Adoptive transfer of CTLA4-Ig-modulated dendritic cells improves TNBS-induced colitis

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32 **Running title: CTLA4-Ig-modulated dendritic cell ameliorates experimental colitis**

33

34 **Abstract**

35 Dendritic cells (DCs) play a crucial role in balancing immune responses, and in that
 36 sense the interactions between the B7-1 and B7-2 molecules expressed on DCs and
 37 CD28 and CTLA-4 on helper T cells are fundamental. While coupling of B7 and CD28
 38 molecules activates immune responses, binding of B7 to CTLA4 results in its blockade.
 39 CTLA4-Ig fusion protein, a competitor molecule of the B7-CD28 interaction, has been
 40 used for the development of immunological tolerance both experimentally and in
 41 patients. Here, we evaluated the effects of adoptive transfer of bone marrow-derived
 42 dendritic cells (BMDCs) pulsed with CTLA4-Ig in TNBS-induced colitis. CTLA4-Ig-
 43 modulated BMDCs or naïve BMDC were administered intravenously to BALB/c mice
 44 prior to TNBS rectal instillation. Five days later, spleens and colon segments were
 45 removed for immunological and histological analysis. Our results showed that the

adoptive transfer of CTLA4-Ig-modulated BMDCs was able to reduce the severity of inflammation caused by the administration of TNBS, in view of tissue integrity and reduced leukocyte infiltration in the colon segments of the treated mice compared to controls. Non-specific spleen cell activation in vitro showed a reduction in the frequency of CD4⁺ IL-17⁺ T cells and CD4⁺ IFN- γ ⁺ T cells as well as IL-9 secretion in cultures. To our knowledge, this is the first description of the beneficial effects of treatment with CTLA4-Ig modulated BMDC in experimental colitis.

Keywords: colitis, dendritic cell, immune modulation, CTLA4-Ig, tolerance, inflammation, inflammatory bowel disease.

1. Introduction

Two distinct signals are required for the activation of the adaptive immune response. The first signal is established by the binding of antigen-specific receptors on the surface of T lymphocytes (TCR) and antigenic fragments associated with MHC molecules on the surface of antigen-presenting cells (APCs). The second signal comes from the engagement between co-stimulatory molecules, among which the CD28 expressed on the surface of T lymphocytes and B7-1 (CD80) and B7-2 (CD86) on the surface of APCs are prominent. The low affinity interaction between CD80/CD86 and CD28 is essential to promote the activation, proliferation and survival of lymphocytes. The production of specific antibodies by B lymphocytes and the increase of phagocytic cell activities are the most evident results of this initial activation (1–4).

As the immune response proceeds and the antigen triggering this response is eliminated, a cell surface glycoprotein called CTLA4 (CD152) begins to be expressed at low levels in activated T cells. CTLA-4 binds with high affinity to the costimulatory molecules CD80 and CD86 in dendritic cells (DCs), thus initiating the reduction of the specific immune response(5–9).

Altered responses against self-antigens are at the origin of autoimmune diseases. Inflammatory bowel disease (IBD) is a group of immune-mediated diseases characterized by severe inflammation of the digestive tract (10). The etiology of IBD is still unknown, but the most plausible hypothesis is that it is due to the combination of genetic and environmental factors, particularly disturbances of the microbiota, leading to an aberrant inflammatory response of the host (11–13). Several immunomodulatory

drugs such as azathioprine and mycophenolate (inhibitors of T-cell proliferation), monoclonal antibodies, such as OKT3 (depletes and blocks T cells), and cyclosporine, tacrolimus and glucocorticoids (blockage of cytokine production) have been applied with relative success in the control of autoimmune diseases. However, most of them can lead to complications related to the onset of opportunistic infections as well as nephrotoxicity. Due to the serious side effects of nonspecific anti-inflammatory drugs and broad-spectrum immunosuppressive drugs routinely employed in the treatment of autoimmune diseases, current studies are looking at ways to manipulate immune system to reduce the need for these substances (10). Thus, new therapeutic approaches aimed at inhibiting immune responses in a more natural way have been developed in the last two decades. Among these new approaches, one of the best studied involves the use of CTLA4-Ig, a competitor molecule of the B7/CD28 interaction. In principle, its use would allow the development of immune tolerance to autoantigens by naturally blocking the activation of specific T lymphocytes (14–19).

