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Role of regulatory T cells in irinotecan-induced intestinal mucositis

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ABSTRACT

Intestinal mucositis (IM) is a common side effect of irinotecan-based chemotherapy. The involvement of inflammatory mediators, such as TNF-α, IL1-β, IL-18 and IL-33, has been demonstrated. However, the role of adaptive immune system cells, whose activation is partially regulated by these cytokines, is yet unknown. Thus, we investigated the role of regulatory T cells (Tregs) in irinotecan-induced IM. C57BL/6 mice were injected with saline or irinotecan (75 mg kg^{-1}, i.p.), once a day for 4 days, and euthanized at day 1, 3, 5 or 7 following the first dose of irinotecan. For Treg depletion, the mice were pretreated with a low single dose of cyclophosphamide (100 mg kg^{-1}, i.p). Intestinal lamina propria lymphocytes were harvested and purified by Percoll gradient. Treg and Th17 cells were identified by flow cytometry. Blood leukocyte count was obtained and ileum samples were collected for histopathological analysis and myeloperoxidase assay. IM caused an accumulation of Tregs and Th17 cells over time. Treg depletion exacerbated intestinal damage, diarrhea, neutrophil infiltration and animal mortality, despite a reduction in Th17 cell number. The frequency of other Th cells increased and was positively correlated with neutrophil infiltration. Tregs showed a negative correlation with neutrophils and the frequency of non-regulatory Th cells. In conclusion, Tregs are important in the control of intestinal damage induced by irinotecan, and their depletion showed a deleterious effect on IM. Activation of these cells appears to be a compensatory mechanism for intestinal inflammation.

1. Introduction

Irinotecan is a semi-synthetic prodrug that inhibits topoisomerase I and is active as the first-line therapy for several cancer types, including colorectal cancer (Campbell et al., 2016). Intestinal mucositis is a common side-effect of cancer chemotherapy and is present in approximately 50–80% of patients, depending on the treatment regime (Touchefeu et al., 2014). Diarrhea is the main sign of mucositis, which negatively affects the quality of life of patients and increases health care costs (Andreyev et al., 2014; Touchefeu et al., 2014; Van Sebille et al., 2015; Ribeiro et al., 2016). Among patients with cancer using irinotecan, 6–47% present with more severe forms of diarrhea, including grades 3 and 4 (Andreyev et al., 2014).

Unfortunately, the pathophysiology of intestinal mucositis has not been fully elucidated. It has been speculated that the first insult is due to a direct cytotoxic effect of Irinotecan and its active metabolite SN-38, which causes apoptosis of intestinal crypt cells (Ribeiro et al., 2016). Cell injury releases damage-associated molecular patterns (DAMPs), which are recognized by pattern-recognition receptors such as toll-like receptors (TLR) on innate immune cells and epithelial cells contributing to pro-inflammatory cytokine production (Newton and Dixit, 2012; Wong et al., 2015; Ribeiro et al., 2016). Furthermore, breakdown of the epithelial intestinal cell lining enables bacterial translocation to the intestinal lamina propria, which also activates immune and epithelial cells (Lee et al., 2014; Wong et al., 2015; Ribeiro et al., 2016), amplifying neutrophil influx and tissue damage (Wong et al., 2015; Ribeiro

Abbreviations: IM, intestinal mucositis; Treg, regulatory T cells; Th, T helper cell; DAMPs, damage-associated molecular patterns; TLR, Toll Like Receptors; DSS, dextran sodium sulfate; MPO, myeloperoxidase; FOXP3, Forkhead box P3; ROR, related orphan receptor

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et al., 2016). Despite the role of inflammatory mediators, for instance, TNF-α, IL-1β, IL-18, IL-33, and the innate immune system in intestinal mucositis (Melo et al., 2008; Lima-Júnior et al., 2012; Lima-Júnior et al., 2014; Guabiraba et al., 2014; Wong et al., 2015), the involvement of adaptive immune cells in this model has not yet been evaluated.

In this context, the balance between the T helper 1 (Th1), Th2 and/or Th17 inflammatory reaction and the T regulatory (Treg) cell anti-inflammatory response appears to be critical (Matricon et al., 2010). The contribution of T cells has been shown in other models of inflammatory bowel disease, such as Crohn's disease, ulcerative colitis, dextran sodium sulfate (DSS)-induced colitis and others (Podolsky, 2002; Hartog et al., 2015; Gálvez, 2014; Ueno et al., 2015). In Crohn's disease, the IL-18- and IL-12-driven Th1 cell response in the intestine plays a pathogenic role in the injury process. This finding is accompanied by a decrease in the frequency of Tregs (Podolsky, 2002). However, the increase in Tregs is related to an amelioration of the disease mediated by dietary non-digestible polysaccharides (Hartog et al., 2015). In addition, the adoptive transfer of murine Tregs improves the symptoms of colitis induced by DSS (Hsu et al., 2013).

Considering that Tregs can suppress a wide range of immune cells, which is important for maintaining homeostasis (Sakaguchi et al., 2009), we investigated the role of Tregs in the pathogenesis of experimental irinotecan-induced intestinal mucositis.

2. Materials and methods

2.1. Animals

C57BL/6 male mice, weighing 20–22 g, were randomly divided into experimental groups and maintained in a temperature-controlled room under a dark-light cycle, with water and food provided *ad libitum*. This mouse strain was selected due to the adequate reproducibility of experimental model of intestinal mucositis induced by irinotecan. Moreover, most of previous data on the pathophysiology of mucositis was obtained with this mouse strain (Lima-Júnior et al., 2012; Lima-Júnior et al., 2014; Wong et al., 2015). Mice were randomly divided into experimental groups (n = 10 animals/group). Due to the animal mortality of about 50% in the groups that were injected with irinotecan, the results are shown for 6–10 mice per group, which is represented in each figure. This paper adheres to the principles for transparent reporting and scientific rigor of preclinical research as set out in the UK Concordat on Openness on Animal Research, the USA NIH Guidelines on reporting preclinical research, the ARRIVE Guidelines, and the Guidelines for the Care and Use of Laboratory Animals (McGrath et al., 2010). All efforts were made in order to minimize animal suffering. In the survival study, the animals were monitored twice daily for fourteen days following the first injection of irinotecan. During the experiment, the animals succumb due to the treatment and its consequences, including diarrhea. Those animals that showed signs of imminent death, including piloerection, reduced locomotion, inability to maintain upright position, ataxia, tremor and altered breath frequency were euthanized by ketamine/xylazine overdose (> 100/10 mg kg^{-1}, s.c., União Química, São Paulo, Brazil) followed by cervical dislocation. Pain relievers or anesthesia were not used in our experiments since those agents directly interfere with the production of inflammatory mediators and/or alter the gastrointestinal transit and mask the diarrheic events in this animal model. At the end of the survival experiment, live animals were euthanized by ketamine/xylazine overdose (> 100/10 mg kg^{-1}, s.c., União Química, São Paulo, Brazil) followed by cervical dislocation. The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Federal University of Ceará (Number: 75/2013).

