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The *N*-Methyl-D-Aspartate Receptor Antagonist Dextromethorphan Improves Glucose Homeostasis and Preserves Pancreatic Islets in NOD Mice

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ABSTRACT

For treatment of type 1 diabetes mellitus, a combination of immune-based interventions and medication to promote beta-cell survival and proliferation has been proposed. Dextromethorphan (DXM) is an *N*-methyl-D-aspartate receptor antagonist with a good safety profile, and to date, preclinical and clinical evidence for blood glucose-lowering and islet-cell-protective effects of DXM have only been provided for animals and individuals with type 2 diabetes mellitus. Here, we assessed the potential anti-diabetic effects of DXM in the non-obese diabetic mouse model of type 1 diabetes. More specifically, we showed that DXM treatment led to five-fold higher numbers of pancreatic islets and more than two-fold larger alpha- and beta-cell areas compared to untreated mice. Further, DXM treatment improved glucose homeostasis and reduced diabetes incidence by 50 %. Our data highlight DXM as a novel candidate for adjunct treatment of preclinical or recent-onset type 1 diabetes.

Introduction

Standard treatment of type 1 diabetes mellitus is the administration of exogenous insulin, either through multiple daily insulin injections or through continuous subcutaneous insulin infusion (CSII) [1]. Over the last two decades, advanced insulin delivery systems, glucose monitoring devices and new insulin analogues have been developed to improve glycemic control, while minimizing the risk of hypoglycemia and reducing treatment-associated burden [2–4]. Although remarkable improvements in glycemic control and cardiovascular outcomes have been observed, still, across all age groups, the majority of individuals with type 1 diabetes does not reach target HbA1c [5, 6]. Among insulin-treated adult individuals with type 1 diabetes, worldwide only 15 % achieve HbA1c levels below 7 % without episodes of severe hypoglycemia or diabetic ketoacidosis [7]. Yet, individuals with type 1 diabetes are at elevated risk of cardiovascular disease and mortality, culminating in a loss of life-expectancy of 17.7 and 14.2 years in women and men, respectively, in those diagnosed before 10 years of age [8].

In individuals with type 1 diabetes, residual beta cell function associates with better glycemic control, lower risk for severe hypoglycemia and a reduced rate of microvascular disease progression [9]. Therefore, preserving beta cell function, that is, the ability to secrete C-peptide after disease onset, or preventing the symptomatic onset of type 1 diabetes in at-risk individuals, is a major goal of modern diabetes management [10].

Although type 1 diabetes is primarily considered a T-cell mediated disease, B-cells and other antigen-presenting cells (e. g., monocytes/macrophages, dendritic cells) as well as components of the innate immune system, including soluble inflammatory factors (e. g., TNFα), are involved in the immunopathology of type 1 diabetes. Beta cells are furthermore thought to be particularly vulnerable to autoimmune destruction and to amplify disease development by provoking an immune response that drives their own dysfunction and demise [11, 12]. Therefore, multiple targets for immune-based intervention strategies exist to prevent or reverse type 1 diabetes. Some progress has recently been achieved with teplizumab, a non-Fcγ receptor-binding/non-activating monoclonal anti-CD3 antibody [hOKT3γ (Ala-Ala)], in delaying the onset of type 1 diabetes in at-risk individuals [13]. However, to date, no single immunomodulatory therapy is able to induce long-lasting C-peptide preservation and clinical remission in a majority of treated individuals. More invasive approaches, for example, islet transplantation, represent an attractive alternative to immunomodulatory interventions, but are associated with serious adverse events and are reserved for a rather small proportion of individuals yet given the limited number of organ donor supply [14].

In recent years, pancreatic *N*-methyl-d-aspartate receptors (NMDARs) have been increasingly recognized as potential novel targets for diabetes management as they are involved in the regulation of insulin release, glucose homeostasis and pancreatic islet cell viability [15–20]. Particularly, we demonstrated that under diabetogenic conditions, inhibition of pancreatic NMDAR with dextromethorphan (DXM) promotes islet cell survival, both *in vitro*, suggesting the activation of islet cell intrinsic protective mechanisms, and *in vivo,* in the type 2 diabetic mouse model *db/db* [15]. We therefore hypothesized that DXM could also be beneficial in an animal model of human type 1 diabetes, that is, in NOD mice.

Notably, NMDAR are also expressed by several types of immunocompetent cells, such as lymphocytes and neutrophils, and NMDAR signaling has been shown to differentially affect their polarization, proliferation and survival [21–27]. Furthermore, preclinical and clinical studies indicate that DXM has anti-inflammatory and immunomodulatory properties [28–37]. For example, DXM treatment inhibits lipopolysaccharide (LPS)-induced functional maturation of murine and human dendritic cells (DC), reduces their production of proinflammatory cytokines, chemokines and oxidative stress, and impairs the ability of LPS-induced murine DCs to activate antigen-specific T-cell responses [34, 36]. In murine models of chronic immune-mediated inflammatory disorders, DXM was shown to slow disease progression and alleviate symptoms by decreasing the generation of proinflammatory cytokines [36, 38]. In human individuals with autoimmune rheumatoid arthritis, treatment with DXM as add-on to disease-modifying-antirheumatic drugs significantly reduced circulating levels of proinflammatory cytokines, including TNFα and IL-6 [36]. Thus, we wished to investigate whether NMDAR antagonists provide any kind of protection for pancreatic islets in the NOD mouse model of type 1 diabetes, one of the most commonly used rodent models of human type 1 diabetes [39].

Material and Methods

Mouse models

For *in vitro* experiments with isolated islets, male C57BL/6 J mice older than 9 weeks were purchased from Janvier (France). Female non-obese diabetic mice (NOD/ShiLtJ, 001976) were purchased from The Jackson Laboratory (USA). NOD mice were maintained under specific pathogen-free conditions and housed in rooms with a controlled temperature of 22 °C, a humidity of 55 %, and a 12:12-hour light/dark cycle. 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, Germany) approved all animal experiments (Az. 84–02.04.2014.A268).

Long-term treatment of NOD mice and glucose tolerance test

From the age of 4 weeks onwards, NOD mice were treated with either normal drinking water (control mice) or with 3 g/l DXM in their drinking water. Mice were either treated up to an age of 10 weeks (prediabetic mice), until diabetes development (diabetic mice), or up to an age of 30 weeks in case diabetes did not develop (non-diabetic mice). Random blood glucose levels were measured weekly. Mice with blood glucose levels exceeding 250 mg/dl on two consecutive days were considered diabetic and directly sacrificed (diabetic mice). The glucose tolerance test was performed with prediabetic NOD mice fasted overnight for 16 hours. These mice had received DXM via the drinking water for 6 weeks. Glucose was intraperitoneally injected (1.5 mg glucose per gram of bodyweight), and glucose concentrations were measured in blood collected from the tail before and at 5, 30, 90, and 120 minutes after glucose injection.