To test these approaches prior to being screened in humans, several experimental models are available. In relation to IBD, experimental models of colitis induced by chemical or biological agents that mimic the main characteristics of human disease are currently used (20–22). Colitis induced by instillation of 2,4,6-trinitrobenzenesulfonic acid (TNBS) in BALB/c mice, for example, generates a relatively mild inflammation of the intestinal mucosa and slight weight loss, with reestablishment of the animal within a few days after instillation. Such characteristics make this one of the experimental models most used in the study of colitis modulators(23,24).

It is well known that DCs can interfere in the balance between immunity and tolerance. However, few clinical applications have been successful so far (25–27). On the other hand, some experimental studies have shown that CTLA4-Ig, a soluble chimeric fusion protein (CD152/Fc), can block the B7/CD28 signaling pathway by competition with CD80 and CD86 molecules expressed in DCs, thereby reducing responses autoimmune and graft rejection (16,28).

In this work, we evaluated the effects of the adoptive transfer of CTLA4-Ig-modulated bone marrow-derived dendritic cells (BMDC_{CTLA4-Ig}) on the inflammatory response observed in TNBS-induced colitis in BALB/c mice. BMDC_{CTLA4-Ig} and control BMDCs (BMDC_{naïve}) were administered intravenously for three consecutive days prior to instillation of TNBS. Five days later, the spleen and colon segments were removed for immunological and histological analysis.

2. Material and methods

2.1. Animals

BALB/c female mice (20–25g) at four weeks of age were obtained from the Multidisciplinary Center for Biological Research (CEMIB) of the University of Campinas (UNICAMP), Campinas, SP, Brazil. They were maintained in specific pathogen-free environment at 25° C ±1 and photoperiod of 12/12 hours. Mice were fed with autoclaved Nuvilab CR-diet (Colombo, PR, Brazil) and water *ad libitum* for 2-4 weeks before being used in experiments. Mouse manipulation were carried out in accordance with the ‘Guide for the Care and Use of Laboratory Animals’, as promoted by the Brazilian College of Animal Experimentation (COBEA) and approved by the

Ethics Committee for Animal Experimentation of University of Campinas (CEUA/UNICAMP Protocol #3077-1). All experimental procedures were performed under anesthesia (ketamine and xylazine) and all efforts were made to minimize animal suffering. Each experimental group consisted of at least five animals. Assays were repeated at least two times. Mice were monitored daily for signs of colitis such as rectal swelling, rectal bleeding, soft stools as well as weight loss.

2.2. Bone marrow dendritic cells

Bone marrow dendritic cells (BMDCs) were generated from bone marrow precursors as described elsewhere. Briefly, bone marrow cells were flushed from femurs and tibias of naïve BALB/c mice with RPMI 1640 medium (Sigma) containing 10% fetal bovine serum (FBS, Cultilab, Brazil), 20 µg/mL gentamicin solution (Sigma). Bone marrow cells were seeded in six-well plates (Corning, USA) at a density of 2×10^6 white cells/well in RPMI-10% FBS containing 20ng/mL mouse recombinant granulocyte macrophage colony-stimulating factor (mrGM-CSF) (Biosource, USA) and then incubated at 37° C in 5% CO₂. On days 3 and 6, the culture medium was replaced with fresh medium containing GM-CSF(29). On the eighth day of culture, the differentiated cells were collected, pelleted by centrifugation at 200 g, for 10 min, resuspended in RPMI-10% FBS containing 40ng/mL CTLA4-Ig (CD152/Fc chimera, non-cytolytic, from mouse, Sigma C4358), plated in 24-well culture plates at a density of 2×10^6 cells/well and cultured for an additional 24 hours (BMDC_{CTLA4-Ig}). Cells cultured in the absence of CTLA4-Ig were used as control (BMDC_{naïve}).

2.3. Phenotypic profile of BMDC

The phenotypic characteristics of the BMDCs were evaluated by flow cytometry as previously described by our group (30) and others (31–33)(33). For this, the cells were labeled with anti-mouse CD11c-APC (Clone: HL3), anti-mouse MHC-II-PE (130-091-368, Miltenyi Biotec), anti-mouseCD80-FITC (Clone: 16-10A1), anti-mouseCD86-FITC (Clone: GL1) and anti-mouse CD40-FITC (Clone: 3/23) according to the manufacturer's instructions (BD Bioscience, USA). All controls were performed using irrelevant isotype staining. The readings were performed using the FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ, USA) flow cytometer, using FCS Express 5 Plus, Research Edition software.

