**Interleukin-33 Ameliorates Experimental Colitis through Promoting Th2/Foxp3+ Regulatory T-Cell Responses in Mice**

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Crohn’s disease (CD) is characterized by the activation of Th1 and Th17 cells and deficiency of regulatory T cells (Tregs), leading to intestine tissue injury and destruction. As a novel cytokine of the interleukin (IL)-1 family, the role and underlying mechanisms of IL-33 in CD remain poorly understood. Here, we assess the effects and mechanisms of IL-33 on the trinitrobenzene sulfonic acid (TNBS)-induced experimental colitis that mimics human CD. We found that IL-33 levels were increased in the TNBS-treated mice, whereas recombinant IL-33 (rIL-33) administration substantially ameliorated TNBS-mediated colonic tissue injury and clinical symptoms of colitis. The protective effect of rIL-33 was partly associated with the markedly increased induction of Th2-type cytokines. Importantly, rIL-33 treatment resulted in prominently upregulated Foxp3 expression in the TNBS-treated mice, and depletion of Tregs significantly abrogated the impact of IL-33 on reducing the development of colitis. Notably, the level of CD103+ dendritic cells (DCs), which promotes development of Tregs, is also increased in mesenteric lymph node and lamina propria of rIL-33–treated mice. The impact of rIL-33 on CD103+ DC induction was the result of indirectly upregulating intestine epithelial cells that produce thymic stromal lymphopoietin and retinoic acid but do not directly act on DCs. In conclusion, our data provide clear evidence that IL-33 plays a protective role in TNBS-induced colitis, which is closely related to a Th1-to-Th2/Treg switch. Thus, IL-33 is a promising candidate for the development of new treatments for CD.

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ated with the activation and production of type II cytokines (15,16). However, by switching Th1/Th17 to Th2 type immune response, IL-33 can reduce the development of atherosclerosis and inhibit graft rejection, which are mainly mediated by Th1 and Th17 response (17,18). In addition, IL-33 shows immunomodulatory effects on dendritic cells (DCs) (19), which play a fundamental role in the homeostasis of the gut (20). Moreover, CD103+ DCs, the primary source of indoleamine 2,3-dioxygenase (IDO) in the gut, can be differentiated by retinoic acid, thymic stromal lymphopoietin (TSLP) and transforming growth factor (TGF)-β1 and promote development of Foxp3+ Tregs in mesenteric lymph node (MLN) and lamina propria (21,22).

Although IL-33 levels are elevated in human CD (23,24), the functional relevance of increased IL-33 production during intestine inflammation remains unclear. Our data here show that IL-33 expression is also significantly increased in inflamed tissues of mice with TNBS-induced colitis that resembles the human CD, and recombinant IL-33 (rIL-33) treatment led to a striking improvement in both the clinical and histopathological aspects of the colitis. The mechanisms of the effects predominantly depend on Treg expansion and partly associated with Th2-type immune bias via upregulating CD103+ DCs and ST2L+ CD4+ T cells.

**MATERIALS AND METHODS**

**Animals and Cell Lines**

Female 6- to 8-wk-old BALB/c mice were purchased from the Institute of Experimental Animal, Chinese Academy of Medical Sciences (Beijing, China). The mice were housed in the specific pathogen-free (SPF) facility at the Tongji Medical College for at least 1 wk before inclusion in experiments. All of the studies were performed in accordance with the Tongji Medical College Animal Care and Use Committee guidelines. The RAW264.7 cell line was obtained from ATCC (Manassas, VA, USA).

**Production of Recombinant Mouse IL-33 Protein and Polyclonal Anti-Mouse IL-33 Antibody and Assessment of Their Activity**

Expression and purification of mouse rIL-33–glutatione S-transferase (GST) were carried out as previously described (18). Removal of the GST tag and endotoxin and the production of polyclonal anti-mouse IL-33 antibody are described in the supplementary materials. The activity of antibody was confirmed (Supplementary Figure S1).

