

# World Journal of *Gastroenterology*

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## Basic Study

# Impact of hyperglycemia on autoimmune pancreatitis and regulatory T-cells

Franz-Tassilo Müller-Graff, Brit Fitzner, Robert Jaster, Brigitte Vollmar, Dietmar Zechner

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**Author contributions:** FTMG and BF performed experiments; RJ evaluated histological sections; DZ designed and performed experiments; FTMG and DZ wrote the manuscript; all authors interpreted the results and revised the manuscript.

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## Abstract

### AIM

To evaluate the influence of hyperglycemia on the progression of autoimmune pancreatitis.

### METHODS

We induced hyperglycemia by repetitive intraperitoneal (ip) injection of 50 mg/kg streptozotocin in MRL/MpJ mice, which develop autoimmune pancreatitis due to a genetic predisposition. We compared the extent of inflammation (histological score, CD3<sup>+</sup> lymphocytes, CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-cells, Foxp3<sup>+</sup> T-helper cells) in the pancreas of hyperglycemic and normoglycemic mice. We also analyzed the number of leukocytes, lymphocytes, granulocytes and monocytes in the blood. In addition, we determined the percentage of CD3<sup>+</sup> lymphocytes, CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-cells, Foxp3<sup>+</sup> T-helper cells, Foxp3<sup>+</sup> CD25<sup>+</sup> T-helper and Foxp3<sup>+</sup> T-helper cells in the spleen by flow cytometry.



## RESULTS

Treatment with streptozotocin caused a strong induction of hyperglycemia and a reduction in body weight ( $P < 0.001$ ). Severe hyperglycemia did not, however, lead to an aggravation, but rather to a slight attenuation of autoimmune pancreatitis. In the pancreas, both the histological score of the pancreas as well as the number of CD3<sup>+</sup> lymphocytes ( $P < 0.053$ ) were decreased by hyperglycemia. No major changes in the percentage of CD8<sup>+</sup> T-cells, CD4<sup>+</sup> T-cells, Foxp3<sup>+</sup> T-helper cells were observed between hyperglycemic and normoglycemic mice. Hyperglycemia increased the numbers of leukocytes ( $P < 0.001$ ), lymphocytes ( $P = 0.016$ ), granulocytes and monocytes ( $P = 0.001$ ) in the blood. Hyperglycemia also moderately reduced the percentage of CD3<sup>+</sup> lymphocytes ( $P = 0.057$ ), significantly increased the percentage of Foxp3<sup>+</sup> T-helper cells ( $P = 0.018$ ) and Foxp3<sup>+</sup> CD25<sup>+</sup> T-helper cells ( $P = 0.021$ ) and reduced the percentage of Foxp3<sup>+</sup> T-helper cells ( $P = 0.034$ ) in the spleen.

## CONCLUSION

Hyperglycemia does not aggravate but moderately attenuates autoimmune pancreatitis, possibly by increasing the percentage of regulatory T-cells in the spleen.

**Key words:** Autoimmune disease; Diabetes; Treg; FoxP3; Autoimmune pancreatitis; MRL/MpJ mice

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**Core tip:** Temporary or sustained hyperglycemia can be observed in about 42%-66% of patients with autoimmune pancreatitis. However, it is unknown to what extent hyperglycemia has an influence on the course of this disease. This preclinical study demonstrates that hyperglycemia does not lead to an aggravation but rather an attenuation of autoimmune pancreatitis. Thus, this result might have the clinical implication that a tight adjustment of blood glucose concentration in patients with autoimmune pancreatitis is not needed, because it might not have a beneficial effect on the progression of this disease.

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## INTRODUCTION

Autoimmune pancreatitis (AIP) is a chronic and progressive inflammatory disease of the pancreas with an assumed autoimmune etiology, which was first described by Sarles *et al.*<sup>[1]</sup> in 1961. The disease is classified by two different histopathological patterns called lympho-

plasmacytic sclerosing pancreatitis (LPSP; AIP type 1) and idiopathic duct centric pancreatitis (ICDP; AIP type 2). AIP presents as a versatile disease with cholestasis, obstructive jaundice and pancreatic swelling. Besides the lymphoplasmacytic infiltrate, fibrosis is often pronounced, and a strong response to steroids can be seen. Besides these similarities, the two types differ in other characteristics such as serological markers, in particular elevated levels of IgG4 in LPSP, other organ involvement, or disease relapse<sup>[2-4]</sup>. The differentiation to pancreatic neoplasia is difficult, which sometimes can lead to unnecessary pancreatectomy<sup>[5]</sup>.

The inflammatory infiltrate in the pancreas during AIP is mainly composed of lymphocytes and plasma cells as well as some eosinophil and neutrophil granulocytes and dendritic cells. Frequently, T-cells are observed, in particular CD4<sup>+</sup>-T-helper cells and CD8<sup>+</sup> cytotoxic cells<sup>[6]</sup>. In addition, regulatory T cells (Tregs) can be found, which are relevant for the immunological self-tolerance by suppressing autoreactive lymphocytes<sup>[7]</sup>. A specific marker for these cells is the transcription factor forkhead-box-protein P3 (FoxP3), which has a major influence on the development of regulatory T-cells<sup>[8,9]</sup>. Increased numbers of these cells were found locally in the pancreas as well as in the peripheral blood during AIP<sup>[7,10]</sup>.