2.2. Drugs

Irinotecan hydrochloride (Evoterin, Evolabis, São Paulo, Brazil, 100 mg ampoule), Cyclophosphamide (Genuxal, Baxter, Halle/Westfalen, Germany, 200 mg ampoule) and sterile saline were used.

2.3. Induction of experimental intestinal mucositis

Experimental intestinal mucositis was induced according to the protocol of Ikuno et al. (1995), which was adapted for our experimental conditions. Briefly, the mice received irinotecan (75 mg kg^{-1}/day, i.p) for four days or saline (3.5 mL kg^{-1}, i.p.) as a control. For the depletion of regulatory T cells, the mice received a low single dose of cyclophosphamide (100 mg kg^{-1}, i.p), 2 h before the first administration of irinotecan. The diarrhea score and body weight were measured on all days until the animal was euthanized. After anesthesia and blood collection *via* the retro-orbital plexus, the mice were euthanized on day 1, 3, 5 or 7 by cervical dislocation. The blood leukocyte count was performed using a cell counter and expressed as cells × 10^3/L of blood. The small intestine was removed for lymphocyte isolation, and ileum samples were stored for morphometric and histopathological analysis and myeloperoxidase (MPO) activity assay. To study animal survival, the mice were observed until the 14th day following the first injection of irinotecan.

2.4. Diarrhea assessment

Diarrhea was blinded evaluated on all days and scored as described by Kurita et al. (2000): 0-normal stool or absent; 1-slightly wet and soft stool; 2-wet and unformed stool with moderate perianal staining of the coat; and 3-watery stool with severe perianal staining of the coat.

2.5. Isolation of lamina propria lymphocytes from the small intestine

The isolation of mouse lymphocytes from the small intestine was performed according to the protocol of Sheridan and Lefrançois (2012). To achieve this, the small intestine was removed and placed in a buffer containing Hanks balanced salt solution, HEPES-bicarbonate and 5% fetal bovine serum (CMF buffer) at 4 °C. The remaining fat and connective tissues were carefully removed for optimal lymphocyte yield. Next, Peyer's patches were removed and discarded. After removal of fecal matter and mucus, the intestine was opened longitudinally along its entire length and cut into pieces of 0.5 mm. The intestinal pieces were washed in CMF buffer until the supernatants were clear. The remaining tissue was incubated twice with 25 mL EDTA buffer 1 (Hanks balanced salt solution, HEPES-bicarbonate, 10% fetal bovine serum and EDTA 0.1 Mm) in 50-ml Erlenmeyer flask on a magnetic stirrer for 20 min at 37 °C and ~220 rpm. The supernatant was discarded, and the tissue was then incubated twice with 25 mL EDTA buffer 2 (Hanks balanced salt solution, HEPES, L-glutamine, penicillin/streptomycin, and gentamycin EDTA 1.3 mM) on a magnetic stirrer for 30 min at 37 °C and ~220 rpm. The supernatant was discarded, and after washing, the tissue was digested enzymatically with collagenase solution for 45 min at 37 °C and ~220 rpm.

The entire contents of the Erlenmeyer flask were decanted into a 70-μm cell strainer, and the pellet resultant was washed twice with 10 mL of culture medium. Lymphocytes were obtained in the 44% to 67% interface of the Percoll gradient. The viable cells were quantified using Trypan blue dye exclusion.

2.6. Immunophenotyping

The antibodies were previously titrated, and the best concentration, 1:100 dilution, was chosen based on the highest percentage of positive cells and mean fluorescence intensity (MFI). To identify the frequency of regulatory T cells and Th17 cells, the lymphocytes of intestinal lamina propria, 1 × 10^6 cells/tube, were incubated with anti-CD3 FITC/APC (145-2C11 clone), anti-CD4 PerCP (RM4-5 clone), anti-CD25 FITC (3C7 clone), anti-FOXP3 PE (FJK-16s clone), anti-ROR-gt PE (AFKJS-9

