GASTROINTESTINAL, HEPATOBILIARY, AND PANCREATIC PATHOLOGY

Inflammasome Activation Is Reactive Oxygen Species Dependent and Mediates Irinotecan-Induced Mucositis through IL-1 β and IL-18 in Mice

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Irinotecan is a useful chemotherapeutic for the treatment of various cancers. Irinotecan treatment is associated with mucositis, which clearly limits the use of the drug. Mechanisms that account for mucositis are only partially known. This study assessed mechanisms and the role of inflammasome activation in irinotecan-induced mucositis. Mucositis in mice was induced by irinotecan injection in C57BL/6 wild-type, *gp91phox*^{-/-}, *il-18*^{-/-}, *casp-1*^{-/-}, and *asc*^{-/-} mice once a day for 4 consecutive days. In some experiments, mice received apocynin to inhibit NADPH oxidase (NOX), IL-1 receptor antagonist, or IL-18 binding protein to prevent activation of IL-1 and IL-18 receptors, respectively. Mice were euthanized 7 days after the beginning of irinotecan treatment, and small intestines were collected for analysis. Irinotecan treatment resulted in increased IL-1 β and IL-18 production in ileum and NOX-2—dependent oxidative stress. *gp91phox*^{-/-} and apocynin-treated mice had diminished oxidative stress and less severe mucositis. Furthermore, treatment with apocynin decreased caspase-1 activation and IL-1 β and IL-18 production in the ileum. *asc*^{-/-} and *casp-1*^{-/-} mice also had less intestinal injury and decreased IL-1 β and IL-18 production. Finally, both the absence of IL-18 and IL-1 β resulted in reduced inflammatory response and attenuated intestinal injury. NOX-2—derived oxidative stress mediates inflammasome activation and inflammasome-dependent production of IL-1 β and IL-18, which mediate tissue injury during irinotecan-induced mucositis in mice. (*Am J Pathol* 2014, 184: 2023–2034; <http://dx.doi.org/10.1016/j.ajpath.2014.03.012>)

Irinotecan (CPT-11) is a chemotherapeutic agent used as a first- or second-line treatment for several solid tumors.^{1–3} It is a prodrug that is converted into its active form (SN-38) after hydrolysis in the liver by carboxylesterase enzymes.⁴ This drug is a potent topoisomerase I inhibitor,^{5,6} an effect that is essential to kill cancerous cells. However, topoisomerase I inhibition also leads to death of healthy cells with high mitotic rates, such as basal cells of the intestine.⁷ Therefore, treatment with irinotecan is associated with several adverse effects, such as leukopenia, diarrhea, and mucositis.^{8,9} It is thought that mucositis arises from lesions of the basal cells in the gastrointestinal tract due to chemotherapy or radiotherapy, resulting in mucosal damage, an intense inflammatory reaction, and

consequent ulceration.^{7,10–12} Irinotecan may induce mucosal damage directly; DNA damage due to topoisomerase I inhibition activates several pathways involved in apoptosis and inflammation.⁷ However, the mechanism by which irinotecan induces mucositis is not well described, and it is likely that the inflammatory response contributes to the drug-induced mucosal damage. It has been reported that chemotherapy leads to enhanced production of reactive oxygen species

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(ROS) and reactive nitrogen species, which induce cell death,^{13–15} but it is not clear whether these species are necessary for the inflammatory responses after irinotecan treatment.

Recently, it has been reported that ROS may promote inflammation by activating multiprotein cytoplasmic complexes called inflammasomes.^{16,17} Inflammasomes are composed of one of several NOD-like receptor (NLR) proteins, including NLRP1, NLRP3, and the non-NLR family member AIM2. NLRs function as sensors of endogenous or exogenous damage-associated molecules and lead to caspase-1 activation and subsequent cleavage of pro-IL-1 β and pro-IL-18 cytokines into their mature forms.¹⁸ Importantly, IL-1 β is one of the various inflammatory mediators reported to be released during mucositis.^{13,19–21} However, whether the inflammasome is involved in IL-1 β production and the role played by IL-18 or IL-1 β in irinotecan-induced mucositis have not been reported. Thus, our aim was to assess the role of ROS for inflammasome activation in irinotecan-induced mucositis. In addition, we also investigated the role of inflammasome-derived IL-1 β and IL-18 for irinotecan-induced mucositis severity. We found that ROS derived from NADPH oxidase (NOX)-2 are crucial for caspase-1 activation and consequent IL-1 β and IL-18 production during mucositis. We also found that the absence of inflammasome complex components leads to diminished intestinal injury, attenuation of body weight loss, and blunted inflammatory response after mucositis induced by irinotecan. In addition, we found that IL-1 β and IL-18 production plays a crucial role in mucositis.

