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ORIGINAL ARTICLE

Interleukin-1 receptor antagonist attenuates cyclophosphamide-induced mucositis in a murine model

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Abstract
Purpose Cyclophosphamide is a cytotoxic chemotherapy drug that causes severe damages to hematopoietic and gastrointestinal systems. The aim of this study is to evaluate the protective effects of recombinant human interleukin-1 receptor antagonist (rhIL-1Ra) on chemotherapy-induced mucositis (CIM) in a murine model of cyclophosphamide chemotherapy.
Methods In *single* chemotherapy models, equal numbers of gender-matched Balb/c mice were administered intraperitoneal injections of rhIL-1Ra at a dose of 1 mg/kg/day or vehicle for 5 continuous days, followed by single intraperitoneal injection of cyclophosphamide at doses of 100, 300, 400 or 550 mg/kg. In *multiple cycles of* chemotherapy models, mice were administered rhIL-1Ra or vehicle for 5 days, followed by cyclophosphamide injection at a dose of 300 mg/kg. The course has been repeated for 2 or 3 times with a 1-month break in between. In *continuous* chemotherapy models, mice were administered rhIL-1Ra or vehicle for 5 days, followed by cyclophosphamide injections at doses of 150 or 200 mg/kg/day for 3 days. Body weight and diarrhea were observed in each model. Intestinal morphology was observed in mice received 300 or 400 mg/kg cyclophosphamide chemotherapy.
Results CIM was induced by cyclophosphamide in a dose-dependent manner. RhIL-1Ra attenuated CIM with reduced body weight loss, diarrhea, intestinal injuries and mortality after CY chemotherapy.
Conclusions The pretreatment with rhIL-1Ra effectively protected murine gastrointestinal system from clinically relevant cyclophosphamide regimens. The identification of these protective effects of rhIL-1Ra highlights clinical values of this protein for the prevention of CIM.

Keywords Recombinant human interleukin-1 receptor antagonist · Cyclophosphamide · Chemotherapy · Mucositis

Abbreviations

rhIL-1Ra	Recombinant human inteuleukin-1 receptor antagonist
CY	Cyclophosphamide
CIM	Chemotherapy-induced mucositis
CID	Chemotherapy-induced diarrhea
BW	Body weight

Introduction

Cytotoxic drugs are used in cancer chemotherapy, but they may also cause different injuries especially to the tissues with high proliferating rate, including the bone marrow and the epithelia of the gastrointestinal tract [1–4].

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Cyclophosphamide (CY) has been used in chemotherapy for its ability to kill dividing cells, such as in the treatment of leukemia [5, 6]. It targets all cells but functions best against fast proliferating cells by cross-linking DNA with its bioactive metabolite, phosphoramide mustard [7, 8]. Although CY has been reported to be less toxic to primitive cells such as hematopoietic progenitors and intestinal crypt stem cells, it still damages these cells at high doses and causes suppression of bone marrow and apoptosis of gastrointestinal system [4, 9–12].

Mucositis is a pathological damage induced by chemotherapy. It affects the alimentary tract and can be classified into oral and gastrointestinal mucositis [13]. Chemotherapy-induced mucositis (CIM) could be divided into different stages. First, chemotherapy drugs cause damages to the DNA and generate reactive oxygen species (ROS); the drugs and ROS will then induce apoptosis and up-regulate inflammatory cytokines. These cytokines produce further tissue damages, amplify signaling cascades and finally cause ulceration including the loss of mucosal integrity and the production of extremely painful lesions [14]. Before the healing stage, CIM may result in severe body weight (BW) loss and chemotherapy-induced diarrhea (CID). As one of the severest complications of gastrointestinal CIM, CID occurs in 50–80% of patients experiencing chemotherapy and leads to malnutrition, electrolyte imbalance and immune attenuation [15–17]. Some proteins have been evaluated for their benefits to cure mucositis including interleukin-1 (IL-1), IL-11, IL-15, R-spondin 1, transforming growth factor-beta 3 (TGF-β3) and keratinocyte growth factor (KGF) [18–23]. However, despite the attempts to develop therapeutic measures of CIM, only 3 drugs have been recommended to treat CIM currently: loperamide, deodorized tincture of opium and octreotide [24]. No protein is recommended in the treatment of CIM.