2.4. Adoptive transfer of BMDC and colitis induction

Three doses of 1×10^6 BMDC_{CTLA4-Ig} or BMDC_{naïve} were injected intravenously into naïve syngeneic mice on days 5, 3 and 1 before the colitis induction (Fig 1). Colitis was induced by intrarectal administration of a single dose of 2,4,6-trinitrobenzenesulphonic acid (TNBS), as described elsewhere, with modifications. Briefly, mice were anesthetized with and instilled with 100µL of 1.0mg/mL TNBS (2,4,6-trinitrobenzenesulfonic acid; Sigma, USA) dissolved in 50% ethanol into the lumen of the colon. To ensure that TNBS enter entire colon, mice were held in a vertical position for 30s. Two control groups of mice that did not receive DC were used: 1) animals inoculated intrarectally with 100µL 50% ethanol in saline; 2) animals inoculated intrarectally with 100µL of 1.0mg/mL TNBS dissolved in 50% ethanol.

[Insert Fig 1]

2.5. Evaluation of clinical symptoms of TNBS-induced colitis

Animals of all groups were weighed daily until sacrifice on the fifth day following the instillation of TNBS. Weight variation was calculated as percentage, considering the weight at day zero as 100%. Clinical symptoms such as diarrhea, rectal prolapse, bleeding and cachexia were registered and assigned as scores, ranging from 0 to 2, with 0: no change, 1: slight change (liquid feces, inflammation in the anus, mucus liberation and weakness), and 2: severe change (diarrhea, rectal prolapse, bleeding, and cachexia) and these values were mean to each animal. Data are presented as the mean $\pm$ Standard Error of the Mean (S.E.M.)

2.6. Histological analysis of the colon

The mice were euthanized five days after induction of colitis. Two portions of the colon distant 1-2 cm (P1 = proximal1) and 2-3 cm (P2 = proximal 2), respectively, of the anal sphincter were removed, fixed in 4% buffered formalin, dehydrated with ethanol solutions, and embedded in paraffin (Paraplast Plus Sigma P3683). Slices of 5 μ m were cut into a microtome (Leica - model Jung Biocut 2035) and mounted on clean glass slides. The specimens were then dewaxed, rehydrated and stained with hematoxylin and eosin (Merck). As the distal portions of the intestine are unaffected by treatment with TNBS, sections of these segments were not examined.

The P1 and P2 segments of the colon were evaluated by microscopy for the presence of folds, hemorrhage, gauge and leukocyte infiltrate. The data were represented by scores corresponding to the sum of the values attributed to the presence and characteristics of the folds in the mucosa (0 for normal folds, 1 for slightly altered folds, 2 for deformed

187 folds, 3 for very deformed folds, 4 for dorsal folds reduction , 5 for lack of folds);
 188 bleeding (0 for no bleeding, 1 for bleeding present, 2 for large bleeding); mucosal
 189 dilatation (0 for absence of voids, 1 for apparent voids, 2 for large voids); and
 190 lymphocytic infiltrate in the mucosa, submucosa and mesentery (0-none; diffuse
 191 inflammatory infiltrate-1; 2-considerable inflammatory infiltrate with submucosal
 192 disorganization; 3-intense infiltrate) as previously described 5. Thickening of the colon
 193 wall was measured in micrometers using the Infinity Analyze Nikon H600L (100X). The
 194 final scores represent the mean $\pm$ S.E.M (23,34).

195 Histomorphometric analysis was performed on sections of P1 and P2 segments prepared
 196 for immunoperoxidase reactions using the following antibodies: anti-CD3 (T
 197 lymphocytes) and anti-F4/80 (macrophages) and anti-Ly-6c NIM (neutrophils) (35,36).
 198 Peroxidase-conjugated secondary antibodies (Sigma) and diaminobenzidine (DAB;
 199 Sigma) were used in the development of reactions (37,38). After counter-staining with
 200 hematoxylin/eosin, the tissues were observed under light microscopy for counting the
 201 labeled cells. Evaluations were done in a double-blind fashion and the quantification of
 202 labeled cells was performed in five random fields in each specimen. Sections P1 and P2
 203 of at least three animals in each group were evaluated, making a total of 15
 204 measurements per group, in each experiment. Results were expressed as mean $\pm$ S.E.M.
 205 Three independent experiments were performed (24,39,40).