Materials and Methods

Animals

Wild-type (WT) and gene-deficient (*il-18*^{−/−} and *gp91phox*^{−/−}) male C57BL/6 mice (6 to 8 weeks old) were obtained from the Immunopharmacology Animal Facility of the Institute of Biological Sciences (Federal University of Minas Gerais, Belo Horizonte, Brazil). Apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC) knockout (*asc*^{−/−}) mice were obtained from the Bioscience Unit Ribeirão Preto, Medical School, University of São Paulo (FMRP/USP), Ribeirão Preto, Brazil). Caspase-1 knockout mice (*casp-1*^{−/−}) mice and their controls were bred and obtained from the Transgenose Institute of the National Center for Scientific Research (Orléans, France). All animals were housed under standard conditions in separate cages with free access to commercial chow and water. Experiments received prior approval by the animal ethics committees of the Federal University of Minas Gerais and National Center for Scientific Research.

Drugs and Reagents

The following drugs were used: irinotecan hydrochloride (CPT-11; Camptosar, Eurofarma, Brazil; 100-mg ampule)

and IL-1 receptor antagonist (IL-1Ra; Biogen, Inc., Geneva, Switzerland). Apocynin (NOX-2 inhibitor) was purchased from Sigma-Aldrich (St. Louis, MO). IL-18 binding protein (IL-18BP) was purchased from R&D Systems (Minneapolis, MN).

Experimental Intestinal Mucositis

The experimental intestinal mucositis in mice was induced as previously described²² and modified for our experimental conditions. Saline or irinotecan (75 mg/kg) was administered i.p. once per day for 4 consecutive days. Mice were weighed daily. Seven days after beginning irinotecan treatment, mice were euthanized by cervical dislocation. The small intestine was removed to measure the length, and ileum was used to further analysis. To evaluate the role of the IL-1 receptor, WT or *il-18*^{−/−} mice were treated with 4 mg/kg of IL-1Ra or saline s.c. 8 hours before the first dose of irinotecan. Subsequently, mice were treated every 8 hours during the following 7 days. Another WT mouse group received one i.p. injection of 20 mg/kg of apocynin 24 hours before the first dose of irinotecan and a daily i.p. injection of apocynin throughout the experimental period. Another group received 250 μ g/kg of IL-18BP daily 1 hour before irinotecan treatment during 4 consecutive days.

To analyze the role of IL-1 β and IL-18 in the later phase of mucositis, mice were kept alive for 21 days after the beginning of irinotecan treatment. In these experiments, mice received irinotecan and were treated with 4 mg/kg IL-1Ra (8 hours before the first dose of irinotecan and subsequently every 8 hours for 21 days) or vehicle (saline, as for the IL-1Ra treatment). In parallel experiments, *il-18*^{−/−} mice were given irinotecan and evaluated for 21 days. Control WT or *il-18*^{−/−} mice received saline (the vehicle for irinotecan). Because there was no difference between WT and the *il-18*^{−/−} mice not exposed to irinotecan (given vehicle only), these groups were pooled and are represented together for ease of visualization.

Cytokine Measurement

The levels of the cytokines IL-1 β and IL-18 in ileum samples were measured as previously described.¹³ The cytokine concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using commercially available kits from R&D Systems and MBL International Corporation (Woburn, MA), respectively. Experiments were reproduced three times, and one representative experiment is presented in the [Results](#) section.

