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# Gadolinium chloride improves the course of TNBS and DSS-induced colitis through protecting against colonic mucosal inflammation

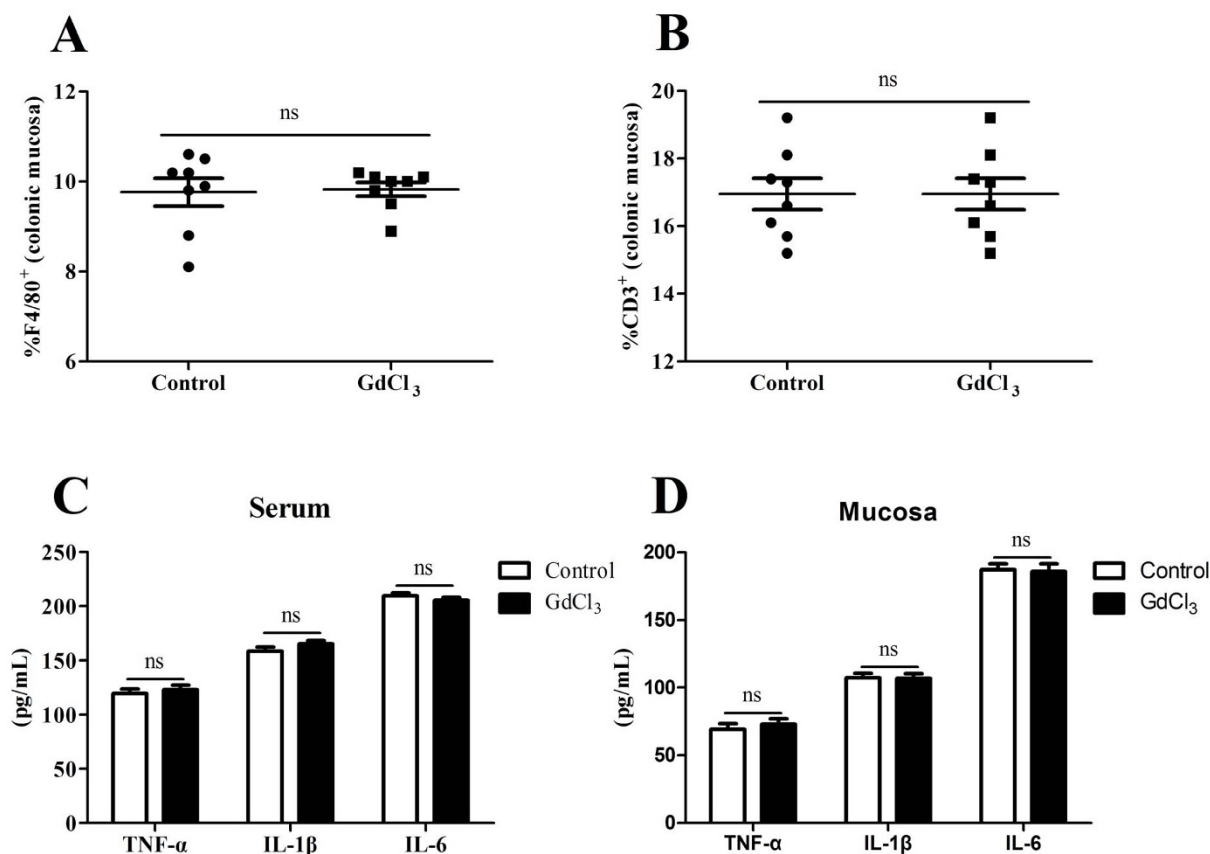
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Inflammatory macrophages in colonic mucosa are the leading drivers of the pathology associated with inflammatory bowel disease (IBD). Here we examined whether gadolinium chloride (GdCl<sub>3</sub>), a macrophage selective inhibitor, would improve the course of 2,4,6-trinitro benzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS)-induced colitis in mice and the potential mechanisms were investigated. By giving GdCl<sub>3</sub> to colitis mice through intravenous or intrarectal route, we found that GdCl<sub>3</sub> markedly ameliorated the colitis severity, including less weight loss, decreased disease activity index scores, and improved mucosal damage. To investigate the potential mechanisms, flow-cytometric analysis was performed to detect the proportion of mucosal macrophages in colon. The results showed that GdCl<sub>3</sub> had no macrophage depletion effect in colonic mucosa, but significantly suppressed TNBS and DSS-induced TNF $\alpha$ , IL-1 $\beta$  and IL-6 secretions. Also, Western blotting analysis indicated that NF- $\kappa$ B p65 expression was significantly attenuated in the mucosa in colitis mice with GdCl<sub>3</sub> treatment. Then, the anti-inflammatory activity of GdCl<sub>3</sub> was confirmed in LPS-stimulated RAW 264.7 cells that GdCl<sub>3</sub> might down-regulate the production of proinflammatory cytokines by macrophages through inhibition of the NF- $\kappa$ B signaling pathway. Therefore, intervention with mucosal inflammatory macrophages may be a promising therapeutic target in IBD.

Inflammatory bowel disease (IBD) comprises Crohn's disease and ulcerative colitis that cause chronic and remittent-relapsing intestinal inflammation of all or part of the intestinal tract<sup>1,2</sup>. Although the aetiology of IBD remains unclear, it is thought to result from dysregulation of the mucosal immune responses to intestinal bacterial antigens in genetically predisposed individuals<sup>1,3</sup>. Currently, treatment options for IBD are mainly focused on suppressing mucosal immune responses, including the use of 5-aminosalicylic acid (5-ASA) agents, steroids, antimicrobials, and some immunomodulators<sup>4</sup>. However, although they are reasonably successful in many patients, still a great number cannot achieve remission, highlighting the need for novel therapeutic targets.

Infiltration and activation of macrophages in colon are central features of IBD, and inflammatory macrophages in mucosa are thought to play an essential role in the pathogenesis of IBD<sup>2,5-7</sup>. In IBD and experimental colitis, monocytes in blood are recruited to the mucosa and differentiate into activated macrophages that produce pro-inflammatory cytokines, such as tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interleukin (IL)-1 and IL-6<sup>2,7,8</sup>. Activation of NF- $\kappa$ B is thought as a strong inducer of these proinflammatory cytokine expressions<sup>9,10</sup>, and activated NF- $\kappa$ B has been demonstrated in colonic macrophages of patients with IBD. Alteration in cytokine production by macrophages is one major component of the pathology of IBD. Therefore, strategies for targeting inflammatory mucosal macrophages may be important for developing new therapeutics. However, studies on intervention with mucosal macrophages in colon are really limited, which might mainly due to the limited intervention methods.