A frequent and important complication of AIP is diabetes mellitus (DM). The rate of DM in patients with AIP varies in the literature between 42%-66.7%<sup>[11,12]</sup>. Of those, 34%-43% already have DM before AIP, 52%-57% show a simultaneous occurrence and 9%-14% develop DM after starting a steroid therapy<sup>[13-15]</sup>. These findings suggest that both pathologies are present simultaneously in many patients. A correlation between hyperglycemia and pancreatitis in patients is well recognized, which is in general explained by the fact that pancreatitis can trigger DM<sup>[16,17]</sup>. Investigations into whether DM or transient hyperglycemia can enhance the development of a pancreatitis in return are rare. Previous preclinical studies demonstrate that diabetes dramatically aggravates the course of cerulein-induced acute and chronic pancreatitis<sup>[18,19]</sup>. Thus, the purpose of this present study was to address the question of whether diabetes can influence the progression of AIP and to analyze which aspects of this disease are affected by diabetes. We chose to use MRL/MpJ mice, an animal model widely used to study AIP. These mice spontaneously develop AIP with an incidence of 74% in female and 36% in male animals at the age of 34-38 wk<sup>[20]</sup>.

## MATERIALS AND METHODS

### Animals

All mice were bred in the local SPF facility (the health of the animal stock is routinely checked according to FELASA guidelines) and were exposed to a 12 h artificial light/dark cycle. The mice were caged in groups and were allowed access to water and standard laboratory chow *ad libitum*. Only female MRL/MpJ mice of the

same age (Sham: 210/204-260, STZ: 210/204-224 median/interquartile range in days) were either sham- or streptozotocin-treated (STZ) in the laboratory. Hyperglycemia was induced by intraperitoneal (ip) injection (between 8:00 and 18:00) of 50 mg/kg STZ (Sigma-Aldrich, Steinheim, Germany) on five consecutive days (day 1-5). All control mice were sham-treated with 50 mmol/L sodium citrate pH 4.5 instead of STZ. Animals were allocated in a non-random manner matching the age of both treatment groups. Tissue was harvested on day 113-116 after first STZ injection. For sampling blood and tissue at the indicated time points the animals were anesthetized (by ip injection) with 90 mg/kg ketamine (Bela-Pharm, Vechta, Germany) and 8 mg/kg xylazine (Bayer Health Care, Leverkusen, Germany). The order in which animals were treated and blood and tissue was sampled was arbitrary. All experiments were executed in accordance with the German legislation, the EU-directive 2010/63/EU and approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern.

#### **Histological and immunofluorescence staining**

On the day of tissue harvesting, the rostral half of the pancreas was placed lengthwise in a biopsy cassette, fixed in 4% phosphate-buffered formalin, and embedded in paraffin. The second half was embedded in Tissue-Tek OCT Compound (Science Services GmbH, Munich, Germany). Paraffin sections of 4 µm thickness were cut and stained with hematoxylin and eosin to evaluate the organ. The histology of the pancreas was investigated by light microscopy (Olympus Corporation, Tokyo, Japan). The severity of autoimmune pancreatitis was determined by scoring the degree of inflammatory cell infiltration and destruction of the parenchyma as described by Kanno *et al.*<sup>[20]</sup> (0 = no inflammation, healthy organ; 1 = mild inflammation, mononuclear cells present in the interstitium but no destruction of parenchyma; 2 = moderate inflammation, focal destruction of parenchyma with mononuclear cell infiltration; 3 = moderate and diffuse or severe but focal inflammation, diffuse destruction of parenchyma with residues of intact parenchyma; 4 = severe and diffuse inflammation, extended mononuclear cell infiltrates with destruction of acini and replacement by adipose tissue)<sup>[20,21]</sup>. The score of two to three sections was averaged to receive a final score by at least two independent reviewers in a blinded manner. In case of discrepancies, a third investigator was consulted for a joint review.

Immunofluorescence was performed using the antibodies eFluor660 conjugated rat anti-CD3 (50-0032; dilution: 600x; eBioscience, Frankfurt, Germany), FITC conjugated rat anti-CD4 (11-0041; dilution: 1200 x; eBioscience), FITC conjugated rat anti-CD8 (ab25676; dilution: 200 x; Abcam, Cambridge, United Kingdom) and rabbit anti-FoxP3 (ab54501; dilution: 2100 x; Abcam) with subsequent secondary antibody Dylight594 goat-anti-rabbit (Dianova, Hamburg, Germany). Immuno-

fluorescence was evaluated on scanned sections with a Leica DMI 4000B scanning microscope using a 40 × objective (Leica Microsystems GmbH, Wetzlar, Germany).

#### **Flow cytometry**

To evaluate subtypes of T-cells, single-cell suspensions were isolated from the spleen of STZ- and sham-treated mice. A total of  $1 \times 10^6$  leukocytes were stained in a volume of 100 µL PBS pH 7.4 (10010-015, Thermo Fisher Scientific GmbH) containing 0.1% BSA (A9418-10G, Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) and appropriate antibodies. These antibodies were eFluor660 conjugated rat anti CD3 (50-0032; dilution: 100 x; eBioscience, Frankfurt, Germany), FITC conjugated rat anti-CD4 (11-0041; dilution, 200 x; eBioscience), PE conjugated rat anti-CD8a (BD Cat 553032; dilution: 100 x; BD Bioscience, Heidelberg, Germany) and PE-Cy7 conjugated rat anti-CD25 (25-0251; dilution: 200 x; eBioscience). Regulatory T-cells were evaluated by staining with FITC conjugated rat anti CD4 and PE conjugated mouse anti-FOXP3 (130-093-014; dilution, 10 x; Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. Data were recorded using a BD FACSCalibur and analyzed using the BD CellQuest Pro software (both Becton Dickinson, NJ, United States).

