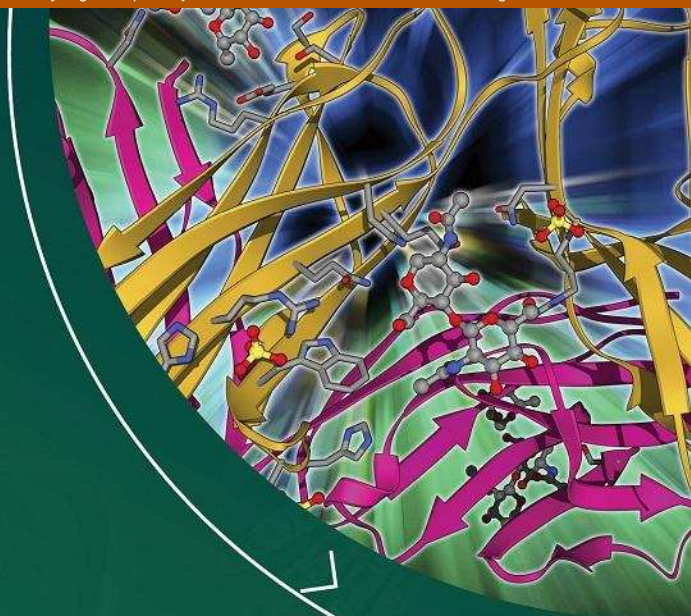
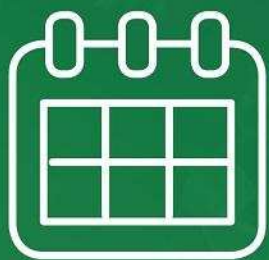


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# Protective effects of carbonyl iron against multiple low-dose streptozotocin-induced diabetes in rodents

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Particulate adjuvants have shown increasing promise as effective, safe, and durable agents for the stimulation of immunity, or alternatively, the suppression of autoimmunity. Here we examined the potential of the adjuvant carbonyl iron (CI) for the modulation of organ-specific autoimmune disease—type 1 diabetes (T1D). T1D was induced by multiple low doses of streptozotocin (MLDS) that initiates beta cell death and triggers immune cell infiltration into the pancreatic islets. The results of this study indicate that the single in vivo application of CI to MLDS-treated DA rats, CBA/H mice, or C57BL/6 mice successfully counteracted the development of insulinitis and hyperglycemia. The protective action was obtained either when CI was applied 7 days before, simultaneously with the first dose of streptozotocin, or 1 day after MLDS treatment. Ex vivo cell analysis of C57BL/6 mice showed that CI treatment reduced the proportion of proinflammatory F4/80<sup>+</sup>CD40<sup>+</sup> M1 macrophages and activated T lymphocytes in the spleen. Moreover, the treatment down-regulated the number of inflammatory CD4<sup>+</sup>IFN- $\gamma$ <sup>+</sup> cells in pancreatic lymph nodes, Peyer's patches, and pancreas-infiltrating mononuclear cells, while simultaneously potentiating proportion of CD4<sup>+</sup>IL17<sup>+</sup> cells. The regulatory arm of the immune system represented by CD3<sup>+</sup>NK1.1<sup>+</sup> (NKT) and CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> regulatory T cells was potentiated after CI treatment. In vitro analysis showed that CI down-regulated CD40 and CD80 expression on dendritic cells thus probably interfering with their antigen-presenting ability. In conclusion, particulate adjuvant CI seems to suppress the activation of the innate immune response, which further affects the adaptive immune response directed toward pancreatic beta cells.

## KEYWORDS

adjuvant, carbonyl iron, T helper 17, T regulatory cells, type 1 diabetes

## 1 | INTRODUCTION

Type 1 diabetes (T1D) is a chronic autoimmune disease characterized by progressive destruction of insulin-producing pancreatic beta cells and hyperglycemia. A variety of immune cells including dendritic cells, macrophages, natural killer (NK) cells, T and B cells have been shown to participate in diabetes pathogenesis (Greeley et al., 2002). Macrophages and dendritic cells (DC) are among the earliest invading cells in

the pancreas and they attract T and B lymphocytes that continue beta cell destruction (Nikolic, Geutskens, van Rooijen, Drexhage, & Leenen, 2005). In the setting of overactive innate immune responses, a therapy that could retune innate immune responses might have beneficial downstream effects on adaptive immunity as well.

Any substance that can enhance specific immune response to co-inoculated antigen is called an adjuvant (Israeli, Agmon-Levin, Blank, & Shoenfeld, 2009). So far, several 100 natural and synthetic compounds

have been identified to have adjuvant activity (Petrovsky & Aguilar, 2004). The use of particulate adjuvants offers an interesting opportunity to enhance, or alternatively suppress the immune responses (Langridge, Dénes, & Fodor, 2010). Several mechanisms of adjuvant-induced immune response have been proposed: the mode of antigen delivery, activation of innate immunity via pattern recognition receptors such as Toll-like (Madan-Lala, Pradhan, & Roy, 2017) or NOD-like receptors (Girardinet al., 2003), increasing the antigen uptake by antigen-presenting cells (APC), altering the cytokine expression by secondary stimulation of APC (Perriconeet al., 2013). As strong activators of immune response, adjuvants' use has been mandatory in the induction protocols of many experimental models of autoimmune diseases, such as experimental autoimmune encephalomyelitis (EAE), neuritis, uveitis, thyroiditis, and orchitis (reviewed in Billau & Matthys, 2001; Stratmann, 2015). However, opposing effects were also observed as exposure to complete Freund's adjuvant (CFA) was shown to suppress the severity of EAE in rats and T1D development in BB rats and NOD mice (Kies & Alvord, 1958; Lee, Qin, Priatel, & Tan, 2008; Sadelain, Qin, Lauzon, & Singh, 1990; Sadelain, Qin, Sumoski, et al., 1990). It has been reported that live *Mycobacterium avium* or bacillus Calmette-Guérin (BCG) can also suppress T1D development in NOD mice (Martins & Aguas, 1999; Yagi et al., 1991). Furthermore, recent clinical studies have revealed that BCG vaccination exerted anti-inflammatory effects in diseases such as multiple sclerosis or T1D (Lagranderie & Guyonvarc'h, 2014; Ristori et al., 2014). Although all of the mentioned adjuvants suppress autoimmune response, their mechanisms of action may vary from inhibition of dendritic cell maturation and autoreactive T cell generation, selective elimination of autoreactive T cells, to stimulation of Th2 cell, secretion of immunosuppressive cytokines, and activation of T regulatory cells (Treg).

Carbonyl iron (CI) is a form of metallic iron characterized by spherical particles. It possesses adjuvant properties as the immunization of rats with CI and spinal cord homogenate (in the absence of bacterial components) promotes the infiltration of granulocytes and NK cells into the CNS and activation of microglia that leads to EAE development (Miljkovic, Momcilovic, Stanojevic, Rasic, & Mostarica-Stojkovic, 2011; Staykova, Liñares, Fordham, Paridaen, & Willenborg, 2008). Interestingly, adjuvant activity was not found in other forms of metallic iron, or other metallic iron suspensions such as iron oxide or iron-dextran (Levine & Sowinski, 1970). However, the potential role of CI as a modulator of organ-specific autoimmunity has not been investigated so far. To define the role of CI in T1D pathogenesis, we have evaluated the impact of this agent in the animal model of T1D, where the disease was induced by multiple low doses of streptozotocin (MLDS) and determined the possible mechanism of CI action.

## 2 | MATERIALS AND METHODS

### 2.1 | Materials and reagents

All chemicals, unless otherwise noted, were obtained from Sigma-Aldrich (St. Louis, MO), and cell culture reagents were from Life Technologies (Grand Island, NY). CI (Sigma-Aldrich, Steinheim, Germany) was prepared as an aqueous suspension.