Interleukin-1 receptor I (IL-1RI) is expressed by intestinal epithelial cells [25]. IL-1 binds to IL-1RI as an agonist and stimulates the expansion of bone marrow progenitor cells and enhances the recovery of murine hematopoiesis after chemotherapy [26–29]. The expressions of IL-1α and IL-1β are significantly increased in mucosa of inflammatory bowel disease, indicating the relationships between these cytokines and mucositis [30]. On the contrary, interleukin-1 receptor antagonist (IL-1Ra), functioning as IL-1RI's antagonist, binds to IL-1RI while induces no intracellular signals [31]. IL-1Ra inhibits bone marrow cells from entering S-phase, and the lowered cycling status of hematopoiesis will then protect bone marrow from chemotherapy [4, 32]. Although IL-1Ra is secreted by normal intestinal mucosa and the secretion is enhanced in inflamed mucosa, the relationship between IL-1Ra and gastrointestinal CIM is not reported yet [33].

The aim of this study is to evaluate the protective effects of recombinant human IL-1Ra (rhIL-1Ra) on CIM in a murine model of CY chemotherapy. In this report, we show that the toxicity of CY leads to the symptoms of CIM including the BW loss, CID and morphological changes in the intestinal tract featured by the injuries of villi and crypts, in a dose-dependent manner. We have further proven that the pretreatment with rhIL-1Ra reduces CIM and mortality after CY chemotherapy. To the best of our knowledge, this is the first report of the protective effects of rhIL-1Ra on CIM.

Materials and methods

Animals

Specific pathogen-free gender-matched Balb/c mice (Slac, Shanghai, China) aged 8–12 weeks were fed in individual ventilated caging systems at 23 ± 5°C with 12-h cycled light and dark environment, being allowed free access to sterilized water and food. All studies adhered to "Principles of laboratory animal care" (NIH publication No. 85–23, revised 1985) and the guidelines of Animal Care and Use Committee of School of Pharmacy of Shanghai Jiao Tong University.

The purification of rhIL-1Ra

The coding sequence of secreted form of human IL-1Ra (GI: 186385) was cloned from human liver cDNA library by PCR using sense 5′-GGAATTC CATATG CGACC CTCTGGGAGAAAATCC-3′ and antisense 5′-CGC G GATCC TTACTCGTCCTCCTGGAAGTAGA-3′ primers (Sangon, Shanghai, China). The underlined were digestion sites for Nde I and BamH I (Toyobo, Dalian, China), respectively. The obtained fragment was packed into pET11a vector (Novagen, Darmstadt, German), and the vector was then chemically transformed into the competent *E. coli* BL21 (DE3) bacteria. RhIL-1Ra was expressed under the induction with 1 mM isopropyl-beta-D-thiogalactopyranoside. The quality and bioactivity of the protein were characterized by methods as previously described [32, 34–36].

CIM models

In order to establish CIM models and to verify the protective effects of rhIL-1Ra, 3 strategies of CY treatments had been designed to mimic the clinical application, including *single*, *multiple cycles* and *continuous* CY chemotherapy.

In *single* CY chemotherapy, equal numbers of mice were first administered intraperitoneal (i.p.) injections of rhIL-1Ra at a dose of 1 mg/kg/day or endotoxin-free normal saline (NS) vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 100 ($n = 12$/group), 300 ($n = 12$/group), 400 ($n = 16$/group) or 550 ($n = 10$/group) mg/kg (indicated as 100-CY, 300-CY, 400-CY or 550-CY later).

In *multiple cycles of* CY chemotherapy, equal numbers of mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at a dose of 300 mg/kg. The course of rhIL-1Ra and CY injections had been repeated for 2 ($n = 8$/group) or 3 ($n = 17$/group) times with a 1-month break in between (indicated as 2 × CY or 3 × CY later).

To further verify the protective effects of rhIL-1Ra against chemotherapy, a third regimen had been designed as *continuous* CY chemotherapy. Equal numbers of mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by i.p. injections of CY at doses of 150 ($n = 13$/group) or 200 mg/kg/day ($n = 15$/group) for 3 continuous days (indicated as 150 × 3-CY or 200 × 3-CY later).

