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RESEARCH PAPER

Compound A, a selective glucocorticoid receptor agonist, inhibits immunoinflammatory diabetes, induced by multiple low doses of streptozotocin in mice

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BACKGROUND AND PURPOSE

Type 1 diabetes is a multifactorial inflammatory disease that develops as a result of deregulated immune responses, causing progressive autoimmune destruction of insulin-producing beta cells of pancreas. 2-((4-acetoxyphenyl)-2-chloro-N-methyl) ethylammonium chloride, compound A (CpdA), is a selective glucocorticoid receptor (GR) agonist that displays strong anti-inflammatory and immunomodulatory activities. We investigated the therapeutic effectiveness of CpdA in a pharmacological model of type 1 diabetes in mice.

EXPERIMENTAL APPROACH

The utility of CpdA in diabetes prevention was evaluated *in vivo* through its prophylactic administration to male C57BL/6 mice that received multiple low doses of streptozotocin for immunoinflammatory diabetes induction. The effect of CpdA on disease development was studied by measuring blood glucose and insulin level, histopathological examination, determination of the nature of infiltrating cells, pro- and anti-inflammatory cytokine production, and signalling pathways.

KEY RESULTS

Prophylactic *in vivo* therapy with CpdA conferred protection against development of immunoinflammatory diabetes in mice by dampening the M1/Th1/Th17 immune response and switching it towards an anti-inflammatory M2/Th2/Treg profile, thus preserving beta cell function.

CONCLUSIONS AND IMPLICATIONS

Anti-diabetic properties of CpdA are mediated through modulation of immune cell-mediated pathways, but without triggering adverse events. These findings provide basic information for the therapeutic use of selective GR agonists in the amelioration of islet-directed autoimmunity.

Abbreviations

CpdA, compound A, 2-((4-acetoxyphenyl)-2-chloro-N-methyl) ethylammonium chloride; GR, glucocorticoid receptor; M, macrophages; MLDS, multiple low dose streptozotocin; PC, peritoneal cells; PLNC, pancreatic lymph node cells; PMC, pancreatic infiltrating mononuclear cells; RORγt, retinoic acid-related orphan receptor γt; SC, spleen cells; STZ, streptozotocin; T1D, type 1 diabetes; T-bet, T-box expressed in T-cells; Treg, regulatory T cells.

Tables of Links

TARGETS
Nuclear hormone receptors
Glucocorticoid receptor (NR3C1)
RORγt receptor, (NR1F3)

LIGANDS
IFN-γ
IL-4
IL-17

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013a,b,c).

Introduction

Type 1 diabetes (T1D) is a multifactorial inflammatory disease, characterized by progressive autoimmune destruction of pancreatic beta cells, eventually resulting in insulin deficiency and hyperglycaemia (Eizirik *et al.*, 2009). The triggering of the full autoimmune response in T1D depends on a crosstalk between beta cells and innate and adaptive arms of the immune system. A key component of this process is T-cell deregulation to a Th1/Th17 response with secretion of inflammatory cytokines that promote a self-perpetuating autoimmune cascade leading to overt disease (McKenzie *et al.*, 2006; Csorba *et al.*, 2010; Haskins and Cooke, 2011; Shao *et al.*, 2012).

Epidemiological studies indicate a global increase in incidence and thus prevalence of T1D (Forlenza and Rewers, 2011). Therefore, a major goal for the prevention and/or reversal of the disease is to find means to preserve or potentiate beta cell mass and function, prolonging the lifespan of present beta cells. One of the potential therapeutic approaches could be induction of an anti-inflammatory Th2 (Muller *et al.*, 2002) and/or regulatory T-cell (Treg) response that can suppress the activation of the immune system and thereby prevent excessive inflammation and/or autoimmunity (Piccirillo *et al.*, 2008; Zhang *et al.*, 2012).

One of the critical determinants of whether diabetes ultimately ensues is the reaction to, and by, the beta cell to the inflammatory environment. Glucocorticoids (GC) remain the most effective treatment for a variety of inflammatory/autoimmune disorders because of their potent immunosuppressive (local and systemic) and anti-inflammatory properties (Boumpas *et al.*, 1991). However, the balance between safety and efficacy is critical, as prolonged treatment with GC may result in severe metabolic side effects (Schäcke *et al.*, 2002). These side effects are considered to arise mainly from the transactivation of GC receptors (Kleiman and Tuckermann, 2007). In that respect, it has been suggested that GC receptor ligands that dissociate transrepression from transactivation may be identified by selecting compounds that prevent dimerization of GC receptors (Dewint *et al.*, 2008; De Bosscher, 2010). One of such GC receptor ligands is compound A (CpdA), 2-((4-acetoxyphenyl)-2-chloro-N-methyl) ethylammonium chloride, a synthetic analogue of a hydroxyphenylaziridine precursor found in the African shrub *Salsola tuberculatiformis* Botschantzev (De Bosscher *et al.*, 2005). Recently, it has been shown that the exposure of immune cells to CpdA down-modulates pro-inflammatory gene expression via transrepression mechanism (Liberman *et al.*, 2012; De Bosscher *et al.*, 2014). Experimental evidences indicate that CpdA attenuates several inflammatory disorders, such as zymosan-induced acute inflammation (De Bosscher *et al.*, 2005), autoimmune neuritis (Zhang *et al.*, 2009), experimental autoimmune encephalomyelitis (Wüst *et al.*, 2009; van Loo *et al.*, 2010), rheumatoid arthritis (Gossye *et al.*, 2009), collagen-induced arthritis (Dewint *et al.*, 2008; Rauner *et al.*, 2013), inflammatory bowel disease (Reuter *et al.*, 2012), asthma (Reber *et al.*, 2012). The aim of the present study was to assess the anti-inflammatory potential of CpdA and to evaluate the efficacy and the underlying mechanism of action of CpdA in a preclinical model of T1D in mice (King, 2012). We opted for a pharmacological model of the disease, induced by multiple low doses of streptozotocin (MLDS diabetes; Weide and Lacy, 1991; Kanitkar *et al.*, 2008). One of the advantages of this MLDS model is the fact that hyperglycemia and insulitis are observed in a relatively short period of time (2–3 weeks after the last streptozotocin injection) in a high percentage of animals (Nikolic *et al.*, 2014). Our results indicate that CpdA is indeed efficacious in MLDS diabetes, through the specific inhibition of immunoinflammatory processes through a variety of pathways, and includes a shift in the cytokine balance that favours anti-inflammatory IL-4 and IL-10.

Methods

Animals

All animal care and experimental procedures complied with directive 2010/63/EU and were approved by the institutional

animal experimentation committee (application NO. 03-01/14). All studies involving animals are reported in accordance with the ARRIVE guidelines for reporting experiments involving animals (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). A total of 66 animals were used in the experiments described here.