Gadolinium chloride (GdCl<sub>3</sub>), a rare earth metal, is widely used experimentally<sup>11,12</sup>. The role of GdCl<sub>3</sub> in macrophage elimination has been widely proven in liver of experimental animals, indicating a preventive or therapeutic effect in liver injury<sup>12-14</sup>. GdCl<sub>3</sub> is also employed as a macrophage selective inhibitor *in vivo*<sup>11</sup>. It has been reported that GdCl<sub>3</sub> has no depletion effect in tissue macrophages in lung, but it decreases the expression of TNF $\alpha$  and IL-6 after LPS stimulation in rat<sup>15</sup>. However, the effect of GdCl<sub>3</sub> on mucosal macrophages in colon remains largely unknown.



**Figure 1** | GdCl<sub>3</sub> had no macrophage depletion effect in colonic mucosa. After intravenous treatment of GdCl<sub>3</sub> (10 mg/kg body weight) for 1 day, mice were killed, and the proportion of mucosal macrophages in colon and the cytokine expression levels in serum and colonic mucosa were evaluated. (A) Proportion of mucosal macrophages in colon. (B) Proportion of CD3<sup>+</sup> cells in colonic mucosa. (C) Cytokine expression of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in serum. (D) Cytokine levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in colonic mucosa. (ns: no significance).

Experimental animal models are crucial tools that provide a road map allowing us to probe the pathogenesis of diseases and to test emerging therapeutic strategies. 2,4,6-trinitro benzene sulfonic acid (TNBS) and dextran sodium sulfate (DSS) colitis models are canonical IBD models that the onset of inflammation is immediate and the procedure is relatively straightforward; therefore, the two models have been frequently used to evaluate the efficacy of potential therapeutic agents<sup>16,17</sup>.

The present study was performed to investigate the protective role and the potential mechanisms of GdCl<sub>3</sub> in TNBS- and DSS-induced colitis. We studied the elimination effect of GdCl<sub>3</sub> on mucosal macrophages. The colitis severity that included body weight loss, disease activity index scores, and mucosal damage, was evaluated, and the protein secretion of cytokines and the activity of NF- $\kappa$ B signal pathways were also studied in colitis mice treated with GdCl<sub>3</sub>. Also, the role of GdCl<sub>3</sub> in macrophages was further investigated in activated RAW 264.7 cells *in vitro*.

## Results

**GdCl<sub>3</sub> has no elimination effect on mucosal macrophages in colon in mice.** The effect of GdCl<sub>3</sub> on mucosal macrophages in colon is still unknown. In this study, we evaluated the proportion of mucosal macrophages in colon in mice after intravenous administration of GdCl<sub>3</sub>. It was revealed that intravenous treatment of GdCl<sub>3</sub> at each concentration could not deplete macrophages in colonic mucosa. We then used GdCl<sub>3</sub> at a concentration of 10 mg/kg body weight for 1 day before animal treatment for further study in accordance with the method described previously<sup>15,18</sup>.

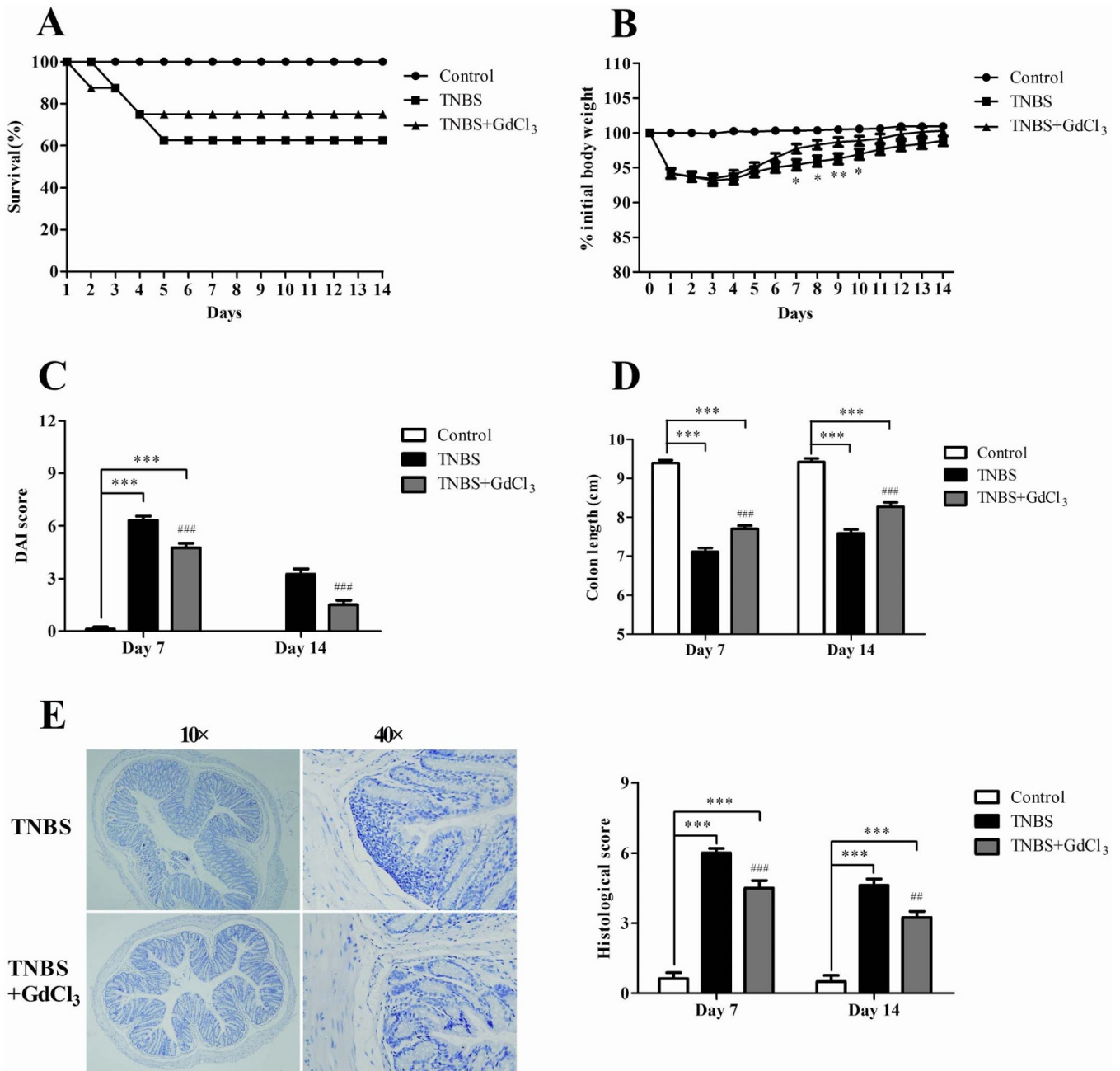
Figure 1A showed that the proportion of macrophages in colonic mucosa was not altered after GdCl<sub>3</sub> treatment (10 mg/kg body

