Anti-mouse CD52 Treatment Ameliorates Colitis through Suppressing Th1/17 Mediated Inflammation and Promoting Tregs Differentiation in IL-10 Deficient Mice

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Recent studies suggested that excessive T helper (Th)1/17 cells concomitant with regulatory T cell deficiency might play important roles in Crohn’s disease. Anti-cluster of differentiation 52 (CD52) monoclonal antibody (mAb), which aims on CD52 antigen on mature immunocytes, has both T cell depletion and immunosuppressive activities. In this study, we evaluated the therapeutic effects and possible mechanisms of anti-CD52 treatment on interleukin-10 (IL-10) deficient mouse. Anti-mouse CD52 mAb was administered to C3H/HeJ (IL-10−/−) C3H.IL-10−/− mice intraperitoneally 20 µg per week for 2 weeks. The disease activity index, body weight, the histological grading of colitis, and levels of tumor necrosis factor (TNF)-α, interferon (IFN)-γ, IL-17 and IL-6 in colon were quantified after treatment. In addition, CD25, Forkhead box P3 (Foxp3) and transforming growth factor (TGF)-β gene as well as the percentage of CD25+Foxp3+ T cells in colon were also measured. The severity of colitis in IL-10−/− mice was significantly decreased by the treatment, with improvement of colon histological grade. The treatment also decreased the TNF-α, IFN-γ, IL-17 and IL-6 levels in colon. Furthermore, the treatment up-regulated the mRNA expression of CD25, Foxp3 and TGF-β gene as well as the percentage of CD25+Foxp3+ T cells in colon lamina propria mononuclear cells (LPCMs) of IL-10−/− mice. Our data might indicate that anti-CD52 treatment could ameliorate the colitis of C3H.IL-10−/− mice and it might be related to the suppression of Th1/17 related inflammation and the promotion of regulatory T cell differentiation. Thus, our data reveals that anti-CD52 treatment may hold potential for clinical applications for Crohn’s disease treatment.

Key words  T cell; interleukin-10 (IL-10) deficient mouse; Crohn’s disease; anti-cluster of differentiation 52 (CD52) monoclonal antibody

Crohn’s disease (CD), one of the two major clinically defined forms of inflammatory bowel disease, is an immune-mediated relapsing systemic inflammatory disease mainly affecting the gastrointestinal tract with extra-intestinal manifestations.⁷ Although the exact pathogenesis of CD is complex, it’s believed that CD might be triggered by environmental factors and resulted in a disturbed innate and adaptive immune response towards a diminished diversity of commensal microbiota.⁵ Researches declare that CD is a T-helper (Th1/Th17 type inflammatory reaction, with exorbitant secretion of numbers of effector cytokines such as interferon (IFN)-γ, interleukin (IL)-17 and tumor necrosis factor (TNF)-α as well as the unmatched changes of the T cell response.⁴ The imbalance of effector T cells versus regulatory T cells (Tregs) in gastrointestinal tract mucosa is a hallmark of CD.⁵ And genome-wide association studies support the imbalance model by linking loci that are crucially involved in Tregs and Th1/Th17 differentiation to CD.⁶ Cluster of differentiation 52 (CD52) is a cell-surface glycoprotein mainly expresses on mature lymphocytes. Anti-CD52 monoclonal antibody (known as campath-1) can rapidly produce a profound lymphopenia mainly by the cytotoxicity, leading to long-lasting changes in adaptive immunity. The humanized Campath-IH has been licensed for chronic lymphocytic leukemia (CLL)⁷ and been used successfully in several autoimmune diseases, such as arthritis, multiple sclerosis, vasculitis⁸ as well as in organ transplantation.⁹ And in several trials, Campath-IH was found that it could promote increasing of Tregs.⁴,¹⁰–¹²

Our previous studies demonstrated that anti-CD52 treatment could improve histological inflammation score,¹³ inhibit epithelial apoptosis and tight junction permeability⁴ in IL-10 deficient mice. In this study, we mainly investigated the therapeutic effects on colitis and potential mechanisms relating to Th1/17 related inflammation and regulatory T cells of treatment with anti-mouse CD52 monoclonal antibody in IL-10 deficient mouse model which mimics human CD.

MATERIALS AND METHODS

Animals and Immunotherapy  C3H/HeJ.Bir.IL-10−/− (C3H.IL-10−/−) mice and C3H/HeJ (wild type) mice were obtained from the Jackson Laboratory (Bar Harbor, ME, U.S.A.). All the mice were maintained at the Model Animal Research...
Center of Nanjing University (Nanjing, China) in a specific-pathogen-free (SPF) animal facility. All mice received humane care in accordance with the law concerning the protection and control of animals in China. C3H/IL-10−/− mice were used because they develop colitis more severely than other strains.