206 **2.7. Spleen cell proliferation**

207 Spleens were collected aseptically from mice of all experimental groups, macerated
 208 individually, suspended in lyses buffer and pelleted by centrifugation at 200 g for 10

min. Cell concentrations were adjusted to 1×10^6 cells/mL in RPMI medium (Sigma, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil). After washing, spleen cell suspensions were incubated with $25 \mu\text{M}$ carboxyfluoresceinsuccinimidyl probe ester (CFSE) in RPMI-10% FBS at room temperature for 5 min, according manufacturer's recommendations (Invitrogen, USA). Cells were then pelleted by centrifugation and suspended in fresh medium. To determine the maximum uptake of CFSE, aliquots of each cell suspension were fixed with 1% formaldehyde in PBS and analyzed by flow cytometer. Then $100 \mu\text{L}$ aliquots of each suspension of CFSE-labeled cells were seeded in duplicate in 96-well plates (Corning) and incubated in the presence of $2.5 \mu\text{g} / \text{ml}$ ConA for 72 hours at 37°C . Cultures of cells conducted in the absence of Con-A were used as controls.

The proliferation of T lymphocytes in the cultures was assessed at the gate of $\text{CD4}^+\text{CFSE}^+$ cells. Acquisitions were performed with FACSCalibur flow cytometer (FACSCalibur flow cytometer, BD Becton Dickinson, San Jose, CA)(34,41). The results were analyzed with the FCS Express Plus Research Edition software (FCS Express Launcher). Results were expressed as proliferation index (fold change) calculated in relation to that of the control group (24,34).

In parallel, cultures of CFSE-unlabeled spleen cells from mice of all experimental groups were conducted to measure the levels of cytokines released in the supernatants after Con-A stimulation as described below.

2.8. Phenotypic profile of T-cells

The frequencies of $\text{TCD4}^+\text{CD25}^+ \text{Foxp3}^+$ (Treg cells), $\text{TCD4}^+\text{IL17}^+$, $\text{TCD4}^+\text{IFN}\gamma^+$ and

TCD4⁺IL-10⁺ cells in the spleen cell cultures were assessed by flow cytometer. Briefly, cell suspensions were washed and initially stained with anti-CD3 APC (clone 145-2C11, BD #553066), anti-CD4-PE (Clone GK1.5) and anti-CD25-FITC (Clone 7D4). Then, cells were permeabilized by the addition of fixation/permeabilization buffer (Cytofix/Cytoperm fixation/permeabilization kit, Becton-Dickinson, BD). Suspension was stained with anti-Foxp3-APC (clone FJK-16S), anti-IL-17-APC (clone eBIO17B7) or Alexa Fluor 647 (Clone TC11-18410), anti-IFN- γ -APC (Clone XMG1.2) and IL-10-APC (Clone JESS-16E3), 647 (Clone Q21-378), according to manufacturer's instructions. Controls were performed with irrelevant isotype staining. Acquisitions were performed with FACScalibur flow cytometer and analyzes were done with the FCS Express 5 Plus, Research Edition software (23,34)

2.9. Determination of Th1, Th2, Th17 and Th9 cytokines

IL-2, IL-4, IL-6, IL-10, IL-17A, IFN- γ and TNF- α were quantified in culture supernatants of spleen cells by flow cytometer, using Multiplex CBA kit (BD Cytometric Bead Array Th1/Th2/Th17, San Diego, USA) according to manufacturer's instructions. Fluorescence were acquired in FACScalibur cytometer and analyzed with FCAP Array TM Software Version 3.0 (BD). IL-9 determination was assayed with CBA flex set (BD Cytometric Flex Set Th9, San Diego, USA) (23,34)

2.11. Statistical analysis

The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA). The statistical significance of differences between control and experimental groups were determined by one-way ANOVA, followed by Bonferroni's

test for multiple comparisons or unpaired Student's t-test. The results were expressed as mean $\pm$ Standard Error of the Mean (S.E.M). Values were considered significant at $P < 0.05$. All data presented are representative of at least three independent experiments.

