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

Lisiery Negrini Paiatto[1], Fernanda Guimarães Drummond Silva[2], Áureo Tatsumi Yamada[3], Wirla Maria Silva Cunha Tamashiro[4]; Patricia Ucelli Simioni[1, 4, 5, 6] *

[1]Department of Biomedical Science, Faculty of Americana, FAM, 13477-360, Americana, SP, Brazil

[2]Department of Food, School of Nutrition, Federal University of Ouro Preto, 35400-000, Ouro Preto, MG, Brazil.

[3]Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas (UNICAMP), 13083-862, Campinas, SP, Brazil.

[4]Department of Genetics. Evolution, Microbiology and Immunology, Institute of Biology, UNICAMP, 13083-862, Campinas, SP, Brazil.

[5]Postgraduate Program in Biological Science (Cellular and Molecular Biology), Department of Biochemistry and Microbiology, Institute of Biosciences, Univ Estadual Paulista, UNESP, 13506-900 Rio Claro, SP, Brazil

[6]Department of Biomedical Science, Faculty of Americana, FAM, 13477-360, Americana, SP, Brazil.

Correspondence should be addressed to Patricia Ucelli Simioni psimioni@gmail.com

E-mail Address:

Lisiery Negrini Paiatto: lisy_paiatto@hotmail.com

Fernanda Guimaraes Drummond Silva: fer.dru@gmail.com

Aureo Tatsumi Yamada: yamadat3g@gmail.com

Wirla Maria da Silva Cunha Tamashiro: wirlatam@unicamp.br

Patricia Ucelli Simioni: psimioni@gmail.com

Full postal address: Rua Joaquim Boer, 733, Department of Biomedical Science, Faculty of Americana, FAM, 13477-360, Americana, SP, Brazil.

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

Abstract

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

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

53 **Keywords: colitis, dendritic cell, immune modulation, CTLA4-Ig, tolerance,**
54 **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

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

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

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

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

112 **2. Material and methods**

113 **2.1. Animals**

114 BALB/c female mice (20–25g) at four weeks of age were obtained from the
115 Multidisciplinary Center for Biological Research (CEMIB) of the University of
116 Campinas (UNICAMP), Campinas, SP, Brazil. They were maintained in specific
117 pathogen-free environment at 25º C ±1 and photoperiod of 12/12 hours. Mice were fed
118 with autoclaved Nuvilab CR-diet (Colombo, PR, Brazil) and water *ad libitum* for 2-4
119 weeks before being used in experiments. Mouse manipulation were carried out in
120 accordance with the 'Guide for the Care and Use of Laboratory Animals', as promoted
121 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% CO_2. 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 x 10^6 cells/well and cultured for an additional 24 hours ($BMDC_{CTLA4\text{-}Ig}$). Cells cultured in the absence of CTLA4-Ig were used as control ($BMDC_{naïve}$).

2.3. Phenotypic profile of BMDC

144 The phenotypic characteristics of the BMDCs were evaluated by flow cytometry as
145 previously described by our group (30) and others (31–33)(33). For this, the cells were
146 labeled with anti-mouse CD11c-APC (Clone: HL3), anti-mouse MHC-II-PE (130-091-
147 368, Miltenyi Biotec), anti-mouseCD80-FITC (Clone: 16-10A1), anti-mouseCD86-
148 FITC (Clone: GL1) and anti-mouse CD40-FITC (Clone: 3/23) according to the
149 manufacturer's instructions (BD Bioscience, USA). All controls were performed using
150 irrelevant isotype staining. The readings were performed using the FACSCalibur
151 (Becton-Dickinson, Franklin Lakes, NJ, USA) flow cytometer, using FCS Express 5
152 Plus, Research Edition software.

153 **2.4. Adoptive transfer of BMDC and colitis induction**

154 Three doses of $1x10^6$ $BMDC_{CTLA4\text{-}Ig}$ or $BMDC_{naïve}$ were injected intravenously into
155 naïve syngeneic mice on days 5, 3 and 1 before the colitis induction (Fig 1). Colitis was
156 induced by intrarectal administration of a single dose of 2,4,6-trinitrobenzenesulphonic
157 acid (TNBS), as described elsewhere, with modifications. Briefly, mice were
158 anesthetized with and instilled with 100µL of 1.0mg/mL TNBS (2,4,6-
159 trinitrobenzenesulfonic acid; Sigma, USA) dissolved in 50% ethanol into the lumen of
160 the colon. To ensure that TNBS enter entire colon, mice were held in a vertical position
161 for 30s. Two control groups of mice that did not receive DC were used: 1) animals
162 inoculated intrarectally with 100µL 50% ethanol in saline; 2) animals inoculated
163 intrarectally with 100µL of 1.0mg/mL TNBS dissolved in 50% ethanol.

164 [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 ± 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 5µm 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 ± 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 ± 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 $1x10^6$ cells/mL in RPMI medium (Sigma, USA) supplemented with 10% fetal bovine serum (Cultilab, Campinas, Brazil). After washing, spleen cell suspensions were incubated with 25μM 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 100μL 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 μg / 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 CD4+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 $TCD4^+CD25^+$ $Foxp3^+$ (Treg cells), $TCD4^+IL17^+$, $TCD4^+IFN\gamma^+$ and

231 TCD4^{+}IL-10^{+} cells in the spleen cell cultures were assessed by flow cytometer. Briefly,
232 cell suspensions were washed and initially stained with anti-CD3 APC (clone 145-
233 2C11, BD #553066), anti-CD4-PE (Clone GK1.5) and anti-CD25-FITC (Clone 7D4).
234 Then, cells were permeabilized by the addition of fixation/permeabilization buffer
235 (Cytofix/Cytoperm fixation/permeabilization kit, Becton-Dickinson, BD). Suspension
236 was stained with anti-Foxp3-APC (clone FJK-16S), anti-IL-17-APC (clone eBIO17B7)
237 or Alexa Fluor 647 (Clone TC11-18410), anti-IFN-γ-APC (Clone XMG1.2) and IL-10-
238 APC (Clone JESS-16E3), 647 (Clone Q21-378), according to manufacturer's
239 instructions. Controls were performed with irrelevant isotype staining. Acquisitions
240 were performed with FACScalibur flow cytometer and analyzes were done with the
241 FCS Express 5 Plus, Research Edition software (23,34)

242 **2.9. Determination of Th1, Th2, Th17 and Th9 cytokines**

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

249 **2.11. Statistical analysis**

250 The statistical analysis was performed using GraphPad Prism 5 (GraphPad Software,
251 San Diego, CA, USA). The statistical significance of differences between control and
252 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 ± 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 (BMDCnaïve), 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 $BMDC_{CTLA4\text{-}Ig}$ 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 $BMDC_{CTLA4\text{-}Ig}$, as shown in Figure 2B.

[Insert Fig 2]

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

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

[Insert Figure 3]

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

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

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

306 As shown in Figure 5C, IL-4 was present at levels detectable only in spleen cell culture
307 supernatants from mice pretreated with $BMDC_{CTLA4\text{-}Ig}$. Significantly elevated IL-10
308 levels were found in supernatants from splenic cell cultures of mice pretreated with
309 $BMDC_{naïve}$ or $BMDC_{CTLA4\text{-}Ig}$, compared to controls (Figure 5E). However, IL-6 levels
310 were also higher in spleen cultures from $BMDC_{naïve}$-pretreated mice compared to the
311 other groups (Figure 5B). Higher IL-9 levels were detected in splenic cell culture
312 supernatants from control mice that received only intrarectal ethanol. In mice treated
313 with $BMDC_{CTLA4\text{-}Ig}$ prior to TNBS instillation, however, levels of IL-9 were
314 significantly lower than in all other groups (Figure 5G). There were no significant
315 differences in the levels of IL-2, IL-17, TNF-α and IFN-γ (Figure 5 A, D, F, H,
316 respectively).