weight) for 1 day in mice ( $9.76 \pm 0.31\%$  vs  $9.83 \pm 0.15\%$ ,  $p > 0.05$ ). No difference in the proportion of CD3<sup>+</sup> cells in colonic mucosa was observed between GdCl<sub>3</sub>-treated mice and control mice ( $p > 0.05$ ; Figure 1B). In serum and colonic mucosa tissue, the expression profiles of TNF $\alpha$ , IL-1 $\beta$  and IL-6 were not obviously elevated after GdCl<sub>3</sub> treatment ( $p > 0.05$  for all three cytokine types; Figures 1C, D). Thus, GdCl<sub>3</sub> could not deplete macrophages in colonic mucosa and also would not induce inflammation *in vivo*.

## GdCl<sub>3</sub> is protective against colitis induced by TNBS and DSS.

To evaluate the potential role of GdCl<sub>3</sub> in colitis *in vivo*, we used two murine models of colitis induced by TNBS and DSS. In TNBS colitis, GdCl<sub>3</sub> was given to mice on day 3 of TNBS application intravenously. The mortality of TNBS colitis mice was improved by about 20% after GdCl<sub>3</sub> treatment (Figure 2A). Treatment with GdCl<sub>3</sub> resulted in prominent protection from colitis as assessed by body weight, disease activity index (DAI) scores, colon length and histopathological damage of the colon. Control mice presented weight loss after TNBS administration, whilst colitis mice treated with GdCl<sub>3</sub> showed markedly less body weight loss (Figure 2B). GdCl<sub>3</sub> significantly improved DAI scores and shortening of colon length in TNBS colitis (Figures 2C, D). Histopathological analysis of the colon, examined on day 7 and 14, showed marked crypt architecture damage, inflammatory cell infiltration and ulceration in colitis mice. GdCl<sub>3</sub> treatment significantly improved these damages of colon as assessed by the histopathological scores (Figure 2E).

Intrarectal treatment of GdCl<sub>3</sub> began on day 3 of TNBS application and continued until day 7. Colitis was also significantly ameliorated after GdCl<sub>3</sub> administration as assessed by changes of body weight, DAI scores, and mucosal damage, which were similar to mice



**Figure 2 | GdCl<sub>3</sub> was protective against colitis in TNBS-treated mice.** After induction of TNBS colitis, GdCl<sub>3</sub> was given to mice intravenously on day 3. After colitis induction for 7 days or 14 days, mice were killed, and the colitis severity was evaluated. (A) The survival rate. (B) The percentage of initial body weight. (C) The disease activity index (DAI). (D) The colon length. (E) The histopathological evaluation. (\* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; ## $P < 0.01$ , ### $P < 0.001$  comparing TNBS + GdCl<sub>3</sub> to TNBS).

treated with GdCl<sub>3</sub> intravenously, and there was no significant difference between mice treated with GdCl<sub>3</sub> through intravenous route and intrarectal route.

Mice were exposed to 3% DSS in drinking water for 7 days in DSS colitis. GdCl<sub>3</sub> was administered to mice through either intravenous or intrarectal route similar to the treatment in TNBS colitis mice. The results revealed that GdCl<sub>3</sub> treatment also resulted in striking protection from DSS-induced colitis (Table 1).

**GdCl<sub>3</sub> suppresses TNBS- and DSS-induced proinflammatory cytokine secretions.** To evaluate whether the protection from colitis induced by TNBS and DSS in mice with GdCl<sub>3</sub> treatment was associated with a reduction in the production of proinflammatory cytokines, expression levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in serum

and colonic mucosa of vehicle and mice treated with GdCl<sub>3</sub> were detected using ELISA.

In TNBS colitis mice treated with GdCl<sub>3</sub> intravenously, the proportion of macrophages and CD3<sup>+</sup> cells in colonic mucosa was not altered on both day 7 and day 14 (Figures 3A, B). On day 7, TNF $\alpha$ , IL-1 $\beta$  and IL-6 protein levels were significantly increased in serum and colonic mucosa obtained from mice treated with TNBS (Figures 3C, D). On day 14, expression profiles of TNF $\alpha$ , IL-1 $\beta$  and IL-6 were a little down-regulated (Figures 3C, D). But GdCl<sub>3</sub> treatment significantly reduced TNF $\alpha$ , IL-1 $\beta$  and IL-6 levels in serum and colonic mucosa on day 7 as compared to those of vehicle control mice (Figures 3C, D). A significant suppression by GdCl<sub>3</sub> treatment of TNF $\alpha$ , IL-1 $\beta$  and IL-6 levels in serum and colonic mucosa was also noted on day 14 (Figures 3C, D).

