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Short Communication

Inflammatory intestinal damage induced by 5-fluorouracil requires IL-4

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ABSTRACT

Background: 5-Fluorouracil (5-FU) induces intestinal mucositis, which is characterized by epithelial ulcerations in the mucosa and clinical manifestations, such as pain and dyspeptic symptoms. Cytokines participate in the inflammatory and functional events of intestinal mucositis. IL-4 is an important mediator of intestinal inflammation, with either anti-inflammatory or pro-inflammatory functions, depending on the model of intestinal inflammation. This study aimed to evaluate the role of IL-4 in 5-FU-induced intestinal mucositis.

Methods: IL-4$^{+/+}$ or IL-4$^{-/-}$ mice (25–30 g) were intraperitoneally injected with 5-FU (450 mg/Kg) or saline (C). After 3 days, the mice were sacrificed and the duodenum was evaluated for epithelial damage, MPO activity and cytokine concentration.

Results: 5-FU induced significant damage in the intestinal epithelium of IL-4$^{+/+}$ mice (reduction in the villus/crypt ratio: control = 3.31 ± 0.21 μm, 5-FU = 0.99 ± 0.10 μm). However, the same treatment did not induce significant damage in IL-4$^{-/-}$ mice (5-FU = 2.87 ± 0.19 μm) compared to wild-type mice. 5-FU-induced epithelial damage increased the MPO activity (neutrophil number) and the level of pro-inflammatory cytokines (IL-4, TNF-α, IL-1β and CXCL-8) in the duodenum. These results were not observed in IL-4$^{-/-}$ mice treated with 5-FU.

Conclusion: Our data suggest that IL-4 participates as a pro-inflammatory cytokine in a 5-FU-induced intestinal damage model and suggests that IL-4 antagonists may be novel therapeutics for this condition.

1. Introduction

Intestinal inflammation causes significant alterations in the structure of the mucous membrane, such as polymorphonuclear infiltration with subsequent damage to the epithelial barrier [1]. Cytokines can induce apoptosis, further contributing to barrier dysfunction during intestinal inflammation [2].

An important problem in oncology clinics is the cytotoxic effect of cancer chemotherapy on the gastrointestinal tract. For example, mucositis is a major oncological problem caused by the cytotoxicity of chemotherapy. The antimetabolite agent 5-fluorouracil (5-FU) has been used in the treatment of a range of cancers and can induce intestinal damage, referred to as intestinal mucositis [3]. Soares et al. [4] demonstrated that in animal models, intestinal mucositis induced by 5-FU is associated with neutrophil infiltration, increased pro-inflammatory cytokine levels and, importantly, delayed gastric emptying.

IL-4 is a critical mediator of intestinal inflammation. It regulates gastrointestinal smooth muscle during inflammatory processes [5] and functions as either an anti-inflammatory or pro-inflammatory molecule depending on the model of intestinal inflammation [6]. Additionally, the pro-inflammatory effect of IL-4 in intestinal inflammation may be caused by increased production of IFN-γ [7]. Several studies indicate that IL-4 is an important cytokine in allergic intestinal disease and the development of pathological Th2 responses [8]. Thus, the aims of this study were to evaluate the role of IL-4 in intestinal mucositis induced by 5-FU and to examine the underlying mechanisms of 5-FU-induced mucositis.

2. Materials and methods

2.1. Mice

Wild-type (WT) and IL-4$^{-/-}$ (Il^{4tm1Nnt}/J) C57BL/6 mice from the same genetic background (6–8 weeks old, n = 8 animals/group) were kept in a temperature-controlled room with water supplied *ad libitum*. All protocols were approved by the local ethics committee.

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2.2. Mouse model of intestinal mucositis induced by 5-FU

Mice were intraperitoneally injected with a single dose of 5-FU (450 mg/kg) (ICN Pharmaceuticals) or saline as a control (vehicle) and sacrificed after 3 days. Samples of the duodenum were removed for *histological analysis* and stored at −70 °C in a solution containing protease inhibitors, which were required for the future evaluation of cytokine levels (IL-4, TNF-α, IL-1β and CXCL-8) and MPO activity.

2.3. Determination of leukocyte number

Mice were anesthetized, and blood samples were collected. The total number of white cells was determined after dilution in Turk's solution using a Neubauer chamber. The results are expressed as the number of leukocytes per mL of sample.

2.4. Intestinal morphometry and histopathology

Segments of duodenum were fixed and stained with hematoxylin and eosin. Measurements of villus heights and crypt depths were performed via microscopy. 10 intact and well-oriented villi and crypts were measured and averaged for each sample. The microscopy analysis was double-blinded.