317 [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

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

346 CTLA4-Ig is able to selectively modulate T cell activation by binding to CD80/CD86
347 costimulatory molecules in DCs (9,45). It is already known that direct administration of
348 CTLA4-Ig affects the functioning of DCs through the IDO pathway, promoting a
349 regulatory phenotype and consequently inducing the increase in the population of
350 $CD4^{+}CD25^{+}Foxp3^{+}$ T cells (9,46,47). In the murine model of arthritis, treatment with
351 CTLA4-Ig was able to reduce the expression of CD80/CD86 molecules on DC and
352 suppressed the inflammatory response associated with the disease (47). Our results did
353 not show significant changes in CD80 and CD86 expression after the BMDCs were
354 treated with CTLA4-Ig. This may be related to the origin of dendritic cells, i.e.
355 differentiated dendritic cells from bone marrow precursors, and to the doses of CTLA4-
356 Ig used in this work. Moreover, we used the recombinant protein to modulate the
357 BMDCs to be transferred adoptively to mice rather than administering it directly to the
358 animals.

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

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

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

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

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

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

401 Examination of effector $CD4^+$ T cell populations in splenic cell cultured in the presence
402 of Con-A showed that treatment with $BMDC_{CTLA4\text{-}Ig}$ resulted in a significant reduction
403 in the frequency of IL-17^+ and IFN-γ^+ cells and in the elevation of $CD4^+$ IL-10^+ and
404 $CD4^+$ $Foxp3^+$ T cells. Frequency of GATA-3 expressing cells was higher in the splenic
405 cell cultures of mice treated with CTLA4-Ig modulated BMDCs. However, $TCD4^+$ cells
406 expressing the Th1/Treg cell associated RORc and T-bet transcription factors did not
407 show significant variations between the different treatments.

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

410 exerted by the ability of Treg cells to accumulate in inflamed areas and to adapt to the
411 environment, being particularly critical in tissues repeatedly exposed to the presence of
412 microbes and environmental aggressions such as the gastrointestinal tract and skin
413 (54,55). It has been shown that the canonical Th2 transcription factor GATA3 is
414 selectively expressed in Treg residing in barrier sites including the gastrointestinal tract
415 and the skin, being fundamental to maintain high levels of Foxp3 expression in various
416 polarized or inflammatory settings (56). Corroborating these data, we observed a
417 significantly higher frequency of Treg cells in spleen cell cultures from mice receiving
418 only TNBS and those from mice pretreated with $BMDC_{CTLA4\text{-}Ig}$. However, a significant
419 increase in cells expressing both Foxp3 and GATA-3 was observed only in the group
420 that received $BMDC_{CTLA4\text{-}Ig}$, indicating its influence in promoting a more efficient
421 control of the inflammatory response induced by TNBS.

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

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

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

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

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

455 50% ethanol is not as innocuous as its use has resulted in some changes of an
456 inflammatory nature such as elevated TNF-α, IL-9 and IL-17 levels in the
457 corresponding splenic cell cultures, although important differences were observed in the
458 instillation of TNBS/50% ethanol compared to ethanol instillation alone.

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

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

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

488 **Data Availability**

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

491 **Conflict of Interest**

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

494 **Funding Statement**

495 Grant (#2013/20258-2) and fellowships (#2014/16701-0, #2014/08591-0,
496 #2014/086192, #2015/09326-1) were obtained from São Paulo Research Foundation
497 (FAPESP) and Coordination for the Improvement of Higher Education Personnel
498 (CAPES).

REFERENCES

1. Metzler B, Burkhart C, Wraith DC. Phenotypic analysis of CTLA-4 and CD28 expression during transient peptide-induced T cell activation in vivo. Int Immunol [Internet]. 1999;11(5):667–75. Available from: https://www.ncbi.nlm.nih.gov/pubmed/10330272

2. Butte MJ, Keir ME, Phamduy TB, Sharpe AH, Freeman GJ. Programmed Death-1 Ligand 1 Interacts Specifically with the B7-1 Costimulatory Molecule to Inhibit T Cell Responses. Immunity [Internet]. 2007;27(1):111–22. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2707944/

3. Munn DH, Sharma MD, Mellor AL. Ligation of B7-1/B7-2 by Human CD4+ T Cells Triggers Indoleamine 2,3-Dioxygenase Activity in Dendritic Cells. J Immunol [Internet]. 2004 Mar 19 [cited 2015 Mar 16];172(7):4100–10. Available from: http://www.ncbi.nlm.nih.gov/pubmed/15034022

4. Koorella C, Nair JR, Murray ME, Carlson LM, Watkins SK, Lee KP. Novel regulation of CD80/CD86-induced phosphatidylinositol 3-kinase signaling by NOTCH1 protein in interleukin-6 and indoleamine 2,3-dioxygenase production by dendritic cells. J Biol Chem [Internet]. 2014 Mar 14 [cited 2015 Feb 27];289(11):7747–62. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3953285&tool=pmcentrez&rendertype=abstract

5. Cutolo M, Soldano S, Montagna P, Sulli A, Seriolo B, Villaggio B, et al. CTLA4-Ig interacts with cultured synovial macrophages from rheumatoid arthritis patients and downregulates cytokine production. Arthritis Res Ther [Internet]. 2009;11(6):R176. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3003520/

6. Perez N, Karumuthil-Melethil S, Li R, Prabhakar BS, Holterman MJ, Vasu C. Preferential costimulation by CD80 results in IL-10-dependent TGF-beta1(+) -adaptive regulatory T cell generation. J Immunol [Internet]. 2008 May 15 [cited 2014 Dec 29];180(10):6566–76. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2435403&tool=pmcentrez&rendertype=abstract

7. Ueda H, Howson JMM, Esposito L, Heward J, Snook H, Chamberlain G, et al. Association of the T-cell regulatory gene CTLA4 with susceptibility to autoimmune disease. Nature [Internet]. 2003 May 29 [cited 2015 Oct 16];423(6939):506–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/12724780

8. Abram DM, Fernandes LGRL, Ramos Filho AACS, Simioni PUPU, Moitinho Abram D, Fernandes LGRL, et al. The modulation of enzyme indoleamine 2,3-dioxygenase from dendritic cells for the treatment of type 1 diabetes mellitus. Drug Des Devel Ther [Internet]. 2017 Jul [cited 2018 Sep 21];2171–8. Available from: https://www.dovepress.com/the-modulation-of-enzyme-indoleamine-23-dioxygenase-from-dendritic-cel-peer-reviewed-article-

542 DDDT

543 9. Cutolo M, Nadler SG. Advances in CTLA-4-Ig-mediated modulation of
544 inflammatory cell and immune response activation in rheumatoid arthritis.
545 Autoimmun Rev [Internet]. 2013 May [cited 2015 Feb 18];12(7):758–67.
546 Available from: http://www.ncbi.nlm.nih.gov/pubmed/23340277

547 10. De Mattos BRR, Garcia MPG, Nogueira JB, Paiatto LNLN,
548 Albuquerque CG, Souza CL, et al. Inflammatory Bowel Disease: An Overview
549 of Immune Mechanisms and Biological Treatments. Mediators Inflamm
550 [Internet]. 2015 Jan 4 [cited 2015 Sep 8];2015:1–11. Available from:
551 http://www.hindawi.com/journals/mi/aa/493012/

