Immunobiology 218 (2013) 338–352

Contents lists available at SciVerse ScienceDirect

Immunobiology

journal homepage: www.elsevier.de/imbio

Dynamic changes of the Th17/Tc17 and regulatory T cell populations interfere in the experimental autoimmune diabetes pathogenesis

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ARTICLE INFO

Article history:
Received 21 December 2011
Received in revised form 2 May 2012
Accepted 16 May 2012

Keywords:
Streptozotocin
Th17 cells
Tc17 cells
Treg cells
Type 1 diabetes

ABSTRACT

A balance between proinflammatory (Th17 and Tc17) and anti-inflammatory (regulatory T cells) subsets of T cells is essential to maintain immunological tolerance and prevent the onset of several autoimmune diseases, including type 1 diabetes. However, the kinetics of these subsets and disease severity during the streptozotocin (STZ)-induced diabetes course has not been determined. Thus, susceptible C57BL/6 mice were administrated with multiple low doses of STZ and we evaluated the frequency/absolute number of these T cell subsets in the pancreatic lymph nodes (PLNs) and spleen and Th1, Th17, Treg cytokine production in the pancreatic tissue. At different time points of the disease progression (6, 11, 18 and 25 days after the last STZ administration), the histopathological alterations were also evaluated by H&E and immunohistochemistry staining. During the initial phase of diabetes development (day 6), we noted increased numbers of $CD4^+$ and $CD8^+$ T cells in spleen and PLNs. At the same time, the frequencies of Th17 and Tc17 cells in PLNs were also enhanced. In addition, the early augment of interferon gamma (IFN-γ), tumoral necrosis factor (TNF-α), IL-6 and IL-17 levels in pancreatic tissue correlated with pancreatic islet inflammation and mild β-cell damage. Notably, the absolute number of Treg cells increased in PLNs during over time when compared to control group. Interestingly, increased IL-10 levels were associated with control of the inflammatory process during the late phase of the type 1 diabetes (day 25). In agreement, mice lacking the expression of IL-17 receptor (*Il17r*) showed impairment in STZ-induced diabetes progression, reduced peri-insulitis and beta cells preservation when compared with wild-type mice. Our findings suggest that dynamic changes of pathogenic Th17/Tc17 and regulatory T cell subsets numbers is associated with early strong inflammation in the pancreatic islets followed by late regulatory profile during the experimental STZ-induced diabetes course.

Introduction

Millions of people worldwide are affected by type 1 diabetes (T1D) and its short or long-term complications, which are life-threatening conditions, are implicated in high health care and social costs (World Health Organization; Daneman 2006). T1D is characterized by an autoimmune response in which cellular immunity plays a pivotal role in the selective destruction of insulin-producing (β) beta cells, thus leading to an impairment in glucose metabolism. Several experimental models of diabetes have been developed and allow the study of type 1 diabetes, including spontaneous (NOD, non-obese diabetic mice; BB, biobreeding rats) and chemically induced disease (STZ, streptozotocin; cyclophosphamide, alloxan) (Rees and Alcolado 2005).

Abbreviations: Th17, IL-17-producing T helper cells; Tc17, IL-17-producing cytotoxic T cells; Treg, regulatory T cells; STZ, streptozotocin; H&E, hematoxilin and eosin; PLNs, pancreatic lymph nodes; T1D, type 1 diabetes; NOD, non-obese diabetic; BB, biobreeding; EAE, experimental autoimmune encephalomyelitis; i.p., intraperitoneally; GLUT2, glucose transporter 2; NOD/SCID, non-obese diabetic/severe combined immunodeficiency; KO, knockout; *Il17r* KO, IL-17 receptor knockout; WT, wild-type.

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0171-2985/$ – see front matter © 2012 Elsevier GmbH. All rights reserved.
http://dx.doi.org/10.1016/j.imbio.2012.05.010

Multiple low doses of streptozotocin are used to induce insulin-deficient diabetes, which is characterized by pancreatic islets destruction in susceptible rodents, resembling human type 1 diabetes (Like and Rossini 1976; Leiter 1982). Several studies demonstrated that this model is mediated by immune destruction, since the administration of T cell-depleting antibodies (anti-CD4, anti-CD8) attenuates the disease (Herold et al. 1987) and while athymic *nu/nu* mice completely fail to respond to STZ (Nakamura et al. 1984). Moreover, transfer of splenocytes derived from STZ-injected mice promotes hyperglycemia development, a process that is abolished when transferred splenocytes are depleted of T cells (Paik et al. 1980). Additional evidences also showed that increased MHC expression is mediated by IFN-γ (Cockfield et al. 1989; Klinkhammer et al. 1989); lymphoproliferation is observed after STZ administration (Klinkhammer et al. 1988); and CD28/B7 costimulation regulates autoimmune diabetes. In this last report, mice lacking CD28 did develop neither hyperglycemia nor insulitis or expressed IFN-γ mRNA following STZ injection (Herold et al. 1997).

Recently, it has been suggested that an imbalance between immune regulatory and pathogenic pathways are implicated in autoimmune disease progression (Sakaguchi 2004; Campbell and Koch 2011). Impairment in regulatory T cells (Treg) number or function has been associated with the development of several autoimmune diseases like multiple sclerosis, rheumatoid arthritis, and type 1 diabetes (Viglietta et al. 2004; Ehrenstein et al. 2004; Lindley et al. 2005). In addition, newly diagnosed and long standing type 1 diabetes patients exhibit low numbers of $CD4^+CD25^+$ (Treg) cells compared with health individuals (Kukreja et al. 2002). In contrast, other findings suggest that patients with type 1 diabetes display normal frequencies of $CD4^+CD25^+Foxp3^+$ T cells (Brusko et al. 2007), but a defective suppressive capacity of these cells was observed (Lindley et al. 2005; Brusko et al. 2005). Moreover, the spontaneous diabetes onset in non-obese diabetic (NOD) mice can also be associated with altered $CD4^+CD25^+$ T cell numbers in thymus and spleen (Wu et al. 2002). Indeed, NOD mice also have defects on immune regulation due to a reduced Treg suppressive capacity (Gregori et al. 2003; Pop et al. 2005; Tritt et al. 2008). Conversely, Th17 cells produce proinflammatory cytokines like IL-17A and IL-17F, which perpetuate inflammation and are involved in the pathogenesis of several autoimmune diseases in both patients and experimental models, including multiple sclerosis and rheumatoid arthritis (Honorati et al. 2001; Rohn et al. 2006; Tzartos et al. 2008). In this context, repeated administration of IL-23 enhances the diabetogenic process in STZ-induced diabetes model, suggesting a possible role for Th17 in diabetes pathogenesis (Mensah-Brown et al. 2006). In accordance, there are some recent evidences that showed increased frequencies of IL-17-producing lymphocytes in children with new onset (Marwaha et al. 2010) and patients with long-term (Bradshaw et al. 2009) type 1 diabetes. On the other hand, studies involving animal models of type 1 diabetes report conflicting results. Zhang and colleagues showed that IL-12 administration prevent diabetes development in NOD mice by suppressing the pathogenic IL-17-producing cells (Zhang et al. 2011). However, different studies showed that Th17 cells induce diabetes only after conversion into Th1 cells (Martin-Orozco et al. 2009; Bending et al. 2009). In parallel, cytotoxic T cells ($CD8^+$ T cells) can also produce IL-17 and this population is called Tc17 cells (He et al. 2006; Kondo et al. 2009; Huber et al. 2009), which was demonstrated to participate in several pathologic conditions, such as infection, cancer and autoimmune inflammation (Ciric et al. 2009). In fact, Tc17 cells are involved in the pathogenesis of experimental colitis, experimental uveitis, EAE and lupus (Tajima et al. 2008; Peng et al. 2007; Huber et al. 2009; Henriques et al. 2010). However, the role of Th17 and Tc17 cells in progression of experimental type 1 diabetes are still not fully elucidated.

Although some findings have demonstrated the involvement of an autoimmune response in the pathogenesis of STZ-induced diabetes, the kinetics and frequency of pathogenic (Th17 and Tc17 cells) and regulatory (Treg cells) cells during disease course were not completely defined yet. Then, we aimed to determine the dynamic changes of Th17/Tc17 and Treg cell populations as well as its correlation with the pancreatic inflammation and β-cell damage in the experimental T1D. Therefore, this current study provides important contribution to point future approaches for testing novel diabetes therapies.

Materials and methods

Induction of experimental diabetes and blood glucose monitoring

C57BL/6 male mice, *Il17r* KO mice, at 10 weeks of age were daily injected intraperitoneally (i.p.) with 40 mg/kg of STZ (Sigma–Aldrich, St. Louis, MO) for 5 consecutive days. STZ was diluted in sodium citrate buffer, pH 4.5, and injected in animals within 15 min of the preparation. Blood samples were taken from the tail vein of non-fasted mice, and glucose levels determined with a glucometer system Accu-Chek Go (Roche Diagnostics, Abbott Park, IL, USA). Mice were considered diabetic when glycemia was above 250 mg/dl after two consecutive determinations. The control group received only sodium citrate buffer (vehicle) intraperitoneally (i.p.). All animal procedures were approved by the Ethics Committee for Animal Research (CETEA, no. 07/53940-0) of the School of Medicine of Ribeirão Preto, at the University of São Paulo.