3. Results

The effects of adoptive transfer of BMDCs on TNBS-induced colitis

Dendritic cells were differentiated in vitro from precursors collected from the bone marrow of BALB/c mice by their culture in the presence of recombinant GM-CSF for eight days. Differentiated bone marrow DCs (BMDCs) were then incubated in the presence or absence of recombinant CTLA4-Ig for 24 hours before being employed in adoptive transfer assays. We observed that modulation of BMDCs with CTLA4-Ig did not modify the expression pattern of the CD11c, MHC class II, CD40, CD80 and CD86 molecules on the surface of these cells compared to CTLA4-Ig untreated BMDCs (BMDC_{naïve}), as can be seen in Supplementary Figure 1 (Supp. Fig 1).

Weight loss in TNBS-induced colitis is generally mild (about 10%) and recovery of body weight is usually observed as early as the fourth day after drug administration in BALB/c mice. As can be seen in Figure 2A, the adoptive transfers of BMDC_{CTLA4-Ig} or BMDC_{naïve} did not result in any significant improvement in weight loss observed in the first days after TNBS instillation and, as expected, by the fourth day all mice had already recovered. However, the other clinical signs of the disease (diarrhea, rectal prolapse, soft stools, and hemorrhagic stools) were significantly reduced by previous treatment with BMDC_{CTLA4-Ig}, as shown in Figure 2B.

274 [Insert Fig 2]

275 As can be seen in Figure 3A, administration of TNBS induced a strong inflammatory
276 reaction that affected the regions closest to the rectum (both P1 and P2 segments), with
277 a large number of infiltrated leukocytes. However, previous treatment with
278 BMDC_{CTLA4-Ig} significantly reduced leukocyte infiltration caused by instillation of
279 TNBS.

280 The cells present in the infiltrate consisted mainly of macrophages (Figure 3B), while
281 neutrophils were rarely found. It was also observed that the colonic tissue of mice
282 treated with BMDC_{CTLA4-Ig} showed a significant reduction in the number of infiltrated
283 macrophages when compared to the tissues of mice without previous treatment with
284 BMDCs. Pretreatment with BMDC_{naïve} did not modify the inflammatory process
285 induced by the instillation of TNBS. On the other hand, the adoptive transfer of
286 BMDC_{CTLA4-Ig}, but not BMDC_{naïve}, was able to prevent thickening of the colon wall,
287 particularly in the P1 region, as shown in Figure 3C.

288 [Insert Figure 3]

289 **The effects of adoptive transfer of BMDCs on immune response of colitic mice**

290 The effects of the adoptive transfer of BMDC_{CTLA4-Ig} on the immunological functions of
291 lymphocytes from colitic mice are shown in Figures 4 and 5. The proliferative response
292 of T lymphocytes was significantly lower in cultures of spleen cells from mice
293 pretreated with BMDC_{CTLA4-Ig} than in controls groups (Figure 4A). The frequencies of
294 Treg cells (CD25⁺ Foxp3⁺ T cells) in cultures of spleen cells of mice previously treated

with BMDC_{CTLA4-Ig} as well as those receiving TNBS alone were significantly higher than in the other groups, as shown in Figure 4B. The frequency of CD4⁺ T cells producing IFN- γ and IL-17 was significantly lower in the cultures of spleen cells from mice pretreated with BMDC_{CTLA4-Ig}, compared to the other groups (Fig 4, C and E). On the other hand, the frequency of CD4⁺ T cells producing IL-10 was higher in the cultures of spleen cells from mice pretreated with BMDC_{CTLA4-Ig} and, interestingly, lower in the cultures of spleen cells from mice pretreated with BMDC_{naïve} compared with the other groups (Figure 4G). No significant differences were observed between the experimental groups in relation to the intracellular labeling of ROR γ c and T-bet transcription factors (Fig 4D, 4F). However, GATA3 factor labeling was higher in spleen cells from mice treated with BMDC_{CTLA4-Ig} (Fig. 4H).

As shown in Figure 5C, IL-4 was present at levels detectable only in spleen cell culture supernatants from mice pretreated with BMDC_{CTLA4-Ig}. Significantly elevated IL-10 levels were found in supernatants from splenic cell cultures of mice pretreated with BMDC_{naïve} or BMDC_{CTLA4-Ig}, compared to controls (Figure 5E). However, IL-6 levels were also higher in spleen cultures from BMDC_{naïve}-pretreated mice compared to the other groups (Figure 5B). Higher IL-9 levels were detected in splenic cell culture supernatants from control mice that received only intrarectal ethanol. In mice treated with BMDC_{CTLA4-Ig} prior to TNBS instillation, however, levels of IL-9 were significantly lower than in all other groups (Figure 5G). There were no significant differences in the levels of IL-2, IL-17, TNF- α and IFN- γ (Figure 5 A, D, F, H, respectively).