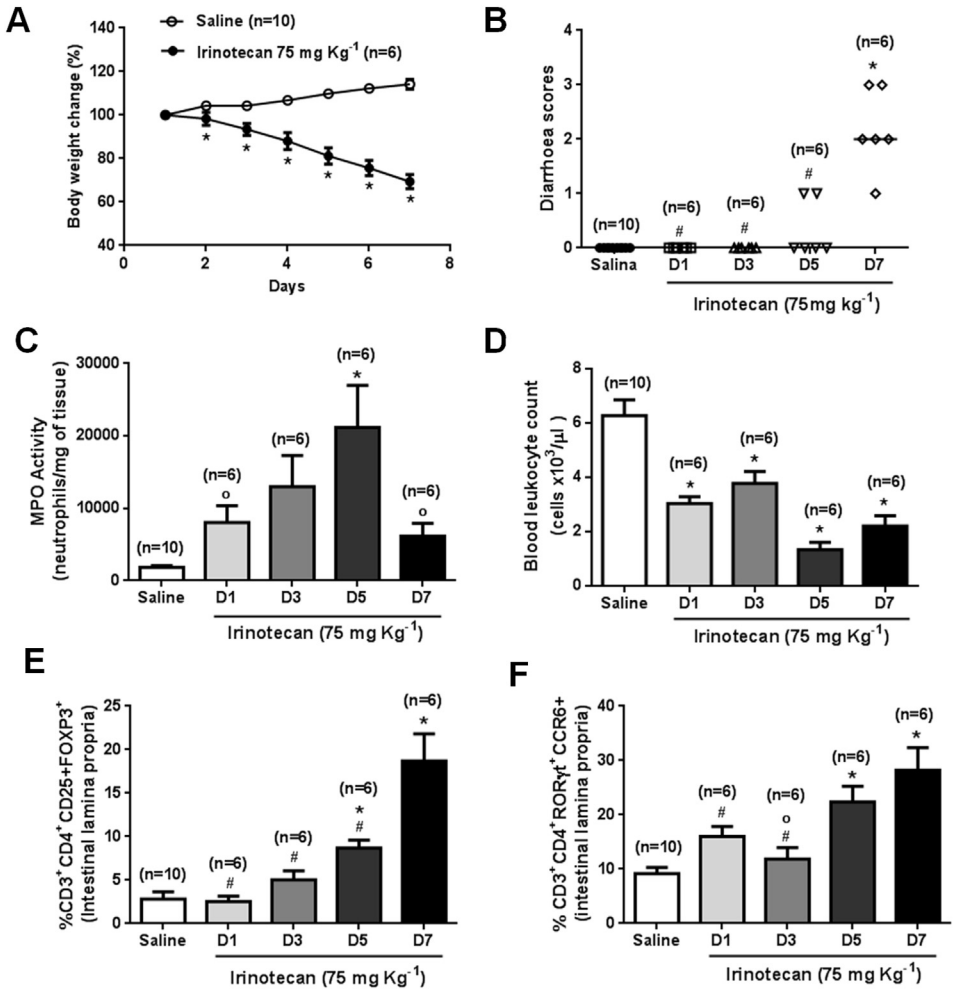

Fig. 1. Irinotecan induces intestinal mucositis in a time-dependent manner. Mice were injected with irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce intestinal mucositis and were euthanized on day 1 (D1), D3, D5 or D7. The control group received only saline as a vehicle. Irinotecan-injected mice showed progressive loss of body weight (Panel A), severe diarrhea (Panel B), and intestinal accumulation of neutrophils (MPO activity), which peaked at day 5 (Panel C). However, neutrophil infiltration was markedly reduced on day 7 (Panel C). The animals also presented with leukopenia (Panel D) and increased frequency of Tregs (Panel E) and Th17 (Panel F) in the lamina propria of the small intestine. $^{*}P < 0.05$ *vs.* the saline group; $^{\circ}P < 0.05$ *vs.* D5; $^{\#}P < 0.05$ *vs.* D7.

clone) (all of BD Biosciences, USA) and CCR6 Alexa 647 (29-2L17 clone, Biolegend, USA) or with their isotype controls for 30 min at 4 °C in the dark. Intracellular labeling of FOXP3 and ROR-γt was performed according to the specifications of the manufacturer. After several washes, the cells were fixed in 1% paraformaldehyde and stored at 4 °C for up to 24 h. The data were collected using a FACSCalibur flow cytometer (BD Biosciences), with a measurement of up to 100,000 events per sample in the lymphocyte gate. The results were analyzed using the FlowJo program (version 7.6.5; Treestar US, Ashland, OR). The quadrants were defined based on isotype controls.

2.7. Myeloperoxidase activity assay (MPO)

Ileum samples were resuspended in a buffer containing 0.02 M $NaPO_4$, 0.1 M NaCl and 0.015 M Na_2 EDTA (pH 4.7), homogenized and centrifuged at 800 × *g* for 15 min at 4 °C. The pellet was lysed with hypotonic solution (0.2% NaCl buffer). After a centrifugation step, the pellet was suspended in 300 µL of 0.05 M NaPO4 buffer, pH 5.4, containing 0.5% hexadecyltrimethyl-ammonium bromide (HTAB, Sigma) and homogenized. The reaction was developed with the addition of a color reagent tetramethylbenzidine (1.6 mM) and enzyme substrate H_2O_2 (0.5 mM). The reaction was stopped with a 2 M H_2SO_4 solution, and the reading was performed at 450 nm in a spectrophotometer. The absorbance was plotted in a standard curve of mouse peritoneal neutrophils processed in the same manner. The data obtained were expressed as the number of neutrophils/mg of tissue.

2.8. Histopathological and morphometric analyses

The ileum samples were fixed in 10% neutral buffered formalin, dehydrated, and embedded in paraffin. Sections of 5 µm thickness were obtained for hematoxylin-eosin staining (H&E) and subsequent examination by light microscopy for histopathological and morphometric analyses. The length of the intestinal villi and crypt was measured using ImageJ 1.4 software (NIH–National Institute of Health, Bethesda, MD, USA) for the villus/crypt ratio. Ten villi were measured per slice. Mucosal damage was also assessed in a blind manner using the histopathological score system described by MacPherson and Pfeiffer (1978) (Score 0-normal histological findings; Score 1-mucosa: villus blunting, loss of crypt architecture, sparse inflammatory cell infiltration, vacuolization and edema. Muscle layer: normal; Score 2-mucosa: villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization and edema. Muscle layer: normal; Score 3-mucosa: villus blunting with fattened and vacuolated cells, crypt necrosis, intense inflammatory cell infiltration, vacuolization and edema. Muscle layer: edema, vacuolization and neutrophilic infiltration).

2.9. Statistical analysis

One-way ANOVA followed by Tukey's test was used for parametric data, which were expressed as the mean ± standard error of the mean (S.E.M.). The non-parametric data were expressed as the median values and analyzed by Kruskal-Wallis followed by Dunn's test. The Pearson Test was used for the correlations analysis. The Mantel-Cox log rank test

was used to determine differences between survival curves. Statistical significance was accepted when $P < 0.05$. The statistical analysis was performed with the GraphPad Prism software version 6.0 (La Jolla, CA, USA).

3. Results

3.1. Irinotecan injection induced intestinal mucositis accompanied by an accumulation of regulatory T cells and Th17 lymphocytes over time

The induction of intestinal mucositis was characterized by a significant body weight change starting from the second day, with an approximate 30% reduction observed on the seventh day compared to the control group (Fig. 1A). Diarrhea was also observed mainly on the seventh day of mucositis development (Fig. 1B). However, neutrophil infiltration peaked at day 5, which was reduced at day 7 (Fig. 1C). In addition, irinotecan-injected animals showed sustained leukopenia over the entire experimental period when compared to saline (Fig. 1D).

Treg frequency in intestinal lamina propria, which is characterized by positivity for CD3, CD4, CD25 and FOXP3 markers, showed a three-fold and a seven-fold increase, respectively, on days 5 and 7 in irinotecan-injected animals compared to mice that received saline (Fig. 1E). The increase in Treg frequency on the seventh day occurred only in the $CD25^+$ subset of cells. As observed in Fig. 3A, no differences between the frequency of $CD3^+CD4^+FOXP3^+$ Tregs, which included $CD25^+$ and $CD25^-$ cells, compared to saline were found. Furthermore, we observed a reduction in the percentage of $CD25^-$ Tregs (Fig. 3C). The mean fluorescence intensity of CD25 also increased compared to the control group (Fig. 3B, D and E).