2.5. Intestinal MPO activity

Myeloperoxidase activity in the duodenum (a quantitative measurement of neutrophil infiltration) was assayed as previously described [9]. The results were calculated by comparing the optical density of the duodenum tissue with a standard curve of neutrophil (>95% purity) numbers.

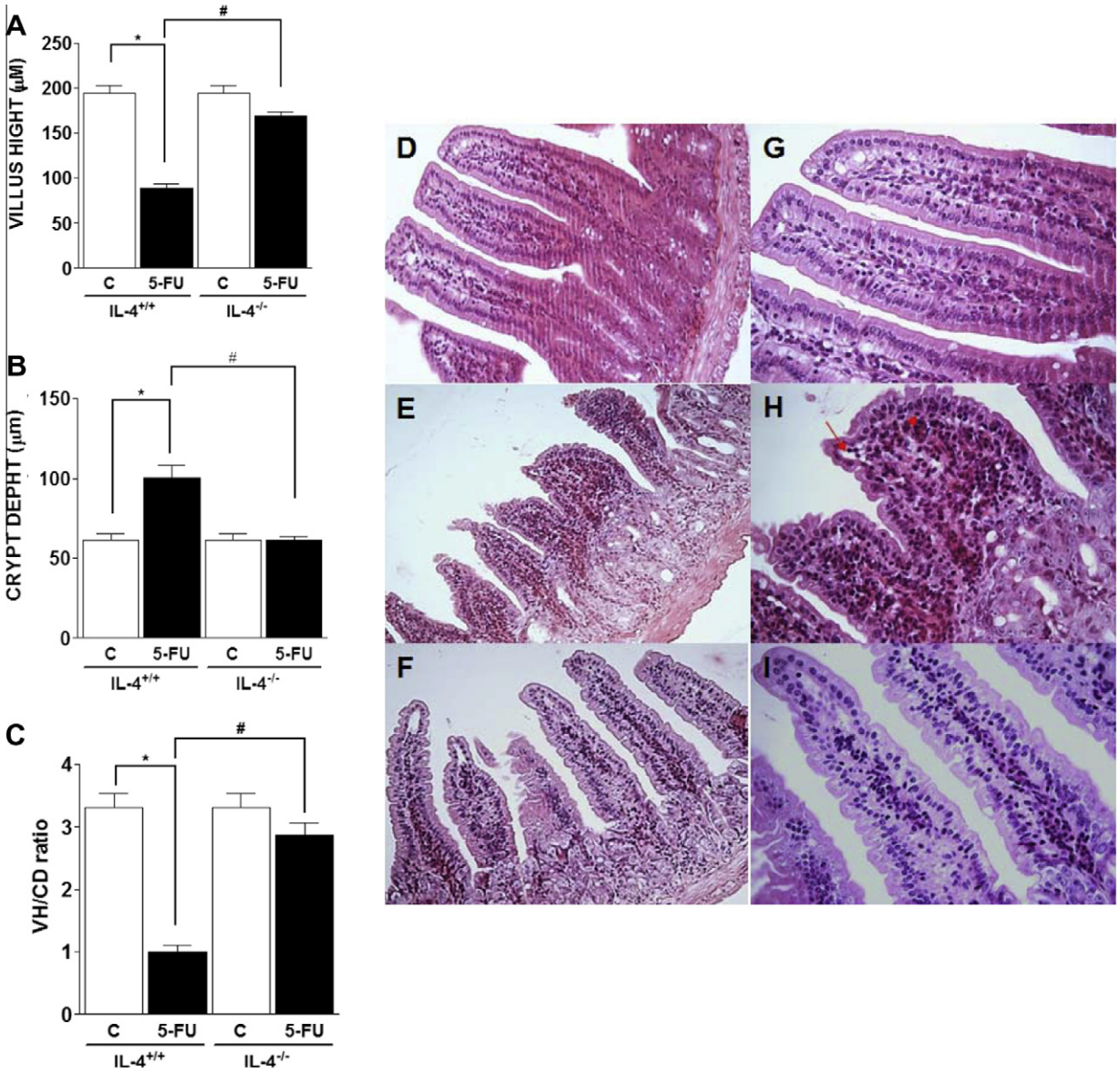

Fig. 1. Histopathological analysis. IL-$4^{+/+}$ ($n = 8$) and IL-$4^{-/-}$ mice ($n = 8$) were pre-treated with saline (C) or 5-FU (450 mg/kg). After 3 days, segments of duodenum were taken for measurement of villus height (panel A), crypt depth (panel B) and villus/crypt ratio (panel C). Control mice showing normal villi and crypts (panel D and G). Wild-type mice + 5-FU (panel E and H), showing: shortened villi recovered with fattened and vacuolated cells (*arrow*), and inflammatory cell infiltration in the lamina propria (*arrowhead*), loss of normal crypt architecture (panel E and H). IL-$4^{-/-}$ mice + 5-FU (panel F and I), showing preservation of the villi and crypts. H&E staining (panels D, E, F; 100X and G, H, I; 400X). The values were reported as mean ± S.E.M. $^{*}p < 0.05$ compared to control (C), $^{\#}$ $p < 0.05$ compared to 5-FU + IL-$4^{+/+}$. Analysis of variance and Bonferroni's *post-hoc* test.