Table 1 | The role of GdCl<sub>3</sub> in DSS colitis mice

	Control		DSS		DSS + GdCl <sub>3</sub>	
	Day 7	Day 14	Day 7	Day 14	Day 7	Day 14
Survival (%)	100	100	50	50	75	75
%initial body weight	100.4 ± 0.50	101.0 ± 0.56	92.16 ± 2.17	97.16 ± 2.01	95.45 ± 1.91*	98.33 ± 1.78
DAI score	0.13 ± 0.35	0	6.88 ± 1.26	3.50 ± 1.20	5.25 ± 1.04*	2.25 ± 0.89*
Colon length	9.40 ± 0.20	9.43 ± 0.26	6.85 ± 0.28	7.31 ± 0.24	7.19 ± 0.24*	7.60 ± 0.22*
Histological score	0.63 ± 0.74	0.50 ± 0.76	6.13 ± 0.99	4.63 ± 0.92	4.50 ± 0.93**	2.88 ± 0.83*
Serum TNF-α (pg/mL)	124.1 ± 16.32	126.1 ± 10.70	340.3 ± 40.26	215.9 ± 12.90	239.2 ± 30.29*	127.1 ± 17.07**
Serum IL-1β (pg/mL)	167.0 ± 6.61	168.3 ± 13.18	390.6 ± 32.11	295.5 ± 18.45	281.6 ± 27.26**	186.1 ± 18.49**
Serum IL-6 (pg/mL)	206.3 ± 4.55	206.3 ± 21.28	522.1 ± 60.97	432.4 ± 27.32	384.0 ± 45.58*	271.1 ± 31.88**
%F4/80 <sup>+</sup> cells in mucosa	9.76 ± 0.87	9.75 ± 0.68	14.03 ± 0.90	12.93 ± 1.15	13.95 ± 0.88	12.87 ± 1.13
%CD3 <sup>+</sup> cells in mucosa	16.95 ± 1.32	16.89 ± 1.21	20.27 ± 0.84	18.47 ± 1.39	19.05 ± 0.79	17.36 ± 1.31
Mucosal TNF-α (pg/mL)	72.3 ± 13.94	74.6 ± 6.47	562.2 ± 19.76	450.3 ± 17.64	366.0 ± 8.74***	291.2 ± 16.98***
Mucosal IL-1β (pg/mL)	105.3 ± 9.67	106.6 ± 17.75	688.3 ± 11.86	364.2 ± 22.94	449.1 ± 10.65***	364.2 ± 22.94***
Mucosal IL-6 (pg/mL)	191.2 ± 14.90	193.2 ± 28.11	730.3 ± 33.70	620.4 ± 33.58	593.1 ± 28.62**	505.3 ± 33.50*

GdCl<sub>3</sub> (10 mg/kg body weight) was given to DSS colitis mice on day 3 after colitis induction through intravenous administration (DSS + GdCl<sub>3</sub>), while PBS was given to the control DSS colitis mice (DSS). On day 7 or day 14, mice were killed, and clinical symptoms, mucosal damage and colonic inflammation were evaluated. All data were expressed as mean values SEM ± SD.

\**P* < 0.05,

\*\**P* < 0.01,

\*\*\**P* < 0.001 comparing DSS + GdCl<sub>3</sub> to DSS.

The proportion of mucosal macrophages and CD3<sup>+</sup> cells in colon was also not changed in TNBS colitis mice treated with GdCl<sub>3</sub> intrarectally. TNBS-induced expression levels of TNFα, IL-1β and IL-6 were significantly decreased after intrarectal administration of GdCl<sub>3</sub>, which were not different from those in mice treated with GdCl<sub>3</sub> intravenously.

In DSS colitis mice, GdCl<sub>3</sub> was administrated to mice through either intravenous or intrarectal route. The results indicated that GdCl<sub>3</sub> also showed an anti-inflammatory property in serum and colonic mucosa (Table 1).

**GdCl<sub>3</sub> reduces proinflammatory cytokine production by LPS-stimulated RAW 264.7 cells.** The anti-inflammatory activity of GdCl<sub>3</sub> was also confirmed in LPS-stimulated RAW 264.7 cells *in vitro*. To define the experimental dose range of GdCl<sub>3</sub> for *in vitro* use, its effect on cell viability was assessed by MTT assay. GdCl<sub>3</sub> did not exert a toxicity in RAW 264.7 cells at a concentration ranging from 10 to 200 μM (Figure 4A). Flow-cytometric analysis also showed a lack of pro-apoptotic effect of GdCl<sub>3</sub> on the cell (data not shown). GdCl<sub>3</sub> at a concentration of 100 μM was used for further studies based on a previous study<sup>19</sup>. In RAW 264.7 cells, TNFα, IL-1β and IL-6 production was markedly increased in the culture supernatant after LPS stimulation, whereas the levels of these proinflammatory cytokines were significantly reduced by GdCl<sub>3</sub> (Figures 4C, D and E).

**GdCl<sub>3</sub> suppresses NF-κB activation in mucosa in colitis mice and also in LPS-stimulated RAW264.7 cells.** Nuclear factor kappa B (NF-κB), a transcription factor, plays an essential role in inflammation. Activation of macrophages is regulated by NF-κB activation<sup>20</sup>. In TNBS colitis mice, the NF-κB p65 expression in colonic mucosa was significantly attenuated with GdCl<sub>3</sub> treatment through intravenous route on both day 7 and day 14. (Figures 5A and B) Intrarectal administration of GdCl<sub>3</sub> also decreased the NF-κB p65 expression in colonic mucosa in TNBS colitis mice, which was similar to mice treated with GdCl<sub>3</sub> intravenously. In DSS colitis mice, NF-κB activation in mucosa was also suppressed with GdCl<sub>3</sub> treatment. Also, *in vitro* studies showed that GdCl<sub>3</sub> markedly reduced NF-κB p65 expression in LPS-stimulated RAW 264.7 cells (Figure 5B). Therefore, GdCl<sub>3</sub> may down-regulate the secretion of proinflammatory cytokines by macrophages through suppression of NF-κB activation.

