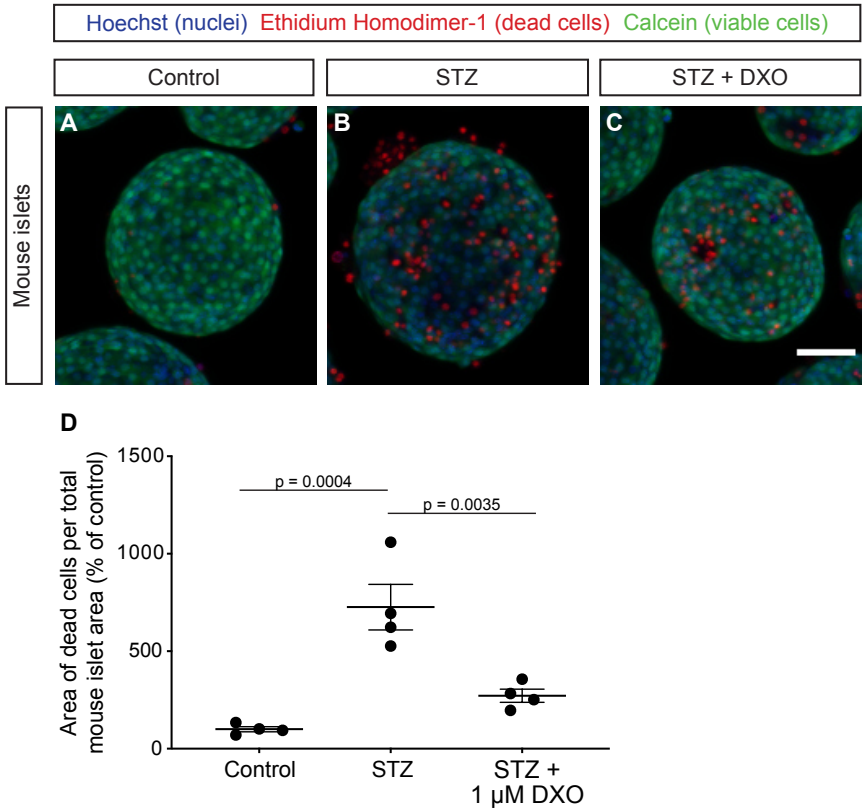

Figure 4: DXO protects mouse islets against STZ-induced cell death *in vitro*. (A-C), Representative LSM images of isolated mouse islets (n ≥ 54 islets per condition) pretreated for 24 h with or without 1 µM DXO before 2 mM STZ was added for another 24 h; blue: Hoechst, all cell nuclei; red: Ethidium Homodimer-1, dead cells; green: Calcein, viable cells; scale bar: 50 µm. (D), Quantification of area of dead cells per mouse islet area (n = 4 for each condition with ≥ 12 islets per batch) shown as percentage of control. Data are presented as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test.

A critical step in the development of new drugs is the translation of promising preclinical results to human studies. Therefore, we were wondering whether DXO is also able to protect human islets against STZ-induced cell death. Thus, we performed similar live cell imaging experiments using isolated human pancreatic islets. Human islets were preincubated with 10 µM DXO for 24 h before 50 mM STZ was added for 24 h to trigger cell death (Figure 5 A-C). Analysis of human islets stained for dead cells clearly showed that a high STZ concentration of 50 mM triggered significantly more cell death compared to control islets (Figure 5 D, $p \leq 0.0001$). Although a higher STZ concentration was required to induce cell death in human islets compared to mouse islets, DXO treatment still resulted in a significant reduction of dead cells compared to STZ treatment alone (Figure 5 D, $p \leq 0.0001$).

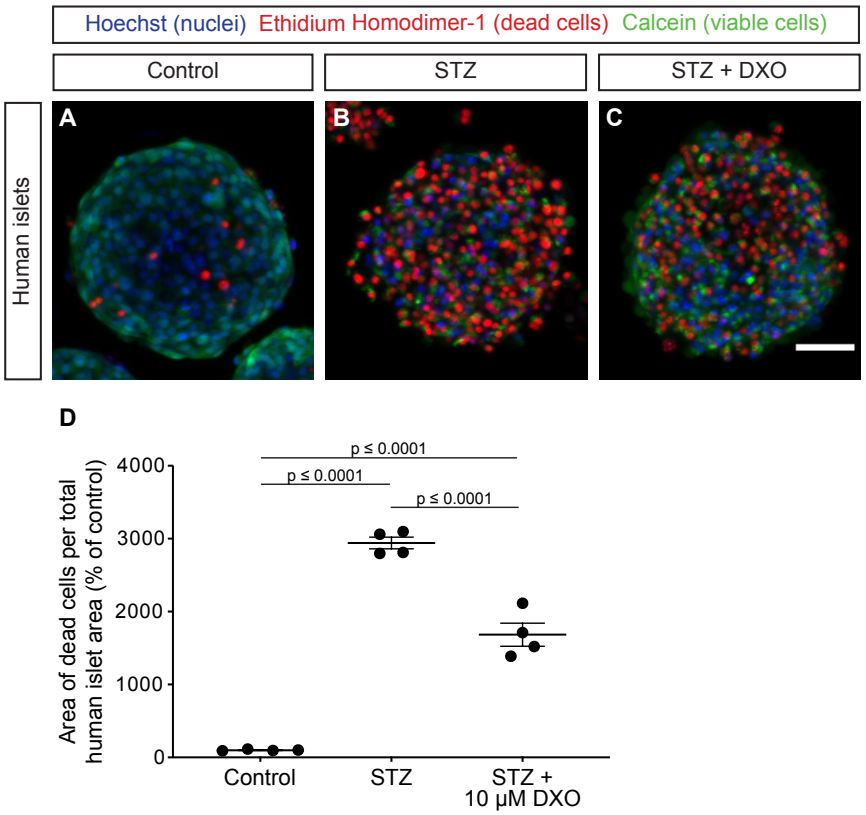

Figure 5: DXO protects human islets against STZ-induced cell death *in vitro*. (A-C), Representative LSM images of isolated human islets (n ≥ 59 islets per condition) treated for 24 h with 50 mM STZ in the presence or absence of 10 µM DXO; blue: Hoechst, all cell nuclei; red: Ethidium Homodimer-1, dead cells; green: Calcein, viable cells; scale bar: 50 µm. (D), Quantification of area of dead cells per human islet area (n = 4 for each condition with ≥ 13 islets per batch) shown as percentage of control. Human islet donor: 57-year-old male, BMI 35.90 kg/m^2. Data are shown as mean ± SEM. Statistical significance was determined using one-way ANOVA followed by Tukey's multiple comparison test.

To sum up, the experiments showed that DXO, besides the already known protective effects against cytokine-induced cell death, is able to convey protection in mouse and human islets against STZ, which is a beta cell-specific toxin commonly used to model T1DM. This observed protection against STZ strongly indicates that DXM/DXO has beta cell protective characteristics that could be particularly useful for the treatment of T1DM.

5.2. Anti-diabetic effects of DXM long-term treatment in NOD mice

Based on our promising *in vitro* data, we conducted a preclinical *in vivo* long-term study using the T1DM NOD mouse model, which spontaneously develops diabetes and whose course of disease shares many similarities with human T1DM such as presence of autoantibodies and insulitis before disease onset [55, 59]. We treated these mice with DXM via the drinking water for several weeks to investigate whether DXM long-term treatment has an impact on diabetes onset and incidence. To be specific, from 4 weeks of age on, NOD mice received either 3 g/L DXM continuously or ordinary drinking water. Different cohorts of mice were studied at different ages in order to track disease progression via immunohistochemical analysis. The prediabetic

cohort (see Figure 6, green) was studied at 10 weeks of age after 6 weeks of DXM treatment. This prediabetic cohort should provide insights into mechanisms that occur prior to diabetes manifestation, thereby helping to elucidate the anti-diabetic effects of DXM. In contrast, the incidence cohort was used to observe effects of DXM on diabetes manifestation and incidence. This cohort was followed until 30 weeks of age and comprised 26 weeks of DXM treatment (see Figure 6, blue), if mice had not become diabetic earlier. Mice were considered diabetic when blood glucose levels exceeded 250 mg/dL for two consecutive days. These mice were then directly sacrificed at this point of the study in order to analyze the state of beta cell demise at diabetes onset (see Figure 6, red).

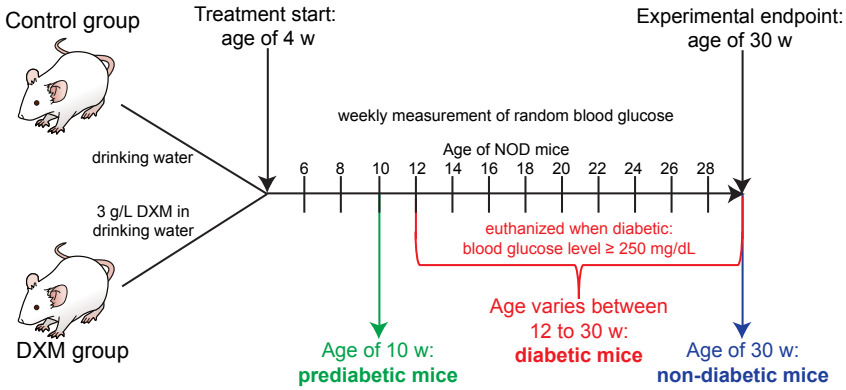

Figure 6: Schematic timeline of the experimental setup to treat NOD mice with DXM *in vivo*. 4-week-old NOD mice permanently received either ordinary drinking water (control group) or 3 g/L DXM via drinking water (DXM group). Mice were euthanized at the age of 10 weeks (prediabetic mice; green), at diabetes onset, defined as blood glucose concentrations ≥ 250 mg/dL on two consecutive days (diabetic mice; red), or following a maximum of 26 weeks of DXM treatment at 30 weeks of age (non-diabetic mice; blue). Parts of this figure were illustrated by Y. Koh.

In order to monitor disease state of NOD mice continuously, random blood glucose levels were measured weekly. At the beginning of the study, blood glucose levels did not differ between treatment groups, but this began to change when the first mice showed rising blood glucose levels and eventually became diabetic. Indeed, the first control mouse became diabetic at an age of 12 weeks, whereas the first DXM-treated NOD mouse did not become diabetic until an age of 17 weeks revealing a delay of diabetes onset for several weeks in the DXM-treated group (Figure 7 A). By the time the first DXM-treated mouse became diabetic, 3 out of 12 control mice had already been diagnosed as diabetic, unveiling that there was not only a delay of diabetes onset, but also a positive effect on diabetes incidence mediated by DXM treatment in NOD mice. At an age of 30 weeks, which was the defined end point of the study, 8 out of 12 control mice (66.7%) were considered diabetic, whereas this was only true for 4 of 12 DXM-treated NOD mice (33.3%) (Figure 7 A). In summary, DXM treatment of NOD mice caused a delay in the onset of diabetes by approximately 5 weeks and a 50% reduction of diabetes incidence in 30-week-old NOD mice (Figure 7 A, $p = 0.0915$). Accordingly, blood glucose

concentrations of DXM-treated NOD mice were permanently lower than blood glucose concentrations of control mice from 15 weeks of age throughout the study (Figure 7 B). At diabetes onset, this blood glucose lowering effect was even more prominent. Although all mice were considered diabetic, meaning that blood glucose levels of all mice exceeded 250 mg/dL, DXM-treated NOD mice still had significantly lower blood glucose concentrations at diabetes manifestation (Figure 7 C, p = 0.0270) suggesting that DXM treatment might improve glycemic control.

Altogether, it can be stated that DXM treatment reduced blood glucose levels, delayed diabetes onset, and overall reduced diabetes incidence in the T1DM NOD mouse model.

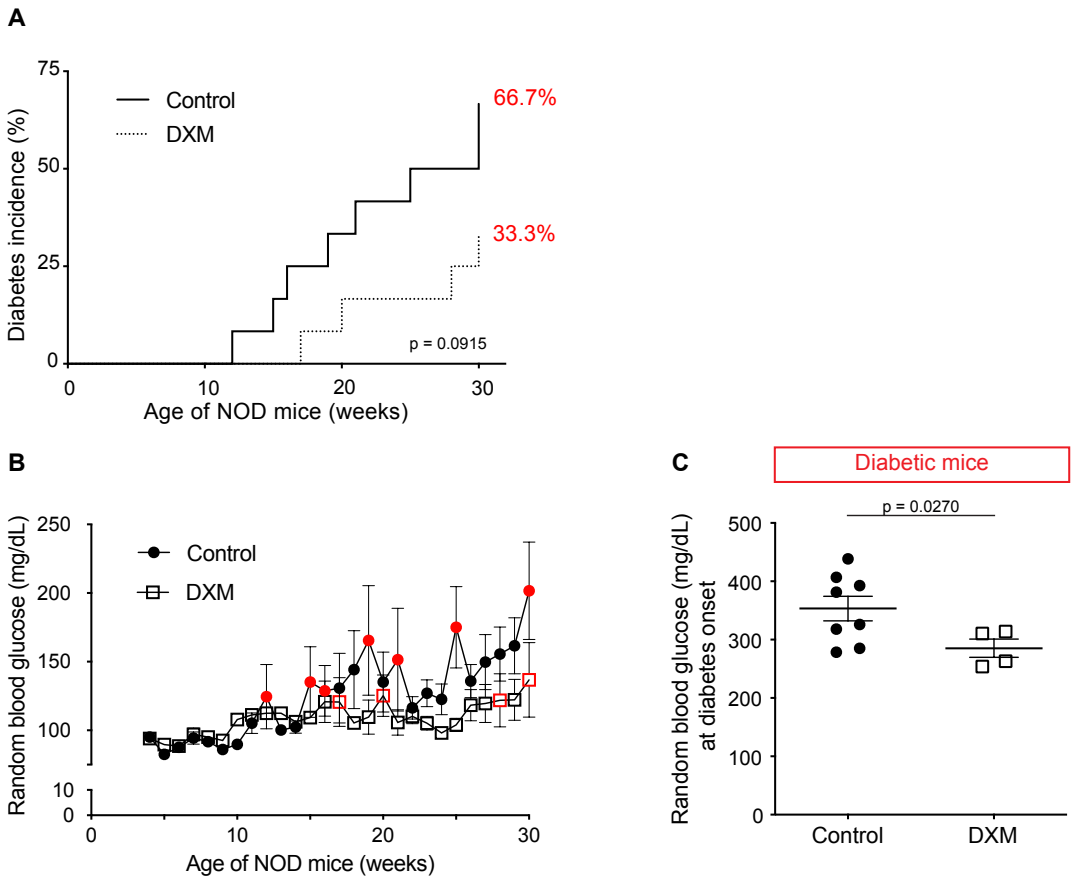

Figure 7: DXM treatment delays diabetes onset, reduces diabetes incidence, and lowers blood glucose concentrations in NOD mice. (A), Kaplan-Meier curve showing diabetes incidence in DXM-treated NOD mice compared to untreated control NOD mice (n = 12 mice per treatment group, p = 0.0915). (B), Random blood glucose concentrations of NOD mice treated as described in (A) throughout the *in vivo* study (n = 12 mice each at study begin). Marked in red are timepoints when mice became diabetic and were sacrificed, so that the following timepoints only refer to the remaining mice. (C), Random blood glucose levels of either untreated or DXM-treated NOD mice at diabetes onset (n ≥ 4 mice per treatment group, p = 0.0270). Statistical significance was determined by (A) Mantel-Cox log-rank test and (C) unpaired Student's t-test. Data in (B) and (C) are presented as mean ± SEM.

5.2.1. DXM long-term treatment leads to increased beta and alpha cell areas in NOD mice

Based on our hypothesis that DXM treatment conveys protection against the progressive beta cell destruction in NOD mice, we wondered whether the observed anti-diabetic effects were related to changes in beta and alpha cell mass of NOD mice. In the natural course of disease in NOD mice, beta cell mass declines due to beta cell destruction and eventually leads to hyperglycemia because the remaining beta cells are unable to compensate for the lost insulin secretory capacity [197, 198]. Since DXM treatment improved hyperglycemia and reduced diabetes incidence in our *in vivo* study, we investigated whether this was caused by effects on the remaining beta and/or alpha cell mass.

In order to analyze the beta, as well as the alpha cell mass, with disease progression, we immunohistochemically stained pancreatic sections for insulin and glucagon at different ages. A total of three different timepoints were analyzed for insulin- and glucagon-positive areas: 10-week-old prediabetic NOD mice, NOD mice at diabetes onset with differing ages (12 to 30 weeks old), and 30-week-old non-diabetic NOD mice that had not become diabetic during the study. For each mouse, four pancreatic sections of different pancreatic depths were analyzed. Mice of the prediabetic cohort were treated with DXM for 6 weeks before their pancreas was harvested and used for immunohistochemical analysis. However, no difference in either beta or alpha cell area per total pancreatic nuclei area was revealed in 10-week-old prediabetic NOD mice after 6 weeks of continuous DXM treatment. The islets still exhibited normal architecture, with most insulin-positive cells located at the center and glucagon-positive cells located rather on the periphery of islets (Figure 8 A, B), as described in the literature [6]. The insulin-positive area comprised around 1.3% of pancreatic nuclei area, regardless of DXM treatment (Figure 8 C). The proportion of glucagon positive area was lower at 0.36% of pancreatic nuclei area (Figure 8 D).

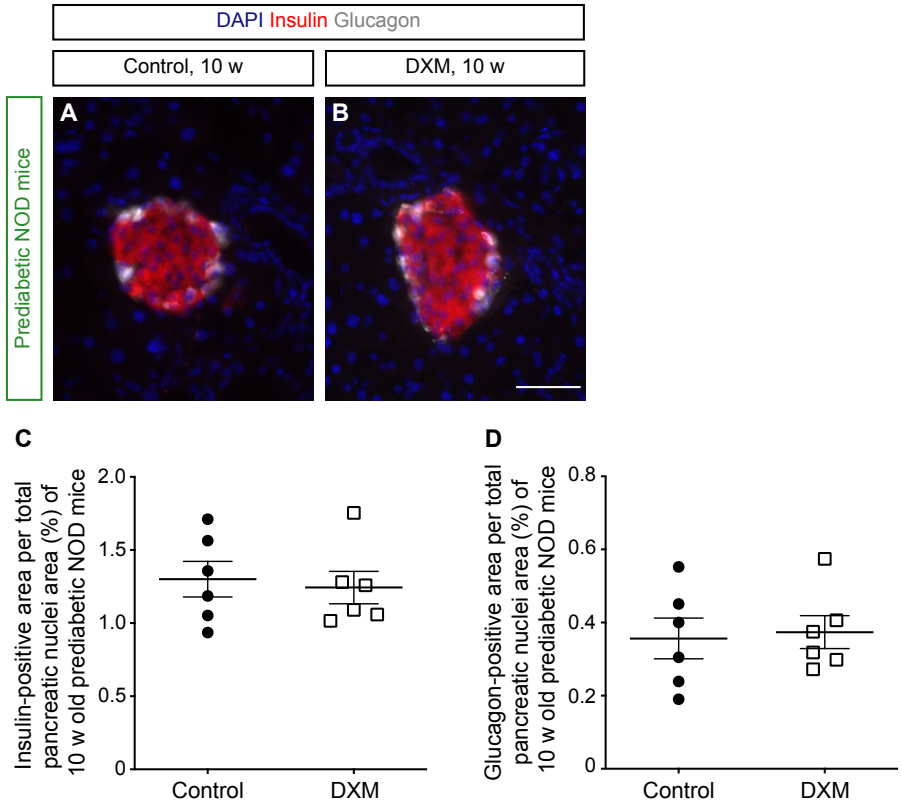

Figure 8: Beta and alpha cell areas are not altered by DXM treatment in prediabetic NOD mice. (A, B), Representative fluorescence microscopic images of pancreatic islets (n ≥ 60 islets per mouse) of either (A) untreated or (B) DXM-treated 10-week-old prediabetic NOD mice; blue: DAPI; red: insulin; grey: glucagon; scale bar: 50 µm. (C, D), Quantification of (C) insulin- ($p = 0.7362$), and (D) glucagon-positive area ($p = 0.8119$) per total pancreatic nuclei area (≥ 60 islets per mouse with n = 6 mice per treatment group) of prediabetic NOD mice treated as described in (A, B). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

Analysis of beta and alpha cell areas in NOD mice at diabetes onset revealed that many of the islet cells must have been gone before disease manifests, as insulin-positive area per total pancreatic nuclei area was on average less than 0.5% in all mice. Still, mice treated with DXM displayed higher remaining insulin- and glucagon-positive areas. The insulin-positive area of DXM-treated NOD mice was, on average, almost twice as high as that of control mice (Figure 9 C, $p = 0.0513$). Additionally, the glucagon-positive area was increased by 50% in DXM-treated NOD mice (Figure 9 D, $p = 0.0611$). This observation was even more stunning considering the fact that DXM-treated NOD mice were older than mice of the control group at diabetes onset. On average, diabetic control mice were 21.0 weeks old, whereas DXM-treated NOD mice were on average 23.8 weeks old at diabetes onset. Islets of diabetic mice displayed more irregular shapes than islets of prediabetic mice, due to the ongoing insulitis, but still not all islets were infiltrated (Figure 9 A, B). In addition, in both treatment groups, there were some pseudoatrophic islets which contained only glucagon-positive cells, but no insulin-positive ones. However, the amount of these islets did not differ between treatment groups (data not shown).