552 11. Corridoni D, Arseneau KO, Cominelli F. Inflammatory bowel disease.
553 Immunol Lett [Internet]. 2014 Oct [cited 2016 Jan 12];161(2):231–5. Available
554 from:
555 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4401421&tool=pmce
556 ntrez&rendertype=abstract

557 12. Aujnarain A, Mack DR, Benchimol EI. The role of the environment in
558 the development of pediatric inflammatory bowel disease. Curr Gastroenterol
559 Rep [Internet]. 2013 Jun [cited 2014 Dec 12];15(6):326. Available from:
560 http://www.ncbi.nlm.nih.gov/pubmed/23640032

561 13. Tontini GE. Differential diagnosis in inflammatory bowel disease colitis:
562 State of the art and future perspectives. World J Gastroenterol [Internet]. 2015

563 Jan 7 [cited 2015 Jan 10];21(1):21. Available from:
564 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4284336&tool=pmce
565 ntrez&rendertype=abstract

566 14. Khan A, Fu H, Tan LA, Harper JE, Beutelspacher SC, Larkin DFP, et al.
567 Dendritic cell modification as a route to inhibiting corneal graft rejection by the
568 indirect pathway of allorecognition. Eur J Immunol [Internet]. 2013 Mar [cited
569 2015 Jun 12];43(3):734–46. Available from:
570 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3615172&tool=pmce
571 ntrez&rendertype=abstract

572 15. Najafian N, Sayegh MH. CTLA4-Ig: a novel immunosuppressive agent.
573 Expert Opin Investig Drugs [Internet]. 2000;9(9):2147–57. Available from:
574 http://www.ncbi.nlm.nih.gov/pubmed/11060799

575 16. Steurer W, Nickerson PW, Steele AW, Steiger J, Zheng XX, Strom TB.
576 Ex vivo coating of islet cell allografts with murine CTLA4/Fc promotes graft
577 tolerance. J Immunol [Internet]. 1995;155(3):1165–74. Available from:
578 http://www.ncbi.nlm.nih.gov/pubmed/7543517

579 17. Yang DF, Qiu WH, Zhu HF, Lei P, Wen X, Dai H, et al. CTLA4-Ig-
580 modified dendritic cells inhibit lymphocyte-mediated alloimmune responses and
581 prolong the islet graft survival in mice. Transpl Immunol [Internet]. 2008;19(3–
582 4):197–201. Available from:
583 http://www.ncbi.nlm.nih.gov/pubmed/18667318%5Cnhttp://ac.els-
584 cdn.com/S0966327408000294/1-s2.0-S0966327408000294-

585 main.pdf?_tid=1f04f6c0-df97-11e3-befe-
586 00000aab0f6b&acdnat=1400532771_9f1bf87c9da1bf8eeaae21083b16bd8f

587 18. Gilson CR, Milas Z, Gangappa S, Hollenbaugh D, Pearson TC, Ford
588 ML, et al. Anti-CD40 monoclonal antibody synergizes with CTLA4-Ig in
589 promoting long-term graft survival in murine models of transplantation. J
590 Immunol [Internet]. 2009 Aug 1 [cited 2015 Jun 16];183(3):1625–35. Available
591 from:
592 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2828346&tool=pmce
593 ntrez&rendertype=abstract

594 19. … ASITS in D, 2019 undefined. Disease Interception in Autoimmune
595 Diseases: From a Conceptual Framework to Practical Implementation.
596 books.google.com [Internet]. [cited 2019 Feb 20]; Available from:
597 https://books.google.com.br/books?hl=pt-
598 BR&lr=&id=uNyGDwAAQBAJ&oi=fnd&pg=PA1&dq=ctla4-
599 ig+tolerance+colitis&ots=NeTRlZ1bvJ&sig=3wf0H2u9tjKcXGtdgXrPzM9xPyI

600 20. Elson CO, Sartor RB, Tennyson GS, Riddell Ii RH, Gonçalves CCM,
601 Hernandes L, et al. Insights from advances in research of chemically induced
602 experimental models of human inflammatory bowel disease. World J
603 Gastroenterol. 1995;13(42):5581–93.

604 21. Oh SY, Cho K-A, Kang JL, Kim KH, Woo S-Y. Comparison of
605 experimental mouse models of inflammatory bowel disease. Int J Mol Med
606 [Internet]. 2014 Feb [cited 2016 Jan 6];33(2):333–40. Available from:

http://www.ncbi.nlm.nih.gov/pubmed/24285285

22. Gerlach K, Hwang Y, Nikolaev A, Atreya R, Dornhoff H, Steiner S, et al. TH9 cells that express the transcription factor PU.1 drive T cell-mediated colitis via IL-9 receptor signaling in intestinal epithelial cells. Nat Immunol [Internet]. 2014 Jul 8 [cited 2014 Dec 3];15(7):676–86. Available from: http://www.ncbi.nlm.nih.gov/pubmed/24908389

23. Paiatto LN, Silva FGD, Simioni PU, Yamada ÁT, Tamashiro WMSC, Simioni PU. Adoptive transfer of dendritic cells expressing CD11c reduces the immunological response associated with experimental colitis in BALB / c mice. Chamaillard M, editor. PLoS One [Internet]. 2018 May 8 [cited 2018 Sep 21];13(5):1–15. Available from: http://dx.plos.org/10.1371/journal.pone.0196994

24. Paiatto LNLN, Silva FGDFGDFGD, Bier J, Brochetto-Braga MR, Yamada ÁTÁT, Tamashiro WMSCWMSCSC, et al. Oral tolerance induced by OVA intake ameliorates TNBS-induced colitis in Mice. Ashour HM, editor. PLoS One [Internet]. 2017 Jan 18 [cited 2017 Jan 24];12(1):e0170205. Available from: http://dx.plos.org/10.1371/journal.pone.0170205

25. Matteoli G, Mazzini E, Iliev ID, Mileti E, Fallarino F, Puccetti P, et al. Gut CD103+ dendritic cells express indoleamine 2,3-dioxygenase which influences T regulatory/T effector cell balance and oral tolerance induction. Gut [Internet]. 2010 May [cited 2013 Jun 7];59(5):595–604. Available from: http://gut.bmj.com/cgi/doi/10.1136/gut.2009.185108

628 26. Mayer L, Kaser A, Blumberg RS. Dead on arrival: understanding the
629 failure of CTLA4-immunoglobulin therapy in inflammatory bowel disease.
630 Gastroenterology [Internet]. 2012 Jul [cited 2014 Dec 12];143(1):13–7. Available
631 from:
632 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3392152&tool=pmce
633 ntrez&rendertype=abstract

634 27. Qualls JE, Tuna H, Kaplan AM, Cohen D a. Suppression of experimental
635 colitis in mice by CD11c+ dendritic cells. Inflamm Bowel Dis [Internet]. 2009
636 Feb [cited 2014 Nov 24];15(2):236–47. Available from:
637 http://www.ncbi.nlm.nih.gov/pubmed/18839426

638 28. Linsley PS, Brady W, Urnes M, Grosmaire LS, Damle NK, Ledbetter
639 JA. CTLA-4 is a second receptor for the B cell activation antigen B7. J Exp Med
640 [Internet]. 1991;174(3):561–9. Available from:
641 http://www.ncbi.nlm.nih.gov/pubmed/1714933%0Ahttp://www.pubmedcentral.ni
642 h.gov/articlerender.fcgi?artid=PMC2118936