Immunohistochemistry

Pancreata from NOD mice were collected and cryopreserved with sucrose after fixation in paraformaldehyde. Cryo-sections (12 µm) from 4 different depths within the pancreas were made for each mouse per slide. Antibodies used for stainings are listed in **Supplementary Table 1 S**. Cell nuclei were counterstained using DAPI (Sigma Aldrich, 1/1000).

Imaging and Image Analysis

For quantification of insulin- and glucagon-positive areas, as well as determination of islet numbers, images were acquired blinded using a Zeiss Apotome Axio Observer.Z1 microscope equipped with an AxioCam MRm and 10×/0.45 as well as 20×/0.8 Plan Apochromat objective lenses (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). Overview images of pancreatic sections were obtained via tile scans using the 10× objective. Each islet consisting of more than 5 insulin-positive cells was additionally imaged using the 20× objective lens. For all other immunohistochemical analyses, the images of all islets per mouse containing more than 5 insulin-positive cells were obtained using a Zeiss LSM 710 equipped with a 40×/1.4oil DIC M27 Plan Apochromat objective lens (Carl Zeiss Microscopy GmbH, Oberkochen, Germany).

Acquired images were analyzed blinded using FIJI (ImageJ) [40]. 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 images of pancreatic sections. CD4- and CD8-positive areas were determined by RenyiEntropy thresholding and normalized to total pancreatic nuclei area determined by Otsu thresholding of images of pancreatic sections. To determine cleaved caspase-3-positive areas of islets, the positive area was measured by RenyiEntropy thresholding within the islet. Islet area used for normalization was defined by Li thresholding of insulin area. Ki67-positive cell nuclei were counted manually and normalized to islet area defined by Li thresholding of insulin area.

Insulitis scoring

Pancreatic sections were stained for CD45-positive cells. Analyses were done blinded. Images were acquired using a Zeiss Apotome Axio Observer.Z1 microscope equipped with an AxioCam MRm and a 20×/0.8 Plan Apochromat objective lens (Carl Zeiss Microscopy GmbH, Oberkochen, Germany). The CD45-positive area was measured by RenyiEntropy or Triangle thresholding and normalized to islet area including the surrounding CD45-positive immune cells and scored as follows. Score 0: no immune cells are present or < 2 % of islet area is infiltrated; score 1: less than 10 % of islet area is infiltrated, but immune cells surround the islets (peri-insulitis); score 2: 10–50 % of the islet area is infiltrated; score 3: 50–90 % of the islet area is infiltrated; score 4: > 90 % of the islet area is infiltrated.

Isolation of mouse pancreatic islets

Mouse pancreatic islets were isolated from C57BL/6 J mice older than 9 weeks according to the protocol by Yesil et al. with few minor changes [41]. Pancreatic tissues were enzymatically digested with Liberase TL Research Grade (Roche, Basel, Switzerland) at 37 °C for 16 minutes. Afterwards, digestion was stopped with DMEM + GlutaMAX (1 mg/ml glucose) supplemented with 15 % heat-inactivated FBS. Several washing and filtering steps were performed, before islets were separated from exocrine tissues by gradient centrifugation at 1200 *g* for 25 minutes and collected from the interphase between Lymphoprep and DMEM. Finally, islets were washed twice with islet medium (Connaught Medical Research Laboratories medium 1066 (CMRL) supplemented with 15 % heat-inactivated FBS, 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µM β-mercaptoethanol, 0.15 % $NaHCO_3$, 10 mM glucose). All further assays with isolated islets were performed after overnight culture in islet medium at 37 °C, 5 % CO_2, and a humidified atmosphere.

In vitro treatment of isolated mouse pancreatic islets

Isolated mouse pancreatic islets were incubated in the presence or absence of 1 µM dextrorphan (DXO), the main demethylated metabolite of DXM, for 24 hours at 37 °C and 5 % CO_2 to analyze chemokine expression under the influence of DXO. Furthermore, to analyze chemokine expression under inflammatory conditions, islets were treated for 24 hours with a mixture of cytokines consisting of 1000 U/mL recombinant mouse TNFα (R & D Systems, Minneapolis, Canada), 1000 U/ml recombinant mouse IFNγ (Thermo Fisher Scientific, Waltham, USA) and 50 U/ml recombinant mouse IL-1β (R & D Systems, Minneapolis, Canada) in the presence or absence of 1 µM dextrorphan (DXO). DXO-treated islets were pretreated with 1 µM DXO for 1 hour before cytokine mixture was added.

Isolation of RNA

RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instruction after islets were centrifuged for 2 minutes at 3300 rpm and 4 °C. An additional DNA digestion step was included in the RNA isolation process using RNase-free DNase Kit (Qiagen, Hilden, Germany).

Quantitative real-time PCR

To measure gene expression levels, isolated RNA was transcribed into cDNA using SuperScript II Reverse Transcriptase according to manufacturer's protocol. Quantitative real time PCR (qPCR) was performed using Brilliant III Ultra-Fast SYBR Green QPCR Master Mix on QuantStudio 1 (Thermo Fisher Scientific, Waltham, USA). Samples were analyzed according to the comparative ΔCt method as described previously [42]. Used primer sequences are listed in **Supplementary Table 2 S**.

Cytokine measurements in supernatants

Supernatants of islets were used to determine concentrations of secreted CCL2 using Mouse CCL2 Quantikine ELISA (MJE00b, R & D Systems, Minneapolis, Canada) according to manufacturer's instruction and measured on GloMax Discover Microplate Reader (Promega, Walldorf, Germany).