The percentage of Th17 cells increased on the fifth day (a two-fold increase) and on the seventh day of mucositis development compared to saline (three-fold increase) (Fig. 1F). Intestinal damage also increased over time, peaking on the seventh day, as confirmed by histopathology (Fig. 2A), morphometric analysis (Fig. 2B) and histopathological scores (Fig. 2C).

3.2. Low dose of cyclophosphamide depletes Tregs in the small intestine

As shown in Fig. 4, a single injection of CYC at a low dose 2 h before the first dose of irinotecan caused a significant reduction (92%) in lamina propria intestinal Tregs on the seventh experimental day compared to mice receiving IRI only (Fig. 4A and B). In addition, CYC + IRI-treated animals showed a partial reduction (approximately 40%) in the frequency of Th17 cells (Fig. 4A and C). We also observed a significant depletion of systemic Tregs seven days after CYC administration, as demonstrated in spleen samples of mice pretreated with CYC compared to mice receiving irinotecan only (CYC + IRI: 1.4 ± 0.6 *vs.* IRI: 7.3 ± 2.8, $P < 0.01$, data not shown).

3.3. Treg depletion enhances irinotecan-induced intestinal mucositis

Fig. 5A shows that Treg-depleted animals receiving irinotecan presented a reduced survival (100% mortality on the ninth day) compared to non-depleted mice that were also injected with irinotecan (50% survival until the end of the follow-up period). In addition, signs of clinical irinotecan-related intestinal mucositis were more prominent in mice pretreated with CYC (Fig. 5B–E). The CYC + IRI-treated mice showed a more pronounced weight loss than those receiving IRI only (Fig. 5B). In addition, the severity of diarrhea and neutrophil accumulation was enhanced in the CYC + IRI group (Fig. 5C and D, respectively). Furthermore, all of the groups that received irinotecan presented marked leukopenia compared with the saline-injected group (Fig. 5E). The enhancement of mucositis severity was confirmed by histopathological analysis (Fig. 6). IRI-treated mice presented a significant reduction in the villus/crypt ratio compared with the saline group, which was potentiated in CYC + IRI-injected animals (Fig. 6A–C). The injection of CYC alone did not cause a loss of body weight, alterations in systemic leukocyte count, animal mortality, diarrhea, inflammatory infiltration or histopathological damage (Figs. 5 and 6) compared to the saline group.

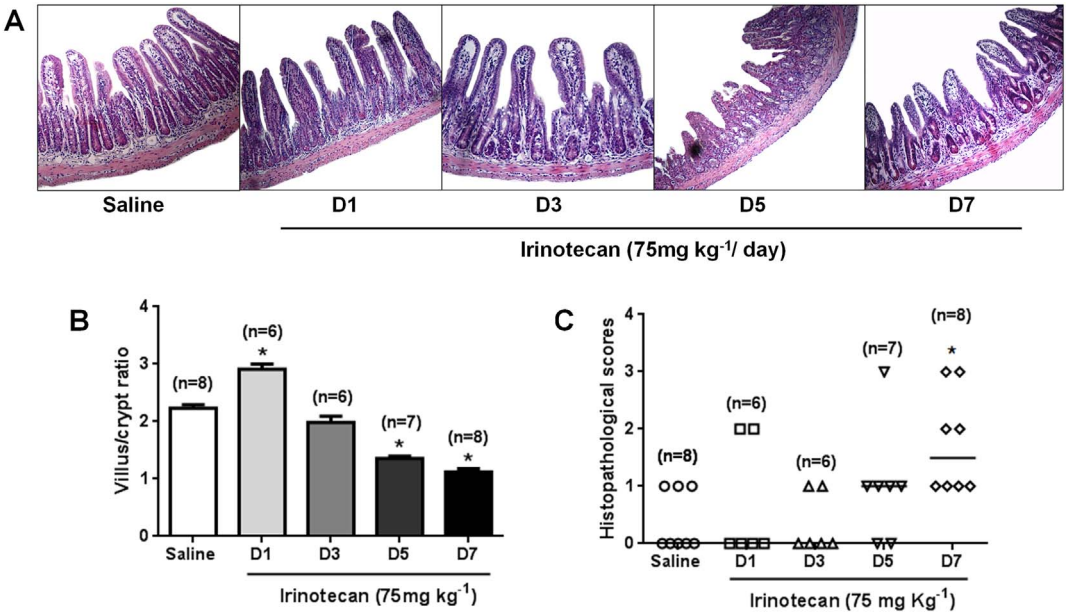

Fig. 2. Intestinal injury induced by Irinotecan. Mice were injected with irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce intestinal mucositis and were euthanized on day 1 (D1), D3, D5 or D7. The control group received only saline as a vehicle. Ileal samples were collected and processed for histopathology and morphometric analyses. The mice that received irinotecan showed progressive intestinal injury (Panel A) characterized by a reduction in the villus/crypt ratio (Panel B). The damage was also assessed by a semi-quantitative analysis (Panel C). $^*P < 0.05$ *vs.* the saline group.