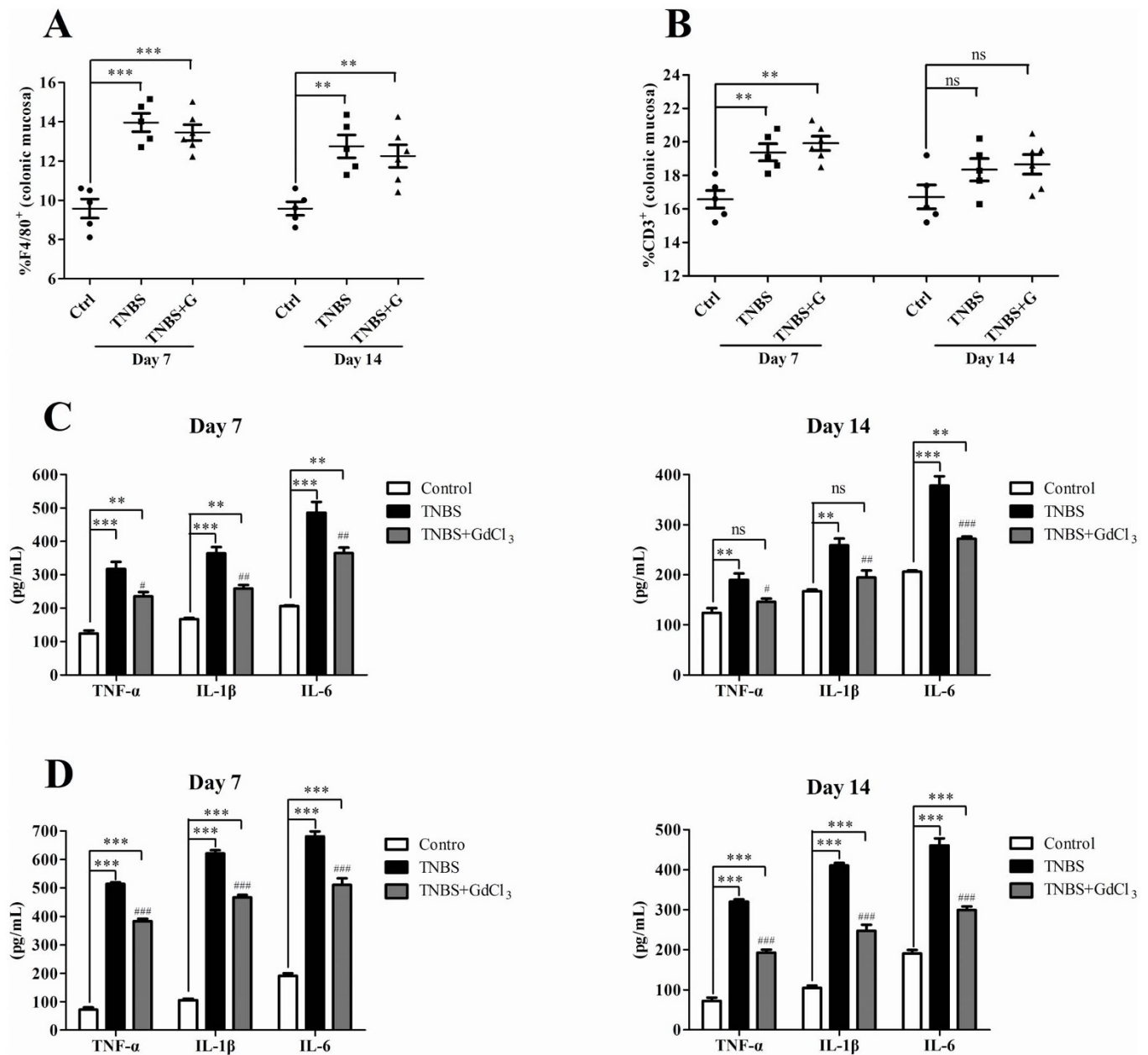
## Discussion

IBD is a chronic inflammatory disease with rising incidence worldwide, and alteration in cytokine production by inflammatory macrophages is one major component. GdCl<sub>3</sub> is a macrophage selective inhibitor, and has been proved to exert anti-inflammatory effect in lung and liver<sup>11,15</sup>. Here, for the first time, we showed a protection effect on colitis severity through an anti-inflammatory property of GdCl<sub>3</sub> in colonic mucosa *in vivo* by suppressing the proinflammatory cytokine secretions by macrophages.

In this study, we demonstrated a protection against colitis induced by TNBS and DSS in mice with GdCl<sub>3</sub> treatment, exhibiting improved weight loss, DAI scores, and mucosal damage. To investigate the protective mechanism of GdCl<sub>3</sub> in colitis, we first evaluated whether GdCl<sub>3</sub> had a macrophage eliminating role in mucosal macrophages in colon in mice. Previous studies had indicated that GdCl<sub>3</sub> treatment efficiently depleted Kupffer cells in the liver<sup>11,21,22</sup>. Wehner, et al.<sup>23</sup> had showed that intravenous treatment with chlodronate liposomes and GdCl<sub>3</sub> led to a significant depletion of 52% muscularis macrophages in colon in rat. However, the result of our study showed an absence of macrophage depletion effect of GdCl<sub>3</sub> in colonic mucosa in mice.

To evaluate whether the protection against colitis was associated with down-regulated production of proinflammatory cytokines, expression levels of TNFα, IL-1β and IL-6 in serum and colonic mucosa of vehicle and mice treated with GdCl<sub>3</sub> were detected. Kono, et al.<sup>15</sup> reported that GdCl<sub>3</sub> had no depletion effect in tissue macrophages in lung, but it decreased the expression of TNFα and IL-6 in lung after LPS stimulation in rat. Results of our study revealed that the level of TNFα, IL-1β and IL-6 in colonic mucosa was markedly reduced in colitis mice with GdCl<sub>3</sub> treatment. Thus, GdCl<sub>3</sub> had an anti-inflammatory effect in colonic mucosa in colitis mice, possibly through suppressing the proinflammatory cytokine secretion by inflammatory macrophages.

It has been reported that the role of GdCl<sub>3</sub> in lung injury might owe to the inhibition of production of inflammatory mediators by the Kupffer cells<sup>15</sup>. In our study, it was revealed that intravenous administration of GdCl<sub>3</sub> induced remission in colitis mice. Thus it was possible that not only the reduced proinflammatory cytokine production by macrophages in mucosa but also the inhibition of production of inflammatory mediators by the Kupffer cells played a role in the remission of colitis. However, the present study revealed that GdCl<sub>3</sub> treatment through intrarectal route showed a similar colitis-protective effect in colitis mice, and no significant difference



**Figure 3** | GdCl<sub>3</sub> treatment reduced TNBS-induced TNF $\alpha$ , IL-1 $\beta$  and IL-6 expressions. After induction of TNBS colitis, GdCl<sub>3</sub> was given to mice intravenously on day 3. And mice were killed on day 7 or day 14. The amount of mucosal macrophages in colon and the levels of cytokines in serum and colonic mucosa were evaluated. (A) Proportion of mucosal macrophages in colon. (B) Proportion of mucosal CD3<sup>+</sup> cells in colon. (C) Cytokine expression of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in serum. (D) Cytokine levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in colonic mucosa. (Ctrl, control; G, GdCl<sub>3</sub>) (ns: no significance; \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  comparing TNBS + GdCl<sub>3</sub> to TNBS).

was found in colitis mice treated with GdCl<sub>3</sub> through the two routes. These results further supported that GdCl<sub>3</sub> induced remission in colitis mice through reducing proinflammatory cytokine production by inflammatory macrophages in colonic mucosa.