Statistical Analysis

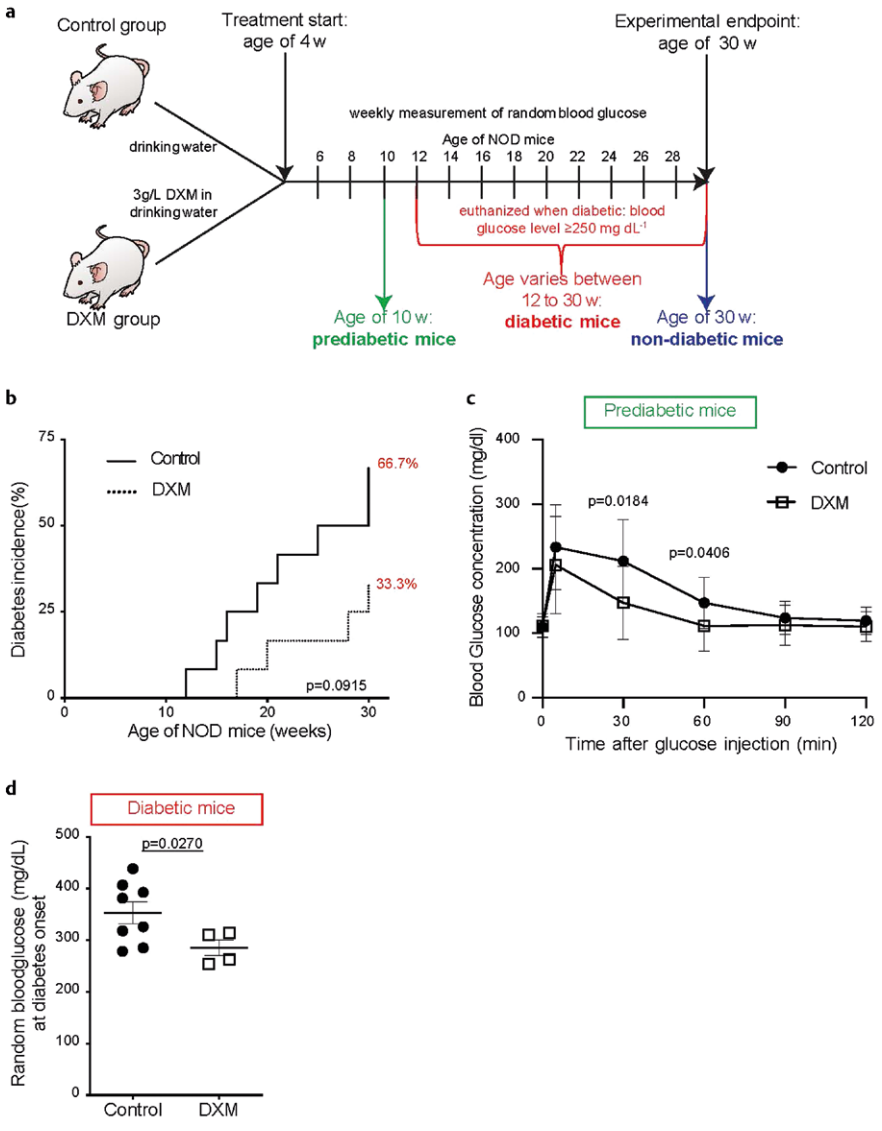
Software Prism 9 (GraphPad Inc.) or Excel (Microsoft Corporation) were used for calculations. For the comparison of two groups, either paired two-tailed Student's *t*-test or unpaired Student's *t*-test with Welch correction was performed as stated in figure legends. The statistical evaluation of diabetes 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) or ± standard de-

viation (SD; glucose tolerance test), except for paired data, diabetes incidence curve and insulitis score.

Results

Dextromethorphan delays diabetes onset and improves glucose homeostasis in NOD mice

To investigate the effect of DXM on blood glucose homeostasis and diabetes incidence in an animal model of type 1 diabetes, female NOD mice were continuously treated with either normal drinking water (control) or DXM via drinking water (DXM, 3 g/l) starting at the age of 4 weeks. Random blood glucose concentrations were measured weekly. Diabetes onset was defined by a confirmed blood glucose concentration ≥ 250 mg/dl on two consecutive days. For subsequent analyses, control mice and DXM-treated mice were sacrificed at three different time points (▶ **Fig. 1a)**, either at the age of 10 weeks before diabetes onset (prediabetic cohort), at diabetes onset (diabetic cohort), or at the age of 30 weeks in the case diabetes did not develop (non-diabetic cohort). In NOD mice, treatment with



▶ **Fig. 1** In the type 1 diabetes mouse model NOD, treatment with dextromethorphan delays diabetes onset and improves glucose tolerance. **a**: Schematic timeline of the experimental setup: 4 week-old female NOD mice continuously received either normal drinking water (control group) or 3 g/l dextromethorphan via drinking water (DXM group); mice were euthanized at the age of 10 weeks (prediabetic mice: green), at diabetes onset, defined as a random blood glucose concentration ≥ 250 mg/dl on two consecutive days (diabetic mice: red), or following a maximum of 26 weeks of DXM treatment at the age of 30 weeks (non-diabetic mice: blue). **b**: Kaplan–Meier curve indicating the incidence of diabetes in DXM-treated NOD mice compared to control NOD mice (n = 12 mice per treatment group). **c**: Blood glucose concentrations of 10–11 week-old female NOD mice during an intraperitoneal glucose tolerance test (1.5 mg g^{-1} body weight) either treated or untreated with DXM via the drinking water starting at the age of 4 weeks (n = 11 control and n = 12 DXM-treated mice). **d**: Random blood glucose concentrations of either DXM-treated or control NOD mice at diabetes onset (n = 8 control and n = 4 DXM-treated mice). Statistical significance determined by Mantel–Cox log-rank test, p = 0.0915 (**b**), multiple Student's *t*-test (**c**), and unpaired Student's *t*-test (**d**). Data in (**c**) and (**d**) are presented as mean ± SD (**c**) and mean ± SEM (**d**).