[Insert Figures 4 and 5]

4. Discussion

Experimental colitis induced by TNBS instillation is characterized by chronic inflammation of the gastrointestinal tract of mice with features overlapping those seen in inflammatory bowel diseases in humans. Literature data have shown the importance of oral tolerance and treatments with tolerogenic dendritic cell for the reduction of colitis damages. In this sense, previous studies of our group have shown that oral tolerance to OVA albumin as well as the adoptive transfer of dendritic cells from OVA-tolerant mice is able to reduce the damage caused by TNBS-induced colitis in syngeneic animals (23,24). We have also shown that flaxseed protein hydrolysates and phenolic fractions were able to ameliorate TNBS-induced colitis in BALB/c mice. Treatments with flaxseed protein fractions reduced inflammation of the intestinal mucosa in TNBS-induced colitis in BALB/c mice, as well as the proliferation of their splenic cells in response to Con-A, the frequency of Th1 and Th17 cells, and the levels of inflammatory cytokines in culture supernatants. In addition, the administration of phenolic compounds from flaxseeds prevented intestinal inflammation and increased the frequency of Treg lymphocytes in splenic cell cultures of BALB/c mice with colitis (34). The present work expanded these findings, demonstrating that the adoptive transfer of bone marrow-derived dendritic cells modulated with CTLA4-Ig, a recombinant mouse protein which binds with both B7-1 and B7-2 molecules, can improve clinical signs of the TNBS-induced colitis in BALB/c mice. It also shows that after transfer of CTLA4-Ig-modulated BMDCs, spleen T lymphocytes from mice with colitis show a more reduced proliferative response to Con-A accompanied by a reduction in the frequency of inflammatory cells secreting IL-17 and IFN-gamma as well as expansion of cells that produce IL-10 in the cultures. Our data showing an improvement in colitis with

CTLA4-Ig-modulated BMDCs corroborates with previous data showing that adoptive transfer of DCs modulated with dexamethasone and Vitamin D3 (42,43) or IL-10-modulated DC (44) protects severe combined immune deficient (SCID) mice from weight loss and pathologies associated with wasting diseases and colitis.

CTLA4-Ig is able to selectively modulate T cell activation by binding to CD80/CD86 costimulatory molecules in DCs (9,45). It is already known that direct administration of CTLA4-Ig affects the functioning of DCs through the IDO pathway, promoting a regulatory phenotype and consequently inducing the increase in the population of CD4⁺CD25⁺Foxp3⁺ T cells (9,46,47). In the murine model of arthritis, treatment with CTLA4-Ig was able to reduce the expression of CD80/CD86 molecules on DC and suppressed the inflammatory response associated with the disease (47). Our results did not show significant changes in CD80 and CD86 expression after the BMDCs were treated with CTLA4-Ig. This may be related to the origin of dendritic cells, i.e. differentiated dendritic cells from bone marrow precursors, and to the doses of CTLA4-Ig used in this work. Moreover, we used the recombinant protein to modulate the BMDCs to be transferred adoptively to mice rather than administering it directly to the animals.

It is well known that the instillation of TNBS causes severe changes in the distal portion of the large intestine, due to the inflammatory process triggered by the immune response to the drug. Administration of TNBS to rats, for example, results in increased expression of fibrosis-associated proteins such as phospho-p38, phospho-SMAD2/3, and PPAR γ (48). In agreement with previous studies, we observed that the instillation of TNBS caused significant histological changes in the large intestine segments of

BALB/c mice, particularly in the P2 segment (2 to 3 cm of the anal sphincter). These changes were characterized by a thickening of the colon and intense inflammatory infiltrate consisting mainly of macrophages.