Myeloperoxidase Assay

The extent of the neutrophil accumulation in the ileum tissue was measured by the quantification of the myeloperoxidase (MPO) activity, a neutrophil enzyme marker, as previously described.²³ Briefly, a portion of the animals' ileums was removed and snap frozen in liquid nitrogen. On thawing and processing, the tissue was assayed for MPO activity by

Figure 1 Irinotecan induces intestinal mucositis in mice. Mice were exposed to irinotecan treatment during 4 days and euthanized 3 days after the end of treatment (day 7 of the experiment). **A:** Weight loss was assessed from the days 1 to 7. **B and C:** On day 7, small intestine shortening was measured (**B**), ileum was removed, and neutrophil influx was assessed by MPO activity (**C**). **D:** The ileum was isolated and fixed for H&E staining, and ileum injury was assessed by histopathologic analysis. The line above control indicates a score of 0. **E and F:** The increase in IL-1 β (**E**) and IL-18 (**F**) production was evaluated by ELISA. Results are the means $\pm$ SEM of 3 to 10 animals in each group. * $P < 0.05$ compared with the control group.

measuring the change in optical density at 450 nm using tetramethylbenzidine (Sigma-Aldrich). Results were expressed as relative units that denote activity of MPO compared with casein-elicited murine peritoneal neutrophils processed in the same way.

Western Blott Analysis

The cleaved form of caspase-1 was detected in supernatants of ileum homogenates using a Western blotting assay with rat anti-caspase-1 p20 monoclonal antibody clone 4B4 (Genentech, South San Francisco, CA), as previously described.²⁴ Protein levels of caspase-1 were quantified by using densitometric analysis LabImage 1D software (LabImage, Leipzig, Germany). The protein levels were normalized by levels of β -actin in the same sample, and results are expressed as caspase-1/ β -actin ratio in arbitrary units.

Histopathologic Analysis

Ileum samples from adult euthanized mice were obtained at day 7 after mice were exposed to irinotecan. Afterward, they were immediately fixed in 10% buffered formalin for 24 hours and embedded in paraffin. Tissue sections (5 μ m thick) were stained with H&E and evaluated under a microscope (Olympus, Tokyo, Japan) adapted with a digital camera (Moticon, Munich, Germany). The height of the mucosal villous or crypt depth of each sample was measured using digitalized images obtained with a 10 $\times$ objective of a light microscope (Olympus) adapted with a digital camera (Moticon) and the villus/crypt ratio calculated. The histologic

score was obtained based on the intensity of mononuclear and polymorphonuclear infiltrates in the lamina propria, changes in the architecture of the mucosa, decreased villus height, and ulceration. For each parameter, the changes were graded according to the following scale: 0, absent; 1, mild; 2, moderate; and 3, intense. The inflammation score was represented by numbers from 0 (normal) to 12 (highly altered). The histologic analysis was performed by a single examiner (M.A.R.) masked to the experimental groups status. None of control mice had any alteration in histopathologic analysis, and their scores were zero. The results were expressed as the means $\pm$ SEM of the inflammation score for each experimental group.²⁵

Quantification of Thiobarbituric Acid Reactive Substances

The total thiobarbituric acid-reactive substance (TBARS) levels in the ileum, an index of malonyldialdehyde production, were determined as previously described.²⁶

Quantification of Reduced Glutathione Levels

The reduced glutathione (GSH) levels were measured as previously described.²⁷ Briefly, ileum was homogenized, mixed with trichloroacetic acid, and kept on ice for 30 minutes to allow protein precipitation and then centrifuged. Then 30 μ L of the clear supernatant were mixed with 270 μ L of phosphate buffer 0.1 mol/L (pH 8.5) and 5 μ L of 5,5-dithiobis-(2-nitrobenzoic acid) in methanol. The absorbance of the reaction solution was measured at 415 nm.