The anti-inflammatory property of GdCl<sub>3</sub> was also confirmed *in vitro* in RAW 264.7 cells with LPS stimulation. Results of our study indicated that GdCl<sub>3</sub> exerted no cytotoxic or pro-apoptotic effect in RAW264.7 cells, which is consistent with a previous report<sup>19</sup>. Production of TNF $\alpha$ , IL-1 $\beta$  and IL-6 was markedly reduced in the culture supernatant in LPS-stimulated cells with GdCl<sub>3</sub> treatment. Therefore, GdCl<sub>3</sub> also had an anti-inflammatory effect on activated macrophages *in vitro*, down-regulating the production of proinflammatory cytokines.

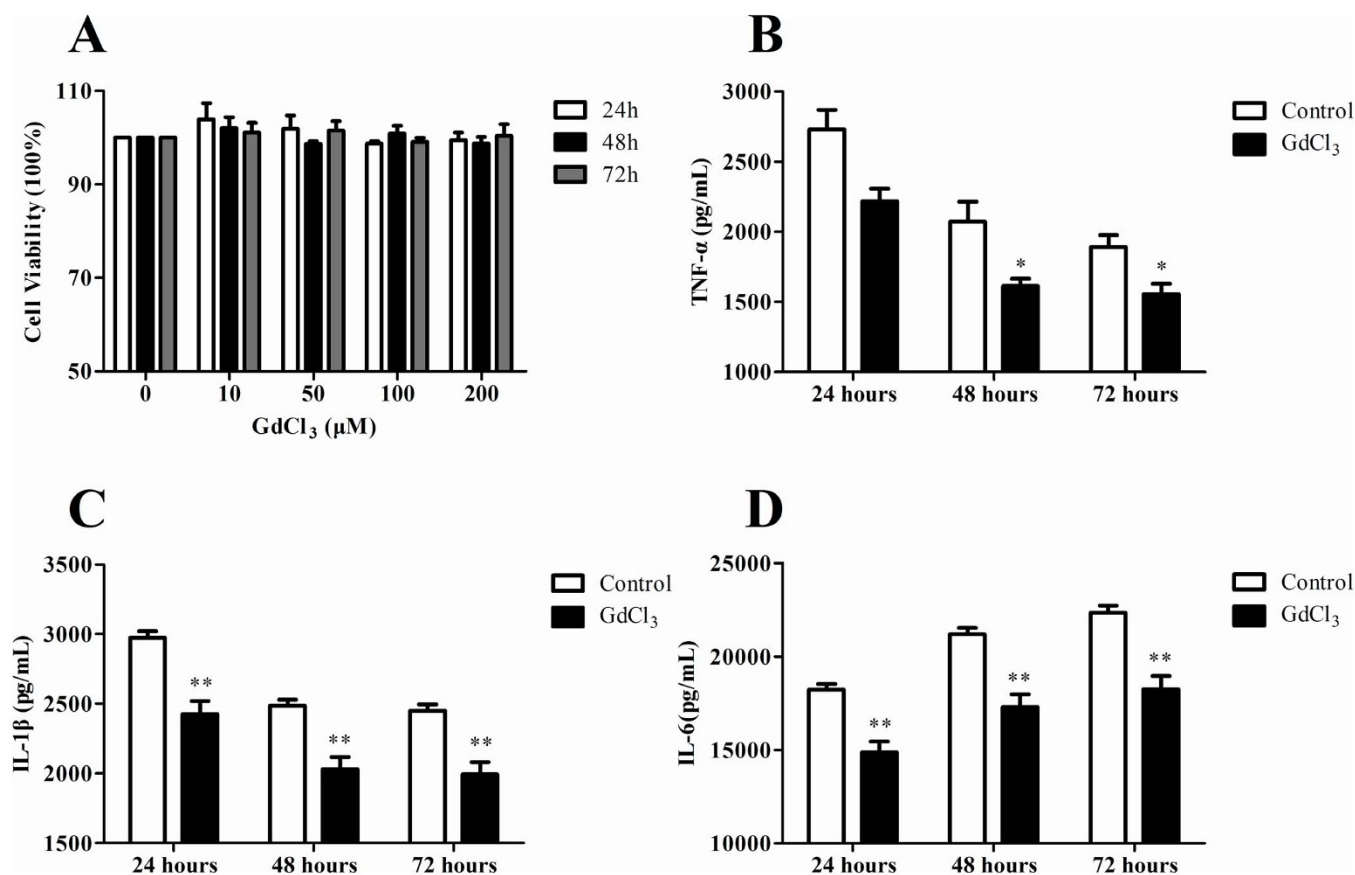
Furthermore, western blot analysis showed that the expression of NF- $\kappa$ B p65 was significantly attenuated in colonic mucosa in colitis

mice treated with GdCl<sub>3</sub>. In *in vitro* studies, GdCl<sub>3</sub> also decreased NF- $\kappa$ B p65 expression in activated RAW 264.7 cells. Thus, GdCl<sub>3</sub> may improve the colitis severity through down-regulated secretion of proinflammatory cytokines in macrophages via inactivation of NF- $\kappa$ B signal pathway.

In summary, GdCl<sub>3</sub> markedly improves the colitis severity in experimental colitis through suppression of NF- $\kappa$ B activation with reduced production of proinflammatory cytokines by mucosal macrophages in colon. Therefore, it provides us a new and bright prospect of promising therapeutics in IBD.

## Methods

**Animal treatment.** Male C57BL/6J mice weighing 20–25 g (aged 8–10 weeks) were used in this study. All mice were purchased from Beijing HFK Bioscience Company.



**Figure 4** | GdCl<sub>3</sub> decreased proinflammatory cytokine production in LPS-stimulated RAW264.7 cells. RAW264.7 cells were treated with GdCl<sub>3</sub> in various concentrations (0–200 μM) for required incubation times to evaluate the cell viability. And then cells were stimulated with LPS (100 ng/mL) with or without GdCl<sub>3</sub> (100 μM). At the end of incubation, the production of cytokines was determined. (A) Changes in cell viability. (B) Cytokine production of TNF $\alpha$ . (C) Cytokine production of IL-1 $\beta$ . (D) Cytokine production of IL-6. (\* $P < 0.05$ , \*\* $P < 0.01$ ).

Animals were housed on a temperature- ( $20 \pm 1^\circ\text{C}$ ) and light-controlled cycle (12 hours) with free access to standard laboratory chow and tap water. All procedures were approved by the Animal Care and Use Committee of Shandong University, and were performed in accordance with the Animal Management Rules of the Chinese Ministry of Health.