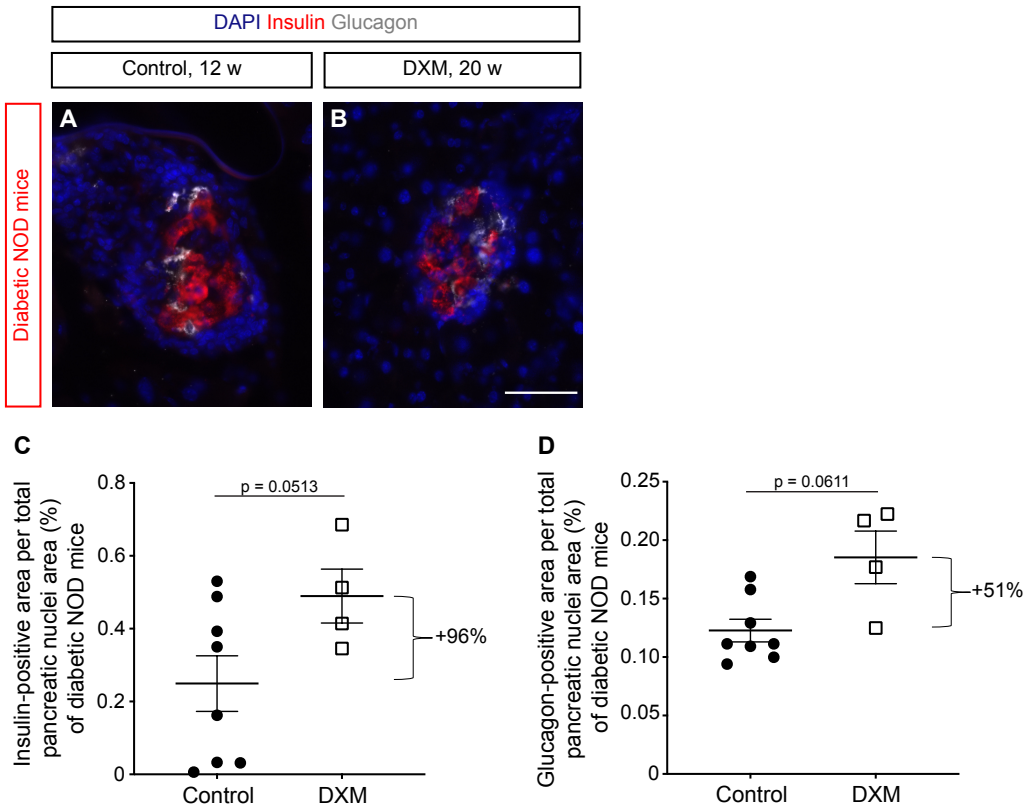

Figure 9: Remaining beta and alpha cell areas are higher in DXM-treated NOD mice at diabetes onset. (A, B), Representative fluorescence microscopic images of pancreatic islets (n ≥ 10 islets per mouse) of either (A) untreated (age 12 weeks) or (B) DXM-treated (age 20 weeks) diabetic NOD mice; blue: DAPI; red: insulin; grey: glucagon; scale bar: 50 µm. (C, D), Quantification of (C) insulin- (p = 0.0513) and (D) glucagon-positive area (p = 0.0611) per total pancreatic nuclei area (≥ 10 islets per mouse with n ≥ 4 mice per treatment group) of diabetic NOD mice treated as described in (A, B). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM. Analysis was performed by Anna Hamacher.

In older, 30-week-old non-diabetic NOD mice which had been treated with DXM for 26 weeks, the effect of DXM on islet cells was even more pronounced. The insulin-positive area per total pancreatic nuclei area was approximately 0.26% in 30-week-old non-diabetic control mice, whereas this was increased by 119% to 0.58% in DXM-treated mice (Figure 10 C, p = 0.0500). Similar effects were observed regarding the glucagon-positive area which was about 0.06% in control mice, but remained at 0.15% with DXM treatment showing an increase of 150% (Figure 10 D, p = 0.0153). As seen in islets of diabetic mice, the shape of some islets was quite irregular in non-diabetic NOD mice due to immune cell infiltration (Figure 10 A, B). Furthermore, insulitis, which appeared in the form of DAPI-positive cell nuclei around islets, was heterogeneous in both treatment groups. Some islets were massively infiltrated, whereas adjacent islets were sometimes not infiltrated at all. On some pancreatic sections, there were even huge infiltrated areas covering more than one islet so that it was difficult to tell whether it was one large islet or several small islets connected by one big layer of lymphocytes.

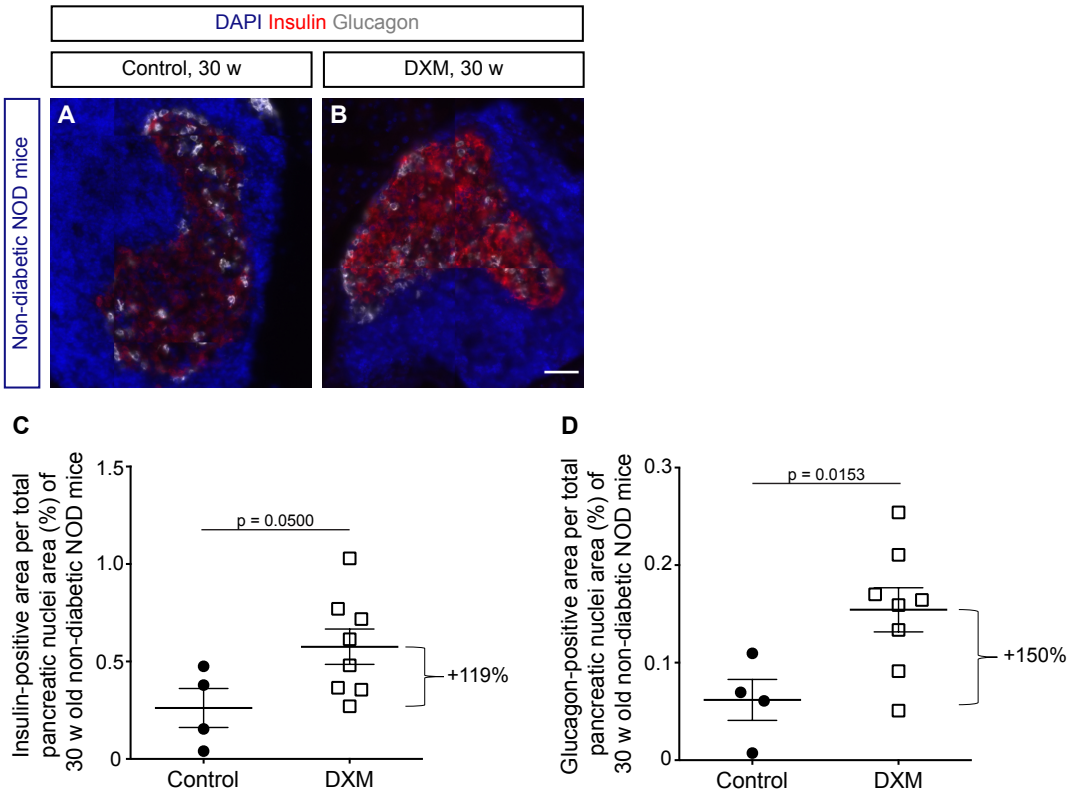

Figure 10: In 30-week-old non-diabetic NOD mice, DXM treatment leads to increased remaining beta and alpha cell areas. (A, B), Representative fluorescence microscopic images of pancreatic islets ($n \geq 2$ islets per mouse) of non-diabetic NOD mice either (A) untreated or (B) DXM-treated; blue: DAPI; red: insulin; grey: glucagon; scale bar: 50 µm. (C, D), Quantification of (C) insulin- ($p = 0.0500$) and (D) glucagon-positive area ($p = 0.0153$) per total pancreatic nuclei area (≥ 2 islets per mouse with $n \geq 4$ mice per treatment group) of non-diabetic NOD mice treated as described in (A, B). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

In summary, the analysis of insulin- and glucagon-positive areas revealed a long-term effect of DXM treatment on beta and alpha cell mass in diabetic, as well as non-diabetic NOD mice, but not in 10-week-old prediabetic NOD mice. Precisely, in diabetic and non-diabetic NOD mice, DXM treatment resulted in an insulin-positive area that was twice as high, and also an increased glucagon-positive area.

5.2.2. Long-term treatment with DXM leads to higher remaining islet numbers in NOD mice during T1DM progression

There are two possible explanations for the observed increase in beta and alpha cell mass upon DXM treatment. First, DXM treatment results in larger islets. Second, DXM treatment does not change the size of islets, but increases their number. Mere comparison of insulin- and glucagon-positive area cannot distinguish whether there are more or larger islets. Hence, the following analysis was performed to answer this question. As a measure of the islet size, the sum of insulin and glucagon-positive areas per islet was calculated. Additionally, pancreatic sections stained for insulin and glucagon were used to count islet numbers. Islets were

counted, if they contained at least five insulin-positive cells. Pseudoatrophic islets that no longer contained insulin were not included. These analysis were performed on the same slides and at the same three timepoints (prediabetic, diabetic, and non-diabetic mice) as analysis of insulin- and glucagon-positive areas to track progression of disease.

Counting the pancreatic islets of 10-week-old prediabetic NOD mice revealed little difference in the number of islets between treatment groups (Figure 11 A, B), consistent with the previously mentioned findings that insulin- and glucagon-positive area did not differ at this age. There was only a tendential increase of 17% in the number of islets counted per total pancreatic nuclei area when NOD mice were treated with DXM (Figure 11 C, $p = 0.1222$). Overall, islet numbers were high, and on average 89 islets distributed on four pancreatic sections were counted per mouse (data not shown), which is typical for this early state of T1DM. On the contrary, the islet size of prediabetic NOD mice tended to be reduced in NOD mice treated with DXM (Figure 11 D, $p = 0.2177$). Islets of control NOD mice were on average 7,171 μm^2 large, whereas the size was reduced by 16% in DXM-treated NOD mice to on average 6,008 μm^2 (Figure 11 D).

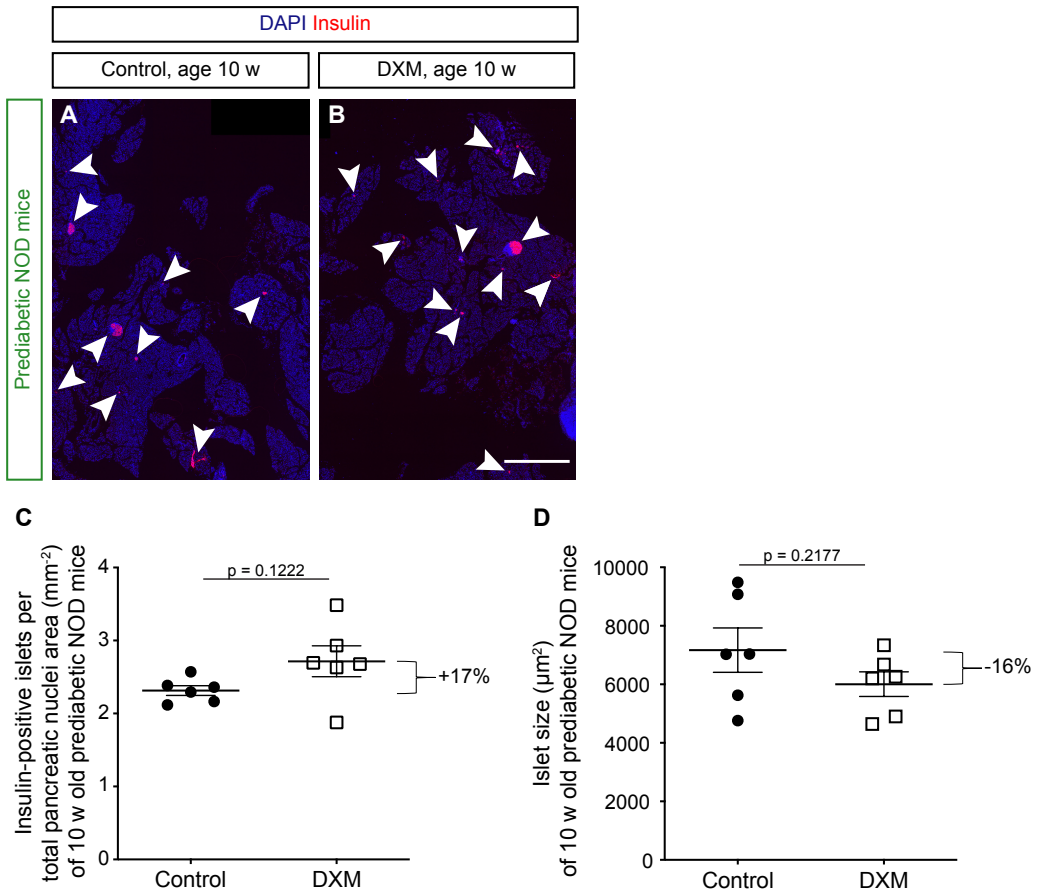

Figure 11: Islet numbers are slightly increased, whereas islet sizes are numerically reduced in DXM-treated prediabetic NOD mice. (A, B), Representative fluorescence microscopy images of pancreatic sections (n = 4 cross-sections per mouse) of (A) untreated or (B) DXM-treated prediabetic NOD mice; blue: DAPI; red: insulin; arrow heads mark pancreatic islets; scale bar: 1000 µm. (C), Islet number per total pancreatic nuclei area (p = 0.1222) and (D), Islet size (µm^2) (p = 0.2177) of prediabetic NOD mice (≥ 60 islets in 4 cross-sections per mouse with n = 6 mice per treatment group,). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM.

As expected according to the NOD mouse model used, islet numbers were massively reduced at diabetes onset in both treatment groups. Stunningly, however, the extent of islet number reduction was still different. NOD mice treated with DXM displayed higher remaining islet numbers compared to control mice (Figure 12 A, B). In control NOD mice, islet numbers per total pancreatic nuclei area had decreased to 0.6 islets/mm^2 pancreatic nuclei area, which was only about 25% of the average islet number in prediabetic control NOD mice. Nonetheless, in NOD mice treated with DXM, the islet number was only decreased to 1.0 islets/mm^2 which was 72% higher than in control mice at diabetes onset (Figure 12 C, p = 0.0026). This indicated that DXM might slow down islet cell destruction. In total, islets numbers per mouse were low and demonstrated the progressive destruction of islet cells. In control NOD mice on average 15 islets distributed on four sections were left at diabetes onset, whereas in DXM-treated NOD mice on average 26 islets remained (data not shown). Size of the islets, defined as sum of insulin- and glucagon-positive area, did not differ between treatment groups at diabetes onset

(Figure 12 D, p =0.9229), suggesting that there are rather more than larger islets in DXM-treated diabetic NOD mice.

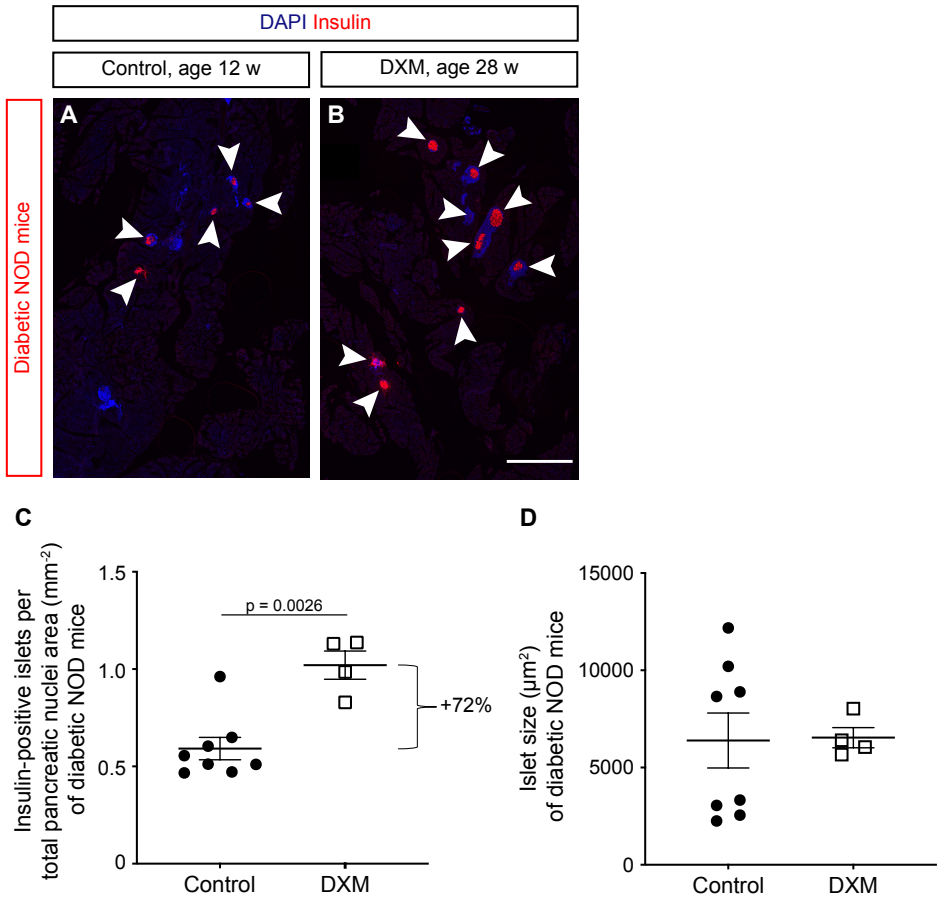

Figure 12: At diabetes onset, islet numbers are increased, whereas islet sizes remain unaltered in NOD mice treated with DXM. (A, B), Representative fluorescence microscopy images of pancreatic sections (n = 4 cross-sections per mouse) of (A) untreated (age 12 weeks) or (B) DXM-treated (age 28 weeks) NOD mice at diabetes onset; blue: DAPI; red: insulin; arrow heads mark pancreatic islets; scale bar: 1000 µm. (C), Islet number per total pancreatic nuclei area (p = 0.0026) and (D), Islet size (μm^2) (p = 0.9229) of diabetic NOD mice (≥ 10 islets in 4 cross-sections per mouse with n ≥ 4 mice per treatment group). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM. Analysis was performed by Anna Hamacher.