BW observation

BW was determined once a day before and after chemotherapy in all experiments except for 400-CY. In *multiple cycles of* CY chemotherapy, mice were coded, and each mouse was weighed individually. The relative BW was calculated for each mouse as "BW of the mouse post-chemotherapy/its BW at the beginning of the study (day 0)". The relative BW was then scored by a modified method [37] as follows: 0, no loss of BW (>90% of the original weight); 1, slight loss (80–90%); 2, moderate loss (70–80%); 3, severe loss (60–70%); 4, huge loss (<60% or death occurred). The scoring was conducted from day 1 to 13 after CY chemotherapy. The incidences of the loss of BW scores were determined by counting observations with that score.

CID observation

CID was observed once a day after 400-CY, 550-CY and 200 × 3-CY chemotherapy. No CID was observed in other experiments. The CID was scored as follows: 0, no diarrhea; 1, slight diarrhea (slightly wet and soft stool); 2, moderate diarrhea (wet and unformed stool with moderate perianal staining of the coat); 3, severe diarrhea (watery stool with severe perianal staining of the coat) [38]. The scoring was conducted when diarrhea happened: from day 1 to day 4 after 400-CY, from day 1 to day 10 after 550-CY and from day 1 to day 17 after 200 × 3-CY chemotherapy. The incidences of CID scores were determined by counting observations with that score.

Intestinal histology

Intestinal histology was investigated as described [18]. Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 300 or 400 mg/kg. They were then killed at days 1.5, 3, 7 and 11 post-chemotherapy, and at day 0 before the chemotherapy began. The segments of the jejunum were fixed in 4% polyformaldehyde and embedded with paraffin. Four cross sections for each mouse were prepared. The slides were stained with hematoxylin and eosin staining. The lengths (depths) and areas of 10 randomly selected villi or survived crypts on each cross section were measured using Nikon NIS Element BR 3.0 (40 villi or crypts for each mouse). Survived crypts were identified as crypts containing more than 10 cells.

The pathologic mucositis of intestine were scored as follows [39]: 0, normal mucosal villi; 1, development of subepithelial space, usually at the apex of the villi with capillary congestion; 2, extension of the subepithelial space with moderate lifting of the epithelial layer from the lamina propria; 3, massive epithelial lifting down the sides of the villi; 4, ulceration at the villous tips or denuded villi with dilated capillaries and increased cellularity of the lamina propria; 5, degradation and disintegration of the lamina propria, hemorrhage and ulceration. Percentage of ulceration was determined by calculating the length of ulcer area alongside the circumference in the cross section of intestine and 4 cross sections for each mouse were determined.

Statistical analysis

Results were expressed as mean ± standard deviation (SD). Significances were determined by two-tailed Student's *t*-test or Wilcoxon test. Survival experiments were analyzed with the log-rank test and expressed as the Kaplan–Meier survival curves by SAS (SAS Institute Inc., USA). Other statistical significances were determined as declared. All comparisons in "Results" part between control and rhIL-1Ra–treated mice were arranged as "control vs. rhIL-1Ra".

Results

Protein expression and purification

RhIL-1Ra was purified as described. Only protein with purity >98%, endotoxin <0.2 E.U./μg and bioactivity

comparable to commercialize protein was used in animal experiments (Fig. S1).

RhIL-1Ra reduced the loss of BW after CY chemotherapy

To establish the robust murine model of CIM, we first investigated the dose–response effects of CY regimens on the loss of BW. In *single* CY chemotherapy, the loss of BW was increased as the CY doses were increased; further, the nadirs of mean BW were delayed as the CY doses were increased, indicating lengthened injury durations (control group, Table 1). This dose–response was not identified in *multiple cycles of* CY chemotherapy, since the loss of BW not worsened as CY chemotherapy was repeated (control group, Table 2).