### 2.2 | Animals and treatment groups

Male C57BL/6 and CBA/H mice at 8–10 weeks of age and male Dark Agouti (DA) rats (6–8 week of age) were bred and kept in the animal facility of the Institute for Biological Research "Sinisa Stankovic," University of Belgrade. Their usage and all experimental procedures were approved by the Ethic Committee at the Institute for Biological Research "Sinisa Stankovic" (App. No 2-27/10-01-189) in accordance with the Directive 2010/63/EU. Rodents were kept in conventional conditions with an underlying 12-hr day/night rhythm and free access to standard rodent chow and water. Animals were randomly assigned to control or CI groups. All groups were comprised of 7–10 animals.

### 2.3 | T1D induction and treatment protocols

Immunoinflammatory T1D was induced in male C57BL/6 and CBA/H mice and DA rats with MLDS (40 mg/kg bw in C57BL/6 and CBA/H, 20 mg/kg bw in DA rats), i.p. for 5 consecutive days as described previously (Stosic-Grujicic et al., 2001). Animals were treated with a single i.p. injection of CI (25 mg/kg bw, treatment group) or PBS as a vehicle (control group). Day 0 was the day of the first streptozotocin (STZ) injection. CI was administered in early prophylactic (7 days before first STZ injection), prophylactic (at the same day as the first STZ injection), and therapeutic (5 days after the first STZ shot) manner. Rodents were monitored for the development of hyperglycemia by weekly measurements of blood glucose levels, using a glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany). Animals were considered hyperglycaemic if their blood glucose level was higher than 10 mmol/l in non-fasted animals. Toxic diabetes was induced in male C57BL/6 mice with a single i.p. injection of high dose of STZ (200 mg/kg bw). Animals were simultaneously treated intraperitoneally with CI (25 mg/kg bw). Glycemia levels were measured 3 days after STZ injection.

### 2.4 | Cell preparation

Ex vivo analysis was performed on MLDS-induced C57BL/6 mice on day 15 of post-diabetes induction and after prophylactic CI treatment. Animals were killed by cervical dislocation and peritoneal cells (PC) were collected by lavage of peritoneal cavity with cold PBS containing heparin (5 U/ml). Single cell suspensions from spleen, pancreatic lymph nodes, Peyer's patches, mesenteric lymph nodes, and pancreas-infiltrating mononuclear cells (PIMC) were obtained as previously described (Nikolic et al., 2014; Stanislavljević et al., 2017).

The obtained cell pellet was re-suspended in fluorescence-activated cell sorting buffer (PBS with 1% BSA, pH 7.4) and  $1 \times 10^6$  cells per sample were used for flow cytometry analysis. Alternatively, spleen cells (SC) were re-suspended in RPMI-1640 medium (with 25 mmol/L HEPES, 2 mmol/L L-glutamine) supplemented with 5% FCS (PAA Chemicals, Pasching, Austria), penicillin/streptomycin and 5  $\mu$ M  $\beta$ -mercaptoethanol and seeded at a density of  $5 \times 10^6$  cells per ml in 24-well culture plates. After 48 hr supernatants were collected and stored at  $-20^\circ\text{C}$  until their analysis for cytokine production.

For in vitro generation of dendritic cells (DC), monocytes were isolated from femur-derived marrow of C57BL/6 mice. Bones were flushed aseptically with RPMI1640 + 10% FBS and cells were centrifuged, erythrocytes lysed with RBC lysis buffer (eBioscience, San Diego, CA) and cells resuspended in RPMI1640 supplemented with 20% FBS, 4 mM L-glutamine and 0.02 mM Na-pyruvate. Obtained cells were cultivated with 20 ng/ml GM-CSF (Peprotech, London, UK) for 7 days with medium change every second day. After 7 days these cells can be designated as immature DC. For gaining mature DC, cells were treated with 100 ng/ml LPS (Sigma-Aldrich) for 24 hr. For flow cytometry analysis,  $1 \times 10^6$  immature DC, mature DC or immature DC stimulated with 100 ng/ml LPS were seeded in 24-well plates and treated with 250  $\mu$ g/ml CI for 24 hr.

CD4<sup>+</sup> T cells were obtained from single cell suspensions of C57BL/6 lymph nodes (cervical, mesenteric, pancreatic) by magnetic separation. Briefly,  $70 \times 10^6$  cells were washed in cold PBS and resuspended in cold PBS 3% FCS with biotin-labelled rat anti-mouse IgG2b $\kappa$  CD4 (eBioscience). After 15 min incubation on ice, cells were washed and resuspended in PBS 0.5% BSA 2 mM EDTA with streptavidin-labeled magnetic beads (BD Biosciences, Franklin Lakes, NJ) for 15 min on +4 °C. After that, cells were exposed to magnetic field and excess solution was removed. Remained cells were washed and resuspended in RPMI1640 + 5% FCS. For detection of IFN- $\gamma$  production,  $2 \times 10^6$  CD4<sup>+</sup> T cells were co-cultivated with  $1 \times 10^6$  mature DC in 24-well plates and treated with 250  $\mu$ g/ml CI for 24 hr, stained and analyzed on flow cytometer. For detection of signaling pathways important for IFN- $\gamma$  production,  $10 \times 10^6$  cells isolated from lymph nodes (cervical, mesenteric, pancreatic) of C57BL/6 mouse were seeded in 24-well plates, stimulated with 10 ng/ml recombinant mouse IL-12 (R&D Systems, Abingdon, UK) and treated with 250  $\mu$ g/ml CI for 48 hr. After that, cells were appropriately stained and analyzed on flow cytometer.

## 2.5 | Histological analysis

To assess the incidence of inflammatory changes and the degree of islet cell destruction, histologic sections of pancreata (5  $\mu$ m thick, at least 200  $\mu$ m between sections) were stained with Mayer's haematoxylin (Bio-Optica) and examined by light microscopy (Zeiss Imager Z1, AXIO, Oberkochen, Germany). Insulinitis scoring was performed by examining at least 20 islets per pancreas and graded in a blinded fashion as follows: grade 0, intact islet; grade 1, peri-islet infiltrate; grade 2, heavy intra-islet infiltrate. Results are expressed as a percentage of graded islets out of the total number of islets.

## 2.6 | Measurement of cytokine levels

Cytokine concentration in 48 hr SC culture supernatants was determined by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine IL-17, IL-4, IL-10, and IFN- $\gamma$  (eBioscience). Absorbance was measured by LKB 5060-006 microplate reader (LKB Instruments, Vienna, Austria) at 450 and 570 nm.

## 2.7 | Assessment of insulin

An ELISA kit (Mercodia, Uppsala, Sweden) was used according to the manufacturer's instructions to determine insulin concentration in the sera of non-fasted mice.

## 2.8 | Measurement of intracellular nitric oxide and nitrite accumulation

4-Amino-5-methylamino-2'-7'-difluoro-fluorescein diacetate (DAF-FM diacetate; Molecular Probes, Leiden, The Netherlands) was used as an indicator of intracellular nitric oxide (NO). Briefly, PC cells were incubated with 2  $\mu$ M DAF-FM diacetate for 1 hr at 37 °C, washed, and then incubated for 15 min at 37 °C in phenol red- and serum-free RPMI-1640 for the completion of de-esterification of intracellular diacetates. The cells were then washed and PBS was added. Green (FL1) fluorescence emission was measured with Partec CyFlow Space and analyzed by FlowMax software (Partec, Görlitz, Germany).