**Induction of Colitis**

TNBS-induced mice colitis was induced in female BALB/c mice as described elsewhere (25). Mice were lightly anesthetized, and then 0.1 mL of a 2.5% (w/v) TNBS (Sigma-Aldrich) solution in 50% ethanol was slowly administered into the colon, and 50% ethanol alone served as the control. Mice received control phosphate-buffered saline (PBS) or rIL-33 (2 μg/d), anti-IL-33 (300 μg/d) polyclonal antibody and control IgG diluted in PBS daily by delivering intraperitoneally at the time of TNBS inoculation until d 4. In the therapeutic model, rIL-33 was injected intraperitoneally into mice from d 2 after TNBS inoculation to d 5. For blockage of IL-4 activity, a neutralizing anti-IL-4 anti-
body (11B11; Biolegend, San Diego, CA, USA) or control IgG (200 μg/mouse) was given to TNBS-treated mice 1 h before rIL-33 administration. Depletion of regulatory T cells has been previously reported (26) and was confirmed as described in Supplementary Figure S2. Anti-CD25 antibody (PC61; Biolegend) or control IgG (200 μg/mouse) was injected 24 h before IL-33 administration.

Histological and Immunohistochemical Analysis

The mice were sacrificed by cervical dislocation. Macroscopic assessment of inflammation was scored as in a previous study (25). Subsequently, samples of colon tissues were prepared for tissue sections and then stained with hematoxylin and eosin. Histological analysis was performed as described in a previous report (27). Immunohistochemical and immunofluorescence staining was performed by an established technique (28). The sections were stained in PBS containing antibodies against IL-33 and F4/80 (Abcam, Cambridge, MA, USA) and were then incubated with fluorescein isothiocyanate (FITC)- and rhodamine-conjugated secondary antibody and examined by an Olympus confocal microscope.

Immunoblots

Colon protein extraction was performed to immunoblot assay conducted as described previously (25). The primary antibody included rabbit anti-IL-33 (Alexis Biochemical, Lausen, Switzerland), rat anti-IL-1R/ST2 (R&D, Minneapolis, MN, USA), rat anti-Foxp3 and rat anti-IDO (Biolegend), rat anti-IFN-γ, rabbit anti-IL-17 and rabbit anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After incubating with secondary antibody–conjugated horseradish peroxidase, immunoreactivity was developed by an enhanced chemiluminescence system (Pierce).

Quantitative Real-Time Reverse Transcriptase–Polymerase Chain Reaction

Total RNA was extracted from colon tissues or intestine epithelial cells (IECs) and used for analyzing the expression levels of the gene of interest. Details of the procedures used for the quantitative reverse transcriptase–polymerase chain reaction (RT-PCR) are described in the Supplementary Materials and Methods.

Generation of Mouse BMDCs, Isolation of LPMCs and IECs and T-cell/BMDC Coculture

Mouse bone marrow–derived cells (BMDCs) were propagated from bone marrow cells, as described previously (28). All recombinant cytokine in vitro experiments were obtained from Peprotech (London, UK). Lamina propria mononuclear cells (LPMC) and IEC isolation was prepared as described in the Supplementary Materials and Methods. BMDCs were conditioned with supernatants from IECs treated with rIL-33 or PBS. Then the BMDCs were cocultured with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled CD4+CD25− T cells purified by CD4 MACS-negative selection and followed by CD25 MACS-positive selection (Mil-
tenyi Biotech, Bergisch Gladbach, Germany). A soluble anti-CD3 (0.5 μg/mL) was added to the T-cell/BMDC cultural medium. The cells and supernatants were harvested after 5 d.

Flow Cytometry
The MLN lymphocytes, LPMCs and BMDCs were obtained, and the cells were incubated with fluorescence-conjugated monoclonal antibodies in staining buffer. The Foxp3 staining was performed in accordance with the manufacturer’s instructions (eBioscience, San Diego, CA, USA). Antibodies used for flow cytometry were as follows: FITC-conjugated anti-CD4, anti-ST2L; phycoerythrin (PE)-labeled anti-CD4 and CD103; allophycoerytin (APC)-labeled CCR7 and Foxp3; and PE/cy7-conjugated CD11c. All antibodies were purchased from eBioscience, except ST2L, which was obtained from MD Bioscience (St. Paul, MN, USA).