Animal experiments

Male C57BL/6 mice 8–12-week-old weighing 20–24 g were obtained from the animal facility of the Institute for Biological Research 'Sinisa Stankovic' (Belgrade, Serbia). The mice were randomly divided into four groups with seven mice in each group, and were used for diabetes monitoring *in vivo*: (i) multiple low dose streptozotocin (STZ)-induced diabetic mice that received vehicle alone (MLDS group); (ii) MLDS-induced diabetic mice that were treated with CpdA (MLDS + CpdA group); (iii) toxic STZ-induced diabetic mice that received single i.p. injection of 200 mg·kg^{-1} STZ and were treated with vehicle alone (STZ group); (iv) toxic STZ-induced diabetic mice that were treated with CpdA in the same schedule as the MLDS + CpdA group (STZ + CpdA group). Day 0 was defined as the first injection of STZ. At day 49 after MLDS induction, when the disease is already established, the mice were killed, and blood samples were taken from the retro-orbital plexus for measuring the insulin content. Serum insulin levels were determined by ELISA. At the time of death, pancreata were removed for histological analysis. The MLDS group (i) and the MLDS + CpdA group (ii) with four mice in each group were formed additionally and used for *ex vivo* analysis by day 16 of diabetes post-induction.

Autoimmune (MLDS) diabetes was induced as described previously (Cvetkovic *et al.*, 2005). Briefly, STZ was dissolved in citrate buffer, pH 4.5, and administered i.p. at a dose of 40 mg·kg^{-1} daily for 5 consecutive days. Toxic non-immune diabetes was induced in mice by a single i.p. injection of 200 mg·kg^{-1} STZ in citrate buffer, as described previously (Stošić-Grujičić *et al.*, 2001). To evaluate the effect of CpdA, the drug was administered i.p. every other day at a dose of 5 mg·kg^{-1} day^{-1} for 2 weeks, starting with first STZ injection. The effective *in vivo* dose of CpdA was chosen on the basis of previous studies (Wüst *et al.*, 2009).

Clinical evaluation of diabetes

The development of the disease in MLDS-induced diabetes was evaluated by measuring blood glucose level and body weight change on a weekly basis. Blood was collected by tail snipping to measure glucose levels using a glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany).

Assessment of insulin

Insulin concentration in the sera of non-fasted mice was determined by an ELISA kit (Mercodia, Uppsala, Sweden) according to the manufacturer's instructions.

Histology and immunohistochemistry

Pancreata were fixed in formalin and embedded in paraffin. The paraffin sections (5 µm) were prepared from different levels (200 µm apart), stained with Mayer's haematoxylin and examined for the presence of mononuclear cell infiltration by light microscopy with a ×40 objective. Immunohistochemical staining for nitrotyrosine was performed as previously described (Cvjetićanin *et al.*, 2010). Briefly, for antigen retrieval tissue sections were treated with 0.1% trypsin for 30 min at 37°C. After blocking endogenous peroxidase, slides were incubated with rabbit anti-nitrotyrosine (1:500) antibody, followed by the Rabbit ExtrAvidin peroxidase staining kit according to the manufacturer's instructions. Staining was developed with diaminobenzidine (DakoCytomation, Carpinteria, CA, USA), and sections were counterstained with haematoxylin. Representative islets were photographed using a Leica photomicroscope (Leitz, Wetzlar, Germany) at ×400 magnification.

Ex vivo *cell preparations*

Pancreas, spleen, pancreatic lymph nodes (PLN) and resident peritoneal cells (PC) were collected from individual mice, given MLDS, and treated with either CpdA or its vehicle, on day 16 after the first injection of STZ. Total PC were harvested by washing the peritoneal cavity with PBS. Solid organs were mechanically disrupted, passed through a 40 µm nylon mesh filter (BD Bioscience, Bedford, MA, USA) and the suspension of splenocytes (SC) and PLN cells (PLNC) was collected by centrifugation. Erythrocytes were lysed using lysis buffer (eBioscience, San Diego, CA, USA). To assess the numbers of mononuclear cells (PMC) infiltrating the pancreas, pancreata were processed by collagenase type V digestion, and digests were passed through a 20 µm cell strainer.

Ex vivo *lymphoproliferative response*

To assess the capacity of lymphocytes to proliferate, SC (5×10^5 per well) and PLNC (3×10^5 per well) were cultured in 96-well microplates (Sarstedt, Numbrecht, Germany) in culture medium for 24 h in the presence of [^{3}H]-thymidine (1 µCi per well, ICN, Costa Mesa, CA, USA). Incorporated radioactivity in triplicate cultures was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA, USA).

Determination of cytokine secretion ex vivo

Cell culture supernatants were obtained by culturing the cells obtained from individual mice for 48 h in 24-well culture plates in 1 mL of a culture medium (5×10^6 SC mL per well, 3×10^6 LNC mL^{-1} per well, or 1×10^6 PC mL^{-1} per well) in 24-well culture plates (Sarstedt). Cytokine concentrations in cell culture supernatant fractions were determined by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Samples were analysed in duplicate for murine IL-1β, TNF, IL-17 (BD Biosciences), IL-6, IL-10, IL-2 (eBioscience), IFN-γ and IL-4 (R&D Systems, Minneapolis, MN, USA).

Immunofluorescence analysis

The cell phenotype of individual mice was assessed by flow cytometry using the following antibodies: F4/80-FITC (rat IgG2a κ), CD40-APC (Armenian hamster IgM), CD11b-FITC (rat IgG2κ), CD4-PE (rat IgG2b κ), CD4-FITC (rat IgG2b κ), CD8-PE (rat IgG2a κ), CD25-PE (rat IgG1), (all from eBioscience) or anti-mouse CD206-PE (goat IgG) (R&D Systems). Treg were detected by the Mouse Regulatory T-cell Staining Kit (eBioscience) according to the manufacturer's instructions.

Table 1
Primer pairs sequences

Gene	Primer pairs	GenBank acc. no.
β-actin	5′-CCAGCGCAGCGATATCG-3′ 5′-GCTTCTTTGCAGCTCCTTCGT-3′	NM_007393.3
IFN-γ	5′-CATCAGCAACAACATAAGCGTCA-3′ 5′-CTCCTTTTCCGCTTCCTGA-3′	NM_008337.3
IL-17	5′-GGGAGAGCTTCATCTGT-3′ 5′-GACCCTGAAAGTGAAGGG-3′	NM_010552.3
IL-4	5′-ATCCTGCTCTTCTTTCTCG-3′ 5′-GATGCTCTTTAGGCTTTCC-3′	NM_021283.2
RORγt	5′-CCGCTGAGAGGGCTTCAC -3′ 5′-TGCAGGAGTAGGCCACATTACA -3′	NM_011281.1

For intracellular cytokine staining, cells were stimulated with phorbolmyristate acetate (400 ng·mL^{-1}), ionomycin (800 ng·mL^{-1}) and brefeldin A (eBioscience) (5 μM) for 4 h. After incubation cells were harvested and stained for surface markers with rat anti-mouse IgG2bκ CD4-FITC antibody (eBioscience). After staining, cells were fixed in 2% paraformaldehyde, permeabilized and stained with the following antibodies: anti-mouse IFN-γ-PE (rat IgG1κ), IL-4-PE (rat IgG1κ), IL-10-PE (rat IgG2b), anti-human/mouse T-box expressed in T-cells (T-bet)-PE (mouse IgG1κ), GATA-3-eFluor®660 (rat IgG2bκ) and retinoic acid-related orphan receptor γt (RORγt)-PE antibody (rat IgG2a) (all from eBioscience), or IL-17-PE (rat IgG1κ) (BD Biosciences). Isotype-matched controls were included in all experiments. Cells were analysed on a PartecCyFlow Space cytometer using FloMax software (Partec, Münster, Germany).