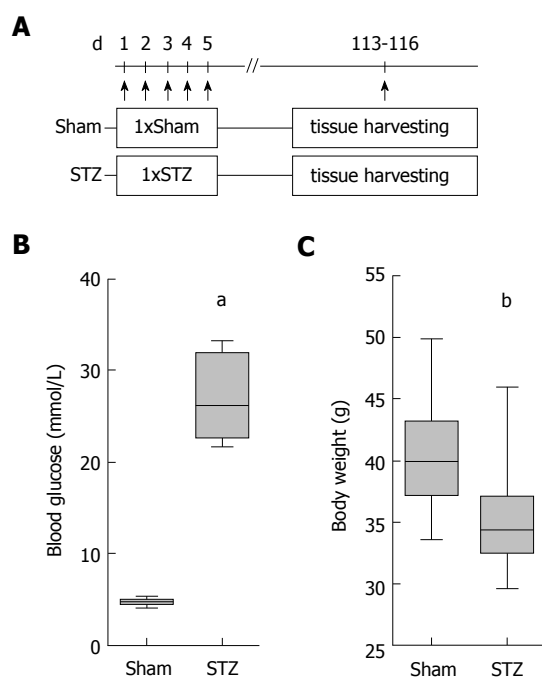
#### **Analysis of the blood**

Progression of hyperglycemia was monitored with the blood glucose meter Contour (Bayer Vital, Leverkusen, Germany). A differential blood cell count was performed with the automated hematology analyzer Sysmex KX 21 (Sysmex Cooperation, Kobe, Japan). To assess acinar cell damage, lipase and amylase activity in blood plasma was analyzed using the Cobas c111 spectrophotometer (Roche Diagnostics, Mannheim, Germany).

#### **Statistical analysis**

Data are presented as box plots indicating the median, the 25<sup>th</sup> and 75<sup>th</sup> percentiles in the form of a box, and the 10<sup>th</sup> and 90<sup>th</sup> percentiles as whiskers. Graphs and statistics were made by using SigmaPlot software version 12.0. Three independent replications (always containing animals of both treatment groups) were done for each experiment. The statistical methods of this study were reviewed by Dr. Anne Glass of the Rostock University Medical Center.

The primary outcome was defined at the beginning of the study as number of T-Lymphocytes per field in the pancreas. No sample size calculation was used. Mice that did not develop hyperglycemia after treatment with STZ and mice that died during this long term experiment (an identical number of mice in both treatment groups) were excluded from the analysis. For data acquisition and description, the ARRIVE guidelines were respected. The significance of differences was evaluated using a Mann Whitney rank-sum test. Differences with  $P \leq 0.05$  were considered to be significant. Differences with  $P \leq 0.08$  were considered to indicate a tendency.



**Figure 1** Experimental protocol of the hyperglycemic autoimmune pancreatitis model. 28-40 wk old- MRL/MpJ mice were intraperitoneally (ip) injected with streptozotocin on day 1-5 (group: STZ), while one age-matched control cohort was ip injected with the appropriate vehicle (group: Sham). The tissue was harvested on day 113-116; (A) Treatment with STZ increased the blood glucose concentration; (B) and reduced the body weight on day 113-116; (C) Box plots indicate the median, the 25<sup>th</sup> and 75<sup>th</sup> percentiles in the form of a box, and the 10<sup>th</sup> and 90<sup>th</sup> percentiles as whiskers. The number of animals evaluated was  $n = 19$  (Sham) and  $n = 17$  (STZ). Differences between the indicated cohorts are indicated as significant. <sup>a</sup> $P < 0.001$ ; <sup>b</sup> $P = 0.009$ . STZ: Streptozotocin-treated.

## RESULTS

### Influence of hyperglycemia on the course of autoimmune pancreatitis

In order to evaluate the influence of hyperglycemia on the course of AIP, we used MRL/MpJ mice due to their spontaneous development of AIP. Distinct cohorts were sham-treated or, in order to induce hyperglycemia, ip injected with STZ for five days and the pancreas was evaluated on day 113-116 (Figure 1A). STZ was effective since it increased the blood glucose concentration on day 22 (Sham: 4.6/4.1-5.5 median/interquartile range in mmol/L. STZ: 22.1/18.2-25.5 median/interquartile range in mmol/L) until the end of the experiment (Figure 1B) and reduced the body weight (Figure 1C). To investigate the severity of AIP, a histological score with numbers from zero to four was used (Figure 2A). Thirteen weeks of hyperglycemia caused a slight decrease in the severity of the histological score and therefore a moderate, non-significant, improvement of AIP when compared to normoglycemic mice (Figure 2B). Little difference was seen in the pancreas to body weight ratio (Figure 2C). Hyperglycemia did not increase the lipase (Figure 2D) or the amylase activity (Figure 2E).