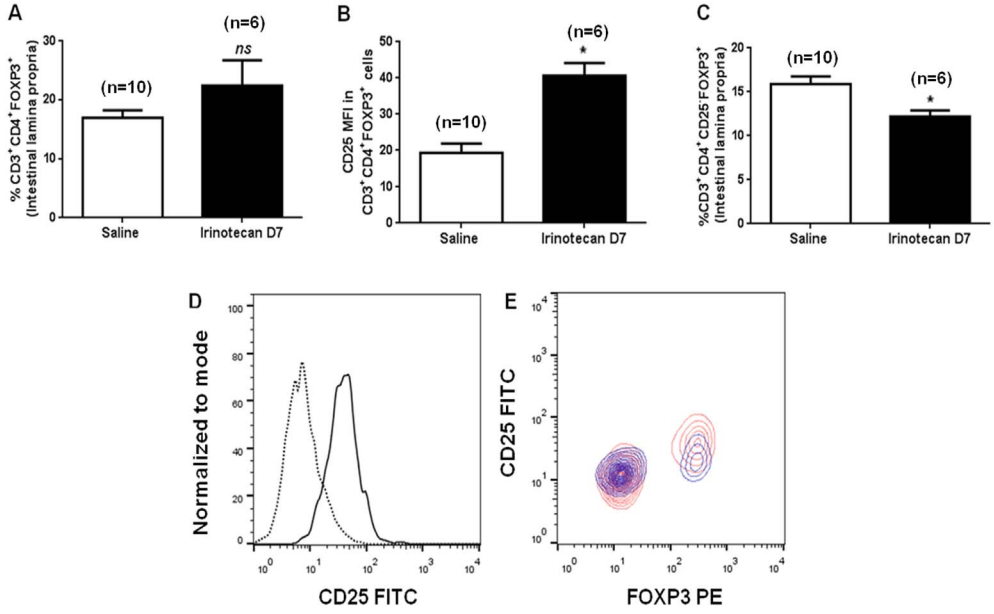

Fig. 3. Irinotecan increases membrane CD25 expression in intestinal regulatory T cells. Mice received irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce mucositis and were euthanized on day seven (D7). The control group received only saline as a vehicle. Lamina propria lymphocytes were isolated, and the frequencies of total Tregs ($CD3^+CD4^+FOXP3^+$), which includes the $CD25^+$ and $CD25^-$ fraction (A), and $CD25^-$ Tregs ($CD3^+CD4^+CD25^-FOXP3^+$) (C) were evaluated. The increase in CD25 expression (Mean of Fluorescence Intensity-MFI) is observed in Panels B and D, with the dotted line and solid line representing saline and irinotecan, respectively. Panel E summarizes this information, with saline represented by the blue line and irinotecan by the red line. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.4. Depletion of regulatory T cells increases the frequency of other T helper cell subsets

As described in Fig. 7, a negative correlation between the percentage of Tregs and the number of neutrophils (Fig. 7B), but not between Th17 and neutrophils (Fig. 7D), in the intestine on the seventh experimental day was observed in mice receiving irinotecan. In contrast, no correlation was found between Tregs or Th17 and neutrophils in saline-treated mice (Fig. 7A and C). The frequency of non-regulatory $CD3^+CD4^+CD25^-FOXP3^-$ cells was also investigated. As shown in Fig. 7I, the frequency of the lymphocyte subset was reduced on the seventh day of mucositis development compared to saline. However, the frequency of non-regulatory T cells increased in CYC-treated animals (Fig. 7J). These cells showed a positive correlation with infiltrating neutrophils in mice with intestinal mucositis (Fig. 7F), which was not observed in the control group (Fig. 7E). We also observed a

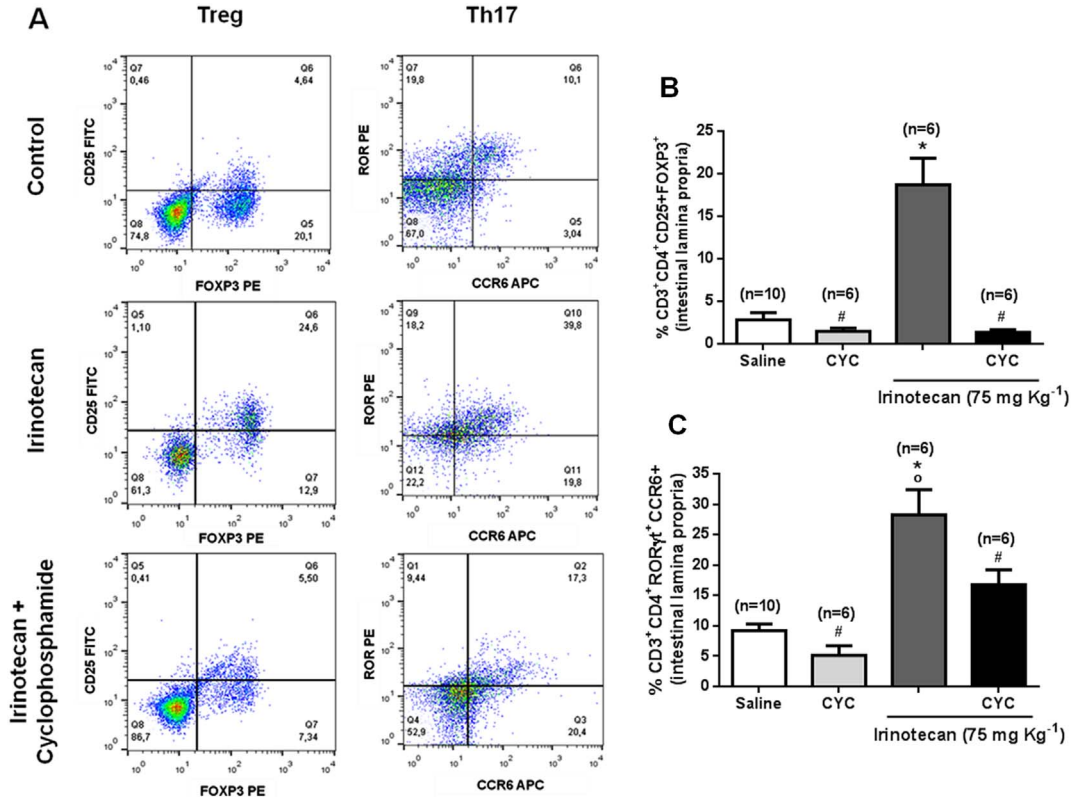

Fig. 4. Low dose cyclophosphamide depletes regulatory T cells. Mice were injected with saline or irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce mucositis and were euthanized on the seventh day. Tregs were depleted by the administration of cyclophosphamide (CYC, 100 mg kg^{-1}, i.p, single dose) 2 h before the first dose of irinotecan. Lamina propria lymphocytes were isolated to determine the frequency of Tregs and Th17 cells. A representative dot plot of each experimental group is demonstrated in Panel A. The frequency of intestinal Tregs and Th17 cells is showed in Panels B and C, respectively. $^*P < 0.05$ *vs.* Saline; $^{\#}P < 0.05$ *vs.* Irinotecan; $^{\circ}P < 0.05$ *vs.* CYC only.