These findings in diabetic mice were corroborated by analyzing pancreatic sections of non-diabetic NOD mice. The islet number per total pancreatic nuclei area of 30-week-old control NOD mice was likewise strongly decreased compared to prediabetic mice showing that disease has progressed. However, the mice were still able to control their blood glucose levels, so they were not yet classified as diabetic in this study. More interestingly, comparing the islet number of 30-week-old non-diabetic NOD mice with or without DXM treatment revealed a huge difference (Figure 13 A, B). NOD mice treated with DXM for 26 weeks showed a five-fold higher islet number compared to control mice of the same age which was an increase of more than 400% (Figure 13 C, p = 0.0030). The islet number in the DXM group was decreased to 1.2 islets/mm^2 which was a 50% reduction compared to prediabetic mice. In contrast, islet

numbers in the control group had even decreased to 0.2 islets/mm^2 (Figure 13 C). In general, islet numbers were very low in the control group with on average 12 islets distributed on 4 sections per mouse. Still, in DXM-treated 30-week-old non-diabetic NOD mice total remaining islet numbers were higher with on average 61 islets on 4 pancreatic sections per mouse (data not shown). This aspect once again supports the idea that DXM treatment leads to more, but not larger islets. In line with this, islet size of DXM-treated NOD mice was not larger but even smaller than in the control group (Figure 13 D, p = 0.0445), ultimately proving that DXM does not increase the size of islets in NOD mice. Interestingly, islet size in prediabetic and diabetic NOD mice varied between 6,000 and 7,000 µm^2 in this study (Figure 11 D, Figure 12 D). However, the averaged islet size of 30-week-old non-diabetic control NOD mice was approximately two-fold larger with 12,579 µm^2, whereas the islet size of DXM-treated 30-week-old NOD mice remained with 6,554 µm^2 in a range similar to the islet sizes of the other cohorts (Figure 13 D).

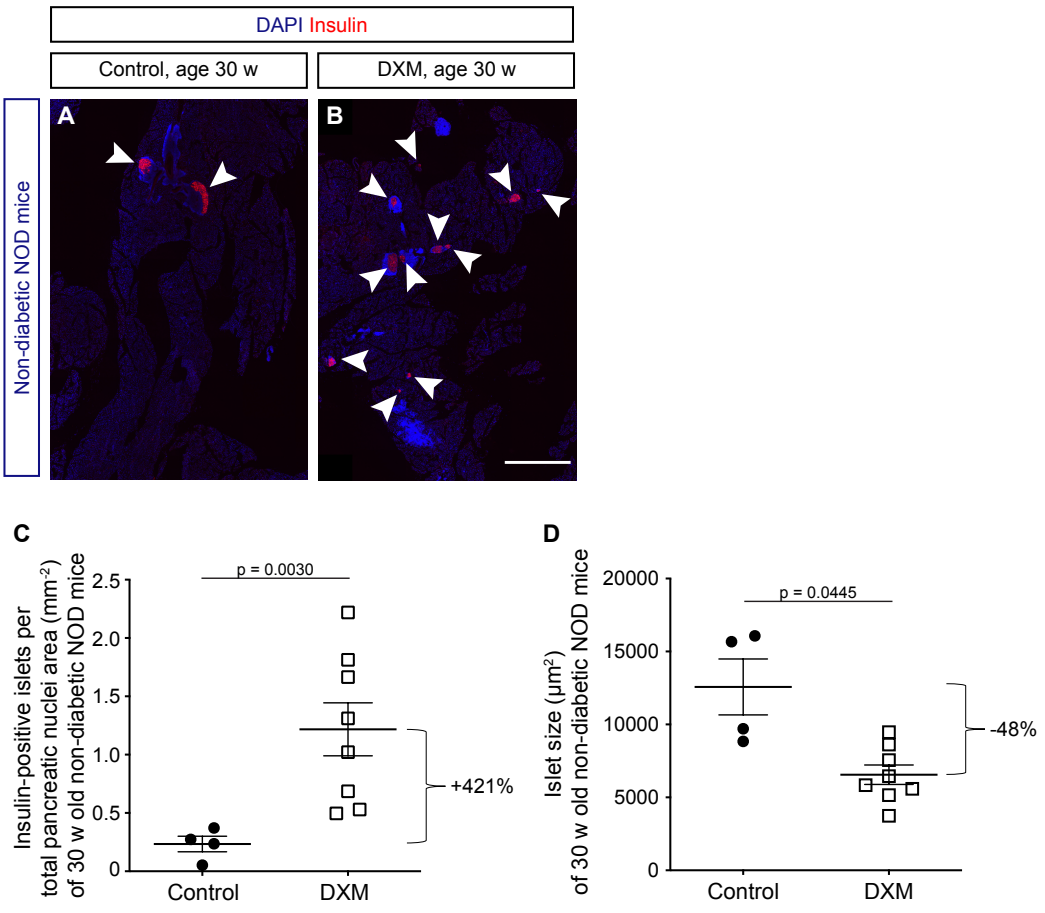

Figure 13: Remaining islet numbers of DXM-treated 30-week-old non-diabetic NOD mice are higher, whereas islet sizes are reduced. (A, B), Representative fluorescence microscopy images of pancreatic sections (n = 4 cross-sections per mouse) of 30-week-old non-diabetic NOD mice either (A) untreated or (B) treated with DXM; blue: DAPI; red: insulin; arrow heads mark pancreatic islets; scale bar: 1000 µm. (C), Islet number per total pancreatic nuclei area (p = 0.0030) and (D), Islet size (µm^2) (p = 0.0445) of non-diabetic NOD mice (≥ 2 islets in 4 cross-sections per mouse with n ≥ 4 mice per treatment group). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM.

As a conclusion, it can be stated that DXM treatment in NOD mice not only leads to higher insulin- and glucagon-positive areas, but moreover to higher remaining islet numbers rather than larger islets in mice of the same age during progression and manifestation of T1DM. The strongest effects on islet numbers were observed in the non-diabetic NOD cohort, in which DXM treatment lasted the longest with 26 weeks. The islet size in prediabetic and diabetic NOD mice was not significantly altered by DXM treatment, but we observed that islets in 30-week-old non-diabetic control NOD mice on average became larger. However, this increase in islet size was not found in islets of DXM-treated non-diabetic NOD mice.

5.2.3. Long-term treatment with DXM protects against progressive islet cell destruction in NOD mice

In order to explore how DXM treatment increases islet numbers and insulin, as well as glucagon areas in NOD mice, further experiments are needed. On the one hand, the observed effects may be explained by protective effects of DXM. On the other hand, it is possible that DXM induces proliferation of islet cells, which would also lead to increased insulin-, glucagon-positive areas and islet numbers.

In summary, comparing the islet numbers per total pancreatic nuclei area in the different cohorts, it became evident that islet numbers decreased with age, as expected. Strikingly, DXM treatment led to increased islet numbers at all timepoints examined. While the increase of islet numbers in prediabetic 10-week-old NOD mice under DXM treatment was with 17% quite small this effect became stronger as the mice got older and the treatment lasted longer, finally resulting in a five-fold increased number of islets in 30-week-old non-diabetic NOD mice. Based on the fact that islet numbers declined progressively in the control group and that this decline was not as severe in DXM-treated NOD mice (Figure 14), it seemed likely that DXM conveys partial protection of islet cells against the progressive destruction.

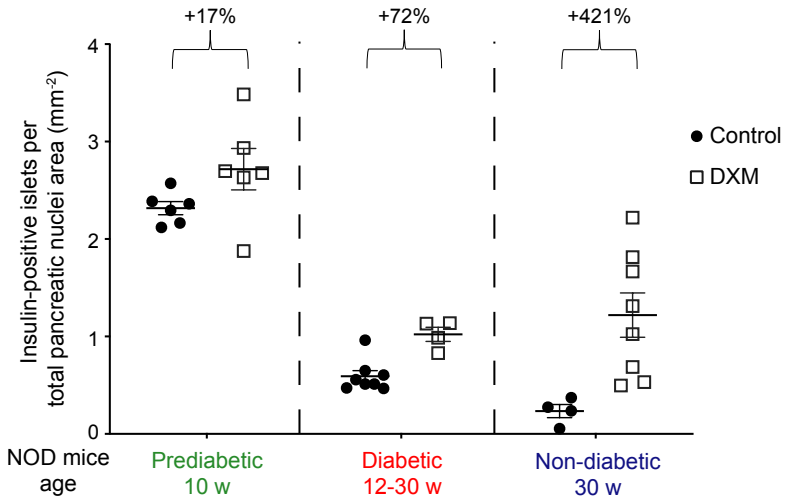

Figure 14: The progressive decline of islet numbers in the NOD mice is partially prevented by DXM treatment. Summary of islet numbers per total pancreatic nuclei area in different NOD mice cohorts either untreated or treated with 3 g/L DXM in their drinking water from an age of 4 weeks on (4 cross-sections per mouse with n ≥ 4 mice per treatment group). Dashed lines mark different cohorts analyzed separately. Data are shown as mean ± SEM.

To support this idea, further experiments were performed. Pancreatic sections of NOD mice treated continuously with or without DXM were stained for the apoptosis marker CC3. All three different timepoints were analyzed to investigate whether DXM exerts a sustained effect and thus continuously prevents islet cell death from the start of treatment.

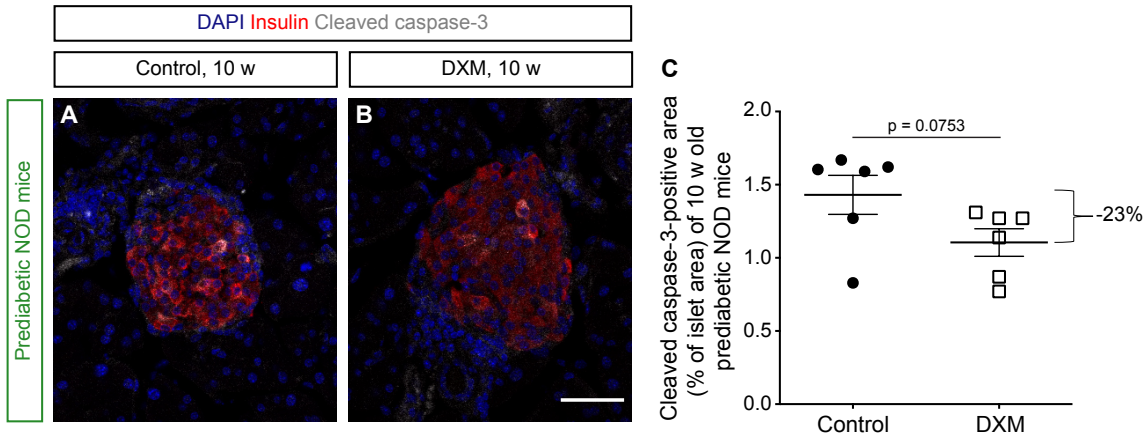

Figure 15: Islet cell survival is improved in prediabetic NOD mice by DXM treatment. (A, B), Representative LSM images of pancreatic islets of 10-week-old prediabetic NOD mice either (A) untreated or (B) continuously treated with 3 g/L DXM for 6 weeks (n ≥ 47 islets per mouse); blue: DAPI; red: insulin; grey: cleaved caspase-3; scale bar: 50 µm. (C), Cleaved caspase-3 positive area per islet area (%) of prediabetic NOD mice (≥ 47 islets per mouse with n = 6 mice per treatment group, p = 0.0753). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM.

Although islet numbers and insulin-, as well as glucagon-areas were only slightly altered after 6 weeks of DXM treatment, the expression of the apoptosis marker CC3 within islets was

surprisingly reduced by 23% in prediabetic NOD mice treated with DXM compared to the control group (Figure 15 A, B). Whereas islets of control NOD mice showed 1.43% CC3-positive area, DXM-treated NOD mice only displayed 1.10% CC3-positive area per islet area (Figure 15 C, p=0.0753).

This reduction of CC3 expression became also evident when comparing islets of diabetic NOD mice treated with or without DXM (Figure 16 A, B). Similar to the prediabetic cohort, apoptosis was reduced in islets of diabetic NOD mice treated with DXM by 31% (Figure 16 C). Interestingly, the proportion of CC3-positive area per islet area was similar to that observed in prediabetic mice albeit diabetes manifestation. In diabetic control NOD mice, we found 1.51% CC3-positive area per islet area, which was reduced to 1.03% in DXM-treated NOD mice at diabetes onset. It should however be noted that the data displayed a high standard deviation concerning the expression of CC3 indicating that islet cell death did not occur to the same extent in all mice.

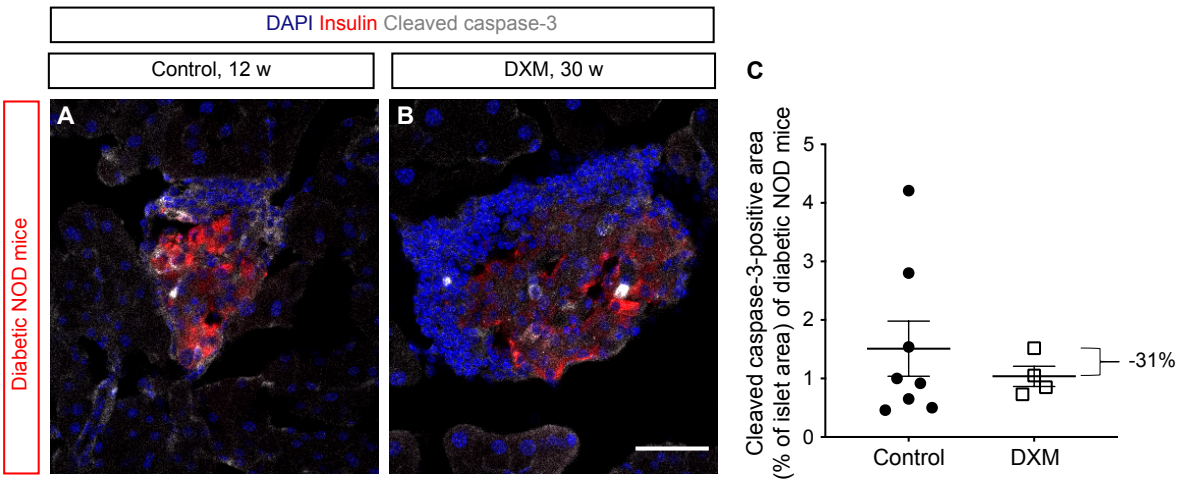

Figure 16: Apoptosis is reduced in diabetic NOD mice treated with DXM. (A, B), Representative LSM images of pancreatic islets of diabetic NOD mice either (A) untreated (age 12 weeks) or (B) continuously treated with 3 g/L DXM (age 30 weeks) (n ≥ 3 islets per mouse); blue: DAPI; red: insulin; grey: cleaved caspase-3; scale bar: 50 µm. (C), Cleaved caspase-3 positive area per islet area (%) of diabetic NOD mice (≥ 3 islets per mouse with n ≥ 4 mice per treatment group, p = 0.3704). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM.

In contrast to the previous results, a reduction in cell death was not found comparing the expression of CC3 in islets of 30-week-old non-diabetic NOD mice between treatment groups (Figure 17 A, B). Irrespective of DXM treatment, 30-week-old NOD mice showed on average approximately 1.6% CC3-positive area per islet area (Figure 17 C). However, it is important to keep in mind that there were only a few islets left in these mice, especially in the control group. Of note, no insulin-positive islet at all was found in one mouse of the control group, although four sections of different depths within the pancreas were examined, demonstrating how difficult this analysis was in the control group of the 30-week-old non-diabetic cohort.

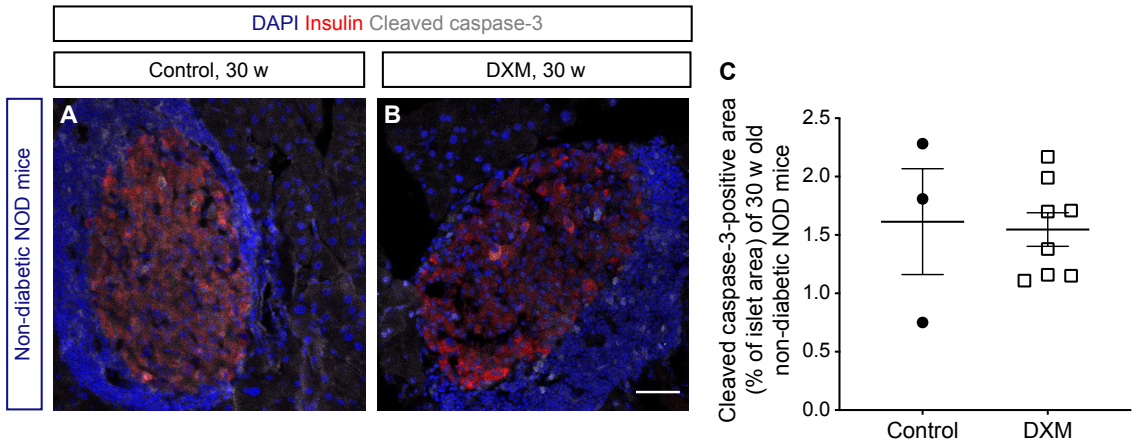

Figure 17: Islet cell death is not changed by DXM treatment in non-diabetic NOD mice. (A, B), Representative LSM images of pancreatic islets of 30-week-old non-diabetic NOD mice either (A) untreated or (B) continuously treated with 3 g/L DXM ($n \geq 3$ islets per mouse); blue: DAPI; red: insulin; grey: cleaved caspase-3; scale bar: 50 µm. (C), Cleaved caspase-3 positive area per islet area (%) of non-diabetic NOD mice (≥ 3 islets per mouse with $n \geq 4$ mice per treatment group, $p = 0.8983$). Statistical significance was determined by unpaired Student's t-test. Data are presented as mean ± SEM.

In summary, all obtained results point to a DXM-mediated reduction in islet cell death. However, the possibility that DXM may induce proliferation should not be neglected. Therefore, pancreatic sections of prediabetic mice were stained for the proliferation marker Ki67 to count proliferating islet cells (Figure 18 A, B). However, quantification of proliferating islet cells in prediabetic NOD mice revealed no difference irrespective of DXM treatment (Figure 18 C), further supporting the hypothesis that DXM tends to reduce islet cell death which results in increased islet numbers.

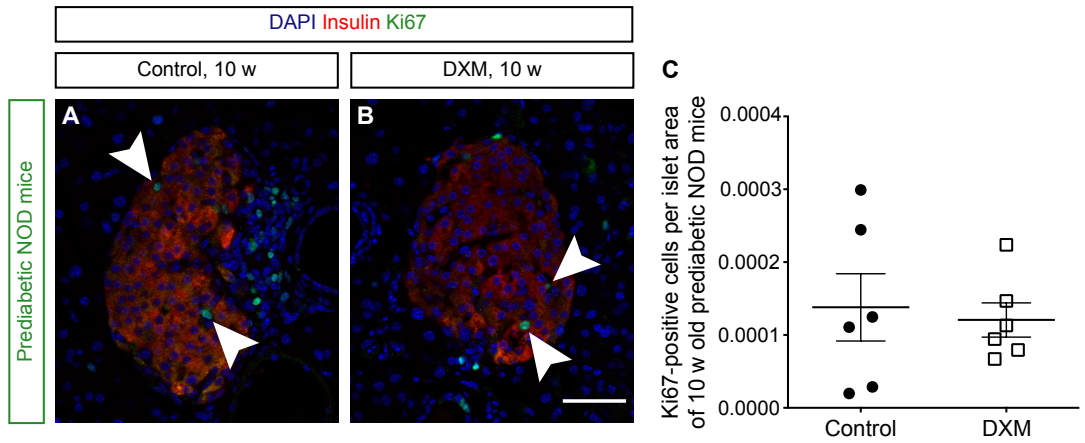

Figure 18: Proliferation is not induced by DXM treatment in prediabetic NOD mice. (A, B), Representative LSM images of pancreatic islets of 10-week-old prediabetic NOD mice either (A) untreated or (B) continuously treated with 3 g/L DXM for 6 weeks ($n \geq 38$ islets per mouse); blue: DAPI; red: insulin; green: Ki67; arrow heads mark proliferating cells; scale bar: 50 µm. (C), $Ki67^+$ cells per islet area of 10-week-old prediabetic NOD mice (≥ 38 islets per mouse with $n = 6$ mice per treatment group, $p = 0.7490$). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

In conclusion, analysis of islet numbers together with the results concerning apoptosis and proliferation of NOD mice treated with DXM demonstrated that the anti-diabetic effects of DXM are rather conveyed by preventing or delaying progressive islet destruction than induction of proliferation.