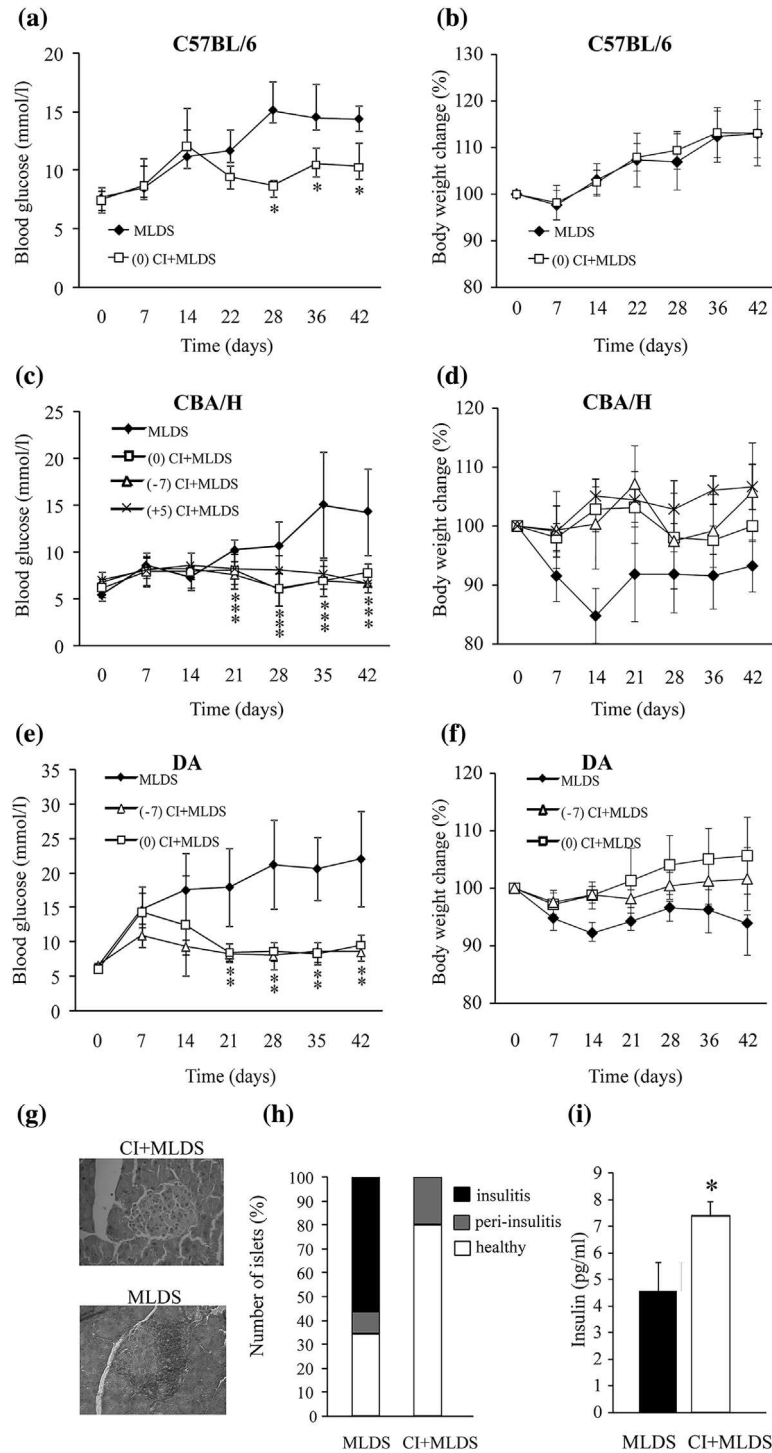
Nitrite accumulation, an indicator of NO release in the supernatant, was detected by Griess reaction as described previously (Stojanovic, Saksida, Nikolic, Nicoletti, & Stosic-Grujicic, 2012).

## 2.9 | Suppressor assay by PC-LNC co-cultivation

PC were isolated on day 15 from MLDS-induced mice treated or not with CI. Each PC suspension was a pool from three to five animals. Cells ( $10^4$  cells/well) were co-cultured with cervical lymph node cells freshly isolated from normal untreated mice (LNC,  $5 \times 10^5$  cells/well), in 96-well flat-bottomed microculture plates in 0.2 ml of standard RPMI-1640 medium in the presence of 1  $\mu$ g/ml concanavalin A (ConA). After 48 hr cell culture supernatants were collected for determination of NO production, while cells were pulsed with 1  $\mu$ Ci [<sup>3</sup>H] thymidine (ICN, Costa Mesa, CA) for another 18 hr for determination of cell proliferation. Incorporated radioactivity in triplicate cultures was measured in a liquid scintillation counter (Beckman Coulter, Fullerton, CA). The magnitude of the proliferative response was expressed as mean counts per minute (cpm) in triplicate co-cultures  $\pm$  SD.

## 2.10 | Immunofluorescence analysis

The phenotype of SC, PLN, PIMC, and PP, as well as the phenotype of pooled lymph nodes and bone marrow-derived dendritic cells in in vitro experiments was assessed by flow cytometry using the anti-mouse antibodies specific for rat anti-mouse IgG2a F4/80 FITC (eBioscience), Armenian hamster anti-mouse IgM CD40 APC (eBioscience); goat anti-mouse IgG CD206-PE (R&D), mouse anti-mouse IgG NK1.1 (BD Pharmingen, Franklin Lakes, NJ), Armenian hamster anti-mouse IgG CD3 PE, CD80 PE-Cyanine 5, CD11c PE-Cyanine 5 (eBioscience), mouse anti-mouse IgG2b $\kappa$  CD86 PE-Cyanine 5 (eBioscience), rat anti-mouse IgG2a CD4 FITC (eBioscience), rat anti-mouse IgG2b $\kappa$  MHC II FITC (eBioscience), rat anti-mouse IgG1 $\lambda$  CD25 PE, rat anti-mouse IgG2 $\kappa$  FoxP3 PE-Cyanine 5 (eBioscience). Treg were detected by the Mouse Regulatory T cell Staining Kit, according to the manufacturer's instructions (eBioscience). Prior to intracellular cytokine staining, cells were stimulated



**FIGURE 1** Effects of CI on T1D clinical and histological parameters in different rodent species. (a) Blood glucose of male C57BL/6 mice injected i.p. for 5 consecutive days with MLDS (40 mg/kg/day) (◆) and treated with single i.p. injection of CI (25 mg/kg) concomitantly with the first STZ (□). (b) Body weight of C57BL/6 after T1D induction. (c) Blood glucose of male CBA/H mice injected i.p. for 5 consecutive days with MLDS (40 mg/kg/day) (◆) and treated with single i.p. injection of CI (25 mg/kg) given either 7 days before (△), concomitantly with the first STZ (□), or 5 days after the first STZ (×). (d) Body weight of CBA/H mice after T1D induction. (e) Blood glucose of male DA rats injected i.p. for 5 consecutive days with MLDS (20 mg/kg/day) (◆) and treated with single i.p. injection of CI (25 mg/kg) 7 days before (△), or concomitantly with first STZ (□). (f) Body weight of DA rats after T1D induction. (g) Representative examples of the light microscopic evaluation of the pancreatic islets. (h) Quantitative histology score of pancreatic islets immune cell infiltrations at day 42 after disease induction. Grade 0 (white bars) represents islets with well-preserved morphology and without mononuclear cell infiltrates; Grade 1 (grey bars) represents moderate mononuclear cell infiltrates. Grade 2 (black bars) represents islets with abundant mononuclear cell infiltrates, necrosis, atrophy, and loss of islet margins. (i) Serum insulin level was measured at day 42 after T1D induction. Results from one representative out of three experiments are presented as mean ± SD ( $n = 7-10$  animals per treatment). \* $p < 0.05$  refers to corresponding MLDS animals

with phorbol myristate acetate (PMA, 100 ng/ml) and ionomycin (400 ng/ml) (both from Sigma–Aldrich) in the presence of Brefeldin A (eBioscience) (5  $\mu$ M) for 4 hr, stained with anti-CD4 antibody, fixed in 2% paraformaldehyde, permeabilized with Permeabilization buffer (eBioscience) and then stained for the intracellular cytokines with the following antibodies: rat anti-mouse IgG1 $\kappa$  IFN- $\gamma$  PE, IgG1  $\kappa$  IL-4 PE, IgG1 $\kappa$  IL-17 PE (BD Biosciences). Cells were also stained for transcriptional factors with the following antibodies: mouse anti-mouse IgG1 $\kappa$  Tbet PE, rabbit anti-mouse IgG p-ERK, ERK, p-IkB- $\alpha$ , I kB- $\alpha$ , p-p38 (Cell Signaling Technology, Boston, MA), followed by incubation with biotin-linked goat anti-rabbit IgG (Sigma–Aldrich), and subsequent incubation with FITC-streptavidin (BioLegend, San Diego, CA). Isotype-matched controls were included in all experiments (eBioscience). Stained cells were detected on Partec CyFlow Space and analyzed by FlowMax software (Partec, Görlitz, Germany). Analysis was performed only on live cells that were gated on FSC versus SSC plot. All other gating strategies are explained in Figure legends.