Experimental design

Pancreata, pancreatic lymph nodes (PLNs), and spleens were harvested on several point times (6, 11, 18, or 25 days) after the last STZ (experimental group) or sodium citrate buffer (control group) administration.

Histology and immunohistochemistry

Pancreata were removed and fixed in 10% neutral buffered formalin, embedded in paraffin and the sections (5 μm) stained with hematoxylin-eosin (H&E). Immunohistochemistry reactions were performed on sections (5 μm) of formalin-fixed tissue. The sections were dewaxed, rehydrated, and incubated with Peroxidase-Blocking Reagent (DAKO Cytomation, Fort Collins, CO) to block endogenous peroxidase. Then, the slides were incubated with PBS + BSA 1% to block unspecific staining. Next, rabbit monoclonal anti-mouse insulin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-mouse glucagon antibody (Santa Cruz Biotechnology, Santa Cruz, CA) were applied to the sections, followed by incubation with LSAB^{TM}+ Kit/HRP (DAKO Cytomation, Fort Collins, CO). The slides were stained with diaminobenzidine (DAB) according to the manufacturer's instructions (DAKO Cytomation, Fort Collins, CO). Finally, the sections were counterstained with hematoxylin, mounted and analyzed.

Isolation of cells from spleen and pancreatic lymph nodes

The spleen was placed into a Petri dish and mashed using the plunger end of the syringe. Cell suspension was collected and erythrocyte lysis was performed using Tris 0.17 M and NH_4Cl 0.16 M buffer. RPMI 1640 medium (Gibco, Grand Island, NY, USA), supplemented with 10% FBS, 2 mmol/l L-glutamine and 100 U/ml penicillin and streptomycin (Gibco, Grand Island, NY, USA) was added to the tube containing splenocytes and centrifuged at 300 *g* for 10 min at 4 C. Then, the supernatant was discarded and the pellet resuspended in RPMI 1640 medium. Pancreatic draining

lymph nodes (PLNs) were collected and placed in the cell strainer. Using the plunger end of the syringe, pancreatic lymph nodes were mashed through the cell strainer into the Petri dish containing RPMI 1640 medium. Then, cell suspension was collected and centrifuged 300 *g* for 10 min at 4 C.

Flow cytometry analysis of extracellular markers

Flow cytometry analysis of T cell populations were performed on 1×10^6 cells/tube in 100 μl PBS. First, cell suspension was incubated with 100 μl of rabbit normal serum 5% for 30 min to block unspecific binding. Next, fluorochrome-conjugated primary antibodies for CD3, CD4, CD8, CD25 and their control isotypes (BD Pharmingen, San Diego, CA, USA) were added and incubated for 30 min in the dark. All monoclonal antibodies were used at concentrations recommended by the manufacturer. Next, cells were washed with PBS, resuspended and analyzed using a FACS Calibur flow cytometer (FACS Calibur, Cell Quest; BD Biosciences, San Jose, CA, USA).

Foxp3 expression, IL-17 and IFN-γ production by intracellular staining

After extracellular antigen staining, cells were incubated with FACS Lysing solution (BD Pharmingen, San Diego, CA, USA) for 10 min in the dark. Then, cells were washed and resuspended in FACS Permeabilizing solution (BD Pharmingen, San Diego, CA, USA) for 10 min. Next, the expression of the transcription factor Foxp3 was assessed by incubating with PE-conjugated anti-mouse Foxp3 monoclonal antibody (BD Pharmingen, San Diego, CA, USA). Cell suspensions were washed, resuspended and analyzed using a FACS Calibur flow cytometer. IL-17 and IFN-γ production was evaluated in spleen and PLNs cells after *in vitro* re-activation with PMA (25 ng/ml) and ionomycin (1 mg/ml; Sigma–Aldrich, St. Louis, MO, USA) together with monensin, at 10 mg/ml (Sigma–Aldrich, St. Louis, MO, USA) for 4 h. Later, cells were stained for extracellular antigens and cell fixation and permeabilization was performed as described above. The cytokine was detected using a rat anti-mouse IL-17 or rat anti-mouse IFN-γ monoclonal antibody (BD Pharmingen, San Diego, CA, USA). Cells were analyzed using a FACS Calibur flow cytometer and data were obtained for 100,000 events/sample (spleen) or 50,000 events/sample (PLNs) using Cell Quest Pro software (BD Pharmingen, San Diego, CA, USA).

Cytokine levels in pancreatic tissue

Pieces of pancreas were removed, weighted and placed into a tube containing 500 μL of Complete Protease Inhibitor Cocktail (Roche Diagnostics, Abbott Park, IL, USA). Pancreatic tissue was homogenized using a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, NY, USA) and IL-23, IL-17, IL-6, TNF-α, IFN-γ, IL-10 levels were detected by ELISA, according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA). The results were expressed as mean ± SEM of nanograms per gram of pancreatic tissue.

Statistical analysis

To achieve the interest objectives was proposed an Unbalanced ANOVA for Two-Way Design with Interaction (Kutner 1974), belonging to the class of general linear models. Comparisons were made with post-test by orthogonal contrasts. The results were obtained with support of the SAS® 9 by PROC GLM. Data are presented as mean ± SEM and p values <0.05 were considered statistically significant.

Results

Hyperglycemia and histopathological changes during chemically induced diabetes

After five consecutive injections of STZ (40 mg/kg), mice became hyperglycemic (301.0 ± 105.34 mg/dl) at day 10. During the disease course, blood glucose levels increased, reaching higher glycemic levels at day 21 until the last period analyzed (Fig. 1A). To verify the dynamic of the inflammatory process after STZ administration, we evaluated the cellular influx in the pancreatic islets at different times (6, 11, 18 and 25 days after diabetes induction) by H&E staining. At day 6, an inflammatory infiltrate was observed around the islets, characterized as peri-insulitis (Fig. 1C). Further, the inflammatory process progressed and more inflammatory cells were found inside the islets (insulitis) on days 11 and 18 (Fig. 1D and E, respectively). On day 25, the inflammatory response in the pancreatic islets was resolved (Fig. 1F), but their morphology was abnormal, exhibiting stellate shape instead of the round shape observed in the control group (Fig. 1B). Thus, our results showed that STZ administration induces a progressive inflammatory response in the pancreatic islets resulting in profound alterations in their morphology and architecture.

β-Cell destruction and α-cell expansion during chemically induced diabetes

To evaluate insulin and glucagon-producing cells ratio during diabetes progression, we detected the expression of these hormones by immunohistochemistry. STZ-administrated mice showed gradual loss of insulin-producing β-cells, from day 6 to day 18 (Fig. 2B–D). At day 25 (Fig. 2E), only few β-cells were found, as compared to mice injected with vehicle (control group; Fig. 2A). On the other hand, glucagon-producing α-cell density remained unaltered until day 11 (Fig. 3B and C) when compared with control group (Fig. 3A). However, we observed an abnormal expansion of these cells at day 18 (Fig. 3D). Later (at day 25), glucagon-producing α-cells were located in a central region of pancreatic islets, which was accompanied by a disruption of the pancreatic islet architecture (Fig. 3E). Overall, our results demonstrated an extensive damage of the β-cell mass correlated with marked expansion of α-cells during the course of the STZ-induced diabetes.

Lymphocyte accumulation and early upregulation of Th17 and Tc17 populations during chemically induced diabetes

The immune mediated etiology of the STZ-induced diabetes is supported by evidences such as prevention of the disease using anti-CD3 antibody and disease onset after splenocytes transfer from diabetic mice to health recipients (Müller et al. 2002). These observations indicate that T cells play a central role in the diabetes progression in this experimental model. Based on these observations, we aimed to determine the frequency and absolute number of helper T cells ($CD3^+CD4^+$) and cytotoxic T cells ($CD3^+CD8^+$) in the spleen and PLNs of control and diabetic group, by flow cytometry. As shown in Figs. 4 and 5, there was an early increase in the frequency and absolute numbers of splenic $CD3^+CD4^+$ (Fig. 4A and E) and $CD3^+CD8^+$ T cells (Fig. 5A and E) 6 days after the STZ administration compared to controls, besides no significant increase in these populations in the spleen at days 11, 18 and 25 was detected (Figs. 4B–D, F–H; 5B–D, F–H). On the other hand, the frequency of $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells in PLNs from STZ-administrated mice was similar to the control group in all periods analyzed (Figs. 4A–D; 5A–D), with exception of day 6, when an enhanced number of $CD3^+CD4^+$ (Fig. 4E) and $CD3^+CD8^+$ cells (Fig. 5E) was observed in comparison to controls.