Immunoblot analysis

SC (5×10^6) obtained from individual mice were disrupted in lysis buffer and processed for immunoblot analysis as previously described (Saksida *et al.*, 2012). The following antibodies were used: P-STAT3 and STAT3, P-STAT4 and STAT4 (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA), followed by incubation with secondary sheep anti-mouse antibody horseradish peroxidase (HRP)-linked (GE Healthcare, Buckinghamshire, UK) or polyclonal anti-rat HRP (eBioscience). Detection was performed using chemiluminescence (ECL, GE Healthcare) and photographs were obtained by X-ray (Kodak, Rochester, NY, USA). Densitometry was performed with Scion Image Alpha 4.0.3.2 (Scion Corporation, Frederick, MD, USA).

RNA isolation and real-time PCR

Total RNA from SC, PLNC and PMC was isolated with TRI Reagent solution (Applied Biosystems, Woolston, UK). RNA (1 μg) was reverse transcribed using RevertAid M-MuLV reverse transcriptase and random hexamer primers (Fermentas, Vilnius, Lithuania). PCR amplification was carried out in real-time PCR machine (Applied Biosystems, UK), using a SYBRGreen PCR master mix (Applied Biosystems) as described previously (Stosić-Grujicić *et al.*, 2009). The primer sequences used are listed in Table 1. The amount of each gene was calculated as $2^{-(C_{ti}-C_{ta})}$, where C_{ti} is the cycle threshold of the gene of interest and C_{ta} is the *β-actin* cycle threshold.

Data analysis

The data are presented as mean ± SD for each experimental protocol. Statistical analysis of differences was made using one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons, or Student's *t*-test, as appropriate. Statistical evaluation of the results was made with Statistica version 6.0 (StatSoft, Tulsa, OK, USA) and conducted at the 0.05 significance level. All experiments on cells were repeated at least three times, while in animal experiments, the number of independent experiments is indicated in the Figures, *n* referring to the number of mice in each group. In the flow cytometry analysis, the figures shown are representative of at least three different experiments performed on different days.

Materials

2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethyl-ammonium chloride (CpdA) was a kind gift from Dr. Kathleen Van Craenenbroeck (LEGEST – University of Gent, Gent, Belgium). It was synthesized and characterized, as described by Louw *et al.* (1997). For *in vivo* application, a fresh stock solution of lyophilized CpdA was prepared in sterile double distilled H_2O.

Unless otherwise stated, all other reagents were obtained from Sigma-Aldrich (St Louis, MO, USA). Cell culture experiments used RPMI-1640 medium (25 mM HEPES, 2 mM L-glutamine) supplemented with 5% fetal calf serum (FCS, PAA Chemicals, Pasching, Austria), 5 μM·L^{-1} of β-mercaptoethanol, 100 U·mL^{-1} penicillin and 100 mg·mL^{-1} streptomycin (complete medium).

Results

The effect of CpdA on MLDS-induced diabetes

To evaluate the ability of CpdA to interfere with immunoinflammatory diabetogenic pathways, mice treated with MLDS for 5 days were treated i.p. every other day for 2 weeks with

5 mg·kg^{-1} body weight of the drug. The group of control mice that were challenged with MLDS and were treated with the vehicle, developed persistent hyperglycaemia that started 2 weeks after the first injection of the STZ (Figure 1A). In contrast, treatment with CpdA resulted in durable suppression of hyperglycaemia that persisted when CpdA was no longer present (Figure 1A). Consistent with this finding, at day 49 after MLDS induction, higher systemic insulin concentrations were found in the sera of CpdA-treated compared with vehicle-treated MLDS mice (1.37 ± 0.15 ng·mL^{-1} vs. 1.02 ± 0.23 ng·mL^{-1}, respectively, P = 0.0435). Importantly, prolonged treatment with the drug appeared to be well tolerated by the mice as judged by their behaviour and general appearance and normal food consumption (not shown). Accordingly, there were no significant differences in body weight gain between these two groups of mice (Figure 1B). Importantly, and in contrast to MLDS-induced diabetes, CpdA did not influence the non-immune 'toxic' form of diabetes induced by a single high dose (200 mg·kg^{-1} b.w.) of STZ, as blood glucose levels in CpdA-treated mice were not different from those in vehicle-treated mice (31.9 ± 2.4 ng·mL^{-1}and 27.4 ± 10.0 ng·mL^{-1}, respectively; P = 0.4983).