2.6. Detection of cytokines (IL-4, TNF-α, IL-1β and CXCL-8) in the duodenum

Cytokine concentrations in the duodenum (IL-4, TNF-α, IL-1β and CXCL-8) were determined by enzyme-linked immunosorbent assay (ELISA) using protocols supplied by the manufacturer (R&D Systems, Minneapolis, USA). The results are expressed as picograms/mL.

2.7. Statistical analysis

The results are reported as means ± standard error of the mean (S.E.M.) for each group. ANOVA was performed followed by Bonferroni's test. Values were considered significant at $p < 0.05$.

3. Results

The 5-FU-treated IL-4$^{-/-}$ mice exhibited leukopenia (1585.00 ± 247.30 cells/mL), similar to wild-type mice (2513.00 ± 166.30 cells/mL) with the same treatment. 5-FU-treated wild-type mice were significantly different from untreated wild-type mice in terms of leukocyte number (7173.00 ± 1032.50 cells/mL) ($p < 0.05$). However, IL-4$^{-/-}$ mice displayed attenuated 5-FU-induced intestinal mucositis. As shown in Fig. 1, 5-FU treatment caused a significant shortening of villi (panel A), an increase in crypt depth (panel B), and a decrease in the villus height/crypt depth ratio (panel C) in wild-type mice.

Fig. 1 presents duodenum photomicrographs that demonstrate the 5-FU-induced intestinal mucositis in wild-type animals. This condition was characterized by the loss of normal crypt architecture, shortened villi with fattened, vacuolated cells and inflammatory cell infiltration into the lamina propria (panel E). 5-FU-treated IL-4$^{-/-}$ mice (panel F) demonstrated preservation of the villi and crypts when compared to 5-FU-treated wild-type mice (panel D).

An increase in IL-4 concentration (101.67 ± 11.10 pg/mL) was observed in 5-FU-treated wild-type mice compared to untreated wild-type mice (43.68 ± 14.70 pg/mL). In addition, as shown in Fig. 2, neutrophil infiltration in the duodenum in 5-FU-treated IL-4$^{+/+}$ mice was 2-fold greater than that in treated IL-4$^{-/-}$ mice. 5-FU treatment resulted in increased TNF-α, IL-1β and CXCL-8 concentrations in the duodenum (1.6-, 3.2- and 1.6-fold higher the concentrations than those in the control group, respectively). In contrast, 5-FU treatment did not increase cytokine expression in mice lacking IL-4.

4. Discussion

In the present study, we observed that 5-FU induced intestinal mucositis with a concomitant increase in IL-4 concentration in wild-type mice compared to untreated wild-type mice. Furthermore, knockout of IL-4 efficiently prevented the pathological alterations of 5-FU-induced mucositis (a decrease in the villus/crypt ratio, neutrophil infiltration and an increase in the level of the cytokines TNF-α, IL-β and CXCL-8) in the duodenum of the mice. Despite reports of both anti- and pro-inflammatory effects for IL-4 depending on the animal model, we demonstrated that this cytokine is pro-inflammatory in an intestinal mucositis model.

Cytokines, such as TNF-α, IL-β and CXCL-8, are critical mediators of intestinal inflammation. Williams [10] reported that the inflammatory cytokines TNF-α and IL-β contribute to the severity and maintenance of injury in intestinal mucositis. CXCL-8 is another important chemokine in the intestinal inflammatory process, as it is recognized as a powerful neutrophil chemotactic factor [11]. Interestingly, IL-4 has either anti- or pro-inflammatory effects in