Figure 2 Oxidative stress induced by irinotecan is dependent on NOX-2 and is pivotal to tissue injury associated with mucositis. **A** and **B**: After irinotecan treatment, mice had increased lipid peroxidation, as measured by increased levels of TBARS (**A**), and decreased levels of reduced GSH (**B**). These parameters were evaluated by colorimetric techniques after protein precipitation compared with the control group. The WT (white bars) mice treated with apocynin or *gp91phox*^{-/-} (black bars) had decreased TBARS levels (**A**), whereas the level of reduced GSH was increased only in *gp91phox*^{-/-} mice (**B**). **C** and **D**: Irinotecan treatment in *gp91phox*^{-/-} mice or WT mice treated with apocynin was associated with attenuation in body weight loss (**C**) and small intestine shortening (**D**). **E**: *gp91phox*^{-/-} or WT mice treated with apocynin presented decreased mucosal damage and ulceration associated with mucositis as assessed in histologic slides, which resulted in a decreased histologic score compared with irinotecan-injected WT mice. Control mice did not have any histopathologic alteration and had a score of zero. The line above control indicates a score of 0. Results are the means $\pm$ SEM of three to eight animals in each group. * $P < 0.05$ compared with the control group; † $P < 0.05$ compared with the WT irinotecan-treated group. Scale bar = 100 μ m (**E**). Apo, apocynin; Irin, irinotecan; Vehic, vehicle for apocynin.

Statistical Analysis

The results were analyzed using GraphPad Prism 4 software (GraphPad Inc., San Diego, CA). Data are presented as means $\pm$ SEM and compared using one-way analysis of variance with posttest Newman-Keuls multiple comparisons. Student's *t*-test was used to assess whether the means of two groups were statistically different from each other. $P < 0.05$ was considered statistically significant.

Results

NOX-2–Derived ROS Is Involved in Caspase-1 Activation and Tissue Injury Induced by Irinotecan

Our results indicate that treatment with irinotecan for 4 days is associated with body weight loss from day 2 of treatment

(Figure 1A). On day 7, mice were euthanized, and analysis of the small intestine revealed shortening of the intestinal length (Figure 1B) and increase in neutrophil influx to the ileum, as assessed by MPO assay (Figure 1C). Consistent with the findings from the MPO assay, histopathologic analysis revealed neutrophil infiltration in the ileum and further marked changes in tissue architecture and ulceration (Supplemental Figure 1A), leading to an important increase in the histopathologic score (Figure 1D). Interestingly, the inflammatory response in the ileum due to irinotecan treatment was associated with an increase in IL-1 β (Figure 1E) and IL-18 (Figure 1F) production.

Next, we evaluated the oxidative stress in ileum of irinotecan-treated mice by assessing levels of TBARS and levels of reduced GSH in tissues. Irinotecan-induced mucositis led to a marked increase in TBARS levels (Figure 2A) and reduced GSH levels (Figure 2B) in the ileum. Experiments in

Figure 3 ROS from NOX-2 are associated with caspase-1 activation and IL-1 β /IL-18 production. **A:** Both *gp91phox*^{-/-} or WT mice treated with apocynin had reduced cleavage of caspase-1 compared with WT mice, as indicated by Western blotting. **B** and **C:** Apocynin treatment was associated with decreased IL-1 β (**B**) and IL-18 (**C**) production induced by irinotecan. Results are the means $\pm$ SEM of 3 to 11 animals in each group. * P < 0.05 compared with the control group; † P < 0.05 compared with the WT Irinotecan-treated group. Apo, apocynin; C, control (without irinotecan treatment); Irin, irinotecan; *phox*^{-/-}, *gp91phox*^{-/-}; V or vehicle, vehicle for apocynin.

gp91phox^{-/-} mice or apocynin-treated mice revealed no increase in TBARS production after irinotecan in these mice (Figure 2A). Furthermore, *gp91phox*^{-/-} mice, but not apocynin-treated animals, presented a partial reversion of the decrease in GSH levels (Figure 2B). These results suggest that NOX-2–derived ROS are associated to oxidative stress during irinotecan-induced mucositis.

We then evaluated the role of NOX-2–derived ROS for disease progression and tissue damage induced by irinotecan. *gp91phox*^{-/-} and apocynin-treated mice had diminished weight loss on day 7 after administration of irinotecan (Figure 2C) and no intestinal shortening (Figure 2D) compared with WT mice given irinotecan. Furthermore, damage in the mucosa architecture, ulceration, and consequently the histopathologic score were decreased in the absence of NOX-2 (Figure 2E). Our results clearly indicate the important role played by ROS for irinotecan-induced experimental mucositis in mice.