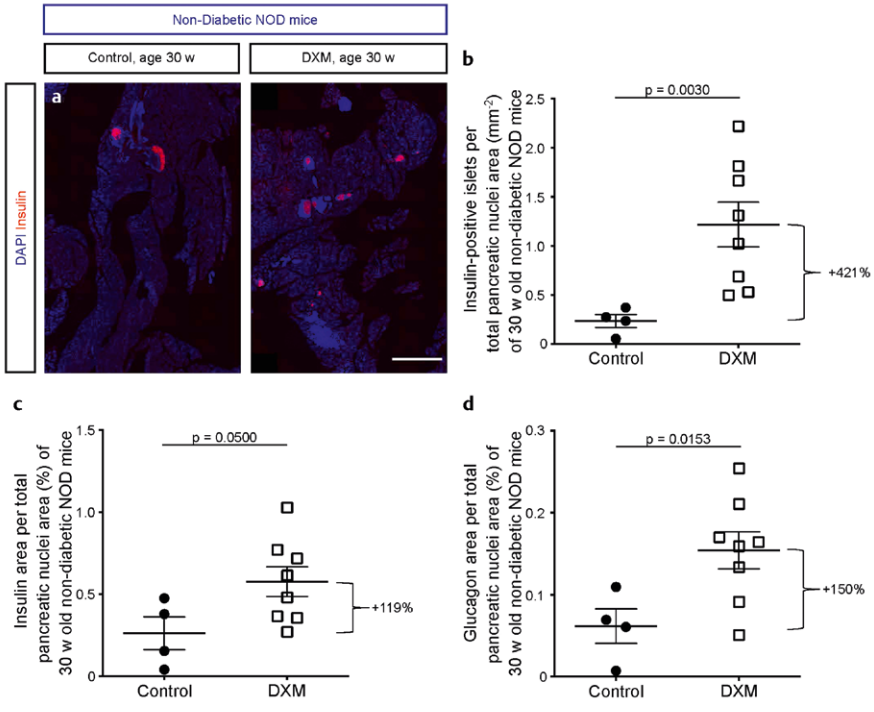
DXM delayed diabetes onset by approximately 5 weeks and reduced diabetes incidence by 50 % until 30 weeks of age (▶ **Fig. 1b**), albeit the effect was not statistically significant (p = 0.09). Precisely, at the age of 30 weeks, 8 of 12 control NOD mice (67 %) had developed diabetes, corresponding to diabetes incidence rates previously described in NOD mice [43], while only 4 of 12 DXM-treated NOD mice (33 %) had developed diabetes (▶ **Fig. 1b**). Furthermore, DXM treatment significantly enhanced glucose tolerance in prediabetic NOD mice 30 and 60 minutes after glucose injection (▶ **Fig. 1c, p** < 0.05 at both time points), and significantly lowered blood glucose concentrations at diabetes onset (▶ **Fig. 1d**, p = 0.027).

Dextromethorphan preserves pancreatic islets in NOD mice

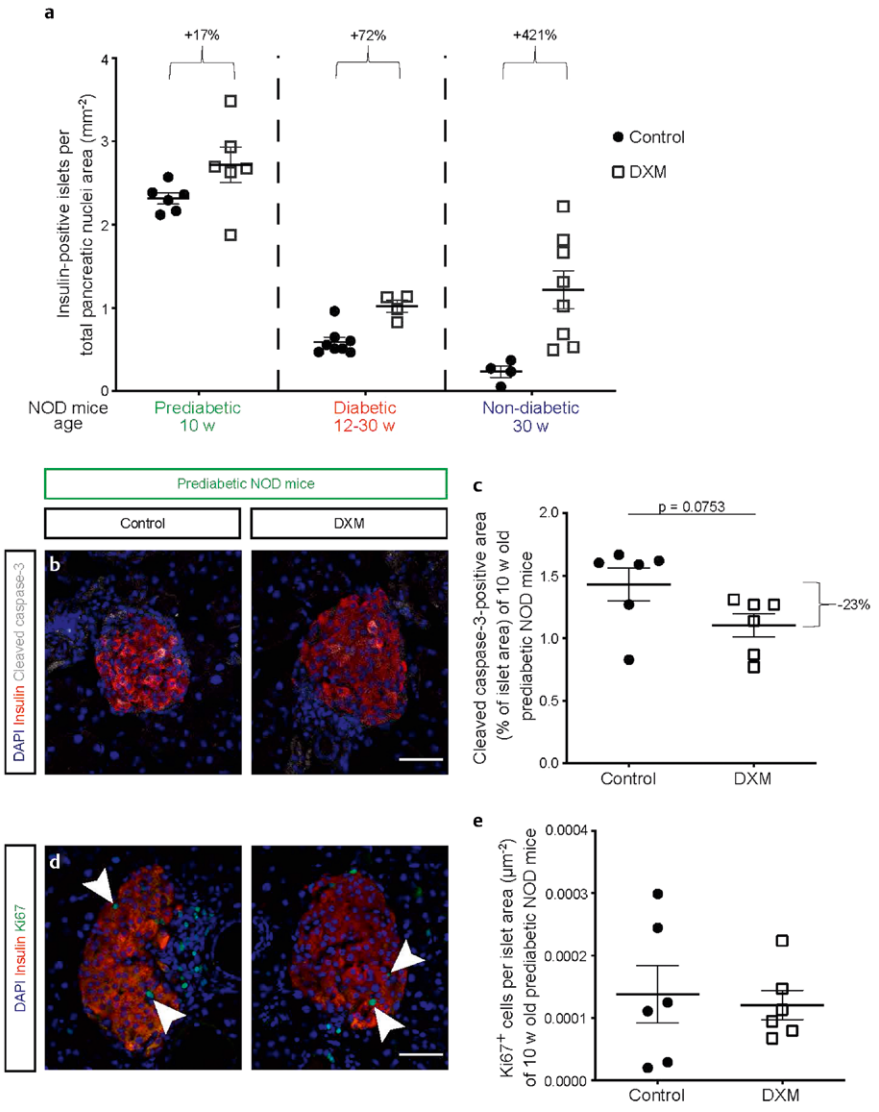
To investigate the effect of DXM on islet numbers, as well as alpha and beta cell areas, pancreata were obtained from prediabetic, diabetic and non-diabetic DXM-treated NOD mice and control NOD mice. For immunohistochemical analyses, pancreatic sections were stained for insulin, glucagon and cell nuclei.

In all three cohorts, that is, in the prediabetic, diabetic and non-diabetic cohort, treatment with DXM resulted in an increased number of pancreatic islets per pancreatic nuclei area compared to control NOD mice (▶ **Fig. 2a,b**; **Suppl. Fig. 1S a,b; Suppl. Fig. 2S a,b**). Notably, the most prominent effect was seen in those mice that did not become diabetic. Among 30 week-old non-diabetic NOD mice, the number of pancreatic islets was five-fold higher in DXM-treated mice compared to control mice, corresponding to a significant increase in pancreatic islets of more than 400 % (▶ **Fig. 2b**, p = 0.003). Furthermore, both, alpha and beta cell areas were more than twice as large in the pancreata of DXM-treated compared to control non-diabetic NOD mice (▶ **Fig. 2c**, p = 0.050 and ▶ **Fig. 2d**, p = 0.015). Even among those NOD mice that developed diabetes, alpha and beta cell areas at diabetes onset were larger in DXM-treated mice compared to control mice, although the effect was not statistically significant (**Suppl. Fig. 2S c**, p = 0.051 and **Suppl. Fig. 2S d**, p = 0.061), whereas we found no difference between prediabetic DXM-treated and control NOD mice (**Supp. Fig. 1S c,d)**. Note that in the diabetic cohort, islet numbers as well as alpha and beta cell areas were higher in DXM-treated NOD mice compared to controls, although the mean age of DXM-treated mice was higher than that of untreated controls (24 weeks versus 21 weeks, data not shown).