### Evaluation of local pancreatic inflammation during autoimmune pancreatitis

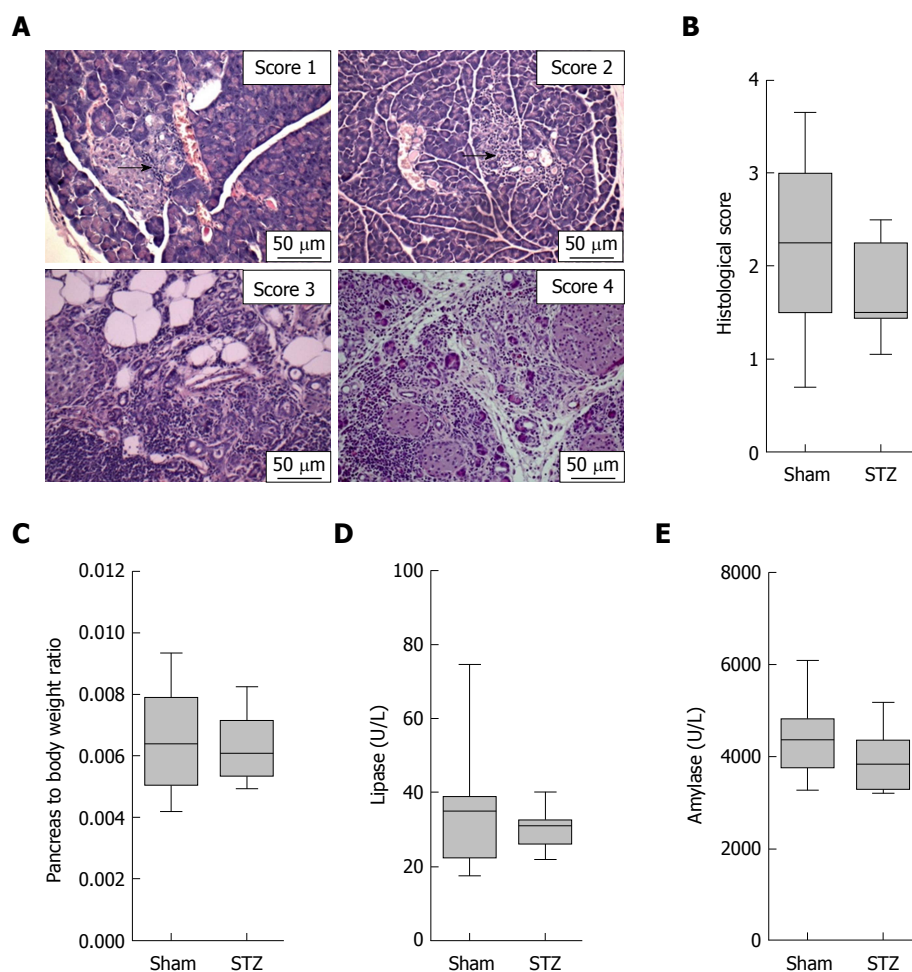
To evaluate the composition of the inflammatory infiltrates in the pancreas, we differentiated between T-lymphocytes (CD3<sup>+</sup> cells), T-helper cells (CD3<sup>+</sup>CD4<sup>+</sup> cells) and cytotoxic T-cells (CD3<sup>+</sup>CD8<sup>+</sup> cells) by immunofluorescence staining (Figure 3A and B). T-Lymphocytes (defined as number of CD3<sup>+</sup> per field) were decreased in diabetic mice compared to non-diabetic mice (Figure 3C). STZ had almost no influence on the percentage of cytotoxic T-cells (CD8<sup>+</sup>CD3<sup>+</sup> cells  $\times 100$  divided by the number of CD3<sup>+</sup> cells) (Figure 3D), the percentage of T-helper cells (CD4<sup>+</sup>CD3<sup>+</sup> cells  $\times 100$  divided by the number of CD3<sup>+</sup> cells) (Figure 3E) and the percentage of regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells  $\times 100$  divided by the number of CD4<sup>+</sup> cells) (Figure 3F). Thus, no increase, but rather a reduction in the number of CD3<sup>+</sup> leukocytes, was observed in the pancreas of hyperglycemic mice.

### Influence of hyperglycemia on leukocytes during autoimmune pancreatitis

To analyze the systemic effect of hyperglycemia during AIP, we sampled blood on the day of tissue harvesting. A significant increase in the number of leukocytes (Figure 4A), lymphocytes (Figure 4B), as well as granulocytes plus monocytes (Figure 4C), was observed in hyperglycemic mice compared to normoglycemic animals.

Since these major differences in the number of immune cells were seen in the blood, we investigated whether hyperglycemia also had an influence on immune cells in the spleen. For this purpose, we analyzed the splenic cells by fluorescent activated cell sorting, which visualized lymphocytes, their stained subpopulations of T-helper cells (CD3<sup>+</sup>CD4<sup>+</sup> cells) and cytotoxic T-cells (CD3<sup>+</sup>CD8<sup>+</sup> cells) as well as regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells) (data not shown and Figure 5A). The quantification of these cells showed a tendentious decrease in the percentage of T-lymphocytes (defined as number of CD3<sup>+</sup> cells  $\times 100$  divided by the number of lymphocytes) in mice treated with STZ compared to controls (Figure 5B). STZ also modestly increased the percentage of cytotoxic T-cells (CD8<sup>+</sup>CD3<sup>+</sup> cells  $\times 100$  divided by the number of CD3<sup>+</sup> cells) (Figure 5C), and reduced those of T-helper cells (CD4<sup>+</sup>CD3<sup>+</sup> cells  $\times 100$  divided by the number of CD3<sup>+</sup> cells) (Figure 5D). Hyperglycemia significantly increased the percentage of regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells  $\times 100$  divided by the number of CD4<sup>+</sup> cells) (Figure 5E). Interestingly, hyperglycemia also significantly increased the percentage of CD25 expressing regulatory T-cells (FoxP3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> cells  $\times 100$  divided by the number of CD4<sup>+</sup> cells) (Figure 5F) compared to normoglycemic mice, whereas the percentage of FoxP3<sup>+</sup> T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells  $\times 100$  divided by the number of CD4<sup>+</sup> cells) was significantly reduced in STZ-treated mice (Figure 5G). Thus, a major influence of hyperglycemia on regulatory T-cells was