## 2.11 | Statistical analysis

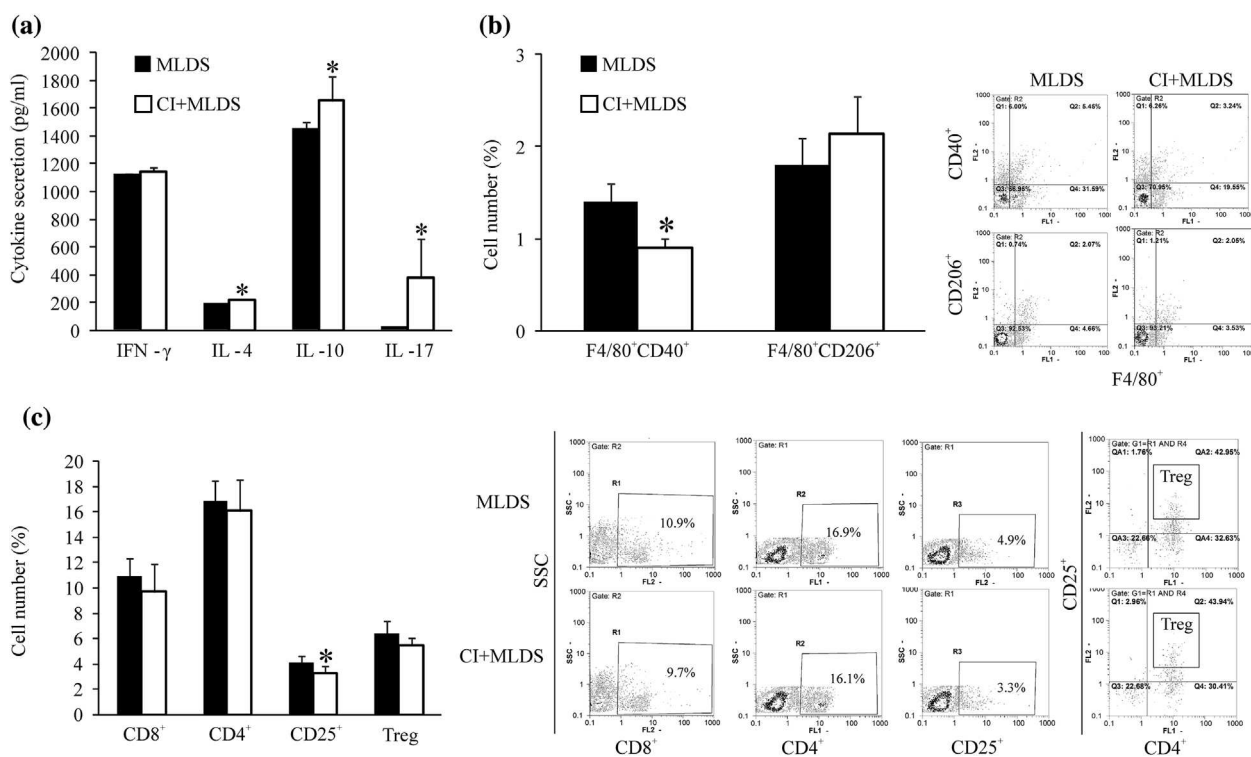
Data are presented as means  $\pm$  SD. Statistical analysis was performed using Statistica 6.0 (StatSoft Inc., Tulsa, OK) software. Statistical comparisons were made by one-way ANOVA followed by Student–Newman–Keuls test

for multiple comparisons or by Student's *t*-test (two tailed), as appropriate. *p* value less than 0.05 was considered to be statistically significant.

## 3 | RESULTS

### 3.1 | CI reduces clinical parameters of MLDS-induced diabetes

To investigate the effect of adjuvant CI on the development of murine T lymphocyte-dependent diabetes, C57BL/6 mice susceptible to diabetes induction were inoculated with CI given as a single intraperitoneal injection at a start of diabetes induction with MLDS. Strikingly, whereas blood glucose level in control MLDS-induced mice gradually increased, in mice inoculated with CI after the initial rise, glucose level rapidly decreased resulting in the restoration of euglycaemia (Figure. 1a). The suppressive effect of the CI treatment has been maintained for at least an additional month (data not shown) and seemingly, CI treatment was well tolerated judging by animal behavior, body weight gain (Figure 1b), normal food, and water consumption and general appearance. Since the disease may differ in some aspects in individual inbred strains, CBA/H mice, which have lower basal blood glucose level in comparison to C57BL/6 mice, were examined as well. In order to avoid interference with STZ, CI was injected either 7 days before the first (pre-treatment), or 1 day after the last STZ injection (post-treatment).



**FIGURE 2** Effects of CI on the cellular changes in the spleen induced by MLDS in C57BL/6 mice. (a) Ex vivo cytokine secretion from SC isolated from MLDS and CI + MLDS mice determined by ELISA. (b) Percentage of pro-inflammatory F4/80<sup>+</sup>CD40<sup>+</sup> and anti-inflammatory F4/80<sup>+</sup>CD206<sup>+</sup> macrophages within spleen in MLDS and CI + MLDS treated animals measured by flow cytometry. Only live cells were gated and subjected to analysis. (c) Distribution of T cells within spleen of mice treated with MLDS or CI + MLDS measured by flow cytometry. Live cells were gated. Treg cells were defined as FoxP3<sup>+</sup> cells within CD4<sup>+</sup>CD25<sup>high</sup> cells. Representative dot plots are displayed along with graphs. Results from one representative out of three experiments are presented as mean  $\pm$  SD (*n* = 7–10 animals per treatment). \**p* < 0.05 refers to corresponding MLDS animals

Inoculation of CI (irrespective of delivery time) to CBA/H mice provided a measurable level of protection (Figure 1c) similar to that obtained in C57BL/6 mice. Also, CI treatment did not interfere with the weight gain of the animals (Figure 1d). Because rats are more sensitive than mice cells to diabetogenic doses of streptozotocin (Stosic-Grujicic et al., 2001), the effect of CI in DA rats was further investigated. As in the mouse, a significant suppressive effect had been observed and was similar whether CI treatment started before, or simultaneously with MLDS application (Figure 1e). In addition, body weight gain was not affected by CI treatment (Figure 1f). Thus, in both rat and mouse models of MLDS T1D, the protective effect of single dose of CI was substantial and long-lasting with limited variations of blood glucose levels throughout the entire 42-days follow-up period.