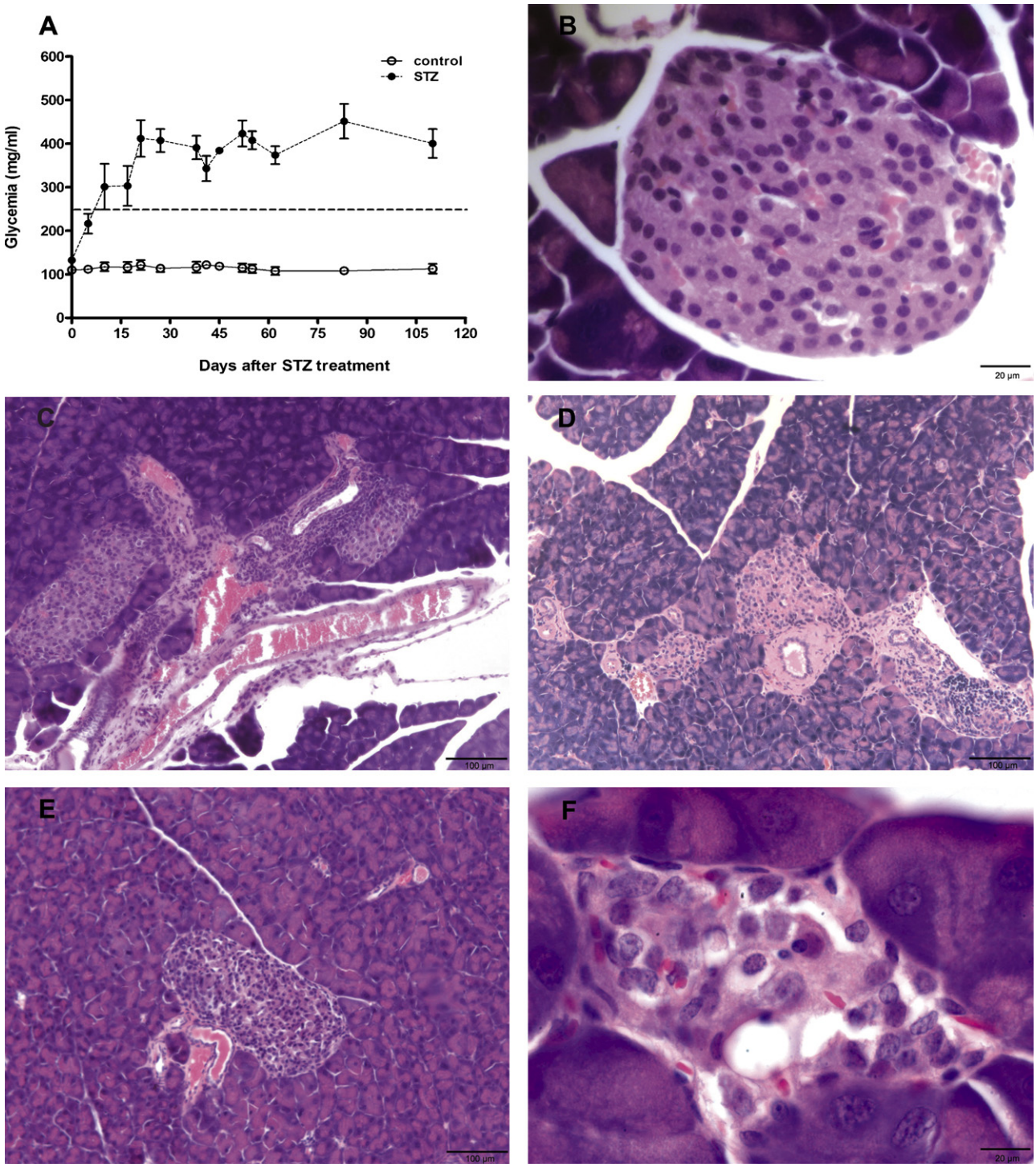

Fig. 1. Hyperglycemia and islet histological alterations during chemically induced diabetes. Diabetes was induced in male C57BL/6 mice by administration of 5 consecutive doses of STZ (40 mg/kg). (A) Blood samples were collected from tail vein and analyzed by glucosimeter. (B–F) Pancreata from control and STZ-injected mice were collected at different periods and histological features were evaluated by H&E staining. Representative H&E-stained islets from control (B) or diabetic mice at different time course: at 6 days (C); 11 days (D); 18 days (E) and 25 days (F) after the last STZ injection.

Since IL-17 responses are crucial to the pathogenesis of a plenty of autoimmune diseases, we examined the frequency of IL-17-producing T cells (Th17 and Tc17 cells) in spleen and PLNs from control and STZ-injected mice. As seen in Fig. 6E, we detected an increased frequency of splenic Tc17 ($CD3^+CD8^+IL\text{-}17^+$) cells during the initial phase of diabetes (6 days after STZ), although we did not found alteration in Th17 ($CD3^+CD4^+IL\text{-}17^+$) population at this period (Fig. 6A). In contrast, the percentage of both CD4 and CD8 populations producing IL-17 tended to be elevated in PLNs on days 11 and 18 in STZ-administrated mice compared to control group (Fig. 6B, C, F and G), in addition to a special increase at day 6 (Fig. 6A and E), when differences were statically significant. No differences in these cell populations were observed at day 25 (Fig. 6D and H).

Thus, we noted that Th17 and Tc17 subsets in spleen and PLNs cells were predominantly elevated in the first days of disease progression (Fig. 6A and E), suggesting that an early IL-17 production

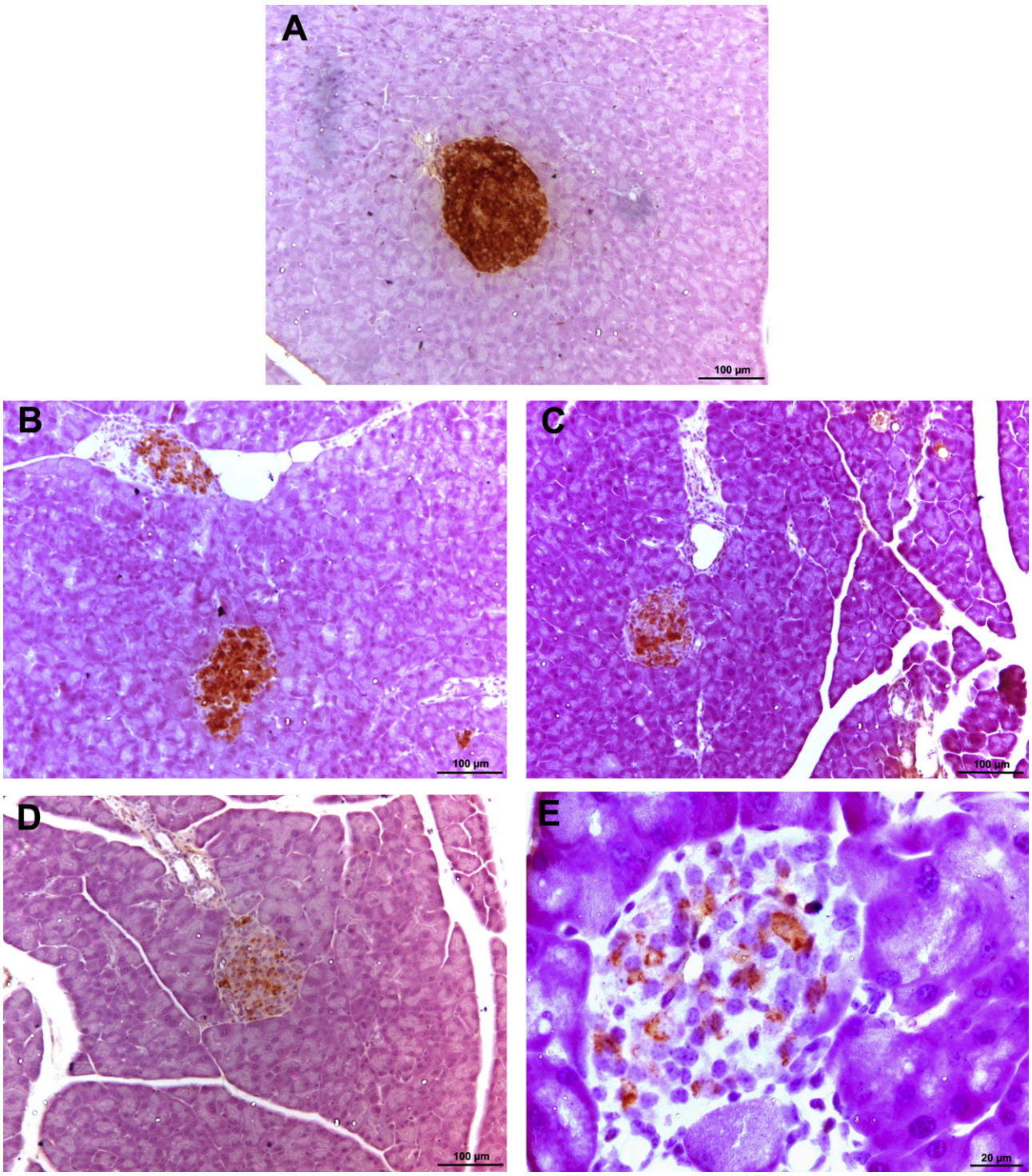

Fig. 2. Insulin-producing β-cell destruction during chemically induced diabetes development. Pancreata were isolated from control (A) or STZ-injected mice at 6 days (B), 11 days (C), 18 days (D) and 25 days (E) after the last STZ injection. Pancreatic tissue sections were analyzed by immunohistochemistry assay with anti-insulin antibody (A–E) to evaluated insulin granule content into pancreatic islets. Representative images are shown.

in local lymphoid tissue may be related to T1D outcome in this experimental model.