We then evaluated the protective effects of rhIL-1Ra on the loss of BW. In *single* CY chemotherapy, the loss of BW was generally reduced by the pretreatment with rhIL-1Ra. The mean BW of rhIL-1Ra–treated mice were 1.9, 7.2 and 9.3% higher than control at day 5, and 3.8, 7.2 and 15.7% higher than control at the control's nadirs post-chemotherapy (Table 1). In experiments when mice received CY injections at a dose of 300 mg/kg for 1, 2 or 3 times (indicated as 300-CY, 2 × CY or 3 × CY), each mouse was weighed individually once a day, and the loss of BW was scored as described. RhIL-1Ra significantly reduced the incidences of the loss of BW above grade 1, 2 or 3 after 300-CY chemotherapy, reduced the incidences above grade 1 or 2 as well as eliminated those of grade 2, 3 and 4 after 2 × CY chemotherapy, and reduced the incidences above grade 3 or 4 after 3 × CY chemotherapy. Importantly, rhIL-1Ra effectively reduced the incidences of severe BW loss (grade 3 or 4). When compared to *single* CY chemotherapy at 300 mg/kg (300-CY), the loss of BW in *multiple cycles of* CY chemotherapy (2 × CY or 3 × CY) did not worsen, and the protective effects of rhIL-1Ra were similar in these three CY regimens (Table 2).

Further, rhIL-1Ra shortened the durations of the loss of BW after CY chemotherapy, especially when CY was administered at high doses. When treated with rhIL-1Ra, the nadirs of BW happened at day 6 after 550-CY chemotherapy, and at day 7 after 3 × CY chemotherapy, when compared to day 9 and day 13 in control, respectively. In general, rhIL-1Ra reduced the severity and duration of the loss of BW after *single* and *multiple cycles of* CY chemotherapy.

RhIL-1Ra reduced the incidences of CID after CY chemotherapy

When administered CY at a dose of 300 mg/kg for 1, 2 or 3 times, mice did not develop CID. However, CID was a dominant toxicity of higher doses of CY chemotherapy and was induced in a dose-dependent manner (the mean CID score was 0.313 and 1.514 in 400-CY and 550-CY, respectively. Control group, Table 3). We evaluated the protective effects of rhIL-1Ra on CID. After 400-CY chemotherapy, slight diarrhea was observed in control group. RhIL-1Ra reduced CID above grade 1 significantly. After 550-CY chemotherapy, much severer diarrhea was noticed in control group. RhIL-1Ra attenuated not only slight CID (grade 1) but also the moderate and severest one (grade 2 and 3). The mean diarrhea scores were significantly reduced by rhIL-1Ra, either (Table 3). RhIL-1Ra not only reduced the severity of CID but also shortened the durations of it by reducing the late diarrhea (Fig. 1). The CID durations were shortened by rhIL-1Ra from 3 days to

Table 1 RhIL-1Ra reduced the loss of BW after single CY chemotherapy

Experiments	Groups	Day 0	Day 5		BW at the control group' nadir		
		BW (g)	BW (g)	The loss of BW (%)	Nadir (day)	BW (g)	The loss of BW (%)
100-CY	Control	24.44 ± 2.95	23.19 ± 3.00	5.1	3	22.77 ± 3.14	6.8
	IL-1Ra	22.93 ± 2.73	23.63 ± 4.14	ND		23.63 ± 1.03	ND
300-CY	Control	23.73 ± 1.37	18.29 ± 1.36	22.9	5	18.29 ± 1.36	22.9
	IL-1Ra	23.75 ± 1.67	19.60 ± 1.58*	17.5		19.60 ± 1.58*	17.5
550-CY	Control	21.04 ± 1.18	15.78 ± 1.15	25.0	9	15.40 ± 3.25	26.8
	IL-1Ra	21.06 ± 0.95	17.24 ± 0.99*	18.3		17.82 ± 0.83	15.5

Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 100, 300 or 550 mg/kg (indicated as 100-CY, 300-CY or 550-CY, respectively). Mice were weighed once a day, and the mean BW was determined, presented as mean ± SD. The loss of BW was calculated as "100% (the mean BW post-chemotherapy/the mean BW at day 0)". RhIL-1Ra significantly reduced the loss of BW after single CY chemotherapy

ND not detectable (the mean BW was not decreased post-chemotherapy)