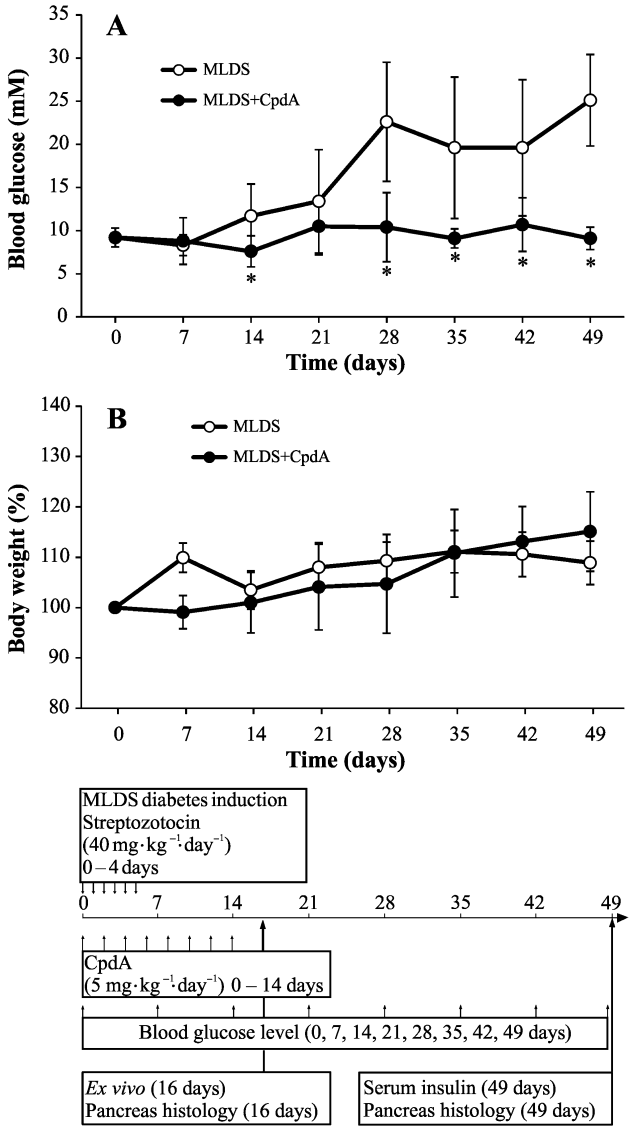

Figure 1

CpdA treatment reduces hyperglycaemia of MLDS-induced C57BL/6 mice. (A) Blood glucose levels measured in male C57BL/6 mice injected i.p. for five consecutive days with MLDS (40 mg·kg^{-1}·day^{-1}), and treated with vehicle (MLDS) or 5 mg·kg^{-1} day^{-1} of CpdA (MLDS + CpdA) as described in Methods. (B) Percent change in body weight from the start of the experiment of mice as described in (A). Day 0 was defined as the first injection of STZ. Experimental timeline is given below. Results from one representative out of three experiments with similar results are presented as mean ± SD (n = 7 mice per group). Statistical comparisons were made by one-way ANOVA followed by Student-Newman-Keuls test for multiple comparisons. *P < 0.05 refers to corresponding MLDS mice.

Histopathological analysis of the pancreata of CpdA-treated and control vehicle-treated MLDS mice revealed significant differences. While the islets of control diabetic mice exhibited insulitis and progressively lost beta cell mass and clear margins (Figure 2A, C, E), CpdA treatment protected against mononuclear infiltration (Figure 2B, D, F). Also, islets from CpdA treated mice (Figure 2B) showed few or no cells positive for IL-1β compared with islets from MLDS-induced mice (Figure 2A). Consequently, nitrotyrosine formation was not evident in islets of CpdA-treated mice compared with those of diabetic mice (Figure 2D vs. Figure 2C). Quantitative analysis of mononuclear cell infiltration at day 49 after diabetes induction revealed that the islets of CpdA-treated animals consistently contained fewer mononuclear cells compared with diabetic MLDS controls (Figure 2G), resulting in the higher number of intact islets.

CpdA modulates peripheral immune responses in vivo

PC, SC and PLNC were harvested on day 16 after MLDS injection from mice that have been treated with either CpdA or its vehicle, and the effect of the drug on T helper (Th) and macrophage (M) differentiation was analysed *ex vivo*.

Phenotype analysis of PC showed that CpdA significantly reduced the percentage of F4/80$^+$CD40$^+$ (pro-inflammatory M1) macrophages, while the proportion of the alternatively activated CD206$^+$ (M2 polarized) macrophages increased (Figure 3A). Moreover, CpdA treatment successfully reduced the production of macrophage-derived pro-inflammatory mediators IL-1β, TNF and IL-6 by PC, but up-regulated IL-10 secretion (Figure 3B), further indicating that CpdA favoured M2 macrophage polarization *in vivo*. A similar trend of macrophage polarization was observed within the SC population (Figure 3C). In addition, SC isolated from CpdA-treated mice were less efficient producers of IL-1β and IL-6 (Figure 3D) compared with control diabetic mice.

We next analysed the effect of CpdA on activation and proliferation of lymphocytes. As evaluated by [^{3}H]-thymidine uptake, attenuated proliferative activity was found in the spleens (Figure 4A) and draining lymph nodes of CpdA-treated mice (Figure 4C), when compared with MLDS-induced control mice. Consistent with this, CpdA reduced the absolute number of cells, as well as the number of CD4$^+$ cells in the spleen (Figure 4B) and PLN (Figure 4D). Furthermore, the proportion of lymphocytes bearing an early activation marker CD25 was lower in the cell populations

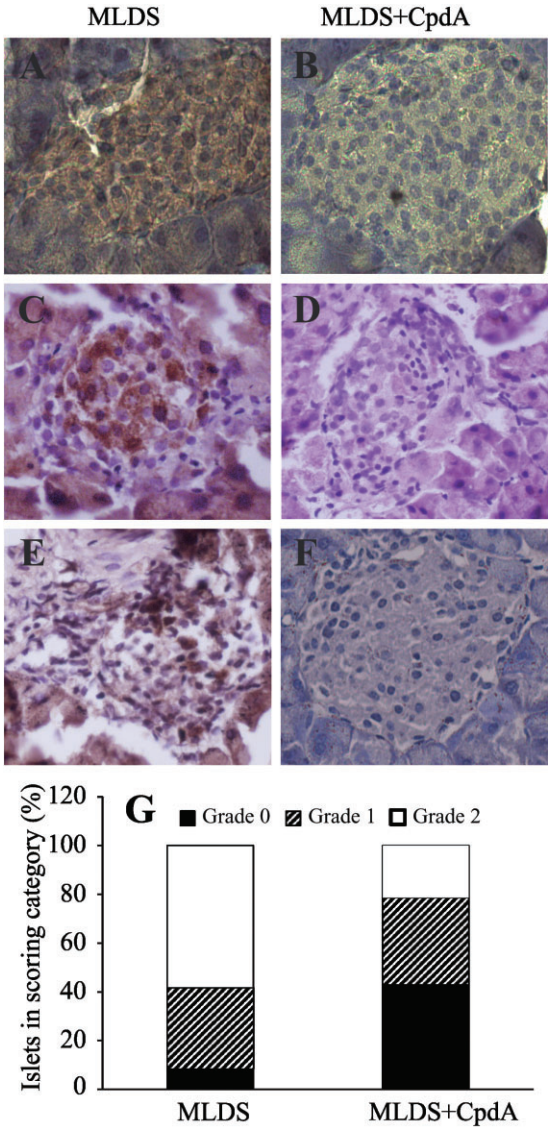

Figure 2

CpdA treatment reduces destructive insulitis of MLDS-induced mice. (A, C) Representative image of pancreatic islet in MLDS-treated mice by day 16 after disease induction: IL-1β (A) and nitrotyrosine (C) immunostaining. Note moderate mononuclear cell infiltrates and positive staining for IL-1β and nitrotyrosine. (E) Pancreatic islet in MLDS-treated mice at the study endpoint (day 49). Note abundant mononuclear cell infiltrate, atrophy and loss of islet margins. (B, D) Typical islet of CpdA-treated MLDS-induced mice by day 16 after disease induction and (F) at the study endpoint (day 49). Note the well-preserved morphology without mononuclear cell infiltration and without positive staining for IL-1β (B) and nitrotyrosine (D). (G) Quantitative histology score of pancreatic islets at the study end point (day 49). Data are presented as mean percentages of total pancreatic islets ± SD (n = 7 mice per group).

derived from CpdA-treated mice (Figure 4E and G). However, the frequency of $CD4^+CD25^+$ $Foxp3^+$ T regulatory cells was similar in both experimental groups (Figure 4F and H).