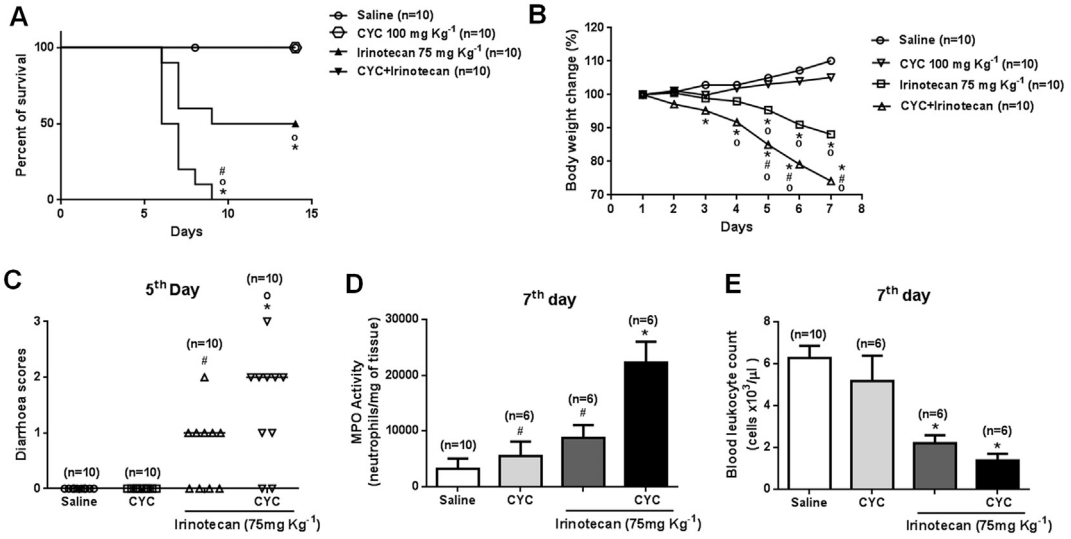

Fig. 5. Cyclophosphamide enhances the severity of irinotecan-induced intestinal mucositis. Mice were injected with saline or irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce mucositis and were euthanized on the seventh day. Tregs were depleted by the administration of cyclophosphamide (CYC, 100 mg kg^{-1}, i.p, single dose) 2 h before the first dose of irinotecan. The percentage of animal survival was monitored until the 14th day. CYC injection enhanced animal mortality (Panel A), loss of body weight (Panel B), severity of diarrhea (Panel C), MPO activity (D) and leukopenia (Panel E). $^{*}P < 0.05$ *vs.* Saline; $^{\#}P < 0.05$ *vs.* CYC + Irinotecan; $^{\circ}P < 0.05$ *vs.* CYC only.

strong negative correlation between the frequency of Tregs and non-Treg cells on the seventh day of intestinal mucositis (Fig. 7H), which was not observed in mice that received saline (Fig. 7G).

4. Discussion

In the present study, we showed for the first time the role of Tregs in controlling intestinal mucositis induced by irinotecan, since depletion of these cells exacerbated tissue damage and increased animal mortality.

Tregs have been described to suppress immunity against several stimuli, including commensal bacteria and self-antigens (Kanamori et al., 2016). These cells are frequently found in the intestinal mucosa and impair excessive effector T-cell responses and maintain organ homeostasis (Pabst, 2013; Omenetti and Theresa, 2015). In the present study, a higher frequency of Tregs was found in the lamina propria of the small intestine of mice with mucositis, which correlates with disease severity and diarrhea. Several previous studies have reported a higher frequency of Tregs in intestinal inflammatory diseases (Reikvam et al., 2011; Lord, 2015; Sznurkowska et al., 2016). However, in murine models of colitis induced by DSS or trinitrobenzenicsulfonic acid (TNBS), the frequency of Tregs in the gut was reduced and correlated with tissue damage (Kang et al., 2015; Zou et al., 2015). In our study, the increase in frequency of Tregs can be a compensatory mechanism to counterbalance the inflammation mediated by neutrophils and other Th cells.

We observed, particularly in the saline group, an important population of $FOXP3^{+}$ T cells that did not express the marker CD25.

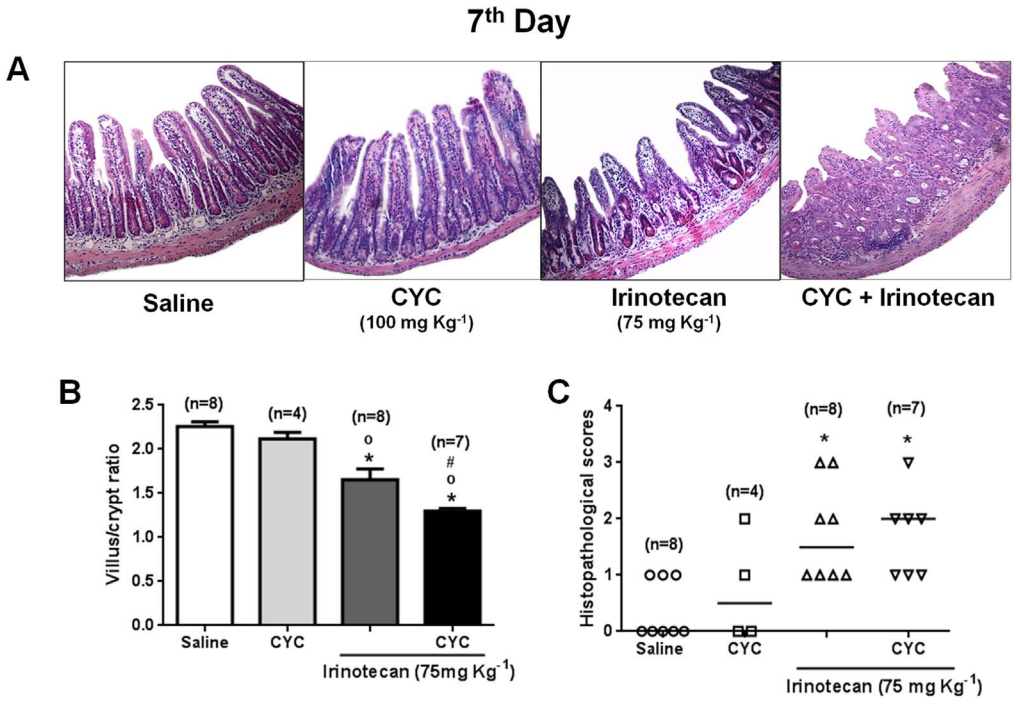

Fig. 6. Intestinal injury is potentiated in regulatory T cell-depleted mice. Mice were injected with saline or irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce mucositis and were euthanized on the seventh day. Tregs were depleted by the administration of cyclophosphamide (CYC, 100 mg kg^{-1}, i.p, single dose) 2 h before the first dose of irinotecan. Ileum samples were collected and processed for histopathology and morphometric analyses. Mice receiving CYC + Irinotecan showed a more pronounced reduction in the villus/crypt ratio (Panels A and B) compared to the animals receiving irinotecan only. Panel C depicts histopathological scores. $^{*}P < 0.05$ *vs.* the saline group. $^{\circ}P < 0.05$ *vs.* CYC only and $^{\#}P < 0.05$ *vs.* Irinotecan.