To prove that CI interfered with autoimmune process during T1D development and not with glycemia per se, CI was administered to C57BL/6 mice that received toxic dose of STZ (200 mg/kg bw). Indeed, mice in both control group (treated with STZ + PBS) and in STZ + CI-treated group developed hyperglycemia 72 hr after STZ injection ( $21.9 \pm 3.4$  vs.  $21.1 \pm 10.2$  mmol/L, respectively,  $p = 0.876$ ), indicating that CI was unable to alter glycemia in animals with completely destroyed pancreatic islets.

### 3.2 | CI treatment prevents insulinitis and preserves insulin production in MLDS-treated C57BL/6 mice

Consistent with the clinical findings, pathohistological analysis of the pancreata of CI-treated and vehicle-treated diabetic mice revealed substantial differences. In contrast to islets of vehicle-treated diabetic mice that exhibited varying degrees of mononuclear cell infiltration (insulinitis) and progressively lost clear margins due to beta cell destruction, the majority of pancreatic islets from CI-treated animals appeared normal, with little or no signs of inflammation or destruction (Figure 1g). Quantitative analysis of mononuclear cell infiltration of pancreas performed at the end of the study (42 days post-MLDS) revealed that CI-treated mice had significantly increased grade 0 (normal) and significantly decreased grade 2 islets (with insulinitis) compared with control diabetic mice (Figure 1h). Notably, CI appeared to preserve not only the number, but also the function of pancreatic beta cells, judging by the higher systemic insulin concentrations in the sera of CI-treated compared with vehicle-treated MLDS mice at day 42 after T1D induction (Figure 1i). Overall, these data indicated that CI treatment preserved islets from autoimmune attack, thus enabling appropriate regulation of plasma glucose level.

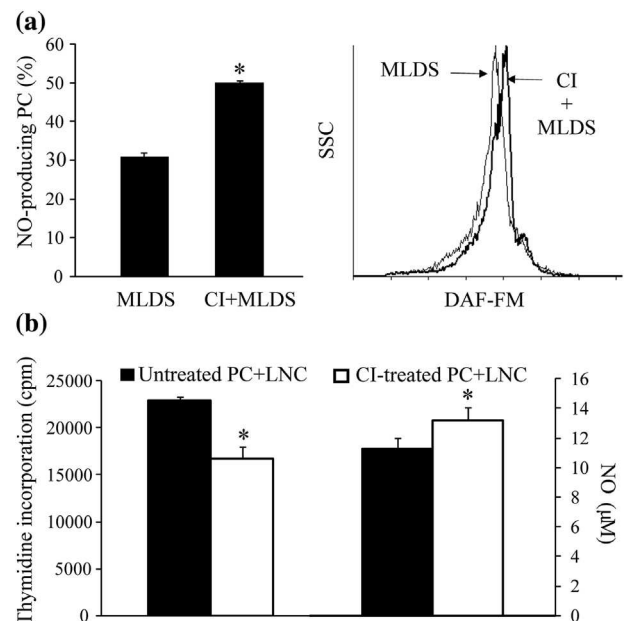
### 3.3 | The effect of CI treatment on the immune response in the spleen

To elucidate the mechanisms by which systemic CI treatment prevents disease onset when given simultaneously with the disease induction, ex vivo studies were performed on peripheral lymphoid organs of C57BL/6 mice during early progression of the MLDS-induced diabetes (15 days after diabetes induction). Spleens were collected from CI-treated or vehicle-treated group of mice, and cytokine release was assayed ex vivo without further stimulation. The data summarized in Figure 2a clearly show that SC isolated from CI-treated mice released increased amounts

of anti-inflammatory cytokines IL-4 and IL-10, compared with control diabetic animals. Interestingly, while secretion of pro-inflammatory IFN- $\gamma$  was not changed after CI treatment, IL-17 secretion was up-regulated (Figure 2a). Phenotype analysis of splenocytes (Figure 2b) showed that CI significantly reduced the percentage of F4/80<sup>+</sup>CD40<sup>+</sup> (pro-inflammatory M1) macrophages, while the proportion of the alternatively activated CD206<sup>+</sup> (M2 polarized) macrophages was not significantly changed. Although the proportion of M1 was down-regulated, their production of pro-inflammatory cytokines remained the same as in MLDS-derived splenic macrophages (MLDS vs. MLDS + CI: IL-1 $\beta$ – $91 \pm 23$  pg/ml vs.  $99 \pm 18$  pg/ml, TNF– $2309 \pm 76$  pg/ml vs.  $2200 \pm 110$  pg/ml, and IL-6– $1904 \pm 148$  pg/ml vs.  $1951 \pm 92$  pg/ml). Also, while CI did not affect the relative sizes of both CD4<sup>+</sup> and CD8<sup>+</sup>T cell populations, as well as the proportion of cells with Treg markers (FoxP3<sup>+</sup> cells within CD4<sup>+</sup>CD25<sup>high</sup> cell population), it produced a significant decrease in the number of activated CD4<sup>+</sup>CD25<sup>low</sup> T cells (Figure 2c).

### 3.4 | CI treatment induces in vitro NO-mediated suppressor function of peritoneal cells

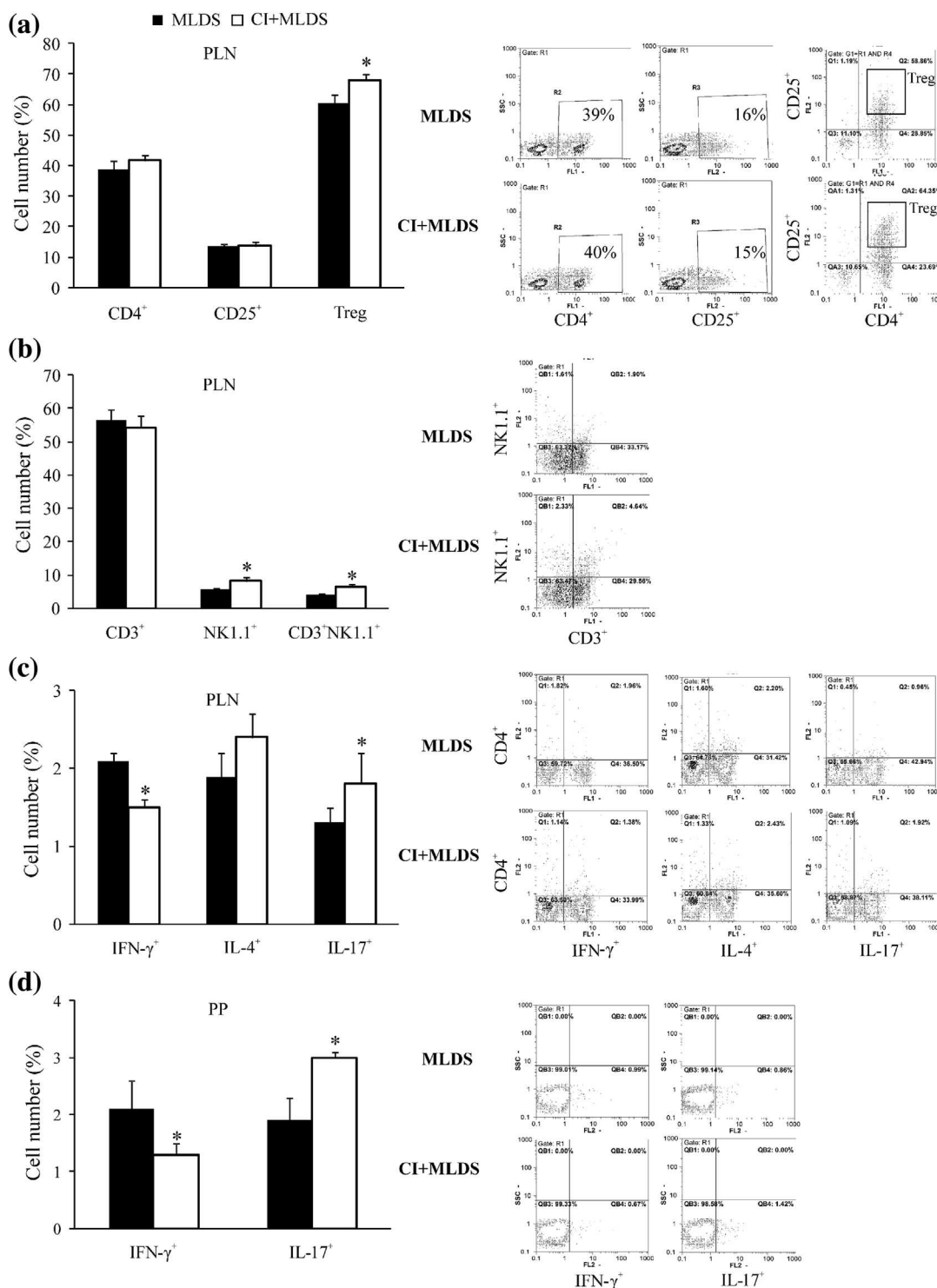
Since our results indicated that CI lowers the number of pro-inflammatory macrophages in the spleen, we further investigated