* $P < 0.05$ represented significant differences between the rhIL-1Ra–treated mice and control (two-tailed Student's *t*-test)

Table 2 RhIL-1Ra reduced the incidences of the loss of BW

Experiments	Groups	Incidence of the loss of BW[a] (%)				Mean score[b]
		Score 1 + 2 + 3 + 4	Score 2 + 3 + 4	Score 3 + 4	Score 4	
300-CY	Control	69.9	33.0	5.8	3.9	1.126
	IL-1Ra	48.2**	14.3**	0.9*	0.9	0.643**
2 × CY	Control	81.6	12.6	1.9	1.0	0.971
	IL-1Ra	23.1**	0.0**	0.0	0.0	0.231**
3 × CY	Control	57.3	31.8	11.8	8.2	1.091
	IL-1Ra	53.3	23.0	4.4*	2.2*	0.830

Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at a dose of 300 mg/kg. The course of rhIL-1Ra and CY injections had been performed for 1 ($n = 12$/group), 2 ($n = 8$/group) or 3 ($n = 17$/group) cycles (indicated as 300-CY, 2 × CY or 3 × CY). Each mouse was weighed individually once a day and the loss of BW was scored as described. RhIL-1Ra significantly reduced the incidences of the loss of BW after CY chemotherapy

* $P < 0.05$, ** $P < 0.01$ represented significant differences between the rhIL-1Ra–treated mice and control

[a] Significances were determined by Fisher's exact test

[b] Significances were determined by Wilcoxon test (SAS)

Table 3 RhIL-1Ra reduced the incidences of CID

Experiments	Groups	Incidence of CID[a] (%)			Mean score[b]
		Score 1 + 2 + 3	Score 2 + 3	Score 3	
400-CY	Control	25.0	6.3	0.0	0.313
	IL-1Ra	9.4*	4.7	1.6	0.156*
550-CY	Control	82.9	42.9	25.7	1.514
	IL-1Ra	30.6**	6.9**	4.2**	0.417**

Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 400 ($n = 16$/group) or 550 mg/kg ($n = 10$/group, indicated as 400-CY or 550-CY). CID was observed once a day and scored as described. RhIL-1Ra significantly reduced CID after high doses of CY chemotherapy

* $P < 0.05$, ** $P < 0.01$ represented significant differences between the rhIL-1Ra–treated mice and control

[a] Significances were determined by Fisher's exact test

[b] Significances were determined by Wilcoxon test (SAS)

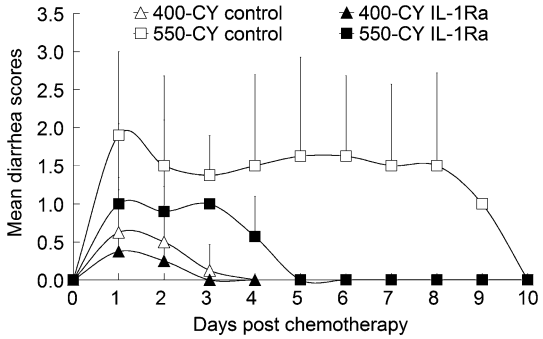

Fig. 1 RhIL-1Ra reduced CID after CY chemotherapy. Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 400 ($n = 16$/group) or 550 mg/kg ($n = 10$/group, indicated as 400-CY or 550-CY). The scoring of CID was conducted once a day as described. RhIL-1Ra shortened the durations of CID by reducing the incidences of late diarrhea. Each point represented the mean ± SD

2 days after 400-CY chemotherapy and from 9 days to 4 days after 550-CY chemotherapy.

RhIL-1Ra reduced the mucositis of mice after *continuous* CY chemotherapy

We further evaluated the effects of rhIL-1Ra after *continuous* CY chemotherapy. BW and CID were determined once a day post-chemotherapy. The results revealed that higher doses of *continuous* CY chemotherapy resulted in severer CIM, including the worsened loss of BW and the presence of CID. Similar to the results derived from *single* and *multiple cycles of* CY chemotherapy, rhIL-1Ra attenuated the intestinal CIM including reduced loss of BW and CID, as well as reduced mortality after *continuous* CY chemotherapy (Fig. S2).