GdCl<sub>3</sub> (5, 10, 15, 20 mg/kg body weight; dissolved in 0.1 mL PBS as a vehicle) was administrated to mice via the tail vein. The PBS vehicle (0.1 mL) was administrated as a control. Mice were sacrificed for detection of the amount of mucosal macrophages in colon after GdCl<sub>3</sub> treatment for 1 day, 2 days and 3 days.

**Colitis induction and design of treatment.** Two well-established colitis models were used in this study. The TNBS colitis was induced by rectal administration of TNBS (2 mg in 50% ethanol, 0.1 mL in total) (Sigma-Aldrich, St. Louis, MO) via a polyethylene catheter inserted 2 to 3 cm from the anus. An equivalent volume of PBS was instilled into control mice. The DSS colitis was induced by an intake of 3% (w/v) DSS (40,000 MW) (MP Biomedicals, Solon, OH) dissolved in drinking water for 7 consecutive days, and then was replaced with regular water for another 7 days. Control mice received only drinking water. GdCl<sub>3</sub> (10 mg/kg body weight) was given to mice on day 3 of TNBS and DSS application through intravenous route or begun on day 3 of TNBS and DSS application and continued until day 7 through intrarectal route. Control mice received equivalent administration of PBS. Mice were sacrificed on day 7 or day 14 after induction of colitis.

**Evaluation of colitis progression.** Body weights were recorded daily. Severity of colitis was assessed by the disease activity index (DAI) based on weight loss, stool bleeding and stool consistency in accordance with the method described previously<sup>24,25</sup>. The DAI was scored on a scale of 0–4 for each parameter and then summed up for each mouse and each group.

Mice were killed on day 7 or day 14 with colons removed. Colons were measured and cut into sections. Histopathological studies were performed on paraffin-embedded, 4 μm thick distal colon sections, stained with haematoxylin and eosin. Histology was scored microscopically in a blinded fashion<sup>26,27</sup> as shown in Table 2.

**Flow cytometric analysis of mucosal macrophages in colon.** Colons were dissected from euthanized mice, and the fecal contents and mesenteric tissues were removed. Under a Leitz dissection microscope, the colonic mucosa was gently peeled off the

underlying layers, using a pair of fine dissection forceps. The mucosa were then cut into pieces of about 5 mm, and placed into  $10 \text{ mg}\cdot\text{mL}^{-1}$  collagenase I in DMEM supplemented with 10% FBS at  $37^\circ\text{C}$  for 60 min. At the end of the reaction, the enzymatic action was blocked with 5 mM EDTA at  $37^\circ\text{C}$  for 10 min. The suspension was filtered on nylon mesh (70 μm), and cells were harvested after centrifugation for 5 min at 1000 g and resuspended in PBS. The cells were incubated for 20 min at  $4^\circ\text{C}$  in the dark with the antibodies, and then were detected using a BD flow cytometer. The following antibody clones were used: F4/80-FITC, which had been used previously<sup>24,28</sup>, and CD3-FITC from eBioscience (San Diego, CA).

**Measurement of cytokine levels in serum and colonic mucosa.** After collection, peripheral blood was centrifuged for 5 min at 1500 g, and serum was collected. Colonic mucosa was cut into pieces and was initially homogenised in a prepared ice-cold 100 mM Tris-HCl buffer, pH 7.0, containing a cocktail of protease inhibitors (Beyotime, Shanghai, China) supplemented with 1 mM phenylmethanesulfonyl fluoride. Levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in serum and colonic mucosa were measured by ELISA kits (KYM, Beijing, China) according to the manufacturer's recommendations.

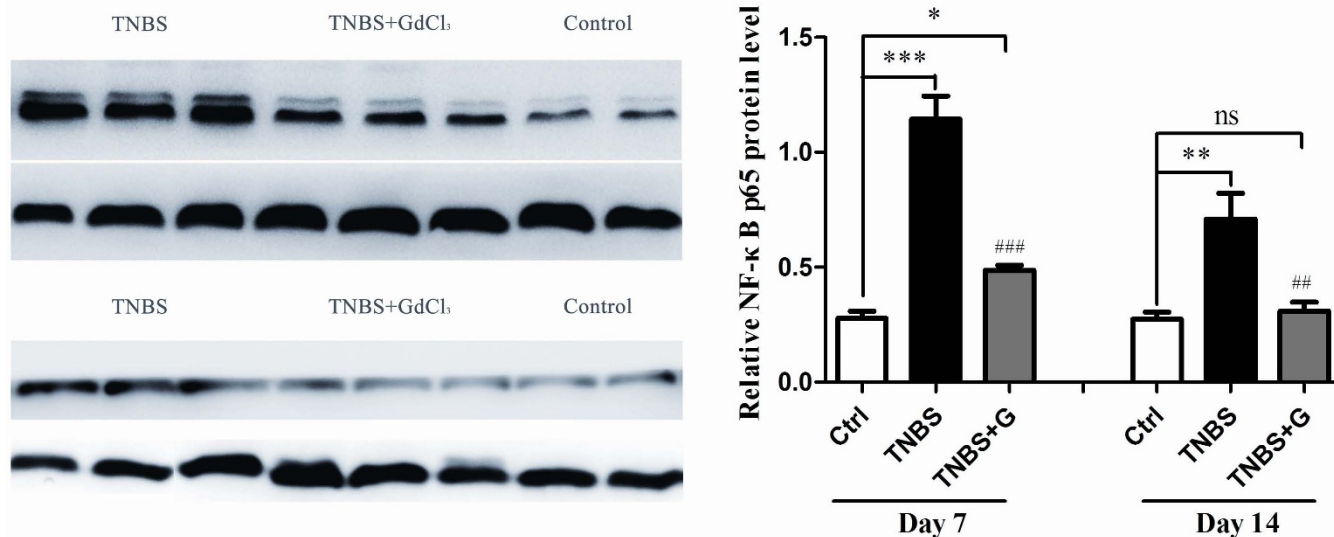
**Cell culture and treatment.** RAW264.7 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco by Invitrogen, CA, USA), containing 10% fetal bovine serum (FBS, Gibco). Cells were seeded onto the 96-well plate with  $5.0 \times 10^3$  cells per well. After 12 hours, cells were treated with gadolinium chloride (GdCl<sub>3</sub>, Sigma-Aldrich, St. Louis, MO) in various concentrations (0–200 μM) for required incubation times (24 hours, 48 hours and 72 hours).