**FIGURE 3** Effects of CI treatment on PC function of diabetic C57BL/6 mice. (a) Intracellular NO production in PC of MLDS-treated or CI + MLDS-treated C57BL/6 mice measured by flow cytometry. Representative dot plot is shown on the right hand side. (b)  $1 \times 10^4$  PC cells from MLDS or CI + MLDS-treated C57BL/6 mice were co-cultured with  $5 \times 10^5$  untreated LNC in the presence of  $1 \mu\text{g/ml}$  ConA. Cell proliferative response was measured by [ $^3\text{H}$ ] thymidine incorporation after 72 hr (left) and NO release into 48 hr co-culture supernatants was measured by Griess reaction (right). Results from one representative out of three experiments are presented as mean  $\pm$  SD ( $n = 7$ –10 animals per treatment). \* $p < 0.05$  refers to corresponding MLDS-treated animals

the effect of CI on the peripheral immune cells in other compartments. Results showed that macrophages from peritoneal cavity of CI-treated MLDS-induced mice contained significantly more intracellular NO in comparison with cells from control animals

(Figure 3a). To examine whether CI-induced up-regulation of peripheral NO production influences T cell activation, we performed a suppression assay of the conventional T cell response to a mitogen. To do this, PC isolated from CI-treated or control

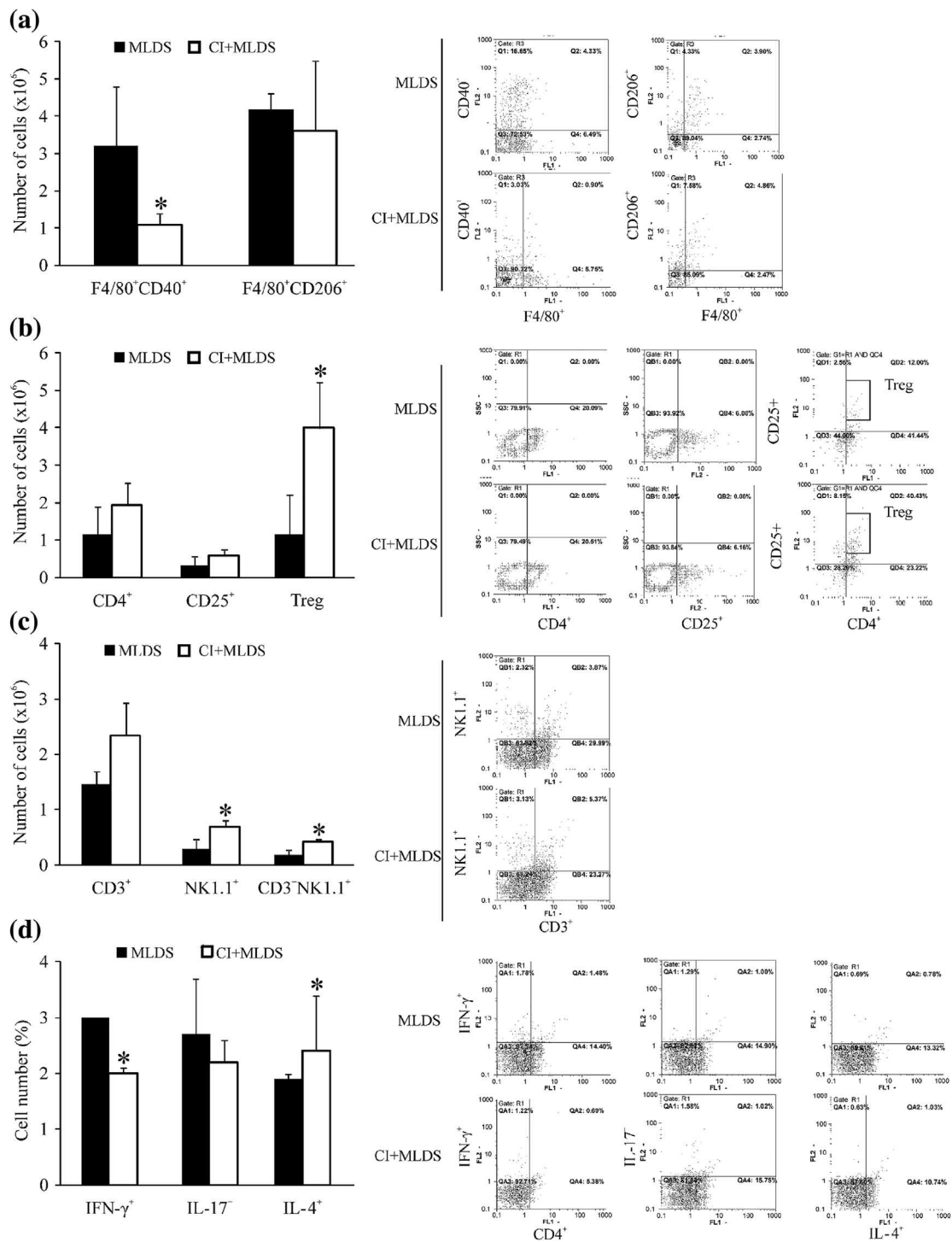


**FIGURE 4** CI treatment of MLDS-induced C57BL/6 mice alters immune cell composition of PLN and PP. (a–c) Distribution of T cells within PLN of MLDS and CI + MLDS treated C57BL/6 mice measured by flow cytometry. (d) Percentage of IFN- $\gamma$ <sup>+</sup> and IL-17<sup>+</sup> PP cells isolated from MLDS and CI + MLDS-treated C57BL/6 mice measured by flow cytometry. Live cells were gated. Representative dot plots are displayed along with the graphs. Results from one representative out of three experiments are presented as mean  $\pm$  SD ( $n = 7$ –10 animals per treatment). \* $p < 0.05$  refers to corresponding MLDS-treated animals



MLDS-induced mice were co-cultured with ConA-stimulated lymphocytes derived from control C57BL/6 mice. As shown in Figure 3b, LNC proliferative response to mitogen was lower in the presence of CI-treated PC when compared to vehicle-treated PC.

In contrast, CI-derived PC released higher concentrations of NO than cells isolated from vehicle-treated mice suggesting that lowered T cell proliferation was a consequence of the active suppression by CI-induced macrophage-derived NO.