We next studied the expression of several prototypical Th cytokines in the spleen and draining lymph nodes. In both organs, CpdA treatment successfully attenuated mRNA expression of pro-inflammatory cytokines IFN-γ and IL-17, and significantly up-regulated the expression of anti-inflammatory IL-4 (Figure 5A, C), thus shifting the balance towards an anti-inflammatory cytokine profile. A similar profile regarding pro- versus anti-inflammatory cytokines was found by measuring cytokine concentration in supernatants of PLNC (Figure 5B) and SC cultures (Figure 5D). Furthermore, CpdA was found to impede Th17 development through down-regulation of its transcription factor, RORγt (Figure 5E, F), while P-STAT3 protein expression was not changed (Figure 5G). Interestingly, CpdA reduced Th1-related STAT-4 activation (Figure 5G), but did not affect T-bet protein expression that is crucial for Th1 cell differentiation (Figure 5F). However, CpdA significantly up-regulated Th2-associated transcription factor GATA3 (Figure 5F).

CpdA reduces the influx of pathogenic cells into the pancreas, and expression of inflammation-related molecules

The absolute number of PMC harvested by day 16 of diabetes post-induction was significantly reduced when derived from CpdA-treated mice (Figure 6A). Examination by flow cytometry revealed significant influx of $CD11b^+$, $CD4^+$, $CD8^+$, as well as activated $CD4^+CD25^+$ mononuclear cells in the islets of MLDS-induced mice, but infiltration of all of these leukocyte subtypes was greatly reduced by CpdA treatment (Figure 6A). Interestingly, CpdA increased the proportion of $FoxP3^+$ regulatory T cells within the pancreas (Figure 6B).

Quantitative real-time PCR analysis of PMC revealed that CpdA treatment markedly depressed the expression of IFN-γ mRNA and significantly up-regulated IL-4 gene expression (Figure 6D). Similarly, the number of IFN-γ^+ and $IL\text{-}17^+$ cells within PMC was reduced by CpdA, whereas the cells expressing IL-4 protein were not significantly influenced (Figure 6C).

Discussion and conclusions

In this study, we have demonstrated for the first time that prophylactic administration of CpdA confers protection against development of immunoinflammatory diabetes in mice. The beneficial effects of CpdA are mediated through restraining immunoinflammatory (M1/Th1/Th17) responses at a systemic level, as well as within the endocrine pancreas. Moreover, CpdA switched cytokine production towards an anti-inflammatory M2/Th2/Treg profile.

Macrophages are the first cells to infiltrate the islets in experimental and human T1D (Jörns *et al.*, 2014). Two major populations have been defined: the classically activated M1 macrophages, which initiate insulitis and destruction of β cells, and alternatively activated M2 macrophages, which dampen the immune response and limit inflammation (Espinoza-Jiménez *et al.*, 2012). Thus, the balance between activation and polarization of M1 and M2 macrophages is important for disease progression. We have shown here that the capacity of macrophages to produce inflammatory mediators is diminished by CpdA. In addition, CpdA reduced the proportion of M1 cells in different peripheral compartments and attenuated their accumulation within pancreas.