Enzyme-Linked Immunosorbent Assay
Blood samples were collected by cardiac puncture and placed at room temperature 30 min before centrifugation. The serum was stored at –80°C until analyzed. The MLN lymphocytes of mice were stimulated by anti-CD3/anti-CD28 antibody (eBioscience) in vitro. Then the supernatants were harvested after 72 h culture. The levels of IFN-γ, IL-4, IL-5, IL-10, IL-13 and IL-17 cytokines were determined by enzyme-linked im-

Figure 3. rIL-33 treatment induces Th2 cytokines and inhibits Th1 cytokines in mice with TNBS-induced colitis. Mice instilled with TNBS were injected intraperitoneally with rIL-33 or PBS daily. The serum, MLN cells and LPMCs were collected from mice killed d 4 after TNBS inoculation. (A) Cytokines (IL-4, IL-5, IL-13, IFN-γ and IL-17) in sera were determined by ELISA (pg/mL). (B) MLN cells were stimulated with α-CD3/α-CD28 and the cultural supernatants were harvested, followed by ELISA analysis of cytokines indicated above (ng/mL). (C) Total mRNA was extracted from colonic tissues to analyze the expression of IL-4, IL-5, IL-13, IFN-γ, IL-17, IL-6, IL-10 and TNF-α by real-time PCR. The data are presented as mean ± SD (n = 6–8/group). (D) MLN cells and LPMCs were obtained at indicated time points and pooled; membrane expression of ST2L in the CD4+ subset was detected by flow cytometry. Data are shown as the mean ± SD (n = 6–8/group) and are representative of three independent experiments; *p < 0.05, #p < 0.01.
munosorbent assay (ELISA) kits (eBio-science) according to the manufacturer’s instructions.

Statistical Analysis

The data are presented as means ± standard deviation (SD). Statistical differences were determined by the Student t test. Two-sided probability (p) values <0.05 were considered significant.

All supplementary materials are available online at www.molmed.org.

RESULTS

IL-33 Is Upregulated in Th1/Th17-Mediated Murine TNBS-Induced Colitis

In recent studies, increased expression of IL-33 was demonstrated in human CD (23, 24). Consistently, in this study, a markedly increased expression of IL-33, IFN-γ and IL-17 was observed in TNBS-induced colitis when compared with the control group (Figures 1A, B). In addition, accompanied by severe colonic tissue destruction in TNBS-treated mice (Figure 1C), an abundance of IL-33 was expressed in the cytoplasm of infiltrated cells in the lamina propria and submucosa, which showed morphological characteristics of macrophages of TNBS-treated mice, whereas the colonic epithelial cells showed a slight staining (Figure 1D). Furthermore, we observed that the majority of the IL-33–producing cells in lamina propria and submucosa were F4/80 macrophages by performing immunofluorescence double staining (Figure 1E). In vitro, IL-33 mRNA and protein were present in both the RAW 264.7 cell line and primary peritoneal macrophages (Figure 1F). These data suggest that the major source of IL-33 in colonic tissues of TNBS-treated mice was the infiltrated macrophages.

IL-33 Reduces the Development of Experimental Colitis in Mice

Next, we sought to determine the role of IL-33 in TNBS-induced colitis. rIL-33, PBS, anti-IL-33 antibody or control IgG was given intraperitoneally daily at the time of TNBS administration, respectively. Compared with the PBS, antibody and IgG group, rIL-33–treated mice displayed a markedly lower loss of original weight (p < 0.05) (Figure 2A). In addition, the antibody group exhibited more severe weight loss than the IgG group, although the changes between them were not significant (Figure 2A). Moreover, lower morphological scores and a reduced inflammatory cell infiltration of mucosa and submucosa were observed in rIL-33–treated mice (Figures 2B–D). Next, we determined whether IL-33 is therapeutic in TNBS-induced colitis by treating with rIL-33 or PBS on d 2 after TNBS inoculation. In contrast to PBS administration, rIL-33 treatment caused significant weight regain and considerably alleviated inflammation in mice with established colitis (Figures 2E, F). These data clearly indicate that IL-33 potently ameliorated the development and bowel pathology of TNBS-induced colitis.