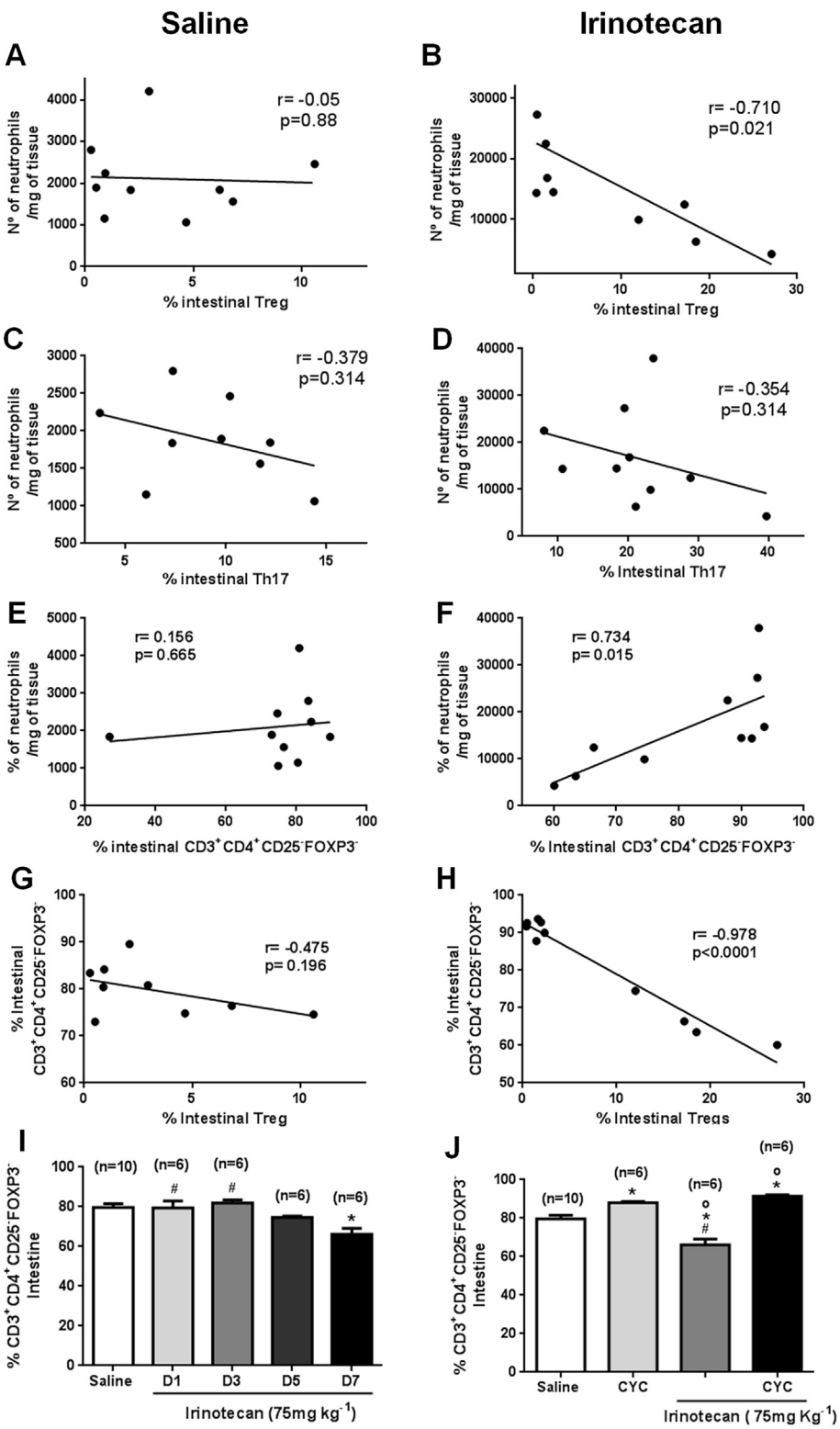

Fig. 7. Depletion of regulatory T cells increases intestinal accumulation of other Th immune cells. Mice received irinotecan (75 mg kg^{-1}, i.p, once a day for four days) to induce mucositis and were euthanized on day 1 (D1), D3, D5 or D7. Tregs were depleted by the administration of cyclophosphamide (CYC, 100 mg kg^{-1}, i.p, single dose) 2 h before the first dose of irinotecan. The control group received only saline as a vehicle. Lamina propria lymphocytes were isolated to determine the frequency of T cells. Tregs (Panel B), but not Th17 cells (Panel D), showed a negative correlation with infiltrating neutrophils in mice with intestinal mucositis. No correlation was observed in saline-injected mice (Panels A and C). Other Th cells, characterized by CD3 and CD4 expression and lack of CD25 and FOXP3 positivity, were identified in all experimental groups (Panels I and J). These cells showed a positive correlation with neutrophils (Panel F) and a strong negative correlation with Tregs in mice receiving Irinotecan (Panel H), which was not observed in the control group (Panel D and G).

However, Tregs of mice with intestinal mucositis showed an upregulation of CD25 expression. According to Zelenay et al. (2005), $CD25^{-}FOXP3^{+}$ T cells are a reservoir of committed regulatory T cells that upregulate CD25 expression upon changes in tissue homeostasis. The study reported that expression of CD25 occurs as a signature of Treg activation and not only of T effector cells (Zelenay et al., 2005). Here, we propose that intestinal Tregs are activated after administration of irinotecan and recover expression of CD25.

To evaluate the role of Tregs on the pathogenesis of intestinal mucositis, a single low dose of cyclophosphamide was administered prior to irinotecan injection. Lutsiak et al. (2005) showed that cyclophosphamide at 100 mg kg^{-1} selectively enhanced apoptosis of Tregs and decreased homeostatic proliferation of these cells, with no effect on other immune cells. In the present study, we observed a long-lasting reduction in the number of Tregs detected in the gut and spleen of mice until the seventh day after administration of cyclophosphamide. An

injection of only cyclophosphamide did not change the leukocyte count, gut integrity or animal weight gain, suggesting no detectable toxic effect of the drug. Interestingly, Treg-depleted mice injected with irinotecan showed aggravated intestinal mucositis, as detected by worsened intestinal inflammation and diarrhea and 100% mortality. Consistent with this finding, the protective role of Tregs in other animal models of intestinal inflammation was previously demonstrated. In colitis induced by DSS, depletion of Tregs with anti-CD25 antibodies also induced more severe intestinal damage (Wang et al., 2015). In addition, adoptive transfer of Tregs prevented the development of colitis (Liu et al., 2003; Mottet et al., 2003).