Sustained increase of the regulatory T cell population during chemically induced diabetes

Because the development of T1D depends on the imbalance between effector and regulatory mechanisms, we next aimed to investigate if STZ-induced diabetes was also dependent on altered Treg population. Then, we quantified the frequency and absolute number of $CD4^+CD25^+Foxp3^+$ cells in the spleen and PLNs after diabetes induction. As seen in Fig. 7, no significant differences were found in the spleen during diabetes progression when compared to controls. On the other hand, the percentage of $CD4^+CD25^+Foxp3^+$ T cells was increased in STZ-group compared to control group on day 6 (Fig. 7A). During the disease progression, the frequency of this cell population tended to be higher in STZ-injected mice than in control mice, but these

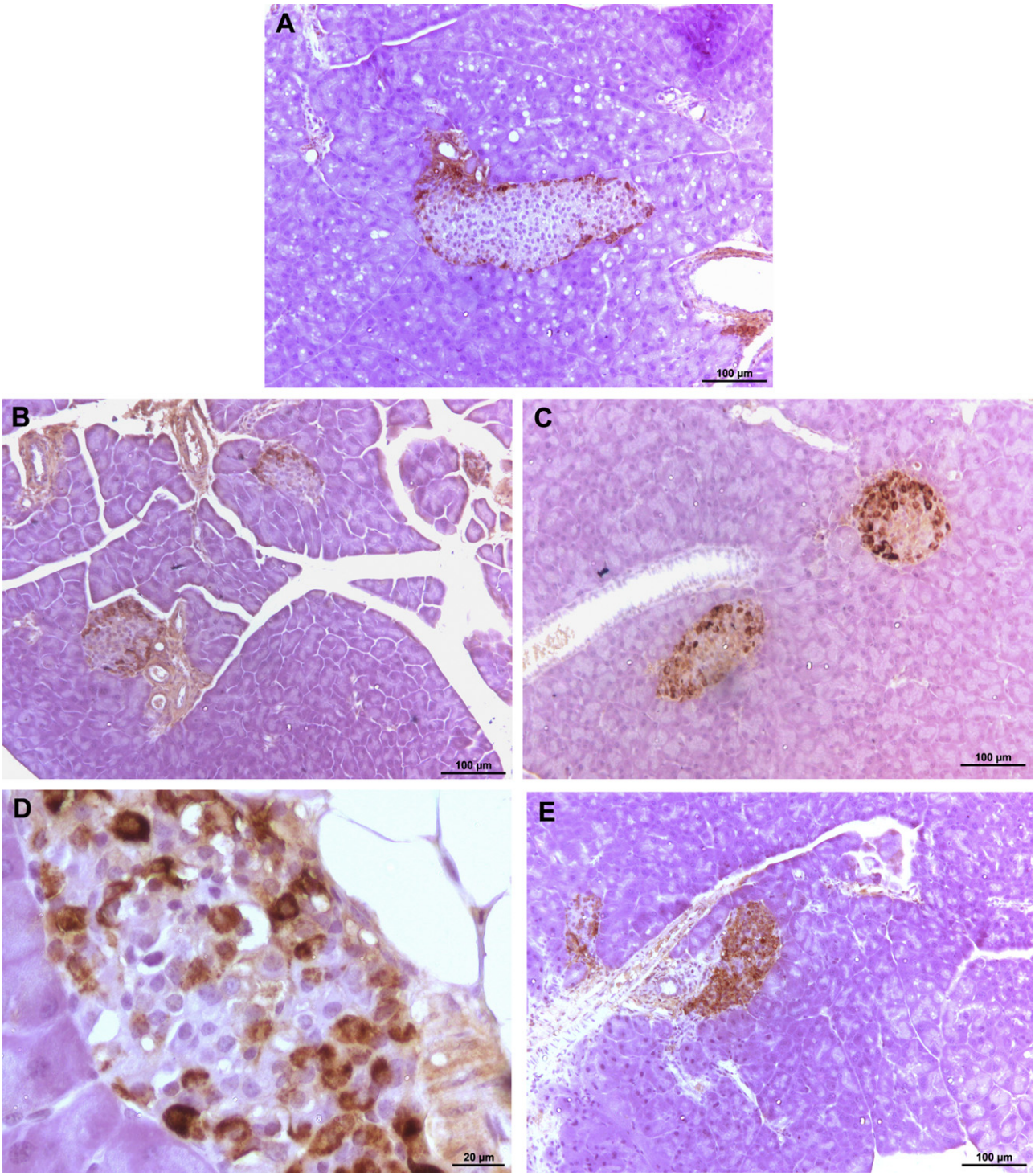

Fig. 3. Glucagon-producing α-cell expansion during chemically induced diabetes development. Pancreata were isolated from control (A) or STZ-injected mice at 6 days (B), 11 days (C), 18 days (D) and 25 days (E) after the last STZ administration. Pancreatic tissue sections were analyzed by immunohistochemistry with anti-glucagon antibody (A–E) to evaluated glucagon positive cells in the islet pancreatic. Representative images are shown.

differences were not significant (Fig. 7B–D). We also observed that the absolute number of $CD4^+CD25^+Foxp3^+$T cells were significantly higher in PLNs from STZ-administrated mice on day 6 (Fig. 7E), 18 (Fig. 7G) and 25 (Fig. 7H) relative to control groups. Despite not to be significant, we also noted a trend of increase in frequency and absolute number of these cells at day 11 in diabetic mice (Fig. 7B and F). These findings suggest that the diabetes course after STZ administration was characterized by an early increase in the regulatory T cell population in pancreatic lymph nodes which was maintained during disease progression relative to health mice.

Th1 and Th17-related cytokines are counterbalanced by IL-10 production

As described before, diabetes development depends on the presence of inflammatory cells that destroy pancreas in an inflammatory milieu where the presence of inflammatory cytokines is

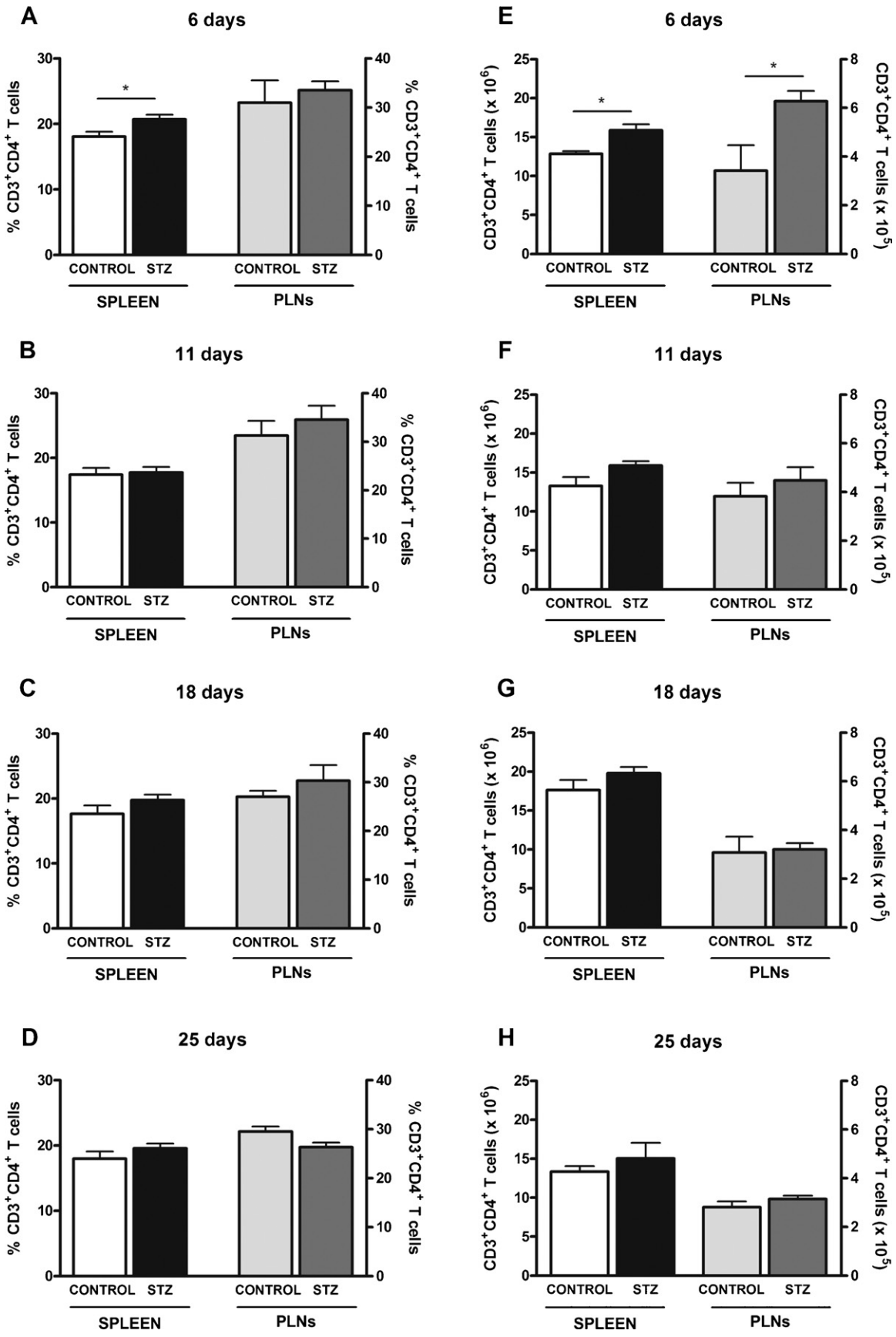

Fig. 4. Frequency and total numbers of $CD4^+$ lymphocytes during chemically induced diabetes development. Cells from spleen and pancreatic lymph nodes (PLNs) were obtained from control (vehicle) and STZ-injected mice at 6, 11, 18 and 25 days after the last STZ injection. The percentage of T helper cells ($CD3^+CD4^+$) in the spleen (left bars) or PLNs (right bars) was determined by flow cytometry (A–D). The absolute number of T helper cells from spleen and PLNs is shown in (E)–(H). Bars represent means ± SEM. Vehicle-injected mice (CONTROL, $n = 4$), STZ-injected mice (STZ, $n = 6$). The results are representative of two independent experiments. *Statistically significant differences ($p < 0.05$).