The literature shows that in the TNBS-induced colitis the adaptive immune response is predominantly Th1 type, characterized by an increase in IFN γ -producing T cells. In protocols for weekly administration of TNBS for six consecutive weeks, an influx of T cells was observed around the third day to two weeks after instillation of the drug, infiltrating the lamina itself and the submucosal layer of the large intestine and supporting chronic colitis (49). In TNBS single-dose protocols such as that used in this study, lymphocyte migration to the lamina propria begins about one week after instillation of the drug (50). Since the animals were euthanized on the fifth day after TNBS administration, this cell type was virtually absent in our histological preparations. Likewise, a reduced number of neutrophils were observed in P1 and P2 preparations since the maximum migration of these cells occurs within the first 48 hours after the instillation of TNBS. As expected, in the time elapsed between administration of TNBS and the euthanasia of the animals for histological analysis, macrophages were the most abundant cells in the inflammatory infiltrate, particularly in the P2 segment of the colon of animals receiving TNBS alone, in a typical hypersensitivity reaction, as described in figures 3.

Our results show that the adoptive transfer of CTLA4-Ig-modulated BMDC was able to significantly prevent colon thickening in the P2 portion of the large intestine as well as the infiltration of macrophages in response to instillation of TNBS. Data from the literature indicate that the intense leukocyte infiltrate in the intestinal mucosa may be

responsible for tissue necrosis and changes associated with colitis symptoms (51). Lesions in the colon mucosa may be associated with the release of significant amounts of free radicals, due to the abundance of activated macrophages attracted to the lesion site (52,53). Thus, our results indicate that DCs modulated in vitro with the recombinant CTLA4-Ig protein constitute at least one more natural therapeutic alternative for the treatment of these disorders.

In order to evaluate the influence of the adoptive transfer of CTLA4-Ig-modulated BMDCs on the immune response of TNBS-treated mice, we examined the proliferative responses, the effector CD4⁺ T cell profiles and the release of cytokines in cultures of spleen cells collected on the fifth day after induction of colitis and stimulated in vitro with Con-A. Data presented here (Fig 4) show that spleen cells from animals of all groups proliferated in response to Con-A, but such ability was significantly lower in splenic cells from mice pretreated with CTLA4-Ig-modulated BMDCs.

Examination of effector CD4⁺ T cell populations in splenic cell cultured in the presence of Con-A showed that treatment with BMDC_{CTLA4-Ig} resulted in a significant reduction in the frequency of IL-17⁺ and IFN- γ ⁺ cells and in the elevation of CD4⁺ IL-10⁺ and CD4⁺ Foxp3⁺ T cells. Frequency of GATA-3 expressing cells was higher in the splenic cell cultures of mice treated with CTLA4-Ig modulated BMDCs. However, TCD4⁺ cells expressing the Th1/Treg cell associated RORc and T-bet transcription factors did not show significant variations between the different treatments.

Treg cells play a key role in the control of immune responses to autoantigens as well as on those that act upon pathogens, commensals, tumors, and grafts. Such control is

exerted by the ability of Treg cells to accumulate in inflamed areas and to adapt to the environment, being particularly critical in tissues repeatedly exposed to the presence of microbes and environmental aggressions such as the gastrointestinal tract and skin (54,55). It has been shown that the canonical Th2 transcription factor GATA3 is selectively expressed in Treg residing in barrier sites including the gastrointestinal tract and the skin, being fundamental to maintain high levels of Foxp3 expression in various polarized or inflammatory settings (56). Corroborating these data, we observed a significantly higher frequency of Treg cells in spleen cell cultures from mice receiving only TNBS and those from mice pretreated with BMDC_{CTLA4-Ig}. However, a significant increase in cells expressing both Foxp3 and GATA-3 was observed only in the group that received BMDC_{CTLA4-Ig}, indicating its influence in promoting a more efficient control of the inflammatory response induced by TNBS.

Although the frequencies of IL-17 and IFN- γ -secreting T cells were reduced in splenic cell cultures of mice pretreated with BMDC_{CTLA4-Ig}, no significant differences were observed in the levels of these cytokines in spleen cell culture supernatants from mice pretreated with either BMDC_{CTLA4-Ig} or BMDC_{naïve}. On the other hand, IL-4, whose production is controlled by GATA-3 expression, was detected only in spleen cell cultures of BMDC_{CTLA4-Ig} treated mice. In the spleen cell cultures from mice pretreated with BMDC_{naïve} it was possible to observe the higher levels of IL-6, but it was also the one that presented the highest levels of IL-10, whose production is controlled by the transcription factor Foxp3. Thus, while splenic cell cultures of BMDC_{CTLA4-Ig} pretreated mice had higher levels of IL-4, those of mice pretreated with BMDC_{naïve} had higher levels of IL-10. The significance of these findings still needs further investigation.