A negative correlation between the number of Tregs and neutrophils suggested that the activation of Tregs might limit the accumulation of these cells in the intestine, in an attempt to control the tissue damage. Indeed, depletion of Tregs caused an increase in neutrophil infiltration and had a deleterious effect, potentially by activating a prominent inflammatory response. Atkinson et al. (2016) also observed intense neutrophil infiltrations in mice that were depleted of Tregs in an arthritis model. In colitis induced by DSS, depletion of Tregs increased cellular infiltration in the colon (Boehm et al., 2012).

In addition to the increase in the frequency of Tregs, in the present study, a higher number of Th17 cells on the fifth and seventh days of mucositis development were detected. Th17 cells are involved in the pathophysiology of several inflammatory bowel diseases, such as Crohn's disease, ulcerative colitis (Gálvez, 2014; Ueno et al., 2015) and different murine colitis models (Leppkes et al., 2009; He et al., 2012; Lim et al., 2016). These cells produce cytokines, such as IL-17A and IL-17F, which induce the expression of inflammatory mediators and recruitment of neutrophils to inflammatory sites *via* direct and indirect mechanisms (Pelletier et al., 2010). The involvement of Th17 cells in experimental mucositis induced by irinotecan has not been previously suggested. However, its role has not yet been clarified.

Unexpectedly, depletion of Tregs with cyclophosphamide did not increase Th17 cell frequency. However, a reduction of approximately 40% in the gut was detected, despite increased intestinal damage. Chen et al. (2011) showed that Treg depletion in mice expressing diphtheria toxin receptor under the control of the Foxp3 promoter resulted in a lower frequency of Th17 cells in blood and draining lymph nodes. The studies showed that Tregs promoted Th17 development by the consumption of IL-2, thereby reducing the upregulation of CD25 and activation of the STAT5 pathway (Chen et al., 2011). Furthermore, Tregs exhibit plasticity and could differentiate into IL-17-producing cells that express the related orphan receptor, ROR-γt, and CCR6 (Koenen et al., 2008). Most likely, the Treg repertoire also contributed to the development of a part of the Th17 cells in our model.

Although Treg depletion coincided with a reduction in Th17 cell number, we observed higher intestinal neutrophil infiltration. This finding suggests the involvement of other immune cells in neutrophil recruitment to the intestine. Interestingly, we did not observe a correlation between the number of intestinal neutrophils and the percentage of Th17 cells. However, when other Th cells ($CD3^+$ $CD4^+$, negative for Treg and Th17 markers) were evaluated, we found a positive correlation between those cells and neutrophils. In addition, Tregs showed a strong negative correlation with other Th cells and depletion of Tregs resulted in an increase in Th cell frequency, suggesting that non-regulatory T cells could be controlled by Tregs in the context of mucositis. Importantly, in addition to alterations in the frequency of Tregs and Th17 cells, irinotecan-induced intestinal mucositis caused animal body weight loss, diarrhea, intestinal neutrophil infiltration, leukopenia, reduction of the villus/crypt ratio and intestinal damage, as shown elsewhere (Lima-Júnior et al., 2012; Melo et al., 2008; Lima-Júnior et al., 2014; Wong et al., 2015). Interestingly, we showed the mucositis peak on the seventh experimental day with the exception of neutrophil infiltration, which peaks on day 5. However, it is still not clear which T cell subset mediates such a deleterious response during mucositis. Nevertheless, the participation of IL-18, a key cytokine involved in Th1 and Th2 differentiation, has been reported to mediate intestinal mucositis development (Lima-Júnior et al., 2014).

Notably, irinotecan-induced intestinal mucositis should not be regarded as the consequence of a benign disease or simply another form of chemically-induced mucosal inflammation, such as colitis associated with the injection of dextran sodium sulfate (DSS) and trinitrobenzene sulfonic acid (TNBS). However, one important limitation of the present study is that mucositis was induced in non-tumor-bearing mice, since the presence of cancer cells may alter the immune response. Nevertheless, local and systemic effects of irinotecan injection on some cells of the immune system were shown, increasing the knowledge of the complex underlying mechanisms related to this life-threatening side effect.

Considering the possible involvement of adaptive immune cells in the pathogenesis of intestinal mucositis (Lima-Júnior et al., 2014), stimulation of Tregs might be a strategy to limit disease progression. Conversely, in cancer patients, such approach is not suitable, due to the potential enhancement of tumor progression and the occurrence of metastases by the inhibition of antitumor innate and adaptive immune responses (Curiel, 2008; Elkord et al., 2010; Mougiakakos et al., 2010). Such findings indicate that targeting the immune system is a double-edged sword.

In the clinical setting, low dose of cyclophosphamide, known as metronomic chemotherapy, has been used as adjuvant treatment in association with conventional chemotherapy for advanced tumors (Colleoni et al., 2016; Perroud et al., 2016). The advantages of metronomic cyclophosphamide-based regimens include the reduction of Tregs (Madondo et al., 2016) and the anti-angiogenic effect (Zhang et al., 2006), which impair tumor growth and show a low-toxicity profile. Taking into account that the low dose of cyclophosphamide aggravated preclinical irinotecan-related intestinal mucositis, caution is required in order to identify potential severe gastrointestinal toxicities in clinical trials that include the combination of these drugs in oncology.

In conclusion, Tregs are critical cells for controlling irinotecan-related intestinal mucositis, which might be associated to other T cell subsets. In our opinion, the modulation of the immune system to synergize with antitumor chemotherapy is currently a milestone in oncology, but efforts to the adequate management of side effects, such as mucositis, are to be specially considered to improve patients' quality of life. Understanding the pathogenesis of anticancer drug toxicities is essential for obtaining better clinical outcomes, which is the main contribution of this study.

Author contributions

Study design: CF, MHLPS and RCPLJ. Performed the Experiments: CF, CWSW, HAM, CMSS, MAT, NRPS, AGFC and RBF. Data analysis: *CF*, MHLPS, PRCA, LMCC and RCPLJ. Interpretation of the results: CF, MHLPS, PRCA, LMCC and RCPLJ. Wrote the paper: CF and RCPLJ. All the authors revised and approved the paper.

Conflicts of interest

The authors declare that there is no conflict of interest regarding the publication of this study.

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