ROS has been suggested to be involved in inflammasome activation in several pathologic conditions.^{28–31} Next we

evaluated whether ROS was involved in inflammasome activation during irinotecan-induced mucositis in mice. Inhibition of NOX by treatment with apocynin or use of *gp91phox*^{-/-} mice was associated with a decrease in caspase-1 activation (Figure 3A). More importantly, inhibition of NOX activation with apocynin caused inhibition of IL-1 β (Figure 3B) and IL-18 (Figure 3C) production in the ileum of mice exposed to irinotecan. These results suggest that NOX-2–derived ROS are involved in both inflammasome activation and IL-1 β /IL-18 production during irinotecan-induced mucositis. Interestingly, in the absence of inflammasome components there is a decrease in TBARS levels (data not shown), suggesting that inflammasome activation feeds back to the system and enhances ROS production.

The Crucial Role Played by the Inflammasome Complex and Its Derived Cytokines, IL-1 β and IL-18, in Irinotecan-Induced Mucositis in Mice

Both IL-1 β and IL-18 are important proinflammatory mediators that are generated at sites of injury by coordinated pathways: proinflammatory stimuli induce expression of inactive cytokine proforms, whereas inflammasome complexes control cytokine maturation and release.^{32–34} Thus, because irinotecan induced inflammasome activation and an increase in IL-1 β and IL-18 levels, we hypothesized that the inflammasome complex may play a role in irinotecan-induced damage. Using *asc* or *casp-1*–deficient mice, we found that mucositis induced by irinotecan treatment was dependent on inflammasome activation, as seen by lack of body weight loss after irinotecan treatment (Figures 4A and 5A), abrogation of intestine shortening (Figures 4B and 5B), and decrease in neutrophil influx (Figures 4C and 5C) compared with irinotecan-treated WT mice. The histopathologic analysis revealed that *asc*^{-/-} and *casp-1*^{-/-} mice had reduced inflammatory cell infiltrate, less damage in the mucosa architecture, and, therefore, less ulceration and consequently a decrease in histologic score (Figures 4D and 5D). Interestingly, lack of ASC or caspase-1 was associated with abrogation of IL-1 β (Figures 4E and 5E) and IL-18 (Figures 4F and 5F) production. Together, these results indicate that activation of ASC/caspase-1 plays an important role in the inflammatory response and IL-1 β and IL-18 production in irinotecan-induced mucositis.

Next we assessed the role of IL-1 β and IL-18 in this mucositis model. Experiments in mice that lack IL-18 or in mice treated with the soluble IL-1Ra suggest that both cytokines are important for the damage induced by irinotecan (Figure 6). In the absence of IL-18 or IL-1 receptor activation, mice had reductions in body weight loss after day 5 of treatment (Figure 6A), intestinal shortening (Figure 6B), and neutrophil influx into the ileum (Figure 6C) compared with irinotecan-treated WT mice. In addition, histologic analysis of the ileum revealed that both the absence of IL-18 or blockade of IL-1 receptor resulted in reductions in inflammatory cell infiltration, damage in the architecture of

Figure 4 A: ASC contributes to IL-1 β and IL-18 production and is associated with ileum damage induced by irinotecan. *asc*^{-/-} mice have attenuation of body weight loss on days 6 and 7 after irinotecan treatment compared with WT mice. B and C: On day 7, there was less intestinal shortening (B) and decreased neutrophil influx (C). D: Absence of ASC resulted in a reductions in inflammatory cell influx, mucosal damage, and ulceration; these alterations resulted in a decrease in histologic score when compared with irinotecan-injected WT mice. The line above control indicates a score of 0. E and F: Control mice did not have any histopathologic alteration and had a score of 0. *asc*^{-/-} mice had a decrease in IL-1 β (E) and IL-18 (F) production. Results are the means $\pm$ SEM of three to nine animals in each group. * P < 0.05 compared with the control group; † P < 0.05 compared with the WT irinotecan-treated group. Scale bar = 100 μ m (D). Irin, irinotecan.

the mucosa, and ulceration. Overall, these changes resulted in reduced histopathologic score (Figure 6D). Both strategies were able to decrease oxidative stress induced by irinotecan treatment, as assessed by decrease of TBARS and increase of GSH (Supplemental Figure 2, A and B, respectively).