**FIGURE 5** CI treatment suppresses pro-inflammatory response in pancreatic mononuclear infiltrates of MLDS-induced C57BL/6 mice. (a) Number of M1 and M2 macrophages within PMI isolated from MLDS and CI + MLDS-treated mice assessed by flow cytometry. (b and c) Distribution of T cells within PMI of MLDS and CI + MLDS treated cells measured by flow cytometry. (d) Proportion of IFN- $\gamma$ <sup>+</sup>, IL-4<sup>+</sup>, and IL-17<sup>+</sup> cells within PMI of MLDS and CI + MLDS treated mice measured by flow cytometry. Live cells were gated. Representative dot plots are displayed along with the graphs. Results from one representative out of three experiments are presented as mean  $\pm$  SD ( $n = 7-10$  animals per treatment). \* $p < 0.05$  refers to corresponding MLDS-treated animals

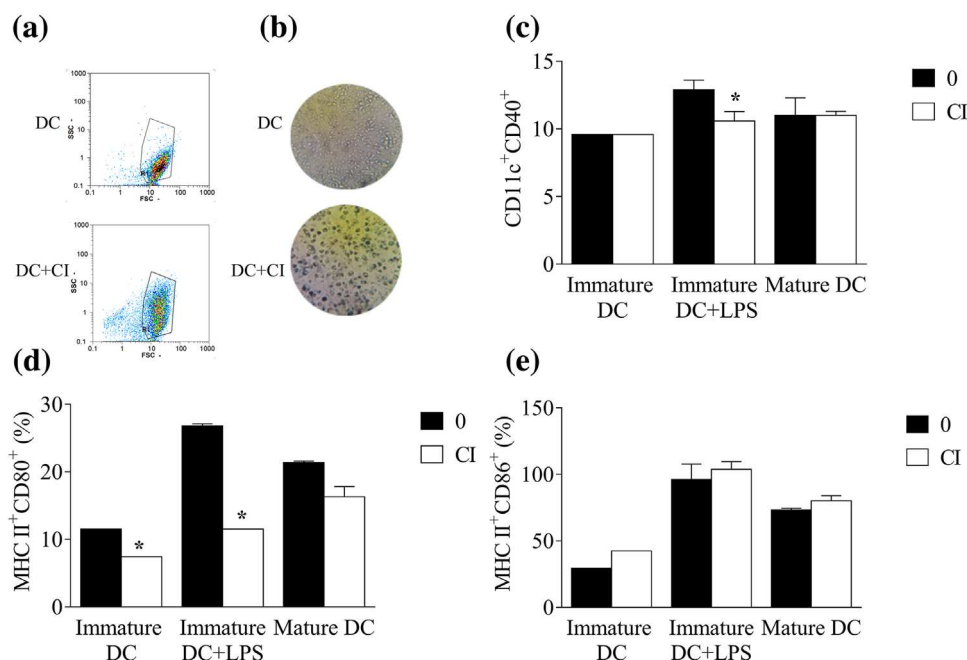
### 3.5 | CI modulates cell phenotype in pancreatic lymph nodes and Peyer's patches

Since the initial events at the level of lymph nodes draining the target organ might have the major influence on the disease output, we analyzed different immune cell subsets in pancreatic lymph nodes. To determine the mechanism of suppression mediated by CI, we initially examined the possible role of Treg cells in conferring protection from T1D. Flow cytometry analysis revealed that CI treatment significantly increased the number of FoxP3<sup>+</sup> cells within CD4<sup>+</sup>CD25<sup>high</sup> T lymphocytes, without changing the proportion of CD4<sup>+</sup>T cells (Figure 4a). Next we analyzed another possible subset of cells that might be involved in protection from T1D—NKT cells, which are heterogeneous subset of T cells that show both properties of T cells and that of natural killer cells (NK) (Lehuen et al., 1998). The results indicated that CI treatment significantly elevated number of NKT cells in PLN compared to control MLDS-treated mice (Figure 4b). We also analyzed CD4<sup>+</sup> cell subsets Th1, Th2, and Th17 cells that produce signature cytokines IFN- $\gamma$ , IL-4, and IL-17, respectively. The data from these experiments suggest that the treatment of mice with CI antagonized the pro-inflammatory response (IFN- $\gamma$ ), while it had no effect on anti-inflammatory (IL-4) response, at least in the PLN (Figure 4c). Interestingly, CI treatment significantly elevated the number of Th17 cells (Figure 4c). Similar effects of CI were observed in gut-associated lymphoid tissue, Peyer's patches, where the treatment of MLDS-treated mice with CI significantly decreased the number of IFN- $\gamma$  producing cells which was accompanied with significant increase

in IL-17-producing cells (Figure 4d). Together, these data indicate protective role of CI in T1D development through the shift from pro-inflammatory Th1 towards protective Treg/NKT response.

### 3.6 | CI treatment inhibits infiltration of immune cells and suppresses pro-inflammatory response in the pancreas

As infiltration of multiple immune cells into islets (insulinitis) is the major T1D trait in both mice and humans (Eizirik, Colli, & Ortis, 2009), we performed flow cytometry analysis of PIMC. Composition of infiltrates clearly showed that CI treatment prevented islet destruction by modulating both innate and adaptive immunity towards protective anti-inflammatory response in the target tissue. Number of M1 macrophages, key drivers of disease activity in the effector phase of T1D, was significantly decreased in CI treated mice (Figure 5a). Also, CI treatment altered the composition of T cells, with significant increase in the number of protective Tregs and NKT cells within the pancreatic infiltrates (Figures 5b and 5c). We further analyzed PIMC for the production of IFN- $\gamma$ , IL-4, and IL-17 and showed that CI treatment significantly decreased number of pro-inflammatory IFN- $\gamma$ -producing cells with simultaneous elevation in the number of anti-inflammatory IL-4-producing cells, while it had no significant effect on the number of IL-17-producing cells (Figure 5d). Therefore, CI treatment successfully suppressed pro-inflammatory response in target tissue by skewing it towards protective Treg/Th2/NKT response.



**FIGURE 6** The effect of CI on the expression of co-stimulatory molecules on immature and mature dendritic cells. Flow cytometry FSC versus SSC plots (a) and light microscopy image (b) showing C57BL/6 bone marrow-derived dendritic cells (DC) that engulfed CI after 24 hr *in vitro* incubation. Proportion of immature DC, mature DC, and immature DC ( $1 \times 10^6$  cells) stimulated with LPS (100 ng/ml) that express CD11c (marker for dendritic cells) and CD40 (c) MHC class II and CD80 (d) and MHC class II and CD86 (e) in the presence or absence of CI (250  $\mu$ g/ml), measured by flow cytometry. Live cells were gated. Results from one representative out of three experiments are presented as mean  $\pm$  SD. \* $p < 0.05$  refers to untreated DC

In addition to the down-regulatory effect of CI on the number of IFN- $\gamma$ -producing cells *in vivo*, *in vitro* results indicate that CI affected IFN- $\gamma$  expression per cell (Supplementary Figure S1A) and that this effect was probably mediated by the reduction of Tbet (key transcription factor for IFN- $\gamma$ ) (Supplementary Figure S1B) and NF- $\kappa$ B expression (Supplementary Figure S1C). Seemingly, p38 (Supplementary Figure S1D) and ERK mitogen-activated protein kinase (Supplementary Figure S1E) were not involved in the observed IFN- $\gamma$  reduction exerted by CI. These results suggest that CI interfered with IFN- $\gamma$  production through reduction of transcription factors relevant for optimal expression of this cytokine.

### 3.7 | CI down-regulates expression of co-stimulatory molecule on dendritic cells *in vitro*

The obtained results indicate that CI modulated the T cell-mediated immune response during T1D pathogenesis. However, it is reasonable to assume that CI acts first on phagocytic cells due to its particulate nature. To investigate whether CI exerts direct effect on maturation and DC function, we have treated DC *in vitro* with 250  $\mu$ g/ml CI at different time points during the maturation process. As deduced by flow cytometry FSC vs SSC plots (Figure 6a) or light microscopy (Figure 6b), DC phagocytized CI after 24 hr of incubation. Results indicate that CI did not affect the expression of co-stimulatory molecules in immature DC (Figure 6c–e). However, it significantly interfered with the DC maturation process (LPS-provoked) regarding CD40 and CD80 co-stimulatory molecules expression (Figures 6c and 6d), while the expression of CD86 remained the same as before CI treatment (Figure 6e). Mature DC were more resistant to CI-mediated effect since only CD80 expression was reduced (Figure 6d). These results indicate that CI might interfere with DC maturation and subsequent stimulation of T cells.