Next, we aimed to reveal the mechanisms underlying pancreatic islet cell preservation upon DXM treatment. When directly comparing the prediabetic, diabetic and non-diabetic cohorts, we observed a marked age-dependent decline in pancreatic islet numbers per pancreatic nuclei area in control NOD mice (▶ **Fig. 3a**). In DXM-treated NOD mice, however, the number of pancreatic islets also declined initially from 10 weeks of age until diabetes onset, but stopped to decline thereafter in those mice that did not develop diabetes (▶ **Fig. 3a**). These data suggest that in NOD mice, DXM



▶ **Fig. 2** DXM treatment increases islet numbers, as well as beta- and alpha-cell areas in NOD mice. **a**: Representative fluorescence microscopy images of pancreatic sections from 30 week-old non-diabetic NOD mice, either untreated (control) or continuously treated with DXM over 26 weeks (DXM); blue: DAPI; red: insulin; scale bar: 1000 µm (≥ 16 cross-sections from n ≥ 4 mice per treatment group). (**b**) Islet numbers, (**c**) beta- and (**d**) alpha-cell areas within pancreatic sections of 30 week-old non-diabetic NOD mice treated as described in (**a**) (≥ 16 sections from n ≥ 4 mice per treatment group). Statistical significance determined by unpaired Student's *t*-test. Data are shown as mean ± SEM.



▶ **Fig. 3** DXM treatment numerically reduces the number of apoptotic islet-cells but has no effect on islet-cell proliferation in NOD mice. **a**: Islet numbers per total pancreatic nuclei area in 10 week-old prediabetic, 12–30 week-old diabetic and 30 week-old non-diabetic NOD mice, either untreated (control) or continuously treated with DXM since 4 weeks old (DXM), dashed lines indicate separate cohorts (n ≥4 mice per treatment group). **b**: Representative LSM images of pancreatic islets from 10 week-old prediabetic NOD mice treated as described in (**a**); blue: DAPI; red: insulin; grey: cleaved caspase-3 (≥47 islets per mouse from n = 6 mice per treatment group). **c**: Cleaved caspase-3-positive area per islet area (≥47 islets per mouse from n = 6 mice per treatment group). **d**: Representative LSM images of pancreatic islets from mice treated as described in (**b**); blue: DAPI; red: insulin; green: Ki67; arrow heads: Ki67-positive cell nuclei within pancreatic islet (≥38 islets per mouse from n = 6 mice per treatment group). **e**: Number of Ki67-positive cells per islet area (≥38 islets per mouse from n = 6 mice per treatment group). Scale bars: 50 µm. Statistical significance determined by unpaired Student's *t*-test comparing treatment groups. Data are shown as mean ± SEM.

confers a partial protection against the progressive autoimmune-mediated destruction of pancreatic islets. In line with these observations, the number of apoptotic cells within pancreatic islets, as determined by cleaved caspase-3-positive area, tended to be lower upon treatment with DXM, particularly in the prediabetic cohort (▶ **Fig. 3b,c**, p = 0.075 and **Suppl. Fig. 3S a**,**b**). In contrast, no difference in islet cell proliferation was determined using the proliferation marker Ki67 (▶ **Fig. 3d,e**).

There was no difference in the number of apoptotic islet cells between 30 week-old DXM-treated and control NOD mice, respectively (**Suppl. Fig. 3S c**,**d**), suggesting that DXM did not confer unspecific protection, but islet cell protection in the setting of diabetes development, ultimately culminating in the observed significant increase of pancreatic islets in the non-diabetic cohort (▶ **Fig. 2b** and ▶ **3a**).

Dextromethorphan had a small, albeit non-significant effect on the extent of insulitis in prediabetic and non-diabetic NOD mice

To determine the effect of DXM on the immune cell infiltration of pancreatic islets (insulitis), pancreatic sections of all three cohorts were stained for the common leukocyte antigen CD45, and insulitis was scored as described previously [44]. Precisely, the CD45-positive area was normalized to the islet area and the extent of insulitis was determined depending on the percentage of islet area infiltrated by CD45-positive immune cells, ranging from no infiltration (grade 0) to > 90 % of immune-cell infiltration (grade 4).

In the prediabetic cohort, the mean insulitis score, which was assessed blinded for treatment groups, was numerically lower in DXM-treated NOD mice compared to control NOD mice, however, the effect was not statistically significant (▶ **Fig. 4a–c**, mean insulitis score of 0.72 in DXM-treated mice vs. 0.98 in controls, p = 0.43). Similarly, among those NOD mice that did not develop diabetes, the extent of immune cell infiltration was lower in DXM-treated NOD mice compared to control NOD mice (**Suppl. Fig. 4S d–f**, mean insulitis score of 1.52 in DXM-treated mice vs. 1.82 in controls, p = 0.35), while there was clearly no difference in insulitis between the two groups at diabetes onset (**Suppl. Fig. 4S a–c)**.

Since type 1 diabetes is a T-cell mediated disease, and in NOD mice $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells are required for diabetes development and beta cell destruction in particular [45], we additionally stained pancreatic sections of prediabetic NOD mice for CD4 and CD8 to specifically assess T-cell infiltration of pancreatic islets. The number of both, infiltrating $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells, was numerically reduced by about 60 % upon DXM treatment (▶ **Fig. 4d–f**). Although the effect was not statistically significant, there was a clear trend for DXM to reduce the number of infiltrating T-lymphocytes, particularly $CD8^+$ T-cells (p = 0.08).