643 29. Simioni PUPUPU, Fernandes LGRLGRL, Gabriel DLL, Tamashiro
644 WMSCMSC. Effect of aging and oral tolerance on dendritic cell function.
645 Brazilian J … [Internet]. 2010 Jan [cited 2015 Mar 27];43(1):68–76. Available
646 from: http://www.ncbi.nlm.nih.gov/pubmed/19967261

647 30. Simioni PUPUU, Fernandes LGRGRLG, Tamashiro WMWMSCMWM.
648 Downregulation of L-arginine metabolism in dendritic cells induces tolerance to
649 exogenous antigen. Int J Immunopathol Pharmacol [Internet]. 2017 Nov 30 [cited

650 2016 Dec 2];30(1):44–57. Available from:
651 https://www.ncbi.nlm.nih.gov/pubmed/27903843

652 31. Lutz MB, Kukutsch N a., Menges M, Rössner S, Schuler G, Rößner S, et
653 al. Culture of bone marrow cells in GM-CSF plus high doses of
654 lipopolysaccharide generates exclusively immature dendritic cells which induce
655 alloantigen-specific CD4 T cell anergy in vitro. Eur J Immunol [Internet]. 2000
656 Apr [cited 2015 Feb 17];30(4):1048–52. Available from:
657 http://www.ncbi.nlm.nih.gov/pubmed/10760792

658 32. Simioni PUPUU, Fernandes LGRGRLG, Tamashiro WMSCMWM, Lutz
659 MB, Kukutsch N a., Menges M, et al. An advanced culture method for generating
660 large quantities of highly pure dendritic cells from mouse bone marrow. Eur J
661 Immunol [Internet]. 2017 Nov 1 [cited 2015 Feb 6];30(1):1048–52. Available
662 from: http://www.ncbi.nlm.nih.gov/pubmed/10037236

663 33. Lutz MB, Kukutsch N, Ogilvie AL, Rössner S, Koch F, Romani N, et al.
664 An advanced culture method for generating large quantities of highly pure
665 dendritic cells from mouse bone marrow. J Immunol Methods [Internet]. 1999
666 Feb 1 [cited 2015 Feb 6];223(1):77–92. Available from:
667 http://www.ncbi.nlm.nih.gov/pubmed/10037236

668 34. E Silva FGD, Paiatto LN, Yamada AT, Netto FM, Simioni PU,
669 Tamashiro WMSC. Intake of Protein Hydrolysates and Phenolic Fractions
670 Isolated from Flaxseed Ameliorates TNBS-Induced Colitis. Mol Nutr Food Res
671 [Internet]. 2018 Aug 9 [cited 2018 Aug 16];e1800088. Available from:

http://doi.wiley.com/10.1002/mnfr.201800088

35. Masterson JC, McNamee EN, Fillon SA, Hosford L, Harris R, Fernando SD, et al. Eosinophil-mediated signalling attenuates inflammatory responses in experimental colitis. Gut [Internet]. 2014 Sep 10 [cited 2015 Mar 9];gutjnl-2014-306998-. Available from: http://gut.bmj.com/content/early/2014/09/10/gutjnl-2014-306998.long

36. Zingarelli B, Szabó C, Salzman AL. Reduced oxidative and nitrosative damage in murine experimental colitis in the absence of inducible nitric oxide synthase. Gut [Internet]. 1999 Aug [cited 2015 Mar 9];45(2):199–209. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1727621&tool=pmcentrez&rendertype=abstract

37. van der Marel S, Majowicz A, Kwikkers K, van Logtenstein R, te Velde AA, De Groot AS, et al. Adeno-associated virus mediated delivery of Tregitope 167 ameliorates experimental colitis. World J Gastroenterol [Internet]. 2012 Aug 28 [cited 2015 Feb 18];18(32):4288–99. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3436043&tool=pmcentrez&rendertype=abstract

38. Shin J-S, Cho E-J, Choi H-E, Seo J-H, An H-J, Park H-J, et al. Anti-inflammatory effect of a standardized triterpenoid-rich fraction isolated from Rubus coreanus on dextran sodium sulfate-induced acute colitis in mice and LPS-induced macrophages. J Ethnopharmacol [Internet]. 2014 Dec 2 [cited 2015 Feb

18];158 Pt A:291–300. Available from: http://www.ncbi.nlm.nih.gov/pubmed/25446582

39. Neurath MF, Fuss I, Kelsall BL, Stüber E, Strober W. Antibodies to interleukin 12 abrogate established experimental colitis in mice. J Exp Med [Internet]. 1995 Nov 1 [cited 2016 Dec 2];182(5):1281–90. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7595199

40. Neurath MF, Fuss I, Kelsall BL, Presky DH, Waegell W, Strober W. Experimental granulomatous colitis in mice is abrogated by induction of TGF-beta-mediated oral tolerance. J Exp Med [Internet]. 1996 Jun 1 [cited 2015 Feb 18];183(6):2605–16. Available from: http://www.ncbi.nlm.nih.gov/pubmed/8676081

41. Thomé R, Fernandes LGRLGRL, Mineiro MFMFM, Simioni PUPU, Joazeiro PPPP, Tamashiro WM da SCWMSC. Oral tolerance and OVA-induced tolerogenic dendritic cells reduce the severity of collagen/ovalbumin-induced arthritis in mice. Cell Immunol [Internet]. 2012 Nov [cited 2015 Feb 5];280(1):113–23. Available from: http://linkinghub.elsevier.com/retrieve/pii/S0008874912002201

42. Horton C, Shanmugarajah K, Fairchild PJ. Harnessing the properties of dendritic cells in the pursuit of immunological tolerance [Internet]. Vol. 40, Biomedical Journal. 2017. p. 80–93. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6138597/

715 43. Pedersen AE, Schmidt EGW, Gad M, Poulsen SS, Claesson MH.
716 Dexamethasone/1α-25-dihydroxyvitamin D3-treated dendritic cells suppress
717 colitis in the SCID T-cell transfer model. Immunology [Internet]. 2009 Jul [cited
718 2016 Dec 2];127(3):354–64. Available from:
719 http://www.ncbi.nlm.nih.gov/pubmed/19019085

720 44. Pedersen AE, Gad M, Kristensen NN, Haase C, Nielsen CH, Claesson
721 MH. Tolerogenic dendritic cells pulsed with enterobacterial extract suppress
722 development of colitis in the severe combined immunodeficiency transfer model.
723 Immunology [Internet]. 2007 Aug [cited 2013 Jul 24];121(4):526–32. Available
724 from: http://www.ncbi.nlm.nih.gov/pubmed/17428312

725 45. Rochman Y, Yukawa M, Kartashov A V, Barski A. Functional
726 characterization of human T cell hyporesponsiveness induced by CTLA4-Ig.
727 PLoS One [Internet]. 2015 Jan [cited 2015 Jun 15];10(4):e0122198. Available
728 from:
729 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4393265&tool=pmce
730 ntrez&rendertype=abstract

731 46. Mayer E, Hölzl M, Ahmadi S, Dillinger B, Pilat N, Fuchs D, et al.
732 CTLA4-Ig immunosuppressive activity at the level of dendritic cell/T cell
733 crosstalk. Int Immunopharmacol [Internet]. 2013 Mar [cited 2013 Jun
734 23];15(3):638–45. Available from:
735 http://dx.doi.org/10.1016/j.intimp.2013.02.007

736 47. Ko HJ, Cho M La, Lee SY, Oh HJ, Heo YJ, Moon YM, et al. CTLA4-Ig

737 modifies dendritic cells from mice with collagen-induced arthritis to increase the
738 CD4+CD25+Foxp3+ regulatory T cell population. J Autoimmun.
739 2010;34(2):111–20.