**Cell viability and apoptosis of RAW 264.7 cells with GdCl<sub>3</sub> treatment.** At the end of incubation of GdCl<sub>3</sub> in RAW264.7 cells, 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT, Amresco, Solon, OH, USA) in PBS was added to each well to reach a final concentration of 0.5 mg/mL and the cells were further incubated at  $37^\circ\text{C}$  for 4 hours. Then the supernatant was removed, and 150 μL DMSO was added to dissolve the formazan. Absorbance was measured at 490 nm on a microplate reader (ThermoFisher Scientific, San Jose, CA). The viabilities of treated cells were expressed as the percentage of control cells, which was assumed to be 100%.

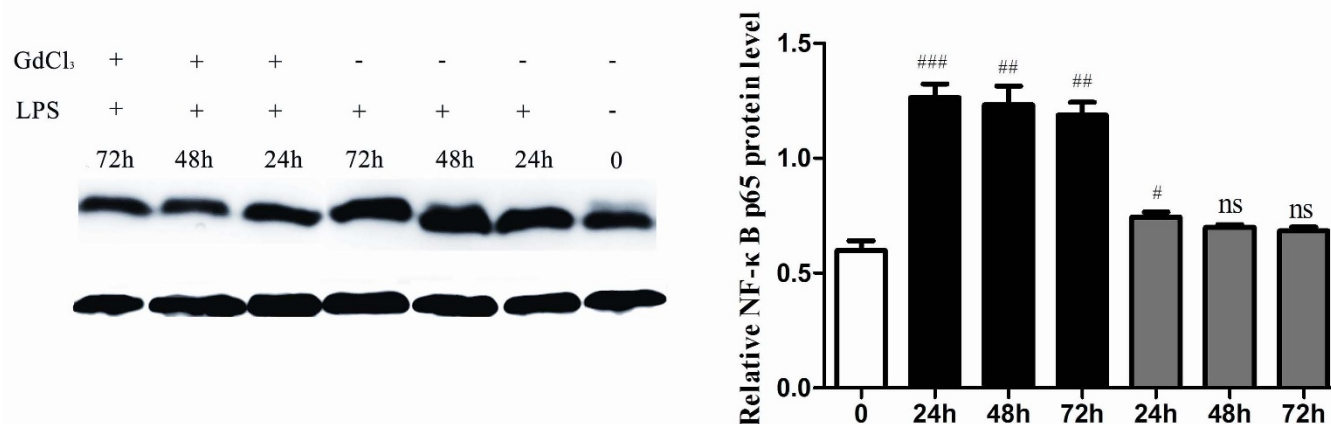
After treatment with GdCl<sub>3</sub> for 24 hours, RAW264.7 cells were trypsinized. The cell pellets were obtained after centrifugation at 1000 rpm for 5 min. Cells were



A



B



**Figure 5 | GdCl<sub>3</sub> reduced NF-κB p65 expression in mucosa in colitis mice and also in LPS-stimulated RAW264.7 cells. (A)** Western blot analysis of protein level of NF-κB p65 in colitis mice. **(B)** Western blot analysis of protein level of NF-κB p65 in LPS-stimulated RAW264.7 cells. (Ctrl, control; G, GdCl<sub>3</sub>) (ns: no significance; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ ; # $P < 0.05$ , ## $P < 0.01$ , ### $P < 0.001$  comparing TNBS + GdCl<sub>3</sub> to TNBS in (A); ## $P < 0.01$ , ### $P < 0.001$  comparing to the control in (B)).

washed twice with cold PBS and then were resuspended in 100  $\mu$ L 1  $\times$  Binding buffer at a concentration of  $1.0 \times 10^6$  cells/mL. Staining of FITC Annexin V and PI was according to the manufacture's instruction (FITC Annexin V apoptosis detection kit II, BD pharmingen™, San Diego, CA). The apoptotic rate was analyzed by flow cytometry.

**LPS treatment and cytokine analysis.** Cells ( $3.0 \times 10^4$ ) in 500  $\mu$ L medium were added to 24-well plates. After 12 hours, LPS at a concentration of 100 ng/mL with or without GdCl<sub>3</sub> (100  $\mu$ M) was added to the wells. The supernatants were collected

24 hours, 48 hours and 72 hours after stimulation. The production levels of TNF $\alpha$ , IL-1 $\beta$  and IL-6 in the supernatant were detected by ELISA.

**Western blot analysis.** Total protein was extracted from mucosal samples of mice and from RAW 264.7 cell lysates in radioimmunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Shanghai). Protein was quantified by using a BCA protein quantification kit (Beyotime). An amount of 20  $\mu$ g total protein from each sample was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (0.22  $\mu$ m pore;

**Table 2 | Grading of the histological evaluations**

Scores	Crypt architecture	Inflammatory cell infiltration	Ulceration
0	normal	no infiltration	intact epithelium
1	<30% reduction of crypt length	infiltration of inflammatory cells in lamina propria	ulceration limited to lamina propria
2	>30% reduction of crypt length and loss of goblet cells	extending into the submucosa	extending to submucosa
3	complete loss of crypts	transmural infiltration	transmural ulceration



Millipore, Bedford, MA, USA). After being blocked with 5% skim milk powder diluted in TBS containing 0.1% Tween-20 for 1 h, the membrane was incubated with primary antibodies (anti-NF- $\kappa$ B p65 monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Horseradish peroxidase-conjugated secondary antibodies (Zhongshan Gold Bridge, Beijing, China) were probed the next day, and an enhanced chemiluminescent substrate (Millipore) was used to detect the protein bands. Densitometry of protein bands was quantified by use of Quantity One 4.6.2 (Bio-Rad Laboratories, Hercules, CA, USA).

**Statistical analysis.** In mice, the nonparametric Mann-Whitney test was used to determine statistical differences between two groups. One-way ANOVA was performed to compare three groups. If the ANOVA analysis was significant, the Newman-Keuls test was applied for comparison between each two groups. In RAW264.7 cells, cytokine concentrations and NF- $\kappa$ B p65 expressions were compared using the Student's *t* test. All data were analyzed with GraphPad Prism 5.01 (Graphpad Software, San Diego, CA, USA). Differences were considered statistically significant at  $P < 0.05$ .

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## Author contributions

C.D. performed the experiments, analyzed the data and wrote the paper. P.W., Y.B.Y., F.X.C. and J.L. performed the experiments and analyzed the data. Y.Q.L. provided advice in designing experiments and writing the paper. All authors have reviewed the manuscript.

## Additional information

**Competing financial interests:** The authors declare no competing financial interests.

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