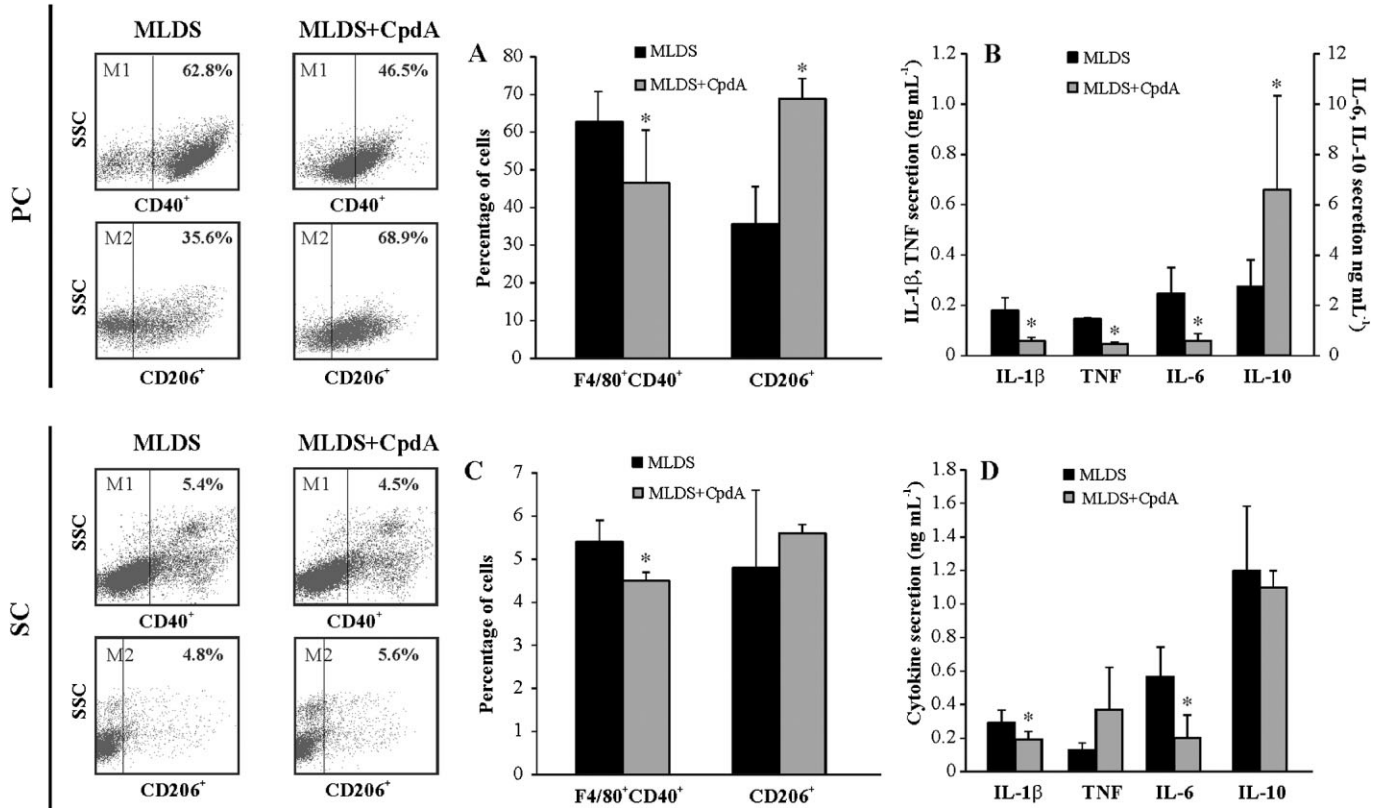

Figure 3

CpdA treatment of MLDS-induced mice alters macrophage differentiation and cytokine generation in the peritoneal cells and spleen. PC (upper panel) and SC (lower panel) were isolated on day 16 after diabetes induction from MLDS and CpdA-treated mice (MLDS + CpdA) or vehicle-treated MLDS-induced mice (MLDS). Day 0 was defined as the first injection of STZ. (A, C) The proportion of $F4/80^+CD40^+$ and $CD206^+$ macrophages was assessed by flow cytometry. Representative flow cytometry profiles are presented. (B, D) Cytokine concentration was determined by ELISA in the PC-derived 24 h culture supernatants (B) or SC-derived 48 h culture supernatants (D). Results from one representative out of three experiments with similar results are presented as mean ± SD ($n = 4$ mice per group). *$P < 0.05$ refers to corresponding MLDS mice.

Moreover, the anti-inflammatory effects of CpdA are associated with macrophage polarization towards alternatively activated $CD206^+$ (M2 polarized) phenotype. In line with this, it has been reported that stimulation with classic GC receptor ligands can induce distinct M2 polarization states with immunoregulatory properties (Mosser and Edwards, 2008). Furthermore, *in vitro* stimulation with IL-4, IL-10 and TGF-β may induce an immunosuppressive M2r macrophage phenotype with the potent ability to halt ongoing autoimmune disease processes in NOD mice (Parsa *et al.*, 2012). Besides a direct effect of CpdA on macrophage polarization, the increased production of IL-4 and IL-10, seen upon CpdA treatment, might be involved in the further recruitment and maintaining of M2 macrophages. Our findings further supported the role for CpdA in macrophage switch towards an anti-inflammatory type, as shown in the EAN autoimmune model (Zhang *et al.*, 2009).

Helper T-cell differentiation is another component that defines the nature of the developing autoimmune responses (Freiesleben De Blasio *et al.*, 1999; Steinman, 2007). It is well documented that GC-treated antigen-presenting cells either fail to present antigen to T-cells, preferentially bias T-cell responses towards a Th2-cell phenotype, or induce the development of regulatory T-cells that can inhibit immune responses (Franchimont, 2004). Moreover, application of CpdA led to a decreased production of Th1 and Th17 cytokines in several experimentally induced T-cell-mediated autoimmune diseases (see De Bosscher *et al.*, 2010). Our mechanistic studies confirmed similar down-modulating effects on these two signature cytokines at both gene and protein levels in peripheral compartments, as well as in the pancreas. The overall consequence was a reduced influx of autoreactive T-cells into the pancreas. As the initiation of the autoimmune response occurs within the draining PLN, and thereafter activated islet-specific T-cells migrate to the pancreas (Hoglund *et al.*, 1999), it can be speculated that CpdA dampened the pro-inflammatory milieu required for entry of auto-aggressive cells into the target tissue, or it directly affected T-cell migration. It has been previously reported that CpdA inhibits the master Th1 transcription factor, T-bet, while inducing GATA-3 (the master Th2 transcription) activity *in vitro* (Liberman *et al.*, 2012). Our data add to these observations by demonstrating the stimulatory effect of CpdA on $GATA\text{-}3^+$ cell population and Th2-type cytokine production in murine T1D. Although CpdA in our system did not interfere with the proportion of Th1 cells, it successfully