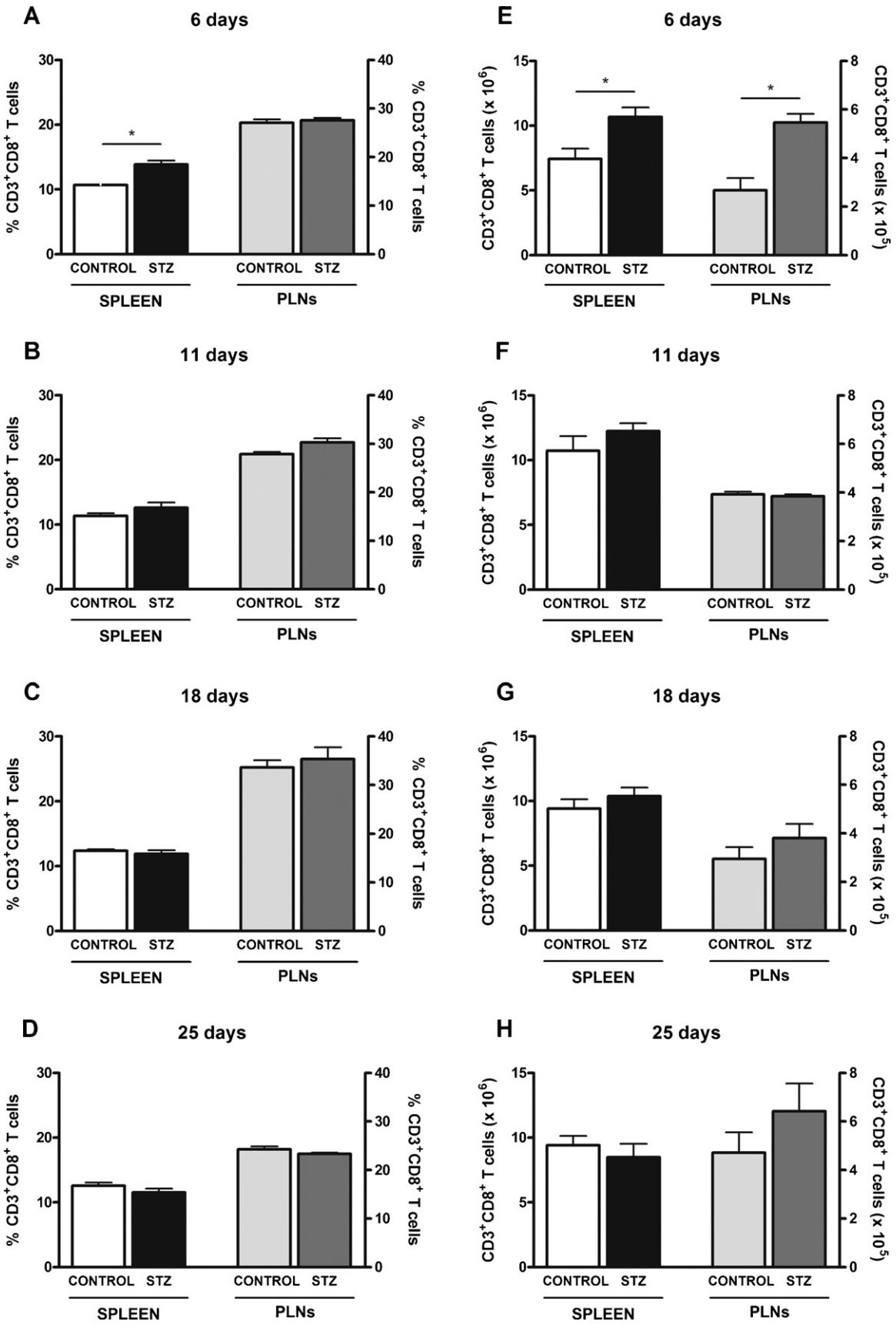

Fig. 5. Frequency and total numbers of $CD8^+$ lymphocytes during chemically induced diabetes development. Cells from spleen and pancreatic lymph nodes (PLNs) were obtained from control (vehicle) and STZ-injected mice at 6, 11, 18 and 25 days after the last STZ injection. The percentage of cytotoxic T cells ($CD3^+CD8^+$) in the spleen (left bars) or PLNs (right bars) was determined by flow cytometry (A–D). The absolute number of cytotoxic T cells from spleen and PLNs is shown in (E)–(H). Bars represent means ± SEM. Vehicle-injected mice (CONTROL, $n = 4$), STZ-injected mice (STZ, $n = 6$). The results are representative of two independent experiments. *Statistically significant differences ($p < 0.05$).

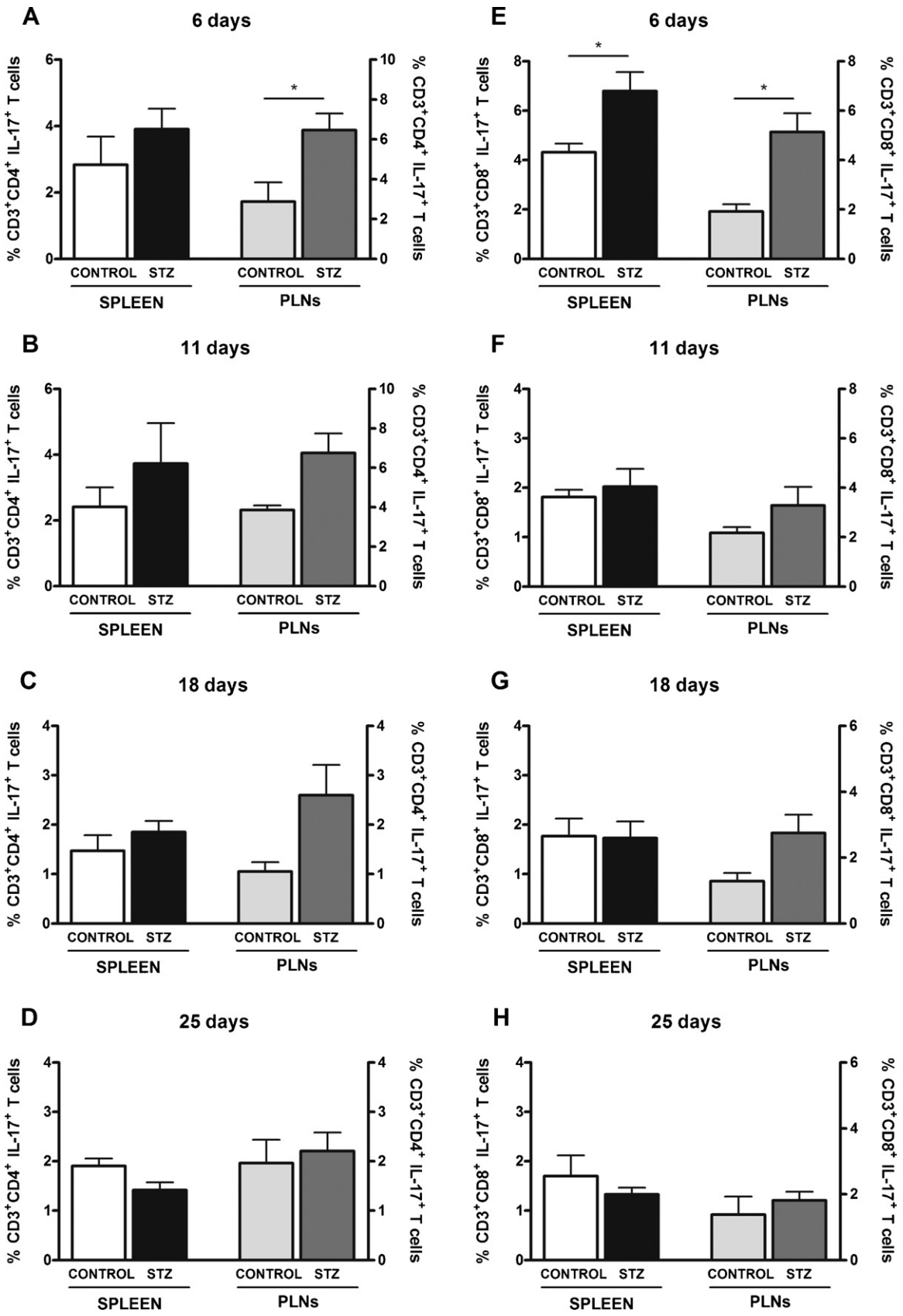

Fig. 6. Frequency of Th17 and Tc17 cells during chemically induced diabetes development. Cells from spleen and pancreatic lymph nodes (PLNs) were obtained from control (vehicle) and STZ-injected mice at 6, 11, 18 and 25 days after the last STZ injection. IL-17 production by $CD3^+CD4^+$ (A–D) and $CD3^+CD8^+$ (E–H) T cells was analyzed after stimulation (PMA + ionomycin), intracellular staining and visualized by flow cytometry. The frequency of $CD3^+CD4^+IL\text{-}17^+$ (Th17) or $CD3^+CD8^+IL\text{-}17^+$ (Tc17) cells in the spleen (left bars) and PLNs (right bars) was determined. Bars represent means ± SEM. Vehicle-injected mice (CONTROL, $n = 4$), STZ-injected mice (STZ, $n = 6$). The results are representative of two independent experiments. *Statistically significant differenc1es ($p < 0.05$).