**Figure 2 Evaluation of autoimmune pancreatitis.** A: Representative images of the histological scores of autoimmune pancreatitis. Arrows point at small inflammatory infiltrates embedded in healthy surrounding tissue; B: hyperglycemia (STZ) reduced the histological score slightly compared to normoglycemic controls (sham); little differences are observed in the pancreas to body weight ratio (C), lipase activity (D), and amylase activity (E). The number of animals evaluated was  $n = 19$  (sham) and  $n = 17$  (STZ) in Panel B and C and  $n = 19$  (Sham) and  $n = 15$  (STZ) in D and E. Scale bar = 50  $\mu\text{m}$ . STZ: Streptozotocin-treated.

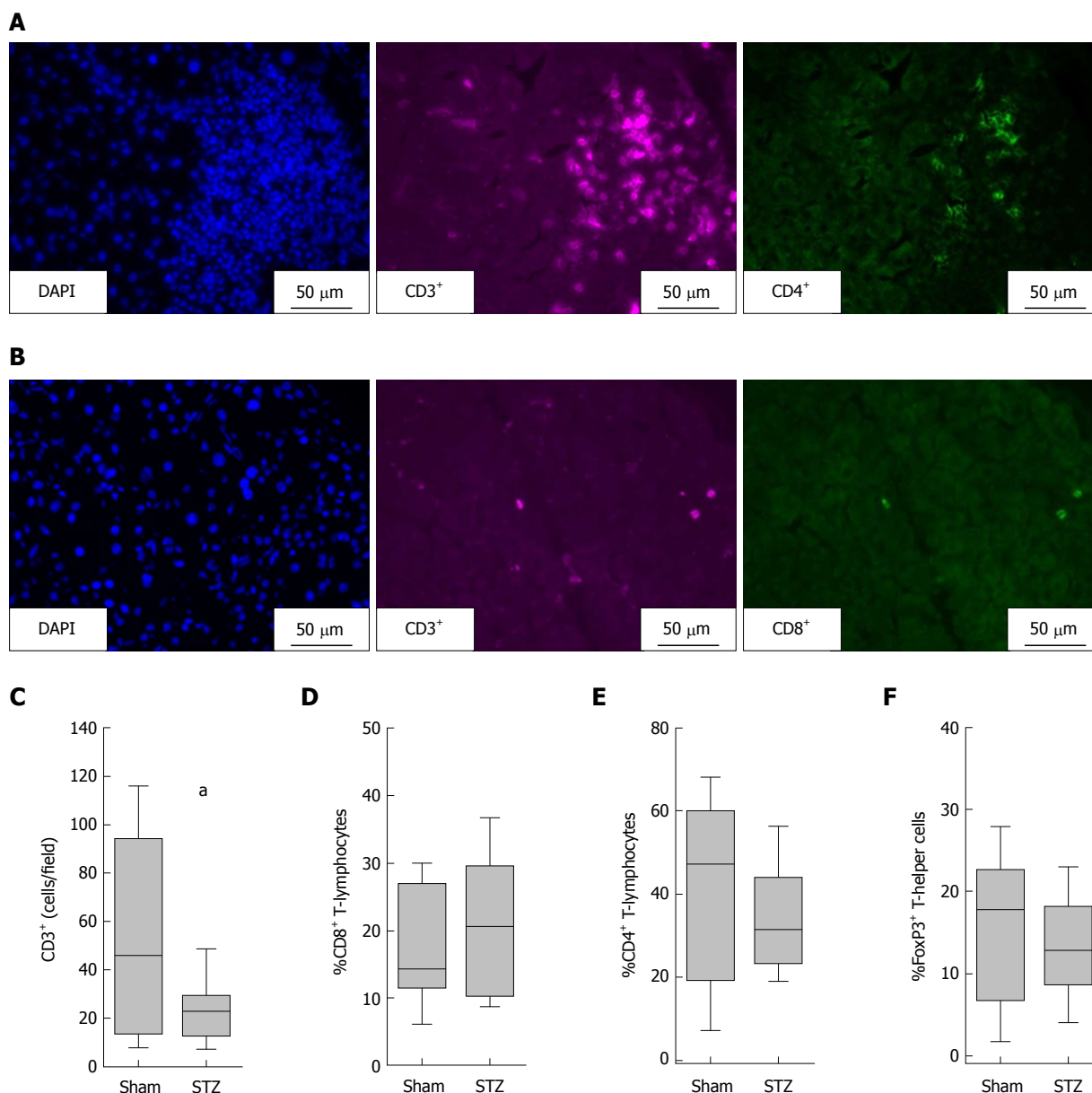
observed in the spleen.