Dextrorphan reduced the expression of chemokines in pancreatic islets

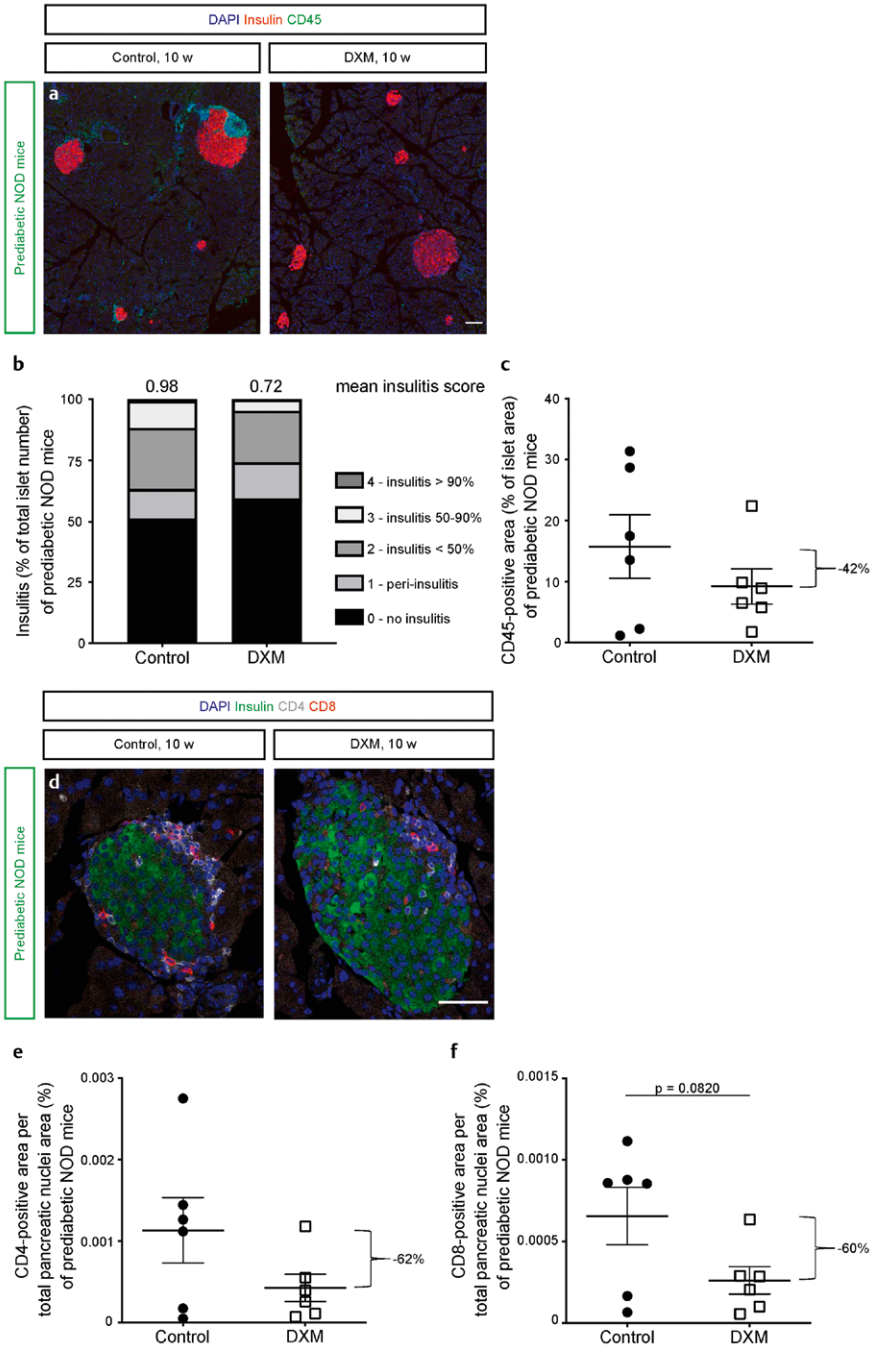
In NOD mice, continuous treatment with DXM numerically decreased the number of infiltrating $CD4^+$ T-helper cells and $CD8^+$ cytotoxic T-cells in pancreatic islets by around 60 % (▶ **Fig. 4d–f**). To investigate whether DXM downregulates the expression of chemokines in pancreatic islets, thus causing reduced immune cell attraction, we incubated isolated mouse pancreatic islets with dextrorphan (DXO), the main metabolite of DXM, and assessed mRNA expression levels of several chemokines by qRT-PCR. DXO treatment significantly reduced mRNA expression of C-X3-C motif chemokine ligand 1 (*Cx3cl1*) and C-X-C motif chemokine ligand 16 (*Cxcl16*), and numerically decreased mRNA expression of C-X-C motif chemokine ligand 1 (*Cxcl1*), C-X-C motif chemokine ligand 2 (*Cxcl2*) and C-C motif chemokine ligand 2 (*Ccl2*) in pancreatic islets (▶ **Fig. 5a**). Cytokines are known to upregulate the expression of chemokines in pancreatic islets [46], and they are directly implicated in type 1 diabetes immunopathology. Therefore, we next investigated whether DXO also affects cytokine-induced chemokine expression of pancreatic islets. As expected, treatment of pancreatic islets with TNFα, IL-1β and IFN-γ increased mRNA expression of *Cxcl1*, *Cxcl2*, *Cxcl16* and *Ccl2* (▶ **Fig. 5b)**. However, treatment with DXO decreased cytokine-induced mRNA expression of all chemokines assessed. Particularly, DXO significantly reduced the expression of *Ccl2* in pancreatic islets by 80 % compared to cytokine treatment alone. Similar results were obtained when mRNA expression was normalized to different housekeeping genes (**Suppl. Fig. 5S a–d**). We furthermore assessed the expression level of CCL2 protein in the supernatant of pancreatic islets treated with TNFα, IL-1β and IFN-γ in the presence or absence of DXO. DXO significantly decreased cytokine-induced CCL2 protein expression in supernatants of pancreatic islets by 50 % (▶ **Fig. 5c**). These data indicate that under inflammatory conditions, DXO downregulates the expression of chemokines in pancreatic islets, known to be associated with immune cell attraction in diabetes development [47–51].

Discussion and Conclusions

Initial intervention strategies to maintain or regenerate beta cells in individuals with type 1 diabetes were limited to individuals with recent-onset diabetes that lack sufficient beta cell mass and thus failed to reach clinically relevant endpoints. Understanding the different stages of type 1 diabetes has allowed for the identification of pre-symptomatic individuals months to years before diabetes onset, and has enabled intervention in the high-risk population. Recently, the monoclonal anti-CD3 antibody teplizumab was approved by the FDA for the delay of stage 3 type 1 diabetes in adult and pediatric individuals ≥ 8 years of age who currently have stage 2 type 1 diabetes. However, although a 14-day course of daily intravenous teplizumab infusions significantly delayed median time to diagnosis of clinical diabetes by about two years, still, after a median follow-up period of 2.5 years, 50 % of individuals in the teplizumab group had developed stage 3 type 1 diabetes [13, 52, 53]. Novel concepts therefore employ combined approaches that target different components of type 1 diabetes immunopathology to enhance the effects on beta cell survival and diabetes progression [10]. It has particularly been suggested to combine immune-based interventions with compounds primarily targeting the beta cells to enhance beta cell survival [54].