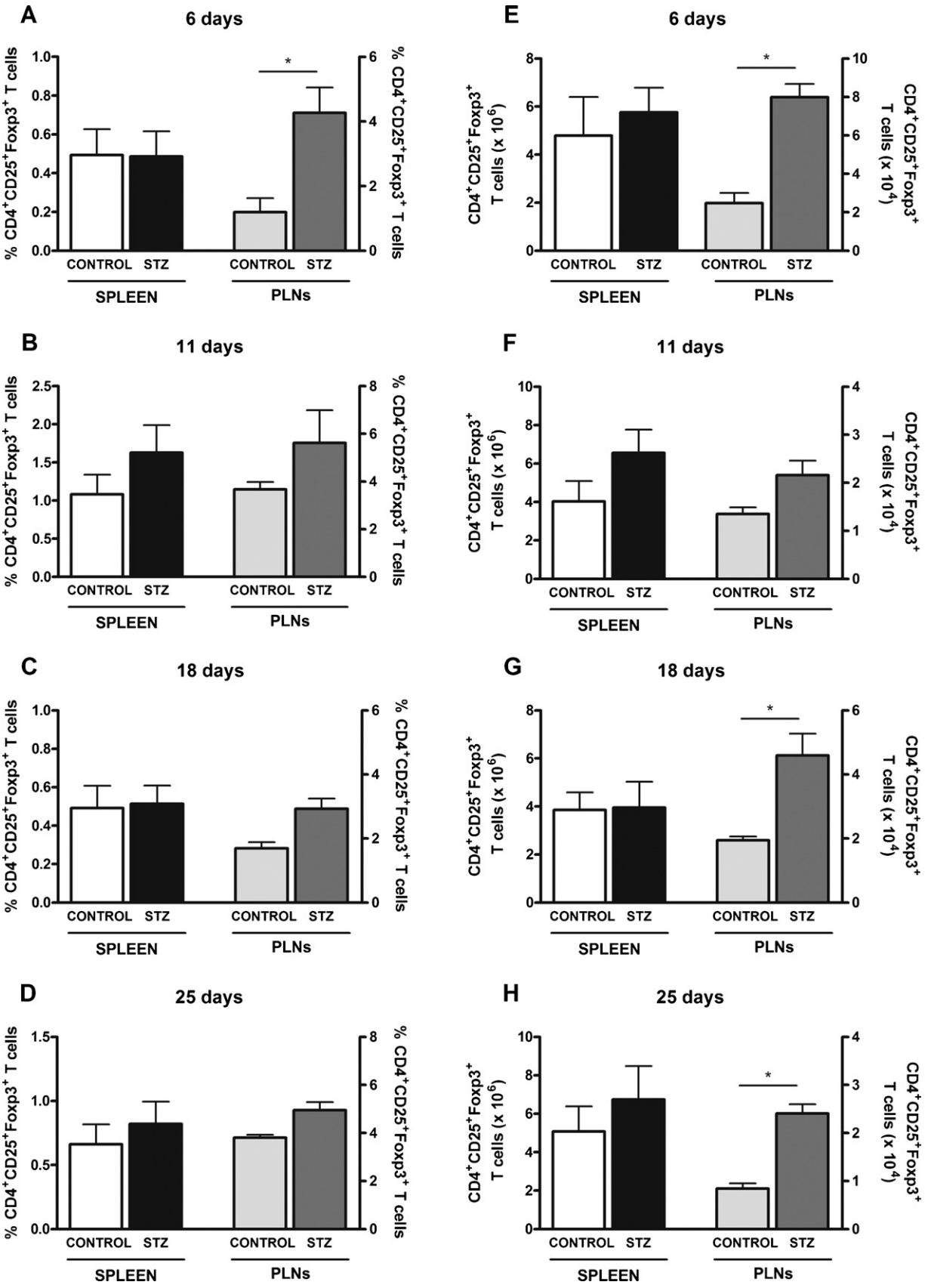

Fig. 7. Frequency and total numbers of regulatory ($CD4^+CD25^+Foxp3^+$) T cells during chemically induced diabetes development. Cells from spleen and pancreatic lymph nodes (PLNs) were obtained from control (vehicle) and STZ-injected mice at 6, 11, 18 and 25 days after the last STZ injection. In (A)–(D) are represented the frequency of $CD4^+CD25^+Foxp3^+$ T cells from spleen (left bars) and PLNs (right bars) and the total number of Tregs are represented in (E)–(H). Bars represent means ± SEM. Vehicle-injected mice (CONTROL, $n = 4$), STZ-injected mice (STZ, $n = 6$). The results are representative of two independent experiments. *Statistically significant differences ($p < 0.05$).

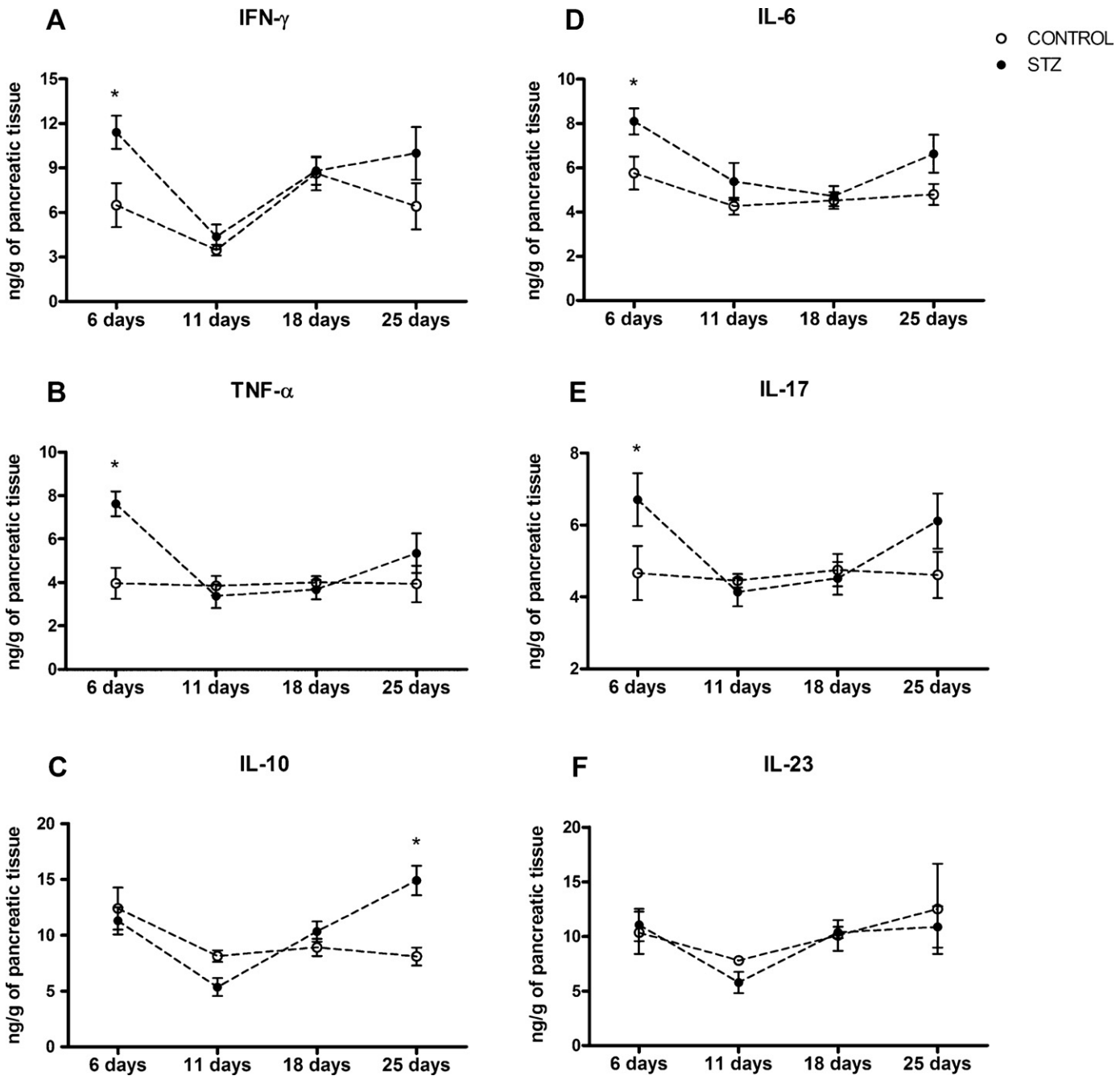

Fig. 8. Proinflammatory and anti-inflammatory cytokine profile during chemically induced diabetes development. Pancreata were obtained from control (vehicle) or STZ-injected mice at 6, 11, 18 and 25 days after the last STZ injection. The samples were weighted and homogenized in the presence of proteases inhibitor. The levels of IFN-γ (A), TNF-α (B), IL-10 (C), IL-6 (D), IL-17 (F) and IL-23 (E) were measured by ELISA method. The cytokine concentrations are represented by nanograms of protein per gram of pancreatic tissue. Bars represent means ± SEM. Vehicle-injected mice (CONTROL, $n = 4$; open circles), STZ-injected mice (STZ, $n = 6$; dark circles). The results are representative of two independent experiments. *Statistically significant differences ($p < 0.05$).