## DISCUSSION

In order to test the influence of hyperglycemia on the course of AIP, we compared the progression of AIP in diabetic and non-diabetic mice. Surprisingly, experimental hyperglycemia didn't cause an aggravation of AIP in MRL/MpJ mice, but moderately improved autoimmune pancreatitis (Figures 2B and 3C). This is in contrast to our previous studies, where hyperglycemia lead to a major aggravation of cerulein-induced acute and chronic pancreatitis<sup>[18,19]</sup>. This might be explained by the differences in the pathogenesis of these two distinct types of pancreatitis. It is currently controversial whether cerulein-induced pancreatitis is mediated by premature activation of trypsinogen or by persistent activation of the NF- $\kappa$ B pathway<sup>[22,23]</sup>. In contrast, AIP is suggested to be caused by an autoimmunological event, which leads to inflammatory infiltration of the organ<sup>[1-4]</sup>. A beneficial effect of hyperglycemia on autoimmune pancreatitis seems to be counterintuitive, since hyperglycemia has been demonstrated to have a deleterious effect on many

organs. However, hyperglycemia can also have an inhibitory effect on the immune system. One could speculate that this impact on the immune system may have a beneficial effect on the progression of some autoimmune diseases.

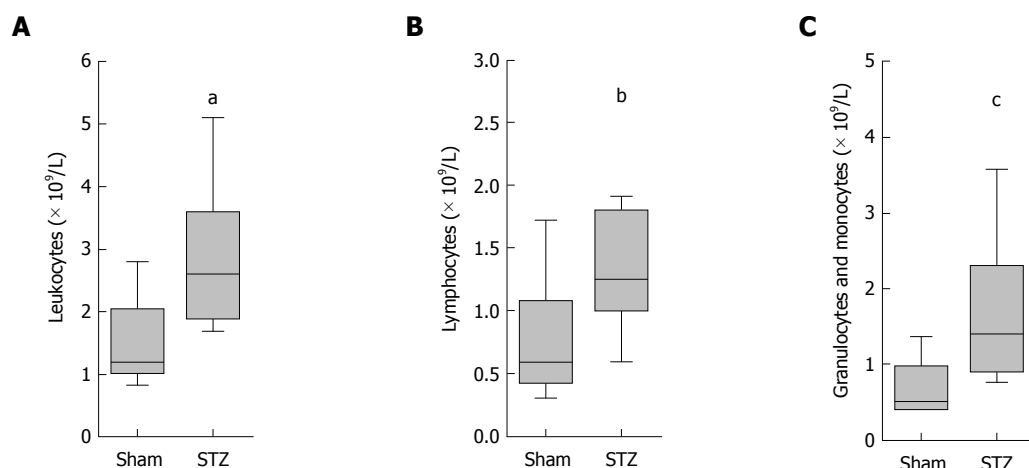
Our study has several limitations. For example, we cannot completely exclude that instead of high glucose concentration in the blood, the drug we used for inducing hyperglycemia (STZ) influences AIP directly. However, in our opinion it is very unlikely that the alkylating agent STZ, which has been demonstrated to be cytotoxic to cells, directly cures AIP. We also limited our study to evaluate the effect of sustained hyperglycemia on AIP and can therefore not conclude if a transient hyperglycemia also has beneficial effects on the progression of AIP. Another limitation of this study is the use of a histological score, which was defined by the ordinal numbers 0 to 4. Comparing the median of the histological score allows, therefore, only a restricted presentation of complex changes in the histology of the pancreas<sup>[20]</sup>. We observed that the median of this score was reduced from 2.25 in normoglycemic to 1.50 in hyperglycemic mice (Figure 2B). However, this simple comparison underestimates the



**Figure 3** Evaluation of the local pancreatic inflammation during autoimmune pancreatitis. Representative images of T-helper cells (CD3<sup>+</sup>CD4<sup>+</sup> cells) (A); and cytotoxic T-cells (CD3<sup>+</sup>CD8<sup>+</sup> cells) (B); hyperglycemia induced by STZ decreased the number of T-lymphocytes (defined as number of CD3<sup>+</sup> per field) (C); little differences are observed in the percentage of cytotoxic T-lymphocytes (CD8<sup>+</sup>CD3<sup>+</sup> cells  $\times$  100 divided by the number of CD3<sup>+</sup> cells) (D); T-helper cells (CD4<sup>+</sup>CD3<sup>+</sup> cells  $\times$  100 divided by the number of CD3<sup>+</sup> cells) (E); and regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells  $\times$  100 divided by the number of CD4<sup>+</sup> cells) (F). The number of animals evaluated was  $n = 19$  (Sham) and  $n = 17$  (STZ). Differences between the indicated cohorts are indicated as tendency, <sup>a</sup> $P = 0.053$ . STZ: Streptozotocin-treated.

observed changes. The same data can also be presented as the percentage of mice with a histological score of  $\geq 3$ . Under normoglycemic conditions, 42% of mice (8 from 19 mice) received a histological score of  $\geq 3$ . Under hyperglycemic conditions, only 6% (1 from 17 mice) received a score of  $\geq 3$ . These high scores were assigned when moderate diffuse or severe focal inflammation plus a diffuse destruction of the acini (score 3) or severe and diffuse inflammation plus extended destruction of acini (score 4) was observed<sup>[20,21]</sup>. This impressive difference in the percentage of mice with a high histological score suggests that hyperglycemia reduces the damage to the parenchyma, which might indirectly reduce local inflammation. Alternatively, this observation could also

suggest that hyperglycemia reduces inflammation and thus minimizes the damage to the parenchyma. Due to the following observations, we prefer the second option. We found increased numbers of regulatory T-cells and decreased FoxP3<sup>+</sup> T-cells in the spleen. This is consistent with data in another preclinical study, where it has been described, that decreased numbers of regulatory T-cells and increased numbers of FoxP3<sup>+</sup> T-cells in the spleen lead to an increased severity of AIP<sup>[24]</sup>. In both studies, the numbers of regulatory T-cells and FoxP3<sup>+</sup> T-cells in the spleen correlate with the severity of AIP. Indeed, one study demonstrated a suppressive effect of transferred Tregs on autoimmune pancreatitis in MRL/MpJ mice<sup>[25]</sup>. However, the observed reduction in the histological score



**Figure 4** Diabetes increased the number of leukocytes in the blood. Treatment with STZ raised the number of leukocytes (A); lymphocytes (B); as well as granulocytes and monocytes (C). The number of animals evaluated was  $n = 19$  (Sham) and  $n = 14$  (STZ). Differences between the indicated cohorts are indicated as significant, (A)  $^aP < 0.001$ ; (B)  $^bP = 0.016$ ; (C)  $^cP = 0.001$ . STZ: Streptozotocin-treated.

was not significant<sup>[25]</sup>.