Age and sex matched C3H/IL-10−/− mice at 12 weeks of age with established colitis and wild type mice were used for the experiments of anti-mouse CD52 administration. The mice were assigned to the anti-mouse CD52 treated IL-10−/− mice group (anti-mouse CD52 group), phosphate buffered saline (PBS) treated IL-10−/− mice group (Control group) and PBS treated wild type group (normal group). There were 6 mice per group. The drug administration of anti-CD52 monoclonal antibody was performed as previously described. Briefly, each mouse in anti-mouse CD52 group was injected intraperitoneally with 20 µg anti-mouse CD52 monoclonal antibody (clone BTG-2G) (MBL, Nakaku Nagoya, Japan) dissolved with PBS. One week later, the drug was administrated again with the same dose. The normal and control group mice received an equivalent quantity of PBS at the same time. The clinic symptoms were observed after the treatment. One and three weeks after the final drug administration, the mice were sacrificed for the following tests.

Disease Activity Index (DAI) The clinical manifestation of colitis was quantified with the DAI according to the methods described previously. Briefly, the ruffled fur, occult fecal blood determined by the Fecal Occult Blood Card (Jiancheng Biotechnology Co., Nanjing, China), rectal prolapsed (<1 cm for one point or >1 cm for two points), soft stool and diarrhea were the score points of DAI. All the points were added to a final DAI score with a range from 0 to 6.

Histopathology The colon samples were collected and fixed in paraformaldehyde (PFA). Samples were paraffin-embedded, sectioned, and stained with hematoxylin and eosin. Grading of intestinal inflammatory score was determined as described previously with the scores ranged from 0 to 4.

Cytokine-Specific Enzyme-Linked Immunosorbent Assay (ELISA) Cytokine determination in colon mucosa was performed as previously described, briefly, protein extracts were obtained by homogenization of colonic segments (0.5 mg tissue/mL) in homogenization buffer consisting of a protease inhibitor (Roche Diagnostics, Mannheim, Germany). Samples were centrifuged at 20000×g for 30 min at 4°C. Supernatant was aliquoted and stored at −80°C for cytokine examination. TNF-α, IFN-γ, IL-17 and IL-6 concentrations in colonic protein extracts were measured using ELISA Kit (Invitrogen, Vienna, Austria) according to the manufacturer's recommendations. Values were expressed as pg/mg protein.

Isolation of Mononuclear Cells from Colon Lamina Propria Lamina propria mononuclear cells (LPMCs) of colon were isolated according to the methods described previously with some modifications. Briefly, the colon of each mouse was immediately harvested after sacrifice, cut longitudinally and washed with cold RPMI1640 containing 5% fetal calf serum (FCS), then cut into pieces to incubate in the solution contains 1 mM ethylenediaminetetraacetic acid (EDTA) (Sigma-Aldrich, St. Louis, MO, U.S.A.) and 1 mM dithiothreitol (Sigma) for 30 min at 37°C. Subsequently, the remained tissues were digested for 30 min in the presence of 40 U/mL collagenase II (Sigma) and 10 µg/mL deoxyribonuclease (DNase) I (Boehringer Mannheim, Mannheim, Germany). The suspensions were passed through a 40-µm nylon mesh separately and then centrifuged through a 40/70% discontinuous Percoll (Pharmacia, Uppsala, Sweden) gradient at 400×g and 20°C for 25 min without breaking. The cells from the interface were collected. All the collected mononuclear cells were washed and resuspended with RPMI1640 for the following FACS analysis.

FACS Analysis Fluorescein isothiocyanate (FITC)-conjugated anti-mouse CD4, Allophycocyanin (APC)-conjugated anti-mouse CD25 and Phycoerythrin (PE)-conjugated anti-mouse Foxp3 (eBioscience, San Diego, CA, U.S.A.) were used for the stain of Tregs according to the protocols supplied by manufacturer. Briefly, 1×10⁶ cells were suspended in 100 µL flow staining buffer with saturating amounts of fluorochrome-conjugated anti-CD4/CD25 for the staining of cell surface antigens, then the cells were incubated with Fixation/Permeabilization buffer and stained with PE-conjugated anti-mouse Foxp3. All the flow cytometric analyses were acquired using the FACScan flow cytometer (Becton Dickinson, San Jose, CA, U.S.A.), and the data were analyzed using the FlowJo software (Tree Star, U.S.A.). Unstained cells and cells stained with isotype-matched control antibodies served as controls.