740 48. Loeuillard E, Bertrand J, Herranen A, Melchior C, Guérin C, Coëffier M,
741 et al. 2,4,6-trinitrobenzene sulfonic acid-induced chronic colitis with fibrosis and
742 modulation of TGF-β1 signaling. World J Gastroenterol [Internet]. 2014 Dec 28
743 [cited 2016 Jan 5];20(48):18207–15. Available from:
744 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=4277958&tool=pmce
745 ntrez&rendertype=abstract

746 49. Wu F, Chakravarti S. Differential Expression of Inflammatory and
747 Fibrogenic Genes and Their Regulation by NF- B Inhibition in a Mouse Model of
748 Chronic Colitis. J Immunol [Internet]. 2007 Nov 2 [cited 2016 Jan
749 5];179(10):6988–7000. Available from:
750 http://www.jimmunol.org/content/179/10/6988.full

751 50. Miura S, Hokari R, Tsuzuki Y. Mucosal immunity in gut and lymphoid
752 cell trafficking. Ann Vasc Dis [Internet]. 2012 Jan [cited 2016 Jan 7];5(3):275–
753 81. Available from:
754 http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3595844&tool=pmce
755 ntrez&rendertype=abstract

756 51. Guo X, Wang WP, Ko JKS, Cho CH. Involvement of neutrophils and
757 free radicals in the potentiating effects of passive cigarette smoking on
758 inflammatory bowel disease in rats. Gastroenterology [Internet]. 1999 Oct 10

759 [cited 2016 Jan 5];117(4):884–92. Available from:
760 http://www.gastrojournal.org/article/S0016508599703471/fulltext

761 52. Seo HG, Takata I, Nakamura M, Tatsumi H, Suzuki K, Fujii J, et al.
762 Induction of nitric oxide synthase and concomitant suppression of superoxide
763 dismutases in experimental colitis in rats. Arch Biochem Biophys [Internet].
764 1995 Dec 1 [cited 2016 Jan 5];324(1):41–7. Available from:
765 http://www.ncbi.nlm.nih.gov/pubmed/7503557

766 53. Yue G, Lai PS, Yin K, Sun FF, Nagele RG, Liu X, et al. Colon epithelial
767 cell death in 2,4,6-trinitrobenzenesulfonic acid-induced colitis is associated with
768 increased inducible nitric-oxide synthase expression and peroxynitrite
769 production. J Pharmacol Exp Ther [Internet]. 2001 Jun [cited 2016 Jan
770 5];297(3):915–25. Available from:
771 http://www.ncbi.nlm.nih.gov/pubmed/11356911

772 54. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic
773 self-tolerance and negative control of immune responses. Annu Rev Immunol
774 [Internet]. 2004;22:531–62. Available from:
775 http://arjournals.annualreviews.org/doi/full/10.1146/annurev.immunol.21.120601
776 .141122?url_ver=Z39.88-
777 2003&rfr_id=ori:rid:crossref.org&rfr_dat=cr_pub%3Dpubmed

778 55. Campbell DJ, Koch MA. Phenotypical and functional specialization of
779 FOXP3+ regulatory T cells. Nat Rev Immunol [Internet]. 2011;11(2):119–30.
780 Available from: http://www.nature.com/doifinder/10.1038/nri2916

781 56. Wohlfert EEA, Grainger JRJ, Bouladoux N, Konkel JE, Oldenhove G,
782 Ribeiro CH, et al. GATA3 controls Foxp3+ regulatory T cell fate during
783 inflammation in mice. J Clin … [Internet]. 2011;121(11):4503–15. Available
784 from: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3204837/

785 57. Kaplan MH. Th9 cells: Differentiation and disease. Immunol Rev
786 [Internet]. 2013;252(1):104–15. Available from:
787 https://onlinelibrary.wiley.com/doi/full/10.1111/imr.12028?sid=nlm%3Apubmed

788 58. Elyaman W, Bradshaw EM, Uyttenhove C, Dardalhon V, Awasthi A,
789 Imitola J, et al. IL-9 induces differentiation of TH17 cells and enhances function
790 of FoxP3+ natural regulatory T cells. Proc Natl Acad Sci U S A [Internet]. 2009
791 Aug 4 [cited 2016 Aug 5];106(31):12885–90. Available from:
792 http://www.ncbi.nlm.nih.gov/pubmed/19433802

793 59. Gerlach K, McKenzie AN, Neurath MF, Weigmann B. IL-9 regulates
794 intestinal barrier function in experimental T cell-mediated colitis. Tissue barriers
795 [Internet]. 2015 Apr 3 [cited 2016 Sep 29];3(1–2):e983777. Available from:
796 http://www.ncbi.nlm.nih.gov/pubmed/25838986

797 60. Bishehsari F, Magno E, Swanson G, Desai V, Voigt RM, Forsyth CB, et
798 al. Alcohol and Gut-Derived Inflammation. Alcohol Res Curr Rev [Internet].
799 2017;38(2):e1-9. Available from:
800 https://www.arcr.niaaa.nih.gov/arcr382/article01.pdf

801 61. Berman D, Parker SM, Siegel J, Chasalow SD, Weber J, Galbraith S, et

al. Blockade of cytotoxic T-lymphocyte antigen-4 by ipilimumab results in dysregulation of gastrointestinal immunity in patients with advanced melanoma. Cancer Immun [Internet]. 2010 Jan [cited 2016 Jan 5];10:11. Available from: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2999944&tool=pmcentrez&rendertype=abstract

62. Daud A. Current and Emerging Perspectives on Immunotherapy for Melanoma. Semin Oncol [Internet]. 2015 Dec [cited 2015 Nov 27];42:S3–11. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26598057

63. Quirk SK, Shure AK, Agrawal DK. Immune-mediated adverse events of anticytotoxic T lymphocyte-associated antigen 4 antibody therapy in metastatic melanoma. Transl Res [Internet]. 2015 Nov [cited 2016 Jan 5];166(5):412–24. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26118951

64. Kawalec P, Mikrut A, Łopuch S. Systematic review of the effectiveness of biological therapy for active moderate to severe ulcerative colitis. J Gastroenterol Hepatol [Internet]. 2014 Jun [cited 2016 Jan 6];29(6):1159–70. Available from: http://doi.wiley.com/10.1111/jgh.12563