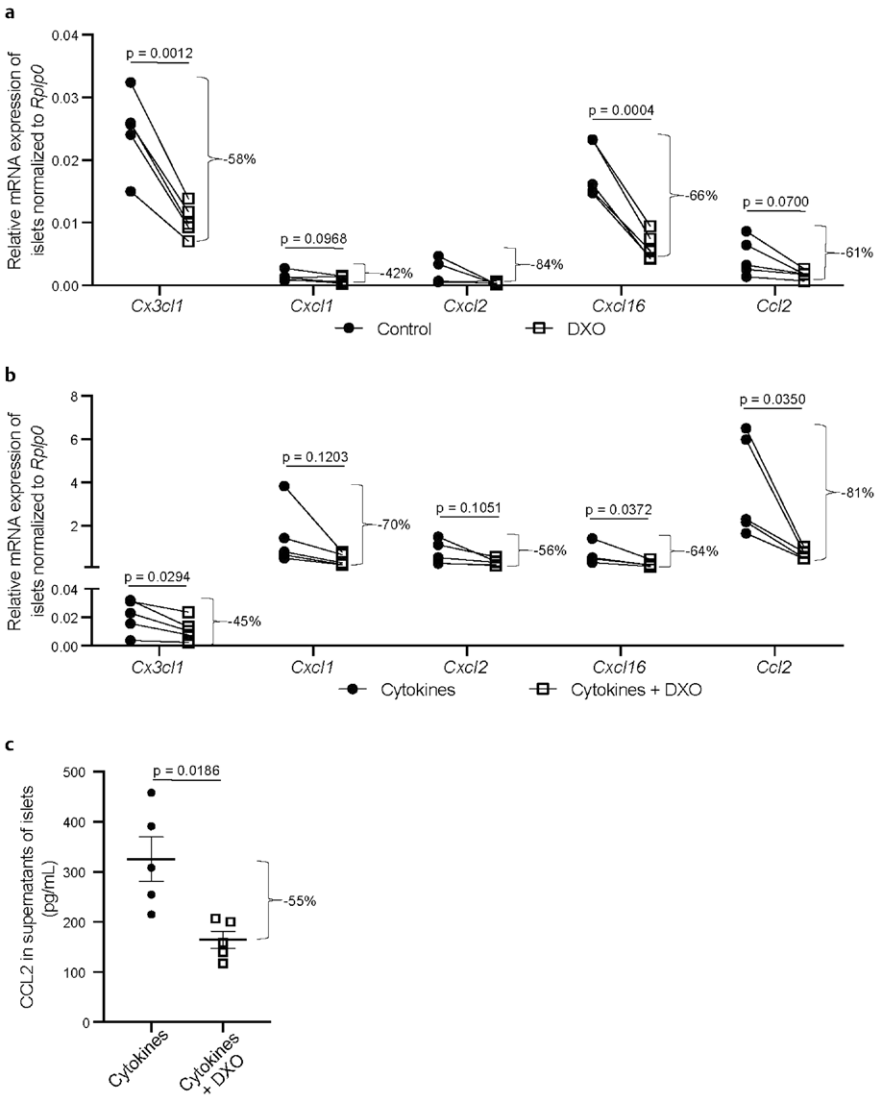
In recent years, we and others demonstrated that the NMDAR antagonist DXM increases insulin secretion and lowers blood glucose level in mice and individuals with type 2 diabetes [15–20]. We furthermore demonstrated that under diabetogenic conditions, NMDAR antagonists are beneficial for mouse and human islet cell survival. Particularly, DXM and its active metabolite DXO were shown to preserve beta cell mass in the type 2 diabetic mouse model *db/db* and to protect isolated human pancreatic islets against cytokine-mediated islet cell death *in vitro* [15]. Moreover, preclinical and clinical studies indicate anti-inflammatory and immunomodulatory properties of DXM in distinct chronic inflammatory and autoimmune diseases [28–37]. We therefore aimed to investigate potential islet cell protective and immunomodulatory effects of DXM in a rodent model of spontaneous autoimmune diabetes.

Here, for the first time we provide *in vivo* evidence that DXM improves glucose homeostasis and delays diabetes onset in NOD mice, the most commonly used mouse model of human type 1 diabetes. More importantly, continuous application of DXM via the drinking water led to five-fold higher numbers of pancreatic islets and more than twofold larger alpha and beta cell areas compared to untreated mice.



▶ **Fig. 4** DXM treatment reduces the number of infiltrating T-cells, particularly CD8-positive T-lymphocytes. **a**: Representative fluorescence microscopy images of pancreatic islets from 10 week-old prediabetic NOD mice either untreated (control) or DXM-treated for 6 weeks (DXM); blue: DAPI; red: insulin; green: CD45; scale bar: 100 µm (≥ 60 islets per mouse from n = 6 mice per treatment group). **b**: Insulitis score and (**c**) CD45-positive area per islet area of the same mice (≥ 60 islets per mouse from n = 6 mice per treatment group). **d**: Representative LSM images of pancreatic islets from mice treated as described in (**a**); blue: DAPI; green: insulin; red: CD8; grey: CD4; scale bar: 50 µm (≥ 40 islets per mouse from n = 6 mice per treatment group). **e**: CD4- and (**f**) CD8-positive area per total pancreatic nuclei area of the same mice (≥ 40 islets per mouse from n = 6 mice per treatment group). Statistical significance determined by unpaired Student's *t*-test comparing treatment groups. Data in (**c**), (**e**) and (**f**) are shown as mean ± SEM.

In line with previous findings our data further support a role for DXM in promoting islet cell survival: notably, although we found a marked variability of caspase-3 staining between different cohorts (data not shown) and among NOD mice of a single cohort, corresponding to the substantial variation in their progression to diabe-



▶ **Fig. 5** DXO treatment decreases the expression of chemokines in pancreatic islets. **a**, **b**: Relative mRNA expression of *Cx3cl1*, *Cxcl1*, *Cxcl2*, *Cxcl16*, and *Ccl2* normalized to the expression of *Rplp0* of either untreated islets compared to islets treated for 24 hours with 1 µM DXO (**a**) or of islets treated for 24 hours with cytokines (IFNγ, IL-1β, TNFα) in the presence or absence of 1 µM DXO (**b**) (n = 5 independent experiments). **c**: Concentration of CCL2 in supernatants of islets treated as described in (**b**) (n = 5 per condition). Statistical significance determined by paired (**a**, **b**) or unpaired (**c**) Student's *t*-test comparing treatments with or without DXO.

tes, we observed a clear trend for DXM to reduce islet cell apoptosis in prediabetic NOD mice (p = 0.075).