The presence of cells expressing the transcription factor PU.1, a regulator of the development of Th9 cells, has been observed in the intestinal lamina propria of patients with ulcerative colitis and Crohn's disease (57). Although we did not examine the frequency of Th9 cells in the splenic cell cultures of the different groups studied here, we found that the production of IL-9 in the supernatants of the spleen cell cultures from mice treated with BMDC_{CTLA4-Ig} was significantly more reduced than in other cell cultures, including those from mice that received only the vehicle.

IL-9 is a cytokine that may act differently on Th17 cells or Treg cells, as an inducer or regulator of tissue inflammation. IL-9 associated with TGF- β may drive the differentiation of Th17 cells. In turn, Th17 cells can secrete IL-9, which affects inflammatory response *in vivo*. IL-9 also acts *in vitro* on FoxP3⁺CD4⁺ Treg cells, increasing their suppressive function. This activation occurs by signaling pathways associated with transcription factors STAT3 and STAT5 (58).

Reports show that in addition to cytokines released by Th1 and Th17 cells, IL-9 is also involved in T cell-mediated experimental colitis, promoting mucosal ulceration and chronic inflammation. In this way, Th9 cells represent a potential target for the treatment of chronic intestinal inflammation (22,59).

It is known that acute and chronic intake of alcohol produces sensitive changes in the intestinal mucosa, contributing to the installation or worsening of IBD already installed, both in humans and in experimental models (60). The literature, however, does not report on possible changes due to the use of 50% alcohol as a control of the instillation of TNBS dissolved in this vehicle. However, we have observed that the instillation of

50% ethanol is not as innocuous as its use has resulted in some changes of an inflammatory nature such as elevated TNF- α , IL-9 and IL-17 levels in the corresponding splenic cell cultures, although important differences were observed in the instillation of TNBS/50% ethanol compared to ethanol instillation alone.

The blockade of the CTLA4 molecule is already described as a potential therapy for tumor treatment (61–63). However, studies related to the blockade of this molecule by the direct administration of CTLA4-Ig for the treatment of inflammatory bowel diseases did not present promising results (64). In this context, the use of CTLA4-Ig-modulated dendritic cells, instead of the direct application of this inhibitor, may be a clinical alternative to treat patients with IBD. Studies have shown that the CTLA4-Ig fusion protein affects the functioning of DCs through the IDO pathway, promoting a regulatory phenotype in this population (9,26,47). Dendritic cell therapies for immunomodulation have been presented as a therapeutic option under study, due to the great advance in the use of these cellular populations in the treatments of autoimmune diseases (65,66). Wang and colleagues observed that BMDCs generated from mouse bone marrow and stimulated with GM-CSF have a mature DC cell profile and can be used in antitumor immunity studies. Adherent cells from these cultures have macrophage properties and may be used to induce tolerance, whereas mixed cells may potentiate tolerogenicity or pro-tumorigenic responses. Immature DCs have a strong migration and capture capabilities, while mature DCs activate naïve T cells and express high levels of costimulatory adhesion molecules and cytokines (67).

Taken together, our results allow us to conclude that adoptive transfer of CTLA4-Ig-modulated BMDC improves clinical signs of TNBS-induced colitis. Histological

analysis of intestinal segments showed that the adoptive transfer of CTLA4-Ig-modulated BMDC reduced the infiltration of inflammatory cells, particularly macrophages, and improved tissue damage in the colon. Adoptive transfer of CTLA4-Ig-modulated BMDC was also able to alter the immunological profile of activated splenic cells in vitro. Spleen cell culture of CTLA4-Ig-modulated BMDC-pretreated mice showed a reduction in the frequency of CD4⁺ T cells producing IFN- γ and IL-17 and IL-9 secretion, as well as increased frequency of Treg cells and IL-10 production. To our knowledge, this is the first description of the beneficial effects of treatment with CTLA4-Ig modulated BMDC in experimental colitis at the histological and immunological level.

Data Availability

The research data used to support the findings of this study are included within the article.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Figures

Figure 1

836 Figure 2:

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839 Figure 3:

840 Figure 4:
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843 Figure 5

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845 Supplementary figure 1

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