We observed major differences between hyperglycemic and normoglycemic mice when analyzing immune cells in the blood and the spleen in MRL/MpJ mice. We noticed (1) increased percentage of regulatory T-cells and (2) decreased percentage of FoxP3<sup>+</sup> T-cells in the spleen, as well as an (3) increased number of leukocytes in the blood of STZ-treated mice (Figures 4A-C and 5E-G). These findings suggest that hyperglycemia might rather influence the overall immune system and only indirectly affect AIP itself. This interpretation is consistent with preclinical studies, which analyzed the influence of hyperglycemia on regulatory T-cells in mice not suffering from AIP. In these animals, hyperglycemia also increased the number of CD4<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> regulatory T-cells in lymph nodes and the spleen of mice<sup>[26,27]</sup>.

For treating AIP in the clinic, guidelines recommend to adjust blood glucose concentration before starting a steroid therapy<sup>[28,29]</sup>. This is mainly based on publications suggesting that a preexisting DM before the onset of AIP is worsening in 75% of patients after the start of a steroid therapy<sup>[28,29]</sup>. However, our preclinical study shows that hyperglycemia does not have a negative influence on AIP. Critical attitudes towards adjusting the blood glucose concentration in patients suffering from AIP have been previously published. For example, in one third of all cases, DM even worsened after insulin therapy<sup>[15]</sup>. Moreover, it was reported that treatment of diabetes with insulin led to hypoglycemic attacks in 10 from 50 AIP patients<sup>[13]</sup>. In addition, several clinical studies could demonstrate that DM improved in many cases immediately after steroid therapy<sup>[15,30]</sup>. These clinical studies argue against a tight control of blood glucose in AIP patients. Although our study has the limitation to be only a preclinical study on mice, it also suggests that an aggressive adjustment of blood glucose concentration might not be necessary as a prerequisite for the treatment of AIP. For final clarification of this issue, a clinical study evaluating if a tight adjustment of blood glucose in addition to steroid therapy is beneficial or harmful to patients

with AIP might need to be pursued.

## ARTICLE HIGHLIGHTS

### Research background

In about 42%-66% of patients with autoimmune pancreatitis, hyperglycemia can be observed. Thus, hyperglycemia is a frequent and important complication of this disease. However, it is not known if it merely reflects the severity of pancreatitis or whether it can also influence the progression of autoimmune pancreatitis.

### Research motivation

For treating autoimmune pancreatitis in the clinic, guidelines recommend to adjust blood glucose concentration before starting a steroid therapy. However, critical attitudes towards adjusting the blood glucose concentration in these patients have also been published. For example, in one third of all cases, hyperglycemia even worsened after insulin therapy, and treatment of diabetes with insulin sometimes leads to hypoglycemic attacks in patients. In order to decide how important a tight adjustment of blood glucose concentration in patients with autoimmune pancreatitis is, we need to know if hyperglycemia has a positive or negative influence on the progression of this disease.