not adequately counterbalanced by regulatory mechanism. Moreover, it is known that dual Th17/Th1 response plays a major role in autoimmune diabetes *via* IFN-γ and IL-17 production. For this reason, we evaluated the Th1, Th17 cytokine profile and anti-inflammatory cytokines in pancreas and correlated the results with pancreatic islet damage. Fig. 8A and B shows clearly increased levels of Th1 cytokines (IFN-γ and TNF-α) at day 6 after diabetes induction. In addition, we also observed significant changes in Th17 cytokine concentrations, as IL-6 and IL-17, which were elevated in pancreas from STZ mice compared to controls at this time (Fig. 8D and E). We did not observe alterations in IL-23 concentration during all period analyzed (Fig. 8F). In the further periods (at 11, 18 and 25 days), we did not note differences regarding these proinflammatory cytokines (Th1 and Th17 pattern) between inflamed and control animals (Fig. 8A, B, D, E). In parallel, we verified that IL-10 concentrations increased at day 25 in the pancreas of diabetic mice (Fig. 8C). Thus, our data indicate that Th1 and Th17 cytokine upregulation in the early phase is counterregulated by a regulatory cytokine profile in the pancreatic islets at the final phase of the autoimmune diabetes course.

IL-17 is related to disease severity in chemically induced diabetes

To examine the role of IL-17 during the initial phase of diabetes development, we evaluated diabetes development in mice lacking the IL-17 receptor (*Il17r* KO) 6 days after the last dose of STZ.

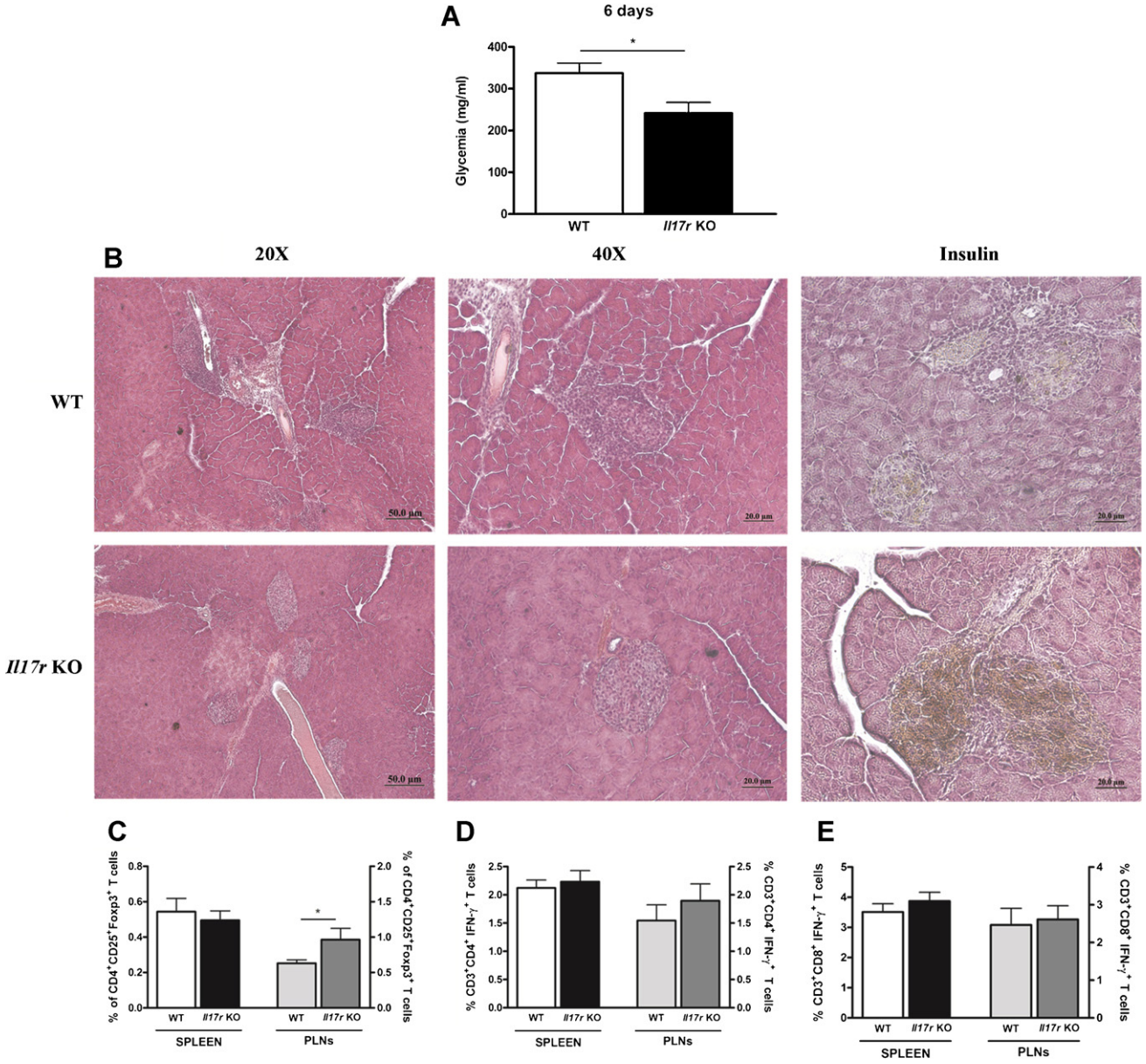

Fig. 9. IL-17 signaling is involved in STZ-induced diabetes development. Diabetes was induced in C57BL/6 WT and *Il17r* KO mice by administration of 5 consecutive doses of STZ (40 mg/kg). On day 6, blood samples were collected from tail vein and analyzed the glucose levels (A). Pancreata from WT (upper panel) and *Il17r* KO STZ injected-mice (lower panel) were evaluated by histological analysis after H&E staining (B, left and middle panels) or by immunohistochemistry for insulin detection (B, right panel). The frequency of regulatory $CD4^+CD25^+Foxp3^+$ T cells (C), $CD3^+CD4^+IFN\text{-}\gamma^+$ (D) and $CD3^+CD8^+IFN\text{-}\gamma^+$ cells (E) were evaluated by flow cytometry in spleen (left bars) and PLNs (right bars). Bars represent means ± SEM. C57BL/6 mice (WT, $n = 6$), *Il17r*-deficient mice (*Il17r* KO, $n = 6$). *Statistically significant differences ($p < 0.05$).

In this period, we observed that *Il17r* KO mice showed decreased levels of blood glucose when compared to diabetic wild-type mice (241 ± 25.65 mg/dl *versus* 337.2 ± 24.06 mg/dl, respectively) (Fig. 9A). To assess whether this resistance was accompanied by a reduced islet inflammation, pancreatic sections from *Il17r* KO and WT were analyzed. Histological analyses showed that diabetic WT mice exhibited typical islet inflammation, whereas *Il17r* KO islets revealed none or few inflammatory cells (Fig. 9B, left and middle panel). In addition, we observed a preservation of beta cells mass in *Il17r* KO mice, demonstrated by insulin staining when compared to WT mice (Fig. 9B, right panel).

To explore the possibility that alterations in regulatory T cells or IFN-γ-producing T cells might play a role in the resistance of the *Il17r* KO mice, we assessed the frequency of these cell subsets in the spleen and PLNs. We observed that *Il17r* KO mice exhibited increased frequencies of regulatory T cells ($CD4^+CD25^+Foxp3^+$) in PLNs when compared to WT mice (Fig. 9C). However, we did not observe differences in $CD3^+CD4^+$ IFN-γ^+ or $CD3^+CD8^+$ IFN-γ^+ populations in spleen and PLNs (Fig. 9D and E, respectively). Thus, differences in disease resistance cannot be explained by alterations in IFN-γ-producing T cells. However, the IL-17 signaling appears to counterregulate the regulatory T cell induction and contributes to initiation of autoimmune diabetes.

Discussion

The association of genetic predisposition and environmental factors (such as viral infections, early infant diet or environmental toxins) account for the development of the human type 1 diabetes (Atkinson and Eisenbarth 2001; Daneman 2006). Based on this evidence, NOD mice represent a limited model to study the pathogenic mechanisms of autoimmune diabetes, since the factors other than

genetics are not equally involved in this model. Thus, an animal model of chemically induced-autoimmune diabetes was proposed by the administration of streptozotocin (STZ) in susceptible rodents (Like and Rossini 1976). Recently, this model has been attractive for its minor influence in genetic background, as new cases of type 1 diabetes are sporadic and occur in families with no previous history of diabetes (Sun et al. 2005).

STZ is a toxin produced by *Streptomyces achromogenes* that promotes selective β-cell destruction, *via* GLUT2 transporter (Lenzen 2008), releases β-cells antigens and induces pancreatic inflammation, acting as an environmental factor for disease development. Indeed, our results showed a progressive inflammatory response in the pancreatic islets after STZ administration, resulting in profound morphologic alteration, loss of β-cell mass and α-cell proliferation. The dynamics of the inflammatory process and insulin content reduction intimately correlated with the hyperglycemia development after STZ administration. As expected, during the process of insulin-producing β-cell destruction, there was a hyperplasia of glucagon-producing α-cells, which means a compensatory mechanism that is also observed in NOD mice and in patients with type 1 diabetes (O'Reilly et al. 1997; Brown et al. 2008).