5.2.4. Insulitis in prediabetic and non-diabetic NOD mice is improved by DXM long-term treatment

Since it is known that the autoimmune attack is one of the most important factors in the manifestation of T1DM, we wondered whether insulitis in NOD mice might be affected by the systemic application of DXM via drinking water. In fact, there is good evidence in the literature that NMDAR are expressed on lymphocytes and that their function is differentially regulated by these receptors [199-205]. In particular, it has already been described that the tested drug DXM reduces the expression of inflammatory cytokines such as TNFα, IL-6, and IL-17A in the context of an autoimmune disease and inhibited dendritic cell-mediated activation $CD4^+$ T lymphocytes *in vitro* [177, 184]. However, to our knowledge, nothing is known about the influence of DXM on the immune response in T1DM.

To address this question, pancreatic sections of NOD mice were stained for the leucocyte common antigen CD45 along with insulin and glucagon to visualize the islets and all infiltrating leucocytes. Thus, we were able to describe the degree of insulitis by calculating the infiltrated islet area and classifying the islets as described previously [206]: Score 0 means that no immune cells were present. Score 1 represented islets with peri-insulitis. Islets of score 2 displayed between 10 and 50% of infiltrated islet area. An infiltrated islet area between 50 and 90% was designated as score 3, and the highest score 4 represented islets in which more than 90% of the islet area was infiltrated.

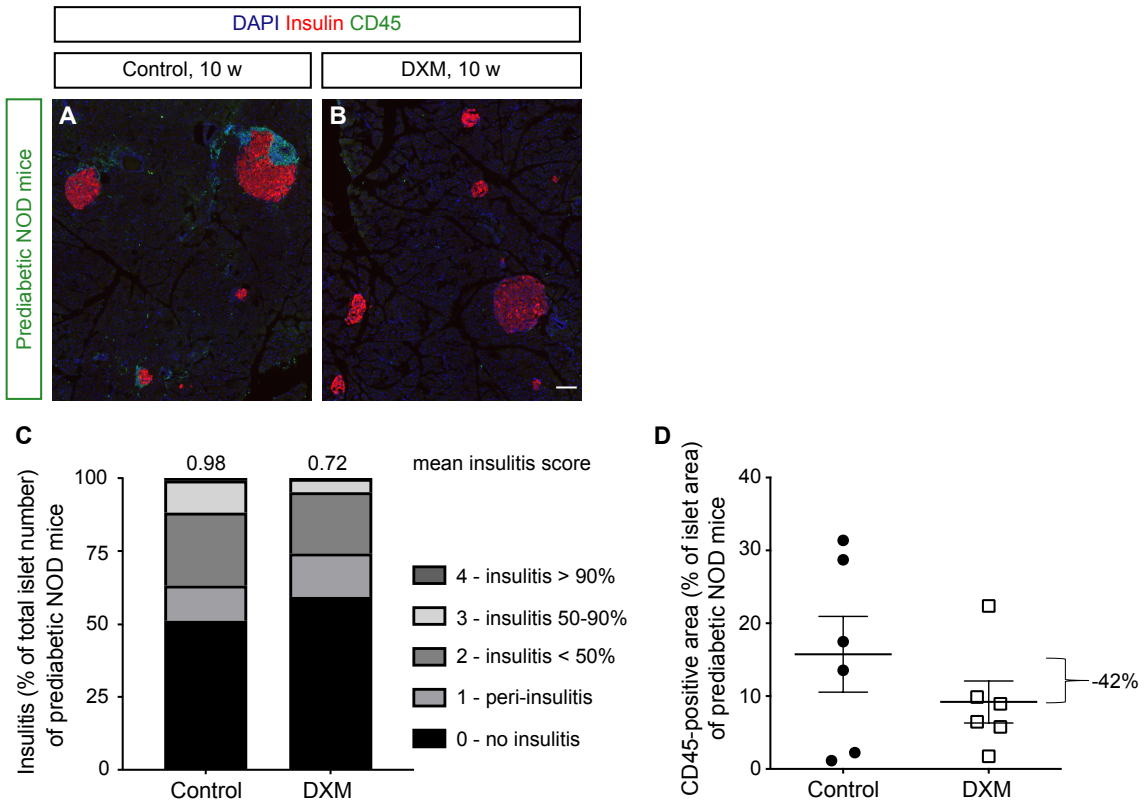

Figure 19: Insulitis is reduced in prediabetic NOD mice treated with DXM. (A, B), Representative fluorescence microscopy images of pancreatic islets of 10-week-old prediabetic (A) control or (B) DXM-treated NOD mice (n ≥ 60 islets per mouse); blue: DAPI; red: insulin; green: CD45; scale bar: 100 µm. (C), Number of islets per insulitis category (% of total islet number) and mean insulitis score of prediabetic NOD mice. (D), CD45-positive area in % of islet area of 10-week-old prediabetic NOD mice (≥ 60 islets per mouse with n = 6 mice per treatment group, p = 0.3042). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

Analyzing the grade of insulitis in prediabetic NOD mice revealed that DXM reduced the amount of infiltrating immune cells (Figure 19 A, B). The mean insulitis score was decreased from 0.98 in the control group to 0.72 in the DXM-treated group, describing that there were more islets in the classes representing low grades of insulitis or even no infiltration (Figure 19 C). Moreover, this trend toward less infiltration in the islets of prediabetic NOD mice treated with DXM was also apparent when comparing the CD45-positive area per islet area with and without DXM treatment, which unveiled on average a 42% reduced infiltrated islet area in NOD mice treated with DXM (Figure 19 D).

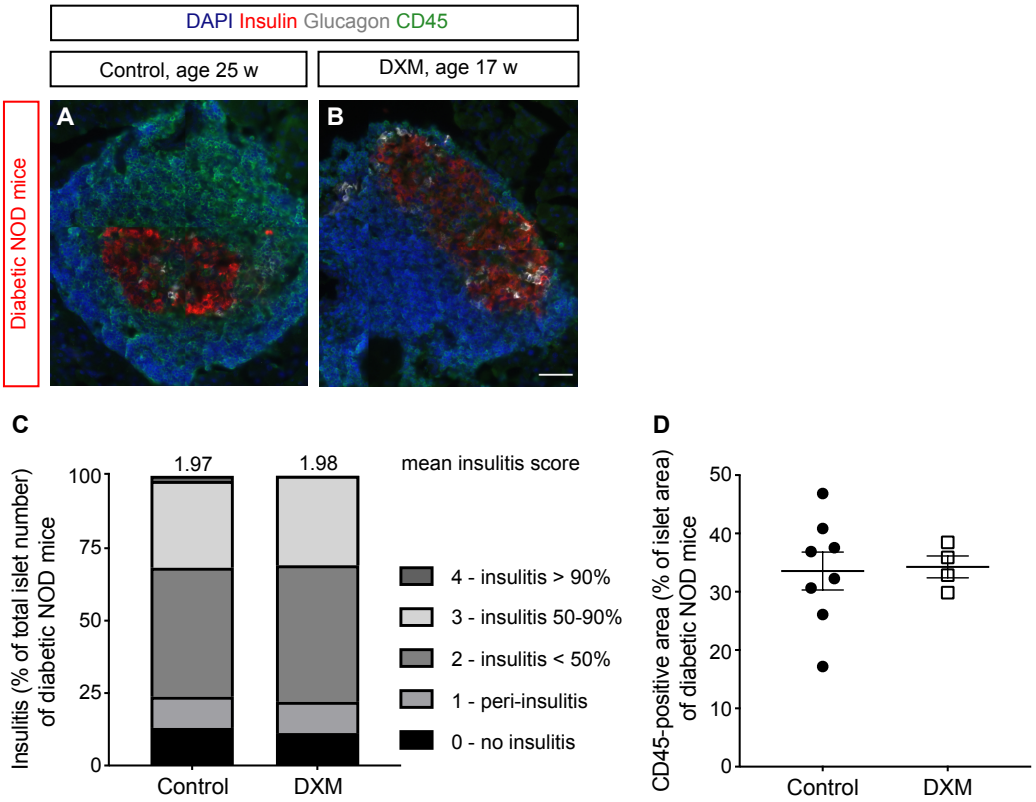

Figure 20: Insulitis in NOD mice at diabetes onset is not altered by DXM treatment. (A, B), Representative fluorescence microscopy images of pancreatic islets of diabetic (A) control (age 25 weeks) or (B) DXM-treated (age 17 weeks) NOD mice (n ≥ 10 islets per mouse); blue: DAPI; red: insulin; grey: glucagon; green: CD45; scale bar: 50 µm. (C), Number of islets per insulitis category (% of total islet number) and mean insulitis score of NOD mice at diabetes onset. (D), CD45-positive area in % of islet area of diabetic NOD mice (≥ 10 islets per mouse with n ≥ 4 mice per treatment group, p = 0.8545). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

In contrast to the results of prediabetic NOD mice, no difference in insulitis was found when islets of untreated NOD mice were compared with islets of DXM-treated NOD mice at diabetes onset (Figure 20 A, B). Islets were heavily infiltrated, as indicated by an average insulitis score of approximately 2 (Figure 20 C), meaning that on average between 20 and 50% of the islet area was infiltrated, whereas in prediabetic NOD mice most islets showed only peri-insulitis. Hence, irrespective of DXM treatment, around 33% of islet area was infiltrated by $CD45^+$ cells (Figure 20 D). Of note, in diabetic control NOD mice islets of category 4 representing islets with more than 90% infiltrated area were found, whereas none of the islet of mice treated with DXM belonged to this category.

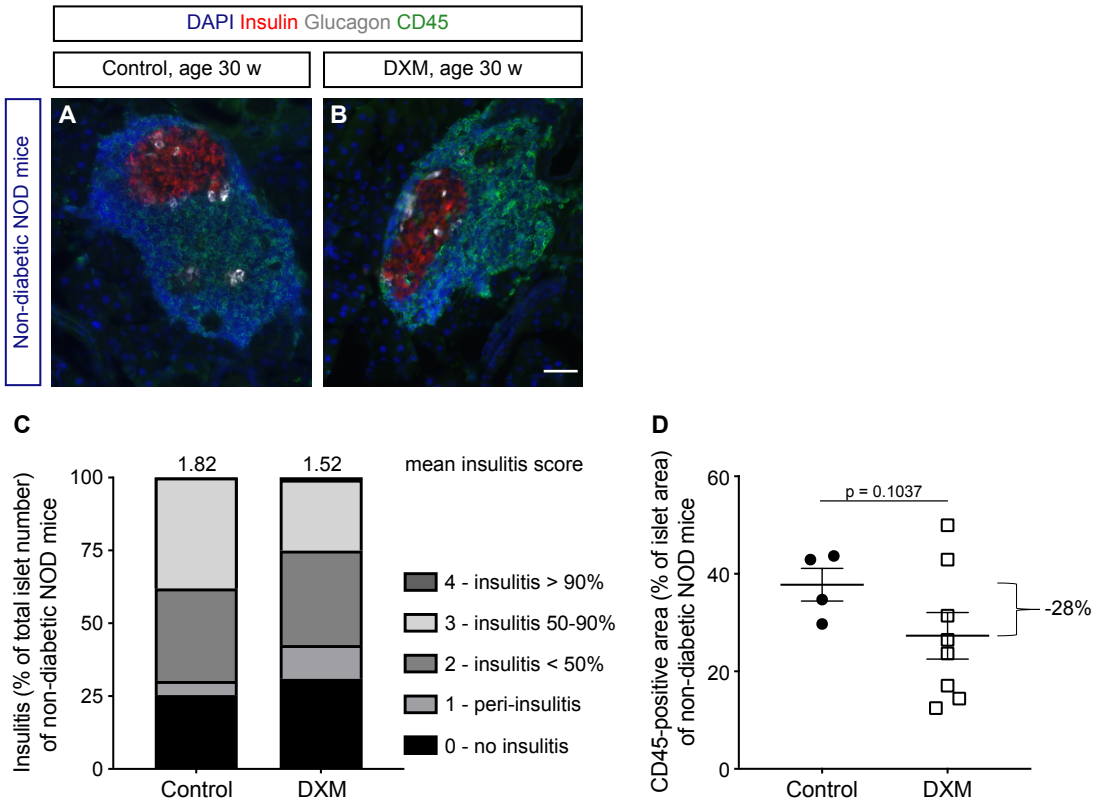

Figure 21: Islets of non-diabetic NOD mice treated with DXM are less infiltrated. (A, B), Representative fluorescence microscopy images of pancreatic islets of 30-week-old non-diabetic (A) control or (B) DXM-treated NOD mice ($n \geq 2$ islets per mouse); blue: DAPI; red: insulin; grey: glucagon; green: CD45; scale bar: 50 µm. (C), Number of islets per insulitis category (% of total islet number) and mean insulitis score of non-diabetic NOD mice. (D), CD45-positive area in % of islet area of 30-week-old non-diabetic NOD mice (≥ 2 islets per mouse with $n \geq 4$ mice per treatment group, $p = 0.1037$). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

Although no difference was observed in the diabetic cohort, this was not true for the non-diabetic cohort. Overall, islets of non-diabetic NOD mice were stronger infiltrated than islets of the prediabetic cohort (Figure 21 A, B). But, despite the fact that non-diabetic mice were on average older than diabetic mice, insulitis was not as severe in non-diabetic NOD mice as in diabetic NOD mice. The mean insulitis score for non-diabetic mice treated with DXM was 1.52, whereas this score was 1.82 for NOD mice of the control group (Figure 21 C). Furthermore, comparison of the CD45-positive islet area between treatment groups exhibited a 28% reduction in infiltrated area in DXM-treated NOD mice (Figure 21 D, $p = 0.1037$). As a conclusion, the performed analyses showed that DXM reduced insulitis in prediabetic and non-diabetic NOD mice, but not when diabetes had become manifest.

5.2.5. Presence of T cells is reduced in NOD mice treated with DXM

Because it has been shown that the overall amount of infiltrating immune cells is reduced in prediabetic and non-diabetic NOD mice treated with DXM, we wondered whether there might be differences in the immune cell subsets present in the immune cell infiltrate. Especially, T

lymphocytes are highly relevant to disease progression in both humans and NOD mice [35, 58, 64]. Therefore, we analyzed pancreatic sections of prediabetic NOD mice for the presence of different subsets of T lymphocytes. Four pancreatic sections of different pancreatic regions were stained per mouse for $CD4^+$ T helper cells and $CD8^+$ cytotoxic T cells (Figure 22 A, B). By quantifying the CD4-positive area around islets, we found that fewer $CD4^+$ immune cells infiltrated the islets of DXM-treated NOD mice. More specifically, the CD4-positive area per total pancreatic nuclei area was reduced by 62% in islets after DXM treatment (Figure 22 C, $p = 0.1498$). Similar results were obtained regarding the amount of infiltrating $CD8^+$ T cells. CD8-positive area per total pancreatic nuclei area was reduced to a similar extent as the CD4-positive area in the DXM group compared to islets of control NOD mice (Figure 22 D, $p = 0.0820$).

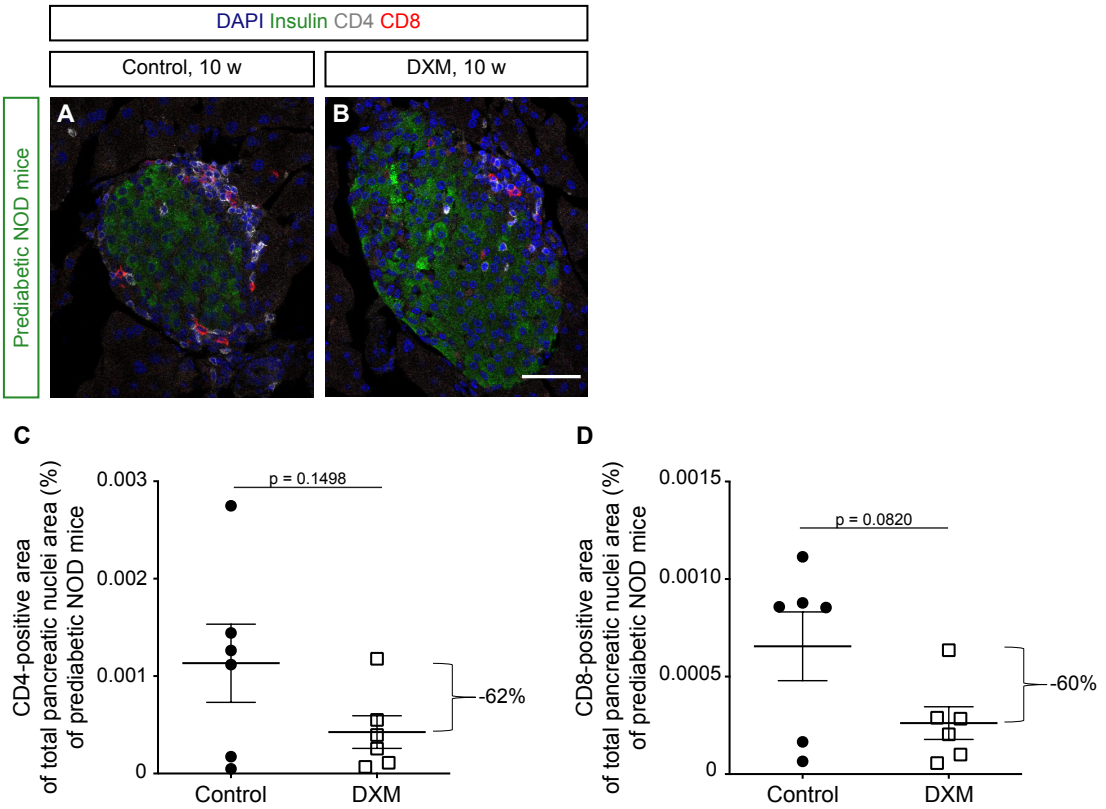

Figure 22: Amount of infiltrating $CD4^+$ and $CD8^+$ T cells is reduced in NOD mice by DXM treatment. (A, B), Representative LSM images of pancreatic islets of prediabetic (A) control or (B) DXM-treated NOD mice ($n \geq 40$ islets per mouse); blue: DAPI; green: insulin; grey; CD4; red: CD8; scale bar: 50 µm. (C), CD4- ($p = 0.1498$) and (D), CD8-positive area ($p = 0.0820$) per total pancreatic nuclei area of prediabetic NOD mice (≥ 40 islets per mouse with $n = 6$ mice per treatment group). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

We further questioned whether DXM not only decreased the amount of $CD4^+$ and $CD8^+$ T cells, but also altered the number of anti-inflammatory $FoxP3^+$ Tregs. Thus, in order to determine the proportion of Tregs in $CD4^+$ cells, four pancreatic sections per prediabetic NOD mouse were stained for FoxP3, CD4, and insulin to identify islets (Figure 23 A, B). Counting $FoxP3^+$

cell nuclei in the infiltrating immune cells around islets revealed that, on average, around 6% of $CD4^+$ T cells were Tregs (Figure 23 C). Yet, the amount of Tregs was not changed by DXM treatment.