## 4 | DISCUSSION

This study shows that the intraperitoneal administration of CI suppresses T1D development in MLDS-treated C57BL/6 mice. The observed effect of CI on T1D was neither strain nor species restricted, judging by its ability to suppress hyperglycemia in both CBA/H mice and DA rats. CI exerted its immunomodulatory activity through modulation of macrophage activity and by boosting the regulatory arm of the immune response.

The propensity of adjuvants to block autoimmunity has been already shown in many cases (Kies & Alvord, 1958; Lee et al., 2008; Sadelain, Qin, Lauzon, et al., 1990; Sadelain, Qin, Sumoski, et al., 1990). In this study, a single injection of CI (administered alone) provided long-lasting protection from MLDS-triggered T1D in animals. Similar results were obtained with single CFA injection given to NOD mice (Lee et al., 2008; Tian et al., 2009). Compared to these studies that hypothesize the inhibitory effect of the adjuvant on autoreactive cytotoxic T cells, NK, and NKT, we assumed that CI acts first on phagocytic cells. Indeed, our study showed profound effect of CI on macrophage polarization and suppressor function, in terms of lowering the proportion of pro-inflammatory M1 macrophages in spleen and pancreatic infiltrates, and by enhancing their capacity to suppress T cell proliferation *in vitro*. The influence of the iron on favoring macrophage anti-inflammatory

phenotype was shown in the other study performed by Corna et al. (2010). Decrease in M1 macrophages after CI treatment probably led to a reduction in the number of activated T cells (CD4<sup>+</sup>CD25<sup>low</sup>) observed in the spleen. This effect might be mediated by NO that is produced by the remaining M1 macrophages. NO was already shown to exhibit direct cytostatic effects on T cells and APC or through depletion of L-arginine during the iNOS reaction (Bingisser, Tilbrook, Holt, & Kees, 1998; Bogdan, 2011). NO can preferentially down-regulate Th1-type cytokines resulting in a Th2 bias (Taylor-Robinson, 1997). Also, the CI-mediated interference with IL-12 production by phagocytes could also lead to less Th1 reactivity in the presence of NO (Mattner et al., 1993). Our *in vitro* experiments confirm that NO might interfere with T cell-mediated response since macrophages from CI-treated animals reduced ConA-stimulated T cell proliferation and thus diminished clonal size of autoreactive T cells. In addition to macrophages, dendritic cells might also be the primary target for CI. *In vitro* experiments confirmed that dendritic cells engulfed CI and consequently reduced the expression of co-stimulatory molecules CD40 and CD80. Therefore, the potential of dendritic cells to activate autoreactive T cells was significantly down-regulated (at least observed *in vitro*) or they had become tolerogenic under persistent signaling provided by the presence of CI (Liu & Cao, 2015).

Different T cell subsets have different roles in T1D pathogenesis. Th1 and Th17 lymphocytes are thought to be generally involved in the promotion of the disease, while Th2 and Treg cells suppress T1D development (Bluestone, Herold, & Eisenbarth, 2010). Inhibition of Th1 signature cytokine IFN- $\gamma$  by CI found in regional lymphoid tissues PLN and PP, as well as in the pancreas seems to be a valid explanation for the beneficial effect of the CI in T1D. This is in line with the animal studies that used specific IFN- $\gamma$  inhibitors for the therapy of autoimmune diabetes induced in DP-BB rat or C57BL/6 mice or NOD mice (Cockfield, Ramassar, Urmsen, & Halloran, 1989; Nicoletti et al., 1996, 1997). Interesting finding in this study was that mice treated with CI had elevated number of Th17 cells in both PLN and PP. This could also be a positive outcome of CI treatment since it was shown that in some settings Th17 protected animals against T1D (Candon et al., 2015; Kriegel et al., 2011). Furthermore, IL-17 silencing did not prevent T1D development in NOD mice (Joseph, Bittner, Kaiser, Wiendl, & Kissler, 2012) again suggesting that Th17 cells might not be involved in T1D pathogenesis or even might exert protective role. In support to this, it was reported that the rise in the level of IL-17 after adjuvant therapy in NOD mice has a protective effect on T1D (Nikoopour et al., 2010). However, the role for IL-17 in T1D is still controversial as other data suggest that therapeutic effects of adjuvant treatment may be partially due to suppressing Th17 commitment (Gao et al., 2010), and that blockade of IL-17 may prevent autoimmune diabetogenesis (Kuriya et al., 2013). The regulatory role of Th17 is especially true within the intestine where these cells participate in the maintenance of intestinal homeostasis rather than inducing inflammation (Zhao et al., 2017). It has been shown recently that both pathogenic Th17 cells and non-pathogenic regulatory Treg17 cells can be generated from CD4<sup>+</sup> T cells under appropriate polarization conditions (Bellemore et al., 2015; Volchenkov, Nygaard, Sener, & Steen Skålhegg, 2017). These studies support our findings that not all Th17 cells are pathogenic or their up-regulation in the gut immune

system is merely a reaction to a change in microbiota composition that occurs during T1D development (Costa et al., 2016).

Inducible Tregs develop from CD4<sup>+</sup>T cells in the lymphoid tissues in response to specific antigens in the presence of TGFβ and IL-10 and in the absence of pro-inflammatory cytokines such as IFN-γ, IL-1, IL-6, and IL-12 (Vignali, Collison, & Workman, 2008). Present study showed that CI treatment provoked the increment of CD4<sup>+</sup>Foxp3<sup>+</sup> T cells frequency and secretion of their signature cytokine IL-10. Again, the possibility that CI stimulated DC tolerogenic phenotype might be one of the reasons for increased regulatory T cell development. These data suggest that CI prevents the onset of hyperglycemia in mice through the suppression of effector T cells function by the regulatory arm of the immunity.

Deficiency in NKT contributes to the pathogenesis of autoimmune diabetes in NOD mice as they are able to suppress the development of autoreactive T cells (Yang, Bao, & Yoon, 2001). NKT produce various cytokines, such as IL-4, IFN-γ, IL-17, and IL-10 (Novak & Lehuen, 2011). Our study showed that CI treatment elevated number of NKT cells in PLN and PIMC. It is therefore intriguing to speculate that treatment by CI favors protective cytokine secretion profiles (Th2 inclined) in NKT cells. Also, observed elevated IL-17 production could stem from iNKT cells (Cua & Tato, 2010) and therefore could be utilized to suppress the adaptive immunity. This is not in accordance with adjuvant effect of CI observed in EAE model where the CI treatment induced EAE by expanding NK and NKT cell population (Miljkovic et al., 2011). Different CI effects in vivo could reflect differences between disease models or differences in the CI delivery route.

In conclusion, we have provided evidence that CI is effective inhibitor of T1D development. This inhibitory effect might be due, at least in part, to the suppression of innate immune components that could lead to impaired activation and proliferation of T lymphocytes (especially Th1). On the other side, there is a substantial increase in the regulatory arm of the immune system after CI treatment. This notion may have therapeutic implications not only for T1D, but also for other autoimmune diseases with Treg defects, suggesting that CI may serve as a useful therapeutic for reducing inflammation and ameliorating autoimmunity.

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## CONFLICTS OF INTEREST

Author declare no conflict of interest.

## AUTHORS' CONTRIBUTIONS

SSG and MMS initiated the study; MV, TS, IS, ND, and SSG conducted the experiments, analyzed the results, and drafted the manuscript; all the authors reviewed the manuscript.

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