To confirm the results obtained in mice genetically deficient for *Il-18*, we treated WT mice with IL-18BP. IL-18BP treatment was also able to decrease the damage induced by irinotecan, as assessed by improvement in body weight loss (Supplemental Figure 3A) and in intestinal shortening

(Supplemental Figure 3B). In addition, there was reductions in neutrophil influx (Supplemental Figure 3C), histologic damage (Supplemental Figure 3D), and IL-1 β production (Supplemental Figure 3E) when compared with vehicle-treated mice.

Previous studies have found that IL-18 deficiency may impair epithelial reconstitution.³⁵ To evaluate whether IL-1 β or IL-18 were necessary for epithelial recovery in our model, we evaluated the effects of blocking these pathways for 21 days after irinotecan treatment. On day 21 after irinotecan treatment, there was full recovery of WT mice, as

Figure 5 Caspase-1 contributes to IL-1 β and IL-18 production and is associated with ileum damage induced by irinotecan. **A:** *Casp-1* mice have attenuation of body weight loss on days 5, 6, and 7 after irinotecan treatment compared with WT mice. **B** and **C:** On day 7, there was less intestinal shortening (**B**) and decreased neutrophil influx (**C**). **D:** Absence of caspase-1 resulted in reductions in inflammatory cell influx, mucosal damage, and ulceration; these alterations resulted in a decrease in histologic score when compared with irinotecan-injected WT mice. Control mice do not have any histopathologic alteration and had a score of 0. The line above control indicates a score of 0. **E** and **F:** *casp-1*^{-/-} mice had a decrease in IL-1 β (**E**) and IL-18 (**F**) production. Results are the means $\pm$ SEM of 3 to 10 animals in each group. * $P < 0.05$ compared with the control group; $^{\dagger}P < 0.05$ compared with the WT irinotecan-treated group. Scale bar = 100 μ m (**D**). Irin, irinotecan.

seen by normalization of intestinal length, MPO levels, and histopathologic score. There was also partial recovery of weight loss at day 21 in mice given irinotecan. Similar results were obtained in *il-18*^{-/-} mice and in WT mice treated with IL-1Ra (Supplemental Figure S4). These results suggest that in our model neither IL-1 β nor IL-18 affected recovery significantly.

Interestingly, *Il-18*-deficient mice had a significant decrease in IL-1 β production (Figure 6E); however, the blockade of IL-1 receptor was also associated with decrease in IL-18 production (Figure 6F). Together, these results

indicate that production of IL-1 β and IL-18 are essential for tissue damage after irinotecan treatment. We also evaluated whether IL-1 β and IL-18 act synergistically. To test this, we used *Il-18*-deficient mice treated with IL-1Ra. All parameters assessed indicate that blockade of either IL-1 β or IL-18 was sufficient to attain full inhibitory effects (Figure 6). Together with data indicating that blockade or absence of one of the cytokine prevents the production of the other, these results suggest that IL-1 β and IL-18 are crucial and likely play cooperative but not synergistic roles during irinotecan-induced mucositis.

Figure 6 IL-1 β and IL-18 play important but redundant roles in mucositis induced by irinotecan. Treatment with irinotecan induced less body weight loss (A), attenuation of intestinal shortening (B), and a decrease in neutrophil influx (C) in *il-18*^{-/-} (black bars) or WT (white bars) mice treated with IL-1Ra compared with WT irinotecan-treated mice. D: Both IL-1 receptor activation blockade and absence of IL-18 resulted in reductions in inflammatory cell infiltration, mucosal damage, ulceration, and histologic score induced by mucositis. Control mice did not have any histopathologic alteration and had a score of 0. The line above control indicates a score of 0. E: Blockade of IL-1 receptor or absence of IL-18 decreased IL-1 β production. F: IL-1Ra treatment reduced IL-18 production. *il-18*^{-/-} mice do not produce any level of IL-18 as measured by ELISA (data not shown). All parameters assessed had no additional inhibitory effects when both cytokines were blocked. Results are the means $\pm$ SEM of three to eight animals in each group. * $P < 0.05$ compared with the control group; $^{\dagger}P < 0.05$ compared with the WT irinotecan-treated group. Scale bar = 100 μ m (D). ND, not detectable.