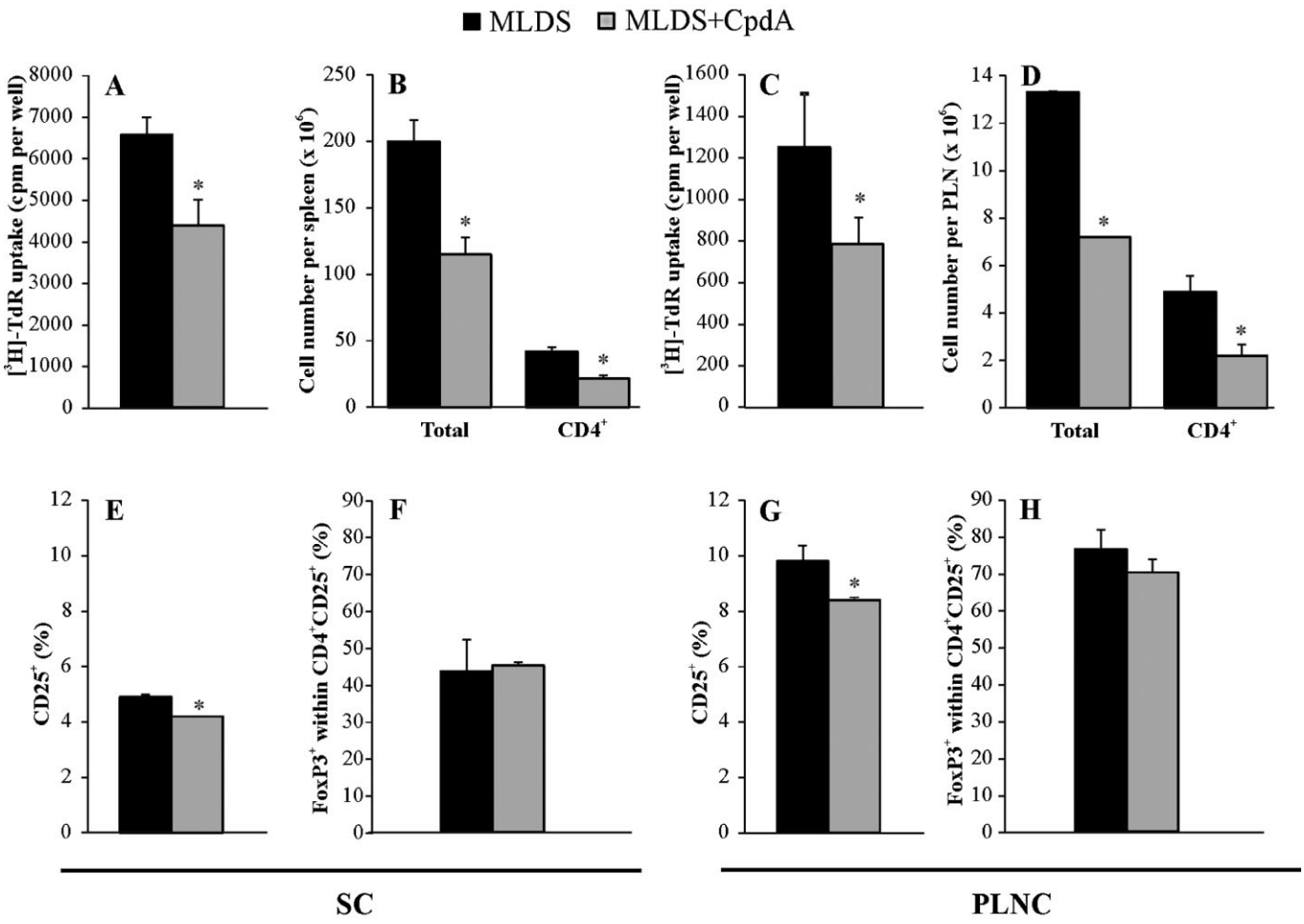

Figure 4

Effect of CpdA on cellular changes in the spleen and draining lymph nodes induced by MLDS treatment. SC (left panels) and PLNC (right panels) were isolated on day 16 post-diabetes induction from MLDS and CpdA-treated mice (MLDS + CpdA) or vehicle-treated MLDS-induced mice (MLDS). (A) Incorporation of [^{3}H]-thymidine in SC (5×10^5 per well) cell cultures was determined as described in Methods. (B) Total number of cells per spleen obtained by conventional cell counting, and number of CD4$^+$ cells obtained by flow cytometry. (C) Incorporation of [^{3}H]-thymidine in PLNC (3×10^5 per well) cell cultures. (D) Total number of cells per PLN obtained by conventional cell counting, and number of CD4$^+$ cells obtained by flow cytometry. Flow cytometric analysis of SC (E, F) and PLNC (G, H). Results from one representative out of three experiments with similar results are presented as mean ± SD (n = 4 mice per group). *P < 0.05 refers to corresponding MLDS mice.

dampened Th1-mediated immune response. This suggests that during T1D induction CpdA affected only the function of Th1, not their differentiation. However, CpdA successfully repressed both Th17 differentiation and effector function.

Treg regulate the priming of autoreactive T-cells by limiting their expansion and differentiation (Roncarolo and Battaglia, 2007). Diabetic mice with less FoxP3$^+$ cells had as expected, a significant infiltration of mononuclear cells, which would include T effector cells responsible for autoimmune diabetes (Hill and Sarvetnick, 2002). Using a model of autoimmune neuritis in rats, Zhang *et al.* (2009) reported that CpdA increased FoxP3$^+$ Treg cells in lymph nodes and peripheral blood of rats. Interestingly, in our study, the proportion of CD4$^+$CD25$^+$ FoxP3$^+$ Treg was not changed in peripheral immune organs upon *in vivo* treatment of MLDS-induced mice with CpdA, but was significantly increased within the pancreas, which points to the local regulation to favor disease outcome. This increase in the Treg number within the pancreas could be a direct result of CpdA-mediated repression of pathogenic leukocyte differentiation or its ability to neutralize pro-inflammatory cytokines that can render Treg defective (Bayry *et al.*, 2008). However, we do not have any direct evidence that Treg are expanded by CpdA treatment, or become trapped within the pancreas.

The effectiveness of CpdA in suppressing clinical symptoms of T1D correlated with amelioration of the degree of inflammation in the pancreas. Therefore, another mechanism of action by which CpdA protects against diabetes might be by acting at a stage when lymphocytes enter the pancreas and its subsequent cascade of local inflammatory events. We have also found that CpdA treatment attenuated nitrotyrosine expression, a key histological parameter of oxidative and nitrosative stress, which underlie beta cell damage and death. However, the drug did not protect mice from