RhIL-1Ra reduced the injuries of the morphology of intestine after high doses of CY chemotherapy

CY decreased the mean BW of mice at all doses and caused CID at higher doses, indicating damages to the morphology of intestine. We then evaluated the damages to intestinal morphology induced by CY chemotherapy and the protective effects of rhIL-1Ra against it. After 300 mg/kg CY chemotherapy, villi lengths decreased in control group. RhIL-1Ra prevented this decrease at every checked time points, and the villi areas were also protected at day 3 and day 11 post-chemotherapy. After 400 mg/kg CY

chemotherapy, the structures of intestine were more severely injured. RhIL-1Ra protected crypts' areas at days 7 and 11 post-chemotherapy, and protected villi lengths and areas at day 11, significantly (Fig. 2a, b, c). Histological grading revealed the protected morphology of intestine in rhIL-1Ra–treated mice after both 300 and 400 mg/kg CY chemotherapy (Fig. 2d).

More importantly, 400 mg/kg CY chemotherapy led to severe damages to crypts including the death of crypts and the progression of ulcer. As reported, due to the ability of aldehyde dehydrogenase (ALDH) in transforming CY into carboxyphosphamide (non-toxic) [8], the high expression level of ALDH in intestinal crypts would reduce the toxicity of CY [40]. We found that the toxicity of CY at similar doses to other cytotoxic agents was weaker, resulting in high crypts survival, which was in accordance with previous publications [12, 41]. We also identified that the survival of crypts was significantly increased by rhIL-1Ra at day 1.5 post-chemotherapy (73.9 ± 27.2% vs. 96.2 ± 2.3%, Fig. 3a).

When ulcer developed, the intestinal structures of villi and crypts were injured and a whole damaged area was observed. The progression of ulcer was observed at day 3 and day 7 post-chemotherapy in control group, but not observed in rhIL-1Ra–treated mice (11.7 ± 14.0% vs. 0% at day 3 and 25.0 ± 20.7% vs. 0% at day 7, Fig. 3b). RhIL-1Ra effectively prevented the death of crypts and ulceration of intestine against high doses of CY chemotherapy.

RhIL-1Ra showed no effects on normal mice

RhIL-1Ra was found to suppress normal hematopoiesis by inhibiting bone marrow cells from entering S-phase [32]. We analyzed whether continuous i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day for 5 days would affect normal murine intestine or not. In the above-mentioned experiments, the averages of BW of rhIL-1Ra–treated mice and control were compared, and no significances were noticed. Inspections on the morphology of the intestine revealed no significant changes, too (day 0, Fig. 2). In another experiment, mice were injected with increasing doses of rhIL-1Ra at 0, 0.1, 0.3, 1, 3 and 9 mg/kg/day for continuous 5 days, and no tendencies had been observed between the protein doses and the mean BW ($n = 4$/group, data not shown). No diarrhea was observed in each experiment.