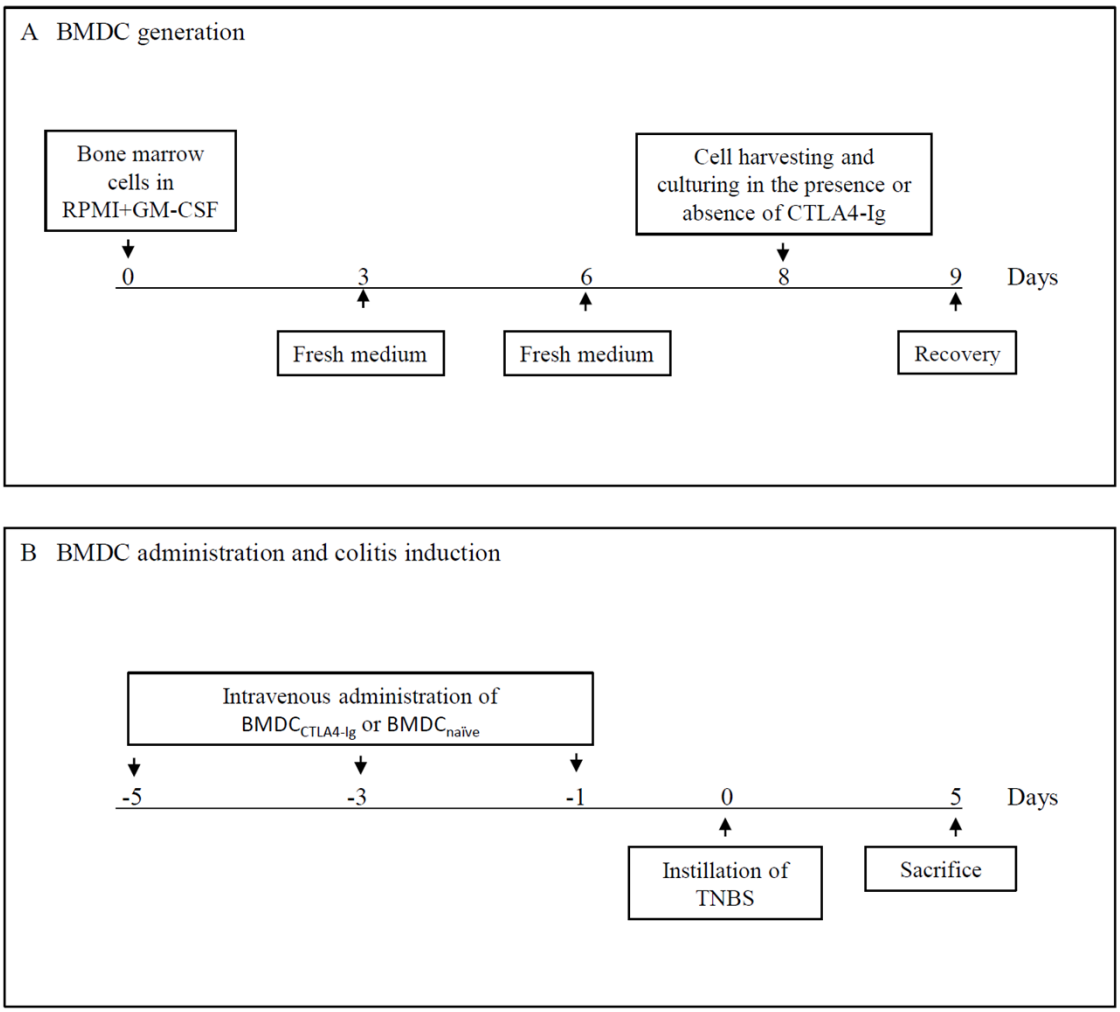
65. Cools N, Petrizzo A, Smits E, Buonaguro FM, Tornesello ML, Berneman Z, et al. Dendritic cells in the pathogenesis and treatment of human diseases: a Janus Bifrons? Immunotherapy [Internet]. 2011 Oct [cited 2015 Feb 17];3(10):1203–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/21995572

823 66. Harden JL, Egilmez NK. Indoleamine 2,3-Dioxygenase and Dendritic
824 Cell Tolerogenicity [Internet]. Immunological Investigations NIH Public Access;
825 Aug 27, 2012 p. 738–64. Available from:
826 http://www.ncbi.nlm.nih.gov/pubmed/23017144

827 67. Wang J, Dai X, Hsu C, Ming C, He Y, Zhang J, et al. Discrimination of
828 the heterogeneity of bone marrow-derived dendritic cells. Mol Med Rep.
829 2017;16(5):6787–93.