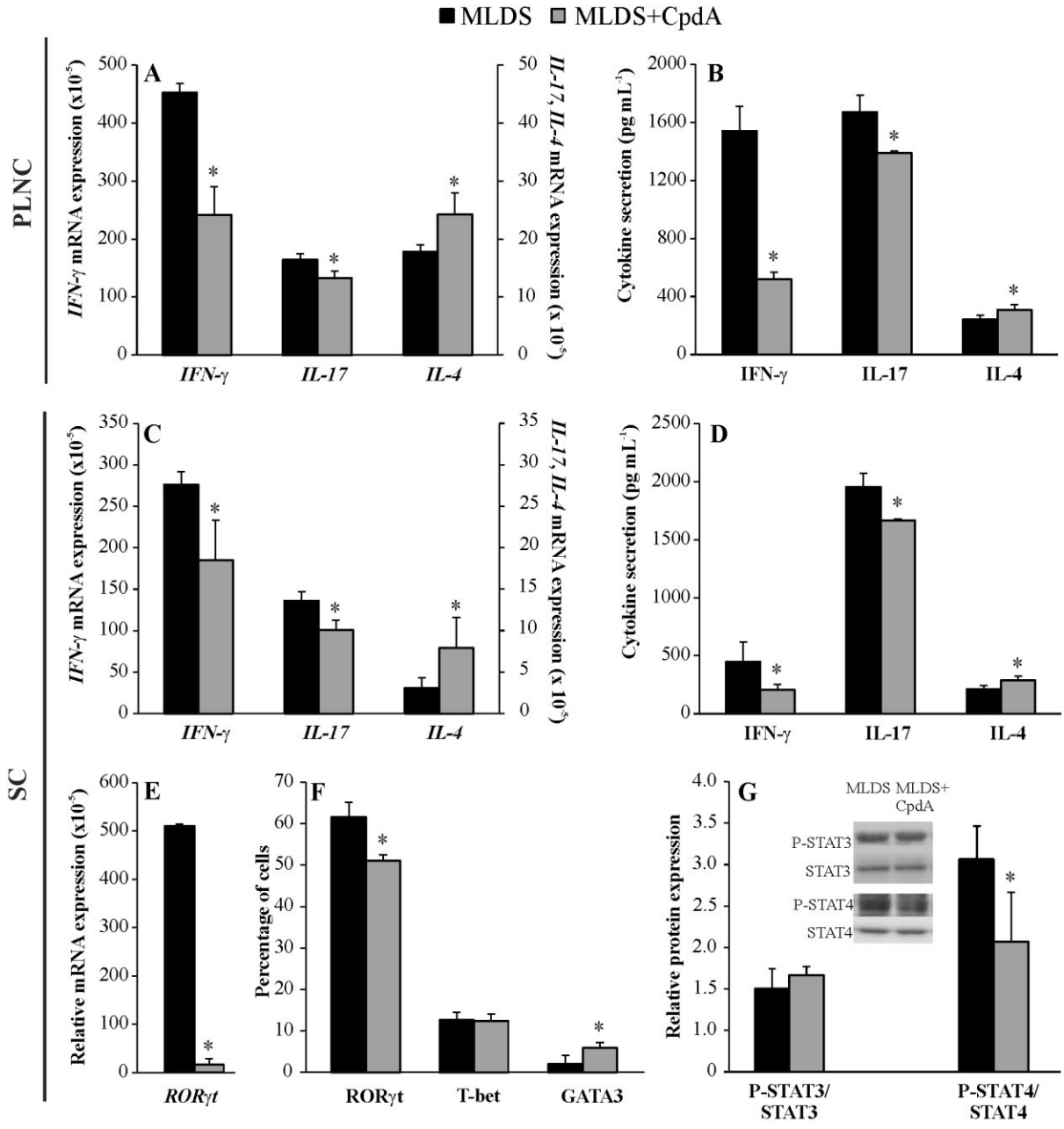

Figure 5

CpdA treatment of MLDS-induced mice shifts the immune response in the spleen and PLNC from proinflammatory Th1/Th17 to Th2 anti-inflammatory response. (A) Cytokine mRNA expression in the PLNC was measured by RT-PCR, as described in Methods and presented relative to *β-actin*. (B) Cytokine concentrations in the 48 h PLNC-derived culture supernatant fractions were determined by ELISA. (C) Cytokine mRNA expression in the SC presented relative to *β-actin*. (D) Cytokine concentrations in the 48 h SC-derived culture supernatant fractions determined by ELISA. (E) *RORγt* mRNA expression in the SC presented relative to *β-actin*. (F) The proportion of RORγt$^+$, T-bet$^+$ and GATA-3$^+$ cells was analysed by flow cytometry from a total SC population. (G) Protein expression of indicated molecules in the SC was determined by immunoblot. Representative blots are shown. Results from one representative out of three experiments with similar results are presented as mean ± SD ($n = 4$ mice per group). *$P < 0.05$ refers to corresponding MLDS group.

single high-dose streptozotocin induced ('toxic') diabetes, pointing to the immune-mediated effects of the drug.

CpdA possesses anti-inflammatory properties via selective transrepression through the glucocorticoid receptor and therefore has been predicted to cause fewer side effects than classical GCs (De Bosscher *et al.*, 2005; 2010; Newton and Holden, 2007; Schäcke *et al.*, 2007). However, very recent data assume that CpdA, due to its differential transcription factor targeting profile, may potentially aggravate an inflammatory response instead of resolving it (De Bosscher *et al.*, 2014). The present study demonstrates that apart from a reduced risk for diabetes induction, CpdA has potent

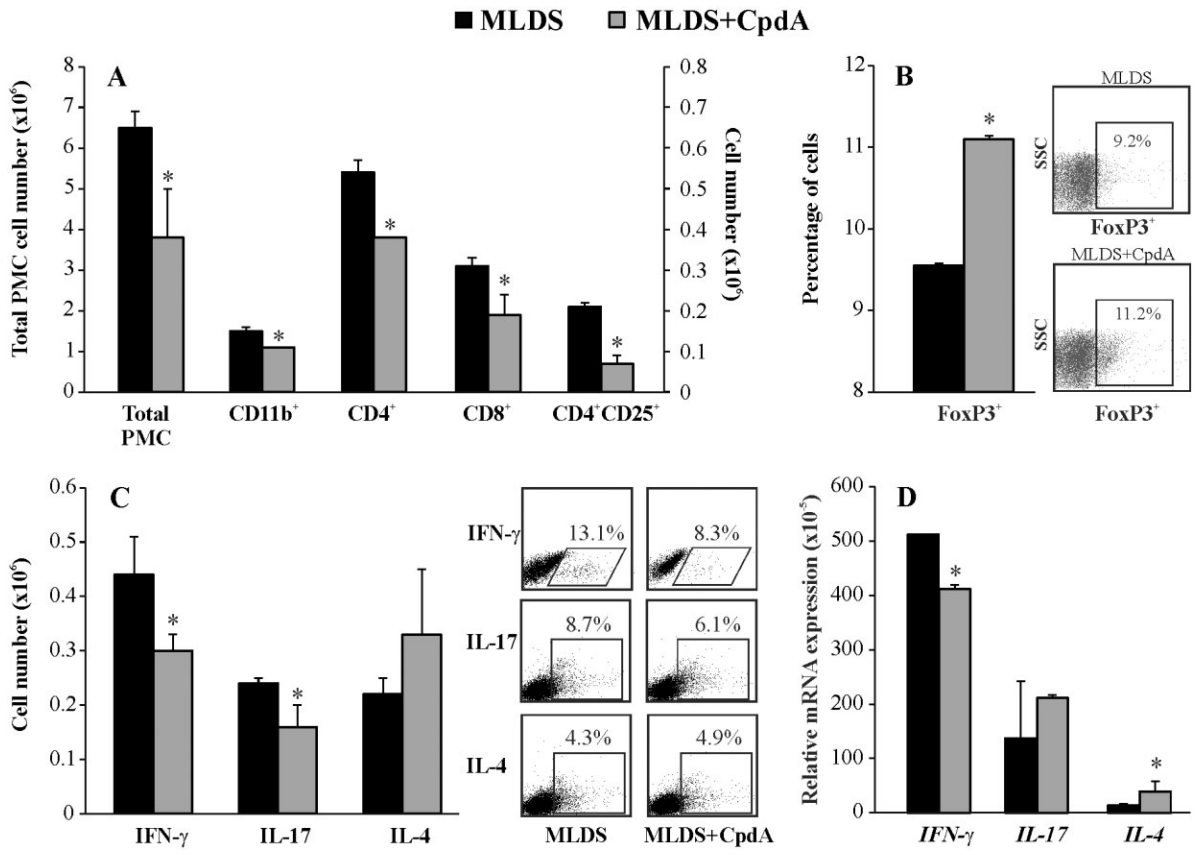

Figure 6

CpdA ameliorates infiltration of pathogenic Th1/Th17 cells into pancreatic islets. PMC were isolated from CpdA-treated or vehicle-treated MLDS-induced mice. (A–C) Cells were counted and analysed *ex vivo* by flow cytometry. Quantitative analysis is expressed as a number of positive cells for each cellular marker indicated (A), as a percentage of Foxp3$^+$ cells (B), or as a number of positive cells for each cytokine indicated (C). Representative flow cytometry profiles of isolated PMC are also presented. (D) Cytokine mRNA expression was measured by RT-PCR and presented relative to *β-actin*. Results from one representative out of three experiments with similar results are presented as mean ± SD ($n = 4$ mice per group). *$P < 0.05$ refers to corresponding MLDS group.

anti-diabetic properties with multi-level immunomodulating characteristics. This makes CpdA an attractive candidate to investigate for future therapeutic applications. However, it needs to be mentioned that the narrow therapeutic window of CpdA hampers its potential use for the treatment of inflammatory disorders in humans due to possible adverse effects that may occur if CpdA is misdosed (Wüst *et al.*, 2009).

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Author contributions

T. S., M. V., I. N., I. S. and S. S. G. performed the experiments and analysed the data. G. H. provided CpdA and edited the manuscript. S. S. G. conceived and designed the study and wrote the paper.

Conflicts of interest

None.

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