Quantitative Real-Time PCR (qPCR) Total RNA of colon tissue was isolated with RNeiso Plus (TaKaRa, Dalian, China) and reversed transcribed by RT reagent Kit (TaKaRa) according to manufacturer’s instructions. The primers were as follows: Foxp3, 5′-CCC AGG AAA GAC GAC ACC AACTT-3′ and 5′-TTCTCA CAA CCAG GGC CACTT-3′; CD4, 5′-AAAG TGA CCTCTCA GTCCGG GTA-3′ and 5′-GGTTAG AGACC TTAGAGT GGCT-3′; CD25, 5′-CCTC CAATGA CAA ATCG AGA AGC-3′ and 5′-ACT CTG TCTC TCTC ACAC AAATG AT-3′, transforming growth factor (TGF)-β, 5′-CCG CAC AACGC CATCTAG T-3′ and 5′-CCCCG AAA TGT CTCG AGCT TATG A-3′; Beta-actin, 5′-GAG AAG ATCTGG GCAC CAC ACC-3′ and 5′-GCATAC AGG GAC AGC ACAG C-3′. The qPCR was performed with the Step One Real-Time PCR system (ABI, Carlsbad, CA, U.S.A.) using the SYBR Premix Ex Taq II kit (TaKaRa). All the process was according to manufacturer’s direction and PCR reactions were done in duplicate and relative expression was calculated using the 2−ΔΔCt method after normalizing beta-actin expression for each sample.

Statistical Analysis SPSS version 17.0 software (SPSS, Inc., Chicago, IL, U.S.A.) was used to perform the statistical analyses. The Mann–Whitney U test with Bonferroni correction was used for multiple comparisons of non-parametric data (the DAI score and histopathology score) for statistical significance. And for the parametric data, Student’s t test was used to compare two groups, and one-way ANOVA was used for multiple comparisons followed by Tukey post-hoc test. Statistical significance was defined as p<0.05. The GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, U.S.A.) software was used for figures drawing.

RESULTS Anti-CD52 Treatment Reduced Disease Activity in IL-10−/− Mice The onset and severity of colitis of IL-10−/− mice were dramatically different among strains and laboratories. We chose the 12 weeks old C3H/HeJ background mice as animal model for the measurement for the first week. The body weight of mice was measured for the first week started colitis. The first drug
administration, the result showed that the IL-10−/− mice spontaneously developed chronic colitis with weight loss, while the anti-CD52 mAb treated group grew more steadily. One, 2 and 3 weeks after the final administration, the body weights of anti-mouse CD52 group mice were significantly higher than the control mice, while the normal group got the similar outcome. (Fig. 1). DAI, which is defined by ruffled fur, fecal blood, rectal prolapse, stool form and diarrhea, was used to analyze the normal group, the control group had higher mean DAI values (Fig. 2b), while the anti-mouse CD52 group showed a significant reduction of DAI compared to the control group three weeks after the treatment. **p<0.01, compared to the Control group. (Fig. 2).

**Anti-CD52 Treatment Lowered Histopathology Scores of Colitis in IL-10−/− Mice** Remarkable findings in the histopathologic effect of anti-CD52 treatment were found three weeks after the administration on mouse colon. The anti-mouse CD52 group mice which received anti-CD52 treatment showed notable depletion of inflammatory cells in the lamina propria of colon mucosae (Fig. 3c), while the control group mice showed the massive infiltration with inflammatory cells, disruption of mucosal and muscular architecture and ulcers penetrated the whole intestinal wall (Fig. 3b). The histopathology scores were calculated and confirmed the effect of the anti-CD52 treatment. Compared to control group, the normal group showed noticable depletion of inflammatory cells in the lamina propria of colon mucosae (Fig. 3c).