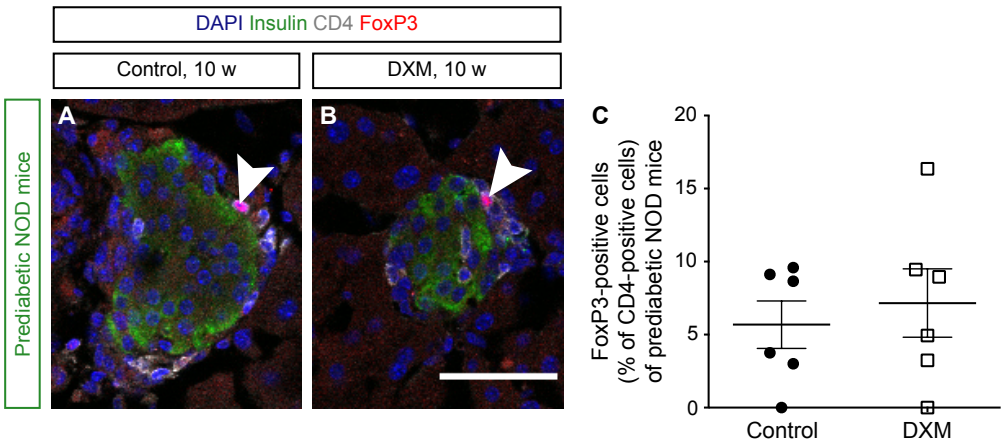

Figure 23: Number of infiltrating $FoxP3^+$ regulatory T cells is not influenced by DXM treatment. (A, B), Representative LSM images of pancreatic islets of prediabetic (A) control or (B) DXM-treated NOD mice ($n \geq 43$ islets per mouse); blue: DAPI; green: insulin; grey; CD4; red: FoxP3; scale bar: 50 µm. (C), Number of FoxP3-positive cells per CD4-positive cells (%) of prediabetic NOD mice (≥ 43 islets per mouse with $n = 6$ mice per treatment group, $p = 0.6184$). Statistical significance was determined by unpaired Student's t-test. Data are shown as mean ± SEM.

5.3. Anti-inflammatory effects of DXO

DXM has been reported to have anti-inflammatory properties *in vitro* and *in vivo* under various pathogenic conditions [176-184]. Therefore, and since T1DM is a disease in which inflammation is a key factor in pathogenesis, we wondered whether the described anti-inflammatory properties of DXM or rather its active metabolite DXO are involved in mediating the observed anti-diabetic effects in T1DM.

5.3.1. DXO reduces the expression of chemokines in pancreatic islets

In the course of the last years' research, it was revealed that beta cells are not innocent victims, but participate in their own destruction by worsening inflammation [68, 69]. Indeed, islets of T1DM patients are known to secrete increased amounts of chemokines that may attract immune cells and exacerbate destructive insulitis [42, 207, 208]. Since our data from NOD mice treated with DXM revealed less infiltrating immune cells, particularly T lymphocytes, we aimed to investigate whether this might be due to reduced chemotaxis towards pancreatic islets because of lower chemokine levels. Chemokines of interest were selected on the basis of RNA sequencing data obtained in previous studies of our lab which indicated that the expression of specific chemokines is downregulated by DXO treatment compared to cytokine treatment alone (unpublished data).

In order to prove this hypothesis, we investigated, as a first step, the expression of the chemokines C-X3-C motif chemokine ligand 1 (*Cx3cl1*), C-X-C motif chemokine ligand 1 (*Cxcl1*), C-X-C motif chemokine ligand 2 (*Cxcl2*), C-X-C motif chemokine ligand 16 (*Cxcl16*), and *Ccl2* under standard islet culture conditions. Precisely, we treated mouse islets isolated from wild-type mice with 1 µM DXO for 24 h and quantified the expression of *Cx3cl1*, *Cxcl1*, *Cxcl2*, *Cxcl16*, and *Ccl2* on mRNA level by RT-qPCR. It was found that the expression of all tested chemokines under these conditions is relatively low. But more interestingly, we revealed that the expression of all these chemokines was further reduced by the parallel treatment with DXO. The highest expressed chemokine was *Cx3cl1,* whose expression was significantly downregulated by DXO by over 29% (Figure 24 A-C, *Rplp0*: -58%, p = 0.0012; *Gapdh*: -34%, p = 0.0074, *Hprt*: -29%, p = 0.0262). The chemokine with the second highest expression in our experiment was *Cxcl16*. However, DXO treatment of islets decreased the expression of *Cxcl16* by about 50% (Figure 24 A-C, *Rplp0*: -66%, p = 0.0004; *Gapdh*: -51%, p = 0.0025, *Hprt*: -46%, p = 0.0081). The chemokines *Cxcl1*, *Cxcl2*, and *Ccl2* were expressed at lower levels, but still DXO treatment further reduced the expression of all three chemokines numerically (Figure 24 A-C).

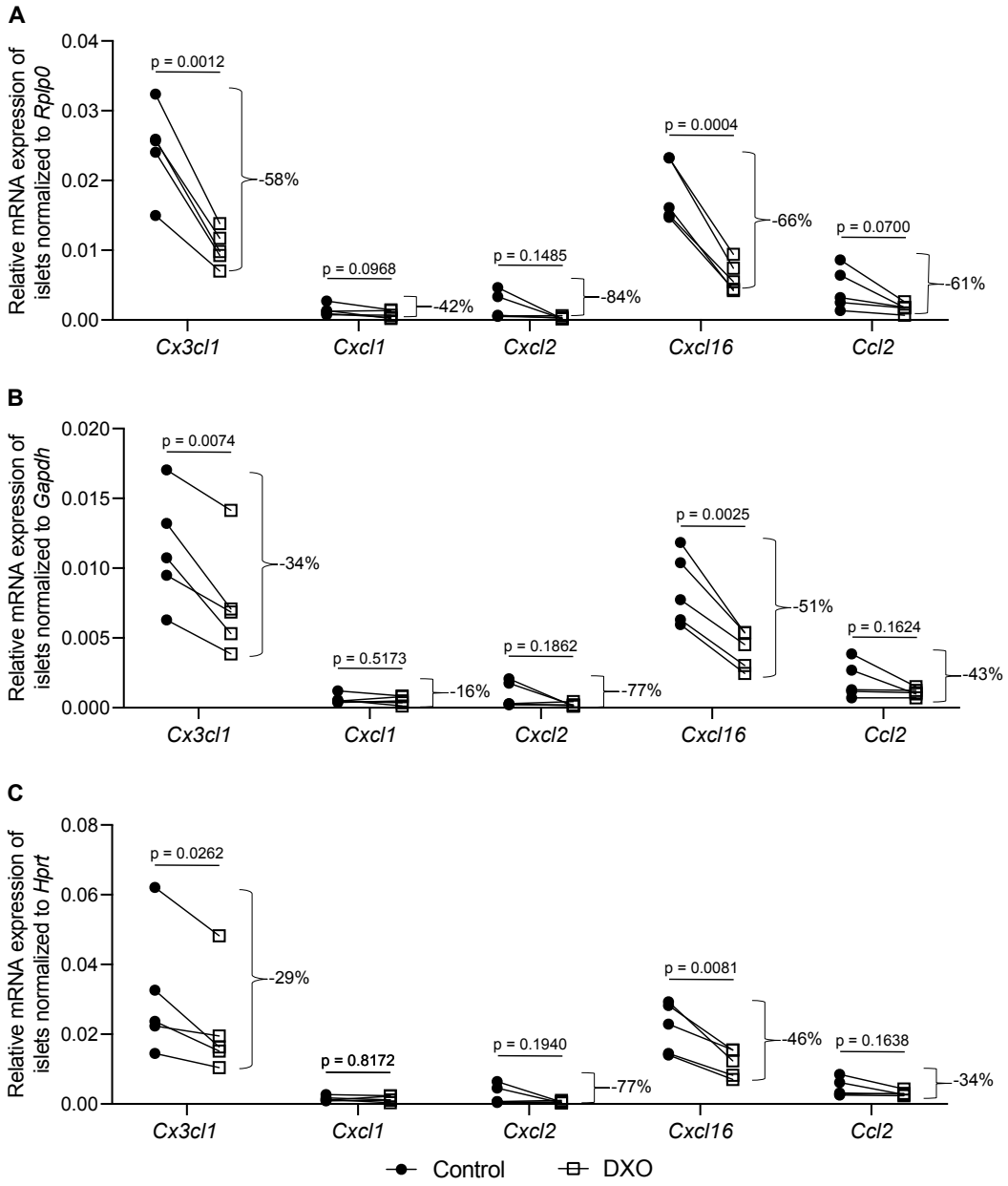

Figure 24: DXO reduces the expression of several chemokines in pancreatic mouse islets *in vitro*. Relative mRNA expression of *Cx3cl1*, *Cxcl1*, *Cxcl2*, *Cxcl16,* and *Ccl2* normalized to the expression of (A) *Rplp0*, (B) *Gapdh* and (C) *Hprt* in isolated mouse islets after 24 h of culture in the presence or absence of 1 µM DXO (n = 5 independent experiments). Statistical significance was determined by paired Student's t-test.

Because it is known that islet cells are stimulated under inflammatory conditions to secrete increased amounts of chemokines [71, 72, 209, 210], we attempted to mimic an inflammatory environment by using a cytokine mixture consisting of IL-1β, IFNγ and TNFα. Thus, isolated mouse islets were pretreated with 1 µM DXO for 1 h and then cytokines were added for another 24 h before RNA was harvested and supernatants collected for analysis of islet chemokine expression and secretion under inflammatory conditions.

The expression of all tested chemokines except for *Cx3cl1* was induced upon cytokine treatment as expected. Strikingly, DXO was still able to reduce the expression of *Cxcl1*,

Cxcl16, and *Ccl2* under inflammatory conditions. Only the expression of *Cx3cl1* and *Cxcl2* was not conclusively altered, but remained overall rather unchanged under DXO treatment considering all three housekeeping genes (Figure 25 A-C). The expression of the other chemokines was more clearly affected by treating the islets with DXO. In fact, islets treated with DXO expressed over 37% less *Cxcl1* with similar tendencies for all three housekeeping genes (Figure 25 A-C, *Rplp0*: -70%, p = 0.1203; *Gapdh*: -43%, p = 0.0559; *Hprt*: -37%, p = 0.1190). Moreover, the *Cxcl16* expression was determined to be significantly downregulated by DXO to a comparable extent under resting conditions (Figure 24), as well as in the presence of cytokines (Figure 25 A-C, *Rplp0*: -64%, p = 0.0372; *Gapdh*: -36%, p = 0.0187; *Hprt*: -30%, p = 0.0199). The highest expressed chemokine under inflammatory conditions in our experiment was *Ccl2*. Notably, DXO affected the expression of this chemokine likewise the most, significantly reducing its expression by over 60% (Figure 25 A-C, *Rplp0*: -81%, p = 0.0350; *Gapdh*: -62%, p = 0.0026; *Hprt*: -61%, p = 0.0370).

The obtained results showing reduced expression of *Ccl2* under DXO treatment were further confirmed by measuring CCL2 levels in the supernatant of cytokine-treated pancreatic mouse islets by ELISA. Remarkably, the amount of secreted CCL2 in the supernatant of islets treated with cytokines and DXO was reduced by 55% compared to islets treated with cytokines alone (Figure 25 D, p = 0.0185). This indicates that DXO downregulates the expression of CCL2 on mRNA, as well as protein levels. Summing up, these *in vitro* experiments suggest that DXO improves inflammation in islets by reducing the expression of several chemokines, especially under inflammatory conditions.

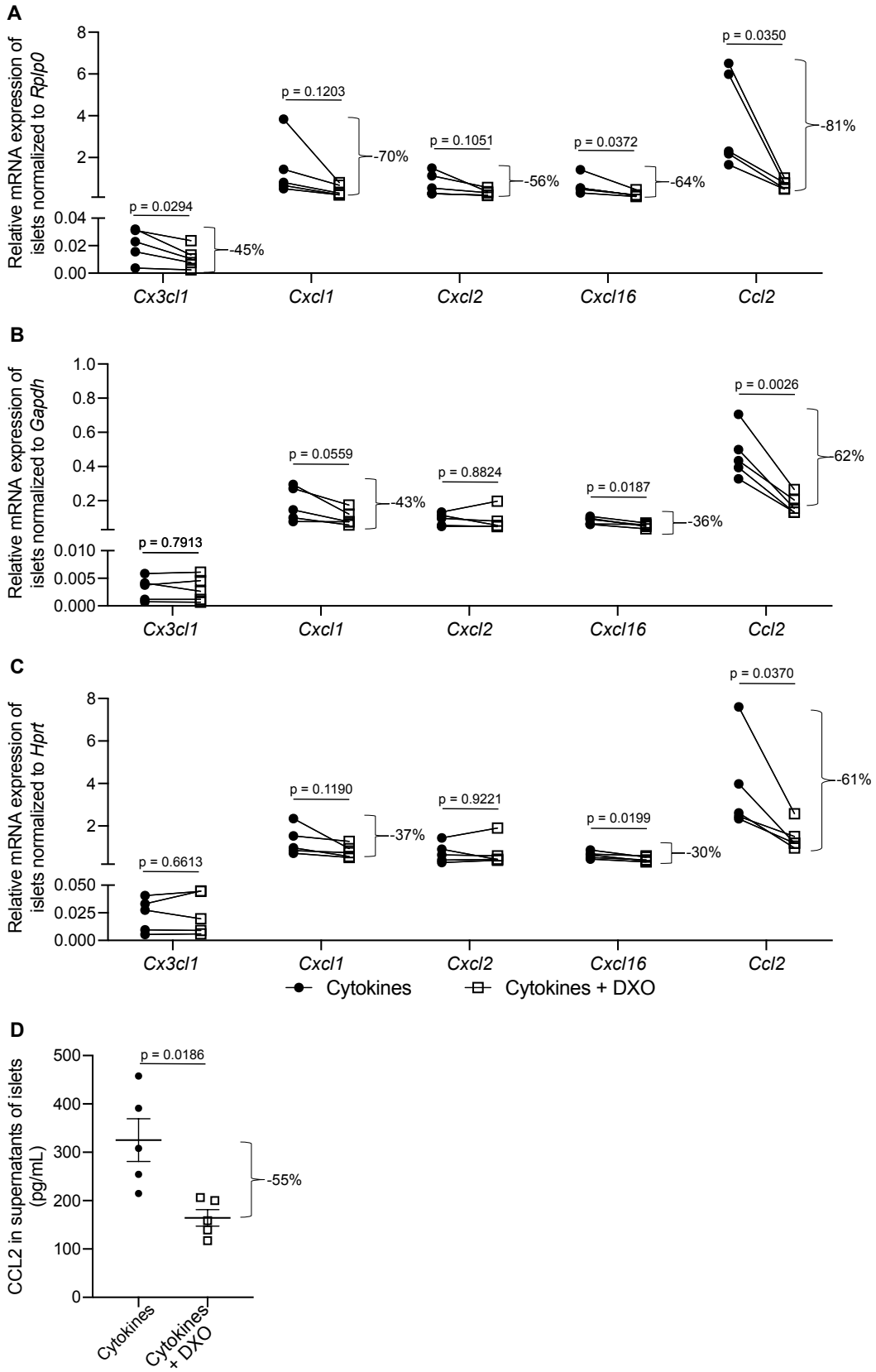

Figure 25: DXO reduces the expression of chemokines in pancreatic islets under inflammatory conditions *in vitro*. Relative mRNA expression of *Cx3cl1*, *Cxcl1*, *Cxcl2*, *Cxcl16*, and *Ccl2* normalized to the expression of (A) *Rplp0*, (B) *Gapdh*, and (C) *Hprt* in isolated mouse islets treated with a cytokine mixture for 24 h in the presence or absence of 1 µM DXO (n = 5 independent experiments). (D) Concentration of CCL2 (pg/mL) in supernatants of islets under conditions as described for (A, B, C). Statistical significance was determined by paired (A, B, C) or unpaired (D) Student's t-test.

6. Discussion

6.1. Effects of DXM on T1DM pathogenesis in the NOD mouse

The major goal of this thesis was to investigate whether DXM could be a potential novel candidate for the treatment of T1DM. To address this question, we performed a long-term study with the commonly used T1DM NOD mouse model. These mice develop autoimmune diabetes spontaneously and exhibit many characteristics comparable to the human disease pathogenesis, like the presence of autoantibodies, insulitis, and similarities in genes responsible for disease susceptibility [55, 59, 211].

By treating NOD mice continuously from 4 weeks of age, we showed that long-term DXM administration delays diabetes onset and results in a reduced diabetes incidence of 50% in 30-week-old NOD mice (Figure 7). Precisely, 67% of control NOD mice had become diabetic which is consistent with the incidence of diabetes reported in the literature for this mouse model [212], whereas only 33% of NOD mice treated with DXM were diabetic at 30 weeks of age (Figure 7). Furthermore, there was a delay of 5 weeks between the first control NOD mouse becoming diabetic and the first DXM-treated mouse.

We started to treat the NOD mice from 4 weeks of age onwards, since it is known that the immune cell infiltration leading to beta cell destruction starts at this age in NOD mice [59, 213]. Additionally, based on our previous *in vitro* findings demonstrating that DXO protects islet cells against cytokine-induced cell death [170], we assumed that DXM might be most effective in the prevention of disease when the majority of beta cells are still present. Thus, DXM should be able to exert its protective properties on islet cells. Furthermore, evidence from the literature suggests that DXM is able to alter the immune response of various cell types [175-178, 181, 183, 184]. Therefore, an early initiation of treatment might ameliorate, or, at best, suppress the autoimmune response, thereby preventing immune cell infiltration, and eventual beta cell destruction. Moreover, we performed a previous study in our lab in which treatment of NOD mice started when mice were older. In these mice only minor effects regarding diabetes incidence and onset were found (data not shown), indicating that an early treatment begin might be necessary for the effectiveness of DXM in NOD mice. With regard to a future translation to the human system, this aspect suggests that DXM might be able to prevent, or at least delay, T1DM, but probably may not lead to diabetes remission, once disease has manifested. Concludingly, DXM could rather be indicated for primary prevention, which is prevention of disease onset in individuals at risk [120], than secondary or tertiary prevention.

This assumption is also supported by the finding that DXM treatment only partially improved glycemic control of NOD mice in this study. Stronger effects of DXM on blood glucose levels were shown in both, *db/db* mice and humans [170, 173], and were therefore expected in this study as well. However, it should be noted that we might underestimate the effect of DXM on blood glucose levels in NOD mice due to our experimental design. The study design stipulated

that mice were excluded from the study as soon as their blood glucose levels increased and exceeded the threshold of 250 mg/dL. Thus, blood glucose levels might actually have been more different, if all mice had continued to be included. Nonetheless, comparing blood glucose levels of diabetic NOD mice between treatment groups revealed significantly lower blood glucose levels in DXM-treated NOD mice (Figure 7) indicating better glycemic control at least in these mice. Therefore, we cannot completely exclude that DXM might additionally be beneficial in secondary prevention of T1DM by stimulating insulin secretion and improving blood glucose levels as already seen in the context of T2DM [170, 173]. To prove this hypothesis, further studies investigating the effects of DXM in NOD mice with manifest diabetes should be conducted. In these studies treatment of NOD mice should start at later ages, when the autoimmune destruction of beta cells has already begun and blood glucose levels started to rise. If DXM is still able to lower blood glucose levels in this context, this could indicate that DXM might indeed also be useful for secondary prevention by improving beta cell function.