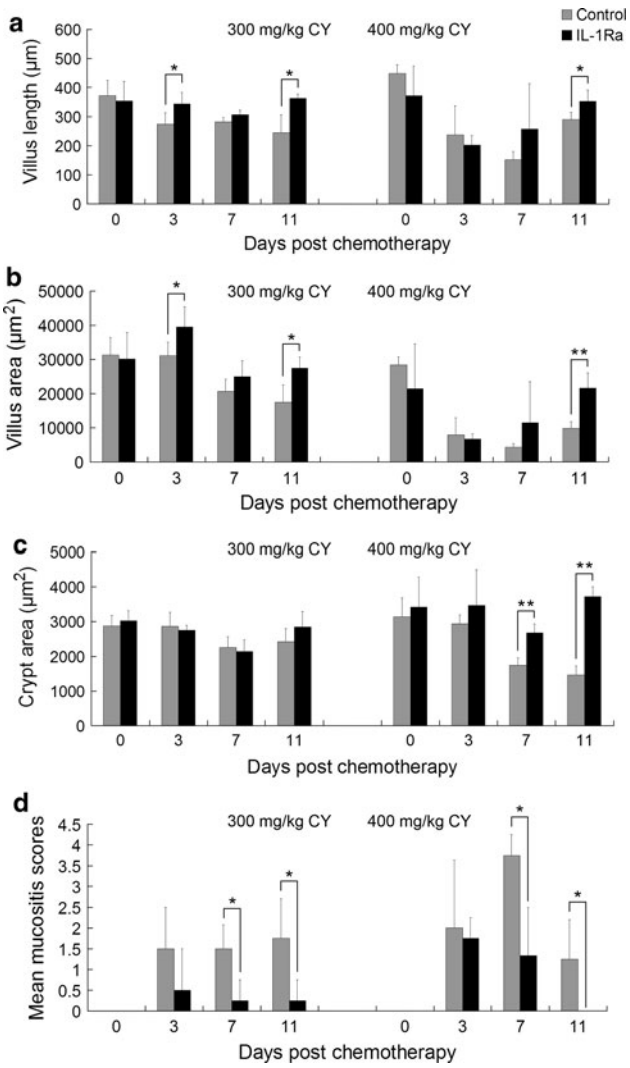

Fig. 2 RhIL-1Ra protected the morphology of intestine after CY chemotherapy. Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at doses of 300 or 400 mg/kg. The morphology of intestine was examined at days 3, 7 and 11 post-chemotherapy and at day 0 after the final rhIL-1Ra or NS injection. Villi and crypts were measured and mucositis was scored as described. **a** and **b** The administration of 400 mg/kg CY resulted in severer damages to intestinal villi than 300 mg/kg CY. RhIL-1Ra reduced the injuries of villi lengths (**a**) and areas (**b**) induced by both 300 and 400 mg/kg CY chemotherapy. **c** The administration of 400 mg/kg CY resulted in severer damages to intestinal crypts than 300 mg/kg CY. RhIL-1Ra reduced the injuries of crypt areas induced by 400 mg/kg CY chemotherapy. **d** The administration of 400 mg/kg CY resulted in severer CIM than 300 mg/kg CY. RhIL-1Ra reduced CIM after both 300 and 400 mg/kg CY chemotherapy. Each *bar* represented the mean ± SD. $n = 4$/group. $^*P < 0.05$, $^{**}P < 0.01$ represented significant differences between the rhIL-1Ra–treated mice and control (**a–c** two-tailed Student's *t*-test; **d** Wilcoxon test)

Discussion

CY is a non-cell-cycle-specific cytotoxic chemotherapy agent. In this report, we first identify the dose-dependent toxicity of CY on intestine. In *single* CY chemotherapy, when CY doses are increased, the loss of BW of mice increased, so as its duration is lengthened. CID is only observed in mice treated with high doses of CY and is