**Anti-CD52 Treatment Decreased the Pro-inflammatory Cytokine Expressions in Colon of IL-10−/− Mice** CD is usually considered as a Th1/Th17 type of inflammatory reaction. To determine the therapeutical effect of anti-CD52 mAb in IL-10−/− mouse model, the TNF-α, IFN-γ, IL-17 and IL-6 expressions in colon tissue were examined by ELISA. Remarkable increase expressions were observed in control group compared to normal group (TNF-α 88.84±12.18 pg/mg, p<0.01; IFN-γ 59.92±19.58 pg/mg, p<0.01; IL-17 171.56±18.82 pg/mg, p<0.01; IL-6 64.33±13.45 pg/mg, p<0.01). However, in the anti-mouse CD52 group, significant decreases in the percentage of CD4+ T cell increased in the control group when compare to the normal group (21.80±0.87% versus 13.02±0.58%, p<0.01). Anti-CD52 treatment dramatically decreased the level of these cytokines compared to the control (TNF-α 26.25±5.54 pg/mg, p<0.01; IFN-γ 25.66±8.09 pg/mg, p<0.01; IL-17 14.86±2.96 pg/mg, p<0.01; IL-6 19.84±3.21 pg/mg, p<0.01) (Fig. 4).

**Anti-CD52 Treatment Promoted the CD25 Foxp3+ Regulatory T Cell Response in IL-10−/− Mice** It has showed that CD4+ T-helper type 1 and T-helper type 17 cells and the inflammation mediated by them are important in CD, while the mature lymphocyte lysis effect of the anti-CD52 mAb was reported in many former studies. Next we tested the depletion of CD4+ T cells in the mononuclear cells in colon tissue three weeks after the anti-CD52 treatment using the flow cytometry. The FACS results showed that after the development of colitis, the percentage of CD4+ T cell increased in the control group when compared to the normal group (8.03±1.64% versus 21.80±0.87%, p<0.01) after the treatment.

Fig. 1. The Body Weight Changes of Mice from the First Anti-CD52 Treatment in Interleukin-10 Deficient Mouse Model

One, 2 and 3 weeks after the final administration, the body weights of anti-mouse CD52 group mice were significantly higher than the control mice, while the normal group got the similar outcome. Values were presented means±S.E.M. (n=6 per group). NS p>0.05, *p<0.05, **p<0.01, compared to the Control group.

Fig. 2. The Changes of DAI Three Weeks after Anti-CD52 Treatment in IL-10 Deficient Mouse Model

(a) Rectal prolapse and stool form of mice in each group. (b) Compared to normal group, the control group had higher DAI values, while the anti-mouse CD52 group showed a significant reduction of DAI compared to the Control group three weeks after the treatment. **p<0.01.
Foxp3-expressing Tregs are believed to have anti-inflammatory activity through the suppression of the Th17 response. And CD25+Foxp3+ Tregs are believed to attenuate the severity of ileitis in a Crohn’s-like mouse model. So we tested the CD25+Foxp3+ Tregs in colon LPMCs by FACS, additionally the mRNA expressions of CD4, CD25, Foxp3 and TGF-β were also examined. The result showed that the percent of CD25+Foxp3+ Tregs in anti-mouse CD52 group was much higher compared to the control group (8.65±0.33% vs. 3.77±0.27%, p<0.01) after receiving the anti-CD52 treatment. (Fig. 6) And the mRNA expressions levels of CD25, Foxp3 and TGF-β in anti-mouse CD52 group were increased, while the mRNA expression of CD4 decreased as expected compared to the control group (Fig. 7).

DISCUSSION

CD is one of the two major forms of inflammatory disease (IBD) and it may affect any part of the whole gastrointestinal tract from mouth to anus. Although it is believed to be due to a combination of abnormal gut microbiota, immune response dysregulation, environmental changes and gene variants, a full understanding of CD pathogenesis is still unclear. However, the exaggerated immune responds to the gut microbial anti-
gens have been reported to play important roles in beginning and development of CD. Usually, CD was assumed to be mediated mainly by Th1 cells, and the concentration has recently moved to the differentiation and balance of Th17/Tregs which has recently been showed to affect in CD.\textsuperscript{23,24} Th17 and Treg cells play an important role in the immune homeostasis, IL-17-releasing Th17 cells are well known for its primarily pro-inflammatory function, while Tregs show anti-inflammatory activity by the suppression of Th17 response, and the TGF-β and IL-10 secreted by Tregs are the main regulatory factors which are also believed could suppress the differentiation of pro-inflammatory T cells.\textsuperscript{25} As Th17 cells are important in intestinal inflammation, it has been aimed as therapeutic target to interfere the intestinal inflammatory. Zhang \textit{et al.} found IL-17R knockout mice were significantly protected against 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced colitis.\textsuperscript{26} Sandborn \textit{et al.}\textsuperscript{27–29} got promised results in patients with CD with anti-IL12/23p40 antibody (ustekinumab), which inhibits IL-23 mediated Th17 responses. The efficiency of vidofludimus in IBD patients was separately confirmed by Fitzpatrick \textit{et al.}\textsuperscript{30} and Herrlinger \textit{et al.},\textsuperscript{31} which could ameliorate experimental colitis by decreasing IL-17A and IL-17F levels via down-regulation of nuclear factor (NF)-κB and signal transducer and activator of transcription 3 (STAT3) pathway. Although the secukinumab, a mAb directed against IL-17A, was ineffective and associated with more adverse events than pla-