In summary, our study revealed that DXM affects T1DM pathogenesis in the NOD mouse resulting in delayed and partially prevented disease onset. These effects were accompanied by improved control of blood glucose levels, especially in NOD mice with manifest diabetes indicating augmented residual beta cell function.

6.1.1. Immune-modulatory effects of DXM in T1DM

Immune-competent cells are known to express NMDARs and these receptors were described to differentially regulate the immune responses of certain immune cells [199-204, 214, 215]. Therefore, effects of systemically applied DXM on the insulitis in T1DM, besides effects on beta cell function, were hypothesized. A recent study demonstrating positive effects of DXM in a mouse model of the autoimmune disease rheumatoid arthritis, as well as in human patients, further underlined this hypothesis [184]. Moreover, the NMDAR antagonist memantine was shown to decrease the pancreatic expression of proinflammatory cytokines, such as TNFα and IL-1β, in a mouse model of T2DM using high-fat diet in combination with STZ [174].

Indeed, we were able to demonstrate that oral application of DXM modulates the immune response in T1DM. Long-term DXM treatment improved insulitis in prediabetic NOD mice as observed by more islets of DXM-treated mice showing no infiltrating immune cells or only peri-insulitis (Figure 19). Similar results were obtained regarding the number of infiltrating immune cells in 30-week-old NOD mice, although insulitis was more severe compared to prediabetic mice (Figure 21). However, no effect on the insulitis could be found in NOD mice with manifest diabetes (Figure 20) indicating that DXM might delay early immune cell infiltration, but is not able to prevent further immune cell infiltration once the autoimmune attacks on pancreatic beta cells have progressed. Still, the numbers of infiltrating T lymphocytes, which are known to be

crucial in beta cell destruction in T1DM pathogenesis [58], were particularly reduced by DXM treatment in prediabetic NOD mice. Infiltrating immune cells of DXM-treated NOD mice comprised over 60% less $CD8^+$ and $CD4^+$ T lymphocytes than infiltrating immune cells in control NOD mice (Figure 22). On the contrary, the proportion of $FoxP3^+$ Tregs per $CD4^+$ T helper cells was not affected by DXM treatment (Figure 23), although they are considered to play an essential role in disease initiation [75, 216]. However, it was demonstrated that it is not the amount of Tregs which is altered in T1DM patients, but rather their function [75], which we did not assess in this study.

Of note, in the prediabetic mice cohort, two mice showed nearly no signs of insulitis, regardless of whether we obtained the overall infiltrating immune cells ($CD45^+$) or T cells ($CD4^+$/ $CD8^+$). Since the analyses were done blinded, it was only afterwards that we noticed it was the same two mice in all analyses that did not exhibit insulitis. One could speculate that these two mice might never have become diabetic, since it is known for the NOD mouse model that not all mice progress to manifest T1DM. If we compare these results to our observed incidence of 67% in control NOD mice, 2 out of 6 mice might display the usual proportion of diabetes-resistant mice in this strain.

In summary, the results imply that systemic DXM application likely mediates its effects on T1DM progression in the NOD mouse by affecting two major contributors of the disease. DXM not only improves beta cell function and survival, but also slightly modulates the autoimmune response in T1DM.

6.1.2. Beta cell protective effects of DXM in T1DM

A major advantage of DXM over existing treatments for T1DM, which we were able to confirm in this study, is that it has beta cell protective properties. This characteristic of DXM was first demonstrated in the study by Marquard et al., in which DXM protected mouse, as well as human islets against cytokine-induced cell death *in vitro*, and additionally increased the number of islets in *db/db* mice *in vivo* [170]. Here, we could now further verify that DXM is able to confer protection in the context of T1DM.

We showed for the first time that DXO protects mouse and human islets *in vitro* against the beta cell toxin STZ (Figure 4, Figure 5). Higher concentrations of STZ were required to induce islet cell death in human islets, because they express lower levels of GLUT-2, which is the glucose transporter responsible for the uptake of STZ. Thus, human islets are considered to be partially resistant against STZ-mediated damage [217-219] and still, DXO was able to significantly enhance islet cell survival in human islets treated with a high dose of STZ (Figure 5).

Furthermore, our *in vivo* study with NOD mice revealed that long-term DXM treatment preserved pancreatic islet numbers, as well as beta and alpha cells. At all the timepoints

investigated, islet numbers were increased in DXM-treated NOD mice compared to age-matched control mice (Figure 11, Figure 12, Figure 13). Whereas only a tendential increase of the remaining number of islets was found in prediabetic NOD mice when treatment had been rather short with 6 weeks (Figure 11), tremendous effects were observed in 30-week-old non-diabetic mice, when the treatment had lasted the longest with 26 weeks. These mice had an even five-fold increased islet number (Figure 13). Thus, these analyses indicated that the observed effect of DXM on islet numbers might be an additive effect over time. In summary, islet numbers declined with age in control NOD mice as expected for this mouse model (Figure 14) [197, 220]. But this decline was not as strong in DXM-treated NOD mice suggesting a partial protection of islet cells. Supporting evidence for this effect of islet cell preservation came from the finding that insulin- and glucagon-positive areas were increased in diabetic (Figure 9) and non-diabetic NOD mice treated with DXM (Figure 10) as well, but not in prediabetic mice (Figure 8).

To rule out the possibility that DXM treatment increased insulin- and glucagon-positive areas in NOD mice based on the formation of hyperplastic islets, the islet size was determined. In prediabetic and diabetic NOD mice, there was no significant difference in the size of islets determined as sum of insulin- and glucagon-positive area (Figure 11, Figure 12). But, islets of DXM-treated prediabetic NOD mice tended to be smaller (Figure 11). In both cohorts, islet size was found to be on average in a range between approximately 6,000 and 7,000 μm^2 which is comparable to other reports about the size of murine islets [221-223]. To our surprise, islets of 30-week-old non-diabetic control NOD mice were on average two-fold larger with an islet size of approximately 12,000 μm^2, whereas islets of DXM-treated mice of the same age did not show a similar pattern. They still displayed an average islet size of approximately 6,500 μm^2 (Figure 13) and thus fitted well to our results obtained from younger NOD mice, as well as results obtained by others [221-223]. Interestingly, a recent study by Boldison et al. reported that in NOD mice, which are naturally resistant to the development of T1DM, the frequency of smaller islets was increased [224]. In line with that, pancreatic islets of recent-onset T1DM patients were found to be increased in size [225]. Furthermore, larger islets were described to be associated with stronger immune cell infiltration [224, 226, 227] suggesting that an increased islet size might be a marker of disease progression and a disadvantage regarding insulitis and thus islet cell survival. In summary, the analysis of islet size revealed that DXM does not increase the size of islets in NOD mice. On the contrary, DXM in fact even decreased the islet size when comparing mice of the same age and disease state which might contribute to the protective effects of DXM.

Another hint on the protective characteristics of DXM was given by determining the amount of cell death via immunohistochemical staining of pancreatic sections for the apoptosis marker CC3. Interestingly, the proportion of CC3-positive area per islet area was similar at all three

timepoints assessed and overall quite low which might be due to a fast clearance of apoptotic cells [228, 229]. However, in prediabetic NOD mice treated with DXM, cell death was reduced by approximately 23% (Figure 15), whereas proliferation remained unchanged (Figure 18). Assuming a reduction of one fourth over time, it is conceivable that this reduced cell death rate leads to increased islet numbers in the end, as observed in DXM-treated NOD mice at later ages. However, this effect on apoptosis could not be clearly demonstrated in diabetic and non-diabetic NOD mice (Figure 16, Figure 17). Only a numerical reduction was found in diabetic NOD mice treated with DXM (Figure 16). Yet, it should be noted that analysis of pancreatic islets at these timepoints is difficult due to the small number of remaining islets in both treatment groups, but especially in control NOD mice. This low number favors variations between data obtained from different mice. Nonetheless, these data corroborate the hypothesis that DXM protects islet cells against the autoimmune-mediated destruction in T1DM.

Although the protective mechanism induced by DXM is not yet resolved, the presented experiments allow some conclusions to be drawn. In particular, the numbers of cytotoxic $CD8^+$ T lymphocytes and $CD4^+$ T helper lymphocytes were reduced in DXM-treated NOD mice (Figure 22). Thus, modulating the immune system will likely contribute to the protective effects of DXM, as T lymphocytes are crucial for beta cell destruction in the context of T1DM [58, 63]. However, protection must be enhanced by a yet unknown beta cell-intrinsic effect. Otherwise, the observed DXM-mediated protection of isolated islets against the beta cell toxin STZ *in vitro* would not be explicable without the presence of immune cells (Figure 4, Figure 5). This implies that, besides reducing immune cell infiltration, DXM must also induce pathways in beta cells making them resistant to different cell death stimuli, such as cytokines [170] and STZ. Since STZ mediates its toxicity via multiple mechanisms, that is DNA alkylation, release of nitric oxide, and generation of reactive oxygen species (ROS) [230-232], several protective mechanisms are possible and need to be elucidated in subsequent studies as outlined in more detail in chapter 6.3. Nonetheless, these islet cell protective characteristics of DXM make it a promising new treatment option, because none of the drugs currently on the market to treat T1DM is able to sustainably prevent the progressive decline in beta cell mass by improving beta cell survival [233, 234].

6.1.3. Anti-inflammatory effects of DXO on pancreatic islets

Another effect of DXO we assessed in this study, is its ability to improve the inflammatory microenvironment in pancreatic islets. For instance, DXO, as active metabolite of DXM used for *in vitro* experiments, lowered the mRNA expression of several chemokines in isolated pancreatic islets. Chemokines are known to be secreted by murine and human islets, especially in an inflammatory milieu as it occurs during T1DM development [71, 72, 209, 210].

Additionally, it was shown that plasma levels of several inflammatory chemokines are elevated in patients with T1DM [62]. Their main function is to attract immune cells to the site of secretion, which in T1DM are pancreatic islets. The infiltrating immune cells then aggravate the autoimmune attack and mediate beta cell death [42, 73]. This mechanism renders blockage of chemokines an interesting approach for the prevention of T1DM by breaking the vicious circle of immune cell infiltration, inflammation, and beta cell damage [73, 235]. However, inhibiting single chemokines only showed modest effects in previous studies, because chemokines and their receptors are expressed redundantly [62, 236]. Nonetheless, using transgenic mice expressing the pan-chemokine inhibitor M3, a crucial role for chemokines in T1DM development was proven [236, 237]. Mice treated with multiple low doses of STZ (MLDS), as well as NOD mice, whose beta cells expressed M3, showed reduced immune cell infiltration and were overall protected from developing T1DM [236, 237].

It is known that DXM can affect the expression of individual chemokines in some immune cells, such as macrophages and dendritic cells [176, 177]. But here, we now showed for the first time that DXO is also able to decrease the expression of several chemokines, such as *Cx3cl1*, *Cxcl1*, *Cxcl2*, *Cxcl16*, and *Ccl2* in pancreatic islets (Figure 24, Figure 25). All these chemokines are somehow associated with T1DM as stated in the following and are thus thought to play a role in disease pathogenesis.

For example, *Cx3cl1* is known to attract cytotoxic T cells, NK cells, and monocytes [238]. Additionally, it was shown to be significantly elevated in blood samples from T1DM children at disease onset compared to healthy probands [239]. Under standard mouse islet culture conditions, *Cx3cl1* was the highest expressed chemokine in our experiments. DXO reduced its expression significantly by more than 30%, but its expression was not altered conclusively, when islets were additionally treated with cytokines (Figure 24, Figure 25). To our surprise, mRNA expression of *Cx3cl1* was not induced by cytokine treatment in mouse islets at all, which is contradictory to previous reports using human islets [71, 72], but consistent with the finding that *Cx3cl1* expression is decreased in prediabetic NOD mice compared to BALB/c mice [71]. This might suggest that in mice CX3CL1 is rather involved in early attraction of immune cells than in chemoattraction when disease has progressed and inflammation is present.

In contrast, expression of *Cxcl1* and *Cxcl2* was strongly upregulated by treating mouse islets with IL-1β, IFNγ, and TNFα. Whereas the expression of *Cxcl1* was numerically reduced by DXO treatment irrespective of the presence of cytokines, *Cxcl2* expression remained fairly unchanged by adding DXO when its expression was stimulated with cytokines (Figure 24, Figure 25). Notably, CXCL1 has been associated with T1DM, as its plasma levels were shown to be elevated in patients with T1DM [240, 241]. In general, the two chemokines CXCL1 and CXCL2 mainly attract neutrophils which are among the first cells recruited to sites of inflammation and involved in further activation and attraction of cells of the innate and adaptive

immune system [234, 242]. Blocking their receptor, the C-X-C motif chemokine receptor 2 (CXCR2), improves insulitis and prevents T1DM in NOD mice and MLDS-treated mice [242, 243]. In the latter model, inhibition of CXCR1 and CXCR2 even induced diabetes remission in already diabetic NOD mice [243], so that the promising CXCR1/CXCR2-inhibitor ladarixin was recently tested in a phase 2 human trial with recent-onset T1DM patients. Indeed, positive effects of ladarixin in subgroups were identified, but primary endpoints were not met [244].

Another chemokine, which was shown to be associated with T1DM in the MLDS mouse model, is CXCL16 [245]. Moreover, it was identified as a potential candidate for the insulin-dependent diabetes susceptibility 4 (*Idd4*) locus in NOD mice [246], whereas its receptor CXCR6 is located in the insulin-dependent diabetes mellitus 22 (IDDM22) disease locus in humans [62]. Expression of *Cxcl16* was strongly affected by DXO. Under standard culture conditions, its expression was reduced by around 50%. This down-regulation of *Cxcl16* upon DXO treatment still persisted when chemokine expression was induced by cytokines (Figure 24, Figure 25).

However, the mRNA expression of a different chemokine was even stronger regulated by adding DXO to murine islets than the ones mentioned before. This chemokine is called CCL2 or monocyte chemoattractant protein 1 (MCP-1). Its expression and secretion was already demonstrated to be affected by DXM in macrophages and dendritic cells [176, 177], but this is the first evidence of DXO affecting CCL2 expression in pancreatic islets. *Ccl2* mRNA expression was strongly enhanced by the used cytokine mixture resulting in the highest expressed chemokine in pancreatic islets in our experiments. Yet, DXO reduced mRNA expression of *Ccl2* by more than 60% (Figure 25). These results were further confirmed by measuring CCL2 protein levels which revealed 55% less secreted CCL2 in supernatants of islets treated with DXO (Figure 25). CCL2 mainly attracts APCs (monocytes/ macrophages, dendritic cells, B cells), but other immune cells such as T cells, NK cells, basophils, and neutrophils are recruited as well [247]. Besides chemoattraction, CCL2 is described to influence leucocyte behavior, such as polarization, secretion of effector molecules, and survival [247]. In NOD mice, CCL2 expression increases with disease progression [248], and genetic deletion of its receptor *Ccr2* delays diabetes onset [249]. With regard to human T1DM, several studies demonstrated elevated plasma levels of CCL2 in T1DM patients [250, 251], especially in recent-onset T1DM patients [252, 253]. CCL2 was also identified to play a decisive role in islet transplantation [254, 255]. In fact, low levels of CCL2 secretion were crucial for long-term insulin independence of human patients indicating improved survival of the grafted islets [254].

As a conclusion, our data demonstrate that DXO reduced the expression of several chemokines in pancreatic islets relevant in T1DM disease pathogenesis. By this, DXO improves the inflammatory environment in pancreatic islets and likely affects the recruitment of further immune cells which might eventually ameliorate the autoimmune destruction of beta

cells. This locally reduced expression of several chemokines in pancreatic islets might actually be an explanation for the observed reduced amount of infiltrating immune cells in DXM-treated NOD mice. In particular, the number of T lymphocytes was reduced upon DXM treatment in NOD mice. Although not all investigated chemokines are known to directly attract T lymphocytes, they still might have an impact on chemotaxis of T lymphocytes to pancreatic islets due to the complex interplay of innate and adaptive immune responses [74, 242]. For instance, Diana et al. showed that usage of a CXCR2 antagonist, which primarily inhibits neutrophil recruitment, additionally decreased the amount of diabetogenic $CD8^+$ T lymphocytes within islets [242]. However, further experiments are necessary to mechanistically link differentially expressed and secreted chemokines in pancreatic islets *in vitro* with the observed improved insulitis in NOD mice *in vivo* upon DXM treatment. For this, chemotaxis assays using supernatants of untreated or DXM-treated islets to attract different lymphocyte subsets could be a useful tool. Nonetheless, the novel finding that DXO reduces the expression of several chemokines in pancreatic islets *in vitro* contributes to our knowledge on the effects of DXM/DXO in the context of T1DM and helps to understand mechanisms occurring in NOD mice treated with DXM.

6.2. DXM as potential new agent for the treatment of T1DM

Currently available treatment options for T1DM try to restore blood glucose homeostasis by increasing insulin secretion or by exogenous insulin administration, although a major effort has been made in recent years to explore potential preventive treatments aiming to maintain beta cell mass. Focusing on preventive strategies was enabled by newly gained knowledge that led to the ability to screen for autoantibodies before disease onset, as well as genetic identification of individuals at risk for developing T1DM [39, 48, 256]. In line with that, new stages of T1DM were introduced by a consensus statement of the ADA, JDRF, and Endocrine Society which classifies people at risk to develop T1DM with present autoantibodies, but otherwise without further symptoms, already as stage 1 [40]. In order to be able to treat these patients, new treatment options preventing beta cell destruction are urgently needed. The importance of restoring or preserving beta cell mass was shown in the Diabetes Control and Complications Trial (DCCT). This trial demonstrated that preserving endogenous insulin secretion, as a measure of remaining beta cell mass, by intensive insulin treatment, positively affects glycemic control and also prevents long-term complications, thus overall improving disease outcome [257].

Although there are drugs currently under investigation showing promising results to preserve beta cell mass, none of them has yet proven prolonged prevention of disease manifestation in a human trial [233]. The most promising candidate for T1DM prevention so far is the anti-CD3 antibody teplizumab. Recently published results of an extended follow-up after a median of

923 days, showed that 50% of probands receiving a single 14-days intervention course with teplizumab remained diabetes-free, whereas this was only true for 22% in the control group. The median time to diagnosis was delayed by approximately 33 months [258]. Of note, teplizumab exerts its effects by exhaustion of T cells, but not by improving beta cell function itself. This might be a gap DXM could be able to fill, since, after all, 50% of those individuals treated with teplizumab still develop diabetes after more than 2 years [140, 258].

In fact, in recent years, it was suggested that effective sustainable prevention of T1DM might need a combination of both, an improvement of beta cell function and simultaneous immune modulation [106, 141, 259]. Since our results demonstrate that DXM improves beta cell function and survival, it should therefore be considered as a potential new candidate for the adjunct treatment of T1DM. Interestingly, DXM beneficially affects beta cells in the context of T1DM directly and indirectly by distinct pathways. First, DXM was shown to directly improve glycemic control by increasing glucose-stimulated insulin secretion of beta cells without inducing hypoglycemic events [170, 173]. Second, DXM increased beta cell survival *in vitro* and *in vivo*. Last but not least, DXM improved inflammation of pancreatic islets and reduced the number infiltrating immune cells, thereby also enhancing beta cell function and survival. Still, because effects of DXM on insulitis were only modest, to properly suppress autoimmune-mediated destruction of beta cells in individuals at risk, it might be advantageous to combine DXM with a strong immune-modulatory treatment like the anti-CD3 antibody teplizumab.