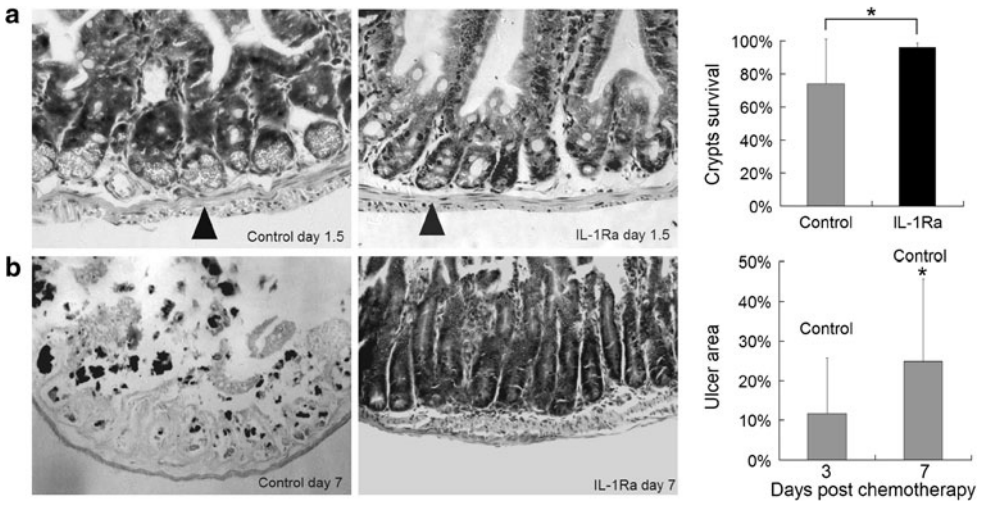

Fig. 3 RhIL-1Ra protected the crypts of intestine after CY chemotherapy. Mice were administered i.p. injections of rhIL-1Ra at a dose of 1 mg/kg/day or NS vehicle for 5 continuous days, followed by single i.p. injection of CY at a dose of 400 mg/kg. The crypts were examined at day 1.5, 3 and 7 post-chemotherapy. **a** Representative hematoxylin and eosin stained cross sections of intestine, indicating that rhIL-1Ra increased crypts survival at day 1.5 post-chemotherapy. Paneth cells were indicated with *arrowhead*. Original magnification: 400×. **b** Representative hematoxylin and eosin stained cross sections of intestine, indicating the progression of ulcer in control group at days 3 and 7 post-chemotherapy. No ulceration was observed in rhIL-1Ra–treated mice. Original magnification: 400×. Each *bar* represented the mean ± SD. $n = 4$–10/group. $^*P < 0.05$ represented significant differences between the rhIL-1Ra–treated mice and control (Wilcoxon test)

increased and prolonged by the increased CY doses. In *continuous* CY chemotherapy, mice treated with 200 × 3-CY develop severer BW loss when compared to mice treated with 150 × 3-CY; and CID is only observed in mice treated with 200 × 3-CY. These evidences indicate that CY causes CIM in a dose-dependent manner. However, the severity of mucositis induced by *multiple cycles of* CY chemotherapy is similar to *single* CY chemotherapy at the same dose. To further evaluate the toxicity of CY, inspections on the morphology of intestine were performed, and much severe mucositis and ulceration in mice treated with 400 mg/kg CY were identified when compared to mice treated with 300 mg/kg CY.

We then prove the protective effects of rhIL-1Ra on intestine against clinically relevant CY regimens. The pretreatment with rhIL-1Ra reduces the severity of BW loss in *single*, *multiple cycles* and *continuous* CY chemotherapy, as well as reduces the durations of it in high doses of CY chemotherapy. These protective effects are further proved by the effects of rhIL-1Ra on reducing the severity and shortening the duration of CID after high doses of CY chemotherapy. To identify the mechanisms of these protective effects, the morphology of intestine is examined. RhIL-1Ra reduces the injuries of intestine featured by protected intestinal villi and crypts as well as attenuated pathological mucositis. We then conclude that the protected status of intestine benefits the health of mice from the toxicity of CY chemotherapy.

As reported previously, TGF-β protects murine intestine crypts when applied prior to irradiation, possibly because of the suppressive effects of TGF-β on cell cycling and proliferating [22, 42]. Interestingly, in our previous report, rhIL-1Ra was proved to suppress bone marrow cells' cycling by inhibiting them from entering S-phase [32]. We assume that the pretreatment with rhIL-1Ra leads to similar suppression on intestine crypts as TGF-β. This suppression would then explain the protective effects of rhIL-1Ra on intestine. Although the effects of rhIL-1Ra on BW and the morphology of intestine are not identified in normal mice in this report, we hypothesize that the administration of rhIL-1Ra alters the microenvironment of murine intestine and changes the growth status of intestine epithelial cells. This alteration leads to the protected status of murine crypts against CY chemotherapy. Nevertheless, it remains to be seen more specific researches to identify the mechanism of rhIL-1Ra on the cell-cycling status of murine intestine crypt cells.

RhIL-1Ra was approved by Food and Drug Administration for treating rheumatoid arthritis and could be safely administrated for 3 years to healthy volunteers, with only few hematologic effects [43, 44]. RhIL-1Ra was identified to protect murine hematopoiesis against CY or 5-Fu chemotherapy [4, 32]. In this report, we prove that rhIL-1Ra protects murine intestine against CY chemotherapy. Our data strongly suggest that rhIL-1Ra may be used to improve the effects of cancer treatment by reducing the

adverse side effects such as bone marrow suppression and diarrhea, and allowing for dose escalation of chemotherapy agents, which equals to improved cure rates.

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