IL-33 Inhibits Th1 and Promotes Th2 Profile in TNBS-Induced Colitis

To explore whether rIL-33–induced amelioration of TNBS-induced colitis correlated with reduction of IFN-γ and IL-17 in TNBS-induced colitis, we analyzed the cytokines of sera and MLN cells from colitis mice treated with PBS or rIL-33. As expected, TNBS-treated mice receiving rIL-33 exhibited markedly decreased expression of IFN-γ and increased Th2-type cytokine production.
of IL-5 and IL-13 compared with PBS controls (Figures 3A, B). Consistently, similar results were observed in the colonic tissues by real-time PCR analysis (Figure 3C). Moreover, an increased expression of IL-4 protein in colonic tissues, the critical Th2 cytokine for counterregulating Th1 function, was also observed by immunoblot (Supplementary Figure S3A). Unexpected, IL-17 expression was not changed between two groups. Next, we sought to confirm whether the IL-33-induced Th2 immune deviation was correlated with ST2L+CD4+ T cells, which are thought to play an important role in the generation of Th2-type cytokines. As expected, the proportion of CD4+ST2L+ in LPMCs and MLN cells of mice treated with rIL-33 was higher when compared with PBS treatment, especially in LPMCs \( (p < 0.05) \) (Figure 3D). Taken together, these results indicate that rIL-33 administration of rIL-33 switches Th1 to Th2 immune profile in the mice with TNBS colitis through ST2L+CD4+ T cells.

### IL-33 Induces Regulatory T Cells by Expanding CD103+IDO+ Tolerogenic DCs in Mice with TNBS Colitis

It has been demonstrated that Foxp3+ Tregs reduce the development of TNBS-induced experimental colitis via IL-10 \( (29) \). In line with an increased expression of IL-10 (Figure 3C), an increase of Foxp3+ Tregs and IDO expression were observed in IL-33-treated mice with TNBS-induced colitis (Supplementary Figure S3). Interestingly, CD103+ DCs, the primary source of IDO in the gut and
involved in Foxp3⁺ Treg development via IDO (21), were remarkably increased in MLN lymphocytes and LPMCs of IL-33–treated mice (Figures 4A–C). As expected, we also observed that CD103⁺ DCs from MLN highly express IDO and CCR7 and might represent a lamina propria–derived migratory population (Figure 4D). Taken together, these results suggest that IL-33–induced amelioration of colitis might also act through the induction of CD103⁺ IDO⁺ DCs and development of regulatory T cells.

**IL-33 Indirectly Promotes Development of Tolerogenic CD103⁺ DCs and Tregs via Activating IECs**

To further support our contention that the induction of CD103⁺ DC and Tregs might be mediated by IL-33, the primary BMDCs were used to investigate the mechanism. Consistently, CD103⁺ BMDCs were also predominantly expressing IDO. Unexpectedly, rIL-33 has no direct effect on the promotion of CD103⁺CD11c⁺ tolerogenic DCs (Supplementary Figure S4). Previous studies have shown that IECs promote gut homeostasis and maintenance of tolerance via educating DC through inducing tolerogenic CD103⁺ DC subpopulation by TSLP, retinoic acid and TGFβ1 (20,22). Therefore, we hypothesized that IL-33 may indirectly promote the development of CD103⁺ DCs through activating IECs. As expected, ST2L was expressed on mice primary IECs (Figure 5A), and the IECs from rIL-33–treated mice with TNBS-induced colitis displayed highly increased mRNA expressions of TSLP and aldehyde dehydrogenase 1A1 (ALDH1A1) involved in the conversion of retinal to retinoic acid (Figure 5B). Furthermore, the supernatants of primary IECs from TNBS-treated mice with IL-33 administration significantly promoted the proportion of CD103⁺ DCs (Figure 5C). Collectively, these results showed that IL-33 treatment indirectly favors the induction of CD103⁺ DCs, partly through enhancing IECs to produce TSLP and ALDH1A1 but not directly act on DCs.

To further confirm whether IL-33 suppresses the development of TNBS-induced mice through promoting generation of Tregs by expanding CD103⁺ DCs, we isolated colonic epithelial cells from mice with TNBS-induced colitis and incubated them with rIL-33. Consistent with above results, IL-33–treated IECs remarkably increased expression of TSLP and ALDH1A1 in vitro (Figure 5D), and the supernatants significantly augmented the CD103⁺ DCs when compared with the PBS group (Figure 5E). Next, we examined whether the induction of the epithelial cell–conditioned DCs have capacity to promote Treg differentiation. As expected, BMDCs conditioned by the supernatants of IL-33–treated IECs markedly promoted Treg differentiation and inhibited T-cell proliferation (Figure 5F).