Discussion

Mucositis is a major adverse effect associated with chemotherapy and radiotherapy during antineoplastic treatment.^{8,9} We report that treatment with the chemotherapeutic agent irinotecan induces mucositis, which is in agreement with

previous studies reporting this association in both humans and mice.^{13,14,36} More importantly, we define the crucial role played by ASC/caspase-1 activation and consequent IL-1 β and IL-18 production for establishment of the inflammatory response in irinotecan-induced mucositis. Furthermore, our results indicate the important participation of ROS in

Figure 7 ROS-dependent inflammasome activation mediates irinotecan-induced mucositis through the control of IL-1 β and IL-18 release. Our data revealed that irinotecan treatment was associated with cell damage and increase of NOX-2-derived ROS. ROS production increased the cleavage of caspase-1 and consequently IL-1 β and IL-18 release. In addition, there was an increase in neutrophil influx and amplification of tissular injury.

mediating inflammasome activation and consequent proinflammatory cytokine production and tissue injury during irinotecan-induced mucositis (Figure 7).

Mucositis is characterized by intense tissue damage, which is associated with the production of several molecules, including mitochondrial and oxidative stress products that may function as damage-associated molecular patterns.³⁷ Oxidative stress occurs after irinotecan treatment, as indicated by the increase in TBARS and the decrease in GSH. In our model, the oxidative stress is likely dependent on NOX-2 because markers of oxidative stress were virtually normalized in *gp91phox*-deficient mice, a heme-binding subunit of the superoxide-generating NOX-2, necessary for the function of this complex. Results in which mice were treated with apocynin, a NOX inhibitor, concur with the idea that NOX-derived ROS are important for inflammasome activation induced by irinotecan. Apocynin had little effect on GSH level, likely because higher doses of the compound would be necessary to attain full inhibition of ROS production. In addition, *gp91phox*^{-/-} mice or apocynin-treated WT mice presented attenuated weight loss and less intestinal injury. These results are in accordance with recent studies that found that the severity of mucositis induced by fluorouracil in NOX-1-deficient mice was reduced and ROS production was decreased.³⁸ It has also been reported that Gp91phox is involved and contributes to the development of experimental inflammatory bowel

disease in a mouse model of dextran sodium sulfate-induced colitis.³⁹ Therefore, NOX-2-mediated ROS production seems to be pivotal for chemotherapeutic-induced mucositis.

The generation of ROS is proposed to be a common step in inflammasome activation, largely based on the observation that caspase-1 activation is blocked by ROS inhibitors.^{17,31,40} Hence, inflammasome activators induce ROS, and these species can be blocked by chemical scavengers that suppress inflammasome activation.⁴¹ Thus, we next sought to assess the participation of ROS in inflammasome activation in the ileum of irinotecan-treated mice. The sources of ROS relevant for inflammasome activation are currently under intense debate, but Gp91phox has been implicated in this process.¹⁷ Our data suggest that ROS from NOX-2 are involved in the activation of caspase-1 because of the observation that there is increased expression of caspase-1 in WT mice with mucositis but decreased expression of the enzyme in apocynin-treated WT mice or *gp91phox*^{-/-} mice. Furthermore, the inhibition of NOX by apocynin was associated with decrease in caspase-1 activation and abrogation of IL-1 β and IL-18 production in irinotecan-induced mucositis.