Some studies already proved that combinatory approaches can be more successful than using one drug alone like the combination of an anti-IL21 antibody with the GLP-1 agonist liraglutide in newly diagnosed patients with T1DM [159]. In this study, it was demonstrated that after 54 weeks of treatment, the combination of anti-IL21 and liraglutide lead to a significantly smaller decrease in MMTT-stimulated C-peptide levels compared to placebo, whereas each of the two drugs alone failed to do so. Other current studies use a combinatory approach as well, for example GAD as immune-modulating agent was combined with the γ-amino butyric acid (GABA) as beta cell regenerative drug (NCT02464033) [260]. However, using more than one drug might salvage the risk of increasing adverse side-effects or even provoking unintended drug interactions. One advantage of DXM here over newly invented drugs is that it is already well-characterized and considered to be safe, because it has been in clinical use for decades, even in pediatric patients [164].

Moreover, as an additional benefit of DXM compared to existing drugs, it seems noteworthy to mention that literature suggests that NMDAR antagonists and particularly DXM may improve diabetes-associated long-term complications [187-191]. For instance, DXM was able to relieve leg pain intensity in a phase 3 clinical trial for diabetic neuropathic pain [191]. Furthermore, in a phase 1/2 proof-of-concept study, DXM as treatment for diabetic macular edema decreased vascular leakage [190]. Most evidence is, however, found concerning diabetic cardiovascular

diseases. DXM improved endothelial function, measured as FMD, in two human studies, one of them investigating the effects of DXM in heavy smokers [187, 188]. Additionally, as add-on to amlodipine monotherapy, low-dose DXM treatment reduced mean systolic blood pressure in patients where standard monotherapy did not achieve sufficient control of blood pressure [186]. Because cardiovascular diseases are the main cause of death in DM patients [30], having a new anti-diabetic class of drug that not only prevents T1DM in the first place, but also prevents complications in the long-run harbors a great potential.

As a conclusion, it can be stated that DXM unifies different advantages over existing drugs for the prevention of T1DM and should therefore be considered as novel adjunct agent in future studies. DXM improves beta cell function and survival, so that it could be used as combinatory treatment with other immune-modulating drugs to obtain synergistic effects on disease prevention. Additionally, it is a commonly used substance und thus known to be well tolerated. Last but not least, DXM might have the potential to prevent diabetic long-term complications so that T1DM patients would greatly benefit from this new approach of T1DM prevention.

6.3. Outlook on future studies

Although this study provides evidence that DXM is an interesting potential candidate for the treatment of T1DM, some questions remain unanswered and need to be further investigated to achieve the ultimate goal of bringing a new adjunct treatment for the prevention of human T1DM on the market.

First, one limitation of this study we need to overcome by future studies is that we only used one *in vivo* animal model. More specifically, usage of the NOD mouse as the only preclinical model for T1DM is questioned due to the limited translation of results obtained in this model to human clinical studies. Numerous studies demonstrated the effectiveness of drugs in the prevention of T1DM in the NOD mouse, but then results could often not be fully reproduced in human trials [55, 59]. This might partially be explained by parameters like the route of administration, dosing, and timing of intervention which cannot easily be transferred from the NOD mouse to the human body [59]. Nonetheless, using the NOD mouse model can still reveal essential hints on the effectiveness of new drugs, if used according to suggested standards [211]. More importantly, it gives the opportunity to access tissues during the studies which cannot be obtained from human patients and thereby provides essential mechanistical insights [55]. Thus, the NOD mouse remains the most commonly used rodent model of T1DM and still has its eligibility in today's T1DM preclinical research. However, before translating the promising results of this study into humans, therapeutic efficacy of DXM should be confirmed by testing its effectiveness in a second animal model. Since our *in vitro* data on beta cell protection of DXO against STZ-induced cell death are promising, it is reasonable to believe, that DXM could prevent diabetes development in a T1DM animal model using STZ as well.

Additionally, a recent publication demonstrated that a modification of DXO was able to numerically increase beta cell areas and tendentially improve blood glucose levels in a mouse model where beta cells were destroyed using one single high dose of STZ [261]. Therefore, it should be investigated whether DXM is able to prevent diabetes manifestation or even induce remission in a MLDS model which is considered to better mimic human T1DM than one single dose of STZ, as it involves islet inflammation and insulitis [196]. If DXM proves its effectiveness in this model, maybe even in larger animal models, it should of course at some point be tested in a human trial. Because DXM already demonstrated anti-diabetic effects in two human clinical trials [170, 173], albeit in the context of T2DM, and we have furthermore shown that it is able to convey islet cell protection in human islets, hopes are high that the results of this preclinical study can be translated to humans.

Second, another issue which needs to be addressed in subsequent experiments, is that the protective mechanisms of DXM have not been fully elucidated yet. Based on this study, we concluded that in the context of T1DM, DXM protects islet cells not only via beta cell-intrinsic effects, but probably also by modulating the autoimmune response. However, our *in vitro* experiments with STZ further confirmed that DXO must mediate effects on the beta cells themselves. Still, it is not clear how DXM confers these. One possible mechanism could be the relief of oxidative stress. STZ is known to partially mediate its toxicity via oxidative stress [230, 231]. Additionally, reactive oxygen species (ROS) participate in beta cell death during T1DM development [262, 263]. For instance, diabetic patients show signs of increased oxidative stress, while simultaneously having decreased antioxidant defense mechanisms [264, 265]. Particularly, beta cells are vulnerable to oxidative stress as they naturally possess only low levels of anti-oxidant enzymes [262, 266]. In line with that, overexpression of anti-oxidant genes, as well as systemic application of anti-oxidants, ameliorates autoimmune-mediated destruction of beta cells in mouse models of T1DM [63, 263]. Interestingly, systemic application seems to be more successful than local genetic overexpression in this context [263]. This might be due to the fact that ROS also participate in the autoimmune response and are crucial for proper activation of initial infiltrating immune cells, but also for driving the adaptive immune response [263, 266]. Thus, if we could show that DXM reduced oxidative stress, this might explain its protective properties on the basis of islet cells, but it could also help understand the mechanisms underlying modulation of immune cells in the NOD mouse model. Indeed, in various preclinical and also clinical studies, DXM was able to reduce oxidative stress by inhibiting the NADPH oxidase (NOX) whose function is to generate ROS [176, 180, 183, 185, 187]. Strikingly, NOX2-deficient NOD and STZ-treated mice, which are lacking an essential subunit for proper function of NOX, displayed a delayed diabetes onset, showed reduced immune cell infiltration, and improved beta cell survival [267, 268]. This proved an essential role for NOX in T1DM. Adoptive transfer of splenocytes from NOX2-

deficient NOD mice into NOD-*scid* (severe combined immunodeficient) mice, which resulted in delayed T1DM onset as well, suggested that the effects are rather mediated by an insufficient autoimmune response than by improving beta cell function and survival [269]. Thus, additional characterization of the effects of DXM on immune cells may also contribute to uncover the protective mechanisms.

In fact, NMDAR are expressed on lymphocytes and were demonstrated to be involved in the regulation of immune responses [199-204, 214, 215]. Therefore, it would be interesting to analyze whether DXM treatment not only reduces the amount of infiltrating immune cells as shown in this study, but also alters their proliferation or activation status. Moreover, the question whether DXM regulates the expression and secretion of cytokines involved in driving the autoimmune response in T1DM should be addressed, as for specific immune cell subsets, like macrophages and dendritic cells, an influence of DXM on the secretion of inflammatory cytokines such as IL-6 and TNFα was reported [175-177]. To sum up, this study raised additional questions on the protective mechanisms of DXM. Therefore, the effects of DXM on pancreatic islets and likewise the immune cells need to be further characterized. One possible approach in elucidating the protective mechanism should include an investigation on whether DXM relieves oxidative stress either in pancreatic islets or in certain immune cells.

This question is not less relevant because oxidative stress also plays a decisive role in micro- and macrovascular diabetic long-term complications, especially in the pathogenesis of atherosclerosis and cardiovascular diseases. Thus, testing the hypothesis whether DXM improves diabetic long-term complications is a third aspect which should be addressed in future studies. Actually, using apolipoprotein E (ApoE)-deficient mice, a common model to study human atherosclerosis [270], Liu et al. already demonstrated that DXM by reducing oxidative stress improved atherosclerosis [176]. It should, however, still be investigated whether this effect of DXM can be confirmed in a model of diabetes-accelerated atherosclerosis. For this, mice with either ApoE- or low-density lipoprotein receptor-deficiency, in which diabetes is induced with STZ treatment, have been established [271]. Additionally, STZ treatment can be utilized to mimic diabetic nephropathy, retinopathy or neuropathy [272-274]. Thus, by assessing the correct parameters like blood pressure, FMD or other disease-specific biochemical markers, we could use STZ-induced beta cell destruction in different mouse strains to challenge our hypothesis whether DXM is able to improve the outcome of diabetes-associated long-term complications [188, 275].

A fourth aspect which is worth future investigations concerns the side effects induced by DXM treatment. Although DXM is considered safe and well tolerated, it still does induce some mild adverse side-effects like dizziness, fever, and nausea [276]. If using higher doses than recommended, DXM can even induce psychoactive effects and is therefore mis-used as a recreational drug [276]. These effects are associated with the ability of DXM to pass the blood-

brain barrier (BBB), where it targets the central nervous system (CNS). A recent study of our lab was successful in modifying the chemical structure of DXO to prevent passage of the BBB, whereas still preserving the anti-diabetic effects such as increasing glucose-stimulated insulin secretion and improving beta cell survival [261]. However, these new entities have not been tested in a long-term *in vivo* study so far. Therefore, it would be very interesting to investigate whether the new molecule Lam39M or one of its further developments exerts similar or even greater anti-diabetic effects compared to DXM in NOD mice.

In summary, additional studies, as outlined in this chapter, are necessary before being able to suggest DXM usage in T1DM patients. Nevertheless, this work could help to lay the foundation for the development of a new preventive T1DM strategy based on this promising molecule.

6.4. Conclusion

The aim of this study was to answer the question whether DXM could be a potential drug for the treatment of T1DM. By performing *in vitro* experiments with isolated islets and an *in vivo* long-term study with NOD mice, we showed for the first time that DXM, in addition to previous reports of its anti-diabetic effects in T2DM [170, 173], is indeed able to partially prevent the progressive destruction of beta cells by improving beta cell function and survival.

In particular, DXM improved control of blood glucose levels, delayed diabetes onset for several weeks, and overall reduced diabetes incidence by preserving residual beta cell mass in NOD mice. This was likely accomplished by improving the inflammation of pancreatic islets, as indicated by reduced expression of chemokines and tendentially reduced numbers of infiltrating lymphocytes. More precisely, the number of immune cells effective in beta cell destruction, such as cytotoxic T lymphocytes and T helper cells, were reduced in DXM-treated NOD mice. Moreover, based on our *in vitro* experiments showing islet cell protection of DXO against STZ-induced cell death, we came to the conclusion that DXM, besides modulating the immune response, preserves islets by inducing islet cell-intrinsic protective mechanisms.

As a final conclusion, the data presented suggest that DXM harbors the potential to be used as preventive treatment for T1DM. However, as outlined in chapter 6.3, a lot of question remain unanswered. Further studies are required to fully elucidate the effects of DXM in T1DM und its underlying mechanisms. Moreover, because most of the data obtained to date originate from mice studies, we can only assume that similar effects will be observed, once DXM can be tested in a human trial. Hence, for the final goal of approval of DXM for the treatment of human T1DM, a lot of efforts need to be undertaken first, but the study presented here may provide the basis for future research.

In fact, novel approaches are urgently needed due to a complete lack of clinically relevant preventive treatment options for T1DM to this day. Former studies trying to prevent T1DM onset often failed in achieving sustainable results. Therefore, new concepts for T1DM

prevention were proposed recently, which are based on the combination of more than one drug. The idea is to use two different kinds of drugs so that one should halt the autoimmune attack by modulating the immune system and the other one should improve beta cell function and survival. Together, this approach is thought to preserve pancreatic islets sustainably, thus enhance endogenous insulin secretion and prevent disease onset at all. Great progress has been made in recent years concerning the development of an immune-modulating agent. However, drugs improving beta cell function and survival are still missing. Based on this study, we propose that DXM could fill this gap and thus has to be further evaluated as adjunct treatment for the prevention of T1DM.

7. Publications

The main part of this study is in preparation for submission (publication date unknown):

Wörmeyer L, Hamacher A, Eberhard D, Mayatepek E, Meissner T, Lammert E, Welters A
The N-methyl-D-aspartate receptor antagonist dextromethorphan preserves pancreatic islets and reduces diabetes incidence in NOD mice

Other publications:

2021: Scholz O, Otter S, Welters A, Wörmeyer L, et al. (2021) **Peripherally active dextromethorphan derivatives lower blood glucose levels by targeting pancreatic islets.** Cell Chem Biol. 10.1016/j.chembiol.2021.05.011

2017: Welters A, Kluppel C, Mrugala J, Wörmeyer L et al. (2017) **NMDAR antagonists for the treatment of diabetes mellitus-Current status and future directions.** Diabetes Obes Metab 19 Suppl 1: 95-106. 10.1111/dom.13017

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9. Supplementary information

The following Fiji/ImageJ Macro scripts were developed by A. Hamacher for the analysis of fluorescence images and were used for quantifications mentioned in this thesis. Syntax highlighting was performed with https://tohtml.com/

ASPIQ_Set_ROI_V12 Script

```
#@ String (visibility=MESSAGE, value="Choose the images to be processed:") topMsg
#@ File (label = "Input directory", style = "directory") input
#@ File (label = "Output directory", style = "directory") output
#@ String (label = "File suffix", value = ".czi") suffix
#@ Boolean(label = "Continue from manual breakpoint", value = false) continueBP
#@ Boolean(label = "Show all channels for validation", value = false) allWindows
#@ Boolean(label = "Live Cell Imaging", value = false) LiveCell
#@ String (visibility=MESSAGE, value="Color allocation per channel for RGB output:") colMsg
#@ String(label = "C1", choices={"blue", "red", "green", "gray", "cyan", "magenta", "yellow"}, style="listBox")
setChan1
#@ String(label = "C2", choices={"green", "red", "blue", "gray", "cyan", "magenta", "yellow"}, style="listBox")
setChan2
#@ String(label = "C3", choices={"red", "green", "blue", "gray", "cyan", "magenta", "yellow"}, style="listBox")
setChan3
#@ String(label = "(optional) C4", choices={"gray", "red", "green", "blue", "cyan", "magenta", "yellow"},
style="listBox") setChan4
#@ String (visibility=MESSAGE, value="Settings for ROI detection:") scriptMsg
#@ String (label = "Allowed Circularity", value = "0.05-1.00") set_circularity
#@ String(label = "Detect ROI based on:", choices={"merged channels", "single channel"},
style="radioButtonHorizontal") roiDetect
#@ String(label = "If single channel detection, use:", choices={"C1", "C2", "C3", "C4"}, style="listBox") setRoiChan
#@ String(label = "Segmentation Threshold:", choices={"Otsu", "Li"}, style="listBox") setSegmentThr
#@ String (visibility=MESSAGE, value="Note: Standard is Otsu, Li preferred for Live Cell Imaging") SegThrMsg
#@ Boolean(label = "Gaussian Blur Filter", value = false) set_gaussianFilter
#@ Double (label = "Gaussian Sigma", value = "2.0") set_gaussianSigma
#@ Boolean(label = "Histogram mode scaling", value = false) set_histoscale
#@ Boolean(label = "Slight Background Substraction", value = false) BGsubstract
#@ String(label = "If BG substraction, on channel:", choices={"C1", "C2", "C3", "C4", "all"}, style="listBox")
setBGsubChan

/*		Last update:		28th February 2020
 *		Script author:		Anna Hamacher, HHU
 *
 *		Macro to process multiple images in a folder to
 *		- automatically segment the image (detect the pancreatic islets)
 *		- in case of faulty segmentation, allow a manual correction
 *		- re-run this macro to save ROIs after manual correction
 *		So far, apotome images can cause problems, if the bio-format importer of Fiji fails to stitch the tiles.
 *		After completion of the ROI setting, the ASPIQ_Measure Macro needs to be run.
 */

// Check if input and output directories are different
if (input == output) {
		showMessage("Please choose an output directory that differs from the input directory and re-run the
script. Script cancelled...");
		exit();
}

// General settings
separator = File.separator;
myChan = newArray(setChan1, setChan2, setChan3, setChan4);

// Main function
processFolder(input);

function processFolder(input) {
```

```
		list = getFileList(input);
		Array.sort(list);
		for (i = 0; i < list.length; i++) {
			// ignore subfolders for processing
			if(endsWith(list[i], suffix) && !(File.isDirectory(input + separator + list[i])))
				if (i == 0 && continueBP) {
					saveROI(input, output, list[i]);
				} else {
					processFile(input, output, list[i]);
				}
		}
}

function processFile(input, output, file) {
		print("Processing: " + input + separator + file);

		run("Bio-Formats Importer", "open=[" + input + separator + file + "] autoscale color_mode=Composite
rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT stitch_tiles series_1");
		imageTitle = getTitle(); // needed for more than 1 tile (string incl. "#1")
		imageId = getImageID();
		totalSlices = nSlices();

		// Perform max intensity projection in case of live cell z-stack images
		if (LiveCell && totalSlices > 4) {
			run("Z Project...", "projection=[Max Intensity]");
			Stack.setDisplayMode("composite");
			totalSlices = nSlices();
			close(imageTitle);
			selectWindow("MAX_" + imageTitle);
			rename(imageTitle);
		}

		// Decision of ROI detection on merged channels or a single channel
		if (roiDetect == "single channel") {
			run("Duplicate...", "duplicate channels=" + substring(setRoiChan, 1) + " title=[Working
Copy "+ setRoiChan + "]");
		}
		// Clear ROIs
		roiManager("reset");
		selectWindow(imageTitle);
		// Keep original image open for cross-reference
		run("Duplicate...", "duplicate");
		selectWindow(imageTitle);
		// Change format to 8-bit
		run("8-bit");
		run("Split Channels");
		for (i=1; i <= totalSlices ; i++)  {
			selectWindow("C" + i + "-" + imageTitle);
			run("8-bit");
			// Improve contrast based on histogram mode in merged RGB image
			// Useful in case of stainings with much background noise (e.g. CD45)
			if (set_histoscale) {
				List.setMeasurements;
				// Get value "mode" and "max" from histogram function
				histogramMode = List.getValue("Mode");
				histogramMax = List.getValue("Max");
				setMinAndMax(histogramMode, histogramMax);
			}

			// Perform a slight background substraction in case of very noisy stainings
			if (BGsubstract) {
				if (setBGsubChan == "all" || substring(setBGsubChan, 1) == i) {
					run("Subtract Background...", "rolling=150");
				}
			}
		}
		// System colours: C1=red, C2=green, C3=blue, C4=gray, C5=cyan, C6=magenta, C7=yellow
		// Color assignment for RGB output image
		sysChan = newArray(myChan.length);
```

```
		for (c = 0; c < myChan.length; c++ ){
			if (myChan[c] == "red") { sysChan[c] = "c1"; }
			else if (myChan[c] == "green") { sysChan[c] = "c2"; }
			else if (myChan[c] == "blue") { sysChan[c] = "c3"; }
			else if (myChan[c] == "gray") { sysChan[c] = "c4"; }
			else if (myChan[c] == "cyan") { sysChan[c] = "c5"; }
			else if (myChan[c] == "magenta") { sysChan[c] = "c6"; }
			else if (myChan[c] == "yellow") { sysChan[c] = "c7";}
		}
		// Merging channels to RGB image for ROI detection
		if (totalSlices == 3) { // Merge only channel 1-3
			run("Merge Channels...", sysChan[0] + "=[C1-" + imageTitle + "] " + sysChan[1] + "=[C2-" +
imageTitle + "] " + sysChan[2] + "=[C3-" + imageTitle + "] keep");
		} else { //merge channel 1-4
			run("Merge Channels...", sysChan[0] + "=[C1-" + imageTitle + "] " + sysChan[1] + "=[C2-" +
imageTitle + "] " + sysChan[2] + "=[C3-" + imageTitle + "] " + sysChan[3] + "=[C4-" + imageTitle + "] keep");
		}
		if (roiDetect == "single channel") {
			selectWindow("Working Copy "+ setRoiChan);
		} else {
			selectWindow("RGB");
			run("Duplicate...", "title=[Working Copy of RGB]");
			selectWindow("Working Copy of RGB");
		}

		// Thresholding with filtering and noise reduction
		run("8-bit");
		run("Enhance Contrast...", "saturated=0.3");
		if (set_gaussianFilter) {
			run("Gaussian Blur...", "sigma=" + set_gaussianSigma + " scaled");
		}

		setOption("BlackBackground", true);
		setAutoThreshold(setSegmentThr + " dark");
		run("Convert to Mask");
		run("Remove Outliers...", "radius=5 threshold=50 which=Bright");
		run("Fill Holes");
		/*if (LiveCell) {
				run("Watershed");
		};*/
		run("Analyze Particles...", "size=1200-Inifity circularity=" + set_circularity + " exclude include add");

		nROIs = roiManager("count");
		// Run watershed if no ROI detected (e.g. touching edges, lower circularity)
		if (nROIs == 0) {
			print ("Invalid operation, no ROI defined, trying Watershed!"); // Debug
			run("Watershed");
			run("Analyze Particles...", "size=900-Inifity circularity=" + set_circularity + " exclude include
add");
			// Re-check the number of ROIs after watershed
			nROIs = roiManager("count");
		}

		// Use function Windows > Tile for the user to see all images at the same time for confirmation of ROIs
		if (allWindows) {
			run("Tile");
		}
		// End the automated islet detection, present result to the user for approval
		selectWindow("RGB");
		roiManager("Deselect");
		roiManager("Show All");

		// Ask user if ROIs are ok or need manual correction
		if (getBoolean("Are the suggested ROIs ok?")) {
			showMessage("Saving ROIs and overlay tiff...");
			saveROI(input, output, file);
			}
		else {
```

```
                        showMessage("Please adjust the ROIs manually and re-run the script with <continue from manual breakpoint = yes>");
                        exit(); // End macro for manual correction
                }
}

function saveROI(input, output, file) {

                setBatchMode(true);
                resultDir = output + separator + file + "_results";
                // Create the image specific result directory, if it doesn't exist yet
                if (!File.isDirectory(resultDir)) {
                        File.makeDirectory(resultDir);
                }

                // Save RGB-Overlay for easier scroll through
                // roiManager("Update") needs to be done while editing ROIs manually!
                selectWindow("RGB");
                // Make sure only the updated existing ROI(s) gets flattened
                roiManager("Deselect");
                roiManager("Show All");
                roiManager("Show None");
                roiManager("Show All");
                run("From ROI Manager");
                run("Flatten");
                selectWindow("RGB-1");
                saveAs("Tiff", resultDir + separator + file + "_RGB-Overlay.tif");

                // Save all ROIs to zip for later reference
                roiManager("Deselect");
                roiManager("Save", resultDir + separator + file + "_RoiSet.zip");

                // Move the original image to the result subfolder, proceed with the list of images in the main directory
                File.rename(input + separator + file, resultDir + separator + file);

                saveScriptSettings(resultDir, file);

                setBatchMode(false);
                run("Close All");
}

function saveScriptSettings (resultDir, file) {

                logSettings = File.open(resultDir + separator + file + "_settings.txt");

                print(logSettings, "PARAMETER \t\t\t\t VALUE");
                print(logSettings, "================================================================================================");
                print(logSettings, "Filename \t\t\t\t" + file);
                print(logSettings, "Live Cell Imaging \t\t\t" + boolean2txt(LiveCell));
                print(logSettings, "Channel 1 \t\t\t\t" + setChan1);
                print(logSettings, "Channel 2 \t\t\t\t" + setChan2);
                print(logSettings, "Channel 3 \t\t\t\t" + setChan3);
                print(logSettings, "Channel 4 (optional) \t\t\t" + setChan4);
                print(logSettings, "Circularity \t\t\t\t" + set_circularity);
                print(logSettings, "ROI detection based on \t\t\t" + roiDetect);
                print(logSettings, "If single channel detection, used \t" + setRoiChan);
                print(logSettings, "Segmentation Threshold \t\t\t" + setSegmentThr);
                print(logSettings, "Gaussian Blur Filter \t\t\t" + boolean2txt(set_gaussianFilter));
                print(logSettings, "Gaussian Sigma \t\t\t\t" + set_gaussianSigma);
                print(logSettings, "Histogram mode scaling \t\t\t" + boolean2txt(set_histoscale));
                print(logSettings, "Background Substraction \t\t" + boolean2txt(BGsubstract));
                print(logSettings, "BG substraction on channel \t\t" + setBGsubChan);
                print(logSettings, "Timestamp \t\t\t\t" + File.dateLastModified(resultDir + separator + file + "_RoiSet.zip"));

                File.close(logSettings);
}
```

```
function boolean2txt(pValue) {
		if (pValue == 0) {
			return "false"
		}
		else {
			return "true"
		}
}
```