831 Figures

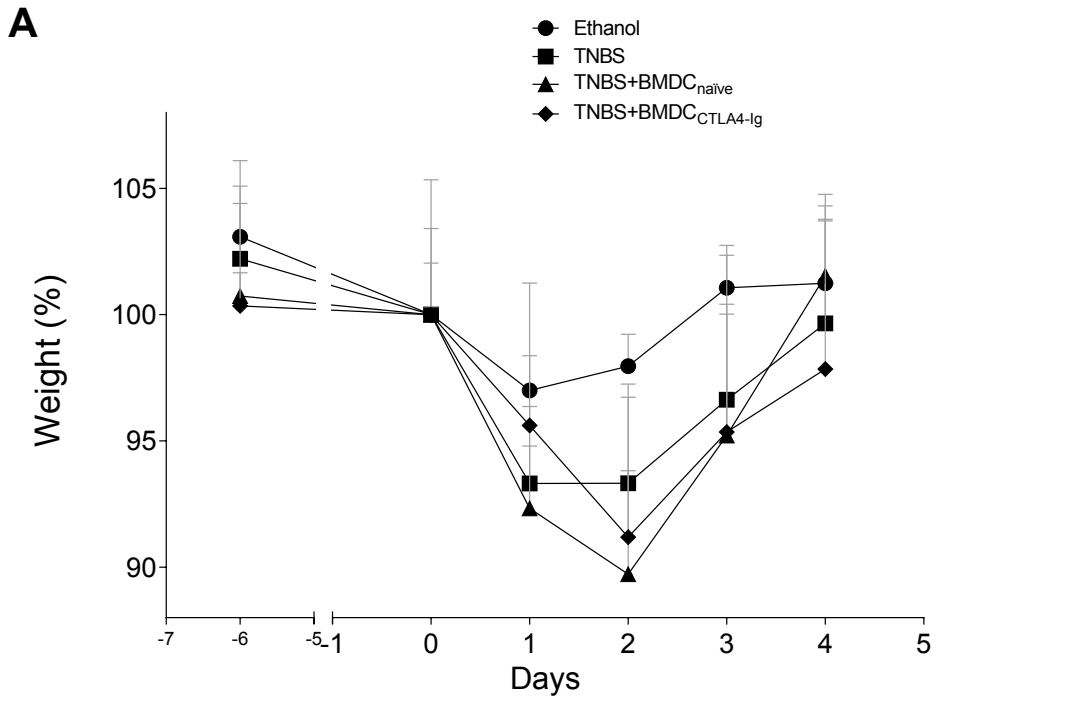
832 Figure 1

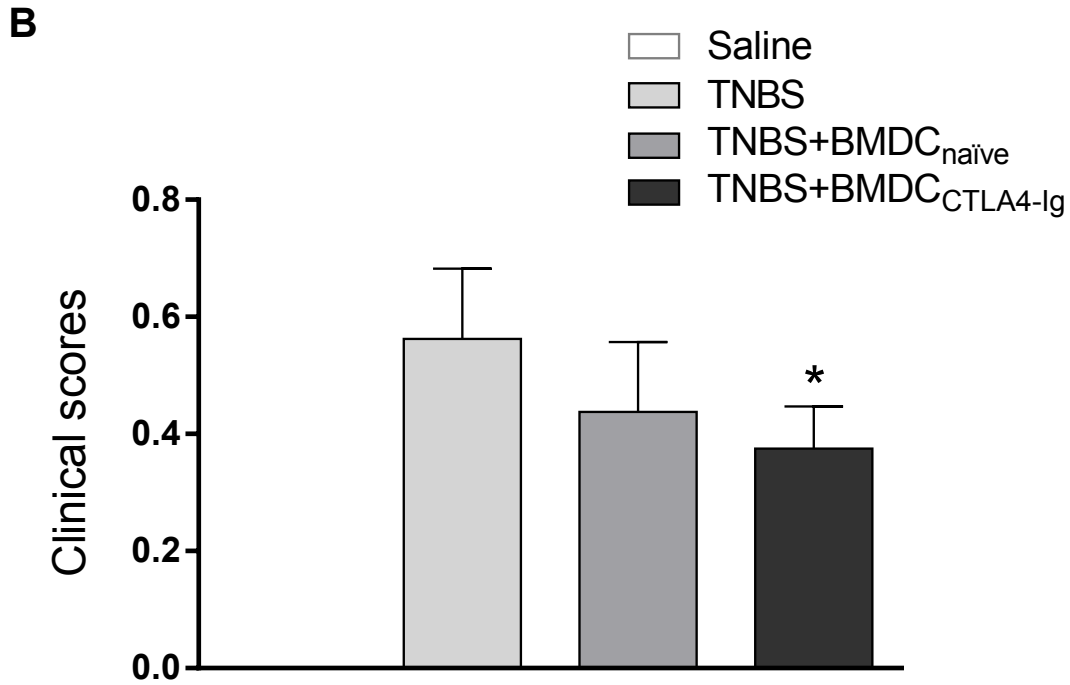


833

834

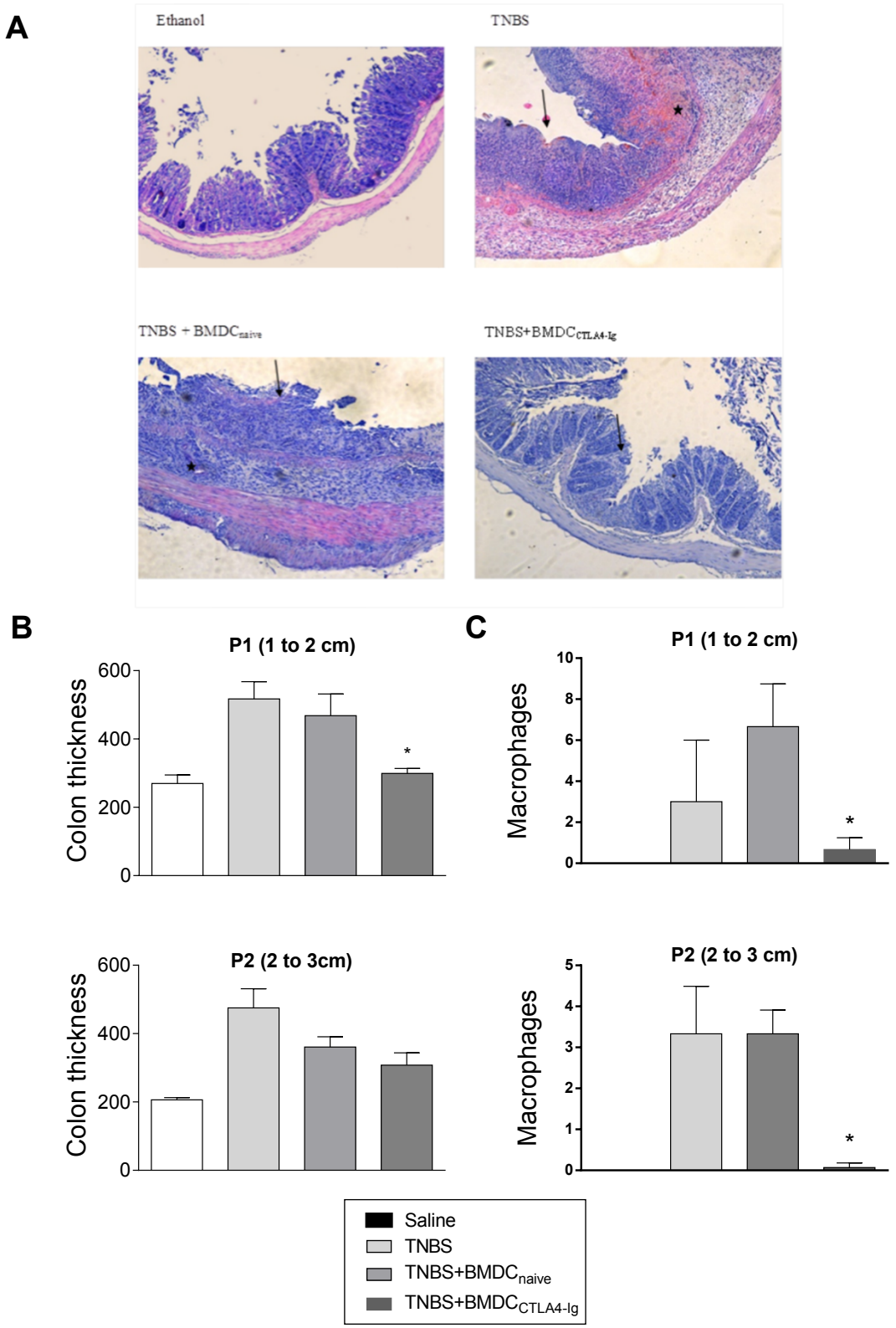
836 Figure 2:





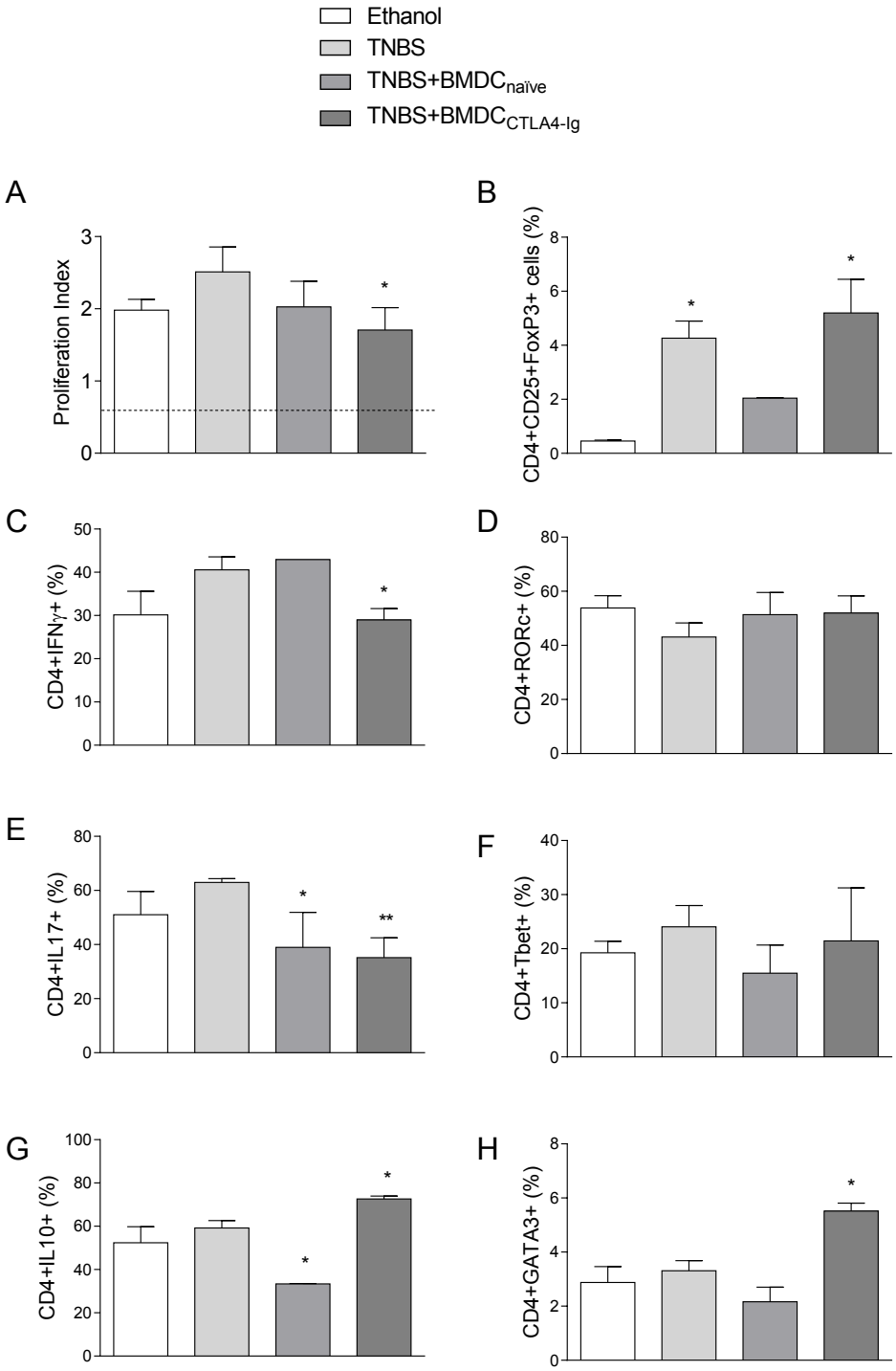
837

839 Figure 3:



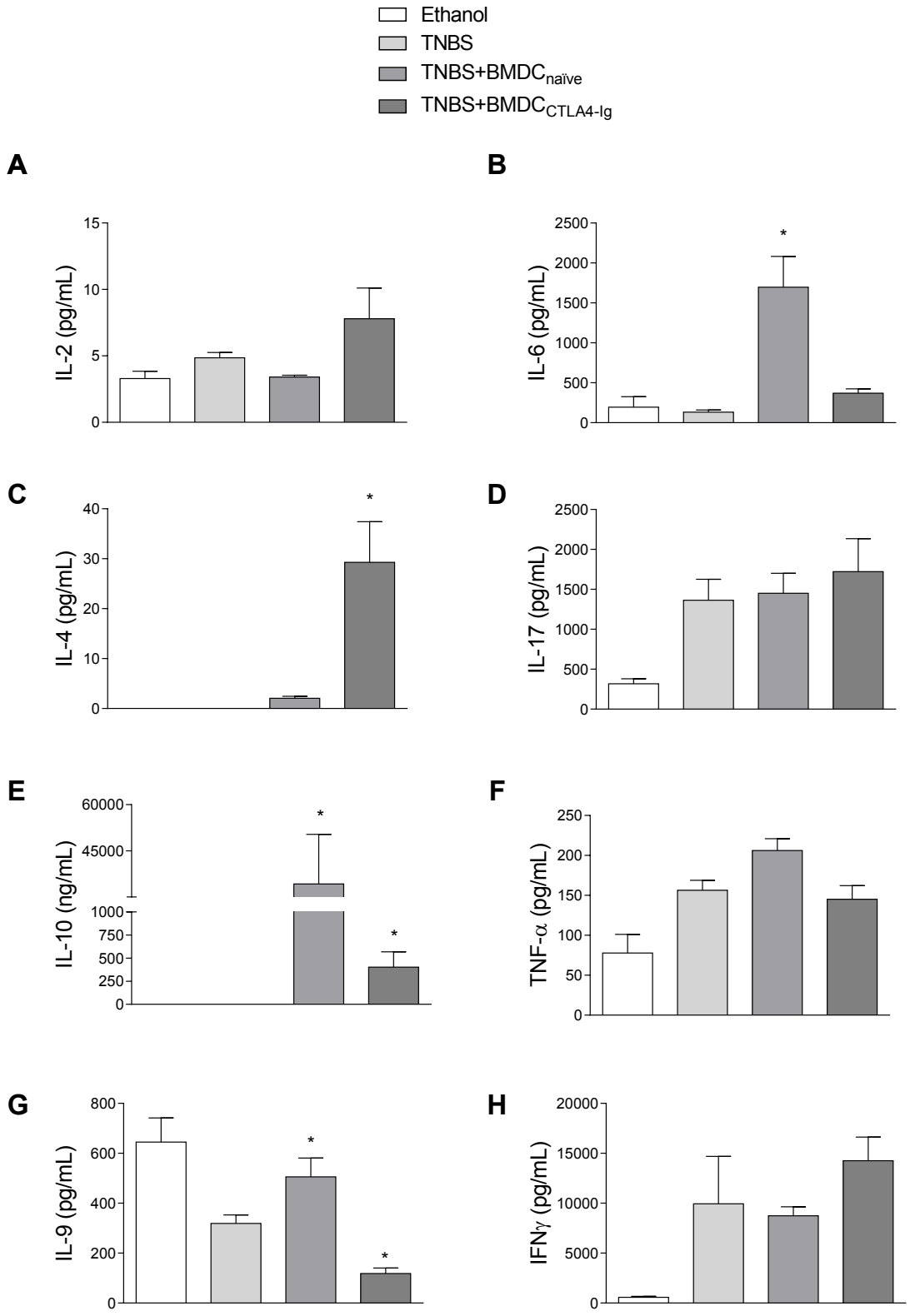
840 42

841 Figure 4:



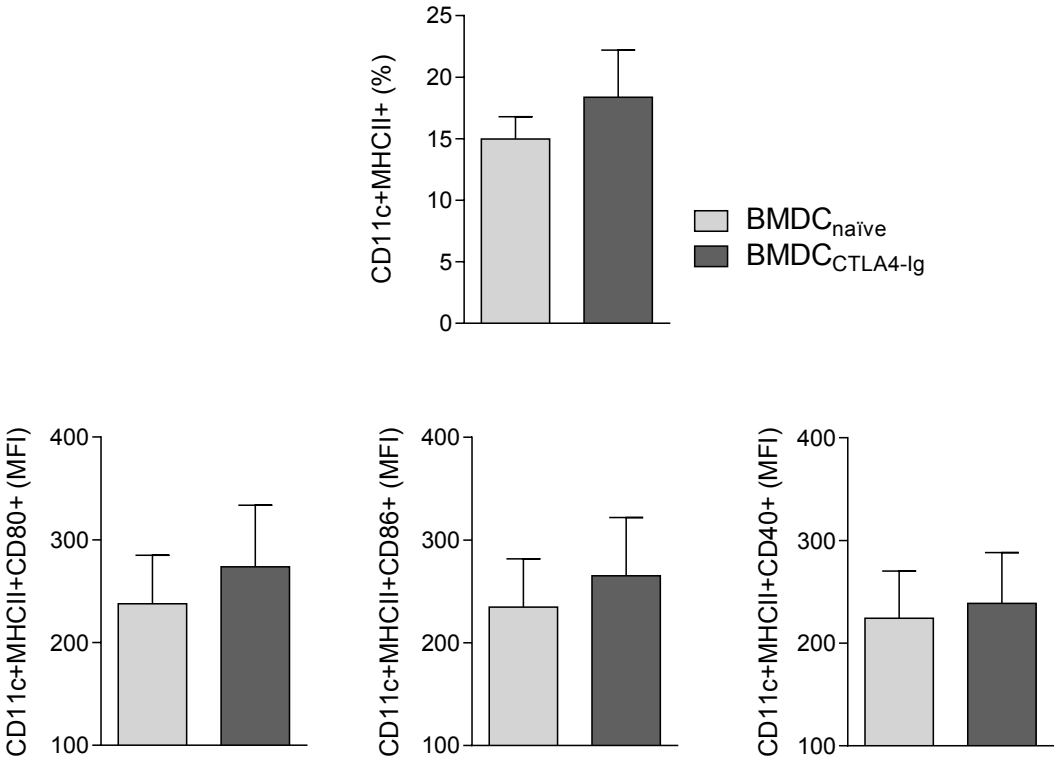
842

843 Figure 5



844

845 Supplementary figure 1



846

847

848