The intestinal environment is a common habitat for millions of bacteria that compose the indigenous microbiota. In mucositis, it is likely that direct epithelial damage and associated dysbiosis may expose several molecules known as microbe-associated molecular patterns from local bacteria.^{42,43} Both damage-associated molecular patterns and microbe-associated molecular patterns have been described as important inducers of inflammasome activation,³⁴ and it is possible that activation and assembly of inflammasome complexes may play an important role in mucositis. In this context, we have performed some experiments to assess the role of the microbiota in this model of mucositis. Our results indicate that germ-free mice exposed to chemotherapeutics had little mucosal damage, suggesting an important role for commensal bacteria in our model (data not shown). However, we have previously reported that sterile and infectious inflammatory responses are greatly suppressed in germ-free mice.^{44,45} Moreover, there is evidence that irinotecan may undergo metabolism by bacteria.⁴⁶ Therefore, the lack of response to irinotecan in germ-free mice is not simple to interpret, and we are currently performing further studies to resolve these issues. Inflammasome activation leads to ASC and caspase-1 oligomerization and consequent caspase-1 activation. Notably, the absence of ASC or caspase-1 was associated with a reduction in disease severity and a significant decrease in IL-1 β and IL-18 production induced by irinotecan treatment. As previously described, the inhibition of NOX-2 is associated with a decrease of caspase-1 activation and IL-1 β /IL-18 production. On other hand, in the absence of inflammasome components, there was less oxidative stress. Together these data link ROS production to caspase-1 activation and cleavage of pro-IL-1 β and IL-18 and suggest an important amplification loop between inflammasome activation and ROS production. Therefore, this

is the first study, to our knowledge, to demonstrate that ASC/caspase-1 is necessary for IL-1 β and IL-18 production during irinotecan-induced mucositis. More importantly, this study is unique in that it indicates that this pathway is crucial for mucositis after irinotecan injection. These results are in agreement with previous studies that have found that ASC and caspase-1 are involved in gut inflammatory events, such as colitis induced by dextran sodium sulfate.^{47–49}

The importance of proinflammatory cytokines processed by caspase-1 for the development of irinotecan-induced mucositis was also demonstrated in the present work. Treatment with IL-1Ra was associated with significant decrease in irinotecan-induced injury. The protective effect of the IL-1 receptor in our model is in accordance with previous studies that found that administration of recombinant IL-1Ra after chemotherapy reduces the lethal toxic effects and intestinal mucosal damage by fluorouracil and enhance intestinal recovery in mice.⁵⁰ Interestingly, the protective effect of IL-1Ra was associated with decrease in intestinal apoptosis induced by fluorouracil, relieving mucosal dysfunction in the small intestine and facilitating recovery of the intestinal mucosa.

Inhibition of IL-18 by the use of deficient mice or IL-18BP was associated with a decrease in mucositis signals. Despite some studies reporting that the blockade of IL-18 signaling decreased the severity of colitis due to decreased influx of inflammatory cells and reduced proinflammatory cytokine production in the large intestine,⁵¹ the work presented here is the first study, to our knowledge, assessing the role of IL-18 in the mucositis model. However, neither IL-1 β nor IL-18 inhibition was associated with improvement in epithelial recovery in irinotecan-induced injury on a later phase of mucositis. Notably, we found that blockade of either IL-1 β or IL-18 was associated with a marked decrease of the other cytokine on the seventh day after mucositis induction. It suggests that one cytokine may control the release of the other one at earlier time points. We have also found that blockade of the IL-1 receptor in *il-18*^{−/−} mice did not potentiate protection of mice to irinotecan-induced mucositis, probably because IL-1 β production is already diminished in *il-18*^{−/−} mice. Altogether, these data demonstrate that IL-1 and IL-18 are pivotal for chemotherapy-induced mucositis.

The absence of inflammasome system components or IL-1/IL-18 signaling was accompanied by a reduction in neutrophil recruitment. Neutrophils are known to contribute to the pathogenesis of several inflammatory disorders by release of proteolytic enzymes, inflammatory mediators, and ROS.^{52–55} Thus, at least in part, the protection afforded by the absence of inflammasome components may be secondary to the decrease in neutrophil recruitment into tissue. However, the role of neutrophils in mucositis remains to be elucidated.

Together, these data support that NOX-2–derived oxidative stress mediates inflammasome activation, proinflammatory cytokine production, and tissue injury during

irinotecan-induced mucositis (Figure 7). Our results suggest new therapeutic targets that could be useful for the treatment of mucositis associated with chemotherapeutic agents.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/10.1016/j.ajpath.2014.03.012>.

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