ASPIQ_Measure_V9 Script

```
#@ String (visibility=MESSAGE, value="Choose the images to be processed:") topMsg
#@ File (label = "Main directory", style = "directory") workingDir
#@ String (label = "File suffix", value = ".czi") suffix
#@ Boolean(label = "Live Cell Imaging", value = false) LiveCell
#@ String (visibility=MESSAGE, value="Choose threshold per channel:") thrMsg
#@ String(label = "C1", choices={"Otsu", "Triangle", "Default", "Li", "Moments", "Minimum", "Huang",
"RenyiEntropy", "Yen", "IsoData", "Intermodes", "MaxEntropy"}, style="listBox") setThr1
#@ String(label = "C2", choices={"Otsu", "Triangle", "Default", "Li", "Moments", "Minimum", "Huang",
"RenyiEntropy", "Yen", "IsoData", "Intermodes", "MaxEntropy"}, style="listBox") setThr2
#@ String(label = "C3", choices={"Otsu", "Triangle", "Default", "Li", "Moments", "Minimum", "Huang",
"RenyiEntropy", "Yen", "IsoData", "Intermodes", "MaxEntropy"}, style="listBox") setThr3
#@ String(label = "(optional) C4", choices={"Otsu", "Triangle", "Default", "Li", "Moments", "Minimum", "Huang",
"RenyiEntropy", "Yen", "IsoData", "Intermodes", "MaxEntropy"}, style="listBox") setThr4
#@ String(label = "Thresholding mode:", choices={"global", "local"}, style="radioButtonHorizontal") setThrMode
#@ String (visibility=MESSAGE, value="Note: global = on whole image; local = only on ROI area") thr2Msg
#@ String(label = "Thresholding on:", choices={"each channel", "single channel"}, style="radioButtonHorizontal")
channelSelect
#@ String(label = "If single channel, use:", choices={"C1", "C2", "C3", "C4"}, style="listBox") setThrChan
#@ Boolean(label = "Slight Background Substraction", value = false) BGsubstract
#@ String(label = "If BG substraction, on channel:", choices={"C1", "C2", "C3", "C4", "all"}, style="listBox")
setBGsubChan
#@ String (visibility=MESSAGE, value="Note: Background substraction removes information, use with caution!")
bgs2Msg

/*		Last update:		28th February 2020
 *		Script author:		Anna Hamacher, HHU
 *		Modification:		Laura Wörmeyer, HHU (normalisation to pancreatic nuclei area, #164-180)
 *
 *		Macro to process multiple images in a folder to
 *		- measure specific thresholds inside this area for each channel
 *		- save the results in a directory for evaluation of the automation and reproducibility.
 *		So far, apotome images can cause problems, if the bio-format importer of Fiji fails to stitch the tiles.
 */

scriptStart = round(getTime()/1000);

// General settings
separator = File.separator;
myThr = newArray(setThr1, setThr2, setThr3, setThr4);

// Main function
run("Clear Results");
processFolder(workingDir);

function processFolder(workingDir) {
	list = getFileList(workingDir);
	Array.sort(list);
	for (i = 0; i < list.length; i++) {
		if(File.isDirectory(workingDir + separator + list[i])){
			processFolder("" + workingDir + separator + list[i]);
		}
		else if(endsWith(list[i], suffix)){
			processFile(workingDir, list[i]);
		}
	}
}

function processFile(workingDir, file) {
	print("Processing: " + workingDir + separator + file);

	setBatchMode(true);
	run("Bio-Formats Importer", "open=[" + workingDir + separator + file + "] autoscale
color_mode=Composite rois_import=[ROI manager] view=Hyperstack stack_order=XYCZT stitch_tiles series_1");
	imageTitle = getTitle(); // needed for more than 1 til (string incl. "#1")
	imageId = getImageID();
	totalSlices = nSlices();
```

```
// Perform max intensity projection in case of live cell z-stack images
if (LiveCell && totalSlices > 4) {
		run("Z Project...", "projection=[Max Intensity]");
		Stack.setDisplayMode("composite");
		close(imageTitle);
		selectWindow("MAX_" + imageTitle);
		rename(imageTitle);
}

// Decision of measurement on each or only a single channel
if (channelSelect == "each channel") {
		iGlobal = 1;
		totalSlices = nSlices();
} else {
		iGlobal = substring(setThrChan, 1);
		totalSlices = iGlobal;
}
// Change image to 8-bit
run("8-bit");
run("Split Channels");
for (i=iGlobal; i <= totalSlices ; i++)  {
		selectWindow("C" + i + "-" + imageTitle);
		run("8-bit");

		// Perform a slight background substraction in case of very noisy stainings
		if (BGsubstract) {
			if (setBGsubChan == "all" || substring(setBGsubChan, 1) == i) {
				run("Subtract Background...", "rolling=150");
			}
		}
}
// Clear ROIs
roiManager("reset");
// Import predefined ROIs
roiManager("Open", workingDir + file + "_RoiSet.zip");
nROIs = roiManager("count");
if (nROIs < 0) {
		print (file + ": Invalid operation, no ROI defined!"); // Debug
		exit();
}
// Set measurement paramaters, don't limit to threshold
run("Set Measurements...", "area area_fraction display redirect=None decimal=4");
// First measure total area of each ROI
for (j=0; j < nROIs; j++) {
		roiManager("Deselect");
		selectWindow("C" + iGlobal + "-" + imageTitle);
		roiManager("Select", j);
		run("Measure");
		roiManager("Deselect");
		}
// Measurement of white area, limit to threshold now
run("Set Measurements...", "area area_fraction limit display redirect=None decimal=4");
// Measure the area of each ROI on each specific channel threshold
if (setThrMode == "local") {
		// Perform local thresholding by selecting the ROIs before thresholding
		for (j=0; j < nROIs; j++) {
			for (i=iGlobal; i <= totalSlices ; i++)  {
				selectWindow("C" + i + "-" + imageTitle);
				run("Select All");
				// Convert image for thresholding and measure the relevant area
				// This needs to be done per channel and per ROI
				thrWindow = "C" + i + " with Threshold " + myThr[i-1] + "_roi" + j;
				run("Duplicate...", "title=[" + thrWindow + "]");
				selectWindow(thrWindow);
				roiManager("Select", j);
				roiName = Roi.getName();
				setAutoThreshold(myThr[i-1] + " dark");
				setOption("BlackBackground", true);
				run("Convert to Mask");
```

```
                                                roiManager("Deselect");
                                                selectWindow(thrWindow);
                                                roiManager("Select", j);
                                                run("Measure");
                                                // Save each mask with ROI
                                                run("Flatten");
                                                saveAs("Tiff", workingDir + file + "_C" + i + "_Threshold_" +
myThr[i-1] + "_roi_" + roiName + ".tif");
                                        }
                                }
                        }
                        else {
                                // Perform global thresholding on whole image
                                for (i=iGlobal; i <= totalSlices ; i++)  {
                                        selectWindow("C" + i + "-" + imageTitle);
                                        run("Select All");
                                        // Convert image for thresholding and measure the relevant area
                                        // This needs to be done per channel and per ROI
                                        thrWindow = "C" + i + " with Threshold " + myThr[i-1];
                                        run("Duplicate...", "title=[" + thrWindow + "]");
                                        selectWindow(thrWindow);
                                        setAutoThreshold(myThr[i-1] + " dark");
                                        setOption("BlackBackground", true);

                                        run("Convert to Mask");
                                        for (j=0; j < nROIs; j++) {
                                                roiManager("Deselect");
                                                selectWindow(thrWindow);
                                                roiManager("Select", j);
                                                run("Measure");
                                        }
                                        // Save each mask
                                        roiManager("Deselect");
                                        saveAs("Tiff", workingDir + file + "_C" + i + "_Threshold_" + myThr[i-1] + ".tif");
                                }
                        }
                        /*
                        // Normalisation to pancreatic nuclei area
                        // Calculate overlap of C1 and C2, works only for global thresholding
                        if (LiveCell) {
                                if (setThrMode == "global") {
                                        imageCalculator("Add create", imageTitle + "_C1_Threshold_" + myThr[0] +
".tif", imageTitle + "_C2_Threshold_" + myThr[1] + ".tif");
                                        run("Convert to Mask");
                                        // selectWindow("Result of " + imageTitle + "_C1_Threshold_" + myThr[0] +
".tif");
                                        rename("Merged C1 and C2 of " + imageTitle + ".tif");
                                        for (j=0; j < nROIs; j++) {
                                                roiManager("Deselect");
                                                roiManager("Select", j);
                                                run("Measure");
                                        }
                                        roiManager("Deselect");
                                        saveAs("Tiff", workingDir + file + "_C1_add_C2" + ".tif");
                                }
                        }
                        */
                        run("Close All");
                        setBatchMode(false);
}

// Save the results to an overall file
timestamp = round(getTime()/1000);
selectWindow("Results");
saveAs("Results", workingDir + separator + "Overall_Quantification_Results_" + timestamp + ".csv");

logSettings = File.open(workingDir + separator + "Overall_Quantification_Results_" + timestamp + "_settings.txt");

print(logSettings, "PARAMETER \t\t\t\t VALUE");
```

```
		print(logSettings,
"=============================================================================================
==============");
		print(logSettings, "Live Cell Imaging \t\t\t" + boolean2txt(LiveCell));
		print(logSettings, "Threshold channel 1 \t\t\t" + setThr1);
		print(logSettings, "Threshold channel 2 \t\t\t" + setThr2);
		print(logSettings, "Threshold channel 3 \t\t\t" + setThr3);
		print(logSettings, "(Optional) Threshold channel 4 \t\t" + setThr4);
		print(logSettings, "Thresholding mode \t\t\t" + setThrMode);
		print(logSettings, "Thresholding on \t\t\t" + channelSelect);
		print(logSettings, "If single channel, used \t\t" + setThrChan);
		print(logSettings, "Background Substraction \t\t" + boolean2txt(BGsubstract));
		print(logSettings, "BG substraction on channel \t\t" + setBGsubChan);
		print(logSettings, "Timestamp \t\t\t\t" + File.dateLastModified(workingDir + separator +
"Overall_Quantification_Results_" + timestamp + ".csv"));

File.close(logSettings);

// Get the total runtime of the script in seconds and display to user
scriptEnd = round(getTime()/1000);
totalRuntime = scriptEnd - scriptStart;
showMessage("ASPIQ Measure Script ended, runtime: " + totalRuntime + "s");

function boolean2txt(pValue) {
		if (pValue == 0) {
			return "false"
		}
		else {
			return "true"
		}
}
```

List of abbreviations

A

ADA	American Diabetes Association
ADP	Adenosine diphosphate
AF488	Alexa fluor 488
ANOVA	Analysis of variance
APC	Antigen presenting cell
ApoE	Apolipoprotein E
ATG	Anti-thymocyte globulin
ATP	Adenosine triphosphate
AUC	Area under the curve

B

BBB	Blood-brain barrier
BMI	Body mass index
BSA	Bovine serum albumin

C

$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CC3	Cleaved caspase-3
CCL2	CC-chemokine ligand 2
CD	Cluster of differentiation
cDNA	Complementary DNA
CGM	Continuous glucose monitoring
CMRL	Connaught Medical Research Laboratories
CNS	Central nervous system
CSII	Continuous subcutaneous insulin infusion
C_T	Cycle threshold
CX3CL1	C-X3-C motif chemokine ligand 1
CXCL1-16	C-X-C motif chemokine ligand 1-16
CXCR1/2/16	C-X-C motif chemokine receptor 1/2/16

D

DAISY	Diabetes Autoimmunity Study in the Young
DAPI	4’,6-Diamidino-2-Phenylindole
DCCT	Diabetes Control and Complications Trial
DFG	Deutsche Forschungsgemeinschaft

DIPP	Type 1 Diabetes Prediction and Prevention Study
DiRECT	Diabetes Remission Clinical Trial
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic acid
DXM	Dextromethorphan
DXO	Dextrorphan

E

ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicine Agency
ER	Endoplasmic reticulum

F

FBS	Fetal bovine serum
FDA	U.S. Food and Drug Administration
FINDIA	Finnish Dietary Intervention Trial for the Prevention of Type 1 Diabetes
FMD	Flow-mediated dilation

G

GABA	γ-Amino butyric acid
GAD65	Glutamate decarboxylase 65
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte-colony stimulating factor
GLP-1	Glucagon-like peptide-1
GLUT-1/2	Glucose transporter 1/2
GTT	Glucose tolerance test
GWAS	Genome wide association studies

H

HbA1c	Glycated hemoglobin
HEPES	(4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HLA	Human leucocyte antigen
HPRT	Hypoxanthine-guanine phosphoribosyltransferase

I

IA-2	Insulin-associated antigen 2

Idd4	Insulin-dependent diabetes susceptibility 4
IDDM22	Insulin-dependent diabetes mellitus 22
IDF	International Diabetes Federation
IFNγ	Interferon γ
IIDP	Integrated Islet Distribution Program
IL-1 - 21	Interleukin 1 - 21
i.p.	Intraperitoneally

J

JDRF	Juvenile Diabetes Research Foundation

K

KRH	Krebs Ringer HEPES

L

LANUV	Landesamt für Natur, Umwelt und Verbraucherschutz
LSM	Laser scanning microscope

M

MCP-1	Monocyte chemoattractant protein 1
MHC	Major histocompatibility complex
MLDS	Multiple low dose streptozotocin
MMTT	Mixed meal tolerance test
mRNA	Messenger ribonucleic acid

N

NIDDK	National Institute of Diabetes and Digestive and Kidney Diseases
NIH	National Institutes of Health
NK	Natural killer
NMDAR	N-methyl-D-aspartate receptor
NOD	Non-obese diabetic
NOX	NADPH oxidase

O

o/n	Overnight
oGTT	Oral glucose tolerance test

P

PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PP	Pancreatic polypeptide

Q

qPCR	Quantitative real-time polymerase chain reaction

R

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RRP	Readily releasable pool
RT	Room temperature

S

Scid	Severe combined immunodeficiency
SEM	Standard error of the mean
SGLT-2	Sodium-glucose linked transporter 2
SPF	Specific pathogen free
STZ	Streptozotocin

T

T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus
TCA	Tricarboxylic acid cycle
TEDDY	The Environmental Determinants of Diabetes in the Young
Teff	Effector T cell
TNFα	Tumor necrosis factor α
Treg	Regulatory T cell

V

VGCC	Voltage-gated calcium channel

W

WHO	World Health Organization

Z

ZnT8	Zinc transporter 8

Statutory declaration

„Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.“

Ich habe die Dissertation weder in der hier vorgelegten, noch in einer ähnlichen Form, bei einer anderen Fakultät eingereicht und habe bisher keine Promotionsversuche unternommen.

Düsseldorf, den 28.09.2021

Laura Wörmeyer

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