Fig. 6. The Percentage of CD25$^{+}$Foxp3$^{+}$ Regulatory T Cell among Colon LPMCs Three Weeks after the Anti-CD52 Treatment

(A) FACS results of the CD25$^{+}$Foxp3$^{+}$ regulatory T cell in colon LPMCs. (B) The percentage of CD25$^{+}$Foxp3$^{+}$ regulatory T cell among colon LPMCs of IL-10 deficient mice, the anti-mouse CD52 group was 8.65±0.33% and the Control group was 3.77±0.27%. Values were presented means±S.E.M. (n=6 per group). **p<0.01.

Fig. 7. The mRNA Expression Levels of CD4, CD25, Foxp3 and TGF-β in Colon Tissues Three Weeks after the Anti-CD52 Treatment

Three weeks after the treatment, the mRNA transcription levels of CD4, CD25, Foxp3 and TGF-β were evaluated by qPCR. The results showed that the levels of CD25, Foxp3 and TGF-β in anti-mouse CD52 group were up-regulated by the anti-CD52 administration compared to Control group, while the CD4 mRNA expression level decreased after the treatment. Values were presented means±S.E.M. (n=6 per group). *p<0.05, **p<0.01.
the development of experimental colitis. And in another trial, the treatment to inhibit STAT3, which is important in the differentiation of Th17 cells, was proved to attenuate intestinal inflammation. And in an experimental colitis model, Treg cells were declared to prevent intestinal inflammation and reduced the expression of Th17-related cytokines. Meanwhile the regulatory T cell therapy and for CD was logical process in IL-10 −/− mice and CD52mAb plays in the process. IL-12/23 pathway, TGF-β important in this procedure. However, further studies focus on Th1/Th17 inhibition and TGF-β regulation, the TGF-β presents on the surface of all T and B lymphocytes, monocytes and eosinophils, but not hematological precursors, can lyse the cells after binding to its receptor through numerous mechanisms and rapidly produce a profound lymphopenia, particularly the low levels of CD4 T cells. And Campath-1, which specifically targets CD52, a most abundant glycoprotein presents on the surface of mature lymphocytes, particularly the TGF-β scores of colitis and decreased the percentage of CD4 T cell in colon lamina propria, as well as the expressions of TNF-α, IFN-γ, IL-17 and IL-6 in IL-10−/− mice. Furthermore anti-CD52 treatment also could increase the percentage of CD25+Foxp3 Tregs in colon LPMC s and up-regulate the mRNA expression of CD25, Foxp3 and TGF-β. Recent results provide evidence that anti-CD52 treatment ameliorates the intestinal inflammation of IL-10−/− mice and it might relate to the suppressing Th1/Th17 type inflammation and promoting regulatory T-Cell response. However, the exact mechanisms of Th1/Th17 inhibition and Tregs promotion is still unclear, we hypothesize that the directly depletion of mature lymphocytes by CD52 mAb might play the role like chemotherapy but not to the stem cell, then the redifferentiation from naive T cell might be something like the stem cell transplants. Because of the empty of pro-inflammatory cells and cytokines by CD52 mAb, a normalizing T-cell balance might reach in the re-differentiation, the TGF-β secreted by Tregs could suppress the differentiation of Th1/Th17 cells in IL-10−/− mice and it might be important in this procedure. However, further studies focus on IL-12/23 pathway, TGF-β pathway and other pathways related to T cell regulation should be carried out to certify the roles which CD52mAb plays in the process.

In summary, the current study, has suggested anti-CD52 therapy may reverse the clinic symptoms and tissue pathological process in IL-10−/− mouse model. It may relate to the depletion of CD4+ T cells in colon tissue, inhibition of Th1/Th17 mediated inflammation and the promotion of Tregs response. Therefore, the anti-CD52 mAb may be a firenew way for the treatment of CD patients. However, further studies are required to determine the long-term maintaining effects and adverse reactions of anti-CD52 treatment.

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Conflict of Interest The authors declare no conflict of interest.

REFERENCES