**IL-33–Mediated Reduction of Experimental Colitis Development Mainly Relies on Treg Expansion**

Both Th2 deviation and Treg expansion play a protective role in the reduction of TNBS-induced colitis development. Therefore, the roles of Th2 deviation and Treg expansion induced by IL-33 treatment were explored in mice with TNBS-induced colitis. Anti-IL-4 or anti-CD25 antibody was used to neutralize IL-4 activity and deplete CD25⁺ Tregs, respectively. Specifically, Treg depletion evidently abolished the protective role of IL-33 when compared with the IgG group. Furthermore, anti-IL-4 antibody treatment partly reversed the IL-33 effect (Figure 6). Thus, these data showed that IL-33 reduced the development of TNBS-induced colitis mainly depending on Treg function.

**DISCUSSION**

This report is the first to document the role of IL-33 in a mouse model of human CD. Current results provide evidence for a novel mechanism by which IL-33 ameliorates the intestinal inflammation via promoting a switch from intestinal Th1 to Th2/Treg responses. Both Th2 deviation and Treg expansion play a protective role in the reduction of TNBS-induced colitis development. Therefore, the roles of Th2 deviation and Treg expansion induced by IL-33 treatment were explored in mice with TNBS-induced colitis. Anti-IL-4 or anti-CD25 antibody was used to neutralize IL-4 activity and deplete CD25⁺ Tregs, respectively. Specifically, Treg depletion evidently abolished the protective role of IL-33 when compared with the IgG group. Furthermore, anti-IL-4 antibody treatment partly reversed the IL-33 effect (Figure 6). Thus, these data showed that IL-33 reduced the development of TNBS-induced colitis mainly depending on Treg function.
defined. Here, we show that IL-33 was markedly increased in the mice with TNBS-induced colitis, which mimics human CD. Previous studies have also demonstrated that increased expression of IL-33 plays a pathological role in the development of ulcerative colitis (24). Interestingly, rIL-33 treatment had a significant beneficial effect on Th1/Th17-mediated experimental colitis, and there was a deleterious consequence with the anti-IL-33 antibody, although without reaching significance. The reason for this result might be that the endogenous IL-33 in the colonic tissues is induced by TNBS, insignificant in relation to the inflammatory response raised.

Previous work has demonstrated that administration of IL-33 to mice leads to histological changes in the lung and gastrointestinal tract, including increased mucus production, epithelial cells hyperplasia and hypertrophy. The histological changes are expected to be linked to enhanced Th2-type response (12). Conversely, in this study, we found that mice treated with rIL-33 reduced the progression of TNBS-induced pathology. Likewise, TSLP and IL-25, shown to develop inflammatory response raised.

Recently, the imbalance of Treg/Th1–Th17 cell response was implicated in colitis (8–10). It was shown that an impaired Treg function is observed in ST2−/− mice (46), suggesting that IL-33/ST2 signaling might be involved in the development and function of Tregs. Indeed, we observed that IL-33 administration caused prominent induction of Tregs and suppressive cytokine IL-10. However, the expression of inflammatory cytokine IL-17 was not downregulated, which could be a result of IL-33 being a potent inducer of Th17 response (31). In parallel with upregulated expression of Foxp3, the tolerogenic CD103+ID0+ DCs, which play a crucial role in reducing the pathological progression of CD and experimental colitis (21,22), were markedly increased in both MLN and lamina propria of rIL-33–treated mice. These results indicate that administration of rIL-33 favors Treg function probably by promoting CD103+ DC differentiation. Indeed, in this study, treatment of mice with rIL-33 resulted in increased ALDH1A1 and TSLP expression in IECs, which is critically implicated in the induction of tolerogenic CD103+ DC (22). As expected, our in vitro data showed that rIL-33 indirectly promoted the differentiation of tolerogenic CD103+ DCs and Tregs via acting on IECs. In addition, TSLP was also reported to induce Th2 immune type response (39).

CONCLUSION

In conclusion, our studies demonstrate that rIL-33 substantially ameliorates the development of TNBS-induced experimental colitis by a Th1-to-Th2/Treg switch, specifically depending on Treg expansion. These results suggest that IL-33 might offer an alternative treatment to our current approaches of managing CD.

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DISCLOSURE

The authors declare that they have no competing interests as defined by Molecular Medicine, or other interests that might be perceived to influence the results and discussion reported in this paper.

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