### Research objectives

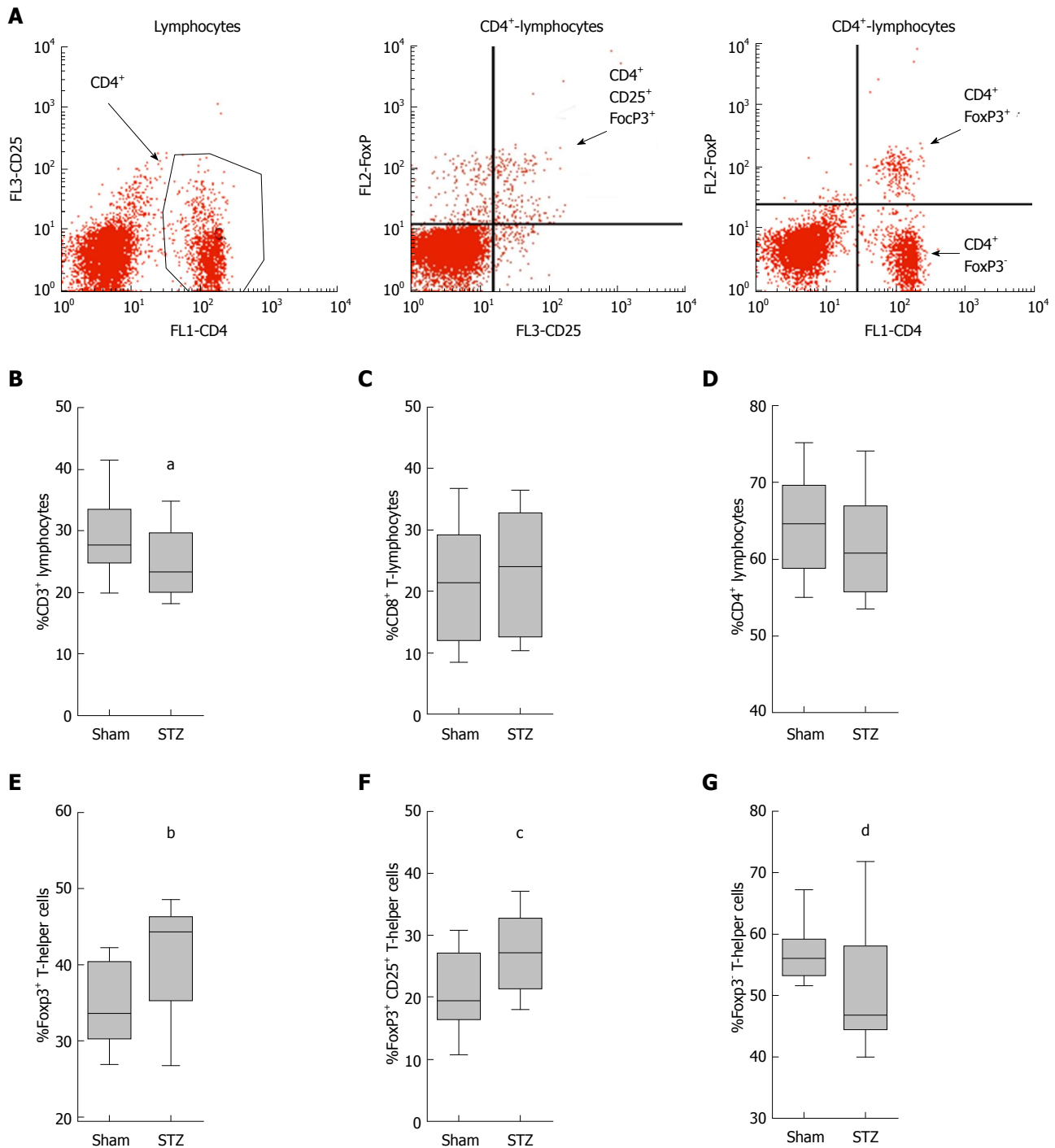
The purpose of this present study was to address the question of whether diabetes can influence the progression of autoimmune pancreatitis and to analyze which aspects of this disease are affected by hyperglycemia.

### Research methods

We chose to use MRL/MpJ mice, an animal model widely used to study autoimmune pancreatitis. These mice spontaneously develop autoimmune pancreatitis. We induced hyperglycemia by repetitive intraperitoneal (ip) injection of streptozotocin in female mice and compared the extent of inflammation in the pancreas of hyperglycemic and normoglycemic animals. We also analyzed the number of immune cells in the blood. In addition, we determined the percentage of T-cells, especially regulatory T-cells in the spleen.

### Research results

Surprisingly, experimental hyperglycemia did not cause an aggravation of autoimmune pancreatitis, but moderately improved autoimmune pancreatitis. We noticed an increased percentage of regulatory T-cells in the spleen, as well as an increased number of leukocytes in the blood of hyperglycemic mice. These findings suggest that hyperglycemia might rather influence the overall immune system and only indirectly affect autoimmune pancreatitis itself.



**Figure 5** Evaluation of leukocytes in the spleen during autoimmune pancreatitis. Representative images of stained T-helper cells and their distinction between regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells), CD25 expressing regulatory T-cells (CD4<sup>+</sup>CD25<sup>+</sup> FoxP3<sup>+</sup> cells) and FoxP3<sup>-</sup> T-cells (FoxP3<sup>-</sup> CD4<sup>+</sup> cells) (A); application of STZ decreased the percentage of T-lymphocytes (defined as number of CD3<sup>+</sup> cells  $\times$  100 divided by the number of lymphocytes) (B); little differences are observed in the percentage of cytotoxic T-cells (CD8<sup>+</sup>CD3<sup>+</sup> cells  $\times$  100 divided by the number of CD3<sup>+</sup> cells) (C); and T-helper cells (CD4<sup>+</sup>CD3<sup>+</sup> cells  $\times$  100 divided by the number of CD3<sup>+</sup> cells) (D); STZ increased the percentage of regulatory T-cells (FoxP3<sup>+</sup>CD4<sup>+</sup> cells  $\times$  100 divided by the number of CD4<sup>+</sup> cells) (E); and CD25 expressing regulatory T-cells (FoxP3<sup>+</sup> CD25<sup>+</sup> CD4<sup>+</sup> cells  $\times$  100 divided by the number of CD4<sup>+</sup> cells) (F); the percentage of FoxP3<sup>-</sup> T-cells (FoxP3<sup>-</sup>CD4<sup>+</sup> cells  $\times$  100 divided by the number of CD4<sup>+</sup> cells) was decreased (G). The number of animals evaluated was  $n = 19$  (sham) and  $n = 17$  (STZ). Differences between the indicated cohorts are indicated as tendency, (B) <sup>a</sup> $P = 0.057$  or as significant, (E) <sup>b</sup> $P = 0.018$ ; (F) <sup>c</sup> $P = 0.021$ ; (G) <sup>d</sup> $P = 0.034$ . STZ: Streptozotocin-treated.

### Research conclusions

Our preclinical study on mice supports the idea that an aggressive adjustment of blood glucose concentration might not be necessary as a prerequisite for the treatment of patients with autoimmune pancreatitis.

### Research perspectives

A clinical study that evaluates whether a tight adjustment of blood glucose is

beneficial or harmful to patients should be pursued.

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