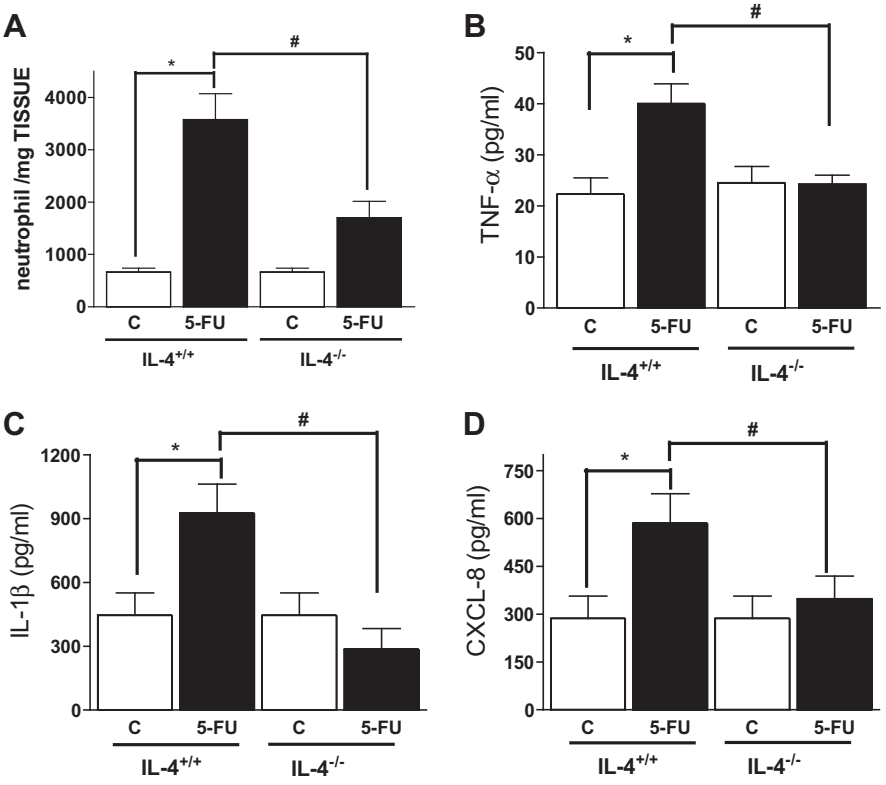

Fig. 2. Neutrophil infiltration and concentration of TNF- α, IL-1β, CXCL-8. MPO activity and cytokines concentration were evaluated by colorimetric method. Panel A shows MPO activity of IL-4$^{+/+}$ and IL-4$^{-/-}$ mice pre-treated with saline (C) or 5-FU. Panel B, C and D, respectively, show concentration of TNF- α, IL-1β and CXCL-8 of IL-4$^{+/+}$ and IL-4$^{-/-}$ mice 5-FU pre-treated or saline (C). The values were reported as mean ± S.E.M. $^{*}p < 0.05$ compared to saline (C), $^{\#}p < 0.05$ compared to 5-FU + IL-4$^{+/+}$. Analysis of variance and Bonferroni's *post-hoc* test. We used $n = 8$ for all groups.

several intestinal inflammation models [6]. IL-4 cDNA (Ad5IL-4) transfection in mice promotes colitis, which is mast cell and T-cell independent but monocyte and granulocyte dependent [6]. In the same study, TNF-α was also found to be an important mediator of colitis. In oxazolone-induced colitis, a similar pro-inflammatory effect of IL-4 was observed [12]. The production of cytokines, such as IFN-γ, may mediate the pro-inflammatory effect of IL-4 in the intestinal inflammation model [7].

We observed that IL-$4^{-/-}$ mice experienced less severe intestinal injury compared to wild-type mice when treated with 5-FU. In addition, these deficient mice demonstrated reduced neutrophil infiltration and a decrease in TNF-α, IL-1β and CXCL-8 in the duodenum. These data suggest that IL-4 may play an important pro-inflammatory role in 5-FU-induced intestinal mucositis. The observation that IL-$4^{-/-}$ mice experience a lesser reduction in the villus height/crypt depth ratio in the duodenum and an improvement in the morphology of the analyzed intestinal segments supports our hypothesis that reducing these inflammatory parameters by removing IL-4 improves the outcome of 5-FU-induced mucositis.

It has been demonstrated that IL-4 promotes the migration of leukocytes, including neutrophils, via increased CCL2 expression or by an increase in the expression of inflammatory proteins, such as sTNFR1 [13]. However, the mechanisms listed above were not evaluated in our study. Moreover, it was reported that IL-4 is responsible for increased IL-8 expression and delays in neutrophil apoptosis, contributing to an increase in the population of these cells [14]. When inflammation is induced in the intestinal mucosa, IL-$4^{-/-}$ mice show less damage, diminished neutrophil activity and a lesser increase in TNF-α mRNA compared to wild-type mice [8].

In conclusion, our study establishes a role for IL-4 in 5-FU-induced intestinal mucositis. This study suggests that treatment with IL-4 antagonists may be a logical novel therapeutic strategy for this condition.

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