The mechanisms underlying the observed beneficial effects of DXM in NOD mice, however, remain unclear and require further investigations. Since NMDAR are also known to be involved in the regulation of human T-cell responses [22, 23, 26], we additionally assessed the effect of DXO on chemokine expression pattern of pancreatic islets under inflammatory conditions *in vitro,* as well as the effect of DXM treatment on inflammatory islet infiltration and islet T-lymphocyte infiltration in NOD mice. Chemokines are known to augment diabetes progression by attracting more immune cells to already inflamed islets, thereby triggering a vicious circle of immune cell infiltration, inflammation, beta cell death and cytokine secretion [55–57]. Notably, in isolated pancreatic islets, DXO numerically decreased basal and cytokine-induced expression levels of all chemokines investigated, and significantly reduced cytokine-induced *Ccl2* expression and CCL2 protein release from pancreatic islets *in vitro*. CCL2 acts as a chemoattractant for leucocytes and has been shown to impact T-cell responses under physiological and pathological conditions, including during diabetes development [58–60]. For example, in NOD mice, genetic deletion of the Ccl2 receptor CC chemokine receptor 2 (*Ccr2*) delays recruitment of inflammatory cells and thus diabetes onset [61]. In addition, pharmacological inhibition of CCR2 has proven anti-inflammatory, antidiabetic and islet cell protective effects in animal models of type 2 diabetes [62]. Furthermore, isolated human pancreatic islets express and release functional CCL2,

and a low level of islet CCL2 secretion has been shown to be associated with insulin independence and graft survival after human islet transplantation in individuals with type 1 diabetes [63].

Although the *in vitro* data indicated attenuated immune cell attraction to pancreatic islets upon DXO treatment, *in vivo* we observed only a modest, non-significant effect on overall insulitis as assessed by CD45-positive islet area. Precisely, DXM numerically decreased the CD45-positive islet area and the mean insulitis score in prediabetic and non-diabetic NOD mice, while increasing the proportion of islets with no insulitis or peri-insulitis. The effect was, however, not statistically significant, yet given the large variability observed among control mice with two mice presenting almost no inflammatory infiltrate. Notably, we observed somewhat stronger effects when specifically assessing T-cell infiltration of pancreatic islets. Islet infiltration of CD4- and CD8-positive T-lymphocyte was reduced by 60 % in DXM-treated NOD mice compared to control mice, and there was particularly a clear trend for DXM to reduce the number of infiltrating $CD8^+$ T-cells ($p = 0.08$). $CD4^+$ and $CD8^+$ T-cells are required for diabetes development, and non-depleting CD4 and CD8 antibodies were previously shown to induce long-lasting diabetes remission in recent-onset diabetic NOD mice [39, 45, 64].

We conclude that the beneficial effects of DXM in the NOD mouse model of type 1 diabetes are mediated via distinct pathways that require further elucidation. The fact, that DXO and its derivatives promote murine and human islet cell survival under diabetogenic conditions, including upon cytokine or streptozotocin treatment *in vitro* [17, 19], points towards islet-cell intrinsic pathways conferring protection. However, the *in vitro* data on the effect of DXO on islet chemokine expression under inflammatory conditions, and the *in vivo* data on the effect of DXM on inflammatory islet infiltration, particularly CD8 + T-cell infiltration, support previous findings on the potential of DXM to modify immune responses.

Notably, DXM is in clinical use for more than 50 years and sold over-the-counter in several cough and cold preparations [16, 65]. Therapeutic doses of DXM are safe, even in pediatric individuals [66]. Moreover, as reviewed previously, there is considerable preclinical and clinical evidence for DXM to be angio-, retino- and nephroprotective [67, 68]. For example, altered glutamate metabolism and NMDAR signaling contribute to neuroretinal dysfunction and death, and in diabetic rats, long-term treatment with the NMDAR antagonist memantine improved retinal function and protected against streptozotocin-induced retinal ganglion cell loss [69]. Interestingly, chronic exposure of pancreatic islets to DXO was recently shown to upregulate the expression of *fibroblast growth factor 21* (*FGF21*) [70], while in rodent models of type 1 diabetes the FGF21 analog PF-05231023 reversed diabetes-induced photoreceptor dysfunction and retinal inflammation [71]. In humans DXM was shown to improve flow-mediated vasodilation, a measure for endothelial function, and to attenuate vascular oxidative stress [31, 67]. However, high doses of DXM are psychoactive, and DXM has been misused for recreational purposes [72]. We have therefore recently designed novel derivates of DXM with reduced central nervous side effects while maintaining their peripheral anti-diabetic effects [17].

One limitation of our study is the use of the NOD mouse to model human type 1 diabetes. Although disease pathogenesis in the NOD mouse shares many features with human disease progression, previous studies indicated that results from NOD mice are not always translatable to the human system. However, the NOD mouse is still the most commonly used rodent model for type 1 diabetes with important implications for human trials, when preclinical studies are conducted according to standards as proposed by Atkinson [73]. Notably, we have already demonstrated in two clinical trials that DXM exerts anti-diabetic effects in humans with type 2 diabetes [15, 19]. Moreover, pancreatic islets from human donors were shown to be protected against cytokine-induced cell death when incubated with DXO [15], further increasing the likelihood of successful translation of our results into the human system.

In conclusion, the strength of our study lies in the first *in vivo* demonstration of the therapeutic potential of DXM in a murine model of human type 1 diabetes. Particularly, for the first time we demonstrate a role for DXM in supporting islet homeostasis in type 1 diabetes. With the approval of teplizumab, the first disease-modifying therapy in type 1 diabetes, a paradigm shift in the management of type 1 diabetes is emerging. However, there is still no robust therapy available that fully controls autoimmunity and restores immune tolerance. The ultimate therapy should therefore concomitantly address autoimmunity and preserve beta cell mass. Further rodent studies are now required to better elucidate the mechanism underlying the observed beneficial effects of DXM in type 1 diabetes, and to investigate whether DXM might be a suitable novel candidate for adjunct treatment of preclinical or recent-onset type 1 diabetes, for example, in combination with immune-based interventions such as teplizumab.

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Conflict of Interest

A.W., T.M., E.M. and E.L. declare the following competing financial interests: these authors are inventors of the US patent 10,464,904 entitled “Dextrorphan-derivatives with suppressed central nervous activity”; and T.M. and E.L. are inventors of the US patent 9,370,511 entitled “Morphinan-derivatives for treating diabetes and related disorders.”

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