The role of the immune system in the pathogenesis of STZ-induced diabetes has been investigated in the last years. Previous studies showed evidence that this model is mediated by immunological mechanism (Paik et al. 1980; Nakamura et al. 1984; Herold et al. 1987; Klinkhammer et al. 1988; Klinkhammer et al. 1989; Cockfield et al. 1989; Müller et al. 2002; Nicoletti et al. 2003). In agreement, we observed increased numbers of $CD4^+$ and $CD8^+$ T cells in spleen and pancreatic lymph nodes of diabetic mice, suggesting a systemic and local expansion of these T cell subsets during the initial phase of disease development. An increase of $CD4^+$ T cells was also observed in Balb/c mice after STZ administration (Zou et al. 2008). In addition, the important role of CD8 T cell during STZ-induced diabetes is supported by the fact that the administration of anti-CD8 monoclonal antibodies resulted in protective effect (Harlan et al. 1995). Thus, probably STZ promotes inflammation followed by β-cell destruction and release of its antigens, which reach local lymph nodes and the spleen, priming and activating the $CD4^+$ and $CD8^+$ T cell populations. Although the autoimmune nature of STZ-induced chemically diabetes model is accepted, the potential involvement of other T cell subsets (Treg, Th17 and Tc17 cells) in autoimmunity development has not been completely investigated.

IL-17-secreting $CD4^+$ (Th17) and $CD8^+$ (Tc17) T cells play an important pathogenic role in several models of autoimmune diseases (Honorati et al. 2001; Rohn et al. 2006; Peng et al. 2007; Tzartos et al. 2008; Tajima et al. 2008; Huber et al. 2009; Henriques et al. 2010). However, the frequency and functional role of IL-17 in autoimmune diabetes is less clear. Thus, we examined the potential alteration in these populations during the course of STZ-induced diabetes. Interestingly, we observed increased frequencies of Th17 and Tc17 cells in PLNs and spleen (only Tc17 cells) during the initial phase of disease development. Moreover, we detect significant alterations in IL-6 and IL-17 levels in the pancreatic tissue on day 6 after the last STZ injection. We also analyzed the Th1 cytokines after STZ-diabetes induction and we observed elevated concentrations of IFN-γ and TNF-α in the pancreatic tissue s in this time point. Taking into account these results, both populations of Th1 cells and IL-17-producing T cells (Th17/Tc17 population) seem to be important in STZ-induced autoimmune diabetes although only a few data is currently available regarding the role of IL-17 in this model (Wang et al. 2011).

Therefore, the involvement of Th17/Tc17 cells in diabetes pathogenesis still requires further investigation and controversial data are present in the literature. There are some specific reports showing a central role of these cells in the disease development in NOD, but not in STZ-induced diabetic mice. Accordingly, Zhang and colleagues showed that IFN-γ induced by IL-12 administration prevents diabetes by inhibiting pathogenic IL-17 production in NOD mice (Zhang et al. 2011). Likewise, the administration of anti-IL-17 or anti-IL-25 antibodies reduced islets T-cell infiltrates, decreased autoantibody levels and increased the frequency of Treg cells in NOD mice (Emamaullee et al. 2009). Moreover, mice lacking IL-21 signaling showed decreased numbers of Th17 cells that resulted in reduced pancreatic inflammation and decline of diabetes incidence in NOD mice (Spolski et al. 2008). Taken together, these findings suggest that Th17 cells are involved in the pathogenesis of autoimmune diabetes in the genetically determined model.

On the other hand, some studies showed a dispensable role of Th17 cells in disease pathogenesis in NOD mice. Joseph and colleagues showed that the loss of IL-17 had no effect in the frequency of spontaneous or cyclophosphamide-induced diabetes (Joseph et al. 2012). Van and colleagues observed that NOD mice treated with all-trans retinoic acid inhibited diabetes development by suppressing IFN-γ producing CD4 and CD8 T cells, without affecting Th17 population, suggesting that under these conditions, a presumably Th17 response is not enough to promote the type 1 diabetes onset (Van et al. 2009). Otherwise, two different studies demonstrated that generated Th17 cells could induce diabetes in NOD/SCID mice. The authors observed that the transferred Th17 cells were converted into IFN-γ producing cells, adopting a Th1 phenotype (Martin-Orozco et al. 2009; Bending et al. 2009).

Based on these evidences, we can argue that the main function of IL-17-producing cells in diabetes progression is not well established yet. It is not clear whether these cells are involved in the initiation of autoimmune response or if they contribute directly to the active phase of disease development. It is not established if these cells migrate to the pancreas and secrete IL-17 to promote beta cell destruction or if Th17/Tc17 cells are preferentially involved in directing the immune response in the secondary lymphoid tissues. Another possibility is that Th17 can also cooperate with Th1/CD8 cells in diabetes pathogenesis.

Then, we also investigated the role of IL-17-producing cells in STZ-induced diabetes using mice lacking the IL-17 receptor (*Il17r* KO). Six days after STZ administration the *Il17r*-deficient mice exhibited low blood glucose levels, no inflammatory cells into the islets, insulin-producing cell mass preservation and increased frequency of Treg cells in PLN when compared to wild type mice. Thus, we can suggest that IL-17 signaling is involved in the initiation of the early events of beta cell destruction in our STZ-induced diabetes model, maybe modulating effector responses and the regulatory T cell population.

Regulatory T cells play an essential role in autoimmunity prevention and impairment in their number or suppressive capacity is related to type 1 diabetes pathogenesis (Kukreja et al. 2002; Lindley et al. 2005; Brusko et al. 2005). In this context, we observed increased absolute numbers of Treg ($CD4^+CD25^+Foxp3^+$) cells in PLNs at days 6, 18 and 25 after the last STZ injection when compared to controls. Similarly, Fallarino and colleagues showed that the percentage of Treg cells was also increased in PLNs after 16 days of the diabetes induction with STZ (Fallarino et al. 2009). Zhen and colleagues also showed increased frequencies of $CD4^+CD25^+Foxp3^+$ T regulatory cells in the spleen, peripheral blood lymphocytes, peripheral and mesenteric lymph nodes from diabetic mice as result of the effects of long-term hyperglycemia. On the other hand, $CD4^+CD25^+$ Tregs obtained from mice with diabetes displayed defective immunosuppressive functions (Zhen et al. 2011).

In our study, we found augmentation of both regulatory and IL-17 producing cells in the STZ-induced diabetes. We cannot exclude that the increased number of Treg cells was caused by the increase in IL-17-producing T cells in PLNs. Higher numbers of resistant Th17 and Tc17 cells could stimulate the proliferation or accumulation of Treg cells in pancreatic lymph nodes. In accordance, some reports

showed that human and murine Th17 cells seem to be more resistant to suppression by regulatory T cells than Th1 cells (Evans et al. 2007; Flores-Borja et al. 2008; Stummvoll et al. 2008; Huter et al. 2008). However, our results can also be related to the administration of STZ, which promote an intense inflammatory process in the islets and is very toxic to beta cells (Like and Rossini 1976; Leiter 1982). As a consequence, the number of Treg cells can be increased in pancreatic lymph nodes in an attempt to control this damage of pancreatic tissue. In this view, one work reported that STZ led to a relative increase of Tregs frequencies in the spleen, peripheral blood and also in lymph nodes, but these cells retain their suppressive capacity *in vitro* (Muller et al. 2008). Although plausible, we still do not know if the Tregs found in our study, especially in front of IL-17 responses are indeed functional and further studies are still necessary to unravel this issue.

One of the main mechanisms used by Tregs to suppress the inflammatory response is the induction of cytokines such as IL-10. Our data showed that IL-10 was increased to high levels in the late disease phase (at day 25). The presence of this cytokine in the pancreatic homogenate at this time point correlated to the attenuation of the inflammatory infiltrate in the pancreatic islets, indicating a late deviation of the immune response to a regulatory phenotype. However, the delay and the reduced IL-10 levels at the beginning of the disease onset may favor permanent tissue destruction and hyperglycemia. In agreement, McGeachy and colleagues showed that the natural resolution of autoimmune pathology in the experimental autoimmune encephalomyelitis (EAE) model correlated with the accumulation of IL-10-producing T cells within the central nervous system (McGeachy et al. 2005). Thus, the elevated IL-10 production in the pancreatic microenvironment and the increased numbers of Treg cells in the PLNs during the late disease phase indicate possible immunoregulator effects as a compensatory mechanism in this STZ-induced experimental diabetes model, although not completely effective to avoid β cell destruction and to induce restoration of the organ functionality.

In conclusion, we demonstrated that the frequency of Th17/Tc17 and Treg cells are differentially modulated during the course of STZ-induced diabetes while the lack of IL-17 signaling affected diabetes development and disease severity. Then, a deeper understanding of the pathogenic/protective immune mechanisms involved in autoimmune diabetes model may allow the identification of molecular and cellular targets related to disease pathogenesis and preclinical assessment of potential therapeutic approaches.

Acknowledgements

This study was financially supported by FAPESP, CAPES and CNPq. We are grateful to Giuliana Bertozi Francisco for assistance with ELISA assays, and the staff of School of Medicine of Ribeirão Preto Animal Facility for mice supply. We thank Alessandra Almeida for correcting the manuscript.

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